LABORATORY MANUAL IN MICROBIOLOGY

SECOND EDITION

by

THE SRI LANKA COLLEGE OF MICROBIOLOGISTS

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FOREWORD

Sri Lanka College of Microbiologists has once again contributed to an echoing need in the laboratory service in the country by preparing the second edition of the 'Laboratory Manual in Microbiology'.

Today, healthcare activities are very much dependent on laboratory test results. Thus reliable laboratory results have become an asset for patient management and control of diseases in hospitals and community.

The guidelines provided by the second edition of the 'Laboratory Manual in Microbiology' is timely and appropriate as patients seeking healthcare have increased in numbers, as well as in their quest for quality. Therefore laboratory staff should be properly qualified and correctly guided to elicit reliable laboratory data. In keeping with Mahinda Chinthana, the Government has taken several actions to boost the tourism industry. Health-tourism is one such area identified, where foreigners come for affordable and reliable medical care. Our hospitals should be geared to meet such future challenges.

I take this opportunity to congratulate the College for a work well done.

Dr. U. A. Mendis
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PREFACE

Microbiology laboratories play a vital role in screening and confirming the diagnosis of infectious diseases both in the hospital settings as well as in the community. Data generated are utilized for patient management and for disease specific control activities.

Almost ten years ago the Sri Lanka College of Microbiologists developed a 'Laboratory Manual in Microbiology' for hospital laboratories as a standard practice guideline for conducting most of the common and epidemiologically relevant tests in state and private sector laboratories. Since then several developments have taken place in health-care management in the country. Requirements and expectations for quality have become wider, especially as laboratories are seeking accreditation for better performance. National External Quality Assurance Schemes in Microbiology have brought about inter-laboratory comparisons, placing less supervised laboratories at a disadvantage. The necessity of an updated standard operating procedure guideline was strongly felt. The Sri Lanka College of Microbiologists appreciating this current need undertook the task of revising and updating the existing manual with the participation of all stake-holders. This manual is the result of a collective collaboration of the microbiology stake-holders in the country. The manual will be available for use by hospital laboratories, university laboratories, microbiologists, MLT schools and post-graduate trainees in microbiology.

On behalf of the College I wish to express my sincere gratitude to all contributors and especially the devoted editorial board comprising of Drs Kumudu Karunaratne, Malka Dassanayake, Thamara Wijesuriya and Kanthi Nanayakkara together with Ms Priyanga Opatha, secretary, SLCM who worked tirelessly to complete this large task.

I am very grateful to the Ministry of Health for providing the funds for printing of the manual to be available for state sector laboratories and to Dr U. A Mendis, Director General of Health Services for the continued support extended to the College.

Dr. Pranitha Somaratne
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BACTERIOLOGY

STANDARD OPERATING PROCEDURE FOR BLOOD AND BONE MARROW CULTURES

Types of specimens

- Blood by venepuncture
- Blood through intra vascular catheters in suspected catheter related blood stream infections (Refer SOP on infections associated with intra vascular catheters)
- Bone marrow obtained by biopsy (sternal / iliac crest)

Bone marrow culture should be reserved for culture for specific pathogens such as *Brucella*, *Salmonella*, *Listeria*, *Mycobacteria* and fungi. It adds a little to detection of most other bacteria in the blood. Routine cultures of bone marrow without a specific indication should be discouraged.

Introduction

Laboratory diagnosis of bacteraemia and fungaemia depends on blood cultures, which are probably the most important cultures performed by the microbiology laboratory. Because the culture methods are so sensitive, the procedure of collection must be aseptically performed to prevent contamination of specimen with skin commensals.

Drawing blood from two different sites will help to identify contamination. Ideally one set of blood culture refers to one anaerobic blood culture bottle and aerobic blood culture bottle inoculated with blood from a single venepuncture.

