

**BASIC PROTOCOLS**  
**FOR LEVEL A LABORATORIES**

FOR THE PRESUMPTIVE  
IDENTIFICATION OF

*Brucella species*

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**Credits: *Brucella* Species**

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## I. General Information

### A. Description of organism

*Brucella* is a fastidious, aerobic, small, gram-negative coccobacillus.

### B. History

Brucellosis is a zoonotic infection, with four species being recognized as causing infection in humans: *Brucella abortus* (cattle), *Brucella melitensis* (goats, sheep, and camels), *Brucella suis* (pigs), and *Brucella canis* (dogs).

The disease has been known by several terms, including Malta fever, undulant fever, Rock of Gibraltar fever, and Bang's disease. Brucellosis is named after David Bruce, a British army medical doctor, who isolated *Brucella melitensis* from the spleen of a dead British soldier on the island of Malta in 1887. Following the institution of measures to prohibit the consumption of goat milk, the number of cases of brucellosis declined. Alice Evans, an American scientist who did landmark work on pathogenic bacteria in dairy products, was central in gaining acceptance of the pasteurization process to prevent brucellosis.

In 1954, *Brucella suis* became the first biological agent to be weaponized by the United States in the days of its offensive biological warfare program. The infective dose for these organisms is very low if acquired via the inhalation route, which makes them a potentially effective bioterrorism agent and also makes them a hazard in the clinical microbiology laboratory.

### C. Geographic distribution

There are between 50 and 100 cases of *Brucella* infection in humans each year in the United States. Infections are seen in essentially two patient populations. The first is individuals who work with animals which have not been vaccinated against brucellosis. This patient population includes farmers, veterinarians, and slaughterhouse workers. *B. abortus* (cattle) and *B. suis* (pigs) are the agents most likely to cause infections in this group of individuals. They become infected either by direct contact with or aerosolization from infected animal tissues.

Brucellosis is also seen in individuals who ingest unpasteurized dairy products contaminated with *Brucella*. This is most likely to occur in individuals who travel to or migrate from rural areas of Latin American and the Middle East, where disease is endemic in dairy animals, particularly goats and camels. *B. melitensis* is the most common agent seen in this patient population.

#### **D. Clinical presentation**

*Brucella* can cause both acute and chronic infections. The symptoms of brucellosis are non-specific and systemic, with fever, sweats, headache, anorexia, back pain, and weight loss being frequent. The chronic form of the disease can mimic miliary tuberculosis with suppurative lesions in the liver, spleen, and bone. The organism is often included in the differential diagnosis of fevers of unknown origin. It has a mortality of 5% in untreated individuals.

## II. Procedures: *Brucella* species

- A. General:** The procedures described below function to rule out suspected *Brucella* species using specimens and isolates.
- B. Precautions:** All patient specimens should be handled while wearing gloves and gowns and working in a biosafety cabinet. Subcultures should be performed in a biosafety cabinet and incubated in 5 to 10% CO<sub>2</sub>. Plates should be taped shut, and all further testing should be performed only in the biosafety cabinet.
- C. Specimens**
1. Blood or bone marrow
  2. Spleen, liver, joint fluid or abscesses are occasionally sources of *Brucella* ssp.
  3. Serum (at least 1ml)-For serologic diagnosis, an acute-phase specimen should be collected as soon as possible after onset of disease. A convalescent-phase specimen should be collected > 14 days after the acute specimen.

