Characterisation of experimental Q fever infection and the detection of Coxiella burnetii antigens in urine

Thesis

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Characterisation of experimental Q fever infection and the detection of *Coxiella burnetii* antigens in urine

by

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of

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Abstract

Coxiella burnetii, the aetiological agent of Q fever, causes acute, chronic or asymptomatic disease in humans. Routine clinical diagnosis of acute and chronic Q fever primarily relies on serodiagnosis. Isolation of the organism is rarely attempted, as C. burnetii is known to be amongst the most infectious of bacteria.

This thesis aims to define and characterise a relevant experimental model of Q fever and establish whether C. burnetii-specific antigens appear in the urine of experimental animals. It is further intended to characterise any antigens excreted and assess urinary antigen detection as a method for the laboratory diagnosis of Q fever.

Experimental C. burnetii infection was established in the guinea pig using Lane strain (a previously uncharacterised British isolate from heart tissue of a patient suffering from Q fever endocarditis). The aerosol route of delivery resulted in a more severe disease compared to delivery by the intraperitoneal route and C. burnetii organisms were shown to persist in heart tissues for at least thirteen weeks following aerosol infection.

A capture ELISA assay was developed with a detection limit of 80 ng antigen ml\(^{-1}\) (equating to approximately 800 organisms ml\(^{-1}\)). C. burnetii-specific antigens appeared in the urine of animals, infected by aerosol with approximately 1000 organisms and 1-10 organisms, at 1 and 10 days post-infection, respectively. During subsequent characterisation of the urinary antigens in severely diseased guinea pigs, viable C. burnetii organisms were detected and a C. burnetii-specific immunoreactive protein with an approximate molecular weight of 62 kDa was demonstrated. Evidence also suggested that LPS was present in the urine.

The appearance of antigen in the urine was used as a marker of infection to assess the efficacy of doxycycline and ciprofloxacin in the treatment of experimental Q fever.

Preliminary studies supported other data (febrile response, serology and PCR) which suggested that doxycycline was more effective than ciprofloxacin in the treatment of acute experimental Q fever.
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I am indebted to the following people for their generous scientific and technical contributions to this thesis. Dr. Graham Hall for histopathology analysis and Mr. Roger Cook and Mrs. Susan Gray for expert histology skills, Mr. Barry Dowsett and Miss. Sheila Davies for electron microscopy and photography, Mrs. Katherine West for the molecular biology studies, Mr. Julian Howells, Mrs. Wendy Freemantle and Dr. David Rutter for help and advice throughout.

Finally, I would like to thank my parents and family and especially my wife Margaret for their patience, support and continued encouragement throughout.
Dedication

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Margaret and Anna
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CHAPTER 1

General Introduction to Q Fever and Coxiella burnetii

1.1 Historical Background

In August 1935, Dr. E.H. Derrick, director of the Laboratory of Microbiology and Pathology at Queensland Health Department in Australia, was asked to investigate an outbreak of undiagnosed febrile illness amongst abattoir workers in Brisbane. He discovered that guinea pigs when inoculated with either blood or urine from these patients became febrile, but was unable to make a diagnosis and therefore called the illness "Q (for Query) fever" (Derrick, 1937). Sir MacFarlane Burnet, a virologist and director of the Walter and Eliza Hall Institute, was then approached by Derrick in an attempt to identify and isolate the cause (Burnet & Freeman, 1937). From a sample of infected liver sent to him by Derrick, Burnet was able to infect guinea pigs and mice and identify a "typical rickettsiae" which was unlike typhus, scrub typhus or spotted fever rickettsiae.

At about the same time, an organism believed to be a rickettsia, and capable of passing through a bacteriological filter, had been isolated from the tick Dermacentor andersoni near Nine Mile Creek in Montana by Dr. Herald Cox and Dr. Gordan Davis at the Rocky Mountain Laboratory in Montana (Davis & Cox, 1938). Following a visit to this laboratory in 1938 to review their findings, a Dr.
Rolla Dyer (identified later as patient X) became ill with retroorbital pains, fever, chills and sweats; symptoms similar to those described by Derrick at the Brisbane outbreak. An organism was isolated from his blood by inoculation and passage in guinea pigs. After staining infected spleens from these animals and performing cross-immunity tests between isolate (X) from Dyer and murine typhus, epidemic typhus and Rocky Mountain Spotted Fever (RMSF) to exclude them as possible causes, the same organism was identified as that isolated from the ticks by Cox and Davis.

Burnet subsequently sent Dyer spleen tissue from mice that had been experimentally infected with the original Q fever organism from the Brisbane outbreak and Dyer demonstrated that this organism was identical to those isolated from the ticks and his blood, and hence linked the Nine-Mile agent to Australian Q fever (Dyer, 1938). Cox named this organism *Rickettsia diaporica*, (Cox, 1939) to describe the rickettsial features of the organism with the ability to pass through bacteriological filters and Derrick named the organism *Rickettsia burneti* (Derrick, 1939).

The highly infectious nature of *Rickettsia burneti* gradually became evident during the 1940’s as several outbreaks were identified at laboratories, meat packing premises and amongst troops based in Greece, Italy and Corsica. During the same decade sheep, goats and cows were identified as a reservoir for the organism and
raw milk was demonstrated as a potential source of infection (Huebner et al., 1948).

By 1948, it had become apparent that *R. burnetii* possessed properties which made it distinct from other rickettsioses and it was eventually placed into a separate genus, called *Coxiella* with the single species *burnetii* (Philip, 1948)

### 1.2 The Organism

*C. burnetii* is an obligate intracellular pleomorphic coccobacillus with a Gram-negative cell wall. It measures approximately 0.3 by 1.0µm and has a complex developmental cycle involving vegetative growth by binary fission and the production of endogenous-like spores by unequal cell division. Two distinct cell types have been identified: a large-cell variant and a small-cell variant. These two cell types are characterised by their ability to metabolise exogenously supplied substrates, the peptidoglycan content of their cell walls, size, and sensitivity to osmotic shock (McCaul, 1991). The developmental cycle begins by attachment of the small-cell variant to the host cell surface, then ingestion into the hosts’ phagosome where fusion of the primary lysosome and the subsequent acid environment activates vegetative growth. At this stage, *C. burnetii* cells become larger and more elongated (large-cell variant) and are more susceptible to osmotic lysis and are capable of metabolising exogenous substrates. As infection of the cell progresses, asymmetrical division or sporogenesis of the cells begins to occur,
possibly as a result of exhaustion of available nutrients. Once released from the mother cell, continued development results in the original, resistant and metabolically inactive small-cell variant. The host cell eventually lyses and these small-cell variants of *C. burnetii* are released into the extracellular environment (McCaul, 1991). This cycle was confirmed by studies *in vitro* in the yolk sac of chick embryonated eggs and in cell culture and *in vivo* in the heart valves of endocarditic patients (McCaul *et al.*, 1994).

The formation of endogenous spores enables *C. burnetii* to be resistant to a range of stresses including acid pH and lysosomal enzymes within the eukaryotic cell, and dessication, UV-light and temperature in the external environment (McCaul *et al.*, 1981).

*C. burnetii* exhibits phase variation which was initially identified by immunological methods. Two different phases of the organism have been identified: phase I and phase II. The naturally occurring virulent phase I organism when passaged several times in embryonated chicken eggs gradually convert to the avirulent phase II organism. This process is thought to be mutational in origin and results in the loss of enzymes required for the synthesis of lipopolysaccharide (LPS) (Williams, J. C. & Waag, 1991) and although no morphological changes occur, differences have been demonstrated in the sugar content (Marrie, 1995). This phase change has been compared to the smooth-to-rough phase variations seen in members of the family Enterobacteriaceae. Organisms such as *Escherichia*...
coli and Salmonella typhimurium exhibit phase variation and conversion from the smooth (virulent) form to the rough (avirulent) form affects the amount and distribution of proteins in the outer membrane. Thus, major envelope proteins which are localised in the outer membrane are reduced by the mutational conversion from smooth to rough LPS. A similar finding was demonstrated in C. burnetii where the $^{125}$I-labelling of surface proteins in the outer membrane was decreased in phase II organisms as compared to phase I organisms (Williams, J. C. & Waag, 1991).

Other differences between the two phases include the differential uptake by phagocytes. Phase II organisms are taken up more readily than phase I organisms, and contain about one-tenth of the extractable amount of LPS than that found in phase I organisms. It is this fact which may explain the reduced rate of uptake of phase I organisms by phagocytes (Baca & Paretsky, 1983).

1.3 Taxonomy

C. burnetii is the sole species of its genus, and although significant differences between the various isolates are known, these are not sufficiently great to subdivide these strains into more than one species (Mallavia et al., 1991).

On the basis of 16S rRNA sequence data, C. burnetii falls into the gamma subdivision of the Proteobacteria (formerly known as purple bacteria), which are
distantly related to *Legionella*. Other bacteria in this subdivision include *Chromatium vinosum, Escherichia coli, Legionella pneumophila,* and *Pseudomonas aeruginosa*. The only other rickettsia placed in the gamma subdivision is *Wolbachia persica*. Like *C. burnetii*, *W. persica* was first isolated in chicken embryos from a tick, *Argas persicus*. Both *C. burnetii* and *W. persica* grow within the phagolysosome of an infected host cell and each has an ecological niche as an intracellular parasite. Unlike *C. burnetii* however, growth of *W. persica* has only been accomplished in yolk sacs of fertile hens eggs, cell culture and arthropods but not in vertebrates (Weiss *et al.*, 1991).

Other rickettsia such as *Rickettsia prowazekii, Rickettsia typhi* and *Rickettsia rickettsii* fall into the alpha subdivision of the proteobacteria and show very close sequence homology and evolutionary distance between each other (Weisburg *et al.*, 1989).

Apart from sharing the same phylum in the classification system, there are other similarities which are shared by *Legionella* species and *C. burnetii*. Both organisms are intracellular parasites, *Legionella* being facultative and *Coxiella* being obligate. Both have adapted, however, to a site specific microenvironment within the interior of eukaryotic cells. *Legionella*, in order to survive intracellularly inhibit phagosome-lysosome fusion (Horwitz & Maxfield, 1984), whereas *Coxiella* has adapted to the acid and lysosomal enzymes within the phagolysosome to survive (Moulder, 1985).
C. burnetii exhibits phase variation (see section 2.2) where phase II organisms are laboratory-derived and avirulent. A similar loss of virulence can be detected in *Legionella* after continued passage on buffered charcoal yeast extract media, or single passage on Mueller-Hinton media (Jepras *et al*., 1985).

*Legionella* infection can present in two forms: Pontiac fever, a self-limiting disease which is usually less severe, and acute disease which presents as a pneumonia, and which is more severe and can result in death. *C. burnetii* displays similar variation in pathogenesis with disease ranging from acute self-limiting infection to chronic disease resulting in endocarditis, hepatitis and often death (Marrie, 1990a).

Structurally, *Coxiella* and *Legionella* both contain lipopolysaccharide (LPS), phospholipid and protein as major constituents in their cell walls, and each have very similar fatty acid profiles (Tzianabos, 1981).

1.4 The Disease in Animals

*C. burnetii* is a zoonotic infection and affects a wide variety of animals. This represents the transmission of the organism within the environment, from tick-borne transmission to wildlife such as rodents and birds, to the aerosol transmission more commonly associated with livestock such as sheep, cows and goats. The transmission of Q fever to humans is primarily from infected livestock. Evidence for this is provided by the high incidence of Q fever among abattoir workers,
farmers and veterinarians and the correlation between increasing dairy herd infection rates and human Q fever statistics in a number of countries (Marrie, 1990b). In addition, sporadic outbreaks of Q fever have occurred as a result of indirect exposure to contaminated straw, manure and dust from farm vehicles (Salmon et al., 1982) and a number of studies have implicated residency in a dairy area as a risk factor for Q fever (Huebner and Bell, 1951).

*Coxiella* infection in animals rarely produces illness but results in various responses to the organism. In sheep, organisms concentrate in the placentas and can be responsible for abortions and consequent spread of *C. burnetii* by aerosol transmission from parturient material. Some evidence suggests that *Coxiella* infection in sheep is transient (Abinanti et al., 1953; Williams, J. C., 1991). Infected sheep, two months after lambing, placed with healthy animals for five months were found no longer infectious After the second lambing, no *C. burnetii* were isolated from their blood, placenta or body secretions (Lang, 1990). Other studies which have concentrated on the measurement of the humoral antibody response by complement fixation (CF) in sheep demonstrated a steady decline in titre over twenty weeks until all animals tested negative (Lang, 1990).

In cows and goats, infection by *C. burnetii* has increased over recent years (Williams, J. C., 1991). Unlike the transient infection in sheep, evidence suggests that *C. burnetii* infection in cows and goats is passed from generation to generation creating a reservoir for the organism.
In goats, *Coxiella* has been isolated from the mammary gland, spleen, kidney, uterus, liver, lung and heart and also from whey indicating that goats shed this organism. Observations from America indicated that naturally-infected and experimentally-infected cows were asymptomatic during *C. burnetii* infection. Evidence from France, Switzerland, and Germany demonstrated that cows infected with *C. burnetii* have higher rates of abortion, excretion of the organism in milk and weak off-spring (Lang, 1990).

The disease has been documented in a large number of animal species including cats and dogs and wildlife as well as livestock. In all these animals, however, coxiellosis is usually asymptomatic (Williams, J. C., 1991).

1.5 The Disease in humans and treatment

The development of a specific immune response follows vaccine administration or infection by *C. burnetii* in both humans and guinea pigs (Williams *et al.*, 1986a, Williams J.C. and Waag D. M., 1991). A specific humoral immune response can act in a number of ways to aid inactivation and clearance of an invading microorganism. Secretory IgA can interfere with the adherence of the microorganism to mucosal surfaces. This however, has not been investigated in Q fever infections. Serum IgG, IgM and IgA can act as neutralising antibodies, however this mechanism is ineffective in *C. burnetii* infections. Opsonisation,
where specific immunoglobulin G, M and complement bind to the surface of a microorganism, may enhance *C. burnetii* infection by facilitating uptake within phagocytes where replication within the phagolysosome and subsequent dissemination throughout the host can occur. The presence of immune antibody however, as well as facilitating uptake of *C. burnetii* by macrophages also accelerates the appearance of a cellular immune response.

The stimulation of cell-mediated immunity in humans is known to be the primary method of resistance to infection by *C. burnetii*. *C. burnetii*-infected euthymic mice were able to clear organisms from peripheral blood and spleen, fourteen days post-infection. In contrast, *C. burnetii* could be detected in the spleen of athymic (nude) mice for sixty days post-infection, even though both types of animals produced similar levels of antibody (Hall et al., 1981).

The primary characteristic of cell mediated immunity against *C. burnetii* in both guinea pigs and humans is the stimulation of differentiation and proliferation of lymphocytes leading to the appearance of bactericidal macrophages. Such activated macrophages present a more inhospitable intracellular environment for *C. burnetii* growth and hence infection can be controlled (Kishimoto, R. A. and Burger, G. T., 1977 and Williams J.C. and Waag D. M., 1991).

In humans, Q fever can be separated into three broad categories: the asymptomatic, the acute and the chronic (Baca & Paretsky, 1983). Of those individuals exposed to *C. burnetii* during an outbreak and on whom serological tests were performed, approximately 50% did not develop any clinical symptoms. Those individuals that do show clinical symptoms usually have an incubation
period of two to six weeks following exposure. The typical symptoms include an acute onset of high fever, chills with rigours, severe headache, retroorbital pain and general malaise. Some patients also suffer from vomiting, chest pain, diarrhea and coughing. The most consistent symptoms are high fever, usually greater than 38.5°C, hepatomegaly and splenomegaly (Meiklejohn et al., 1981). Unlike other rickettsial diseases, no rash is usually observed in C. burnetii infections.

Acute Q fever is usually described as an atypical pneumonia, although pneumonia is rarely seen in countries where Q fever is endemic. In other countries, however, pneumonia is the primary symptom. This discrepancy may be as a result of variation in host response, organism variation or differences in the route of exposure (Marrie, 1995).

Hepatitis, another symptom of acute Q fever, demonstrates varying rates of prevalence throughout the world, from 10% to 65% of cases (Reimer, 1993). The majority of cases of acute Q fever however, are self-limiting with symptoms resolving in 1 to 2 weeks. Approximately 1% of those individuals that are infected may not clear the organism entirely and can ultimately go on to develop chronic Q fever.

Chronic Q fever can present with a number of clinical manifestations but the most serious result of infection is endocarditis. It has been suggested that specific strains such as the Priscilla strain, originally isolated from a placenta of a goat, are responsible for chronic Q fever and other strains such as the Nine Mile strain
originally from ticks cause acute Q fever. Alternative theories propose that chronic Q fever results from an earlier asymptomatic infection or result from immunosuppression or pre-existing valvular damage (Williams, J. C., 1991).

Cases of Q fever endocarditis are prevalent in countries where acute Q fever is well known. However, the proportion of patients who suffer from Q fever endocarditis, out of the total number of Q fever cases reported, shows substantial country-to-country variation and may be due to the variation in diagnostic services available. Of those patients diagnosed as suffering from Q fever endocarditis, the majority reported pre-existing valvular abnormalities (Raoult et al., 1990a). In the U.K., Q fever is estimated to be responsible for approximately 1-2% of all cases of endocarditis.

Treatment of acute Q fever is usually successful and a number of antibiotics including lincomycin, erythromycin, chloramphenicol and several tetracyclines (Yeaman & Baca, 1990). Treatment of chronic Q fever however, is less successful with the most serious complication being endocarditis (Yeaman & Baca, 1990). Replacement of infected valves is often undertaken; however, replacement valves are often found to be re-colonised by C. burnetii at a later date. Re-infection is probably from organisms from an extracardiac site such as the liver or may be reactivation of organisms residing in the heart tissue itself. Antibiotic treatment of endocarditis with tetracyclines, the drug of choice, has been reported as being bacteriostatic rather than bacteriocidal and treatment must be prolonged. C. burnetii has been isolated from cardiac valves following prolonged treatment with
tetracycline and penicillin (Yeaman & Baca, 1990). The majority of patients suffering from Q fever endocarditis die from primary endocarditis, relapse or reinfection or heart failure resulting from persistent *C. burnetii* infection. Therefore, prognosis for a chronic Q fever patient is poor.

The differential response to treatment of patients with acute or chronic Q fever strengthens the argument that different strains may be responsible for each. The early diagnosis of *C. burnetii* infection may therefore help the efficient management of the disease (Fournier et al., 1998).
Between 100 and 200 cases of human Q fever are encountered annually in the UK (Aitken, I. D., 1987). The majority are sporadic but occasionally large outbreaks occur. Such outbreaks in the UK are shown in table 1.1.

### Table 1.1 Outbreaks of Q fever in the United Kingdom

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of cases</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Somerset</td>
<td>5</td>
<td>Jorm, L. R., <em>et al.</em>, 1990</td>
</tr>
<tr>
<td>Solihull</td>
<td>147</td>
<td>Smith, G., 1989</td>
</tr>
<tr>
<td>Oxfordshire</td>
<td>25</td>
<td>Winner, S. J. <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>Gwent, South Wales</td>
<td>29</td>
<td>Salmon, M. M.<em>et al.</em>, 1981</td>
</tr>
<tr>
<td>Staffordshire</td>
<td>No information</td>
<td>Brown, G. L. <em>et al.</em>, 1968</td>
</tr>
</tbody>
</table>

*C. burnetii* persists and is transmitted within the environment by a number of routes. These are summarised in Fig 1.1. The primary reservoir for transmission to man is by aerosol from domestic animals such as sheep, goats and cows and especially from parturient products. The placenta of infected ewes can contain up to $10^{12}$ organisms per gram, with the blood, urine and amniotic fluid also heavily contaminated (Marrie, 1990b). Q fever can survive in aerosols for long periods of time and is very resistant to dessication and UV radiation (Williams, J. C., 1991). During air-sampling experiments, *C. burnetii* was detected long distances from
parturient ewes and has been shown to have a very low infective dose (one organism can initiate infection) (Tigertt et al., 1961).

In scientific institutions where sheep have been used for research purposes, employees became infected from using a lift that transported infected sheep. In addition, a number of cases of Q fever were reported along the route taken to transport the sheep to the laboratory (Meiklejohn et al., 1981; Hall, C. J. et al., 1982) and (Curet & Paust, 1972).

The ingestion of raw milk or unpasteurised goats cheese has also been shown to result in seroconversion but not infection. This was demonstrated in a number of trials where human volunteers drank C. burnetii infected milk and a small percentage of them developed specific antibodies (Editorial, 1950).

The other pathways of transmission of C. burnetii are less important in the transmission to man; however, they play an important role in the perpetuation of C. burnetii in the environment. Infected ticks can spread the organism via percutaneous transmission to domestic animals and wildlife such as deer and rodents, while infected tick faeces may be aerosolised presenting an additional method of transmission (Marrie, 1990b).

C. burnetii has been isolated or identified by seroconversion in man and livestock in most parts of the world. The rate of human cases of Q fever is not consistent in some parts of the world with the rate of infection in animals in these parts. A
possible explanation for this could be strain variation resulting in less infective variants, or the organisms are transmitted more easily from some animal vectors than others.

Fig. 1.1 Epidemiology of Q fever

1.7 Clinical diagnosis of *C. burnetii* infection

Diagnosis of *C. burnetii* is based on serology by either immunofluorescence (IFA), ELISA or microagglutination techniques. Antibodies to phase II antigens are detected during the acute phase of infection, followed by a rise in antibody titre to phase I antigens. Immunoglobulin levels can persist for many years, whilst the presence of specific IgM may indicate a recent or an active infection. Elevated
levels of both IgG and serum IgA to phase I antigens are indicative of Q fever endocarditis and that high titres of IgG and IgA to phase I and II antigens are highly suggestive of a chronic infection (Peacock et al., 1983). The ratio of phase I to phase II IgG antibodies is therefore characteristic of the type of infection. Hence, a ratio of phase I: phase II IgG antibodies of $< 1$ is indicative of acute infection, a ratio of phase I: phase II IgG antibodies of $> 1$ suggests the infection is chronic.

Occasionally guinea pig inoculation is used to confirm C. burnetii-induced endocarditis. Macerated heart valves are injected intraperitoneally into guinea pigs, and sera tested by ELISA for phase II specific antibodies three weeks later (Williams, J. C. et al., 1986a; Fournier et al., 1998). Embryonated eggs and tissue culture, using shell vials, with subsequent confirmation by IFA, have also been described for the isolation of C. burnetii from clinical samples. Such methods, however, may isolate the more virulent or aggressive strains and select against the less virulent strains (Waag et al., 1991).

The direct detection of C. burnetii is hazardous, requiring specialised containment facilities and trained personnel, consequently this is rarely attempted in the clinical diagnostic laboratory. More recently, polymerase chain reaction (PCR) techniques have been applied to the laboratory diagnosis of suspected C. burnetii cases (Stein & Raoult, 1992; Willems et al., 1993). This technique has proven to be more sensitive than standard culture techniques and can be performed on a wide range of clinical samples including blood, cardiac valve biopsy and liver biopsy. In
addition, milk, placental tissue and aborted specimens from animals have been tested and proven to be positive for the presence of *C. burnetii* DNA (Fournier et al., 1998).

1.8 **Aim of this study**

This study aimed to define and characterise a relevant experimental model of Q fever and establish whether *C. burnetii*-specific antigens were present in the urine of experimental animals. It was further intended to characterise any urinary antigens excreted and determine whether the detection of *C. burnetii*-specific antigens in the urine, could provide an additional or alternative method of diagnosis and assessment of cases of Q fever.
CHAPTER 2

Development of an Animal Model of *C. burnetii* Infection

2.1 Introduction

Animal models are usually divided into two groups: screening and discriminative (Wilson & Raynor, 1993). Screening models are usually used for testing antibiotics, where large numbers of animals are involved for statistical analysis and the similarity between the infection profile and clinical situation plays a secondary role. Discriminative models are more relevant to the human disease, where route of infection, symptoms, pathology and state of immunocompetence are similar.

The present study required a discriminative model, where disease progression and pathology was similar to the disease in humans, to provide relevant urine samples to study the excretion of specific antigens in urine.

Previous work, using urine from guinea pigs experimentally infected with Legionnaires’ disease demonstrated the usefulness of an animal model that accurately modelled the human disease (Williams and Featherstone, 1988). Such a model enabled the strain and infective dose to be controlled and urine and tissue samples could be obtained at known times post-infection. The use of urine from clinical cases of Q fever provide no information on the initial infective dose.
received or the infective strain. In addition, no longitudinal study involving tissues and serum for pathological and serological analysis could be achieved.

The guinea pig is highly susceptible to infection with *C. burnetii* however to date, no animal model has been described that accurately reflects the chronic disease seen in humans. This highlights one of the limitations in the use of animal models for the study of *C. burnetii* infection. The guinea pig in particular has limited use for the study of detailed cellular immune responses as few reagents are available. It has been suggested that the relative ratio of IgG subclasses may play an important role in the progression of an acute disease to the chronic form in humans (Camacho, *et al.* 1995) however such distinctions are limited in guinea pigs.

**2.1.1 Experimental infection in the guinea pig.**

The first published account describing the identification of *C. burnetii* involved its isolation in guinea pigs infected with blood and urine obtained from clinical cases of Q fever (Derrick, 1937). Specific *C. burnetii* phase II antibodies appeared two to three weeks post infection confirming the diagnosis of Q fever.

