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Review

Q fever: the neglected biothreat agent

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Coxiella burnetii is the causative agent of Q fever, a disease with a spectrum of presentations from the mild to fatal, including chronic sequelae. Since its discovery in 1935, it has been shown to infect a wide range of hosts, including humans. A recent outbreak in Europe reminds us that this is still a significant pathogen of concern, very transmissible and with a very low infectious dose. For these reasons it has also featured regularly on various threat lists, as it may be considered by the unscrupulous for use as a bioweapon. As an intracellular pathogen, it has remained an enigmatic organism due to the inability to culture it on laboratory media. As a result, interactions with the host have been difficult to elucidate and we still have a very limited understanding of the molecular mechanisms of virulence. However, two recent developments will open up our understanding of *C. burnetii*: the first axenic growth medium capable of supporting cell-free growth, and the production of the first isogenic mutant. We are approaching an exciting time for expanding our knowledge of this organism in the next few years.

Taxonomy

In 1935, in two near concurrent incidences on two different continents, a previously undescribed organism was identified. In Brisbane, Australia, E. H. Derrick had been tasked to investigate a febrile illness of unknown aetiology in abattoir workers, which led to him naming the disease Q (for query) fever (Derrick, 1937). His experimental work showed that he was able to transmit the fever to guinea pigs in blood and urine from infected patients, but was unable to isolate or identify the infectious agent. He sent a guinea pig liver to Burnet and Freeman. Their investigation noted 'intracellularly situated bodies which appear to be typical Rickettsiae' in smears from the spleens of infected mice (Burnet & Freeman, 1983). They therefore named the organism *Rickettsia burnetii*. Meanwhile, on the other side of the Pacific in Montana, USA, a tick transmission study examining Rocky Mountain spotted fever recovered an unknown infectious agent capable of passing through filters. When injected into guinea pigs, this agent produced unexpected clinical signs (Davis & Cox, 1938). This agent, named the Nine Mile agent, displayed properties of both bacteria and viruses, and they were unable to grow the organism on axenic medium. Later, Cox reported a breakthrough in the ability to culture the organism in tissue culture and embryonated eggs (Cox & Bell, 1939). A laboratory-acquired infection with the Nine Mile agent in Montana led to the discovery that the Australian and American groups were investigating the same organism. It was proposed to rename the organism *Coxiella burnetii* to credit both groups for their pioneering research into this newly identified infectious agent.

The historical classification of the aetiological agent of Q fever as a *Rickettsia* was due to the shared characteristics of being an obligate intracellular organism and having a tick reservoir. However, the restructuring of the family *Rickettsiaceae* based on genetic differences resulted in the organism becoming a member of the family *Coxiellaceae* in the order *Legionellales*. More recently, 16S rRNA sequencing placed *Coxiella* into the gamma subdivision of Proteobacteria, with *Legionella*, *Francisella* and *Rickettsiales* being its closest, although rather distant, relatives (reviewed by Maurin & Raoult, 1999). Molecular techniques have subsequently shown considerable variation between isolates, although there does appear to be clustering based upon geographical origin (Maurin & Raoult, 1999).

Bacteriology

C. burnetii is a small (0.2 to 1 μm) Gram-negative highly pleomorphic coccobacillus. The organism displays a typical Gram-negative cell wall structure, but does not stain reliably with Gram stain; for this reason the Gimenez stain has been used historically (Gimenez, 1964).

Similarly to *Chlamydia*, *Coxiella* has a unique intracellular lifestyle with two distinct morphological forms, the large cell variant (LCV) and the small cell variant (SCV) (McCaul & Williams, 1981). They can be differentiated by size, morphology, peptidoglycan content and resistance to physical disruption. SCVs are the environmental extracellular form of the organism. They are 0.2 to 0.5 μm long, compact and typically rod-shaped with an electron-dense core bounded by cytoplasmic and outer membranes (McCaul & Williams, 1981). They are metabolically inactive, and show a high degree of resistance to chemical agents and physical conditions, such as osmotic pressure and sonic disruption (McCaul & Williams, 1981), which

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confers the ability to survive for prolonged periods in the environment. In contrast, LCVs resemble Gram-negative bacteria, can exceed 1 μm in length, but are more pleomorphic than the SCVs. They possess a thinner cell wall, with a more dispersed filamentous nucleoid region. They are metabolically active, and are the intracellular form of the organism. McCaul and Williams reported a 'spore-like particle' in the polar regions of some LCVs and thus they hypothesized that an endogenous spore was a part of the developmental cycle of *C. burnetii* (McCaul & Williams, 1981). Further work showed that the spore-like particle did not stain with spore stains, and was not detected by tests for dipicolinic acid, a traditional spore marker (McCaul, 1991). It has been hypothesized that the 'spores' develop into SCVs, although these particles have never been isolated and purified, and thus this has not been proven as yet.

C. burnetii enters cells passively (Baca *et al.*, 1993), and resides within a parasitophorous vacuole (PV), which has been described as a structure similar to a secondary lysosome (Heinzen *et al.*, 1999). The PV is an acidic environment (pH 4.7–4.8), and the low pH appears to trigger the conversion of SCVs to LCVs (Hackstadt & Williams, 1981). Morphogenesis from SCV to LCV occurs during an initial lag phase with no increase in bacterial number (Coleman *et al.*, 2004), before replication to high levels until it reaches stationary phase at around 6 days post-infection (Coleman *et al.*, 2004). Intracellular growth is relatively slow, with a doubling time of approximately 8–12 h (Baca & Paretsky, 1983).

