Epidemiology, Diagnosis and Prevention of Q fever in Queensland

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Abstract

Coxiella burnetii is the causative agent of the zoonotic disease, Q fever. The disease Q fever is diagnosed globally, however Australia, and in particular Queensland, has the highest rates of notifications in the world.

This study is the first comprehensive investigation of Q fever in Queensland, with a special focus on paediatric infections. At the time of commencement, very limited data were available on the incidence of Q fever in Queensland, the risk factors associated with infection, and the presentation of the disease in children.

Analysis of 6,797 notified Queensland Q fever cases, confirmed the disease to be mainly confined to rural populations, yet there were a noteworthy number of cases reported from urban and non-rural communities. Alarmingly there were 235 notifications from children in Queensland. Globally, Q fever is considered to be under-reported and therefore a seroprevalence study was performed to truly assess the rate of exposure in the Queensland population.

This seroprevalence study highlighted a wider exposure to the organism, with a substantial number of people being exposed from non-rural communities, along with an increased prevalence in children. Further investigations were performed to identify the sources of exposure, especially in “low risk” populations using PCR. This study identified a number of potential sources of infection to humans including domestic pets, flying foxes and dust.

Q fever is an infectious disease presenting with a wide variety of symptoms which may obscure the clinician’s diagnostic approach and treatment. This thesis investigated specific cases of Q fever in both children and adults where there were unusual presentations, including whole families infected, and a serve case in which the patient had complete organ failure. These cases showed the need for greater understanding and awareness of the disease and also demonstrated that indirect transmission of infection from a family member working in a high risk occupation, to other family members may occur.

Prevention of Q fever in the population through vaccination is highly desirable. To ensure adequate vaccine coverage, genotypic characterisation of the *Coxiella* strains circulating in the population is necessary. This study determined the range of different genotypes

detected, and found new, novel genotypes circulating in Queensland, as well as genotypes that were unique to Australia.

Q fever is a vaccine preventable disease in Australia and has had Federal Government funding to protect workers in high risk occupations. Yet the data generated in this thesis highlighted the need for the vaccine strategy to be revised so that children and family, members of at risk workers are included. The current vaccine has many limitations, including that it requires extensive pre-screening procedures to prevent previously exposed subjects from having an adverse reaction upon vaccination. The vaccine can induce a hyper-sensitised reaction in some subjects, eliciting symptoms of Q fever.

As part of this thesis a cell mediated immunity assay was developed to enable vaccine candidates to be screened for previous exposure without having to be re-exposed to the bacteria in the widely applied skin test. This pre-screening tool will allow children to be screened without the potential for an adverse hypersensitive reaction to occur, and should facilitate the wider administration of the vaccine to this younger age group.

In summary, the results presented in this Thesis, address the knowledge gap regarding the epidemiology and clinical impact of Q fever in the Queensland population. It presents evidence of other populations at risk, which may help to formulate an improved vaccination strategy for Queenslanders, and help shape the Public Health approach to the management of this serious disease.

Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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- ***I am finished now.***

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Abbreviations

µL	Microliter
µM	Micromolar
µm	Micrometre
Ab	Antibodies
AGRF	Australian Genome Research Facility
AMI	Antibody mediated immunity
BAL	Bronchoalveolar lavage
BFV	Barmah Forest virus
bp	Base pairs
CDC	Centers for Disease Control and Prevention
CDNA	Communicable Diseases Network Australia
CFS	Chronic fatigue syndrome
CFT	Complement fixation test
CI	Confidence interval
CMI	Cell mediated immunity
CMV	Cytomegalovirus
CO_2	Carbon dioxide
CRP	C-reactive protein
CSL	Commonwealth Serum Laboratory
CT	Cycle threshold
CYS	Chicken yolk sac
DET	Detected
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
E/LFTs	Electrolytes and liver function tests
EBV	Epstein Barr virus
EDTA	Ethylenediaminetetra acetic acid
EHV	Equine herpes virus
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme-Linked ImmunoSpot
FAM	Carboxyfluorescein
FBC	Full blood count
FITC	Fluorescein isothiocyanate
g	Gravitational acceleration
g	Gram
GP	General practitioner

GS	Gold standard
H_2O	Water
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HGDI	HunterTGaston discrimination index
HREC	Human Research Ethics Committee
HRP	Horseradish peroxidase
HVS	High-volume air sampler
IFA	Immunofluorescence assay
IFNTγ	InterferonTγ
IL	Interleukin
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMVS	Institute of Medical and Veterinarian Science
IS	Insertion sequence
IV	Intravenous
kDa	Kilodalton
km	Kilometre
LCV	Large cell variant
LFTs	Liver function tests
LPS	Lipopolysaccharide
M	Molar
M/C/S	Microscopy, culture and sensitivity
M:F	Male:female ratio
Min	Minutes
ml	Millilitres
MLVA	Multilocus VNTR analysis
mm	Millimetres
MREC	Medical Research Ethics Committee
MST	Multi-space sequence typing
n	Number
N/A	Not available
ND	Not done
NDT	Not detected
nm	Nanometre
NNDSS	National Notifiable Disease Surveillance System
NOCS	Notifiable Conditions System

NQFMP	National Q fever Management Program
NSW	New South Wales
^{o}C	Degrees Celsius
OD	Optical density
PBMC’s	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemagglutinin
pmol	Picomolar
Pos	Positive
PUO	Pyrexia of unknown origin
QF PCR	Q fever polymerase chain reaction test
QFS	Post Q fever fatigue syndrome
QLD	Queensland
RBC	Red blood cell
RBWH	Royal Brisbane and Women’s Hospital
RCH	Royal Children’s Hospital
rpm	Revolutions per minute
RPMI	Roswell Park Memorial Institute
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RRV	Ross River virus
RT	Real-time
s	Seconds
SASVRC	Sir Albert Saksewski Virus Research Centre
SCV	Small cell variant
SDs	Statistical divisions
SLA	Statistics local areas
SNP	Single nucleotide polymorphism
SSD	Statistical subdivisions
tAB	Total antibodies
TMB	Tetramethylbenzidine
µg	Micrograms
UK	United Kingdom
USA	United States of America
VNTR	Variable number of tandem repeats
WBC	White blood cells

Chapter 1

Q Fever - Review of the Current Literature

1.1. Introduction

Q fever is a zoonosis caused by the intracellular, gram negative bacterium *Coxiella burnetii,* and has a worldwide distribution with the exception of New Zealand and Antarctica [1–3] (Figure 1.1). Human infection with *C. burnetii* results in the disease known as Q fever. The disease was first described in Brisbane, Australia in abattoir workers and has been a notifiable disease in Australia since 1952[4]. The disease in humans is thought to be contracted by direct contact with large ruminants: cattle, sheep or goats and is hence a disease largely associated with animal handlers. The organism *C.burnetii* can survive for long periods of time in the soil and dust as a result of the dissemination from infected animals. Natural forces such as wind and dust movement, along with animal transportation have long been considered to be secondary sources through which humans can be exposed to *C. burnetii* and go on to acquire Q fever [5,6].

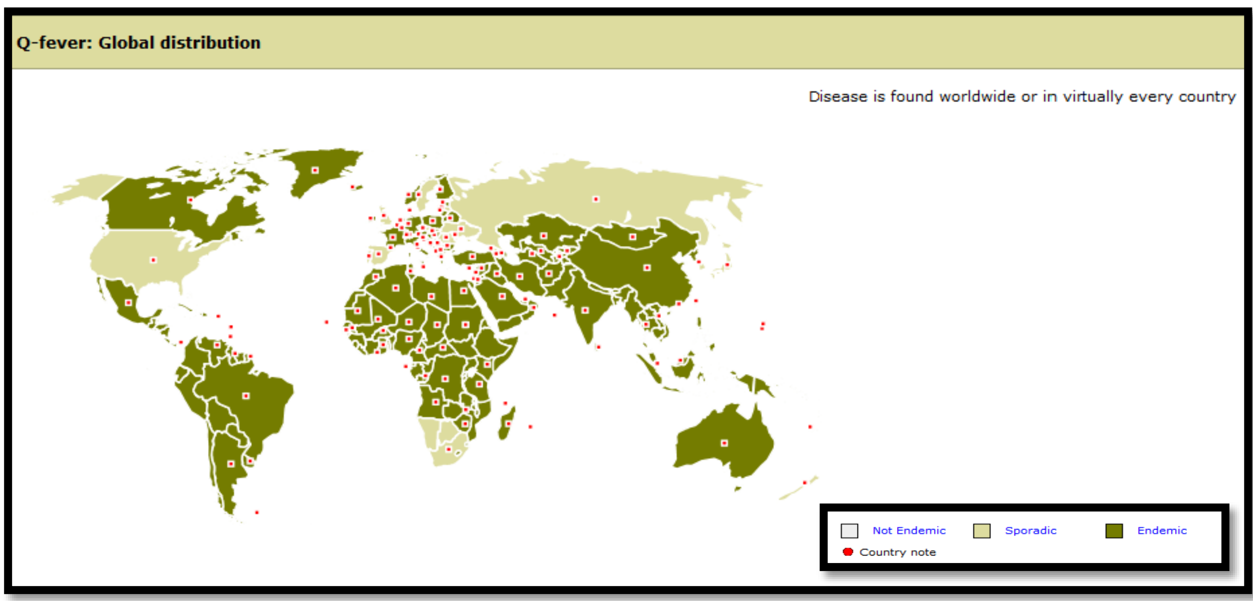

Figure 1.1: Map showing the global distribution of Q fever as at 2014[7]

1.2. History of Q Fever

"Query" fever or Q fever was first identified by an Australian scientist Dr Edward H Derrick in 1936. Dr Derrick was the pathologist in charge at the Royal Women's and Brisbane Hospital. He was investigating a disease outbreak associated with a severe febrile illness in nine abattoir workers residing and working in Brisbane. However, at the time this new disease "Query fever" was identified, the organism responsible was unable to be identified[8]. Dr Derrick sent emulsions of infected guinea pig livers to Frank MacFarlane Burnet in Victoria, who was able to infect healthy guinea pigs, monkeys and mice in his

laboratory, and by employing new staining techniques, was able to identify large numbers of rickettsial-like organisms[9].

Subsequently, Cox et al. identified cells containing a rickettsia-like organism from guinea pigs in Montana, USA, which had contracted a febrile illness after being fed on by ticks. This agent was named “Nine Mile”, and caused a febrile illness in guinea pigs that were inoculated with blood from the diseased animals[10]. The organism was able to be cultured in chicken embryonated eggs[11]. It was through a series of immunological studies and the accidental infection of a visiting scientist that the Q fever agent and the organism isolated from Montana ticks were recognized as being the same organism[12].

In further collaborative studies on Q fever and the rickettsia-like organism isolated in Montana, it was confirmed that five of the guinea pigs which had recovered from Q fever were subsequently immune to the newly identified organism isolated from ticks. Furthermore, serum taken from a patient infected from Montana ticks was able to neutralise the infectivity of the Q fever agent[12]. The organism identified was named *Rickettsia diaporica* in the U.S and *Rickettsia burnetii* in Australia, but after further characterization studies it was renamed *Coxiella burnetii* in 1948 to honor the two scientist Cox and Burnet for their dedication [11–14].

1.3. Bacteriology of *Coxiella burnetii*

1.3.1. Classification

C. burnetii was initially classified in the class of α-proteobacteria due to the similarities it shared with the family *Rickettsiaceae*. The bacterium is small in size, 0.3μm x 1 μm, is pleomorphic and has a poor staining affinity with commonly used microbiological stains[9,11]. However, there were other characteristics that set it apart from the rickettsiae. *Coxiella* resides within phagolysosomes and is able to withstand greater exposure to physical and chemical agents than is observed for the ricketsia[15,16].

With the development of molecular techniques, and in particular with genome sequencing, it was revealed that *Coxiella* has a much closer genetic relationship to *Legionella pneumophila* than the rickettsia. This led to *Coxiella* being assigned to the γ-subdivision of proteobacteria[17]. *C. burnetii* is the only species belonging to the genus *Coxiella* based on the sequencing of the 16s rRNA gene in which all strains examined showed >99% homology[18].

1.3.2. The Infectious Agent

C. burnetii is an obligate, intracellular, gram negative coccobacillus responsible for Q fever disease. On infection, this organism resides within host macrophages and replicates in the placenta and reproductive tissues of infected animals. *Coxiella* has the unique characteristic of being able to genetically change via a chromosomal deletion, resulting in a change of the O-antigen polysaccharide (LPS) in the cell wall membrane which in turn alters its antigenic phase[19].

1.3.3. Phase Variation

Phase variation occurs among many species of pathogenic bacteria, and allows the organism to evade the host cells immune system.

A virulent phase I lipopolysaccharide (LPS) structure of the bacterium (wild-type), is responsible for natural infection. The phase I stage of the organism slowly changes after several reproductive passages to produce phase II, containing a chromosomal alteration which has genetic deletions in the gene responsible for LPS biosynthesis. Phase II is avirulent and has an altered lipopolysaccharide antigenic structure which prevents the organism from reverting back to phase I and allows the organism to be phagocytosed more readily by host cells than phase I[20,21]. Typical phase variation is observed in both animal and human infections. It is the irreversible avirulent phase II form that causes acute disease in man[22].

1.3.4. Intracellular Interaction of Coxiella and Phagocytes

Cellular uptake of the *Coxiella* bacterium varies based on the phase variants. The phase II form of the bacteria is more readily phagocytosed than phase I [22]. The internalization of the bacteria is linked to integrin CR3 which is expressed on the surface of the monocytes and macrophages. Phase II organisms engage another integrin, $\alpha_v\beta3$, which in turn triggers activation of CR3 via a protein IAP[23]. Adherence of the phase I bacteria causes a rearrangement of the actin cytoskeleton changing the physical shape of the membrane of the host cell[24]. These protrusions contain $\alpha_v\beta3$ but not the CR3 and so the interaction between integrins does not occur (Figure 1.2)[23].

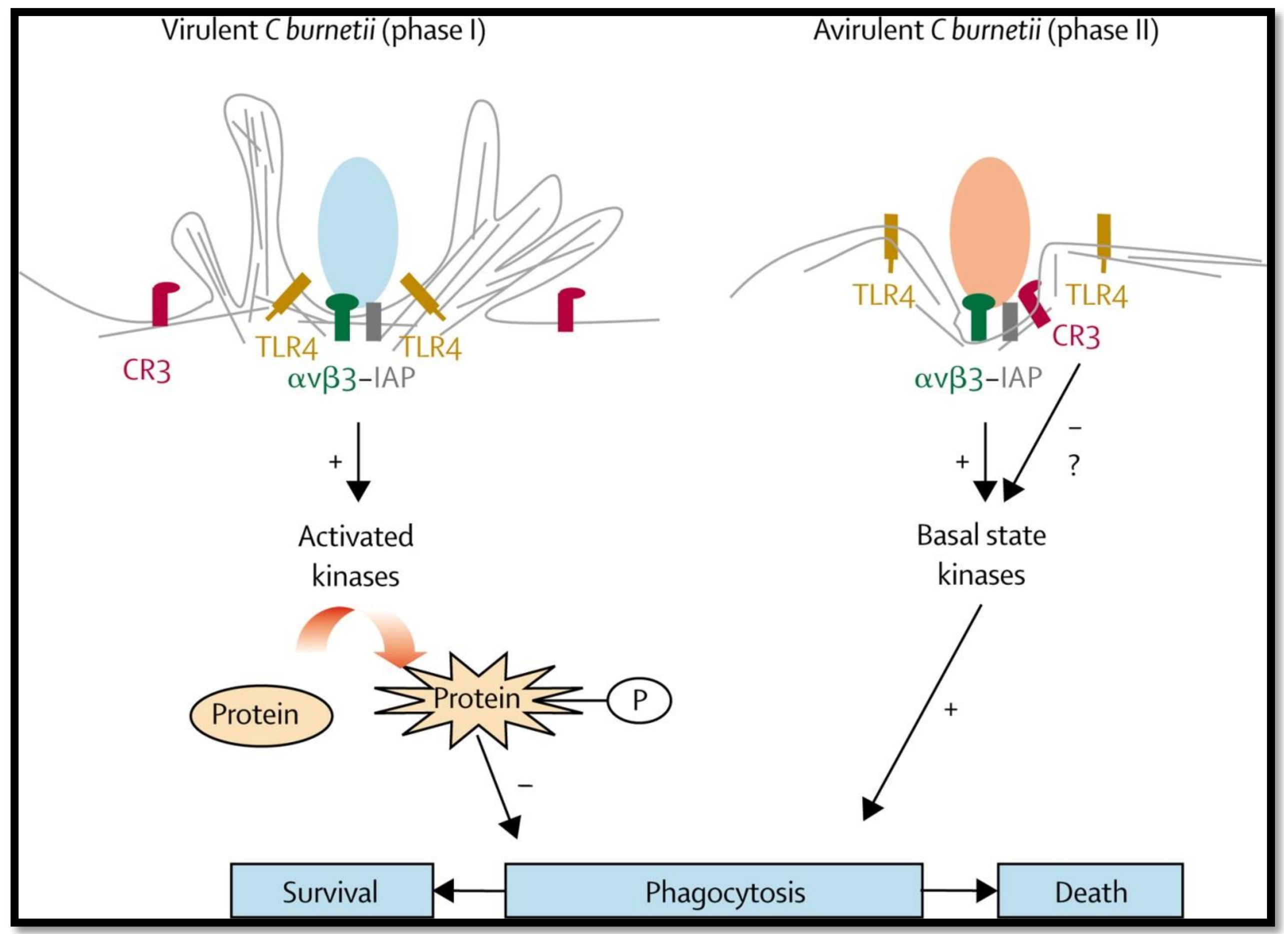

Figure 1.2: Diagram showing phagosome uptake and internalisation of *Coxiella* highlighting the different pathways initiated by the two phase variations of *Coxiella*[26].

Other non-phagocytic cells involved in the uptake of the bacteria also show variation in the rates of internalization. The mechanism though to be responsible is the LPS structure which is different for each phase. The phase I LPS was shown to impair the uptake of this phase of the *Coxiella* compared to the uptake of phase II organism without the LPS[27].

Once inside the phagocyte *Coxiella* are contained within a phagosome, a membrane bound vacuole. In the normal cellular destruction of foreign agents the phagosome matures, becoming acidic and fuse with secondary lysosomes to form phagolysosomes. This is the host cell's natural defense mechanism by which most foreign agents are destroyed[28]. However, this is not the case with *Coxiella*. The *Coxiella* bacterium causes the phagosome to form a large compartment known as a parasitophorous vacuole which acts as the perfect acidic environment in which *Coxiella* can replicate[29]. This acidic, bacteria filled, vacuole remains non-toxic to the host cell (Figure 1.3)[30].

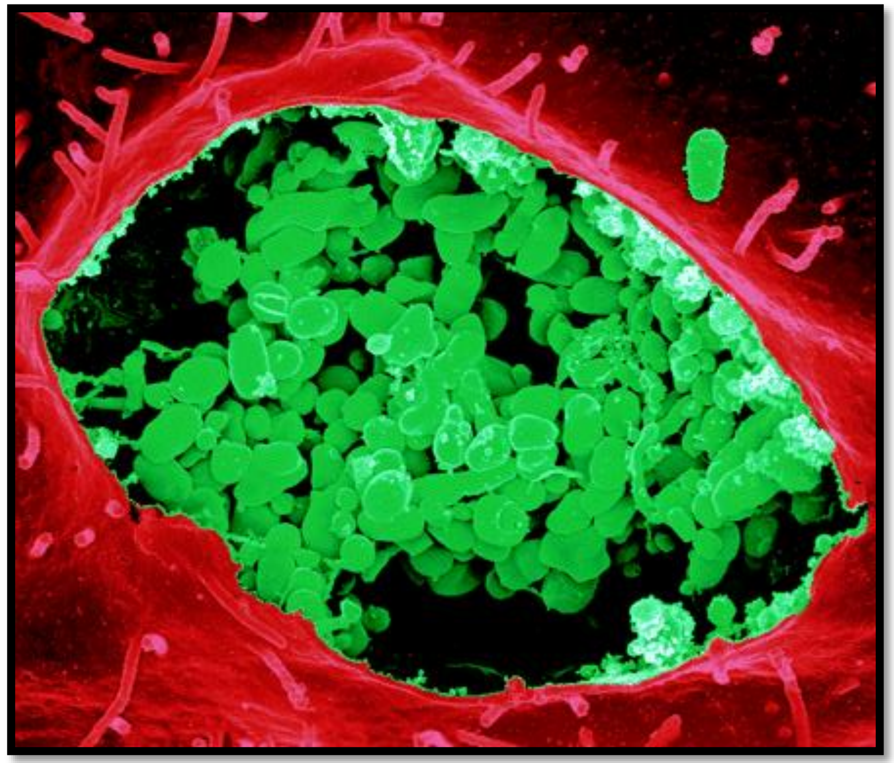

Figure 1.3: Electron micrograph of a *Coxiella*-infected cell and the lumen of the parasitophorous vacuole revealing the organisms inside[31] (*Image reproduced with permission by Elizabeth Fischer of the Rocky Mountain Laboratories Microscopy Unit*).

1.4. *Coxiella burnetii* and Human Infection

C.burnetii is an extremely infectious pathogen. The disease Q fever can be induced through the inhalation or invasion of a single bacterium[32]. The extremely high infectivity, the ability to withstand harsh environmental conditions, and the potential to cause severe disease in man, has deemed this organism to be considered as a biological terrorist agent. It has been listed as a Category B biological warfare agent by the Centre's of Disease Control and Prevention[33]. *C.burnetii* fulfils all of the requirements for a biological weapon: it consistently causes disease; it can be produced on a mass scale; it remains stable under production, storage, transportation and delivery conditions; it can readily be disseminated into the environment; and it can remain viable in the environment for years. Its classification as a category B biological agent rather than Class A is due to its inability to cause large-scale fatalities as is the case with category A agents such as smallpox, anthrax, botulism and the viral hemorrhagic fevers[33].

1.4.1. Routes of Transmission

Q fever in humans is contracted by the inhalation of contaminated dust particles or bacterium-containing aerosols shed from infected animals and their products[34]. This makes Q fever infection to be primarily an occupational disease associated with animal handlers and workers, including veterinarians, abattoir workers, meat handlers, shearers, tanners, and farmers. There have been other routes of transmission reported, including human to human cases, but these are rare. Also, cases have been reported as a result of sexual transmission, and cases in mortuary assistants attending an autopsy in which the patient died as a result of Q fever. There was also a case of an obstetrician contracting the disease after delivering an aborted foetus from an infected pregnant women[35–37].

Although inhalation of the organism is the most common route for *Coxiella* transmission there are other routes by which the organism can gain entry into the human body, such as vertical transmission from an infected mother to her unborn child[38,39]. The ingestion of contaminated dairy products from infected animals is also a known source of transmission to humans[34,40], and ticks have also been identified as transmission vectors as demonstrated by the original isolation of *C.burnetii* from a tick[41,42].

1.4.2. Groups at Risk of Q fever Infection

The transmission routes for infection with Q fever largely originate in animal sources. This has made Q fever disease an occupational hazard for persons working with large ruminants, in particular, cattle, sheep and goats[26]. There are reports of people from communities who have been infected with Q fever as the result of direct or indirect transmission from parturient animals, via wind, exposure to animal transporters, and stock sale yard sand from living close to these environmental exposures [5,43–48]. There are people at risk of Q fever who do not work regularly with animals yet may come in contact with areas where animals and or their products have previously been. These also include people working as contractors or maintenance workers, and visitors to farms or animal fairs/shows, in fact anywhere where animals or their products are present[49].

Infections are not only restricted to those working with cattle, sheep and goats. There have been many outbreaks in which other host animals have been identified as the source of infection. Parturient cats and their kittens have been implicated in many outbreaks of Q fever in Canada and Nova Scotia[50–54].

1.4.3. Pathogenesis

Although discovered over 60 years ago, and despite control measures being implemented, Q fever still remains a serious public health issue and a significant burden on health care resources[55,56]. Q fever disease may present as either acute or chronic infection, based on the clinical manifestation, with the prognosis of chronic infection ranging from highly favorable to potentially fatal. The spectrum of clinical syndromes range from an asymptomatic, acute "flulike" illness, acute hepatitis or acute pneumonia, to chronic endocarditis and or chronic hepatitis. Host factors, such as age, gender and certain medical conditions, may influence the disease presentations[57]. Age has been identified as a risk factor for Q fever, with a higher risk associated with increasing age. This was demonstrated in a Q fever outbreak in Switzerland in 1983, showing that subjects older than 15 years had a five times greater risk of contracting the disease[58]. Similarly, a study in Greece showed an increase of confirmed clinical cases with an increase in age[59].

Although Q fever has rarely been reported in children younger than 15 years, it is likely that globally, the disease is under reported in this population[60]. This is largely due to the non-specific and varied clinical presentations that occur in children infected with *Coxiella*. However, when symptoms do present in children they are similar to those observed in adults [61–66]. Q fever disease largely affects males at a ratio of up to 5.3:1 compared to females[67]. Predisposing conditions for the development of chronic Q fever disease include pregnancy, immunosuppression and preexisting heart valve lesions [49].

1.4.4. Clinical Disease States of Q fever

The large majority of all *C. burnetii* infections are asymptomatic or self-limiting, and resolve with very little impact to the patients, and often requiring no formal medical treatment. It is only when extensive laboratory investigations into Q fever are performed, often retrospectively, that it becomes evident exposure has occurred in these patients. Of those patients that acquire acute Q fever, 10-30% go on to develop chronic Q fever, in which 2% are diagnosed with endocarditis. The diverse range of clinical manifestations of the disease, gives rise to an increase in misdiagnosed cases which in turn increases the mortality and morbidity associated with the disease.

1.4.4.1. Acute Q fever

Acute Q fever infections are often asymptomatic, subclinical or cause an extremely mild disease. It usually manifests after a 1-3 week incubation period depending on the

infectious dose received. It has been reported that up to 60% of patients diagnosed with acute Q fever are asymptomatic[32,34,62,66]. The other 40% are symptomatic patients showing a number of varied clinical manifestations ranging from fevers, sweats and headaches to hepatitis and pneumonia, and of these 2% are hospitalised[68] . The classical presentation of Q fever is a “flu-like” illness with a rapid and severe fever lasting longer than seven days. However, this mimics symptoms by many other infections. This may be accompanied with severe headaches, myalgia, rigors, arthralgia, and general fatigue, reflecting the cytokine cascade of the acute phase response and the developing cellular immune response[69]. Infection may also present as pneumonia or acute hepatitis. The illness generally subsides within two to six weeks with some patients reporting bouts of extended fatigue[69].

In 1973 Derrick et al. noted that in 173 patients diagnosed with Q fever the length of the fever varied with age, showing that fever duration increased with increasing age of the patient[70]. Approximately 5% of acute Q fever patients require hospitalisation[68].

1.4.4.2. Chronic Q fever

Approximately 5 % of acute Q fever cases go on to develop chronic Q fever[72]. People may become chronically infected without having being previously diagnosed with acute disease, and chronic Q fever may manifest months or years after an acute infection[72]. In these chronic infections, *C. burnetii* multiply in host macrophages, producing a permanent rickettsaemia, characterised by high levels of persistent antibodies. The heart is the most commonly affected organ followed by the liver. Clinically, chronic Q fever presents as endocarditis in 60-70% of cases, but the arteries, bones and liver may also be affected[68]. Endocarditis usually occurs in patients with underlying heart valve damage or immunocompromised patients and it generally results in cardiac failure, mitral valve and or aortic valve dysfunction[73]. Chronic Q fever may also present with low grade fevers which are remittent, malaise, weakness, fatigue, night sweats, chills and weight loss. Hepatomegaly, renal insufficiency and splenomegaly are often seen in patients suffering from chronic Q fever over a long period of time[74].

Other less frequently observed complications associated with chronic Q fever are vascular infections, chronic hepatitis and chronic pulmonary infections[68,75,76]. In addition, osteoarticular infections may occur, and can be of three types, osteomyelitis, osteoarthritis and aortic graft infection with adjacent spinal osteomyelitis[68,77,78].

1.4.4.3. Post Q fever Fatigue Syndrome (QFS) and Chronic Fatigue

Chronic fatigue syndrome (CFS) following Q fever was first described in Australian abattoir workers[79]. These patients presented with prolonged fatigue well after the acute phase of the illness[70,79,80]. Whilst controversial, chronic fatigue has now been accepted by the courts in Australia as a sequelae of Q fever infection, and it is estimated that post-QFS occurs in approximately 20% of acute cases[80].

Patients that suffer from QFS continue to experience chronic fatigue-like symptoms for over 12 months post-acute illness, and some for much longer, with up to ten years post infection[79]. Symptoms vary with acute and chronic disease presentation, with the most common being debilitating fatigue, nausea, headaches, blurred vision, night sweats and joint pain[79,81].

Retrospective diagnosis of QFS has been reported following Q fever outbreaks in the UK, Canada and now The Netherlands[82–84]. These studies have validated the initial reports of CFS as a recognized consequence of Q fever disease.

1.4.4.4. Q fever and Pregnancy

Despite numerous associations between Q fever infections and adverse outcomes of pregnancies in animals, relatively little has been documented about outcomes of pregnancy and Q fever infections in humans[76]. This may be due to a lack of awareness of Q fever as a serious human pathogen by obstetricians, resulting in an underestimate of the incidence of the disease [85].

In 2007 a review of Q fever in pregnancy involving 38 human cases, demonstrated that Q fever in pregnancy was associated with a high morbidity and mortality[86]. The study showed that spontaneous abortion occurred in 26% of Q fever related pregnancies, with 5.3% of pregnancies resulted in intrauterine death, 45% resulted in premature birth and 5.1% reported intrauterine growth retardation[86]. In another study, of 53 pregnant women with Q fever, it was identified that more than 50% of these mothers developed a Q fever serological profile consistent with chronic Q fever, compared to a 5% conversion rate in the general population[86]. This study also highlighted the link between placentitis and obstetric complications. Contracting Q fever during pregnancy, results in long term risk of developing chronic Q fever disease, along with adverse outcomes for the unborn child.

1.5. Diagnosis of Q fever

1.5.1. Serological Methods

The diagnosis of Q fever is based on the detection of specific antibodies produced against the organism *C.burnetii* following exposure. Until relatively recently, the complement fixation test (CFT) and the micro agglutination assay were the methods employed in the detection of antibodies against *C.burnetii*[87]. However, the indirect immunofluorescence assay (IFA) is now considered the reference method or "Gold standard" for the diagnosis of Q fever by serology[88,89]. This has proven to be the most sensitive and specific for the detection of *Coxiella* antibodies (Table1.1)[89].

The IFA method can differentiate between the different classes of antibodies produced by a host in response to Q fever infection. Testing of serum for the presence of acute Q fever antibody testing is generally performed on paired sera. These samples are ideally taken 14-21 days apart and the change in antibody levels can be directly compared. An alternate method is the detection of the acute antibody marker immunoglobulin M (IgM), also by IFA, which is indicative of a current infection[90]. This IFA method is subjective and there have been studies performed to measure inter-laboratory variation, with one showing only a 35% agreement rate between laboratories[91].

There are other systems designed to measure antibody levels which also have high sensitivity and specificity such as the enzyme linked immunosorbant assay (ELISA). This assay can be automated and reduce turnaround times, and produces a measurable end point which allows for standardization of the assay results across laboratories[92].

Only phase I organisms are virulent towards humans, however, serologically the anti-phase II antibodies are the first to be detected in an acute infection using the previously described methods. High levels of specific anti-phase I antibodies are normally associated with a chronic Q fever profile, whereas specific anti-phase II antibodies dominate during acute Q fever infections. This is largely due to the phase II antigens being more immunogenic than the phase I surface components[93–95]. Phase I antibody may be detected following initial infection in conjunction with a phase II antibody titre. However, the phase I titre seldom exceeds the phase II titre.

As seen with many other infectious diseases, IgM antibodies are the first to appear and generally the first to be detected. These acute markers are usually detectable within one week after the onset of symptoms and may be detectable for up to 17 weeks[96]. Immunoglobulin G (IgG) may be detectable at the same time as IgM. However, it has been shown that the titres of IgG are lower in the first week post infection than IgM titres. The IgG levels peak, and on average can persist for years, even life long, as opposed to the short, higher titred production of IgM[95,97]. The presence of immunoglobulin A (IgA) to phase I antigen strongly correlates with endocarditis and is indicative of a chronic infection[74,97]. However, some experts dismiss the value of phase I IgA detection as a definitive marker for chronic Q fever and state that only IgG and IgM titres should be assessed when considering acute and chronic Q fever[98]. The detection of phase II IgA has been noted in many studies at low titres, but questions regarding its role as a diagnostic marker continue[89,95,99–102].

Table 1.1: Sensitivity and specificity of the various serological methods used to diagnose acute Q fever.

Test	Sensitivity	Specificity	Reference
Micro-agglutination	82%	97%	Nguyen 1996[103]
Complement Fixation	78%	99%	Peter 1985[104]
Immunofluorescence	98%	100%	Slaba 2005[98]
ELISA	84%	99%	Waag 1995[96]

1.5.2. Molecular Methods

Molecular techniques have been employed in the detection of many bacterial and viral pathogens and have become the gold standard of testing for many infectious agents[105]. Serological tests have proven to be inadequate for detecting Q fever infections in the very early phase of the disease, when antibody levels are low or developing[106]. The development of molecular methods such as the polymerase chain reaction (PCR) and the continuing advances made by molecular diagnostics such as sequencing whole genomes, have vastly improved the early and accurate diagnosis of acute Q fever, along with the

ability to confirm chronic cases of Q fever. Rapid and accurate diagnostic methods such as nucleic acid detection, of which PCR is the most widespread are imperative in identifying the biology and pathogenesis of this organism in order to reduce the morbidity and mortality associated with Q fever disease.

There have been many PCR assays designed for the detection of *Coxiella* DNA, and these have targeted different areas of the organism's genome[107–111]. The target most often chosen to detect *Coxiella* DNA is the repetitive insertion element sequence *IS1111*, which has multiple copies throughout the *Coxiella* genome[112]. The *com1* gene is the other commonly selected gene target which encodes for a 27-kDa outer membrane protein[113]. PCR has also allowed the detection of Q fever disease from a variety of different sample types including serum. Acute Q fever is often diagnosed by PCR in the very early stages of disease using sera that has tested negative to *Coxiella* antibodies[107]. PCR has also been used widely for the detection of *Coxiella* DNA in chronic Q fever patient samples. These generally are tissue samples taken directly from infected organs which highly loaded with bacteria; for example: heart valves in the cases of endocarditis[69]. PCR has already improved the turnaround times for diagnosis and treatment of many Q fever sufferers.

1.5.3. Isolation of Coxiella by Culture

Culturing of *Coxiella* is no longer employed as a diagnostic tool for Q fever disease in many laboratories due the difficulties and dangers working with the organism. *Coxiella* can be isolated from clinical samples using a shell vial centrifugation and inoculation onto Human Embryonic Lung cell layers[114]. Isolation can also be achieved by inoculating patient samples into embryonated chicken eggs and animal models, including guinea pigs and mice.

1.5.4. Microarray Technology

C. burnetii infects the macrophages and induces an immune response. Different pathogens trigger specific pattern-recognition receptors that are expressed on the surface of leukocytes[115–117]. Microarray technology can be used to analyse different gene expression patterns in the leucocytes from patients who have been exposed to specific pathogens such as *C. burnetii*. This type of technology has already brought a new perspective to the diagnosis and prognosis in cancer. The gene expression patterns produced from the leukocytes have led to a better understanding of the mechanisms behind the cancer[118], and have been used to identify genes regulated by the immune

response[117,119]. These specific patterns are "signatures" of the leukocytes and are indicative of disease.

1.6. Treatment and Prevention

1.6.1. Treatment of Q fever Infections

Treatment of Q fever remains difficult due to its varied clinical presentation and misdiagnosis of infection. Most acute infections are subclinical and recover spontaneously without medical intervention. However, when a positive diagnosis of acute Q fever is made treatment should commence immediately and continue for 2-3 weeks[69]. Doxycycline is the antibiotic of choice because of its ability to readily permeate cell membranes and destroy the intracellular organism within a few weeks[120].

Chronic Q fever patients have a much poorer prognosis[73]. This disease persists for longer periods of time and has a mortality rate of up to 60%[121]. Endocarditis is the main clinical manifestation of chronic Q fever and is much more difficult to treat effectively, requiring a combination of antibiotics, usually:

a) Doxycycline in combination with quinolones for at least 4 years; or

b) Doxycycline in combination with hydrochloroquine for 1.5 to 3 years.

These treatment regimens are some of the lengthiest reported for bacterial infections[121], and relapse of the disease may occur after the termination of treatment. Therefore clinical and biological evaluation should be performed at least annually for the rest of the patient's life[68]. Often surgery is the only treatment in the case of endocarditis requiring the removal and replacement of damaged heart valves in conjunction with antibiotic treatment.

1.6.2. Vaccine

Derrick's early work on Q fever and the discovery of *C. burnetii* as the aetiological agent, led to the development of a crude vaccine[122,123]. This vaccine was imperative to reduce the high number of infections among abattoir workers and laboratory personal.

During initial vaccine development, the antigenic structure and nature of *C. burnetii* was unknown, and it was later proven that the potency and the efficacy of the vaccine were dependent upon the antigenic phase of the organism[124]. Vaccines developed with phase I organisms have a potency of 100-300 times that of cellular vaccines produced using the

phase II organism[124]. In Australia, a whole cell vaccine against Q fever "Q Vax®" was manufactured by the Commonwealth Serum Laboratory (CSL), Australia, and was licensed for use in 1989. This vaccine was implemented for wide-spread use into a government funded vaccination program with the aim to substantially reduce the number of Q fever infections associated with occupational outbreaks. This nationally available vaccination program was called the National Q Fever Management Program (NQFMP). The vaccine is recommended for use in at risk candidates aged 15 years or older, and is not administered to children younger than 15 years. However, there is a compelling case to include children in a wider vaccination program, but the efficacy and safety for this lower age group is not known[125]. Australia is the only country in the world that has a licensed vaccine for the prevention of Q fever in humans.

1.6.2.1. Adverse Reactions Associated with Vaccination

The vaccine was trialed as a preventative measure in personnel employed in four South Australian abattoirs during 1981-8. During this time over 4000 vaccines were administered to workers and it was shown to have an efficacy of 100% with protection lasting at least 5 years[123]. However, there were many adverse reactions associated with the administration of the vaccine. Vaccinees very commonly showed local tenderness (48%) and erythema (33%) at the site of injection. General symptoms occurred commonly in 10% of vaccinees and may include transient headaches, flu-like symptoms, fevers, chills and sweats[125]. There was also evidence of more significant adverse reactions which were documented for an estimated 130, 000 individuals vaccinated from 1989-2004[126]. These reactions included intensified local reactions at the site of injection, which may occur in individuals previously immunologically sensitized from either a subclinical infection or from repeated vaccination. Other reactions observed were painless hardened lumps which took months to heal, sterile abscesses requiring excision and drainage before resolving, and systemic symptoms mimicking post QFS[125,127]. It was recognized that these adverse reaction could be avoided by testing for preexisting immunity[123,127–129]. While antibodies are required to clear extracellular organisms, sensitization of lymphocytes to the Q fever antigens, and their subsequent secretion of lymphokines, is the mechanism that clears the intracellular infection and provides immunogenic memory.

1.6.2.2. Pre-vaccination Sero-profiling

In 1983, an initial investigation of pre-vaccination profiling by skin test was initiated using the Q-fever phase I antigen on 74 subjects[128]. The results revealed that 38 (51.3%)

developed erythema less than 8 mm, and 36 (48.6%) had erythema greater than or equal to 10 mm's. Of these, only 14 subjects had positive serology or evidence of past exposure. All skin test-positive individuals and one skin test-negative individual developed mild local reactions.

A trial was initiated in volunteer abattoir workers in South Australia to assess the safety of the vaccine[130], and to survey any adverse effects after vaccination. Adverse reactions were limited by pre-assessing the vaccination candidate's serological results and cell-mediated immunity via skin tests. Q fever antibodies were measured using complement fixation and immunofluorescence assays. Seronegative subjects were skin tested by intradermal inoculation of Q fever vaccine containing 0.02µg of purified organism. The sites of injection were examined 5-7 days after inoculation, and an indurated area of 7mm or more was regarded as a positive reaction[131]. The results of these studies by Marmion et al. and Ascher et al., prompted the introduction of routine Q fever skin tests to aid in the sero-profiling of pre vaccination candidates by directly measuring the candidate's cell mediated immune response[128,130].

1.6.2.3. Limitations of Sero-profiling

In Australia, pre-vaccination sero-profiling is mandatory when vaccination against Q fever is considered using the current vaccine [125]. However, there are potential difficulties that may be encountered when testing for prior immunity. Using antibody titres as an indicator of immunity and hence prior infection, may not eliminate the risk of adverse reactions as specific antibody levels start to diminish after an acute infection or previous exposure. Therefore antibody absence may not always reflect the immune status of the vaccine candidate[132,133]. Skin tests however, are able to provide an accurate indication of cell mediated immunity, and provide an insight into the likelihood of adverse reactions following vaccination. These tests however, carry have a degree of uncertainty and subjectivity as the interpretation of results is based on individual judgment[125]. This requires that skin tests must be performed correctly and only by experienced, trained personnel. With this in consideration, skin testing is very time consuming as there is a 7-10 day window before the results can be read and interpreted. This in turn results in the skin test being costly and having the potential to be misinterpreted, especially if performed incorrectly[49,128].

It would be advantageous and cost effective to develop a vaccine that is less of a risk to candidates that have been previously exposed to *C.burnetii* and thereby reduce the risk and time associated with a Q fever vaccination. Recent Q fever vaccines that are currently being trialed include the chloroform methanol residual (CMR) extracted phase I whole cell vaccine, which unfortunately has not correlated well serologically with immune status [132,134,135]. However, it is proposed that this newly developed vaccine carries the appropriate antigens needed to prime a host response and this is enhanced by the use of a booster vaccine which stimulates the immune system to produce detectable IgG antibody levels, which were previously not detectable after initial vaccination. There have been other vaccines developed that have been unsuccessful in providing protective immunity. These included a fusion protein vaccine comprised of a *C.burnetii* outer membrane protein I and heat shock protein B, but this vaccine provided only minimal protection[136]. The protective mechanisms of the immune response to *Coxiella* exposure are still not fully understood and this has hampered the development of a safer, protective vaccine.

1.7. Epidemiology

The disease Q fever, is a zoonosis reported worldwide excluding New Zealand and Antarctica[2,136,137]. The causative agent, *C. burnetii,* has a wide range of animal reservoirs, and has been detected in all the animal kingdoms, with domestic ruminants the most frequent source of human infections[138]. Generally, *C.burnetii* infections in animals are asymptomatic, however, in mammals the infections can lead to abortion and stillbirths[139], in which case the organism is found in large numbers in products of conception and birthing by-products[139]. Animals may also shed the organism in milk, faeces, and urine, and it may be present in large numbers in wool, due to infestations with ticks and their faeces[140]. For humans, exposure to these animal products is considered to be the major source of infection. Transmission of *Coxiella* and Q fever infection in humans is through inhalation of particulate matter generated from infected animals during parturition or through the inhalation of air borne contaminated dust [5,31,141,142]. Due to the highly resistant nature of this organism it may persist in the environment for weeks or months, with a common source of dissemination by the wind[5,42,45,143]. The latter would account for Q fever infections in humans without direct animal contact. There are also a small number of infections that occur as a direct result of ingestion of contaminated milk[144], and even fewer cases of Q fever result from tick bites[66]. Although very rare, human to human spread has

been documented, with early cases contracted during an autopsy, one documented case of sexual transmission and transmission from bone marrow transplants and blood transfusions[33,34,145].

Although at greatest risk of Q fever infection are those in direct contact with farm animals, there is also a significant risk associated with laboratory personnel working with infected specimens and laboratory animals[146]. For those workers at risk of Q fever infection, access to an appropriate and comprehensive vaccine strategy is of the utmost importance and should be part of their Occupational Health and Safety consideration.

1.7.1. Q Fever Notifications

The true prevalence of Q fever is difficult to assess as many countries are not vigilant with disease recognition or reporting cases. In some countries Q fever is a notifiable condition, including Australia, USA, Germany, France, United Kingdom and Netherlands. The cases of Q fever that have been reported for these countries over the past 60 years have been compared in Figure 1.4. Australia has constantly reported on average more notifications than any other country in the world, with the exception of The Netherlands during a Q fever outbreak in 2007 which saw over 4, 000 cases reported[147]. There have been over 370 outbreaks of Q fever worldwide since 1954, with over 28,508 cases reported[7]. Table 1.2 highlights some of the countries that have encountered Q fever outbreaks[7].

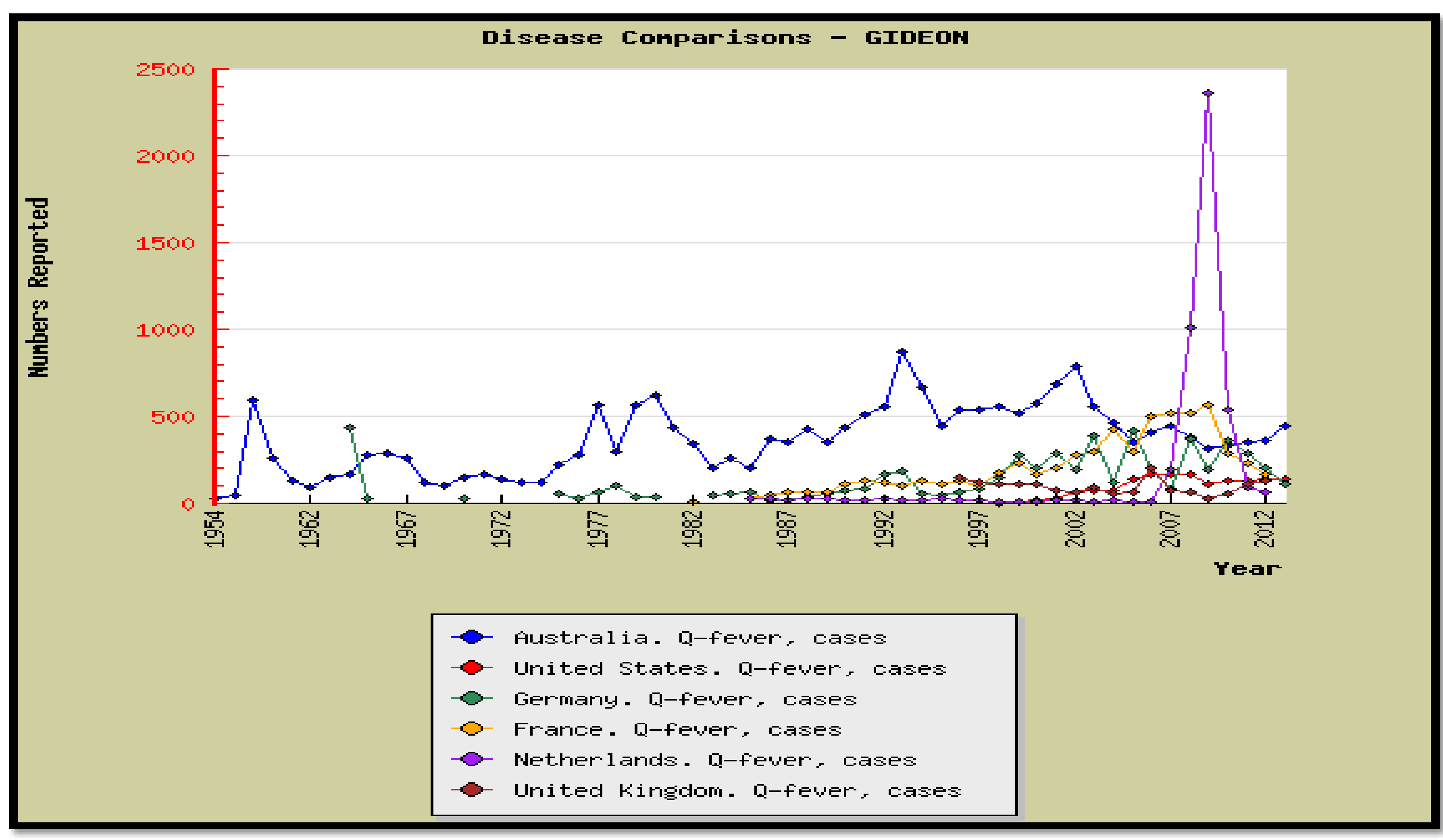

Figure 1.4: Q fever Notifications from Australia, USA, Germany, France, The Netherland and the United Kingdom for the years 1954 to 2013[7].

Table 1.2: Summary of the number of Q Fever outbreaks reported around the world from 1940-2014.

Countries	Outbreaks
Worldwide Outbreaks	6
Australia	7
Bosnia and Herzegovina	3
Bulgaria	2
Canada	6
Croatia	4
Cyprus	1
Czech Republic	2
France	1
Germany	9
Israel	1
Italy	2
Madagascar	1
Netherlands	1
Poland	1
Romania	1
Russian Federation	1
Serbia and Montenegro	1
Spain	1
Taiwan	1
United Kingdom	5
United States	3
Grand Total	60

1.7.1.1. Australian Outbreaks

There have been 11 significant outbreaks of Q fever reported in Australia between 1959 and 2013 (Table 1.3). Details of each of the outbreaks, the state of Australia in which it occurred along with the numbers of confirmed cases that occurred in each outbreak.

Table 1.3: Summary of Australian Q fever Outbreaks from 1954 to 2013

Year	Outbreak Details
1959	An outbreak of Q-fever in Queensland was associated with sheep contact [148]
1962	An outbreak was reported in a meat-works in South Australia [149]
1969	An outbreak in a Brisbane "meat works" affected 7.9% of workers[150]
1979	An outbreak (110 cases) was reported at an abattoir in Victoria[151,152]
1998	An outbreak (29 confirmed and 8 suspect cases) was reported at an abattoir in New South Wales. [153,154]
2004	An outbreak (9 confirmed and 6 suspect cases) was reported among farmers in South Australia. [44]
2005	An outbreak (5 cases) was reported among persons involved in calving activities, in New South Wales. [155]
2006	An outbreak (4 cases) was reported among workers at a cosmetics factory [156]
2006	An outbreak (27 cases) was reported in rural South Australia[107]
2007	An outbreak (5 cases confirmed, 1 possible fatal case) was associated with an abattoir in South Australia[157]
2013	Two separate outbreaks were reported in two separate veterinary hospitals (2cases and 3 case)[158,159]

In 2012, Australia reported 358 confirmed case of Q fever disease, or 1.6 cases per 100 000 population, and the majority of these cases were reported from Queensland and New South Wales.

As previously noted, Australia has one of the highest reported incidence rates of Q fever in the world, with rates 2, 3 and 6 times higher than in France, the European Union and the United Kingdom (UK) respectively[56]. Based on very little data available, Queensland and Northern New South Wales have the highest notification rates in Australia (Figure 1.5). In Australia, Q fever became a notifiable disease in 1977, and since then, Q fever infections are extensively monitored as shown by notification numbers[67,127].

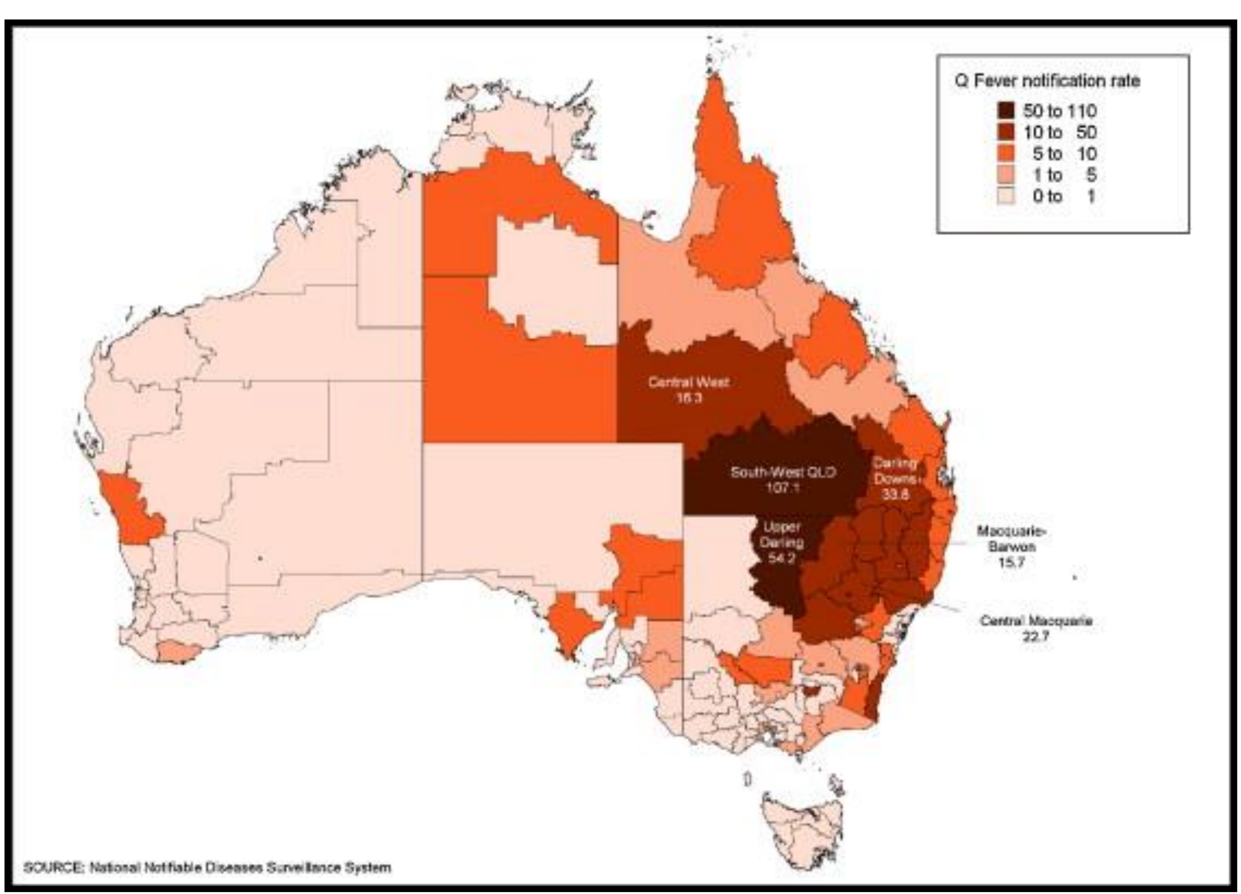

Figure 1.5: Australian Q fever notification rates (per 100,000) by ABS Statistical Subdivision (2006) (Australian Bureau of Statistics)[56].

1.7.2. Seroprevalence

1.7.2.1. Worldwide Prevalence

Q fever cases and epidemics have been reported in many countries around the world; however,despite the global prevalence of *C. burnetii*, there have been few large population-based studies examining the epidemiology of this infection. Much of the literature states that there is a lack of prevalence studies and that the prevalence within populations are still very much undetermined. Also, there is little information regarding the distribution of Q fever infection of humans[25,65,67,145,158,159] .

As far back as 1955 it was documented that Q fever was known to exist in 51 countries[159]. In 2007, both Britain and the USA, reported that annually there are approximately 100 human cases of Q fever identified in those countries, but diagnosis remains problematic[160]. Published seroprevalence studies are limited and generally performed in an "at risk population" or performed on small patient populations. With this in mind, those studies that have been conducted give prevalence rates ranging from 18% to 37% from blood donors in Morocco, Tunisia, Zimbabwe, Nigeria and North Africa[1]. In 2007, a study in Albacete, Spain, reported prevalence rates of phase II antibodies to be 23.1%, with 0.3% of participants (blood donors) having positive IgM titers[161]. Turkey has recently (2008) reported a seroprevalence of anti-phase II IgG of 32.3%, with 2.8% being IgM positive[162]. In both these studies it was shown that men were more frequently seropositive than women and this difference was not related to occupational exposure to animals. In Tianjin, People's Republic of China, seroprevalence rates of 6.4% for *C. burnetii* were reported in farm workers in 2008[163].

Despite this high seroprevalence for *C. burnetii*, there have been few large population-based studies performed examining the epidemiology of this infection. Northern Ireland in 2008 performed an extensive population based study in order to gain a more accurate insight into the seroprevalence of Q fever. *C. burnetii* phase II specific IgG antibodies were measured by enzyme-linked immunosorbent assay in stored serum from 2,394 randomly selected subjects, aged 12-64, collected in 1986 - 1987. The overall prevalence of *C. burnetii* antibody was 12.8%. The prevalence was slightly higher in males than in females (14.3% versus 11.2%). Sero-positivity was low in children (<10%), increasing to 19.5% and 16.4% respectively, in males and females in the 25-34 age group. This prevalence remained fairly consistent with increasing age. Sero-positivity among farmers, was 48.8%,

which was significantly higher than the general population. More sero-positive than sero-negative women had a history of a miscarriage or still-birth (19.5% versus 9.8%). The authors estimated that 20% of Q fever infections in Northern Ireland occurred in farmers[164].

Recently, The Netherlands experienced large population outbreaks of Q fever, in particular in Noord Brabant. This area has a large goat farming industry, and recorded large numbers of herd abortions on some farms, which were blamed for the outbreaks. The notification rates of Q fever in The Netherlands up until the recent outbreaks in 2006 were on average 17 cases annually. The recent outbreaks saw notification rates rise almost ten-fold in 2007 to 168 confirmed case and then to a staggering 1000 registered cases reported in 2008[165]. This outbreak raised an awareness for clinicians worldwide, to consider Q fever as a possible cause, when assessing patients with “flu-like” illnesses, and or those in contact with ruminant and or parturient animals.

1.7.2.2. Seroprevalence in the Australian Population

The seroprevalence of *C.burnetii* in Australia is largely unknown and there are no recent published data on the seroprevalence of Q fever in either the rural or urban populations at either a national or state level. The studies that have been documented generally target the groups with high occupational hazards such as veterinarians, farmers and abattoir workers[166].

Studies performed in North-Western Australia have not reported any confirmed cases of Q fever for 15 years up to and including 2002. Yet, during a Q fever vaccination study targeting this area, 39 of 59 (66%) subjects showed serological evidence of previous exposure. It was suggested that there was a low incidence of adult infections due to the high level of exposure to *Coxiella* during childhood, resulting in asymptomatic infections[169]. The seroprevalence surveys that have been performed were often difficult to compare since researchers use different populations, different diagnostic tests and cut-off criteria for these assays[168]. No recent seroprevalence studies have been published for the Queensland population.

1.8. Q fever in Children

Although Q fever has historically been regarded as an occupational hazard among adults in high risk occupations, an increasing number of reports of children being infected with *Coxiella* and contracting Q fever have emerged. These infections often present as an atypical pneumonia, which has a high rate of morbidity, and therefore an accurate diagnosis is important to institute rapid and appropriate treatment[106]. There is the continual, underlying consensus that Q fever in children has been rarely reported and most probably remains under diagnosed[6,59,61,63–65,68,169,170]. Most of the literature on Q fever in children centers on specific case reports [63,170,171]. Even with the limited data published on seroprevalence, seems to indicates a high level of exposure in children[60]. A study form Africa reported that the seroprevalence rate was higher in children <5 years of age (16.9%) than that of adults (8.9%)[61].

1.8.1. Disease States

Historically this disease has been mainly regarded as an occupational illness of adults. With this in mind, Q fever is often not considered for children presenting with “flu-like illnesses”, fever, headache, pneumonia, fatigue or hepatitis. Q fever infections in children occur through the same routes as that described earlier for adults

1.8.1.1. Acute Q fever

Q fever can result in a multi-system disease[68]. The clinical presentation of the acute disease, in children is predominantly a self-limiting, febrile illness as described for adults[60]. Other syndromes that have been noted in children with acute Q fever are myocarditis, central nervous system infections including atypical pneumonia, encephalitis, meningitis and pericarditis, hepatitis, haemophagocytosis and rhabdomyolysis. In Switzerland a study was done during an outbreak of Q fever, and it showed that only 12% (10 out of 80) paediatric cases confirmed with Q fever, were symptomatic compared to 64% of adults with the disease[172]. It was noted that the older children (11-14 year olds) tended to be more symptomatic than the younger children[60]. This correlates with adult infections as there is an increase in noted symptoms with an increase in age. There is greater disease severity also associated with an increase in age[164,173]. The literature shows that infections occur equally between boys and girls; this however,is not mimicked in the adult population where studies report that men are more often diagnosed with Q fever than women[60,63,66].

Q fever has been identified as one of the major infectious agents responsible for atypical pneumonias in children. Other organisms responsible for atypical pneumonia include *Mycoplasma pneumonia*, Legionella and *Chlamydia pneumoniae* which, together with *Coxiella*, may be responsible for over 40% of these infections[174].

1.8.1.2. Chronic Q fever

There are very few reported cases of chronic Q fever in children in the literature. There have only been two recorded clinical manifestations of chronic Q fever that occur in children, Q fever endocarditis and osteomyelitis. It is thought that children who suffer from chronic Q fever are likely to have a specific immunological defect, resulting in a delayed clearance of the organism[170].

A clinical review of Q fever in children reported that endocarditis in 4 out of 5 reported cases was coupled with an underlying congenital heart condition[60]. All the cases presented revealed vegetations on examination of echocardiograms and were identified serologically as having chronic Q fever. All the children in the recognised case reports had prolonged symptoms for several months before a diagnosis was made. There are six documented cases of osteomyelitis in children due to chronic Q fever to date[61,170]. The majority of children had bone lesions for up to 5 years before the diagnosis of Q fever was established and appropriate treatment could commence.

1.8.2. Treatment of Q fever in Children

As is the treatment for adults, children require prolonged courses of tetraxycycline, rifampicin, ciprofloxacin and hydroxychloroquine to combat the disease. Treatment may be recommended for up to 3 years for chronic Q fever endocarditis and even longer for children with chronic osteomyelitis[121]. There is prolonged follow-up in Q fever patients to avoid and or identify possible later relapses of the disease[73].

1.8.3. Vaccination of Children

In Australia a whole cell vaccine against Q fever, Q Vax® was trialed and released for use by CSL in 1989 for those aged 16 years and over[125]. The vaccine was designed to protect the "at risk population", that is, those that had a high occupational risk of contracting Q fever. The majority of these vaccine candidates were 16 years or over and hence children were not included in vaccine trials. The lower age limit for the Q Vax® is not known, as there is limited safety and efficacy data with regard to children under 16 years of age[63,125].

With increasing numbers of notifications in children, especially in South East Queensland, it seems likely that the vaccine should be made more widely available to include this age group. Although the current literature reports far more cases of Q fever in adults than children, studies of seroprevalence in children are limited. However, those that do contain data on children, indicate that they are frequently exposed to *C.burnetii,* and published data suggests that there is an increase in symptomatic infections of Q fever with age[60,66,166]. The lack of information regarding Q fever infection in children may be attributed to the a number of factors, a) symptomatic Q fever infection increases with age, and therefore is more rare in children than adults b) the exposure to *C.burnetii* in children is different from adults or c) Q fever is often not considered and diagnosed as a childhood infection[60].

1.9. Thesis Summary

At the commencement of this study, there was considerable lack of information regarding the epidemiology of *Coxiella burnetii* in Queensland, and to some extent Australia. A national vaccine strategy was in place, but this strategy was based on only fragmentary historical data gathered more than 20 years ago, and focused on adult males working in high risk occupations. Also, children were not considered for Q fever vaccination, and there had been no information about the risk or incidence of Q fever in children. Clearly, such data are vital if Australia is to develop a comprehensive vaccine strategy that includes all community groups at risk, including children.

