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Guidelines for the diagnosis of tick-borne bacterial diseases in Europe

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ABSTRACT

Ticks are obligate haematophagous acarines that parasitise every class of vertebrate (including man) and have a worldwide distribution. An increasing awareness of tick-borne diseases among clinicians and scientific researchers has led to the recent description of a number of emerging tick-borne bacterial diseases. Since the identification of *Borrelia burgdorferi* as the agent of Lyme disease in 1982, 11 tick-borne human bacterial pathogens have been described in Europe. Aetiological diagnosis of tick-transmitted diseases is often difficult and relies on specialised laboratories using very specific tools. Interpretation of laboratory data is very important in order to establish the diagnosis. These guidelines aim to help clinicians and microbiologists in diagnosing infection transmitted by tick bites and to provide the scientific and medical community with a better understanding of these infectious diseases.

Keywords *Borrelia*, guidelines, Lyme disease, review, *Rickettsia*, tick-borne disease

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INTRODUCTION

Ticks are obligate haematophagous acarines that parasitise every class of vertebrate (including man) and have a worldwide distribution. An increasing awareness among clinicians and scientific researchers of tick-borne diseases has led to the recent description of a number of emerging tick-borne bacterial diseases. Since the identification of *Borrelia burgdorferi* as the agent of Lyme disease in 1982, 11 tick-borne bacterial pathogens of man have been described in Europe [1]. This review focuses on the available diagnostic tools and provides guidelines for the diagnosis of these diseases. These guidelines aim to help clinicians who encounter a suspected tick-transmitted disease, and microbiologists who wish to standardise methods for diagnosis, collate microbiological, clinical and epidemiological information, and develop case definitions. More details on the diseases, the limitations of diagnostic tools, and the ticks themselves can be found elsewhere [2].

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RICKETTSIOSES

Agents and vectors

Tick-borne rickettsioses are caused by obligate intracellular bacteria belonging to the spotted fever group within the genus *Rickettsia* [3]. It has been demonstrated for numerous rickettsiae that these bacteria are maintained in ticks through transtadial (from larva to nymph to adult) and transovarial (from one generation to another through ovaries) transmission. Thus, ticks may act as reservoirs for these bacteria, and the geography of tick-borne rickettsioses is determined by the distribution of ticks. The role of vertebrates as reservoirs in maintaining zoonotic foci has yet to be determined. When transmitted to man, the pathogenic rickettsiae multiply in endothelial cells and cause a vasculitis, which is responsible for the clinical and laboratory abnormalities that occur in rickettsioses [4].

Until recently, Mediterranean spotted fever (MSF) was considered to be the only rickettsiosis prevalent in Europe. This disease, also known as 'boutonneuse' fever, is caused by *Rickettsia conorii*

and is transmitted by the brown dog tick *Rhipicephalus sanguineus*. However, five new autochthonous tick-borne rickettsioses have been described in Europe during the last decade. These new rickettsioses include infections caused by '*Rickettsia mongolotimonae*' (southern France) [5,6], *Rickettsia slovaca* (throughout Europe) [7,8], *Rickettsia helvetica* (throughout Europe) [9], *R. conorii* Israel strain (Sicily and Portugal) [10] and *R. conorii* Astrakhan strain (Astrakhan, Kosovo) [11]. In addition, African tick-bite fever (ATBF) caused by *Rickettsia africae* is now recognised frequently as a travel-associated illness in travellers returning to Europe [12,13]. A new spotted fever caused by *Rickettsia parkerii* was reported in the USA in 2004 [14].

Each *Rickettsia* sp. has one or several tick vectors, and it is the geographical distribution, seasonal activity, host-seeking behaviour or tendency of these arthropods to bite humans that underlies the epidemiology of the disease [2]. In addition to the organisms described above, some other rickettsiae that have yet to be implicated in human disease have been isolated from ticks [4].

Clinical and standard laboratory features

Typically, the clinical symptoms of tick-borne rickettsioses begin 6–10 days after a tick bite, and include fever, headache, muscle pain, rash (Fig. 1), local lymphadenopathy and a characteristic inoculation eschare ('tache noire') at the site of the tick bite (Figs 1 and 2) [4]. However, these major clinical signs vary with the rickettsial species involved (Table 1). Non-specific haematological and biochemical findings include thrombocytopenia, leukocyte count abnormalities and elevated hepatic enzyme levels [15,16]. Further studies of atypical cases have also led to the description of new clinical syndromes caused by organisms previously considered as 'rickettsiae of unknown pathogenicity', including *R. slovaca* and *R. helvetica* [8,9,17].

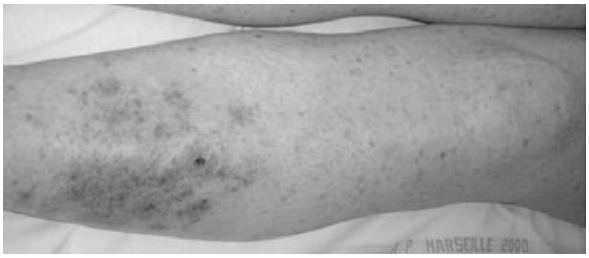

Fig. 1. Maculopapular rash of Mediterranean spotted fever. Note the inoculation eschar surrounded by a purpuric lesion.

Fig. 2. Typical eschar ('tâche noire') of tick-transmitted rickettioses. A scalp location of the eschar indicates tick-borne lymphadenopathy caused by *Rickettsia slovaca*.

Collection and preservation of samples

Before antimicrobial therapy, a minimum of 5 mL of blood should be collected for culture in either heparin- or citrate-containing tubes (leukocyte cell buffy coat), or EDTA tubes for molecular diagnosis. EDTA anticoagulant should be avoided for culture, as it is harmful to the cell monolayers used for recovery of rickettsiae. Heparinised blood can also be used for immunocytological detection of rickettsiae in circulating endothelial cells [18]. When culture or molecular diagnosis (based on PCR) is delayed for > 24 h, samples (plasma, buffy coat or whole blood) should be frozen at − 70°C or in liquid nitrogen for isolation procedures, or at − 20°C for molecular diagnosis.

For serological diagnosis, 10 mL of blood should be collected early in the course of the disease, and a second sample should be obtained 2 weeks later. If a four-fold rise in antibody titre is not observed, collection of a third sample after 4–6 weeks should be considered. Sera can be preserved at or below − 20°C for several months without degradation of the antibodies. An alternative, inexpensive and convenient method for collecting, storing and transporting blood samples suitable for serological testing is to collect a few

Table 1. Geographical distribution and symptoms of the tick-borne rickettsioses in Europe

					Clinical features				
Diseases	**Rickettsia**	**Vector(s)**	**Distribution**	**Epidemiological features**	**Fever**	**Diffuse rash**	**Eschar**	**Enlarged local nodes**	**Fatality rate without treatment**
Mediterranean spotted fever	*R. conorii*	*Rhipicephalus sanguineus*	Mediterranean area[a]	Urban (2/3) Rural (1/3) Late spring and summer	100%	97% (10% purpuric)	72%	Rare	1–2.5%
TIBOLA	*R. slovaca*	*Dermacentor marginatus* *D. reticulatus*	From western Europe to central Asia	Spring, autumn and winter	24%	Low (8%)	Yes (scalp)	Cervical (44% painful)	
Israeli spotted fever	*R. conorii* Israel	*Rh. sanguineus*[b]	Portugal, Sicily		Yes	100%	No	No	< 1%
Astrakhan fever	*R. conorii* Astrakhan	*Rh. pumillio* *Rh. sanguineus*	Astrakhan, Kosovo		Yes	100%	23%	No	
Unnamed	*R. mongolotimonae*	Unknown	Southern France[c]		Yes	Yes	Yes (may be multiple)	No	
Unnamed	*R. helvetica*	*Ixodes ricinus*	From northwestern Europe to central Asia		Yes	?	?	?	?
African tick bite fever	*R. africae*	*Amblyomma hebraeum* *A. variegatum*	Sub-Saharan Africa West Indies[d]	Imported disease Safari Grouped cases or epidemic	81–92%	42–50% (50% vesicular)	53–98% (55% multiple)	49–57%	

TIBOLA, Tick-borne lymphadenopathy.
[a]Cases may occur in northern countries, as brown dog ticks survive easily in homes and the human environment.
[b]*R. conorii* Israel has not yet been detected in ticks in Portugal, and the vector in this country is not yet known.
[c]Few cases reported.
[d]Imported from sub-Saharan Africa and the West Indies.

drops of blood on to blotting paper [19]. Skin biopsy specimens, collected before antimicrobial therapy (preferably from the site of tick attachment), are particularly valuable clinical samples that can be used for culture, immunohistochemistry and PCR. If processing of biopsy material is delayed, the sample should be preserved frozen at −70°C for culture and PCR, or in formalin for immunohistochemistry.

Tools available for diagnosis

Serology

Serological tests are the easiest methods for the diagnosis of tick-borne rickettsioses. The various techniques available have been reviewed elsewhere, but microimmunofluorescence (MIF) is accepted widely as the reference method [20]. MIF should be used to detect IgM and IgG in acute and convalescent sera. Currently, at the Unité des Rickettsies in Marseilles, IgG titres ≥128 and/or IgM titres ≥64 are considered indicative of infection by *R. conorii* in suspected cases of MSF, whereas IgG titres ≥64 and/or IgM titres ≥32 are considered indicative of infection by other *Rickettsia* spp. in other rickettsioses. IgM and IgG are usually both detected 7–15 days after disease onset. However, the IgG and the IgM responses to *R. africae* are delayed in ATBF patients, with median seroconversion times of 28 and 25 days after the onset of symptoms for IgG and IgM, respectively [21]. Doxycycline treatment within 7 days of the onset of disease has been shown to prevent the development of antibodies to *R. africae* [21]. Thus, the sensitivity of MIF is low in the early stages of ATBF, and it is recommended that sera collected 4 weeks after the onset of illness should be used for the definitive serological diagnosis of *R. africae* infection in patients suspected of having this syndrome [21]. Serological responses also appear to be slower to develop in patients infected with *R. slovaca*, but this aspect requires further investigation, as only a small number of patients have been studied.

