

**Level A Laboratory Procedures for Identification of *Brucella* Species**

- I. General:** The procedures described below are designed to rule out (presumptively identify) *Brucella* spp. from clinical specimens or isolates.
- II. Precautions**
- A.** These procedures should be performed in microbiology laboratories that utilize Biological Safety Level 2 (BSL 2) practices.
- B.** If *Brucella* spp. is suspected:
1. All work should be performed in a biological safety cabinet (BSC) with gloves.
  2. Because of the highly infectious nature of some *Brucella* spp. (BSL 3), consultation with the state public health laboratory is recommended if *Brucella* spp. is suspected.
  3. Specimens with suspected *Brucella* spp. should be labeled as such.
- C.** Refer to Procedure for Laboratory Safety and Decontamination.
- III. Specimen**
- A. Acceptable specimen types**
1. Blood or bone marrow: *Brucella* spp. are most often isolated from these specimens.
  2. Serum: 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.
  3. Spleen, liver, or abscess: *Brucella* spp. are occasionally isolated from these sources. Selective media can be used for isolation of *Brucella* spp. from specimens with mixed flora (see below).
- B. Rejection criteria**
1. Use established laboratory criteria.
  2. Dried specimens should be referred to your state public health laboratory.
  3. Environmental/nonclinical samples and samples from announced events are not processed by Level A laboratories; submitter should contact the state public health laboratory directly.
- C. Transport and storage**
1. Serum: Follow standard laboratory protocols. Preferably send at least 1 ml, refrigerated.
  2. Specimens for culture: Specimens should be inoculated into appropriate culture media within 2 h of collection. If this is not possible, specimens should be refrigerated (2-8°C) until inoculation. Tissue must be kept moist; add several drops of sterile saline if necessary.
- IV. Materials**
- A. Reagents**
1. Gram stain reagents
  2. Oxidase reagent (1% tetramethyl-p-phenylenediamine)

3. Urea agar (Christensen's)
4. XV requirement (XV disks/strips, satellite test, or other to differentiate from *Haemophilus* spp.)

**B. Media**

1. General nutrient agar: 5% sheep blood agar (SBA), tryptic soy agar base, or equivalent
2. Chocolate agar (CA)
3. MacConkey agar (MAC)
4. Thayer-Martin agar or equivalent
5. Blood culture bottles

**C. Equipment/supplies**

1. Blood culture instrument (optional)
2. Light microscope with 10X, 40X, and 100X objectives and 10X eyepiece
3. Microscope slides, 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 Centers for Disease Control and Prevention, the Department of Health and Human Services, the United States Army, or the Federal Bureau of Investigation.

**V. Quality control:** Perform and document all quality control efforts according to standard laboratory procedure/protocol.

**VI. Procedure:** Refer to Fig. A1a and A1b.

**A. Stains and smears:** Gram stain

1. Procedure: Perform Gram stain procedure/QC per standard laboratory protocol.
2. Characteristics: *Brucella* spp. have a characteristic Gram-stain morphology that is extremely helpful in differentiating them from other gram-negative organisms. *Brucella* cells appear as tiny, gram-negative coccobacilli. Refer to Fig. A2.

**B. Cultures**

1. Blood cultures, bone marrow, and tissue specimens: Process and subculture using standard laboratory protocol (using media such as 5% SBA, CA, MAC, and/or Thayer-Martin agar). Note: Growth on MAC will be negative or poor for most *Brucella* spp. Thayer-Martin can be used as a selective medium for *Brucella* spp. as appropriate.
2. Incubation of cultures
  - a. Temperature: 35-37°C
  - b. Atmosphere: CO<sub>2</sub> enriched, incubator humidity should be sufficient to prevent plates from drying out with prolonged (>7 days) incubation. Humidity may also be maintained by wrapping the plates with gas permeable tape.
  - c. Length of incubation
    - (1) Blood cultures: For suspected brucellosis cases at least 21 days, with blind subculturing every 7 days. Follow by terminal subculturing of negative blood cultures and holding sealed plates for 7 additional days. Perform all culture manipulations (e.g., subculturing or smear preparation for Gram stains) with gloves in a BSC.

