LABORATORY RESPONSE NETWORK (LRN) SENTINEL LEVEL CLINICAL LABORATORY PROTOCOLS FOR SUSPECTED BIOLOGICAL THREAT AGENTS AND EMERGING INFECTIOUS DISEASES

GENERAL INTRODUCTION, RECOMMENDATIONS AND BIOCHEMICAL PROCEDURES

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II. General Overview of the LRN and Recommendations

The primary function of the Laboratory Response Network (LRN) for Biological Threat Preparedness and Response is to provide rapid detection of biothreat and emerging agents of infectious diseases. Due to laboratory precautions and processing requirements, education of healthcare providers on the importance of notifying the laboratory when one of these agents is suspected is recommended. To this end, the LRN Sentinel Clinical Laboratories play a significant and vital role in fulfilling this function by providing prompt rule-out or referral service. A Sentinel Clinical Laboratory is formally defined as a laboratory that is "certified to perform high complexity testing under the Clinical Laboratory Improvement Amendments of 1988 (CLIA) by the Centers for Medicare and Medicaid Services (CMS) for the applicable Microbiology specialty or the laboratory is a Department of Defense (DoD) Laboratory certified under the DoD Clinical Laboratory Improvement Program or the laboratory is a veterinary medical diagnostic laboratory that is fully accredited by the American Association of Veterinary Laboratory Diagnosticians (AAVLD). Laboratory in-house testing includes Gram stains and at least one of the following: lower respiratory tract, wound or blood cultures. Sentinel Level Clinical Laboratories have been, and will continue to be, an integral part of the LRN through their active participation as partners with the public health community which is vital to surveillance and responses for endemic and emerging pathogens, including novel threats such as pandemic influenza.

The LRN Sentinel Level Clinical Laboratory Testing Protocols have been developed for the purpose of promoting uniformity and standardization of testing among clinical laboratories. The tests and phenotypic characteristics described for each agent are conventional manual tests commonly performed in the laboratory and allow the clinical laboratory to rapidly rule-out suspicious agents or refer them to the designated LRN Reference Laboratory. The American Society for Microbiology (ASM), in partnership with the Association of Public Health Laboratories (APHL) and the Centers for Disease Control and Prevention (CDC, serves as the lead agency for maintaining the protocols and making them available to the Sentinel Level Clinical Laboratory community. The current edition is compliant with the Clinical Laboratory Standards Institute (CLSI) format based on current information and recommendations of the APHL Sentinel Laboratory Partnerships and Outreach Subcommittee. These protocols reflect the standard practices for specimen processing as well as agent specific guidance. In addition to promoting standardization and uniformity of testing, adherence to, and maintaining the highest level of safety practices, is emphasized in the respective protocols. Please take note of the following revisions and/or additions that are contained within each protocol:

1. DO NOT USE AUTOMATED OR KIT-Based SYSTEMS for identification and susceptibility testing if you see any of the following characteristics:

    - Lack of growth on MacConkey agar
    - Slow growing, tiny Gram negative rods

The respective libraries (data bases) associated with these systems have limited accuracy for biothreat agents and often result in misidentification. The respective protocols are to be utilized for meeting the primary objective of "rule-out or refer".

2. Guidelines for selection, collection, transport, and handling of appropriate specimens are updated for each agent with greater emphasis on Specimen Type for each designated agent.

3. Guidance regarding Chain-of-Custody compliance in which the laboratory is encouraged to follow its institutional policy criteria in the event that a biothreat or emerging infectious agent is suspected and requires referral to your designated LRN Reference Laboratory.

4. Guidance is included for the safe and effective destruction of specimen material and organisms.

5. Testing of *Francisella tularensis, Brucella* spp., *Burkholderia* spp., *Bacillus anthracis,* and *Yersinia pestis* must be performed using BSL-3 or BSL-2 with BSL-3 precautions. Because these organisms may be detected in blood cultures, the same precautions should be used when working up positive cultures.

6. Guidance is provided for notification of- and reporting to your designated LRN Reference Laboratory in accordance with your local and state reporting requirements.

7. Where appropriate, guidance regarding therapy with supporting references is included.

8. The designation, Sentinel Clinical Laboratory, has replaced the former Level A designation, and the more recent Basic or Advanced LRN Laboratory.

9. Updated/current web links are included.

10. The Safety/Biosafety section in each protocol includes the current web link to the Biosafety in Microbiological and Biomedical Laboratories (BMBL) publication.

11. Biochemical test procedures are described and included at the end of this document.

Every effort has been made to provide the Sentinel Level Clinical Laboratory Community with practical, easy-to-follow, and reliable methods designed to rule-out or refer suspicious biothreat or emerging infectious disease agents.

