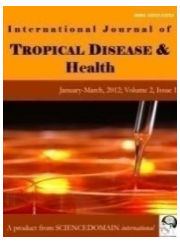

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# Current and Future Trends in the Clinical Diagnosis of Rickettsioses *Sensu lato*

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***Authors' contributions***

*This work was carried out in collaboration between all authors. Authors SM and DTG contributed equally and share first authorship. All authors read and approved the final manuscript.*

***Review Article***

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## ABSTRACT

**Aims:** The aim of this review is to present Rickettsioses *sensu lato*, with emphasis on their current and future clinical diagnosis. The review presents the conditions, the agents that cause them, and the current gold standards on their diagnosis in national and international reference centres. Additionally, this review covers the various emerging technologies available in the diagnosis of Rickettsioses and discusses their potential for future use as gold standards in the diagnosis of these diseases.
**Introduction:** The introduction presents Rickettsioses *sensu lato* and gives a broad overview of the conditions they cause, the issues associated with their current diagnosis and the need for their improved, earlier and more accurate diagnosis, in order to prevent current issues with false negatives, misdiagnosis or delay in the diagnosis associated with these conditions, which often renders them grave or lethal.
**Main Body:** The main body of the review presents in independent sections Rickettsias, Ehrlichia, Anaplasma, Bartonella and Coxiella and the conditions associated with each of these bacteria. Spotted fever, endemic typhus, human granulocytic anaplasmosis, human monocytic ehrlichioses, bartonellosis and Q-fever are some of the conditions associated with this group of proteobacteria. The emphasis is on the clinical diagnosis of these conditions and an overview of the current practice, gold standards in reference

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laboratories and improvements in these methodologies is presented. The last part of the review focuses on novel technologies in bacterial detection and their application specifically on Rickettsioses *sensu lato*, demonstrating how these technologies are being applied in this field and how they could improve current standards and resolve issues associated with the clinical diagnosis of rickettsioses.

**Conclusion:** Rickettsioses *sensu lato* are conditions associated with proteobacteria historically included in the Rickettsiaceae family, able to cause a number of conditions, often grave or lethal. One of the major issues associated with poor clinical outcome is the lack of early and accurate differential diagnostic methodologies. Current methods, including serological and molecular biology techniques have various advantages and disadvantages, which new technologies available or currently in development may be in a position to resolve and the issues associated with the institution of such technologies.

*Keywords: Rickettsia; Ehrlichia; Anaplasma; Bartonella; Coxiella; clinical diagnosis.*

## 1. INTRODUCTION

Significant advances in bacterial detection and clinical diagnosis of bacterial diseases have been achieved in the last decade and have opened the path for future developments. However, a lot of bacterial diseases remain misdiagnosed and, despite advances in antibiotics and the availability of curative interventions, they can often lead to serious conditions and death, with diagnosis arriving in very late stages or even post-mortem. One such group of bacteria, where methods for accurate and early diagnosis remain necessary are the "rickettsias *sensu lato",* here referred to as proteobacteria historically included in the Rickettsiaceae family, before phylogenetics investigations that placed genera *Bartonella* and *Coxiella* in other taxonomic subdivisions [1,2]. Besides these, the genus *Orientia*, causing scrub typhus, has recently been removed from the genus *Rickettsia* and classified as a separate genus in the Rickettsiaceae family. *Orientia* is shown to be endemic in eastern Asia and western Pacific, and is transmitted by mites, and is thus not included in this review, as is not *Neorickettsia* and *Wolbachia*.

Various diseases are attributed to "rickettsias *sensu lato*", including rickettsioses of the spotted fever group (SFG), recrudescent typhus, typhus transmitted by cat fleas, rickettsioses varicelliform, ehrlichiosis, Q fever and bartonellosis, transmitted by arthropods, like mites, ticks, lice and fleas, and are found in many parts of the world. *Rickettsia* and *Ehrlichia* genus are $\alpha$-proteobacteria of the subgroup 1 and *Bartonella* of the subgroup 2, whereas *Coxiella burnetii* belongs to the $\gamma$-proteobacteria. Although initially the above species all belonged to the order *Rickettsiales,* family *Rickettsiaceae*, they are now reorganised and considered as individual families of bacteria. The family *Bartonellaceae*, as well as *Coxiella burnetii*, were removed from the order *Rickettsiales*, which includes now two families, the *Anaplasmataceae* and *Rickettsiaceae.* However, they are often still studied within the field of rickettsiology and for the purpose of this review we will consider them jointly, particularly due to the many common characteristics they present, both in the way they are transmitted and in many of the symptoms they cause [1,2].

Rickettsioses, especially SFG, are often misdiagnosed for other endemic diseases, such as dengue fever or leptospirosis, in tropical countries and, thus, often result in grave or fatal outcome [3-5]. This in part is due to the similarity of symptoms with other common endemic diseases, but also in part due to the nature of the diagnostic assays currently employed as gold standards in the diagnosis of these conditions, based on serological assays for the

presence of patient antibodies against these bacteria. As patient antibodies, IgG or IgM, can take up to two weeks to appear, early serological examination may lead to false negative results and two evaluations are necessary. Yet, until the second evaluation takes place to confirm seropositivity, symptoms may have advanced significantly. Thus, in this review, we are looking at "rickettsias *sensu lato*", including, besides the genus *Rickettsia*, the *Ehrlichia, Anaplasma, Bartonella* and *Coxiella* genus with a focus on the current diagnostic standard and an evaluation of future trends and their applicability and viability as laboratory methods that could have a wider application in the early and accurate diagnosis of these bacterial infections.

## 2. RICKETTSIOSES CAUSED BY THE GENUS *RICKETTSIA* AND THEIR DIAGNOSIS IN HUMANS

Rickettsioses are zoonoses caused by obligate intracellular bacteria of the genus *Rickettsia* in the order *Rickettsiales*. Rickettsias are Gram-negative, non-motile bacteria, with endothelial cells as their primary target. Their life cycle involves arthropod vectors like ticks, fleas, lice and mites [6]. Members of this genus may be classified into four groups: (i) typhus group (TG), which includes *Rickettsia typhi* and *Rickettsia prowazekii*; (ii) spotted fever group (SFG), based mainly on phenotypic and serological features with more than 25 different species including *Rickettsia rickettsii, Rickettsia conorii, Ricketsia africae,* and *Rickettsia parkeri*; (iii) ancestral Rickettsiae group, which includes *Rickettsia belli* and *Rickettsia canadensis* and (iv) transitional group, with *Rickettsia akari, R. autralis* and *R. felis* [7-10].

Rickettsial pathogens are widely distributed throughout the world, causing emerging and re-emerging infectious diseases. These zoonoses are among the oldest known vector-borne diseases. Their global distribution varies according to the density and distribution of the arthropod vector and the population density of the reservoir hosts [6,11]. SFG are increasingly being identified among international travellers. Two percent of imported fevers are caused by rickettsioses and 20% of these patients are hospitalized [12].

The case definition of confirmed SFG includes both clinical evidence and laboratory confirmation. The clinical features of this rickettsiosis consist of an acute undifferentiated febrile illness, often accompanied by headache, myalgia and nauseas, and a maculopapular or vesicular rash may be observed a few days after the onset of illness [13-15]. When a patient develops a febrile illness, the suspicion for tick-borne illness should be high in those who live or travel to endemic areas. Outdoor activities, whether recreational or occupational, particularly in areas with high-uncut grass, weeds, and low brush, can increase the risk for tick bites and thus the chance for disease onset [16].

Clinical disease develops prior to an antibody response, so patients presenting clinical evidence of disease due to rickettsial infections, should be empirically treated with appropriate antibiotics, regardless of the outcome of initial laboratory testing [11]. Doxycycline is the treatment of choice for adults and children. Resistance to doxycycline has not yet been reported. Chloramphenicol may be used in cases of life-threatening allergy to doxycycline [17]. Rickettsial organisms are naturally resistant to many antimicrobial drugs routinely used as treatment for acute fevers, including β-lactams. Fluoroquinolones and newer macrolides are useful options of treatment [12]. Several species of SFR have been confirmed as human pathogens and the number is rising. Some examples are: *R. rickettsii, R. parkeri, R. conorii, R. africae, R. felis, R. japonica, R. helvetica, R. montanensis, R. rhipicephalis and R. sibirica*.

Similar SFG, epidemic typhus, caused by *Rickettsia prowazekii*, is also usually associated with more severe symptoms, which typically begin suddenly, and high mortality in the absence of specific of treatment. Regarding to endemic typhus, caused by *Rickettsia typhi* and occasionally *R. felis*, its clinical manifestations are also similar to those of SFG, but are less severe and rarely lead to death [6,18].

