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Vector-Borne and Zoonotic Diseases

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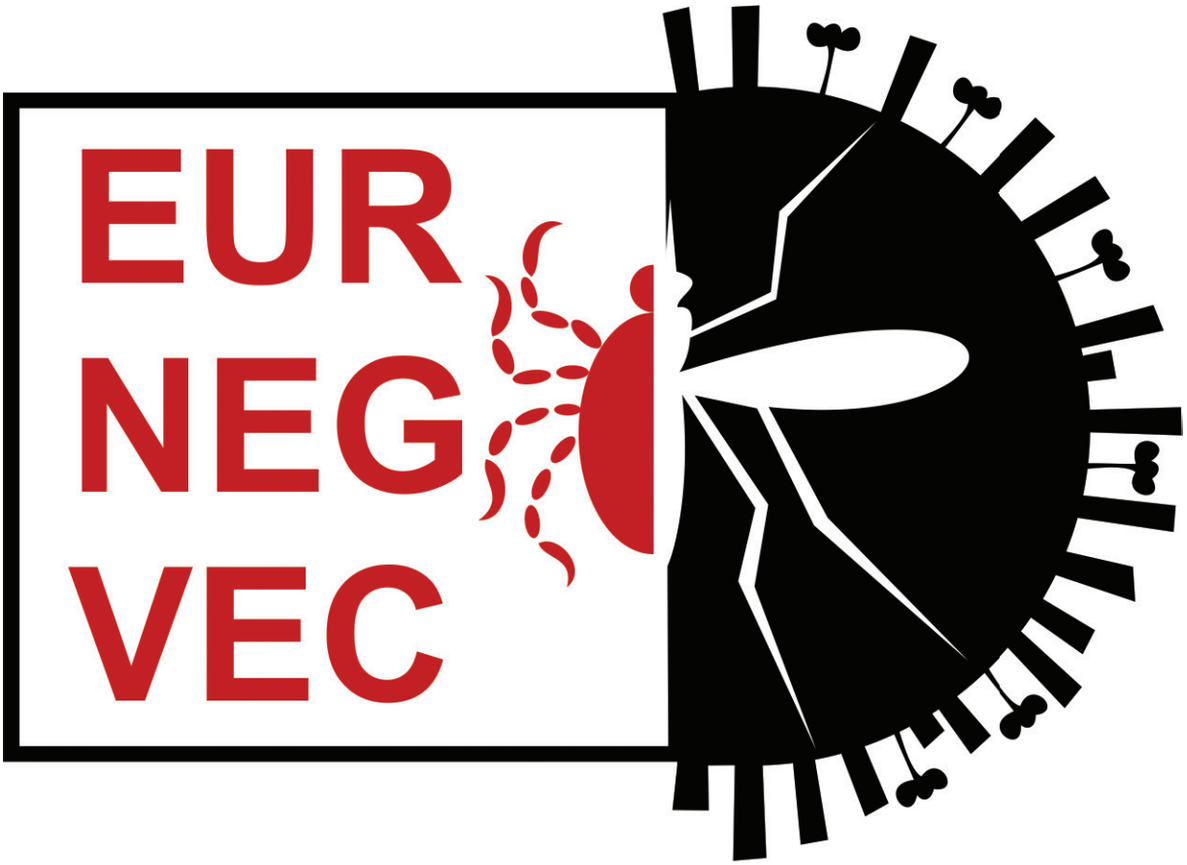
Guidelines Issue



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EDITORIAL

European Network for Neglected Vectors and Vector-Borne Infections COST Action Guidelines: What Is This About and What Is This For?

Remi N. Charrel,[1,2] Laetitia Lempereur,[3] Andrei D. Mihalca,[4] and Muriel Vayssier-Taussat[5]

COST IS THE NETWORKING MECHANISM to support European Cooperation in Science and Technology between member countries and beyond. The main objective of the COST TD1303 action (European Network for Neglected Vectors and Vector-Borne Infections, EurNegVec, http://eurnegvec.org) is to establish a powerful transboundary network of partner institutions across Europe that are involved in education and research related to arthropod-transmitted infectious diseases of man and animals. This network addresses the growing importance of vector-borne diseases at a time of global change, all integrated under the one health concept, and reflecting the complexity and demands of current high-end research. Currently the action includes 34 participating countries, with representatives from 58 institutions, assigned as experts in five working groups (WGs). In addition, there are 10 more international partner countries associated with the action such as United States, South Africa, Brazil, or Argentina.

Among the five WGs that constitute EurNegVec COST action, the WG2 (comprising 80 members from 26 countries), dedicated to ''Barcoding, molecular diagnosis, and next-generation sequencing,'' has taken the initiative to produce these extensive guidelines that are being published together in a special issue of *Vector-Borne and Zoonotic Disease*. A total of 58 international experts have been involved in the writing of these guidelines.

Despite the recent advances and technological progress, the detection of vector-borne pathogens remains highly challenging and is frequently problematic for newcomers in the field, such as PhD students and postdoctoral fellows. Therefore, we have selected different pathogens for which detection remains highly challenging and for which expertise was available among the participants of the WG2. Short notes ($\sim$2000 words) focusing on the different techniques allow direct detecting of vector-borne pathogens. Each publication includes a short fact sheet, different technical approaches for direct detection of vector-borne pathogens (this part will include the pros and cons of each technique), gaps of knowledge, and expert recommendations.

The guidelines are intended to support young researchers in the field of vector-borne and zoonotic diseases. They do not aim at being exhaustive, but rather practical for field activities and laboratory or experimental work. The topics selected for these nine guidelines were based on the long-standing expertise of WG2 members. They do not intend to cover all the fields of interest and to fill all the gaps. Five guidelines are focusing on bacteria, two on sandfly-borne viruses, and two on protozoan parasites.

When possible, young scientists were in charge of collating the bibliographic knowledge and were coached by more experienced researchers for providing experience-based information that is rarely published because it looks like cooking recipes or because it is considered as know-how and is hardly divulged.

We have designed these minireviews as operational guidelines. We do hope that they will help young researchers (and less young) who are beginning in the field of vector-borne pathogens to use techniques that work without getting lost, using the tricks that are not given in conventional publications and avoiding those that do not work.

Address correspondence to:
Remi Charrel
Unite des Virus Emergents
Faculte de Medecine de Marseille
27, Boulevard Jean Moulin
13005 Marseille cedex 05
France

E-mail: remi.charrel@univ-amu.fr

[1]U190-IRD French Institute of Research for Development, U1207-INSERM Institut National de la Santé et de la Recherche Médicale, EHESP French School of Public Health, EPV UMR_D 190 ''Emergence des Pathologies Virales,'' & IHU Méditerranée Infection, APHM Public Hospitals of Marseille, Aix Marseille University, Marseille, France.
[2]Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.
[3]Faculty of Veterinary Medicine, Center for Fundamental and Applied Research for Animal and Health (FARAH), Laboratory of Parasitology and Parasitic Diseases, University of Liege, Liege, Belgium.
[4]Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine, Cluj Napoca, Romania.
[5]UMR BIPAR, Animal Health Laboratory, ANSES, INRA, ENVA, Maisons Alfort Cedex, France.

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Diagnosing Borreliosis

Sally J. Cutler,[1] Nataliia Rudenko,[2] Maryna Golovchenko,[2] Wibke J. Cramaro,[3] Josiane Kirpach,[3] Sara Savic,[4] Iva Christova,[5] and Ana Amaro[6]

Abstract

Borrelia species fall into two groups, the Borrelia burgdorferi sensu lato (Bbsl) complex, the cause of Lyme borreliosis (also known as Lyme disease), and the relapsing fever group. Both groups exhibit inter- and intraspecies diversity and thus have variations in both clinical presentation and diagnostic approaches. A further layer of complexity is derived from the fact that ticks may carry multiple infectious agents and are able to transmit them to the host during blood feeding, with potential overlapping clinical manifestations. Besides this, pathogens like Borrelia have developed strategies to evade the host immune system, which allows them to persist within the host, including humans. Diagnostics can be applied at different times during the clinical course and utilize sample types, each with their own advantages and limitations. These differing methods should always be considered in conjunction with potential exposure and compatible clinical features. Throughout this review, we aim to explore different approaches providing the reader with an overview of methods appropriate for various situations. This review will cover human pathogenic members of Bbsl and relapsing fever borreliae, including newly recognized *Borrelia miyamotoi* spirochetes.

Keywords: Lyme disease, *Borrelia* species, diagnosis, relapsing fever

Introductory Remarks

Borrelia species fall into two groups, the *Borrelia burgdorferi* sensu lato (Bbsl) complex, the cause of Lyme borreliosis (LB; also known as Lyme disease [LD]), and the relapsing fever group. Both groups exhibit inter- and intraspecies diversity and thus have variations in both clinical presentation and diagnostic approaches. A further layer of complexity is derived from the fact that ticks may carry multiple infectious agents and are able to transmit them to the host during blood feeding, with potential overlapping clinical manifestations. Besides this, pathogens like *Borrelia* have developed strategies to evade the host immune system, which allows them to persist within the host, including humans.

Appropriateness of different diagnostics vary with both time, clinical course and causative species, each additionally having strengths and limitations. These differing methods should always be considered in conjunction with potential exposure and compatible clinical features. Throughout this review, we aim to explore different approaches providing the reader with an overview of methods appropriate for various situations. This review will cover human pathogenic members of Bbsl and relapsing fever borreliae, including newly recognized *Borrelia miyamotoi* spirochetes.

Detection of *Borrelia* in the Arthropod Vector

Various methods can be applied to detect the presence of *Borrelia* in vectors. Widely used approaches that demonstrate significant sensitivity, specificity and reliability include the following: multiple formats of PCRs, mostly nested PCR that target different genomic loci, selection of which depends on the sample origin (template); reverse-line blotting, based on hybridization of amplified selected *Borrelia* genes with spirochete-specific probes; and multilocus sequences analysis and multilocus sequence typing, based on the sequence analysis of amplified fragments of spirochete genome or microscopy with stained spirochetes in tick midgut or salivary glands (Aguero-Rosenfeld et al. 2005, Margos et al. 2011). The most recently applied techniques

[1]School of Health, Sport & Bioscience, University of East London, London, United Kingdom.
[2]Biology Centre CAS, Institute of Parasitology, Ceske Budejovice, Czech Republic.
[3]Department of Infection and Immunity, Luxembourg Institute of Health, Esch-sur-Alzette, Luxembourg.
[4]Scientific Veterinary Institute ''Novi Sad,'' Rumenacki put 20, Novi Sad, Serbia.
[5]Department of Microbiology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.
[6]National Institute for Agrarian and Veterinarian Research (INIAV), Lisboa, Portugal.

include next-generation sequencing (NGS) and proteomic approaches.

Cultivation of *Borrelia* in commercial Barbour-Stoenner-Kelly (BSK) or home-made modified Kelly- Pettenkoffer (MKP) media, which for a long time is considered to be a gold standard in LB diagnostics, is still widely used, but is rather time-consuming and challenging. The culture-negative cases do not necessarily mean the absence of spirochetes in a sample. The failure to culture the spirochetes might be caused by multiple vector-, spirochete-, media-, or cultivation condition-related factors (Cerar et al., 2008, Ružić-Sabljić et al. 2014, Rudenko et al., 2016).

Nowadays, the priority of all used techniques is redirected from simple detection of pathogen in either environmental sample or clinical sample, to simultaneous detection and identification of spirochete species (or possible coinfection agents). Considering the high possibility of the presence of multiple pathogens in tick vectors, the other question is whether to use singleplex or multiplex formats for their detection/identification. Fluidic microarrays allow the assessment of multiple tick-borne pathogens simultaneously (Vayssier-Taussat et al. 2013).

Use of proteomic methods to detect presence of some relapsing fever *Borrelia* in the hemolymph of ticks provides additional options for borrelial detection in vectors (Fotso Fotso et al. 2014).

These methods provide invaluable research tools and facilitate epidemiological studies, but their clinical relevance is debatable. Detection of a pathogen in the vector does not imply that it has been successfully transmitted to the host upon which the tick has fed. Transmission dynamics are complex and multifactorial, and beyond the scope of this review. Home-use diagnostic kits are available and allow individuals to test collected ticks for the presence of Lyme borreliae. The reliability of these tests has been highly debated. Tick bites are frequently unnoticed and might only demonstrate that you have been in a risk environment, but do not necessarily correlate with any infectious consequences. That is why, the use of such tests is of limited value for diagnosis, but can be useful for epidemiological studies.

Recommendation

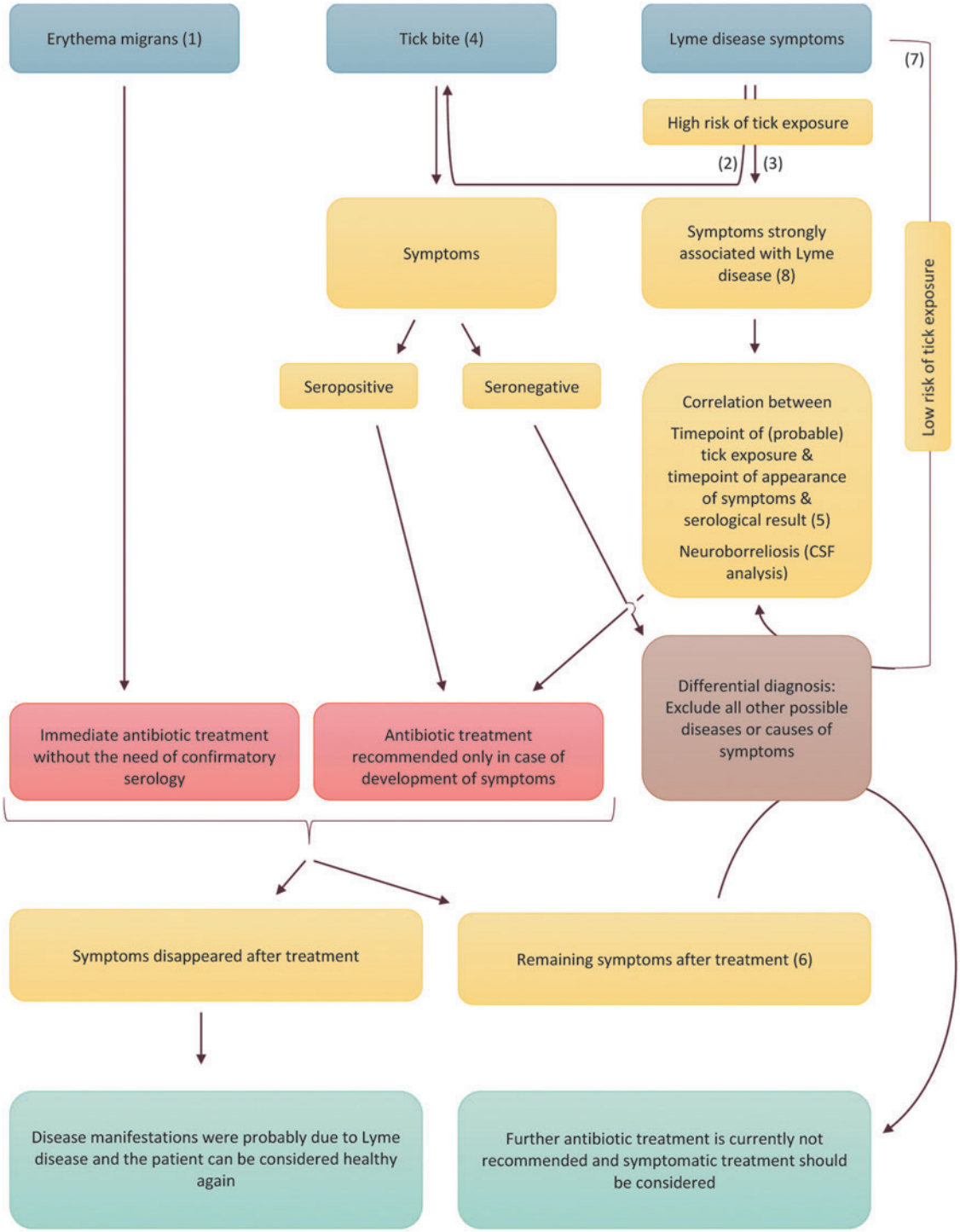
Tick testing as supportive data for identification of LB endemic regions; correct selection of PCR target based on the final goal of tests and sample nature; reanalysis of tested sample targeting different genomic loci; and to consider the

FIG. 1. No diagnostic tests currently exist that provide a yes or no result for acute Lyme borreliosis (LB), thus clinical signs still remain the major factor for deciding whether antibiotic treatment is necessary. In case of unclear symptoms, the risk of tick exposure and serological tests should be considered to support the diagnosis. Represented in *blue* are three possible scenarios for which LB should be considered: the patient presents with the characteristic skin manifestation erythema migrans (EM) or a recent tick bite. A third possibility is that the patient's symptoms might be compatible with LB. As can be readily deduced from this schematic representation (*yellow*: clinical decision), EM is the least complicated case and should be treated (*red*) immediately without need for further testing. The situation gets more complicated if the patient cannot remember a tick bite [which can occur in up to 2/3 of cases (Hofhuis et al. 2013)] and/or has nonspecific symptoms. *Green*: Final outcome. (1) EM: Incubation time between 3 days and 1 month. *Red* skin lesion that might, in some cases, be associated with slight itching or burning and that expands around the site of the tick bite. EM can be distinguished from a simple tick bite-induced irritation of the skin by the fact that it has a minimum diameter of 5 cm. EM is often associated with nonspecific symptoms like fatigue, headache, fever, or malaise and can occur at different locations on the same patient (multiple EM) (Godar et al. 2015). (2) In case a patient presents with symptoms that have been associated with, but are not clearly specific, for Lyme disease (LD), an assessment of the risk of prior tick exposure should be done. For this purpose, the following questions might be considered: Does the patient pay attention to ticks? Did the patient maybe notice in the recent past an itching and scratched something small off from his body? Does the patient have pets that often have ticks? How much time does the patient spend outdoors in the green? Has the patient recently been on holidays in a risk area? Season or weather conditions supporting high activity of ticks (might also be interesting to exclude other possible infections)? (3) Try to estimate, based on the symptoms (early or late stage), the time point of infection and check if the season and/or weather conditions have been such that at the possible time point that infection ticks might have been active. Ticks are active during wet, not too hot, seasons of the year. For more information on factors affecting tick activity please refer to reference (Medlock et al. 2013). (4) If a patient shows up with a tick bite, appropriate and early removal of the tick can prevent transmission of LD, however, since the transmission efficiency and kinetics depends on the *Borrelia* strain (Crippa et al. 2002), an early transmission cannot reliably be excluded (Kahl et al. 1998), and the patient should be monitored for the development of symptoms and treatment considered only if such appear. In case the tick has been damaged or removed late, a short-term prophylactic antibiotic (oral or cutaneous) treatment might be considered (Warshafsky et al. 2010, Piesman and Hojgaard 2012, Piesman et al. 2014). However, due to the small time period during which this method is efficacious and the high number of patients who need to be treated for a successful outcome (Hofhuis et al. 2013), controversial opinions exist on this procedure. (5) Please consider, in this study, the fact that patients are not necessarily protected after a first course of LD and reinfection can occur (Nadelman and Wormser 2007, Khatchikian et al. 2014, Shapiro 2015). In this case, the interpretation of serological results might be complicated. (6) In case of persistent flu-like symptoms after appropriate treatment of EM, consider coinfections with other tick-borne pathogens (Godar et al. 2015). Make sure that treatment has been done in the correct way, otherwise consider retreatment with an appropriate method. In case of a post-treatment chronic course of LD, other possible reasons for the symptoms should be excluded. (7) Make sure that the symptoms have only occurred after potential exposure to a tick bite and they did not already exist before the tick exposure. In case of nonspecific disease manifestations, ask the patient if he might recall symptoms similar to EM in the past. (8) To have a better overview of the symptoms that are frequently associated with LD consult for example Stanek et al. (2012) and Koedel, et al. (2015).

presence of coinfection with multiple pathogens as highly possible.

Clinical Diagnosis of LB and Supportive Diagnostic Strategies

A reliable clinical diagnosis of LB is only evident to the nonexpert physician when a typical erythema migrans (EM) is present (Stanek and Strle 2003). Since the large majority of LB symptoms have minimal diagnostic value because of their lack of specificity, diagnosis of LB might be challenging for general practitioners in patients without EM (Strle and Stanek 2009). Generally, there exists a tendency toward overdiagnosis of chronic LD (Sigal 1996, Koedel et al. 2015, Czupryna et al. 2016). Although different diagnostic approaches (mentioned later) have been explored, to date, the only recommended supportive tests used are serological confirmation. Serological results alone are insufficient to distinguish whether the patient suffers from an acute or reinfection that needs treatment, or is only seropositive because of a past infection. This might be especially problematic for individuals who are frequently



exposed to ticks and therefore have a high risk of reinfection. However, even in low-risk areas, the positive predictive value of serological tests can be very low (Lantos et al. 2015), meaning that, clinical manifestations still remain crucially important criteria for a reliable diagnosis of the disease. Factors that need to be integrated for a reliable diagnosis are therefore the occurrence of compatible symptoms, serological results, and risk of tick exposure. Figure 1 provides an overview of different important steps in the diagnosis of LB.

To Date, Only Serological Tests are Recommended to Support the Diagnosis of LB in the Absence of EM

In cases where EM is clearly evident, serological tests are not needed and treatment should start immediately (Stanek et al. 2012). In patients who do not develop EM, serological tests are recommended to support the diagnosis (Aguero-Rosenfeld et al. 2005). Initial problems with the specificity and sensitivity of serological tests have resulted in controversial statements on their efficacy to support diagnosis of acute LB. Recently, serological tests have been optimized switching from a single *Borrelia* strain cell extract to the use of a combination of more precisely chosen recombinant antigens or synthetic peptides (Fang Ting et al. 2000, Goettner et al. 2005). Previously, a two-tier test approach, in which the presence of antibodies is first tested by a highly sensitive ELISA and, in case of a positive result, further confirmed by a highly specific immunoblot, was recommended (Branda et al. 2010, Koedel et al. 2015).

Noteworthy, the reported accuracy of ELISAs and immunoblots varies throughout Europe and a recent study revealed no overall benefit of two-tiered tests over single tests (Leeflang et al. 2016). Only early-stage patients (symptoms <6 weeks) might still be seronegative, as they have not developed antibodies yet. Therefore, diagnosis of LB should be reevaluated in seronegative late-stage patients (Stanek et al. 2012). Low antibody titers have been observed after antibiotic treatment, indicating that the induced B cell immune response is probably not very long-lived and robust. Especially, patients where *Borrelia* took longer to disseminate seem to develop long-lived antibody titers less efficiently (Hammers-Berggren et al. 1994, Aguero-Rosenfeld et al. 1996, Nowakowski et al. 2003, Elsner et al. 2015). Recent mouse studies have shown that *Borrelia* have a direct effect on the mouse B cell response (Hastey et al. 2012, 2014, Elsner et al. 2015a, 2015b). However, the underlying mechanism in humans requires further investigation. Showing the induction of strain-specific immunity (but not cross-protective), mouse and human studies together (Khatchikian et al. 2014) may explain reinfection of LB. Consequently, previous *Borrelia* infections must be taken into account when considering serological testing (Nadelman and Wormser 2007).

Despite the described improvement of these tests, we still face the problem of nonstandardization and inappropriate application of current serological tests (Ang et al. 2011, Muller et al. 2012, Markowicz et al. 2015, Leeflang et al. 2016). Different (in-house) assays and result interpretation remain a major problem (Fallon et al. 2014) that should be solved in the future by the implementation of a universal and worldwide (or Europe/USA wide) diagnostic standard test, or as a minimum, use of internationally agreed standards and participation in quality control schemes. However, the problem remains (especially among high-risk groups) to distinguish between an acute and a resolved infection. Future studies should therefore focus on the development of new strategies that would allow a yes or no result.

Noteworthy, serological tests should not be used as a proof of efficacy of the antibiotic treatment. Although antibody titers generally decrease after antibiotic treatment; however, patients may remain seropositive for years after the infection in the absence of active disease (Hammers-Berggren et al. 1994, Aguero-Rosenfeld et al. 1996, Lomholt et al. 2000, Kalish et al. 2001, Glatz et al. 2006, Kowalski et al. 2010). Instead, the disappearance of symptoms is a more reliable sign of cure.

When neuroborreliosis is suspected, detection of intrathecally produced anti-*Borrelia* antibodies significantly supports the diagnosis. However, results might be negative at early stages and more often in children (Christen et al. 1993). Measurement of *Borrelia*-specific antibodies in cerebrospinal fluid (CSF) cannot be used to assess the efficiency of treatment (Koedel et al. 2015).

Since antibiotic treatment is generally considered efficient, differential diagnosis is crucial in case of a chronic course of the disease (Halperin 2015, 2016, Hjetland et al. 2015, Markowicz et al. 2015, Rebman et al. 2015, Wills et al. 2016). A chronic course has been observed in patients infected by *Borrelia*, viral and nonviral pathogens, such as Epstein–Barr virus (glandular fever), *Coxiella burnetii* (Q fever), or Ross River virus (epidemic polyarthritis) (Hickie et al. 2006, Galbraith et al. 2011, Aucott et al. 2013, Katz and Jason 2013), and the underlying causes are not clear. In this context, the general health status and/or the lifestyle of the patient should also be considered. In general, immunocompromised or otherwise not completely healthy patients might be at higher risk to develop chronic symptoms after treatment. Patients with hematological malignancies, for example, seem to suffer more often from disseminated disease and more frequently require retreatment (Maraspin et al. 2015). In nonimmunocompromised cases, where symptoms continue to persist even after appropriate antibiotic treatment, it is currently not recommended to prolong the treatment. Clinical studies have shown that the risk of side effects outweighs any potential therapeutic benefits (Klempner et al. 2001, Krupp et al. 2003, Koedel et al. 2015). In these cases, coinfections with other tick-borne diseases or other possible causes of the symptoms should be excluded (Belongia 2002, Swanson et al. 2006, Berghoff 2012, Godar et al. 2015) and symptomatic treatment considered (Koedel et al. 2015). Only in late neuroborreliosis is prolongation of the antibiotic treatment justifiable in cases of persistent CSF lymphocytic pleocytosis (Koedel et al. 2015).

In rare cases, *Borrelia* can cause problems with the heart and vascular system, and might be considered an underlying cause of stroke-like symptoms in patients who otherwise have no obvious risk for cardiovascular diseases (Zajkowska et al. 2015, Allen and Jungbluth 2016). Full description of LB clinical manifestations and their diagnosis have been recently reviewed by Stanek et al. (2011).

When encountering a tick bite, correct and early removal of the tick is a good way to reduce probability of infection. In Europe, only about 2% (Wilhelmsson et al. 2016) and in USA, about 1% (Heymann and Ellis 2012) of patients

bitten by a tick develop LB. Detection of spirochete DNA in ticks alone does not necessarily mean successful pathogen transmission, which is why the value of this test has limited diagnostic value for LB (ESGBOR 2013), but is useful for epidemiological studies (Reye et al. 2010) to define risk areas. In this context, NGS is a new emerging technique that allows screening of the same tick in parallel for various tick-borne pathogens, with the potential of getting more detailed information about coinfections of ticks and identification of new, yet unrecognized pathogens (Vayssier-Taussat et al. 2013, Michelet et al. 2014). As transmission of *Borrelia* (and indeed other pathogens) depends on the length of tick attachment, measurement of scutal and coxial indexes can indicate the duration of attachment (Kahl et al. 1998, Crippa et al. 2002, Gray et al. 2005, Meiners et al. 2006, Tijsse-Klasen et al. 2011). In the absence of an EM and the presence of other LB-related symptoms, seroconversion can be used for supportive diagnosis. However, in the absence of symptoms, seroconversion is no indication for antibiotic treatment as a study in a Swiss risk group demonstrated that only 2% of patients who seroconverted developed clinical LB (Fahrer et al. 1991). Thus, as tick bite is a poor predictor of disease, treatment is advisable only upon appearance of LB symptoms.

Recommendation

Clinical diagnosis alone, given a history of potential exposure and presence of EM, can be sufficient; however, clinical interpretation should generally be made in conjunction with supporting laboratory findings to reach a reliable diagnosis.

Alternative Strategies Explored for the Diagnosis of LB but Not on the List of Recommended Tests (ECDC 2016)

Direct detection of *Borrelia* in the peripheral blood, other body fluids, or tissues by microscopy or molecular methods can be used as strong additional evidence in the diagnosis of LB, but might have limited significance when used alone (Aguero-Rosenfeld et al. 2005). The sensitivity of PCR on skin biopsies is significantly higher than some other molecular tools; however, recognition of the EM itself is the best diagnosis for LB (Aguero-Rosenfeld et al. 2005). Nevertheless, this provides useful research data regarding strain prevalence and virulence, and provides insights into deciphering pathogenesis of LB (Strle et al. 2013). Cultivation of *Borrelia* from patient samples might be an alternative method to detect viable *Borrelia*, but is both time-consuming and challenging (Rudenko et al. 2016). As such, cultivation is best reserved as a research tool.

Lymphocyte transformation tests (LTT) have been explored for their potential to overcome the diagnostic gap in LB patients without EM, but before seroconversion and in reinfected seropositive patients. This assay measures lymphocyte proliferation *in vitro* after stimulation with *B. burgdorferi*-specific antigens. Currently, results are contradictory and consequently LTT is not recommended as a routine diagnostic tool (Mygland et al. 2010, Dessau et al. 2014). T cell ELISPOT is another *in vitro* stimulation assay currently explored and improved (Jin et al. 2013). More direct methods measuring peripheral blood levels of specific cell subpopulations (CD57+) cells (Marques et al. 2009) or antigen-reactive cells (Tario et al. 2015) by flow cytometry and direct measure of CXCL13 levels in the CSF or metabolites within serum (Molins et al. 2015) are also not at a point yet to be used reliably for clinical diagnosis. CD57 cell counts seem not to be reliable as a validation study found no difference between patients and healthy controls (Marques et al. 2009). Demonstration of CSF CXCL13 as an activation marker is not specific for LB, its absence is believed to have some value in excluding neuroborreliosis (Rupprecht et al. 2014), and it might become a valuable supportive tool to estimate treatment efficiency in case of neuroborreliosis (Senel et al. 2010, Schmidt et al. 2011, Koedel et al. 2015). Problems with human leukocyte antigen (HLA) types and identification of epitopes for antigen-specific T cell staining are challenges that need to be addressed to validate the potential of *Borrelia*-specific T cell counts in peripheral blood to support diagnosis of LB. Metabolite measurement is a future strategy under investigation, but needs further validation.

Generally, the detection of *Borrelia* DNA within ticks as well as other methods discussed above should be considered valuable research tools providing useful information about the epidemiology of tick-borne diseases in general and LB particularly. As with serological methods, their value is lower when used alone. Combination of diagnostic tests and clinical signs provides a more robust and timely diagnosis of disease. In any case, interpretation of test results and clinical diagnosis of LB remains controversial and should currently be restricted to experts.

Development and application of new molecular tools allow the detection and differentiation among LB or relapsing fever spirochetes, clearly separating *B. burgdorferi* sensu lato spirochetes from recently described *B. miyamotoi* (Margos et al. 2008, Rudenko et al. 2009, Venczel et al. 2015). Combination of multilocus PCR with electrospray ionization and mass spectrometry has recently been investigated for the detection and genotyping of *Borrelia* species in whole blood (Eshoo et al. 2012).

Recommendation

These tests are valuable research tools providing useful information about the patient's immune response, but interpretation for clinical diagnosis has not been clearly shown and should currently be restricted to specialized laboratories.

Diagnostics Within Symptomatic Animals

Veterinary infections are less well-documented and benefit from laboratory confirmation to ensure correct diagnosis. This is particularly important as EM lesions have not been reported in animals and clinical signs are often common to several pathologies. As for human cases, serology is the primary diagnostic approach used, sometimes supported by the use of PCR. Despite the absence of EM, cardiac and neurological signs and lameness have been reported among companion animals (Hovius et al. 1999, Krupka and Straubinger 2010, Agudelo et al. 2011). Most veterinary cases have focused upon lameness in dogs with positive serology, although this does not necessarily establish borrelial causality for this condition. Rapid immunochromatographic tests are often used in veterinary private practice to aid diagnosis; however, these assays have not necessarily undergone the

rigorous quality control applied to human serodiagnostic tests (Savić et al. 2010).