If your laboratory uses Mass Spectrometry (MALDI-TOF or bioMerieux) for bacterial identification and if the manufacturer provides your facility with an alternate tube extraction method, it is recommended that the resulting extract be filtered using a 0.2 µ (or less) filter. This additional step is recommended to reduce the risk of laboratory contamination with viable bacteria and spores.

Using automated systems, including Mass Spectrometry (MALDI-TOF, bioMerieux) technology may result in exposure to dangerous pathogens, and could result in erroneous identification, Ex: *Bacillus anthracis* misidentified as *B. cereus*; *Yersinia pestis* misidentified as *Y. pseudotuberculosis*.

NOTE: Sentinel Clinical Laboratories do not require registration with the Select Agent Program to conduct diagnostic testing for Select Agents, both Tier I and non-Tier 1. Testing for Select Agents may be performed by laboratories as long as the laboratory destroys any residual specimen and destroys or transfers the confirmed select agent within 7 days of receipt of confirmed identification. Reporting of all identified Select Agents is still required; laboratories will need to complete Form 4. If the organism is transferred following identification, then the laboratory must also complete Form 2. For further guidance and access to the necessary forms, consult with your designated LRN Reference Laboratory or refer to the CDC Division of Select Agents and Toxin website at: www.selectagents.gov.

III. GLOSSARY OF TERMS

GLOSSARY

AAVLD: American Association of Veterinary Laboratory Diagnosticians
APHL: Association of Public Laboratories
APHIS: Animal and Plant Health Inspections Service
ASM: American Society for Microbiology

BAP/SBA: Blood Agar Plate/Sheep Blood Agar
BCYE: Buffered Charcoal Yeast Extract
BHI: Brain Heart Infusion
BMBL: Biosafety in Microbiological and Biomedical Laboratories
BSC: Biological Safety Cabinet
BSL: Biosafety Level

CDC: Centers for Disease Control and Prevention
CHA: Cysteine Heart Agar
CHOC: Chocolate Agar
CLIA: Clinical Laboratory Improvement Amendment
CLSI: Clinical Laboratory Standards Institute
CMPH: Clinical Microbiology Procedures Handbook
CMS: Center for Medicare and Medicaid Services

CNA: Colistin Nalidixic Acid Agar

DoD: Department of Defense
DoT: Department of Transportation

EIA: Enzyme Immunoassay
EMB: Eosin Methylene Blue Agar

FBI: Federal Bureau of Investigation
FERN: Food Emergency Response Network

KIA: Kligler's Iron Agar

LRN: Laboratory Response Network

MAC: MacConkey Agar
MH: Mueller-Hinton Agar
MMWR: Morbidity and Mortality Weekly Report

NASBA: Nucleic Acid Sequence-based amplification

PCR: Polymerase Chain Reaction
PPE: Personal Protective Equipment

RIDTs: Rapid Influenza Diagnostic Tests
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction

SEB: Staphylococcal Enterotoxin B

TM: Thayer Martin Agar
TSB: Tryptic Soy Broth
TSI: Triple Sugar Iron agar

USDA: United States Department of Agriculture

VIG: Variola Immunoglobulin

IV. Websites

- www.selectagents.gov
- http://www.cdc.gov/biosafety/publications/bmbl5/
- http://emergency.cdc.gov/agent/agentlist.asp
- http://www.cdc.gov/flu/h2n2bsl3.htm
- http://www.asm.org/index.php/guidelines/sentinel-guidelines
- http://www.bt.cdc.gov/lrn

- http://www.bt.cdc.gov/training/
- www.asm.org
- http://www.bt.cdc.gov/agent/smallpox/
- http://wwwnc.cdc.gov/eid/
- http://www.cdc.gov/qfever
- http://www.aphl.org/Pages/default.aspx

V. Biochemical Procedures

Disclaimer: Names of vendors or manufacturers may be provided as examples of suitable product sources; their inclusion does not imply endorsement by the American Society for Microbiology or protocol authors.

ANTIMICROBIAL DISK TESTS FOR IDENTIFICATION

I. PRINCIPLE

Polymyxin B and colistin disks are useful to separate species of non-fermenting bacilli, which can be susceptible (*Pseudomonas*) or resistant (*Burkholderia*). Susceptible organisms generally produce a zone of inhibition around the disk and strains that are resistant have no zone. Other antimicrobial agents, such as penicillin and amoxicillin-clavulanic acid can be used for the identification to the species level of various bacteria because of their usual susceptibility to the agents, however in these cases, the actual zone size must be measured.

II. MICROORGANISMS TESTED

A. **Polymyxin B or Colistin:** Used as part of the tests used to rule out *B. mallei* and *B. pseudomallei*.
B. **Penicillin**: Used as an alternate to demonstrate the presence of a beta-lactamase in *Francisella*
C. **Amoxicillin-clavulanic** acid is used to separate *Burkholderia pseudomallei* from the other species of *Burkholderia* (3, 4).

