

AperTO - Archivio Istituzionale Open Access dell'Università di Torino

## Guidelines for the Detection of Rickettsia spp

### **This is the author's manuscript**

*Original Citation:*

*Availability:*

This version is available <http://hdl.handle.net/2318/1632966> since 2017-06-21T15:44:42Z

*Published version:*

DOI:10.1089/vbz.2016.1966

*Terms of use:*

Open Access

Anyone can freely access the full text of works made available as "Open Access". Works made available under a Creative Commons license can be used according to the terms and conditions of said license. Use of all other works requires consent of the right holder (author or publisher) if not exempted from copyright protection by the applicable law.

(Article begins on next page)

This is the author's final version of the contribution published as:

Portillo, Aránzazu; De Sousa, Rita; Santibáñez, Sonia; Duarte, Ana; Edouard, Sophie; Fonseca, Isabel P.; Marques, Cátia; Novakova, Marketa; Palomar, Ana M.; Santos, Marcos; Silaghi, Cornelia; Tomassone, Laura; Zúquete, Sara; Oteo, José A. Guidelines for the Detection of Rickettsia spp. VECTOR BORNE AND ZOONOTIC DISEASES, VECTOR BORNE AND ZOONOTIC DISEASES. 17 (1) pp: 23-32.  
DOI: 10.1089/vbz.2016.1966

The publisher's version is available at:  
<http://online.liebertpub.com/doi/10.1089/vbz.2016.1966>

When citing, please refer to the published version.

Link to this full text:  
<http://hdl.handle.net/2318/1632966>

## Guidelines for the Detection of *Rickettsia* spp.

Aránzazu Portillo,<sup>1</sup> Rita de Sousa,<sup>2</sup> Sonia Santibáñez,<sup>1</sup> Ana Duarte,<sup>3</sup> Sophie Edouard,<sup>4</sup> Isabel P. Fonseca,<sup>3</sup> Cátia Marques,<sup>3</sup> Marketa Novakova,<sup>5,6</sup> Ana M. Palomar,<sup>1</sup> Marcos Santos,<sup>3</sup> Cornelia Silaghi,<sup>7</sup> Laura Tomassone,<sup>8</sup> Sara Zúquete,<sup>3</sup> and José A. Oteo<sup>1</sup>

<sup>1</sup>Center of Rickettsiosis and Arthropod-Borne Diseases, Hospital San Pedro-CIBIR, Logroño, Spain.

<sup>2</sup>National Institute of Health Dr. Ricardo Jorge, A ´guas de Moura, Portugal.

<sup>3</sup>Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal.

<sup>4</sup>Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Institut Hospitalo-Universitaire Méditerranée-Infection, Marseille, France.

<sup>5</sup>Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

<sup>6</sup>CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.

<sup>7</sup>National Centre for Vector Entomology, Institute of Parasitology, University of Zurich, Zurich, Switzerland.

<sup>8</sup>Department of Veterinary Sciences, University of Torino, Grugliasco, Italy.

## Abstract

The genus *Rickettsia* (Rickettsiales: Rickettsiaceae) includes Gram-negative, small, obligate intracellular, nonmotile, pleomorphic coccobacilli bacteria transmitted by arthropods. Some of them cause human and probably also animal disease (life threatening in some patients). In these guidelines, we give clinical practice advices (microscopy, serology, molecular tools, and culture) for the microbiological study of these microorganisms in clinical samples. Since in our environment rickettsioses are mainly transmitted by ticks, practical information for the identification of these arthropods and for the study of *Rickettsia* infections in ticks has also been added.

**Keywords:** diagnosis, reservoir host, *Rickettsia*, tick(s)

The genus *Rickettsia* (class  $\alpha$ -Proteobacteria; order Rickettsiales; family Rickettsiaceae) includes Gram-negative, small, obligate intracellular, nonmotile, pleomorphic coccobacilli bacteria transmitted by arthropods. Mainly transmitted by ticks nowadays, other arthropods like fleas, lice, or mites are also involved as vectors in Europe. Probably, mosquitoes may act as vectors in the transmission of some species of *Rickettsia* in other regions of the planet (e.g., *Rickettsia felis*) (Merhej et al. 2014, Dieme et al. 2015, Portillo et al. 2015). There are several classifications of *Rickettsia* spp. The most widely used divides this genus into the spotted fever group (SFG) and typhus group (TG), according to antigenic and genetic particularities.

There are several circulating *Rickettsia* species and Candidatus to *Rickettsia* spp. in Europe where rickettsioses are well documented (Portillo et al. 2015) (Table 1). The main clinical manifestations of a rickettsial syndrome in humans are fever, rash, and eschar with different combinations (Faccini-Martínez et al. 2014). However, they are not pathognomonic. Whenever a rickettsiosis is suspected, an early antimicrobial treatment must be started before confirming the infection. Confirmatory assays provide information to retrospectively validate the accuracy of the clinical diagnosis and contribute to the epidemiological knowledge of the pathogenic circulating species.

Due to the increasing knowledge and interest in these pathogens, the European Neglected Vectors network (COST TD1303 EURNEGVEC) understands that the establishment of guidelines to detect *Rickettsia* spp. in

vectors (mainly in ticks) and to support the diagnosis of rickettsioses in the clinical practice will be a helpful tool for the study of these bacteria.

These guidelines are organized in different sections that comprise microscopy, serological, molecular, and culture assays for the diagnosis of *Rickettsia* infection. Since tick-borne rickettsioses are the main rickettsioses present in Europe, advices for the tick collection and identification are offered in Supplementary Data (Supplementary Data are available online at [www.liebertpub.com/vbz](http://www.liebertpub.com/vbz)) and in Supplementary Table S1.

Storage requirements of samples (including arthropods) to improve profitability of diagnosis are detailed in Table 2.

### **Microscopy: Stains and Immunohistochemical Assays for the Detection of *Rickettsia* spp.**

The microbiological characteristics of *Rickettsia* spp. do not allow their visualization in the tissues of affected patients using classical stains like Gram. Thus, immunohistochemical (IHC) assays will be necessary to visualize rickettsiae in the affected tissues. Moreover, *Rickettsia* spp. are not available in sufficient amount to be detected in blood smears in cases of human rickettsiosis since these bacteria are located inside cells within the tissues (Woods and Walker 1996).

