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

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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 burnetti, 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 status 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 diarrhoea-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
fast on cell-free and semi-solid agar-based culture media (e.g. Staphylococcus aureus), whereas others require the support of liq-
uid media sometimes with balanced gaseous phases included (e.g. Clostridiods difficile and many other anaerobic organisms). In the
most complicated culture tests, bacteria may require the intra-
cellular compartment of eukaryotic host cells to provide the right
environment for replication (e.g. Rickettsia spp.) (Valaříková et al.
2016). The combination of such methodologies has been defined as ‘culuromics’ (meaning microbial cultivation techniques us-
ing 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 tech-
ology to be used is strongly dependent on the nature and qual-
ity of the biological specimen obtained as well as the metabolic
characteristics of the infectious organism involved. Tissue biops-
ies, 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 mi-
crobiology laboratory (van Belkum and Rochas 2018). It has to
be stated explicitly that cultivation tests have significant short-
comings in sensitivity and specificity and also affect the timeli-
ness of downstream testing for antimicrobial susceptibility.
For the latter, rapid replication of bacterial pathogens is a prerequi-
site (Anton-Vazquez et al. 2021) and slow or no growth on solid
media combined with the absence of minimal inhibitory concen-
trations (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 algo-
rithms available to identify infections caused by such pathogens.
Several of such algorithms will be presented throughout the cur-
rent manuscript.

Next to culture, the microbiology laboratory is usually
equipped with tools and expertise for immunological and molecu-
lar testing although this varies depending on geographic localiza-
tion and financial support (Vandenberg et al. 2021). Both bacteria-
derived antigens (e.g. for detection of infections caused by Le-
gionella pneumophila) and serum antibodies (e.g. for detection of
infections caused by Borrelia burgdorferi) can be diagnostically tar-
geted. 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 clini-
cally. Indirect immune testing uses patient sera for the detec-
tion 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 visu-
alize bacterial cells directly in clinical specimens or cultured ma-
terial using in situ protocols for detection of bacterial antigens,
either surface located or intracellular ones, although these diag-
nostic 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 hybridiza-
tion 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 re-
action (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. burneti, Orientia spp. and
Rickettsia spp., we will describe the epidemiology, clinical features of
disease, diagnostic approaches currently in use, recent develop-
ments and shortcomings still to be addressed (see summary Ta-
ble 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 in-
tracellular bacterial species that reside within cytoplasmic vac-
uoles 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 rel-
ance 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 symp-
tomatic 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. can-
not 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         | Worldwide, human   | Worldwide,      | 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 |
| distribution         | infections reported from North America, Europe and Asia | B. bacilliformis restricted to South America |             |               |                |
|                      |                    |                 |             |               |                |
| Disease prevalence   | USA 2017: 5,762    | 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 |
| worldwide/Europe     | cases/Europe and Asia rare |             |             |               |                |
|                      |                    |                 |             |               |                |
| Zoonotic species of  | USA: white-footed | High risk for  | Several mammalian hosts, mainly rodents (especially rats and mice) |                |                |
| host                 | mouse; Europe:     | transmission:   |             |               |                |
|                      | under debate       | livestock (e.g. sheep, goats, cows) |             |               |                |
|                      |                    | Low risk for   |             |               |                |
|                      |                    | transmission:   |             |               |                |
|                      |                    | wild animals and pets |             |               |                |
|                      |                    |                 |             |               |                |
| Main vectors         | Ixodes ricinus     | Mainly airborne | Leptotrombidium spp. (L. pallidum, L. scutellare, L. deliensis and others), exceptionally other mite species |                |                |
|                      | complex            | transmission but the role of ticks as a vector remain to be elucidated |             |               |                |
|                      |                    |                 |             |               |                |
| **Growth behavior and pathogenicity** |                    |                 |             |               |                |
| Growth-specific      | Obligate           | 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 | Obligate intracellular: cell culture |
| requirements         | intracellular: cell culture |             |             |               |                |
| Extracellular        | 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 |
| bacterial products   |                    |                 |             |               |                |
|                        | A. phagocytophilum | Bartonella spp. | C. burnetii | Orientia spp. | Rickettsia spp. |
|------------------------|-------------------|----------------|-------------|---------------|----------------|
| **Principal bacterial** |                   |                |             |               |                 |
| adhesins               | AipA, Asp14,      | B. henselae:  | 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) |
|                        | OmpA, Msp2        | B. quintana:  |             |               |                 |
|                        |                   | Vomps.        |             |               |                 |
|                        |                   | B. bacilliformis: Brp |             |               |                 |
| **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 | Primary targets: macrophages, monocytes and dendritic cells, acute Q-fever: lung, liver, placentas, 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) |
| Microbiological diagnostics | A. phagocytophilum | Bartonella spp. | C. burnetii | Orientia spp. | Rickettsia spp. |
|----------------------------|-------------------|----------------|-------------|--------------|----------------|
| 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 | 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 |

