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Implications of laboratory diagnosis on brucellosis therapy

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Brucellosis is a worldwide zoonosis with a huge economic impact on animal husbandry and public health. The diagnosis of human brucellosis can be protracted because the disease primarily presents as fever of unknown origin with unspecific clinical signs and symptoms. The isolation rate of the fastidious etiologic agent from blood cultures is low, and therefore laboratory diagnosis is mainly based on serologic and molecular testing. However, seronegative brucellosis patients have been described, and antibody titers of diagnostic significance are difficult to define. Whether the molecular detection of *Brucella* DNA in clinical samples should be followed by long-term antibiotic treatment or not is also a matter of debate. The aim of this article is to review and discuss the implications of laboratory test results in the diagnosis of human brucellosis on disease therapy.

KEYWORDS: antibiotic therapy • *Brucella* • culture • human brucellosis • laboratory diagnosis • molecular detection • serologic tests

Brucellosis is one of the world's most widespread bacterial zoonoses, leading to tremendous economic losses in endemic regions and serious complaints in affected patients. The infection may be transmitted by direct animal contact, but is usually acquired through the consumption of contaminated food products of animal origin, mainly via unpasteurized goat's milk and cheese. Furthermore, brucellosis is the most common bacterial laboratory-acquired infection worldwide [1].

Although many national and international programs have been established to eradicate the pathogen and control its spreading in animal husbandry, brucellosis is still a re-emerging disease. The surveillance of animal brucellosis is difficult due to bacterial persistence in wildlife and environmental reservoirs, with consecutive spill-over to domestic animals [2].

Brucellosis is caused by members of the genus *Brucella* (*B.*), which are gram-negative, facultative intracellular coccobacilli that were historically differentiated by their preferred animal host, varying pathogenicity and a few selected phenotypic traits. The genus comprises six classical species: *B. melitensis* bv 1–3 (primarily isolated from sheep and goats); *B. abortus* bv 1–6 and 9 (from cattle and other Bovidae); *B. suis* bv 1–3 (from pigs), bv 4 (from reindeer), bv 5 (from small rodents); *B. canis* (from dogs); *B. ovis* (from

sheep); and *B. neotomae* (from desert wood rats). Recently, two novel species of marine origin, *B. pinnipedialis* (isolated from seals) and *B. ceti* (from dolphins and whales) [3], *B. microti* isolated from the common vole (*Microtus arvalis*) [4], red foxes (*Vulpes vulpes*) [5] and from soil [6], and *B. inopinata* isolated from a breast implant wound of a 71-year-old female patient [7] have been described. In the past, a lot of atypical *Brucella* strains arose. These could represent novel species or lineages of already described species, for example various *Brucella* strains originating from wild native rodent species in North Queensland, Australia [8], a novel *Brucella* isolate in association with two cases of stillbirth in non-human primates [9], and a *B. inopinata*-like strain (BO2), which was isolated from a lung biopsy of a 52-year-old Australian patient suffering from chronic destructive pneumonia [10].

Physicians' awareness of the infection is very poor in many countries, and most cases correctly identified are clinically advanced. Because of its protean clinical manifestations, human brucellosis can be easily confused with other infectious and noninfectious diseases, leading to diagnostic delays and late onset of therapy. The isolation of the fastidious organisms is often unsuccessful or takes a long time, which is why the presumptive clinical diagnosis is usually confirmed by

serologic tests. However, seronegative cases, cross-reactivity of anti-*Brucella* antibodies with many other clinically relevant bacteria, poorly defined cutoffs in serologic test systems, and so on can make the interpretation of the titers measured very difficult. Alternatively, molecular techniques can be used for the laboratory diagnosis of human brucellosis, but the detection of *Brucella* DNA does not prove an active infection with viable bacteria, and therefore does not effectively support therapeutic decision making.

The scope of this article is to review up-to-date laboratory techniques in the diagnosis of human brucellosis and to discuss if positive serologic or molecular tests should result in long-term antibiotic therapy.

Global epidemiology of brucellosis

Approximately half a million human brucellosis cases are annually reported, but the official figures do not fully reflect the number of people infected. Many cases remain unrecognized due to inaccurate diagnosis, and are thus treated as other diseases or as 'fever of unknown origin'. According to the estimates of the WHO, the true incidence is 10–25-times higher than the reported figures indicate [11]. Surveillance data reveal a smaller gap between notified and actual cases, but the difference in these figures is obviously caused by a variable case definition.

Since new species continuously emerge, and the currently known *Brucella* spp. keep on adapting to environmental changes, the epidemiology of brucellosis remains obscure. On the one hand, improved diagnostic strategies and transboundary actions for a better surveillance helped to eradicate and control the disease in formerly endemic regions. On the other hand, alterations in socioeconomic and political systems, increasing globalization including international animal trade and worldwide tourism, and a decreasing awareness by practitioners and public health authorities led to the re-emergence of new endemic foci [12].

At present, *B. melitensis* is by far the main cause of clinically apparent disease in humans worldwide, although the distribution of ovine/caprine brucellosis is geographically limited [13]. Ovine/caprine brucellosis is highly prevalent in countries surrounding the Mediterranean Sea and the Arabian Gulf, in Central Asia and parts of Latin America, especially Mexico, Peru and northern Argentina [14–16]. In addition, *B. melitensis* infections in sheep have been noted in sub-Saharan Africa [17]. In the USA, Canada, Northern Europe, Australia, New Zealand and Southeast Asia, *B. melitensis* is not enzootic, and only sporadic incursions have been reported. In southern European countries the epidemiological situation is less favorable [18], and the Balkan Peninsula is still a major hotspot. These endemic regions are presumably an important source of disease distribution throughout Europe through illegal imports of contaminated food products and international tourism [19]. The highest annual incidence rates are reported from countries of the Middle East, such as Syria, Iraq, Iran and Saudi Arabia [12].