III. MEDIA, REAGENTS AND SUPPLIES

A. Disks: store a small supply at 4°C (store stock at –20°C).
   1. Polymyxin B 300 U or colistin 10 µg
   2. Penicillin 10 U
   3. Amoxicillin-clavulanic acid 20/10 µg
B. Media
   1. BAP or CHOC
   2. Mueller Hinton agar (MH)
C. Supplies

1. Swabs
2. Broth and McFarland standard for inoculum

IV. QUALITY CONTROL

A. Perform quality control with each new lot or shipment of disks used for identification with a susceptible strain prior to using for identification tests. Testing should be repeated monthly for disks that are stored at 4°C, rather than –20°C.

B. Strains, media and zones size requirements (2). Quality control for the disks is performed on MH agar even if the isolate is not tested on this agar.

Antimicrobial Agent	Test Organisms	I. Zone Size 6 mm disk
Polymyxin B 300U	*Pseudomonas aeruginosa* ATCC 27853	**14-18 mm**
	Escherichia coli ATCC 25922	**13-19 mm**
Colistin 10 µg	*Pseudomonas aeruginosa* ATCC 27853	**11-17 mm**
	Escherichia coli ATCC 25922	**11-17 mm**
Penicillin 10U	*Staphylococcus aureus* ATCC 25923	**26-37 mm**
Amoxicillin-clavulanic acid 20/10 mg	*Escherichia coli* ATCC 35218	**17-22 mm**

V. PROCEDURE

A. Prepare a No. 0.5 McFarland suspension of the organism and inoculate MH in 3 directions as for a disk susceptibility test (1). If the organism does not grow on MH, BAP or CHOC can be used solely for identification purposes.
B. Place disks on agar
C. Incubate 18-24 h at 35° C in non-CO_2 incubator.

VI. INTERPRETATION (1)

A. Polymyxin B
Any zone of inhibition is susceptible
B. Colistin
Any zone of inhibition is susceptible
C. Penicillin (4)
Zone ≤ 28 mm is resistant

Zone ≥ 29 mm is susceptible

D. Amoxicillin-clavulanic acid

Zone ≥ 18 mm is susceptible

VII. REPORTING

A. *Burkholderia* susceptible to amoxicillin-clavulanic acid are likely to be *B. pseudomallei* and are not *B. cepacia* (3, 4).

B. Non-fermenting gram negative rods that are polymyxin B or colistin resistant can be *Burkholderia*.

C. *Francisella tularensis* is penicillin resistant.

D. A gram negative rod that is penicillin susceptible and polymyxin B resistant or does not grow on MAC, may be a Bacillus that did not retain crystal violet in the Gram stain.

VIII. LIMITATIONS

A. Resistance of *Burkholderia pseudomallei* to amoxicillin-clavulanic acid was present in 2 of 4021 patients who presented with melioidosis in Thailand (4), however during their treatment an additional 14 isolates became resistant. If the organism is weaponized, it could be modified to be a resistant strain. The utility of the disk test is to minimize errors when an automated or kit system reports an identification of *B. cepacia*. If the isolate is susceptible to amoxicillin-clavulanic acid, it is not *B. cepacia* and may be *B. pseudomallei.*

B. *B. anthracis* is susceptible to penicillin, however, if the organism is weaponized, it could be modified to be a resistant strain.

IX. REFERENCES

1. CLSI. 2012. *Performance Standards for Antimicrobial Disk Susceptibility Tests; Approved Standard-11th edition;* M2-A11. CLSI, Wayne, PA.
2. CLSI. 2013. *Performance Standards for Antimicrobial Susceptibility Tesings; Twenty-third Informational Supplement;* M100-S23. CLSI, Wayne, PA.
3. Hodgson. K., C. Engler, B. Govan, N. Ketheesan, and R. Norton. 2009. Comparison of routine bench and molecular diagnostic methods in identification of *Burkholderia pseudomallei J. Clin. Microbiol.* 47: 1578-1580
4. Wuthiekanun, V, P. Amornchai, N. Saiprom, N. Chantratita, W. Chierakul, G. Koh, W. Chaowagul, N. Day, D. Limmathurotsakul, and S Peacock. 2011. Survey of antimicrobial resistance in clinical *Burkholderia pseudomallei* isolates over two decades in Northeast Thailand. Antimicrob. Agents *Chemother*. 55:5388-5391

CATALASE TEST

I. PRINCIPLE

Bacteria that synthesize the enzyme, catalase, hydrolyze hydrogen peroxide into water and gaseous oxygen, which results in the liberation of gas bubbles. The test is useful in the initial characterization of most bacteria (1, 2). For safety purposes, it is recommended that this test be performed in a BSC, covered petri dish or tube to ensure the containment of aerosols that are produced when the test organism generates a positive result (production of bubbles).