1Not bona fide proven.
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 automatatable 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 a significant proportion 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 automatatable 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).

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 1. A. phagocytophilum. (A) A. phagocytophilum Webster strain grown in HL60 cells (arrow), magnification x1000. Scale bar: 2 μm. (B) IFA for detection of anti-A. phagocytophilum IgG antibodies, magnification x400. Cutoff titer for IgG ≥ 1.64. Scale bar: 20 μm.

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. koehleri and B. vinsonii subsp. berkoffii might also represent important human pathogenic bacteria (Breitschwerdt et al. 2010a, b), therefore more diagnostic development is required also for these neglected pathogens.

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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).
best. This, in combination with the relatively low bacteremia level in the patients, affects the sensitivity of the diagnostic cultivation procedures, which is \(\sim 20-31\%\) 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 2. B. henselae. (A) B. henselae colonies on Columbia blood agar plate (cultivation time: 8 days, 37°C, 5% CO2). 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.
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 chuito*, 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 endosomes, 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 fromuffy 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-
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, Scar) followed by a phylogenetic analysis of the amplicon sequences is essential (Chitimia-Dober 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

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**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                                                 |

**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 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. helongiangensis | 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 (after 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.

| General information | A. phagocytophilum | Bartonella spp. | C. burnetii | Orientia spp. | Rickettsia spp. |
|---------------------|-------------------|----------------|-------------|--------------|----------------|
| 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. rickettsiae: 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 Skin biopsy: after appearance of eschar (5–12 days after tick bite); blood: during febrile stage of disease |
| Time for isolation | 1–2 weeks of incubation | Weeks of incubation | 1–2 weeks of incubation | Weeks of incubation | |
| 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. Giemena 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., Rickettsia 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. |

| 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 | IS111 | 16S-rRNA, 47kD, 56 kD or groEL genes |
| **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 | Citrate synthetase gene (gltA) ompA, ompB, 23S-5S interspacer region |
| **Drawbacks** | Cannot be grown on cell-free media. Slow growth | Direct detection from peripheral blood: limited value (except for B. baviiiformis). Tissue specimens: invasive medical procedures. Sample enrichment: limited by bacterial slow growth. | 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. |
| **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. | 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 lead to the ultimate design and manufacturing of tests that withstand the highest control criteria and measures. 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.

