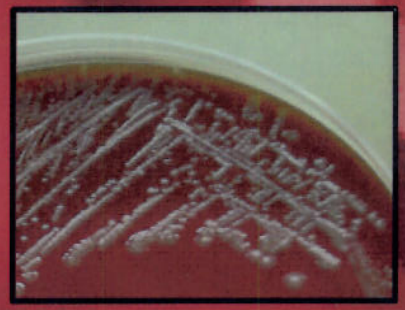
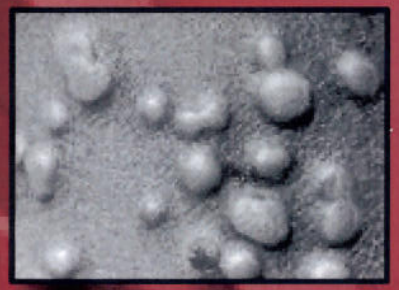
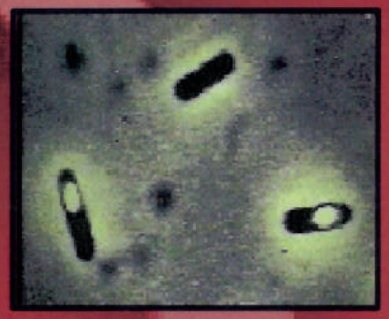
B – SAFE Bench Cards







Basic Select Agent Flow Chart & Evaluation

Laboratory Response Network (LRN) Reference Laboratories
State of Alaska

Lab	Location	Daytime	Afterhours
Alaska State Public Health Laboratory	Anchorage	907-334-2100	855-222-9918

Packaging and shipping requirements must be met for all samples and are the responsibility of the shipper.

Information can be found at
http://iata.org and
http://hazmat.dot.gov

Additional information on select agents and bioterrorism can be found at the CDC website http://www.bt.cdc.gov

‡ Photograph provided by CDC.

This publication was supported, in part, by cooperative agreement number U90/CCU216988 from the Centers for Disease Control and Prevention (CDC). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the CDC.

Bacillus anthracis

Colony Morphology

- Grows well on Blood Agar (BA); will not grow on MacConkey (MAC) agar
- 2-5 mm on BA at 24 h
- Flat or slightly convex with irregular borders that have comma-shaped protrusions
- Colonies have a ground-glass appearance
- Non-hemolytic on BA
- Tenacious colonies

Gram Stain

- Large gram-positive rod (1-1.5 x 3-5 μm)
- Long chains in liquid culture but may include single cells or short chains
- Spores are subterminal or central

Additional Information

- Can be misidentified as: *Bacillus megaterium* and other *Bacillus* spp.
- Biosafety Level 2 agent (Use BSL3 for large volume or high titer culture)
- Infectious Dose: <10,000 spores
- Transmission: Inhalation, ingestion, direct contact with skin
- Contagious: No

Acceptable Specimen Types

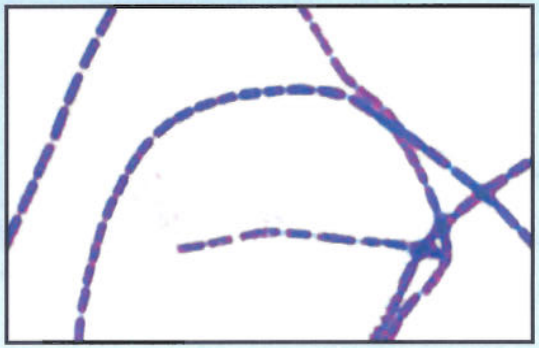
- Swab of vesicular fluid from cutaneous lesion
- Sputum (≥ 1 ml)
- Whole blood: 5-10ml blood in EDTA, and/or Inoculated blood culture bottle
- Stool (> 5g)

24 h growth on BA

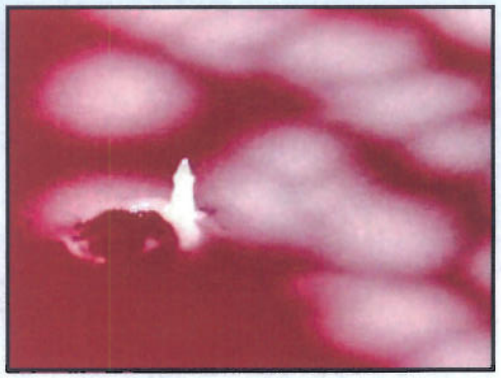
‡

Comma-shaped colonies

‡



Gram stain



Tenacity on BA

Sentinel Laboratory Rule-Out of *Bacillus anthracis*

Grey, ground-glass,
tenacious colonies at 24 h on BA at 35/37°C
Non-hemolytic on BA

↓

Large gram-positive rods
(chaining in liquid culture)

↓

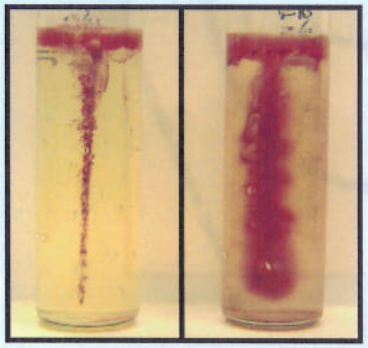
Perform all additional work in a certified Class II Biosafety Cabinet

*Motility: Non-Motile
(use semi-solid media rather than wet mount;
2,3,5-triphenyltetrazolium chloride indicator)
*Catalase: Positive

*Motility and Catalase: Appearances of test results are not agent-specific. Photos represent typical reactions

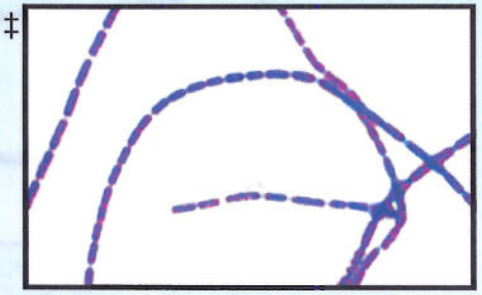
B. anthracis colonies at 24 h on BA

Motility



Non-Motile Motile

Gram stain



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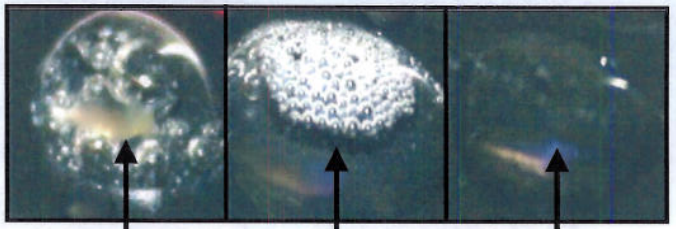


