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CULTURE-BASED ID FOR AGENTS OF BIOTERRORISM IN THE ERA OF MALDI- TOF MS

The microbiologist's way...

When a culture comes along

You must sniff it

Before the lid is off too long

You must sniff it

Your nose just can't be wrong

You must sniff it



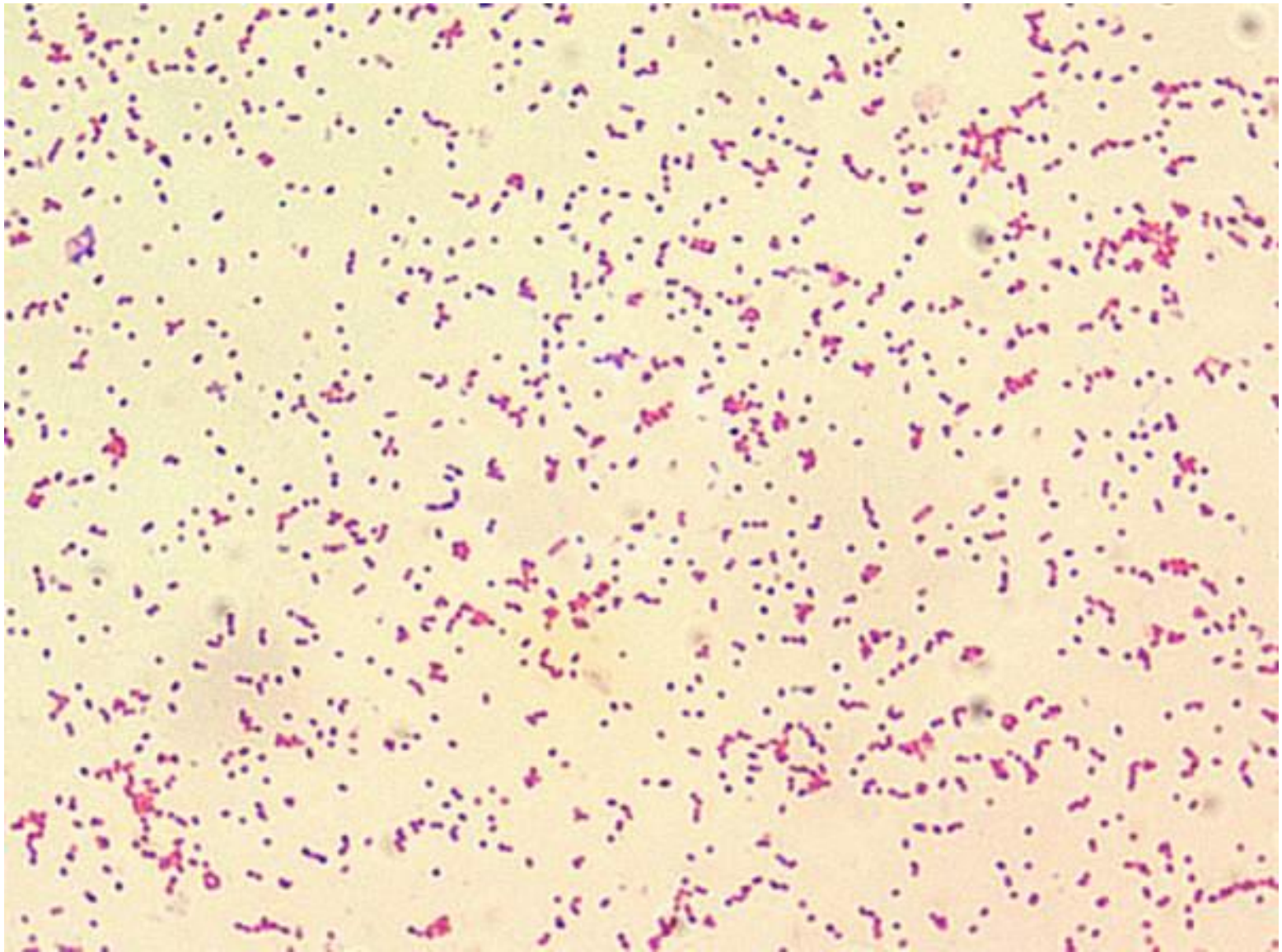
Case 1

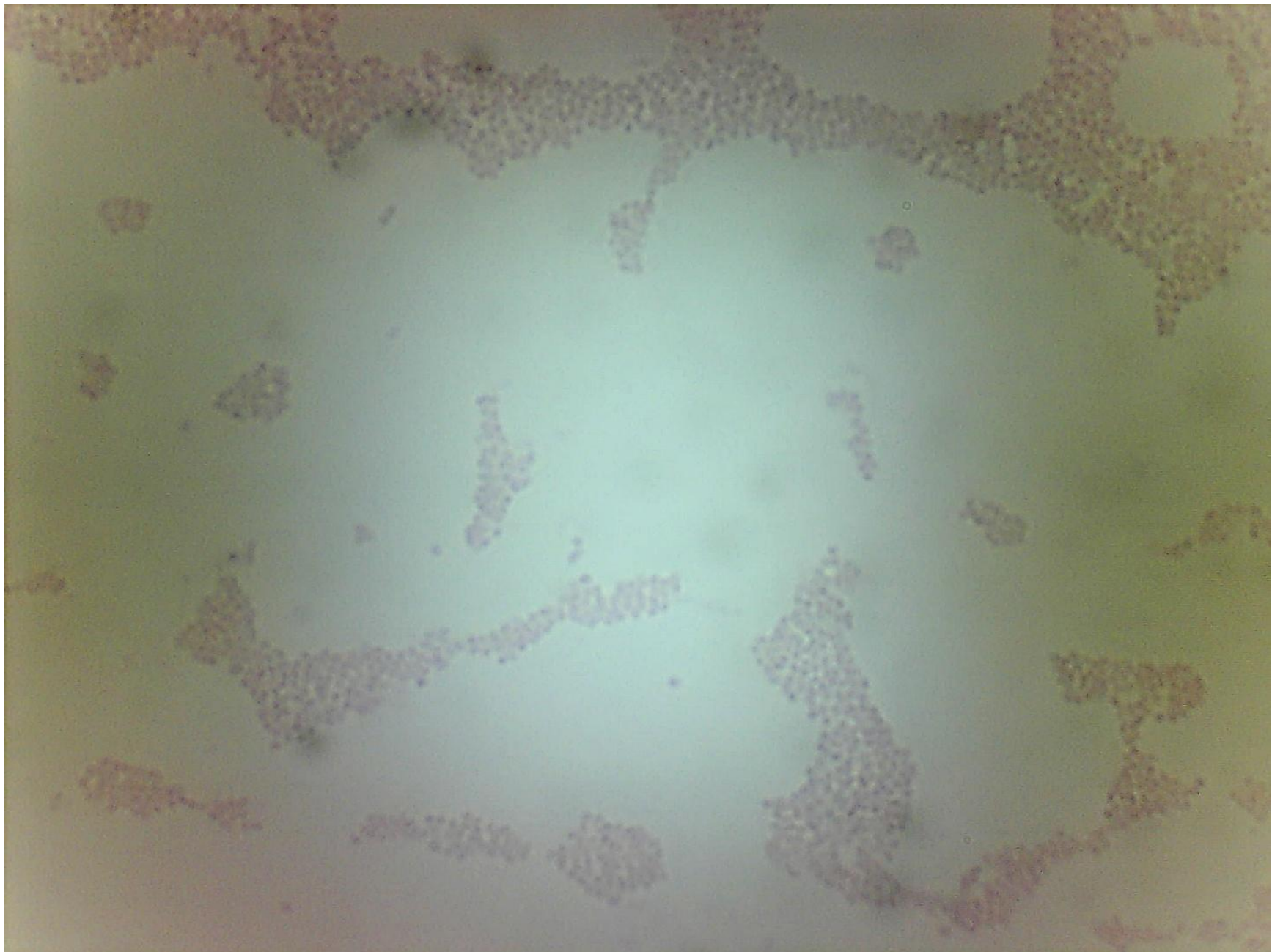
- 47 yo male
- No significant past medical history
- 4 days redness and swelling on the right lower neck and sternoclavicular region
- Right sided shoulder pain 2 months prior
- No fevers, chills, night sweats
- Travel to Mexico 4 months ago, no farm contact
- Took a course of TMP-SMZ and cephalexin 1 month prior

Microbiology Work-Up

- 10 cc purulent neck mass fluid sent to micro for culture
- After 5 days: Growth of 1 colony on anaerobic culture— aerobic chocolate plate
- After 10 days: Growth on aerobic plates