II. MICROORGANISMS TESTED

Young (18 h old, if possible) colonies of bacteria growing on agar media, preferable BAP or CHOC

III. REAGENTS AND SUPPLIES

A. 3% Hydrogen peroxide reagent. Store at 2-8^{O}C.

B. Supplies
   1. Glass slide
   2. Sterile wooden sticks or plastic or platinum loops or wires.

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of reagent, prior to being put into use. Frequency of further quality control testing is determined by local, state and federal guidelines.

B. Organisms:

Staphylococcus aureus ATCC 25923 - Catalase-positive
Streptococcus pyogenes ATCC 19615 - Catalase-negative

V. PROCEDURE

A. Using a wooden stick, plastic loop, or platinum loop, touch the center of a well isolated colony growing for less than 24 h on non-selective media. If growth is not visible until 48 h, the test can be performed on older growth.

B. Transfer to a clean glass slide.
   1. Be sure transferred growth is visible to the naked eye on slide.
   2. If colony is from BAP, use care not to pick up blood.

C. Place one drop of peroxide reagent on slide and observe immediately for effervescence.
   1. Use a magnifying lens if necessary.
   2. Hold over dark background to enhance detection of bubbles.

D. Discard slide into sharps container.

VI. INTERPRETATION

A. Positive test- Immediate appearance of bubbles
B. Weak-reaction- Immediate appearance of one or two bubbles
C. Negative test- No bubbles or a few bubbles after 20 sec

VII. REPORTING

A. *Bacillus* spp are catalase-positive and *Clostridium* are catalase-negative.
B. The test is useful to separate among the fastidious gram-negative rods.

VIII. LIMITATIONS

A. Red blood cells contain catalase. To avoid false positive results, do not pick up blood agar with colony. If colony does not easily pick up or grow well, repeat the test from CHOC, which does not interfere with assay.
B. Do not test from Mueller-Hinton agar (2).
C. Selecting colonies with some metal bacteriological loop materials will yield false positive results; platinum loops do not yield false positive results
D. Because the enzyme is present in viable cultures only, non-viable cultures may give false negative results.
E. Do not reverse the order of adding the reagent to the colony; false-negative results can occur.
F. Do not mix the reagent and the colony.

IX. REFERENCES

1. Levin, M. and D. Q. Anderson. 1932. Two new species of bacteria causing mustiness in eggs. J. Bacteriol. 23:337-347.
2. MacFaddin J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 78-97. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.

BETA-LACTAMASE TEST

I) PRINCIPLE

Beta-lactamases are enzymes produced by many clinically significant bacteria and are major mediators of bacterial resistance to beta-lactam agents. Routine beta-lactamase tests are based on visual detection of the end products of beta-lactamase hydrolysis, which is demonstrated with a colorimetric reaction. These tests primarily include the chromogenic cephalosporin method, the acidimetric method, and the iodometric method. Not every method is satisfactory for detecting beta-lactamase produced by all of the bacteria for which the test is useful. Cefinase is recommended for detection of penicllin resistance in most bacteria.

In the cefinase test, the disk is impregnated with the chromogenic cephalosporin nitrocefin. This compound exhibits a very rapid color change from yellow to red as the amide bond in the beta lactam ring is hydrolyzed by a beta-lactamase. When a bacterium produces this enzyme in significant quantities, the yellow-colored disk turns red in the area where the isolate is smeared.

II) SPECIMEN

A) Use the disks for rapid testing of isolated colonies of fastidious gram negative rods

B) Use several colonies of similar colony morphology grown overnight (18 to 24 h) on nonselective medium (e.g., BAP or CHOC)

II) QUALITYCONTROL

A) QC strains
   1) *Staphylococcus aureus* ATCC 29213—positive
   2) *Haemophilus influenzae* ATCC 10211 or *S. aureus* ATCC 25923 beta lactamase (-) negative

B) Perform QC each day the test is performed and record results on QC form. Less frequent quality control may be accepted by state and federal guidelines.

III) MATERIALS

A) Media and reagents (1)
   1) Cefinase disks (BD Microbiology Systems, Cockeysville, MD) Store at 2 to 8°C. Bring to room temperature before use.
   2) Sterile distilled water

B) Supplies
   1) Glass slides or an empty petri plate.
   2) Sterile Pasteur pipettes
   3) Sterile wooden applicator sticks or inoculating loops

IV) PROCEDURE

A) Dispense the required number of disks onto a clean microscope slide or an empty petri plate.

B) Moisten each disk with 1 drop of sterile distilled water. Do not saturate.
C) With a sterile loop or applicator stick, smear several colonies onto the disk surface.
D) Observe disk for color change. Positive results usually appear within 15 s to 5 min. If no color change occurs within 5 min, the test is negative.

V) RESULTS

A) Positive: yellow changes to red where inoculated.
B) Negative: no change in color occurs.

VI) REPORT

Report positive results either as "penicillin-resistant" or "beta-lactamase positive" and report negative results as "beta-lactamase negative."