To address this knowledge gap, the sections described in the following research Chapters of this study sought to address these specific aims:

Chapter 2: General Materials and Methods

In this section of the study, a comprehensive range of laboratory tools was developed to detect *Coxiella* and to determine the presence of *Coxiella* specific antibodies in clinical specimens.

Chapter 3: Analysis of Q fever Notification Data in Queensland

There were no previous studies that had analysed Q fever notification data for the Queensland population. The broad assumption was that Q fever was predominantly a

disease associated with rural communities. However, this assumption appeared to be based on anecdotal evidence rather than published scientific analysis.

The study in this Chapter did examine the comprehensive Queensland notification data available between the years 1984 to 2014, aiming to determine which communities and population groups report the greater incidence of disease. A particular emphasis was be to examine the rate of disease in children.

Chapter 4: Seroprevalence of *Coxiella burnetii* in Humans and Animals in Queensland

Although the analysis of notification data is important to identify the incidence of disease, such data do not give an indication of the rate of exposure to *Coxiella* in the general population. Also, there were no data to indicate if domestic and native animals are exposed to the bacterium.

The study in this Chapter sought to determine the level of exposure to *Coxiella* in both humans and animals by determining seroprevalence in these groups of subjects. Such data was be compared to the notification data from Chapter 3, to determine if the risk of Q fever disease is confined to specific population groups, or if the risk of exposure is wider than assumed.

Chapter 5: Potential Environmental Sources of Q fever Infection for Humans Residing in Queensland.

The acquisition of Q fever disease in Queensland is considered to be higher than most other populations, yet there is little published data to identify the potential sources of infection to the Queensland population.

The study described in this Chapter did examine a range of animal, dust and soil samples to determine the presence of *Coxiella*, and identify those that may pose a risk for infection to the human population.

Chapter 6: Factors Contributing to the Potential Under-Diagnosis of Q fever in Queensland

The incidence of Q fever as gathered by health statistics relies on accurate diagnosis of the disease in the laboratory. However, many different diagnostic algorithms are used with

different performance characteristics in terms of sensitivity and specificity. In addition, Q fever may present with a wide range of symptoms, and therefore is often not considered in the wider clinical context.

This study did examine the diagnostic algorithm used by most laboratories in Queensland and determine the rate of successful diagnosis associated with these. In addition, it investigated samples submitted for the laboratory diagnosis of infections which present with symptoms that may be due to a *Coxiella* infection, yet have not been tested for this disease. This data provided an estimate of the level of under-reporting of Q fever, which has a direct impact on the accurate estimation of disease incidence in Queensland.

Chapter 7: Clinical Case Studies of Q fever from Queensland

The clinical diagnosis of Q fever infection is often difficult, particularly when the patient presents outside the clinical context normally associated with the disease. To highlight the diverse range of factors that lead to infection, many not normally associated with traditional *Coxiella* acquisition, a number of case studies was examined, with a particular emphasis on the manifestation of Q fever infections in children.

Chapter 8: Molecular Typing of *Coxiella burnetii* in Queensland Samples

At the time of commencement of this study, there were no data regarding the molecular epidemiology of *Coxiella burnetii* in Queensland, and only very limited data regarding Australia. Identifying genotypes is important for studies in bacterial evolution and pathogenesis, and have an important impact on the design and development of vaccines, particularly in the local context.

The study in this Chapter applied genotypic analysis to identify *Coxiella* strains circulating in Queensland, and examine their relationship to other Australian genotypes, and to those that have been reported globally.

Chapter 9: Development of a Cell Mediated Immunity Assay to Determine Previous Exposure to *Coxiella burnetii*

One of the serious limitations of vaccination for Q fever in humans has been the range of adverse reactions that are associated with pre-vaccination screening using the skin test. This is particularly the case for children. Secondly, the skin test, and the determination of

Coxiella antibody status are notoriously problematical, and do often not provide an accurate assessment of previous exposure to the bacterium.

This study established and validated a method to measure the cell mediated immunity in vaccine candidates, pre-vaccination, by measuring IFN- ɣ in a cytokine release assay. This method has many advantages over existing pre-screening methods, and is highly applicable for measuring pre-existing exposure in children. The use of this method may allow the wider introduction of the vaccine to population groups that are now excluded because of inadequate pre-screening protocols.

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Chapter 2

General Materials and Methods

2.1. Ethics Approvals:

All studies described in this thesis were carried out at the Queensland Paediatric Infectious Diseases Laboratory, located at the Sir Albert Saksewski Virus Research Centre (SASVRC), Royal Children's Hospital (RCH), Brisbane, Queensland.

Ethics approvals were obtained for all studies from the following institutions:

- Royal Children's Hospital by the RCH Human Research Ethics Committee (HREC)
- University of Queensland by the Medical Research Ethics Committee (MREC)
- Director General, Pathology Queensland, Queensland Health - For the Release of Confidential Information for the Purposes of Research under the provision of Section 280 of the Public Health Act 2005

The research performed for this thesis and the reporting of findings resulting from the research, was conducted within the guidelines set out for research as stated in the *National Statement on Ethical Conducted in Human Research (2007),* and the Australian Government's National Health and Medical Research Councils guidelines stated in the *Australian Code for the Responsible Conduct of Research.*

This was in addition to the ethical guidelines enforced at *Queensland Health* under the *Research Management Policy* and the *Research Management Policy Implementation Standard – Ethical and Scientific Review of Human Research (2010).*

All work was conducted as a Queensland Health employee, and was performed with adherence to the *Queensland Health Code of Conduct Policy (2011).*

As a student of the University of Queensland, the research performed was also conducted within the recommendations by The University as part of the *Responsible Conduct of Research Policy (2011)* including codes of conduct, authorship, student integrity and misconduct.

Ethics was requested and granted for the following projects:

2.1.1. Determining the Seroprevalence of Any Infectious and/or Potentially Infectious Agent Including Viruses and Bacteria in the Population.

Reference HREC 2008/087

2.1.2. Review of Q Fever Notifications and Vaccine Coverage in Queensland

Reference HREC/08/QRCH/66 and Queensland Health RD001050

2.1.3. Comparison of Laboratory Diagnostic Methods for the Identification of Q Fever

Reference HREC/08/QRCH/88

2.1.4. Comparison of the Cellular Immune Response and the Humoral Immune Response in People Naturally Infected or Vaccinated Against Q fever.

Reference HREC/10/QRCH/17 and UQ 2010000431

2.1.5. Prevalence of Q fever in the Environment in Queensland

Reference HREC/11/QRCH/21.

2.1.6. A Review of Q fever Cases from Queensland Health.

Reference Permission granted letter dated: 8th January, 2014 by Professor John Pearn (Chair) Children's Health Services Queensland Human

2.2. General Methods Applied Throughout the Thesis:

2.2.1. DNA Extraction for *Coxiella burnetii* PCR:

Nucleic acids were extracted from a wide variety of samples for the screening of *Coxiella burnetii* using PCR. The samples were extracted using commercially available kits from Qiagen (Brisbane, Australia). Before DNA sample extraction commenced, a known volume of equine herpes virus (EHV) standard, equivalent to $1x10^4$ copies of EHV DNA, was added to each of the different sample types. This step is performed to examine the efficiency and reproducibility of the various extraction processes by comparing and monitoring Crossing Threshold (CT) values for the EHV standard PCR [1,2]. Nucleic acids were then extracted using the methods below for each of the various sample types; using either the Mini stool kit (Qiagen, Brisbane, Australia), the QIAampDNA Mini kit (Qiagen, Brisbane, Australia) or the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia)[3].

2.2.1.1. Extraction of Blood, Milk and Urine

Both human and animal blood, milk and urine samples were extracted using the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia) as per the manufacturer's instructions. Briefly, 200µL of each sample was spiked with 5µL of EHV standard and was processed using the QIAampDNA Mini kit (Qiagen, Brisbane, Australia), a spin column DNA extraction method with DNA being eluted in a final volume of 100uL.

2.2.1.2. Extraction of Serum Samples

Serum samples from humans were processed in batches of 95 samples and one water sample as a negative control. Samples were extracted using the QIAxtractor semi-automated system (Qiagen, Brisbane, Australia) according to the manufacturer's instructions. The QIAxtractor DNA procedure involves the specific binding of nucleic acids to silica membranes using simple bind, wash, and elution steps.

During the process, 200µL of serum is loaded to a lysis plate and cell lysis is performed using lysis and binding buffers. The DNA released adheres to the silica filter matrix which is then washed using alcohol wash buffers to remove contaminants (PCR inhibitors) This is followed by the addition of elution buffer to alter the charge in solution which releases the bound DNA from the matrix into a capture plate in a 100µL final volume. These were stored in 96 well racks (Matrix, Thermo Scientific, Australia) and stored at minus 80°C.

2.2.1.3. Extraction of Animal Stool Samples

Animal stool samples were extracted using the QIAamp DNA Stool Mini kit (Qiagen, Brisbane, Australia) following the manufacturer's recommendations. Briefly, one pellet of sample (approximately 1.5-2.0 g) was added to kit ASL buffer for cell lysis and heated to 95°C for 15 minutes. Inhibitors were removed by absorption using the InhibitEX tablets (Qiagen, Brisbane, Australia) followed by centrifugation. DNA in the supernatant was then extracted using the QIAamp spin columns as described in the kit insert and DNA was eluted in a final volume of 100µL.

2.2.1.4. Extraction of Whole Tick Samples

Tick samples were extracted whole using the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia). Briefly, ticks were placed on glass slides and the blood and tissue was released by teasing out the tick with a scalpel blade. All parts of the tick were added to 200µL of tissue lysis buffer containing 20µL of proteinase K and incubated overnight at

56°C on a mechanical rocker at low speed for cell lysis. The samples were then treated as per manufacturer's instruction. Briefly, 200µL of lysed tick sample spiked with 5µL of standard EHV and was processed using an ethanol buffer and a spin column DNA extraction method with the DNA being eluted in a final volume of 100µL.

2.2.1.5. Extraction of Environmental Soil Samples

Nucleic acids in soil samples were extracted as by a previously published protocol by Fitzpatrick et al. and Kersh et al., using commercially available extraction kits [4,5]. Soil samples were pre-treated prior to DNA extraction. Briefly, 5g of soil was mixed with phosphate buffer saline (PBS) and put on a mechanical rocker at room temperature for one hour. Samples were then centrifuged for 5 minutes at 123g/800 rpm to remove large particles and the supernatant was retained for further centrifugation at 20,000g to pellet bacteria. The pellet was resuspended in 1ml of PBS and used in the DNA extraction protocol previously described for the QIAamp DNA Stool kit (Qiagen, Brisbane, Australia), where the cellular material was lysed and then inhibitors were removed before continuing with the column extraction method and eluting the DNA in a final elution volume of 100µL.

2.2.1.6. Extraction of Environmental Dust Samples

Atmospheric dust samples are collected on Whatman glass fibre filter paper with a nominal pore size of 1.6µm from a high-volume air sampler (HVS), and were placed in sterile tubes containing 5 ml of sterile water. These were incubated at room temperature on a mechanical rocker overnight to release bacterial particles, followed by DNA extraction. Briefly, 200µL from the 5ml dust suspension was processed using the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia) following the manufacturer's method for blood extraction using the spin column method. The DNA was eluted in a final volume of 100µL of water for PCR.

Dust swabs were processed as previously described by Kersh et el [5]. Briefly, swabs were placed in 1ml of sterile PBS and vortexed for 1 minute to release dust and bacteria trapped in the swab. A volume of 200µL was then processed using the QIAamp DNA Stool kit (Qiagen, Brisbane, Australia) as per the previously published protocol by Kersh et al in which the PBS containing dust and bacteria are lysed, treated for inhibitors, followed by a silica spin column extraction method. Extracted DNA was eluted in 100µL of water for PCR.

2.2.2. Real-time (RT) PCR Methods

2.2.2.1. Coxiella burnetii PCR

Real-time PCR for the detection of *C. burnetii* was performed using two individual PCR assays with specific primers and probes targeting two different gene targets in order to increase specificity of the result. The first gene was that of the repetitive transposon-like element *IS1111* of the transposase gene, which can be repeated up to 20 times throughout the *Coxiella* genome. The second gene target was the *Coxiella* Outer membrane (*com1*) gene, coding for the 27kD protein. Both these assays were previously described by Klee et al. (2006a) and Lockhart et al. (2011)(see Table 2.1) [6,7].

Briefly, each reaction mix consisted of 12.5 µL of Quantitect Probe PCR Mix (Qiagen, Brisbane, Australia), 10 pmol of each *com1* primers or *IS1111* primers, 5 pmol of *com1* probe or *IS1111* probe and 5 µL of template DNA in a final reaction volume of 25 µL.

Amplification was performed in a ABI7500 (Applied Biosystems, Australia) or a Rotor-Gene Q or a Rotor-Gene 6000 (Qiagen, Brisbane, Australia) using the following cycling conditions: 15 minutes incubation at 95°C, followed by 50 cycles of 95°C for 15 seconds and 60°C for 1 minute. Previous validation had shown that these instruments gave identical results.

All primers and probes were synthesised by GeneWorks (Hindmarsh ,Australia). Lyophilised primers and probes were reconstituted to a standard 200µM stock concentration with sterile, distilled water and were stored in a dedicated PCR set up laboratory in a -20°C freezer. Working stocks of primers and probes were made fresh as and when required for PCR testing. The following PCR procedures were followed for all Q fever and EHV reactions (Table 2.2 and 2.3).

2.2.2.2. EHV Real-Time PCR

EHV real-time PCR was performed on all extracted samples. Briefly, EHV PCR mix consisted of 10 pmol each primer; EQHSV-F and EQHSV-R, and 4 pmol of probe EQHSV (Table 2.1), and 12.5 ml of Quantitect Probe master mix (Qiagen, Brisbane, Australia). This assay was performed in a ABI 7500 (Applied Biosystems) or a Rotor-Gene Q or a Rotor-Gene 6000 (Qiagen, Brisbane, Australia) using the following cycling conditions: 15-min incubation at 95°C, followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

C.burnetii was considered to be present in a sample if a positive result was obtained for either gene target.

Table 2.1: List of *C.burnetii* primers and probe sequences used for PCR assays throughout this thesis including the extraction control primers EQHSV

Target Gene	Name	Oligonucleotide Sequence	Reference
***IS1111* Transposase Gene**	Cox-F	GTC TTA AGG TGG GCT GCG TG	Klee (2006)[6]
	Cox-R	CCC CGA ATC TCA TTG ATC AGC	
	Cox-Probe	FAM-AGC GAA CCA TTG GTA TCGGAC GTT TAT GG-BHQ	
***com1* Outer Membrane Gene**	Com1-F	AAA ACC TCC GCG TTG TCT TCA	Lockhart (2007)[7]
	Com1-R	GCT AAT GAT ACT TTG GCA GCG TAT TG	
	Com1-Probe	FAM AGA ACT GCC CAT TTT TGG CGG CCA -BHQ1	
Equine Herpes Virus	EQHSV-F	GAT GAC ACT AGC GAC TTC GA	Rockett (2011)[1] Schuller (2010)[8]
	EQHSV-R	AGG GCA GAA ACC ATA GAC A	
	EQHSV-Probe	FAM-TTT CGC GTG CCT CCT CCA G-BHQ-1	

Table 2.2: Preparations of working stock primers (1:20 of 200M stock) and probes (1:10 of 200M stock)

Reagents	Stock Concentration	x1	Final Concentration
H_2O		450 µL	
Specific Forward Primer	[200µM]	25 µL	[10µM]
Specific Reverse Primer	[200µM]	25 µL	[10µM]
Total Volume		500 µL	
Reagents	**Stock Concentration**	**x1**	**Final Concentration**
H_2O		90 µL	
Specific Probe	[100µM]	10 µL	[10µM]
Total Volume		100 µL	

Table 2.3: Composition of general PCR reaction mix used for PCR reactions in all the studies described throughout this thesis

Reagents	Working Concentration	x1(μL)	Final Concentration
H_2O		6.25	
Quantitect Probe PCR Mix (Qiagen)		12.50	
Specific Probe	[20μM]	0.25	[0.2μM]
Specific Primers	[10μM]	1.0	[0.4μM]
DNA		5.0	
Total		25.0	

2.2.3. Immunofluorescence Assay for Q fever (IFA)

2.2.3.1. The General Principle of IFA:

The IFA method used throughout this thesis was an adaptation of the IMVS IFA methodology employed by Marmion et al. (personal communication).

Dilutions of the test serum were placed onto microscope slides previously coated with phase I and phase II organisms of *C.burnetii*. If the serum contained specific antibody, the antibodies adhered to the *Coxiella* antigens during the initial incubation step. After washing to remove non-specific antibodies, fluorescein-labelled anti-human class specific immunoglobulin conjugate was added. During the second incubation step, the conjugate adhered to the patient antibodies bound to the bacterial antigens in the initial incubation step. Further washing to remove excess unbound conjugate was performed. The slides were dried and mounting fluid added before examining the slides on a fluorescent microscope. If the serum tested contained Q fever antibodies, the organisms with the antibodies bound showed apple- green fluorescence as illustrated in Figure 2.1.

2.2.3.2. Specimen of Choice for IFA

The sample of choice for the IFA assay is serum stored at 2-8°C for 5-7 days or stored at -20°C.

2.2.3.3. Reagents and Materials used in IFA

1. Teflon coated microscope slides with 24 wells from Menzel-Gläser (Thermo Fisher Scientific Inc , Australia, Cat # MIC040).
2. Virion /Serion (Wurzburg, Germany) *C. burnetii* phase I (Cat # 1227) and phase II (Cat # 1123) organisms lyophilised. Stored at 2-8 ^{0}C.
3. PBS -phosphate buffered saline pH7.2
4. 3% Chicken yolk sac (CYS) in PBS (IMVS - Adelaide, South Australia) -
   i. Stock solution stored at –20 ^{0}C.
   ii. Working solution (0.5% suspension): Used for serum dilution and antigen preparation.
5. Controls - Positive and negative control sera, pooled from previously characterised specimens.
6. Anti-human IgG (Sigma - Cat # 982041020 GDF) and anti-human IgM (Sigma -Cat # 983031020 MAF), both FITC conjugated. Stored at 2-8 ºC. Fresh dilutions were made for each test batch.
7. Evans Blue dye – for counterstaining cells; 1 drop of dye (10% solution) to 5 ml of PBS, stored at 2-8 ºC; also used in conjugate dilutions.
8. Buffered glycerol mounting medium pH 8.0 – 8.4.

2.2.3.4. Equipment and Apparatus:

1. 37°C incubator
2. Microscope: Nikon Eclipse E600; Excitation peak wavelength = 490 nm, Emission peak wavelength = 520 nm
3. Wash station with magnetic stirrer
4. Microtitre plates with 96 U bottom wells for serum dilutions

2.2.3.5. Slide Preparation:

1. Clean microscope slides with methanol and rub with tissue to remove any residue if required, label slides.
2. Reconstitute the vial of phase I and phase II CF antigens with 1 ml of distilled H_2O as per manufacturer's instructions. Mix well.
3. Dilute the rehydrated commercial antigens 20 times with 0.5% CYS.
4. Require 3ml of working antigen suspension to make 25 (24 well) slides.

For example: 150μL reconstituted antigen
500 μL 3% egg yolk sac
2350 μL distilled water

5. Add 4μL of the suspensions to each well and allow to air dry.
6. When fully dry, immerse the slides in methanol for 5 minutes, to fix the organisms.
7. Remove slides from the methanol and allow to dry for 10 minutes.
8. Place the slides into labelled boxes for storage at - 20°C

2.2.3.6. Screening Assay Method

1. Remove the required number of slides from the storage box in the freezer and place in a humid chamber whilst preparing serum dilutions. Ensure the slides are well dried by placing in the drying chamber for a few minutes before adding specimen dilutions.
2. Use a microtitre plate to prepare specimen and control dilutions.
3. **For IgG testing** - for patient and control sera, prepare 1:10 dilutions by pipetting 10μL serum to 90μL of 3% CYS in dedicated microtitre plate.
4. **For IgM testing** - for patient and control sera, add 10μL of serum to 70μL of RF Absorbent plus 20μL of 3% CYS in dedicated microtitre plate.
5. Label the slides with control and specimen dilution numbers, phase type and specific immunoglobulin. Load 10μL of each dilution (controls and sample) to the appropriate slides.
6. Incubate for 30 minutes at 37°C in a moist chamber.
7. Wash all slides in fresh PBS buffer, employing a brief rinse followed by two 5-minute washes in wash station with stirrer, and finally a brief rinse into distilled water.
8. Gently blot all slides before air-drying.
9. Calculate the amount of each conjugate needed, allowing for 10 μL / well. Conjugate dilutions are on each reagent bottle; dilute in PBS with Evans blue 1/100 counterstaining of the FITC IgG (previously determined for specific lot number)
10. Add 5-10 μL of specific conjugate to the appropriate wells; incubate for 30 minutes at 37 °C.
11. Repeat the washing and drying steps.

Apply mounting fluid and coverslip for each slide and examine the slides using a fluorescent microscope at a magnification of x100 for apple green fluorescence (Figure2.1)

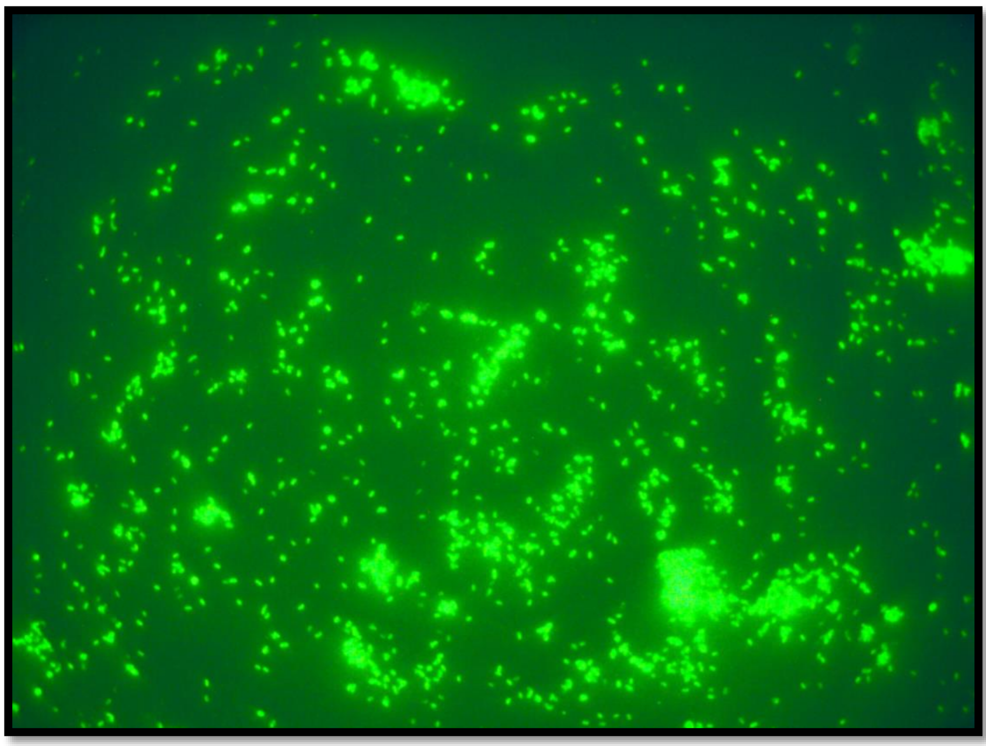

Figure 2.1: Image: Fluorescence observed of *Coxiella* phase II bacteria following staining with positive human IgG antibodies (100x magnification).

2.2.4. Commercial CFT Antigen: *Coxiella burnetii* whole cell bacteria

Whole cell bacteria suspensions (*C.burnetii* - Nine Mile Strain) were used in the preparation of both IFA slides, and as a positive control for all PCR assays. The bacteria were obtained commercially from Virion /Serion (Wursburg,Germany – Table 2.3) in the form of whole cell bacteria, formalin inactivated phase I and II from the Nine Mile strain. This source of whole cell bacteria was used as a control to validate PCR assays, and strain typing methods.

Table 2.4: *Coxiella burnetii* antigen used in IFA; catalogue numbers - Source: Virion /Serion (Wursburg,Germany).

Antigen	Catalogue Number
***Coxiella burnetii* (phase I)**	1227
***Coxiella burnetii* (phase II)**	1123

2.2.5. Commercial ELISA Kit Method for Q fever Antibody Screening

The Panbio (Brisbane, Australia) *Coxiella burnetii* (Q fever) ELISA kits were used for the qualitative detection of IgG and IgM antibodies to *C.burnetii* in serum. This is a commonly used tool in the clinical diagnosis of Q fever as well as a tool for assessing immune status in potential Q fever vaccination candidates. The presence of Q Fever IgM antibodies is highly suggestive of recent or active Q fever infection. Presence of Q fever IgG antibodies indicates previous or resolving infection.

2.2.5.1. Principle of the Q fever ELISA

Diluted patient serum containing IgM or IgG class antibodies bind with *C. burnetii* antigen (Henzerling strain), which has been fixed to the polystyrene surface of a micro-well in a microtitre plate. After washing, to remove all unbound antibodies and excess serum, peroxidase conjugated, anti-human IgG or IgM class-specific antibody, is added to the wells which in turn bind to the immobilised patient's *C.burnetii* antibodies. After a second washing to remove unbound conjugate, a colourless chromogenic substrate, tetramethylbenzidine (TMB) is added. On incubation this produces a blue colour reaction, which is stopped using acid producing a yellow end colour. The optical density of the final colour can be read with an ELISA plate reader.

In the IgM assay, patient's samples are initially treated with absorbent solution designed to reduce non-specific reactions by removing competing IgG antibody that may cause false negatives and rheumatoid factor that may cause false positive reactions.

2.2.5.2. Specimens for Analysis

The sample of choice is serum stored at 2-8°C for 5-7 days or stored at -20°C.

2.2.5.3. Reagents Used

2.2.5.3.1. The Q Fever IgG ELISA Test Kit (Panbio QFB01G).

Contains:

- *C. burnetii* Phase-II (Henzerling strain) Antigen Coated Microwells (12x8 wells).
- Serum Diluent: Two bottles 50ml (pink) Tris buffered saline. Ready for use.
- Positive Control: Red capped vial, 200μL of human serum.
- Negative Control: Green capped vial, 200μL of human serum.
- Cut-off Calibrator: Yellow capped vial, 400μL of human serum.

- Conjugate: 15ml (green) Horseradish peroxidase conjugate sheep anti-Hu IgG.

2.2.5.3.2. The Q Fever IgM ELISA Test Kit (Panbio QFB01M)

Contains:

- C. burnetii Phase-II (Henzerling strain) Antigen Coated microwells (12x8 wells).
- Serum Diluent IgM: One bottle 22ml (pink) Tris buffered saline. Ready for use.
- Serum IgG Absorbent: 22ml (blue) goat anti-human IgG (precipitating antibody to remove competing IgG antibody and rheumatoid factor)
- Positive Control: Black capped vial, 200µL of human serum.
- Negative Control: White capped vial, 200µL of human serum.
- Cut-off Calibrator: Orange capped vial, 400µL of human serum.
- Conjugate: 15ml (yellow) Horseradish peroxidase conjugate sheep anti-Hu IgM.
- Generic Reagents for both assays:
- Substrate: Tetramethylbenzidine (TMB) 15ml with hydrogen peroxide.
- Stop Solution: 15 ml of 1M Phosphoric Acid.
- 20X Wash Buffer Concentrate: 60 ml of phosphate buffered saline with detergent. Prepare a working solution by diluting the 20X solution to make up a final volume of 1200 ml with distilled water. Make up in Wash Bottle A and store the remaining portion in a reagent bottle at room temperature for future use. Label the bottle with the lot number and expiry date.

Procedure:

- Each test serum, control and cut-off calibrator must be diluted 1:100. Use a clean microtitre plate to prepare the initial dilutions.
  NB: IgG and IgM dilution procedures are different.

Q Fever IgG

- Add 90µL of kit Serum Diluent to the required number of wells of a microtitre plate.
- Add 10µL of each patient serum, controls or cut-off calibrator to give a 1:10 dilution.
- Add 180µL of Serum Diluent to the required number wells of a microtitre plate. Using a multichannel pipette transfer 20µL from each well of the previous step to the Serum Diluent in the second ELISA plate to give a 1:100 dilution.

Q Fever IgM

- Add 90µL of Serum Diluent to the required number of wells of a microtitre plate.
- Add 10µL of each patient serum, controls or cut-off calibrator to give a 1:10 dilution.
- Add 180µL of Serum Absorbent to the required number of wells of a microtitre plate. Using a multichannel pipette transfer 20µL from each well of the previous step to the Serum Absorbent in the ELISA plate to give a 1:100 dilution.

General steps for both IgG and IgM ELISA

1. Seal the wells, and incubate the plate for 30 minutes at 37°C.
2. Wash the plate with working Wash Solution 6 times using wash procedure outlined below. Turn the plate upside down and tap firmly on a paper towel to remove excess wash solution.
3. Add 100µL of the appropriate IgG or IgM conjugate to each well.
4. Reseal and incubate the plate for 30 minutes at 37°C.
5. Wash the plate as described previously.
6. Add 100µL of TMB substrate to each well.
7. Reseal the plates; incubate for 10 minutes at room temperature.
8. A blue colour will develop.
9. Add 100µL of stop solution to each well. The blue colour will change to yellow.
10. Read each reaction in dual wavelength mode at 450nm and 620nm within 30 minutes.

Washing Procedure

1. Discard contents of plate in appropriate waste container
2. Fill wells with wash buffer using a suitable squeeze bottle. (Avoid bubbling of wash buffer as this may reduce wash efficiency)
3. Discard wash buffer from wells immediately
4. Refill wells with wash buffer and discard immediately
5. Repeat step 3 another 4 times (This will make a total of six washes)
6. After the final wash, discard contents of wells and tap the plate on blotting paper to ensure all wash buffer is removed.

Reading Results

The mean cut-off absorbance value is determined. Those cut-off values lying more than 10% outside the mean should not be used and the mean cut-off value must be recalculated. The cut-off is also corrected with the calibration factor noted on the specification sheet accompanying each kit (lot specific).

Cut-off value = Mean absorbance of calibrators x calibration factor.

Panbio units (PBU) = Sample Absorbance x 10 times Cut-off value

Run Validity

For an assay to be valid, the control samples must be within a range of predetermined values. Acceptable values for positive and negative controls, the cut-off calibrator and positive/cut-off ratio are provided in the specification sheet accompanying each kit.

Result Interpretation

The upper limit for negative results is 9 PBU. Test sera with less than 9 PBU are considered Non-Reactive for IgG or IgM antibodies to Q fever.

The lower limit for positive results is 11 PBU. Test sera with greater than 11 PBU are considered Reactive for IgG or IgM antibodies to Q fever.

Sera tested for IgM, and IgG with values between 9 and 11 PBU, should be repeated as the result is deemed ***Equivocal*** (Table2.5).

Table 2.5: Interpretation of Panbio ELISA kit results for test sera

PBU	Result	Interpretation
<9	Negative	No detectable IgM antibody to Q Fever.
9 - 11	*Equivocal*	Presence or absence of detectable levels of IgG antibody to Q Fever cannot be determined. The test should be repeated.
>11	Positive	Detectable levels of IgM antibody to Q Fever.

2.2.5.4. Equipment and Apparatus Used in the Assay

1. Wash bottles
2. ELISA Plate Reader: Molecular Devices Spectramax 340PC384 plate reader
3. 37°C Incubator
4. Microtitre plate with 96 U bottom wells for serum dilutions
5. ELISA plate sealers

2.2.6. Performance Characteristics of the IFA and ELISA for IgG Antibody Detection

The IFA and ELISA methods were used throughout this study to determine IgG seroprevalence to *Coxiella*. Therefore the performance characteristics of these assays were established.

2.2.6.1. Serum Samples Used to Determine Performance Characteristics

De-identified serum samples (n=2122) collected from patients with non-Q fever related infections were obtained from public and private pathology laboratories. Queensland Health Pathology Services – Central Laboratory at Herston, and the Toowoomba Hospital servicing the highest risk rural region in Queensland (postcode 4350)– provided serum samples collected between October 2008 and June 2009. De-identified sera were also obtained from a private pathology laboratory for previous investigation into allergies in children. These samples were collected from January to May, 2007 and from January to April 2008, and were used to determine the performance parameters of the IFA and ELISA.

2.2.6.2. Evaluation of the IFA and ELISA Methods for Screening Human Sera

Of the 2,122 specimens examined, there were 98 (4.6%) that were positive for *Coxiella* phase II IgG by both screening methods (IFA and Panbio ELISA) and 1997 (94.1%) samples that were not detected by either method. There were 27 (1.3%) discordant results (Table 2.6). Further testing was performed on the 27 discordant results using the commercially available IFA (Focus Diagnostics, Cypress, USA). Of the 21 IFA positive, ELISA negative samples: 11 were confirmed to be positive. Of the 6 IFA negative/ELISA positive results, none were confirmed as positive (Table 2.7). This resulted in a total of 109 positive specimens for further analysis: 98 with both screening assays positive, and 11 positive following subsequent discrepant testing.

Table 2.6: Summary of results for IFA versus ELISA screening on 2122 serum samples, showing 27 discrepant results which are highlighted in red.

Method Result	IFA Detected	IFA Not Detected
ELISA Detected	98	*6*
ELISA Not Detected	*21*	1997

Table 2.7: Summary of the discordant results from serology screening and confirmation testing by commercial IFA assay as the reference standard showing 11 results confirmed as positive

Test Results	Discrepant Results	Focus IFA Detected Result
IFA Detected/ ***ELISA Not Detected***	21	11
ELISA Detected/ ***IFA Not Detected***	6	0

From the above the number of true positives was determined as 109, and the number of true negatives as 2013. Using this algorithm for defining positive and negative results, the performance characteristics of the IFA and ELISA were established and shown in Table 2.8.

Table 2.8: Performance parameters for the IFA (IMVS) and indirect ELISA assays used to determine the seroprevalence in human sera. * Positive Likelihood Ratio was determined

	IFA (IMVS Method)		ELISA	
Sensitivity	100%	96 – 100%	95.6%	90 -99%
Specificity	99.5%	99 – 100%	99.7%	99 – 100%
Positive Predictive Value	91.6%	85 – 96%	95.6%	90 - 99%
Negative Predictive Value	100%	99 – 100%	99.7%	99 – 100%
Positive Likelyhood Ratio*	202.3	109 – 375	385.9	160 - 927

$$\textbf{LR+} = \frac{\text{Probability of an individual with the condition having a positive test}}{\text{Probability of an individual without the condition having a positive test}}$$

LR+ = > 10 ; Large and often conclusive increase in the likelihood of disease.

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These results showed that sensitivity and specificity of the two assays were highly comparable, and supported the results previously published by Herremans et al[9].

2.3. Significant Outcomes from this Chapter

- The IFA and ELISA proved to be sensitive and specific methods for the detection of *Coxiella* antibodies
- Both the assays have a high positive and negative predictor value, ensuring a high correlation with previous exposure to *Coxiella*
- Due to the reliability and performance parameters met, these assays will be used throughout the studies for the detection of *C.burnetii* antibodies

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Chapter 3

Analysis of Q fever Notification Data in Queensland

Articles published as a result of work done in this chapter:

Tozer, SJ., Lambert, SB., Sloots, TP. and Nissen, MD. A Comprehensive Analysis of Q fever Notifications in Queensland Between 1984 – 2014. *Communicable Diseases Intelligence Journal*, (Submitted for Publication 27 July 2015)

3.1. Introduction

3.1.1. Surveillance of Q Fever in Australia

Since 1917, Australia has had a national notifiable disease surveillance system for monitoring infectious diseases, in which data were collected by individual states and territories under public health legislation. It was the responsibility of the medical practitioners to notify the relevant health authorities of certain communicable diseases and other infectious agents. The data were collated and results were distributed to medical professionals via medical journals such as the Medical Journal of Australia and the Commonwealth Departments' Journal of Health along with the Commonwealth Year Book [1]. The National Notifiable Disease Surveillance System (NNDSS) was implemented in 1990 under the Communicable Disease Network, Australia, in which more than 50 communicable diseases were monitored. This system, enforced under current Public Health legislation, requires that local health authorities notify the relevant State or Territory Health Departments. This reporting system distributes patients' de-identified records to the Australian Government Department of Health on a daily basis. Subsequently, information is made available to the Commonwealth agencies via the Communicable Diseases Intelligence Journal and a fortnightly reports from the Communicable Diseases Network Australia (CDNA) (http://www.health.gov.au/cdnareport).

There are many levels involved with disease surveillance within Australia. The responsibility at a national level is to provide assistance in prevention and control of public health issues involving communicable diseases along with implementing strategic measures in the event of public health outbreaks and to minimise the impact of communicable disease transmission within the immediate region.

For Q fever, Australia has been collecting data since 1952, focusing on gaining a more in-depth understanding of the disease epidemiology and subsequent disease burden[2] . The data are collected by the Communicable Disease Departments in each Australian state and territory. In Queensland, these data are collated and managed using the Notifiable Conditions System (NOCS), and compiled data are then provided to the NNDSS. A national case definition has been developed to allow recognition of Q fever cases and its documentation (Table 3.1)[3].

Table 3.1: Case definition and laboratory parameters used to diagnose Q fever[3]

Q fever Case definition

National definition from January 2004

Only confirmed cases are notifiable. Confirmed cases require either laboratory definitive evidence or laboratory suggestive evidence together with clinical evidence.

a. Laboratory definitive evidence
   - Detection of *Coxiella burnetii* by nucleic acid testing; *or*
   - Seroconversion or significant increase in antibody level to phase II antigens in paired sera tested in parallel in absence of recent Q fever vaccination.
b. Laboratory suggestive evidence
   - Detection of specific IgM in the absence of recent Q fever vaccination.
c. Clinical evidence
   - A clinically compatible disease.

Q fever has a wide diversity in the presentation of symptoms, including infections which may be asymptomatic. As a result, it is a disease that is underreported in Australia and the rest of the world [4,5].

In Australia, the disease Q fever is most frequently identified in patients residing in rural Queensland and northern New South Wales who typically have contact with large ruminants [6]. The disease is of public health concern, with the potential to cause extensive outbreaks, as recently occurred in The Netherlands[7]. Hence, surveillance for the disease is of utmost importance and should be continuous, with the results made available to veterinary and health professionals world-wide in order to reduce wide-spread disease and to be able to limit or at least control potential outbreaks.

The continual collection of Q fever disease data in Australia via NOCS, has highlighted the importance of identifying and monitoring those at risk of contracting the disease, and hence provides valuable information for the initiation of extensive screening programs and vaccination strategies within Australia. These strategies led to the establishment of the National Q fever Management Program (NQFMP)[8] in the year 2000, which was an Australian and world-first in the prevention of Q fever disease.

The NQFMP sought to raise education and awareness of those at risk of Q fever in high risk areas, along with providing medical staff with clinical and diagnostic background. The program included extensive Q fever pathology screening of at risk subjects followed by vaccine administration, and was initiated in 2001 in Queensland South Australia, Victoria and Western Australia as stage 1. The second stage of the program, stage 2, commenced in 2002 in the Australian Capital Territory and New South Wales. The Northern Territory reported very few cases, and opted out of the Federal Government program[8]. This program was funded nationally and was concluded at different times between June 2004 and December 2006 in different jurisdictions[6]. It was designed to identify subjects at "high risk" through extensive analysis of the national notification data over time. Such analysis highlighted that abattoir workers and farm workers were most at risk and should receive the vaccine immediately, and that veterinarians and other animal handlers (tanners, boners, butchers and shearers) should be offered the vaccine as a second stage to the program.

3.1.2. Australian Demographics and Q Fever

Australia has a total population of 23,625,600 inhabitants as of June 2014, and has one of the highest rates of Q fever disease globally, excluding outbreak episodes. Between 1954 and 2014 Australia had over 20,000 clinically confirmed cases of Q fever reported to the national surveillance units, despite this being a vaccine preventable disease[9] (Figure 3.1).

Australia has three times as many Q fever cases as are reported in the European Union, and records more than six times the number of cases diagnosed in the United Kingdom[6]. In addition, Australia had the highest annual notification rate recorded in 1993 at 4.9 cases per 100,000 persons. However, a rapid decrease followed the implementation of the NQFMP in 2000, after which rates dropped by more than 50% in the subsequent years of 2005-2006[6].

Laboratory diagnosis of Q fever in patients in Australia has been ongoing since 1936 and results have been included on the national surveillance register for over 60 years. There have been a number of notable outbreaks of Q fever recorded nationally during this time, highlighting once again the importance of the surveillance program (Table 3.2).

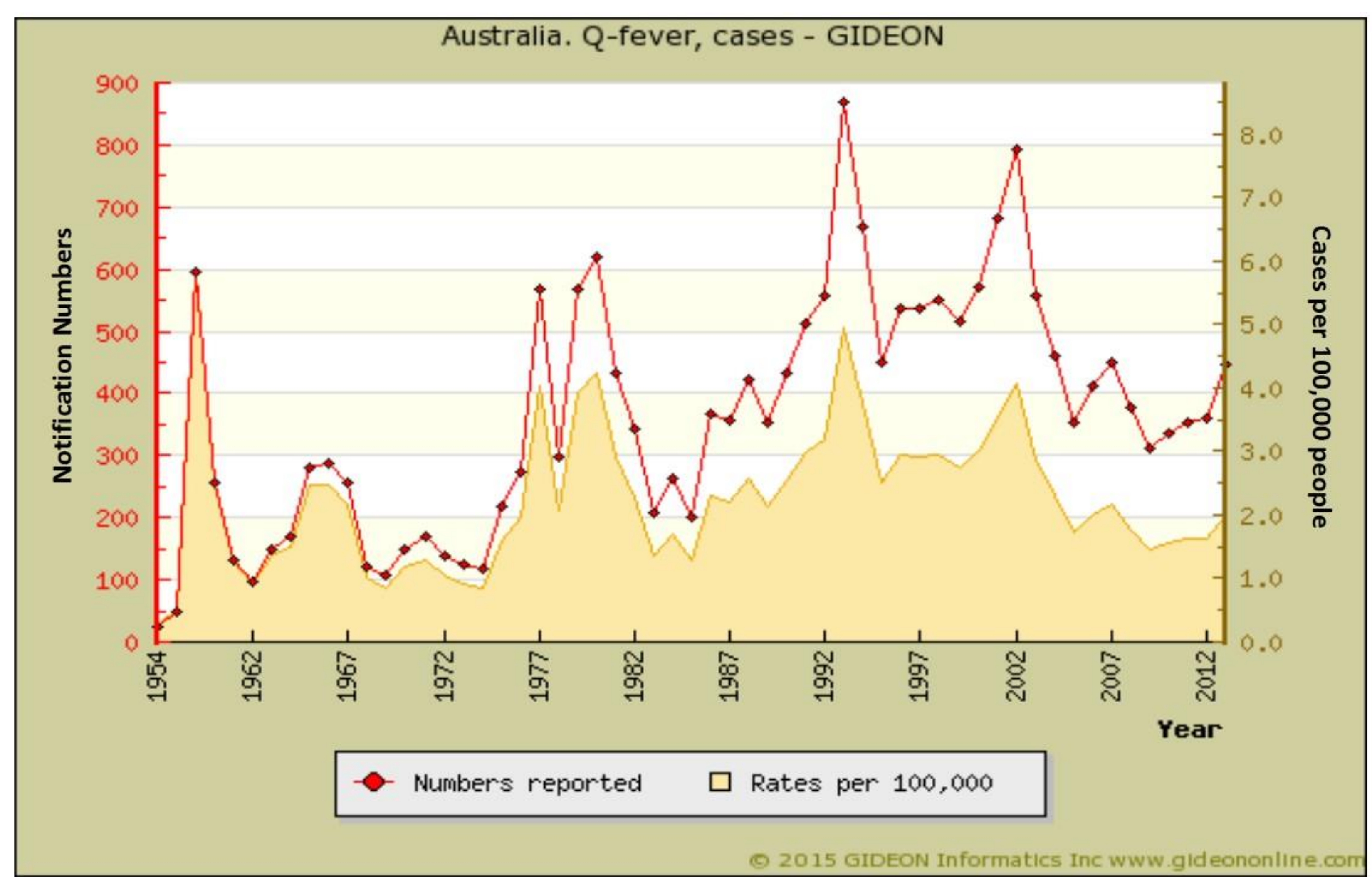

Figure 3.1: Australian Q fever notifications for 1954 to 2014. These data show Q fever rates that are twice those reported in France, a country that invests a large amount of time and resources into *Coxiella* research[52].

Table 3.2: Small and medium size outbreaks of Q fever in Australia

Year	Event	Reference
1957	An outbreak (52 cases) was reported in a meat-works in Adelaide, South Australia.	Beech[10]
1959	An outbreak of Q-fever in Queensland was associated with sheep contact.	Derrick [11]
1969	An outbreak in a Brisbane "meat works" affected 7.9% of workers.	McKelvie[12]
1979	An outbreak (110 cases) was reported at an abattoir in Victoria.	Buckley[13]
1998	An outbreak (29 confirmed and 8 suspect cases) was reported at an abattoir in New South Wales.	No Author[14]
2004	An outbreak (9 confirmed and 6 suspect cases) was reported among farmers in South Australia.	Gilroy[15,16]
2005	An outbreak (5 cases) was reported among persons involved in calving activities, in New South Wales.	Rodriguez[17]
2006	An outbreak (4 cases) was reported among workers at a cosmetics factory.	Wade[18]
2006	An outbreak (27 cases) was reported in rural South Australia.	Turra[19]
2007	An outbreak (5 cases confirmed, 1 possible fatal case) was associated with an abattoir in South Australia.	ProMED[20]
2013	An outbreak was reported among cats in a veterinary hospital.	Kopecny[21]

3.1.3. Queensland Demographics and Q Fever

Queensland has over 20% of Australia’s total population, and geographically is the second largest state in Australia with an area of 1,730,648 square kilometres. It is the third most populous state with over 4.56 million inhabitants as of 2013. This equates to a population density of 2.6 persons/km^2 [22]. South-East Queensland, including the Statistical Divisions (SDs) of Brisbane, Gold Coast, Sunshine Coast and West Moreton has 3 million inhabitants residing in this sector, which is approximately two-thirds of Queensland's population (Figure 3.2)[23]. Queensland is classified into 452 postcodes of which 293 are classified as “rural”. 81% of Queensland’s land is used for farming and agriculture[24].

To date there are no extensive data on the rates of Q fever in Queensland in the literature, and although statistical data has been gathered, this has not been analysed and published. Two previous publications exist, presenting snap-shot data reviewing the national rates of Q fever disease over very short time periods. These data suggest that Queensland may contribute a large percentage of cases to the national disease figures [6,25].

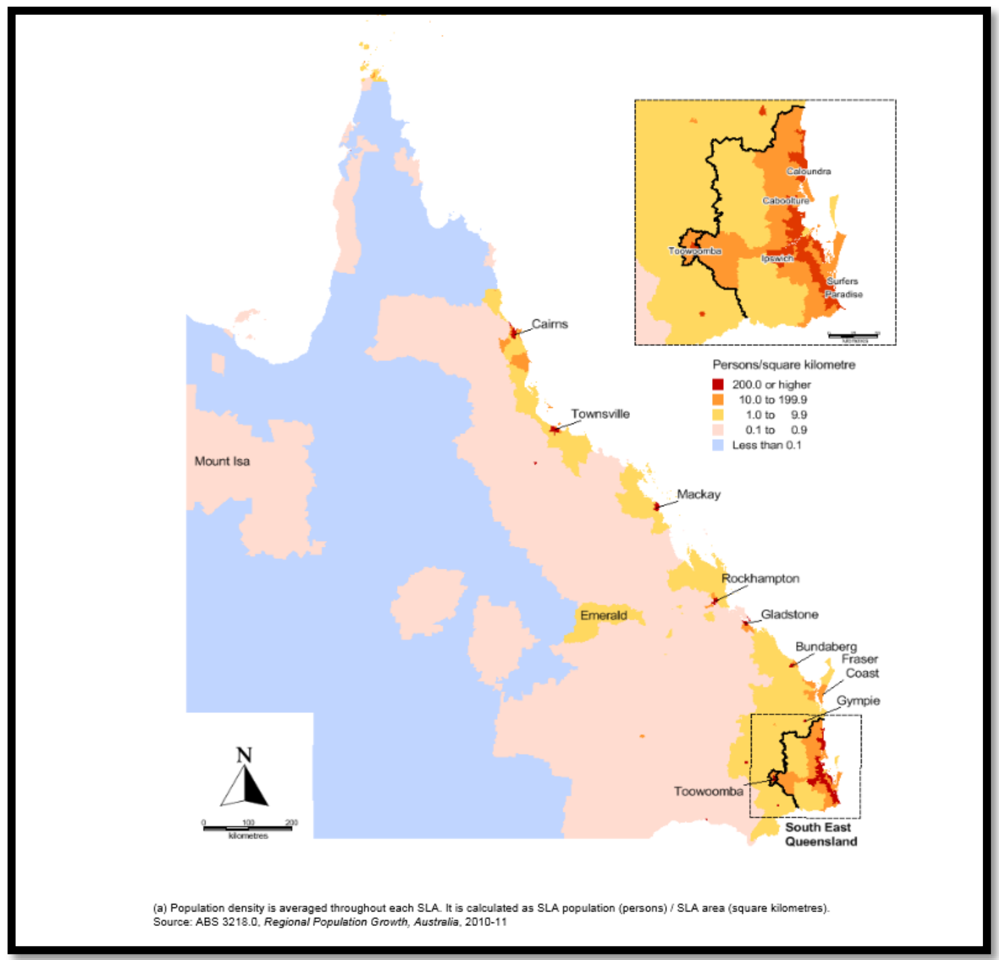

Figure 3.2: Population density for Queensland at 2011[26]

3.1.3.1. Age Distribution of the Queensland Population

Globally, Q fever has been reported primarily in adults and was rarely reported in children except in unique cases[27]. Also, Q fever disease is mainly diagnosed in abattoir workers, farmers and vets and hence the disease is associated with the age groups of the "working" population (20 to <60 year olds)[28]. Australia has approximately 12,521,000 people in this working age group or 55% of the Australian population.

In 2010, the average age group of Queenslanders was between 35 -39 years old with the median age at 36.2 years. The median age of Queensland males was 35.5 years and for Queensland females it was 36.9 years. In Queensland, 54% of the population were aged 20 to <60 years as of 2012[29]. Approximately 20% of Queensland's population or 901,452 persons are children under 15 years of age (Table 3.3).

Table 3.3: Distribution of age groups in the Queensland population at 2014 [29]

Queensland	
Age group (years)	**Total Persons**
0–4	309 885
5–9	297 766
10–14	294 736
15–19	305 448
20–24	328 037
25–29	332 950
30–34	309 774
35–39	313 356
40–44	330 119
45–49	309 744
50–54	304 866
55–59	268 547
60–64	247 851
65–69	205 768
70–74	145 046
75–79	104 437
80–84	78 654
85–89	48 094
90–94	19 796
95–99	4 594
100 and over	591
All ages	4 560 059

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3.1.3.2. Queensland Population and Gender

In 2012 the Australian population had a male to female ratio of 0.989:1 [26], and in Queensland this ratio was 1:1, with the ratio only varying in the elderly age groups (Table 3.4).

Previous published data for Australia show that Q fever predominantly affects males[6,25]. An Australian review of cases between June 1962 and June 1981 showed that all but one of 111 consecutive Q fever cases occurred in males, and medical review showed that the single case reported in a woman may have originated outside Australia[28,30]. Of the 111 cases reviewed, 93% of the infected males were abattoir workers. During 1991-1994, the male to female ratio of Q fever disease in the 20 to 50 year old age group, was 5:1[25]. Based on these data the working age group referred to by many Q fever publications is gender specific and emphasizes Q fever as a disease that effects *working age men* in occupations involving contact with animals[28].

3.1.3.3. Queensland Primary Industries

Previous notification data for Australia (2006)[6] have shown that cases of Q fever have largely been diagnosed from patients in geographical locations with a high percentage of livestock usage, and where agriculture is the primary occupation. Queensland livestock is widely distributed and consists predominantly of cattle and sheep. In 2010, Australia had more than 2.4 million head of dairy cattle, over 25 million head of beef cattle, and over 68 million head of sheep with total livestock commodities valued at AU $19,073 million[31]. Queensland has nearly three times the number of cattle, including dairy cattle, as it does sheep with the rural farming industry being responsible for livestock commodities worth AU $4,653 million (Table 3.5) [31]. Grazing occupies approximately 82% of the total area of land usage, and Queensland is the leading beef-producing state in Australia with a large percentage of its population working both directly and indirectly in the livestock and or beef industries (Figure 3.3)[32].

Sheep farming has been one of Queensland's main primary industries since the 1840's. The current sheep belt covers an area of about 55 million hectares, with a large supply of lambs for the meat industry being raised on farms on the Darling Downs.

Table 3.4: Queensland population by age and gender for 2014.

Queensland			
Age group (years)	**Females**	**Males**	**Ratio**
0–4	150 837	159 048	1.1
5–9	144 600	153 166	1.1
10–14	143 682	151 054	1.1
15–19	149 288	156 160	1.0
20–24	162 741	165 296	1.0
25–29	165 016	167 934	1.0
30–34	154 896	154 878	1.0
35–39	157 713	155 643	1.0
40–44	166 923	163 196	1.0
45–49	156 430	153 314	1.0
50–54	154 155	150 711	1.0
55–59	135 291	133 256	1.0
60–64	123 847	124 004	1.0
65–69	102 461	103 307	1.0
70–74	72 918	72 128	1.0
75–79	54 981	49 456	0.9
80–84	44 273	34 381	0.8
85–89	29 659	18 435	0.6
90–94	13 368	6 428	0.5
95–99	3 409	1 185	0.3
100+	488	103	0.2
All ages	2 286 976	2 273 083	1.0

Table 3.5: Queensland livestock distribution in 2010.

Queensland Livestock 2009-2010	Total Numbers
Sheep and lambs	3 622 141
Dairy cattle	162 200
Meat cattle	11 193 348
Other livestock – Goats	49 141

3.1.4. Seasonal Conditions and Q Fever

During any given season, Australia and Queensland experience a widely divergent weather pattern across the continent, consistent with desert, temperate and tropical conditions. Queensland's average temperature over the past 10 years was 23.4 °C with an average annual rainfall of 656 mm over the state.

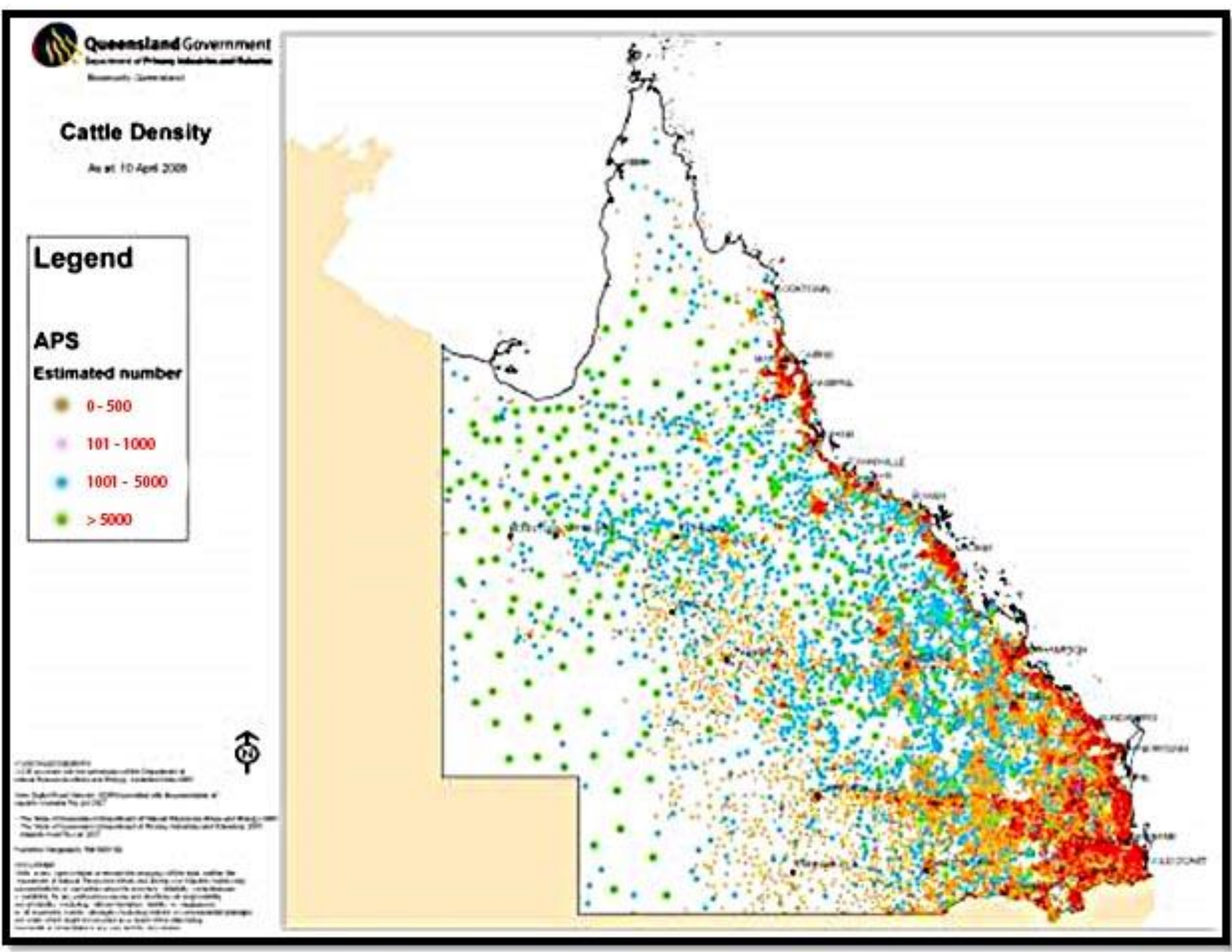

Figure 3.3: Geographical distribution of cattle numbers in Queensland per property as at April 2008[32].
(Blue and green indicate cattle numbers, red and orange are population densities).

In dry areas of Queensland the weather phenomenon may also include the initiation of dust storms which carry dust particles, including bacteria, into the atmosphere. These may be carried over large distances[33]. Atmospheric dust particles are between 0.001 - 40 µM in size, and settle at a rate depending on their size. Bacteria are between 0.3 – 60 µM and are considered to be medium sized particles which can take days to years to settle out of

the atmosphere[34]. Dust storms may occur locally, or may be widely distributed to diverse geographical locations by favourable atmospheric conditions [35].

Coxiella burnetii is known to infect humans via airborne transmission, and previous studies have detected *C. burnetii* DNA in inhalable dust samples, demonstrating that low levels of the bacterial DNA were present in inhalable sized fractions[36].

Australian data showed that Q fever may be acquired in any given month throughout the year, yet many studies report a correlation between the onset of disease with spring and the birthing of new animals[37]. The seasonal presentation of Q fever is still being studied globally, and research in France has examined the correlation between wind and Q fever cases in that country [38]. However, Australian cases recorded and reviewed from Townsville, highlighted increases in notifications after seasonal rainfall and increasing animal populations following the wet season; hence the disease was thought to be associated with rainfall rather than the season of Spring and/or the birthing season[39].

3.1.5. Vaccination and Q Fever

Q fever is a vaccine preventable disease, and there has been a commercially available vaccine in Australia from the Commonwealth Serum Laboratories (Victoria, Australia) since 1989. The vaccine, Q Vax®, has been targeted at workers in high risk occupations, in particular those working in abattoirs, and has contributed to the reduction of Q fever cases nationally in workers in "at risk" occupations[40]. The national financial impact of Q fever disease on the community was estimated (1993-1994) at AU$1million or 1700 weeks of lost work, with financial estimates of AU$3,800 – AU$ 7,000 per patient[41]. The costs associated with an individual case of chronic Q fever have been estimated at AU$20,500, and in 1997 there were workers compensation claims paid of AU$1.1million dollars[42].

3.2. Specific Aims Addressed in this Chapter:

There has been only limited examination of the incidence of Q fever in the Queensland population. These data are important in order to assess (i) the true incidence of disease over an extended period of time, (ii) the correlation with specific population groups, (iii) the identification of risk factors for infection, and (iv) the formulation of an effective vaccination strategy.

This chapter examines Q fever notification data collected for the Queensland population for the year 1984-2014. Specifically the analysis addressed the following:

- The temporal distribution of Q fever among the Queensland population
- Identifying the populations most at risk of infection
- The spatial distribution of Q fever notifications within Queensland
- Distribution of Q fever notification with gender and age groups
- Q fever in children
- Correlation between Q fever and Queensland weather patterns
- Association between notifications and known risk factors

3.3. Methods

3.3.1. Data Used in the Analysis

Notification data forwarded to the NOCS, Department of Health, Queensland, between 1970 and 2014 were used in the analyses described in this Chapter. Notification data for Q fever have been collected since 1970; however,extensive data have only been collected since 1984. So far there has been no publication regarding the analysis of these data.

These data included a unique identification number, demographic details such as time period for onset of disease, patient age, gender and geographical location of residence using the Australian Bureau of Statistics Local Areas (SLA) and postcodes. Specific employment history and animal contact was requested by NOCs but was not always obtained or provided, or was frequently included as an “uncertain” category in the reporting of Q fever cases. The data regarding employment and exposure risks have been included but are limited.

3.3.2. Analysis of the Data

The NOCS data were compared to national data previously published for Australia, which was limited in scope, and even less data have been generated and published for Queensland. Data available for comparison have been restricted to snap-shots of either Australian notifications or discrete data generated from other states.

All Q fever notification data were based on laboratory confirmation of disease. There have been a number of different laboratory testing methods used over time to determine disease status, but details of these methods were not included in the data sets obtained and were not included in the analyses.

3.4. Results

3.4.1. Temporal Distribution of Q Fever Notifications

From the years 1970 to 2014, 18,669 cases of Q fever were reported in Australia. For the same time period 6,797 cases, or 36.4% (95% Confidence Interval (CI) 35.7%-37.1%) of the total number of national cases, were recorded in the Queensland NOCS (Figure 3.4). During the last 31 years (1984-2014), a vaccine for Q fever has been available, and more comprehensive data collection has occurred. During this period Australia has recorded 14,443 notifications, for which Queensland reported 6,794 cases or 47% (95% CI 46.2% to 47.9%) of the total notifications.

Notification data collected since 1970, show that three peaks of disease occurred in Australia in 1979-80 (which included an outbreak in Victoria of 110 cases), in 1993-94 (during limited vaccine usage) and 2001-02 (when the NQFMP had already been implemented nationally). Comprehensive Queensland data were available from 1984 onwards and on analysis showed coincident peaks for the years 1993 and 2001 with other Australian outbreaks. In 2001 the number of cases in Queensland totalled 444 or 65% of the national total, and occurred at a time when the Q fever vaccine had been available for 10 years and recommended to the high risk groups in the community.