Bovine brucellosis has been successfully eradicated in Canada, Australia, Japan and Northern Europe, whereas *B. abortus* is still widely distributed among cattle in sub-Saharan Africa [20]. Sweden, Denmark, Finland, Germany, the UK (except

for Northern Ireland), Austria, The Netherlands, Belgium and Luxembourg were granted brucellosis-free status in the EU [21]. Norway and Switzerland are also considered 'officially free from bovine and ovine/caprine brucellosis'.

National surveillance programs monitoring the prevalence of porcine brucellosis in livestock are scarce, but outbreaks on pig-rearing farms are observed in most areas where pigs are kept outdoors. In Asia, South America (predominantly in Argentina), the southeastern states of the USA, and in Queensland, Australia, the human pathogenic biovars *B. suis* bv 1 and bv 3 are isolated from wild boars, feral swine and domestic pigs [2,22]. In Europe, *B. suis* bv 2 is the most frequently isolated biovar in porcine brucellosis, but has only exceptionally been described as the causative agent of human brucellosis.

None of the above-mentioned human pathogenic *Brucella* species has been globally controlled or finally eradicated. Brucellosis is still a regionally emerging disease, and cannot be easily contained by border controls [23].

Clinical manifestations & treatment of human brucellosis

Human brucellosis is characterized by protean clinical manifestations, and almost every organ system can be affected. Cutaneous, hematologic, gastrointestinal, genitourinary, respiratory, osteoarticular, cardiovascular and neurologic disorders may occur [24]. After an incubation period varying from a few weeks to several months, the acute infection usually presents as a feverish flu-like disease. As the fever may wax and wane, human brucellosis was formerly named 'undulant fever'. Owing to the wide spectrum of its clinical manifestations, brucellosis mimics many other infectious, as well as noninfectious diseases and, therefore, clinical and laboratory diagnosis is frequently delayed or even missed. Brucellosis patients initially suffer from headaches, arthralgia and myalgia, fatigue, malaise, weight loss, chills and sweating. The acute stage of the disease is usually accompanied by bacteremia and the spreading of *Brucella* to various organ systems, mainly to the reticuloendothelial tissues, for instance, liver, spleen, skeletal and hematopoietic system. Consequently, major clinical findings are hepatomegaly and splenomegaly [25]. Since brucellae are able to survive and replicate in mononuclear phagocytic cells, human brucellosis is frequently characterized by focal complications, prolonged and chronic courses, primary treatment failures and relapses. Osteoarticular manifestations, for example spondylitis, sacroiliitis and arthritis, are known to be the most frequent focal complications [24,25]. Endocarditis and neurobrucellosis are responsible for the majority of fatal outcomes. Although these focal complications can be life-threatening, the overall case fatality rate of human brucellosis is low (<1%).

Brucellosis is both a preventable and curable infectious disease. Primary aims and objectives of an adequate antibiotic treatment are shortening the natural course of a symptomatic disease, decreasing the incidence of complications and preventing relapse. However, the versatility of the clinical picture often results in misdiagnosis or extensive diagnostic delays, which increases both complication and case fatality rates [26].

Although essential drug resistance has not yet been described in *Brucella* isolates, neither monotherapy nor short-term antibiotic regimens are considered to be adequate in the treatment of human brucellosis [27]. A prolonged application of antibiotic drugs progressively decreases the risk of primary treatment failure and relapse. In particular, patients suffering from focal disease such as endocarditis or spondylitis may require long-term antibiotic therapy and additional surgical interventions.

The most widely used antibiotic regimens are based on oral doxycycline (DOX) 100 mg twice a day in combination with rifampin (RIF) 600–900 mg/day in a single oral dose over a 6-week course. Instead of rifampin, streptomycin (STR) 1 g (15 mg/kg/day) can be administered intramuscularly once daily for 2–3 weeks [28]. The aminoglycoside streptomycin can be replaced by gentamicin (GENTA) in multidrug regimens for brucellosis without loss of efficacy [29]. Although the overall failure rate (mainly due to a high relapse rate) is significantly higher in patients treated with DOX-RIF compared with DOX-STR (relative risk 2.80 [95% CI: 1.81–4.36], 13 trials) [29], the former is also recommended as first line-therapy in human brucellosis [27]. Major reasons for the preference of the DOX-RIF regimen are the oral application and fewer adverse effects. Triple combinations including DOX, RIF and GENTA proved to be more effective than DOX only combined with an aminoglycoside [29]. However, triple therapy has to be critically evaluated, especially for patients suffering from acute disease without focal complications. In the treatment of children <8 years old, tetracycline is contraindicated and should be replaced by trimethoprim-sulfamethoxazole (TMP-SMX; co-trimoxazole) in dual drug regimens [30]. In exceptional circumstances, TMP-SMX might be used as a monotherapy for a prolonged period of time (up to 6 months) [29].

In clinical practice, the recommended regimens cannot be applied universally but must be individualized. The key to a successful treatment remains a matter of continuance of antibiotic therapy and a matter of specific regimens.

It is difficult to compare the various antibiotic regimens in the therapy of human brucellosis because double-blind, placebo-controlled, multicenter clinical trials are still lacking, but also because cases are not clearly defined. In particular diagnostic criteria for case definition and successful therapy are extremely variable. Hence, brucellosis patients may comprise culture-proven cases, cases showing significant anti-*Brucella* antibody titers, a significant rise in titers or seroconversion in several serologic tests, cases based on the detection of *Brucella* DNA in blood, tissues and body fluids, or even seronegative cases with a presumptive clinical diagnosis of brucellosis and epidemiological coherence.