A number of experimental animals have been described which proved to be susceptible to *C. burnetii*. Each show a characteristic pathology, aetiology and virulence.

*C. burnetii* infection of guinea pigs by the intraperitoneal route produces pyrexia of 40° C or greater from 5 to 12 days post infection (Lang, 1990). The incubation time has been shown to be dependant on the virulence of the organism, the dose
received and route administered. The average incubation time was eight days, for
guinea pigs injected intraperitoneally with one organism, compared to eleven days
for those animals administered one organism by the respiratory route. The dose-to-
incubation relationship, based on febrile response, observed in guinea pigs is
similar to that observed in humans when *C. burnetii* was given by the respiratory
route (Williams, J. C. & Thompson, 1991).

Various methods have been adopted for the measurement of the humoral immune
response to experimental infection of guinea pigs with phase I *C. burnetii* by either
the intraperitoneal or respiratory route of infection. Earlier methods included
microagglutination (Johnson *et al.*, 1976; Urvolgyi & Brezina, 1978; Williams, J.
C. *et al.*, 1986a) and complement fixation (Kazar *et al.*, 1977; Urvolgyi & Brezina,
1978; Williams, J. C. *et al.*, 1986a). More recently, immunofluorescence and
ELISA have been utilised for the determination of antibody response to infection
in guinea pigs (Williams, J. C. *et al.*, 1986a). Using the above procedures, a trend
in the time of onset of the humoral immune response has been reported, but the
appearance of antibodies has been variable and dependant on the sensitivity of the
test used.

Following intraperitoneal inoculation with *C. burnetii* phase I whole cells,
antibodies directed against phase II antigens appear first, at approximately day 6
post-infection, when tested by ELISA, and day 8 post-infection by other standard
tests i.e. immunofluorescence (IFA), microaglutination assay (MAA) and
complement fixation (CF) (Williams, J. C. et al., 1986b). Antibodies to phase I antigens appear by day 8 post-infection by ELISA, following intraperitoneal injection of virulent Nine Mile organisms (Williams, J. C. et al., 1986b). However, when the less virulent M-44 strain of organism was injected, no antibodies were detected against phase I antigens until 14 weeks post-infection (Johnson et al., 1977).

Following infection with *C. burnetii* phase I, certain pathological changes take place in the guinea pig. (Kishimoto & Burger, 1977) reported interstitial pneumonia at 5 days post-infection after aerosol challenge with Henzerling strain, which became pronounced and was characterised by extensive exudation of fibrin, neutrophils, macrophages and lymphocytes by 9-11 days post-infection. Liver and spleen granulomas were present by day 9 post-infection persisting in the spleen until day 20 post-infection and in the liver until day 29 post-infection. Limited lymphoreticular myocarditis and epicarditis were reported by day 9 post-infection.

Following intraperitoneal challenge with the less virulent M-44 strain, pathology in the guinea pigs resembled that seen with Henzerling infection; however, the severity of the lesions were less and splenitis was less severe. Myocarditis was reported in approximately one third of the infected animals, which had resolved after day 15 post-infection (Johnson et al., 1977).
C. burnetii is not completely cleared in the guinea pig and disease can be reactivated by pregnancy, or reduction of immunity by chemicals such as cyclophosphamide or methyl prednisolone to cause further acute infection (Johnson et al., 1977). The guinea pig displays characteristics therefore, of an overt acute disease following either intraperitoneal or aerosol challenge with C. burnetii phase I.

2.1.2 Other experimental infection models

Infection of mice with C. burnetii phase I produces a specific inflammatory and humoral response but with varying morbidity and mortality. A study conducted by (Scott et al., 1987) showed that of 47 inbred strains of mice, 33 were resistant (no morbidity or mortality), 10 showed intermediate sensitivity and 4 were sensitive. It was found that 1000-fold fewer organisms were required to produce an LD50 in susceptible mice compared to resistant mouse strains.

An asymptomatic infection model in mice has been described, where the infection process is characterised by long-lasting multiplication of C. burnetii at specific sites but without apparent systemic or metabolic changes taking place. Changes such as anorexia, pyrexia, hypoglycemia, lipidosis of hepatocytes and loss of glycogen are common to Coxiella infection in the guinea pig but do not occur in mice. The relatively slow multiplication rate of C. burnetii in mice may be due, in part, to cell-mediated immunity, and also to the relative tolerance of mice to the
toxic effects of *C. burnetii* LPS. The relative tolerance or resistance to *C. burnetii* infection in mice is genetically linked. Sensitive A/J mice died even though they were able to mount an humoral immune response to *C. burnetii* but only a limited cellular immune response. Resistant C57BL/6J or C3H/HeN mice, however, showed no overt signs of disease, but did show significant tissue lesions as well as humoral and cellular immune responses (Williams, J. C., 1991) and (Williams, J. C. & Cantrell, 1981). The inability of A/J mice to mount an effective cell-mediated immune response correlated with a decrease in the number of lesions, and consequently death.

High numbers of live or dead *C. burnetii* organisms or their LPS are required to kill mice. Splenomegaly has been observed in mice either vaccinated or infected with *C. burnetii*. Splenomegaly is a dose-dependant response and the proliferation of B-cells is correlated directly to the proliferation of *C. burnetii* within the spleen cells. Splenomegaly has therefore been used, to evaluate the initial infective dose in some studies (Khavkin, 1990).

Immunocompromised balb/cJ (H-2^d^) mice infected intraperitoneally with Nine Mile *C. burnetii* phase I were shown, at ten days post infection, to have *C. burnetii*-induced valvular endocarditis as a result of disseminated *C. burnetii* infection. At 150 days post infection, all cardiac valves were shown by immunocytochemistry to be clear of antigen. This work suggested that using an acute disease-causing strain, valvular endocarditis could only be demonstrated
immediately after infection and only as part of a disseminated infection (Atzpodien et al., 1994).

Euthymic (normal) mice demonstrated clearance and reduction in the number and size of lesions in the spleen, liver and lung two weeks after aerosol infection, compared to athymic (nude) animals. This suggested that clearance of organisms was dependant, at least in part, on thymus-derived lymphocytes (Hall, W. C. et al., 1981).

Cynomolgus monkeys proved highly susceptible when exposed to $10^5$ mouse median infectious intraperitoneal doses (MIPID$_{50}$) of C. burnetii phase 1 Henzerling strain given as a small particle aerosol (Gonder et al., 1979). Signs of illness and pathological changes, such as pneumonia and detectable antibodies to C. burnetii, were demonstrated at day 9 and 7, respectively. Organisms were demonstrated by IFA in lungs, liver, spleen, testes, kidney and heart by day 14 post-infection. Animals exposed to a lower infectious dose of C. burnetii ($10^3$ MIPID$_{50}$) showed no clinical evidence of infection and no organisms were isolated from peripheral blood; however specific antibodies were detected by IFA on day 21 post-infection.
2.1.3 Aim of this chapter

The object of this study was to establish a discriminative experimental infection of *C. burnetii* in guinea pigs, using a previously uncharacterised British isolate (Lane) delivered via the aerosol route. The airborne route of infection will be compared to the intraperitoneal route of infection in terms of the clinical symptoms, serology and histology, and the resulting disease profile of this strain of *C. burnetii* will be characterised.
2.2 Materials and methods

2.2.1 Isolation of C. burnetii

*C. burnetii* Lane strain, originated from a patient suffering from endocarditis with high IgG and IgA anti-*C. burnetii* whole cell phase I antibody titres. Isolation of *C. burnetii* Lane strain was performed by Dr. David Rutter. Fragments of aortic valve (received at CAMR) were homogenised in 20 ml PBS and allowed to settle. The upper 10 ml of the suspension was collected and 2.5 ml injected intraperitoneally into each of four Dunkin Hartley guinea pigs. Seroconversion confirmed the presence of *C. burnetii*, three weeks post-inoculation.

Twenty, 6-day old embryoated eggs were inoculated via the air sac, with the remaining heart valve suspension, which had been made up to 20 ml with PBS. Each yolk sac received 0.5 ml of suspension. All eggs were candled daily. After 14 days, or when 50% of the previous days eggs were dead, yolk sacs were harvested (5 yolk sacs into 5ml saline) and homogenised in 70 ml PBS for 1 minute. Two further passages in embryoated hens eggs were performed. Each yolk sac was inoculated with 0.1 ml of the first egg passage, diluted 0.2 ml in 5ml PBS. When 50% of the previous days eggs had died, the remaining eggs were harvested (as above).
2.2.2  *C. burnetii* whole cell antigen preparation

Approximately 70 ml of homogenate was produced, of which 20 ml was added to 100 ml of 1% formal-saline. The suspension was stored for at least one week to inactivate the *Coxiellae* then centrifuged at 6,000g for one hour and resuspended in 1% formal-saline and homogenised for 2 minutes. An equal volume of ether was added to the suspension, shaken and left to separate overnight at 4°C. The aqueous layer was removed and re-extracted with ether until a clear interface between ether and aqueous layer was achieved. Residual ether was removed by evaporation and the suspension washed and resuspended in PBS with 0.08% sodium azide to 1/10 of the original volume. The final suspension was examined for purity by transmission electron microscopy. A non-formalised suspension of *C. burnetii* (Lane strain), phase I organisms was used for all animal experiments.

2.2.3  Phase conversion

Conversion from phase I organisms to phase II organisms involved treatment with potassium iodide based on the methods of (Fiset et al., 1969) and (Schramek *et al.*, 1972). Briefly, a purified inactivated suspension of phase I organisms at a final concentration of 1mg/ml was treated with 0.01M potassium iodide for 4 hours at 45°C. The reaction was stopped by addition of a 1/10 volume of 10% glucose.
Antigen was washed twice by centrifugation at 11,000 rpm in PBS pH 7.2 and resuspended to the original volume in peptone water, prior to dilution to 1mg/ml.

2.2.4 Enumeration of *C. burnetii*

A total count was performed on purified antigen preparation by phase contrast microscopy. Organisms treated with formalin were diluted 1:2 in water and placed onto a Helber counting chamber. Using a 40 x objective lens, the number of organisms per graticule square were counted and 20 squares in total were counted.

Applying the formula:

Number of organisms ml\(^{-1}\) = 2n \times 10^7 \times \text{dilution}

where n = average number of organisms per square (20 squares counted)
and dilution = the original dilution of antigen preparation prior to count.

An approximate count of the number of organisms present in the original egg preparation was calculated, allowing for the dilution factors during preparation.

2.2.5 Safety and containment

The guidelines for working with hazard group 3 pathogens, recommended by the Health and Safety Executive, were adhered to throughout all procedures involving manipulation of live *C. burnetii* (Advisory Committee on Dangerous Pathogens, 1995). Namely, class III safety cabinets within a containment level III laboratory
facility, for all laboratory procedures. Animal studies in conjunction with C. burnetii were undertaken within a ACDP category III animal containment facility, which included a complete change of clothes into disposable garments, use of personal respiratory protection and showering out on exiting from the room. Additional safety precautions undertaken, whilst infectious aerosols of C. burnetii were generated and delivered to animals included the complete containment of the Henderson apparatus (see Section 2.2.6) and the use of a downdraught post-mortem table on which the animals were placed for infection.

2.2.6 Aerosol generation and respiratory infection

Home Office Ethics Committee approval for animal experimentation was obtained. Female Dunkin-Hartley guinea pigs (barrier reared) weighing 300-400g were exposed to bacterial aerosols containing particles ≤ 5μm in diameter, generated using a three jet Collison spray at 65% relative humidity in a Henderson-type apparatus (Henderson, 1952; Druett, 1969). Calculated inhaled and retained doses were based on previous findings with a variety of bacterial species (Fitzgeorge et al., 1983; Lever et al., 1995; Williams, A. & Lever, 1995; Cooper et al., 1996; McBride et al., 1998) and (Bracegirdle et al., 1994). Initial spray suspensions contained approximately 10⁹ organisms/ml (high dose) and 10⁶ organisms/ml (low dose). Guinea pigs were allowed to breath the aerosol for 5 minutes. Twelve animals were administered high dose and twenty animals administered a low dose of C. burnetii by aerosol. Rectal temperatures and weights were recorded daily.
Serum samples were obtained by cardiac puncture whilst the animals were under CO₂, and urine obtained by manual expression of the bladder. Tissue samples for histology were taken on day nine, of animals administered a high dose of *C. burnetii*, by aerosol (group 1) i.e. when animals were moribund. Guinea pigs that received a low dose of *C. burnetii* by aerosol (group 3) were killed when moribund or at day 92 post-infection.

### 2.2.7 Intraperitoneal infection

Eighteen guinea pigs were inoculated intraperitoneally with 0.1ml suspension containing approximately 10⁹ organisms/ml (high dose, group 2). Six guinea pigs were inoculated with 0.1ml of a suspension containing approximately 10⁶ organisms/ml (low dose, group 4). Animals administered a high dose of *C. burnetii* were killed at 28 days post-infection and those administered a low dose; 92 days post-infection. Tissue samples for histology were taken approximately every four-seven days, from animals administered a high dose of *C. burnetii*. Tissue samples from guinea pigs that received a low dose of *C. burnetii* were taken at post mortem (day 92 post-infection).

### 2.2.8 Urine samples

Urine was obtained from infected guinea pigs by manual expression of the bladder. Samples were taken every other day until day nine and twenty eight post-infection.
from guinea pigs administered high dose \textit{C. burnetii} by aerosol (group 1) and intraperitoneally (group 2) respectively. Urine samples were removed from guinea pigs in the low dose groups (groups 3 and 4) approximately weekly up to 50 days post infection. Urine from four animals was removed each day and pooled.

2.2.9 Polyclonal antisera preparation

Antiserum to \textit{C. burnetii} was obtained by immunising three New Zealand White rabbits with whole inactivated \textit{C. burnetii} phase II Lane strain. After a preliminary blood sample had been taken, each rabbit was injected with 500 µl of antigen (approximately $1.0 \times 10^8$ organisms/ml) in incomplete Freund's adjuvant (Difco). Immunisation was distributed between two intramuscular (hind legs, 2 x 200 µl) and two dorsal subcutaneous (2 x 50 µl) sites. A boost was given with the same dose after two weeks and blood was taken after a further two weeks and assayed by direct ELISA. A further boost was administered after 10 weeks. Two weeks following this, the rabbits were exsanguinated under pentobarbitone anaesthesia.

Sera with the highest titre was chosen and the immunoglobulin G (IgG) fraction obtained by Protein G affinity chromatography (Pharmacia) following manufacturers instructions. Briefly, the column was equilibrated with 20 mM phosphate buffer (pH 7.0) and 200 µl samples of sera in the same buffer were injected onto the column. Separation took place at a flow rate of 1.0 ml min$^{-1}$ and the eluting buffer was 0.1 M glycine pH 2.7. Samples were collected in 1 ml
fractions with 50µl addition of 1M tris buffer pH 9.0 to neutralise the low pH conditions created by the elution buffer. Eluted fractions were tested for anti-C. burnetii phase I, II and LPS I IgG antibodies by indirect ELISA (see Section 2.2.10).

2.2.10 Indirect ELISA

The method of (Cowley et al., 1992) was used. Sterile 96-well microtiter plates (Pro-bind; Falcon) were coated with 0.1 ml of antigen suspension (C. burnetii whole cell, phase I or phase II) at 1/800 and 1/2000 respectively in coating buffer (0.04M sodium carbonate, 0.06M sodium bicarbonate in sterile distilled water, pH 9.6). Plates were then incubated shaking for 1 hour at 37°C, and washed three times (Wellwash 4; Denley) with 0.3 ml volumes of PBS (pH 7.2) containing 0.1% (vol/vol) Tween 20 (PBST). Guinea pig test serum was added at 1/100 diluted in PBST +10% horse serum (HS) and double diluted out. A guinea pig positive and negative control serum was included as well as a blank control (PBST). Plates were incubated, shaking, for 30 minutes at 37°C, then washed three times as before and 0.1 ml anti-guinea pig IgG-HRP (1/2000) (Nordic) added.

The presence of specific IgM was assayed as described above, with the exception that each serum sample was pretreated with goat anti-guinea pig IgG (Sigma) at 1/2000 dilution in (PBST) to remove any possible interference from rheumatoid
factor. Anti-guinea pig IgM-HRP (1/2000) (Nordic) was then added. Plates were incubated for 30 minutes at 37°C shaking and washed three times in PBST before addition of 100µl of TMB substrate, consisting of one TMB tablet (Sigma) dissolved in 10ml of citrate buffer (Sigma), and left for 15 minutes to develop. The reaction was stopped by the addition of 25µl 2M sulphuric acid and the OD read immediately at 450 nm.

For the assay of specific IgA in infected guinea pig serum, the above method was employed except guinea pig test sera was initially diluted 1/20 in PBST+ 10% HS and then double diluted out. A sheep anti-guinea pig IgA antibody (Nordic) was then used at 1/2000, incubated for 30 minutes shaking and rabbit anti-sheep IgG (H and L) peroxidase conjugate (Nordic) at 1/2000 added and incubated as detailed above. The substrate step was as above.

2.2.11 DNA extraction from C. burnetii-infected guinea pig tissue

DNA was purified from tissues using the QIAamp tissue kit (Qiagen Ltd, Crawley) following manufacturer's instructions. Approximately, 25 mg of tissue was cut into small pieces and added to a microfuge tubes (1.5ml) containing 180 µl of tissue lysis buffer (guanidium thiocyanate based). Proteinase K was added (20 µl) and the suspension vortexed and incubated at 55 ºC until completely lysed. Lysis buffer was then added (200 µl) and sample was vortexed and incubated at 70 ºC for 10 minutes. Ethanol (210 µl) was added and the sample mixed by vortexing. The
mixture was then applied to a QIAamp spin column and centrifuged at 6000 x g for 1 minute and filtrate was discarded. The spin column was then washed twice with wash buffer (500 µl) by centrifugation as above and filtrate again discarded. DNA was then eluted from the spin column by addition of 200 µl of distilled water heated to 70 °C followed by centrifugation as above.

2.2.12 PCR analysis of *C. burnetii*-infected samples

Primers were designed to target a 495 bp DNA sequence within the superoxide dismutase gene sequence published by (Heinzen *et al.*, 1990). The primers were,

Cox 3 [5'-GAATTACCGGATTTGCCC-3']

Cox 4 [5'-CGGGTATCGATGTAATAG-3'].

Each PCR reaction was set up in sterile pyrogen free tubes. PCR was performed in a total volume of 50 µl containing 2 µl of template (extracted sample), 5 pmoles of each primer, 200 µM of each dNTP (NBL) and 5µl of x 10 buffer. Tubes were placed on ice for 5 minutes before 2.6 units of a Taq and Pwo polymerase mix (Boehringer Mannheim Expand High Fidelity PCR System ) was added. Tubes were briefly flicked and spun to mix and returned to ice. Each reaction was overlaid with two drops of mineral oil. The PCR reaction took place in a Biometra Thermocycler. Reaction tubes were incubated at 94°C for 2 minutes followed by 40 cycles of 94°C denaturation for 1 minute, 50°C annealing for 2 minutes and 72°C extension for 30 seconds followed by a final extension of 72 °C for 7
minutes. PCR products were resolved on 2% agarose gels stained with ethidium bromide and viewed under U.V. light.
2.3 Results

2.3.1 Mortality and Clinical Symptoms

Guinea pigs infected by high dose *C. burnetii* by aerosol (group 1) became febrile (> 40.0 °C) beginning at day 2 post-infection. Pyrexia continued, in those animals that survived, until day 9 post-infection, when all remaining animals were killed. Fifty percent of animals had died by 9 days post-infection and the remainder were moribund and therefore sacrificed. Guinea pigs infected by high dose, *C. burnetii* intraperitoneally (i.p.) (group 2), became febrile from day 4 post-infection and pyrexia lasted until day 8 post-infection, when temperatures returned to those seen in non-infected animals (Fig 2.1). No mortality was observed in animals infected i.p. (Table 2.1). Whilst febrile, guinea pigs were inactive and food and water intake was reduced, affecting their weight and general condition. Weights in aerosol-infected animals in particular decreased rapidly from day 2 post-infection and continued to decrease in survivors, until termination of the experiment at day 9 post-infection. Weights of animals infected i.p. increased gradually, at an equivalent rate to non-infected animals until day 4 post-infection. Weights then decreased for four days, coinciding with the febrile response, after which weights gradually increased in-line with non-infected animals.

Animals infected with low dose by aerosol (group 3) and low dose i.p. (group 4) had a milder form of the disease with fewer fatalities, less pronounced weight loss and pyrexia, although still higher than non-infected controls. In both cases,
infection by the aerosol route induced a greater level of disease. Weights of guinea pigs decreased significantly from day 1 to 3 post-infection but increased gradually thereafter. However, weights in the aerosol group remained lower than non-infected control animals throughout the course of the experiment. Pyrexia of >40°C was evident in animals administered low dose C. burnetii by the aerosol route from day 14 post-infection which continued to day 28 post-infection when measurements were stopped. Pyrexia was also evident, beginning at day 1 post-infection, in i.p.-infected animals where temperatures remained above those detected in non-infected control animals. Peak temperatures in these animals, however, remained lower than aerosol-infected animals throughout the 28 days measurements (Fig 2.2).

Table 2.1 Mortality in guinea pigs infected by the aerosol and intraperitoneal routes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Estimated Retained dose (ergs/ml)</th>
<th>Route</th>
<th>Day post-infection</th>
<th>No. dead/total</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>$10^3$</td>
<td>Aerosol</td>
<td>9</td>
<td>12/12</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>$10^8$</td>
<td>I. P.</td>
<td>28</td>
<td>0/14</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>$10^1$</td>
<td>Aerosol</td>
<td>92</td>
<td>8/20</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>$10^5$</td>
<td>I. P.</td>
<td>92</td>
<td>0/14</td>
<td>0</td>
</tr>
</tbody>
</table>
Figure 2.1  Mean temperature elevation in °C (a) and weight in grams (b) of non-infected control, high dose aerosol (group 1) and intraperitoneally-infected (group 2) guinea pigs. Values shown represent the mean of four animals.

(a) Temperature

(b) Weight
Figure 2.2 Mean temperature elevation in °C (a) and weight in grams (b) of non-infected control, low dose aerosol (group 3) and intraperitoneally-infected (group 4) guinea pigs. Values shown represent the mean of four animals.

(a) Temperature

(b) Weight
Humoral Immune Responses - high dose (groups 1 and 2) animals.

Immunoglobulin M anti-\textit{C. burnetii} titres in guinea pigs challenged i.p. and by aerosol began to rise by day 6 post-infection and IgG titres by day 8 post-infection. Intraperitoneally-infected animals however, produced approximately 100 times greater anti-\textit{C. burnetii} IgM and IgG titres than aerosol-infected animals by day 9 post-infection. At the time of sacrifice, all immunoglobulin levels were rising (Fig 2.3 a and b). Anti-\textit{C. burnetii} phase I IgG and IgM antibodies in animals challenged by both aerosol and intraperitoneal routes of infection showed no response above background.
Figure 2.3 Guinea pig serum immunoglobulin M and G anti-*C. burnetii* phase II whole cell antibodies. Data is the mean of two animals.

(a) Aerosol-infection

(b) Intraperitoneal-route
2.3.3 Humoral Immune Response - low dose (groups 3 and 4).

Infection by i.p. inoculation with $10^3$ fewer organisms, produced significantly reduced levels of anti-*C. burnetii* phase II IgM and IgG antibody responses compared to levels detected in animals that received a higher challenge dose. A dose response was therefore evident.

Anti-*C. burnetii* phase II IgM in low dose i.p.-challenged guinea pigs appeared at day 5-6 and peaked at day 19 post-infection. By day 25 post-infection, no IgM was detectable. In contrast, anti-*C. burnetii* phase II IgM levels appeared later, at day 10 post-infection, and peaked at day 28 post-infection, in animals challenged with low dose *C. burnetii* by aerosol (group 3). By day 42 post-infection, IgM was no longer detectable. Anti-*C. burnetii* phase II IgG antibody levels in aerosol and i.p. challenged animals reached similar levels up to day 50 post-infection. By day 92 post-infection, levels continued to rise in i.p.-infected animals but had begun to decrease in aerosol infected animals (fig 2.4a and b).

Levels of anti-*C. burnetii* serum IgA antibodies to phase II cells were greater in aerosol-infected animals. Levels appeared by day 25 post-infection, reaching a maximum by day 40 post-infection and decreasing to low levels by day 92 post-infection. In contrast, levels of anti-*C. burnetii* serum IgA antibodies in i.p.-infected animals (group 4) remained at low levels until day 50 post-infection, and
then gradually increased until the end of the experiment at day 92 post-infection (fig 2.4c).

Anti-*C. burnetii* IgG and serum IgA antibody response to phase I antigens in i.p. and aerosol-infected groups were similar. IgG levels peaked at day 50 post-infection and serum IgA levels were continued to rise up to 92 days post-infection (Fig 2.5a and b).

Figure 2.4 Mean titres against *C. burnetii* phase II whole cells, determined by ELISA, from guinea pigs infected by low dose aerosol (group 3) and intraperitoneal infection (group 4). Each point represents the mean value of four animals

(a) IgM
b) IgG

![Graph showing antibody titre over days post infection for IgG.]

(c) IgA

![Graph showing antibody titre over days post infection for IgA.]