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 >10⁴–10⁵ 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 (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 (Schulz 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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References

Aistleitner K, Jeske R, Wölfel R et al. Detection of Coxiella burnetii in heart valve sections by fluorescence in situ hybridization. J Med Microbiol 2018;67:537–42.
Alsmark CM, Frank AC, Karlberg EO et al. The louse-borne human pathogen Bartonella quintana is a genomic derivative of the zoonotic agent Bartonella henselae. Proc Natl Acad Sci USA 2004;101:9716–21.
Andersson SGE, Stothard DR, Fuerst P et al. Molecular phylogeny and rearrangement of rRNA genes in Rickettsia species. Mol Biol Evol 1999;16:887–95.
Angelakis E, Mediannikov O, Jos S-L et al. Candidatus Coxiella masiliensis infection. Emerg Infect Dis 2016;22:285–8.
Anitharaj V, Stephen S, Pradeep J et al. Serological diagnosis of acute scrub typhus in Southern India: evaluation of InBios scrub typhus
detect IgM rapid test and comparison with other serological tests. J Clin Diagn Res 2016;10:DC07–10.

Anton-Vazquez V, Hine P, Krishna S et al. Rapid versus standard antimicrobial susceptibility testing to guide treatment of bloodstream infection. Cochrane database Syst Rev 2021.5 CD013235.

Arraga-Alvarado CM, Parra OC, Hegarty BC et al. Molecular evidence of Anaplasma platys infection in two women from Venezuela. Am J Trop Med Hyg 2014;91:1161–5.

Bakken JS, Dumler JS. Human granulocytic anaplasmosis. Infect Dis Clin North Am 2015;29:341–55.

Berglund EC, Ehrenborg C, Vinnere Pettersson O et al. Bartonella grahamii in micro-populations of woodland rodents. BMC Genomics 2010;11:152.

Bergmans AM, Feeters MF, Schellekens JF et al. Pitfalls and fallacies of cat scratch disease serology: evaluation of Bartonella henselae-based indirect fluorescence assay and enzyme-linked immunosorbent assay. J Clin Microbiol 1997;35:1931–7.

Blacksell SD, Bryant NJ, Paris DH et al. Scrub typhus serologic testing with the indirect immunofluorescence method as a diagnostic gold standard: a lack of consensus leads to a lot of confusion. Clin Infect Dis 2007;44:391–401.

Breitschwerdt EB, Maggi RG, Lantos PM et al. Bartonella vinsonii subsp. berkhoffii and Bartonella henselae bacteremia in a father and daughter with neurological disease. Parasit Vectors 2010;3:29.

Breitschwerdt EB, Maggi RG, Robert Mozayeni B et al. PCR amplification of Bartonella koehlerae from human blood and enrichment blood cultures. Parasit Vectors 2010;3:76.

Brooke RJ, Kretzschmar MEE, Mutters NT et al. Human dose response relation for airborne exposure to Coxielia burnetii. BMC Infect Dis 2013;13:488.

Brouqui P, Bacellar F, Baranton G et al. Guidelines for the diagnosis of tick-borne bacterial diseases in Europe. Clin Microbiol Infect 2004;10:1108–32.

Bruneval P, Choucair J, Paraf F et al. Detection of fastidious bacteria in cardiac valves in cases of blood culture negative endocarditis. J Clin Pathol 2001;54:238–40.

Caponetti GC, Pantanowitz L, Marconi S et al. Evaluation of immunohistochemistry in identifying Bartonella henselae in cat-scratch disease. Am J Clin Pathol 2009;131:250–6.

Caputo A, Fournier P-E, Raoult D. Genome and pan-genome analysis to classify emerging bacteria. Biol Direct 2014;9:14.

Carcopino X, Raoult D, Bretelle F et al. Managing Q fever during pregnancy: the benefits of long-term cotrimoxazole therapy. Clin Infect Dis 2007;45:548–55.

Centers for Disease Control and Prevention (CDC). Ehrlichiosis and Anaplasmosis | 2008 Case Definition. 2021.

Charretier Y, Daouvalder O, Franceschi C et al. Rapid bacterial identification, resistance, virulence and type profiling using selected reaction monitoring mass spectrometry. Sci Rep 2015;5:13944.

Charretier Y, Schrenzel J. Mass spectrometry methods for predicting antibiotic resistance. Proteomics Clin Appl 2016;10:964–81.