Relapsing Fever Diagnostics

Clinical diagnosis of relapsing fever infections

In general, the clinical presentation of relapsing fever borreliosis is significantly distinct from that of LB. The possible exception to this being the appearance of a skin rash that challenges the previously believed ''pathognomonic'' EM, caused by the borrelial agent carried by *Amblyomma americanum* ticks in the United States, known as STARI (Masters et al. 2008, Borchers et al. 2015).

Human infection by recently described *B. miyamotoi* usually results in fever and associated flu-like signs (headache, chills, fatigue, myalgia), occasionally with neurological complications such as meningoencephalitis (Fonville et al. 2014, Krause et al. 2015).

Relapsing fever, as its name suggests, results in relapsing febrile episodes interspersed by afebrile periods. This is often accompanied by jaundice, muscle pain, headaches, and sometimes involvement of major organs (Borgnolo et al. 1993). This clinical picture can often be mistaken for other infections such as malaria that tend to overlap geographically in many endemic regions (Lundqvist et al. 2010).

Laboratory Diagnostics for Relapsing Fever

Microscopy

Although for LB, microscopy is not suitably sensitive for detection, this has been the diagnostic gold standard for detection of many relapsing fever spirochetes. Darkfield examination of unstained wet preparations, Giemsa or silver-stained blood or tissue sections, or immunofluorescence methods has been successfully used. Despite its frequent use, even relapsing fever can be difficult to detect using microscopy with some species such as *B. crocidurae* typically producing lower blood burdens than others, like *B. duttonii*. For such cases, a centrifugation step to concentrate the sample can be beneficial (Larsson and Bergström 2008). Furthermore, detection is restricted to times of febrile episodes when spirochetes are present at detectable levels. On a cautionary note, various artifacts can share the size and helical shape of spirochetes when viewed by darkfield microscopy, but tend to not show the typical gyrating spirochete-characteristic movement. Microscopy will not provide information regarding the infecting species.

Recommendation

Microscopic methods lack both sensitivity and specificity, but can add value when used in conjunction with other methods. Sample concentration can offer distinct benefits.

Cultivation

Cultivation methods for detection of *Borrelia* have been particularly challenging, some members of the genus being particularly refractory to cultivation (Cutler et al. 1994), while others are cultivable, but only in a complex medium. Huge advances were made with the formulation of BSK medium with a commercial variant BSK-H supporting the growth of LB strains (Barbour 1984). Relapsing fever strains appear more diverse in their requirements. *B. miyamotoi* for instance appears to prefer the MKP medium (Wagemakers et al. 2014) or high serum concentrations (Margos et al. 2015). On a cautionary note, these preferences might reflect batch variations of composite ingredients that can vastly influence performance of these ''home-made'' media (Cutler personal observation). Collectively, cultivation should be considered a low-yield procedure, but vital for recovery of much-needed strains for research purposes (Ružić-Sabljić et al. 2014).

Animal inoculation or xenodiagnosis (allowing infected ticks to feed upon a test animal) has been used for primary recovery of isolates before cultivation in an axenic medium (Schwan et al. 2012, Naddaf et al. 2015). It must be remembered that some species are refractory to growth in most animal models, such as *B. recurrentis*.

Recommendation

Cultivation is low yield, time-consuming, and expensive, and thus poorly suited to support diagnosis. Nevertheless, it still has a vital role for recovery of isolates for research purposes.

Serological Diagnosis

For the relapsing fever group, specific serology can be undertaken using GlpQ protein as antigen. GlpQ is absent from LB species, thus facilitating its specificity for diagnostic purposes (Fritz et al. 2013). Alternatively, BipA can also serve as a differential antigen present in relapsing fever spirochetes, but absent from the LB group (Lopez et al. 2010). As acutely presenting patients may not have had sufficient time for seroconversion, serology is best reserved for retrospective diagnosis.

Polymerase Chain Reaction

PCR provides a valuable diagnostic approach in acutely ill patients (Mediannikov et al. 2014). This overcomes the poor sensitivity of microscopy and can either be used to diagnose relapsing fever borreliosis or to further characterize the infecting spirochete. The absence of *GlpQ* in LB species makes it a specific target for detection of relapsing fever spirochetes (Takano et al. 2014). Other assays can either speciate specific relapsing fever borreliae or be designed to detect a single member of the relapsing fever clade such as *B. miyamotoi* (Elbir et al. 2013, Reiter et al. 2015). The limitation of this approach is having an appropriate sample that is likely to contain spirochetal DNA. Blood collected during febrile episodes and CSF samples have given good results (Gugliotta et al. 2013). Furthermore, in highly relapsing fever endemic areas, it is possible to have positive PCR results unrelated to current clinical pathology (Cutler et al. 2010).

Recommendation

PCR can provide useful supporting information, but multiple available assays must be properly standardized, and are hampered by sample timing, type, and quality.

Next-Generation Sequencing

NGS offers huge potential and data have only recently been forthcoming, limiting comprehensive appraisal at this stage. With the exception of dermatoborreliosis, in this study,

the challenge is which diagnostic sample type to investigate for LB in the absence of focal lesions. Sensitivity can be further improved, especially among high levels of host DNA. Care should be taken to avoid bias when using target enhancement strategies to amplify low-copy-number targets. Data analysis represents an additional computational challenge. NGS methods combined with bioinformatics tools might overcome the limitations of culture-connected techniques or some molecular protocols. However, the extreme diversity of spirochetes from *B. burgdorferi* sensu lato complex reduces the usefulness of NGS as it does not differentiate the pathogenic to human spirochete strains from those that were never connected with human LB. In addition, this offers a means of assessing rank abundance, evolving genomic profiles such as those corresponding to vector adaptations (Gatzmann et al. 2015), and fluctuations over time providing valuable insights into host–microbial interactions (Strandh and Råberg 2015).

To date, enrichment techniques can only partially overcome sensitivity problems caused by the giant excess of host DNA (vector, endosymbiont, and other microbial DNA) compared to the low proportion of target DNA (borrelial DNA in ticks is <0.01% of total DNA within field-collected nymphal ticks) (Carpi et al. 2015). This can impact, upon successful detection, only about a third of infected ticks revealing positive *Borrelia* NGS data (Carpi et al. 2015).

Recommendations

NGS offers huge potential and data have only recently been forthcoming, limiting comprehensive appraisal at this stage. Sensitivity can be further improved, especially among high levels of host DNA. Care should be taken to avoid bias when using target-enhancement strategies to amplify low-copy-number targets. Data analysis represents an additional computational challenge.

Fact Sheets and Resources

Several excellent fact sheets have been produced by ECDC to provide information on LB and tick-borne relapsing fever. Furthermore, more specific resources can be obtained from European study group for LB (ESGBOR; www.escmid.org/research_projects/study_groups/esgbor).

Knowledge Gaps and Future Perspectives

The poor sensitivity of direct detection methods coupled with the poor predictive value of indirect serological methods, particularly in less typical clinical presentations, presents a significant diagnostic challenge. Serology is further challenged by the requirement for sufficient time in order for the host to produce antibody responses to enable detection. Detection of the host response to infections provides a particularly attractive prospect for LB where organism loads are typically low. Indeed, levels of CXCL13 have shown promise for neuroborreliosis, but require further validation (Senel et al. 2010, Schmidt et al. 2011). It is possible that signature biomarker profiles might have value, but whether this would vary too much between individuals or indeed with differing genetic variants of borreliae awaits investigation. Another diagnostic approach under exploration is based on targeted proteomics. By selected reaction monitoring mass spectrometry, specific *Borrelia* proteins can be detected and quantified in skin biopsies (Schnell et al. 2015). The powerful new emerging technologies provide insights into our understanding of the dynamic interactions of borreliae with their vector, host, and other organisms, with the possibility of disclosing opportunities for future intervention.

Concluding Remarks

During these brief guidelines, we have attempted to highlight the strengths and limitations of various diagnostic methods used to diagnose borrelial infection. No single approach is suitably robust for this purpose, thus making interpretation challenging. Furthermore, laboratory diagnostics need to be viewed in conjunction with potential exposure and compatible clinical features.

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Address correspondence to:
Sally J. Cutler
School of Health, Sport & Bioscience
University of East London
London E15 4LZ
United Kingdom

E-mail: s.cutler@uel.ac.uk

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Guidelines for the Direct Detection of *Anaplasma* spp. in Diagnosis and Epidemiological Studies

Cornelia Silaghi,[1,*] Ana Sofia Santos,[2,*] Jacinto Gomes,[3] Iva Christova,[4] Ioana Adriana Matei,[5] Gernot Walder,[6] Ana Domingos,[7] Lesley Bell-Sakyi,[8] Hein Sprong,[9] Friederike D. von Loewenich,[10] José A. Oteo,[11] José de la Fuente,[12,13] and J. Stephen Dumler[14,15]

Abstract

The genus *Anaplasma* (Rickettsiales: Anaplasmataceae) comprises obligate intracellular Gram-negative bacteria that are mainly transmitted by ticks, and currently includes six species: *Anaplasma bovis*, *Anaplasma centrale*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma platys,* and *Anaplasma ovis.* These have long been known as etiological agents of veterinary diseases that affect domestic and wild animals worldwide. A zoonotic role has been recognized for *A. phagocytophilum*, but other species can also be pathogenic for humans. *Anaplasma* infections are usually challenging to diagnose, clinically presenting with nonspecific symptoms that vary greatly depending on the agent involved, the affected host, and other factors such as immune status and coinfections. The substantial economic impact associated with livestock infection and the growing number of human cases along with the risk of transfusion-transmitted infections, determines the need for accurate laboratory tests. Because hosts are usually seronegative in the initial phase of infection and serological cross-reactions with several *Anaplasma* species are observed after seroconversion, direct tests are the best approach for both case definition and epidemiological studies. Blood samples are routinely used for *Anaplasma* spp. screening, but in persistently infected animals with intermittent or low-level bacteremia, other tissues might be useful. These guidelines have been developed as a direct outcome of the COST action TD1303 EURNEGVEC (''European Network of Neglected Vectors and Vector-Borne Diseases''). They review the direct laboratory tests (microscopy, nucleic acid-based detection and in vitro isolation) currently used for *Anaplasma* detection in ticks and vertebrates and their application.

Keywords: *Anaplasma* spp., direct diagnosis, in vitro isolation, microscopy, PCR, ticks, vertebrate hosts

[1]National Center for Vector Entomology, Vetsuisse Faculty, University of Zurich, Zurich, Switzerland.
[2]Center for Vector and Infectious Diseases Research, National Institute of Health Doutor Ricardo Jorge, Águas de Moura, Portugal.
[3]Animal Health and Production Unit, National Institute for Agrarian and Veterinary Research, Oeiras, Portugal.
[4]Department of Microbiology, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria.
[5]Department of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Agricultural Sciences and Veterinary Medicine Cluj-Napoca, Cluj-Napoca, Romania.
[6]Department of Hygiene, Medical Microbiology and Social Medicine, Innsbruck Medical University, Innsbruck, Austria.
[7]Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical, Universidade Nova de Lisboa, Lisboa, Portugal.
[8]The Pirbright Institute, Ash Road, Pirbright, Woking, Surrey, United Kingdom.
[9]Center for Infectious Disease Control, National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands.
[10]Department of Medical Microbiology and Hygiene, University of Mainz, Medical Center, Mainz, Germany.
[11]Infectious Diseases Department, Center of Rickettsioses and Arthropod-Borne Diseases, Hospital San Pedro- CIBIR, Logroño, Spain.
[12]SaBio. Instituto de Investigación de Recursos Cinegéticos, IREC-CSIC-UCLM-JCCM, Ciudad Real, Spain.
[13]Department of Veterinary Pathobiology, Center for Veterinary Health Sciences, Oklahoma State University, Stillwater, Oklahoma.
[14]Departments of Pathology and Microbiology and Immunology, University of Maryland, School of Medicine, Baltimore, Maryland.
[15]Department of Pathology, Uniformed Services University for the Health Sciences ''America's Medical School,'' Bethesda, Maryland.
*These authors contributed equally to this work.

Introduction

Members of the genus *Anaplasma* (Rickettsiales: Anaplasmataceae) are non-motile, obligate intracellular Gram-negative bacteria residing in membrane-bound cytoplasmic vacuoles of the host cell where they form agglomerates called morulae (Latin *mōrus* = mulberry). Following the reorganization of the order Rickettsiales in 2001, the genus *Anaplasma* contains at least six species: *Anaplasma bovis*, *Anaplasma centrale*, *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Anaplasma platys,* and *Anaplasma ovis* (Dumler et al. 2001, 2005). In future, recently identified agents such as *Anaplasma odocoilei* and *Anaplasma capra* could be included (Tate el al. 2013, Li et al. 2015). *Anaplasma* spp. are etiologic agents of veterinary diseases affecting domestic ruminants, equines, dogs, and cats worldwide. *A. phagocytophilum* is also regarded as an emerging human pathogen with growing importance in the Northern Hemisphere (Jin et al. 2012, Bakken and Dumler 2015). Reports of human infections caused by other species such as *A. platys*, *A. ovis,* and the putative *A. capra,* suggest a broader medical relevance of this taxon (Chochlakis et al. 2010, Maggi et al. 2013, Arraga-Alvarado et al. 2014, Breitschwerdt et al. 2014, Li et al. 2015).

Anaplasma exhibit a biological cycle involving infection of both invertebrate and vertebrate hosts. Ticks are regarded as primary vectors, with different cell types targeted by these agents in a replication cycle, including invasion of salivary glands and transmission in saliva released during blood feeding. Alternative transmission routes include mechanical transfer by other hematophagous arthropods or fomites such as contaminated veterinary instruments, and transfusion-transmitted infections (Leiby and Gill 2004, Aubry and Geale 2011, Renneker et al. 2013, Shields et al. 2015). *Anaplasma* spp. display unique cell tropisms in vertebrate hosts and depending on the species, different cells of the hematopoietic lineage are specifically infected (erythrocytes, monocytes/macrophages, granulocytes, or platelets). The most important biological, ecological, and epidemiological features of *Anaplasma* spp. are shown in Table 1.

Anaplasmosis is a challenging disease in terms of diagnosis because clinical presentation may vary greatly depending on the agent involved, the affected host, and other factors such as immune status and coinfections (Kocan et al. 2010, Gaunt et al. 2010, Aubry and Geale 2011, Renneker et al. 2013, Bakken and Dumler 2015). The substantial economic impact associated with livestock infection, the zoonotic potential, and the risk of transfusion-transmitted infection, determines the need for accurate direct laboratory tests. Although histopathological investigations can provide suggestive diagnoses, and immunohistochemical stains can provide more definitive information, these guidelines review the most frequent and currently used methods for the direct detection of *Anaplasma* in ticks, humans, and other animals and their application in laboratory diagnosis and surveillance.

Microscopy

In vertebrates, microscopical observation of blood smears has traditionally been used for diagnosing clinical anaplasmosis and to a lesser extent for surveillance purposes, for example, wildlife screening and identification of reservoirs.

Peripheral blood smears directly prepared after fingerstick or superficial vein puncture or from venous blood collected into anticoagulant, obtained during the early acute phase of symptoms and before initiation of effective antimicrobial therapy, are best for visualization of bacteria in both animals and humans. This time frame for sample collection is crucial for all direct tests, including molecular detection and in vitro cultivation, as it covers the stage of infection when sufficient numbers of bacteria are present in the circulating blood. For leukocytotropic species (*A. bovis* and *A. phagocytophilum*), buffy coat smears are preferred to regular whole blood preparations, as due to leukopenia very few infected leukocytes may be present. Representing a leukocyte- and platelet-enriched fraction, buffy coat is also considered useful for detection of *A. platys* morulae within platelets (Eddlestone et al. 2007). In any case, bacterial detection is best achieved if smears are prepared immediately after blood collection. Alternatively, anticoagulated samples can be refrigerated and processed preferably within 24–48 h. At *post-mortem* examination, tissue impressions or smears (spleen, liver, kidney, heart, lung and, in particular, blood vessels) can be performed in an attempt to visualize erythrocytotropic *Anaplasma* spp. (Kocan et al. 2010, OIE 2015). After being dried and fixed in methanol, smears are stable for at least 2 months. Differential staining is achieved with Eosin Azure (Romanovsky)-type dyes such as Giemsa and Diff-Quik; *Anaplasma* morulae typically appear in the host cells as dark blue to purple formations by light microscopic examination at 400× or 1000× magnification. Open access reference images can be found in the literature of *A. bovis* (Liu et al. 2012), *A. centrale* (Bell-Sakyi et al. 2015), *A. marginale* (Kocan et al. 2003), *A. phagocytophilum* (Annen et al. 2012, Henniger et al. 2013), *A. platys* (Dyachenko et al. 2012), and *A. ovis* (Yasini et al. 2012).

Light microscopy is the most inexpensive and quickest laboratory test, but also the least sensitive, and is highly dependent on examiner experience, relative quantity of target cells, bacteremia levels, the degree of neutropenia, monocytopenia, thrombocytopenia, or anemia and infection status. It is commonly used for the erythrocytotropic *Anaplasma* spp. with good results in recently acquired infections (with the examination of up to 100 microscopic fields, ~ 100,000 cells), except in cases of severe anemia (Potgieter and Stoltsz 1994). However, it has limited value in persistently infected animals that usually present low-level bacteremia (Eriks et al. 1989, Palmer et al. 1998, Kocan et al. 2010). For *A. platys*, light microscopy presents low sensitivity due to the cyclic character of thrombocytopenia and the low percentage of infected cells (between 0.5% and 5%); therefore, it is recommended to examine between 2,000 and 20,000 platelets (Kontos et al. 1991, Chang et al. 1996, Brown et al. 2006, Eddlestone et al. 2007). For the granulocytotropic species, morulae can also be sparsely distributed and difficult to detect, particularly in human samples from which at least 800–1,000 granulocytes should be examined (Aguero-Rosenfeld 2002), although at least one study demonstrates identification in all human cases after examination of only 200 granulocytes (Rand et al. 2014). For ruminants, the examination of 400 granulocytes are generally regarded as sufficient to detect infected leucocytes in recent disease, but blood smears from persistently infected animals may give negative results (Stuen et al. 2002, 2006). Thus, a negative result does not rule out infection and microscopy should

Table 1. Biological, Ecological, and Epidemiological Features of *Anaplasma* Species

Species	*Ixodid tick, confirmed or potential vectors*[a]	*Affected vertebrates (rare cases)*	*Target cell in vertebrates*	*Disease*	*Geographic distribution*
Anaplasma bovis	*Haemaphysalis* spp. (*H. longicornis, H. punctata, H. concinna, H. megaspinosa, H. langrangei, H. leporispalustris*); Less frequent in: *Dermacentor* spp. (*D. occidentalis, D. andersoni*), *Rhipicephalus* spp. (*R. sanguineus; R. turanicus; R. evertsi*)	Domestic ruminants (dogs)/several wild ruminant species	Monocytes	Bovine ''ehrlichiosis''	Worldwide, excluding Australia
Anaplasma centrale	*Rhipicephalus simus*	Cattle	Erythrocytes	Anaplasmosis	South Africa In South America, Australia, Africa, the Middle East, and South-East Asia as vaccine strain
Anaplasma marginale	Up to 20 species involved[b] Examples are: *Dermacentor* spp. (*D. andersoni, D. variabilis*), *Rhipicephalus* spp. (*R.* [*B.*] *annulatus*, *R.* [*B.*] *microplus*, *R. sanguineus, R. simus*)	Cattle/wild ruminants	Erythrocytes	Bovine anaplasmosis	Worldwide in tropical and subtropical regions of the New World, Europe, Africa, Asia, and Australia
Anaplasma ovis	*Dermacentor* spp. (*D. marginatus, D. andersoni, D. occidentalis D. niveus*), *Haemaphysalis* spp. (*H. concinna, H. punctata*), *Hyalomma* spp., *Ixodes ricinus, Rhipicephalus* spp. (*R. bursa, R. sanguineus*)	Domestic small ruminants/wild ruminants (humans)	Erythrocytes	Ovine anaplasmosis	Mainly tropical and subtropical regions, also Mediterranean
Anaplasma platys	*Rhipicephalus* spp. (*R. sanguineus, R. turanicus, R. evertsi*)	Dogs (cats, humans, ruminants)/wild canids (other wild animals)	Platelets and platelet precursors	Infectious cyclic thrombocytopenia	Worldwide in tropical and subtropical areas few reports from Africa, not reported from northern part of the world (Canada, Northern Europe, and Russia)
Anaplasma phagocytophilum	Up to 20 species involved[c] Examples are: *Ixodes* spp. (*I. ricinus, I. persulcatus, I. pacificus, I. scapularis, I. ovatus*), *Dermacentor* spp. (*D. variabilis, D. occidentalis, D. silvarum*), *Haemaphysalis concinna*	Domestic ruminants, horses, dogs, cats, humans/several wild animals	Neutrophils (rarely eosinophils, basophils, and monocytes)	Tick-borne fever, equine, canine, feline, and human granulocytic anaplasmosis	Europe, North America, Asia rare reports from Africa and South America

[a]Not all are experimentally proven; this list also includes tick species in which *Anaplasma* spp. have been detected by PCR only.
[b]Reviewed in detail by Kocan et al. (2004).
[c]Reviewed in detail by Stuen et al. (2013).

always be combined with other laboratory diagnostic tests, complemented by the screening of other sample types if persistent infections are suspected, as discussed below. Moreover, false-positive interpretations can occur due to Döhle and Howell–Jolly bodies, other inclusions, contaminant particles or platelets, and nuclear fragments superimposed on leukocytes. In addition, agent identification can be misinterpreted in hosts known to be affected by different *Anaplasma* spp. or related agents with identical cell tropism. For example, wild ruminants can be infected by the erythrocytotropic species *A. marginale*/*A. centrale* and *A. ovis* worldwide, by the granulocytotropic *A. phagocytophilum* and *Ehrlichia ewingii* in North America, and by the monocytotropic *A. bovis*, *Ehrlichia chaffeensis*, and *Ehrlichia canis* in North America and in the Far East or *Ehrlichia ruminantium* in Africa that can also target granulocytic leukocytes as well as endothelial cells (Dumler et al. 2005). The identification of the causative agent in uncommon infections or atypical locations or hosts can also be limited if relying solely on light microscopy.

Other light and electron microscopic techniques (transmission electron microscopy, scan electron microscopy, confocal microscopy) have been used to study host samples for specific research purposes. The same is true for the microscopic detection of *Anaplasma* in vectors, which has been most useful for life cycle investigation rather than for diagnosis and epidemiological studies.

Molecular Detection

Both experimental and field studies have pointed out the utility of molecular laboratory diagnosis for sensitive and specific identification of *Anaplasma* infections (Eriks et al. 1989, Palmer et al. 1998, Eddlestone et al. 2007, Haigh et al. 2008, Hing et al. 2014). The growing number of high-performance molecular protocols with increased potential for automation and multiplex detection has resulted in these becoming indispensable laboratory tools.

Anticoagulated whole blood and buffy coat are the best samples for molecular screening of *Anaplasma*-infected human and nonhuman vertebrates. Ethylenediaminetetraacetic acid or citrate are preferred to heparin as anticoagulant, since the latter is considered to interfere with PCR (Hebels et al. 2014, Sánchez-Fito and Oltra 2015). Spleen samples are equally good and highly recommended for persistently infected animals, especially in wildlife studies or as an additional sample to rule out intermittent or low-level bacteremia in blood-negative individuals (Eddlestone et al. 2007). Other samples reported in the literature with variable results for *Anaplasma* screening include serum/plasma, liver, lung, lymph nodes, bone marrow, and skin biopsies (Massung et al. 1998, Eddlestone et al. 2007, Gaunt et al. 2010, Blaňarová et al. 2014, Szekeres et al. 2015). To increase the sensitivity of *Anaplasma* detection in clinical cases, it is important to pay attention to the previously mentioned time frame for sample collection (as mentioned in the Microscopy section). Again, samples should be processed as soon as possible after collection, at least to a stage at which they can be maintained below −20°C until required (*e.g.*, buffy-coat separation, preparation of aliquots with volume/weight suitable for nucleic acid extraction) to avoid repeated freeze and thaw cycles.

For studies of prevalence of *Anaplasma* spp. in vectors, it is advisable to use questing (unfed) ticks. In the case of species that are difficult to obtain in a questing state, as for example the one-host ticks *Rhipicephalus* (*Boophilus*) *microplus* and *R.* (*B.*) *annulatus*, it should always be kept in mind that tick positivity could either result from the remnant of infected host blood meal or from an established infection in the tick tissues (Estrada-Peña et al. 2013). Since transovarial transmission of *Anaplasma* spp. is not known to occur in naturally infected ticks, questing larvae are not useful in prevalence studies. However, it is important to note that attached larvae with PCR-positive results might be of potential value for the identification of infected hosts.

Ticks should be identified to species level by examining morphological characters before being processed for molecular analysis. Otherwise, any conclusion drawn regarding vector–pathogen associations could be subject to substantial errors (Estrada-Peña et al. 2013). Identification can also be confirmed by molecular methods, by sequencing 12S or 16S rRNA gene fragments (Mangold et al. 1998, Beati and Keirans 2001). However, tick-derived sequences in the GenBank database are still far from being comprehensive, preventing accurate classification based solely on molecular tools in many cases.

Ticks can be used freshly for DNA extraction, with only short-term storage at 4°C. For partially fed ticks removed from hosts, no more than 1–2 days is recommended, but for unfed ticks this might be as long as a month; alternatively, unfed ticks can be kept for longer at 12–16°C, 85% relative humidity. For longer time periods, samples can be immersed in RNAlater or ≥70% ethanol and maintained at 4°C or frozen immediately at −20°C or −80°C. Decontamination of the tick surface should be performed before DNA extraction by a sequence of 5-min immersions in sterile distilled water/PBS, 70–80% ethanol, and again water/PBS, ending by air drying (or drying on sterile filter paper). Ticks should be handled with sterile forceps in between each step and after decontamination.

Molecular testing of blood, tissue samples, and ticks (or other arthropods) is usually performed on total DNA. Extraction can be manual or automatic, with ready-to-use commercial kits or prepared solutions, but in any case it is highly recommended to include DNA purification to remove PCR inhibitors, particularly in the case of blood and tick samples (Schwartz et al. 1997). For fully engorged female ticks, DNA should be extracted from only one half of the specimen after longitudinal bisection, to avoid excess sample and to prevent PCR inhibition due to the high erythrocyte concentration during extraction. Prior disruption or homogenization of ticks and tissue samples is very important for efficient DNA extraction and can be performed using automatic homogenizers (*e.g.*, Precyllis or Mixer Mill benchtop units) or manually, using sterile tools, such as scalpel blades or pestles (one per sample). For these samples, if an enzymatic digestion step is included it should last at least 1 h at 56°C or can be extended to an overnight incubation, facilitating organization of work. Several commercial kits have been used by the authors in diagnosis and surveillance studies with good results (Table 2 footnote). Less expensive protocols using the TRI Reagent protocol (Sigma-Aldrich) and alkaline hydrolysis with 1.25% ammonium solution (Schouls et al. 1999) have also been used in tick surveillance studies. The former is a time-consuming protocol, although it yields good-quality DNA and also enables protein and RNA isolation. The alkaline hydrolysis method is easy to perform and uses intact ticks, but results in a

Table 2. PCR Assays for Detection of *Anaplasma* spp. DNA in Tick and Host Materials

Organism	*Target gene*	*Method*	*Primers/Probe 5′-3′*	*Reference*
Anaplasma spp.[a]	*rrs*, 345 bp	Conventional	EHR16SR: GGTACCYACAGAAGAAGTCC EHR16SD: TAGCACTCATCGTTTACAGC	Parola et al. (2000)
	rrs, 421 bp	Conventional	16SANA-F: CAGAGTTTGATCCTGGCTCAGAACG 16SANA-R: GAG TTT GCC GGG ACT TCTTCT GTA	Stuen et al. (2003)
A. centrale/A. marginale/ A. ovis	*msp4*, 842 bp	Conventional	MSP43: CCGGATCCTTAGCTGAACAGGAATCTTGC MSP45: GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	de la Fuente et al. (2002)
A. centrale	*msp*3 989 bp	Conventional	Acent_F: CCAGAAGGTGAAGGAGAAGAT Acent_R: AAGCATTTACAGGAAAGGAA GC	Shkap et al. (2009)
	groEL 77 bp	Real-time	AC-For: CTATACACGCTTGCATCTC AC-Rev: CGCTTTATGATGTTGATGC AC-Probe: Texas Red ATCATCATTCTTCCCCTTTACCTCGT BHQ2	Decaro et al. (2008)
A. marginale	*msp1ß* 95 pb	Real-time	AM-forward: TTGGCAAGGCAGCAGCTT AM-reverse: TTCCGCGAGCATGTGCAT AM-probe: 6FAM TCGGTCTAACATCTCCAGGCTTTCAT BHQ1	Carelli et al. (2007)
A. phagocytophilum/ A. platys	*groEL,* 442 bp	Nested	1st amplification HS1: TGGGCT GGTAMTGAAAT HS6: CCICCIGGIACIAYACCT TC 2nd amplification HS43: ATWGCWAARGAAGCATAGTC HS45: ACTTCACGYYTCATAGAC	Sumner et al. (1997)
	Rrs 98 bp	Real-time	EPlat-19f: CGGATTTTTGTCGTAGCTTGCTAT EPlat-117r: CCATTTCTAGTGGCTATCCCATACTACT EPlat-55p-S1: FAM-TGGCAGACGGGTGAGTAATGCATAGGA-BHQ1	Teglas et al. (2005)
A. phagocytophilum	*msp4*, 849 bp	Conventional	MSP4AP3: TTAATTGAAAGCAAATCTTGCTCCTATG MAP4AP5: ATGAATTACAGAGAATTGCTTGTAGG	de la Fuente et al. (2005)
	rrs, 497 bp	Nested	1st amplification ge3a: CACATGCAAGTCGAACGGATTATTC ge10r: TTCCGTTAAGAAGGATCTAATCTCC 2nd amplification ge9f: AACGGATTATTCTTTATAGCTTGCT ge2: GGCAGTATTAAAAGCAGCTCCAGG	Massung et al. (1998)
	msp2; 77 bp	Real-time	ApMSP2f: ATGGAAGGTAGTGTTGGTTATGGTATT ApMSP2r: TTGGTCTTGAAGCGCTCGTA ApMSP2p: FAM TGGTGCCAGGGTTGAGCTTGAGATTG TAMRA or BHQ	Courtney et al. (2004)
A. platys	*rrs*, 359 bp	Conventi onal	EPLAT5: TTTGTCGTAGCTTGCTATGAT EPLAT3: CTTCTGTGGGTACCGTC	Murphy et al. (1998)

Examples of commercial kits for total DNA extraction that have been widely used by the authors with good results in diagnosis and surveillance studies include: QIAamp and DNeasy Kits (Qiagen, Hilden, Germany; manual/automated protocol for both blood and tissue), High Pure PCR Template Preparation Kit (Roche, Basel, Switzerland; manual protocol for blood), MagCore Genomic DNA kits (RBCBioscience, New Taipai City, Taiwan; automated protocols for blood or tissue) and Maxwell® 16 LEV Blood DNA Kit (Promega GmbH, Madison, Wisconsin; automated protocol).

[a]This protocol also amplifies other Anaplasmataceae species.

msp, major surface protein.

