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Review Article

Contemporary diagnostics for medically relevant fastidious microorganisms belonging to the genera *Anaplasma*, *Bartonella*, *Coxiella*, *Orientia* and *Rickettsia*

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One sentence summary: Some zoonotic and vector-transmitted bacteria are strongly adapted to the infected host hindering pathogen cultivation and further identification: an overview of the current and future perspectives for diagnosis of medically relevant fastidious Gram-negative bacteria.
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Abstract

Many of the human infectious pathogens—especially the zoonotic or vector-borne bacteria—are fastidious organisms that are difficult to cultivate because of their strong adaption to the infected host culminating in their near-complete physiological dependence on this environment. These bacterial species exhibit reduced multiplication rates once they are removed from their optimal ecological niche. This fact complicates the laboratory diagnosis of the disease and hinders the detection and further characterization of the underlying organisms, e.g. at the level of their resistance to antibiotics due to their slow growth. Here, we describe the current state of microbiological diagnostics for five genera of human pathogens with a fastidious laboratory lifestyle. For *Anaplasma* spp., *Bartonella* spp., *Coxiella burnetii*, *Orientia* spp. and *Rickettsia* spp., we will summarize the existing diagnostic protocols, the specific limitations for implementation of novel diagnostic approaches and the need for further optimization or expansion of the diagnostic armamentarium. We will reflect upon the diagnostic opportunities provided by new technologies including mass spectrometry and next-generation nucleic acid sequencing. Finally, we will review the (im)possibilities of rapidly developing new *in vitro* diagnostic tools for diseases of which the causative agents are fastidiously growing and therefore hard to detect.

Keywords: slow-growing bacteria, *in vitro* diagnostics, serology, mass spectrometry, PCR diagnostics, whole-genome sequencing

Introduction

Infections have a major impact on daily life and are often subject to media attention. Some infectious agents have even reached a 'prominent celebrity state' and are well known to the general public and physicians. Such diseases have a major effect on human health and strongly impact health and the economic prosperity of societies (Serra-Burriel et al. 2020). SARS-CoV-2 is a contemporary viral example but also the influenza virus and some of the diarrhea-inducing agents (Norovirus, *Salmonella* spp., *Escherichia coli*) are commonly acknowledged by politicians, media and people. Still, a large variety of disease-causing agents does not share this level of celebrity or level of awareness. Such diseases can be prevalent on a global or regional level, can cause significant morbidity and mortality, and may suffer from underdeveloped diagnostic and therapeutic strategies. Some diseases may therefore go underdiagnosed and, even when recognized, be suboptimally and sometimes even poorly treated. In such cases, reliable diagnostics will lead to better medical care but also increased public and—even more important—medical awareness (Peeling and Mabey 2010). For that reason, there is a clear need for improvement of laboratory technologies suited for sensitive and specific detection of such pathogen species. In addition, clinical symptom-based algorithms need to be implemented in teaching programs at the level of general practitioners, medical students and other categories of health professionals (e.g. local health authorities) and must be combined with microbiological algorithms reflecting optimal laboratory testing.

Classical diagnostic technology

For many bacterial pathogens, the microbial growth characteristics are widely different. Some may grow unproblematically and

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fast on cell-free and semi-solid agar-based culture media (e.g. *Staphylococcus aureus*), whereas others require the support of liquid media sometimes with balanced gaseous phases included (e.g. *Clostridioides difficile* and many other anaerobic organisms). In the most complicated culture tests, bacteria may require the intracellular compartment of eukaryotic host cells to provide the right environment for replication (e.g. *Rickettsia* spp.) (Valáriková et al. 2016). The combination of such methodologies has been defined as 'culturomics' (meaning microbial cultivation techniques using different incubation conditions, both at the biological and the physical level, adapted composition of the medium, temperature, osmolarity, oxygen, other gas pressures, etc.) (Caputo et al. 2019). Choice of the classical culture-based laboratory cultivation technology to be used is strongly dependent on the nature and quality of the biological specimen obtained as well as the metabolic characteristics of the infectious organism involved. Tissue biopsies, for instance, may require different conditions than liquid samples or superficial swab specimens. Still, culture technologies are very much at the heart of the routine diagnostic medical microbiology laboratory (van Belkum and Rochas 2018). It has to be stated explicitly that cultivation tests have significant shortcomings in sensitivity and specificity and also affect the timeliness of downstream testing for antimicrobial susceptibility. For the latter, rapid replication of bacterial pathogens is a prerequisite (Anton-Vazquez et al. 2021) and slow or no growth on solid media combined with the absence of minimal inhibitory concentrations (MICs) gets in the way of antibiotic susceptibility testing of these pathogens. Still, for infections caused by organisms that fail to grow or are highly fastidious, there are various laboratory algorithms available to identify infections caused by such pathogens. Several of such algorithms will be presented throughout the current manuscript.

Next to culture, the microbiology laboratory is usually equipped with tools and expertise for immunological and molecular testing although this varies depending on geographic localization and financial support (Vandenberg et al. 2021). Both bacteria-derived antigens (e.g. for detection of infections caused by *Legionella pneumophila*) and serum antibodies (e.g. for detection of infections caused by *Borrelia burgdorferi*) can be diagnostically targeted. For most if not all of the fastidious organisms, indirect immunoassays (detecting specific antibodies) and direct nucleic acid amplification tests have been developed and validated clinically. Indirect immune testing uses patient sera for the detection of antibodies specific to the pathogen in question. This can be done using immunofluorescence assays (IFAs), enzyme-linked immunosorbent assay (ELISA) formats and western blotting. Furthermore, immunological staining methods can be used to visualize bacterial cells directly in clinical specimens or cultured material using *in situ* protocols for detection of bacterial antigens, either surface located or intracellular ones, although these diagnostic approaches stay experimental.

For molecular diagnostics, nucleic acids have to be extracted from clinical material. If the specimen is rich in bacterial cells, tests may detect the pathogen directly, such as *in situ* hybridization assays although this is widely an experimental approach (Aistleitner et al. 2018). In most cases, however, pathogen-specific nucleic acids have to be amplified *in vitro* by polymerase chain reaction (PCR) or other methods to reach concentrations applicable in more classical or generic nucleic acid detection methods (Chen and Kontoyiannis 2010).

In the separate sections below, we describe the state of affairs for classical and innovative diagnostics for five bacterial genera that elicit relatively hard to identify but serious infections. For the genera *Anaplasma* spp., *Bartonella* spp., *C. burnetii*, *Orientia* spp. and *Rickettsia* spp., we will describe the epidemiology, clinical features of disease, diagnostic approaches currently in use, recent developments and shortcomings still to be addressed (see summary Table 1). Finally, the route toward improved diagnosis, antimicrobial susceptibility testing and epidemiological studies will be defined.

Anaplasma spp.

Members of the genus *Anaplasma* are Gram-negative obligate intracellular bacterial species that reside within cytoplasmic vacuoles of their host cells. *Anaplasma* spp. infect hematopoietic cells of vertebrates and are primarily transmitted by ticks although other transmission routes do occur (Pritt et al. 2019).

Description of the genus *Anaplasma*

According to the List of Prokaryotic names with Standing in Nomenclature (Parte et al. 2020), the genus *Anaplasma* contains five confirmed species: *Anaplasma bovis*, *A. centrale*, *A. marginale*, *A. ovis* and *A. phagocytophilum*. *Anaplasma platys* is another well-known species but its nomenclatural status is uncertain because it is noncultivable and its name has not been validly published yet (Parte et al. 2020). '*Anaplasma capra*' is a newly described organism but the approval of its species designation is still pending (Zhou et al. 2010).

All valid *Anaplasma* spp. are of veterinary importance causing disease in domestic and farm animals (Rar et al. 2021). According to current knowledge, only *A. phagocytophilum* is of worldwide relevance for human health although DNA of other *Anaplasma* spp. have been amplified from human blood (Chochlakis et al. 2010; Maggi et al. 2013; Arraga-Alvarado et al. 2014; Lu et al. 2019) (see Table 1 for a general overview of *A. phagocytophilum*). In China, '*Anaplasma capra*' DNA was detected in the blood of 28 symptomatic patients after tick bites and it was isolated via cell culture from three of them (Li et al. 2015). The main vectors are ticks of the *Ixodes ricinus* complex (Rar et al. 2021).

Clinical findings in human disease

Human granulocytic anaplasmosis caused by *A. phagocytophilum* is a nonspecific febrile illness occurring 1–2 weeks after tick bite (Bakken and Dumler 2015). Typical clinical symptoms comprise fever, headache, myalgias and arthralgias (Ismail and McBride 2017). The treatment of choice is doxycycline. The prognosis is good with a lethality below 1% (Ismail and McBride 2017).

Current diagnostics and drawbacks

Due to their obligate intracellular replication, *Anaplasma* spp. cannot be grown on cell-free media. Their isolation depends on cell culture systems of which the most common ones comprise the human promyelocytic leukemia HL60 cell line or different tick cell lines (Silaghi et al. 2017). In principle, cell culture is a suitable method to detect *A. phagocytophilum* (Pritt et al. 2019). However, the procedure is laborious and results cannot be expected within the first week of inoculation (Dumler et al. 2007). Therefore, most diagnostic laboratories do not provide this method routinely.

Although *Anaplasma* spp. are Gram-negative bacteria, they are not detectable by Gram-staining. Direct examination is possible by Giemsa- or Wright-stained peripheral blood smears (see Fig. 1A). However, the sensitivity of microscopy is limited and strongly depends on the experience of the investigator.

