CHAPTER 2.1.12.
Q FEVER

SUMMARY

Query (Q) fever (or Coxiellosis) is a zoonosis that occurs in most countries. Humans generally acquire infection through airborne transmission from animal reservoirs, especially from domestic ruminants, but other domestic and wildlife animals (pets, rabbits, birds, etc.) can be involved. The causal agent is the obligate intracellular bacterium, Coxiella burnetii, which displays different morphological forms in its developmental cycle. Some forms can survive extracellularly and even accumulate in the environment. Because this microorganism is classified as a Group 3 pathogen, handling viable C. burnetii must be done in biosafety level 3 facilities.

In humans, the disease exhibits a large polymorphism. Q fever occurs in either an acute form or a severe chronic form following an early infection that may go unnoticed. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In Australia, a vaccine (named Q-Vax) is available for professionally exposed population groups.

In domestic ruminants, Q fever is mostly associated with sporadic abortions or outbreaks of abortions and dead or weak offspring, followed by recovery without complications. Moreover, data also suggests that Q fever plays a role in infertility or problems such as metritis in cattle. Coxiella burnetii infection persists for several years, and is probably lifelong. Sheep, goats and cows are mainly subclinical carriers, but can shed bacteria in various secretions and excreta.

Identification of the agent: For laboratory diagnosis in the context of serial abortions and/or stillbirths, samples can be taken from the placenta, vaginal discharges and tissues of aborted fetuses (liver, lung or stomach content). For investigation of bacterial shedding, samples can be taken from vagina, milk, colostrum and faeces.

As an obligate intracellular bacterium, Coxiella burnetii can be isolated by inoculation of specimens into conventional cell cultures, embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues, faeces, milk or environmental samples contaminated with various microorganisms.

The bacteria can be visualised in stained tissue or vaginal mucus smears using a microscope with an oil-immersion objective lens. Because it is acid resistant, the bacteria can be stained by several methods: Stamp, modified Ziehl–Neelsen, Gimenez, Giemsa and modified Koster. Because of lack of specificity, a positive finding is presumptive evidence of Q fever.

To date, demonstration of the agent by immunohistochemical staining, by in-situ hybridisation or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. No specific antibodies for immunochrometry are commercially available, but PCR kits are proposed for ruminants and can be used easily in suitably equipped laboratories. PCR is considered to be a useful and reliable test for screening large numbers and various types of samples. Currently, PCR has become the tool of choice for Q fever diagnosis.

Two PCR-based typing methods have been described recently, MLVA (multi-locus variable number of tandem repeats analysis) and multispacer sequence typing (MST), permitting the typing of C. burnetii without the need for isolation of the organism.

Serological tests: A number of tests can be used, particularly the indirect immunofluorescence (IFA) test, the enzyme-linked immunosorbent assay (ELISA), and the complement fixation test (CFT). The presence of specific IgG antibodies provides evidence of a recent C. burnetii infection or a past exposure. ELISAs are preferred for practical reasons and for their higher sensitivity.
Requirements for diagnostic biologicals and vaccines: Serological antigens are based on the two major antigenic forms of *C. burnetii*: phase I, obtained from spleens after inoculation of laboratory animals, and phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Currently available commercial tests allow the detection of phase II or of both phases II and I anti-*C. burnetii* antibodies.

Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I *C. burnetii* should be considered protective.

An inactivated phase I vaccine (named Coxevac) is commercially available. Repeated annual vaccination, particularly of young animals, are recommended in at-risk areas.

**A. INTRODUCTION**

Q fever is widely distributed throughout the world with the exception of New Zealand. Although Q fever is present in virtually all ‘animal kingdoms’, including arthropods, the disease affects mostly humans, cattle, sheep and goats (Arricau-Bouvery & Rodolakis, 2005; EFSA, 2010; Lang, 1990; Maurin & Raoult, 1999). The aetiological agent, *Coxiella burnetii*, is a Gram-negative obligate intracellular bacterium, adapted to thrive within the phagolysosome of the phagocyte. It has been historically classified in the *Rickettsiaceae* family; however, phylogenetic investigations, based mainly on 16s rRNA sequence analysis, have shown that the *Coxiella* genus is distant from the *Rickettsia* genus in the alpha subdivision of *Proteobacteria* (Drancourt & Raoult, 2005). *Coxiella burnetii* has been placed in the *Coxiellaceae* family in the order *Legionellales* of the gamma subdivision of *Proteobacteria*. The complete genome sequencing of *C. burnetii* has been achieved and confirms its systematic position (Seshadri et al., 2003). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form (Coleman et al., 2004; Heinenz et al., 1999). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants described from in-vitro studies: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) measuring 0.2 µm wide and between 0.5 and 2 µm long or 0.4 to 0.7 µm diameter (Coleman et al., 2004; Heinenz et al., 1999). The SDC and SCV represent the small morphological variants of the bacterium likely to survive extracellularly as infectious particles, a trait that is important for persistence in the environment and transmission (ECDC 2010; EFSA, 2010; Kersh et al. 2010). Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the attenuated phase II, obtained by repeated in-ovo or in-vitro passages. An LPS (lipopolysaccharide) change occurs during serial passages: phase I cells, with full-length LPS O-chains, change to intermediate phases with decreasing LPS O-chain lengths and then to phase II, with truncated LPS. Thus, the long phase I LPS contains the phase II part. The latter has been described as a major immunogenic determinant. Currently available commercial tests allow the detection of at least the anti-*C. burnetii* phase II antibodies, which appear to be present whatever the infection stage or form. In contrast, vaccination is effective with a phase I vaccine but not with a phase II vaccine (Arricau-Bouvery et al., 2005; EFSA, 2010; Krauss, 1989). In general, the genomes of *C. burnetii* isolates from a wide range of biologically and geographically diverse sources are highly conserved, but notable polymorphism occurs such as rearrangements of syntenic blocks (Beare et al., 2009). This genomic plasticity might contribute to different phenotypes and is of great interest for genotyping methods.