TABLE 3. COMMONLY USED AND POTENTIALLY USEFUL CELL LINES FOR *ANAPLASMA* SPP. ISOLATION AND CULTIVATION

Cell line	*Medium and supplementation*	*Culture conditions*
DH82 (ATCC® CRL-10389)		
Type: canine macrophage-like	MEM medium	Cells grow as adherent monolayer
Origin: dog with malignant histiocytosis Wellman et al. (1988)	10% or 5%[a] heat-inactivated FBS	Incubation at 37°C with 5% CO_2 atmosphere
	2 mM L-glutamine,	Can also be grown at 32°C
	1 mM sodium pyruvate	Change of 1/3 medium volume every 2–3 days
	0.1–1 mM nonessential amino acids	Subculture once a week is advised for stock cultures
HL60 (ATCC CCL-240)		
Type: human promyelocyte	RPMI-1640 medium	Cells grow in suspension
Origin: human with promyelocytic leukemia Collins et al. (1977)	5% or 2%[a] FBS	Incubation at 37°C with 5% CO_2 atmosphere
	2 mM L-glutamine	Change medium every 2–3 days, adjusting cell concentration to 4×10^5 cell/mL or to 2×10^5 cell/mL[a]
IDE8 (ATCC CRL-11973)		
Type: tick cell line	L-15B medium Munderloh and Kurtti (1989)	Cells grow in loosely adhered layers
Origin: *Ixodes scapularis* embryos Munderloh et al. (1994)	5% FBS	Incubation at 32°C (at 34°C[a]) in sealed container, under normal atmospheric conditions
	10% tryptose phosphate broth 0.1% bovine lipoprotein concentrate 100 IU/mL penicillin[b] 100 μg/mL streptomycin Additional supplementation[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Change of 3/4 medium volume once or twice a week.
ISE6 (ATCC CRL-11974)		
Type: tick cell line	L-15B300 medium Munderloh et al. (1999)	Cells grow in loosely adhered layers
Origin: *Ixodes scapularis* embryos Kurtti et al. (1996)	5% FBS 10% tryptose phosphate broth	Incubation at 32°C (at 34°C[a]) in sealed container, under normal atmospheric conditions
	0.1% bovine lipoprotein concentrate 2 mM L-glutamine 100 IU/mL penicillin[b] 100 μg/mL streptomycin Additional supplementation[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Change of 3/4 medium volume once or twice a week.
IRE/CTVM20[c]		
Type: tick cell line	1:1 mixture of L-15 (Leibovitz) medium and L-15B medium Bell-Sakyi (2004)	Cells grow predominantly in suspension
Origin: *Ixodes ricinus* embryos Bell-Sakyi et al. (2007)	15% FBS	Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
	10% tryptose phosphate broth 0.05% bovine lipoprotein concentrate 2 mM L-glutamine 100 IU/mL penicillin[b] 100 μg/mL streptomycin	Change 3/4 of medium volume once a week.
IRE/CTVM19[c]		
Type: tick cell line	L-15 (Leibovitz) medium 20% FBS	Cells grow predominantly in suspension

(*continued*)

Table 3. (Continued)

Cell line	*Medium and supplementation*	*Culture conditions*
Origin: *Ixodes ricinus* embryos Bell-Sakyi et al. (2007)	10% tryptose phosphate broth	Incubation at 28–32°C, in sealed container, under normal atmospheric conditions
	2 mM L-glutamine 100 IU/mL penicillin[b] 100 μg/mL streptomycin	Change 3/4 of medium volume once a week.
RAE25[c]		
Type: tick cell line	L-15B medium Munderloh and Kurtti (1989)	Cells grow in loosely adherent sheets and clumps
Origin: *Rhipicephalus appendiculatus* embryos Kurtti and Munderloh (1982)	5% FBS	Incubation at 32°C, in sealed container, under normal atmospheric conditions
	10% tryptose phosphate broth 0.1% bovine lipoprotein concentrate 2 mM L-glutamine 100 IU/mL penicillin[b] 100 μg/mL streptomycin Additional supplementation[a]: 0.1% $NaHCO_3$ 10 mM HEPES Adjust to pH 7.5	Change 3/4 of medium volume once a week.

[a]For isolation attempts and propagation of infected cells.
[b]Uninfected cultures can be maintained with antibiotics (penicillin and streptomycin) if required, whereas infected cultures can be supplemented with an antimycotic (amphotericin B) if required for the first few weeks to minimize fungal contamination, but antibiotics should be avoided if the target *Anaplasma* sp. is known or suspected to be sensitive to penicillin or streptomycin.
[c]Available from the Tick Cell Biobank http://tickcells.pirbright.ac.uk
FBS, fetal bovine serum.

crude DNA extraction, and elimination of PCR inhibitors and long-term DNA storage stability are not guaranteed.

Evaluation of the extraction process is an important requirement. Negative controls (*i.e.*, sterile water) should be included in each group of samples during extraction, to monitor the occurrence of contamination by DNA carryover. Quality and quantity of nucleic acids can be ascertained with a spectrophotometer and degradation of DNA by gel electrophoresis. PCRs targeting host (*e.g.*, β-actin, albumin, human β-globin) or vector (*e.g.*, *Ixodes cox* gene, 12S or 16S rRNA genes) housekeeping genes can also be performed to validate extraction and to confirm the absence of PCR inhibitors (Ausubel et al. 1998, Mangold et al. 1998, Beati and Keirans 2001, Schwaiger and Casinotti 2003). Alternatively, validation can be achieved by spiking the samples with nonrelated bacterial suspensions before extraction, and subsequently targeting the corresponding DNA by specific PCR. *Bacillus thuringiensis* commercial suspensions are widely used for this purpose (De Bruin et al. 2011).

Despite being closely related species, few molecular approaches have been designed to target the entire *Anaplasma* genus. One of the reasons is that some *Anaplasma* spp. are ecologically divergent and not found in the same hosts or vectors. Moreover, broader-range PCR assays are usually less sensitive and prone to selectively amplifying the predominant *Anaplasma* spp. or genetically similar agents (*e.g.*, other members of the Anaplasmataceae or other alphaproteobacteria) that might be present in the samples in higher concentrations. However, genus-specific primers could be used when, for example, nothing is known about the *Anaplasma* species present in a given area. Furthermore, broad-range PCRs are indispensable when attempting to identify clades within the *Anaplasma* genus.

Sensitivity of molecular detection depends on several factors such as: (1) sample nature and quality, that is, plasma and serum usually present much lower bacterial loads than blood due to the intracellular nature of *Anaplasma*; (2) the genomic copy number of target genes, for example, the major surface protein (*msp*) families include multiple copy genes; and (3) the amplicon length (*e.g.*, short sequences are generally preferred to long ones for screening). Among the most targeted genes of *Anaplasma* spp. are those for 16S rRNA (*rrs*), heat shock protein (*groEL*), citrate synthase (*gltA*), and major surface proteins (*msp1*, *msp2*, *msp4*, *msp5*). Protocols targeting some of these genes are suggested in Table 2. For molecular screening, the sensitive multicopy *msp* approaches are preferred over single copy genes, whereas for sequence comparison and database crossmatch, conservative or moderately conservative *rrs* and *groEL* strategies are regarded as the best choice. Nested PCRs are now generally replaced by less time-consuming and more sensitive real-time assays. However, further confirmation of positive results by sequencing is still highly advised. In most cases, this requires an additional conventional PCR, as real-time PCR targets that are most suitable for screening are usually very short (<150 bp), yielding sequence data of limited phylogenetical value.

For the confirmation of a potential new strain/variant/species it is strongly encouraged to rely on a multilocus approach and, if possible, in vitro isolation. Generally, DNA sequences from fully characterized *Anaplasma* variants are a reliable source for typing (Huhn et al. 2014, Guillemi et al. 2015).

In Vitro Isolation

Isolation and in vitro propagation of *Anaplasma* species provides a valuable tool for the study of their biology. It is,

however, less used in direct routine diagnosis and surveillance as the previously mentioned approaches. Still, it is of great value for proving the etiology in atypical/new clinical occurrences or fatal cases. It is also an indispensable procedure to claim discovery of a new bacterial species, enabling proper taxonomic classification and the attribution of a formal scientific name (Cabezas-Cruz et al. 2012, Zweygarth et al. 2013). Due to ethical issues regarding animal experimentation, there is a strong incentive for isolation and propagation of *Anaplasma* in continuously cultured cell lines.

In vitro culture of *Anaplasma* spp. in mammalian cells relies on the availability of cellular systems that are similar to or could mimic the intracellular environment found in natural host cells. This was first achieved for the granulocytotropic *A. phagocytophilum* using the human promyelocytic leukemia cell line HL-60 (ATCC CCL-240) (Goodman et al. 1996). Since then, HL-60 cells have been routinely used for *A. phagocytophilum* culture, although other mammalian cell lines are also reported to sustain its growth. These include the human monocytic THP-1 (ATCC TIB-202) and microvascular endothelial HMEC-1 and MVEC cell lines, the bovine corneal BCE C/D1-b (ATCC CRL-2048) cell line and the Rhesus monkey retina choroid RF/6A (ATCC CRL-1780) endothelial cell line (Munderloh et al. 2004, Garcia-Garcia et al. 2009). Continuous growth of the intraerythrocytic *A. marginale* was also achieved in both BCE C/D1-b and RF/6A cells, but only after establishment in tick cell lines (Munderloh et al. 2004). Additionally, HL-60 and THP-1 cells have enabled the isolation of the newly reported zoonotic *A. capra* (Li et al. 2015).

As an alternative to mammalian-derived cells, tick cell lines are a valuable tool for the cultivation of *Anaplasma* species. They can be a good option for the isolation of *Anaplasma* variants found in vectors, but with low or unknown pathogenicity for vertebrates (Massung et al. 2007). Moreover, these cells have already proven their value for bacteria that target mammalian cells that are difficult to continuously propagate in vitro, such as the intraerythrocytic and intrathrombocytic *Anaplasma* spp. (Munderloh et al. 1994, 2003). The continuous culture of *A. marginale* was first achieved in an *Ixodes scapularis* embryo-derived cell line (IDE8) (Munderloh et al. 1994, 1996). Since then, many isolates have been established in the IDE8 cell line, as well as in other tick cell lines (Munderloh et al. 2004, Zivkovic et al. 2010), making possible a more intense study of *A. marginale* biology, as reviewed by Blouin et al. (2002) and Passos (2012). Very recently, continuous cultures of the vaccine strain of *A. centrale* were established for the first time in *Rhipicephalus appendiculatus* RAE25 and *Dermacentor variabilis* DVE1 cell lines (Bell-Sakyi et al. 2015). *A. phagocytophilum* cell cultures have also been established in several tick-derived cell lines, including *I. scapularis*-derived IDE8 and ISE6, *Ixodes ricinus*-derived IRE/CTVM19 and IRE/CTVM20 (Munderloh et al. 1996, Woldehiwet et al. 2002, Silaghi et al. 2011, Dyachenko et al. 2013, Alberdi et al. 2015), *R. appendiculatus*-derived RAE25 and *I. ricinus*-derived IRE11 (Bell-Sakyi, unpublished data). ISE6 cells were also valuable for the isolation of potentially new thrombocytotropic *Anaplasma* closely related to *A. platys* (Munderloh et al. 2003, Tate et al. 2013).

As mentioned for the other direct diagnostic techniques, blood collected from animals or humans in the proper time frame is also the best inoculum for in vitro culture (as mentioned in the Microscopy section). Furthermore, growth of *Anaplasma* can also be attempted from vertebrate tissue samples or fresh ticks, after maceration, fragmentation, or dissection in culture medium. However, especially for techniques which do not discard the tick exoskeleton, contamination with environmental bacteria and fungi can be a problem for establishment of the culture. In this case, special attention should be given to external surface decontamination of the tick, as previously mentioned for molecular testing, adding an extra 5-min immersion in 0.1% benzalkonium chloride (Sigma) before the ethanol step to ensure decontamination. Samples of 0.1–0.5 mL anticoagulated whole blood, buffy coat, or tissue samples can be inoculated straight into small cell culture flasks (12.5- or 25-cm^2 capacity) or flat-sided tubes (Nunc) and maintained according to the respective cell line culturing conditions (Table 3). Every 2–7 days, fresh medium should be added to cultures and, in the case of mammalian cells growing in suspension, cell concentration adjusted. As tick cells tolerate high cell densities and can survive for many months without subculture, there is no need to adjust cell density and inoculated cultures can be maintained for the long periods (12 weeks or more) required for adaptation of some *Anaplasma* spp. to in vitro growth (Silaghi et al. 2011, Dyachenko et al. 2013, Bell-Sakyi et al. 2015). Cultures should also be periodically evaluated by microscopy to detect any microbial growth. Infection can be assessed by direct observation of cytocentrifuged culture aliquots after staining with Eosin Azure-type dyes as described above.

In vitro culturing is a demanding task in terms of time and expertise, and only a limited number of research institutions are currently able to perform it. Even so, this might not be a constraint on its use for direct diagnosis at least for some agents such as *A. phagocytophilum* that can be successfully cultured from infected blood kept for up to 18 days under refrigerated conditions (Kalantarpour et al. 2000). Thus, biological samples can be transported under refrigerated conditions to a referral laboratory, where the appropriate assays can be carried out.

Conclusion

In summary, for the direct detection of *Anaplasma* spp. in blood and tissue samples, ticks or other vectors, molecular methods are preferred. Specific real-time PCRs offer several advantages over conventional PCR assays for screening purposes, but the confirmation of sequence identity is still often required. Approaches targeting multiple genes can be very useful for phylogeny and taxonomy studies. Other direct methods such as microscopy or in vitro isolation are mostly reserved for research applications, such as experimental studies, transmission trials etc., but can also contribute in specific diagnostic/surveillance investigations and in identifying and characterizing novel *Anaplasma* spp.

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Address correspondence to:
Ana Sofia Santos
Centre for Vector and Infectious Diseases Research
National Institute of Health Doutor Ricardo Jorge
Avenida da Liberdade 5
Águas de Moura 2965–575
Portugal

E-mail: ana.santos@insa.min-saude.pt

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Guidelines for the Detection of *Rickettsia* spp.

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

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 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) and in Supplementary Table S1.

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

[1]Center of Rickettsiosis and Arthropod-Borne Diseases, Hospital San Pedro-CIBIR, Logroño, Spain.
[2]National Institute of Health Dr. Ricardo Jorge, Águas de Moura, Portugal.
[3]Center for Interdisciplinary Research in Animal Health, Faculty of Veterinary Medicine, University of Lisbon, Lisbon, Portugal.
[4]Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes, Institut Hospitalo-Universitaire Méditerranée-Infection, Marseille, France.
[5]Department of Biology and Wildlife Diseases, Faculty of Veterinary Hygiene and Ecology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.
[6]CEITEC VFU, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.
[7]National Centre for Vector Entomology, Institute of Parasitology, University of Zurich, Zurich, Switzerland.
[8]Department of Veterinary Sciences, University of Torino, Grugliasco, Italy.

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.

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*

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 2–8°C for several days.
EDTA, ethylenediaminetetraacetic acid; PCR, polymerase chain reaction; CSF, cerebrospinal fluid; IFA, indirect immunofluorescence assay; IHC, immunohistochemical assay.

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, IgG titers ≥ 64 and IgM titers ≥ 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).

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*)

TABLE 5. (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 μg/mL streptomycin).
DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MEM, minimum essential media.

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 45 min to 1 h in the cell culture medium (minimal essential medium supplemented with 4% heat-inactivated fetal calf serum and 2 mM 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 cm^2 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. *Ixode*s, *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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Address correspondence to:
José 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

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Critical Aspects for Detection of *Coxiella burnetii*

Marcella Mori,[1,2] Katja Mertens,[3] Sally J. Cutler,[4] and Ana Sofia Santos[5]

Abstract

Coxiella burnetii is a globally distributed zoonotic γ-proteobacterium with an obligatory intracellular lifestyle. It is the causative agent of Q fever in humans and of coxiellosis among ruminants, although 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 medium 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.

Keywords: *Coxiella burnetii*, direct diagnosis, PCR, isolation, axenic culture, genome sequencing

Introduction

Coxiella burnetii is a globally 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). *C. burnetii* possesses several remarkable features, including the 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).

C. 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 facilitates spread (Kruszewska and Tylewska-Wierzbanowska 1997, Alsaleh et al. 2011). *C. 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-Wierzbanowska 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

[1]Bacterial Zoonoses of Livestock, Operational Directorate Bacterial Diseases, Veterinary and Agrochemical Research Centre, CODA-CERVA, Brussels, Belgium.
[2]Belgian Reference Centre for *Coxiella burnetii* and *Bartonella*, Brussels, Belgium.
[3]Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute for Bacterial Infections and Zoonoses, Jena, Germany.
[4]University of East London, London, United Kingdom.
[5]Centre for Vector and Infectious Diseases Research, National Institute of Health Doutor Ricardo Jorge, Águas de Moura, Portugal.

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.

Real-time 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 before antibiotic treatment are most useful (Anderson et al. 2013). Serum, urine, and throat swabs have also proven to be valuable for *C. burnetii* screening (Klaassen et al. 2009). In more protracted infections, tissue samples from focal regions of infection should be investigated, that is, valvular material from endocarditis, aneurism, or vessel fragments in vascular infections, and bone biopsies in osteomyelitis. 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 month following vaccination), PCR will not discriminate between the vaccine and wild-type strains (Hermans et al. 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 (Bittar et al. 2014, Tozer et al. 2014, González-Barrio et al. 2015b). 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 bacteremia and intermittent shedding can also occur, thus the collection of different sample types obtained during longer sampling periods serves 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/fetal and valvular/vascular material) to very low agent loads in environmental samples, milk samples, and usually in blood samples. For DNA amplification, several real-time 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 multicopy *IS1111* repetitive element is often used for agent's detection as this provides increased sensitivity when compared with 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* real-time 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 elements, possibly explaining their biological differences (Beare et al. 2009). Recently, along with the development of powerful sequencing platforms, the numbers of sequenced genomes have blossomed to more than 40, 26 being publically available (D'Amato et al. 2014, 2015, Karlsson et al. 2014, Sidi-Boumedine et al. 2014, Walter et al. 2014, Hammerl et al. 2015). Despite the large number of genome records for *C. burnetii* since 2003, only nine genomes are fully sequenced and annotated as closed circular genomes, the remainder are available as fragmented scaffolds, contigs, or whole genome shotgun sequences in various genome databases (www.patricbrc.org/portal/portal/patric/Home, www.ncbi.nlm.nih.gov/genome/genomes/543).

Obtaining 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 for complete removal of host DNA. Classical DNA isolation methods are suitable (as cited for real-time 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 2 Mb). Whole genome sequencing is becoming more affordable, but data analyses remain time-consuming and require specific knowledge and extra funding. Although 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.

TABLE 1. MOST CITED REAL-TIME ASSAYS (PUBMED) FOR *COXIELLA BURNETII* OR Q FEVER OVER THE LAST 3 YEARS

Target gene	*Method*	*Application*	*Fragment amplified (bp)*			*Reference*
			<100	*100–200*	*>200*	
Multiplex						
IS1111, *com1*	TaqMan	Detection, quantification	✓			De Bruin et al. (2013)
IS1111, *com 1*, *icd*	TaqMan	Detection, quantification		✓		De Bruin et al. (2011)
Singleplex						
IS1111	TaqMan	Detection	✓			Harris et al. (2000), Loftis et al. (2006), Schneeberger et al. (2010), Tilburg et al. (2010), Niemczuk et al. (2011), Schets et al. (2013), Di Domenico et al. (2014), Niemczuk et al. (2014)
	TaqMan	Detection		✓		Brouqui et al. (2005), Kim et al. (2005), Fournier et al. (2010), Tilburg et al. (2010)
	TaqMan	Detection			✓	Klee et al. (2006), Barkallah et al. (2014)
	Hyprobe	Detection	✓			Panning et al. (2008)
	Hyprobe	Detection			✓	Stemmler and Meyer (2002)
IS30a	TaqMan	Detection, quantification		✓		Brouqui et al. (2005), Mediannikov et al. (2010)
icd	TaqMan	Detection, quantification	✓			Klee et al. (2006)
com1	TaqMan	Detection, quantification	✓			Marmion et al. (2005), Kersh et al. (2010a) and (2010b), Lockhar et al. (2011), Schets et al. (2013)
	SYBR green	Detection, quantification	✓			Cooper et al. (2007)
	Hyprobe	Detection, quantification		✓		El-Mahallawy et al. (2016)
16S rRNA	TaqMan	Detection, quantification	✓			Marmion et al. (2005)
	SYBR green	Detection, quantification	✓			Millán et al. (2016)
groES and groEL	TaqMan	Detection, quantification	✓			Elsa et al. (2015)
	TaqMan	Detection, quantification		✓		Bond et al. (2016)
ompA	TaqMan	Detection	✓			Jaton et al. (2013)
CBU_678	SYBR green	Detection				Kersh et al. (2010a)
CBU_686	SYBR green	Detection				Kersh et al. (2010a)
dotA	TaqMan	Quantification	✓			Coleman et al. (2004)
rpoS	TaqMan	Quantification	✓			Coleman et al. (2004)

Most of the real-time PCR diagnostic tests described are in-house assays, specifically adapted for detection of *C. burnetii* DNA in various sample types. Commercial PCR diagnostic tests are also available, but not included in this table as information on components (PCR Master Mix reagents, primer and probe sequences, PCR product lengths) is often omitted due to patent constraints.

The choice of the most appropriate typing option may depend on the research objectives. The simplest and direct tests (lacking further sequencing), with 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 of the number of repeats (Klaassen et al. 2009, Tilburg et al. 2012a), and single-nucleotide-polymorphism (SNP) genotyping (Hornstra et al. 2011, Huijsmans et al. 2011). Both typing approaches were used for the Dutch outbreak investigation (Klaassen et al. 2009, Huijsmans et al. 2011, Tilburg et al. 2012a). Presently, these methods are reviewed toward harmonization and standardized nomenclature (http://mlva.u-psud.fr/mlvav4/genotyping/view.php, Hornstra et al. 2011, Huijsmans 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 scales. Multispacer 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

Although 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 from clinical samples enable 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. Furthermore, technical expertise and availability of suitable laboratory biosafety level 3 facilities are essential. Handling and processing of samples or cultures with a high bacterial load bear the risk of generating contaminated aerosols and sets that 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, Lockhart et al. 2012, Santos 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% CO_2 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 20–40×). Monthly assessment of culture aliquots should also be undertaken with initial cytoconcentration, stained by Gimenez, and examined by microscopy (by immersion at 1000×) for the characteristic tightly packed *C. burnetii* vacuoles (Gimenez 1964). Positive findings should be confirmed by PCR (see above Real-time PCR section).

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, and ticks, etc. Fluids are directly inoculated, while 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 prerequisite is the absence of microbial contaminants, which is challenging when working with postmortem or aborted tissues, ticks, and environmental samples. Ticks can be surface decontaminated by serial passages in bleach 10% and/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 and exposed to frozen–thaw cycles and low-speed centrifugation, with the resulting supernatant subjected to sequential filtration, using 1 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 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 with 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-day mice, with volumes complying with ethical requirements. Successive injections (up to three) 5–7 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 before injection. Following inoculation, the host should be monitored for clinical signs and by indirect serology (Mori et al. 2013), or postmortem evaluation, at 3–5 weeks postinfection. The spleen, liver, and lungs are preferred organs for *C. burnetii* monitoring by either microscopy or real-time PCR. Infection is typically accompanied by measurable splenomegaly caused by massive *C. burnetii* propagation.

Propagation of Bacterial Isolates

Embryonated egg inoculation

Propagation of highly concentrated *C. burnetii* cultures is achieved through the 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, 7-day-old, specific, pathogen-free chicken eggs are candled to locate the yolk sac. Once identified, the edge of the air sac should also be localized 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 5 postinjection 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 ($\sim$4.5) and antimicrobial 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, which 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% CO_2 and 2.5% O_2 achieved by the use of a dual-gas incubator or alternatively using an anaerobic pouch in case of a monogas incubator (Omsland et al. 2009, 2011). ACCM-D supports the biphasic transition from the SCV to the replicative large cell variant 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 and 100 GE/mL (genome equivalents), depending on the quality of the sample (authors' experience). The impact of repeated axenic propagation on virulence remains to be fully elucidated (Kersh et al. 2011, Kuley et al. 2015).

Coxiella-like Organisms

Initially, the *Coxiella* genus was thought to comprise solely *C. burnetii* species, but is now recognized to contain other members, namely *Coxiella cheraxi* and novel *Coxiella*-like organism identified in birds and in nonvertebrate species. *C. cheraxi* was first isolated in 2000 from connective and hepatopancreatic tissues of a dead crayfish, displaying inclusion bodies with *Rickettsia*-like gram-negative bacteria (Tan and Owens 2000). The partial 16S rDNA, *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,

Table 2. PCR* Assays (PubMed) Used for Detection of *Coxiella*-Like Bacteria

		Fragment amplified (bp)			
Target gene	*Method*	*<100*	*100–500*	*>500*	*Reference*
sodB	Conventional PCR + sequencing		✓		Stein and Raoult (1992), Papa et al. (2016)
				✓	Tan and Owens (2000)
16S rRNA	Real-time PCR + sequencing	✓			Liu et al. (2013)
	Conventional PCR + sequencing		✓		Almeida et al. (2012)
	Conventional PCR + sequencing			✓	Bernasconi et al. (2002), Mediannikov et al. (2003), Duron et al. (2014), Wilkinson et al. (2014), Duron et al. (2015), Al-Deeb et al. (2016), Machado-Ferreira et al. (2016)
23S rRNA	Conventional PCR + sequencing			✓	Noda et al. (1997), Duron et al. (2015)
groEL	Conventional PCR + sequencing			✓	Duron et al. (2014), Duron et al. (2015)
icd	LAMP PCR + sequencing			✓	Al-Deeb et al. (2016)
com1	Conventional PCR + sequencing		✓		Raele et al. (2015)
	Conventional PCR + sequencing			✓	Tan and Owens (2000)
fusA	Conventional PCR + sequencing		✓		Zhong (2012)
rpoB	Conventional PCR + sequencing			✓	Duron et al. (2015)
dnaK	Conventional PCR + sequencing			✓	Duron et al. (2015)

*Amplified fragments were either directly sequenced or cloned into plasmid vectors before sequencing.

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 among 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 codivergence 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 previously mentioned in the above Real-time PCR section. Table 2 summarizes PCR assays used to screen samples for *Coxiella*-like bacteria.

Conclusion

Direct detection of *C. burnetii*, although challenging, fulfills 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.

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No competing financial interests exist.

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Address correspondence to:
Ana Sofia Santos
Centre for Vector and Infectious Diseases Research
National Institute of Health Doutor Ricardo Jorge
Av da Liberdade 5
Águas de Moura 2965-575
Portugal

E-mail: ana.santos@insa.min-saude.pt

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Guidelines for the Isolation, Molecular Detection, and Characterization of *Bartonella* Species

Ricardo Gutiérrez,[1] Muriel Vayssier-Taussat,[2] Jean-Philippe Buffet,[2] and Shimon Harrus[1]

Abstract

Bartonellae are fastidious, facultative, intracellular vector-borne bacteria distributed among mammalian reservoirs worldwide. The pathogenic potential of many *Bartonella* spp. has increased the interest in these bacteria and advanced their research. Isolation of *Bartonella* spp. is laborious using classical bacteriological methods and requires specific conditions and prolonged incubation periods. In contrast, molecular methods for detection of *Bartonella* DNA are considered as more practical and sensitive than the former. Among the molecular methods, the use of real-time PCR assays for primary screening of *Bartonella* spp., followed by several molecular confirmatory assays, using either conventional or real-time PCR, is recommended. Although primary isolation of *Bartonella* is a laborious task, we encourage its application to all PCR-positive samples as this is the most reliable proof for the presence of live bacteria. Moreover, a successful trial will enable a broader molecular characterization and speciation of isolated colonies. The present guideline gathers and summarizes recommendations, including advantages and limitations of isolation and molecular detection of *Bartonella* from mammalian and arthropod samples.

Keywords: *Bartonella*, diagnosis, vector-borne

Introduction

Bartonella organisms are vector-borne, gram-negative, facultative intracellular bacteria, which establish long-lasting intraerythrocytic infections in adapted reservoirs (Boulouis et al. 2005, Chomel et al. 2009). They are distributed worldwide and have been isolated from several mammalian species, including humans and domestic and wild animals (Vayssier-Taussat et al. 2009, Kosoy 2010). Due to their hemotropic lifestyle, bartonellae are typically transmitted by blood-sucking arthropods within mammalian reservoir communities. Sand flies, lice, fleas, biting flies (*e.g.*, Hippoboscidae, Muscidae), and ticks are among the arthropods associated with *Bartonella* transmission and/or infection (Cotté et al. 2008, Chomel et al. 2009, Tsai et al. 2011). Special attention has been given to this bacterial genus due to the pathogenicity exhibited by many of its species, including the two anthropogenic bartonellae (*Bartonella bacilliformis* and *Bartonella quintana*) and the zoonotic species (*e.g.*, *Bartonella henselae*, *Bartonella grahamii*, *Bartonella elizabethae*, *Bartonella koehlerae* and *Bartonella rochalimae*) (Daly et al. 1993, Avidor et al. 2004, Eremeeva et al. 2007, Lydy et al. 2008, Irshad and Gordon 2009, Chomel and Kasten 2010, Ramdial et al. 2012, Oksi et al. 2013).

The diagnosis of *Bartonella* spp. infection has been considered a challenging task due to the difficulty to isolate these bacteria in vitro, requiring specific conditions that will be discussed below. These characteristics limited not only the detection of infected animals but also the identification of the species involved since they are inert to most classical biochemical assays (Regnery et al. 1992, Clarridge et al. 1995, Bermond et al. 2000). Thus, molecular detection assays (PCR) were rapidly adopted to improve their sensitivity and facilitate the identification. Moreover, reservoirs and vectors are commonly infected with more than one *Bartonella* species or variants, thus the detection of a particular *Bartonella* genotype may represent just a portion of the infection repertoire, most probably the dominant genotype (Gurfield et al. 1997, Abbot et al. 2007, Gutiérrez et al. 2014b). Yet, the sole application of molecular methods can represent an obstacle for accurate identification and description of novel *Bartonella* spp. Therefore, the expertise of both molecular and culture isolation methods is essential and complementary in the diagnosis of *Bartonella* spp.

[1]Koret School of Veterinary Medicine, The Hebrew University of Jerusalem, Rehovot, Israel.
[2]INRA, UMR BIPAR INRA, Anses, ENVA 14 rue Pierre et Marie Curie, Maisons-Alfort, France.

Accumulative experience in *Bartonella* research has led to significant improvements of molecular and culture isolation practices, which have provided a more exhaustive determination of *Bartonella* among animal samples. The present guidelines intend to gather and summarize practical recommendations for both techniques applied on mammalian and arthropod samples. Special attention is given to the handling of samples collected from the wild.

Sample Collection and Processing

A proper collection of a sample from an animal is the first essential step for successful detection of bartonellae. Due to their hemotropism, blood is the ideal sample source for *Bartonella* diagnosis from reservoir animals (Kosoy et al. 1999, Schulein et al. 2001). However, it should be noted that bacteremia can be cyclic (Morick et al. 2013) and therefore low blood bacterial levels, below the detection limit of the applied assay, can represent false-negative results. If possible, several tests over time should be performed to overcome this potential limitation. Other tissue samples such as the spleen and liver could be obtained at necropsy or clinical autopsies (Guptill et al. 1997, Maruyama et al. 2004, Angelakis et al. 2009, Morick et al. 2009). When blood is not available such as in the case of carcasses, the spleen is probably the most pertinent internal organ for isolation and detection of *Bartonella* spp. since it has been demonstrated experimentally that bartonellae are retained and filtered in this organ (Deng et al. 2012). In incidental hosts, *Bartonella* species are unable to produce durable bacteremia (Vayssier-Taussat et al. 2009), in such cases, the screening of tissues (*e.g.*, spleen, liver, lymph nodes, and skin lesions) is recommended. Recruited samples should be transported and kept at 4°C until their subsequent analysis. When samples are not processed in the day of collection, they should be kept frozen (−20°C or −80°C) until use.

When isolation of *Bartonella* organisms is intended, it is extremely important to minimize contamination by skin-associated bacteria and other environmental contaminants due to the slow-growing characteristic of bartonellae. Shaving and disinfection of the mammalian's skin with ethanol 70% before sample collection is required when the sample is collected through venipuncture. For small mammalians, such as rodents, other bleeding procedures include retro-orbital bleeding, tail bleeding, and cardiac puncture (Hui et al. 2007). However, most of these techniques require anesthetics and need to be performed by trained practitioners. Blood samples need to be collected in sterile anticoagulated tubes. EDTA tubes have been widely used as the preferred collection tubes and have shown to maintain the viability of *Bartonella* spp. for a long term (Brenner et al. 1997). Importantly, all sampling procedures, collection techniques, frequency, and the maximum volume of sample allowed for a particular animal species must be approved by the institutional corresponding animal care authorities.