Detection of *Rickettsia* spp. in ticks can be achieved by microscopic examination after Giménez or Giemsa stain. *Rickettsia* spp. take on a characteristic red color in the cytoplasm of the infected cells. Nevertheless, they are indistinguishable from other rickettsiae using this type of stain. The hemolymph of viable ticks (or the salivary glands extract if ticks are frozen) is the main source to study the presence of rickettsiae. Hemolymph is smeared onto a microscope slide, stained, and examined for the presence of bacteria (hemocyte test) (Brouqui et al. 2004). For epidemiological studies, it is worth mentioning that costs can be reduced if *Rickettsia* screening is performed by PCR only in ticks that stain positive.

Staining methods such as Giemsa or Giménez stains or immunofluorescence assays can be used to detect rickettsiae in cell culture. Acridine orange staining is recommended to detect the viable microorganisms in cell lines. IHC assays using monoclonal or polyclonal antibodies have allowed the identification of pathogenic *Rickettsia* spp. in tissues of ill patients, in formalin-fixed paraffin embedded biopsy or autopsy tissue specimens (Dumler et al. 1990, Paddock et al. 1999, 2008, Lepidi et al. 2006). Indirect immunoperoxidase and immunofluorescence staining have been used to demonstrate rickettsiae in human tissues by immunofluorescence or immunoenzyme methods (Woods and Walker 1996, Rozental et al. 2006).

The main benefit of the IHC staining lies in its relative sensitivity (around 70%), specificity (100% in experienced hands), and the speed (generally within 24 h within receipt at the laboratory) at which an infection caused by *Rickettsia* sp. can be confirmed. Another advantage of IHC techniques is that they can be applied to specimens obtained years or even decades earlier. However, these technical approaches are limited to very few research laboratories because monoclonal antibodies are not easily available. In addition, the acquisition of the sample (e.g., tissue) is typically more complex than collection of blood or serum, and immunologic reagents are generally group specific rather than species specific.

### **Serological Assays**

Diagnosis of rickettsial infections and rickettsial diseases is more often made by serological tests because they are the easiest methods, and equipments are available in most clinical microbiology laboratories. Currently, the most common used in-house and commercial tests are enzyme-linked immunosorbent assay

(ELISA), indirect immunoperoxidase assay (IPA) (most common in Asia), and, overall, indirect immunofluorescence assay (IFA). The last one is considered the reference method.

IFA is based on the detection of antirickettsial antibodies that bind to fixed antigens (e.g., *Rickettsia conorii*) on a slide and is detected by a fluorescein-labeled antisppecies-specific (e.g., human or dog) G or M immunoglobulin (La Scola and Raoult 1997).

Detection of antibodies depends on the timing of collection of the blood sample. In general, patients with rickettsiosis lack detectable antibodies in the first 7–10 days of illness. Immunological response caused by *Rickettsia africae* infection might even be more delayed (>25 days) by comparison with other *Rickettsia* species (Fournier et al. 2002). A presumptive clinical diagnosis of rickettsiosis should be confirmed by testing two sequential serum samples taken at least 2–6 weeks apart. Confirmation of a recent or current infection can be demonstrated by seroconversion, or a fourfold or greater rise in antibody titer between acute and convalescent samples. Local serologic studies are useful to determine the prevalence of antibodies against *Rickettsia* spp. in a certain area, and they should be taken into account to recommend the cutoffs. In regions where Mediterranean spotted fever is endemic, a cutoff value for IgG titers  $\geq 128$  and IgM titers  $\geq 32$  is considered indicative of infection by *R. conorii*, whereas for nonendemic countries, IgG titers  $\geq 64$  and IgM titers  $\geq 32$  are considered indicative of infection by *Rickettsia* spp. (Brouqui et al. 2004). Single titers of IgG  $\geq 64$  or IgM  $\geq 32$  antibodies should not be considered as indicative of active infection. There is an extensive serologic cross-reactivity within *Rickettsia* groups (SFG, TG), and thus, a positive titer only indicates exposure to a rickettsial species. Moreover, antibody cross-reactions related to infections caused by other pathogens have been described, and false-positive IgM antibodies can be observed when rheumatoid factor is present. Since serology by IFA is only suitable for discrimination between SFG- and TG-*Rickettsia*, and not for species, some reference laboratories have developed techniques such as microimmunofluorescence (MIF) to simultaneously detect the presence of antibodies against several antigens in a single well. A rickettsial antigen is considered to represent to a species of *Rickettsia* when titers of IgG and/or IgM antibody against the antigen are, at least, two serial dilutions higher than titers of IgG and/or IgM antibody against another rickettsial antigen (Brouqui et al. 2004).

To identify the infecting *Rickettsia* sp. by discriminating cross-reacting antibodies between two or more antigens, cross-adsorption assay has been successfully developed for patients with rickettsiosis. First of all, the serum of the patient is mixed separately with the bacteria involved in the cross-reaction and then tested against each of these antigens. Cross-adsorption results in the disappearance of both homologous and heterologous antibodies when adsorption is performed with the bacterium responsible for the cross-reaction (La Scola et al. 2000). This technique is accurate, but it is limited. Indeed, this assay is very expensive and time-consuming because a large number of species of *Rickettsia* are required.

The western blot (WB) assay could be useful in the microbiological diagnosis of rickettsioses since it is positive earlier in the course of the disease by detection of antibodies reactive with lipopolysaccharide (early occurring antibodies). This assay is also helpful for the confirmation of the diagnosis because it detects late occurring antibodies against specific protein antigens located in the rickettsial outer membrane. WB, particularly in conjunction with sera that have been cross-absorbed, can be also used to identify the infecting rickettsial species, but the technique is only suited to reference laboratories (Teyssere and Raoult 1992).

Serological assays are also used to perform seroepidemiological studies in populations. They give information about the prevalence of the infection in the studied populations and the potential risks of acquiring the infection in the area. In these studies, distribution of samples in pools helps to reduce the costs.

## Molecular Methods

Molecular methods based on PCR have enabled the development of sensitive, specific, and rapid tools for the detection and identification of *Rickettsia* spp. in human and animal specimens, including ticks and other arthropods.

Different types of specimens can be used for molecular diagnosis of rickettsioses, such as whole blood, buffy coat, skin or eschar biopsies, and eschar swabs. Other samples like organ tissues, cerebrospinal fluid (CSF), or pleural fluid could also be used (Table 2). Collection of patient specimens should be performed early in the course of the infection and before the patient initiates specific treatment. PCR appears to be more useful for detecting rickettsiae in eschars and skin or organ biopsies than in acute blood since typically low numbers of rickettsiae circulate in the blood in the absence of advanced disease or fulminant infection (Walker and Ismail 2008). In specific situations in which there are no other options, molecular detection can be also performed from plasma, serum, paraffin-embedded tissues, or even fixed slide specimens (Denison et al. 2014).

