Identification of potential reservoirs of Q fever in Queensland, Australia

Thesis submitted by

Alanna Elizabeth COOPER BBiomedSc (Hons)
James Cook University

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James Cook University

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ETHICS DECLARATION

The research presented and reported in this thesis was conducted within guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human (1999)* and Joint *NHMRC/AVCC Statement and Guidelines on Research Practice (1997)*, the *James Cook University Policy on Experimentation Ethics. Standard Practices and Guidelines (2001)* and the *James Cook University Statement and Guidelines on Research Practice (2001).*

The proposed research methodology received clearance from the James Cook University Experimentation Ethics Review Committee.

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STATEMENT ON THE CONTRIBUTION OF OTHERS

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ABSTRACT

Q fever is a zoonotic disease caused by the bacterium, *Coxiella burnetii*. The bacterium has a wide host range and human infections are most commonly contracted following contact with infected livestock. Australian surveys have shown an increased prevalence of human disease in recent years, with an increase in cases involving patients with no known contact with the typical reservoir species. The aim of this project was to determine the seroprevalence of *C. burnetii* in livestock, companion animals, feral animals and native wildlife and their ability to act as reservoirs of Q fever in Queensland, Australia. Due to the unavailability of secondary antibodies for native Australian marsupials, this project also aimed to develop a phage display library of chicken recombinant antibodies (CRAbs) for a variety of Australian native marsupials and determine their effectiveness as secondary antibodies for epidemiological studies and pathogen surveillance in wildlife populations. This project also aimed to determine the prevalence of *C. burnetii* in the ticks and blood of Australian native marsupials to determine their potential capacity to act as reservoirs of Q fever.

Prior to the development of diagnostic tools for the detection of antibodies to *C. burnetii* in animals, an appropriate *C. burnetii* isolate needed to be selected for the purpose of antigen production. A comparison of virulence and ability to induce seroconversion was performed in mice and guinea pigs, which resulted in the selection of the Australian Cumberland Q fever isolate. A series of quantitative reverse transcriptase PCRs were developed to determine the antigenic phase of the isolate and were validated against traditional methods for determining antigenic phase. ELISAs were then developed for the detection of antibodies to both *C. burnetii* antigenic phases and validated using sera from infected and uninfected mice and guinea pigs. The ELISAs developed in this study are the first known use of an Australian Q fever isolate as antigen.

The ELISAs developed were then modified for use with bovine, canine, feline, porcine and human sera. A total of 1,835 bovine, 1,522 human, 127 dingo, 201 domestic dog, 49 domestic cat, 31 feral cat, 19 feral pig and 16 feral fox samples were tested for antibodies to *C. burnetii*. Seroprevalence was found to be highest in

foxes (43.8%; 95% CI 42.5-48.1%) and feral cats (38.7%; 38.7-40.6%). Similar seroprevalence was found in beef cattle (16.8%; 16.78-16.80%), domestic dogs (both currently (21.8%; 21.6-22.1%) and retrospectively (16.0%; 15.9-16.2%) and wild dogs/dingoes (17.3%; 17.2-17.5%). Seroprevalence was relatively low in domestic cats (6.1%; 6.1-6.5%) and the human population (3.5%; 3.48-3.50%). No significant difference was found between seroprevalence in domestic dogs and dingoes. However, the difference between seroprevalence in domestic cats and feral cats was statistically significant ($P<0.05$).

In order to produce recombinant secondary phage-displayed antibodies for Australian native marsupials, the technique was initially optimised and validated using a murine model. Purified murine IgG was used to immunise domestic chickens and reverse transcriptase PCR was used to amplify genes encoding the heavy and light chain immunoglobulin. These were then inserted into a phage-display vector and used to create libraries of chicken recombinant antibody (CRAb). This library was then screened for phage-displayed antibodies binding to murine IgG. Selected CRAbs were then characterised in ELISA and DNA sequences obtained. The selected CRAbs were then validated in ELISA using the sera of mice infected with *C. burnetti* and uninfected mice.

Using the optimised method for the production CRAbs against IgG, further libraries were produced for macropods (*Macropus sp*.), common northern bandicoot (*Isoodon macrourus*) and brushtail possum (*Trichosurus vulpecula*). Selected CRAbs were also characterised in ELISA and DNA sequences obtained. Each CRAb was tested for cross-reactivity against the IgG of the other species. The CRAb raised against murine IgG in the initial optimisation experiments was found to bind to the IgG of all species tested. This CRAb was used in subsequent serological testing to simplify development of ELISAs as only this CRAb would need to be amplified for the production of ELISA conjugate.

The phage-displayed CRAb was compared to competitive ELISA, standard indirect ELISA and complement fixation in serological testing on serum samples from a variety of Australian native marsupials. A total of 500 macropod, 56 brushtail possum and 52 common northern bandicoot samples were tested for antibodies to

C. burnetii. Seroprevalence was found to vary significantly between sites ($P<0.01$) and regions ($P<0.01$). Seroprevalence was highest in bandicoots (26.9%; 95% CI 26.6-27.7%), followed by macropods (25.6%; 25.6-25.7%) and possums (19.6%; 19.5-20.2%). Agreement between the ELISA methods used was poor and it is thought that this was due to a combination of immunoglobulin isotype subclass and antigen epitope specificity. The heterogeneity of serological responses in native marsupials made the ultimate validation of phage-displayed CRAbs in ELISA difficult, as they could not be directly compared to another test in field surveys.

A qPCR was successfully developed for the detection of the *Coxiella*-specific *com1* gene in tick extracts and whole blood collected from Australian native marsupials. A total of 323 ticks were collected from 34 bandicoots, 14 macropods and one human. Whole blood was collected from 35 bandicoots, 31 macropods and two possums. The detection of the *com1* gene indicated the presence of *C. burnetii* in both the ticks (15.5% pools) and whole blood (24.2%) of bandicoots and a variety of macropods in northern Queensland. *Coxiella burnetii* was also detected in the whole blood of one of the two possums tested. The additional detection of a PCR product with regions of DNA homologous with the *com1* gene in the ticks and whole blood of these species indicated the presence of an, as yet, unidentified tick-borne agent.

This project demonstrated the prevalence of antibodies to *C. burnetii* in the serum of a variety of animals, including livestock, domestic animals, feral animals and native Australian marsupials. In addition, PCR assays detected the presence of *C. burnetii* in both ticks and whole blood of native Australian marsupials. The serological and molecular assays performed in this study demonstrated the potential for a wide variety of animals to act as reservoirs of Q fever in Queensland, Australia.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)
ANOVA	Analysis of variance
bp	Base pair
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary deoxynucleic acid
com1	*Coxiella* outer membrane protein 1
CRAb	Chicken recombinant antibody
DAB	Diamino-benzidine tetra-hydrochloride
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotidetriphosphate
EDTA	Ethyline-diamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
g	Gravity
HRP	Horseradish peroxidase
IgG	Immunoglobulin Isotype G
kb	Kilobase
LPS	Lipopolysaccharide
M	Molar
Mb	Mega base
mg	Milligram
mL	Millilitre
mM	Millimolar
mRNA	Messenger ribonucleic acid
ng	Nanogram
nm	Nanometre
nM	Nanomolar
NMI	Nine Mile I *Coxiella burnetii* isolate
NMII	Nine Mile II *Coxiella burnetii* isolate
NMII/C4	Nine Mile II clone four *Coxiella burnetii* isolate

ORF	Open reading frame
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PVDF	Polyvinylidene difluoride
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RPM	Revolutions per minute
RNA	Ribonucleic acid
scFv	Single chain variable fragment
V_H	Heavy chain immunoglobulin gene
V_L	Light chain immunoglobulin gene
WCA	Whole cell antigen
µm	Micrometre
µL	Microlitre

CHAPTER ONE

INTRODUCTION

Coxiella burnetii is the causative agent of the zoonotic disease, Q fever. The disease was first recognised in Australia in 1937 (Derrick, 1937). It has since been recognised worldwide, with the exception of New Zealand (Hilbink *et al.*, 1993). Acute Q fever is characterised by a febrile disease lasting for two to three weeks with fatigue, chills and headaches. Presentation can vary from a relatively mild influenza-like illness to hepatitis or pneumonia, making accurate and timely diagnosis difficult (Maurin and Raoult, 1999). While Q fever is not a notifiable disease in many countries, these factors have resulted in difficulty assessing the prevalence and impact of Q fever.

Coxiella burnetii is an obligate intracellular pathogen with worldwide distribution and wide host range (Babudieri, 1959). In the natural lifecycle of the organism, it is transmitted between wild animals and their ticks. The bacterium can also be transmitted to livestock and other domestic animals via ticks. Once in livestock, *C. burnetii* primarily infects the female reproductive tract, resulting in the shedding of a spore-like form of the organism in parturient fluids and milk (Lang, 1990). The bacterium can also be shed in urine and faeces. Infection of animals with *C. burnetii* is referred to as coxiellosis, which is often asymptomatic, but can result in reproductive failure (Lang, 1990). The spore-like form of *C. burnetii* is highly resistant to extremes of temperature, pressure, UV radiation and desiccation (Maurin and Raoult, 1999). In this form it can be transmitted between livestock via inhalation of aerosols without, the aid of ticks. Inhalation of contaminated aerosols is the primary means by which Q fever is contracted. As a result, individuals of greatest risk of infection include veterinarians, farmers, abattoir workers, dairy workers and laboratory personnel working with *C. burnetii* and infected animals. Infected fomites can also be spread on the wind, potentially exposing humans and animals that have no direct contact with infected animals (Hawker *et al.*, 1998).

Australian surveys have shown an increased prevalence of human disease in recent years (Garner *et al*, 1997). Although this has been attributed to several factors there is little current data on potential reservoirs of human infection. The most well known reservoirs for human infection with *C. burnetii* are livestock such as cattle, sheep and goats. Domestic animals such as dogs and cats have also been attributed to Q fever outbreaks. Epidemiological studies conducted in Europe and North America have demonstrated increased prevalence in livestock populations (Lang, 1990). Q fever has been described as a re-emerging pathogen of increasing importance as a public health issue (Arricau-Bouvery and Rodolakis, 2005). In order to produce data on the epidemiology of Q fever and determine the risk of infection a variety of methods have been used in the attempt to detect and monitor Q fever.

The experimental work outlined in this thesis aimed to determine the potential for livestock, companion animals, feral animals and native wildlife to act as reservoirs of Q fever in Queensland, Australia. Standard indirect ELISAs were developed for the detection of antibodies to *C. burnetii* in non-native animals. However, the development of diagnostic tests for native wildlife was more problematic due to a lack of diagnostic reagents for these animals.

Wildlife has been involved in the epidemiology of many zoonoses and functions as a major reservoir for the transmission of the aetiological agents to domestic animals and humans (Bengis *et al.*, 2004; Kruse *et al.*, 2004). It has been suggested that human encroachment into wildlife habitat has resulted in increased transmission of pathogens between wildlife, domestic animals and humans (Cleaveland *et al.*, 2001). Also, international livestock movement and modern agricultural practices have resulted in an emergence of zoonoses in areas previously unaffected (Daszak *et al.*, 2000). This increased transmission of pathogens is thought to be responsible for the emergence of diseases of importance to both human and animal health. In order to determine and manage the risks associated with zoonoses and emerging infectious diseases, effective surveillance for the relevant pathogens is essential.

Emerging infectious diseases are classified as newly recognised diseases, diseases with increasing incidence (also referred to as re-emerging infectious diseases), diseases with increasing virulence and diseases that were previously unknown in a particular location (Jones *et al.*, 2008). It is estimated that 75% of emerging infectious diseases are zoonotic in origin (Woolhouse and Gowtage-Sequeria, 2005). The emergence of new infectious diseases is associated with various factors, including climate change, changing human demographics and behaviour, technology and industry, economic development and changes in land usage, international travel and trade, microbial adaptations and failure of public health initiatives (Daszak *et al.*, 2000; Polley and Thompson, 2009).

Regular surveillance for evidence of potential zoonotic infections is a primary requirement for maintaining the safety of the Australian population. In order to predict the risk of infection within a population, and thus prepare timely strategies relating to public education, vaccination or prophylactic treatment options, it is imperative to determine the source(s) of possible infection (Chomel *et al.*, 2007). However, the current capacity to detect, identify and predict potential zoonotic outbreaks in Australia, and in particular the tropical north, is compromised due to the lack of data regarding host populations in this area and the unavailability of standardised rapid diagnostic reagents to produce such data.

Screening techniques, such as ELISA rely on the availability of anti-species antibodies conjugated to a fluorochrome for detection. These antibodies are readily available only for animal species commonly used in laboratory research. To effectively study infection or exposure in other species, it is currently necessary to create individual custom antibodies, a time consuming, laborious and sometimes expensive task. Competitive ELISA has been used as a generic detection system, but requires blocking antibodies specific for the pathogen being investigated (Soliman *et al*, 1992). Phage display is an alternate method to traditional monoclonal and polyclonal antibody production. The technique uses the chicken for immunisation and selection of antibodies (Sapats *et al*, 2006). As mammalian proteins are absent in chickens, it allows for the production of an

expanded immune response, not limited by immunological tolerance. Phage display of antibody fragments can be accomplished using essentially any species.

The experimental work outlined in this thesis aimed to develop a phage display library of chicken recombinant antibodies (CRAbs) for the IgG of a variety of Australian native mammals. To determine the effectiveness of CRAbs as secondary antibodies for epidemiological studies and pathogen surveillance in wildlife populations, these antibodies were compared to competitive ELISA and standard indirect ELISA in a seroprevalence survey for the endemic zoonosis, Q fever (*Coxiella burnetii*). In this study, these recombinant phage-displayed, anti-species antibodies (CRAbs) were used in an ELISA format to determine the prevalence of *C. burnetii* exposure in these animals in northern Queensland. With the data obtained from this work, investigations to determine the relationship between seroprevalence in wildlife and livestock and human infection were conducted. Investigations were also conducted to determine the prevalence of *C. burnetii* in ticks found on native wildlife and their role in transmission.

CHAPTER TWO

LITERATURE REVIEW

2.1 Brief History

In 1934, sporadic cases of fever with a typhoid-like initial presentation came to the attention of Australian medical practitioners after all the patients were found to have worked in Brisbane abattoirs (Derrick, 1972). Originally, the disease was named 'abattoir fever'. However, this term was deemed unsuitable after confirmed cases occurred outside of abattoirs on cattle properties and dairy farms. The term Q fever (for query fever) was proposed by Edward Holbrook Derrick of the Laboratory of Microbiology and Pathology of the Queensland Health Department (Derrick, 1937). After initial attempts to isolate the aetiological agent failed, it was speculated that the causative agent was viral. However, in subsequent investigations, intracellular vacuoles filled with *Rickettsia*-like organisms were observed in infectious material provided by Derrick, leading to confirmation of a bacterial agent (Burnet and Freeman, 1937).

Simultaneously, research into Rocky Mountain spotted fever at the Rocky Mountain Laboratory, Montana resulted in the discovery of a febrile disease transmitted by ticks that was distinct from Rocky Mountain spotted fever (Davis and Cox, 1938). The organism was dubbed the Nile Mile agent after the location from which the infected ticks were collected. Herald Cox characterised the Nine Mile agent as a rickettsial organism and was the first to propagate it in embryonated eggs (Cox, 1939). The connection between the Q fever and Nine Mile agents was not made until a laboratory acquired infection occurred at the Rocky Mountain Laboratory in 1938. The then Director of the National Institutes of Health, Rolla Dyer became infected and the subsequent febrile illness was able to be reproduced in guinea pigs using Dyer's blood (Dyer, 1938). Spleens infected with the Australian Q fever agent were made available to

Dyer after it was suspected the two organisms were related. Subsequent experiments with the Q fever agent demonstrated cross-immunity when it was found to provide protection from challenge with the Nine Mile agent (Dyer, 1938). Originally named *Rickettsia burnetii* in Australia and *Rickettsia diaporica* in the U.S., the Q fever agent was renamed *Coxiella burnetii* in honour of both Cox and Burnet in 1948 (Phillip, 1948).

2.2 Bacteriology

Coxiella burnetii is a small Gram-negative bacterium with dimensions ranging from 0.2 to 0.4 µm wide and 0.4 to 1µm long (Maurin and Raoult, 1999). It is an obligate intracellular pathogen with replication occurring in the phagolysosome. The *C. burnetii* bacterium is a highly pleomorphic coccobacillus, the morphology of which is dependent on the developmental phase of the life cycle (Heinzen and Samuel, 2001). There are two major forms; the large cell variant (LCV) and the small cell variant (SCV). The LCV is the metabolically active form that multiplies within the host cell (Figure 2.1), while the SCV is a highly resistant spore-like form that can remain dormant in the environment for extended periods (McCaul, 1991). This ability to retain viability outside of a host is not seen among other rickettsial organisms and is unique to *C. burnetii* (Derrick, 1972).

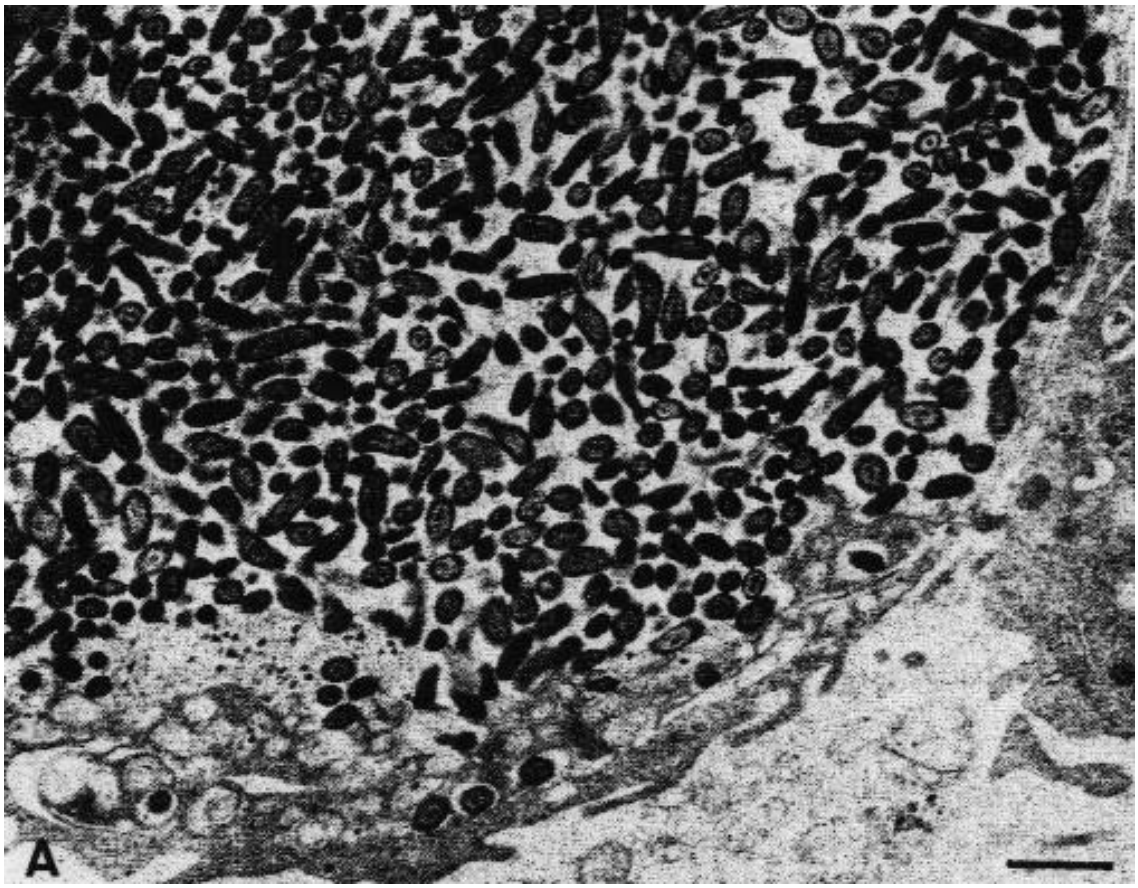

Figure 2.1: Electron micrograph showing *Coxiella burnetii* proliferating in an intracellular vacuole.
Image (in J774 macrophage) demonstrates pleomorphism of both cell width and type. Bar indicates 1,000nm. From McCaul, 1991.

Although originally classified as a Rickettsia, phylogenic analysis supported the creation of a new genus after 16s rRNA sequence analysis indicated *C. burnetii* belonged to the gamma subdivision of the *Proteobacteria*. This subdivision is distinct from the *Rickettsia* genus, which belongs to the alpha-1 subdivision (Stein *et al.*, 1993). In addition, *C. burnetii* was found to be phenotypically distinct from *Rickettsia sp* in that it was stable in the environment, could be transmitted by aerosol and required an acidic pH for metabolism (Baca and Paretsky, 1983). According to 16s rRNA analysis, the closest relative of *C. burnetii* is the Family *Legionellaceae*. Both *C. burnetii* and *L. pneumophila* (Fields *et al.*, 2002) are intracellular bacteria that reproduce within membrane-bound vacuoles. There is significant homology in genes associated with intracellular survival, such as *mip* (macrophage infectivity potentiator), the product of

which is associated with survival within macrophages (Mo *et al.*, 1995). *Coxiella burnetii* and *L. pneumophila* were also found to possess similar type IV secretory systems, which are involved in transport of macromolecules and the exchange of genetic material between bacterial cells (Zamboni *et al.*, 2003). However, *C. burnetii* is distinct from *L. pneumophila* in that the latter is a facultative intracellular bacterium and can multiply extracellularly (Fields *et al.*, 2002). The clinical presentation following infection is also different.

2.2.1 Phase variation and pathogenesis

It is currently believed that following inhalation of *C. burnetii*-contaminated aerosols, alveolar macrophages are the first cells to be infected in acute Q fever (Maurin and Raoult, 1999). Myeloid dendritic cells have been found to constitute a protective niche for *C. burnetii* (Shannon et al., 2005b). The mechanism of entry into phagocytic cells has been found to be different depending on the phase variation of *C. burnetii*. The organism is infectious when in phase I, whereas it is non-infectious in phase II (Amano and Williams, 1984). Phase variation is characterised by transition of lipopolysaccharide (LPS) from a smooth form with full-length O-side chains in phase I to a rough form with truncated O-side chains in phase II. The changes in LPS following phase variation are thought to be irreversible due to chromosomal DNA deletions (Hoover *et al.*, 2002). However, phase I organisms have been recovered following back-passage in animals of *C. burnetii* considered to be in phase II following passage in cell culture (Stoker and Fiset, 1956; Kazar *et al.*, 1975). Inability to revert back to phase I from phase II has only been demonstrated in isolates that have been plaque purified to a homogenous phase II culture (Ormsbee and Marmion, 1990).

Several Phase II clones of the Nine Mile isolate have been observed to have large deletions of 26 to 31.5 kb in the LPS operon (Denison *et al.*, 2006). In contrast, other isolates, such as the Henzerling RSA 331, M44 and Australian QD do not demonstrate these deletions in phase II and have been found to express genes related to O-antigen

synthesis, albeit at lower level than phase I. Phase variation is thought to be post-translationally regulated in these isolates (Denison *et al.*, 2006). Phase II cells are not thought to occur naturally, but can be produced following repeated passage of virulent phase I *C. burnetii* in hosts lacking a functional immune response, such as embryonated eggs and cell culture (Heinzen and Samuel, 2001). Conversely, experiments performed by (Kordova *et al.*, 1970a; Kordova *et al.*, 1970b) indicated *C. burnetii* of both phases was present in organisms maintained exclusively *in vivo* in a guinea pig model. These findings suggest *C. burnetii* may be present as a mixed population and phase variation may be due to selection pressure under the different conditions.

There has been no difference detected in growth kinetics between the phase variants of *C. burnetii*. However, the truncation of O-antigen in the LPS following conversion to phase II enables the two virulence phases to be distinguished by their antigenic properties (Amano and Williams, 1984). The immune response is mounted against phase II antigens first, followed by phase I antigens. Only phase II-specific antibodies are produced following vaccination with phase II cells, whereas, both phase II and phase I-specific antibodies are produced following vaccination or infection with phase I cells (Stoker and Fiset, 1956). Both phase I and phase II epitopes have been found to be present in phase I cells (Williams *et al.*, 1984). This was demonstrated by phase II epitopes being revealed in phase I cells following chloroform-methanol extraction. It is thought that while phase II epitopes are present in phase I cells, the phase I LPS sterically hinders antibody binding to phase II epitopes due to its extended carbohydrate structure (Hackstadt, 1990).

Both phase I and phase II *C. burnetii* enter eukaryotic host cells via the leucocyte response integrin (LRI or $\alpha_v\beta_3$)/ integrin associated protein complex (IAP) (Mege *et al.*, 1997) (Figure 2.2). Phase II organisms differ in that they also engage complement receptor 3 (CR3) (Capo *et al.*, 1999). Phase I *C. burnetii* internalisation involves activation of toll-like receptor four (TLR-4), whereas phase II does not (Honstettre *et al.*, 2004; Raoult *et al.*, 2005). It is currently believed that the cytoskeletal rearrangements

caused by adherence of phase I *C. burnetii* to monocytes may result in reduced internalisation by restricting binding to CR3 (Meconi *et al*., 1998). This process, while reducing internalisation of phase I organisms, prevents the rapid destruction via the phagolysosomal pathway that is seen with phase II organisms which are readily internalised (Mege *et al.*, 1997). Internalisation was found to be microfilament-dependent and directed by the *C. burnetii* organism (Meconi *et al.*, 1998). However, the ligands responsible could not be identified and internalisation was passive with no difference between the internalisation rates of viable and inactivated organisms (Baca *et al.*, 1993).

In myeloid dendritic cells, infection with virulent phase I *C. burnetii* does not result in maturation (Shannon *et al.*, 2005b). Nonetheless, dendritic cell maturation is not actively inhibited, as subsequent infection with avirulent phase II *C. burnetii* or treatment with *Escherichia coli* LPS results in maturation. Infection of dendritic cells with avirulent phase II *C. burnetii* results in toll-like receptor 4-dependent maturation and increased IL-12 and TNF production (Shannon *et al.*, 2005b). This phenomenon was further demonstrated with three different phase I *C. burnetii* isolates (Shannon *et al.*, 2005a). It is thought that the LPS present in phase I *C. burnetii* masks toll-like receptor ligands from recognition by dendritic cells, enabling the organism to replicate intracellularly without inducing dendritic cell maturation and cytokine production. This effect is not LPS chemotype specific, as infection of dendritic cells with isolates synthesising different LPS chemotypes were equally deficient in stimulating maturation and cytokine production (Shannon *et al.*, 2005a).

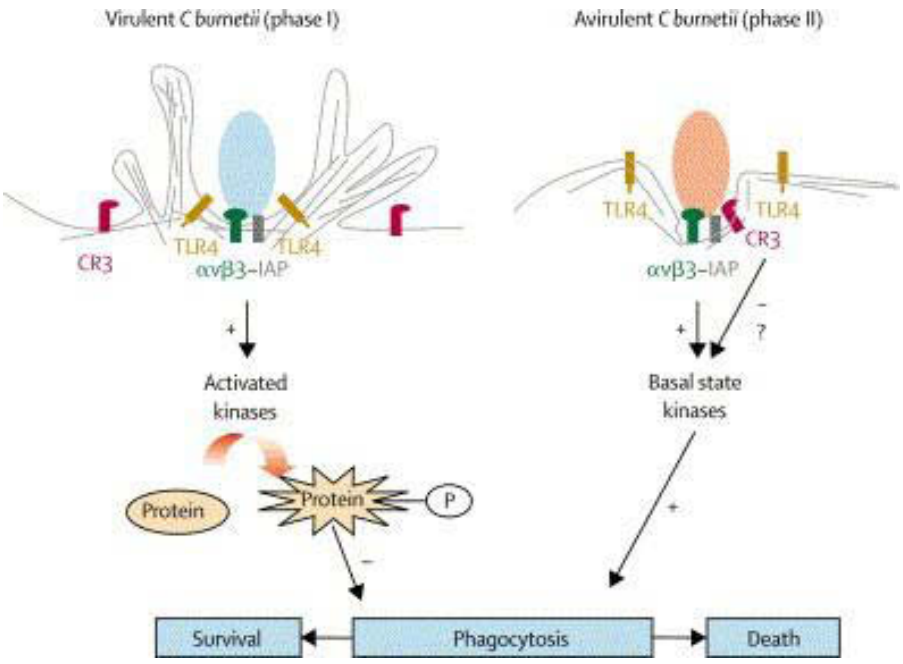

Figure 2.2: Differential internalisation of phase I and phase II *Coxiella burnetii*.
Phase I internalisation involves TLR-4 activation, whereas phase II does not. TLR-4 recognises LPS and usually results in activation of the innate immune response. It is not normally associated with phagocytosis. From Raoult *et al*, 2005.

The demonstration of the possibility of natural infection with a mixed phase populations of *C. burnetii* (Kordova *et al.*, 1970a; Kordova *et al.*, 1970b) in conjunction with the demonstration of the rapid internalisation and destruction of phase II cells by macrophages (Raoult *et al.*, 2005), may provide an alternative explanation for the seemingly contradictory antibody response to *C. burnetii* infection. If natural infection occurs with a mixed population, the rapid destruction of phase II cells may explain the presentation of associated antigens before those of phase I cells. This possibility has not yet been considered and genetic determination via either chromosomal deletions or post translational modifications remains the accepted explanation of the mechanisms of phase variation.

2.2.2 Intracellular lifecycle

Once internalised, the vacuole containing *C. burnetii* of either phase proceeds through the endocytic pathway to form a late phagosome (Maurin *et al.*, 1992). The bacterium is acidophilic and requires the low pH of the phagolysosome for uptake of nutrients such as glutamate (Hackstadt and Williams, 1983), proline (Hendrix and Mallavia, 1984) and the synthesis of nucleic and amino acids. The pH in the phagosome is approximately 4.8, which activates the metabolic processes of the *C. burnetii* bacterium (Howe and Mallavia, 2000). The phagosome fuses with lysosomes, and rapid multiplication of *C. burnetii* occurs (Figure 2.3). The replication of *C. burnetii* results in the production of relatively large intracellular vacuoles (Heinzen *et al.*, 1996). A recent study found that the generation and maturation of these vacuoles involves the induction of the autophagic pathway (Gutierrez *et al.*, 2005). In addition, it was found that the *C. burnetii* organism subverted the autophagic pathway in order to provide the necessary nutrients for the conversion of SCVs to LCVs.

Maturation of the lysosomes to phagolysosomes is delayed, which is thought to allow for the conversion of the environmentally stable small cell variant (SCV) of *C. burnetii* to the metabolically active large cell variant (LCV) (Howe and Mallavia, 2000). This process is thought to be modulated by *C. burnetii* through interaction with the autophagic pathway, resulting in delayed fusion with the lysosome (Romano *et al.*, 2007). Both SCVs and LCVs are thought to divide by binary fission. However in LCVs, a spore-like form is produced at the pole (McCaul, 1991). The spore-like form is thought to convert to an SCV, which is subsequently released by the host cell. It is not known exactly how the process occurs, or the factors which induce SCV formation. Yet, it is thought that lysis of the host cell results in the release of SCVs into the extracellular environment.

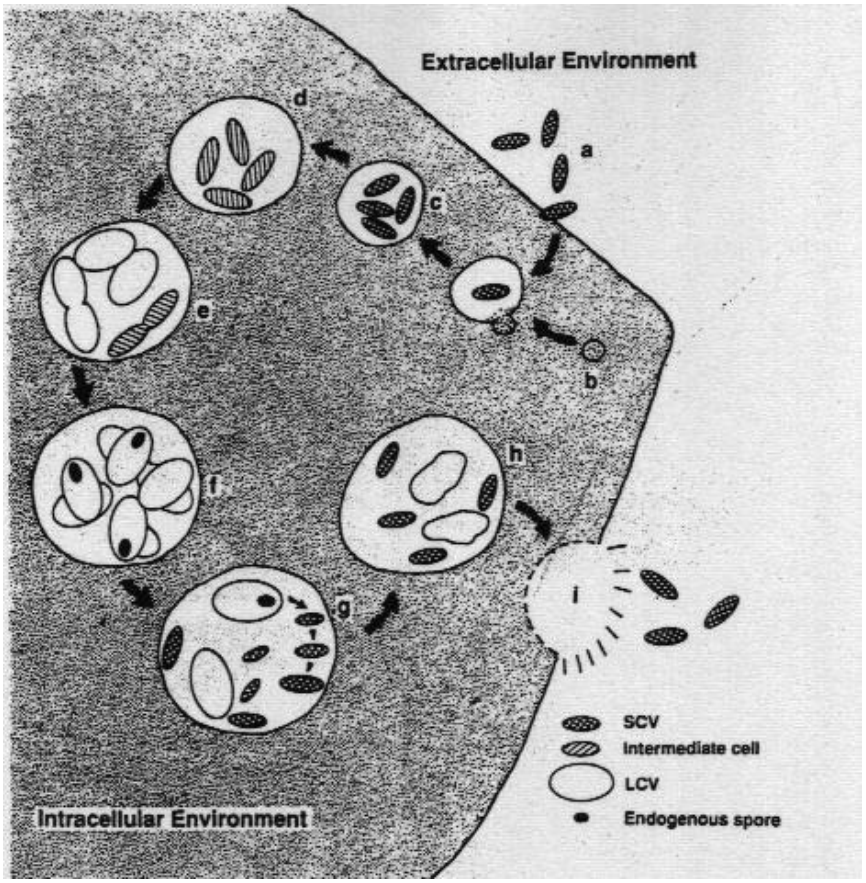

Figure 2.3: Diagram of the life cycle of *Coxiella burnetii*.
(a) SCV attaches and is ingested; (b) fusion of phagosome containing SCV with early lysosome; (c) activation of metabolism; (d) intermediate cell with SCV morphology but loss of resistant characteristics; (e) LCV development with typical binary fission; (f) sporogenesis signalled (exact mechanism unknown); (g) spore develops resistant properties of SCV; (h) SCV released into vacuolar space following disintegration of mother cell; (i) lysis of host cell, releasing *C. burnetii* and spores into extracellular environment. From McCaul, 1991.

2.2.3 Axenic growth

Recent studies reported sustained axenic metabolism (Omsland *et al.*, 2008) and replication (Omsland *et al.*, 2009) was possible in a cell-free medium. Expression microarrays, genomic reconstruction and metabolite typing were used to determine the conditions required axenic growth of *C. burnetii*. From these experiments *C. burnetii* was determined to be microaerophilic with a preferred oxygen content of 2.5%. A complex medium termed acidified citrate cysteine medium (ACCM) with a pH of 4.5

was developed to support protein synthesis. A further ACCM agar was also developed, enabling the culture of single colonies of *C. burnetii*. This culture method has since been used to grow *C. burnetii* axenically in two investigations of *C. burnetii* pathogenesis (Chen *et al.*, 2010; Hill and Samuel, 2011). It is thought that the development of axenic culture media will; enable improved investigation of factors required for intracellular growth and pathogenesis of *C. burnetii,* enable improved diagnostic techniques for the isolation of *C. bur*netii from clinical samples, facilitate clonal isolation and genetic tool development, aid in vaccine development and enable host-cell free production of *C. burnetii* for diagnostic and research purposes (Omsland *et al.*, 2009).

2.2.4 Virulence factors

Coxiella burnetii has been found to possess several virulence factors that enhance the organism's ability to evade the immune response and survive within host cells. The major virulence determinants reported to date include acid activation, LPS and the immunomodulatory complex (Williams *et al.*, 1991). One important adaptation of *C. burnetii* that enables it to survive within host cells is the ability to multiply within the phagolysosome. The environment within the phagolysosome, while rich in nutrients, is highly acidic and normally microbicidal. The ability to survive and multiply within this environment is thought to enhance *C. burnetii*'s ability to evade the host's immune system. Survival within the phagolysosome requires mechanisms to control the pH homeostasis across the cell membrane. Proton concentration is essential in energy production for all organisms for the formation of an energetically favourable proton gradient. Normally such a low pH would be microbicidal; however, *C. burnetii* was found to control cell membrane permeability to protons via an as yet undefined mechanism (Thompson, 1991).

2.2.4.1 Lipopolysaccharide

The lipopolysaccharide present in phase I *C. burnetii* cells has an important role in protecting the organism from microbicidal effects of the phagolysosome (Williams *et al.*, 1991). As a result, phase I cells are more virulent than phase II cells, which have truncated LPS molecules. Phase II cells can still survive and multiply within the phagolysosome. However, they are more easily destroyed by the host cell (Raoult *et al.*, 2005). Successive passage in immuno-incompetent cells results in a continual decrease in virulence relative to the original phase I cells (Kazar *et al.*, 1974).

Several *C. burnetii* isolates deviate from this characteristic of diminishing virulence. Examples include the M44 isolate which can be reactivated by immunocompromisation, Nine Mile clone 4 isolate which can survive in guinea pigs and produce an immune response, and the intermediate LPS 'crazy' Nine Mile 514 isolate which has intermediate virulence (Williams *et al.*, 1991). This deviation suggests factors other than LPS are acting as virulence determinants.

2.2.4.2 Immunomodulatory complex

Coxiella burnetii infection results in adverse tissue reactions, which can also be observed following injection of inactivated bacteria in the form of a vaccine (Damrow *et al.*, 1985). Infection also results in hypo-responsiveness of lymphocytes to mitogens and recall antigens. The adverse reactions and reduced lymphocyte activity are thought to be due to interaction of the organism's microbial immunomodulatory complex (IMC) with the host immune response (Waag and Williams, 1988). Originally, it was hypothesised that LPS played a role in the activity of the IMC and was responsible for adverse tissue reactions due to the correlation between phase variation and the ability to cause immunosuppression (Baca and Paretsky, 1983). However, subsequent experiments with the injection of different isolates of *C. burnetii* with variable LPS characteristics did not result in immunosupression (Waag and Williams, 1988). In addition, injection of phase I organism subfractions containing LPS were found to enhance the immune

response to *C. burnetii* (Williams *et al.*, 1991). These findings indicated that LPS did not have a significant role in immunosupression by the IMC. Expression of the truncated LPS phenotype as seen in phase II cells correlated with a decrease in IMC activity (Waag and Williams, 1988). This suggests LPS and IMC component synthesis might be genetically linked and differences in gene expression may be responsible for both the reduction in immunosuppression and virulence of *C. burnetii*.

Immunomodulatory complexes of *C. burnetii* cell components, antigens, LPS and genomic DNA have been found to persist in human Q fever patients decades post-infection (Marmion *et al.*, 2009). These non-infectious and non-degradable complexes have been found to stimulate cytokine production in mice and THP-1 macrophages and stimulate inflammatory responses in hyperimmunised guinea pigs (Sukocheva *et al.*, 2010). It is thought that these complexes may be involved in post Q fever fatigue syndrome as significantly greater amounts of IMC were recovered from post Q fever fatigue syndrome patients (Sukocheva *et al.*, 2010).

2.2.4.3 Macrophage infectivity potentiator analogue

Another potential virulence factor of *C. burnetii* is a homologue of a macrophage infectivity potentiator (CbMip) (Cianciotto *et al.*, 1995; Mo *et al.*, 1995). In infections with *L. pneumophila*, Mip is associated with the organism's ability to infect and survive within macrophages (Cianciotto and Fields, 1992). In *C. burnetii*, the function of Mip is undefined. Furthermore, it is localised to the cytoplasm, periplasmic space and outer surface and is thought to have a similar function to that of Mip in *L. pneumophila* (Mo *et al.*, 1995; Mo *et al.*, 1998).

2.2.3 Genetics

2.2.3.1 The genome of *Coxiella burnetii*

The genome size of *C. burnetii* varies between different isolates, ranging from 1.5 to 2.4 Mb (Willems *et al.*, 1998). The Nine Mile isolate has a genome size of 2.1 Mb. It was originally debated as to whether *C. burnetii* had a linear or circular genome. Some experiments suggested the genome may be linear due to the inability of its putative origin of replication (*oriC*) locus to initiate DNA synthesis (Suhan *et al.*, 1994). This hypothesis was supported by an inability to clone the ends of two fragments obtained by digestion of phase I Nine Mile strain DNA with *Not*I restriction enzyme into a cloning vector (Willems *et al.*, 1998). Subsequently, the sequencing of the *C. burnetii* genome and further analysis confirmed the organism had a circular genome (Seshadri *et al.*, 2003).

Restriction fragment length polymorphism (RFLP) analyses resulted in the division of *C. burnetii* isolates into six genomic groups (I to VI) (Hendrix *et al.*, 1991). The heterogeneity between isolates leading to this subdivision was thought to be due to repeat regions in the genome of some isolates. An extensive microarray based comparison of the whole genomes of 24 *C. burnetii* isolates confirmed the RFLP based groupings and identified a further two distinct genomic groups (Beare *et al.*, 2006). Heterogeneity between isolates was found to be due mainly to deletions of open reading frames rather than the presence of repeat regions.

Investigations into the metabolism and intracellular survival of *C. burnetii* have led to the sequencing and expression of related genes in the organism. To date, twelve *C. burnetii* chromosomal genes have been functionally expressed in *E. coli* (Table 2.1).

Table 2.1: Functionally Expressed *Coxiella burnetii* genes

Gene	*Function*	*Reference*
gltA	*citrate synthase*	*(Heinzen and Mallavia, 1987)*
sodB	*superoxide dismutase*	*(Heinzen et al., 1992)*
htpA	*14-kDa heat shock protein*	*(Vodkin and Williams, 1988)*
htpB	*62-kDa heat shock protein*	*(Vodkin and Williams, 1988)*
com1	*27-kDa surface antigen*	*(Hendrix et al., 1990)*
pyrB	*aspartate carbamoyl transferase*	*(Hoover and Williams, 1990)*
qrsA	*sensor protein*	*(Mo and Mallavia, 1994)*
dnaJ	*heat shock protein*	*(Zuber et al., 1995b)*
mucZ	*capsule induction protein*	*(Zuber et al., 1995a)*
serS	*seryl-tRNA synthase*	*(Maurin and Raoult, 1999)*
algC	*phosphomanno-mutase*	*(Maurin and Raoult, 1999)*
sdh	*succinate dehydrogenase*	*(Heinzen et al., 1995)*

2.2.3.2 Plasmids

There are four main plasmids harboured by *C. burnetii* designated QpH1, QpRS, QpDV and QpDG (Mallavia, 1991). Another, as yet undesignated plasmid has been found in a Chinese *C. burnetii* isolate (Jager *et al.*, 2002). The plasmids range in size from 36 to 42 kb, 30 kb of which is conserved between plasmid types (Mallavia, 1991). These plasmids are associated with particular genomic groups. In contrast, some isolates do not contain a plasmid and others contain multiple copies or have plasmid DNA integrated into the chromosome. Recent research has suggested that the plasmids QpH1 and QpDG may be identical (Jager *et al.*, 2002). Originally, the plasmid type carried by *C. burnetii* isolates was thought to correlate with the clinical presentations of Q fever (Samuel *et al.*, 1985). It was suggested that particular plasmids may encode virulence factors that correlated with acute or chronic presentation of Q fever. However, this study only focused on a small group of 20 isolates and subsequent studies involving a geographically wider spread of isolates did not support this hypothesis (Stein and Raoult, 1993). A later study confirmed the conclusion of Stein and Raoult (1993) and found no correlation between plasmid type and disease presentation (Thiele and Willems, 1994). The significance of the plasmids remains undefined.

2.3 Clinical Presentations of Q fever

Q fever (Derrick, 1937) is a zoonotic disease caused by the obligate-intracellular bacterium, *Coxiella burnetii*. In animals the disease is generally asymptomatic and referred to as coxiellosis (Lang, 1990). However, the disease has been reported to be responsible for abortion and low reproduction rates in some domestic animals (Waldhalm *et al.*, 1978; Sanford *et al.*, 1994; Stein *et al.*, 2000). As reviewed by Angelakis and Raoult (2010), Q fever presents as two forms, either acute or chronic in humans. Due to the varying manifestations of the disease in humans it is thought to be under-diagnosed and under-reported (Parker *et al.*, 2006).

2.3.1 Acute Q fever

Acute Q fever has an incubation period of two to three weeks with an abrupt onset of symptoms including fever, fatigue, chills myalgia and headaches (Maurin and Raoult, 1999). The febrile period usually lasts for less than two weeks with fever increasing to a plateau of 39-40°C within 2 to 4 days with a rapid return to normal after 5 to 14 days (Angelakis and Raoult, 2010). In untreated patients fever may last from 5 to 57 days (Derrick, 1973). Due to the similarities in clinical presentation, mild cases of Q fever may be mistaken for influenza, resulting in under-reporting of infection. Moreover, the majority of Q fever cases are believed to be asymptomatic (Maurin and Raoult, 1999). The major clinical presentations include atypical pneumonia, hepatitis and arthralgias. Rarer presentations include myocarditis, pericarditis, meningoencephalitis and rashes (Maurin and Raoult, 1999). Clinical manifestations of acute Q fever, as reviewed by Angelakis and Raoult (2010) are summarised in Table 2.2.

2.3.2 Chronic Q fever

Chronic Q fever is a rare complication of Q fever, with approximately 0.2% of acute Q fever cases progressing to chronic form (Maurin and Raoult, 1999). The major clinical presentation of chronic Q fever is endocarditis, accounting for 60 to 70% of cases. It is usually associated with individuals with existing heart conditions and is fatal without treatment (Maurin and Raoult, 1999). Other presentations include vascular, osteoarticular and pulmonary infections and chronic hepatitis (Brouqui *et al.*, 1993). Persistent infection with *C. burnetii* is common in animals, with recrudescence following stress, pregnancy or immuno-suppression (Maurin and Raoult, 1999). This has also been observed in human cases (Harris *et al.*, 2000). Another complication of Q fever is chronic fatigue syndrome, which is not due to chronic infection but rather a complication seen in convalescing patients (Ayres *et al.*, 1998). The mechanisms of chronic fatigue syndrome are, as yet unknown. However, it has been proposed that persistent non-infectious, non-biodegradable complexes of *C. burnetii* cellular components may have a role in post Q fever fatigue syndrome (Marmion *et al.*, 2009; Sukocheva *et al.*, 2010). Greater amounts of these complexes have been found to be present in samples from post Q fever fatigue patients and have been demonstrated to have immunomodulatory effects on macrophages and cytokine production (Sukocheva *et al.*, 2010). Clinical manifestations of chronic Q fever, as reviewed by Angelakis and Raoult (2010) are summarised in Table 2.2.

Table 2.2 Clinical manifestations of Q fever

FORM	MANIFESTATIONS	SYMPTOMS
Acute	Febrile illness	Severe headaches
		Myalgia
		Arthralgia
		Cough
	Pneumonia	Non-productive cough
		Acute respiratory distress
		Pleural effusion
	Hepatitis	Hepatomegaly
		Granulomas
		Jaundice
	Cardiac involvement	Myocarditis
		Pericarditis
	Neurologic signs	Meningoencephalitis
		Encephalitis
		Lymphocytic meningitis
		Peripheral neuropathy
Chronic	Endocarditis	Aortic and mitral valve involvement
		Prosthetic valve involvement
		Heart failure
	Vascular infection	Aortic aneurism
	Osteoarticular infections	Osteomyelitis
		Osteoarthritis
	Chronic fatigue syndrome	Fatigue
		Myalgia
		Arthralgia
		Night sweats
	Chronic hepatitis	

2.3.3 Q fever in pregnancy

It has been demonstrated that Q fever during pregnancy is associated with high morbidity and mortality (Carcopino *et al.*, 2007). In pregnant women, *C. burnetii* infection results in localisation of the bacteria in the uterus and mammary glands (Angelakis and Raoult, 2010). Infection during the first trimester results in spontaneous abortion of the foetus, whereas infection during the second or third trimesters can result in premature delivery and low birth weight. Contracting Q fever during pregnancy also results in increased long-term risk of developing chronic Q fever.

2.4 Diagnosis of Q Fever

2.4.1 Culture

Coxiella burnetii can be detected in clinical samples following isolation employing shell vial centrifugation (Marrero and Raoult, 1989). Clinical specimens are inoculated onto HEL cell monolayers in shell vials and centrifuged to enhance *C. burnetii* attachment and penetration. Gimenez (Gimenez, 1965) or immunofluorescence staining (Brezina and Kovacova, 1966) is performed to visualise *C. burnetii* by microscopic examination in cell monolayers.

2.4.2 Molecular assays

2.4.2.1 Polymerase chain reaction (PCR)

Coxiella burnetii genes targeted for diagnostic PCR include the isocitrate dehydrogenase gene (*icd*) (Klee *et al.*, 2006), superoxide dismutase gene (*SodB*), 16s rDNA genes, *com1* and the *IS1111a* repetitive element (Hoover *et al.*, 1992). Conventional PCR

(Mullis and Faloona, 1987) has been used to detect *C. burnetii* DNA in both cell culture and clinical samples (Stein and Raoult, 1992). However, this technique has the disadvantage in that it is not quantitative. As the insertion sequence 1111a (*IS1111a*) is a transposable element, it is often present in multiple copies within the *C. burnetii* genome and is thought to be the most sensitive target for PCR (Fournier and Raoult, 2003). Consequently, the transposable nature of this gene makes it somewhat unsuitable for routine diagnostic PCR due to the variation in copy number and possibility of false negatives if insertion has not occurred. In addition, the variation in copy number between isolates makes this PCR target unsuitable for quantification. While the use of single copy genes may be slightly less sensitive, the possibility of false negatives would be reduced. PCR targets such as *sodB* and *icd* would be the most ideal targets, particularly for RT-PCR and quantitative PCR, due to their necessity for intracellular survival and metabolism and their highly conserved nature (Klee *et al.*, 2006).

While standard PCR does not distinguish between viable and non-viable organisms it has the advantage in being able to detect an organism early in an illness before antibody titres are able to be detected (Graves *et al.*, 2006). Conversely, a comparison between PCR and serology for the detection of *C. burnetii* in serum demonstrated a sensitivity of only 18% using a nested PCR targeting the *htpAB*-associated repetitive element (Fournier and Raoult, 2003). This finding indicates PCR may not be effective for the diagnosis of Q fever, particularly when performed on sera. This is not surprising as *C. burnetii* is an intracellular pathogen and is unlikely to be found in great quantities in serum. Better results may have been obtained if the PCR was performed on leukocytes separated from whole blood. Another study found a PCR targeting the *IS1111* gene of *C. burnetii* in serum had sensitivity of 67% and enabled diagnosis of Q fever in 4 days compared to 17 days with serological diagnosis (Turra *et al.*, 2006). However, this study was performed on a much smaller cohort of patients. A more recently developed PCR assay, the light-cycler nested PCR (LCN-PCR) targets a 20-copy *htpAB*-associated element as has been used in the diagnosis of both acute and chronic Q fever (Fenollar and Raoult, 2007).

While real-time PCR has been increasingly adopted as a first-line diagnostic test for acute Q fever in human patients, potential problems with the method have become apparent. Recently, the use of real-time PCR for the *IS1111a* element in clinical samples was complicated by the discovery of contamination of commercial PCR master mix with *C. burnetii* DNA (Tilburg *et al.*, 2010). It is thought that the presence of compounds of animal origin commonly used in PCR, such as bovine serum albumin (BSA) were responsible for the contamination. As cattle are known reservoirs of *C. burnetii* it is thought that BSA from infected animals may be responsible for the presence of *C. burnetii* DNA in unopened commercial PCR master mixes.

2.4.2.2 DNA probes

Coxiella burnetii is also detected in tissue samples, particularly paraffin embedded sections using labelled DNA probes. Most probes are designed to detect *C. burnetii* 16s rDNA (Frazier *et al.*, 1992). Probes can be either biotinylated or labelled with a fluorescent marker. While this technique is not routinely used in the diagnosis of acute Q fever due to the requirement for infected tissue samples; it has proved useful for detecting *C. burnetii* in heart valves following replacement to determine whether *C. burnetii* was present, particularly when no other pathology could be detected (Bruneval *et al.*, 2001).

2.4.3 Immunohistochemistry

Immunohistochemical methods of detection of *C. burnetii* are particularly useful in chronic Q fever cases (Fournier *et al.*, 1998). Often in Q fever endocarditis, vegetations are very small or absent and therefore undetectable by echocardiography (Maurin and Raoult, 1999). Detection is performed after tissue fixation and paraffin embedding (Fournier *et al.*, 1998). Many laboratories use formalin fixation, however Bouin's fixative has been found to provide better visualisation due to reduced cross-linkage of antigens (Baumgartner *et al.*, 1988). Detection techniques include immunoperoxidase

staining (figure 2.4), capture ELISA and immunosorbent fluorescent assays (ELIFA) and immunofluorescent monoclonal (Thiele *et al.*, 1992) or polyclonal tests (Muhlemann *et al.*, 1995). A new method termed autoimmunohistochemistry was recently proposed for the detection of *C. burnetii* in cardiac valves from suspected Q fever endocarditis patients (Lepidi *et al.*, 2006).

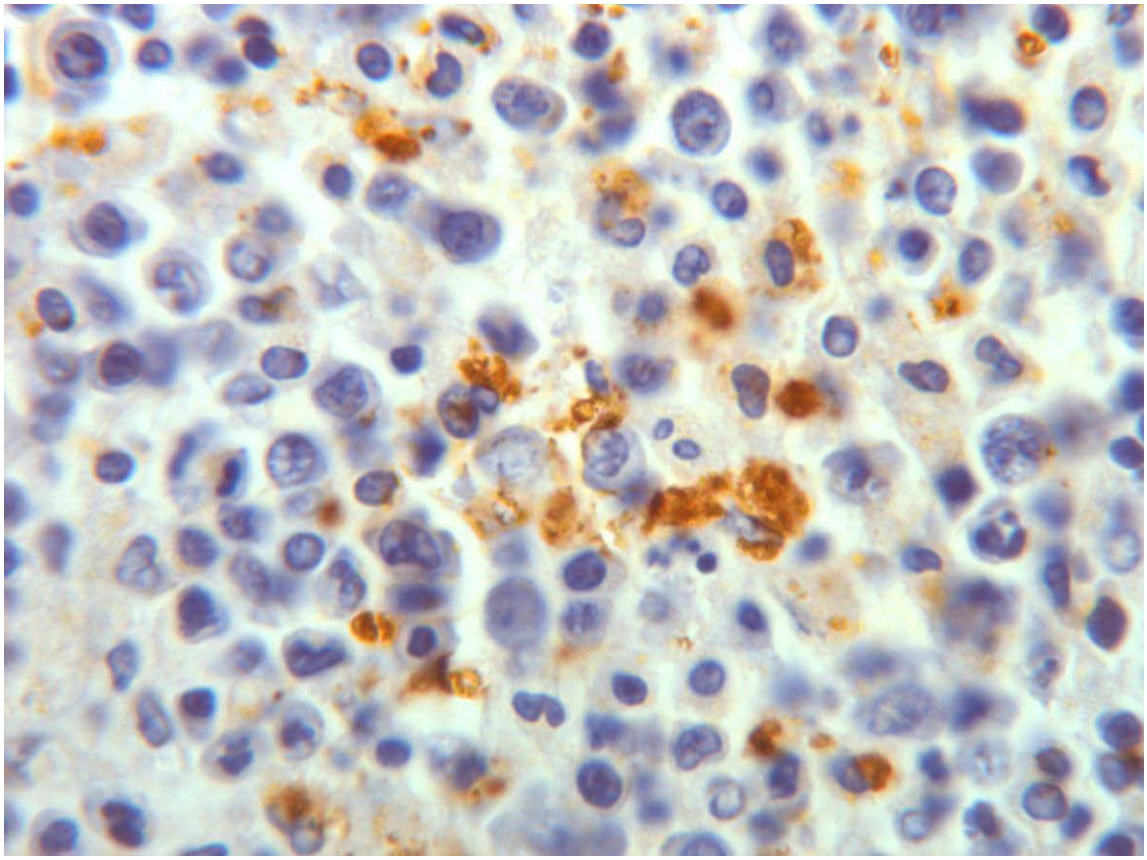

Figure 2.4 Immunoperoxidase stain of NBF-fixed paraffin embedded section of A/J strain mouse spleen showing *Coxiella burnetii*.
Stain was performed using DAB with human anti-*Coxiella* antibody at a dilution of 1:200 and HRP-conjugated goat anti-human IgG monoclonal antibody at a dilution of 1:2,000. Magnification × 1,000.

2.4.4 Serology

Serology remains the mainstay for diagnosis of Q fever due to the difficulty in culturing *C. burnetii* and the lack of appropriate facilities for handling the organism (Fournier *et al.*, 1998). The most reliable serological assays include complement fixation (Peter *et al.*, 1985), microagglutination (Nguyen *et al.*, 1996), enzyme-linked immunosorbent assays (ELISA) (Waag *et al.*, 1995; Field *et al.*, 2002) and indirect immunofluorescence (Field *et al.*, 1983; Peacock *et al.*, 1983). Indirect immunofluorescence has been the reference method for serological diagnosis since 2000 (Field *et al.*, 2000). The sensitivity, specificity, positive predictive value and cost have to be considered when choosing a diagnostic test to ensure it is appropriate for the purpose (Fournier *et al.*, 1998).

Commercial kits are available for complement fixation assays, indirect immunofluorescence assays and ELISA (Fournier *et al.*, 1998). The serological tests developed to date are based upon phase I or phase II antigens as well as immunoglobulin subsets (Maurin and Raoult, 1999). Predictive titres also vary greatly depending on the type of test used. In addition, individual antibody titres and the period over which antibodies can be detected vary between individuals and can be difficult to interpret. Generally, the criteria for diagnosis of acute Q fever is a four-fold or greater increase in antibody titre between the acute phase and convalescent serum samples (Waag *et al.*, 1991). The criterion for diagnosis of chronic Q fever is elevated anti-phase I IgG (Fournier *et al.*, 1998). However, serology has its disadvantages in that false negatives can occur if serum is taken too early during the course of the illness (Graves *et al.*, 2006). It is estimated that only 40% of suspected Q fever infections are diagnosed on the first test. In addition, there is also high variability between individual antibody titres (Fournier and Raoult, 2003). This can be illustrated by the variation in results obtained from only two patients in a recent investigation (Leung-Shea and Danaher, 2006). Results varied considerably between laboratories, even those using the same serological test (Table 2.3). Several tests indicated chronic infection according to the standard threshold titres despite being performed on relatively early samples.

Table 2.3: Variation in serological results in two cases of Q fever

PATIENT	WEEKS AFTER ONSET	TEST	TITRES	
			PHASE I	PHASE II
1	8	Focus Diagnostics IFA IgG	1:2048	1:128
1	8	Focus Diagnostics IFA IgM	1:128	1:512
1	8	Noncommercial IFA IgG	1:4096	1:4096
2	5	Focus Diagnostics IFA IgG	1:128	<1:16
2	5	Focus Diagnostics IFA IgG	<1:64	1:4096
2	5	Focus Diagnostics IFA IgM	1:16	1:64

Adapted from Leung-Shea and Danaher (2006)

2.4.4.1 Complement fixation

Complement fixation (CF) tests have historically been used to detect antibody responses to *C. burnetii* antigens (Stallman, 1965; Cowley *et al.*, 1992). The assay is based on the ability of antigen-antibody complexes to bind complement (Waag *et al.*, 1991). Red blood cells (RBC) in combination with specific antibodies are used as indicators of a positive result as the RBCs will fail to be lysed if bacterial immune complexes sequester the available complement. The CF test is highly specific for *C. burnetii* (Fournier *et al.*, 1998) and is useful for determining previous exposure to the organism as complement fixing antibodies persist long after initial infection (Lennette *et al.*, 1952; Murphy and Field, 1970). Nevertheless, the CF test is less sensitive than other tests routinely used for diagnosing Q fever and fails to detect early infection (Fournier *et al.*, 1998). A CF titre of 1:40 to phase II is considered to be diagnostic for acute Q fever (Guigno *et al.*, 1992). Whereas, a titre of 1:200 to phase I is considered to be diagnostic for chronic Q fever (Fournier *et al.*, 1998). In addition, the subjective nature of the test, demonstration of anti-complementary activity in some serum samples (Schmidt and Harding, 1956; Lang, 1988) and lack of standardisation between laboratories (Field *et al.*, 2000) have lead to the replacement of the CF test with alternative methods (Waag *et al.*, 1991).

2.4.4.2 Microagglutination

Several types of agglutination assays have been developed for the diagnosis of Q fever with the microagglutination assay (MAA) being the most commonly used (Waag *et al.*, 1991). The MAA is based on the binding of anti-*Coxiella* antibodies to *C. burnetii* cells, forming a cell-antibody complex (Lennette *et al.*, 1952). The advantage of the MAA is that it selects for IgM antibodies making it superior to the CF test for the early diagnosis of acute Q fever (Riemann *et al.*, 1979a). However, as with the CF test, the MAA is also subjective and is not standardised, with thresholds for positive reactions varying between studies from ≥ 1:4 (Riemann *et al.*, 1979a) to 1:64 (Nguyen *et al.*, 1996). In addition, large amounts of antigen are required and more sensitive assays have subsequently been developed for detecting the presence of anti-*Coxiella* antibodies.

2.4.4.3 Indirect immunofluorescence

The indirect immunofluorescence assay (IFA) is currently the reference method for the diagnosis of Q fever (Fournier *et al.*, 1998). The method involves fixing formalin-inactivated *C. burnetii* cells to microscope slides followed by probing with serial dilutions of patient sera and a secondary antibody conjugated to a fluorescent dye. Titres of anti-phase II IgG and anti-phase II IgM of ≥1:200 and ≥1:50 respectively are used as the threshold for the diagnosis of acute Q fever with IFA (Tissot-Dupont *et al.*, 1994). For the diagnosis of chronic Q fever, the threshold is a titre of anti-phase I IgG of ≥ 1:800. Conversely, another study found that the use of single threshold values for the IFA may result in both false positive and false negative results (Setiyono *et al.*, 2005). This group found that some confirmed Q fever patients were missed with the existing threshold, while some patients with pneumonia of different aetiology were included. They proposed that titres for anti-phase II IgM falling between ≥1:32 and ≤1:64 and anti-phase II IgG between ≥1:64 and ≤1:128 be tested with additional serological assays to eliminate false positives and false negatives. Another study in Denmark (Villumsen *et al.*, 2009) found that existing recommended threshold titres resulted in poor specificity with other clinically related diseases. They proposed threshold titres based on

local baseline titres of anti-phase II IgM of ≥1:256 and anti-phase II IgG of ≥1:1,024 be adopted. In addition, a further study conducted in the Netherlands (Blaauw *et al.*, 2011) found that increasing the threshold titres to these levels resulted in improved agreement between the IFA and ELISA. However, it was unclear whether the improvement in specificity resulted in a substantial loss of sensitivity.

IFA is more sensitive and specific than either the MAA or CF tests and allows for the identification of different antibody classes against both phase I and II antigens (Waag *et al.*, 1991). Nonetheless, there is some debate as to whether the IFA is superior to the ELISA for the diagnosis of Q fever (Devine, 1998). Fournier *et al* (1998) describe IFA as the simplest and most accurate serological technique. Conversely, IFA cannot be automated and, as it also requires microscopic examination, it is not suitable for large scale screening and can be subjective (Field *et al.*, 2000). A number of studies have found the ELISA to be more sensitive, reproducible and easier to perform (Peter *et al.*, 1987; Waag *et al.*, 1995; D'Harcourt *et al.*, 1996; Field *et al.*, 2000).

2.4.4.4 Enzyme-linked immunosorbent assays (ELISA)

In the ELISA, phase I or II *C. burnetii* antigens are bound to microtitre plates and probed with patient sera (Waag *et al.*, 1991). Reactive antibodies are detected with enzyme conjugated anti-sera. The assay is easily automated and the use of electronic plate readers eliminates the inherent subjectivity of other serological tests for Q fever such as IFA. The ELISA is a useful in both serological surveys and the serodiagnosis of Q fever (Uhaa *et al.*, 1994). Proposed threshold titres for the diagnosis of acute Q fever with the ELISA consist of ≥1:1,024 and ≥1:512 for anti-phase II IgG and IgM respectively (Waag *et al.*, 1995). For diagnosis of chronic Q fever an anti-phase I IgG titre of ≥1:128 has been proposed as the threshold.

While Fournier *et al* (1998) describe the ELISA as more laborious and difficult to interpret than IFA, other reviewers describe the ELISA as the method of choice for Q fever diagnosis (Waag *et al.*, 1991; Byrne, 1997). Comparisons of the ELISA against

the IFA (Field *et al.*, 1983; Peter *et al.*, 1987; Waag *et al.*, 1995; D'Harcourt *et al.*, 1996; Field *et al.*, 2000) have demonstrated a high degree of agreement between the two assays and the ability of the ELISA to detect antibody responses earlier than the IFA would seem to contradict the statements of Fournier *et al* (1998). It is likely that the ELISA will replace the IFA as the reference diagnostic method for Q fever. Nonetheless, there is some discrepancy between the antigens used in the ELISA. Many ELISAs for Q fever use formalin-inactivated whole cells as antigen (Cowley *et al.*, 1992; Uhaa *et al.*, 1994; Field *et al.*, 2002) while others have used sonicated *C. burnetii* cells (Williams *et al.*, 1986; Peter *et al.*, 1987). Williams *et al* (1986) compared whole cell antigen to disrupted *C. burnetii* antigens prepared by various methods. The study found whole cell antigen to be most effective and the authors recommended this form of antigen be used in all ELISAs for Q fever to standardise assays. An ELISA developed by Peter *et al* (1987) using sonicated *C. burnetii* detected antibodies in 95.4% of Q fever patients. Another ELISA using whole cell antigen had sensitivities of 80 and 84% for anti-phase II IgG and IgM antibodies respectively (Waag *et al.*, 1995). A further assay using whole cell antigens was found to have a sensitivity of 97.8% (Behymer *et al.*, 1985). However, this assay was developed for screening animal sera. Further comparisons would be required to determine the optimum antigen preparation for use in ELISA for detection of anti-*Coxiella* antibodies.

Table 2.4: Sensitivity and specificity of common serological tests for Q fever

TEST	SENSITIVITY	SPECIFICITY	REFERENCE
Microagglutination	81.6%	98.6%	(Nguyen *et al.*, 1996)
Complement fixation	77.8%	99%	(Peter *et al.*, 1985)
Immunofluorescence	58.4%	92.2%	(Tissot-Dupont *et al.*, 1994)
ELISA	84%	99%	(Waag *et al.*, 1995)

2.5 Prevention and Treatment

2.5.1 Vaccines

The highly infectious nature of *C. burnetii* and its status as an occupational hazard for those in the meat and livestock industries, veterinarians and laboratory workers has lead to many attempts to develop effective vaccines (Marmion, 1967; Ormsbee and Marmion, 1990). Many vaccine attempts have involved the use of immunogenic *C. burnetii* proteins (Ormsbee and Marmion, 1990). However, these vaccines elicit a predominately humoral response. The intracellular lifecycle of *C. burnetii* has restricted the effectiveness of such vaccines, due to the requirement for an effective cellular response. As a result, whole cell inactivated vaccines have been found to be more effective and hence form the basis for the only commercially developed vaccines against Q fever.

