Brucellosis (*Brucella sp.*)

Laboratory case definition

The Public Health Laboratory Network (PHLN) has developed standard case definitions for the diagnosis of key diseases in Australia. This document contains the laboratory case definition for *brucella sp*.

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1 PHLN Summary Laboratory Definition

1.1 Condition

Brucellosis

1.1.1 Definitive Criteria

a Isolation of *Brucella* species from a sterile site;
OR
b Seroconversion or significant increase in *Brucella* antibody level in acute and convalescent sera.

1.1.2 Suggestive Criteria

A single high titre of specific *Brucella* antibody.

2 Introduction

Brucellosis was first described by Bruce in 1887 with the discovery of the bacterial species subsequently named *Brucella melitensis.* A type of febrile illness characterised by regular remissions or intermissions has been recognised in the Mediterranean region for centuries. Many names have been applied to it, often relating to the localities in which it was particularly prevalent. Malta fever, Mediterranean fever, Gibraltar fever or Rock fever and undulant fever are probably the best known.

The cause of the disease was obscure until Bruce reported numerous small coccal organisms in stained sections of spleen from a fatally infected soldier. He named the organism *Micrococcus melitensis.* Eventually the connection was made between *M. melitensis* and the organism described by Bang (1897) as the cause of contagious abortion in cattle. The genus name *Brucella* was proposed in recognition of Bruce's discovery.

An increasingly complex pattern of strains has subsequently emerged in which specific members of the genus *Brucella* have been linked to a particular primary animal host. The potential range of interactions with humans has increased with each new species because each *Brucella* species has distinctive epidemiological features.

The genus *Brucella* is currently divided into 6 species, *B. abortus, B. melitensis, B. suis, B. canis, B. ovis* and *B. neotomae.* Three of these have been present in Australia, *B. abortus* in cattle, *B. suis* in pigs and *B. ovis* in sheep.

Brucellosis is a zoönosis, and with few exceptions infections in humans result from direct or indirect contact with animal sources. Rare instances of person-to-person transmission have been recorded, either in circumstances implicating sexual contact or by the transfer of tissue, including blood and bone marrow. Another more frequent source of infection is laboratory-acquired brucellosis, caused by exposure to *B. melitensis, B. abortus, B. suis,* and *B. canis* as a result of aerosols generated during the manipulation of cultures. Several studies have suggested that in the past, brucellosis was acquired in the laboratory more frequently than any other bacterial disease (Topley and Wilson).

Most cases of infection caused by *B. abortus, B. melitensis, B. suis* and *B. canis* arise from occupational or domestic contact with infected animals or with an environment contaminated by their discharges. Farmers and their families, abattoir workers, butchers and veterinarians are particularly at risk. Infected animals that have recently aborted or given birth present the greatest hazard: infection may occur through ingestion or inhalation, or contamination of the conjunctiva or skin with discharges. The main source of infection of the general population is dairy produce prepared from infected milk (Topley and Wilson).

In Australia, since eradication of *B. abortus* from cattle, the highest incidence of infection is in humans who kill and handle the carcasses of feral pigs (especially in Queensland and Northern NSW) among which *B. suis* infection is endemic. Otherwise most human cases of brucellosis are imported from endemic countries (Mediterranean region, Middle East and Latin American Countries) or occur in laboratory workers handling cultures from infected patients.

The genus itself is highly homogenous with all members showing >90% homology in DNA-DNA pairing studies, thus classifying *Brucella* as a mono-specific genus (Verger and Grayon). However the proposed classification where all types would be regarded as biovars of *B. melitensis* has not been adopted on the grounds that it would be very confusing.

More recently restriction fragment–length polymorphism (RFLP) has been used to distinguish between species within the genus *Brucella* and has supported host preference as a basis for classifying the brucellae.

PCR of selected sequences followed by restriction analysis has provided further evidence of polymorphisms in a number of genes including the omp 2, dna k, htr and ery genes.

The old nomenclature (Table 1) has been retained with the former species names being used as the nomen species. Within these, seven biovars of *B. abortus* are recognised, three for *B. melitensis* and five for *B. suis.*

Table 1. Recognised species and biotypes with host range and species endemic to Australia

Species	Biotypes	Host	Present in Australia
B. abortus	1–9	Cattle,dogs, horses sheep and man	No
B. suis	1–5	Pigs, cattle, dogs, hares and man	Yes biotype 1 only
B. melitensis	1–3	Sheep, goats, cattle and man	No
B. ovis		Sheep	Yes
B. canis		Dogs and man	No
B. neotomae		Desert wood rat	No

3 Laboratory Diagnosis/Tests

The variable symptoms, the paucity of distinctive physical signs and the occurrence of subclinical and atypical infections make the clinical diagnosis of brucellosis in humans particularly difficult.