Samples (including arthropods) and storage requirements for a better diagnostic performance are detailed in Table 2. When the clinical sample is a tick, you can proceed following the instructions of the Supplementary Data about ticks. DNA extraction methods are detailed in Table 3.

Molecular detection strategies have been mainly based on recognition of sequences from different targets such as 16S rRNA (*rrs*) gene (Weisburg et al. 1991, Márquez et al. 1998) and other protein-coding genes: 17-kDa protein (*htr*) (Labruna et al. 2007), citrate synthase (*gltA*) (Regnery et al. 1991), and surface cell antigen (*sca*) autotransporter family, including the outer membrane proteins, *ompA* (Regnery et al. 1991, Roux et al. 1996) and *ompB* (Roux and Raoult 2000, Choi et al. 2005), and the surface cell antigens, *sca4* (Sekeyova et al. 2001) and *sca1* (Ngwamidiba et al. 2006). The most useful primers in clinical and epidemiological practice are shown in the Table 4.

The 16S rRNA and 17-kDa gene sequences lack discriminatory power compared to other genes. The *gltA*, *ompA*, and *ompB* genes have been the most widely used targets for species identification in human diagnosis and in vectors (Parola et al. 2005). For the identification of a *Rickettsia* species, we must take into account that *gltA* gene is present in all of them. However, *ompA* is specific for SFG *Rickettsiae* with some exceptions (e.g., *Rickettsia helvetica*), but it is not present in TG, *Rickettsia canadensis*, or *Rickettsia belli* (Roux et al. 1996). The *ompB* region can be detected in all *Rickettsia* spp., except for *R. canadensis* and *R. belli* (Roux and Raoult 2000). Moreover, the *sca1* gene is present in at least 20 currently validated *Rickettsia* species (Ngwamidiba et al. 2006). Although this region has been less frequently reported as a PCR target than the *omp* genes, it can be useful for identification and phylogenetic analysis of these bacteria.

The guidelines for taxonomic classification and identification of a new rickettsial species suggest the characterization by, at least, these five genes: *rrs*, *gltA*, *ompA*, *ompB*, and *sca4* (Raoult et al. 2005). Regular PCR assays are frequently used for the characterization or detection of DNA of *Rickettsia* spp. from culture, arthropods, or eschar biopsies. However, the use of nested PCR technique for human specimens such as blood, buffy coat, or plasma with a low level of rickettsiemia is advisable to increase the analytical sensitivity (Fournier and Raoult 2004, Choi et al. 2005, De Sousa et al. 2005, Santibáñez et al. 2013) (Table 4). A comparative study on PCR detection for *Rickettsia* in different human and animal samples showed that nested PCR sensitivity depends not only on the type of the sample but also on the target gene (Santibáñez et al. 2013). The nested PCR technique should be performed by specialized and trained personnel and in specific laboratory rooms due to the risk of DNA amplicon contamination. Sequences obtained from positive amplicons must be edited (with a specific software) and compared with those available in the GenBank database from the National Center for Biotechnology Information (NCBI), <http://blast.ncbi.nlm.nih.gov/blast.cgi>

Real-time PCR for *Rickettsia* spp. and species-specific detection has been developed. This type of assays offers the advantages of speed, reproducibility, quantitative capability, and low risk of contamination compared to conventional PCR (Stenos et al. 2005, Wölfel et al. 2008, Angelakis et al. 2012) (Table 4).

To avoid contaminations, a “suicide” PCR was developed by the National Reference Laboratory for Rickettsioses in Marseille. This modified PCR was based on selecting two primer sequences for each assay that had never been previously used in the laboratory. The main disadvantage was the increase of the costs since each primer pair was thrown after a single reaction (Fournier and Raoult 2004).

The use of positive and negative controls is essential for PCR assays. DNA of a species that is not expected to be present in the area (e.g., *Rickettsia amblyommii* in Europe) is recommended as a template.

### **In Vitro Culture**

The isolation of *Rickettsia* spp. requires suitable techniques that must be performed only in specialized laboratories. Samples must be handled as highly pathogenic in biosafety level 3 laboratories (BSL-3), since it is unknown which rickettsia is present in the sample. *Rickettsia* spp. can be isolated from clinical specimens and from infected arthropods. The most useful human specimens are blood (total blood or buffy coat, collected preferentially on heparin or citrate) and swab eschars (La Scola and Raoult 1997, Bechah et al. 2011). Skin biopsies and sterile fluids (e.g., CSF) can be also used (Brouqui et al. 2004). Human specimens should be collected as soon as possible in the course of the disease and before the administration of effective antimicrobials if it is possible. It is recommended to freeze the samples at -80°C to preserve the viability of the bacteria if they are not immediately inoculated in cells (Angelakis et al. 2012). Since in vitro culture requires sterile conditions, it is advisable to use a closed blood collection system (e.g., vacutainer) to avoid contaminations with other bacteria and fungi that can complicate cell culture isolation attempts. Skin/eschar specimens should be disinfected for 10 min in 70% ethanol before inoculation in cell culture and then rinsed with sterile distilled water. For arthropods, an additional initial step for disinfection is recommended (Supplementary Data). Embryonated chicken eggs and animal inoculation have been widely used in the past to isolate and propagate *Rickettsia* spp. Nevertheless, nowadays, shell vial cell culture technique is the most disseminated methodology for *Rickettsia* isolation (Angelakis et al. 2012). The centrifugation-shell vial system, previously used for virus isolation, was adapted for the culture of *R. conorii* from human blood (Marrero and Raoult 1989). This technique is based on the inoculation of clinical specimens on confluent cell monolayer seeded in a shell vial tube (La Scola and Raoult 1996). The centrifugation step after the inoculation of the sample enhances the adhesion and the penetration of the bacteria in cells. The small surface area at the bottom of the tube allows enhancing the ratio of the number of bacteria to the number of cells for a more efficient recovery. The specimens are inoculated in the shell vial tube and centrifuged at 700 g for 45min to 1 h in the cell culture medium (minimal essential medium supplemented with 4% heatinactivated fetal calf serum and 2mM glutamine, without antibiotics) at 4°C. After centrifugation, the cell culture medium is discarded and one milliliter of fresh culture medium is added. Shell vials are incubated at 28–34°C depending on the selected cell line with or without 5% CO<sub>2</sub> atmosphere. *Rickettsia* culture in mammalian cell lines usually grows at 32°C. *Rickettsia* growth is usually detected by the cytopathic effect and Giménez staining and/or immunofluorescence assay revelation using specific polyclonal antibodies from immune animals after 7 days (range 3–30 days) of incubation of the shell vial (Gouriet et al. 2005, Lagier et al. 2015). A successful culture of *Rickettsia* spp. can be detected from 3 days to several weeks in some cases. The phenotypic characters for the identification of rickettsiae are insufficient. Consequently, definitive identification of the bacteria must be performed by PCR and sequencing. When rickettsiae growth is observed, subculture should be done. The shell vial is harvested and inoculated into 25 cm<sup>2</sup> flask to establish the isolate. In clinical practice, when there is a concern about significant sample contamination, antibiotics (0.2% penicillin–streptomycin) and 1% fungizone