Q fever is a zoonosis. In humans, the infection can manifest as an acute, chronic or subclinical form (ECDC, 2010; Maurin & Raoult, 1999). Diagnosis and the treatment is often delayed because of the various and nonspecific clinical expressions. The acute forms commonly include a self-limiting febrile episode, pneumonia or granulomatous hepatitis. The main clinical manifestation of chronic Q fever is endocarditis in patients with valvulopathies, vascular infections, hepatitis or chronic fatigue syndrome. The acute form resolves quite quickly after appropriate antibiotic therapy, but the chronic form requires prolonged antibiotic therapy (for 2 years or more), coupled with serological monitoring. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal. Moreover, *C. burnetii* infection of pregnant women can provoke placentitis and leads to premature birth, growth restriction, spontaneous abortion or fetal death. Overall, the chronic disease is more likely to develop in immunocompromised individuals. The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. Awareness for Q fever is increased during human outbreaks, which are generally temporary and rarely comprise more than 300 acute Q fever cases. However, the largest community outbreaks of Q fever ever reported emerged in 2007 in the Netherlands. In the subsequent years, peak incidence from February to September has increased and the geographical area has expanded progressively. The country reported 982 and 2305 confirmed cases in 2008 and 2009, respectively. However, the factors leading to outbreaks are not fully understood (ECDC, 2010; EFSA, 2010).

Domestic ruminants are considered the main reservoirs for *C. burnetii*, but cats, dogs, rabbits, birds, etc., have also been reported to be implicated in human disease/infection. There is clear epidemiological and experimental evidence that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals, their reproductive tissues or other animal products, like wool (Arricau-Bouvery & Rodolakis, 2005; ECDC, 2010; Maurin & Raoult, 1999). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown a significant transmission to humans by food. Indeed, there are epidemiological indications of sero-conversion but no association with clinical Q
fever in humans. Q fever seems also very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission or blood transfusion is possible. In animals, vertical transmission and sexual transmission could occur but their importance is not known. Finally, arthropods, principally ticks, may be involved in Q fever transmission. The risk of transmission seems to be linked to wildlife animals. It could be associated with bites as well as with contaminated dust from dried excrement.

In cows, ewes and goats, Q fever has been associated mostly with late abortion and reproductive disorders such as premature birth, dead or weak offspring (Arricau-Bouvery & Rodolakis, 2005; Lang, 1990). Moreover, C. burnetii might be associated with metritis and infertility in cattle. Given the lack of specificity of these latter signs, it is not recommended to retain them for clinical diagnosis of Q fever (EFSA, 2010). Domestic ruminants are mainly subclinical carriers but can shed bacteria in various secretions and excreta. In the environment, C. burnetii can survive for variable periods and can spread. The levels of bacterial contamination in the environment have been tackled using quantitative PCR (polymerase chain reaction) for detection of C. burnetii DNA, but a rapid test assessing viability is required (EFSA, 2010; Kersh, 2010). For now, the lack of knowledge on shedding patterns among ruminants has made the determination of Q fever status difficult. Concomitant shedding into the milk, the faeces and the vaginal mucus may be rare (Guatteo et al., 2007; Rousset et al., 2009a). The vaginal shedding at the day of kidding may be the most frequent (Arricau-Bouvery et al., 2005). Within herds or flocks experiencing abortion problems caused by C. burnetii, most of animals may be shedding massive numbers of bacteria whether they have aborted or not. The global quantities are thus clearly higher than within subclinically infected herds/flocks. At the parturitions following an abortion storm, higher bacterial discharges were measured among the primiparous compared with the other females (Guatteo et al., 2008; Rousset et al., 2009b). Moreover, the shedding may persist several months, describing either an intermittent or a continuous kinetic pattern. Animals with continuous shedding patterns might be heavy shedders. These latter animals seem to mostly exhibit a highly-seropositive serological profile (Guatteo et al., 2007).

Diagnosis of Q fever in ruminants, including differentiating it from other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). At present, direct detection and quantification by PCR and serological ELISA (enzyme-linked immunosorbent assay) should be considered as methods of choice for clinical diagnosis (Sidi-Boumedine et al., 2010). Proposals have been recently elaborated for the development of harmonised monitoring and reporting schemes for Q fever, so as to enable comparisons over time and between countries (EFSA, 2010; Sidi-Boumedine et al., 2010). However, no gold standard technique is available and efforts are encouraged both for the validation of the methods and for development of reference reagents for quality control, proficiency and harmonisation purposes (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). The Q fever diagnostic tests are also required for epidemiological surveys of ‘at risk’ and suspected flocks in limited areas (following recent outbreaks in development of reference reagents for quality control, proficiency and harmonisation purposes (see Chapter 1.1.4 Principles of validation of diagnostic assays for infectious diseases). The Q fever diagnostic tests are also required for epidemiological surveys of ‘at risk’ and suspected flocks in limited areas (following recent outbreaks in treatment of Q fever). Treatment is limited but there is a need for a better surveillance system. In human epidemic situations, active surveillance of acute Q fever is the best strategy for avoiding chronic cases. Measures for the control of animal Q fever should be implemented, particularly for domestic ruminants. Only a combination of measures is expected to be effective. Long-term options include preventive vaccination, manure management, changes to farm characteristics, wool shearing management, a segregated kidding area, removal of risk material, visitor ban, control of other animal reservoirs and tick control. The culling of pregnant animals, a temporary breeding ban, stamping out, identifying and culling shedders and controlling animal movements are considered as suitable options in the case of human outbreaks.