Q-VAX (Commonwealth Serum Laboratories) is the only commercially available Q fever vaccine suitable for human vaccination (Marmion, 2007). It is prepared from virulent phase I Henzerling isolate *C. burnetii*, which is subsequently formalin inactivated (Ormsbee and Marmion, 1990). Q-VAX was first developed in 1972 and was grown in chicken eggs. It was licensed for use in Australia in 1987 (Parker *et al.*, 2006). The production process has not been modified since and the organism is still grown in eggs, making vaccine production both time-consuming and expensive. The expense of production led to the intention of phasing out production of Q-VAX by CSL by 2007 (Marmion, 2007). Subsequent pressure from the Australian Federal Government has lead to the commitment to continue production until an alternative vaccine can be developed.

Following its introduction, a retrospective study of Q-VAX efficacy found that none of over 2,000 abattoir employees vaccinated had developed Q fever after five years (Ackland *et al.*, 1994). The only exceptions were in two individuals thought to be incubating the disease at the time of vaccination. In contrast, out of the 1,365 unvaccinated employees surveyed, there were 55 Q fever cases. A more recent

investigation of the national Q-VAX program found uptake of almost 100% in abattoir workers and 43% in primary producers (Gidding *et al.*, 2009). Notification rates for Q fever declined by 50% between 2002 and 2006, with similar trends in hospitalisations.

Immune individuals can have localised adverse reactions such as abscesses, due to the cellular nature of the vaccine (Parker *et al.*, 2006). The most common side effect reported is erythema and tenderness at the inoculation site. Headaches, shivering, and flu-like symptoms have been reported in 10-18% of vaccinees (Marmion *et al.*, 1990). During 2001-2004 a total of 86 individuals were notified to the Therapeutic Goods Administration Australia, as a result of an adverse effect following Q fever vaccination (Gidding *et al.*, 2009). Of these, eight (9.3%) required hospitalisation with one case being life-threatening. As a result of the possibility of adverse reactions, pre-vaccination screening is now required to ensure naturally immune individuals are not vaccinated (Kermode *et al.*, 2003). Pre-vaccination screening consists of a clinical history, skin test and serology. Q-VAX is only administered if there is no history of Q fever and both the skin and serological tests are negative (Parker *et al.*, 2006). The vaccine cannot be administered to children under the age of fifteen. Generally, Q fever is rarely reported in children and children who are exposed to *C. burnetii* are less frequently symptomatic following infection (Maltezou and Raoult, 2002). However, another study conducted in rural Ghana indicated Q fever may be an important under-diagnosed childhood disease, due to clinical manifestations similar to other childhood diseases and malaria (Kobbe *et al.*, 2008). A recent study performed in Queensland, Australia found that seroprevalence in children under the age of 15 was 2.5% (Parker *et al.*, 2010). Nonetheless, it is expected that children living in rural areas are exposed to *C. burnetii* and there is a need for a vaccine that is safe for such children.

Due to the possibility of adverse reactions in some individuals, acellular vaccines have been proposed. There are two existing acellular vaccines; a trichloroacetic acid (TCA) extracted vaccine from the former Czechoslovakia (Chemovaccine) (Camacho *et al.*, 2000) and a chloroform-methanol residue (CMR) from the USA (Waag *et al.*, 2002). To date, none of these vaccines are commercially available. The acellular vaccines are

thought to be as effective as Q-VAX with fewer adverse reactions. In a comparative efficacy study between Q-VAX and CMR, only a 100 µg dose of CMR resulted in significant increase in lymphocyte proliferation following challenge compared to the standard 30 µg dose given of Q-VAX. In addition, a proliferative lymphocyte response following vaccination with CMR could no longer be detected after four weeks (Waag *et al.*, 2002). In contrast, following Q-VAX immunisation 85% of vaccinees converted to a proliferative lymphocyte response within six weeks (Izzo *et al.*, 1988) and proliferative responses could still be observed up to eight years following vaccination. The TCA vaccine was also treated with chloroform-methanol, which resulted in a vaccine with even fewer incidences of adverse reactions. However, this vaccine had decreased immunogenicity in comparison to the unaltered TCA vaccine and the protective effect was never evaluated in human subjects (Maurin and Raoult, 1999).

2.5.2 Antibiotic therapy

Several classes of antibiotics are active against *C. burnetii*, including tetracyclines, fluoroquinolones, macrolides and rifampin (Raoult, 1993). Standard treatment for acute Q fever is two to three weeks with doxycycline (a tetracycline), ofloxacin (a fluoroquinolone) or clarithromycin (a macrolide). Clarithromycin is a relatively new macrolide compound that has demonstrated activity against *C. burnetii* in antibiotic activity assays (Morovic, 2005). As tetracyclines have adverse effects in pregnant women and children under the age of eight, co-trimoxazole has been recommended as an alternative (Maltezou and Raoult, 2002). Guidelines for the treatment of Q fever are summarised in Table 2.5.

Chronic Q fever requires prolonged combination antibiotic therapy with doxycycline usually used in combination with rifampin or co-trimoxazole (Raoult, 1993). Other combinations include doxycycline with perfloxacin or ofloxacin. As of 1999, therapy for Q fever endocarditis is continued medication with a combination of doxycycline and a fluoroquinolone for at least three years (Maurin and Raoult, 1999). Co-trimoxazole has

been recommended as an alternative to tetracyclines in pregnant women and children under the age of eight due to contraindication of tetracyclines in these patients (Nourse *et al.*, 2004).

Table 2.5: Guidelines for the treatment of Q fever

FORM	PATIENT GROUP	TREATMENT	DURATION	REFERENCE
Acute	Adults	Doxycycline (100mg/day)	14 days	Maurin & Raoult (1999)
		Fluoroquinolones (600mg /day)	14-21 days	Maurin & Raoult (1999)
		Perfloxacin (400mg/day)	14-21 days	Maurin & Raoult (1999)
		Rifampin (1200mg/day)	21 days	Raoult (1993)
	Pregnant	Trimethoprim (320mg/day) and Sulphamethoxazole (1600mg)	>5 weeks	Carcopino *et al* (2007)
	Children	Trimethoprim (320mg/day) and Sulphamethoxazole (1600mg)	21 days	Maltezou & Raoult (2002)
Chronic	Adults	Doxycycline (100mg/day) and Hydroxychloroquine (600mg)	>18 mths	Carcopino *et al* (2007)
	Children	Trimethoprim (320mg/day) and Sulphamethoxazole (1600mg)	>18mths	Nourse *et al* (2004)

2.6 Transmission of Q fever

2.6.1 Human infection

Human infection with *C. burnetii* is most commonly acquired via the respiratory route (Marrie, 1990). Contaminated aerosols can be acquired from the parturient fluids of infected animals (Woldehiwet, 2004) and dispersed by wind (Hawker *et al.*, 1998;

Tissot-Dupont *et al.*, 1999; Tissot-Dupont *et al.*, 2004). The spore-like SCV form is highly resistant to heat, drying, extremes of pH, disinfectants and UV radiation (Babudieri, 1959; Scott and Williams, 1990). In addition, *C. burnetii* is highly stable in the environment, remaining viable for months (Williams *et al.*, 1991) and possibly years (van Woerden *et al.*, 2004). Those most at risk of contracting Q fever include veterinarians, farmers, abattoir workers, dairy workers and laboratory personnel working with *C. burnetii* and infected animals (Maurin and Raoult, 1999). Less common routes of infection include ingestion of unpasteurised milk containing *C. burnetii*, human to human transmission, percutaneous transmission and tick bites, as ticks are a natural reservoir of *C. burnetii* in the wild (Marrie, 1990). Human to human transmission has been reported in several people who attended the autopsy of a Q fever patient (Harman, 1949), a bone marrow transplant recipient (Kanfer *et al.*, 1988) and one sexually transmitted case (Milazzo *et al.*, 2001). However, human to human transmission of Q fever is very rare (Marrie, 1990).

2.6.2 Livestock infection

Coxiellosis in livestock is transmitted between animals by means of fomites that can be spread by wind and inhaled by other livestock (Lang, 1990). Coxiellosis is predominately asymptomatic in animals with transmission usually occurring via the respiratory route, without respiratory pathology. *Coxiella burnetii* is thought to originally be transmitted to livestock via ticks, which form part of the natural transmission cycle of the organism. Many animals become persistently infected, yet do not demonstrate the endocarditis or hepatitis seen in human chronic infection. Rather, infection is localised to the female reproductive system and is found in both the uterus and mammary glands. This infection is usually sub-clinical, although can be associated with abortions and reproductive disorders (To *et al.*, 1998a; Bildfell *et al.*, 2000; Cabassi *et al.*, 2006). This infection usually has no adverse affects on the animal or foetus (Lang, 1990). However, massive amounts of *C. burnetii* can be present and result in the shedding and spread of the organism in the environment. The transmission of *C. burnetii*

in livestock provides the main source of direct and/or indirect infection of humans, with ruminants such as cattle, sheep and goats being among those most commonly associated with transmission of *C. burnetii* to humans. A recent critical review of the prevalence of *C. burnetii* infection in domestic ruminants found a wide range in all three species (Guatteo *et al.*, 2010). Evidence of *C. burnetii* infection in domestic ruminants was found in all five continents. Prevalence was higher in cattle, with a mean prevalence for animal and herd level of 20.0% (n=36 studies) and 37.7% (n=27 studies) respectively, compared with 15.0% (n=26 studies) and 25.0% (n=12 studies) found in sheep and goats.

2.6.3 Role of livestock in human infection

2.6.3.1 Cattle

Cattle are the most common source of human *C. burnetii* infections in most countries. This is thought to be due to cattle being more likely than goats or sheep to be persistently infected with *C. burnetii* (Lang, 1990). Subsequently, it has been found that correlations exist between seropositivity for *C. burnetii* in cattle and human cases of Q fever (Enright *et al.*, 1971c; Niang *et al.*, 1998). Cattle have been found to shed *C. burnetii* in their milk, urine, faeces and parturient fluids. Q fever is rarely contracted orally in humans, and in most cases, where *C. burnetii* is ingested in contaminated milk the individuals demonstrate seroconversion only, with no clinical signs. Pasteurisation effectively prevents transmission of *C. burnetii* in milk. Increased seropositivity for *C. burnetii* in humans has been correlated with the use of manure in gardening (Psaroulaki *et al.*, 2006). The shedding of *C. burnetii* into the environment results in increased risk of infection on cattle properties and in stock yards and along stock routes. Contact with infected placentas has been associated Q fever cases, particularly in abattoir workers. The ability of *C. burnetii* to survive for extended periods in the environment means it can be spread from areas with infected livestock to urban areas on the wind (DeLay *et al.*, 1950; Tissot-Dupont *et al.*, 2004). Serological surveys have

demonstrated that coxiellosis is increasing in cattle populations worldwide (Lang, 1990). In Japan seroprevalence rose from 2.3% (n=3,072) in the 1950s to 29.5% (n=2,063) in 1992 (Hirai and To, 1998). The corresponding seroprevalence in meat-processing workers rose from 2.9% (n=756) to 11.2% (n=107). The prevalence of *C. burnetii* in dairy cattle in Canada was found to have increased from 2.3% (n=4567) in 1964 to 66.8% (n=199) in 1984 (Lang, 1989). Testing of bulk milk tanks from dairy herds in the US demonstrated that between 2001 and 2003, 93.2-94.3% (n=316) of samples were PCR positive for *C. burnetii* (Kim *et al.*, 2005). The increasing seropositivity for *C. burnetii* in cattle worldwide indicates a potential increase in animals shedding the organism into the environment, and increased opportunity for transmission to humans.

In epidemiological studies conducted overseas during the last ten years, the seroprevalence of *C. burnetii* in cattle populations varied according to geographic location. Seroprevalence rates were reported as 6.2% (n=5,182) in Northern Ireland (McCaughey *et al.*, 2010), 6.7% (n=626) in Spain (Ruiz-Fons *et al.*, 2010), 7.9% (n=1,656) in Albania (Cekani *et al.*, 2008), 10.8% (n=93) in Iran (Khalili and Sakhaee, 2009), 14.3% (n=784) in the Central African Republic (Nakoune *et al.*, 2004a), 24% in Newfoundland (n=75) (Hatchette *et al.*, 2002) and Cyprus (n=75) (Psaroulaki *et al.*, 2006) and 25.6% (n=414) in Korea (Kim *et al.*, 2006). Previous serological investigations of prevalence of *C. burnetii* infection in Australian cattle demonstrated it was not common in beef cattle in Western Australia (n=329) (Banazis *et al.*, 2010) and South Australia (n=10 herds) (Durham and Paine, 1997), dairy cattle in Victoria (n=1,576) (Hore and Kovesdy, 1972) and beef and dairy cattle in New South Wales (n=700) (Forbes *et al.*, 1954) with seroprevalence of less than 1% in all four studies.

2.6.3.2 Sheep

Sheep primarily transmit *C. burnetii* to humans via infected placental products, and only shed the organism during parturition or abortion (Lang, 1990). However, *C. burnetii* can be carried in the fleece and has been associated with the contraction of Q fever at wool processing plants where live animals are not present. Seropositivity fluctuates annually

in sheep, with peaks during the lambing season (Enright *et al.*, 1971b). High winds during this period are associated with an increased incidence of Q fever in nearby human populations (Tissot-Dupont *et al.*, 1999). Similar to findings in cattle, seroprevalence in sheep has been found to be increasing. In Canada, rates were found to have increased from 3.1% (n=293) in 1997 to 23.5% in 2000 (n=34) (Hatchette *et al.*, 2002). In The North Rhine-Westphalia region of Germany, seroprevalence had increased from 0.0% (n=2,199) in 1960 to 57% (n=100) in 1999 (Hellenbrand *et al.*, 2001). In epidemiological studies conducted overseas during the last ten years, the seroprevalence of *C. burnetii* in sheep populations varied according to geographic location. Seroprevalence rates were reported as 40.0% (n=90) in Mexico (Salinas-Meledez *et al.*, 2002), 22.5% (n=89) in Egypt (Mazyad and Hafez, 2007), 20.0% (n=151) in Turkey (Kennerman *et al.*, 2010), 18.9% (n=420) in Cyprus (Psaroulaki *et al.*, 2006), 12.3% (n=1,022) in Ireland (McCaughey *et al.*, 2010), 11.8% (n=1,379) in Spain (Ruiz-Fons *et al.*, 2010), 11.0% (n=142) in Chad (Schelling *et al.*, 2003), 10.4% (n=615) in Greece (Pape *et al.*, 2009) and 3.1% (n=350) in Albania (Cekani *et al.*, 2008).

The ability of parturient animals to transmit *C. burnetii* to humans is illustrated by several cases where pregnant animals gave birth near urban areas or at agricultural fairs (Lyytikainen *et al.*, 1998; Porten *et al.*, 2006). A large outbreak of 322 cases in 2005 was linked to a herd of sheep kept near a housing area (RKI, 2005). An earlier outbreak in 2003 of 299 cases was linked to a sheep that had lambed at a farmer's market (Porten *et al.*, 2006). The attack rate was found to be 20%, with 25% of cases requiring hospitalisation. Unlike cattle, sheep are not normally persistently infected and, if they remain unexposed to other reservoirs, can be expected to clear infection (Lang, 1990). Therefore, they have less implication than other ruminants in the transmission and maintenance of *C. burnetii* in the environment.

2.6.3.3 Goats

In countries where cattle and sheep are not the main sources of meat and milk, goats are more associated with transmission of *C. burnetii* to humans (Lang, 1990; Serbezov

et al., 1999). Goats have been found to be susceptible to persistent infection with *C. burnetii* and are capable of maintaining the organism in the environment. Routes of transmission are similar to those seen with cattle, as goats also shed the organism in the milk, faeces and births products. Goats have been associated with an ongoing epidemic of Q fever in the Netherlands (Schimmer *et al.*, 2009).

In epidemiological studies conducted overseas during the last ten years, the seroprevalence of *C. burnetii* in goat populations varied according to geographic location. Seroprevalence rates were reported as 65.8% (n=76) in Iran (Khalili and Sakhaee, 2009), 48.2% (n=420) in Cyprus (Psaroulaki *et al.*, 2006), 35.0% (n=60) in Mexico (Salinas-Meledez *et al.*, 2002), 18.7% (n=443) in Albania (Cekani *et al.*, 2008), 16.8% (n=72) in Egypt (Mazyad and Hafez, 2007), 13.0% (n=134) in Chad (Schelling *et al.*, 2003), 9.3% (n=54) in Ireland (McCaughey *et al.*, 2010), 8.7% (n=115) in Spain (Ruiz-Fons *et al.*, 2010) and 6.5% (n=61) in Greece (Pape *et al.*, 2009).

2.6.4 Role of companion animals in human infection

While the greatest risk factors for the contraction of Q fever include contact with domestic ruminants, some cases cannot be associated with these risk factors. These include cases that are contracted in urban environments where there is little or no contact with the usual reservoirs of human infection. Other domestic animals, such as companion animals have demonstrated the ability to transmit *C. burnetii* to humans (Lang, 1990). In the absence of the usual risk factors it is thought that these animals constitute an important reservoir of *C. burnetii* in urban environments.

2.6.4.1 Dogs

Dogs have been found to shed *C. burnetii* in their milk and urine for 30 and 70 days respectively (Babudieri, 1959). Outbreaks have also been associated with parturient animals (Laughlin *et al.*, 1991; Buhariwalla *et al.*, 1996). Coxiellosis is readily

established in dogs following bites from infected ticks (Lang, 1990). The organism can be vertically transmitted in ticks, allowing the organism to persist in the absence of other hosts. Transmission of *C. burnetii* from ticks to dogs and occasionally to humans may constitute an alternative reservoir of Q fever in semi-urban and urban environments. Few seroprevalence studies have been performed on dogs. One survey of dogs belonging to the French military in various countries found seroprevalences of 11.6% (n=43) in Senegal, 9.8% (n=348) in France, 8.3% (n=12) in Ivory Coast, 5.2% (n=19) in French Guyana and 0.0% (n=7) in Martinique (Boni *et al.*, 1998). While a serosurvey in Slovakia found a seroprevalence of 11.5% (n=366) (Kovacova *et al.*, 2006). Another survey in Italy found seroprevalence of 8.0% (n=283) in dogs in Sicily (Torina *et al.*, 2007). A comprehensive survey of over 12,000 serum samples in New Zealand found no seropositives, and is considered an important indication of the absence of *C. burnetii* from New Zealand (Hilbink *et al.*, 1993).

2.6.4.2 Cats

Cases of Q fever acquired from cats are more commonly reported than those from dogs (Lang, 1990). As with domestic dogs, Q fever outbreaks have been associated with parturient animals (Kosatsky, 1984; Langley *et al.*, 1988). Seroprevalence in domestic cats can be relatively high with reported rates of 14.2% (n=310) in Japan (Komiya *et al.*, 2003), 13% in Zimbabwe (Matthewman *et al.*, 1997), 12.9% (n=201) in Canada (Higgins and Marrie, 1990) and 8.6% (n=11) in Korea (Komiya *et al.*, 2003). Rates can be much higher in stray animals, with 41.7% (n=36) of stray cats sampled in Japan testing positive for anti-*Coxiella* antibodies (Komiya *et al.*, 2003). These results, in conjunction with the many reported cases of Q fever acquired from cats indicate they may be an important reservoir of *C. burnetii*.

2.7 Wildlife Reservoirs of Q fever

2.7.1 Ticks

Early investigations into Q fever demonstrating its rickettsial appearance in tissue sections (Burnet and Freeman, 1937) and transmission by ticks (Cox, 1939) lead to the search for other arthropod vectors of *C. burnetii* (Lang, 1990). *Coxiella burnetii* has been isolated from various tick species that feed on a wide variety of vertebrate hosts (Table 2.6). The natural cycle of *C. burnetii* is thought to exist between wild mammals and their ticks. Ticks also represent a self-perpetuating reservoir for *C. burnetii*, in that the organism is vertically transmitted in some species (Pandurov and Zaprianov, 1975; Weyer, 1975; Daiter, 1977). The host promiscuity of many species of ticks that feed on wild animals results in the transmission of *C. burnetii* to domestic animals in endemic areas. However, transmission between livestock does not require ticks (Babudieri, 1959).

Table 2.6: Tick species known to transmit *Coxiella burnetii*

VECTOR	MAIN HOSTS	COUNTRY	REFERENCE
Dermacentor andersoni	various mammals, humans	USA	(Davis and Cox, 1938)
Dermacentor occidentalis	rodents, large mammals	USA	(Cox, 1940)
Dermacentor marginatus	various mammals	Europe	(Rehacek *et al.*, 1975)
Amblyomma americanum	various mammals	USA	(Cox, 1940)
Amblyomma cayennense	various mammals	Brazil, USA	(Sanders *et al.*, 2008)
Amblyomma triguttatum	kangaroo	Australia	(Pope *et al.*, 1960)
Hyalomma savignyi	ruminants	Europe	(Parker *et al.*, 1950)
Hemaphysalis leporispalustris	rabbit	USA	(Cox, 1940)
Hemaphysalis humerosa	bandicoot	Australia	(Smith and Derrick, 1939)
Ixodes holocyclus	bandicoot	Australia	(Smith, 1942)
Ixodes ricinus	various mammals	Europe	(Rehn and Radvan, 1957)
Otobius megnini	livestock, dogs, humans	USA	(Jellison *et al.*, 1948)
Rhipicephalus sanguineus	dogs, various mammals	Worldwide	(Parker and Sussman, 1949)
Ornithodoros moubata	humans	Africa	(Weyer, 1975)

2.7.2 Wildlife

Wild animals and the tick species which feed on them form the natural transmission cycle and reservoir of *C. burnetii* (Babudieri, 1959). Many serological surveys and bacterial isolations have indicated the extent of wildlife coxiellosis. In Australia, bandicoots (Smith and Derrick, 1939; Smith, 1942) and kangaroos (Pope *et al.*, 1960) were found to be reservoirs. Some of their ticks were capable of transmitting *C. burnetii* and were found to feed on both cattle and humans. A retrospective serological survey for anti-*Coxiella* antibodies performed on 160 kangaroo sera found a seropositivity rate of 11.8% (Stallman, 1965). However, approximately 50% of the samples were anti-complementary resulting in difficulty in determining the true extent of coxiellosis in the animals. A more recent survey of kangaroos in Western Australia found seropositivity of 33.5% (n=343) (Banazis *et al.*, 2010).

In Japan serological surveys were performed on a wide variety of wild animals and birds, as well as domestic birds. High prevalence of anti-*Coxiella* antibodies were detected in Japanese black bears (78%; n=36), Hokkaido deer (69%; n=61), Japanese hares (63%; n=8) and Japanese deer (56%; n=72) (Ejercito *et al.*, 1993). The high seropositivity rates indicated these animals may be potential sources of animal and human infection. Surveys on wild and domestic birds demonstrated overall prevalence of 19% (n=863) and 2% (n=1,951) respectively (To *et al.*, 1998b). Seropositivity in some species of wild birds, particularly scavengers such as carrion crows (37%; n=173) and jungle crows (35%; n=258) is noticeably higher than that of other wild birds. Seropositivity was higher in areas adjacent to livestock, and was thought to be due to feeding on animal remains and parturient material. As such, these animals are not thought to be primary reservoirs of Q fever, but may be responsible for transmission to other areas.

A survey of rodents in India demonstrated seroprevalence in rats, mice, shrews and bandicoots of 12.4 (n=105), 14.3 (n=7), 14.3 (n=42) and 13.3% (n=15) respectively (Yadav *et al.*, 1979). Inoculation of guinea pigs with pooled samples from rats, shrews

and bandicoots resulted in the isolation of *C. burnetii*. This indicates that a small percentage of these animals have not only been exposed to *C. burnetii*, but are actively infected with the organism and are potentially excreting the organism into the environment. Rodents were also identified as a reservoir of Q fever in the UK where wild rats were found to have prevalence of up to 53% (n=225) on farms with endemically infected livestock (Webster *et al.*, 1995). No ticks were found on the rats and it was suggested the animals were infected via the livestock transmission cycle of inhalation of aerosols. *Coxiella burnetii* was detected in rats year-round, suggesting the animals act as persistent reservoirs. The relatively high prevalence of coxiellosis in rodents is thought to be a source of infection for animals that prey upon rodents, particularly cats (Webster *et al.*, 1995). This was also demonstrated in other animals which prey upon rodents. In another study conducted in India, anti-*Coxiella* antibodies were detected in 23% (n=48) of snakes, 40% (n=5) of pythons and 12.5% (n=16) tortoises (Yadav and Sethi, 1979). The bacterium was also isolated from the visceral organs of one of two pythons examined.

In Slovakia, wild ruminant species were screened for antibodies to phase II and I antigens (Dorko *et al.*, 2009). Seropositivity for phase II antigen in Cameroun goats and Carpathian goats, Cameroun sheep, fallow deer and mouflon were 70 (n=17), 37.5 (n=8), 28.3 (n=60) and 25% (n=4) respectively. For phase I antigen seropositive rates were 12.5% in Cameroun sheep, 5% in fallow deer and 0% in goats and mouflon. In the Czech Republic, a survey of mouflon, fallow deer (n=4), red deer (n=24), roe deer (n=33), wild boars (n=34) and hares (n=48) found seropositives rates of 100, 50, 25, 6, 6, and 0% respectively with an overall prevalence of anti-*Coxiella* antibodies of 12% (Hubalek *et al.*, 1993).

In Canada, four wildlife species were screened for antibodies to *C. burnetii* phase I and II antigens (Marrie *et al.*, 1993). Seropositivity for the phase I antigen in hares (n=730), moose (n=243), deer (n=68) and raccoons (n=42) was 49, 16.5, 1.5 and 7.1% respectively. For phase II antigen, seropositive rates were 12, 11.5, 4.4 and 9.5% respectively. These results indicated coxiellosis was relatively common in the hare,

moose and raccoon. Also, the higher seropositivity for phase I antigens in hares and moose may indicate these animals are persistently infected with *C. burnetii*. However, this possibility was not addressed in the study and no attempt was made to isolate the organism from these animals. A deer was suggested to have been the initial reservoir of *C. burnetii* in a Q fever outbreak in Canada (Laughlin *et al.*, 1991).

In the US, antibodies were detected by CF test in a wide variety of animals including coyotes (78%; n=27), foxes (55%; n=22), brush rabbits (53%; n=30), jackrabbits (39%; n=251), deer (22%; n=342), chipmunks (22%; n=9) and skunks (21%; n=19) (Enright *et al.*, 1971a). Seroprevalence in various species of mice ranged from 9 to 31%. Like the Australian study, a high proportion of samples were anti-complementary for some species. One study in California found the presence of agglutinating antibodies in rodents (n=759) and birds (n=583) (Riemann *et al.*, 1979a). Seropositivity ranged from 2 to 11% (3% average) in the rodent species screened and 7 to 68% (20% average) in the birds. A further study in the same area found seropositive rates of 57% (n=7) in native black-tailed deer and 51% (n=152) in exotic axis and fallow deer (Riemann *et al.*, 1979b). Seropositivity was found to be higher in areas with infected livestock, and it was suggested that the livestock were the reservoir for infection of the wildlife. Another study in Texas found agglutinins in coyotes (3% to phase I, 31.8% to phase II), opossums (0% to phase I, 12.1% to phase II) and raccoons (27.3% to phase I, 45.5% to phase II) (Randhawa *et al.*, 1977). Therefore, as the microagglutination assay selects for IgM antibodies, which are only present early in infection, the true seroprevalence may have been higher. In Idaho, seropositivity in black bears was found to be 6.2% (n=265) (Binninger *et al.*, 1980). Conversely, in Alaska where there is only a small livestock industry, seroprevalence in native Dall sheep was found to be 80% (n=15) (Zarnke, 1983). Other animal species were not tested, which would yield more data on which to hypothesise. Even so, it would appear that the primary reservoir of Q fever varies in different locations.

In some locations, the primary source of *C. burnetii* infection in humans is thought to be wildlife, rather than domestic animals as is usually seen. This is particularly evident in

French Guiana where the prevalence of antibodies to *C. burnetii* was found to be only 1.7% (n=355) in cattle (Gardon *et al.*, 2001). Antibodies were not detected in any of the sheep, goats, pigs and cats surveyed. Seroprevalence in dogs was found to be 12.3% (n=57). However, there was no statistically significant difference between seropositivity in dogs or Q fever patients and control subjects. It was found that proximity to the forest and sighting bats and other wild animals close to homes were risk factors. Serological testing of several species of wild animals indicated *C. burnetii* exposure in the common opossum (25%; n=4), native spiny rats (15.4%; n=26) and four-eyed opossums (11.1%; n=36). Despite the association between bats and increased Q fever incidence, antibodies were not detected in the two species tested. Tick bites were found to be commonly reported in French Guiana and they were proposed as a vector. Nevertheless, there was no significant difference between Q fever patients and control subjects who reported tick bites.

2.8 Detection and Surveillance for Q fever

It is estimated that approximately 60% of known human pathogens and 75% of emerging infectious diseases are of zoonotic origin (Cleaveland *et al.*, 2001). While Q fever has been recognised since 1937, epidemiological studies have demonstrated an increased prevalence in livestock populations (Lang, 1990). As a result, Q fever has been described as a re-emerging pathogen of increasing importance as a public health issue (Arricau-Bouvery and Rodolakis, 2005). In order to produce data on the epidemiology of Q fever and to determine the risk of infection a variety of methods have been used in the attempt to detect, monitor and control Q fever.

2.8.1 Screening methods

2.8.1.1 Serological surveying

Serological surveys have provided the bulk of epidemiological data on Q fever (Lang, 1990). However, no standardised technique has been employed and there is a large degree of variability between the serological tests and positive thresholds used. This makes accurate comparison of epidemiological data difficult. Many early studies employed either microagglutination assay or complement fixation tests. These methods, while highly specific, lack the sensitivity of ELISA and IF testing. The use of less sensitive tests for serological surveys may result in the underestimation of true prevalence in animal populations. The MAAs developed for *C. burnetii* would not be suitable for assessment of the extent of *C. burnetii* infection in livestock and wildlife as they select for IgM responses (Riemann *et al.*, 1979a). Therefore, MAAs would only be effective in the detection of early infections and unable to identify persistently infected animals responsible for shedding of *C. burnetii* into the environment. While complement fixing antibodies persist for extended periods following initial infection (Murphy and Field, 1970), the CF test has demonstrated a failure to detect the presence of antibodies in some animal species (Lang, 1988; Arricau-Bouvery and Rodolakis, 2005). It also demonstrated a high proportion of anti-complimentary results (Stallman, 1965; Enright *et al.*, 1971a), particularly when samples were taken in the field and were unable to be analysed promptly. These disadvantages indicate the CF test for *C. burnetii* may no longer be suitable for use in epidemiological studies on livestock and wildlife.

Antibody based immunoassays, such as the IF and ELISA have better sensitivity than the MAA and CF tests (Fournier *et al.*, 1998). However, the IF cannot be automated and, as it requires the manual scanning of test slides it would be unsuitable for large scale serological surveys. As a result, the ELISA has been identified as the method of choice for epidemiological surveys (Behymer *et al.*, 1985; Peter *et al.*, 1987). Nonetheless, the ELISA does have disadvantages in that it requires *C. burnetii* antigens which must be cultured under PC3 conditions and specific enzyme conjugated anti-species antibodies

for detection. Antigen preparation can be avoided by the availability of commercially produced *C. burnetii* phase I and II antigens (Fournier *et al.*, 1998). Anti-species antibodies are available for many domestic animals, enabling serological testing on these species. In contrast, secondary antibodies are not available for wildlife and must be produced using either polyclonal production is an unrelated species or monoclonal production using hybridomas. Polyclonal antibody production requires the continual boosting and bleeding of the host animal with purified immunoglobulin. This process can be time-consuming and sometimes require terminal bleeding of the host animal. The technique for producing and amplifying monoclonal antibodies is also time-consuming and expensive. An alternative method is the use of enzyme conjugated protein A or G, both of which bind specifically to the Fc region of IgG subclasses (Ejercito *et al.*, 1993). However, this method is not species specific and protein A and G have variable binding with the IgG of different species. As a result, ELISA using this detection method would not be as effective as polyclonal or monoclonal anti-species antibodies. Competitive ELISA can be used in instances where secondary antibodies are not available for the target species; nevertheless, the technique has rarely been used in serological surveys for *C. burnetii*. Only one instance could be identified in literature, where a competitive ELISA (cELISA) was developed for detection of antibodies to *C. burnetii* in camel sera (Soliman *et al.*, 1992). In this study, the method was compared to ELISA using peroxidase-conjugated protein A and found to be more sensitive for the detection of antibodies to *C. burnetii*. The cELISA was also compared to both standard indirect ELISA and IFA for the detection of antibodies to *C. burnetii* in species with available secondary antibodies. The competitive ELISA was found to be more sensitive than either assay, with the ELISA found to be more sensitive than the IFA (Soliman *et al.*, 1992).

2.8.1.2 Molecular detection

Active shedding of *C. burnetii* has been described in seronegative animals (Berri *et al.*, 2001). While this may be due to the relatively low sensitivity of some serological tests, anti-complementary activity or the prozone effect, it has implications for the monitoring

of Q fever. Serological tests are disadvantaged in that they determine exposure, not presence of an organism. Moreover, molecular techniques are only useful in active infections and cannot fully determine the epidemiology of infectious diseases. In the study by (Berri *et al.*, 2001), 24% of parturient sheep were ELISA positive, 32% IF positive and 44% were PCR positive. This indicated that approximately 20% of animals tested were shedding *C. burnetii* despite being seronegative. However, five weeks post-partum, 47% of sheep were ELISA positive and 6% were PCR positive. These findings may indicate that recently infected animals are capable of shedding the organism before a detectable IgG response is present. As the authors did not consider using an anti-IgM assay, it is unknown whether early infection could be detected serologically with comparable sensitivity to the PCR assay. PCR assays directed against the IS1111 repetitive transposable region have been used to detect *C. burnetii* in genital swabs, milk and faecal samples of both cattle and sheep (Berri *et al.*, 2000; Guatteo *et al.*, 2006). Therefore, this assay has been proposed as a means of monitoring *C. burnetii* excretion and controlling the spread of the organism in livestock. PCR assays have been used to detect *C. burnetii* in ticks and wildlife (Smetanova *et al.*, 2006). An oligonucleotide-chip based assay has also been developed for detecting *C. burnetii* and other tick-borne bacteria in ticks (Blaskovic and Barak, 2005).

2.8.2 Control measures

The ability to control Q fever is impeded by the presence of wild reservoirs of infection and the vertical transmission of *C. burnetii* in the tick vector. Measures have been proposed to reduce the spread of the organism among livestock, thereby reducing the risk of human infection (Arricau-Bouvery and Rodolakis, 2005). These measures include the use of specific location or facility for animal parturition which can be disinfected along with associated instruments. In addition, placental material should be disposed of immediately to prevent dissemination of *C. burnetii* into the environment. In Australia, it is recommended that at risk personnel should be vaccinated against Q fever (Parker *et al.*, 2006). However, in countries where vaccination is not available it is

recommended that abattoir workers exercise caution when removing mammary glands and internal organs (WHO, 1986). Due to the shedding of *C. burnetii* in milk it is advised that milk be pasteurised using the high-temperature, short-time (HTST) method at 72°C for 15 s (Arricau-Bouvery and Rodolakis, 2005) to inactivate the organism. As *C. burnetii* can be shed in faeces and manure has been implicated in infection, it is recommended that manure be covered or treated with lime or 4% calcium cyanamide before use. While the efficiency of this method in inactivating *C. burnetii* has not been fully evaluated, it is still advisable (OIE, 2004). To prevent the spread of *C. burnetii* to uninfected livestock populations, it has been proposed that both serology and PCR be performed on new additions to detect shedding beasts. In order to prevent re-infection of *C. burnetii*-free livestock, it is advised that livestock be protected from tick infestation with regular dipping or spraying with acaricidal products (WHO, 1986).

Commercial *C. burnetii* vaccines are available in France and Slovakia (Maurin and Raoult, 1999). Vaccines based on phase I and II *C. burnetii* have been produced, with phase I whole cell vaccines being the most effective (Arricau-Bouvery *et al.*, 2005). The phase II vaccine available in France has been shown to be poorly protective, as goats that had been vaccinated were implicated in an outbreak of Q fever (Fishbein and Raoult, 1992). Specifically, the vaccines are only protective in animals that are uninfected at the time of vaccination (Maurin and Raoult, 1999). Trials using phase I based vaccines have reported mixed results in the reduction of *C. burnetii* shedding. One study found that vaccination of unexposed heifers protected them from infection from a naturally infected herd and prevented shedding of the organism (Sadecky *et al.*, 1975). Others found that shedding, while greatly reduced, was not completely prevented in challenged, vaccinated sheep (Brooks *et al.*, 1986; Lang *et al.*, 1994) and cattle (Biberstein *et al.*, 1977). However, these animals were given unnaturally high challenge doses and it was thought that under normal circumstances the vaccine would be effective (Lang *et al.*, 1994). Vaccination of animals is not widely used as there has been no comprehensive evaluation of the effectiveness of vaccine prophylaxis in its ability to prevent animal and human infection (Maurin and Raoult, 1999). In contrast, the use of the phase I based vaccine, Bodibion (Bioveta, Slovakia) in Slovakia significantly

reduced the incidence of human Q fever (Serbezov *et al.*, 1999). Widespread vaccination of animals has not been adopted in any other countries. While vaccination may provide an effective means of preventing *C. burnetii* infection in animals, the only effective vaccines to date have been produced from inactivated phase I bacteria. Consequently, this prevents the distinction between vaccinated and naturally infected animals (Arricau-Bouvery and Rodolakis, 2005).

Several studies have investigated the application of antibiotic therapy on reducing *Coxiella*-related abortions and persistent shedding of the organism. The use of oxytetracycline in a sheep flock with *Coxiella*-related reproductive problems was found to reduce the number of abortions, shedding of *C. burnetii* and transmission of the organism (Berri *et al.*, 2005). Furthermore, antibiotic therapy did not fully eliminate abortion and shedding in this study. Another study on persistently infected cattle found that treatment with chlortetracycline resulted in dramatic reduction in shedding of *C. burnetii* in milk and following parturition (Behymer *et al.*, 1977). However, this study employed the inoculation of mice and subsequent staining for the organism in splenic tissue, a method which may be less sensitive than modern PCR-based detection techniques. In addition, this study reported that oral treatment with chlortetracycline may suppress rather than eradicate *C. burnetii*. Further investigations would be required, particularly in conjunction with real time PCR to establish effective antibiotic dosages for livestock. Therefore, it would be necessary to determine whether continual antibiotic therapy is a cost-effective means of reducing *C. burnetii* shedding in animals (Arricau-Bouvery and Rodolakis, 2005).

More drastic control measures were adopted in the Netherlands following a Q fever epidemic beginning in 2007 (Roest *et al.*, 2011). In the 2007-2009 period there were 3,523 human Q fever cases reported. This scale of outbreak was due to the proximity to aborting goats and the relatively high susceptibility of the human population. In the 2005 to 2009 period, *C. burnetii*-related abortions in goats and sheep were reported on 30 properties. Initial control measures involved restricting access to affected farms and voluntary vaccination of goats with Coxevac®, an as yet unregistered phase I vaccine

for ruminants. Phase I animal vaccines have been found to reduce the number of abortions and excretion of *C. burnetii* in challenged pregnant goats that are uninfected prior to receiving the vaccine (Arricau-Bouvery *et al.*, 2005). These vaccines have also been found to reduce *C. burnetii* excretion in infected goat herds, particularly in young animals vaccinated prior to onset of the breeding season (Rousset *et al.*, 2009b). However, the effect of vaccination in infected goats is unknown, and these animals may continue to shed coxiellae (Roest *et al.*, 2011). Vaccination was later made mandatory in affected areas in 2008 and extended into neighbouring areas in 2009. In 2009, more stringent, mandatory hygiene protocols were introduced for goat and sheep farms in the Netherlands, regardless of Q fever status. These protocols involved vermin control, compulsory rendering of aborted foetuses and placentas, strict manure handling rules and improved farm hygiene. Manure handling measures included bans on removing manure from deep litter stables for one month post kidding season, mandatory covering of manure during storage and transport and mandatory underplowing of manure when spread on farm land or storage of manure for at least three months. Improved farm hygiene measures included prevention of dust and aerosol formation, protective industrial clothing, use clean delivery equipment and clean bedding material. Later in 2009, additional control measures were included, with Q fever being notifiable on goat and sheep farms with bulk milk tank PCR positivity and abortion rates of greater than 5%. Removal of animals from and visiting Q fever-positive properties was banned. Bulk milk tank monitoring using PCR was initially to be performed every two months, but was increasing to every two weeks. Despite all the control measures adopted, it was believed that vaccination program may not be sufficient to reduce *C. burnetii* excretion in the 2010 kidding season due to failure to vaccinate all animals prior to the 2009 breeding season (Roest *et al.*, 2011). It was believed that these animals constituted a continuing reservoir of *C. burnetii* that would hinder control efforts. Due to this, a decision was made to cull all pregnant goats on Q fever positive properties which began in late 2009 and ended mid 2010. A ban on breeding dairy goats and sheep was in place during this period. The effectiveness of the measures adopted is unclear due to the lag time of the measures and potential contribution of environmental contamination with *C. burnetii*. Fewer Q fever cases were reported in 2010, and it is believed this may be

due to the control measures implemented. This outbreak highlighted the importance of monitoring and use of surveillance systems in assessing and controlling Q fever.

2.9 Summary

Coxiella burnetii has almost worldwide distribution with a broad spectrum of potential hosts including mammals, reptiles, birds and arthropods (Babudieri, 1959). Zoonotic pathogens capable of infecting both wildlife and domestic animals, such as *C. burnetii* are more likely to form a reservoir of infection for the human population (Chomel *et al.*, 2007). As such, it is important to effectively monitor and control zoonotic agents with overlapping host populations to reduce the impact on both animal and human health. The surveillance for *C. burnetii* in the animal population is hampered by variation in diagnostic and detection techniques between laboratories. The lack of readily available secondary antibodies for potential wild reservoir species is also an issue in determining the extent of coxiellosis in the environment and its impact on transmission of *C. burnetii*. Standardised protocols and thresholds for determining positivity should be established to enable meaningful comparison of data between surveys performed in different locations.

Effective control of the transmission of *C. burnetii* is made difficult by the existence of wildlife reservoirs and the vertical transmission of the organism in arthropod hosts. In addition, properties of *C. burnetii* such as the resistance to extreme environmental conditions, persistence in the environment, ease of dispersal via aerosol and high infectivity contribute to difficulty in controlling spread of the pathogen. However, the transmission of the organism between livestock could be reduced with the widespread adoption of recommendations that would limit spread of fomites. These measures would indirectly reduce transmission to humans. Additionally, the development of effective vaccines that could be administered to domestic animals would reduce shedding of the

organism, and if widely adopted, result in considerable reduction in coxiellosis and Q fever prevalence.

Further data is required to determine the implications of carriage of *C. burnetii* in animals for human and animal health and potential outbreaks of Q fever. The current situation, with the use of different serological assays and PCR targets complicates direct comparison of prevalence data and epidemiological investigations. The universal adoption of specific serological and molecular assays for the diagnosis and detection of *C. burnetii* in animals is required to establish prevalence data that is comparable worldwide.

CHAPTER THREE

GENERAL MATERIALS AND METHODS

3.1 *Coxiella burnetii* Isolates

3.1.1 Nine Mile II clone 4

Nine Mile II clone four (NMII/C4) was obtained by plaque purification of the Nine Mile isolate of *C. burnetii* following 304 guinea pig passages, 90 embryonated egg passages, one tissue culture passage and four embryonated egg passages (Denison *et al.*, 2007). The isolate was originally isolated from ticks in Montana, USA in 1935 (Davis and Cox, 1938). NMII/C4 isolate antigens were used in optimisation and validation of ELISA. This phase II isolate was obtained from the Australian Rickettsial Reference Laboratory, Geelong and maintained in cell culture.

3.1.2 Arandale

The phase I Arandale isolate was obtained from the Australian Rickettsial Reference Laboratory, Geelong. The isolate was a clinical isolate from a patient who contracted Q fever after assisting in a goat birthing. The phase I variant was maintained by animal passage. The phase II variant was maintained in Vero cell culture.

3.1.3 Cumberland

The phase I Cumberland isolate was obtained from the Australian Rickettsial Reference Laboratory, Geelong. The isolate was a clinical isolate from a patient who contracted

Q fever through contact with beef cattle. The phase I variant was maintained by animal passage. The phase II variant was maintained in Vero cell culture.

3.2 Animals

3.2.1 Mice

Six-10 week old A/J mice of either sex were used in animal infection experiments and animal passage of phase I *C. burnetii*. All mice were supplied by the James Cook University Small Animal Breeding Unit, Townsville. They were housed in the School of Veterinary and Biomedical Sciences PC3 small animal facility at James Cook University and provided with pellet food and water *ad libitum*. Mice were used under James Cook University Animal Ethics Approval number A1139.

3.2.2 Guinea pigs

Outbred guinea pigs between ages of 20 and 40 weeks of either sex were used in animal infection experiments with *C. burnetii*. All guinea pigs were supplied by the James Cook University Small Animal Breeding Unit, Townsville. They were housed in the School of Veterinary and Biomedical Sciences PC3 small animal facility at James Cook University and provided with pellet food and water *ad libitum*. Guinea pigs were supplemented with vitamin C, dissolved in drinking water. Guinea pigs were used under James Cook University Animal Ethics Approval numbers A1422 and A1139.

3.2.3 Chickens

Embryonated eggs of white leghorn chickens were obtained from the James Cook University breeding colony. They were incubated in a humidified chamber at 37°C with

automated turning for approximately 21 days until hatch. Chicks were provided with water and starter crumble *ad libitum*. They were brooded for approximately one week before being moved to an enclosure physically separated from other chickens. Chickens were raised for six weeks prior to commencement of experimental work. Chickens were used under James Cook University Animal Ethics Approval number A1205.

3.3 Human Ethics

3.3.1 Q fever patients

Blood was collected in 6 mL vacutainers from Q fever patients by a trained phlebotomist following informed consent. Ethics approval (#60/05) was provided by the Townsville Health Service District Institution Ethics Committee.

3.3.2 Blood donors

Serum was isolated from blood collected by the Australian Red Cross Blood Service (ARCBS) in Townsville during routine blood donations. Ethics approval (#2010#2) was provided from the Australian Red Cross Blood Service Ethics Committee on the condition that no further indentifying information regarding the donors was provided.

3.4 Propagation of *Coxiella burnetii*

3.4.1 Vero cell culture

For conversion to and maintenance of the phase II variant, all isolates of *C. burnetii* were propagated in Vero cell culture. Cultures were performed in 25 cm^2 cell culture flasks in DMEM (Invitrogen, Australia) with 2 mM L-glutamine, 4.5 g L^{-1} glucose, 110 mg L^{-1} sodium pyruvate (Invitrogen, Australia) and 5% FBS (Invitrogen, Australia). Cells were incubated at 37°C with 5% CO_2 until host cell budding was apparent. Cells were passaged weekly. The monolayer was disrupted using a sterile cell scraper, transferred to sterile falcon tube and cells pelleted by centrifugation at 500×*g* for 10 min at room temperature in sealable swing out buckets. Vero cells were resuspended in fresh media and split into further 0.2 µm filter capped 25 cm^2 cell culture flasks. A record of passage number was maintained. Vero cells were resuspended in DMEM with 20% FBS and 10% DMSO (Sigma, Australia) for cryopreservation in cryovials (Nunc, Denmark) in liquid nitrogen.

3.4.2 Embryonated chicken egg culture

To obtain purified stocks of *C. burnetii*, the bacterium was propagated in embryonated chicken eggs. Prior to inoculation, eggs were cleaned and treated with iodine tincture. A hole was made above the air sac with a sterile 22 gauge needle. A 100 µL aliquot of organism diluted in PBS, pH 7.4 was inoculated into the yolk sac of five-six day old embryonated eggs with 1.5 inch 25 gauge needle and 1 mL syringe through the air sac. The hole was sealed with cyanoacrylate glue and incubated at 37°C and 60% relative humidity for seven days with regular turning in an incubator (Brinsea, UK). Prior to extraction, the egg shell was swabbed with iodine tincture and a small incision was made in the egg shell with a sterile 18 gauge needle, after which the egg shell was carefully removed. Contents of egg were carefully tipped into a sterile dish and contents of the yolk sac were removed with an 18 gauge needle and 10 mL syringe. Yolk sacs were

pooled and homogenised with three parts PBS, pH 7.4 (Appendix A). Yolk sac homogenates were diluted 100 fold in PBS, pH 7.4 with 500 U mL^{-1} penicillin and 0.5mg mL^{-1} streptomycin and inoculated into further five-six day old embryonated chicken eggs for passage. *Coxiella burnetii* can be passaged using this method for a maximum of six times before conversion to the phase II variant (Stoker, 1953). Approximately 10 µL yolk sac homogenates were subcultured on blood agar and incubated at 37°C aerobically and under 5% CO_2 for 24 hrs to test for contamination. A further 10 µL yolk sac homogenate was applied to a glass slide in a smear for Gimenez staining (Section 3.5) to test for purity.

3.4.3 Propagation of *Coxiella burnetii* in A/J mice

For maintenance of the phase I variant, Arandale and Cumberland isolates of *C. burnetii* were passaged in A/J mice. Six-10 week old A/J mice of either sex were inoculated *i.p.* with 100 µL *C. burnetii* suspension in DMEM with 3/4 inch 27 gauge needles and 1 mL syringes. Suspensions of *C. burnetii* were obtained from either original organ macerates provided by the Australian Rickettsial Reference Laboratory or from passage in cell culture (Section 3.3.1), embryonated chicken eggs (Section 3.3.2) or previously infected mice (Section 3.3.3). Mice were euthanised by CO_2 asphyxiation after four days and the spleen removed. Spleens were homogenised and passed through a sterile stainless steel strainer. Spleen homogenates were diluted in PBS, pH 7.4. *Coxiella burnetii* was extracted according to the protocol described in Section 3.6.2. Purified *C. burnetii* was resuspended in either standard DMEM for further animal passage or DMEM with 10% glycerol for cryopreservation in o-ring sealed microtubes (Astral Scientific, Australia) at -80°C.

3.5 Bacterial Extraction and Antigen Production

3.5.1 Vero cell culture

All propagation and manipulation of *C. burnetii* was performed under PC3 conditions. Vero cell monolayers infected with *C. burnetii* were disrupted using a sterile cell scraper, transferred to a sterile falcon tube and cells pelleted by centrifugation at 500 ×*g* for 10min at 20°C in sealable swing out buckets. Vero cells were resuspended in PBS, pH 7.4 and sonicated for two 40 sec bursts at 20 kHz with a 30 sec break with a microtip sonicator (Misonix, USA) in a class II biological safety cabinet. *Coxiella burnetii* was separated from cell debris by centrifugation at 550 ×*g* at 20°C for 10 min. The resultant supernatant was layered over 25% sucrose with a 2:3 ratio of supernatant to sucrose, then centrifuged at 2,250 ×*g* at 20°C for 20 min. The supernatant was discarded and the *C. burnetii* pellet resuspended in PBS, pH 7.4. *Coxiella burnetii* was inactivated with 1.6% formalin for 48 hrs at 4°C. Inactivation was confirmed by inoculation of uninfected vero cell monolayers and absence of viable *C. burnetii* by both microscopic examination and RT-PCR for the *com1* gene (Sections 4.3.2.3 and 3.7.2). RT-PCR is used as standard PCR will detect non-viable *C. burnetii*, whereas RT-PCR can detect the expression of genes in viable cells. Inactivated *C. burnetii* were pelleted at 10,000 ×*g* and 20°C for 10 min and washed three times in sterile ddH_2O. The inactivated cells were resuspended in sterile ddH_2O, after which they were referred to as phase II or phase I antigen for Cumberland (CII or CI) and Arandale (AII or AI) strains respectively. Whole cell antigen was stored at -70°C. For lysates, purified *C. burnetii* were sonicated for five 2 min bursts at 20 kHz with 30 sec breaks with a microtip sonicator (Misonix, USA) in a class II biological safety cabinet. Antigen was stored at -70°C in o-ring sealed microtubes (Astral Scientific, Australia).

3.5.2 Mouse spleen

Mice infected with *C. burnetii* were euthanised by CO_2 asphyxiation after four days and the spleen removed. Spleens were homogenised and passed through a sterile stainless steel strainer. Spleen homogenates were diluted in PBS, pH 7.4 and sonicated as described in Section 3.4.1. *Coxiella burnetii* was separated from cell debris as described in Section 3.4.1. *Coxiella burnetii* was not inactivated if intended for further animal passage, embryonated chicken egg culture, Vero cell culture or cryopreservation.

3.5.3 Embryonated chicken eggs

A small incision was made in the egg shell as described in Section 3.3.3. Contents of egg were carefully tipped into a sterile petri dish and contents of the yolk sac were removed with an 18 gauge needle and 10 mL syringe. Yolk sac contents were pooled and homogenised with three parts PBS, pH 7.4. The lipid supernatant was discarded and the remaining yolk sac homogenate centrifuged at 500 ×*g* for 30 min at 20°C. The pellet was resuspended in PBS, pH 7.4 and the centrifugation repeated. *Coxiella burnetii* was separated from cell debris as described in Section 3.4.1. *Coxiella burnetii* was inactivated with 1.6% formalin for 48 hrs at 4°C. Inactivated *C. burnetii* were pelleted at 10,000 ×*g* and 20°C for 10 min and washed three times in sterile ddH_2O. The inactivated cells were resuspended in sterile ddH_2O, after which they were referred to as phase II or phase I antigen for Cumberland (CII or CI) and Arandale (AII or AI) strains respectively. Antigen was stored at -70°C in o-ring sealed microtubes (Astral Scientific, Australia).

3.6 Gimenez Staining

Gimenez staining was performed on smears of pelleted *C. burnetii* following extraction and purification to confirm purity. Briefly, approximately 20 µL *C. burnetii* suspension was applied to a glass slide in a smear, air dried then heat fixed. Gimenez stain (Appendix A) was applied to cover the smear and incubated at room temperature for 1 min. The stain was then washed off with tap water and counter-stained with 0.8% malachite green solution (Appendix A) for 9 sec. The slide was washed off again, dried and examined under oil immersion for the presence of host cell or bacterial contamination.

3.7 Quantitative PCR for *com1* gene in *Coxiella burnet*ii

3.7.1 Standard curve construction

To quantify *C. burnetii* at each passage, a standard curve was generated using genome equivalents (GE) of *C. burnetii* NMII/C4. This strain was selected as the full genome sequence, genome size and GC content have been determined previously. Copies of the single-copy *com1* gene were calculated as follows:

Copies of *com1* gene:

$$\frac{\text{X ng NMII/C4 DNA} \times 6.022\times10^{23}}{1{,}969{,}275 \text{ bp} \times 1\times10^{9} \times 650}$$

3.7.2 Quantitative PCR

To quantify *com1*, PCR reactions of 20 µL reaction volume were set up (Table 4.2) for a 36-well rotor in a RotorGene 3000 (Corbett Research, Australia). A primer set targeting the *com1* gene in *C. burnetii* was sourced from the literature (Marmion *et al.*, 2005). Primers were resuspended in TE buffer (Amasco, USA) to a stock concentration of 100 pm μL^{-1} and stored at -20°C. Standards constructed as described previously (Section 3.7.2) were included, as were positive and no-template controls. Cycling conditions for quantitative real time PCR consisted of an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 10 sec at 94°C, annealing of primers for 10 sec at 62°C, extension for 20 sec at 72°C. A melt curve analysis was then performed with an increase in temperature from 72°C to 95°C in 1°C increments.

Table 3.1: Real time PCR reagents

REAGENT	CONCENTRATION
ddH_20	to 720 µL
Immomix (Bioline)	1×
forward primer (Sigma Genosys)	300 nM
reverse primer (Sigma Genosys)	300 nM
SYTO-9 (Invitrogen)	10 µM
template DNA	10-30 ng

CHAPTER FOUR

OPTIMISATION OF ANTIGEN PREPARATION FOR SEROLOGICAL SCREENING FOR *COXIELLA BURNETII* AND COMPARISON OF ISOLATE VIRULENCE

4.1 Introduction

Coxiella burnetii exists in two antigenically different phases, which are characterised by transition of lipopolysaccharide (LPS) from a smooth form with full-length O-side chains in phase I to a rough form with truncated O-side chains in phase II. In human Q fever, differences in titres of immunoglobulin subsets to phase I or phase II antigens form the basis for diagnosis of acute or chronic Q fever (Maurin and Raoult, 1999). A variety of serological tests are used to detect antibodies, the most common being immunofluoresence assay (IFA) and ELISA. The IFA test has become the most commonly used diagnostic test for Q fever. Nonetheless, there is some debate as to whether the IFA is superior to ELISA (Devine, 1998). A recent study conducted in the Netherlands (Blaauw *et al.*, 2011) found that the use of IFA or ELISA resulted in different estimates of seroprevalence in the same population of 27.3% and 16.2% respectively. Of the two assays the ELISA was found to be more specific and IFA more sensitive. Agreement between the assays only improved when the threshold for positive values in IFA was increased 8-fold (Blaauw *et al.*, 2011). Generally, ELISA is more suited to seroprevalence studies due to the potential for automation. However, ELISA has not been standardised for antigen preparation or positive threshold.

While several studies have found ELISA to be the most practical assay for use in seroprevalence surveys for *C. burnetii*, there is some discrepancy between the antigens used in between different laboratories. Many Q fever ELISAs use formalin-inactivated

whole cells as antigen (Cowley *et al.*, 1992; Uhaa *et al.*, 1994; Field *et al.*, 2002), while others have used sonicated *C. burnetii* cells (Williams *et al.*, 1986; Peter *et al.*, 1987). Williams *et al* (1986) compared whole cell antigen to disrupted *C. burnetii* antigens prepared by various methods. The study found whole cell antigen to be most effective and the authors recommended this form of antigen be used in all ELISAs for Q fever to standardise assays. Further comparisons would be required to determine the optimum antigen preparation for use in ELISA for detection of anti-*Coxiella* antibodies in animals.

It has been demonstrated that animals infected with *C. burnetii* produce antibodies to both phase I and phase II antigens (Marrie *et al.*, 1993). However, how this relates to disease state is unclear. It is therefore important to test for antibodies to both antigenic phases of *C. burnetii* in animal enzyme immunoassays to determine exposure. Historically, complement fixation tests (CFT) were used for serological testing for *C. burnetii* in animal sera. More recent studies have indicated ELISA was more sensitive than CFT for the detection of antibodies in animals (Rousset *et al.*, 2007; Rousset *et al.*, 2009a). Commercial animal coxiellosis ELISAs consist of a mixture of phase I and phase II antigen. Two commercial ELISA kits were available for the detection of antibodies to *C. burnetii* in ruminants, the CHECKIT Q Fever Antibody ELISA Test Kit (IDEXX Laboratories, USA) and the Q Fever Serum Screening ELISA (Pourquier Institute, France). Both ELISAs used mixed antigenic phase II and I whole cell antigens from the Nine Mile isolate and were developed and validated in Europe. Recently, the Pourquier Institute became a subsidiary of IDEXX Laboratories and the IDEXX Q fever Ab Test is now the only commercially available test kit for the detection of antibodies to *C. burnetii* in ruminants.

In a recent Australian study conducted in Western Australia (Banazis *et al.*, 2010), a commercial coxiellosis ELISA developed in Europe (IDEXX CHEKiT Q fever ELISA) was used. In this study very low numbers of seropositive samples were detected in beef cattle and sheep using this ELISA despite relatively high numbers of seropositive samples being detected in kangaroo samples from the same areas using an in-house

ELISA. After the introduction of the use of both commercially available Q fever ELISAs at the Department of Primary Industries Laboratory in Victoria, Australia it was found that results obtained with these ELISAs did not correlate with CFT results from positive and negative samples provided by the Australian National Quality Assurance Programme (Kittelberger *et al.*, 2009). The contradictory results were confirmed by the Investigation and Diagnostic Centre-Animal Health Laboratory in New Zealand (Kittelberger *et al.*, 2009). Differences in antigen production, such as the use of whole cell antigen, lysates or chemical extracts may account for variation in ELISA results. Also, it may be possible that there are antigenic differences between *C. burnetii* isolates from Europe and Australia that may account for the variations in ELISA results.

One of the problems in producing *C. burnetii* antigen for use in serological tests is the difficulty in determining the precise phase state of the antigen. Traditionally, complement block titration has been the technique of choice for differentiating between phase states. In this method, antigen is combined with the serum of animals infected with phase I or phase II *C. burnetii*. As antibodies will be raised to both phases in natural infection, and phase II only following infection with a phase II strain, antigen can be differentiated by whether the antigen preparation reacts with one or both serum groups. However, complement fixation based tests are disadvantaged in that they are subjective and there can be high levels of anti-complementary activity (Schmidt and Harding, 1956; Lang, 1988).

The changes in LPS following phase variation are thought to be irreversible due to chromosomal DNA deletions (Hoover *et al.*, 2002). However, phase I organisms have been recovered following back passage in animals of *C. burnetii* considered to be in phase II following passage in cell culture (Stoker and Fiset, 1956; Kazar *et al.*, 1975). Inability to revert back to phase I from phase II has only been demonstrated in strains that have been plaque purified to a homogenous phase II culture (Ormsbee and Marmion, 1990). Strains that have not been plaque purified, such as the Henzerling RSA 331, M44 and Australian QD do not demonstrate deletions in phase II and have been found to express genes related to O-antigen synthesis, albeit at lower level than phase I

(Denison *et al.*, 2006). Therefore, it may be possible to determine phase state according to expression levels of genes associated with LPS synthesis.

Virulence is highly variable among *C. burnetii* strains, with strains such as Nine Mile I, Luga and S causing high febrile reactions and lethal infection while strains such as Priscilla cause low febrile reactions and non-lethal infection when used to infect mice or guinea pigs at a similar dose (Kazar *et al.*, 1993). Restriction fragment length polymorphism (RFLP) analyses have resulted in the division of *C. burnetii* isolates into six genomic groups (I to VI) (Hendrix *et al.*, 1991). The heterogeneity between strains leading to this subdivision was thought to be due to repeat regions in the genome of some isolates. An extensive microarray based comparison of the whole genomes of 24 *C. burnetii* strains confirmed the RFLP based groupings and identified a further two distinct genomic groups (Beare *et al.*, 2006). These groupings were further validated by the application of multi-locus variable nucleotide tandem repeat analysis (VDTR) (Svraka *et al.*, 2006). Virulence and acute or chronic disease manifestation are thought to be related to genotype (Russell-Lodrigue *et al.*, 2009).

To date, very little comparison of the virulence of Australian Q fever isolates has been performed. The relative ability of *C. burnetii* isolates to cause disease may have an effect on their suitability for use as antigen in ELISA, particularly with isolates that result in no acute disease in animal models, such as Priscilla and Dugway. Two Australian Q fever strains of clinical origin were isolated recently from acute Q fever patients at the Hunter Area Pathology Unit in conjunction with the Australian Rickettsial Reference Laboratory. The Arandale isolate came from a patient that acquired Q fever following assistance with a birthing goat. The Cumberland isolate came from a patient who was thought to have contracted Q fever following contact with beef cattle. This thesis has suggested the Arandale isolate is significantly less virulent than the Cumberland isolate in animal models of infection.

The experimental work outlined in this chapter aimed to determine the optimal antigen preparation for use in animal ELISAs for *C. burnetii* exposure. This experimental work

also aimed to produce an alternative method for determining the antigenic phase state of *C. burnetii* used as antigen. A comparison of the relative virulence of two Australian Q fever isolates (Cumberland and Arandale) was performed in mice and guinea pigs to determine suitability for use as antigen in animal ELISAs for *C. burnetii* exposure.

4.2 Aims

The specific aims for the work described in this chapter were to:

1. Investigate differences in virulence in two Australian *C. burnetii* isolates and determine an optimal isolate for use in antigen preparation for anti-*C. burnetii* antibody detection in ELISA;
2. Investigate whether antigen phase variation could be determined using qRT-PCR;
3. Determine the optimal antigen preparation for anti-*C. burnetii* antibody detection in ELISA; and
4. Develop ELISAs for the detection of antibodies to *C. burnetii* in human and animal sera.

4.3 Materials and Methods

4.3.1 Comparison of virulence in Australian *Coxiella burnetii* isolates

4.3.1.1 Arandale isolate in mouse model

Arandale isolate *C. burnetii* was passaged once in six A/J mice and extracted as described in Sections 3.4.3 and 3.5.2. DNA was extracted using High Pure PCR Template Preparation kit (Roche, Germany) according to manufacturer's instructions for bacterial DNA. Dose determination was performed using *com1* qPCR assay as described previously in Section 3.7. One group of 10 female, 12 week old C57/Bl6 mice were inoculated *i.p.* with 100 µL of live Arandale isolate at 1×10^7 genome equivalents (GE) using 27 gauge needles and 1 mL syringes. Another group of 10 female, 12 week old C57/Bl6 mice were inoculated *i.p.* with 100 µL PBS using 27 gauge needles and 1 mL syringes. Mice were maintained for two weeks, euthanised with CO_2 and blood collected via cardiac puncture. Serum was separated by centrifugation at 1,400 ×*g* for 10 min at room temperature. Indirect ELISA was performed against NMII/C4 whole cell antigen (NMII) at 25 µg mL^{-1} using mouse sera at a dilution of 1:100 and rat anti-mouse IgG (BD Pharmingen, Australia) at a dilution of 1:1,000. Plates were incubated at 37°C for 1 hr for each reagent and washed three times with PBS-T (Appendix A) between each incubation step. Binding was visualised with ABTS (Tropbio, Australia) at 37°C for 30 min and optical density determined at 414/494nm. The murine ELISA for detecting anti-*C. burnetii* antibodies was developed and optimised in a previous study (Cooper, 2006). Mann-Whitney U tests were used determine whether there were statistically significant differences between optical density values for infected and uninfected animals. Mann-Whitney U tests were also used to determine whether there were statistically significant differences between optical density values for animals infected with the different *C. burnetii* isolates.