3.4.2. Geographic Location

Q fever notifications were recorded in both rural communities and urban cities of Queensland, and included data from 386 of the 452 postcode divisions in Queensland. This equates to more than 85% of the State's regions. Figure 3.5 shows the geographical distribution of the 6,749 Q fever notifications across Queensland for the past 31 years from 1984 to 2014. Metropolitan Brisbane, the capital of Queensland, with a central business postcode of 4000, recorded 128 notifications, being the 6th highest notification rate of the State. There were 69 postcodes that have recorded 50 notifications or more for the 31 year period (Table 3.6). The postcode/geographical region with the highest notification

rate recorded was postcode 4350 with 337 notifications. This corresponded to the regional city of Toowoomba in the District of the Darling Downs. The regions of Queensland with the second highest notifications recorded were postcodes 4470, 4455 and 4487 (Table 3.6).These postcodes correspond to the district of South West Queensland and include the rural towns of Charleville, St George and Roma (Figure 3.6).

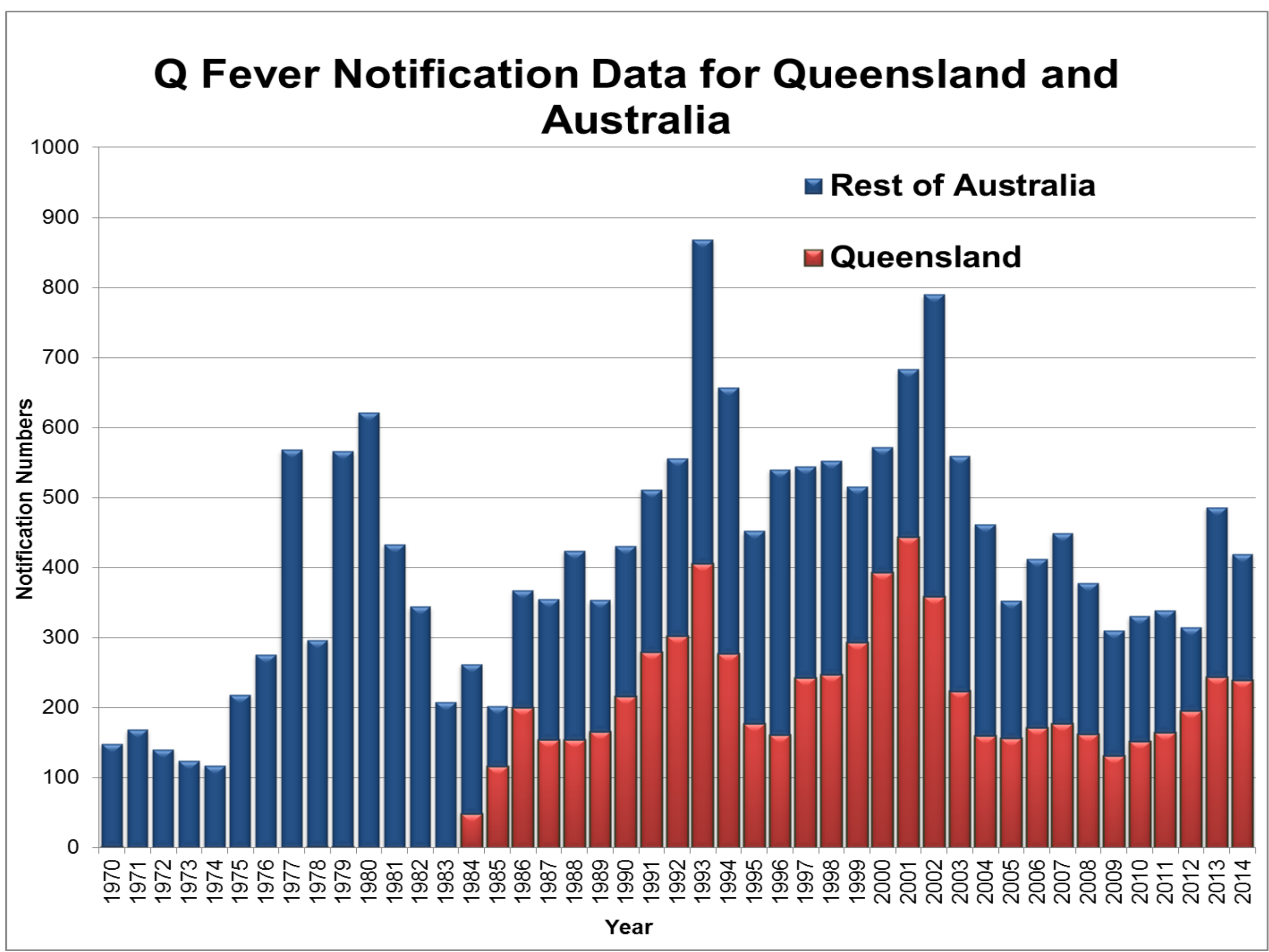

Figure 3.4: Q fever notification data for Queensland and the rest of Australia from 1970-2014. *NB: Data was collected from other Australian States before 1970. Queensland only began recording complete notification data in 1984*

A preferred method to directly compare infections or disease rates within communities is an estimation of number of infections per capita. However, the Australian Bureau of Statistics did not have specific population numbers for specific postcodes. It also did not have population numbers for every year in which Q fever notifications were collected. The population numbers were based on census years and these occur at 5 year intervals.

For this reason the notifications were expressed as a single whole number. This is the next best estimate of Q fever incidence, and in fact the only method possible with the data available.

Table 3.6: Postcodes of Queensland notification data highlighting the regions with more than 50 notifications for the 31 year period (1984 to 2014).

Postcode	Notifications
4350	337
4470	179
4455	166
4487	159
4370	147
4000	128
4570	126
4305	116
4285	115
4700	114
4421	106
4390	95
4207	93
4352	93
4490	92
4610	85
4816	75
4701	73
4472	72
4810	70
4872	70
4870	69
4465	66
4702	66
4306	61
4102	57
4343	57
4405	57
4740	57
4800	55
4605	54
4730	54
4883	52

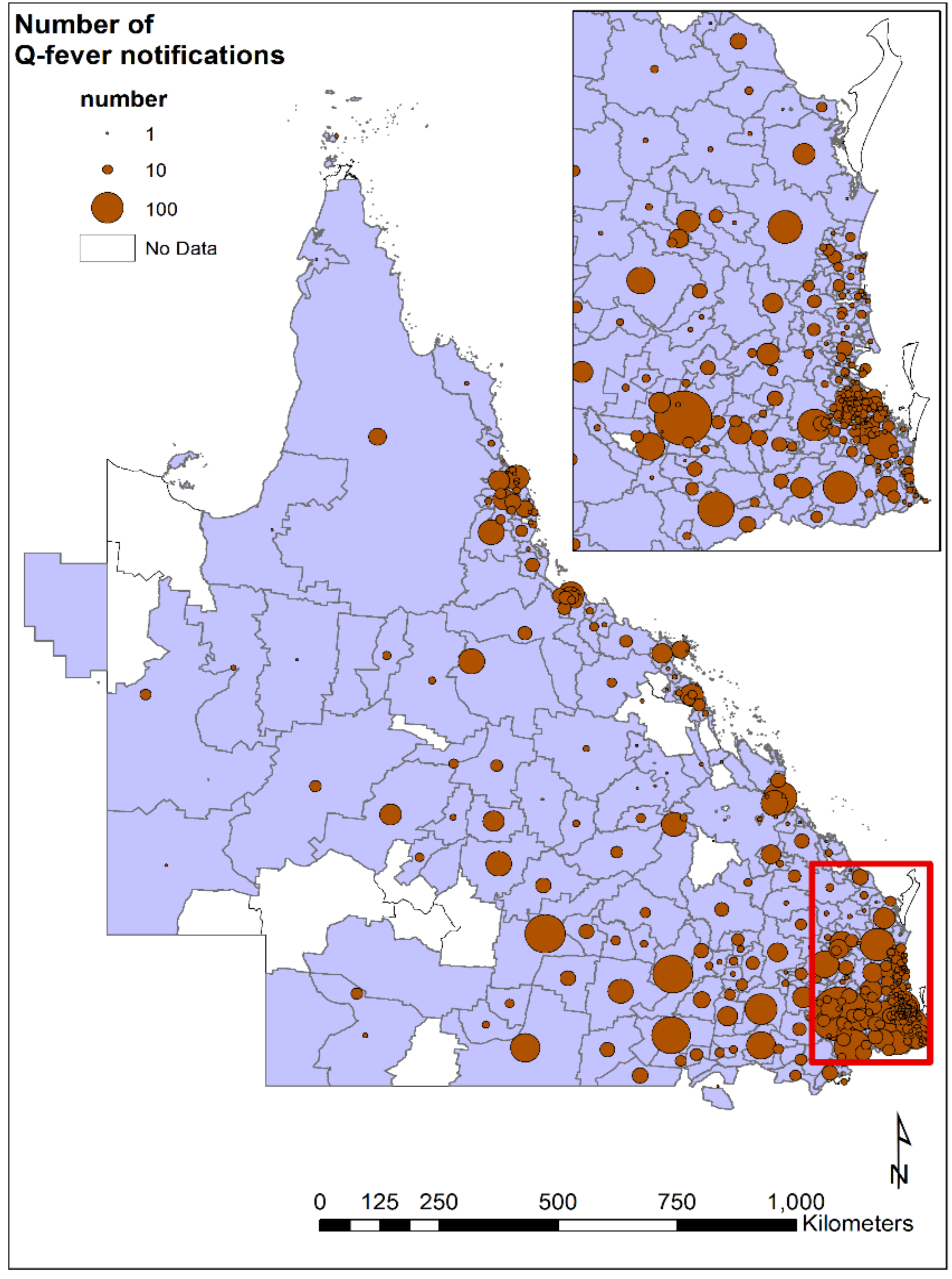

Figure 3.5: Queensland map showing the distribution of Q fever notifications as a visual for 1984 to 2014 by postcode.

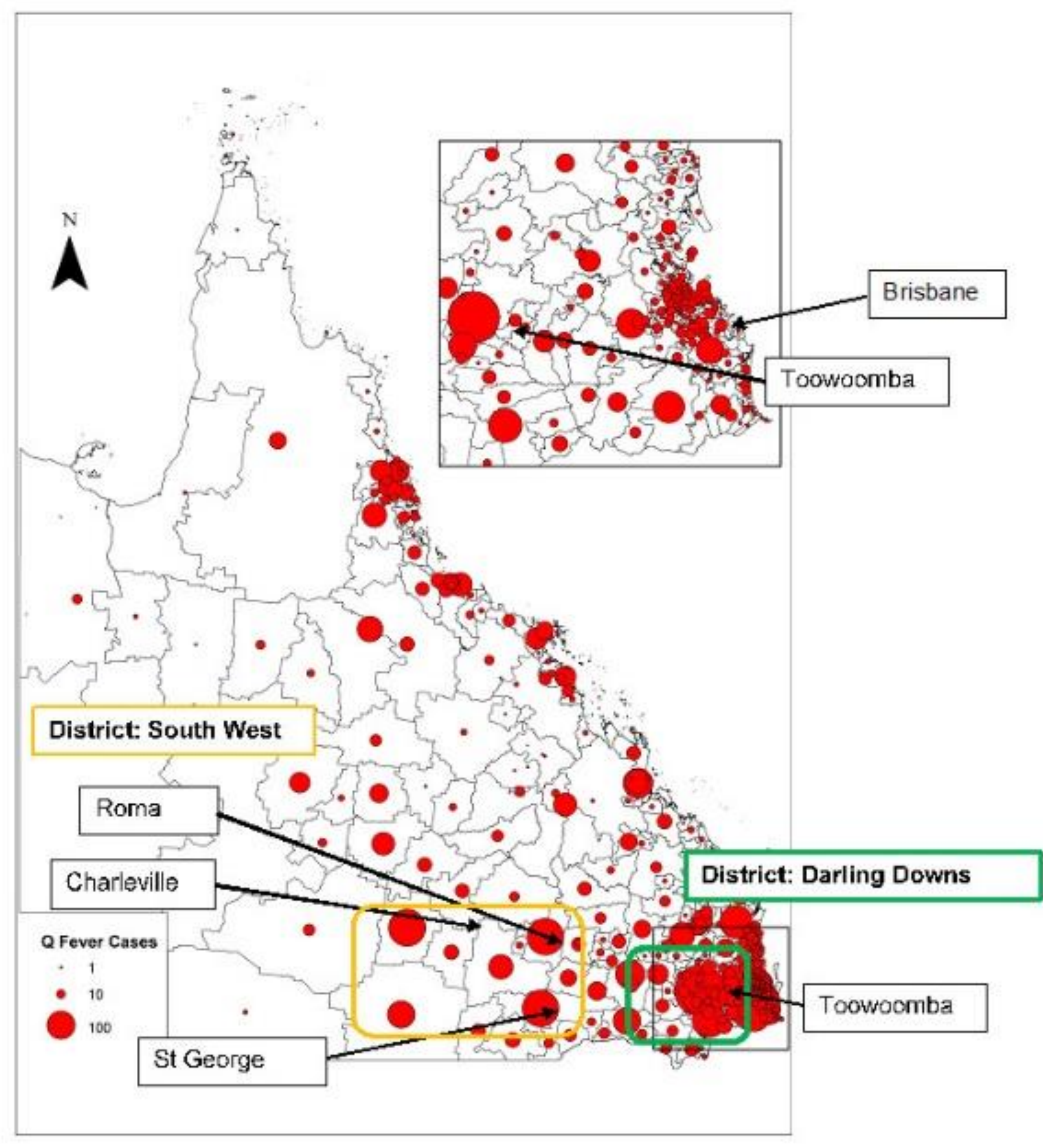

Figure 3.6: map pf Queensland showing visually Queensland's postcodes with the highest notifications over a 31 year period (1984 to 2014)

3.4.3. Gender

Although Q fever is usually associated with men working in "at risk" occupations, it is a disease that may also affect women. In Queensland, meaningful data was only recorded from 1984 onwards and showed that the rate of infection varied markedly between the two genders (Table 3.7). Over the period 1984-2014, there were a total of 6,794 notifications, 5,417 (79.7%) from men and 1,370 (20.1%) notifications from women, with 7 (0.2%) notifications where the gender data was not provided. This gave an overall ratio of 4.0:1 (M:F) for all of the Queensland Q fever notifications. Over this period (1984 to 2014), the annual ratio of male to female notifications has declined from 8.6:1 (Male 103: Female 12) in 1985 to a ratio of 2.4:1 (Male 114: Female 48) in 2008.

From 1984 to 1990 the numbers of notifications for males and females remained similar, giving a range of M:F ratios from 8.6 – 6.0 (average = 6.8). However, from 1991 onwards the numbers of notifications for both males and females rose substantially, with a greater rise in the number of cases reported in females, resulting in a decrease in the M:F ratio between 1992 and 2002 (average = 4.4) (Figure 3.7). Since 2002, the numbers of notifications has declined in both males and females, giving a range of M:F ratios of 2.4 – 4.5 (average = 2.9).

The notable exceptions were the years 1997 and 2000, when the M:F ratios were considerably greater than the preceding and the subsequent year (Table 3.7). Interestingly, in 1997 the Q fever vaccine registry was introduced along with a large scale education program including the Q fever Information Kit for the Australian Meat Industry which specifically targeted men to heighten awareness of the disease, and 2000 saw the introduction of the NQFMP in Queensland, with a program of large scale vaccination of men in "at risk" occupations.

Stage 1 of the NQFMP (2001-2002) coincided with record numbers of notification in females, nearly doubling those previously recorded. The average annual notification in women prior to the NQFMP in 2001 was 36.6 notifications, which increased by over 260% to an average of 95 cases per year in the first years of stage 1. Following the roll out of stage 2 of the NQFMP in 2002, which expanded the vaccination program to include farmers, their employees and unpaid family members working on farms, the number of notifications in both males and females began to decline (Table 3.7). This decline has been sustained with a M:F ratio of 2.6 in 2014.

Table 3.7: Annual Queensland Q fever notifications from 1984 to 2014 showing the distribution throughout the different genders. Numbers for each gender, ratios and total numbers are shown.

Year	Male	Female	Ratio (M:F)	Total Number
1984	36	12	3.0	48
1985	103	12	8.6	115
1986	169	25	6.8	199
1987	135	19	7.1	154
1988	135	19	7.1	154
1989	142	23	6.2	165
1990	185	31	6.0	216
1991	240	39	6.2	279
1992	250	52	4.8	302
1993	329	75	4.4	405
1994	230	46	5.0	276
1995	143	33	4.3	176
1996	120	40	3.0	160
1997	208	34	6.1	242
1998	195	52	3.8	247
1999	235	57	4.1	292
2000	338	54	6.3	392
2001	347	96	3.6	443
2002	262	95	2.8	358
2003	174	49	3.6	223
2004	116	43	2.7	159
2005	123	33	3.7	156
2006	140	31	4.5	171
2007	126	51	2.5	177
2008	114	48	2.4	162
2009	95	36	2.6	131
2010	108	43	2.5	151
2011	118	46	2.6	164
2012	146	49	3.0	195
2013	183	60	3.1	243
2014	172	67	2.6	239
Grand Total	5417	1370	4.0	6794

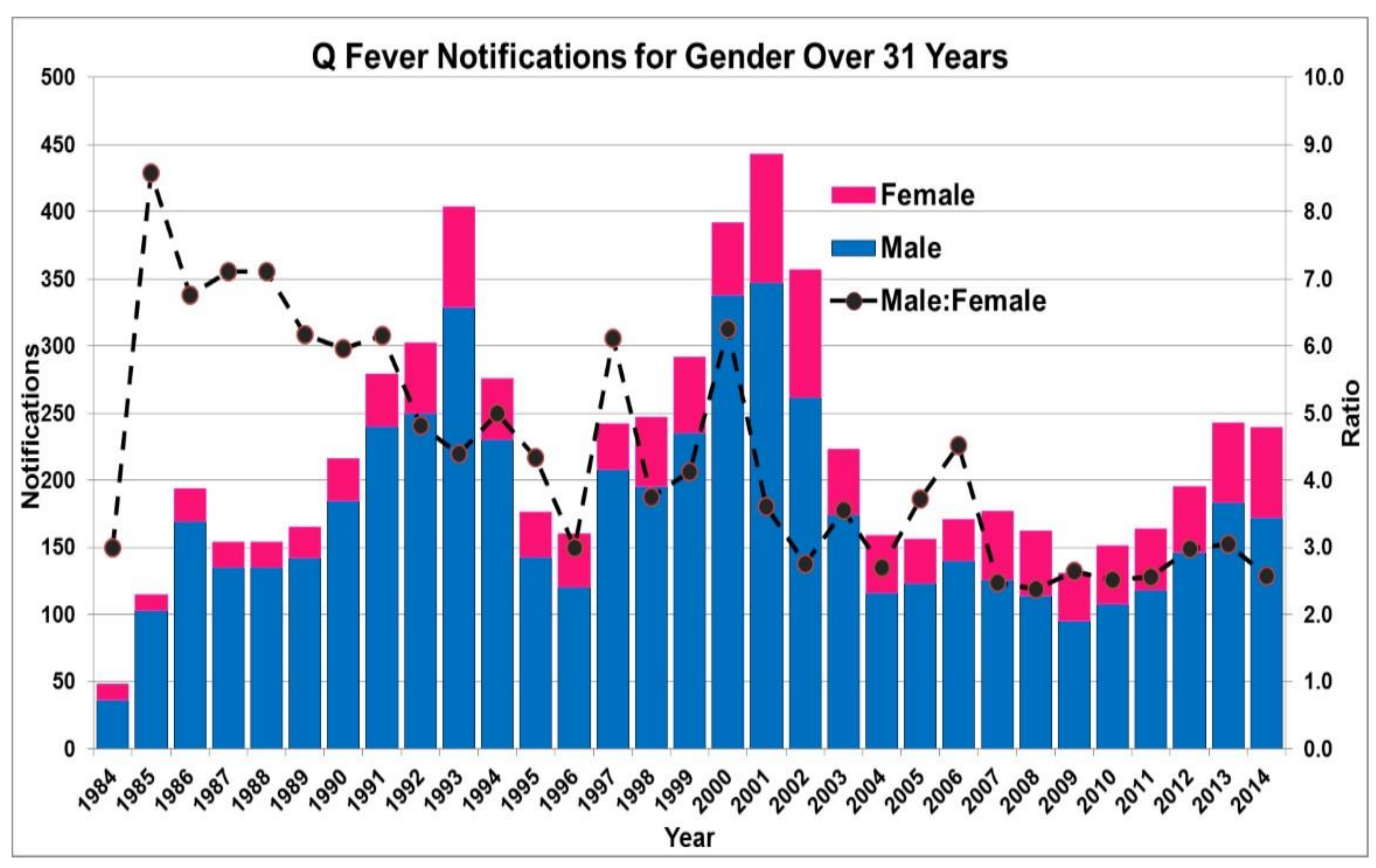

Figure 3.7: Temporal distribution of Q fever notifications from 1984 to 2014 highlighting ratio between different genders over time

3.4.4. Q Fever and Age Distribution

3.4.4.1. Queensland Data in the Australian Context

Since the implementation of the National Notifiable Disease Surveillance System (NNDSS) in 1990 under the auspices of the Communicable Diseases Network Australia; limited data analyses has been performed and published linking national Q fever notifications with age for the general Australian population, nor has there been previous analysis of paediatric cases [43]. However, the limited data available for Australian notifications have focused on short discrete time periods including 1991-1994, which covers post vaccine development, and the period 2006-2007, which is post implementation of the NQFMP[44,45].

In this study, the analysis of Queensland notifications for the same years (1991-1994), showed that the majority of Queensland cases, 76.5% (965 of 1261) were from subjects aged between 16-45 years of age (a 30 year age bracket). This was a slightly younger age group than was identified in the national notifications (20-50 year olds; 70% of notifications)[44,45]. Within the Queensland age groups, the highest numbers of total notifications were from 21-25 year olds, with 196 cases recorded, equating to 16% of the total Queensland notifications. The average number of notifications for the 21-25 year olds in Queensland for 1991-1994 was 49, compared to national data which showed an average of 70 cases per year being reported from this same age group during the 1991-1994 period.

Analysis of the 2006-2007 Queensland notification data showed that the age range with the highest notifications occurred in 26-59 year age group, accounting for over 66% (252 of 384) of the Queensland total notifications during this time. This was very similar to Australian national data of 69% (587 of 852) in this age bracket. Analysis of specific age groupings for 2006-2007 of Queensland data showed that the 46-50 year old age group had the highest number of recorded notifications, with 43 or 12% of the total notifications during this time period (Table 3.8). The Australian data did not discriminate in 5 year brackets.

Table 3.8: Queensland notifications for the two periods published 1991-1994 and 2006-2007, showing age distribution

Age Groups	1991-1994	Average Notification /year	2006-2007	Average Notification /year
00-04	4	1.3	2	2
05-09	7	3.5	7	3.5
10-14	10	2.5	8	4
15-19	149	37.3	17	8.5
20-24	204	51.0	26	13
25-29	171	42.8	25	12.5
30-34	170	42.5	25	12.5
35-39	127	31.8	29	14.5
40-44	121	30.3	39	19.5
45-49	103	25.8	44	22
50-54	63	15.8	41	20.5
55-59	47	11.8	33	16.5
60-64	34	8.5	23	11.5
65-69	14	3.5	14	7
70-74	10	2.5	8	4
75-79	12	4.0	4	2
80+	15	5.0	3	1.5
Grand Total	1262	315.5	348	174

The comparison between the two time periods analysed, 1991-1994 (post vaccine development) and 2006-2007 (post NQFMP implementation), showed a significant shift in age groups recording maximum numbers of notifications. This was seen in both the published national data and the Queensland data[44,45].

3.4.4.2. Analysis of All Queensland Data (1984 – 2014)

There has been no previous in depth analysis of Q fever notifications in Queensland. Data in this study showed notifications in all age groups over time with a total of 6,794 notifications recorded over the 31 year period. The average age of patients confirmed with Q fever infection was 38.8 years, with a median age of 38.0 years and an age range of <1 months to 98 years old (Table 3.9). The age group that had the most notifications were those aged between 40-45 years of age with 736 notifications (11% of the total number of notifications for the 31years analysed). The age groups that had the least number of notifications were 0-4 year olds with a total of 26 notifications.

Queensland has seen a clear shift in the age groups reporting Q fever notifications for the period 1984-2014 (Figure 3.8). Prior to 2001 and the NQFMP, the majority of notifications occurred in the 15-39 year olds with >61% (2332 from 3822), with an average notification age of 35.1 years. During the NQFMP implementation the age groups reporting the highest notifications were the 30-59 year olds, with 827 from 1334 notifications or >62% and an average of 42.1 years old. The age groups showing the highest notifications post NQFMP, from 2006 onwards are the 35-64 year olds with 1026 from 1633 notifications, and an average age of 44.3 years. Figure 3.8 shows the distribution of notifications over time with relation to the various age groups, highlighting a clear shift in the average age of Q fever notifications with time.

Q fever notifications rates peaked between 1991-1994 with a total of 1261 notifications, and an average of 315.5 notifications per year during this time. A second peak in notifications in Queensland was seen post vaccination program implementation, between 2000 and 2002 with 1193 notifications being recorded and an average of 398 cases reported each year.

These data show that Q fever primarily occurs in older age groups, but a significant number of cases were reported in children less than 16 years of age. This finding warrants more detailed analysis of notifications in children.

Table 3.9: All Queensland notification data for years 1984-2014, showing distribution of gender among the notifications and in relation to age groups

Age Groups in years	Females	Males	Ratio	Unknown	Total
00-04	9	17	1.9	-	26
05-09	18	34	1.9	-	52
10-14	33	58	1.8	-	91
15-19	83	513	6.2	-	596
20-24	98	620	6.3	-	718
25-29	102	581	5.7	-	683
30-34	125	566	4.5	1	692
35-39	139	562	4.0	1	702
40-44	158	578	3.7	-	736
45-49	159	506	3.2	-	665
50-54	135	421	3.1	-	556
55-59	122	374	3.1	-	496
60-64	83	247	3.0	-	330
65-69	54	156	2.9	-	210
70-74	16	69	4.3	-	85
75-79	17	50	2.9	-	67
80+	19	65	3.4	5	89
Grand Total	1370	5417	4.0	7	6794

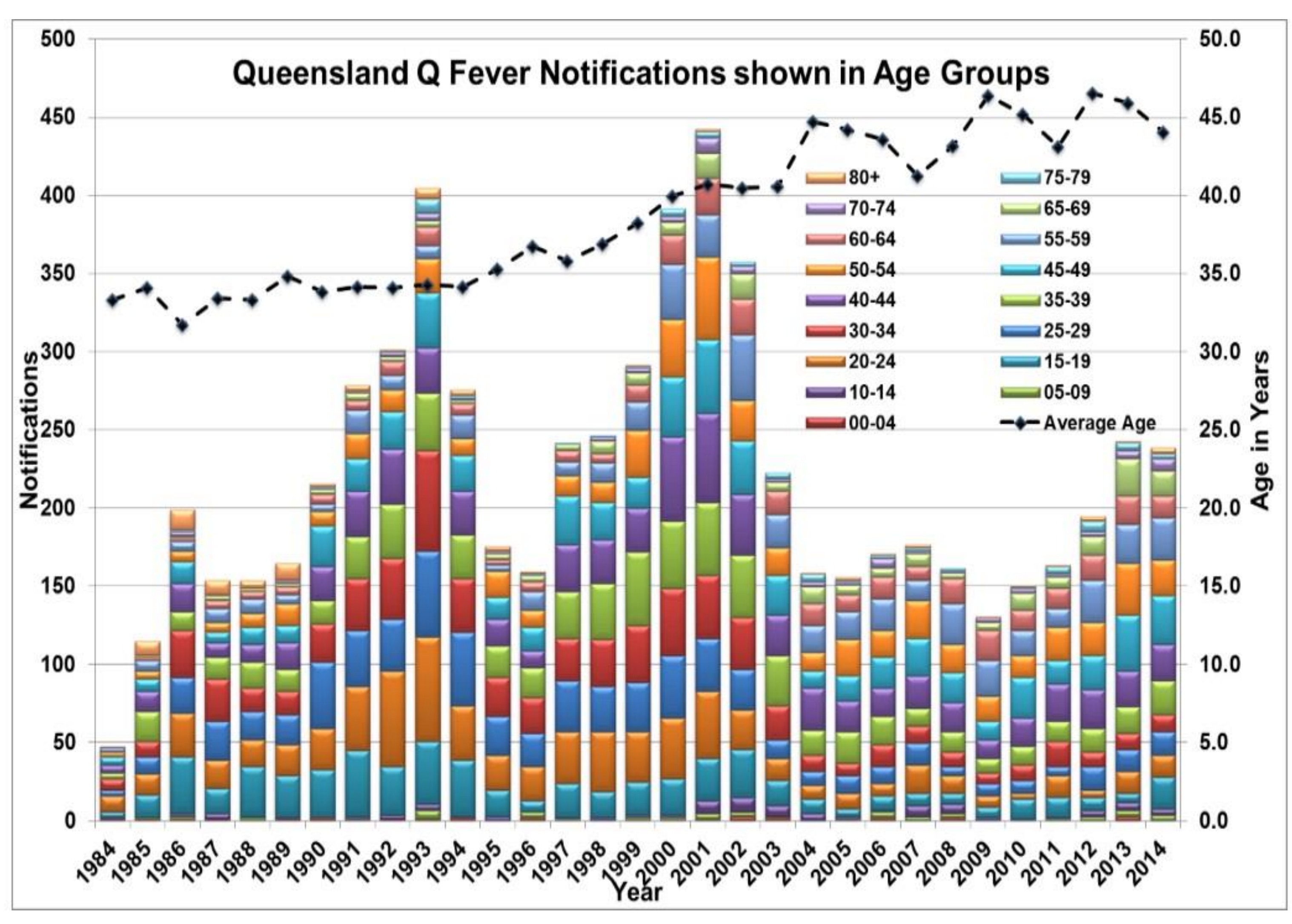

Figure 3.8: Temporal Distribution of Q Fever Notifications in Age Groups for the 31 year period 1984-2014

3.4.4.3. Q Fever in the Paediatric Population

In Queensland there were 235 reported cases of Q fever in children under 16 years of age over the study period 1984-2014. The average age was 10.8 years, the median age was 12.0 years (Figure 3.9), and the average number of cases reported per year was 7.6.

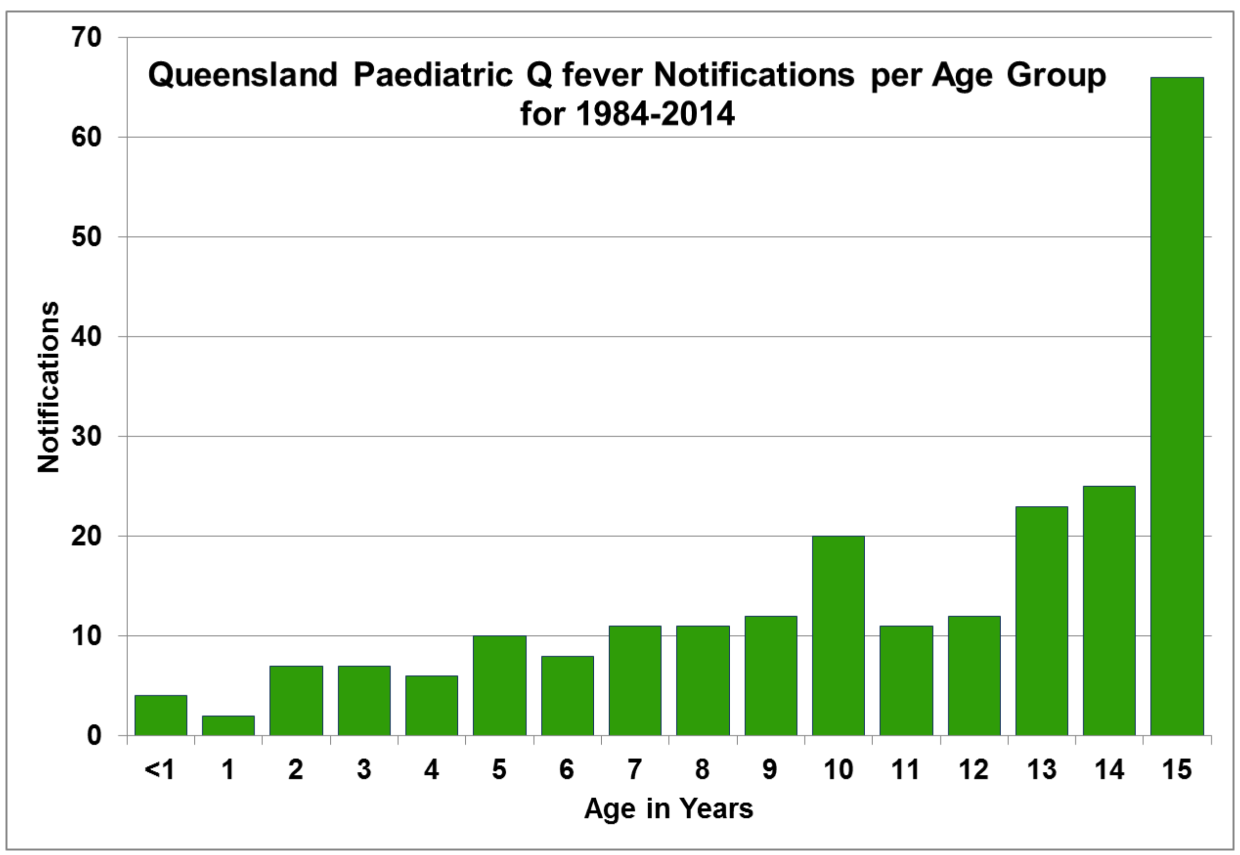

Figure 3.9: Queensland paediatric notifications per age group (1984-2014)

From the year 2000 the average annual number of children reported with Q fever has slightly increased from 6.1 cases in the period 1984-2000 to 8.7 cases in the period 2001-2012. In 2001 and 2002, 19 and 18 paediatric cases were recorded respectively, and a single case was reported in 2010 (Figure 3.10).

The average age of infection per year studied ranged from 5.5 years to 14.5 years. However, the number of notifications, when analysed per individual age group, showed that there was an increase in disease recorded with increase in age. The older children of 10 years and above were responsible for 67 % (157 notifications) of the total number of Q fever infections recorded between 1984 and 2014 (Figure 3.10).

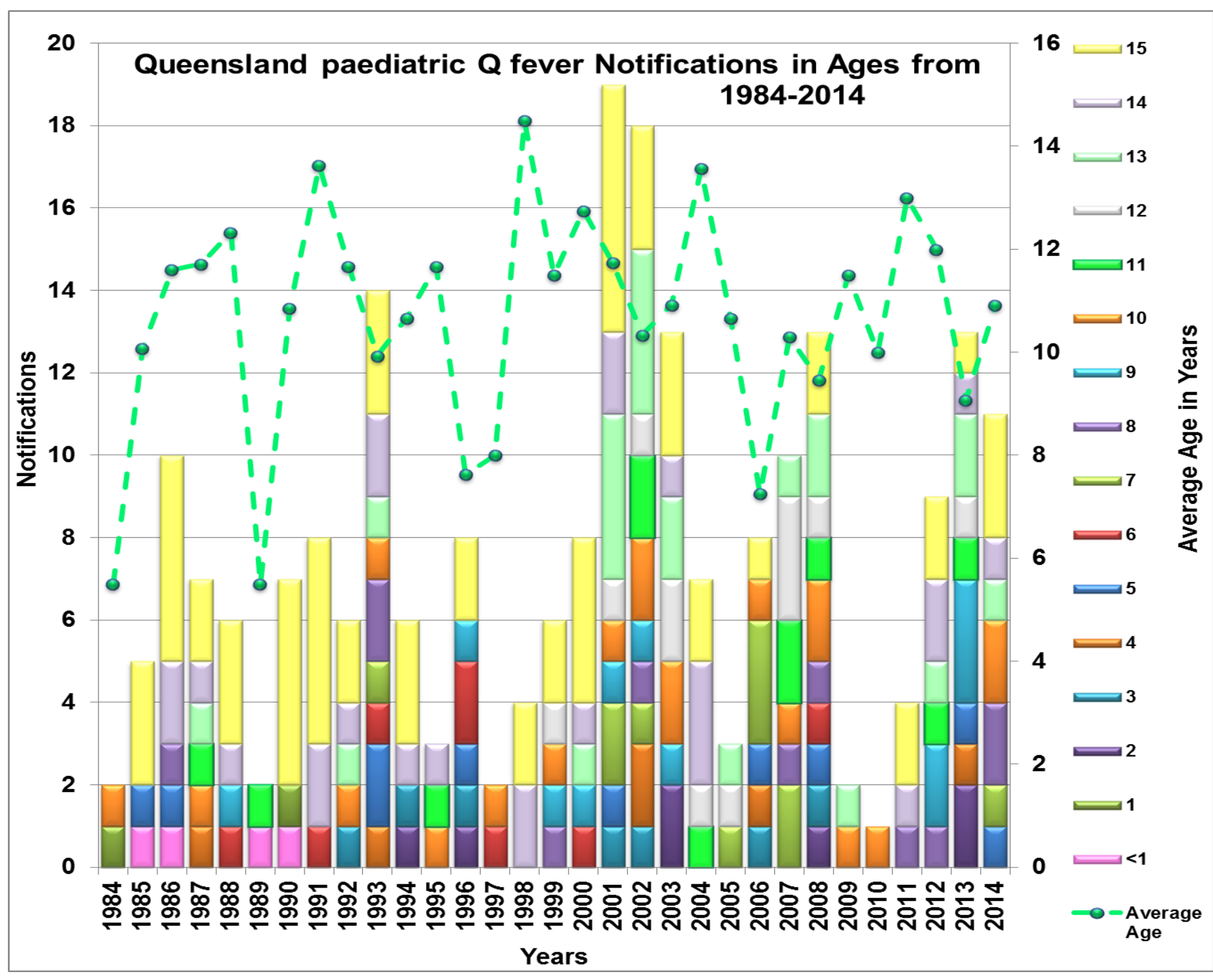

Figure 3.10: Paediatric Q fever notifications for the years 1984 – 2014, shown in ages with the average age highlighted.

The Q Vax® vaccine has not been licensed for use in children under 16 years old, and therefore does not influence these numbers of notifications. During the implementation of the vaccine program in adults, there were 60 paediatric notifications during the 5 year period (2001-2005), resulting in an average of 14.5 paediatric cases identified each year of the campaign.

3.4.4.4. Paediatric Q Fever Cases and Geographic Location

The 235 paediatric notifications recorded were identified using postcodes and comprised 85 geographical locations in Queensland (Figure 3.11). The areas that recorded more than 4 notifications and their associated postcodes are shown in Table 3.10, and those areas in Queensland with more than 8 paediatric notifications are highlighted in Figure

3.12. Most of the paediatric notifications were from the Darling Downs and South West districts of Queensland, similar to the adult cases. However, a substantial number of paediatric notifications were recorded from Brisbane metropolitan postcodes.

Table 3.10: Queensland postcodes with paediatric Q fever notifications greater than 4 for the period 1984-2014.

Postcode	Notifications
4285	4
4468	4
4478	4
4700	4
4870	4
4000	5
4350	5
4455	5
4610	5
4305	6
4570	6
4406	7
4490	7
4421	8
4465	10
4470	10
4487	11
4390	13

Marker	Notifications	Location
1	4	Queensland 4468, Australia
2	4	Queensland 4487, Australia
3	4	Queensland 4700, Australia
4	4	Cairns QLD 4870, Australia
5	5	Brisbane QLD 4000, Australia
6	5	Queensland 4350, Australia
7	5	Queensland 4610, Australia
8	6	Queensland 4305, Australia
9	6	Queensland 4570, Australia
10	7	Queensland 4406, Australia
11	7	Queensland 4490, Australia
12	8	Queensland 4421, Australia
13	10	Queensland 4465, Australia
14	10	Queensland 4470, Australia
15	13	Queensland 4390, Australia
16		Queensland 4488, Australia
17	4	Queensland 4285, Australia

Figure 3.11: Number of Queensland Q fever notifications shown per location (postcode). The marker colours shown are representative of the number of notifications per location.

Legend: Yellow = 4 notifications; Grey = 5 notifications; Fawn = 6 notifications; Aqua = 7 notifications; Pink = 8 notifications; Green = 10 notifications; Red = 13 notifications.

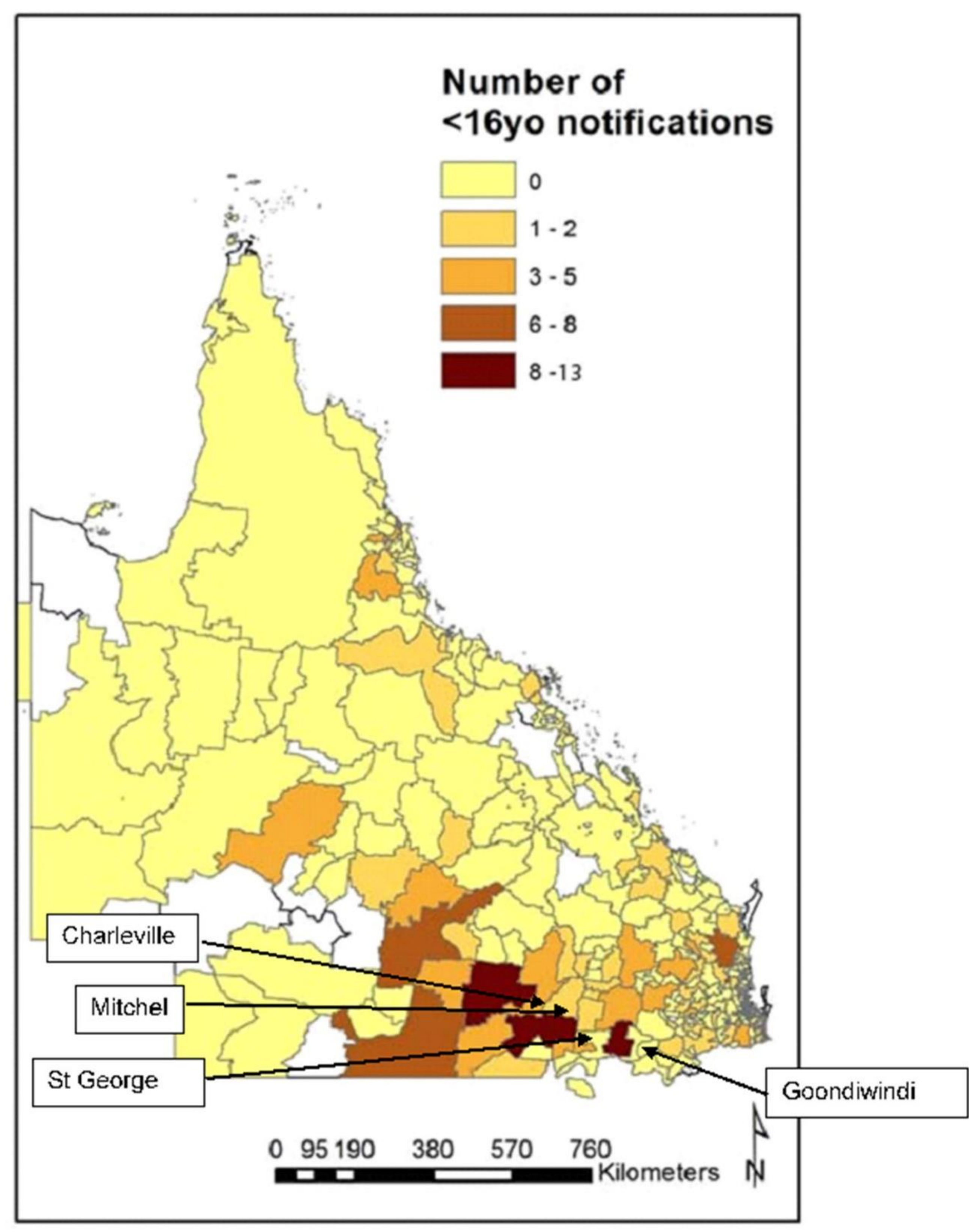

Figure 3.12: Geospatial display of areas with more than 8 paediatric Q fever notifications mapped

3.4.4.5. Gender and Paediatric Q Fever Cases

Q fever notifications in the paediatric population for the total period between 1984 and 2014 show an average male to female notification ratio of 2.3:1. In Q fever notifications occurring in children under 6 years old, the gender ratio varied from 1:0 (<1year old) up to 7:1 (6 year olds) with an average ratio over the 6 years of 1.9:1 (Male:Female). The children aged between 6 and 15 showed an overall Male:Female ratio of 2.4:1, and were generally dominated by Q fever cases reported in boys, except for the 3 and 11 year olds, where there were more notifications from girls (Figure 3.13). As the children reached a working age of 15 years there was a substantial increase in Q fever notifications recorded from males.

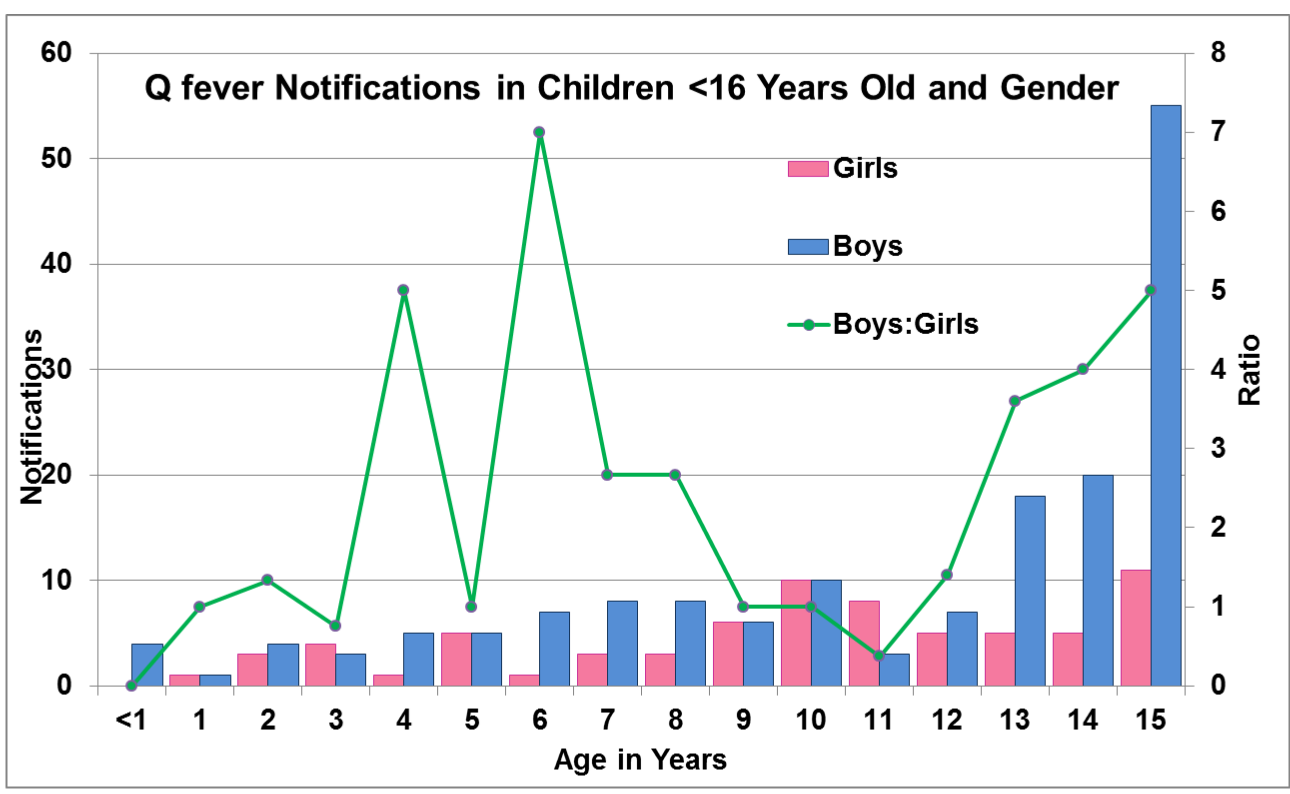

Figure 3.13: Distribution of paediatric Q fever cases among the various ages for the years 1984- 2014, showing the ratio of male to female notifications

The increasing number of notifications in adolescent males is reflected in the <16 years total age group data collected between 1984 and 2014, which shows a preponderance of notifications from males for most years (Figure 3.14). Only in 2007 and 2013, were there

more notifications reported in females than males. Interestingly there were 5 years with no reports of Q fever in girls at all, and thus ratios could not be calculated (Figure 3.14).

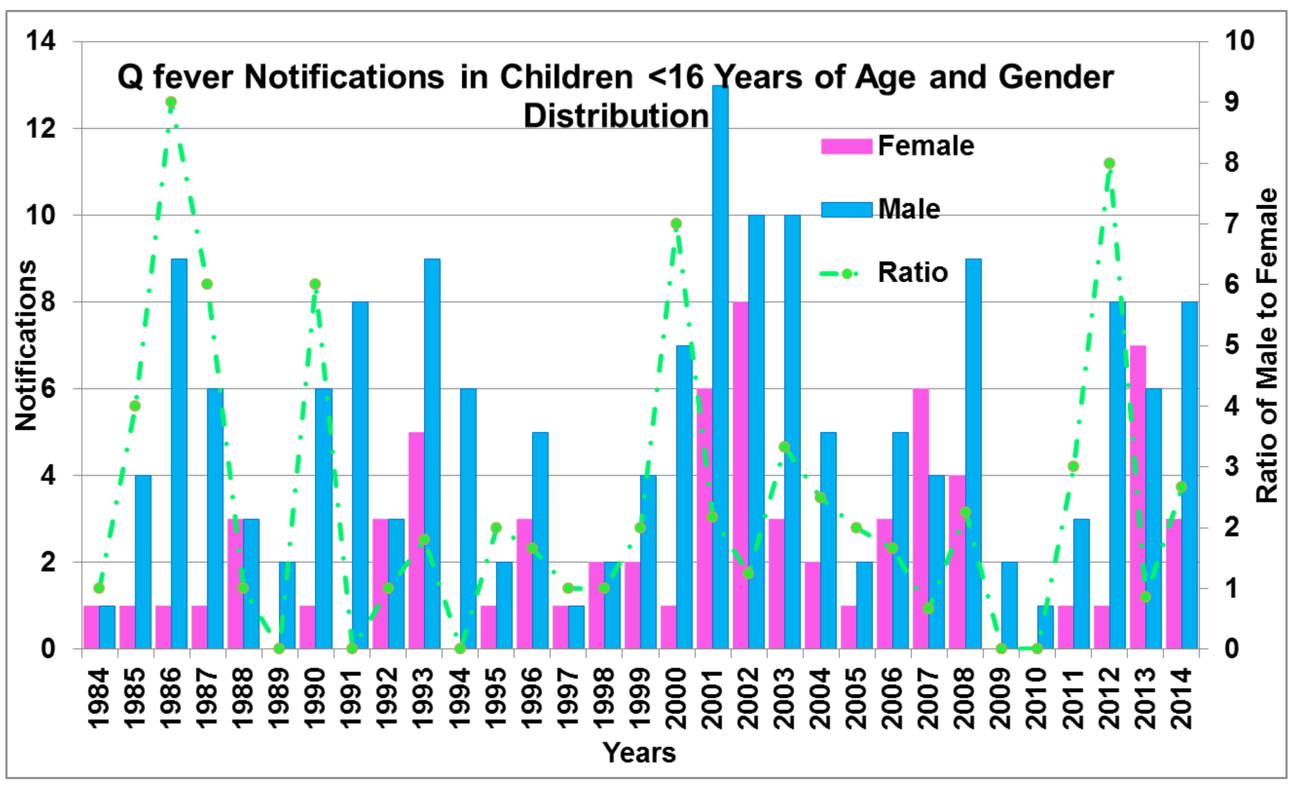

Figure 3.14: Q fever notifications from children aged less than 16 years of age for the each of the 31 years analysed 1984-2014, along with the ratio of male to female notifications

3.4.5. Seasonality of Q Fever Infection

Q fever notifications were recorded in all months of every year from 1984 to 2014. On average, an increase in notifications was recorded for the months of March, April and May which corresponded to autumn in Queensland and spring in the Southern Hemisphere, and generally follows a period of increased rainfall during summer (Figure 3.15).

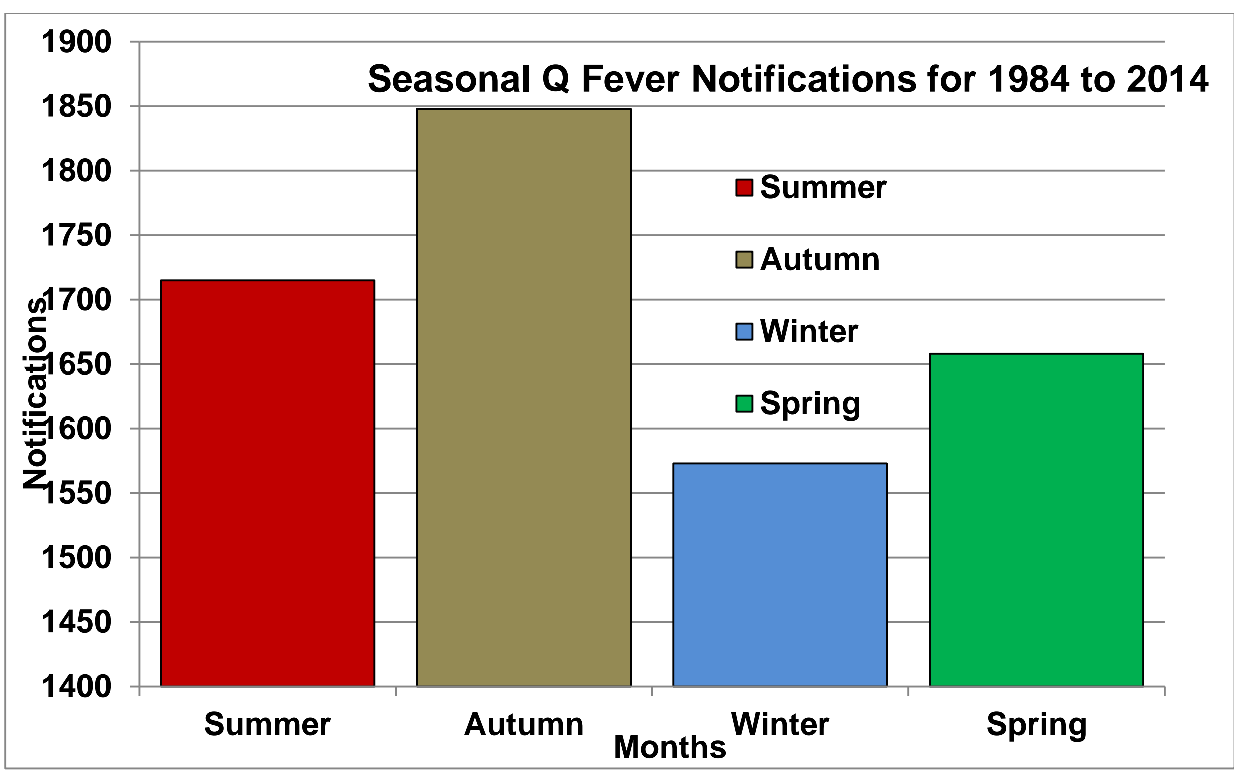

Figure 3.15: Seasonal breakdown of Q fever notifications for the years 1984-2014

However, there was no statistical correlation between Q fever notifications and average annual rainfall for Queensland, (p value was >0.23 r(29) = 0.22) with only a slight decrease in the number of notifications recorded during a time of lower rainfall. Queensland consistently records Q fever notifications more frequently and in higher numbers in the months preceding the state's highest average rainfalls (Figure 3.16).

Interestingly, increases in Q fever notifications were observed in the years of 1992-1994 and in the years 2001-2003 which coincided with a significant decrease in average annual rainfall for the state, and Australia as a whole. These were periods of widespread drought with increased dust activity, during which the highest number of Q fever notifications were recorded (Figure 3.17).

There was no correlation identified between notification data and recorded dust events in Queensland with p value = >0.73 r(29) =0.07. Nor was there any significance calculated between Q fever notifications and average temperatures p value= >0.66 r(29) =0.08.

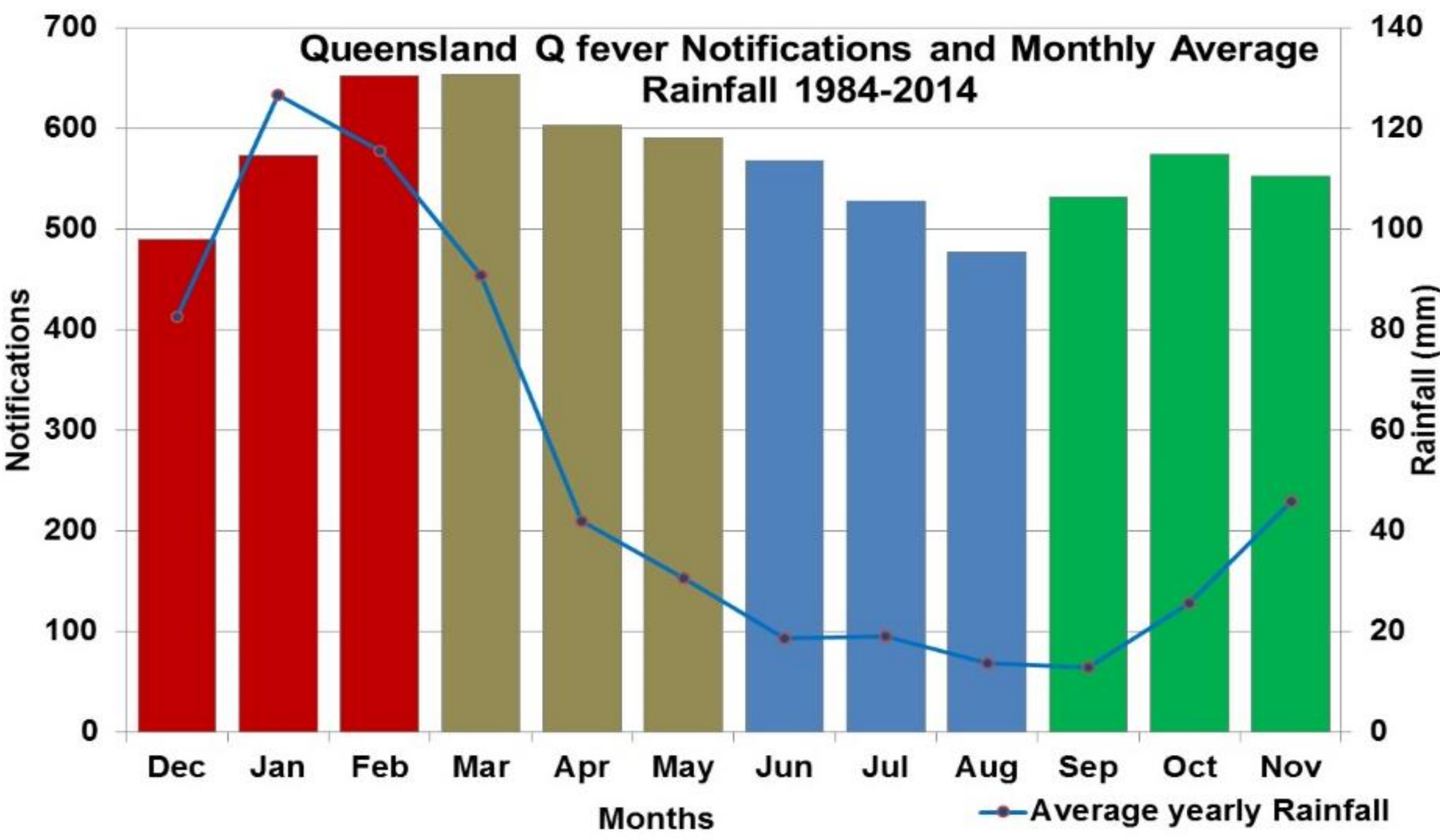

Figure 3.16: Queensland notifications of Q fever and average monthly rainfall for the period 1984 to 2014

● Summer; ● Autumn; ● Winter; ● Spring.

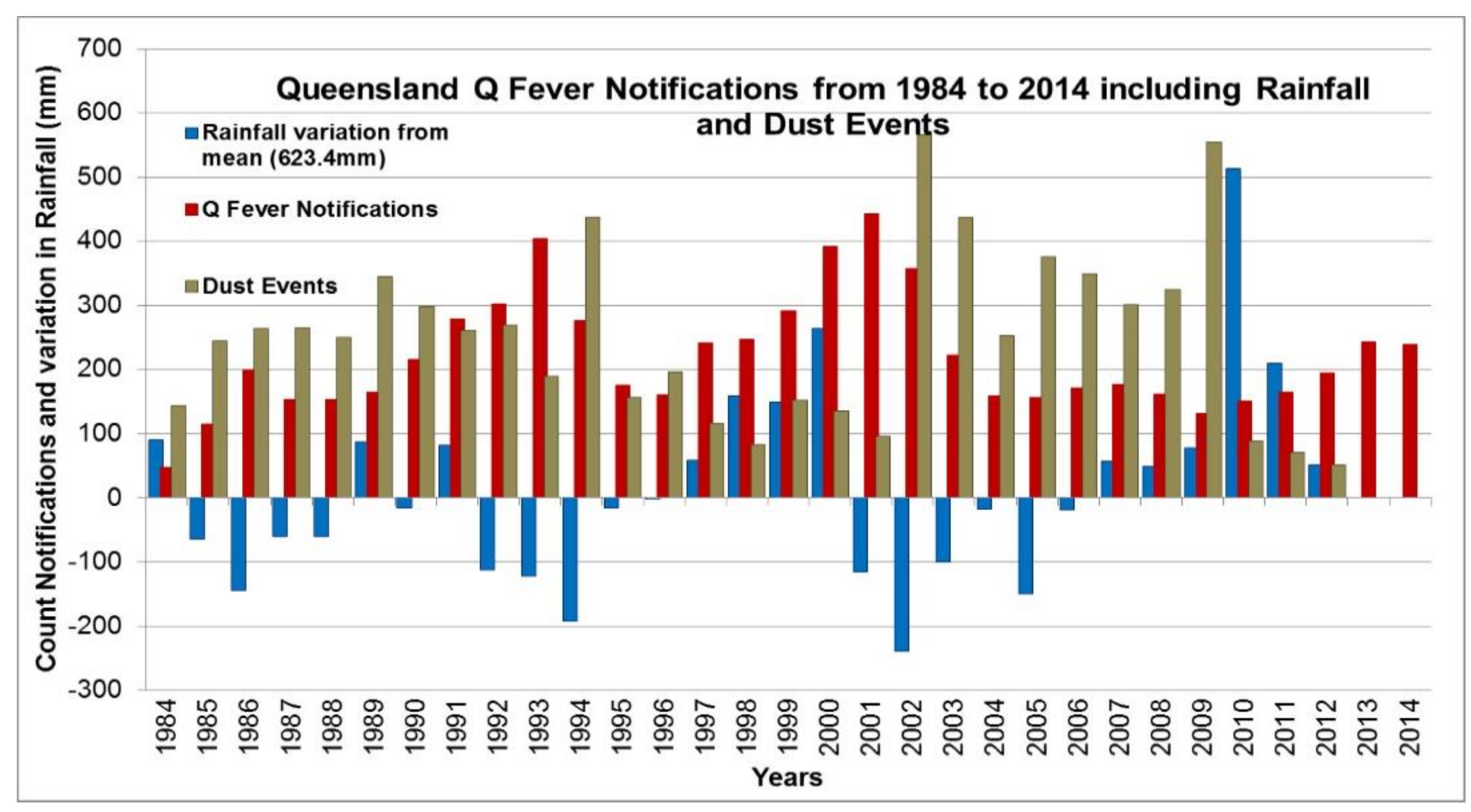

Figure 3.17: Queensland notifications and average rainfall for the years 1984-2014

3.4.6. Identification of Risk Factors

The collection of NOC data included questionnaires regarding 25 categories of potential exposure risks associated with the disease, Q fever. However, these data were not always complete or included in the information sent to the Queensland Department of Health.