Regarding biosafety and biosecurity, C. burnetii is extremely hazardous to humans. Because of its ability to cause incapacitating disease in large groups of people, its likely low infectious dose, resistance in the environment, and aerosol route of transmission, C. burnetii is currently considered a potential agent of bioterrorism and is classified by the Center for Disease Control and prevention as a group B biological agent (Drancourt & Raoult, 2005; Kersh et al., 2010). Appropriate precautions must be taken with this risk group 3 agent. Live culture or contaminated material from infected animals must only be handled in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 Biosafety and biosecurity in the veterinary microbiology laboratory and animal facilities.

### B. DIAGNOSTIC TECHNIQUES

1. **Identification of the agent**

_Coxiella burnetii_ can be demonstrated in various ways, depending on the type of sample and the purpose of investigations (Samuel & Hendrix, 2009; Sidi-Boumedine et al., 2010). The ability to detect and quantify _C. burnetii_...
DNA by real-time PCR has dramatically enhanced diagnostic and study approaches. For laboratory diagnosis in the context of serial abortions and stillbirths, samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. The diagnosis should always include a differential investigation of major abortive agents. Early detection of a Q fever storm of abortions in a herd or flock and correct measures is essential to deal with both farm-based and environmental route of infection. A positive case is a herd or flock with clinical signs (abortion and/or stillbirth) for which the presence of the agent has been confirmed. If possible, vaginal swabs at the day of parturition (or taken less than 8 days after) should be collected in order to limit the number of false-negative PCR results. When difficulties in interpretation of diagnostic results are encountered, an association with a positive serological result at the herd or flock level is useful. Milk from the tank or individual milk or colostrum, vaginal and faecal samples can be taken for investigating bacterial shedding. However, detection of shedders is still fastidious as the shedding dynamics are not well known (EFSA, 2010; Guatteo et al., 2007; Kim et al., 2005; Rousset et al., 2009a). Testing bulk tank milk or pooled individual samples (i.e. vaginal swabs and/or milk samples) should be assessed in terms of the relationships with the intra-herd or flock prevalence of shedding.

a) Isolation of the agent

For specific laboratory investigations, it may be necessary to isolate the agent. Where microscopic examination has revealed large numbers of C. burnetii combined with a low contamination rate with other bacteria, direct isolation by inoculation of embryonated chicken eggs or cell culture is possible (Maurin & Raoult, 1999; Samuel & Hendrix, 2009). For example, a portion of placenta is homogenised in phosphate-buffered saline (PBS) containing antibiotics (streptomycin 100–200 µg/ml and penicillin or gentamicin 50–100 µg/ml). After low-speed centrifugation, dilutions of the supernatant fluid are inoculated into 6 to 7-day-old embryonated chicken eggs via the yolk sac. Eggs are preferably from specific pathogen free (SPF) hens. Embryos that die during the first 5 days after inoculation are discarded. The yolk sacs are harvested after 10–15 days of incubation. Stained smears of the yolk sac wall are examined to ensure the absence of bacterial contamination and to determine the presence of C. burnetii. PCR analysis can also be used to detect the presence of C. burnetii. Further passages may be required to obtain an isolate in pure culture.

A cell microculture system from a commercially available method used for virus culture, the shell vial cell culture, has been adapted for isolating strict or facultative intracellular bacteria, including C. burnetii. Such a method was described for C. burnetii in 1990 (Raoult et al., 1990). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm² cover-slip within a shell vial. Various cell lines may be used to allow the observation of characteristic vacuoles of C. burnetii multiplication. Centrifugation for 1 hour at 700 g enhances the attachment and penetration of bacteria into the cells. Three shell vials are used for the same sample, and by day 3, 10 and 21, the cytopathic effect (CPE) – C. burnetii characteristic vacuoles in cells – are examined using an inverted microscope. After 10 days, detection of growing C. burnetii within the cells is achieved directly on the cover-slip inside a shell vial by a direct immunofluorescence assay with polyclonal anti-C. burnetii antibodies and an appropriate secondary antibody conjugated to fluorescein isothiocyanate (FITC). Cells of the remaining shell vial are harvested and transferred in a 25 cm² culture flask. Incubation can be conducted for 3 months, with a culture medium change once a week. The infection can be monitored by microscopy of Gimenez-stained cells cyto-centrifuged from the culture supernatant and by PCR analysis of the culture supernatant. When the CPE observations and Gimenez staining or PCR results are positive, a passage in a 75 cm² culture flask is performed. Culture supernatant is then inoculated on confluent layers of Vero cells or L929 mouse fibroblasts in a 150 cm² culture flask in order to establish a C. burnetii isolate. This method was developed for humans but could be adapted for animals.