- (2) Primary plated cultures: 7 days, read daily.
3. Colony characteristics: *Brucella* spp. will appear as punctate colonies after 48 h of incubation. Colonies are nonpigmented and nonhemolytic. All suspicious colonies should be examined by Gram stain, oxidase, and urea test.

### C. Biochemical reactions/tests

1. Oxidase test
  - a. Principle: Used to detect the presence of oxidase enzymes associated with the cytochrome respiratory system. The reagent is a dye that changes color in the presence of oxidase enzymes.
  - b. Specimen: Performed on samples of actively growing colonies (18-24 h) from SBA or equivalent plates.
  - c. Reagents and materials
    - (1) 0.5% tetramethyl-p-phenylenediamine (Sigma; catalog #T7394).  
Note: It is important to use the tetramethyl-based reagent, as opposed to the dimethyl-based reagent; the dimethyl-based may result in false-negative reactions.
    - (2) Combine 50 mg of powder with 10 ml of distilled water.
    - (3) Store in a brown dropper bottle. The solution is stable 1 week at 2-8°C, or indefinitely when frozen at -20°C.
    - (4) Whatman #1 filter paper or equivalent
    - (5) Disposable plastic loop
  - d. Procedure (can be performed using other validated laboratory protocol)
    - (1) Place 1 to 2 drops of oxidase reagent on a piece of Whatman #1 filter paper.
    - (2) Using a disposable plastic loop, mix a loopful of the organisms from an 18-24 h plate culture into the reagent on the paper.
    - (3) Observe for the development of a light to dark blue color within 10 sec of inoculation.
  - e. Characteristics
    - (1) Positive result: Development of blue color within 10 sec of inoculation.
    - (2) Negative result: No development of blue color within 10 sec of inoculation.
    - (3) Caution: Some metals in bacteriological loops produce false-positive reactions. Platinum-iridium wire (85% platinum: 15% iridium) loops are satisfactory. If another kind of loop is used, it should be tested first by exposing it to the oxidase reagent on filter paper.
  - f. Quality control
    - (1) Control strains
      1. Positive control: *Pseudomonas aeruginosa* ATCC 35032 or equivalent will produce a blue color within 10 sec.
      2. Negative control: *Escherichia coli* ATCC 25922 or equivalent will NOT produce a blue color within 10 sec.
    - (2) Method control: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be assayed on each day of testing.
    - (3) Resolving out-of-control results: Check media, reagents, equipment, and controls; replace or correct as appropriate. Document corrective actions taken and repeat test.
2. Urease test (Christensen's method)

- a. Principle: Used to determine an organism's ability to hydrolyze urea, forming ammonia by action of the enzyme urease. The presence and rate of urease activity are useful in the differentiation of *Brucella* spp.
- b. Specimen: Actively growing culture of the test organism from non-selective media.
- c. Reagents and materials
  - (1) Pre-made agar slants of this medium can be obtained from commercial sources (BD Bioscience; catalog #221096 or 221097; or Remel; catalog #65210-A).
  - (2) Urea agar
    1. Solution A: Bacto-urea agar base 29 g (Difco/BD Bioscience; catalog #0140) (composed of Bacto-peptone, 1 g; Bacto-dextrose, 1 g; sodium chloride, 5 g; monopotassium phosphate, 2 g; urea [Difco/BD Bioscience], 20 g; and Bacto-phenol red, 0.012 g); distilled water 100 ml. Mix to dissolve and filter sterilize.
    2. Solution B: Bacto-agar 15 g; distilled water 900 ml. Autoclave solution B at 121°C for 15 min, and cool to 50°C. Then add solution A, gently mix, and aliquot in 5-ml amounts into 15 x 125-mm screw-cap tubes and slant. Allow the agar to harden at room temperature; store the slants at refrigerator temperature (2-8°C).
  - (3) Timing device that can measure up to 15 min
  - (4) Sterile inoculating loop
- d. Procedure
  - (1) At least 30 min before setting up the test, remove the urea tubes from storage and allow them to equilibrate to room temperature.
  - (2) Using a sterile inoculating loop, transfer a loopful of test organism to the surface of the urea agar slant.
  - (3) Incubate at 35-37°C in ambient atmosphere.
  - (4) Set timer for 15 min.
  - (5) After 15 min incubation, observe the inoculated slant for a color change to pink in the inoculated area. If no color change is observed, replace the slant in the incubator and observe it again after 24 h incubation.
- e. Characteristics
  - (1) Positive result: Development of a pronounced pink color in the agar. All *Brucella* spp. should produce a positive reaction after overnight incubation.
  - (2) Negative result: Absence of pink color
- f. Quality control
  - (1) Control strains
    1. Positive control: *Proteus vulgaris* ATCC 8427 or equivalent will produce a pink color within 24 h.
    2. Negative control: *Enterobacter aerogenes* ATCC 13048 or equivalent will NOT produce a pink color within 24 h.
  - (2) Method control: Perform the test with fresh cultures of the control strains using the same method as with unknowns. Control strains should be tested on receipt of each new batch/lot of medium.
  - (3) Resolving out-of-control results: Check media, reagents, equipment, and controls; replace or correct as appropriate. Document corrective actions taken and repeat test.
- g. Limitations of the procedure: Other gram-negative organisms, particularly *Bordetella bronchiseptica* and some *Haemophilus influenzae* biogroups, may produce a rapid urease reaction. Gram stain morphology, X and V