VII) PROCEDURE NOTES

For some bacteria there is a direct correlation between a positive beta-lactamase reaction and resistance to specific beta-lactam drugs that might be prescribed for treatment of infections caused by them.

A) Microdilution MIC tests with penicillin may fail to detect penicillin-resistant (beta-lactamase-positive) staphylococci among some isolates that produce small amounts of beta-lactamase. An induced-beta-lactamase test is needed to confirm an isolate as penicillin susceptible (2).
B) *Bacillus* spp. do not react well in this test. Resistance is best detected by a penicillin disk or MIC (3)

VIII) REFERENCES

1) **Becton Dickinson Microbiology Systems.** 1996. BBL paper disks for the detection of beta-lactamase enzymes. Product 88-0973-1. Becton Dickinson Microbiology Systems, Cockeysville, MD.
2) **Clinical and Laboratory Standards Institute.** 2012. *Performance Standards for Antimicrobial Disk Susceptibility Tests,* 11th ed. Approved standard M2-A11. **CLSI**, Wayne, PA.
3) **Clinical and Laboratory Standards Institute**. 2010. *Methods for Antimicrobial Dilution and Disk Susceptibility Testing of Infrequently Isolated or Fastidious Bacteria.*; Approved Guideline, Second edition. M45A2, CLSI, Wayne, PA

INDOLE TEST

I. PRINCIPLE

The ability of an organism to split indole from the amino acid tryptophan is due to the presence of tryptophanase. Indole, if present, combines with the aldehyde in the reagent to produce a pink to red violet quinoidal compound (benzaldehyde reagent) or a blue to green color (cinnamaldehyde reagent). In the rapid spot test, indole is detected directly from the colony growing on a medium rich in tryptophan (2).

II. MICROORGANISMS TESTED

Fresh growth of a gram-negative rod on medium that does not contain dyes and contains tryptophan, e.g. BAP or CHOC

III. MEDIA, REAGENTS and SUPPLIES

A. Rapid spot indole:

1. Prepare or purchase either 5% *p*-dimethylaminobenzaldehyde or 1% paradimethylaminocinnamaldehyde in 10% (vol/vol) concentrated HCl. (1, 3)
2. Store indole reagents in dark at 4° C.

B. **Supplies**

1. A sterile loop, swab or stick for harvesting.
2. Filter paper (optional)

IV. QUALITY CONTROL

A. Perform QC on each new lot or shipment of reagent prior to being put into use. Perform more frequent quality control as indicated by local, state and federal regulations and the package insert for commercial tests.

B. Organisms:
Escherichia coli ATCC 25922 (indole-positive)
Pseudomonas aeruginosa ATCC 27853 (indole-negative)

V. PROCEDURE

II. Use one of the methods below:

1. Moisten filter paper with reagent. Using a wooden stick, rub portion of colony onto paper.
2. Sweep the colony onto a swab. Add drop of indole reagent to the colony swab.
3. Add reagent directly to the colony growing on the agar surface.

VI. INTERPRETATION

A. The development of a brown-red to purple-red color (benzaldehyde reagents) or blue color (cinnamaldehyde reagent) within 20 sec. indicates the presence of indole.

B. A negative test is colorless or slightly yellow.

VII. REPORTING

A. *Escherichia coli* is indole-positive, as are many other *Enterobacteriaceae, Vibrio, Aeromonas, Plesiomonas* and *Pasteurella*.

B. Several fastidious gram-negative rods are indole-positive, such *Cardiobacterium hominis* and *Pasteurella bettyae.*

VIII. LIMITATIONS

A. Detectable indole will diffuse to colonies within 5 mm of a 2 to 3 mm colony, giving false-positive results

B. Do not use media that contain dyes (e.g. EMB, MAC).

C. Growth medium must contain an adequate amount of tryptophan. **Do not use Mueller-Hinton agar** for test, because tryptophan is destroyed during the acid hydrolysis of casein.

D. Do not use a plate with a nitrate disk to perform indole test, as nitrate can interfere with spot indole test by inducing false negative results.

E. If rapid indole test is negative, the isolate could be positive in the more sensitive tube test.

F. For fastidious gram-negative rods, such as *Cardiobacterium hominis,* a heavy inoculum is necessary.

IX. REFERENCES

1. Bale, M. J., S. M. McLaws, and J. Matsen. 1984. The spot indole test for identification of swarming *Proteus.* Am. J. Clin. Pathol. **83**: 87-90.
2. MacFaddin J. F. 2000. *Biochemical Tests for the Identification of Medical Bacteria,* 3rd ed., p. 221-232. The Lippincott, Williams & Wilkins Co., Philadelphia, PA.
3. Vracko, R. and J.C. Sherris. 1963. Indole-spot test in bacteriology. Am. J. Clin. Pathol. 39: 429-432

MOTILITY TESTS

I. PRINCIPLES

The motility test is used to detect the presence of flagella on bacteria, allowing them to travel in and out of the microscopic field or beyond their initial inoculation in agar (2). For the wet preparation, a light inoculum of an organism in a drop of broth is suspended on a clean glass slide, a coverslip is added and the culture is observed microscopically for motility. Occasionally the organism is incubated in the broth prior to examination. **Note:** Because of the risk for aerosol exposure, the slide motility test is not recommended.

