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GetCampy_Laboratory Procedures V.1

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We are still developing and optimizing this protocol

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Keywords: Campylobacter, gastroenteritis, genomics, pathogen surveillance, campylobacter spp, campylobacter, enteric bacteria, campylobacter identification, getcampy-laboratory procedure, getcampy-laboratory procedures this standard operating procedure, key enteric pathogen, biochemical test, salmonella typhimurium, negative enteric pathogen, tsi test distinguishes glucose, fermentation, sugar fermentation, members of the enterobacteriaceae family, enterobacteriaceae family, exclusion of key enteric pathogen, anaerobic condition, anaerobic conditions within slanted agar tube, agar slants for the biochemical identification, indole production, miu test, biochemical identification, lactose, key metabolic trait, relevant aetiologies of diarrhoeal disease, hydrogen sulfide production, diarrhoeal disease

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Disclaimer

All protocols intended for research use only.

Abstract

This standard operating procedure (SOP) outlines the preparation, quality control, and application of Motility-Indole-Urease (MIU) agar and Triple Sugar Iron (TSI) agar slants for the biochemical identification of Gram-negative enteric pathogens. These assays are routinely used to differentiate members of the Enterobacteriaceae family and other related organisms based on key metabolic traits, including motility, urease activity, indole production, sugar fermentation, gas production, and hydrogen sulfide generation.

The MIU test evaluates motility, indole production, and urease activity through stab inoculation and biochemical reactions, while the TSI test distinguishes glucose, lactose, and sucrose fermentation, alongside gas and hydrogen sulfide production, through differential aerobic and anaerobic conditions within slanted agar tubes. These tests facilitate the identification and exclusion of key enteric pathogens such as *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Shigella flexneri*, and *Salmonella Typhimurium*.

While *Campylobacter spp.* are the primary focus of the GETCampy project, these biochemical tests do not apply to *Campylobacter* identification due to its distinct physiology. Instead, MIU and TSI are retained within the diagnostic workflow to ensure comprehensive exclusion or detection of co-isolated enteric pathogens from clinical and animal samples. This supports a One Health surveillance framework, ensuring all relevant aetiologies of diarrhoeal disease are considered.

Detailed procedures cover media preparation, pH adjustment, sterilisation, inoculation, incubation, interpretation, and quality control with reference strains to ensure the reliability of results. These SOPs complement *Campylobacter*-specific methodologies by maintaining diagnostic capacity for a broad range of enteric bacteria.

Image Attribution

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Materials

Figure 1. Specimen accessioning workflow

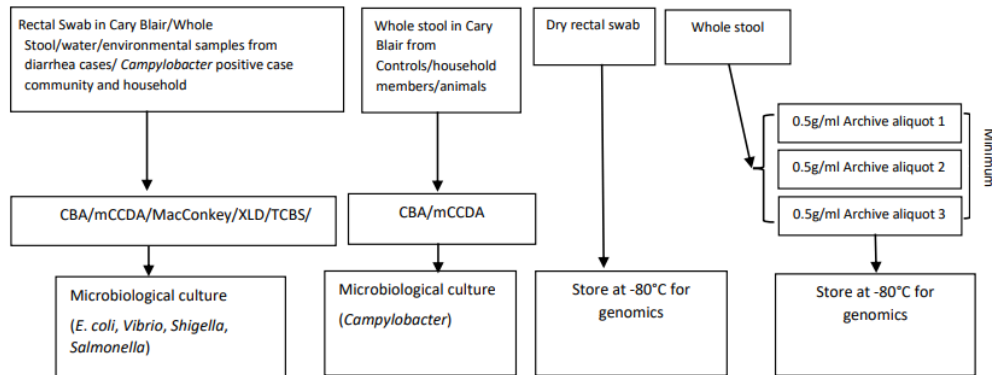


Figure 1: Specimen accessioning workflow.

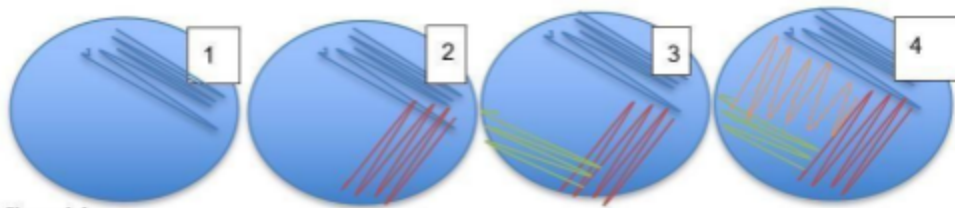


Figure 2: 4-streak quadrant method for plate streaking.

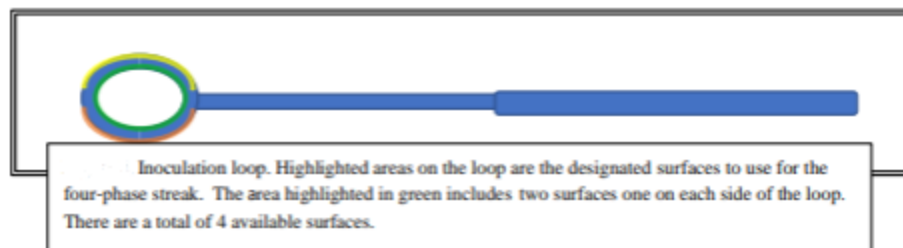


Figure 3: Plastic loop highlighted for streak plating.

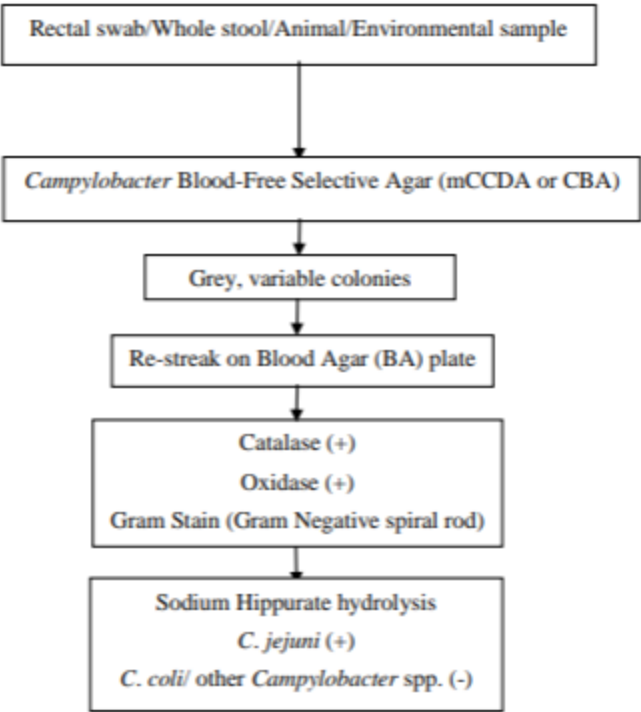


Figure 4: Workflow for identification of *Campylobacter* spp.

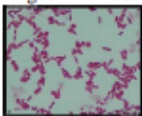
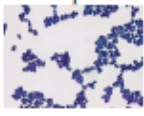
Table 1. Expected QC Results		
Testing Frequency	Once a week	
Inoculum	<i>E. coli</i> ATCC 25922	<i>Staphylococcus aureus</i> ATCC 25923
Number of slides	1	1
Specification	Positive Control	
Gram Staining Results	-	+
Appearance	Bright Pink-rods 	Dark Purple-cocci 

Table 1. Quality control results for known organisms.

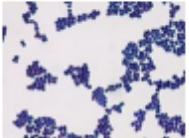
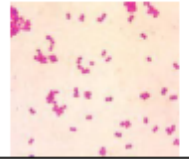
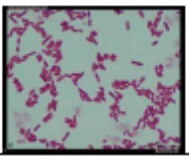
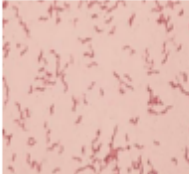
Gram Result	Description	Appearance	Organism
Positive, Cocci	Dark purple, spherical shaped		Possible <i>Staphylococcus</i> , <i>Streptococcus</i>
Negative, Cocci	Bright pink, spherical shaped		Possible <i>Neisseria</i>
Negative, Bacilli	Bright pink, rod shaped		Possible <i>Aeromonas</i> spp., <i>E. coli</i> , <i>Salmonella</i> spp., <i>Shigella</i> spp. <i>Vibrio</i> spp. (Gram negative rods).
Negative, "S" shaped	Bright pink, "S" shaped		Possible <i>Campylobacter</i> spp.