Chen SCA, Kontoyiannis DP. New molecular and surrogate biomarker-based tests in the diagnosis of bacterial and fungal infection in febrile neutropenic patients. Curr Opin Infect Dis 2010;23:567–77.

Chitimia-Dobler L, Rieß R, Kahl O et al. Ixodes inopinatus: occurring also outside the Mediterranean region. Ticks Tick Borne Dis 2018;9:196–200.

Chochlakis D, Ioannou I, Tslelentis Y et al. Human Anaplasmosis and Anaplasma ovis variant. Emerg Infect Dis 2010;16:1031–2.

Chomel BB, McMillan-Cole AC, Kasten RW et al. Candidatus Bartonella merieuxii, a potential new zoonotic Bartonella species in Canids from Iraq. PLoS Negl Trop Dis 2012;6:e1843.

Clark NJ, Soares Magalhães RJ. Airborne geographical dispersal of Q fever from livestock holdings to human communities: a systematic review and critical appraisal of evidence. BMC Infect Dis 2018;18:218.

Cocking JH, Deberg M, Schupp J et al. Selective whole genome amplification and sequencing of Coxiella burnetii directly from environmental samples. Genomics 2020;112:1872–8.

Dangel I, Koempf D, Fischer SF. Comparison of different commercially available enzyme-linked immunosorbent assays with immunofluorescence test for detection of phase II IgG and IgM antibodies to Coxiella burnetii. J Clin Microbiol 2020;58:e00951–19.

Dugat T, Loux V, Marthey S et al. Comparative genomics of first available bovine Anaplasma phagocytophilum genome obtained with targeted sequence capture. BMC Genomics 2014;15:973.

Dumler JS, Madigan JE, Pusterla N et al. Ehrlichioses in humans: epidemiology, clinical presentation, diagnosis, and treatment. Clin Infect Dis 2007;45:545–51.

Elliott I, Batty EM, Ming D et al. Oxford Nanopore MinION sequencing enables rapid whole genome assembly of Rickettsia typhi in a resource-limited setting. Am J Trop Med Hyg 2020;102:408–14.

Elliott I, Pearson I, Dahal P et al. Scrub typhus ecology: a systematic review of Orientia in vectors and hosts. Parasit Vectors 2019;12:513.

Elliott I, Thangnimitchok N, de Cesare M et al. Targeted capture and sequencing of Orientia tsutsugamushi genomes from chiggers and humans. Infect Genet Evol 2021;91:104818.

Fournier P-E, Couderc C, Buffet S et al. Rapid and cost-effective identification of Bartonella species using mass spectrometry. J Med Microbiol 2009;58:1154–9.

Fournier P-E, Marrie TJ, Raoult D. Diagnosis of Q fever. J Clin Microbiol 1998;36:1823–34.

Fournier P-E, Raoult D. Suicide PCR on skin biopsy specimens for diagnosis of rickettsioses. J Clin Microbiol 2004;42:3428–34.

Garcia M, Fares-Gusmao R, Sapsford K et al. A Zika reference panel for molecular-based diagnostic devices as a US food and drug administration response tool to a public health emergency. J Mol Diagnostics 2019;21:1025–33.

Garcia-Quintanilla M, Dichter AA, Guerra H et al. Carrion’s disease: more than a neglected disease. Parasites Vectors 2019;12:141.

Ge Y, Rikihisa Y. Subversion of host cell signaling by Orientia tsutsugamushi. Microbes Infect 2011;13:636–48.

Geebelen L, Lernout T, Tersago K et al. Detection of fastidious bacteria in cardiac valves in cases of blood culture negative endocarditis. J Clin Pathol 2001;54:238–40.

Guo J, Sun Y, Luo X et al. De novo transciptome sequencing and comparative analysis of Haemaphysalis flava Neumann, 1897 at larval and nymph stages. Infect Genet Evol 2019;75:104008.