The situation is complicated further by phase I and phase II forms. Phase variants display different LPS lengths with phase I organisms producing a full-length LPS with O antigen sugars, and phase II organisms producing a truncated LPS without O antigen (Schramek & Mayer, 1982). This phase variation has been compared to the smooth and rough LPS variation found in *Enterobacteriacae*, with phase II equivalent to the rough LPS phase (Hackstadt *et al.*, 1985), and is often, but not always, associated with chromosomal deletion of genes involved in LPS biosynthesis (Hoover *et al.*, 2002; Denison *et al.*, 2007). The phase I form is isolated from infected hosts, but not the phase II form. Only following serial passage in eggs or tissue culture can the phase II form be obtained (Fiset, 1957). Microscopically the two forms are indistinguishable, but the impact on the serological response is significant. The response to the phase II antigen is much higher during acute infection than chronic infection, whereas titres to phase I antigen are higher during chronic infection compared to acute infection (outlined in the publication by Olson *et al.*, 2006). Although the phase II is attenuated for growth in primary mouse macrophages, the two forms do not differ for growth in other cells, including continuous cell lines and primary guinea pig macrophages (reviewed by Howe *et al.*, 2010)

C. burnetii has a single circular chromosome (Seshadri *et al.*, 2003). PFGE has indicated that the genome size ranges from 1500 to 2400 kb (Willems *et al.*, 1998). The complete genome of the Nine Mile reference strain has been sequenced and the circular chromosome was found to be 1 995 275 bp in length with a mol% G+C content of 42.6 % (Seshadri *et al.*, 2003). Other isolates of *C. burnetii* have also been sequenced, and have been shown to possess genomes of very similar length. The Nine Mile strain also possesses a 37.4 kb plasmid, QpH1 (Samuel *et al.*, 1983). Three other related plasmids carried by other strains of *C. burnetii* have also been described, designated QpDG, QpDV and QpRS (Valková & Kazár, 1995; Mallavia, 1991). The plasmids share significant regions of homology, but also have plasmid-specific sequences, which can be used to differentiate the plasmids (Jäger *et al.*, 2002; Lautenschläger *et al.*, 2000). Plasmidless strains possess plasmid-homologous sequences integrated into the chromosome (Willems *et al.*, 1997; Savinelli & Mallavia, 1990), which implies a critical function for the core plasmid genes. *C. burnetii* shows a similarity in lifestyle to *Rickettsia* and *Chlamydia*, but differs significantly with regard to genetic structure. For example, the proportion of pseudogenes is lower than seen in many other intracellular pathogens, suggesting that genome reduction may have started relatively recently in *Coxiella* (Seshadri *et al.*, 2003). Also, compared to the many other obligate intracellular pathogens, *C. burnetii* possesses many copies of insertion sequence (IS) elements. Interestingly, the IS elements are only located in the chromosome of the Nine Mile genome, with no copies in the plasmid (Seshadri *et al.*, 2003). Multiple copies of IS elements appear to mediate genomic plasticity in *C. burnetii* (Beare *et al.*, 2009b), similarly to that seen in other intracellular pathogens that are relatively recently emerged, such as *Yersinia pestis* (Parkhill *et al.*, 2001). Unravelling the pathogenic mechanisms involved in the host–pathogen interactions has been difficult, due to the inability to genetically manipulate *C. burnetii.* This has been circumvented by using surrogate hosts to assess various functions, such as *Escherichia coli* (Suhan *et al.*, 1994) and *Legionella pneumophila* (Zamboni *et al.*, 2003; Zusman *et al.*, 2003). Recently, however, *Himar1* transposon mutagenesis was employed to create an *ftsZ* mutant in *Coxiella* (Beare *et al.*, 2009a), a method that was previously used to create mutants in the obligate intracellular organisms *Anaplasma phagocytophilum* (Felsheim *et al.*, 2006) and *Rickettsia prowazekii* (Liu *et al.*, 2007).

Culture

Due to the high infectivity of the pathogen and the disease that ensues, the organism should only be handled under bio-safety level 3 containment. Historically, cultivation of the organism was performed in guinea pigs. This practice is not as widely used in modern science; however, it is an excellent procedure for isolating *C. burnetii* from contaminated samples, and passage through guinea pigs ensures isolation of phase I organisms from mixed cultures (Maurin & Raoult, 1999). Spleen extracts from the infected animals can then be propagated in embryonated eggs. Yolk

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sac inoculation of 5–7-day-old embryonated eggs, with incubation for 10–12 days, produces a high yield in the yolk sac, specifically the yolk sac membrane, with lower numbers found within the tissues of the embryo (reviewed by Baca & Paretsky, 1983). The yolk sac is then harvested. During their biowarfare offensive programme, however, the US harvested the entire egg minus shell (Tigertt *et al.*, 1961). *C. burnetii* can infect a range of cells, including monocytes and macrophages, and cell lines, including macrophages, fibroblast and epithelial cells (reviewed by Maurin & Raoult, 1999). Indeed, antibiotic susceptibility profiling has usually been undertaken in cell culture models, but results obtained in these screens were not always replicated in the clinic (Levy *et al.*, 1991). Care must be taken with repeated passage through eggs or cell lines as phase II variants can arise as mentioned above.

