

Q FEVER

SUMMARY

Query (Q) fever is a zoonosis that occurs in most countries. Humans acquire infection from animal reservoirs, especially from domestic ruminants. Q fever is a highly infectious disease, which is due to the proliferation of *Coxiella burnetii*, a small and pleomorphic bacterium measuring 0.3–1.5 μm long \times 0.2–0.4 μm wide. As an obligate intracellular bacterium, *C. burnetii* can be grown only in embryonated eggs or cell cultures or, when necessary, in inoculated laboratory animals. It occurs in two antigenic forms: the pathogenic phase I, found in infected animals or humans, and the avirulent phase II, obtained by repeated passages in embryonated eggs or in cell cultures. Because this microorganism is extremely hazardous, handling viable *C. burnetii* must be done in facilities that meet the OIE requirements for Containment Group 3 pathogens.

In humans, Q fever occurs in either an acute form (self-limiting febrile episode, pneumonia, hepatitis) or a severe chronic form (endocarditis) following an early infection that may be passed 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 some countries, a vaccine is available for professionally exposed population groups.

The signs of Q fever in cattle include abortion, dead or weak offspring, retained placenta, metritis, and infertility. In small ruminants, Q fever is often associated with sporadic abortions or outbreaks of abortions followed by recovery without complications. *Coxiella burnetii* infection persists for several years, and is probably life long. Sheep, goats and cows are mainly asymptomatic carriers, but can shed massive numbers of bacteria at parturition, and intermittently in various secretions and excreta. Domestic animals, such as dogs, cats, rabbits, birds, etc., are also susceptible to infection and should be considered as possible sources of infection for animals and humans.

Identification of the agent: For laboratory diagnosis, samples can be taken from the placenta, vaginal discharges, and liver, lung or stomach contents of aborted fetuses, and from milk, colostrum and faeces.

The bacteria can be visualised in stained tissue 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. This finding is presumptive evidence of Q fever, but coupled with serological tests, clinical findings and no other infectious abortive agents, it may be sufficient to establish a diagnosis of the disease at the flock or herd level.

To date, demonstration of the agent by immunohistology using specific antibodies or by polymerase chain reaction (PCR) has proven to be more specific and sensitive than classical staining methods. No specific antibodies for immunochemistry are commercially available, but PCR can be done in suitably equipped laboratories. PCR is considered a useful test for screening large numbers of samples and various types of samples. Furthermore, samples can be heat inactivated and therefore ensure the safety of laboratory personnel.

Coxiella burnetii can be isolated by inoculation of specimens into conventional cell cultures or embryonated chicken yolk sacs or laboratory animals. Inoculation of laboratory animals (guinea-pig, mouse, hamster) is helpful in cases requiring isolation from tissues contaminated with various microorganisms or in order to obtain phase I *Coxiella* antigens.

Serological tests: The diagnosis of Q fever often relies on serology. A number of tests can be used, particularly the indirect immunofluorescence test, the enzyme-linked immunosorbent assay, and the complement fixation test. Currently, commercially available tests allow the detection of anti-

C. burnetii phase II antibodies. The presence of specific IgG antibodies provides evidence of a recent *C. burnetii* infection or a past exposure.

Requirements for vaccines and diagnostic biologicals: Several inactivated vaccines against Q fever have been developed, but only vaccines containing or prepared from phase I *C. burnetii* should be considered protective. Repeated annual vaccination is recommended in heavily infected areas, particularly of young animals.

An inactivated phase I vaccine is commercially available in Slovakia. A recent study has proved its efficacy with regard to both abortion and *C. burnetii* shedding in experimentally vaccinated then challenged pregnant goats, but information on its safety is lacking.

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 (27, 30). 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 (60). *Coxiella burnetii* has now been placed in the Coxiellaceae family in the order Legionellales of the gamma subdivision of Proteobacteria (26). The complete genome sequencing of *C. burnetii* has been achieved recently and confirms its systematic position (47). Unlike rickettsiae, *C. burnetii* produces a small, dense, highly resistant spore-like form that is highly stable in the environment, a trait that is important for transmission (31). This ability has been attributed to the existence of *C. burnetii* developmental cycle variants: large-cell variants (LCV), small-cell variants (SCV), and small dense cells (SDC) (10, 17, 48). The SDC and SCV represent the forms of the bacteria likely to survive extracellularly as infectious particles. Another essential characteristic is that *C. burnetii* has two antigenic forms: the pathogenic phase I, isolated from infected animals or humans, and the avirulent phase II, obtained *in ovo* or *in vitro*. 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. The LPS phase variation is accompanied by a permanent chromosomal deletion that makes impossible cell reversion from phase II to phase I (56).