The interpretation of serological data can be confounded by the cross-reactivity that occurs among the spotted fever group rickettsiae. Tests for a single antigen do not allow a definitive conclusion regarding the causative agent [22],

although tests for several antigens on the same slide may allow the causative agent to be identified from its comparatively higher antibody level [12,23]. Western blotting will yield false-positive results because of cross-reacting antibodies, which are directed mainly against lipopolysaccharide, but is useful in detecting antibodies early in the course of MSF. Western blotting, particularly in conjunction with sera that have been cross-absorbed, can also be used to identify the infecting rickettsial species, but the technique is only suited to reference laboratories [24] (Fig. 3). Table 2 shows a comparison of data regarding the diagnostic value of the above-mentioned serological tests. Table 3 sets out the diagnostic criteria used for interpreting the results of the standard MIF procedure. Immunodetection methods may also be used to identify rickettsiae in biopsy specimens and arthropods.

Cultivation

Isolation of rickettsiae is of great importance, as the ultimate diagnostic goal is recovery of the bacterial agent from a tick or a patient. Spotted fever group rickettsiae are strictly intracellular, short Gram-negative bacilli that retain basic fuchsin when stained by the method of Gimenez [25]. Rickettsiae can be grown *in vitro* in tissue cell cultures, but isolation attempts must be performed only in Biosafety Level 3 laboratories. Rickettsiae are isolated most commonly from blood (decanted plasma, collected on heparin or citrate anticoagulant), skin biopsy specimens (with the inoculation eschar being the best specimen to use) or ticks. The centrifugation shell vial technique, first developed for cytomegalovirus culture and early antigen detection, has been adapted for the isolation of *R. conorii*; in most cases, cultured microorganisms are detectable 48–72 h post-inoculation [26]. In a study based

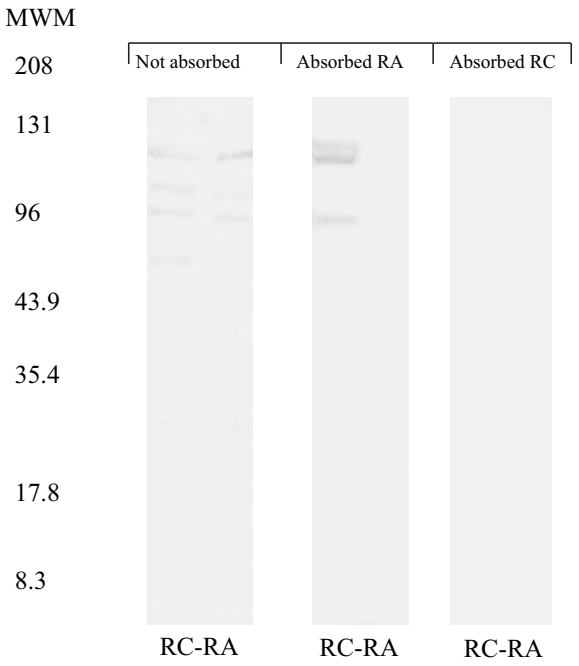

Fig. 3. Western blotting and cross-absorption study to differentiate between *Rickettsia conorii* (RC) and *Rickettsia africae* (RA) infection. Once absorbed with RA, the antibodies that remain are those directed against RC; all antibodies disappeared following absorption with RC, indicating that antibodies contained in the sera were directed specifically against *R. conorii*. MWM, molecular size marker.

Table 2. Value of serological tests in the diagnosis of rickettsial diseases

Disease	Test	Se	Sp	PPV	NPV	Ref.
Rocky Mountain spotted fever[a]	IFA	100	–	–	–	[172]
Rocky Mountain spotted fever[b]	IFA	–	–	–	–	[173]
	≥ 1/64	84.6	100			
	≥ 1/32	97.4	99.8			
Mediterranean spotted fever[c]	IFA ≥ 1/40	–	–	–	–	[174]
	Sampling day 5–9	46				
	Sampling day 20–29	90				
	Sampling after day 29	100				
African tick bite fever[d]	–	–	–	–	–	[12]
	IFA	26	100	100	–	
	CA	83	100	100	–	
	WB	53	100	100	–	
	IF + CA + WB	56				

CA, Cross-absorption; IFA, immunofluorescence assay; WB, Western blot; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

[a]Sixty paired sera, including specimens with stationary titres (5%) and four-fold rising titres (95%).

[b]Study of 417 sera from 178 patients who very probably did not have rickettsial infections, and 88 sera from 41 patients who very probably had Rocky Mountain spotted fever.

[c]One hundred and eighty-four sera tested.

[d]Four hundred and fourteen patients tested for *R. africae*, with 39 confirmed by PCR or culture, and 81 considered positive on the basis of IFA + WB + CA.

Table 3. Standard procedure used at the Unité des Rickettsies for the diagnosis of rickettsioses when cross-reactions are noted between several rickettsial antigens in the microimmunofluorescence test [20]

1. A rickettsial antigen is considered to be implicated in the infection when titres of IgG and/or IgM antibody against this antigen are at least two serial dilutions higher than titres of IgG and/or IgM antibody against other rickettsial antigens
2. When differences in titres between several antigens are <2 dilutions, Western blot assays are performed. A rickettsial antigen is then considered to be implicated in the infection when acute sera show an exclusive reactivity with the specific proteins of this antigen
3. When Western blots are non-contributive, cross-absorption studies are performed when IgG/IgM titres are ≥128/32. Specific diagnostic criteria after cross-absorption studies are: (1) immunofluorescence serology positive for a single antigen; or (2) Western blot assay showing exclusive reactivity with the specific proteins of an antigen

on specimens collected from 157 suspected MSF cases before the prescription of antimicrobial agents, *R. conorii* was isolated from 59% of patients with a concurrent MIF titre of ≤1/32 [20]. In 34 of these patients, *R. conorii* was detected as soon as day 3 after the tick bite, i.e., before seroconversion. However, when samples were not inoculated on the day of sampling, but held at room temperature or at 4°C overnight before inoculation, no culture was positive. When the shell vial technique is used, the small surface area (1–2 cm) of the coverslips on which cell monolayers are established enhances the ratio of rickettsiae to cells, thereby allowing better recovery. Vero or L929 cells have been shown to be more suitable than HEL or MRC5 cells for the isolation of rickettsiae. Nevertheless, the use of HEL or MRC5 cells is more convenient, as once a monolayer is established, contact inhibition prevents further division, and the cells can then be used for prolonged incubation. Centrifugation of the shell vial following the introduction of material suspected of containing rickettsiae is critical for the sensitivity of the technique, because it enhances rickettsial attachment and penetration of the cell monolayer. Detection of rickettsiae within the cells can be achieved by microscopic examination after Gimenez staining, immunodetection or PCR. If immunofluorescence is negative after 20 days, the culture is considered negative. If immunofluorescence is positive, parallel shell vials are inoculated on to confluent monolayers of HEL cells in culture flasks, in an attempt to obtain isolates of *Rickettsia* spp.

Molecular methods

Molecular methods based on PCR have enabled the development of sensitive, specific and rapid tools for both the detection and identification of rickettsiae in blood, skin biopsy specimens (the 'tache noire' being the most useful specimen) and even ticks. Primer sets targeting various rickettsial genes have been described (Table 4) and can be used in any laboratory with suitable facilities.

Case definition

A flow chart outlining laboratory methods for the diagnosis of rickettsioses is presented in Fig. 4. Case definitions and diagnostic scores have been established for ATBF (Table 5) and MSF (Table 6), but not for other less established rickettsial diseases.

ANAPLASMOSIS (EHRLICHIOSES)

The agent and its vector

Human (granulocytic ehrlichiosis) anaplasmosis is an acute infectious disease caused by *Anaplasma phagocytophilum* (formerly human granulocytic ehrlichia, *Ehrlichia phagocytophila*, *Ehrlichia equi*) and was first characterised in the northern USA in 1994 [27]. This bacterium belongs to the order Rickettsiales within the α-Proteobacteria [28]. *A. phagocytophilum* is an obligate intracellular pathogen that parasitises the granulocytes of man and animals. Although this agent has been associated only recently with disease in man, its role in the aetiology of tick-borne fever in European livestock has been recognised since the 1930s [29]. The bacterium has been detected by PCR in *Ixodes* ticks from most European countries, including France [30], Slovenia [31], Switzerland [32,33], The Netherlands [34], northern Italy [35], Bulgaria [36], southern Norway [37], the Baltic region of Russia [38], southern Germany [39,40], the UK [41] and Spain [42]. The prevalence of *A. phagocytophilum* in ticks ranged from 2% to 45% [30,34].

The first European case of human anaplasmosis was reported in 1995 in Slovenia [43]. Although serological evidence of the disease has been reported frequently in most European countries [42,44–53], few acute cases have been confirmed by observation of a four-fold increase in specific antibodies, the presence of morulae in circulating granulocytes, or PCR-based detection of the organism in the blood. To our knowledge, only 22 confirmed cases have been reported to date, comprising one case from The Netherlands [54], two from Sweden [55,56], 13 from Slovenia [43,57–60], one from Spain [61], one from Austria [62], three from Poland [63] and one from France [64].

Clinical and laboratory characteristics

Human anaplasmosis is an acute tick-borne infection. Most patients recall a tick bite before the onset of their illness. The incubation period ranges from 5 to 21 (median, 11) days. Most cases of human granulocytic ehrlichia occur between

Table 4. DNA targets that have been exploited for detection of rickettsia in samples of infected patients

Gene used and species	Forward and reverse primers	Method	Sample	Ref.
Citrate synthase (*gltA*) All species	RpCS.877p: GGGGACCTGCTCACGGCGG RpCS.1258n: ATTGCAAAAAGTACAGTGAACA	Regular PCR	Skin	[175]
Outer-membrane protein A (*ompA*)	Rr190.70p: ATGGCGAATATTTCTCCAAAA Rr190.602n: AGTGCAGCATTCGCTCCCCCT	Regular PCR	Skin	[175]
All species except *R. helvetica*, *R. australis*, *R. bellii*, *R. canadensis*,	Rr190.70p: ATGGCGAATATTTCTCCAAAA Rr190.701n: GTTCCGTTAATGGCAGCATCT	Regular PCR		
ompA	AF1F: CACTCGGTGTTGCTGCA AF1R: ATTAGTGCAGCATTCGCTC AF2F: GCTGCAGGAGCATTTAGTG AF2R: TATCGGCAGGAGCATCAA	'Suicide PCR'[a]	Serum	[12]
	AF3F: GGTGGTGGTAACGTAATC AF3R: CGTCAGTTATTGTAACGGC AF4F: GGAACAGTTGCAGAAATCAA AF4R: CTGCTACATTACTCCCAATA	'Suicide PCR'[a]		
Outer-membrane protein B (*ompB*) All species except *R. helvetica*, *R. bellii*, *R. massiliae*	BG1-21: GGCAATTAATATCGCTGACGG BG2-20: GCATCTGCACTAGCACTTTC	Regular PCR	Skin	[175]

[a]Note that primers used for this nested PCR are used only once in a particular laboratory, with no positive control. Other pairs of primers can be designed each time that a new screening is performed; these can be selected to target other rickettsial genes.