Isolation & identification of *Brucella* spp. from clinical samples

Definitive diagnosis of human brucellosis requires the isolation of the etiologic agent from blood, bone marrow or other tissues and body fluids. The bacterial isolation rates are variable depending on the stage of disease, previous use of antibiotics, the clinical specimen and, last but not least, the culture methods [31]. Since the number of circulating bacteria in the blood of brucellosis patients

is assumed to be low, the successful recovery of *Brucella* is highly dependent on the total volume of the sample. The time-to-detection inversely correlates with the concentration of viable organisms in the blood sample [32]. Hence, multiple blood sampling in acute brucellosis cases and sampling material from infected sites in patients suffering from focal complications may help to confirm an active infection by bacterial isolation. Bacteremia is an early event in the pathogenesis of *Brucella* infections, and isolation rates are much higher in acute cases presenting with symptomatic disease of less than 2 weeks [33]. Because bacteremic patients more often present with fever and chills than nonbacteremic patients [34], the recovery rate of brucellae can be improved through blood samples taken in the pyrexial phase. In acute brucellosis cases, the sensitivity of culturing *Brucella* spp. from blood may vary from 80 to 90%, whereas in chronic cases bacteriologic confirmation is less successful, ranging from 30 to 70% depending on the technical approach [35,36]. Hence, successful recovery of brucellae from blood samples depends on both the stage of disease and culture techniques. In highly developed nonendemic countries diagnosis is often missed despite modern and suitable technologies because chronic courses frequently occur due to a lack of clinical suspicion, whereas in endemic countries diagnosis is often missed because laboratories are poorly equipped.

Using bone marrow cultures instead of blood cultures further increases the recovery rate at any stage of disease (TABLE 1), and the mean time to detection can be significantly shortened [37,38]. Bone marrow culture also proved to be more sensitive in detecting *Brucella* spp. in patients who underwent antibiotic pretreatment. Although bone marrow aspiration and biopsy can be painful, this procedure might be of value in particular cases such as serologically negative patients suffering from fever of unknown origin if brucellosis has to be suspected due to the patient's medical history and clinical presentation [37].

Brucella spp. grow on most standard media, for example blood agar, chocolate agar, trypticase soy agar and serum-dextrose agar. Bovine or equine serum (2–5%), which is needed for growth by various strains, is routinely added to the basal medium. Blood cultures should be incubated at 35–37°C in air supplemented with 5–10% CO₂. Since the isolation of *Brucella* from clinical samples is hampered by its slow growth, culturing the fastidious bacterium may take several days or even weeks before visible, punctate, nonpigmented and nonhemolytic *Brucella* colonies may appear. Colonies of smooth brucellae are raised, convex, circular, translucent and 0.5–1 mm in diameter. The oxidase- and urease-positive *Brucella* spp. are very small, faintly stained Gram-negative coccobacilli that microscopically look like 'fine sand'.

To maximize the recovery rate from clinical specimens, broth culture methods are traditionally used for primary enrichment, incubation periods are prolonged in presumptive cases and blind subcultures are regularly performed. However, previous antibiotic therapy in feverish patients may prevent or delay the recovery of brucellae from clinical specimens, especially from usually sterile body fluids in which the bacterial inoculum is often small. In the past decades, various technical improvements (e.g., the biphasic Castañeda method, automated systems and yield-optimizing

Table 1. Diagnostic yield of culture techniques, serologic tests and molecular methods in the laboratory diagnosis of human brucellosis depending on the stage of disease.

Diagnostic methods (n)	Diagnostic yield depending on the stage of disease (duration of clinical symptoms) (%)				Ref.
	<i>Acute brucellosis</i> (≤8 weeks)	<i>Subacute brucellosis</i> (8–52 weeks)	<i>Chronic brucellosis</i> (≥52 weeks)	<i>Relapse</i>	
<i>Whole blood culture</i>					
Brucella broth and subcultures (50)	66.6	23.5	0.0		[106]
Castañeda biphasic medium (88)	42.3	41.7	11.0	8.3	[35]
Castañeda biphasic medium (103)	54.7	36.4	28.6		[38]
Castañeda biphasic medium (148)	71.8		33.3		[42]
Castañeda biphasic medium (50)	83.3	40.0	25.0		[37]
Lysis centrifugation (88)	48.1	58.3	22.2	16.6	[35]
Lysis centrifugation (148)	90.9		74.1		[42]
<i>Bone marrow culture</i>					
Brucella broth and subcultures (50)	83.3	52.0	33.0		[106]
Castañeda biphasic medium (50)	97.2	90.0	50.0		[37]
Castañeda biphasic medium (103)	92.2	72.7	64.3		[38]
<i>Serology</i>					
Tube agglutination (50)	91.7	70.0	75.0		[37]
Serum agglutination (92)	21.0	50.0	27.5		[67]
Commercial IgM- and IgG-ELISA (92)	49.1	66.6	82.7		[67]
<i>Molecular detection</i>					
Real-time quantitative PCR (18)	100			100	[95]

methods such as lysis centrifugation) have gradually increased the sensitivity of culture methods and have significantly shortened the time to detection for *Brucella* spp. in clinical samples [32].

The classic biphasic Castañeda method, which is based on a solid and a liquid phase in the same blood culture bottle, avoids the need for repeated subcultures. However, the recovery time of brucellae from blood can still take up to 30 days. In contrast, automated blood culture systems significantly reduce the time to detection. Hence, the etiologic agent can be isolated from blood of infected patients within ≤4 days using BACTEC™ (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) or BacT/Alert™ (bioMérieux Inc., Durham, NC, USA), which continuously monitor the CO₂ release of potentially growing microorganisms [39]. Furthermore, the recovery rate for pathogenic microorganisms including *Brucella* spp. from sterile body fluids is higher using automated blood culture systems [40]. Despite the better performance of automated culture systems compared with solid culture media and semi-automated systems, a prolonged incubation period and periodic subcultures for at least 4 weeks are still recommended to reliably exclude a *Brucella* infection [32].

Enrichment of the bacteria using the blood clot culture technique or lysis centrifugation increases the isolation rate of brucellae from blood samples [35,41]. The lysis centrifugation method shows the highest yield among the well-established culture techniques

independent of the stage of disease (TABLE 1) and the mean time to detection both in blood and sterile body fluids can be significantly reduced by several days [35,40,42]. Using lysis centrifugation, the mean detection time ranges from 2 to 3 days, and most of the *Brucella* isolates are recovered before bacterial growth occurs in conventional cultures [42]. The BACTEC Myco/F-Lytic system successfully combines lytic activity and automation [43].