From the 6,794 notifications recorded in Queensland since 1984 to 2014, there were 6,312 instances of risk identified, with some notifications identifying multiple risk factors, and more than 5% of all notifications were associated with a known risk factor for Q fever. Meat processing and abattoir environment exposure were two of the most documented risk factors, and were identified in 5.0% of Queensland Q fever cases for the 31 years analysed. In all, 14% of all Q fever notifications had a risk factor documented (Table 3.11).

Table 3.11: Risk factors identified as part of the notification process associated with Q fever disease

Q FEVER RISK FACTORS IDENTIFIED	AT RISK	NOT AT RISK	UNKNOWN	NO RECORD	GRAND TOTAL	% AT RISK	% AT RISK NOTIFICATIONS
At Risk Q Fever	348	1066	118	5610	1184	29.4	5.1
Abattoir Exposure	333	1010	109	5675	1119	29.7	5.0
Visitor To Abattoir	75	213	17	6564	230	32.6	1.1
Work Inside Abattoir	178	583	40	6171	623	28.6	2.6
Inside Abattoir Duties	39	55	13	6726	68	57.4	0.6
Other Exposure In Abattoir	35	84	5	6705	89	39.3	0.5
Work In Grounds Abattoir	77	212	19	6563	231	33.3	1.1
Contract Worker	74	201	16	6577	217	34.1	1.1
Assist Observe Animal Birth	334	1033	74	5687	1107	30.2	4.9
Skinning Meat Process	337	1035	77	5682	1112	30.3	5.0
Shooting Hunting	336	1037	74	5683	1111	30.2	4.9
Work With Wool	339	1042	75	5677	1117	30.3	5.0
Work In Shearing Shed	315	970	69	5755	1039	30.3	4.6
Work In Wool Processing	308	967	67	5760	1034	29.8	4.5
Work With Straw Animal Bedding	338	1036	72	5686	1108	30.5	5.0
Attend Saleyard Animal Show	334	1034	68	5692	1102	30.3	4.9
Work With Animal Manure	338	1037	71	5686	1108	30.5	5.0
Live On Farm	74	88	11	6695	99	74.7	1.1
Visit Farm	341	1037	84	5673	1121	30.4	5.0
Launder Clothes Animal Worker	306	966	71	5757	1037	29.5	4.5
Exposed To Livestock Transport	339	1041	74	5679	1115	30.4	5.0
Consume Unpasteurised Milk	341	1049	91	5654	1140	29.9	5.0
Exposure To Paddock Dust	324	1035	90	5669	1125	28.8	4.8
Live Work Within 1km Abattoir	318	1026	90	5678	1116	28.5	4.7
Live Work Within 300m Bush	132	1247	21	5526	1268	10.4	1.9
Totals	6312	20104	1516	148230	21620	14.5	

3.5. Discussion

Q fever disease in Australia continues to impact on rural communities and veterinarians despite the availability of an effective vaccine and the implementation of a large scale national vaccination campaign. However, despite this awareness, both in Australia and the rest of the world, there is a shared consensus that the disease is underreported and often misdiagnosed[46].

In Australia, over time, there has been an increase in Q fever notifications both nationally and in Queensland. This has included the period of implementation of the national Q fever vaccine program. Interestingly, Queensland continues to record the highest proportion of notifications in Australia suggesting that either (i) a larger proportion of the workforce is engaged in risk-related employment, or (ii) there is increased laboratory testing and screening in Queensland compared to other Australian states which results in the identification of more cases. Alternatively, there may be other risk factors, not work related, that predispose Queensland residents to infection.

The Queensland notification data supported previous observations that Q fever was widespread in rural populations. There have been regions where notifications have been consistently recorded over time and these have been in areas of high concentrated beef production along with sheep and cattle rearing areas such as Goondiwindi, Roma, St George and Charleville. These were areas with increased numbers of people in direct occupational contact with farm animals. Over time, Toowoomba has recorded the highest number of notifications for any specific geographical location in Queensland, yet it was not a location of great farming activity or where large numbers of livestock resided. The population of Toowoomba were mainly employed in healthcare, retail and education (38%) with only 6% of the community working in the agricultural sector with an associated occupational risk of contracting Q fever. However, Toowoomba has close proximity to cattle rearing areas, and is the main corridor for livestock transport for shipping centres in Brisbane and interstate. In addition, there are a number of rural and metropolitan abattoirs that are supplied via livestock road trains passing through Toowoomba, and hence the dispersion of contaminated dust and the direct depositing of contaminated animal secretions may occur. This may contribute to the high numbers of notifications recorded from this region.

A high rate of Q fever notification was also observed for the metropolitan area of Brisbane, with the 6th highest rate of notification. The risk factors associated with these infections were not clear, but are likely to involve factors other than those associated with close proximity to ruminants or employment.

Queensland was responsible for over 50% of the national Q fever notified cases since the collection of Q fever data began in 1952, and continues to report cases from all over the state. It recorded a continual increase over time in Q fever notifications, with the highest number of cases recorded during the implementation of the vaccine program. This may have been the result of an increase in Q fever screening and a heightened awareness of the disease by medical practitioners and other health professionals. In Queensland, there has been a decline in notifications and under-recognition of Q fever since the vaccine program ended in 2005 with the number of yearly average notifications decreasing. This may simply be a reflection of the lapse in awareness of the disease or as a direct impact of the effectiveness of the vaccine program. The number of cases identified post Q fever vaccine program in Queensland were still high compared to national disease rates, illustrating that Queenslanders were still very much at risk of contracting the disease, and probably reflects the large number of Queenslanders in at risk occupations.

Although Q fever occurred mainly in males, high levels of infection were recorded in females in the years 1997 and 2001. This coincided with the introduction of the Q fever vaccination program and the NQFMP respectively. These increases may have been due directly to a heightened awareness of the disease by health professionals and therefore a higher rate of diagnosis, or perhaps by the incursion of females into previously male dominated roles such as meat packing and processing. Also, it is possible that with increased vaccination, pre-vaccine screening identified cases that had not previously been detected and therefore added to the overall notification numbers.

The notification data showed that Q fever disease affects all age groups including children and elderly. However nationally, including in Queensland, over the last 20 years there has again been an upward shift in the average age groups reporting Q fever disease, and this coincided with the implementation of the national vaccine program. The average age of persons identified as having Q fever in Queensland prior to the vaccine campaign in 2000 was 36.6 years old. This comprised the traditional working age group of 16-45 year olds, an age group that potentially had the highest occupational exposure risks. The average

age of notification post implementation of the vaccine program was 43.4years and the group recording the highest notifications came from the 36-60 year olds. This may be due to an increase in awareness of Q fever among these age groups or it may reflect the number of cases of chronic Q fever being reported as a result of previously identified or asymptomatic, acute Q fever episodes and an ongoing Q fever sequelae. These data were consistent with previous observations of an increase in exposure to *C.burnetii* with an increase in age.

The occurrence of Q fever has been reported in children in Australia and internationally. However, the awareness of Q fever in children is rare, and the disease is often not recognised and subsequently under-reported in this population, or reported as unusual case studies. Queensland has reported some of the highest rates of Q fever disease in children in the world, excluding the recent outbreak in The Netherlands. In Australia, rural children are often involved in farm duties including maintaining animals and pasture enclosures, and are often present at the birthing of farm and stock animals. As a result they are commonly exposed to the *Coxiella* bacteria. The increase in responsibilities and duties that commonly occur with increasing age for the children living on farms and rural properties is reflected in the increasing number of Q fever cases reported in children as they get older. Also, for younger children, the potential to have secondary exposure to animal products and secretions from the parents or their work clothing and footwear, may account for the number of paediatric cases recorded from this age group in rural Queensland. To highlight this some clinical case studies were examined in Chapter 7. Potentially, any or a combination of these factors may help explain the higher rates of Q fever seen in Queensland among the paediatric population, than that recorded in other areas.

In the Northern Hemisphere, Q fever disease primarily occurs in spring or the “birthing” season[47]. However, in Queensland and Australia as a whole, the majority of notifications occur in the autumn months, directly following large rain periods or the wet season. This corresponded to the same global time period as spring in the Northern Hemisphere but did not correlate to the birthing seasons in Australia. These data suggest that Q fever was a disease seen globally in the beginning of any given year and was not linked to the spring season. Queensland experiences its wet season in January and February or the season of summer, while in Europe the wet season begins in the same months during winter. This

suggested that Q fever is a disease that is influenced by rainfall. With increased rainfall comes new vegetation and food supply for many animals, and perhaps this is the link between Q fever disease and climate.

Even though no direct correlation was found between Q fever notification and annual rainfall patterns, it was interesting to observe that the two peak periods of Q fever disease occurred during drought conditions in 1992-1994 and 2001-2003. During these periods of drought, high levels of dust and dust storms were recorded in the country, and these may have contributed to the widespread dissemination of *C.burnettii* from rural areas to the country as a whole. Similar observations in soil dissemination have previously been reported in the USA[48] .

This extensive notification data analysis showed that there were many factors that influenced the occurrence of Q fever disease in Queensland. However, the data confirmed that Q fever infection was not exclusively confined to the rural population, and that other groups at risk included those in metropolitan areas, and children in both regions. So far, the true extent of exposure to *C.burnetii* by residents in Queensland has not been extensively tested, and requires further examination by a retrospective serological survey of the population to determine the true rate of exposure. Also, there is a need to determine other risk factors associated with infection, particularly those that are responsible for infections in metropolitan populations. These may include exposure to animals other than farm animals, such as domestic pets and native fauna, or perhaps environmental exposure through soil and dust particles[49–51].

Finally, Q fever may present with a wide range of clinical signs and symptoms. It is likely therefore that the disease is not recognised in areas and populations that are not normally considered to be at risk, particularly those in metropolitan locations. Also, the laboratory diagnosis of infection has traditionally been based on the immunofluorescent antibody assay which detects antibody developed following infection. This gives a retrospective diagnosis but may not be optimal to detect early acute infections. The newly evolving molecular diagnostic methods may provide an improvement in some areas of Q fever diagnosis and are being implemented more in the general screening for disease. Given this, it is possible therefore that the exposure rates described above based on notification data may in fact be under-reporting the true level of Q fever disease in Queensland, and by association in Australia.

3.6. Significant Outcomes from this Chapter

- This was the first comprehensive study examining Q fever notifications in Queensland over an extended time period (1984 – 2014)
- There was a higher notification rate of Q fever than expected in the urban population, who are traditionally considered to be at low risk of infection
- Despite a national government funded vaccine program, Q fever disease in Queensland still had one of the highest rates of notification in the world
- There was evidence of a substantial rate of Q fever notifications among children in Queensland, indicating that the disease was not restricted to the at risk adult population

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Chapter 4

Seroprevalence of *Coxiella burnetii* in Humans and Animals in Queensland

Articles published as a result of work done in this chapter:

Tozer, S. J., Lambert, S. B., Sloots, T. P. & Nissen, M. D. Q fever seroprevalence in metropolitan samples is similar to rural/remote samples in Queensland, Australia. Eur. J. Clin. Microbiol. Infect. Dis. 30, 1287–1293 (2011).

4.1. Introduction

Epidemiological studies provide insights into the state of knowledge of particular infectious agents within defined populations. The study of disease and the impact a specific disease has on a population is often investigated using prevalence studies. A cross sectional study of *Coxiella burnetii* exposure in the Queensland population using serological based methods and hence a seroprevalence study will help to ascertain the true exposure rate of *Coxiella* in this population and help to predict the potential disease burden for the community.

Current epidemiological data published for Q fever is largely produced using serological methods[1]. There are however, no standardised methods that have been established for sero-profiling of Q fever, and the methods used vary greatly and have different thresholds for determining positivity. Early investigations employed micro-agglutination assays and or complement fixation tests which proved to be highly specific but lack the sensitivity associated with the newer techniques of ELISA and IFA screening. These less sensitive techniques of micro-agglutination and complement fixation assays may result in an underestimation of the true prevalence of *Coxiella* throughout the community. Immunoassays measuring antibody levels such as the ELISA and IFA have a high level of sensitivity, and in the case of the ELISA this method can be automated for more accurate results[2,3].

C.burnetii is widely shed into the environment by infected animals via birth product, urine, faeces, and milk, and can also be transmitted via ticks which draw blood contaminated with *Coxiella* from an infected animal. Both avian and mammalian species are able to become infected with *Coxiella* and hence are potential sources of *Coxiella* exposure to humans[4]. These infected animals generally do not exhibit any signs or symptoms of disease, and it is only a subset of infected animals that may present with late abortions and reproductive difficulties[5]. Traditionally Q fever infections in human have been widely associated with parturient animals and the exposure of humans to infectious birth products.

4.1.1. Prevalence of Q Fever in Humans

Q fever cases and local epidemics have been reported around the globe, yet, despite the global prevalence of *Coxiella* and Q fever disease, there have been very few large population-based studies investigating the seroepidemiology of this infection in humans[4,6–11]. Seroprevalence studies have been performed in both animals and humans to assess the potential infection rates, while other studies have been used to estimate disease burden within populations[12]. Recently seroprevalence studies for Q fever have been performed following large outbreaks in The Netherlands and Germany. These highlight the potential risks associated with Q fever disease and illustrate the epidemiological inconsistencies associated with such outbreaks[13].

4.1.1.1. Global Prevalence of Q Fever

Australia identified the first cases of Q fever[14], and since then every country in the world has reported cases of Q fever with the exception of New Zealand[4]. There have since been numerous outbreaks of Q fever globally with early reports from the United States of outbreaks in meat packing plants in Texas and Chicago in the late 1940's[15,16] and more recently in US troops returning from Afghanistan[17]. There were over 300 cases of Q fever reported in Canada as a result of secondary exposure to parturient cats which was identified as a single outbreak event[18], and Israel reported an acute Q fever outbreak in a school [19]. The United Kingdom has reported outbreaks of Q fever associated with sheep and the dispersion of *Coxiella* via wind[20]. In addition, there have been many countries throughout Europe that have experienced outbreaks of Q fever including Switzerland[21,22], Italy[23] and Poland[24]. These outbreaks have highlighted the extent of *Coxiella* dispersion throughout the world and hence the different populations exposed to the disease.

Four European countries have been associated with the largest outbreaks of Q fever since 1982: The Netherlands, Germany, Bulgaria and France[13]. These reports identified goats and sheep as the main sources of infection to humans, and highlighted that these animals were potential sources responsible for large community outbreaks. These investigations also identified that cattle do not play a major role in the dissemination of *Coxiella* into the environment within these countries, as abortions in cattle were less frequent and hence the transmission of *Coxiella* to humans from this source appeared to be limited[5,13]. The large scale outbreaks that occurred in The Netherlands, Germany, Bulgaria and France have initiated the need for further investigations into the extent of exposure that these

populations, and potentially other nations have to *Coxiella*. To truly assess this extent of exposure in defined populations, seroprevalence studies have been performed in these countries.

4.1.1.2. Seroprevalence Studies

Seroprevalence rates in the general population of 14% were reported for France and 2.4% for The Netherlands (Table 4.1). Higher seroprevalence rates have been reported from these countries in “at risk” populations. These include reports from The Netherlands of up to 24%, 22% reported in Germany and up to 38% in Bulgaria. These populations were investigated after Q fever outbreaks and showed that whole communities were considered to be at risk of infection. The true extent of exposure in these communities as a result of the outbreaks were assessed using seroprevalence studies [13]. Globally, seroprevalence of Q fever varies greatly. Different countries report different rates of disease with outbreaks occurring sporadically and not necessarily limited to large farming nations or at risk populations (Table 4.1). The extent of exposure needs to be identified for individual countries, states, districts or provinces for a true assessment of exposure and to estimate the potential disease burden.

4.1.1.3. Seroprevalence Studies in Australia

Australia has on average, the highest, annual notifications of Q fever cases in the world[25] and yet there are very few epidemiology data available from this Q fever endemic country. Only limited seroprevalence studies have been performed in Australia, and as a result there are limited nationwide seroprevalence data regarding the true exposure rate of *Coxiella* in the Australian population. These studies have been restricted to small numbers and limited to only six of the eight states and territories. Only nine seroprevalence studies into Q fever exposure have been performed since the first study in 1980, which was more than thirty years after the disease was first described in Queensland. Until recently, these studies have focused on different “high risk” or “at risk” populations, including animal handlers, meat workers, abattoir workers and rural communities or populations, and reported seroprevalence rates from 7%-66% (Table 4.2) Many of these studies have involved small cohorts and have been from very diverse geographical locations around Australia. Also they were limited to adolescents and adults. To date, there have been few comprehensive studies in Australia which assessed the general population for *Coxiella* exposure.

Table 4.1: Seroprevalence reported in different countries around the world over time.

Published	Country	Seropositive	Sample	Positives	Population	Ages	Author
1986	Switzerland	7-32%	5446	381-1743	following outbreak	adults	Dupuis[21]
1995	England	19%	730	143	farming	10-70	Thomas[26]
1995	Nova Scotia	15%	492	72	general	18-70	Marrie[27]
2000	Taiwan	4.2%	616	26	general	adults	Ko[28]
2001	Germany	22%	1651	51	general (including outbreaks)	adult	Hellenbrand[29]
2006	Barcelona	15%	216	33	general	00-91	Cardenosa[30]
2006	E. Turkey	20%	92	18	cattle farmers	adults	Seyitoglu[31]
2006	Cyprus	53%	583	307	general	all	Psaroulaki [32]
2008	Ankara	32%	601	194	general	adults	Kilic[33]
2008	Ireland	13%	2394	306	general	12-64	McCaughey[34]
2009	USA	3%	4437	133	general	>20yrs	Anderson[35]
2010	Northern Turkey	12%	407	50	general	>5yrs	Gozalan[36]
2012	Greece	62%	159	445	at risk	adult	Vranakis[37]
2012	Greece	49%	493	242	general	adult	Vranakis[37]
2012	Netherlands	12%	543	66	general	adults	Hogema[38]
2013	Kenya	31%	246	76	-	-	Knobel[39]
2013	Gambia	8.3%	796	66	general	Childre n<16	Van der Hoek[40]
2014	Bulgaria	18%	5207	937	at risk group	adult	Georgiev [41]
2014	France	14%	578	81	following outbreak	adult	Georgiev [41]
2014	Netherlands	2.4%	5 654	136	general	adult	Georgiev [41]
2014	Germany	22%	255	56	following outbreak	adult	Georgiev [41]

4.1.1.4. Seroprevalence in the Queensland Population

Queensland reports, on average, 215 cases of Q fever a year compared to the national average of 467 cases, therefore over 46% of Australia's laboratory confirmed cases of Q fever are contracted in Queensland. However, given the wider understanding that the disease is misdiagnosed or under-reported, the true incidence of the disease may be underestimated along with the extent of true exposure in this population[42].

The first published epidemiology study performed in Queensland was in Brisbane in 1980 among meat workers as part of a retrospective study spanning 10 years from 1968 to 1977. This showed an average incidence rate for *Coxiella* of 1% per annum, with an increase to 7.9% following a work-place related outbreak in 1969[43]. The first Queensland sero-prevalence study was performed on this population involving 139 meat workers showing a rate of 15.8% [43].

Table 4.2: Results of seroprevalence rates reported in Australia

Published	Region	Sero-positive	Sample (n)	Positive (n)	Population	Ages	Author
1980	Brisbane Queensland	16%	139	22	meat workers	adult	McKelvie[43]
1984	Adelaide South Australia	45%	1922	875	abattoir	adults	Marmion[44]
1999	NSW	11%	829	89	abattoir	adults	Casolin[45]
2000	NSW	27%	1417	394	cattle handlers	adults	Hutson[46]
2001	Central Queensland	19%	265	49	rural/ farming	12-79	Taylor[47]
2003	Kimberly WA	66%	59	39	rural/ farming	16-65	Mak[48]
2005	Victoria	20%	9196	1859	abattoir	10-72	Greig[49]
2010	South West Queensland	7%	447	29	rural	<25yrs	Parker[50]

Central Queensland has high numbers of notifications of Q fever each year, and in 2001 the Central Queensland Rural Division of General Practice undertook a large study and vaccination program for rural community residents. The participants in this study were of adolescent or adult age, largely from a rural background, with the majority having resided on farms or worked with ruminants, thus having a potentially high level of exposure or risk associated with the acquisition of Q fever disease. This population had a seroprevalence of 18.5% [47].

A recent study performed in a select population of under 25 year old subjects residing in South West Queensland, the geographical “hot spot” for Q fever in Australia, highlighted the exposure in children and the younger rural population in potentially high risk communities, showing a rate of 7% from the 447 subjects investigated[50].

Coxiella exposure in the general Queensland population remains largely unknown, and there is a distinct lack of knowledge regarding those populations considered not at risk and those residing in urban or metropolitan areas with low rates of Q fever notification. Table 4.2 shows the results of seroprevalence studies that have been conducted in Australia, highlighting the limited data that currently exists for a nation that has one of the highest rates of notification per capita.

4.1.2. Seroprevalence in Animals

Coxiellosis may occur in a variety of animal species throughout the world including mammals, birds, reptiles, amphibians, insects and fish[51]. The transmission of *Coxiella* between animal species and within species, in particular between livestock, creates potential sources of infection for humans. The level of potential risk of disease may be assessed by investigating the previous exposure rates in these animal species by use of seroprevalence studies.

There are very limited data generated describing the seroprevalence of *Coxiella* in Australian animals. The studies that have been performed are listed in Table 4.3 and highlight the need for further more extensive investigations in animal populations from regions with high Q fever notifications.

4.1.2.1. Seroprevalence in Livestock

4.1.2.1.1. Cattle

In most countries cattle are one of the main sources of transmitting *Coxiella* to humans and causing Q fever disease. Correlations between the sero-positivity in cattle and humans has previously been established[52], and there were varying seroprevalence rates from different countries and within different countries around the world depending on geographical location[1]. Rates varied from 6 % in Northern Ireland[53], 7% in Spain[54], up to 11% in Iran[55], 24% in Cyprus[32], 26% in Korea, Bulgaria had 21% prevalence rate, Germany showed 19% among cattle while France had 15% within herd prevalence and up to 73% for between herd prevalence and The Netherlands reported 21% within herd rates and up to 37% for between herd prevalence[13]. Australia had reported rates of <1% from cattle in Western Australia[56], South Australian[57], Victoria and New South Wales[58]. Recently there has been a prevalence study of cattle in Central Queensland showing rates of 17%[59].

Table 4.3: Animal seroprevalence studies performed in Australia

State	Species	Sero-positive (%)	(n)	Year Published	Author
Queensland	Dogs	21.8	201	2011	Cooper [59,77,81]
	Cats	38.7	31		
	Foxes	43.8	16		
	Pigs	22	50		
	Bandicoots	23.9	46		
	Dingos	17.3	127		
	Possums	10.7	56		
	Cattle	16.8	308		
	Macropods	20.8	500	2012	Cooper[85]
Eastern Australia	Cats	0-9	712	2015	Shapiro[82]
Western Australia	Cattle and Sheep	0.5	379	2009	Banazis[56]
	Kangaroos	33.5	343		
	Kangaroos	24.3	1017	2011	Potter[86]
	Bandicoots	8.6	35	2011	Bennett[87]

4.1.2.1.2. Sheep

Small ruminants have also been regarded as potential sources of infection to humans as they carry *Coxiella* in birth products and their fleece[60–62]. The prevalence rates in sheep also vary depending on geographical location. Rates in sheep from various countries include 40% from Mexico[63], 23% in Egypt[64], 20% in Turkey with flocks having an 80% seroprevalence rate[65], Cyprus reports 19% of sheep tested positive[32], 12% in Ireland[53] and Spain[54]and 3% in Albania[66]. The four European countries investigated after large outbreaks showed up to 57% of sheep screened in Bulgaria were positive, 20% in France, 9% in Germany and 4 % in The Netherlands[13].

In Australia there has only been one sero-survey conducted involving sheep co-residing with cattle in Western Australia, which has one of the lowest notification rates of Q fever in humans in Australia. The rate detected among these sheep was 1%[56].

4.1.2.1.3. Goats

Goats may have persistent *Coxiella* infections and can shed the bacteria in milk, urine and faeces along with high bacterial loads in parturient animals[1]. Goats have recently been associated with large outbreak of Q fever in The Netherlands[67]. As with cattle and sheep the rates of exposure in goats globally, vary with geographical location. Seroprevalence rates have been reported in Iran of 66%[55], 49% of goats in Cyprus[32], 35% seen in Mexico[63], 17% reported in Egypt[64], 9% in both Ireland[53] and Spain[54]. Following large outbreaks in Europe, Bulgaria reported up to 40% sero-positivity in goats, 88% was reported in France, 3% in Germany and 8% in The Netherlands.

Australia also recorded an outbreak of Q fever in meat processors following exposure to feral goats during slaughtering[68,69]. Following this outbreak a seroprevalence study of *Coxiella* in feral goats was performed, showing that 52% were positive for *Coxiella*-specific antibodies[70].

4.1.2.2. Domestic Pets

It is well documented that Q fever disease is predominantly associated with large ruminants, however, there have been numerous cases and outbreaks that have been related to domestic pets including felines and canines[71,72]. In the cases of Q fever disease where no direct ruminant contact can be recorded, it is thought that perhaps these domestic animals may be the reservoir of the disease and hence the sources of infection to humans in urban or non-rural environments.

4.1.2.2.1. Canines

Dogs have long been companion animals for humans as well as working animals to aid farmers, yet few seroprevalence studies in dogs have been performed throughout the world. A French study showed that military dogs used in various countries had rates of 12% when stationed in Senegal, 10% from France, 8% in the Ivory Coast and 5% in Guyana. The dogs that resided in Martinique showed no exposure to *Coxiella* while residing there[73]. Slovakia showed rates of 12% among canines screened[74] while a study in Sicily showed a rate of 8% [75]. A large study performed in New Zealand with over 12,500 serum samples from dogs located in various geographical areas throughout New Zealand showed no seropositive samples. This formed part of a major study into Q fever in New Zealand and is an important finding which seems to confirm the absence of *Coxiella* in that country [76].

Recently in 2011, Australia reported the first investigation into the seroprevalence in dogs, with a study performed in Townsville, one of Australia's major beef/cattle producing regions and known for high notification of Q fever in humans. This study showed a rate of 22% among domestic dogs from Townsville[77] and hypothesised that dogs may be a potential source of transmission for *Coxiella* to humans.

4.1.2.2.2. Felines

Cats have been widely associated with outbreaks of Q fever throughout the world and have been responsible for causing Q fever disease in humans more commonly than dogs[1]. Seroprevalence studies have been initiated following such outbreaks to determine the extent of exposure in cats and to aid in predicting the potential risk of disease to humans. Japan reported rates of 14% among domestic cats, while stray cats had up to 42% seropositivity rates[78]. Canada, which has had several outbreaks of Q fever linked to domestic cats, reported 13% of cats screened had been exposed to *Coxiella*[79]. The United Kingdom recently reported 62% of cats screened for *Coxiella* antibodies were positive in 2014 [80].

Australia has limited studies regarding cats and *Coxiella*. However, Australia has reported seroprevalence rates among feral cats trapped in Central Queensland of 39%[81]. A recent study performed on feral, domestic, shelter and cattery felines from the east coast of Australia showed varying exposure rates from 0-9%. The cattery felines had 9% exposure rates while the domestic pets had 1% and the other two groups had no seropositive results recorded[82]. These animals may play a significant role in the chain of transmission of *Coxiella* to other wildlife and domestic animals in the region.

4.1.2.3. Native Animals

4.1.2.3.1. Flying Foxes

In Australia, flying foxes have been identified as a potential source of infection to humans or other animals for a large number of diseases, including Hendra virus and Lyssa virus. To date, there has only been one study investigating the prevalence of *Coxiella* in flying foxes in Australia. This is the study described in Chapter 5 of this thesis and forms the basis of a publication on which the Chapter is based[83].

However, there have been no reports of seroepidemiology studies investigating *Coxiella* and flying foxes, but there was a report of *Coxiella* seroprevalence in Phyllostomus and

Molossus, both are genera of bats. This study was in French Guiana and no antibodies were detected[84].

4.2. Specific Aims Addressed in This Chapter

The previous chapter, examined the notification data for Q fever in Queensland, and showed that the disease was far more widespread than previous reports suggested. It was not just restricted to the rural population, but showed significant notification rates from both rural and urban populations.

Also, it showed that, based on notification data, children in both rural and urban settings had a considerable rate of exposure to the disease.

At the time of commencement of this study, there were very limited data regarding the seroprevalence of Q fever in the Queensland population. Also, acquisition of the disease was largely associated with animal contact, specifically ruminants with some limited evidence that native animals might be a source of infection.

The study in this chapter therefore sought to address the following specific aims:

- To assess the seroprevalence of *Coxiella burnetii* antibodies in serum samples collected from a generally healthy population in Queensland, and compare those from rural and urban populations, in order to assess the population with greater exposure.
- To determine such seroprevalence in a subset of serum samples collected from children, to assess if children are at risk of contracting the disease.
- To develop and apply competitive ELISA and IFA assays to investigate the seroprevalence in animal species. Competitive assays needed to be developed because specific anti-species conjugates were not available for many of the animals to be tested.
- To determine the level of *C.burnetii* exposure in serum samples collected from a range of animals, including ruminants and farm animals, native animals and domestic pets, using the serological assays above.

4.3. Methods

4.3.1. Serum Samples Collected

4.3.1.1. Human Serum Samples

The Queensland Q fever notification data reviewed in Chapter 3 highlighted that Q fever was predominantly a rural disease. This was taken into consideration and over-sampling of sera from rural areas was deliberately performed. The investigation into Q fever exposure in paediatric age-groups (children younger than 16 years of age), was sought to be better defined by over-sampling of sample numbers from this age-group also. Subjects in this age group are currently not eligible for vaccination in Australia. Sample demographics were collated and only sera from patients with a current Queensland residential postcode were included in the seroprevalence analysis.

In the serum bank of 2122 serum samples described in Chapter 2 (section 2.2.5.1.) there were a total of 1988 serum samples from subjects residing in Queensland. Geographical distribution was determined using the Bureau of Statistics Standard Geographical Classification System. Statistical Subdivisions (SSD) were defined as socially and economically homogeneous regions characterized by identifiable links between the inhabitants[88]. Two distinct geographical populations were established based on patient's postcodes and SSD. SSD's beginning with the code 305 were considered to be an "Urban" population and the rest of the patient population from Queensland was considered to be "Rural" [89]. When samples were collated based on postcodes, it was shown that there was a representative sample from each of the 39 Statistical Subdivisions within Queensland.

Limited demographic data were available on each de-identified sample, being restricted to date of collection, date of birth, age, sex and postcode. The age distribution amongst samples in the serum bank was 0 – 92 years of age, with an average age of 37.6 years and a median age of 37 years. The paediatric populations were defined as patients being younger than 16 years of age.

These 1988 samples were screened for Q fever phase I and II IgG antibodies to determine the seroprevalence of the Queensland population to *C.burnetii.*

4.3.1.2. Animal Samples

A collection of canine and feline serum samples conveniently acquired from the University of Queensland, School of Veterinarian Science in 2011 was used in this study. These samples were collected from animals presenting to the University of Queensland Veterinary Clinic located at the St Lucia and Gatton campuses. There were 628 serum samples obtained. These samples did not have any clinical data attached. There were 470 dog sera of which 250 were male dogs of which 50% were de-sexed and 220 female sera of which 30% were de-sexed. There were 158 serum samples obtained from cats in which 88 were from males with 72% being de-sexed males and 70 were from females with 76% being de-sexed.

Serum collected from flying foxes were also obtained from Queensland's Centre for Emerging Infectious Diseases and Biosecurity. These sera were included in the study as *Coxiella* DNA had previously been detected using molecular methods in pooled flying fox urine collected from colonies in close proximity to humans (Chapter 5 of this thesis)[83]. There were 50 of these serum samples included in this study.

4.3.2. Laboratory testing

Human serum samples were analysed using the standard IFA and ELISA methods as previously described with anti-human FITC or anti-human HRP as the detector (Chapter 2 – sections 2.2.3. & 2.2.4.). For some animal species, species-specific conjugates were available, and sera from these animals were examined using the standard indirect IFA as applied to the human sera. However, for a significant number of animal species, such a species-specific conjugate was not available, and a competitive ELISA, and a competitive IFA method were developed to test the sera from these animals.

4.3.2.1. Screening Methods for Human Serum Samples

In Australia, Q fever testing is largely performed using serological methods, with only very few laboratories now introducing molecular techniques. The gold standard for Q fever screening is considered to be the immunofluorescence assay (IFA)[4], which is used as a diagnostic screening tool for confirmation testing and a tool to determine past exposure. This assay is used widely due to its high sensitivity and specificity rates (Chapter 2 - section 2.2.5.2.)[4,8,9].

In this study, serum samples were screened for Q fever IgG antibodies using two screening assays:

(1) a modified, in-house IFA method (Institute of Medical and Veterinary Science - IMVS, Adelaide)[90] The IFA method was able to measure all serological markers IgA, IgG, and IgM for both phase I and phase II of *Coxiella*. For this study, only phase II IgG antibodies were examined. A full description for the method has been described in Chapter 2 under General Methods (Section 2.2.3).

(2) a commercially available ELISA method (ALERE Panbio Ltd, Brisbane, Queensland, Australia). The ELISA kit was able to detect IgG antibodies against phase II *C.burnetii* organisms only (Chapter 2 - Section 2.2.4). Results were recorded as positive, negative, and equivocal according to manufacturer's instructions. For the purpose of this study an equivocal result was repeated and if still equivocal it was recorded as a negative for the ELISA assay.

A third assay was used for discrepant analysis of discordant results that occurred between the two screening assays. This was a commercially available IFA kit assay (Focus Diagnostics, Cypress, California, USA) and specimens were recorded as detected or not detected. The assay was performed as per the manufacturer's instructions and had a documented sensitivity of 100% and specificity of 99% when compared to the IMVS IFA method and the complement fixation test (CFT).

A specimen that was positive in the two screening assays was considered a true positive, and the commercial IFA was not performed. Where a specimen was positive in only one of the screening assays, the commercial IFA was performed and the result in this assay was considered the final result.

4.3.2.2. Indirect IFA for Testing Animal Sera

Sera collected from dogs and cats were screened using IFA slides prepared as before for human screening, using the Nine Mile strain of *Coxiella*. The IFA methodology employed was as previously described in Chapter 2 (Section 2.2.3.). The specific conjugates used were anti-dog IgG (H+L) - F(ab')2 fragment-FITC (Sigma-Aldrich, St Louis, USA) for the detection of dog specific antibodies, and anti-Cat IgG (H+L), F(ab')2 fragment-FITC (Sigma-Aldrich, St Louis, USA) for cat antibody detection, both conjugates were used at a working concentration of 1:500.

4.3.2.3. Competitive ELISA for the Detection of Coxiella in Animal Sera

4.3.2.3.1 Principle of the Competitive ELISA Method

Indirect ELISA assays and species-specific conjugates to detect the antibodies of different animal species were not commercially available. As a result, a variation of the standard ELISA method was developed specifically for this study to detect *Coxiella* antibodies in animals; the competitive ELISA. The general principle of the competitive ELISA assay is shown in Figure 4.1.

The central principal of the competitive ELISA is a competitive binding process executed by the animal sample antibody and a competitor antibody. The procedures of competitive ELISA are different in some respects compared with the indirect ELISA method described in Chapter 2 (section 2.2.4.) for the detection of *Coxiella* antibodies in human sera.
A simplified list of the steps involved in the competitive ELISA is as follows:

1. Primary animal serum is incubated in the ELISA plate which is coated with *Coxiella* substrate antigens.
2. After removal of unbound animal antibodies by washing, the competitor antibody (e.g. anti-*Coxiella* mouse serum) is added to the 96-well ELISA plate and incubated.
3. Unbound competitor antibody is removed by washing the plate. (The more anti-*Coxiella* antibodies in the animal serum, the less competitor antibody will be able to bind to the antigen in the well, hence "competition.")
4. An enzyme conjugated anti-competitor species antibody (e.g. anti-mouse HRP conjugate) is added, incubated and washed to remove unbound conjugate.
5. A substrate is added, and the bound enzymes elicit a chromogenic signal.

For competitive ELISA, the higher the sample antibody concentration, the weaker the eventual signal. The major advantage of a competitive ELISA is the ability to test serum samples from animal species for which a species-specific conjugate is not available.

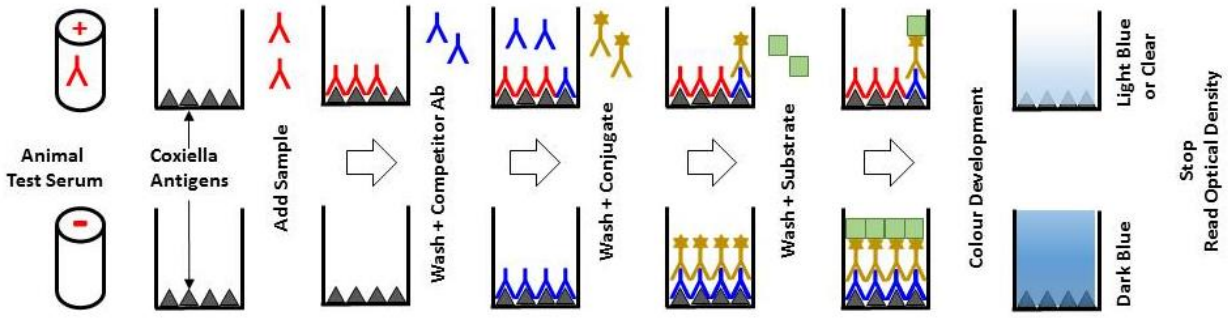

Figure 4.1 Competitive ELISA principle, describing the reaction with Positive and Negative sera.
⅄ = positive animal serum; ⅄ = competitor antibody (CA); ⅄ = anti CA-conjugate HRP; ■ = substrate

4.3.2.3.2. Titration of Inhibitor Antibody

Known positive sera from different animal species were tested in the competitive ELISA using the commercially available human ELISA screening kits. Previously confirmed pooled human positive sera was used as the competitor antibody. These initial trials proved to deliver a high background absorbance in the assay and it was determined that human serum was not acceptable for the use as a competitor in the competitive ELISA.

Coxiella-positive mouse sera, provided by Dr Alana Cooper (James Cook University, Townsville) at high titre was then used as the competitor antibody in the competitive ELISA method. A checker board titration was performed to determine the optimal concentration of competing mouse sera to be used, with the appropriate anti-mouse conjugate.

4.3.2.3.3. Optimisation of Reagents

Q Fever Phase II IgG ELISA plates (ALERE Panbio Ltd, Brisbane, Queensland, Australia) were blocked with 10% skim milk at 37ºC for one hour, and washed three times with wash buffer provided in the kit. Positive mouse sera were diluted using kit dilution buffer from 1:1 to 1:20,000 and 100µL was loaded into the plate (Figure 4.2), and incubated for 1 hour at 37ºC. The plate was washed three times using kit wash solution and 100µL of goat anti-mouse IgG specific HRP-conjugate (Sigma-Aldrich, Missouri, USA) diluted 1:100 to 1:102,400 was loaded across the plate as per Figure 4.2. The plate was incubated at 37ºC for one hour. The plate was then washed three times using kit wash provided. 100 µL of tetramethylbenzidine (TMB) substrate was added and incubated at room temperature in the dark for 30 minutes. The substrate reaction was stopped by the addition of 100 µL of 2M sulphuric acid, producing a final yellow colour. Optical densities in the plate were then

determined using the Molecular Devices Spectramax 340PC384 plate reader at wavelength 450nm.

Anti-mouse IgG HRP Conjugate

Positive Mouse Serum

	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400	Negative
Negative												
1:100												
1:500												
1:1000												
1:2000												
1:5000												
1:10 000												
1:20 000												

Figure 4.2: Configuration of ELISA plate for optimisation of competing antibodies and conjugate for the screening of animal sera to determine previous exposure to *Coxiella* antigens.

Optimal results in the checkerboard titration of the competitor mouse sera and the anti-mouse conjugate was established as a dilution factor of 1:500 for the mouse serum, and a 1:200 dilution of the conjugate (Figure 4.3). These dilutions were subsequently used in the competitive ELISA.

4.3.2.3.4. Determination of Assay Parameters

The level of competition required for assigning positive and negative values was determined using known positive rabbit sera obtained from a commercial source (R-Biopharm, Laboratory Diagnostics Pty Ltd, Kurnell NSW, Australia). Dilutions of the rabbit sera were analysed using the IFA method described in Section 4.3.2.1 above with the substitution of anti-rabbit IgG fluorescein isothiocyanate (FITC) as a species-specific conjugate, and a dilution end point was determined. Based on this, the rabbit serum was diluted from 1:100 to1:1000,000 (see Figure 4.4) and loaded onto the indirect ELISA plates as per the described method above. Rabbit-specific HRP-conjugated goat anti-rabbit IgG (Sigma-Aldrich, Missouri USA) was used in the ELISA as the detector.

The end-point titre of positive rabbit sera determined to give an equivocal or negative result in the indirect ELISA was 1:50,000 (Figure 4.4). Titration of this antibody by IFA showed an end point of one titre higher at 1:100,000.

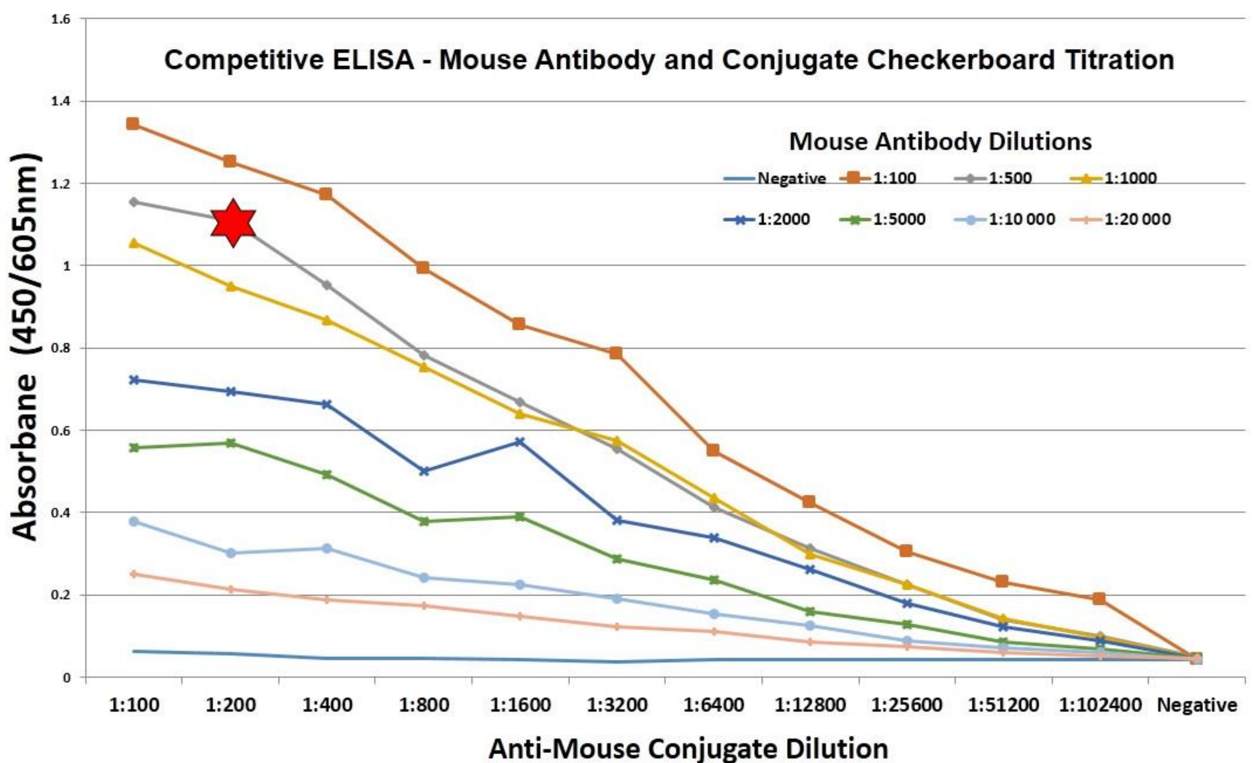

Figure 4.3: Competitive ELISA mouse antibody and conjugate checkerboard titration. Optimal dilutions were determined.

Shows combination of anti-mouse antibody and conjugate dilution chosen for subsequent use in the competitive ELISA.

The positive rabbit serum dilutions were then re-screened using the competitive ELISA assay with positive mouse sera used as a competitor antibody and detection of this antibody using HRP-conjugated goat anti-mouse IgG (Sigma-Aldrich, Missouri USA). Negative human and rabbit sera were also screened using the assay and gave identical results. As the supply of *Coxiella*-negative human sera was more plentiful, this was used as the negative control throughout the subsequent experiments.

Using the competitive ELISA, dilutions of positive rabbit serum ranging from 1:100 – 1:10,000 which have been predetermined to be positive by indirect IFA, showed absorbance values of less than 2.0 OD's (Figure 4.5). While negative human sera screened with the competitive ELISA gave absorbance values of >2.0 indicating no competition for binding to the antigen.

From the results above, it was determined that the following conditions for optimal assay performance would be applied in the competitive ELISA used in this study:

- Test animal serum dilution of 1:10
- Competitors mouse serum dilution of 1:500
- Anti-mouse conjugate dilution of 1:500

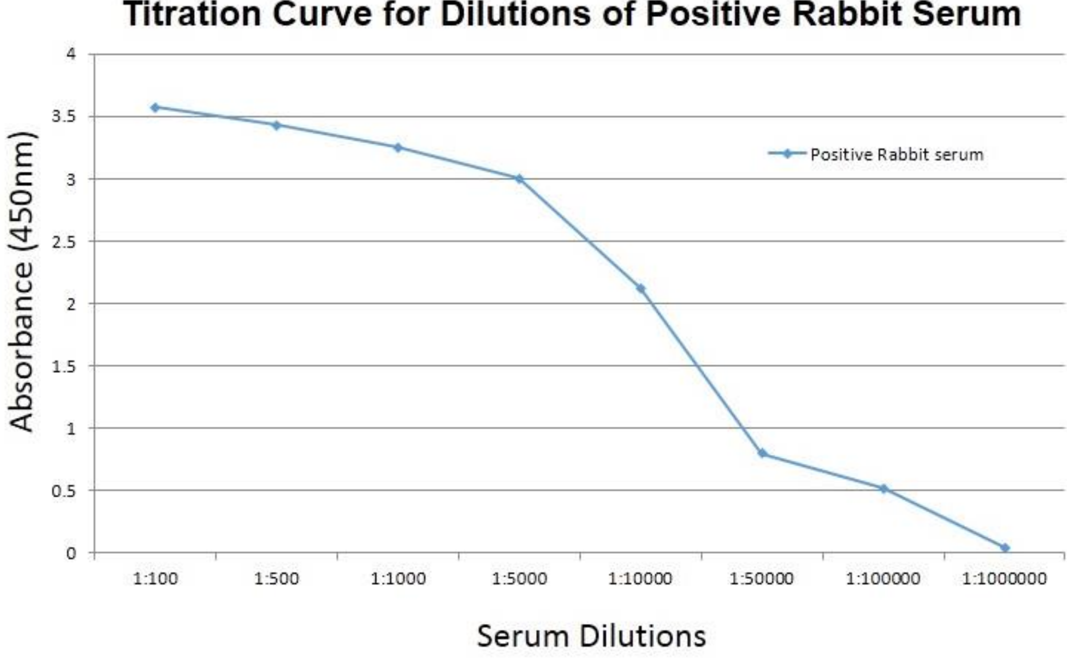

Figure 4.4: Positive rabbit serum dilutions in ELISA using species specific HRP-conjugated anti-rabbit IgG to determine end point titre absorbance of a positive serum

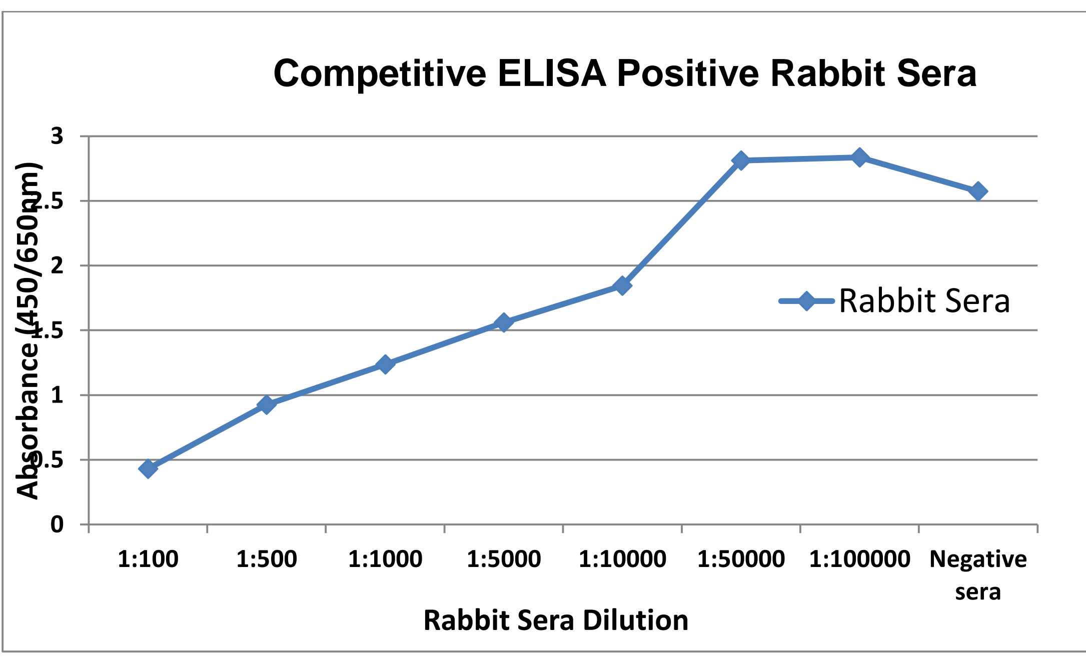

Figure 4.5: Positive rabbit serum dilutions in competitive ELISA using mouse sera as competitive antibody to determine cut off point titres absorbance

4.3.2.3.5. Calculation of Results

Using the above parameters, it was determined that if the difference in OD between the negative sera and the test sera calculated as a percentage was 40% or above, then the sample was deemed positive.

Calculation: $\frac{\text{(OD Negative Serum – OD Test Sera) x100}}{\text{(OD Negative serum)}}$ **= % of Competition**

Known positive and negative animal sera from different species were finally used to verify the competitive ELISA. Results are shown in Figure 4.6 and Table 4.4.

4.3.2.3.6. Optimisation of the Competitive IFA Using Animal Sera

The indirect IFA was performed using the same method previously described in the general methods in Chapter 2 (Section 2.3.3). The competitive IFA method used animal serum undiluted and diluted 1:5 in 3% Chicken yolk sac (IMVS Adelaide, South Australia). The 5µL of test serum was loaded to the slide and incubated for 30 minutes at 37°C, washed 2 times for 5 minutes in PBS-tween, air dried, and 5µL of competitive mouse sera diluted 1:1000 was loaded. This was incubated for 20 minutes at 37 ^{o}C. The sera were washed as before and slides air dried. The slides were then loaded with 8µL of FITC conjugate diluted 1:500 in PBS with 1% Evans blue to each well and incubated for 30 minutes at 37°C before being washed for 5 minutes in 2x PBS-tween and air dried. Slides were then mounted with coverslips and read using a fluorescent microscope as previously described. Figure 4.7 shows the results obtained in competitive IFA for positive and negative *Coxiella* animal serum.

4.3.2.4. Screening Methods for Animal Serum

<u>Competitive ELISA:</u> Animal test sera were screened using the competitive ELISA method described above. This method used the commercially available Panbio *Coxiella* ELISA plates from (ALERE Panbio Ltd, Brisbane, Queensland, Australia), which were coated with antigens derived from the Henzerling strain of Q fever. These plates were deliberately chosen so that the substrate antigen used was the same as the indirect ELISA used to test human sera. This allowed a direct comparison of human seroprevalence rates with animal rates knowing there was no discrepancy's due to different strain types of *Coxiella* as has been identified previously [91] .

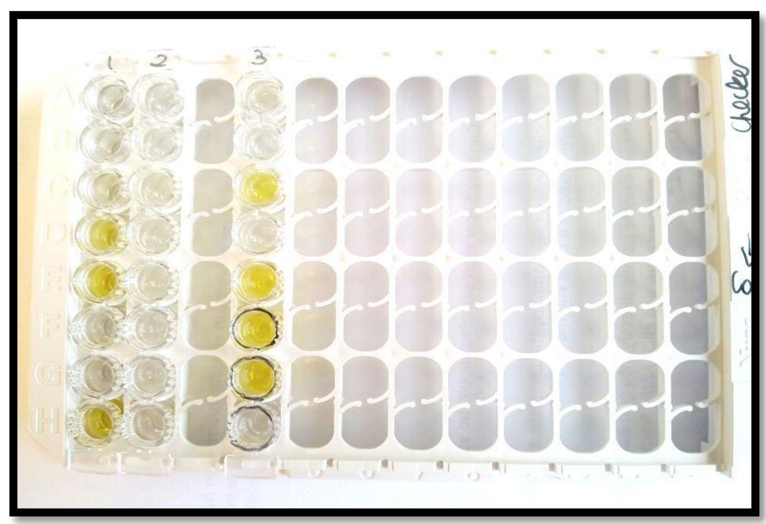

Figure 4.6: Competitive ELISA plate image of positive and negative animal sera

Table 4.4: Competitive ELISA optical densities at 450nm and the % of competition between test sera and detector sera.

Column 1 Serum Sample Loaded	OD 450nm	Colour	% Competition	Detector Antibody
Human Positive (Titre:256)	0.086	Clear	97.0	Mouse Detector (1:500)
Human Positive (Titre:512)	0.095	Clear	96.7	
Human Positive (Titre:64)	1.159	Faint Yellow	59.7	
Human Equivocal	2.614	Yellow	9.0	
Human Negative	3.875	Yellow	-34.8	
Rabbit Positive (1:10)	0.163	Clear	94.3	
Rabbit Positive (1:100)	0.101	Clear	96.5	
Rabbit Positive (1:1000)	1.737	Yellow	39.5	
Column 2 Serum Sample Loaded				
Human Positive(Titre:256)	0.072	Clear	N/A	No Detector (Diluent)
Human Positive (Titre:512)	0.072	Clear	N/A	
Human Positive (Titre:64)	0.035	Clear	N/A	
Human Equivocal	0.076	Clear	N/A	
Human Negative	0.058	Clear	N/A	
Rabbit Positive (1:10)	0.055	Clear	N/A	
Rabbit Positive (1:100)	0.054	Clear	N/A	
Rabbit Positive (1:1000)	0.054	Clear	N/A	
Column 3 Serum Sample Loaded				
Horse Positive	1.642	Faint Yellow	42.9	Mouse Detector (1:500)
Horse Positive	0.047	Clear	N/A	No Detector (Diluent)
Cat Equivocal	3.747	Yellow	-30.4	Mouse Detector (1:500)
Cat Equivocal	0.101	Clear	N/A	No Detector (Diluent)
Mouse Positive	2.870	Yellow	N/A	Mouse Detector (1:500)
Mouse Positive	2.893	Yellow	N/A	No Detector (Diluent)
Diluent	2.960	Yellow	N/A	Mouse Detector (1:500)
Diluent	0.058	Clear	N/A	No Detector (Diluent)

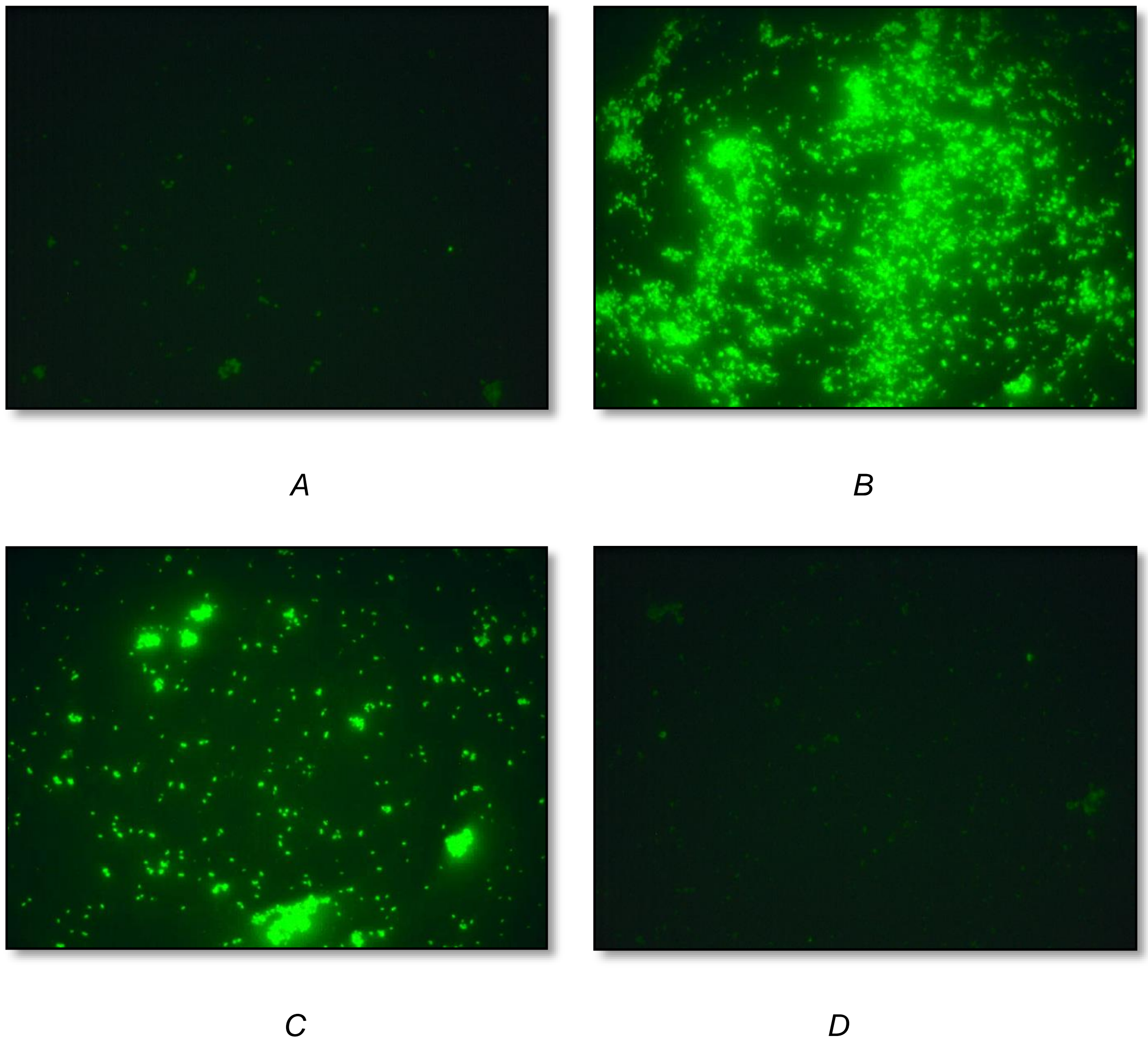

Figure 4.7: Competitive IFA Images taken under the Nikon Fluorescent Microscope

A: *Coxiella phase II antibody* ***positive*** *rabbit serum in competitive IFA with mouse detector serum*

B: *Coxiella phase II antibody* ***negative*** *rabbit serum in competitive IFA with mouse detector serum*

C: *Coxiella phase II antibody* ***negative*** *flying fox serum in competitive IFA with mouse detector serum*

D: *Coxiella phase II antibody* ***positive*** *flying fox serum in competitive IFA with mouse detector serum*

Indirect IFA and Competitive IFA: The indirect IFA used to screen animal sera was the same method as described in Chapter 2 (section 2.2.3.) with the substitution of species-specific FITC conjugates to detect antibodies. The competitive IFA was performed as described in section 4.2.2.2.5 above.

4.3.3. Data Analysis

Patient demographic data were analysed based on date of collection, date of birth/age, sex and postcode. Two distinct geographical populations were defined based on patients' postcode sorted into the 39 statistical subdivisions (SSD) within Queensland. On analysis of the research data 95% confidence intervals were calculated using Stata 10 (Stata Corp, College Station, Texas, USA).

4.4. Results

4.4.1. Seroprevalence of Q fever in Human Sera from Queensland

Serum samples (N=1988) were screened for both *Coxiella* phase I and phase II IgG antibodies using the IFA method. Of these, 103 were Q fever phase II IgG-positive, giving a seroprevalence in the total sample population of 5.2% (95% CI: 4.3%-6.2%).

Examining the seroprevalence in 1182 serum samples collected from the rural population only, the prevalence rate was 5.3% (95% CI: 4.6%-6.6%), compared to a seroprevalence of 5.0% (95% CI: 3.7%-6.7%) in 806 serum samples from an exclusively urban population.

Of the 63 seropositive males identified, 36 (57%) were from rural Queensland, and from the 40 seropositive samples from females, 27 (68%) were from rural communities (Table 4.5).

1144 serum samples were collected from adults (older than 15 years), with 92 (8.0%) of these positive for Q fever IgG. 649 of these samples were collected from a rural population and showed a seropositivity of 8.8% (57/649). Similarly, the 495 samples from the urban adult population showed a seropositivity of 7.1% (35/495). An increase in seropositivity with increasing age was noted in this sample population (Figure 4.8 and Table 4.5).

Of the 103 positive samples above, 77 (75%) also had antibodies to *Coxiella* phase I. These results were detected from all age groups with 50/77 (64.9%) positive results from males, and 27/77 (35%) from females.

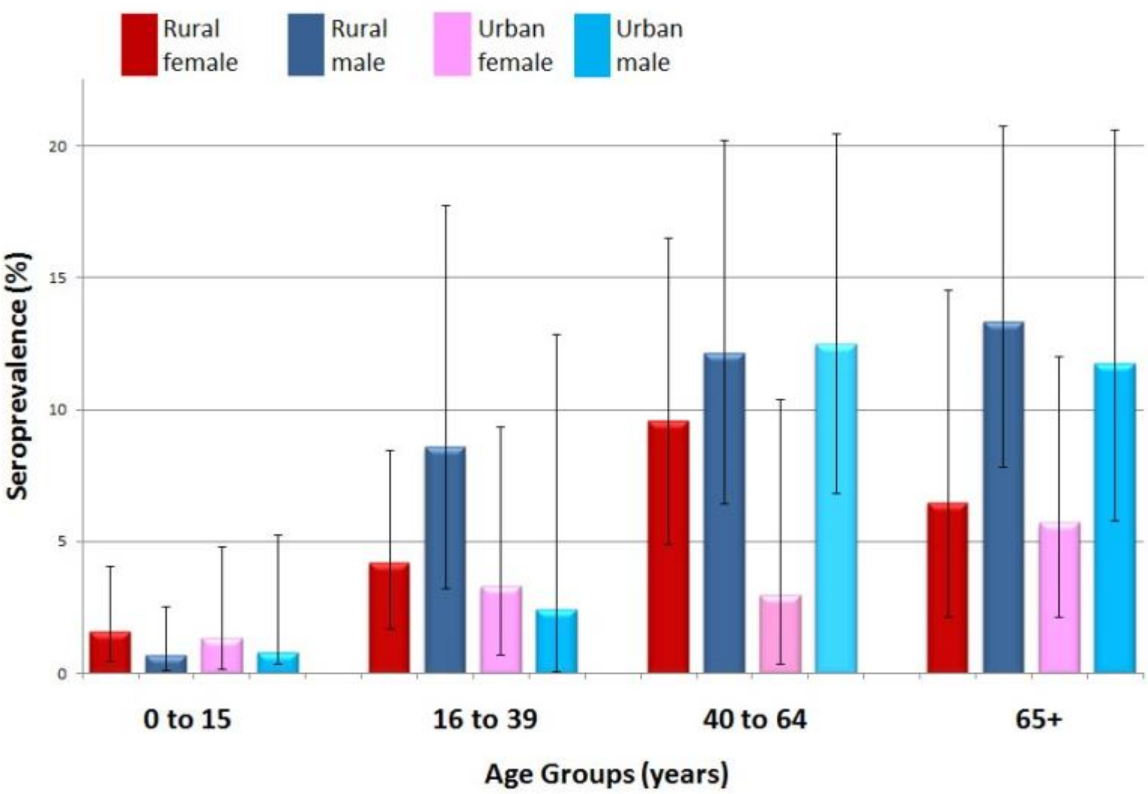

Figure 4.8: Distribution of seropositive results with age groups in rural and urban populations.