Diseases caused by agents of the *Rickettsia* genus can be diagnosed in a number of different ways; each approach has its advantages and its limitations. The specific laboratory confirmation could be either detection of specific DNA by PCR, demonstration of the organism in cell culture, or a fourfold rise in antibody titres on paired samples taken 2-4 weeks apart.

Rickettsiae are isolated most commonly from blood, skin biopsy specimens and autopsy tissue fragments or arthropods, especially ticks. Rickettsiae culture must be performed only in biosafety level 3 facilities, which are limited to Reference Centers or few research laboratories in the world [19,20].

Rickettsial antibodies can be detected by complement fixation, latex agglutination, enzyme-linked immunosorbent assay and immunofluorescence assay (IFA). IFA is the gold standard for serological diagnostic of rickettsial infections and it is performed worldwide [21]. Diagnostic criteria of recent infection by IFA test are either a 4-fold increase of immunoglobulin IgG or IgM titres in paired samples drawn $\geq$ seven/10 days apart, or elevated IgG and/or IgM titres in single samples consistent with recent infection. A diagnostic IFA titre of < 64 is not considered positive. A titre of > 64 is considered exposure or probable acute infection without rising titre. Titres should be interpreted based on the background seroprevalence of endemic area [17]. The main limitation with serological diagnosis include a usually negative result in the acute phase when patients first seek medical care, poor sensitivity in cases treated with doxycycline, and an inability to distinguish among various rickettsial species caused by cross-reaction [12].

The greatest challenge to clinicians is not therapy but the difficult diagnosis during the early phase of infections [18]. The diagnosis of rickettsiosis can be missed because of these nonspecific initial clinical presentations and the absence of specific laboratory confirmation [22]. Serological diagnosis is usually retrospective; antibody increase takes 15-26 days, thus limiting the clinical impact of diagnosis [23].

Molecular diagnosis using polymerase chain reaction (PCR) targeting various rickettsial genes has been developed to accelerate the diagnosis of rickettsiosis and allow early species diagnosis. While some PCR target several species, others are designed to detect only a single rickettsial species. The five genes usually targeted by PCR for detection and diagnosis are citrate synthase *glt*A*,* gene D *sca*4, the 17kDa lipoprotein precursor antigen gene 17kDa, and genes for outer membrane proteins A and B *omp*A and *omp*B [21,24]. Other targeted genes include 16S ribosomal RNA *16S rDNA*, serine peptidase *htr*A [20]. It should be noted that, in contrast to SFG rickettsiae, all rickettsiae belonging to TG (*R. prowazekii* and *R. typhi*) lack the *ompA* gene [25].

Rickettsial diagnosis based on PCR has been used extensively [20,24,26-31]. PCR primer sets, targeting various rickettsial genes, have been described and can be used in any laboratory with suitable facilities. Rickettsiae can be detected from clinical samples including skin biopsies, autopsy tissue fragments, blood and sera. Modifications of the PCR technique

led to its improvement and the development of the nested PCR (nPCR), which increased the sensitivity of PCR to the level of detecting 1-10 genomic equivalents per reaction [32].

Santibáñez and co-workers [20] evaluated the effectiveness of PCR methods to detect *Rickettsiae* from clinical samples. They determined the sensitivity and usefulness of molecular diagnosis targeting the *16S rDNA, htr*A*, glt*A*, omp*A and *omp*B genes by PCR. They performed single and sequential (nested or semi-nested) PCR assays. The samples tested were collected from patients in the early phase of the illness before antibiotic therapy. For single PCR assays, the greatest sensitivity to detect rickettsial DNA in clinical samples was obtained using *glt*A (33.3%). Higher sensitivity was achieved using sequential *omp*B PCRs (83.3%). They recommend performing *glt*A and *omp*B PCR assays, followed in positive samples by *omp*A PCR and nucleotide sequence analysis for species identification. The *omp*B PCR detects a high percentage of positive samples and it is effective as a first screening. The *omp*A PCR assay is an accurate method to diagnose and to implicate a new *Rickettsia* species. Combination of three sequential PCR assays (*omp*A, *omp*B and *glt*A) achieved 100% sensitivity [20]. Similarly, Sekeyova et al. [33] monitored clinical samples for *Rickettsia, Bartonella, Borrelia, Coxiella, Anaplasma, Franciscella* and *Diplorickettsia* from patients from Slovakia. They evaluated the seroprevalence by multiple-antigen IFA and confirmed the results with PCR. Serum samples from 50 hospitalized patients with suspected tick-borne diseases were evaluated. As a result, 32% were positive by IFA for spotted fever group rickettsia, but only 10% were confirmed by PCR. The discrepancy between IFA and PCR might be due to the sensitivity linked to the time of collection of the serum samples [33]. Therefore, these assays can provide high accuracy and sensitivity and have been used for the molecular diagnosis of rickettsioses, as well as to identify *Rickettsia* species in new clinical patients.

Newer molecular assays are also available. Pan et al. [18] evaluated the potential of loop-mediated isothermal amplification (LAMP) targeting the *omp*B gene to detect SFG rickettsiae early infection. LAMP is a highly sensitive and specific technique that under isothermal conditions (60-65$^{o}$C) can generate up to $10^{9}$-fold amplification in less than one hour. They compared the sensitivity of the *omp*B LAMP assay and general PCR. They found the limits of detection of LAMP and PCR for the *omp*B gene were 5 and 625 copies per reaction, respectively. Thus, the LAMP assay is 125-fold more sensitive than conventional PCR. They also evaluated the clinical applicability of the LAMP assay with clinical samples previously positive by serology or real-time PCR and compared the results of LAMP with nested PCR protocols. LAMP detected 8 of 10 confirmed cases while nested PCR detected none of the positive samples [18]. Thus, LAMP assay is a reliable test and could be an ideal choice for development as a rapid and cost-effective means of detecting SFG rickettsiosis.

Renvoisé et al. [34] evaluated the widespread use of real-time PCR for rickettsial diagnosis. They reported two years of their experience at the French National Reference Center (FNRC) with molecular diagnosis for rickettsial diseases using real-time PCR. They designed a new set of primers and probes to detect TG *Rickettsia,* and SFG *Rickettsia*, such as *R. conorii, R. slovaca, R. africae* and *R. australis.* Specificity was tested *in silico* using blastN analysis on GenBank and *in vitro* using a panel of 30 rickettsial strains. Sensitivity was determined by 10-fold serial dilution. Primers and probes both sensitive and specific were routinely used for diagnosis of rickettsial infections at FNRC. Among the positive clinical samples, 68.9% (31/45) were cutaneous biopsies, 17.8% (8/45) were cutaneous swabs, 4.4% (2/45) were total blood samples and 8.9% (4/45) were serum samples [34]. Based on these findings, it appears that real-time PCR could also be an option that can be

easily implemented in laboratories that have molecular facilities and its widespread use is inexpensive and reduces the delay of rickettsial diagnosis.

Angelakis et al. [35] performed a comparison between cell culture techniques and PCR for the diagnosis of *Rickettsia* infections. They analyzed skin biopsies and ticks collected from patients with suspected *Rickettsia* infections. They identified the presence of *Rickettsia* spp. in skin biopsies and ticks using molecular methods and cell culture. Culture methods were less sensitive than PCR. Culture sensitivity was low in patients under antibiotic treatment because of the high susceptibility of *Rickettsia* to antimicrobial agents. They found a positive correlation between the bacterial copies and the isolation success. Early antibiotic treatment, prior to skin biopsies, reduced the sensitivity of both methods tested [34]. As a result of these studies, it is suggested that PCR would be a much more promising as a reference laboratory technique than cell cultures that are less sensitive and much more demanding, and can also be affected by previous treatments.

A duplex real-time PCR, targeting the DNA of any rickettsial species and TG *Rickettsia* in clinical samples (skin biopsies) has also been developed [23]. The test was sensitive for at least 10 DNA copies per reaction and exhibited good reproducibility. The results from clinical samples allowed an early diagnosis of spotted fever in two cases and recognition of murine typhus in another. Despite the limited number of samples tested, the clinical experience with the duplex real-time PCR assay is encouraging. The recognition of typhus group rickettsia is clinically and epidemiologically relevant, as these infections may be associated with worse prognosis than spotted fever.