Microbiology Work-Up

- Gram negative cocobacilli—faintly staining
- Rapid urease + in <30 min
- Oxidase positive
- Subculture grew on SBA, CHOC, but not on MAC in 24 hrs
- MALDI-TOF MS for ID
- Sent to IDPH for further identification



Final ID

- Final ID= *Brucella melitensis*
 - PCR and additional confirmatory testing
- Patient therapy changed to doxycycline and rifampin
- Hold plates 14 days to “r/o Brucella” on original requisition
- Exposure investigation initiated with Occupational Health
- Brucella serology was negative

Brucella

- *B. abortus*, *B. melitensis*, *B. suis*, *B. canis*
- Cause of brucellosis
- One of the world's most common zoonoses
- Common infection route through eating or drinking unpasteurized/raw dairy products or direct contact with livestock
- Symptoms appear 1 to 3 weeks after exposure and include fever, sweats, malaise, headache, and back pain—can be sub-acute
- Chronic untreated—can lead to abscesses in the liver, spleen, heart valves, brain, or bone; osteoarticular complications;
- Mortality rate is low (1 to 2%)—endocarditis

Case 2

- 65 yo male
- Dysuria and urinary frequency and no response to 4 days of nitrofurantoin
- Travel to rural Thailand for about 1 month prior to presentation
- Suffered a myocardial infarction, hospitalized for 7 days prior to returning to the US.

Microbiology Work Up

- Urine specimen received in the lab for culture
- After 24 h
 - Growth of small colonies on SBA
 - No growth evident on MAC
 - Initially reported as 1,900 CFU/ml of Gram-positive flora.
- At 48 h
 - Pure culture of gray, medium-sized colonies was growing equally well on SBA and MAC.
 - Oxidase-positive GNR, non-hemolytic, non-pigmented
- MALDI-TOF MS for ID
- Sent to WSPHL for confirmation

Final ID

- Final ID= *Burkholderia pseudomallei*
 - Species specific PCR assay
- No evidence of wrinkled colonies during initial evaluation
- Exposure investigation initiated with Occupational Health

Burkholderia pseudomallei

- Cause of melioidosis
- Develops after bacterial inoculation or inhalation, often in relation to occupational exposure
- Most commonly reported in northeast Thailand and northern Australia but also occurs in other parts of Asia, South America, and the Caribbean
- Independent risk factors: diabetes mellitus, excess alcohol consumption, chronic renal failure, and chronic lung disease
- Symptoms may be exhibited many years after exposure, commonly in association with an alteration in immune status
- Spectrum from rapidly life-threatening sepsis to chronic low-grade infection
- Common presentation is sepsis associated with bacterial dissemination; concomitant pneumonia and liver and splenic abscesses
- May also occur in bone, joints, skin, soft tissue, or the prostate
- Mimic those of many other diseases like TB
- Mortality rate is 50% in northeast Thailand (35% in children) and 19% in Australia

Cases in the US

- Three primary sources of human brucellosis in the United States
- 80-140 cases reported annually
 - Consumption of unpasteurized dairy products consumed in or imported from a country where brucellosis is endemic
 - Contact with meat or tissues of infected wild animals
 - Laboratory exposures to *Brucella* isolates
 - Most frequently reported lab associated bacterial infection
- Melioidosis is rare in the United States
 - 0–5 cases are reported annually
 - Most cases occur in travelers returning from disease-endemic areas

Why Are They Select Agents?

- Readily aerosolized
- Infective dose of 10 to 100 organisms
- Patients often present with nonspecific signs and symptoms so clinicians are unaware
- Laboratory workers may not be familiar with the organism
- Poor diagnostics

High Risk Exposure

- Any individual who worked with a *B. pseudomallei* isolate and
 - The presence of any predisposing condition without proper personal protective equipment (PPE): diabetes mellitus; chronic liver or kidney disease; alcohol abuse; long-term steroid use; hematologic malignancy; neutropenia or neutrophil dysfunction; chronic lung disease (including cystic fibrosis); thalassemia; any other form of immunosuppression
 - Needlestick or other penetrating injury with implement contaminated with *B. pseudomallei*
 - Splash event leading to contamination of mouth or eyes
 - Generation of aerosol outside biologic safety cabinet (e.g., sonication, centrifuge incident)
 - Bite or scratch by experimental animal infected with *B. pseudomallei*