Days Post Infection

Antibody Titre

Days Post Infection
Figure 2.5 Mean titres (IgG and IgA) against *C. burnetii* phase I whole cells, determined by ELISA, from guinea pigs infected by low dose aerosol (group 3) and intraperitoneal infection (group 4). Each point represents the mean of four animals.

(a) IgG

(b) IgA
2.3.4 Histopathology and immunocytochemistry

2.3.4.1 Group 1- aerosol challenge (high dose)

Lungs from guinea pigs infected with *C. burnetii* revealed an extensive exudative pneumonia causing widespread consolidation, characterised by focal congestion, haemorrhage and observable emphysema (Fig. 2.6). In addition, diffuse exudation of macrophages and neutrophils were observed in the alveoli and respiratory bronchioles. Around many of the arteries, perivascular oedema and cuffs of lymphocytes were detected (Fig. 2.7). Specific staining confirmed the presence of *C. burnetii* antigen associated with the cellular infiltrate (Fig. 2.8).

In the liver, vacuolation of the periportal hepatocytes was thought to be due to the presence of intracellular lipid. Foci of hepatocyte necrosis with macrophage infiltration was common to all animals studied (Fig. 2.9). Small brown intracytoplasmic bodies were seen in degenerate hepatocytes in three foci, these being interpreted as evidence of *C. burnetii* invasion. Immunocytochemical analysis of liver sections identified areas of specific staining within hepatocytes (Fig. 2.10).
Fig. 2.6  Haemotoxylin and eosin (H+E) stained section of guinea pig lung 9 days after infection with a high dose, by aerosol, of C. burnetii (group 1). Magnification x 480.

Fig. 2.7  Haemotoxylin and eosin (H+E) stained section of guinea pig lung 9 days after infection with a high dose, by aerosol, of C. burnetii (group 1). Magnification x 480.
Fig. 2.8 Immunocytochemical analysis of guinea pig lung, 9 days after infection with a high dose by aerosol, of *C. burnetii* (group 1). Sections were immunostained with anti-*C. burnetii* whole cell phase II IgG antibody. *C. burnetii* antigen is stained brown (indicated by arrow), and the tissue counterstained with haematoxylin. Magnification x 480.
Fig. 2.9 Haemotoxylin and eosin (H+E) stained section of guinea pig liver 9 days after infection with a high dose, by aerosol, of *C. burnetii* (group 1). L, indicates the presence of intracellular lipid and N, indicates an area of hepatocyte necrosis. Magnification x 480.

Fig. 2.10 Immunocytochemical analysis of guinea pig liver, 9 days after infection with a high dose, by aerosol, of *C. burnetii* (group 1). *C. burnetii* antigen is indicated by the arrow. Magnification x 480.
Thrombi were evident in the heart adherent to both atrio-ventricular and aortic valves in six (50%) guinea pigs. Focal myocardial necrosis was also observed in the heart tissue of one animal (Fig. 2.11).

Immunocytochemical investigation of tissue at 9 days post-infection, confirmed the presence of *C. burnetii*. Specific staining was observed within myocardial cells (Fig. 2.12). In addition, examination of areas of specific staining by scanning electron microscopy identified individual bacteria (Fig. 2.13a, b, c and d).

Spleens were often enlarged, particularly in the red pulp, as a result of macrophage infiltration. Giant cells with multiple or giant nuclei were also common features in the red pulp (Fig. 2.14).

No histopathological abnormalities were detected in the kidneys of any animal.
Fig. 2.11  Haemotoxylin and eosin (H+E) stained section of guinea pig heart 9 days after infection with a high dose, by aerosol, of *C. burnetii* (group 1). Arrows, indicate foci of necrosis within the myocardium. Magnification x 50.

Fig. 2.12  Immunocytochemical analysis of guinea pig heart, 9 days after infection with a high dose, by aerosol, of *C. burnetii* (group 1). Magnification (large picture) x480, and (inset) x 550.
Fig. 2.13 a and b  Scanning electron micrographs of *C. burnetii* cells within de-waxed sections of guinea pig heart 9 days after infection with a high dose of *C. burnetii* by aerosol (group 1).

a)

b)
Fig. 2.13 c and d  Scanning electron micrographs of *C. burnetii* cells within de-waxed sections of guinea pig heart 9 days after infection with a high dose of *C. burnetii* by aerosol (group 1).
Fig. 2.14  Haemotoxylin and eosin (H+E) stained section of guinea pig spleen 9 days after infection with a high dose, by aerosol, of C. burnetii (group 1). The arrow indicates a giant cell within the red pulp. Magnification x 480.
Group 2 - intraperitoneal challenge (high dose)

Lungs of eleven guinea pigs (78%) challenged by the intraperitoneal route showed focal acute pneumonia and pneumonitis. Focal thickening of alveolar walls by macrophage-like cells was also noted, although this was seen in non-infected controls and therefore could not be ascribed to infection by *C. burnetii* (Fig. 2.15).

The hearts of seven animals (50%) that had been infected, presented with focal chronic endocarditis and/or myocarditis which was characterised by lesions composed of small foci of lymphocytes in endocardial or myocardial connective tissue (Fig.2.16). No histopathological abnormalities were observed in any spleens, and regenerated tubules were observed in the cortex of kidneys of all animals. This was regarded as mild focal damage.

In the liver, focal necrosis, focal granulomas and perilabular macrophages were common observations in all animals. Focal granulomas in the liver were composed of small numbers of macrophages and lymphocytes, while the presence of macrophage-like cells in portal areas was also common (Fig. 2.17a and b).
Fig 2.15 (H+E) stained section of guinea pig lung 15 days after a high dose intraperitoneal injection of *C. burnetii* (group 2). Magnification x 480.

Fig. 2.16 (H+E) stained section of guinea pig heart 15 days after a high dose intraperitoneal injection of *C. burnetii* (group 2). The left atrioventricular valve is shown, infiltrated by lymphocytes and macrophages with sloughing of the endocardium. Magnification x 120.
Fig. 2.17 a (H+E) stained section of guinea pig liver 9 days after a high dose intraperitoneal injection of *C. burnetii* (group 2). The portal area is infiltrated by lymphocytes. Magnification x 480.

Fig. 2.17 b (H+E) stained section of guinea pig liver 15 days after a high dose intraperitoneal injection of *C. burnetii* (group 2). A focus of necrotic hepatocytes, lymphocytes macrophages and fibroblasts. Magnification x 500.
2.3.4.3 Group 3 - aerosol challenge (low dose)

Post mortem tissue taken from twelve guinea pigs 92 days post-infection, showed less severe lung and liver pathology but more extensive heart and kidney involvement. In particular, heart sections revealed thrombosis and endocarditis, indicating active disease, in at least four animals with additional observations of vacuolation and fibrosis present in one animal. The left atrio-ventricular valve showed evidence of thrombosis and endocarditis consistent with active disease (Fig 2.18). In addition, sub-acute and chronic areas of inflammation were observed at the base of the aorta and the chronic inflammation affected the left atrial wall.

Areas of vacuolation and fibrosis in the myocardium were features consistent with healed lesions. Immunohistochemical analysis demonstrated the presence of C. burnetii antigen, by specific staining, within myocardial cells in one animal (Fig. 2.19). Scanning electron microscopy confirmed that individual bacteria were present in areas of specific staining (Fig. 2.20a and b).
Fig. 2.18  (H+E) stained section of guinea pig heart, 92 days after infection with a low dose, by aerosol, of *C. burnetii* (group 3). Arrow indicates thrombosis present in the left atrioventricular. Magnification x480.

Fig. 2.19  Immunocytochemical analysis of guinea pig heart, 92 days after infection with a low dose, by aerosol, of *C. burnetii* (group 3). *C. burnetii* antigen is stained brown (indicated by arrows). Magnification x480.
Fig. 2.20 a and b  Scanning electron micrographs of de-waxed sections of guinea pig heart 92 days after a low dose aerosol infection of *C. burnetii* (group 3).

a) [Image]

b) [Image]
Foci of interstitial fibrosis and nephritis with tubule regeneration in the cortex which was indicative of chronic infection was evident in the kidneys of four animals studied (Fig. 2.21).

Liver pathology was confined primarily to small foci of lymphocytes and areas of necrotic hepatocytes encircled by collagen, in three animals, indicating healed foci of earlier damage (Fig. 2.22).

No abnormalities were detected in lung or spleen tissue from guinea pigs examined 92 days post-infection (Fig. 2.23). However, immunohistochemical staining of lung sections revealed that four infected animals examined, showed specific evidence of *C. burnetii* involvement (Fig. 2.24).
Fig. 2.21 (H+E) stained section of guinea pig kidney 92 days after infection with a low dose, by aerosol, of C. burnetii (group 3). The heavily stained area (N) indicates chronic interstitial nephritis in the cortex. Magnification x 350.

Fig. 2.22 (H+E) stained section of guinea pig kidney 92 days after infection with a low dose, by aerosol, of C. burnetii (group 3). Arrow indicates a small group of lymphocytes. Magnification x 500.
Fig. 2.23 (H+E) stained section of guinea pig lung 92 days after infection with a low dose, by aerosol, of *C. burnetii* (group 3). No abnormalities were detected. Magnification x 500.

Fig. 2.24 Immunocytochemical analysis of guinea pig lung, 92 days after infection with a low dose, by aerosol, of *C. burnetii* (group 3). *C. burnetii* antigen is stained brown and the tissue counterstained with haemotoxylin. Magnification x 480.
2.3.5 **Histopathology and immunocytochemistry summary**

Tissues examined from animals that received high dose aerosolised *C. burnetii* showed no abnormalities in the trachea or kidneys. Lesions were seen in the heart and spleen and severe pathology was seen in the lungs and liver. Immunocytochemical staining showed that the heart and lung were the primary targets of aerosolised infection. The liver and spleen were also involved during the acute phase of infection.

Tissues from animals infected intraperitoneally with high doses of *C. burnetii* (group 2) and examined at 28 days post-infection demonstrated mainly liver pathology. No evidence of *C. burnetii* antigen could be detected, in any organ, in this group of animals as tested by immunocytochemistry.

Evidence of heart and lung involvement remained during the convalescent phase in animals that received low doses of aerosolised *C. burnetii* (group 3). Less severe lung and liver pathology but more extensive heart and kidney involvement was observed in tissues examined ninety two days post aerosol challenge with low dose *C. burnetii*. 
C. burnetii-specific DNA was detected in all tissues obtained from animals challenged with high levels of C. burnetii by either the aerosol or i.p. routes (group 1 and 2), nine days post-infection. Alternatively, only heart and lung proved positive for C. burnetii DNA at ninety-two days post-infection in guinea pigs receiving low levels of C. burnetii by both the aerosol and i.p. routes of infection (groups 3 and 4) (Table 2.2).
Table 2.2  Detection of *C. burnetii* DNA in guinea pig tissues following aerosol and intraperitoneal infection. Tissues from two animals were tested.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>High Dose (groups 1 and 2)</th>
<th>Low Dose (groups 3 and 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 9 post-infection</td>
<td>Day 92 post-infection</td>
</tr>
<tr>
<td>Heart</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Kidney</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: + = present      - = absent
The object of this part of the study was to establish a discriminative animal model of *C. burnetii* infection which accurately reflected the disease in humans. *C. burnetii* infection of experimental animals can be categorised as inapparent or apparent infections. The guinea pig is an example of an experimental laboratory animal which is highly susceptible to *C. burnetii*, where morbidity and mortality are characteristics of the disease. Alternatively, *C. burnetii* infection in the mouse is a model of inapparent infection, (unless infected with large numbers of *C. burnetii*), and the infectious process is characterised by long-lasting multiplication of the pathogen in infectious foci, without apparent systemic and metabolic changes (Khavkin, 1990). The guinea pig was selected in this study for a number of reasons. Firstly, clinical symptoms, mortality and serology could be used as parameters of infection. Secondly, much previous work had been conducted on the guinea pig by other groups, providing comparative data. Thirdly, the guinea pig had been used routinely in this laboratory for the confirmation by serology, of the presence of *C. burnetii* in clinical material (heart valves).

The aerosol route of infection is the most common and relevant mode of transmission of *C. burnetii* to humans (Baca & Paretsky, 1983) and was used in this study to deliver a mono-dispersed infectious aerosol of *C. burnetii* to guinea pigs.
A number of parameters were monitored during infection including mortality, pyrexia, anorexia, histopathology, immunocytochemistry, serology and direct antigen detection.

2.4.1 Mortality and clinical signs.

Previous workers have shown that mortality was directly related to the virulence of the strain of *C. burnetii* and the initial infective dose received (Ormsbee et al., 1978; Kazar et al., 1993b). In this study all animals that received high dose *C. burnetii* (group 1) were observed to be in distress with pyrexia and dehydration, and therefore killed at day nine post-infection. Mortality for this group was therefore classed as 100%. Animals administered low dose *C. burnetii* by aerosol (group 3), suffered 40% mortality. Therefore, a dose response was observed. Mortality figures in low dose animals (group 3) however, are higher than other workers have reported with comparable febrile reaction data (Kishimoto & Burger, 1977; Kazar et al., 1993b). (Kazar et al., 1993b), reported that mortality and a strong febrile reaction in guinea pigs was characteristic of infection, when an inhaled retained dose of approximately $10^6$ egg infectious dose 50 (EID$_{50}$) organisms of acute causing strains (Luga and Nine Mile) and chronic causing strain (S) of phase I *C. burnetii* were administered. However, no mortality and a reduced febrile reaction was observed, with similar doses to that used in the present study, when *C. burnetii* strain Priscilla (a chronic disease causing strain) was given by aerosol. A similar pattern of virulence emerged when Priscilla, Nine Mile and S
strains of *C. burnetii* were given by the intraperitoneal route to guinea pigs and Balb/c mice.

Other workers have reported a febrile response in guinea pigs following inhalation of *C. burnetii* Henzerling strain (a strain associated with acute disease) (Kishimoto & Burger, 1977), and Qi Yi (a strain associated with chronic disease) (Fan et al., 1982). However, no mortality following infection was reported by these groups.

The characteristic febrile response observed in guinea pigs infected by the aerosol route during this study, was similar to those reported by (Kazar et al., 1993b) using Nine Mile strain and (Kishimoto & Burger, 1977) using Henzerling strain. In all studies, by day seven post-infection there is an elevation of temperature, although initial infective doses are quoted as $6.6 \times 10^6 \text{ EID}_{50}$ by (Kazar et al., 1993b) and $10^4$ mouse median infective doses by (Kishimoto & Burger, 1977).

(Kazar et al., 1993b) found very little variation in the febrile response of guinea pigs given *C. burnetii* Luga and Nine Mile strains (both associated with acute disease) and *C. burnetii* strain S (associated with chronic disease), but found that the *C. burnetii* Priscilla strain produced no significant febrile reaction. (Kazar et al., 1993b) concluded that their results supported and extended previous work by (Moos & Hackstadt, 1987) which suggested that the Priscilla strain associated with chronic disease was less virulent in guinea pigs than the Nine Mile strain associated with acute disease. However, further strains of *C. burnetii* phase I
organisms would need to be tested to determine if strains associated with human endocarditis (chronic disease-associated strains), proved less pathogenic than strains associated with acute disease.

The present study suggests that, based on febrile reaction, the Lane strain (isolated from a heart valve from a patient suffering from endocarditis) is at least as virulent for guinea pigs as Nine-Mile strain or Henzerling strain when given by aerosol. This study also questions the statement by (Waag et al., 1991) that strains isolated from humans suffering from endocarditis are highly infective but poorly pathogenic for guinea pigs. This study has shown that when given as a small particle aerosol (< 5 µm) the Lane strain is highly virulent for guinea pigs even at low doses.

2.4.2 Route of infection

The aim of this section of the work was to establish an experimental *C. burnetii* infection in the guinea pig. Much research has been conducted on guinea pigs and mice infected by *C. burnetii* via the intraperitoneal (I.P.) route including (Tigertt et al., 1961; Paretsky et al., 1964; Heggers et al., 1975; Johnson et al., 1976, 1977; Gonder et al., 1979; Scott et al., 1987; Williams, J. C. et al., 1990), and the first isolation experiments of *C. burnetii* (Derrick, 1937) therefore the I.P. route of infection was included in this study for direct comparative purposes with previous work. In addition, the Lane strain used in this study was an uncharacterised strain
of *C. burnetii*. Previous workers, when evaluating the comparative virulence of different strains of *C. burnetii* delivered organisms by both the aerosol and I.P. routes of infection (Kazar et al., 1993b). Both routes of infection were therefore chosen in this study to obtain the maximum amount of information on the virulence of the Lane strain.

The route of infection has been shown to be important to the pathogenesis of *C. burnetii* within the host (Marrie et al., 1996) and (La Scola et al., 1997). (Williams, J. C. et al., 1990) demonstrated that spleen weights, and the number of infectious *C. burnetii* organisms within spleens, were significantly greater in mice infected by the intraperitoneal route than those infected by the aerosol route. Intraperitoneally-infected guinea pigs showed reduced mortality and length of febrile reaction compared to animals infected with a comparable dose via the aerosol route (Kazar et al., 1993b). In addition, (Marrie et al., 1996) demonstrated that I.P.-challenged mice developed hepatosplenomegaly and interstitial pneumonia, whereas mice challenged via the intranasal (I.N.) route developed only pneumonia.

In the present study, no mortality was observed in guinea pigs given high or low dose *C. burnetii* by the I.P. route, and the febrile reaction observed in both dose groups was less severe and, in the case of the low dose animals, less prolonged. Guinea pigs given *C. burnetii* I.P. in the present study would have received significantly higher numbers of organisms than those challenged by aerosol. Based on previous studies with guinea pigs using *Legionella pneumophila*, *Bacillus*
\textit{anthracis, Listeria monocytogenes} and \textit{Mycobacterium tuberculosis}, the inhaled, retained dose is routinely between four and six log colony forming units (CFU) less than the total number of viable organisms originally in the nebuliser (personal observation). This would be equivalent to an inhaled retained dose of approximately $10^{3-5}$ organisms/ml and $10^{0-2}$ organisms/ml for groups 1 and 3 respectively. Therefore, the number of organisms received by guinea pigs in the I.P. groups would be significantly higher than the number of organisms retained by guinea pigs in the aerosol groups.

The dose levels chosen in this study do not provide an ideal range of dose responses. An high and low dose of \textit{C. burnetii} administered by the aerosol route produced 100% and 40% mortality respectively. The inclusion of a range of challenge doses, centred around the low dose challenge, would have provided a more precise set of detailed data, as a greater number of animals would have survived for comparison. In addition a more realistic human infective dose (1-10 organisms), could have been achieved. Febrile response data from guinea pigs challenged with a high and a low dose of \textit{C. burnetii} by the I.P. route indicated that a dose level intermediate between the high and low would have produced a more informative dose response.

As the number of animals available for studying are limited and animal containment level 3 working conditions are restrictive, future studies should concentrate on a greater range of dose levels but administered by one route only.
2.4.3 Serological response

The ELISA is the serological method of choice due to greater sensitivity and reproducibility (Peter et al., 1987) and specific IgM, IgG and IgA can be distinguished more easily than using IFA and agglutination techniques. The detection of anti-\textit{C. burnetii} antibodies provides the best indication of exposure to, and infection by \textit{C. burnetii} and is true for both experimental animals and humans. The serological response, measured by ELISA, against whole cell phase I and II antigens in our study demonstrated how route of infection and dosage can produce different responses.

The serology results in the present study followed a similar trend to those reported elsewhere (Ormsbee et al., 1968; Kazar et al., 1977; Williams, J. C. et al., 1986a) for experimental infection in the guinea pig. Procedures such as immunofluorescence (IFA), complement fixation (CF) and microagglutination assay (MAA), which are less sensitive than ELISA, were employed in previous studies, and hence detection of immunoglobulin occurred slightly later than in the present study.

Guinea pigs given high dose \textit{C. burnetii} by I.P. injection produced an approximately ten-fold stronger serological reaction than those animals given high dose \textit{C. burnetii} by aerosol. As mentioned earlier, animals challenged by aerosol
would be expected to receive less organisms (4-6 log$_{10}$ organisms ml$^{-1}$), than animals challenged by the I.P. route. The serological response to whole cell phase II antigens (QII) demonstrated that over the same period both IgG and IgM levels were significantly reduced in the high dose aerosol-infected animals. Both routes, however, showed levels of IgM against QII to be higher throughout the duration of the experiment than levels of IgG. By day nine post-infection, the titre of IgG directed against QII began to rise. No humoral response directed against whole cell phase I (QI) antigen was detected in those animals given high dose *C. burnetii* by either route.

Titres (IgM and IgG) reached higher levels sooner, in animals given high dose *C. burnetii* by I.P compared with animals given low dose I.P. A similar dose response was observed for IgM titres of aerosol-infected animals. However, IgG titres in aerosol-infected animals over the early days post-infection showed no such dose response. (Kazar *et al.*, 1977) reported a similar finding in that immunoglobulin levels were higher in those guinea pigs given a low (10$^3$ EID$_{50}$) intraperitoneal injection of phase I Nine Mile strain than those given a high (10$^8$ EID$_{50}$) injection by the same route.

The I.P. route of infection in animals given a low dose of *C. burnetii* produced an IgM peak titre which appeared earlier, and was greater than that produced by the aerosol route. This could represent a dose response. In contrast, virtually identical peak IgG titres, at similar days post-infection were detected for both routes in low
dose animals and, therefore, no dose response or route-dependant variation was observed in IgG titres.

No IgM was detected against QI antigen in our study which is consistent with work reported by (Kazar et al., 1977) following I.P. infection of guinea pigs. Immunoglobulin G and A titres to QI antigen in guinea pigs given low dose (groups 3 and 4) were detected in our study at approximately twenty five and forty days respectively, post-infection. Appearance of these immunoglobulins appeared to be independant of route and therefore dose.

Many workers have reported on the value of humoral immune response to C. burnetii infection in humans and its applications for serodiagnosis including (Peter et al., 1987, 1988; Cowley et al., 1992; Dupont et al., 1994; Uhaa et al., 1994; Waag et al., 1995) and (Dupuis et al., 1985). In acute infection, specific IgM immunoglobulins against QII antigens are detected within seven days following exposure (Dupuis et al., 1985). Immunoglobulin G levels to QII antigens rise after seven days, reaching a peak three to four weeks post infection. Approximately three weeks following onset of illness, a maximum serological titre is reached which may continue for eleven to seventeen weeks. Specific antibodies to QII and QI antigens may develop as the acute illness progresses to chronic disease which may occur from three to twenty years later (Peacock et al., 1983).
Chronic Q fever in humans is characterised serologically by persistently elevated IgG and IgA titres to QII and QI. When the titres of immunoglobulins to QI and QII antigens are measured, a ratio of anti-QII to anti-QI of > 1 represents acute Q fever, a ratio of ≥1 represents granulomatous hepatitis and a ratio of ≤ 1 is indicative of Q fever endocarditis (Peacock et al., 1983).

In the present guinea pig study, only IgA levels to QI antigen were higher at the end of the experiment than IgA levels to QII antigen. No high IgG titre to QI was detected. These titres levelled out by day ninety two post-infection. In guinea pigs, based on the above ratios, the serodiagnosis would be suggestive of granulomatous hepatitis. This assumes, however, that the humoral immune response in guinea pigs is comparable to that seen in humans.

(Camacho et al., 1995) reported that elevated levels of subclasses IgG1 and IgG3 were present in chronic human cases of Q fever and that a combination of decreasing IgG2 and increasing IgG3 levels may be predictive of disease progression from acute to chronic. Further studies of immunological subclasses in our guinea pig model may prove useful to determine the disease state at various times post infection.
2.4.4 Host response to infection

Although useful as a means of serodiagnosis, the humoral immune response plays a minor role in the defence against infection by *C. burnetii*. The primary defence against infection is cell mediated immunity (CMI) (Hinrichs & Jerrells, 1976; Kishimoto & Walker, 1976; Kishimoto et al., 1977). It is possible that the reduced IgM and IgG response in animals given *C. burnetii* by the aerosol route was a consequence of the lower retained dose as discussed earlier. However, mortality in the aerosol group would suggest that dose alone was not the only important factor. Following phagocytosis, *C. burnetii* resides within the acidic vesicles of the phagocytes. *C. burnetii* antigens are presented by MHC class II molecules on the cell surface where CD4 T cells activate both B cells for a humoral immune response and T cells for a cell-mediated immune response. (Williams, J. C. et al., 1982; Harding et al., 1988) and (Mims, C. A. et al., 1995). Those animals that received high dose *C. burnetii* by the I.P. route mounted a strong IgM and IgG response, and animals in the low dose by I.P also mounted a strong IgM response. The production of IgG and IgM by the host controls infection by a process of opsonisation. IgM and IgG promote the phagocytosis of *C. burnetii* by binding specifically to antigens via the Fab variable region of the antibody molecule which in turn facilitates binding to Fc and complement receptors present on polymorphs and macrophages, and increases the development of a CMI. The relative lack of an effective antibody response may explain why mortality was higher in animals
infected by the aerosol route compared to those infected by the intraperitoneal route.