Table 2. Gram stain interpretation for *Campylobacter* spp. and other enteric pathogens.

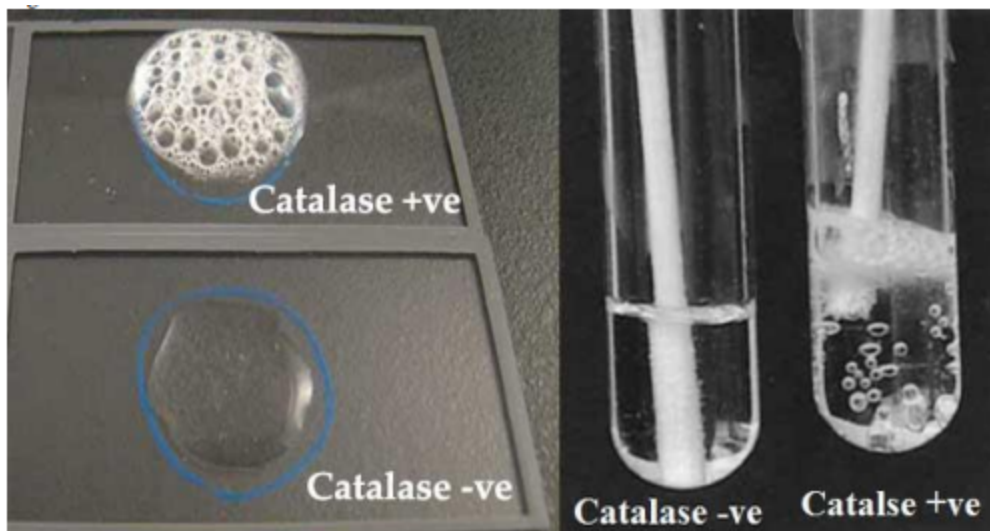


Figure 5. Results of a positive and negative catalase test with quality control organisms.

Expected QC Results			
Testing Frequency	• Upon Preparation • Monthly • Anytime there is sign of suspected contamination		
Incubation Temp/Time	37°C ± 2°C for 18-24 hours		
Inoculum	Retained Media w/o inoculation	<i>E. coli</i> ATCC 25922	<i>Shigella flexneri</i> ATCC 12022
Batch Size	1	1	1
Specification	Sterility	Growth Promotion/ Chemical Confirmation	
Result	No growth	+	-
Lysine Decarboxylation Medium	Purple	Purple	Yellow
Expected Result in LDC Tube			

Table 3. Quality control results for known organisms.

Interpretation of oxidase test		
Lysine decarboxylation Interpretation	Description	Appearance
Positive	Appearance of a purple color on the inoculum after 18-24 hours incubation	
Negative	No color change of the inoculum.	

Table 4. Lysine decarboxylation test interpretation for (+) and (-).

Interpretation of oxidase test		
Oxidase Interpretation	Description	Appearance
Positive	Appearance of a dark blue color on the inoculum within 60 seconds.	
Negative	No color change of the inoculum.	

Table 5. Oxidase test interpretation for (+) and (-).

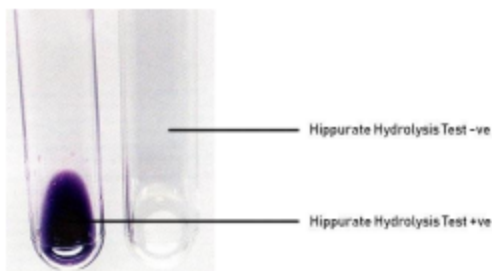


Figure 6. Sodium Hippurate Hydrolysis test (+) and (-) interpretation.

QC Testing				
Testing Frequency	• Upon Preparation • Monthly • Anytime there is sign of suspected contamination			
Incubation Temp/Time	37°C ± 2°C for 18-24 hours			
Inoculum	Retained Media w/o Inoculation	<i>E. coli</i> ATCC 25922	<i>Klebsiella pneumoniae</i> ATCC 13882	<i>Proteus mirabilis</i> ATCC 25933
Batch Size	1	1	1	1
Specification	Sterility	Growth Promotion/Chemical Confirmation		
Motility Results	-	+	-	+
Indole Results	-	+	-	-
Urease Results	-	-	+/- (weak positive)	+

Table 6. Quality Control testing for motility, urease, indole results.



Figure 7. Potential interpretation of motility, urease, and indole test.

Test	Result	Interpretation
Indole	+	Pink color appearing at the interface of the Kovac's and the MIU
	-	Reagent medium interface does not become pink remains the color of the reagent
Motility	+	Cloudiness extending out from the primary stab into the medium
	-	Growth only along the stab line, the remainder of the tube is clear
Urease	+	A color change from yellow-orange to pink-red
	-	No color change indicates a negative reaction

Table 7. Interpretation of unknowns for motility, urease, and indole test



Figure 8. Pattern described for TSI slant inoculation.

Expected QC Results					
Testing Frequency	• Upon Preparation • Monthly • Anytime there is sign of suspected contamination				
Incubation Temp/Time	37°C ± 2°C for 12-24 hours				
Inoculum	Retained Media w/o Inoculation	<i>E. coli</i> ATCC 25922	<i>Proteus mirabilis</i> ATCC 12453	<i>Shigella flexneri</i> ATCC 12022	<i>Salmonella</i> Typhimurium ATCC 14028
Batch Size	1	1	1	1	1
Specification	Sterility	Growth Promotion			
Passing Slant Result	No growth	Yellow	Red/black	Red	Red
Passing Butt Result	No growth	Yellow	Yellow/black	Yellow	Yellow/black
Gas (+/-)	-	+	-	-	+
H ₂ S (+/-)	-	-	+	-	+
Colony Image on TSI Agar Slant					

Table 8. Quality Control results of known organisms.



Interpretation		
Red/Pink: Alkaline (K) Yellow: Acid (A) Bubbles: Gas (G) Black precipitate: H ₂ S		
Result (Slant/Butt)	Symbol	Interpretation
Red/Yellow	K/A	Glucose fermentation only, peptone catabolized
Yellow/Yellow	A/A	Glucose and Lactose and/or sucrose fermentation
Red/Red	K/K	No fermentation, peptone catabolized under aerobic and/or anaerobic conditions
Yellow/Yellow + bubbles	A/A, G	Glucose and lactose and/or sucrose fermentation, gas produced
Red/Yellow + bubbles	K/A, G	Glucose fermentation only, gas produced
Red/Yellow + bubbles + black precipitate	K/A, G, H ₂ S	Glucose fermentation only, gas produced, H ₂ S produced
Yellow/Yellow + bubbles + black precipitate	A/A, G, H ₂ S	Glucose and lactose and/or sucrose fermentation, gas produced, H ₂ S produced
Red/Yellow + black precipitate	K/A, H ₂ S	Glucose fermentation only, H ₂ S produced
Yellow/Yellow + bubbles + black precipitate	A/A, H ₂ S	Glucose and lactose and/or sucrose fermentation, H ₂ S produced

Table 9. Interpretation of TSI slant results for unknown bacteria.

Specimen Reception and Accessioning (1 of 6)

1 PURPOSE

This procedure provides instruction for reception and accessioning specimens collected from participants and environmental samples enrolled in the GETCampy-Africa study upon arrival in the laboratory.

RESPONSIBILITIES

The selected laboratories should adhere to the established protocols outlined and agreed upon by the investigators of this study. The head of the laboratory or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed.