## D. Materials

### 1. Media

- a. Blood, bone marrow or joint fluid culture. Choose one of the following.
  - i. Standard liquid blood culturing system
  - ii. Biphasic system such as
    - a. Septi-Chek BBL Septi-Chek (B-D Microbiology Systems, Cockeysville, MD)
    - b. PML biphasic (PML Microbiologicals, Inc., Wilsonville, OR)
  - iii. Lysis-centrifugation system of ISOLATOR (Wampole Laboratories, Cranbury, NJ)
- b. Media for subculturing of positive blood culture bottles
  - i. Sheep blood agar (BAP)
  - ii. Chocolate agar (CHOC)
  - iii. MacConkey (MAC) or EMB agar

### 2. Reagents

- a. Gram stain
- b. Catalase (3% hydrogen peroxide)
- c. Oxidase (0.5 tetramethyl-p-phenylenediamine)
- d. Urea agar (Christensen's) or rapid urea disks (3) (Remel, Inc; Key Scientific; or Hardy Diagnostics)
- e. Culture of *Staphylococcus aureus* ATCC 25923

### 2. Equipment and supplies

- a. Blood culture instrument (optional)

- b. 35°C incubator with 5-10% CO<sub>2</sub>
- c. Light microscope with 100X objective and 10X eyepiece
- d. Microscope slides, coverslips, disposable bacteriologic inoculating loops

**Disclaimer:** Names of vendors or manufacturers are provided as examples of suitable product sources; inclusion does not imply endorsement by the American Society for Microbiology, or any other contributor.

**E. Quality Control:** Perform quality control of media and reagents according to package inserts, NCCLS document M22-A2, and CLIA standards, using positive and negative controls. Do not use *Brucella* spp. as a control organism, due to its infectious nature.

**F. Stains and smears:**

B. Procedures:

1. Aseptically inoculate liquid blood culture bottles with maximum amount of blood or body fluid per manufacturers' instructions. Incubate at 35°C. (See below for subculture method).
  - i. Incubate non-automated broth blood cultures for 21 days, with blind subculturing every 7 days, followed by terminal subculturing of negative blood cultures and holding sealed plates for 7 additional days.
  - ii. Incubate automated systems for 10 days and perform terminal subcultures at 7 days to increase yield (10).

**NOTE:** Isolation of *Brucella* is often delayed compared to other bloodstream pathogens, with peak isolation occurring at 3 to 4 days compared to 6 to 36 h for most other pathogens. Although incubation time of 21 days with weekly or terminal blind subculture are advocated, careful studies in *Brucella*-endemic areas using the BACTEC 9240 system (B D Division Instrument Systems, Sparks, MD) suggest that a maximal incubation time of 10 days is sufficient for reliable recovery of this organism, with 93% of 97 patient isolates being detected in 5 days (1). For the BacT Alert system (Biomérieux Inc., Hazelwood, MO), terminal subcultures at 7 days increased yield (10). Lysis-centrifugation has been shown to be less sensitive than broth-based systems for pediatric specimens (11). There is very limited published data with the ESP system (TREK Diagnostics, Westlake OH), so its effectiveness in the recovery of *Brucella* is unknown (10).

2. For tissues, inoculate BAP, CHOC and MAC or EMB and incubate for up to 7 days at 35°C in a humidified incubator with 5 to 10% CO<sub>2</sub>. Humidity may be maintained by placing a pan of water in the bottom of the incubator or by wrapping the plates with gas permeable tape.

**NOTE:** *Brucella* has been responsible for many laboratory-acquired infections (4,6,8). If *Brucella* is suspected or the Gram stain shows a small, gram-negative coccobacillus, avoid aerosols and perform subcultures in a biosafety cabinet. Plates should be taped

shut, and all further testing should be performed only in the biosafety cabinet, using Biosafety level III practices (2).