growth factor requirements, and motility are useful characteristics for ruling out these other species. *Brucella spp.* are non-motile.

3. Catalase reagent (3% hydrogen peroxide): This test is NOT recommended if *Brucella spp.* is suspected because of the generation of aerosols; this test MUST be performed in a BSC (if performed).

## **VII. Interpretation and reporting**

### **A. Presumptive identification criteria** (Refer to Table A1)

1. Colony morphology on SBA: *Brucella spp.* will appear as punctate colonies after 48 h incubation. Colonies are non-pigmented and non-hemolytic. All suspicious colony types should be examined by Gram stain and urea test.
2. Gram stain morphology: *Brucella spp.* have a characteristic Gram-stain morphology that is extremely helpful in differentiating them from other gram-negative organisms. *Brucella* cells appear as tiny, faintly stained coccobacilli.
3. Oxidase test (Kovac's modification) positive
4. Urease test (Christensen's method) positive

### **B. Reporting and appropriate actions**

1. Level A laboratories should consult with state public health laboratory director (or designate) prior to or concurrent with testing if *Brucella spp.* is suspected by the physician.
2. Immediately notify state public health laboratory director (or designate) and state public health department epidemiologist/health officer if *Brucella spp.* 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.
3. Immediately notify physician/infection control according to internal policies if *Brucella spp.* cannot be ruled out.
4. Preserve original specimens pursuant to a potential criminal investigation and possible transfer to an appropriate 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. Start chain of custody documentation if appropriate.
5. Obtain guidance from the state public health laboratory as appropriate (e.g., requests from local law enforcement or other local government officials).
6. If *Brucella spp.* is ruled out, proceed with efforts to identify using established procedures.