ABBREVIATIONS

- **CBA:** *Campylobacter* Blood Agar
- **TCBS:** Thiosulfate–citrate–bile salts–sucrose agar
- **XLD:** Xylose Lysine Deoxycholate agar
- **mCCDA:** Modified Charcoal Cefoperazone Deoxycholate Agar

2 SAFETY

Rectal swabs/stool specimens may contain microorganisms that are hazardous to the health and well-being of personnel handling the specimens, their colleagues, and their families. Laboratory safety guidelines including those listed below must be followed along with institutional and governmental regulations. The following safety precautions are recommended for every stage of the rectal swab/stool/animal/environmental or other sample work-up.

- 2.1
 - Always employ universal precautions.
 - Utilize techniques and /or equipment that will protect the operator from aerosols.
 - Handle all pathogenic bacteria and rectal swab/stool/animal or environmental samples inside of a biological safety cabinet.
 - Do not put anything in the mouth during sample or culture manipulations.
 - Ensure that appropriate Personnel Protective Equipment (PPEs) such as gloves, laboratory coats etc., are always worn during stool or culture manipulations.
 - Wash hands with disinfectant soap and water after handling or working with samples or cultures.
 - Disinfect work surfaces before and after use.
 - As much as is feasible use disposable equipment, decontaminate reusable instruments after use.
 - Decontaminate biological waste before disposal.
 - Dispose of toxic chemical waste as prescribed by your institution.

3 EQUIPMENT

- -80°C freezer
- Refrigerator (4°C)
- Biological Safety Cabinet II

MATERIALS

- Cooler box containing thermometers to monitor temperature and specimens.
- Stool/environmental specimens in appropriate containers.
- Rectal/fecal swab in Cary-Blair (CB)
- Cryogenic vials (Sarstedt 2.0ml screw cap microtube or equivalent)
- Storage boxes for cryogenic vials and swabs (Nalgene or equivalent)
- Barcoded labels

4 PROCEDURES

Specimen Receipt:

Review the information on the sample labels and ensure that the information on the labels match that on the collection form CRF14a/CRF14b (Rectal swab/stool/environmental). If a physical copy is not available, review the participant data entered in the Stool Collection Form in REDCap.

Take note of the sample types recorded on the form/in REDCap versus actual samples delivered to the lab.

If any discrepancies between records are identified, immediately contact the clinical/field staff to harmonize the information. Continue processing the sample and record any changes made on the relevant document before inputting the data into the data management system.

Complete CRF15a/CRF15b (Specimen reception Form) using the following steps:

- **(1):** Record the Date, Participant ID, time of reception, and temperature at arrival in the laboratory logbook.
- **(2):** Ensure that the identification numbers and other information on the labels of all samples (dry swab/stool/animal/ environmental) match.
- **(3):** Ensure that the specimens are properly labeled, packaged and tightly shut. Ensure that the specimen containers are intact. If the integrity of the sample has been compromised, please make a comment in CRF15a/CRF15b (Specimen reception Form).
- **(4):** Ensure that samples are transported within 2-4°C.
- **(5):** Ensure that the specimens are delivered to the lab within 12 hours of collection.
- If all criteria are met **(1-5)** the specimen is acceptable for accession. If any criteria are not met, contact the clinical/ field staff immediately to inform them and to resolve any discrepancies. If discrepancies can be resolved, proceed with sample processing. If discrepancies cannot be resolved, the sample should be discarded.

- 4.1 Either use the Participant ID to label the samples or create a Specimen ID for each sample using the specified numbering system. If possible, generate a barcoded label with the Participant ID/Specimen ID, date and specimen type. Place the label on the relevant vials/swabs. Prepare enough labels for all the possible sample types and make the following aliquots (see Figure 1):

- Rectal swab
- Whole stool aliquot 1
- Whole stool aliquot 2 (when available)
- Whole stool aliquot 3 (when possible)
- Environmental sample aliquot 1
- Environmental sample aliquot 2 (when possible)
- Environmental sample aliquot 3 (when possible)

Log the unique identification number in the appropriate data management system.

Note: aliquots 2 and 3 are optional.

4.2 Specimen processing for stool culture:

Note: the 'Time processed in lab' must be less than or equal to 12 hours after sample collection. If the time exceeds 12 hours, record on CRF15a/CRF15b (Specimen reception Form) and continue processing.

1. Inoculate samples on culture media and incubate media for bacteriological culture as per SOP0002 (Campylobacter culture) and SOP0004 (Other enteric bacterial pathogens culture).
2. Record the date and time of plating the samples onto the primary isolation media and aliquoting of stool and environmental samples (as needed).
3. After inoculation of primary isolation media, store the swabs and aliquots of stool and environmental samples in the refrigerator (2-8°C) for one week before discarding in case of suspected problems with primary isolation and the need to repeat the culture.

4.3 Specimen processing for storage of samples:

Note: Sample collection to storage at minus 80°C must be ≤12 hours. If the time exceeds 12 hours, record on CRF 15a/15b, and continue processing.

Whole stool/animal/environmental sample storage:

1. Aliquot 0.5 g or 0.5 ml (+/-0.02 g or ml) into a cryogenic vial that is compatible (Sarstedt 2.0 screw cap micro tube or equivalent) for repeat or future testing if needed.
2. Store samples at -80°C.

5 DOCUMENTATION

- All completed forms must be reviewed and signed by the appropriate supervisor after which they will be loaded onto the EpiCollect database within one week.

- All deviations to this protocol must be documented and reviewed by the appropriate supervisor.
- Any quality incidents must also be documented and reviewed by the appropriate supervisor.
- All forms and documentation must be filed appropriately for future reference.

Specimen Storage of Bacterial Isolates (2 of 6)

6 INTRODUCTION

TSB Broth with 15% glycerol is recommended for the preservation of microorganisms by freezing. Glycerol is needed to maintain viability of microorganisms and to protect cell membranes. Glycerol acts as a cryoprotective agent and allows for long-term bacterial preservation. Any isolates that have been biochemically confirmed as bacterial pathogens must be stored.

PURPOSE

This protocol describes the procedure for freezing isolates for storage. Isolates collected should be biochemically confirmed *Campylobacter*, *E. coli*, *Salmonella*, *Shigella* and *Vibrio* spp.

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The selected laboratories should adhere to the established protocols outlined and agreed upon by the investigators of this study. The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the use of this technique.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

7 MATERIALS

- TSA or other non-selective agar plates
- Cryovials containing 0.5 ml of TSB with 15% glycerol
- Freezer boxes
- Sterile inoculating loops (Fisher, Cat # 12-544-1 or equivalent)
- Cryolabels or markers

EQUIPMENT

- Biological safety cabinet II
- Freezer $-80^{\circ}\text{C} \pm 10^{\circ}\text{C}$
- Incubator, $35-37^{\circ}\text{C} \pm 2^{\circ}\text{C}$

- Vortex

8 PROCEDURES

Confirmed bacterial pathogens should be stored for future use according to the following instructions:

1. Subculture on TSA
2. Using a sterile loop pick an isolated colony of the identified bacterial pathogen.
3. Subculture the confirmed bacterial pathogens on fresh TSA plate using a 4-streak method.
4. Invert and incubate the plate for 18-24 hours at $37 \pm 2^{\circ}\text{C}$.

Storage

1. Label 2 cryovials containing 0.5 ml of TSB with 15% glycerol for each sample ID with a confirmed bacterial pathogen. Labels should contain a barcode (where barcode labels are available), patient ID, Organism name and date of storage.
2. Within a BSC, using a sterile loop collect 2-3 loopfuls of bacteria from each organism.
3. Place the loopful of bacteria into the cryovial containing 0.5 ml TSB with 15% glycerol.
4. Mix well by vortex and freeze immediately at -80°C .
5. Record location in a storage map.

