

CHAPTER 2.1.16.

Q FEVER

SUMMARY

Definition of the disease: Query (Q) fever (or Coxiellosis) is a zoonosis that occurs in most countries. Humans generally acquire infection through air-borne 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. All manipulations with potentially infected or contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis

Description of the disease: In humans, the disease exhibits a large polymorphism. Q fever occurs either as 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 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 suggest 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 (spleen, liver, lung or stomach content). For investigation of bacterial shedding, samples can be taken from vagina, milk and colostrum.

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 only presumptive evidence of Q fever and confirmatory tests should be carried out.

Demonstration of the agent by immunohistochemical staining, by in-situ hybridisation or by polymerase chain reaction (PCR) is more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry 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 are becoming widely used, 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. Moreover, SNP genotyping (single nucleotide polymorphism) has been recently described.

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.

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.

Requirements for vaccines: 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 is commercially available. Repeated annual vaccination, particularly of young animals, is recommended in at-risk areas.

A. INTRODUCTION

1. Definition of the disease and transmission routes

Q fever (or Coxiellosis) is widely distributed throughout the world with the exception of New Zealand. The causal agent, *Coxiella burnetii*, is present in virtually all animal kingdoms, including arthropods, but the disease affects mostly humans, cattle, sheep and goats (EFSA, 2010; Lang, 1990). Domestic ruminants are considered the main reservoirs of *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 exposure in the vicinity of infected animals, their reproductive tissues or other animal products, like wool (ECDC, 2010). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, but no good evidence has shown significant transmission to humans by food. Q fever also seems 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.

2. Description of the causal pathogen

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 of 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). 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 rearrangement of syntenic blocks (Beare *et al.*, 2009). This genomic plasticity might contribute to different phenotypes and is of great interest for genotyping methods (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form (Heinzen *et al.*, 1999; Minnick & Raghavan, 2012). 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 (Heinzen *et al.*, 1999; Minnick & Raghavan, 2012). The SDC and SCV represent the small morphological variants of the bacteria 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 (EFSA, 2010; O'Neil *et al.*, 2013).

3. Description of the disease in humans

Q fever is a zoonosis. In humans, the infection can manifest as an acute, chronic or subclinical form (Anderson *et al.*, 2013; ECDC, 2010). Diagnosis and the treatment is often delayed because of the various and nonspecific clinical

expressions. The acute forms commonly range from a self-limiting flu-like syndrome to pneumonia or granulomatous hepatitis that may require hospitalisation. The main clinical manifestations of chronic Q fever are endocarditis, valvular, vascular or aneurismal infections, hepatitis, pneumonia 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 lead to premature birth, growth restriction, spontaneous abortion or fetal death. Overall, the chronic disease is more likely to develop in individuals with high risk factors (e.g. immuno-compromised or valvulopathies). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. Q fever affects all ages but is mostly reported in those aged 30–60 years. Awareness for Q fever is increased during human outbreaks, which are generally temporary and rarely comprise more than 300 acute cases. However, the largest community outbreaks ever reported emerged in 2007 in the Netherlands. In subsequent years, the peak incidence from February to September has increased and the geographical area has expanded progressively. The country reported more than 4000 human cases with a hospitalisation rate of 20%, and it is expected to result in more cases of chronic Q fever among risk groups in the coming years (ECDC, 2010; EFSA, 2010). The losses caused by this epidemic have been estimated to be approximately 307 million euros (van Asseldonk *et al.*, 2013).

4. Description of the disease in animals

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 (EFSA, 2010; 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 rely on 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 to evaluate the infectious risk in the environment (EFSA, 2010; Kersh, 2010). For now, the lack of knowledge of 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). In herds or flocks experiencing abortion problems caused by *C. burnetii*, most animals may be shedding massive numbers of bacteria whether they have aborted or not. The global quantities are thus clearly higher than in subclinically infected herds/flocks. At the parturitions following an abortion storm, higher bacterial discharges were measured among the primiparous compared with the other females (de Cremoux *et al.*, 2012; Guatteo *et al.*, 2008; Rousset *et al.*, 2009b). Moreover, the shedding may persist for several months, following either an intermittent or a continuous kinetic pattern. Animals with continuous shedding patterns might be heavy shedders. These latter animals seem mostly to exhibit a highly-seropositive serological profile (Guatteo *et al.*, 2007). Importantly, shedding and serological responses are associated at the group level but not at the individual level.