With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary as a filtration system. Biocontainment level 3 requirements are recommended for holding experimentally infected rodents (see Chapter 1.1.2). Mice and guinea-pigs are the most appropriate laboratory animals for this purpose (Scott et al., 1987). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status can be monitored. This method should be performed in conjunction with serological tests on other guinea-pigs or mice that have been inoculated with the same samples. Sera are collected 21 days after inoculation. A positive result confirms a diagnosis of C. burnetii infection. If pyrexia develops, the animal is killed and the spleen is removed for isolation of the agent by inoculation into embryonated chicken eggs or in cell cultures. Microscopic examination of C. burnetii can be done using impressions and staining of the collected spleens. Alternatively, the process can be simplified by performing PCR for detection of C. burnetii DNA (see below) on spleens. In the mouse model, the spleens can be systematically collected around 9 days post-inoculation.

b) Staining

In a case of an abortion suspected to have an infectious origin, smears of placental cotyledon are prepared on microscope slides. Lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be

1 Sterilin, Bibby Sterilin Ltd, Stone, Staffordshire ST15 0SA, United Kingdom.
used in the same manner. These could be stained according to several methods: Stamp, Gimenez, Macchiavello, Giemsa and modified Koster (Gimenez, 1964; Quinn et al., 1994; Samuel & Hendrix, 2009). The first three techniques give the best results. These methods are close to the modified Ziehl–Neelsen method involving basic fuchsin to stain bacteria. For example, the Stamp staining method is performed with 0.4% basic fuchsin solution, followed by rapid decolouration with 0.5% acetic acid solution, and counterstaining with 1% methylene blue or malachite green solution. The smears are examined microscopically with an oil-immersion objective lens (×500 or more). The Stamp method is preferred in veterinary laboratories while the Gimenez method is widespread for monitoring infected cultural cells in research laboratories. Gimenez is fastest because an acidic solution is not included for differentiation. Coxiella burnetii are characterised by a very large number of thin, pink-stained coccobacillary bacteria against a blue or green background. They may sometimes be difficult to detect because of their small size, but this is compensated for by their large numbers; often inclusions within the host cells appear as red masses against the blue or green background. Attention must be taken in the interpretation of the results as, microscopically, C. burnetii can be confused with Chlamydia abortus or Brucella spp. However, using the same staining procedure, Chlamydia have sharper outlines, are round, small and may resemble globules. Brucella spp, are larger (0.6–1.5 µm long × 0.5–0.7 µm wide), more clearly defined and stain more intensely. Control positive slides of C. burnetii, Chlamydia abortus and Brucella must be used for comparison. Diagnosis made on the basis of microscopy, coupled with positive serological results, is usually adequate for routine purposes. When biological staining is inconclusive, one of the other methods may be used as a confirmatory test.

### c) Specific detection methods

Detection of C. burnetii in samples can also be achieved by specific immunodetection (capture ELISA, immunohistochemistry), in-situ hybridisation or DNA amplification (Jensen et al., 2007; Samuel & Hendrix, 2009; Thiele et al., 1992). Immunohistochemistry may be used with paraffin-embedded tissues or on acetone-fixed smears (Raout et al., 1994). The method is an indirect immunofluorescence or immunoperoxidase assay using polyclonal C. burnetii specific antibodies produced in laboratory animals (rabbit or guinea-pig). An anti-species (rabbit or guinea-pig) anti- IgG conjugate, labelled with FITC or peroxidase, is then used to visualise the bacteria. Control positive slides of C. burnetii antigen should be available for comparison. No specific antibodies for immunohistochemistry are commercially available.

Fluorescent in-situ hybridisation using specific oligonucleotide probes targeting 16s rRNA may be used on paraffin embedded tissues, especially placenta samples (Jensen et al., 2007).

PCR methods have been used successfully to detect C. burnetii DNA in cell cultures and biological samples. The PCR methods of C. burnetii detection are generally performed for the health investigations of ruminant herds or flocks prone to abortions (Sidi-Boumedine et al., 2010). Nevertheless, as the number of C. burnetii is likely to be lower in milk, colostrums and faeces than in abortion material, PCR can be used for analysis of this large diversity of samples. Before performing the PCR, biological samples can be inactivated, for ensuring the safety of laboratory personnel, by heating at 90°C for 30–60 minutes, depending of the samples’ nature, their size or their weight. This technique can be performed in suitably equipped laboratories using primers derived from various targets, such as multicopy insertion sequence IS1111 (accession number M80806), the most largely employed (Berri et al., 2000). The use of these primers for the amplification of this sequence allows the sensitivity of the test to be increased and this because of the presence of several copies in the Coxiella genomes. The other target genes reported to be used in the PCR for specific C. burnetii identification are: superoxide dismutase (socB) gene (accession number M74242); com1 encoding a 27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat shock proteins (hpaA and hpaB) (accession number M20482); isocitrate dehydrogenase (icd) (accession number AF069035); and macrophage infectivity potentiator protein (cbmip) (accession number U14170).