Low Risk Exposure

- Any individual who worked with a *B. pseudomallei* isolate and
 - Inadvertent opening of the lid of an agar plate growing *B. pseudomallei* outside a biologic safety cabinet
 - Inadvertent sniffing of agar plate growing *B. pseudomallei* in the absence of contact between worker and bacterium
 - Splash event leading to visible contact of *B. pseudomallei* with gloved hand or protected body, in the absence of any evidence of aerosol
 - Spillage of small volume of liquid culture (<1mL) within a functioning biologic safety cabinet
 - Contamination of intact skin with culture

Post-Exposure Follow-up

- Serologic testing on all workers exposed at 1, 2, 4, and 6 weeks post-exposure in addition to baseline
- Active surveillance for febrile illness among all workers exposed for 3 weeks
- The appropriate choice of antimicrobial agent and optimal duration of therapy are unknown
- 3 weeks prophylaxis should be offered to both high and low risk groups
 - Trimethoprim-sulfamethoxazole (160 mg/800 mg) twice daily
 - Doxycycline (100 mg) twice daily or Amoxicillin–clavulanic acid (500 mg/125 mg) three times daily

High Risk Exposure

- Any individual who worked with a *Brucella* isolate
 - Sniffed or opened culture plate
 - Mouth pipetted specimen material
 - Worked in Class II biosafety cabinet or on open bench without using BSL-3 precautions
- 5 foot radius of work with *Brucella* even if it DID NOT involve widespread aerosol generating procedures (vortexing, sonicating, etc)

Post-Exposure Follow-up

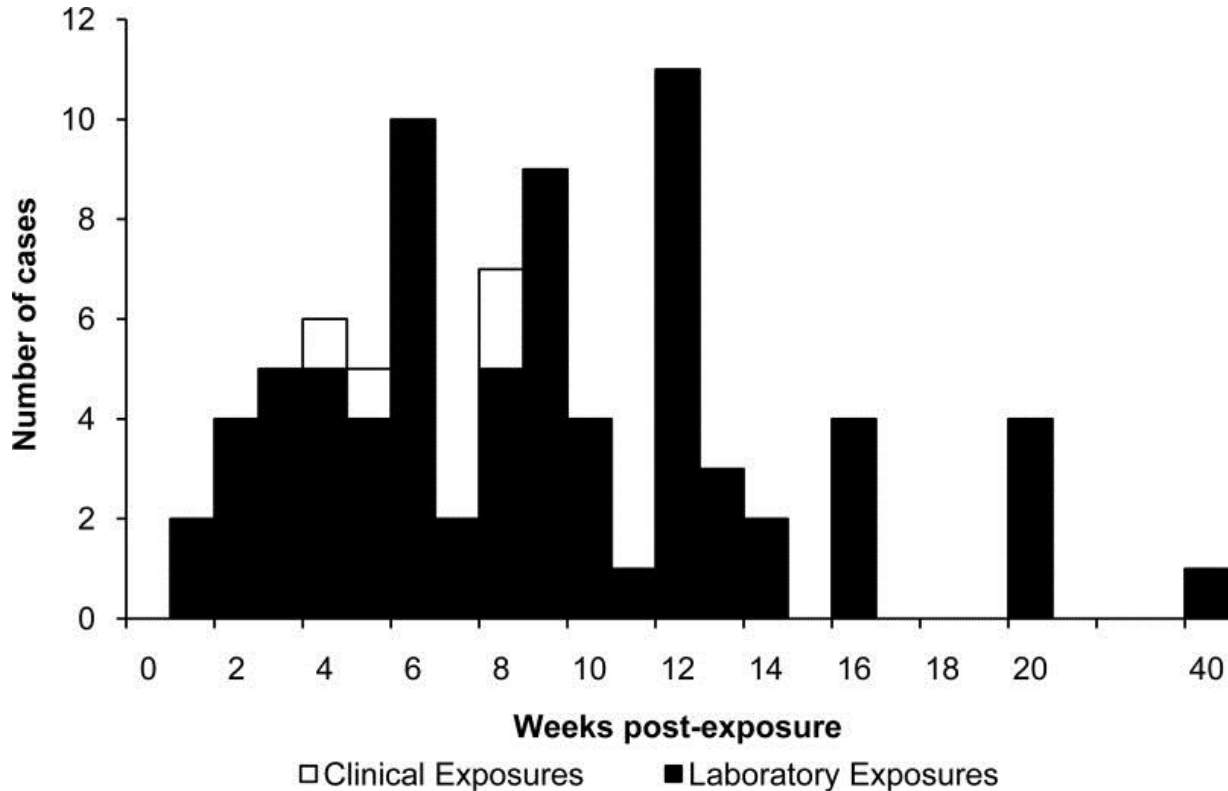
- Serologic testing on all workers exposed at 6, 12, 18, and 24 weeks post-exposure in addition to baseline
- Active surveillance for febrile illness among all workers exposed for 6 months
- 3 weeks prophylaxis for high risk group
 - doxycycline 100 mg twice daily and rifampin 600 mg once daily
 - Trimethoprim-sulfamethoxazole (160 mg/800 mg) once daily