4.3.1.2 Cumberland isolate in mouse model

Cumberland isolate *C. burnetii* was passaged once in six A/J mice and extracted as described in Sections 3.4.3 and 3.5.2. DNA was extracted using High Pure PCR Template Preparation kit (Roche, Germany) according to manufacturer's instructions for bacterial DNA. Dose determination was performed using *com1* qPCR assay as described previously in Section 3.7. One group of 10 female, 12 week old C57/Bl6 mice were inoculated *i.p.* with 100 µL of live Cumberland isolate at 1×10^7 GE using 27 gauge needles and 1 mL syringes. Another group of 10 female, 12 week old C57/Bl6 mice were inoculated *i.p.* with 100 µL PBS using 27 gauge needles and 1 mL syringes. Mice were maintained for two weeks, euthanised with CO_2 and blood collected via cardiac puncture. Serum was separated by centrifugation at 1,400 ×*g* for 10 min at room temperature. Indirect ELISA was performed as described previously in Section 4.3.1.1.

4.3.1.3 Arandale isolate in guinea pig model

Arandale isolate *C. burnetii* was passaged once in 30 A/J mice and extracted as described in Sections 3.4.3 and 3.5.2. This was used as the infective material for guinea pigs. DNA was extracted using High Pure PCR Template Preparation kit (Roche, Germany) according to manufacturer's instructions for bacterial DNA. Dose determination was performed using *com1* qPCR assay as described previously in Section 3.7. Four groups of four guinea pigs were inoculated *i.p.* with 100 µL of live Arandale isolate at 1×10^5, 1×10^6, 1×10^7 and 1×10^8 GE using 27 gauge needles and 1 mL syringes. Doses of 1×10^9 GE could not be achieved with the Arandale isolate as it failed to replicate well in animal passage. A further group of four guinea pigs received sham injections of PBS using 27 gauge needles and 1 mL syringes. Rectal temperatures were recorded at day zero, two, three, four, six, eight, 10, 11, 13 and 15 for each animal. At the conclusion of the trial, guinea pigs were euthanised with CO_2 and blood collected via cardiac puncture. Serum was separated by centrifugation at 1,400 ×g for 10 min at room temperature. Indirect ELISA was performed against NMII/C4 whole cell antigen at 25 µg mL^{-1} using guinea pig sera at a dilution of 1:100 and goat anti-guinea pig IgG

(ABD Serotec, USA) at a 1:5,000 dilution. Plates were incubated at 37°C for 1 hr for each reagent and washed three times with PBS-T (Appendix A) between each incubation step. Binding was visualised with ABTS (Tropbio, Australia) at 37°C for 30 min and optical density determined at 414/494nm. Mann-Whitney U tests were used determine whether there were statistically significant differences between optical density values for infected and uninfected animals. Mann-Whitney U tests were also used to determine whether there were statistically significant differences between optical density values for animals infected with the different *C. burnetii* isolates. The Kruskal-Wallis test was used determine whether there were statistically significant differences between maximum temperature values for infected and uninfected animals and between animals infected with the two *C. burnetii* isolates.

4.3.1.4 Cumberland isolate in guinea pig model

Cumberland isolate *C. burnetii* was passaged in 30 A/J mice and extracted as described in Sections 3.4.3 and 3.5.2. DNA was extracted using High Pure PCR Template Preparation kit (Roche, Germany) according to manufacturer's instructions for bacterial DNA. Dose determination was performed using *com1* qPCR assay as described previously in Section 3.7. Five groups of three guinea pigs were inoculated *i.p.* with 100 µL of live Cumberland isolate at 1×10^5, 1×10^6, 1×10^7, 1×10^8, 1×10^9 GE respectively using 27 gauge needles and 1 mL syringes. A further group of four guinea pigs received sham injections of PBS using 27 gauge needles and 1 mL syringes. Rectal temperatures were recorded at day zero, and each successive day for eight days for each animal. At the conclusion of the trial (14 days post inoculation), guinea pigs were euthanised with CO_2 and blood collected via cardiac puncture. Serum was separated by centrifugation at 1,400 ×*g* for 10 min at room temperature. Indirect ELISA was performed as described previously in Section 4.3.1.3.

4.3.2 Identification of phase differentiation

4.3.2.1 Passage of *Coxiella burnetii*

To investigate phase differentiation during serial passage in cell culture, passage of the Cumberland isolate of *C. burnetii* for a total of 15 passages was performed as described in Section 3.4.1. Four 25 cm^2 0.22 µm filter cap cell culture flasks were used at each passage with the excess cells from each flask passage used for DNA and RNA extraction.

4.3.2.2 Extraction of *Coxiella burnetii* DNA

Extraction of DNA from Cumberland isolate *C. burnetii* using a HighPure™ PCR Template Preparation Kit (Roche Diagnostics, Germany) DNA extraction kit according to the manufacturer's instructions for extraction of bacterial DNA. Purified DNA was stored at -20°C.

4.3.2.3 Extraction of mRNA and cDNA synthesis

Extraction of RNA from Cumberland isolate *C. burnetii* using Trisure™ RNA extraction solvent (Bioline, Australia) according to the manufacturer's instructions. Any remaining DNA was digested with DNaseI for 30 mins at 37°C. First strand cDNA synthesis was performed with reverse transcriptase (Fermentas, USA) and random hexamers (Sigma-Genosys, Australia). Initial reaction mixes were prepared with 0.1-5 µg RNA, 0.5 µg random hexamers and molecular biology grade water (Sigma, Australia) to a volume of 11 µL. The reaction mixes were incubated at 70°C in a Mastercycler® gradient thermocycler (Eppendorf, Germany) for 5 min then chilled on ice. The following reagents were then added in order; 1× reaction buffer (Fermentas, USA), 1mM DNTPs, 20 U ribonuclease inhibitor (Fermentas, USA) and molecular biology grade water to a volume of 19 µL. The reactions were then incubated at 37°C for 5 min, after which 200 U RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, USA) was

added. Reaction were incubated for a further 60 min at 42°C, stopped by heating to 70°C for 10 min then chilled on ice.

4.3.2.4 Primer design

Primers (Sigma Genosys, Australia) were designed for the real time amplification of 14 genes present in the O-Ag biosynthesis region of *C. burnetii* (Table 4.1). The genes selected were previously investigated in several isolates of *C. burnetii* by Denison *et al* (2006). These genes were CBU680 (UDP-glucose/GDP-mannose dehydrogenase), CBU686 (acetoin dehydrogenase), CBU688 (GDP-fucose synthetase), CBU689 (GDP-mannose 4,6-dehydratase), CBU690 (glycosyl transferase), CBU692 (dehydrogenase β subunit), CBU694 (glycosyl transferase), CBU696 (pleiotropic regulatory protein), CBU697 (pleiotropic regulatory protein), CBU703 (O-Ag ABC transporter), CBU704 (O-Ag ABC transporter), CBU830 (polysaccharide biosynthesis), CBU844 (capsular polysaccharide biosynthesis protein I) and CBU1661 (ADP-heptose LPS heptosyltransferase II). A primer set targeting the *com1* gene in *C. burnetii* was sourced from the literature (Marmion *et al.*, 2005). Primers were resuspended in TE buffer (Amasco, USA) to a stock concentration of 100 pm μL^{-1} and stored at -20°C.

Table 4.1: Primer pairs used in analysis of O-Ag biosynthesis during phase variation

Primer Name	Sequence 5'-3'	Product Size
CBU680-for	CTGGCTGAGTTGGCATCC	98 bp
CBU680-rev	CGGTTCTAAATACGCATACTGA	
CBU686-for	ACCACCGATCTAGCAGTCAGTA	122 bp
CBU686-rev	GCCGCAACCAGCAGTAACG	
CBU688-for	GCCTGCTGCCACCTTATG	90 bp
CBU688-rev	GATGCTAATTTCTTTACCACTTC	
CBU689-for	GTTTAATCCTCGCAGCCCTTAT	97 bp
CBU689-rev	GCCTGTTGACGCATGAAGC	
CBU690-for	CGGGTAATCAGAGAAATGC	130 bp
CBU690-rev	ATCACACCATTCATCAACTC	
CBU692-for	CAAACCATAGGCATCTTCAG	123 bp
CBU692-rev	CGCTATTGTGGGTAGAGG	
CBU694-for	TCGTCGTCACACAAATAAATG	130 bp
CBU694-rev	AGGATGAGCGTATTCGTTAC	
CBU696-for	CTTGGACAATAGCACCTACGGA	113 bp
CBU696-rev	CACAGGCACTGACGCATTAGC	
CBU697-for	CGTATTGGCTGTGGTAATC	107 bp
CBU697-rev	ATTAGAAGTCATTCGCTATCG	
CBU703-for	CGCTACAAACGAACCCTTATAG	106 bp
CBU703-rev	TGATTTCACGGTGCCACAAAG	
CBU704-for	GGGCGGGTAACTTCATTATTC	109 bp
CBU704-rev	GCTCCTGCTTTGTCATTCC	
CBU830-for	TTAACGACGAACAACACC	115 bp
CBU830-rev	AATCAGGCAGAAGATAAGG	
CBU844-for	CTACATAAGCGTAAGGATTAGT	114 bp
CBU844-rev	CTGACCGACAAGGAATGAC	
CBU1661-for	GCAATCCATAACGCATCTCAC	118 bp
CBU1661-rev	GAACGAGGCTACGACCAAG	

4.3.2.5 Standard curve construction

The standard curve for the *com1* gene was constructed as described in Section 3.7.1. Further standard curves were generated for the O-Ag biosynthesis region genes using

plasmids. DNA was amplified using conventional PCR with cycling conditions of an initial denaturation for 5 min at 94°C, followed by 35 cycles of denaturation for 30 sec at 94°C, annealing of primers for 20 sec at 62°C, extension for 30 sec at 72°C, with a final extension at 72°C for 5 min. DNA was electrophoresed at 200 V for 15 min in 2% agarose with bands excised and gel purified using a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan) according to the manufacturer's instructions. PCR products were ligated into TA vector using a TA vector cloning kit (Real Genomics, Taiwan) and used to transform DH5α *E. coli* HIT cells (Real Genomics, Taiwan) according to the manufacturer's instructions. White colonies were inoculated into 5 mL LB (Appendix A) and incubated overnight at 37°C with shaking at 150 RPM. Plasmid minipreps were performed using an RBC kit (Real Biotech, Taiwan) according to the manufacturer's instructions. Gene copy numbers were calculated using the following formulae:

Quantitation of plasmid DNA in fmol:

$$\frac{\text{X ng plasmid DNA } \mu\text{L}^{-1}}{0.66 \text{ ng fmol}^{-1} \text{ kbp}^{-1} \times (2.73 \text{ kbp} + \text{insert size}) \text{ kbp}}$$

Conversion to copies:

$$\frac{\text{X fmol } \mu\text{L}^{-1} \times 1 \text{ mol} \times 6.02\times10^{23} \text{ copies mol}^{-1}}{1\times10^{12} \text{ fmol}}$$

4.3.2.6 Quantitative PCR

To quantify *C. burnetii* and expressed genes, PCR reactions of 20 µL reaction volume were set up (Table 4.2) for a 36-well rotor in a RotorGene 3000 (Corbett Research, Australia). Standards constructed as described previously (Sections 3.7.1 and 4.3.2.5) were included, as were positive and no-template controls. Cycling conditions for

quantitative real time PCR consisted of an initial denaturation for 5 min at 94°C, followed by 40 cycles of denaturation for 10 sec at 94°C, annealing of primers for 10 sec at 62°C, extension for 20 sec at 72°C. A melt curve analysis was then performed with an increase in temperature from 72°C to 95°C in 1°C increments.

Table 4.2: Real time PCR reagents

REAGENT	CONCENTRATION
ddH_20	to 720 µL
Immomix (Bioline)	1×
SYTO-9 (Invitrogen)	10 µM
forward primer (Sigma Genosys)	300 nM
reverse primer (Sigma Genosys)	300 nM
template DNA	10-30 ng

4.3.3 Complement block titration

To determine antigenic phase of *C. burnetii*, serum from A/J mice infected with Phase I Cumberland isolate *C. burnetii* (anti-Phase I serum) and serum from A/J mice infected with Phase II NMII/C4 isolate *C. burnetii* (anti-Phase II serum) was diluted 1:10 in veronal buffer (Virion\Serion, Germany). Commercial control sera for phase I and phase II were included as anti-sera control (Virion\Serion, Germany). Endogenous complement was inactivated by incubation of diluted sera for 30 min at 56°C.

A complement block titration was set up according to the template in Figure 4.1 in a round bottom 96-well plate (Nunclon, Germany). Anti-phase I and anti-phase II serum was serially diluted across the plate in 25 µL aliquots. Phase I and Phase II antigen were added in 25 µL aliquots to each half of the plate. Complement was applied to each well of the plate. Complement controls were prepared by serially diluting complement in 25 µL aliquots across the bottom row of the plate. The plate was covered and incubated overnight at 4°C.

A 1:1 dilution of haemolytic anti-sheep erythrocyte serum (Virion\Serion, Germany) and 1% sheep erythrocyte suspension was prepared and incubated at 37°C for 30 min. The overnight plate was pre-warmed at 37°C for 15 min and 50 µL of serum/erythrocyte suspension was applied to each well of the plate. The plate was then incubated at 37°C for 15-30 min with shaking at 10 min intervals. Incubation was ceased when the complement controls for two and one units displayed complete haemolysis, after which the plate was centrifuged at 1,000 ×*g* for 5 min at room temperature. Wells with >50% inhibition of haemolysis were considered to be positive.

A control complement block titration plate was performed simultaneously with commercial phase I and phase II antigen (Virion\Serion, Germany).

		Phase I Antigen						Phase II Antigen					
	SC	1:10	1:20	1:40	1:80	1:160	SC	1:10	1:20	1:40	1:80	1:160
PI Serum												
PII Serum												
CC		2	1	0.5	0.25			2	1	0.5	0.25	

Figure 4.1: Complement block titration layout.
Serial dilutions of sera were combined with each antigen and complement, and then tested for inhibition of haemolysis with the addition of anti-erythrocyte serum and erythrocytes. SC represents serum control for anti-complementary activity and CC represents complement controls.

4.3.4 Optimisation of antigen preparation for use in ELISA

A 96-well NUNC™ Maxisorp plate was coated with both *C. burnetii* whole cell antigen and lysate (Section 3.3.1), diluted 1:1 with PBS/bicarbonate coating buffer (Appendix A) according to the template in Figure 4.2. The plate was incubated uncovered overnight at 37°C. The plate was washed three times with PBS-T (Appendix A), coated with 50 µL post-coating buffer (TropBio, Australia), incubated at room temperature for 1 hr then washed again. Human patient sera positive and negative for *C. burnetii* were applied at a dilution of 1:100 in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated goat anti-human IgG (Thermo Scientific, USA) at 1:5,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Absorbance was measured in a Multiskan Ascent plate reader at 414/494 nm. Statistical analyses were performed using single Mann-Whitney U tests.

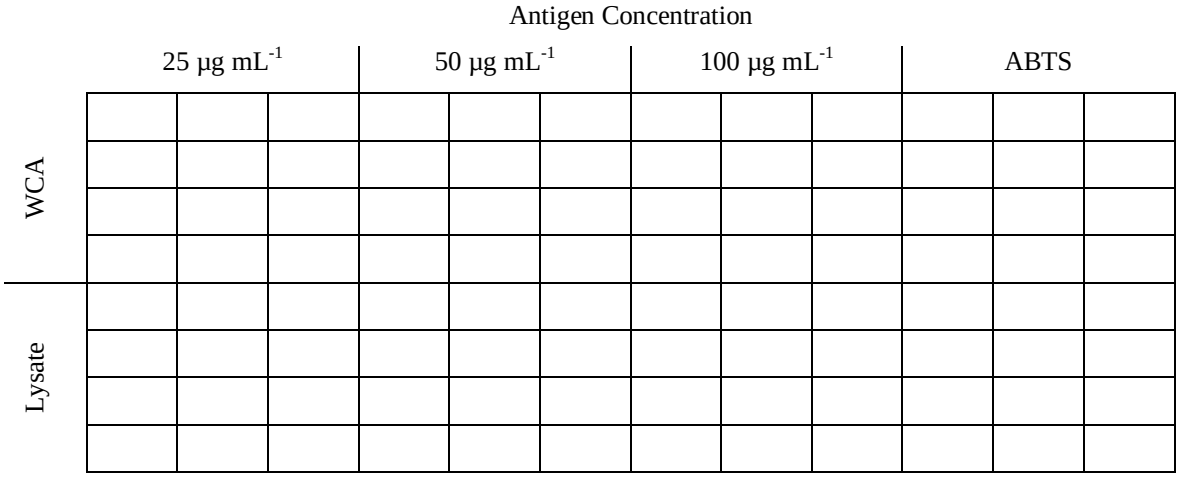

Figure 4.2: ELISA template to determine optimal antigen preparation for use in ELISA. Plate was coated with both antigen preparations at several concentrations and probed with both *C. burnetii* positive and negative sera.

4.4 Results

4.4.1 Comparison of virulence in Australian *Coxiella burnetii* isolates

4.4.1.1 Mouse model

In comparison of serological responses, there was a significant difference in absorbance values for sera from Cumberland infected animals, compared to that of sham inoculated animals in indirect ELISA ($P<0.01$) (Figure 4.3). There was no significant difference between infected and uninfected animals with Arandale. Absorbance values of sera from animals infected with Cumberland are significantly higher than those of animals infected with Arandale ($P<0.05$).

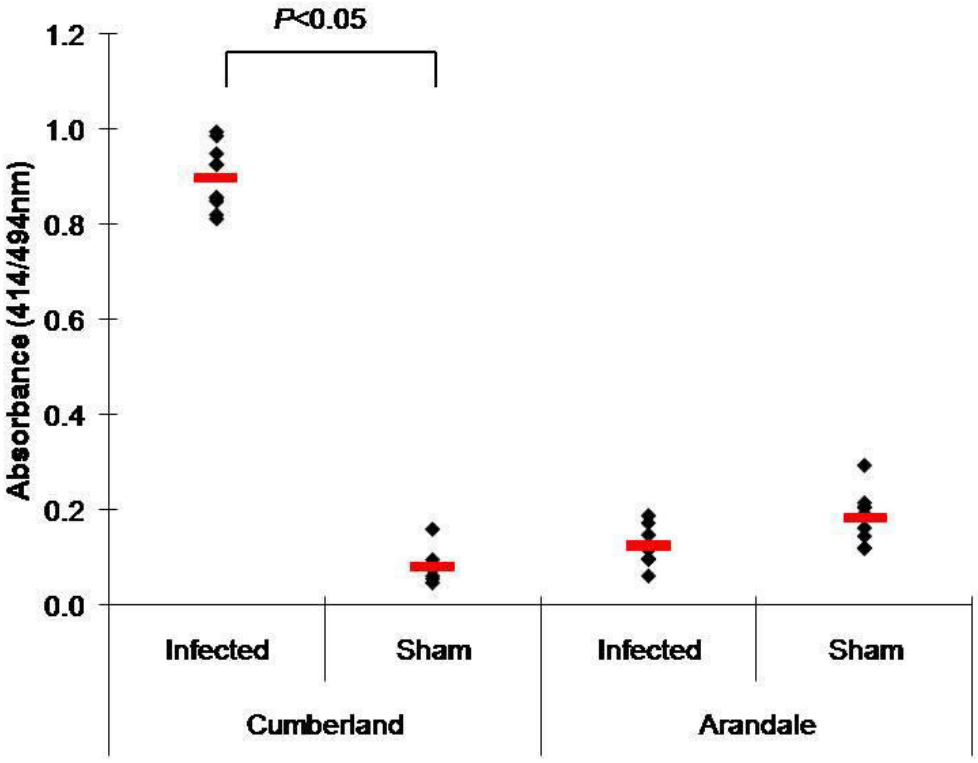

Figure 4.3: Comparison of serological response in ELISA from animals infected with Cumberland or Arandale isolate *Coxiella burnetii*.
Average serological response of infected and sham inoculated C57/Bl6 mice to *C. burnetii* antigen in indirect ELISA following infection with either Cumberland or Arandale isolate *C. burnetii*. Red lines represent mean for each group.

4.4.1.2 Guinea pig model

Guinea pigs inoculated with the Arandale isolate only exhibited a febrile response with rectal temperature measurement exceeding 39.5°C when inoculated with 1×10^{8} GE (Figure 4.4). Animals infected with Arandale did not exhibit morbidity during the trial. Morbidity was characterised by ruffled fur and lethargy. In contrast, guinea pigs inoculated with the Cumberland isolate demonstrated higher average temperatures (Figure 4.4), with some individuals recording temperatures between 39°C and 39.9°C during the trial. The group of guinea pigs inoculated with the highest dose of

Cumberland (1×10^9 GE) exhibited morbidity from days two to six post infection before recovering (not included in Figure). There was no correlation between dose and temperature reached, with no significant difference between the average temperatures for infected and uninfected animals.

In comparison of serological responses, guinea pigs inoculated with Cumberland demonstrated seroconversion at all inoculum doses (Figure 4.4). Guinea pigs inoculated with Arandale did not demonstrate seroconversion at any inoculum dose, the exception being a dose of 1×10^8 GE, with no significant difference determined between the serological response of sham animals and any of the inoculum groups except 1×10^8 GE. The maximum temperatures recorded for each inoculum group were significantly higher for animals infected with Cumberland compared to animals infected with Arandale ($P<0.05$) (Figure 4.4).

When sera from individual infected animals was compared to that of sham inoculated animals in indirect ELISA there was a significant difference in absorbance values for animals infected with Cumberland ($P<0.01$) (Figure 4.5). There was no significant difference between infected and uninfected animals with Arandale. Absorbance values of sera from animals infected with Cumberland were significantly higher than those of animals infected with Arandale ($P<0.05$).

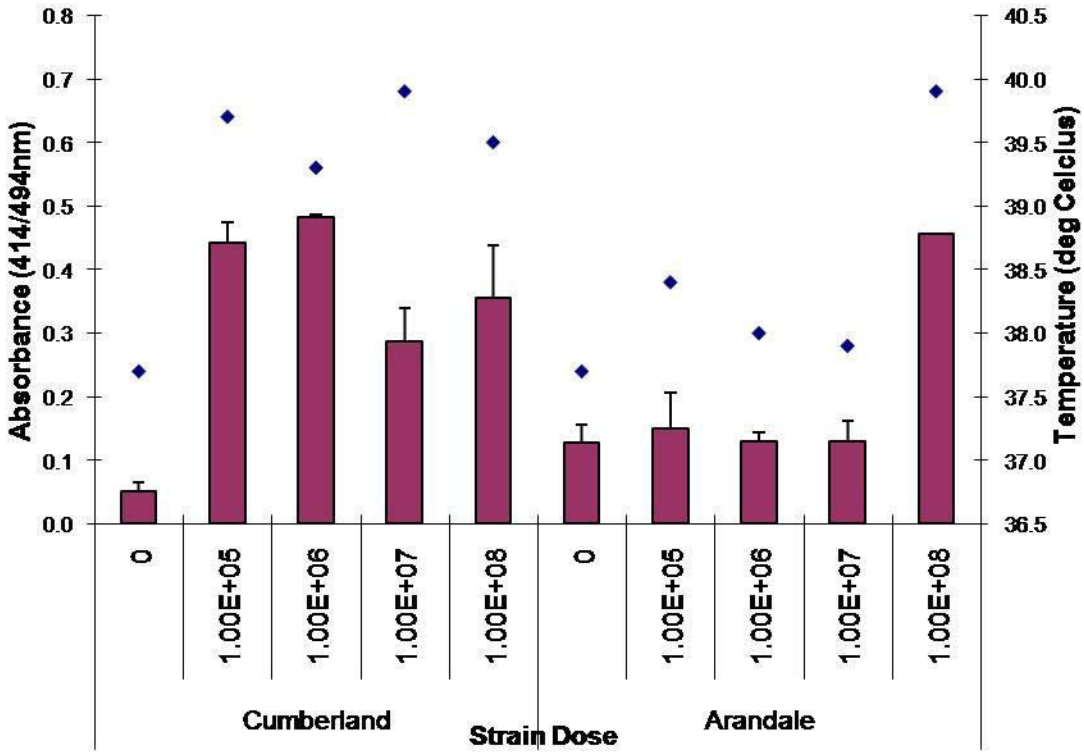

Figure 4.4: Comparison of serological conversion and febrile response elicited by Cumberland and Arandale isolates of *Coxiella burnetii* in guinea pigs.
Guinea pigs were inoculated with varying doses of *C. burnetii* genome equivalents (GE) from a virulent (Cumberland) and low virulence (Arandale) isolate. Rectal temperatures were taken, with the average maximum readings indicated by blue diamonds (Max Temp). At the conclusion of the experiment guinea pigs were sacrificed and serum collected for serology. Indirect ELISA was performed against *C. burnetii* antigen with average absorbance reading for each dosage group denoted by vertical bars.

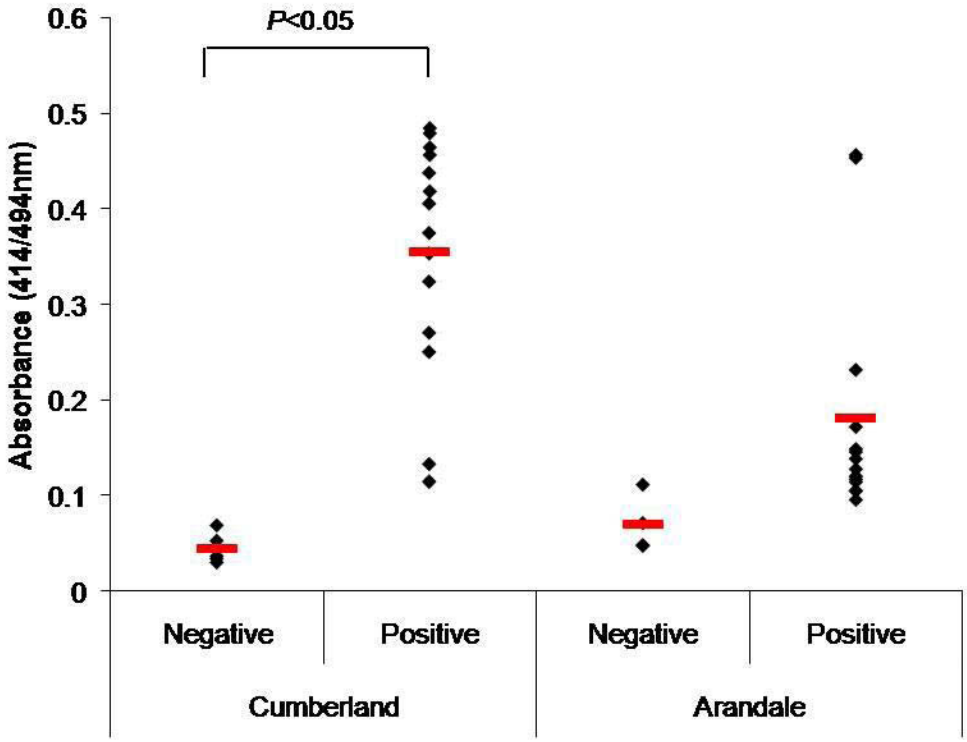

Figure 4.5: Comparison of serological response in ELISA from animals infected with Cumberland or Arandale isolate *Coxiella burnetii*.
Average serological response of infected and sham inoculated guinea pigs to *C. burnetii* antigen in indirect ELISA following infection with either Cumberland or Arandale isolate *C. burnetii*. Red lines represent the mean for each group.

4.4.2 Phase determination using PCR

A summary of expression level changes over the course of 15 cell culture passages is outlined in Table 4.3. Each arrow represents a single-fold increase or decrease in expression. A net reduction in expression was observed for the O-antigen biosynthesis genes selected for investigation over the course of 15 passages of Cumberland *C. burnetii* in vero cell culture (Figure 4.6). The greatest reduction in expression levels occurred during the first five passages. The greatest reductions in expression were observed for CBU 688, CBU 689, CBU 694, CBU 703 and CBU 830. The *com1* gene

was expressed at a higher level at P_0, prior to cell culture passage, reflecting a higher replication rate for *C. burnetii* when in phase I. The expression of *com1* dropped to a plateau during cell culture passage, with a slight reduction by 15 passages reflecting a reduction in the replication rate for *C. burnetii* when in phase II. Several genes demonstrated increases in gene expression during passage 10 to 15 compared to passages 5 to 10. However, these increases did not result in a return to expression levels present in earlier passages. The purpose of the correction in gene expression in these genes compared to earlier reductions is unknown.

Table 4.3: Summary of expression level changes during passage through vero cell line

Gene	Function	P0-P5	P5-P10	P10-P15
CBU680	UDP-glucose/GDP-mannose dehydrogenase	↓↓↓	↓	↑
CBU686	acetoin dehydrogenase	↓↓↓	↓	↑
CBU688	GDP-fucose synthetase	↓↓↓	↓↓↓	↑↑
CBU689	GDP-mannose 4,6-dehydratase	↓↓↓	↓↓↓	↑
CBU690	glycosyl transferase	↓↓↓	↓↓	↑
CBU692	dehydrogenase β subunit	↓↓↓	↓↓	↑
CBU694	glycosyl transferase	↓↓↓	↓↓↓	↑↑
CBU696	pleiotropic regulatory protein	↓↓↓	↓	No change
CBU697	pleiotropic regulatory protein	↓↓↓	↓↓	↑
CBU703	O-Ag ABC transporter	↓↓↓	↓↓	↑
CBU704	O-Ag ABC transporter	↓↓↓	↓↓	↑
CBU830	polysaccharide biosynthesis	↓↓↓	↑	No change
CBU844	capsular polysaccharide biosynthesis protein I	↓↓↓	↓↓	↑
CBU1661	ADP-heptose LPS heptosyltransferase II	↓↓↓	↓↓	↑

[nb] ↑increasing expression, ↓decreasing expression, - no expression

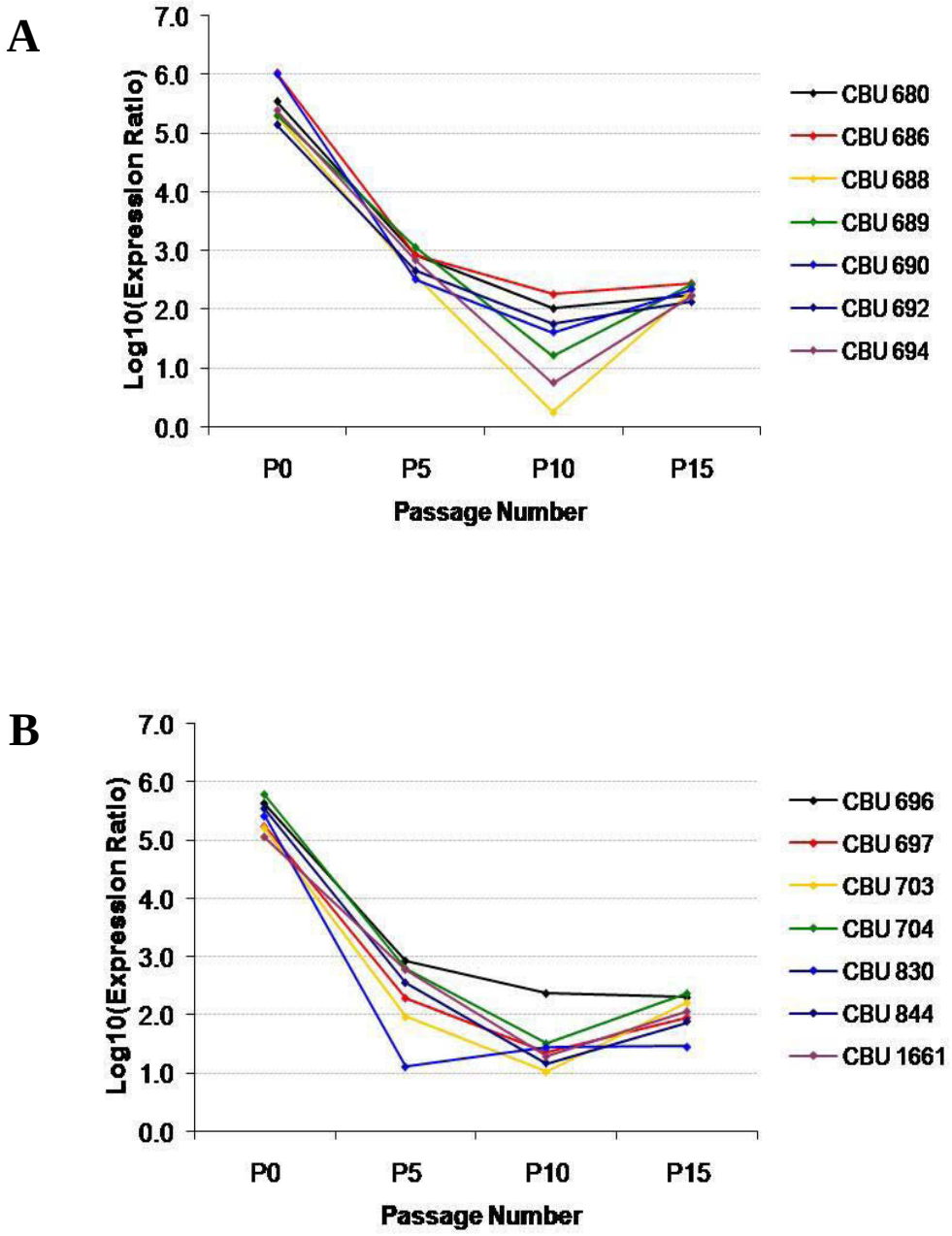

Figure 4.6: Expression of various genes in the O-antigen biosynthesis region.
Expression of the first (A) and second (B) set of 7 of 14 genes as a ratio of bacterial genome equivalents (GE) at successive passages in vero cell culture. The gene *com1* was used as the housekeeping gene/positive control.

4.4.3 Phase determination using complement block titration

Cumberland isolate *C. burnetii* antigen extracted and purified from cell culture at passage 15 was determined to be in phase II by complement block titration. Cumberland isolate *C. burnetii* antigen extracted from embryonated chicken egg culture at passage one was determined to be in phase I by complement block titration. Control commercial antigen demonstrated similar titres to the Cumberland isolate in-house antigen.

4.4.4 Optimisation of antigen preparation for use in ELISA

There was no significant difference between optimal antigen concentration for whole cell antigen or lysate (Figure 4.7). Also, there was no significant difference between the absorbance values obtained for positive sera against either antigen (Figure 4.8). However, there was a greater difference between the absorbance values for positive and negative sera when whole cell antigen was used (Figure 4.8). Use of lysate as ELISA antigen resulted in greater background absorbance than that observed with whole cell antigen. As a result of this comparison, whole cell antigen was selected as the antigen preparation for use in further ELISA optimisation.

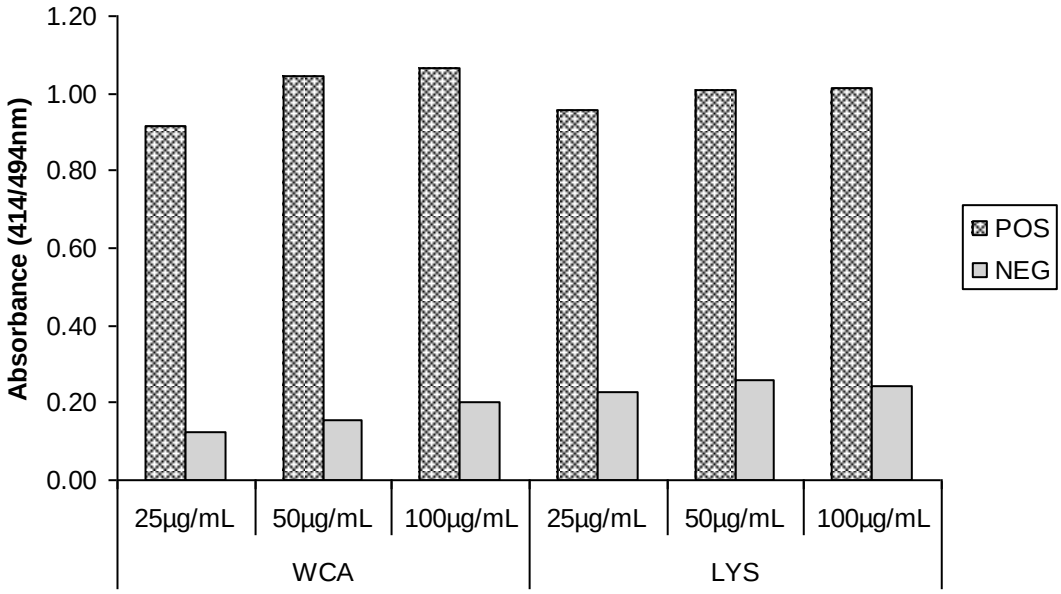

Figure 4.7: Comparison of antigen preparation in ELISA with two *Coxiella burnetii* positive sera and two negative sera from human Q fever patients and unexposed controls.
Two antigen preparations at various concentrations were probed with pooled human *C. burnetii* positive and negative sera to determine the optimal antigen preparation.

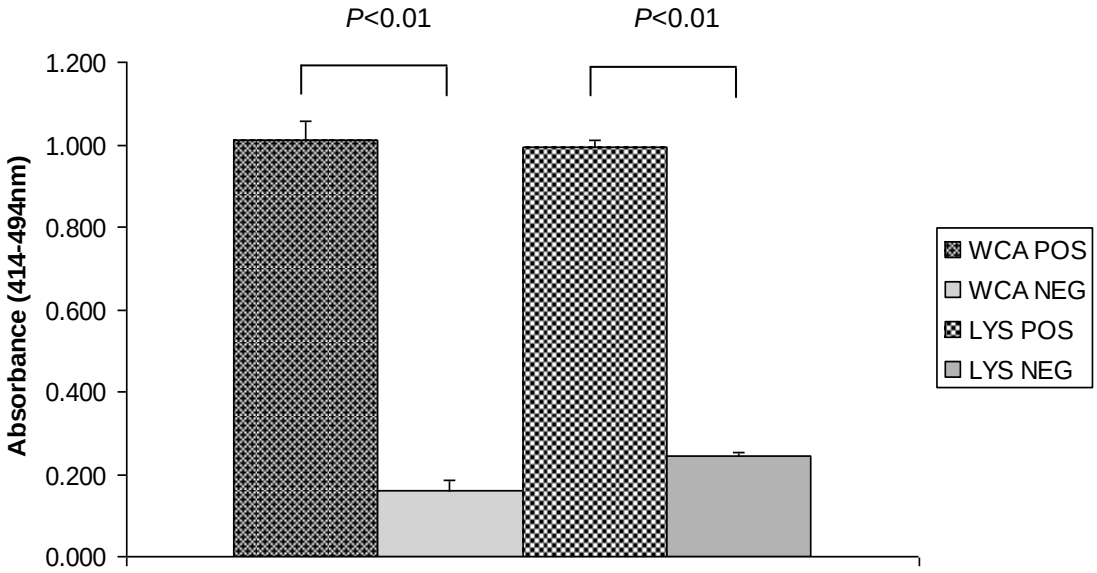

Figure 4.8: Determination of optimal antigen preparation.
Comparison between whole cell antigen and lysate with *C. burnetii* positive and negative human sera. The mean reaction of positive and negative sera against either antigen at all concentrations was compared to determine which preparation resulted in greatest differentiation between positive and negative sera.

4.5 Discussion

The combined febrile and serological response data obtained from animal experiments conducted using the two Australian *C. burnetii* isolates, indicated the Arandale isolate was considerably less virulent than the Cumberland isolate and a poor inducer of seroconversion in mice as well as guinea pigs. The Cumberland isolate was therefore considered more suitable for use as an inoculum for generating positive reactor sera for use in ELISA as positive control sera or as indicator sera in competitive ELISA.

Differences in isolate virulence are thought to be genogroup specific (Russell-Lodrigue *et al.*, 2009). *Coxiella burnetii* isolates can be separated into six different genomic groups based upon RFLP analysis and plasmid type (Hendrix *et al.*, 1991; Jager *et al.*, 1998). A study by Russell-Lodrigue, *et al* (2009) was performed on a group I isolates Nine Mile, African and Ohio, group IV isolates Priscilla and P, group V isolates G and S and a group VI isolate Dugway. They found group I isolates produced severe acute disease in guinea pigs, while group V isolates produced mild to moderate acute disease and group IV and VI isolates produced no acute disease. In SCID mice, all isolates produced clinical signs of infection, with the most severe produced by group I isolates. In immunocompetent CB-17 mice, only mild disease was produced with all isolates, with noticeably ruffled fur produced by group I isolates. Mice infected with group I isolates had significantly higher splenic loads of *C. burnetii* than mice infected with the other isolates. Group I isolates also resulted in a greater inflammatory response, followed by groups V, IV and VI. Antigens from *C. burnetii* were diffusely distributed throughout the organs of SCID mice but were rarely detected in CB-17 mice.

These results indicate that the Cumberland isolate may be a group I isolate as even mid-level doses of 10^5 bacteria were capable of eliciting a fever in guinea pigs. This isolate also resulted in noticeably ruffled fur in A/J mice and severe splenomegaly on observation. In contrast, the Arandale isolate only produced fever in guinea pigs at very high doses of 10^8 bacteria and did not result in outward clinical signs in mice, however did result in splenomegaly. This indicates the Arandale isolate may be a group VI or IV isolate. Further investigation of the Cumberland and Arandale isolates by RFLP analysis and PCR for the different plasmid types is needed to determine which genomic group the isolates belong to.

Genes associated with the biosynthesis of *C. burnetii* LPS were shown to down-regulate during passage in Vero cell culture. Of these 14 genes, nine are located in the deleted region of the Nine Mile phase II strain genome. These genes include CBU680 (UDP-glucose/GDP-mannose dehydrogenase), CBU686 (acetoin dehydrogenase), CBU688 (GDP-fucose synthetase), CBU689 (GDP-mannose 4,6-dehydratase), CBU690

(glycosyl transferase), CBU692 (dehydrogenase β subunit), CBU694 (glycosyl transferase), CBU696 (pleiotropic regulatory protein) and CBU697 (pleiotropic regulatory protein). The greatest reduction in expression levels occurred during the first five passages with a net reduction occurring over the course of 15 passages. A minimum of five cell culture passages has been established as necessary to ensure a *C. burnetii* isolate is in phase II antigenically (Stoker, 1953). The results of the expression level analysis supported this finding. Passaged *C. burnetii* was confirmed to be in phase II by complement block titration. From the results, it was concluded that a reduction in the expression ratio of the O-Ag biosynthesis genes to *C. burnetii* genome equivalents to below three would be indicative of a phase II antigenic phenotype. The greatest reductions in expression were observed for CBU688, CBU689, CBU694, CBU703 and CBU830. Of these, the first two are known to be associated with the bio-synthesis of virenose, a unique sugar present in the LPS of phase I *C. burnetii*. The pronounced reduction in the expression of genes in the virenose-coding region is particularly indicative of the shift to the phase II antigenic state. The use of these four genes in PCR-based determination of *C. burnetii* antigenic phase would be a less subjective and time-consuming method than complement block titration or other serological means of antigenic phase determination.

The ELISAs developed as a result of the experimental work described in this chapter, were for the detection of antibodies to phase II and phase I antigen separately. Whole cell antigen was determined to be the optimal antigen type for ELISA production. These ELISAs were optimised using sera from individuals confirmed to have Q fever and individuals un-exposed to *C. burnetii*. The ELISAs were further optimised and validated using murine and cavine sera from animals experimentally infected with *C. burnetii* and un-infected controls. The ELISAs were effective in detecting the development of antibodies to both antigenic phases of *C. burnetii* in animal and human sera. The ELISAs developed in this study differed from commercially available Q fever ELISAs in that they enable separate detection of phase II and phase I antigens. They are also the first known use of an Australian clinical Q fever isolate in ELISA. This was an important development due to the potential problems identified with using commercial

Q fever tests of European origin on Australian ruminant sera (Kittelberger *et al.*, 2009). In this study (Kittelberger *et al.*, 2009), there was good to excellent agreement between the two commercial ELISAs when used on samples from the Netherland and Germany. However, there was no agreement between the two tests when used on a small number of bovine samples from Queensland. Initially, there was also poor agreement between the two tests when used on positive and negative ovine control sera from the Australian National Quality Assurance Programme inter-laboratory proficiency testing. The positive sera were obtained from naïve sheep repeatedly vaccinated with Q-Vax (CSL, Australia). Later experiments indicated the discrepancy was due to a problem with the batch of test kit used. This indicated commercial Q fever ELISA kits may not perform consistently and would require additional kit-independent controls and inter-laboratory proficiency testing (Kittelberger *et al.*, 2009). Preliminary experiments performed using these commercial tests in-house also demonstrated an inability to detect antibodies in known positive sera from human Q fever patients and infected mice (data not shown). These results indicate the commercially available Q fever ELISAs may not be suitable for use on animal sera of Australian origin.

In conclusion, a qRT-PCR based method of tracking and identifying phase variation and type was developed for an Australian Q fever isolate. This method is yet to be used on other *C. burnetii* isolates. However, the reduction of expression of genes directly associated with phase I LPS indicates the method would be able to be applied to other isolates. A comparison of isolate virulence found that Australian clinical isolates differed in virulence for both mice and guinea pigs and indicated the presence of multiple genotypes in endemic *C. burnetii* isolates. These comparisons indicated some isolates may be unsuitable for use in the preparation of diagnostic reagents. In addition, ELISAs were developed for the separate detection of antibodies to phase II and I antigens. These ELISAs also represent the first known use of an Australian *C. burnetii* isolate as antigen. The development of ELISAs using an Australian isolate in both antigenic phases will enable more precise investigations of *C. burnetii* seroprevalence in both non-native and native animal populations, and the human population in Australia.

CHAPTER FIVE

DETECTION OF ANTIBODIES TO *COXIELLA BURNETII* IN NON-NATIVE ANIMALS AND HUMANS IN NORTHERN QUEENSLAND

5.1 Introduction

Coxiella burnetii is the aetiological agent of Q fever (Angelakis and Raoult, 2010). The natural reservoir of *C. burnetii* exists between wild animals and their ticks, with tick-borne transmission to livestock and other domestic animals also occurring (Lang, 1990). However, once established in livestock, tick-borne transmission is no longer required for the continuation of infection. *Coxiella burnetii* primarily infects the female reproductive tract in livestock, resulting in the shedding of the bacterium in parturient fluids and milk. The bacterium can also be excreted in urine in faeces. Environmental contamination with infectious material from livestock has been associated with outbreaks of Q fever, especially following wind-borne spread in areas where livestock are bred, held or transported (Hawker *et al.*, 1998; Porten *et al.*, 2006). Inhalation of infectious fomites is the primary source of human infection (Maurin and Raoult, 1999). Less commonly, infection can be acquired from the consumption of infected animal products such as unpasteurised milk and cheeses.

The domestic reservoir, consisting of cattle, sheep and goats, is considered the major source of transmission of *C. burnetii* infection to humans in Australia. Infections in animals are usually sub-clinical, although infection of sheep and goats are sometimes associated with abortions and reproductive disorders. Sporadic abortion in cattle has also been reported (Cabassi *et al.*, 2006). Seropositivity is believed to be seasonal in livestock, particularly sheep as serological studies indicated the presence of recurring

annual cycles of antibody prevalence in response to *C. burnetii* exposure during lambing (Enright *et al.*, 1971b).

Outbreaks of Q fever in Australia have predominantly been associated with abattoirs beginning in 1934, when sporadic cases of fever with a "typhoid-like" presentation came to the attention of Australian medical practitioners (Derrick, 1973). All affected individuals were found to work in an abattoir, and the disease was initially named "abattoir fever". In a more recent series of cases spanning a 20-year period in Southern Australia, 92% of the 111 reported Q fever cases were abattoir workers (Spelman, 1982). One study investigating Q fever infection in a southern Queensland meatworks over a ten year period (1968 to 1977) revealed an average annual incidence of 1% for the nine years excluding 1969, when an outbreak with a 7.9% incidence occurred. Results indicated that cattle formed the major source of *C. burnetii*, and that the highest risk of infection was on the cattle slaughter floor (McKelvie, 1980). In North Queensland, a study of Q fever cases reported during 1994 to 2006, 22% of acute and 33% of chronic Q fever patients reported exposure to cattle (Gale *et al.*, 2007). This trend extends internationally, where an occupation in meat processing industries has been associated with increased risk of Q fever infection (Maurin and Raoult, 1999).

In epidemiological studies conducted worldwide during the last six years, the seroprevalence of *C. burnetii* in cattle populations varied according to geographic location. Seroprevalence rates were reported as 6.2% in Northern Ireland (McCaughey *et al.*, 2010), 7.9% in Albania (Cekani *et al.*, 2008), 10.75% in Iran (Khalili and Sakhaee, 2009), 14.3% in the Central African Republic (Nakoune *et al.*, 2004), 24% in Newfoundland (Hatchette *et al.*, 2002) and Cyprus (Psaroulaki *et al.*, 2006) and 25.6% in Korea (Kim *et al.*, 2006). Previous serological investigations of prevalence of *C. burnetii* infection in Australian cattle demonstrated it was not common in beef cattle in Western Australia (Banazis *et al.*, 2010) and South Australia (Durham and Paine, 1997) or dairy cattle in Victoria (Hore and Kovesdy, 1972) with seroprevalence in all three studies found to be less than 1%. Although, the incidence of Q fever in humans is much higher in Queensland compared to these states (NSDSS, 2008), no record of

serological surveys performed on beef cattle in the State of Queensland could be identified.

The State of Queensland has the largest beef cattle herd in Australia and is also the largest producer and exporter of beef cattle (Admans, 2010). Beef cattle production represents the largest agricultural industry in the State and was valued at $3.4 billion in 2007 to 2008 (Australian Bureau of Statistics, 2009). The Queensland beef cattle herd is estimated to be 12.2 million head, 96% of which is managed on 14,568 specialised beef cattle properties (Admans, 2010).

Dogs and cats have been implicated in Q fever outbreaks in Canada, although no such transmissions have yet been reported in Australia. A family dog was implicated in a Q fever outbreak which occurred following birthing (Buhariwalla *et al.*, 1996). In this report, three members of the family contracted the disease after contact with an infected parturient bitch and her four puppies, all of which died soon after birth. A study of dogs belonging to the French military reported a seroprevalence of 9.7% (Boni *et al.*, 1998). Infected parturient cats and exposure to stillborn kittens have been reported as the cause of several outbreaks of Q fever in residential areas of Nova Scotia, Canada (Kosatsky, 1984; Langley *et al.*, 1988; Marrie *et al.*, 1988b). A high prevalence of *C. burnetii* antibodies was found in a Japanese study of cats, with 14.2% seroprevalence in pet cats and 41.7% in stray cats (Komiya *et al.*, 2003).

In Australia, feral animals and wild dogs are distributed in both remote and peri-urban areas. These animals may be involved in the natural cycle of *C. burnetii* in wildlife. With increased population growth in northern Queensland there is increasing urban development into bushland. This provides a potential conduit for the transmission of Q fever from wild and feral animals to domestic animals and humans.

In epidemiological studies conducted worldwide during the last 10 years, the seroprevalence of *C. burnetii* in human populations varied according to geographic

location. Seroprevalence rates were reported as 1.5% (n=205) in rural Korea (Kim *et al.*, 2006), 3.1% (n=4,437) in the United States (Anderson *et al.*, 2009), 4.2% (n=616) in Taiwan (Ko *et al.*, 2000), 12.8% (n=2,394) in northern Ireland (McCaughey *et al.*, 2008), 13.5% (n=407) in Turkey (Gozalan *et al.*, 2010), 15.3% (n=216) in north eastern Spain (Cardenosa *et al.*, 2006) and 52.7% (n=583) in Cyprus (Psaroulaki *et al.*, 2006).

To determine the risk of infection, with a microbial organism, the sources of potential infection and routes of transmission must be identified. Consequences associated with such a lack of knowledge were highlighted by an outbreak of Q fever in an Australian abattoir slaughtering feral goats (Buckley, 1980). Poor understanding of the transmission of *C. burnetii* and potential for goats as a reservoir resulted in failure to employ methods to reduce the generation of aerosols. The capacity to identify such potential sources of Q fever infection in Australia, particularly the tropical north, is compromised due to the lack of data regarding reservoir populations. The experimental work outlined in this chapter aimed to establish the prevalence of anti-*C. burnetii* antibodies in non-native animals and the human population in northern Queensland. This study also aimed to determine the potential of non-native animals to act as reservoirs for human Q fever infection.

5.2 Aims

The specific aims for the work described in this chapter were to:

1. Develop ELISAs for the detection of antibodies to phase II and I *C. burnetii* antigens in bovine, canine, feline and porcine sera.
2. Determine prevalence of anti-*C. burnetii* antibodies against both antigenic phases in beef cattle in various statistical divisions of Queensland;
3. Determine prevalence of anti-*C. burnetii* antibodies against both antigenic phases in domestic dogs and cats in Townsville, Queensland;
4. Determine prevalence of anti-*C. burnetii* antibodies against both antigenic phases in dingoes, feral cats, foxes and feral pigs in northern Queensland and south-east Queensland;
5. Determine prevalence of anti-*C. burnetii* antibodies against phase II *C. burnetii* antigen in human population in Townsville, Queensland;
6. Determine whether human incidence of Q fever has any correlation with seroprevalence in animals in various regions.

5.3 Materials and Methods

5.2.1 Blood collection and serum separation

5.2.1.1 Beef cattle serum

Blood samples were obtained immediately following slaughter of beef cattle at an abattoir in the Townsville Shire District. The catchment area of this abattoir includes the regions of Northern, Far Northern, North Western Queensland and the Northern Territory. The abattoir has a daily processing capacity of 900 beasts. Samples (n=730) were collected during 2006 to 2007 on four separate occasions approximately six

months apart, with 150-200 samples collected randomly on each occasion. Samples were stratified by cattle property. This ensured a representative spectrum of the northern Queensland beef industry was covered with animals originating from 17 different cattle properties in eight districts. Only properties where greater than 10 samples were obtained were included in the study.

A further sample set was obtained in collaboration with Professor Michael McGowan of the University of Queensland which was collected for a different study involved with beef cattle seroprevalence surveys. Randomly selected samples (n=1,344) were collected during 2008 to 2009 from breeders and heifers originating from 46 different Queensland beef cattle properties in 24 districts. A statistically valid sample size was calculated for each property based on the total number of cattle present in each mob. Sera was separated from blood by centrifugation at room temperature and stored at -20ºC.

5.2.1.2 Domestic dog serum

Blood samples were collected during 2006 to 2007 from dogs attending suburban veterinary practices for routine procedures by licensed veterinarians after obtaining the written consent of the pet-owners. A total of 2ml of blood was drawn into a clot-activated vacutainer from live, anesthetized animals during ovariohysterectomy or orchiectomy. Dogs sampled were generally considered to be "well", with no apparent signs of illness. Following blood collection and centrifugation, serum was obtained and stored at -70°C until collection was completed. Each serum sample was accompanied by a questionnaire to be completed by owners of the pet from which the blood was drawn, and included factors relating to the residential area the pet was from, its food habits and degree of exposure to ticks, domestic farm animals and wildlife.

A retrospective cohort of 100 canine serum samples were obtained from a series collected and stored during 1984 to 1985 by veterinarians undertaking an unrelated study (Smith, 1986).

5.2.1.3 Wild dog and fox serum

Samples were obtained for this study from a pest control program. Wild dogs/dingoes were captured humanely by professional trappers (Mark Goullet of *Ferals Out*, Burpengary, Queensland and Russell Warner of *Townsville City Council Pest Control*) using rubber padded leg-hold traps. All wild dogs were shot at close range in the head within several hours of capture with a 0.22 calibre pistol. Blood samples were collected via cardiac puncture with 18 gauge needles and 20 mL syringes and transferred to 10 mL heparinised vacutainers (Figure 5.1). The approximate age, sex and capture area were recorded for each animal. Samples were centrifuged at 1,400 ×*g* for 10 min at room temperature and the serum collected and stored at -70°C until tested.

5.2.1.4 Domestic cat serum

Serum samples were sourced from a serum bank compiled from blood samples collected from Veterinary Clinics in North Queensland. Blood samples were collected between February and July 2006. The age, sex, origin and animal contact details for each animal were recorded in a database.

A further sample set was obtained from sera collected as part of an earlier study (Speare and Tinsley, 1987). These domestic cat samples were originally obtained from veterinary clinics in the Townsville region. However, no other data was available for these samples.

5.2.1.5 Feral cat serum

Feral cats were captured humanely by professional trapper (Mark Goullet of *Ferals Out*) using either rubber padded leg-hold traps or small animal cage traps. All feral cats were shot at close range in the head within several hours of capture with a 0.22 calibre pistol. Blood samples were collected via cardiac puncture with 18 gauge needles and 10 mL syringes and transferred to 10 mL heparinised vacutainers (Figure 5.1). The approximate

age, sex and capture area were recorded for each animal. Samples were centrifuged at 1,400 ×*g* for 10 min at room temperature and the serum collected and stored at -70°C until tested.

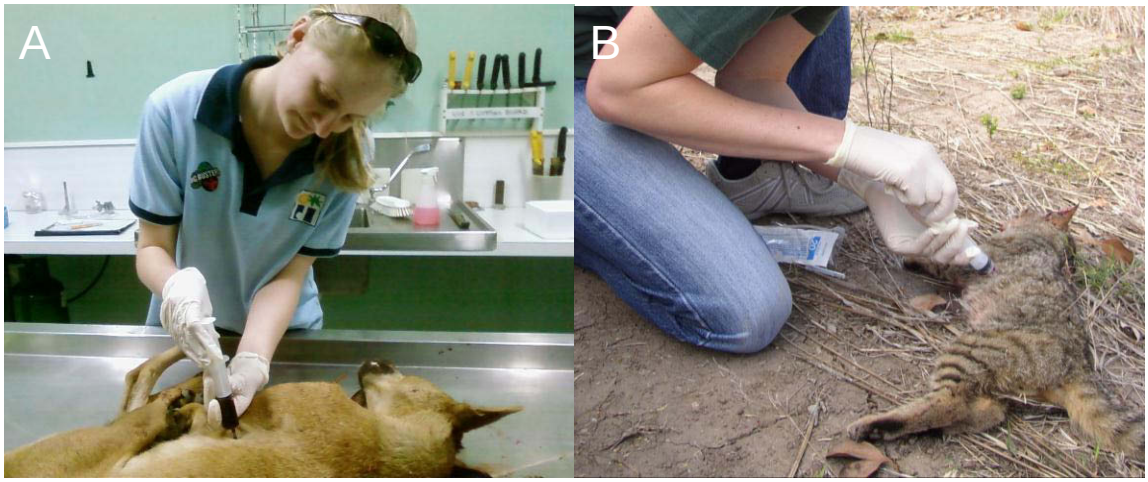

Figure 5.1: Blood sample collection from wild dogs (A) and feral cats (B).
Blood samples were collected from recently deceased animals via cardiac puncture with 1.5" 18 gauge needles and either 20 mL or 10 mL syringes for dogs or cats respectively.

5.2.1.6 Feral pig serum

Feral pigs were captured humanely by volunteer and professional trappers involved in an eradication program (Geoff Sloman of *Hunting and Conservation Queensland* and Mark Goullet of *Ferals Out*). Blood samples were collected immediately following slaughter in 6 mL heparinised vacutainers. The age, sex and capture area were recorded for each animal. Samples were centrifuged at 1,400 ×*g* for 10 min at room temperature and the serum collected and stored at -70°C until tested.

5.2.1.7 Human serum

Serum was obtained from blood collected by the Australian Red Cross Blood Service (ARCBS) in Townsville during routine blood donations. The samples were obtained from 1,522 randomly selected donors who donated blood to the ARCBS during 2005 to

2007. All donor samples were collected by the Townsville branch of the ARCBS which covers the entire Townsville shire catchment area, which is located in the Northern Region. Ethics approval (ARCBS #2010#2) was provided from the Australian Red Cross Blood Service Ethics Committee on the condition that no further indentifying information regarding the donors was obtained.

5.2.2 ELISA Optimisation

5.2.2.1 Bovine ELISA

Initially, 30 samples from an initial collection of 520 were randomly selected and screened using the reagent concentrations used for murine sera (Section 4.3.3.1). These consisted of 25 µg mL^{-1} antigen, 1:100 test sera and 1:1,000 conjugate. From this, *C. burnetii*-positive and negative samples were selected to pool for further optimisation. Checkerboard ELISAs (Figure 5.2) were set up in a 96-well NUNC™ Maxisorp plates (Nunclon, Australia) for both phase I and phase II antigen. Antigen concentration was tested at 25 µg mL^{-1}, 50 µg mL^{-1} and 100 µg mL^{-1}. Bovine *C. burnetii*-positive sera were tested at dilutions of 1:50, 1:100, 1:200 and 1:400. HRP-conjugated rabbit anti-bovine IgG (Serotec, UK) were tested at serial 2-fold dilutions from 1:1,000 to 1:8,000. Antigen samples were diluted with 50 µL coating buffer (TropBio, Australia) and incubated uncovered overnight at 37°C. The plate was washed three times with PBS-T (Appendix A), coated with 50 µL post-coating buffer (TropBio, Australia), incubated at room temperature for 1 hr then washed again. Bovine *C. burnetii*-positive sera were applied at dilutions described in 50 µL aliquots and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated rabbit anti-bovine IgG (Serotec, UK) was applied at the dilutions described and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. The plate was read in a Multiskan Ascent plate reader (Labsystems, USA) at 414/494 nm.

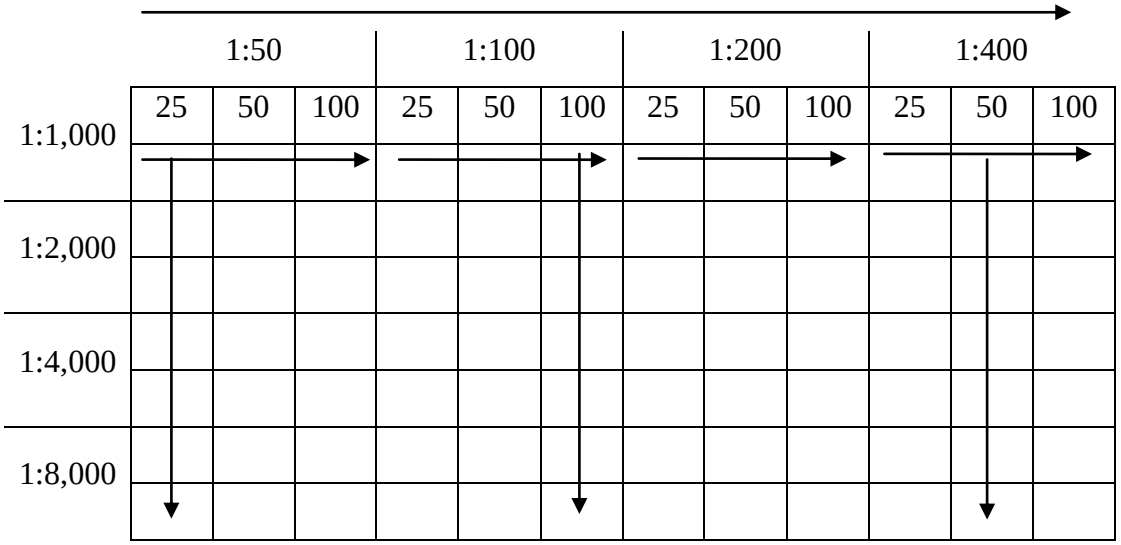

Figure 5.2: Layout of ELISA for optimisation of reagent concentrations for screening of exposure to *Coxiella burnetii*.
Bovine sera dilutions were grouped from left to right, secondary antibody from top to bottom and antigen concentrations from left to right within each group.

For determination of threshold for seropositivity, the 96-well NUNC™ Maxisorp plates were coated with 50 µL of either phase I or phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Bovine sera (three negative and three positive) were applied at dilutions of 1:50 and 1:100 in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated rabbit anti-bovine IgG (Serotec, UK) was applied at a dilution of 1:1,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm.

5.2.2.2 Canine ELISA

Initially, 30 samples from an initial total of 100 were randomly selected and screened using the reagent concentrations used for murine sera (Section 4.3.1.1). These consisted of 25 µg mL^{-1} antigen, 1:100 test sera and 1:1,000 conjugate. From this, *C. burnetii*-positive and negative samples were selected to pool for further optimisation. Checkerboard ELISAs were set up in a 96-well NUNC™ Maxisorp plates (Nunclon, Australia) for both phase I and phase II antigen as described previously (Figure 5.2). Canine *C. burnetii*-positive serum was tested at dilutions of 1:100, 1:200, 1:400 and 1:800. HRP-conjugated sheep anti-canine IgG (Serotec, UK) was tested at serial 2-fold dilutions from 1:1,000 to 1:8,000. Antigen was diluted 1:1 with 50 µL PBS/bicarbonate coating buffer to a final concentration of 100µg mL^{-1} and incubated overnight at 37°C. The plate was coated with 100 µL post-coating buffer (Tropbio, JCU), incubated at room temperature for 2 hr then dried. Canine *C. burnetii*-positive and negative serum was applied at dilutions described in 50 µL aliquots and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated sheep anti-canine IgG (Serotec, UK) was applied at the dilutions described and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader (Labsystems, USA) at 414/494 nm. Positive samples threshold determination was performed as described for bovine ELISA (Section 5.2.2.1) with reagent concentrations optimised for canine sera.

5.2.2.3 Feline ELISA

Initially, 15 samples from an initial total of 49 were randomly selected and screened using the reagent concentrations used for murine sera (Section 4.3.3.1). These consisted of 25 µg mL^{-1} antigen, 1:100 test sera and 1:1,000 conjugate. From this, *C. burnetii*-positive and negative samples were selected to pool for further optimisation. Checkerboard ELISAs were set up in a 96-well NUNC™ Maxisorp plates (Nunclon, Australia) for both phase I and phase II antigen as described previously (Figure 5.2).

Feline *C. burnetii*-positive serum was tested at dilutions of 1:100, 1:200, 1:400 and 1:800. HRP-conjugated goat anti-feline IgG (Serotec, UK) was tested at serial 2-fold dilutions from 1:1,000 to 1:8,000. Plates were coated with antigen as described previously (Section 5.2.2.2). Feline *C. burnetii*-positive and negative serum was applied at dilutions described in 50 µL aliquots and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated goat anti-feline IgG (Serotec, UK) was applied at the dilutions described and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader (Labsystems, USA) at 414/494 nm. Positive sample threshold determination was performed as described for bovine ELISA (Section 5.2.2.1) with reagent concentrations optimised for feline sera.

5.2.2.4 Porcine ELISA

Initially, eight samples from a total of 16 were randomly selected and screened using the reagent concentrations used for murine sera (Section 4.3.3.1). These consisted of 25 µg mL^{-1} antigen, 1:100 test sera and 1:1,000 conjugate. From this, *C. burnetii*-positive and negative samples were selected to pool for further optimisation. Checkerboard ELISAs were set up in a 96-well NUNC™ Maxisorp plates (Nunclon, Australia) for both phase I and phase II antigen as described previously (Figure 5.2). Porcine *C. burnetii*-positive serum was tested at dilutions of 1:100, 1:200, 1:400 and 1:800. HRP-conjugated rabbit anti-porcine IgG (Serotec, UK) was tested at serial 2-fold dilutions from 1:1,000 to 1:8,000. Plates were coated with antigen as described previously (Section 5.2.2.2). Porcine *C. burnetii*-positive and negative serum was applied at dilutions described in 50 µL aliquots and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated rabbit anti-porcine IgG (Serotec, UK) was applied at the dilutions described and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader (Labsystems, USA) at 414/494 nm. Positive samples threshold

determination was performed as described for bovine ELISA (Section 5.2.2.1) with reagent concentrations optimised for porcine sera.