If the number of culturable brucellae is expected to be very low, for example in clinical specimens such as pus, shell vial culture may be an alternative method that allows the isolation of the facultative intracellular pathogens [44].

Colonies suspicious for *Brucella* spp. can be confirmed by the slide agglutination test using undiluted polyvalent *Brucella* anti-serum (anti-S [smooth] serum) mixed with a saline suspension of colonies. Further identification of *Brucella* species and biovars is usually based on CO₂ requirement, H₂S production, urease activity, agglutination with monospecific sera (A and M), selective inhibition of growth on media containing dyes such as thionin or basic fuchsin and phage typing [31,45]. Since these procedures are time-consuming, hazardous and subject to variable interpretation, they are not suited for clinical microbiological laboratories. Using commercially available biochemical tests such as API 20 NE® (bioMérieux, Nürtingen, Germany), *Brucella* spp. may be misidentified, for example, as *Psychrobacter phenylpyruvicus*

(formerly *Moraxella phenylpyruvica*) [46] or *Ochrobactrum anthropi* [47]. Recently, a semi-automated metabolic biotyping system (Micronaut™; Merlin Diagnostika, Bornheim-Hersel, Germany) based on a selection of 93 different substrates was developed for the identification of *Brucella* and the differentiation of its species and biovars [48]. This novel technology may replace or at least complement time-consuming tube testing, especially in case of atypical strains. However, subtyping of *Brucella* spp. is not necessary to arrive at a decision on therapeutic measures. In contrast, the rapid identification of the genus *Brucella* is crucial in order to initiate antibiotic treatment early in the course of disease, thus preventing chronic courses and focal complications.

The development of new diagnostic techniques that facilitate the rapid detection of brucellae directly from culture and minimize the risk of laboratory infection is of great practical importance. A direct urease test on positive blood cultures, which are suggestive of *Brucella* spp., can accelerate the presumptive diagnosis of human brucellosis and unmask *Brucella* bacteremia despite contaminating organisms within blood cultures [49,50]. Fluorescence *in situ* hybridization (FISH) using *Brucella*-specific probes is also a valuable tool for the identification of cultured isolates and for the direct detection of brucellae in positive blood cultures [51]. Hence, the bacteria can be rapidly identified without the need for further subcultures and phenotypical tests. A novel up-converting phosphor technology-based lateral flow assay quantitatively detected *Brucella* from both pure culture and spiked tissue samples [52]. Quite recently, matrix-assisted laser desorption/ionization mass spectrometry and surface enhanced laser desorption/ionization mass spectrometry proved to be of benefit in the direct identification of members of the genus *Brucella* from culture plates and blood culture bottles [53,54]. However, comprehensive databases including characteristic protein profiles of *Brucella* species are missing, which currently restricts the use of these upcoming technologies in clinical microbiology laboratories.

Bacteria isolated during relapse show the same antimicrobial susceptibility pattern as isolates obtained in the primary episode of the disease, and most of the relapsed cases respond well to a repeated course of the standard antibiotic therapy [55]. Hence, antibiotic drug resistance does not essentially contribute to treatment failures and relapses, and *in vitro* susceptibility testing of *Brucella* isolates is therefore of questionable clinical significance.

Serological diagnosis in brucellosis patients

Since culture techniques are time-consuming, hazardous and not sensitive, most physicians rely on the indirect proof of *Brucella* infections based on high or rising titers of specific antibodies. Furthermore, serologic tests are not only used for the primary diagnosis of human brucellosis, but also for treatment follow-up. However, the usefulness of serology in treatment follow-up is just minimally proven.

Antibody profiles & serologic testing in the course of disease

The predominance of IgM isotype antibodies in the first week after inoculation is usually followed by a switch to IgG in the second week and a continuous rise in titers of both subtypes that

peaks within 4 weeks [56]. Early in the course of the disease, serologic tests can be negative and, therefore, laboratory testing should be repeated after 1–2 weeks in clinically suspicious cases.

Sequential serologic testing also allows the monitoring of treatment response. Antibody titers usually decline after the beginning of an adequate antibiotic treatment, but significant titers may persist for several months or even years, despite therapeutic success and negative blood cultures [57,58]. This fact complicates the differentiation between active infection and a history of brucellosis or an immune memory without clinical relevance as a result of repeated exposure to the etiologic agent. Raised titers may consequently lead to unnecessary long-term antibiotic therapy.

Due to missing laboratory diagnostic criteria for definite cure, persistent antibody titers in the course of follow-up are difficult to interpret. Patients with active disease cannot be easily differentiated from people with past brucellosis by serologic test results. On the one hand, the sole detection of anti-*Brucella* antibodies does not provide evidence for the presence of the pathogen. On the other hand, high titers during post-treatment follow-up are often related to high titers during the initial disease phase and are not always a sign of primary treatment failure, chronic disease or relapse. Particularly in endemic regions, a large proportion of the population may have persistent specific antibodies due to continuous exposure to *Brucella*. The evaluation of the background prevalence in healthy individuals is therefore important to determine reliable cutoff values for serological methods in endemic and nonendemic regions.

A rapid fall of IgG antibody titers is a prognostic indicator for a successful therapy, whereas persisting high IgG titers after treatment can be a sign of active disease [59]. Antibody titers decrease more slowly in patients suffering from focal complications [58], and a relapse is characterized by a second peak of anti-*Brucella* IgG and IgA, but not IgM immunoglobulins.

The serologic response in the course of brucellosis is mainly based on antibodies directed to smooth lipopolysaccharides (s-LPS). Most classical serologic tests, along with commercially available ELISA, make use of bacterial extracts containing high concentrations of s-LPS, and thus exhibit a reliable detection of agglutinating and/or non-agglutinating antibodies. Since the immunodominant epitope of the *Brucella* O-polysaccharide is similar to that of various clinically relevant bacteria, for example *Yersinia enterocolitica* O:9, *Salmonella urbana* group N, *Vibrio cholerae*, *Francisella tularensis*, *Escherichia coli* O:157 and *Stenotrophomonas maltophilia*, cross-reactivity may occur and the specificity of LPS-based assays can be low [56].