Indirectly and retrospectively, infection with *A. phagocytophilum* is confirmed by a 4-fold rise in IgG antibody titer between acute

Table 1. General overview on distribution, ecology, pathogenicity, disease entities and microbiology procedures.

	A. phagocytophilum	*Bartonella* spp.	*C. burnetii*	*Orientia* spp.	*Rickettsia* spp.
Epidemiology					
Geographical distribution	Worldwide, human infections reported from North America, Europe and Asia	Worldwide, *B. bacilliformis* restricted to South America	Nearly worldwide	Asia-pacific region ('Tsutsugamushi Triangle' between Japan, India, Australia). Anecdotally in Arabian peninsula (Dubai) and South America (Chile, Peru), suspected cases from Africa	Spotted fever rickettsioses group and murine typhus worldwide; epidemic typhus Africa, America
Disease prevalence worldwide/Europe	USA 2017: 5.762 cases/Europe and Asia rare	Seroprevalence ~5–10%, ~9 infections per 100 000 inhabitants per year	0.2 infections per 100 000 inhabitants per year (EU)	Seroprevalence (6 countries across Asia): ~9–28%. Estimated 1 million cases globally per year	Spotted fever in Africa second most important cause of febrile disease after malaria; murine typhus endemic mainly in port areas; epidemic typhus causes outbreaks in highlands with poor hygiene standards
Zoonotic species of host	USA: white-footed mouse; Europe: under debate	*B. henselae*: cats *B. quintana*: none *B. bacilliformis*: none	High risk for transmission: livestock (e.g. sheep, goats, cows) Low risk for transmission: wild animals and pets	Several mammalian hosts, mainly rodents (especially rats and mice)	Spotted fever group: mainly mammalia, also birds (Aves) and reptiles (Reptilia). Murine typhus: many rodent species (especially *Rattus rattus*, *Rattus norvegicus*), cats (*Felis catus*), opossums (*Didelphius* spp.). Epidemic typhus: humans (*Homo sapiens*), flying squirrels (*Glaucomys* spp.) Rickettsialpox: mice (*Mus musculus*)
Main vectors	*Ixodes ricinus* complex	*B. henselae:* cat flea (*Ctenocephalides felis*), tick (*Ixodes* spp.)[1] *B. quintana:* body lice (*Pediculus humanus corporis*) *B. bacilliformis:* sandfly (*Lutzomyia verrucarum*)	Mainly airborne transmission but the role of ticks as a vector remain to be elucidated	*Leptotrombidium* spp. (*L. pallidum*, *L. scutellare*, *L. delicense* and others), exceptionally other mite species.	Typhus: Body lice (*Pediculus humanus corporis*). Murine typhus: fleas (*Xenopsylla cheopis*). Spotted fever: Ticks (*Rhipicephalus sanguineus*, *Amblyomma* spp., *Dermacentor* spp., *Ixodes* spp.). Rickettsialpox: Mites (*Liponyssoides sanguineus*)
Growth behavior and pathogenicity					
Growth-specific requirements	Obligate intracellular: cell culture	Hemin-dependent, slow-growing, agar, cell cultures (e.g. Vero, HeLa-229, endothelial cells), special liquid media	Host cell-dependent growth; axenic growth in special acidified media with oxygen-reduced atmosphere (agar, liquid culture).	Obligate intracellular: cell culture (e.g. Vero cells, L929 mouse fibroblasts).	Obligate intracellular: cell culture
Extracellular bacterial products	Unknown	*B. henselae:* BafA *B. bacilliformis:* deformation factor (unidentified; 'deformin'), hemolysin (unidentified)	Unknown	Ankyrin-repeat-containing proteins (Anks, T1SS substrates), deubiquitylase	Rickettsial 'toxin' postulated but never identified

Table 1. Continued

	A. phagocytophilum	*Bartonella* spp.	*C. burnetii*	*Orientia* spp.	*Rickettsia* spp.
Principal bacterial adhesins	AipA, Asp14, OmpA, Msp2	*B. henselae*: BadA. *B. quintana*: Vomps. *B. bacilliformis*: Brp	OmpA (dual role: adhesin and invasin)	*O. tsutsugamushi*: type-specific antigen (TSA)-56, ScaA-C autotransporter proteins	OmpB, OmpA; rickettsial adhesin rADR2; cell surface antigens (Sca1, Sca2)
Infectivity and cell/organ tropism					
Minimum infection dose	Unknown	Unknown	1-15 bacteria	Unknown	10-100 bacteria
Type of cell/tissue affected	Neutrophils	Endothelial cells, epithelial cells, erythrocytes lymph nodes, heart valves, liver, spleen, eyes[1]	Primary targets: macrophages, monocytes and dendritic cells acute Q-fever: lung, liver, placenta chronic Q-fever: heart valves	Dendritic and Langerhans cells, monocytes/ macrophages, endothelial (epithelial) cells. Tissue tropism: skin (inoculation), lung, brain, kidney, heart, liver, spleen (dissemination)	Endothelial cells
Bacterial load in tissue	Unknown	Unknown, probably low (bacterial cultivation from tissue only on very rare occasions successful)	Unknown, probably low (bacterial cultivation from tissue works best with tissue samples from chronic Q-fever patients)	Unknown in humans, probably strain-dependent; in mouse models: lung>heart> brain>liver	Unknown
Clinical entities					
Most well-known clinical sign(s)	Fever, headache, myalgias, arthralgias	*B. henselae*: cat scratch disease (lymphadenopathy). *B. quintana*: five-day fever ('trench fever'). *B. henselae* and *B. quintana*: bacillary angiomatosis, endocarditis. *B. bacilliformis*: hemolytic anemia (Oroya fever), verruga peruana	Acute Q-fever: pneumonia, hepatitis, placentitis chronic. Q-fever: endocarditis chronic hepatitis chronic vascular infections	Eschar at the site of mite bite (<10 to >90% of patients); often unspecific (fever, headache, cough, rash, lymphadenopathy). Progression to pneumonia, acute respiratory distress syndrome, acute kidney failure, encephalitis, myocarditis, pancreatitis, hepatitis, etc.	Fever, constitutional symptoms, spots on skin including palmar and plantar areas; black ulcer at the location of entry of rickettsiae into skin (mainly in tick-borne rickettsioses)

Table 1. Continued

	A. phagocytophilum	*Bartonella* spp.	*C. burnetii*	*Orientia* spp.	*Rickettsia* spp.
Microbiological diagnostics					
Biological samples used for diagnostic	Peripheral blood for PCR, blood smear and cell culture; serum/plasma for serology	*B. bacilliformis*: peripheral blood for blood smears and PCR. All: biopsies for PCR and culture, serum/plasma for serology (less used for *B. bacilliformis* infections)	Peripheral blood for PCR, biopsies for PCR or staining, serum/plasma for serology	Peripheral blood for qPCR; eschar swab or biopsy for qPCR; serum/plasma for serology	Skin biopsy of eschar for PCR; EDTA-blood for PCR in typhus, murine typhus; serum/plasma for serology
Need for prompt diagnosis Critical: up to 48 h. Medium: days. Normal: days to weeks	Medium	*B. bacilliformis*: critical. *B. henselae*: medium. *B. quintana*: medium	*C. burnetii*: medium	*O. tsutsugamushi*: medium	Rocky Mountain spotted fever: critical. Mediterranean spotted fever: critical. Typhus: critical. Murine typhus: medium. Other spotted fever group: normal

[1]Not *bona fide* proven.

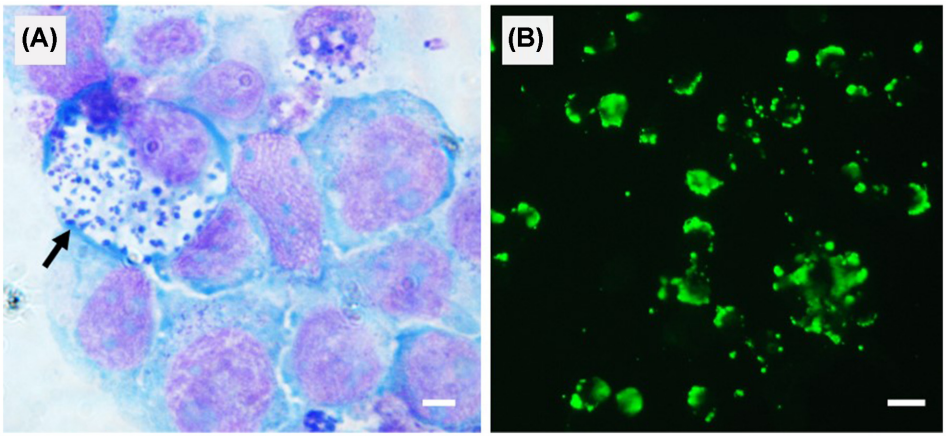

Figure 1. *A. phagocytophilum*. **(A)** *A. phagocytophilum* Webster strain grown in HL60 cells (arrow), magnification ×1000. Scale bar: 2 μm. **(B)** IFA for detection of anti-*A. phagocytophilum* IgG antibodies, magnification ×400. Cutoff titer for IgG ≥ 1:64. Scale bar: 20 μm.

and convalescent phase sera (Centers for Disease Control and Prevention (CDC) 2021). IgM testing is not recommended because of its nonspecificity. Although widely performed, serology is useless to diagnose an acute *A. phagocytophilum* infection at the time of patient presentation (Pritt et al. 2019). IFA is the most used method to detect *A. phagocytophilum* antibodies. However, its analysis is not automatable and results are highly observer dependent (see Fig. 1B). Further, confounding serological cross-reactivity with *Ehrlichia* spp., *Rickettsia* spp. and *Coxiella burnetii* exists (Ismail and McBride 2017; Pritt et al. 2019).

