Title:
CRITICAL ASPECTS FOR DIRECT DIAGNOSIS OF *COXIELLA BURNETII*
FOR CLINICAL AND VETERINARY SAMPLES

Running title:
*Coxiella burnetii* direct diagnosis

Authors:
Marcella Mori¹², Katja Mertens³, Sally J Cutler⁴, Ana Sofia Santos⁵

Affiliations:
¹Bacterial Zoonoses of Livestock, Operational Directorate Bacterial Diseases, Veterinary and Agrochemical Research Centre, CODA-CERVA, Brussels, Belgium, E-mail: Marcella.Mori@codacerva.be
²Belgian Reference Centre for *Coxiella burnetii* and *Bartonella*
³Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Bacterial Infections and Zoonoses, Jena, Germany, E-mail: Katja.Mertens@fli.bund.de
⁴University of East London, London, E15 4LZ, UK, E-mail: s.cutler@uel.ac.uk
⁵Centre for Vector and Infectious Diseases Research, National Institute of Health Doutor Ricardo Jorge, Av da Liberdade 5, 2965-575 Águas de Moura, Portugal, E-mail: ana.santos@insa.min-saude.pt

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Abstract:

_Coxiella burnetii_ is a global distributed zoonotic γ-proteobacterium with an obligatory intracellular lifestyle. It is the causative agent of Q fever in humans and of coxiellosis amongst ruminants, albeit the agent is also detected in ticks, birds and various other mammalian species. Requirements for intracellular multiplication together with the necessity for biosafety level 3 facilities restrict the cultivation of _C. burnetii_ to specialized laboratories. Development of a novel media formulation enabling axenic growth of _C. burnetii_ has facilitated fundamental genetic studies. This review provides critical insights into direct diagnostic methods currently available for _C. burnetii_. It encompasses molecular detection methods, isolation and propagation of the bacteria and its genetic characterization. Differentiation of _C. burnetii_ from _Coxiella_-like organisms is an essential diagnostic prerequisite, particularly when handling and analyzing ticks.
INTRODUCTION

*Coxiella burnetii* is a global distributed zoonotic γ-proteobacterium whose economic and health importance has recently been underscored following the largest ever reported outbreak, which has occurred in the Netherlands (Roest et al. 2011). *Coxiella burnetii* possesses several remarkable features including ability for proliferation within phagolysosome-like vacuoles of mononuclear phagocytes, a biphasic developmental life cycle, and a lipopolysaccharide phase variation (van Schaik et al. 2013). Infections can either be asymptomatic or result in clinical disease. In humans, the disease is known as Q fever and varies from uncomplicated and self-limited febrile illness (acute Q fever) to long-lasting usually focal disease (chronic Q fever), which may result in fatality (Maurin and Raoult 1999, Million and Raoult 2015). Known as coxiellosis in animals, the disease predominantly manifests as reproductive disorder (Agerholm 2013). Sporadic or clustered cases, and large outbreaks have been described worldwide in both humans and animals (Smith 1989, Gilroy et al. 2001, Amitai et al. 2010, Roest et al. 2011, Georgiev et al. 2013).

*Coxiella burnetii* can infect ticks, birds and mammals. Ticks are regarded as important vectors for agent transmission between wild animals and for amplification of enzootic cycles to the domestic environment (Cutler et al. 2007, Boarbi et al. 2015). Aerogenic transmission following environmental contamination has been demonstrated between flocks/herds and has resulted in human outbreaks (Hawker et al. 1998), however direct contact between and with infected animals additionally facilitate spread (Kruszewska and Tylewska-Wierzbanowska 1997, Alsaleh et al. 2011). *Coxiella burnetii* is excreted in vast numbers during normal parturition as well as abortion. Once aerosolized, the bacteria can be transmitted over long distances by the wind. During the biphasic developmental life cycle *C. burnetii* develops highly resistant “spore-like”
structures, known as small cell variants (SCVs) providing long lasting environmental stability. Other body fluids and secretions are also infectious and may facilitate both vertical and sexual transmission (Kruszewska and Tylewska-Wierzbanska 1997, Maurin and Raoult 1999, Milazzo et al. 2001, Miceli et al. 2010, Agerholm 2013). Small domestic ruminants are the most frequently infected species and are considered as the primary source of human infections.