In patients presenting clinical symptoms similar to brucellosis and a compatible epidemiological background and/or inconclusive serologic test results using standard methods based on smooth-*Brucella* antigens, canine brucellosis should be excluded [60]. The serological diagnosis of canine brucellosis requires the use of specific antigen preparations, since *B. canis* strains do not share the cross-reacting LPS antigen with other *Brucella* spp. As there is still no standardized reference antigen used in serologic tests, the antigen preparation may influence the serological diagnosis of human brucellosis.

Numerous serological methods for the detection of *Brucella*-specific antibodies are available. The technical details of current serologic tests in the diagnosis of human brucellosis have been previously described in-depth [45,56,61]. The most popular serologic tests in the diagnosis of human brucellosis are serum agglutination test (SAT), Rose Bengal test (RBT), Coombs' test (CT) and ELISA. According to their overall accuracy in clinical settings, these test systems can be ranked, such as ELISA > RBT > SAT > CT [56].

The complement fixation test, which is widely used as a confirmatory test for the serological diagnosis of animal brucellosis, is not frequently applied to diagnose human disease in clinical laboratories. There is comparably little experience in the use of fluorescence polarization assay in the laboratory diagnosis of human brucellosis [62,63], whereas this test has been widely used for the surveillance of animal brucellosis.

The SAT, RBT & lateral flow assay

The SAT is generally accepted as the reference method in the serological diagnosis of human brucellosis [56]. However, the classical tube agglutination test (Wright test) is labor-intensive and time-consuming, making its applicability in routine laboratories with a large turnover of blood samples questionable. More practicable formats of this method are slide, plate and card agglutination.

In endemic countries the RBT, which is a card test using *B. abortus* strain 1119-3 (USDA) antigen suspension (8%) stained with Rose Bengal dye buffered to pH 3.65 ± 0.05, has been traditionally used as rapid screening test in emergency departments. The diagnostic gain of the RBT is excellent in patients without previous exposure to *Brucella* or a history of brucellosis, but poor in patients who were repeatedly exposed to the etiologic agent or were formerly infected [64]. The RBT therefore requires confirmation with a more specific test such as ELISA. Alternatively, the titrated RBT testing diluted sera may help to correctly identify patients in a high-risk population with potential previous exposure. In nonendemic countries, specificity of diagnostic tests is less of a concern provided that follow-up sera can easily be requested in order to re-evaluate one-off low-level positive titers as 'non-diagnostic' [65]. In addition, a high pre-test probability based on clinical signs and symptoms may also increase the likelihood of a true positive serologic test result in patients with suspected brucellosis [64]. Although the interpretation of SAT and RBT are largely subjective, there is a considerable agreement between test results both among different laboratories and with other serologic tests.

An alternative easy-to-perform method for rapid field or bedside testing in poor rural areas where well-equipped laboratories are not available is the lateral flow assay. The *Brucella* lateral flow assay proved to be slightly more sensitive in detecting low levels of specific IgG or IgM antibodies than SAT [66].

SAT titers ≥1:160 are generally considered consistent with active brucellosis if accompanied by a compatible clinical course in patients with a history of potential exposure. However, the cutoff values for relevant titers in agglutination tests are still controversially discussed. A high prevalence of *Brucella* antibodies in the healthy population decreases specificity, and titers ≥1:320 may be more specific in endemic areas. SAT suffers from high

false-negative rates in complicated and chronic cases. In the early course of the disease, even bacteremic patients may present with titers ≤1:160 [33,35]. A fourfold or higher rise in *Brucella* agglutination titer between acute- and convalescent-phase serum specimens obtained at least 2 weeks apart may prove the infection.

Hence, a single agglutination titer ≤1:160 cannot be of diagnostic significance, and some brucellosis cases that are often seronegative in the acute stage of disease might be overlooked without serological testing of paired sera [67] or performing more than a single serologic test [68]. A combination of various serological assays including different test methods may help to warrant quality performance because false-negative results due to low antigen quality or poor technical standards can be avoided.

The definite cure of a patient correlates well with lower SAT titers. Therefore, brucellosis patients should be followed-up, both clinically and serologically [69]. However, extended serological follow-up periods might not be reasonable in patients who are clinically well. Significant SAT titers are found in 3–5% of the clinically cured brucellosis cases 2 years after successful antibiotic treatment [69], and these figures may be even higher in different populations, after another antibiotic regimen, using other serological tests, and so on.

Coombs' test & Brucellacapt®

The classical CT is most commonly used as an extension of SAT to detect incomplete, blocking or nonagglutinating antibodies. CT is the most appropriate serologic test to detect the slight changes in anti-*Brucella* antibody titers in chronic courses and during relapse [58].

A major drawback of the classical tests, such as SAT and CT, is that they are labor-intensive and time-consuming. Brucellacapt® (Vircell, Santa Fé, Granada, Spain), a single-step immunocapture assay for the detection of total anti-*Brucella* antibodies, is a valuable alternative to the CT. The simplicity of Brucellacapt makes it suitable as a second complementary test. Patients with persistent disease more frequently present with higher titers at admission, a slower regression during follow-up and never reach titers ≤1:320 in the Brucellacapt [59]. Particularly in relapsed cases, titers determined by Brucellacapt and by CT decrease more slowly and show several peaks compared with SAT [58]. These changes are more evident in bacteremic relapses. The decrease in titers after successful treatment and clinical cure of patients is more pronounced and rapid in Brucellacapt than in SAT and CT. Hence, Brucellacapt titers are a good marker of infection activity especially useful in the follow-up of patients. However, in some cases of relapse and chronic disease, only slight changes in low-affinity antibodies are observed, which are better detected by CT [58].

Enzyme-linked immunoassay

The results obtained using commercially available ELISA kits show very good concordance with SAT and CT for detecting *Brucella* antibodies [70,71]. The ELISA can therefore be reliably used in the diagnosis of human brucellosis. Especially in chronic and past brucellosis, the ELISA is more sensitive than SAT (TABLE 1). In acute cases, however, agglutination tests show the same results and are less expensive.