The most reliable and timely method to diagnose an *A. phagocytophilum* infection is the amplification of pathogen DNA via PCR from EDTA-anticoagulated acute phase whole blood. The most widely used targets are the 16S rRNA and the *msp2* genes (Matei et al. 2019). Sequencing of the amplicons is encouraged because 16S rRNA gene-based assays tend to amplify nucleic acids from several *Anaplasma* spp. (von Loewenich, unpublished). This includes for example the DNA of *A. phagocytophilum*, *A. ovis* and *A. platys*. Positive *msp2* PCR results must be verified by a second method targeting another gene because a significant proportion is false positive due to so far unknown reasons (Razanske et al. 2019).

Bartonella spp.

The genus *Bartonella* spp. comprises ∼45 species of facultative, intracellular, Gram-negative bacteria. Two habitat types are mainly used by *Bartonella* spp.: the gut of obligately blood-sucking arthropod vectors (fleas, sand flies and body lice) and the bloodstream of mammalian hosts where they induce acute or persistent intravascular infections (Okaro et al. 2017). Due to the vector-restricted distribution, some *Bartonella* spp. are geographically restrained. This is the case for *B. bacilliformis* that is transmitted by the sandfly *Lutzomyia verrucarum*, only present in the Andean regions of South America. Others, such as *B. henselae* and *B. quintana* appear to have worldwide dissemination. Identified vectors are the cat flea *Ctenocephalides felis* and human lice *Pediculus humanus corporis*, respectively. The role of ticks as vectors for *Bartonella* spp. remains unclear. The clinically most important species are *B. bacilliformis* that is causative of 'Carrion's disease', the zoonotic cat-transmitted species *B. henselae*, causative of 'cat scratch disease' (CSD), and the human restricted *B. quintana* causative of 'trench fever' (Okaro et al. 2017) (see Table 1 for a general overview of human pathogenic *Bartonella* spp.). There is increasing evidence that *B. koehlerae* and *B. vinsonii* subsp. *berkhoffii* might also represent important human pathogenic bacteria (Breitschwerdt et al. 2010a,b), therefore more diagnostic development is required also for these neglected pathogens.

Epidemiology and disease

Carrion's disease is a biphasic life-threatening human illness caused by *B. bacilliformis* presumably after bites of infected sand flies. In the acute stage of the disease, febrile and progressive anemia caused by bacterial invasion to the erythrocytes leads to hemolysis ('Oroya fever') with a lethality up to 90% if untreated but even under appropriate antibiotic therapy still up to 10%. In the chronic disease stage, nodular angioproliferative cutaneous lesions appear after weeks to months of the infection ('verruga peruana'). The prognosis for recovery is good at this stage of the disease (Garcia-Quintanilla et al. 2019).

CSD occurs after infection with *B. henselae*. Infected fleas transmit bacteria to cats, which are the main bacterial reservoir. The transmission to humans, the incidental host species, occurs after a cat scratch or bite. CSD is characterized by lymphadenopathy often accompanied by prolonged fever. The prevalence of *B. henselae* is highest in warm and humid places where cat fleas are more abundant, while there is a seasonal pattern of CSD in temperate regions with most cases seen in autumn and winter (Okaro et al. 2017).

Trench fever is a human louse-borne disease caused by infection with *B. quintana*. Because of recurrent bacteremia, periodic fever occurs approximately every 5 days (also known as 'five-day fever'). Severe stages of infection can result in endocarditis or the vasculoproliferative disease bacillary angiomatosis (this is also the case for *B. henselae*). *B. quintana* infections represent the most prevalent vector-borne illness among urban homeless and marginalized people in the United States and Europe (Leibler *et al.* 2016).

Current diagnostics and drawbacks

Blood and tissue specimens (e.g. from enlarged lymph nodes or various other organs) can be used for primary isolation of *Bartonella* spp. The diagnostic sensitivity using conventional or advanced culture techniques (e.g. microaerophilic incubation on Columbia blood-agar plates (see Fig. 2A) and shell-vial endothelial cell culture) are hampered due to the fastidious nature and slow growth characteristics of the bacteria (doubling time ∼24 h), leading to its identification typically after weeks of incubation at

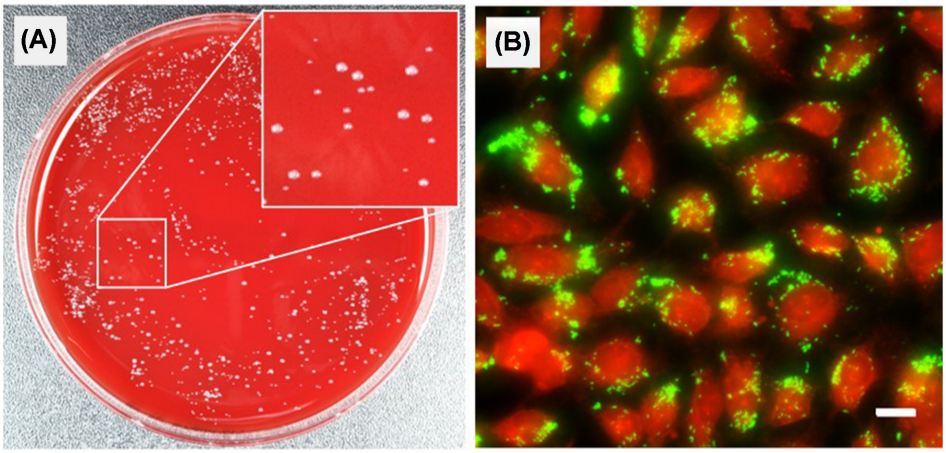

Figure 2. *B. henselae*. **(A)** *B. henselae* colonies on Columbia blood agar plate (cultivation time: 8 days, 37°C, 5% CO_2). Insert: enlarged picture detail. **(B)** IFA for detection of anti-*B. henselae* IgG antibodies. Vero cells were infected with the *B. henselae* and used for detection of IgG. IgG titers of <64/<80 are evaluated as negative, titers >256/320 as positive and titers in between at threshold level. Scale bar: 20 μm.

best. This, in combination with the relatively low bacteremia level in the patients, affects the sensitivity of the diagnostic cultivation procedures, which is ~20–31% for blood and tissue samples from *Bartonella* spp. endocarditis patients (Houpikian and Raoult 2005).

Most *Bartonella* spp. are biochemically inert, oxidase and catalase-negative and do not produce acid from carbohydrates. Gram-staining works poorly for *Bartonella* spp., whereas Gimenez and Warthin–Starry staining have been shown to represent better alternatives. However, genus-specificity is not guaranteed (e.g. from histopathological samples such as valvular tissues) (Lepidi et al. 2000; Bruneval et al. 2001). Conversely, immune-histochemical staining of affected tissue using monoclonal or polyclonal *Bartonella* spp. antibodies or fluorescence *in situ* hybridization (FISH) for detection of ribosomal RNA have to some extent improved identification to the species level (Caponetti et al. 2009; Mallmann et al. 2010). Even though the specificity for *Bartonella* spp. identification in both approaches has shown improvement, their sensitivity is still insufficient, preventing their application as routine diagnostic technologies.

Accepted methods for laboratory diagnosis are serology and PCR. IFA is widely used for *Bartonella* spp. serodiagnosis (see Fig. 2B). Seeking for automated workflows, ELISA-based approaches with improved sensitivity and specificity as well as augmented throughput have been established (Jost et al. 2018). This method also tackled the previously reported cross-reactivity of IFA (e.g. *Chlamydia* spp. and *C. burnetii*, causative agents of endocarditis as well) (Bergmans et al. 1997; Rahimian et al. 2006). Moreover, PCR-based detection has been proven to be a specific approach to detect *Bartonella* spp. infections (e.g. via amplification of the 16S-rRNA and riboflavin synthase *ribC* gene sequences) (Jensen et al. 2000; Hobson et al. 2017). Despite its specific performance, direct PCR diagnostics from blood is of limited value for patients with a very low bacteremia level, while in the case of tissue specimens (e.g. biopsies), the sample collection requires invasive medical procedures that are often avoided by doctors.

Efforts have been made to support the enrichment of *Bartonella* spp. from human samples. Some novel approaches using liquid growth media are available (Maggi et al. 2005; Riess et al. 2008), thereby possibly improving the sensitivity of culture-based diagnosis (Maggi et al. 2011). A diagnostic combination for the detection of *Bartonella* spp. from animal and human specimens using pre-enrichment media and digital droplet PCR technology has been recently suggested (Maggi et al. 2020). Additionally, matrix-assisted laser desorption time of flight mass spectrometry (MALDI-TOF MS) has proven to be an accurate and reproducible tool for rapid and inexpensive identification, but not for diagnostic detection of *Bartonella* spp. (Fournier et al. 2009). Although improvement has been made in the specificity, sensitivity and reproducibility of diagnostic tools, the efficacy of current strategies still relies on culture pre-enrichment and is negatively influenced by slow bacterial growth.