Catalase

Positive Weak Positive Negative

No → Continue laboratory identification procedures

Yes → Contact Alaska State Public Health Laboratory 907-334-2100 (business hrs) or 855-222-9918 (PHL On-call)

Brucella species

Colony Morphology

- Growth seen on Blood Agar (BA) and Chocolate Agar (CA)
- Non-hemolytic, non-pigmented small grey colonies; may require 48-72 h for discrete colonies to become evident
- CO_2 enhances growth
- Slow grower; punctate colonies (0.6 µm - 1.5 µm) in 48 h
- Clear or white colonies on MacConkey (MAC) agar at 48 h

Gram Stain

- Tiny, often faintly stained gram-negative coccobacilli (0.4 µm x 1.0 µm)
- May be mistaken for cocci
- May retain crystal violet stain in blood culture smears

Biochemical/Test Reactions

- Urea: Positive
- Oxidase: Positive
- Motility: Non-Motile

Additional Information

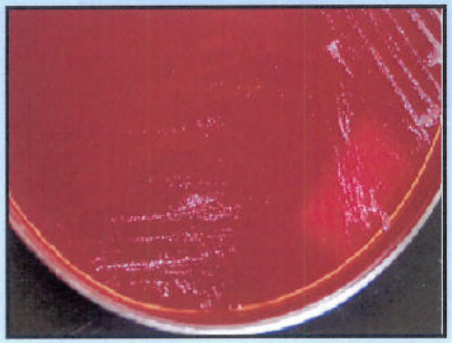
- May be misidentified as: *Moraxella* spp., *Micrococcus* spp., *Corynebacterium* spp., "slow growing" *Staphylococcus* spp., *Oligella ureolytica, Bordetella bronchiseptica, Haemophilus* spp., *Pasteurella* spp. by automated systems
- Biosafety Level 2/3 agent (once *Brucella* is suspected, work should only be done in a certified Class II Biosafety Cabinet)
- Transmission: Inhalation, consumption of unpasteurized dairy products, direct contact with skin

Acceptable Specimen Types

- Whole blood: 5-10 ml blood in EDTA, and/or Inoculated blood culture bottle
- Liver or spleen biopsy, or abscess
- Lymph node or bone marrow aspirate (1ml)
- Synovial fluid, CSF (≥ 1ml)

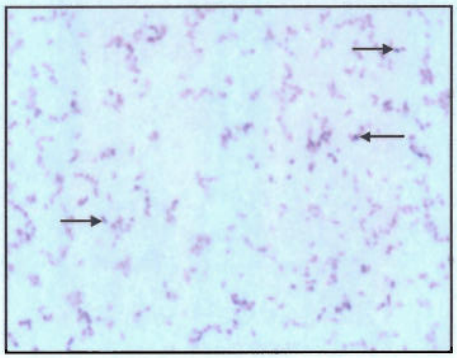
Brucellosis is a commonly acquired laboratory infection; all work on suspect *Brucella* spp. cultures should be performed at a minimum under BSL2 conditions with BSL3 practices.

Growth on BA at 48 h



Growth on CA at 48 h

Gram stain



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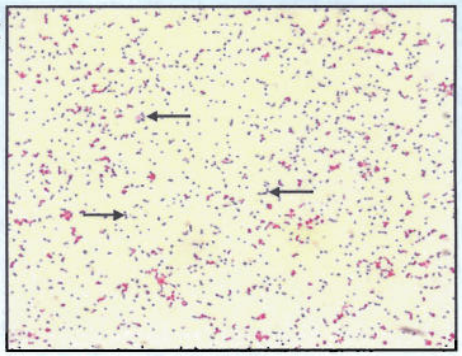


As indicated by the arrows, some gram-negative coccobacilli may retain crystal violet stain

Sentinel Laboratory Rule-Out of *Brucella* spp.

Fastidious organism; growth on BA and CA
Punctate, grey, shiny, circular, convex colonies at 48 - 72 h at 35/37°C
Non-hemolytic; non-pigmented

↓

Tiny, gram-negative coccobacilli

↓

Perform all additional work in a certified Class II Biosafety Cabinet

*Oxidase: Positive
*Urease: Positive (can be within minutes)
*Motility: Non-Motile
(use semi-solid media rather than wet mount; 2,3,5-triphenyltetrazolium chloride indicator)
X and V Factors (optional): No Requirement
Satellite growth (optional): Negative

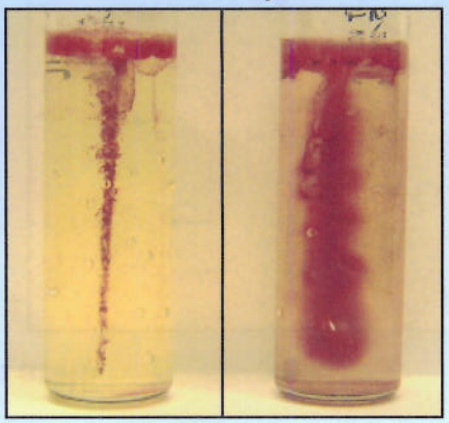
*Oxidase, Urease, and Motility: Appearances of test results are not agent-specific. Photos represent typical reactions

No	Yes
Continue laboratory identification procedures	Contact Alaska State Public Health Laboratory 907-334-2100 (business hrs) or 855-222-9918 (PHL On-call)