(amphotericin B) can be added to the monolayer and removed after 48 h. According to some authors, the success of *Rickettsia* isolation is higher from skin biopsies than from blood (Vestris et al. 2003). However, the success of the culture clearly depends on the timing of the blood collection after the onset of the disease (3–5 days).

*Rickettsia* spp. can infect and grow in a variety of different cell types, although the most frequently used are Vero cells. The different cellular lines used for the isolation of *Rickettsia* spp. are shown in Table 5. *R. conorii* can grow faster when isolated in L929 compared to Vero-E6 cell line (Balraj et al. 2009). The temperature may be more important for the successful isolation of some *Rickettsia* species rather than the cell line (Milhano et al. 2010, Santibáñez et al. 2015). Some species such as *R. felis*, *Rickettsia monacensis*, or *Rickettsia raoultii* are only successfully isolated and maintained at 28°C. Arthropod-derived cell lines usually require richer medium with more supplements compared to mammalian cell lines.

Tick cell lines have the advantage to reproduce partially the natural environment of the rickettsiae and allow an incubation temperature ranged between 28 and 34°C. However, the culture of these cells is long and delicate. *Ixodes*, *Dermacentor*, and *Rhipicephalus sanguineus* tick cell lines are the most frequently used for isolation of *Rickettsia* spp. (Bell-Sakyi et al. 2007). Mosquito cell lines (C6/36) are permissive to multiple arthropod-borne pathogens, including *Rickettsia* from SFG and TG. These cells have been successfully used for the isolation of *R. felis*, *Rickettsia montanensis*, *Rickettsia peacockii*, and *Rickettsia typhi* (Uchiyama 2005, Horta et al. 2006, Lagier et al. 2015).

The isolation of *Rickettsia* is primordial to describe a new *Rickettsia* species. Culture remains also very important for the study of pathogen physiology, genetic descriptions, and antibiotic susceptibility and for the improvement of diagnostic tools (Parola et al. 2013). The plaque assay became the reference method to test the antibiotic susceptibility of rickettsiae (Rolain et al. 1998), but consistent results were also obtained with microplaque colorimetric assay and culture combined with quantitative PCR (Rolain et al. 2002). To date, the conception of an axenic medium enabling the growth of *Rickettsia* remains a challenge. The future development of these axenic media would allow significant progress, thus facilitating genetic manipulation and understanding the pathogenicity of *Rickettsia* spp. (Singh et al. 2013).

## Acknowledgments

This article has been prepared as a direct outcome of the networking under WG2 of the COST Action TD1303: EurNegVec—European Network for Neglected Vectors and Vector-Borne Infections.

## Author Disclosure Statement

No competing financial interests exist.

## References

- Alexandre N, Santos AS, Bacellar F, Boinas FJ, et al. Detection of *Rickettsia conorii* strains in Portuguese dogs (*Canis familiaris*). Ticks Tick Borne Dis 2011; 2:119–122.
- Angelakis E, Richet H, Rolain JM, La Scola B, et al. Comparison of real-time quantitative PCR and culture for the diagnosis of emerging Rickettsioses. PLoS Negl Trop Dis 2012; 6:e1540.
- Balraj P, Vestris G, Raoult D, Renesto P. Comparison of *Rickettsia conorii* growth within different cell lines by real-time quantitative PCR. Clin Microbiol Infect 2009; 15(Suppl 2):294–295.



- Bechah Y, Socolovschi C, Raoult D. Identification of rickettsial infections by using cutaneous swab specimens and PCR. *Emerg Infect Dis* 2011; 17:83–86.
- Bell-Sakyi L. *Ehrlichia ruminantium* grows in cell lines from four ixodid tick genera. *J Comp Pathol* 2004; 130:285–293.
- Bell-Sakyi L, Zwegarth E, Blouin EF, Gould EA, et al. Tick cell lines: Tools for tick and tick-borne disease research. *Trends Parasitol* 2007; 23:450–457.
- Brouqui P, Bacellar F, Baranton G, Birtles J, et al. Guidelines for the diagnosis of tick-borne diseases in Europe. *Clin Microbiol Infect* 2004; 10:1108–1132.
- Choi YJ, Jang WJ, Kim JY, Ryu JS, et al. Spotted fever group and typhus group rickettsioses in humans, South Korea. *Emerg Infect Dis* 2005; 11:237–244.
- De Sousa R, Ismail N, Doria-Nobrega S, Costa P, et al. The presence of eschars, but not greater severity, in Portuguese patients infected with Israeli spotted fever. *Ann N Y Acad Sci* 2005; 1063:197–202.
- Denison AM, Amin BD, Nicholson WL, Paddock CD. Detection of *Rickettsia rickettsii*, *Rickettsia parkeri*, and *Rickettsia akari* in skin biopsy specimens using a multiplex real-time polymerase chain reaction assay. *Clin Infect Dis* 2014; 59: 635–642.
- Dieme C, Bechah Y, Socolovschi C, Audoly G, et al. Transmission potential of *Rickettsia felis* infection by *Anopheles gambiae* mosquitoes. *Proc Natl Acad Sci U S A* 2015; 112: 8088–8093.
- Dumler JS, Gage WR, Pettis GL, Azad AF, et al. Rapid immunoperoxidase demonstration of *Rickettsia rickettsii* in fixed cutaneous specimens from patients with Rocky Mountain spotted fever. *Am J Clin Pathol* 1990; 93:410–414.
- Faccini-Martínez AA, García-Alvarez L, Hidalgo M, Oteo JA. Syndromic classification of rickettsioses: An approach for clinical practice. *Int J Infect Dis* 2014; 28:126–139.
- Fournier PE, Jensenius M, Laferl H, Vene S, et al. Kinetics of antibody responses in *Rickettsia africae* and *Rickettsia conorii* infections. *Clin Diagn Lab Immunol* 2002; 9:324–328.
- Fournier PE, Raoult D. Suicide PCR on skin biopsy specimens for diagnosis of rickettsioses. *J Clin Microbiol* 2004; 42: 3428–3434.
- Gouriet F, Fenollar F, Patrice JY, Drancourt M, et al. Use of shell-vial cell culture assay for isolation of bacteria from clinical specimens: 13 years of experience. *J Clin Microbiol* 2005; 43:4993–5002.
- Horta MC, Labruna MB, Durigon EL, Schumaker TT. Isolation of *Rickettsia felis* in the mosquito cell line C6/36. *Appl Environ Microbiol* 2006; 72:1705–1707.
- Jado I, Escudero R, Gil H, Jiménez-Alonso MI, et al. Molecular method for identification of *Rickettsia* species in clinical and environmental samples. *J Clin Microbiol* 2006; 44:4572–4576.
- Kidd L, Maggi R, Diniz PP, Hegarty B, et al. Evaluation of conventional and real-time PCR assays for detection and differentiation of spotted fever group *Rickettsia* in dog blood. *Vet Microbiol* 2008; 129:294–303.
- La Scola B, Raoult D. Diagnosis of Mediterranean spotted fever by cultivation of *Rickettsia conorii* from blood and skin samples using the centrifugation-shell vial technique and by detection of *R. conorii* in circulating endothelial cells: A 6-year follow-up. *J Clin Microbiol* 1996; 34:2722–2727.
- La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: Current approaches to diagnosis of old and new rickettsial diseases. *J Clin Microbiol* 1997; 35:2715–2727.