3.1 Culture for *Brucella*

Isolation of a *Brucella* sp. is the most certain means of reaching a diagnosis. It is most likely to be successful during the acute phase of the infection.

3.1.1 Media

For optimal isolation of *Brucella* sp. 3–5 mL of blood should be inoculated into 20—100 mL of serum dextrose broth, tryptone soya broth or trypticase–soy broth, or more commonly, a commercial automated blood culture broth, and incubated at 37° C in air enriched with 5–10% CO_2. Selective media are not necessary for the culture of human blood samples taken with aseptic precautions but should be used if samples of excreta or contaminated tissues are to be examined. The liquid media can be made selective by the addition of an antibiotic mixture containing: amphotericin B 1 mg L^{-1}, bacitracin 25 mgL^{-1}, cycloheximide 100 mg L^{-1}, d-cycloserine 100 mg L^{-1}, nalidixic acid 5 mg L^{-1}, polymyxin B 6 mg L^{-1}, and vancomycin 20 mg L^{-1}.

When liquid media are used for enrichment or primary isolation, subcultures should be made every 3–5 days on a suitable solid medium such as horse blood agar or serum dextrose agar, which is then incubated at 37° C in air enriched with 5–10% CO_2. The need for frequent subculture can be avoided by use of a 2-phase system in which both the solid and liquid phase are contained in the same bottle. Even with this system, subcultures to fresh solid medium should be made immediately growth appears to minimise the dissociation of smooth cultures to the non-smooth colony variant (Topley and Wilson, Corner and Alton)

For human diagnosis blood cultures are now invariably performed using an automated commercial blood culture system. These systems are very sensitive and usually signal within 5 days without blind subcultures being needed. (Yagupsky, 1999, 2004)

3.1.2 Suitable specimens

Samples of any body fluid, or of tissue collected surgically or at necropsy, may be cultured. Blood is the material most commonly examined, but bone marrow may be more likely to give positive results. Blood cultures are best performed on samples collected during a febrile episode, preferably while the temperature is rising. The isolation rate is improved if several samples are taken over 24 hours (Topley and Wilson).

3.1.3 Test sensitivity

The procedure outlined above is capable of achieving isolation rates of up to 85% from patients with acute *B. melitensis* infection.

3.1.4 Test specificity

No mathematical data available

A diagnosis of brucellosis is confirmed by the isolation of small Gram-negative cocco-bacilli from blood or other tissues, a suitable history and confirmed by specific biochemical tests. Biotyping at a reference laboratory can provide additional information on the aetiology of the isolate. The isolation of *Brucella* sp is notifiable in all States of Australia.

3.1.5 Predictive values

A negative culture does not exclude the diagnosis of brucellosis.

3.1.6 Suitable acceptance criteria

No growth occurs after incubation of Blood Agar or Serum Dextrose Agar (SDA) plates for 24 hours at 37° C in 5–10% CO_2.

After incubation of Blood Agar for 48 hours at 37° C in 5–10% CO_2 colonies are 1 mm, non-haemolytic and have a metallic sheen. After 48 hours incubation on SDA *Brucella* spp are slightly larger than on blood agar and are cream coloured.

Some *Brucella* sp. will grow without increased CO_2 concentration whilst other species have a requirement of 5–10% CO_2 for growth. *Brucella* sp. will not grow anaerobically.

3.1.7 Suitable internal controls

A properly documented, relevant, quality control program for each type and batch of medium used

3.1.8 Suitable test validation criteria

Isolation of a *Brucella* sp. confirmed by biochemical and phenotypic parameters. Diagnosis supported by a clinical history and serology.

3.1.9 Suitable external QC program

No suitable external QC program exists.

3.1.10 Special considerations

Extreme care must be exercised when working with brucellae as humans are highly susceptible to brucellosis and laboratory infections are not uncommon. Cultures of these organisms and materials containing them, including clinical specimens, should only be handled with proper precautions by persons who are adequately trained and experienced in microbiological techniques.
Since *Brucella* spp are highly transmissible in the laboratory all isolates from sterile sites should be handled in a safety cabinet until their identity is known and blood culture bottles should always be sampled in safety cabinets.

If *Brucella* spp are suspected cultures should be handled in a PC3 facility or referred to a laboratory with appropriate facilities. All manipulations involving live *Brucella* cultures should be done in exhaust protective cabinets of a design which enables the operator to be physically separated from any sources of contamination.