Q fever is a zoonosis. In humans, the infection has an acute, chronic and subclinical form (38). The acute forms commonly include a self-limiting febrile episode, pneumonia, and granulomatous hepatitis. The main clinical manifestation of chronic Q fever is endocarditis in patients with valvulopathies. In the absence of any appropriate antibiotic treatment, complications of the chronic form may be severe to fatal (11). Moreover, *C. burnetii* infection of pregnant women can provoke placentitis and often leads to premature birth, growth restriction, spontaneous abortion or fetal death (36). The infection is endemic in many areas leading to sporadic cases or explosive epidemics. Its incidence is probably greater than reported. The epidemiology of Q fever suggests that the infection is principally transmitted by inhalation of desiccated aerosol particles, and through contact with infected animals and their reproductive tissues (29, 30). Ingestion has been often suggested, particularly through the consumption of dairy products derived from contaminated raw milk, and even possibly following pasteurisation (16, 38, 53). Q fever is very rarely transmissible from person to person, although exposure during childbirth, through sexual transmission and blood transfusions, is possible (12, 32). In animals, in addition to respiratory and digestive routes, vertical transmission and sexual transmission can occur (25, 58). Arthropods, principally ticks, may be involved in Q fever transmission.

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, metritis and infertility (27). Nevertheless, serological responses in a given species or even the isolation of *C. burnetii* do not necessarily correlate with expression of the clinical disease (18, 27, 44). Indeed, the agent may persist in infected animals, be shed intermittently in milk, faeces, urine, and may be present in blood. The agent can be recovered in very high numbers in birth products (placenta, amniotic fluid and fetus), and non-pregnant animals are less of a risk. 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 (23, 29, 30, 51). Thus, infected domestic and wild animals usually shed the agent with no outward signs of disease, and should be regarded as possible sources of infection for humans.

Abortions in ruminants have usually been investigated to determine if Q fever is present as it may affect the health of humans or other animals. Diagnosis of Q fever, and other abortive diseases, traditionally has been made on the basis of microscopy on clinical samples, coupled with positive serological results, is usually adequate for this purpose (27, 41, 43). The Q fever diagnostic is also required for epidemiological surveys of 'at risk' and suspected flocks in limited areas (following recent outbreaks in humans or animals), or for export purpose. However, identification of *C. burnetii* shedders and asymptomatic carriers is not currently practised (4, 5, 7, 21).

Generally, when several animals are seropositive, an appropriate intervention is advised. The measures could be adapted according to the seroprevalence and epidemiological context. Proper pasteurisation of milk products must be ensured (53). The amount of agent can be reduced in the environment by regular cleaning and disinfection of animal facilities, with particular care of parturition areas, using 10% sodium hypochlorite. Pregnant animals must be kept in separate pens, and placentas and aborted fetuses must be removed quickly and disposed properly to avoid being ingested by dogs, cats or wildlife. Spreading manure from contaminated farms in suburban areas and gardens should be avoided. While the efficiency of fermentation by composting or decontamination with a chemical treatment (such as 0.4% calcium cyanamid or lime) for inactivating *C. burnetii* need to be evaluated, these methods are still recommended. In order to acquire and maintain *Coxiella*-free livestock, introduction of animals, regrouping of flocks, contacts with wildlife and infestation by ticks should be minimised. These methods may be effective in controlling disease but exposed animals may remain infected. Although vaccines for animal Q fever have been developed, there are not commercially available in most countries.