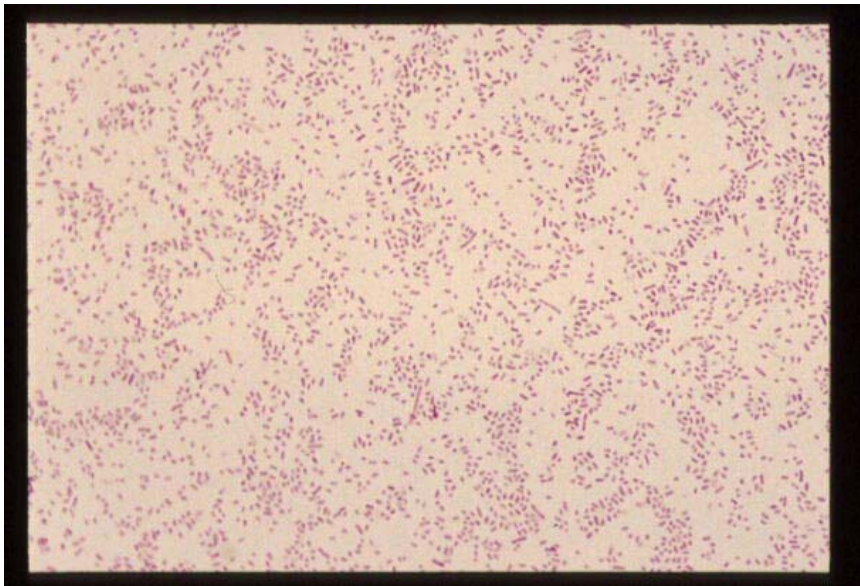
3. Gram stain suspicious colonies or positive blood culture bottles.

*Brucellae* are small (0.4 by 0.8  $\mu\text{m}$ ), gram-negative coccobacilli that can be visualized directly from positive blood culture bottles or Gram stains of colonies from primary media. (See Figure 1).

4. Subculture suspicious blood cultures to BAP, CHOC and MAC or EMB.

5. Incubate plates at 35°C in a humidified incubator with 5 to 10% CO<sub>2</sub>.

Figure 1. Gram stain shows a small, gram-negative coccobacillus.



#### G. Further testing

1. Perform the following biochemical tests in a biological safety cabinet if the above criteria are met. All reactions are positive for *Brucella* spp.
  - a. Oxidase
  - b. Catalase
  - c. Urea
    - i. Observe for color change to pink at 15 min, 2 h and up to 72 h.
    - ii. Reactions of small numbers of strains are delayed up to 72 h on Christensen's agar (See Figure 2).



**WARNING:** The identification of *Brucella* species should not be attempted with commercial identification systems.

2. *Haemophilus* can be confused with *Brucella*; however *Haemophilus* do not grow on BAP. When in doubt, differentiate between these two genera by performing a satellite test. Inoculate a blood agar plate, followed by cross-streaking or spotting with *Staphylococcus aureus* ATCC 25923. After 24-48 h of incubation in 5% CO<sub>2</sub>, *Haemophilus* demonstrate satellite growth around the *S. aureus*, while *Brucella* growth is not limited to the area around the staphylococcus.
3. Other organisms that can be confused with *Brucella* species because they are urease positive are *Oligella ureolytica* (usually found only in the urine), *Psychrobacter phenylpyruvicus*, *Psychrobacter immobilis*, and *Bordetella bronchiseptica* (motile) (5,9) (See Table 1).