Urease

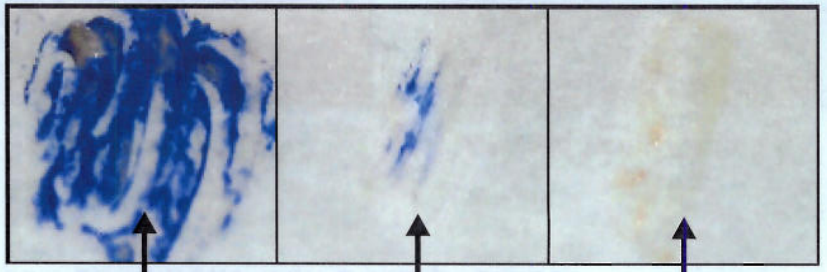
Negative Positive

Motility



Non-Motile Motile

Oxidase



Positive Weak Positive Negative

Burkholderia mallei

Colony Morphology

- Pinpoint to small grey colonies at 24 h that may become smooth, grey, and translucent at 48 h on Blood Agar (BA)
- No growth or colorless to light pink colonies on MacConkey (MAC) agar

Gram Stain

- Faintly staining gram-negative rods or slightly curved coccobacilli (1-3 µm x 0.3 µm)
- May be arranged singly, end to end pairs, in parallel bundles, or letter form, irregularly arranged

Biochemical/Test Reactions

- Oxidase: Negative or variable
- TSI: No change
- Motility: Non-Motile

Additional Information

- May be misidentified as: Other nonfermenting gram-negative bacilli by automated ID systems
- Infectious Dose: 10 colony forming units
- Biosafety Level 2 agent
  (Use BSL3 for large or high titer volume culture)
- Transmission: Inhalation, wound contamination, direct contact with infected animals
- Contagious: No infections have been reported

Acceptable Specimen Types

- Abscess material, tissue aspirate, tissue biopsy
- Whole blood: 5-10 ml blood in EDTA, and/or
  Inoculated blood culture bottle
- Urine (≥ 5 ml)

‡ BA-24 h

‡ BA-48 h

‡ Gram stain

Burkholderia pseudomallei

Colony Morphology

- Smooth, creamy-white colonies at 24 h that may become dry or mucoid at 48 h on Blood Agar (BA)
- Heavy growth at 42°C
- Pink or colorless colonies after 24 h that may remain pink or turn colorless at 48 h on MacConkey (MAC) agar

Gram Stain

- Gram-negative rods 1-3 µm x 0.3 µm
- Possible bipolar staining
- May appear as long thin bundles or irregularly arranged

Biochemical/Test Reactions

- Oxidase: Positive
- TSI: No change or slight oxidation (picture on back of card)
- Motility: Motile

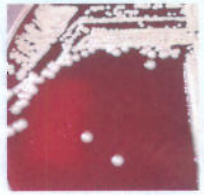
Additional Information

- May be misidentified as: *Chromobacterium violaceum* and other nonfermenting gram-negative bacilli by automated ID systems
- Infectious Dose: 10 colony forming units
- Biosafety Level 2 agent
  (Use BSL3 for large or high titer volume culture)
- Transmission: Inhalation, wound contamination

Acceptable Specimen Types

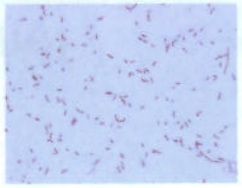
- Abscess material, tissue aspirate, tissue biopsy
- Whole blood: 5-10 ml blood in EDTA, and/or
  Inoculated blood culture bottle
- Urine (≥ 5 ml)

BA-24 h



BA-48 h

Gram stain



Sentinel Laboratory Rule-Out

Burkholderia mallei

Small grey-white colonies on BA (48 h)
Colorless colonies on MAC (72 h)

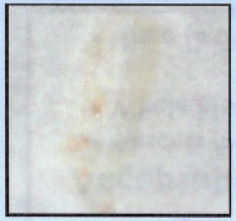
Gram-negative coccobacilli

Perform all additional work in a certified Class II Biosafety Cabinet

*Oxidase: Negative or Variable
TSI: No Change
*Motility: Non-Motile
(use semi-solid media rather than wet mount; 2,3,5-triphenyltetrazolium chloride indicator)

*Oxidase and Motility: Appearances of test results are not agent-specific. Photos represent typical reactions

Oxidase



Negative

TSI

No Change

Motility

Non-Motile

Burkholderia pseudomallei

Creamy-white colonies on BA
May become dry or mucoid on BA (48 h)
Pink to colorless colonies on MAC (24 h)

Gram-negative, bipolar staining rods

Perform all additional work in a certified Class II Biosafety Cabinet

*Oxidase: Positive
TSI: No Change or Slight Oxidation
*Motility: Motile
(use semi-solid media rather than wet mount; 2,3,5-triphenyltetrazolium chloride indicator)

*Oxidase and Motility: Appearances of test results are not agent-specific. Photos represent typical reactions

Oxidase

Positive

TSI

Slight Oxidation

Motility

Motile

Yes

No

Continue laboratory identification procedures

Contact Alaska State Public Health Laboratory 907-334-2100 (business hrs) or 855-222-9918 (PHL On-call)

No

Continue laboratory identification procedures

Francisella tularensis

Colony Morphology

- Aerobic, fastidious, requires cysteine for growth
- Grows poorly on Blood Agar (BA)
- Chocolate Agar (CA): tiny, grey-white, opaque colonies, 1-2 mm ≥48hr
- Cysteine Heart Agar (CHA): greenish-blue colonies, 2-4 mm ≥48h
- Colonies are butyrous and smooth

Gram Stain

- Tiny, 0.2–0.7 μm pleomorphic, poorly stained gram-negative coccobacilli
- Mostly single cells

Biochemical/Test Reactions

- Oxidase: Negative
- Catalase: Weak positive
- Urease: Negative

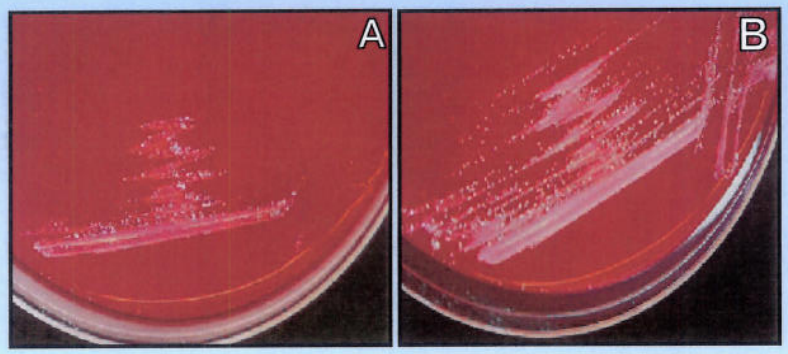
Additional Information

- Can be misidentified as: *Haemophilus influenzae*, *Actinobacillus* spp. by automated ID systems
- Infective Dose: 10 colony forming units
- Biosafety Level 3 agent (once *Francisella tularensis* is suspected, work should only be done in a certified Class II Biosafety Cabinet)
- Transmission: Inhalation, insect bite, contact with tissues or bodily fluids of infected animals
- Contagious: No