Bacterial Culturing from Sample Collection - *Campylobacter* spp. (3 of 6)

9 INTRODUCTION

The major reason for the culture of rectal swab/stool/animal and environmental samples is the isolation and identification of agents of microbiological origin that might be implicated in causing diarrhoeal diseases. This protocol focuses on the diarrhoea causing Gram-negative bacteria *Campylobacter* species. In this study, *Campylobacter* species will be fully identified and further differentiated into *Campylobacter jejuni*, *Campylobacter coli* or other *Campylobacter* species for all MAD cases, healthy controls, household members, animal and environmental samples. To isolate *Campylobacter* species, specimens are inoculated onto *Campylobacter* Blood-Free Selective Agar (Modified CCDA). Cary Blair (CB) media will be used as transport media for the rectal swab and aliquots of whole stool, if collected. After the specimen is plated on mCCDA media, it must be incubated in an environment that provides the optimum conditions for growth of *Campylobacter* species. The optimum growth temperature for *Campylobacter* spp. is 42°C in microaerophilic gas mixture conditions (5% Oxygen, 10% Carbon dioxide and 85% Nitrogen).

PURPOSE

This protocol describes the procedure of clinical microbiology culture for isolating

Campylobacter species.

RESPONSIBILITIES

The laboratories should adhere to the established protocols outlined and agreed upon by the investigators of this study. The head of the laboratory or his/her designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed. The head of the laboratory team or his/her designee is also responsible for reviewing planned deviations with study investigators prior to their use, documenting deviations accordingly and ensuring that accurate methodology is employed so that if *Campylobacter* species is present in a rectal swab/stool/animal/environmental specimen it will be isolated and accurately identified.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

9.1 EQUIPMENT

- Refrigerator (4°C)
- Incubator (42°C)
- Biological Safety Cabinet II
- -80°C freezer

MATERIALS

- Supplies for culture
- Sterile swabs (Fisher Cat # 14-959-102 or equivalent)
- Bacteriological inoculating loops (Nunc or equivalent)
- Bacteriological needle (Nunc or equivalent)
- *Campylobacter* Blood-Free Selective Agar (mCCDA) (Oxoid Cat # CM0739), CCDA selective supplement (Oxoid Cat #SR0155E) or *Campylobacter* blood agar (Oxoid Cat # CM06890) supplemented with *Campylobacter* Growth Supplement (Oxoid Cat # SR0232E) and 5% sheep blood.
- Catalase reagent (3% Hydrogen Peroxide)
- Oxidase reagent (1% tetramethyl-p-phenylenediamine dihydrochloride)
- Gram stain
- Frosted microscope slides
- Fresh Stool Specimen/Rectal Swab/animal/environmental swab
- Stool-containing swab in Cary-Blair medium
- Whole stool
- Environmental sample
- Animal sample
- Trypticase Soy broth with 15% TSB glycerol in cryovials

9.2 PROCEDURES

NOTE 1: Ensure that the culture procedures are well recorded for each PID, and isolate processed throughout the stool culture workflow.

NOTE 2: Perform Quality Control (QC) for all culture media upon preparation and if required.

NOTE 3: Ensure that all culture media and reagents pass quality control test before use.

Inoculation and incubation of primary media:

- If no media control test has been performed within 30 days of isolation, perform QC testing concurrently with the rectal swab/stool/environmental sample culture.
 - Label the back of freshly prepared plates with the culture medium type (mCCDA or *Campylobacter* blood agar), sample identification number for each specimen and date of plating.
1. Use one (1) *Campylobacter* Blood-Free Selective Agar (mCCDA) plate supplemented with CCDA selective supplement (Oxoid Cat #SR0155E) or *Campylobacter* blood agar, CBA, (Oxoid Cat # CM06890) supplemented with *Campylobacter* Growth Supplement (Oxoid Cat # SR0232E) and 5% sheep blood.
- Inoculate all primary isolation media from CB and whole stool/animal/environmental samples using the following instructions.
1. Make a smear of the specimen on the mCCDA or CBA using either the CB swab or a 10µL loop to inoculate whole stool samples onto mCCDA or CBA plates.
 2. Streak each plate using the four-phase streak method for isolation.
 3. Use the appropriate rectal swab to perform the first streak.
 4. Use a sterilized loop for the remaining three quadrants. Figure 2 below describes the four-phase streak method.
 5. Use a new surface of the loop for each quadrant if using a plastic loop or sterilize loops between quadrants if using metal loops.
 6. See Figure 3.
 7. Each numbered quadrant (2-4) indicates the point of use for a newly sterilized loop. See Appendix A for overview of workflow.
 8. Invert and incubate mCCDA or CBA agar plates for 48 hours in microaerophilic gas mixture conditions (5% Oxygen, 10% Carbon dioxide and 85% Nitrogen) at 42°C.
 9. If no colonies are observed after 48 hours incubation, the sample is considered negative for *Campylobacter* spp.

Identification:

1. After 48 hours incubation examine plates for growth.
2. Using a sterile loop select and pick colonies suggestive of *Campylobacter* spp. following Figure 4, streak them on fresh Blood Agar (BA) plates and incubate at 42°C for 24 hours.

3. After incubation, perform biochemical tests on pure colonies as outlined in Figure 4 to identify, confirm, and differentiate *Campylobacter spp.*
4. Store *Campylobacter spp.* in Trypticase Soy broth with 15% TSB glycerol stocks in cryovials isolates at -80°C for sequencing.

9.3 DOCUMENTATION

- All completed forms for culture results must be reviewed and signed by the appropriate supervisor after which they will be loaded onto the EpiCollect database within one week.
- All deviations to this protocol must be documented and reviewed by the appropriate supervisor.
- Any quality incidents must also be documented and reviewed by the appropriate supervisor.
- All forms and documentation must be filed appropriately for future reference.

Gram Staining (4 of 6)

10 INTRODUCTION

The Gram stain is one of the most commonly used staining procedures in microbiology to identify bacteria. It helps to divide bacteria into two groups based on their cell wall and the ability to retain the primary stain, crystal violet. The thick cell wall of Gram-positive bacteria contains a thick peptidoglycan whereas Gram-negative bacteria have a thin layer of peptidoglycan beneath the outer membrane.

The Gram stain assay uses four reagents crystal violet (basic purple dye), iodine, alcohol (acetone alcohol) and safranin (counter stain). When the crystal violet primary stain is added to a heat-fixed bacterial smear, it enters the cytoplasm and stains the cell wall of both Gram-positive and Gram-negative bacteria purple. Iodine forms a complex with the crystal violet (crystal violet-iodine complex) that is too large to escape through the cell wall of bacteria with thick layers of peptidoglycan. The decolorizer alcohol dehydrates the Gram-positive cell making it difficult for the crystal violet-iodine complex to escape. Alcohol or acetone (decolorizer), which is added after the iodine, dissolves the outer membrane of Gram-negative bacteria and forms pores in the thin peptidoglycan, thus facilitating the easy escape of the crystal violet-iodine complex. Gram-positive bacteria remain purple after the alcohol de-staining while Gram-negative bacteria lose the purple color and are subsequently stained by the red dye safranin.

PURPOSE

This protocol describes the procedure for performing Gram's staining, a procedure for the determination of the Gram reaction and cellular morphology of bacteria and helps to differentiate bacteria into Gram-positive and Gram-negative bacteria. For the GETCampy study, our main interest is to observe Gram negative straight and spiral rods, characteristic of *Aeromonas spp.*, *Campylobacter spp.*, *Salmonella spp.*, *Shigella spp.* and *Vibrio spp.* This test is preliminary and considered optional for characterization of

other target bacteria except for *Campylobacter* spp.

GUIDANCE

All modifications to this procedure must be documented clearly.