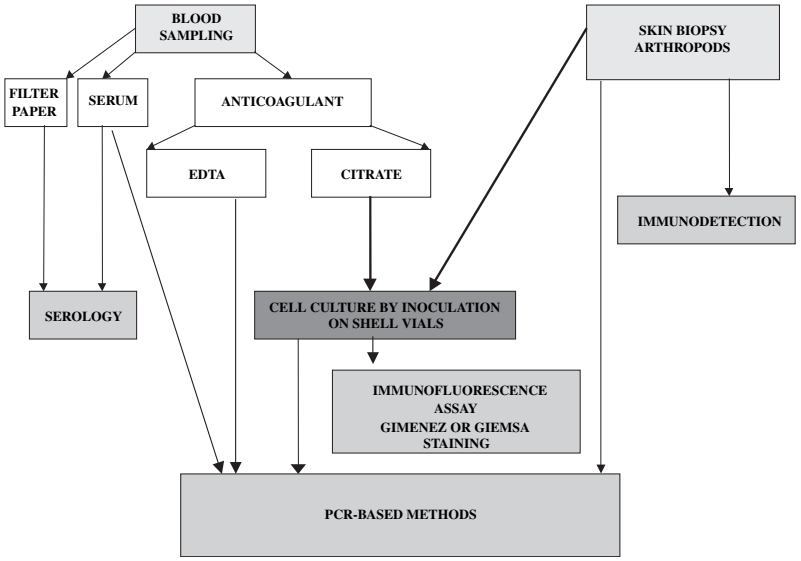

Fig. 4. Collection of samples and diagnostic tools for use in the diagnosis of rickettsial diseases transmitted by tick bite.

April and October, peaking in July. Patients present with an acute non-specific febrile illness characterised by high-grade fever (> 38.5°C), chills, severe headache and generalised myalgias and/or arthralgias. Significantly more infections have been reported in males than in females, with an age range of 11–73 years. The duration of fever is 2–11 days (median, 10 days). Less common symptoms include nausea, abdominal pain, diarrhoea and cough. Skin rash and confusion are very infrequent findings. The results of physical examination are often unremarkable. Interestingly, several cases of human anaplasmosis have presented as atypical pneumonitis [60,63,64].

Approximately one-half of patients with acute anaplasmosis require hospitalisation. Complications are unusual, and opportunistic infections have not yet been described in European patients. Clinical features and laboratory findings reported in patients from Europe are similar to those reported from the USA, although the disease appears to be generally milder and to resolve sooner, even in the absence of specific antimicrobial therapy [57,59,65,66]. No fatal infections or long-term sequelae have been reported. No chronic forms of the illness have yet been documented in Europe, although *A. phagocytophilum* DNA has been demonstrated in the convalescent blood of

Table 5. Diagnostic criteria for African tick bite fever (ATBF); a patient is considered to have ATBF when criteria A, B or C are met

A. Direct evidence of *R. africae* infection by culture and/or PCR
or
B. Clinical and epidemiological features highly suggestive of ATBF, such as multiple inoculation eschars and/or regional lymphadenitis and/or a vesicular rash and/or similar symptoms among other members of the same group of travellers coming back from an endemic area (sub-Saharan Africa or French West Indies)
and
Positive serology against spotted fever group rickettsiae
or
C. Clinical and epidemiological features consistent with a spotted fever group rickettsiosis such as fever and/or any cutaneous rash and/or single inoculation eschar after travel to sub-Saharan Africa or French West Indies
and
Serology specific for a recent *R. africae* infection (seroconversion or presence of IgM ≥ 1:32), with antibodies to *R. africae* greater than those to *R. conorii* by at least two dilutions, and/or a Western blot or cross-absorption showing antibodies specific for *R. africae*

Table 6. Diagnostic criteria for Mediterranean spotted fever caused by *Rickettsia conorii*

Criteria	Score[a]
Epidemiological criteria	
Stay in endemic area	2
Occurrence in May–October	2
Contact (certain or possible) with dog ticks	2
Clinical criteria	
Fever > 39°C	5
Eschar	5
Maculopapular or purpuric rash	5
Two of the above criteria	3
All three of the above criteria	5
Non-specific laboratory findings	
Platelets < 150 G/L	1
SGOT or SGPT > 50 U/L	1
Bacteriological criteria	
Blood culture positive for *Rickettsia conorii*	25
Detection of *Rickettsia conorii* in a skin biopsy	25
Serological criteria	
Single serum and IgG > 1/128	5
Single serum and IgG > 1/128 and IgM > 1/64	10
Four-fold increase in two sera obtained within a 2-week interval	20

SGOT, serum glutamate–oxaloacetate transaminase; SGPT, serum glutamate–pyruvate transaminase.
[a]A positive diagnosis is made when the overall score is ≥ 25.

one patient on the 22nd day after the onset of illness and 16 days after defervescence [59].

Haematological and biochemical tests can be helpful for diagnosis, but the findings are non-specific. The most common laboratory abnormalities are thrombocytopenia (90%) and leukopenia (70%), usually accompanied by abnormal liver function, manifesting as mild increases in serum concentrations of aspartate aminotransferase, alanine aminotransferase and, most frequently, lactate dehydrogenase. An elevated concentration of C-reactive protein has been found in almost all patients with acute human granulocytic ehrlichia. Other less common laboratory findings are anaemia, and elevated serum creatinine and blood urea nitrogen levels. In general, all laboratory abnormalities found in European patients resolve within 14 days of initial presentation.

Acute human anaplasmosis has a clinical and laboratory presentation remarkably similar to the initial phase of infection by tick-borne encephalitis virus, which is an endemic disease in northern, central and eastern Europe. Comparison of clinical signs, symptoms and laboratory findings in patients with these two syndromes has, however, revealed several significant differences [67]. The possibility of human anaplasmosis should be suspected each time a patient in an endemic region presents with an undifferentiated febrile illness occurring after a tick bite or tick exposure.

Collection and preservation of samples

For isolation of the agent, blood should be collected during the acute phase of the disease when circulating infected neutrophils are at their highest concentration [68]. The blood should be collected, preferably on EDTA, and kept either at room temperature [69] for not more than 48 h or frozen at −20°C before inoculation [70]. Samples of citrate-anticoagulated blood containing *A. phagocytophilum* were shown to be infective for 10 days if stored at room temperature, or for 13 days if stored at 4°C [68]. Although heparin-anticoagulated blood has been used successfully to infect animals, routine use of anticoagulant should be avoided because of its ability to compromise PCR-based detection methods. Transportation of clinical material to the laboratory should be performed as quickly as possible at room temperature, or at −20°C if transport times are expected to be > 48 h.

For serology, at least two sera should be collected, one during the acute phase and one after 15–21 days during the convalescent phase. Sera should be kept at 4°C or frozen at −20°C. Information on the duration and kinetics of the antibody response following acute anaplasmosis is limited in Europe, and is based entirely on a report from Slovenia. Significant antibody titres were detectable in 40% of patients for 2 years after the initial presentation [65].

Diagnosis of infection by observation of infected phagocytes in blood smears is best achieved if smears are prepared immediately after blood collection. Smears should be air-dried and preserved at room temperature.

Diagnostic tools

Serology

Detection of circulating antibodies is assessed by indirect immunofluorescence antibody testing. The antigens used in these assays vary (Table 7). *A. phagocytophilum* antigen, grown intracellularly, is usually coated on to glass slides, fixed with cold acetone or alcohol, and preserved either at −20°C or in light-protected paper. Alternatively, cell-free antigen may be prepared by purification of infected cell culture, and preserved either frozen or at 4°C in solution with sodium azide 1% w/v in phosphate-buffered saline. In the latter case, the slide can be prepared on the day of use by the addition of antigen with a pen-nib, followed by fixation in either cold acetone or alcohol [71].

Cultivation

Isolation of *A. phagocytophilum* should only be attempted in a Biohazard Level 3 laboratory. The promyelocytic HL-60 leukaemia cell line (ATCC CCL240) is the most widely used cell line for growing *A. phagocytophilum* [69]. These cells are maintained in antibiotic-free RPMI-1640 medium, supplemented with 2 mM glutamine and fetal bovine serum 20% v/v, and incubated at 37°C in CO_2 5%. Fresh blood (100 μL) [69], or 0.5 mL of the leukocyte fraction of previously frozen (− 20°C) EDTA–blood [70], can be inoculated into a 25-cm^2 flask containing HL-60 cells at a density of 2×10^5 cells/mL. Infection can be assessed by Giemsa staining of CytoSpin cells. Infection is usually detectable as observable morulae at days 3–7 post-inoculation [70].