- La Scola B, Rydkina L, Ndiokubwayo JB, Vene S, et al. Serological differentiation of murine typhus and epidemic typhus using cross-adsorption and Western blotting. *Clin Diagn Lab Immunol* 2000; 7:612–616.
- Labruna MB, Pacheco RC, Nava S, Brandao PE, et al. Infection by *Rickettsia bellii* and *Candidatus "Rickettsia amblyommii"* in *Amblyomma neumanni* ticks from Argentina. *Microb Ecol* 2007; 54:126–133.
- Lagier JC, Edouard S, Pagnier I, Mediannikov O, et al. Current and past strategies for bacterial culture in clinical microbiology. *Clin Microbiol Rev* 2015; 28:208–236.
- Lepidi H, Fournier PE, Raoult D. Histologic features and immunodetection of African tick bite fever eschar. *Emerging Infect Dis* 2006; 12:1332–1337.
- Màrquez FJ, Muniain MA, Soriguer RC, Izquierdo G, et al. Genotypic identification of an undescribed spotted fever group Rickettsia in *Ixodes ricinus* from southwestern Spain. *Am J Trop Med Hyg* 1998; 58:570–577.
- Marrero M, Raoult D. Centrifugation-shell vial technique for rapid detection of Mediterranean spotted fever *Rickettsia* in blood culture. *Am J Trop Med Hyg* 1989; 40:197–199.
- Merhej V, Angelakis E, Socolovschi C, Raoult D. Genotyping, evolution and epidemiological findings of *Rickettsia* species. *Infect Genet Evol* 2014; 25:122–137.
- Milhano N, de Carvalho IL, Alves AS, Arrube S, et al. Coinfections of *Rickettsia slovaca* and *Rickettsia helvetica* with *Borrelia lusitaniae* in ticks collected in a Safari Park, Portugal. *Ticks Tick Borne Dis* 2010; 1:172–177.
- Munderloh UG, Jauron SD, Fingerle V, Leitritz L, et al. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. *J Clin Microbiol* 1999; 37:2518–2524.
- Ngwamidiba M, Blanc G, Raoult D, Fournier PE. Sca1, a previously undescribed paralog from autotransporter protein-encoding genes in *Rickettsia* species. *BMC Microbiol* 2006; 6:12.
- Paddock CD, Finley RW, Wright CS, Robinson HN, et al. *Rickettsia parkeri* rickettsiosis and its clinical distinction from Rocky Mountain spotted fever. *Clin Infect Dis* 2008; 47: 1188–1196.
- Paddock CD, Greer PW, Ferebee TL, Singleton J Jr, et al. Hidden mortality attributable to Rocky Mountain spotted fever: Immunohistochemical detection of fatal, serologically unconfirmed disease. *J Infect Dis* 1999; 179:1469–1476.
- Parola P, Paddock CD, Raoult D. Tick-borne rickettsioses around the world: Emerging diseases challenging old concepts. *Clin Microbiol Rev* 2005; 18:719–756.
- Parola P, Paddock CD, Socolovschi C, Labruna MB, et al. Update on tick-borne rickettsioses around the world: A geographic approach. *Clin Microbiol Rev* 2013; 26:657–702.
- Portillo A, Santibáñez S, García-Alvarez L, Palomar AM, et al. Rickettsioses in Europe. *Microbes Infect.* 2015; 17:834–838.
- Raoult D, Fournier PE, Ereemeeva M, Graves S, et al. Naming of Rickettsiae and rickettsial diseases. *Ann N Y Acad Sci* 2005; 1063:1–12.
- Regnery RL, Spruill CL, Plikaytis BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. *J Bacteriol* 1991; 173:1576–1589.
- Rolain JM, Maurin M, Vestris G, Raoult D. In vitro susceptibilities of 27 rickettsiae to 13 antimicrobials. *Antimicrob Agents Chemother* 1998; 42:1537–1541.