Haaas CT, Roe JK, Pollara G et al. Diagnostic ‘omics’ for active tuberculosis. BMC Med 2016;14:37.

Hawker JI, Ayres JG, Blair I et al. A large outbreak of Q fever in the West Midlands: windborne spread into a metropolitan area? Commun Dis Public Health 1998;1:180–7.

Hechmey KE, Stevens RW, Sasowski S et al. Discrepancies in Weil–Felix and microimmunofluorescence test results for Rocky Mountain spotted fever. J Clin Microbiol 1979;9:292–3.

Heppell CW, Egan JR, Hall I. A human time dose response model for Q fever. Epidemiics 2017;21:30–8.

Hobson C, Le Brun C, Beaureuelle C et al. Detection of Bartonella in cat scratch disease using a single-step PCR assay kit. J Med Microbiol 2017;66:1596–601.

Houplikian P, Raoult D. Blood culture-negative endocarditis in a reference center. Medicine (Baltimore) 2005;84:162–73.
Huang HN, Diarra AZ, Pham QL et al. Morphological, molecular, and MALDI-TOF MS identification of ticks and tick-associated pathogens in Vietnam. PLoS Negl Trop Dis 2021;15:e0009813.

Ihnatko R, Shaw E, Toman R. Proteome of Coxiella burnettii. In: Toman R, Heinzen RA, Samuel JE et al. (eds) Coxiella Burnetti. Recent Advances and New Perspectives in Research of the Q Fever Bacterium. Vol. 984. Dordrecht: Springer. 2012, 105–50.

Ismael N, McBride JW. Tick-borne emerging infections. Clin Lab Med 2017;37:317–40.

Jensen WA, Fall MZ, Rooney J et al. Rapid identification and differentiation of Bartonella species using a single-step PCR assay. J Clin Microbiol 2000;38:1717–22.

Jiang J, Marienau KJ, May LA et al. Laboratory diagnosis of two scrub typhus outbreaks at Camp Fuji, Japan in 2000 and 2001 by enzyme-linked immunosorbent assay, rapid flow assay, and Western blot assay using outer membrane 56-kD recombinant proteins. Am J Trop Med Hyg 2003;69:60–6.

Jiang N, Tansukawat ND, Gonzalez-Macia L et al. The prevalence of serotypes in circulating endothelial cells: a 6-year follow-up. Mod Pathol 2011;24:2715–27.

Körner S, Makert GR, Ulbert S et al. Improvement of the diagnostic sensitivity of tick-borne Bartonella henselae antibodies in human serum. J Clin Microbiol 2018;56:e01329–18.

Kelly DJ, Fuerst PA, Ching W et al. Scrub typhus: the geographic distribution of phenotypic and genotypic variants of Orientia tsutsugamushi. Clin Infect Dis 2009;48:S203–30.

Kim YJ, Yeo S-J, Park S-J et al. Development of a specific and sensitive enzyme-linked immunosorbent assay as an in vitro diagnostic tool for detection of Bartonella henselae antibodies in human serum. J Clin Microbiol 2018;56:e01329–18.

Kock F, Hauptmann M, Osterloh A et al. Orientia tsutsugamushi is highly susceptible to the RNA polymerase switch region inhibitor Corallopyronin A in vitro and in vivo. Antimicrob Agents Chemother 2018;62:e01732–17.

Körner S, Makert GR, Uhlert S et al. The prevalence of Coxiella burnetti in hard ticks in Europe and their role in Q Fever transmission revisited: a systematic review. Front Vet Sci 2021;8:1–16.

La Scola B, Raoult D. Diagnosis of Mediterranean spotted fever by cultivation of Rickettsia conorii from blood and skin samples using the centrifugation-shell vial technique and by detection of R. conorii in circulating endothelial cells: a 6-year follow-up. J Clin Microbiol 1996b;34:2722–7.