Following growth of the organism, purification is laborious and time-consuming, involving several differential centrifugation steps, followed by density-gradient centrifugation steps. This process has been evaluated and improved over time (Cockrell *et al.*, 2008; Williams *et al.*, 1981; Davis & Patrick, 1965; Ormsbee, 1962), but still remains an involved process.

A massively important development reported recently was the first cell-free laboratory medium for the growth of *C. burnetii*. Omsland *et al.* (2009) developed an acidified citrate cysteine medium. By evaluating the organism's metabolic requirements by transcriptomics, metabolic typing and pathway analysis, a complex medium was developed. The acidic medium (pH 4.75) and low oxygen tension (2.5 %) were designed to mimic conditions in the PV. A heavy starting inoculum was required, but a 3 $\log_{10}$ increase was detected after 6 days culture. Moreover the medium and environment facilitates the transition from SCV to LCV. This is an enormous breakthrough that will greatly facilitate studies into the biology and pathogenesis of *Coxiella*.

Ecology, epidemiology and vectors

Human Q fever has been described in countries around the world with the exception of New Zealand (Hilbink *et al.*, 1993). As it is not a notifiable disease in many countries, the geographical distribution of the organism is extrapolated from serological surveys and investigated outbreaks (Maurin & Raoult, 1999). It is also considered that the actual incidence of human infection is under-reported due to the difficulty in diagnosis and asymptomatic infections that can only be identified by seroconversion (Raoult, 2009). It is believed to be ubiquitous in the environment as was shown recently in a 3 year study across the USA (Kersh *et al.*, 2010), where geographically diverse areas, both agricultural and urban, were sampled. The organism has reservoirs in a wide range of wild and domestic animals, including mammals, birds and arthropods (reviewed by Maurin & Raoult, 1999), although the true extent of the reservoirs is unknown. Of greatest relevance to human disease are domestic ruminants, which are the most common source of human infections (Dupuis *et al.*, 1987; Fishbein & Raoult, 1992; Marrie *et al.*, 1985).

C. burnetii infection of livestock is termed coxiellosis, a chronic but often symptomless disease. The uterus and mammary glands are sites of chronic infection in females, and this is associated with abortions in goats and sheep, and infertility in cattle (Palmer *et al.*, 1983; Rady *et al.*, 1985; To *et al.*, 1998). *C. burnetii* is shed from milk, urine and faeces. At birth, the placenta contains vast quantities of *Coxiella*, which are released into the environment. Farming practices can facilitate environmental spread, such as transport of infected animals and the spreading of contaminated manure onto fields (Enserink, 2010). This, together with the environmental stability of the organism, pose a difficulty in containing outbreaks, as has been noted in the recent outbreak in the Netherlands (Enserink, 2010).

A range of arthropods, including ticks, have been shown to be able to be colonized via ingestion of contaminated blood feeds. These ticks release significant quantities of *Coxiella* in their faeces. While experimental transmission between guinea pigs has been achieved via tick bite (Smith, 1940, 1941), arthropod vectors are not considered essential to the natural cycle of infection in livestock that live closely together and likely contract the organism from close contact with other infected animals (Babudieri, 1959). However, ticks may play an important role in transmission in the wild, for example between birds (Stein & Raoult, 1999; Babudieri, 1959).

Decontamination and control measures

C. burnetii is known to be resistant to physical stresses, such as elevated temperature, desiccation, osmotic shock and UV light, and to chemical stresses, such as disinfectants, which contributes to its stability in the environment. In the UK, Health Protection Agency guidelines (at www.hpa.org.uk/deliberate_accidental_releases/biological) suggest the use of 2 % formaldehyde, 1 % Lysol, 5 % hydrogen peroxide, 70 % ethanol or 5 % chloroform for decontamination of surfaces, and spills of contaminated material should be dealt with immediately using hypochlorite (5000 p.p.m. available chlorine), 5 % peroxide or phenol-based solutions. However, they also state that 'It is impossible to decontaminate large areas of a potentially contaminated environment'. Formaldehyde vapour was ineffective without humidity control (despite inactivating *Bacillus* spores under these conditions), but effective when a high relative humidity was maintained (Scott & Williams, 1990). Although areas thought to be contaminated could be assessed for presence of *C. burnetii* DNA by PCR (Kersh *et al.*, 2010; Fitzpatrick *et al.*, 2010), determining viability would be difficult, and combined with the organism's resistance to chemical disinfectants, assessing decontamination efficacy would be difficult.

Tick control and good hygiene procedures can reduce the incidence in livestock (reviewed by Angelakis & Raoult,

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2010). Contaminated bedding and high risk materials, such as aborted fetuses, should be buried with lime or incinerated. Manure from infected herds should be treated with lime or calcium cyanide before spreading on land. In addition, spreading on a relatively calm day will reduce the risk of wind transmitting infectious material for large distances. Separating infected animals from the herd and restricting animal movements from infected areas may also help to reduce spread.

The recent Q fever outbreak in the Netherlands has shown how difficult it is to contain an outbreak in livestock due to airborne transmission (Enserink, 2010). Control measures during this ongoing outbreak have included the culling of infected animals and pregnant livestock in infected areas. Unfortunately, there is no means to quickly identify infected from healthy animals and vaccination is ineffective in infected animals. Vaccination of naive animals does not completely prevent infection, but does reduce abortion rates, which curtails the spread of the organism. A widespread programme of cattle vaccination in Slovakia significantly reduced the local incidence of Q fever (Kovácová & Kazár, 2002). Antibiotic treatment of infected animals does not stop shedding and as such is ineffective in controlling the disease spreading (Astobiza *et al.*, 2010).