Finally, it is important to remember that *C. burnetii* is extremely hazardous to humans, and laboratory infections are common. Because of its ability to cause incapacitating disease in large groups of people, its low infectious dose, resistance in the environment, and aerosol route of transmission, *C. burnetii* is considered a potential agent of bioterrorism and is classified by the CDC as a group B agent (9). 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 microbiological 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 diagnosis (11, 41, 43). Samples should be collected from aborted fetuses, placenta and vaginal discharges soon after abortion or parturition. Milk from the tank, individual milk or colostrum, and faeces samples can also be taken.

a) Staining

In a case of an abortion suspected of being caused by an infection, smears are prepared on microscope slides of placental cotyledon (42). 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 (14, 35, 41, 42, 44). 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 in human diagnosis. 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 due to their small size (0.3–1.5 µm long × 0.2–0.4 µm wide), 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 *Chlamydophila abortus* or *Brucella* spp. However, using the same staining procedure, *Chlamydophila* 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*, *Chlamydophila 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 (43). When biological staining is inconclusive, one of the other methods (below) may be used as a confirmatory test.

b) Specific detection methods

Detection of *C. burnetii* in samples can also be achieved by specific immunodetection (capture enzyme-linked immunosorbent assay [ELISA], immunohistochemistry), or DNA amplification (8, 11, 55). Immunohistology may be used with paraffin-embedded tissues or on acetone-fixed smears (37). The method is an indirect immunofluorescence or immunoperoxidase assay using polyclonal *C. burnetii* antibodies, either a well characterised antiserum of human origin or a specific antiserum produced in laboratory animals (rabbit or guinea-pig). An anti-species (human, rabbit or guinea-pig) anti-IgG conjugate, labelled with fluorescein isothiocyanate (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.

Polymerase chain reaction (PCR) methods have been used successfully to detect *C. burnetii* DNA in cell cultures and biological samples. 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 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 (accession number M80806), the most popular employed (4, 19). 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* genome. The level of detection of the conventional trans-PCR is related to the analysed samples (for example 1–500 bacteria/ml of milk sample or 1 bacteria/mg of faeces). 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 (htpA and htpB) (accession number M20482); isocitrate dehydrogenase (icd) (accession number AF069035); and macrophage infectivity potentiator protein (cbmp) (accession number U14170). Different primers used in PCR can be obtained on the web site (http://ifr48.timone.univ-mrs.fr/Fiches/Fievre_Q), regularly updated by the French CNR.

The real-time PCR provides an additional means of detection and quantification (21, 22, 52). As the conventional PCR, various target genes are used: IS1111 (accession number M80806); com1 (accession number AB004712); and isocitrate dehydrogenase (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 (IS) varied widely (between 7 and 110) depending of the isolates (22). Whereas the use of this sequence could increase the sensitivity of the test, it may not be accurate for quantification.

Ready-to-use kits are commercially available and can detect the bacteria in several samples. However, there is an urgent need for the development of a molecular method for the assessment of bacterial viability, especially in milk samples. The development of a multiplex PCR constitutes another technique for screening all infectious abortive agents.

c) 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 (18). 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 5-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 be used to confirm 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 culture¹, has been adapted for isolating strictly or facultatively intracellular bacteria, including *C. burnetii*. Such a method was described for *C. burnetii* in 1990 (11, 39). Suspensions of samples are inoculated into human embryonic lung (HEL) fibroblasts grown on a 1 cm² cover-slip within a shell vial. 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 HEL 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 anti-species conjugated to 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 (50).

With heavily multi-contaminated samples, such as placentas, vaginal discharges, faeces, or milk, the inoculation of laboratory animals may be necessary. Biocontainment level 3 requirements are recommended for holding experimentally infected rodents (see Chapter 1.1.2). Mice and guinea-pigs are the most

1 Sterilin, Bibby Sterilin Ltd, Stone, Staffordshire ST15 05A, United Kingdom.

appropriate laboratory animals for this purpose (45). Following intraperitoneal inoculation with a dose of 0.5 ml per animal, body temperature and antibody status are monitored. This method should always 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* is done using impressions and staining of the collected spleens. Alternatively, PCR may be performed on spleens systematically collected 7–9 days post-inoculation (8).

Although characterisation of isolates seems necessary for understanding the varying epidemiology of Q fever in different geographical areas, no discriminatory typing methods are available currently. For this purpose, efforts should focus on genetic typing methods. High resolution molecular typing methods that are currently being developed and evaluated offer promise of mapping progression of clones and contact traceback studies. Studies using multi-spacer typing (15) and variable number tandem repeat typing (2, 54) have demonstrated high levels of resolution among *C. burnetii* isolates. Techniques for genotyping *C. burnetii* have been described.