## 3. HUMAN GRANULOCYTIC ANAPLASMOSIS, HUMAN MONOCYTIC EHRLICHIOSIS AND THEIR DIAGNOSIS

Intracellular bacteria of the Anaplasmataceae family cause ehrlichiosis and anaplasmosis. *Ehrlichia* and *Anaplasma* species are transmitted through the bite of an infected nymph, or adult tick vector that had been previously infected in larval or nymph stage while feeding on a rickettsemic animal, known as a reservoir host. Their agents are maintained in nature through enzootic ticks as well as wild and domestic animals [11,36].

The causative agents of human monocytic ehrlichiosis (HME) and human granulocytic anaplasmosis (HGA) are small, Gram-negative, obligate intracellular bacteria that have tropism for specific leukocytes. HME has an affinity for monocytes and HGA preferentially infects granulocytes. They replicate within vacuoles in these leukocytes forming microcolonies called morulae, which allows the organisms to avoid phagocytosis to facilitate their survival [11,37,38].

Clinical presentations of ehrlichiosis and anaplasmosis are similar and nonspecific. HGA and HME are acute febrile tick-borne diseases. Fever is followed by headache, myalgia and arthralgia. Less common symptoms include nausea, abdominal pain, diarrhoea and cough. A rash may be present in 10-30% of cases of HME but is uncommon in HGA, present in less than 10% of infected patients. Neurologic symptoms, including meningitis and meningoencephalitis, have been reported in approximately 20% of patients with HME. Laboratory findings include leukopenia, thrombocytopenia, elevated serum aminotransferase levels, and elevated creatinine levels [37,38,39].

The diseases are considered as emerging and the number of reported cases has been growing due to better diagnostic techniques and surveillance programs [36]. Accurate

diagnosis of many tick-borne diseases is hampered owing to similar clinical manifestations, overlapping geographical distributions and shared vectors. Laboratory confirmation can be carried out using a number of microbiological, serological and molecular techniques [37].

Direct visualization of bacterial inclusions, termed morulae, in the cytoplasm of infected circulating leukocytes can allow rapid diagnosis of *Ehrlichia* spp. and *Anaplasma* spp. [11,37,40]. Morulae detected in neutrophils are indicative of infection by *Anaplasma phagocytophilum*, while detection in monocytes delineates infection by *Ehrlichia chaffeensis* [37]. However, the low level of morulae, the short duration of rickettsemia, and the need of an experienced microscopist limit the utility of this approach.

Recovery of *E. chaffeensis* and *A. phagocytophilum* in mammalian cell culture can also be used to diagnose infection. *A. phagocytophilum* is usually cultivated in the human promyelocytic leukemia cell line HL-60 and the canine histocytic cell line DH28 is employed for culturing *E. chaffeensis* [37,41,42]. Limitations of cell culture as a diagnostic tool include the need of an antibiotic-free environment for growing these cell lines and technical staff trained in cell culture techniques. Besides, with this approach, it can take several days to obtain a positive result.

The indirect immunofluorescence assay (IFA) is the gold standard method proposed by the World Health Organization Collaborating Centre for Rickettsial Reference and Research. Antibodies in the serum bind to fixed antigens on a slide and are detected by a fluorescein-labelled conjugate. The greatest limitation of IFA is the need of a pair of serum samples from both acute and convalescent stages of the illness; which is not applicable as an early test for infection [18]. Nonspecificity can occur in IFA due to cross-reactivity. The Centers for Diseases Control and Prevention recommend that diagnoses based on serological tests should be confirmed by molecular methods [40].

A number of European countries have been confirmed as a hotspot for HGA and rickettsial diseases. Thus, Cochez and co-workers [43] presented results from a 10 years (2000-2009) study of *A. phagocytophilum* sero-surveillance in Belgium. Serum samples from 1350 patients were tested using an IFA IgG and IgM antibody test kit, according to the manufacturer's specifications (Focus Diagnostic, CA, USA). In total, 111 confirmed cases were detected. All cases had a history of tick bite, fever, and initially showed a titre of at least 64, which increased to 256 or higher in their follow-up sample. Based on these findings, the authors suggest that Belgium is a hot spot for HGA infections [43].

A number of clinical samples were also evaluated in France and positive results were identified both with spp. and *Ehrlichia* spp. during acute infection. Several conserved genes have been employed as PCR targets, including the *rrs* (16S rRNA) and *gro*ESL (heat shock operon). Koebel and co-workers [44] highlight the PCR assay on EDTA-blood samples as the diagnostic test of choice during acute phase of diseases. A Taqman-based real-time PCR was applied to amplify a 73-bp fragment from the *A. phagocytophilum msp2/p44* gene. They report confirmation of three consecutive clinical cases with HGA by Real-time PCR in eastern France [44]. Similarly, Edouard et al. [45] also reported five new human cases of *A. phagocytophilum* infection diagnosed by serology and Real-Time PCR. Serum specimens were tested with microimmunofluorescence assays using a large panel of antigens, including *A. phagocytophilum.* Serum samples were also screened with primers and probes targeting 102-bp of DNA polymerase I of *A. phagocytophilum*. A positive result was confirmed using primers and probes targeting a 92-bp fragment of the glutamyl tRNA amidotransferase gene.

Clinical samples were obtained retrospectively (2000-2010) from the archives of the National Reference Centre for Rickettsioses, Marseille, France [45].

Weil et al. [46] described a tick-associated pathogen panel (TAPP) that includes a PCR assay for the detection of the organisms that cause HGA, other human ehrlichioses (*A. phagocytophilum, E. chaffeensis, and E. ewingii*), as well as *Borrelia burgdorferi* and *Babesia microti*, the causative agents of Lyme disease and babesiosis, respectively. The PCR targeted *HSPD1*, an open reading frame gene segment of the heat shock protein operon (*gro*ESL). From 692 samples tested, 33 presented an HGA-positive result. Serological assays were not included in this study [46]. The results of the authors would also suggest the potential of PCR for bacterial detection in the evaluation of more than one organism, even though they have not presented serological evidence for comparison.

Other currently proposed molecular detection methods include LAMP to detect *A. phagocytophilum* in clinical samples [47]. They developed a LAMP assay based on a conserved region in the *msp2* gene. The limits of detection of LAMP compared to PCR were 25 copies of *msp2* gene and 625 copies, respectively. Forty-two clinical samples were tested by LAMP and results were compared with nested-PCR and Real-time PCR. Twenty-six tested positive by LAMP assay while only one and three samples tested positive by nested-PCR and Real-time PCR, respectively. Thus, as shown earlier for rickettsias, LAMP is a rapid, simple, sensitive, and cost-effective assay for detecting *A. phagocytophilum* in clinical samples.

## 4. *BARTONELLA* AND ITS DIAGNOSIS

*Bartonella* species are small, fastidious, intracellular, Gram-negative rod-shaped bacteria belonging to $\alpha$-Proteobacteria subgroup 2, with a worldwide distribution, which cause an increasingly large number of infectious diseases in humans and animals [48]. In 1993, Brenner and colleagues proposed to unify *Bartonella* and *Rochalimaea* genera and renamed some species as *Bartonella quintana, B. henselae, B. vinsonii* and *B. elizabethae*. As a result of this unification, the transfer of these organisms from the *Rickettsiaceae* family to the *Bartonellaceae* family was required and, at present, more than 31 species and subspecies are recognized [49].

Several hematophagous insects have been implicated in *Bartonella* transmission, including sand flies [50], the human body louse [51], the cat flea [52] and, potentially, ticks [53,54]. *Bartonella* infections have been encountered in all species surveyed, which extend to members of different orders of mammalian, including carnivores, primates, ungulates, rodents, and bats. It is believed that the vector preference for certain hosts can influence the transmission of these organisms and that is responsible for the association of a given *Bartonella* sp. with a specific host, i.e., *B. henselae, B. clarridgeiae and B. koehlerae* with cats, *B. alsatica* with wild rabbits, *B. baciliformis* and *B. quintana* with humans [55,56].

Bartonellae have been recognized as agents causing human disease, including *B. bacilliformis* (agent of Oroya fever and verruga peruana), *B. henselae* (agent of cat scratch disease CSD, bacillary angiomatosis, bacillary peliosis, endocarditis) and *B. quintana* (agent of trench fever, bacillary angiomatosis, bacteremia and endocarditis) and *B. elizabethae* (agent of endocarditis), among others [55]. The *Bartonella* spp. infection can cause great diversity of clinical manifestations in humans, including recurrent fever and fever of unknown origin, malaise, fatigue, insomnia, loss of memory, psychiatric disorders, lymphadenopathy,

splenomegaly, angiomatosis and bacillary peliosis, endocarditis, hepatitis, osteomyelitis, encephalitis, meningitis, and other neuroretinites [57,58].