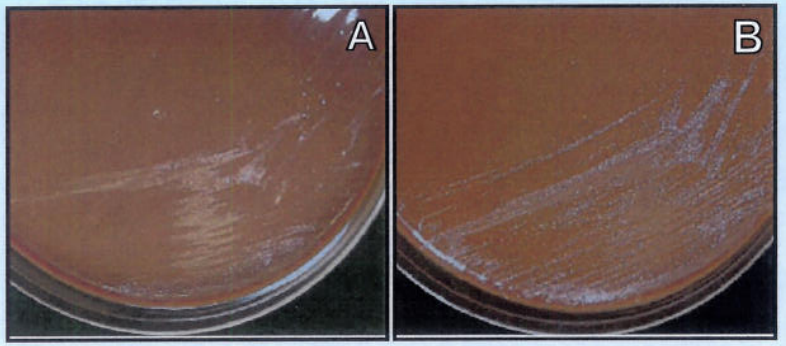
Acceptable Specimen Types

- Tissue biopsy
- Whole blood: 5-10 ml blood in EDTA, and/or Inoculated blood culture bottle
- Swab of lesion in transport media

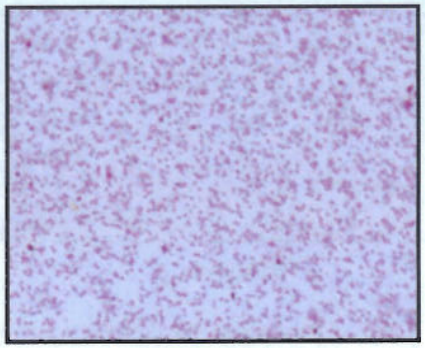
Tularemia is a commonly acquired laboratory infection; all work on suspect *F. tularensis* cultures should be performed at minimum under BSL2 conditions with BSL3 practices.



Growth on BA (A) 48 h, (B) 72 h



Growth on CA (A) 48 h, (B) 72 h



Gram stain

Sentinel Laboratory Rule-Out of *Francisella tularensis*

Little to no growth on BA >48 h

↓

Small, grey-white opaque colonies on CA after ≥48 h at 35/37°C

↓

Tiny, pleomorphic, faintly stained, gram-negative coccobacilli (red, round, and random)

↓

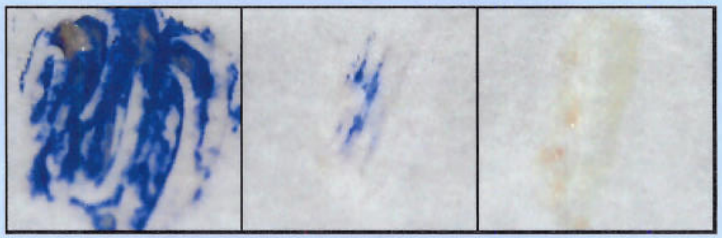
Perform all additional work in a certified Class II Biosafety Cabinet

*Oxidase: Negative
*Catalase: Weak positive
*Urease: Negative

*Oxidase, Catalase, and Urease: Appearances of test results are not agent-specific. Photos represent typical reactions

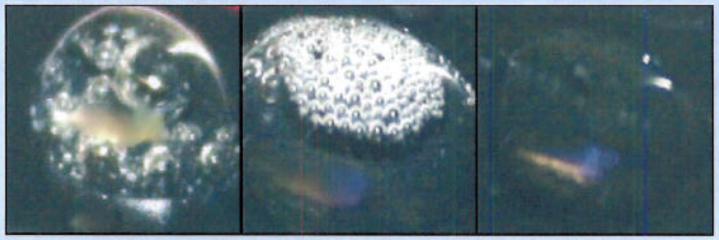
No	Yes
Continue laboratory identification procedures	Contact Alaska State Public Health Laboratory 907-334-2100 (business hrs) or 855-222-9918 (PHL On-call)

Oxidase



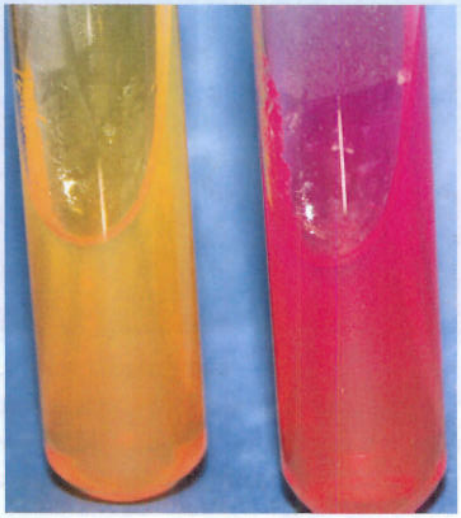
Positive | Weak Positive | Negative

Catalase



Positive | Weak Positive | Negative

Urease



Negative | Positive

Yersinia pestis

Colony Morphology

- Grey-white translucent colonies in 24 h on Blood Agar (BA) and Chocolate Agar (CA) at ambient and 35/37°C (growth faster at 28°C)
- 1-2 mm colonies in 48 h that may be opaque and yellow
- "Fried egg" or "hammered copper" appearance on BA in older cultures
- Clear or white colonies on MacConkey (MAC) agar at 48 h

Gram Stain

- Gram-negative rods (0.5 - 0.8 x 1- 3 μm)
- Giemsa stain: Bipolar staining
- Gram stain: Bipolar staining may be poor