5.2.2.5 Human ELISA

Human ELISA was optimised in a previous study (Cooper, 2006). Concentrations of 100 µg mL^{-1} antigen, 1:100 test sera and 1:1,000 conjugate were considered to be the best tested for the assessment of antibodies to *C. burnetii* in human sera.

5.2.3 Screening of serum samples for antibodies to *Coxiella burnetii* using ELISA

5.2.3.1 Bovine ELISA

NUNC™ 96-well Maxisorp plates were coated with 100 µL of phase I or phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:100 in 100 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed as described previously, after which 50 µL of HRP-conjugated rabbit anti-bovine IgG (Serotec, UK) at 1:1,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. The S/P% was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 30% were considered to be negative. Samples with an S/P% of between 30% and 50% were considered to be weak positives; those between 50% and 70% were considered to be positive and those greater than 70% were considered to be strongly positive.

5.2.3.2 Canine ELISA

NUNC™ 96-well Maxisorp plates were coated with 100 µL of phase I or phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed as described previously, after which 50 µL of HRP-conjugated sheep anti-canine IgG (Serotec, UK) at 1:2,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. The S/P% was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 50% were considered to be negative. Samples with an S/P% of between 50% and 75% were considered to be positives; those greater than 75% were considered strongly positive.

5.2.3.3 Feline ELISA

NUNC™ 96-well Maxisorp plates were coated with 100 µL of phase I or phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed as described previously, after which 50 µL of HRP-conjugated goat anti-feline IgG (Serotec, UK) at 1:2,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. The S/P % was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 50% were

considered to be negative. Samples with an S/P% of between 50% and 75% were considered to be positives; those greater than 75% were considered strongly positive.

5.2.3.4 Porcine ELISA

NUNC™ 96-well Maxisorp plates were coated with 100 µL of phase I or phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed as described previously, after which 50 µL of HRP-conjugated rabbit anti-porcine IgG at 1:5,000 (Serotec, UK) was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. The S/P% was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 50% were considered to be negative. Samples with an S/P% of between 50% and 75% were considered to be positives; those greater than 75% were considered strongly positive.

5.2.3.5 Human ELISA

NUNC™ 96-well Maxisorp plates were coated with 100 µL of phase II antigen at 100 µg mL^{-1} in coating buffer and incubated overnight at 37°C. Plates were coated with 100 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at 1:100 dilutions in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed as described previously, after which 50 µL of HRP-conjugated goat anti-human IgG (Chemicon International, USA) at 1:1,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan

Ascent plate reader at 414/494 nm. The S/P% was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 50% were considered to be negative. Samples with an S/P% of between 50% and 75% were considered to be positives; those greater than 75% were considered strongly positive.

5.2.4 Statistical analyses

Mann-Whitney U tests were used to determine whether differences in optical density values for positive and negative sera were statistically significant. Mann-Whitney U tests were also used to determine whether there was any statistically significant difference between seropositivity according to age. Risk Ratio and Odds Ratio calculations were performed where greater than five samples were available for tested factors. Pearson Chi-Squared tests were used to identify factors with positive associations with seropositivity. Single factor ANOVA tests were used to determine whether differences in beef cattle seropositivity at various times of year were statistically significant. Spearman Rank Correlation analysis was used to determine whether any correlation existed between seroprevalence in beef cattle and human Q fever notifications and incidence.

5.3 Results

5.3.1 Optimisation of ELISA

5.3.1.1 Bovine ELISA for detection of antibodies to *Coxiella burnetii*

Optimal reagent concentrations for the bovine ELISA were determined to be 100 µg mL^{-1} antigen for both phase II and I antigen, 1:100 test sera and 1:1,000 conjugate (Figures 5.3, 5.4 and 5.5).

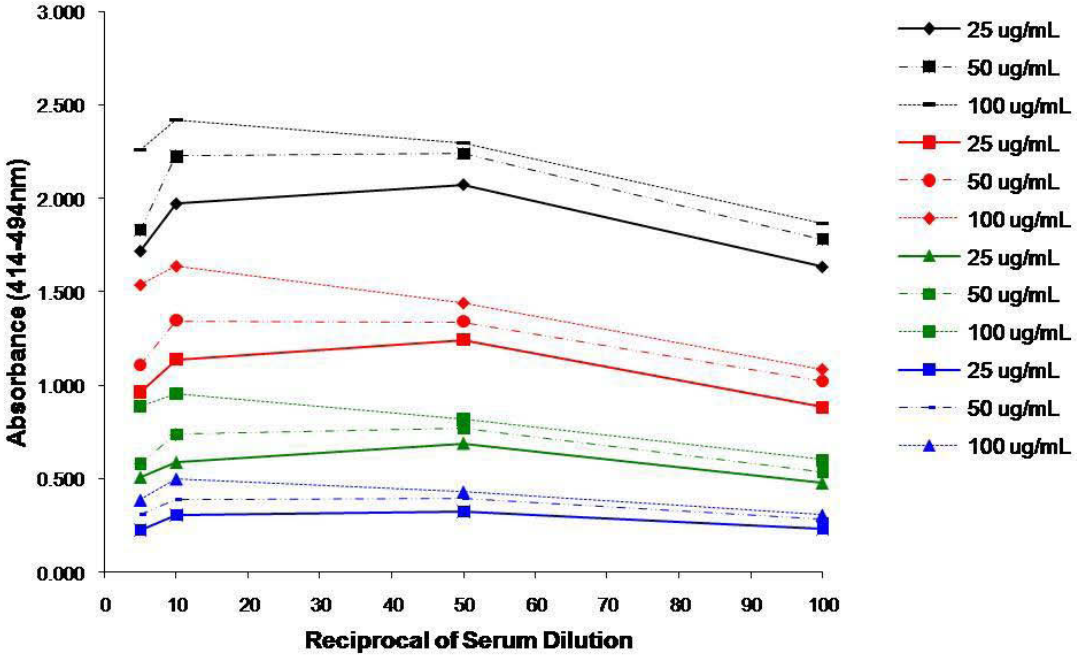

Figure 5.3: Optimisation ELISA for bovine sera against phase II antigen.
C. burnetii-positive bovine serum was tested at several dilutions against three concentrations of *C. burnetii* antigen. Secondary antibody was tested at 1:1,000 (black), 1:2,000 (red), 1:4,000 (green) and 1:8,000 (blue) respectively.

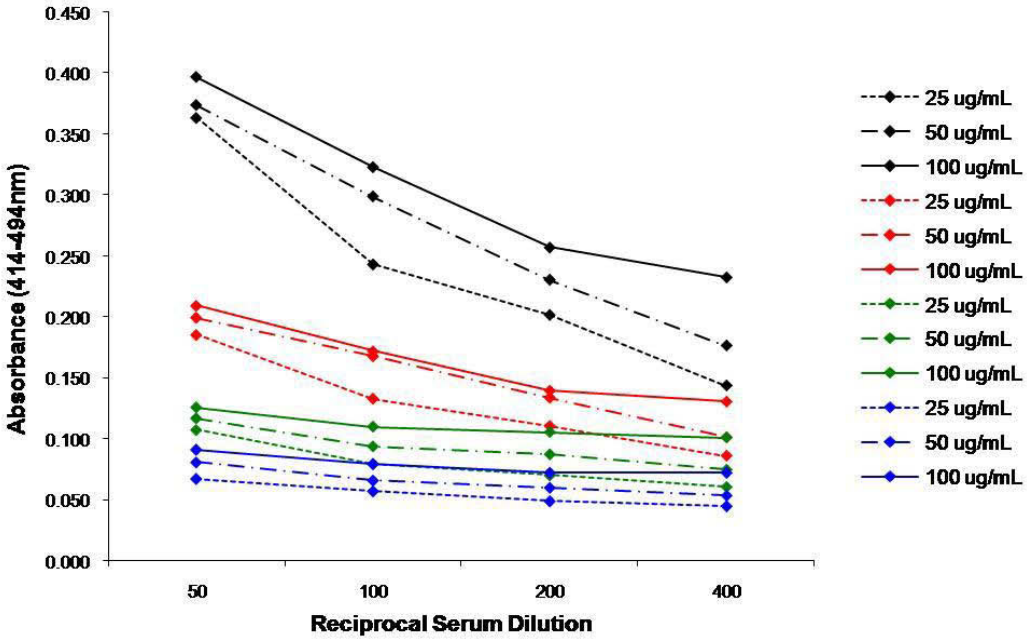

Figure 5.4: Optimisation ELISA for bovine sera against phase I antigen.
C. burnetii-positive bovine serum was tested at several dilutions against three concentrations of *C. burnetii* antigen. Secondary antibody was tested at 1:1,000 (black), 1:2,000 (red), 1:4,000 (green) and 1:8,000 (blue) respectively.

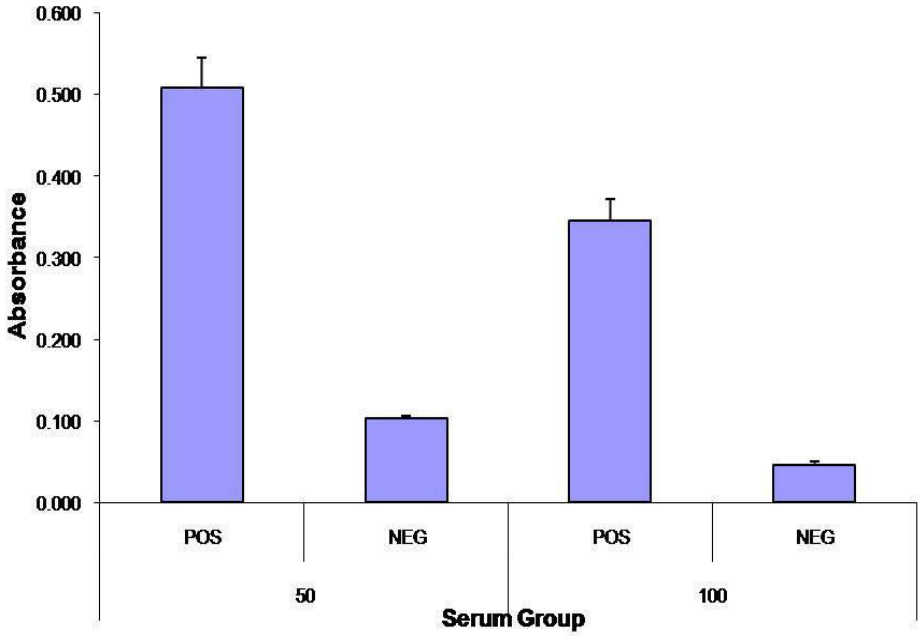

Figure 5.5: Bovine ELISA positive sample threshold determination.
C. burnetii-positive and negative bovine sera were tested at both 1:50 and 1:100 against *C. burnetii* antigen to determine best reagent concentrations for distinction between samples.

5.3.1.2 Canine ELISA for detection of antibodies to *Coxiella burnetii*

Optimal reagent concentrations for the canine ELISA were determined to be 100 µg mL^{-1} antigen for both phase II and I antigen, 1:100 test sera and 1:1,000 conjugate (Figure 5.6).

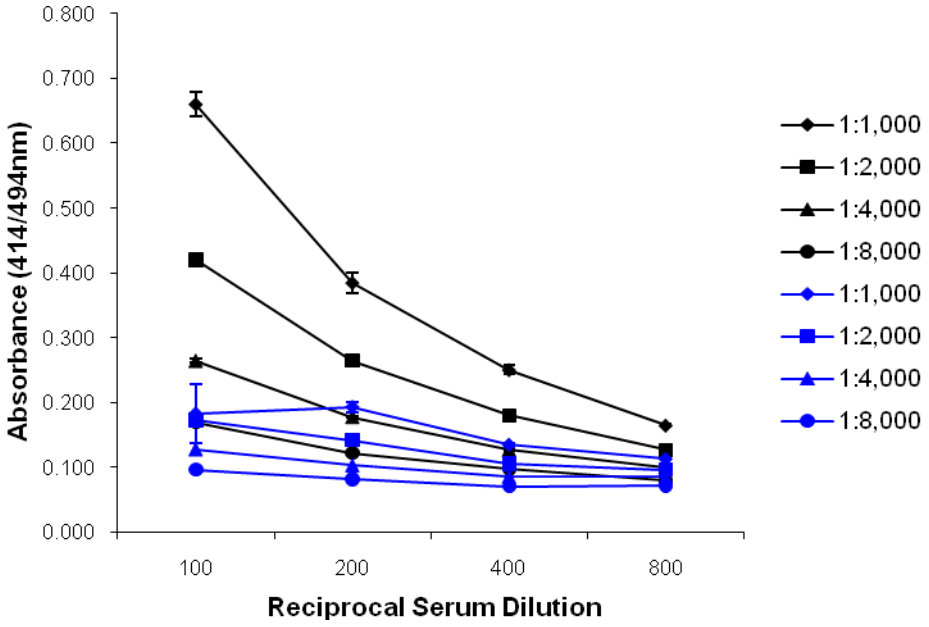

Figure 5.6: Canine serum optimisation ELISA.
C. burnetii-positive (black) and negative (blue) serum was tested at serial 2-fold dilutions from 1:100 to 1:800 with secondary antibody at serial 2-fold dilutions from 1:1,000 to 1:8,000 (legend). Error bars represent SEM.

5.3.1.3 Feline ELISA for detection of antibodies to *Coxiella burnetii*

Optimal reagent concentrations for the feline ELISA were determined to be 100 µg mL^{-1} antigen for both phase II and I antigen, 1:100 test sera and 1:1,000 conjugate (Figure 5.7).

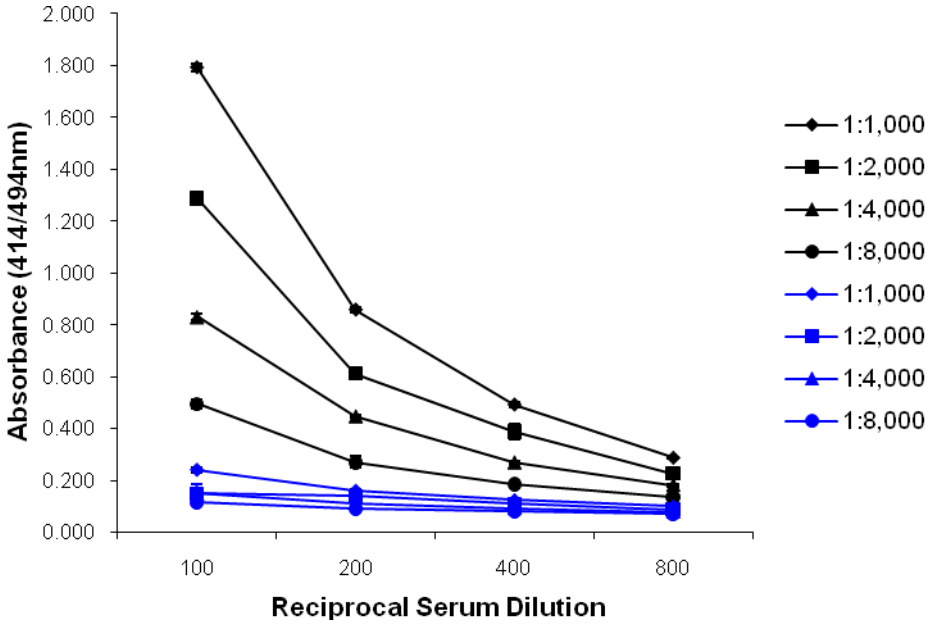

Figure 5.7: Feline serum optimisation ELISA.
C. burnetii-positive (black) and negative (blue) serum was tested at serial 2-fold dilutions from 1:100 to 1:800 with secondary antibody at serial 2-fold dilutions from 1:1,000 to 1:8,000 (legend). Error bars represent SEM.

5.3.1.4 Porcine ELISA for detection of antibodies to *Coxiella burnetii*

Optimal reagent concentrations for the porcine ELISA were determined to be 100 µg mL^{-1} antigen for both phase II and I antigen, 1:100 test sera and 1:1,000 conjugate (Figure 5.8).

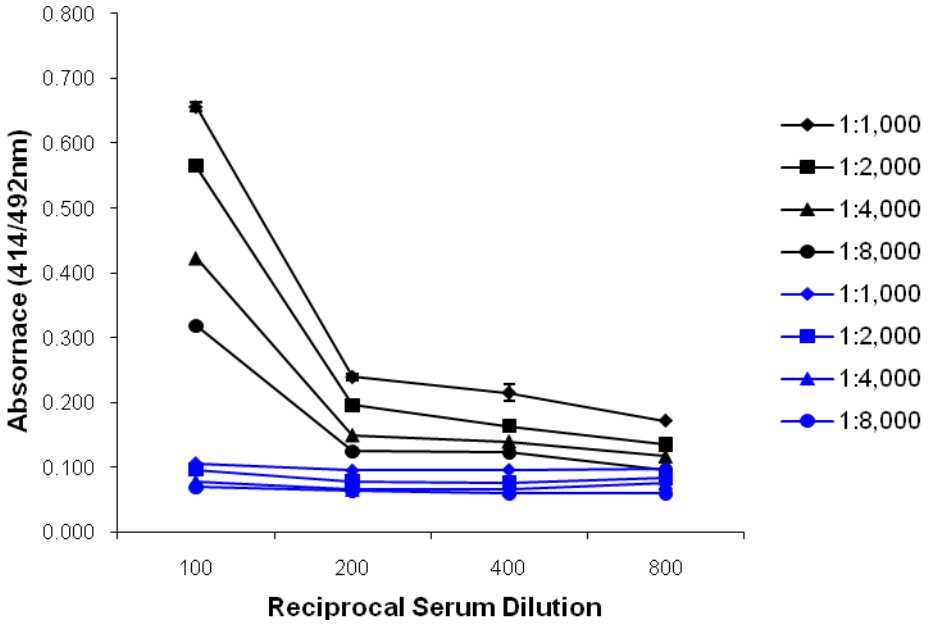

Figure 5.8: Porcine serum optimisation ELISA.
C. burnetii-positive (black) and negative (blue) serum was tested at serial 2-fold dilutions from 1:100 to 1:800 with secondary antibody at serial 2-fold dilutions from 1:1,000 to 1:8,000 (legend). Error bars represent SEM.

5.3.2 Bovine ELISA for the screening of beef cattle sera for antibodies to *Coxiella burnetii*

5.3.2.1 Screening of beef cattle sera from abattoir

Of the 720 abattoir samples collected, 67 (9.3%) and 34 (4.7%) were positive for antibodies to phase II and phase I *C. burnetii* antigen respectively. Of the seropositive samples, 28% (n=201) were positive for both phase II and phase I, with 82.3% of beef cattle positive for phase II also positive for phase I. Seropositivity to either or both antigenic phases was 10.1% (n=73). Of the sera positive for antibodies to phase II antigen, 64% (n=43) were classed as weakly positive, 15% (n=10) positive and 21% (n=14) strongly positive. For the sera positive for antibodies phase I antigen, 20% (n=7) were classed as weakly positive, 50% (n=17) positive and 20% (n=7) strongly positive. Seropositive samples were returned from 40% of the properties. Approximately 70% of the phase II and 80% of the phase I seropositive samples came from a single property in the Townsville Shire recording a high seroprevalence of 26.7% and 13.7% for phase II and phase I respectively. The cattle from which the abattoir sera were collected originated from a total of 17 northern Queensland cattle properties. These properties were located in 8 different districts, of which only those with greater than 10 samples per property were included in statistical analyses. There was no statistically significant difference between seropositivity cattle under and over two years of age.

5.3.2.2 Screening of beef cattle sera from breeders and heifers

Of the 1,345 samples tested, 134 (9.9%) and 145 (10.7%) were positive for antibodies to phase II and phase I *C. burnetii* antigen respectively. Of these, 2.6% were seropositive for both phase II and phase I. Seropositivity to either or both antigenic phases was 20.8% (n=280). Of the sera positive for antibodies to antibodies to phase II antigen, 46% (n=62) were classed as weakly positive, 30% (n=40) positive and 24% (n=32) strongly positive. For the sera seropositive for phase I antigen, 53% (n=77) were classed as weakly positive, 26% (n=38) positive and 21% (n=30) strongly positive. The cattle from

which the sera were collected originated from a total of 46 cattle properties. These properties were located in 24 different districts. Seropositive samples were returned from 78.2% of the properties. Seropositivity ranged from 0.0 to 65.4% for antibodies to phase II antigen and from 0.0 to 46.7% for antibodies to phase I antigen.

5.3.2.3 Overall seropositivity in Queensland beef cattle sampled

Overall seropositivity to phase II and phase I *C. burnetii* antigens in beef cattle sampled (n=1,835) in Queensland was 10.0% (n=184) and 9.2% (n=169) respectively. Seropositivity to either or both antigenic phases of *C. burnetii* was 16.8% (n=308). Samples were obtained from 58 mobs on 56 beef cattle properties located in 20 districts across Queensland. These districts covered most of the statistical divisions of the State of Queensland with the exception of the south east corner. A breakdown of beef cattle seropositivity by statistical division is included in Table 5.1. With the sera positive for antibodies to phase II antigen, 46% were classed as weakly positive, 30% positive and 24% strongly positive. Of the sera positive for antibodies to phase I antigen, 53% were classed as weakly positive, 26% positive and 21% strongly positive. Seropositivity was variable both between and within many of the districts and regions sampled (Table 5.1). The greatest variation was observed in the Fitzroy, Central West and Mackay regions.

5.3.2.4 Seasonality of seropositivity in beef cattle sampled

Seropositivity to both antigenic phases of *C. burnetii* was lower in earlier months of the year (March/April), higher in mid-year months (May/June) and increasingly higher in later months of the year (August/September) (Figure 5.9). Similar trends were seen for seropositivity to phase II and phase I antigen (Figure 5.9).

5.3.2.5 Q fever notifications and incidence in the human population

Annual Q fever notifications in Queensland, represented 34.3% (n=159) of notifications in Australia in 2004, 44.4% (n=156) in 2005, 41.7% (n=170) in 2006, 39.1% (n=174) in

2007 and 43% (n=159) in 2008. The incidence per 100,000 population per year in Queensland was the highest in the country and approximately double the incidence for the country combined (Figure 5.10). Incidence rates for the statistical divisions of the State of Queensland from which cattle samples originated were variable (Table 5.1). Q fever incidence rates in South West and Central West Queensland were the highest in the State. There was no correlation between seropositivity in beef cattle and Q fever notifications or incidence by district or region. Notifications for Q fever in Townsville have increased during 2004 to 2008, with five reported during 2004, seven during 2005, six during 2006, 11 during 2007 and 13 in 2008. Total cases reported during the period numbered 42 with a cumulative incidence of 5.3 per 100,000 population. The location of each statistical division in the State of Queensland is displayed in Figure 5.11 overlaid with the beef cattle seropositivity data, Q fever notifications during 2004 to 2008 and cumulative Q fever incidence data for the period for each division.

Table 5.1: Cattle seropositivity and human Q fever notification and incidence data per 100,000 population by Queensland Statistical Division (Region)

REGION	# CATTLE REGION	# CATTLE SAMPLES	SEROPOSITIVE CATTLE 2006-2009 PHASE II (%)	PHASE I (%)	OVERALL (%)	HUMAN NOTIFICATIONS 2004-2008 (POPULATION)	HUMAN INCIDENCE 2004-2008 PER 100,000
FAR NORTH	756,400	133	35 (26.4%)	17 (13.1%)	41 (31.1%)	76 (292,308)	5.2
NORTHERN	1,073,600	539	65 (12.1%)	27 (5.0%)	80 (14.8%)	56 (219,608)	5.1
NORTHWEST	1,915,400	350	33 (9.4%)	46 (13.1%)	71 (20.3%)	3 (33,333)	1.8
MACKAY	1,232,200	30	0 (0.0%)	10 (33.3%)	10 (33.3%)	39 (165,957)	4.7
CENTRAL WEST	1,012,600	88	8 (9.1%)	10 (11.4%)	14 (15.9%)	21 (11,290)	37.2
FITZROY	2,354,600	55	3 (5.5%)	9 (16.4%)	11 (20.0%)	38 (211,111)	3.6
WIDE BAY/BURNETT	939,400	245	12 (4.9%)	9 (3.7%)	21 (8.6%)	39 (288,889)	2.7
DARLING DOWNS	1,390,800	240	24 (10.0%)	25 (10.4%)	42 (17.5%)	190 (233,129)	16.3
SOUTH WEST	1,146,800	155	4 (2.6%)	15 (9.7%)	18 (11.6%)	119 (26,154)	91.0
TOTAL	11,821,800	1,835	184 (10.0%)	168 (9.2%)	308 (16.8%)	581 (2,427,340)	4.7

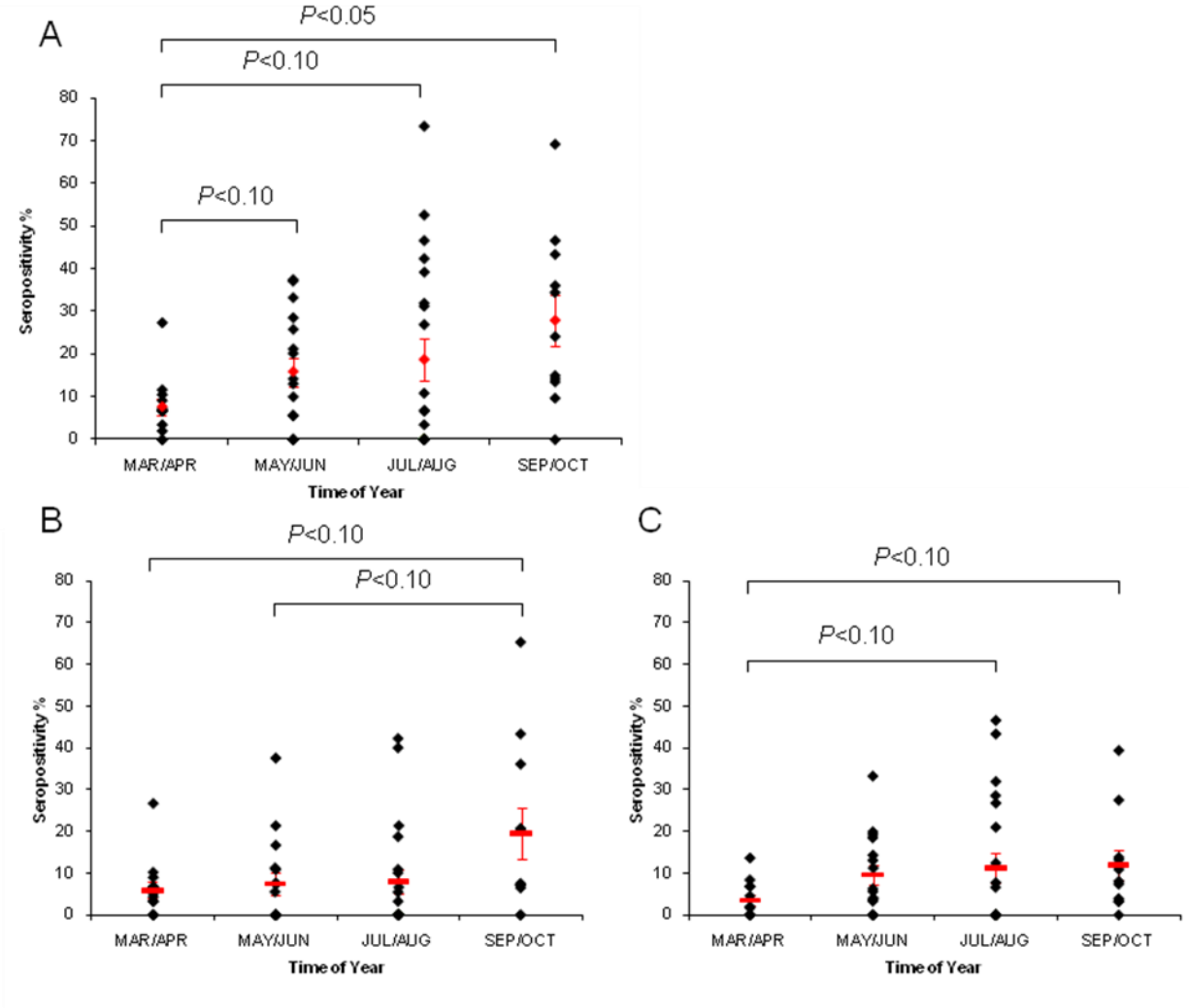

Figure 5.9: Seasonality of seropositivity to *Coxiella burnetii* antigens in beef cattle in the state of Queensland.

A. Seropositivity to either or both antigenic phases in various months of the year. **B.** Seropositivity to phase II antigen in various months of the year. **C.** Seropositivity to phase I antigen in various months of the year. Red horizontal bars represent mean for each period and red vertical bars represent standard error of the mean for each period. Black bars represent degree of statistical significance between periods.

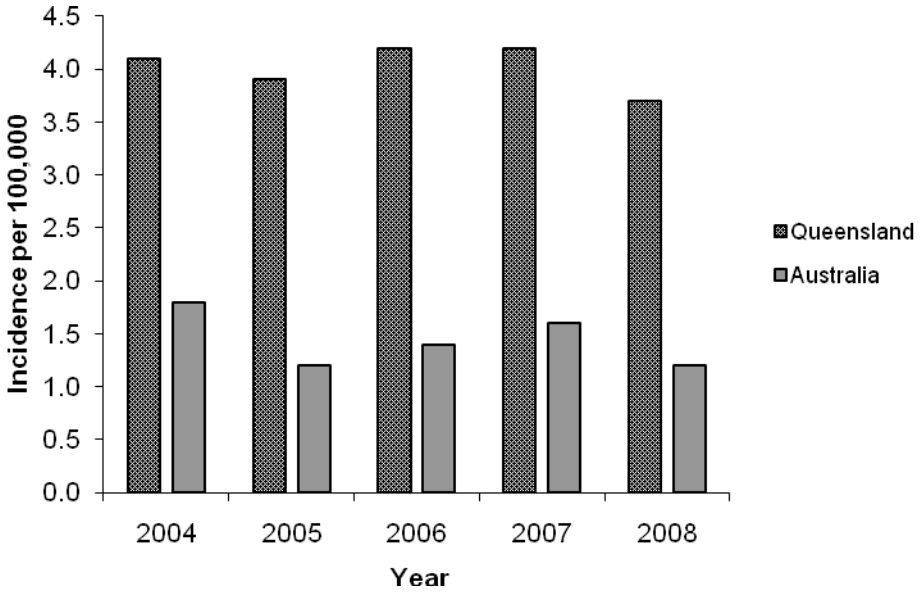

Figure 5.10: Q fever incidence in Queensland and Australia 2004 to 2008.
Incidence of Q fever per 100,000 population for the State of Queensland and the rest of Australia over the period 2004 to 2008. Human incidence data obtained from the National Notifiable Diseases Surveillance Scheme, Department of Health and Aging, Australian Federal Government.

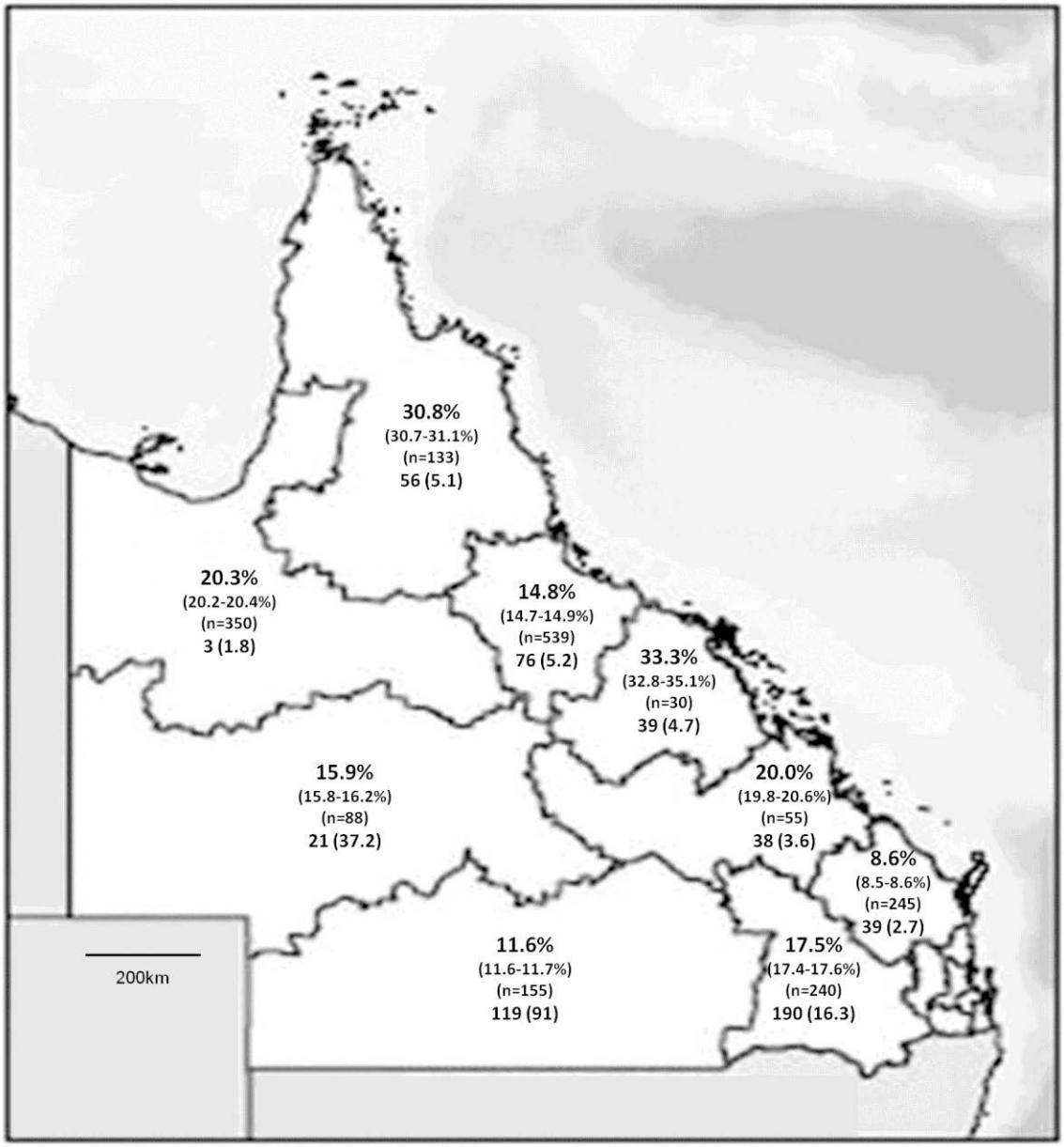

Figure 5.11: Relative seropositivity of beef cattle in sampled Queensland statistical divisions.

Data in each statistical division denotes seroprevalence in beef cattle, number of samples, Q fever notifications and cumulative incidence per 100,000 population for 2004 to 2008 period respectively (in brackets). Map of Queensland from Queensland Treasury, 2009.

5.3.2 Canine ELISA for the screening of sera for antibodies to *Coxiella burnetii*

A total of 201 domestic, 127 wild dog and 16 fox serum samples were screened for the presence of anti-*C. burnetii* phase I and II antibodies.

Of the 101 domestic dog samples collected during 2006 to 2009, seroprevalence for phase II and phase I antigens was 12.8% (n=13) and 10.9% (n=11) respectively with an overall seroprevalence of 21.8% (n=22). Of the samples seropositive for phase II antigens, three (23.1% of positive samples, 2.9% of samples) were also seropositive for phase I antigens. A summary of seroprevalence as determined by S/P% is listed in Table 5.2. Using Chi-squared tests, the only factor found to have a positive association with seropositivity against phase II antigen (S/P%≥50%) was contact with wildlife (Relative Risk 2.3, Odds Ratio 3.0, $P<0.05$) (Table 5.3). Factors found to have a positive association with seropositivity against phase I antigen (S/P%≥50%) in domestic dogs included contact with wildlife (RR 2.4, OR 2.9, $P<0.05$), contact with ticks (RR 2.7, OR 2.9, $P>0.05$) and contact with farm animals (RR 2.2, OR 2.4, $P>0.05$) (Table 5.3). Factors with a positive association with seropositivity against either or both phase II and phase I antigens included contact with wildlife (RR 2.2, OR 2.8, $P<0.01$), contact with farm animals (RR 1.9, OR 2.4, $P<0.05$) and contact with pregnant animals (RR 1.9, OR 2.3, $P<0.05$) (Table 5.3).

Of the 100 domestic dog samples collected during 1984 to 1985 seroprevalence for phase II and phase I antigens was 10.0% (n=10) and 8.0% (n=8) respectively with an overall seroprevalence of 16.0% (n=16). Of the samples seropositive for phase II antigen, two (20.0% of positive samples, 2.0% of samples) were also seropositive for phase I antigen. Overall, seroprevalence in the 201 domestic dogs sampled was 18.9% (n=38) with 11.4% (n=23) for phase II and 9.5% (19) for phase I. A summary of seroprevalence as determined by S/P% is listed in Table 5.2. Only age and sex data were available for the earlier sample set and risk associations were not determined.

In the dingo samples collected (n=127) seroprevalence for phase II and phase I antigens was 12.6% (n=16) and 8.7% (n=11) respectively with an overall seroprevalence of 17.3% (22). Of the samples seropositive for phase II antigen, five (22.7% of positive samples, 3.9% of samples) were also seropositive for phase I antigen.

A total of 328 canine samples were tested for antibodies to phase II and phase I antigen. Overall seroprevalence for all dogs was 18.3% (n=60) with 11.9% (n=39) for phase II and 9.1% (n=30) for phase I. Of the samples seropositive for phase II antigen, two (28.6% of positive samples, 12.5% of samples) were also seropositive for phase I antigen. The only factor associated with seropositivity in dingoes was origin, with samples originating from south-eastern Queensland more likely to be seropositive for phase II (RR 2.5, OR 2.9, χ^2 4.8) or both antigens (RR 2.8, OR 3.6, χ^2 9.8) than samples originating from northern Queensland. The difference in seropositive samples between south-eastern Queensland and northern Queensland was statistically significant for phase II antigen (P<0.05) and both antigens (P<0.05).

In the 16 foxes sampled, seroprevalence was determined to be 43.8% (n=7) overall and 37.5% (n=6) and 18.7% (n=3) for phase II and phase I respectively.

Table 5.2: Summary of seropositivity in domestic dogs from both cohorts and dingoes

COHORT	**SEROPOSITIVITY % (n)**				
	Negative (S/P%<50)	Positive (S/P%≥ 50)		High Positive (S/P%≥ 75)	
		Phase II	Phase I	Phase II	Phase I
2006-2009	77.2 (78)	12.9 (13)	10.9 (11)	5.9 (6)	7.9 (8)
1984-1985	84.0 (84)	10.0 (10)	8.0 (8)	2.0 (2)	1.0 (1)
Dingo	82.7 (105)	12.6 (16)	8.7 (11)	1.6 (2)	2.4 (3)

Table 5.3: Relative Risk, Odds Ratio and Chi Squared values for various factors associated with seropositivity in domestic dogs

CATEGORY	RR	OR	CHI^2
ALL PII POSITIVE DOGS (S/P% >50%)			
CONTACT WITH WILDLIFE	2.3	3.0	5.6*
ALL PI POSITIVE DOGS (S/P% >50%)			
CONTACT WITH WILDLIFE	2.4	2.8	3.0*
EXPOSURE TO TICKS	2.7	2.9	2.1
CONTACT WITH FARM ANIMALS	2.2	2.4	2.3
EITHER OR BOTH PII & PI (S/P% >50%)			
CONTACT WITH WILDLIFE	2.2	2.8	6.0*
CONTACT WITH FARM ANIMALS	1.9	2.4	4.3*
CONTACT WITH PREGNANT ANIMALS	1.9	2.3	3.7*

* Represent statistically significant CHI^2 critical value $P<0.05$

5.3.3 Feline ELISA for the screening of sera for antibodies to *Coxiella burnetii*

A total of 49 domestic and 31 feral cat serum samples were screened for the presence of anti-phase II and phase I *C. burnetii* antibodies. A total of three seropositive samples were identified in domestic cats, with an overall seroprevalence of 6.1%. Seroprevalence for phase II and phase I was 6.1% (n=3) and 0% respectively. In feral cats a total of 12 seropositive samples were identified, with an overall seroprevalence of 38.7%. Seroprevalence for phase II and phase I was 29.0% (n=9) and 22.6% respectively (n=7). The differences in seroprevalence between feral cats and domestic cats was statistically significant for phase II ($P<0.01$) and phase I ($P<0.01$) antigens and overall seroprevalence ($P<0.01$).

Overall seroprevalence in the 80 feline samples was determined to be 18.8% overall (n=15), 15.0% for phase II (n=12) and 8.8% for phase I (n=7). Age, sex and origin data were only available for feral cat serum samples. Factors associated with seropositivity in feral cats were origin, sex and age (Table 5.4). Statistically significant factors included origin (P<0.05) and sex (P<0.05).

Table 5.4: Relative Risk, Odds Ratio and Chi Squared values for various factors associated with seropositivity in feral cats

CATEGORY	RR	OR	CHI^2
ALL PII POSITIVE FERAL CATS (S/P% >50%)			
MALE	5.1	8.0	5.4*
SOUTH-EAST QLD ORIGIN	2.6	3.5	2.8
AGE	2.3	3.0	1.2
ALL PI POSITIVE FERAL CATS (S/P% >50%)			
SOUTH-EAST QLD ORIGIN	3.7	4.6	2.4
EITHER OR BOTH PII & PI (S/P% >50%)			
SOUTH-EAST QLD ORIGIN	3.7	6.7	8.0*
MALE	3.2	5.6	6.2*

* Represent statistically significant CHI^2 critical value P<0.05

5.3.4 Porcine ELISA for the screening of sera for antibodies to *Coxiella burnetii*

A total of 19 feral pig serum samples were screened for the presence of anti-phase II and phase I *C. burnetii* antibodies. A total of four seropositive samples were identified, with an overall seroprevalence of 21.1%. Seroprevalence for phase II and phase I was 15.8% (n=3) and 10.5% (n=2) respectively.

5.3.5 Human ELISA for the screening of sera for antibodies to *Coxiella burnetii*

Of the 1,522 human serum samples tested, 3.5% (n=53) were positive for antibodies to phase II *C. burnetii* antigen. Of these 40.7% (n=22) were classified as weakly positive, 33.3% (n=18) as positive and 26% (n=14) as strongly positive. As IgG to phase I *C. burnetii* antigen are only elevated in chronic Q fever, phase I antigen was not tested for human samples.

5.3.6 Summary of seroprevalence for all species sampled

Seroprevalence for either or both phase II and phase I antigens for each species surveyed are listed in Table 5.5. Seroprevalence was highest in foxes and feral cats. Similar seropositivity was found in beef cattle, domestic dogs (both currently and retrospectively) and wild dogs/dingoes. Seroprevalence was relatively low in domestic cats and the human population. There was no significant difference between seroprevalence in domestic dogs and dingoes. However, the difference between seroprevalence in domestic cats and feral cats was statistically significant.

Table 5.5: Seroprevalence for either or both phase II and phase I antigens in selected species

SPECIES	SEROPREVALENCE (95% CI)	NUMBER SAMPLED
Beef cattle (*Bos primigenius indicus*)	16.8 (16.78-16.80)	1,835
Wild dog/dingo (*Canis lupus dingo*)	17.3 (17.2-17.5)	127
Domestic dog (*Canis lupus familiaris*)	21.8 (21.6-22.1)	101
Domestic dog* (*Canis lupus familiaris*)	16.0 (15.9-16.2)	100
Domestic cat (*Felis catus*)	6.1 (6.1-6.5)	49
Feral cat (*Felis catus*)	38.7 (38.0-40.6)	31
Feral pig (*Sus scrofa*)	21.1 (20.7-23.5)	19
Fox (*Vulpes vulpes*)	43.8 (42.5-48.1)	16
Human (*Homo sapiens*)	3.5 (3.48-3.50)	1,522

*Denotes 1984-1985 cohort

5.4 Discussion

In this study antibodies to both phase II and phase I *C. burnetii* antigens were detected using ELISA. In human serology, elevated levels of antibodies to phase II antigen compared to phase I antigen are indicative of acute infection. Alternatively, elevated levels of antibodies to phase I antigen compared to phase II antigen are indicative of chronic infection. The development of antibodies to each antigenic phase of *C. burnetii* in animal infection has not been fully established (McQuiston and Childs, 2002). However, some studies have suggested the presence of antibodies to phase II antigen in animal sera is indicative of recent infection (Lackman *et al.*, 1962; Sidwell and Gebhardt, 1962). Serological tests for the presence of antibodies against *C. burnetii* in animals are unable to determine whether an animal is actively shedding the organism (McQuiston and Childs, 2002). Animals can remain seropositive for long periods after the initial infection has been cleared and some can seroconvert without shedding

C. burnetii. Alternatively, animals may begin to shed the organism prior to the production of antibodies and some infected animals never demonstrate seroconversion (McQuiston and Childs, 2002).

The current study demonstrated that the seroprevalence of *C. burnetii* in the bovine population sampled was 16.8% (95% CI 16.78-16.80%). While this is within the range of reported seroprevalences of *C. burnetii* in cattle in other parts of the world, it is still considered high. In the canine population sampled, seroprevalence for all dogs was 18.3% (95% CI 18.2-18.4%). There was no significant difference between seroprevalence in domestic dogs and wild dogs. In the feline population sampled, seroprevalence for all cats was 18.8% (95% CI 18.6-19.1%). There was a significant difference ($P<0.01$) between seroprevalence in domestic and feral cats, with seroprevalence in feral cats considerably higher for both antigenic phases. Seroprevalence in the relatively small numbers of feral pigs and foxes was 21.1% (95% CI 20.7-23.5%) and 43.8% (95% CI 42.5-48.1%) respectively.

Levels of seropositivity to *C. burnetii* antigens in the beef cattle tested varied according to the time of year. Seasonality of antibody levels has been demonstrated in sheep in North America (Enright *et al.*, 1971b). Antibody levels were found to increase during the lambing season due the shedding of *C. burnetii* into the environment. This effect is unlikely to be seen in beef cattle as year-round breeding occurs in most areas. However, as large quantities of *C. burnetii* can be shed during parturition it may be possible for infection of other species to occur, particularly in areas where primary production of both cattle and sheep are concentrated. However, sheep are unlikely to be a factor in seasonality of antibody levels in beef cattle in Queensland. This is reflected by the similarity in antibody trends in beef cattle in Queensland in both the northern and southern regions despite the differences in lambing season between the regions. Generally, the areas south of the southern regions lamb during July to September whereas areas north lamb during February to April (Wilson and Maxwell, 2003). The rise in seropositivity to *C. burnetii* in the later months of the year may be due to the increase in tick populations. In Queensland, ticks are predominately in the nymphal

phase during winter, with the emergence of adults and increase in tick numbers occurring during spring (Macleod, 2009).

The relationship between the geographical distribution of seroprevalence in cattle and human Q fever cases varies between studies. In Northern Ireland, no relationship was found between seroprevalence in cattle and human clinical cases (McCaughey *et al.*, 2010a). It was suggested that this may be due to differences between seroprevalence and susceptibility of the human population. This was supported by a study performed in Cyprus where, while the human incidence was only 1.2 per 100,000 population per year, the seroprevalence in the human population was 52.7% (Psaroulaki *et al.*, 2006). These findings suggest that high seroprevalence in the human population results in low susceptibility for Q fever infection. A further study reported high rates of seroconversion in previously seronegative humans and indicated most cases were sub-clinical (Loukaides *et al.*, 2006). In the current study, there was no correlation between beef cattle seroprevalence and either human Q fever notifications or human Q fever incidence. This may reflect the possibility of animals other than cattle also acting as the reservoirs of infection in some cases. In the current study the seroprevalence in the human samples tested in the Townsville region was 3.5%. The relatively low seroprevalence in this region may be related to the relatively high cumulative incidence for Q fever of 5.1 per 100,000 population over the 2004 to 2008 period.

The evidence of *C. burnetii* infection in beef cattle may have public health implications due to the proximity of beef cattle properties and residential areas in regional Queensland. This proximity also results in the transport of livestock through urban areas, particularly in the Townsville region where both a large abattoir and international port are located. Housing shortages have resulted in residential areas expanding into traditionally rural farming areas. There has also been an increase in semi-rural living and hobby farming in northern Queensland. These developments would increase the exposure of the human population and companion animals to wildlife and livestock.

Although cattle, sheep or goats are the traditional sources of human infection, it has been suggested that domestic pets may be responsible for an increasing number of outbreaks of Q fever. This potential reservoir was investigated in the current study due to an increasing number of reports of small animal veterinarians with no known exposure to ungulates being diagnosed with Q fever (personal communication). It has been proposed that in some regions, these pets may actually be more commonly implicated than domestic ruminants in the transmission of disease to humans (Marrie *et al.*, 1988a). Following experimental infection, cats shed *C. burnetii* in their urine for up to two months (Babudieri, 1959). Exposure to contaminated litter or gardens may therefore be a potential source of human exposure. Although not reported, the same may apply to domestic dog waste. Although an epidemiological role of dogs in Q fever has been demonstrated (Buhariwalla *et al.*, 1996), there is still a paucity of information on the subject.

The prevalence of *C. burnetii* seropositivity in canine samples collected at suburban veterinary practices in 2006 to 2007 was 21.8% (95% CI 21.6-22.1%). In the retrospective study on the canine serum samples collected in 1983 to 1984, seroprevalence was 16.0% (95% CI 15.9%-16.2%). Seroprevalence in dingoes was 17.3% (95% CI 17.2-17.5%). The canine samples in this study tended to be positive for either phase II or phase I antigen, with a small number of samples positive for both. This differs from human Q fever serology where seroconversion to the various antigens post-infection is relatively well characterised (Maurin and Raoult, 1999), with an initial rise in antibodies to phase II antigen, followed by antibodies to phase I antigen. However, the process of seroconversion is not well characterised in animals and seropositivity to either or both antigenic phase of *C. burnetii* has been shown to vary between species (Enright *et al.*, 1971a; Marrie *et al.*, 1985; Marrie *et al.*, 1993). The increasing incidences of human Q fever in what are traditionally considered to be low risk populations may be due to transmission of *C. burnetii* from companion animals.

While developing the questionnaires for the pet-owners, it was hypothesised that factors such as farm animal contact, wildlife contact, contact with ticks, living near farms or

having pet-owners who worked closely with other animals would have a positive correlation with seropositivity to *C. burnetii* in the pets. This hypothesis was supported by the higher risk ratios and odds ratios associated with some of these factors. In particular, contact with wildlife, farm animals (ruminants) and pregnant animals were found to have a positive association with seropositivity. These positive associations were consistent with previously observed transmission routes of *C. burnetii*. The positive association with seropositivity in dingo samples originating from south-eastern Queensland indicated these animals may be a potential reservoir for Q fever in peri-urban areas in this region. Studies involving GPS tracking of dingoes in this region indicated animals regularly ranged into urban areas (Allen, 2006).

While domestic cats do not appear to be a potential reservoir of *C. burnetii* in northern Queensland, their feral counterparts may constitute a more significant reservoir. The potential for feral cats as a reservoir of *C. burnetii* is considerably greater in south-eastern Queensland, where seroprevalence in these animals was greater than 50%. The seroprevalence in domestic and feral cats determined in the current study was consistent with that of feline surveys conducted internationally (Komiya *et al*., 2003).

As only 19 feral pig and 16 fox serum samples were collected, only preliminary conclusions could be drawn from the seropositivity results for these species. The fox samples taken in this study consisted of by-catch of wild dog/dingo control works, as foxes were not the target species of the eradication programs. However, the high seroprevalence in fox sera sampled indicates further investigation of this species as a reservoir for Q fever may be warranted. The incidence of feral pig incursion in urban areas has been increasing in Queensland (Mitchell, 2002). Feral pigs also constitute the most popular game animal in Queensland (McGaw and Mitchell, 2003). The detection of antibodies to *C. burnetii* in these animals indicates they may be a potential reservoir for Q fever for recreation and professional pig hunters, as well as primary producers who engage in feral pig eradication measures.

The current study demonstrated that the seroprevalence of *C. burnetii* in the human population sampled was approximately 3.5% (95% CI 3.48-3.50%). This is within the range of reported seroprevalences of *C. burnetii* in humans in other parts of the world. This seropositivity level is similar to levels determined in a larger study in the United States (Anderson *et al.*, 2009). A recent survey of individuals under 25 years of age in South West Queensland found seroprevalences of 6.5% (95% CI 4.5-9.2%), with 2.5% (95% CI 1.0-5.5%) in children under 15 and 11.0% (95% CI 7.4-16.0%) in those aged 15 to 24 (Parker *et al.*, 2010). A further study conducted in the Hunter region of New South Wales found an overall seroprevalence of 7% (Islam *et al.*, 2011). However, these studies were both performed in areas with relatively high Q fever incidence.

This is the first known study to use an Australian *C. burnetii* isolate as a source of antigen in ELISA. In a recent Western Australian study (Banazis *et al.*, 2010), a commercial Q fever ELISA developed in Europe (IDEXX CHEKiT Q fever ELISA) was used to test the beef cattle sera. This study reported very low numbers of seropositive samples in beef cattle and sheep samples despite relatively high numbers of seropositive samples being detected in kangaroo samples from the same areas. A different antigen preparation was used to develop the kangaroo ELISA. Differences in antigen production such as the use of whole cell antigen, lysates or chemical extracts may account for variation in ELISA results. Also, it may be possible that there are antigenic differences between *C. burnetii* isolates from Europe and Australia that may account for the discrepancies in ELISA results published by the Western Australian group. Therefore, the use of local isolates for antigen production in ELISA may eliminate these discrepancies between results obtained using European isolates.

In conclusion, the ELISA technique developed in this study enabled large numbers of animal sera to be screened efficiently at a relatively low cost per sample. As there are no studies published on *C. burnetii* prevalence in this region, this study is a step forward towards understanding the seroepidemiology of Q fever in Queensland. Further investigation of additional potential reservoirs such as other livestock and wildlife is needed to determine their role in incidences of human Q fever.

CHAPTER SIX

OPTIMISATION AND VALIDATION OF PHAGE DISPLAY FOR PRODUCTION OF RECOMBINANT ANTI-SPECIES ANTIBODIES

6.1 Introduction

Secondary antibodies are essential for immunoassays to detect antibody responses against pathogens, thereby enabling diagnosis of current and prior infections. Although anti-species or secondary antibodies are readily available for most domestic animals, for more exotic animals anti-species antibodies are not commercially available. This lack of availability of secondary antibodies for exotic animals requires the use of alternative methods to monitor zoonoses in these populations. Surveillance for zoonotic diseases with non-domesticated hosts is often performed via the use of sentinel animals. These animals are usually domestic animals for which secondary antibodies are commercially available, such as cattle, dogs, pigs and chickens (Ward *et al.*, 1995; Broom and Whelan, 2005; Duncan *et al.*, 2005). Sentinel animals are maintained and regularly screened for exposure in areas where wildlife reservoirs of diseases of interest are located.

Alternatively, monoclonal or polyclonal antibodies can be produced against species immunoglobulins for the purposes of performing serological surveys in those animals. Historically, the hybridoma technique (Kohler and Milstein, 1975) has been used for the generation of monoclonal antibodies. Practical applications of this method have been used in several serological surveys in wildlife (Marrie *et al.*, 1993; Gardon *et al.*, 2001). The technique is based on the immortalisation and subsequent cloning of specific antibody producing lymphocytes (Schmitz *et al.*, 2000). Lymphocytes isolated from animals, usually mice, immunised with the antigen of choice, are fused with myeloma cells. The hybridomas are then screened for clones producing the appropriate antibody.

Monoclonal antibodies are then produced in ascites in mice, from which they are affinity purified and labelled if necessary. This technique is expensive, time consuming and increasingly difficult to justify due to animals welfare issues. Polyclonal antibodies are more commonly used as secondary antibodies in serological surveys as they are more easily generated. However, they require the maintenance of an animal over an extended period with occasional boosting, or the performance of a terminal bleed. Phage display is a relatively new technique, and has been developed for the production of recombinant monoclonal antibodies as an alternative to hybridoma technology (McCafferty *et al.*, 1990; Gram *et al.*, 1992).

Phage display is a molecular technique used to display proteins on the surface of filamentous bacteriophages (Smith, 1985); (Figure 6.1). Phage display involves the genetic engineering of the genes encoding the antigen binding regions of immunoglobulins into filamentous bacteriophages to produce recombinant antibodies (McCafferty *et al.*, 1990). Recombinant antibodies can be expressed in two forms, antigen binding fragments (Fab) and single chain variable fragments (scFv). This is performed by immunising an animal host with the antigen of choice, then extracting the mRNA from mononuclear cells expressing antigen specific antibodies. Reverse transcriptase PCR (RT-PCR) is then used to amplify the genes encoding the antigen binding regions. Recombinant antibodies are more commonly constructed using murine or human derived antibodies. However, recombinant antibody libraries constructed using chickens (*Gallus gallus*) are technically less difficult to construct due to the immunoglobulin gene diversification mechanism unique to birds (Thompson and Neiman, 1987; McCormack *et al*, 1993). Diversity of the immunoglobulin response in birds is generated by immunoglobulin gene rearrangement (Thompson and Neiman, 1987). The advantage of producing chicken recombinant antibodies (CRAbs) is that the chicken possesses a single functional immunoglobulin variable region for both the heavy and light chain genes (McCormack *et al.*, 1993).

Following amplification, immunoglobulin genes are inserted into a phagemid vector containing the necessary genetic components to integrate into the genome of a

filamentous bacteriophage (usually M13 derived); (Smith *et al.*, 2004). The phage library is then produced using an *Escherichia coli* host. Successive rounds of panning are required to select reactive antibodies. The panning process removes non-specific clones while enriching the specific clones. Phage display antibodies can be bound to the required antigen, and then reproduced by infection of *E. coli*. As *E. coli* cultures can be grown overnight, rather than the weeks to months required for cloning hybridomas, this technique significantly reduces the traditionally time-consuming nature of monoclonal antibody production (Schmitz *et al.*, 2000). Once produced, phage displayed antibodies can potentially be used in any of the applications conventional monoclonal antibodies are currently applied to.

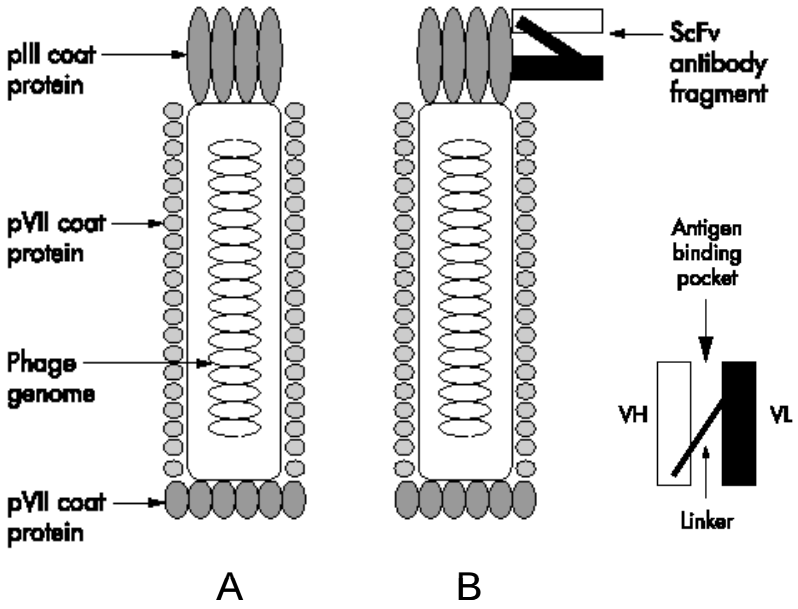

Figure 6.1: Phage-displayed scFv antibody fragments.
Representation of the standard M13 filamentous bacteriophage (A) and with expression of a single chain heavy and light chain variable region fragment (scFv) as a fusion to the pIII coat protein (B). Taken from (Smith *et al.*, 2004).

To date phage displayed antibodies have only been used as primary antibodies (Sapats *et al.*, 2006; Morar *et al.*, 2007; Tan *et al.*, 2007) in serological screening and neutralisation assays. Since monoclonal antibodies against immunoglobulins can also be

produced using the hybridoma technique, it stands to reason that secondary antibodies could be produced using phage display technology. These antibodies could potentially be used in serological tests for the detection and surveillance of zoonotic diseases in exotic animals for which secondary anti-species antibodies are not readily available.

The experimental work outlined in this chapter aimed to determine whether phage display was an effective tool for the production of secondary antibodies (conjugate) for use in indirect ELISAs. A mouse model was initially used to optimise the technique and validate chicken recombinant antibodies (CRAbs) against commercially available secondary antibodies.

6.2 Aims

The specific aims for the work described in this chapter were to:

1. Determine whether recombinant chicken secondary antibodies can be produced using phage display; and
2. Validate recombinant secondary antibodies in ELISA by comparison with commercially available secondary antibodies.

6.3 Materials and Methods

6.3.1 Blood collection and serum separation

Forty five male 12 week old BALB/c mice were euthanised by CO_2 asphyxiation and exsanguinated by cardiac puncture. The blood was pooled and left to clot for

approximately 30 min. The serum separated by centrifugation at 1,400 $\times g$ at room temperature for 10 min. A total of 10 mL of serum was collected.

6.3.2 IgG purification

6.3.2.1 Liquid chromatography

To purify murine IgG for the immunisation of chickens, murine serum was diluted 1:1 in binding buffer (Appendix A) and filtered through a 0.45 µm filter (Sarstedt, Germany). The serum was then loaded onto a 1 mL HiTrap protein G column (GE Healthcare, Australia) in an AKTAdesign™ liquid chromatography system (GE Healthcare, Australia). IgG was eluted using a linear gradient of elution buffer (Appendix A) at a rate of 1 mL min^{-1} at room temperature. Fractions of 500 µL were collected in 2 mL sample tubes contained 100 µL neutralisation buffer (Appendix A). AKTAdesign™ analysis software was used to determine fractions containing IgG. Fractions containing IgG were pooled and the concentration was determined using a BCA protein assay kit (Quantum Scientific, Australia). Purified IgG was lyophilised overnight using a freeze dryer (Dynavac, Australia).

6.3.2.2 Immunoblotting

To confirm purification of murine IgG, polyvinylidene difluoride (PVDF) membranes (Bio-Rad, USA) were cut to size, soaked in methanol for 30 sec and then rinsed with PBS-T (Appendix A). Aliquots of eluted IgG were blotted onto PVDF and bound for 30 min at room temperature. The membranes were blocked with blocking buffer (Appendix A) for 30 min at room temperature. Membranes were washed with PBS, and then incubated with rabbit anti-mouse IgG_1 or IgG_{2a} (BD Pharmingen, Australia) antibodies at a dilution of 1:5,000 for 1 hr at room temperature. The membranes were washed with PBS and then developed with DAB (Sigma, Australia) for 5 min.

6.3.3 Chicken immunisation

To induce the expression of Ig genes with specificity for murine IgG, two male and one female, six week old white leghorn chickens were immunised with 100 µg murine IgG emulsified with Freund's incomplete adjuvant (Sigma, USA) using 27 gauge needles and 1 mL syringes. Two boosters of 100 µg murine IgG with adjuvant were given at 14 and 28 days post initial immunisation. Chickens were provided with commercial feed and water *ad libitum* and monitored daily. Chickens were used under James Cook University Animal Ethics Approval number A1205.

6.3.4 Extraction and purification of messenger RNA

6.3.4.1 Messenger RNA from peripheral blood mononuclear cells

Whole blood was collected from chickens via wing stab with 25 gauge needles and 3 mL syringes then transferred to 10 mL tubes containing 5 mL 3% tri-sodium-citrate. Peripheral blood mononuclear cells (PBMC) were collected following purification over Ficoll-Paque (GE Healthcare, USA). Buffy coats were washed in RPMI (Invitrogen, USA) and then subjected to RNA extraction using a Total RNA Extraction Kit (Real Genomics, Taiwan) according to the manufacturer's instructions. RNA was quantified using a UV biophotometer at 260/280 nm (Eppendorf, Germany) and stored at -20°C. DNase I treatment was performed with 1× DNase I reaction buffer with $MgCl_2$ (Fermentas, USA), 1 unit ug^{-1} RNA DNase I (Fermentas, USA) and molecular biology grade water (Sigma, Australia) to a total volume of 10 µL.

6.3.4.2 Messenger RNA from splenic mononuclear cells

Chickens were euthanised by CO_2 asphyxiation. Excised spleens were passed through a sterile stainless steel strainer into transfer medium (Appendix A) in a stainless steel dish. The suspension was transferred to a 10 mL plastic centrifuge tube and tissue debris was

allowed to settle for 10 min at room temperature. The cell suspension was then layered over 3 mL Ficoll-Paque Plus (Amersham-Pharmacia, USA) and centrifuged at 500 ×*g* at 20°C for 20 min. Splenic mononuclear cells (MNC) were collected from the interface with a sterile Pasteur pipette and washed twice at 500 ×*g* at 20°C for 10 min in transfer media. Mononuclear cells were resuspended in 100 µL lysis buffer (Real Genomics, Taiwan). RNA extraction was performed using a Total RNA Extraction Kit (Real Genomics, Taiwan) according to the manufacturer's instructions with the DNA residue degradation protocol included. Ribonuclease inhibitor (Fermentas, USA) was added to the purified mRNA to prevent degradation. RNA was quantified using a UV biophotometer at 260/280 nm (Eppendorf, Germany) and stored at -20°C.

6.3.5 Complementary DNA synthesis and RT-PCR of V_H and V_L genes

6.3.5.1 Complementary DNA synthesis

To convert mRNA into cDNA, first strand cDNA synthesis was performed with reverse transcriptase (Fermentas, USA) and oligo dTT primers (Sigma-Genosys, Australia). Initial reaction mixes were prepared with 0.1-5 µg RNA, 0.5 µg oligo dTT primer and molecular biology grade water (Sigma, Australia) to a volume of 11 µL. The reaction mixes were incubated at 70°C in a Mastercycler® gradient thermocycler (Eppendorf, Germany) for 5 min then chilled on ice. The following reagents were then added in order; 1× reaction buffer (5×) (Fermentas, USA), 1mM DNTPs (Fermentase, USA), 20 U ribonuclease inhibitor (Fermentas, USA) and molecular biology grade water to a volume of 19 µL. The reactions were then incubated at 37°C for 5 min, after which 200 U RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, USA) was added. Reactions were incubated for a further 60 min at 42°C, stopped by heating to 70°C for 10 min then chilled on ice.