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 (CF) test (11). Three older serological tests are no longer used in routine diagnosis: the microagglutination technique, the capillary agglutination test and the indirect haemolysis test. A high-density particle agglutination (HDP) test has been evaluated (33). Serological assays are suitable for screening herds, but interpretation at the individual animal level can be difficult. Indeed, animals may remain seropositive for several years following an acute infection, some animals may shed *C. burnetii* and pose a risk for infection prior to the development of antibodies, and some infected animals seem not to seroconvert (1, 6, 8). Serological cut-off titres used to diagnose Q fever are given below; interpretation of the results requires at least ten animals (aborted or not). Both serological responses and bacterial evidence are necessary for establishing the presence of the infection.

a) Indirect immunofluorescence test

In human medicine, the IFA adapted as a microimmunofluorescence technique is the reference method for the serodiagnosis of Q fever (11, 57). The procedure can be adapted to perform an immunoperoxidase assay. Some commercial CF test antigens are suitable, but antigens prepared for the diagnosis in humans are preferred (11). This method of preparation has been demonstrated to yield antigens with the highest sensitivity for *C. burnetii* antibody detection. 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 (57). 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.

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 immunoglobulins.

o Antigen preparation

Antigen should only be prepared in facilities that meet the requirements for Containment Group 3 pathogens as outlined in Chapter 1.1.2 of this *Terrestrial Manual*.

Phase II *C. burnetii* Nine Mile are grown in confluent layers of Vero or L929 cells in 150 cm² culture flasks with minimal essential medium (MEM) supplemented with 2 mM L-glutamine and 4% fetal bovine serum. The infection is monitored by microscopic examination of Gimenez-stained cells scraped from the bottoms of the flasks. 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* Nine Mile 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 5–6 days of age, the microorganism is inoculated into the yolk sac of the embryonated eggs, which are harvested after death of the embryo at 12–15 days. 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 between 5 and 10 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 ($\sim 500\text{ g}$) 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 yolk passage (EP6).

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

o **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.

o **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 $\times 400$ or more.

o **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.

The reaction is considered to be positive if there is obvious immunofluorescence at the 1/160 dilution and upwards. In human medicine, this method is used to determine antibodies against phases I and II in the IgG,

IgM, and IgA fractions allowing acute and chronic Q fever to be differentiated. Rheumatoid factor absorbant is used for remove IgG before the determination of IgM and IgA. Screening of the sera is performed with phase II antigen, and positive sera are tested subsequently for the presence of the different classes of Ig directed against phases I and II antigens. However, neither phases I and II antibody responses nor Ig classes responses have been well studied in domestic animals.

b) Complement fixation test

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 CF test is specific but less sensitive than the ELISA or IFA (11, 34). Seroconversion is detected later by the CF test than by the IFA or ELISA, but CF antibodies can persist for long periods after illness, and the CF test gives excellent results for routine diagnosis at the flock level for abortive diseases (41, 43). The CF test is still largely used by many 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 strain³. In France, this method has been standardised (AFNOR NFU47-006).

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.

o 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.

o 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.

o Test procedure

- i) Make twofold dilutions of decomplexed 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.

² Dade Behring, Marburg, Germany.

³ Virion, Zürich, Switzerland.

- 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.

o **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 evolutive phase of the infection.

c) **Enzyme-linked immunosorbent assay**

This technique has a high sensitivity and a good specificity (11, 40, 59). It is easy to perform in laboratories that have the necessary equipment (a spectrophotometer) and reagents. The ELISA tends to replace the IFA and CF tests as the test of choice because it is convenient for large-scale screening and, particularly for veterinary diagnosis, as it is a reliable technique for demonstrating *C. burnetii* antibody in various animal species (20, 49). It requires a relatively pure antigen. Antigens prepared for the CF test may be used for coating the plates. 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.

o **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'-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid)] for peroxidase); hydrogen peroxide.

o **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.

o **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 30–40%	doubtful serum
Ab >40%	positive serum

C. REQUIREMENTS FOR VACCINES AND DIAGNOSTIC BIOLOGICALS

1. Vaccine

Vaccination is the most logical strategy for preventing Q fever in exposed subjects and livestock. A *C. burnetii* vaccine can only be prepared by trained staff only working in adequate conditions of protection (at a minimum in a biosafety level 3 laboratory). It is recommended to obtain the vaccine from manufacturers capable of completing and certifying tests for safety, inactivation and sterility.