Biochemical/Test Reactions

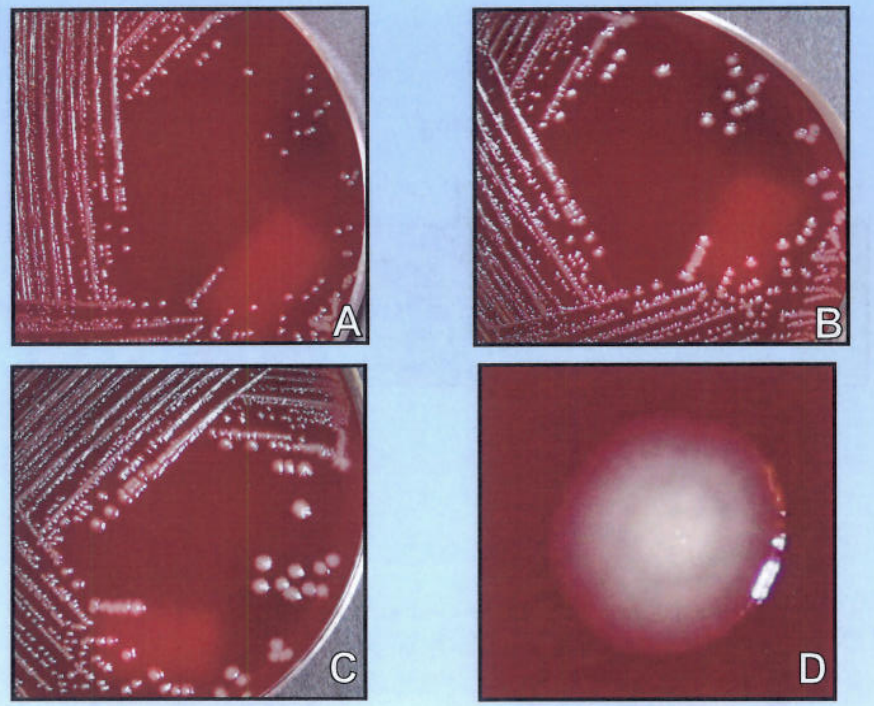
- Flocculent or "stalactite" growth in broth (35/37°C)
- Non-Motile at 25°C - 35°C
- Catalase: Positive
- Oxidase, Urease, and Indole: Negative

Additional Information

- Can be misidentified as: *Shigella* spp., $H_2S(-)$ *Salmonella* spp., *Acinetobacter* spp., or *Yersinia pseudotuberculosis* by automated ID systems
- Biosafety Level 2 agent (Use BSL3 for large volume or high titer culture)
- Infective Dose: <100 colony forming units
- Transmission: Inhalation, flea bite
- Contagious: Yes (only pneumonic form)

Acceptable Specimen Types

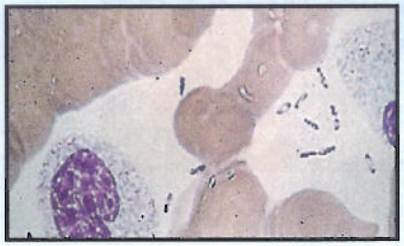
- Bronchial wash/tracheal aspirate (≥ 1 ml)
- Whole blood: 5-10 ml blood in EDTA, and/or Inoculated blood culture bottle
- Aspirate or biopsy of liver, spleen, bone marrow, lung, or bubo



Yersinia pestis growth on BA at (A) 48 h, (B) 72 h, (C) 96 h, (D) 96 h "Fried egg"

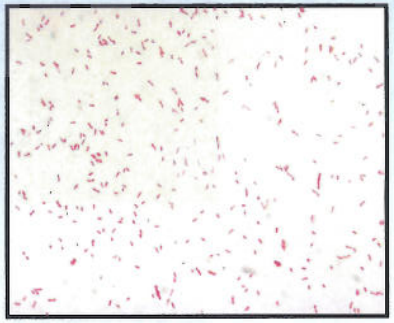
Giemsa stain (100X from blood culture): note *Y. pestis* bipolar appearance

‡



Gram stain: note that bipolar staining may be poor

‡



Sentinel Laboratory Rule-Out of *Yersinia pestis*

Grey-white translucent, non-hemolytic colonies on BA or CA (24 h)
Yellow and opaque (48 h) raised, irregular, "fried egg" or "hammered copper" shiny appearance (48-72 h)
Small, non-lactose fermenters on MAC (≥ 48 h)
Slow growing at 35/37°C (prefers 28°C)
Growth in broth culture at 48 h: Clumped, flocculent, or stalactite

↓

Gram-negative rods
Bipolar staining (resembling closed safety pin) may be evident with Gram stain but more apparent with Giemsa or Wayson stain

↓

Perform all additional work in a certified Class II Biosafety Cabinet

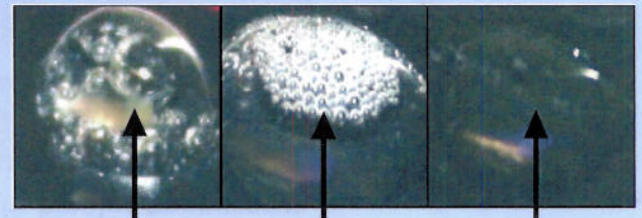
*Catalase: Positive
*Motility: Non-Motile at 25° C and 35/37° C
(use semi-solid media rather than wet mount; 2,3,5-triphenyltetrazolium chloride indicator)
*Urease: Negative
*Oxidase: Negative
Indole: Negative

*Catalase, Motility, Urease, and Oxidase: Appearances of test results are not agent-specific. Photos represent typical reactions

No → Continue laboratory identification procedures

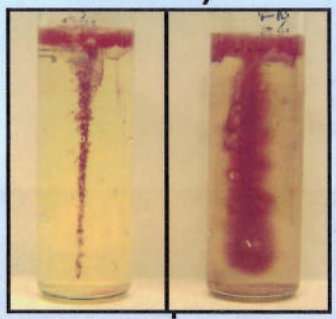
Yes → Contact Alaska State Public Health Laboratory 907-334-2100 (business hrs) or 855-222-9918 (PHL On-call)