Animal models

Q fever in man can manifest as either acute or chronic disease, thus there has been a search for animal models that mimic both human disease profiles. As Q fever is a disease arising from inhalation of infectious organisms, the most appropriate model should be an animal susceptible to inhalation challenge and that develops a disease similar to that seen in man. The guinea pig is one of the more familiar models of Q fever, being used for the first isolation of the pathogen. The route of infection, i.e. intranasal versus intraperitoneal, alters the pattern of pathology (La Scola *et al.*, 1997). An aerosol challenge model has been developed. In this model, the LD_{50} was calculated as $2 \times 10^{5.7}$ *C. burnetii*, although the infectious dose was significantly lower with animals receiving as few as 20 organisms developing fever (Russell-Lodrigue *et al.*, 2006). Guinea pigs can also develop a latent infection that can be reactivated following immunosuppression (Sidwell *et al.*, 1964a, b). Generally, the guinea pig is regarded as a model of acute Q fever in humans.

Mice have also been used as an infection model, but develop a chronic infection compared to the more acute guinea pig model, although endocarditis does not develop in normal adult mice. Similarly to the guinea pig, intranasal or intraperitoneal challenge does not result in clinical signs although pathology develops with high bacterial loads in the liver and spleen (Burnet & Freeman, 1937; Scott *et al.*, 1987), and the pathology varies with the challenge route (Marrie *et al.*, 1996). Mice are chronically infected for months, with prolonged shedding of bacteria in faeces and urine. The susceptibility of the mice is dependent on the mouse strain (Scott *et al.*, 1987), and resistant strains can be rendered susceptible by loss of the thymus, with the mice again developing a chronic infection (Kishimoto *et al.*, 1978). Also, A/J mice defective in various immunological processes are highly susceptible to *C. burnetii* (Scott *et al.*, 1987). Transgenic mice constitutively expressing interleukin 10 have been proposed as an improved murine model for chronic Q fever (Meghari *et al.*, 2008). Cardiac valve damage has been reported in mice immunosuppressed with cyclophosphamide prior to challenge (Atzpodien *et al.*, 1994), and in pregnant mice inoculated intraperitoneally with *C. burnetii* (Stein *et al.*, 2000).

A non-human primate model based on the cynomolgus and rhesus macaques infected by the aerosol route has also been reported (Gonder *et al.*, 1979; Waag *et al.*, 1999). Animals developed pneumonia and fever 4–7 days post-challenge, and thus represent the closest representation of the disease in man. However, although a non-human primate model would be required for licensing new medical countermeasures, such as vaccines, it would be unethical to perform most basic research in such a model. Thus, the guinea pig and mouse remain the most common laboratory animal models, despite their limitations, and can be supplemented with observations gained with infected egg and cell culture models.

Disease in humans

Q fever was first documented in abattoir workers in Queensland, Australia. The lack of a diagnosis based on vague febrile symptoms led to the illness being designated Query (Q) fever (Derrick, 1937). Although *C. burnetii* infection can result in outcomes from asymptomatic seroconversion to death, fever and pneumonia are the typical clinical manifestations, although other complications including hepatitis and endocarditis can also develop (reviewed by Maurin & Raoult, 1999). Typically, infection in humans arises by inhalation of infectious aerosols produced by farm animals and pets, although infection has also been shown to arise by ingestion of infected dairy products (reviewed by Angelakis & Raoult, 2010). Although, as described above, ticks are important vectors for the transmission of *C. burnetii* between wild animals, they do not transmit the infection to humans (Kazár, 1996). Person-to-person transmission is rare but has been documented. For example *C. burnetii* has been identified in the semen of infected males, and this has resulted in sexual transmission of the pathogen (Miceli *et al.*, 2010; Milazzo *et al.*, 2001). Chronic Q fever can develop many months or years after infection, manifesting in the majority of cases as culture-negative endocarditis (Gami *et al.*, 2004), and more rarely as osteomyelitis, osteoarthritis, hepatitis and other diverse manifestations (reviewed by Angelakis & Raoult, 2010). One chronic outcome to acute Q fever is post-Q fever fatigue syndrome. This syndrome develops in approximately 15 % of patients recovering from acute

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infection, can be disabling in severity and can persist for up to a decade (Marmion *et al.*, 1996; Ayres *et al.*, 1996, 1998). The fatigue syndrome has been attributed to a dysregulation of cytokine production, induced by persistent antigens including LPS and proteins, rather than persistent latent *Coxiella* (Marmion *et al.*, 2009).

C. burnetii has been identified by organizations such as the World Health Organization (WHO), the United Nations and the Australia Group as an agent of concern (WHO, 2004). Indeed it featured in offensive weapons programmes in the USA and former Soviet Union in the last century (reviewed by Waag, 2007). In the event of a biological attack, it is likely that the pathogen will be delivered as an aerosol. Inhalation of *C. burnetii* results in pneumonic disease, and the infectious dose is as low as 1–10 c.f.u. (Tigertt *et al.*, 1961). Following inhalation, symptoms can develop after 10 to 90 days, depending on the dose. Lower doses often result in an asymptomatic outcome or mild cases characterized by a non-productive cough, fever and minimal abnormalities to normal breathing sounds. However, acute pneumonic Q fever can result in respiratory distress. The mortality rate ranges from 0.5 to 1.5 % (Tissot Dupont *et al.*, 1992). In a study to estimate the effect of Q fever used as a bioweapon, the WHO estimated that if 50 kg *C. burnetii* were aerosolized over an urban area with 500 000 inhabitants, there would be 125 000 cases of acute illness, 9000 cases of chronic Q fever and 150 fatalities (WHO, 1970). During the ongoing outbreak of Q fever in the Netherlands, as of November 2009, 2293 human cases had been confirmed, including 6 deaths, showing that the mortality rate used by WHO in their assessment erred on the conservative side, so the impact of a bioweapon attack with Q fever may in fact be more severe. In 2010, there were a further 421 human cases, and 5 potential fatalities, reported up to June 2010 by the Netherlands authorities, indicating that the stringent control measures of culling and vaccination of livestock may be having an impact and bringing the outbreak under control.