In the tube test, semi-solid motility media is inoculated in a straight line down through the center of a tube (3). Motile organisms will migrate out from the line of inoculation causing visible turbidity throughout the tube. Non-motile organisms will grow only along the line of inoculation. Other substrates may be added to the medium, which allow simultaneous testing of other biochemical reactions that aid in the identification of microorganisms.

II. MICROORGANISMS TESTED

Enterobacteriaceae, *Listeria*, *Bacillus*, other gram-positive rods, glucose non-fermenting gram-negative rods, and any other organism where motility is useful for the identification

III. MEDIA, REAGENTS AND SUPPLIES

A. MEDIA, REAGENTS

1. Broth medium for wet preparation.
    a. TSB or BHI broth
    b. Saline or distilled water

NOTE: Cleary (1) reported that some motile organisms can become immobile in distilled water.

2. Motility tube media
    a. SIM – combination of sulfide for H_2S detection, indole, and motility test medium
    b. Motility test medium with or without with TTC (triphenyltetrazolium chloride, a colorless vital dye incorporated into the medium, which turns red as it is reduced when incorporated into bacteria. The dye allows easier visualization of the bacteria).

B. SUPPLIES

1. 22 x 22 mm coverslips and microscope slides
2. Phase contrast or bright field microscope
3. Sterile inoculating needle or sticks.

IV. QUALITY CONTROL

A. Test each new lot or shipment of tube media prior to use with a positive and negative reacting organism and for sterility.

B. Organisms:

Escherichia coli	**ATCC** 25922	+
Klebsiella pneumoniae	**ATCC** 13883/ **ATCC** 27736	-

C. Competence of technologists in the hanging drop test is validated by testing known motile enterococci or *Listeria* in the broth assay.

V. PROCEDURE

A. Wet Mount Preparation

1. Inoculum
   a. Use <u>fresh</u> growth from an agar plate and suspend isolated colonies in broth. Use a light inoculum (not visibly turbid).
      (i) It is acceptable to suspend the organism in a small amount of medium for an initial wet mount, but follow with incubation of a larger amount of broth media, if the result is negative.
      (ii) Choosing the medium
         (a) Use any broth which does not contain carbohydrate and will support the growth of the organisms (BHI, Nitrate broth).
         (b) Broth works best for *Bacillus* spp. (1).
         (c) Use 0.5 ml of BHI or TSB for enterococci.
         (d) Saline can be used for gram-negative rods.
         (e) For problem organisms, streak an agar slant and then carefully add a few drops of BHI or nutrient broth to the base of the slant covering just a bit of the streaked slant. After overnight incubation, use a drop of the BHI broth for the wet mount (this works very well for *Bacillus* sp.).
2. Examination:
   a. While wearing gloves, place a small drop of fresh liquid on the center of a microscope slide, add coverslip. Allow organisms to 'settle" for a minute.
   b. Observe under the high power (40X)
      (i) For a light microscope, decrease the light by closing the diaphragm.
      (ii) Preferably, use a phase-contrast microscope.

3. For all organisms negative for motility by initial wet mount, repeat the wet mount after incubation in broth or test by tube method.
      (i) Incubate for 24 h at 30°C for non-fermenting gram-negative rods
      (ii) Other organisms may be incubated at temperatures optimal for their growth, usually 35°C.

B. Tube media for *Enterobacteriaceae*, non-fermenting gram-negative rods.

1. With a sterile inoculating wire, pick an isolated colony and stab the medium straight down through the center to a depth of one-half inch for small tubes and one inch for larger tubes.
2. Incubate at
   a. 35°C for *Enterobacteriaceae* for 24 h.
   b. 30°C for non-fermenting gram-negative rods for 24 h.
   c. If there is a question regarding a negative result, incubate at 25°C.

VI. INTERPRETATION:

A. Wet Mount Preparation

Directional purposeful motility is a positive test. Motile organisms change position with respect to one another. Brownian movement (random jiggling or shaking due to molecular bombardment), where the organisms remain in the same relative position with respect to each other, should not be mistaken for true motility (4).

B. Tube media

1. Diffuse growth outward away from stab line or turbidity of the media is a positive test.
2. A clear tube (the same as the uninoculated media) with growth only along the line of inoculation indicates that the organism is non-motile.
3. In media with TTC, the red color forms in the area of bacterial growth. Motile organisms produce a pink color that diffuses from the stab line. Organisms that are non-motile produce a pinkish-red pigment that is confined to the stab line.