*C. burnetii* delivered via the aerosol route was shown to be more infective than by intraperitoneal inoculation. A possible hypothesis for this observation could be that alveolar macrophages may be more numerous or efficient at phagocytosing organisms presented to them than peritoneal macrophages. Once organisms are intracellular they are inaccessible to immunoglobulins, and extracellular organisms are cleared from the infection site by a transient influx of polymorphonuclear leukocytes (PMNs). Intracellular *C. burnetii* are stimulated to multiply by the acidic conditions (Williams, J. C., 1991), and destroyed host cells release microorganisms and their components, which prime the humoral and CMI response as described. Lymphocytes and polymorphonucleocytes (PMN) are usually observed in the vicinity of destroyed host cells. *C. burnetii* circulates either freely in the plasma, on the surface of blood cells or within macrophages released from infectious foci. Secondary infectious foci then result in other organs around the body. This was demonstrated by intratesticular challenge of guinea pigs with *C. burnetii* and the subsequent demonstration of infectious foci in the lungs, liver, spleen and kidneys (Khavkin, 1990). It was also demonstrated that from infectious foci, *C. burnetii* could be released into the outer environment such as the urine and bile from infected glomeruli and kidney stroma and hepatocytes.
The present study suggests that systemic spread of *C. burnetii* occurred throughout organs of the body in a set pattern of infection which was dose-dependent, and route dependent. In the high dose animals administered *C. burnetii* by aerosol, the most severe pathology was observed in the lungs, followed by the spleen, liver, and heart. The kidneys showed no abnormalities. In animals receiving low *C. burnetii* by aerosol, the heart, liver and kidneys showed lesions and abnormalities but no pathology was seen in the lungs or spleen. Molecular (PCR) and immunohistochemical studies confirmed that clearance of organisms occurred more rapidly in animals administered *C. burnetii* by intraperitoneal injection. No antigen could be detected in tissue sections by immunohistochemical methods or by PCR after day 12 post infection in high dose I.P. animals. In contrast PCR analysis revealed the presence of organisms in the lungs and immunohistochemistry and scanning electron microscopy showed organisms to be present in heart tissue at 92 days post-infection in aerosol-infected animals. These observations suggest a resolving systemic infection with prolonged clearance in animals that received low dose *C. burnetii* by aerosol.

In the earliest attempts to identify the agent responsible for the fever observed in humans, experimental mice and rats were found to develop splenomegaly and hepatomegaly, following I.P. inoculation of infectious material (Burnet & Freeman, 1937). In the present study, only guinea pigs that received a high dose of *C. burnetii* showed splenomegaly and hepatomegaly. Those animals given low dose of *C. burnetii* and examined ninety two days later, showed no splenomegaly.
and virtually no hepatic involvement. This pattern of pathology was reported by (Heggers et al., 1975) and (Paretsky et al., 1964) in guinea pigs following I.P. injection of $10^6$ median embryo lethal doses of Nine Mile phase I cells and $10^{5.6}$ egg LD$_{50}$ Nine Mile phase I cells, respectively. Similar self-limiting lesions in the spleen and liver of guinea pigs were reported by (Johnson et al., 1977) following I.P. inoculation of a low virulence strain (M-44) C. burnetii. In their experiment, lesions in spleen and liver were minimal and confined to the early phase of infection. The reduction in severity and pathological observations were attributed to the less virulent strain (M-44) used.

In experimental mice, splenomegaly has been shown to be proportional to infective dose (Hackstadt & Williams, 1981; Scott et al., 1987) and occurs in both sensitive (A/J) strains of mice and resistant (C57BL/6J) strains. Aerosol infection of athymic nude mice with C. burnetii resulted in a progressive infection and splenomegaly, and also occurred in euthymic mice similarly infected. Euthymic mice however, were able to clear the infection (Hall, W. C. et al., 1981).

In the present study, it was impossible to determine if animals in the low dose group developed splenomegaly or hepatomegaly as no sequential samples were taken from this group. Based on similar work performed elsewhere, the low dose animals may have developed splenomegaly and hepatomegaly. The remaining small foci of lymphocytes, necrotic hepatocytes and neutrophils observed in three out of the twelve animals could represent residual infection prior to complete
 clearance. The spleens in low dose guinea pigs showed no lesions or abnormalities suggesting clearance may have occurred earlier during the infection.

Present studies suggested that the kidneys appeared to be the last organ, of those examined, to become infected. Animals given high dose *C. burnetii* were killed before the kidneys became involved. The kidneys of guinea pigs given low dose *C. burnetii* by aerosol remained with foci of infection after ninety two days post-infection; however, immunocytochemical staining and PCR proved negative for specific antigen.

The pathology found in the heart of animals given high dose *C. burnetii* was consistent with (Heggers *et al.*, 1975; Powanda *et al.*, 1978) and (Johnson *et al.*, 1977) where epicarditis and myocarditis were observed. Low dose *C. burnetii* animals demonstrated pathology of the heart tissues, probably as a result of the widespread disseminated infection mentioned earlier. However, no other studies in guinea pigs have reported pathology in the heart, or identified antigen by immunocytochemical staining so long after infection.

**2.4.5 Persistence of *C. burnetii* within the host**

Although the guinea pig is highly susceptible to infection by *C. burnetii*, the infectious foci are cleared of microorganisms faster and more effectively in the guinea pig than in mice (Khavkin, 1990). In both mice and guinea pigs, however,
clearance is incomplete and acute infection can be reactivated in the immunocompromised host or during pregnancy (Atzpodien et al., 1994); (Kazar and Kovacova, 1983); (Tokarevich, 1979).

In human cases of *C. burnetii* infection, the organism is able to remain dormant and unaffected by the immune system for long periods of time. It has been suggested that the spore-like formation of *C. burnetii* enables the microorganism to survive in a dormant state. The developmental cycle of *C. burnetii* consists of both vegetative growth and spore formation and the appearance of different antigens in the cell walls provides a mechanism of evasion from the immune response (McCaul, 1991). Aerosol delivery of organisms may inadvertently select for small cell variants which are phagocytosed and express fewer antigens on the surface of the phagocyte and consequently avoid detection by antibodies. This may be a possible reason why aerosol-infected animals showed a reduced serological response. The small cell variant, which is more stable in the environment, would be the most likely cell to be transmitted to humans and animals aerogenically in the natural environment, and may explain why the route of infection determines the eventual disease outcome (McCaul, 1991).

The specific cell type within which the microorganisms reside may also be responsible for the survival of cells (Khavkin, 1990). Cells able to retain *C. burnetii* organisms within them would be long-lived non-phagocytic cells rather than short-lived mononuclear phagocytes. Such cells may be stromal cells,
belonging to the lymphoid tissues called limbocytes. These cells are non-phagocytic, non-haematopoietic and relatively long-lived. Ultra-structural studies on stromal cells within the spleen and lymph nodes containing *C. burnetii* bear similarities to limbocytes. Other microorganisms such as *Mycobacterium leprae* and *Toxoplasma gondii* are found in non-phagocytic cells such as muscle, neural and Schwann cells when acute infection converts to chronic and latency (Khavkin, 1990).

It is possible that lesions seen in heart tissues of guinea pigs given low dose *C. burnetii* are due to organisms that have avoided clearance by the CMI responses, or alternatively these organisms are the remnants of a disseminated infection. Immunocompromised mice were shown to have endocarditis of the atrioventricular and semilumbar valves ten days post I.P injection of Nine Mile phase I organisms. At one hundred and fifty days post-infection, however, cardiac valves were clear of antigen and lesions (Atzpodien et al., 1994). Guinea pigs are able to clear infection more rapidly than mice; therefore it would be expected that heart tissues in the guinea pig would be free of *C. burnetii* before one hundred and fifty days.

Immunocytochemistry and PCR studies in the guinea pig model correlated well with the histological findings. Antigen was detected in the lungs, heart, liver and spleen of animals tested from the high dose group. Only PCR was able to detect antigen in the kidneys from these animals. This may have been due to greater sensitivity of the PCR compared to immunological techniques.
The histology of the liver and spleen would have predicted a greater quantity of antigen present than was detected. However, the observed CMI response could have been directed against organisms which had subsequently been cleared or insufficient quantities of antigen may have been present to be detected immunocytochemically. Alternatively, the progression of disease may have been less advanced in those animals tested for antigen compared to those processed for histology.

Electron microscopical analysis demonstrated that antigen detected by immunohistochemical techniques in heart sections from 9 days post infection, in the high dose group, and 92 post-infection, in the low dose group, were whole organisms. Such antigen detection, particularly in the heart and lungs has not been reported elsewhere in experimental infections as most studies in the guinea pig have been confined to the acute response.

Immunohistological demonstration of *C. burnetii* from human heart valves has been reported (Brouqui *et al.*, 1994). These authors concluded that the absence of granulomas in a number of their patients, but the presence of *C. burnetii*, suggested persistence by the organisms due to an aberrant host immune response. It was proposed that during acute infection, *C. burnetii*-infected monocytes circulated within the host and attached to previously damaged heart valves where persistence eventually led to chronic infection.
In the present guinea pig infection studies, organisms were shown to persist in the heart and lung for long periods of time after histological evidence suggested clearance had occurred. This supports previous findings that infection can be reactivated by pregnancy and irradiation in experimental animals and may explain why reactivation can occur in humans (Yeaman & Baca, 1990).

(Kazar & Kovacova, 1983) also reported that, during acute infection in mice and guinea pigs, large numbers of \( C. \) \( \text{burnetii} \) were present in spleen and livers. Persistence of organisms was noted in kidneys and reproductive tracts for longer intervals after the acute phase of infection. Immunocytochemically, this was not found to be the case in our study; however, lesions were observed histologically in the kidneys of some animals in the low dose group long after spleen pathology indicated no abnormalities.

In conclusion, this study has presented evidence that route of infection greatly affects the virulence of \( C. \) \( \text{burnetii} \). A previously uncharacterised strain of \( C. \) \( \text{burnetii} \) (Lane), a British isolate of low passage history, isolated from a human suffering from endocarditis, was shown to be highly virulent when given to guinea pigs by the aerosol route. This is contradictory to the suggested theory that human endocarditic causing strains are less pathogenic for guinea pigs.

The serological response elicited in guinea pigs was characteristic of an acute phase reaction and supports previously published data for acute \( C. \) \( \text{burnetii} \).
infection in both guinea pigs and humans. No data has been published previously on the IgA response in \textit{C. burnetii} infected guinea pigs and hence the serology data presented here extends this information.

This study has also corroborated previously published results indicating persistence of \textit{C. burnetii} within organs after clinical signs suggested clearance. Persistence of organisms in heart tissue, thirteen weeks after low dose aerosol infection in the guinea pig, has not been reported elsewhere.

The guinea pig study represents a well characterised model system of acute human Q fever. This model can therefore be used to study the pathogenesis of respiratory Q fever and to provide samples for diagnostic research. In the following chapter, an assay for the detection of Q fever infections by detection of specific antigens in urine was developed. Urine, obtained from animals infected with \textit{C. burnetii} by aerosol, provided samples with which to validate the assay in place of urine from human Q fever cases.
CHAPTER 3

Development of an Assay for the Detection of Antigen in Urine from *C. burnetii*-Infected Guinea Pigs.

3.1 Introduction

3.1.1 Diagnosis of *C. burnetii* infection

The primary objectives for laboratory diagnosis of infectious diseases, including Q fever, are early, accurate and rapid identification of the causative organism, enabling suitable patient management. As outlined in the general introduction (Section 1.7) current procedures for the clinical diagnosis of disease rely on direct isolation of the microorganism or, serodiagnosis. Direct isolation of organisms however, including *C. burnetii*, are not always achievable or reliable from body fluids such as sputum, blood and urine during various stages of the disease, as bacteraemia may be transient. In addition, organisms may be bound by serum antibodies, sequestrated into the reticuloendothelial system, metabolised, or at low levels within the individual (Baca & Paretsky, 1983; Reimer, 1993).

3.1.2 The detection of specific antigens in urine for the clinical diagnosis of disease

The object of an immunoassay which detects *C. burnetii*-specific antigens in urine is to identify infected patients from non-infected for the clinical diagnosis of *C.
burnetii. The assay should provide a positive or negative result based on an established cut-off value. The clinical sensitivity measures how well the assay detects those patients with infection and is the proportion of true positives that are correctly identified.

The clinical specificity measures how well the assay correctly identifies those patients who do not have the infection and is defined as the proportion of true negatives that are correctly identified. Sensitivity and specificity, however do not provide information on the probability of the assay giving the correct diagnosis in a given population. Such information is provided by the positive predictive and negative value of the assay. The positive predictive value is therefore the proportion of patients with a positive result that actually have the disease and the negative predictive value is the proportion of patients with a negative result who do not have the disease.

The bulk of research into antigen detection in body fluids has concentrated on site specific fluids such as cerebrospinal fluid in cases of bacterial meningitis, and sputum in pneumonia cases. As early as 1917, however, specific capsular polysaccharide was detected in the urine of rabbits with pneumococcal bacteremia (Dochez & Avery, 1917), and a large number of infectious diseases have been diagnosed by the detection of specific antigen in urine (Table 3.1).
Table 3.1  Infections diagnosed by urinary antigen detection.

<table>
<thead>
<tr>
<th>Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chlamydia trachomatis</em></td>
<td>(Chernsky et al., 1989)</td>
</tr>
<tr>
<td></td>
<td>(Lebrun et al., 1983)</td>
</tr>
<tr>
<td><em>Aspergillus spp.</em></td>
<td>(Rogers et al., 1990)</td>
</tr>
<tr>
<td><em>Brucella abortus</em></td>
<td>(Limet et al., 1988)</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em></td>
<td>(Magnarelli et al., 1994)</td>
</tr>
<tr>
<td><em>Trypanosoma cruzi</em></td>
<td>(Corral et al., 1989)</td>
</tr>
<tr>
<td><em>Streptococcus pneumoniae</em></td>
<td>(Farrington &amp; Rubenstein, 1991)</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>(Chaicumpa et al., 1992)</td>
</tr>
<tr>
<td><em>Mycobacterium leprae</em></td>
<td>(Sharma et al., 1992)</td>
</tr>
<tr>
<td><em>Mycobacterium avium</em></td>
<td>(Sippola et al., 1993)</td>
</tr>
</tbody>
</table>

The above studies proved the potential of urinary antigen detection as a method of rapid and early diagnosis of infectious disease.
Such conclusions have been particularly validated in the case of Legionnaires' disease. Experimental infection of guinea pigs with *L. pneumophila* has shown that specific breakdown products of this organism are shed into the urine 2 days post-infection coinciding with the onset of disease symptoms (Williams, A. & Featherstone, 1988), and before any detectable antibody response (approximately 7-10 days post-infection). In addition, it has been demonstrated that the antigen appearing in the urine of a guinea pig model of Legionnaires' disease is lipopolysaccharide (LPS), and is identical to the antigen in urine of human cases of Legionnaires' Disease (Williams, A. & Lever, 1995).

Detection of specific antigens in urine has the potential, therefore, to provide a specific, rapid, sensitive and early diagnostic test with a non-invasive sample collection procedure enabling drug therapy to be targeted to specific pathogens earlier, thereby improving the efficiency of the treatment. It also offers several advantages over either direct isolation of organisms or detection of specific antibodies. It is a non-invasive procedure to collect samples and is adaptable to automated routine testing in laboratories where specialised equipment is not always available for the isolation of infectious agents. It is also a rapid method of detection, where tests can be performed in a matter of hours using well established reagents and methodologies. Furthermore, it has the potential to be adapted to the 'dipstick' format for use outside the laboratory, for example, in general practitioners surgeries or the Third World. The procedure also yields large volumes
of fluid which is easily concentrated 20-50 fold enabling low levels of antigen to be concentrated (Coonrod, 1983).

It has been documented previously (Derrick, 1937; Marrie, 1990b), and (Baca & Paretsky, 1983) that infected animals and humans shed *C. burnetii* in urine, therefore the detection of organisms or *C. burnetii*-specific antigens would provide direct evidence of infection. The development of an experimental animal model for acute *C. burnetii* infection, described in the previous chapter, provided appropriate urine samples with which to study the appearance of *C. burnetii*-specific antigens in infected guinea pig urines over time.

### 3.1.3 Aim of this chapter

The aim of this element of the work was to determine if *C. burnetii*-specific components are excreted in the urine of experimentally-infected guinea pigs. It is intended to develop an ELISA to detect this urinary antigen and determine the appearance of the antigen in the urine post-infection, and relate this to the infective dose. It will then be determined whether urinary antigen detection is a potential method of diagnosis for Q fever. The experimental strategy is outlined below in Fig. 3.1.
Fig. 3.1  Outline of strategy for development of assay

- Reagents
  - Standardisation of Reagents
  - Reactivity of Antibodies
  - Sensitivity of Antibodies

Guinea pig urine

- Direct ELISA
- Capture ELISA

Test infected urines
3.2 Materials and methods

3.2.1 Preparation and concentration determination of C. burnetii phase I lipopolysaccharide (LPS)

The method of (Hitchcock & Brown, 1983) was followed. A wet preparation (approximately 0.5g) of C. burnetii phase I organisms were resuspended in 10ml of 0.0625M Tris buffer containing 2% SDS and heated at 100°C for 10 minutes. Sample was cooled to 55°C and proteinase K added at 0.15mg/ml and incubated at 55°C for 3 hours, then at 20°C for 18 hours. Sodium dodecyl sulphate (SDS) was added at 0.02g/ml and heated to 100°C for 10 minutes. Sample was centrifuged at 12,000g for 30 minutes at 20°C and the LPS precipitated by addition of three volumes of isopropanol at 4°C for 30 minutes. The sample was then centrifuged again at 12,000g for 30 minutes at 4°C and the pellet resuspended in 10 ml of distilled water. The precipitation and centrifugation step was repeated twice, then the LPS suspension was digested with RNase A and DNase I (Boehringer Manheim) both at 0.01mg/ml and 37°C for 30 minutes. Proteinase K at 0.01mg/ml was then added and incubated at 55°C for 3 hours, then at 20°C for 18 hours. Following another LPS precipitation stage with three volumes of isopropanol at 4°C for 30 minutes, the sample was centrifuged at 12,000g for 30 minutes and the resultant pellet resuspended in 1 ml distilled water and lyophilised or left in solution. The concentration of LPS present in solution was determined by an assay based on 3-deoxy-D-manno-2-octulosonate (KDO) to give an approximate value as to the concentration of LPS present in the sample.
3.2.2 KDO assay

This method was based on that of (Weissbach & Hurwitz, 1959). Briefly 0.025M periodic acid was dissolved in 0.0625M concentrated sulphuric acid and 0.4 ml of this solution was added to a set of tubes prepared using standard KDO (Sigma) in the range of 1-15ug/0.2 ml and the test sample. These were left to oxidise at room temperature for 20 minutes then 0.6 ml of 2% sodium arsenite in 0.5M HCl was added and mixed rapidly. Following this 2.5ml of 0.3% thiobarbituric acid in 0.01M HCl was added and heated for 20 minutes at 100°C until the tubes developed a pink colouration which was read at 548 nm whilst still warm.

3.2.3 Direct ELISA

3.2.3.1 Titration of antisera

Materials, buffers and incubation times were as detailed in section 2.2.10. Coating antigens were: C. burnetii phase I stock antigen diluted 1/200, phase II stock antigen diluted 1/2000 and LPS I diluted to a concentration of 0.65µg ml⁻¹. The optimum working dilution of C. burnetii phase I (QI) and phase II (QII) was determined using reference serum obtained from a patient suffering from C. burnetii-confirmed endocarditis as primary antibody. Each antigen was diluted appropriately to give an absorbance reading of 1.0, when read at 450nm, after reaction with the reference serum.
Serial dilutions of rabbit anti-\textit{C. burnetii} phase II polyclonal antibody and a monoclonal antibody 7H12G (prepared by Dr. P. Doyle, Queens University, Belfast, against inactivated \textit{C. burnetii} whole cell phase II) were added to determine optimal titres. An experiment to determine the sensitivity of the polyclonal antibody was performed where the coating antigen (LPS I) was serially diluted and antibody added at 3 dilutions of 1/1000, 1/2000 and 1/4000. Antibodies were detected with anti-mouse HRP (Sigma) whole molecule (1/2000) or anti-rabbit HRP (Sigma) whole molecule (1/2000). Detection was with TMB substrate as detailed in Section 2.2.10.

3.2.3.2 Detection of antigen in spiked urines

LPS I was serially diluted in non-infected guinea pig urine or PBS. The dilutions were coated on an ELISA plate and tested in the direct ELISA with rabbit polyclonal antibody at 1/1000 dilution. The urine and PBS with no LPS added were used as controls.

3.2.4 Capture ELISA

3.2.4.1 Purification of IgG from rabbit polyclonal serum

A Protein G, sepharose column in conjunction with an FPLC system (Pharmacia) was used to purify the IgG fraction from the rabbit anti-\textit{C. burnetii} serum. Serum
was loaded on the column in 1ml aliquots, a pH gradient of 7.0 to 2.7 was applied, and 1 ml fractions were collected. The fractions were tested in a direct ELISA against *C. burnetii* phase II whole cell antigen coated on the plates at 1/2000 dilution. Fractions were diluted 1/1000. Fractions which reacted with the phase II antigen were re-tested in the ELISA but serially diluted to determine optimum titres of IgG.

### 3.2.4.2 Optimisation of capture ELISA

The purified IgG was used to coat ELISA plates and experiments to determine optimum conditions for binding were performed. Incubation times of 2 or 4 hours at 37°C or overnight at 4°C were compared. Varying dilutions of IgG and the detector monoclonal antibody-HRP conjugate were tested. A series of controls were also run in the ELISA to determine non-specific and cross-reactions in each layer of the assay. These were:

+ve control – QII antigen diluted 1/2000 in PBS

-ve control – PBS in place of QII antigen

-ve control – PBS in place of monoclonal detector antibody

-ve control – Non-infected guinea pig urine in place of the antigen and PBS in place of the monoclonal detector antibody

-ve control – PBS only

+ve control – QII antigen diluted 1/2000 in non-infected guinea pig urine.
3.2.4.3 Detection of *C. burnetii*-specific antigen in infected guinea pig urine

Urines obtained from guinea pigs infected with *C. burnetii* by the aerosol and intraperitoneal routes (see section 2.1) were tested in the capture ELISA. The positive control was *C. burnetii* QII antigen diluted 1/2000. Non-infected guinea pig urine was used as a negative (background). The cut-off level was defined as twice the SD above background levels (Wood and Wreghitt, 1990).

3.2.4.4 Biotin-extravidin capture assay

The capture ELISA was modified to increase sensitivity by replacing the monoclonal HRP conjugate with biotin-conjugated, anti-mouse antibody in conjunction with extravidin peroxidase. A series of controls were run as for the peroxidase assay to determine non-specific reactions. These were:

-ve control – No coating antibody, QII as antigen

-ve control – No coating antibody, -ve urine as antigen

-ve control – No coating antibody, PBS as antigen

-ve control – PBS as antigen

-ve control – inappropriate IgG as coating antibody, PBS as antigen

+ve control – QII as antigen

-ve control – negative urine as antigen.
The detection limit of the assay was determined by testing serial dilutions of a stock suspension of QII whole organisms diluted in non-infected guinea pig urine. The stock suspension contained approximately $1 \times 10^9$ organisms ml$^{-1}$, and 1 µl (i.e. $1 \times 10^6$ organisms) of this was added to the first well in the dilution series.
3.3 Results

3.3.1 Characterisation of reagents

Antigens used throughout the course of this study were *C. burnetii* phase I and II whole cells (both formalin inactivated) and lipopolysaccharide prepared from *C. burnetii* phase I whole cell (LPS I). The working dilution for QI and QII control antigens was 1/200 and 1/2000, respectively. The concentration of KDO present in the LPS I preparation was used to determine the concentration of LPS. Typically, KDO constitutes 5% of LPS molecules and could provide, therefore, a measure of the concentration of LPS present. KDO standards were prepared and the LPS I preparation was tested. The concentration of KDO in the test was approximately 12.60 µg/0.2 ml, or 63.0 µg/ml (Table 3.2).
Table 3.2  Determination of KDO in LPS I preparation

<table>
<thead>
<tr>
<th>Test</th>
<th>Concentration KDO (ug/0.2 ml)</th>
<th>Absorbance (548nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard 1</td>
<td>1.25</td>
<td>0.073</td>
</tr>
<tr>
<td>Standard 2</td>
<td>2.50</td>
<td>0.160</td>
</tr>
<tr>
<td>Standard 3</td>
<td>5.00</td>
<td>0.351</td>
</tr>
<tr>
<td>Standard 4</td>
<td>10.00</td>
<td>0.604</td>
</tr>
<tr>
<td>Standard 5</td>
<td>15.00</td>
<td>1.143</td>
</tr>
<tr>
<td>Test (1/20 dilution)</td>
<td>12.60</td>
<td>0.630</td>
</tr>
</tbody>
</table>

Two antibodies were produced for use in the development of an ELISA, a rabbit polyclonal and a mouse monoclonal antibody (ascites fluid, 7H12G) both raised against *C. burnetii* inactivated phase II antigen. The reactivity of each antibody was evaluated by direct ELISA (Table 3.3). The anti-*C. burnetii* polyclonal rabbit sera reacted against inactivated *C. burnetii* phase I whole cell antigen (QI), inactivated whole cell phase II antigen (QII) and lipopolysaccharide prepared from QI (LPS I). The monoclonal antibody reacted against QII only (Table 3.3).

Table 3.3  Reactivity of polyclonal and monoclonal antibodies in direct ELISA with various antigens

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Polyclonal antibody</th>
<th>Monoclonal antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>QI</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>QII</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>LPS I</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
The rabbit anti-\textit{C. burnetii} phase II polyclonal sera reacted in direct ELISA to a dilution of 1/4000 against LPS I preparation. The optimum antibody dilution was 1/1000 against a KDO (LPS) concentration of 1.25\(\mu\)g/ml (Fig 3.2).