Eight decades after the first description of Q fever cases, diagnosis remains challenging. Case confirmation in humans and appropriate surveillance of animals depend mostly on the interest of the involved clinician/veterinarian and their diagnostic capabilities, mostly relying upon serology. In this review direct laboratory detection tests for *C. burnetii* will be reviewed, especially molecular diagnostic methods and recent improvements in pathogen isolation methods.

**REALTIME PCR**

DNA amplification is most frequently used for direct detection of *C. burnetii*. This enables investigation of all sample types from vertebrates to ticks and environmental samples such as dust, soil and water. For acute human cases, whole-blood or buffy-coat aliquots collected in EDTA or citrate at onset of symptoms and prior to antibiotic treatment are most useful (Anderson et al. 2013). Serum, urine and throat swabs have also proven valuable for *C. burnetii* screening (Klaassen et al. 2009). In more protracted infections, tissue samples from focal regions of infection should be investigated, i.e valvular material from endocarditis, aneurism or vessels fragments in vascular infections, bone biopsies in osteomyelitis. In these cases, recent antibiotic use is not a limiting factor (authors’ experience). For livestock, aborted material (placental material and fetal
organs), milk, vaginal swabs, feces and more rarely semen have proven to be valuable. On a cautionary note, if the herd has been recently vaccinated (first months following vaccination), PCR will not discriminate between the vaccine and wild type strains (Hermans 2011). As *C. burnetii* is shed intermittently, consecutive samples are preferred to single collections. Bulk tank milk is recommended for herd monitoring rather than individual samples because of its ease of collection, cost effectiveness, reduced contamination and sensitivity for evaluation of the pathogen at the herd level. However a single collection is not sufficient for detection of *C. burnetii* in flocks with low numbers of infected animals. Therefore two to three samples (collected two to three months apart) are more informative (Boarbi et al. 2014). For wildlife screening, blood, urine, feces, vaginal, cloacae and anal swaps can be useful (Bitar et al. 2014, Tozer et al. 2014, González-Barrio et al. 2015a). In case of dead animals (hunted, road-killed, euthanized, etc.) other samples such as spleen, lung, and liver should also be considered. As for domestic animals, short *bacteraemia* and intermittent shedding can also occur thus the collection of different sample types obtained during longer sampling periods, serve to overcome seasonal fluctuations of *C. burnetii* in wildlife (González-Barrio et al. 2015a).

For DNA extraction, fresh or frozen samples are preferable, although paraffin-embedded tissues have also been used successfully for the identification of chronic Q fever patients (Costa et al. 2015). DNA extraction protocols vary from column to magnetic particle-based methods. In either case, PCR general guidelines should be rigorously followed to limit sample cross-contamination that might occur when high *C. burnetii* loads are present. Bacterial numbers are highly variable, with massive *C. burnetii* burdens in persistently infected tissue samples (as placental/foetal and valvular/vascular material) to very low agent loads in environmental samples, milk samples and usually in blood samples. For DNA amplification, several realtime PCR protocols targeting
different genes are described in the literature as reviewed in Table 1. These have superseded previously used conventional and nested PCRs that are prone to cross-contamination. The multi-copy IS1111 repetitive element is often used for agent’s detection as this provides increased sensitivity when compared to other targets, but since the exact copy number is unknown for most of the strains, except for C. burnetii Nine Mile I with 20 copies per genome, it cannot be used for quantification (Klee et al. 2006, Tilburg et al. 2010). When results are equivocal (Ct values 35 or greater), additional confirmation using another target or a different region within the same gene should be considered. Furthermore, when investigating arthropod vectors, it must be remembered that the specificity of the IS1111 realtime PCR might be compromised through detection of Coxiella-like variants (Elsa et al. 2015). Confirmation of findings can be verified when necessary by sequencing.