VII. REPORTING:

A. *Bacillus* spp. should be motile. Lack of motility could indicate *B. anthracis.*

B. *Acinetobacter* species are non-motile.

C. Non-fermenting gram-negative rods and *Enterobacteriaceae* vary in their motility.

VIII. LIMITATIONS:

A. *Bacillus* species are best tested directly from a fresh plate. If a fresh plate is not available, inoculate a plate and incubate for 4 h. Then perform wet mount.

B. Excessive heat on a microscope slide can affect the results.

C. False negative reactions may occur if bacterial flagella are damaged due to heating, shaking, or other trauma. Such environmental shock will render the organism non-motile.

D. Some microorganisms do not produce flagellar proteins at 35-37°C but do so at 22°C.

E. TTC may be inhibitory to certain fastidious bacteria.

II. REFERENCES

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OXIDASE TEST

I. PRINCIPLE

In the presence of atmospheric oxygen, a bacterium's intracellular cytochrome oxidase enzymes oxidize the phenylenediamine reagent (an electron acceptor) to form a deep purple compound, indol phenol (2). The test is useful in the initial characterization of gram negative bacteria (2).

II. MICROORGANISMS TESTED

A. Fresh isolates of aerobic or facultatively anaerobic, or microaerobic gram-negative rods and cocci growing on BAP, MH, Brucella, BHI or CHOC agars

B. Restrictions

1. Do not use media with dyes, such as EMB or MAC.
2. Do not test organisms growing on media that contains glucose.

III. REAGENTS AND SUPPLIES

A. Reagents

1. Kovacs' reagent (1)

NOTE: Other formulas exist, but Kovacs' is the most sensitive reagent.

a. For 0.5 - 1% solution, dissolve 0.1 gm (size of a pea) *N, N, N, N* tetramethyl-*p*-phenylenediamine dihydrochloride in 10 ml sterile distilled water.
b. Mix well and allow to sit for 15 min.
c. Made fresh daily (preferred method) or store aliquots of the reagent in foil wrapped test tubes at –20°C. Remove from the freezer and thaw before use.
d. Discard unused portion daily.

2. Dried filter paper disks or strips impregnated with reagent
3. Disposable glass ampules (available from most vendors).

B. Supplies

1. Filter paper or swab
2. Disposable Petri dish
3. Sterile wooden sticks or plastic or platinum loops or wires.
4. De-ionized water

IV. QUALITY CONTROL

A. Do not use once the pale purple color of the reagent or the filter paper begins to darken.

B. Perform QC on each new lot of reagent, prior to being put into use. Perform more frequent quality control as indicated by local, state and federal regulations and the package insert for commercial tests.

C. Organisms:

Pseudomonas aeruginosa ATCC 27853 (oxidase-positive)
Escherichia coli ATCC 25922 (oxidase-negative)

V. PROCEDURE

A. Filter paper method

1. Preparations:
    a. Place a small square of Whatman #1 filter paper in a Petri dish and moisten with 1 or 2 drops of prepared Kovacs' oxidase reagent **or**
    b. Place impregnated dry disk or strip in Petri dish and moisten with deionized water.
2. Methods of testing
    a. Pick an isolated colony with a stick and smear onto Kovacs' dampened filter paper. Observe paper for purple color.
    b. For fastidious bacteria, swipe colony onto white cotton swab and rub onto the dampened filter paper. Observe swab for purple color.
    c. Touch moistened filter paper to colony and observe for purple color development.

B. Plate method

1. Drop a few drops of reagent directly on top of a few suspected colonies. Do not flood entire plate because bacteria covered by the reagent generally are not viable for subculture.
2. Expose the colonies to air by tilting the culture after flooding with oxidase reagent to allow oxygen to reach the colonies.
3. Observe colony for purple color. Ignore any discoloration of surrounding medium.

NOTE: Colonies tested by this method are quickly non-viable. Subculture immediately.

VI. INTERPRETATION

A. Positive test

1. Development of a deep blue to purple color in 10-30 sec is a positive reaction.
2. Development of the color in 30-60 sec is a weak positive reaction.
3. Do not read after 60 sec.

B. Negative test is no color change in 60 sec.

VII. REPORTING

To avoid misidentifications, perform oxidase test on all **gram-negative rods, except those that swarm. Most commercial kits do not** include an oxidase test as part of their biochemical reactions, but require the user to record the result as part of their identification scheme. If the test is omitted, errors in identification can be made. In addition, oxidase testing can aid in rapid identifications, avoiding the need for costlier kit identifications (3).

Gram-negative diplococci should give a positive reaction, since all members of the genus *Neisseria* are oxidase-positive. *Moraxella* spp. which are either gram-negative diplococci or coccobacilli are also oxidase-positive.