The low aerosol infectious dose of Q fever for man can be stated with some confidence as, unlike many pathogens, classical biothreat agents have been used to deliberately infect humans, providing information on infectious dose, incubation period and development of symptoms. These experiments were performed in the USA on conscientious objectors, who wished to serve their country without taking up arms. The experiments were collectively referred to as Operation Whitecoat and one of the pathogens included was *C. burnetii*. In addition to understanding the disease and the pathogen, Operation Whitecoat also aimed to evaluate medical countermeasures against threat pathogens. The studies involving Q fever were the first conducted during Operation Whitecoat, and were referred to as CD-22. In January 1955, volunteers were exposed to indoor aerosols of *C. burnetii* at the US Army's research facility at Fort Deitrick (Frederick, USA). The aim of these studies was to determine the minimal infectious dose for humans and evaluate the efficacy of vaccination. Young adult male volunteers were exposed to aerosolized Q fever produced as an egg slurry (Tigertt *et al.*, 1961). After inhalation of 10 l volumes of the aerosol, the volunteers were monitored for the development of a persistent fever over 100°F (Table 1), after which they were treated with oxytetracycline to clear the infection. It was also found that oral dosing with the same therapy post-exposure, but before the development of symptoms, was effective in preventing disease (Tigertt *et al.*, 1961). Vaccination was able to protect against even extremely high challenge levels. Subsequently in July of the same year the volunteers, together with guinea pigs and primates, were exposed to Q fever under conditions mimicking a biological attack. To this end, aerosols of Q fever were released 3000 feet (914.4 m) away from the test subject, at night, in the desert at the Dugway Proving Ground in Utah. The test was aimed to deliver 150 infectious units to each individual (Tigertt *et al.*,1961). Individuals were treated with antibiotics at the end of the study, and all recovered. Considering the chronic sequelae associated with Q fever, longer term follow up was conducted on those volunteers, but no sequelae were identified (Pittman *et al.*, 2005).

Due to the wide range of presentations of disease and difficulties in culturing the pathogen on laboratory media, the clinical diagnosis of *C. burnetii* is most commonly reliant on seroconversion detected by immunofluorescence assays (reviewed by Maurin & Raoult, 1999). However, a lag in seroconversion during the early stages of infection can confound diagnosis. Thus, PCR-based methods have been developed for direct detection in clinical samples

Table 1. Infection of guinea pigs and humans with *C. burnetii* following inhalational exposure as reported by Tigertt *et al.* (1961)

Dose of *C. burnetii* (guinea pig infecting dose)	Seroconversion of guinea pigs (positive/exposed)	Time to onset of fever (days)	Seroconversion of man (positive/exposed)	Time to onset of fever (days)
1	0/29	12.5	0/2	17
10	1/26	Not shown	4/5 (two subclinical infections)	16
10^2	4/24	11	7/8	15
10^3	22/27	9	4/5	13.5
10^4	28/28	8.5	4/4	10.5
10^5	24/24	7	2/2	10

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(Fenollar & Raoult, 2007; Klee *et al.*, 2006; Fenollar *et al.*, 2004; Schneeberger *et al.*, 2010). Recently, serological and molecular methods were compared in the on-going outbreak in the Netherlands (Schneeberger *et al.*, 2010): real-time PCR was effective at detecting *C. burnetii* DNA during the early stages of infection, but an immunofluorescence assay was more reliable once an antibody response developed as the immune response resulted in circulating organism being suppressed below detectable levels.

Laboratory evaluation of antibiotic susceptibility has been complicated by the inability to culture *C. burnetii* on laboratory media. As such, complex assays involving egg infections and cell culture models have been employed, but have given somewhat confusing and contradictory results (reviewed by Maurin & Raoult, 1999). Also, promising performance *in vitro* is not always replicated in the clinic, for example, as was seen for fluoroquinolones (Levy *et al.*, 1991). Therapeutic regimens are dependent upon whether the patient is suffering from acute or chronic Q fever. Doxycycline (200 mg daily for 14 days) is the current drug of choice for acute Q fever, although other antibiotics such as chloramphenicol and co-trimoxazole have also been suggested as potential therapies. However, as most cases of acute Q fever are self-limiting it has been difficult to establish the clinical efficacy of such regimens, although it appears that therapy is of most benefit if initiated in the first 3 days of illness (Olson *et al.*, 2006). Treatment of chronic Q fever requires protracted regimens, for periods of up to several years. Relapse is common upon withdrawal of the therapy. Maurin & Raoult (1999) recommended patients with endocarditis be treated with doxycycline/chloroquine for at least 18 months, or doxycycline/ofloxacin for at least 3 years. The therapy can also be supported by valve replacement surgery (Fernández-Guerrero *et al.*, 1988; Kristinsson & Bentall, 1967).