6.3.5.2 Reverse Transcriptase-PCR

Primer sequences (Table 6.1) for amplification of the V_H and V_L genes were obtained from published literature (Sapats *et al.*, 2003; Sapats *et al.*, 2006) and confirmed by Basic Local Alignment Search (NCBI, USA). Primers (Sigma-Genosys, Australia) were resuspended in TE buffer (Amasco, USA) to a stock concentration of 100 pm μL^{-1} and stored at -20°C. Five rounds of 10 × 50 µL PCR reactions were carried out with reagents at arbitrary concentrations (Table 6.2).

Table 6.1: Primers used to amplify V_H and V_L genes

Primer Name	Primer Sequence
V_H forward (Asc)	TTAGCTGGGCGCGCCGTGACGTTGGACGAGTC
V_H reverse (Xba)	GAACCGCCTCCACCATCTAGAGAGGAGACGATGACTTCGG
V_L forward (Sal)	GGCGGTGGCGGGTCGACAGCGCTGACTCAGCCGTCCTCG
V_L reverse (Not)	AGTTACTGGAGCGGCCGCACCTAGGACGGTCAGGG

Sapats *et al*. (2003), Sapats *et al*. (2006)

Table 6.2: PCR reagents

Reagent	Working Concentration
molecular biology grade H_20 (Sigma-Aldrich)	to 500 µL
10× reaction buffer (RBC)	1×
dNTPs (RBC)	200 µM
forward primer (Sigma Genosys)	300 nM
reverse primer (Sigma Genosys)	300 nM
RealHi *Pfu* blend polymerase (RBC)	0.03U μL^{-1}
Template DNA	10-30 ng

6.3.5.3 Amplification V_H gene

To amplify the V_H gene, cycling conditions consisted of an initial denaturation for 10 min at 94°C, followed by three sets of six cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 83°C, 82.5°C and 82°C respectively; extension for 1 min at 72°C, 20 cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 76.5°C and extension for 1 min at 72°C. The reaction was held at 4°C and 10 µL top up reagents (Table 6.3) was added to each reaction. A further five cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 72°C, extension for 1 min at 72°C, and a final cycle for 10 min at 72°C were performed.

6.3.5.4 Amplification V_L gene

To amplify the V_L gene, cycling conditions consisted of an initial denaturation for 10 min at 94°C, followed by three sets of six cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 75°C, 74.5°C and 74°C respectively; extension for 1 min at 72°C, 20 cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 66.5°C and extension for 1 min at 72°C. The reaction was held at 4°C and 10 µL top up reagents (Table 6.3) was added to each reaction. A further five cycles of denaturation for 15 sec at 94°C, annealing of primers for 30 sec at 62°C, extension for 1 min at 72°C, and a final cycle for 10 min at 72°C were performed.

Amplification was repeated five times for each gene, with the products pooled each time. V_H and V_L products were purified using a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan) according to the manufacturer's instructions.

Table 6.3: Top-up reagents

Reagent	Working Concentration
molecular biology grade H_20 (Sigma-Aldrich)	to 100 µL
dNTPs (RBC)	200 µM
forward primer (Sigma Genosys)	300 nM
reverse primer (Sigma Genosys)	300 nM
RealHi *Pfu* blend polymerase (RBC)	0.03U μL^{-1}

6.3.6 Half library construction

6.3.6.1 Vector amplification

The phagemid vector, pCANTAB-link (Sapats *et al.*, 2003) was amplified using a plasmid maxiprep kit (Bio-Rad, USA). Succinctly, 500 mL Luria Bertani (LB) broth (Appendix A) with 100 µg mL^{-1} ampicillin was inoculated with 100 µL transformed DH5α *E. coli* HIT cells (Real Biotech, Taiwan) and incubated overnight in a shaking incubator at 30°C and 150 RPM. The overnight culture was centrifuged at 4,000 ×*g* at room temperature for 10 min and phagemid extracted according to the manufacturer's instructions.

6.3.6.2 Restriction digestion of vector and inserts

To produce half libraries containing the V_H and V_L genes separately, the vector was digested with 10 units each of the restriction enzymes AscI and XbaI for the V_H library and SalI and NotI for the V_L library. Digests were performed in 100 µL volumes with 10 µg DNA at 37°C for 1 hr. The PCR products for the V_H and V_L genes were digested with AscI/XbaI and SalI/NotI respectively. Digests were performed in 100 µL volumes with 10 units of each restriction enzyme and 10 µg DNA at 37°C for 1 hr. Digested vectors were separated in a 2% agarose gel at 200 V for 20 min then excised. Excised

vector and PCR products were run through a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan) according to the manufacturer's instructions.

6.3.6.3 Ligation of vectors and inserts

The digested vectors and inserts were quantified and two ligation reactions (AscI/XbaI digested vector with AscI/XbaI digested PCR product and SalI/NotI digested vector with SalI/NotI digested PCR product) were set up with a 1:3 vector/insert molar ratio. Ligation reactions were incubated at 12°C for 3 hr. The ligation reactions were cleaned up using a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan).

6.3.6.4 Transformation of competent cells

To insert the phagemid containing either V_H or V_L genes into host cells, ligation reactions were electroporated into electrocompetent XL1β *E. coli* cells (Eppendorf, USA). Five separate electroporations were performed for each ligation reaction. Electrocompetent cells were thawed on ice, separated into 100 µL aliquots and incubated with 5 µL aliquots of ligation reaction on ice for 1 min. Cells were pulsed for 5 ms at 1.80 kV. Super optimal broth with catabolite repression (SOC, Appendix A) was added in 1 mL aliquots and transferred to a 50 mL falcon tube. A further 3 mL SOC was added to the pooled cells and incubated in a shaking incubator for 1 hr at 37°C and 250 RPM in a shaking incubator. Culture in aliquots of 1 µL, 10 µL and 100 µL was plated out on SOBAG agar (Appendix A) in order to count library sizes. Super optimal broth with ampicillin and glucose (SOBAG) agar plates were incubated overnight at 30°C. The remaining culture was added to 200 mL SOBAG medium and incubated overnight at 30°C and 170 RPM in a shaking incubator. Plasmid FastIon ™ Midipreps (Real Biotech, Taiwan) were performed according to the manufacturer's instructions. Vector containing the V_H fragments was referred to as V_H-link and vector containing the V_L fragments was referred to as V_L-link

6.3.7 Combined library construction

6.3.7.1 Restriction digests of half libraries and inserts

To produce libraries containing both V_H and V_L genes, the V_H-link phagemid was digested with NotI and SalI in 100 µL with 10 units of each enzyme and 10 µg DNA at 37°C for 1 hr. The V_L-link phagemid was digested with AscI and XbaI in 100 µL with 10 units of each enzyme and 10 µg DNA at 37°C for 1 hr. The PCR products for the V_H and V_L genes were digested with AscI/XbaI and SalI/NotI respectively. Digests were performed in 100 µL volumes with 10 units of each enzyme and 10 µg DNA at 37°C for 1 hr. Digested vectors were separated in a 2% agarose gel at 200 V for 20 min then excised. Excised vector and PCR products were cleaned by running through a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan) according to the manufacturer's instructions.

6.3.7.2 Ligation of inserts and production of combined library

To ligate V_H and V_L inserts into the respective half libraries, the digested vectors and inserts were quantified and two ligation reactions (V_H-link with SalI/NotI digested PCR product and V_L-link with AscI/XbaI digested PCR product) were set up with 1:5 vector/insert molar ratio. Ligation reactions were incubated at 12°C for 3 hr. The ligation reactions were cleaned using a HiYield Gel/PCR DNA Fragment Purification Kit (Real Genomics, Taiwan). The ligation reactions were electroporated into electrocompetent XL1β *E. coli* as previously described (Section 6.3.6).

6.3.8 Expression phage display library

To express the entire phage display library for panning against murine IgG, the overnight culture of electroporated cells in SOBAG agar was diluted to 0.5 OD_{600} using 2×YT-ATG (Appendix A), then incubated at 37°C for 1 hr. M13KO7 bacteriophage was

added at an M.O.I. of 5 and incubated at 37°C for 1 hr. The suspension was centrifuged at 1,000 ×*g* at 20°C for 20 min, resuspended in 10 mL 2×YT-AKT (Appendix A) and incubated overnight in a shaking incubator at 37°C and 250 RPM. Cells were pelleted by centrifugation at 1,000 ×*g* at 20°C for 20 min and the supernatant containing recombinant phage was filtered through a 0.45 µm filter. The phage were precipitated by the addition of 2 mL PEG/NaCl (Appendix A), mixed and incubated on ice for 45 min, followed by centrifugation at 10,000 ×*g* at 4°C for 20 min. Phage were resuspended in PBS and filtered through at 0.45 µm filter.

6.3.9 Library panning

In order to pan the phage-display library against murine IgG for the selection of reactive chicken recombinant antibodies (CRAbs), immunotubes™ (Nunclon, Denmark) were coated with 100 µg mL^{-1} murine IgG (Section 6.3.2) in 4 mL of carbonate buffer overnight at 4°C on a rotary wheel. Tubes were washed three times with PBS, pH 7.2 (Appendix A), filled with blocking buffer and incubated for 1 hr at room temperature on a rotary wheel, then washed three times with PBS. Precipitated recombinant phage was diluted 2:3 (v/v) in blocking buffer with 0.1% Triton X-100 and incubated for 15 min at room temperature. Tubes were filled with diluted phage solution and incubated for 2 hr at 37°C on a rotary wheel. Tubes were washed five times with PBS-T. Log phase XL1β *E. coli* (Invitrogen, USA) were added to tubes and incubated at 37°C for 1 hr at 250 RPM.

To generate phage displaying scFv, phage rescue was performed by adding 100 µg mL^{-1} ampicillin, 20 µg mL^{-1} tetracycline, 2% glucose (v/v) and 4×10^{10} pfu M13K07 bacteriophage. The culture was incubated at 37°C for 1 hr at 250 RPM. Cells were pelleted by centrifugation at 1,000 ×*g* at 20°C for 10 min and resuspended in 10 mL 2×YT-AKT and incubated overnight in a shaking incubator at 37°C and 250 RPM. Cells were pelleted by centrifugation at 1,000 ×*g* at 20°C for 20 min and the supernatant containing recombinant phage was filtered through a 0.45 µm filter. PEG precipitation

was performed as previously described (Section 6.3.8). Two further panning steps were performed with 10 and 20 PBS washes respectively. Log phase XL1β *E. coli* from the final panning round were converted to glycerol stocks and stored at -80°C.

6.3.10 Recombinant antibody screening

6.3.10.1 Preparation of master plates

To prepare master plates for the bulk screening of phage-displayed CRAb binding to murine IgG, glycerol stocks of the panned material were plated out on SOBAG agar at serial dilutions from 10^0 to 10^{-3}. Individual well-isolated colonies were inoculated into 400 µL aliquots of 2×YT-ATG in a 96-well cluster tube plate (Whatman, UK) and incubated at 30°C overnight at 250 RPM in a shaking incubator. For phage rescue of the single clones, 50 mL of 2×YT-ATG containing 2.5×10^{10} pfu of M13K07 bacteriophage was separated into 400 µL aliquots in a fresh 96-well cluster tube plate and inoculated with 40 µL of overnight culture from each tube of the previous set of cluster tubes. The plate was incubated at 37°C for 2 hr at 150 RPM in a shaking incubator. The plate was centrifuged at 1,500 ×*g* at 20°C for 20 min and the supernatants discarded. Double strength YT-AKT was added to each tube in 400 µL aliquots and incubated at 37°C overnight at 250 RPM in a shaking incubator. The plate was centrifuged again at 1,500 ×*g* at 20°C for 20 min and 300 µL of each supernatant was transferred to a 96-well plate. Blocking buffer (75 µL) was added to each tube and incubated at room temperature for 10 min.

6.3.10.2 Screening ELISA

To screen the selected phage-displayed CRAb binding to murine IgG, ELISA plates were coated with 100 µL of 100 µg mL^{-1} murine IgG in coating buffer (TropBio, Australia) overnight at 4°C. Plates were washed three times with PBS-T. Diluted phage stock was applied in 100 µL aliquots and incubated at 37°C for 1 hr with shaking. The

plates were washed again and 100 µL of 1:4,000 anti-M13 HRP conjugate (GE Healthcare, USA) was added and incubated at 37°C for 1 hr with shaking. The plates were again washed and 100 µL of ABTS was added and incubated for 20 min at 37°C, and absorbance measured at 414/494 nm in a Multiskan Ascent plate reader (Labsystems, USA). A set of antigen negative plates was included for each ELISA to eliminate any polypropylene-binding phage antibodies.

6.3.10.3 Amplification and sequencing of positive CRAbs

To obtain sequences of the strongly binding phage-displayed CRAbs, positive phage stock was inoculated into 5mL log phase XL1β *E. coli* culture in 2×YT-A (Appendix A) and incubated overnight at 37°C and 250 RPM. Plasmid minipreps were performed using an RBC kit (Real Biotech, Taiwan) according to the manufacturer's instructions. For each phagemid preparation, a 15 µL aliquot of 100 ng μL^{-1} DNA was prepared in an o-ring sealed microcentrifuge tube. All sequencing was performed commercially by Macrogen, Inc (Korea) using BigDye Terminators (ABI, USA). To construct consensus sequences, three forward and three reverse sequences were obtained for plasmids for three clones of CRAb phagemid. All consensus sequence construction was performed using Sequencher™ Version 4.7 (Gene Codes Corporation, USA). Sequence alignments were performed using Genedoc 2.7.0 (Biology Software Net, USA).

6.3.11 Production of soluble CRAbs

6.3.11.1 Transformation of HB2151 cells

To produce soluble scFv, phagemids from strongly binding CRAbs were transferred to host cells that would enable soluble expression. Positive phage detected using the ELISA protocol described in Section 6.3.10.2 was used to infect HB2151 *E. coli*. A 2 µL aliquot of phage supernatant was used to infect 100 µL log phase HB2151 *E. coli* cells (GE Healthcare, USA), incubated at 37°C for 1 hr then plated out on SOBAG-N

(Appendix A) agar. Single colonies were inoculated into 10 mL 2×YT-AG (Appendix A) and incubated overnight at 30°C with shaking at 250 RPM.

6.3.11.2 Soluble antibody expression

To produce soluble scFv from strongly binding CRAbs, the overnight culture was added to 200 mL super broth with ampicillin and glucose (SB-AG) (Appendix A) and incubated at 30°C for 1 hr at 250 RPM. Cultures were centrifuged at 4,000 ×*g* at 20°C for 10 min in 250 mL bottles in a Suprafuge 22 centrifuge (Heraeus Sepatech, Germany). The supernatant was discarded and the cells resuspended in 250 mL of SB-AI (Appendix A) and incubated at 30°C for 4-5 hr at 250 RPM. The culture was again centrifuged at 4,000 ×*g* at 20°C for 10 min with the supernatant discarded.

For periplasmic extracts, the cell pellets were resuspended in 20 mL chilled 1×TES (Appendix A), then combined with 30 mL 0.2×TES and vortexed to induce osmotic shock. The solution was chilled on ice for 30 min then centrifuged at 10,000 ×*g* at 4°C for 20 min in an Optima L-90 ultracentrifuge (Beckman Coulter, UK). The supernatant containing soluble antibodies was retained and dialysed against PBS, pH 7.2 overnight at 4°C. Concentrated soluble antibodies were stored at -20°C for further use.

For whole cell extracts, the cell pellets were resuspended in 50 mL PBS, pH 7.2 and lysed by freeze/thawing 5 times. Cell debris was pelleted by centrifugation at 2,000 ×*g* at 20°C for 20 min and supernatant transferred to a fresh tube and stored at -20°C for further use.

6.3.12 Production of phage-displayed CRAbs

To produce larger volumes of phage-displayed CRAbs, positive CRAbs were inoculated into 5 mL YT-A (Appendix A) and incubated overnight at 37°C with shaking at 250 RPM. The overnight culture was added to 50 mL YT-AG and incubated to log

phase at 37°C and 250 RPM. M13KO7 bacteriophage was added at an M.O.I. of 5 and incubated at 37°C for 1 hr. Cultures were centrifuged at 2,000 ×*g* at 20°C for 10 min, the supernatant discarded and the cells resuspended in 50 mL of YT-AKT (Appendix A) and incubated at 37°C overnight at 250 RPM. The culture was again centrifuged at 4,000 ×*g* at 20°C for 10 min with the supernatant centrifuged until a cell pellet was no longer produced. The supernatant was combined with PEG/NaCl (Appendix A), chilled and precipitated as described in Section 4.3.8. Phage were resuspended in TNE (Appendix A) and stored at 4°C until used.

6.3.13 Validation of CRAbs in ELISA

6.3.13.1 Infection of mice with *Coxiella burnetii*

Two groups of 10 female C57BL/6 strain mice at nine weeks of age were maintained in separate cages under PC3 conditions with food and water *ad libitum*. One group of 10 mice was inoculated with 1×10^4 live Cumberland isolate *C. burnetii*. The second group was inoculated with PBS. Mice were checked twice daily for two weeks, after which they were euthanised by CO_2 asphyxiation. Blood was collected via cardiac puncture and serum separated by centrifugation at 1,400 ×*g* for 10 min.

6.3.13.2 Optimisation of ELISA

A 96-well NUNC™ Maxisorp plate was coated with NMII/C4 antigen (50 µL of 25µg mL^{-1}), diluted 1:1 with coating buffer and incubated uncovered overnight at 37°C. Plates were washed three times with PBS-T, coated with 100 µL post-coating buffer, incubated at room temperature for 1 hr then dried. Murine sera from animals inoculated with live and formalin-inactivated *C. burnetii* and PBS were tested at dilutions of 1:20, 1:50 and 1:100. Sera were applied in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed as described previously. Anti-murine IgG CRAb C2 was applied

in 50 µL aliquots at a dilution of 1:5 and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL HRP-conjugated anti-M13 (GE Healthcare, USA) was applied at a dilution of 1:1,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied to the second plate and incubated at 37°C for 20 min. Plates were read in a Multiskan Ascent plate reader at 414/494 nm. A further checkerboard ELISA was performed with CRAb C2 tested at dilutions of 4:5, 3:4, 1:2, 1:4, 1:5, and 1:8. Anti-M13 was tested at 1:1,000, 1:2,000, 1:4,000 and 1:5,000. Statistical analyses were performed using Kruskal-Wallis tests to determine whether differences in optical density between infected, vaccinated and un-infected animals were statistically significant.

6.3.13.3 Validation ELISA

ELISAs were performed 96-well NUNC™ Maxisorp plates coated with NMII/C4 antigen (50 µL of 25µg mL^{-1}), diluted 1:1 with coating buffer and incubated uncovered overnight at 37°C. Plates were washed three times with PBS-T, coated with 100 µL post-coating buffer, incubated at room temperature for 1 hr, flicked out and dried. Murine sera were applied at a dilution of 1:20 in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed as described previously. Anti-murine IgG CRAb was applied at a titre of 1:5 in 100 µL aliquots and incubated at 37°C for 1 hr. The wells were washed again as described previously and HRP-conjugated anti-M13 applied at a dilution of 1:4,000 in 100 µL aliquots, then incubated at 37°C for 1 hr. The wells were washed again as described previously, after which 100 µL ABTS was applied incubated at 37°C for 20 min.

A standard indirect ELISA was performed with the same plates, antigen and sera. Murine sera were applied at a dilution of 1:100 in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed as described previously. HRP-conjugated rat anti-mouse IgG_{2a} (BD Pharmingen, Australia) was applied at a dilution of 1:1,000 in 50 µL aliquots and incubated at 37°C for 1 hr. The wells were washed again as described previously after which 100 µL ABTS was applied and

incubated at 37°C for 20 min. Plates were read in a Multiskan Ascent plate reader at 414/494 nm. Statistical analyses were performed using the Kruskal-Wallis test to determine degree of statistical significance between the ability of the commercial and phage-displayed ELISAs to distinguish between infected and un-infected animals.

6.4 Results

6.4.1 IgG purification

A total of 18 mg IgG was purified from 10 mL of pooled murine serum from multiple mice. An elution profile was obtained each batch of murine IgG (Figure 6.2).

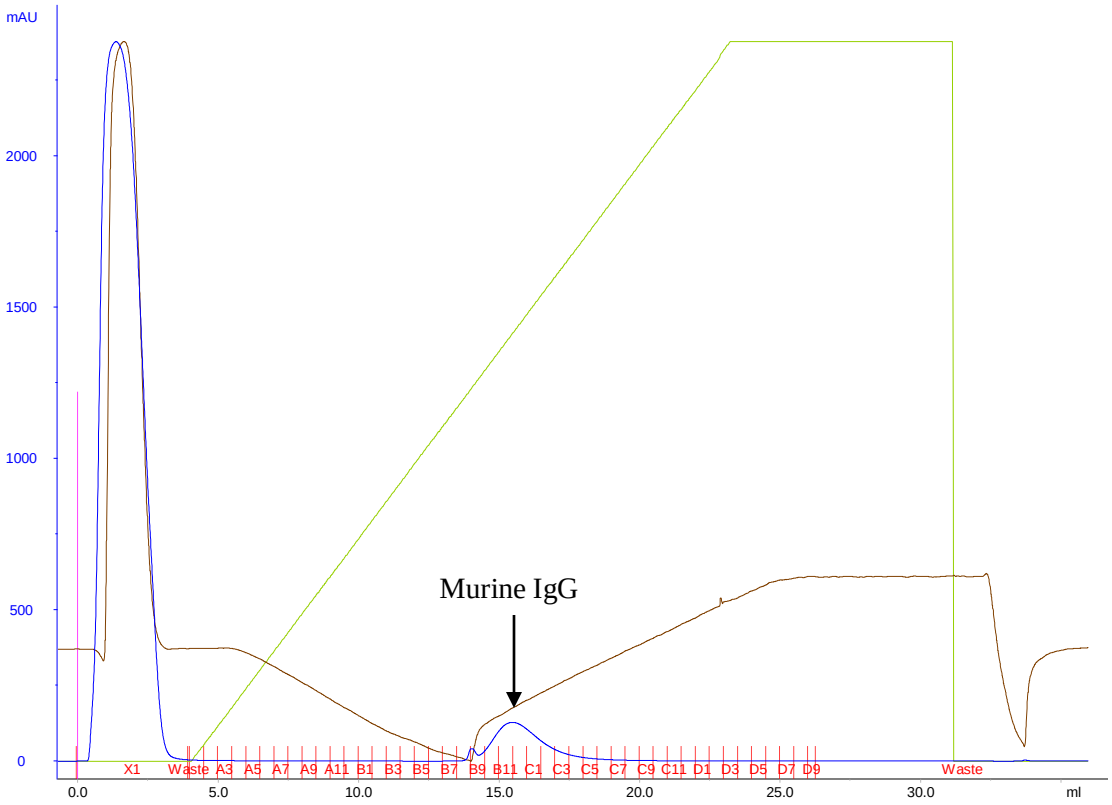

Figure 6.2: Chromatograph demonstrating elution of murine IgG.
Murine IgG purified over a protein G column using 0.1M glycine. Major plots include fractions (red), absorbance (blue), conductivity (brown) and elution buffer concentration (green). Murine IgG eluted over approximately 10 fractions from 50-60% 0.1M glycine (shown with arrow).

6.4.1.1 Immunoblotting

The presence of murine IgG was confirmed using an immunoblot (Figure 6.3). Both IgG_1 and IgG_{2a} were detected in the purified IgG. This result indicated more than one IgG subclass was able to be purified using the protein G affinity column. It also indicated murine IgG was consistently eluted at similar concentrations from sera using the affinity column.

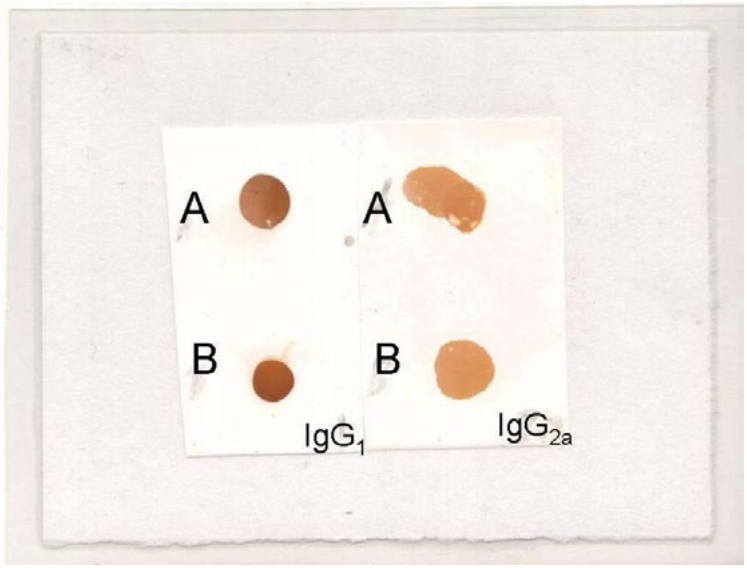

Figure 6.3: Immunoblots demonstrating purification of murine IgG from sera using protein G affinity column.
Pooled fractions from two separate IgG purifications (A and B) were blotted onto PVDF membrane then probed with HRP-conjugated anti-murine IgG_1 (left) and IgG_{2a} (right) antibodies and stained with DAB to confirm the presence of IgG and consistency of elution.

6.4.2 Library construction

A total of 3.2×10^6 and 5.2×10^6 clones were produced from the V_H and V_L half libraries respectively. For the combined library, 4.5×10^6 clones were produced for panning.

6.4.3 Panning and selection of CRAbs

A total of 95 CRAbs from the panned library were stored as glycerol stocks. Of these six CRAbs were positive for anti-murine IgG in phage-displayed format. None of the phage-displayed CRAbs bound significantly to the antigen negative plates. The CRAbs which reacted most strongly (A2, B3, C2 and D8) to the murine IgG were selected for further mass expression. CRAbs were unable to be tested in soluble form as the HRP-conjugated anti-E tag antibody (GE Healthcare, USA) required for their detection was discontinued by the manufacturer. However all CRAbs were expressed in bulk as phage-displayed antibodies and demonstrated binding to murine IgG. Of these, CRAb C2 was found to be the highest binder (Figure 6.4). None of the CRAbs demonstrated cross-reactivity with *C. burnetii* antigen.

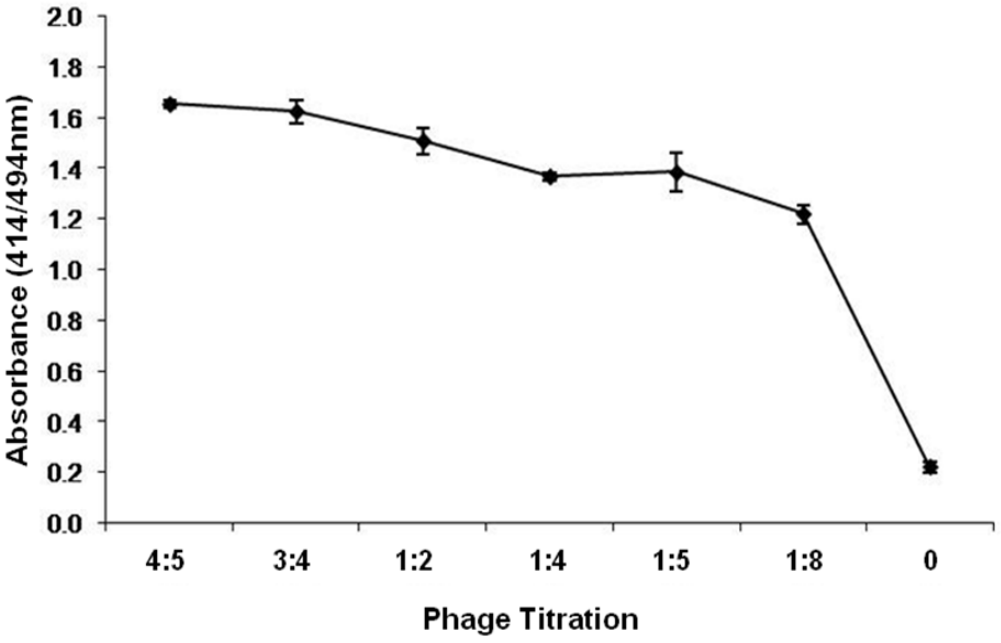

Figure 6.4: Titration of anti-murine IgG CRAb C2.
CRAb C2 was tested at various dilutions against murine IgG in ELISA format. Phage was detected with HRP-conjugated anti-M13 antibody. Titration series is not continuous. Error bars represent standard deviation.

6.4.4 Validation of CRAbs in ELISA

6.4.4.1 ELISA Optimisation

The variance between *C. burnetii* positive and negative murine sera was able to be distinguished with anti-murine IgG CRAb C2. Difference in absorbance between positive and negative sera was statistically significant at all serum dilutions tested ($P<0.01$). However, the greatest difference between positive and negative samples was obtained at a serum dilution of 1:20 (Figure 6.5). According to the results of the checkerboard ELISA, optimal dilutions of CRAb and anti-M13 antibody were determined to be 1:5 and 1:4,000 respectively (Figure 6.6).

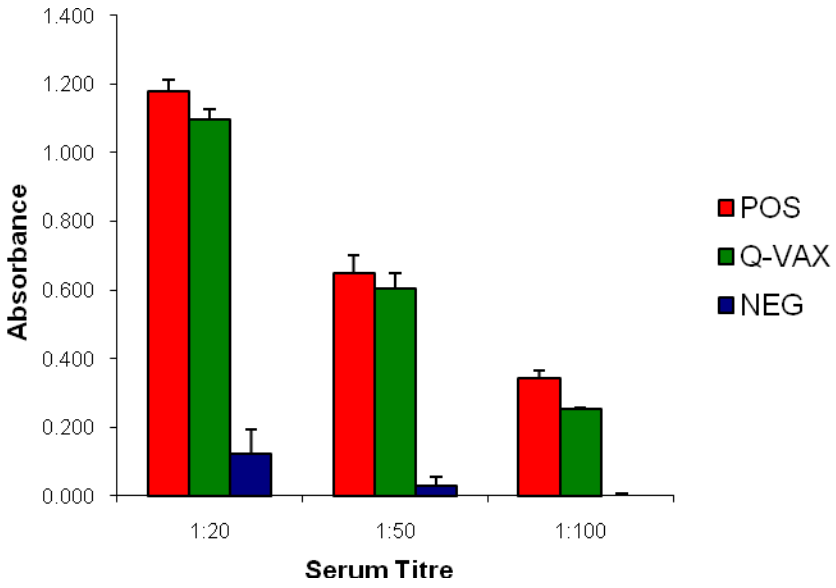

Figure 6.5: Optimisation of anti-murine IgG CRAb C2 in ELISA.
Pooled serum samples from three groups of mice; infected (POS), vaccinated (Q-VAX) and uninfected (NEG) were tested in *C. burnetii* ELISA using CRAb C2 and anti-M13 antibody for detection. Sera were tested at several dilutions to determine the optimal dilutions for the distinction between positive and negative samples.

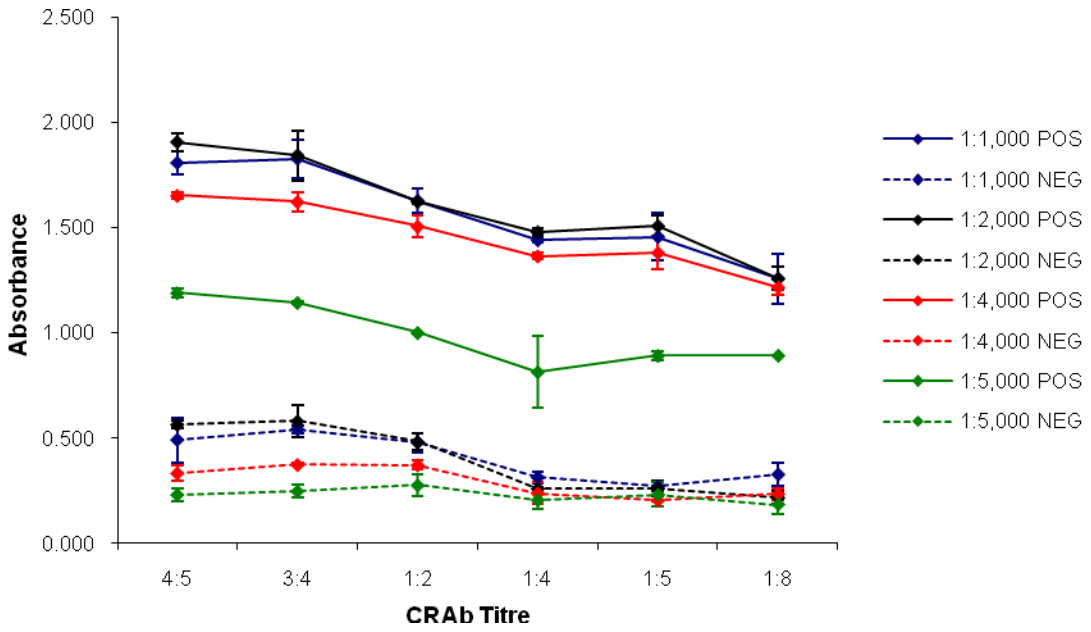

Figure 6.6: Optimisation of anti-murine IgG CRAb C2 in ELISA.
Pooled *C. burnetii* positive and negative serum samples from mice were tested in ELISA using CRAb C2 and anti-M13 antibody at various titres for detection. Titration series is not continuous.

6.4.4.2 Validation ELISA

All infected mice seroconverted following infection with *C. burnetii*, as demonstrated by indirect ELISA using conventional commercial antibodies. All PBS immunised animals had minimal absorbance readings in comparison to readings for positive animals ($P<0.01$). All *C. burnetii* immunised animal sera were also detected by phage-displayed antibody. No false positives were detected and sera from PBS immunised animals had minimal absorbance readings in comparison to readings for *C. burnetii* immunised animal sera ($P<0.01$). Absorbance readings generated using recombinant antibodies were significantly higher than those generated using commercial antibodies ($P<0.01$; Figure 6.7).

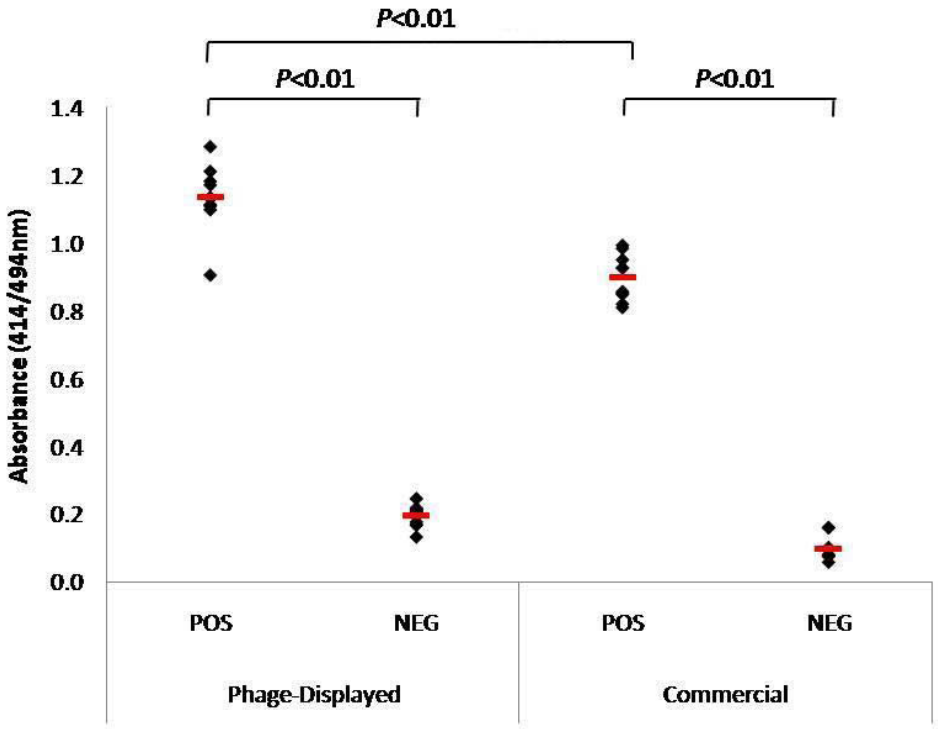

Figure 6.7: Validation of phage displayed antibodies by indirect ELISA.
Determination of *C. burnetii* antibody levels by ELISA comparing recombinant phage-displayed anti-murine IgG antibodies to commercial anti-murine IgG antibodies. Secondary antibody produced by phage display was efficient in detecting the presence of *C. burnetii* antibodies in immunised mice. Parallel bars represent mean for each group.

6.4.5 Sequencing of positive CRAbs

Sequencing of the positive CRAbs revealed a truncation of the light chain variable region Ig gene sequence of approximately 200 bp in two of the CRAbs (B3 and D8). This truncation did not appear to reduce binding efficacy to murine IgG in ELISA. However, a CRAb with a full length light chain Ig gene sequence was used in the validation ELISA (C2). Further investigation of the truncation phenomenon demonstrated the successive truncation of the light chain Ig gene sequence in the library during panning. Full light chain Ig gene sequence was found to be present by restriction digest in the phage display library prior to panning. Similar restriction digests from the

library at successive panning rounds revealed truncation and loss of the light chain Ig gene sequence. While all four CRAbs appeared to have full length heavy chain variable region Ig gene sequences, they did not match to heavy chain Ig gene sequences currently available on Genbank. However, alignment of heavy chain Ig gene sequences from CRAb phagemids with currently available sequences demonstrated homology of approximately 50% (Figure 6.8). All four sequenced CRAbs had identical heavy chain Ig gene sequences (Figure 6.9).

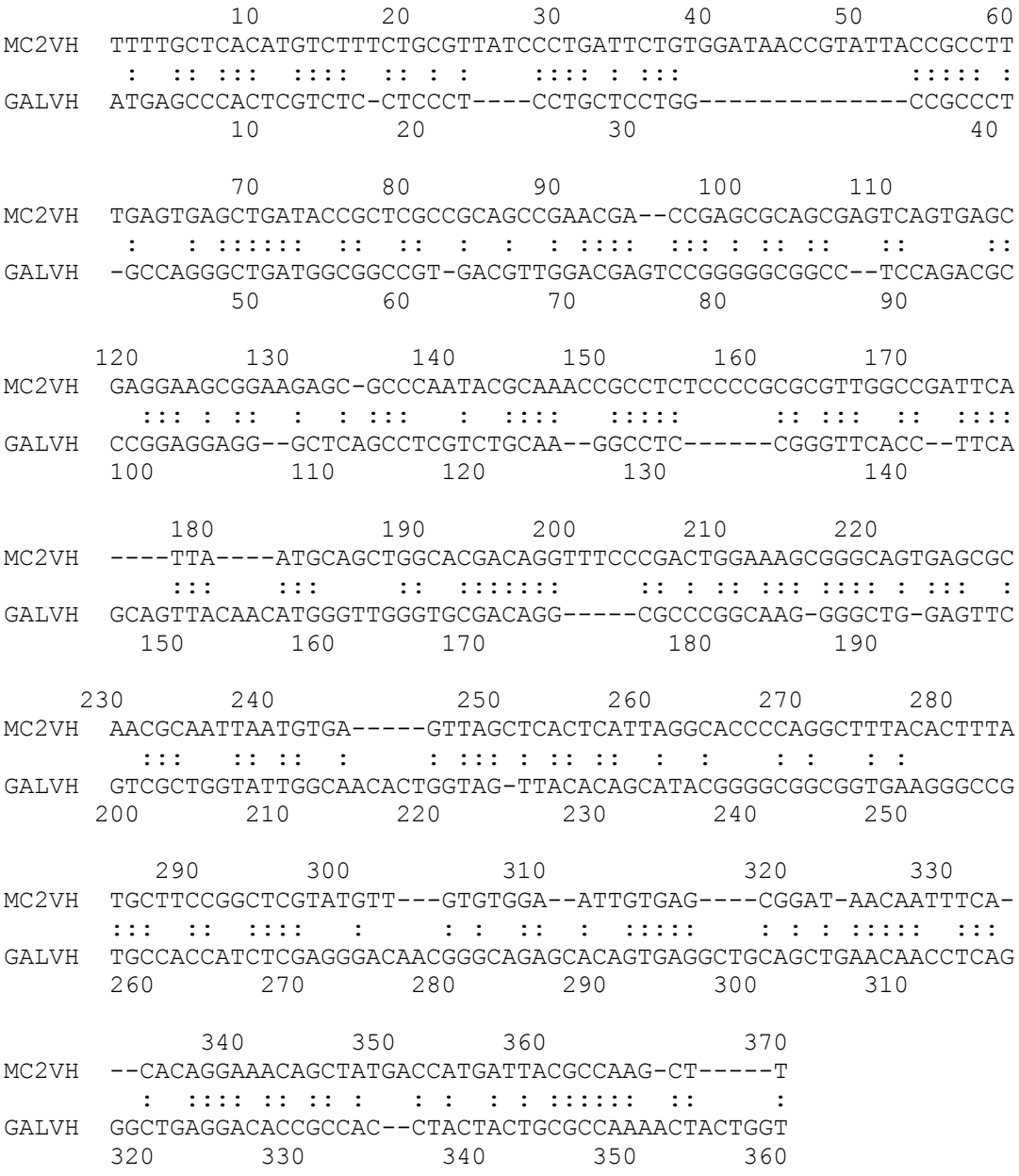

Figure 6.8: Alignment of DNA sequence for amplicon Type 2 against DNA sequence for *com1*.

Alignment of two sequences was performed using Align Pairwise DNA Alignment Algorithm. Identity of 49.4% was demonstrated between the two sequences. Top sequence is heavy chain Ig gene sequence for CRAb MC2 and bottom is heavy chain Ig gene sequence for *Gallus gallus*.

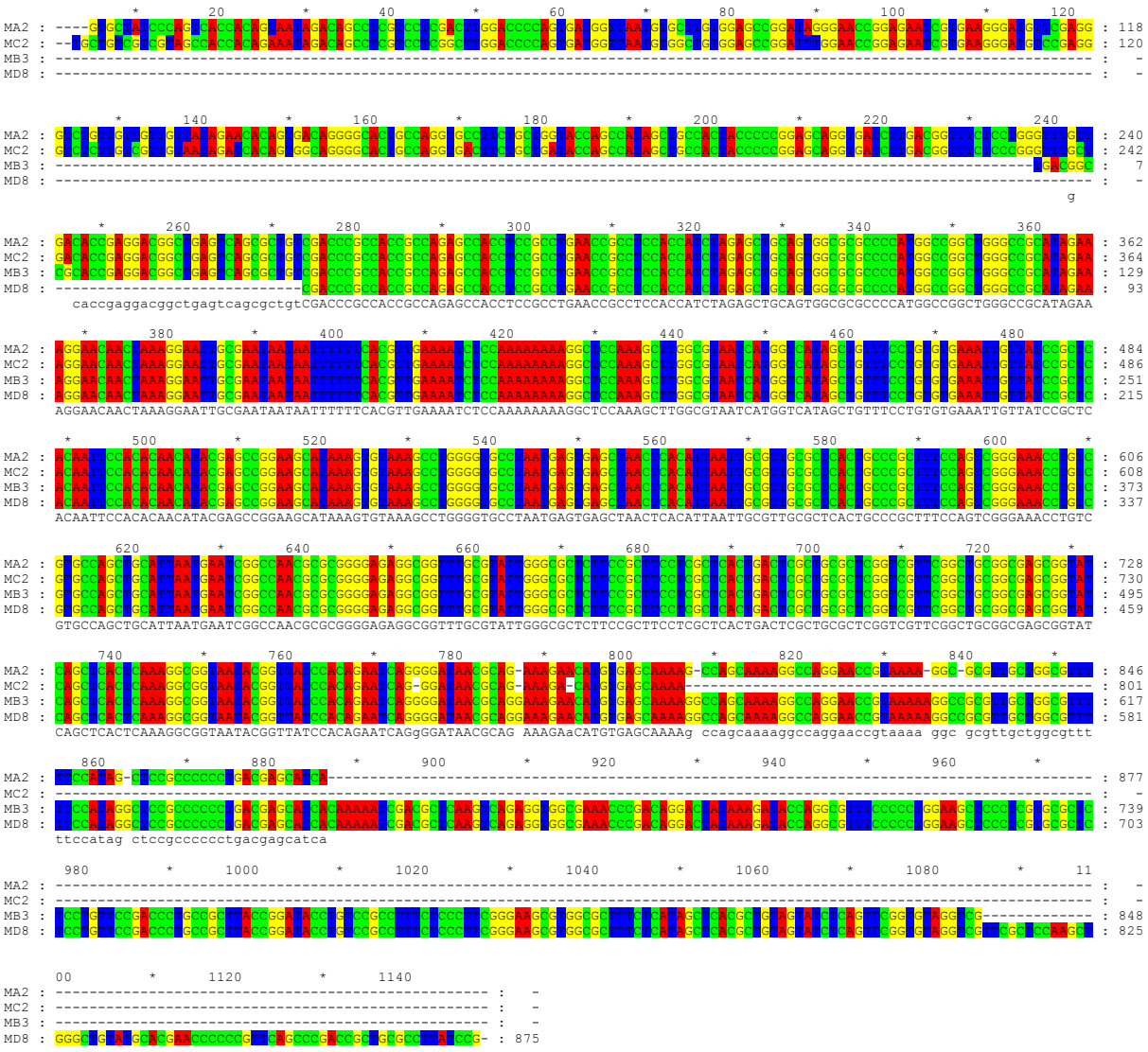

Figure 6.8: Anti-murine IgG CRAb sequence alignment.
Sequences obtained for CRAbs positive for murine IgG were aligned using Genedoc (Biology Software Net, USA). CRAb sequences are aligned 5'-3' from light chain to heavy chain. Post 800 bp includes phagemid vector sequence.

6.5 Discussion

Phage display has been established as an alternative method to hybridoma production of antibodies with a defined specificity (Dall'Acqua and Carter, 1998; Fernandez, 2004; Smith *et al,* 2004). However, it has not been assessed as an alternative to polyclonal antibodies. While phage display is initially more time consuming than polyclonal antibody production in host animals, it eliminates the need to maintain animals for subsequent boosting and bleeding. Recombinant phage-displayed antibodies can be generated with two overnight growth periods, rather than the weeks to months required producing polyclonal antibodies. In addition, the technique ensures conformity of antigen binding between batches as recombinant antibodies are essentially monoclones.

Recombinant phage-displayed antibodies produced according to the experimental work described in this chapter have been shown to be effective when used as secondary antibodies in ELISA. Recombinant anti-murine IgG antibodies were effective in detecting serological exposure to a pathogen (*C. burnetii*) with identical sensitivity and specificity to that achieved using commercial polyclonal anti-murine IgG antibodies. The recombinant antibodies generated using the methods optimised in the work described in this chapter elicited greater absorbance readings than those of the commercial secondary antibodies. This may be due to the greater surface area available for anti-M13 antibody binding on the phage. Greater sensitivity may be possible with HRP conjugation to the phage surface, which would also eliminate an incubation step in the ELISA protocol.

Sequencing of the recombinant antibodies demonstrated a truncation of the light chain variable region Ig gene sequence in some CRAbs. The cause of the truncation could not be established. However, the use of XL1 blue *E. coli* cells should have prevented insert truncation as these cells are *recA* deficient, a phenotype designed to improve insert stability. Additionally, the original glycerol stocks of the XL1 blue *E. coli* cells used for the production of the phage display library were prepared from colonies grown on

minimal media to ensure retention of the F-minus episome. This ensured transformation of the host cells by a single phagemid to reduce the potential for recombination events.

Preliminary DNA sequencing of PCR products prior to library construction indicated the PCR products were of correct length and expected heavy and light chain variable region Ig gene sequence. Alignment of heavy chain sequence from the CRAbs generated in the current study with heavy chain sequence obtained from Genbank (NCBI, USA) indicated approximately 50% homology between the sequences. As the chicken has a high degree of Ig gene diversification, it may be possible that this sequence does represent chicken heavy chain variable region sequence, but is simply atypical.

The absence of a typical variable region heavy chain Ig gene sequence and truncation events may be due to over-selection occurring during the panning process of the library. Over-selection is a phenomenon that occurs when the point at which positive CRAbs are present during panning is exceeded and the majority of CRAbs have similar affinity for the antigen, resulting in the selection pressure for binding affinity to the antigen losing effectiveness (Clackson and Lowman, 2004). Once this occurs, the expression level and valency of the CRAbs can begin to drive selection, which results in unusual CRAbs being selected. Valency may explain the binding of the positive CRAbs despite the unexpected heavy chain Ig gene sequence. In original Amersham Biosciences protocols for the Expression Module/Recombinant Phage Antibody System (Amersham, 1996) from which subsequent protocols were developed, only one round of panning is performed. It may be necessary to revert to a single panning round with a greater number of washes to avoid truncation events and over-selection in successive rounds of panning for the production of recombinant antibodies in future.

Recombinant phage-displayed secondary antibodies could potentially be applied to any application which polyclonal antibodies are currently designed for. The phage display technique can be used to generate antibodies specific for the immunoglobulin classes of animal species not currently available commercially. The ability to generate anti-immunoglobulin antibodies for exotic animals and wildlife would be of great value

in veterinary diagnostics, serological screening, zoonotic disease monitoring and biosecurity. This was evaluated through construction of anti-IgG antibodies for Australian native animals and the comparison of their effectiveness in serological screening of an endemic zoonotic pathogen, *C. burnetii* to be described in subsequent chapters.

CHAPTER SEVEN

PHAGE DISPLAY FOR PRODUCTION OF RECOMBINANT ANTI-SPECIES ANTIBODIES FOR SELECTED AUSTRALIAN NATIVE MARSUPIALS

7.1 Introduction

Zoonoses are diseases for which the aetiological agent is transmitted between vertebrate animals and humans (Hugh-Jones *et al.*, 1995). Vertebrate animals are the natural reservoir for the agents of zoonoses. These agents may be transmitted directly from animals to humans or indirectly by vectors or fomites. Of the 1,407 species of organisms pathogenic to humans, 58% are of zoonotic origin (Woolhouse and Gowtage-Sequeria, 2005). Pathogens that are transmitted between wild and domestic animals and humans are of particular importance to public health, livestock industries and wildlife conservation (Cleaveland *et al.*, 2001). Zoonotic diseases can be particularly serious for public health if they frequently spill over from their animal reservoirs or are easily transmitted between humans once introduced.

Wildlife has been involved in the epidemiology of many zoonoses and functions as a major reservoir for the transmission of the aetiological agents to domestic animals and humans (Kruse *et al.*, 2004; Thompson *et al.*, 2009). It has been suggested that human encroachment into wildlife habitat has resulted in increased transmission of pathogens between wildlife, domestic animals and humans (Cleaveland *et al.*, 2001). Also, international livestock movement and modern agricultural practices have seen an emergence of zoonoses in areas previously unaffected (Daszak *et al.*, 2000). Climate change is another factor that is expected to alter the distribution of wildlife and vectors, thereby facilitating the transmission of infectious agents (Bengis *et al.*, 2004). Climate

change effects, combined with land clearing and urbanisation, will lead to increased interactions between humans, wildlife and the pathogens they carry. Climate change also has the potential to alter boundaries for spatial distributions, host-parasite interactions, life cycle phenologies and patterns of infection and disease (Polley and Thompson, 2009). Increased transmission of pathogens in wildlife is thought to be responsible for the proliferation in diseases of importance to both human and animal health. In order to determine and manage the risks associated with zoonoses and emerging infectious diseases, effective surveillance for the relevant pathogens is crucial.

Secondary antibodies are essential for immunoassays to detect antibody responses against pathogens, thereby enabling diagnosis of infections and in seroepidemiological studies. Anti-species or secondary antibodies are readily available for most domestic animals. However, for non-domestic animals, such as native Australian wildlife, anti-species antibodies are not commercially available.

The experimental work outlined in this chapter aimed to produce chicken recombinant antibodies (CRAbs) against several native animal species IgG, including macropods (*Macropus sp*), brushtail possum (*Trichosurus vulpecula*) and common northern bandicoot (*Isoodon macrourus*) for use as diagnostic reagents in ELISA.

Each of these species are common in semi-rural, peri-urban and urban areas in Australia and have been associated with various zoonotic diseases such as hydatid disease, leptospirosis and Q fever (Stevenson and Hughes, 1988). Of these, macropods and bandicoots have been identified as reservoirs of Q fever in Australia in early studies (Derrick and Smith, 1940; Pope *et al.*, 1960). However, no current data on the epidemiology of *C. burnetii* in these species is available in the state of Queensland.

Macropods are marsupials belonging to the Family Macropodidae, which includes kangaroos, wallabies, pademelons and bettongs. Common species of macropod in Queensland include the eastern grey kangaroo (*Macropus giganteus*), agile wallaby (*Macropus agilis*) and common wallaroo (*Macropus robustus*). These animals are

known reservoirs of various zoonotic diseases including, Q fever, leptospirosis, hydatid disease, salmonellosis, rickettsial diseases, dermatophytes and several arboviruses including Barmah Forest and Ross River viruses (Stevenson and Hughes, 1988).

The common brushtail possum (*Trichosurus vulpecula*) belongs to the Family Phalangeridae and is one of the most common marsupial species found in urban areas. The species is common throughout Queensland. Brushtail possums are known reservoirs of various zoonotic diseases including, leptospirosis, mycobacteriosis, dermatophytes and rickettsial diseases (Stevenson and Hughes, 1988).

The common northern or northern brown bandicoot (*Isoodon macrourus*) belongs to the Family Peramelidae. It is a marsupial found on the eastern coast of Queensland extending into northern New South Wales. Bandicoots are nocturnal and are occasionally found in urban areas, where evidence of foraging can be seen in suburban lawns. These animals are known reservoirs of various zoonotic diseases including, Q fever, lyme disease, salmonellosis and rickettsial diseases (Stevenson and Hughes, 1988).

7.2 Aims

The specific aims for the work described in this chapter were to:

1. Produce a chicken recombinant antibody library against macropod (*Macropus sp*.) IgG;
2. Produce a chicken recombinant antibody library against brushtail possum (*Trichosurus vulpecula*) IgG;
3. Produce a chicken recombinant antibody library against common northern bandicoot IgG (*Isoodon macrourus*) IgG;
4. Select and characterise positive CRAbs against IgG of each species using ELISA; and
5. Test cross-reactivity of clones against IgG of each other species in ELISA.

7.3 Materials and Methods

7.3.1 Blood collection and serum separation

Initial blood samples for the isolation of generic serum from kangaroos and wallabies were obtained from animals at the Billabong Sanctuary, Townsville. Samples were taken by a veterinarian with the permission of the Sanctuary Manager, Bob Flemming. Blood samples from bandicoots and possums were obtained from animals trapped around the Veterinary and Biomedical Sciences precinct at James Cook University, Townsville using weight triggered 60 cm × 30 cm × 30 cm cages. Animals were released at the place of capture following sampling. Additional samples were also supplied by wildlife veterinarians at the Aachilpah Veterinary Clinic, Townsville. The venupuncture site, needle gauge, syringe volume and collection volume for animal species sampled are

listed in Table 7.1. All sampling was performed under James Cook University Animal Ethics Approval (A1205).

Table 7.1: Blood collection from native animal species

Species	Venupuncture Site	Needle Gauge	Syringe Volume (mL)	Sample Volume (mL)
Kangaroo	Lateral caudal	25	5	5
Wallaby	Lateral caudal	25	5	5
Bandicoot	Distal cephalic	25	1	1
Possum	Distal cephalic	25	3	3

7.3.2 IgG purification

To purify IgG for the immunisation of chickens, serum from each species was diluted 1:1 in binding buffer (Appendix A) and filtered through a 0.45 µm filter. The serum was then loaded onto a 1 mL HiTrap protein G column (GE Healthcare, Australia) in an AKTAdesign™ liquid chromatography system (GE Healthcare, Australia). IgG was eluted using a linear gradient of elution buffer (Appendix A) at a rate of 1 mL min^{-1}. Fractions of 500 µL were collected in 5 mL sample tubes containing 100 µL neutralisation buffer (Appendix A). AKTAdesign™ analysis software was used to determine fractions containing IgG, which were pooled and the concentration determined using a BCA protein assay kit (Quantum Scientific, Australia). Purified IgG was lyophilised overnight using a freeze dryer (Dynavac, Australia).

7.3.3 Chicken immunisation

To stimulate the expression of chicken Ig genes, two (1 male, 1 female) six week old white leghorn chickens per animal species were immunised with 100 µg IgG emulsified with Montanide™ ISA 206 (Seppic, France) adjuvant. Two boosters of 100 µg IgG with

adjuvant were given at 14 and 28 days post initial immunisation. Chickens were provided with commercial feed and water *ad libitum* and monitored daily. Chickens were used under James Cook University Animal Ethics Approval (A1205).

7.3.4 Extraction and purification of mRNA

Whole blood was collected from chickens by wing with 25 gauge needles and 10 mL syringes then transferred to 10 mL tubes containing 2 mL 3% tri-sodium-citrate. Collection of PBMC was performed following purification of blood over Ficoll-Paque (GE Healthcare, USA). The buffy coat was washed in RPMI (Invitrogen, USA), and then subjected to RNA extraction using a Total RNA Extraction Kit (Real Genomics, Taiwan) according to the manufacturer's instructions. RNA was quantified using a UV biophotometer at 260/280 nm (Eppendorf, Germany) and stored at -20°C. DNase I treatment was performed with 1× DNase I reaction buffer with $MgCl_2$ (Fermentas, USA), 1 unit ug^{-1} RNA DNase I (Fermentas, USA) and molecular biology grade water (Sigma, Australia) to a total volume of 10 µL.

7.3.5 Complementary DNA synthesis and RT-PCR of V_H and V_L genes

To convert mRNA into cDNA for the production of the phage-display library, first strand cDNA synthesis and RT-PCR of V_H and V_L genes was performed for each species set as described in Section 6.3.5.

7.3.6 Library construction and expression

To produce phage-display libraries for the IgG of each species, initially half libraries, followed by full libraries were constructed as described in Sections 6.3.6 and 6.3.7. Expression of phage displayed CRAbs was performed as described in Section 6.3.8.

In order to increase the probability of selecting a macropod-specific CRAb, the construction of the kangaroo and wallaby full libraries was refined. Heavy and light chain variable fragment genes from both species were inserted into the half libraries for both species to produce hybrid kangaroo/wallaby libraries as well as pure kangaroo and wallaby libraries. All full libraries were then combined following the transformation process.

7.3.7 Library panning and CRAb selection

To select CRAbs with binding to each species IgG, library panning was performed as described in Section 6.3.9 with antigen substituted according to each anti-species set. The protocol was later refined in that a single panning round was performed with 20 PBS washes prior to re-infection of host *E. coli* and phage rescue. CRAbs produced using both methods were tested in ELISA.

A master plate for each species set was prepared as described in Section 6.3.10.1. ELISA plates were coated with 100 µL of 100 µg mL^{-1} IgG in carbonate buffer overnight at 4°C. Plates were washed three times with PBS-T. Phage stock diluted in 2% skim milk was applied in 50 µL aliquots and incubated at 37°C for 1 hr with shaking. The plates were washed again and 50 µL of anti-M13 HRP conjugate was added at a dilution of 1:5,000 and incubated at 37°C for 1 hr with shaking. The plates were again washed and 100 µL of ABTS was added and incubated for 10 min at 37°C then read at 414/494 nm in a Multiskan Ascent plate reader (Labsystems, USA). Positive CRAb phagemids were sequenced as described in Section 6.3.10.3.

7.3.8 Production of soluble CRAbs

To produce soluble CRAbs unattached to phage, soluble scFv for each species set were produced as described in Section 6.3.11. Soluble CRAbs were first used from whole cell

extracts and detected with HRP-conjugated anti-E tag antibody. The use of soluble antibodies for detection could not be continued for further work due to discontinuation of the HRP-conjugated anti-E tag antibody by the manufacturer. All further optimisation and screening was performed with phage-displayed antibodies.

7.3.9 Production of phage-displayed CRAbs

Phage-displayed CRAbs were produced for characterisation in ELISA. Phage-displayed CRAbs were produced as described in Section 6.3.12.

7.3.10 Sequencing of CRAbs

To obtain sequences of CRAb phagemids, positive phage stock was inoculated into 5mL log phase XL1β *E. coli* culture in 2×YT-A (Appendix A) and incubated overnight at 37°C and 250 RPM. Plasmid minipreps were performed using an RBC kit (Real Biotech, Taiwan) according to the manufacturer's instructions. For each plasmid preparation, a 20 µL aliquot of 100 ng μL^{-1} DNA was prepared in an o-ring sealed microcentrifuge tube. All sequencing was performed by Macrogen, Inc (Korea) using BigDye Terminators (ABI, USA). To construct consensus sequences, three forward and three reverse sequences were obtained for three clones of each CRAb phagemid. All consensus sequence construction was performed using Sequencher™ Version 4.7 (Gene Codes Corporation, USA). Sequence alignments were performed using Genedoc 2.7.0 (Biology Software Net, USA).

7.3.11 Testing of phage-displayed CRAbs in ELISA

To test phage-displayed CRAbs against species IgG in ELISA, 96-well NUNC™ Maxisorp plates were coated with IgG of each species (50 µL of 50µg mL^{-1}), diluted 1:1

with coating buffer and incubated uncovered overnight at 37°C. Plates were coated with 100 µL post-coating buffer and incubated at room temperature for 1 hr. Phage were diluted in 2% skim milk and applied dilutions of 3:4, 1:2, 1:4 and 1:5 in 50 µL aliquots in triplicate and incubated at 37°C for 1 hr. The wells were washed four times with PBS T (Appendix A). HRP-conjugated anti-M13 antibody was applied in 50 µL aliquots at a dilution of 1:5,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 100 µL ABTS was applied and incubated at 37°C for 20 min. Plates were read in a Multiskan Ascent plate reader at 414/494 nm.

7.3.12 Determination of species cross-reactivity

To determine cross-reactivity of CRAbs, phage displayed CRAbs from each species master plate were tested against the IgG of the other species in ELISA according to the protocol described in Section 7.3.11. Coating concentration for various species IgG was limited due to relative amount of purified IgG in stock. Selected strongly positive CRAbs were tested separately against purified IgG from each species.

7.4 Results

7.4.1 IgG purification

IgG was successfully purified from all species selected. Yield and binding efficiency of IgG differed between species. Elution profiles for each species did not differ significantly between runs (Figures 7.1-7.4). Elution of IgG for all species occurred between 50 and 60% concentration of 0.1M glycine, with the exception of bandicoot IgG which eluted with 30% 0.1M glycine. The cumulative average yield of IgG per millilitre of serum for each species is listed in Table 7.2.

Table 7.2: IgG yield per millilitre serum

Species	IgG Yield
Agile wallaby	7 mg mL^{-1}
Eastern grey kangaroo	0.7 mg mL^{-1}
Common brushtail possum	0.6 mg mL^{-1}
Common northern bandicoot	0.6 mg mL^{-1}

7.4.1.1 Purification of kangaroo IgG

A total of 9.9 mg IgG was purified from 14 mL of pooled kangaroo serum from 20 eastern grey kangaroos. Compared to the elution profile for murine IgG (Section 6.4.1), kangaroo IgG was eluted in greater quantity and over a narrower range of elution buffer concentration. An example of the elution profile for kangaroo IgG is provided in Figure 7.1.

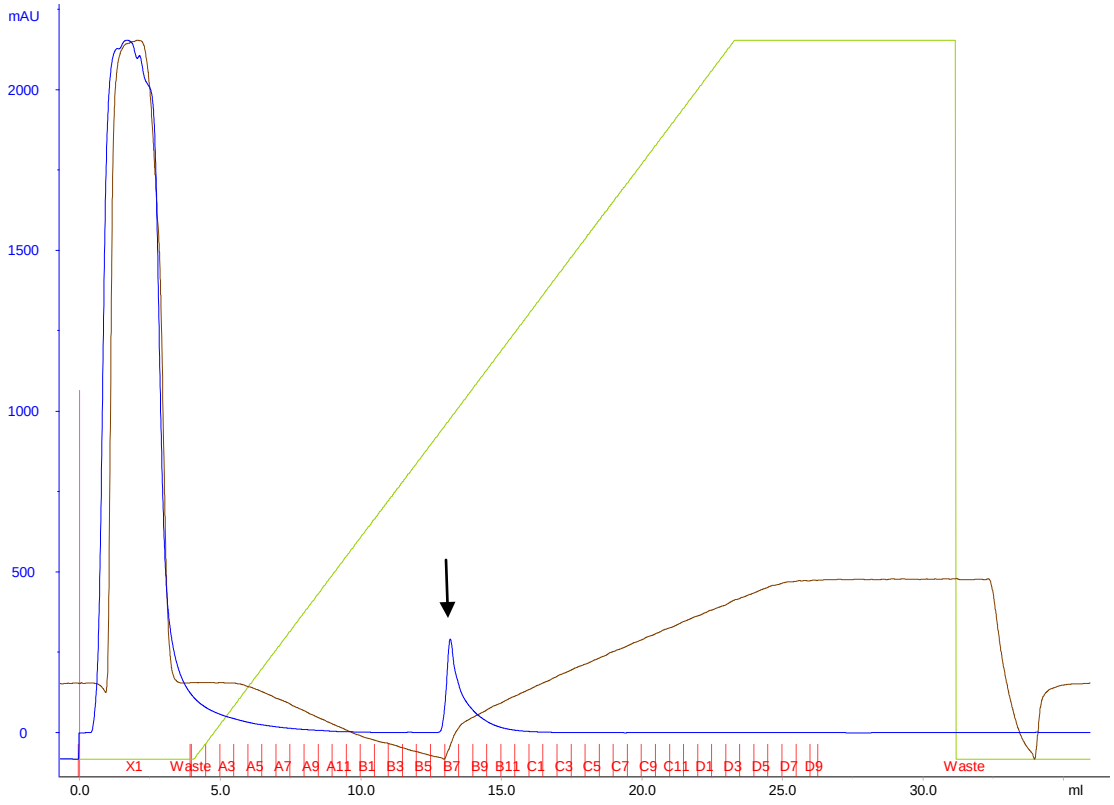

Figure 7.1: Chromatograph demonstrating elution of kangaroo IgG.
Kangaroo IgG purified over a protein G column using 0.1M glycine. Major plots include absorbance (blue), conductivity (brown) and elution buffer concentration (green). IgG eluted over approximately five fractions from 50-60% 0.1M glycine (arrow).