Coxiella

Coxiella is a genus of obligate intracellular, nonmotile, pleomorphic, Gram-negative bacterial species. Although the genus *Coxiella* consists of *C. burnetii* and several *Coxiella*-like bacterial species, only *C. burnetii* is a relevant zoonotic pathogen (see Table 1 for a general overview of *C. burnetii*). Some *Coxiella*-like bacteria are tick endosymbionts with little to none or unclear human pathogenicity. '*Candidatus* Coxiella massiliensis' is suspected to cause scalp eschar and neck lymphadenopathy after a tick bite (Angelakis et al. 2016). *C. burnetii* causes Q-fever, a disease occurring around the world. This species has a broad spectrum of host species among livestock, wild animals and pets leading to an extensive zoonotic reservoir (Fournier et al. 1998).

C. burnetii exhibits a physiological peculiarity in that it shows a biphasic developmental cycle (McCaul and Williams 1981) similar to that of *Chlamydia* spp. The metabolically active and replicating large cell variant (LCV) is observed during infection of a suitable host cell. The dormant and resilient small cell variant (SCV) is observed under less favorable conditions and exhibits a strong environmental tenacity. The SCV shows spore-like characteristics and is one of the main reasons for the increased infectivity of *C. burnetii* compared to many other human pathogenic bacteria that are less persistent in the environment (see Fig. 3A). It easily spreads by airborne or droplet transmission, which occurs mainly by inhalation of SCV-contaminated dust or aerosols. The median infectious dose (ID50) may be as low as 1–15 SCVs (Brooke et al. 2013; Heppell et al. 2017). The main cause of human infection is indirect contact with infected small ruminants via airborne or droplet transmission routes, which is possible up to a distance of 10 km and in extreme conditions even up to 18 km (Hawker et al. 1998; Clark and Soares Magalhães 2018). The role of ticks as a disease-carrying vector has not yet been fully elucidated (Körner et al. 2021).

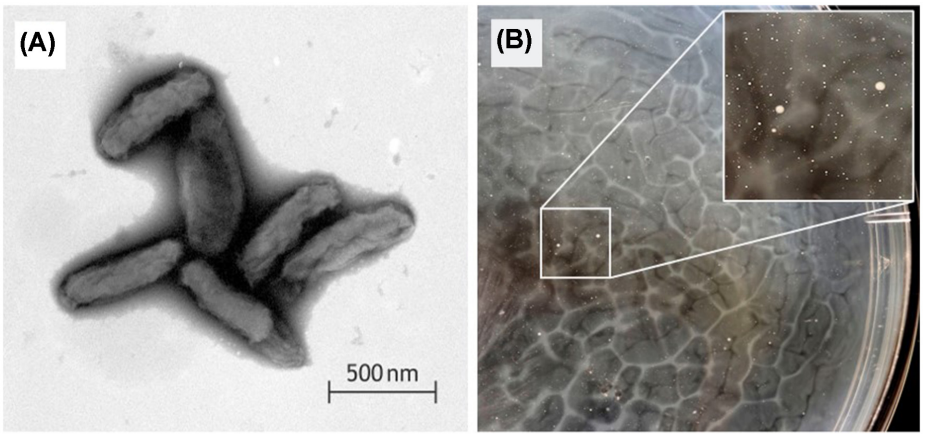

Figure 3. *C. burnetii*. **(A)** Transmission electron microscopy (negative staining) of *C. burnetii* RSA439, showing one LCV and five SCVs. Photography courtesy of: Dr E. Liebler-Tenorio, Friedrich-Loeffler-Institut, Jena, Germany. **(B)** *C. burnetii* RSA 439 colonies on acidified citrate cysteine medium (ACCM)-2 agar plate (cultivation time: 6 days, 37°C, 5% CO_2, 2.5% O_2). Insert: enlarged picture detail. Photography courtesy of: Dr K. Mertens-Scholz, Friedrich-Loeffler-Institut, Jena, Germany.

Epidemiology and disease

In humans, Q-fever manifests as a flu-like illness with the potential to develop severe complications, e.g. pneumonia or even hepatitis (Maurin and Raoult 1999). The incubation period is around a median of 18 days with 95% of cases occurring between 7 and 32 days after transmission (Todkill et al. 2018). Symptoms in humans are often nonspecific and vary depending on age, which renders accurate diagnosis difficult and makes careful anamnesis necessary. Among the most common symptoms are fever, fatigue, chills, intense headaches, myalgia, sweats and cough (Maurin and Raoult 1999). Therefore, Q-fever should be considered in patients showing a fever of unknown origin paired with respiratory symptoms. Most patients recover without requiring treatment but in ∼1% of the cases, the disease progresses into a chronic form possibly resulting in life-threatening endocarditis if left untreated. Patients diagnosed with valvulopathies (diseases of the cardiac valves), an aneurysm, those who received a vascular graft or a prosthetic valve, immunocompromised patients and pregnant women are at high risk of developing a chronic infection. Chronic Q-fever during pregnancy may significantly increase the possibility of premature birth, spontaneous abortions or stillbirth (Carcopino et al. 2007).

Current diagnostics and drawbacks

Blood, serum or tissue samples are used for diagnostic purposes. The diagnostic gold standard for Q-fever is IFA, which works in almost all stages of the disease and requires a serum sample. The detection of specific classes of antibodies against *C. burnetii* is required since the pathogen shows a phase-specific antigen variation of its lipopolysaccharide (LPS) that can be used to differentiate between acute and chronic but also past infections. Phase I organisms have a 'smooth' (full length) LPS and are virulent. The full length LPS is a major virulence factor of *C. burnetii*. Phase II organisms with a 'rough' LPS are avirulent and are obtained by *in vitro* cultivation of the pathogen. The transition from 'smooth' to 'rough' LPS is associated with genome mutations and deletions and is hence irreversible. The acute phase is characterized by high IgM antibody titers against the phase I and II antigens. IgM antibodies are observed ∼2–3 weeks after the onset of symptoms and may remain observable for years. Phase II IgG-antibodies can be detected soon after and may also persist for years. If a transition into a chronic form occurs, high antibody titers against the phase I antigen become detectable. Patients with high titers of phase I IgG-antibodies and those who are at high risk of developing a chronic infection (e.g. relevant preexisting medical condition) should hence be monitored via serology.

Alternatives to IFA include ELISA. A major drawback of the IFA is that it is very time consuming and comes with a high workload. The ELISA on the other hand has a reduced sensitivity compared to the IFA and shows significant variation in results depending on the manufacturer of the test (Dangel et al. 2020). Therefore, the ELISA is mainly used as a screening method (e.g. in outbreak situations) and positive samples are validated via IFA. Cross-reactions of antibodies against *C. burnetii* and other bacteria are unlikely, but cannot be ruled out for *Legionella* spp. or *Bartonella* spp. (La Scola and Raoult 1996a; Musso and Raoult 1997).

Quantitative PCR detection of *C. burnetii* is possible during the acute and chronic phase using whole blood or tissue samples as the origin of nucleic acids. The Insertion Sequence IS1111 has been the main target for PCR diagnostics as it has multiple copies per genome, rendering the PCR highly sensitive. Another approach for tissue sample testing (e.g. heart valve samples) is the newly developed *C. burnetii*-specific FISH that could be combined by a set of additional probes, e.g. for *Bartonella* spp. (Aistleitner et al. 2018; Prudent et al. 2018) or immune-histochemistry. Both techniques could be particularly useful for the detection of *C. burnetii* in patients with endocarditis or vascular infections (Prudent et al. 2018).

Several studies have also unraveled the *C. burnetii* proteome making an identification via MALDI-TOF MS possible. Although this method finds almost no diagnostic usage in this field to date, it could become a powerful diagnostic tool if it would be able to differentiate between phase I and phase II or SCV- and LCV-type organisms (Ihnatko et al. 2012).

Cultivation of *C. burnetii* is particularly hazardous since it is easily transmitted by aerosols while also having a high environmental tenacity, making it a *BSL*-3 agent requiring a specially equipped diagnostic laboratory. This poses a major challenge to the cultivation of *C. burnetii* for diagnostic purposes. Furthermore, as *C. burnetii* is an obligate intracellular bacterial species, it can only be cultured in a cell culture-based system or in acidified media with an oxygen-reduced atmosphere (Sanchez et al. 2018), which may be challenging for diagnostic laboratories (see Fig. 3B).

In conclusion and even though the diagnostic toolbox for *C. burnetii* is quite large, the main diagnostic techniques remain the IFA, ELISA and PCR. Still, the IFA remains the accepted gold standard technology, but testing is driven by local expertise and preferences.

Orientia spp.

Orientia tsutsugamushi is a vector-borne, zoonotic, Gram-negative, obligate intracellular bacterium from the family of Rickettsiaceae. Many different strains and serotypes exist (e.g. the 'prototype' strains Gilliam, Karp and Kato) (Kelly et al. 2009). A diverging *Orientia* species, *Orientia chuto*, was isolated from a traveler returning from Dubai (Elliott et al. 2019). Recently, a new endemic focus of autochthonous scrub typhus infections has emerged in Chile, where another distinct *Orientia* species, *Candidatus* Orientia chiloensis, was identified (Elliott et al. 2019) (see Table 1 for a general overview of *Orientia* spp.).

Epidemiology and Disease

O. tsutsugamushi is transmitted to humans during cutaneous feeding of trombiculid mite larvae (chiggers), the genus *Leptotrombidium* spp. being the major vector (Elliott et al. 2019). Related to the distribution of vector mites, scrub typhus is endemic across the tropical regions of Southeast and East Asia, India, Australia and China. However, indigenous *Orientia* infections have also been sporadically reported from the Middle East and Latin America (Elliott et al. 2019).