In addition to the adult samples above, 844 samples were from children under the age of 16. Of these paediatric samples, 533 (63.1%) were from children in rural communities and 311 (36.8%) from those in urban settings. Of the 844 samples tested, 11 showed previous exposure to Q fever; 5 boys and 6 girls, giving a seroprevalence in the Queensland paediatric population of 1.3% (95% CI: 0.7%-2.3%).

Six (6) seropositive children, 4 girls and 2 boys were from rural Queensland, and 5 seropositive samples were from children living in urban locations, with 2 positive samples from girls and 3 from boys.

Table 4.5: Results and percentages of the Q fever sero-positives described by gender, location, and age-group**.**

	Sample (n)	Seropositive (n, %, 95%CI)
Sex		
Female	1020	40 ***3.9%*** (95% CI 2.9-5.3)
Male	968	63 ***6.5%*** (95% CI 5.1-8.2)
Location		
Metropolitan Brisbane	806	39 ***4.8%*** (95% CI 4.0-6.0)
Rural	1182	64 ***5.4%*** (95% CI 4.4-6.4)
Age-group		
0 to 15	844	11 ***1.3%*** (95% CI 0.7- 2.3)
16 to 39	371	17 ***4.6%*** (95% CI 2.9- 7.2)
40 to 64	385	38 ***9.9%*** (95% CI 7.3- 13.3)
65+	388	37 ***9.5%*** (95% CI 7.0- 12.9)
Total Sera	**1988**	**103** ***5.2%*** **(95% CI 4.3-6.2)**

4.4.2. Seroprevalence of Q fever in Animals from Queensland

Of the 628 animal sera that were screened for *Coxiella* phase II IgG antibodies in both the competitive ELISA assay and the IFA or competitive IFA, 14 samples were identified as positive. This gave an overall seroprevalence amongst all the animals screened of 2.1% (95% CI = 1.0% - 3.1%).

There was a higher seroprevalence recorded in the cat population of 2.5%, (4/158) (95%CI = 0.8% - 6.6%). These four samples were positive in all three assays (the competitive ELISA and both the indirect ELISA and indirect IFA using species specific conjugates.

The dog sera showed and overall seroprevalence rate of 1.9 % (9/470; 95% CI = 1.0-3.7%) using a combination of assays. The IFA assay using species-specific anti-dog conjugate - FITC showed greater sensitivity and detected nine positive sera in dogs. There were 6/470 (1.3%; 95% CI = 0.5% - 2.8%) sero-positive samples detected among the dogs screened with the competitive ELISA.

Analysis of the 50 flying fox sera returned one positive result by the competitive IFA (Figure 4.6). This gave a seroprevalence among the flying foxes of 2.0% (95% CI = 0.1% - 16.7%).

A summary of seroprevalence in animal sera and the assays used is shown in Table 4.6.

Table 4.6: Seropositivity determined in animal sera from three species using Indirect IFA and competitive ELISA and IFA assays**.**

		Seropositive Samples			
Species	**Number (n)**	**Competitive ELISA (%)**	**Species Specific IFA (%)**	**Competitive IFA (%)**	**Total (%)**
Dog	470	6 (1.3)	9 (1.9)	ND	9 (1.9)
Cat	158	4 (2.3)	4 (2.3)	ND	4 (2.3)
Flying Fox	50	0	ND	1 (2.0)	1 (2.0)
Total		10 (1.5)	13 (2.1)	1	14 (2.1)

ND- Not Done

4.5. Discussion

It is known that Q fever infections in humans are acquired through the inhalation of contaminated particles released by infected animals in their products, and hence the disease is more frequently recorded in rural settings, where close contact with ruminant animals is common. In 2006, Queensland reported a notification rate of 107 Q fever infections per 100,000 people, with the majority of these in South West Queensland, a rural area with a large agricultural base[50]. This fact was supported by notification data for the Queensland population in this study, which showed that the highest numbers of confirmed Q fever cases occurred in rural communities, with a ratio of 5.5:1.0 of rural to urban notified cases.

However, this difference was not observed in the seroprevalence results for these two populations in this study. The seroprevalence of Q fever in rural samples tested was 5.3% which was very similar to the 5.0% for the urban population. These results were also at odds with other studies [6,92–96], and reflect a significant level of exposure in the urban Queensland population possibly without clinical manifestation, compared to more overt clinical disease described in the rural population. This may be the consequence of heightened awareness of disease in the rural communities, with more Q fever laboratory testing being performed, thereby resulting in a greater notification rate. However, it may also be the result of the encroachment of urban housing at the outskirts of cities, utilising land previously used for rural (farming) purposes, in particular cattle grazing and abattoir sites. An additional possibility may be that Queensland cities are intermittently enveloped by dust storms originating in rural areas, containing Q fever bacteria on dust particles which may have resulted in further undiagnosed cases in urban areas [92].

However, the current paradigm in Australia is that the vaccine is only made available to rural populations, or workers in high risk occupations as these are considered to be most at risk of exposure. The findings from this study clearly show that exposure to *Coxiella* is more widespread, and that it may be advisable to extend the vaccination program to include the population more generally. Before this may happen however, further studies must be conducted to assess the scope of risk factors for the general population.

The notification data described in Chapter 3, and the observations made by others showing that males are more often diagnosed with Q fever than females[7,97,98] was generally

supported by the results of the seroprevalence from this study. This presumably is due to the fact that occupational exposure is the primary cause of infection and occurs predominantly in the male workforce. Although the seroprevalence data supported this finding, the ratio of male to female exposure to Q fever was less pronounced. This may be explained by an increasing need and interest from rural women to be involved in animal handling occupations that may have previously been performed by men, and hence increasing their risk of disease. Alternatively, perhaps women are exposed from contaminated material at a lower dose, carried by their male partners, and go on to develop an asymptomatic infection with the subsequent development of an immune response.

The seroprevalence of 8.0% observed in the Queensland adult population was low compared to some other seroprevalence studies performed in Australia and overseas (Tables 4.1, 4.2) which showed values ranging from 3% to 66%. However, most of these other studies were performed on "at risk" populations only, such as rural workers or a general population of adult age. This study however, investigated samples taken from the general Queensland population which were not associated with outbreaks of the disease, and included subjects with a varying range of ages including children.

There have been a limited number of previous seroprevalence studies that investigated Q fever in a paediatric population. These previous studies have been limited by either small numbers of samples, and/or being drawn from a high risk population. The seroprevalence of 1.3% in the paediatric sample set tested here, reflected results reported by others in a recent study of children younger than 16 years of age from South West Queensland. These showed a seroprevalence rate of 2.5% from 237 samples examined[50]. However, these children were known to reside in a high risk area[11,50].

Overall, the seroprevalence results in this study supported the hypothesis that currently the greatest risk of Q fever infection in Queensland is for males living or working in a rural environment. Although the seroprevalence in children was significantly lower than adults, of concern was the observation that the average annual notification rate of Q fever in Queensland children has increased nearly 4-fold over the last 7 years. The reasons for this are unclear but the introduction of the NQFMP may have increased awareness of the disease and the use of diagnostic screening. This highlights the need to closely examine public health policy which may prevent or limit the acquisition of Q fever in the paediatric

population, and vaccination for children and adolescents in high-risk settings should be strongly considered.

In animals the disease process has not been as well-defined as in humans[99]. The presence of antibodies in animal sera cannot determine the infectious state of the animal, as antibody production may continue long after the animal was initially infected. Also, some animals shed the organism prior to the production and detection of antibodies, while other animals never show any seroconversion after exposure and infection[99].

The detection of antibodies in the companion animals screened in this study raised many questions regarding the levels of exposure and shedding that may be occurring from this potential source of infection. The seroprevalence among dogs was 1.9% from the 470 companion animals screened. This was within the various seroprevalence rates reported in other countries throughout the world, however, compared to data reported by Cooper et al (Townsville, Australia) in 2011[77], this was a very conservative rate. This may simply be related to the geographical location, or perhaps differences in sampling or test methods. The animals screened in the study described in this Chapter resided in South East Queensland and the animals investigated by Cooper et al[77] were from Central Northern Queensland, particularly Townsville, which is an area that reports high numbers of Q fever notifications. Differences in the seroprevalence rates reported may also be due to different strains of *Coxiella* used as substrate antigens in the assays used to screen for antibodies, resulting in differences in test results. To address this potential limitation, this study specifically used the commercially available kit, which used the Henzerling strain in the preparation of antigen coating on the ELISA plates, thus ensuring that the most consistent results were obtained.

The 2% antibody detection rate observed among cats from South East Queensland, was again within the range of seroprevalence rates reported from other countries (Table 4.3). Cats, particularly domesticated and parturient cats, are known reservoirs of infection and may be responsible for transmission of the bacteria to humans, as illustrated by outbreaks of Q fever involving many individuals, which have been directly linked to cats[71,100]. Recent studies performed in New South Wales, Australia, identified that 1% of domestic companion animals (dogs and cats) were seropositive for *Coxiella* antibodies (Table 4.3). The seroprevalence results above showed that cats carry evidence of a significant level of

exposure, and cats may be the cause of an increasing rate of spread of the disease to humans,

These seroprevalence results have illustrated that domestic cats and dogs have a substantial level of exposure to *C.burnetii* and therefore may pose a potential threat of infection to their owners and to the communities in which they reside. Also, cats may travel some distance within a 24 hour period, enhancing the potential for spread of *Coxiella* over a greater area.

Finally, the evidence showing that flying foxes have also been exposed to *Coxiella* is a significant finding. Flying foxes are very mobile, sometimes flying hundreds of kilometres per night. Therefore, if these animals are a reservoir for *Coxiella* infection, this will have a significant implication for the wider spread of Q fever disease, not only for humans, but also other animals, particularly farm animals and native fauna.

4.6. Significant Outcomes from this Chapter

The results of this study showed:

- This was the first study to show that the seroprevalence for *Coxiella* in the human urban population was comparable to the rural population of Queensland.
- Similarly, the findings showed that children residing in Queensland are an emerging group at risk of Q fever infection.
- The demonstration that flying foxes may be a potential reservoir of *Coxiella* was novel, and warrants further investigation to assess the potential for these animals to be a source of widespread infection.
- A competitive ELISA was developed to test for the presence of *Coxiella* antibodies in animals. This assay may be used in future studies to conduct a further, more comprehensive, survey of *Coxiella* seroprevalence in animals, particularly native fauna.

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Chapter 5

Potential Environmental Sources of Q fever Infection for Humans Residing in Queensland.

Articles published as a result of work done in this chapter:

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5.1 Introduction

Q fever disease is a worldwide zoonosis with the infectious agent having been detected in every country with the exception of New Zealand[1]. The distribution of *Coxiella* is widespread throughout the entire animal kingdom with detections in many species[2]. However, large domestic ruminants are the main reservoirs of infection to humans[3]. Queensland notification data shown in Chapter 3 were consistent with data reported around the world showing that Q fever notifications were recorded predominantly in rural regions and in those subjects that were exposed to large ruminants. However, the seroprevalence data generated for the Queensland population in Chapter 4 showed a different pattern of exposure to that observed in the notification data reported in Chapter 3. These data showed that Q fever exposure occurred at the same rates in rural communities as it did in urban settings, and implied that there may be other sources of *Coxiella burnetii* infection[4].

5.1.1. Animal Infections

Coxiella infects animals asymptomatically, often residing persistently within the host's macrophages. Female animals frequently have chronic infection and harbour high bacterial loads in their reproductive organs, including the mammary glands and the uterus. There have been reports of abortions and still births associated with *Coxiella* infection, as well as animals born with low birth weight and failure to thrive. Animals with high loads of *Coxiella* in birth products and the products of conception expel the bacteria along with the offspring and birthing products directly into the environment. Concentrations of the organisms expelled have been reported as high as 10^9 *Coxiella* organisms per gram of placenta [2]. Therefore the risk of infection to humans is especially high in large facilities housing ruminants, such as barns, dairies, abattoirs and their surrounding areas.

Furthermore, *Coxiella* organisms are extremely hardy and able to withstand harsh conditions for long periods of time. The result is that they can survive for several months in areas which have had animals residing [5].

5.1.2. Transmission to Humans

5.1.2.1. Ruminants as a Source of Infection

The common route of transmission associated with infections in animal handlers or subjects with direct animal contact is through the inhalation of contaminated aerosols or direct contact with contaminated material. However, there are many infections that occur in humans residing in low risk communities such as in urban settings or where no animal contact has been reported. The primary source for the dispersal of *Coxiella* bacteria to these areas of low risk is thought to be wind, and the transportation of infected animals through these areas. Both of these modes of Q fever transmission have been implicated in previous outbreaks of disease[6–9].

5.1.2.2. Ticks as a Source of Infection

Part of the natural route of transmission for *Coxiella* to animals includes the transmission via ticks, which may act as a reservoir for the bacteria[2]. Ticks harbour *Coxiella* in their digestive tract and expel the organism via faecal excrement. Tick faeces contains high loads of the bacteria, which is responsible for contaminating the skin and wool of the host on which ticks have been feeding[10]. In addition, ticks may be the source of infection for native and domestic animals. *Coxiella* has been isolated from many tick species around the world. In Australia *Coxiella* has been detected in ticks removed from a variety of native animals which were widely distributed geographically[11].

5.1.3. Human Infections

Q fever infections in humans may present as a variety of clinical syndromes and are classified as either acute or chronic. Acute Q fever infections may be asymptomatic or present as a self-limiting febrile illness such as pneumonia or hepatitis[12–14]. Chronic Q fever disease follows an acute episode resulting most commonly in endocarditis or may manifest as a chronic hepatitis or osteomyelitis; with the latter commonly present in paediatric infections[3,15,16]. Initially infection may be subclinical or asymptomatic in patients where there is no direct contact with large ruminants, and often in these cases the patient can go undiagnosed and untreated until a time when a diagnosis of chronic Q fever is made[17,18]. This may be one of the explanations why the disease is considered to be under-diagnosed in areas considered to be of low risk. Yet recent seroprevalence data

show a significant level of exposure in these low risk communities, which warrants further investigation.

The risk of contracting Q fever in a rural setting has been extensively reported worldwide and has been recently investigated in Australia. Direct correlations between animal handlers contracting Q fever from large ruminants has been identified and well documented[10,19–22]. Publications have identified many species of animals from around the world as being hosts for *Coxiella*[2,10]. There have been few prevalence studies from Australian remote and urban areas that have shown that a number of animal hosts, both domestic and native; have been exposed to *Coxiella*, with these animals producing detectable antibodies.[21,23–26].

5.1.4. Presence of Coxiella in the Environment

Coxiella is shed directly into the environment from infected animals and is highly stable for many months and even years[27]. The Netherland has recently endured an exceptionally large epidemic with over 4,000 cases of Q fever reported from 2007 to 2010. This outbreak was the result of infected pregnant goats in local surrounding goat farms shedding billions of organisms into the environment. With less than 10 organisms required to seed infection in humans, the dispersal of *Coxiella* via various transmission routes resulted in many people being exposed and contracting Q fever [28–30]. It was established that those residing within a 5 kilometre radius were at greatest risk of contracting Q fever from the infected goat farms, and was directly related to the dust generated from these farms[31]. It also highlighted that Q fever infection was due to the dispersion via wind and the movement of contaminated bedding of animals shedding *Coxiella.* In Australia, the majority of the country is arid and often windy which is perfect for the formation of dust. Australia has been recognized and often referred to as the Southern Hemisphere's largest source of dust, with the central Australian basin, which expands into the Northern Territory and South Australia, spanning much of inland Queensland and New South Wales. Australia is the globe's eighth largest source of atmospheric dust[32,33].

5.2. Specific Aims Addressed in this Chapter

The results in chapters 3 and 4 indicated a discrepancy between notification rates and evidence of previous exposure to Q fever in the Queensland urban population. Chapter 4 showed evidence of *Coxiella* exposure in animals including domestic pets. This evidence points to a more widespread source of *Coxiella* infection for humans, which is mostly likely associated with environmental exposure.

Specifically this addressed the following:

- An examination by PCR of a diverse range of samples from a large number of animal species to determine the presence of *C.burnetii* DNA.
- To examine soil and dust samples collected from various geographic locations in Queensland for the presence of *C.burnetii* DNA, and identify these as possible sources of infection.

5.3. Materials and Methods:

5.3.1. Samples

It is hypothesised that environmental exposure plays a large role in the dissemination of *Coxiella burnetii* in the community. To determine the potential source of exposure for humans in Queensland, different sample banks of environmental samples were examined. The species sample types tested in this study are shown in Table 5.1.

5.3.1.1. Blood, Urine and Milk Samples

In total, 515 blood samples were collected from 20 different animal species residing in 91 different geographical locations. 704 urine samples from 10 animal species in 90 locations, submitted for investigations not including Q fever, were conveniently obtained from private animal pathology providers. There were 7 milk samples collected from bovines kept on a farm property at Gatton, Queensland.

In addition, 90 DNA extracts from flying fox urine were obtained from the Queensland Centre for Emerging Infectious Diseases, Biosecurity Queensland. These samples were collected via large tarpaulin urine collection pools set up directly under flying fox colonies. The colonies were from Cedar Grove, Slacks Creek and the Gold Coast, in South East Queensland. These samples were specifically collected for the screening of Hendra virus and were negative for this virus by molecular testing.

Table 5.1: Species sample types collected for *C.burnetii* investigation. * Ticks were removed from 2 species. ǂ Urine was obtained as DNA extracts from pooled collections.

Species	Specimen Collected					
	Total Samples	Blood	Faeces	Milk	Ticks*	Urine
Avian	1	1	-	-	-	-
Bilby	1	1	-	-	-	-
Bovine	12	4	-	7	-	1
Canine	614	201	-	-	40	373
Caprine	1	1	-	-	-	-
Dolphin	2	2	-	-	-	-
Donkey	1	1	-	-	-	-
Elephant	2	2	-	-	-	-
Equine	126	112	-	-	-	14
Feline	319	132	-	-	-	187
Ferret	1	1	-	-	-	-
Flying-Foxes	90ǂ	-	-	-	-	90ǂ
Kangaroo	3	3	-	-	-	-
Koala	99	26	43	-	-	30
Lemur	2	2	-	-	-	-
Porcine	7	7	-	-	-	-
Possum	9	1	-	-	5	3
Quoll	1	1	-	-	-	-
Reptile	3	3	-	-	-	-
Tiger	9	7	-	-	-	2
Wallaby	5	-	4	-	-	1
Wombat	10	7	-	-	-	3
Total	1318	515	47	7	45	704

5.3.1.2. Faecal Samples

Faecal samples (N=47) were collected from 43 koalas and 4 wallabies, residing in South East Queensland. These samples were obtained by Australia Zoo between October 2010 and December 2011 from animals brought to the clinic for trauma or treatment of injury.

5.3.1.3. Animal Ticks

This sample pool included ticks removed from domestic and native animals presented to a local Brisbane veterinary surgery in December 2011. There were 45 ticks from 5 possums

and 40 canines. All ticks were classified as *Ixodeos holocyclus*, commonly known as "paralysis ticks".

5.3.1.4. Soil and Dust Samples

Approximately 50 grams of soil was collected from 151 locations throughout South East Queensland. These included a random collection from local Brisbane residences, and direct sampling from highway surrounds taken by cattle trucks in the Darling Downs, South West Queensland, which is considered to be the Q fever "hot spot" in Australia. These samples were collected from January 2011 through to October 2011 and were from 78 geographical locations.

Dust samples were provided by DustWatch Northern Atmospheric Environment Research Centre, Griffith University, Nathan, Queensland. Atmospheric dust collection involved the use of high volume air samplers (HVS) and monitors that were maintained and operated by selected Dust Watchers throughout Queensland. The HVS instrument is able to trap the total suspended sediment onto a glass fibre filter paper (Whatman GF/A) with a nominal pore size of 1.6 µm. A total of 72 dust samples collected at 2 geographical locations between 1988 and 2009 were tested for *Coxiella*. These collection points were (1) South East Queensland; 35 samples from 20 dust events, and (2) Charleville with 37 samples from 2 dust events.

In addition, swab samples of dust collected from 50 domestic vacuum cleaners in 32 geographical locations of Queensland were also tested. These samples were collected by local residents between January 2011 and October 2011.

5.3.2. Laboratory Testing

5.3.2.1. DNA extraction:

Nucleic acids were extracted from the various samples using commercially available kits. Before DNA extraction, equine herpes virus (EHV) was added to each of the different sample types to examine the efficiency and reproducibility of the various extraction processes by monitoring real-time PCR Cycle Threshold (CT) values. Nucleic acid was then extracted using the methods below for each sample type[34,35].

Animal blood, milk and urine samples were extracted using the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia) as per the manufacturer's instructions. Briefly, 200µL of

sample was spiked with EHV before processing with a final dilution of DNA in 100uL of water.

5.3.2.1.1. Flying Fox Urine

Pooled flying fox urine was extracted using the automated KingFisher Duo Prime Purification System by ThermoScientific (Thermo Fisher Scientific, USA), in conjunction with the KingFisher Cell and Tissue DNA extraction kits (Thermo Fisher Scientific, USA) as per the manufacturer's instructions.

5.3.2.1.2. Faecal Samples

Faecal samples were extracted using the Mini stool kit (Qiagen, Brisbane, Australia) following the manufacturer's recommendations. Briefly, one pellet of faecal sample (approximately 1.5-2.0 g) was added to ASL buffer for cell lysis at 95°C. Inhibitors were removed by absorption using the kit InhibitEX tablets followed by centrifugation. The DNA in the supernatant was then extracted using the QIAmp spin columns as described in the kit insert and DNA was eluted in a 100uL volume of water.

5.3.2.1.3. Tick Samples

Tick samples were extracted using the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia). Ticks were placed on glass slides and engorged blood and tissue was released with a scalpel blade. These were added to 200µL of tissue lysis buffer containing 20µL of proteinase K and incubated overnight at 56°C on a mechanical rocker at low speed. The samples were then treated with the same extraction kit and method as the blood, milk and urine samples above.

5.3.2.1.4. Soil Samples

Nucleic acids in soil samples were extracted as previously described [36,37]. Soil samples were pre-treated prior to DNA extraction. Briefly, 5g of soil was mixed with phosphate buffered saline (PBS) and put on a mechanical rocker at room temperature for one hour. Samples were centrifuged for 5 minutes at 123g and the supernatant was retained and further centrifuged at 20,000g to pellet cellular material. The pellet was resuspended in 1ml of PBS and used in the DNA extraction protocol previously described with the QIAamp DNA Stool kit (Qiagen, Brisbane, Australia).

5.3.2.1.5. Dust Samples

Dust collected on Whatman glass fibre filter paper was placed in sterile tubes containing 5ml of sterile water. These were incubated at room temperature on a mechanical rocker overnight to release bacterial particles. Briefly, 200µL of the dust suspension was processed with the DNeasy Blood and Tissue kits (Qiagen, Brisbane, Australia) using the manufacturer's method for blood extraction. The DNA extract was collected in a final volume of 100 µL of water for PCR.

Dust swabs were processed as previously described by Kersh et el [37]. Swabs were placed in 1.0ml of sterile PBS and vortexed. A volume of 200µL was processed using the QIAamp DNA Stool kit (QIAGEN Australia) as per the previously published protocol. DNA was eluted in 100µL of water for PCR.

5.3.2.2. Real Time PCR Method

Real-time PCR for *C.burnetii* was performed targeting the repetitive *IS1111* gene and the outer membrane protein *com1* gene as previously described in Chapter 2 (section 2.2.2.1).

EHV real-time PCR was described in Chapter 2 (section 2.2.2.2) of this thesis and was performed on all extracted samples.

5.4. Results

5.4.1. Animal Samples

Screening by real-time PCR showed a total of 85 of 1318 animal samples were positive by either gene target, giving an overall incidence of 6.2% (CI 5.0%-7.5%). Positive results were confined to 6 of 22 different animal species, from 28 different geographical locations throughout Queensland. Table 5.2 shows *C. burnetii* PCR results for the 6 different species with positive results and the various samples types screened.

Of the 515 whole blood samples from 20 different species, 34 (6.6%; CI 4.8%-9.1%) samples had a positive PCR result in either gene target tested. Of these 14 (2.7%; CI 1.6%-4.5%) were positive for both targets leaving 20, (3.9%; CI 2.5%-5.9%) that were positive in a single *Coxiella* gene target. These were 18 extra *IS1111* detection and 2 extra detections by the *com1* target. These detections comprised 5 different species residing in 16 different locations.

Table 5.2: Positive PCR detections within a variety of sample types from individual animal species. Only those species recording a positive result are shown.

Species	Total Samples Screened	Species Detection Rate	Specimen types			
			Faecal	Urine	Blood	Ticks
Avian	1	100%	0	0	1	0
Flying Fox	90	7.8%	0	7	0	0
Canine	574	5.6%	0	26	3	3
Equine	127	11.8%	0	1	14	0
Feline	319	7.8%	0	12	13	0
Koala	99	5.1%	1	1	3	0
TOTAL	1210		1	47	34	3

In urine samples, 40 of 614 (6.5%; CI 4.8%-8.8%) were PCR positive for the *IS1111* gene target. In addition, 28 (4.6%; CI 3.2%-6.5%) of these were positive with the second *com1* target. These were collected from 4 species in 12 various Queensland locations. There was additional testing on DNA samples from 90 pooled flying fox's urine which showed 7 (7.8%; CI= 3.8%-15.2%) were positive. Detections occurred as single target detections, with the *IS1111* gene target giving one positive result and the *com1* target identifying 6 positives. There were no detections among the 7 milk samples screened.

Only one koala faecal sample of the 43 koala faecal samples tested was positive for *Coxiella* DNA (2.1%; CI 0.5%-10.9%) in the *IS1111* gene target only. None of the 4 wallaby samples had detectable *Coxiella* DNA. Of the 45 tick samples tested, 3 had detectable *Coxiella* DNA. This was distributed with 2 ticks having detections in both *IS1111* and *com1* genes and an additional detection in the *com1* gene. This was a 6.7% (CI 2.4%-18.0%) detection rate among the ticks removed from metropolitan/urban animals. (Table 5.3).

Table 5.3: Table of different sample types collected from various animals and the numbers tested. Dual targets *IS1111* and *com1* were used and the numbers expressed as percentages.

Sample	Total Number	Locations Postcodes	PCR Detections		
			Either Target	*IS1111*	*com1*
Blood	515	91	34 (6.6%)	32	16
Urine	704	90	47 (6.8%)	41	34
Faeces	47	2	1 (2.1%)	1	0
Ticks	45	1	3 (6.7%)	2	3
Milk	7	1	0	-	-
Soil	151	78	3 (2.0%)	3	2
Dust (filters)	72	2	5 (6.9%)	5	1
Dust (swabs)	50	32	1 (2%)	1	1

Detection of *C.burnetii* occurred in animals sampled from 28 different locations within South East Queensland. The highest incidence, 18.5% (CI of 10.4%-30.9%), was in a semi-rural location in South East Queensland (postcode 4285; south west of Brisbane) followed by 8.8% (CI of 4.9%-15.4%) in the Gold Coast hinterland (postcode 4211). Samples from a nearby urban location (postcode 4127; Springwood, south of Brisbane) showed an incidence of 8.3% (CI of 4.2% - 14.1%).

5.4.2. Soil and Dust Samples

Of the 151 soil samples collected from South East Queensland, 3 (2.0%; CI 0.7% - 5.7%) contained detectable levels of *Coxiella* DNA. Two of these were positive by both PCR targets, with an additional detection in the *IS1111* gene target. The positive samples were collected from urban areas where there were no large ruminant animals residing.

The 72 dust samples collected by the HVS showed that 5, (6.9%; CI 3.1%- 15.2%) of the samples had detectable *Coxiella* DNA present. These detections occurred in the *IS1111* gene with one sample having a dual detection with the *com1* gene. There were 2 positive samples collected from Brisbane and 3 from Charleville. These positive dust samples were collected between 2001 and 2005. There was a single detection (2.0%; CI 0.5%-10.4%), of *Coxiella* DNA from the household vacuum cleaner swabs. This sample had

detectable DNA in both the *IS1111* and *com1* gene targets. This positive swab sample was obtained from Taigum (postcode 4018), close to the Brisbane CBD which is a semi-rural area that now has high density housing on its boundaries.

Table 5.4: Summary table of results for PCR testing of *C.burnetii* in soil and dust samples collected in Queensland from specific postcodes.

Sample	Total Number	Different Locations (Postcodes)	Detections		
			Either Target	***IS1111***	***com1***
Soil	151	78	3 (2.0%)	3	2
Dust (HSV filters)	72	2	5 (6.9%)	5	1
Dust (swabs)	50	32	1 (2%)	1	1

5.5. Discussion

Traditionally, the acquisition of Q fever has been linked to close contact with ruminants. However, recent reports from Australia show that the number of cases of Q fever lacking any known direct contact with any animal is increasing [17,18,38]. Furthermore, seroprevalence studies in Australia have highlighted the significant increase of exposure to *Coxiella* in supposably “low risk” communities and geographical locations [4,26,39].

This study sought to identify potential sources for *Coxiella* exposure to humans in Queensland, and found an overall incidence of 6.2% (CI 5.0%-7.5%) amongst the 1318 animal samples screened. Also, detections in environmental samples ranged from 2 to 6.9% (CI =0.5%-15.2). Overall *C.burnetii* DNA was identified in all sample types tested from a variety of species residing in different geographical location throughout Queensland.

The high prevalence (6.7%) of *Coxiella* found in the urine of domestic animals (cats 6.4% and dogs 6.9%) suggested that pets may play a significant role in the transmission of *Coxiella* in the domestic non-rural setting. Perhaps a likely pathway may be the spread of *Coxiella*-contaminated urine into the environment by these animals, followed by transfer to

the susceptible host through direct contact or aerosolation of contaminated soil or dust through gardening or mowing. The high rate of carriage in domestic dogs has been confirmed by seroprevalence studies showing that 22% of dogs were previously exposed to *Coxiella*, again highlighting the potential risk to animal owners and those who have contact with the animals [25].

Parturient cats have been identified as the primary source of isolated outbreaks of Q fever on a number of occasions [40,41]. However, the results of this study highlighted the potential for humans to be exposed to *C.burnetii* not only via parturient animals and their birth products, but also to healthy animals performing normal activities.

Screening of 122 native animals showed that 4.1% (CI 1.8%-9.2%) had detectable *Coxiella* DNA, in blood, urine or faeces, suggesting these animals as a potential source of infection. However, the predominant species with detectable organisms were koalas (5.1%). These were the only species tested in significant numbers, and the prevalence in other native animals is likely to increase with a larger sample population. This is supported by the findings in pooled flying fox urine samples (N=90) which showed that 7.8% were positive for *Coxiella* DNA, suggesting that flying fox urine may be another source of transmission to either native or domestic animals and humans. Analogous to the current theory of Hendra virus transmission from flying foxes to horses, in this study 11.8% of 126 horses tested were positive for *Coxiella* DNA. These animals resided in close proximity to established flying fox roosts.

It is postulated that ticks may be another source of bacterial transmission particularly amongst the native animal population. However, there is still considerable uncertainty if ticks themselves are infected or merely reflect the positive status of animals from which they derived their blood meal. In this study 45 ticks removed from both domestic and native animals were screened for *Coxiella* by PCR, showing a detection rate of 6.7%, with those removed from dogs having the greater detection rate (7.5%). Ticks shed high loads of bacteria in their faeces and saliva and may be another potential source for direct human exposure to *Coxiella* [42].

Although Q fever disease in Queensland is frequently reported in rural communities, there has been a significant increase in detection of the disease in urban populations where no direct animal to patient contact can be identified [4,17]. This is supported by the similarity in seroprevalence status between rural and urban populations reported in Chapter 4 of this

thesis. These observations cannot be attributed to animal spread alone, and other environmental factors clearly need to be considered.

The inhalation of contaminated aerosols and dust particles as the source of human infection has also previously been reported [6,20,43–45], and a recent study in the USA described 24% of soil samples contained detectable *Coxiella* DNA [37]. This contrasted with the results of this study in which only 2% of soil samples had a detectable level of the Q fever organism. These differences may just reflect sampling difference related to different geographic locations, or perhaps the differences in stock density and population density and distribution in the USA compared to a much sparser Australia. The samples in this study that did have detectable levels of *Coxiella* DNA were from "low risk areas" and coincided with positive samples collected from animals in the same region.

During dry weather cycles, soil is frequently dispersed as dust, and hence dust may be an important mechanism for the dispersal of *Coxiella* organisms to both rural and urban regions as previously suggested by Kersh et el[37]. Examination of dust samples using HVS and domestic vacuum cleaners showed the presence of *Coxiella* DNA with an overall detection rate of 4.9%. The direct sampling of dust via HSV gave a 6.9% detection rate, representing positive results from both "high and low" risk areas. This confirms that contaminated dust is a likely source of exposure to *Coxiella* in "low risk areas" and is perhaps responsible for asymptomatic disease and the development of high seroprevalence among low risk communities.

This study specifically targeted samples from Queensland because of the high seroprevalence described previously in areas that were considered low risk [4]. There were many publications, using serological methods, that have reported that both domestic and native animals have significant exposure rates to *Coxiella*, yet the presence of the bacteria in these animals has not been widely studied. This study was the first to investigate a diverse range of sample types collected from a variety of animal species identifying those as potential vectors in the transmission of Q fever to humans. In addition, data for soil and dust highlighted the important role these may play in the dispersal of *Coxiella*, not only as a source of infection for humans, but also as part of the transmission cycle in animals. This may help to explain the significantly high exposure rate in low risk populations which do not have direct animal contact.

5.6. Significant Outcomes from this Chapter

- *Coxiella burnetii* was detected in a range of animals and ticks in South East Queensland.
- Importantly, bacterial DNA was present in domestic dogs and cats, identifying these animals as a potential source of infection.
- This is the first study which has identified flying foxes as harbourers of *Coxiella*, which has significant implications for the wider spread of the bacteria in the community.
- Another unique finding by this study was the presence of *Coxiella* in environmental dust and soil samples collected in Australia.

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Chapter 6

Factors Contributing to the Potential Under-Diagnosis of Q fever in Queensland

6.1. Introduction

Q fever was first discovered at the Royal Brisbane and Women's hospital at Herston, Queensland in 1936 by Edward Derrick. He investigated a febrile illness in nine abattoir workers and was successfully able to transfer the infectious agent (*Coxiella burnetii*), via blood samples from the infected workers to guinea pigs and replicate the disease in the animal model[1]. Further investigation by Frank Macfarlane-Burnet found that blood from the infected tissue of the inoculated guinea pigs was able to agglutinate when mixed with blood from convalescing patients, and so the beginning of a laboratory diagnostic system in which to identify and confirm the disease Q fever, began[2].

6.1.1. Bacteria

C.burnetii is an intracellular, pathogenic, gram negative coccobacilli, which was renamed from the order of *Ricketsiales* to the more genetically related order of *Legionallales* in recent years. The intracellular nature of the organism decreases the utility of traditional microbiological methods, such as isolating the bacteria by culture, which can be used to identify an acute infection. The extreme infective nature of *C.burnetii* also poses an occupational hazard to diagnostic laboratory staff and hence traditional microbiology methods are not readily employed to determine infection in humans. *C.burnetii* is the only intracellular, pathogenic bacteria able to reside and replicate inside a phagosome, while evading the innate and adaptive immune response of its host. This in turn decreases the effectiveness of diagnostic tools, such as serology, for laboratory confirmation of the presence of the organism[3].

6.1.2. Variants

This organism has a complex life cycle and is able to exist in two distinct forms, a small cell variant (SCV) which is the extracellular, survival form that is able to withstand harsh environmental conditions such as heat, desiccation and UV radiation[4]. The SCV invades a host generally by the inhalation of the organism from the environment. The host macrophages are able to engulf the bacteria, which lead to transformation of the SCV to the large cell variant (LCV). The LCV is metabolically different and has different surface proteins to the SCV. Also *Coxiella* is biphasic, meaning that it has the ability to alter its

antigenic properties and structural lipopolysaccharide molecules resulting in two distinct serological variants, phase I and phase II[5,6]. It is these distinct phases that elicit a different antibody response in the infected host, which forms the basis of immunological testing and laboratory confirmation of infection[1,2].

6.1.3. Diagnostic Methods

Usually, the microbiological diagnosis of Q fever relies upon serology, and the most commonly used serological techniques to determine disease status in patients with acute Q fever include the complement fixation testing (CFT), immunofluorescence assays (IFA) and enzyme linked immunosorbant assays (ELISA)[7]. However, although highly reliable, serology provides only indirect evidence of infection, and antibodies are absent in the early phases of the disease. Recent advances in technology have allowed for the use of specific molecular techniques such as PCR to play a role in the diagnosis of Q fever, particularly in early acute infection[8]. However, the utility of molecular detection methods in the diagnosis of acute Q fever has not been widely tested.

Q fever disease can be either acute or chronic and may be asymptomatic or present with a variety of clinical manifestations including, fevers, chills, severe headaches, pneumonia, hepatitis, osteomyelitis and fatigue syndromes[9]. There is a strong belief that the true numbers of Q fever cases are under-diagnosed and hence under-reported in many countries[10]. This is supported by the findings in previous chapters of this thesis which highlight a difference between Q fever case notifications (Chapter 3) and the exposure rates of *Coxiella* in the Queensland population as determined in the seroprevalence study shown in Chapter 4. Many countries, including Australia, only report clinical cases of Q fever disease after a laboratory confirmation has been obtained. However, accurately identifying cases of Q fever clinically presents a diagnostic dilemma due to the widely varying clinical presentations of the disease[10,11]. The initial infection with *C.burnetii* will only produce symptoms in approximately half of the patients infected, with about 2-5 % of these patients going on to develop chronic Q fever[12,13]. Regardless of the clinical manifestation of a *C.burnetii* infection and disease severity, seroconversion will occur in most cases. It is this immune response, produced by the different immunoglobulin subsets as a direct result of exposure to the two distinct phases of *C.burnetii,* that has allowed for differentiation and clinical diagnoses of both acute and chronic Q fever disease and hence lead to appropriate treatment and management of the distinctly different disease states[14].

Acute Q fever in humans occurs approximately 14-21 days after initial inhalation of organisms, resulting in the presentation of immediate clinical signs of infection. These may include severe fevers reaching peaks as high as 40 °C and lasting for extended periods if untreated. Fatigue, myalgia and headaches are also commonly reported from patients[15] (see Appendix 6.1 at the end of this Chapter). It is any, or combinations of these symptoms that complicate clinical diagnosis, as many of these manifestations occur in a variety of other respiratory illnesses, such as influenza. This contributes to the misdiagnosis and under- reporting of Q fever infections[16].

After initial exposure to the virulent phase I of the organism in the host, the bacteria in this phase continue to metabolise and multiply within the macrophage until modifications occur to the structural lipopolysaccharide (LPS). This results in an antigenic shift to form phase II, which is an avirulent form of the bacteria. This phase II is highly antigenic and one to which the host cells mount a specific immune response [17]. It is by the indirect measurement of the immune response and the production of phase-specific antibodies that Q fever disease diagnosis may occur.

First infection with *C.burnetii* results in bacteraemia during which the bacteria may be isolated from the blood, followed by the induction of a specific immunoglobulin M (IgM) response against phase II antigen epitopes expressed on the cell surface. IgM antibodies may be detected as soon as seven days after onset of symptoms. The specific immunoglobulin G (IgG) response, directed against phase II of the organism, occurs approximately seven days post exposure, and reaches a peak level at 3 – 4 weeks post infection. The development of antibodies directed against phase I organisms may also be detected during the acute phase, but these are at notably lower titres (Figure 6.1).

Chronic Q fever is a secondary disease that occurs in 2-5 % of patients who have suffered acute infections. This form of the disease transpires months or even years after initial infection, and may persist for years. It may result in death of the patient, depending on the manifestation of the disease, before a definitive diagnosis can be made. Serological methods have been employed over time to identify cases of chronic Q fever, which were characterised by an increase in phase I antibodies. Typically, the heart is the organ most commonly affected during chronic Q fever resulting in endocarditis. This is generally associated with patients who have an underlying heart valve defect, or in patients who are immunocompromised. During chronic Q fever infection, bacteria multiplying within the host

macrophages produce a permanent state of rickettsemia and induce production of elevated levels of antibodies to phase I in the host. Clinically this low-level, persistent infection with *C.burnetii* produces an array of nonspecific symptoms, which in turn may delay a definitive diagnosis and appropriate treatment, resulting in increased morbidity and mortality[8]. With the introduction of PCR, chronic Q fever diagnosis has become more sensitive and specific. However, the sample of choice is heart valve tissue which requires invasive cardio surgery for the patient. The initiation of such a procedure depends on clinical awareness by medical experts that Q fever may be involved; otherwise laboratory investigation still remains problematic

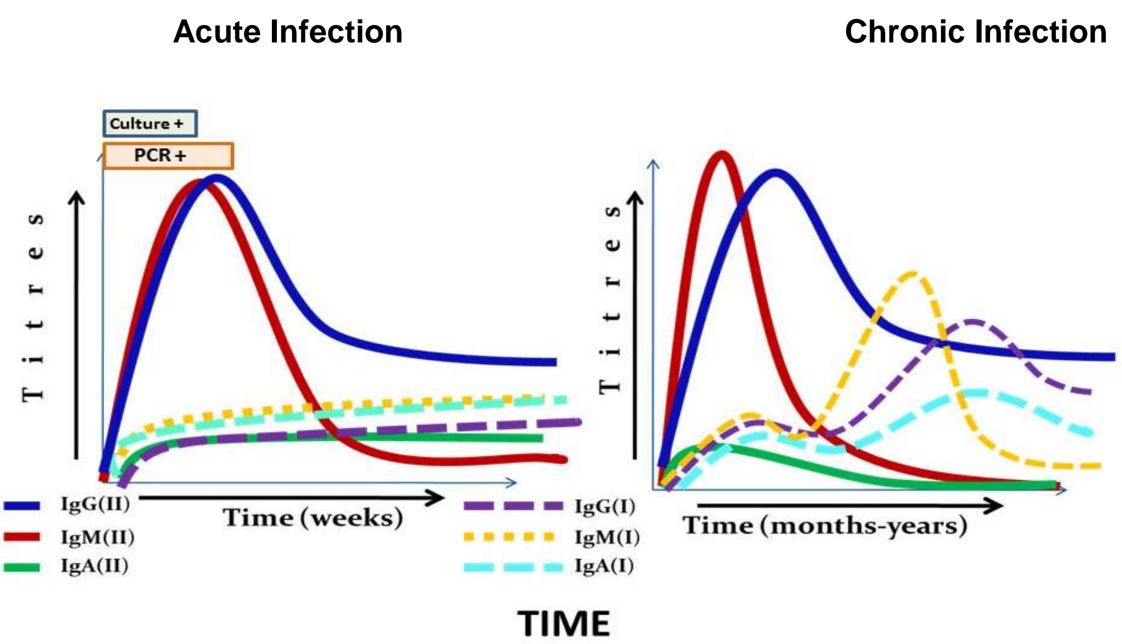

Figure 6.1: Temporal serological profiles for immunoglobulins A, G and M in acute and chronic Q fever in humans. The interval for PCR and culture positivity is shown early in acute infection.

In Queensland and the rest of Australia, strict guidelines have been established to accurately confirm cases of Q fever. The Queensland Health Communicable Diseases branch, together with the Public Health Laboratory Network, has specific clinical case definitions by which practitioners are able to notify the NOCS departments of Q fever cases. Clinical evidence of the disease is required for reporting cases but in the absence of laboratory confirmation, this alone is not sufficient for notification of the disease. It is a requirement by the Communicable Disease Control Units of Queensland Health

(Queensland Government) that all Q fever cases be confirmed by definitive laboratory evidence[18].

Despite the many advances in serological test methods over the past decade, the accurate diagnosis of Q fever by serology is often still problematical. The reference ("gold standard") method for acute Q fever diagnosis remains the IFA despite the number of different "in house" and commercial ELISA assays and the advances in molecular methods including strain typing and nucleic acid detection[19]. However, the knowledge of *C.burnetii* biology and its pathogenesis remains limited, and particularly the reasons that the disease is under-diagnosed need to be explored.

6.2. Specific Aims Addressed in this Chapter

The overall aim of this study was to determine if the current diagnostic algorithm employing serological tests only for the laboratory confirmation of acute Q fever in Queensland was adequate, or perhaps failed to diagnose early infections, and therefore contributed to the perceived under-reporting of disease as may be inferred from previous data.

Also, the diagnosis of Q fever may not be considered in some patients with general non-specific symptoms, or may be clinically diagnosed as a different disease. This study will also examine samples from patients with laboratory investigations not including Q fever, yet presenting with similar clinical symptoms. This will provide a measure of the potential rate of under-reporting that may result in cases where a definitive laboratory diagnosis was not made.

Specifically the aims for this study were:

- To compare the clinical utility of serological methods (IFA IgM and ELISA IgM) and the molecular method of PCR as tools to diagnose acute Q fever infections, and determine the optimal diagnostic algorithm for their use.
- Use PCR to examine the presence of *C.burnetii* in various sample types collected from patients with similar clinical presentations as Q fever, such as respiratory illness, flulike illnesses and hepatitis.

- Investigate serum samples submitted for the investigation of pyrexia of unknown origin (PUO), atypical pneumonia, and respiratory infections in order to establish undiagnosed Q fever disease status in these patient populations

6.3. Methods

6.3.1. Diagnostic Assays

To determine if assay sensitivity might be a causative factor for the under-reporting of acute Q fever in Queensland, three assays were applied to investigate potential assay sensitivity issues. These were:

1. IgM phase II Immunofluorescence assay (IFA): this assay is considered the "gold standard" for the diagnosis of acute Q fever in Australia.
2. Commercially available Q Fever phase II IgM ELISA Test: Panbio EQFB01M; Panbio ELISA Assays (Alere, Australia). This assay is widely used by many laboratories in Australia.
3. PCR for the detection of *C.burnetii* DNA using dual gene targets for the *IS1111* gene and the outer membrane protein *com1* gene[20]. PCR is not routinely used in Queensland to diagnose Q fever infections.

The principles of these three methods have previously been described in detail in Chapter 2 (General Materials and Methods).

The widely considered "gold standard" laboratory method for diagnosing acute Q fever is serology, with the immunofluorescence assay (IFA) for the detection of IgM antibodies currently regarded as the reference method for acute Q fever screening [21]. The set of samples described in section 6.3.2. below were screened for IgM antibodies using the recommended IgM phase II IFA method (Chapter 2; section 2.2.3.6.) and the commercially available IgM ELISA method above (Chapter 2; section 2.2.4.3.2.), and compared with PCR for their ability to diagnose acute Q fever disease.

6.3.2. Samples Screened

6.3.2.1. Serum Samples Submitted for the Diagnosis of Acute Q Fever Infection

This study used a sample set of sera that was obtained from Queensland Health over a three-year period. This included samples submitted from years 2008, 2009 and 2011. These samples were submitted to Queensland Health for the specific investigation of acute Q fever disease.

There were 275 serum samples that were collected and stored; from this a 200uL aliquot of serum was extracted for nucleic acid testing. These samples were selected randomly from a larger serum pool available at the Pathology Queensland laboratory, and had been submitted for the investigation of Q fever. Demographic data were available for all of these samples.

The samples were collected from 100 females and 175 males, and comprised an age range of 0.4 - 87.3 years, with a mean age of 47.0 years and a median age of 47.5 years. The females were 12.1 - 87.3 years of age with an average age of 46.9 years and the median age 46.5 years. The male population selected had an age range of 0.4 - 83.0 years with the average age being 47.1 years and a median age of 47.7 years. There were nine samples from patients under 16 years of age. The age range for these was 0.4 - 15.5 years with an average age of 11.2 years and the median age of 12 years. The paediatric samples screened were from 7 males and 2 females.

The postcode with the most requests for Q fever disease investigation was 4816, which was Townsville, with 10 requests, and the surrounding areas of Townsville had 16 requests for Q fever testing. The region with the second highest number of requests for Q fever was 4880, Mareeba, in the northern tropical hinterland. The surrounding areas including Cairns had 13 requests.

The clinical notes at the time of sample collection were examined. The most frequently occurring clinical symptoms were fever/temperature in 39 patients, headaches in 11, pneumonia in 9, “flu-like illness” in 9, PUO in 9, myalgia in 8 patients.

From clinical notes provided, nine patients were considered to be in a high risk group (ie: animal contact or employment risk). Of the 275 samples, 15 patients had a clinical follow-

up for Q fever investigation or for the development of chronic Q fever, and 97 patients had nothing recorded in the clinical history or clinical notes.

6.3.2.2. Bronchoalveolar Lavage Samples

A set of bronchoalveolar lavage (BAL) samples were collected from 100 patents in 2006 for the investigation of a lower respiratory disease etiological agent. This sample type represents a direct sampling of the lung cell surface, and hence any foreign material causing respiratory disease can be collected. Also, alveolar macrophages are found lining the airways, and these cells are responsible for engulfing *C.burnetii*, if inhaled, so BAL are an ideal sample type to detect *Coxiella* in the lung.

These samples were obtained from patients aged 0.2 – 82.6 years of age, with a mean age of 39.3 and a median age of 48.3 years. The samples were from 42 females and 58 males, all presenting with respiratory disease. Included in the 100 samples were BAL from 13 transplant recipients who were all receiving immunosuppressive treatment. According to the clinical notes recorded on sample submission, there were nine patients who recorded a cough, two patients with a febrile illness and one patient with "flu like illness".

6.3.2.3. Respiratory Samples for Flu-Like Illness

Q fever is a disease with clinical manifestations that may mimic "flu like illness". Clinical presentation may include high fevers, chills, sweats and headaches. With seasonal outbreaks and continually circulating newly emerging strains of influenza both globally and nationally, "flu like illness" is readily identified in Queensland. However, often these patients are clinically diagnosed without laboratory confirmation. Considering that some of these infections may be manifestation of Q fever infection that are not correctly diagnosed, a sample set submitted for laboratory confirmation of respiratory viral illnesses, including "flu like illness", was included in this study for testing of *C.burnetii*. These samples were negative for infectious agents by all previous laboratory investigations.

A total of 1385 samples were collected and submitted to Queensland Health Pathology Departments for the PCR diagnosis of viral respiratory disease during the year 2008. These included 708 nasal pharyngeal aspirates, 21 lavages, 631 throat swabs, 4 tissue samples and 21 nasal washings. The samples were from body sites including the bronchial tract, lung, nasal cavity and throat. The samples were collected from patients aged 0-95 years with a mean age of 21.6 years and a median age of 4 years. There were

840 samples from children under the age of 16 years. There were approximately 80 samples from each month of the year with the exception of February, March and April which had at least 157 samples.

These samples were examined in this study for *C.burnetii* DNA using previously described methods (Chapter 2 – section 2.2.2.1).

6.3.2.4. Samples for the Diagnosis of Atypical Pneumonia

Atypical pneumonia is commonly reported in patients diagnosed with acute Q fever[22]. In Queensland however, patient serum samples submitted to a pathology provider with the specific request for the investigation of "atypical pneumonia" or "atypical serology" do not have *C.burnetii* included in the investigation. In Queensland laboratories, the current "atypical pneumonia" screen only includes *Mycoplasma pneumoniae, Legionella pneumoniae* and *Chlamydia pneumoniae.* Yet in the literature, it is commonly reported that the most common cases of atypical pneumonia are caused by three zoonotic pathogens, *Chlamydia psittaci* (psittacosis), *Francisella tularensis* (tularemia), and *Coxiella burnetii* (Q fever) [22].

There were 374 serum samples collected from Pathology Queensland during the month of April 2009. These samples were submitted for the specific test request of "atypical serology" or "atypical pneumonia testing" and were only screened for *Mycoplasma pneumoniae, Legionella pneumoniae* and *Chlamydia pneumoniae.*

These samples were from patients aged 1 to 95 years of age, with the average age of 49 years and a median age of 51 years. Of the 374 samples, there were 44 samples from children under the age of 16 years of age. These 374 samples were tested for the presence of *Coxiella* DNA by PCR.

6.3.2.5. Extraction of Coxiella DNA from Samples for PCR Analysis

For BAL and respiratory samples, nucleic acids were extracted from 200µL of original sample using the Magna Pure automated extractor (Roche Diagnostics, Australia), according to the manufacturer's instructions. The DNA was eluted in a final volume of 50µL and stored at -20°C.

The nucleic acids from serum samples submitted for the diagnosis of atypical pneumonia were processed by aliquoting 200µL of sample into a sample preparation plate for

extraction using the Reagent Pack VX (Qiagen Brisbane, Australia) in the QIAxtractor (Qiagen Brisbane, Australia) as per the manufacturer's instructions for the extraction of DNA and RNA. The DNA was eluted in a final volume of 50µL and stored at -20°C.

6.4. Results

6.4.1. Comparison of Serological Assays and PCR for the Diagnosis of Acute Q Fever

Of the 275 samples tested there were a total of 59 (21.5%) patients that had a positive result in any of the screening assays for acute Q fever markers (Table 6.1). There were 34 patients that were PCR positive in either gene target and 33 patients that had positive IgM serology markers for acute disease. 23 samples were positive by PCR only, with 2 samples only detected by ELISA and 3 by IFA only.

Table 6.1: Positive results for each of the three assays employed in testing 275 serum samples submitted for acute Q fever.

Assay	No Positive	% of Positives
PCR	34	58%
IFA (IgM)	25	43%
ELISA IgM)	23	39%
TOTAL Positives detected	59	21.5% (59/275)

PCR was able to detect the greatest number of patients with acute Q fever. The ELISA and IFA detected similar numbers of acute Q fever infections with 23 and 25 detections respectively.

When compared to the "gold standard" IFA method, the ELISA showed a sensitivity of 64% and a specificity of 97%, which was in concordance with that reported by Herremans et al. for the detection of *Coxiella* IgM antibodies[23]. These values compared to a sensitivity for PCR of 24% and specificity of 89% (Table 6.2).

Table 6.2: Comparison of diagnostic methods (sensitivity and specificity) for acute infection using IFA as the reference Gold Standard (* GS)

		Serology		Molecular	Performance*	
	Total Detected	**IFA (GS*) Detected**	**ELISA Detected**	**PCR Detected**	**Sensitivity %**	**Specificity %**
Assay				**Results**		
IFA IgM (*GS)	**25**	**25**	16	6	-	-
ELISA (IgM)	**23**	16	**23**	5	64	97
PCR	**34**	6	5	**34**	24	89

However, the PCR did not detect many of the patients that were positive by IFA or ELISA. Yet PCR detected 23 positive patients that were not detected by the serological methods. These PCR positive results were primarily from patients with early, acute infections.

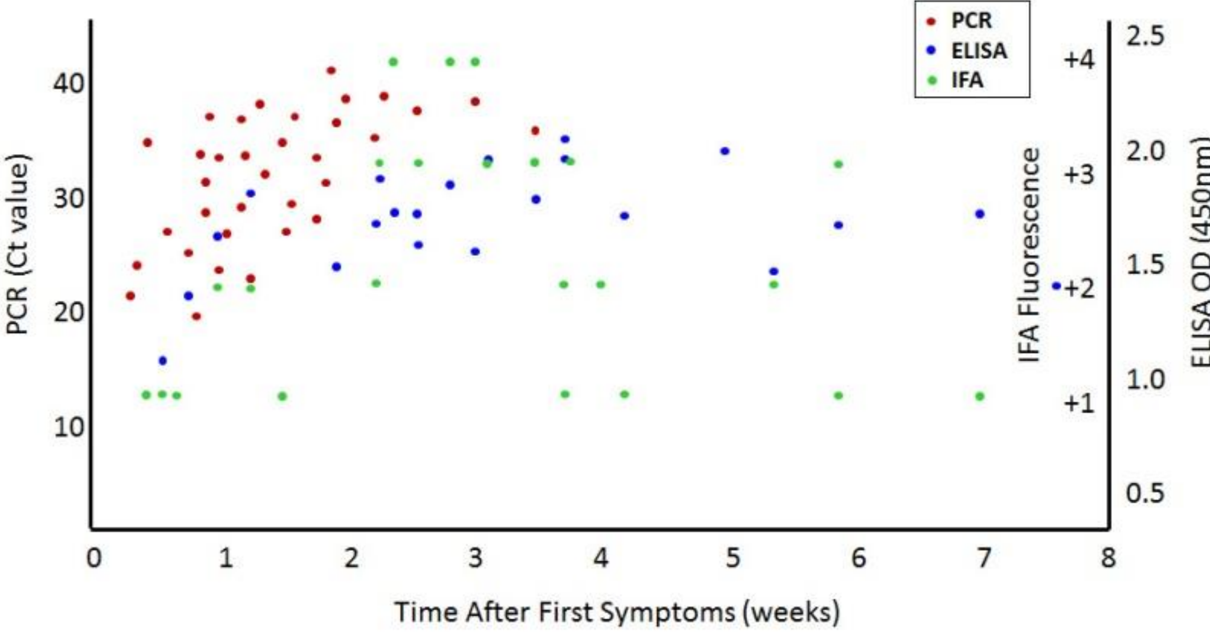

Figure 6.2: A scatter graph showing positive detections for *Coxiella* DNA by PCR (●), and positive IgM antibody detections by IFA (●) and ELISA (●). Positive results are displayed by reactivity in the assay used with real-time PCR results shown as Ct value, and the results of IFA as fluorescence intensity (+1 to +4), and ELISA as the OD of the colour reaction at 450 nm.

6.4.2. Analysis of Bronchoalveolar Lavage (BAL) Samples

Only one of the 100 BAL samples tested positive for *Coxiella* DNA (Figure 6.3). The patient sample was identified as a BAL washing from a 48 year old male who was admitted to hospital post renal transplantation. The patient had a history of lingual lung lesions and complained of left pleural pain. He was investigated for respiratory viruses including influenza virus A and B, parainfluenza viruses 1, 2 and 3, adenovirus, human metapneumovirus, herpes simplex virus 1 and 2, cytomegalovirus and also for *Pneumocystis jiroveci* using PCR. Results for all these were negative and a diagnosis was not recorded in this case.

On examination of this sample with the *Coxiella* dual target PCR, it showed a positive result for the *com1* gene only, producing a CT value of 40.7. This patient resided in Toowoomba, postcode 4350, which is an area of high Q fever notification (Chapter 3).

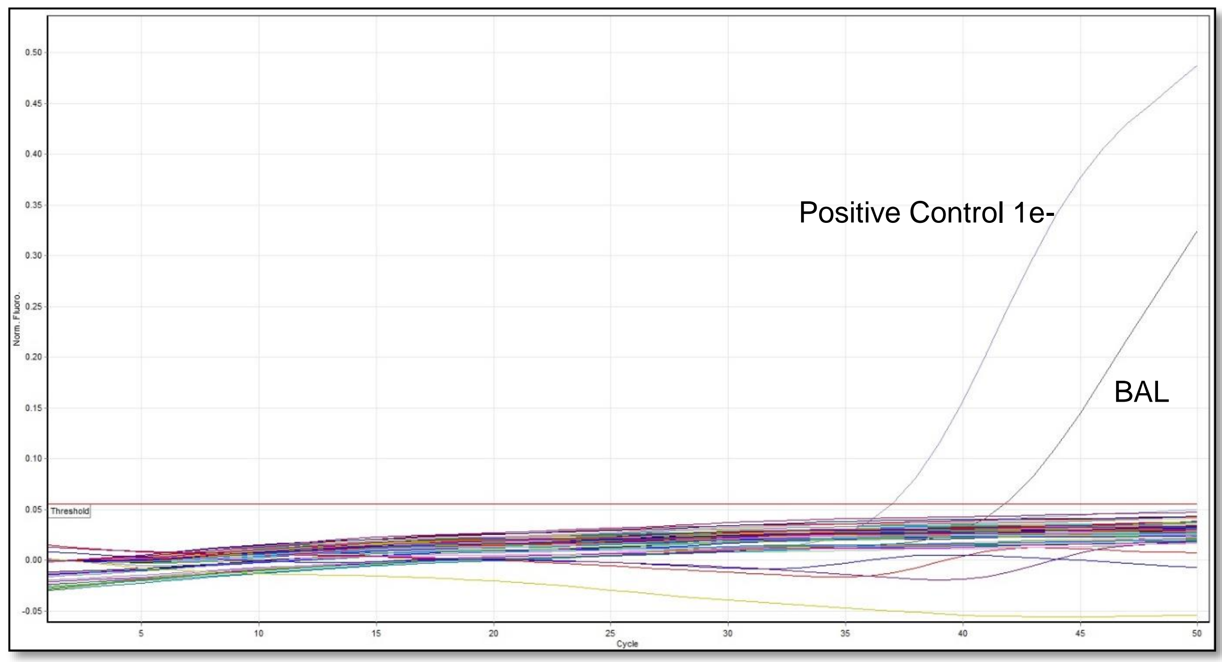

Figure 6.3: Qiagen RotorGene real-time PCR results for bronchoalveolar lavage samples screened using dual targets *com1* and *IS1111* genes**.**

6.4.3. Results for Samples Submitted for the Investigation of Respiratory Disease

Of the 1385 respiratory samples tested, there were 12 (0.9%) PCR positive results using the *com1* and *IS1111* gene target. Three samples were positive by the *com1* assay only, and 9 samples were detected by both targets. Limited clinical data were available for these 12 patients, but 2 samples were collected from children. One child was less than 12 months old and the other was 7 years old. None of these patients showed any evidence in their clinical histories of follow-up laboratory investigations, and a diagnosis of Q fever was not recorded.

6.4.4. Samples for the Investigation of Atypical Pneumonia

374 serum samples were screened for acute markers of Q fever infection, including the dual gene target PCR and IgM serological markers with both the IFA and the ELISA. Nine patients (2.4%) were identified as having one or more marker positive for Q fever.

The PCR detected six patients with Q fever in the *com1* gene only, with CT values between 36 and 40 (Figure 6.4). None of these samples gave a positive PCR result with the *IS1111* target. The *IS1111* target was expected to be more sensitive as there are

multiple copies in the bacterial genome. However, the results above may reflect the susceptibility of the assay to strain variation as previously described by Cooper et al(Cooper, 2011).