## **VIII. Limitations**

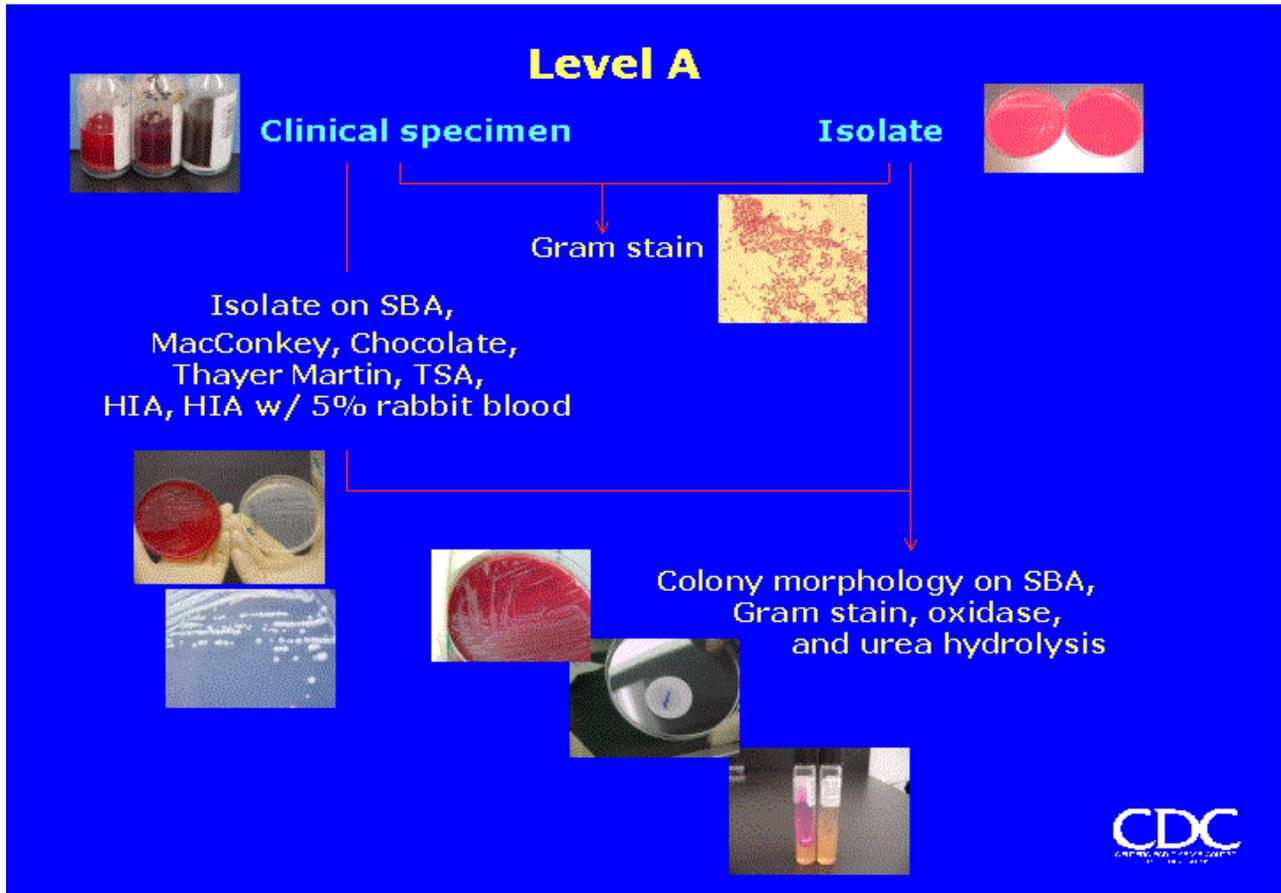
- A.** The identification of *Brucella* species should not be attempted with commercial identification systems because of the potential of generating aerosols and the lack of accuracy in identification.
- B.** To differentiate *Brucella spp.* from *Haemophilus spp.*, a satellite test should be performed by inoculating a blood agar plate, and then cross-streaking with *Staphylococcus aureus* ATTC 25923. After 24-48 h of incubation in enriched CO<sub>2</sub> atmosphere, *Haemophilus spp.* should have satellite growth around the *S. aureus*, while *Brucella spp.* do not exhibit satellite growth. XV disks or strips are a suitable alternative to *S. aureus* culture.