Arthropod specimens can be collected from the mammalian hosts by inspecting carefully the mammalian's fur, using a hairbrush or toothbrush and clean tweezers. If possible, all specimens observed on the host should be collected (or at least 10–20 when present). If the goal is DNA detection only, the ectoparasites can be collected into tubes containing sterile 70% ethanol and transported or stored at room temperature (with the lid covered to prevent ethanol volatilization). When bacterial isolation is intended, the arthropod should be kept alive until further processing in the laboratory. For this purpose, the arthropods can be collected in a flask/tube with a small portion of vegetation (*e.g.*, pieces of grass) or wet paper to maintain high humidity during transportation.

Isolation of *Bartonella* Organisms

Bartonellae are fastidious bacteria characterized by slow-growing rates. Isolation of *Bartonella* species from natural reservoir hosts is relatively easy, while more difficult from incidental hosts. They require blood-enriched media under humid and increased carbon dioxide (CO_2) atmospheres (5%). The incubation periods for primary isolations of *Bartonella* vary across species and animal host source. Visible *Bartonella* colonies from primary isolations can be obtained as soon as 3–5 days, but usually require longer periods of up to 5–6 weeks (Maurin et al. 1994, Brenner et al. 1997, Kosoy et al. 1997, Breitschwerdt et al. 2001). Subculturing of isolated colonies usually requires shorter incubation periods, ranging from 4 to 10 days (fully grown colonies) at their optimal temperatures (between 27°C and 37°C, depending on the *Bartonella* species), and 5% CO_2 and high humid atmosphere.

Samples can be directly plated on the appropriate agar media, but certain pretreatments have shown to enhance *Bartonella* isolation. When blood samples are used, lysis of the erythrocytes by lysis-centrifugation or freezing–thawing techniques has been shown to promote the release of intraerythrocytic bartonellae (Welch et al. 1992, Brenner et al. 1997, Heller et al. 1997). Additionally, infected cells can be concentrated by centrifugation to increase the chances of isolation (Chomel et al. 1996). Moreover, to reduce the overgrowth and impact of coinfecting bacteria (common in wild animals), blood and/or tissue samples can be diluted and homogenized (1:2–1:16) in liquid media, such as brain heart infusion (BHI) broth, before the seeding of a sample in an agar medium. This liquid solution can be supplemented with 5% amphotericin to reduce potential fungal contamination (Kosoy et al. 1997, Bai et al. 2011).

For isolation of *Bartonella* species from arthropod samples, it is recommended to pretreat the specimens before cultivation in agar media. Decontamination of superficial bacteria from the arthropod with 70% ethanol or ethanol–iodine solutions, followed by sterile water or phosphate-buffered saline (PBS) washes (incubations of 5–10 min), results in reduction of contaminants and does not prevent the isolation of *Bartonella* (Koehler et al. 1994, Flexman et al. 1995, La Scola et al. 2001, Dehio et al. 2004, Kernif et al. 2014). Then, the arthropod can be homogenized in a liquid medium (*e.g.*, Luria-Bertani, BHI broths) and subsequently plated on agar as described below.

A variety of agar media have been used for the isolation of bartonellae. Columbia, Brucella, BHI, and Trypticase soy-based agars, all supplemented with 5% blood (usually rabbit or sheep), and chocolate agar are the most common solid media used for *Bartonella* isolation (Koehler et al. 1992, Regnery et al. 1992, Schwartzman et al. 1993, Kosoy et al. 1997, Ellis et al. 1999, Dehio et al. 2004). In a pioneering study, Koehler et al. (1992) compared the use of different media for the isolation of *Bartonella* spp. from human

samples. The authors highlighted the effectiveness of chocolate agar, which promoted its use in following studies. Notably, a previous report described the contamination of sheep blood, with *Bartonella*, used as a supplement for culture medium (Bemis and Kania 2007). Therefore, it is crucial to check the sterility of blood used for preparation of each new batch of media. For this aim, incubation of noninoculated control plates (negative controls) for at least 6 weeks, at optimal conditions, is recommended. Due to the extended incubation periods, it is crucial to prevent overdrying of the agar and contaminations by sealing the plates, using semipermeable membranes such as commercial shrink seals.

The phenotype of *Bartonella* colonies varies according to the *Bartonella* species. A particular species may present different colony morphologies during isolation. Moreover, variation according to the level of passage in agar plates is commonly observed. Primary colonies can be deeply invaginated or raised, cauliflower-like or circular, gray or creamy, smooth or rough-firm, adherent or nonadherent, and/or embedded in the surface of the agar (Regnery et al. 1992, Kosoy et al. 1997). The most evident phenotypic characteristic that may assist in the selection of potential *Bartonella* colonies is their slow-growing rate during incubation. Accordingly, once *Bartonella* colonies appear in the agar plate, they usually continue to grow slowly and produce small size changes over several days.

Various liquid media have been described for the isolation and culture of *Bartonella* species. Commercial broth media supplemented with hemin or histidine–hematin were first reported to support *B. henselae* and *B. quintana* growth (Schwartzman et al. 1993, Wong et al. 1995, Chenoweth et al. 2004). Later, the use of media bases for the maintenance of insect cell cultures has provided the most promising broths for *Bartonella* cultures. The *Bartonella*-Alpha-Proteobacteria growth medium (BAPGM) (Maggi et al. 2005) and Schneider's insect-based liquid medium supplemented with fetal calve serum and sucrose (Riess et al. 2008) have demonstrated their capability to serve as proper media for isolation of several *Bartonella* species. A combination of liquid culture, followed by isolation on blood-based agar media, has also been shown to be successful for the primary isolation of *Bartonella* spp. from human and dog samples (Breitschwerdt et al. 2007, Duncan et al. 2007). However, since these media are not selective for *Bartonella* spp. only, overgrowth of coinfecting bacteria can limit their use for primary isolations.

Molecular Detection of *Bartonella* spp. DNA

DNA extraction from animal samples

When choosing the DNA extraction method, two major aspects need to be considered: the presence of high concentrations of PCR inhibitors in the animal blood and tissue samples (Al-Soud and Radstrom 2001) and the efficacy of the DNA extraction method. To overcome the former, many commercial blood and tissue-based kits include PCR inhibitor neutralizers. The ability to amplify DNA from the extracted sample needs to be evaluated and confirmed when a new method is chosen, for instance, by targeting a host-associated locus (Roux and Raoult 1999, Halos et al. 2004, Morick et al. 2011). Second, the *Bartonella* loads in blood samples from animal reservoirs may be very low, representing a small proportion of infected erythrocytes, less than 5% (Harms and Dehio 2012). In such cases, mechanical and biochemical lysis combined methods and/or prolonged incubations in the buffer lysis (Roux and Raoult 1999) can enhance the recovery of the *Bartonella* DNA from the blood samples. Additionally, a pre-enrichment culture step of the *Bartonella* in liquid medium before DNA extraction has also shown to enhance the *Bartonella* DNA detection from clinical samples (Duncan et al. 2007, Bai et al. 2010).

For arthropod samples, a superficial cleaning step before DNA extraction is critical to remove potential biological contaminants and remnants of the ethanol used for storage and transportation. Washes usually include an immersion in fresh sterile ethanol 70% for 5–10 min, followed by two to three immersions of sterile water or PBS. After the washing steps, the arthropod can be processed for DNA extraction using tissue-based DNA extraction protocols (*e.g.*, commercial kits, phenol–chloroform protocols), using single individual arthropods or pools (2–20 individuals) depending on the size of the specimens. The use of arthropod pools increases the chances of detection of *Bartonella* DNA; however, it prevents the capability to determine coinfections with several *Bartonella* spp. in single arthropods and restricts prevalence determination. For small specimens (*e.g.*, fleas, lice, and mites), maceration of the whole arthropod with a sterile pestle in a sterile tube containing small volume of PBS ($\sim$50–100 μL) is recommended to avoid DNA losses. For larger arthropods, such as hard ticks, mincing the specimens into small pieces, by a bead beater or a sterile scalpel, and separating the exoskeleton by centrifugation, facilitate the mechanical destruction of the internal arthropod tissues and reduce the saturation of silica columns with excess exoskeleton (Halos et al. 2004, Harrus et al. 2011). To increase the efficiency of DNA extraction from the arthropod tissues, long incubation (over 2 h) in the buffer lysis is usually needed. In hard ticks, the presence of low quantities of the targeted DNA can be overcome by DNA preamplification protocols before the specific PCR (Michelet et al. 2014) or by using nested PCR assays (Cotté et al. 2008).

Amplification of Bartonella *DNA*

Amplification of *Bartonella* DNA from animal and human samples by PCR-based techniques has been extensively assessed. Conventional PCR assays were initially described for direct detection of *Bartonella* DNA from samples of clinical cases (Relman et al. 1990, Anderson et al. 1994, Birtles et al. 2000). However, when conventional PCR is used for the detection of *Bartonella* spp. in subclinical reservoirs, the sensitivity of the assays is usually limited. This is attributed to the low *Bartonella* loads in wild animals, which may result in false-negative results. Therefore, the use of more sensitive techniques, such as nested and real-time PCR assays, has improved the sensitivity of *Bartonella* diagnosis (Rampersad et al. 2005, Diaz et al. 2012, Gutiérrez et al. 2013). The disadvantage of the latter assay is the small size of the amplicons, limiting its specificity. Thus, confirmatory real-time PCR assays targeting additional loci or the combination with conventional PCR assays for larger amplicons are recommended.

Many conserved and housekeeping loci have been developed as targets for the characterization and molecular detection of *Bartonella* organisms (see Table 1 for recommended targets). Among them, the citrate synthase gene (*gltA*) and the

TABLE 1. AUTHOR-RECOMMENDED PCR TARGETS AND PRIMERS REQUIRED FOR THE DETECTION AND CHARACTERIZATION OF *BARTONELLA* SPP.

Bartonella *locus*		*Primers*	*Approximate size (bp)*	*Assay type*	*Reference*
Targets recommended for molecular characterization of isolates					
Citrate synthase	*gltA*	443F and 1210R; Bhcs.781p and Bhcs.1137n	340–370	Conventional PCR	Norman et al. (1995); Birtles and Raoult (1996)
β subunit of the RNA polymerase	*rpoB*	1400F and 2300R	825	Conventional PCR	Renesto et al. (2001)
16S-23S Internal transcribed spacer	ITS	321s and 983as	621–704	Conventional PCR	Maggi et al. (2005)
16S ribosomal RNA gene	*16S*	p24E and p12B	280	Conventional PCR	Bergmans et al. (1995)
Riboflavin synthase	*ribC*	Barton-1 and Barton-2	588	Conventional PCR	Johnson et al. (2003)
NADH dehydrogenase gamma subunit	*nuoG*	F and R	346	Conventional PCR/real-time PCR	Colborn et al. (2010)
Cell division protein	*ftsZ*	Bfp1 and Bfp2	900	Conventional PCR	Zeaiter et al. (2002)
60 kDa Heat shock protein	*groEL*	Hs233 and Hs1630	720	Conventional PCR	Marston et al. (1999)
Targets recommended for direct molecular detection (from blood/tissue/arthropod)					
mtRNA	*ssrA*	ssrAF and ssrAR	350	Real-time PCR	Diaz et al. (2012)
16S-23S Internal transcribed spacer	ITS	321s and H493as	200	Real-time PCR	Maggi et al. (2005); conditions for real-time PCR described in Gutiérrez et al. (2013)
β subunit of the RNA polymerase	*rpoB*	600F and 800R	200	Real-time PCR	Morick et al. (2009)

RNA polymerase β-subunit gene (*rpoB*) are the most commonly used targets for the identification of *Bartonella* spp. due to their potent discriminatory power (La Scola et al. 2003), their relative stability as housekeeping genes, and their extensive GenBank database. However, homologous recombination events have been reported within the *gltA*, potentially leading to some species misidentification (*e.g.*, in rodent-associated bartonellae) (Paziewska et al. 2011, Buffet et al. 2013). Recommendations for the identification of *Bartonella* variants are included in the Experts' Advice section below.

The sensitivity to detect positive samples varies considerably among the described PCR assays. Notably, many of the described primers in the literature were developed for the amplification and characterization of *Bartonella* isolates (from colonies). Consequently, once applied for direct detection of *Bartonella* DNA, cross-reaction with the host gDNA and/or with coinfecting microorganisms has been reported (Maggi and Breitschwerdt 2005, Colborn et al. 2010, Gutiérrez et al. 2013). Therefore, nonspecific amplification can mislead the diagnosis of *Bartonella* if the amplicons obtained are not sequenced or characterized. Although sequencing of positive amplicons is preferable, other techniques such as high-resolution melting (HRM) analysis can assist in rapid discrimination of *Bartonella* DNA amplicons according to their melt profiles (Morick et al. 2009, Gutiérrez et al. 2013).

Coinfection of hosts with more than one *Bartonella* species or genotype is a well-known phenomenon (Gurfield et al. 1997, Abbot et al. 2007, Chan and Kosoy 2010, Gutiérrez et al. 2014b). Taking into consideration that PCR-based assays are biased toward the most predominant species, in samples containing several species or genotypes, the molecular detection of a particular species does not rule out the presence of other coinfecting *Bartonella* spp. It has been observed that amplification of additional loci can lead to the detection of distinctly related *Bartonella* spp. DNA, suggesting coinfection (Gutiérrez et al. 2013, 2014a). The latter phenomenon can occur due to different primer-annealing sensitivities among the primers used that may favor detection of one of the species over the other (Whiley et al. 2008). Another possibility is that the diverse DNA sequences are originated from a single recombinant strain, as has been described earlier in rodent-associated bartonellae (Harrus et al. 2009, Paziewska et al. 2011, Buffet et al. 2013). Although both scenarios have been demonstrated, the former seems to be a more common event. Ideally, to characterize coinfecting *Bartonella* genotypes by molecular methods, multiple amplicons of a single-locus target (*e.g.*, *gltA*) can be sequenced, using cloning libraries (in *Escherichia coli* vectors) or 454-pyrosequencing platforms (Abbot et al. 2007, Gutiérrez et al. 2014b). Furthermore, in cases where the *Bartonella* spp. involved in a particular animal population have been well characterized and seem homogeneous worldwide (*e.g.*, feline-associated and bovine-associated bartonellae), species-specific primers may be developed to test the potential coinfecting spp. in previously screened positive samples (Bereswill et al. 1999, Rolain et al. 2003, Cherry et al. 2009). The development of species-specific primers is currently facilitated with the availability of the complete and/or partial genomes of many *Bartonella* spp.

In wild animals, such as carnivores, rodents, bats, and their associated ectoparasites, the detection of yet uncharacterized

Bartonella spp. and genotypes is a common phenomenon (Diniz et al. 2009, Inoue et al. 2009, Morick et al. 2010, Bai et al. 2012). To prevent the erroneous relationship of loci from different genotypes (during coinfection) with one uncharacterized strain, independent description of the amplicons is recommended. Only further characterization of isolated colonies will eventually guarantee accurate identification.

The advantages and limitations of the use of molecular detection assays for *Bartonella* diagnosis have led to the following recommendations: (1) real-time PCR should be chosen over conventional PCR assays when *Bartonella* infection is assessed among animal hosts; (2) characterization (sequencing) of the amplicons obtained is indispensable; and (3) additional loci preferably of longer length should be targeted to confirm positive samples, detect false-negative samples, and to potentially detect coinfecting *Bartonella* spp. Furthermore, when a new screening of samples is planned, evaluation of the sensitivity and specificity of different primers to the intended samples is recommended. Ideally, the assay should be standardized so as to quantify the detection limit of the assay selected.

Molecular Detection Versus Culture Isolation

The culture isolation of a *Bartonella* species from an infected animal will always be the preferred direct method for the diagnosis and characterization of the species involved. However, despite the improvements in culturing methods, these assays are still laborious, time-consuming, and their sensitivity to detect *Bartonella*-positive samples from wild animals is considerably low. In contrast, molecular diagnostic methods offer a more rapid, specific, and sensitive tool to determine the *Bartonella* infections. Studies that have compared both methods have demonstrated the higher sensitivity of real-time PCR assays over isolation in more than twofold of the positive samples (Mietze et al. 2011, Diaz et al. 2012, Gutiérrez et al.

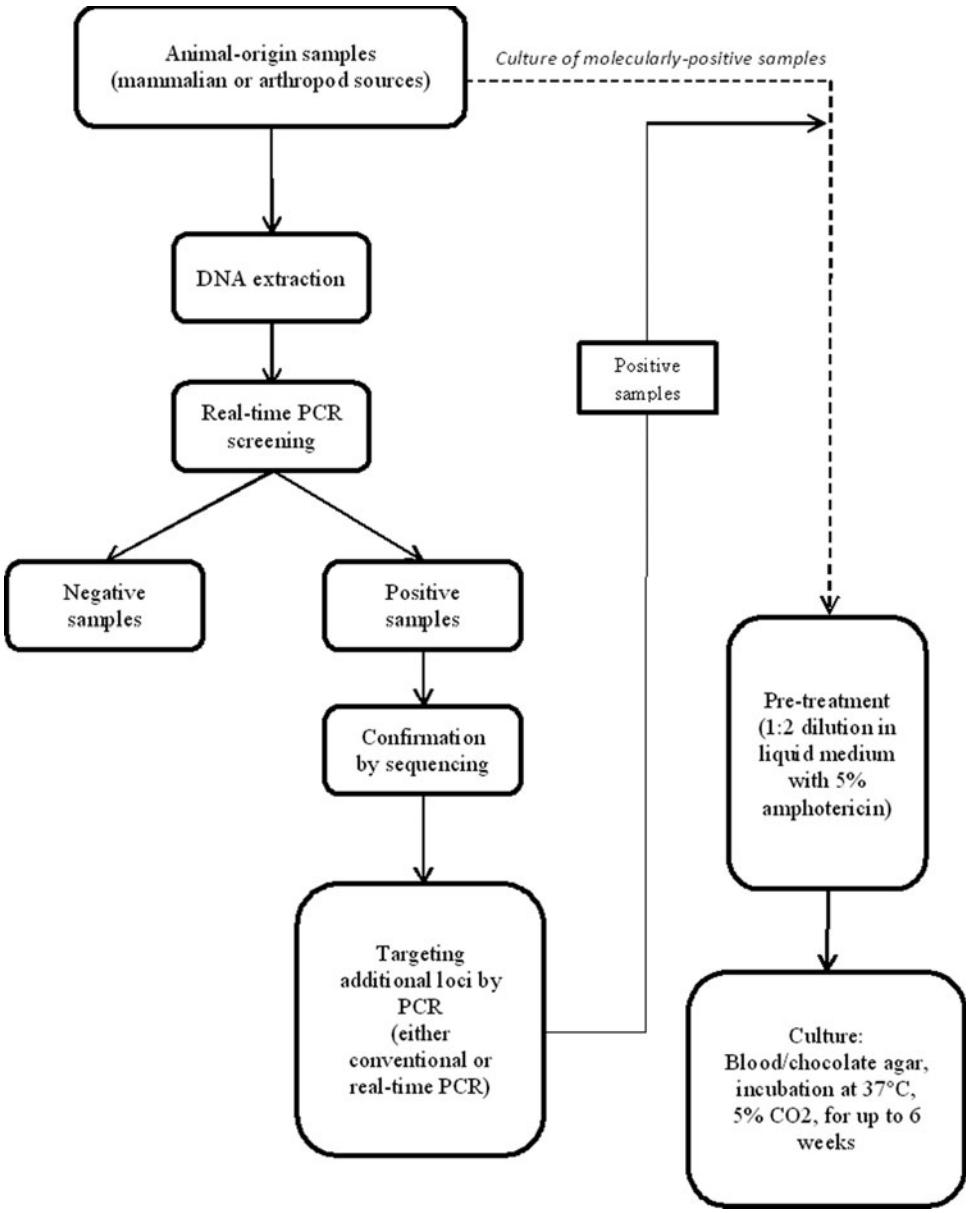

FIG. 1. Recommended workchart for detection of *Bartonella* in animal samples. Those should be prepared for DNA extraction, followed by molecular screening for *Bartonella* DNA, preferably by real-time PCR assay. Positive amplicons need to be characterized, preferably by sequencing. Then, *Bartonella*-positive samples should be screened for additional loci. Blood and/or tissue samples confirmed to be PCR positive can be assessed for *Bartonella* isolation. Pretreatment of the samples before seeding in agar plates is recommended.

2013). Additionally, different *Bartonella* spp./strains present different growing rates in culture, significantly biasing the infection description in coinfected animals, using this sole methodology. Thus, applying the appropriate PCR targets, followed by a characterization of the obtained DNA sequences, molecular methods can represent more sensitive and discriminatory assays for the detection of *Bartonella* within animal populations. Moreover, we encourage the attempt of culture isolation of DNA-positive samples since isolated colonies enable a broader molecular characterization and speciation of the infecting bacteria. Figure 1 illustrates the recommended work-chart in the detection of *Bartonella* in animal samples.

Experts' Advice on the Identification of *Bartonella*

The high genetic diversity of *Bartonella* species makes the identification of *Bartonella* isolates or uncultured organisms a challenging task. La Scola et al. (2003) proposed a criterion based on the sequencing of 327-bp *gltA* and 825-bp *rpoB* fragments. Accordingly, if an isolate showed similarities lower than 96.0% and 95.4% for the latter genes (of the validated species), respectively, it was proposed to be considered a new species. However, using these housekeeping loci and additional ones (*e.g.*, ITS, *ribC*, *groEL*), many isolates or uncultured bartonellae sequences have shown to represent variants of the validated *Bartonella* spp. (with sequence similarities between 95% and 99%), as well as more distinctly related variants (similarities below 95%), and/or cases of mixed origin species (*i.e.*, isolates carrying two or more loci closely related to different validated *Bartonella* spp.). Moreover, due to the frequent coinfection of hosts with more than one *Bartonella* species, the identification of *Bartonella* needs to be carefully addressed. Thus, in the case of isolates, we recommend to identify all *Bartonella* isolates with at least three to five loci and use only well-isolated colonies (*i.e.*, at least two repassages in agar plates from single colonies). Moreover, phylogenetic analysis using concatenated sequences is recommended to support the differentiation and diagnosis of newly isolated variants (Harrus et al. 2009, Buffet et al. 2013). In addition, we recommend the isolation of several colonies (three to five colonies) from the primary isolation plates to facilitate the detection of potential coinfections. When direct molecular detection (*i.e.*, uncultured organisms) is performed for the identification of the host-infecting *Bartonella*, screening of at least two to three loci is highly recommended. The latter will allow a more robust confirmation of positive samples and potentially identify coinfecting bartonellae. With the constant decrease in costs of whole-genome sequencing (WGS) techniques, future characterization of new *Bartonella* species and variants will require the use of WGS.

Conclusions

As *Bartonella* bacteria emerge constantly and their importance in public health increases, rapid diagnostic tools are required. We suggest the use of real-time PCR assays for the initial screening of *Bartonella* spp., followed by several molecular confirmatory assays targeting several loci (preferably of longer fragments than those amplified by the real-time assay). In addition, we encourage the use of culture isolation as it denotes the presence of live bacteria. Moreover, the isolated colonies enable a broader molecular characterization and accurate speciation of the bacteria.

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Address correspondence to:
Shimon Harrus
Koret School of Veterinary Medicine
The Hebrew University of Jerusalem
PO Box 12
Rehovot 76100
Israel

E-mail: shimon.harrus@mail.huji.ac.il

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Guidelines for the Detection of *Babesia* and *Theileria* Parasites

Laetitia Lempereur,[1] Relja Beck,[2] Isabel Fonseca,[3] Cátia Marques,[3] Ana Duarte,[3] Marcos Santos,[3] Sara Zúquete,[3] Jacinto Gomes,[4] Gernot Walder,[5] Ana Domingos,[6] Sandra Antunes,[6] Gad Baneth,[7] Cornelia Silaghi,[8] Patricia Holman,[9] and Annetta Zintl[10]

Abstract

The genera *Babesia* and *Theileria* (phylum Apicomplexa, order Piroplasmida) are mainly transmitted by Ixodid ticks in which the sexual part of their life cycle followed by sporogony takes place. They include protozoan parasites that infect erythrocytes of a variety of vertebrate hosts, including domestic and wild animals, with some *Babesia* spp. also infecting humans. *Babesia* sporozoites transmitted in the tick's saliva during the bloodmeal directly infect erythrocytes, where they asexually multiply to produce pear-shaped merozoites in the process of merogony; whereas a pre-erythrocytic schizogonic life stage in leukocytes is found in *Theileria* and precedes merogony in the erythrocytes. The wide spectrum of *Babesia* and *Theileria* species and their dissimilar characteristics with relation to disease severity, transmission, epidemiology, and drug susceptibility stress the importance of accurate detection of babesiosis and theileriosis and their causative agents. These guidelines review the main methods currently used for the detection of *Babesia* and *Theileria* spp. for diagnostic purposes as well as epidemiological studies involving their vertebrate hosts and arthropod vectors. Serological methods were not included once they did not indicate current infection but rather exposure.

Keywords: *Babesia*, diagnosis, in vitro culture, PCR, *Theileria*

Introduction

The genera *Babesia* and *Theileria* (phylum Apicomplexa, order Piroplasmida) are protozoan parasites that infect erythrocytes of a variety of vertebrate hosts, including domestic and wild animals, with some *Babesia* spp. also infecting humans. The parasites are transmitted by Ixodid ticks. In the arachnid host, sexual reproduction takes place followed by sporogony. Resulting sporozoites are transmitted in the tick's saliva during the bloodmeal. *Babesia* spp directly infect erythrocytes, where they asexually multiply to produce pear-shaped merozoites in the process of merogony. In contrast, *Theileria* spp undergo a pre-erythrocytic schizogonic life stage in leukocytes that precedes merogony in the erythrocytes (Uilenberg 2006, Hunfeld et al. 2008).

Babesia species have traditionally been categorized according to their vertebrate hosts and morphology into large ($\sim$3–5 μm) and small forms (0.5–2.5 μm) based on the size of their merozoites when viewed by light microscopy in stained blood smears. More recent molecular studies involving gene sequencing and phylogenetic analyses have uncovered a much broader diversity among *Babesia* species, even those of a similar size and infecting the same animal host. Such studies have shown that babesial species actually group into several discrete clades that separate what appear to be identical parasites by light microscopy, thus enforcing the importance of the use of molecular techniques for species identification (Criado-Fornelio et al. 2004, Schnittger et al. 2012, Yabsley and Shock 2012). In the past decade, several new pathogenic species of *Theileria* have been identified and

[1]Laboratory of Parasitology and Parasitic Diseases, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium.
[2]Laboratory for Parasitology, Croatian Veterinary Institute, Zagreb, Croatia.
[3]Faculty of Veterinary Medicine, Centre for Interdisciplinary Research in Animal Health, University of Lisbon, Lisbon, Portugal.
[4]National Institute for Agrarian and Veterinary Research, Oeiras, Portugal.
[5]Department of Hygiene and Medical Microbiology, Innsbruck Medical University, Innsbruck, Austria.
[6]Global Health and Tropical Medicine, Instituto de Higiene e Medicina Tropical (IHMT), Lisbon, Portugal.
[7]Koret School of Veterinary Medicine, Hebrew University, Rehovot, Israel.
[8]National Centre for Vector Entomology, Institute of Parasitology, University of Zurich, Zurich, Switzerland.
[9]Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, Texas.
[10]UCD Veterinary Sciences Centre, University College Dublin, Belfield, Dublin, Ireland.

pathogenic strains of other previously low-pathogenic species have emerged (Morrison 2015).

Clinical babesiosis and theileriosis are usually associated with anemia due to erythrocyte destruction and hemolysis, with lymph node enlargement characteristic of theileriosis. Infected hosts may suffer from acute onset of fever and lethargy, and the disease may progress to severe fatal organ failure. On the other hand, animals may become chronic carriers of infection, often without manifesting clinical abnormalities. *Babesia* species possess variable degrees of virulence, with some species able to cause severe disease whereas others only induce mild-to-moderate illness. Different species are transmitted by different tick vectors, and they may, therefore, be limited geographically to the regions where their vectors are prevalent. Furthermore, drug susceptibility differs among species and especially between the small and large forms of parasites (Solano-Gallego and Baneth 2011, Mosqueda et al. 2012).

The wide spectrum of *Babesia* and *Theileria* species and their species-specific characteristics with relation to disease severity, transmission, epidemiology, and drug susceptibility underline the importance of accurate identification of the causative agents. These guidelines review the main methods currently used for the detection of *Babesia* and *Theileria* spp. for diagnostic purposes as well as epidemiological studies involving their vertebrate hosts and arthropod vectors. Serological methods were not included, because they indicate exposure, rather than current infection and often lack species specificity.

Microscopic Examination

Detection of Babesia *in blood smears*

Sensitivity and specificity of parasite identification based on microscopical examination of blood smears are dependent on the experience and proficiency of the examiner. Nevertheless, this technique is a cheap and easy method that is readily available in all standard laboratories and possibly even in the field for the detection of acute cases. Moreover, knowledge of the endemic species in an area and differentiation between ''large'' and ''small'' *Babesia* species can help to narrow down the identity of the detected piroplasms (Table 1). Most commonly, thin blood smears are air-dried, fixed (absolute methanol, 2 min), and stained in a 1:20 dilution of Giemsa stain (40 min). Other stains such as Romanowsky, Diff-Quik, and Wright's can also be used. Sensitivity may be improved by using ''thick'' blood smears. However, identification of the parasite outside the host cell and in the presence of the many artefacts produced by this method is challenging. Species that accumulate in capillaries and tissues, such as *Babesia bovis*, are more readily detected in capillary blood (collected from the ear tip or nail bed) or in crush smears of certain organs such as brain (gray matter of the cerebral cortex), spleen, liver, or kidneys (Figueroa et al. 2010). All other species are best diagnosed from anticoagulated venous blood.

With regard to human infections, in areas where malaria is abundant, differentiation between *Babesia* and *Plasmodium* spp. can be challenging. The main differences are that *Babesia*-infected erythrocytes lack hemozoin deposits (although these are not always present in infections with young *Plasmodium* trophozoites either) and occasionally present tetrade or ''Maltese cross'' formations, which are never observed in *Plasmodium* spp. infection. In contrast, the latter may exhibit distinctive schizonts and gametocytes (Kjemtrup and Conrad 2000).

Detection of Theileria *in blood and tissue samples*

The same protocols are used to fix and stain *Theileria* parasites in thin blood/lymph node or spleen smears. In erythrocytes, *Theileria* merozoites are predominantly rod shaped and up to 2.0 μm long and 1.0 μm wide. Round, oval, and ring-shaped forms also occur. Multiple parasites per erythrocyte are common. In the cytoplasm of lymphocytes, two types of schizonts (Koch's blue bodies) can be found: macroschizonts and microschizonts, both about 8.0 μm, containing up to 8 and 36 small nuclei, respectively (Urquhart et al. 1996).

Microscopic detection in ticks

Although sporozoites of both *Babesia* and *Theileria* species can be detected in whole tick salivary glands (SG) using the Feulgen technique or in SG histological sections, after staining with basic dye, they can only be differentiated by polymerase chain reaction (PCR). However, it provides a quick and easy method for screening a pasture before introducing cattle onto it.

For this purpose, unfed or 4–5 day engorged ticks are embedded in a small Petri dish in a 1 cm-diameter circle of melted paraffin, with the tick's dorsal surface facing up. To lift the scutum, an incision is made with a scalpel blade around the margin of the body, starting and ending at the base of the capitulum. This facilitates removal of the gut and exposure of the SG (Edwards et al. 2009). Trachea fragments are removed, and the whole SG are immersed in physiological saline solution. SG are then fixed for 15–30 min in Carnoy's fluid (Marx and Stern 2003) followed by dehydration overnight in absolute ethanol. Using a small brush, samples are washed in a small Petri dish, stained for 2 h with Feulgen's reagent, washed again, dehydrated, and cleared with xylol. Infected acini that appear as Feulgen-positive bodies (DNA red-purple and cytoplasm green) can be quantified by immersion in xylol or methyl salicylate in a Petri dish or after slide mounting in Canada balm or DPX using a stereomicroscope at magnifications of ×500 or higher. Other, more time-consuming staining methods such as green-methyl pyronine, toluidine blue, or hematoxylin-eosin (histological sections) can also be used.