- Rolain JM, Stuhl L, Maurin M, Raoult D. Evaluation of antibiotic susceptibilities of three rickettsial species including *Rickettsia felis* by a quantitative PCR DNA assay. *Antimicrob Agents Chemother* 2002; 46:2747–2751.
- Roux V, Fournier PE, Raoult D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. *J Clin Microbiol* 1996; 34: 2058–2065.
- Roux V, Raoult D. Phylogenetic analysis of members of the genus *Rickettsia* using the gene encoding the outer-membrane protein rOmpB (ompB). *Int J Syst Evol Microbiol* 2000; 50: 1449–1455.
- Rozental T, Eremeeva ME, Paddock CD, Zaki SR, et al. Fatal case of Brazilian spotted fever confirmed by immunohistochemical staining and sequencing methods on fixed tissues. *Ann NY Acad Sci* 2006; 1078:257–259.
- Santibáñez S, Portillo A, Palomar AM, Bell-Sakyi L, et al. Isolation and maintenance of *Rickettsia raoultii* in a *Rhipicephalus sanguineus* tick cell line. *Microbes Infect* 2015; 17: 866–869.
- Santibáñez S, Portillo A, Santibáñez P, Palomar AM, et al. Usefulness of rickettsial PCR assays for the molecular diagnosis of human rickettsioses. *Enferm Infec Microbiol Clin* 2013; 31:283–288.
- Sekeyova Z, Roux V, Raoult D. Phylogeny of *Rickettsia* spp. inferred by comparing sequences of ‘gene D’, which encodes an intracytoplasmic protein. *Int J Syst Evol Microbiol* 2001; 51:1353–1360.
- Singh S, Eldin C, Kowalczywska M, Raoult D. Axenic culture of fastidious and intracellular bacteria. *Trends Microbiol* 2013; 21:92–99.
- Stenos J, Graves SR, Unsworth NB. A highly sensitive and specific real-time PCR assay for the detection of spotted fever and typhus group rickettsiae. *Am J Trop Med Hyg* 2005; 73: 1083–1085.
- Teyssie N, Raoult D. Comparison of Western immunoblotting and microimmunofluorescence for diagnosis of Mediterranean spotted fever. *J Clin Microbiol* 1992; 30:455–460.
- Uchiyama T. Growth of typhus group and spotted fever group rickettsiae in insect cells. *Ann N Y Acad Sci* 2005; 1063: 215–221.
- Vestris G, Rolain JM, Fournier PE, Birg ML, et al. Seven years’ experience of isolation of *Rickettsia* spp. from clinical specimens using the shell vial cell culture assay. *Ann N Y Acad Sci* 2003; 990:371–374.
- Walker DH, Ismail N. Emerging and re-emerging rickettsioses: Endothelial cell infection and early disease events. *Nat Rev Microbiol* 2008; 6:375–386.
- Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal DNA amplification for phylogenetic study. *J Bacteriol* 1991; 173:697–703.
- Wölfel R, Essbauer S, Dobler G. Diagnostics of tick-borne rickettsioses in Germany: A modern concept for a neglected disease. *Int J Med Microbiol* 2008; 298:368–374.
- Woods GL, Walker DH. Detection of infection or infectious agents by use of cytologic and histologic stains. *Clin Microbiol Rev* 1996; 9:382–404.

Address correspondence to:

Josè A. Oteo

Department of Infectious Diseases

Center of Rickettsioses and Arthropod-Borne Diseases

Hospital San Pedro-Center of Biomedical Research from La Rioja (CIBIR)

C/Piqueras, 98, Logroño, La Rioja 26006, Spain

E-mail: [jaoteo@riojasalud.es](mailto:jaoteo@riojasalud.es)

Table 1. *Rickettsia* spp. Present in Europe

<i>Rickettsia</i> species	Confirmed or potential vectors	Disease
<i>Rickettsia aeschlimannii</i>	<i>Hyalomma marginatum</i> , <i>Hyalomma anatolicum excavatum</i> , <i>Hyalomma rufipes</i> , <i>Haemaphysalis punctata</i> , <i>Haemaphysalis inermis</i> , <i>Ixodes ricinus</i> , <i>Rhipicephalus sanguineus</i> , <i>Rhipicephalus turanicus</i> , <i>Rhipicephalus bursa</i>	Unnamed
<i>Rickettsia conorii</i> subsp. <i>conorii</i>	<i>Rh. sanguineus</i>	Mediterranean spotted fever
<i>R. conorii</i> subsp. <i>indica</i>	<i>Rh. sanguineus</i>	Indian tick typhus
<i>R. conorii</i> subsp. <i>israelensis</i>	<i>Rh. sanguineus</i>	Israeli tick typhus
<i>R. conorii</i> subsp. <i>caspia</i>	<i>Rhipicephalus punilio</i> , <i>Rh. sanguineus</i>	Astrakhan fever
<i>Rickettsia helvetica</i>	<i>I. ricinus</i>	Unnamed
<i>Rickettsia massiliae</i>	<i>Rh. sanguineus</i> , <i>Rh. turanicus</i>	Unnamed
<i>Rickettsia monacensis</i>	<i>I. ricinus</i>	Unnamed
<i>Rickettsia sibirica</i> subsp. <i>mongolitimonae</i>	<i>Hy. anatolicum</i> , <i>Rhipicephalus pusillus</i> , <i>Hy. marginatum</i>	LAR
<i>Rickettsia slovacae</i>	<i>Dermacentor marginatus</i> , <i>Dermacentor reticulatus</i>	DEBONEL/TIBOLA/SENLAT <sup>a</sup>
<i>Rickettsia raoultii</i>	<i>D. marginatus</i> , <i>D. reticulatus</i>	DEBONEL/TIBOLA/SENLAT <sup>a</sup>
<i>Rickettsia felis</i>	<i>Ctenocephalides felis</i>	Flea borne spotted fever
<i>Rickettsia typhi</i>	<i>Xenopsylla cheopis</i> , <i>C. felis</i>	Murine or endemic typhus
<i>Rickettsia akari</i>	<i>Liponyssoides sanguineus</i>	Rickettsialpox
<i>Rickettsia prowazekii</i>	<i>Pediculus humanus corporis</i>	Epidemic typhus
<i>Rickettsia africae</i> <sup>b</sup>	<i>Amblyomma variegatum</i> , <i>Amblyomma hebraeum</i>	African tick bite fever
<i>Rickettsia hoogstraalii</i>	<i>Ha. punctata</i> , <i>Haemaphysalis sulcata</i>	Only detected in ticks
Candidatus <i>Rickettsia rioja</i>	<i>D. marginatus</i>	DEBONEL/TIBOLA/SENLAT <sup>a</sup>
Candidatus <i>Rickettsia barbariae</i> ( <i>Rickettsia</i> PoTiRb 169)	<i>Rh. bursa</i> , <i>Rh. turanicus</i>	Only detected in ticks
<i>Rickettsia</i> sp. strain Davousti	<i>I. ricinus</i> , <i>Ixodes lividus</i>	Only detected in ticks
Candidatus <i>Rickettsia kotlanii</i>	Ixodid tick	Only detected in ticks
Candidatus <i>Rickettsia siciliensis</i>	<i>Rh. turanicus</i>	Only detected in ticks
Candidatus <i>Rickettsia vini</i>	<i>Ixodes arboricola</i> , <i>I. ricinus</i>	Only detected in ticks
Candidatus <i>Rickettsia tarasevichiae</i>	<i>Ixodes persulcatus</i>	Unnamed
<i>Rickettsia lusitaniae</i> sp. nov	<i>Ornithodoros erraticus</i>	Only detected in soft ticks

<sup>a</sup>Tick-borne lymphadenopathy/Dermacentor-borne necrosis erythema lymphadenopathy/scalp eschar and neck lymphadenopathy.