7.4.1.2 Purification of wallaby IgG

A total of 28 mg IgG was purified from 4 mL of pooled wallaby serum from five agile wallabies. Compared to the elution profile for murine IgG (Section 6.4.1), wallaby IgG was eluted in greater quantity and over a narrower range of elution buffer concentration. An example of the elution profile for wallaby IgG is provided in Figure 7.2.

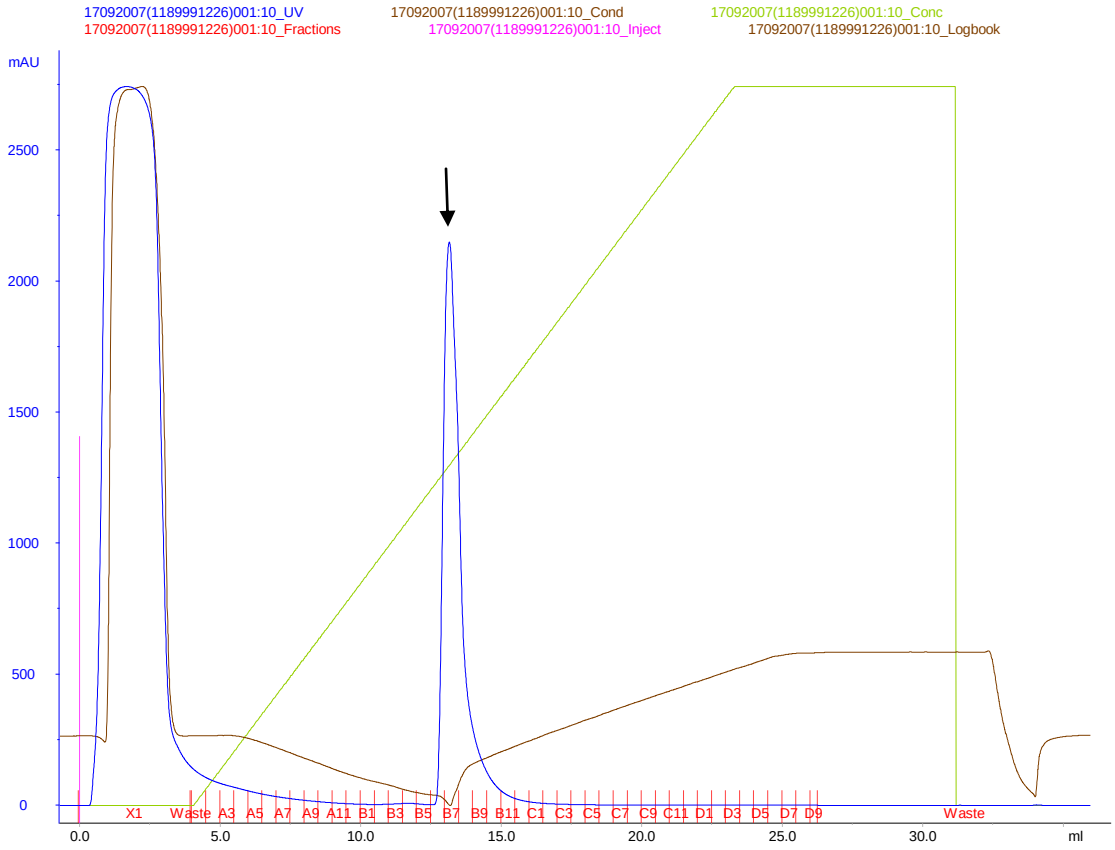

Figure 7.2: Chromatograph demonstrating elution of wallaby IgG.
Wallaby IgG purified over a protein G column using 0.1M glycine. Major plots include absorbance (blue), conductivity (brown) and elution buffer concentration (green). IgG eluted over approximately five fractions at 50% 0.1M glycine (arrow).

7.4.1.3 Purification of possum IgG

A total of 5 mg IgG was purified from 8 mL of pooled possum serum from 12 brushtail possums. Compared to the elution profile for murine IgG (Section 6.4.1), possum IgG was eluted in greater quantity and over a narrower range of elution buffer concentration. In addition, the peak absorbance of the eluted IgG was lower than wallaby IgG. An example of the elution profile for possum IgG is provided in Figure 7.3.

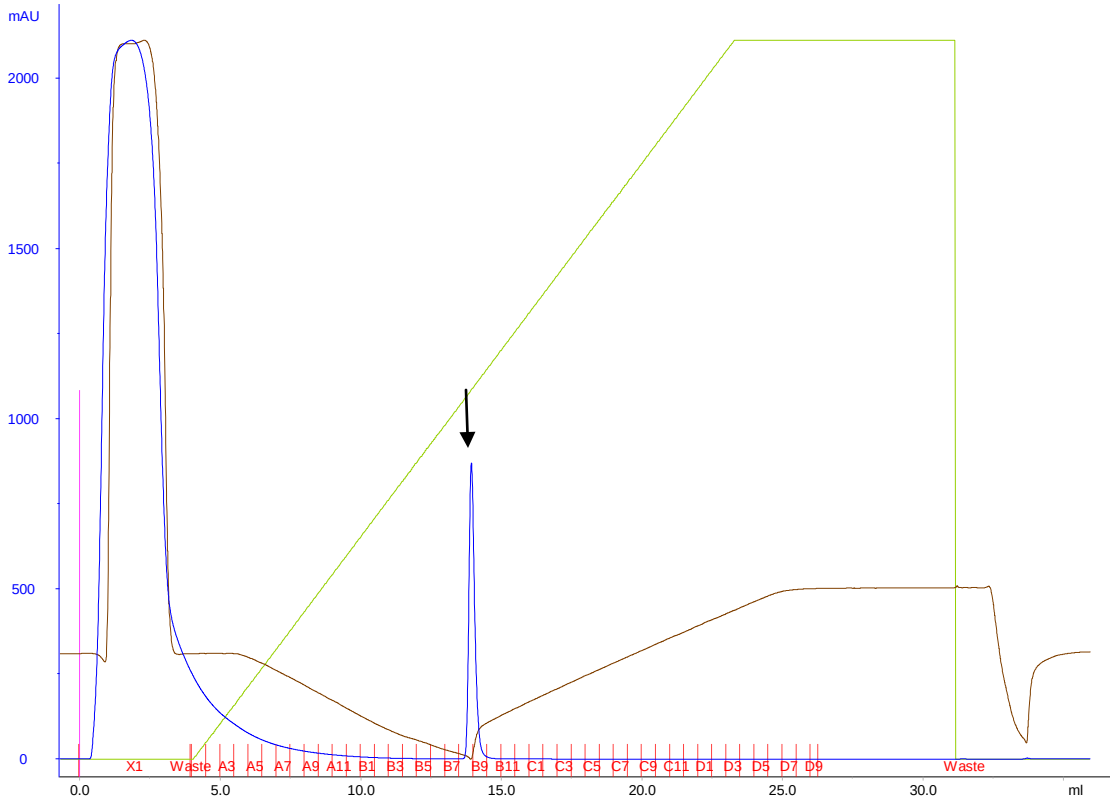

Figure 7.3: Chromatograph demonstrating elution of possum IgG.
Possum IgG purified over a protein G column using 0.1M glycine. Major plots include absorbance (blue), conductivity (brown) and elution buffer concentration (green). IgG eluted over approximately two fractions at 50% 0.1M glycine (arrow).

7.4.1.4 Purification of bandicoot IgG

A total of 5.5 mg IgG was purified from 10 ml of pooled serum from 30 common northern bandicoots. The elution profile for bandicoot IgG was different to that of the other species in that it was eluted at a lower glycine concentration. The bandicoot IgG was eluted over a narrow range of glycine concentration. An example of the elution profile for bandicoot IgG is provided in Figure 7.4.

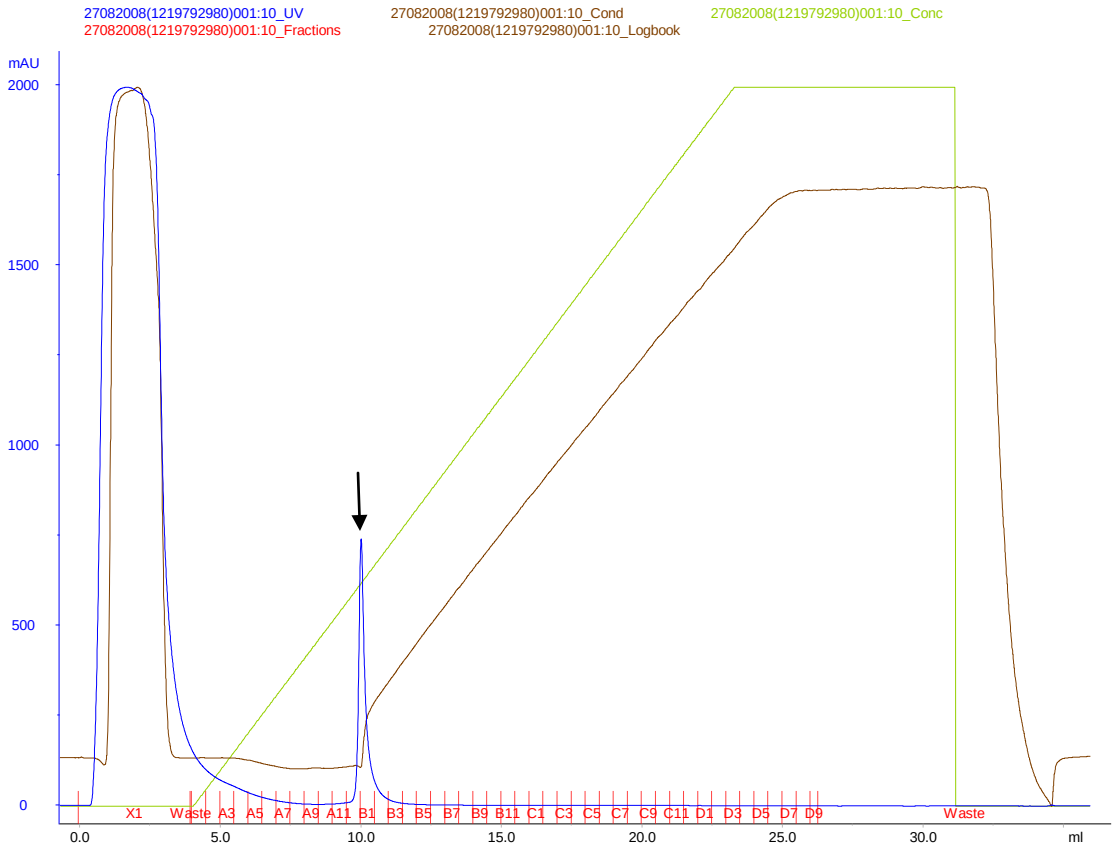

Figure 7.4: Chromatograph demonstrating elution of bandicoot IgG.
Bandicoot IgG purified over a protein G column using 0.1M glycine. Major plots include absorbance (blue), conductivity (brown) and elution buffer concentration (green). IgG eluted over approximately 3 fractions at 30% 0.1M glycine.

7.4.2 Library expression

7.4.2.1 Half libraries

The number of clones produced for the half libraries of V_H and V_L genes for each species is listed in Table 7.3.

Table 7.3: Size of half libraries for various animal species

Species	V_H Library	V_L Library
Eastern grey kangaroo	4.3×10^5	2.8×10^4
Agile wallaby	1.2×10^5	5.4×10^5
Brushtail possum	3.5×10^4	3.6×10^5
Common bandicoot	1.5×10^5	7.5×10^5

7.4.2.2 Combined libraries

The number of clones produced for the combined, full libraries of V_H and V_L genes for each species is listed in Table 7.4.

Table 7.4: Size of combined libraries for various animal species

Species	Library Size
Eastern grey kangaroo	7.5×10^5
Agile wallaby	7.5×10^5
Brushtail possum	1.0×10^5
Common bandicoot	1.2×10^5

7.4.3 Panning and selection of chicken recombinant antibodies

7.4.3.1 Selection of CRAbs for kangaroo IgG

Of the 95 CRAbs screened for binding to kangaroo IgG as phage-displayed antibodies after three panning rounds, 91.5% had greater than double background absorbance and were considered positive. Of those, three CRAbs (KE1, KF10 and KG6) demonstrated very strong binding of greater than eight times background absorbance and were selected for further characterisation (Figure 7.5). As the kangaroo and wallaby libraries had been constructed together, all 95 CRAbs panned against kangaroo IgG were also tested against wallaby IgG. Of these, 22.1% were positive, with the same three CRAbs demonstrating binding of greater than three times background absorbance.

Of the 95 CRAbs screened for binding to kangaroo and wallaby IgG after a single panning round of 20 washes, 6.3% were positive. Of those, the highest binding CRAb to both species IgG (W1.4) was selected for further characterisation.

7.4.3.2 Selection of CRAbs for wallaby IgG

Of the 95 CRAbs screened for binding to wallaby IgG as phage-displayed antibodies, 10.5% had greater than double background absorbance and were considered to be positive. Of those, five (WA1, WD8, WE1, WF2 and WG9) demonstrated binding of greater than three times background absorbance. All 95 CRAbs panned against wallaby IgG were also tested against kangaroo IgG. Of those, 98.9% were positive, with three (WD8, WF2 and WG9) demonstrating very strong binding of greater than eight times background absorbance. These CRAbs were also among the highest binders to wallaby IgG and were selected for further characterisation.

7.4.3.3 Selection of CRAbs for possum IgG

Of the 95 CRAbs selected for binding to possum IgG 20% were positive (greater than double background absorbance). Of those, four (PD8, PE8, PH5 and PH6) demonstrated binding of greater than three times background absorbance.

7.4.3.4 Selection of CRAbs for bandicoot IgG

Of the 95 CRAbs selected for binding to bandicoot IgG 13.7% had greater than double background absorbance and were considered to be positive. Of those, three (BA12, BF12 and BH5) demonstrated binding of greater than three times background absorbance.

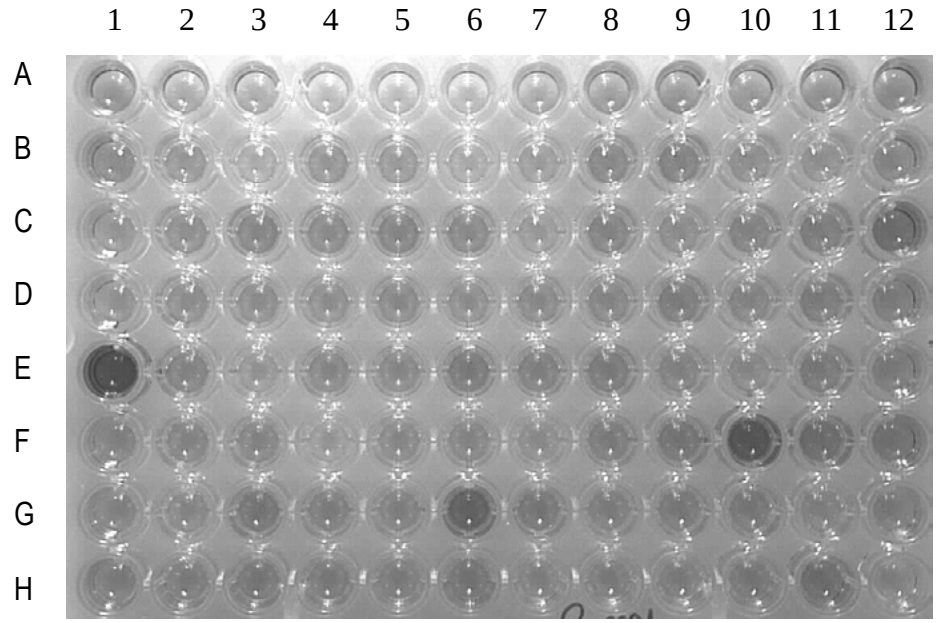

Figure 7.5: Example of screening ELISA with anti-kangaroo CRAbs.
Plate was coated with kangaroo IgG (100µg ml^{-1}) then probed with CRAbs transferred from cluster plate. No CRAb was grown in well H1 in order to provide a reference for background absorbance. The strongest binding CRAbs in this example were in wells E1, F10 and G6 (Designated K to refer to CRAbs raised against kangaroo IgG).

7.4.4 Production of phage-displayed CRAbs and ELISA testing

Of the strongly binding anti-kangaroo CRAbs identified in the screening process, CRAbs KG6 and KD8 were poorly amplified in culture. CRAb KE1 demonstrated the highest binding to macropod IgG (Figure 7.6). Of the four anti-bandicoot CRAbs selected for further characterisation, BH5 was the strongest binder. Of the four anti-possum IgG CRAbs selected for further characterisation, PE8 was the strongest binder.

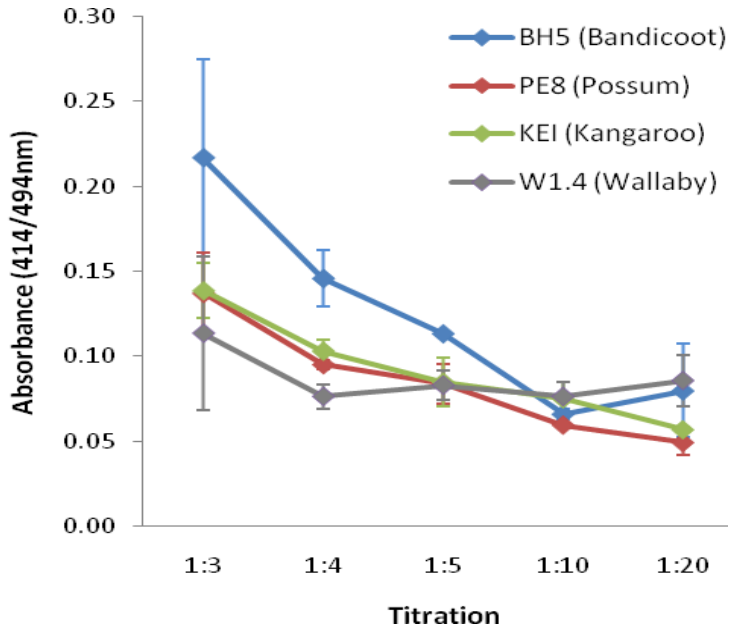

Figure 7.6: Titration of phage-displayed CRAbs against species IgG.
High binding phage-displayed CRAbs identified in the initial screening process were produced individually and tested against species IgG. Of these the highest binders are shown as a titration. BH5 represents CRAb raised against bandicoot IgG, PE8 represents CRAb raised against possum IgG, KE1 represents CRAb raised against kangaroo IgG and W1.4 represents CRAb raised against wallaby IgG. Titrations are not continuous. Error bars represent standard deviation.

7.4.5 Sequencing data

Sequencing of the positive CRAbs revealed a truncation of the light chain variable region Ig gene sequence of approximately 200 bp in three CRAbs (BH5, PE8 and W1.4). Chicken recombinant antibody WD8 had a smaller truncation of 60 bp in the light chain Ig variable region gene and 10 bp in the heavy chain variable region Ig gene sequences. CRAbs BH5 and PE8 also had truncations in the heavy chain variable region Ig gene sequences by 30 and 60 bp respectively. These truncations did not appear to reduce binding efficacy to IgG in ELISA.

As described previously (Section 6.4.5), the truncation phenomenon demonstrated the successive truncation of the light chain Ig gene sequence in the library during panning. Full light chain Ig gene sequence was found to be present by restriction digest in the phage display library prior to panning. A reduction in the number of panning rounds did not result in CRAbs without truncations (clone W1.4).

Of the sequenced positive CRAbs, most had heavy and light chain variable region Ig gene sequence that matched to expected *Gallus gallus* DNA sequences in BLAST search. The exception was CRAb PE8, which, as with sequences described previously in Section 6.4.5, had heavy chain variable region Ig gene sequences that did not match with currently available sequences for chicken heavy chain variable region Ig gene. The sequence for the heavy chain variable region Ig gene in CRAb PE8 was similar to that of the CRAbs raised against murine IgG (Section 6.4.5) and had homology with expected heavy chain Ig gene sequences of approximately 50%.

Sequence alignments of all CRAbs are included in Figure 7.7, with sequence alignments of CRAbs raised against macropod IgG included in Figure 7.8.

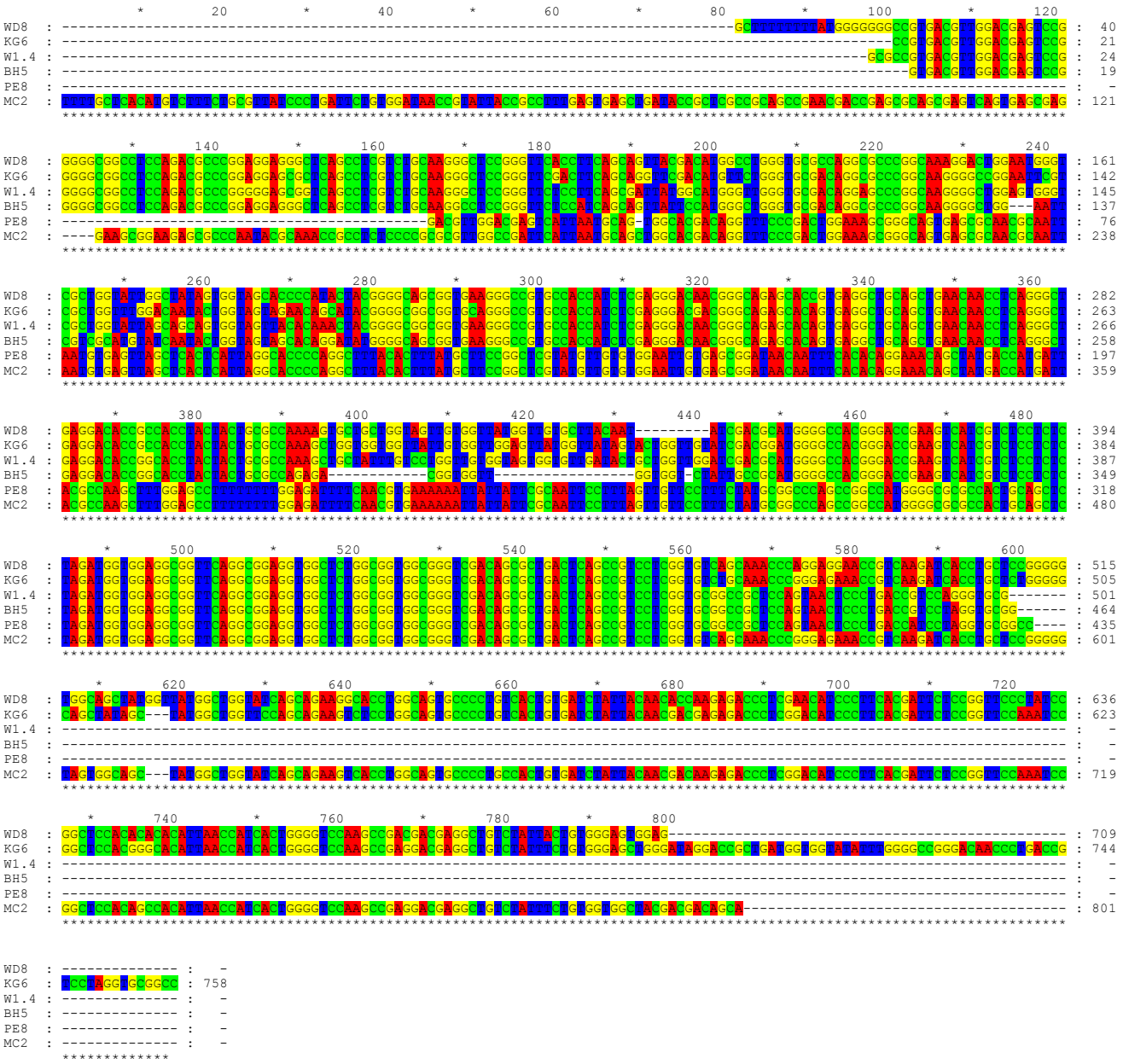

Figure 7.7: Anti-species IgG CRAb sequence alignment.
Sequences were aligned using Genedoc (Biology Software Net, USA). CRAb sequences are aligned 5'-3' from light chain to heavy chain. Post 800 bp includes phagemid vector sequence.

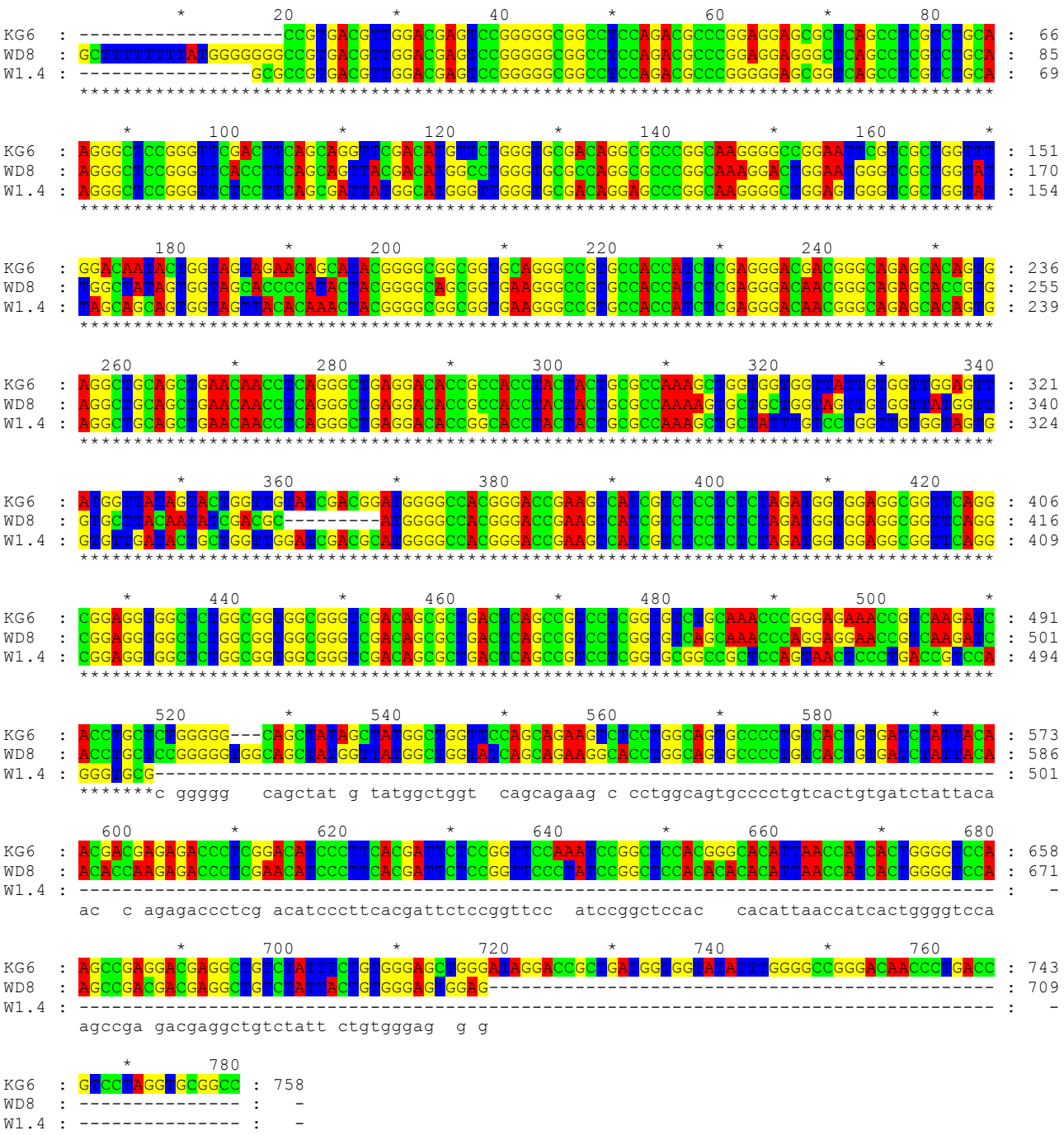

Figure 7.8: Anti-macropod IgG CRAb sequence alignment.
Sequences were aligned using Genedoc (Biology Software Net, USA). CRAb sequences are aligned 5'-3' from light chain to heavy chain. Post 800 bp includes phagemid vector sequence.

7.4.6 Determination of species cross-reactivity

Most of the anti-species phage-displayed CRAbs produced cross-reacted against the IgG of other native species tested. The exception was KE1, a CRAb raised against macropod IgG which only bound to macropod IgG. However, the other CRAbs raised against different species IgG bound to macropod IgG with greater strength (Figure 7.9). The CRAb with the greatest degree of cross-reactivity was MC2 which was raised against murine IgG. This CRAb bound to all species IgG with greater intensity than the CRAbs raised specifically for those species (Figures 7.9-7.11). For possum IgG, both CRAbs originally raised against murine (MC2) and bandicoot (BH5) IgG bound with greater intensity than the CRAb originally raised against possum IgG (PE8) (Figure 7.10). For bandicoot IgG, the CRAb originally raised against bandicoot IgG (BH5) was the second highest binder after MC2 (Figure 7.11).

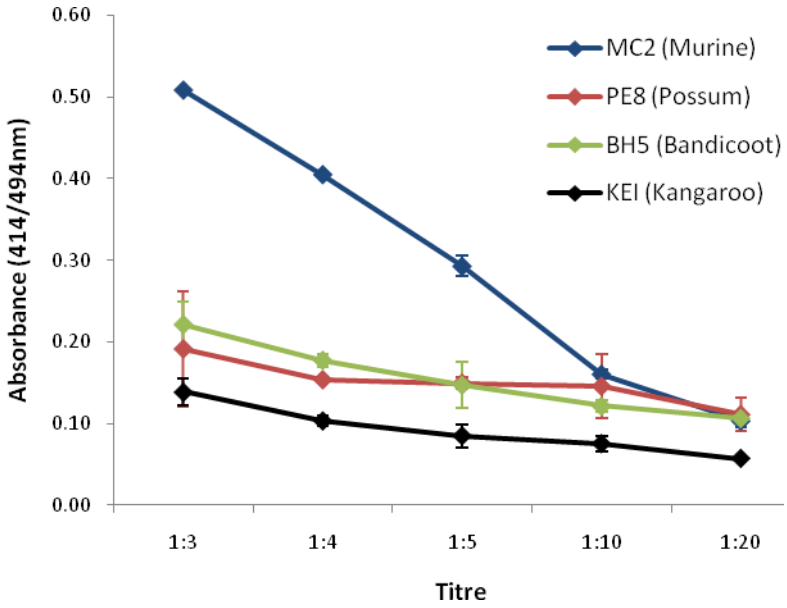

Figure 7.9: Cross-reactivity of selected anti-species phage-displayed CRAbs against macropod IgG.
Anti-species phage-displayed CRAbs were tested at various dilutions against 25 µg mL^{-1} macropod IgG. MC2 represents CRAb raised against murine IgG, PE8 represents CRAb raised against possum IgG, BH5 represents CRAb raised against bandicoot IgG and KE1 represents CRAbs raised against kangaroo IgG. Error bars represent standard deviation. Titrations are not continuous.

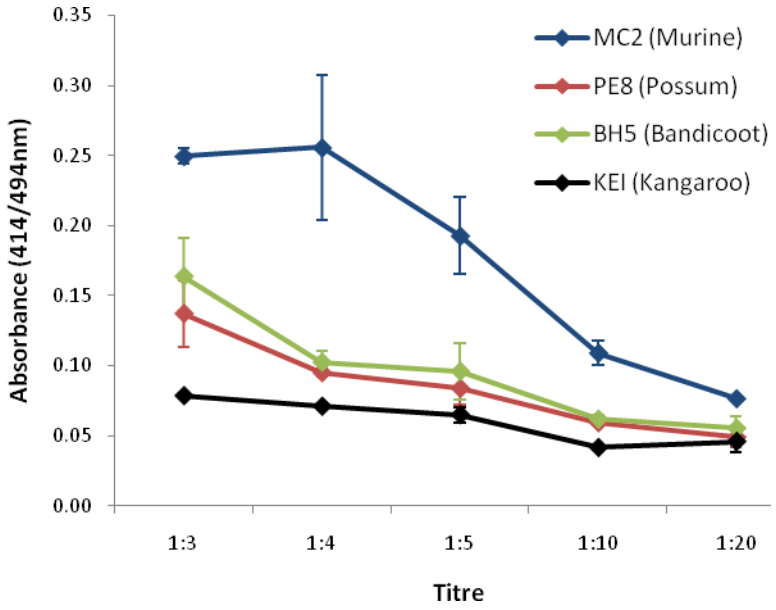

Figure 7.10: Cross-reactivity of selected anti-species phage-displayed CRAbs against possum IgG.

Anti-species phage-displayed CRAbs were tested at various dilutions against 25 µg mL^{-1} possum IgG. MC2 represents CRAb raised against murine IgG, PE8 represents CRAb raised against possum IgG, BH5 represents CRAb raised against bandicoot IgG and KE1 represents CRAbs raised against kangaroo IgG. Error bars represent standard deviation. Titrations are not continuous.

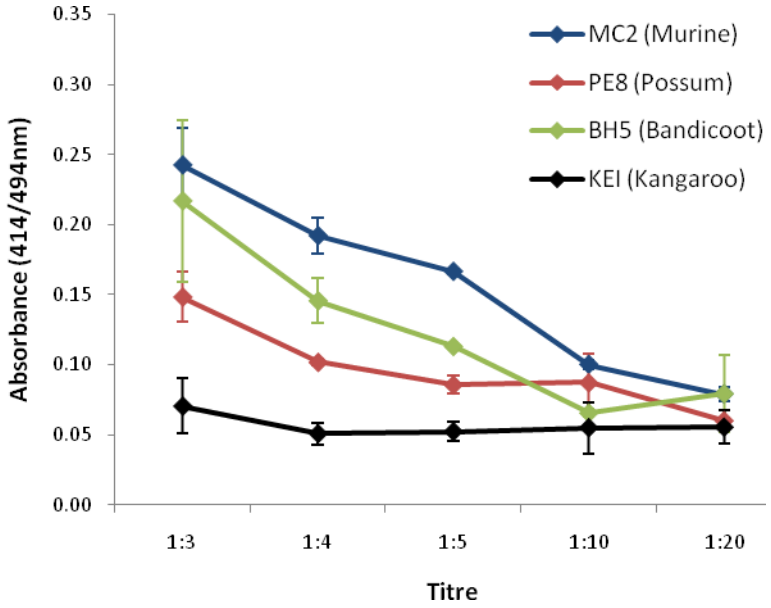

Figure 7.11: Cross-reactivity of selected anti-species phage-displayed CRAbs against bandicoot IgG.
Anti-species phage-displayed CRAbs were tested at various dilutions against 25 µg mL^{-1} bandicoot IgG. MC2 represents CRAb raised against murine IgG, PE8 represents CRAb raised against possum IgG, BH5 represents CRAb raised against bandicoot IgG and KE1 represents CRAbs raised against kangaroo IgG. Error bars represent standard deviation. Titrations are not continuous.

7.5 Discussion

The development of phage displayed recombinant antibodies may improve the current capacity to detect, identify and predict potential zoonotic outbreaks in Australia, particularly where wildlife reservoirs are suspected. The northern tropics of Australia are considered to be one the most significant potential entry points of non-endemic diseases and an important area for animal surveillance. Currently, commercially produced secondary antibodies are not available for native species and alternative methods for serological screening have to be employed as a result. These methods include

competitive ELISA, neutralisation assays and the use of sentinel animals. All of these methods have disadvantages that could be eliminated if secondary antibodies were available for the species of interest. Competitive ELISAs require the production of specific indicator antibodies for each pathogen of interest, neutralisation assays can require the production of relatively large amounts of the pathogen of interest and sentinel animal programs require the maintenance of domestic animals in remote regions. If phage displayed recombinant antibodies prove to be an effective means of producing secondary antibodies they could be used to produce indirect ELISAs for pathogens of interest that would be more reliable than currently available methods. Phage displayed recombinant antibodies would also be valuable in the epidemiological evaluation and monitoring of endemic zoonoses, such as *C. burnetii*.

Recombinant phage-displayed antibodies raised against the purified IgG of selected species according to the experimental work described in this chapter were shown to bind to the purified IgG of each species. The majority of selected CRAbs demonstrated cross-reactivity with the IgG of the other selected species. While this result was expected with macropod IgG, it was less expected for possum and bandicoot IgG. This result indicated that there is a relatively high degree of conservation of structure in the IgG of various native Australian mammals. The strong cross-reactivity of the recombinant phage-displayed antibody raised against murine IgG (MC2) with the IgG of the other species indicated this clone had binding affinity for a highly conserved region of mammalian IgG.

As with the sequencing results obtained for reactive CRAbs previously during optimisation (Section 6.4), sequencing of the recombinant antibodies demonstrated truncations of the light chain variable region Ig gene sequence, with truncations of the heavy chain variable region Ig gene sequence also present in some CRAbs. Subsequently, it was found that CRAbs with higher binding efficacy and full length heavy and light chain variable region Ig gene sequences were poorly amplified in *E. coli* compared to those with truncated chain variable region Ig gene sequences. The truncation may be a response to the relative toxicity of the recombinant antibody to the

E. coli host cell. However, the use of XL1 blue *E. coli* cells should have prevented this effect as these cells are *recA* deficient, a phenotype designed to improve insert stability.

As discussed previously (Section 6.5), over-selection of the library may be responsible for the apparent lack of a true heavy chain Ig gene sequence. It was suggested previously (Section 6.5) that it may be necessary to revert to a single panning round, with a greater number of washes to avoid truncation events and over-selection during panning for the production of recombinant antibodies. However, reducing the number of panning rounds did not result in CRAbs without truncations. The poor amplification of CRAbs with full length variable region Ig gene sequences indicates the relative toxicity of the recombinant antibody product may be a more important selection pressure than the binding affinity.

While recombinant phage-displayed anti-IgG antibodies were produced for each of the selected native animal species, it was demonstrated that the anti-murine IgG antibody selected for validation in ELISA previously (Section 6.4) had greater binding affinity against all the selected species IgG. This result would simplify development of subsequent indirect ELISAs as only one CRAb would have to be amplified for the production of ELISA conjugate. A universal CRAb with reactivity to the IgG of multiple native Australian marsupial species has the potential to improve the development of serological tests for detecting antibody responses to a variety of pathogens in these animals.

CHAPTER EIGHT

DETECTION OF ANTIBODIES TO *COXIELLA BURNETII* IN NATIVE AUSTRALIAN MARSUPIALS

8.1 Introduction

Wild animals and the tick species which feed on them form the natural transmission cycle and reservoir of *C. burnetii* (Babudieri, 1959). Many serological surveys and bacterial isolations have indicated the extent of wildlife coxiellosis worldwide. In Australia, bandicoots (Smith and Derrick, 1939; Smith, 1942) and kangaroos (Pope *et al.*, 1960; Banazis *et al.*, 2010) have been found to be reservoirs. In early studies *C. burnetii* was isolated from the common northern bandicoot, *Isoodon macrourus* in Queensland (Derrick and Smith, 1940; Derrick, 1961). Guinea pigs and mice have been shown to develop bacteraemia following inoculation with organ macerates from bandicoots. Bandicoots were associated with an outbreak of Q fever in Queensland in 1958, where there was no association with any other potential reservoir species (Derrick, 1961). In the following 50 years, no further work has been performed on the role of bandicoots in the epidemiology of Q fever.

Serological evidence of *C. burnetii* infection has also been demonstrated in several macropod species, including red kangaroo (*Macropus rufus*), eastern grey kangaroo (*Macropus giganteus*) (Pope *et al.*, 1960) and western grey kangaroo (*Macropus fuliginosus*) (Banazis *et al.*, 2010). Seropositivity for phase II and phase I antigens was found to be 33% and 29% respectively in *Macropus rufus* and 12% and 6% in *Macropus giganteus* (Pope *et al.*, 1960). A retrospective serological survey for anti-*Coxiella* antibodies performed on 160 kangaroo and wallaby sera found a seropositivity rate of 11.8% (Stallman, 1965). However, approximately 50% of these samples were anti-complementary, resulting in a difficulty when determining the true

extent of coxiellosis in the animals. More recently, a survey was performed on kangaroo sera in Western Australia (Banazis *et al*., 2010). This survey found a seropositivity rate of 33.5% and detected *C. burnetii* DNA in 12.2% of faecal samples, indicating kangaroos may pose a significant threat for zoonotic transmission of *C. burnetii*. Evidence of active *C. burnetii* infection in macropods is not well established, with only a single demonstration of bacteraemia following inoculation of mice with the blood of a naturally infected animal (Pope *et al.*, 1960). Yet, macropods have been associated with Q fever cases in kangaroo shooters and chiller box workers (Parker *et al.*, 2006).

Many species that are reservoirs for leptospirosis are also reservoirs for Q fever. To date, no evidence of *C. burnetii* has been identified in possums. However, possums have been identified as potential reservoirs of leptospirosis in Australia (Slack *et al.*, 2006) and New Zealand (Hathaway *et al.*, 1981). Serologically leptospirosis-positive possums have been identified in major suburban areas in Australia (Eymann *et al.*, 2007). Therefore, there may be potential for possums to act as reservoirs of Q fever.

Q fever has been described as a re-emerging pathogen of increasing importance as a public health issue (Arricau-Bouvery and Rodolakis, 2005). In order to produce data on the epidemiology of Q fever and determine the risk of infection a variety of methods have been used in the attempt to detect, monitor and control Q fever. Australian surveys have shown an increased prevalence of Q fever in humans in recent years (Garner *et al.*, 1997). Although this has been attributed to several factors there is no current data on potential reservoirs of human infection. Australian studies investigating native wildlife as reservoirs of infection in Queensland are outdated (Derrick, 1937; Smith and Derrick, 1939; Pope *et al*., 1960).

8.2 Aims

The specific aims for work described in this chapter were to:

1. Determine the seroprevalence of *C. burnetii* in macropods in several locations with ELISA using recombinant phage-displayed antibodies, competitive ELISA and standard indirect ELISA,
2. Determine the seroprevalence of *C. burnetii* in brushtail possums and common northern bandicoots in the Townsville Region using recombinant phage-displayed antibodies and competitive ELISA,
3. Compare ELISA using recombinant phage-displayed antibodies, competitive ELISA and standard indirect ELISA for the detection of antibodies to *C. burnetii* in the native animals selected,
4. Determine factors associated with seropositivity in native Australian marsupials and;
5. Determine whether any relationship exists between seroprevalence in native Australian marsupials, seroprevalence in beef cattle and human Q fever incidence.

8.3 Materials and Methods

8.3.1 Sample collection

8.3.1.1 Samples obtained by trapping

Animals were trapped according to procedures used by the Queensland Parks and Wildlife Service. All care was taken to reduce stress on the animals. Blood samples (equivalent to less than 0.5% of the body weight to a maximum 2 mL) collected from each identified animal were taken from the tail vein or other suitable site (Table 8.1).

Following blood collection, animals were released at the site at which they were captured. Whole blood was allowed to clot and centrifuged at 1,400 ×*g* for 10 min at room temperature. Serum removed from the samples was frozen at -20°C prior to analysis.

Table 8.1: Live blood collection details for various animal species

Species	Venupuncture Site	Needle Gauge	Syringe Size (mL)	Blood Volume (mL)
Macropods (*Macropus sp.*)	Lateral caudal	23	5	2
Bandicoot (*Isoodon macrourus*)	Cephalic	25	3	1
Possum (*Trichosurus vulpecula*)	Cephalic	25	3	1

8.3.1.2 Samples obtained from veterinary clinic

Blood samples (equivalent to less than 0.5% of the body weight to a maximum 2 mL) collected from each identified animal were taken from the tail vein or other suitable site during routine examination of animals received at the Aachilpah veterinary clinic. Reasons for presentation were variable, with the majority consisting of native animals presented for physical injuries such as vehicle strike and wounds due to mauling by domestic animals. Whole blood was allowed to clot and centrifuged at 1,400 ×*g* for 10 min at room temperature. Serum removed from the samples was frozen at -20°C prior to analysis.

8.3.1.3 Samples obtained post-mortem

Blood samples were collected via cardiac puncture from deceased animals shot by licensed kangaroo shooters during routine hunting expeditions. Animals were killed by cranial shot according to Queensland regulations. Whole blood was allowed to clot and centrifuged at 1,400 ×*g* for 10 min at room temperature. Serum removed from the samples was frozen at -20°C prior to analysis.

8.3.1.4 Additional samples provided

Additional samples were sourced to provide seroprevalence data for multiple regions. One additional sample set was provided by Dr Abbey Potter, University of Murdoch, consisting of 180 macropod samples obtained from a related study in south-western West Australia (Banazis *et al.*, 2010). Another sample set was provided by Dr Tamsin Barnes, University of Queensland, consisting of 200 macropod samples obtained from unrelated studies in south western Queensland (Barnes *et al.*, 2007; Barnes *et al.*, 2008).

8.3.1.5 List of species sampled

Serum samples were obtained for 15 species of native Australian marsupial. Of these, 13 were macropods. Species tested are included in Table 8.2.

Table 8.2: List of species samples, source and sample methods in the current study

SITE	SPECIES	SAMPLES (n)	DATE (MM/YY)	SAMPLE METHOD
Warwick (SQ)	*P. penicillata*	64	11/04-08/05	trap
	M. robustus	13	02/04-06/04	*p.m.*
	M. rufogriseus	9	02/04-06/04	trap
	M. giganteus	8	02/04-06/04	*p.m.*
Injune (SQ)	*M. giganteus*	83	06/04-05/05	*p.m.*
Roma (SQ)	*M. giganteus*	23	06/04-05/05	*p.m.*
Townsville (NQ)	*I. macrourus*	50	04/07-07/10	trap
	T. vulpecula	47	04/07-07/10	trap
	M. agilis	16	unknown	*p.m.*
	M. giganteus	13	unknown	*p.m.*
	M. antilopinus	9	unknown	*p.m.*
	M. dorsalis	8	unknown	trap
	T. stigmatica	5	04/07-07/10	trap
	P. penicillata	4	unknown	trap
	A. rufescens	4	04/07-07/10	trap
	M. parryi	2	unknown	trap
Richmond (NQ)	*M. giganteus*	5	04/07-07/10	*p.m.*
	M. rufus	5	04/07-07/10	*p.m.*
	M. robustus	3	04/07-07/10	*p.m.*
	M. dorsalis	1	04/07-07/10	*p.m.*
Greenvale (NQ)	*M. giganteus*	12	04/07-07/10	*p.m.*
	M. agilis	5	04/07-07/10	*p.m.*
Ayr (NQ)	*T. vulpecula*	9	04/07-07/10	trap
Malanda (NQ)	*I. macrourus*	2	04/07-07/10	trap
Longreach (WQ)	*M. giganteus*	17	unknown	*p.m.*
	M. dorsalis	1	unknown	trap
Thurles park (WQ)	*M. rufus*	7	unknown	*p.m.*
	M. giganteus	1	unknown	*p.m.*
Winton (WQ)	*M. giganteus*	2	unknown	*p.m.*
Preston Beach (WA)	*M. fuliginosus*	60	06/07-11/07	*p.m.*
Capel (WA)	*M. fuliginosus*	37	06/07-11/07	*p.m.*
Nannup (WA)	*M. fuliginosus*	34	06/07-11/07	*p.m.*
Whiteman Park (WA)	*M. fuliginosus*	32	06/07-11/07	*p.m.*
Eneabba (WA)	*M. fuliginosus*	17	06/07-11/07	*p.m.*

8.3.2 Optimisation of ELISA

8.3.2.1 Competitive ELISA

Competitive ELISA (cELISA) was used to detect antibodies to *C. burnetii* in the serum of animals where no secondary antibodies specific for their IgG were available. NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Known positive and negative sera from experimentally infected mice were tested at dilutions of 1:10, 1:20, 1:40 and 1:80 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Indicator serum was then tested at dilutions of 1:50, 1:100, 1:200 and 1:400 for both antigenic phases and incubated at 37°C for a further 1 hr. The wells were washed three times with PBS-T (Appendix A) after which 50 µL HRP-conjugated rabbit anti-bovine Ig (Serotec, UK) at 1:1,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. Statistical analyses were performed using the Mann-Whitney U test. Using reagent dilutions optimised for murine sera, a random selection of 30 macropod, 10 bandicoot and 10 possum sera were screened to select positive and negative sera for species-specific optimisation using the same methods as the murine sera.

8.3.2.2 Indirect polyclonal ELISA

Indirect polyclonal ELISA was used to detect antibodies to *C. burnetii* in the serum of animals using polyclonal antibodies specific for their IgG raised in domestic chickens. NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Positive and negative sera (selected using cELISA) were then tested at dilutions of

1:50 and 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. The wells were washed three times with PBS-T (Appendix A) after which 50 µL of chicken anti-macropod IgG polyclonal antibody was tested at dilutions of 1:125, 1:250, 1;500 and 1:1,000 for both antigenic phases and incubated at 37°C for a further 1 hr. The wells were washed four times with PBS-T (Appendix A) after which 50 µL HRP-conjugated rabbit anti-chicken IgY (Jackson, USA) was tested at dilutions of 1:1,000, 1:2,000, 1:4,000 and 1:8,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. Statistical analyses were performed using the Mann-Whitney U test.

8.3.2.3 Indirect phage-displayed CRAb ELISA

Indirect phage-displayed CRAb ELISA was used to validate this technique by comparing it to the two previously described methods (8.3.2.1 and 8.3.2.2) for the detection antibodies to *C. burnetii* in the serum of animals. NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Known positive and negative sera from experimentally infected mice were tested at dilutions of 1:25, 1:50 and 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. The wells were washed four times with PBS-T (Appendix A) after which 50 µL of phage displayed CRAb MC2 was applied at a dilution of 1:4 in 2% skim milk and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated anti-M13 antibody (GE Healthcare, USA) was applied at a dilution of 1:5,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. Statistical analyses were performed using the Mann-Whitney U test. Using reagent dilutions optimised for murine sera, a random selection of 30 macropod, 10 bandicoot and 10 possum sera were

screened to select positive and negative sera for species-specific optimisation using the same methods as the murine sera.

8.3.3 Screening of native Australian marsupial serum for antibodies to *Coxiella burnetii* using ELISA

8.3.3.1 Competitive ELISA

NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:10 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. Indicator serum was then applied at a dilution of 1:200 for both antigenic phases and incubated at 37°C for a further 1 hr. The wells were washed three times with PBS-T (Appendix A) after which 50 µL HRP-conjugated rabbit anti-bovine Ig (Serotec, UK) at 1:1,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. A reduction in optimal density of ≥ 70% from that of the indicator serum alone was considered to be positive. Results of duplicates for each sample were averaged to produce a mean result for each animal.

8.3.3.2 Indirect polyclonal ELISA

NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:100 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in

duplicate. The wells were washed three times with PBS-T (Appendix A) after which 50 µL of chicken anti-macropod IgG polyclonal antibody was applied at a titre of 1:250 for both antigenic phases and incubated at 37°C for a further 1 hr. The wells were then washed four times with PBS-T (Appendix A) after which 50 µL HRP-conjugated rabbit anti-chicken IgY (Jackson, USA) at 1:2,000 was applied and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. The S/P% was calculated for each sample using the following formula: S/P% = (OD sample – OD negative control) ÷ (OD positive control – OD negative control) × 100. Sera with an S/P% less than 50% were considered to be negative. Samples with an S/P% of between 50% and 75% were considered to be positives; those greater than 75% were considered strongly positive. Results of duplicates for each sample were averaged to produce a mean result for each animal.

8.3.3.3 Indirect phage-displayed CRAb ELISA

NUNC™ 96-well Maxisorp plates were coated with 50 µL of phase I or phase II antigen at a dilution of 1:100 in coating buffer and incubated overnight at 37°C. Plates were coated with 50 µL post-coating buffer, incubated at room temperature for 2 hr then dried. Sample sera were applied at a dilution of 1:50 in 50 µL aliquots in duplicate and incubated at 37°C for 1 hr. Positive and negative control sera were also included in duplicate. The wells were washed four times with PBS-T (Appendix A) after which 50 µL of phage-displayed CRAb MC2 was applied at a dilution of 1:4 in 2% skim milk and incubated at 37°C for 1 hr. The wells were washed as described previously, after which 50 µL of HRP-conjugated anti-M13 antibody (GE Healthcare, USA) was applied at a dilution of 1:5,000 and incubated at 37°C for 1 hr. The wells were washed again, after which 50 µL ABTS was applied and incubated at 37°C for 30 min. Optical density readings were obtained using a Multiskan Ascent plate reader at 414/494 nm. Average absorbance readings greater than two standard deviations above that of the average absorbance reading for the negative control were considered to be positive. Results of duplicates for each sample were averaged to produce a mean result for each animal.

8.3.4 Complement fixation

As insufficient purified possum and bandicoot IgG was available for polyclonal production in domestic chickens, complement fixation tests were trialled as replacement for the indirect polyclonal ELISA for these species. Serum from possums and bandicoots was diluted 1:10 in veronal buffer (Virion\Serion, Germany). Commercial control sera for phase I and phase II were included as anti-sera control (Virion\Serion, Germany). Endogenous complement was then inactivated by incubation of diluted sera for 30 min at 56°C.

Test sera and control sera were serially diluted across the plate in 25 µL aliquots. Phase I and Phase II antigen were added in 25 µL aliquots to each half of the plate. Complement was then applied to each well of the plate. Complement controls were prepared by serially diluting complement in 25 µL aliquots across the bottom row of the plate. The plate was covered and incubated overnight at 4°C.

A 1:1 dilution of haemolytic anti-sheep erythrocyte serum (Virion\Serion, Germany) and 1% sheep erythrocyte suspension was prepared and incubated at 37°C for 30 min. The overnight plate was pre-warmed at 37°C for 15 min and 50 µL of serum/erythrocyte suspension was applied to each well of the plate. The plate was then incubated at 37°C for 15-30 min with shaking at 10 min intervals. Incubation ceased when the complement controls for two and one units displayed complete haemolysis, after which the plate was centrifuged at 1,000 ×*g* for 5 min. Wells with >50% inhibition of haemolysis were considered to be positive.

8.3.5 Statistical analyses

8.3.5.1 General statistical methods

Seropositivity percentage was calculated by dividing the number of positive samples by the total number of samples and multiplying by 100. Comparisons between groups were performed using the Mann-Whitney U test. Post-hoc power analysis was performed on bandicoot and possum agreement data to ensure sufficient sample size was present.

8.3.5.2 Statistical analysis of macropod data

An ordinal logistic generalised linear model was constructed using SPSS Statistics 19 (IBM, USA) to identify factors associated with seropositivity for each antigenic phase separately, and seropositivity to either or both antigens combined in macropod samples. All data was transformed into numerical values prior to statistical analysis. Type III analysis was performed with Wald Chi-Square test and 95% Profile Likelihood Confidence Intervals calculated. Cross-tabular analysis and Pearson Chi-Squared Tests were also performed for factors potentially associated with seropositivity for either or both antigenic phases of *C. burnetii*. Factors modelled included, sex, age, species, sample site and region. Age was subjectively categorised as adult (approximately three years and older) or juvenile (under three years including pouch young) based on size and apparent sexual maturity. Species were categorised according to phylogenetic similarity and were divided into genera and sub-genera (Table 8.3). The eastern grey kangaroo and western grey kangaroo samples were considered separately despite their phylogenetic similarity due to the geographical separation of these species. Sample sites were grouped according to location, with each site considered to be within a 100 km radius of the major locality in the area (Figure 8.1). Sites and Regions included in the analyses are included in Table 8.4.

Table 8.3: Species categories for statistical analyses of macropod samples

GROUP	SPECIES INCLUDED
Subgenus *Notamacropus* (Species 6)	*Macropus agilis* (Agile wallaby)
	Macropus dorsalis (Black-striped wallaby)
	Macropus parryi (Whiptail wallaby)
	Macropus rufogriseus (Red-necked wallaby)
Subgenus *Osphranter* (Species 5)	*Macropus robustus* (Common wallaroo)
	Macropus antilopinus (Antilopine kangaroo)
	Macropus rufus (Red kangaroo)
Subgenus *Macropus* 1(Species 1)	*Macropus giganteus* (Eastern grey kangaroo)
Subgenus *Macropus* 2 (Species 7)	*Macropus fuliginosus* (Western grey kangaroo)
Genus *Petrogale* (Species 4)	*Petrogale penicillata* (Brush-tailed rock wallaby)
Genus *Thylogale* (Species 2)	*Thylogale stigmatica* (Red-legged pademelon)
Genus *Aepyprymnus* (Species 3)	*Aepyprymnus rufescens* (Rufous bettong)

Table 8.4: Sample collection sites and corresponding regions included in statistical analyses of macropod samples

REGION	SITE (STATISTICAL DIVISION)	LOCALITIES INCLUDED
Western Australia (Region 4)	South Western Coast (Site 7)	Capel
		Preston Beach
	South Western Inland (Site 8)	Nannup
	Western Coast (Site 9)	Eneabba
	Perth District (Site 11)	Whiteman Park
Southern Queensland (Region 3)	South West Queensland (Site 12)	Roma
		Injune
	Darling Downs (Site 14)	Warwick
Western Queensland (Region 2)	Western Queensland (Site 2)	Longreach
		Winton
		Thurle's Park
Northern Queensland (Region 1)	Northern Queensland (Site 1)	Townsville
	Northern Queensland (Site 6)	Greenvale
	North West Queensland (Site 5)	Richmond

Figure 8.1: Origin of macropod samples included in the survey.
Sample sites were grouped according to location, with each site considered to be within a 100 km radius of the major locality in the area

8.3.5.3 Statistical analysis of possum and bandicoot data

Bandicoot and possum data were modelled separately from the macropod data due to the differences in samples size and reduced number of collection sites. An ordinal logistic generalised linear model was constructed using SPSS Statistics 19 (IBM, USA) to identify factors associated with seropositivity for each antigenic phase separately, and seropositivity for either or both antigens combined in bandicoot and possum samples as described previously (Section 8.3.6.3). Factors modelled included, sex, age, lactating, species, sample site and region. Age was categorised as adult for adult animals and

juvenile for sub-adult and pouch young. Locations were considered to be within a 100 km radius of the major locality in the area (Table 8.5). Sites within locations included in the analyses are listed in Table 8.5. All locations were situated in the Northern Statistical Division of Queensland.

Table 8.5: Sample collection sites and corresponding regions included in statistical analyses of bandicoot and possum samples

Location	Sites Included
Townsville (Location 1)	School of Veterinary and Biomedical Sciences (Site 1)
	Townsville General Hospital (Site 1)
	Gulliver (Site 5)
	Fairfield Waters (Site 5)
	Wulguru (Site 6)
	Cleveland (Site 7)
	Bushland Beach (Site 4)
Burdekin (Location 2)	Ayr (Site 2)
	Giru (Site 2)
Atherton Tablelands (Location 3)	Malanda (Site 3)
	Ravenshoe (Site 3)

8.3.5.4 Seropositivity in native marsupials, beef cattle seroprevalence and human Q fever incidence

Spearman Rank Correlation analyses were used to determine whether any correlation existed between seropositivity in native marsupials and seropositivity beef cattle or human Q fever notifications and incidence.

8.4 Results

8.4.1 Blood sample collection

A total of 33 macropod samples were collected in north Queensland in the current study. A further set of 87 samples were obtained from an unrelated study in northern (59 samples) and western Queensland (28 samples) conducted during 1982 to 1985. Of the northern Queensland samples, 59 were collected in the Townsville region, 17 at Greenvale and 13 at Richmond.

A sample set of 180 macropod samples were obtained from a related study in south-western West Australia (Banazis *et al.*, 2010). In addition, a sample set of 200 macropod samples were obtained from unrelated studies in south western Queensland (Barnes *et al.*, 2007; Barnes *et al.*, 2008). In total, 500 macropod samples were tested.

A total of 56 common brushtail possum samples were collected in north Queensland in the current study with 47 collected in the Townsville region and nine in the Burdekin.

A total of 52 common northern bandicoot samples were collected in north Queensland in the current study with 50 collected in the Townsville region and two on the Atherton tablelands.

8.4.2 Optimisation of ELISA

8.4.2.1 Competitive ELISA

Optimal reagent dilutions were found to be 1:10 test sera, 1:200 indicator sera and 1:1,000 conjugate. Species thresholds for positive samples were determined to be a ≥70% reduction in optical density compared to indicator sera for macropods and bandicoots and a ≥50% reduction for possums.

8.4.2.2 Indirect polyclonal ELISA

Optimal reagent dilutions were found to be 1:100 test sera, 1:250 polyclonal sera and 1:2,000 conjugate. Threshold for positive samples was determined to be an S/P% ratio of ≥50%.

8.4.2.3 Indirect phage-displayed CRAb ELISA

Optimal reagent dilutions were found to be 1:50 test sera, 1:4 CRAb MC2 and 1:5,000 conjugate. Threshold for positive samples was determined to be an average optical density of two standard deviations above that of the average for the negative control.

8.4.3 Screening ELISA

8.4.3.1 Detection of antibodies to *C. burnetii* in macropod sera using three ELISA methods

Reactivity to phase II and phase I antigens in serum samples varied both within and between (phage-displayed, cELISA and indirect) the ELISA methods performed. Seropositivity determined by using each ELISA method for both antigenic phases is displayed in Table 8.6. The number of samples determined to be positive for antibodies to both phase II and phase I antigens varied between regions (Table 8.7). The number of samples determined to be positive for antibodies against either or both phase II and phase I using the different ELISA methods also varied between regions (Table 8.8).

Agreement within each ELISA method was variable, with greatest agreement phase II and I seropositivity using the cELISA (50.0%; 49.4-51.1%), followed by the polyclonal indirect ELISA (22.2%; 22.0-22.9%) and the phage-display indirect ELISA (12.5%; 12.4-13.2%) respectively. Overall, 38% of macropod samples testing positive for antibodies to phase II antigen, also tested positive for phase I antigen. This differed by

region, with 54% of samples positive for phase II antigen also reacting against phase I antigen in the northern Queensland samples, 50% in western Queensland samples and 45% in southern Queensland samples, with only 22% of samples from Western Australia reacting against both antigens.

Agreement between ELISA methods was minimal, with only 13 of the 500 samples (2.6%; 2.59-2.61%) determined to be positive using more than one ELISA method and only two of the 500 samples (0.4%; 0.39-0.40%) determined to be positive using all three ELISA methods. Overall seropositivity in macropod samples in the various regions tested, as determined using all three ELISA methods is displayed in Table 8.9.

Of the northern Queensland samples tested, those collected during 2007 to 2010 had greater seroprevalence for both phase II and phase I antigen than those collected during 1982 to 1985 using all three ELISA methods (Table 8.10). Overall seropositivity in macropods in northern Queensland for the two different time period cohorts, as determined using all three ELISA methods is also displayed in Table 8.10. Seroprevalence for phase II ($P<0.01$), phase I ($P<0.05$) and both antigens ($P<0.01$) in macropods sampled in northern Queensland during 2007 to 2010 was significantly higher than that in macropods sampled during 1982 to 1985.

Using cross-tabular analyses with Pearson Chi-Squared Test on all macropod sample data, factors found to have greater than expected counts of seropositive samples, and thereby a positive association with seropositivity at statistically significant levels included Site ($P<0.01$) and Region ($P<0.01$). For seropositivity to either phase II or phase I antigen, factors included Site ($P<0.01$) and Sex ($P<0.05$) for phase II and Site ($P<0.01$) and Region ($P<0.01$) for phase I.

Using the generalised linear model on all macropod sample data, statistically significant factors associated with seropositivity to phase II antigen included Sex ($P<0.05$), Site ($P<0.01$) and Region ($P<0.01$) with male animals ($P<0.05$), animals from Richmond

(P<0.05), Whiteman Park (P<0.05) and animals from northern Queensland (P<0.01) found more likely to be positive.

Statistically significant factors associated with seropositivity to phase I antigen included Site (P<0.01) and Region (P<0.01) with animals from Townsville (P<0.05), Richmond (P<0.05) and Greenvale (P<0.01) and animals from northern Queensland (P<0.01) found more likely to be positive.

Using the generalised linear model on all macropod sample data, statistically significant factors associated with seropositivity to either or both *C. burnetii* antigens were similar to those for each antigen individually. These factors included Sex (P<0.05), Site (P<0.01) and Region (P<0.01). Within these it was found that male animals (P<0.05), animals from Richmond (P<0.01), Greenvale (P<0.05), South Western Coast (P<0.05), Whiteman Park (P<0.05) and animals from northern Queensland (P<0.01) were more likely to have antibodies to *C. burnetii*. Cross-tabulation analyses and generalised linear model outputs for macropod data are included in Appendix B.