GENOME AND GENETIC CHARACTERIZATION

The first whole genome sequence of C. burnetii, from the Nine Mile RSA 493 reference strain, isolated in 1935 from an infected group of ticks (Dermacentor andersoni), was released in 2003. The sequence spans 1,995,275 base pairs and was obtained using the random shotgun method (Seshadri et al. 2003). Four years later, a second genome was published, strain Henzerling RSA 331, isolated from blood of an infected patient in Italy in 1945 (“J. Craig Venter Institute”-CVI, 2007). Later, three additional strains, « K » and « G » derived from human endocarditis and the « Dugway » rodent strain were published (Beare et al. 2009). Comparative analysis of these genomes highlighted their diversity regarding pseudogene content and number of insertion sequence (IS) elements, possibly explaining their biological differences (Beare et al. 2009). Recently, along with the development of powerful sequencing platforms, the
numbers of sequenced genomes has blossomed to more than 40, 26 being publically available (D'Amato et al. 2014, Karlsson et al. 2014, Sidi-Boumedine et al. 2014, Walter et al. 2014, D'Amato et al. 2015, Hammerl et al. 2015). Despite the large number of genome records for *C. burnetii* since 2003, only 9 genomes are fully sequenced and annotated as closed circular genomes, the remainder are available as fragmented scaffolds, contigs or whole genome shot gun sequences in various genome databases (https://www.patricbrc.org/portal/portal/patric/Home, https://www.ncbi.nlm.nih.gov/genome/genomes/543).

To obtain high quality and host cell-free DNA from an intracellular organism for deep sequencing analyses is a challenging task but has benefitted more recently from the use of axenic cultivation. When using *in vitro* cell cultures or embryonated hen eggs particular care should be taken to complete removal of host DNA. Classical DNA isolation methods are suitable (as cited for realtime PCR). However, bioinformatic filters are required to subtract the host genome sequence. Depending on the degree of host DNA contamination (sometimes in excess of 60%), additional sequencing may be required to obtain a complete genomic coverage for *C. burnetii* (median genome length 2Mb). Whole genome sequencing is becoming more affordable, but data analyses remains time consuming and requires specific knowledge and extra funding. Though still not used in routine diagnostics, access and use of whole genome sequence data are steadily increasing and tools for outbreak investigations and trace-back studies applicable in routine diagnostic laboratories will become available. Till then, traditional genotyping approaches are the best choice. Genotyping methods for *C. burnetii* were fully revised elsewhere (Massung et al. 2012) and therefore will be only briefly described in this review.
The choice on the most appropriate typing option may depend on the research objectives. The simplest and direct tests (lacking further sequencing), with a good discriminatory power and lowest DNA demands are mostly used for rapid tracking of outbreaks. Examples include the multiple-locus variable-number tandem repeat analysis (MLVA), particularly applicable when adapted to capillary electrophoresis for estimation the number of repeats (Klaassen et al. 2009, Tilburg et al. 2012a), and single-nucleotide-polymorphism (SNP) genotyping (Huijsmans et al. 2011, Hornstra et al. 2011). Both typing approaches were used for the Dutch outbreak investigation (Klaassen et al. 2009, Tilburg et al. 2012a, Huijsmans, 2011). Presently, these methods are reviewed towards harmonization and standardized nomenclature (http://mlva.u-psud.fr/mlvav4/genotyping/view.php, Huijsmans et al. 2011, Hornstra et al. 2011).

A more robust and conservative typing system, preferably supported by large databases and broadly accepted/used would provide the best overall option for eco-epidemiological investigations and data integration, at both local and global scale. Multi-spacer sequence typing (MST) is a good example of this case (Glazunova et al. 2005, Tilburg et al., 2012b). It has the advantage of using standardized nomenclature and genotypes can be identified using a web-based MST database (http://ifr48.timone.univ-mrs.fr/mst/coxiella_burnetii/), enabling comparison of results between laboratories.

