Update on laboratory diagnosis of human brucellosis

George F. Araj*

Department of Pathology and Laboratory Medicine, American University of Beirut Medical Center, PO Box 11-0236, Beirut 1107-2020, Lebanon

ARTICLE INFO

Keywords:
Brucellosis
Diagnostic tests
Immunossay
Serologic tests

ABSTRACT

The persistent worldwide prevalence of human brucellosis causes serious public health concerns and economic loss to communities. The multisystem involvement and the protean and unusual clinical presentations of the disease pose significant diagnostic challenges. The clinical features are non-specific and can overlap with a wide spectrum of other infectious and non-infectious diseases, leading to brucellosis being labelled the 'disease of mistakes'. Protracted chronicity and serious complications can result and mislead physicians onto a path of costly laboratory and radiological investigations. To reach a diagnosis clinicians must use a wide range of non-specific routine haematological and biochemical tests in addition to Brucella-specific assays. The latter are microbiological (culture), serological (e.g. slide or tube agglutination, Coombs test, immunocapture agglutination, Brucellacapt, immunochromatographic lateral flow, enzyme-linked immunosorbent assays and the indirect fluorescent antibody test) and molecular (e.g. polymerase chain reaction (PCR) and real-time PCR). Each of these tests has advantages and limitations, and thus requires careful interpretation. Since brucellosis can have several presentations and phases (acute, subacute, chronic, relapsed, active and inactive), the search for reliable, discriminatory diagnostic and prognostic markers, especially for monitoring disease evolution, are ongoing. Although much progress has been made, further challenges remain to the accurate diagnosis of this historic but still common global zoonotic disease.

© 2010 Elsevier B.V. and the International Society of Chemotherapy. All rights reserved.

Brucellosis is a zoonotic disease whose aetiological agent was first identified by Sir David Bruce in the 1860s [1,2]. Despite being long recognized, the disease continues to be prevalent and afflicts humans and domestic and other animals in many countries around the world. Most affected are the Eastern Mediterranean basin, the Middle East, the Arabian peninsula, Mexico, Central and South America, Central Asia and the Indian subcontinent [3,4].

The disease results in a wide range of significant veterinary and public health problems, and economic loss. Increased business and leisure travel to endemic countries has led to diagnostic challenges in areas where brucellosis is uncommon, especially when the presentation is unusual [5–8].

Because of the low infectious transmittable dose (≤10² organisms), the protracted pathogenesis and disease, and the possibility of it being used in biological weapons, *Brucella* was classified as a category B 'select agent' [9,10].

1. *Brucella* species

*Brucella* spp. are small (0.5–1.5 μm), facultative, intracellular Gram-negative coccobacilli that lack capsules, flagellae, endospores or native plasmids. They are aerobic, do not ferment sugars and are positive in a few oxidative metabolic tests. They can grow on a wide range of culture media and colonies generally appear after 24–48 h incubation [11,12].

To date, six terrestrial and three marine *Brucella* species have been recognized: *B. melitensis* (preferred hosts are goats, sheep, camels), *B. abortus* (cattle, buffalo), *B. suis* (swine and a range of wild animals), *B. canis* (dogs), *B. ovis* (sheep), *B. neotomae* (desert and wood rats) and *B. delphini*, *B. pinnipediae* and *B. cetaceae* from marine mammals (e.g. seals, whales, dolphins). The first four species can infect humans, with *B. melitensis*, *B. abortus* and *B. suis* causing the most disease in both humans and animals [13,14]. *Brucella* spp. associated with marine animals have been reported to cause disease in humans [15–17].

2. Antigenic components

Several antigenic components have been identified and determined to be involved in a variety of roles, including pathogenesis and the immune response, and are potentially useful in diagnosis of the disease. Lipopolysaccharide (LPS) is the major antigen and can exist in two partially shared antigenic epitopes: A (*B. abortus*) and M (*B. melitensis*). The O-specific side chains of the LPS molecule are considered the cause of the reported cross-reactions in both the agglutination and complement fixation tests between smooth

Protein antigens such as cytoplasmic, periplasmic and outer membrane structural proteins (e.g. Omp 25) are also recognized by the immune system during infection and are potentially useful in diagnostic tests [12,19,20]. Others, such as ribosomal proteins (e.g. L7/L12) and fusion proteins, have demonstrated a protective effect against *Brucella* based on antibody- and cell-mediated responses [12,21]. Such antigenic molecules may be useful in potential vaccines.