The mite bite can be followed by the formation of an eschar, a localized cutaneous inflammation of 0.5–2 cm in size. The eschar usually presents with a central black crust that is surrounded by an erythematous area. Its appearance is highly variable, with a prevalence of 1–97% in patients, depending on strains and geographical location (Xu et al. 2017). The typical black crusts are formed after 6–8 days after onset of symptoms and resolve after 2 weeks, leaving a scar-like macule.

Symptoms of scrub typhus range from mild and unspecific symptoms such as headache, fever, rash and generalized lymphadenopathy to acute encephalitis syndrome, acute respiratory distress syndrome, acute kidney failure, interstitial pneumonia or myocarditis (Rajapakse et al. 2017). If diagnosis and treatment are delayed, complications may be fatal. In the natural course of scrub typhus, lethal complications usually occur after 2–3 weeks of high fever. In the absence of treatment, mortality rates of 0–70% were reported, with a median of 6%, while mortality for treated patients is 1.4% (Xu et al. 2017). Despite antimicrobial treatment, *O. tsutsugamushi* enters a state of latent persistence in humans and rodents, probably for life, but it remains susceptible to the antibiotics previously used (Kock et al. 2018).

Tropism and replication

Orientia spp. is known to infect macrophages, monocytes and dendritic cells, which are the primary cellular targets in the skin (Paris et al. 2012). In lethal cases, endothelial cells were demonstrated to be infected (Moron et al. 2001). *Orientia* spp., after escaping from the endosome, replicate cytosolically in the perinuclear region (Fig. 4A). Triggered by unknown signals, *Orientia* spp. migrates to the cell membrane to leave the cell via a virus-like budding process (Fig. 4B) (Ge and Rikihisa 2011).

Genotypic variability across geographical regions

Antigenic variability of *O. tsutsugamushi* is largely caused by variations within its type-specific antigen, a protein of 56 kD (TSA56). Between strains, the similarity may be 60–80% at the amino acid level and can be <50% in one of the four variable domains (Ohashi et al. 1992). Multiple strains of *O. tsutsugamushi* may be detected within a single patient.

Current diagnostics and drawbacks

O. tsutsugamushi cannot be diagnosed by classic routine bacteriology. The laboratory diagnosis of scrub typhus, therefore, relies on serological and molecular approaches. *O. tsutsugamushi* can be isolated from blood samples via shell-vial cell culture, but mainly for research purposes. Resistance to doxycycline has been observed in anecdotal studies but cannot be tested and confirmed routinely.

The IFA is regarded as the gold standard for detection of IgM and IgG antibodies in scrub typhus patients, but it is largely restricted to reference laboratories. Cutoff titer, strain(s) used and background seroprevalence affect the sensitivity and specificity of the test. Due to limited cross-reactivity between strains, laboratories usually select pools of antigens from multiple strains (Blacksell et al. 2007). In regions of high endemicity, the background rates of IgG seropositivity can reach 50% (Blacksell et al. 2007). Positive IgM results are often interpreted as indicative of acute infections. However, IgM responses above the cutoff may persist for >1 year after acute infection. For a definite diagnosis, a 4-fold or more increase of antibody titers in paired acute and convalescent sera has therefore been suggested (Blacksell et al. 2007).

For routine clinical use, ELISAs employing whole-cell antigens from the Gilliam, Karp and Kato strains are available (Jiang et al. 2003). Recently developed ELISAs contain recombinant bacterial proteins (e.g. the 22-, 47- and 56 kD antigens). Employing a combination of recombinant antigens has increased sensitivity and specificity for IgG and IgM detection (Kim et al. 2013). The Weil–Felix agglutination test, based on cross-reactivity of scrub typhus sera to *Proteus mirabilis* OXK, has been discontinued due to insufficient sensitivity and specificity (Kelly et al. 2009). IgM rapid diagnostic tests (RDT), e.g. lateral flow assays using recombinant TSA56 from more than three different strains, allow a cost-effective point of care diagnosis of scrub typhus with high sensitivity and specificity (Anitharaj et al. 2016).

Real-time PCR-based detection of *O. tsutsugamushi* targets either conserved regions of the ribosomal 16S rRNA gene or organism-specific genes including the *47kD*, *56kD* or *groEL* genes. *O. tsutsugamushi* DNA is detectable from serum, plasma or whole blood (Paris and Dumler 2016), but only in 25–65% of acute scrub typhus patients. Detection from buffy coats or eschar swabbing, where possible, appears as an alternative with increased sensitivity.

Rickettsia spp.

Rickettsioses are caused by members of the family of Rickettsiaceae. This family contains two genera, *Orientia* spp. and *Rickettsia* spp. (Murray et al. 2016). Members of the genus *Rickettsia* comprise >30 obligate intracellular Gram-negative bacterial species (Parola et al. 2013). All known pathogenic *Rickettsia* spp. are vector-borne and transmitted by blood-sucking arthropods (ticks, fleas, lice or mites) (see Table 1 for a general overview of *Rickettsia* spp.). A number of *Rickettsia* spp. are thought to be endosymbionts in arthropods (Tomassone et al. 2018). Pathogenic *Rickettsia* spp. may infect vertebrates and multiply mainly in en-

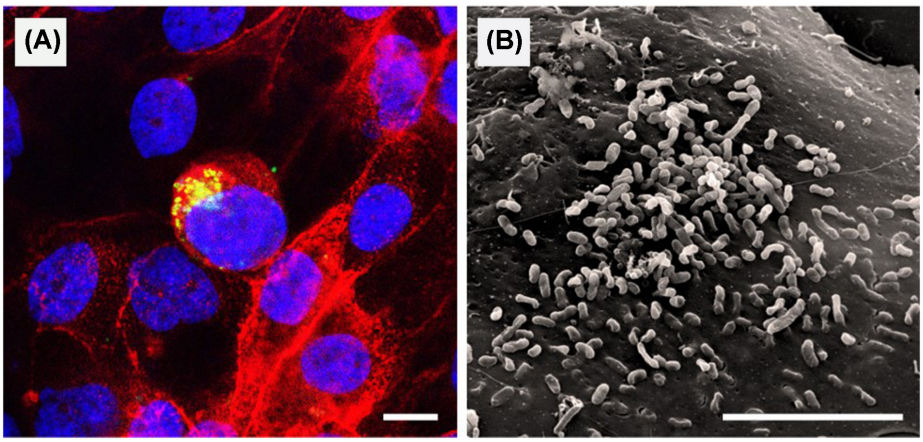

Figure 4. *O. tsutsugamushi.* **(A)** Indirect immunofluorescence of L929 cells infected with *O. tsutsugamushi* Karp (6 days postinfection). Green: *O. tsutsugamushi*; blue: nuclei; red: actin. Scale bar: 10 μm. **(B)** Scanning electron microscopy of *O. tsutsugamushi* budding from infected L929 cells (day 9 postinfection). Scale bar: 10 μm.

Table 2. *Rickettsia* spp.: groups and species.

Rickettsial group	Prominent members
Typhus group	*R. prowazekii*, *R. typhi*
Spotted fever group	*R. rickettsii*, *R. conorii*, *R. africae*
Ancient group	*R. bellii*, *R. peacockii*
Transitional group	*R. felis*, *R. australis*, *R. akari*

dothelial cells and thereby cause increased cellular and vascular permeability, petechial bleeding in the skin, mucous membranes and inner organs. This will ultimately cause symptomatic disease (spotted fever, typhus) (Parola et al. 2013). There is now good evidence that *Rickettsia* spp. evolved from extracellular bacteria by genome reductive evolution (degradation or loss of some essential genes). It was shown that essential genes of the energy metabolism are missing in *Rickettsia* spp. making them dependent on the cellular metabolism (Salje 2021). The rickettsial species with the highest pathogenicity (*R. prowazekii*, *R. typhi*, *R. rickettsii*) have the smallest genomes and therefore it seems that the extent of loss of genes coincides with increasing pathogenicity in vertebrates (Andersson et al. 1999). *Rickettsia* spp. can be grouped into several groups (see Table 2).

Epidemiology and disease

Rickettsia spp. cause different and particular clinical entities (Parola et al. 2013). These are caused by the increased permeability of the endothelial cell layer of infected organs (and sometimes the extremities) resulting in vascular leakage, bleeding and circulatory disorders. In members of the spotted fever group, an eschar can be sometimes found at the location of the arthropod bite. After an incubation period of 3–10 days, the acute stage is characterized by fever, constitutional symptoms, and petechial bleeding on the skin (spots typically also on palmar and plantar areas). Louse-borne typhus ('epidemic typhus') may show a fatality rate of up to 20%. Among the most common tick-borne rickettsioses is African tick-bite fever. The etiologic agent, *R. africae*, is transmitted by ticks of the genus *Amblyomma* in sub-Saharan Africa and the Caribbean. Another rickettsiosis, which might have been dispersed with cats and their fleas to all continents except Antarctica is flea-borne spotted fever, caused by *R. felis*. Among the clinically more severe forms of rickettsioses, Mediterranean spotted fever shows an especially severe course in patients with certain genetic blood disorders (Raoult et al. 2004; Parola et al. 2013). The fatality rate of Rocky Mountain spotted fever increases with the delay of treatment of >48 h after the beginning of symptoms. Most rickettsioses cause infection with an acute course, only *R. prowazekii* causes a chronic form of rickettsiosis. This chronic form is named Brill–Zinsser disease, an exacerbation of the clinical symptoms years to decades after the primary infection (Raoult et al. 2004).

Rickettsial diseases occur in most parts of the world. Especially spotted fever variants transmitted by various tick species are globally distributed and mostly associated with a single or very few closely related tick species (Parola et al. 2013). One important exception is *R. rickettsii*, which is prevalent in Northern and Southern America and is transmitted under different specific ecological conditions by ticks from different genera (*Amblyomma*, *Dermacentor*, *Rhipicephalus*) (see Table 3).