Guidelines for the production of veterinary vaccines are given in Chapter 1.1.8 Principles of veterinary vaccine production. The guidelines given here and in Chapter 1.1.8 are intended to be general in nature and may be supplemented by national and regional requirements.

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 (28). Phase I vaccines are more 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, as the phase II vaccines are 100 times less effective against the colonisation of mouse spleen than phase I vaccines (13). An inactivated phase I vaccine is commercially available in Slovakia for vaccination of cattle. 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 during 10 years, together with improved veterinary control of domestic animal transport within the country (46).

This vaccine consists of highly purified antigen prepared from Nine Mile strain in the phase I (egg passage 2 to egg passage 6) and inactivated by formaldehyde. 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 (3). Ideally, vaccine efficacy must be demonstrated by tests on all the target species.

In the case of vaccination on already infected animals, there is a lack of information on possible adverse effects and on the shedding of the Q fever agent. Consequently, 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 (24). To date, no data are available for comparing the cost–benefit of this strategy with a nonselective strategy in the control of Q fever.

2. Diagnostic biologicals

See Section B.2.a (Antigen preparation).

REFERENCES

1. ADESIYUN A.A., JAGUN A.G., KWAGA J.K. & TEKDEK L.B. (1985). Shedding of *Coxiella burnetii* in milk by Nigerian dairy and dual purposes cows. *Int. J. Zoonoses*, **12**, 1–5.
2. ARRICAU-BOUVERY N., HAUCK Y., BEJAOU A., FRANGOULIDIS D, BODIER C., SOURIAU A. MEYER H., NEUBAUER H., RODOLAKIS A. & VERGNAUD G. (2006). Molecular characterization of *Coxiella burnetii* isolates by infrequent restriction site-PCR and MLVA typing. *BMC Microbiol.*, **6**, 38.
3. ARRICAU-BOUVERY N., SOURIAU A., BODIER C., DUFOUR P., ROUSSET E. & RODOLAKIS A. (2005). Effect of vaccination with phase I and phase II *Coxiella burnetii* vaccines in pregnant goats. *Vaccine*, **23**, 4392–4402.
4. BERRI M., LAROUCAU K. & RODOLAKIS A. (2000). The detection of *Coxiella burnetii* from ovine genital swabs, milk and fecal samples by the use of a single touchdown polymerase chain reaction. *Vet. Microbiol.*, **72**, 285–293.