Molecular Detection

Samples and storage

Both blood and spleen are the most commonly selected samples that are suitable for molecular detection of *Babesia* and *Theileria* spp. in vertebrate hosts. Samples should be stored at –20°C unless they are used within 72 h, in which case they should be refrigerated at +4°C. Spleen samples can also be stored in 70% ethanol. Under field conditions, blood samples can be dried onto FTA cards, which allows easy transportation and long-term storage (Rahikainen et al. 2016).

Prevalence studies of *Babesia* and *Theileria* in ticks should be based on analysis of questing ticks collected from the environment, as positivity in ticks collected from hosts may simply represent a remnant of the most recent bloodmeal rather than an active infection in the tick. All stages of ticks can be used for epidemiological studies, but it should be

Table 1. Morphological Description and Geographic Distribution of *Babesia* and *Theileria* Species Occurring in Different Vertebrate Hosts

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Species reported from humans					
Babesia divergens	"Small" 1.9×0.8 μm (piriforms)	Up to 80%	Ring stages characterized by big vacuoles; merozoites subcentral; tetrad stages; and polyparasitism (5–8 parasites per RBC) common	Throughout Europe and North Africa	Zintl et al. (2003); Figueroa et al. (2010)
Babesia venatorum (EU1)	"Small" 1–2.5 μm (ring stages)	1–30%	Ring forms most common; tetrad stages present but rare; position in RBC's generally peripheral	Cases reported from Italy, Austria, Germany, and China	Häselbarth et al. (2007); Herwaldt et al. (2003); Jiang et al. (2015)
Babesia microti	"Small" 1–2.5 μm	4.5%	"Characteristic" ring and piriforms; parasites usually present singly or in pairs	Germany (single case); USA	Hildebrandt et al. (2007); Smith et al. (2014)
Babesia Duncani	"Small"	6%	Round to oval, with some piriform, ring, and ameboid forms	USA	Conrad et al. (2006)
Bovine SPP					
Babesia bigemina	"Large" 2.5–3.5 μm (ring stages) 2.5–4.5 μm (piriforms) (may extend to the full diameter of the RBC)	Up to 40%	Single forms, elongated or amoeboid with fine cytoplasmic filaments; paired piriforms typically at an acute angle	Tropical and subtropical regions of all continents, including Southern Europe	De Vos and Potgieter (1994); Bock et al. (2004); Mosqueda et al. (2012); Figueroa et al. (2010)
Babesia bovis	"Small" 1–1.5 μm (ring stages) 1.5–2.4 μm (piriforms)	1–0.1% (highest in capillary blood)	Single forms, generally round or oval; paired piriforms, usually but not always at an obtuse angle and less common than vacuolated signet ring forms; amoeboid forms rare	As for *B. bigemina* but less widespread	De Vos and Potgieter (1994); Bock et al. (2004); Mosqueda et al. (2012); Figueroa et al. (2010)
B. divergens	"Small" 1.5–1.8 μm (ring stages) 1.5–1.9×0.4–1.1 μm (piriforms)	Up to 30–45%	Widely divergent paired organisms at the very edge of RBC's, may cause slight protrusions of the erythrocyte membrane; tetrad formations and polyparasitism infrequent	Europe and North Africa	Zintl et al. (2003)
Babesia major	"Large" 1.8 μm (ring stages) 2.6×1.5 μm (piriforms)	No information available	Piriforms characteristically paired at an acute angle; position typically central	Europe	Bock et al. (2004); Figueroa et al. (2010)
Theileria parva	No information available	No information available	Schizonts ("Koch bodies") typically with 12 nuclei (up to 80 reported from in vitro cultures) Piroplasms predominantly round or oval; division by binary fission may result in two or four daughter cells, the latter typically in the shape of a cross	East Africa	Morrison (2015)

(*continued*)

Table 1. (Continued)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Theileria annulata	1–15 μm (average 8, max. 27 μm) (schizonts in macrophages/monocytes)	May reach 95%	Schizonts (''Koch bodies'') typically with 12 nuclei (up to 80 reported from in vitro cultures)	North Africa, Mauritania, Sudan, Near and Middle East, central Asia, Indian subcontinent, southern Europe (Portugal, Spain, the Balkans)	Mehlhorn (2008); Uilenberg (1981); Darghouth et al. (2010); Pipano and Shkap (2004)
	In RBC's: 0.5–2 μm (round stages); 0.5 × 1.6 μm (comma shapes); parasites observed during the acute stage smaller than those seen during the chronic stage of infection		Piroplasms predominantly round or oval; division by binary fission may result in two or four daughter cells, the latter typically in the shape of a cross		
Theileria buffeli/ orientalis complex	Highly variable	No information available	Variable morphological features; predominantly bacilliform; piroplasms sometimes associated with a ''bar'' or ''endoerythrocytic veils''; spiny deformation of RBC's during acute infection	Asia, Mediterranean basin, western Europe, Australia, North Africa, North America?	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004a)
Canine SPP					
Babesia canis	''Large'' 2.5–3 × 5 μm (piriforms) 2–4 μm (amoeboid forms)	1%	Piriform, usually paired; polyparasitized RBC's common (up to 16 parasites/RBC)	Europe, Asia	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Purnell (1981)
Babesia vogeli	''Large'' 2.5 × 4.5 μm	No information available	Piriform, single or paired	Tropical and subtropical regions of all continents, including southern Europe	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Figueroa et al. (2010)
Babesia rossi	''Large'' 2.5 × 4.5 μm	No information available	Piriform, single or paired	Africa; reported mostly from southern Africa and also from Nigeria and Sudan	Jacobson (2006); Solano-Gallego and Baneth (2011)
Babesia gibsoni	''Small'' 1.2 × 3.2 μm (ring stages) 1.4–2 μm (piriforms) 2–2.5 μm (amoeboid forms) (no more than one eighth the diameter of the host erythrocyte)	No information available	Pleomorphic with oval and signet rings, single or in pairs; tetrads observed occasionally	Southeast Asia, USA, South America, Europe	Solano-Gallego and Baneth (2011); Ayoob et al. (2010a); Purnell (1981)
Babesia vulpes	''Small'' 2.5 × 1 μm	No information available	Typically single	Northern Spain, Portugal, Croatia, Sweden	Baneth et al. (2015); Ayoob et al. (2010a); Zahler et al. (2000)

(*continued*)

Table 1. (Continued)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Babesia conradae	"Small"	No information available	Morphologically not distinguishable from other canine small *Babesia* spp. by light microscopy	California	Kjemtrup et al. (2006)
Cervine SPP					
Babesia capreoli	"Small" (1–3 μm), larger in fallow than in roe deer RBC's	20%–25% in roe and reindeer	Round to oval of various sizes; paired piriforms divergent and peripheral; tetrads and polyparasitism (≥ 8 parasites per RBC) present in roe, not in fallow deer	Throughout Europe	Malandrin et al. (2010)
B. divergens		Isolated cases in reindeer		Throughout Europe and North Africa	Wiegmann et al. (2015)
B. venatorum	No information available	Deer	No information available	Throughout Europe	Herwaldt et al. (2003)
Equine SPP					
Babesia caballi	"Large" 1.5–3 μm (ring stages) 2–5 $\times$ 1–1.5 μm (piriforms)	$\leq 0.1\%$	Piriforms typically in pairs with an acute angle between them; RBC's rarely with >2 parasites	Africa, South and central America and southern USA, southern Europe, Asia	Bock et al. (2004); Wise et al. (2013); De Waal and Heerden (2004)
Theileria equi	"Small" 2–3 μm in diameter	1–5% (severe cases may exceed 20%)	Polymorphic (oval, round, elliptical, or piriform); typically two or four piriforms per RBC; distinctive Maltese cross formation	Southern Europe Africa, Asia, America	Wise et al. (2013); Mehlhorn (2008); De Waal and Heerden (2004)
Feline SPP					
B. canis	"Large" 3 $\times$ 5 μm	No information available	Piriform, singlet and pairs	Spain, Portugal	Ayoob et al. (2010b)
Babesia felis	"Small" 0.9 $\times$ 0.7 μm	Determined by immunocompetence, chronicity of infection, and concurrent disease	Single or paired annular body (signet rings), pair-shaped forms, and rarely tetrads	Africa, South Asia, Europe	Ayoob et al. (2010b)
Babesia lengau	"Small" 1.1 $\times$ 1.9 μm	No information available	Found in the blood and also in brain capillaries associated with feline cerebral babesiosis	Africa	Bosman et al. (2013)
Babesia presentii	"Large" 2.5 $\times$ 1.4 μm	1–5%	Single ring forms and paired merozoite forms, with some cells containing four merozoites not arranged as tetrads	Israel	Baneth et al. (2004)

(*continued*)

Table 1. (Continued)

Babesia/Theileria *species*	*Size*	*Typical parasi-taemia (%) in clinical cases*	*Characteristic features*	*Geographic distribution*	*Reference*
Ovine and caprine SPP					
Babesia motasi	"Large" 2.2–3.8 × 2 μm (piriforms) (occupying two thirds or more of the RBC)	No information available	Usually piriform, singly or in pairs, generally at an acute angle; ring, oval, elongated, or budding forms less frequent; forms with two nuclei common	Europe, particularly the Mediterranean basin, Africa, Asia, America? (Cuba?)	Bock et al. (2004); Figueroa et al. (2010); Yeruham and Hadani (2004)
B. ovis	"Small" 1–2.5 μm	5–7.5%	Mostly oval or piriform, paired forms usually at an obtuse angle; position in RBC's typically marginal; tetrads uncommon; all forms with single nucleus	Mediterranean basin, Balkans, Kazakhstan, Kirghizstan, Turkmenistan, Iraq, Iran	Bock et al. (2004); Yeruham and Hadani (2004)
Theileria lestoquardi (syn. *T. hirci*)	10–20 μm (average 8 μm) (schizonts in macrophages/ monocytes) In RBC's: 0.6–2 μm (round stages); 1.6 μm (comma shapes)	Parasitaemia typically much higher than in *Theileria ovis*	Round, oval, or rod shaped	Balkans, Mediterranean basin, Middle East, North and East Africa, Asia	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004b)
T. ovis (syn. *Theileria recondita, Theileria sergenti*)	As for *T. lestoquardi*	<2% (of RBC's)		Widespread in Africa, Asia, and Europe	Mehlhorn (2008); Darghouth et al. (2010); Lawrence (2004b)
Porcine SPP					
Babesia perroncitoi	"Small" 0.7–2 μm (ring stages) 1.2–2.6 × 0.7–1.9 μm (piriforms)	Up to 60%	Mostly pleomorphic and annular (some oval, quadrangular, and piriforms also occur), usually single, sometimes ≥2 per cell	Southern Europe (Italy), West and Central Africa, China, Vietnam	Bock et al. (2004); Figueroa et al. (2010); De Waal (2004)
Babesia trautmanni	"Large" 2.5–4 × 1.5–2 μm (piriforms)	Up to 60%	Paired forms, usually oval or pirifoms; single forms, typically amoeboid or round; 1–4 parasites per RBC	Southern Europe, former USSR, Africa	Bock et al. (2004); Figueroa et al. (2010); De Waal (2004)

RBC, red blood cell.

remembered that *Theilera* species do not undergo a transovarial transmission in the vector tick whereas some *Babesia* species do, and therefore larvae are not recommended for detection of *Theileria* species (Schnittger et al. 2012). Whenever epidemiological studies in ticks are performed, it is of utmost importance to identify the collected ticks to species level to gain valid epidemiological data and to avoid drawing wrong conclusions regarding host–pathogen associations.

Ticks can be stored in 70% ethanol at room temperature or +4°C, or they can be frozen at −20°C or −80°C depending on the intended usage. Analyses may be performed on partial or whole ticks, individually or pooled, depending on the study design.

DNA, RNA, and protein extraction

Before DNA extraction, whole ticks may be split into two longitudinal halves using a sterile scalpel blade. One half can then be processed further, whereas the other could be stored as a back-up sample. Longitudinal slicing is especially recommended for fully engorged ticks, as the relatively large amount of sample can interfere with the DNA extraction process. Individual organs may also be dissected and analyzed to address more specific research questions: Analysis of SG is recommended for assessing pathogen transmission capacity; analysis of ovaries for investigating vertical transmission; and midgut analysis to track pathogens after meals of infected blood. SG, ovaries, and midgut are isolated using the method described earlier. The gut is a dark red, spider-shaped structure. On removal of the gut, ovaries appear as an inverted U-shaped structure distal to the rectal sac (Edwards et al. 2009).

Before extraction, all tick tissue samples, whether whole or dissected parts, as well as host spleen samples, should be homogenized either manually with a sterile scalpel blade or automatically with a device such as the Tissue Lyser (Qiagen) or Precyllis® (Halos et al. 2004). Afterward, samples are directly placed in DNA extraction buffer (according to the chosen commercial kit) or in RNA later® or Trizol® if RNAs and proteins are also to be extracted.

Total DNA extraction of all tissues mentioned earlier and blood can be performed using a commercial DNA extraction kit, although other methods using Proteinase K have been described (Boom et al. 1990). Extracted DNA can be stored at +4°C (short term) or −20°C (long term) until further use. The quality and quantity of extracted DNA can be evaluated using gel electrophoresis, spectrophotometry, or partial gene amplification of conserved genes of the tick or vertebrate host (18S, β-actin, ITS, or others).

PCR analysis

Several conventional and real-time PCR assays have been described for detection of *Babesia* and *Theileria* spp. in vertebrate and tick hosts. These techniques are usually more sensitive than microscopy (Wang et al. 2015) and, depending on gene target and size, they may permit identification to genus or species level and/or phylogenetic analysis. Genus-specific assays are recommended for epidemiological studies when several piroplasm species may be present, or for diagnostic purposes when species-specific assays fail, but piroplasms are suspected. Finally, genus-specific assays are used when a piroplasm that cannot be further identified is found. In the latter case, full sequence analysis of the 18S rRNA should be attempted. On the other hand, species-specific molecular methods are used for diagnostic purposes or for epidemiological studies of the distribution of a particular piroplasm species.

Table 2 lists primers used for genus and species-specific detection and identification of *Babesia* and *Theileria* spp. Molecular targets include the 18S rRNA gene, HSP70, ITS1, CCTeta, Ema-1, and Tams, with the 18S gene being, by far, the most commonly used target gene. Nuclear ribosomal rRNA genes are frequently used as targets for species identification (Chae et al. 1998, Katzer et al. 1998, Allsopp and Allsopp 2006), because of their conserved nature and repetitive arrangement within the genome that provide ample amounts of template DNA for PCR. Although a high degree of 18S rRNA gene sequence conservation has been reported between *Babesia* and *Theileria* species, it has been recommended that the complete 18S rRNA gene be amplified, particularly when dealing with new organisms, to ensure that genetic variation is not overlooked (Herwaldt et al. 2003, Hunfeld et al. 2008, Bhoora et al. 2009).

Tams 1 encodes a major polypeptide that is located on the surface membrane of merozoite and piroplasm stages of *Theileria annulata*. Because of the level of diversity within the gene, the primers listed may not detect all *T. annulata* genotypes, potentially leading to an underestimation of the prevalence of this pathogen (Katzer et al. 2006, Santos et al. 2013).

Alternative molecular methods include reverse line blot, which allows simultaneous screening of large numbers of pathogens using probe hybridization (Gubbels et al. 1999, Hurtado 2015), and modern high-throughput screening methods such as the microfluidic real-time PCR system (Fluidigm) (Michelet et al. 2014) and Next Generation sequencing (Bonnet et al. 2014).

In Vitro Culture

With the widespread use of molecular tools, traditional methods such as in vitro culture are used less frequently in current research. It could be argued, however, that without detailed information on parasite biology and its interaction with the host, molecular data are of limited use. Cell culture-based research is an important tool for gaining such information, for allowing for easy manipulation of conditions, and for reducing the need for animal experimentation.

The intra-erythrocytic stage of a great number of *Babesia* species has been established in in vitro culture (Table 3). In contrast, most successful in vitro culture systems for *Theileria* spp. involve the pre-erythrocytic schizont stage in leukocytes (Table 4).

In vitro culture of Babesia *and erythrocytic* Theileria *stages*

The earliest in vitro methods used to grow *Babesia* parasites relied on suspension cultures adapted from systems originally developed for *Plasmodium* (Trager and Jensen 1976). However, these required large volumes of reagents and extensive manipulations and were quickly replaced by microaerophilous stationary-phase systems, which are characterized by reduced O_2 tension in the atmosphere, and a static layer of erythrocytes settled at the bottom of the culture unit (Levy et al. 1981). Since then, a variety of methods have

Table 2. Primers Designed for Polymerase Chain Reaction and Real Time PCR Detection of *Babesia/Theileria* Species

Organism	*Target gene*	*Amplicon size (bp)*	*Primers*	*Reference*
Apicomplexa	18S rRNA	1700	CryptoF: AACCTGGTTGATCCTGCCAGT CryptoR: GCTTGATCCTTCTGCAGGTTCACCTAC	Herwaldt et al. (2003)
Babesia/Theileria spp.	18S rRNA	411–452	BJ1: GTCTTGTAATTGGAATGATGG BN2: TAGTTTATGGTTAGGACTACG	Casati et al. (2006); Lempereur et al. (2011)
Babesia/Theileria spp.	18S rRNA	389–426	BTV4_Fow: CACAGGGAGGTAGTGACAAG BTV4_Rev: AAGAATTTCACCTCTGACAG	Schnittger et al. (2004)
Babesia spp.	18S rRNA	422–440	BabsppF1: GTTTCTGMCCCATCAGCTTGAC BabsppR: CAAGACAAAAGTCTGCTTGAAAC	Hilpertshauser et al. (2006)
B. venatorum	18S rRNA	91	Bab_EU_RNA18S_F: GCGCGCTACACT GATGCATT Bab_EU_RNA18S_R: CAAAAATCAATC CCCGTCACG Bab_EU_RNA18S_P: CATCGAGTTTAAT CCTGTCCCGAAAGG	Michelet et al. (2014)
B. divergens	Hsp 70	83	Bab_di_hsp70_F: CTCATTGGTGACGCCGCTA Bab_di_hsp70_R: CTCCTCCCGATAAGCCTCTT Bab_di_hsp70_P: AGAACCAGGAGGCCCGT AACCCAGA	Michelet et al. (2014)
Babesia microti/ Babesia duncani	ITS1	930–950	BABITS_F: GGTGAACCTGCRGAAGGATC BABITS_R: TCTKCCGCTTARTTATATGC	Wilson et al. (2015)
B. microti	CCTeta	145	Bab_mi_CCTeta_F: ACAATGGATTTTCC CCAGCAAAA Bab_mi_CCTeta_F: GCGACATTTCGGCAA CTTATATA Bab_mi_CCTeta_P: TACTCTGGTGCAATGA GCGTATGGGTA	Michelet et al. (2014)
Babesia ovis	18S rRNA	549	OvisB_Fow: TGG GCA GGA CCT TGG TTC TTC OvisB_Rev: CCG CGT AGC GCC GGC TAA ATA	Aktas et al. (2005)
T. equi	Ema-1	115–270	Ema 1-f: GAGTCCATTGACCACCTCAC Ema 1-r: GTGCCTGACGACAGTCTTTGG Ema 1-p: TCGACAAGCAGTCCGAGCACA	Ueti et al. (2003)
Babesia caballi	Ema-1	700	BC 48-f: ACGTACACGAATGTTGATGAGTT BC 48-r: AGAGCGAATAATCTGCTTAAGTGC BC 48-p: TGATGCCCCAGCCGAACAACCCAG	Heim et al. (2007)
Theileria spp.	18S rRNA	230	F: GGTAAT TCCAGC TCC AATAG R: ACC AAC AAA ATAGAACCA AAG TC	Sibeko et al. (2008)
T. annulata	*Tams1*	319	Tams1F: CCAATTCGAGACCTACTACGATG Tams1R: CCACTTRTCGTCCTTAAGCTCG	Santos et al. (2013)

been developed, some of which are detailed in Table 3. As the table indicates, there are some differences of opinion between laboratories on the optimum culture conditions for any given species. This may be due to regional differences in parasite strains and their specific requirements or variations in the suitability of sera and red blood cells derived from donor animals (Canning and Winger 1987). The methods described here are based on culture systems originally developed by Holman et al. (1993a). In our experience, it supports the isolation and maintenance of many *Babesia* species, including some of the most fastidious members of the genus, as well as *Theileria equi* and *T. uilenbergi*, the only two *Theileria* spp. that have been successfully maintained in the erythrocytic stage (Holman et al. 1994a, Zweygarth et al. 1995, Miranda et al. 2006).

Given appropriate culture conditions, the parasites usually reach optimal growth within one or two subcultures after isolation, suggesting that a period of adaptation and/or selection is not required (Levy et al. 1981, Canning and Winger 1987).

(1) Donor red blood cells:

Anticoagulants have an inhibitory effect, and best culture growth is usually achieved with defibrinated erythrocytes. However, red blood cells collected into anticoagulant can be used if they are subjected to thorough washing before use.

(a) Preparation of defibrinated erythrocytes:

Defibrinated red blood cells are prepared by collecting blood from a donor animal directly into an air-tight sterile conical flask containing glass beads (0.5 to 1 cm in diameter) and swirling gently and continuously until a clot has formed on top of the liquid. After centrifugation (500 *g*, 10 min, 4°C), and removal of the serum and buffy layer (upper third of the cell pellet), the lower half of the remaining pellet is carefully withdrawn, transferred to an equal volume of Puck's saline (Puck's saline [g/L]: NaCl: 8; KCl: 0.4; $MgSO_4 \cdot 7H_2O$: 0.154; $CaCl_2 \cdot 2H_2O$: 0.016; $Na_2HPO_4 \cdot 7H_2O$: 0.29; KH_2PO_4: 0.15; glucose: 1.10; phenol red: 0.0012.) (with 2% extra glucose), and stored at 4°C. The shelf-life of red blood cells in Puck's saline ranges from about 2 to 5 weeks depending on

Table 3. Culture Conditions Developed for Various *Babesia* spp. and Erythrocytic *Theileria* Merozoites

Species	*RBC compatibility*	*Serum compatibility*	*Suitable culture media*	*Reference*
B. bigemina	Bovine (5–10% PCV)	20–50% adult bovine serum	Medium 199 in Hank's (with 2.2 g/L $NaHCO_3$) or in Earle's balanced salt solution (with 1.4 g/L $NaHCO_3$) or HL-1 with 2 mM L-glutamine	Canning and Winger (1987); Vega et al. (1985); P. Holman (personal communication)
B. bovis	Bovine (PCV most commonly 5%, 9% in older publications)	20–40% adult bovine serum	Medium 199 in Hank's balanced salt solution with 15 mM HEPES (pH 7); Medium 199 in Earle's balanced salt solution with 20–26 mM TES (pH 7), ±2 mM L-glutamine; or RPMI 1640 with 15 mM HEPES (pH 7) or HL-1 with 2 mM L-glutamine	Canning and Winger (1987); Timms et al. (1983); Palmer et al. (1982); Ristic and Levy (1981); Holman et al. (1993b); P. Holman (personal communication)
	Can be adapted to grow in horse, rabbit, and deer RBC's	Horse and rabbit serum (cervine serum is inhibitory)		
B. caballi	Equine (5% or 9% PCV)	20–40% adult horse serum	HL-1 with 2 mM L-glutamine; or RPMI 1640 with 20 mM HEPES	Holman et al. (1993a); Avarzed et al. (1997)
B. canis	Canine (2% PCV)	10–20% adult dog serum	RPMI 1640 with 20–40 mM HEPES (±0.6 g/L reduced glutathione) (pH 7.2–7.4)	Schetters et al. (1997); Canning and Winger (1987)
B. capreoli	Exclusively in roe deer (7.5% PCV)	20% FCS	RPMI 1640	Malandrin et al. (2010)
B. divergens	Good growth in bovine, human RBC's, moderate growth in sheep (5–7.5% PCV), slow growth in roe deer RBC's, very poor growth in horse RBC's	10–30% bovine (calf and adult) or sheep serum (horse serum was always inhibitory, and human serum was sometimes found to be inhibitory)	HL-1 medium with 2 mM L-glutamine; or RPMI 1640	Zintl et al. (2002); Malandrin et al. (2004); Grande et al. (1997)
B. gibsoni	Canine (10% PCV)	20–40% adult dog serum	HL-1 medium with 15 mM HEPES, 2.2 g/L $NaHCO_3$, 2 mM L-glutamine, 0.2 mM hypoxanthine; or RPMI 1640 with 25 mM HEPES, 2 mM L-glutamine, 1 mM pyruvic acid, and 24 mM $NaHCO_4$	Zweygarth and Lopez-Rebollar (2000); Sunaga et al. (2002)
T. equi	Equine (10% PCV)	20% FCS or 40% horse serum	HL-1 with 1 g/L AlbuMAX 1, 1–2% HB101, 2 mM L-glutamine, ±16 μM thymidine (pH 7.3–7.4); or Medium 199 (with Hank's salts), 10 mM TAPSO, 2 mM L-glutamine, and 0.2 mM hypoxanthine (or 0.2 mM guanosine or adenosine)	Holman et al. (1994a, 1998); Zweygarth et al. (1995)
T. uilenbergi	Sheep (5–10% PCV)	20% sheep serum	HL-1 with 0.75% chemically defined lipid concentrate	Miranda et al. (2006)

FCS, fetal calf serum; PCV, packed cell volume.

Table 4. Culture Conditions for *Theileria* Schizonts

Species	*Host cells*	*Feeder layer*	*Serum compatibility*	*Suitable culture media*	*Reference*
T. annulata	Bovine (ovine and caprine) macrophages, dendritic cells, and B cells	Not required	20% FCS, normal bovine or goat serum	RPMI 1640; Leibovitz L-15	Brown (1987); Sharma et al. (1998); Tretina et al. (2015); Brown et al. (1998); Shkap et al. (1996)
T. lestoquardi (syn. *T. hirci*)	Ovine and caprine lymphocytes (mostly B cells)	Not specified	Ovine	Eagle's MEM based on HBSS, with lactalbumin hydrolysate and yeast extract	Hooshmand and Hawa (1975); Brown et al. (1998); Hooshmand et al. (1993)
T. parva	Bovine (and buffalo) B and T lymphocytes	BESP cells	10–20% (heat inactivated) FCS or newborn calf serum	RPMI 1640 with 20 mM HEPES and 2 mM L-glutamine or Eagle's MEM with 0.01 g/L L-asparagine or modified Leibovitz's L-15 medium (pH 6.8) with 10% tryptose phosphate	Jongejan et al. (1984); Malmquist et al. (1970); Stagg et al. (1974); Tretina et al. (2015); Kurtti et al. (1981)
Theileria taurotragi	Eland lymphocytes and apparently monocytes/ macrophages	BESP cells	20% FCS	Eagle's MEM	Stagg et al. (1976)
Theileria sp. (buffalo)	Buffalo lymphocytes	Bovine aortic endothelia cells	10% heat-inactivated FCS	RPMI 1640 with Glutamax; 25 mM HEPES, 2 g/L sodium bicarbonate; 0.2 mM hypoxanthine	Zweygarth et al. (2009a)
Theileria sp. (roan antelope)	Antelope lymphocytes	Not specified	10% heat-inactivated FCS	RPMI 1640, 25 mM HEPES, 2 g/L sodium bicarbonate; 0.2 mM hypoxanthine	Zweygarth et al. (2009b)

BESP, bovine embryonic spleen.

the donor species and is indicated by the degree of hemolysis in the supernatant.

(b) Preparation of erythrocytes collected into anticoagulant:

After centrifugation (500 *g*, 10 min, 4°C) and removal of the plasma and buffy layer, the cell pellet is washed twice in Dulbecco's phosphate-buffered saline (DPBS) (pH 7.2) with 15 mM EDTA, and once in Puck's saline (with 2% extra glucose) with complete removal of the buffy layer after each wash. Subsequently, the bottom third of the pellet is carefully withdrawn and added to an equal volume of Puck's saline (with 2% extra glucose). Shelf-life and recommended storage conditions are as described earlier.

(2) Culture media:

As first described by Holman (1993a) and later confirmed in several other in vitro systems (Holman et al. 1994a, 1994b, 1994c, Zweygarth and Lopez-Rebollar 2000, Zintl et al. 2002), HL-1 medium (BioWhittaker UK) supports the growth of *Babesia and Theileria* spp. extremely well. This medium, which was originally designed as a serum-free, chemically defined medium for in vitro cultivation of hybridoma and other cells of lymphoid origin, is usually supplemented with serum from an appropriate naïve host animal. Heat inactivation of the serum is not necessary. In fact, early workers suggested that complements assisted erythrocyte invasion by the parasite (Chapman and Ward 1977). For many *Babesia* spp., excellent culture growth has been achieved with fetal calf serum (FCS)-supplemented medium. Notable exceptions are *Babesia bigemina*, *B. bovis*, *Babesia divergens*, and *Babesia caballi*, which grow better in the presence of normal serum.

(3) Culture initiation:

In our experience, culture isolations have a better chance of success if they are initiated using infected red blood cells rather than merozoites separated from the host cell, as the latter have reduced viability and tend to clump together. In most cases, infected red blood cells are collected in small volumes from an infected animal, and because the amount is small, the blood is mixed with anticoagulant rather than defibrinated. After centrifugation (500 *g*, 10 min, 4°C), plasma and the buffy layer are removed. The cells are then washed twice in five volumes of cold DPBS with 15 mM EDTA and once in DPBS without EDTA (with removal of the buffy layer after each centrifugation). Although thorough washing is essential to remove all traces of host plasma (including potential immune mediators) and, in the final step, anticoagulant, it is important to proceed gently, particularly if dealing with species that increase red blood cell fragility, such as *B. divergens* and *B. bigemina* (Canning and Winger 1987). Subsequently, 0.05 to 0.1 mL of washed infected erythrocytes are added to 1 to 1.1 mL HL-1 medium (supplemented with 2 mM L-glutamine and serum) in each well of a lidded 24-well plate. The exact amount of infected red blood cells that is required to initiate the culture depends on the parasitaemia in the sample. It is also dependent on whether the infected red blood cells are derived from the same or a different species, as mixing of heterologous material can lead to hemolysis, thereby leading to a decrease in the proportion of red blood cells or packed cell volume (PCV). If necessary, the red blood cell volume in the culture wells is adjusted to the desired PCV (Table 1) by the addition of donor erythrocytes. Standard concentrations of antibiotics may also be added to control the growth of bacterial and fungal contaminants.

(4) Culture maintenance:

Cultures are maintained in 24-well plates in a total volume of 1.1 to 1.2 mL culture mix per well consisting of HL-1 medium (supplemented with 2 mM L-glutamine and serum) and donor erythrocytes at a PCV of between 1% and 10%. It is important to note that different species have specific preferences for PCV (Table 3), as erythrocyte concentrations lower or higher than the optimum can suppress culture growth. Depending on requirements, it is, of course, possible to either scale up or down culture systems by changing the well size; however, care must be taken to maintain the same PCV and surface-to-volume ratio. Good culture growth requires a low O_2 concentration in the erythrocyte layer (Rodriguez et al. 1983). In new or slow-growing cultures, this is usually achieved by incubating the cells in a microaerophilous atmosphere consisting of 5% CO_2, 2% O_2, and 93% N_2 (provided by commercial suppliers) in airtight modular incubation units (or hypoxia chamber, Billups-Rothenberg). The chambers are equipped with an inflow that can be connected to the gas cylinder and an outflow. After flushing of the chamber with the special gas mix, both openings are sealed and the chamber is placed in the incubator. Humidity is provided by pipetting a small amount of sterile water into the bottom of the chamber. In well-established, fast growing cultures, the metabolism of parasites is sufficient to deplete the O_2 concentration in the erythrocyte layer. As a result, these cultures can be maintained at 5% CO_2 in air [in fact, in some established culture systems, transfer to CO_2 in air has been reported to boost in vitro growth (Avarzed et al. 1997)]. It is important to ensure, however, that the depth of the medium is maintained at a level that does not allow for complete replenishment of O_2 in the settled layer (around ≥ 6.2 mm but optimization may be required). The supernatant is replaced daily with fresh medium, and parasite growth is monitored by microscopic examination of fixed and Giemsa-stained smears prepared from the erythrocyte layer. Optimal subculturing and maintenance conditions are determined by allowing a culture to ''grow out'' and recording percentage of infected red blood cells daily. Subcultures should be carried out during optimum exponential growth by transferring resuspended cultures to freshly prepared wells to achieve parasitaemias of $\leq 1\%$. Optimum incubation temperatures vary slightly depending on the normal body temperature of the respective host species.