Current diagnostics and drawbacks

The first choice of diagnosis of rickettsial disease is the detection of the pathogen either in clinical samples and/or in the biting arthropods if available from the patient (La Scola and Raoult 1997; Brouqui et al. 2004). For rickettsioses of the spotted fever group, a skin biopsy of the eschar is the clinical material of choice for the detection of the pathogen. The skin biopsy may be used for detection by real-time PCR or by cultivation of the pathogens using cell culture (e.g. Vero cells, tick cells; see Fig. 5A) (La Scola and Raoult 1996b). Cultivation is usually performed by specialized laboratories under BSL-3 conditions, so culture for routine clinical microbiology laboratories is beyond the scope. For molecular detection, several PCR tests have been published that mostly target the citrate synthetase gene (*gltA*), which is conserved in most rickettsial species (Fournier and Raoult 2004). In typhus, murine typhus and scrub typhus, the detection of the pathogens from EDTA blood is the method of choice. For the identification of the *Rickettsia* spp. to species level, the amplification and sequencing of at least three different genes (among them, e.g. *ompA*, *ompB*, 23S-5S spacer region, *Sca4*) followed by a phylogenetic analysis of the amplicon sequences is essential (Chitimia-Dobler et al. 2018). For the identification of the pathogen in the arthropod vectors mainly real-time PCRs are used (Springer et al. 2020).

The classical method to diagnose rickettsioses is the detection of antibodies by serological methods. Again, the Weil–Felix test

Table 3. Disease and epidemiology of *Rickettsia* spp.

Disease	Pathogen	Geographic distribution	Vector	Clinical symptom 'rash' in % of patients
Typhus	*R. prowazekii*	South America, Africa	*Pediculus humanus corporis*	80%
Murine typhus	*R. typhi*	Worldwide	*Xenopsylla cheopis*, fleas	60%
Rocky Mountain spotted fever	*R. rickettsii*	North America, South America	Ticks	90%
Mediterranean spotted fever	*R. conorii*	Europe, Africa, Asia	*Rhipicephalus sanguineus*	95%
Siberian tick typhus	*R. sibirica*	Asia	Ticks	95%
Japanese spotted fever	*R. japonica*	Far Eastern Asia, Japan	Ticks	95%
Flinders Island spotted fever	*R. honei*	Australia, Asia	Ticks	75%
Queensland tick typhus	*R. australis*	Eastern Australia	Ticks	95%
Far Eastern spotted fever	*R. heilongjiangensis*	Eastern Asia	Ticks	90%
African tick-bite fever	*R. africae*	Sub-Saharan Africa, Caribbean	*Amblyomma* ticks	50%
Rickettsialpox	*R. akari*	North America, Europe, Asia	Mites	100%
Flea-borne spotted fever	*R. felis*	Worldwide	Fleas	75%
Tick-borne lymphadenopathy (TIBOLA), Dermacentor-borne necrosis erythema and lymphadenopathy (DEBONEL)	*R. slovaca*	Europe, Asia	*Dermacentor* ticks	5%

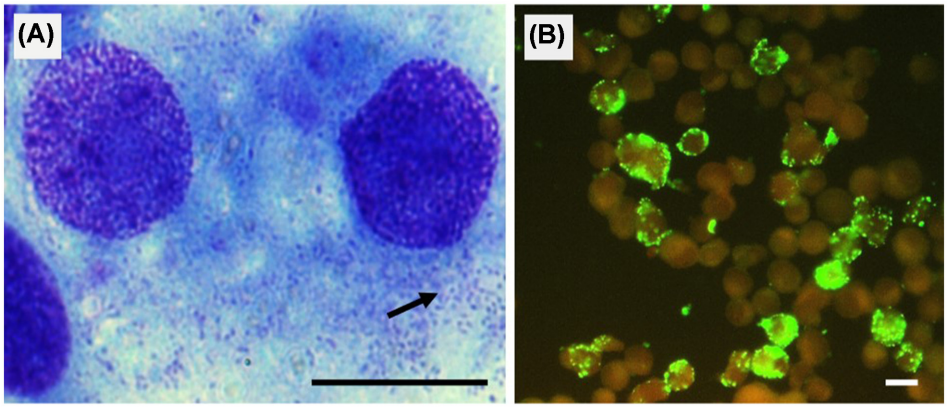

Figure 5. *Rickettsia* spp. **(A)** *R. africae* (arrow) in Vero cells (Romanowsky staining). Scale bar: 10 μm. **(B)** IFA for detection of anti-*R. conorii* (spotted fever group) IgG antibodies. Vero cells were infected with the *R. conorii* and used for detection of IgG. IgG titers of ≥64 are classified as positive. Scale bar: 10 μm.

should no longer be used (Hechemy et al. 1979). Meanwhile, the preferred serological assay performed in most laboratories is IFA, either conventional or in the micro-immunofluorescence format (MIF), infected cell culture material is used as antigen. IgG and IgM can be detected in sera/plasma of patients earliest at 10–14 days (for African tick-bite fever up to 25 days) after the start of symptoms (Brouqui et al. 2004). A titer of >64 (IgG) or >32 (IgM) is usually indicative of a recent or postacute rickettsiosis (see Fig. 5B). Usually, a 4-fold or more increase of titer in two consecutive serum samples would confirm acute rickettsiosis. IgM may not be indicative of an acute rickettsiosis as it is sometimes persisting over months and often cross-reacts with other bacterial pathogens.

By testing against multiple rickettsial antigens in MIF a differentiation of antibodies against particular rickettsial species may be possible by detecting significant titer differences between the species. However, by IFA and MIF no precise differentiation of spotted fever group *Rickettsia* spp. antibodies is possible (Brouqui et al. 2004). Also, no differentiation between typhus and murine typhus is possible using serology but there is only low cross-reactivity between the members of typhus and spotted fever groups. In some specialized reference laboratories, an absorption western blot technology is used for the differentiation of antibodies against particular *Rickettsia* spp. (La Scola et al. 2000). Meanwhile also ELISA formats for the detection of IgG and IgM antibodies against *Rickettsia* spp. are commercially available. Comparison with the standard IFA shows that they exhibit acceptable sensitivity and specificity rates. However, IFA remains the standard test for detection of antibodies against *Rickettsia* spp.

More complete and innovative diagnostics

As can be gathered from the five genus-specific sections above and Summary Table 4 there is no easy answer to the questions that surround the adequate laboratory diagnosis of fastidious microbial pathogens. For all genera, culture is cumbersome, and—even if successful—there are significant drawbacks based on biosafety risks associated with handling large or even low numbers of pathogens (e.g. *C. burnetii* and *Rickettsia* spp.) (Peng et al. 2018).

Table 4. Overview on details in clinical microbiology of medically relevant fastidious microorganisms.

	A. phagocytophilum	*Bartonella* spp.	*C. burnetii*	*Orientia* spp.	*Rickettsia* spp.
General information					
Type of intracellular bacteria	Obligate	Facultative	Obligate (bi-phasic developmental cycle)	Obligate	Obligate
Clinically most representative species and disease	*A. phagocytophilum:* Human granulocytic anaplasmosis	*B. bacilliformis:* Carrion's disease. *B. henselae:* Cat scratch disease. *B. quintana:* Trench fever	*C. burnetii:* Q-fever	*O. tsutsugamushi*: Scrub typhus	*R. prowazekii* and *R. typhi:* Typhus group. *R. rickettsia:* Spotted fever group
Diagnostics					
Primary isolation	Blood	Blood, tissue specimens (e.g. lymph nodes, heart valves)	Blood and tissue samples (e.g. valve samples, requires BSL-3 lab)	Blood (research purpose only, requires BSL-3 lab)	Arthropod Spotted fever group: Tissue (skin biopsy of the eschar). Typhus group: blood
Time for isolation	1–2 weeks of incubation	Weeks of incubation	1–2 weeks of incubation	Weeks of incubation	Skin biopsy: after appearance of eschar (5–12 days after tick bite); blood: during febrile stage of disease
Cultivation methods	Cell cultures (human promyelocytic leukemia HL60 cell line; different tick cell lines)	Columbia agar plates. Rare: shell-vial cell cultures.New: special liquid media (BAPGM-, BaLi-medium)	Cell cultures (Vero E6 cells) or axenic in acidified media with oxygen reduced atmosphere (ACCM)	Cell cultures (Vero E6 cells, L929 mouse fibroblasts)	Various tick cell lines; shell-vial cell cultures (Vero E6 cells)
Biochemical identification	n/a	Inert (oxidase, and catalase-negative, do not produce acid from carbohydrates). Production of peptidases.	n/a	n/a	n/a
Detection by microscopy	Not stainable by Gram.Giemsa- or Wright-stain: ok (peripheral blood smears, limited by observer expertise)	Gram staining: poor. Gimenez and Warthin–Starry stainings: ok. Giemsa staining (*B. bacilliformis*-blood smears). Immunohistochemistry or FISH: experimental use	Gram staining: Gram-variable. Gimenez staining: ok Immunohistochemistry and FISH: experimental use	Gram staining: not possible. Immunohistochemistry, or FISH: HC, direct IF and fluorescent probes in blood and tissue specimens for experimental use	n/a
Serology-based methods	IFA gold standard (cross-reactivity with *Ehrlichia* spp., *Rickettisa* spp. and *C. burnetii*). IgM not used independently because of unspecificity	IFA, ELISA (new) cross-reactivity with *Mycoplasma* spp. *Chlamydia* spp. and *C. burnetii* (also cause endocarditis)	IFA gold standard using serum samples for the detection of IgM and IgG antibodies against both phase I and II antigens. ELISA: lack of sensitivity, used as a screening method. Cross-reactivity with *Legionella* spp. or *Bartonella* spp.	IFA gold standard, detection of IgM and IgG antibodies in scrub typhus patients. ELISA: whole-cell antigen, surface proteins antigens with increased sensitivity and specificity Rapid tests: anti-IgM antibodies against 56 kD antigen.	IFA: IgG and IgM could persist for months and cross-reactivity with other bacteria. ELISA: commercially available.