5. Differential diagnosis in ruminants

Diagnosis of Q fever in ruminants, including differentiating it from other causes of abortion, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results (Lang, 1990). At present, no gold standard technique is available, but direct detection and quantification by PCR and serological ELISA (enzyme-linked immunosorbent assay) should be considered as the methods of choice for clinical diagnosis (Niemczuk *et al.*, 2014; Sidi-Boumedine *et al.*, 2010). Proposals have been 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). Q fever diagnostic tests are also required for epidemiological surveys of at risk and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for exchanges between herds or flocks. Thus, efforts are encouraged both for the validation of the methods for each purpose given (see Table 1), and for development of reference materials for quality control, proficiency and harmonisation purposes (see Chapter 1.1.6 *Principles and methods of validation of diagnostic assays for infectious diseases*).

6. Zoonotic risk and biosafety requirements

Concerns about the risks posed by Q fever have been raised in Europe, where the European Commission requested scientific advice and risk assessment for humans as well as animals (ECDC, 2010; EFSA, 2010). The main conclusions were that the necessary actions to stop an outbreak must be carried out by health authorities together with veterinary authorities at the national and the local levels. The overall impact of *C. burnetii* infection on public health 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. Among these options, 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 ticks could be used. Moreover, the culling of pregnant animals, a temporary breeding ban, identifying and culling shedding herds or flocks and controlling animal movements may have a role in the face of human outbreaks.

Because of its ability to cause incapacitating disease in large groups of people, its resistance in the environment as a pseudo-spore and its natural spread as an aerosol, *C. burnetii* is currently considered a potential agent of bioterrorism and is classified by the Centers for Disease Control and Prevention as a group B biological agent (Drancourt & Raoult, 2005; Kersh *et al.*, 2010). Regarding biosafety and biosecurity, *C. burnetii* is extremely hazardous to humans. Q fever is thus a recognised occupational zoonosis. All laboratory manipulations with live cultures or potentially infected/contaminated material must be performed at an appropriate biosafety and containment level determined by biorisk analysis (see Chapter 1.1.4 *Biosafety and biosecurity: Standard for managing biological risk in the veterinary laboratory and animal facilities*). Precautions must be taken with both phase I and phase II *C. burnetii*. Even if the phase II bacteria are considered avirulent, phase I bacteria may be present in a phase II preparation. In particular, it is advised to wear full coverage protective clothing and a class 3 filtering face piece (FFP3) respiratory protection and to handle infectious and potentially infectious material with two pairs of gloves, inside a biological safety cabinet (BSC). Centrifugation of infected materials must be carried out in closed containers placed in sealed safety cups, or in rotors that are unloaded in a BSC. The use of needles, syringes, and other sharp objects should be strictly limited. After all manipulations where there is a known or potential exposure to aerosols of viable *C. burnetii*, showers must be taken when leaving the laboratory. Sporicidal disinfectants are recommended. An appropriate serological survey would help when following up the evolution of immune status of the laboratory personnel. In some countries, vaccination is practised for occupationally exposed people, such as abattoir workers, veterinarians and laboratory personnel. Phase I vaccines are effective, but vaccination is contraindicated for individuals who had seroconverted or had been exposed to *C. burnetii* prior to immunisation.

B. DIAGNOSTIC TECHNIQUES

Table 1. Test methods available for the diagnosis of Q fever and their purpose

Method	Purpose					
	Population freedom from infection	Individual animal freedom from infection prior to movement	Contribute to eradication policies	Confirmation of clinical cases	Prevalence of infection – surveillance	Immune status in individual animals or populations post-vaccination
Agent identification						
PCR	+++	n/a	+++	+++	++	+ ¹
Culture	+	n/a	+	–	+	–
Staining	+	n/a	+	+	+	–
Genotyping	n/a	n/a	n/a	n/a	++	n/a
Detection of immune response						
ELISA	+++	n/a	+++	++	+++	+++
IFA	++	n/a	++	++	++	++
CFT	–	n/a	–	++	+	+

Key: +++ = recommended method; ++ = suitable method; + = may be used in some situations, but cost, reliability, or other factors severely limits its application; – = not appropriate for this purpose; n/a = not applicable.
 Although not all of the tests listed as category +++ or ++ have undergone formal validation, their routine nature and the fact that they have been used widely without dubious results, makes them acceptable.
 PCR = polymerase chain reaction; ELISA = enzyme-linked immunosorbent assay;
 IFA = indirect immunofluorescence assay; CFT = complement fixation test.