ELISA is an excellent method for screening sera for *Brucella* antibodies, and the primary detection of IgM titers may be sufficient for the diagnosis of brucellosis in most patients presenting with symptoms suggestive of acute disease. However, IgM antibodies cannot be reliably detected in all patients suffering from brucellosis. False-negative IgM results might be obtained due to an excess of IgG antibodies, and false-positive results due to the presence of rheumatoid factor [65,72]. Hence, rheumatoid factor should be eliminated by absorption routinely before testing for anti-*Brucella* IgM antibodies to rule out a false-positive result [72]. If only a single subgroup of immunoglobulins is quantified using ELISA, many patients will be tested false-negative [68]. At least IgG and IgM antibodies have to be determined to reliably diagnose human brucellosis and classify the stage of disease [67,73].

Molecular detection of brucellae in clinical specimens

In the laboratory diagnosis of human brucellosis, PCR has proven to be more sensitive than blood culture and more specific than serologic tests, both in acute and chronic disease. Furthermore, work on DNA decreases the risk of laboratory-acquired infections due to highly infectious live cultures.

Numerous PCR methods have been developed for the direct identification of cultured brucellae, and a few of these techniques have proven valuable for the diagnosis of human brucellosis [74,75]. *Brucella* DNA is detectable in various clinical specimens, including serum, whole blood and urine samples, different tissues, cerebrospinal fluid, synovial or pleural fluid, and pus [76–81]. Due to their easy availability, whole blood and serum samples are currently preferred for molecular diagnosis of human brucellosis. Whether the serum fraction is superior to whole blood for molecular detection of the etiologic agent, or vice versa, is still controversially discussed [82,83]. The concentration of PCR inhibitors is lower in serum samples, but the small number of circulating bacteria, for example in chronic courses or after antibiotic treatment, may result in the absence of target DNA leading to false-negative results.

Because antibiotic regimens are independent of the disease-causing species in human brucellosis, the detection of *Brucella* by genus-specific PCR is basically adequate for rapid diagnosis and initiation of therapy. Although various target sequences have been used for the identification of the genus *Brucella* [31], in clinical settings the majority of PCR assays target the *bcsp31* gene, which codes for a 31-kDa immunogenic outer membrane protein conserved among all *Brucella* spp. [84]. A genus-specific approach targeting the *bcsp31* gene appears to be suitable for screening, as false-negative results caused by rare species and biovars may be avoided. However, a second gene target is mandatory to confirm a primary molecular diagnosis. The use of more than one molecular marker may increase sensitivity and specificity [85]. 16S rRNA gene sequencing can be a reliable tool for the rapid confirmatory identification of *Brucella* spp., and their differentiation from closely related microorganisms [86]. Differential PCR assays may additionally allow species-specific surveillance. For this purpose, a conventional multiplex PCR assay suitable for the identification of all *Brucella* species and the vaccine strains, *B. abortus*

RB51, *B. abortus* S19 and *B. melitensis* Rev1, has recently been developed [87,88]. In a worldwide multicenter study, the so-called Bruce-ladder PCR has proven to be useful for the rapid identification of *Brucella* strains in basic microbiology laboratories [89].

In routine microbiology laboratories, real-time PCR will allow a more rapid high-throughput screening of samples, making test results available within a few hours [85,90]. Genus-specific real-time PCR assays have been successfully applied to various human specimens. Inhibitory effects may occur in clinical samples [91], but an internal amplification control can help to unmask PCR inhibition [85]. Despite the high analytical sensitivity of real-time PCR assays, the generally small number of bacteria in clinical samples is still a challenge in the molecular diagnosis of human brucellosis. Basic sample preparation methods, therefore, have to reduce inhibitory effects caused by matrix components and should also concentrate the DNA. The simple boiling of serum to process a bacterial DNA template does not prevent the presence of PCR inhibitors, but various commercial kits, such as the QIAamp™ DNA Mini Kit (Qiagen Inc., Valencia, CA, USA) and the UltraClean™ DNA BloodSpin Kit (MO BIO Laboratories Inc., Carlsbad, CA, USA) have been successfully used to extract *Brucella* DNA from whole blood, serum and tissue samples [92–94]. However, comprehensive evaluation studies on DNA extraction methods in different human samples are currently lacking.

Using well-established *Brucella* real-time PCR assays, approximately five bacteria per reaction can be detected [85,95]. Sensitivity can be further increased by testing several replicates of the purified DNA in parallel or using the *IS711* insertion element of *Brucella* as a target sequence that is found in multiple copies within *Brucella* chromosomes [96,97].

In the past decade, a lot of clinical studies have been conducted to evaluate the usefulness of PCR methods in the course of human brucellosis, beginning with the incubation period throughout post-treatment follow-up. The qualitative molecular detection of *Brucella* DNA virtually proves acute brucellosis or a history of the disease. After initiating antibiotic therapy, the DNA load in blood samples clearly decreases, coinciding with the disappearance of symptoms, but remains low-level positive during follow-up even in asymptomatic patients [95]. Comparable with conventional microbiological methods, clearly defined criteria to establish the success of treatment or to predict relapse are also missing in the molecular diagnosis of human brucellosis.

Quantitative real-time PCR is a valuable tool in the initial diagnosis of the symptomatic nonfocal disease in patients, for whom the classical microbiological methods fail [98]. In addition, active and past *Brucella* infections can be differentiated [99]. Using single-step conventional PCR assays for post-treatment follow-up, the detection of *Brucella* DNA in blood samples is supposed to be a sign of relapse, whereas a negative PCR result proves a successful treatment outcome [82,100]. In contrast, using real-time PCR techniques, *Brucella* DNA can be detected in the majority of brucellosis patients throughout treatment and follow-up, despite appropriate antibiotic therapy and apparent clinical recovery [92,94,95]. Bacterial DNA load constantly decreases after the end of treatment. However, in a significant number of patients, *Brucella*

DNA remains detectable for several months or even years after clinical cure and in the absence of any symptoms indicative of chronic disease or relapse [94]. Hence, clinical response to antibiotic treatment does not seem to be equivalent to pathogen eradication in brucellosis patients, a phenomenon that may be explained by survival and persistence of brucellae in human macrophages. Due to the low bacterial replication rate, the patient's immune system is able to control transient bacteremia. Furthermore, modern real-time PCR assays reveal a higher diagnostic yield than conventional techniques, leading to the sensitive detection of nonviable or phagocytosed microorganisms. However, *Brucella* DNA-negative patients can also relapse [95].