**CULTIVATION**

Though cultivation is not usually required for a definitive diagnosis, it is valuable when new clinical presentations or atypical epidemiological situations in association with a *C. burnetii* infection occur. Isolation and propagation of strains from clinical samples enables phenotypic and genotypic characterization using molecular typing methods or deeper genetic analyses such as whole genome sequencing. Cultivation is also of
paramount importance to build strain collections to aid further research. It is laborious, time consuming, and success largely depends upon sample quality, freshness and pathogen load. Further technical expertise and availability of suitable laboratory biosafety level 3 (BSL3) facilities are essential. Handling and processing of samples or cultures with a high bacterial load bear the risk of generating contaminated aerosols and sets involved personnel at risk as demonstrated by several laboratory-acquired infections (Johnson and Kadull 1966, Curet and Paust 1972, Hall et al. 1982, Graham et al. 1989, Wurtz et al. 2016). Despite this, increasing numbers of isolates are now available.

ISOLATION FROM CLINICAL SAMPLES

In vitro isolation

Several in vitro cell lines support *C. burnetii* replication, including those from macrophage, (P388D1, J774, DH82) fibroblast (L929, HEL) and epithelial lineages (Vero E6) (Maurin and Raoult 1999, Mediannikov et al. 2010, Santos et al. 2012). The human embryonic lung fibroblast cell line – HEL is one of the most widely used as it is easy to maintain, preserves monolayer integrity during prolonged incubations, and is highly susceptible to infection (Gouriet et al. 2005, Lagier et al. 2015). The canine malignant histiocytic macrophage cell line - DH82 (ATCC CRL-10389), traditionally used for culturing other mononuclear leucocytes targeting bacteria, such as *Ehrlichia canis* and *E. chaffeensis*, has been increasingly adopted as an in vitro system for *C. burnetii* (Mediannikov et al. 2010, Santos et al. 2012, Lockhart et al. 2012, Cumbassa et al. 2015). In vitro isolation is usually performed using the shell-vial technique (Gouriet et al. 2005, Santos et al. 2012). Cultures are incubated at 37°C and 5% CO₂ atmosphere for 2 months possibly extending up to 4-5 months, with periodical evaluation of microbial growth using either light or fluorescence microscopy. During this period, supplementation by
partial replacement of culture medium is required with a frequency adapted according to the cell line in use. Fetal bovine serum (FBS) concentration can be reduced to 5% (v/v) in culture medium to decrease cell proliferation and maintain monolayer longevity. Appearance of parasitophorous vacuoles can be checked directly using an inverted microscope (magnification 20 to 40X). Monthly assessment of culture aliquots should also be undertaken with initial cytoconcentration, stained by Giménez and examined by microscopy (by immersion at 1000X) for the characteristic tightly packed \( C.\ burnetii \) vacuoles (Giménez, 1964). Positive findings should be confirmed by PCR (see section above).

Various fresh or frozen samples (< -80°C) can be used with the shell-vial technique, including anticoagulated whole-blood, buffy-coat, other biological fluids, tissue biopsies or necropsies, ticks etc. Fluids are directly inoculated whilst tissue samples should be macerated with a pestle or disrupted with a scalpel in culture medium before being inoculated into the shell-vial. An important pre-requisite is the absence of microbial contaminants, which is challenging when working with post-mortem or aborted tissues, ticks and environmental samples. Ticks can be surface decontaminated by serial passages in bleach 10% or alcohol 70%, and rinsed in sterile water before further manipulations. For placenta, fetal and other samples that are associated with high \( C.\ burnetii \) loads (Ct values <25), a tissue homogenate filtration step can increase recovery. Briefly, samples are homogenized in FBS-free medium, exposed to frozen-thaw cycles and low speed centrifugation, with the resulting supernatant subjected to sequential filtration, using 1µm and 0.45 µm syringe filters, and directly inoculated into shell-vials. During the initial days of cultivation, a broad spectrum antibiotic-antifungal cocktail containing 10,000 units/mL of penicillin, 10,000 µg/mL of streptomycin, and 25 µg/mL of Fungizone® (amphotericin B) can be added to culture medium to limit unwanted microbial growth.
**In vivo isolation**