Pathogenesis and immunity

Following inhalation, the organism must first invade before subsequently causing systemic infection. The alveolar macrophage has been proposed as the primary target. It has been suggested that one factor contributing to the attenuation of phase II forms versus phase I forms is the nature of the interaction between the organism with host cells (Mege *et al.*, 1997). However, recently it has been shown that the replication of phase I and phase II cells is very similar in human monocyte-derived macrophages (Howe *et al.*, 2010). Once inside the cell, the organism resides in a modified vacuole with late endosome-lysosome characteristics, the PV (Berón *et al.*, 2002; Heinzen *et al.*, 1996; Howe & Mallavia, 2000; Romano *et al.*, 2007). Similarly to a wide range of Gram-negative pathogens, the LPS is a dominant virulence factor of *C. burnetii*. Indeed, it has been suggested that the structure of the LPS may influence acute versus chronic outcomes (Hackstadt, 1990; Baca & Paretsky, 1983). LPS contributes to the uptake of virulent strains by a Toll-like receptor 4-dependent mechanism (Honstettre *et al.*, 2004) and the two phases appear to interact with different surface receptors on macrophages (Capo *et al.*, 2003). However, LPS does not appear to play a role in survival within the PV, as both phase I and II cells were observed within PVs, and even within the same PV of co-infected cells (Howe *et al.*, 2010).

In acute cases in humans, Q fever presents as pneumonia and hepatitis (reviewed by Maurin & Raoult, 1999). It is not known how *C. burnetii* infects systemically from the lungs, but most likely it is trafficked by infected phagocytic cells, and transient bacteraemia has been observed late in the incubation period. Kupffer cells are considered the primary target in the liver (reviewed by Maurin & Raoult, 1999).

Once phagocytosed, the organism must resist killing following fusion of the lysosome. *C. burnetii* is well-adapted to this niche, being an acidophilic organism, and requiring an acidic environment for many metabolic processes (Chen *et al.*, 1990; Hackstadt & Williams, 1983; Hendrix & Mallavia, 1984; Zuerner & Thompson, 1983). Acidification of the phagolysosome upon phagocytosis has been proposed as a possible trigger for activation of SCVs to form LCVs. The LCV is the intracellular, metabolically active form, which can undergo a division process, with similarities to sporulation, to generate SCVs, the metabolically inactive, highly resistant form released extracellularly to infect new susceptible cells (McCaul *et al.*, 1991). A few proteins important for intracellular survival have been identified, including superoxide dismutase, catalase and macrophage infectivity potentiator (CbMip) (Mo *et al.*, 1995; Akporiaye & Baca, 1983). The CbMip protein shows homology to the FKBP family with peptidyl-prolyl-*cis*/*trans* isomerase activity. Several intracellular pathogens have been shown to produce FKBP-like proteins that have been shown to be important in pathogenesis, apparently by modulating cellular responses (reviewed by Hacker & Fischer, 1993). CbMip is a secreted protein, and export is triggered *in vitro* by an acid pH (Redd & Thompson, 1995), similarly to conditions that would be experienced in the phagolysosome. Interrogation of the genome sequence data from *C. burnetii* Nine Mile phase I RSA493 revealed little in the way of pathogenicity loci (Seshadri *et al.*, 2003). However, a type IV secretion system was identified, similar to the Dot/Icm system of *L. pneumophila* (Vogel & Isberg, 1999). *C. burnetii* encodes 23 of the 26 Dot/Icm proteins identified in *L. pneumophila* (Vogel, 2004). Mutation of some genes of the *L. pneumophila* secretion system can be complemented by some of the corresponding genes from *C. burnetii*, but not in all cases (Vogel, 2004; Zusman *et al.*, 2003; Zamboni *et al.*, 2003).The range of effectors identified that are proposed to be secreted by this system in *L. pneumophila* is large, with over 70 proteins identified (Ninio & Roy, 2007), whereas effectors of *C. burnetii* remain elusive although attempts to identify them are under way (reviewed by Voth & Heinzen, 2009). In *Legionella* the Icm/Dot system acts to prevent lysosome fusion, and then has profound effects on diverse cellular