**Figure 2. Weak urea reaction of *B. melitensis* at 24 h. on Christensen's agar**



## H. Interpretation and reporting

### 1. Major characteristics of *Brucella*

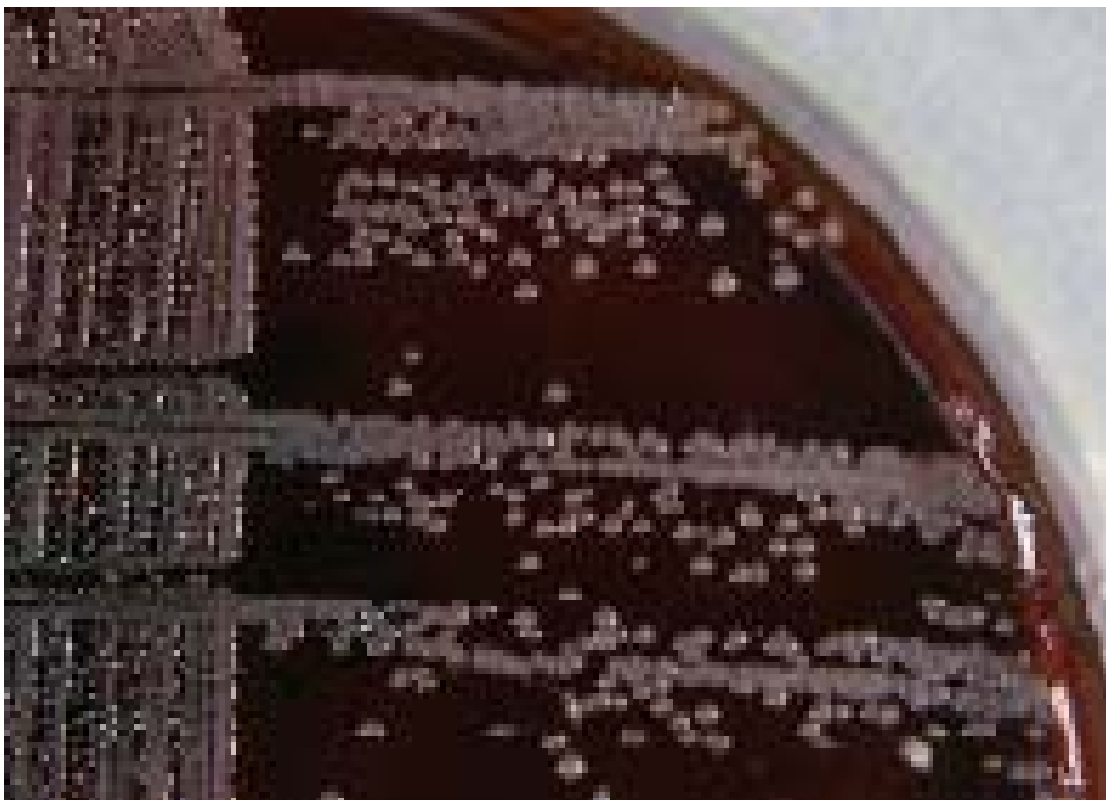
- a. Small, gram-negative coccobacilli
- b. Grows only in aerobic blood culture bottles after 2-4 days.
- c. Grows on BAP and CHOC but not on MAC or EMB
  - i. Colony morphology on BAP: *Brucella* will appear as punctate colonies after 48 h (See Figure 3)
  - ii. Colonies are non-pigmented and non-hemolytic
- d. Positive for oxidase, catalase, and urea

**NOTE:** Confirmatory identification is made by agglutination with specific antiserum, generally in a reference or public health laboratory.

### 2. Presumptive identification of *Brucella* species

- a. *Brucella* species will grow on subculture after 48 h of incubation in 5 to 10% CO<sub>2</sub> on CHOC and BAP.
- b. The organism does not grow on MAC, which will allow it to be separated from some other gram-negative coccobacilli.
- c. The colonies typically show “dust-like” growth after overnight incubation, and a minimum of 48 h is necessary to get sufficient growth for further identification.
- d. Colonies are smooth, convex, and raised with an entire edge (i.e., they have no distinguishing features). (See Figure 3)

**Figure 3. Colony of *Brucella* on subculture at 48 h on BAP**



### 3. Reporting/appropriate action

- a. Level A laboratories should consult with state public health laboratory director (or designate) prior to or concurrent with testing if *Brucella* species is suspected by the physician.
- b. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *Brucella* cannot be ruled out and a bioterrorist event is suspected. The state public health laboratory/state public health department will notify local FBI agents as appropriate.
- c. Immediately notify physician/infection control according to internal policies if *Brucella* species cannot be ruled out.
- d. Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate Laboratory Response Network (LRN) laboratory. FBI and state public health laboratory/state public health department will coordinate the transfer of isolates/specimens to a higher-level LRN laboratory as appropriate.
- e. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials).
- f. If *Brucella* species is ruled out, proceed with efforts to identify using established procedures.
- g. **Do not process nonclinical (environmental or animal specimens). Restrict processing to human clinical specimens only. Nonclinical specimens should be directed to the state public health laboratory.**