The real-time PCR provides an additional means of detection and quantification (Kim et al., 2005; Klee et al., 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used: IS1111; IS30; com1; and icd. To quantify the bacteria in biological samples using the real-time PCR, it is recommended to amplify a unique and specific sequence. Indeed, recent data show that the number of the insertion sequence (IS1111) varied widely (between 7 and 110) depending on the isolate (Klee et al., 2006). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification when different strains are involved. It is nevertheless sufficiently informative for high quantities of bacteria (i.e. >10⁶ per vaginal swab) for abortive diagnosis (Sidi-Boumedine et al., 2010). Regarding complex matrices, the DNA eluates should be evaluated for their ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target).

Different primers and probes used in PCR can be obtained on the web site (http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q.htm#htoc22), regularly updated by the French Reference National Center for human Q fever. Ready-to-use kits are commercially available and can detect the bacteria in various sample types. However, there is an urgent need for the development of a molecular method for the assessment of bacterial
viability, especially in milk samples and environmental samples. The development of a multiplex PCR or microarray constitutes other techniques for screening all infectious abortive agents in a single assay.

d) Genotyping methods

Q fever epidemiology is complex as represented by its wide host range, its capacity to resist in the environment and its multifactorial air-borne transmission. Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, assessment of discriminatory typing methods for molecular epidemiology are in progress (Chmielewski et al., 2009; Klaassen et al., 2009; Sidi-Boumdedine et al., 2009). These tools are very useful for epidemiological investigation, particularly to clarify links regarding source of infection, for better understanding the epidemiological emerging factors, and to a lesser extent for evaluating control measures.

Several typing methods have been used for the characterisation of C. burnetii strains, such as restriction endonuclease of genomic DNA (Hendrix et al., 1991), PFGE (pulsed-field gel electrophoresis) (Heinzen et al., 1990; Jager et al., 1998), and sequence and/or PCR-RFLP (restriction fragment length polymorphism) analysis of icd, com1 and mucZ genes. More recently, two PCR-based typing methods have been described, MLVA (multi-locus variable number of tandem repeats analysis) (Arricau-Bouvery et al., 2006; Svraka et al., 2006) and multiphaser sequence typing (MST) (Glazunova et al., 2005) that permit the typing of C. burnetii without the need for isolation of the organism.

To date, MLVA and MST are considered to be the most discriminating methods for C. burnetii, allowing the identification of up to 36 distinct genotypes. Moreover, databases have been established http://minisatellites.u-psud.fr/MLVAnet/ and http://ifr48.timone.univ-mrs.fr, respectively for MLVA and MST. The availability of such databases allows interlaboratory comparisons to be made easily and this will lead to a better understanding of the propagation of the C. burnetii isolates. Furthermore, their use in the characterisation of field samples or isolates is increasing (Chmielewski et al., 2009; Klaassen et al., 2009) and efforts to produce a standardised scheme for MLVA (based on common decisions for allele calling and marker panels to be used) are in progress and should be available in the near future (Sidi-Boumdedine et al., 2009).

2. Serological tests

Among the various techniques that can be employed, the three most often used are: the indirect immunofluorescence assay (IFA), the ELISA and the complement fixation test (CFT). Three older serological tests are no longer used in routine diagnosis: the microagglutination technique, the capillary agglutination test and the indirect haemolysis test. Overall, ELISAs are preferred for practical reasons. Currently, no IFA is commercially available for ruminants. The production of the commercial antigen for CFT will probably be interrupted in the future. Numerous reports showed a weak sensitivity of CFT compared with other methods (EFSA, 2010; Kittelberger et al., 2009; Rouset et al., 2007; 2009a). Serological tools allowing specific antibodies detection in sera from different animal species (not only ruminants) should be developed (Jasper et al., 1994; Soliman et al., 1992).

The presence of specific IgG anti-C. burnetii antibodies provides evidence of a recent infection as well as a past exposure. Serological assays are suitable for screening herds or flocks, but the interpretation at the individual animal level is not possible. Indeed, a significant proportion of animals shedding C. burnetii bacteria and even some Q fever aborted animals are found to be seronegative (Arricau-Bouvery et al. 2005; Gatteo et al., 2007; Rouset et al., 2007, 2009a). Serological cut-off values used to diagnose Q fever are given by the suppliers; It was proposed that interpretation of the results requires at least six ewes or goats and ten cows (those aborted in priority). Both serological responses and bacterial evidence are often necessary for establishing the presence of the infection.

a) Indirect immunofluorescence assay (IFA)

In human medicine, the IFA adapted as a micro-immunofluorescence technique is the current method for the serodiagnosis of Q fever (Tissot-Dupont et al., 1994). The procedure can be adapted to perform an immunoperoxidase assay. Briefly, both phase I and phase II C. burnetii antigens are used; phase II antigen is obtained by growing C. burnetii Nine Mile reference strain in cell culture, while phase I antigen is obtained from the spleens of laboratory animals inoculated with phase II C. burnetii in cell cultures. A few phase I cells may still be present in the phase II population and can be selected and propagated within animals. Antigen is diluted, dropped on to the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection, acute and chronic, have different serological profiles: during acute Q fever, IgG antibodies are elevated against phase II only whereas during chronic Q fever, high levels of IgG antibodies to both phase I and II of the bacteria are observed (Tissot-Dupont et al., 1994). In addition, antigen-spot slide wells may be purchased from a supplier providing the phase II form, or the phase I and II forms of
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C. burnetii. These can be adapted by replacing the human conjugate by a conjugate adapted to the animal species.