Fig 3.2  Sensitivity of polyclonal rabbit sera, in direct ELISA, at various dilutions against different coating concentrations of KDO (LPS I)
The optimum working dilution for the monoclonal antibody ranged from 1/200-1/6400 (fig 3.3).

Fig 3.3 Reactivity of monoclonal antibody (ascitic fluid) for *C. burnetii* whole organisms phase I, II and LPS I in direct ELISA. Background was defined as background mean plus two standard deviations (2 SD). Points represent the mean of two readings.
3.3.2 Development and optimisation of assay

3.3.2.1 Direct ELISA on Urine

A direct ELISA, using non-infected guinea pig urine spiked with various concentrations of LPS I, was performed before testing urine from *C. burnetii* infected guinea pigs. At 1/1000 dilution, the polyclonal rabbit serum reacted with coated LPS at concentrations from 1.25 µg/ml to 0.01 µg/ml with peak reaction occurring at 0.75 µg/ml. In contrast, serum did not detect LPS diluted in non-infected guinea pig urine at any of the concentrations tested (Fig 3.4). To overcome the reaction inhibition, a capture ELISA format was attempted using rabbit polyclonal antibody as a capture antibody and monoclonal antibody (7HG12) as detector antibody.

**Fig 3.4** Detection of LPS I-spiked urine samples by direct ELISA with rabbit anti-*C. burnetii* whole serum
3.3.2.2 Capture ELISA on urine

3.3.2.2.1 Optimisation of the capture assay

Purified IgG was prepared from the rabbit anti-\textit{C. burnetii} polyclonal serum characterised above. The first stage in the development of the capture ELISA was to determine the adsorption of the coated, purified IgG to the microtitre plate. The effect of temperature and coating time was studied on adsorption of the IgG over a range of coating dilutions. Incubation at 37\textdegree C for four hours, and overnight incubation at 4\textdegree C, produced high absorbance readings, and hence efficient binding of the IgG to the microtitre plate, over all dilutions of coated IgG tested. Lower absorbance values, and therefore less binding of IgG to the plate, was achieved with a two hour incubation at 37\textdegree C (Fig 3.5). Four hour incubations at 37\textdegree C were used, therefore, for all future experiments.

\textbf{Fig 3.5} Variation of coating avidity of rabbit IgG on ELISA plate with incubation time.
3.3.2.2  Optimum IgG coating and monoclonal detector antibody dilution determination

A chequerboard assay was performed, to determine the optimum coating dilution of IgG and monoclonal detector antibody, for the next stage in the development of the capture ELISA. IgG was coated at a range of dilutions on a microtitre plate and inactivated C. burnetii whole cell phase II (QII) antigen, diluted 1/2000 in non-infected guinea pig urine, was added. Monoclonal detector antibody at various dilutions was added. The optimum working dilution for both coating IgG and monoclonal detector antibody was 1/200 (Fig 3.6). In addition, this experiment confirmed that the coated IgG and the monoclonal detector antibody were capable of capturing and detecting QII antigens diluted in non-infected guinea pig urine. Hence the reaction inhibition observed in the direct ELISA (Section 3.3.2.1.) had been overcome.
Controls (in triplicate) were performed, using the capture ELISA, to determine background values and eliminate non-specific reactivity (Fig 3.7). Controls 1 and 6, where QII was diluted in PBS and non-infected guinea pig urine, respectively, served as positive controls. Both produced absorbance values of between 0.3 and 0.4 absorbance units. Various negative controls were tested (2, 3, 4 and 5). PBS replaced the QII antigen (experiment 2), PBS replaced the monoclonal detector antibody (experiment 3) and only PBS was coated on the plate in control 5. Control experiment 4 contained coating IgG with non-infected guinea pig urine added. Absorbance values for all the negative controls remained below 0.1 absorbance units indicating that levels of non-specific reactivity were low.
Control experiments to determine background and levels of non-specific reactivity of the capture ELISA.

- Control 1 = Capture ELISA with QII diluted 1/2000 in PBS.
- Control 2 = Capture ELISA with PBS as antigen.
- Control 3 = Capture ELISA with PBS added instead of monoclonal detector antibody.
- Control 4 = Capture ELISA with non-infected guinea pig urine and no monoclonal detector antibody.
- Control 5 = PBS negative control.
- Control 6 = Capture ELISA with QII diluted 1/2000 in non-infected guinea pig urine.
3.3.2.3 Evaluation of infected guinea pig urines in the capture assay.

Urine from four guinea pigs infected with *C. burnetii*, either by the aerosol route (group 1 and 3), or by the intraperitoneal route (group 4), were pooled and tested in the capture ELISA for *C. burnetii* specific reactivity.

Reactivity was detected (above background levels of pooled non-infected guinea pig urine), from 3 days post-infection in animals challenged with high dose (1000 organisms ml\(^{-1}\)) *C. burnetii* by aerosol (group 1). Levels of *C. burnetii*-specific urinary antigen peaked at 6 days post-infection, but remained at detectable levels in the urine until the animals were killed at 9 days post-infection (Fig 3.8).

Animals challenged with a low dose of *C. burnetii* (1-10 organisms ml\(^{-1}\)), by the aerosol route (group 3) and low dose (10\(^5\) organisms ml\(^{-1}\)) by the intraperitoneal route (group 4), proved positive for *C. burnetii*-specific urinary antigen (above background levels) between 3 to 50 days post-infection. Considerable fluctuation in levels of urinary antigen between sample days was observed; however, levels were generally greater up to 24 days post-infection, and gradually reduced by 50 days post-infection (Fig 3.9). Levels of urinary antigen, throughout the course of the experiment, were lower in urine from guinea pigs infected intraperitoneally, compared to those animals infected by aerosol, except at 23 days post-infection. Comparable levels of urinary antigen was detected in urine of guinea pigs infected
by the aerosol route with either high or low dose of *C. burnetii*. Consequently, no
dose response was observed.

**Fig 3.8** Capture ELISA using urine from guinea pigs, infected by
aerosol, with high challenge dose (group 1). Points indicate the mean of three
samples. The standard deviation from the mean is indicated by error bars.
and background was defined as the mean of non-infected urine plus two
standard deviations (2 SD).
Capture ELISA using urine from guinea pigs, infected by aerosol and intraperitoneal routes, with low challenge dose (groups 3 and 4). Points indicate the mean of three samples. The standard deviation from the mean is indicated by error bars and background was defined as the mean of non-infected urine plus two standard deviations (2 SD).
3.3.2.4 Detection of *C. burnetii*-specific antigen in the urine of infected guinea pigs using the biotin-extravidin assay.

Levels of reactivity achieved with the capture ELISA described in Section 3.3.2.3 were low (although above background controls). To amplify the reaction, biotin-conjugated anti-mouse antibody was used in conjunction with extravidin-peroxidase, as a further modification to the capture ELISA. Extravidin has four binding sites for biotin; therefore, it is possible to increase the number of enzyme molecules bound per antigen.

Control experiments were carried out to determine background levels of absorbance (Fig 3.10). Non-infected guinea pig urine gave the highest non-specific absorbance (control 7, Fig 3.10). Absorbance values were less than 0.2 for all other controls with very little variation between replicates. Negative control backgrounds were higher than in the original capture ELISA; however, reactivity of the QII positive control was significantly higher using the biotin-extravidin assay (1.0 absorbance units compared to 0.3 in the original capture assay).
Control experiments to determine background and non-specific reactivity of the biotin-extravidin capture assay

Control 1= No IgG coating antibody, QII and all other reagents added.
Control 2= No IgG coating antibody, non-infected urine and all other reagents added.
Control 3= No IgG coating antibody, PBS and all other reagents added.
Control 4= Capture ELISA with PBS as antigen.
Control 5= Non-specific IgG as coating antibody and PBS as antigen, all other reagents added.
Control 6= Capture ELISA with QII whole cells as antigen.
Control 7= Capture ELISA with non-infected urine added.
The detection limit of the biotin-extravidin capture ELISA was determined (Fig 3.11). Stock suspensions of whole phase II organisms (original concentration of 1mg/ml which was equivalent to approximately $1 \times 10^9$ organisms ml$^{-1}$) were initially diluted 1/100 so that each microtitre well contained approximately $1 \times 10^7$ organisms ml$^{-1}$ or $1 \times 10^6$ organisms/well. The detection limit cut-off for the capture ELISA was at dilution of 1/12800 of the stock suspension which was equivalent to approximately 800 organisms ml$^{-1}$ or 80 organisms/well (8ng of antigen/well).

**Fig 3.11** Detection limit for *C. burnetii* phase II whole organisms in the biotin-Extravidin capture ELISA. Each point represents the mean of two readings and background is defined as the mean of uninfected guinea pig urine plus 2 SD.
Urine obtained from *C. burnetii* infected guinea pigs (groups 1 and 3) were tested in the biotin-extravidin assay. Evidence of *C. burnetii*-specific urinary antigen at 1 day post-infection was detected and continued to the end of the experiment at 8 days post-infection in urine from animals infected with high dose by aerosol (group 1). Levels of urinary antigen were low, compared to the QII positive control, with the peak amount of antigen appearing at 6 days post-infection (0.4 absorbance units). The addition of biotin-extravidin to the assay amplified the reactivity and in addition, the standard deviation from the mean was less than the variation detected by the original capture ELISA (Fig 3.8 compared to Fig 3.12).

Specific urinary antigen was evident at 10-15 days post-infection, and continued to be detected until 43 days post-infection, in those animals given low dose by aerosol (group 3). By 50 days post-infection, reactivity dropped below background levels. A peak in antigen excretion (0.5 absorbance units) was detected at 34 days post-infection, and a greater deviation from the mean was detected throughout the experiment compared to urines obtained from group 1 animals (Fig 3.9 compared to Fig 3.13). Less fluctuation in levels of antigen detected between sample times was evident, however, compared to values obtained by the original capture assay, for the same experiment. The absorbance values, and hence the amount of antigen detected were low, however, compared to the QII positive control.
Fig 3.12 Capture ELISA using urine from guinea pigs, infected by aerosol, with high challenge dose (group 1). Points indicate the mean of six replicates. The standard deviation from the mean is indicated by error bars and background is defined as the mean of non-infected guinea pig urine plus 2 SD.
Capture ELISA using urine from guinea pigs, infected by aerosol, with low challenge dose (group 3). Points indicate the mean of four replicates. The standard deviation from the mean is indicated by error bars and background is defined as the mean of non-infected guinea pig urine plus 2 SD.
3.4 Discussion

3.4.1 Assay Development.

Characterisation of the polyclonal rabbit antiserum confirmed that although animals were immunised with inactivated phase II antigens, immunoglobulins reactive against lipopolysaccharides and whole phase I organisms were also produced. In contrast, the monoclonal antibody proved to be specific for phase II antigens only. Consequently, the polyclonal antibody was used initially in the direct ELISA, to ensure that all potential antigens in the urine could be detected.

The simplest and most rapid format for ELISA is the direct ELISA. Sample is adsorbed directly onto the microtitre plate and specific detector antibody, either directly conjugated with an enzyme or using a conjugated anti-species antibody, is added afterwards. The direct ELISA was used as the standard diagnostic assay for the detection of specific human anti-\textit{C. burnetii} immunoglobulins at CAMR and was found to be as sensitive and specific as IFA or dot-immunoblotting (Cowley \textit{et al.}, 1992). The direct ELISA was initially chosen therefore, as it was sensitive and compatible with existing equipment and procedures. Direct ELISA, however, can be less sensitive and specific than other formats, but this is dependant upon the sample being tested, and the antibody used (Kemeny, 1991).

In this study, reaction inhibition in direct ELISA occurred when non-infected guinea pig urine was introduced into the system. This suggested that urine was
either inhibiting the binding of antigen to the microtitre plate, or inhibiting antibody-antigen binding. The reaction between the rabbit polyclonal antibody and the anti-rabbit conjugated antibody may also have been inhibited. Reaction inhibition in ELISA, by urine, has been described previously. (Yamanaka et al., 1992) reported an unknown substance in urine that interacted with the Fc portion of the immunoglobulin molecule, and (McKeating et al., 1986) showed that β2-microglobulin bound to viral envelope protein, masking the antigenic determinants. It is possible, therefore, that similar components were responsible for the lack of reaction in spiked urine samples in this study.

A capture ELISA or double antibody sandwich format was attempted to overcome the reaction inhibition. In this format, IgG was passively adsorbed onto plastic microtitre wells by hydrophobic adsorption. Coating the solid phase (microtitre well) with IgG from whole rabbit antiserum has been shown to reduce non-specific absorbance (Williams, A. & Featherstone, 1988; Wood & Wreghitt, 1990).

The optimum concentration of the reactants in an ELISA is related to the assay incubation time and temperature. Optimum incubation time for the coating IgG antibody was determined before the optimum working dilution, as the amount of antigen that can be bound to antibody is proportional to the concentration of free antibody. The sensitivity of the ELISA would be reduced, therefore, if the capture antibody concentration was limited (Yolken, 1982). Optimum dilutions of both capture IgG and detector monoclonal antibody were determined using a chequer-
board assay. Absorbance values, using a standard dilution of QII whole cell antigen as a positive control however, were comparatively low. This could be because monoclonal antibodies are directed at specific epitopes on a large molecule whereas polyclonal antibodies react at various sites on one molecule. Also monoclonal antibodies directed against high-molecular-weight protein antigens can have low affinity constants (Yolken, 1982), (Frankel & Gerhard, 1979). The result, however, is that monoclonal antibodies can be less sensitive than polyclonal antibodies. This may explain the low sensitivity of the monoclonal antibody and, therefore, the capture ELISA used in this study. Although the sensitivity of the original ELISA for QII was low, absorbance values for negative controls were significantly lower. Non-specific reactions between reagents, and between reagents and non-infected guinea pig urine were also low proving that the capture format had overcome the original reaction inhibition.

The detection limit calculated for the biotin-strepavidin assay based on dilution of QII whole cells was 80 ng antigen ml⁻¹ or 800 organisms ml⁻¹. This is less sensitive than that reported for a number of other capture ELISA-based assays, but higher than some alternative techniques such as agglutination and dot-ELISA for urinary antigen detection in human and experimental animals. Table 3.3 provides some comparative examples reported elsewhere.
### Table 3.3 Detection limits of various urinary antigen assays

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antigen</th>
<th>Detection limit</th>
<th>Assay</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. typhi</em></td>
<td>Vi antigen</td>
<td>1 ng/ml</td>
<td>ELISA</td>
<td>(Barrett et al., 1982)</td>
</tr>
<tr>
<td><em>L. pneumophila</em></td>
<td>lipopolysaccharide</td>
<td>10 ng/ml</td>
<td>ELISA</td>
<td>(Williams, A. &amp; Featherstone, 1988)</td>
</tr>
<tr>
<td><em>B. abortus</em></td>
<td>lipopolysaccharide</td>
<td>0.1 ng/ml</td>
<td>ELISA</td>
<td>(Limet et al., 1988)</td>
</tr>
<tr>
<td><em>C. burnetii</em></td>
<td>whole cells</td>
<td>10^5 cells/ml</td>
<td>dot-blot</td>
<td>(Tylewska-Wierzbanowska &amp; Kruszewska, 1993)</td>
</tr>
<tr>
<td><em>N. meningitidis</em></td>
<td>polysaccharide</td>
<td>&gt; 100 µg/ml</td>
<td>agglutination</td>
<td>(Suwanagool et al., 1986)</td>
</tr>
<tr>
<td><em>H. influenzae</em></td>
<td>polysaccharide</td>
<td>6 ng/ml</td>
<td>agglutination</td>
<td>(Kaldor et al., 1979)</td>
</tr>
</tbody>
</table>

#### 3.4.2 Assay Findings

Excretion of *C. burnetii* has been shown to occur, via urine, following secondary infection by *C. burnetii* in the glomeruli and kidney stroma of guinea pigs (Khavkin, 1990). In this study, *C. burnetii* DNA was detected at 9 days post infection, in the kidneys of animals given high dose *C. burnetii*, and histological
evidence of infection was detected, 92 days post-infection, in low dose animals (Chapter 2). Such evidence, therefore, suggested whole organisms may have been excreted into the urine during infection, and that the capture assay described may have detected these organisms. Bacteriemia, characterised by a febrile response, occurred much sooner in animals administered high dose C. burnetii compared to animals given low challenge dose (Chapter 2), consequently, secondary infection sites in the kidney would be expected to occur much sooner in animals challenged with a high dose of C. burnetii. The detection of urinary antigen by day 1 post-infection in high dose animals compared to day 10-15 post-infection in low dose animals provided further evidence that the assay detected whole organisms, excreted into the urine, due to kidney damage.

The monoclonal detector antibody, in the capture ELISA, was specific for a C. burnetii phase II epitope only, however C. burnetii phase I cells or antigens would be expected to be excreted into the urine of infected guinea pigs. It was established that the monoclonal antibody was unreactive against C. burnetii whole phase I cells, but was able to detect a C. burnetii-specific antigen in guinea pig urine. This suggested that the urinary antigen was not whole phase I cells, but partially degraded cells where the phase II epitope was exposed. (Williams, J. C. & Stewart, 1984) and (Williams, J. C. & Waag, 1991) showed that immune sera from mice immunised with phase II cells detected an 86 kDa protein antigen, present in soluble detergent extracts of both phase I and II cells grown in vitro. This antigen was not detected, however, by sera from mice infected or immunised with phase I
cells. Therefore although the antigen was synthesised by both phase I and II cells, it was inaccessible to the murine immune system, when challenged with viable or killed phase I cells. Likewise, the monoclonal antibody in this study may be directed against a phase II antigen, which was present on both phase I and II cells, but only exposed on phase I cells after processing through an animal. Other antigens may be excreted during infection, such as LPS or QI specific antigens, but these would not be detected by the assay as the monoclonal detector antibody was only capable of detecting QII antigens.

The antigen detected in this assay, therefore, may be a degradation product resulting from the intracellular digestion of the organism in the phagolysosome and subsequently released during host cell lysis. In the present study there was an increase in the level of antigen detected, beginning at thirty days post infection, which coincided with the end of the febrile response in those animals given low dose *C. burnetii* by aerosol (chapter 2). In addition, specific immunoglobulin levels rose in animals administered a low dose of *C. burnetii*, beginning at ten days post infection, to a peak at approximately thirty five days after challenge. Since it is known that specific immunoglobulin promotes the uptake of organisms by phagocytes (Kazar *et al.*, 1975); the rise in antigen excretion detected at this time may represent antigen released from infected phagocytes.

The detection of urinary antigen was related to the initial infective dose received by the animals. The appearance of the antigen in urine closely followed the onset of
clinical signs. Similar results were reported during studies of experimental Legionnaires' disease in guinea pigs (Williams, A. & Featherstone, 1988) and brucellosis in mice (Limet et al., 1988). Following an acute (and lethal) aerosol challenge of *L. pneumophila* in guinea pigs, levels of *L. pneumophila*-specific antigen detected in the urine of infected animals rose sharply after day 1 post infection, to a peak at death on day 3 post-infection. Antigen was detected initially 3 days post-infection after a lower (or chronic) infective dose of *L. pneumophila* was administered and peaked at day 6 post-infection. Antigen continued to be detected in guinea pig urine until day 17 post-infection. In experimental brucellosis in mice, *B. abortus* antigens were detected in urine for 85 days following an intraperitoneal injection of $2 \times 10^6$ cfu, with the maximum quantities of antigen appearing in the first days after infection.

In summary, this chapter has described the development of a capture ELISA, and demonstrated by use of the ELISA, that guinea pigs experimentally infected with *C. burnetii* excreted specific antigen into their urine. The appearance of antigen in the urine was dependant on the initial infective dose and appears at approximately the same time as the IgM and IgG response.

The demonstration of specific antigen in urine means that urinary antigen detection could be used as a method for the diagnosis of acute Q fever. It is not known if this antigen is excreted in human acute cases of Q fever, although the guinea pig model described in chapter 2 showed the guinea pig to be a relevant model for human
acute Q fever. Relevant human acute and chronic Q fever urine samples need to be tested to determine if antigen is present in human urine, and if disease state affects the excretion of the antigen.

This assay also has potential application for testing the excretion of \textit{C. burnetii} antigen in the urine of other animal species such as cows, sheep and goats. Valuable epidemiological data could then be obtained during sporadic outbreaks as well as determining infection within herds and flocks.

This chapter concentrated on the development of an assay to detect antigen in the urine of guinea pigs experimentally infected with \textit{C. burnetii}. Such an assay was developed, however the nature of the antigen in urine was not investigated. Evidence was presented which suggested that the antigen detected was not whole organisms but a degradation product. The next part of the investigation is to characterise the antigen appearing in the urine.
CHAPTER 4

Characterisation of the antigen in urine from \textit{C. burnetii}-infected guinea pigs.

4.1 Introduction

Acute \textit{C. burnetii} infections are characterised by extensive bacteriemia which results in the formation of numerous infectious foci throughout the body. From an initial primary infection site, secondary infectious foci are established. \textit{C. burnetii} organisms enter the blood and lymph systems from an infection site where they circulate freely in the plasma or are attached to the surface of blood cells, or are contained within macrophage-type cells released from infectious foci (Khavkin, 1990). Organisms may be released into the urine from these secondary infection sites in the kidney, or degradation products of the organisms may be solubilised, thus enabling the passage of these components across the serous membranes into the urine (Coonrod, 1983). This has been reported in experimental infections in guinea pigs where glomeruli and kidney stroma were infected, resulting in excretion of whole \textit{C. burnetii} into the urine (Khavkin, 1990). In addition, whole organisms were detected up to 14 days post-infection, in urine obtained from experimental \textit{C. burnetii} infection in mice (Kruszewska & Tylewska-Wierzbanowska, 1992) and (Parker \textit{et al.}, 1951) reported the presence of whole
viable *C. burnetii* in dried guinea pig urine and noted the ability of the organism to survive for 49 days in the urine.

Initial isolation studies for *C. burnetii* involved the injection of urine from infected abattoir workers into guinea pigs. A subsequent febrile response, and detection of organisms in the spleen of guinea pigs, indicated that organisms were present in three out of seven individuals' urine towards the end of the illness or during convalescence (Burnet & Freeman, 1937; Derrick, 1937). The human cases indicated that organisms had spread from the primary infection site (probably the lungs) to a secondary infection site in the kidneys where whole organisms had subsequently been excreted into the urine.

Following entry into the host, *C. burnetii* adheres to host cells such as alveolar macrophages and granulocytes. After internalisation into the cell by phagocytosis, phagosome-lysosome fusion takes place and *C. burnetii* multiplies within the phagolysosomal space of phagocytic cells (Hackstadt & Williams, 1981).

The organism is able to resist a number of host antimicrobial activities, generated by the initial oxidative burst of the granulocytes and mononuclear phagocytes following phagocytosis, by the production of specific enzymes such as superoxide dismutase (SOD) and catalase (Akporiaye & Baca, 1983; Beaman & Beaman, 1984). SOD converts the toxic superoxide anions to hydrogen peroxide, and catalase then converts this to oxygen and water. In addition, certain intracellular
parasites such as *Legionella micdadei*, *Mycobacterium leprae* and *Leishmania donovani* as well as *C. burnetii* have been shown to produce acid phosphatase (ACP) which inhibits toxic oxygen metabolites being generated by the host cell (Baca et al., 1993, 1994). Although *C. burnetii* is adapted to survive intracellularly by the mechanisms outlined above, the eventual lysis of the vacuole releases further organisms into the environment as well as lysosomal contents, soluble antigens and fragments of the organism (McCaul, 1991).

*C. burnetii* undergoes a complex developmental cycle, within the host phagolysosome. Initially, the infective stage of the organism (the small resting cell, SRC) undergoes development, activated by the acid conditions within the phagolysosome, to small and large vegetative cells (Williams, J. C. & Waag, 1991). A number of these vegetative cells, depending on the prevailing conditions, may then produce endogenous spores. It is proposed that the spores mature at the time of exit from the mother cell and go on to produce the next generation of SRC. This cycle has been compared to the growth phases seen in normal bacteria. The activation of the SRC to vegetative forms is comparable to the lag phase. The exponential phase is the continued reproduction of large and small vegetative cells. The stationary phase results in no net increase in bacterial cell numbers because of the death of old vegetative large cells and renewal by vegetative small cells. Finally, the death phase is characterised by the death of old large vegetative cells and the lysis of mother cells liberating endogenous spores which mature to form SRC. Throughout this cycle, antigenic variation occurs in the cell wall of the
organism as determined by monoclonal antibodies directed against LPS and protein epitopes (McCaul et al., 1991). In particular a 29.5 kDa protein was identified which was present in large and small vegetative cells and the mother cells but not identified in the SRC or the spore. Consequently, the phagolysosome is filled with an heterologous population of C. burnetii including SRC, vegetative small and large cells, mother cells, and mother cells containing endogenous spores. The phagolysosome also contains cell walls, degraded large cells and soluble bacterial products (Williams, J. C. & Waag, 1991). An imbalance may occur where excessive substrates (bacterial products) are supplied to the lysosome for the lysosomal enzymes to degrade. The resultant accumulation of bacterial cells and soluble products may then influence the degradative capacity of the lysosome (Pfeifer, 1987). This enlargement of the phagolysosome has been reported in Q fever infections (Janigan & Marrie, 1990; Khavkin, 1990) and in continuously infected cells in tissue culture (Baca, 1989). An engorged cell will then occasionally lyse and spill the cell contents into the extracellular environment.