Likelihood of recovering a pathogen increases as the volume of blood increases. Optimal blood to broth ratio of 1:5 to 1:10 should be maintained. Addition of SPS (Sodium Polyanethol Sulphonate) at a concentration of 0.025 to 0.05% will increase isolation rate but it can be inhibitory to certain bacteria.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment Arrange to store blood culture bottles in wards/units or provide from laboratory on a 24 hour basis.
Recommended total volume and numbers of blood cultures Store unused manual blood culture bottles at room temperature (not refrigerated) Follow the manufactures instructions for the storage of unused automated blood culture bottles	Volume of blood cultured is crucial to increase the sensitivity • Neonates (< 4 kg): 1 ml per bottle • Children: 3 – 5 ml of blood per bottle • Adults: 6 – 10 ml of blood per bottle • Maintain blood to broth ratio of 1:5 to 1:10 • For commercially available blood culture bottles, follow manufacturer's instructions on volume of blood to be inoculated depending on the type of bottle. • Only aerobic blood culture is currently routinely available in Sri Lankan laboratories. • Bone marrow should be inoculated directly into a blood culture broth as for blood culture.

Fungal blood culture service is available at the Mycology Department, MRI Tel:011 2698725 Anaerobic blood culture service available from Bacteriology Department, MRI Tel: 011 2691350	**IMPORTANT:** • When acute sepsis or another infection (osteomyelitis, meningitis, pneumonia, pyelonephritis) requires immediate institution of antimicrobial therapy, draw two blood cultures of maximum volume from different anatomical sites before starting antibiotics. • For fever of unknown origin, infective endocarditis, or other continuous bacteraemia or fungaemia, draw a maximum of three blood cultures with maximum volume. • When it is required to draw blood cultures from patients on antimicrobial therapy, culture should be collected when antimicrobial agents are at their lowest concentration (just before the next dose).
Skin antisepsis and collection of blood from venepuncture (clinical teams should be provided with this information)	**Collection of peripheral blood for culture:** • Select a different venepuncture site for each blood culture. • Do not draw blood from a vein into which an intravenous solution is being infused. 1. Select the vein to be used for venepuncture. 2. Clean with 70% ethyl alcohol for 1 minute and allow to dry. 3. Wipe concentrically starting at the center with 7.5% povidone iodine. 4. Allow iodine to dry for 2 minutes and avoid touching the site. 5. Label the bottles with the patient identification details, date, time and site of collection. 6. Wipe the top of the blood culture bottle with 70% ethyl alcohol after removing the cello tape or the cap covering the lid. Allow to dry completely usually for 30 to 60 seconds. Do not open the lid. 7. Wash hands with soap and water and wear sterile gloves before collecting blood. 8. Use a disposable sterile needle and syringe and draw the blood in the volumes as indicated above taking aseptic precautions. Use a new sterile needle if the first attempt is not successful. 9. Take precautions for the prevention of sharps injuries. Apply safety device to protect the phlebotomist from needle exposure if available. 10. Inoculate into the blood culture bottles carefully. Inoculate first the aerobic bottle and then the anaerobic bottle with no more than the recommended blood volumes indicated above. 11. Thoroughly mix bottles to avoid clotting. 12. After phlebotomy, dispose needles and syringes into a sharp bin. 13. Remove iodine from the skin with a 70% alcohol swab.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	• Do not refrigerate blood cultures. • Specimen should be sent to laboratory immediately with accompanying properly filled Microbiology request form giving patient identification details, clinical details and date, time and site of collection. • If immediate transport is not possible, blood cultures can be stored at room temperature in the ward. Blood cultures taken after hours should be kept at room temperature and sent to the laboratory the following morning. • Refer to manufacturer's instructions for the appropriate method to store commercial blood culture bottles prior to incubation in automated blood culture systems.

REJECTION CRITERIA

1. Specimen leaking from container (likely contamination of sample)
2. Broken or cracked bottles

   In 1&2, inform ward / responsible clinician **before** discarding the specimen. Document persons involved and action taken and request repeat sample.

3. Patient details on request form does not match those on bottle
4. Unlabelled specimen

   In 3&4 inform medical officer – DO NOT DISCARD BLOOD CULTURE
   Ward staff must authenticate any changes in writing.

   Labeled blood cultures are not rejected even if medium is expired, volume or number of bottles is insufficient, or bottles were received >12 hours after collection. Document deficiency, as well as the effect on the reliability of culture result, in report. Educate the staff to ensure that cultures are collected appropriately.