Table 4. Continued

	A. phagocytophilum	*Bartonella* spp.	*C. burnetii*	*Orientia* spp.	*Rickettsia* spp.
PCR-based detection	16S-rRNA and *msp2* gene (confirmation by sequencing)	16S-rRNA, riboflavin synthase gene (*ribC*), and 16S-23S rRNA intergenic region sequences and others	IS1111	16S-rRNA, *47kD*, *56 kD* or *groEL* genes	Citrate synthetase gene (*gltA*) *ompA*, *ompB*, 23S-5S interspacer region
Novel approaches	Metagenomics NGS	Combination from liquid culture and PCR droplet digital PCR technology. MALDI-TOF MS for species identification	MALDI-TOF MS: to differentiate between phase I and phase II or SCV- and LCV-type organisms	Metagenomic NGS probe-based sequence capturing	MALDI-TOF MS from infected ticks
Drawbacks	Cannot be grown on cell-free media. Slow growth	Direct detection from peripheral blood: limited value (except for *B. bacilliformis*). Tissue specimens: invasive medical procedures. Sample enrichment: limited by bacterial slow growth.	Diagnostic test available: time-consuming, reduced sensitivity. Cultivation limited (BSL-3 laboratory)	Diagnostic test available: gold standard tests IFA limited sensibility and specificity. In highly endemic regions, acute and subsided infections cannot be reliably differentiated using IgM	Cultivation limited (BSL-3 laboratory)
Aspects already solved with current diagnostic methods	PCR-procedures relatively well established	Sensitivity and specificity of IFA satisfactory; PCR procedures well established	n/a	Rapid tests: IgM detection with high specificity and sensitivity. Limitations: variations of local strains.	Elimination of cross-reactions between groups by preabsorbing
Possible improvement	Specificity of serologic tests, confirmation of specific PCR amplification by sequencing	Direct pathogen detection: noninvasive patient sampling, better sample pre-enrichment, faster cultivation. Serology: improve of cross-reactivity	Sensitivity and specificity of serology, PCR and cultivation tests.	Faster, sensitive direct pathogen detection from blood or tissue samples/swabs. Short time cultivation outside BSL-3 labs for improved direct detection. Increased sensitivity and specificity of serology. Improved tests for acute vs subsided infection in highly endemic regions	Higher specificity of routine serological assays (ELISA) for differentiation of antibodies against different *Rickettsia* spp.

n/a: not available.
NGS: next-generation sequencing.

The technologies left, immune assays and PCR testing, both suffer from problems with sensitivity, specificity, availability of reagents and instruments, and significant cross-reactivity between microbial antigens or high homology of nucleic acid sequences. Hence, alternative methods -or optimized versions of existing ones- need to be made available for diagnostics in routine laboratories satisfying product development and quality control procedures (see the Text box).

Text box:

DIAGNOSTIC PRODUCT DEVELOPMENT

Diagnostic detection and characterization of pathogenic microorganisms is a science in itself, governed by its own medical subspecialty ('clinical microbiology'). The initial design of diagnostic tools as such and the subsequent research and development process are highly regulated and subject to a variety of risks analysis and quality control procedures. The test development roadmap and diagnostic decision-making should lead to the ultimate design and manufacturing of tests that withstand the highest control criteria and measures (Lathrop *et al.* 2016; Garcia *et al.* 2019). Test design and intellectual property are usually intrinsically interwoven although in many cases old-fashioned trade secrets are important as well. As a minimum, at the end of the development process, and sometimes earlier as well, a test should be subjected to conditions of routine use. This is usually done in the clinical laboratory setting where the test will be exploited once the test kit will be finalized. At this stage, all concepts of the test need to be locked and secured. The availability of well-characterized positive and negative samples, as well as the time-to-results of the reference methods, may extend the timelines of the study. While fast-track for regulatory clearance may be allowed exceptionally in the case of pandemics (as for the COVID-19), this is not the general case.

Modern test development should also consider sustainability issues. Limiting the amount of plastics used and the impacts of production processes and wastes (e.g. by using smaller boxes, assuring longer shelf life and increased test stability) are important for environmental protection, as well as providing for easier transport and handling of the tests by customers.

Once tests have been designed, validated and verified, various additional hurdles need to be conquered. Continuous quality assessment during the production of a test is mandatory and complex. In big *in vitro* diagnostics companies, it has been calculated that ~20% of the production workload is partaking in quality monitoring. This ranges from assessing the chemical purity of raw ingredients to defining in detail the shelf life of a test. All quality parameters need to be aligned with the preset quality criteria for a test. Not meeting such criteria can have huge legal and thereby financial consequences but it may also limit the availability of tests to customers and patients. It has to be realized that with the implementation of artificial intelligence and machine learning approaches, also the routing of product development will change and be modernized in the near future.

Diagnostic testing is at the heart of the global battle against infection and should be taken very seriously. The performance of a test needs to be uniform at a global level. This implies that, in principle, tests should be functional all over the world. Obviously, test costs are also extremely important and a hard-to-overcome obstacle between global versus more restricted use. In remote settings, a point-of-care test format may be important since the flexibility of use is a 'must' when centralized, modern facilities are lacking.

For fundamental improvement in diagnostic efficacy, there are two crucial factors requiring attention: (i) on the medical doctor's side there must be an increase of awareness (via continuous medical education) and knowledge about infections caused by these pathogens, symptoms and correct patient sampling (Lamont et al. 2020). The patient sample should be accompanied by suitable medical information to properly guide the diagnostic laboratory. In addition, (ii) on the laboratory side, there should be clear guidelines for the diagnostics of these pathogens based on current technologies that offer the highest sensitivity and specificity.

Still, the basic message of the five species-specific sections above is that significant shortcomings in the available test portfolio are observed and obvious. New approaches that might be useful for filling in some of the diagnostic gaps must be discussed. These considerations will mostly be technical in nature since real clinical studies have not yet been executed in much detail with the technologies we discuss below. This needs a clear priority setting in the near future although we realize that these methodologies may not all comply with the WHO ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid, equipment-free, delivered) (Okeke et al. 2011; Jiang et al. 2021). Even though the following methods may sound technically appealing, their implementation may require solutions to relatively simple but practically difficult-to-overcome problems.

Mass spectrometry

MALDI-ToF MS has become the gold standard method for bacterial identification over the past decade. Its specificity is significantly better than any other method (except for nucleic acid sequencing strategies). These, however, are much more time-consuming and costly than MALDI-ToF MS and hence noncompetitive at the current state of affairs. This implies that MALDI-ToF MS is the method of choice for bacterial identification where $>10^4$–10^5 cells are available for testing. In many cases, and this accounts not only for infections caused by fastidious organisms but for all human pathogens, even the best and most appropriate clinical specimens contain a very small number of pathogens (Geebelen et al. 2022). This might result in false-negative diagnostic tests and this should always be considered in the interpretation of test results and the design of a therapeutic approach. For fastidious organisms, this amount of bacterial input is problematic and presents two major blocking items. First, obtaining sufficient biomass is a problem as conventional culture (e.g. on solid agar-based media) is usually not suitable. Also, if pathogen isolation depends on host cell-containing culture media, host cell presence could affect the generation of clean species-specific spectra in the MALDI-ToF MS. Second, there will be significant biohazard and possible contamination of expensive key equipment can occur. This implies that only inactivated biomass can be analyzed if it is available at sufficient quantity and purity. Therefore, MALDI-ToF MS is unlikely to become widely available any time soon for routine clinical microbiology testing of fastidious bacterial species.

Scientific publications on the use of MALDI-ToF MS for species-identification of fastidious bacteria are relatively rare. There are no papers that deal with the proteomic detection and identification of *Anaplasma* spp. and *Orientia* spp. that are also absent from the databases of commercially available routine MALDI-ToF MS taxonomy systems. Studies are available where different species of ticks are identified by MALDI-ToF MS (Yssouf et al. 2013) and then the *Anaplasma* spp. present are subsequently detected by molecular means (Huynh et al. 2021). Similar studies have been published for ticks carrying *Bartonella* spp., *C. burnetii* and *Rick-*

ettsia spp. (Sevestre et al. 2021). For *Orientia* spp. and *C. burnetii* protein fingerprints of activated macrophages have been reported to be specific for the bacterial species used for their activation. In these cases, MALDI-ToF MS allowed indirect detection of these species based on bacterial indicator peaks present in the MS spectra (Ouedraogo et al. 2012). For *Rickettsia* spp., there is a single study that shows the successful distinction of infected from non-infected ticks directly based on the MALDI-ToF MS spectra via unidentified protein peaks in the profiles derived from the tick extracts. At best, this is another form of indirect detection of *Rickettsia* spp. but an important illustration of the diagnostic power and practical feasibility of MALDI-ToF MS in this field (Yssouf et al. 2015). For *C. burnetii*, the successful MS-based discovery of species-specific protein biomarkers has been reported but this has not been translated into a pragmatic routine identification approach yet (Shaw et al. 2004). *Bartonella* spp., however, can be successfully identified at species level using MALDI-ToF MS. Various species can be distinguished from cultures and this is really nearing routine application (Fournier et al. 2009). In conclusion, most fastidious organisms are hard to identify using MALDI-ToF MS although proof of principle assays have been developed for some species. Interestingly, the MALDI-ToF MS technology can be used to define whether or not ticks are infected or colonized with the fastidious pathogens we discuss here (Yssouf et al. 2015).

