Diagnosis of Brucellosis in Humans: a Review

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Abstract

Diagnosis of brucellosis presents a challenge to the clinician and diagnostic lab alike. It is one of those diseases with “protean” manifestations and can mimic other diseases clinically thus posing a challenge to clinical diagnosis. Thus lab diagnosis is essential. In the lab, the “gold standard” remains isolation in culture but this takes a long time, requires biosafety level 2 during handling and is one of the highly reported hazards to laboratory personnel. Diagnosis therefore is most commonly done by serological tests, but these are not the most sensitive and specific and require standardization for different populations depending on level of endemicity. Newer diagnostic approaches such as PCR are still being studied and have not yet been approved for diagnostic purposes. In this review, these different approaches are tackled.

Key words: Diagnosis, brucellosis, disease, biosafety, PCR

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Introduction

Brucellosis is not an emerging disease but rather one that is overlooked by the majority of the scientific community. It is one of the most important zoonotic diseases mainly transmitted from cattle, sheep, goats, pigs and camels through direct contact with blood, placenta, fetuses or uterine secretions, or through consumption of contaminated raw animal products (especially unpasteurized milk and soft cheese). Half a million new cases are reported worldwide each year, but according to the World Health Organization, these numbers greatly underestimate the true incidence of human disease. This is because, unlike other zoonotic diseases such as campylobacteriosis which is worldwide (Ugboma et al., 2012), brucellosis is more of a Mediterranean disease. In endemic areas, brucellosis causes high economic loss and has serious public health consequences. Worldwide, \textit{Brucella melitensis} is the most prevalent species causing human brucellosis. Thus, accuracy and short turnaround time are required for the diagnostic tests (Joint WHO/FAO/OIE, 2004).

Human brucellosis has a wide spectrum of clinical manifestations, earning it a place alongside syphilis and tuberculosis as one of the “great imitators” (Andriopoulos et al., 2007; Mantur et al., 2006; Giannakopoulos et al., 2006; Mantur et al., 2004; Barrosa Garcia et al., 2002). Thus, the diagnosis of human brucellosis cannot be made solely on clinical grounds. Furthermore, since the disease constitutes a serious infection necessitating treatment with a prolonged course of antibiotics, accuracy and short turnaround time are required for the diagnostic tests (Solera et al, 1997).

Culture

The only conclusive evidence of Brucella infection is the recovery of the bacteria from the patient. The isolation and identification of Brucella offers a definitive diagnosis of brucellosis and may be useful for epidemiological purposes and to monitor the progress of a vaccination program in animals (WHO publications, 2006).

Blood culture is the gold standard in the diagnosis of bacterial infections, including brucellosis. Although Brucella can be isolated from bone marrow, cerebrospinal fluid, wounds, pus, etc., blood is the material most frequently used for bacteriological culture. However, Brucella represents a risk to personnel handling it in the laboratory and the biphasic method of Castaneda which uses both solid and liquid medium in the same container, limits the need for subculture and thus reduces the risk of laboratory acquired infection. Serum dextrose broth with corresponding solid phase is often recommended but Brucella will grow on most high quality peptone based media used for blood culture. Incubation should be performed in air supplemented with 5% CO2.

Although the biphasic Ruiz-Castañeda system is the traditional method for the isolation of Brucella spp from clinical samples (Yagupsky, 1999; Ganado & Bannister, 1960; Ruiz-Castañeda, 1954), it has now largely been replaced by automated culture systems—such as the lysis centrifugation method (WHO publications, 2006; Cockerill et al., 2004; Mantur & Mangalgi, 2004), with increased sensitivity and reduced culture times (Durmas et al., 2003; Bannatyne et al., 1997; Navas et al., 1993).

The newer semiautomatic methods (BACTEC 9204 and BacT/Alert) shorten considerably the time taken for detection; the presence of Brucella can be detected with these methods by the third day of incubation. Earlier systems (BACTEC NR 730) failed to detect an appreciable number of samples which were positive by conventional blood culture systems. Conventional Castaneda blood cultures are seldom positive before the fourth day of incubation. The majority of blood cultures are positive between the seventh and 21st day, and only 2% are positive after the 27th day. For this reason, incubations should be carried out for at least 45 days before rejecting a blood culture as negative for Brucella.