The genus *Bartonella* has pathogenic characteristics, such as the ability to invade and lyse red blood cells. Besides erythrocytes, the endothelial cells represent another target of *Bartonella* in their mammalian hosts [56]. Current opinion is that these cells serve as a primary niche for bartonellae prior to them entering the bloodstream. After five days, these bacteria are released into the bloodstream and then are able to invade erythrocytes, where occurs their intracellular replication [59].

Antimicrobials have been used widely in the treatment of bartonellosis. A study using azithromycin in CSD showed a benefit in lymph node regression in 30 days, as compared with placebo [60]. In immunocompromised patients the antimicrobials indicated are erythromycin, clarithromycin, azithromycin, doxycycline, either co-administered or not with rifampicin, and the period of treatment ranges from 4-6 weeks [60,61]. In the acute febrile phase of Carrión's disease the preferred treatment has been chloramphenicol [62], but oral ciprofloxacin has been increasingly and successfully used [61].

Several laboratory methods must be used in bartonellosis diagnostic to avoid false negatives; there is no gold standard [63]. Diagnostic techniques for infections with *Bartonella* spp. include serology by immunofluorescence (IFA) to detect antibodies against the bacteria in the patient's serum, culture of the pathogen, histopathological examination of lymph nodes or tissue biopsy of skin, liver or other affected organs, and molecular biology techniques, especially PCR, to amplify *Bartonella* spp. genes from patient's tissue fragments or blood [64,65].

Serology is particularly important because it allows the rapid identification of *Bartonella* spp. [64,66]. But it is limited by cross-reactions between the different species of *Bartonella*, and also between genera such as *Coxiella* and *Chlamydia* [67,68]. Many studies have shown that serological differentiation between *B. henselae* and *B. quintana* through IFA is impossible, since cross-reactivity between these species is very high (95%). The heterogeneity among strains and genotypes of *Bartonella* spp., might result in differences between the parameters of laboratory analysis and subjectivity of the readings of the results of IFA, which could result in false positives [69,70]. Despite these limitations, IFA remains the gold standard for the diagnosis of infection [68,71].

ELISA method has been proposed as an alternative to IFA. Several ELISA protocols use sonically disrupted *B. henselae* (whole cell-proteins) or the putative outer membrane proteins (OMPs) as the antigens [72,73]. However, studies have demonstrated low sensitivity of ELISA-based serodiagnosis when compared with IFA. Thus, researchers have focused their efforts on the improvement of the antigen preparation for use in IgG ELISA. Tsuruoka et al. [73] found that sarcosine-soluble proteins of *B. henselae* are significantly more specific than whole-cell or sarcosine insoluble proteins as antigens for IgG ELISA, indicating an improvement in accuracy of diagnosis of Bartonella infection [73].

The diagnosis of *Bartonella* infection should be confirmed by culturing the organism from aseptically obtained patient samples, including blood, CSF, lymph nodes, or other tissue aspiration samples, ocular exudates, and from surgical biopsies [58]. The liquid culture of *Bartonella* spp. is necessary to increase the sensitivity of detection of bacteraemia through molecular methods and is one of the most used methods of diagnosis worldwide [74,75,76]. The isolation of most species of *Bartonella* in blood agar plates requires a long incubation

period (6-8 weeks) at 35°C in a water saturated atmosphere containing 5% $CO_2$. The development of a new liquid culture medium called BAPGM (*Bartonella* Alpha-Proteobacteria Growth Medium) that allows the growth of at least seven *Bartonella* species enabled the improvement of this method as diagnosis. It is now widely used as a pre-enrichment stage and, combined with molecular methods, increased the success and sensitivity of culture for diagnosis in both animals and humans [74-77]. However, to date, there is no consensus regarding the best culture medium or best antigen to be used in the diagnosis of bartonellosis.

Likewise, a consensus has not been achieved on the best gene to be used for primer development and optimal conditions to be used in PCR. Species-specific PCR has been useful, especially when a particular diagnosis is already suspected. Thus, several studies describe regions of the 16*S rRNA* gene, the 16S-23S rRNA intergenic spacer region-encoding gene (ITS), citrate synthase gene (*glt*A), the riboflavin synthase (*rib*C), the 60-kDa heat shock protein gene *(gro*EL*)*, the N-terminal region of the cell division protein gene (*FtsZ)* or gene of the beta subunit of RNA polymerase (*rpo*B) as the most efficient and promising primer targets for detection of different species of *Bartonella* [48,74].

Besides the set of primers that determine the region to be amplified and therefore the sensitivity of the reaction, the type of PCR also influences the success of the diagnosis. The nested PCR can greatly increase the sensitivity of detection, as does the real-time PCR [76,78]. A semi-nested PCR has also been designed to amplify the gene of a 31-kDa major protein (*Pap*31) associated with the bacteriophage harboured in *B. henselae* [79]. The main advantages of this technique are that it is easy and reliable, culture independent and almost all bacteria can be detected in a single reaction [67]. A limitation of the system is the quality of DNA of some clinical materials, high host DNA concentrations that interferes with DNA amplification of the *Bartonella* target and potential DNA contamination [64].

An important study conducted by La Scola et al. [80] using sequences available in the GeneBank database, compared seven gene targets, specifically the 16S *rDNA, glt*A*, gro*EL*, rpo*B*, ftsZ, rib*C and the internal transcribed spacer 16S-23S (ITS). This comparison demonstrated the relative discriminatory power of each gene examined, and only two genes – *glt*A *and rpo*B – were able to clearly differentiate all species and subspecies of *Bartonella* analysed [80].

Newer molecular approaches in microbiology are available for characterization and typing of *Bartonella* genotypes [81]. The most widely used are pulse field gel electrophoresis (PFGE) [82], multilocus sequence typing (MLST) [83,84], multispacer typing (MST) and multilocus variable number tandem repeat analysis (MLVA) [81].

## 5. Q FEVER AND *COXIELLA BURNETII* DIAGNOSIS IN HUMANS

*Coxiella burnetii* is a small, obligate intracellular, gram-negative, pleomorphic bacterium of the order Legionellales and is the cause of Q fever. *Coxiella burnetii* is very stable in the environment in its sporoid form. In natural infections, *Coxiella* has a tropism for cells of the mononuclear phagocytic class, such as lung alveolar macrophages, Kupffer cells of the liver and, more rarely, pneumocytes, fibroblasts and endothelial cells [85].

*Coxiella burnetii* exists in two antigenic phases, called phase I and phase II. In nature, the *C. burnetii* phase I expressed antigen is observed in humans, infected animals and arthropods and it is the infectious form of the bacterium. The phase II variant is obtained after several

passages in embryonated eggs or cell cultures and is less virulent [86]. The abnormally high resistance against chemical and physical factors, due to endosporous formation, is one of the most impressive attributes of *C. burnetii*. It enables this organism to persist in the environment for long periods of time and remain infectious. The surviving ability of the organism, its resistance to heat, desiccation and many disinfectants, justifies its high infectivity, a fact that makes this proteobacteria a possible weapon of bioterrorism and an agent classified as category B. Studies show that a single inhaled organism may produce clinical disease [87,88].

Cattle, sheep and goats are the primary reservoirs of *C. burnetii*. Infection is known in a wide variety of other animals, including other species of ruminant domestic animals as well as wild animals [2,89]. The reservoirs of *C. burnetii* include mammals, birds and arthropods, especially ticks [90]. *Coxiella burnetii* does not usually cause clinical symptoms in these animals, although abortion in goats and sheep may be related to infection by this microorganism that is excreted in the milk, urine and feces of infected animals. During the time of birth of the animals, *C. burnetii* is present in great quantity in the amniotic fluid and placenta. Transmission to humans usually occurs through inhalation of contaminated aerosols from urine, feces, milk, amniotic fluid, placenta, abortion products, wool, or less commonly by drinking raw milk from infected animals [89]. This broad spectrum of unique reservoirs and resistance of *C. burnetii* to environmental factors makes, as mentioned previously, tracing the source of infection very difficult [91].

Infection with *C. burnetii* may present clinical manifestations of acute or chronic Q fever, a disease with a wide spectrum of clinical manifestations, ranging from limited febrile illness, pneumonia, hepatitis and other forms of infections, such as endocarditis, and meningoencephalitis [92,93]. The acute Q fever in humans is asymptomatic in almost 60% of infected people. Among the 40% of symptomatic patients, the majority (38% of 40%) undergo a mild disease without requiring hospitalization. In symptomatic patients, the onset is usually abrupt, with high fever, fatigue, chills and headaches. The most frequent clinical manifestation of acute Q fever is probably a self-limiting febrile disease associated with intense headache. Atypical pneumonia is also a frequent clinical presentation and clinical symptoms can vary from asymptomatic pneumonia, diagnosed by chest radiography, and rarely severe pneumonia resulting in acute respiratory failure. Hepatitis is another common presentation of acute Q fever detected biochemically by increased levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosphatase. Hepatomegaly may be clinically detectable, but jaundice is rare [2].