3. Transmission

Ingesting unpasteurized animal milk (goat, sheep, cow or camel) or its products, e.g. soft cheese, account for most cases worldwide [4,5,22]. Occupational infection (mostly in veterinarians, workers in clinical, research and production laboratories, and abattoir workers) is primarily associated with respiratory, conjunctival and skin routes of infection, e.g. through inhalation, sprays and aerosols; abrasions, accidental inoculation or cuts; and mishandling and misidentification of the organism [23].

4. Virulence and pathogenesis

The virulence and pathogenesis of *Brucella* infection and the bacterium’s avoidance of the immune system remain to be clarified and resolved [24]. The incubation period is variable, but generally is 1–4 weeks. Its intracellular survival within polymorphonuclear and mononuclear phagocytes, escaping phagosome–lysosome fusion and the immune response, is facilitated by factors including its ability to produce urease, which offers protection from stomach acid, *Brucella*-containing vacuoles, where the bacteria can survive, LPS and Cu/Zn superoxide dismutase. The pathogen is then transferred via the lymph nodes into the circulation to seed different organs and body systems, ultimately manifesting the varied clinical signs and symptoms.

5. Clinical features

The clinical diagnosis of brucellosis remains a considerable challenge, and to the unwary physician the diagnosis becomes a protracted problem for months and sometimes years. This difficulty can be attributed to several aspects of infection, including the prolonged and variable incubation period, its frequent presentation via the lymph nodes into the circulation to seed different organs and body systems, ultimately manifesting the varied clinical signs and symptoms.

6. Focal complications and conservative cost estimates

The most commonly encountered focal complications are osteoarticular (10–70%, mostly joints), genital in both males (6–8%) and females (2–5%), neurological (3–5%), cardiac (1–3%), pulmonary (1–2%) and renal (<1%). Mortality is very low (<1%) and almost exclusively results from cardiac complications [5,6,27–33]. It is noteworthy that in some patients who have undergone even simple surgeries, like cholecystectomy and hernia repair, unexplained protracted fever thereafter has proved to be reactivated brucellosis.

Very basic cost estimates based on reasonable charges for one of the hospitals in Lebanon pertaining to in-patient diagnosis and treatment of a patient with brucellosis, depending on the clinical signs and/or infection site are as follows: fever US$ 900–1000; skeletal/joint US$ 950–1200; respiratory US$ 1200–1300; central nervous system, US$ 3800–4200; and cardiac US$ 3900–4400.

7. Laboratory diagnosis

The diagnosis of a patient with possible brucellosis requires the combination of several approaches, including medical history, clinical examination, routine haematological and biochemical laboratory tests, radiological investigation and, most importantly, established and newly available *Brucella*-specific culture, serological and molecular tests, as described in this article.

The routine haematological investigations used in the diagnosis of brucellosis are mainly complete blood count, erythrocyte sedimentation rate and liver function tests. Generally, the findings in these tests are not specific in the diagnosis of human brucellosis, are variable and can overlap with other diseases [5].

The mainstay of the diagnosis of human brucellosis is specific laboratory tests. Knowledge of the advantages and limitations of each test are warranted for their appropriate application and interpretation. Several tests are available, as discussed below.

7.1. Culture

Culture, when positive, provides the definitive diagnosis and is considered the gold standard in the laboratory diagnosis of brucellosis. Though hazardous, it is essential for determining antimicrobial susceptibility and performing strain typing. The yield and speed of recovery are dependent on the culture method used and the type and volume of specimen used. For example, the conventional method using biphasic Ruiz–Castaneda bottles requires a long incubation time (6 weeks) and its yield is variable (40–90% in acute cases vs. 5–20% in chronic, focal and complicated cases) [5,12,34,35].

Automated continuously monitored blood culture systems such as Bactec (BD Diagnostics, Sparks, MD, USA) and BacTAlert (bioMérieux, Durham, NC, USA) give higher yields than the conventional culture method and expedite the detection of bacterial growth (majority recovered within 1 week). There is no need to incubate bottles longer than 10–14 days [35,36]. Bone marrow cultures result in 15–20% higher yields than peripheral blood cultures [34,35]. Rarely, some patients with brucellosis will have a positive blood culture in the absence of positive serology [6,35].

