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Guidelines for the Detection of *Rickettsia* spp.

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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 α-Proteobacteria; order Rickettsiales; family Rickettsiaceae) includes Gramnegative, 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 intothe 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-Martìnez 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 R*ickettsia* 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) 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 immunoalkaline phosphatase and immunoperoxidase 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 antispecies-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 ‡ 128 and IgM titers ‡ 32 is considered indicative of infection by *R. conorii*, whereas for nonendemic countries, IgGtiters ‡ 64 and IgMtiters ‡ 32 are considered indicative of infection by *Rickettsia* spp. (Brouqui et al. 2004). Single titers of IgG ‡ 64 or IgM ‡ 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 (Teysseire 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, Màrquez 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 isadvisable 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_2 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 cm2 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).

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Table 1. *Rickettsia* spp. Present in Europe

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

[a]Tick-borne lymphadenopathy/Dermacentor-borne necrosis erythema lymphadenopathy/scalp eschar and neck lymphadenopathy.

[b]*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)

Specimen	*Collection method*	*Time and transport temperature*	*Preservation*	*Microbiological assay*
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[a] >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

[a]If prevented from drying out, live ticks can be kept at 2–8C 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

Specimen	*Method*	*Comments*
Blood (whole blood, buffy coat, plasma, and serum)	Commercial kits: DNeasy® Blood kit (Qiagen) or similar (manual or automated)[a]	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)[a]	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)[a]	High quality DNA Fast and reproducible Very expensive
Ticks[b]/Hemolymph/Portion of a tick leg[c]	Commercial kits: DNeasy Tissue kit or similar (manual or automated)[a]	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

[a]Automated systems can be also used: MagCore nucleic acid extraction (MagCore), NucliSens Easymag (Biomerieux), or similar.

[b]Adult ticks are individually processed (half specimen cut lengthwise), and nymphs and larvae are processed in pools (for prevalence studies).