Table 8.6: Seropositivity for *Coxiella burnetii* in macropods using three ELISA methods

Collection Site	Samples	Seropositivity (n)								
		Phase II (%)			Phase I (%)			Combined* (%)		
		A	B	C	A	B	C	A	B	C
Southern Queensland	200	16 (8.0)	11 (5.5)	5 (2.5)	13 (6.5)	2 (1.0)	9 (4.5)	17 (8.5)	11 (5.5)	12 (6.0)
Northern Queensland	92	15 (16.3)	7 (7.6)	8 (8.7)	19 (20.7)	9 (9.8)	8 (8.7)	21 (22.8)	10 (10.9)	14 (15.2)
Western Queensland	28	1 (3.6)	0 (0.0)	2 (7.1)	1 (3.6)	0 (0.0)	1 (3.6)	1 (3.6)	0 (0.0)	2 (7.1)
Western Australia	180	15 (8.3)	30 (16.8)	7 (3.9)	9 (5.0)	9 (5.0)	5 (2.8)	21 (11.7)	33 (18.4)	12 (6.7)
Total	500	47 (9.4)	48 (9.6)	22 (4.4)	42 (8.4)	20 (4.0)	23 (4.6)	60 (12.0)	54 (10.8)	40 (8.0)

[A] competitive ELISA, [B] indirect ELISA using polyclonal anti-macropod IgG, [C] phage-displayed CRAb ELISA, *Combined represents antibodies to either or both phase II and phase I antigen

Table 8.7: Seropositive macropod samples reacting against both phase II and phase I antigen within ELISA methods

Collection Site	Seropositive Samples (%)			
	cELISA	Indirect	Phage	>1 ELISA
Southern Queensland	12 (70.6)	2 (18.2)	2 (16.7)	2 (5.3)
Northern Queensland	13 (61.9)	6 (60.0)	2 (14.3)	5 (13.5)
Western Queensland	1 (100)	0 (0.0)	1 (50.0)	0 (0.0)
Western Australia	3 (14.3)	4 (19.0)	0 (0.0)	4 (8.0)
Total	28 (22.4)	12 (9.6)	5 (4.0)	13 (10.4)

[nb] cELISA represents competitive ELISA, indirect represents ELISA using polyclonal anti-macropod IgG, phage represents phage-displayed CRAb ELISA and > ELISA represents more than one ELISA method

Table 8.8: Seroprevalence in macropods for either or both phase II and phase I antigens within ELISA methods

Collection Site	Seropositive Macropods (%)			
	cELISA	Indirect	Phage	All ELISAs
Southern Queensland	17 (8.5)	11 (5.5)	12 (6.0)	38 (19.0)
Northern Queensland	21 (22.8)	10 (10.9)	14 (15.2)	37 (40.2)
Western Queensland	1 (3.6)	0 (0.0)	2 (7.1)	3 (10.7)
Western Australia	21 (11.7)	33 (18.4)	12 (6.7)	50 (27.8)
Total	60 (12.0)	54 (10.8)	40 (8.0)	128 (25.6)

[nb] cELISA represents competitive ELISA, indirect represents ELISA using polyclonal anti-macropod IgG, phage represents phage-displayed CRAb ELISA

Table 8.9: Overall seroprevalence for macropods determined by all ELISA results

Collection Site	Samples	Seropositive Samples (n)		
		Phase II (%)	Phase I (%)	Combined* (%)
Southern Queensland	200	31 (15.5)	24 (12.0)	38 (19.0)
Northern Queensland	92	26 (28.3)	31 (33.7)	37 (40.2)
Western Queensland	28	3 (10.7)	2 (7.1)	3 (10.7)
Western Australia	180	39 (21.7)	22 (12.2)	50 (27.8)
Total	500	99 (19.8)	79 (15.8)	128 (25.6)

[nb] *Combined represents antibodies to either or both phase II and phase I antigen

Table 8.10: Seroprevalence for *Coxiella burnetii* in macropod serum samples from retrospective and current northern Queensland sample sets using three ELISA methods

Time Period	Samples	Seropositive Samples (n)								
		Phase II (%)			Phase I (%)			Combined* (%)		
		A	B	C	A	B	C	A	B	C
1982-1985	59	4 (6.8)	3 (5.1)	3 (5.1)	4 (6.8)	4 (6.8)	5 (8.5)	6 (10.2)	4 (6.8)	7 (11.9)
Overall		10 (16.9)			13 (22.0)			17 (28.8)		
2007-2010	33	11 (33.3)	4 (12.1)	5 (15.2)	7 (20.7)	5 (15.2)	3 (9.1)	15 (45.5)	6 (18.2)	7 (21.2)
Overall		16 (48.5)			18 (54.5)			20 (60.6)		

[A] cELISA, [B] indirect ELISA using polyclonal anti-macropod IgG, [C] phage-displayed CRAb ELISA, *Combined represents antibodies to either or both phase II and phase I antigen

Table 8.11: Seroprevalence of anti-*C. burnetii* antibodies in macropod species sampled

SITE	SPECIES	SAMPLES (n)	POSITIVE (n)	SEROPREVALENCE (95% CI)
Warwick (SQ)	*P. penicillata*	64	13	20.3% (20.1-20.8%)
	M. robustus	13	3	23.1% (22.7-27.2%)
	M. rufogriseus	9	0	0.0% (0-3.8%)
	M. giganteus	8	2	25.0% (24.6-33.1%)
Injune (SQ)	*M. giganteus*	83	17	20.5% (20.3-20.9%)
Roma (SQ)	*M. giganteus*	23	3	13.0% (12.9-14.5%)
Townsville (NQ)	*M. agilis*	16	4	25.0% (24.5-28.3%)
	M. giganteus	13	5	38.5% (37.4-43.7%)
	M. antilopinus	9	2	22.2% (21.9-28.9%)
	M. dorsalis	8	2	25.0% (24.6-33.1%)
	T. stigmatica	5	2	40.0% (38.9-57.0%)
	P. penicillata	4	1	25.0% (24.8-45.1%)
	A. rufescens	4	0	0.0% (0.0-15.1%)
	M. parryi	2	0	0.0% (0.0-42.1%)
Richmond (NQ)	*M. giganteus*	5	4	80.0% (74.3-99.9%)
	M. rufus	5	1	20.0% (19.9-34.3%)
	M. robustus	3	2	66.7% (63.5-99.7%)
	M. dorsalis	1	1	100% (97.5%-100%)
Greenvale (NQ)	*M. giganteus*	12	7	58.3% (56.0-65.4%)
	M. agilis	5	3	60.0% (57.1-78.9%)
Longreach (WQ)	*M. giganteus*	17	3	17.6% (17.4-20.2%)
	M. dorsalis	1	0	0.0% (0.0-97.5%)
Thurles park (WQ)	*M. rufus*	7	0	0.0% (0.0-5.9%)
	M. giganteus	1	0	0.0% (0.0-97.5%)
Winton (WQ)	*M. giganteus*	2	0	0.0% (0.0-42.1%)
Preston Beach (WA)	*M. fuliginosus*	60	12	20.0% (19.8-20.5%)
Capel (WA)	*M. fuliginosus*	37	19	51.4% (50.4-53.2%)
Nannup (WA)	*M. fuliginosus*	34	6	17.6% (17.4-18.7%)
Whiteman Park (WA)	*M. fuliginosus*	32	13	40.6% (39.9-42.5%)
Eneabba (WA)	*M. fuliginosus*	17	0	0.0% (0.0-1.1%)

Table 8.12: Factors associated with seropositivity to *C. burnetii* in macropods

FACTOR	Relative Risk	Odds Ratio	*P* (χ^2)
SEROPOSITIVITY TO PHASE II ANTIGEN			
Greenvale origin	2.7	3.9	<0.05
Whiteman Park origin	2.3	3.0	<0.05
Northern Queensland origin	1.5	1.6	<0.01
Male	1.5	1.6	<0.05
SEROPOSITIVITY TO PHASE I ANTIGEN			
Greenvale origin	9.2	20.8	<0.01
Richmond origin	6.5	11.1	<0.05
Northern Queensland origin	7.7	10.3	<0.01
SEROPOSITIVITY TO EITHER/BOTH PHASE II/I ANTIGEN			
Greenvale origin	3.4	6.8	<0.01
Capel origin	2.6	3.8	<0.05
Whiteman Park origin	2.1	2.8	<0.05
Northern Queensland origin	1.9	2.3	<0.01

8.4.3.2 Detection of antibodies to *Coxiella burnetii* in possum sera using two ELISA methods

Reactivity to phase II and phase I antigens in serum samples varied within, and between (phage-displayed and cELISA) the ELISA methods performed. Seropositivity determined using each ELISA method for both antigenic phases is displayed in Table 8.13. The number of samples determined to be seropositive for both phase II and phase I antigens varied between regions (Table 8.13). The number of samples determined to be seropositive for either or both phase II and phase I antigens using the different ELISA methods also varied between regions (Table 8.13).

Agreement within ELISA methods was variable, with good agreement between phase II and I seropositivity using the cELISA and poor agreement using the phage-display indirect ELISA. Using the competitive ELISA, five of six (83%; 77.4-99.9%) possum samples testing positive for antibodies to phase II antigen were also positive for phase I antigen. However, using the phage-displayed recombinant antibody indirect ELISA, only one of five (20%; 19.9-34.3%) samples positive for phase II antigen also reacted against phase I antigen.

Agreement between ELISA methods was poor, with none of the 56 samples determined to be positive using both ELISA methods. Overall seropositivity in possum samples in the two regions tested, as determined using both ELISA methods is displayed in Table 8.14. As all possum serum samples tested displayed anti-complementary activity, despite pre-incubation to degrade endogenous complement, seroprevalence could not be determined using complement fixation.

Using cross-tabular analyses with Pearson Chi-Squared Test, none of the factors analysed were found to have greater than expected counts of seropositive samples, and thereby a statistically significant association with seropositivity. Using the generalised linear model, none of the factors tested were found to have statistically significant associations with seropositivity to either or both *C. burnetii* antigens or to each antigen separately.

Table 8.13: Seroprevalence for *Coxiella burnetii* in possums using two ELISA methods

	Seropositive Samples (n)					
	Phase II % (%)		**Phase I (%)**		**Combined (%)**	
Site (n)	CRAb	cELISA	CRAb	cELISA	CRAb	cELISA
Townsville (47)	4 (8.5)	6 (12.8)	1 (2.1)	5 (10.6)	6 (12.8)	6 (12.8)
Burdekin (9)	0 (0.0)	1 (11.1)	0 (0.0)	1 (11.1)	0 (0.0)	1 (11.1)
Total (56)	4 (7.1)	7 (12.5)	1 (1.8)	6 (10.7)	6 (10.7)	7 (12.5)

[nb] CRAB represents phage-displayed CRAb ELISA and cELISA represents competitive ELISA

Table 8.14: Overall seroprevalence in possums determined by both ELISA methods

	Seropositive Samples (n)		
Site (n)	**Phase II (%)**	**Phase I (%)**	**Combined (%)**
Townsville (47)	9 (19.1)	5 (10.6)	9 (19.1)
Burdekin (9)	1 (11.1)	1 (11.1)	2 (22.2)
Total (56)	10 (17.9)	6 (10.7)	11 (19.6)

[nb] Combined represents antibodies to either or both phase II and phase I antigen

8.4.3.3 Detection of antibodies to *Coxiella burnetii* in bandicoot sera using two ELISA methods

Reactivity to phase II and phase I antigens in serum samples varied within, and between (phage-displayed and cELISA) the ELISAs performed. Seropositivity determined using each ELISA method for both antigenic phases is displayed in Table 8.15.

Agreement within ELISAs was variable, with good agreement between phase II and I seropositivity using the cELISA and poor agreement using the phage-display indirect ELISA. Using the competitive ELISA, eight of 10 (80%; 75.6-89.7%) bandicoot

samples positive for antibodies to phase II antigen were also positive for phase I antigen. However, using the phage-displayed recombinant antibody indirect ELISA, none of the seropositive samples reacted against both antigens (0.0%; 0.0-23.6%).

Overall seropositivity in bandicoot samples tested, as determined using both ELISA methods is displayed in Table 8.15. Agreement between ELISAs was poor, with none of the 46 samples found to be seropositive using both ELISA methods (0.0%; 0.0-0.2%). Seroprevalence could not be determined using complement fixation, as all bandicoot serum samples tested displayed anti-complementary activity, despite pre-incubation to degrade endogenous complement.

None of the factors analysed using cross-tabular analyses with Pearson Chi-Squared Test were found to have greater than expected counts of seropositive samples. Also, none of the factors tested using the generalised linear model were found to have statistically significant associations with seropositivity to either or both *C. burnetii* antigens or to each antigen separately. Cross-tabulation analyses and generalised linear model outputs for possum and bandicoot data are included in Appendix B.

Table 8.15: Seroprevalence for *Coxiella burnetii* in bandicoots using two different ELISA methods

	Seropositive Samples (n)		
ELISA Type (n)	**Phase II % (%)**	**Phase I (%)**	**Combined (%)**
Phage Display (52)	2 (3.8)	1 (1.9)	3 (5.8)
cELISA (46)	8 (17.4)	10 (21.7)	11 (23.9)
Total (52)	12 (23.1)	11 (21.2)	14 (26.9)

[nb] Combined represents antibodies to either or both phase II and phase I antigen

8.4.3.4 Seroprevalence in native marsupials, beef cattle seroprevalence and human Q fever incidence

The location of each statistical division in the State of Queensland is displayed in Figure 8.2 overlaid with the macropod and beef cattle seroprevalence data, Q fever notifications during 2004 to 2008 and cumulative Q fever incidence data for the period in each division. Possum and bandicoot seroprevalence data for the northern region is also included. There was a correlation ($P<0.05>0.025$) between seropositivity in macropods and Q fever incidence by statistical division. There was no correlation between seropositivity in macropods and beef cattle or seropositivity in macropods and Q fever notifications.

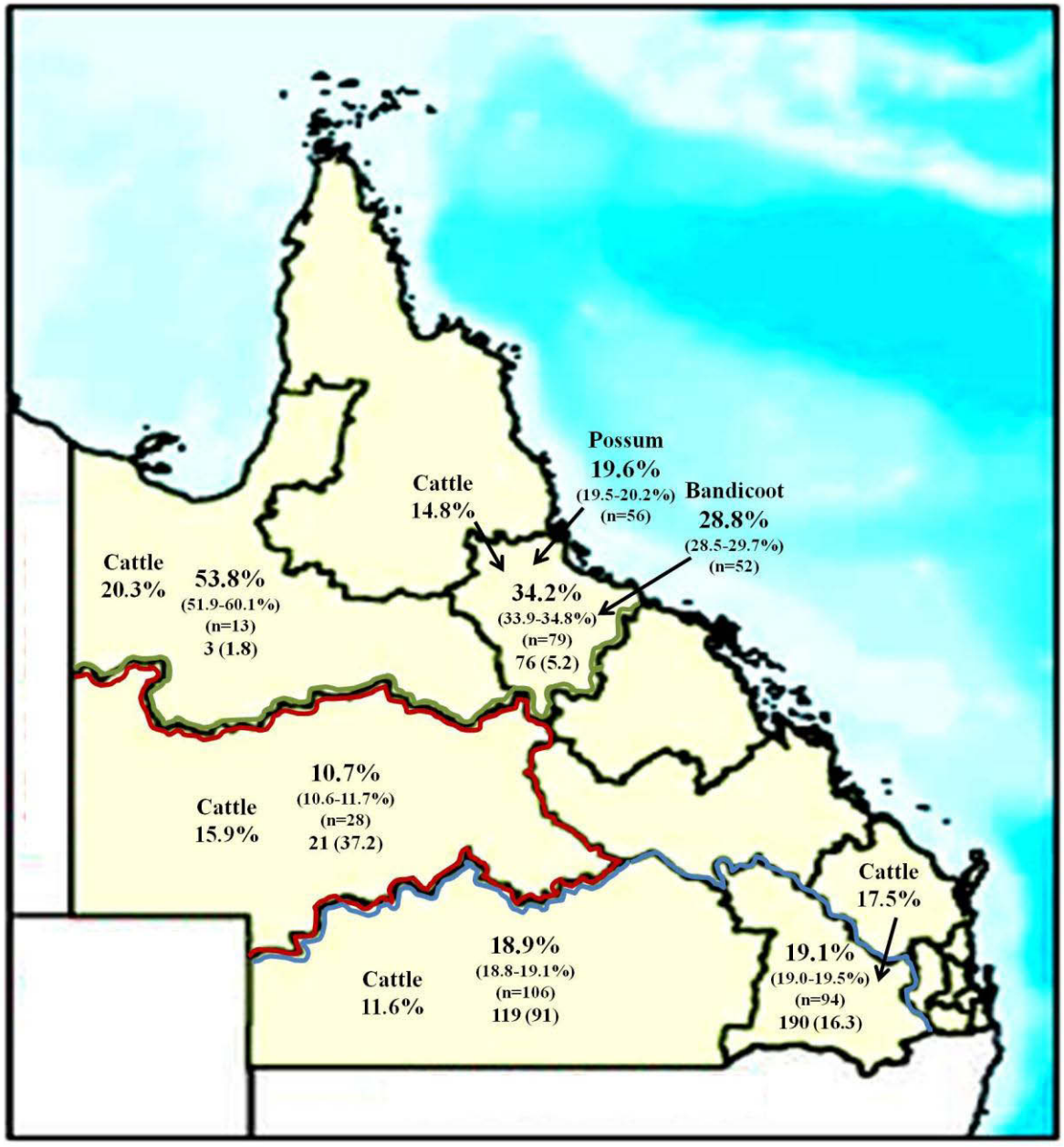

Figure 8.2: Relative seroprevalence of native Australian marsupials in sampled Queensland statistical divisions.

Data in each statistical division denotes seroprevalence in macropods, 95% confidence limits, number of samples, Q fever notifications and cumulative incidence per 100,000 population for 2004 to 2008 period respectively (in brackets). Seroprevalence in beef cattle in the statistical divisions is listed separately. For the Northern statistical division, possum and bandicoot data is also included. Coloured lines represent borders of regions used in statistical analyses with green, red and blue representing Northern, Western and Southern Queensland respectively. Map of Queensland from Queensland Treasury, 2009.

8.5 Discussion

The current study demonstrated that the seroprevalence of *C. burnetii* in the macropod populations sampled from northern Queensland (n=120), southern Western Australia (n=180), southern Queensland (n=200) and western Queensland (n = 28) were 30.8% (95% CI 30.6-31.2%), 27.8% (95% CI 27.7-28.0%), 19.0% (95% CI 18.9-19.1%) and 10.7% (95% CI 10.6-11.7%) respectively. Overall seroprevalence in the 500 macropod samples tested was 25.6% (95% CI 25.6-25.7%). Sites with significantly higher seroprevalence included Greenvale and Richmond in northern Queensland with 58.8% (95% CI 56.9-63.6%), and 46.2% (95% CI 44.7-51.9%) respectively and Capel and Whiteman Park in southern Western Australia with 43.2% (95% CI 42.5-44.9%) and 37.5% (95% CI 36.8-39.3%) respectively. Species with relatively high seroprevalence included *M. giganteus*, *M. fuliginosus*, *M. robustus* and *M. dorsalis*. However, seroprevalence varied between sites for the same species. Seroprevalences found in the current study are similar to those reported in previous studies in Queensland (Pope *et al.*, 1960) and Western Australia (Banazis *et al.*, 2010). In the brushtail possum and common northern bandicoot populations sampled, seroprevalence was found to be 19.6% (95% CI 19.5-20.2%) and 26.9% (95% CI 26.6-27.7%) respectively.

Several sites were found to have higher than expected numbers of seropositive macropods ($P<0.05$). These included Richmond (QLD), Greenvale (QLD), Preston Beach and Capel (WA) and Whiteman Park (WA). Northern Queensland as a whole also had higher than expected numbers of seropositive animals. The only factor associated with increased likelihood of seropositivity was Sex, with male animals more likely to be seropositive than females ($P<0.05$). No factors were found to be associated with seropositivity in possums and bandicoots in the current study. However, much smaller sample sets from fewer sites were tested for these species. Both sample sets were collected predominately within the Townsville and Burdekin districts (Northern Statistical Division). Further serosurveys of possums and bandicoots with a larger sample size are needed to provide a more comprehensive understanding of the epidemiology of *C. burnetii* in the region.

A correlation was found to exist between seroprevalence in macropods and Q fever incidence according to statistical division in Queensland ($P<0.05$). No correlation was found to exist between seroprevalence in macropods and Q fever notifications or seroprevalence in beef cattle by statistical division. Additionally, the detection of antibodies to *C. burnetii* in such a wide range of species indicates no one species is primarily associated with human Q fever cases in Queensland. These data also indicate that no one species is associated with seroprevalence in livestock.

Seropositivity varied for phase II and phase I antigens depending on the ELISA method used. Combined seropositivity for either or both phase II and I antigens also varied between ELISA methods. In Q fever patients, seroconversion to the various antigens post-infection is relatively well characterised, with an initial rise in antibodies to phase II antigen generally followed by antibodies to phase I antigen (Maurin and Raoult, 1999). Differences in titres of immunoglobulin isotypes to phase II or phase I antigens also form the basis for diagnosis of acute or chronic Q fever. Antibodies to phase II and I antigen of IgM isotype, and antibodies to phase II of IgG and IgA isotypes are associated with acute Q fever; whereas, antibodies to phase I of IgG and IgA isotypes are associated with chronic Q fever (Capo *et al.*, 1998; Fournier and Raoult, 1999; Camacho *et al.*, 2000). However, the process of seroconversion is not well characterised in animals and seropositivity to either or both antigenic phase of *C. burnetii* has been shown to vary between species (Enright *et al.*, 1971a; Marrie *et al.*, 1985; Marrie *et al.*, 1993). Some studies have suggested the presence of antibodies to phase II antigen in animal sera is indicative of recent infection (Lackman *et al.*, 1962; Sidwell and Gebhardt, 1962). In the current study, seropositivity was generally higher for phase II antigen than phase I antigen, with the exception of the northern Queensland macropod cohort. This trend was found with all ELISA methods used.

Seropositivity was similar for phase II and phase I antigen using the cELISA for all species tested, with many of these samples testing positive for both antigens. This ELISA method also resulted in the highest seropositivity values for the various species.

The greater number of positive samples detected by the cELISA is thought to be due to the choice of indicator sera and conjugate. Strongly reacting bovine sera was used as the indicator, combined with anti-bovine Ig conjugate, as bovine sera was found to have no cross-reactivity with the IgG of other species tested. As the conjugate was not immunoglobulin isotype specific, it would be able to detect bovine immunoglobulin of all potential isotypes. It is hypothesised that due to this factor, prevention of the indicator sera from binding to *C. burnetii* antigens by the test sera would indicate the presence of any potential immunoglobulin isotypes in the test sera. Immunoglobulin isotypes associated with Q fever in human serology include IgM and IgG predominately, but also IgA (Angelakis and Raoult, 2010). Therefore, it is thought that the seropositivity determined using the cELISA, may represent antibodies to *C. burnetii* from these three immunoglobulin isotypes. However, the only antibodies that would be detected in the test sera using cELISA are those that bind to the same epitopes in the antigen preparation as the indicator sera. This may explain the poor kappa agreement between the ELISA methods.

The polyclonal indirect ELISA was only used for macropod samples as this was the only animal group with sufficient purified IgG available for the production of polyclonal secondary antibodies. Seropositivity was higher for phase II antigen than phase I, and, of these a reasonable number of samples tested positive for both antigens. The phage-displayed CRAb ELISA detected fewer antibodies to *C. burnetii* than the other ELISA methods. This is most likely due to the phage-displayed CRAb being essentially a monoclonal antibody. Using this ELISA there were also very few samples found to be seropositive for both antigens. Also, distinction between the absorbance values of positive and negative samples was only two standard deviations. This is likely due to the use of a CRAb originally designed for binding to murine IgG. Although this CRAb demonstrated binding to the IgG of other species, it was not as strong as that of some CRAbs identified following the panning process for each species. Unfortunately, the CRAbs that demonstrated strong binding to species IgG in initial screening ELISAs were poorly amplified in culture and could not be reliably produced in bulk for further characterisation (Section 7.4).

Agreement between the ELISA methods was poor and it is thought that this was due to a combination of immunoglobulin isotype subclass and antigen epitope specificity. Purified IgG obtained through chromatography using protein G was shown to contain multiple IgG subclasses in immunoblotting on murine IgG (Section 6.4) and it is likely that all IgG subclasses were also present in the macropod, possum and bandicoot purified IgG. As in human serum from Q fever patients and vaccinees, IgG_1 appeared to be the predominant subclass present. In human Q fever patient sera, IgG_1 and IgG_3 are the immunoglobulin subclasses found to be associated with acute and chronic Q fever infection (Capo *et al.*, 1998), whereas IgG_1 and IgG_2 are associated with responses to vaccination (Camacho *et al.*, 2000). A similar trend was also demonstrated in cattle; where high IgG_2 levels and low IgG_1 were found in vaccinated animals, with the reverse detected in naturally infected animals (Schmeer *et al.*, 1986). However, previous studies of infection in mice by the author (Cooper, 2006) found that high levels of anti-*C. burnetii* IgG_2 and only threshold levels of IgG_1 were present, with significantly higher levels of IgG_2 in mice infected with *C. burnetii* compared to vaccinated mice. It can be difficult to extrapolate human IgG subclasses to their animal homologues as they are not always equivalent in biological function in all species. In human Q fever patient sera, IgG_1 and IgG_3 antibodies are directed against protein epitopes, whereas IgG_2 antibodies are directed against carbohydrate epitopes (Janeway *et al.*, 2008). As the predominant epitopes in *C. burnetii* are lipopolysaccharides (Hackstadt, 1990) the expected isotype of specific anti-*C. burnetii* antibodies would be IgG_2. However, antibody responses to protein antigens have also been found to be present in *C. burnetii* (Blondeau *et al.*, 1990). It is thought that the unexpected lack of IgG_2 antibodies to *C. burnetii* in human infection is due to the inability of this isotype to fix complement (Capo *et al.*, 1998). As both IgG_1 and IgG_3 are capable of fixing complement, it is thought that they are important in enhancing the uptake of *C. burnetii* by macrophages and are produced in response to *C. burnetii* for this purpose (Capo *et al.*, 1998).

It is thought that the positive samples detected by the phage-displayed CRAb ELISA may represent specific IgG_2 antibody. This is due to the phage-displayed CRAb ELISA producing similar results to indirect ELISA using specific IgG_2 antisera when used on

sera from *C. burnetii* infected mice. This may also explain the relatively lower number of seropositive samples detected using this ELISA method, as other animal studies indicated IgG_1 antibodies are more prevalent in natural infection (Schmeer *et al.*, 1986). Conversely, it is thought that the positive samples detected by the polyclonal indirect ELISA may mainly represent IgG_1 antibody as this would have been the predominant isotype present in the inoculum used to produce the polyclonal antibody. The agreement between the ELISA methods is also thought to be due to epitope differences, as IgG_1 is protein specific and IgG_2 carbohydrate specific. As whole cell antigen preparations were used in the three ELISA methods, both protein and liopolysaccaride antigens would be present.

In summary, three ELISA methods were developed for the detection of antibodies to *C. burnetii* in native Australian marsupials. As can be seen in the current study, the choice of ELISA method and conjugate greatly affects the detection of antibodies. This phenomenon made the ultimate validation of phage-displayed CRAbs in ELISA difficult, as they could not be directly compared to another test in field surveys. Antibody responses to *C. burnetii* in the animals tested were highly heterogeneous, a finding which was consistent with human Q fever serology. A similar discrepancy in the estimation of seroprevalence in a population was demonstrated in a recent human study using two different serological assays (Blaauw *et al.*, 2011). The heterogeneity of the antibody response to *C. burnetii* infection complicates serological investigation and epidemiological studies. In serosurveys, the cELISA developed in the current study would be of greatest advantage, whereas, in isotype specific studies the other assays may be of greater worth. Further work would be required in animal serology in order to determine the pattern of antibody production in response to *C. burnetii*. Such patterns may differ between species and may indicate relative recentness of infection and whether animals are chronically infected. Additional work would also be required to establish the identity of the immunoglobulin subsets detected in the current study, as isotype-specific and subclass-specific regents are not currently available for native Australian marsupials. However, this work would require the experimental infection of Australian native marsupials with *C. burnetii*, a process that is unlikely to be approved

under existing animal ethics regulations. In reality, this work may never be possible and may not be completely necessary as ultimately, the aim of this project was to detect circulating antibodies to *C. burnetii* in native Australian marsupials. The detection of antibodies of multiple isotypes and subclasses, while confusing, is still evidence of prior infection with *C. burnetii* in these species.

In conclusion, it was found that antibodies to *C. burnetii* were detected in all native marsupial species tested in this study. This result indicates these animals are potential reservoirs of *C. burnetii*. The correlation between seropositivity in macropods and Q fever incidence suggests these animals may be acting as reservoirs of Q fever. However, no correlation was found to exist between seropositivity in macropods and seropositivity in beef cattle. The increasing incidence of human Q fever cases where no contact with more typical reservoir species is present may be attributable to contact with atypical reservoirs, such as marsupials. Housing shortages in Queensland have resulted in residential areas expanding into wildlife habitats throughout the State. There has also been an increase in demand for semi-rural housing estates in northern Queensland. These developments would increase the exposure of the human population and companion animals to wildlife. In addition, some native species such as brushtail possums and bandicoots have adapted to urban habitats and are regularly observed on suburban properties. The close association these species have with human habitation, combined with the evidence of exposure to *C. burnetii* may have important public health implications. While the detection of antibodies to *C. burnetii* in marsupials gives an indication of previous infection with the bacterium, molecular detection of *C. burnetii* circulating in these species would confirm their ability to act as reservoirs of Q fever. Further investigation of native marsupials using molecular techniques such as PCR, for the presence of *C. burnetii* would be required to verify their status as potential reservoirs of Q fever.

CHAPTER NINE

DETECTION OF *COXIELLA BURNETII* DNA IN TICKS AND HOST SPECIES

9.1 Introduction

Wild animals and the tick species which feed on them form the natural transmission cycle and reservoir of *C. burnetii* (Babudieri, 1959). The bacterium has been isolated from various tick species that feed on a wide variety of vertebrate hosts. *Coxiella burnetii* is vertically transmitted from adult to nymph during egg production in some tick species, resulting in a self-perpetuating reservoir for *C. burnetii* (Pandurov and Zaprianov, 1975; Weyer, 1975; Daiter, 1977). The host promiscuity of many species of tick that feed on wild animals results in the transmission of *C. burnetii* to domestic animals in endemic areas. However, transmission between livestock does not require ticks, as inhalation of infected fomites produced by other livestock is sufficient for transmission (Babudieri, 1959).

Coxiella burnetii has been detected in a variety of tick species in Australia (Table 9.1). Early investigations demonstrated the presence of *C. burnetii* in *Haemaphysalis humerosa* (Smith and Derrick, 1939) and *Ixodes holocyclus* (Smith, 1942) collected from bandicoots (*Isoodon macrourus*) in south-eastern Queensland. The transmission of *C. burnetii* by these tick species was also demonstrated in bandicoots (Derrick and Smith, 1940; Smith, 1942). While *H. humerosa* is primarily a bandicoot tick that is rarely associated with other species, *I. holocyclus* is more promiscuous. This tick represents a potential vector for the transmission of *C. burnetii* from natural hosts to domestic animals, livestock and humans. Another tick species of importance as a reservoir for *C. burnetii* is *Amblyomma triguttatum*. This tick is primarily found on macropodids, but is also promiscuous in host species and has a wide distribution across

Queensland, Northern Territory, Western Australia, New South Wales (Roberts, 1970) and South Australia (McDiarmid *et al.*, 2000). In more recent studies, *C. burnetii* has been detected in another Australian tick species not previously investigated, *Bothriocroton auruginans* (Vilcins *et al.*, 2009). However, this tick species is unlikely to be an important vector for *C. burnetii* due to its limited host range in wombats. *Coxiella burnetii* has been found to be present in the gut lumen and epithelial lining of Australian ticks examined (Smith, 1942). Moreover, transovarial passage of *C. burnetii* has not been observed in Australian tick species investigated. While tick species known to be potential vectors of *C. burnetii* are capable of feeding on humans, this route of infection is a rare source of Q fever cases (Lang, 1990).

Table 9.1: Tick species associated with *Coxiella burnetii* in Australia[nb]

VECTOR	HOSTS
Amblyomma triguttatum	Macropods, livestock, feral animals, domestic animals, humans
Haemaphysalis humerosa	Bandicoots, rats, possums, antechinus
Ixodes holocyclus	Bandicoots, macropods, livestock, feral animals, domestic animals, humans
Bothriocroton auruginans	Common wombat
Ornithodorus gurneyi	Macropods, dogs, cattle, rodents, humans

[nb] From Roberts, 1970

As *C. burnetii* replicates primarily in the digestive tract of ticks, it is expelled in the faeces during feeding, which can result in heavy contamination of the skin of host animals with Coxieallae (Lang, 1990). This mechanism of transmission has been the suspected source of infection in Q fever epidemics (Derrick *et al.*, 1959; Pope *et al.*, 1960). Dried faeces from infected ticks have been found to harbour large quantities of phase I *C. burnetii* and remain infective for approximately two years (Stoker and Marmion, 1955). In addition, it has been demonstrated that ticks infected with *C. burnetii* can remain infected for several years and for the lifespan of the tick in some cases (Stoker and Marmion, 1955).

While investigations of tick reservoirs of *C. burnetii* have been performed in Australia previously, none have been performed since the 1960s and none have been performed in northern Queensland. Both the potential host and the tick species associated with *C. burnetii* transmission in past investigations are present in northern Queensland and current data is required in determining their potential role in Q fever epidemiology in the region. The experimental work outlined in this chapter aimed to determine whether *C. burnetii* was present in ticks and the blood of host animals in northern Queensland.

9.2 Aims

The specific aims for the work described in this chapter were to:

1. Develop protocols to detect the presence of *C. burnetii* DNA in ticks and blood samples from Australian native animals, and;
2. Investigate the potential relationship between *C. burnetii* load in ticks, presence in blood samples and seropositivity in Australian native animals in northern Queensland.

9.3 Materials and Methods

9.3.1 Optimisation of DNA extraction and quantitative PCR for *com1* gene

A preliminary experiment was performed with 10 *H. humerosa* nymphs, 10 *H. humerosa* adults, 10 *I. holocyclus* nymphs and 1 *I. holocyclus* adult collected from three common northern bandicoots (*Isoodon macrourus*) to ensure the extraction method and subsequent PCR were capable of detecting *C. burnetii* DNA. Ticks were processed

according to the method described in Section 9.3.3 and the genomic extract was then tested for presence of *C. burnetii* DNA using the qPCR described in Section 3.7. Tick genomic extracts were tested in duplicate at neat, 1/10 and 1/100 dilutions to determine whether PCR inhibition was present.

9.3.2 Collection of ticks from Australian native marsupials

In subsequent collections, up to 10 ticks in either or both nymphal and adult stages were collected from each animal during blood collection (Sections 8.3.1.1 and 8.3.1.3) using forceps. Ticks were placed in absolute ethanol in 5 mL sample tubes. The species and area where the animals were trapped was recorded. Ticks were then identified to species level using a key (Roberts, 1970).

9.3.3 Extraction of DNA from ticks

In order to extract DNA to determine the presence of *C. burnetii*, ticks were treated individually due to size or engorgement, or pooled according to host animal. Ticks were washed with 70% ethanol, air dried for 10 min on sterile paper and then finely diced with a sterile scalpel blade on a sterile glass slide. DNA was extracted using a HighPure™ PCR Template Preparation Kit (Roche Diagnostics, Germany) DNA extraction kit according to the manufacturer's instructions. DNA was eluted with molecular biology grade water (Sigma, Australia) preheated to 70°C. Purified DNA was then stored at -20°C prior to further analysis.

9.3.4 Collection of whole blood from Australian native marsupials

Blood collection was performed as described in Sections 8.3.1.1 and 8.3.1.3. A 200 µL aliquot was removed from each sample prior to centrifugation and stored at -20°C for subsequent DNA extraction.

9.3.5 Extraction of DNA from whole blood of Australian native marsupials

In order to extract DNA from whole blood to determine the presence of *C. burnetii*, approximately 200 µL frozen whole blood samples were thawed. DNA was extracted using a HighPure™ PCR Template Preparation Kit (Roche Diagnostics, Germany) DNA extraction kit according to the manufacturer's instructions for extraction of DNA from tissue. DNA was eluted with molecular biology grade water (Sigma, Australia) preheated to 70°C. Purified DNA was stored at -20°C for subsequent analysis.

9.3.6 Quantitative PCR for *com1* gene

To quantify the *com1* gene in DNA extractions, PCR reactions with a volume of 10 µL were prepared for a 72-well rotor in a RotorGene 6000 (Corbett Research, Australia) as described previously (Section 3.7.2). Each sample was tested in duplicate. Standards constructed as described previously (Section 3.7.1) were included, as were positive and no-template controls. Cycling conditions for qPCR consisted of an initial denaturation for 10 min at 94°C, followed by 40 cycles of denaturation for 10 sec at 94°C, annealing of primers for 10 sec at 62°C and extension for 20 sec at 72°C. A melt curve analysis was then performed with an increase in temperature from 72°C to 95°C in 1°C increments.

9.3.7 Sequencing of PCR products

To sequence the PCR products, PCR reactions with a volume of 20 µL were prepared for a 36-well rotor in a RotorGene 6000 (Corbett Research, Australia) as described previously (Section 3.7.2). Representative samples for each melt curve profile were amplified in ten tubes each. PCR products were quantified using a NanoPhotometer™ (Implen, Germany) and diluted to 100 ng μL^{-1} in 40 µL aliquots in o-ring sealed microfuge tubes. Sequencing of PCR products was performed by Macrogen Inc, Korea using *com1* primers provided.

9.3.8 Statistical analyses

Chi-squared tests were performed to determine whether there was any association between the presence of DNA in ticks, presence of DNA in blood samples from native marsupials and seropositivity in the same animals.

9.4 Results

9.4.1 Preliminary experiment for detection of the *com1* gene in ticks

A total of 10 *H. humerosa* nymphs, 10 *H. humerosa* adults, 10 *I. holocyclus* nymphs and 1 *I. holocyclus* adult were tested for the presence of the *Coxiella*-specific gene *com1* by using the qPCR described previously (Section 3.7). The nymphs and adults of each species were treated as separate pooled samples, with a total of four pooled samples tested. The *com1* gene was detected in all four pooled samples, with the greatest numbers of copies detected in *H. humerosa* nymphs, followed by *I. holocyclus* adult, *H. humerosa* adults and *I. holocyclus* nymphs respectively (Figure 9.1). Melt curve analysis indicated the *com1* amplicon from the four pooled samples was similar to that

of the *com1* amplicon from Nine Mile II clone four *C. burnetii* (standard curve). The results of the PCR also indicated that the DNA extracts from large, fully engorged ticks would need to be diluted 1/10 to avoid inhibition of the PCR.

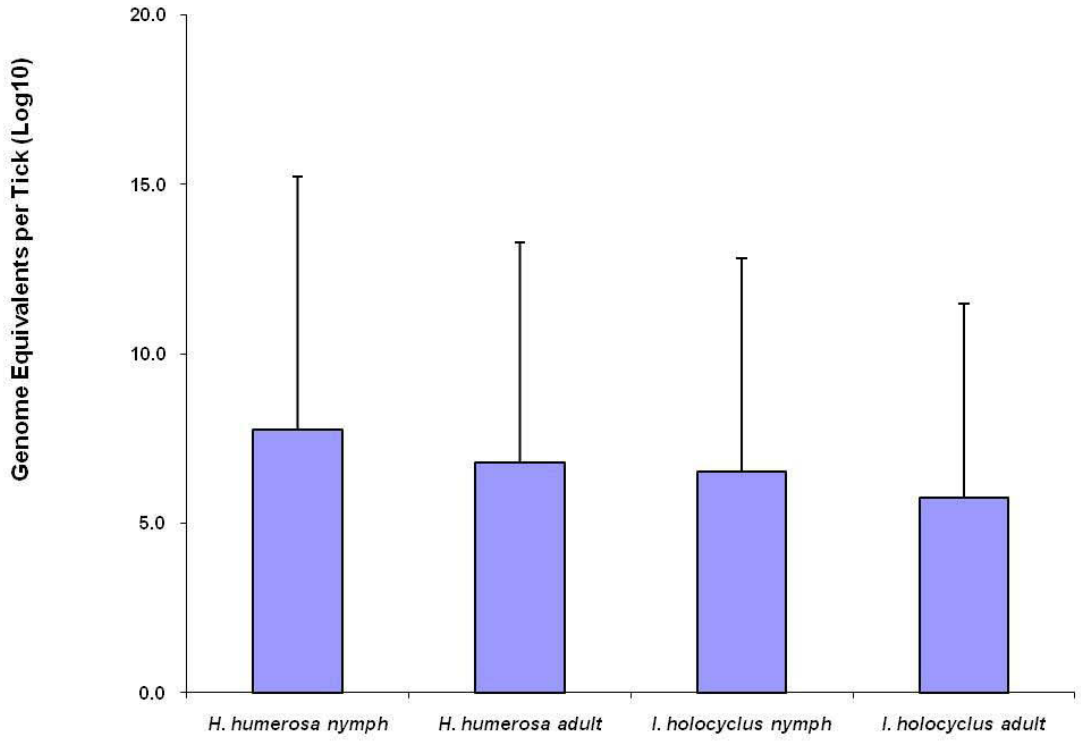

Figure 9.1: Quantification of *com1* gene in tick samples collected from *Isoodon macrourus*. Copy numbers of the *com1* gene were calculated according to the *com1* standard curve and corrected for dilution and number of ticks per pool. Error bars represent standard error of the mean.

9.4.2 Collection of ticks from Australian native marsupials

A total of 280 ticks were collected from 34 common northern bandicoots (*I. macrourus*). Of these, 250 were identified as *H. humerosa* and 30 as *I. holocyclus*. These were

divided according to host animal into 38 pools of *H. humerosa* and three pools of *I. holocyclus*.

A total of 43 *A. triguttatum* specimens were collected from nine eastern grey kangaroos (*M. giganteus*), four agile wallabies (*M. agilis*) and one rufous bettong (*A. rufescens*). All *A. triguttatum* samples were tested separately due to the size of the specimens. Several *I. holocyclus* specimens were also collected from a human subject and combined in one pooled sample.

9.4.3 Collection of whole blood from Australian native marsupials

Whole blood was collected from 35 common northern bandicoots (*Isoodon macrourus*), 17 eastern grey kangaroos (*Macropus giganteus*), five agile wallabies (*Macropus agilis*), four red kangaroos (*Macropus rufus*), three common wallaroos (*Macropus robustus*), two brushtail possums (*Trichosurus vulpecula*), one black striped wallaby (*Macropus dorsalis*) and one rufous bettong (*Aepyprymnus rufescens*).

9.4.4 Detection of the *com1* gene in tick samples using qPCR

Melt curve analysis identified the amplification of two different PCR products. Of these, the melt curve of one product was identical to that of the *com1* product amplified in the standard curve. The melt curve of the second amplified product demonstrated a right shift indicating the product was significantly different to the *com1* sequence of the first product and the standard curve product amplified from Nine Mile I clone four isolate (NMI/C4) DNA. The amplification product with a melt curve identical to NMI/C4 *com1* was designated Type 1, and the amplification product with the atypical melt curve was designated Type 2 (Figure 9.1). As both amplification products were present in some samples, precise quantification of *com1* copies could not be performed. Amplification products were instead described as present or absent for each amplicon type.

Amplicons of either type were detected in 100% of the 42 pooled samples of both *H. haemaphysalis* and *I. holocyclus* collected from bandicoots. Amplicon Type 1 (*com1*) was detected in one of three (30%) *I. holocyclus* and none of 38 (0%) *H. haemaphysalis* pooled samples collected from bandicoots. Amplicon Type 2 (atypical) was detected in two of three (66%) *I. holocyclus* and all 38 (100%) *H. haemaphysalis* pooled samples collected from bandicoots (Table 9.2).

Of the *Amblyomma triguttatum* samples collected from macropods, amplicons of either Type were detected in 39 of 43 (90.7%) samples. Amplicon Type 1 was detected in 12 of 43 (27.9%) macropod tick samples and Type 2 was detected in 19 of 43 (44.1%) samples. Both amplicon types were detected in eight samples (18.6%) and four samples (9.3%) were negative. A breakdown of amplicon types detected in the ticks collected from macropods is included in Table 9.2.

Amplicon Type 1 was detected in the *I. holocyclus* pooled sample collected from a human subject.

No association was found between the detection of DNA in the tick samples collected from a host animal and seropositivity in serum of the host animal; or detection of DNA in the tick sample and detection of DNA in the whole blood sample for each animal.

9.4.5 Detection of the *com1* gene in blood samples using qPCR

Melt curve analysis of amplified products from blood samples also identified two different PCR products. As with the amplicons detected in tick samples, the melt curve of one product was identical to that of the *com1* product amplified in the standard curve and the other demonstrated a right shift indicating the product was significantly different to the *com1* sequence (Figure 9.1). The two products were designated Type 1 and Type 2 respectively according to their product as described previously (Section 9.4.3). As both

amplification products were present in some samples, precise quantification of *com1* copies could not be performed, products instead described as merely present or absent.

Amplicons of either type were detected in 18 of 35 (51.4%) samples collected from bandicoots. Amplicon Type 1 was detected in six (17.1%) bandicoot blood samples and amplicon Type 2 in four (11.4%) samples. Both amplicon types were detected in eight samples (22.8%) and 17 samples (48.6%) were negative. A summary of amplicon types detected in the whole blood samples collected from bandicoots is included in Table 9.2.

Of the blood samples collected from macropods, amplicons of either Type were detected in 15 of 31 (48.4%) samples. Amplicon Type 1 was detected in 10 (32.3%) macropod blood samples and Type 2 was detected in two (6.5%) samples. Both amplicon types were detected in three samples (9.7%) and 16 samples (51.6%) were negative. A summary of amplicon types detected in the whole blood samples collected from macropods is included in Table 9.2.

Amplicons of either Type were detected in both (100%) samples collected from brushtail possums. Amplicon Type 1 was detected in one (50%) possum blood sample and amplicon Type 2 in the other samples (50%). A summary of amplicon types detected in the whole blood samples collected from possums is included in Table 9.2.

No association was found between the detection of DNA in the whole blood sample and seropositivity in the serum or the detection of DNA in the whole blood samples and detection of DNA in ticks.

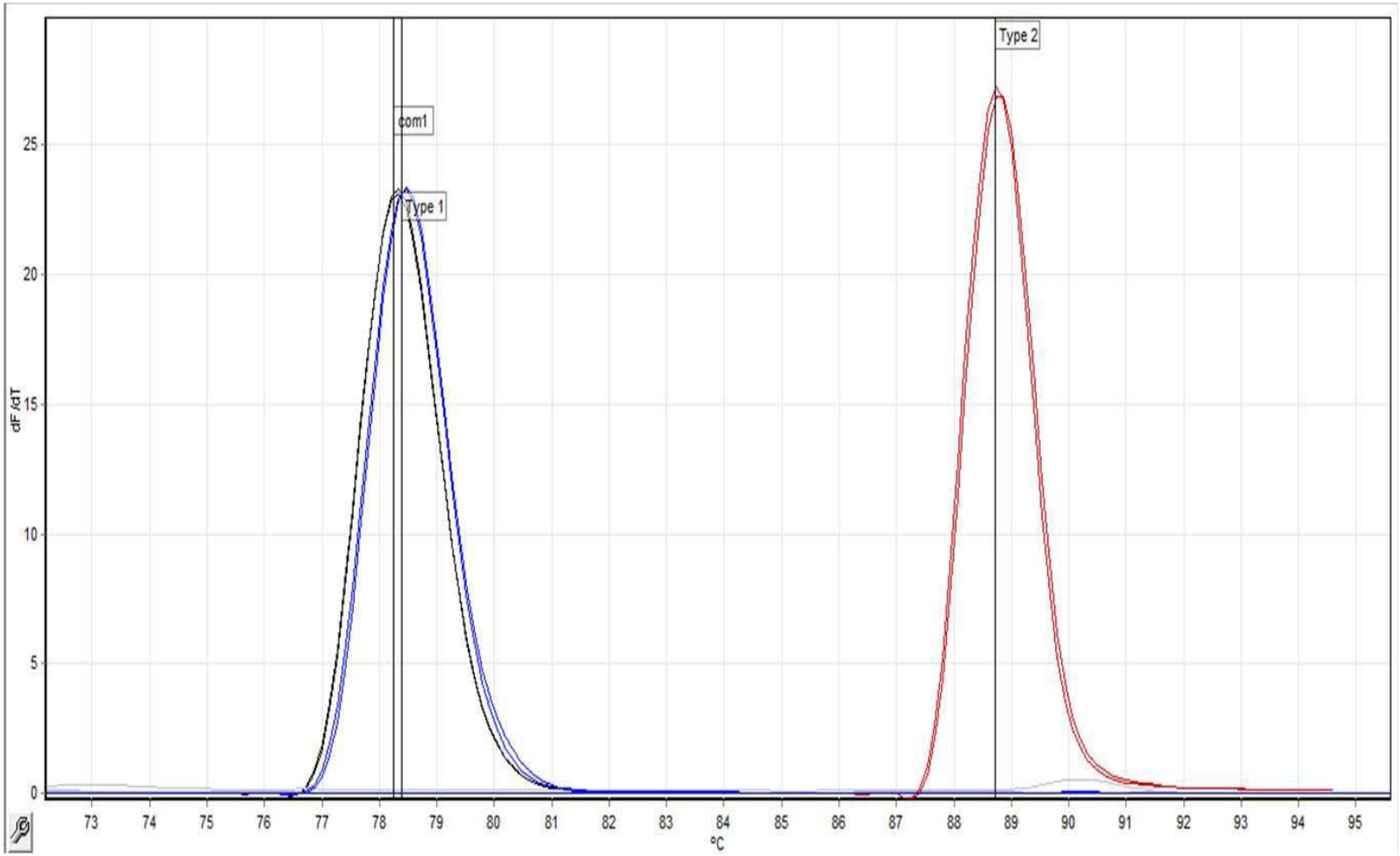

Figure 9.1: Melt curves of amplicons generated during qPCR performed on DNA extracts from ticks and whole blood collected from Australian native marsupials.

Black represents melt curve of *com1* amplicon from NMII/C4 DNA used as standard. Blue represents melt curve of amplicon Type 1. Red represents melt curve of amplicon Type 2.

Table 9.2: Summary of amplicon types detected in ticks and whole blood collected from Australian native marsupials

Host Animal		Ticks Collected		Amplicon Types Detected					
				Type 1		Type 2		Both Types	
Species	**Number**	**Species**	**Number**	**Tick**	**Blood**	**Tick**	**Blood**	**Tick**	**Blood**
Common northern bandicoot (*Isoodon macrourus*)	35	*Haemaphysalis humerosa*	250	0 (0%)	6 (17.1%)	250 (100%)	4 (11.4%)	0 (0%)	8 (22.9%)
		Ixodes holocyclus	30	10 (33.3%)		20 (66.7%)		0 (0%)	
Eastern grey kangaroo (*Macropus giganteus*)	17	*Amblyomma triguttatum*	31	12 (38.7%)	6 (35.3%)	13 (41.9%)	2 (11.8%)	4 (12.9%)	0 (0%)
Agile wallaby (*Macropus agilis*)	5	*Amblyomma triguttatum*	6	0 (0%)	1 (20%)	2 (33.3%)	0 (0%)	3 (50%)	2 (40%)
Red kangaroo (*Macropus rufus*)	4	No ticks collected	0	N/A	1 (25%)	N/A	0 (0%)	N/A	0 (0%)
Common wallaroo (*Macropus robustus*)	3	*Amblyomma triguttatum*	4	0 (0%)	1 (33.3%)	4 (100%)	0 (0%)	0 (100%)	0 (0%)
Black-striped wallaby (*Macropus dorsalis*)	1	No ticks collected	0	N/A	1 (100%)	N/A	0 (0%)	N/A	0 (0%)
Rufous bettong (*Aepyprymnus rufescens*)	1	*Amblyomma triguttatum*	2	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (50%)	1 (100%)
Brushtail possum (*Trichosurus vulpecula*)	2	No ticks collected	0	N/A	1 (50%)	N/A	1 (50%)	N/A	0 (0%)

9.4.6 Sequencing of PCR products

Sequencing of amplicon types 1 and 2 determined they were 75 bp and 190 bp respectively. Amplicon Type 1 was of expected length for the target sequence, whereas Type 2 was considerably longer. Sequencing results indicated that amplicon Type 1 sequence matched the sequence for *com1* from Nine Mile I *C. burnetii* (AE016828.2). Agreement between Type 1 and Nine Mile I *com1* sequence was 98%. Amplicon Type 2 did not have any significant matches to existing DNA sequences in GenBank. Alignment of amplicon Type 2 with Nine Mile I *com1* sequence indicated there were several homologous regions (Figure 9.2). However, overall agreement between the two sequences was 41%. Alignment of both amplicon types was performed against a *com1* DNA sequence obtained from a *C. burnetii* isolate found in ticks in Western Australia (HM804027.1). Agreement between this sequence and Type 1 and 2 amplicons was 98% and 41% respectively (Figure 9.3).

```
EMBOSS_com I      50 catcaccagtggc--aggcaatcctcatggcaatgttacattggttgaat
                     |||..|..|||.|  ||.|.|.||||
EMBOSS_Type2      1  CATGGCTCGTGCCCAAGCCGACCCTC------------------------

EMBOSS_com I      98 ttttcgattatcaa-----tgtggccattgcaaagccatgaattctgtta
                     .|||||.|...|.|     |    ||||||.||.|||
EMBOSS_Type2      27 GTTTCGCTACGCCACCCCTT----CCATTGAAACGCC-------------

EMBOSS_com I     143 ttcaagcta----------tcgtgaaacaaaataaaaacctccgcgttgt
                      |.||||||          ||.||   |.|.|.||||.|
EMBOSS_Type2      60 -TGAAGCTACGCCGCAGGCTCCTG---CGAGAGAAAATC-----------

EMBOSS_com I     183 cttcaaagaact--gcccatttttggcggccaatcgcaatacgctgccaa
                               |||  ||.||||    |||| ||||||    ||||
EMBOSS_Type2      95 ---------ACTGGGCTCATT----GCGG-CAATCG----ACGC------

EMBOSS_com I     231 agtatcatt----agcagcc-gcta-----aacaaggaaaatattatgct
                          |.||    ||.|.|| ||||     .|.|.|||      |.||.|
EMBOSS_Type2     121 -----CCTTCTCCAGGACCCGGCTACGCACGAGACGGA------TCTGGT

EMBOSS_com I     271 ----------------ttccacgacgcgctgct    287
                                     ||||||||||||||||
EMBOSS_Type2     160 TGCCGCGATCTACCACTTCCACGACGCGCTGCT    192
```

Figure 9.2: Alignment of DNA sequence for amplicon Type 2 against DNA sequence for *com1*

Alignment of two sequences was performed using EMBOSS Pairwise Alignment Algorithms. Identity between the two sequences was determined to be 41.0%.

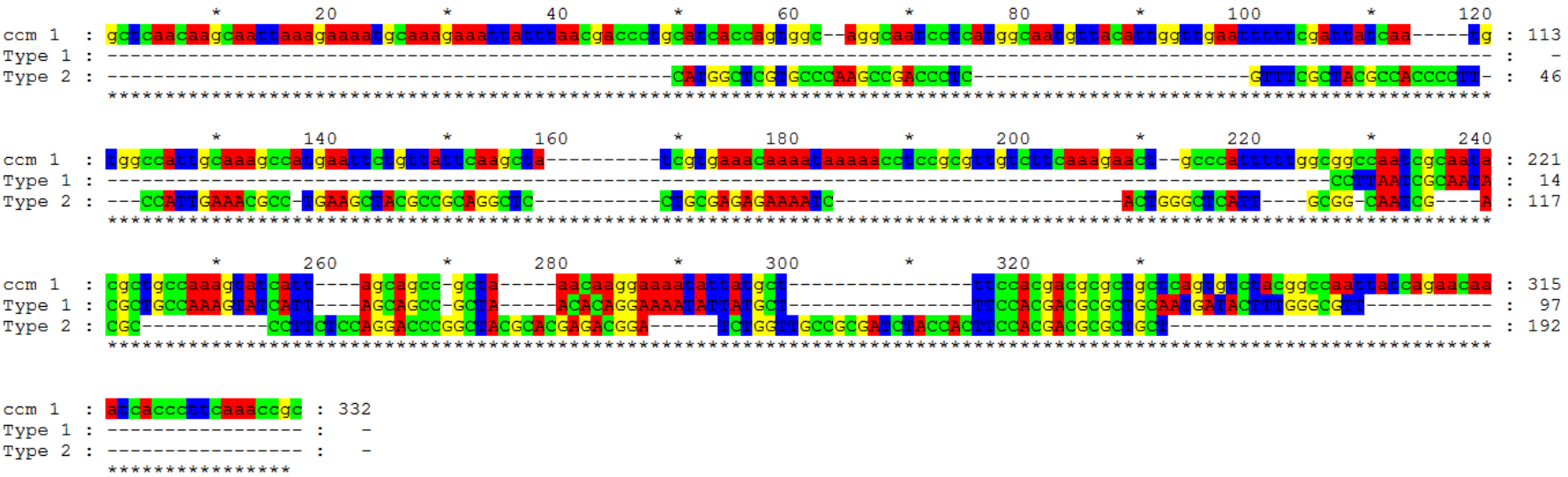

Figure 9.3: Alignment of DNA sequence for amplicons Type 1 and Type 2 against DNA sequence for *com1*
Sequences obtained for Type 1 and Type 2 were aligned with *com1* using Genedoc (Biology Software Net, USA). PCR product sequences are aligned from 5'-3'.

9.5 Discussion

A qPCR was successfully developed for the detection of the *com1* gene in tick extracts and whole blood collected from Australian native marsupials. The detection of the *com1* gene indicated the presence of *C. burnetii* in both the ticks and whole blood of bandicoots and a variety of macropods in northern Queensland. The identification of a PCR product with regions of conserved DNA with the *com1* gene in the ticks and whole blood of these species indicated the presence of an, as yet, unidentified tick-borne agent. The presence of *C. burnetii* in both the ticks and whole blood of Australian native marsupials suggests these animals are capable of acting as reservoirs of Q fever in northern Queensland.

The quantitative PCR developed for the detection of the *Coxiella*-specific *com1* gene in the current study was successfully used to detect the *com1* gene in genomic extracts from ticks collected from bandicoots. These findings were consistent with previous studies conducted in Australia. The current study also confirmed the presence of *C. burnetii* in the ticks of various macropod species via the detection of *C. burnetii* DNA. This study represents the first known detection of *C. burnetii* in ticks collected from agile wallabies. The current study also represents the first known detection of *C. burnetii* in the blood of the agile wallaby, common wallaroo and rufous bettong.

The amplification of PCR products other than that of the typical *com1* sequence was unexpected. The preliminary experiment performed on both *H. humerosa* and *I. holocyclus* specimens did not indicate the presence of the atypical amplicon. The primer sequences specific for the *com1* target sequence were present in the atypical amplicon, in addition to several homologous regions of DNA sequence. The DNA sequence for the atypical amplicon did not match any DNA sequences currently available on GenBank. This prevented non-specific binding by the *com1* primers from being predicted prior to qPCR. The amplification of the atypical amplicon also prevented precise quantification of *com1* genomes in the samples. The atypical amplicon was detected in both the whole blood sample and tick samples from four common

northern bandicoots and one eastern grey kangaroo. The detection of this amplicon in both tick and whole blood samples indicates it may be DNA sequence from another, as yet unidentified tick-borne agent. The identification of homologous regions in DNA sequence between the atypical amplicon and *com1* indicate it may be a *Coxiella*-like agent. As it was not identified in the tick samples collected during 2007, but was identified in samples collected during 2009 to 2010, the unidentified agent may be present seasonally or have emerged relatively recently in the tick and host species tested. However, as a relatively low number of ticks were collected from only three *I. macrourus* for the preliminary experiment conducted in 2007, this possibility could not be investigated further.

The atypical amplicon was favourably amplified over that of *com1* from *H. humerosa* specimens. Yet, the amplification of genuine *com1* sequence from the whole blood of bandicoots indicated *C. burnetii* is circulating in this species. The amplification of both amplicons in some bandicoot blood samples indicate this species is exposed to both *C. burnetii* and the unidentified agent. The favourable amplification of the atypical amplicon may indicate this agent is more abundant in *H. humerosa* and thereby more likely to be amplified in PCR. Analysis of the amplicon types detected in *A. triguttatum* specimens indicated the presence of both amplicon types separately and in combination, sometimes on a single animal. The amplification of both amplicons, either separately or simultaneously in some macropod blood samples indicate these species are exposed to both *C. burnetii* and the unidentified agent. While none of the possums sampled in this study were found to harbour ticks, both Type 1 and Type 2 amplicons were detected in the whole blood of this species. This finding indicated possums are exposed to both *C. burnetii* and the unidentified agent. However, further investigation of the carriage rate in possums is required as only two animals were sampled in the current study.

While *C. burnetii* DNA was detected in the blood and ticks of the native Australian marsupials surveyed in this study, there was no association between the presence of *C. burnetii* DNA in either ticks or blood and seropositivity. There was also no association found between the presence of *C. burnetii* DNA in ticks and presence of

C. burnetii DNA in blood. However, this result was not unexpected, as bacteraemia is often transient in animals (Mantovani and Benazzi, 1953) and not necessarily coincidental with seroconversion (McQuiston and Childs, 2002).

The results suggested that the unidentified agent represented by the atypical amplicon was more abundant in ticks than *C. burnetii*, as *com1* was more likely to be detected in whole blood than tick samples. These results also indicated that the unidentified agent was less commonly transmitted than *C. burnetii* as it was present in a greater proportion of ticks than *C. burnetii*, yet was present in a similar proportion of blood samples tested. Further investigation is warranted to formally identify the unknown tick-borne agent detected in this study.

The detection of *C. burnetii* DNA in both ticks and whole blood of native Australian marsupials indicates transmission of *C. burnetii* between these species is occurring in northern Queensland. This detection also indicates these species may be reservoirs of *C. burnetii* in the region. Both *A. triguttatum* and *I. holocyclus* have promiscuous host ranges and the detection of *C. burnetii* DNA in these species indicates they may be capable of transmitting *C. burnetii* to a wide range of host species, including livestock, domestic animals and their feral counterparts.

In conclusion, the detection of *C. burnetii* in both the ticks and whole blood of Australian native marsupials confirmed these animals were capable of acting as reservoirs of Q fever in northern Queensland. The confirmation of the presence of *C. burnetii* in these species indicates they may be the source of human Q fever cases where no contact with typical reservoir species was reported. The detection of *C. burnetii* in bandicoots and possums is particularly important, due to the adaptation of these species to urban environments. The detection of *C. burnetii* in macropods in the region may also have public health implications due to these animals often ranging into outlying suburban areas that border bushland. The evidence of *C. burnetii* in macropod ticks, in addition to the findings of another Australian study indicating macropods shed

C. burnetii in their faeces (Banazis *et al.*, 2010), suggests these animals may act as a vector of *C. burnetii* to domestic animals and the human population.

CHAPTER TEN

GENERAL DISCUSSION

The experimental work outlined in this thesis aimed to determine the potential for livestock, companion animals, feral animals and native wildlife to act as reservoirs of Q fever in Queensland, Australia. The detection of circulating antibodies in relation to pathogens in the sera of animals is indicative of prior infection, and thereby potential reservoir status, due to the possibility of shedding of the organism into the environment or transmission to arthropod vectors during infection. In order to undertake serological screening for exposure to *C. burnetii* in animal sera, diagnostic tools, specifically ELISA-based methods had to be developed. While a kit for the detection of *C. burnetii* in ruminant sera is commercially available, there have been problems associated with its use (Kittelberger *et al.*, 2009). In addition, the IDEXX Q fever Ab Test (IDEXX Laboratories, USA) has not been evaluated for use in animals other than ruminants. The test is also relatively expensive and only enables the testing of approximately 180 samples per kit. In order to test large numbers of animal sera, from ruminants and other species in an economically feasible manner, it was necessary to develop in-house screening protocols. Due to potential differences in antigenicity of *C. burnetii* isolates between continents, it was determined that the use of an Australian isolate for antigen preparation would be critical. Also, due to *C. burnetii* possessing two antigenic phases that can be important in determining whether infections are acute or chronic, it was necessary to be able to distinguish between antigenic phases in antigen preparations.

Virulence of *C. burnetii* isolates has been found to have a substantial effect on the immune response of animals (Russell-Lodrigue *et al.*, 2009). Therefore, it was important to select an Australian *C. burnetii* isolate of appropriate virulence to induce antibody production in animals and for antigen preparations to be used in ELISA. A comparison of isolate virulence between two recently identified Australian clinical isolates found that they differed in both mice and guinea pigs. This difference in virulence indicated the

potential presence of multiple circulating *C. burnetii* genotypes in Australia. These comparisons also indicated some isolates may be unsuitable for use in the preparation of diagnostic reagents, as they may not result in seroconversion in animals. As a result, these isolates, when used in antigen preparations for serological tests would be unlikely to detect circulating antibodies in animal sera.

In order to identify antigenic phase and track phase variation according to the differential gene expression, a qRT-PCR based method was developed for an Australian *C. burnetii* isolate. The method was validated by comparison with complement block titration, the traditional method for determining antigenic phase. While the method is yet to be used on other *C. burnetii* isolates, the reduction of expression of genes directly associated with phase I LPS indicates the method may be applicable for other isolates.

Following the selection of an appropriate Australian *C. burnetii* isolate (Cumberland), and the identification of both antigenic phases of the isolate, ELISA protocols were developed for the separate detection of antibodies to both phase II and I antigens. The ELISAs developed in this study enabled large numbers of animal and human sera to be screened at a relatively low cost per sample. These ELISAs also represented the first known use of an Australian *C. burnetii* isolate as antigen. The development of ELISAs using an Australian isolate in both antigenic phases will enable more precise investigations of *C. burnetii* seroprevalence in both non-native and native animal populations, and the human population in Australia.

Phase II and I specific ELISAs were optimised and validated using the sera of experimentally infected mice and guinea pigs and clinically confirmed human Q fever patients. The ELISAs were subsequently adapted for testing sera from humans, beef cattle, domestic dogs and cats; feral cats, pigs, dingoes and foxes. These ELISAs were relatively simple to develop due to the availability of commercially produced conjugate for these species. However, the adaptation of the ELISAs for use with native wildlife was more problematic due to a lack of diagnostic reagents for these animals. A relatively new technique, phage display was proposed as a potential solution for producing

conjugate specific for IgG from Australian native marsupials. As this method had not previously been employed for the production of secondary antibodies, it was initially optimised and validated using a mouse model. The method was then used to produce a phage display library of chicken recombinant antibodies (CRAbs) for a variety of Australian native marsupials. To determine the effectiveness of CRAbs as secondary antibodies, the use of these antibodies in indirect ELISA was compared to conventional ELISA methods. These methods included competitive ELISA and standard indirect ELISA using polyclonal antibodies.

While phage-displayed CRAbs were able to be validated using experimentally infected mice, validation of this method in native Australian marsupials proved problematic due to the heterogeneity of antibody response to *C. burnetii* in these animals. The heterogeneous nature of antibody responses to *C. burnetii* may preclude the use of phage-displayed CRAbs in ELISA for these species as the CRAbs are essentially monoclones and thereby limited in the range of immunoglobulin subsets detected. The results of this study indicate phage-display for the production of secondary recombinant antibodies may not be the solution to producing diagnostic reagents for wildlife as originally hypothesised. It was originally thought that the use of polyclonal antibodies against the IgG of native Australian marsupial species would be inferior to phage-displayed antibodies due to the potential requirement for follow-up boosting and bleeding of the host animal. However, this was not found to be the case when using domestic chickens (*Gallus gallus*) to produce the polyclonal antibodies. This species was found to produce sufficient anti-macropod IgG antibody for the testing of over 500 macropod samples from a single pooled blood sample from two immunised birds. The potential to use the eggs of immunised birds for even greater volumes of specific antibody indicates chickens would be an ideal host for the production of diagnostic reagents for wildlife.

Antibodies to *C. burnetii* were detected in all non-native and native species tested in this study. The seroprevalence of antibodies to *C. burnetii* varied between species, with high seroprevalence of greater than 20% found in foxes, feral cats, bandicoots, macropods

and feral pigs; medium seroprevalence of greater than 10% found in possums, domestic dogs, dingoes and beef cattle; and low seroprevalence of less than 10% found in domestic cats and humans. Seroprevalence of greater than 30% was found in foxes and feral cats. While the greatest risk factors for the contraction of Q fever include contact with domestic ruminants, some cases cannot be associated with these risk factors. These include human Q fever cases contracted in urban environments where there is little or no contact with the usual reservoirs of human infection. The relatively high seroprevalence found in foxes and feral cats warrants further investigation, as these species are found in peri-urban areas and often range into urban areas. Similarly, the seroprevalence found in bandicoots and macropods is also noteworthy as these species are also observed in peri-urban and urban areas.