¹ Confirmation of immune status should be accompanied by tests for the absence of vaginal shedding of the organism.

Clearly, a confirmed positive identification of *C. burnetii* from an individual animal would support a diagnosis. However as a general principle, the methods for the diagnosis of Q fever allow only an interpretation at the population level and not at the individual level. Moreover, laboratory test results should be interpreted in the context of herd or flock history (abortions, vaccination, movement and introduction, etc.).

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. Individual vaginal, milk or colostrum samples or milk from the tank can be taken for investigation of bacterial shedding. However, detection of shedders is still difficult to achieve as the shedding dynamics are not well known (de Cremoux *et al.*, 2012; EFSA, 2010; Guatteo *et al.*, 2007; Rousset *et al.*, 2009a). Indeed, the PCR cannot be relied on to determine the infection status because of the variability of shedding by animals (different shedding routes, potentially intermittent shedding). Serological analyses may be carried out using ELISA, indirect immunofluorescence assay (IFA) or complement fixation test (CFT). Several published works showed that the relative sensitivity is lowest for the CFT, but conversely it has a high specificity for the high levels of anti-*C. burnetii* antibodies generated in a Q fever aborted herd or flock (Emery *et al.*, 2014; Horigan *et al.*, 2011; Kittelberger *et al.*, 2009; Niemczuk *et al.*, 2014; Rousset *et al.*, 2007; 2009a). IFA has the disadvantage of being less reproducible between operators, and therefore between laboratories. Although the ELISA methods are, not fully validated and harmonised, they are robust and can be automated and are recommended for routine serological testing of animals for Q fever.

A serological survey is a good way to evaluate prevalence. The presence of specific 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 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 (de Cremoux *et al.*, 2012; Guatteo *et al.*, 2007; Rousset *et al.*, 2007, 2009a). Sampling should target a representative number of animals (in particular from different age categories). Sampling strategy should take into account the possibility of a low prevalence if no prevalence data are available in the studied area. Alternatively, testing bulk tank milk (BTM) or pooled individual samples (i.e. vaginal swabs or milk samples) can be used for prevalence estimation, but must be assessed in relationship to the intra-herd or intra-flock shedding prevalence. For example, PCR analyses of BTM have been performed every 2 months since 2009 in the Netherlands to monitor a herd or flock with proven clinical status.

The herd or flock status can be assessed serologically by ELISA investigation of all animals (or a significant sample). However, some discordant results can be observed using different ELISA kits (Horigan *et al.*, 2011). One option is to use at least three kits to determine the status of a serum. Available serological methods do not, unfortunately, distinguish between infected and vaccinated ruminants. Analysis by PCR in BTM or individual samples (vaginal swabs, preferably at the time of parturition) is required and may need to be repeated if the purpose is to determine free status. Individual animals can only be assessed as free if the herd or flock is free and has no serological or clinical history of Q fever. It is difficult to ensure that the status of the animal has not changed over time because transmission is by air.

PCR is the most reliable tool for the diagnosis of infectious abortions (EFSA, 2010; Sidi-Boumedine *et al.*, 2010). 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. Early detection of a Q fever storm of abortions in a herd or flock and implementation of the correct measures are essential to the handling of both farm-based and environmental route of infection. The confirmation of clinical cases should always include a differential investigation of major abortive agents and target at least two aborted animals. The interpretation of results is possible only at the group level. 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 abortion (or taken less than 8 days after) should be collected in order to limit the number of false-negative PCR results. Effectively, the vaginal bacterial load may decrease progressively after abortion or parturition. In the placenta, at least three cotyledons should be tested for *Coxiella* as colonisation can be heterogeneous. Bacterial quantification is helpful on vaginal or placental swabs, as high levels are more likely to be associated with clinical cases. The fetal organs may provide useful samples, but negative results can be questionable. Bacteria are likely to spread to different organs (spleen, lung, liver, stomach contents, etc.) depending on the progression of the infection, so that the absence in one organ cannot exclude its presence somewhere else.