Alternative MS technologies may be more suited for the analysis of small amounts of microbial biomass (in the order of hundreds of cells). These approaches can detect species-specific peptides at ultimate sensitivity and specificity. The major problem associated with this approach is that the equipment (e.g. Quadrupole Time-of-flight (QTOF) MS, Ion Trap (ORBITRAP) MS, etc.) is expensive, is selectively suited for the analyses of single specimens at a time, and requires 30–60 min per analysis (Charretier and Schrenzel 2016). Again, this is not (yet) compatible with the diagnostic need of a routine diagnostic laboratory and hence is also not suited for screening purposes although a presumably infected patient would clearly benefit from a definitive diagnosis. On the other hand, all innovative MS methods can also be applied to host materials and full blood or skin proteomes can be differentiated using various MS approaches (Haas et al. 2016). MS may offer excellent sensitivity and specificity but, in the end, every sample turns into a project, taking an amount of time for analysis that is incompatible with adequate clinical care. This leads to the conclusion that—when fastidious pathogens can be successfully cultured—their identification by MS can be implemented successfully as long as this would not compromise biosafety (e.g. by using inactivated bacteria). In addition, new MS technologies will continue to mature, and once available to diagnostic laboratories with an affordable high-quality protocol such tests will successively replace MALDI-ToF MS. However, this perspective is still at least a decade away from clinical practice (Charretier et al. 2015).

Genome sequencing technology

Next-generation sequencing allows for rapid and affordable elucidation of primary sequences for complete microbial genomes. Such whole-genome sequencing (WGS) approaches allow for the mapping of all genes and regulatory regions important for the coordinated expression of the genome. This implies that WGS can be used for clinical diagnostics. First, it allows for the sequencing of all nucleic acid molecules in a clinical specimen, and whether the molecules are of host or pathogen origin does not make a difference as long as these sequences can be filtered later in the workflow (Maljkovic Berry et al. 2019). Essentially, all pathogens, fastidious ones included, can be detected in a sample extract (given that a significant number of reads are detectable). This opens new broad-spectrum diagnostic avenues. In the field of the detection of meningitis-causing pathogens, the value of this methodology has already been demonstrated (Wilson et al. 2019). Also, this methodology can be used to generate complete inventories of the various microbiomes that exist in and on eukaryote bodies. Mapping these microbial communities in full detail will have a diagnostic impact and will help to develop health-promoting measures by aimed intervention in the resident microbiota, for instance by supplementation of organisms that are absent or underrepresented. Second, if nucleotide sequences of complete pathogen genomes have been solved, existing but also new antibiotic resistance genes, virulence factors or genes defining metabolic pathways might be detected. Such genetic subcatalogs can be used to direct optimal treatment of the infected host. Third, when whole-genome sequences are known for various isolates from within a single species, these can then be compared for (non)identity. This will help to define the genetic population structure of a species as well as provide a framework for epidemiological tracing of infectious agents to improve infection control measures, e.g. in hospitals to identify or to exclude outbreak scenarios. Although bioinformatic pipelines are not in broad use in routine clinical microbiology, they are getting more and more involved in scientific infection control measures (Schultze et al. 2021) and are expected to gain access to routine laboratories soon.

For the fastidious organisms targeted here, several more or less detailed genome studies have been published. Comparative genome studies were presented for *A. phagocytophilum* and methods for purifying *Anaplasma* genomes from infected cell lysates were developed (Dugat et al. 2014). For *O. tsutsugamushi*, a successful genome capturing method was also developed that will surely lead to the elucidation of many more genomes in the near future (Elliott et al. 2021). This will help to refine the taxonomic positioning of the species and will also reveal potentially important new targets for more specific diagnostic tests. After the first publication of a rickettsial genome in 2004 (Merhej and Raoult 2011), ~130 publications followed, describing basic microbiological and infection-related features of the species including many focusing on species definition and taxonomic positioning. Although many genomes have been shared for *Rickettsia* spp. and their biology has been studied in better detail subsequently, this has as yet not generated novel diagnostic tools ready for routine use. Interestingly, it has been shown that Oxford Nanopore long-read technology can be used well for genome sequencing of *Rickettsia* spp. (Elliott et al. 2020). This technology is close to be compatible with point of care diagnostics also in resource-limited settings. Therefore, this development will closely be followed by diagnostic microbiologists worldwide. For *C. burnetii*, many genome studies have been published as well and the research topics were comparable for those of the other fastidious bacterial species discussed before in this section. In this case, putative genome testing useful for environmental detection of the species has been developed that is based on a selective whole-genome amplification methodology (Cocking et al. 2020). This shows that genome sequencing may be on the verge of a diagnostic breakthrough. Finally, for *Bartonella* spp., after the publication of the first genome in the year 2004 (Alsmark et al. 2004), further genomes were published in 2010 representing strains from woodland rodents (Berglund et al. 2010). New species were suggested based on genome sequence data (Chomel et al. 2012) and comparative virulome studies were performed (Tay et al. 2018), but as shown for other fastidious bacterial species, there have been no major new developments in the field of routine

medical diagnostics as yet. In conclusion, WGS has improved our understanding of the taxonomy and pathogenicity of fastidious pathogens. Translation of this knowledge in new diagnostic approaches is still lagging behind but new strategies are being unveiled continuously. This is bound to have a serious impact on the diagnostic field.

Other OMICS technologies

Besides the 'culturomic', proteomic and genomic technologies outlined in the previous sections, a variety of additional high-throughput methodologies have been developed. Lipidomics assembles the global analyses of biological lipid molecules, whereas glycomics covers the complete study of all sugar and polysaccharide molecules in a test sample (Veenstra 2021). Both technologies have not been applied for routine microbiologic diagnostics yet. Transcriptomics catalogs all genes that are expressed under a given condition, whereas metabolomics represents the accumulated technology suited for mapping type and concentration of small molecules, including cellular metabolites (Guo et al. 2019). Both technologies might help to identify pathogen's adaption to the host environment (e.g. upregulation of efflux pumps resulting in antimicrobial resistance, adaption to different oxygen levels in tissues, etc.). All these, and a few more, technologies are still at an experimental research stage and not yet suited for broad application in the microbiology diagnostic laboratory setting. However, as happened very rapidly with MS-driven proteomics, these technologies will be introduced progressively in clinical laboratories, initially for complex, individual and mostly rare cases.

Concluding remarks

Fastidious pathogens need highly specific laboratory methods for detection, biological amplification, and further phenotypic and molecular characterization. Many fastidious pathogens can be cultivated to a certain extent, but the conditions required are too complex to be maintained by routine diagnostic microbiology laboratories. In addition, biological amplification leading to positive cultures often poses increased biohazard risks. This is reflected by the need to develop specialized national expert laboratories sometimes even focusing on a single pathogen species. Serological tests are sometimes a useful option but often complicated by lack of sensitivity, specificity and availability (of reagents). Further, confounding cross-reactivity between pathogen species is often observed. This puts immunological testing at a distinct disadvantage, although in some cases serology is still considered as the gold standard for *in vitro* diagnostics (IVD). Essentially, molecular testing using nucleic acid amplification suffers from the same drawbacks although sensitivity and specificity are considered satisfactory. However, this technology requires intact pathogens or their remaining DNA to be present in certain quantities, so valid diagnosis may be hampered by sampling errors (e.g. non-precise fine needle aspiration). Hence, optimal and high-quality testing is not available for the human pathogen genera discussed herein. In consequence, although incremental improvement in the existing tests will surely continue, a significant diagnostic paradigm shift toward the use of new technology such as MS and WGS is not to be expected soon. Newer 'omics' technologies promise test improvements but it will take many years for the technologies to lead to IVD-approved tests that are also affordable and available where they are needed most—in the clinical microbiology laboratories worldwide. Fastidious organisms will continue to pose significant diagnostic challenges over the years to come.

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Authors' contributions

All authors contributed to this manuscript with their specific expertise and writing. DJV, AVB and VAJK coordinated the writing of this manuscript. Authors did contribute to the specific sections of their particular expertise (FDVL: *Anaplasma* spp.; DJV and VAJK: *Bartonella* spp.; SFF and MK: *C. burnetii*; CK and SUS: *Orientia* spp.; GD: *Rickettsia* spp.; AVB and SO: sections on development of new diagnostics).

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Conflict of interest statement. AVB and SO are employees of bioMérieux, a company designing, developing and marketing tests in the domain of infectious diseases. The company was not involved in the design of the current study and the opinions expressed are those of the authors and may be different from formal company opinions and policies. VAJK is an inventor on a pending patent (a method for inducing an immune response against *B. bacilliformis* in a patient; application EP21158777.9) and he holds also other patents in the field of *Bartonella* (modulation of angiogenesis by *B. henselae* (German patent 10351627.1-41; European patent 1680499; US patent 7638489); composition for cultivating sophisticated bacteria (German patent 102008022333.6-09; European patent 09737819.4; US patent 12/911396)).

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