Twofold dilutions of the serum under test are placed on immunofluorescence slides with wells previously coated with one or two antigens. If specific antibodies are present, they are fixed by the antigen on the slide. The complex is then detected by examination with a fluorescence microscope following the addition of the fluorescent conjugate recognising the species-specific immunoglobulins.

- **Materials and reagents**
  
  Microscope equipped for fluorescence, humidified incubator, washing basin.

  Slides suitable for the antigen are necessary. The latter may be either prepared in the laboratory or purchased from a supplier (see above). The method described is adapted from the BioMérieux kit, and is given as an example. Ready-to-use slides contain 12 wells per slide, each of 7 mm diameter, coated with phase II antigen obtained from culture on Vero cells and can be stored at 4°C or –20°C.

  Concentrated fluorescent conjugate, to be diluted when required with PBS + 1% Evans blue at the dilution recommended by the manufacturer.

  PBS, buffered glycerine, Evans blue dye 1% solution.

- **Test procedure**
  
  i) Inactivate the sera under test for 30 minutes at 56°C, then dilute serially from 1/40 to 1/640 in PBS.
  
  ii) Allow the previously antigen-coated slides to warm to room temperature. Do not touch the wells.
  
  iii) Add 20 µl of each serum dilution to the wells. Add negative and positive control sera. To one well, add 20 µl of PBS to serve as antigen control.
  
  iv) Incubate in a humid chamber for 30 minutes at 37°C. Wash the slide twice with PBS for 10 minutes each. Rinse with distilled water and air dry.
  
  v) Add to the wells, including the controls, 20 µl of the conjugate directed against the appropriate species (e.g. FITC-labelled rabbit anti-goat or anti-sheep IgG[H+L]), freshly diluted in PBS + Evans blue. Incubate in a humid chamber for 30 minutes at 37°C. Rinse with distilled water and air-dry. Add a few drops of buffered glycerine and cover with a cover-slip. Examine under a fluorescence microscope at magnification ×400 or more.

- **Interpretation of the results**
  
  A positive reaction will consist of small brilliant points against a dark background. Verify that the conjugate by itself and the negative control serum give a negative result (absence of small brilliant points). Nonspecific fluorescence usually takes the form of spots of irregular shape. The positive control must give the known titre with ± one dilution.

  Values for interpretations have to be validated.

b) **Complement fixation test (CFT)**

This cold fixation micromethod of the type developed by Kolmer is performed with 96-well U-bottomed microtitre plates. The test detects complement-fixing antibodies present in the serum. The CFT is specific but less sensitive than the ELISA or IFA (Kittelberger et al., 2009; Rousset et al., 2007; 2009a). The CFT is still used by laboratories in many countries. This method often uses antigen in phase II prepared from a mixture of two strains (Nine Mile and Henzerling)\(^2\) or antigen in phase I and II mixture prepared from Nine Mile strain\(^3\).

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and sheep erythrocytes, sensitised by the anti-sheep erythrocyte serum, are added. Fixation of the complement by the antigen/antibody complex during the first step does not permit lysis of erythrocytes; in contrast, if there are no complement-fixing antibodies, the complement induces the lysis of the sensitised erythrocytes. Then the haemolysis rate is inversely proportional to the level of specific antibodies present in the sample serum.

- **Reagents**
  
  Veronal/calcium/magnesium buffer (VB), pH 7.2.

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2 Dade Behring, Marburg, Germany.
3 Virion, Zürich, Switzerland.
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The haemolytic system: a mixture of equal parts of a 2% suspension of sheep erythrocytes in VB; and haemolytic serum diluted to a specified titre in VB.

Complement: commercial freeze-dried preparation or fresh guinea-pig serum.

Antigen: use commercial antigens at the titre recommended by the manufacturer if the antigen titration is performed with this method.

Positive and negative control sera.

- Pretitrations
  i) Dilute the sheep erythrocytes to a final concentration of 2% in VB.
  ii) Titrate the haemolytic serum on a microplate: 25 µl of complement at a known haemolytic concentration (e.g. 1/30); 25 µl of increasing dilutions of haemolytic serum + 2% sheep erythrocytes. Include controls without complement. Incubate for 30 minutes at 37°C. Establish the dilution equivalent to 2 haemolytic units.
  iii) Dilute the antigen as recommended by the manufacturer. The antigen may also be titrated: make increasing dilutions of antigen (25 µl horizontally) and a positive serum of known titre (25 µl, vertically). Add 25 µl of the suspension of sensitised erythrocytes and incubate for 30 minutes at 37°C. The antigen titre is the highest dilution producing a positive reaction with the highest serum dilution. Verify the absence of anticomplementary activity of the antigen at different dilutions.
  iv) Titrate the complement on a microplate: serially dilute the complement or guinea-pig serum in VB, for example from 1/15 to 1/200. To each well containing 25 µl of this dilution, add 25 µl of antigen and 25 µl of the haemolytic system. Incubate for 30 minutes at 37°C and establish the dilution equivalent to 2 haemolytic units of complement.

- Test procedure
  i) Make twofold dilutions of decomplemented sample sera from 1/10 to 1/320 in six wells and in four additional wells at dilutions from 1/10 to 1/80 to detect anticomplementary activity (25 µl per well).
  ii) Add 25 µl of diluted antigen or 25 µl of VB to control serum wells.
  iii) Add 25 µl diluted complement to all wells. Cover the plate with plastic adhesive film and incubate for 18 hours at 4°C.
  iv) Remove the plates from the refrigerator, allow them to reach room temperature, and add 25 µl of freshly prepared haemolytic system. Incubate at 37°C for 30 minutes. Centrifuge the plates at 500 g for 5 minutes at 4°C. Examine the controls and read the results.