<sup>b</sup>*R. africae* could be present in travellers who return from endemic areas (Sub-Saharan Africa and Guadalupe Island) and it has been detected in the European area of Turkey.

LAR, lymphangitis-associated rickettsiosis.

Table 2. Preservation and Storage of Samples for Detection of *Rickettsia* spp. (and for Tick Identification When Applicable)

<i>Specimen</i>	<i>Collection method</i>	<i>Time and transport temperature</i>	<i>Preservation</i>	<i>Microbiological assay</i>
Whole blood/buffy coat	EDTA or citrate tube (3–5 mL)	<24 h, 2–8°C >24 h, at least –20°C	>24 h, at least –20°C	PCR
Whole blood/buffy coat	Heparin tube (3–5 mL)	>24 h, dry ice	To process immediately or freeze –80°C	Culture
Serum/plasma	Serum separator tube/ anticoagulant tube	<24 h, 2–8°C	>24 h, at least –20°C	IFA/PCR
Other body fluids (CSF, pleural fluid) (not preferred specimens)	Sterile tube	<24 h, 2–8°C >24 h, at least –20°C	>24 h, at least –20°C To process immediately or freeze –80°C	PCR Culture
Skin or eschar biopsy and autopsy organ tissue	Sterile tube Tissue should be sent dry	>24 h, at least –20°C >24 h, dry ice	>24 h, at least –20°C To process immediately or freeze –80°C	PCR Culture
Eschar swab	Sterile tube. Swab should be sent dry	24–72 h, 2–8°C	2–8°C	PCR/culture
Tick	Tube	24–48 h, 2–8°C <sup>a</sup> >48 h, at least –20°C >48 h, 70%/absolute ethanol >48 h, dry ice	>48 h, at least –20°C >48 h, at least –20°C >48 h, 70%/absolute ethanol To process immediately or freeze –80°C	PCR/culture PCR/culture PCR PCR/culture
Formalin-fixed tissue paraffin-embedded tissue	Tube/cassette	Room temperature	Room temperature	PCR/IHC
Hemolymph	Slide	Immediately, Room temperature	Room temperature	PCR/stain

<sup>a</sup>If prevented from drying out, live ticks can be kept at 2–8°C for several days.

EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; IFA, indirect immunofluorescence assay; IHC, immunohistochemical assay.

Table 3. DNA Extraction Methods

<i>Specimen</i>	<i>Method</i>	<i>Comments</i>
Blood (whole blood, buffy coat, plasma, and serum)	Commercial kits: DNeasy <sup>®</sup> Blood kit (Qiagen) or similar (manual or automated) <sup>a</sup>	High quality DNA Fast and reproducible Very expensive
Other body fluids (CSF, pleural fluid)	Commercial kits: QIAamp DNA kit (Qiagen) or similar (manual or automated) <sup>a</sup>	High quality DNA Fast and reproducible Very expensive
Skin or eschar biopsies, eschar swabs, and internal organs	Commercial kits: DNeasy Tissue kit (Qiagen) or similar (manual or automated) <sup>a</sup>	High quality DNA Fast and reproducible Very expensive
Ticks <sup>b</sup> /Hemolymph/Portion of a tick leg <sup>c</sup>	Commercial kits: DNeasy Tissue kit or similar (manual or automated) <sup>a</sup>	High quality DNA Fast and reproducible Very expensive
	Ammonium hydroxide	Fast and simple Low-cost method Many variations exist
	Phenol and chloroform	High-quality DNA Time-consuming Expensive Potentially health hazardous chemical Modified version with isothiocyanate

<sup>a</sup>Automated systems can be also used: MagCore nucleic acid extraction (MagCore), NucliSens Easymag (Biomerieux), or similar.

<sup>b</sup>Adult ticks are individually processed (half specimen cut lengthwise), and nymphs and larvae are processed in pools (for prevalence studies).

<sup>c</sup>Only for tick identification studies (not for *Rickettsia* infection studies in ticks).

Table 4. PCR Protocols for DNA Detection of *Rickettsia* spp. in Clinical Samples and Ticks.