5. BERRI M., ROUSSET E., HECHARD C., CHAMPION J.L., DUFOUR P. & RODOLAKIS A. (2005). Progression of Q fever and *Coxiella burnetii* shedding in milk after an outbreak of enzootic abortion in a goat herd. *Vet. Rec.*, **150**, 548–549.
6. BERRI M., SOURIAU A., CROSBY M., CROCHET D., LECHOPIER P. & RODOLAKIS A. (2001). Relationships between the shedding of *Coxiella burnetii*, clinical signs and serological responses of 34 sheep. *Vet. Rec.* **148**, 502–505.
7. BERRI M., SOURIAU A., CROSBY M. & RODOLAKIS A. (2002). Shedding of *Coxiella burnetii* in ewes in two pregnancies following an episode of *Coxiella* abortion in a sheep flock. *Vet. Microbiol.*, **85**, 55–60.
8. BOUVERY N.A., SOURIAU A., LECHOPIER P. & RODOLAKIS A. (2003). Experimental *Coxiella burnetii* infection in pregnant goats: excretion routes. *Vet. Res.*, **34**, 423–433.
9. CENTER FOR DISEASE CONTROL AND PREVENTION (2000). Biological and chemical terrorism: strategic plan for preparedness and response. Recommendations of the CDC Strategic Planning Workgroup. *MMWR Recomm. Rep.*, **49**, 1–14.
10. COLEMAN S.A., FISCHER E.R., HOWE D., MEAD D.J. & HEINZEN R.A. (2004). Temporal analysis of *Coxiella burnetii* morphological differentiation. *J. Bacteriol.*, **186**, 7344–7352.
11. FOURNIER P.E., MARRIE T.J. & RAOULT D. (1998). Diagnosis of Q fever. *J. Clin. Microbiol.*, **36**, 1823–1834.
12. FOURNIER P.E. & RAOULT D. (2003). Comparison of PCR and serology assays for early diagnosis of acute Q fever. *J. Clin. Microbiol.*, **41**, 5094–5098.
13. GAJDOSOVA E., KOVACOVA E., TOMAN R., SKULTETY L., LUKACOVA M. & KAZAR J. (1994). Immunogenicity of *Coxiella burnetii* whole cells and their outer membrane components. *Acta Virol.*, **38**, 339–344.
14. GIMENEZ D.F. (1964). Staining rickettsiae in yolk-sack cultures. *Stain. Technol.*, **39**, 135–140.
15. GLAZUNOVA O., ROUX V., FREYLIKMAN O., SEKEYOVA Z., FOURNOUS G., TYCZKA J., TOKAREVICH N., KOVACAVA E., MARRIE T. & RAOULT D. (2005). *Coxiella burnetii* genotyping. *Emerg. Infect. Dis.*, **11**, 1211–1217.
16. HATCHETTE T.F., HUDSON R.C., SCHLECH W.F., CAMPBELL N.A., HATCHETTE J.E., RATNAM S., RAOULT D., DONOVAN C. & MARRIE T.J. (2001). Goat-associated Q fever: a new disease in Newfoundland. *Emerg. Infect. Dis.*, **7**, 413–419.
17. HEINZEN R.A., HACKSTADT T. & SAMUEL J.E. (1999). Developmental biology of *Coxiella burnetii*. *Trends Microbiol.*, **7**, 149–154.
18. HO T., HTWE K.K., YAMASAKI N., ZHANG G.Q., OGAWA M., YAMAGUCHI T., FUKUSHI H. & HIRAI K. (1995). Isolation of *Coxiella burnetii* from dairy cattle and ticks, and some characteristics of the isolates in Japan. *Microbiol. Immunol.*, **39**, 663–671.
19. HOOVER T.A., VODKIN M.H. & WILLIAMS J.C. (1992). A *Coxiella burnetii* repeated DNA element resembling a bacterial insertion sequence. *J. Bacteriol.*, **174**, 5540–5548.
20. JASPERS U., THIELE D. & KRAUSS H. (1994). Monoclonal antibody based competitive ELISA for the detection of specific antibodies against *Coxiella burnetii* in sera from different animal species. *Zentralbl. Bakteriol.*, **281**, 61–66.
21. KIM S.G., KIM E.H., LAFFERTY C.J. & DUBOVI E. (2005). *Coxiella burnetii* in bulk tank milk samples, United States. *Emerg. Infect. Dis.*, **11**, 619–621.
22. KLEE S.R., TYCZKA J., ELLERBROK H., FRANZ T., LINKE S., BALJER G. & APPEL B. (2006). Highly sensitive real-time PCR for specific detection and quantification of *Coxiella burnetii*. *BMC Microbiol.*, **6**, 2.
23. KOSATSKY T. (1984). Household outbreak of Q-fever pneumonia related to a parturient cat. *Lancet*, **2**, 1447–1449.
24. KRAUSS H. (1989). Clinical aspects and prevention of Q fever in animals. *Eur. J. Epidemiol.*, **5**, 454–455.
25. KRUSZEWSKA D. & TYLEWSKA-WIERZBANOWSKA S. (1997). Isolation of *Coxiella burnetii* from bull semen. *Res. Vet. Sci.*, **62**, 299–300.