7.1.1. Identification and typing systems

*Brucella* spp. colonies can be recovered from clinical specimens on different media, e.g. blood and chocolate agar, within 24–48 h of aerobic incubation or under 5–10% CO2 incubation at 37 °C. In the clinical microbiology laboratory a few tests can be done for the presumptive identification of the pathogen, namely showing Gram-negative cocccobacilli, positive oxidase and catalase tests, and positive slide agglutination reaction with *Brucella*-specific
7.2. Brucella serology tests

Serological assays are the most commonly relied upon tests in the laboratory diagnosis of brucellosis. A wide range of in-house and commercial serological tests and formats have been used for investigating patients with brucellosis [39–41]. Since there is no standardized reference antigen it is important to note that the source of the antigen used, commercial or otherwise, can influence the test result. Moreover, the detection of antibodies to infections with B. canis and B. ovis requires the use of major outer membrane protein antigens because these strains exist in a rough colony form and do not share cross-reacting antigens with the other Brucella spp. [42]. A brief description of the most commonly used diagnostic tests follows.

7.2.1. Rose Bengal slide agglutination test

This test is based on the agglutination reaction of serum with a suspension of whole B. abortus cells stained with Rose Bengal dye and buffered at pH 3.65 to inhibit non-specific agglutinins [12,43]. Though this antigen is the most reliable in the slide agglutination test, other commercially available stained antigens that are mostly used for serum agglutination tests can be used for slide agglutination, but variable results can be observed depending on the source of the antigen. To avoid this the antigen should be obtained from reliable sources, mainly reference laboratories such as the National Veterinary Services Laboratories, Ames, IA, USA and the Veterinary Laboratories Agency, Surrey, UK. The test is simple to perform, rapid (within 5–10 min) and has relatively good results in diagnosing patients with acute brucellosis, but gives a high rate of false-negative results in chronic and complicated cases [44].

7.2.2. Serum agglutination test (SAT)

Although this test was introduced over a century ago, in 1897, it remains a cornerstone of the serodiagnosis of brucellosis. The test is performed in tubes by reacting a known standardized volume and concentration of whole Brucella cell suspension with a standardized volume of doubling serum dilutions, usually ranging from 1:20 to 1:1280. The suspension mixture is incubated in a water bath at 37 °C for 24 h, and agglutination at the bottom of the tubes is examined visually. The highest serum dilution showing more than 50% agglutination is considered the agglutination titre [11]. Commercial cell suspensions could vary in quality and thus each cell lot should be quality controlled with known positive and negative standard sera before accepting it for clinical testing. SAT measures total Brucella antibody (IgG, IgM and IgA).

7.2.3. Microagglutination test (MAT)

This is essentially a miniaturized format of SAT performed in microtitre plates (V- or U-bottomed). Its advantage over SAT is that it uses smaller volumes of serum and reagents, and can test multiple samples at the same time.

The agglutination tests show good performance in diagnosing patients with acute brucellosis. However, they suffer from high false-negative rates in complicated and chronic cases, are liable to false-positive findings because of cross-reactions, and both are cumbersome to set up.

7.2.4. Indirect Coombs (anti-human globulin) test

The Coombs test is an extension of SAT, used for the detection of incomplete, blocking or non-agglutinating IgG [11]. Those SAT tubes containing serum dilutions and whole B. abortus or B. melitensis cells (as antigens) that were negative after incubation for 24 h are centrifuged, the supernatant decanted and the cell pellet resuspended and washed with phosphate-buffered saline, repeated three times. Standardized anti-human globulin reagent (anti-IgG) is added to the last pelleting in each test tube. The pellet is resuspended and incubated in a water bath at 37 °C for 24 h. Agglutination can be determined visually, as for SAT, by using an agglutinoscope or a drop on a slide examined under the microscope. The test is good for complicated and chronic cases and misses around 7% of cases compared with ELISA [45].

7.2.5. Brucellacapt (Vircell, Granada, Spain)

This test is based on an immunocapture agglutination technique and in a single step detects non-agglutinating IgG and IgA antibodies, as well as agglutinating antibodies. The test is performed according to the manufacturer’s instructions: a specified volume of each serum dilution is added to a microplate with U-shaped wells pre-coated with anti-human immunoglobulin. Then a whole-cell, formaldehyde-killed, coloured B. melitensis antigen suspension is added. The plate is incubated for 18–24 h at 37 °C before reading visually. Positive reactions show agglutination over the bottom of the well. Negative reactions present a pellet in the centre of the bottom of the well [46]. Brucellacapt offers a valuable alternative to the Coombs test, since it shows similar sensitivity and specificity, similar performance (especially in diagnosing complicated and chronic cases), and most importantly is more rapid and less cumbersome to carry out [46].