#### I. Limitations

1. *B. abortus*, *B. melitensis*, and *B. suis* are all oxidase-positive organisms. *B. canis* isolates may be oxidase-variable.
2. Using the Christensen's tube test, urea hydrolysis can be observed in as early as 15 min incubation with *B. suis* strains and within 1 day of incubation with most strains of *B. abortus*, and *B. melitensis*. Some *B. melitensis* strains take even longer to be positive.
3. Do not attempt to identify tiny gram-negative rods that do not grow on MAC or EMB using a commercial identification system because of their lack of accuracy and danger of aerosols.
4. Because there are a number of urea-positive, fastidious tiny gram-negative rods, the definitive identification of *Brucella* is generally performed by a reference or state health department laboratory. However, isolation of an organism with the characteristics of *Brucella* listed in this procedure from a blood or normally sterile site is most likely *Brucella*.
5. In vitro susceptibility testing is not helpful. Tetracyclines (doxycycline) are the most active drugs and should be used in combination with streptomycin (or gentamicin or rifampin, if streptomycin is unavailable) to prevent relapse.

### III. References

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- 11. Yagupsky, P., N. Peled, J. Press, O. Abramson, and M. Abu-Rashid. 1997.**  
Comparison of BACTEC 9240 Peds Plus medium and Isolator 1.5 Microbial Tube for the detection of *Brucella melitensis* from blood cultures. J. Clin. Microbiol. **35**:1382–1384.

Flowchart

**SAFETY: As soon as *Brucella* is suspected, perform ALL further Work in a BioSafety Cabinet (BSL3)**

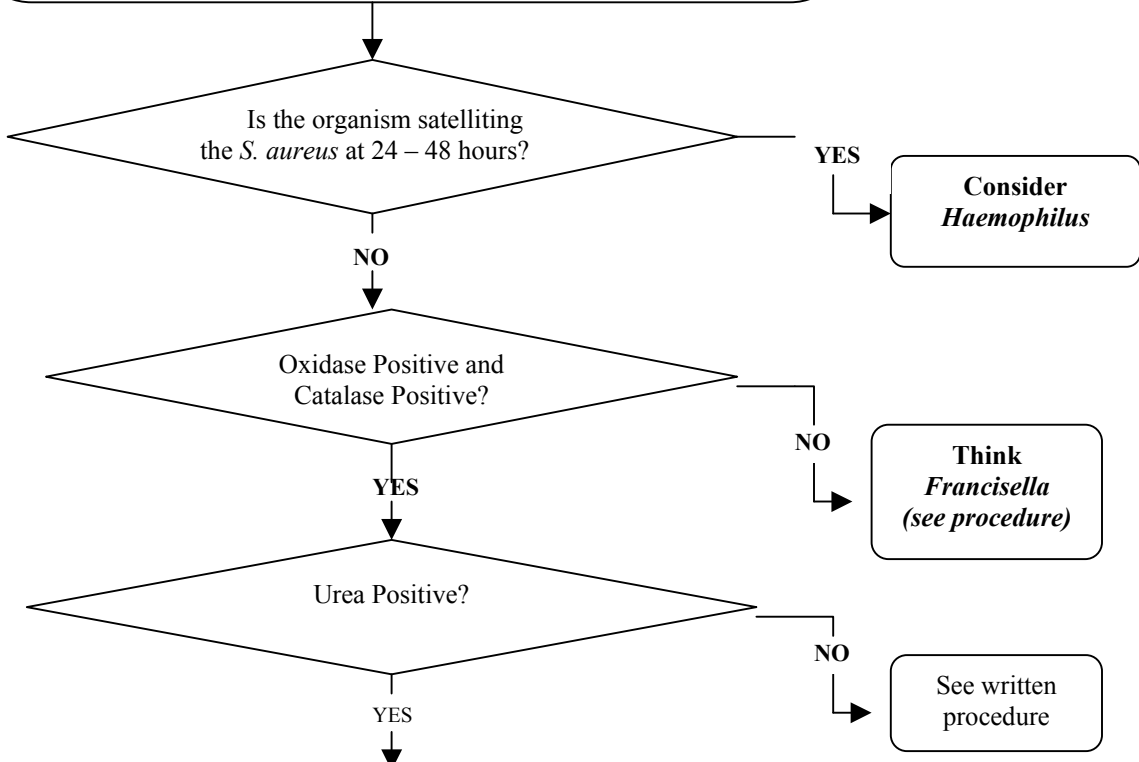
**Major Characteristics of *Brucella* Species**  
**Morphology:** Small (0.4 x 0.8um), Gram-negative coccobacillus  
Visible on Gram stain of positive blood culture broth