RESPONSIBILITIES

The participating laboratories should adhere to the established protocols outlined and agreed upon by the investigators of this study. The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the use of this technique.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

11 MATERIALS

- Sterile Saline (Fisher, Cat # 48212-270)
- Inoculation Loop (Fisher, Cat # NC9980207)
- Plastic Pasteur pipette
- Frosted glass slide
- Microscope slide (Fisher, Cat # 12-544-1)
- Fresh bacterial culture
- Filter Paper
- Forceps
- Staining Tray
- Water

Gram staining Reagents Kit (BD, Cat # 212539)

1. Crystal Violet (BD, Cat # 212525)
2. Iodine (BD, Cat # 212542)
3. Gram Decolorizer (BD, Cat # 212527)
4. Safranin (BD, Cat # 212531)

Quality Control Organisms

- *Escherichia coli* ATCC 25922
- *Staphylococcus aureus* ATCC 25923

12 PROCEDURES

Quality control procedure

- Heat-fixed smear preparation
- Label a clean glass slide each with control strains of *E. coli* and *S. aureus*.
- Add 2 small drops of sterile saline to the slide. This can be done by using a plastic Pasteur pipette or using an inoculation loop to transfer the saline to the slide.
- With an inoculation loop or needle, pick up a small amount of the QC organisms (*E. coli* ATCC 25922 and *S. aureus* ATCC 25923). Note: To obtain a successful Gram

stain only a small number of bacteria is required. Simply touching a colony should be sufficient to avoid a dense smear which will prevent proper visualization.

- Suspend the bacteria in the saline and spread the mixture over a wider area of the slide.
- Air dry the bacterial specimen smear on the slide.
- When slides are completely dry, heat fix the bacterial specimen by passing the slide slowly over the flame twice.
- Heat fixing kills cells adheres them to the slide.
- Cells will be rinsed off the slides if they are not heat fixed properly.
- Be careful not to overheat the slides in this procedure. Overheating ruptures and distorts the cell wall.
- After heat-fixing is complete proceed to the Gram stain procedure below.

Gram stain procedure

1. Place a microscope slide containing labeled heat-fixed and cooled smear on a staining rack.
2. Flood the smear with crystal violet and allow it to rest for one minute.
3. Using a gentle flow of water wash the crystal violet off the slide.
4. Flood the slide with iodine and allow it to remain for one minute.
5. Wash the slide with gentle flow of water.
6. Hold the slide at an angle with forceps and pour alcohol from the top of the slide so that it runs over the entire smear. Wash the slide with acetone/alcohol until blue stain stops running off the smear (approximately 10 seconds). **Note:** De-staining for too long will result in all bacteria appearing Gram-negative.
7. Immediately wash the slide with water.
8. Flood the slide with the counter stain safranin for three minutes.
9. Wash with water.
10. Blot the slide with filter paper or allow the slide to air dry and read under the microscope under oil immersion (100X magnification).

INTERPRETATION

- **Table 1** shows the expected results for the QC organisms.
- **Table 2** shows the interpretation for *Campylobacter* spp. and other enteric pathogens.

13 NOTES

- There are many modifications of the Gram stain. Therefore, always follow the manufacturer's instructions and document the method used.
- New batches of stains must be tested by using quality control organisms before use for testing unknown specimens. Run control tests once a week alongside unknown specimens.
- Bacteria could be over-decolorized therefore it is important to decolorize smears individually.

Biochemical Tests (5 of 6)

14 Catalase Test

14.1 INTRODUCTION

Catalase is an enzyme that is found in most living aerobic and facultative anaerobic bacteria which used it to decompose peroxides formed by the oxidative breakdown of sugars. The buildup of peroxides is toxic to the bacterial cells. Catalase decomposes hydrogen peroxide into oxygen and water. Strict safety precautions should be employed because aerosols are released during the performance of this test.

PURPOSE

This protocol describes the procedure for detecting the presence of the enzyme catalase in bacteria.

GUIDANCE

All modifications to this procedure must be documented clearly.

RESPONSIBILITIES

The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the preparation of the Catalase reagent and use of this test.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

14.2 MATERIALS

- 3% Hydrogen peroxide
- Glass slide
- Plastic Pasteur pipette
- Plastic bacteriological loop
- Isolated bacterial colony on fresh bacterial agar plate

Quality Control Organisms

- *Escherichia coli* ATCC 25922 (Catalase negative)
- *Streptococcus pneumoniae* ATCC 49619 (Catalase negative)
- *Staphylococcus aureus* ATCC 25923 (Catalase positive)

EQUIPMENT

- Incubator

PROCEDURES

Quality control procedure:

1. Using a sterile Pasteur pipette place a drop of 3% hydrogen peroxide on a clean glass slide.
2. Use a sterile bacteriological loop to pick up a small amount of the QC organisms (*E. coli* ATCC 25922, *S. pneumoniae* 49619 and *S. aureus* ATCC 25923) from an overnight culture plate.
3. Place the plastic bacteriological loop with the bacterial colony on the 3% hydrogen peroxide on the glass slide.
4. Observe for bubbles.

Testing unknown organisms/ isolates:

1. Using a sterile Pasteur pipette place a drop of 3% hydrogen peroxide on a clean glass slide.
2. Use a sterile bacteriological loop to pick an isolated colony of unknown bacteria from an overnight culture plate.
3. Place the plastic bacteriological loop with the bacterial colony on the 3% hydrogen peroxide on the glass slide.
4. Observe for bubbles

INTERPRETATION

- Positive result: production of bubbles within 10 seconds of contact between bacterium and 3% hydrogen peroxide.
- Negative result: No bubbles

See Figure 5 for reference images of catalase (+) and (-).

14.3 NOTES

- The test can be done using a test tube.
- New batches of reagents must be tested by using quality control organisms before use for testing unknown specimens.
- Run control tests once a week alongside unknown specimen.
- A positive catalase test produces many aerosols, as such, caution must be exercised in the performance of the test.
- Catalase activity is present only in viable cells therefore, do not use cells older than 24 hours because false negative results might occur.
- Red blood cells demonstrate catalase activity, therefore do not pick colonies from blood agar plates or a false positive reaction can occur.
- Colonies from chocolate agar can be used for catalase testing.
- Hydrogen peroxide may cause irritation in the skin, respiratory tract and eyes.

- Nichrome wire bacteriological inoculating loops can cause a false positive result when placed in hydrogen peroxide.

15 Lysine Decarboxylase Test

15.1 INTRODUCTION

The lysine decarboxylase test is used primarily for the identification of both fermentative and non-fermentative Gram-negative bacteria. It is especially important in the differentiation of *Salmonella spp.* and *Shigella spp.* from other members of the family *Enterobacteriaceae* and for the identification and differentiation of *Aeromonas spp.* and *Vibrio spp.* Bacteria may produce enzymes that permit them to utilize certain amino acids, such as lysine, as a nutrient source. Such enzymes break down amino acids via removal of a carboxyl group resulting in the generation of alkaline metabolic byproducts. When the amino acid lysine is decarboxylated it forms carbon dioxide and cadaverine. In this assay, the medium contains lysine as the potential substrate for lysine decarboxylase (if produced by the test bacterium). Glucose is the fermentable carbohydrate and pyridoxal phosphate is a coenzyme. Taylor's modification of the medium shows an improved performance over the formulation described by Falkow. This is achieved by omitting peptone from the medium, thus eliminating false positives. Taylor's modification makes the medium relatively easy to control without the requirement of anaerobic culture and low pH. During the initial stages of incubation, fermentation of glucose by the organism, with acid production, results in a color change to yellow. On further incubation, if lysine is decarboxylated to cadaverine, there will be an alkaline reaction. The color then changes to purple (positive). If the color remains yellow, the reaction is negative.