La Scola B, Raoult D. Laboratory diagnosis of rickettsioses: current approaches to diagnosis of old and new rickettsial diseases. J Clin Microbiol 1997;35:2715–27.

La Scola B, Raoult D. Serological cross-reactions between Bartonella quintana, Bartonella henselae, and Coxiella burnetti. J Clin Microbiol 1996a;34:2270–4.

La Scola B, Rydkina L, Ndiokubwayo JB et al. Serological differentiation of murine typhus and epidemic typhus using cross-absorption and Western blotting. Clin Diagn Lab Immunol 2000;7:612–6.

Lamont RF, van den Munchhof EH, Lief BM et al. Recent advances in cultivation-independent molecular-based techniques for the characterization of vaginal eubiosis and dysbiosis. Fac Rev 2020;9:21.

Lathrop JT, Jeffery DA, Shea YR et al. US food and drug administration perspectives on clinical mass spectrometry. Clin Chem 2016;62:41–7.

Leibler JH, Zakhour CM, Gadboke P et al. Zoonotic and vector-borne infections among urban homeless and marginalized people in the United States and Europe, 1990–2014. Vector Borne Zoonotic Dis 2016;16:435–44.

Lepidi H, Fournier P-E, Raoult D. Quantitative analysis of valvular lesions during Bartonella endocarditis. Am J Cardiol Pathol 2000;114:880–9.

Li H, Zheng Y-C, Ma L et al. Human infection with a novel tick-borne Anaplasma species in China: a surveillance study. Lancet Infect Dis 2015;15:663–70.

Lu M, Li F, Liao Y et al. Epidemiology and diversity of Rickettsiales bacteria in humans and animals in Jiangsu and Jiangxi provinces, China. Sci Rep 2019;9:13176.

Maggi RG, Duncan AW, Breitschwerdt EB. Novel chemically modified liquid medium that will support the growth of seven Bartonella species. J Clin Microbiol 2005;43:2651–5.

Maggi RG, Kempf VAJ, Chomel BB et al. Bartonella. In: Versalovic J, Caroll KC, Funke G et al. (eds) Manual of Clinical Microbiology. 10th edn. Washington, D.C.: American Society of Microbiology, 2011, 786–98.

Maggi RG, Mascarelli FE, Havenga LN et al. Co-infection with Anaplasma platys, Bartonella henselae and Candidatus Mycoplasma haemotoparvum in a veterinarian. Parasit Vectors 2013;6:103.

Maggi RG, Richardson T, Breitschwerdt EB et al. Development and validation of a droplet digital PCR assay for the detection and quantification of Bartonella species within human clinical samples. J Microbiol Methods 2020;176:106022.

Malyjovic Berry I Melendrez MC, Bishop-Lilly KA et al. Next generation sequencing and bioinformatics methodologies for infectious disease research and public health: approaches, applications, and considerations for development of laboratory capacity. J Infect Dis 2019;221:529–307.

Mallmann C, Siemoneit S, Schmiedel D et al. Fluorescence in situ hybridization to improve the diagnosis of endocarditis: a pilot study. Clin Microbiol Infect 2010;16:767–73.

Matei IA, Estrada-Peña A, Cutler SJ et al. A review on the eco-epidemiology and clinical management of human granulocytic anaplasmosis and its agent in Europe. Parasit Vectors 2019;12:599.

Maurin M, Raoult D. Q fever. Clin Microbiol Rev 1999;12:518–53.

McCaul TF, Williams JC. Developmental cycle of Coxiella burnetti: structure and morphogenesis of vegetative and sporogonic differentiations. J Bacteriol 1981;147:1063–76.

Merhej V, Raoult D. Rickettsial evolution in the light of comparative genomics. Biol Rev 2011;86:379–405.

Moron CG, Popov VL, Feng HM et al. Identification of the target cells of Orientia tsutsugamushi in human cases of scrub typhus. Mod Pathol 2001;14:752–9.