*In vivo* isolation using rodent models, mice or guinea pigs, has proven particularly well suited for contaminated samples, such as environmental (such as, ticks, etc.) or veterinary field samples including as milk or products of conception. Inoculation of the sample into a vertebrate host provides a buffer against unwanted microbial contamination. Furthermore, *in vivo* models are essential for maintenance of the native virulent form (phase I) of *C. burnetii*. The mouse strain OF1 is the genetic lineage frequently used for isolation, because of its relative sensitivity compared to either BALB/c or C57/BL6 mice (authors’ experience). Milk samples should be decreamed first by simple decantation. Inoculum being aspirated from just under the fat layer can be directly injected intraperitoneally into adult (> 50 days mice), with volumes complying with ethical requirements. Successive injections (up to three) five to seven days apart can be used where material permits and low microbial load is suspected (Ct values >32). For abortive material, tissues should be macerated and diluted at least twice in physiological water or PBS prior to injection. Following inoculation, the host should be monitored for clinical signs and by indirect serology (Mori et al. 2013), or post mortem evaluation, at 3 to 5 weeks post-infection. The spleen, liver and lungs are preferred organs for *C. burnetii* monitoring by either microscopy or realtime PCR. Infection it typically accompanied by measurable splenomegaly caused by massive *C. burnetii* propagation.

**PROPAGATION OF BACTERIAL ISOLATES**

**Embryonated eggs inoculation**

Propagation of highly concentrated *C. burnetii* cultures is achieved through use of yolk sac infection. This method was historically used for direct isolation but it is no longer
recommended in favor of *in vitro* or *in vivo* protocols (see above). Nonetheless, it remains useful for massive propagation in specific settings (vaccine production, fundamental studies) and therefore the protocol will be briefly reviewed. Surface disinfected seven-day old specific-pathogen-free (SPF) chicken eggs are candled to locate the yolk sac. Once identified, the edge of the air sac should also be localised and marked on the eggshell. Inoculation with a suspension containing *C. burnetii* infected material is injected through a hole drilled few mm above the marked air sac. Inoculation material might arise from *in vitro* or *in vivo* isolation procedures (see paragraphs above), including cell culture suspensions or macerated mouse organs. The latter might require a 1:2 to 1:10 dilution in physiological water or PBS prior injection. The eggshell holes are sealed with scotch tape or solvent-free glue and the eggs are incubated at 35-37°C until day 21. Bacterial growth may result in death of the embryo, but only eggs dying after day five post-injection are collected. Once opened, the yolk sac should be harvested by detachment, washed several times in physiological water or PBS and then macerated and processed for further use.

**Axenic media**

Over the last decades our understanding has evolved regarding the physiological and structural characteristics of the destructive phagolysosomal-like compartment with its acidic pH (~4.5) and anti-microbial factors, such as hydrolytic and proteolytic enzymes, yet it is this same environment that provides the required intracellular niche of *C. burnetii*. Early studies demonstrated the necessity of an acidic pH for metabolic activation (transport of nutrients, glucose and glutamate, and intracellular replication) (Hackstadt and Williams 1981). Understanding this acid activation and the ability to decipher the metabolic pathways of *C. burnetii* by genome analyses, led to the
development an axenic medium, namely Complex Coxiella Medium (CCM), that supports metabolic activity of *C. burnetii* (Omsland et al. 2008, Omsland and Heinzen 2011). This axenic medium has subsequently been refined to its third generation formulation, the defined Acidified Citrate Cysteine Medium (ACCM-D) which contains amino acids, glutamine as carbon source and methyl-β-cyclodextrin to sequester inhibitory metabolites (Omsland et al. 2011). It has a low pH of 4.75 and cultivation requires specific microaerophilic atmosphere conditions of 5% CO₂ and 2.5% O₂ achieved by the use of a dual-gas incubator or alternatively using an anaerobic pouch in case of a mono-gas incubator (Omsland et al. 2009, 2011). ACCM-D supports the bi-phasic transition from the SCV to the replicative large cell variant (LCV) of *C. burnetii* (Sandoz et al. 2016). Typically, there is an initial lag phase of 2 days, followed by an exponential phase until day 8 and transition into stationary phase. The second generation formula, ACCM-2, has occasionally been used for direct isolation of *C. burnetii* from *in vivo* experimental or clinical samples (Omsland et al. 2011, Boden et al. 2015). ACCM-2 or ACCM-D may not support growth of all *C. burnetii* strains and therefore axenic cultivation is more frequently used for amplification of bacteria from cell culture, or inoculation of macerated organs into mice. The sensitivity of axenic cultivation has been estimated to fall between 10 to 100 GE/ml (genome equivalents), depending on the quality of sample (authors’ experience). The impact of repeated axenic propagation on the virulence remains to be fully elucidated (Kersh et al. 2011, Kuley et al. 2015).