Molecular detection

PCR-based methods are not yet standardised and may yield discrepant results. Detection of *A. phagocytophilum* DNA has been achieved from blood and from acute-phase sera (before day 21) of infected patients [72]. Few techniques have been evaluated properly, but several PCR targets have been described (Table 8). In a study involving cases of human anaplasmosis where infection had been confirmed by seroconversion, PCR targeting a fragment of the *epank* gene detected DNA in all patients, while PCR targeting a 16S rRNA gene fragment failed to do so [73]. The detection limits of 13 published PCR assays have been assessed, and those targeting fragments of the *msp2* homologues or the 16S rRNA gene were found to be the most sensitive [74]. Discrepancies in PCR sensitivity might be related to the length of the amplicon and the primer used. Whichever DNA target is chosen, sequencing of PCR products is mandatory to confirm their identity. The use of broader-range, genus-specific PCRs is not recommended, as knowledge of the true diversity of *A. phagocytophilum* or the *Anaplasma* genus is, as yet, very limited [34,75]. However, until issues concerning sensitivity and specificity of the various assays have been better addressed, the best-

Table 7. Serological tests available for testing *Anaplasma phagocytophilum* antibody in man

Laboratory	IFA	(Se/Sp) IgG, (Se/Sp) IgM	Antigen source	Commercial test	Ref.
Unité des Rickettsies, France[a]	IgG, IgM	(80/92.7), (40/94.1)	HGE Webster strain (human isolate)	No	[71]
Kalmar County Hospital, Sweden[b]	IgG	Not evaluated	SMA 308 (Swedish isolate)	No	[176]
Institute for Microbiology and Immunology, Slovenia[c]	IgG	Not evaluated	USG3 (tick isolate)	No	[65]
University of Zurich, Switzerland[d]	IgG	Not evaluated	*A. phagocytophilum* (Swiss strain)	No	[47]
MRL Diagnostics, USA[e]	IgG, IgM	86.6/92.7, 33/98	HGE 1 (human isolate)	Yes	[46] [71]
Pettenkofer-Institute, Germany[f]	IgG, IgM	Not evaluated	HGE (human isolate)	No	[71]

IFA, immunofluorescence assay; Se, sensitivity; Sp, specificity.

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[c]Institute for Microbiology and Immunology, Medical Faculty, Ljubljana, Slovenia. E-mail: tatjana.avsic@mf.uni-lj.si.

[d]Department of Veterinary Internal Medicine, University of Zurich, Winterthurerstrasse 260, CH-8057 Zurich, Switzerland

[e]MRL Diagnostics, Cypress, CA, USA.

[f]Max von Pettenkofer-Institute, Consiliary Laboratory for Ehrlichiae, University of Munich, Pettenkofer-Strasse 9a, D80336 München, Germany. E-mail: Bettina.Wilske@mvp-bak.med.uni-muenchen.de.

Table 8. DNA targets that have been used for the detection of *Anaplasma phagocytophilum* in samples from infected patients

Gene used	Forward and reverse primers	Method	Sample	Sensitivity/specificity	Ref.
16S rRNA	GE9f, 5′-AACGGATTATTCTTTATAGCTTGCT-3′ GE10r, 5′-GGAGATTAGATCCTTCTTAACGGAA-3′	Regular PCR	Blood	86%/100%	[43,72,177]
16S rRNA	EC9, 5′-AAGGATCCTACCTTGTTACGACTT-3′ EC12, 5′-AATCTAGATTAGATACCCT(A/T/G)GTAGTCC-3′ Ge9f, 5′-AACGGATTATTCTTTATAGCTTGCT-3′ Ge10r, 5′-GGAGATTAGATCCTTCTTAACGGAA-3′	Nested	Serum	Not evaluated	[178,179]
Epank1	LA6, 5′-GAGAGATGCTTATGGTAAGAC-3′ LA1, 5′-CGTTCAGCCATCATTGTGAC-3′	Regular PCR	Blood	95%/100%	[73]
HGE 44	Not published	Regular PCR	Blood	Not evaluated	[54]
P44 (*msp2*) paralog sequence	P44f1-5′-TTGATCTTGAGATTGGTTACG-3′ P44r1-5′-GGCAGATCATCATAAACRCC-3′ P44f2-5′-CAAGGGTATTAGAGATAGT-3′ P44r2-5′-AAACTGAACAATATCCTTAC-3′	Nested suicide	Serum	Not evaluated	[64,180]
GroESL	HS1, 5′-TGGGCTGGTA(A/C)TGAAAT-3′ HS6, 5′-CCICCIGGIACIA(C/T)ACCTTC-3′ HS43, 5′-AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC-5′ HS45, 5′-ACTTCACG(C/T)(C/T)TCATAGAC-3′	Nested	Blood	Not evaluated	[57,181]

evaluated methods are recommended for clinical application.

Case definition

There have been too few reported cases of acute human ehrlichioses to allow the precise definition of the clinical spectrum of human anaplasmosis in Europe. The case definition summarised in Table 9 is based on the epidemiological, clinical and laboratory characteristics of reported cases in Europe, as well as the diagnostic tools described above.

LYME BORRELIOSIS

The agent and its vector

Lyme disease is a multisystem disease caused by bacteria termed *Borrelia burgdorferi* sensu lato (sl). *B. burgdorferi* sl is a spirochaete, helically shaped, motile, 20–30 μm in length and 0.2–0.3 μm in width [76]. Within *B. burgdorferi* sl, three human pathogens are recognised, namely *B. burgdorferi* sensu stricto (ss), *Borrelia afzelii* and *Borrelia garinii* [77,78]. Only *B. burgdorferi* ss is encountered in North America, while all three species exist in Europe. Another species, *Borrelia lonestari* [79], has been reported to cause human disease [80], and other species have been described recently, including *Borrelia japonica* [81], *Borrelia andersonii* [82], *Borrelia lusitaniae* [83], *Borrelia miyamotoi* [84], *Borellia turdae* [85], *Borrelia tanukii* [85] and *Borrelia valaisiana* [86]. It has been suggested, but not yet demonstrated, that the last of these may be a human pathogen.

Table 9. Proposed case definition for human anaplasmosis

Confirmed human anaplasmosis
1. Febrile illness with a history of a tick bite or tick exposure
   and
2. Demonstration of *Anaplasma phagocytophilum* infection by seroconversion or ≥4-fold change in antibody titre[a]
   or
3. Positive PCR result with subsequent sequencing of the amplicons demonstrating *Anaplasma*-specific DNA in blood
   or
4. Isolation of *A. phagocytophilum* in blood culture

Probable human anaplasmosis
1. Febrile illness with a history of a tick bite or tick exposure
   and
2. Presence of stable titre of *A. phagocytophilum* antibodies in acute and convalescent sera if titre >4-fold above the cut-off value[a]
   or
3. Positive PCR result without sequence confirmation[b]
   or
4. Presence of intracytoplasmic morulae in a blood smear

[a]By immunofluorescence assay, with use of either intracellular or purified antigen in a reference laboratory or the MRL Diagnostics (Cypress, CA, USA) kit.
[b]With use of the species-specific primers listed in Table 8.

Ixodes ticks transmit all species belonging to *B. burgdorferi* sl. Tick larvae and nymphs feed primarily on small rodents and birds, whereas adult ticks feed on a variety of mammals (deer, domestic and wild carnivores, and larger domestic animals). The feeding time of *Ixodes* ticks (in Europe, primarily *I. ricinus*) is long (several days to >1 week); during this period, the ticks are dispersed on a scale that depends on the range of their hosts. Birds, particularly migratory sea birds, can transport ticks (*Ixodes uriae*) over very long distances, and thus distribute borreliae (especially *B. garinii*) worldwide [87]. There is an association between certain *B. burgdorferi* sl spp. and specific vertebrate hosts, e.g., between *B. afzelii and* small rodents, and between *B. garinii*, *B. valaisiana* and birds [88].

Clinical and laboratory characteristics

B. burgdorferi ss, *B. afzelii* and *B. garinii* are associated with different clinical manifestations

of Lyme disease [89–92]. Three stages are usually described: stage I, early/localised infection; stage II, early/disseminated infection; and stage III, late/persistent infection. The initial clinical presentation (stage I) involves skin lesions—erythema migrans (EM), the pathognomonic sign associated with all three species—that occur early at the site of the tick bite in the form of a gradually extending erythematous lesion (Fig. 5). Other secondary ring-shaped lesions develop in certain cases. A rare, but typical, skin lesion is borrelia lymphocytoma, a bluish-red tumour-like skin infiltrate, most often localised at the ear lobe, the nipples and areola mammae. Joint lesions occur between a few weeks and 2 months after disease onset in the form of polyarthralgia or true arthritis, usually in the knee (80% of untreated patients in the USA) (stage II). It has been proposed that Lyme arthritis is associated more frequently with *B. burgdorferi* ss infections, but this hypothesis remains controversial [92].

Neurological manifestations (associated most frequently with *B. garinii*) show considerable variety, ranging from isolated meningitis to encephalopathy, and include meningoradiculitis and lesions of pairs of cranial nerves (usually resulting in facial paralysis such as Bell's palsy). In general, these manifestations are of late onset, but they may also be observed during the EM period. Neurological manifestations are reported more frequently in Europe. In the weeks following the onset of disease, cardiac involvement is observed in 5% of cases, usually with electrocardiographic signs of myocarditis (conduction disorder and, sometimes, atrioventricular block). Acrodermatitis chronica atrophicans (ACA) is associated with *B. afzelii*, and is generally observed (stage III) several years after EM. ACA begins with a characteristic bluish-red discoloration, often with a doughy infiltration, and then progresses to atrophy of the skin, and is sometimes complicated by sclerodermic changes.

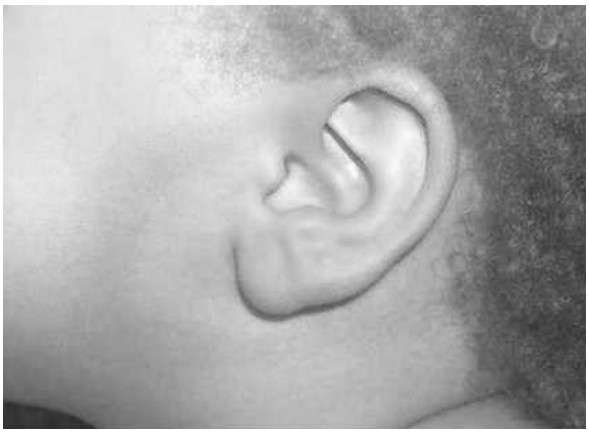

Fig. 5. The pathognomonic sign associated with Lyme borelliosis, which occurs early at the site of the tick bite in the form of a gradually extending erythematous lesion termed erythema migrans.