Culture suspensions should not be centrifuged in ordinary centrifuges unless these are fitted with aerosol-proof rotors and tube assemblies. The tubes or bottles should only be filled and emptied in a safety cabinet. The use of glass tubes or bottles should be avoided.

Attention should be paid to the use of techniques which minimise aerosol production. Thus, pipettes should be filled and emptied using rubber bulbs or battery operated pipetters, care being taken to avoid contaminating these and to prevent frothing or bubbling during the procedure.

Laboratory staff who have handled *Brucella* spp on an open bench should seek medical advice for consideration to be offered antibiotic prophylaxis with doxycycline (Fiori *et al*).

3.2 Identification of *Brucella* sp.

There are two phases in the identification of *Brucella* species.

A: A: Diagnostic laboratories – presumptive identification of a *Brucella* species is usually made in a routine clinical laboratory. Laboratories should confirm that the presumptive isolate is not Haemophilus. Brucella is a PC3 level organism and because of the potential risk to laboratory personnel any suspect isolate should be sent to the National Brucella Reference Laboratory at the Australian Animal Health Laboratory for confirmation and biotyping.

B: Reference Laboratory – all presumptive isolates should be confirmed as *Brucella* spp and biotyped.

Conventional biochemical tests

3.2.1 Suitable specimen

A pure culture on solid medium (Blood Agar or Serum Dextrose Agar).

3.2.2 Staining

Gram'S stain—*Brucella* species are small Gram-negative rods that often appear coccobacillary.

Modified Ziehl-Neelsen—Some of the *Brucella* species are partially acid –fast in that they are not decolourised by 0.5 per cent acetic acid in the modified Ziehl-Neelsen stain. In this stain the carbol fuchsin is retained and the brucellae appear as red-staining coccobacilli.

3.2.3 Test details

For routine identification, a few biochemical tests, together with colony morphology and staining properties, will presumptively identify the isolate as a *Brucella* sp. In summary, the brucellae are non-motile, catalase-positive, oxidase-positive hydrolyse urea, reduce nitrate and are indole negative. Using known positive antisera (e.g. "Murex" *Brucella abortus* and *Brucella melitensis* specific antisera) a rapid slide-agglutination test can be used to presumptively identify *Brucella* sp.

3.2.4 Test sensitivity

No mathematical data available.

3.2.5 Test specificity

The isolation of a Brucella-like organism must be confirmed by both specific biochemical and serological tests. Full biotyping should then be carried at a reference laboratory.

3.2.6 Predictive values

A negative culture does not exclude the diagnosis of brucellosis.

3.2.7 Suitable test criteria

On blood agar *Brucella* sp are non-haemolytic colonies that take at least 48 hours to grow. They do not grow anaerobically. Organisms that morphologically and biochemically fit the *Brucella* species.

3.2.8 Suitable internal controls

Each batch of biochemical substrate tested with an appropriate positive and negative control organism. Results of all testing recorded and the results maintained.

3.2.9 Suitable validation criteria

Correct biochemical and phenotypic reactions exhibited by reference strains of *Brucella* sp.

3.2.10 Suitable external QC program

IFM Quality Services veterinary microbiology check sample program.

IFM Quality Services, PO Box 351, Moorebank, NSW 1875, Australia.

3.2.11 Kits/automated systems for biochemical identification

None suitable.

3.2.12 Molecular identification

Several PCR targets for assays to identify *Brucella* spp have been described including *dnaK,* 16S rRNA, the 16S-23S intergenic spacer region, *omp2,* and *bcsp31*(gene for an immunogenetic membrane protein specific for *Brucella* genus). (Probert et al, 2004). Further analysis of amplification products of some of these can be used to differentiate among the nomospecies. For example, polymorphisms in the *dnaK* gene have been used to differentiate *B. melitensis* from the other *Brucella* species. The entire dnak gene is amplified and the product digested with *Eco*RV. *B. melitensis* has one *Eco*RV site whilst all the other *Brucella* species have two *Eco*RV sites (Cloeckaert). 16S sequencing cannot differentiate among nomospecies. Recently a real-time multiplex PCR, based on *bcsp3,1* was developed to identify cultures of presumptive *Brucella* species (Probert, 2004).

3.3 Biotyping

Biotyping of presumptive *Brucella* spp isolates is carried out by determining the sensitivity of the organisms to selected aniline dyes (Thionin, Basic Fuchsin, Thionin Blue and Safranin O) and Penicillin. Ability of the organism to grow in the presence of Erythritol, production of H_2S, requirement of CO_2 for growth, ability to hydrolyse urea, agglutination with antisera produced against A and M Brucella antigens and sensitivity to lysis using the Tbilisi and Weybridge phages.