Q fever can be considered chronic with the persistence of clinical manifestations for more than 6 months after the beginning of the symptoms. It occurs in approximately 5% of patients infected with *C. burnetii* and may develop insidiously over months to years after the acute illness. Patients with cardiovascular abnormalities are at higher risk of developing chronic infection. Normally, the heart is the organ most commonly involved, followed by arteries, bones and liver. Endocarditis usually occurs in patients with previous valvular lesions, or those who are immunocompromised, months or years later, and primarily with the involvement of the aortic valve [86]. Granulomatous hepatitis with a more protracted course can also be observed in some patients and the diagnosis is only possible by liver biopsies. Renal involvement with glomerulonephritis has also been described in Q fever [91,94,95]. Thus, endocarditis, vascular infections, osteoarticular infections, chronic hepatitis, chronic lung infections, chronic fatigue syndrome, prematurity in pregnancy and abortion are some of the clinical presentations that result from chronic infection. Coxielemia event results in permanent production and hence very high levels of antibodies in the circulation.

The difference in clinical presentation of Q fever can be explained by: (i) the route of infection by *C. burnetii*, including aerosol, or gastrointestinal tract, (ii) the dose of inoculation of *C. burnetii*, (iii) the variant of infective *C. burnetii*, and (iv) host factors, including immune status of the infected patient [2].

In contrast to acute Q fever in human, animal infection with *C. burnetii* is in most cases so amazingly asymptomatic. This fact implies that the term coxiellose is considered a more appropriate than animal Q fever. In animals during acute phase, *C. burnetii* can be found in the blood, lungs, spleen and liver, while during the chronic phase *C. burnetii* is presented as a persistent release of the organism in feces and urine [96].

The reservoirs of *C. burnetii* are numerous, including mammals, birds and biological vectors (ticks). Forty species of ticks or more are naturally infected with *C. burnetii*, but are not important in the maintenance of infections in domestic animals and in humans [2]. *Coxiella burnetii* multiplies in intestine cells of ticks and a large number of viable organisms are eliminated through feces. Contaminated leather and wool become vehicles to transmission to people either by direct contact or through the dry feces, inhaled as dust particles suspended in the air [96]. Although it appears that wild rodents are an important reservoir, the most commonly identified source of human infection is farm animals such as cattle, goats and sheep. Pets, including cats, dogs and rabbits can also be potential sources of urban outbreaks. It is suspected that cats are a major reservoir of *C. burnetii* in urban areas. In Canada, studies have shown that 6-20% of cats have anti-*C. burnetii* antibodies [86].

Infected animals eliminate *C. burnetii* through urine, feces and milk products. The occurrence of infection reactivation in female mammals during pregnancy can result in abortions in goats and, to a lesser extent, in sheep, and cause reproductive problems in cattle. *C. burnetii* is located in the uterus and mammary glands of infected animals. High concentrations of *C. burnetii* (up to $10^9$ bacteria per g of tissue) have been found in placentas, as well as in the milk of infected animals, although this form is probably less effective in spreading Q fever [2,86,96].

Q fever is a major public health problem in many countries especially in France [97] but also in Spain [98] and the Netherlands, where more than 3,500 cases were reported in the last three years [99-103]. *Coxiella burnetii* is responsible for 5-8% of cases of infective endocarditis in the south of France and Q fever cases occur in 50 per 100,000 inhabitants in this area [2,66,90,104,105,106]. More recent data show that throughout the French territory, the annual incidence of acute Q fever and endocarditis is 2.5/100,000 and 0.1/100,000 persons, respectively [97].

Since the first publication on the emergence of Q fever in the Netherlands, thousands of cases have been reported [101,107-115] and the study of small ruminants demonstrated that the same genotype *C. burnetii* identified in these animals was responsible for the epidemic of Q fever in the Netherlands [101]. Yet another study, conducted by Klaassen et al. [116], identified five genotypes of *C. burnetii* in six patients and six animals from three different regions of the Netherlands [116]. Based on the Q fever epidemic occurred in the Netherlands, the application and importance of an efficient surveillance system, aimed at early identification, has been emphasized [101,113].

The first case of Q fever in Brazil was described in 1953, and, despite being a worldwide zoonosis, remains neglected. Q fever is not a notifiable disease, perhaps because of the lack of human and animal clinical histories. Incidence and epidemiology remains unknown

and the scarce information on the serological evidence in humans and animals is restricted to the states of Bahia, Minas Gerais, Rio de Janeiro and São Paulo [117-123]. The first case of Q fever in Brazil, confirmed by molecular analysis, was reported in the rural area of Itaboraí, Rio de Janeiro State, in 2008, when the patient reported a contact with products of goats' abortion [124]. Further study confirmed the presence of the antigen in the milk of goats and the serum of two dogs that had been fed non-pasteurized milk from these goats, confirming the origin of the source of infection of the patient [125]. According to Rozental et al. [126], the occurrence of Q fever in urban areas confirms the need to include its diagnosis in clinical cases compatible with a history of contact with childbirth or abortion material of pets like dogs and cats [126].

As with all zoonotic diseases, the animal disease control will influence the level of disease observed in humans. Appropriate strategies of tick control and good hygiene practices can reduce environmental contamination. Fluid and foetal membranes infected, aborted foetuses and contaminated bedding materials should be incinerated or buried after disinfection. Furthermore, manure must be treated with lime or calcium cyanide 0.4% before being spread on the fields, which must be done in the absence of wind to prevent the spread of the microorganism over long distances. Treatment of animals with antibiotics may be performed to reduce the number of abortions and the releasing *C. burnetii* in offsprings. Although it can be expensive, infected animals should be removed from or placed in confinement herds separated at birth. Workers from the animal industry should be fully informed about the risk factors for contracting Q fever and laboratories should be provided with adequate safety devices and equipment [96].

In relation to animals, the antibiotic treatment using tetracycline during the last month of pregnancy can reduce the number of abortions and the number of released *C. burnetii* in offspring. The efficacy of this treatment has never been assessed accurately but has not been proven to prevent abortion or completely eliminate the release of *C. burnetii* in new-borns [127]. Similarly, regular pasteurization at 72°C for 15 seconds or sterilization of milk from infected flocks is recommended, even though oral is not the major route of transmission of *C. burnetii* [128].

As it has become clear above, the main characteristic of Q fever is its clinical polymorphism, and thus diagnosis can only be made by systematic tests. Clinically, it is likely that factors such as the route of infection and the size of the inoculum determine, in humans, the expression of infection by *C. burnetii*. In fact, the airway is associated with pneumonia, and the gastrointestinal tract with hepatitis [96], while high inoculations are associated with myocarditis [2].

With respect to laboratory diagnosis, Q fever may be detected by serological or molecular methods as well as isolation and histopathology associated with immunohistochemistry techniques. In cases of acute Q fever, antibody levels of phase II are usually higher than those of phase I, often by several orders of magnitude, and usually are first detected during the second week of infection. In the chronic phase the situation is reversed and antibodies to phase I antigens of *C. burnetii* have higher titres, requiring long time to appear and thus indicate continued exposure to the bacteria. In this context, high levels of antibodies to phase I samples later on, in combination with constant or falling levels of phase II antibodies and other signs of inflammatory disease may suggest chronic Q fever. It is known that antibodies against antigens of phase I and II persist for months or years after initial infection [2,96]. Since the clinical diagnosis is difficult, in most cases the diagnosis of Q fever depends

on serological tests. A variety of serological techniques are available, but the indirect microimmunofluorescence test became the reference technique [96].

A real-time PCR assay for the detection of *C. burnetii* DNA in serum samples, targeting the IS*1111* transposase sequences (also known as *htp*AB-associated repetitive element) [129], was used in the Netherlands and in Brazil to diagnose acute Q fever early in infection to avoid delay in treatment that can lead to increased hospital admission rates and prolonged morbidity. Researchers found the latest time point after onset of disease in which *C. burnetii* could be detected by PCR of serum samples was day 17 [126,130]. During the last years, several PCR-based diagnostic assays were developed to detect *C. burnetii* DNA in cell cultures and in clinical samples. These assays used conventional PCR, nested PCR or real-time PCR conditions with Light-Cycler, SYBR Green or TaqMan chemistry [96,131]. The Light-Cycler Nested PCR (LCN-PCR), a rapid nested PCR assay that uses serum as a specimen and the Light-Cycler as a thermal cycler, targeting a multicopy 20-copy *htp*AB-associated element sequence, has been adapted for the diagnosis of both acute and chronic Q fever [96,132]. The LCN-PCR assay may be helpful in establishing an early diagnosis of chronic Q fever [90, 96]. Due to its high sensitivity and specificity, the repetitive element IS*1111* is the best target gene for the detection of *C. burnetii* in patients with active Q fever [132], although the complete sequences of the genome of *C. burnetii* have recently become available, allowing a large choice of DNA targets.