The sensitivity of blood culture depends on several factors, particularly the phase of the disease and previous use of antibiotics (Doganay & Aygen, 2003; Gotuzzo et al., 1986). For instance, in acute cases, the sensitivities of the Ruiz-Castañeda method and lysis centrifugation have been reported as high as 80% and 90%, respectively, but as low as 30% and 70%, respectively, in chronic cases (Mantur & Mangalgi, 2004; Gaviria-Ruiz & Cardona-Castro, 1995; Gotuzzo et al., 1986). Bone marrow cultures may provide a higher sensitivity,
yield faster culture times, and may be superior to blood cultures when evaluating patients with previous antibiotic use (Deepak et al., 2003; Doganay & Aygen, 2003; Gaviria-Ruiz & Cardona-Castro, 1995; Gotuzzo et al., 1986). Brucella can also be cultured from pus, tissue samples, and cerebrospinal, pleural, joint, or ascitic fluid (Doern, 2000; Etemadi et al., 1984). Since brucellosis constitutes one of the most common laboratory-acquired infections, special care should be taken when using the lysis centrifugation method to avoid infection from contaminated aerosols (Yagupsy & Baron, 2005; Noviello et al., 2004; Robichaud et al., 2004; Miller et al., 1987).

**Serological testing**

In the absence of culture facilities, the diagnosis of brucellosis traditionally relies on serological testing with a variety of agglutination tests such as the Rose Bengal test, the serum agglutination test, and the antiglobulin or Coombs’ test. In general, the Rose Bengal test is used as a screening test, and positive results are confirmed by the serum agglutination test (Ruiz-Mesa et al., 2005; Al Dahouk et al., 2003). In reality, serological testing is the most commonly used method of brucellosis diagnosis.

The Rose Bengal plate test can be used as a sensitive rapid screening test but the results should be confirmed by bacteriological and other serological tests. Should the screening test prove negative in the face of a history and clinical presentation, it is advisable to check the result using additional tests. The sensitivity of the Rose Bengal test is very high, however, and false-negative results are rarely observed (Ruiz-Mesa et al., 2005; Serra & Vinas, 2004). The specificity of the assay is also fairly high, and in unexposed populations, false positive results are rare. Differences in the quality of the reagent used and disagreement in the interpretation of results might add to variability of test results (Maichomo et al., 1998; Blasco et al., 1994).

**Serum tube agglutination test**

This test, developed by Bruce, measures antibodies against smooth lipopolysaccharide (LPS); it remains the most popular test tool for the diagnosis of brucellosis. These antibodies tend to persist in patients long after recovery; therefore, in endemic areas, high background values could occur that may affect the diagnostic value of the test (Ariza et al., 1992). Furthermore, the brucella smooth lipopolysaccharide antigen tends to show cross-reactivity with other Gram-negative bacteria such as *Yersinia enterocolitica* O:9, *Vibrio cholerae*, *Escherichia coli* O: 157, and *Francisella tularensis*, increasing the possibility of false-positive results (Nielsen et al., 2004).

As with other serological tests, the sensitivity and specificity of the confirmatory agglutination tests for brucellosis depend on the cut-off value used, and on the background level of reactive antibodies in the population. By doing the test on a serial dilution of the samples with results judged positive above a certain titre, the specificity and positive predictive value of a positive test result could be increased by selecting a higher cut-off value for areas where brucellosis is endemic. However, by selecting a higher cut-off value the sensitivity decreases and because patients with acute brucellosis and those with persisting and relapsing disease may present with low antibody levels, the interpretation of test results and diagnosis of these patients by serological testing might not be straightforward (Franco et al., 2007). The sensitivity of the serum agglutination test of can range from 47-1% to 84-6% depending on the endemicity and cutoff value used (Mantecon et al., 2006).

If collection of a follow-up sample is feasible, the sensitivity of the test could be increased by testing paired serum samples and looking for seroconversion, or a fourfold increase in titre. Demonstration of seroconversion or a significant increase in antibody titre provides strong supportive evidence for the infection and this may be observed by testing a follow-up sample collected a few weeks to several months after the initial diagnosis. To exclude the possibility of cross-reactive IgM antibodies, the 2-mercaptoethanol test for measuring specific agglutinating IgG antibodies is sometimes used; results are compared with the serum agglutination test titre and reactivity in the 2-mercaptoethanol test is taken as evidence for the presence of specific IgG antibodies.