3.3.1 Suitable and unsuitable specimens

Isolates that biochemically and morphologically fit into the *Brucella* spp.
Isolates must be smooth cultures.

3.3.2 Test details

Thionin, basic fuchsin, thionin blue, and safranin O dyes of appropriate dilutions, erythritol and penicillin are all prepared and added to separate aliquots of dextrose agar to which foetal calf serum is added. Plates are poured no more than 24 hours prior to use.
A 48–72 hour culture of the organism to be tested is checked for smoothness using crystal violet.

If the culture is smooth a heavy inoculum is then spread onto the test plates as well as 2 slopes which are incubated under aerobic and air enriched with 5% CO_2 and to determine if the isolate requires CO_2 for growth. A lead acetate impregnated paper strip is added to the inside cap of the CO_2 tube to determine if H_2S is produced. The test organism is added to urea broth to determine if the organism hydrolyses urea. A lawn culture of the isolate is made and the Tbilisi and Weybridge phages are added at a dilution that will cause confluent lysis (routine test dilution) in sensitive strains of *Brucella* spp. All cultures are incubated at 37° C.

The urea broth is examined for evidence of hydrolysis every 15 minutes for 2 hours.
The slope containing the lead acetate strip is examined daily for evidence of H_2S production. All other plates are examined after 3 days and sensitivity to the dyes, and penicillin are recorded. The organisms ability to grow in the presence of erythritol is determined are its sensitivity to the 2 phages and its requirement for CO_2 for growth.

3.3.3 Test sensitivity

Depends on the isolate. Rough strains are not sensitive to the Tbilisi and Weybridge phages.

3.3.4 Test specificity

Test is specific for *Brucella* species.

3.3.5 Predictive values

None available.

3.3.6 Suitable test acceptance criteria

All controls work as expected.

3.3.7 Suitable internal controls

WHO reference strains of *Brucella suis* biotype 1, *Brucella abortus* biotype 2 and *Brucella melitensis* biotype 1 are set up with each unknown.

3.3.8 Suitable original test validation criteria

Brucella biotype confirmed by biochemical and phenotypic parameters.

3.3.9 Suitable external quality assurance programs

None available.

3.3.10 Special considerations

Brucella cultures growing in vitro may undergo changes in colonial morphology which are accompanied by alterations in antigenic structure, phage susceptibility and virulence. This process is termed dissociation and is particularly likely to occur when smooth strains are grown in static liquid culture. The variants produced may range from grossly aberrant mucoid or rough forms to others which are transitional between the extreme stages; intermediate or smooth-intermediate forms (Corbel *et al*).

Testing cultures with either crystal violet or acriflavine prior to testing for phage susceptibility or agglutination with mono-specific antisera is necessary to detect variants which may not be susceptible in these tests.

The Tbilisi and Weybridge phages have no affect on any non-smooth species of Brucella. Any reaction with A or M antisera should be ignored.

3.4 Nucleic Acid Detection

PCR assays for the direct detection of brucella in human samples have a significant safety advantage over culture methods. Several targets for PCR tests have been described overseas including *bcsp31*(gene for an immunogenetic membrane protein specific for *Brucella* genus). Queipo-Ortuno et al, 2005, described a real-time PCR assay to detect *Brucella* spp in serum in patients from an endemic area in Spain (serum was found to be less inhibitory than whole blood). They reported a

sensitivity for the assay of 94.6% and specificity of 94.6%. The corresponding sensitivity of blood culture was 65%. PCR tests are not yet available commercially and not in routine use in Australia.

3.5 Serological tests

Several tests are in routine use in Australia, the Serum Agglutination Test (SAT), Rose Bengal, dithiothreitol test (DTT), Coombs antihuman globulin test (AHG), CFT and EIA. The antigen used in all tests should be derived from smooth cells of brucella rather than rough cells which are known to increase the incidence of cross reactions with other organisms. The most important cross reaction occurs with *Yersinia enterocolitica* O:9 which has LPS O-chain antigenic determinants in common with *Brucella.* All serological tests for brucella presently used in Australia for diagnosing human disease are affected by this cross reaction. Only tests using antigen suspensions free of LPS are specific for brucella. This has been successfully achieved using *n*-lauroylsarcosine (Erdenebaatar et al 2003) Counterimmunoelectrophoresis has been used to measure antibodies against soluble antigens but is presently unavailable in Australia.