When difficulties in interpretation of diagnostic results are encountered, an association with a positive serological result at the herd or flock level is useful. ELISA, IFA as well as CFT methods may be used for testing clinical cases but it is essential to define the test characteristics (sensitivity, specificity, accuracy around the cut-off, and reproducibility) under local conditions. Serological cut-off values used to diagnose Q fever are given by kit suppliers. Interpretation of the results requires samples from at least six ewes or goats and ten cows (with priority to those that have aborted).

Determination of the immune status of populations post-vaccination should be based on the more sensitive tests (ELISA or IFA); if possible, it should be linked to PCR testing of vaginal swabs collected at parturition. If the infection pressure is high, vaccination may only limit the magnitude of infection and shedding without inducing solid protection. The combination of seroconversion with the absence of vaginal shedding, at the following parturition, is indicative of immune protected status.

1. Identification of the agent

1.1. 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 (Samuel & Hendrix, 2009). To achieve isolation, a concentration above 10^5 bacteria per ml is recommended.

Embryonated chicken eggs: 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* and to monitor the process of isolation. Further passages may be required to obtain an isolate in pure culture.

Cell 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 (trypsinisation is not used). 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.

Laboratory 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. Experimentally infected rodents must be housed in appropriate biosafety and containment conditions, determined by biorisk analysis (see Chapter 1.1.4). 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.

1.2. Staining

In the case of an abortion having a suspected infectious origin, smears of placental cotyledon are prepared on microscope slides. Spleen, lung, liver and abomasal contents of the aborted fetus or vaginal discharge may be 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 diagnostic 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. The staining method is rapid. The limit of detection is high ($>10^5$ bacteria/ml) and appropriate to the clinical diagnostic purpose as high levels of bacteria are present in samples found positive. 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 of clinical cases 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 specific methods may be used as a confirmatory test. PCR methods are preferred.

1.3. 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). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (Raoult *et al.*, 1994). The method is an indirect immunofluorescence or immunoperoxidase assay using specific polyclonal *C. burnetii* 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 immunochemistry are commercially available.

Fluorescent *in-situ* hybridisation (FISH) 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. To ensure the safety of laboratory personnel, biological samples can be inactivated prior to carrying out the PCR by heating at 90°C for 30–60 minutes, depending on the nature of the samples, their size or their weight. The inactivation process must be checked and validated under local conditions, before use. The PCR 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 due to 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 (*sodB*) gene (accession number M74242); *com1* encoding a 27 kDa outer membrane protein (accession number AB004712); heat shock operon encoding two heat shock proteins (*hspA* and *hspB*) (accession number M20482); isocitrate dehydrogenase (*icd*) (accession number AF069035); and macrophage infectivity potentiator protein (*cbmip*) (accession number U14170). Some primer and probe sequences can be obtained on the web site of the French national reference centre for human Q fever².

The real-time PCR provides an additional means of detection and quantification (Klee *et al.*, 2006; Stemmler & Meyer, 2002). As with the conventional PCR, various target genes are used: for example 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 and accurate for high quantities of bacteria (i.e. $>10^4$ per vaginal swab) for abortive diagnosis (Sidi-Boumedine *et al.*, 2010). Regarding complex matrices, the DNA eluates should be evaluated for their

2 At: http://iffr48.timone.univ-mrs.fr/Fiches/Fievre_Q.html#toc22

ability to inhibit a PCR by adding an internal DNA control (such as a GAPDH sequence target) or an external control.

Ready-to-use kits are commercially available and can detect the bacteria in various sample types. Specific quantitative methods based on PCR kits have been validated for diagnosis of abortions according to a French standard for real-time PCR validation (Rousset *et al.*, 2012). An external reference material of quantified bacteria is available from the French national reference laboratory either for method validation or for a control chart to routinely monitor quality of the assays.