Collection and preservation of samples

When culture of borreliae is being attempted, collection and preparation of specimens under aseptic conditions is essential. Body fluids should be transported without any additives, and biopsy specimens should be placed in a small quantity of sterile saline or suitable culture medium [92]. Samples should reach the laboratory as quickly as possible after collection (within 2–4 h). Before specimens are collected and transported, the laboratory should be contacted so that details related to methodology can be agreed upon. If postal transport is unavoidable, overnight delivery is recommended.

Specimens used for diagnosis of Lyme borreliosis

Serum is among the samples of choice (Table 10), as serology is used widely for the diagnosis of

Table 10. Specimen types used for the diagnosis of Lyme disease [92]

	Specimens for	
Clinical manifestation	**Direct pathogen detection**	**Antibody detection**
Stage I (early/localised) (days to weeks after tick bite)		
Erythema migrans	Skin biopsy specimen	Serum
Stage II (early/disseminated) (weeks to months after tick bite)		
Multiple erythemata	Skin biopsy specimen	Serum
Borrelial lymphocytoma	Skin biopsy specimen	Serum
Lyme carditis	Endomyocardial biopsy	Serum
Neuroborreliosis	CSF	Paired serum/CSF[a]
Ophthalmoborreliosis		Serum
Stage III (late/persistent) (months to years after tick bite)		
Arthritis	Synovial fluid, synovial biopsy	Serum
Acrodermatitis chronica atrophicans	Skin biopsy specimen	Serum
Chronic neuroborreliosis	CSF	Paired serum/CSF[a]

[a]Paired serum and CSF specimens are obtained on the same day for CSF antibody/serum antibody index determination.
CSF, cerebrospinal fluid.

Lyme disease. In suspected cases of neuroborreliosis, cerebrospinal fluid (CSF) and a concurrent serum sample should be obtained for determination of cell counts/differentials, protein content and intrathecal immunoglobulin synthesis. Laboratory confirmation of neuroborreliosis is achieved most readily by demonstration of borrelia-specific, intrathecal antibody production. CSF can also be used for culture or PCR; however, detection rates with these approaches are only 20% [93]. PCR investigation of synovial fluid or, preferably, a synovial biopsy specimen, is recommended for patients in whom Lyme arthritis is suspected [94,95]. The high protein permeability of the synovium means that synovial fluid and serum display roughly equivalent antibody titres. Skin biopsy specimens are the best material from which to attempt isolation of *B. burgdorferi*, and spirochaetes can be isolated from most untreated patients with early and late skin borreliosis (EM and ACA, respectively). The success rate for culture is highest for patients with EM (up to 86%), with biopsy specimens taken close (within 4 mm) to the expanding border of the lesion [96]. Without treatment, *B. burgdorferi* can persist for long periods of time in the skin, as shown by isolation of the organism from a 10-year-old acrodermatitis lesion [97]. If available, other biopsy materials from the heart, brain and eye can be investigated for detection of borreliae. Ticks are often tested for borreliae as part of epidemiological studies to assess the risk for human populations in a given geographical area (see concluding section of these guidelines).

Table 11. Humoral immune response in Lyme disease [182]

Stages	Immune response
I: Early/localised infection	Initial IgM response (evolving from approximately the third week after infection), followed by IgG antibodies (evolving from approximately the sixth week after infection). Patients are often seronegative. IgM antibodies are sometimes undetectable in cases of short duration
II: Early/disseminated infection	Immune response similar to stage I, but with IgG antibodies now predominant. Intrathecal antibody production in neuroborreliosis
III: Late/persistent infection	Usually high IgG antibody titres in arthritis and acrodermatitis. IgM antibodies usually undetectable. Intrathecal antibody production in chronic neuroborreliosis

Diagnostic tools

Serology

Specific antibodies are often not detectable in the primary stage of the infection with the use of currently available test methods, but IgG antibodies are frequently present in the second stage (Table 11). Most cases of neuroborreliosis additionally show intrathecally produced antibodies in CSF [98,99]. Patients with stage III illness typically have high IgG titres, especially if suffering from arthritis or ACA [100,101]. However, in late manifestations, IgM can be detected in only some patients (10–40%, depending on the test method used) [102]. Intrathecal antibodies are produced in cases of chronic neuroborreliosis, and detection of these is the most important microbiological diagnostic criterion, as direct detection methods may not succeed and IgM antibodies are regularly not found.

Although assays allowing detection of a specific immune response against *B. burgdorferi* are used widely, a positive antibody assay in the absence of the corresponding clinical symptoms is not sufficient for the diagnosis of Lyme borreliosis. Serological diagnosis can be impaired by cross-reactivity of borrelial antigens with antigens of other pathogens, by the delayed appearance, or even permanent lack, of a measurable immune response in the early stage of Lyme borreliosis, and by the absence of a marker of disease activity in late manifestations.

Many insufficiently evaluated commercial kits for the detection of antibodies against *B. burgdorferi* are currently available, with different serological methods and test antigens being used. These kits have heterogeneous diagnostic properties that are often unknown to both the user (laboratory) and the requesting clinician. Although a complement fixation test is available commercially, it is not recommended for the diagnosis of Lyme borreliosis. The underlying principle of the complement fixation test suggests that this method is not sensitive enough to serve as a screening assay. The indirect immunofluorescence antibody assay can also be used for detection of specific IgG and IgM antibodies. If sera are preabsorbed with Reiter treponemes, the validity of the indirect immunofluorescence antibody assay corresponds to that of a second-generation assay, as improved specificity is achieved compared with the test with non-absorbed sera. However, in the early stage of infection, absorption with Reiter treponemes may in some cases entail a loss of diagnostic sensitivity. Enzyme

immunoassay allows differentiation between the immunoglobulin classes of antibodies. The distinction between IgM and IgG is of diagnostic relevance. Commercial tests for the detection of IgA antibodies are available, but as there are no convincing data to demonstrate that measurement of this parameter provides clinically relevant information, these assays cannot be recommended. Overall, second- or third-generation enzyme immunoassays are more likely to provide diagnostically useful results than other serological screening approaches for Lyme disease.

Western blotting is used currently as a confirmatory assay in the serodiagnosis of Lyme borreliosis, but is usually only employed following a positive screening assay. In order to interpret the individual reactivity patterns of patient sera correctly, it is necessary to be familiar with the stage-dependent diagnostic significance of immunogenic proteins. For example, in the diagnosis of early Lyme borreliosis, the intensity of at least some of the immunoblot bands (p41, OspC) must be taken into account. Therefore, each assay requires precise internal controls that allow semi-quantitative evaluation of blot intensities. Whole-cell antigen assays are available, in which electrophoretically separated whole-cell lysates from borreliae are employed, with the borrelial proteins transferred to blot membranes. Suppliers also offer recombinant antigen assays in which, analogously, selected genetically engineered *Borrelia* proteins are used. The advantage of whole-cell lysate Western blots resides in the fact that a greater number of immunoreactive proteins can be detected. The main disadvantage is the difficulty of distinguishing specific from cross-reactive bands, and examination by an expert is therefore required. In recombinant Western blots, specific proteins that can be assigned unambiguously to blot bands are selected. Another advantage is that homologous proteins of different species can be combined [103–105]. Recombinant blots have long been considered to have poorer sensitivity than whole-cell lysate blots (provided that the latter are performed under standardised conditions with a strain that expresses the diagnostically relevant immunodominant proteins). However, the sensitivity of the recombinant Western blot has now been improved significantly by the addition of recombinant VlsE, a protein that is primarily expressed only *in vivo* [106]. The decisive criterion qualifying the Western blot as a suitable confirmatory assay is thus reliable identification of the immunoreactive bands. Numerous tests, using antigens of various strains or genospecies of *B. burgdorferi* sl, are available commercially. In principle, only tests allowing unequivocal distinction of diagnostically relevant bands should be used. A further prerequisite is reliable specification of these bands by the manufacturer, i.e., based on their identification with monoclonal antibodies (Fig. 6).

There is no single optimum test for the serodiagnosis of Lyme borreliosis. The existing methods must therefore be combined logically in order to achieve the highest possible diagnostic efficiency. A stepwise diagnostic protocol is recommended in which a screening assay, as defined above, is used as the first step (Fig. 7). If the result of the screening assay is positive or borderline, a confirmatory Western blot should be used. A two-tier protocol has also been recommended in the USA

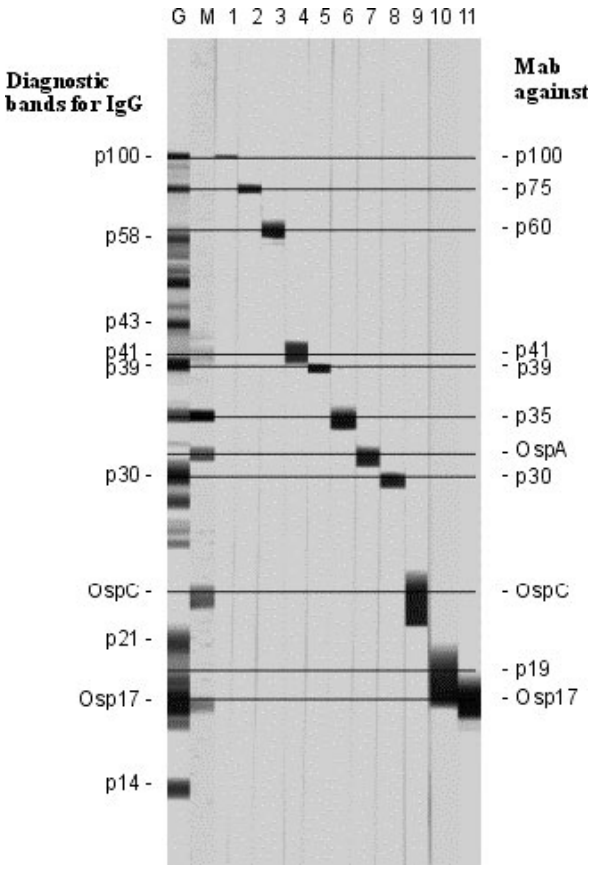

Fig. 6. Standardisation of immunoblots with reference sera. G, IgG; M, IgM; 1–11, monoclonal antibodies. The antigen used was *Borellia afzelii* (Pko Strain) [92].