The detection of the *Coxiella*-specific *com1* gene in both the ticks and whole blood of bandicoots and in a variety of macropods confirms the presence of *C. burnetii* in these species in northern Queensland. The presence of an, as yet, unclassified tick-borne agent was identified by the detection of a PCR product with regions of conserved DNA with the *com1* gene in the ticks and whole blood of bandicoots and macropods sampled in this study. The presence of *C. burnetii* in both the ticks and whole blood of Australian native marsupials confirms these animals are capable of acting as reservoirs of Q fever in northern Queensland.

In conclusion, serological tests were developed for the detection of antibodies to *C. burnetii* in a variety of animals including; livestock, domestic animals, feral animals and native Australian marsupials. These tests were successfully used to determine the seroprevalence of *C. burnetii* in the animals sampled. In addition, PCR assays were developed for the detection of the *Coxiella*-specific gene *com1* in ticks and whole blood of native Australian marsupials. These assays then detected the presence of *C. burnetii* in both ticks and whole blood of these species. The serological and molecular assays performed in this study demonstrate the potential for a wide variety of animals to act as reservoirs of Q fever in Queensland, Australia. The evidence of infection or exposure in a wide range of domestic and native animals may explain the incidences of Q fever in

humans with no known contact with typical livestock reservoirs. This data also demonstrates the need for greater awareness of potential atypical reservoirs acting as sources of Q fever. As no studies have been published recently on *C. burnetii* prevalence in Queensland, this study represents an important progression in understanding the epidemiology of Q fever in the region.

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APPENDIX A

BUFFERS AND SOLUTIONS

A 1.1 General Solutions

10× Phosphate Buffered Saline PBS-A
NaCl 80 g L^{-1}; KCl 2 g L^{-1}; Na_2HPO_4 11.48 g L^{-1}; KH_2PO_4 2 g L^{-1}
Dissolve in ddH_2O, adjust to pH 7.4 with 6 M HCl and sterilise by autoclaving

50× TAE buffer
Tris-HCl 242 g L^{-1}; Glacial acetic acid 57.1 mL; 0.5 M EDTA pH 8.0 100 mL, Add ddH20 to 1000 mL and sterilise by autoclaving

PBS-T wash buffer
1× PBS-A with 0.05% Tween-20

A 1.2 SDS-PAGE Solutions

1× running buffer
Tris[hydroxymethyl]-methylamine 12.1 g L^{-1}; HEPES 23.8 g L^{-1}; SDS 1 g L^{-1}
Dissolve in ddH_2O

2× reducing buffer
SDS 0.4 g ; glycerol 2 mL; 0.05% bromophenol blue 2 mL; 0.5 M Tris-HCl, pH 6.8 2.5 mL; 2-β mercaptoethanol 0.2 mL; ddH_2O to 10 mL

A 1.3 Immunoblotting Buffers

1× transfer buffer
Tris[hydroxymethyl]-methylamine 3 g L^{-1}; bicine 4.08 g L^{-1}; methanol 100 mL; 10% SDS 5 mL
Dissolve in ddH_2O and add SDS prior to use

1× blocking buffer
Skim milk powder 0.5 g; Tween 0.05%
Dissolve in 50 mL PBS

A 1.4 Molecular Biology Solutions

2×YT media
Tryptone 17 g L^{-1}; yeast extract 10 g L^{-1}; NaCl 5 g L^{-1}; ddH2O to 1L. For agar 15 g L^{-1} technical agar added.

2×YT-T media
2×YT media with 30 µg mL^{-1} tetracycline

2×YT-ATG media
2×YT media with 100 µg mL^{-1} ampicillin; 30 µg mL^{-1} tetracycline; 2% glucose

2×YT-AKT media
2×YT media with 100 µg mL^{-1} ampicillin; 50 µg mL^{-1} kanamycin; 30 µg mL^{-1} tetracycline

2×YT-AI media
2×YT media with 100 µg mL^{-1} ampicillin; 1 mmol L^{-1} IPTG

SOBAG media
Tryptone 20 g L^{-1}; yeast extract 5 g L^{-1}; NaCl 0.5 g L^{-1}; ddH_2O to 900mL. For agar 15 g L^{-1} technical agar added. After cooled to 55°C, 100 µg mL^{-1} ampicillin; 2% glucose; 10 mmol L^{-1} $MgCl_2$

SOBAG-N media
SOBAG media with 100 µg mL^{-1} nalidixic acid

SB media
Tryptone 35 g L^{-1}; yeast extract 20 g L^{-1}; NaCl 5 g L^{-1}
Dissolve in ddH_2O and adjust to pH 7.5

SB-AG media
SB media with 100 µg mL^{-1} ampicillin; 2% glucose

SB-AI media
SB media with 100 µg mL^{-1} ampicillin; 1 mmol L^{-1} IPTG

1M glucose
Glucose 180 g L^{-1}; Dissolve in ddH_2O and filter sterilise

1× TES buffer
Tris[hydroxymethyl]-methylamine 0.2 mol L^{-1}; EDTA 0.5 mmol L^{-1}; sucrose 0.5 mol L^{-1}
Make up in 1L ddH_2O and filter sterilise
LB media
Tryptone 10 g L^{-1}; yeast extract 5 g L^{-1}; sodium chloride 5 g L^{-1}

Dissolve in ddH_2O, adjust to pH 7.5 with 1 M NaOH. For agar add technical agar 15 g L^{-1}

SOC media
Tryptone 2 g; yeast extract 0.5 g; 1 mol L^{-1} sodium chloride 1 mL; 1 mol L^{-1} potassium chloride 0.25 mL. Make up to 98 mL with ddH_2O and adjust to pH 7.0. After autoclaving add filter sterilised 2 mol L^{-1} Mg^{2+} stock (1 mol L^{-1} $MgCl_2.6H_2O$ + 1 mol L^{-1} $MgSO_4.7H_2O$) 1 mL and 2 mol L^{-1} glucose 1 mL

TNE buffer
Tris[hydroxymethyl]-methylamine 10 mmol L^{-1}; EDTA 1 mmol L^{-1}; pH 8.0; sodium chloride mmol L^{-1}

A 1.5 Immunoglobulin Purification Buffers

Binding buffer
Sodium phosphate 3.12 g L^{-1}
Dissolve in ddH_2O, adjust to pH 7.0 and sterilise by filtration (0.2 µm filter)

Elution buffer
Glycine 7.5 g L^{-1}
Dissolve in ddH_2O, adjust to pH 2.9 with 6 M HCl and sterilise by filtration (0.2 µm filter)

Neutralisation buffer
Tris[hydroxymethyl]-methylamine 24.2 g; ddH_2O to 200 mL, adjust to pH 9.0 with 6 M NaOH and sterilise by filtration (0.2 µm filter)

A 1.6 DNA Extraction Buffers

TE buffer
Tris[hydroxymethyl]-methylamine 10 mmol L^{-1}; EDTA 1 mmol L^{-1}; pH 8.0

TNE buffer
Tris[hydroxymethyl]-methylamine 10 mmol L^{-1}; sodium dodecyl sulphate 0.5%; calcium chloride 1 mmol L^{-1}

CTAB solution
Sodium chloride 0.7 mol L^{-1}; cetyl trimethyl-ammonium bromide 10%

A 1.7 Gimenez Staining Solutions

Phosphate buffer
Sodium di-hydrogen phosphate 3.5 mL 0.2 mol L^{-1}; di-sodium hydrogen phosphate 15.5 mL 0.2 mol L^{-1}; distilled water 19 mL; pH 7.45.

Gimenez stock solution
Basic fuschin 10 g, ethanol 95% (v/v) 100 mL, aqueous phenol 4% (w/v) 250 mL, distilled water 650 mL.

Gimenez working solution
Phosphate buffer, pH 7.45 10 mL; Gimenez stock solution 4 mL; filtered.

APPENDIX B

Statistical Analyses

Crosstabs

B 1.1 Phase II Seropositivity in macropods

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
SexNum * PhaseIINum	436	87.2%	64	12.8%	500	100.0%
AgeNum * PhaseIINum	500	100.0%	0	.0%	500	100.0%
SiteNum * PhaseIINum	500	100.0%	0	.0%	500	100.0%
SpeciesNum * PhaseIINum	496	99.2%	4	.8%	500	100.0%
RegionNum * PhaseIINum	472	94.4%	28	5.6%	500	100.0%

SexNum * PhaseIINum

Crosstab

			PhaseIINum		
			1.00	2.00	Total
SexNum	1.00	Count	55	172	227
		Expected Count	46.9	180.1	227.0
	2.00	Count	35	174	209
		Expected Count	43.1	165.9	209.0
Total		Count	90	346	436
		Expected Count	90.0	346.0	436.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	3.719[a]	1	.054		
Continuity Correction[b]	3.276	1	.070		
Likelihood Ratio	3.750	1	.053		
Fisher's Exact Test				.059	.035
Linear-by-Linear Association	3.711	1	.054		
N of Valid Cases	436				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 43.14.

b. Computed only for a 2x2 table

AgeNum * PhaseIINum

Crosstab

			PhaseIINum		
			1.00	2.00	Total
AgeNum	1.00	Count	89	367	456
		Expected Count	88.5	367.5	456.0
	2.00	Count	8	36	44
		Expected Count	8.5	35.5	44.0
Total		Count	97	403	500
		Expected Count	97.0	403.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.046[a]	1	.831		
Continuity Correction[b]	.000	1	.989		
Likelihood Ratio	.046	1	.829		
Fisher's Exact Test				1.000	.508
Linear-by-Linear Association	.046	1	.831		
N of Valid Cases	500				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.54.

b. Computed only for a 2x2 table

SiteNum * PhaseIINum

Crosstab

			PhaseIINum		
			1.00	2.00	Total
SiteNum	1.00	Count	10	52	62
		Expected Count	12.0	50.0	62.0
	2.00	Count	3	25	28
		Expected Count	5.4	22.6	28.0
	5.00	Count	7	6	13
		Expected Count	2.5	10.5	13.0
	6.00	Count	7	10	17
		Expected Count	3.3	13.7	17.0
	7.00	Count	25	72	97
		Expected Count	18.8	78.2	97.0
	8.00	Count	2	32	34
		Expected Count	6.6	27.4	34.0
	9.00	Count	0	17	17
		Expected Count	3.3	13.7	17.0
	11.00	Count	12	20	32
		Expected Count	6.2	25.8	32.0
	12.00	Count	18	88	106
		Expected Count	20.6	85.4	106.0
	14.00	Count	13	81	94
		Expected Count	18.2	75.8	94.0
Total		Count	97	403	500
		Expected Count	97.0	403.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	36.347[a]	9	.000
Likelihood Ratio	36.743	9	.000
Linear-by-Linear Association	.448	1	.503
N of Valid Cases	500		

a. 3 cells (15.0%) have expected count less than 5. The minimum expected count is 2.52.

SpeciesNum * PhaseIINum

Crosstab

			PhaseIINum		
			1.00	2.00	Total
SpeciesNum	1.00	Count	35	128	163
		Expected Count	31.9	131.1	163.0
	2.00	Count	2	3	5
		Expected Count	1.0	4.0	5.0
	4.00	Count	8	60	68
		Expected Count	13.3	54.7	68.0
	5.00	Count	6	30	36
		Expected Count	7.0	29.0	36.0
	6.00	Count	7	37	44
		Expected Count	8.6	35.4	44.0
	7.00	Count	39	141	180
		Expected Count	35.2	144.8	180.0
Total		Count	97	399	496
		Expected Count	97.0	399.0	496.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	5.406[a]	5	.368
Likelihood Ratio	5.524	5	.355
Linear-by-Linear Association	.035	1	.851
N of Valid Cases	496		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is .98.

RegionNum * PhaseIINum

Crosstab

			PhaseIINum		
			1.00	2.00	Total
RegionNum	1.00	Count	24	68	92
		Expected Count	18.3	73.7	92.0
	3.00	Count	31	169	200
		Expected Count	39.8	160.2	200.0
	4.00	Count	39	141	180
		Expected Count	35.8	144.2	180.0
Total		Count	94	378	472
		Expected Count	94.0	378.0	472.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.988[a]	2	.083
Likelihood Ratio	4.989	2	.083
Linear-by-Linear Association	.768	1	.381
N of Valid Cases	472		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.988[a]	2	.083
Likelihood Ratio	4.989	2	.083
Linear-by-Linear Association	.768	1	.381
N of Valid Cases	472		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 18.32.

B 1.2 Phase I Seropositivity in macropods

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
SexNum * PhaseINum	436	87.2%	64	12.8%	500	100.0%
AgeNum * PhaseINum	500	100.0%	0	.0%	500	100.0%
SiteNum * PhaseINum	500	100.0%	0	.0%	500	100.0%
SpeciesNum * PhaseINum	496	99.2%	4	.8%	500	100.0%
RegionNum * PhaseINum	472	94.4%	28	5.6%	500	100.0%

SexNum * PhaseINum

Crosstab

			PhaseINum		
			1.00	2.00	Total
SexNum	1.00	Count	35	192	227
		Expected Count	36.4	190.6	227.0
	2.00	Count	35	174	209
		Expected Count	33.6	175.4	209.0
Total		Count	70	366	436
		Expected Count	70.0	366.0	436.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.142[a]	1	.706		
Continuity Correction[b]	.061	1	.805		
Likelihood Ratio	.142	1	.706		
Fisher's Exact Test				.794	.402
Linear-by-Linear Association	.142	1	.706		
N of Valid Cases	436				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 33.56.

b. Computed only for a 2x2 table

AgeNum * PhaseINum

Crosstab

			PhaseINum		
			1.00	2.00	Total
AgeNum	1.00	Count	69	387	456
		Expected Count	69.3	386.7	456.0
	2.00	Count	7	37	44
		Expected Count	6.7	37.3	44.0
Total		Count	76	424	500
		Expected Count	76.0	424.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.019[a]	1	.891		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.019	1	.891		
Fisher's Exact Test				.828	.516
Linear-by-Linear Association	.019	1	.891		
N of Valid Cases	500				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 6.69.

b. Computed only for a 2x2 table

SiteNum * PhaseINum

Crosstab

			PhaseINum		
			1.00	2.00	Total
SiteNum	1.00	Count	13	49	62
		Expected Count	9.4	52.6	62.0
	2.00	Count	1	27	28
		Expected Count	4.3	23.7	28.0
	5.00	Count	6	7	13
		Expected Count	2.0	11.0	13.0
	6.00	Count	10	7	17
		Expected Count	2.6	14.4	17.0
	7.00	Count	12	85	97
		Expected Count	14.7	82.3	97.0
	8.00	Count	5	29	34
		Expected Count	5.2	28.8	34.0
	9.00	Count	0	17	17
		Expected Count	2.6	14.4	17.0
	11.00	Count	5	27	32
		Expected Count	4.9	27.1	32.0
	12.00	Count	9	97	106
		Expected Count	16.1	89.9	106.0
	14.00	Count	15	79	94
		Expected Count	14.3	79.7	94.0
Total		Count	76	424	500
		Expected Count	76.0	424.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	46.704[a]	9	.000
Likelihood Ratio	40.009	9	.000
Linear-by-Linear Association	2.558	1	.110
N of Valid Cases	500		

a. 5 cells (25.0%) have expected count less than 5. The minimum expected count is 1.98.

SpeciesNum * PhaseINum

Crosstab

			PhaseINum		
			1.00	2.00	Total
SpeciesNum	1.00	Count	25	138	163
		Expected Count	25.0	138.0	163.0
	2.00	Count	0	5	5
		Expected Count	.8	4.2	5.0
	4.00	Count	13	55	68
		Expected Count	10.4	57.6	68.0
	5.00	Count	7	29	36
		Expected Count	5.5	30.5	36.0
	6.00	Count	9	35	44
		Expected Count	6.7	37.3	44.0
	7.00	Count	22	158	180
		Expected Count	27.6	152.4	180.0
Total		Count	76	420	496
		Expected Count	76.0	420.0	496.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	4.358[a]	5	.499
Likelihood Ratio	5.053	5	.409
Linear-by-Linear Association	.203	1	.652
N of Valid Cases	496		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is .77.

RegionNum * PhaseINum

Crosstab

			PhaseINum		
			1.00	2.00	Total
RegionNum	1.00	Count	29	63	92
		Expected Count	14.6	77.4	92.0
	3.00	Count	24	176	200
		Expected Count	31.8	168.2	200.0
	4.00	Count	22	158	180
		Expected Count	28.6	151.4	180.0
Total		Count	75	397	472
		Expected Count	75.0	397.0	472.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	20.897[a]	2	.000
Likelihood Ratio	18.200	2	.000
Linear-by-Linear Association	17.041	1	.000
N of Valid Cases	472		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 14.62.

B 1.3 Seropositivity for either or phase II and phase I in macropods

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
SexNum * CombinedNum	436	87.2%	64	12.8%	500	100.0%
AgeNum * CombinedNum	500	100.0%	0	.0%	500	100.0%
SiteNum * CombinedNum	500	100.0%	0	.0%	500	100.0%
SpeciesNum * CombinedNum	496	99.2%	4	.8%	500	100.0%
RegionNum * CombinedNum	472	94.4%	28	5.6%	500	100.0%

SexNum * CombinedNum

Crosstab

			CombinedNum		
			1.00	2.00	Total
SexNum	1.00	Count	67	160	227
		Expected Count	59.9	167.1	227.0
	2.00	Count	48	161	209
		Expected Count	55.1	153.9	209.0
Total		Count	115	321	436
		Expected Count	115.0	321.0	436.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.403[a]	1	.121		
Continuity Correction[b]	2.078	1	.149		
Likelihood Ratio	2.413	1	.120		
Fisher's Exact Test				.129	.075
Linear-by-Linear Association	2.398	1	.122		
N of Valid Cases	436				

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.403[a]	1	.121		
Continuity Correction[b]	2.078	1	.149		
Likelihood Ratio	2.413	1	.120		
Fisher's Exact Test				.129	.075
Linear-by-Linear Association	2.398	1	.122		
N of Valid Cases	436				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 55.13.

b. Computed only for a 2x2 table

AgeNum * CombinedNum

Crosstab

			CombinedNum		
			1.00	2.00	Total
AgeNum	1.00	Count	112	344	456
		Expected Count	114.0	342.0	456.0
	2.00	Count	13	31	44
		Expected Count	11.0	33.0	44.0
Total		Count	125	375	500
		Expected Count	125.0	375.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.532[a]	1	.466		
Continuity Correction[b]	.299	1	.584		
Likelihood Ratio	.514	1	.473		
Fisher's Exact Test				.469	.286
Linear-by-Linear Association	.531	1	.466		
N of Valid Cases	500				

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.532[a]	1	.466		
Continuity Correction[b]	.299	1	.584		
Likelihood Ratio	.514	1	.473		
Fisher's Exact Test				.469	.286
Linear-by-Linear Association	.531	1	.466		
N of Valid Cases	500				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 11.00.

b. Computed only for a 2x2 table

SiteNum * CombinedNum

Crosstab

			CombinedNum		
			1.00	2.00	Total
SiteNum	1.00	Count	16	46	62
		Expected Count	15.5	46.5	62.0
	2.00	Count	3	25	28
		Expected Count	7.0	21.0	28.0
	5.00	Count	8	5	13
		Expected Count	3.3	9.8	13.0
	6.00	Count	10	7	17
		Expected Count	4.3	12.8	17.0
	7.00	Count	31	66	97
		Expected Count	24.3	72.8	97.0
	8.00	Count	6	28	34
		Expected Count	8.5	25.5	34.0
	9.00	Count	0	17	17
		Expected Count	4.3	12.8	17.0
	11.00	Count	13	19	32
		Expected Count	8.0	24.0	32.0
	12.00	Count	20	86	106

		Expected Count	26.5	79.5	106.0
	14.00	Count	18	76	94
		Expected Count	23.5	70.5	94.0
Total		Count	125	375	500
		Expected Count	125.0	375.0	500.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	39.859[a]	9	.000
Likelihood Ratio	41.146	9	.000
Linear-by-Linear Association	2.035	1	.154
N of Valid Cases	500		

a. 3 cells (15.0%) have expected count less than 5. The minimum expected count is 3.25.

SpeciesNum * CombinedNum

Crosstab

			CombinedNum		
			1.00	2.00	Total
SpeciesNum	1.00	Count	41	122	163
		Expected Count	41.1	121.9	163.0
	2.00	Count	2	3	5
		Expected Count	1.3	3.7	5.0
	4.00	Count	14	54	68
		Expected Count	17.1	50.9	68.0
	5.00	Count	8	28	36
		Expected Count	9.1	26.9	36.0
	6.00	Count	10	34	44
		Expected Count	11.1	32.9	44.0
	7.00	Count	50	130	180
		Expected Count	45.4	134.6	180.0
Total		Count	125	371	496

Crosstab

			CombinedNum		
			1.00	2.00	Total
SpeciesNum	1.00	Count	41	122	163
		Expected Count	41.1	121.9	163.0
	2.00	Count	2	3	5
		Expected Count	1.3	3.7	5.0
	4.00	Count	14	54	68
		Expected Count	17.1	50.9	68.0
	5.00	Count	8	28	36
		Expected Count	9.1	26.9	36.0
	6.00	Count	10	34	44
		Expected Count	11.1	32.9	44.0
	7.00	Count	50	130	180
		Expected Count	45.4	134.6	180.0
Total		Count	125	371	496
		Expected Count	125.0	371.0	496.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	2.295[a]	5	.807
Likelihood Ratio	2.269	5	.811
Linear-by-Linear Association	.144	1	.704
N of Valid Cases	496		

a. 2 cells (16.7%) have expected count less than 5. The minimum expected count is 1.26.

RegionNum * CombinedNum

Crosstab

			CombinedNum		
			1.00	2.00	Total
RegionNum	1.00	Count	34	58	92
		Expected Count	23.8	68.2	92.0
	3.00	Count	38	162	200
		Expected Count	51.7	148.3	200.0
	4.00	Count	50	130	180
		Expected Count	46.5	133.5	180.0
Total		Count	122	350	472
		Expected Count	122.0	350.0	472.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.166[a]	2	.004
Likelihood Ratio	11.056	2	.004
Linear-by-Linear Association	2.735	1	.098
N of Valid Cases	472		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	11.166[a]	2	.004
Likelihood Ratio	11.056	2	.004
Linear-by-Linear Association	2.735	1	.098
N of Valid Cases	472		

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 23.78.

B 1.4 Seropositivity for phase II in possums and bandicoots

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Sexnum * PIInum	108	100.0%	0	.0%	108	100.0%
Agenum * PIInum	108	100.0%	0	.0%	108	100.0%
Lactnum * PIInum	108	100.0%	0	.0%	108	100.0%
Sitenum * PIInum	108	100.0%	0	.0%	108	100.0%
Locationnum * PIInum	108	100.0%	0	.0%	108	100.0%
Speciesnum * PIInum	108	100.0%	0	.0%	108	100.0%

Sexnum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Sexnum	1.00	Count	11	48	59
		Expected Count	12.0	47.0	59.0
	2.00	Count	10	31	41
		Expected Count	8.4	32.6	41.0
	3.00	Count	1	7	8
		Expected Count	1.6	6.4	8.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.822[a]	2	.663
Likelihood Ratio	.844	2	.656
Linear-by-Linear Association	.022	1	.883
N of Valid Cases	108		

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 1.63.

Risk Estimate

	Value
Odds Ratio for Sexnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Agenum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Agenum	1.00	Count	19	75	94
		Expected Count	19.1	74.9	94.0
	2.00	Count	3	11	14
		Expected Count	2.9	11.1	14.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.011[a]	1	.916		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.011	1	.917		
Fisher's Exact Test				1.000	.578
Linear-by-Linear Association	.011	1	.916		
N of Valid Cases	108				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.85.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Agenum (1.00 / 2.00)	.929	.236	3.664
For cohort PIInum = 1.00	.943	.320	2.779
For cohort PIInum = 2.00	1.015	.758	1.360
N of Valid Cases	108		

Lactnum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Lactnum	1.00	Count	1	5	6
		Expected Count	1.2	4.8	6.0
	2.00	Count	21	81	102
		Expected Count	20.8	81.2	102.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.054[a]	1	.817		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.056	1	.813		
Fisher's Exact Test				1.000	.646
Linear-by-Linear Association	.053	1	.818		
N of Valid Cases	108				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.22.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Lactnum (1.00 / 2.00)	.771	.085	6.962
For cohort PIInum = 1.00	.810	.130	5.043
For cohort PIInum = 2.00	1.049	.724	1.521
N of Valid Cases	108		

Sitenum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Sitenum	1.00	Count	9	35	44
		Expected Count	9.0	35.0	44.0
	2.00	Count	4	7	11
		Expected Count	2.2	8.8	11.0
	3.00	Count	0	2	2
		Expected Count	.4	1.6	2.0
	4.00	Count	1	7	8
		Expected Count	1.6	6.4	8.0
	5.00	Count	5	13	18
		Expected Count	3.7	14.3	18.0
	6.00	Count	1	5	6
		Expected Count	1.2	4.8	6.0
	7.00	Count	0	7	7
		Expected Count	1.4	5.6	7.0
	8.00	Count	1	8	9
		Expected Count	1.8	7.2	9.0
	9.00	Count	0	1	1
		Expected Count	.2	.8	1.0
	10.00	Count	1	1	2
		Expected Count	.4	1.6	2.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.816[a]	9	.656
Likelihood Ratio	8.425	9	.492
Linear-by-Linear Association	.439	1	.507
N of Valid Cases	108		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.816[a]	9	.656
Likelihood Ratio	8.425	9	.492
Linear-by-Linear Association	.439	1	.507
N of Valid Cases	108		

a. 13 cells (65.0%) have expected count less than 5. The minimum expected count is .20.

Risk Estimate

	Value
Odds Ratio for Sitenum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Locationnum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Locationnum	1.00	Count	21	76	97
		Expected Count	19.8	77.2	97.0
	2.00	Count	0	2	2
		Expected Count	.4	1.6	2.0
	3.00	Count	1	8	9
		Expected Count	1.8	7.2	9.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	1.085[a]	2	.581
Likelihood Ratio	1.555	2	.459
Linear-by-Linear Association	.766	1	.381
N of Valid Cases	108		

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .41.

Risk Estimate

	Value
Odds Ratio for Locationnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Speciesnum * PIInum

Crosstab

			PIInum		
			1.00	2.00	Total
Speciesnum	1.00	Count	12	40	52
		Expected Count	10.6	41.4	52.0
	2.00	Count	10	46	56
		Expected Count	11.4	44.6	56.0
Total		Count	22	86	108
		Expected Count	22.0	86.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.453[a]	1	.501		
Continuity Correction[b]	.188	1	.664		
Likelihood Ratio	.453	1	.501		
Fisher's Exact Test				.634	.332
Linear-by-Linear Association	.449	1	.503		
N of Valid Cases	108				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 10.59.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Speciesnum (1.00 / 2.00)	1.380	.539	3.533
For cohort PIInum = 1.00	1.292	.611	2.735
For cohort PIInum = 2.00	.936	.772	1.135
N of Valid Cases	108		

B 1.5 Seropositivity for phase I in possums and bandicoots

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Sexnum * PInum	108	100.0%	0	.0%	108	100.0%
Agenum * PInum	108	100.0%	0	.0%	108	100.0%
Lactnum * PInum	108	100.0%	0	.0%	108	100.0%
Sitenum * PInum	108	100.0%	0	.0%	108	100.0%
Locationnum * PInum	108	100.0%	0	.0%	108	100.0%
Speciesnum * PInum	108	100.0%	0	.0%	108	100.0%

Sexnum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Sexnum	1.00	Count	10	49	59
		Expected Count	9.3	49.7	59.0
	2.00	Count	6	35	41
		Expected Count	6.5	34.5	41.0
	3.00	Count	1	7	8
		Expected Count	1.3	6.7	8.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.166[a]	2	.920
Likelihood Ratio	.170	2	.919
Linear-by-Linear Association	.165	1	.685
N of Valid Cases	108		

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 1.26.

Risk Estimate

	Value
Odds Ratio for Sexnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Agenum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Agenum	1.00	Count	14	80	94
		Expected Count	14.8	79.2	94.0
	2.00	Count	3	11	14
		Expected Count	2.2	11.8	14.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.392[a]	1	.531		
Continuity Correction[b]	.054	1	.816		
Likelihood Ratio	.365	1	.546		
Fisher's Exact Test				.460	.383
Linear-by-Linear Association	.389	1	.533		
N of Valid Cases	108				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 2.20.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Agenum (1.00 / 2.00)	.642	.159	2.595
For cohort PInum = 1.00	.695	.228	2.116
For cohort PInum = 2.00	1.083	.813	1.442
N of Valid Cases	108		

Lactnum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Lactnum	1.00	Count	1	5	6
		Expected Count	.9	5.1	6.0
	2.00	Count	16	86	102
		Expected Count	16.1	85.9	102.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.004[a]	1	.949		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.004	1	.949		
Fisher's Exact Test				1.000	.652
Linear-by-Linear Association	.004	1	.949		
N of Valid Cases	108				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is .94.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Lactnum (1.00 / 2.00)	1.075	.118	9.822
For cohort PInum = 1.00	1.063	.168	6.723
For cohort PInum = 2.00	.988	.684	1.427
N of Valid Cases	108		

Sitenum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Sitenum	1.00	Count	7	37	44
		Expected Count	6.9	37.1	44.0
	2.00	Count	4	7	11
		Expected Count	1.7	9.3	11.0
	3.00	Count	0	2	2
		Expected Count	.3	1.7	2.0
	4.00	Count	1	7	8
		Expected Count	1.3	6.7	8.0
	5.00	Count	2	16	18
		Expected Count	2.8	15.2	18.0
	6.00	Count	1	5	6
		Expected Count	.9	5.1	6.0
	7.00	Count	0	7	7
		Expected Count	1.1	5.9	7.0
	8.00	Count	1	8	9
		Expected Count	1.4	7.6	9.0
	9.00	Count	0	1	1
		Expected Count	.2	.8	1.0
	10.00	Count	1	1	2
		Expected Count	.3	1.7	2.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.670[a]	9	.568
Likelihood Ratio	8.011	9	.533
Linear-by-Linear Association	.455	1	.500
N of Valid Cases	108		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	7.670[a]	9	.568
Likelihood Ratio	8.011	9	.533
Linear-by-Linear Association	.455	1	.500
N of Valid Cases	108		

a. 12 cells (60.0%) have expected count less than 5. The minimum expected count is .16.

Risk Estimate

	Value
Odds Ratio for Sitenum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Locationnum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Locationnum	1.00	Count	16	81	97
		Expected Count	15.3	81.7	97.0
	2.00	Count	0	2	2
		Expected Count	.3	1.7	2.0
	3.00	Count	1	8	9
		Expected Count	1.4	7.6	9.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.561[a]	2	.756
Likelihood Ratio	.885	2	.642
Linear-by-Linear Association	.287	1	.592
N of Valid Cases	108		

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .31.

Risk Estimate

	Value
Odds Ratio for Locationnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Speciesnum * PInum

Crosstab

			PInum		
			1.00	2.00	Total
Speciesnum	1.00	Count	11	41	52
		Expected Count	8.2	43.8	52.0
	2.00	Count	6	50	56
		Expected Count	8.8	47.2	56.0
Total		Count	17	91	108
		Expected Count	17.0	91.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	2.216[a]	1	.137		
Continuity Correction[b]	1.498	1	.221		
Likelihood Ratio	2.236	1	.135		
Fisher's Exact Test				.187	.110
Linear-by-Linear Association	2.195	1	.138		
N of Valid Cases	108				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 8.19.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Speciesnum (1.00 / 2.00)	2.236	.762	6.564
For cohort PInum = 1.00	1.974	.787	4.956
For cohort PInum = 2.00	.883	.747	1.044
N of Valid Cases	108		

B 1.6 Seropositivity for either or both phase II and phase I in possums and bandicoots

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
Sexnum * Combnum	108	100.0%	0	.0%	108	100.0%
Agenum * Combnum	108	100.0%	0	.0%	108	100.0%
Lactnum * Combnum	108	100.0%	0	.0%	108	100.0%
Sitenum * Combnum	108	100.0%	0	.0%	108	100.0%
Locationnum * Combnum	108	100.0%	0	.0%	108	100.0%
Speciesnum * Combnum	108	100.0%	0	.0%	108	100.0%

Sexnum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Sexnum	1.00	Count	13	46	59
		Expected Count	13.7	45.3	59.0
	2.00	Count	10	31	41
		Expected Count	9.5	31.5	41.0
	3.00	Count	2	6	8
		Expected Count	1.9	6.1	8.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.092[a]	2	.955
Likelihood Ratio	.092	2	.955
Linear-by-Linear Association	.084	1	.772
N of Valid Cases	108		

a. 1 cells (16.7%) have expected count less than 5. The minimum expected count is 1.85.

Risk Estimate

	Value
Odds Ratio for Sexnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Agenum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Agenum	1.00	Count	22	72	94
		Expected Count	21.8	72.2	94.0
	2.00	Count	3	11	14
		Expected Count	3.2	10.8	14.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.027[a]	1	.870		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.027	1	.869		
Fisher's Exact Test				1.000	.587
Linear-by-Linear Association	.026	1	.871		
N of Valid Cases	108				

a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.24.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Agenum (1.00 / 2.00)	1.120	.287	4.378
For cohort Combnum = 1.00	1.092	.376	3.177
For cohort Combnum = 2.00	.975	.725	1.310
N of Valid Cases	108		

Lactnum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Lactnum	1.00	Count	1	5	6
		Expected Count	1.4	4.6	6.0
	2.00	Count	24	78	102
		Expected Count	23.6	78.4	102.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.150[a]	1	.699		
Continuity Correction[b]	.000	1	1.000		
Likelihood Ratio	.161	1	.688		
Fisher's Exact Test				1.000	.577
Linear-by-Linear Association	.149	1	.700		
N of Valid Cases	108				

a. 2 cells (50.0%) have expected count less than 5. The minimum expected count is 1.39.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Lactnum (1.00 / 2.00)	.650	.072	5.838
For cohort Combnum = 1.00	.708	.114	4.385
For cohort Combnum = 2.00	1.090	.750	1.583
N of Valid Cases	108		

Sitenum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Sitenum	1.00	Count	11	33	44
		Expected Count	10.2	33.8	44.0
	2.00	Count	4	7	11
		Expected Count	2.5	8.5	11.0
	3.00	Count	0	2	2
		Expected Count	.5	1.5	2.0
	4.00	Count	2	6	8
		Expected Count	1.9	6.1	8.0
	5.00	Count	5	13	18
		Expected Count	4.2	13.8	18.0
	6.00	Count	1	5	6
		Expected Count	1.4	4.6	6.0
	7.00	Count	0	7	7
		Expected Count	1.6	5.4	7.0
	8.00	Count	1	8	9
		Expected Count	2.1	6.9	9.0
	9.00	Count	0	1	1
		Expected Count	.2	.8	1.0
	10.00	Count	1	1	2
		Expected Count	.5	1.5	2.0
Total		Count	25	83	108

Crosstab

			Combnum		
			1.00	2.00	Total
Sitenum	1.00	Count	11	33	44
		Expected Count	10.2	33.8	44.0
	2.00	Count	4	7	11
		Expected Count	2.5	8.5	11.0
	3.00	Count	0	2	2
		Expected Count	.5	1.5	2.0
	4.00	Count	2	6	8
		Expected Count	1.9	6.1	8.0
	5.00	Count	5	13	18
		Expected Count	4.2	13.8	18.0
	6.00	Count	1	5	6
		Expected Count	1.4	4.6	6.0
	7.00	Count	0	7	7
		Expected Count	1.6	5.4	7.0
	8.00	Count	1	8	9
		Expected Count	2.1	6.9	9.0
	9.00	Count	0	1	1
		Expected Count	.2	.8	1.0
	10.00	Count	1	1	2
		Expected Count	.5	1.5	2.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.094[a]	9	.730
Likelihood Ratio	8.237	9	.510
Linear-by-Linear Association	1.045	1	.307
N of Valid Cases	108		

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	6.094[a]	9	.730
Likelihood Ratio	8.237	9	.510
Linear-by-Linear Association	1.045	1	.307
N of Valid Cases	108		

a. 13 cells (65.0%) have expected count less than 5. The minimum expected count is .23.

Risk Estimate

	Value
Odds Ratio for Sitenum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Locationnum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Locationnum	1.00	Count	23	74	97
		Expected Count	22.5	74.5	97.0
	2.00	Count	0	2	2
		Expected Count	.5	1.5	2.0
	3.00	Count	2	7	9
		Expected Count	2.1	6.9	9.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.624[a]	2	.732
Likelihood Ratio	1.075	2	.584
Linear-by-Linear Association	.064	1	.800
N of Valid Cases	108		

a. 3 cells (50.0%) have expected count less than 5. The minimum expected count is .46.

Risk Estimate

	Value
Odds Ratio for Locationnum (1.00 / 2.00)	[a]

a. Risk Estimate statistics cannot be computed. They are only computed for a 2*2 table without empty cells.

Speciesnum * Combnum

Crosstab

			Combnum		
			1.00	2.00	Total
Speciesnum	1.00	Count	14	38	52
		Expected Count	12.0	40.0	52.0
	2.00	Count	11	45	56
		Expected Count	13.0	43.0	56.0
Total		Count	25	83	108
		Expected Count	25.0	83.0	108.0

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	.803[a]	1	.370		
Continuity Correction[b]	.446	1	.504		
Likelihood Ratio	.804	1	.370		
Fisher's Exact Test				.494	.252
Linear-by-Linear Association	.796	1	.372		
N of Valid Cases	108				

a. 0 cells (.0%) have expected count less than 5. The minimum expected count is 12.04.

b. Computed only for a 2x2 table

Risk Estimate

	Value	95% Confidence Interval	
		Lower	Upper
Odds Ratio for Speciesnum (1.00 / 2.00)	1.507	.613	3.707
For cohort Combnum = 1.00	1.371	.685	2.743
For cohort Combnum = 2.00	.909	.737	1.122
N of Valid Cases	108		

Generalized Linear Models

B 2.1 Generalised linear model for phase II seropositivity in macropods

Model Information

Dependent Variable	PhaseIINum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	436	87.2%
Excluded	64	12.8%
Total	500	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	PhaseIINum	1.00	90	20.6%
		2.00	346	79.4%
		Total	436	100.0%
Factor	SexNum	1.00	227	52.1%
		2.00	209	47.9%
		Total	436	100.0%
	AgeNum	1.00	394	90.4%
		2.00	42	9.6%
		Total	436	100.0%
	SiteNum	1.00	33	7.6%
		2.00	1	.2%
		5.00	13	3.0%
		6.00	17	3.9%
		7.00	97	22.2%
		8.00	34	7.8%
		9.00	17	3.9%
		11.00	32	7.3%

		12.00	103	23.6%
		14.00	89	20.4%
		Total	436	100.0%
	SpeciesNum	1.00	139	31.9%
		4.00	59	13.5%
		5.00	23	5.3%
		6.00	35	8.0%
		7.00	180	41.3%
		Total	436	100.0%
	RegionNum	1.00	63	14.4%
		2.00	1	.2%
		3.00	192	44.0%
		4.00	180	41.3%
		Total	436	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	39.102	31	1.261
Scaled Deviance	39.102	31	
Pearson Chi-Square	33.589	31	1.084
Scaled Pearson Chi-Square	33.589	31	
Log Likelihood[a]	-48.722		
Akaike's Information Criterion (AIC)	127.443		
Finite Sample Corrected AIC (AICC)	128.586		
Bayesian Information Criterion (BIC)	188.608		
Consistent AIC (CAIC)	203.608		

Dependent Variable: PhaseIINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
41.689	14	.000

Dependent Variable: PhaseIINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
SexNum	3.236	1	.072
AgeNum	1.209	1	.272
SiteNum	21.963	8	.005
SpeciesNum	2.986	3	.394

Dependent Variable: PhaseIINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [PhaseIINum=1.00]	-2.924	.8841	-4.657	-1.191
[SexNum=1.00]	-.477	.2651	-.996	.043
[SexNum=2.00]	0[a]	.	.	.
[AgeNum=1.00]	-.506	.4598	-1.407	.396
[AgeNum=2.00]	0[a]	.	.	.
[SiteNum=1.00]	-.561	.7110	-1.954	.833
[SiteNum=2.00]	20.282	52051.5720	-101998.925	102039.488
[SiteNum=5.00]	-1.757	.7648	-3.256	-.258
[SiteNum=6.00]	-1.317	.7875	-2.860	.227
[SiteNum=7.00]	-1.165	.7343	-2.604	.275
[SiteNum=8.00]	.448	1.0160	-1.543	2.439
[SiteNum=9.00]	19.395	12447.7117	-24377.671	24416.462
[SiteNum=11.00]	-1.713	.7908	-3.263	-.163

[SiteNum=12.00]	.414	.6696	-.898	1.726
[SiteNum=14.00]	0[a]	.	.	.
[SpeciesNum=1.00]	-.980	.6109	-2.177	.218
[SpeciesNum=4.00]	-.223	.8048	-1.800	1.354
[SpeciesNum=5.00]	-.286	.7837	-1.822	1.250
[SpeciesNum=6.00]	0[a]	.	.	.
[SpeciesNum=7.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [PhaseIINum=1.00]	10.939	1	.001
[SexNum=1.00]	3.236	1	.072
[SexNum=2.00]	.	.	.
[AgeNum=1.00]	1.209	1	.272
[AgeNum=2.00]	.	.	.
[SiteNum=1.00]	.622	1	.430
[SiteNum=2.00]	.000	1	1.000
[SiteNum=5.00]	5.279	1	.022
[SiteNum=6.00]	2.796	1	.095
[SiteNum=7.00]	2.516	1	.113
[SiteNum=8.00]	.194	1	.659
[SiteNum=9.00]	.000	1	.999
[SiteNum=11.00]	4.693	1	.030
[SiteNum=12.00]	.382	1	.536
[SiteNum=14.00]	.	.	.
[SpeciesNum=1.00]	2.573	1	.109
[SpeciesNum=4.00]	.077	1	.782
[SpeciesNum=5.00]	.133	1	.715
[SpeciesNum=6.00]	.	.	.
[SpeciesNum=7.00]	.	.	.
(Scale)			

Dependent Variable: PhaseIINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [PhaseIINum=1.00]	-2.924	.8841	-4.657	-1.191
[SexNum=1.00]	-.477	.2651	-.996	.043
[SexNum=2.00]	0[a]	.	.	.
[AgeNum=1.00]	-.506	.4598	-1.407	.396
[AgeNum=2.00]	0[a]	.	.	.
[SiteNum=1.00]	-.561	.7110	-1.954	.833
[SiteNum=2.00]	20.282	52051.5720	-101998.925	102039.488
[SiteNum=5.00]	-1.757	.7648	-3.256	-.258
[SiteNum=6.00]	-1.317	.7875	-2.860	.227
[SiteNum=7.00]	-1.165	.7343	-2.604	.275
[SiteNum=8.00]	.448	1.0160	-1.543	2.439
[SiteNum=9.00]	19.395	12447.7117	-24377.671	24416.462
[SiteNum=11.00]	-1.713	.7908	-3.263	-.163
[SiteNum=12.00]	.414	.6696	-.898	1.726
[SiteNum=14.00]	0[a]	.	.	.
[SpeciesNum=1.00]	-.980	.6109	-2.177	.218
[SpeciesNum=4.00]	-.223	.8048	-1.800	1.354
[SpeciesNum=5.00]	-.286	.7837	-1.822	1.250
[SpeciesNum=6.00]	0[a]	.	.	.
[SpeciesNum=7.00]	0[a]	.	.	.

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

B 2.2 Generalised linear model for phase I seropositivity in macropods

Model Information

Dependent Variable	PhaseINum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	436	87.2%
Excluded	64	12.8%
Total	500	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	PhaseINum	1.00	70	16.1%
		2.00	366	83.9%
		Total	436	100.0%
Factor	SexNum	1.00	227	52.1%
		2.00	209	47.9%
		Total	436	100.0%
	AgeNum	1.00	394	90.4%
		2.00	42	9.6%
		Total	436	100.0%
	SiteNum	1.00	33	7.6%
		2.00	1	.2%
		5.00	13	3.0%
		6.00	17	3.9%
		7.00	97	22.2%
		8.00	34	7.8%
		9.00	17	3.9%
		11.00	32	7.3%
		12.00	103	23.6%
		14.00	89	20.4%
		Total	436	100.0%

	SpeciesNum	1.00	139	31.9%
		4.00	59	13.5%
		5.00	23	5.3%
		6.00	35	8.0%
		7.00	180	41.3%
		Total	436	100.0%
	RegionNum	1.00	63	14.4%
		2.00	1	.2%
		3.00	192	44.0%
		4.00	180	41.3%
		Total	436	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	35.620	31	1.149
Scaled Deviance	35.620	31	
Pearson Chi-Square	30.419	31	.981
Scaled Pearson Chi-Square	30.419	31	
Log Likelihood[a]	-45.857		
Akaike's Information Criterion (AIC)	121.714		
Finite Sample Corrected AIC (AICC)	122.857		
Bayesian Information Criterion (BIC)	182.879		
Consistent AIC (CAIC)	197.879		

Dependent Variable: PhaseINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
41.244	14	.000

Dependent Variable: PhaseINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
SexNum	.085	1	.770
AgeNum	.331	1	.565
SiteNum	28.034	8	.000
SpeciesNum	3.330	3	.343

Dependent Variable: PhaseINum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [PhaseINum=1.00]	-3.236	1.0152	-5.226	-1.246
[SexNum=1.00]	-.086	.2937	-.661	.490
[SexNum=2.00]	0[a]	.	.	.
[AgeNum=1.00]	-.290	.5041	-1.278	.698
[AgeNum=2.00]	0[a]	.	.	.
[SiteNum=1.00]	-1.561	.8020	-3.133	.011
[SiteNum=2.00]	19.883	57963.7609	-113587.000	113626.767
[SiteNum=5.00]	-1.949	.8354	-3.586	-.311
[SiteNum=6.00]	-2.725	.8935	-4.476	-.974
[SiteNum=7.00]	-.981	.8772	-2.700	.738
[SiteNum=8.00]	-1.218	.9639	-3.107	.671
[SiteNum=9.00]	18.959	14023.7686	-27467.123	27505.040
[SiteNum=11.00]	-1.265	.9567	-3.141	.610
[SiteNum=12.00]	.346	.8181	-1.257	1.949
[SiteNum=14.00]	0[a]	.	.	.
[SpeciesNum=1.00]	-.894	.6053	-2.081	.292
[SpeciesNum=4.00]	-1.442	.8882	-3.183	.299
[SpeciesNum=5.00]	-.878	.8001	-2.446	.690

[SpeciesNum=6.00]	0[a]	.	.	.
[SpeciesNum=7.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [PhaseINum=1.00]	10.162	1	.001
[SexNum=1.00]	.085	1	.770
[SexNum=2.00]	.	.	.
[AgeNum=1.00]	.331	1	.565
[AgeNum=2.00]	.	.	.
[SiteNum=1.00]	3.789	1	.052
[SiteNum=2.00]	.000	1	1.000
[SiteNum=5.00]	5.442	1	.020
[SiteNum=6.00]	9.303	1	.002
[SiteNum=7.00]	1.251	1	.263
[SiteNum=8.00]	1.596	1	.206
[SiteNum=9.00]	.000	1	.999
[SiteNum=11.00]	1.750	1	.186
[SiteNum=12.00]	.179	1	.672
[SiteNum=14.00]	.	.	.
[SpeciesNum=1.00]	2.183	1	.140
[SpeciesNum=4.00]	2.635	1	.105
[SpeciesNum=5.00]	1.205	1	.272
[SpeciesNum=6.00]	.	.	.
[SpeciesNum=7.00]	.	.	.
(Scale)			

B 2.3 Generalised linear model for either or both phase II and phase I seropositivity in macropods

Model Information

Dependent Variable	CombinedNum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	436	87.2%
Excluded	64	12.8%
Total	500	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	CombinedNum	1.00	115	26.4%
		2.00	321	73.6%
		Total	436	100.0%
Factor	SexNum	1.00	227	52.1%
		2.00	209	47.9%
		Total	436	100.0%
	AgeNum	1.00	394	90.4%
		2.00	42	9.6%
		Total	436	100.0%
	SiteNum	1.00	33	7.6%
		2.00	1	.2%
		5.00	13	3.0%
		6.00	17	3.9%
		7.00	97	22.2%
		8.00	34	7.8%
		9.00	17	3.9%
		11.00	32	7.3%
		12.00	103	23.6%

		14.00	89	20.4%
		Total	436	100.0%
	SpeciesNum	1.00	139	31.9%
		4.00	59	13.5%
		5.00	23	5.3%
		6.00	35	8.0%
		7.00	180	41.3%
		Total	436	100.0%
	RegionNum	1.00	63	14.4%
		2.00	1	.2%
		3.00	192	44.0%
		4.00	180	41.3%
		Total	436	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	33.964	31	1.096
Scaled Deviance	33.964	31	
Pearson Chi-Square	28.148	31	.908
Scaled Pearson Chi-Square	28.148	31	
Log Likelihood[a]	-49.205		
Akaike's Information Criterion (AIC)	128.409		
Finite Sample Corrected AIC (AICC)	129.552		
Bayesian Information Criterion (BIC)	189.574		
Consistent AIC (CAIC)	204.574		

Dependent Variable: CombinedNum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
44.257	14	.000

Dependent Variable: CombinedNum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
SexNum	3.009	1	.083
AgeNum	.132	1	.716
SiteNum	25.901	8	.001
SpeciesNum	4.205	3	.240

Dependent Variable: CombinedNum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [CombinedNum=1.00]	-2.622	.8365	-4.261	-.982
[SexNum=1.00]	-.422	.2431	-.898	.055
[SexNum=2.00]	0[a]	.	.	.
[AgeNum=1.00]	-.146	.4010	-.932	.640
[AgeNum=2.00]	0[a]	.	.	.
[SiteNum=1.00]	-1.058	.6860	-2.403	.286
[SiteNum=2.00]	20.153	47219.7688	-92528.893	92569.199
[SiteNum=5.00]	-2.009	.7717	-3.522	-.497
[SiteNum=6.00]	-1.981	.7855	-3.520	-.441
[SiteNum=7.00]	-1.512	.7196	-2.922	-.101
[SiteNum=8.00]	-.787	.8291	-2.412	.838
[SiteNum=9.00]	19.199	11383.1557	-22291.376	22329.774
[SiteNum=11.00]	-1.861	.7771	-3.385	-.338
[SiteNum=12.00]	.314	.6599	-.979	1.607
[SiteNum=14.00]	0[a]	.	.	.
[SpeciesNum=1.00]	-1.104	.5749	-2.231	.023
[SpeciesNum=4.00]	-.949	.7587	-2.436	.538

[SpeciesNum=5.00]	-.422	.7533	-1.898	1.055
[SpeciesNum=6.00]	0[a]	.	.	.
[SpeciesNum=7.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [CombinedNum=1.00]	9.825	1	.002
[SexNum=1.00]	3.009	1	.083
[SexNum=2.00]	.	.	.
[AgeNum=1.00]	.132	1	.716
[AgeNum=2.00]	.	.	.
[SiteNum=1.00]	2.381	1	.123
[SiteNum=2.00]	.000	1	1.000
[SiteNum=5.00]	6.781	1	.009
[SiteNum=6.00]	6.359	1	.012
[SiteNum=7.00]	4.414	1	.036
[SiteNum=8.00]	.900	1	.343
[SiteNum=9.00]	.000	1	.999
[SiteNum=11.00]	5.738	1	.017
[SiteNum=12.00]	.226	1	.634
[SiteNum=14.00]	.	.	.
[SpeciesNum=1.00]	3.689	1	.055
[SpeciesNum=4.00]	1.563	1	.211
[SpeciesNum=5.00]	.313	1	.576
[SpeciesNum=6.00]	.	.	.
[SpeciesNum=7.00]	.	.	.
(Scale)			

Dependent Variable: CombinedNum

Model: (Threshold), SexNum, AgeNum, SiteNum, SpeciesNum

a. Set to zero because this parameter is redundant.

b. Fixed at the displayed value.

B 2.4 Generalised linear model for phase II seropositivity in possums and bandicoots

Model Information

Dependent Variable	PIInum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	108	100.0%
Excluded	0	.0%
Total	108	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	PIInum	1.00	22	20.4%
		2.00	86	79.6%
		Total	108	100.0%
Factor	Sexnum	1.00	59	54.6%
		2.00	41	38.0%
		3.00	8	7.4%
		Total	108	100.0%
	Agenum	1.00	94	87.0%
		2.00	14	13.0%
		Total	108	100.0%
	Lactnum	1.00	6	5.6%
		2.00	102	94.4%
		Total	108	100.0%
	Sitenum	1.00	44	40.7%
		2.00	11	10.2%
		3.00	2	1.9%
		4.00	8	7.4%
		5.00	18	16.7%

		6.00	6	5.6%
		7.00	7	6.5%
		8.00	9	8.3%
		9.00	1	.9%
		10.00	2	1.9%
		Total	108	100.0%
	Locationnum	1.00	97	89.8%
		2.00	2	1.9%
		3.00	9	8.3%
		Total	108	100.0%
	Speciesnum	1.00	52	48.1%
		2.00	56	51.9%
		Total	108	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	19.128	18	1.063
Scaled Deviance	19.128	18	
Pearson Chi-Square	17.162	18	.953
Scaled Pearson Chi-Square	17.162	18	
Log Likelihood[a]	-20.293		
Akaike's Information Criterion (AIC)	68.586		
Finite Sample Corrected AIC (AICC)	73.102		
Bayesian Information Criterion (BIC)	106.136		
Consistent AIC (CAIC)	120.136		

Dependent Variable: PIInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
9.803	13	.710

Dependent Variable: PIInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
Sexnum	1.170	1	.279
Agenum	.003	1	.957
Lactnum	.515	1	.473
Sitenum	3.536	8	.896
Speciesnum	.000	1	.994

Dependent Variable: PIInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [PIInum=1.00]	-1.987	1.3131	-4.560	.587
[Sexnum=1.00]	-1.666	1.8474	-5.287	1.954
[Sexnum=2.00]	-2.266	1.8494	-5.891	1.359
[Sexnum=3.00]	0[a]	.	.	.
[Agenum=1.00]	-.041	.7626	-1.535	1.454
[Agenum=2.00]	0[a]	.	.	.
[Lactnum=1.00]	.898	1.2515	-1.555	3.351
[Lactnum=2.00]	0[a]	.	.	.
[Sitenum=1.00]	1.326	1.8706	-2.341	4.992
[Sitenum=2.00]	.334	2.0586	-3.700	4.369
[Sitenum=3.00]	21.757	36477.8650	-71473.545	71517.059
[Sitenum=4.00]	0[a]	.	.	.
[Sitenum=5.00]	.813	1.5473	-2.219	3.846

[Sitenum=6.00]	1.509	1.8266	-2.071	5.089
[Sitenum=7.00]	21.632	19564.4042	-38323.896	38367.159
[Sitenum=8.00]	2.138	1.8092	-1.408	5.684
[Sitenum=9.00]	21.452	52339.2085	-102561.512	102604.415
[Sitenum=10.00]	0[a]	.	.	.
[Speciesnum=1.00]	.010	1.2121	-2.366	2.385
[Speciesnum=2.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [PIInum=1.00]	2.289	1	.130
[Sexnum=1.00]	.814	1	.367
[Sexnum=2.00]	1.501	1	.220
[Sexnum=3.00]	.	.	.
[Agenum=1.00]	.003	1	.957
[Agenum=2.00]	.	.	.
[Lactnum=1.00]	.515	1	.473
[Lactnum=2.00]	.	.	.
[Sitenum=1.00]	.502	1	.479
[Sitenum=2.00]	.026	1	.871
[Sitenum=3.00]	.000	1	1.000
[Sitenum=4.00]	.	.	.
[Sitenum=5.00]	.276	1	.599
[Sitenum=6.00]	.682	1	.409
[Sitenum=7.00]	.000	1	.999
[Sitenum=8.00]	1.397	1	.237
[Sitenum=9.00]	.000	1	1.000
[Sitenum=10.00]	.	.	.
[Speciesnum=1.00]	.000	1	.994
[Speciesnum=2.00]	.	.	.
(Scale)			

Dependent Variable: PIInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

B 2.5 Generalised linear model for phase I seropositivity in possums and bandicoots

Model Information

Dependent Variable	PInum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	108	100.0%
Excluded	0	.0%
Total	108	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	PInum	1.00	17	15.7%
		2.00	91	84.3%
		Total	108	100.0%
Factor	Sexnum	1.00	59	54.6%
		2.00	41	38.0%
		3.00	8	7.4%
		Total	108	100.0%
	Agenum	1.00	94	87.0%
		2.00	14	13.0%
		Total	108	100.0%
	Lactnum	1.00	6	5.6%
		2.00	102	94.4%
		Total	108	100.0%
	Sitenum	1.00	44	40.7%
		2.00	11	10.2%
		3.00	2	1.9%
		4.00	8	7.4%
		5.00	18	16.7%

		6.00	6	5.6%
		7.00	7	6.5%
		8.00	9	8.3%
		9.00	1	.9%
		10.00	2	1.9%
		Total	108	100.0%
	Locationnum	1.00	97	89.8%
		2.00	2	1.9%
		3.00	9	8.3%
		Total	108	100.0%
	Speciesnum	1.00	52	48.1%
		2.00	56	51.9%
		Total	108	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	16.328	18	.907
Scaled Deviance	16.328	18	
Pearson Chi-Square	15.361	18	.853
Scaled Pearson Chi-Square	15.361	18	
Log Likelihood[a]	-16.975		
Akaike's Information Criterion (AIC)	61.950		
Finite Sample Corrected AIC (AICC)	66.466		
Bayesian Information Criterion (BIC)	99.499		
Consistent AIC (CAIC)	113.499		

Dependent Variable: PInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
10.402	13	.661

Dependent Variable: PInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

	Type III		
Source	Wald Chi-Square	df	Sig.
Sexnum	.096	1	.757
Agenum	.416	1	.519
Lactnum	.004	1	.948
Sitenum	3.421	8	.905
Speciesnum	.000	1	.999

Dependent Variable: PInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

Parameter Estimates

			95% Wald Confidence Interval	
Parameter	B	Std. Error	Lower	Upper
Threshold [PInum=1.00]	-1.434	1.3316	-4.044	1.176
[Sexnum=1.00]	-1.788	1.8608	-5.435	1.859
[Sexnum=2.00]	-1.592	1.8620	-5.241	2.058
[Sexnum=3.00]	0[a]	.	.	.
[Agenum=1.00]	.512	.7940	-1.044	2.068
[Agenum=2.00]	0[a]	.	.	.
[Lactnum=1.00]	.085	1.3178	-2.497	2.668
[Lactnum=2.00]	0[a]	.	.	.
[Sitenum=1.00]	21.661	26077.3850	-51089.074	51132.396
[Sitenum=2.00]	20.627	26077.3850	-51090.109	51131.362
[Sitenum=3.00]	42.007	48814.8389	-95633.319	95717.333
[Sitenum=4.00]	0[a]	.	.	.
[Sitenum=5.00]	2.012	1.6407	-1.203	5.228

[Sitenum=6.00]	1.436	1.8311	-2.152	5.025
[Sitenum=7.00]	21.814	22015.4007	-43127.578	43171.207
[Sitenum=8.00]	1.931	1.7974	-1.592	5.454
[Sitenum=9.00]	21.795	58470.4884	-114578.256	114621.846
[Sitenum=10.00]	0[a]	.	.	.
[Speciesnum=1.00]	-20.310	26077.3849	-51131.045	51090.426
[Speciesnum=2.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [PInum=1.00]	1.160	1	.282
[Sexnum=1.00]	.924	1	.337
[Sexnum=2.00]	.731	1	.393
[Sexnum=3.00]	.	.	.
[Agenum=1.00]	.416	1	.519
[Agenum=2.00]	.	.	.
[Lactnum=1.00]	.004	1	.948
[Lactnum=2.00]	.	.	.
[Sitenum=1.00]	.000	1	.999
[Sitenum=2.00]	.000	1	.999
[Sitenum=3.00]	.000	1	.999
[Sitenum=4.00]	.	.	.
[Sitenum=5.00]	1.504	1	.220
[Sitenum=6.00]	.615	1	.433
[Sitenum=7.00]	.000	1	.999
[Sitenum=8.00]	1.154	1	.283
[Sitenum=9.00]	.000	1	1.000
[Sitenum=10.00]	.	.	.
[Speciesnum=1.00]	.000	1	.999
[Speciesnum=2.00]	.	.	.
(Scale)			

Dependent Variable: PInum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

B 2.5 Generalised linear model for either or both phase II and phase I seropositivity in possums and bandicoots

Model Information

Dependent Variable	Combnum[a]
Probability Distribution	Multinomial
Link Function	Cumulative logit

a. The procedure applies the cumulative link function to the dependent variable values in ascending order.

Case Processing Summary

	N	Percent
Included	108	100.0%
Excluded	0	.0%
Total	108	100.0%

Categorical Variable Information

			N	Percent
Dependent Variable	Combnum	1.00	25	23.1%
		2.00	83	76.9%
		Total	108	100.0%
Factor	Sexnum	1.00	59	54.6%
		2.00	41	38.0%
		3.00	8	7.4%
		Total	108	100.0%
	Agenum	1.00	94	87.0%
		2.00	14	13.0%
		Total	108	100.0%
	Lactnum	1.00	6	5.6%
		2.00	102	94.4%
		Total	108	100.0%
	Sitenum	1.00	44	40.7%
		2.00	11	10.2%
		3.00	2	1.9%
		4.00	8	7.4%
		5.00	18	16.7%
		6.00	6	5.6%

		7.00	7	6.5%
		8.00	9	8.3%
		9.00	1	.9%
		10.00	2	1.9%
		Total	108	100.0%
	Locationnum	1.00	97	89.8%
		2.00	2	1.9%
		3.00	9	8.3%
		Total	108	100.0%
	Speciesnum	1.00	52	48.1%
		2.00	56	51.9%
		Total	108	100.0%

Goodness of Fit[b]

	Value	df	Value/df
Deviance	19.080	18	1.060
Scaled Deviance	19.080	18	
Pearson Chi-Square	16.820	18	.934
Scaled Pearson Chi-Square	16.820	18	
Log Likelihood[a]	-20.683		
Akaike's Information Criterion (AIC)	69.366		
Finite Sample Corrected AIC (AICC)	73.882		
Bayesian Information Criterion (BIC)	106.916		
Consistent AIC (CAIC)	120.916		

Dependent Variable: Combnum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. The full log likelihood function is displayed and used in computing information criteria.

b. Information criteria are in small-is-better form.

Omnibus Test[a]

Likelihood Ratio Chi-Square	df	Sig.
8.980	13	.774

Dependent Variable: Combnum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

a. Compares the fitted model against the thresholds-only model.

Tests of Model Effects

Source	Type III		
	Wald Chi-Square	df	Sig.
Sexnum	.357	1	.550
Agenum	.058	1	.809
Lactnum	.405	1	.524
Sitenum	2.498	8	.962
Speciesnum	.053	1	.817

Dependent Variable: Combnum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

Parameter Estimates

Parameter	B	Std. Error	95% Wald Confidence Interval	
			Lower	Upper
Threshold [Combnum=1.00]	-1.280	1.1111	-3.458	.897
[Sexnum=1.00]	-1.030	1.7051	-4.372	2.312
[Sexnum=2.00]	-1.349	1.7075	-4.696	1.998
[Sexnum=3.00]	0[a]	.	.	.
[Agenum=1.00]	-.182	.7535	-1.659	1.295
[Agenum=2.00]	0[a]	.	.	.
[Lactnum=1.00]	.788	1.2389	-1.640	3.217
[Lactnum=2.00]	0[a]	.	.	.
[Sitenum=1.00]	1.376	1.8606	-2.271	5.023
[Sitenum=2.00]	.748	2.0384	-3.247	4.743
[Sitenum=3.00]	21.999	34993.3276	-68563.663	68607.661
[Sitenum=4.00]	0[a]	.	.	.

[Sitenum=5.00]	.922	1.5412	-2.098	3.943
[Sitenum=6.00]	1.557	1.8209	-2.012	5.126
[Sitenum=7.00]	21.626	18739.1397	-36706.413	36749.665
[Sitenum=8.00]	2.126	1.7997	-1.402	5.653
[Sitenum=9.00]	21.558	49677.3573	-97344.273	97387.389
[Sitenum=10.00]	0[a]	.	.	.
[Speciesnum=1.00]	-.276	1.1961	-2.621	2.068
[Speciesnum=2.00]	0[a]	.	.	.
(Scale)	1[b]			

Parameter Estimates

	Hypothesis Test		
Parameter	Wald Chi-Square	df	Sig.
Threshold [Combnum=1.00]	1.328	1	.249
[Sexnum=1.00]	.365	1	.546
[Sexnum=2.00]	.624	1	.429
[Sexnum=3.00]	.	.	.
[Agenum=1.00]	.058	1	.809
[Agenum=2.00]	.	.	.
[Lactnum=1.00]	.405	1	.524
[Lactnum=2.00]	.	.	.
[Sitenum=1.00]	.547	1	.460
[Sitenum=2.00]	.135	1	.714
[Sitenum=3.00]	.000	1	.999
[Sitenum=4.00]	.	.	.
[Sitenum=5.00]	.358	1	.550
[Sitenum=6.00]	.731	1	.392
[Sitenum=7.00]	.000	1	.999
[Sitenum=8.00]	1.395	1	.238
[Sitenum=9.00]	.000	1	1.000
[Sitenum=10.00]	.	.	.
[Speciesnum=1.00]	.053	1	.817
[Speciesnum=2.00]	.	.	.
(Scale)			

Dependent Variable: Combnum

Model: (Threshold), Sexnum, Agenum, Lactnum, Sitenum, Speciesnum

APPENDIX C

Publications

C 1.1 Cooper A., Hedlefs R., McGowan M., Ketheesan N., Govan B (2011) Serological evidence of *Coxiella burnetii* infection in beef cattle in Queensland, *Australian Veterinary Journal*; 89(7): 260-264.

C 1.2 Cooper A., Ketheesan N., Govan B (2011) Serological evidence of *Coxiella burnetii* infection in dogs in a regional centre, *Australian Veterinary Journal*; (accepted).

C 1.3 Cooper A., Goullet M., Mitchell J., Ketheesan N., Govan B. (2011) Serological evidence of Coxiella burnetii exposure in native marsupials and introduced animals in Queensland, Australia, Epidemiology and Infection; (accepted).

C 1.4 Cooper A., Barnes T., Potter A., Ketheesan N., Govan B. (2011) Determination of Coxiella burnetii seroprevalence in macropods in Australia, Veterinary Microbiology; (accepted).