Catalase



Positive Weak Positive Negative

Motility



Non-Motile Motile

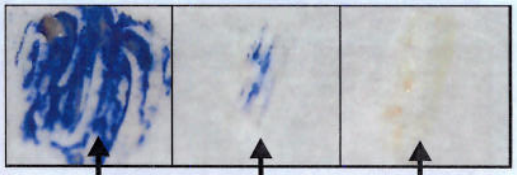
48 h Broth

Y.pestis *Y.pseudo.*

Urease

Negative Positive

Oxidase



Positive Weak Positive Negative

6/06

Smallpox: Differential Diagnosis

General Information

Transmission

- Direct contact with lesions, body fluids or contaminated bedding and towels
- Droplet inhalation (within 6 feet)

Contagious: YES

- With onset of fever and rash
- Once all scabs have fallen off, the patient is no longer contagious

Incubation Period: 12-14 Days

Initial Symptoms

- High fever (101 – 104°F), malaise, head and body aches

Rash Characteristics

- Begins on face which spreads to arms, legs, hands and feet. All lesions on any one part of the body are in the same stage of development
- 3rd day: Rash becomes raised bumps
- 4th day: Become fluid-filled with a depressed center (bellybutton-like)

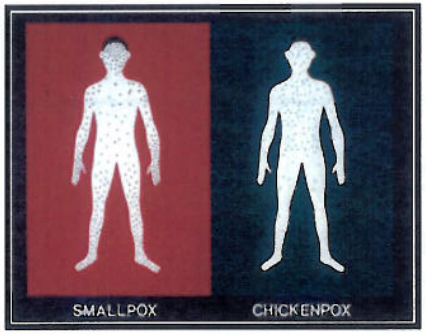
Can be Misidentified as Chickenpox

‡

Smallpox (upper photos): Lesions present on palms of hands and soles of feet

Chickenpox (lower photos): Lesions rarely seen on palms of hands and soles of feet

‡



Lesion Distribution

Specimen Collection Procedures

ONLY vaccinated individuals should perform collection of suspect smallpox specimens

Personal Protective Equipment (PPE): Gloves and N95 Respirator

Specimens to be Collected

DO NOT COLLECT ANY SPECIMENS

Contact the State of Alaska, Division of Public Health, Section of Epidemiology IMMEDIATELY 800-478-0084

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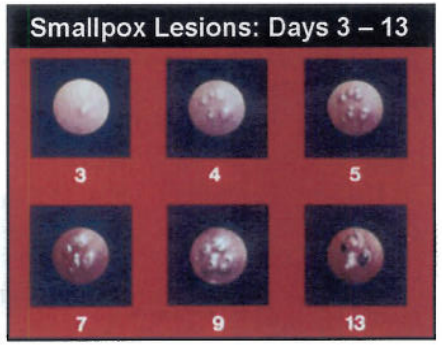
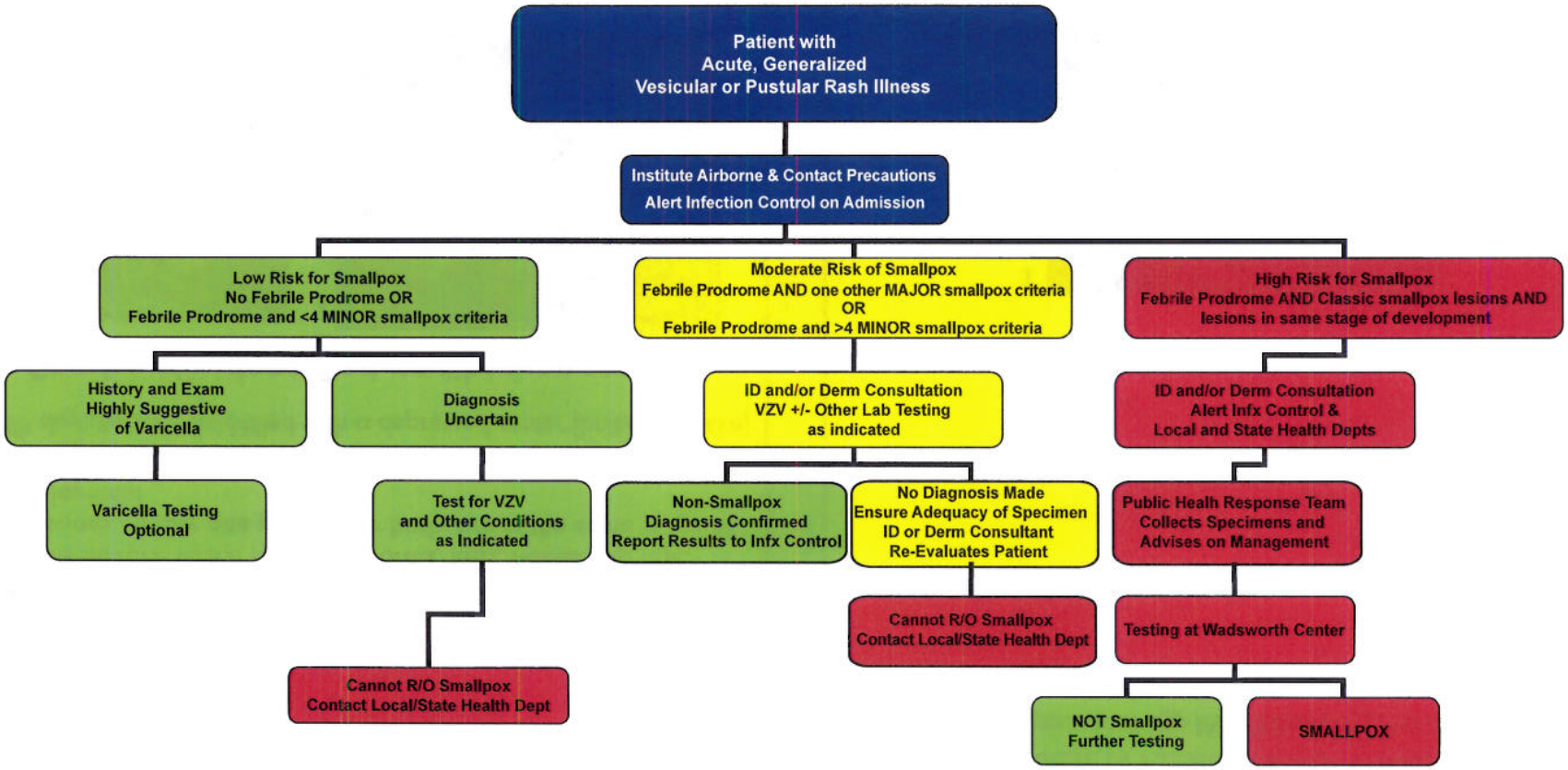