Lab Exposure

- Of 167 exposed workers, 43% developed laboratory acquired brucellosis (LAB)
- About ½ of reported LAB cases are in microbiologists
- Frequently reported symptoms included: fever (71%), arthralgia (58%), headache (58%), fatigue (56%), sweats (45%), malaise (44%), and myalgia (40%)
- Cases do occur despite constant use of a BSC with no recognized lapses in biosafety

Lab Exposure

- Individual incubation periods were identified or calculated for 80 LAB cases
 - The median time to symptom onset was 8 weeks
 - The median time to seroconversion was 11 weeks (reported for 12 workers)



Time course of disease onset following occupational exposure to *Brucella* spp. ($n = 80$).

Table 2. Microbiology biosafety

Agent	BSL for:		Specimen exposure risk	Recommended precautions for level A laboratories	
	Specimen handling	Culture handling		BSL-2	BSL-3
<i>B. anthracis</i>	2	2	Blood, skin lesion exudates, CSF, pleural fluid, sputum, and rarely urine and feces	Activities involving clinical material collection and diagnostic quantities of infectious cultures	Activities with high potential for aerosol or droplet production
<i>Brucella</i> spp. ^a	2	3	Blood, bone marrow, CSF, tissue, semen, and occasionally urine	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures
<i>C. botulinum</i> ^b	2	2	Toxin may be present in food specimens, clinical material (serum, gastric, and feces), and environmental samples (soil, surface water). <i>Toxin is extremely poisonous!</i>	Activities with materials known to contain or potentially containing toxin must be handled in a BSC (class II) with a laboratory coat, disposable surgical gloves, and a face shield (as needed).	Activities with high potential for aerosol or droplet production
<i>F. tularensis</i> ^c	2	3	Skin lesion exudates, respiratory secretions, CSF, blood, and urine; tissues from infected animals and fluids from infected arthropods	Activities limited to collection, transport, and plating of clinical material	All activities involving manipulations of cultures
<i>Y. pestis</i> ^d	2	2	Bubo fluid, blood, sputum, CSF, feces, and urine	Activities involving clinical material collection and diagnostic quantities of infectious cultures	Activities with high potential for aerosol or droplet production

a: Laboratory-acquired brucellosis has occurred by sniffing cultures; aerosols generated by centrifugation; mouth pipetting; accidental parenteral inoculations; sprays into eyes, nose, and mouth; and finally by direct contact with clinical specimens

c: Laboratory-acquired tularemia has been more commonly associated with cultures than with clinical materials or animals. Direct skin or mucous membrane contact with cultures, parenteral inoculation, ingestion, and aerosol exposure have resulted in infection.

Post Exposure Follow Up

- Employees were categorized into high- and low-risk exposure groups.
- Regardless of the exposure risk, all 21 employees were offered antibiotic prophylaxis in *B. pseudomallei* case
- 7 employees offered prophylaxis in *Brucella* case
- Serology testing was performed on exposed individuals, and no employees seroconverted.

**BUT WHAT SHOULD WE BE DOING
NOW?**

Resources

Gilchrist, M. J. R., W. P. McKinney, J. M. Miller, and J. W. Snyder. 2000. **Cumitech 33, Laboratory safety, management, and diagnosis of biological agents associated with bioterrorism.** Coordinating ed., J. W. Snyder. ASM Press, Washington, D.C.