Recently developed techniques such as MLVA and SNP typing have shown promise and improved the discrimination capacity and utility of genotyping methods for molecular epidemiologic studies of this challenging pathogen [133].

Initial typing systems described were based on plasmid types. Restriction fragment length polymorphisms (RFLP) were analyzed with SDS-PAGE, pulsed-field gel electrophoresis (PFGE), and sequence studies of single genome targets like *16s/23s, com1, mucZ, and icd*. They showed different levels of discriminatory power and epidemiological significance, but all suffered with problems of inter- and intra-laboratory reproducibility, hampering their widespread use [133]. Other techniques that are used for plasmid typing (four different plasmids QpH1, QpRS, QpDV, and QpDG are utilized) include Multispacer Sequence Typing (MST), a method based on the analysis of the intergenic regions of genomes, Single Nucleotide Polymorphism (SNPs), Infrequent Restriction Site-(IRS)-PCR, Microarray-Based Whole-Genome Comparisons/Typing and the IS*1111*-insertion sequence, coding for a transposase seen in up to 56 copies in *C. burnetii* genomes [133].

## 6. ALTERNATIVE AND NOVEL APPROACHES TO BACTERIAL DETECTION

### 6.1 Bacterial Imaging

One method that has been growing in the detection of bacterial infections is the use of imaging. This has been achieved by the use of various imaging techniques and includes nuclear medicine approaches, nuclear magnetic resonance (NMR), optical probes, fluorescent and near-infrared imaging. A number of probes have been developed for the imaging of inflammation, including labelled monoclonal and polyclonal antibodies, cytokines, liposomes, leucocytes, antimicrobial peptides and antimicrobial agents among others [134,135]. The advantage of using nuclear medicine approaches is the differentiation between bacterial and viral infections, or inflammation due to infection with microbial pathogens (bacteria, fungus etc.) and inflammation due to immune response where no

microbial invasion is present, as is the case for example in autoimmune disorders [135]. This is achieved through the radiolabelling of antibiotics or other antimicrobial agents and their subsequent use as imaging agents. The advantage of using labelled antimicrobial agents as the localising agent for infection is the selective toxicity of these agents for microbial rather than human targets and the ability to bind selectively to those, offering the potential to rapidly distinguish a particular type of infection and differentiate it from others, thus influencing clinical decisions and therapeutic approaches [135]. Various antibiotics of broad-spectrum activity have been labelled to date, all or most of them with the radionuclide technetium 99m ($^{99m}$Tc), a radionuclide widely used in nuclear medicine. These antibiotics include ciprofloxacin (also known as $^{99m}$Tc-Infecton), sparfloxacin, enroflaxacin and ceftizoxime. Other agents used in bacterial imaging that are not antibiotics include $^{99m}$Tc-ubiquicidin, a cationic synthetic peptide [136,137] and [$^{125}$I] FIAU 1-(2´-deoxy-2´-fluoro-b-D-arabinofuranosyl)-5-[$^{125}$I] iodouracil, a substrate for bacterial thymidine kinase present in most bacteria [138,139].

Magnetic resonance imaging (MRI) is another imaging technique that offers the ability to visualise in real time the bacterial infections *in vivo* and study the impact of antibiotics on the bacterial proliferation and viability. The majority of MRI applications are in the detection of bacterial-associated conditions, such as bacterial meningoencephalitis [140], bacterial pyomyositis [141] or bacterial abscesses [142]. However, labelling of MRI probes has led to the development of MRI imaging directly for bacteria. Thus, *Bifidobacterium longum* and *Clostridium novyi-NT* were labelled with super paramagnetic iron oxide nanoparticles and they were subsequently followed by MRI [143]. Similarly, *P. aeruginosa* was labelled with green fluorescent protein (GFP) and was followed *in vivo* with magnetization transfer contrast MRI with excellent results, as it was shown able to be used to track bacterial proliferation and potentially gene expression *in vivo* [144].

Optical imaging of bacterial infection has also been developed, but it is primarily focused on the use of generic reporters, such as light-emitting enzymes (luciferase) and fluorescent proteins such as GFP. However, more specific probes offer the advantage of specific bacterial recognition. As molecular probes, in similar approaches to those described in nuclear medicine techniques, antibodies, sugars, bacteria-binding peptides and antibiotics have been used with varying results. In addition, a synthetic zinc (II) dipicolylamine (Zn-DPA) coordination complex has been used as affinity group in bacterial imaging studies labelled with a carocyanine dye. The complex has been able to stain the periphery of both Gram-positive (*Staphylococcus aureus*) and Gram-negative (*Escherichia coli*) bacteria, giving clear images of the infection site and with no obvious side effects to the host. This technique would be easy to use and could have applications in infections at sites within the tissue penetration limit of the NIR light, such as skin, throat, urinary tract etc. Furthermore, altering the probes could offer more specific bacterial imaging [145].

Bacterial imaging has already been used in the area of rickettsioses, and in particular in the diagnosis of bartonelloses and cat scratch disease (CSD). It is known that ocular manifestations occur in 5%-10% of patient with CSD and several imaging modalities can be used to assist in the diagnosis and management of ocular CSD. They include colour fundus photography that allows the clinician to monitor the fundus changes in this disease, fluorescein angiography that demonstrates leakage at the optic nerve in CSD neuroretinitis and optical coherence tomography to provide confirmation in early stages of neuroretinitis before the formation of a macula star [146].

## 6.2 Bacterial Identification using Spectroscopic and Spectrometric Techniques

Mass spectrometry has found a number of applications in the identification of bacteria. Some have been used coupled with molecular biology techniques for bacterial typing [147,148], whilst others have been used for the detection of pathogens. MALDI time of flight (TOF) mass spectrometry can offer a robust automated methodology for bacterial analysis, based on the detection of patterns of protein masses and potentially genotyping of single nucleotide polymorphisms for additional accuracy of identification [149].

Eshoo et al. [150] developed a multilocus PCR and electrospray ionization mass spectrometry (PCR/ESI-MS) method for the detection of bacterial tick-borne pathogens, including *Ehrlichia* and *Anaplasma*, in blood specimens. The role procedure of PCR/ESI-MS assay can be completed within six hours. The assay employs 16 primer pairs, including four broad-range primer pairs targeting the 16S and 23S genes of all bacteria. The other primers were selected based on their coverage groups of known tick-borne bacteria. Following PCR, the amplicons are analysed in an electrospray ionization mass spectrometer. Two hundred and thirteen clinical specimens from suspected ehrlichiosis patients were included in the study. This demonstrated the ability of PCR/ESI-MS to correctly diagnose the pathogen responsible for ehrlichiosis and identify it to species level [150].

Fourier transform infrared (FTIR) spectroscopy is another spectroscopic technique that has found extensive use in bacterial detection. It has been used for bacterial analysis since the 1980s, and a lot of different types of bacteria have been investigated by FTIR, including *Listeria, Bacillus, Staphylococcus, Clostridium, E. coli and Lactobacillus* [151]. The authors presented the differential discrimination of *Listeria innocua*, *Listeria welshimeri*, *Escherichia coli* K12, *Escherichia coli* ATCC 29181, *Salmonela cholerasuis*, *Salmonella subterranean*, *Enterobacter sakazakii* and *Enterobacter aerogenes*. They have managed to accurately identify the microorganisms, even in complex backgrounds containing other bacterial populations, and differentiate bacteria even within the same genotype independently of growth phase [151].

Several groups have been specialising on the FTIR detection of pathogens and bacteria in particular. Such works were characterised by the detection and characterisation of particular molecular groups of the bacteria, such as lipopolysaccharides from *E. coli* strains [152], to the identification of bacteria such as *E. coli* O157:H7 and *Salmonella typhimurium* [153]. Additionally, bacteria have been detected by FTIR in complex media, such as *E. coli* O157:H7 in fruit juices [154], ground beef [155] and *Salmonella enterica* serovars in chicken breast [156].