PURPOSE

This protocol describes the test procedure to determine whether a bacterium can use the amino acid lysine as a source of carbon and energy for growth. This biochemical test is particularly useful for differentiation of *Salmonella* and *Shigella spp.*

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the preparation of the Lysine Decarboxylase reagent and use of this test.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

15.2 MATERIALS

Reagents for Preparing Medium

- LDC Dehydrated Agar (Thermofisher, Cat # CM0308S or equivalent)
- Deionized (DI) Water
- Autoclaved bottle
- Stir Bar
- Sterile/autoclavable transparent container with cap to hold 1–5 ml of media

Quality Control Organisms

- *Escherichia coli* ATCC 25922
- *Shigella flexneri* ATCC 12022

EQUIPMENT

- Biological Safety Cabinets (BSC)
- Autoclave
- Incubator
- Refrigerator
- Stir Plate

15.3 PROCEDURES

Media preparation

Prepare the medium according to the manufacturer's instructions. The general steps are:

1. Ensure that the required reagents have not expired.
 2. Measure out half the amount of deionized water needed for the final volume to be prepared and place in an autoclavable bottle. Note: Select a bottle that when filled, will not boil over while autoclaving.
 3. Take out the number of LDC tabs needed for the final desired volume and put them in the bottle. Mix well using a stir bar and stir plate. Ensure that there are no remaining granules of medium remaining before proceeding.
 4. Check and record the manufacturers' recommendation for the pH of the medium and adjust, if necessary, (following the *, **).
 5. Record the final pH
 6. Make up to the final volume with DI water. Mix well.
 7. Autoclave the LDC medium at 121°C and 15 psi.
 8. Allow, medium to cool to 55 °C in a water bath.
 9. In a BSC, aliquot about 0.5–4 ml of the medium into sterile Sarstedt tubes.
 10. Store at 4°C or room temperature.
 11. Follow the manufacturer's recommendation for expiry of prepared media. If no expiry provided, discard and do not use media 90 days after preparation.
 12. Perform QC testing at required intervals or with testing of unknown samples.
- *: Measure the pH using narrow range pH strips or a pH meter.

- **: If the pH is too acidic (low pH), adjust to the required pH using sodium hydroxide. If the pH is too alkaline (high pH), adjust to the required pH using hydrochloric acid.

Quality control:

Quality control testing is only required for media prepared by the laboratory, commercially procured medium without an expiry date, or for commercially procured medium suspected of contamination.

1. Test 3 tubes of lysine decarboxylate medium for growth promotion/chemical confirmation (2 tubes) and sterility (1 tube).
2. Inoculate 2 tubes (separately) with each of the QC organisms *E. coli* ATCC 25922 and *S. flexneri* ATCC 12022 using a bacteriological needle/straight wire. Inoculate the medium by stabbing deep into the bottom of the tube. Note: QC strains should be taken from TSA agar plates.
3. Retain an additional tube of the prepared batch of media.
4. Incubate the inoculated (growth promotion/chemical confirmation QC) and uninoculated (sterility QC) tubes of medium at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 18 - 24 hours.
5. The following day record the results of the QC testing.
6. QC testing should be performed upon preparation, monthly and anytime there is suspected contamination.
7. Both growth promotion/chemical confirmation and sterility must pass to release the batch of media. See Table 3 for expected results.

Testing of unknown samples:

1. Confirm that LDC medium is not expired and has been tested for sterility and growth promotion in the past 30 days. **Note:** If media has expired and/or growth promotion was performed more than 30 days prior, proceed at risk but perform the QC testing along with the test sample(s).
2. Label 1 tube of LDC broth. Label with transport medium, accession number, colony/PID number and date.
3. Using a sterile needle pick growth from an isolated bacterial colony.
4. Inoculate the medium by stabbing deep into the bottom of the tube.
5. Cover each tube and incubate each tube aerobically at $37 \pm 2^{\circ}\text{C}$ for 18-24 hours.
6. Read and record results of LDC medium for each sample. Interpret results using Table 4.

INTERPRETATION

See Table 3 and Table 4 for quality control organisms and testing unknown samples.

15.4 NOTES

All media, even those that have been sterility tested at the time of preparation, should always be checked visually immediately before being inoculated for any change in appearance that could indicate contamination or deterioration

16 Oxidase Test

16.1 INTRODUCTION

The oxidase test is used to determine whether a bacterial strain produces the oxidative enzyme cytochrome oxidase. In the bacterium, cytochrome c is involved in the transfer of electrons from one protein to another. In this test, the substrate is tetramethyl-p-phenylenediamine dihydrochloride, a colorless reagent. If the bacterium produces cytochrome oxidase it will oxidize the colorless substrate to indophenol which is dark blue in color. The test is used primarily for the differentiation of species in the family *Enterobacteriaceae* (oxidase-negative) from oxidase-positive bacilli such as *Aeromonas spp.*, *Campylobacter spp.*, *Pseudomonas spp.*, and *Vibrio spp.*

PURPOSE

This protocol describes the procedure detecting the production of the enzyme cytochrome oxidase by bacteria.

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the preparation of the Catalase reagent and use of this test.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

16.2 MATERIALS

- Dry Filter paper soaked with 1% tetramethyl-p-phenylenediamine dihydrochloride (Thomas Sci, Cat # C929C01)
- Sterile wooden applicator (Fisher, Cat # 23-400-102 or equivalent)
- Bacterial isolate on Tryptic Soy Agar (TSA)

Quality Control organisms

- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853

EQUIPMENT

- Incubator

16.3 PROCEDURES

Quality control procedure:

1. Quality control testing is required each time the test is to be performed.
2. Using a sterile wooden applicator stick touch the top of an isolated bacterial colony of *E. coli* ATCC 25922 (negative control) and *P. aeruginosa* ATCC 27853 (positive control) grown overnight on a non-selective media such as TSA.
3. Transfer the collected culture to the filter paper by rolling the wooden applicator stick onto the soaked filter paper.
4. Examine for the development of a color change (darker blue for positive) within 60 seconds.

Testing unknown organisms/ isolates:

1. Using a sterile wooden applicator stick touch the top of an isolated bacterial colony grown overnight on a non-selective media such as TSA.
2. Transfer the collected culture to the filter paper by rolling the wooden applicator stick onto the soaked filter paper.
3. Examine for the development of a color change (darker blue for positive) within 60 seconds.

INTERPRETATION

See Table 5 for visual interpretation of an oxidase (+) and (-).

NOTES

- Read the test within a minute of application because the reagent can become oxidized after lengthy exposure to air, thereby giving a false positive result.
- Nichrome wired loops and needles loops give a false positive result, if necessary, use platinum or plastic loops.
- Comparison to known positive and negative controls is particularly important in this assay to determine that the application of reagent has been conducted correctly. Incorrect timing or amount of reagent can result in false results.

17 Sodium Hippurate Hydrolysis Test

17.1 INTRODUCTION

The ability of *Campylobacter jejuni* to produce Hippurate hydrolase is used to differentiate this species from other *Campylobacter* species. Hippurate hydrolase is an amidohydrolase which cleaves the benzoyl group from Hippurate (N-benzoyl glycine). In sodium Hippurate the by-products are glycine and sodium benzoate. Ninhydrin is an indicator that reacts preferentially with the α -amino group of the substances resulting in a purple-blue color. In the Hippurate hydrolysis test, ninhydrin reacts with the glycine

residue to produce a purple color. Thus, a positive reaction results in the media changing to a dark blue-purple color. Interpretation of this test is facilitated by comparison with uninoculated media.

PURPOSE

This protocol describes a procedure for differentiating *Campylobacter jejuni* from other species of *Campylobacter*.

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the preparation of the sodium Hippurate solution reagent and use of this test.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

17.2 MATERIALS

- 1% solution of sodium Hippurate preparation:
 1. Aseptically, dissolve 1g of sodium Hippurate in 100 mL of water.
 2. Dispense 0.4 mL into sterile cryotubes with stoppers.
 3. Store at -20°C until ready for use.
- Preparation of Ninhydrin solution
 1. Prepare a 1:1 mixture of acetone and butanol.
 2. Dissolve 0.350 g of ninhydrin in 10 mL of the 1:1 solution of acetone and butanol
- Sterile 10µL bacteriological inoculating loop
- 48- hour growth of *Campylobacter* spp.
- Pipette.

Quality Control organisms

- *Campylobacter jejuni* ATCC 33560 or ATCC 33291
- *Escherichia coli* ATCC 25922

EQUIPMENT

- Incubator

17.3 PROCEDURES

Quality control procedure

Quality control testing is required each time the test is to be performed.