26. LABRENZ M. & HIRSCH P. (2003). The genus *Coxiella*. In: Bergey's Manual Of Systematic Bacteriology, Garrity G., Boone D.R. & Castenholz R.W., eds. Springer-Verlag, New York, USA.
27. LANG G.H. (1990). Coxiellosis (Q fever) in animals. In: Q Fever. Volume I: The Disease, Marrie T.J., ed. CRC Press, Boca Raton, USA, 23–48.
28. MARMION B.P., ORMSBEE R.A., KYRKOV N.V., WRIGHT J., WORSWICK D.A., IZZO A.A., ESTERMAN A., FEERY B. & SHAPIRO R.A. (1990). Vaccine prophylaxis of abattoir-associated Q fever: eight years' experience in Australian abattoirs. *Epidemiol. Infect.*, **104**, 275–287.
29. MARRIE T.J. (1990). Epidemiology of Q fever. In: Q Fever. Volume I: The Disease, Marrie T.J., ed. CRC Press, Boca Raton, USA, 49–70.
30. MAURIN M. & RAOULT D. (1999). Q fever. *Clin. Microbiol. Rev.*, **12**, 518–553.
31. MCCAUL T.F. & WILLIAMS J.C. (1981). Developmental cycle of *Coxiella burnetii*: structure and morphogenesis of vegetative and sporogenic differentiations. *J. Bacteriol.*, **147**, 1063–1076.
32. MILAZZO A., HALL R., STORM P.A., HARRIS R.J., WINSLOW W. & MARMION B.P. (2001). Sexually transmitted Q fever. *Clin. Infect. Dis.*, **33**, 399–402.
33. NGUYEN S.V., TO H., MINAMOTO N., OGAWA M., YAMAGUCHI T., FUKUSHI H. & HIRAI K. (1997). Evaluation of the high-density agglutination test for *Coxiella burnetii* antibodies in animals. *Clin. Diagn. Lab. Immunol.*, **4**, 676–680.
34. PETER O., DUPUIS G., PEACOCK M.G. & BURGENDORFER W. (1987). Comparison of enzyme-linked immunosorbent assay and complement fixation and indirect fluorescent-antibody tests for detection of *Coxiella burnetii* antibody. *J. Clin. Microbiol.*, **25**, 1063–1067.
35. QUINN P.J., CARTER M.E., MARKEY B. & CARTER G.R. (1994). Bacterial pathogens: microscopy, culture and identification. In: Clinical Veterinary Microbiology. Wolfe Publishing, Mosby-Year Book Europe Limited, 21–30.
36. RAOULT D., FENOLLAR F. & STEIN A. (2002). Q fever during pregnancy: diagnosis, treatment, and follow-up. *Arch. Intern. Med.*, **162**, 701–704.
37. RAOULT D., LAURENT J.C. & MUTILLOD M. (1994). Monoclonal antibodies to *Coxiella burnetii* for antigenic detection in cell cultures and in paraffin-embedded tissues. *Am. J. Clin. Pathol.*, **101**, 318–320.
38. RAOULT D., TISSOT-DUPONT H., FOUCAULT C., GOUVERNET J., FOURNIER P.E., BERNIT E., STEIN A., NESRI M., HARLE J.R. & WEILLER P.J. (2000). Q fever 1985–1998. Clinical and epidemiologic features of 1,383 infections. *Medicine (Baltimore)*, **79**, 109–123.
39. RAOULT D., VESTRIS G. & ENEA M. (1990). Isolation of 16 strains of *Coxiella burnetii* from patients by using a sensitive centrifugation cell culture system and establishment of the strains in HEL cells. *J. Clin. Microbiol.*, **28**, 2482–2484.
40. ROGES G. & EDLINGER E. (1986). Immunoenzymatic test for Q-fever. *Diagn. Microbiol. Infect. Dis.*, **4**, 125–132.
41. RUSSO P. (1997). Infection à *Coxiella burnetii* ou Fièvre Q. In: Manuel pratique de diagnostic de laboratoire des avortements infectieux des petits ruminants, Rodolakis A. & Nettleton P., eds. L'Espace Vétérinaire, Casablanca, Morocco, 103–114.
42. SANCHIS R. (1982). Diagnostic direct des avortements infectieux des petits ruminants. *Rev. Méd. Vét.*, **133**, 351–356.
43. SANCHIS R., RUSSO P., CALAMEL M. & PEPIN M. (1997). Diagnostic différentiel des avortements infectieux des petits ruminants. In: Manuel pratique de diagnostic de laboratoire des avortements infectieux des petits ruminants, Rodolakis A. & Nettleton P., eds. L'Espace Vétérinaire, Casablanca, Morocco, 25–34.
44. SANFORD S.E., JOSEPHSON G.K. & MACDONALD A. (1994). *Coxiella burnetii* (Q fever) abortion storms in goat herds after attendance at an annual fair. *Can. Vet. J.*, **35**, 376–378.
45. SCOTT G.H., WILLIAMS J.C. & STEPHENSON E.H. (1987). Animal models in Q fever: pathological responses of inbred mice to phase I *Coxiella burnetii*. *J. Gen. Microbiol.*, **133**, 691–700.