In addition to the ability to differentiate between different bacterial cells in complex environments, FTIR has provided the possibility to identify treated and un-treated, or live and dead cells of the same bacteria [157]. Bacterial typing and subtyping at the haplotype level has also been described for *Listeria monocytogenes* [158] and *E. coli* O157:H7 isolates [159]. Finally, FTIR detection has been used in sensor development [160,161], amongst a number of other detection techniques, some of which will be briefly mentioned below on the biosensor section for bacterial detection.

FTIR remains a promising methodology that could be of interest in the detection of rickettsiosis, but so far it has been limited to applications in food borne pathogens. However, wider exploitation of this methodology could also offer a rapid and accurate detection and

typing methodology. Detection limit mentioned is 4.8-5.8 log CFU/ml. (about 100,000-500,000 bacteria/ml), when upper acceptable limit for bacteria in milk is 200.000/ml. That would, for example be of potential interest in the detection of *Coxiella* in milk.

## 6.3 Microarrays

One interesting approach to bacterial detection, and rickettsial detection in particular, that may offer differential diagnostic and simultaneous analysis of various samples, could be with the use of microarrays. A number of microarrays have been developed so far, based on the printing of a recognition element on a slide and subsequent detection of a signal. The recognition element can be protein, antibodies, carbohydrates or nucleic acids and different types of arrays have been resulted from the use of the above elements, with different scales of success. Thus, carbohydrate arrays have been described [162], where five different aminofunctionalised monosaccharide derivatives have been printed onto glass slides and the specific binding of fluorescently labelled *E.coli* ORN178 to mannose was observed. Furthermore, the array was shown to be able to differentiate between different strains of *E. coli* with differential affinity to mannose, and a detection limit of $10^5$-$10^6$ cells. On a similar approach, Wang et al. [163] have developed a carbohydrate microarray with 48 microbiol polysaccharides for the specific recognition of carbohydrate-binding antibodies in the serum of patients. They were able to identify the particular type of infection based on the detection of patient antibodies with unique affinity for a specific pathogen polysaccharide, and with this methodology they successfully identified infections from *E. coli* and *Pneumococcus* and can be extended to a number of other pathogenic bacteria [163].

A similar, but somewhat reverse approach is the use of microarrays with lectins, to capture specific carbohydrate chains on the surface of bacterial cells. Most pathogenic bacteria possess cell surface polysaccharide or lipopolysaccharide shells, with crucial functions for their protection from the immune system and host invasion. Exploiting the natural affinity of lectins for bacterially expressed polysaccharides, lectin microarrays have been produced and utilised for bacterial detection. Lectins have been printed on glass slides and incubation with fluorescently labelled bacteria offered profiling of the diverse glycan structures [164,165,166] according to the specific binding of lectin to the lipopolysaccharide. As bacteria can specifically and reproducibly bind to certain lectins, such arrays can offer the potential to specifically differentiate bacterial species, or strains of the same bacterium with differential affinities to the specific lectin [164,167].

Protein microarrays have also been reported and, using antibodies as recognition agents, microarrays were able to detect *E. coli* and *Renibacterium Salmoninarum* [168]. One of the important characteristics of this work was the signal detection by scanning probe microscopy (SPM), whose high resolution imaging demonstrated the high binding selectivity of the antibodies for the specific bacteria, compared to signal from non-specific, control ones [168]. However, the most widely applied microarrays remain DNA microarrays. This has been primarily due to the technological developments that allow synthesis of oligonucleotides on the surface of the array directly and high-density printing. A number of commercial arrays are currently available, such as those available from Agilent, which allow up to a million oligonucleotide probes printed on a single slide. Other commercially available arrays include those from Affymetrix, NimbleGen, CombiMatrix, Oxford Gene Technologies, etc. Bacterial detection based on oligonucleotide arrays has been an active field of research and development for more than one decade, with reports of bacterial detection of a conserved bacterial gene in 2001 [169], species identification [170] and genotyping of bacterial pathogens using epidemiological markers [171-173].

Oligonucleotide arrays have been used in bacterial detection in a number of formats and for a number of applications. In rickettsial diseases, there has been use of microarrays in two different forms. First of all, based on the whole genome sequence of *R. prowazekii*, the Rickettsial Diseases Division of the U.S. Naval Medical Research Center constructed the first rickettsial microarray with all predicted ORFs. The genomic compositions of virulent strain and attenuated strain were studied by co-hybridization on this DNA microarray [174]. They have also deposited a patent for the detection and diagnosis of *R. prowazekii* infection, but this time by measuring the increased or decreased expression of specific human genes following infection, using DNA microarrays and PCR. This method permits the detection of the rickettsial infection and diagnosis of epidemic typhus earlier than other available methods [175]. Another DNA array for *R. prowazekii* has also been generated, which was the first DNA microarray for the analysis of global gene expression changes in *R. prowazekii* under stress conditions [176]. In addition to *R. prowazekii*, other *Rickettsia* microarrays are available based on rickettsial genomic information. The Rickettsia Genome microarray from Agilent Technologies, comprising probes specific to all genes and spacers from *R. prowazekii*, was used by Bechah et al (2010) for the genomic, proteomic and transcriptomic analysis of *R. prowazekii* [177]. The genome of *R. rickettsii* str. 'Sheila Smith' has also been provided and used for the development of a database containing 3205 oligonucleotides that represent the *R. rickettsii* 'Sheila Smith' transcriptome [178]. Within the same database for pre-designed oligonucleotide microarray probes, available to the research community, are included the sequences for specific strains of *R. rickettsii, R. africae*, *R. akari*, *R. bellii*, *R. canadensis*, *R. conorii*, *R. felis*, *R. massiliae*, *R. peacockii*, *R. prowazekii*, and *R. typhi* [178].

Finally, even this very year there has been a further development in bacterial detection by microarrays by Ballarini et al. [179] with the creation of the BactoChip microarray. Whereas the majority of microarrays use the 16S rRNA gene for diagnosis, the BactoChip uses 60-mer probes against an *in silico* identified set of genes. In so doing, BactoChip has been able to distinguish successfully among bacterial species from 21 different genera and determine the species-level relative abundances of 37 clinically relevant bacteria in complex bacterial communities and with a low detection limit of 0.1% [179]. Although "rickettsias *sensu lato*" are not included in this microarray, it is only a matter of time until an array containing rickettsias, either alone or amongst other bacteria with similar clinical manifestations, is created.

Apart from changing the probes on the microarrays and using a variety of molecules that have affinity for different bacterial parts, or using oligonucleotide probes against bacterial genes, there is significant development on signal detection and enhancement. The majority of microarrays are based on fluorescent detection. However, we already saw above the use of SPM for improved signal detection [168]. Other such techniques for improved detection include Resonance-Light Scattering (RLS), Planar-Waveguide Technology (PWT) [180], Infrared detection [181,182] and electrical or electrochemical detection [183].

## 6.4 Biosensors

Biosensor technology holds great promise for the health care market, environmental diagnostics, the food industry and the veterinary sector; harnessing the specificity and sensitivity of biological-based assays packaged into portable and low cost devices which allow the rapid analysis of complex samples in out-of-laboratory environments. Numerous biosensors have been described for bacterial identification, based on a number of detection technologies and recognition elements. Thus, bacterial sensors have been characterised based on their transducer properties, which include surface plasmon resonance (SPR),

amperometric, potentiometric, and acoustic wave sensors [184]. These sensors have been independently reviewed in Zourob, Elwary and Turner (2008) [185] in individual chapters, including SPR [186], Evanescent Wave-based Fluorescent biosensors [187], Fiber Optic biosensors [188], Integrated Deep-Probe Optical Waveguides [189], Interferometric biosensors [190], Luminescence sensors [191], Porous and planar silicon sensors [192], Acoustic wave biosensors [193], Amperometric biosensors [194], Field Effect Transistors [195], Impedance-based sensors [196], Molecular Nanowire transducer-based sensors [197], Magnetic sensors [198], Cantilever sensors [199], Raman spectroscopy [200], and others.

The main biological sensing materials used in biosensor development are the couple antibody/antigen [201]. Yet, a number of other recognition elements are currently used, which include nucleic acid diagnostics [202], aptamers [203], molecularly imprinted polymers [204], phage display methods [205], bacteriophages [206,207]. An excellent representation of bacterial sensors and a review of progress of bacteriophage use in bacterial sensors are presented by Singh et al. [208], where the majority of types of sensors, including that of nucleic acids and antibodies are presented, in addition to bacteriophages, and tables of sensors available for specific microorganisms. Part of nucleic acid based sensors utilise a number of the techniques available in common molecular detection assays, such as PCR or real-time PCR amplification and subsequent detection of amplification products, but in a lab-on-chip format with integrated microfluidic platform systems and various transducer/detection methodologies, reviewed by Lui et al. [209].