PROCESSING OF BLOOD CULTURES

Appearance on receipt	**Note:** Date of collection, turbidity, haemolysis , pellicle formation and "puffballs"
DAY 0	1. On receipt – enter details on specimen register. 2. Mark bottle and request form with the laboratory number for manual cultures. 3. Place it on blood culture tray and keep in the incubator (35°C).
DAY 1	Inspect all blood culture bottles after overnight incubation. **Note turbidity, haemolysis, pellicle formation, and "puffballs"**
Ideally, sub culturing should be done in a safety cabinet to minimize risk of contamination	**A) If no change** • Withdraw 1 ml of blood culture broth using a vacutainer needle, sterile needle and syringe or sterile loop if needles and syringes are not available. • Inoculate: - Blood agar – stab with *S.aureus.* Incubate at 35°C overnight in 5% -10% CO_2. - MacConkey agar - Incubate at 35°C overnight in air - Chocolate agar – Incubate at 35°C overnight in 5% - 10% CO_2
If Gram stain positive - inform Microbiologist / MO / Clinician	**B) If changes present** In addition to inoculation of plates • Place 1 drop of broth onto a clean glass slide and make a smear • Gram stain the smear and examine using battlement technique • If organisms seen, proceed as follows a) Inform Microbiologist / MO b) Inform clinician c) Perform direct sensitivity testing - Gram positive cocci- ABST for streptococci and staphylococci - Gram negative bacilli- ABST for enterobacteriaceae + pseudomonas
DAY 2	1. Read all plates: • No growth – send a report. Re-incubate plates for another 24 hours. • Growth + – Gram stain and identify the isolates as far as possible. - Inform Microbiologist / MO. - Inform Clinician and discuss the significance of the isolate. - Perform ABST.
If growth present - inform Microbiologist / MO / Clinician	
DAY 3-7	2. Re-incubate all bottles which are negative and inspect daily. If evidence of growth detected, subculture and identify. Further reports will be issued only on positives.
DAY 7	Subculture after 7 days of incubation. Read after overnight incubation. Send the final report.

Special incubation periods	**Note:** Laboratory contamination is a recognized problem if bottles are opened for sub culturing. Infective endocarditis 3 weeks Typhoid 2 weeks

Note:

- It is very important to follow the manufacturer's instructions in processing the blood cultures by automated blood culture systems.