Since all nomospecies cross react with each other and *B. abortus* vaccine strains serology is not able to identify the infecting *Brucella* species. Antigen used in serological tests is most commonly whole *B. abortus* cells.

3.5.1 Serum (Tube) Agglutination Test (SAT)

The SAT is considered to be the gold standard serological test and, being simple to perform, is widely used. The antigen used is a saline suspension of washed smooth whole cells of *Brucella abortus,* usually obtained commercially. Commercial antigen preparations can vary in quality and each batch should be standardised against a panel of sera of known titre.

3.5.1.1 Test sensitivity

High—approximately 95% compared with culture. SAT does not detect nonagglutinating antibodies; therefore false negatives can occur.

3.5.1.2 Test specificity

Cross reactions occur with antibodies to *Yersinia enterocolitica* O:9 and *Afipia clevelandensis* (Drancourt et al (1997).

3.5.1.3 Predictive values

High for positive titre especially in acute disease. Since the SAT detects predominantly IgM (but also IgG and IgA) it is often positive in early stages of disease and a seroconversion or rising titre may not be seen. Laboratories are often reluctant to define the titre consistent with infection. In Australia, which has a low incidence of human disease, a diagnostic titre is generally above 1:80. A prozone has been occasionally reported in the literature.

3.5.1.4 Suitable acceptance criteria

A full epidemiological investigation should be made, to support a positive result.

3.5.1.5 Suitable internal controls

Standard positive and negative controls and a positive serum of known titre.

3.5.1.6 Suitable external QC program

NATA/ RCPA QAP for serology.

3.5.2 Rose Bengal Test

The RB is a rapid plate agglutination test which was originally developed to screen cattle for *B. abortus* and is valuable for diagnosis in areas with no laboratory facilities. It is now often used for diagnosing human disease. The test uses a suspension of *B. abortus* smooth cells stained with Rose Bengal dye to detect Brucella agglutinins and has a cross reactivity with other organisms identical to the SAT. The performance characteristics of the Rose Bengal test are very similar to the SAT but it is more sensitive and is very useful as a screening test.

3.5.3 2-Mercaptoethanol Test (2-ME)

This test was designed to measure IgG in patients with chronic brucellosis and high SAT titre where the diagnosis was unclear. Briefly a SAT is performed in the presence of 0.005*M* 2-ME which splits disulphide bonds in IgM removing their ability to agglutinate Brucella antigens. Titres before and after treatment with 2-ME are compared. The test is now offered by only a few laboratories in Australia.

3.5.4 Coombs' Test (AHG)

The Coombs' test detects the presence of nonagglutinating antibodies which may not be detected by other serological tests. It is used to confirm a diagnosis in patients with a convincing history of brucellosis but in whom the SAT and RB tests are negative. Briefly serum is reacted with brucella antigens and antihuman globulin added to agglutinate any antigen – antibody complexes formed. The sensitivity of the Coombs' test is significantly higher than SAT but is laborious to perform.

3.5.5 Complement Fixation Test (CFT)

CFT becomes positive after the SAT and RB. It may be useful diagnosing patients who present late in the disease when titres of the other tests have declined. The antigen used is normally whole smooth cells and its cross reactivity with other organisms is similar to SAT and RB. Very few Australian laboratories now offer this test.

3.5.6 EIA (ELISA)

Several commercial Brucella EIAs have been developed in recent years. An ELISA manufactured in Australia is now used by several large private and public laboratories to screen sera for the presence of Brucella IgG and IgM antibodies. Positive sera or sera from patients with a highly suggestive history but negative EIA are then tested by at least one of the other assays. The antigen is prepared by washing whole Brucella cells and cross reactions are the same as seen in the tests described above. The performance characteristics of the assay may be accessed via the company's website (www.panbio.com.au) and is similar to other EIAs reported in the literature (Araj et al, 1986, Diaz and Moriyon 1989).

3.5.6.1 Test sensitivity

High.

The sensitivity of the IgM ELISA approaches 100% in acute sera. IgM may not be detectable in convalescent sera but can rarely remain positive for 19 months.

The IgG ELISA may be negative in sera collected very early in disease but approaches 100% in convalescent sera.

3.5.6.2 Test specificity

High but will cross react with antibodies to *Yersinia enterocolitica* LPS

3.5.6.3 Predictive values

Negative predictive value is high but positive predictive value is less so. IgG ELISA may detect antibodies after exposure without disease in workers such as veterinarians with extreme exposure risk and remain positive in patients who have recovered from disease.

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