The bacterial DNA load in the course of disease does not differ fundamentally in patients who relapse and those who do not [92,95]. Nevertheless, quantitative real-time PCR may be helpful in the diagnosis of chronic infection. *Brucella* DNA can be detected in asymptomatic subjects with a history of brucellosis, albeit in a smaller proportion than in the group of symptomatic patients suffering from chronic disease [98].

As mentioned above, species identification is not necessary to decide on the antibiotic regimen or the duration of therapy, but further subtyping to the strain level can be helpful to differentiate a new exposure from a relapse, especially in endemic countries. Genetic loci containing variable number of tandem repeats (VNTRs) have recently proven their usefulness in molecular typing of *Brucella* strains, despite the high genetic homogeneity within the genus [101,102]. As a consequence, a multiple locus VNTR analysis assay based on 16 markers (MLVA-16) was developed for diagnostic use in human brucellosis [103]. Identical MLVA-16 genotypes of *Brucella* strains isolated from the same patient before and after first-line therapy may ascertain a relapse, and treatment modifications like a prolonged antibiotic therapy can be implemented [104]. In contrast, genetic fingerprinting may reveal different genotypes in the case of re-infection, and standard therapy can be repeated without losing effectiveness.

Implications of laboratory diagnosis on therapy

Since the treatment of human brucellosis is based on long-term application of multiple antibiotic drugs with a multitude of adverse effects, therapeutic recommendations have to rely on a definite laboratory diagnosis. First of all, only persons with a potential exposure should be tested for brucellosis in order to decrease the likelihood of false-positive results. Patients who require treatment should be clearly defined both before first-line therapy and in the course of follow-up. Although the infection may have been proven already in relapsed and chronic cases, diagnostic criteria must not be weaker.

A patient should be considered to be a brucellosis case if signs and symptoms are compatible with the disease and the presumptive clinical diagnosis is laboratory-confirmed (FIGURE 1); for instance, brucellae can be isolated from blood, other body fluids or tissue samples, the primary agglutination titer exceeds 1:160, seroconversion or a fourfold rise in agglutination titers is monitored in a follow-up serum, or *Brucella* DNA is detected in a clinical specimen.

The isolation of *Brucella* spp. from clinical specimens should promptly initiate therapeutic considerations. Particularly in endemic regions, cultural isolation should be of priority to assure a clinical diagnosis, because the interpretation of agglutinating antibody titers can be confounded by the presence of elevated baseline titers in the population. The cutoff for a positive serologic test result may vary depending on the contact frequency with *Brucella* spp. Oligosymptomatic and asymptomatic, self-limiting episodes of infection are common in human brucellosis, and significant anti-*Brucella* IgG antibody titers may persist over a long period of time after exposure to the etiologic agent, even after successful antibiotic therapy [105]. Therefore, serologic test results should only be considered as supportive evidence for a recent infection and interpreted in the context of both a clinically compatible illness and exposure history. In addition, the prescription of prolonged antibiotic treatment on the basis of a single serologic test result does not seem to be justified. For a reliable serological diagnosis of human brucellosis, at least two different tests based on a highly sensitive method for screening and a more specific method to confirm the primary test result are needed.

Molecular techniques such as quantitative real-time PCR assays have proven to be more efficient than conventional methods in the diagnosis of infections caused by fastidious microorganisms such as *Brucella*. The detection of *Brucella* DNA in blood or tissue samples of symptomatic, so far untreated patients may indicate the necessity for antibiotic therapy despite negative blood cultures or negative serologic tests, whereas asymptomatic patients with a positive PCR result have to be carefully re-evaluated within the clinical context. Furthermore, the significance of DNA detection in obviously cured patients remains obscure.

Expert commentary & five-year view

Due to the lack of a single reliable laboratory test, human brucellosis is a disease which is difficult to diagnose. Time-consuming blood cultures and subsequent phenotypic characterization of the isolate are still the 'gold standard' in the diagnosis of human brucellosis. The low yield of *Brucella* cultures, however, often results in diagnostic delay and the late initiation of appropriate antibiotic therapy. Serology is a more effective means of diagnostic assessment, although the unavailability of internationally standardized tests, high background prevalence of anti-*Brucella* antibodies in endemic countries, long-term persistence of significant antibody titers after successful treatment, cross-reacting antibodies, and so on may hamper laboratory diagnosis. Adequate cutoff points have to be defined for each serologic test system and for different populations living in regions of varying endemicity. Only comprehensive seroprevalence studies in the general population may help to determine the significance of positive serologic test results.

In the near future, molecular techniques may revolutionize the laboratory diagnosis of human brucellosis. Real-time PCR technology meets all requirements for the rapid diagnosis in clinical microbiology laboratories. The drastic reduction of diagnostic delay will have important prognostic implications, especially in life-threatening complications of the disease, such as neurobrucellosis and *Brucella* endocarditis.