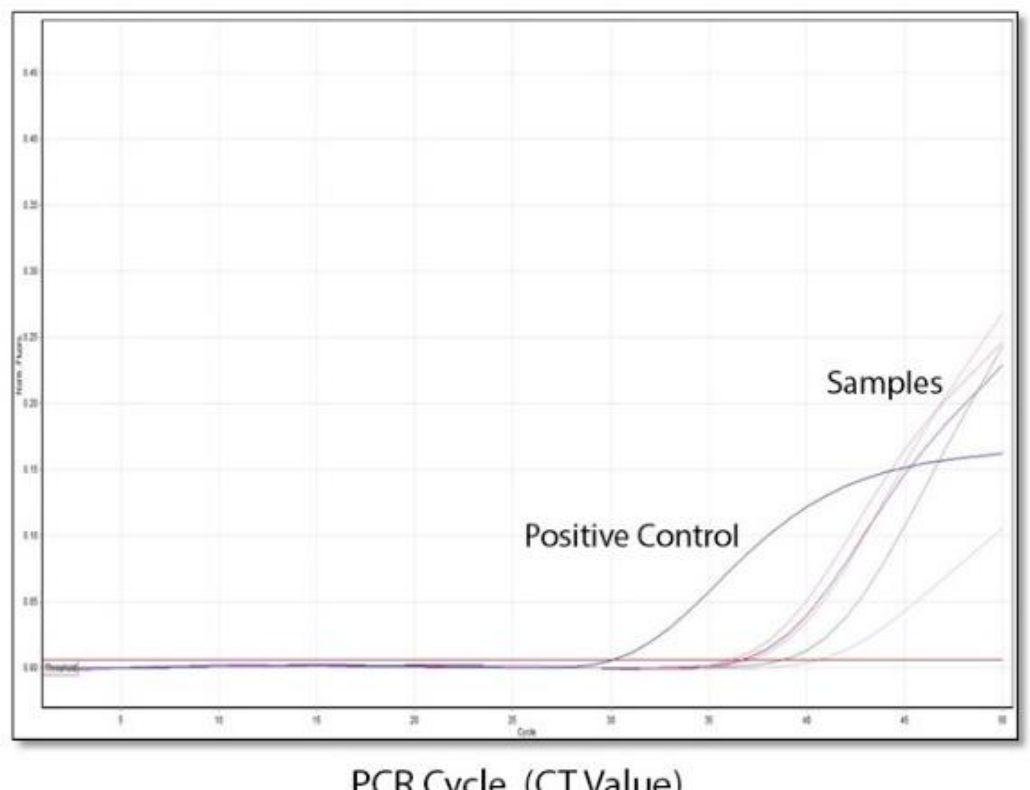

Figure 6.4: Qiagen RotorGene real-time PCR results for atypical pneumonia samples screened using *com1* genes targets.

The serology methods identified six patients as having acute Q fever serology markers (Table 6.3). The IFA phase II IgM was positive in all six patients and positive serology results in the ELISA phase II IgM were seen in three patients. There were only 3 patients with a positive serology marker and a positive PCR result.

Table 6.3: Distribution of positive results for acute Q fever in serum samples submitted for the investigation of atypical pneumonia.

	TOTAL	PCR +	IFA +	ELISA +
PCR Positive	**6**	**6**	3	0
IFA IgM Positive	**6**	3	**6**	3
ELISA IgM Positive	**3**	0	3	**3**

Out of the 374 patient sera tested for atypical pneumonia only 3 were subsequently investigated for Q fever. These three patients showed positive results in all three assays above, including PCR, and their clinical histories showed that they had positive results for the Q fever serology markers on follow up laboratory testing as part of their clinical investigation (Table 6.4). Consequently, these three patients had a definitive diagnosis of acute Q fever recorded and were treated accordingly.

There was one case of atypical pneumonia in which Q fever was considered after the initial negative laboratory results. Here Q fever serology was included in secondary follow up testing using the ELISA IgM commercial kit. This returned a negative result, and no further Q fever serology was requested.

There were five cases of atypical pneumonia where routine Q fever screening was not performed as part of secondary follow up testing. This study found three cases that were identified as acute Q fever using PCR and two with positive IgM serology.

Table 6.4: Atypical pneumonia sample investigation, including testing, patient history and demographics. o/s = overseas.

IFA phase II IgM	PCR Result	ELISA phase II IgM	History	Follow Up	Age	Postcode
DET	**DET**	**DET**	Pneumonia	QF invest requested IFA IgM phase I IFA titre>1280	43.9	4888
DET	**DET**	***Equivocal***	Pneumonia	QF serology request IgM Positive	33.8	4305
DET	**DET**	**DET**	Embolism, ACS, pneumonia, myco-neg,	QF serology request IgM Positive	64.9	4610
NDT	**DET**	NDT	n/a F/up resp NPA BP-neg RSV-POS Myco-neg	Nil – QF not repeated	2.0	4502
NDT	**DET**	NDT	Pneumonia Myco 80 f/up Myco-160 Chlamydia 200 IgA	Nil – QF not repeated	41.9	4160
DET	NDT	NDT	Viral illness Q fever serol-neg,myco-neg,f/up-nil	Nil – QF not repeated	22.5	4029
DET	NDT	NDT	Cough, vomiting	Nil - Pos Mycoplasma	22.4	4680
NDT	**DET**	NDT	Febrile illness 40°C temps	Nil – QF not repeated	75.5	4680
Weak DET	NDT	NDT	Atypical pneumonia Myco,leg,chlamydia -neg,	QF-serology negative	69.4	o/s

6.5. Discussion

Q fever is an important disease particularly in rural Queensland. Chapter 3 of this thesis highlighted that notifications are based on laboratory findings as the Australian national notifiable diseases case definition for Q fever requires only confirmed cases to be reported. Currently in Queensland, a confirmed case requires definitive laboratory evidence in the form of positive nucleic acid detection via molecular methods for *Coxiella burnetii* or a positive serological results for an acute marker, this can be demonstrated presence of IgM antibody, or either a seroconversion or a significant increase in IgG antibody titre to phase II antigens. These scenarios must occur in the absence of a recent Q fever vaccination[24].

In Queensland pathology laboratories today, the method widely employed for the diagnosis of Q fever is serology. The method of choice is ELISA even though the immunofluorecent assay is still considered to be the most sensitive method for diagnosing Q fever using serum samples[23]. The commercially available ELISA from Alere Panbio (Brisbane, Australia) is widely used by Australian laboratories as an initial screening method for acute Q fever, as it is very cost effective, has the capacity for screening large numbers of specimens, and can be fully automated reducing the subjectivity associated with IFA.

The evaluation of the IgM ELISA in this Chapter showed a specificity of 97% but a sensitivity of 64% when compared to the IgM IFA. This is similar to the findings by a large international study evaluating the different diagnostic methods, which showed that ELISA methods for the detection of IgM phase II as acute markers of Q fever diseases had a sensitivity of 60%[23]. This is an important result from this study, because the current regime for initial testing of acute Q fever in Queensland diagnostic laboratories employ the ELISA method only. Clearly, this has a significant impact on the identification of acute Q fever cases in Queensland, and may be the cause of under-reporting of the disease as reflected in the discrepancy between notification rates and seroprevalence (Chapters 3 and 4).

The differences in the specificity results may be related to the substrate antigens used in the ELISA and IFA assays. Different strains of *Coxiella* may be used in preparing the antigens to which antibodies are measured, and this may affect the sensitivity observed

between assays. The Alere Panbio kit used in Queensland has been prepared using the Henzerling strain of *Coxiella,* whereas the IFA assay was prepared using the Nine Mile strain of *Coxiella*.

The reason Q fever infections have been diagnosed by serology is mainly due to the fact the organism is fastidious to grow and is an occupational hazard for laboratory staff. Recently molecular methods have replaced serological techniques for diagnosing many bacterial and viral agents causing disease in humans.

PCR provides a rapid and accurate method for detecting *Coxiella* DNA as a marker of acute Q fever infection. This has a distinct advantage over serological methods, which are often negative during the early phase of the disease, and ideally require examination of paired sera taken 14 days apart to confirm antibody conversion.

At first glance the sensitivity of PCR compared to the gold standard IFA, is poor with only 24% concordance. However, as this study has shown PCR is a more effective tool for the early diagnosis of Q fever when the antibody response is still developing and below the level of detection with serological testing.

The PCR detected 23 cases of acute Q fever that were not diagnosed by serological methods. These were predominantly in samples that were collected early following the presentation of symptoms. Although these results could possibly be attributed to false positive reaction in the PCR, in 11 cases, where further confirmation was available in clinical histories or by presence of Q fever-specific symptoms, serology, or further molecular confirmation, the diagnosis was deemed to be correct.

Early diagnosis and accurate recognition provides a better outcome for the patient, in terms of treatment and may reduce possible Q fever-associated sequelae including chronic Q fever and chronic Q fever fatigue syndrome. This study was conducted on randomly selected samples, and therefore was an observational analysis of a random population. However, it demonstrated that most of the patients in the cohort with suspected acute Q fever, presented to clinical staff early enough in their illness to enable the detection of *Coxiella* DNA before the detection of IgM antibodies. Therefore it would seem highly advantageous for the effective clinical management of patients with Q fever, that PCR be introduced to complement serological testing, particularly in those patients that present within the first 2-3 weeks after the onset of symptoms.

Thus, the results of this study support an improved diagnostic strategy for the early diagnosis of acute Q fever in patients with clinical manifestations suggestive of the disease. Namely:

1. For serum specimens collected in the first 2 weeks of infection, both PCR and serology should be performed;
2. From weeks 3 to 4 after onset, serology should be performed first and PCR should be reserved for seronegative specimens;
3. For serum specimens collected later than 4 weeks after the onset of symptoms, serology but not PCR should be used as diagnostic test.

Also, the diagnosis of Q fever using PCR may be more effective if the samples collected are more appropriate for the infectious agent; that is, if the samples were to be more representative of the cell types or tissues that are directly infected during the primary phase of disease. For example, the bacteria are initially engulfed by macrophages in the respiratory tract[15,26], therefore it may be more appropriate to sample macrophages and perform PCR directly on these infected cells rather than measuring DNA from the breakdown of the bacteria in the blood.

Examining a range of specimens submitted for the laboratory examination for infectious agents other that *Coxiella*, yet presenting with similar symptoms, provided a valuable insight in the possible level of under-recognised Q fever that may occur in the Queensland population.

Bronchoalveolar lavage samples collected in this study provided and excellent sample set to interrogate cellular components of the lung epithelial lining for the presence of *Coxiella*. These samples were specifically collected for the investigation of an etiological agent causing respiratory disease in this cohort of patients. The results showed that one patient was positive for Q fever using PCR, confirming that this was the cause of infection. Although this was only a small sample set, it did indicate that *Coxiella* should be considered as a cause of respiratory disease, particularly in cases where another diagnosis is not provided.

In this same context, samples collected for the investigation of a “flulike illness” were examined for Q fever using PCR, and showed that 0.9% of these were positive. Although this is not considered to be high prevalence, it is of similar magnitude as other recognised

respiratory pathogens such as influenza C and parainfluenza 4. Again, the patients making up this sample cohort were not considered to be infected with *Coxiella*, and the inclusion of an appropriate test to diagnose this may have improved clinical management, or perhaps may have prevented possible clinical complications such as the development of chronic Q fever and chronic Q fever fatigue syndrome.

The most common symptoms of Q fever presentation are those that are commonly associated with atypical pneumonia. For this reason a sample population from patients with this presentation and request for atypical pneumonia investigation were included in this study. Currently, testing algorithms in Queensland do not offer Q fever serology as part of initial screening when a request for atypical serology/pneumonia investigation is made.

Atypical pneumonia is often community acquired, and the aetiological agent is largely unknown. A study into community acquired pneumonia in Australian adults has shown that the most common agents responsible are respiratory viruses (15%), *Streptococcus pneumoniae* (14%), *Mycoplasma pneumoniae* (9%), *Haemophilus influenzae* (5%), Legionella species (3%) and *Chlamydophila (Chlamydia)* species (2%) [27], making up approximately 48% of atypical pneumonia diagnoses. This still leaves a large percentage of patients with pneumonia for which there has been no definitive diagnosis. Atypical pneumonia is considered to be the result of exposure to zoonotic infectious agents, and in Queensland, Q fever has been the zoonotic disease with the highest number of notifications over the last five years. Therefore it seemed highly likely that *Coxiella* may be an important agent of atypical pneumonia.

This study showed that 2.4% of the atypical pneumonia patients with a specific request for atypical investigations were positive for Q fever, yet this test was not included as part of the routine testing regime in Queensland. Over the past 5 years in Queensland Health laboratories there have been 13,338 request for the specific investigation of atypical pneumonia, an average of 2, 668 requests per year. For the same time frame there have been 16,938 requests for the investigation of Q fever disease and from this there were 2,011 cases (12%) identified using the ELISA screening method. If atypical pneumonia investigation were to include Q fever screening as a first line screening option, this could have potentially identified another 2.4% or 320 cases of Q fever over the five years. This data has only been generated from Queensland Health patient populations and has not

taken into consideration the testing occurring in other pathology providers in Queensland. This is notwithstanding the number of extra cases that would be identified if PCR were to be included in the diagnostic algorithm as discussed above.

In summary, the results presented here showed the current diagnostic protocol that is applied in Queensland is adequate to detect the majority of Q fever cases. However, significant improvement in the detection rate could result if routine PCR testing were to be included in the laboratory investigative pathway, and if there were greater clinical awareness that *Coxiella* should be considered in the presentation of respiratory illness, especially, atypical pneumonia.

6.6. Significant Outcomes from this Chapter

- The results of this chapter confirmed that using the current diagnostic algorithm, a substantial number of Q fever infections remain undiagnosed, and that the current diagnostic paradigm must be re-examined.
- Use of IFA testing is recommended in preference to the Alere (Panbio) ELISA kit, which is used extensively throughout Australia. This will provide improved sensitivity in the diagnosis of acute Q fever infection.
- Although PCR is not widely applied in the diagnosis of acute Q fever, its inclusion in diagnostic protocols is highly recommended, especially for the detection of early Q fever infections.
- Clinical awareness of *Coxiella* as a pathogen involved in the wider presentation of respiratory disease must be improved, so that Q fever may be considered as a diagnosis in patients presenting with respiratory symptoms especially atypical pneumonia.

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6.8. Appendix 6.1: Clinical manifestations that may be presented as a result of Q fever infection

	Acute		Chronic		Post -Q fever fatigue syndrome	
Origin	inhalation or ingestion of bacteria		persistent low level infection with Coxiella		post-acute or post-chronic Infection	
Cell Target	macrophages		organs - heart valve, liver, lungs, bone		organs – muscles, lungs general health	
Incubation period	7 - 60 days		weeks - years post initial exposure		weeks Months Years	
Disease	**Manifestation**	**Symptom**	**Manifestation**	**Symptom**	**Manifestation**	**Symptom**
	asymptomatic	none		cardiac valve failure		prolonged malaise
		high fevers (up to 40°C)	endocarditis	vegetations		fever
		severe headache		heart failure		myalgia
	"flu" like illness	general malaise			chronic QFS	arthralgia
		myalgia				night sweats
		chills and/or sweats				osteomyelitis
	non-productive cough	acute pneumonia	chronic pneumonia	fever		osteoarthritis
atypical pneumonia	chest pain			productive cough		
	respiratory distress			wheezing		spontaneous abortion
	fever			night sweats		
	consolidation			chest pain		vascular grafts infections
				breathlessness		

APPENDIX 6.1 (continued): Clinical manifestations that may be presented as a result of Q fever infection

	Acute		**Chronic**		**Post -Q fever fatigue syndrome**	
Origin	inhalation or ingestion of bacteria		persistent low level infection with *Coxiella*		post-acute or post-chronic Infection	
Cell Target	macrophages		organs - heart valve, liver, lungs, bone		organs – muscles, lungs general health	
Incubation period	7 - 60 days		weeks - years post initial exposure		weeks months years	
	Manifestation	**Symptom**	**Manifestation**	**Symptom**	**Manifestation**	**Symptom**
hepatitis	hepatomegaly	chronic hepatitis		hepatomegaly		
	granuloma formation			granulomatous hepatitis		
	jaundice					
myocarditis	chest pain	fatigue		generalised malaise		
	fever			anorexia		
				night sweats		
cutaneous infections	maculopapular or pruritic rash	osteoarticular disease	osteomyelitis			
	erythema nodosum		osteoarthritis			

APPENDIX 6.1 (continued): Clinical manifestations that may be presented as a result of Q fever infection

	Acute		**Chronic**		**Post -Q fever fatigue syndrome**	
Origin	inhalation or ingestion of bacteria		persistent low level infection with coxiella		post-acute or post-chronic infection	
Cell Target	macrophages		organs - heart valve, liver, lungs, bone		organs – muscles, lungs general health	
Incubation period	7 - 60 days		weeks - years post initial exposure		weeks months years	
	Manifestation	**Symptom**	**Manifestation**	**Symptom**	**Manifestation**	**Symptom**
neurological disease	meningoencephalitis	infections during pregnancy	spontaneous abortion			
	meningitis					
	encephalitis	vascular disease	vascular grafts infections			
	cerebellitis					
	neuritis					
	Guillain-Barre syndrome					
	myelitis					
	peripheral neuropathy					
	extrapyramidal disease					
hemophagocytosis	uremic syndrome					
	anemia					
	rhabdomyolisis					
	bone marrow necrosis					
renal disease	glomerulonephritis					

Chapter 7

Clinical Case Studies of Q fever from Queensland

Articles published as a result of work done in this chapter:

Sarah R. Stevenson, John Gowardman, **Sarah Tozer**, Marion L. Woods. Life Threatening Q Fever Infection following Exposure to Kangaroos and Wallabies. April, 2015. British Medical Journal Case Reports. Submitted for publication (April, 2015): Manuscript ID bcr-2015-210808

Tozer SJ, Lambert SB, Sloots TP and Nissen MD. Multiple Presentation of Q Fever in Six Family Members from Rural Queensland, Australia. BMC Clinical Pathology – Case Reports. Manuscript in Preparation

7.1. Introduction

So far in this study, evidence has shown that although notifications of Q fever were predominantly centred on rural communities, significant levels of disease were also reported in urban populations. The fact that Q fever also may occur in those populations that are considered as "low risk" was supported by the evidence of the seroprevalence study described in Chapter 4, which showed that urban populations had similar levels of exposure as those in rural settings, further suggesting that infected subjects had asymptomatic infections, or failed to be correctly considered for the laboratory investigation of Q fever.

Furthermore, Q fever is widely considered to be an occupational illness of adults in close, direct contact with ruminant animals in a rural environment. Therefore, it is not often considered when seeking preliminary diagnosis for a febrile illness, especially in children. Also, children may present with less defined symptoms than adults, and Q fever disease may not be considered as part of the clinical diagnosis for children presenting with acute symptoms[1]. Q fever infections occurring in children, in general are rarely reported, probably misdiagnosed and hence infections are potentially under-reported in this population [1,2].

The documented acquisition of Q fever infection in humans is through inhalation of contaminated aerosols via the respiratory route[3]. Aerosols may be contaminated by the products of parturient animals, including birthing products, and the dispersion of these into the environment [4]. The bacteria can remain highly stable and infectious in the environment for months and perhaps years and are able to withstand many harsh environmental conditions[3]. Other routes of transmission include the ingestion of contaminated dairy products containing *C.burnetii* and transmission from ticks that are infected with the bacteria[5]. There have been isolated cases of human to human transmission in the performance of an autopsy on a Q fever patient, in bone marrow transplant recipients and one sexually transmitted case[6]. However, these modes of transmission are very rare[7].

Other studies, including the findings in Chapter 5 of this thesis, have identified domestic pets as a potential source of infection. Epidemics have been reported in Nova Scotian (Canada) communities which were linked to one single parturient cat that resulted in more

than 30 cases of Q fever[8]. Recently, urban veterinary surgeries have also been associated with small Q fever outbreaks. In Sydney, two separate veterinary clinics recorded an outbreak of Q fever amongst the staff working within the surgery. One clinic performed an emergency feline caesarean, which was determined to be the source of Q fever infection for 9 of 20 staff employed at the clinic. Eight of the staff members were present the day of the caesarean but the 9th patient contracted the disease 24 hours later through simply handling equipment used in the caesarean procedure the previous day. Under the current vaccination guidelines, the veterinarian was the only person vaccinated and hence did not contract Q fever[9]. The second episode also occurred in a Sydney veterinary clinic where a moribund bitch dog underwent an emergency caesarean section. The birth resulted in all the pups being delivered still-born.

There were three nurses present during the birthing episode who aided in the procedures, and one nurse cared for the dog at home during three weeks of convalescence. Four weeks post-caesarean two of the nurses presented with a flu-like illness while the third nurse was hospitalised six weeks later with pericarditis and pericardial effusion along with fevers and rigors. All three nurses tested positive for Q fever serology and the dog was also serologically positive and hence identified as the source of infection. Again, the veterinarian who performed the procedure had been vaccinated against Q fever and did not produce any symptoms[10]. These outbreaks highlight the potential for companion animals to transmit Q fever to humans in urban settings.

Previous data from studies performed as part of this thesis identified a number of Queensland animals that have the potential to transmit *C.burnetii* to humans[11]. Among these were companion animals in particular cats and dogs, along with native animals, that co-reside in suburbia with humans. A significant find in this study was the detection of *C.burnetii* DNA in pooled flying fox urine and “bat spat” from colonies around Brisbane. These animals have the potential to spread the bacteria anywhere along their flight path to the feeding grounds of other animals. Also, Australian native animals such as the possums, bandicoots, grey kangaroos and other macropods have been identified as harbouring *C.burnetii* and hence pose yet another potential source of infection to humans, especially as suburbia begins to encroach into native animal habitats[12] . There is also the potential for any animal to become infected with *C.burnetii* from different tick species that are found throughout Australia[13]. In Chapter 5, it was shown that ticks removed from both

domestic and native animals in Brisbane may be positive for *C.burnetii* DNA, highlighting the potential for any animal to become a carrier of Q fever and shed the bacteria into the human environment[11].

7.2. Specific Aims of This Chapter

The study in this chapter sought to examine cases of Q fever that fell outside the normal paradigm associated with traditional methods of contracting the disease, or those with an unusual clinical presentation. In particular:

- It highlights the complexity associated with recognising Q fever infections and that direct animal contact is not a prerequisite for the acquisition of C.*burnetii* and Q fever disease.
- It also demonstrates the potential for transmission in families, including infection of children from their parents or siblings
- And demonstrates the clinical severity and complications that may occur as a result of infection.

7.3. Methods

7.3.1. Clinical Cases Examined

Unusual presentations of patients with Q fever were identified by the Director of the Infectious Management & Prevention Service at the Royal Children's Hospital-Brisbane, Associate Professor Michael Nissen, and Senior Staff Specialist in Infectious Diseases, Dr Marion Woods at the Royal Brisbane and Women's Hospital. The cases analysed were all from rural Queensland and involved children younger than 16 years of age with unusual clinical histories in which Q fever was the definitive diagnosis, and one adult case.

Specifically these were:

1. Three cases of Q fever in children four years old or younger were the primary focus of infection.
2. One family case investigation initiated by Q fever disease in a thirteen year old girl involving both parents and three siblings.
3. One severe adult case in a 28 year old female which was contracted through presumed occupational exposure in Central Queensland, but for whom a definitive source of infection was not identified.

The majority of these cases had no history of direct animal contact and hence Q fever was not initially considered for diagnostic investigation, and excluded as a possible cause of disease. Those cases which had a recorded history of animal contact had no direct contact with ruminants, and again were considered outside the established paradigm associated with classical Q fever acquisition.

The cases presented here serve as an illustration of issues that may lead to the under-recognition of Q fever in populations that are considered at "low risk", highlighting the need for greater vigilance in considering unusual sources of Q fever infection.

7.3.2. Method of Clinical Case Review

Clinical charts from patients admitted to the Royal Children's Hospital, Brisbane, or the Royal Brisbane and Women's Hospital with a clinical diagnosis of Q fever were reviewed with data collated from the clinical notes and patient histories. Pathology results were reviewed and recorded along with any specialist reports and clinical requests from referring doctors and GPs. All abbreviations, including medical, used in the description of case analysis were standardised and are shown in Table 7.1.

Table 7.1: Medical abbreviations used in this Chapter**.**

Medical Abbreviations			
Abbreviation	**Full term**	**Abbreviation**	**Full term**
GP	General practitioner	IV	Intravenous
LFTs	Liver function Tests	PUO	Pyrexia of unknown origin
E/LFTs	Electrolytes and liver function tests	CFT	Complement fixation test
M/C/S	Microscopy, culture and sensitivity	BFV	Barmah Forest virus
CRP	C-reactive protein	CMV	Cytomegalovirus
FBC	Full blood count	HCV	Hepatitis C virus
WBC	White blood cells	EBV	Epstein Barr virus
RBC	Red blood cell	QF PCR	Q fever polymerase chain reaction test
IgA	Immunoglobulin A	RRV	Ross River virus
IgG	Immunoglobulin G	IFA	Immunofluorescence assay
IgM	Immunoglobulin M	ELISA	Enzyme-linked immunosorbant assay
tAB	Total antibodies	HBV	Hepatitis B virus

7.4. Clinical Case Presentations

7.4.1. Case No 1: Four Year Old Female from Goondiwindi

7.4.1.1. Clinical Presentation

A four year old female child residing in Goondiwindi developed fevers and lethargy over six days before presenting to a General Practitioner in the town. The child had previously been in good health.

Day 1: On presentation at the GP clinic the child was alert but pale, febrile with a temperature of 39.0^{0}C. No rash was detected, and the clinical examination was within normal including; ears, nose, throat, chest and abdomen. As a follow up a chest x-ray was requested, this was also normal. Blood work revealed a lymphocytopaenia along with a thrombocytopenia, E/LFTs were abnormal and the biochemical screen showed a low iron level. Microscopy of urine was normal and blood cultures were negative. Serology investigations were ordered and included CMV, BFV, RRV and Q fever. The child deteriorated overnight with temperatures peaking at 40.7°C and associated rigors.

Day 2: The child represented at the GP with a temperature of 37.6°C, very pale and lethargic, with no rash and no organomegaly. The E/LFTs were still abnormally raised and an provisional diagnosis of a viral infection or Q fever was proposed. The child was sent home and returned 3 days later for GP review and serology results.

Day 5: On review, the child remained pale and lethargic with body temperatures remaining elevated at night. No rash was detected. FBC was normal as were E/LFTs. Q fever was finally diagnosed based on the results of serology (Table 7.2). Treatment with Rulide D tablets (roxithromycin – Sanofi Pharmaceuticals) was commenced and follow up serology was requested in 10 days.

Table 7.2: Case 1 - Results of laboratory investigation for patient in Case 1.

Date of Collection	Serology Test	Result
05 August 2002	CMV IgM	Negative
	BFV IgM	Negative
	EBV IgM	Negative
	Q Fever Phase II IgM	Equivocal
09 August 2002	CMV IgM	Negative
	BFV IgM	Equivocal
	EBV IgM	Positive
	RRV IgM	Negative
	Q Fever Phase II IgM	Positive
	Q Fever Phase II IgG	Positive

7.4.1.2. Summary of Laboratory Investigations

The results of serology performed at first presentation showed an equivocal Q fever IgM result for Q fever Phase II antigens. The diagnosis of Q fever was confirmed by increasing levels of Q fever IgM antibody and the presence of Q fever IgG antibodies in the second blood sample taken 4 days later. Unfortunately the results from follow up serology taken 10 days after discharge from hospital were not available. Two positive IgM antibody detections, to unrelated viruses (BFV and EBV), had the potential in this case to confound the interpretation of results, were it not for the clear seroconversion to Q fever antibody positivity demonstrated between the two samples collected at different times.

7.4.1.3. Case History

A detailed history revealed that the child lived on a large cattle property. The father of the child had recently been diagnosed with acute Q fever and the child's three siblings were also unwell with similar symptoms. This case, almost certainly, is an infection as a result from exposure to a parental contact. However, it is unknown whether the infection was caused by direct personal contact through aerosolised Q fever organisms from the infected father, or by or by transference of infected material acquired by the father during his

occupational activities to the child. Also previous close contact, although not identified on review, could not be discounted. To resolve this molecular typing of the *Coxiella* isolates would be highly desirable, but unfortunately, as these studies in the thesis were based on case reviews, no samples were available on which molecular typing could be performed

7.4.2. Case No 2: Three Year Old Boy from Goondiwindi

7.4.2.1. Clinical Presentation of the 3 Year Old Boy

A previously well three year old boy from Goondiwindi presented to a local General Medical Practitioner's clinic with a 10 day illness.

Day 1: He presented with a fever, cough and headaches. The GP diagnosed a lower respiratory tract infection with a pleural effusion was treated with oral amoxicillin without improvement. He became increasingly unwell and was admitted to Goondiwindi Hospital two days later.

Day 3: On admission to hospital the boy was febrile and dehydrated. A chest x-ray was performed. He was treated with IV fluids and administered IV ceftriaxone for presumptive pneumonia. A specialist examination found him to be febrile with 97% oxygen saturation levels, alert and active with no respiratory distress. His right lung had reduced air entry and his abdomen was distended with a large right upper quadrant mass. The boy was then transferred to Toowoomba Hospital.

Day 4: At Toowoomba Hospital, further investigations were requested including another chest x-ray and pathology blood tests. The repeat chest x-ray showed a large right pleural effusion while ultrasound examination of the chest confirmed a large right effusion and a smaller left effusion. Ascites was also detected by abdominal ultrasound, however,the liver and kidneys appeared normal. Pathology testing including FBC, E/LFTs and blood cultures were performed by a private pathology provider. The FBC revealed a low platelet count and reactive lymphocytes. The child had a markedly elevated C reactive protein (CRP), a low serum iron with elevated E/LFTs, and normal urine M/C/S. Given the seriousness of his condition, the boy was transferred to the Royal Children's Hospital (RCH) in Brisbane, Queensland.

Day 6: On presentation at the RCH, further blood samples were taken for LFTs and serology screening for other infectious agents including; *Mycoplasma pneumoniae*, CMV,

EBV, HBV, *Toxoplasma gondii*, Q fever, Leptospira *spp.* and respiratory viruses including; RSV, influenza A and B, parainfluenzae 1 & 3 and adenovirus. A third x-ray revealed a lung consolidation of the right middle and lower lobes, with a left pleural effusion and ascites. An repeat ultrasound examination showed an enlarged liver.

The results of Q fever serology detected phase II IgM antibodies and an IFA phase I IgM antibodies at a titre of >1280. The *M. pneumoniae* total antibody titre was 320. Blood cultures were subsequently reported as negative. These results confirmed a diagnosis of acute Q fever infection. The IV ceftriaxone was ceased and treatment with oral doxycycline was commenced. The results of serological investigations are shown in Table 7.3. Given these results, a subsequent clinical review of other close family members was instigated.

7.4.2.2. Clinical Review of Family Members

Further follow up of the boy's family revealed that his grandmother had been suffering with pneumonia, which was subsequently diagnosed as due to *M. pneumoniae.* The boy had visited her prior to becoming unwell. Also, it was found that the boy's mother had also been unwell with fevers and had been hospitalised in Toowoomba as well prior to his presentation to the RCH.

Table 7.3: Pathology results of patient described in Case2

Date of Collection	Serology Test	Result
16 July 2003	CMV IgM and IgG	Negative
	BFV IgM and IgG	Negative
	EBV IgM and IgG	Negative
	Mycoplasma total AB	160
	HBV IgM	Negative
	HBC IgM	Negative
	Toxoplasma IgM and IgG	Negative
	Serovars Leptospiorosis	Negative
	Respiratory Virus Panel PCR	Negative
	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Positive
	Q Fever IFA Phase II IgG	80
	Q Fever IFA Phase II IgM	>1280
	Brucella IgM and IgG	Negative
06 August 2003	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Positive
	Q Fever IFA Phase II IgG	320
	Q Fever IFA Phase II IgM	>1280

.7.4.2.3. Clinical Presentation of the Mother

The boy's mother, a 31 year old female, had been unwell for 10 days prior to the child falling ill and was herself admitted to Toowoomba hospital with a severe acute community acquired pneumonia and hepatitis. She had suffered severe headaches, fevers, rigors, photophobia, meningism, myalgia, nausea, vomiting and complained of a nocturnal cough. Her chest x-ray showed consolidation of the right lung. A lumber puncture was performed with no WBCs present and normal cerebrospinal biochemistry glucose and protein concentrations. A FBC revealed low platelet and WBC counts. An atypical pneumonia screen revealed a *M. pneumoniae* antibody titre of 320 and negative serology results for Q fever antibodies. She was commence IV antibiotics. She remained febrile, with worsening headaches intensifying with movement, neck pain, photophobia, nausea and persistent lung consolidation over the next three days. Her symptoms slowly began to improve on days 4, 5 and 6, however, headaches remained as did the lung effusion. Her FBC improved with the WBC count increasing, but she remained febrile. IV therapy was ceased after day 6 (a total of 96 hrs), She was now afebrile and much improved with only mild headaches and discharged after a 9 day in hospital, and 3 days prior to her son becoming unwell.

As a result of the clinical review initiated by her son's diagnosis, she was retested for Q fever antibodies, 9 days after being discharged from hospital (Table 7.4). Her serology results confirmed a diagnosis of acute Q fever and she was treated with oral doxycycline.

7.4.2.4 Summary of Laboratory Investigations

The serology results for this three year old boy confirmed a definitive diagnosis of acute Q fever, with Q fever phase II antibodies for IgM and IgG detected by IFA and ELISA testing. There was additional evidence of a previous infection to *M. pneumoniae*. Follow up serology confirmed diagnosis of Q fever with increasingly antibody titres. Interestingly, his mother was also previously diagnosed with *Mycoplasma* infection, supported by her elevated *Mycoplasma* total antibody titres, and like her son was consistent with her acquiring this infection from her mother (the boy's grandmother) who was previously also diagnosed with *Mycoplasma* pneumonia. Retrospective antibody testing on the mother indicated a resolving acute Q fever infection.

Table 7.4: Case 2- Pathology results for the mother. *NB: Some of these tests were performed retrospectively

Date of Collection	Serology Test	Result
02 July 2003 *	Mycoplasma tAB	<40
	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Negative
11 July 2003 *	Mycoplasma total AB	320
	Mycoplasma IgM	Positive
17 July 2003	Mycoplasma total AB	320
	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Negative
	Q Fever IFA Phase II IgG	40
	Q Fever IFA Phase II IgM	>1280
06 August 2003	Mycoplasma total AB	40
	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Positive
	Q Fever IFA Phase II IgG	>1280
	Q Fever IFA Phase II IgM	>1280

7.4.2.5. Case History

The mother was questioned regarding recent animal contact after the positive diagnosis of Q fever was made for her son. She clearly recalled aiding in the delivery of a calf by the roadside some weeks before becoming unwell. Her son was present at the time, but remained in the car during the birthing. The mother and child had a lengthy drive home. Given this new evidence, it seems likely that the boy was infected as a result of being in close proximity to his mother who presumably carried *Coxiella* infected birth products on her person. It is also possible that the grandmother may have been suffering from acute Q fever as a result of being transported in the same vehicle following the event or by personal contact with her infected daughter. Unfortunately no serology testing for Q fever antibodies was performed on the maternal grandmother to investigate this hypothesis.

7.4.3. Case No 3: A Four Year Old Boy from Nanango

7.4.3.1. Clinical Presentation of the 4 Year Old Boy

This child had a complex past history of congenital heart disease and Klippel-Feil syndrome. He had an unresolving acute illness which was not responding to medical treatment and had no definitive diagnosis. His symptoms included fever, diarrhoea, cough, tachypnoea, hepatomegaly, intermittent swelling in the left scrotum, and a nonspecific erythematous macular rash on the limbs and trunk. In addition he had had been febrile for 14 days and had abnormal E/LFTs and with thrombocytopenia on FBC.

Day 1: The boy was admitted to the Toowoomba Hospital and treated with oral antibiotics which included penicillin and amoxicillin for 7 days. He remained febrile and lethargic and an enlarged liver was palpated on abdominal examination. The child's FBC showed thrombocytopenia but was otherwise normal. His treatment progressed to IV antibiotics. Two days into the admission, he continued to have spiking fevers and developed rigors and seizures. His platelet count continued to plummet, and he was treated with serum immunoglobulins over the next 12 hours. His E/LFTs remained abnormal and blood cultures remained negative. The boy was transferred to the RCH-Brisbane where he was further assessed.

Day 8: At RCH, he presented as alert and afebrile with a clear chest. An echocardiogram was reported as normal with no evidence of endocarditis. On examination, he had a petechial rash, hepatomegaly and mild ascites. His treatment was continued with IV antibiotics. Bloods were drawn for serology and urine collected for M/C/S. That night he became febrile with temperature reaching 38.2^{0}C overnight. The existing treatment continued.

Day 9: Overnight his fevers again returned, peaking at 37.9°C. There was no evidence of pneumonia with a clear chest auscultation. Previous serology testing had detected Q fever IgM antibodies, but this was dismissed as a false positive result due to possible cross reaction with a viral IgM antibody response. Further bloods were drawn with follow up Q fever serology tests requested. A clinical diagnosis of acute Q fever was made based on a decreasing Q Fever CFT titre (Table 7.5) and a suggestive clinical history that included high fevers, headaches, minor LFTs abnormalities and the acute respiratory illness prior to hospitalisation. As a result, treatment was altered to IV ciprofloxacin and

rifampicin. To seek a definitive diagnosis, the presence of *C. burnetii* DNA in the blood by specific PCR was sought. PCR testing demonstrated the presence of *C.burnetii* DNA in all previously collected blood samples confirming a diagnosis of acute Q fever infection. Following the detection of Q fever DNA in blood, retrospective Q fever serology testing revealed the presence of IgM antibodies with increasing levels of IgG, which was a further confirmation of acute Q fever (Table 7.6).

Table 7.5: Case 3 – Summary of pathology results

Date of Collection	Serology Test	Result
05 December 2000	CMV IgM and IgG	Negative
	EBV IgM	Negative
	Toxoplasma IgM and IgG	Negative
	Toxoplasma IgM and IgG	Negative
	Serovars Leptospiorosis	Negative
	Mycoplasma pneumonae total Ab	Negative
	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Negative
	Q Fever total Ab (CFT)	128
	Rickettsia serology IgG	Negative
07 December 2000	Brucella IgM and IgG	Negative
12 December 2000	Bartonella IgM and IgG	Negative
	Q Fever Phase II IgM (ELISA)	Positive
	Q Fever Phase II IgG (ELISA)	Negative
	Q Fever total Ab (CFT)	64

Table 7.6: Case 3 – Summary of retrospective Q fever serology results

Q Fever IFA Serology							
	Phase I				Phase II		
Date	**IgG**	**IgM**	**IgA**	**Total Ab**	**IgG**	**IgM**	**Total Ab**
7 December 2000	ND	ND	-	128	128	512	>128
12 December 2000	ND	80	-	>128	512	512	>128
10 January 2001	ND	320	-	>128	512	128	>128
5 April 2001	64	128	-	>128	2048	ND	>128
25 July 2001	128	128	-	>128	2048	ND	>128
14 March 2006	640	-	160	>512	>1280	ND	512
12 October 2010	320	-	ND	64	>1280	ND	128

7.4.3.2. Summary of Serological Results

The serological profile based on retrospective testing for this child shows a classical pattern of phase II IgM and IgG Q fever antibodies developing over time consistent with acute Q fever infection. This child had an underlying congenital heart condition putting him at risk of developing chronic Q fever infection. He was hence monitored at regular intervals for the development of chronic Q fever infection. Over a ten year period this boy showed clear evidence of the development of chronic Q fever infection with the detection of Q fever phase I IgA antibodies, which is considered a marker of chronic infection, together with increasing titres of Q fever phase I IgG and total Q fever antibodies. This child therefore continues to be clinically monitored for chronic Q fever infection together with his congenital heart condition.

7.4.3.3. Review of Clinical Case and Contact Tracing

In consultation with the boy's mother, possible sources of Q fever exposure were discussed. He came from rural environment. The family had a domestic cat and exposure to farm animals was identified as a possible source. There was no evidence of contact with ticks, and there was no previous history of overseas travel identified. The boy's

mother was a veterinaryy surgeon and recalls attending the delivery of a stillborn goat kid six weeks prior to her son being hospitalised with Q fever. She vividly recalled returning home from the delivery with blood and products from the birth on her overalls and boots. The boy had greeted her by embracing and then sitting in her lap while she was still dressed in her work clothes. This indirect contact is considered the most likely the original source of his infection.

As part of the NQFMP for occupations at risk, the mother had been vaccinated, but her son and the rest of the family were not considered “at risk” and therefore not included in the vaccination program.

7.4.4. Case No 4: An Entire Family from Cunnamulla

The case study presented here was of particular interest, as it involved multiple family members. Family member 1, an eleven year old girl, presented with febrile illness which remained undiagnosed. Only on the diagnosis of acute Q fever in her sibling was a retrospective diagnosis of Q fever infection made in this girl. Subsequently, the infection was found to involve her other siblings, and parents. These cases are here presented and examined.

7.4.4.1. The First Case: An Eleven Year Old Girl – Family Member 1

An eleven year old female had been unwell for several days with symptoms including nausea, a cough which produced clear to white sputum and a tender abdomen. She had presented to her local GP on six previous occasions with no definitive diagnosis.

An eleven year old female had been unwell for several days with symptoms of nausea, a cough productive of clear to white sputum and a tender abdomen. She had presented to her local GP on six previous occasions with no definitive diagnosis.

Day 1: The girl was afebrile when presenting 3 days after her sixth GP visit at her local regional hospital. She was noted to have mild dehydration, a clear chest on examination and soft yet tender abdomen. She was admitted to hospital and treated with IV antibiotics, IV fluids and oral paracetamol. Urine was obtained for a M/C/S and bloods were drawn for laboratory investigations. During that afternoon and evening she became febrile with temperatures reaching 39.8^{0}C. She remained febrile for the next 24 hours and Q fever serology was added to the pathology requests.

Day 2: A FBC showed elevated lymphocytes and her urine was negative for pathogens. She remained febrile and was continued on IV antibiotics. Over the next twenty-four hours her fevers peaked at 40.0^{0}C and she had nausea with vomiting. A chest x-ray was clear. Treatment was changed to IV ceftriaxone while awaiting the results of Q fever serology. A further FBC showed an elevated WBC count with low platelets and RBC count.

Days 3-5: The patient continued to be febrile and continued to complain of nausea. Temperatures intermittently peaked at 38.0^{0}C. She remained on IV ceftriaxone and IV fluids and Q fever serology remained negative. She was discharged 6 days after admission, afebrile but with no definitive diagnosis being made.

7.4.4.2. Serology Testing

Retrospective Q fever serology testing was requested three weeks after hospitalisation of this girl. This decision was based on positive Q fever serology results obtained for her sibling who became ill subsequent to this case. The retrospective serology results showed seroconversion to Q fever antibodies, thereby confirming the diagnosis. The results are shown in Table 7.7.

Table 7.7: Case 4 – Summary of pathology results for family member 1

Date of Collection	Serology Test	Result
3 April 2002	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Negative
6 April 2002	CMV IgM	Negative
	EBV IgM	Negative
	BFV IgM	Negative
	Brucella IgG	Negative
24 April 002	Q Fever Phase II IgM (ELISA)	Reactive
	Q Fever Phase II IgG (ELISA)	Reactive
	Q Fever Phase I Total Ab	<8
	Q Fever Phase II Total Ab	256

7.4.4.3. Family Member 2: An Eight Year Old Male Sibling of Family Member 1

Family member 2 was an eight year old boy and brother of family member 1. He presented to the same hospital where his sister had been an inpatient for six days. He was afebrile with nausea and vomiting, but had a clear chest and soft abdomen on examination. He had a past medical history of upper respiratory tract infections.

He had blood taken for routine laboratory investigations. His FBC showed thrombocytopaenia and all cell types were also below normal limits. His LFT's were elevated and a urine sample submitted for M/C/S showed no growth for pathogens. The boy became febrile overnight with temperatures reaching 40°C. Treatment was commenced including IV gentamicin and IV fluids. The boy remained febrile for the next 48 hours, while his chest remained clear to auscultation.

Subsequent blood samples were taken and on the fifth day of hospitalisation serology showed a seroconversion for Q fever antibodies (Table 7.8). He remained febrile throughout with temperatures up to 38.4^{o}C. The boy's treatment was altered to include IV doxycycline. His 11 year old sibling previously admitted to the same hospital (Family member 1 above), still remained negative for Q fever serology.

Table 7.8: Case 4 – Summary of pathology results for family member 2.

Date of Collection	Serology Test	Result
7 April 2002	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Negative
	CMV IgM	Negative
	EBV IgM	Negative
	BFV IgM	Negative
	Brucella IgG	Negative
10 April 2002	Q Fever Phase II IgM (ELISA)	Reactive
	Q Fever Phase II IgG (ELISA)	Non-Reactive
	Q Fever Phase I Total Ab	<8
	Q Fever Phase II Total Ab	<8

7.4.4.4. Family Member 3: A Thirteen Year Old Female Sibling of Family Members 1 and 2

A female teenage sibling of the two children previously described became unwell 6 days after the onset of symptoms of the first family member. She presented with a similar febrile illness as her brother and sister with high fevers with no obvious focus. She was admitted to the local regional hospital for 5 days where she was treated with IV antibiotics. She remained febrile during the afternoons and evenings with temperatures reaching 39.3°C. She was generally unwell and complained of lethargy, but did not suffer nausea or vomiting. Because one of her siblings was diagnosed with Q fever (Family member 2) she was started on doxycycline as part of her clinical management. She continued to have fevers overnight with source unclear. Q fever serology was requested. Her chest was clear on examination. Treatment was changed to IV ceftriaxone as fevers were not subsiding. She became afebrile 24 hours later and was discharged. Her Q fever serology remained negative while she was an inpatient (Table 7.9).

Table 7.9: Case 4 – Summary of pathology results for family member 3

Date of Collection	Serology Test	Result
10 April 2002	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Negative
24 April 2002	Q Fever Phase II IgM (ELISA)	Reactive
	Q Fever Phase II IgG (ELISA)	Reactive
	Q Fever Phase I Total Ab	<8
	Q Fever Phase II Total Ab	>512

7.4.4.5. Family Member 4: The 31 Year Old Mother of the Three Siblings

The 31 year old mother of the children became unwell 14 days after her first child (Family member 1) presented with first symptoms. She presented to hospital with symptoms similar to her hospitalised children, including; fever, cough and a clear chest on auscultation. She however, complained of severe headaches. A FBC was requested showing a neutrocytopaenia. She too was admitted to hospital and continued to have fevers and headaches. She was treated with IV antibiotics and fluids with blood samples

drawn for Q fever serology (Table 7.10). She remained in hospital for a further 3 days and was discharged with oral medication and with convalescent Q fever serology requested.

Table 7.10: Case 4 – Summary of pathology results for family member 4

Date of Collection	Serology Test	Result
13 April 2002	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Negative
29 April 2002	Q Fever Phase II IgM (ELISA)	Reactive
	Q Fever Phase II IgG (ELISA)	Equivocal
	Q Fever Phase I Total Ab	<8
	Q Fever Phase II Total Ab	>512

7.4.4.6. Family Member 5: A Four Year Old Female Sibling of Hospitalised Children and Daughter to the Hospitalised Mother

This child had presented to the local GP with fever and nausea and milder symptoms similar to those displayed by her other siblings, including fever and nausea. She was not admitted to hospital but did have blood samples taken for Q fever serology. The results of serology testing for this child are shown in Table 7.11.

Table 7.11: Case 4 – Summary of pathology results for family member 5

Date of Collection	Serology Test	Result
29 April 2002	Q Fever Phase II IgM (ELISA)	Negative
	Q Fever Phase II IgG (ELISA)	Reactive
	Q Fever Phase I Total Ab	<8
	Q Fever Phase II Total Ab	64

7.4.4.7. Summary of Serological Results

The infections described in these patients were definitively diagnosed using Q fever serology. A consistent pattern of Q fever IgM positivity with the development of IgG and phase II total antibodies (CFT) was evident in all cases.

7.4.4.8. Review of Clinical Case History

Family member 6 was the father of the children and the husband of the 31year-old female (Figure 7.1). He recalled feeling unwell and having mild symptoms of fever and headaches following the kidding of goats on his property, 6 weeks prior to the display of symptoms in family member 1, his 11 year old daughter. He was not previously vaccinated against Q fever. It was presumed his mild symptoms were consistent with existing low level immunity as the result of constant exposure to *C.burnetii* in his working environment. It is likely that the infection in other family members was the consequence of either direct or indirect contact with infected material from the goats.

A timeline of symptoms and hospitalisation of family members 1-4 who were hospitalised is shown on Figure 7.2.

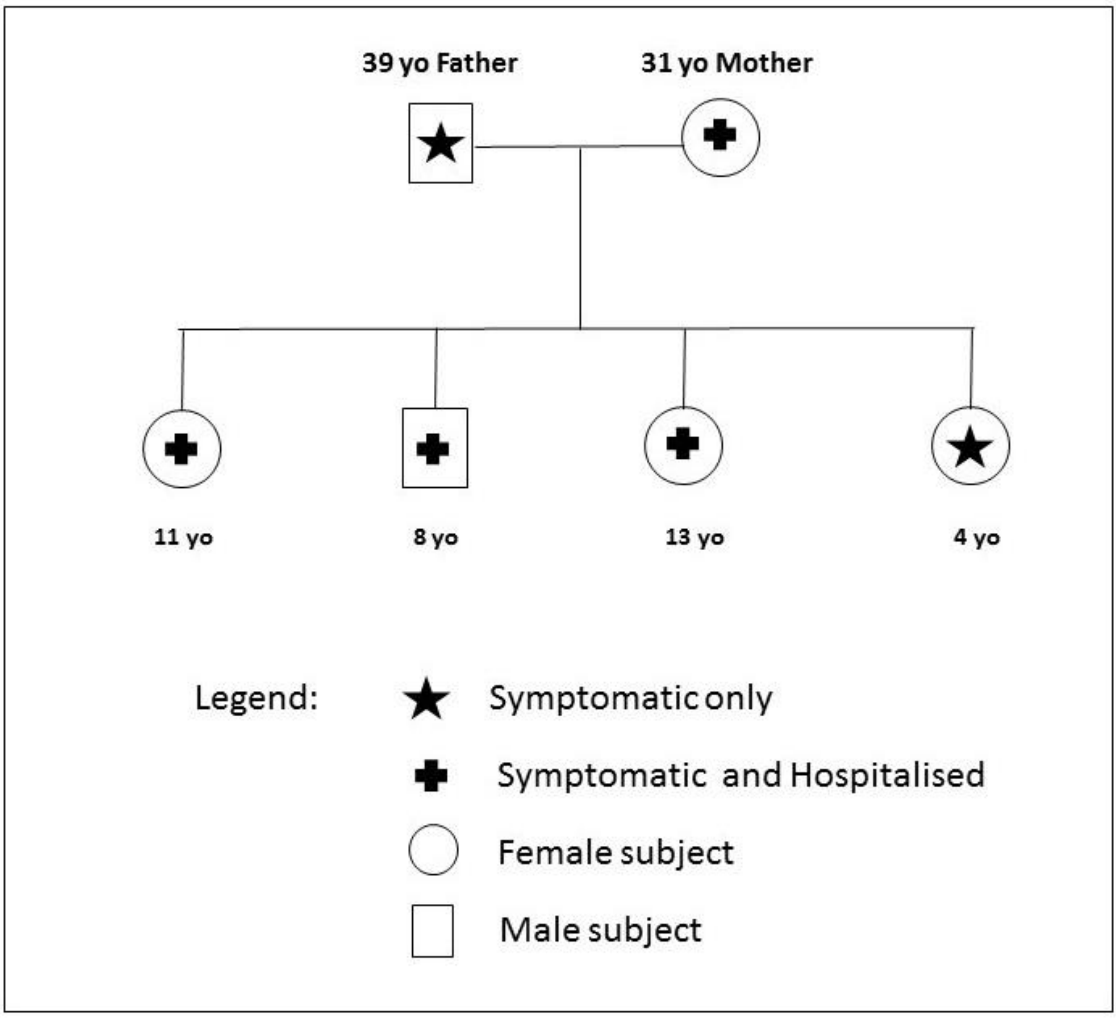

Figure 7.1: Case 4 - Family relationship of clinical cases

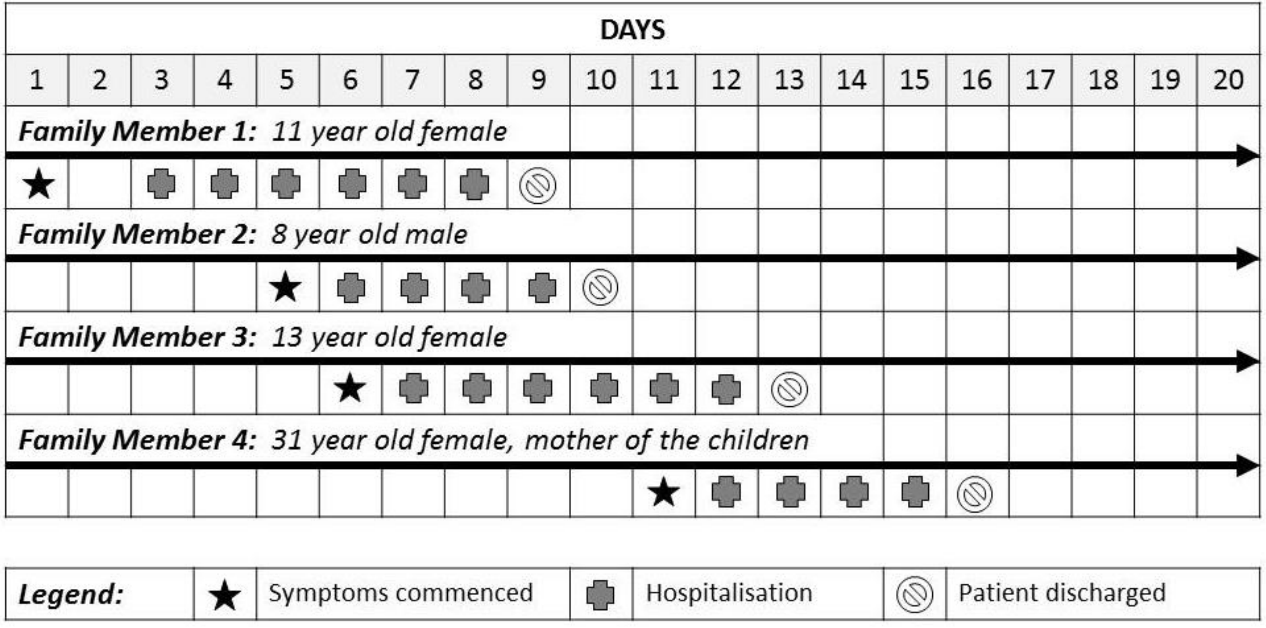

Figure 7.2: Case 4 – Timeline of clinical presentation and medical history for the four family members hospitalized (in days)

7.4.5. Case No 5: A 28 Year Old Female Park Ranger from Central Queensland

7.4.5.1. Clinical Presentation

A previous fit and well 28 year old female with no significant past medical history developed a mild influenza like illness associated with headaches. She presented to a local GP clinic in Rockhampton, Queensland, and was prescribed oral Tamiflu (oseltamivir) with amoxicillin/clavulanic acid. Over the next eight days, her condition worsened with the development of nausea, vomiting and generalised abdominal pain.

Day 1: She presented to the local regional hospital and was admitted. On presentation, she was febrile with a temperature of 37.8°C. She developed acute kidney failure, profound hyperbilirubinemia, mucosal bleeding without any other major haemorrhage being detected. She became hypotensive with haemodynamic instability requiring 8 litres of IV crystalloid fluids. She was ventilated for seven days. Blood samples were taken and showed an abnormal LFTs, thrombocytopenia and disseminated intravascular coagulation. She had negative blood culture results and was treated with IV ampicillin and gentamicin. She was stabilised and transferred to the tertiary intensive care unit at the RBWH.

Day 8: During the transfer she went into vasodilatory shock. On arrival at the RBWH there was evidence of mucosal bleeding but no petechial rash. She had unremarkable

respiratory and cardiac signs and there were no signs of meningitis or neurological disorders. The initial differential diagnosis included the possibility of scrub typhus infection due to the severity of her illness and extensive evidence of mite bites. This was also supported by the likely mite exposure during the performance of her occupation as a Park Ranger, as well as to native animal faeces which may have contained *Rickettsia spp.* A treatment regime with doxycycline was commenced. Her platelet count remained low and she underwent a blood transfusion. She rapidly developed progressive respiratory failure and was intubated and ventilated for seven days. Investigations of her blood including extensive serology testing, confirmed Q fever infection and treatment was altered to IV vancomycin and IV ciprofloxacin. Q fever serology results are shown in Table 7.12.

She remained in hospital for a total of six weeks, of which three weeks were spent in intensive care for the treatment of vasodilatory shock with fluid resuscitation. She was febrile throughout her hospitalisation with temperatures greater than 38.5^{0}C and she continually complained of headaches. She slowly recovered on the treatment regime with the normalization of LFTs and her thrombocytopenia.

7.4.5.2. Summary of Serological Results

The antibody profile for this patient showed increasing levels of phase II IgM and IgG as the disease progressed. On resolution of the infection phase I IgG became positive with maximum antibodies approximately 7 months after the first evidence of infection. IgA antibodies to phase I and II were not detected. Also, at the second sampling timepoint (23/4/2011) the IFA IgG result was positive, yet the ELISA IgG result for this sample was negative, demonstrating that the IFA was more sensitive than the ELISA as previously noted in Chapter 6 (6.3 Discussion)

7.4.5.3. Case History and Contact Tracing

Follow up investigation regarding potential sources of Q fever exposure revealed that, as part of her occupation as a wildlife park ranger, she would regularly remove kangaroo and wallaby carcasses that resulted from road kill. Her last contact with such animals had been 3 weeks prior to her onset of symptoms. Also, three of her work colleagues had been previously unwell with self-limiting influenza-like illnesses.

Table 7.12: Case 5 - *Coxiella burnetii* serology results.

Q Fever Tests	21/4/2011	23/4/2011	30/4/2011	1/6/2011	21/9/2011	17/11/2011
IFA Phase I IgG	<10	<10	10	80	≥1280	≥1280
IFA Phase I IgA	-	-	-	10	10	10
IFA Phase II IgG	<10	160	≥1280	≥1280	≥1280	≥1280
IFA Phase II IgM	40	320	≥1280	≥1280	80	160
IFA Phase II IgA	-	-	-	10	<10	<10
ELISA Phase II IgG	Negative	Negative	REACTIVE	REACTIVE	REACTIVE	REACTIVE
ELISA Phase II IgM	REACTIVE	REACTIVE	REACTIVE	REACTIVE	EQUIVOCAL	Negative
PCR blood	DETECTED*	-	-	-	-	-

Results are expressed as reciprocal titres, reactive, negative, or equivocal. Screening tests included ELISA assay for IgG and IgM antibodies to the phase II antigens of *C.burnetii*, and specific immunofluorescence (IFA) testing for Q fever phase I antibodies IgG and IgA and phase II antibodies IgG, IgM, and IgA. *PCR for *C.burnetii* was positive on two blood collections taken 13.7 hours apart

7.5. Discussion

Although Q fever disease was described over 60 years ago in Queensland, the presentation of this infection still appears to be poorly recognised, with many cases missed or misdiagnosed. This is more than likely because the initial symptoms of Q fever are non-specific and mimic many other infections. The cases presented in this Chapter emphasised the utility of supplemental Q fever serology testing and the importance of detailed patient histories when diagnosing Q fever infections. Moreover, in the majority of cases it was Q fever-specific antibody testing that confirmed a clinical diagnosis, because PCR is not widely used and is not available in regional Queensland laboratories.

As previously indicated, the population considered most at risk are rural farmers, abattoir workers, veterinary surgeons, and large ruminant handlers. As a result of this all these occupational groups were included in the National Vaccination program. However, the majority of clinical cases presented here were in children and an adult considered to be at low risk of infection. These patients were all resident in rural Queensland, contributing to the high rate of notifications previously reported from this sector.

Clearly the cases presented here demonstrate that Q fever can be a very serious disease in children and highlight the complexity associated with its clinical recognition and diagnosis. Often a diagnosis of Q fever in children is not considered and therefore the disease is under-recognised and under-reported as an unidentified cause of some febrile illness.

The children presented in these case studies resided in traditional high risk communities, but consideration of the high risk was only associated with adults directly handing large ruminants. With the rollout of the NQFMP the majority of at risk workers have been targeted and vaccinated, but the children have not been included in this program.

However, children in rural Queensland are at risk of acquiring Q fever, because they are often expected to assist on the farm or rural property on a regular basis with daily contact and exposure to animals and animal products. Yet there is no vaccination program available to them, nor have they been considered from exclusion from the properties during extremely high risk activity such as in times of calving, lambing or kidding. Children are commonly present at these activities and generally encouraged to help with the birthing, caring for and maintenance of the new born animals, including simple tasks such

as “mucking out” stalls or pens. It is these chores or activities that potentially expose such children to high loads of the bacteria and hence the acquisition of Q fever disease.

This risk was demonstrated in two of the paediatric cases presented above, highlighting the source of infection from indirect animal contact. In both cases, the children had no known direct dealings with any animals or their products prior to becoming unwell and it was only post-diagnosis that the potential exposure and risk scenarios were identified. These children were exposed indirectly from other family members, who must now be considered as an important source of infection, and identified the need for preventive strategies, including possibly vaccination, for children of parents working in “high risk” occupations.

The risk of infection to children from secondary sources, including their parents was clearly demonstrated by the case of the 4 year old boy from Nanango who was infected from animal products carried by his mother. It is this indirect animal exposure that may lead to the inhalation of *C.burnetii* from contaminated clothing, footwear or work tools thus placing those who come in close proximity with these articles at high risk of contracting Q fever. This case was particularly interesting as it highlights the progression of acute Q fever to a chronic infection in a child. The presentation of chronic Q fever in children is considered to be extremely rare[14], particularly as the presentation in children primarily occurs as osteomyelitis. In this chronic case, cardiac infection and involvement was considered the likely source but could not be confirmed, which is not uncommon in Q fever endocarditis.

Throughout the review of the cases above it was noted that Q fever was eventually considered in the majority of presentations, and the appropriate serology was requested. However, in these cases the initial serology and antibody results were negative or equivocal therefore a diagnosis of Q fever was dismissed without consideration for follow up serological testing. In two cases, the results of serology testing were confused by positive results for antibodies to other infectious agents. In Case 1 these were IgM positive antibody reactions to BFV and EBV which confounded the recognition of Q fever as the true cause of the disease. Cross reactivity of IgM antibodies is a well-recognised phenomenon in serological testing, and can be attributed to non-specific polyclonal stimulation during the disease process of B-lymphocytes which are primed for IgM antibody production. Such a cascade of IgM molecules to various antigens may lead to non-specific binding and false-positive reactions and thus an incorrect diagnosis.

Even when evidence of acute Q fever infection was indicated by antibody results in Case 2, it was discounted, because the clinical presentation was not in the expected context, and hence the results were considered as an IgM cross-reaction similar to that described above. Here the results of PCR testing for *C.burnetii* DNA were conclusive, and confirmed that during early stages of Q fever infection PCR is the testing method of choice (This was discussed previously in Chapter 6).

The case studies presented emphasise the need for physicians to consider the disease in context, and examine case histories in detail, including family history and circumstance. Also, the initial results of serology and antibody testing should be considered with caution and supplementary or retrospective serology may be recommended. Perhaps in children, the onset of symptoms is rapid and more intense than in adults, and the early serology results are not truly indicative of the disease. In these cases, Q fever PCR testing may be a more accurate tool for diagnosing the infection.