[c]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*
rrs	fD1: AGAGTTTGATCCTGGCTCAG Rc16S.452n: AACGTCATTATCTTCCTTGC	Single PCR	426	Weisburg et al. (1991)
htrA	17 kDa-1: GCTCTTGCAACTTCTATGTT 17 kDa-2: CATTGTTCGTCAGGTTGGCA	Single PCR	434	Labruna et al. (2007)
	17 kDa-3: GCTTTACAAAATTCTAAAAACCATATA 17 kDa-5: TGTCTATCAATTCACAACTTGCC	Single PCR	549	Labruna et al. (2007)
ompA	Rr190.70p: ATGGCGAATATTTCTCCAAAA Rr190.701n: GTTCCGTTAATGGCAGCATCT	Single PCR	631	Roux et al. (1996)
	Rr190.70p: ATGGCGAATATTTCTCCAAAA Rr190.602n: AGTGCAGCATTCGCTCCCCCT	Seminested PCR (with primers Rr190.70p and Rr190.701n)	532	Regnery et al. (1991)
	107F: GCTTTATTCACCACCTCAAC 299R: TRATCACCACCGTAAGTAAAT	Single or nested PCR (with primers Rr190.70p and Rr190.701n)	212	Kidd et al. (2008)
	FW1: CGCAGCGATAATGCTGAGTA RV2: CACCAAAACCATGATTTGCAG	Single or nested PCR (with primers Rr190.70p and Rr190.602n)	397	Alexandre et al. (2011); De Sousa et al. (2005)
ompB	120-M59: CCGCAGGGTTGGTAACTGC 120–807: CCTTTTAGATTACCGCCTAA	Single PCR	833	Roux and Raoult (2000)
	120–2788: AAACAATAATCAAGGTACTGT 120–3599: TACTTCCGGTTACAGCAAAGT	Single PCR	765	Roux and Raoult (2000)
	rompB-OF: GTAACCGGAAGTAATCGTTTCGTAA rompB-OR: GCTTTATAACCAGCTAAACCACC	Single PCR	511	Choi et al. (2005)
	rompB SFG-IF: GTTTAATACGTGCTGCTAACCAA rompB TG-IF: AAGATCCTTCTGATGTTGCAACA rompB SFG/TG-IR: GGTTTGGCCCATATACCATAAG	Single or nested PCR (with primers ropmB-OF and ropmB-OR)	420	Choi et al. (2005)
gltA	CS 415: GCTATTATGCTTGCGGCTGT CS 1220: TGCATTTCTTTCCATTGTGC	Single PCR	806	De Sousa et al. (2005)
	RpCS.877p: GGGGGCCTGCTCACGGCGG RpCS.1258n: ATTGCAAAAAGTACAGTGAACA	Single PCR	381	Regnery et al. (1991)
	RpCS.896p: GGCTAATGAAGCAGTGATAA RpCS.1233n: GCGACGGTATACCCATAGC	Single or nested PCR (with primers RpCS.877p and RpCS.1258n)	337	Choi et al. (2005)
	CS-78: GCAAGTATCGGTGAGGATGTAAT CS-323: GCTTCCTTAAAATTCAATAAATCAGGAT	Single PCR	401	Labruna et al. (2007)
	CS-239: GCTCTTCTCATCCTATGGCTATTAT CS-1069: CAGGGTCTTCGTGCATTTCTT	Single PCR	834	Labruna et al. (2007)
	PanRick_2_for: ATAGGACAACCGTTTATTT PanRick_2_rev: CAAACATCATATGCAGAAA PanRick_3_taq–FAM: CCTGATAATTCGTTAGATTTTACCG–TMR	Real-time PCR	70	Wölfel et al. (2008)
sca1	F1MAX: AAGAGGTYTRTGGATGCGT RMAX: GAYAATATATTATTYTCTTTC	Single PCR	488	Ngwamidiba et al. (2006)
sca4	D1f: ATGAGTAAAGACGGTAACCT D928r: AAGCTATTGCGTCATCTCCG	Single PCR	928	Sekeyova et al. (2001)
23S-5S RNA	RCK/23–5-F: GATAGGTCRGRTGTGGAAGCAC 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: *Cercopithecus aethiops*	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_2 atmosphere
L929 (ATCC CCL-1) Type: Fibroblast Origin: *Mus musculus*	DMEM or MEM 5% or 2%[a] FBS 2 mM L-glutamine, nonessential aminoacids	Cells grow as adherent monolayer Incubation at 37°C with or without 5% CO_2 atmosphere
HUVEC (ATCC CRL-1730™) Type: Endothelial Origin: Umbilical vein from human	Endothelial cell basal medium 10% or 5%[a] FBS 2 mM L-glutamine	Cells grow as adherent monolayer Incubation at 37°C with 5% CO_2 atmosphere
XTC Type: Epithelial Origin: *Xenopus laevis*	Leibovitz medium L-15 5% or 2%[a] FBS 2% tryptose phosphate broth	Cells grow as adherent monolayer Incubation at 28°C without CO_2 atmosphere
C6/36 (ATCC CRL-1660™) Type: Mosquito cell line Origin: *Aedes albopictus*	L-15 medium 5% FBS	Cells grow as adherent monolayer Incubation at 28°C with or without 5% CO_2 atmosphere
ISE6 (ATCC CRL-11974) Type: Tick cell line Origin: *Ixodes scapularis* 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[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Cells grow in loosely adhered layers Incubation at 32°C (at 34°C[a]) in sealed container, under normal atmospheric conditions
RML/RSE[b] Type: Tick cell line Origin: *Rhipicephalus sanguineus*	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[b] Type: Tick cell line Origin: *Dermacentor (Anocentor) nitens* 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[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Cells grow predominantly in suspension Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
DAE 100 T[b] Type: Tick cell line Origin: *Dermacentor andersoni* 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[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Cells grow predominantly in suspension Incubation at 28–32°C, in sealed container, under normal atmospheric conditions

(continued)

Cell-line	*Medium and supplementation*	*Culture conditions*
DAE 15[b] Type: Tick cell line Origin: *D. andersoni* 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[a]: 0.1% $NaHCO_3$ 10 mM 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[b] Type: Tick cell line Origin: *Dermacentor albipictus* 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[a]: 0.1% $NaHCO_3$ 10 mM 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[b] Type: Tick cell line Origin: *Dermacentor variabilis* 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[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Cells grow in loosely adhered layers Incubation at 28–32°C, in sealed container, under normal atmospheric conditions

[a]For isolation attempts and infected cell propagation.

[b]Available from the Tick Cell Biobank, http://tickcells.pirbright.ac.uk

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

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