Target gene	Primers (nucleotide sequence 5'-3')	Method	Amplicon size (bp)	Reference
<i>rrs</i>	Id1: AGAGTTTGATCTGGCTCAG Rel6S.452n: AACGTCATTAATCTCTCTTGC	Single PCR	426	Weisburg et al. (1991)
<i>htrA</i>	17 kDa-1: GCCTTGGCAACTTCATGT 17 kDa-2: CATTGTCGTCAGGTTGGCA 17 kDa-3: GCTTACAAATCTAAAAACCATATA 17 kDa-5: TGTCTATCAATTCACAACTTGCC	Single PCR	434	Labruna et al. (2007)
<i>ompA</i>	Rr190.70p: ATGGGGAATATTTCTCCAAA Rr190.701n: GTTCCGTTAATGGCAGCATCT Rr190.70p: ATGGGGAATATTTCTCCAAA Rr190.602n: AGTGCAGCATCTGCTCCCT 107F: GCITTTATTCACCACTCAAC 299R: TRATCACCAACCGTAAAGTAAAT FW1: CGCAGCGATAATGCTGAGTA RV2: CACCAAAACCATGATTTGCAG	Single PCR Seminested PCR (with primers Rr190.70p and Rr190.701n) Single or nested PCR (with primers Rr190.70p and Rr190.701n) Single or nested PCR (with primers Rr190.70p and Rr190.602n)	631 532 212 397	Roux et al. (1996) Regnery et al. (1991) Kidd et al. (2008) Alexandre et al. (2011); De Sousa et al. (2005)
<i>ompB</i>	120-M59: CCGCAGGTTGGTAACCTGC 120-807: CCTTTAGATTACCGCTAA 120-2788: AAACAATAATCAAGGTACTGT 120-3599: TACTTCGGTTACAGCAAAAGT rompB-OF: GTAACGGGAAGTAATCGTTTCGTAA rompB-OR: GCTTATAAACCAGCTAAACCACC rompB SFG-IF: GTTTAAATAGCTGCTGCTAACCA rompB TG-IF: AAGATCCTTCGTGATGTGCAACA rompB SFG/TG-IR: GTTTGGCCATATACCAATAAG	Single PCR Single PCR Single PCR Single or nested PCR (with primers rompB-OF and rompB-OR)	833 765 511 420	Roux and Raoult (2000) Roux and Raoult (2000) Choi et al. (2005) Choi et al. (2005)
<i>gltA</i>	CS 415: GCTATTA TGCTTGGGCTGT CS 1220: TGCATTTCTTCCATTTGTC RpCS.877p: GGGGGCTGCTCACGGCGG RpCS.1258n: ATTGCAAAAAGTACAGTGAA RpCS.896p: GGCTAATGAAAGCATGATA RpCS.1235n: GCGACGGTATACCCATAGC CS-78: GCAAGTATCGGTGAGGATGTAAT CS-323: GCTTCTTAAAATTCATAAATCAGGAT CS-239: GCTTCTTCATCCTATGGCTATTAT CS-1069: CAGGTCTTCGTGCAITTCIT PanRick_2_for: ATAGGACAACCGTTTATTT PanRick_2_rev: CAAACATCATATGCAGAAA PanRick_3_laq-FAM: CCTGATAATCGTTAGATTTTACCG-TMR	Single PCR Single PCR Single or nested PCR (with primers RpCS.877p and RpCS.1258n) Single PCR Single PCR Real-time PCR	806 381 337 401 834 70	De Sousa et al. (2005) Regnery et al. (1991) Choi et al. (2005) Labruna et al. (2007) Labruna et al. (2007) Wölfel et al. (2008)
<i>scaI</i>	FIMAX: AAGAGGTYTRTGGATGGGT RMAX: GAYAAATATATTATTCITTC	Single PCR	488	Ngwamidiba et al. (2006)
<i>scaH</i>	DIF: ATGAGTAAAAGACGGTAACTT D928r: AAGCTATGCGTCACTCCG	Single PCR	928	Sekeyova et al. (2001)
23S-5S RNA	RCK/23-5-F: GATAGGTCRGTGTGGAAGCAC RCK/23-5-R: TCGGGAYGGGATCGTGTGTTTC	Single PCR	388	Jado et al. (2006)



Table 5. Commonly Used and Potentially Useful Cell Lines for *Rickettsia* spp. Isolation and Cultivation

Cell-line	Medium and supplementation	Culture conditions
VERO E6 (ATCC 1008) Type: Epithelial Origin: <i>Cercopithecus aethiops</i>	MEM 5–10% heat-inactivated FBS 2 mM L-glutamine, nonessential aminoacids	Cells grow as adherent monolayer Incubation at 37°C with or without 5% CO <sub>2</sub> atmosphere
L929 (ATCC CCL-1) Type: Fibroblast Origin: <i>Mus musculus</i>	DMEM or MEM 5% or 2% <sup>a</sup> FBS 2 mM L-glutamine, nonessential aminoacids	Cells grow as adherent monolayer Incubation at 37°C with or without 5% CO <sub>2</sub> atmosphere
HUVEC (ATCC CRL-1730™) Type: Endothelial Origin: Umbilical vein from human	Endothelial cell basal medium 10% or 5% <sup>a</sup> FBS 2 mM L-glutamine	Cells grow as adherent monolayer Incubation at 37°C with 5% CO <sub>2</sub> atmosphere
XTC Type: Epithelial Origin: <i>Xenopus laevis</i>	Leibovitz medium L-15 5% or 2% <sup>a</sup> FBS 2% tryptose phosphate broth	Cells grow as adherent monolayer Incubation at 28°C without CO <sub>2</sub> atmosphere
C6/36 (ATCC CRL-1660™) Type: Mosquito cell line Origin: <i>Aedes albopictus</i>	L-15 medium 5% FBS	Cells grow as adherent monolayer Incubation at 28°C with or without 5% CO <sub>2</sub> atmosphere
ISE6 (ATCC CRL-11974) Type: Tick cell line Origin: <i>Ixodes scapularis</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 10% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow in loosely adhered layers Incubation at 32°C (at 34°C <sup>a</sup> ) in sealed container, under normal atmospheric conditions
RML/RSE <sup>b</sup> Type: Tick cell line Origin: <i>Rhipicephalus sanguineus</i>	Mixture of L-15 (Leibovitz) medium and MEM (Bell-Sakyi 2004) 15% FBS 20% Tryptose phosphate broth 2 mM L-glutamine	Cells grow in loosely adhered layers Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
ANE 58 <sup>b</sup> Type: Tick cell line Origin: <i>Dermacentor (Anocentor) nitens</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 5% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow predominantly in suspension Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
DAE 100T <sup>b</sup> Type: Tick cell line Origin: <i>Dermacentor andersoni</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 5% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow predominantly in suspension Incubation at 28–32°C, in sealed container, under normal atmospheric conditions

(continued)

<i>Cell-line</i>	<i>Medium and supplementation</i>	<i>Culture conditions</i>
DAE 15 <sup>b</sup> Type: Tick cell line Origin: <i>D. andersoni</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 5% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow in loosely adhered layers Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
DALBE 3 <sup>b</sup> Type: Tick cell line Origin: <i>Dermacentor</i> <i>albipictus</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 5% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow predominantly in suspension Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
DVE 1 <sup>b</sup> Type: Tick cell line Origin: <i>Dermacentor</i> <i>variabilis</i> embryo derived	L-15B300 medium (Munderloh et al. 1999) 5% FBS 5% Tryptose phosphate broth 0.1% Bovine lipoprotein concentrate 2 mM L-glutamine Additional supplementation <sup>a</sup> : 0.1% NaHCO <sub>3</sub> 10mM HEPES Adjust to pH 7.5	Cells grow in loosely adhered layers Incubation at 28–32°C, in sealed container, under normal atmospheric conditions

<sup>a</sup>For isolation attempts and infected cell propagation.

<sup>b</sup>Available from the Tick Cell Biobank, <http://tickcells.pirbright.ac.uk>

Uninfected cultures can be maintained with antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin).

DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MEM, minimum essential media.