Interestingly, it was observed that the majority of patients described above had thrombocytopenia which was identified as part of their initial blood investigation in determining a cause of infection. Thrombocytopenia is a common manifestation associated with all tick-borne disease yet in none of the cases described in this Chapter was there any indication of tick involvement in the disease. The Centre for Disease Control (CDC) described that only 25% of Q fever cases present with a thrombocytopenia, but in all the cases above there was a noted decrease in platelets during the patient's initial infection. This may be a manifestation only observed in children, or perhaps in severe Q fever cases such as described in Case 5.

Finally, the risk of infection from indirect exposure to animal products cannot be underestimated, as recently demonstrated by the extensive and costly outbreak of Q fever in The Netherlands. Here the majority of the 4000 recorded cases were indirectly exposed to the bacteria shed from infected goats. In Australia, there is currently a growing industry in goat farming for their milk, and with this, an increasing potential risk for infection of the general population with *C.burnetii.*

Given the evidence presented in the cases above, demonstrating the risk factors associated with Q fever in children, and the growing goat farming industry, it may be prudent for Australian Public Health authorities to consider the wider distribution of the Q fever vaccine, QVax® (bioCSL, Australia), to include, not only those individuals in

occupations “at risk” but also their families and other individuals that have regular close contact. This should also include all staff working at veterinarian clinics where there has been a clear risk identified with the exposure to domestic pets, as well as people in occupations that expose them to native animals such as Park Rangers and government workers that are involved in activities in areas that may have been contaminated by wildlife secretions and tissues. Such a strategy would help in reducing the overall rate of Q fever infections in Australia, and result in considerable savings of the health care dollar.

7.6. Significant Outcomes from this Chapter

- This was the first study highlighting that indirect transmission of Q fever to children must be considered as a realistic source of infection.
- These cases were presented in unusual clinical settings not immediately associated with *Coxiella* infection, as a result the appropriate follow up Q fever testing was not performed.
- *Coxiella* is highly infectious and the increased risks associated with direct or indirect transmission within family groups in rural settings should be considered in the application of an effective vaccination strategy.
- Vaccination should also be considered for individuals in occupations not traditionally associated with risk of Q fever infection such as Park Rangers or those working with wildlife.

7.7. References

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Chapter 8

Molecular Typing of *Coxiella burnetii* in Queensland Samples

Articles published as a result of work done in this chapter:

Tozer SJ, Lambert SB, Vincent G, Nissen MD, Sloots TP. Unique Genotypes of *Coxiella burnetii* recovered from human and environmental samples in Queensland, Australia. BMC Infectious Diseases (Manuscript in Preparation).

8.1. Introduction

Q fever disease and the epidemiology associated with the infection are very complex. The worldwide distribution, reservoirs, routes of transmission and vectors are multifarious and make research and investigations difficult, especially for comparison studies. Research and diagnosis of the disease has improved with the introduction of molecular techniques such as PCR and genome sequencing, and these tools have opened the way forward for investigations into the biology of the bacteria and the disease states causes by the organism.

There are many factors that hinder comparative investigations involving *Coxiella burnetii*. Culturing the organism is hazardous and requires Biosafety Level 3 conditions, because the organism is considered a major biological warfare agent (Category B) by the Centre for Disease Control (CDC, Atlanta, USA). Therefore, comparative analysis using traditional methods have been limited, as sufficient bacteria for these techniques have often not been available, and clinical or environmental samples positive for *Coxiella* are the only samples used in such studies.

Also, culturing of *Coxiella* is difficult and requires enrichment media and animal derived cell culture systems which have long incubation times of up to 7 days. In addition, considering that the organism is highly infectious, culturing and purification of the organism carries considerable risk for laboratory scientists, and therefore has been restricted to laboratories with the appropriate facilities.

Modern techniques of whole genome sequencing allow the entire genetic code of the bacteria to be determined. This permits direct comparisons and evolutionary relationships to be established using bioinformatics.

The effectiveness and real-time monitoring of results made possible by molecular techniques, have allowed for rapid diagnosis of the disease states of *Coxiella*, and have provided an essential tool to terrorism investigation bodies for assessing potential biological warfare threats that employ *Coxiella burnetii*. These tools aid in identifying potential sources of the bacteria.

8.1.1. Taxonomy of *Coxiella burnetii* in the Molecular Era

The bacteria are obligate, intracellular organisms. They are gram negative, non-motile, pleomorphic cocobacillary bacteria, ranging in size from 0.2-0.4μm x 0.4-1μm.. It is these

phenotypic attributes that resulted in the bacteria being originally classified in the phylum of Proteobacteria, order Rickettsiales, categorized with the Rickettsiaceae family, in the genus *Rickettsia* species *burneti*[1]. Philip in 1943 renamed the organism to *Coxiella burnetii* after further cultural and biochemical investigations[1].

However, the introduction of molecular techniques such as PCR and the development of genome sequencing, has made enormous improvements in the correct classification of *Coxiella*. Sequencing of the 16s rRNA gene has allowed for the molecular differentiation between species, as these regions are highly conserved and are often used in phylogenetic analysis, because these genes have slow rates of evolution and mutation. The analysis of the *Coxiella* 16s rRNA showed more genetic diversity between *C.burnetii* and the other Rickettsias, and subsequently it was established that the *Coxiella* are most closely related to the genus *Legionella* and hence *Coxiellae* were reclassified into the order *Legionellales*, family *Coxiellaceae*[2].

C.burnetii is the only species that has been formally recognised in the genus *Coxiella.* There is >99% homology between all the strains sequenced using 16s rRNA comparing gene sharing[3]. A new *Coxiella*, *Coxiella cheraxi,* was isolated from Australian fresh water crayfish and although initially considered a new species, the rRNA, *sodB* and *com1* genes sequenced from this new organism showed a >95% homology with *C.burnetii*[4].

8.1.2. Genome of *Coxiella burnetii*

The first full *Coxiella* genome was sequenced in 2003[5]. This was the Nine Mile Phase I RSA493 isolate which was isolated from a tick host retrieved in Montana in 1935. It produced a 1,995 Kbp circular chromosome. There are currently six *C.burnetii* genomes that have been sequenced in entirety. Nine Mile RSA 493[5] (NC_002971.3), two sequences from human endocarditis patients CbuG_Q212 (NC_011527.1) and CbuK_Q154 (NC_011528.1)[6], a strain isolated from a rodent, the Dugway 5J108-111[6] (NC_009727.1), strain RSA 331 (Henzerling strain) (NC_010117.1), isolated from a patient in Italy in 1945 and recently a strain sequenced from The Netherlands *Coxiella burnetii* Z3055 (NZ_LK937696.1) linked to the large Q fever outbreak in 2007. These sequences show a variation in the *Coxiella* bacterial genome between ~2.0 to 2.2 Mb in length, and display considerable genetic homology between each other when examined with 16s rRNA sequencing[2].

The *Coxiella* genome is comprised of circular chromosomes generally associated with one independent plasmid or the genome contains integrated sequences homologous with plasmids. There have been four plasmid types labelled QpH1[7], QpRS[8], QpDG[9], QpDV[10] along with one plasmid from a Chinese isolate without labelling[11]. Initial studies suggested that there was a correlation between plasmid type and disease state, however, this has since been disproved as plasmid QpH1 was detected in both acute and chronic patients[3]. A recent study suggested that the proteins encoded by the plasmids have an essential role in the modification of the host cell during infection[12].

8.1.3. Typing of *Coxiella* Isolates

C. burnetii is highly infectious, and the variety of potential sources for natural infection from proximity of livestock, consumption of their products, exposure to dust and other sources of infection identified in Chapter 5, make it of paramount importance to be able to investigate the source of any outbreak. The discrimination between different strain types of *C.burnetii* was initially very difficult as the conventional serological methods were unsuccessful[13]. An attempt to use the difference in the lipopolysaccharide banding patterns produced was also unsuccessful in differentiating *Coxiella* isolates[14] and the typing of plasmids gave limited discrimination[8]. Typing of strain isolates was hence based on their description of source, geographical origin and clinical manifestation. However, the great diversity associated with *Coxiella* and the disease states initiated, a broad host range and widespread distribution only increased the importance of strain differentiation for epidemiology studies and clinical diagnostics.

8.1.4. Genotyping of *Coxiella burnetii*

The advancement in molecular technology and sequencing has led to the molecular characterisation of complete bacterial genomes. Whole genome sequencing is the ultimate method for performing direct comparisons between strains. However, even with vastly reduced cost of sequencing technology, it is still not practical, nor financially viable for many laboratories to perform such analyses. This has influenced the development of other typing methods for comparisons between *Coxiella* strains. The earlier typing methods that were used to discriminate strains were created using the restriction fragment length polymorphisms (RFLP)[9,15–17].However, these methods required growth of the organism which is hazardous and lacked inter laboratory reproducibility. Discrimination of *Coxiella* strains based on sequencing the *com1*, *mucZ and isocitrate dehydrogenase*

genes has also been used with limited results[18–21]. The introduction of real-time PCR technology and the availability of whole genomic sequences on public databases has seen a new emergence of genotyping methods. From 2005, highly discriminative assays were developed including multi-space sequence typing (MST), multilocus variable number tandem repeat analysis (MLVA) and single nucleotide polymorphism (SNP).

8.1.4.1. MST- Multispace Sequence Typing

The MST method analyses the intergenic regions of coding genes. These non-coding regions (= "spacer") are used for typing because they are not constrained by selective pressure to encode functional proteins, as are coding genes. This method was first applied in 2005 and was able to separate 173 isolates of *Coxiella* into 30 sequence types. This method showed correlation with both geographical and plasmid clusters that had been previously described[22] and allowed for direct inter laboratory comparisons owing to the resolution power delivered by a sequencing technique. Results were analysed through the use of internet-based data-links that allow comparisons with other strains lodged on a central database (http://ifr48.timone.univ-mrs.fr).

8.1.4.2. Multilocus Variable Number Tandem Repeat (VNTR) Analysis (MLVA)

The use of software to identify naturally occurring tandem repeats enabled the identification of small repeat units recurring at loci throughout the genome, and this can vary between different isolates. The variation between repeat copy numbers is largely due to insertions and deletions that are the result of DNA replication mutations along with recombination mutation events[23]. This technique has been widely used for typing other organisms and has become the reference typing method. Bacteria such as *Neisseria meningitidis*, *Bordetella pertussis, Staphylococcus aureus, Streptococcus pneumonia, Haemophilus influenza, Mycobacterium tuberculosis* and *Yersinia pestis* are differentiated using MLVA.

Genotyping by multilocus VNTR analysis (MLVA) involves the amplification of several variable number tandem repeats (VNTR) loci by PCR. The specific amplicons are sized and converted to a repeat copy number. Profiles are produced comprised of copy number alleles from each locus; these in turn are compared to different strains. For the typing of *Coxiella burnetii* isolates, two MLVA typing schemes have been developed, both in 2006 by Svraka et al[24] and Arricau-Bouvery et al[25] . The method developed by Arricau-Bouvery et al incorporates 17 VNTR loci that could be analysed in 2 separate panels. The first panel of 10 loci was more adaptable for analysis using conventional gel

electrophoresis detection methods as repeat sequences were 9-126 bp in length. The second panel, containing 7 loci, had much smaller repeat units of 6-7 bp in length and were targeted for use using more sophisticated automated, sizing methods such as capillary electrophoresis[25]. The selected loci are deemed to be relatively stable supported by the fact that isolates of the reference strain Nine Mile (RSA493) from different laboratories with widely varied cell culture passages showed no differences[24]. In addition, like the MST-method, a web based data repository has been established facilitating comparative analysis with known strain repeat patterns (http://minis atellites.u-psud.fr/).

8.1.4.3. SNP Single Nucleotide Polymorphism Typing

SNP typing methods have been developed based on mismatches previously identified by MST genotyping. These assays are designed to be rapid and inexpensive using real-time PCR systems. One analysis designed 14 SNP assays to genotype over 40 *Coxiella* isolates which could be divided into 8 distinct genotypes[26]. This method showed good correlation relating to geographic origin, infection characteristics, and plasmid types of isolates.

8.1.4.4. Determination of Most Discriminative Typing Method for Coxiella

The method of choice for genotyping is dependent on the power of discrimination. A numerical value can be assigned between various methods using a modified Simpson's index formula described by Hunter and Gaston[27]. This publication describes an index of discrimination for various typing methods centred on the possibility of two separate strains being characterized as the same genotype. The index is used to compare the various typing methods for *Coxiella* and the method with the highest index is used as the greatest discriminatory method (Table 8.1)[28].

From this index the two current methods with the greatest discriminative power are the MLVA and MST typing methods for *Coxiella.* The MST typing method has been used to perform the most comprehensive genotyping study with 173 isolates from various geographical locations worldwide[22]. However, this study contained isolates mainly from Europe and the discrimination based on geography produced some interesting clustering with all the Canadian strains having the same genotype, with France having 30 different genotypes and yet one of the specific genotypes was isolated on 4 different continents.

There was however, no data from Australian isolates or Q fever patients included in the genotyping analysis[22].

Table 8.1: The Hunter-Gaston Discrimination Indices for five *Coxiella* typing methods available. (The higher the index the greater the discriminative power).

METHOD	AUTHOR	DISCRIMINATION INDEX
MLVA (Panels 1 and 2)	Arricau-Bouvery[25]	0.99
MST	Glazunova[22]	0.92
RFLP	Hendrix[9]	0.87
SNP analysis	Huijsmans[29]	0.85
***com1* sequencing**	Sekeyova[20]	0.54

8.2. Specific Aims Addressed in this Chapter:

Very limited genotypic data was available for *C.burnetii* detected in Queensland. Following the detection of the bacteria in various clinical samples as described in Chapter 5, this study sought to address this knowledge gap, and used MLVA typing to characterise *C.burnetii* in these samples. Specifically the study sought to:

- Determine if different strains of *Coxiella* circulate in Queensland
- Determine if the strains that are present in Queensland are similar to those identified in other parts of Australia, and the world
- Examine if genetically similar strains circulate in the environment as those identified in human infections
- Investigate if there is an association between geographical location and genotype

8.3. Methods

8.3.1. Samples Used for Strain Typing

There were a total of 63 samples that were included as part of the MLVA study. There were 51 samples from *Coxiella*-positive human subjects that were examined by MLVA analysis to determine strain variation of the bacteria. These samples were acquired from the Queensland Public Health laboratory, and comprised samples from 33 males, 16

females and in two the gender was not recorded. The subjects were aged between 5-77 years of age, with an average age of 43.5 years. The sample types included blood, bone, tissue, heart valve and placenta. These samples were collected between 2006 and 2013 from patients residing in Queensland and previously diagnosed with Q fever. These samples were all confirmed *Coxiella* positive using PCR.

There were an additional 12 environmental samples analysed for *C.burnetii* strain variation using MLVA strain typing. These included urine samples from 8 animals: canines, felines and flying foxes. There were 5 dust samples that were identified as containing *Coxiella* DNA which were included in the MLVA typing study and were part of the environmental samples investigated. All these environmental samples were obtained from Queensland locations and were previously identified as PCR positive as described in Chapter 5 as part of the identification of potential sources of infection to populations in Queensland. Demographic data were available for all of these samples.

8.3.2. MLVA Typing Method

The MLVA strain typing was performed using the published methods by Klasson et al[30] which had been modified from a previously published method by Arricau-Bouvery et al (2006)[25]. These methods had been adapted to the MLVA6-Nijmegen method which is widely used, and takes advantage of the six highly variable loci ms23, ms24, ms27, ms28, ms33, ms34. These loci were found to be the most valuable in the outbreak context in The Netherlands and correspond to VNTR panel 2 proposed by Arricau-Bouvery et al[25] with the exception of target ms31.

8.3.2.1 MLVA Typing Using MLVA6 and profiling via Capillary Sequencing Detection

Originally the Queensland samples were screened for the MLVA6 targets including ms23, ms24, ms27, ms28, ms33 and ms34 as previously described [25,30,31]. Table 8.2 shows the 6 loci and the expected amplicon size and number of repeats detected when Nine Mile is used as a control. The clinical samples were processed by conventional PCR using the primers shown in Table 8.3 and products were detected on a 1.5% agarose gel. Figure 8.1 shows the PCR products obtained for human and animal samples screened. These samples were sequenced and the data investigated for repeat units.

Table 8.2: The 6 loci used in the MLVA analysis of Queensland *C.burnetii*-positive samples and the amplicon size and numbers of repeats as observed in Nine Mile strain[25,30,31].

Locus	Amplicon Size (bp)	Forward Primer 5`-3`	Reverse Primer 5`-3`	No. of Repeat Units
ms23	133	CGCMTAGCGACACAACCAC	GACGGGCTAAATTACACCTGCT	9
ms24	26	TGGAGGGACTCCGATTAAAA	GCCACACAACTCTGTTTTCAG	27
ms27	89	TCTTTATTTCAGGCCGGAGT	GAACGACTCATTGAACACACG	4
ms28	111	AGCAAAGAAATGTGAGGATCG	GCCAAAGGGATATTTTTGTCCTTC	6
ms33	104	TCGCGTAGCGACACAACC	GTAGCCCGTATGACGCGAAC	9
ms34	101	TTCTTCGGTGAGTTGCTGT	GCAATGACTATCAGCGACTCGAA	5

Samples were processed by conventional PCR, gel detection and Sanger sequencing as described in 8.2.3.1 above. The final analysis however, was limited to the three loci shown in red as these were the loci that produced better sequencing results.

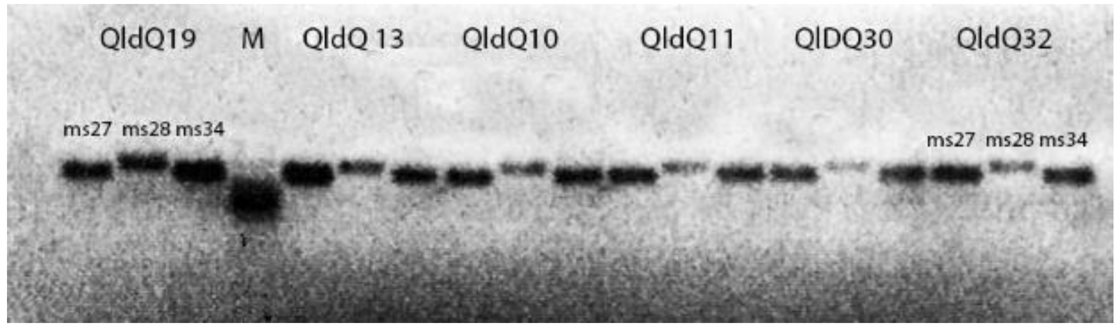

Figure 8.1: Agarose gel showing PCR amplification products obtained for MLVA6 Sanger loci ms27, ms28 and ms34. M= DNA marker

The positive DNA products were "cleaned up" to remove remaining contaminants from the PCR reaction and prepared for Sanger sequencing by AGRF (Australian Genome Research Facility- Brisbane, Australia). Sequence data was generated however, it proved to be difficult to obtain good and consistent sequence data for all 6 loci using this method on the Queensland samples. The analysis was then limited to the 3 loci for which adequate sequence information was available for all samples. These were ms27, ms28 and ms34.

LOCUS	LOCUS NAME	REPEAT UNIT SIZE (BP)	FORWARD PRIMER (5'-3')	REVERSE PRIMER (5'-3')
PANEL 1	ms01	16	GGCTCATTCAATTTTAGCTTCG	AACGTGGGGAAGTTTGTTATTT
	ms03	12	TTGTCGATAAATCGGGAAACTT	TTGTCGATAAATCGGGAAACTT
	ms07	126	CTCTTAGCCATCGCTTACCACT	AACGAAAATTGGTTTGCATTTT
	ms12	126	GAAAATTGGTTTGCGCTCTG	CCTTCTCCCAAGAAGTTTAGCC
	ms20	18	CTGAAACCAGTCTTCCCTCAAC	CTTTATCTTGGCCTCGCCCTTC
	ms21	12	AGCATCTGCCTTCTCAAGTTTC	TGGGAGGTAGAAGAAAAGATGG
	ms22	11	GGGGTTTGAACATAGCAATACC	CAATATCTCTTTCTCCCGCATT
	ms26	9	AGAATCAAACCTGCAAAACCTT	TTGATTATTTTGACTTCGCTGGT
	ms30	18	ATTTCCTCGACATCAACGTCTT	AGTCGATTTGGAAACGGATAAA
	ms36	9	GAAACCAGTCTTCCCTCAACAG	ATAACCGTCATCGTCACCTTCT
PANEL 2	ms23	7	GGACAAAAATCAATAGCCCGTA	GAAAACAGAGTTGTGTGGCTTC
	ms24	7	ATGAGAAAGGATGGAGGGACT	GATAGCCTGGACAGAGGACAGT
	ms27	6	TTTTGAGTAAAGGCAACCCAAT	CAAACGTCGCACTAACTCTCAG
	ms28	6	TAGCAAAGAAATGTGAGGATCG	ATTGAGCGAGAGAATCCGAATA
	ms31	7	GGGCATCTAATCGAGATAATGG	TTTGAGAAAATTTTGGGTGCTT
	ms33	7	TAGGCAGAGGACAGAGGACAGT	ATGGATTAGCCAGCGATAAAA
	ms34	6	TGACTATCAGCGACTCGAAGAA	TCGGTGCGTTAGTGTGCTTATCT
	GV_ms23	7	AATGCTGATAAAAAGGCCAAT	TGAGGTAGGAGCTGAATCTTTGA
	NL_ms23	7	CGCMTAGCGACACAACCAC[a]	GACGGGCTAAATTACACCTGCT
	NL_ms24	7	TGGAGGGACTCCGATTAAAA	GCCACACAACTCTGTTTTCAG

Table 8.3: Primers used in the PCR of recognised VNTR repeat regions for *Coxiella* from Arricau Bouvery et al[25] first description. This table of primers was taken from Vincent et al[28]. These primers were used in the fragment analysis of the Queensland samples performed in . *Primer named with the prefix NL_ are modified primers described by Tilburg et al[32] specifically for use on clinical samples not isolates. The primers highlighted in red were used specifically for the analysis of Queensland samples*

8.3.2.2 MLVA Typing Using 3 Loci and Capillary Electrophoresis Detection

A limitation of the above method was the inability to obtain usable MLVA profiles for some of the samples with low bacterial loads. Capillary electrophoresis detection using fluorescently labelled amplicons has been used successfully to overcome this limitation. Although the use of additional loci may add value when investigating a wider genetic diversity, the use of 3 loci only was deemed to be sufficient for easy and rapid classification of *C. burnetii* into 4 main clusters[33]. This was addressed in this study, by using a set of 3 markers as had been used successfully in the outbreak in The Netherlands to analyse clinical samples[30]. These loci produce very small amplicon targets which are more suitable for analysis by capillary electrophoresis.

The investigation by Vincent[28] into the discriminatory power of each loci using the Hunter-Gaston discrimination index (HGDI) showed that a single loci, ms24 was able to produce a HGDI of 0.83 (0 being no discrimination and 1 having complete discrimination) compared to a HGDI of the total 16 loci of 0.89. There were three loci that gave good discriminative power when used in combination; these were ms24, ms28 and ms33. This considered, further analysis of Queensland samples by MLVA used these loci (ms24, ms28 and ms33) followed by capillary electrophoresis.

Another key to using the capillary electrophoresis method was to ensure the use of controls/standards for all of the loci under investigation. This ensured that results obtained were standardised and consistent across runs of analysis. It is important to ensure that sample sizing is relative to the controls rather than being an absolute value. Therefore, MLVA analysis in this study used a subset of targets from Panel 2 (Table 8.4), and the more sensitive detection method of capillary electrophoresis of fluorescently labelled amplification products for the designated loci. These loci have repeat units much smaller than those in panel 1; repeat units are only 6-7bp in length.

The PCR primers for the loci ms24, ms28 and ms33 were synthesised by Invitrogen (Life Technologies, Victoria, Australia). In order for amplicons to be detected by capillary separation on the 3130 xl Genetic Analyser (Life Technologies, Victoria, Australia), it was necessary for the primers to be fluorescently labelled. In these reactions the forward primer was labelled with the Fluorophore 6-carboxyfluorescein (FAM).

Table 8.4: Specific VNTR loci from *C.burnetii* Nine Mile with the expected amplicon size and the number of repeats determined by Arricau-Bouvery et al[25] for the direct comparison to Queensland samples.

Locus	Amplicon Size (bp)	No. of Repeats
ms01	198	4
ms03	229	7
ms07	1112	8
ms12	1074	8
ms20	402	15
ms21	210	6
ms22	246	6
ms26	127	4
ms30	306	6
ms36	477	4
ms23	157	8
ms24	344	27
ms27	276	4
ms28	276	6
ms31	285	5
ms33	262	9

Reactions were set up comprising 1x PCR Buffer, 1.5 mM of magnesium chloride, 200μM of dNTPs, 1U of recombinant Taq DNA polymerase (Invitrogen, Life Technologies, Victoria, Australia), 400nM each of primer, 15μL, 1μL of DNA and nuclease free water to a total reaction volume of 25μl. Amplification was performed in a Maxygene ThermW1000 thermal cycler (VWR International, Queensland, Australia) or a 7500 Fast Real Time PCR System (Life Technologies, Victoria, Australia) and consisted of a 10 minute hold at 94°C followed by 40 cycles of denaturation at 94°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 1 minute. Amplification was completed by a final extension of 7 minutes at 72°C. To check that amplification had been successful, 5uL of each reaction was run on a 1.8% agarose gel (Figure 8.2).

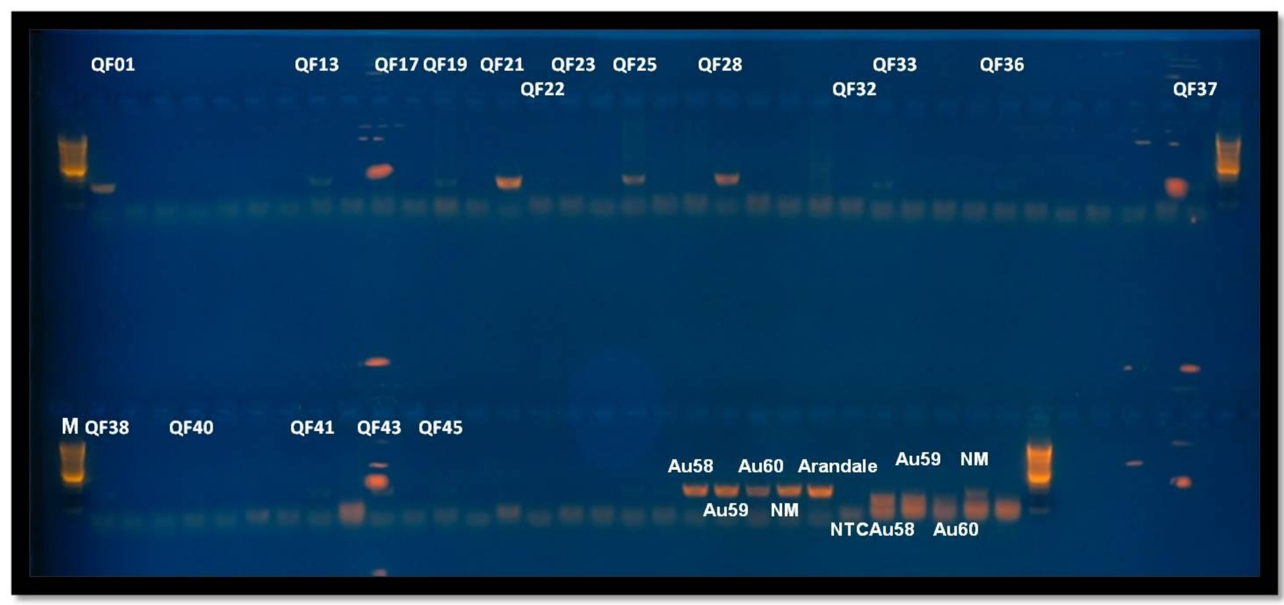

Figure 8.2: Confirmation of PCR product as visualised by gel electrophoresis of the Queensland samples for the loci ms28 along with control samples and the Nine Mile strain

Where a product was visualised following gel electrophoresis, 10ul of the remaining reaction was sent to AGRF (Australian Genome Research Facility- Brisbane, Austalia) for capillary separation using the Applied Biosystems 3730 xl analyser (Life Technologies, Victoria, Australia).The amplicon size was determined by analysing the subsequent data using Peak Scanner v 1.0 (Applied Biosystems). Samples where no products were visualised were also submitted to AGRF for capillary separation. This was because capillary electrophoresis has greater sensitivity and was able to detect amplification products which could not be visualised on the gel.

The number of amplicon repeats present in amplification products of each loci were calculated in relation to the results obtained for the *C. burnetii* Nine Mile control strain (Table 8.5). These MLVA profiles were compared to strains entered on the online database http://mlva.uUpsud.fr/mlvav4/genotyping/index.php, along with strains previously identified from Australia (accessed June 2015). Profiles that showed no correlation with those entered on the database and hence deemed to be novel, were assigned an annotation in the format of: CbQldxx, where xx was a two digit number. Any new MLVA genotypes were allocated genotype names as previously described by Vincent[28] and followed on from the latest Australian genotype discovered.

8.4. Results

8.4.1. MLVA Typing Using MLVA3 Sanger and Loci ms27, ms28 and ms34

Of the 63 samples examined, there were 11 samples for which MLVA genotyping data was obtained using the MLVA3 method with 3 loci. These included 10 samples from humans and one canine sample. When these samples were compared to profiles produced for the Australian isolates previously described by Vincent[28] and those samples lodged on the MLVA databases, there were four novel MLVA genotypes identified from Queensland, and seven samples that had profiles similar to those identified in other Australian isolates (Table 8.5).

Table 8.5: The Queensland samples screened using three discriminative loci and the related MLVA genotypes obtained using the methods described by Arricau-Bouvery et al[25] and Klaasen et al[30]**.**

Sample	Host	Origin	ms27	ms28	ms34	MLVA Genotype	Location/Postcode
Nine Mile	tick	USA	4	6	5		
NOVEL GENOTYPES IDENTIFIED							
QldQ01	**human**	**Queensland**	**3**	**5**	**4**	**Novel CbAu15**	**Blackstone, 4304**
QldQ11	**human**	**Queensland**	2	6	2	**Novel CbAu18**	**Chermside, 4032**
QldQ30	**human**	**Queensland**	**3**	**5**	**1**	**Novel CbAu19**	**Ingham, 4850**
QldQ32	**human**	**Queensland**	**4**	**5**	**3**	**Novel CbAu17**	**Beachmere, 4510**
EXISTING GENOTYPES DETECTED							
QldQ16	human	Queensland	3	5	2	10 CbAu matches*	Townsville, 4814
QldQ02	human	Queensland	3	5	-	11 CbAu matches*	Lowood, 4311
QldQ08	human	Queensland	3	5	-	11 CbAu matches	Marsden, 4132
QldQ13	human	Queensland	3	5	2	10 CbAu matches	Cluden, 4811
QldQ19	human	Queensland	3	5	2	10 CbAu matches	Proserpine, 4800
QldQ25	human	Queensland	3	5	2	10 CbAu matches	Ravenshoe, 4886
Qld305	canine	Queensland	3	5	2	10 CbAu matches	NA
Number of Different Queensland Alleles Detected			3	2	4		

NA = Not available. * Matches 10 or 11 genotypes previously identified[28].

The use of sequence data in alignments was used to determine the number of alleles detected at each of the loci (Figure 8.3). The phylogenetic relationship between the samples genotyped with the MLVA3 Sanger method for the set of 3 loci (ms27, ms28, ms34), using only the alleles as for discrimination show the four novel genotypes (samples QldQ01, QldQ11, QldQ30, and QldQ32) to be similar to the other Queensland genotypes

detected with the exception of sample QldQ32 which had a MLVA genotype more closely related to that of the Dugway rodent strain and the Nine Mile strain.

There were two samples QldQ01 and QldQ32 that were identified by both the first set of genotyping loci and the second set of loci as novel MLVA genotypes. These samples were unique from any other samples or isolates in the MLVA database and were named CbAu15 and CbAu17. The other two novel genotypes detected in samples QldQ11 and QldQ30 were named CbAu18 and CbAu19 using the nomenclature used by Vincent for previously described Australian genotypes[28]. The locations of residence for the patients with these unique genotypes are highlighted in red on the map of Queensland (Figure 8.4).

Interestingly there were three Queensland samples QldQ08, QldQ02 and the novel genotype from sample QldQ30, that had similarities to the D19 human isolate previously identified from Queensland (Figure 8.3)

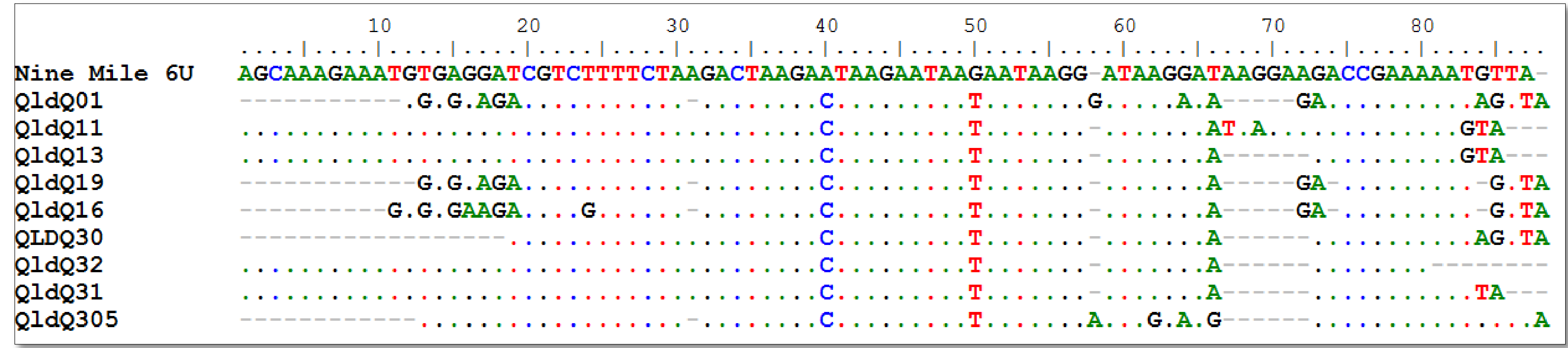

Figure 8.3: The sequence data alignment for some Queensland samples genotyped using MLVA typing at loci ms28 directly comparing the Queensland samples with the reference strain of Nine Mile. This loci shows the Nine Mile strain contains a 7 bp repeat sequence that is detected as 6 units. There was one of the Queensland samples that also has the 7bp 6 unit repeat QldQ11. All the other Queensland samples sequenced had only a 5 alleles at this loci.

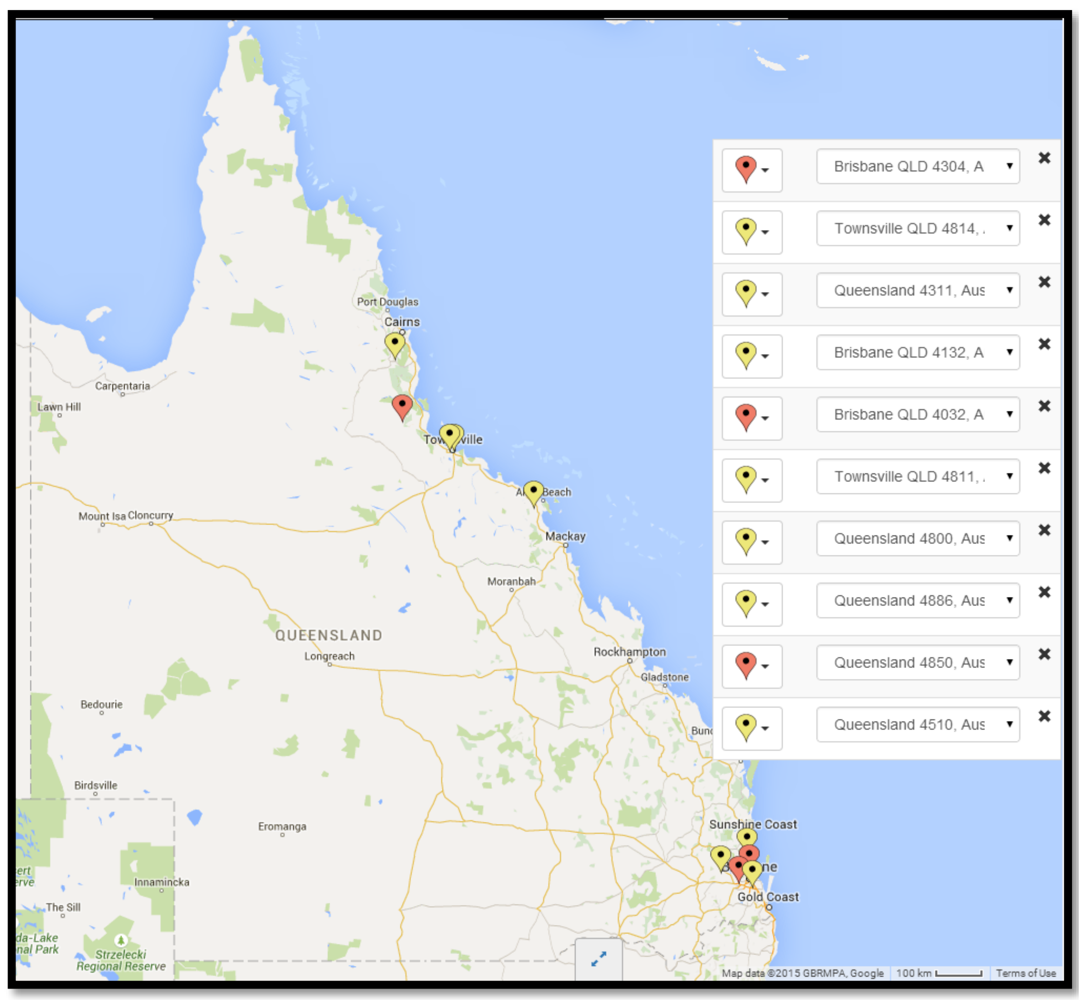

Figure 8.4: The geographical distribution of the Queensland samples genotyped using the three loci ms27, ms28 and ms34.

= Novel genotypes identified in Queensland *= Other genotypes that have previously been described*

8.4.2. MLVA typing using 3 loci and sizing by capillary electrophoresis

Of the 63 original samples, 52 were available for further examination with the 3 loci capillary electrophoresis method. MLVA data was generated for 19 of the 52 Queensland samples using the loci ms24, ms28 and ms33. The raw data for the Queensland samples following capillary electrophoresis identification is shown in Appendix 8.7 at the end of this Chapter, with the amplicon size and the number of repeat units ascertained at each of the specific loci.

There were three samples that produced complete profiles by capillary sequencing, and 16 samples with partial profiles for the three loci screened. These were compared to the 42

Australian isolates previously genotyped[28], and also compared to over 340 global isolates that had been genotyped and uploaded to a public database for reference. (http://mlva.u-psud.fr/mlvav4/genotyping/index.php.) The results of this comparison are shown in Table 8.6.

From the Queensland samples screened, there were 6 different alleles identified for the ms24 loci, a single allele from the ms28 locus and 3 different alleles from the ms33 loci. The three novel genotypes identified by this analysis from Queensland samples have been compared to known Australian genotypes, with 8 of the 14 Australian genotypes sharing similar alleles at the loci ms24, ms28, ms33 (Table 8.6). Of these three, two were also identified by the MLVA6 Sanger method (QldQ01 and QldQ32), and one was newly identified by the capillary method (QldQ28).

A phylogenetic tree with the strains from Queensland and Australia and along with strains commonly referenced such as Nine Mile, Henzerling and Dugway and the inclusion of strains that were obtained from similar sources and sample types were compared. The Queensland samples that had more than one loci with an identified allele for discrimination were displayed in a dendogram. There were 5 Queensland samples collected from humans that showed profiles that were closely related to the MLVA genotypes previously identified from Australian isolates. These were sample QldQ13 related to CbAU01 or CbAU013, sample QldQ21 typed to Australian genotype CbAU05, sample QldQ22 related to CbAU02 and CbAU05, and sample QldQ23 related to CbAU08 and CbAU010 (Figure 8.5). There were three Queensland samples, QldQ01, QldQ28 and QldQ32 collected from human patients that produced novel profiles, and were uniquely different from the Australian isolates previously genotyped. Two of these samples showed closer genetic phylogeny with isolates taken from humans and sheep from both The Netherlands and France. The geographical location of the genotypes identified by the 3 loci capillary electrophoresis method are shown in Figures 8.6 and 8.7.

Table 8.6: Queensland samples with positive alleles at loci ms24, ms28, and ms33.

Sample	Host	Origin	ms24	ms28	ms33	Genotype	Location
Nine Mile	tick	USA	27	6	9		
Henzerling			7	3	4		
CbuG	human	Nova Scotia	8	4	7		
Dugway	rodent	USA	5	4	8		
CbuK	human	USA	9	5	7		
Q177			9	5	7		
Frankrijk070	human	Netherlands	12	5	7		
CWZ155	cattle		12	5	9		
Frankrijk033	sheep	Netherlands	13	5	8		
Frankrijk080	human	Netherlands	13	5	8		
Cb#061	goat		12	5			
Cb#017	sheep	France	10	5	9		
Cb#013	goat	France	10	5	8		
AuQ01		Australia	16	5	5	CbAU01	
AuQ30		Australia	19			CbAU14	
AuQ05		Australia	14			CbAU05	
AuQ29		Australia			6	CbAU13	
AUQ13		Australia	17	5	5	CbAU06	
AUQ10		Australia	17	5	5	CbAU06	
AUQ05		Australia	14	5	4	CbAU05	
AUQ02		Australia	14	5	5	CbAU02	
AUQ08		Australia	15	5	5	CbAU02	
QldQ01	**human**	**Queensland**		**5**	**10**	**Novel CbAu15**	**Blackstone, 4304**
QldQ07	human	Queensland	15			CNBD	Nobby,4360
QldQ13	human	Queensland	16	5		CbAU01/ CbAU013	Cluden,4811
QldQ19	human	Queensland	12	5		3 matches*	Dingo Beach,4800
QldQ20	human	Queensland	16			CNBD	New Farm,4005
QldQ21	human	Queensland	14	5	4	CbAU05	Redcliffe,4020
QldQ22	human	Queensland	14	5		CbAU02/ CbAU05	Black Duck Creek,4343
QldQ23	human	Queensland	15	5		CbAU08/ CbAU010	Pimpama,4209
QldQ25	human	Queensland		5		CNBD	Malandra,4886
QldQ28	**human**	**Queensland**	**10**	**5**	**13**	**Novel CbAu16**	**Aurukun,4871**
QldQ32	**human**	**Queensland**	**13**	**5**	**4**	**Novel CbAu17**	**Beachmere,4510**
QldQ33	human	Queensland		5		CNBD	Ipswich,4305
QldQ40	feline	Queensland		5		CNBD	Numinbah 4211
QldQ44	feline	Queensland		5		CNBD	Daisy Hill,4127
QldQ76	dust	Queensland		5		CNBD	Fitzgibbon,4018
QldQ36	human	Queensland		5		CNBD	NA
QldQ37	human	Queensland		5		CNBD	NA
QldQ38	human	Queensland		5		CNBD	NA
QldQ39	human	Queensland		5		CNBD	NA
Number of Different Queensland Alleles			6	1	3		

*AUQXX are Australian isolates previously described by other studies[28]; CNBD = could not be determined. *Three possible genotype matches were identified from the international database*

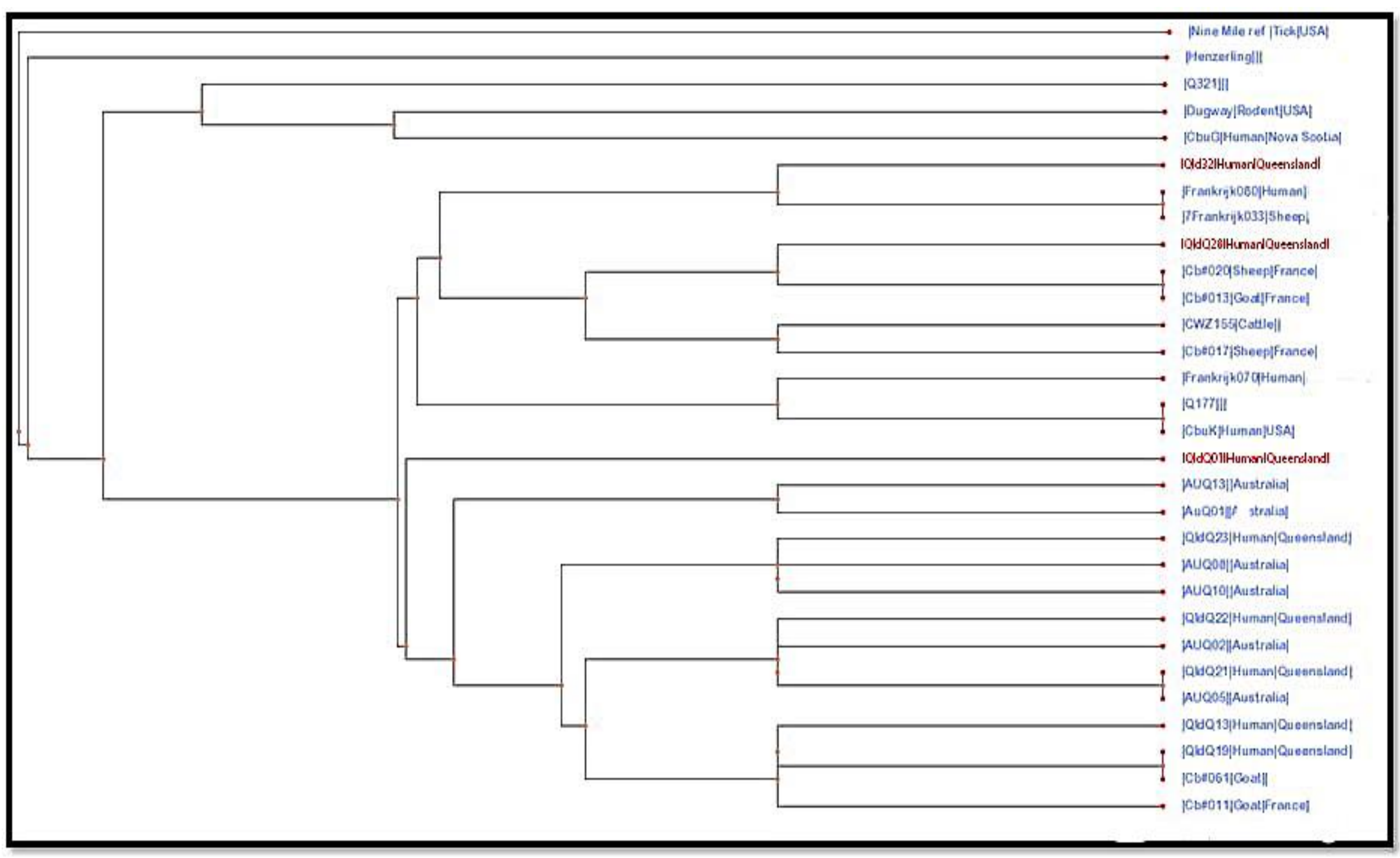

Figure 8.5: Phylogenetic tree displaying the Queensland samples identified using MLVA, highlighting the similarity seen with the Australian isolates and the diversity shown by two samples showing a closer relationship to the French strains

Novel Genotypes identified in Queensland *Other Genotypes that have previously been described*

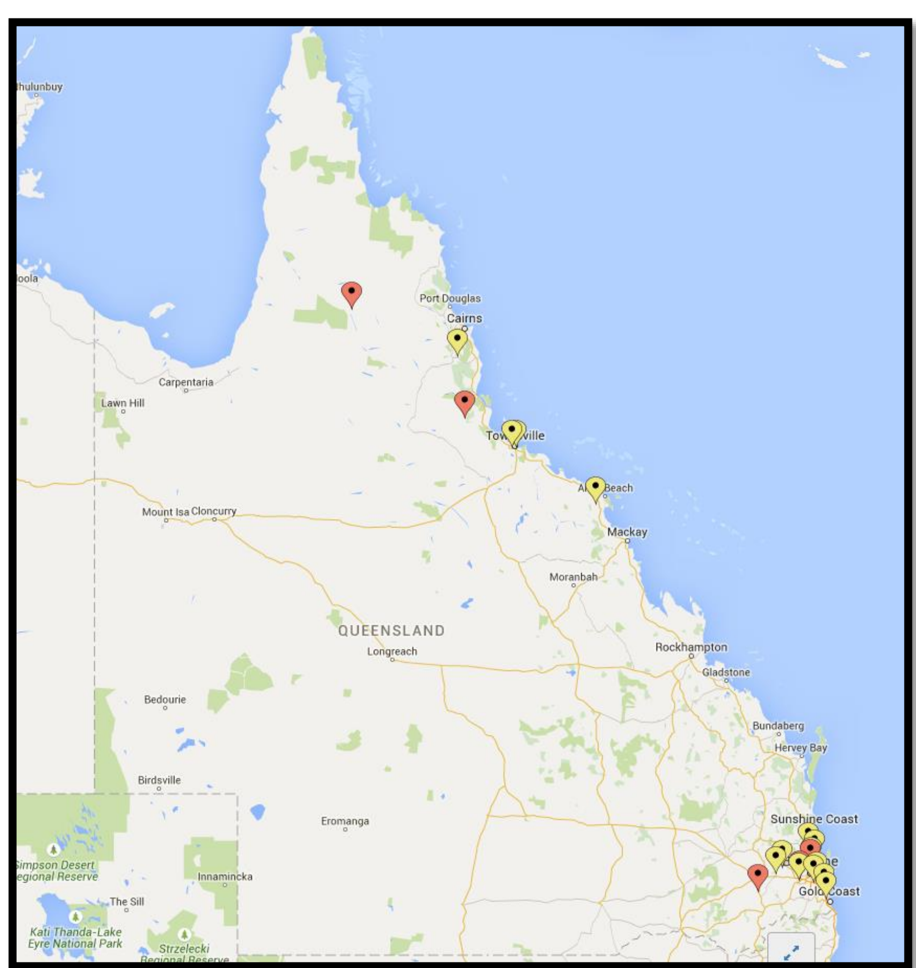

Figure 8.6: Queensland map showing the geographical distribution of the Queensland samples genotyped using MLVA. *There were three novel MLVA types identified from patients residing in Queensland highlighted in the different colours*

Figure 8.7: South East Queensland map showing the geographical distribution of the Queensland samples genotyped using MLVA. *There was one novel MLVA types identified from a patient in South East Queensland highlighted in yellow*

8.4.3. Combined MLVA Results Using Both the MLVA3 (Sanger) Method and the 3 Loci Capillary Electrophoresis Method.

The MLVA data generated from both methods used above were combined and examined. In all, there were 25 samples for which at least one locus could be amplified and sized the number of repeats determined (Table 8.7).

Table 8.7: Summary of combined results for MLVA profiles obtained with MLVA6 Sanger and the capillary electrophoresis methods. * *Matches 3 strains in international MLVA database.*

Sample Name	Source	MLVA3 (Sanger)			Capillary Electrophoresis			Genotype
		ms27	ms28	ms34	ms24	ms28	ms33	
Nine Mile Control		4	6	5	27	6	9	
Samples with at least 3 MLVA loci be either assay								
QldQ01	human	3	5	4	-	5	10	**Novel CbAu15**
QldQ11	human	2	6	2	-	-	-	**Novel CbAu18**
QldQ30	human	3	5	1	-	-	-	**Novel CbAu19**
QldQ32	human	4	5	3	13	5	4	**Novel CbAu17**
QldQ28	human	-	-	-	10	5	13	**Novel CbAu16**
QldQ21	human	-	-	-	14	5	4	CbAU05
QldQ16	human	3	5	2	-	-	-	10 CbAu matches
QldQ13	human	3	5	2	16	5	-	CbAU01/ CbAU013
QldQ19	human	3	5	2	12	5	-	3 matches*
QldQ25	human	3	5	2	-	5	-	10 CbAu matches
Qld305	canine	3	5	2	-	-	-	10 CbAu matches
Samples with at least 2 MLVA loci by either assay								
QldQ02	human	3	5	-	-	-	-	11 CbAu matches
QldQ08	human	3	5	-	-	-	-	11 CbAu matches
QldQ22	human	-	-	-	14	5	-	CbAU02/ CbAU05
QldQ23	human	-	-	-	15	5	-	CbAU08/ CbAU010
Samples with only 1 MLVA locus by either assay								
QldQ07	human	-	-	-	15	-	-	
QldQ20	human	-	-	-	16	-	-	
QldQ33	human	-	-	-	-	5	-	
QldQ40	feline	-	-	-	-	5	-	
QldQ44	feline	-	-	-	-	5	-	
QldQ76	dust	-	-	-	-	5	-	
QldQ36	human	-	-	-	-	5	-	
QldQ37	human	-	-	-	-	5	-	
QldQ38	human	-	-	-	-	5	-	
QldQ39	human	-	-	-	-	5	-	
No of Different Alleles		3	2	5	7	2	4	

Following comparison against >300 strains already in the MLVA database and to 14 unique isolates that have been previously genotyped from Australia, it was shown that this study had identified 5 novel genotypes in Queensland. These novel genotypes together with previously identified Australian genotypes are shown in Table 8.8.

Table 8.8: Queensland novel genotypes compared to previously identified Australian genotypes with similar alleles in the loci ms24, ms28 and ms33

	Strain	Loci					
		ms24	ms28	ms33	ms27	ms28	ms34
Reference	Nine Mile	27	6	9	4	6	9
Australian Genotypes*	CbAU01	16	5	5	3	5	2
	CbAU13	16	5	6	3	5	2
	CbAU10	15	5	4	3	5	2
	CbAU08	15	5	5	3	5	2
	CbAU02	14	5	5	3	5	2
	CbAU05	14	5	4	3	5	2
	CbAU03	13	5	5	3	5	2
	CbAu07	ND	ND	ND	3	6	2
	CbAu09	ND	ND	ND	3	5	3
	CbAU04	13	6	11	3	6	2
Novel Genotypes	CbAu15	-1	5	10	3	5	4
	CbAu16	10	5	13	ND	ND	ND
	CbAu17	13	5	4	4	5	3
	CbAu18	ND	ND	ND	2	6	2
	CbAu19	ND	ND	ND	3	5	1
No. of Different Alleles**		**5**	**2**	**6**	**3**	**2**	**4**

Legend: -1= MLVA typing was performed but no result was obtained as opposed to no allele being detected; ND= MLVA typing not performed due to insufficient sample.

** This is only a selection from the 14 unique Australia genotypes, and are the Australian genotypes that share common alleles with the Queensland novel genotypes*

*** The number of different alleles identified in the Australian and Queensland genotypes*

One of these novel genotypes, identified in specimen QldQ32, had complete profiles for both MLVA assays (3 loci each), and was named CbAu17 following the convention for naming novel Australian isolates described by Vincent[28].

The profile for this sample was generated from blood collected from a 31 year old female, who was a resident of Caboolture (postcode 4510) which is an urban residential town on the outskirts of Brisbane. This patient was working in an abattoir at the time, and was not vaccinated against Q fever. This new genotype was similar to genotypes Cb#013, Cb#017, Cb#020 and Cb#061, which were isolates obtained in France from sheep and goats.

Three other novel genotypes were identified from samples QldQ01, QldQ11 and QldQ30. These gave complete MLVA profiles with the MLVA6 Sanger method but a partial profile for QldQ01 only in the capillary electrophoresis method. The novel genotypes assigned to the *Coxiella* in these samples were CbAu15, CbAu18 and CbAu19 respectively.

Sample Qld01 was a blood sample taken from a 55 year old male residing in Bundamba in the semi-rural suburbs of Ipswich, in South East Queensland. This suburb is within 4 kilometres of the largest meat processing plant the Southern hemisphere. The genotype assigned (CbAu15) showed a MLVA profile that was similar to, but genotypically different from other isolates typed from Australian samples, but closely related to genotypes identified in France from humans, goats and sheep.

The two other unique genotypes CbAu18 and CbAu19 were both detected in blood samples. The first patient QldQ11 with genotype CbAu18 was a 52 year old female who resides in suburban Brisbane and submitted a blood sample for the laboratory investigation of pyrexia of unknown origin, and was subsequently diagnosed with Q fever. The second patient QldQ30 with the unique genotype CbAu19 was a 62 year old male with a history of frequent exposure to ticks and animal products. This man was a farmer on a property north of Townsville (postcode 4810), a rural area with high numbers of annual Q fever notifications.

The last sample from which a unique *Coxiella* genotype was detected was Qld28, which was assigned the name CbAU16. On MLVA analysis this sample gave a profile for the 3 loci (ms24, ms28 and ms33) in the capillary sequencing method, but not in any of the loci used for the MLVA6 Sanger method. This genotype was detected in Q fever PCR positive DNA from a 48 year old Australian indigenous male who was being investigated for the

causative agent of septic shock. This patient resided in the indigenous community of Aurukun (postcode 4871), located in Queensland's north, inland from Cairns.

There was one complete MLVA profile obtained with the 3 loci using the capillary method from sample QldQ21. None of the loci could be characterised from this sample using the MLVA6 Sanger method. This MLVA profile was the same as a previously described Australian isolate genotyped with a novel profile and was named CbAU05. This unique Australian genotype had been previously detected in 4 isolates AuQ05, AuQ09, AuQ39, and AuQ42 described by Vincent[28]. These four isolates were from New South Wales. DNA for this Queensland MLVA analysis had been isolated from the prosthetic heart valve of a 77 year old male patient, who resided in South East Queensland, at Redcliffe (postcode 4020), part of Metropolitan Brisbane.

There were five Queensland samples that gave complete MLVA profiles with the MLVA6 Sanger method and none or partial profiles with the capillary electrophoresis method. Three of these gave identical profiles with the MLVA6 Sanger profile, and had 2 or 1 loci detected by the capillary method (Table 8.7). One of these, sample QldQ13 was classified as either Australian genotype CbAU01 or CbAU013 based on the additional profile obtained with the m24 and m28 loci for this sample. The MLVA profiles for the remaining samples showed possible matches to 10 or 11 different genotypes previously described for Australian isolates.

Sample Qld19 was taken from the placenta of a 29 year old female living at Dingo Beach, in Central Queensland. This lady delivered a stillbirth child near full term. Her previous antenatal laboratory investigation had been normal, and on delivery the results of serology for Q fever were negative for IgM and IgG antibodies. However, a placental biopsy taken 2 days later than the bloods for mothers serology was positive for *C.burnetti* by PCR. This was the sample that was subsequently analysed by MVLA. This sample showed a MLVA profile for 5 loci, ms27, ms28, ms34, ms24 and ms28, which was similar to 3 other genotypes on the international MLVA database. These were Cb#061 isolated from a goat, Frankrijk070 isolated from a human aortic valve in France, and CWZ155 which was isolated from cow's milk in Qatar.

The remainder of the samples showed only partial profiles for either the MLVA6 Sanger method or the capillary electrophoresis method. Of these, two, QldQ22 could be typed to

previously identified Australian genotypes CbAU02 or CbAU05 and sample QldQ23 aligned with either CbAU08 or CbAU010 based on the capillary method, which offered greater discrimination power than the MLVA6 Sanger profile.

There was not enough data generated for the other samples by either method to assign a genotype with any confidence. However, all showed 5 unit repeats on the ms28 locus, which was the value most commonly determined for Australian isolates reported to date.

The location of all genotypes identified by both the MLVA6 Sanger and capillary electrophoresis methods were mapped, and their locations in Queensland are shown in Figure 8.8.

Figure 8.8: Geographic location of all Coxiella genotypes identified in Queensland as part of this study.

Unique genotypes are shown with red markers

8.5. Discussion:

Globally, there have been several Q fever outbreaks where the origin of infection has yet to be identified. This was due to a lack of epidemiological markers available using traditional bacterial typing methods. Discriminative typing methods are essential for the surveillance and evolutionary tracking of *Coxiella*, and a number of different methods have been assessed in order to determine the one to provide appropriate discrimination between strains. This resulted in a detailed analysis of the limited *Coxiella* sequence data available, and the identification of tandem repeat loci which may be used to assess the molecular divergence of *Coxiella* strains. In this study, MLVA was applied as the method of choice, because it provided good discriminatory power, higher than MST, and the profiles generated for the Queensland samples were directly comparable to MLVA profiles recently generated by another group in Victoria, Australia.

MLVA is now widely accepted as a typing tool for *Coxiella* as it provides consistency in results and the power to reveal molecular diversity among isolates globally. This has been aided by the development of international databases for which strains can easily be assessed and compared to other local and global strains.

In MLVA, up to 17 different genomic target-regions have been used for differentiation of *Coxiella* strains, but even so comparison of results from different laboratories remains problematic. However, the results from a recent study in Germany showed that although a combination of 8 MLVA markers provided the highest discriminatory power for attributing *C. burnetii* isolates to genotypes, it was sufficient to use three MLVA markers alone for rapid classification of *C. burnetii* into 4 main clusters[33]. In following this recommendation, this study applied MLVA analysis using two panels each of three loci to analyse the *Coxiella*-positive samples identified in this study.

PCR rather than serology was used extensively to diagnose acute Q fever cases during the large and protracted outbreak in The Netherlands (2007–2010). This led to the possibility of directly typing *Coxiella* from clinical material with its obvious advantages of timesaving and rapid instigation of appropriate control measures. Clinical material usually only contains low amounts of *Coxiella*-specific DNA which makes it very difficult to get reliable typing results. This can be partially overcome by shortening primer lengths to

improve sensitivity. This may not be applicable to all known markers, but a reduced panel of three markers was used successfully in the Dutch outbreak to analyse clinical samples[30]. Four different genotypes could be clearly identified using this method.

The use of MLVA for *Coxiella* typing however, allows for the genomic analyses of strains directly from the clinical or environmental samples by selecting the appropriate panel to use for discrimination[25]. In this study the investigation into the molecular diversity within Queensland was also performed using clinical samples, along with environmental samples containing *Coxiella* DNA. These samples also contained relatively low bacterial load, and the major challenge arose from the very low amounts of *Coxiella*- specific DNA available to provide reliable results. This was addressed by using the shorter primer sets for 2 panels of 3 markers. The first included the loci ms27, ms28 and ms34, and the second panel included the loci ms24, ms28 and ms33 which had been used successfully used in the Dutch outbreak to analyse clinical samples[30].

A previous study in Australia has identified 14 unique MLVA genotypes compared to the global data available on the international database[28]. These genotypes, named CbAu01-14, were identified in bacterial isolates obtained from clinical samples, predominately from New South Wales. These isolates proved to be very different from those detected in the Northern hemisphere, including those identified in The Netherlands outbreak. The Australian isolates were typed using the extensive 17 loci panel and compared to other isolates screened with the same or slightly modified extensive panel. Similarly to the previous observations, it was determined from the Australian data that the same level of discrimination could be obtained by using 3 select loci which resulted in identifying the same degree of diversity using the Hunter and Gaston index[28].