Biosafety in Microbiological and Biomedical Laboratories (BMBL)

www.cdc.gov/od/ohs/biosfty/bmbl4/bmbl4toc.htm

Prevention

- Good lab practices that reduce aerosol production
 - No bunsen burners
 - No “hot looping”
- Don’t sniff the plates
- Risk assessment



Note: UIH Clinical Microbiology Laboratory does not accept stool (or any other specimens) for the recovery of Clostridium botulinum or any specimen for viral culture if Smallpox (Variola Virus) is suspected. Please refer to IDPH for guidance.

Automated Microbiological Instrumentation

- Equipment used for dispensing suspensions into devices that are used for identification and susceptibility testing may create aerosols.
- Recommended that vortexing of such suspensions be performed in a BSC.
- At minimum, the vortexing should be done in test tubes that have vapor-tight, as well as droplet-containing, sealed lids.

Preparedness

- A laboratory working at BSL-2 with an unknown agent should be prepared to shift to BSL-3 when such an agent is identified or suspected.
- Once an organism has been identified as *F. tularensis* or a *Brucella* sp., the organism should be treated as a BSL-3 agent and manipulated solely in the BSC.

Preparedness

Example: Positive Blood Cultures

Any blood culture found to contain Gram negative coccobacilli.

Any blood culture where no organisms were seen on Gram stain.

- Tape plates after inoculation
 - No organisms seen on Gram stain
 - Where there is sufficient evidence of growth after 18-24 hours of incubation, plates can be returned to regular workflow.
 - If organisms are slow growing (>24 hours to recover isolate) proceed to “Organisms Recovered on Agar”
 - Gram negative coccobacilli
 - Remove all plates from the main laboratory to the AFB lab BSL-3 room. Any further manipulation of organisms must be performed in the hood in the negative pressure room with the door closed.
 - Any person working with the organism must wear an N-95 mask and have current certification on record for fit testing of the N-95 mask.
 - Notify a supervisor immediately. An exposure risk assessment must be performed.

Bacillus anthracis

- Gram stain: wide (1.0 mm) gram positive rods
- Non-hemolytic colonies
- Sticky, adheres to agar surface
- Non-motile



Brucella species

- Gram stain: Gram negative coccobacillus
- No growth on MacConkey agar
- Oxidase positive
- Urease positive (rapid)
- Slow growing (>2 days) from primary specimen



Yersinia pestis

- Gram stain: Bipolar (safety pin) Gram negative rods
- Lactose negative on agar
- Oxidase negative
- Indole negative
- Slow growing from primary specimen



Francisella tularensis

- Gram stain: tiny Gram negative coccobacillus
- No growth on MacConkey agar
- Oxidase negative
- Weak catalase
- Urea negative
- Slow growing (>2 days) from primary specimen



Burkholderia pseudomallei/mallei

- Gram stain: Gram negative rod
- Oxidase positive
- Slow growth (>1 day) from primary specimen
- Non-hemolytic
- Non-pigmented
- Non-wrinkled (young growth)



Preparedness

- The use of a BSL-3 facility does little to add protection to the personnel performing the work in the clinical microbiology laboratory.
- Rather, it contains the aerosol within the immediate laboratory space and protects those outside of the space.

MS + BT

- Standard practice is to exclude more commonly encountered bacteria before considering less common and potentially hazardous isolates.
- May take several days, placing personnel at risk of laboratory-acquired infection
- MALDI-TOF MS = fast and accurate : standard practice

MS + BT

1. Can MALDI-TOF MS be used safely in the standard clinical microbiology lab?
2. Can commercial MALDI-TOF MS systems identify agents of bioterrorism?

Bacterial Inactivation

- Chemically extracted in a microcentrifuge tube using 70% ethanol, 70% formic acid, and acetonitrile
- Our data demonstrate that extraction methods without a filtration step do not inactivate all BCC bacterial extracts
- Does not take into account other factors that can affect viability, such as standard inoculum loads or extended incubation times.
- “We recommend to err on the side of caution and confirm sample inactivation of SSBA material before testing with MALDI-TOF MS.”