**COXIELLA-LIKE ORGANISMS**

Initially the *Coxiella* genus was thought to be comprised of solely *C. burnetii* species, but is now recognised to contain other members, namely *Coxiella cheraxi* and novel *Coxiella*-like organism identified in birds and in non-vertebrate species. *C. cheraxi*
was first isolated in 2000 from connective and hepato-pancreatic tissues of a dead crayfish displaying inclusion bodies with *Rickettsia*-like Gram-negative bacteria (Tan and Owens 2000). The partial 16rDNA, *sodB* and *com1* sequences of *C. cheraxi* (strain TO-98) shared highest homology with *C. burnetii* sequences achieving similarity of 96%, 96% and 100%, respectively (Tan and Owens 2000, Cooper et al. 2007). Birds are commonly infected with *C. burnetii* without apparent clinical signs, but in contrast show pathology when infected with *Candidatus Coxiella avium*, a pleomorphic *Coxiella*-like organism multiplying in macrophage vacuoles and leading to inflammation of liver, lung and spleen or systemic infection and death of the host (Shivaprasad et al. 2008, Vapniarsky et al. 2012). Further diversity amongst the genus has been described with reports of *Coxiella*-like organisms as endosymbionts among several species of ticks (Duron et al. 2015), with extremely high (close to 100%) infection frequency. Indeed, it has been postulated that these might represent ancestral species of *C. burnetii* (Duron et al. 2015). The genetic classification of these organisms within the *Coxiella* genus is complex, with common patterns of co-divergence within tick-species (tick species-specific clades) and horizontal gene transfer events complicating the phylogenetic separation (Duron et al. 2015). The genome is further reduced in comparison with that of *C. burnetii* (Smith et al. 2015) and traditional cultivation methods for *C. burnetii* have been unsuccessful to date (Duron et al. 2015). Importantly, several *IS1111* sequence haplotypes are present in *Coxiella*-like tick endosymbionts (Duron 2015), consequently caution is needed to avoid misidentification between *Coxiella*-like bacteria and *C. burnetii*, as previous mentioned in the above realtime PCR section. Table 2 summarizes PCR assays used to screen samples for *Coxiella*-like bacteria.

**CONCLUSION**
Direct detection of *C. burnetii* though challenging, fulfils a much-needed diagnostic gap. Recovery of isolates is essential to address our evolving understanding of this pathogen and to decipher our understanding of the intricate interactions between this microbe and its vertebrate host. This will pave the way for better-targeted intervention and control strategies. Furthermore, direct detection is essential to provide categorical association of emerging clinical sequelae with *C. burnetii* infection. Finally, the discriminatory methods reviewed above furnish us with tools to detect hitherto undescribed species expanding our understanding of the *Coxiella* genus and highlighting potential limitations of our current diagnostic tools.

**DISCLOSURE STATEMENT**

No competing financial interests exist.

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**REFERENCES**


Duron O. The IS1111 insertion sequence used for detection of *Coxiella burnetii* is widespread in *Coxiella*-like endosymbionts of ticks. FEMS Microbiol Lett 2015;362.


Tan CK, Owens L. Infectivity, transmission and 16S rRNA sequencing of a rickettsia, *Coxiella cheraxi* sp. nov., from the freshwater crayfish *Cherax quadricarinatus*. Dis Aquat Organ 2000;41:115-122.


CORRESPONDING AUTHOR:
Ana Sofia Santos. Centro de Estudos de Vectores e Doenças Infecciosas, Instituto Nacional de Saúde Dr. Ricardo Jorge. Avenida da Liberdade 5, 2965-575 Águas de Moura, Portugal; Tel: +351 217 508 125; Fax: +351 217 508 521; e-mail: ana.santos@insa.min-saude.pt