**The antihuman globulin test (AHG) or Coombs test**
The AHG test was developed to overcome the prozone effect which affects SAT and other agglutination tests. It gives positive results when SAT is negative. This is because it measures IgG. For this reason it is considered a better test in long term or chronic brucellosis. However it does not give accurate results in all cases of chronic brucellosis particularly longer term (Barrett & Rickard, 1953).

The AHG or Coombs test was developed by Coombs (1945) to detect "incomplete" Rh agglutinins. It was applied to Brucella by Wilson (1952) in an attempt to disclose non-agglutinating antibodies. A typical method is described by Kerr et al (1968). Two sets of serum dilutions are prepared for each of the SAT and AHG titres. The antigen used is usually killed B abortus which is used at a higher concentration for the AHG titre. For the AHG titre antigen is added to the dilutions in tubes which are held at 37°C for 24 hours and then examined. Tubes with agglutination present are recorded and discarded. The other tubes are centrifuged cold at 2000 rpm for 15 minutes the precipitate retained and the supernatant discarded. The precipitate is re-suspended in buffer with thoroughly washing and re-centrifuged 3 times to remove free human protein. The precipitate thus produced is then re-suspended in saline buffer and anti-human globulin added. The tubes are incubated for 24 hours at 37°C and then examined for agglutination as for the SAT titre.

AHG determines IgG and IgA so it does not work in the earliest stages of acute illness when only IgM is present. It is considered together with ELISA IgG and ELISA IgA the best test for chronic brucellosis although it fails to detect many cases. The main problem with this test is that it is slow and labour intensive. It usually takes at least 3 days to complete the test whereas ELISA IgG and IgA can be done in a few hours.

However, many patients have low levels of agglutinating IgG antibodies and results can easily be misinterpreted. Coombs’ test may be more suitable for confirmation of brucellosis in relapsing patients or patients with persisting disease, but few laboratories have the expertise and equipment to do this very sensitive but complex and demanding technique.

ELISA

ELISA has also become increasingly popular as a well standardized assay for brucellosis. When the detection of specific IgM antibodies is complemented with the detection of specific IgG antibodies, the sensitivity of the ELISA may be high (Mantecon et. al., 2006). The specificity of ELISA, however, seems to be less than that of the agglutination tests. Since ELISA for brucella is based on the detection of antibodies against smooth lipopolysaccharide, the cut-off value may need adjustment to optimise specificity when used in endemic areas, and this may influence sensitivity (Ariza et. al., 1992). Manufacturers need to compare their product performance with culture rather than other commercial ELISAs and cut-off values should be established based on local epidemiological conditions as commercial ELISAs also have not been evaluated under different epidemiological conditions and results should be interpreted with care.

The more recently developed Immunocapture-Aglutination Test (Brucellacapt) (Vircell SL, Granada, Spain) which is a single stage proprietary test kit and is a new form of the agglutination test to test for brucellosis antibodies in sera. The method is: samples of diluted sera are added to wells on a microtiter plate coated with antihuman immunoglobulin, then stained Brucella antigen is added followed by incubation at 37°C for 24 hours. A positive test result is shown by activation of a coating of dye on the surface of the plate. The manufacturer suggests that the test can detect both agglutinating and "incomplete antibodies" and therefore correlates well with the antihuman globulin test. Independent assessment suggests that it only works well for the early stages of brucellosis infection but not for chronic or relapsed brucellosis.

The usefulness of Brucellacapt has been assessed by several studies that concluded that it could be used to give results similar to both SAT and AHG tests but was poor at low dilutions, chronic and relapse cases and subclinical brucellosis (Casanova et. al., 2009; Diaz et. al., 2006; Orduna et. al., 2000)

Rapid point-of-care assays
Additionally, rapid point-of-care assays can enable fast and accessible diagnostic capabilities even in remote areas as the serum agglutination test, Coombs’ test, ELISA, and Brucellacapt all require a well-equipped laboratory, a facility often lacking in health centres of resource-poor countries where the disease is endemic. Rapid tests such as the fluorescent polarisation immunoassay (FPA) for brucellosis and the immunochromatographic brucella IgM/IgG lateral flow assay (LFA), a simplified version of ELISA, have great potential as point-of-care tests (Irmak et. al., 2004; Lucero et. al., 2003). The FPA test is done by incubation of a serum sample with brucella O-polysaccharide antigen linked to a fluorescent probe (Lucero et al., 2003). The sensitivity of this test at the selected cut-off value is 96% for culture-confirmed brucellosis, and the specificity was determined to be 98% for samples from healthy blood donors. The LFA uses a drop of blood obtained by fingerprick, does not require specific training, is easy to interpret, and can be used at the bedside. The components are stabilised and do not require refrigeration for transportation or storage (Hasanjani-Roushan et. al., 2005). The sensitivity and specificity of LFA are high (more than 95%), and the test can be used at all stages of disease. Further studies will be needed to confirm the usefulness of these new point-of-care tests in different clinical settings in endemic areas, with particular attention to the diagnosis of patients with acute and relapsing brucellosis. The requirement of specific equipment and reagents make the FPA test too expensive, but a simple test such as the LFA could be ideal for field testing risk groups during outbreaks (Irmak et. al., 2004). Another useful application for these tests is to screen the contacts of brucellosis patients (Alsubaie et. al., 2005; Almuneef et. al., 2004; Abramson et. al., 1991).