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functions (reviewed by Franco *et al.*, 2009). The genetic intractability of *C. burnetii* has impeded the discovery of effectors but there is gradual evidence accumulating as to the identity and function of some of the effectors. The bioinformatic analysis of the *C. burnetii* genome for features associated with type IV secretion system effectors identified proteins with features normally associated with eukaryotic proteins, indicating that they may have a role in the eukaryotic cell (Voth & Heinzen, 2007). These features included coiled-coil domains, ankyrin repeats, leucine-rich repeats, GTPase domains, ubiquitination-related motifs and tetratricopeptide repeats, which are all normally rare in prokaryotic proteins. The first three features mentioned are usually involved in mediating protein–protein interactions, and could be predicted to act by direct action with host proteins following secretion. Other motifs indicate proteins that could exert their effects by regulating signal transduction pathways. Ankyrin repeat domain-containing proteins (Anks) have been identified in other intracellular pathogens including *L. pneumophila*, *Anaplasma*, *Wolbachia* and *Rickettsia* (reviewed by Voth *et al.*, 2009), and in *L. pneumophila* many of the proteins secreted by the type IV secretion system are Anks. The Anks of *C. burnetii* have been shown to be secreted by the *L. pneumophila* Icm/Dot system (Voth *et al.*, 2009), adding further weight to the hypothesis that these are some of the elusive effectors. As to function, Anks were shown to localize to various sites within the cell, which may give an indication of function: some localized to host microtubules and may thus impede microtubule functions; some localized to mitochondria, possibly to modulate apoptotic functions; while others were identified in the PV membrane, possibly to mediate vesicle fusion for maturation (Voth *et al.*, 2009). Actin is recruited to, and involved in the formation of, the vacuole inhabited by *C. burnetii* (Aguilera *et al.*, 2009). Actin dynamics are often regulated by Rho GTPases, and in HeLa cells the correct formation of the mature *C. burnetii* vacuole appeared to be dependent upon two such proteins, RhoA and Cdc42 (Aguilera *et al.*, 2009). It has been hypothesized that the type IV secretion system effectors may have an effect on Rho GTPases, a theory supported by the observation that inhibition of *C. burnetii* protein synthesis by chloramphenicol inhibits Rho proteins being recruited to the vacuole. It is also supported by observations on other intracellular pathogens, such as *Legionella*, *Leishmania* and *Chlamydia*, where Rho GTPases are similarly important (reviewed by Aguilera *et al.*, 2009). Recently, it has been shown that the secretion machinery is located at the bacterial poles during infection (Morgan *et al.*, 2010).

Phase I *C. burnetii* triggers tumour necrosis factor (TNF) synthesis by infected monocytes (Capo *et al.*, 1996). It has been suggested that the increased TNF may induce expression of the receptors on the macrophage surface, and thus facilitate uptake, which is relatively inefficient (Mege *et al.*, 1997). Somewhat at odds with this, it has also been found that TNF, in conjunction with gamma interferon (IFN-γ), results in the killing of *C. burnetii* in THP-1 monocytes by an apoptotic mechanism (Dellacasagrande *et al.*, 1999). However, although IFN-γ is key to controlling Q fever (Scott *et al.*, 1987), apoptosis is closely controlled by the invading *Coxiella* to maintain viability of the host cell during the lengthy inhabitation (Voth *et al.*, 2007). Infected host cells show decreased caspase activity, induction of a pro-survival transcriptional response, including Akt and Erk 1/2 activation, and decreased release of cytochrome *c* (Voth *et al.*, 2007, 2009; Lührmann & Roy, 2007). In addition to controlling apoptosis, *C. burnetii* modulates autophagy (Gutierrez *et al.*, 2005). The autophagy pathway protein Bectin 1 and the anti-apoptotic protein Bcl 1 are both recruited to the membrane of the vacuole surrounding the *Coxiella* (Vázquez & Colombo, 2010b) and interplay of these proteins is essential for successful infection to be established (Vázquez & Colombo, 2010b), and for correct modulation of both apoptosis and autophagy (Vázquez & Colombo, 2010a).

The immune response to chronic infection is different to that observed in acute infection. For example, peripheral blood lymphocytes from chronically infected individuals do not proliferate when exposed to *C. burnetii* antigens (although they retain the ability to proliferate when exposed to other antigens and mitogens) (Koster *et al.*, 1985b), whereas a response is seen in people recovering from acute Q fever or in vaccinees (Izzo *et al.*, 1988; Koster *et al.*, 1985a). Interestingly, *C. burnetii* phase I cells expressing full-length LPS appear almost invisible to dendritic cells (Shannon *et al.*, 2005), which may be a key factor in the development of chronic infections.

Cell-mediated immune responses are key to controlling Q fever, but the role of humoral responses is not clear. For a range of intracellular pathogens it has been shown that both cell-mediated and humoral responses are required to defend against intracellular pathogens (Casadevall & Pirofski, 2006). During primary acute Q fever, antibodies are key during the bacteraemic phase of acute infection, but are potentially detrimental during chronic infection, where immune complexes can cause pathology (Raoult, 1990). Passive transfer of antibody has been shown to be able to protect animals against subsequent challenge with *C. burnetii* (Burnet & Freeman, 1983), although no protection was seen if antibody was transferred to SCID mice, showing the importance of the cell-mediated response (Zhang *et al.*, 2007). Opsonization resulted in increased uptake *in vitro* by macrophages and dendritic cells, but did not subsequently impact on growth intracellularly (Shannon *et al.*, 2009).

Vaccines

Ever since the discovery of the aetiological agent of Q fever, there have been attempts to produce a safe, effective vaccine. However, only partial success has been achieved, as will be discussed. Four main vaccines have been used in

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humans: a live attenuated strain, M-44; a trichloroacetic acid (TCA) extract; a chloroform-methanol extract; a formalin-inactivated culture extract.

The live attenuated strain designated M-44 was isolated in the former Soviet Union in the 1960s. This was obtained by repeated passage through guinea pigs and mice. In human volunteers, minimal side effects were reported, but issues with long-term persistence in animals raised concerns about safety, including the risk of endocarditis developing in recipients (Freylikhman *et al.*, 2003; Marmion, 1967). Therefore, despite extensive use in Russia, this strain is not used in the West.