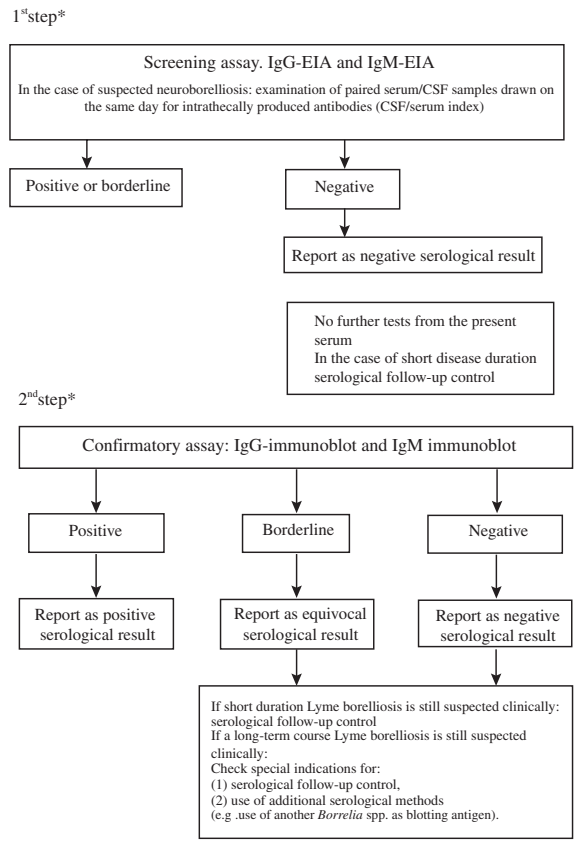

Fig. 7. Stepwise serodiagnosis of Lyme borelliosis [92]. IFA, immunofluorescent assay; IHA, immunohaemagglutination assay.

by the Centers for Disease Control (CDC) [107]. Such an approach, the aim of which is to increase the pre-test probability, and thus the predictive value, of a positive result with each step, can only work if the tests are performed in succession. Following a positive screening assay, the possibility of cross-reactivity with *Treponema pallidum* must be considered, and this can be clarified by means of a *T. pallidum* haemagglutination assay.

Cultivation

This method should be attempted only in certain clinical circumstances (Table 10) and should be carried out by reference laboratories. *B. burgdorferi* can be cultivated in an artificial semi-solid medium [108] (modified Kelly's medium, e.g., MKP medium, described by Preac-Mursic *et al.* [109]), but as this approach is time-consuming and labour-intensive, it is rarely used for the routine diagnosis of Lyme borreliosis. Attempted isolation of borreliae is potentially of most diagnostic value in patients with suggestive clinical presentations, but no detectable antibody response (seronegative Lyme borreliosis), e.g., atypical EM, suspected neuroborreliosis without detection of intrathecal antibodies, or suspected Lyme borreliosis combined with immune deficiencies. Such seronegative cases are often characterised by a short duration of disease. Occasionally, culture attempts may be justified in seropositive patients, e.g., patients with dermatological disease manifestations that cannot be attributed unambiguously to Lyme borreliosis.

Molecular detection

There are numerous publications on the application of PCR-based methods to the diagnosis of Lyme disease [92,110]. However, neither a standardised method for DNA isolation nor a routine PCR method, validated through several clinical-diagnostic studies and carried out in standardised conditions, has been established to date. Furthermore, since a negative PCR result cannot be considered to be indicative of an absence of borreliae, the use of PCR-based methods alone cannot currently be recommended for routine laboratories [92,111]. However, in some circumstances, molecular methods can be valuable additional diagnostic tools, particularly when used in specialised laboratories with sufficient experience and the capacity to sequence amplification products. As mentioned above, no standardised method for the preparation of DNA templates for diagnostic PCRs has been adopted. Widely different lysis and DNA extraction techniques have been used in varying combinations, and no reliable statement on the quality of the different methods can be made at the present time. Therefore, comparison of molecular diagnostic results obtained by different laboratories is not yet possible.

In the PCR-based assays described to date, a variety of different borrelial DNA fragments have been targeted, including *ospA* and *ospB*, genes for the flagellar protein or p66 (clone 2H1), 16S rRNA genes, or the 5S/23S rRNA intergenic spacer region. Use of more than one target sequence is recommended. With the exception of the confirmed high detection rates from synovial fluid or synovial biopsy specimens in Lyme arthritis, the diagnostic sensitivity of molecular methods is not significantly greater than that of culture

Table 12. Sensitivity of direct pathogen detection methods for Lyme borreliosis [92]

Specimen	Sensitivity
Skin (erythema migrans, acrodermatitis)	50–70% when using culture or PCR
CSF (neuroborreliosis, stage II)	10–30% when using culture or PCR
Synovial fluid[a] (Lyme arthritis)	50–70% when using PCR (culture is very rarely positive)

[a]Higher sensitivity in synovial tissue than in synovial fluid.

(Table 12). For example, when skin biopsy specimens and CSF have been used as sources of template DNA, molecular methods have been found to have diagnostic sensitivities of 60% and 25%, respectively [93,112].

More information on the microbiological diagnosis of Lyme borreliosis can be found in recent reviews [92,110] or on the internet (http://www.dghm.org/red/index.html?cname = MIQ, and alpha1.mpk.med.uni-muenchen.de/bak/nrz-borrelia/miqlyme/Frame-miq-Microbiological.html).

Risk assessments

Criteria for the diagnostic assessment of patients with suspected Lyme borreliosis were published in 1996 by the European Union Concerted Action on Lyme Borreliosis (EUCALB) [113] and are summarised in Table 13.

TULARAEMIA

Organism and vectors

Tularaemia is a zoonotic disease caused by *Francisella tularensis*, a small, aerobic, pleiomorphic, catalase-positive, Gram-negative, facultative-intracellular coccobacillus that grows poorly on most ordinary culture media [114,115]. There are three main subspecies or biovars. *F. tularensis* subsp. *tularensis* (or *nearctica* or biovar A) is the most virulent species; it is the predominant subspecies in north America and has been reported recently from central Europe [116]. *F. tularensis* subsp. *palaearctica* (or *holarctica* or biovar B) is a less virulent biovar that occurs in Europe, Asia and north America, while *F. tularensis* subsp. *novicida* (biovar C) is found in north America and has a low virulence [115,117]. Tularaemia occurs both sporadically and in epidemics, and has been reported throughout Europe, with the exception of the UK.

The epidemiology of tularaemia is poorly understood, with evidence of infection having been reported in >250 animal species, including mammals (particularly hares, rabbits and rodents), birds, fish, amphibians, arthropods and parasites [118]. Multiple routes of transmission have been reported. The routes of transmission to man include tick bite (by *Dermacentor* spp. and *Ixodes* spp.), bites of infected mammals, deer flies or mosquitoes, direct contact with infected animals (even through intact skin), inhalation [118–120], ingestion of contaminated water or food [121–124], and occupational exposure [120,123,125]. Individuals with an increased risk of tularemia are laboratory workers, farmers, veterinarians, hunters or trappers, and cooks or meat handlers [117]. Most cases are associated with tick exposure and occur from May to July (65%), whereas those associated with exposure to animals (rabbits, rodents) occur in November or December (33%) and from May to July (22%) [121,125–129].

Clinical and standard laboratory features

The disease varies in severity, depending on the route of inoculation, the aetiological biovar, the number of organs involved and the immune status of the host. The incubation period is 1–21 days (mean, 4–5 days). Classically, tularaemia is a severe illness characterised by a sudden onset of fever, chills, headache, lymphadenopathy, myalgia, malaise and fatigue [117,121,125–127]. Pulse–temperature dissociation is a classic finding (42%) [125]. Six classic types of tularaemia have been described, namely the ulceroglandular (49–75%) (Fig. 8) [117,121,125–127], glandular (12.5–15.9%), oculoglandular (0.5–3.5%), oropharyngeal (0–9.2%), typhoidal (8–14%) and pneumonic (1.5 to >50%) forms.

Differential diagnoses are presented in Table 14. The results of non-specific diagnostic tests are often unremarkable. The leukocyte count is usually normal or slightly elevated, and atypical lymphocytes are sometimes present [130,131]. Serum hepatic aminotransferase levels are often elevated. Urinary analysis may reveal pyuria in some (25%) cases [132]. The results of CSF examination are normal in nearly 70% of cases. When pathology is present, CSF examination may reveal an increased number of mononuclear cells, an elevated protein level, and a decreased glucose level [125,130,131].

Table 13. Criteria for the diagnosis of Lyme disease based on the EUCALB clinical case definitions for Lyme borreliosis [113,182]

Disease	Major clinical criteria (essential)	Minor clinical criteria (supporting)	Laboratory evidence[a] Major criteria (essential)	Laboratory evidence[a] Minor criteria (supporting)
Erythema (chronicum) migrans (EM)	Expanding reddish or bluish-red patch, often with central clearing; advancing edge, typically distinct, intensely coloured, yet not markedly elevated; localised around the point of inoculation or (rarely) disseminated	Preceding tick bite at the same localisation	None	Detection of *B. burgdorferi* by culture or NAT from a skin biopsy specimen, significant rise in specific antibodies[b] or detection of specific IgM
Borrelial lymphocytoma (rare manifestation)	Painless bluish-red nodule or plaque, usually localised at the ear lobe, ear helix, nipple or scrotum, more frequent in children (especially on ear) than in adults	EM, present or preceding	Detection of serum antibodies against *B. burgdorferi* (IgG and/or IgM) *or* significant rise in the IgG antibody titre against *B. burgdorferi*	Histological evidence of a B-cell pseudolymphocytoma
Acrodermatitis chronica atrophicans	Long-standing red or bluish-red lesion, usually on the extensor surfaces of the extremities. Possible initial doughy skin swelling, the lesion becoming eventually atrophic. Possible skin induration over bony prominences	High concentration of specific serum IgG antibodies *or* Characteristic histological evidence for and detection of *B. burgdorferi* by culture *or* NAT from a skin biopsy specimen		
Early neuroborreliosis	Painful meningo-radiculoneuritis with or without facial palsy or paresis of other cranial nerves (Garin–Bujadoux–Bannwarth syndrome). In children, most frequently meningitis, isolated monolateral (occasionally bilateral) facial palsy or paresis of other cranial nerves	EM, present or preceding	Lymphocytic pleocytosis in CSF[c] *and* Detection of intrathecally produced specific antibodies[d] *or* Detection of *B. burgdorferi* by culture or NAT from CSF	Specific oligoclonal bands in CSF; significant rise in specific serum antibodies
Chronic neuroborreliosis (very rare)	Long-standing encephalitis, meningoencephalitis, encephalomyelitis, radiculomyelitis		Detection of intrathecally produced specific antibodies *and* Lymphocytic pleocytosis in CSF[c] *and* Detection of serum antibodies against *B. burgdorferi*	Specific oligoclonal bands in CSF
Lyme carditis	Acute onset of atrioventricular block (degrees II and III), rhythm disturbances, sometimes myocarditis or or pancarditis (tick exposure is assumed irrespective of whether or not a tick bite has occurred)	EM, present or preceding	Detection of serum IgG and IgM antibodies against *B. burgdorferi or* Detection of a significant rise in IgG antibodies against *B. burgdorferi or* Detection of *B. burgdorferi* by culture from a heart biopsy specimen	
Lyme arthritis	1. Recurrent brief attacks of objective joint swelling in one or a few large joints, occasionally progressing to chronic arthritis *and* Exclusion of other causes for the clinical symptoms *or* 2. Recurrent brief attacks of objective joint swelling in one or a few large joints, occasionally progressing to chronic arthritis without history of a confirmed clinical manifestation of Lyme disease		1. High concentration of specific serum serum IgG antibodies *or* Only in conjunction with the clinical major criterion no. 2 2. High concentration of specific serum IgG antibodies *and* Detection of *B. burgdorferi* by NAT *or* culture from synovial fluid and/or synovial fluid biopsy specimen	