46. SERBEZOV V.S., KAZAR J., NOVKIRISHKI V., GATCHEVA N., KOVACOVA E. & VOYNOVA V. (1999). Q fever in Bulgaria and Slovakia. *Emerg. Infect. Dis.*, **5**, 388–394.
47. SESHADRI R., PAULSEN I.T., EISEN J.A., READ T.D., NELSON K.E., NELSON W.C., WARD N.L., TETTELIN H., DAVIDSEN T.M., BEANAN M.J., DEBOY R.T., DAUGHERTY S.C., BRINKAC L.M., MADUPU R., DODSON R.J., KHOURI H.M., LEE K.H., CARTY H.A., SCANLAN D., HEINZEN R.A., THOMPSON H.A., SAMUEL J.E., FRASER C.M. & HEIDELBERG J.F. (2003). Complete genome sequence of the Q-fever pathogen *Coxiella burnetii*. *Proc. Natl Acad. Sci. USA*, **100**, 5455–5460.
48. SESHADRI R. & SAMUEL J.E. (2001). Characterization of a stress-induced alternate Sigma factor, RpoS, of *Coxiella burnetii* and its expression during the development cycle. *Infect. Immun.*, **69**, 4874–4883.
49. SOLIMAN A.K., BOTROS B.A. & WATTS D.M. (1992). Evaluation of a competitive immunoassay for detection of *Coxiella burnetii* antibody in animal sera. *J. Clin. Microbiol.*, **30**, 1595–1597.
50. SPYRIDAKI I., PSAROULAKI A., LOUKAIDES F., ANTONIOU M., HADJICHRISTODOLOU C. & TSELENTIS Y. (2002). Isolation of *Coxiella burnetii* by a centrifugation shell-vial assay from ticks collected in Cyprus: detection by nested polymerase chain reaction (PCR) and by PCR-restriction fragment length polymorphism analyses. *Am. J. Trop. Med. Hyg.*, **66**, 86–90.
51. STEIN A. & RAOULT D. (1999). Pigeon pneumonia in provence: a bird-borne Q fever outbreak. *Clin. Infect. Dis.*, **29**, 617–620.
52. STEMMLER M. & MEYER H. (2002). Rapid and specific detection of *Coxiella burnetii* by LightCycler PCR. In: *Methods and Applications. Microbiology and Food Analysis*, Reisch U., Wittwer C. & Cockerill F., eds. Springer, Berlin, Germany 149–154.
53. SUNG N. & COLLINS M.T. (1998). Thermal tolerance of *Mycobacterium paratuberculosis*. *Appl. Environ. Microbiol.*, **64**, 999–1005.
54. SVRAKA S., TOMAN R., SKULTETY L., SLABA K. & HOMAN W.L. (2006) Establishment of a genotyping scheme for *Coxiella burnetii*. *FEMS Microbiol. Lett.*, **254**, 268–274.
55. THIELE D., KARO M. & KRAUSS H. (1992). Monoclonal antibody based capture ELISA/ELIFA for detection of *Coxiella burnetii* in clinical specimens. *Eur. J. Epidemiol.*, **8**, 568–574.
56. THOMPSON H.A., HOOVER T.A., VODKIN M.H. & SHAW E.I. (2003). Do chromosomal deletions in the lipopolysaccharide biosynthetic regions explain all cases of phase variation in *Coxiella burnetii* strains? An update. *Ann. N.Y. Acad. Sci.*, **990**, 664–670.
57. TISSOT-DUPONT H., THIRION X. & RAOULT D. (1994). Q fever serology: cutoff determination for microimmunofluorescence. *Clin. Diagn. Lab. Immunol.*, **1**, 189–196.
58. VAN MOLL P., BAUMGARTNER W., ESKENS U. & HANICHEN T. (1993). Immunocytochemical demonstration of *Coxiella burnetii* antigen in the fetal placenta of naturally infected sheep and cattle. *J. Comp. Pathol.*, **109**, 295–301.
59. WAAG D., CHULAY J., MARRIE T., ENGLAND M. & WILLIAMS J. (1995). Validation of an enzyme immunoassay for serodiagnosis of acute Q fever. *Eur. J. Clin. Microbiol. Infect. Dis.*, **14**, 421–427.
60. WEISBURG W.G., DOBSON M.E., SAMUEL J.E., DASCH G.A., MALLAVIA L.P., BACA O., MANDELCO L., SECHREST J.E., WEISS E. & WOESE C.R. (1989). Phylogenetic diversity of the Rickettsiae. *J. Bacteriol.*, **171**, 4202–4206.

*

* *