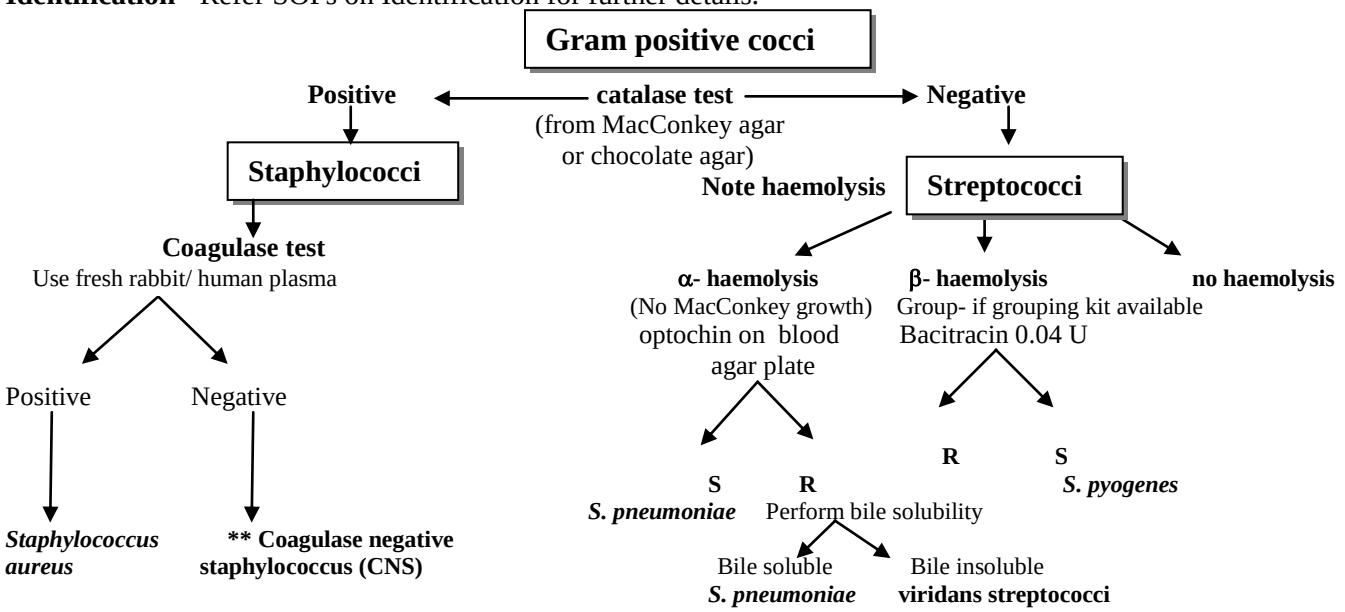
Safety

- Ideally blood cultures should be processed in a biosafety cabinet.
- Always wear gloves, because blood cultures may contain blood borne pathogens.
- Take precautions to prevent needle stick injuries. Never recap sharps. Dispose needles and syringes in puncture proof containers.