1. Thaw a tube of Sodium Hippurate.
2. Using a sterile bacteriological loop pick three to four colonies of growth from a 24-48hour culture of *C. jejuni* ATCC 33560 or ATCC 33291 (positive control) on blood agar and emulsify them in a tube of Sodium Hippurate to form a heavy suspension.
3. Using a sterile bacteriological loop pick three to four colonies of growth from a 24hour culture of *E. coli* ATCC 25922 (negative control) on Tryptic Soy Agar (TSA) and emulsify them in a tube of Sodium Hippurate to form a heavy suspension.
4. Incubate the suspension at 37°C for two hours with intermittent shaking to mix the contents.
5. After incubation, add 0.2mL of ninhydrin reagent. **DO NOT MIX**
6. Re-incubate the tube at 37°C for 10 minutes.
7. Examine the tubes for color change

Testing unknown organisms/ isolates

1. Thaw a tube of Sodium Hippurate.
2. Using a sterile bacteriological loop pick three to four colonies of growth from a 24-48hour culture of the suspected *Campylobacter* strain on blood agar and emulsify them in a tube of Sodium Hippurate to form a heavy suspension.
3. Incubate the suspension at 37°C for two hours with intermittent shaking to mix the contents.
4. After incubation, add 0.2mL of ninhydrin reagent. **DO NOT MIX**
5. Re-incubate the tube at 37°C for 10 minutes.
6. Examine the tubes for color change.

INTERPRETATION

- Positive result: Appearance of a deep purple color.
- Negative result: Light purple or no color change.

See Figure 6 for Sodium Hippurate Hydrolysis (+) and (-).

NOTES

Ninhydrin will temporarily stain the skin blue if it comes into contact.

Growth Agar Tests (6 of 6)

18 Motility, Urease, and Indole Test

18.1 INTRODUCTION

Motility Indole Urea (MIU) medium is a differential medium used primarily for the identification of *Enterobacteriaceae* and is prepared as a deep semi-solid tubed medium. Its main components are peptones, urea, casein enzymic hydrolysate, sodium chloride,

dextrose, and phenol red. Peptones of the medium provide carbon and nitrogen required for the growth of bacteria. Urea is used as a source of nitrogen for those organisms that possess the enzyme, urease. Casein enzymic hydrolysate provides amino acids and other nitrogenous substances. Sodium chloride maintains osmotic equilibrium. Dextrose is a fermentable carbohydrate. Phenol red is the pH indicator that turns pink, red in alkaline conditions. Urease is indicated by a color change of the pH indicator, Phenol Red, from yellow orange (pH 6.8) to red, pink (pH 8.4). The low agar concentration (i.e., 0.2%) is useful for the demonstration of motility of bacteria. The indole production from casein enzymic hydrolysate by the tryptophanase present in test organisms is achieved upon the addition of aldehyde present in Kovac's reagent; shown by the appearance of a pink-red color ring in the tube. The test organisms in MIU agar after incubation show either diffused growth or turbidity extending away from the stab inoculation line in the case of motile organisms while non-motile organisms appear as restricted growth along the stab-line. Organisms that possess urease, hydrolyze urea, and release ammonia and carbon dioxide. Ammonia reacts in solution to form ammonium carbonate, which is alkaline leading to an increase in pH of the test medium. Phenol red present in the medium changes its color from yellow to pink, red in alkaline pH. Indole, skatole, and indole acetic acid are produced from tryptophan present in casein enzymic hydrolysate by the enzyme, tryptophanase. The indole formed reacts with p-dimethyl amino benzaldehyde present in Kovac's reagent to form a quinoidal red, violet compound.

PURPOSE

The Motility Indole Urea (MIU) test is useful for the identification of Gram-negative bacilli especially bacteria of the *Enterobacteriaceae* family. These three tests in a single tube can help to differentiate organisms based on motility, urease, and indole production.

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control (QC) measures are employed in the preparation of the Catalase reagent and use of this test.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

18.2 MATERIALS

Reagents for Preparing Medium

- MIU Dehydrated Medium (Biolab, Cat # MIU20500 or equivalent)
- Deionized (DI) Water
- Autoclavable tube 12×75 mm (Fisher, Cat # 22-171-606 or equivalent)
- Stir Bar

- Hydrochloric Acid
- Sodium Hydroxide
- Urea solution (Fisher, Cat # SA54-1 or equivalent)
- Kovac's reagent (Fisher, Cat # 60-014-33 or equivalent)

Quality Control Organisms

- *Escherichia coli* ATCC 25922
- *Klebsiella pneumoniae* ATCC 13882
- *Proteus mirabilis* ATCC 25933

Inoculum Materials

- Bacterial Isolate(s)
- Bacteriological needle/straight wire (Fisher, Cat # NC9925242)
- Tube of MIU medium (prepared or procured commercially)

EQUIPMENT

- Biological Safety Cabinets (BSC)
- Autoclave
- Incubator
- Refrigerator
- Stir Plate
- Scale

18.3 PROCEDURES

Media preparation:

Prepare the medium according to the manufacturer's instructions. The general steps are:

1. Ensure that the required reagents have not expired.
2. Weigh out the recommended amount of dehydrated medium and place in an autoclavable bottle. Note: Select a bottle that when filled, will not boil over while autoclaving.
3. Make up to ~90% of the final volume with DI water. Mix well using a stir bar and stir plate. Ensure that there are no remaining granules of medium remaining before proceeding.
4. Check and record the manufacturers' recommendation for the Ph of the medium and adjust, if necessary, (following the *, **):
5. Record the final Ph on the Media Preparation Log FRM03106.
6. Make up to the final volume with DI water. Mix well.
7. Autoclave the medium at 121°C for 15 minutes at 15 psi or per manufacturer's recommendation.
8. Allow medium to cool to ~50°C using a water bath set to 50°C, or cool enough to hold with your hand to the bottle without burning.
9. Add 5 ml of sterile 40% urea solution per 95 ml of medium.

10. In a BSC, aliquot medium into tubes to a depth of approximately 3 inches.
 11. Allow to cool in an upright position.
 12. Store at 4°C or room temperature
 13. Follow the manufacturer's recommendation for expiry of prepared media. If no expiry provided, discard and do not use media 90 days after preparation.
 14. Perform QC testing.
- *: Measure the Ph using narrow range Ph strips or a Ph meter.
 - **: If the Ph is too acidic (low Ph), adjust to the required Ph using sodium hydroxide. If the Ph is too alkaline (high Ph), adjust to the required Ph using hydrochloric acid.

Quality control procedure:

Quality control testing is only required for media prepared by the laboratory, commercially procured medium without an expiry date, or for commercially procured medium suspected of contamination.

1. Test 4 tubes of MIU medium for growth promotion/chemical confirmation (3 tubes) and sterility (1 tube).
2. Use a sterile needle/straight wire to pick growth from an isolated bacterial colony.
3. Remove the cap and inoculate 1 of each of 3 tubes with each QC organism (*E. coli* ATCC 25922, *K. pneumoniae* ATCC 13883 and *P. mirabilis* ATCC 25933) using a bacteriological needle/straight wire. Inoculate the slant by stabbing the middle of the MIU Agar to a depth of two-thirds of the agar. **Note:** Do not insert the needle/wire to the bottom of the tube.
4. Retain an additional tube of the prepared batch of media.
5. Incubate the inoculated (growth promotion/chemical confirmation QC) and uninoculated (sterility QC) tubes of medium at 37°C ± 2°C for 18-24 hours.
6. The next day read and record the results of the urease reaction and motility before adding Kovac's reagent.
7. Add 3-4 drops of Kovac's reagent to the surface of the MIU.
8. Interpret indole reaction after one minute.
9. Record the results of the QC testing.
10. QC testing should be performed upon preparation, weekly and anytime there is suspected contamination.
11. Both growth promotion and sterility must pass to release the batch of media. See Table 6. for expected results.

Testing unknown organisms/ isolates:

1. Confirm that MIU medium has not expired and has been tested for sterility and growth promotion in the past 30 days. Note: If media has expired and/or growth promotion was performed more than 30 days prior, proceed at risk but perform the QC testing along with the test sample(s).