Murray GGR, Weinert RA, Rhule EL et al. The phylogeny of Rickettsia using different evolutionary signatures: how tree-like is bacterial evolution? Syst Biol 2016;65:265–79.

Musso D, Raoult D. Serological cross-reactions between Coxiella burnetti and Legionella micdadei. ClinDiagnostic Lab Immunol 1997;4:208–12.

Ohashi N, Nashimoto H, Ikeda H et al. Diversity of immunodominant 56-kDa type-specific antigen (TSAs) of Rickettsia tsutsugamushi. Sequence and comparative analyses of the genes encoding TSAs homologues from four antigenic variants. J Biol Chem 1992;267:12728–35.

Okaro U, Addisu A, Casanas B et al. Bartonella species, an emerging cause of blood-culture-negative endocarditis. Clin Microbiol Rev 2017;30:709–46.

Okeke IN, Peeling RW, Goossens H et al. Diagnostics as essential tools for containing antibacterial resistance. Drug Resist Updat 2011;14:95–106.
Ouedraogo R, Daumas A, Ghigo E et al. Whole-cell MALDI-TOF MS: a new tool to assess the multifaceted activation of macrophages. J Proteomics 2012; 75: 5523–32.

Paris DH, Dumler JS. State of the art of diagnosis of rickettsial diseases. Curr Opin Infect Dis 2016; 29: 433–9.

Paris DH, Phetsouvanh R, Tanganuchitcharntchai A et al. Orientia tsutsugamushi in human scrub typhus eschars shows tropism for dendritic cells and monocytes rather than endothelium. PLoS Neg Trop Dis 2012; 6 e1466.

Parola P, Paddock CD, Socolovschi C et al. Update on tick-borne rickettsioses around the world: a geographic approach. Clin Microbiol Rev 2013; 26 657–702.

Parte AC, Sardà Carbasse J, Meier-Kolthoff JP et al. List of Prokaryotic names with Standing in Nomenclature (LPSN) moves to the DSMZ. Int J Syst Evol Microbiol 2020; 70: 5607–12.

Peeling RW, Mabee D. Point-of-care tests for diagnosing infections in the developing world. Clin Microbiol Infect 2010; 16: 1062–9.

Peng H, Bilal M, Iqbal H. Improved biosafety and biosecurity measures and/or strategies to tackle laboratory-acquired infections and related risks. Int J Environ Res Public Health 2018; 15: 2697.

Pritt B, Dumler JS, Reller ME et al. Ehrlichia, Anaplasma, and related intracellular bacteria. In: Carroll KC, Pfaller MA, Landry ML (eds.). Manual of Clinical Microbiology. 12th edn. Washington, DC, USA. American Society of Microbiology, 2011, 1962–79.

Prudent E, Lepidi H, Angelakis E et al. Fluorescence in situ hybridization (FISH) and peptide nucleic acid probe-based FISH for diagnosis of Q fever endocarditis and vascular infections. J Clin Microbiol 2018; 56: 1–8.

Rahimian J, Raoldt D, Tang YW et al. Bartonella quintana endocarditis with positive serology for Coxiella burnetii. J Infect 2006; 53: e151–3.

Rajapakse S, Weeratunga P, Sivayoganathan S et al. Clinical manifestations of scrub typhus. Trans R Soc Trop Med Hyg 2017; 111: 43–54.

Raoldt D, Woodward T, Dumler JS. The history of epidemic typhus. Infect Dis Clin North Am 2004; 18: 127–40.

Rar V, Tkachev S, Tikunova N. Genetic diversity of Anaplasma bacteria: twenty years later. Infect Genet Evol 2021; 91: 104833.

Razans J, Rosel O, Radzijevskaja J et al. Prevalence and co-infection with tick-borne Anaplasma phagocytophilum and Babesia spp. in red deer (Cervus elaphus) and roe deer (Capreolus capreolus) in southern Norway: Int J Parasitol Parasites Wildl 2019; 8: 127–34.