(5) Storage:

(a) Short-term storage: Once continuous cultures are established, isolates can be switched to ''slow growth'' between experiments by transferring infected blood cells in culture medium to 4°C (Canning and Winger 1987). Although parasitaemia falls off rapidly within the first few days at 4°C, this decline is slowed down by weekly changes of medium. Normal growth resumes once cultures are returned to the incubator.

(b) Cryopreservation: For long-term storage, cultures can be transferred to liquid nitrogen (N_2). Stabilates are prepared from healthy cultures during exponential growth. After centrifugation (500 *g*, 10 min, 4°C) and removal of the supernatant, the pellet is resuspended in an equal volume of ice-cold Puck's saline supplemented with 2% glucose and 20% polyvinylpyrrolidone-40. The suspension is dispensed into cryovials and immediately transferred to −70°C. After 20 to 30 h at −70°C, the cultures are placed into liquid N_2. The cultures are revived by rapid thawing and transferred to freshly prepared culture wells containing complete medium and donor erythrocytes.

In vitro *culture of* Theileria *schizonts in lymphoblastoid or monocytic cells*

This technique exploits the ability of certain *Theileria* species to induce a cancer-like immortalized leukocyte phenotype that can proliferate indefinitely (Brown 1987). During each mitosis, schizonts that reside in the host cell cytosol bind to the host mitotic spindle, ensuring segregation of the parasite into both daughter cells (Tretina et al. 2015). All *Theileria* spp. that have been shown to have the ability to elicit this transformation (*Theileria parva*, *T. annulata*, *Theileria lestoquardi*, *Theileria taurotragi*, *Theileria* sp. [buffalo]) (Sivakumar et al. 2014) have been established in immortalized schizont-infected cell lines (Brown 1987); whereas species such as *T. mutans*, *T. sergenti*, *T. velifera*, and *T. cervi* seem to have, to our knowledge, little or no transforming ability and have never been established in schizont cultures.

(1) Culture initiation

Cultures can be initiated using lymphoid tissue or peripheral blood mononuclear cells (PBMCs) from infected animals or sporozoites isolated from engorged ticks.

(a) Suitable lymphoid tissues include lymph node, spleen, thymus, bone marrow, lung, liver, and kidney (Brown 1987). After washing in complete medium containing antibiotics and preservative-free heparin (10 IU/mL), the tissue is homogenized to produce a cell suspension that is adjusted to a concentration of 2×10^6 cells/mL and plated (10 mL/25 cm^2 flask; 2 mL/2 cm^2 well; or 1 mL/1 cm^2 well). Alternatively, infected PBMCs are isolated from whole blood or the buffy layer either by erythrocyte lysis (in ice-cold 0.17 M ammonium chloride) or by density gradient centrifugation (using Ficoll-Paque, Histopaque, or Lymphoprep, each of which is formulated to a density of 1.077) (Brown 1987, Zweygarth et al. 2009a). Subsequently, isolated PBMCs are washed in EDTA/PBS (pH 7.2) or autologous plasma, resuspended in complete medium, and plated. During acute infections, cultures may be initiated using whole blood instead of PBMCs (Gharbi et al. 2012). Confusion of the cellular origin of the cell line or overgrowth of certain cell populations in the heterogenous cell mixture derived from tissue or blood can be avoided by enriching the cell suspension for monocytes or lymphocytes (depending on the host cell preference of the *Theileria* spp.). This is done by allowing the monocytes in the suspension to attach to the culture flask (''preculture'' for 3 h at 37°C) and discarding or transferring the unattached cells to a new flask (Kurtti et al. 1981). Uninfected lymphocytes have negligible mitotic activity and are eventually diluted out of the cultures when the lymphoblastoid cells are passaged.

(b) Cultures could also be initiated by introducing sporozoites harvested from infected ticks into uninfected mononuclear cells from susceptible animals (Kurtti et al. 1981, Brown 1987, Kimbita et al. 2004). After allowing infected adult female ticks to engorge to stimulate sporozoite maturation, they are surface sterilized using 1% benzalkonium chloride (one wash) and 70% ethanol (three washes). Subsequently, whole ticks or aseptically collected SG (if infection levels are low) are ground in ice-cold culture medium; the supernatant is centrifuged, filtered to remove debris, and used to infect mononuclear cells that are isolated as described earlier.

For most species, pre-established monolayers of supporting feeder cell lines of bovine embryo spleen, bovine embryo thymus, bovine embryo skin, bovine aortic endothelium, or buffalo lung cells (IMR31), together with their conditioned medium, provide unspecified growth factors, greatly enhancing the chances of successful culture initiation (Brown 1987).

(2) Culture maintenance

Although recent studies continue to use traditional cell culture media such as Eagle's MEM, RPMI 1640, and Leibovitz's L-15 medium, it is likely that some of the modern media available today such as HL-1 or Opti-MEM may negate the need for a feeder layer. Generally, media are supplemented with antimicrobials, antimycotics, and FCS, although Sharma et al. (1998) managed to maintain *T. annulata* long-term cultures in media containing normal bovine or goat serum. Cultures are maintained at 37°C in 5% CO_2 in air, and media are changed every other day.

Depending on the host cell, cultures are grown as monolayers or in suspension and monitored using Giemsa-stained cytospin smears (Sharma et al. 1998, Gharbi et al. 2012). Growth parameters include the ''schizont index'' (infected WBC's/examined WBC's), the ''mitotic index'' (mitotic cells/infected cells), and mean schizont nuclear number (number of theilerial particles per cell) (Sharma et al. 1998, Gharbi et al. 2012). As a rule of thumb, schizonts of both *T. annulata* and *T. parva* double every 18–21 h, necessitating subculture every 3 days (after a 10-fold multiplication).

(3) Cryopreservation

Theileria-infected cell lines can be frozen down for long-term storage essentially as described earlier for *Babesia* and *Theileria* merozoite cultures. Freezing media are composed of FCS and DMSO at a final concentration of 7% or glycerol at a final concentration of 10%. As DMSO is very toxic and immediately penetrates cell membranes, the freezing medium should be ice cold and the cryovials should be transferred to −70°C immediately. When glycerol is used, an equilibration time of 30–40 min is required before freezing the cultures. To initiate new cultures, frozen cells are rapidly thawed at 37°C and diluted 1:5 in complete medium before plating.

Xenodiagnosis Using Animal Inoculation

Inoculation of susceptible animals with whole blood from a suspected case is sometimes used to aid the diagnosis of human cases, although compared with molecular methods the procedure is time consuming, expensive, and questionable for ethical reasons. Most rodent laboratory hosts are susceptible to *B. divergens*, particularly if they have been splenectomized (Zintl et al. 2003); whereas infections are most reliably produced in intact Mongolian gerbils (Meriones unguiculatus) (Lewis and Williams 1979). For instance, the causative agent in a case of human babesiosis in Scotland was identified as *B. divergens* by inoculating a splenectomized calf with blood from the patient (Entrican et al. 1979), whereas inoculation of hamsters with whole blood from suspected cases has been used as a diagnostic method for *Babesia microti* (Kjemtrup and Conrad 2000). More recently, to aid the identification of newly discovered zoonotic agents such as *Babesia venatorum* and *Babesia Duncani*, the parasites were isolated in SCID mice and hamsters, respectively (Conrad et al. 2006, Jiang et al. 2015).

Conclusion

The past 50 years have seen the emergence of numerous methods for the detection and identification of *Babesia* and

Theileria parasites in the vertebrate and the tick hosts. Sample characteristics, research facilities, and infrastructure as well as worker experience and expertise will define the most practicable methods under any given set of circumstances. Nevertheless, we would like to urge researchers to consider using at least two independent methods wherever possible, as this would improve our understanding of the biological characteristics of many of the ''new'' species that have been identified, based chiefly on molecular data.

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Address correspondence to:
Laetitia Lempereur
Laboratory of Parasitology and Parasitic Diseases
Faculty of Veterinary Medicine
University of Liège
Quartier Vallée 2
Avenue de Cureghem 6 B43a
Liège 4000
Belgium

E-mail: llempereur@hotmail.com

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A Review of Methods for Detection of *Hepatozoon* Infection in Carnivores and Arthropod Vectors

David Modrý,[1–3] Relja Beck,[4] Kristýna Hrazdilová,[3] and Gad Baneth[5]

Abstract

Vector-borne protists of the genus *Hepatozoon* belong to the apicomplexan suborder Adeleorina. The taxonomy of *Hepatozoon* is unsettled and different phylogenetic clades probably represent evolutionary units deserving the status of separate genera. Throughout our review, we focus on the monophyletic assemblage of *Hepatozoon* spp. from carnivores, classified as *Hepatozoon* sensu stricto that includes important pathogens of domestic and free-ranging canine and feline hosts. We provide an overview of diagnostic methods and approaches from classical detection in biological materials, through serological tests to nucleic acid amplification tests (NAATs). Critical review of used primers for the 18S rDNA is provided, together with information on individual primer pairs. Extension of used NAATs target to cover also mitochondrial genes is suggested as a key step in understanding the diversity and molecular epidemiology of *Hepatozoon* infections in mammals.

Keywords: diagnostics, *Hepatozoon*, PCR

Introduction

The apicomplexan genus *Hepatozoon* (apicomplexan suborder Adeleorina, family Hepatozooidae Wenyon, 1926) includes more than 300 species infecting a wide range of vertebrates from amphibians to mammals (Smith 1996, Baneth 2011). In recent phylogenetic studies, the genus *Hepatozoon* is described as paraphyletic and its split into a number of distinct genera has been suggested (Barta et al. 2012, Karadjian et al. 2015).

The life cycle of *Hepatozoon* sensu lato is heteroxenous, and a range of invertebrates (argasid and ixodid ticks, mosquitoes, fleas, triatomines, tsetse flies, and biting lice) serve as vectors for individual species. The vertebrate host is usually infected by ingestion of a hematophagous arthropod, although infection can be acquired also by intrauterine transmission or by predation. Infection through ingestion of an infected invertebrate vector is unlikely for many *Hepatozoon* s.l. from reptiles, where development can be alternatively completed through a three-host life cycle along an arthropod—paratenic host—reptile axis (Landau 1973; Sloboda et al. 2007).

In cases of infection via ingesting an infected invertebrate host, the sporozoites released from sporocysts within oocysts enter the circulation and migrate to various organs (liver, bone marrow, spleen, kidneys, lungs, intestines, and lymph nodes) where they undergo merogony. When merozoites are released from mature meronts into the bloodstream, they enter the blood cells and transform into gamonts, which are later ingested with the blood meal by a competent vector. Subsequent development includes the production of microgametes and macrogametes (in different parts of vector organs) that fuse and develop to polysporocystic oocysts (Smith 1996, Kim et al. 1998, O'Dwyer 2011).

The taxonomy of *Hepatozoon* s.l. is unsettled and different phylogenetic clades probably represent evolutionary units deserving the status of separate genera. Throughout our review, we focus on the monophyletic assemblage of *Hepatozoon* spp. from carnivores, classified as *Hepatozoon* sensu stricto by Karadjian et al. (2015).

Species of Importance

Hepatozoon spp. has been described in a range of mammalian hosts, mainly carnivores and rodents. While the infections in most mammalian hosts are typically subclinical, species that infect canid and felid carnivores (*Hepatozoon americanus*, *Hepatozoon canis*, *Hepatozoon felis*) are of veterinary importance. Currently, the ubiquitous presence of

[1]Department of Pathology and Parasitology, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.
[2]Biology Centre, Institute of Parasitology, Czech Academy of Sciences, České Budějovice, Czech Republic.
[3]CEITEC-VFU, University of Veterinary and Pharmaceutical Sciences Brno, Brno, Czech Republic.
[4]Department for Bacteriology and Parasitology, Croatian Veterinary Institute, Zagreb, Croatia.
[5]Koret School of Veterinary Medicine, Hebrew University, Rehovot, Israel.

H. canis and closely related species in European foxes is attracting considerable attention (Najm et al. 2014, Hodžić et al. 2015) inasmuch as the main tick vector of this species, *Rhipicephalus sanguineus*, is not endemic in some areas where infection is abundant in local foxes.

H. canis infections in domestic dogs can vary from subclinical to severe, with animals manifesting extreme lethargy, cachexia, and anemia (Baneth and Weigler 1997). Infection with this species or its variants in wildlife canids has not been clearly associated with clinical disease to date. In contrast, *Hepatozoon americanum* infection of domestic dogs is associated with severe disease characterized by muscular pain induced by myositis, severe lameness, and subsequent muscle atrophy. Infections with *H. felis* primarily target striated muscle in domestic cats, but these also are mainly subclinical (Baneth et al. 2013). Nevertheless, infection with this species or a closely related variant has resulted in severe myositis in a wild Pampas gray fox (Giannitti et al. 2012).

In carnivores, vectors have been identified only for two *Hepatozoon* species: *R. sanguineus* and *Amblyomma ovale* for *H. canis* (Baneth et al. 2001, 2007, Forlano et al. 2005) and *Amblyomma maculatum* for *H. americanum* (Mathew et al. 1998). The diversity of *Hepatozoon* species in carnivores is probably more complex than is presently known because considerable DNA heterogeneity in conserved gene sequences of *Hepatozoon* from carnivore hosts is frequently seen (Starkey et al. 2013). However, a lack of data on different manifestations of infection, life cycle variations, antigenic variability, and vector competence constitute a serious drawback for further taxonomic work.

Hepatozoon Detection in Blood, Tissues, and Invertebrate Vectors

Due to their complex life cycle, stages of *Hepatozoon* can be detected in a variety of tissues. In vertebrate hosts, blood is the most commonly used material for intravital diagnostics. Inasmuch as the gamonts are mostly present in white blood cells within mammalian hosts, however, using the buffy coat layer for microscopic and molecular diagnostics increases the sensitivity of detection (Sasanelli et al. 2010, Otranto et al. 2011). Depending on the given *Hepatozoon* species, life stages can be further detected by cytology or biopsy histopathology in the bone marrow, spleen, lymph nodes, or striated muscle. At necropsy, almost any parenchymal tissue can be used for parasite detection by nucleic acid amplification tests (NAATs). The spleen is nevertheless the primary target organ for the parasite's detection (Baneth et al. 2007). However, the ability of some *Hepatozoon* spp. to survive in tissues of hosts accidentally infected by ingestion of other infected hosts (*e.g.*, in bats after presumed ingestion of infected arthropods) complicates the interpretation of PCR positivity in tissues (Pinto et al. 2013, Karadjian et al. 2015). Polysporocystic oocysts (or DNA) of *Hepatozoon* species can also be detected in bloodsucking arthropod vectors. Detection of DNA in questing ticks is highly suggestive for the vectorial role. On the contrary, the presence of *Hepatozoon* DNA in blood-engorged arthropods should not be considered proof of their vectorial capacity unless mature oocysts are demonstrated microscopically (Giannelli et al. 2013).

Diagnosis by Microscopy

The traditional direct diagnostic methods for rapid detection of *Hepatozoon* include thick and thin blood smears and buffy coat smear (Fig. 1). These methods are effective only in animals with high parasitemia. Sensitivity is noticeably diminished for subclinical infections and for those *Hepatozoon* species (such as *H. americanum*) that typically produce very low parasitemia levels during clinical disease (Vincent-Johnson et al. 1997). Romanowsky stains, including Giemsa stains and such commercial rapid stains as Diff-Quick, are suitable for detecting *Hepatozoon* spp. in blood. *Hepatozoon* life stages can be detected also in histopathology sections of tissues demonstrating the architecture of the parasite's tissue stages in a clear manner. Histopathology is also helpful in characterizing inflammatory response to infection with different *Hepatozoon* species in distinctive hosts (Klopfer et al. 1973, Baneth and Weigler 1997, Vincent-Johnson et al. 1997, Baneth et al. 2013).

Serological Tests

Serological assays have been developed for detecting antibodies for *Hepatozoon* spp. in dogs. An indirect fluorescent antibody (IFA) test for anti-*H. canis* antibodies using gamont antigen has been used in epidemiological studies in Israel, Japan, and Turkey (Shkap et al. 1994, Baneth et al. 1996, Inokuma et al. 1999, Karagenc et al. 2006), and an IFA assay using sporozoite antigens derived from ticks has been developed for detecting anti-*H. americanum* antibodies. The latter assay was found to be as sensitive as a muscle biopsy in diagnosing this infection (Mathew et al. 2000). Furthermore, an enzyme-linked immunofluorescence assay (ELISA) for anti-*H. canis* antibodies based on gamont antigen has also been developed (Gonen et al. 2004). A low degree of cross-reactivity was found when using the *H. canis* ELISA with sera of dogs infected with *H. americanum* (Gonen et al. 2004). In the past decade, serological tests have largely been replaced by NAATs.

NAATs for *Hepatozoon* species

Similar to other vector-borne protists, molecular diagnostic approaches relying mainly on the amplification of 18S rRNA gene fragments have prevailed during the past two decades in *Hepatozoon* detection and genetic characterization in mammals, while studies on reptilian *Hepatozoon* s.l. use ITS-1 as an additional or alternative target for both diagnostic PCR assays and phylogenetic analyses (Kim et al. 1998, Boulianne et al. 2007). To date, sequences of the 18S rRNA gene comprise the only available genetic data from *Hepatozoon* species infecting carnivores (Table 1, Fig. 2). The major bulk of sequences originating from different host species are ca 250–700 bp in length, and suggests a monophyletic character of *Hepatozoon* species from carnivores (Criado-Fornelio et al. 2006, Barta et al. 2012, Najm et al. 2014). However, the variability of the 18S rDNA within *Hepatozoon* species combined with the short length of the sequences used in some phylogenetic analyses complicate definitions of species status among *Hepatozoon* spp. (see *e.g.*, Karadijan et al. 2015). Neighbor-joining analysis of partial 18S RNA sequences has revealed five groups of *H. canis* isolates from dogs based on individual point mutations,

Table 1. Examples of Primers for PCR Amplification of 18S Sequences of *Hepatozoon* spp.

Primer name	*Sequence (5′ → 3′)*	*Product size (bp)*[a]	*Nested PCR*[b]	*Specific*[c]	*Reference*[d]
BTHL1	CCA GCA GCC GCG GTA ATT C	380	No	No	Tabar et al. (2008)
BTHL2	CTT TCG CAG TAG TTY GTC TTT AAC AAA TCT				
HepF	ATA CAT GAG CAA AAT CTC AAC	670	No	Yes	Inokuma et al. (2002)
HepR	CTT ATT ATT CCA TGC TGC AG				
HAM-1	GCC AGT AGT CAT ATG CTT GTC	1760	No	No	Criado-Fornelio et al. (2006)
HPF-2	GAC TTC TCC TTC GTC TAA G				
HEP-1	GCG CAA ATT ACC CAA TT	660	No	Yes	Criado-Fornelio et al. (2006)*
HEP-4	TAA GGT GCT GAA GGA GTC GTT TAT				
BT1-F	GGT TGA TCC TGC CAG TAG T	430	No	No	Criado-Fornelio et al. (2003)
BT1-R	GCC TGC TGC CTT CCT TA				
BTH-1F	CCT GMG ARA CGG CTA CCA CAT CT	750	Yes	No	Criado- Fornelio et al. (2003)
BTH-1R	TTG CGA CCA TAC TCC CCC CA				
RLB-F	GAG GTA GTG ACA AGA AAT AAC AAT A	550	No	No	Majláthová et al. (2007) and Gubbels et al. (1999)
RLB-R	TCT TCG ATC CCC TAA CTT TC				
H14Hepa18SFw	GAA ATA ACA ATA CAA GGC AGT TAA AAT GCT	620	Yes	Yes	Hodžic et al. (2015)
H14Hepa18SRv	GTG CTG AAG GAG TCG TTT ATA AAG				
GF2	GTC TTG TAA TTG GAA TGA TGG	610	Yes	No	Zintl et al. (2011)
GR2	CCA AAG ACT TTG ATT TCT CTC				
4558F	GCT AAT ACA TGA GCA AAA TCT CAA	1180	No	Yes	Mathew et al. (2000) and Wozniak et al. (1994)
2773R	CGG AAT TAA CCA GAC AAA T				
Piroplasmid F	CCA GCA GCC GCG GTA ATT	390	No	No	Baneth at al. 2013
Piroplasmid R	CTT TCG CAG TAG TTY GTC TTT AAC AAA TCT				
KIM18SF	GAA ATT AGA GTG TTT C	600	No	No	Williams et al. (2014)
KIMR2	ACC CTA TTT AGC AGG TTA AG				
Piro A1	AGG GAG CCT GAG AGA CGG CTA CC	520	No	No	O'Dwyer et al. (2009) and Jefferies et al. (2003)
Piro B	TTA AAT ACG AAT GCC CCC AAC				
BmF1	GCG ATG TAT CAT TCA AGT TTC TG	1160	Yes	No	Simpson et al. (2005)
BmR1	TGT TAT TGC CTT ACA CTT CCT TGC				
BmF2	ACGGCT ACCACATCTAAGGAAGGC	700	Yes	No	Simpson et al. (2005)
BmR2	TCTCTCAAGGTGCTGAAG GA				
Babesia-F	GTG AAA CTG CGA ATG GCT CA	740	No	No	Oyamada et al. (2005)
Babesia-R	CCA TGC TGA AGT ATT CAA GAC				

[a]Expected approximate size of PCR product for *Hepatozoon* spp. sequence.
[b]Primers used in cited literature in nested PCR protocol.
[c]Yes = primers specific only for *Hepatozoon* spp. sequences; no = primers with broader range of amplified species.
[d]Reference to first use of primers for *Hepatozoon* spp. sequence amplification, original annotation of primers in brackets.
*Sequence of primer HEP-1 was originally published as 5′-CGC GAA ATT ACC CAA TT-3′. As this very probably contains a mistyping error, we suggest here the correct sequence to anneal to *Hepatozoon* 18S.

thereby demonstrating the variability of *H. canis* within the microenvironment in Croatia (Vojta et al. 2009). Foxes from the same country showed less variability (Dežđek et al. 2010).

Molecular detection of *Hepatozoon* spp. is highly influenced by the specificity of the primer pair and the PCR cycle conditions that are used. Most of the primers used are universal enough to amplify either a broad range of apicomplexan protists (*e.g.*, BTHL 1 and 2, BTH-1F and −1R, BmF and R) or piroplasmids (*e.g.*, Piroplasmid F and R, Piro A1 and B, BT1F and R). It should be recognized that several primer pairs amplify *Hepatozoon* 18S despite mismatches in target sequence (GF2 and GR2, BmF and R, Babesia-F and -R) (Table 1). For this reason, it is always recommended to confirm the identity of PCR products by sequencing a representative amount of samples from each particular set. The difference of ca 40 bp in length of the 18S PCR products for piroplasmids and *Hepatozoon* species can be helpful in distinguishing them when universal primers are used (Fig. 3).

The choice of primers should reflect the purpose and design of the particular study. For diagnostic PCR, specific primers amplifying short PCR products are recommended (*e.g.*, H14Hepa18SFw and Rv, Hep-1 and -4, HepF and R). High sensitivity can be achieved using a nested PCR protocol (*e.g.*, a combination of universal BTH-1F and −1R with more specific Hep-1 and -4 or H14Hepa18SFw and Rv; Fig. 2), but

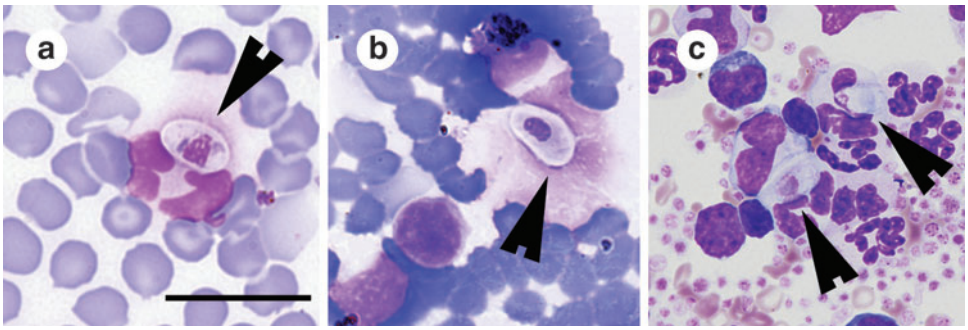

FIG. 1. *Hepatozoon* gamonts in white blood cells manifested in blood smears remain the keystone for clinical diagnosis of infected mammalian hosts. **(a)** *Hepatozoon canis* in a dog from Romania, **(b)** *Hepatozoon felis* in the blood of a Kenyan cat, **(c)** *Hepatozoon* sp. in buffy coat smear from a Romanian red fox. Stained by Giemsa stain **(a, b)** and Diff-Quick **(c)**. Scale bar = 20 μm, all images is same scale. Color images available online at www.liebertpub.com/vbz

this may come at the cost of increased contamination risk. For phylogenetic analyses and species determination, amplification of longer fragments (using, *e.g.*, HAM1-HPFR, 4558F – 2773) is advisable (Table 1).

Quantitative PCR

In recent years, quantitative PCR (qPCR) has been frequently used in studies because it enables quantification and detection of mixed infections from various biological sources, without laborious evaluation of results by gel electrophoresis. This notably has not been the case, however, for studies of *Hepatozoon* species. Only two qPCRs assays targeting the conserved 18S rRNA gene have been developed to estimate the prevalence and intensity of *Hepatozoon* infection in carnivore species (Criado-Fornelio et al. 2007, Li et al. 2008). Using the primers HEP1 and HEP2, Criado-Fornelio et al. (2007) failed to separate species infecting canids and felids by melting peak temperature analysis, while qPCR combined with FRET revealed single target nucleic copies with 100% specificity and enabled differentiation of dog species (*H. canis* and *H. americanum*) from canine blood (Li et al. 2008, Kelly et al. 2013). The first and to date only qPCR protocol based on melting curve analysis that enables differentiation between mixed infection of adeleorinid (*Hepatozoon* s.l.) and eimeriorinid (*Schellackia* and *Lankesterella*) protists was developed to study their presence in reptiles (Maia et al. 2014).

Relevance of Various Detection Techniques for Clinical Diagnosis in Dogs and Cats

The assays used to diagnose *Hepatozoon* infections in dogs and cats include detection of gamonts in blood smears, cytology of tissues, serology, and PCR. While detection by microscopy of blood smears is easy and rapid when parasitemia is sufficient, as in most clinical infections with *H. canis*, it is not sensitive enough to detect *H. americanum* and *H. felis* infections. Furthermore, follow-up treatment for *H. canis* should be carried out using PCR and buffy coat microscopy because detection by microscopy is considerably less sensitive than is PCR (Sasanelli et al. 2010, De Tommasi et al. 2014). A study from Turkey demonstrated that detection of *H. canis* by PCR is far more sensitive than is examination of blood by light microscopy. In that study, the prevalence of infection among 349 dogs was 10.6% according to blood smear evaluation and 25.8% according to blood PCR (Karagenc et al. 2006). Additionally, a comparative study on

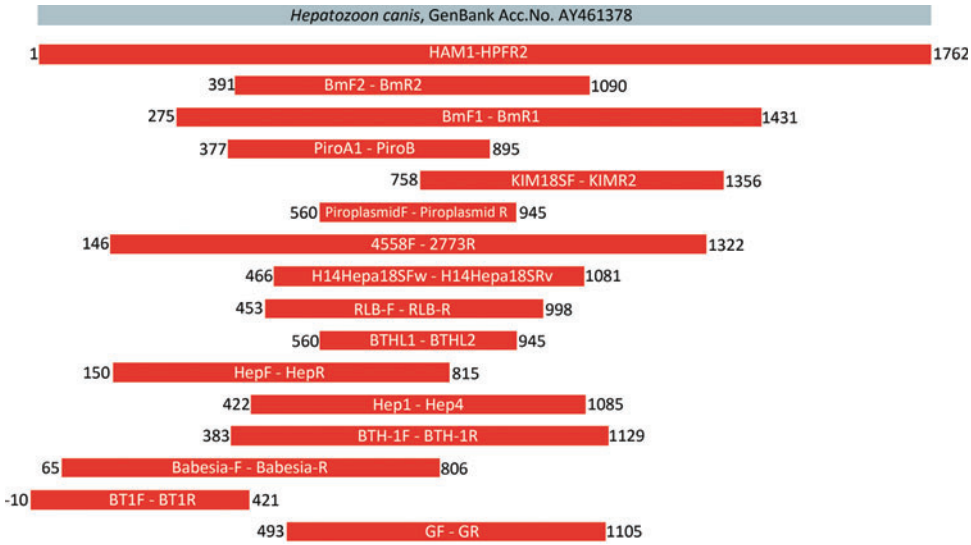

FIG. 2. Length and position of 18S rDNA fragments amplified by various primer pairs. The nucleotide positions are extrapolated according to the reference sequence of *H. canis* AY461378 (shown in *gray*). Color images available online at www.liebertpub.com/vbz

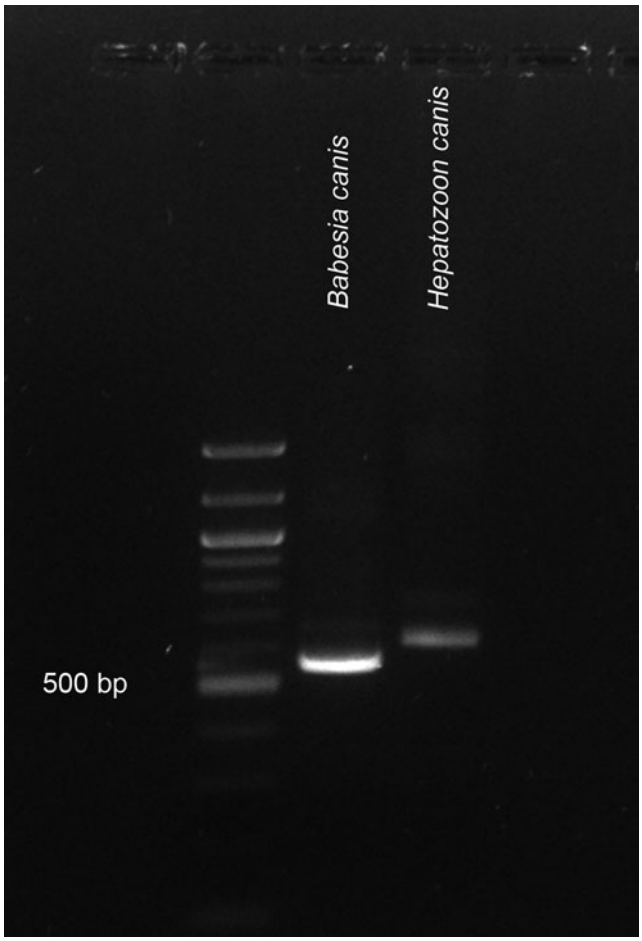

FIG. 3. Demonstration of *H. canis* and *Babesia canis* 18S PCR products amplified by nested PCR using nonspecific primers (BTH-1F, BTH-1R, and GF2, GR2), showing ca 40 bp length difference between the amplicons; 100 bp DNA Ladder (New England BioLabs).

the bone marrow, blood, and buffy coat of dogs infected with *H. canis* has found that buffy coat and blood are the best tissues for detecting *H. canis* infection in dogs using PCR (Otranto et al. 2011). Sensitive and specific real-time qPCR assays are able to detect both *H. americanum* and *H. canis* while distinguishing between the two species (Criado-Fornelio et al. 2007, Li et al. 2008). Serological assays for canine hepatozoonosis are indicative of exposure to the parasite and can be used for epidemiological surveys, but they lack the sensitivity and specificity of real-time PCR.