Patient Evaluation for Smallpox Infections



Major Smallpox Criteria

Febrile prodrome: Occurring 1 - 4 days before rash onset, Fever >101ºF AND at least one of the following: prostration, headache, backache, chills, vomiting or severe abdominal pain

Classic smallpox lesions: Deep-seated, firm/hard vesicles or pustules, may be umbilicated or confluent

Lesions in same stage of development: On any one part of the body all lesions are in the same stage of development (i.e.: all vesicles or all pustules)

Minor Smallpox Criteria

Centrifugal distribution with greatest concentration of lesions on face and distal extremities

First lesions on the oral mucosa, face or forearms

Slow evolution of lesions: macules to papules to pustules

Lesions on the palms and soles

Patient appears toxic or moribund

	SMALLPOX	CHICKENPOX
FEVER	2 to 4 days before rash	At time of rash
RASH ·Appearance ·Developement	 Pocks in same stage Slow	 Pocks in several stages Rapid
·Distribution	More pocks on arms and legs	More pocks on body
·On palms and soles	Usually present	Usually absent
DEATH	Usually 1 in 10 die	Very uncommon

Source: Centers for Disease Control and Prevention (CDC)

Botulinum Toxin

Source
- *Clostridium botulinum*, a large gram-positive, spore-forming, anaerobic bacillus

Characteristics
- Grows anaerobically on Blood Agar and egg yolk plates

Pathogenesis
- Toxin enters nerve terminals and blocks release of acetylcholine, blocking neuro-transmission and resulting in muscle paralysis

Toxicity
- Most lethal of all toxic natural substances
- Groups A, B, E (rarely F) cause illness in humans

Symptoms
- 24-36 h (up to 3 d for wound botulism)
- Progressive skeletal muscle weakness
- Symmetrical descending flaccid paralysis
- Can be confused with stroke, Guillain-Barre syndrome or myasthenia gravis

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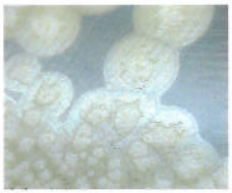
Gram stain

‡ 

Lipase on egg yolk plates

Ricin Toxin

Source
- *Ricinus communis* seeds commonly called castor beans

Characteristics
- Toxin can be disseminated in the form of a liquid, powder or mist

Pathogenesis
- A-chain inactivates ribosomes, interrupting protein synthesis
- B-chain binds to carbohydrate receptors on the cell surface and allows toxin complex to enter cell

Toxicity
- Highly toxic by inhalation, ingestion and injection
- Less toxic by ingestion due to digestive activity and poor absorption
- Low dermal toxicity

Symptoms
- 18-24 h post exposure
- Fever, cough, chest tightness, dyspnea, cyanosis, gastroenteritis and necrosis; death in ~72 h

Ricin plant

Castor beans

Staph Enterotoxin B

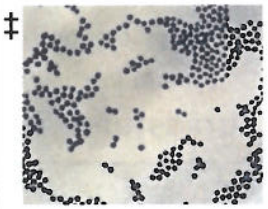
Source
- *Staphylococcus aureus*, a gram-positive cocci

Characteristics
- Appears as grape-like clusters on Gram stain or as small off-white colonies on Blood Agar
- Toxin-producing and non-toxigenic strains of *S. aureus* will appear morphologically identical

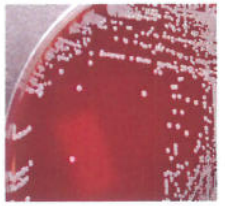
Pathogenesis
- *Staphylococcus* Enterotoxin B (SEB) is a superantigen. Toxin binds to human class II MHC molecules causing cytokine release and system-wide inflammation

Toxicity
- Toxic by inhalation or ingestion

Symptoms
- 4-10 h post-ingestion, 3-12 h post-inhalation
- Flu-like symptoms, fever, chills, headache, myalgia
- Nausea, vomiting, and diarrhea
- Nonproductive cough, chest pain, and dyspnea
- SEB can cause toxic shock syndrome

‡ 

S. aureus Gram stain



Growth on Blood Agar

Botulinum Toxin

Transmission
- Aerosol release
- Food contamination
- Wound contamination
- Toxin not transmitted person to person

Clinical Specimens
- Serum: > 5 ml (10 ml preferred)
- Feces and gastric contents: >10 g
- Clinical swab specimens: Place swabs in an anaerobic transport media
- Autopsy: contents from small and large intestines
- Storage: Refrigerate, preferably in plastic containers

Environmental Samples
- All environmental samples: 100 ml or 2 g
- Food, drinks: Send entire item
- Storage: Refrigerate, preferably in plastic containers

Detection
- Mouse neutralization assay
- Enzyme-linked immunosorbent assays (ELISA)
- PCR

Ricin Toxin

Transmission
- Aerosol release
- Food contamination
- Injection
- Toxin not transmitted person to person

Clinical Specimens
- Serum to test for circulating antibody (>5ml) (Tested at CDC)
- Urine: >5 ml (10 ml preferred)
- Storage: Room temperature, in plastic containers (do not use glass)
- Note: Ricin toxin can be denatured by freezing or excess heat

Environmental Samples
- All environmental samples: 100 ml or 2 g
- Food, drinks: Send entire item
- Storage: Room temperature, in plastic containers (do not use glass)
- Note: Ricin toxin can be denatured by freezing or excess heat

Detection
- Time-resolved fluorescence (TRF)
- PCR
- Ricinine detection by chemical analysis

Staph Enterotoxin B

Transmission
- Aerosol release
- Food contamination
- Toxin not transmitted person to person