4.2 Aim of this chapter

The establishment of a capture ELISA (in Chapter 3), detected C. burnetii-specific immunoreactive product in the urine of experimentally infected guinea pigs. The assay described however, could not differentiate between viable C. burnetii, soluble antigens, or a combination of both. This chapter aims to determine the nature of the antigen shed into the urine of experimental C. burnetii infection and
determine if the antigen can be used as a specific marker of infection. The nature of the antigen will be investigated as shown in the flow diagram below (Fig. 4.1).

Fig 4.1 Strategy of investigation to characterise the antigen in C. burnetii-infected guinea pig urine

whole urine

Molecular (PCR)  Physical (gel filtration)  Viability

animal inoculation (ELISA)  tissue culture (IFA)

immunoreactive fractions (ELISA)

analysis of reactive fractions

Enzyme treatment  Antigenicity  Immunoreactivity  Molecular (PCR)  Biochemical (protein)

(proteinase K)  (animal inoculation)  (immunoblot)

SDS-PAGE

SDS-ELISA

PAGE

(LPS)  (LPS)

IFA  immunoblot  ELISA
4.3 Materials and methods

4.3.1 Viability studies

4.3.1.1 Tissue culture

Samples of lung, liver, spleen and urine from animals infected with a high dose of C. burnetii by the aerosol route were collected and macerated with 10 ml sterile PBS in a class III cabinet within an ACDP level 3 laboratory. L929 cells (obtained from ECCAC, CAMR), in DMEM + 10% foetal calf serum (Gibco), with no antibiotics, at a concentration of $2.0 \times 10^5$ cells ml$^{-1}$ were seeded into duplicate 75cm$^3$ flasks and grown overnight at 37°C in an atmosphere of 5% CO$_2$. Flasks were then inoculated with $10^{-1}$, $10^{-2}$ and $10^{-3}$ dilutions of the above tissue samples and incubated at 37°C in 5% CO$_2$ atmosphere for 14 days. Standard immunofluorescent staining techniques were used to detect the presence of C. burnetii. Briefly, media was decanted from the tissue culture flasks and cells were rinsed with sterile PBS. 5ml of trypsin (Gibco) was added to each flask and cells incubated for 10 mins or until the cell sheet was observed to be lifting. PBS with 0.001% bovine serum albumen (PBS/BSA) (5ml) was added and cell suspension decanted to a centrifuge tube and centrifuged at 3,000 rpm for 5 minutes. The resultant supernatant was discarded and the cell pellet resuspended into 500 µl of PBS/BSA. The method of (Cowley et al., 1992) was then followed using known human positive control serum which was reactive against C. burnetii as detector antibody, and goat anti-human IgG (Sigma) as conjugate.
4.3.1.2 Animal inoculation

Aliquots (0.5 ml) of the above samples were inoculated intraperitoneally (two guinea pigs per sample) and 21 days post-infection, serum was removed by cardiac puncture and tested for the presence of specific anti-\textit{C. burnetii} antibodies by indirect ELISA. Additional guinea pigs were inoculated with urine obtained from non-infected guinea pigs, and non-infected guinea pig sera, was also used as negative controls.

4.3.2 Molecular (PCR)

4.3.2.1 DNA extraction from \textit{C. burnetii}-infected guinea pig urine

QIAamp method for extraction of DNA from body fluids was followed according to the manufacturers instructions (Qiagen Ltd, Crawley). Urine (140 µl) was added to 560 µl of lysis buffer in a 1.5 ml microfuge tube and mixed by vortexing. Tubes were incubated at room temperature (15 °C-25 °C) for 10 minutes then 560 µl of ethanol was added to the sample and mixed by vortexing. To a spin column, 630 µl of the above solution was added and this was centrifuged at 6,000 x g for 1 minute and the filtrate discarded this was repeated twice. The spin column was then washed twice with 500 µl of distilled water by centrifugation at 20,000 x g for 3 minutes. DNA was eluted from the spin column by addition of 200 µl of distilled water heated to 70 °C followed by centrifugation as above.
4.3.2.2 PCR analysis of *C. burnetii*-infected samples

This was as described in Section 2.2.11.

4.3.3 Physical investigation {size exclusion chromatography by fast protein liquid chromatography (FPLC)}

A Superose 6 gel filtration column was used with a fast protein liquid chromatography system (FPLC, Pharmacia). Samples were applied in 200 µl volumes at a flow rate of 1 ml min⁻¹ using PBS as eluent. The column was calibrated at the above conditions using molecular weight markers and according to the manufacturers instructions (Pharmacia). Markers were Dextran Blue, 2,000 kDa; thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; albumen, 69 kDa; ovalbumen, 43kDa; chymotrypsin A, 25 kDa and ribonuclease A, 13.7 kDa.

Urine from guinea pigs administered high and low doses of *C. burnetii* by aerosol was centrifuged at 10,000 rpm for 5 minutes in a microfuge (MSE) and filtered through a 0.1µm syringe-fitting filter (Millipore) and injected onto the column. Fractions were tested in a direct ELISA (Section 4.3.4) for specific reactivity against polyclonal anti-*C. burnetii* whole cell phase II antibody. The ELISA-reactive fractions were pooled and concentrated by freeze drying.

Non-infected guinea pig urine and non-infected guinea pig urine spiked with 1µg/ml and 5µg/ml LPS from *C. burnetii* phase I Lane strain were separated on the
size exclusion column and the eluent was tested by direct ELISA, to serve as controls. Additional control urine from guinea pigs infected with aerosols of either *M. tuberculosi*s or *L. pneumophila* were also separated on the column and tested for reactivity in direct ELISA. Non-infected guinea pig urine with 0.1 mg ml\(^{-1}\) guinea pig albumin (Sigma) and 0.1 mg ml\(^{-1}\) \(\beta_2\)-microglobulin (Sigma) were also separated on the column and subsequent fractions were tested in the direct ELISA.

4.3.4 Direct ELISA

Putative antigen-containing solution was coated directly onto the ELISA plates (100 µl) and incubated, shaking at 37°C for three hours. Coating buffer, if necessary, was used as diluent. Rabbit anti-*C. burnetii* whole cell phase II IgG (1/1000 diluted in PBST + 10% HS) or guinea pig anti-ELISA reactive fractions, (serially diluted in PBST + 10% HS) was incubated at 37°C shaking for two hours. Plates were washed in PBST three times and anti-rabbit HRP conjugate (Sigma), diluted 1/2,000 in PBST + 10% HS or anti-guinea pig IgG (Sigma), diluted 1/2000 in PBST + 10% HS, was added and plates incubated as above. Substrate development and absorbance reading was as described in Section 3.2.3.

4.3.5 Enzymatic treatment of ELISA-reactive fractions

Proteinase K digestion of the ELISA-reactive fractions from *C. burnetii*-infected urines was performed according to the method of (Heckels & Virji, 1988).
Fractions were incubated with proteinase K (Sigma; 100 µg ml\(^{-1}\) in PBS) at a ratio of 1:1 at 60 °C for 60 minutes. Enzyme activity was stopped by denaturation of the proteinase K by boiling at 100 °C for 20 minutes.

4.3.6 Antigenicity studies: immunisation of guinea pigs with ELISA-reactive fractions

Antisera was raised in two guinea pigs by the intramuscular injection of 0.25 ml of concentrated, ELISA-reactive fractions. Two identical booster injections were administered at two weeks intervals and the animals were bled 3 weeks later. Negative control serum was obtained by the injection intramuscularly of one guinea pig with sterile PBS in identical volumes and serum removed as above. Serum reactivity against *C. burnetii* whole cell in phases I and II was investigated by Western blot, ELISA and IFA and reactivity against ELISA-reactive fractions was investigated by Western blotting and ELISA.

4.3.7 Total protein determination

Bicinchoninic acid protein assay kit (Sigma) was used to determine the total protein concentration according to manufacturers instructions.
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of C. burnetii whole cells phase I and II was performed in pre-cast 8-25% acrylamide gradient gels and run on a horizontal gel electrophoresis system (Pharmacia) using the buffer system described by (Laemmli, 1970). All other samples were separated, using the same buffer system as above, in 10% acrylamide vertical slab gels and run using a Hoefer “Mighty-Small” SE200 series apparatus. The stacking gel was 5% acrylamide, and the acrylamide/bisacrylamide ratio was 37.5:1.

Samples were dissociated at 100°C for 5 minutes in 0.0625M Tris HCl buffer pH 6.8 containing 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and bromophenol blue. Samples volumes loaded were 1µl and 10 µl on the Phast gels and slab gels respectively. Molecular weight markers used with the Phast gel system were Sigma, SDS-6H (29, 45, 66, 97.4, 116 and 205 kDa), and Pharmacia (20.1, 30, 43, 67, and 94 kDa) with the slab gels. Electrophoresis was at 250V, 13 mA for 40 minutes with Phast gels and 40mA, constant current for slab gels. Gels were stained for proteins with either Coomassie blue or silver stained (Silver Stain Plus, Bio-Rad) and stained for LPS using a modified version of a method by (Tsai & Frasch, 1981). Briefly, gels were fixed in 40% ethanol and 5% acetic acid in water overnight and then incubated in 0.7% periodic acid in fixative for 5 minutes. Gels were then washed three times for fifteen minutes each wash and agitated for 10 minutes in stain solution, which consisted of solution A: 20% silver nitrate
solution and solution B: 6.7% v/v ammonia and 0.1 M sodium hydroxide, mixed together in the ratio of 1:14 and then diluted 1:3 with water. The gel was washed as before and developed (using developer from the Bio-Rad silver stain kit). The reaction was stopped by addition of 5% acetic acid in water.

Analysis of protein profiles was performed using a BioRad GS690 imaging densitometer in conjunction with the BioRad Multi-Analyst software package.

4.3.9 Western blotting

Separated molecules (protein and LPS) in unstained gels were transferred onto nitrocellulose sheets 0.45 µm pore size (Hybond) using a semi-dry electro-blotter (Bio-Rad) at 3mA/cm² of gel for 30 minutes. Following incubation with 3% gelatin in 50 mM phosphate buffered saline (PBS) pH 7.4 containing 0.05% Tween 20 (PBST) the nitrocellulose was immunostained immediately or stored at -20 °C.

Blots were incubated in primary antibody (rabbit anti-C. burnetii whole cell phase II, diluted 1/1,000 or guinea pig anti-ELISA reactive fractions, diluted 1/10 in PBST) at 37 °C for 2 hours and washed three times in PBST. Bound antibodies were recognised with corresponding anti-species antibodies conjugated to horseradish peroxidase (HRP) (anti-rabbit whole molecule, diluted 1/2,000 {Sigma}, and anti-guinea pig IgG {Sigma}, diluted 1/2,000 in PBST) incubated for 2 hours at 37 °C. Blots were washed three times as before and substrate added. Substrate consisted of 20 mg of 4-chloro-1-naphthol (Sigma) in 1 ml methanol then
100 µl of this solution was added slowly to 10 ml PBS with 5 µl hydrogen peroxide. The reaction was stopped by washing in distilled water.
4.4 Results

4.4.1 Viability studies

4.4.1.1 Animal inoculation

The presence of viable *C. burnetii* organisms in urine and tissue from aerosol-infected guinea pigs was investigated. Macerated tissue and urine from *C. burnetii*-infected guinea pigs was injected intraperitoneally into guinea pigs. Three weeks post-inoculation, sera was tested by ELISA for a rise in anti-*C. burnetii* phase II IgG antibodies. Sera from guinea pigs inoculated with non-infected guinea pig urine served as a negative control as did sera obtained from guinea pigs which received no inoculation (normal guinea pig sera).

Sera from guinea pigs inoculated with macerated liver, spleen and urine (from group 1 animals), contained greater levels of anti-*C. burnetii* phase II IgG antibodies than the negative control sera. Urine from 1 day post-infection produced the greatest level of anti-*C. burnetii* phase II antibodies, followed by spleen and liver taken at 9 days post-infection. Urine taken at 3 and 8 days post-infection produced a similar anti-*C. burnetii* phase II antibody response, which was higher than negative control sera (Fig 4.2). At three weeks post-inoculation, negative control sera contained the same level of anti-*C. burnetii* phase II IgG antibodies as normal guinea pig sera. However, both negative control sera and normal guinea pig sera had higher levels of reactivity than the PBS control.
Fig 4.2 ELISA IgG antibody responses in guinea pigs inoculated with C. burnetii-infected tissue and urine. Values indicate the mean obtained from two animals.

Key to inocula
1= Non-infected guinea pig sera
2= Guinea pigs inoculated with non-infected urine
3= Guinea pigs inoculated with urine from day 1 post-infection (group 1)
4= Guinea pigs inoculated with urine from day 3 post-infection (group 1)
5= Guinea pigs inoculated with urine from day 8 post-infection (group 1)
6= Guinea pigs inoculated with spleen (day 9 post-infection) from group 1 animals
7= Guinea pigs inoculated with liver (day 9 post-infection) from group 1 animals
8= PBS negative control
Cell culture isolation of viable *C. burnetii* from tissue samples of liver, spleen and urine from 1, 3, and 8 days post-infection from group 1 animals was attempted. Flasks inoculated with *C. burnetii* phase I organisms were incubated in parallel to serve as positive controls. Cell cultures inoculated with liver, spleen and urine samples all proved negative for the presence of whole *C. burnetii* following immunofluorescent staining with rabbit anti-*C. burnetii* IgG. In contrast, all positive control cultures inoculated with phase I *C. burnetii* were positive.

**4.4.2 Determination of the presence of *C. burnetii* DNA in urine obtained from *C. burnetii*-infected guinea pigs**

Samples of pooled urine (four animals), from each group of infected guinea pigs (aerosol-infected; groups 1 and 3 and intraperitoneally-infected; groups 2 and 4) were tested for the presence of *C. burnetii* DNA. A range of days was selected from each group to test.

A 495 base pair product was detected for the positive *C. burnetii* DNA control, however, all urine samples tested from all groups proved negative for *C. burnetii* DNA.
4.4.3 Determination of *C. burnetii* components in infected guinea pig urines

A size exclusion column (superose 6) was used to identify *C. burnetii*-specific components in guinea pig urine, other than whole organisms or *C. burnetii* DNA. Whole guinea pig urines, obtained from aerosol infected animals (groups 1 and 3), were filtered through a 0.1 µm filter to remove cell debris and whole *C. burnetii* organisms. Filtered urines were run down a size exclusion column and components of the urine were separated by their retention time through the column. Retention time was directly related to molecular size, hence the higher molecular weight components passed more quickly through the column, leaving lower molecular weight components to be retained on the column for longer. Each fraction from the column was coated directly onto an ELISA plate, and tested for reactivity against a rabbit anti-*C. burnetii* phase II IgG antibody (the polyclonal antibody used in the capture assay in Chapter 3).

The size exclusion column was calibrated, using molecular weight standards, (Table 4.1). The void volume (those molecules which passed through the column and were not separated by molecular size i.e. greater than 2,000 KDa) was determined to be fraction seven.
Table 4.1 Calibration of the size exclusion column (superose 6) using molecular weight standards

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Molecular weight (kDa)</th>
<th>Fraction eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran Blue</td>
<td>2,000</td>
<td>7</td>
</tr>
<tr>
<td>Thyroglobulin</td>
<td>669</td>
<td>12</td>
</tr>
<tr>
<td>Ferritin</td>
<td>440</td>
<td>13</td>
</tr>
<tr>
<td>Catalase</td>
<td>232</td>
<td>15</td>
</tr>
<tr>
<td>Albumen</td>
<td>67</td>
<td>17</td>
</tr>
<tr>
<td>Ovalbumen</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>Chemotrypsin A</td>
<td>25</td>
<td>19</td>
</tr>
<tr>
<td>Ribonuclease A</td>
<td>13.7</td>
<td>19</td>
</tr>
</tbody>
</table>

4.4.3.1 Separation, by size exclusion chromatography, of positive control urines

Urine obtained from non-infected guinea pigs spiked with 1 µg/ml and 5 µg/ml LPS (from phase I C. burnetii) were separated, by size exclusion chromatography, to serve as positive controls. Specific reactivity, by direct ELISA with anti-C. burnetii IgG, was observed in fractions 8 and 11-15 in non-infected guinea pig urine spiked with 1 µg/ml LPS and fraction 1 and 8 in urine spiked with 5 µg/ml LPS (Fig 4.3). No specific reactivity, in direct ELISA with anti-C. burnetii IgG, was observed in any fractions of non-infected guinea pig urine (negative control).
Non-infected guinea pig urine and non-infected guinea pig urine spiked with 1µg/ml and 5µg/ml LPS from *C. burnetii* phase I organisms separated on a superose 6 column, and tested by direct ELISA.

**Fig 4.3**

4.4.3.2 Separation, by size exclusion chromatography, of negative control urines

Negative control urines were obtained from guinea pigs infected by aerosols of *Mycobacterium tuberculosis* and *Legionella pneumophila*. In addition, non-infected guinea pig urine spiked with β2-microglobulin and guinea pig albumin served as negative controls. All controls were separated on the size exclusion column and tested in the direct ELISA.
Reactivity above background levels was demonstrated in the first eight fractions in *L. pneumophila*-infected guinea pig urine and non-infected guinea pig urine spiked with 0.1 mg/ml β₂-microglobulin (Fig 4.4). No reactivity, detected by ELISA, was seen in any fractions obtained from separated *M. tuberculosis* aerosol-infected guinea pig urine or non-infected guinea pig urine spiked with albumen.

**Fig 4.4** Urine from infected control guinea pigs, or non-infected guinea pig urine spiked with albumen or β₂- microglobulin separated by size exclusion chromatography.
4.4.3.3 Separation of urine from *C. burnetii*-infected guinea pigs

Reactivity in fractions 1 and 8-15, depending on the day post infection, was detected by direct ELISA on urine from high dose aerosol-infected (group 1) animals (Fig 4.5). Reactivity was detected in fraction 8 of all urines tested. Fraction 13, reacted strongly in urine obtained at day 6 post infection, as did fractions 8-15 from urine obtained at day 9 post-infection. Fractions 1-3 in urine obtained at day 8 post-infection also showed reactivity in the direct ELISA.

Reactivity was confined primarily to fraction 1 in urines obtained from low dose aerosol-infected guinea pigs (group 3). However fractions 8, 14 and 15 also showed reactivity, primarily in urines from later days post-infection (Fig 4.6). Levels of reactivity, when detected, in low dose aerosol-infected animals (group 3), were lower than levels of reactivity detected in fractions from animals that received a high dose of *C. burnetii* by aerosol (group 1).

The molecular weight of antigens separated by the size exclusion column and appearing in fractions 8-15 ranged from approximately 300 KDa -> 2,000 KDa.
Fig 4.5 Urine from high dose aerosol-infected guinea pigs (group 1), separated by size exclusion chromatography
Fig 4.6 Urine from low dose aerosol-infected guinea pigs (group 3), separated by size exclusion chromatography.

4.4.4 Analysis of ELISA-reactive fractions

4.4.4.1 Protein determination

To obtain larger volumes of sample for analysis, repeated runs using the size exclusion column were performed on samples of urine obtained at 9 days post-infection from high dose aerosol-infected guinea pigs (group 1). After 4 separate runs, ELISA-reactive fractions 8-15 were tested by ELISA, and pooled. Pooled
ELISA-reactive fractions 8-15 (10 ml) were freeze-dried and reconstituted in 300 µl of sterile distilled water. A protein estimation on the concentrated freeze-dried ELISA-fractions was performed (Table 4.2).

**Table 4.2  Total protein determination in concentrated ELISA-reactive fractions**

<table>
<thead>
<tr>
<th>Protein (µg / ml)</th>
<th>Absorbance (562 nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>200</td>
<td>0.252</td>
</tr>
<tr>
<td>400</td>
<td>0.532</td>
</tr>
<tr>
<td>600</td>
<td>0.583</td>
</tr>
<tr>
<td>800</td>
<td>0.943</td>
</tr>
<tr>
<td>1000</td>
<td>1.003</td>
</tr>
<tr>
<td>Concentrated ELISA-reactive fractions</td>
<td>0.192</td>
</tr>
</tbody>
</table>

Total protein in ELISA-reactive fractions was 0.18 mg/ml.

**4.4.4.2 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

Pooled concentrated ELISA-reactive fractions from the size exclusion column were separated on a 10% SDS-PAGE. Silver stain analysis for protein revealed five major bands at approximately 120 kDa, 67 kDa, 62 kDa 40 kDa and 35 kDa (Fig 4.7). In addition, pooled concentrated fraction 1 (from non-infected guinea pig
urine), non-infected guinea pig urine, inactivated *C. burnetii* whole cells phase I and II and LPS I were analysed by SDS-PAGE.

A single band was present in pooled concentrated fraction I at approximately 67 kDa (Fig 4.7), and a number of bands, although fainter, were present in non-infected guinea pig urine (data not shown). *C. burnetii* whole cells phase I and II when silver stained for protein, revealed few distinct bands, and appeared smeared. Two bands were evident however, at 62 kDa and at <29 kDa in phase II organisms (Fig 4.8). Protein silver staining of phase I organisms revealed a protein at approximately 35 kDa and a number of low molecular weight (<29 kDa) minor bands (Fig 4.8). No protein was detected in the LPS I lane (data not shown).

4.4.4.3 Proteinase K treatment of concentrated ELISA-reactive fractions

4.4.4.3.1 SDS-PAGE analysis

Concentrated ELISA-reactive fractions were separated by SDS-PAGE and silver stained for LPS after proteinase K treatment. The SDS-PAGE profile (LPS) revealed one band at approximately 35 kDa (Fig 4.9). Inactivated *C. burnetii* phase I and II whole cells and LPS I underwent identical proteinase K treatment which revealed a similar band at 35 kDa. No bands were detected in non-infected guinea pig urine treated with proteinase K or in proteinase K and buffer only (Fig 4.9).
Fig. 4.7  SDS-PAGE analysis of pooled, concentrated ELISA-reactive fractions from the size exclusion column. Samples were silver stained for protein.

Lane 1= Pooled, concentrated fraction 1 from non-infected guinea pig urine (negative control).
Lane 2= Pooled, concentrated ELISA-reactive fractions.
Lane 3= Molecular weight markers (kDa).
Fig. 4.8  SDS-PAGE analysis of *C. burnetii* inactivated whole phase I and II cells. Samples were silver stained for protein.

Lane 1= Molecular weight markers (kDa).

Lane 2= *C. burnetii* phase II whole cells.

Lane 3= *C. burnetii* phase I whole cells.
Fig. 4.9 SDS-PAGE analysis of *C. burnetii* whole cells phase I, II LPS I and ELISA-reactive fractions, after proteinase K treatment.

Samples were silver stained for LPS.

Lane 1 = Molecular weight markers (kDa).

Lane 2 = *C. burnetii* phase II whole cells.

Lane 3 = *C. burnetii* phase I whole cells.

Lane 4 = Pooled, concentrated ELISA-reactive fractions.

Lane 5 = Non-infected guinea pig urine treated with proteinase K.

Lane 6 = Proteinase K in buffer only (negative control).

Lane 7 = Lipopolysaccharide from *C. burnetii* phase I (LPS I), (positive control).
4.4.3.2 Analysis by ELISA

Concentrated ELISA-reactive fractions were incubated with and without proteinase K to determine if reactivity was due to LPS. LPS, prepared from *C. burnetii* phase I whole cells, was added to PBS and similarly incubated to serve as a positive control. Non-infected guinea pig urine was also incubated with and without proteinase K as a negative control. The reactivity of each sample was then assessed by direct ELISA against rabbit anti-*C. burnetii* phase II antibody. The results are summarised in Fig 4.10. A decrease in reactivity in the ELISA-reactive fractions, was detected after proteinase K treatment, however, some reactivity above background levels (negative controls) was retained. A small reduction in reactivity was detected in LPS controls after proteinase K treatment.
Fig 4.10 Reactivity of ELISA-reactive fractions after proteinase K treatment, as measured by direct ELISA

Key: LPS-K = LPS I with no proteinase K treatment
LPS+K = LPS I with proteinase K treatment.
Frac.-K = ELISA-reactive fractions with no proteinase K treatment.
-ve-K = Non-infected guinea pig urine with no proteinase K treatment.
-ve+K = Non-infected guinea pig urine with proteinase K treatment.

4.4.4.4 PCR analysis of ELISA-reactive fractions

Concentrated ELISA-reactive fractions, proved negative for the presence of C. burnetii DNA. Product was detected in positive controls (organisms from egg yolk extract).
Concentrated ELISA-reactive fractions, LPS I, inactivated phase I and II whole cells and concentrated fraction I from non-infected guinea pig urine (fraction I reacted in the direct ELISA, Fig. 4.4, 4.5, 4.6), were separated by SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were immunoblotted with purified rabbit anti-\textit{C. burnetii} IgG (as used in the capture ELISA in Chapter 3, and the direct ELISA this chapter).