**Molecular Methods**

Polymerase chain reaction (PCR) testing for brucellae is a recent advance with promising potential. It would allow for rapid and accurate diagnosis of brucellosis and overcome the limitations of conventional methodology. Several genus-specific PCR systems using primer pairs that target 16S RNA sequences and the genes of different outer membrane proteins have been developed (Leal-Klevezas et al., 1995; Matar et al., 1996; Queipo-Ortuno et al., 1997; Navarro et al., 1999; and Zerva et al., 2001). Each of these PCR systems produces a discrete DNA product, whose length is identical for and specific to all *Brucella* species.

However, reports on different sensitivities among the different protocols used may presumably depend on the nature of the clinical specimen, the sample preparation procedure, and the duration and stage of illness. Standardization for such protocols still remains a crucial issue before application in clinical diagnostic labs.

Queipo-Ortuno and co-workers found 100% sensitivity and 98.3% specificity using the B4/B5 primer pair amplifying a 223-bp fragment of the *bcsp31* gene, compared with 70% sensitivity for blood culture.

Zerva and colleagues reported that the sensitivity of the B4/B5 primer pair improved from 61% to 94% when serum instead of whole blood samples was used. However, using a modified detection system, Vrioni and co-workers found no improvement in the detection rate by testing whole blood samples.

Baddour and Alkhalifa, 2008 concluded that using the B4/B5 primer PCR with their suggested modification of increasing the number of cycles by 5 is a robust assay, which meets the sensitivity requirements to be used for testing of human blood samples for brucellosis in the diagnostic laboratory. B4/B5 primers able to detect 7 x 10^2 cfu/ml and a sensitivity of 98% compared to culture. Using JPF/JPR primers on the same samples yielded 88.4% sensitivity and only 53.1% sensitivity by F4/R2 primers.

The incorporation of a robust DNA extraction method, such as the diatom-guanidinium isothiocyanate method, which effectively removes inhibitors commonly present in a variety of clinical specimens, may improve sensitivity and reproducibility (Boom et. al., 1990). Mitka and colleagues, 2007 found an almost 100% sensitivity for each of four PCR systems by testing either serum, buffy coat, or whole blood samples from 200 patients with acute brucellosis of whom 74% were culture-confirmed.

PCR could be particularly useful in patients with specific complications such as neurobrucellosis, or other localised infections, since...
serological testing often fails in such patients (Colmenero et al., 2005; Colmenero et al., 2002; Morata et al., 2001).

Real-time PCR systems have been developed that are faster and less prone to contamination and are thus more clinically useful (Colmenero et al., 2005; Queipo-Ortuno et al., 2005; Debeaumont et al., 2005; Redkar et al., 2001; Newby et al., 2003; Probert et al., 2004; Bogdanovich et al., 2004). Comparative analysis of the various real-time PCRs is needed to assess their diagnostic value. However, the high costs of these assays will restrict their use.

Other promising tests include nested PCR, real-time PCR (Navarro et al., 2006; Debeaumont et al., 2005), and PCR-ELISA, but the clinical role for these tests remains to be defined (Mitka et al., 2007; Navarro et al., 2002; Nimri, 2003; Queipo-Ortuno et al., 2005).

Before implementing any such procedures, it is essential to institute efficient containment procedures to prevent contamination of samples with bacterial DNA or amplified replicons from the laboratory environment (WHO, 2006).

**Conclusion**

It seems that accurate and rapid diagnosis of brucellosis in the diagnostic lab still holds challenge and requires additional testing and standardization especially with the development of more recent diagnostic assays.

**References**