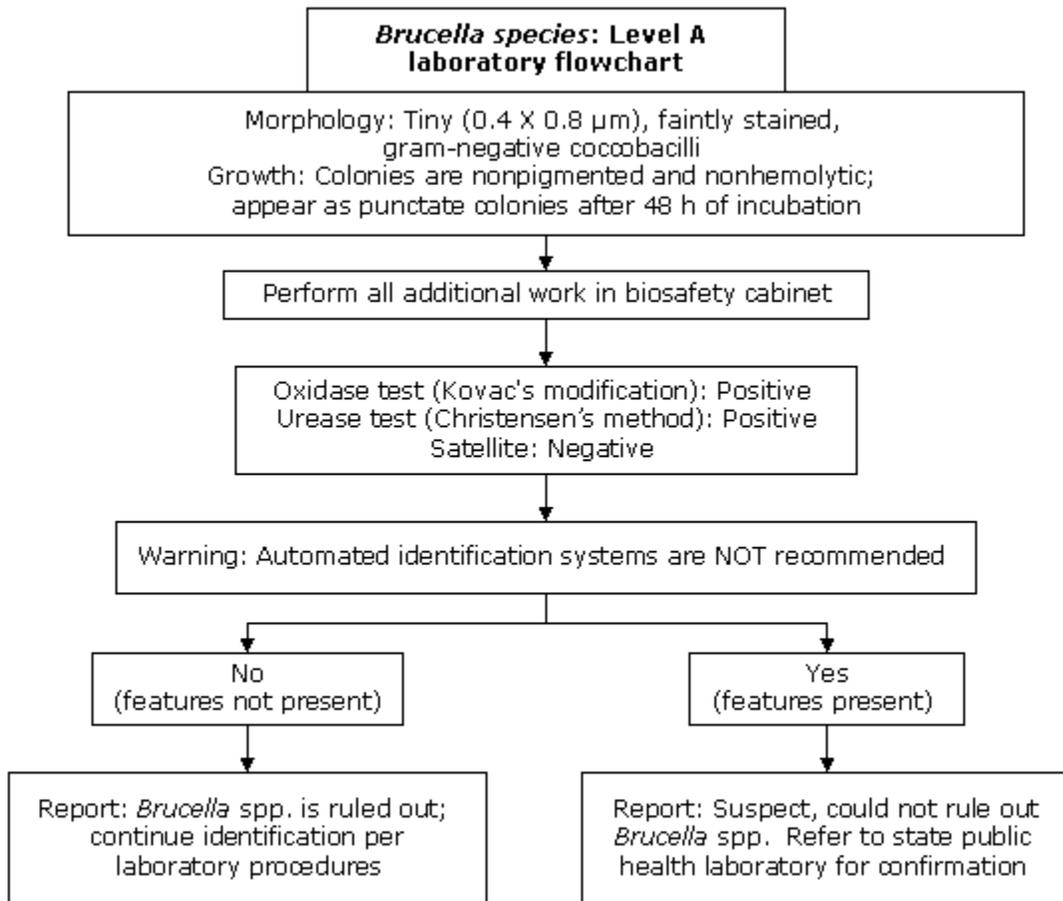
- C. Other organisms that can be confused with *Brucella* species because they are urease positive include *Oligella ureolytica* (usually found only in urine), *Psychrobacter phenylpyruvicus*, *Psychrobacter immobilis*, and *Bordetella bronchiseptica* (motile).