**THINK BRUCELLA**

**Growth:** Subculture positive aerobic blood culture bottle to:  
Sheep Blood Agar (BAP)  
Chocolate Agar (CHOC)  
Incubate in 5 – 10 % CO<sub>2</sub> at 35° C.

Spot BAP with *S. aureus* ATCC 25923 for satellite test

Note poorly growing colonies after 24 hours incubation on BAP and CHOC  
Incubate plates for at least 2 additional days if no growth in 24 hours. Organism does not grow on MAC



**SEND TO LOCAL OR STATE PUBLIC HEALTH LABORATORY**

Inform physician that *Brucella* species cannot be ruled out.

**Antimicrobial therapy:  
Rifampin or Streptomycin plus Doxycycline**

**Table 1. Differentiation of *Brucella* from other urea-positive, oxidase-positive gram-negative coccobacilli<sup>a</sup>**

	<i>Brucella</i> <sup>a</sup>	EO-2, EO-4 <i>Psychrobacter immobilis</i>	<i>Psychrobacter phenylpyruvicus</i>	<i>Oligella ureolytica</i> <sup>a</sup>	<i>Actinobacillus</i> spp. <sup>a</sup>	<i>Bordetella bronchiseptica</i> <i>Ralstonia paucula</i> (IV c2)	<i>Bordetella hinzii</i>	<i>Haemophilus</i> spp. <sup>c</sup>
Gram stain morphology	tiny ccb, stains faintly	small ccb, rods E0-2 in packets	ccb	tiny ccb	ccb, rods	ccb, rods	ccb, rods	ccb
Catalase	+	+	+	+	v	+	+	v
Oxidase	+	+	+	+	+	+	+	v
Urea <sup>b</sup>	+	v	+	+	+	+	14% pos	v
Motility	-	-	-	+,delayed	-	+	+	-
PDA <sup>a</sup>	-	-	+	+	-	V	-	-
Nitrate	+	v	68%	+	+	V	-	NA
Nitrite	-	v	-	+	-	-	-	NA
TSI	Alkaline	Alkaline	Alkaline	Alkaline	Acid/Acid	Alkaline	Alkaline	No growth
MAC-48 h	-,poor	-, poor	-,poor	-,poor	-,poor	+	+	-

<sup>a</sup> Reactions extracted from references 7 and 9; NA, not applicable; v, variable; ccb, coccobacilli; PDA is phenylalanine deaminase. TSI is triple sugar iron agar; MAC is Mac Conkey agar. *O. ureolytica* is primarily a uropathogen. *A. actinomycetemcomitans* is urea -negative and rarely oxidase-positive. Urea-positive *Actinobacillus* are from animal sources.

<sup>b</sup> Use rapid urea test to increase sensitivity.

<sup>c</sup> Only grows on chocolate; or on blood agar associated with staphylococcus colony.

#### IV. **Appendix A:** Change record

1. 29 May 2002

a. Revised Table 1.