- Interpretation of the results
  Titres between 1/10 and 1/40 are characteristic of a latent infection. Titres of 1/80 or above in one or more sera from a group of from five to ten animals reveal an active phase of the infection.

c) Enzyme-linked immunosorbent assay (ELISA)

This technique has a high sensitivity and a good specificity (Kittelberger et al., 2009; Rousset et al., 2007; 2009a). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA is preferred to IFA and CFT, particularly for veterinary diagnosis, because it is convenient for large-scale screening and, as it is a reliable technique for demonstrating C. burnetii antibody in various animal species (Jaspers et al., 1994; Soliman et al., 1992). Ready-to-use kits are commercially available and can detect anti-phase II antibodies or both anti-phase I and II antibodies.

Wells of the microplate are coated with C. burnetii whole-cell inactivated antigen. Diluted serum samples are added to the wells and react to antigens bound to the solid support. Unbound material is removed by washing after a suitable incubation period. Conjugate (horseradish-peroxidase-labelled anti-ruminant Ig) reacts with specific antibodies bound to the antigen. Unreacted conjugate is removed by washing after a suitable incubation period. Enzyme substrate is added. The rate of conversion of substrate is proportional to the amount of bound antibodies. The reaction is terminated after a suitable time and the amount of colour development is measured spectrophotometrically.

- Materials and reagents
  Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with Q fever antigen; microplate reader (spectrophotometer; 405 and/or 450 and/or 492 nm filters); 37°C humidified incubator; 8- and 12-channel pipettes with disposable plastic tips; microplate shaker (optional).
Positive and negative control sera; conjugate (ruminant anti-immunoglobulin labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2’-azonio-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

- **Test procedure**
  i) Dilute the serum samples, including control sera, to the appropriated dilution (usually 1/100) and distribute 0.1 ml per well in duplicate. Control sera are positive and negative sera provided by the manufacturer and an internal positive reference serum from the laboratory in order to compare the titres between different tests.
  ii) Cover the plate with a lid and incubate at room temperature for 30–90 minutes. Empty out the contents and wash three times in washing solution at room temperature.
  iii) Add the appropriate dilution of freshly prepared conjugate to the wells (0.1 ml per well).
  iv) Cover each plate and incubate as in step ii. Wash again three times.
  v) Add 0.1 ml of freshly prepared chromogen substrate solution to each well (for example: TMB in 0.1 M acetic acid and 30% H₂O₂ solution [0.2 µl/ml]; or 0.25 mM ABTS in citrate phosphate buffer, pH 5.0, and 30% H₂O₂ solution [0.1 µl/ml]).
  vi) Shake the plate; incubate according to the manufacturer recommendations, stop the reaction by adding stopping solution to each well, e.g. 0.05 ml 2 M sulphuric acid for TMB or 10% sodium dodecyl sulphate for ABTS.
  vii) Read the absorbance of each well with the microplate reader at 405 nm (ABTS) or 450 nm (TMB). The absorbance values will be used to calculate the results.

- **Interpretation of the results**
  For commercial kits, interpretations and values are provided with the kit. For example: calculate the mean absorbance (Ab) of the sample serum and of the positive (Ab_{pos}) and negative (Ab_{neg}) control sera, and for each serum, calculate the percentage

\[
\frac{\text{Ab}_{\text{pos}} - \text{Ab}_{\text{neg}}}{\text{Ab}_{\text{pos}} - \text{Ab}_{\text{neg}}} \times 100
\]

Interpret the results as follows:
- Ab <30% negative serum
- Ab 30–40% doubtful serum
- Ab >40% positive serum

C. REQUIREMENTS FOR DIAGNOSTIC BIOLOGICALS AND VACCINES

1. **Production of Coxiella burnetii antigen**

Growth and purification of *C. burnetii* should only be performed in facilities that meet the requirements outlined in Chapter 1.1.2. Precautions assigned to Containment Group 3 pathogens must be taken either for phase I or phase II *C. burnetii*. As seen in the Nine Mile reference strain, the LPS phase variation could be accompanied by a permanent chromosomal deletion that makes impossible a reversion from phase II to phase I. However, a variant of the Australia QD isolate producing truncated LPS had no detectable large deletion. The molecular changes that occurred in LPS phase variation are not clearly defined. Even with extensive repeated passage in non-immunologically competent hosts (cultural cells, embryonated eggs), the majority of isolates are non-clonal as growth from a single colony is difficult to establish (Samuel & Hendrix, 2009). The risk of aerosols must be taken into account at all stages when working with viable *C. burnetii*. Sustained serological monitoring of Q fever should be carried out for laboratory personnel.