However, in limiting the number of loci, accurate sizing of the amplicons is extremely important to generate meaningful results. This study examined the use of PCR primers from Panel 2 shown in Table 8.3. In the first method applied, the amplicons generated by these were detected by gel electrophoresis and sized by analysis of the sequencing data generated by Sanger sequencing. This was partially successful for most samples, but for some it was difficult to accurately assign the number of repeats in each amplicon, and often the sequence data generated was difficult to interpret with confidence. Since accurate size determination was important for the calculation of repeat unit numbers, it was decided that gel based analysis did not provide the consistency of results required,

and was not suitable for the analysis of samples with low bacterial loads. Capillary electrophoresis was a more sensitive method that had previously been applied for MLVA amplicon detection and sizing using Panel 2 primers [25]. This method was then used to re-examine the samples in this study for MLVA analysis. The results of the two methods applied in this study were examined together and analysed in context.

In the study described here, there were fifteen MLVA genotypes that could be identified from two loci or more from the Queensland samples screened. Five of these were unique to Queensland, and the remaining ten were similar to previously described Australian genotypes. These stains seemed to cluster together and may represent a novel genomic group. The samples were mainly blood samples collected from both acute and chronic Q fever patients.

One sample QldQ19 showed a MLVA profile consisting of five loci, and matched three possible genotypes identified in goats, cattle and humans. This case involved a stillbirth in a 31 year old female, as a result of Q fever infection, and would seem one of the very rare cases where acute Q fever results in the demise of the human foetus. Although such cases are rare they must be considered as a real threat to the well-being of the unborn child. The diagnosis of infection in this case was made by PCR on placental material after birth, and serology of the mother was negative giving no indication of acute Q fever infection. The origin of infection was not identified in this case[34,35].

There was one patient, QldQ22, which had been vaccinated with Q Vax® two months prior to being diagnosed with acute Q fever. The MLVA profile for 2 alleles that was obtained from a blood sample showed a genotype of either CbAu02 or CbAu05. This proved that the infection was not the result of vaccination. This could be a potential source of infection in some subjects. In such cases the use of MLVA to characterise the infecting organism would definitively determine if such infections were vaccine derived.

One sample with a unique Queensland genotype was derived from an Australian indigenous male living in Northern Queensland at Aurukun. At the 2011 census, this town had a population of 1,295, including 1,193 Indigenous people. Q fever in the indigenous population is considered to be rare[36], however, there have not been many studies to assess the true prevalence in this population.

This study found that a genotype represented by the same MLVA profile (ms27=3; ms28=5; ms34=2) was present in both humans and a dog sample. This highlights that single genotypes can be co-shared between humans and their environment, and indicated that animals and perhaps other environmental sources played an important part in transmission of disease. Unfortunately, many of the animal and other environmental samples examined in this study failed to provide a complete MLVA profile, presumably due to very low bacterial loads, and hence no firm conclusions could be formulated to identify their role the dissemination of disease. A further, more comprehensive investigation will be initiated to examine this question, but this is outside the scope of this present study.

All genotypes identified from Australia, including these recently typed from Queensland, showed a very close relationship to each other. However, they showed a substantial difference from other genotypes associated with disease from other parts of the world. It has been suggested that the strain type that was responsible for the large scale outbreak in The Netherlands was a hyper-virulent strain and that the genotypes that differed by only a single repeat were micro-variants of the original strain, rather that the spread of infection from multiple sources with different strains of *Coxiella*. The same may apply to the Australian strains and genotypes. The rates of infections identified in Chapter 3 were much higher in Queensland than any other part of the world. This may be due to the presence of more pathogenic strains circulating in Australia. Australia is an island geographically separated by sea from the rest of the world. This is ideal for the evolution of novel *Coxiella* strains which may be more pathogenic, as well as the clonal expansion of these in localities within Australia such as regions in Queensland which may be separated by 1000’s of kilometres. Such conditions would allow for variants to exist and to continue evolving over time without impacting on the rest of the country.

To date, no “classical” virulence factors like toxins or pore-forming proteins have been identified among *Coxiella*. This lack of understanding of the virulence mechanisms makes it difficult to develop appropriate tools to recognise organisms with different pathogenic profiles. The application of discriminating methods like MLVA, produces a molecular “signature” and hold promise for providing a means for differentiating isolates. However, the growing technical power of whole genome sequencing has the potential to replace the current loci and target based methods, and especially next-generation sequencing

technology, has the potential to rapidly expand the *Coxiella* genome information database, and our understanding of the biology and epidemiology of this important pathogen.

8.6. Significant Outcomes from this Chapter

This was the first study to examine the molecular epidemiology of *Coxiella burnetii* in Queensland, and examined the genetic profile of bacteria detected in humans, animals and the environment. The findings were summarised as follows:

- The MLVA typing method using three loci could be successfully used to genotype *Coxiella* direct from clinical specimens. In this study both the methods using Sanger sequencing and capillary electrophoresis provided comparable results.
- Unique genotypes of *Coxiella* circulate in Queensland. These are molecularly distinct from Australian isolates and from global isolates currently listed on international databases.
- Potentially the same genotype of *Coxiella* can co-circulated in humans and in dogs, suggesting that these genotypes are widely shared in the environment and confirms that animals may be an important part of the chain of infection.
- The novel Queensland genotypes were widely distributed across Queensland, and were not confined to a specific location.

8.7. References

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8.8. Appendix 8.1: MLVA Raw Data

Data obtained from the 3130 xl Genetic Analyser (AGRF) determining the size of the amplicon and then the unit repeat number within the target loci

Sample	Fragment Size	Peak Height	No of repeats
QF007-ms24	186.103	16465	15
QF013-ms24	193.0346	13026	16
QF019-ms24	164.9403	32539	12
QF020-ms24	193.1607	32711	16
QF021-ms24	178.4346	31699	14
QF022-ms24	178.7287	32568	14
QF023-ms24	186.3721	32564	15
QF028-ms24	151.7328	32707	10
QF032-ms24	172.8587	32737	13
NM-ms24	269.7463	32715	27
NM-ms24	269.4071	32494	27
Arandale-ms24	192.183	31349	16
AuQ05-ms24	178.4532	30471	14
AuQ30-ms24	212.8736	31246	19
D19-ms28	269.5702	13189	5
Qld01-ms28	269.2811	32493	5
Qld13-ms28	269.8906	32651	5
Qld19-ms28	270.0682	32709	5
Qld21-ms28	269.5273	32070	5
Qld22-ms28	269.8036	32531	5
Qld23-ms28	269.6164	32682	5
Qld25-ms28	269.8108	32668	5
Qld28-ms28	269.6634	32564	5
Qld32-ms28	269.8266	32587	5
Qld33-ms28	269.95	32751	5
Qldu40-ms28	270.1376	19083	5
Qld44-ms28	270.0463	11377	5
Qld76-ms28	269.5842	33586	5
Qld36-Ms28	270.0029	32720	5
Qld38-ms28	269.9225	32708	5
Qld39-ms28	270.2321	32726	5
Qld40-ms28	270.0494	32728	5
NM-ms28	274.9772	32146	6
NM-ms28	275.1871	32344	6
Arandale-ms28	269.62	32012	5
Qld01-ms33	270.1003	32353	10
Qld21-ms33	224.1398	32617	4
Qld28-ms33	287.901	32694	13
Qld32-ms33	224.4633	32634	4
NM-ms33	260.4049	32704	9
Arandale-ms33	232.0021	32551	5
Pearson-ms33	239.3747	32566	6

Chapter 9

Development of a Cell Mediated Immunity Assay to Determine Previous Exposure to *Coxiella burnetii*

9.1. Introduction

9.1.1. Host Immune Response

Coxiella burnetii is an intracellular bacterium and typically replicates inside the host cells. The principle host cells involved are the macrophage, monocytes, microglia and dendritic cells[1]. Following infection, the bacteria survive and multiply within the macrophage phagosome created within the cell. The *Coxiella* gene products are able to modify interfaces of the phagolysosome and utilise metabolic products of the autophagic pathway for the bacteria's replication[2]. The initial innate and adaptive immune response is inhibited during the long incubation period within the macrophage, however,as infection progresses, both these are eventually initiated.

It has been well established that adaptive immunity is essential to cure Q fever infection[3]. Patients that are receiving immunosuppressive treatment therefore, are at greater risk of developing chronic Q fever and are unable to mount an effective immune response to the bacteria[1].

The host adaptive immune response can be divided into antibody-mediated immunity (AMI) or cell-mediated immunity (CMI) (Figure 9.1). Studies of the host immune system show that both AMI and CMI are involved in host defences against *Coxiella*. The CMI is believed to be the major immune response, effective against invasion of the intracellular bacteria, and is the major mechanism for control of infection in patients with acute Q fever.

During infection, the phagocytic cells are able to present *Coxiella* antigen-la complexes on their cell surface, resulting in a cascade of T cell activation and the production of cytokines including interleukin 1 (IL-1), IL-2, Tumour Necrosis Factor-α (TNF- α) and interferon-gamma (INF-ɣ)[4].

The role of T cells in the control of *C.burnetii* infection has been well documented in mouse models. One study showed that athymic mice deficient in T cells, when challenged with *C.burnetii,* showed no effect on bacterial invasion and bacterial growth, highlighting the important role of T cells in the infection process[5]. Another study showed SCID mice that are devoid of T and B cells succumb to infection, demonstrating the major role of the adaptive immune response for the control of *C.burnetii* infection[6]. INF-ɣ plays a role in

restoring the ability of the host cell to disperse viable *Coxiella*[7], and the production of INF-ɣ is essential for the early control of infection[8].

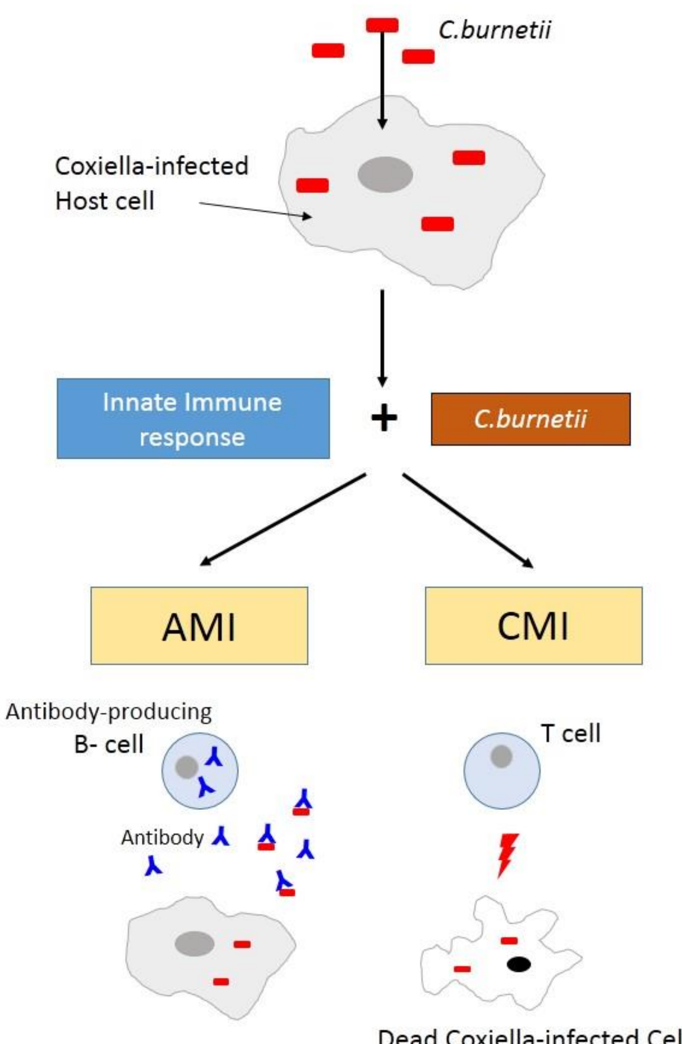


Figure 9.1: The two main classes of adaptive immune response
Lymphocytes respond to a *C.burnetii* infection. In the AMI response, B cells secrete antibodies that neutralize the bacteria. In the CMI response, T cells kill the *Coxiella*-infected cells.
(*Figure adapted from Molecular Biology of the Cell. 4th edition Alberts B et al. New York: Garland Science; 2002.)*

Although the role of T cells and their response to vaccination has not been well studied[9], it has been demonstrated that individuals vaccinated with formalin-inactivated *C.burnetii* exhibit specific lymphoproliferation and IFN- ɣ production in response to the bacterial antigens[10]. As a result of previous vaccination or natural infection, the cellular immune response is persistently sensitised to *Coxiella* and this is the bases for the use of the intradermal skin test as a tool for pre-vaccination screening.

9.1.2. Vaccine

The vaccine Q Vax® (bioCSL, Melbourne, Australia) has been licenced for use in “at risk” populations in Australia since 1989. Q Vax® is a whole cell, phase I, Henzerling strain, formalin inactivated preparation, containing a Thiomersal preservative[11]. It is administered as a 0.5mL dose containing 25µg of *Coxiella burnetii.* The vaccine has a 98% efficacy lasting at least 5 years[12], and was implemented by the NQFMP commencing in 2000, targeting workers in high risk occupations. In the second phase of the program, vaccination was extended to include adults in families of rural workers. However, uptake of the vaccine in other countries around the world has been limited due to the considerable adverse reactions that have been associated with vaccine administration.

9.1.3. Adverse Vaccine Reactions

There were a number of common adverse reactions reported during clinical trials of the vaccine, including local tenderness in about 50% of vaccinees, followed by a third of subjects reporting local erythema. Local oedema was also reported along with transient headaches and “flu-like symptoms. Other presentations included dizziness, aching joints and swollen glands[13]. Some of these reactions, including tenderness, erythema and oedemas at the vaccine administration site, have also been observed with other bacterial vaccines and are not uncommon. The development of short-lived flu-like illness after vaccination is the result of the cellular immune response being stimulated[14]. However, the adverse reactions that may occur in a vaccine candidate previously exposed to *Coxiella*, can be a severe local reaction such as site abscess formation or granuloma, extending to severe generalised adverse systemic reactions including lymphadenopathy and chronic fatigue syndrome (QFS)[15].

There have been several documented cases of severe adverse reactions after Q fever vaccination. One case involved the development of an immune abscess. Within hours of vaccination the site was inflamed, which resulted in the lesion becoming indurated and finally breaking down to form a sinus which was only cured with surgical intervention. The subject then developed QFS. Follow up serology from the abscess exudate showed high *Coxiella* antibody titres. This case highlights the importance of using effective pre-screening techniques to safeguard against adverse reactions and more serious clinical complications.

If vaccine candidates with pre-existing immunity are vaccinated they exhibit 'sensitisation' to Q fever antigens through the release of cytokines by memory T cells. This is the result in a heightened cell mediated immune response, which can be maintained by low-level persistence of the organism or its antigens after infection. Vaccinating these previously exposed persons may lead to severe local inflammatory reactions and general systemic reactions as described above. Such reactions are not inevitable and are unpredictable, and represent re-stimulation of the sensitised CMI response leading to hypersensitivity reactions.

9.1.4. Pre-vaccination Screening

Pre-vaccination screening helps to identify subjects that have been previously exposed to *Coxiella* and hence sensitised to bacterial antigens. These subjects are at risk of experiencing a serious hypersensitivity reaction if they are re-exposed through vaccination[16].

Currently there are three steps used in pre-vaccination screening to identify those previously exposed.

> **1. History:** A detailed medical history to identify previous infections with Q fever and to ensure the subject has not previously been vaccinated for Q fever.
>
> Those identified as having a positive history of Q fever exposure are immediately excluded and require not further investigation.
>
> **2. Skin Test:** An intra-dermal injection of diluted Q Vax® is administered, and in addition
>
> **3. Serological Profile:** A blood sample is collected and screened for phase II IgG antibodies to *Coxiella* by a standard laboratory method such as IFA.

These two testing regimes (steps 2 and 3 above) measure two different immune pathways. The skin test is indicative of the CMI response, and is the primary test[15]. The serological screening detects antibodies previously produced as a result of past exposure and provides additional confirmation of the subject's exposure status, if ambiguous or inconclusive results are obtained with the skin test.

There are a number of factors to consider while using the skin test as a means of identifying previous exposure. Only experienced personnel can perform and interpret skin tests successfully, and there are a number of errors that can complicate the interpretation of skin test results. For instance, important factors are:

- The depth of injection; subcutaneously is too deep, the injection should be intradermal.
- The location on the inner forearm for administering the intradermal injection.
- The length of time between injection and interpreting the results. Early reading of the skin test may result in the erythema being mistaken for a positive reaction.

Serology screening also has some limitations when used as an indicator for previous exposure. The serological markers may decline over time and may in fact be negative in a previously exposed subject[17].

9.1.5. Cell Mediated Immune Response Screening

As already discussed, activation of T cells leads to the direct production of cytokines including the secretion of INF-Ɣ. The direct measurement of INF-Ɣ resulting from the T cell response to stimulating antigens, may be achieved in the Enzyme-Linked ImmunoSpot (ELISPOT) assay, by measuring the amount of secreted cytokine produced, which in turn can be quantified or linked to individual cells. This technique has already been applied in clinical diagnostics, and is currently used for the detection of Tuberculosis. It has also been used extensively in the monitoring of transplant recipients for graft tolerance or rejection.

The ELISPOT assay allows visualization of individual cells that have been stimulated and responded by producing INF-Ɣ. Each coloured spot that develops on the assay membrane is the direct result of the reactive response induced by the *Coxiella* stimulus in a single reactive T cell. Thus, the ELISPOT assay provides both qualitative and quantitative information regarding the CMI response in a subject previously exposed to *Coxiella burnetii.*

9.2. Specific Aims Addressed in this Chapter

Recent studies have investigated INF-ɣ based assays, including a whole-blood INF-ɣ production assay and a *Coxiella* ELISPOT assay, as potential diagnostic tools for Q fever diagnosis, including chronic Q fever. This was the first ever attempt to use CMI in Q fever pre-vaccination screening.

The study in this chapter sought to establish **Proof of Principle**, and to develop and evaluate the use of a *Coxiella* ELISPOT assay for use as a pre-vaccination screening tool.

The aims addressed were:

- To develop an in house ELISPOT method to measure INF-ɣ production from T cells stimulated with *Coxiella*
- To test the ELISPOT method on blood samples collected from subjects of known immune status to *C.burnetii*
- Evaluate the suitability of this ELISPOT assay as an alternative to the intradermal skin test used routinely for assessing *Coxiella* immune status in vaccine candidates

9.3. Methods

9.3.1. Samples

Whole blood samples collected into 5ml Lithium Heparin tubes (Becton Dickinson, North Ryde, Australia) were taken from three previously vaccinated subjects. These comprised:

(i) **Subject S1**: a recently vaccinated subject (<1 year post vaccination).

(ii) **Subject S2**: approximately 5 years post vaccination.

(iii) **Subject S3**: a non-vaccinated, non-exposed subject, to act as a negative control.

(iv) **Subject S4**: this subject was approximately 10 years post vaccination.

The blood samples from these four subjects were processed as described below to isolate the peripheral blood mononuclear cells (PBMC’s) (section 9.2.3).

At the same time, serum samples were collected for the measurement of phase I and II *Coxiella* IgG antibodies.

9.3.2. General Principle of the ELISPOT Assay

The ELISPOT assay is a highly sensitive immunoassay that measures the frequency of cytokine-secreting cells at the single-cell level. Cells are cultured on a surface coated with a specific capture antibody in the presence of stimulus antigen. Cytokines such as INF-ɣ that are secreted by the cells will be captured by the specific antibodies on the surface. After an appropriate incubation time, cells are removed and the secreted molecule is detected using a detection antibody in a similar procedure to that employed by the ELISA. The detection antibody is biotinylated and is followed by a streptavidin-enzyme conjugate step. By using a substrate with a precipitating rather than a soluble product, the end result is visible as spots on the surface. Each spot corresponds to an individual cytokine-secreting cell (Figure 9.2).

ELISPOT ASSAY

1. Antibody coating
Cytokine-specific monoclonal capture antibodies are immobilized on an ethanol-treated PVDF membrane plate.

2. Cell incubation
Cells are added to the wells in the presence or absence of activating stimuli, and then incubated to allow for IFN-Y secretion

3. Detection antibodies
Following removal of the cells and washing of the plate wells, biotinylated IFN-Y -specific detection antibodies are added to the wells

4. Streptavidin-enzyme conjugate
To enable the formation of spots on the membrane, a streptavidin-enzyme conjugate is added to the wells

5. Addition of substrate
Colorimetric substrate is added to the wells and will form an insoluble precipitate when catalyzed by the enzyme; a visible representation of cytokine release by a single activated cell.

6. Analysis
Spots are counted in an automated ELISpot reader, and the frequency of secreting cells is calculated

Figure 9.2: Basic principles of the ELISPOT assay applied for the detection of IFN- ɣ in the study below. (Figure was adapted from *https://www.mabtech.com/knowledge-center/assay-principles/elispot-assay-principle/elispot-step-step*)

9.3.3. T cell Isolation

The PBMC's containing T cells were isolated from heparinised blood using a Ficoll gradient. A patient's blood was diluted 1:2 with RPMI 1640 – L Glutamine (Gibco by Life Technology, Victoria, Australia) in a 50ml conical tube. Ficoll 10ml was layered into a new

50ml conical tube and then 10ml of the patient's diluted blood was gently overlayed using a 5ml pipette to maintain an interface between the Ficoll and the diluted blood. This was spun in a Beckman Coulter Allegra X-22 Series (Victoria, Australia) bench centrifuge at 400g, at room temperature for 30 minutes with both the acceleration and the deceleration reduced to 5 minutes with no brake. This produced an interface by which the buffy coat containing the PBMC's could be visualised as distinct from the plasma and the Ficoll (Figure 9.3)

Figure 9.3: The PBMC interface layer between the plasma and Ficoll after centrifugation

The buffy coat containing the PBMC's was removed and added to a new sterile 50ml conical tube and topped up to 50ml with RPMI L-Glutamine at room temp to wash the cells. The cells were spun at 300g/1200rpm for 12 minutes at room temperature to pellet cells and remove contaminates. The wash step was repeated and the final wash solution media was removed, and cell pellets were resuspended in 10 ml of complete media – (RPMI L-Glutamine + 10% foetal calf serum + 1% antibiotics). White blood cells were counted at a dilution of 1:2 with Trypan Blue (2%), using a haemocytometer. The cells were then adjusted to a concentration of 10^6 cells per well for pre-stimulation with *Coxiella* antigens.

9.3.4. The In House ELISPOT Assay

9.3.4.1 Pre-stimulation of T cells with Coxiella Antigens

Two antigen preparations and the PBMC's from 3 patients, S1, S2, S3, were trialled in pre-stimulating the T cells for the production of IFNɣ. PBMC's were inoculated into a 24 well

tissue culture plate at a concentration of 10^6 cells per well. Two stimulus antigens were evaluated. These were (i) 4 µg/ml and 20 µg/ml final concentration per well of the *Coxiella* whole cell phase I and II combined CFT antigens (Virion/Serion (Wurzburg, Germany), and (ii) the Q Vax® CSL vaccine (Commonwealth Serum Laboratories, Victoria, Australia), at a 50 µg/ml final concentration.

For the initial stimulation of the T cells, the final total volume was made up to 2 ml with RPMI, containing 10% foetal calf serum and antibiotics. The T cell suspensions and stimulus antigens were incubated at 37^0C for 20 hours. The pre-stimulated T cells were washed as previously described and pelleted for seeding into the ELISPOT test plate. The CFT antigens and the Q Vax® were added to the test plate as per Figure 9.4.

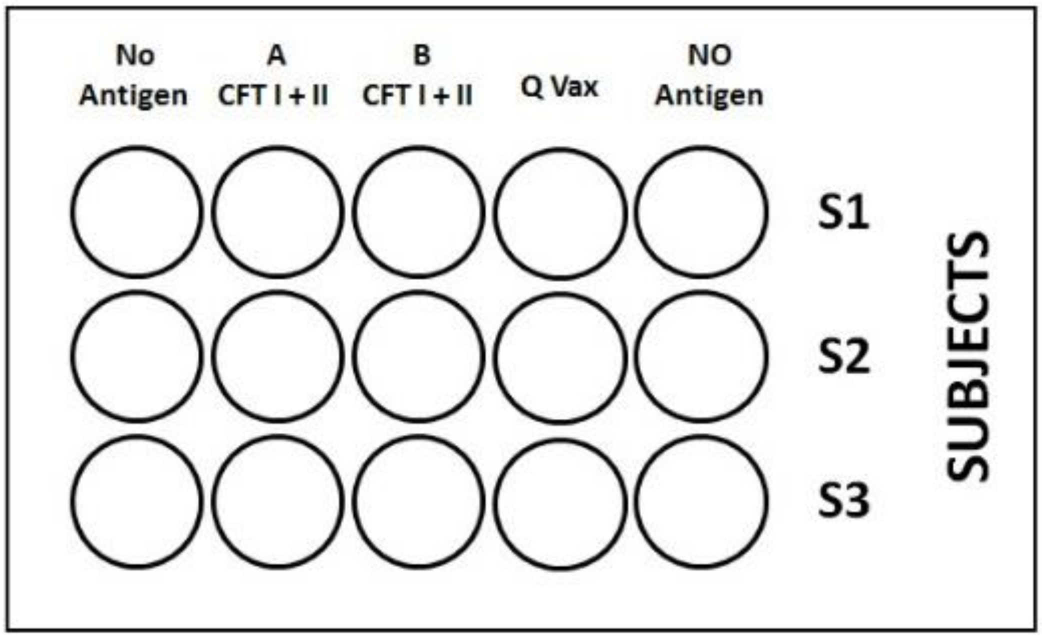


Figure 9.4: Pre-stimulation plate configuration showing antigens and subjects tested. (Final concentrations used: A. CFT phase I and phase II combined = 20 µg/ml; B. CFT phase I and phase II combined = 4 µg/ml and Q Vax® phase I =1µg/ml)

9.3.4.2. Re-stimulation of T cells with Coxiella Antigens

The commercially available MabTech^{plus} ELISPOT plates from Resolving Images (Victoria, Australia) were used in the assay. A re-stimulation of the T cells with *Coxiella* antigens was performed in the ELISPOT plate following the pre-stimulation or priming of the T cells in cell culture as described above.

The plate was prepared as per the manufacturer's recommendations, which included the preparation of the plate by washing with PBS four times before blocking the wells with the same media used as in the pre-stimulation of the T cells. Blocking was for at least 30 minutes at room temperature. The culture media were removed and the addition of the pre-stimulated, primed T cells and stimulus antigen were added to the wells.

The cells were loaded at a concentration of 10^5 cells per well in RPMI with 10% foetal calf serum following by the addition of the *Coxiella* antigen for re-stimulation. The combined CFT phase I and II antigens only (25 µL of each phase), were used as the re-stimulating antigen and added in duplicate wells. The combined CFT phase I and II antigens were loaded to give a final concentration of 20µg/ml of antigen in each well with a final volume of 100µL in the well.

PHA was used as a non-specific T cell stimulator to act as a positive control, and monitor the T cells ability to produce IFN-Ɣ. It was used at a concentration of 10µg/ml per well. The negative well had the addition of T cells with no stimuli, to ascertain a baseline for INF-Ɣ production and to account for any non-specific stimulation (Figure 9.5). The ELISPOT plate was wrapped in aluminium foil to prevent evaporation and incubated at 37ºC with humidity and 5% CO_2 for 14 hours.

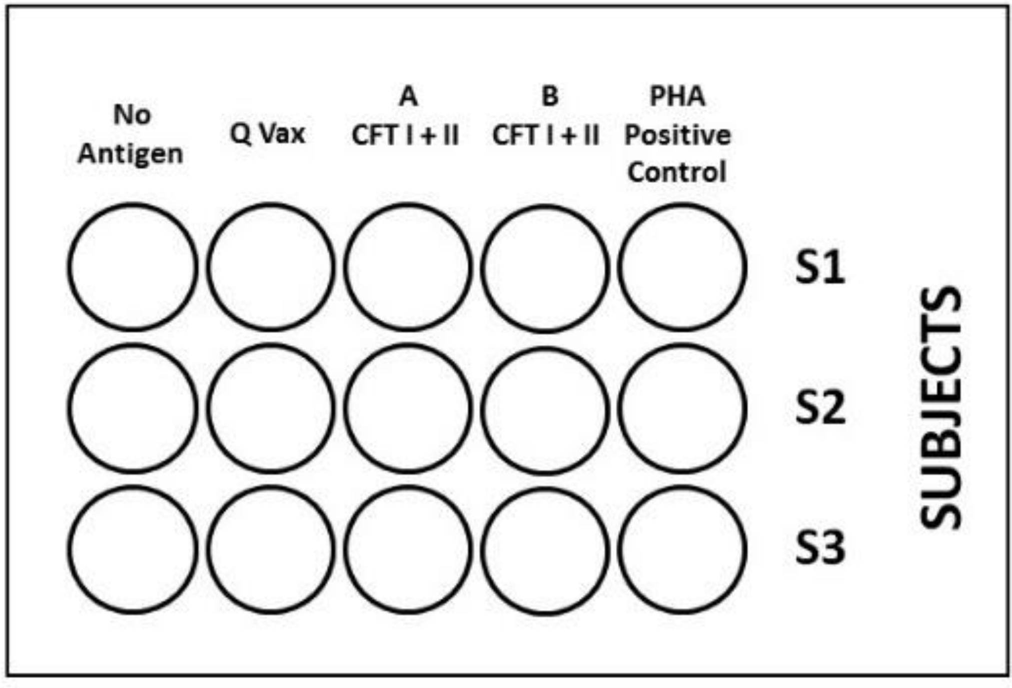


Figure 9.5: Configuration of cells tested with stimulus antigens and control wells.

The “no antigen” well was used as a non-stimulus control, and PHA as a positive control to monitor cytokine release after non-specific stimulation. A and B were duplicate wells with 20µg/ml of combined CFT phase I and II antigens added.

9.3.4.3. Detection of IFN-Ɣ-producing cells

The detection of cells producing IFN-Ɣ was achieved by the removal of the media from the plate and washing the plate 5 times with PBS. The biotin labelled detection antibody provided in the kit was diluted in PBS with 5% foetal calf serum at a concentration of 1µg/ml. 100 uL was added to each well and incubated for 1 hour at room temperature. The plate was washed as before. The substrate solution provided in the kit was loaded to each well and left until the development of spots occurred. The colour production was stopped by washing the wells extensively in tap water. The wells were left to dry completely, away from direct light and the collation of spots was achieved using the ELISPOT plate reader (AID EliSpot Reader, ResolvingIMAGES, Victoria, Australia)

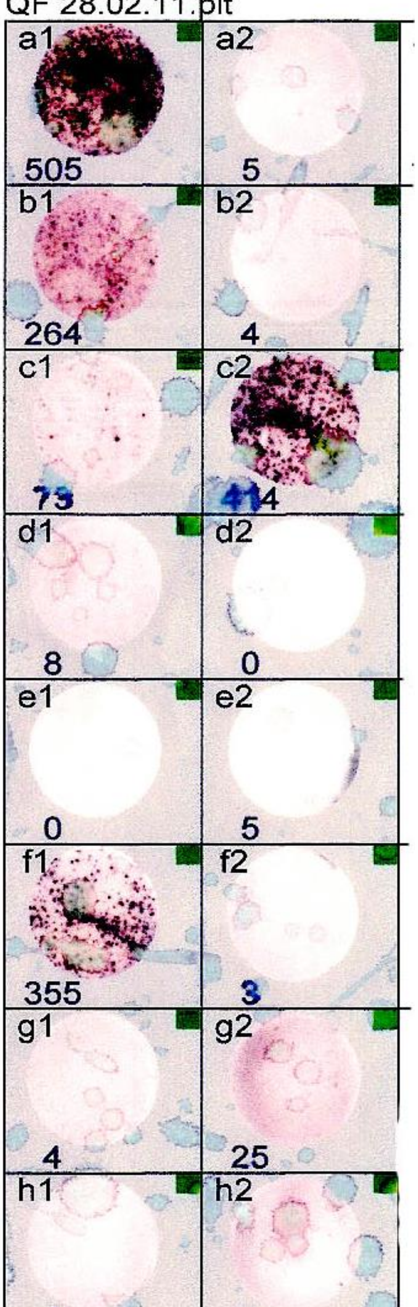
9.4. Results

9.4.1. Results of the Optimisation of Coxiella Stimulation

The combinations of two different antigen stimuli were trialled to ascertain which antigen stimulus produced maximum activation of the T cells to produce measurable INF-Ɣ.

Subject S1, who had been most recently immunised against Q fever showed a marked response to both the CFT I+II antigens that were used in pre-stimulation and re-stimulation (Figure 9.6; Table 9.1). There was a 264-fold increase in the number of INF-Ɣ-producing T cells that responded to the antigens in the plate. The subject (S2) that was vaccinated 5 years prior to testing showed little INF-Ɣ production when challenged with *Coxiella* antigens. The non-vaccinated subject (S4) showed some reactivity in the T cells to the Q Vax® stimuli.

It was determined that the stimuli which initiated the greatest production of INF-Ɣ was the CFT antigen phase I and phase II combination, at a concentration of 20µg/ml. This was used in the ELISPOT testing for the four subjects examined in the study below.



Position	Subject	Stimuli	Cells Producing INF-Ɣ
A1	S1	PHA	505
B1	S1	CFT I+II (20µg/ml)	264
C1	S1	CFT I+II (4µg/ml)	73
D1	S1	Q Vax (1µg/ml)	8
E1	S1	No Stimuli	0
F1	S2	PHA	355
G1	S2	CFT I+II (20µg/ml)	4
H1	S2	CFT I+II (4µg/ml)	0
A2	S2	Q Vax (1µg/ml)	5
B2	S2	No Stimuli	4
C2	S3	PHA	414
D2	S3	CFT I+II (20µg/ml)	0
E2	-	Blank	-
F2	S3	CFT I+II (4µg/ml)	3
G2	S3	Q Vax (1µg/ml)	25
H2	S3	No Stimuli	4

Figure 9.6: Illustration of ELISPOT plate readings from *Coxiella* pre-stimulus trial.

Table 9.1: Details of ELISPOT optimisation of pre-stimulating antigens with PBMC's from three subjects. The number of T cell producing INF-Ɣ is shown. (S1 = Recent vaccination <1 year; S2 = Vaccinated approximately 5 years; S3 = Not previously vaccinated).

9.4.2. Results of ELISPOT Analysis for Four Subjects Tested

PBMC's isolated from the four test subjects S1, S2, S3 and S4 were analysed in the ELISPOT assay described above. Cells were tested in duplicate wells, using 20 µg/ml of stimulus antigen. The number of cells producing INF-Ɣ for each subject and the controls are shown in Table 9.2.

The n-fold scores were calculated for each subject tested to provide an indicator of the level of INF-Ɣ production in activated T cells expressed as the number of coloured spots, using the number of spots produced with non-stimulated T cells as the denominator.

n-fold scores were calculated as follows:

$$\left(\frac{\textbf{T cell count with stimuli}}{\textbf{T cell count NO stimuli}} \right) = \textbf{variation in INF-}\gamma\textbf{ production (n-fold)}$$

The greatest number of T cells to produce INF-Ɣ were from subject S1 who had been most recently vaccinated (<1 year post vaccination), with an average of 275 INF-Ɣ producing cells and an n-fold score of 32. Subject S2 who had been vaccinated approximately 5 years prior to testing had an average n-fold score of 13, and subject S3 who had no prior exposure to *Coxiella* by either natural infection or vaccination showed a low reaction to the stimulus antigens with an average of 55 cells producing INF-Ɣ, and an n-fold score of 3. However, the subject (S4) who had been vaccinated more than 10 years prior, also showed a low number of T cells producing INF-Ɣ; average = 61, which was similar to the non-vaccinated subject. When the n-fold score was calculated for subject S4, this was determined to be 15 which was in fact similar to subject S3 who had been vaccinated for approximately 5 years. These subjects were able to produce higher differential levels of INF-Ɣ than the subject that had never been challenged with *Coxiella*.

The *Coxiella* serology was also performed on these subjects by IFA. Serology for IgG phase I and II showed the non-vaccinated subject (S3) had no evidence of past exposure to *Coxiella*. The recently vaccinated subject, S1 had detectable *Coxiella* IgG antibodies to

phase II, and subject S2, vaccinated 5 years ago, showed a weak antibody response on the IFA to *Coxiella* phase II antigens. Subject S4, vaccinated more than 10 years ago, had no detectable IgG antibodies on the IFA to either phase I or phase II antigens.

Table 9.2: Subjects 1-4 and the numbers of T cell producing INF- ɣ, along with the comparison of stimulated T cells compared to non-stimulated T cells expressed as an n-fold number.

	Stimuli	Cells Producing INF-ɣ	Variation from No Stimuli (n-fold)	Average n-fold score
S1	PHA	336	37	
	CFT I+II (20µg/ml)	230	26	32
	CFT I+II (20µg/ml)	319	35	
	No Stimuli	9	1	
S2	PHA	297	49	
	CFT I+II (20µg/ml)	75	13	14
	CFT I+II (20µg/ml)	89	15	
	No Stimuli	6	1	
S3	PHA	TNTC	~	
	CFT I+II (20µg/ml)	50	3	3
	CFT I+II (20µg/ml)	61	3	
	No Stimuli	18	1	
S4	PHA	118	30	
	CFT I+II (20µg/ml)	61	15	15
	CFT I+II (20µg/ml)	61	15	
	No Stimuli	4	1	

(S1 = Recent vaccination <1 year; S2 = Vaccinated approximately 5 years; S3 = Not previously vaccinated; S4 = Vaccinated for >10 years). TNTC – too numerous to count.

9.5. Discussion

Q fever vaccination with Q Vax® is only available to “at risk” populations in Australia. This vaccine was supported by the Australian government with the implementation of the NQFMP, which saw large numbers of workers in high risk occupations offered access to the vaccine. However, results previously described in Chapters, 3,4 and 5 of this thesis have highlighted that other populations within Australia, including children, were also at risk of contracting the disease, and hence need to be considered in future vaccination strategies.

Candidates with previous exposure to *Coxiella* are at increased risk of hypersensitivity reactions if vaccinated with Q Vax® [18]. Q fever skin testing is traditionally used to measure *in vivo* cell-mediated immunity to *C. burnetii* in pre-vaccination screening as the primary immune response indicator, to exclude individuals with pre-existing immunity. The skin test has major procedural drawbacks, and can be troublesome for the tested subjects, particularly children. Even more important is the response induced by the *in vivo* skin testing. The injection of *C. burnetii* antigens, may cause higher antibody titres and higher *in vitro* levels of IFN- ɣ to *C. burnetii* in participating subjects, and therefore affect subsequent Q fever diagnostics [19].

The measurement of *in vitro* IFN- ɣ and IL-2 to monitor the treatment of chronic Q fever has previously been described[20]. In this setting, the discontinuation of antimicrobial therapy strongly depends on the result of follow up serology. However, in practice the slow serological decline results in longer treatment, and the need for additional biomarkers to monitor treatment of chronic Q fever is evident. In this respect the measurement of CMI, specifically the production of IFN- ɣ from stimulated T cells, against *C.burnetii* plays a pivotal role.

Following the recent outbreak in The Netherlands the demand to monitor patients for the development of chronic Q fever is ever increasing, as this stage of the disease carries a high mortality rate[21]. With over 4000 cases of acute Q fever having been reported in The Netherlands, a diagnostic tool to predict chronic Q fever has become imperative. The use of CMI to measure the cellular response can lead to a more effective diagnosis and treatment response.

The relevance of measuring IFN- ɣ production profiles, as diagnostic correlates of memory T cell responses, has been studied outside the field of Q fever, and is reflective of the immune response induced by intracellular pathogens such as *Coxiella*. Therefore, the CMI response that occurs as a result of natural infection with *Coxiella* was considered to be a more suitable mechanism for measuring previous exposure to *C.burnetii,* than the traditional skin test.

The findings in this study showed that an ELISPOT assay developed to measure IFN- ɣ production in PBMC's was, potentially, a more suitable method to determine pre-existing immunity to *C.burnetii*. The results obtained with a limited number of vaccine exposed subjects, showed a potential for this assay to be implemented as a pre-vaccination screening tool to replace the skin test. The use of the ELISPOT assay for the determination of previous exposure has many advantages over the current skin testing regime. The ELISPOT assay can be repeated in order to ascertain a true exposure profile. This is a limitation of the skin test, which, on first use will initiate a low level immune response with activation of T cells. Subsequent use of the skin test in these subjects may lead to an interpretation of previous exposure, and hence preclude them from effective vaccination against *Coxiella*. There is also the high risk of the vaccine candidate suffering an adverse reaction with the administration of a follow up skin test. This exposure may also give rise to an antibody response which is producing short lived heightened results[19]. The value of being able to simulate a CMI response with *Coxiella* antigens using the ELISPOT assay, allows for perpetual monitoring of an exposure response and hence aid the decision for vaccination.

Although the results of this study were promising, clearly it had some limitations. First of all, only a small number of subjects were studied, and a larger cohort of samples needs to be examined to determine the practical utility of this method for pre-vaccination screening. Such a study has commenced in our laboratory, under my supervision, seeking to determine the immune status of 200 veterinary students and controls before the administration of the Q Vax® vaccine. The results of the ELISPOT will be correlated with serological markers and the results of skin tests. However, this larger study is ongoing, and has not yet generated enough data to be included in the study described in this thesis.

Secondly, this study was performed on the vaccine induced immune response to one strain of *Coxiella*, the Henzerling strain, administered in the Q Vax® vaccine. The CMI

immune response that is produced by naturally infected or exposed subjects may potentially be different to that measured in the CMI ELISPOT assay from a vaccine induced immune response. The response elicited may also vary depending on the strain type of the infecting bacteria. These factors will also need to be further investigated to ensure the ELISPOT method described here will accurately reflect exposure status and help protect vaccine candidates from wrongly receiving the vaccine and hence potentially enduring adverse reactions.

Thirdly, although the level of IFN- ɣ produced was quantifiable in terms of number of cytokine-producing cells, this will vary depending on the individual's effective T cell response. However, by using each subject's own T cells as both a positive control to assess an individual's T cells ability to produce IFN- ɣ with the PHA stimulus, and as a negative control which aids in the assessment of non-specific stimulation and a baseline to which a direct comparison can be made, the level of IFN- ɣ produced as a direct result of the *Coxiella* stimulus can be determined.

Clearly, there is a need to determine the cut-off for the assay to give an indication of the level of expression that is indicative of previous exposure to *Coxiella*. Also, it was shown here that subjects tested, including the naïve subject S3, produced some cytokine release from cells that were not stimulated with antigen, thus potentially confounding the interpretation of results. The background cytokine expression observed was presumably due to non-specific stimulation of the T-cells, or perhaps a low level of previous exposure to *Coxiella* antigens which were not detected by the skin test in subject S3.

For that reason the n-fold score was applied in this study and potentially proved to be a reliable predictor of immune status against *Coxiella* for the subjects examined. From the limited number of subjects investigated, there was a clear increase in n-fold score associated with subjects previously vaccinated against Q fever when compared to the non-vaccinated subject. In fact this indicator proved to be more indicative than relying on the presence of IgG antibodies as an indicator of previous exposure. Subjects S3 who was naïve, and S4 who had been vaccinated approximately 10 years previous, both failed to show a presence of *Coxiella* IgG. Yet S4 showed an n-fold score that was indicative of previous exposure and S3 did not.

To establish an appropriate cut-off is extremely important for determining previous exposure and hence aid in the administration of the vaccine. As shown by the results above, some subjects that are considered naïve to previous exposure may have a low level of IFN- ɣ production which could be the result of previous low level exposure to the bacteria from the environment or perhaps the direct response of the macrophages and T cells to the pre-stimulation of cells with *Coxiella* antigens in a previous skin test. An appropriately validated ELISPOT method would prove invaluable to identify such individuals and would aid in the selection of vaccine candidates.

The ELISPOT method described will aid greatly in identifying those individuals with a CMI response to *Coxiella*, and hence assess their suitability as potential Q Vax® candidates. In addition, the accurate assessment of previous exposure will reduce the number of adverse reactions that may result from vaccinating previously exposed individuals. It also offers a safer method over traditional skin testing, along with reducing the inconsistencies associated with interpreting skin test results. Finally, it would be a major improvement for the pre-vaccination screening of children who are more prone to adverse reactions following skin testing, and suffer with more serious complications. However, the prevention of adverse reactions and truly addressing the lack of confidence surrounding the current pre-vaccination screening, would require the development of a safer vaccine.

9.6. Significant Outcomes from this Chapter

- Assay parameters were determined to assess pre-existing exposure to *Coxiella* antigens using an in house ELISPOT assay.
- The ELISPOT assay successfully identified three subjects who had differing intervals of previous exposure, and proved to be more accurate than the assessment of IgG antibody status.
- The calculation of an n-fold index proved to be a valuable tool for calculating the differential of cytokine release between activated memory T cells and non-specific reactions. The use of this factor allowed the accurate categorisation of previous exposure versus naïve status. To our knowledge this study is the first to use this calculation for the assessment of *Coxiella* immune status.

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Chapter 10

General Discussion

10.1. Epidemiology of *Coxiella burnetii* in Queensland

This study was commenced at a time when there were very little data regarding Q fever in Queensland. The primary aim was to gather sufficient information about the true extend of the disease in the Queensland community, and from this formulate a recommendation for an appropriate vaccination strategy that would benefit the community as a whole.

Specifically, the work performed in this thesis aimed to extend the state of knowledge regarding Q fever in Queensland, and was the first comprehensive study which examined the epidemiology of *Coxiella burnetii* and sought to determine potential sources of infection and factors which might influence the correct diagnosis of the disease, and their impact on determining accurate exposure rates.

Q fever has been reported globally, and this study also confirmed that Q fever disease was a significant health threat in Australia and in particular Queensland. Yet despite this fact, Australia has the only licenced vaccine available for humans, but this is currently restricted to those identified as being at high risk of Q fever, through their occupational practices.

The results of this investigation determined that not only is Q fever an important clinical disease occurring in rural Queensland but that the disease was being reported at a higher than expected rate in cities and urban communities. Queensland reported the highest average yearly notification rates compared to any other location in the world[1]. The results here showed that high rates of notification occurred in both subjects identified as being at high risk of contracting Q fever, and those for which no known risk factors were identified. Alarming were the numbers of increasing notifications that occurred in children from Queensland. These high notification rates might be the result of increased clinical vigilance and/or more widespread laboratory screening for the disease, or simply the presence of higher and wider sources of exposure, such as higher bacterial loads in the Australian environment.

Previous notification data for Queensland have been restricted to only very small “snap shot” studies centred on the introduction of the vaccine and the implementation of the NQFMP. The comprehensive data obtained in this study, assessed a number of factors that could explain the high numbers of notifications that were observed in the Queensland population.

High notifications recently occurred during an outbreak of Q fever in The Netherlands. This country recently endured a large scale epidemic for which the close proximity to infected animals and farms played a key role for the dissemination of the bacteria, resulting in the reporting of thousands of animal and human cases. In Queensland, vast distances span localities and communities, compared to regions in Europe and in particular The Netherlands. Queensland boasts huge geographical separation between its communities, towns and cities. This large physical separation could be considered a control measure which limits the dissemination and distribution of *Coxiella*, leading to less disease and fewer notifications. Nevertheless, in spite of these large geographical distances separating communities, Queensland continues to report large numbers of cases from all over the state consistently with time, in spite of employing large scale, government funded vaccine campaigns targeting at risk populations.

The study in this thesis emphasised the importance that disease awareness played in the recognition and diagnosis of Q fever. Even with the large government campaign for Q fever vaccination and its associated educational program, there was again an increase in notifications and disease. The increase in awareness did not only apply to the medical fraternity, but also involved the rural community generally. However, there was an alarming increase in the number of notifications reported from females at two specific time points in Queensland, which could again be explained by the awareness in the community raised by the education program that was associated with vaccination. Very recently, community awareness has been raised even further in Australia, through media reports on the Australia Broadcasting Commission television programs highlighting the seriousness of Q fever disease in the context of the recent epidemic in The Netherlands. This increased awareness, based on recent findings, including publications that have arisen from the study described in this thesis, will contribute towards the formulation of Public Health policy for an effective vaccination strategy for the population at large.

However, Q fever notification data alone were not a true reflection of the extent of exposure occurring in the Queensland population. This could only be assessed accurately by examining the seroprevalence in the population. To date this study is the first extensive investigation examining the prevalence of *Coxiella* antibodies in the Queensland population.

Taken together, the data from the seroprevalence and notification studies showed surprising results, which have an impact on the perception of the risk of Q fever in the community. Both studies showed that the risk of exposure and subsequent disease is not just confined to rural communities, but also poses a significant risk to people living in urban or metropolitan regions. This fact, together with the observation that high rates of *Coxiella* exposure occurred in low risk populations, raised the question about sources of *Coxiella* exposure, other than those traditionally associated with ruminants and farming activities.

It is widely accepted that large ruminants, such as cattle, sheep and goats, are the most common source of *Coxiella* infection in humans. Also, a recent seroprevalence study in Northern Queensland showed native macropods should be considered as potential sources of infection. This thesis presented data identifying potential sources of *Coxiella* exposure never before explored, and was the first study in Queensland that demonstrated the presence of *Coxiella* DNA in domestic animal samples, and also detected *Coxiella* DNA in ticks removed from both domestic and native animals.

Moreover, it was the first report to identify the presence of *Coxiella* in native flying foxes. Flying-foxes have an important ecological role because their feeding behaviour helps disperse seeds and spread the pollen of native plants. Flying foxes are known to travel long distances and excrete faeces and bat spat (indigestible seeds and fruit in spit or saliva), this increases the distribution of *Coxiella* from those animals that are infected with the bacteria, and may be directly responsible for the spread in the general environment. The close association of flying foxes and horses has been well established during investigations into transmission of the deadly Hendra Virus, and it seems highly likely that the high incidence of *Coxiella* in horses reported in Chapter 5 of this study may be the result of this co-habitation. This general spread of *Coxiella* in the environment would also explain the high detection rate observed in other animal species, and the higher than anticipated exposure observed in humans in low risk environments, such as urban populations where no direct animal contact was recorded. The high rates of Q fever disease reported in this thesis, occurring in populations in Queensland previously not considered for vaccination due to the perceived low risk of exposure, may now be attributed to other sources such as domestic pets and local wildlife.

Queensland, and in particular South East Queensland and the city of Brisbane, enjoy an all year round climate well suited for outdoor living. This in turn sees many Queenslanders

spend time in outdoor rooms, patio and or decks, which in turn provide shelter and living alternatives for local animals and wildlife. The close proximity to these animals can lead to the exposure of subjects to *Coxiella* through the direct contact with infected animal faeces, urine or even birthing products. Also, an increased risk arises from the aerosolization of contaminated soil and dust particles through either the use of vacuum cleaners and garden blowers or through the other cleaning mechanisms such as using high pressure water hoses which create fine spray and mist. With drought conditions prominent over many years in Queensland, the introduction of such devices to remove dust, dirt and unwanted animal products without the use of water, employing wind forces such as blowers has increased. These practices provide the potential for animal owners to distribute or aid in disseminating *Coxiella* from infected domestic animals in addition to the spread by natural forces.

There has been a recent re-emergence of interest in Q fever in the Australian media and again there have been many cases reported where a clinical diagnosis of Q fever has been made in the absence of evidence of animal contact. Queensland is situated in a sub-tropical region which makes perfect climatic conditions for the growth of plants and grasses. This sees many Queenslanders cutting household lawns on a regular basis. This activity can aid in the dispersion of contaminated animal faeces along with creating aerosols from which *Coxiella* can be inhaled. There have been other diseases in which the distribution of pathogens into the general environment has been created using machinery. The recent tunnelling and excavation works performed in Brisbane, directly adjacent to the Royal Children's Hospital at Herston, saw an alarming increase in the number of oncology patients acquiring aspergillus infections. Such similar activities also occur on the outskirts of metropolitan areas with the creation of new housing development. These areas are typically land parcels that have previously been used for grazing cattle or rearing farm animals, and dispersal of soil from these areas carries an increased risk of releasing dust-borne *Coxiella* into the immediate environment.

The infection of Q fever in humans is widely associated with the inhalation of contaminated dust, yet there were no studies in Australia that investigated dust as a potential source of infection. The studies that have been performed have been restricted to environments considered to be at high risk of potential infection such as shearing sheds and barns. Therefore this was the first study to investigate the presence of *Coxiella* in dust collected in

air samplers from a number of locations throughout Queensland, and proved the potential for dust from the general environment as a source of infection to humans.

However, there was a study performed in the USA that showed high levels (24%) of *Coxiella* DNA present in soil samples, yet these results conflicted with low notification rates (51 cases annually) and the results of seroprevalence studies for that country which reported low prevalence rates (2.5%). This disparity may be the result of animal-derived reagents utilised in the assays, such as bovine serum albumin. These reagents were shown previously to be contaminated with *Coxiella* DNA as they are derived from animal origin. This was highlighted recently in The Netherlands outbreak as part of a larger investigation[2],and would explain the discordances seen between high rates of *Coxiella* detected in environmental samples and the low seroprevalence and the low incidence of clinical disease reported in the USA.

10.1.1. Molecular Epidemiology of Coxiella

This was the first study to identify unique genotypes of *Coxiella* present in samples collected in Queensland. There were also genotypes that were closely related to those previously identified in NSW, the state reporting the second highest notification rates globally. In addition, samples were identified with similar allele profiles from both animal sources and from clinical human cases proving that animals play a role in the chain of infection of humans.

The geographical isolation of Australia has allowed for the clonal expansion of unique strains of *Coxiella*. This may have resulted in the development of genotypes with higher pathogenicity or perhaps hyper-virulent strains, as occurred in The Netherlands, and hence may account for the higher rates of disease notified in Australia and Queensland.

10.2. Clinical Presentation and Diagnosis of Q Fever

Q fever is a disease that is under-recognised globally. This also was the case in Queensland, even though the state has the highest notification rate in the world. There are a number of factors that may contribute to this observation, and these were explored in this study. Typically in urban settings of Queensland a patient presenting to a general medical practice with symptoms of flu-like illness would receive a provisional diagnoses of a viral infection, which is self-limiting and the treatment would be rest and recovery. It

would only be upon representation with persisting symptoms that subsequent laboratory investigation be requested for respiratory pathogens only and these generally would not include Q fever screening. In many cases a diagnosis was not made or at worst a misdiagnosis might occur. However, even if Q fever investigations were initiated, the diagnostic algorithm currently includes specific IgM and IgG antibody detection with the ELISA assay, which this study has shown to be less effective than the IFA and PCR at diagnosing acute Q fever.

Also, this study has highlighted the importance to again raise awareness of the disease in the clinical community, and for the need to modify the current diagnostic protocols to include PCR testing for early acute infections which, with the current algorithm, were not detected. Undetected cases may result in more serious medical complications including the development of chronic Q fever or chronic QFS. Chronic QFS is a debilitating and long lasting illness that places a huge burden on the physical and mental health of the patient, along with compounding financial implications associated with long-term health care. It has been calculated that significant annual income is lost to both industry and personal income as a result of Q fever infections, particularly chronic infections and chronic QFS. In fact the largest workers compensation claim in Australia has been awarded to an abattoir worker diagnosed with chronic Q fever.

10.2.1. Unusual Clinical Presentations of Q Fever

Many cases of Q fever are diagnosed in Queensland annually, however, this thesis highlighted the significant impact that the disease can have on individuals, families and the wider community. Chronic Q fever in children is a rare disease and this study described an acute infection in a child that progressed to chronic disease which was complicated by an underlying cardiac condition. The inclusion of children in an effective vaccine program would have prevented extensive medical care required by this patient for the rest of their life.

This study also identified infections occurring in children as a result of indirect exposure to the bacteria. One of these studies included the investigation into a family which all contracted Q fever within 3 weeks of each other. The source of infection was never identified. It seems likely that the male adult may have indirectly infected some family members; however,it seems unlikely that he would have infected all members of the

families given the ages of 3 years to adult. This case study may be a rare case of person to person spread of the infection. There has only been one other case of person to person transmission recorded and that was again among a family[3]. If this were another case of person to person spread, then it highlighted the infectiousness of *Coxiella* and the risk it posed to entire families who have one family member working in a high risk occupation.

Another unusual case identified in this study was the detection of a unique Australian strain of *Coxiella* in the placenta of a woman who delivered a stillborn child. This genotype has only previously been detected in Australia. Reports previously published, have identified the risks associated with pregnant women contracting Q fever, and have included the development of chronic Q fever and the high associated risks of miscarriage and abortion. These data also recognised the significance that *Coxiella* strain types have on the outcome of the pregnancy[4]. The carriage of *Coxiella* during pregnancy, not only poses a risk to the foetus, but also is a potential risk to the medical staff and midwives involved in the care of the patient when termination or delivery is performed. There have been previous cases reported where human to human spread has occurred during a delivery[4,5]. These risks are also factors associated with mortuary staff whilst handling the deceased and preparing autopsies and bodies for burial.

A case of Q fever, with a genotype unique to Queensland, originating from an Australian indigenous male was detected in this study. There have been very limited data reported regarding the rate of *Coxiella* infection in the indigenous population of Australia, and there has been speculation that Q fever in this ethnic group occurs less frequently than in the Caucasian population. The Northern Territory of Australia has the highest indigenous population (30% of the population) and its economy relies heavily on the cattle industry, yet no cases were reported before 2002. There have been 10 cases reported since, with 4 diagnosed in indigenous patients.

The indigenous population of Australia lead an almost nomadic lifestyle, living very close to the land and the animals that share it. From birth, members of this population group are constantly exposed to low levels of the bacteria from the environment and hence may have developed an immune response which prevents the development of acute Q fever. To investigate this hypothesis, a seroprevalence study of *Coxiella* in this population of Australians needs to be conducted.

The need for wider distribution of the vaccine was very clearly demonstrated by a severe case of Q fever detected in a wildlife park ranger working in a Queensland National Park. This patient was extremely ill with life threatening disease requiring intensive medical care, with the subsequent development of medical complications including chronic fatigue syndrome, and faces the prospect of never fully recovering from the disease[6]. This case reinforced the fact that community and medical awareness must be extended to consider the risks of all subjects working with animals, or environments where animals reside, and reinforced the need to extend government funding for the vaccination program, which would prevent such occurrences.

There was no question that Q fever is a serious disease that has a significant impact on the health of many Australians. It is responsible for significant morbidity which can be related directly to a substantial extra burden on the health care system. Many patients are incapacitated for weeks and even months, and with the development of chronic QFS, many go on to endure long term sequelae as a result of Q fever infection. The potential impact of Q fever on the Australian society has recently been brought to the public's attention by a number of media stories aired on television by the Australian Broadcasting Commission following awareness in the media of Q fever and its effect on the population in The Netherlands. With an expanding interest in goat farming, Australia is potentially facing an increased risk of Q fever epidemics in this sector, which in turn poses a substantial risk to the population at large. These media stories highlighted the debilitating nature of both the acute and chronic states of the disease, and featured some of the "hardest" outback men quoting "I would have been happy to die when I was lying in hospital with Q fever. I was that sick I couldn't have cared if I'd died". Another patient interviewed, who contracted Q fever, was subsequently placed on life support and has never fully recovered[6].

10.3. Q fever in Children

At the time of commencement of this study, there was very little known about Q fever in Queensland children and the risks associated with contracting the disease. This study was the first to identify accurately the number of children contracting Q fever, and in doing so has identified an increase in those numbers over time. This study also showed that the disease was not limited to rural children living in close proximity to livestock, but might affect children living in cities equally. Children residing in both high and low risk regions

were being exposed at the same rates. However, children in both locations are not included in the national vaccination strategy, and with a possible increase in *Coxiella* dissemination through dust and wildlife, might be at increased risk of infection as reflected by the increasing numbers of paediatric cases.

Q fever disease is not well understood in the paediatric populations both globally or in Australia. Children are often exposed to the bacteria; however, the rates of infection reported in this group were low. Often children tend to have much closer contact with animals and pets than adults, and this may be how children can be exposed to *Coxiella* and acquire infection. Children, more so than adults, regularly present with symptoms including fever and respiratory like illnesses for which they are given the tentative diagnosis of "flu" or a "flu-like illness and no laboratory investigations are requested. Or the child is under investigation for a respiratory disease for which there is no laboratory conformation and the infectious etiological agent is never identified. However, with the recent reviews of Q fever disease in children, there is a general push for a safe and effective vaccine to be developed as many rural children cannot avoid the potential exposure to the organism from a number of different sources. With this fact in mind, some rural practitioners have chosen to vaccinate younger children who fall outside the recognized target group for vaccination, and who are at high risk of exposure to the disease[7].

The data presented throughout this thesis highlighted the risk of Q fever infection to children. The recent media coverage confirmed this, and specifically describes cases of Q fever in children, even profiling severe cases in which the children had long term medical follow-up and complications. Yet, despite these warnings, a licensed vaccine and a comprehensive vaccine strategy for children is still lacking.

10.4. Preventing Q fever in the Future

The need for a comprehensive vaccination strategy, which encompasses all sectors of the Australian population, is indisputable. Yet, despite having an effective vaccine against *Coxiella*, it is limited to adults at risk of infection and, until this study, there was no data regarding other populations that may be at risk of infection, especially children. This thesis has identified a number of risk factors for the Queensland population in regards to the

transmission of *Coxiella*, and has shown there is a need to protect the children of Queensland who are at risk of the disease, either as a result of geographical or residential location, or because of a family member working in a previously recognised high risk occupation.

The current, licensed vaccine in Australia, Q Vax®, has not been formulated for children, and has been identified as having potentially severe adverse reactions as a consequence of receiving the vaccine. In addition, current pre-vaccination screening is inadequate to assess the true rate of previous exposure in vaccine candidates.

Therefore a successful vaccination strategy would need to address these issues before a comprehensive preventative program can be initiated. This would need to include the development of a new vaccine, perhaps a recombinant antigen, which would limit the side effects associated with the current whole-cell vaccine, and the introduction of a pre-screening procedure that would accurately assess pre-existing immunity. Yet, so far there have been no developments in this area.

The need for a better pre-screening protocol was recognised at the commencement of the study described in this thesis, and it was deemed important to include the development of such an assay in this study. As a result proof of principle was established for an assay which used a cell meditated immunity assay for the assessment of pre-existing *Coxiella* exposure of vaccine subjects. This assay was a major improvement, and will also aid in screening children for *Coxiella* immune status. Also, this assay will prevent potentially dangerous hypersensitivity reactions in subjects with previous *Coxiella* exposure who are subjected to the bacterial antigens during the current skin test administration. Another major advance in using the CMI method is that it would reduce the subjectivity or incorrect interpretation associated with the skin test, and hence improves the pre-screening procedure.

10.5. Conclusions

Never before has a comprehensive study been conducted that identified the true exposure rate to *Coxiella burnetii* in the Queensland population. This thesis has provided a significant contribution to narrowing this knowledge gap, and identified those populations at risk of the disease along with previously unrecognised potential sources of infection.

Also, based on the outcomes from this study, a revised diagnostic approach is recommended that will improve the accurate identification of disease across all population groups. Furthermore, the discovery of novel genotypes in Queensland may aid in better understanding the extremely high numbers of notified Q fever cases in Australia, and will aid in the development of new effective vaccines.

So far, the occurrence of Q fever in children has been largely ignored, but this thesis clearly highlighted that this disease was a serious threat, and that children must be included in any public health preventative measures, including vaccination, that may be introduced in the future. To facilitate this, the cellular immune assay described in this study should be applied and will provide a safer and more effective mechanism for which pre-screening of vaccine subjects can be performed.

Taken together, the comprehensive data presented in this thesis will make a significant contribution to raising the awareness of Q fever in Queensland and Australia, and will help facilitate a more comprehensive approach to a preventative public health strategy that will ultimately lead to more effective control of this serious disease.

10.6. References

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