1.4. Genotyping methods

Q fever epidemiology is complex as represented by its wide host range, its capacity to persist 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 (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011). These tools are very useful for epidemiological investigation, particularly to clarify links regarding source of infection, for better understanding the epidemiological emerging factors, elucidating human outbreaks, 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, PFGE (pulsed-field gel electrophoresis), 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) and multispacer sequence typing (MST) that permit the typing of *C. burnetii* without the need for isolation of the organism. Research continues on the development of new tools, such as single nucleotide polymorphism (SNP), and the comparison of their discriminatory capabilities and informative value.

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://mlva.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 or to identify new emerging strains. Furthermore, their use in the characterisation of field samples or isolates is increasing and efforts to produce a standardised scheme for MLVA, based on common decisions for allele calling and marker panels to be used, should be encouraged so that they can be made available in the near future (Massung *et al.*, 2012; Sidi-Boumedine & Rousset, 2011; Sidi-Boumedine *et al.*, 2009).

2. Serological tests

2.1. Enzyme-linked immunosorbent assay (ELISA)

This technique has a high sensitivity and a good specificity according to comparative evaluations between methods (Emery *et al.*, 2014; Horigan *et al.*, 2011; Kittelberger *et al.*, 2009; Niemczuk *et al.*, 2014; 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 the most robust. Ready-to-use kits are commercially available and can detect mixtures of anti-phase I and II antibodies. The quality control for some ELISA kits was recently improved using an external reference material, available from the French national reference laboratory, showing the standardisation between kit batches.

Coxiella burnetii ELISA antigen is prepared by growth of standard strains in either embryonated hens' eggs or in cell culture, as described below under IFA. 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.

2.1.1. Materials and reagents

Microtitre plates with 96 flat-bottomed wells, freshly coated or previously coated with *C. burnetii* 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 or protein A/G labelled with peroxidase); tenfold concentration of diluent (PBS–Tween); distilled water; substrate or chromogen (TMB [tetramethylbenzidine], ABTS [2,2'-azino-bis-(3-ethylbenzo-thiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

2.1.2. Test procedure

- i) Dilute the serum samples, including control sera, to the appropriate dilution (1/100 or 1/400 depending on the kit used) 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.

2.1.3. 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

$$\frac{Ab - Ab_{neg}}{Ab_{pos} - Ab_{neg}} \times 100$$

Interpret the results as follows:

Ab < 30% negative serum
Ab > 30% positive serum

Prepare a control chart and estimate the measurement uncertainty around the cut-off in order to interpret results close to the cut-off.

2.2. 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. Antigen is diluted, dropped onto the wells of a glass microscope slide, allowed to dry, and fixed with acetone. The two forms of the infection in humans, 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 *C. burnetii*. These can be adapted by replacing the human conjugate by a conjugate adapted to the

animal species. Nevertheless, the interpretation as acute or chronic forms has not been validated for ruminants.

2.2.1. Antigen preparation

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 2–5 weeks in embryonated eggs or cell cultures. An infection in mice can require 7–14 days. Purification of bacteria from host material includes differential centrifugations and takes 1 or 2 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.

To obtain phase I antigen, mice are inoculated with *C. burnetii* grown in cells (mainly in phase II). The spleens are removed 9 days after infection. 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 and white spot patches. 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 immunofluorescence tests.

2.2.2. 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.

2.2.3. Test procedure

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 bound 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.

- i) Dilute the sera 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 x400 or more.

2.2.4. 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 \pm one dilution.

2.3. Complement fixation test (CFT)

As mentioned above the CFT is considered less sensitive than ELISA or IFA and its use for veterinary diagnosis has declined.

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. This method uses antigen in phase I and II mixture prepared from human or Nine Mile strain.

The reaction is done in two stages. Antigen and complement-fixing antibodies are first mixed, and incubated overnight at 4°C. The next day 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.

2.3.1. Reagents

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

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.

2.3.2. 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.

2.3.3. Test procedure

- i) Make twofold dilutions of inactivated 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.

2.3.4. 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. REQUIREMENTS FOR VACCINES

SECTION UNDER STUDY

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NB: There is an OIE Reference Laboratory for Q fever
(see Table in Part 4 of this *Terrestrial Manual* or consult the OIE Web site for the most up-to-date list:
<http://www.oie.int/en/our-scientific-expertise/reference-laboratories/list-of-laboratories/>).
Please contact the OIE Reference Laboratories for any further information on
diagnostic tests, reagents and vaccines for Q fever