Conclusions and Future Perspectives

A plethora of studies employing molecular tools for detecting *Hepatozoon* s.l. in domestic and free ranging mammals have been published in the past decade. To date, both molecular diagnosis and phylogenetic analyses rely solely on nuclear 18S rDNA data. Extending the range of available molecular markers to plastid or mitochondrial genes will greatly improve the understanding of diversity within the genus *Hepatozoon* and intergeneric relationships between individual clades of hemogregarines. The recently published mitochondrial genome of *Hepatozoon* (*Bartazoon*) *catesbianae* (Leveille et al. 2014) opened a range of opportunities in the search for alternative phylogenetic markers and diagnostic primers also for mammalian *Hepatozoon* spp. Inasmuch as the prevalence assessed by PCR in clinically healthy dogs and cats can reach more than 25% in areas of endemic occurrence, and because extremely sensitive diagnostic PCR also detects subclinical carriers, direct demonstration of gamont presence in circulating white blood cells (in blood smears or buffy coat smears) remains an important clinical diagnostic tool.

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Address correspondence to:
David Modrý
Department of Pathology and Parasitology
University of Veterinary and Pharmaceutical Sciences Brno
Palackeho tr. 1946/1
Brno 61242
Czech Republic

E-mail: modryd@vfu.cz

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Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part I: Important Points to Consider Ante Field Work

Nazli Ayhan,[1] Amal Baklouti,[1] Jorian Prudhomme,[2] Gernot Walder,[3] Fatima Amaro,[4] Bulent Alten,[5] Sara Moutailler,[6] Koray Ergunay,[7] Remi N. Charrel,[1] and Hartwig Huemer[8]

Abstract

The purpose of this review is to provide practical information to help researchers intending to perform ''from field to laboratory'' studies on phleboviruses transmitted by sandflies. This guideline addresses the different steps to be considered starting from the field collection of sandflies to the laboratory techniques aiming at the detection, isolation, and characterization of sandfly-borne phleboviruses. In this guideline article, we address the impact of various types of data for an optimal organization of the field work intending to collect wildlife sandflies for subsequent virology studies. Analysis of different data sets should result in the geographic positioning of the trapping stations. The overall planning, the equipment and tools needed, the manpower to be deployed, and the logistics to be anticipated and set up should be organized according to the objectives of the field study for optimal efficiency.

Keywords: arbovirus(es), Bunyaviridae, field studies, sand fly (flies), Toscana virus, vector-borne

How to Determine the Region for Trapping Sandflies to Search for Viruses

Using entomological data

Sandflies show a worldwide distribution in tropical and subtropical, arid/semiarid areas, and temperate zones (Killick-Kendrick 1999). The genera *Phlebotomus* and *Sergentomyia* are present in the Old World, whereas the genus *Lutzomyia* inhabits the New World (NW); these three genera belong to the *Phlebotominae* subfamily within the *Psychodidae* family (Tesh 1988). It is important to know the distribution, abundance, and diversity of sandfly fauna in the study region. For some countries, it is possible to reach old entomological data from the literature that may help to predict the possible sandfly population presence. Recently, the number of sandfly entomological studies has increased all over the world that also facilitates phleboviruses research. In Europe, research projects such as VBORNET (European Network for Arthropod Vector Surveillance for Human Public Health) and Vector-Net, funded by the European Community in the framework of the FP7 and H2020, have recently provided very useful data, updating the outdated historical records. The objectives of VBORNET were to establish a European Network of entomological and public health specialists to assist European Centre for Disease Prevention and Control in its preparedness activities on vector-borne diseases and to provide updated maps reflecting the current presence and circulation of vectors involved in the transmission of vector-borne diseases of human and veterinary importance (www.vbornet.eu/index.php?p=11; http://ecdc.europa.eu/en/activities/diseaseprogrammes/emerging_and_vector_borne_diseases/Pages/VBORNET.aspx). Vector-Net supports the collection of data on vectors and pathogens in vectors, related to both animal and human health.

[1]UMR ''Emergence des Pathologies Virales'' (EPV: Aix-Marseille Univ. - IRD 190 - Inserm 1207 - EHESP), Fondation IHU Méditerranée Infection, APHM Public Hospitals of Marseille, Marseille, France.
[2]Centre IRD, UMR MIVEGEC (IRD 224 - CNRS 5290 – Université Montpellier), Montpellier, France.
[3]GmbH, 9931 Außervillgraten, Austria.
[4]Centre for Vectors and Infectious Diseases Research, National Institute of Health Ricardo Jorge, Águas de Moura, Portugal.
[5]Ecology Section, ESRL Laboratories, Department of Biology, Faculty of Science, Hacettepe University, Ankara, Turkey.
[6]Animal Health Laboratory, UMR BIPAR, ANSES Maisons-Alfort, Paris, France.
[7]Virology Unit, Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey.
[8]Division of Virology, Departments Hygiene, Microbiology and Social Medicine, Innsbruck Medical University, Innsbruck, Austria.

Female individuals of sandflies require a blood source for egg maturation and both female and male individuals need a sugar source for energy. Sandflies' weak flight capability is affected by the wind and windy weathers make conditions difficult for sandflies to achieve the sugar and blood sources (Alexander 2000). After maturation of the eggs, they are laid in the soil that is rich in organic matter such as herbivorous animal feces that provide food for larvae (Feliciangeli 2004). Therefore, it is important to place traps in or near animal housing places due to these requirements. Sandflies are mainly dispersed in rural and periurban areas; thus, collaborating with local veterinarians might help with finding suitable places for setting traps and explain to the local people the aim of the trapping.

Using parasitology data

Besides phleboviruses, sandflies can also transmit the flagellate protozoan *Leishmania* that cause three forms of the disease called leishmaniasis: (1) visceral leishmaniasis, which affects 300,000 people with more than 6.6% lethality rate, (2) cutaneous leishmaniasis, with more than 1 million cases worldwide, (3) and mucocutaneous leishmaniasis with most cases occurring in South America (WHO 2014). Leishmaniasis is listed in the 10 most worrying neglected tropical diseases (www.who.int/neglected_diseases/diseases/en). Funding and manpower supporting research and surveillance of leishmaniasis are considerably higher than those related to sandfly-transmitted viruses: for instance, in PubMed, ''*leishmania*'' keyword retrieved >5000 peer-reviewed articles during the last 5 years, compared with >500 when using the ''*phlebovirus*'' keyword. Thus, it is worth using such data as indirect markers for the presence of sandflies that are vectors of the parasite (Gebre-Michael et al. 2004, Maroli et al. 2013).

Using virology data

Seroprevalence studies performed using the sandfly-borne phlebovirus antigens are of utmost interest to help researchers at the design step of field studies aiming at the detection, isolation, and characterization of viruses transmitted by phlebotomine flies (Fig. 1 and Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/vbz). The seminal study of Tesh et al. (1976) remains a goldmine for phlebovirus-related studies. In this study, the authors have used strains of viruses that belong to the three serocomplexes (Naples, Sicilian, and Salehabad), which are transmitted by sandflies; since they used neutralization tests to assess the prevalence of the selected viruses, pitfalls due to cross-reactivity (observed with methods such as inhibition of hemagglutination, complement fixation, immunofluorescence (IF) assay, or enzyme-linked immuno sorbent assay (ELISA) did not cause biased results. Unfortunately, Toscana virus (TOSV) was not included in this study. In 1978, a symposium entitled ''Arboviruses in the Mediterranean Countries'' was held in the Yugoslavian island of Brac; the corresponding book is of instrumental value for sandfly-borne phleboviruses (Vesenjak-Hirjan et al. 1980). The cross-reactivity between sandfly-borne phleboviruses can be of advantage when seroprevalence studies employ the low-specificity methods aforementioned. Recently, a large number of studies have used ELISA and/or IF techniques (for a review see Alkan et al. 2013). Such results should be used to provide a rough idea of the sandfly-borne virus activity and the level of circulation in a given region (Alkan et al. 2015a). For such purpose, data provided by human and animal studies are of equal importance. During the last decade, existing and novel phleboviruses have been described. Several new phlebovirus detection and isolations have recently been reported globally (Charrel et al. 2009, Zhioua et al. 2010, Papa et al. 2011, 2015, Calzolari et al. 2014, Ergunay et al. 2014, Remoli et al. 2014, Alkan et al. 2015b, Amaro et al. 2015, 2016, Es-Sette et al. 2015, Palacios et al. 2015, Baklouti et al. 2016, Bichaud et al. 2016). The identified viruses could be used as a guide; year, location, and sample used for detection/isolation may give hints for possible other phleboviruses in circulation. For several countries, despite detection or isolation of phleboviruses is lacking, serological studies reveal phlebovirus exposure in human or animal populations through the detection of antibodies (Batieha et al. 2000, Hukić et al. 2009, Venturi et al. 2011, Abutarbush and Al-Majali 2014, Sakhria et al. 2014).

Using medical data

Phleboviral infections demonstrate a seasonal incidence peaking between April and October, depending on the geographical location (Tesh et al. 1976), correlated with the regional sandfly activity (Figs. 2–5). Medical reports on outbreaks in autochthonous or imported populations as well as case reports are indicative of the presence of infected sandflies in specific geographic areas (Supplementary Table S1). The main problem of the clinical diagnosis is the symptoms being nonspecific; thus suspected cases must be confirmed by virological methods to demonstrate either the presence of the virus in blood or cerebrospinal fluid or the seroconversion in two successive serum samples. Since standardized and commercialized assays for the RT-PCR detection of these viruses are lacking and a limited number of commercially available serological tests are available, definitive confirmation is rarely obtained and the majority of probable cases remain unconfirmed.

Naples and Sicilian viruses have identical clinical syndromes, which are fever, headache, malaise, photophobia, myalgia, and retro-orbital pain. Because the fever lasts for 2–3 days, the disease was named as ''3-day fever.'' In contrast, TOSV can cause aseptic meningitis, or meningoencephalitis presenting with headache, fever, nausea, and vomiting in infected individuals (Dionisio et al. 2003, Charrel et al. 2005, 2012, Depaquit et al. 2010). During World War II, a large number of soldiers was affected by sandfly fever (Sabin 1951). Recently, TOSV human case records came from Italy (Serata et al. 2011, Calzolari et al. 2014), France (Dupouey et al. 2014, Marlinge et al. 2014), Portugal (Santos et al. 2007, Amaro et al. 2011), Croatia (Punda-Polić et al. 2012), Turkey (Ocal et al. 2014, Ergunay et al. 2015), Greece (Papa et al. 2014), and Tunisia (Fezaa et al. 2014) (Fig. 3). A large sandfly fever Sicilian virus outbreak recently occurred in Ethiopia (Woyessa et al. 2014). However, due to lack of specific manifestations and reliable differential clinical diagnosis, medical records need to be complemented by virological and microbiological tests for the definitive etiological identification.

Using veterinary data

Although the capacity of sandfly-borne phleboviruses to cause diseases in animals is currently unknown, accumulating data indicate that mammals can be infected with at least

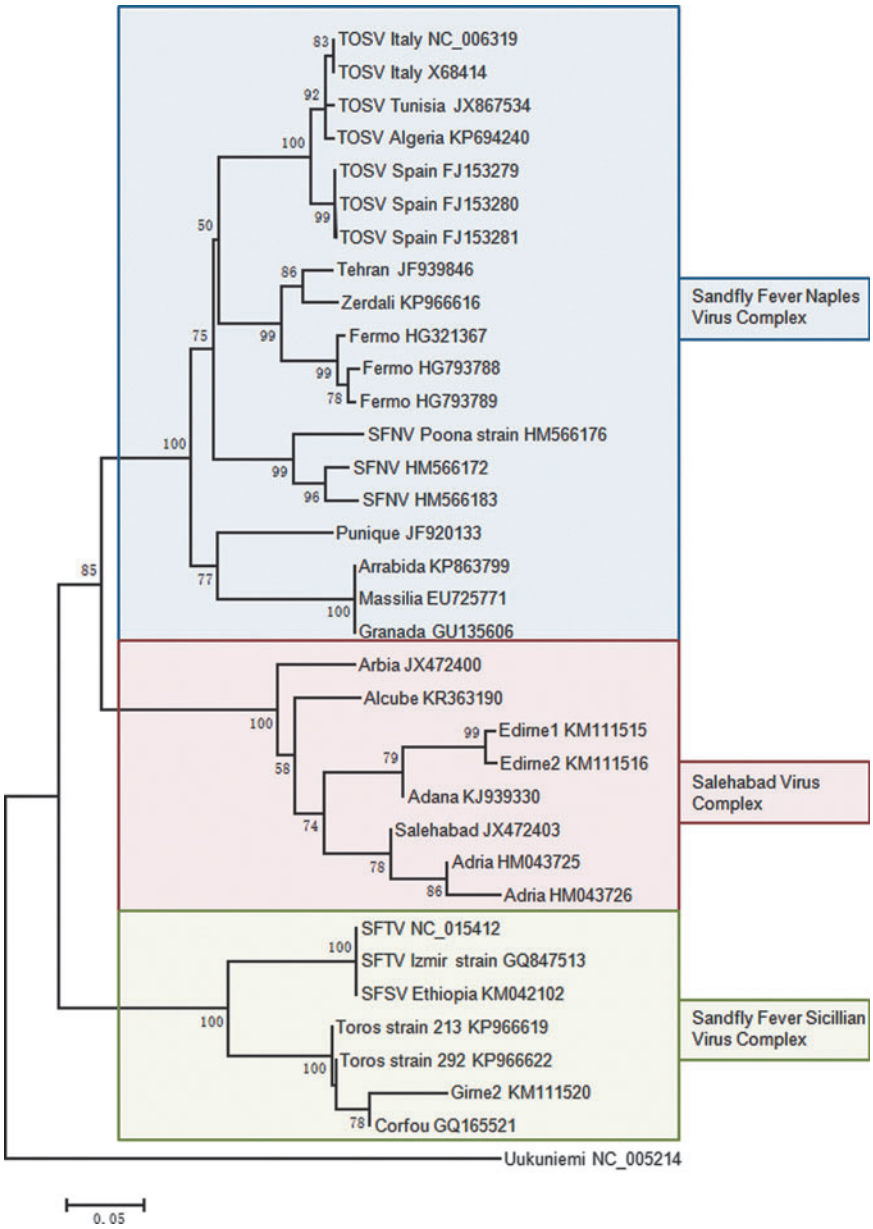

FIG. 1. Phylogenetic analysis of Old World sandfly-borne phleboviruses using a 193-amino acid region in the polymerase protein. Sequences were aligned using the Clustal W program. Distances and groupings were determined by the p-distance method and neighbor-joining algorithm implemented with the pairwise deletion model in the MEGA 6.06 software program. Bootstrap values are indicated and correspond to 500 replications.

some of these viruses (Navarro-Marí et al. 2011, Alkan et al. 2013, 2015b, Sakhria et al. 2014, Dincer et al. 2015, Bichaud et al. 2016, Tahir et al. 2016); accordingly they can serve as sentinels for the presence of the corresponding viruses. There is no undisputable evidence that birds can be infected by sandfly-borne phleboviruses, but few studies have addressed this point.

Using ecological and environmental data

Since the dynamics of sandfly populations is intimately linked to environmental parameters, ecological data are of great importance for an optimal yield of field studies. The organization of field collections requires a deep survey analysis in the study region. The suitable habitats for *Phlebotominae* sandflies need to be determined using climatic and geographic data. Sandflies are small (1.5–3 mm), delicate, nocturnal insects with short distance flight capability. Factors such as yearly, monthly, and daily temperatures can have a major impact on sandfly population size and activity, and therefore can affect the sampling success (Tesh et al. 1976, Alexander 2000). The altitudinal distribution and climatic needs are varying between sandfly species from sea level to 3500 m (Killick-Kendrick 1999, Aransay et al. 2004, Guernaoui et al. 2006a, 2006b, Belen and Alten 2011, Alten et al. 2015). In Spain, *Phlebotomus ariasi* was collected at higher altitudes (600–900 m) from coolest and most humid Mediterranean bioclimatic zone (supra-Mediterranean), whereas *Phlebotomus perniciosus* predominated in the lower altitudes, warmer and drier bioclimatic zones (Aransay et al. 2004). Biogeographic parameters have a huge impact on the species distribution and density (Zhioua et al. 2010, Fares et al. 2015). Rainfall is another factor with a huge impact on sandfly activity; heavy rains could decrease the flight range of the sandflies. In Panama, rainfall amount and distribution were found to correlate with seasonal sandfly density (Chaniotis

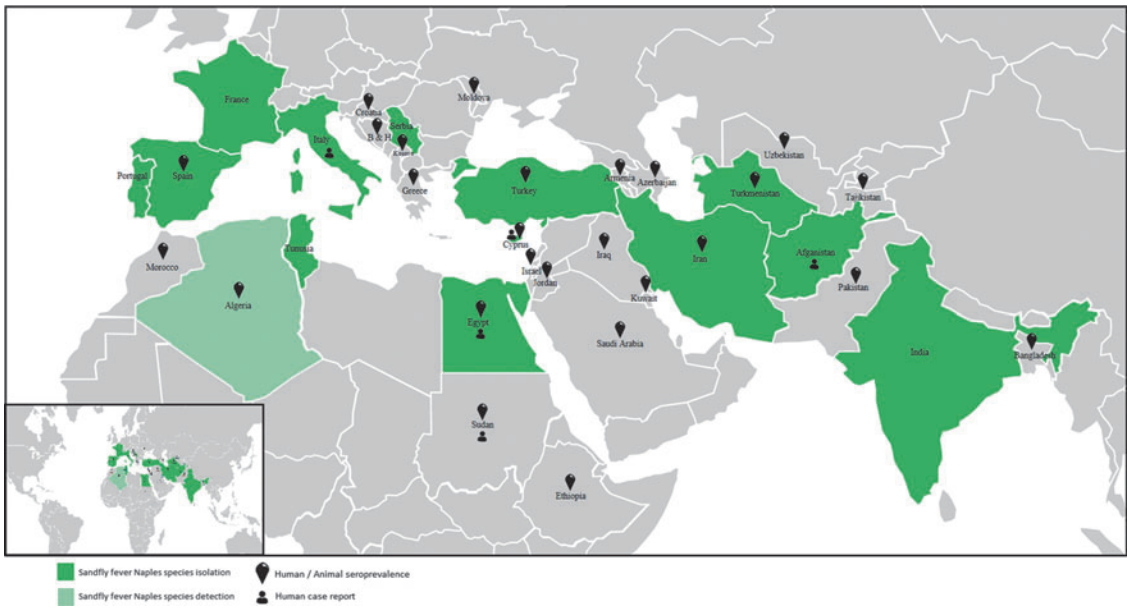

FIG. 2. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the *Sandfly fever Naples* species.

1974). The adult individuals resting sites are animal barns, houses, poultries, caves, tree holes, animal burrows, spaces between rocks, and holes of walls. Heavy rains could flood these resting sites and reduce suitable places for sandflies (Alexander 2000). Old traditional animal husbandry barns with stone construction can shelter bigger sandfly populations than modern new farms, due to providing more resting sites. However, sandfly species differ in their preference for resting sites. For instance, although *Sergentomyia minuta* tend to rest between small rocks, *Phlebotomus mascitii* has special habitat preference, which mainly includes caves (Grimm et al. 1993, Alten et al. 2015).

In addition, insecticides have huge effects on sandflies. In Greece, for instance, due to high-level DDT spraying in

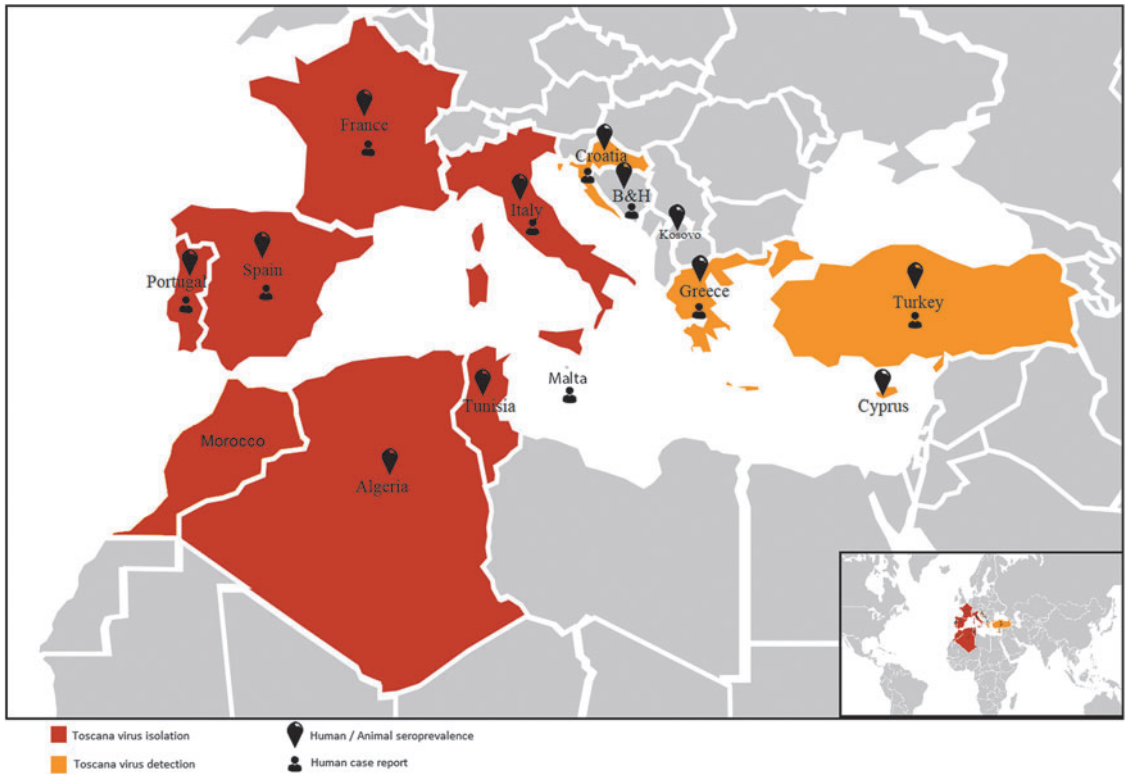

FIG. 3. Countries where data are available for seroprevalence, PCR detection, and virus isolation for Toscana virus.

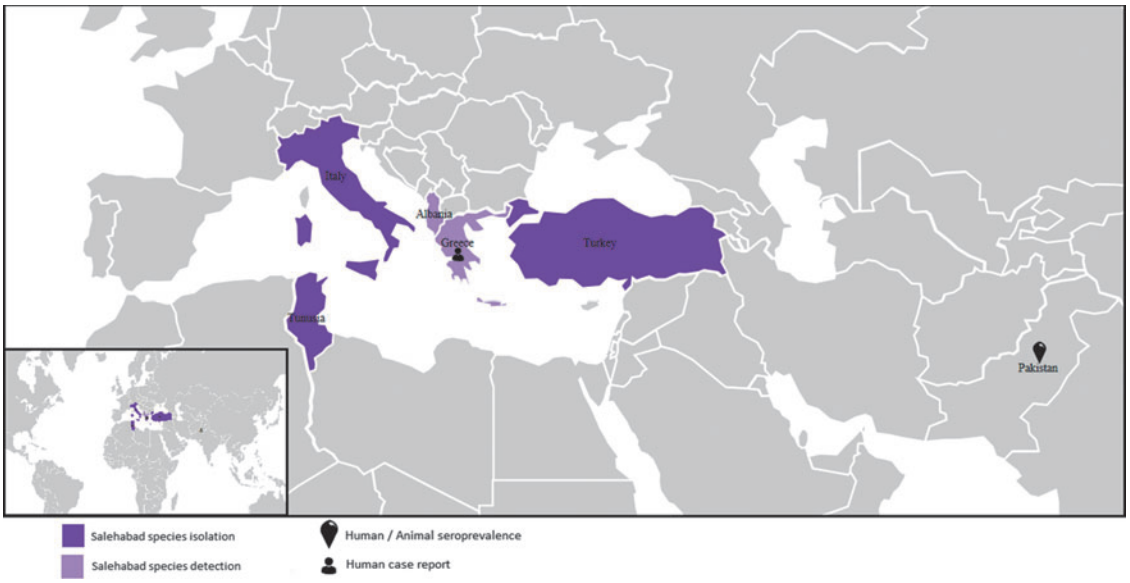

FIG. 4. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the *Salehabad* species.

nation-wide malaria control program, the number of sandflies dramatically decreased in the year 1946 (Hadjinicolaou 1958, Tesh and Papaevangelou 1977). It would be useful to ask the local people in the trapping region if they use insecticides.

How to Organize for Field Collection

The objectives of the study determine the global organization of the field collection, the equipment and tools needed, the manpower to be deployed, the logistics to be anticipated, and the setup. Depending on the aim of the study, the field area can be chosen for specific sandfly species. Until now, Sicilian virus was isolated from *Phlebotomus papatasi* in 1943 by Albert Sabin (Sabin 1951) and following studies show the presence of Sicilian-like viruses in *P. ariasi* in Algeria (Izri et al. 2008, Moureau et al. 2010) and in *Phlebotomus longicuspis*, *P. perniciosus*, and *S. minuta* in Tunisia (Zhioua et al. 2010). Sandfly fever Turkey virus, a variant of the sandfly fever Cyprus virus, which are considered as Sicilian-like phleboviruses, was detected in *Phlebotomus major complex* (Ergunay et al. 2012). Naples virus was isolated from *P. perniciosus* in Italy (Vesenjak-Hirjan et al. 1980) and from *Phlebotomus perfiliewi* in Serbia (Gligic et al. 1982). The first isolation of TOSV was in central Italy in 1971 from *P. perniciosus and P. perfiliewi* (Vesenjak-Hirjan et al. 1980). Consecutive studies show the presence of TOSV in

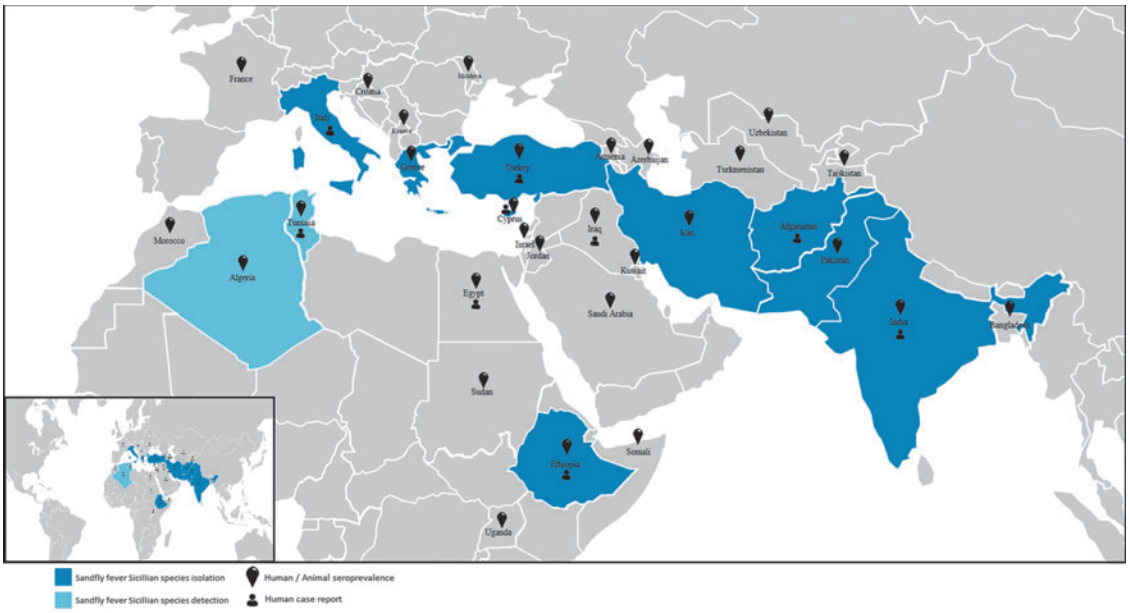

FIG. 5. Countries where data are available for seroprevalence, PCR detection, and virus isolation for viruses belonging to the sandfly fever Sicilian serocomplex.

S. minuta (Charrel et al. 2006). Massilia and Granada viruses were isolated from *P. perniciosus* (Charrel et al. 2009, Collao et al. 2010) and Punique virus was isolated from *P. longicuspis* and *P. perniciosus* (Zhioua et al. 2010). NW Phlebovirus species such as Buenaventura virus, Punta Toro virus, and Leticia virus were isolated from *Lutzomyia sp.* sandflies (Tesh et al. 1974). Despite extensive studies have been done around the Mediterranean area, vector–virus association remains poorly understood. Trapping in the regions that are known as endemic for the target virus could enhance the chances of success and increase the detection rate.

A clear definition of the objectives is of great importance to organize the field campaign in a manner that is suited to fulfilling these objectives. Different strategies depending on the purposes to be served are detailed in the sister review article entitled ''Practical guidelines for studies on sandfly-borne phleboviruses: part II: important points to consider for field work and subsequent virological screening.''

Detection of new viruses is very likely in regions where sandflies are present at high density. In our experience, the larger the number of sandflies the higher the chance to find a new virus. Recent studies have demonstrated that several sandfly-borne phleboviruses that may belong to distinct genetic complexes frequently cocirculate in a given locality (Amaro et al. 2015, Fares et al. 2015, Charrel unpublished data). Cocirculation of several viruses has been showed to be more frequent than initially considered. The outcome of the field campaigns is related to the number of sandflies trapped and tested. Even though there were previous studies in the same region, detection or isolation of novel phleboviruses can still be achieved. Recently, new phleboviruses isolation/detection was achieved from Turkey (Alkan et al. 2015b, Ergunay et al. 2014), Portugal (Amaro et al. 2015), Italy (Remoli et al. 2014), France (Charrel et al. 2009, Peyrefitte et al. 2013), Albania (Papa et al. 2011), and Tunisia (Zhioua et al. 2010), which shows the huge diversity of phleboviruses transmitted by sandflies. Moreover, the differences in the number of naturally infected sandflies depend on the region. The prevalence of the phlebovirus RNA in sandflies (phlebovirus positive pool/total number of tested sandflies) are reported as 1/460 (Charrel et al. 2007, France), 7/798 (Charrel et al. 2009, France), 5/427 (Peyrefitte et al. 2013, France), 4/896 (Amaro et al. 2015, Portugal), 5/1910 (Ergunay et al. 2014, Turkey), 7/900 (Remoli et al. 2014, Italy), 10/1489 (Zhioua et al. 2010, Tunisia) in various efforts. It is assumed that these values more or less reflect the level of virus circulation in a region. Surely, the high number of collection would increase the chance to detect or isolate the virus.

Actually, the majority of studies aiming at virus discovery in field-collected sandflies has resulted in the identification of new viruses when using open-detection techniques (generic PCR assays and cell culture), in contrast with specific techniques (Charrel et al. 2009, Moureau et al. 2010, Zhioua et al. 2010, Alkan et al. 2015b, Bichaud et al. 2016). Such nonspecific techniques have also shown to be capable of isolation and characterization of viruses belonging to the *Flavivirus* genus, not only the *Phlebovirus* genus (Alkan et al. 2015c).

Conclusions

It is unfortunate to address the virus discovery efforts in nature, just as additions to the virology stamp album. It must be recalled that the evidence for TOSV pathogenicity in humans (which is currently the most widespread arthropod-borne virus in Europe with at least 250 million people living in at risk area) was assessed 12 years after the virus was discovered in the field. Besides, the Rockefeller foundation has supported the most eminent arbovirologists to conduct studies of these viruses for more than 30 years. Although there is no doubt that Next Generation Sequencing will reveal many new discoveries about these viruses, the need to isolate and characterize the strains initially identified at their natural habitat, as well as investigating their pathogenic impact, has recognized globally among virologists. Without well-characterized infectious virus strains, serosurveillance or serodiagnosis studies to identify the specific etiological agent responsible for outbreaks or epidemics in susceptible populations cannot be performed. When carried out properly, the neutralization assay is the recognized gold standard for all virological seroepidemiological investigations. The virological ''stamp album'' is and has been for more than 60 years the essential tool with which to conduct these investigations and thence to inform health agencies charged with the responsibility of enabling implementation of the necessary disease control strategies.