2. Label 1 tube of MIU. Label with transport medium, accession number, colony number and date.
3. Using a sterile needle pick growth from an isolated colony.
4. Inoculate the medium by stabbing the middle of the MIU Agar to a depth of two-thirds of the tube.
5. Loosely cover each tube and incubate each plate 18-24 hours, aerobically $37 \pm 2^{\circ}\text{C}$ for 18-24 hours.
6. On the following day read and record the results of the urease reaction and motility before adding Kovac's reagent.
7. Add 3-4 drops of Kovac's reagent to the surface of the MIU.
8. Interpret indole reaction after one minute.
9. Read and record results of MIU medium for each sample. Interpret results using Figure 7 and Table 7. Continue processing all suspected *Shigella* spp. per other enteric bacterial pathogens culture SOP0004

INTERPRETATION

See Table 6, Table 7, and Figure 7 for quality control results and interpretation of unknown samples.

NOTES

All media, even those that have been sterility tested at the time of preparation, should always be checked visually immediately before being inoculated for any change in appearance that could indicate contamination or deterioration.

19 Triple Sugar Agar Slant

19.1 INTRODUCTION

Triple sugar iron agar (TSI) is a differential medium used in the identification of Gram-negative bacteria primarily in the family Enterobacteriaceae. The top of the agar slant is an aerobic environment, while the bottom (butt) of the slant is anaerobic. The media contains a small amount of glucose (0.1%) along with 1% each of lactose and sucrose. Phenol red dye indicates pH, turning yellow in acidic conditions and red in alkaline conditions. Sodium thiosulfate and Ferric Ammonium Citrate react with H_2S (if the bacteria produce it) to form a black precipitate. Comparison of the butt and slant colors along with inspection for gas (cracks in the agar) and hydrogen sulfide production (black precipitate) demonstrates the ability of the bacteria to:

1. Metabolize lactose and/or sucrose (top and bottom yellow)
2. Conduct fermentation to produce acid (any yellow)
3. Produce gas as byproduct of fermentation (cracks)
4. Generate H_2S (black precipitate)

The enteric bacteria are facultative anaerobes. If a bacterial species ferments only glucose, very little acid is generated because there is minimal glucose present. Thus, the butt turns yellow, and the slant remains red because more acid is required to change the slant yellow in the presence of oxygen. If the abundant carbohydrates lactose and/or sucrose are metabolized, more acid is generated, and the slant and butt will become yellow. If the bacterium produces gas as a by-product of fermentation the medium cracks. If hydrogen sulfide is produced the ferrous sulfate forms a black precipitate in the butt.

PURPOSE

This protocol describes the procedure for detecting the bacterial fermentation of three sugars and the production of hydrogen sulfide as characteristics used in the identification of Gram-negative bacteria.

GUIDANCE

Please document any modifications of this procedure clearly.

RESPONSIBILITIES

The selected laboratories should adhere to the established protocols outlined and agreed upon by the investigators of this study. The Laboratory Director or designee is responsible for ensuring that accurate methodology and appropriate quality control measures are employed in the use of this technique.

SAFETY

See "Specimen Reception and Accessioning" for appropriate safety precautions.

19.2 MATERIALS

Medium Preparation Material

- TSI Dehydrated Agar (Remel, Cat #454981 or equivalent)
- Deionized (DI) Water
- 16×150 mm autoclavable tube
- Hydrochloric Acid, HCl (Fisher, Cat # SA54-1 or equivalent)
- Sodium Hydroxide, NaOH (Fisher, Cat # 60-014-33 or equivalent)
- Stir Bar

Quality Control Organisms

- *Escherichia coli* ATCC 25922
- *Proteus mirabilis* ATCC 12453
- *Shigella flexneri* ATCC 12022
- *Salmonella Typhimurium* ATCC 14028

Inoculating Materials

- Clinical specimens
- Bacteriological Needle/straight wire (Fisher, Cat # NC9925242 or equivalent)
- TSI Agar Slant (prepared or procured commercially)

EQUIPMENT

- Biological Safety Cabinets (BSC)
- Autoclave
- Incubator
- Refrigerator
- Stir Plate
- pH Meter

19.3 PROCEDURES**Media preparation:**

Prepare the medium according to the manufacturer's instructions. The general steps are:

1. Ensure that the required reagents have not expired.
2. Weigh out the recommended amount of dehydrated medium and place in an autoclaved bottle.
3. Make up to ~90% of the final volume with DI water. Mix well using an autoclaved stir bar and stir plate. Ensure that there are no remaining granules of medium remaining before proceeding.
4. Check and record the manufacturers' recommendation for the pH of the medium and adjust, if necessary, (following the *, ** below):
5. Record the final pH on the Media.
6. Make up to the final volume with DI water. Mix well.
7. In a BSC, aliquot the medium into tubes. Use approximately 7 ml in a 16×150 mm tube. Loosely cap each tube.
8. Autoclave the loosely capped tubes of medium, at 121°C for 15 minutes or per manufacturer's instructions.
9. Slant the tubed, autoclaved medium so that it has approximately a 3.5 cm butt and a 3 cm slant. It is important that the butt be of sufficient depth. Allow the medium to solidify.
10. Examine the medium for a typical red color. Perform sterility and growth promotion check prior to use.
11. Store at 4°C or room temperature.
12. Follow the manufacturer's recommendation for expiry of prepared media. If no expiry provided, discard and do not use media 90 days after preparation.
13. Perform QC testing.
 - *: Measure the pH using narrow range pH strips or a pH meter.
 - **: If the pH is too acidic (low pH), adjust to the required pH using sodium hydroxide. If the pH is too alkaline (high pH), adjust to the required pH using hydrochloric acid.

Quality control:

1. Test 5 TSI agar slants from each batch for growth promotion (4 slants) and sterility (1 slant).

2. Inoculate 1 slant of the prepared batch with each QC organism (*E. coli* ATCC 25922, *P. mirabilis* ATCC 12453, *S. flexneri* ATCC 12022 and *S. Typhimurium* ATCC 14028) using a bacteriological needle/straight wire (Figure 8). Inoculate the slant by streaking the needle tip over the entire surface of the slant in a "Z" and then stabbing deep into the bottom of the slant. Be sure not to crack the agar during inoculation.
3. Retain an additional slant of the prepared batch of media.
4. Incubate the inoculated (growth promotion QC) and uninoculated (sterility QC) slants of medium at $37^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 12– 24 hours.
5. On the following day record the results of the QC testing on the Media Preparation Log FRM03106.
6. QC testing should be performed upon preparation, monthly and anytime there is suspected contamination.
7. Both growth promotion and sterility must pass to release the batch of media. See Table 8 for expected results.

Inoculation method:

1. Confirm that TSI agar slants are not expired and have been tested for sterility and growth promotion in the past 30 days. Note: If media has expired and/or growth promotion was performed more than 30 days prior, proceed at risk but perform the QC testing along with the test sample(s).
2. Label the TSI agar slants. Label with transport medium, accession number, colony number and date.
3. Pick growth from an isolated colony using a sterile needle.
4. Inoculate 1 TSI Agar slant of the prepared batch with each test organism using a bacteriological needle/straight wire (Figure 8). Inoculate the slant by streaking the needle tip over the entire surface of the slant in a "Z" and then stabbing deep into the bottom of the slant. Be sure not to crack the agar during inoculation.
5. Loosely cover the tube and incubate each slant, aerobically $37 \pm 2^{\circ}\text{C}$ for 12–24 hours.
6. Read and record results of TSI slants for each sample. Interpret results using Table 9. Continue processing all suspected *Shigella* spp. (red or pink slant/yellow butt).

INTERPRETATION

See Figure 8, Table 8, and Table 9 for TSI inoculation and interpretation.

NOTES

- TSI contains 0.1% glucose. Glucose fermenting bacteria will utilize the small amount of glucose in the medium within 12–24 hours of inoculation and the reaction in the slant may revert to the original color under alkaline conditions.
- All media, even those that have been sterility tested at the time of preparation, should always be checked visually immediately before being inoculated for any change in appearance that could indicate contamination or deterioration.



Sequencing of Samples

20 To be updated with appropriate changes from manufacturers' protocols.

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Acknowledgements

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