EM, erythema migrans; NAT, nucleic amplification technique; CSF, cerebrospinal fluid.
[a]When considering the laboratory findings, the method-specific indications listed in the text should be observed.
[b]The levels of specific serum antibodies may increase as a reaction to disease progress or shortly after the beginning of antibiotic treatment; they may decrease when the disease is resolved.
[c]CSF pleocytosis may be absent if only a short time (several days) has elapsed since the onset of the disease, or in children with isolated facial palsy.
[d]Intrathecally-produced antibodies may not be detectable for a short time after the onset of symptoms.

Collection and preservation of sample

All samples from patients with suspected tularaemia must be processed in Category 3 Safety Level laboratories. Biopsy specimens of skin lesions (particularly an inoculation eschar) or lymph node biopsy specimens, as well as blood (heparin-containing vial), pharyngeal or sputum samples, can be used for culture, but must be processed immediately or stored at −80°C for culture. Samples for PCR (with blood obtained on EDTA) may be maintained at −20°C. For serological diagnosis, a blood sample should be collected early in the course of the disease, and again after 2–4 weeks.

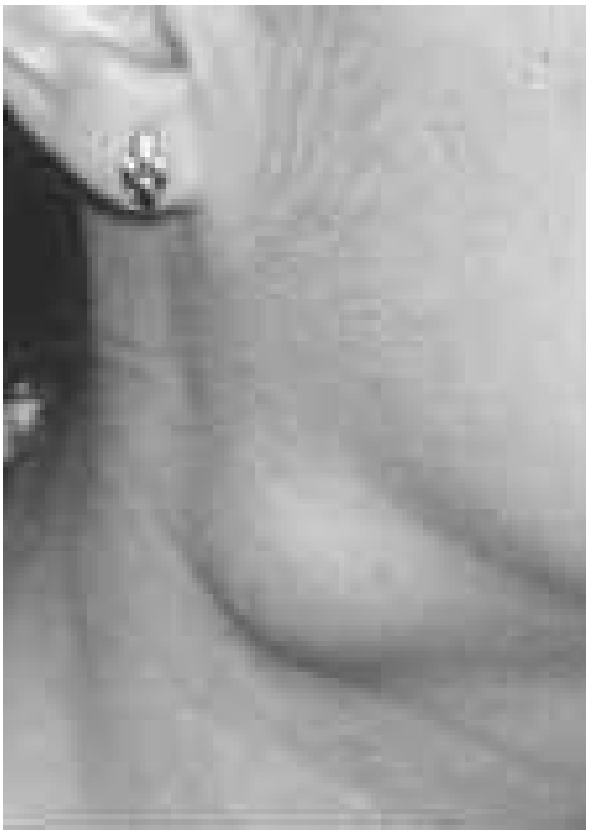

Fig. 8. Tularaemia: detail of the neck showing a typical lymphadenopathy.

Table 14. Differential diagnosis for clinical manifestations of tularaemia

Manifestation	Disease (causative organism if applicable)
Ulceroglandular	Anthrax (*Bacillus anthracis*)
	Cat-scratch disease (*Bartonella henselae*)
Glandular	*Dermacentor* bite rickettsia infection
	TIBOLA (*R. slovaca*)
	Herpes simplex virus infection
	Lymphogranuloma venereum (*Chlamydia trachomatis*)
	Other non-tuberculous mycobacterial infection
	Plague (*Yersinia pestis*)
	Pyogenic bacterial infection
	Rat-bite fever (*Streptobacillus moniliformis*, *Spirillum minor*)
	Sporotrichosis (*Sporothrix schenckii*)
	Syphilis (*Treponema pallidum*)
	Toxoplasmosis (*Toxoplasma gondii*)
	Tuberculosis (*Mycobacterium tuberculosis*)
Oculoglandular	Cat-scratch disease (*Bartonella henselae*)
	Herpes simplex virus infection
	Pyogenic bacterial infection
	Syphilis (*Treponema pallidum*)
Oropharyngeal	Actinomycosis (*Actinomyces* sp.)
	Diphtheria (*Corynebacterium diphtheriae*)
	Extensive necrotic cervical adenopathy
	Infectious mononucleosis (Epstein–Barr virus)
	Lymphoma
	Streptococcal angina (*Streptococcus* sp.)
	Tonsillopharyngitis
	Tuberculosis (*Mycobacterium tuberculosis*)
Typhoidal	Acute gastroenteritis
	Anthrax (*Bacillus anthracis*)
	Brucellosis (*Brucella melitensis*)
	Endocarditis
	Legionnaires' disease (*Legionella pneumophila*)
	Malaria (*Plasmodium* sp.)
	Q fever (*Coxiella burnetii*)
	Rickettsioses
	Typhoid fever (*Salmonella typhi*)
Pneumonic	Anthrax (*Bacillus anthracis*)
	Brucellosis (*Brucella melitensis*)
	Legionnaires' disease (*Legionella pneumophila*)
	Mycoplasmal pneumonia (*Mycoplasma pneumoniae*)
	Psittacosis (*Chlamydia psittaci*)
	Q fever (*Coxiella burnetii*)
	Tuberculosis (*Mycobacterium tuberculosis*)

TIBOLA, tick-borne lymphadenopathy.

Diagnostic tools

Direct examination of sputum is usually not helpful [125]. A false-positive direct fluorescent antibody stain for *Legionella* on a bronchoscopy specimen has been reported [133]. Pleural fluid, when present, is serosanguineous, with a predominance of lymphocytes, and is usually negative following Gram's stain. A high pleural fluid concentration of adenosine deaminase has been described in a tularaemia pleural effusion [134]. If a biopsy specimen is taken from a lymph node or abscess, histopathological examination reveals a combination of abscess and caseous-type necrosis, with infiltration of numerous polymorphonuclear leukocytes [130,135].

Serology

Serology remains the most useful technique for the diagnosis of tularaemia [136–139]. Various techniques have been used, including the standard tube agglutination assay, the microagglutination test, haemagglutination, immunofluorescence and ELISA. With the standard tube agglutination assay, specific antibodies are usually detected 2 weeks after the onset of clinical manifestations, and reach their maximum titres after 3–4 weeks [140]. A single antibody titre of ≥1:160 is considered to be significant. Antibodies are usually detectable within 2 weeks of the onset of symptoms, and peak after 4–5 weeks [114]. The microagglutination assay has been reported to be more sensitive than the standard tube agglutination assay [136]. The cut-off titres have not been well-defined with other serological techniques, and may vary according to geographical region, with higher cut-off titres being required in endemic than in non-endemic areas. Since antibodies may persist for prolonged periods [141], a diagnosis of tularaemia cannot be established firmly on the basis of a single serum titre. A four-fold increase in antibody titre, or a seroconversion between two sera, with the first being collected at the onset of

clinical manifestations and the second during convalescence, are much more specific criteria. Cross-reactions, especially with IgM-type antibodies, have been described between *F. tularensis* and *Francisella philomiragia* [142], *Brucella* spp., *Proteus* OX19 and *Yersinia pestis* [114]. Consequently, the specific *F. tularensis* subspecies of the infecting organism cannot be determined with serology.

Cultivation

A diagnosis of tularaemia has rarely been established by culture, because isolation of the pathogen is both hazardous and insensitive [125]. According to Taylor *et al.* [143], *F. tularensis* is isolated in only *c.* 10% of cases. Isolation is more frequent from immunocompromised patients [144] and from patients with pneumonia (28%) [145]. *F. tularensis* is highly infectious by the aerosol route, and the pathogen should be manipulated in a Biosafety Level 3 laboratory [146]. Vaccination of laboratory personnel working with *F. tularensis* has been recommended [147].

F. tularensis is a fastidious bacterium, but recovery is possible on cystine- or cysteine-enriched medium after incubation for 2–4 days at 37°C in CO_2 5% v/v. Historically, cystine glucose blood agar has been the most widely used medium for isolation of *F. tularensis* [117]. Growth may also be obtained on commercially available chocolate agar supplemented with IsoVitalex (BBL, Cockeysville, MD, USA) or PolyVitex (bioMérieux, Marcy l'Etoile, France), as well as in automated blood culture systems [148–150]. However, routine blood cultures usually (87.5–95%) remain sterile [125,145]. Colonies growing on agar are blue–grey, round, smooth and slightly mucoid, with a small zone of α-haemolysis on blood agar. The isolation of *F. tularensis* with the use of a cell culture system has recently been reported [151]. The cell culture system has several advantages over the axenic medium. It is a more versatile system, allowing isolation of a number of strict and facultative intracellular pathogens that may be responsible for cutaneous lesions and/or lymphadenopathy, and it is safer, as vials can be closed hermetically after inoculation of a clinical sample.