Riess T, Dietrich F, Schmidt K V et al. Analysis of a novel insect cell culture medium-based growth medium for Bartonella species. Appl Environ Microbiol 2008; 74: 5224–7.

Salje J. Cells within cells: Rickettsiales and the obligate intracellular bacterial lifestyle. Nat Rev Microbiol 2021; 19: 375–90.

Sanchez SE, Vallejo-Esqueria E, Omsland A. Use of axenic culture tools to study Coxiella burnetii. Curr Protoc Microbiol 2018; 50 e52.

Schultzte TG, Ferstl PG, Villinger D et al. Molecular surveillance of carbapenem-resistant Gram-negative bacteria in liver transplant candidates. Front Microbiol 2021; 12: 791574.

Serra-Burriel M, Keys M, Campillo-Artero C et al. Impact of multi-drug resistant bacteria on economic and clinical outcomes of healthcare-associated infections in adults: systematic review and meta-analysis. PLoS One 2020; 15 e0227139.

Sevestre J, Diarra AZ, Oumarou HA et al. Detection of emerging tick-borne disease agents in the Alpes-Maritimes region, southeastern France. Ticks Tick Borne Dis 2021; 12: 101800.

Shaw EI, Moura H, Woolfitt AR et al. Identification of biomarkers of whole Coxiella burnetii phase I by MALDI-TOF mass spectrometry. Anal Chem 2004; 76: 4017–22.

Silaghi C, Santos AS, Gomes J et al. Guidelines for the direct detection of Anaplasma spp. in diagnosis and epidemiological studies. Vector Borne Zoonotic Dis 2017; 17: 12–22.

Springer A, Shuaib YA, Isaas MH et al. Tick fauna and associated Rickettsia, Thereloria, and Babesia spp. in domestic animals in Sudan (North Kordofan and Kassala States). Microorganisms 2020; 8: 1969.

Tay ST, Kho KL, Lye SF et al. Phylogeny and putative virulence gene analysis of Bartonella bovis. J Vet Med Sci 2018; 80: 653–61.

Todkill D, Fowler T, Hawker JI. Estimating the incubation period of acute Q fever, a systematic review. Epidemiol Infect 2018; 146: 665–72.

Tomassone L, Portillo A, Nováková M et al. Neglected aspects of tick-borne rickettsioses. Parasit Vectors 2018; 11: 263.

Valáríková J, Sekeyová Z, Škultéty L et al. New way of purification of pathogenic rickettsiae reducing health risks. Acta Virol 2016; 60: 206–10.

van Belkum A, Rochas O. Laboratory-based and point-of-care testing for MSSA/MRSA detection in the age of whole genome sequencing. Front Microbiol 2018; 9: 1–9.

Vandenberg O, Martiny D, Rochas O et al. Considerations for diagnostic COVID-19 tests. Nat Rev Microbiol 2021; 19: 171–83.

Veenstra TD. Omics in systems biology: current progress and future outlook. Proteomics 2021; 21: 2000235.

Wilson MR, Sample HA, Zorn KC et al. Clinical metagenomic sequencing for diagnosis of meningitis and encephalitis. N Engl J Med 2019; 380: 2327–40.

Xu G, Walker DH, Jupiter D et al. A review of the global epidemiology of scrub typhus. PLoS Negl Trop Dis 2017; 11: 1–27.

Yssouf A, Flaudrops C, Drali R et al. Matrix-assisted laser desorption ionization-time of flight mass spectrometry for rapid identification of tick vectors. J Clin Microbiol 2013; 51: 522–8.

Zhou Z, Nie K, Tang C et al. Phylogenetic analysis of the genus Anaplasma in Southwestern China based on 16S rRNA sequence. Res Vet Sci 2010; 89: 262–5.