Bacterial Inactivation

- Panel of Brucella strains
- Direct transfer procedure recommended by the manufacturer
 - matrix solution + solvent mixture
 - air dried for 2 min at room temperature.
- Dried material was recovered with a sterile swab and spread on culture plates
- Incubated for up to 3 weeks
- Viable bacteria were recovered for most strains
- Treating two full loops of Brucella with 70% ethanol did not consistently inactivate all bacteria after 10 min of incubation
 - Ethanol should not be used for short inactivation of Brucella

Bacterial Inactivation

- Vortex-mixing of bacteria in 5 μl of the CHCA matrix solution was systematically very efficient
 - Small volume could not ensure that the liquid would cover all bacteria consistently
 - Matrix is an expensive reagent
- Tested efficiency of the solvent alone
- Two full loops of bacteria of *Brucella* strains that form small, medium, or large colonies (corresponding to 1.8×10^{11} , 9.9×10^{11} , or 2.2×10^{12} CFU, respectively) were vortex-mixed in 200 μl of freshly prepared solvent mixture.
 - No colonies grew for any of the solvent-treated bacteria
- Inactivate up to 2×10^{12} CFU of *Brucella*.

MS + BT

- Standard Bruker Daltonics Biotyper database does not contain select agents, such as *B. anthracis*
- Bruker has developed a database containing 53 spectra from 6 BT agents = Security Relevant Library
 - Requires special clearance for acquisition and use
- Vitek MS IVD database contains spectra for *B. anthracis* and *Yersinia pestis*, but these are not FDA cleared (Unclaimed)
- Vitek MS RUO database v4.12 contains a number of BT agent spectra (including *B. pseudomallei*)

MS + BT

- MALDI-TOF MS diagnostic tests have been reported to incorrectly identify
 - *Y. pseudotuberculosis* as *Y. pestis*
 - Reliable identification of *Brucella* spp. to the genus level only
- Likely that cultures with select agents can be incorrectly, and unknowingly, identified by the software as a species-level match to a BSL-2 near-neighbor-species profile.

Commercial Database ID

- 20 sterilized isolates
 - 70% ethanol exposure time was extended to 10 min to
- Using the Biotyper reference library alone, 18 isolates returned high-quality spectra with results of “no reliable identification”
- Spectra from the two *B. pseudomallei* isolates matched *Burkholderia thailandensis*, with scores of 1.954 and 1.962, which are considered valid scores for genus-level identification (i.e., *Burkholderia* species);
 - Report was accompanied by a comment indicating that *B. thailandensis* is closely related to *B. pseudomallei*.
- Likely to result in “no reliable identification” of *Brucella* species and *F. tularensis*; this has the potential to lead to further work-up of isolates and exposure risk

Bruker Security-Relevant (SR) Library

- Seven *F. tularensis* isolates were identified as *F. tularensis* and two as *Francisella* species
- Five *Brucella* isolates (including one *Brucella suis* isolate) were identified as *B. melitensis* and four as *Brucella* species.
- One *B. pseudomallei* isolate yielded a top match of *Burkholderia mallei*, with a second-best match of *B. pseudomallei*, and the second yielded a top match of *B. pseudomallei*, with a second-best match of *B. mallei*

Database ID

No. Sample Identity	Identification Approach A			Identification Approach B			Identification Approach C		
	Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect	Correct	Partly correct	Incorrect
1 <i>Burkholderia pseudomallei</i> A101-10	9	1 9.5 (86%)	1	9	1 9.5 (95%)	0	10	1 10.5 (95%)	0
2 <i>Francisella tularensis</i> subsp. <i>holarctica</i> Ft 32	4.5	6.5 7.75 (70%)	0	9	1 9.5 (95%)	0	11	0 11 (100%)	0
3 <i>Brucella canis</i> A183-5	3	8 7 (64%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
4 <i>Bacillus anthracis</i> AMES	9	0 9 (82%)	2	9	1 9.5 (95%)	0	11	0 11 (100%)	0
5 <i>Ochrobactrum anthropi</i> A148-11	10	1 10.5 (95%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
6 <i>Yersinia pseudotuberculosis</i> type III	8	0 8 (73%)	3	6	3 7.5 (75%)	1	9	1 9.5 (86%)	1
7 <i>Burkholderia mallei</i> A106-3	9	0 9 (82%)	2	9	1 9.5 (95%)	0	8	3 9.5 (86%)	0
8 <i>Burkholderia thailandensis</i> E125	10	1 10.5 (95%)	0	10	0 10 (100%)	0	11	0 11 (100%)	0
9 <i>Yersinia pestis</i> A106-2	7	3 8.5 (77%)	1	6	4 8 (80%)	0	6	5 8.5 (77%)	0
10 <i>Bacillus thuringiensis</i> DSM350	4	2 5 (45%)	5	10	0 10 (100%)	0	9	2 10 (91%)	0

Lasch, P, et al. Identification of Highly Pathogenic Microorganisms by Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry: Results of an Interlaboratory Ring Trial. J Clin Microbiol. 2015 Aug;53(8):2632-40.