Molecular detection

Several diferent PCR-based methods have been applied to the diagnosis of tularaemia [152–154]. Primers have been used that specifically amplify fragments of a number of genes, including 16S rDNA [152] and a gene encoding a 17-kDa lipoprotein [153,154]. These techniques allow the presence of *F. tularensis* DNA to be demonstrated without requiring the hazardous isolation of this pathogen. However, molecular techniques are more costly than all of the other diagnostic techniques available.

Species and subspecies identification

Several biochemical tests have been described that allow differentiation of *F. tularensis* subspecies and biovars [117]. An agglutination assay in which specific sera are used has also been used for identification of *F. tularensis*, but does not allow identification of the biovar [155]. More accurate differentiation is best achieved with molecular biological techniques. Forsman *et al.* [156,157] and Sandstrom *et al.* [158] reported differentiation of *F. tularensis* subsp. *tularensis* from *F. tularensis* subsp. *palearctica* on the basis of 16S rDNA sequences. Modern PCR-based epidemiological methods have been applied successfully to the characterisation of *F. tularensis* strains, including repetitive extragenic palindromic element PCR, enterobacterial repetitive intergenic consensus sequence PCR and random amplified polymorphic DNA [159]. More recently, amplification of short-sequence tandem repeats has also been reported [160]. These different approaches are now facilitated by the sequencing of the entire *F. tularensis* subsp. *tularensis* Schu S4 genome [161], although recent concerns about the possible use of *F. tularensis* for bioterrorist purposes has resulted in restricted access to the data.

Case definition

Table 15 presents the laboratory criteria defined by the CDC for the serological diagnosis of tularaemia [162].

TICK-BORNE RELAPSING FEVER

Organism and vectors

Relapsing fever, an infectious disease with a sudden onset of high fever with septicaemic signs and symptoms, is characterised by the occurrence of one or more spells of fever after the subsidence

Table 15. Criteria used by the Centers for Disease Control for case definition of tularaemia [162]

Clinical description
An illness characterised by several distinct forms, including the following:
Ulceroglandular (cutaneous ulcer with regional lymphadenopathy)
Glandular (regional lymphadenopathy with no ulcer)
Oculoglandular (conjunctivitis with preauricular lymphadenopathy)
Oropharyngeal (stomatitis or pharyngitis or tonsillitis and cervical lymphadenopathy)
Intestinal (intestinal pain, vomiting, and diarrhoea)
Pneumonic (primary pleuropulmonary disease)
Typhoidal (febrile illness without early localising signs and symptoms)
Clinical diagnosis is supported by evidence or history of a tick or deer fly bite, exposure to tissues of a mammalian host of *Francisella tularensis*, or exposure to potentially contaminated water.
Laboratory criteria for diagnosis
Presumptive
Elevated serum antibody titre(s) to *F. tularensis* antigen (without documented fourfold or greater change) in a patient with no history of tularemia vaccination or Detection of *F. tularensis* in a clinical specimen by fluorescent assay
Confirmatory
Isolation of *F. tularensis* in a clinical specimen *or*
Four-fold or greater change in serum antibody titre to *F. tularensis* antigen
Case classification
Probable
A clinically compatible case with laboratory results indicative of presumptive infection
Confirmed
A clinically compatible case with confirmatory laboratory results

of the primary febrile attack. Tick-borne relapsing fever (TBRF) is encountered throughout the world and is caused by at least 16 different *Borrelia* spp. that are harboured in soft ticks of the genus *Ornithodoros* (*Alectorobius*). TBRF is a serious disease with a mortality rate, if untreated, of up to 5%**.** Each *Borrelia* sp. associated with relapsing fever appears to be specific to its tick vector. In southern Europe, recurrent fever caused by *Borrelia hispanica* has been reported sporadically in the Iberian Peninsula, Greece and Cyprus [163,164]. The organism is transmitted by *Ornithodoros erraticus*, a species that usually parasitises rodents and small mammals in their nests. A novel *Borrelia* sp. associated with TBRF was isolated and characterised in Spain in 1996 [165], while a new species that is related closely to the TBRF-associated species *Borrelia miyamoto* has been detected recently in Sweden in two of 301 *Ixodes* ticks [166]. Many rodents and small mammals serve as natural reservoirs, and borreliae also persist for many years in their long-lived tick vectors.

Clinical and standard laboratory features

TBRF is characterised by acute onset of high fever with chills, headache, myalgia, arthralgia and coughing [92]. Haemorrhage (rarely severe), iritis or iridocyclitis, hepatomegaly or splenomegaly may also occur, and abdominal pain, nausea, vomiting, diarrhoea and photophobia are common in African cases. A rash may occur at the end of the first febrile episode, and neurological findings are frequent and may be severe [92]. Jaundice occurs in 7% of patients, and the case fatality rate is *c.* 2–5%. In general, the primary episode lasts about 3 days and is followed by a second, shorter and milder, episode after a further 7 days. Thereafter, one or more subsequent episodes may occur at intervals of several days, with each episode lasting about 2 days.

Collection and preservation of samples

Blood is the specimen of choice. During febrile attacks, large numbers of borreliae are detected easily by dark-field or bright-field microscopy of a wet-mounted blood sample or a stained blood smear. The level of spirochetaemia may reach 10^6–10^8 cells/mL. Blood from acutely ill patients is also the best source for confirmation by culture. However, the spirochetaemia diminishes with each successive relapse, and visualisation or culture of borreliae is less successful during afebrile periods. Serum is suitable for detection of indirect (antibody) evidence of exposure to borreliae. Specific antibody detection tests have recently been employed to confirm cases of relapsing fever.

Diagnostic tools

Examination of blood

Diagnosis is established by the demonstration of borreliae in the peripheral blood of febrile patients. This test has a sensitivity of 70% when blood smears are examined by dark-field microscopy or stained with Giemsa or Wright stain. Recently, a quantitative buffy coat analysis method has been described as a very sensitive and specific technique for the detection of borreliae in blood [167].

Serology

Serological assays are not readily available, and their diagnostic value is limited because of the antigenic variation shown by the TBRF borreliae [92].

Cultivation

Borrelia dutonii, the agent causing East African TBRF, has been propagated successfully in axenic culture. The spirochaetes are cultivated at 33°C on

BSK II growth medium with added bovine serum albumin fraction V (Sigma, St Louis, MO, USA) without antibiotics, and then maintained by serial passage every 2–3 days [168].

Molecular detection

Nested PCRs targeting fragments of a 16S rRNA-encoding gene or the flagellin gene have been described for the detection of TBRF borreliae in human blood and in ticks [165,166].

TICKS AS TOOLS IN THE DIAGNOSIS OF TICK-BORNE DISEASES

Ticks themselves can be used as tools for the diagnosis of tick-borne bacterial disease in patients. The different steps include the identification of the tick(s) to the species level, and the detection or isolation of infectious disease agents. This strategy is summarised in Table 16.

Identification of the tick

Ticks can be identified to the family, genus and species level with use of the numerous taxonomic keys that are available for ticks from different regions of the world [2]. However, tick identification, particularly when larvae are being studied, can be difficult and may require entomological skills. Molecular methods are currently being developed to identify ticks, and it is expected that such methods will be used more widely in the future, particularly for the differentiation of closely related species [169].

Preservation of the tick

Depending on the studies that are to be performed, ticks can be kept alive in the laboratory at a relative humidity of 85% and, depending on the species, at between 15°C and 25°C. Alternatively, ticks can be kept at 0–5°C in the dark and at 95% relative humidity for up to 3 months before they require feeding. However, keeping ticks alive is a task for specialised laboratories, as their viability is not necessary for pathogen detection. Indeed, freezing ticks to −80°C may be the best storage option, as this will maintain many infecting pathogens in a culturable state. Ticks preserved for long periods of time can still be tested for pathogens with molecular methods, and these approaches can also be used on recently dead ticks, even if they are desiccated or damaged.

Table 16. Strategy for detecting and/or isolating bacteria from ticks [2]

1. Identification of the ticks to the species level
2. Detection of bacteria in ticks with the use of staining tests (haemolymph for viable ticks; salivary glands if ticks were frozen), or PCR-based methods (using one-half of the tick, the other half being kept frozen). PCR may also be done using only ticks that stain positive
3. Sequencing of the amplified PCR fragment and comparison with sequences available in sequence databases
4. If there is 100% similarity between the test sequence and the corresponding sequence of a known organism, the presumptive identification is confirmed
5. If the test sequence appears to be different from all corresponding sequences available, the organism is probably a new strain and should be isolated and characterised from the stored frozen part of the tick

Detection of bacteria in ticks

The haemolymph test is performed on live ticks by amputating the distal portion of a tick leg. Haemolymph that drains from the amputation site is smeared onto a microscope slide, stained and examined for the presence of bacteria. Ticks from which leg segments have been amputated can be kept alive and used for subsequent haemolymph tests or other experiments. Impression smears may be made of salivary glands or ovaries removed from dissected ticks, or the organs may be used for histology. Immunodetection methods may also be used to detect organisms in haemolymph or organ smears. Slides are air-dried and fixed in acetone before being treated with polyclonal or monoclonal antibodies conjugated with immunofluorescent labels.

Isolation and cultivation of bacteria from ticks

Currently, rickettsiae are usually isolated with the use of cell culture systems, with the centrifugation shell vial technique with HEL fibroblasts being the method of choice in most reference laboratories. A drop of haemolymph from a surface-sterilised tick is inoculated on to a confluent monolayer in the shell vials. Dead ticks may also be used after surface disinfection. The tick is macerated in 1 mL of cell culture medium and then inoculated into shell vials or on the specialised BSK II growth medium for borreliae [2].

Molecular detection

Identification strategies based on the DNA sequences of a number of genes have been described for tick-associated bacteria, and also for

viruses and parasites. These approaches have been reviewed in some detail previously [170,171].

CONCLUSIONS

It can be concluded that the aetiological diagnosis of tick-transmitted diseases is often difficult and relies on trained personnel in specialised laboratories using very specific tools. Interpretation of laboratory data is very important in order to establish the diagnosis. The guidelines described above are aimed at helping clinicians and microbiologists to diagnose infection transmitted by tick bites and at providing the scientific and medical community with comprehensive information on these infectious diseases.

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