## IX. References

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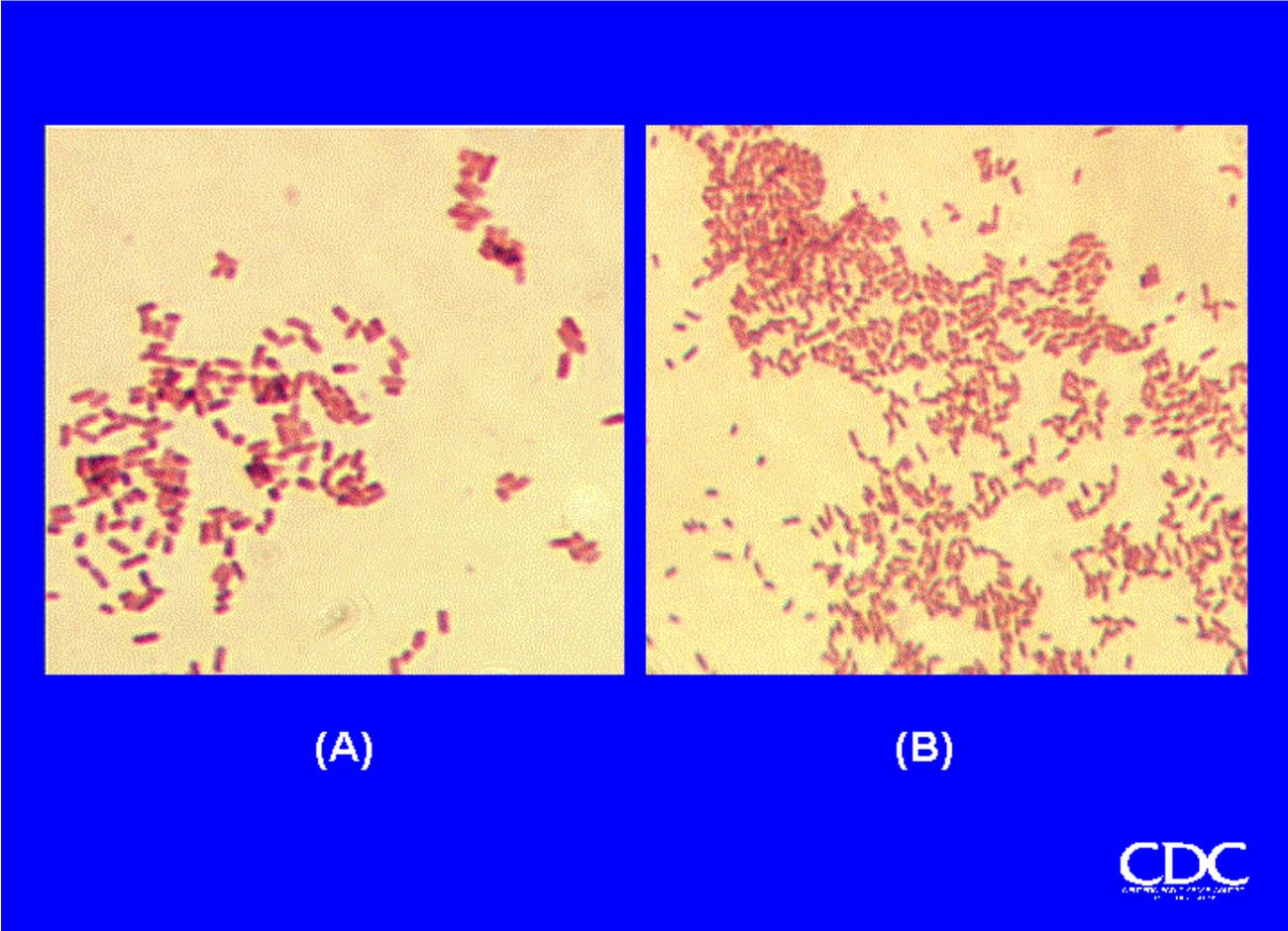
**Figure A1a.** Flowchart of Level A laboratory procedures for isolation and presumptive identification of *Brucella* spp.





**Figure A1b:** Level A flowchart for *Brucella* species

**Figure A2.** Gram stain of pure cultures: *Escherichia coli* (A) and *Brucella abortus* (B)



**Table A1.** Presumptive identification and differentiation of *Brucella* spp. from similar gram-negative bacteria

Test	<i>Oligella</i> <sup>a</sup> spp.	<i>Haemophilus influenzae</i> <sup>b</sup>	<i>Francisella tularensis</i>	<i>Brucella</i> spp.	<i>Acinetobacter</i> spp.	<i>Psychrobacter phenylpyruvicus</i> <sup>c</sup>	<i>Pasteurella</i> spp.	<i>Bordetella bronchiseptica</i> <sup>d</sup>
Specimen Source	urinary tract	various	various	blood, bone marrow	various	various	various	various
Gram stain morphology	tiny coccobacilli	small coccobacilli	tiny coccobacilli	tiny coccobacilli	broad coccobacilli	broad coccobacilli	medium size rods	small to medium rods
Oxidase	+	v	-	+ <sup>e</sup>	-	+	+	+
Urea hydrolysis <sup>f</sup>	+	v	-	+	v	+	v	+

<sup>a</sup> *Oligella* spp. can sometimes be motile.

<sup>b</sup> *Haemophilus* spp. require X and/or V factors for growth, which differentiates them from all other listed genera.

<sup>c</sup> Formerly *Moraxella phenylpyruvica*

<sup>d</sup> *B. bronchiseptica* exhibits vigorous growth at 1 day incubation; motile with peritrichous flagella.

<sup>e</sup> Oxidase: *B. abortus*, *B. melitensis*, and *B. suis* are all oxidase-positive organisms. *B. canis* isolates may be oxidase-variable.

<sup>f</sup> Urea hydrolysis: most *Brucella* isolates vigorously hydrolyze urea. Using the Christensen's tube test, 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*.

+ Greater than or equal to 90% positive.

- Less than or equal to 10% positive.

v Variable, 11-89% positive.