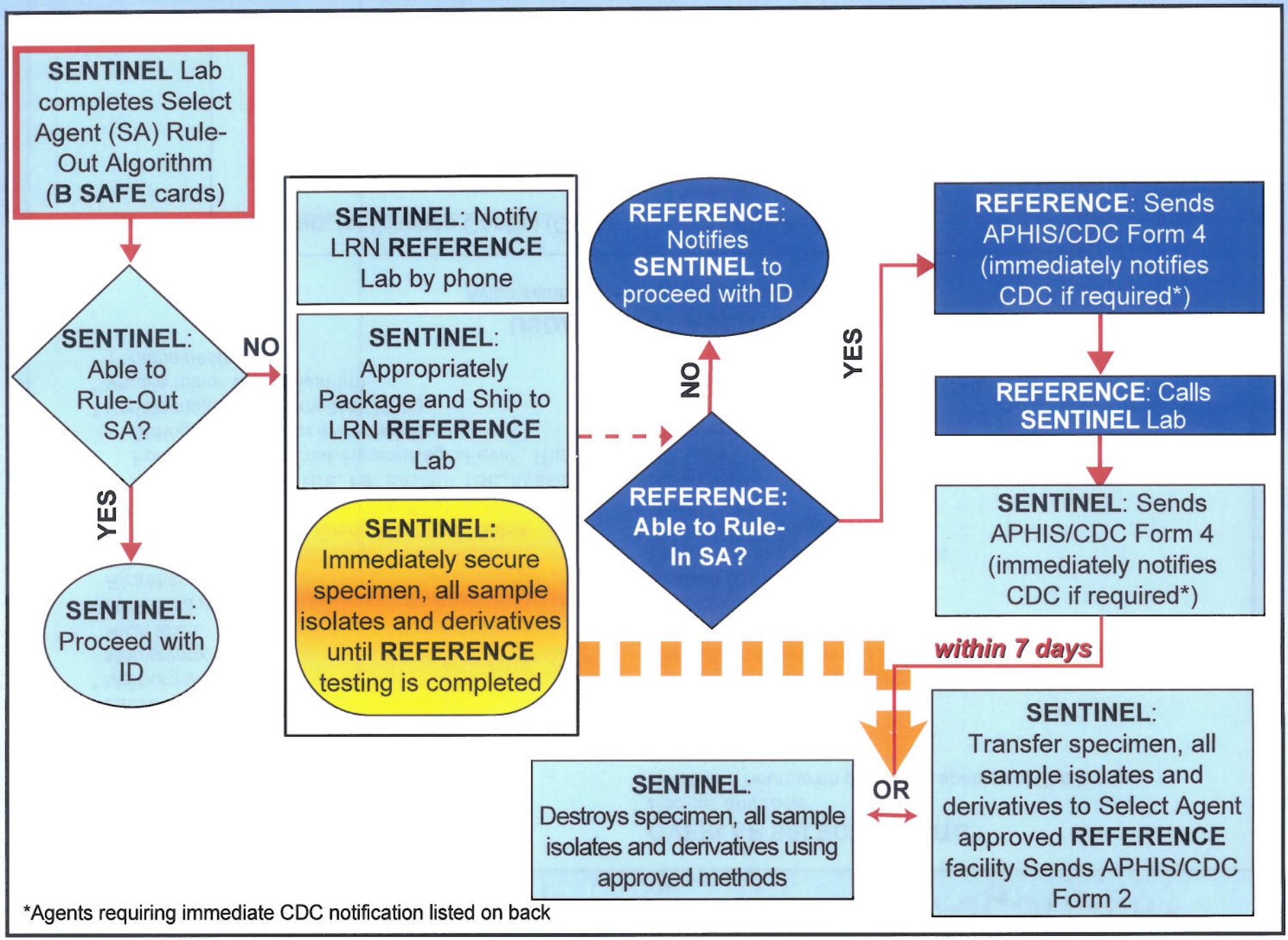
Clinical Specimens
- 5-10 ml blood in EDTA
- Urine: > 5 ml (10 ml preferred)
- Respiratory secretions, or nasal swabs
- Bacterial isolates
- Storage: Refrigerate

Environmental Samples
- All environmental samples: 100 ml or 2 g
- Food, drinks: Send entire item
- Storage: Refrigerate

Detection
- ~~Time-resolved fluorescence (TRF)~~
- Gel diffusion assay
- Latex agglutination test
- ~~PCR~~

If any of these toxins are suspected immediately contact the Alaska State Public Health Laboratory to refer the specimen/sample 907-334-2100 (business hours) or 855-222-9918 (PHL On-Call)

Select Agent Response Algorithm



Select Agents Requiring Response Algorithm◆

HHS SELECT AGENTS

Cercopithecine herpesvirus 1 (Herpes B virus)
Coccidioides posadasii
Crimean-Congo haemorrhagic fever virus
* Ebola virus
* Lassa fever virus
* Marburg virus
Monkeypox virus
Ricin toxin
Rickettsia prowazekii
Rickettsia rickettsii
* South American Haemorrhagic Fever viruses
  Flexal , Guanarito , Junin , Machupo , Sabia
Tick-borne encephalitis complex (flavi) viruses
  Central European TBE, Far Eastern TBE, Kyasanur Forest disease , Omsk Hemorrhagic Fever , Russian Spring and Summer encephalitis
* Variola major virus (Smallpox virus)
* Variola minor virus (Alastrim)
* *Yersinia pestis*

OVERLAP SELECT AGENTS

* *Bacillus anthracis*
* Botulinum neurotoxin producing species of *Clostridium*
Brucella abortus
* *Brucella melitensis*
Brucella suis
Burkholderia mallei (formerly *Pseudomonas mallei*)
Burkholderia pseudomallei (formerly *Pseudomonas pseudomallei*)
Clostridium perfringens epsilon toxin
Coccidioides immitis
Coxiella burnetii
Eastern Equine Encephalitis virus
* *Francisella tularensis*
* Hendra virus
* Nipah virus
* Rift Valley fever virus
Shigatoxin
Staphylococcal enterotoxins
* Venezuelan Equine Encephalitis virus

USDA SELECT AGENTS

Avian influenza virus (highly pathogenic)

Centers for Disease Control (CDC): 404-498-2255

State LRN Reference Lab: 907-334-2100

Local LRN Reference Lab:

Website for APHIS/CDC Forms: http://www.cdc.gov/od/sap/downloads2.htm

◆For a complete listing, refer to http://www.cdc.gov/od/sap/docs/salist.pdf

*Agents requiring immediate CDC notification