Due to the issues identified with persistence of live attenuated strains, various approaches were examined for developing inert vaccines. Generally attention has focussed on the phase I micro-organisms, as these are significantly more effective than the phase II form (Ormsbee *et al.*, 1964). The chloroform-methanol residue (CMR) vaccine was developed in the USA (Fries *et al.*, 1993). Based on the phase I Henzerling strain of *C. burnetii*, it was able to induce protection in animals, including primates exposed to aerosol challenge (Fries *et al.*, 1993; Waag *et al.*, 2002). However, on transition into human volunteer studies the CMR vaccine was found to be overly reactogenic (Fries *et al.*, 1993). Similarly, a soluble TCA extract of phase I Nine Mile strain, containing proteins and LPS, was highly reactogenic in humans (Kazár *et al.*, 1982), and combining TCA and CMR approaches, although producing a less reactive vaccine, resulted in loss of immunogenicity (Kazár *et al.*, 1987).

A formalin-inactivated vaccine prepared by Commonwealth Serum Laboratories named Q-Vax is licensed for use in Australia. The vaccine is prepared from the phase I Henzerling strain. Studies have reported 100 % efficacy in abattoir workers for many years post-vaccination (Ackland *et al.*, 1994; Marmion *et al.*, 1990). However, similarly to the CMR and TCA extract vaccines, Q-Vax induced side-effects in a significant proportion of recipients. The most common adverse reactions reported involved swelling, erythema and tenderness at the site of inoculation, but headaches and flu-like symptoms were reported in up to 18 % of recipients. Individuals who are sero-positive prior to immunization can suffer severe reactions (Bell *et al.*, 1964), and as such should be identified with a pre-vaccination skin-test (Ascher *et al.*, 1983), and for the same reason a single-dose regimen is recommended (Ormsbee & Marmion, 1990).

As a result of the safety considerations regarding immunization with Q-Vax, there is a need to identify an improved vaccine for Q fever; however, there is a significantly lower effort on this than for other bioweapon pathogens, such as anthrax and plague. Recombinant protein subunit vaccines expressed in *E. coli* have shown limited efficacy. However, immunization with identical native proteins isolated directly from *C. burnetii* did result in reduced microbial colonization in non-lethal animal models (Table 2). Two reasons for this discrepancy have been suggested: either post-translational modification in *C. burnetii* results in different antigenicity compared to protein expression in *E. coli*, or the proteins were contaminated with LPS (Shannon & Heinzen, 2009). It is known that LPS purified from phase I organisms can induce protective immune responses in animals (Zhang *et al.*, 2007).

Is *C. burnetii* a real biological threat?

When considering microbes as weapons they can simplistically be divided into lethal agents and incapacitating agents. Lethal agents, such as *Yersinia pestis*, induce an acute disease with a high associated mortality rate. Incapacitating agents make people ill enough that they cannot carry on with normal life for a period of time, but ultimately most people will recover. Q fever belongs primarily to the incapacitating agents. It is considered that a biological attack will affect the largest number of people if disseminated as an aerosol. Q fever has been shown to travel over large distances on the

Table 2. Studies of recombinant and native protein subunits of *C. burnetii*

Antigen	Source	Result	Reference
Heat-shock protein B (HspB)	Recombinant expression in *E. coli*	No protection	Li *et al.* (2005)
29 kDa outer-membrane protein (P1)	Recombinant expression in *E. coli*	No protection	Li *et al.* (2005)
P1	*C. burnetii* Phase I Nine Mile	Reduced splenic colonization in mice following challenge	Williams *et al.* (1990)
P1	Recombinant expression in *E. coli*	No protection	Zhang & Samuel (2003)
Omp, Pmm, HspB, Fbp, Orf 410, Crc, CbMip, MucZ	Recombinant expression in *E. coli*	No protection	Tyczka *et al.* (2005)
Com	Recombinant expression in *E. coli*	No protection	Zhang & Samuel (2003)
CbMip	Recombinant expression in *E. coli*	No protection	Zhang & Samuel (2003)
P28	Recombinant expression in *E. coli*	No protection	Zhang & Samuel (2003)
P1-HspB fusion	Recombinant expression in *E. coli*	Reduced splenic colonization in mice following challenge	Li *et al.* (2005)
67 kDa outer-membrane protein	*C. burnetii* QiYi	Protection observed in mice and guinea pigs	Zhang *et al.* (1994)

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wind during natural outbreaks, and is a relatively resistant organism in the environment. In addition, as described above, the infectious dose for man is extremely low. For this reason, the pathogen attracted attention in offensive programmes of the last century. However, a significant impediment to use of *C. burnetii* is the difficulty in culturing the pathogen. As mentioned previously, the WHO modelled the effects of a release of 50 kg *C. burnetii* over an urban area. However, a typical yield of *Coxiella* reported in the literature is around 1 g wet weight organism from eight dozen eggs (Baca & Paretsky, 1983).

The zoonotic nature of Q fever is a problem for defence. Q fever outbreaks occur throughout the world, and the symptoms are vague and non-specific. To this end, it would be difficult to identify a small-scale deliberate release. For example, during Desert Storm, 19 deployed US troops were hospitalized with acute bilateral pneumonitis in March 2003. However, only one of these was subsequently confirmed as a Q fever case, which was probably acquired enzootically (Mitchell *et al.*, 2007).

However, routine health surveillance would identify a peak in cases, which would not be the normal pattern in endemic areas. An attack would also have implications for agriculture, as infection would occur in both wild and domesticated animals. This could result in secondary cases of infection in man. The post-release sequelae, including on epidemiology, have been discussed in depth by Madariaga *et al.* (2003). As with any biothreat agent, the consequences of use would be significant, but are further complicated for *C. burnetii* by its environmental stability and resistance to chemical decontaminants.

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