2. **Diagnostic biologicals**

An example of *C. burnetii* preparation for IFA serological diagnostic based on phase II and phase I antigens is given below, but other modified protocols are used around the world (Samuel *et al.*, 2009). Significant amounts of *C. burnetii* (>10^{10} bacteria) can be obtained in 2 weeks in embryonated eggs or cell cultures. Purification of bacteria from host material includes differential centrifugations and takes 1 or 2 days. An infection on mice can require 7–14 days.
Phase II C. burnetii Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm² culture flasks at 35°C under 5% CO₂ with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of intracellular vacuoles or by Gimenez-stained cells collected from the supernatants of the flasks. Recent specific real-time quantitative PCR has been extremely valuable in routine monitoring. When a heavy C. burnetii infection is seen, the supernatants of 15 flasks are individually pelleted by centrifugation (5000 g, 15 minutes) resuspended in 1 ml of PBS with 0.1% formaldehyde and incubated for 24 hours at 4°C. After pooling, the remaining cells are broken by sonication. Cellular debris is removed by two successive centrifugation steps (100 g, 10 minutes each). The 15 ml suspension is then centrifuged through 20 ml of PBS with 25% sucrose (6000 g, 30 minutes, without a break). The resulting pellet is washed three times in PBS (6000 g, 10 minutes), resuspended in the smallest possible volume of sterile distilled water, and adjusted to 2 mg/ml by UV spectroscopy. An antibacterial preservative, such as sodium azide at a final dilution of 0.1% or thiomersal at 0.01%, is added. Antigen prepared in this manner is frozen at −20°C.

In order to obtain phase I antigen, mice are inoculated with C. burnetii grown in cells (mainly in phase II). Nine days after infection, the spleens are removed. Each one is ground in 7.5 ml MEM, and inoculated into three 75 cm² culture flasks containing L929 or Vero cell monolayers (2.5 ml per flask). Amplification of phase I C. burnetii is conducted for 4 weeks, with a culture medium change once a week. The infected cells are then harvested and the bacteria are purified as described above (mainly in phase I).

Antigen production can also be performed by culture of C. burnetii in SPF embryonated eggs. At 6–7 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after 10–15 days of incubation. Infected yolk sacs have a characteristic straw-yellow colour. Uninfected yolk sacs are orange in colour and have a viscous consistency. Any embryos that die before 5 days of incubation are discarded. The strain used for egg inoculation is a 1/100 homogenate of yolk sac in PBS containing penicillin (500 International Units/ml) and streptomycin (0.5 mg/ml). The yolk sacs are pooled and homogenised with three parts PBS. The suspension is inactivated with 1.6% formaldehyde for 24 hours at 37°C. The lipid supernatant fluid is discarded. The suspension is then centrifuged at moderate speed (~500 g) for 30 minutes. After removal of the supernatant fluid, more PBS is added and centrifugation is repeated. The final suspension is diluted with PBS. Sodium azide or thiomersal is added as an antibacterial preservative. The abundance of C. burnetii and the absence of bacterial contaminants in homogenates of yolk sacs suspended in PBS are verified by microscopic examination of a smear on a microscope slide, stained by Stamp’s method. In order to obtain phase I antigen, C. burnetii recovered from spleen material of infected laboratory animals can be propagated, as ground spleen extracts are subsequently transferred in the yolk sacs, given that the amount of phase I cells is still high until the sixth egg passage.

Titration of antigen with at least three different known sera (with high, moderate and low titres, respectively) is sufficient to determine the appropriate dilution for further immunoffluorescence tests.

3. Vaccine

The protective antigen is composed of purified particles in phase I with the nontruncated phase I LPS structure. In some countries, vaccination is practised for occupationally exposed people, such as abattoir workers, veterinarians and laboratory personnel. A vaccine inactivated by formaldehyde (Q-VAX, CSL Ltd, Australia), prepared from the Henzerling strain of phase I C. burnetii, received the approval of the Australian authorities in 1989. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or had been exposed to C. burnetii prior to immunisation.

Several vaccines have been developed against animal Q fever. Results converge today towards the use of a phase I vaccine that is helpful against Q fever in combination with other control measures. An inactivated phase I vaccine is commercially available (Coxevac, CEVA, Hungary) for vaccination of ruminants. A review on Q fever in Slovakia suggests that the decrease in the occurrence of human and animal Q fever could be the result of the large-scale vaccination of cattle that was carried out there over a 10 year period, together with improved veterinary control of domestic animal transport within the country (Serbezov et al., 1999). In the Netherlands, a large vaccination programme has been implemented in goat and sheep farms, accompanied by the controlled processing of manure and checks on animal transports, but it is not clear yet whether bacterial shedding by animals is prevented or at least reduced by vaccination. Controlling the epidemic is difficult and can be compromised by the prolonged stability of the bacterium in the environment and the possible role of animal species other than small ruminants (EFSA, 2010).

This vaccine consists of highly purified whole cells prepared from Nine Mile strain in the phase I (egg passage 3 to egg passage 5) and inactivated by formaldehyde. No adjuvant is used. Recently, a French study demonstrated the efficacy of this vaccine through experimental vaccination and challenge of pregnant goats: the vaccine prevented abortion and shedding in milk, and decreased considerably the shedding in the vaginal secretions and faeces (Arricau-Bouvery et al., 2005). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.
In the case of vaccination on already infected animals, some authors believe that it is preferable to select seronegative herds or animals for immunisation, and to continue vaccination over several years in young animals (Krauss, 1989). First follow-up studies on shedding herds or flocks show a contribution of the vaccination against the infection incidence and the shedding levels (Guatteo et al., 2008; Rousset et al., 2009b). Repeated annual vaccination, particularly of young animals, is recommended. However, the duration of immunity is not defined. The development of serological tools distinguishing between infected and vaccinated ruminants (DIVA) would be helpful. To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

REFERENCES


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