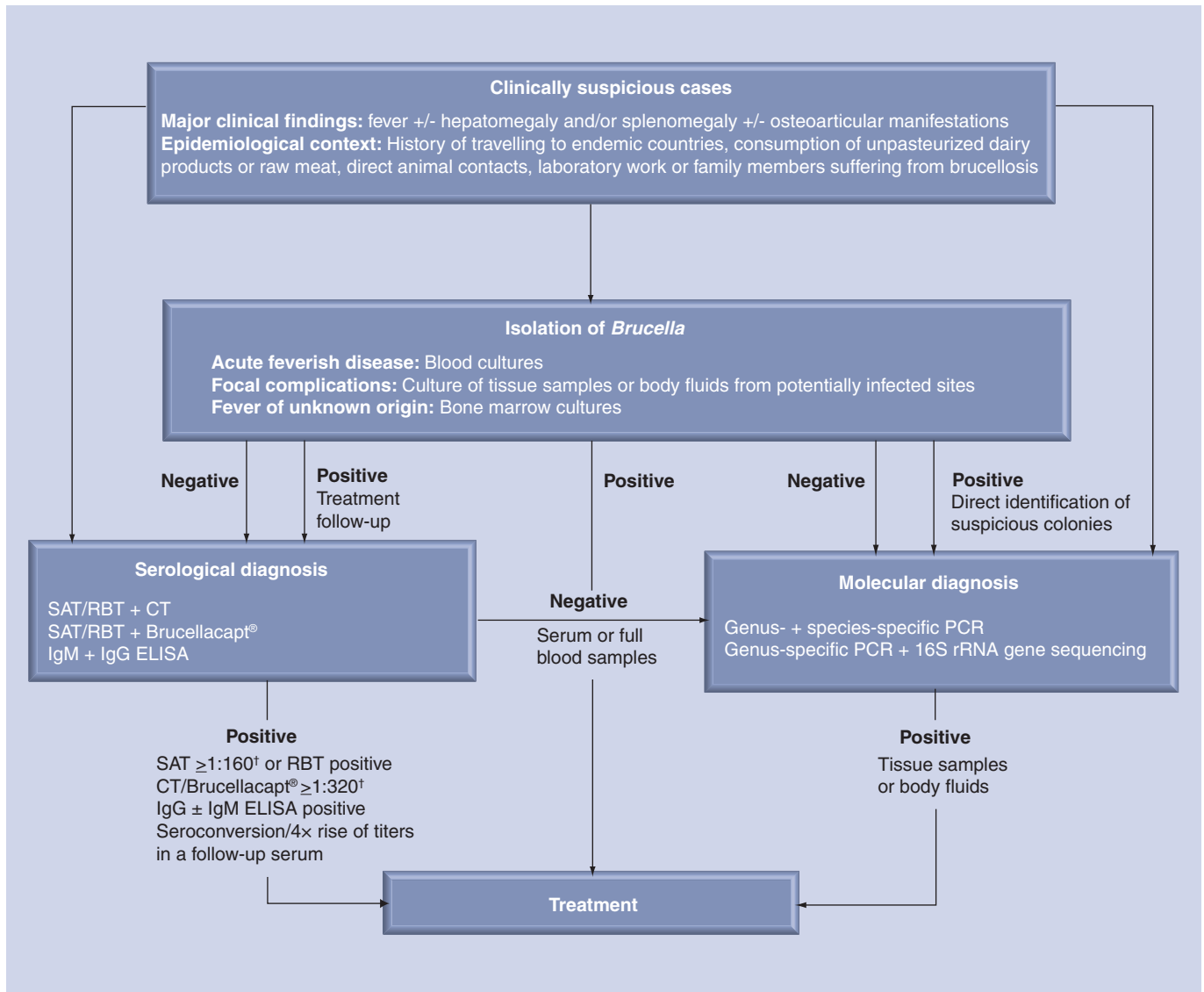


Figure 1. Decision tree in the laboratory diagnosis of human brucellosis.

†Cutoff values may vary in different populations and in patients depending on the stage of disease.

CT: Coombs' test; RBT: Rose Bengal test; SAT: Serum agglutination test.

However, laboratory criteria proving active infection or definite cure have not yet been clearly defined, neither for serologic nor for molecular tests. Studies correlating antibody titers or bacterial DNA load in blood samples with culture findings and clinical outcome, for instance disease severity, tendency to relapse and the need for an enhanced antibiotic treatment, are currently scarce. Furthermore, serologic and molecular tests have to be revalidated in clinical settings in due consideration of the stage of disease. Prolonged follow-up periods are needed to determine whether the decrease of anti-*Brucella* antibody titers and bacterial DNA load is transient or reflects definite eradication of the disease-causing agent.

Last but not least, molecular methods are relatively expensive which is why serological assays may be more adequate for routine laboratories in endemic regions characterized by a lower

socioeconomic status. For this purpose, cheap, simple and rapid point-of-care tests are urgently needed for screening the larger numbers of potential patients in endemic countries.

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Key issues

- Despite being controlled in the livestock of many developed countries, brucellosis is still a regionally emerging zoonotic disease and remains a major public health concern both in endemic and nonendemic regions, considering the globalization of the animal trade and worldwide tourism.
- A fast and reliable diagnosis is of paramount importance for effective treatment, reducing morbidity and mortality associated with human brucellosis. Due to the polymorphic signs and symptoms of the disease, a high index of suspicion is needed to prompt laboratory testing. Since public health services in nonendemic countries are unfamiliar with brucellosis, awareness campaigns are needed.
- Before committing a patient to prolonged antimicrobial therapy for brucellosis, the respective clinical findings, an epidemiological context and a laboratory diagnosis have to merge. Especially a laboratory diagnosis unequivocally proving active infection is indispensable before treatment is initiated. Therefore, effective diagnostic benchmarks to clearly define a case of human brucellosis at different stages of the disease should be elaborated.
- Isolation of *Brucella* from blood, bone marrow or other tissues and body fluids confirms the infection, but culture is frequently unsuccessful, especially in chronic courses of the disease.
- Because of the high risk of laboratory infections when handling cultures and viable brucellae, serological and molecular methods play a key role in the routine diagnosis of brucellosis by clinical microbiology laboratories.
- The interpretation of laboratory test results in the diagnosis of human brucellosis necessitates a detailed understanding of useful methods and their pitfalls.
- The laboratory methods to diagnose human brucellosis have to be internationally standardized, and should undergo a quality assurance system (i.e., harmonization of diagnostic standards in veterinary and human medicine, quality management, hands-on-training, transfer of protocols and periodical ring trials).

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