Gram-negative rods that are oxidase-positive do not belong to the *Enterobacteriaceae*, with the exception of *Plesiomonas shigelloides*, which is both oxidase and indole-positive.

Use this test as a major characteristic for identification of gram-negative rods that are not in the *Enterobacteriaceae* family.

VIII. LIMITATIONS

A. To avoid false positive results
   1. Do not use a nichrome wire to pick colony.
   2. Do not test organisms growing on media that contains glucose or dyes (*e.g.* MAC or EMB)
   3. Do not use if reagent or filter paper is purple.

B. Mixed cultures of *Neisseria* and pseudomonads can give false-negative results, since the pseudomonads can elaborate an inhibitory substance that interferes with the production of oxidase by *Neisseria*.

C. Timing is critical to accurate testing.

D. Some organisms may require several subcultures or colony growth of several days to produce a positive reaction

IX. REFERENCES

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UREASE TEST

I. PRINCIPLE

Urease tests contain urea and a phenol red pH indicator. Many organisms have a urease enzyme, which is able to split urea in the presence of water to release two molecules of ammonia and carbon dioxide. The ammonia combines with the carbon dioxide and water to form ammonium carbonate, which turns the medium alkaline (3), turning the indicator from its original orange-yellow color to bright pink (1).

This test can be used as part of the presumptive identification of *Yersinia* and *Brucella* species.

II. MICROORGANISMS TESTED

The urea test is part of the procedures to rule out:

A. *Yersinia* spp. – urea agar or broth (Christensen)
B. *Brucella* spp. – urea agar or broth, or disks/tablets

III. REAGENTS AND SUPPLIES

A. REAGENTS
   1. Christensen's urea agar
   2. Christensen's urea broth
   3. Urea disks or tablets
B. SUPPLIES
   1. Sterile wooden sticks or loops
   2. Saline or water in small plastic tube for disk test

IV. QUALITY CONTROL

Inspect agar for evidence of freezing, contamination, cracks, and dehydration prior to storage and before use.

Test each new lot or shipment of tablets, disks, or medium with a positive and negative control prior to being put into use.

Organisms – see also manufacturer's package insert

Proteus mirabilis ATCC 12453	= positive control
Escherichia coli ATCC 25922	= negative control

V. PROCEDURE

A. Christensen's urea agar (*Yersinia* or *Brucella*):
   1. Using a sterile stick or loop, heavily inoculate the agar surface with growth from an 18-24 hour pure culture. Do not stab the butt.
   2. Incubate aerobically at 35°C, with **cap loosene**d.
      Examine for color development after 18-24 hours incubation.

B. Christensen's urea broth (*Yersinia* or *Brucella*):
   1. Using a sterile stick or loop, heavily inoculate the broth with growth from an 18-24 hour pure culture.
   2. Incubate aerobically at 35°C with **cap loosene**d.
   3. Examine for color development after 18-24 hours incubation.

C. Urea disks or tablets (*Brucella*)
   NOTE: See also manufacturer's package insert for differences from procedure below.
   1. Add 0.25 ml (5 drops) of saline or water to a small sterile plastic test tube.
   2. Make a heavy suspension in the test tube using growth from an 18-24 hour pure culture.
   3. Add a urea disk to the tube. (some tubes may come with the urea disk/tablet already in the tube).
   4. Incubate aerobically at 35-37°C for 1 to 24 hours and examine for color development.

VI. INTERPRETATION – see also manufacturer's package insert

I. Positive test – development of an intense magenta to bright pink color in 15 minutes to 24 hours.
II. Negative test – no color change or any color other than pink/red.

VII. REPORTING

A. Urea-positive, oxidase-positive gram-negative coccobacilli that do not grow on MAC in 24 h are presumptively identified as *Brucella*, unless they are isolated from urine. ***Immediately transfer cultures to a biosafety cabinet.***
B. Urease-positive, oxidase-positive gram-negative coccobacilli that are isolated from the urinary tract may be *Oligella ureolytica,* and can be differentiated from *Brucella* because they are motile (see motility procedure) and PDA-positive. PDA can be tested by adding a drop of 10% ferric chloride to the tube with the urea-PDA disk. A black to dark green color is a positive reaction.

VIII. LIMITATIONS

A. Some *Brucella* rapidly split urea, while others react slowly.
B. When performing overnight tests from urea agar medium that contains peptone, the alkaline reaction may not be due to urease, but to hydrolysis of peptone by non-glucose fermenting organisms.
C. Urea is light sensitive and can undergo autohydrolysis. Store urea at 2-8°C in the dark.
D. The test is less sensitive if the medium is not buffered.
E. *Y. pseudotuberculosis* and *Y. enterocolitica* give stronger reactions in urea agar or broth when incubated at 25-28°C, but incubation at this temperature is not necessary to demonstrate urease production.
F. Use of glass test tubes may cause false-positive reactions with urea disk tests.

IX. REFERENCES

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