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Address correspondence to:
Remi N. Charrel
UMR ''Emergence des Pathologies Virales''
Faculte de Medecine, 27 blvd Jean Moulin
Marseille 13005
France

E-mail: remi.charrel@univ-amu.fr

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Practical Guidelines for Studies on Sandfly-Borne Phleboviruses: Part II: Important Points to Consider for Fieldwork and Subsequent Virological Screening

Hartwig Huemer,[1] Jorian Prudhomme,[2] Fatima Amaro,[3] Amal Baklouti,[4,5] Gernot Walder,[6] Bulent Alten,[7] Sara Moutailler,[8] Koray Ergunay,[9] Remi N. Charrel,[4,5,10] and Nazli Ayhan[4,5]

Abstract

In this series of review articles entitled ''Practical guidelines for studies on sandfly-borne phleboviruses,'' the important points to be considered at the prefieldwork stage were addressed in part I, including parameters to be taken into account to define the geographic area for sand fly trapping and how to organize field collections. Here in part II, the following points have been addressed: (1) factors influencing the efficacy of trapping and the different types of traps with their respective advantages and drawbacks, (2) how to process the trapped sand flies in the field, and (3) how to process the sand flies in the virology laboratory. These chapters provide the necessary information for adopting the most appropriate procedures depending on the requirements of the study. In addition, practical information gathered through years of experience of translational projects is included to help newcomers to fieldwork studies.

Keywords: arbovirus, *Bunyaviridae*, *Phlebotomus*, phlebovirus, Toscana virus

Introduction

The main goal in any study aimed at phlebovirus detection and isolation must provide suitable conditions to ensure that the collected specimens are processed or preserved shortly after they are trapped. This basic approach ensures optimal yields of positive results for comparative analysis. During his long and brilliant career as one of the most eminent arbovirologists in the immediate aftermath of the Second World War, Dr. Jean Pierre Digoutte established standards for optimization of virus isolation procedures from wild caught specimens: the most important rule is to process viable material rapidly upon collection and to discard dead insects or animals because the time from death to collection is rarely known and may mitigate the isolation or detection processes. In the case of sand flies, these recommendations are particularly appropriate to apply because these tiny insects deteriorate rapidly after death; accordingly they must be stored at an appropriate low temperature after collection. Alternatively, they must be transferred to the laboratory for immediate processing or storage before further analysis. Here we provide an overview of the optimal procedures recommended for studies of phleboviruses transmitted by sand flies. We also provide personal opinions, based on available data, and the personal experience of the authors.

[1]Division for Human Medicine, Austrian Agency for Health and Food Safety (AGES), Vienna, Austria.
[2]Centre IRD, UMR MIVEGEC (IRD 224-CNRS 5290–Universite Montpellier), Montpellier, France.
[3]Centre for Vectors and Infectious Diseases Research, National Institute of Health Ricardo Jorge, Aguas de Moura, Portugal.
[4]UMR ''Emergence des Pathologies Virales'' (EPV: Aix-Marseille University–IRD 190–INSERM 1207-EHESP), Marseille, France.
[5]Fondation IHU Méditerranée Infection, APHM Public Hospitals of Marseille, Marseille, France.
[6]GmbH, Ausservillgraten, Austria.
[7]EBAL-VERG Laboratories, Ecology Division, Department of Biology, Faculty of Science, Science and Engineering Institute, Hacettepe University, Ankara, Turkey.
[8]UMR BIPAR, Animal Health Laboratory, ANSES, Maisons-Alfort, France.
[9]Department of Medical Microbiology, Faculty of Medicine, Hacettepe University, Ankara, Turkey.
[10]Special Infectious Agents Unit, King Fahd Medical Research Center, King Abdulaziz University, Jeddah, Saudi Arabia.

Trapping of Phlebotominae: Factors Influencing Efficacy

The methods chosen for sampling sand flies depend on the main objectives of the study in relation to the target phlebovirus(es). In addition to active collection of samples from humans or animals acting as bait, there are a variety of established mechanical methods for trapping *Phlebotomus* species depending on the specific requirements [for a review, see Killick-Kendrick (1987) and Alexander (2000)]. The most commonly used are sticky traps, light traps, and carbon dioxide (CO_2) traps. However, each of these trap types has advantages and disadvantages and also variations in efficacy (Burkett et al. 2007, Hoel et al. 2010, Junnila et al. 2011, Hesam-Mohammadi et al. 2014, Müller et al. 2015); thus, combining the various traps may be advisable when performing field studies intended to estimate the number and species of sand flies. For readers requiring detailed information, the review written by Alten et al. (2015) is recommended. Many observers have noted that huge number of night-flying insects attracted by light traps appear to be circling the traps and settling on the surrounding vegetation (Hartstack 1991). Thus preferential use of suction traps is observed in most studies of insect flight range and dispersal. Weather conditions, humidity, wind direction and many other factors can also play an important role, but often have not been extensively studied and adapted with the different designs of traps. In most cases, mosquito capture data from light traps can be compared with data obtained from human or animal baits using suction traps, CO_2-baited traps, and collections of resting insects during their inactive daytime period. However, some comparisons show that particular species of biting insects, which are rarely taken in light traps, may be captured by alternative capture methods. Alternative capture methods help to clarify whether closely related species, which are consistently recorded widely at different population levels, reflect a difference in abundance, or differences in trap response solely of the involved species.

Concerning the respective efficacy of trap types for virus isolation/detection, the limited number of comparative studies precludes any conclusions; most of the published studies have used Center for Disease Control and Prevention (CDC) light traps that have enabled virus isolation and/or detection; in the absence of comparative studies, it is now impossible to measure their efficacy relative to other types of traps. Therefore, until more data become available, we have to assume that the different types of traps are not impacting the subsequent virological studies. Thus far, quality of traps has been measured by their capacity to catch the highest number of sand flies.

Nonbaited traps

There are various mechanical techniques available for collecting sand flies using nonattractant traps, including flight trapping by nets or netting screens and/or simple mechanical suction devices. The latter can also be handheld devices that have the advantage of being deployable, thus making use of the experience of the sampler employed to seek the most likely insect resting sites. However, this is a highly stochastic process and may reflect the preferences of the sampler. In complex environments, for example, urban or sylvatic reliance solely on this method may lead to biased estimates of species composition and other distribution parameters. Moreover, statically positioned traps collect only flies within their immediate vicinity. Thus, reliance on these traps alone would give a misleading picture of the tested locality. As there is no ''gold standard'' among the available field-sampling procedures, multiple methods are applied consistently throughout the year. This is considered essential to obtain an approximation of species diversity and density for particular areas.

Sticky traps. Simple sticky traps have been successfully employed in France and the former USSR. These initially consisted of standardized pieces of paper/cards soaked in castor oil that are usually exposed overnight. Other carrier materials such as bottle designs can be used as alternatives to paper. The result of the catch is expressed by the number of sand flies attached to the equivalent of 1 square meter. If placed properly, that is, near likely insect resting sites and human and animal housings, they provide objective means of risk evaluation and also a reliable quantitative method of collection. However, unilluminated sticky papers like other nonattractive traps, for example, unlit, unbaited CDC traps, usually yield relatively low number of sand flies as they only catch flies from their immediate surroundings (Burkett et al. 2007). They are best suited for insect density studies, and because they kill the insect almost immediately, their use for virological studies of sand flies is not ideal. Sticky papers are very cheap and flexible. They can be placed in wind-protected sites and they can be used for complex environmental studies. For example, castor oil paper traps have been placed next to the exit and entry paths of rodents burrows to capture phlebotomines. The choice of trap may also influence the proportion of males or females collected. Sticky traps were found to be more effective than light traps for collecting sand flies entering rodent burrows either to take bloodmeals or for mating (Lahouiti et al. 2014).

Malaise traps. Malaise traps (Fig. 1) are open tent constructions developed by a Swedish entomologist in the 1930s

FIG. 1. Malaise trap.

while observing the high frequency of insects entering his tent. On reaching the apex of the tent, the only way out is through the collecting device that is filled with a killing agent (Malaise 1937). Modified versions using plastic cylinders for sampling and different netting materials were invented in the 1960s [for a review of different constructions, see Townes (1962)], but the basic design principle of the Malaise trap has remained virtually unchanged since its invention. These flight traps can be equipped with different types of collection heads. One type of head enables the use of isopropyl alcohol, which kills the insects rapidly and avoids them becoming damaged. The catch is then removed by unscrewing the bottles hanging under the angled collection heads. Malaise constructions are quite versatile as they can be simply baited, illuminated, or in some cases they can be adapted to house small animals to attract insects. Malaise traps are also relatively insensitive to wind compared with other traps, and they can be used for selective sampling as they provide most of the functions of Shannon traps or Disney traps. However, their tent-like design, the need to transport and assemble ropes, nets and poles together with their obtrusive appearance in the environment have reduced their popularity against competition from many other types of traps.

Shannon traps. They consist of black or white nets or netting screens used to attract sand flies, which can then be captured using manually held suction or other mechanical devices under visible control. Thus they are suitable for preselected catches. Shannon traps can also be illuminated and baited by placing humans or animals next to them. Studies in Brazil have shown that the black nets seem to be more attractive for sand flies and lead to higher yields (Galati et al. 2001). Shannon traps are most effective in a forest environment, where specific insect resting sites are not readily apparent. In some cases, Shannon traps have a tent-like construction with a strong light source. Typically used in the early evening and during the night, sand flies are attracted to the light and walk up the tent side where they can be hand aspirated. Illumination of the Shannon traps has the advantage of enabling sampling to be standardized. To some extent, mosquitoes may display a preference for individual investigators. Thus, ''baiting or repellent effects'' caused by natural or deodorant-induced odors may be considered, although this is not an evidence-based recommendation.

Baited traps

Human/animal landing collection. Landing collections often attract large number of insects, but the effectiveness and overall yield of the catches largely depend on the skill and ''attractiveness to the insect'' of the individual collectors. In addition, collections can also be obtained using domestic animals as bait. This can have the advantage of providing insights into human/animal preferences for ''biting'' behavior of local species and the ecological impact of livestock (Gebresilassie et al. 2015b). One disadvantage of this method is that it may expose the collectors to an increased risk of phlebotomine-transmitted infections, as the sampling is usually conducted in areas of suspected or proven disease prevalence. Studies have shown a strong correlation between sticky trap indices and human baiting. Thus, the simple and inexpensive sticky traps, although lacking an evaluation of individual insect aggressiveness or human/animal preferences, may be regarded as an acceptable substitute for studies of human-landing/biting rates (Hanafi et al. 2007).

Light traps. The use of artificial light has been applied to many different trap designs to attract nocturnal insects. Light traps (Fig. 2) have been widely used with considerable success for more than 50 years especially in the Americas. Owing to their simplicity and cost effectiveness, they have effectively become the ''standard'' method for most investigations. CDC traps, that is, miniature light traps developed by the U.S. Center for Communicable Diseases, now known as the Center for Disease Control and Prevention (CDC), equipped with incandescent or ultraviolet (UV) light, tend to catch significantly more sand flies than unilluminated traps and are effective up to several meters of distance (Killick-Kendrick 1985) (Fig. 3). When equipped with a suction device, they remain lightweight and portable and are more easily standardized than other manually aspirated sampling methods. However, the efficacy for collecting sand flies varies at the inter- and intraspecies levels, by gender and physiological status as a result of significant differences in phototropic and other behavioral characteristics within the same genus. Despite these limitations in collecting blood-fed females, CDC light traps have been shown to catch sufficient proportions of both indoor and outdoor sand flies to justify their recommendation (Dinesh et al. 2008).

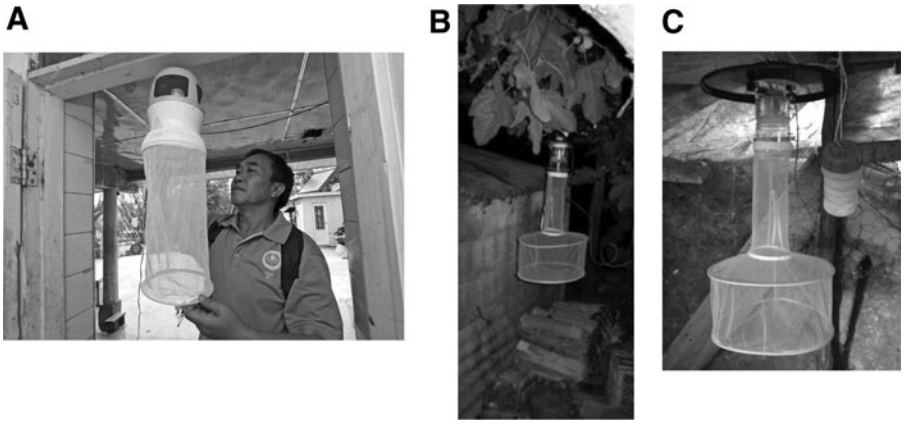

FIG. 2 (**A**) WHO light trap. (**B**) CDC miniature UV light trap, with modified ultrafine mesh in a pig pen, Algarve, Portugal. (**C**) CDC miniature UV light trap, with modified ultrafine mesh in a chicken pen, Algarve, Portugal. CDC, Center for Disease Control and Prevention; UV, ultraviolet.

FIG. 3. CDC miniature light traps, with modified ultrafine mesh and baited with dry ice in a sheep pen, Arrábida, Portugal.

Light intensity, wavelength, and some environmental factors have been shown to influence significantly the efficiency of light traps.

- Light intensity and wavelength: Short wavelengths of UV light may upset the orientation of nocturnal flying insects rather than simply attracting them (Nowinszky 2004); sand flies with compromised orientation are directed toward the light source (Junnila et al. 2011). Influence of moonlight and the lunar cycle has been clearly described (Gebresilassie et al. 2015a). One study showed that light displayed by light emitting diodes can attract sand flies, and that red light seems more effective than blue light (Hoel et al. 2007); this contrasts with results that show no measured differences in the efficacy when using different wavelengths.
- Environmental factors: The influence of environmental factors on the sensitivity and overall yield of light traps has been reported, in particular for exophilic species, that is, those ecologically independent of humans and their domestic environment. This could be because seasonal variations, changing weather conditions, environmental illumination in urban areas, or other factors (Guernaoui et al. 2006a, 2006b). The collection period lasts from before nightfall until just after dawn in outside installations. In endophilic species, that is, those ecologically associated with humans and their domestic environment, these factors are generally better controlled and the traps can be installed for longer time periods in enclosed places such homes or animal housing. Comparing studies of different regions may be difficult because of interspecies variation in the response to light. Only limited information on differences in phototropism of local species is currently available. Light trap catches are also affected by the wind direction (downwind, upwind), especially with sand flies, which because of their lightweight are highly sensitive to wind flow.

Carbon dioxide traps. CO_2 is a very powerful attractant for blood questing sand flies, but for cost as well as technical maintenance/supply reasons, it is used infrequently (Killick-Kendrick 1987). It can be applied in various mechanical sampling devices, mostly suction traps. Its use in combination with CDC light traps is common and ''CO_2–light trap combos'' are also available in several commercial forms that uses CO_2 production either by combustion of propane gas or dry ice (Fig. 4) (Hoel et al. 2010). Another advantage is that propane is less expensive and, in many areas, is much easier to obtain and easily handled compared with dry ice or containers of gaseous CO_2. A convenient workaround has been described when access to dry ice is impossible to obtain. This involves the use of self-fermenting sugar–yeast baits leading to the continuous production of CO_2 in warm climates (Kirstein et al. 2013).

Other baited sand fly collection systems

Sugar based and plant component based. Attractive toxic sugar baits (ATSBs) consisting of fermented ripe fruit have been used successfully as attractants for several mosquito species. Mixed with oral insecticide and sprayed on vegetation or bait stations, they have also been proposed for insect control. A study in the Jordan valley showed that ATSBs may also work for *Phlebotomus papatasi*, reducing local populations at the testing sites significantly (Müller and Schlein 2011). An interesting recent approach combines the attractant activity of sugar and CO_2 by using a sugar–yeast mixture in their trapping systems, continuously producing CO_2 by fermentation. This mixture, applied in 3 V miniature suction traps, has been shown to be of efficacy similar to collecting phlebotomines using light traps (Kirstein et al. 2013). Additional strategies have been tested that include plant material within the traps, mimicking the vegetation of suspected preferred resting sites. Thus, different plants have been identified that have either attractant or repellent

FIG. 4. Carbon dioxide light trap.

features. Addition of water to the traps in dry areas has also shown an enhancement effect for yields of phlebotomines [review see Müller et al. (2015)].

Animal-baited traps. The original Disney trap consisted of an animal cage in which a small animal such as a rodent (rat, guinea pig, or hamster) was placed as bait for insects. The cage was enclosed within a protective construction that denied access to predators. In its unmodified form, this outer area contained sticky papers to trap insects as they approached the caged animal (Disney 1966) (Fig. 5). Initially used with rats, it has been improved in several modified forms and can be used with a variety of small or larger animals known to serve as a blood source for local phlebotomine populations (Dorval et al. 2007). Other animal-baited insect traps suitable for *Phlebotomus* trapping or Leishmaniasis studies include tents or nets housing a goat, sheep, or cattle. Larger domestic animals such as goats appear to be more attractive to *Phlebotomus* species than rodents or chickens, and trapping successes of *Phlebotomus duboscqi* in semi-field environments have been observed to be similar in performance to CO_2-baited CDC light traps (Kasili et al. 2009).

Considerations of general trap design functions. Other trap design functions may often have an unexpected influence on insect-catch efficiency. Using the CDC miniature light/suction traps, updraft modifications of the suction/air stream, representing the equivalent of an ''inverted CDC trap'' deployed with their access point close to the ground, seem to be more effective for trapping sand flies than the classical downdraft designs in open habitats (Kline et al. 2011). One disadvantage of fan-incorporated traps resides in the turbulence generated by the airflow that may prevent fragile insects such as sand flies from entering the trap. Thus, both New Jersey and CDC trap designs used successfully in classic studies in the Americas have been found to be relatively ineffective in trapping European sand fly species in southern France (Rioux and Golvan 1969); the air movement at the fringe of the fan repelled light-attracted flies, before they were drawn in by the airflow of the trap. In more recent studies, the frequent use of ''sticky papers'' has proven its value in complementing suction-operated mini CDC traps for trapping living insects. However, additional sampling methods including handheld suction devices/aspirators clearly help to supplement light trap catches. It is important to underline that ''sticky papers'' are not suitable for virus isolation, and that their interest for viral RNA detection remains to be established.

FIG. 5. Modified Disney trap installed in a forested area, Bela Vista, Brazil.

How to Process the Sand Flies in the Field

As aforementioned, the procedure will depend upon the objectives of the study; accordingly, distinct approaches can be employed.

Virus detection versus isolation of viruses

Techniques used for maintenance and transportation of the sand flies after collection depend on the purpose of the study. The initial technical difference between virus isolation and virus detection approaches starts from the specimen collection step. Virus isolation requires sand flies to be collected alive and maintained either alive or at ultralow temperature from the time of trapping, through the transportation stage, and during storage. For virus detection only, it is possible to identify viral RNA from sand flies stored either under refrigeration or in 70% ethanol, which avoids total dehydration.

Virus isolation

Virus isolation has been the method of choice for direct diagnosis for almost a century. However, it is beginning to be displaced after the discovery of PCR and the development of molecular recovery methods to rescue infectious viruses. Historically, virus isolation was performed using laboratory animals (mice, rhesus monkeys, etc.) and chick embryos. At the beginning of the 1950s, cell cultures started to be used for virus studies, which provide facile working opportunities and easier cytopathic effect (CPE) monitoring (Bichaud et al. 2014). Despite the apparent sensitivity of laboratory-animal inoculation compared with cell cultures, they have been progressively abandoned, largely for ethical reasons. For virus isolation, sandfly material derived either from individual insects or from pooled homogenates is inoculated onto monolayers of cultured cells. The most commonly used cell line is Vero cells because sandfly-borne phleboviruses do not replicate in C6/36 insect cells. *Sandfly fever Naples virus* and *Sandfly fever Sicilian virus* also replicate in LLC-MK2 and BHK21 cells (Karabatsos 1985), but these cell lines have rarely been used in recent studies.

Molecular detection of the viral genomic RNA

For a long time, the paucity of complete genome or individual RNA segment sequences available for phleboviruses has rendered molecular screening difficult, and a limited number of detection assays has been available with unpredictable capacity to detect virus variants. For instance, dedicated RNA primers developed by Valassina et al. (1996, 2003) were unable to amplify genetic variants of Toscana virus, which were subsequently identified as a distinct lineage (lineage B). However, in a pioneer study, Sánchez-Seco et al. (2003) developed a nested PCR system, capable of amplifying all sandfly-borne phleboviruses recognized at the time

of publication. Importantly, this system has revealed its great potential because it enables the detection of novel virus strains.

Qualitative versus quantitative study: individual sand flies versus pools

Ideally sand flies should be studied individually. This increases the sensitivity and optimizes species identification of the sand flies, which can be achieved through gene sequencing. The reduced manipulation required with individual sand flies also decreases the likelihood of virus inactivation. However, this approach requires maximal manpower and high direct and indirect costs. Thus, most studies have relied on pooling of sand flies for virological studies. Nevertheless it is still important to pool sand flies based on sex, trapping site, and trapping date, which provides essential information concerning phlebovirus transmission. Interestingly phlebovirus isolation and/or detection has been achieved from both blood-sucking females and males (Zhioua et al. 2010, Peyrefitte et al. 2013, Remoli et al. 2014, Alkan et al. 2015a, 2015b), implying transovarial, venereal, or both transmission pathways of the viruses within sand fly populations (Tesh and Modi 1987, Tesh et al. 1992). Organizing pools according to trapping site and day is crucial for mapping purposes and to correlate the results with environmental parameters. Finally, blood-fed sand flies could be investigated individually for further possible host investigation with bloodmeal identification. In general, sand fly pool sizes of 20–50 are convenient for most purposes.

Identification and distribution of sand fly species in the trapping region

When robust epidemiological and sand fly species distribution data are available in the region where trapping will take place, the information can be used to optimize the yield of the study. However, sand fly population densities can vary widely both annually and monthly because of changes in climate or population dynamics. The objectives of the study must, therefore, be critically discussed to determine the most suitable sampling strategy. For instance, if the aim is to search for phleboviruses in specific sand fly species, then any robust information concerning distribution of the target sand fly species could enhance the quality of the investigation.

If there is no information concerning the distribution of sand fly species in the collection region, the biology and ecological requirements of the species and the area should be investigated intensively to choose the most suitable places for sample collection. Accordingly, all data on (1) *Leishmania* parasites, (2) human/canine leishmaniasis cases, (3) seroprevalence results for phleboviruses, and (4) previous data indicative of phlebovirus isolation or detection will be invaluable for study planning and should be searched in the peer-reviewed literature and in appropriate databases.

Living versus preserved sand flies

Virus infectivity and viral RNA structural integrity are highly susceptible to adverse climatic conditions, particularly elevated temperatures and extended periods of time before study or preservation. Phlebovirus studies based on phlebotomine sand flies require optimal methods for maintaining viral infectivity and the integrity of viral RNA from the time of field collection until arrival at the investigating laboratory. The optimal conditions to maintain viral infectivity and viral RNA integrity include (1) exclusion of the dead sand flies when traps are harvested, (2) keeping the flies alive as long as possible before processing for virus isolation, (3) relying on dry ice or −80°C cold chain until laboratory processing or permanent storage becomes available. The decision of whether or not to keep the specimens alive or frozen should rely on the facilities available. In cases in which immediate laboratory transfer of the specimens is not anticipated, dry ice or liquid nitrogen could be employed for preserving the cold chain. For PCR detection of the viral RNA genome, sand flies can be preserved either individually or in pools in 70% ethanol without the need for freezing (Bichaud et al. 2014, Remoli et al. 2015).

The need for sand fly species identification

Since sandfly-borne phleboviruses are vectored by sand flies belonging to a variety of different species that have characteristic ecological niches and geographic distributions, entomological identification is critical. Depending on the purpose of the study, species identification can be performed as a complementary task in trapping areas that have resulted in virus detection or isolation. Currently, in the Old World, phleboviruses have been isolated from the following species: *P. papatasi* [Sicilian virus (George 1970), Naples virus (Schmidt et al. 1971), Tehran virus (Karabatsos 1985), Punique virus (Zhioua et al. 2010)], *Phlebotomus longicuspis* [Toscana virus (Es-Sette et al. 2015)], *Phlebotomus sergenti* [Toscana virus (Es-Sette et al. 2015)], *Phlebotomus neglectus* [Corfou virus (Rodhain 1985)], *Phlebotomus perfiliewi* [Naples virus (Gligic et al. 1982), Fermo virus (Remoli et al. 2014), Toscana virus (Verani et al. 1980)], and *Phlebotomus perniciosus* [Toscana virus (Verani et al. 1980, Charrel et al. 2007, Remoli et al. 2016), Massilia virus (Charrel et al. 2009), Alcube virus (Amaro et al. 2015), and Arbia virus (Verani et al. 1988)].

Moreover, viral RNA of phleboviruses has been detected in the following species: *P. papatasi* [Sicilian virus (Moureau et al. 2010)], *P. longicuspis* [Naples-like virus (Moureau et al. 2010)], *P. perfiliewi* [Girne and Edirne virus (Ergunay et al. 2014)], *P. perniciosus* [Toscana virus (Es-Sette et al. 2012), Provencia virus (Peyrefitte et al. 2013), Utique virus (Zhioua et al. 2010)], and *Sergentomyia minuta* [Toscana virus (Charrel et al. 2006)].

Optimal Identification and Processing Procedures for Sand Flies in the Laboratory

Identification of sand flies

Sand fly species morphological identification is based on the morphology of male genitalia and female spermathecae and pharynges according to morphologic taxonomic keys (Lewis 1982, Killick-Kendrick et al. 1991), which need abdominal dissection of the specimen. During virus isolation studies, it is imperative to perform the sand fly identification process on ice to reduce the risk of degradation of the virus and thus to maintain its infectivity. Successful phlebovirus isolation has been accomplished in the morphologically identified samples in several studies (Sabin 1951, Verani

et al. 1980, Gligic et al. 1982, Charrel et al. 2006, Zhioua et al. 2010, Remoli et al. 2014). In some cases, particular sand fly species may not be reliably identified through morphological examination, which requires molecular identification approaches for accurate results (Alten et al. 2015). As molecular methods for identification of sand flies and other insects continue to improve, they almost certainly will ultimately become the method of choice.

The gene regions most commonly used for molecular identification of the sand fly species include mitochondrial cytochrome b, mitochondrial cytochrome c, ribosomal ITS2, nuclear EF-1α, and cytochrome oxidase subunit 1 (Depaquit et al. 2005, Kasap et al. 2013, Alten et al. 2015). Generally speaking, pooling the individual specimens without performing morphological identification increases the probability of successful virus isolation. This is largely because of the reduced time and workload involved in morphological and/or genetic identification that are done at temperatures that are deleterious for viral RNA and virus infectivity. With Next Generation Sequencing (NGS) techniques, it is now possible to perform molecular identification of the sand fly species that are contained in the pools as recently described (Alkan et al. 2016).

Recently, MALDI-TOF (matrix-assisted laser desorption/ionization time-of-flight) mass spectrometry was used for identification of sand fly species using the thorax/wings/legs of the specimen (Dvorak et al. 2014, Mathis et al. 2015, Lafri et al. 2016). Interestingly, since arbovirus replication in the vector is prominent in salivary glands attached to the head, it is possible to separate body parts used for MALDI-TOF identification from body parts used for virus isolation and detection. Such procedures can be easily performed in the field, where distinct body parts can be stored in separate tubes for specific use. Moreover, nucleic acids extracted from head and salivary glands can be used for molecular determination of sand fly species in samples requiring confirmation.

Virus isolation in cell culture or newborn mice

In general, sand fly pools that test positive using molecular methods are used secondarily to inoculate newborn mice intracerebrally (although this approach is gradually being phased out despite producing excellent results) or to seed cell lines that are competent for the replication of sandfly-borne phleboviruses. Naples virus, Sicilian virus, and Toscana virus can replicate in Vero, LLC-MK2, and BHK-21 cells (Karabatsos 1985). Among these lines, Vero cells have been the most frequently used in recent studies (Charrel et al. 2009, Collao et al. 2010, Alkan et al. 2015b, 2016, Amaro et al. 2016, Bichaud et al. 2016). Other cell lines, including monocytic cell lines, have also been used for basic research studies and diagnostic purposes. It is important to underline that during the initial isolation efforts from sand flies, several blind passages may be required before CPE becomes apparent (Alkan et al. 2016). Viral replication can be monitored using molecular detection procedures before CPE becomes obvious.

Nucleic acid extraction: RNA only, RNA+DNA, DNA only

Technically, the yield of RNA and DNA obtained by using RNA only, DNA only, or total nucleic acid kits is suitable for the detection of DNA and RNA microorganisms. Total nucleic acid purification is preferred rather than viral RNA extraction for practical reasons. Indeed, the entomological material is frequently collected during integrated and multidisciplinary projects, in which virological aspects overlap with parasitic or bacterial aspects that demand access to DNA rather than RNA. In addition, it is appropriate to anticipate that the stored material might be screened for DNA viruses in the future. Although PCR inhibitors have rarely been reported to affect virus detection in sand fly-derived material, spiking all samples subjected to extraction with appropriate internal controls should be considered because it enables the monitoring of all steps from nucleic acid purification to PCR (Ninove et al. 2011).

Nucleic acid extraction: manual versus automated

Both methods are equally effective. The choice more or less depends on the availability of equipment in the laboratory. Pooling the sand flies does not appear to affect virus detection rates significantly. Recent reports clearly indicate that pooling does not significantly impact on the isolation of the virus strains. The viral loads in infected sand flies are generally high enough to allow molecular detection and also virus isolation (Zhioua et al. 2010, Alkan et al. 2015a, 2015b, 2016, Amaro et al. 2016; Bichaud et al. 2016).

PCR detection using generic detection systems based on RT-nested PCR protocols

The relatively low number of available complete genomic sequences for viruses in the *Phlebovirus* genus has been a limiting factor in the design of either universal primers for all phleboviruses or group-specific primers (for viruses belonging to the *Sandfly fever Naples* complex, the *Salehabad* species, but also for other groups of phleboviruses belonging to species transmitted by mosquitoes and ticks). Subsequently, few systems have proved their capacity to detect a large array of phleboviruses. Although being far from optimal, most studies aimed at virus discovery have been performed using these PCR assays either singularly or in combination. The corresponding systems are (1) NPhlebo 1S/1R together with the nested NPhlebo 2S/2R described by Sánchez-Seco et al. (2003) located in the polymerase gene and enabling amplification of a primary PCR product ($\sim$560 bp) and of a nested PCR product ($\sim$240 bp), (2) Phlebo forward 1 and 2/Phlebo reverse described by Lambert and Lanciotti (2009) allowing the amplification of a 370-bp PCR product, and (3) SFNV-S1/R1 associated with nested SFNV-S2/R2, which enables detection of all members of the *Sandfly fever Naples virus* species (Charrel et al. 2007).

For PCR detection using species-specific assays

Although the limited number of complete genome sequences has hampered the development of specific assays, several systems have been described in the literature (Weidmann et al. 2008, Cusi and Savellini 2011, Brisbarre et al. 2015). The accumulating number of newly determined sequences justifies verification of these assays to evaluate in silico their capacity to detect all variants and genotypes for which sequences are available. Indeed, some of these systems are based on sequence alignments with a relatively small

number of sequences; the recent increase in sequence data should attract researchers to reconsider these systems for improvement and constant updating.

Conclusions

Isolation and subsequent complete genomic and antigenic characterization still remain the mainstay for identification of novel and well-known viruses. Advances in tNGS techniques, enabling viral metagenomic investigations in a variety of specimens including field-collected vectors, have also accelerated investigations for new viruses. All these approaches rely mainly on the appropriate collection, transfer, and processing of the specimens. As discussed in detail, the choice of methodology in major tasks should be based on the goals of the particular project, the budget, available infrastructure, as well as the experience of the research team, and such an effort definitely requires thorough planning and organization. These studies also facilitate fruitful collaborations among various research domains and are more likely to provide an integrated, holistic view of virus circulation in nature, as emphasized within the One Health concept.

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Address correspondence to:
Remi N. Charrel
UMR ''Emergence des Pathologies Virales''
(EPV: Aix-Marseille University–IRD
190–INSERM 1207-EHESP)
27 Boulevard Jean-Moulin
Marseille 13005
France

E-mail: remi.charrel@univ-amu.fr