Supplemented Database ID

- Intact Cell Mass Spectrometry is a convenient and efficient molecular analytic tool for the first-line diagnosis
 - provided that bacterial culture conditions are well controlled,
 - standardized sample preparation protocols are followed, and
 - databases including high-quality reference spectra are carefully maintained
- Proteins were extracted by the ethanol-formic acid preparation procedure
 - Provided no studies to show that they “killed the bacteria”
 - Culture from blood agar only
- Library supplementation enhances the performance of commercial MALDI-TOF MS libraries
- Given challenges obtaining and working with select agents in the United States = not an easy endeavor.

MALDI-TOF MS ID

Case 1

- VitekMS IVD database identified the organism as *Ochrobactrum anthropi*
- VitekMS RUO database identified the organism as *Brucella* sp.

Case 2

- Bruker Daltonics database v3.3; identified as *Burkholderia thailandensis* with a log score value of 1.864

Mis-Identification

Horvat, RT, et al. (2011). Ribosomal RNA Sequence Analysis of *Brucella* Infection Misidentified as *Ochrobactrum anthropi* Infection. J Clin Microbiol. 49:1165-1168

- *Oligella ureolytica* or *Psychrobacter phenylpyruvicus*= **urease producers**

Another Case In Point

- Urge and reinforce the need for a policy change, as many bacteriology laboratories are about to lose biochemical and morphological identification methods in routine use.
- Important that laboratory directors have mechanisms in place to ensure the proper handling of potential biothreat organisms and confirmation of identification prior to the release of results.
- Relying on MALDI-TOF MS-based identification without a working knowledge base of other characteristics of the pathogen in question can result in undependable and erroneous identifications.
- These other characteristics may include growth characteristics on different media, colony morphology, Gram stain characteristics, motility, urease, oxidase, and results of other common rapid tests.
- Unrestricted, rapid identification of pathogens that are possible biothreats, such as *Brucella* spp., *Bacillus anthracis*, *F. tularensis*, and *Yersinia pestis*, is imperative in order to protect our employees from laboratory infections, to take the appropriate safety precautions, and to serve our patients by allowing diagnosis in a timely manner.

Brucella species

- Gram stain: gram negative coccobacillus
- No growth on MacConkey agar
- Oxidase positive
- Urease positive
- Slow growing (>2 days) from primary specimen
- Identified as *Ochrobactrum anthropi* by VitekMS

Francisella tularensis

- Gram stain: tiny Gram negative coccobacillus
- No growth on MacConkey agar
- Oxidase negative
- Weak catalase
- Urea negative
- Slow growing (>2 days) from primary specimen
- No identification by VitekMS

Burkholderia pseudomallei/mallei

- Gram stain: Gram negative rod
- Oxidase positive
- Slow growth (>1 day) from primary specimen
- Non-hemolytic
- Non-pigmented
- Non-wrinkled (young growth)
- Identified as *Burkholderia* (*species undetermined*) by VitekMS

CAP LPX

- Laboratory Preparedness Exercise (LPX)
- Developed by College of American Pathologists, the Centers for Disease Control and Prevention (CDC), and the Association of Public Health Laboratories (APHL).
- Laboratories will be sent live organisms that either exhibit characteristics of bioterrorism agents or demonstrate epidemiologic importance
- Respond following Laboratory Response Network Sentinel Laboratory Guidelines if a bioterrorism agent is suspected.
- All agents provided are excluded from the CDC's select agent list.
- These may include strains of *Bacillus anthracis*, *Yersinia pestis*, *Francisella tularensis*, and *Brucella abortus* that have been modified and are safe for testing in a laboratory
 - contains a certified Class II Biological Safety Cabinet
 - capable of handling Category A and B agents.

MS + BT

- Better methods are needed
- Prevention + preparedness is the best strategy for clinical labs