One band with a molecular weight of 62 kDa was observed from the ELISA-reactive fractions and one band at 67 kDa was observed in the concentrated fraction I lane. One band was detected at 35 kDa in the LPS I lane (Fig 4.11). A smear was observed in the high molecular weight region of both \textit{C. burnetii} phase I and II whole cells. In addition bands were evident at approximately 35-40 kDa in phase I whole cells and 62 kDa in phase II whole cells (Fig 4.12).
Fig. 4.11  Immunoblot analysis of concentrated, pooled ELISA-reactive fractions, concentrated pooled fraction 1 (from non-infected guinea pig urine) and LPS I using anti-*C. burnetii* phase II IgG antibody.

Lane 1= Molecular weight markers (kDa).
Lane 2= Pooled, concentrated ELISA-reactive fractions.
Lane 3= Pooled, concentrated fraction 1 from non-infected guinea pig urine.
Lane 4= Lipopolysaccharide from *C. burnetii* phase I (LPS I).
Fig. 4.12 Immunoblot analysis of *C. burnetii* whole inactivated phase I and II cells, using anti-*C. burnetii* phase II IgG antibody.

MWM= Molecular weight markers (kDa).

Lane 1= *C. burnetii* whole inactivated phase II cells.

Lane 2= *C. burnetii* whole inactivated phase I cells.
4.4.4.6 Reactivity of polyclonal antiserum raised against ELISA-reactive fractions

Guinea pig serum, raised against concentrated ELISA-reactive fractions from infected guinea pig urine was produced to identify the *C. burnetii*-specific antigen. Whole inactivated phase I and II *C. burnetii* were coated onto an ELISA plate. Guinea pig "anti-ELISA-reactive fractions" antiserum, when tested in ELISA, showed higher levels of reactivity to *C. burnetii* phase I and II organisms than negative control guinea pig sera (Fig 4.13). Reactivity was greater against phase II than I organisms; however the titre of the sera was low.

Fig 4.13 Reactivity of guinea pig "anti-ELISA-reactive fractions" sera in ELISA against QI and QII

![Graph showing reactivity of guinea pig sera against QI and QII](image)
In Western immunoblotting, the guinea pig “anti-ELISA-reactive fractions” serum reacted against a protein with an approximate molecular weight of 62 kDa, which was present in the purified ELISA-reactive fractions (Fig 4.14). No reactivity was detected against *C. burnetii* phase I and II organisms or LPS from phase I organisms using Western immunoblotting or IFA with “anti-ELISA-reactive fractions” serum (data not shown).
Fig. 4.14  Immunoblot analysis of ELISA-reactive fractions using guinea pig anti “ELISA-reactive fractions” antibody.

Lane 1= Molecular weight markers (kDa).

Lane 2= ELISA-reactive fractions.
4.4.4.7 Summary of results

A summary of the results is shown in fig. 4.15 below, based on the strategy outlined at the beginning of this chapter (fig. 4.1).

**Fig. 4.15 Summary of antigen characterisation results**

![Diagram showing the summary of antigen characterisation results](image)

Shaded boxes denote the outcome of the investigation and the brief result.
4.5 Discussion

Chapter Three of this study demonstrated that a capture ELISA could detect C. burnetii-specific antigens in the urine of guinea pigs experimentally infected with C. burnetii. The nature of the antigen detected, however, was not determined. The aim in this section of the study was to characterise the antigen(s) excreted in the urine of guinea pigs during C. burnetii infection.

4.5.1 Determination of whole C. burnetii in urine from infected guinea pigs

Two methods were employed to determine the presence of viable C. burnetii in urine from infected guinea pigs. Intraperitoneal inoculation of suspected C. burnetii-containing material into guinea pigs produces a C. burnetii-specific antibody response. This antibody response is dependant on the multiplication of C. burnetii within the guinea pig and can be produced from an initial inoculum containing as few as 1-10 organisms (Waag et al., 1991) and is therefore highly sensitive. Intraperitoneal inoculation is a standard procedure for the isolation and determination of C. burnetii in tissue samples from human and animal origin, and the guinea pig is the animal of choice due to increased susceptibility to C. burnetii infection. Animal inoculation is particularly useful when potentially contaminated samples such as urine are used. The immune system of the animal is able to clear bacterial contaminants to allow the growth of C. burnetii. The appearance of C.
burnetii-specific titres to phase II whole organisms, in guinea pigs inoculated with urine from guinea pigs infected by aerosol with a high dose of C. burnetii (group 1), suggested that C. burnetii-specific antigen was present in the initial inoculum. In addition, homogenised tissues of liver and spleen from group 1 animals, when inoculated, produced an equivalent anti-C. burnetii phase II antibody response to that seen with urine. Without supportive clinical data however, showing a febrile response indicative of C. burnetii infection, the presence of viable C. burnetii within urine and tissues remains unconfirmed.

Attempts to isolate C. burnetii from urine and homogenated organs in tissue culture were unsuccessful. Samples may not have contained any viable C. burnetii organisms, or the number of organisms in the samples may have been too few to be detected by tissue culture techniques. It has been reported that strains of C. burnetii which were infective for mice were markedly less infective for L929 mouse fibroblast cells (Ormsbee et al., 1978). For tissue culture to be effective, C. burnetii must be taken up by the monolayer of cells. Centrifugation of organisms onto cells in shell vials, giving a 2-to 4-fold increase in adsorbed organisms, has been reported by (Raoult et al., 1990b). In this study, no such centrifugation step was employed and, therefore, the sensitivity of the tissue culture method may have been too low. Negative tissue culture does not exclude the possibility of whole viable organisms being present in the samples, but may indicate that few organisms were present.
The PCR technique, which was successful at detecting \textit{C. burnetii} DNA in tissue samples (section 2.3.6), was applied directly to the detection of \textit{C. burnetii} DNA in urine samples. No \textit{C. burnetii} DNA was detected in any urine samples tested, however, this did not exclude the presence of \textit{C. burnetii} DNA in the samples as a number of factors may have affected the outcome. The concentration of urea in normal human urine ranges from approximately 0.125 M to 0.25 M (Strasinger, 1989). It has been reported that urea at a concentration of 0.5 M can completely inhibit a PCR reaction (Gelfand & White, 1990), however, other workers have reported no such inhibition (Maiwald et al., 1995) for the detection of \textit{L. pneumophila} DNA in guinea pig and human urine. In addition, ammonium chloride (another component of urine) has been reported to inhibit the PCR reaction (Gelfand & White, 1990). Failure to detect a PCR product in urine may be due to either or both these factors. In addition, the detection system used in this study (ethidium bromide staining), although sensitive enough to detect a specific product from infected tissues, may not have been sensitive enough to detect low levels amplified from urine. Silver staining of gels or radiolabelling of PCR products could improve the sensitivity of the technique. Degradation of \textit{C. burnetii} DNA by endogenous DNAases present in urine, resulting in fragments of DNA too small to be amplified by the primers, may also explain the absence of a product. Also, the volume of urine extracted may have been insufficient to yield enough DNA for successful amplification.
In conclusion, animal inoculation studies indicated that C. burnetii-specific antigen was present in urine from animals which received a high dose of C. burnetii by the aerosol route (group 1). Other methods of isolation for whole C. burnetii were unsuccessful.

4.5.2 Determination of C. burnetii components in urine from C. burnetii-infected guinea pigs.

It is possible that as well as whole organisms being excreted into the urine of C. burnetii-infected animals, soluble products of the organism are also excreted. This has been demonstrated in other diseases. Individuals with acquired immunodeficiency syndrome (AIDS) and disseminated Mycobacterium avium disease have been shown to excrete a protein antigen with a molecular weight of 22.5 kDa in their urine. Cytomegalovirus was diagnosed by a monoclonal antibody directed against a 150-kDa protein antigen in the urine of patients (Yamanaka et al., 1992) and dogs experimentally infected with Trypanosoma cruzi were shown to excrete an 80-kDa glycoprotein urinary antigen (Corral et al., 1989). In addition, individuals suffering from leprosy produce a 35-70 kDa cell wall antigen (probably a phosphoglycolipid) in their urine (Sharma et al., 1992).

Size exclusion chromatography has been employed during studies on urine obtained from experimental infection studies on pneumoccocal and Legionella pneumophila infections (Coonrod, 1983) and (Williams, A. & Lever, 1995). Size
exclusion chromatography, followed by a direct ELISA on eluted fractions, incorporates a number of advantages. Firstly, urines are filtered through a 0.1µm filter prior to loading onto the chromatography column. This removes whole C. burnetii organisms and cellular and particular material which may interfere with the ELISA. Secondly, filtering renders the urine safe to work with outside a class III safety cabinet, and thirdly, any inhibitory components, which are able to pass through the filter, are separated from antigen(s) by molecular size on the column. Direct ELISA was used as a final screening method to detect any specific antigen(s) present.

In this study, for comparative purposes, non-infected guinea pig urine spiked with purified LPS from phase I C. burnetii Lane strain was separated by size exclusion chromatography (fig 1). The region of reactivity, detected by direct ELISA, in fractions 8-15 of LPS spiked urines was also detected in urine obtained from guinea pigs in groups 1 and 3 (high and low dose by aerosol, respectively). No such reactivity was evident in non-infected urines, or urines from guinea pigs suffering from other respiratory diseases such as Legionnaires’ disease or Mycobacterium tuberculosis. This indicated that the reactivity seen in fractions 8-15 was specific for C. burnetii infection only and was of a comparable molecular weight to LPS from C. burnetii. This suggested that the antigenic product in guinea pig urine could be an LPS-related component.
Reactivity detected in fraction 1, during the separation of selected urines, was due to a non-specific component, with an approximate molecular weight of 67 kDa, when run under reduced conditions on SDS-PAGE. Gel filtration, however, removed this non-specific component which was not present in fractions 8-15. A non-specific serum component may be present in urine from all guinea pigs in greater or lesser amounts, and could explain why some urines showed no reactivity in fraction 1 whereas other urines did react. The molecule, $\beta_2$-microglobulin, was discussed in Chapter 3 of this study as being a possible reaction inhibitor. Indeed, control experiments of urine spiked with $\beta_2$-microglobulin in this chapter (Fig 4.4) indicated that low levels of cross-reactivity occurred between the anti-C. burnetii IgG antibody and fractions 1-8 of separated urine containing $\beta_2$-microglobulin. This provided further evidence, therefore, that $\beta_2$-microglobulin was a possible cause of the reaction inhibition observed.

The detection of antigenic product in fractions 8-15 was related to the initial challenge dose. Guinea pigs administered the high challenge dose (group 1) demonstrated specific reactivity in fractions 8-15 from day 2 post infection. Animals given the low challenge dose (group 3) showed no specific reactivity in fractions 8-15 until day 5 post infection. Similar findings in experimental brucellosis in mice were reported by (Limet et al., 1988), where the LPS-related antigen in urine appeared sooner in animals challenged with the higher infective dose. Furthermore, a correlation between initial infective dose, disease state and the quantity of antigen eluted in fractions 8-15 was observed. By day nine post
infection, when guinea pigs had severe anorexia, pyrexia and a widespread, disseminated infection (data from chapter 2), large quantities of antigenic product were evident in the urine. Equivalent levels of antigen were not detected in the urine of those guinea pigs challenged with low dose *C. burnetii* (group 3), and probably reflects the less severe infection seen in these animals.

All urines from animals given high infective dose by aerosol (group 1) tested positive for *C. burnetii* antigen, independent of the time post-infection. Until 9 days post-infection, fraction 8 contained the majority of the reactivity, indicating that a higher molecular weight antigen was being excreted into the urine of infected guinea pigs. As infection progressed (by 8 and 9 days post infection), a broader range of molecular weight antigens were detected.

This trend was not seen in animals administered with a low challenge dose (group 3). The molecular weight of the antigens shed into the urine of these animals was lower and independent of time post infection. The range of antigenic molecular weights detected, therefore, was related to the severity of the disease. This suggested that the specific *C. burnetii* antigens detected in the urine of infected animals remained within a defined molecular weight range i.e. 300 kDa- 2,000 kDa. It also indicated that degradation of the antigens may have occurred, or that a greater number of antigenic products were appearing in the urine as infection progressed. (Coonrod, 1983) reported polysaccharides that appeared in urine, and which degraded over time, producing progressively smaller molecular weight
polysaccharides. This polysaccharide degradation, however, occurred after purified polysaccharide had been injected into rats and was not produced as a result of infection. In contrast, (Williams, A. & Lever, 1995) found that in experimental Legionnaires’ disease in guinea pigs, LPS with a consistent molecular weight, was detected in the urine of infected animals throughout the course of infection.

Soluble polysaccharides are produced during growth of pneumococci both in vivo and in vitro and are able to cross serous membranes to be excreted into the urine (Coonrod, 1979, 1983). Other infections also result in specific polysaccharide antigens being excreted in the urine of infected individuals. Some of these are summarised in Table 4.3 below.

Table 4.3  Infections resulting in excretion of specific polysaccharides into urine

<table>
<thead>
<tr>
<th>Organism/ Infection</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Haemophilus influenzae</em> type b</td>
<td>(Kaldor et al., 1979),</td>
</tr>
<tr>
<td></td>
<td>(Suksanong &amp; Dajani, 1977),</td>
</tr>
<tr>
<td><em>Neisseria meningitidis</em></td>
<td>(Feigin et al., 1976),</td>
</tr>
<tr>
<td>pneumococcal pneumonia</td>
<td>(Coonrod, 1974),</td>
</tr>
<tr>
<td></td>
<td>(Coonrod &amp; Rytel, 1973)</td>
</tr>
<tr>
<td>streptococcal infections</td>
<td>(Seigal &amp; McCracken, 1978)</td>
</tr>
<tr>
<td><em>Legionella pneumophila</em></td>
<td>(Williams, A. &amp; Lever, 1995)</td>
</tr>
</tbody>
</table>
Evidence that the antigenic product, identified in urine from C. burnetii-infected guinea pigs, was associated with an LPS molecule was provided by resistance to proteinase K digestion. Silver staining for LPS revealed a common 35 kDa molecule present in C. burnetii phase I and II whole cells, LPS I and concentrated guinea pig reactive fractions. A 35 kDa antigen, was recognised by the rabbit anti-C. burnetii phase II antibody in phase I organisms and LPS I, but no such antigen was detected in C. burnetii phase II organisms or concentrated guinea pig reactive fractions by Western immunoblotting techniques. This study confirmed, therefore, that LPS present on whole phase II cells was sufficiently immunogenic to induce an immune response, in rabbits, to phase I LPS and whole phase I cells, {as previously reported by (Williams, J. C. & Waag, 1991)}. The response, however, was not sufficient to show a reaction with phase II whole cells in Western immunoblotting. C. burnetii phase II whole cells, have been shown to contain approximately ten times less extractable LPS than C. burnetii phase I cells (Baca & Paretsky, 1983), consequently, low sensitivity of the rabbit antibody for LPS or low levels of LPS present in the guinea pig fractions, may explain the lack of reaction.

By the more sensitive ELISA procedure reduced reactivity was detected in guinea pig reactive fractions following incubation with proteinase K. This indicated that a proportion of the epitopes detected by the antibody in ELISA were sensitive to proteinase K. A small amount of reactivity remained which suggested that a proportion of the antigenic determinants in the guinea pig reactive fractions were
proteinase K-resistant and therefore LPS in origin. Likewise, the LPS control showed a reduction in reactivity after incubation with proteinase K, although a significant proportion of the reactivity remained. This was also found to be the case in antigen detected in urine from *L. pneumophila*-infected guinea pigs (Williams, A. & Lever, 1995). This showed that LPS from *C. burnetii* was resistant to proteinase K and that the guinea pig reactive fractions also contained a proportion of resistant antigens. This suggested that there was either (a) two antigenic products detected by ELISA, one of which was protein and the other LPS in origin, or (b) there was only one antigenic product, and this showed reduced reactivity following incubation with proteinase K.

Immunoblots showed that the rabbit anti-*C. burnetii* phase II antibody recognised a 62 kDa antigen, present in *C. burnetii* phase II whole cells and concentrated guinea pig reactive fractions, but not recognised in phase I whole cells or LPS I. (Williams, J. C. *et al.*, 1990) reported a 62-kDa protein which was present in large amounts on the surface of phase II cells, but was not identified using polyclonal antibodies, on the surface of phase I cells. Failure to identify the protein on phase I cells may have been due to the close association of the protein with other molecules which caused stearic hinderance, or failure of the polyclonal antibodies to recognise the native conformation of the protein. Membrane lipids or phase I LPS have been shown to mask protein antigenic determinants, which were subsequently exposed after conversion from phase I to phase II (Williams, J. C. *et al.*, 1986b) and (Williams, J. C. & Waag, 1991). This may explain the lack of
reactivity of the polyclonal antibody against such a 62 kDa protein in phase I organisms in this study.

This evidence suggested that two separate antigens were excreted into the urine of C. burnetii-infected guinea pigs. The first was a protein antigen, of 62 kDa molecular weight, which was detectable by immunoblotting, and was also evident when phase II organisms were separated by SDS-PAGE. The second, was an LPS antigen of approximately 35 kDa molecular weight, which was present in fractions 8-15, phase I organisms and LPS I, but not detectable in fractions 8-15 or phase II cells by immunoblotting, but remained reactive in ELISA after the enzymatic removal of protein.

Polyclonal antiserum was raised in guinea pigs against the concentrated guinea pig reactive fractions to determine the immunogenicity of the C. burnetii-specific antigen. Guinea pigs were immunised with the ELISA-reactive fractions 8-15 to produce only C. burnetii-specific antibodies with no contaminating antibodies against guinea pig components. The serum proved reactive in both immunoblotting and ELISA. Antibodies specific for anti-C. burnetii phase I and II whole cells, and concentrated guinea pig reactive fractions, were demonstrated by ELISA. A 62 kDa antigen was identified when reactive guinea pig fractions were separated by SDS-PAGE and immunoblotted with the fractions 8-15 serum. No reaction, however, was detected against phase I or II cells, but the reaction with the 62 kDa antigen in immunoblots corroborated the results seen earlier, using the rabbit anti-
C. burnetii phase II antibody, and proved this to be the immunodominant antigen in
the guinea pig reactive fractions. Reactivity of the anti-fractions 8-15 serum in
ELISA against C. burnetii phase I and II cells did not provide further evidence as
to the nature of the antigen appearing in guinea pig urine. It did however, prove
that the antigen was C. burnetii-specific and immunogenic in guinea pigs.

The end-point titre of the guinea pig anti-fractions 8-15 serum against C. burnetii
phase I and II, in ELISA was low (between 1/128 -1/256). Corresponding
absorbance levels were also low, which indicated that the sensitivity of the
antiserum was low. This low sensitivity may explain the non-reactivity against
phase I and II cells observed in less sensitive techniques such as immunoblotting
and immunofluorescence. Highly purified antigens, containing concentrations as
low as 50µg of total protein, have been reported as successful at inducing an
immune response in rabbits, and a normal inoculum may contain between 0.1- 5.0
mg of protein (Jurd, 1987). Total protein estimates indicated that protein
concentrations in the reactive fractions contained 0.18 mg/ml; however, only a
small percentage of this total protein content may be C. burnetii-derived. The final
solution, inoculated into guinea pigs, therefore, may have contained sub-optimal
concentrations of C. burnetii material than required, to elicit a strong antibody
response.

In conclusion, this chapter has provided evidence that viable C. burnetii organisms
are present in the urine of aerosol-infected guinea pigs. In addition, a 62 kDa
soluble immunoreactive *C. burnetii*-specific protein antigen is present in the urine of experimentally-infected guinea pigs. Evidence also suggests that LPS is present in the urine. The quantity and appearance of the antigenic product(s) in the urine is related to the initial infective challenge of *C. burnetii*.

A method has been described, using size exclusion chromatography followed by a direct ELISA, to detect these antigens and the next Chapter will investigate the effect of doxycycline and ciprofloxacin on the appearance of the antigenic product described above, in urine. The application of urinary antigen detection as a marker for antibiotic efficacy will then be assessed.
CHAPTER 5

Use of C. burnetii-urinary antigen as a means of assessing the efficacy of doxycycline and ciprofloxacin in the treatment of experimental Q fever in guinea pigs

5.1 Introduction

Three methods of assessing antibiotic efficacy against C. burnetii have been utilised in the past: animal models, chick embryos (eggs) and cell culture (Yeaman and Baca, 1990; Yeaman, et al 1989 and Raoult, et al, 1989). Antibiotic efficacy against experimental infection in animals, is often determined by clinical symptoms, serology and the quantification of organisms in tissues such as the spleen and liver. To obtain such data, homogenated infected tissues are inoculated into either eggs or tissue culture, and the lethality in eggs or cells can be correlated directly to the number of C. burnetii present in the tissue. These figures are expressed in tissue culture infective doses (TCID$_{50}$) or egg infectious doses (EID$_{50}$). Infected embryonated eggs or tissue culture systems for determining C. burnetii antibiotic susceptibility also rely on the comparison of TCID$_{50}$ or EID$_{50}$ between treated and untreated groups.

An experimental infection that accurately models the disease seen in humans can provide the opportunity to determine the efficacy of antibiotic treatment.
Pharmacokinetic and pharmacodynamic data can be obtained and antibiotic concentrations in tissues can be determined and compared to levels that are achievable in humans. Both chick embryos and cell culture, are more convenient, less expensive and require less extensive containment facilities than \textit{in vivo} methods. For facultative intracellular organisms such as \textit{C. burnetii}, however, minimum inhibitory concentration (MIC) and minimum bacteriocidal concentration (MBC) values obtained using \textit{in vitro} techniques often differ markedly from those obtained by \textit{in vivo} methods (Jepras, \textit{et al} 1985).

In Chapter four, \textit{C. burnetii}-specific antigens were identified, that appeared in the urine of \textit{C. burnetii}-infected guinea pigs by the aerosol route. The usefulness of these antigens as a marker of efficacy during treatment of \textit{C. burnetii} infection with doxycycline and ciprofloxacin was investigated. If successful the technique could provide physicians with an early means to monitor antibiotic efficacy during patient management of \textit{C. burnetii} infection.

A similar approach was investigated with urine obtained from humans suffering from Legionnaires’ disease (Kashuba & Ballow, 1996). Such studies showed that by ten days post antibiotic treatment, a small number of patients (4 \%) became urinary antigen negative. In the same study, however, 3 \% of patients remained urinary antigen positive for more than one hundred days after initiation of antibiotic therapy. Results suggested that the use of urinary antigen as a marker of
disease, and subsequently as a method to monitor clearance of infection, was variable.

Acute Q fever in humans, when diagnosed promptly, has been successfully treated with a number of antibiotics including lincomycin, erythromycin, chloramphenicol and the antibiotic of choice, doxycycline. In addition, the fluoroquinolone, ciprofloxacin, has been shown to be effective against C. burnetii strain Nine Mile in a persistently infected tissue culture model (Yeaman and Baca, 1990).

In this study, the efficacy of doxycycline and ciprofloxacin against C. burnetii in a guinea pig model of acute C. burnetii infection was investigated. Guinea pigs were infected with C. burnetii Lane strain by the intraperitoneal route. Febrile response, weight loss, serology and direct antigen detection by PCR were used to monitor the course of infection.

5.2 Aim of Study

The aim of this part of the study was to determine the excretion of C. burnetii antigens in urine over time, obtained from experimentally-infected guinea pigs after antibiotic treatment. The detection of urinary antigen would be assessed as a marker for disease and compared to other disease markers such as febrile response, weight loss, serology and direct antigen detection in tissues. Finally, detection of
C. burnetii-specific antigen in urine would be used as a parameter for assessment of antibiotic efficacy against C. burnetii.
5.3 Materials and methods

5.3.1 Intraperitoneal infection

Guinea pigs were divided into five groups. Group A consisted of eighteen guinea pigs which were inoculated intraperitoneally with 0.1ml suspension containing approximately $10^9$ organisms ml$^{-1}$. Groups B and C each contained fourteen animals, infected as above, and treated with either doxycycline or ciprofloxacin (see Section 5.3.3). The remaining two groups of fourteen animals were not infected but were administered doxycycline or ciprofloxacin at an equivalent dose to above. Animals were killed at 28 days post-infection and tissue and blood samples for PCR analysis and measurement of the serological response were taken from two animals approximately every four-seven days.

5.3.2 Urine samples

Urine was obtained from infected guinea pigs by manual expression of the bladder. Samples were taken at the same sample times as tissue and blood samples (approximately every four-seven days). Urine from four animals was removed each sample day and pooled.
5.3.3 Antibiotic administration

Doxycycline (Pfizer) and ciprofloxacin (Bayer) were administered subcutaneously (0.1ml) immediately post-\textit{C. burnetii} infection, at a concentration of 10 mg kg\(^{-1}\) body weight (Lever \textit{et al.}, 1998). Treatment continued every 12 hours for 5 days post-infection. The dose level of antibiotic was based on previous work performed on antibiotic prophylaxis for experimental inhalation anthrax at CAMR (Jones \textit{et al.}, 1994) and was approximately equivalent to a human dose of 600 mg daily. To prevent the establishment of clinical infection the short-term efficacy of doxycycline and ciprofloxacin treatment immediately after exposure was investigated in this study. The dosage schedule was therefore predetermined based on existing practice.

5.3.4 Urinary antigen detection

Urine samples were treated and separated by size exclusion chromatography as described in Section 4.3.3. Fractions were tested for specific \textit{C. burnetii} reactivity by direct ELISA as previously described (4.3.4). Fractions eluting between 8-15 ml, which proved positive by direct ELISA, were considered to contain \textit{C. burnetii}-specific antigens (Section 4.4.3). Positive fractions were those which had an absorbance value in the direct ELISA greater than twice the standard deviation of non-infected urine (background).
5.4 Results

5.4.1 Clinical signs

A febrile response of greater than $40^\circ$C was detected in infected, untreated animals between 4 and 8 days post-infection (Figs 5.1, 5.2). Ciprofloxacin administered at 10 mg/kg proved ineffective at reducing pyrexia, with temperatures of treated animals remaining in-line with those of untreated controls. However, doxycycline administered at 10 mg/kg reduced pyrexia, between day 4 to 8 post-infection to near normal levels.