7.2.6. Enzyme-linked immunosorbent assay (ELISA)

ELISA is the test of choice for complicated, focal and chronic cases, especially when other tests are negative while the case is under high clinical suspicion. It can reveal total and individual specific immunoglobulins (IgG, IgM, IgA) rapidly (4–6 h) with high sensitivity and specificity [34]. Briefly, the test is carried out in 96-well microtitre plates that are pre-coated/fixied with predetermined Brucella (whole cells or sonicate, purified LPS, protein extracts or other standardized antigen). A standardized volume and dilutions of serum are added to react with the antigen in the wells, and the plates are incubated and washed. An enzyme-conjugated (e.g. alkaline phosphatase, horseradish peroxidase) anti-human IgG, IgM or IgA is added to the last pelleting in each test tube. The pelleting in each test tube is repeated three times. Standardised antihuman globulin reagent is added to the last pelleting in each test tube. The plate is incubated for 18–24 h at 37 °C before reading visually. Cut-off values for seropositive samples can be extrapolated from incorporated negative and positive controls and/or a standardized assay.

ELISA is considered an excellent method for serosurveys [42–47]. Though most of the tests are designed in-house, commercial assays have good performance as well [48]. In addition to the detection of immunoglobulin classes, ELISA can also detect Brucella-specific IgG subclasses and other Brucella immunoglobulins, such as IgE [49,50].

7.2.7. Indirect fluorescent antibody test (IFA)

The test involves fixing (by acetone) a predetermined suspension of whole B. abortus or B. melitensis cells (obtained from different commercial sources or reference laboratories) on acetone-resistant slides. After the addition of doubling serum dilutions, incubation (30 min at 37 °C) and washing in phosphate-buffered saline,
flourescein-labelled antihuman IgG, IgM or IgA is added to the designated circles on the slide, which is incubated (30 min at 37 °C), repeatedly washed, and dried before being mounted with, e.g. Fluoroprep (bioMerieux, France). The slides are read using a fluorescence microscope to determine the titre that is the highest dilution showing positive fluorescence. Positive and negative control sera should be included in each run. The test is rapid (2–3 h) and shows comparable results to ELISA. However, it is subjective in reading, may fail to detect IgA, and differences in reactions to antigens obtained from different manufacturers have been observed [51].

7.2.8. Immunochromatographic lateral flow assay

This assay is run using a composite strip in a plastic device consisting of a nitrocellulose detection strip and a reagent pad. The former contains Brucella LPS antigen, as a Brucella-specific capture probe, and a reagent control applied in distinct lines. The reagent pad contains dried and stabilised detection reagent consisting of a colloidal gold-conjugated antihuman IgG or IgM. The serum sample is added to a sample well, followed by test liquid. The result is read based on positive or negative staining after 10–15 min by visual inspection of the antigen and control lines in the test window. These Brucella-specific IgG and IgM lateral flow assays have been advocated for screening/surveillance of patients with brucellosis in endemic areas and as outbreak and field tests. They are simple, rapid, easy to perform and read, with high (>90%) sensitivity and specificity [52,53].

7.2.9. Interpretation of serological tests

The interpretation of serological test results in relation to exposure, diagnosis and prognosis of the disease necessitates an accurate assessment of the clinical history and current status of the patient, and an understanding of the usefulness and pitfalls of the tests [6,39]. It is also important to understand the evolution of the immune response following infection and treatment, since in a good number of individuals Brucella-specific IgG, and in some cases IgM, can persist for years, despite treatment and cure [5,6,39,54].

The cut-off positive titres in SAT have been under discussion in the literature, being noted at >160 in symptomatic patients. However, an open mind should be kept, since lower and higher values in SAT can be detected in active and asymptomatic cases, respectively [5]. In certain situations, when the clinical suspicion of brucellosis is high and the serology is negative, care should be paid to think of very early disease presentation and repeat testing is warranted in 1–2 weeks. Moreover, the possibility of Br. abortus or B. melitensis infection in such cases should be kept in mind, since serological assays using B. abortus or B. melitensis antigen can miss it, as its diagnosis requires using major outer membrane protein antigen [55,56]. For the diagnosis of neurobrucellosis, ELISA would be the test of choice [57,58].