There have not, as yet, appeared specific sensors for rickettsial diseases in the literature. However, a number of the currently available sensor methodologies would apply to the detection of rickettsias as well as the organisms they were originally designed for, or have used as exemplars. To that effect, a number of patents on sensor development for bacterial detection have already included rickettsias in their list of pathogenic organisms potentially detected by the patented sensor technology. Two such examples are the design of electrochemical sensors including electrode systems with increased oxygen generation [210,211], where the invention describes the development of systems and methods for electrochemical analyte detection based on increased oxygen generation. Though the initial idea is to measure glucose, the patent proposes to cover a number of other 'contemplated analytes', one of which is rickettsia. In a similar case, a transcutaneous analyte sensor is described by Brister et al. [212], for measuring analytes in a host. In that invention, it is specified that the analyte is used in a broad sense, to include, without limitation, reaction products, naturally occurring substances, artificial substances, metabolites and/or reaction products and it subsequently specifies an extensive, but not limiting list of chemicals, products and microorganisms that the sensor could be applied for, including rickettsia, and thus protecting the use of such sensors in rickettsial diseases.

There are, however, two specific references to rickettsial pathogen detection. One is on a document from the US Office of Scientific and Technical Information (OSTI) of the Department of Energy (DOE), where Andy Hatch describes the development of the first ultrasensitive microanalytical diagnostic method for rickettsial pathogens [213]. In the published summary of his report, he states the use of *in situ* polymerised porous polymer monoliths as size exclusion elements for capture and processing of rickettsial infected cells from a sample and as a photopatternable framework for grafting high densities of functionalised antibodies and fluorescent particles. With this, they have achieved selective capture and identification of bacterial cells with at least an order of magnitude improvement in the detection limits of currently available methodologies and reduced detection times

[213]. The second direct reference to the development of rickettsial sensors is again from the US, with the award of a programme for the development of 'Handheld Aptamer-Magnetic Bead-Quantum Dot Sensor for Rickettsiae' from the US Department of Defence (Small Business Innovation Research SBIR website) [214]. In this programme, they have proposed to use a previously developed sensor format for *Campylobacter jejuni* developed by Operational Technologies, that uses a rapid and ultrasensitive one-step plastic adherent DNA aptamer-magnetic bead plus aptamer-quantum dot sandwich assay to detect the bacteria in a handheld battery operated fluorimeter sensor that can be operated under field conditions. The group has now proposed to develop aptamers against a mixture of isolated rickettsias and substitute those used for the *Campylobacter* detection on the same sensor. No further data have been found to date regarding the outcome of this project.

The existence of these two efforts clearly demonstrates the applicability of sensor development for rickettsial diseases and it may prove to be a practical route to an economically viable and rapid diagnostic approach that would offer definitive response without the need for extensive laboratorial work and long waiting times for patient immune response, at least as a first approach, prior to confirmation by molecular techniques.

## 6.5 Aptamers

Aptamers offer an interesting and promising new approach for the detection of bacteria and the development of new assays that could offer a more rapid and accurate detection. They are, in effect, recognition elements that can be selected against any target of interest, bacterial proteins, DNA or entire bacterial cells, and be integrated into any of the potential new methodologies described above. Thus, aptamers have been used in molecular imaging techniques, labelled with radionuclides [215,216] for the diagnostic imaging of disease. Similarly, aptamers have been used in a number of microarray formats and in conjunction with various detection methodologies. For example, RNA aptamers have been used in conjunction with enzymatically amplified surface plasmon resonance imaging for the detection of protein biomarkers [217]. Similarly, aptamers have been used in microarrays for the capture of biomarkers in serum to be analysed and identified by Mass Spectrometry [218]. Aptamers have also been described in biosensor applications, as seen earlier on [203, 219]. In addition to their use in such more novel approaches to detection, they have directly substituted antibodies in more traditional approaches, such as ELISA, and have been used in conjunction with antibodies in sandwich ELISAs [220] and in the bacterial detection of *Francisella tularensis* in what is described as an aptamer-linked immobilized sorbent assay - ALISA [221].

Aptamers have been utilised extensively in the development of sensors for bacterial detection and there is an extensive list of bacteria that have been used as targets of aptamers and aptamer sensor development for detection and bacterial typing. Some examples include *Salmonella* species [222-224], *E. coli* [224,225], *Staphylococcus aureus* [224,226], *Bacillus anthracis* and *Bacillus thuringiensis* [227], *Pseudomonas aeruginosa* [228], *Listeria monocytogenes* [229], *Francisella tularensis* [221], among others.

Despite the number of bacterial targets, both those used in diagnostic sensor development, as those mentioned above, and others used in the development of riboswitches, there have not been as yet any aptamers reported against rickettsias. However, *B. henselae* has been used as negative control, to show that aptamers were specific for *F. tularensis* but they did not bind to the related *Bartonella* [221] and, finally, they have appeared as the target in the development of the first rickettsial sensor by Jonh Bruno [214]. And as aptamers can be

applied in a variety of formats and can substitute antibodies in the more established and well-accepted ELISA and immunofluorescence assays, as well as in the variety of sensor development and microarray approaches, they show a great promise for bacterial diagnosis and detection of rickettsial infections in particular.

## 7. CONCLUSION

It is a big challenge for clinicians to offer an accurate diagnosis of rickettsial diseases during the early phase of infection. The gold standard serodiagnostic, immunofluorescence assay, is usually retrospective. The sensitivity of this method range from 84 to 100%, but it is limited by cross-reactivity. Antibodies increase takes more than 10 days, thus limiting the clinical impact of diagnosis. The isolation of rickettsia organisms in cell culture also take several days. Besides this, laboratory facilities with biosecurity level 3 are required. Molecular methods, based on PCR, for the diagnosis of human rickettsiosis allow rapid detection at the acute phase of infection and identification of bacterial species, even in small amount of the agent. Multiplex or Real-Time PCR can combine the detection of two or more agents of tick-borne infection into a single diagnostic test. Blood, serum, autopsy tissue fragments and skin biopsies specimens can be used as clinical samples to detect the rickettsial infection in molecular methods. The sensitivity of molecular method assays is linked to time of collection of clinical samples, the rickettsiemia level and is limited by the use of antibiotic therapy.

Various new approaches to the traditional assays have offered improvements to serologic and molecular diagnostic methods, which prove to be more sensitive than previous conventional methods. Thus, a novel enrichment liquid culture medium promotes the growth of *Bartonella* species in a shorter period, increasing the bacterial detectable level for PCR detection. The test double amplification (nested PCR), when compared with culture and simple PCR, is a more sensitive and faster method to detect bacteremia in both humans and animals. The use of combined approaches is also often necessary to minimize the chance of negative false results. Emphasizing that there is no gold standard for the diagnosis of bartonellose and the difficulty of detecting bacteremia caused by *Bartonella* spp. increases the need to use different and complementary methods to increase the sensitivity and accuracy of diagnosis.

Various novel methodologies have for a long time being developed by research laboratories on the area of biosensors, microarrays, imaging methods or spectroscopic techniques, but most of them have not yet made it to the market, or at least they have not been accepted as techniques used in reference laboratories worldwide. According to Jeanne Moldenhauer [230], industry has been reluctant to follow scientific and technological advancements in the development of rapid/alternative microbiological methods due to two major reasons. One was the concern that regulators would not recognise or accept these methods in place of traditional methods. The other was that companies would not be allowed to change test limits based upon the test method, i.e. they would use a superior method that was likely to detect more organisms and not be allowed to adjust the limits to accommodate for the sensitivity of the new method [230]. Yet, it is likely that these inhibitions will necessarily be overcome, due to the limitations to current methodologies. Thus, DNA and aptamer microarrays could offer simultaneous detection of multiple parasites with similar broad clinical manifestations, and thus avoid misdiagnosis and wrong treatment, often detrimental for the patient. Similarly, direct ELISA or ALISA (aptamer-based ELISA type assay), if available, could eliminate the time necessary in rickettsioses for the detection of patient antibodies, and the need for double measurements to confirm infection. Finally, biosensor and aptasensor technologies could offer the possibility of early detection with cheap and

approachable diagnostic assays at the clinic or the field, rather than the need for all material to be analysed only at national reference centres. Thus, aptamers and sensor technologies that are currently in development could play a pivotal role in the detection and early treatment of rickettsial disease, thus significantly reducing the death toll associated with these infections and contributing to public health improvement.

## CONSENT

Not applicable.

## ETHICAL APPROVAL

Not applicable.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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