During the course of the infection the guinea pigs were inactive during the febrile phase of illness and reduced their intake of food and water, which affected their weights and general condition. Noticeably, the guinea pigs inoculated with *C. burnetii* that received no antibiotic, did not put on any weight during the first 7 days post-infection, after which there was a gradual weight increase. In contrast, the condition of guinea pigs treated with doxycycline (10 mg/kg) showed no evidence of anorexia and developed normally.

Treatment with ciprofloxacin (10 mg/kg) did not alter the course of disease with all animals showing characteristic signs of *C. burnetii* infection. In addition, the animals showed some distress to the administration of the antibiotics (Figs 5.3, 5.4).
5.4.1.1 Pyrexia

Fig 5.1 Temperature of guinea pigs treated with doxycycline immediately after *C. burnetii* infection. Values represent the mean of four animals.
Fig 5.2  Temperature of guinea pigs treated with ciprofloxacin immediately after *C. burnetii* infection. Values represent the mean of four animals.
5.4.1.2 Weight

Fig 5.3 Weight of guinea pigs treated with doxycycline immediately after *C. burnetii* infection. Values represent the mean of four animals.
Fig 5.4 Weight of guinea pigs treated with ciprofloxacin immediately after *C. burnetii* infection. Values represent the mean of four animals.
5.4.2 Serology

Doxycycline treatment reduced the level of anti-\textit{C. burnetii} IgM phase II antibody responses compared to infected, untreated control animals (Fig 5.5). Treatment with doxycycline delayed the appearance of anti-\textit{C. burnetii} IgM antibodies until 15 days post-infection, compared to 9 days post-infection in infected control animals. Levels then gradually increased to a maximum at 22 days post-infection.

Ciprofloxacin treatment reduced the level of anti-\textit{C. burnetii} IgM phase II antibody responses compared to infected, untreated control animals (Fig 5.6). Anti-\textit{C. burnetii} IgM antibodies appeared at 9 day post-infection, in both treated and untreated animals; however, levels peaked lower and earlier in ciprofloxacin-treated animals compared to infected controls.

Doxycycline treatment greatly reduced the level of anti-\textit{C. burnetii} IgG phase II antibody responses compared to infected, untreated control animals. In the untreated infected controls, anti-\textit{C. burnetii} phase II IgG antibodies appeared on day 9 post-infection, peaking at day 19 post-infection. In comparison, antibodies produced in infected animals treated with doxycycline did not appear until day 15 post-infection. Antibody levels then gradually increased to a level (1:50000) at day 28 post-infection compared to 1:25000 in untreated infected controls (Fig 5.7).

Ciprofloxacin treatment proved less effective than doxycycline when comparing antibody production as a measure of infectivity. Treatment with ciprofloxacin did
not delay the appearance of anti-\textit{C. burnetii} phase II IgG antibodies, but antibody responses were diminished between 13 and 22 days post-infection (Fig 5.8).

\textbf{Fig 5.5} \hspace{1cm} Appearance of anti-\textit{C. burnetii} IgM antibodies in guinea pigs treated with doxycycline immediately after infection. Points indicate the mean of two animals.
Fig 5.6 Appearance of anti-*C. burnetii* IgM antibodies in guinea pigs treated with ciprofloxacin immediately after infection. Points indicate the mean of two animals.
Fig 5.7 Appearance of anti-\textit{C. burnetii} IgG antibodies in guinea pigs treated with doxycycline immediately after infection. Points indicate the mean of two animals.
5.4.3 PCR

*C. burnetii* DNA was not detected in non-infected, antibiotic-treated animals (group A (dox) and group A (cip), Table 5.1). No *C. burnetii* DNA was detected in any organ examined after day 15 post-infection in any tissue in the infected controls (group B, Table 5.1). *C. burnetii* DNA was detected in the heart, lung, liver and spleen of infected animals treated with doxycycline and ciprofloxacin; however, more tissues proved positive in ciprofloxacin-treated guinea pigs (group D) than doxycycline-treated animals (group C), particularly the lung (Table 5.1).
Table 5.1  Detection of *C. burnetii* DNA in tissues from non-infected, infected and antibiotic treated guinea pigs. Data based on two animals per sample point

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Group</th>
<th>3</th>
<th>8</th>
<th>12</th>
<th>15</th>
<th>18</th>
<th>28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>8</td>
<td>12</td>
<td>15</td>
<td>18</td>
<td>28</td>
</tr>
<tr>
<td>Heart</td>
<td>A (dox)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A (cip)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C (dox)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D (cip)</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Lung</td>
<td>A (dox)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>A (cip)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>-</td>
<td>+*</td>
<td>+*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C (dox)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D (cip)</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Liver</td>
<td>A (dox)</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>A (cip)</td>
<td>-</td>
<td>-</td>
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<td>+</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>+*</td>
<td>+</td>
<td>+*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>C (dox)</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>D (cip)</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Spleen</td>
<td>A (dox)</td>
<td>-</td>
<td>-</td>
<td>-</td>
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</tr>
<tr>
<td></td>
<td>A (cip)</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<tr>
<td></td>
<td>B</td>
<td>+*</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>+</td>
</tr>
<tr>
<td></td>
<td>C (dox)</td>
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</tr>
<tr>
<td></td>
<td>D (cip)</td>
<td>+*</td>
<td>+</td>
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</tr>
</tbody>
</table>

Key:

Group A (dox) = Non-infected guinea pigs treated with doxycycline

Group A (cip) = Non-infected guinea pigs treated with ciprofloxacin

Group B = Infected guinea pigs with no antibiotic treatment

Group C = Infected guinea pigs, treated with doxycycline

Group D = Infected guinea pigs, treated with ciprofloxacin

- = No *C. burnetii* DNA detected, + = *C. burnetii* DNA detected and +* = *C. burnetii* DNA detected in one animal only.
Urinary Antigen Detection

Urines were separated on a size exclusion column (as described previously, Chapter 4) and fractions tested for *C. burnetii*-specific antigens using the rabbit anti-*C. burnetii* whole cell phase II antibody, in direct ELISA. Non-infected guinea pig urine was also separated and served as a negative control to provide background absorbance values. Fractions were considered positive for *C. burnetii* antigen if they reacted above background levels in ELISA and eluted in fractions 8-15 (as determined previously in Chapter 4, Fig 4.5 and 4.6). All urines which proved to contain *C. burnetii* antigens eluted in fractions 11-15. Throughout the duration of the study (19 days after infection), no *C. burnetii*-specific antigen was detected in the urine of non-infected guinea pigs. Levels of *C. burnetii*-specific antigen appeared at two days post-infection (the first day tested) in infected, untreated control guinea pigs (Fig 5.9). Between three to nine days post-infection, however, antigen decreased below background levels in these animals. From nine days post-infection until the end of the study, antigen levels increased above background values. Levels decreased slightly at 18 days post-infection, but still remained above background values. Antigen levels in guinea pigs treated with doxycycline remained below background levels throughout the study, except at 5 days post-infection where levels just above background levels were recorded. Antigen levels in guinea pigs treated with ciprofloxacin were greater than those detected in the doxycycline group. Peak values were recorded at five and nineteen
days post-infection, and levels were higher than background at twelve and fifteen days post-infection.

Antigen levels in infected control animals were generally higher than those detected in urine from infected, treated animals. Day five post-infection proved the only exception to this.

Fig 5.9 Detection of C. burnetii-specific urinary antigen in infected and treated guinea pigs. Background was defined as twice the standard deviation of the mean of non-infected urine. Points represent the mean of duplicate tests.
5.5 Discussion

5.5.1 Urinary antigen detection in untreated control animals

Urine from guinea pigs infected intraperitoneally with *C. burnetii* has been shown to contain a *C. burnetii*-specific antigen by direct ELISA on FPLC-separated urine fractions. Animals were febrile from four to eight days post infection, and weight loss was evident throughout this period. In contrast, levels of urinary antigen were at their lowest throughout the same period. Immune responses such as antibody-antigen reactions and a generalised CMI response to an infection are responsible for the febrile response (Mims, C., A et al., 1995). The inflammatory response develops when *C. burnetii* are released from destroyed host cells, and in the guinea pig it is known to coincide with fever and weight loss (Khavkin, 1990). The excretion of *C. burnetii*-specific antigens into the urine may reflect the cyclic nature of the infection in the guinea pig. At one to three days post-infection, organisms from the original inoculum multiply within peritoneal phagocytes and at initial foci of infection in organs such as the liver and spleen. The antigen detected in the urine, therefore, from one to three days post-infection may originate from this initial infection. Four to eight days post infection, extensive ricketsiemia occurs, characterised by the febrile response and the development of the humoral and inflammatory response. The anti-microbial response in the guinea pig may temporarily reduce the quantity of antigen excreted into the urine by the development of circulating immune complexes (as has been reported by {Williams, J. C. et al., 1981}), and by the increased numbers of phagocytic cells. By nine
days post infection, the increase in antigen levels in the urine may represent the increased elimination products of phagocytosis.

After day twelve post-infection, *C. burnetii* DNA was not detected in any organ tested from infected, untreated guinea pigs. This suggests that organs containing sites of infectious foci had successfully been cleared of *C. burnetii*. Urinary antigen, however, continued to be detected up to nineteen days post-infection (the end of the experiment), in infected only animals. This provided further evidence that the *C. burnetii*-specific antigen detected in the urine was an elimination product released from host phagocytic cells following clearance of the organisms from the tissues.

Alternatively, the variation in levels of urinary antigen over time may be simply due to individual day-to-day and animal-to-animal variation. Urine was taken from four guinea pigs and pooled for each sample point. Levels of urinary antigen detected may vary, therefore, depending on small variations in the stage of the disease in each animal, and on other factors such as fluid intake and volume of urine collected. Pooling urine samples, however, would reduce animal-to-animal variation.
5.5.2 Urinary antigen detection in antibiotic treated animals

PCR data, in combination with febrile response and serological evidence, suggested that doxycycline treatment reduced the number of circulating *C. burnetii* organisms, and that the severity of the disease in doxycycline treated animals was reduced. Indeed, levels of urinary antigen from guinea pigs treated with doxycycline was less than those detected in the infected only guinea pigs throughout the experiment, except at five days post-infection. Doxycycline treatment is known to be bacteriostatic for *C. burnetii* (Yeaman & Baca, 1990), and may have delayed the initial appearance of urinary antigen (detected at two to three days post-infection in infected only animals) until five days post-infection in treated animals.

Ciprofloxacin treatment was shown to be ineffective at reducing the number of organisms present (as determined by serological evidence), and the severity of the disease (as determined by weight loss and pyrexia). Levels of urinary antigen detected at all sample points were higher than those levels from doxycycline treated animals. At five and nineteen days post-infection, levels of urinary antigen were higher than untreated controls. Greater variation was detected in the levels of antigen from ciprofloxacin-treated animals, and this may reflect the inherent fluctuation in levels of urinary antigen excreted throughout *C. burnetii* infection as mentioned earlier.
Intraperitoneal infection compared to aerosol infection

Comparison of the excretion of urinary antigen from intraperitoneally-infected guinea pigs (this Chapter) with that from aerosol-infected animals (Chapter 3) was difficult, as the retained dose of each group was different. Guinea pigs challenged with high dose *C. burnetii* by the aerosol route (group 1, Chapter 3) developed severe disease characterised by a febrile response by two days post-infection (Fig 2.1), high levels of mortality, and high, rising anti-*C. burnetii* antibodies (Fig 2.3). Urinary antigen was detected, using the size exclusion column technique, from two days post infection (the earliest sample time) and at every day post infection, when tested, until animals were killed. By nine days post-infection, the high levels of urinary antigen reflected the severity of the disease (Fig 4.5).

A much less severe disease was produced in guinea pigs challenged with a low dose by the aerosol route (group 3, Chapter 3). The febrile response occurred much later (approximately fourteen days post infection, Fig 2.2) and levels of urinary antigen were lower and primarily confined to the third and fourth week post-infection (Fig 4.6). In addition, no antigen was detected in the urine from some days sampled from this group of animals.

In contrast, urinary antigen was detected consistently in the urine of guinea pigs infected with a high dose of *C. burnetii* by the intraperitoneal route (this chapter, Fig 5.10), on all days sampled from day 8 post-infection to the end of the
experiment (day 19 post-infection). In these animals, the febrile response occurred later than in animals which received high dose C. burnetii by aerosol (group 1), but earlier than animals which received a low dose of C. burnetii (group 3). In addition, the disease was less severe (no mortality occurred) in intraperitoneally-infected guinea pigs, but more acute than that seen in animals infected with low dose C. burnetii by the aerosol route. This data suggests, therefore, that the appearance of specific urinary antigen is dependent on the initial infective dose rather than the route of infection. Consequently, consistent excretion of high levels of urinary antigen correlated with a high initial challenge dose. The route of infection was more important in determining the severity of the disease.

This study has confirmed that the combined use of size exclusion chromatography and direct ELISA provides a direct antigen detection method for experimental C. burnetii infection in guinea pigs. Furthermore, after the initial filtering step, the procedure can be performed on the bench without category III containment facilities, and provides an extra parameter with which to measure the efficacy of C. burnetii treatment.

In addition, levels of urinary antigen, in general, supported the febrile response data, serology, and PCR data which indicated that doxycycline was more effective than ciprofloxacin in the treatment of C. burnetii infection in the guinea pig. Variation in levels of urinary antigen from day-to-day (particularly in the levels of urinary antigen from ciprofloxacin-treated animals) suggested that the
determination of antibiotic efficacy could not rely solely on urinary antigen excretion.
CHAPTER 6

Concluding discussion

The aim of the studies presented in this thesis were to determine if *C. burnetii* antigens appeared in the urine in an experimental guinea pig model of Q fever, and if the detection of such antigens could provide the basis of a specific laboratory diagnostic test for Q fever.

To this end a number of stages had to be achieved. Firstly, an experimental guinea pig model was established which accurately represented the characteristic features that are recorded in acute Q fever in humans, in terms of clinical signs, serology and pathology. The primary route of transmission of *C. burnetii* to humans is via the aerosol route (Baca & Paretsky, 1983) and in this study guinea pigs were administered *C. burnetii* in a small particle aerosol. Infective doses for humans can be estimated by the onset time of the febrile response, which is dependent on the initial infective dose (Tigertt et al., 1961), but which normally ranges from 2-30 days (Baca & Paretsky, 1983). In this study guinea pigs that received a low dose by the aerosol route, received a realistic infective dose of *C. burnetii* (approximately 1-10 organisms) as demonstrated by initiation of a febrile response at 14 days post-infection.

The severity of the disease was shown to be dependant on the route of infection. Mortality was a feature of guinea pigs infected with *C. burnetii* by the aerosol
route. In this study, the generation of infectious aerosols was achieved using a Collison nebuliser in conjunction with a contained Henderson apparatus. The primary feature of this system was the ability to generate, and deliver to the alveolus small infectious, monodispersed particles, less than 5 µm with a mean diameter of 1-2µm (Henderson, 1952; May, 1973). In such a system, single droplet nuclei would be delivered to the guinea pigs. The high rates of mortality and the infectivity of the Lane strain used in this study could therefore be due to the delivery system used. Particle size has been shown to be important to the eventual disease outcome for such organisms as *Bacillus anthracis* (Druett et al., 1953) and *Yersinia pestis* (Druett et al., 1956) where aerosol droplets that contained single organisms (approximately 1µm) were more infective (in terms of mortality), than particles larger than 5 µm in diameter. Indeed, *B. anthracis* delivered as small particles (approximately 1 µm) was 17 times more infective for guinea pigs than when the bacteria were delivered in 12 µm particles (Druett et al., 1953). Comparison with work recorded by (Marrie et al., 1996) and (La Scola et al., 1997), where route of infection (intranasal versus intraperitoneal) was investigated, concluded that the route of infection and dose of inoculum determined the eventual disease outcome in acute Q fever. However, intranasal instillation results in flooding of the upper respiratory tract and produces relatively few particles small enough to be deposited in the terminal respiratory bronchioles and alveoli (Fitzgeorge et al., 1983). Other workers who have administered *C. burnetii* by the aerosol route include (Kishimoto & Burger, 1977; Powanda et al., 1978; Williams, J. C. et al., 1986a) and (Kazar et al., 1993b), however, mortality
rates reported by all these workers was less than in this present study. In addition, the delivery systems used, when stated, did not specify the size of the administered particles. The present study has therefore confirmed that *C. burnetii* is more infective when delivered by the aerosol route, and advanced the work of previous workers by suggesting that particle size may play an important role in the severity of the disease.

*C. burnetii* exhibits pleomorphism, existing as both small and large cells, depending on the stage of the developmental cycle (McCaul, 1991). A delivery system, such as employed in the present studies, to deliver an aerosol to guinea pigs, would be more likely to select for smaller particles. A greater number of small cells would therefore be expected in the infectious aerosol delivered to the animals in this study. The small cell variant has been shown to be highly resistant to environmental stresses such as osmotic lysis and chemical disinfectants (McCaul, 1991) and would therefore be the most likely cell-type to be transmitted in aerosols from natural environments to hosts (McCaul *et al.*, 1981). An experimental infection which selects for the small cell variant would more accurately reflect human infection with *C. burnetii* in the environment. Future studies could concentrate, therefore, on the relative infectivity and virulence, in guinea pigs, of the small and large cell variants and the effect of particle size on infectivity.
Aerosol transmission of *C. burnetii* Lane strain proved highly infectious for guinea pigs, persisting in heart tissues for up to thirteen weeks post-infection. Since the Lane strain originated from a patient suffering from chronic Q fever (endocarditis) current findings contradict the theory that strains associated with human endocarditis are highly infective but poorly pathogenic for guinea pigs (Waag *et al.*, 1991). However, this work confirmed the findings of (Kazar *et al.*, 1993a) that strains of *C. burnetii* associated with chronic disease were able, when delivered by the aerosol route, to cause disease in guinea pigs. To extend these findings, a greater number of strains associated with both chronic and acute disease need to be tested in our infection system. In addition, the persistence in tissue of those strains associated with acute disease needs to be investigated in the future.

The specific laboratory diagnosis of *C. burnetii* relies on a number of procedures including serodiagnosis, culture, DNA amplification and immunodetection of *C. burnetii* in tissues (Fournier *et al.*, 1998). In the experimental guinea pig model of *C. burnetii* infection developed in this thesis, it was possible to compare the detection of *C. burnetii*-specific antigen in urine to detection by serology. The capture ELISA described (Chapter 3), proved able to consistently detect *C. burnetii*-specific antigens in the urine of infected guinea pigs that had been administered a low dose of *C. burnetii*, from day 10 post-infection until day 43 post-infection. An anti-*C. burnetii* whole cell phase II antibody response was also evident at day 10 post-infection. The data show that the detection of urinary antigen provides a novel method, using a non-invasive technique, for the rapid
detection of acute experimental Q fever infection. In addition, the detection of *C. burnetii* antigen in urine provides direct evidence of current infection, as do techniques such as PCR, culture and immunodetection (Fournier *et al.*, 1998). A longitudinal study was not performed comparing the detection of urinary antigen with such techniques, as the number of animals involved was prohibitive in terms of cost of animals and practical difficulties of working within a category 3 animal containment facility for long periods of time. However, post mortem samples tested for the presence of *C. burnetii* by PCR and immunodetection (Chapter 2) indicated that antigen was present 92 days post-infection in the heart and lungs of animals administered a low dose of *C. burnetii* by aerosol (group 3). In contrast, no antigen was detected in urine beyond 43 days post-infection suggesting that PCR and immunodetection were more sensitive. Immunodetection, however, requires tissue removal by biopsy, and is only suitable, therefore, in cases of chronic Q fever (Fournier *et al.*, 1998). The detection and diagnosis of Q fever by PCR has been reported from clinical samples (Mallavia *et al.*, 1990; Frazier *et al.*, 1992; Willems *et al.*, 1993; Fritz *et al.*, 1995) and from cows milk (Willems *et al.*, 1994) and provides a sensitive and specific technique for the diagnosis of Q fever (Fournier *et al.*, 1998). However in this study, no *C. burnetii* DNA could be detected in any urine sample, and to date no references to the detection of *C. burnetii* DNA in human urine are present in the literature. This thesis concentrated on the immunological detection of *C. burnetii*-specific antigens in urine, as previous studies with *L. pneumophila* had proved promising (Lebrun *et al.*, 1983; Williams, A. & Featherstone, 1988). In contrast, (Maiwald *et al.*, 1995) described
the detection of *L. pneumophila* DNA in human and guinea pig urine and stated that PCR was more sensitive than ELISA for the diagnosis of legionellosis from urine samples. The development of a PCR urinary test in future studies may therefore prove useful as a more sensitive technique for the diagnosis of Q fever infection.

Previously, it has been reported that whole *C. burnetii* has been detected in the urine of experimentally-infected mice (Kruszewska & Tylewska-Wierzbanska, 1991) and in urine of humans suffering from Q fever (Burnet & Freeman, 1937; Derrick, 1937; Baca & Paretsky, 1983; Tylewska-Wierzbanska & Kruszewska, 1993). This study however, concentrated on the detection of *C. burnetii*-specific antigens, and is the first to report the presence of such antigens in the urine of experimentally-infected animals.

Characterisation of the antigen appearing in urine obtained from *C. burnetii*-infected guinea pigs (Chapter 4) discovered for the first time that urine from guinea pigs that received high dose *C. burnetii* by aerosol contained a *C. burnetii*-specific protein with an approximate molecular weight of 62 kDa. Evidence also suggested that LPS from *C. burnetii* was present in the urine from these guinea pigs. Urine, used to obtain these results, was taken from guinea pigs at day 9 post-infection (when the animals were severely diseased) to maximise the antigenic yield. Therefore, although the 62 kDa protein was present in urine from severely diseased animals, other antigens may be excreted in greater quantities during other
phases of the disease. Indeed, less antigen was detected in urine from guinea pigs infected with a low dose of *C. burnetii* by aerosol, and antigen that was detected in these animals was primarily of a low molecular weight. The detection of low molecular weight antigens, later on post-infection, may represent LPS molecules, as has been reported in various other diseases (Coonrod, 1983). Therefore, the 62 kDa protein antigen may only be a marker for severe disease. Such a theory was supported by gel filtration experiments on urine obtained from guinea pigs infected by the intraperitoneal route in Chapter 5. Antigenic reactivity was confined to lower molecular weight fractions and the infection in these guinea pigs was less severe with no mortality. Therefore, in future studies on *C. burnetii* urinary antigen, an antibody directed against LPS may prove more specific and, therefore, possibly more sensitive. In addition, with a more specific and sensitive antibody, urines from human acute and chronic cases of Q fever could be investigated for the presence of *C. burnetii*-specific antigens.

As well as providing a diagnostic test for suspected cases of Q fever, preliminary data presented in Chapter 5 indicated that detection of *C. burnetii*-specific antigens in urine could provide a rapid and convenient method to monitor the efficacy of antibiotics against Q fever. This has not been attempted before for Q fever, although a similar approach has been described for Legionnaires' disease (Kashuba & Ballow, 1996) and for Schistosoma infections (Deedler et al., 1994). This technique may prove particularly useful for monitoring antibiotic efficacy in chronic cases of Q fever, where numerous treatment regimens may be tried,
treatment is often prolonged and in many cases ineffective (Raoult et al., 1989; Yeaman et al., 1989; Yeaman & Baca, 1990).

Many suspected cases of *C. burnetii* infection encountered in a diagnostic laboratory are of chronic infection (G. Lloyd, personal communication). Most patients suffering from *C. burnetii* endocarditis, present with a low grade fever, intermittent fever or are afebrile. In addition, infectious foci are limited to discrete sites in the heart tissues and are not disseminated throughout the body (Raoult et al., 1990a). It would be expected, therefore, that lower or irregular levels of antigenic product would be excreted into the urine of individuals suffering from chronic Q fever than those suffering from acute *C. burnetii* infection. These studies have conclusively proved that *C. burnetii* antigens appear in the urine during experimental Q fever infections, therefore, future studies should concentrate on the identification of such antigens in the urine of humans suffering from both acute and chronic Q fever.

The future strategy for the clinical laboratory diagnosis of Q fever, could therefore incorporate urinary antigen detection as shown in Fig.6.1.
Fig 6.1 The potential role of urinary antigen detection in the clinical laboratory diagnosis of Q fever

Shaded boxes denote the existing techniques used in the clinical laboratory diagnosis of *C. burnetii* infections.

In conclusion the establishment and characterisation of a relevant experimental *in vivo* model of acute Q fever provided defined samples on which to base the development of a capture ELISA. Subsequent data obtained by the capture ELISA proved that antigens of *C. burnetii* appeared in the urine during experimental acute Q fever and characterisation of the antigens provided data on specific markers of disease. The preliminary investigation of such markers in urine, to determine the efficacy of antibiotic treatment of experimental Q fever, proved of limited value. The detection of such markers in urine, however, could provide a method for the early and rapid diagnosis of Q fever infections.
CHAPTER 7

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