In addition to using Brucella-specific IgG, IgM and IgA in diagnosis, further diagnostic and discriminative markers have been developed to determine Brucella-specific IgE and subclasses of IgG among clinically well-characterized patients. Although not absolute, in acute brucellosis the predominant specific immunoglobulin profile is IgM, IgG, IgA, IgE, IgG1 and IgG3, whereas in chronic brucellosis it is IgG, IgA, IgE and IgG4 [34,49,50,54].

Monitoring the treatment response requires the sequential follow-up of patients to determine the progress of titres in serological tests. A decline indicates a good prognosis, persistent high titres necessitate continuous monitoring, while resurgence of antibody titres most likely indicates relapse or reinfection. In acute cases, slide and tube agglutination titres fall faster than ELISA [55,59–61]. Relapse has also been diagnosed by detecting resurgence of Brucella-specific IgG and IgA antibodies, not IgM [54,55,59,62].

Determination of interleukin (IL) levels has also been considered as a predictor of treatment outcome. A significant post-treatment decline in serum soluble IL-2 receptor alpha levels has been observed compared with pre-treatment levels, and this might be used as a marker of treatment efficacy in brucellosis [63].

Markers for differentiating active from inactive disease are being sought. For example, anti-Brucella cytoplasmic or periplasmic protein antibodies, as determined by ELISA, have been found to increase only in active brucellosis and be a better predictor of cure than anti-LPS antibodies [60,64,65].

In order to achieve the most reliable diagnosis of human brucellosis it is recommended that a laboratory use a combination of two agglutination tests, namely SAT and indirect Coombs or SAT and Brucellacapt, or ELISA for IgG and IgM. This allows the detection of antibodies at different stages of the disease, since in the acute stage any test can be positive whereas in chronic, complicated or focal cases SAT can be negative while Coombs, Brucellacapt and ELISA IgG are positive.

7.3. Molecular assays

Advances in molecular-based technology have been utilised for the laboratory diagnosis of human brucellosis. In-house developed conventional polymerase chain reaction (PCR) and real-time PCR (RT-PCR) assays have been attempted for the direct detection of Brucella from clinical specimens, to monitor treatment response, and for the identification, speciation and differentiation of recovered Brucella spp. The sensitivities of these assays in the direct detection of Brucella from clinical specimens have been quite variable, ranging from 50% to 100%, and specificity has varied between 60% and 98%. This variation might be related to different DNA extraction methods, detection formats and limits, and to different types of specimens used [56,66–75].

The ribosomal 16S–23S internal transcribed spacer region seems to constitute a suitable target in clinical specimens and formalin-fixed paraffin-embedded archived tissue, as well as for the speciation of isolates from culture [56].

Overall, molecular assays have good potential in the investigation of patients with brucellosis. So far, however, further standardisation and optimisation are necessary to achieve consistency and reliability of results before they are incorporated in routine laboratory investigations [76].

8. Conclusions

Many improvements have been made towards better understanding of the clinical, epidemiological, pathogenetic, diagnostic, therapeutic and other aspects of human brucellosis, a historic zoonotic disease. However, several challenges remain to be addressed: (1) to define specific serological diagnostic and prognostic markers, (2) determine purified, specific and relevant antigenic epitope predictors of each disease stage (e.g. acute, active, relapse, chronic, cure), (3) carry out follow-up studies to correlate the course of disease with classes and subclasses of immunoglobulin and (4) design standardized specific nucleic acid probes for amplification technology (e.g. RT-PCR) that would be useful in diagnosis, especially of chronic and complicated cases such as those with central nervous system infections. Collaborative local, regional and international team work is essential for success in overcoming these challenges.

Funding: No funding sources.

Competing interests: None.

Ethical approval: Not required.


Araj GF, Awar GN. The value of ELISA vs negative Coombs findings in the serodiagnosis of human brucellosis. Serodiag Immunother Infect Dis 2007;7:775–86.


Araj GF, Kaufman AF. Determination of Brucella specific IgG, IgM and IgA by ELISA in sera of patients with brucellosis against, B. melitensis major outer membrane proteins (MOMP) and whole cell heat killed (HK) antigens. J Clin Microbiol 1989;27:1909–12.