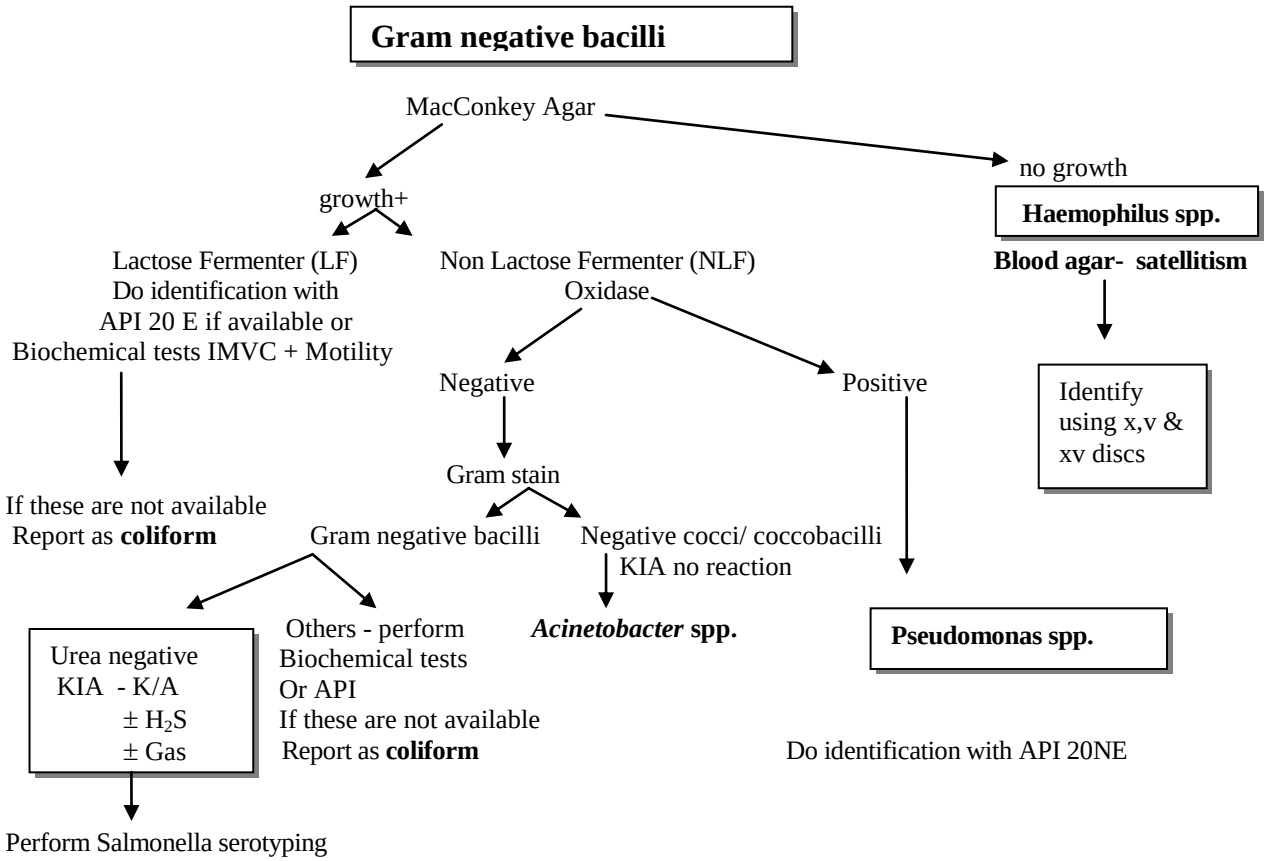
Incubation

- Maintain incubation conditions to allow recovery of microorganisms and maintain rotation or agitation of the media if possible.
- Incubate blood cultures at 35^0C. For manual blood cultures 7 days of incubation is indicated. For automated blood culture systems usually 05 days. Longer incubation needed in special circumstances e.g. Infective endocarditis, typhoid fever, *Brucella* culture. 2 weeks for typhoid fever and 3 weeks for endocarditis for manual blood culture.
- For manual cultures, perform at least one blind subculture to solid agar from visually negative bottles. This has to be performed essentially after overnight incubation.
  Note: Blind subculture of automated systems has little clinical utility.

Identification - Refer SOPs on Identification for further details.



** Inform Microbiologist or MO to decide on significance



ABST

Perform ABST for all isolates.

Do primary sensitivity if organisms are seen in Gram stain of broth culture. Repeat ABST following day with isolate.

REPORTING PROCEDURE

Microscopy	**Microscopy results should be informed to the ward by telephone if positive.**
Culture	**<u>For manual blood cultures</u>** 1. No growth on day 2 report as → Blood culture - No growth after 24 hours of incubation. 2. Positive culture i) report preliminary identity by telephone ii) send final report , including ABST when complete report as → …….. isolated after …….days of incubation ABST : **Note:** **a) Infective endocarditis** Most likely isolate: viridans streptococci Do not do routine sensitivity testing. MIC for penecillin required – if facilities are not available, contact MRI. **b)** ABST should be done and recorded on all blood culture isolates. However, coagulase negative staphylococci, diphtheroids, aerobic spore bearers are frequent contaminants. Inform MO to check on clinical details. Report ABST only if clinically significant.

<u>Coliforms (Enterobacteriaceae)</u>	
1st line	**2nd line**
Ampicillin	Cefepime
Gentamicin	Ticarcillin-clavulanic acid
Amoxicillin-clavulanic acid	Piperacillin-tazobactam
Cefuroxime	Imipenem
Ciprofloxacin	Meropenem
Netilmicin	Amikacin
Cefotaxime or ceftriaxone	Aztreonam
Ceftazidime	Ertapenem

Important:

- **Screening and confirmatory tests for ESBLs in *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca* and *Proteus mirabilis* are indicated.**
- **Screening and confirmatory tests for suspected carbapenemase production is indicated.**

Salmonella typhi* and *Salmonella paratyphi A

Ampicillin
Trimethoprim – sulfamethoxazole
Cefotaxime or Ceftriaxone
Nalidixic acid (report as Ciprofloxacin) } Nalidixic acid R – Ciprofloxacin – R
Ciprofloxacin } Nalidixic acid S – Ciprofloxacin - S
Chloramphenicol

Acinetobacter spp.

1st line	**2nd line**
Gentamicin	Ticarcillin-clavulanic acid
Ceftazidime	Piperacillin-tazobactam
Cefepime	Amikacin
Ciprofloxacin	Imipenem
Trimethoprim-sulfamethoxazole	Meropenem
Tetracycline	Cefoperazone – sulbactam
Netilmicin	Ampicillin - sulbactam
	Colistin – recommended method in CLSI is MIC

Pseudomonas aeruginosa and Pseudomonas spp.

1st line	**2nd line**
Ceftazidime	Piperacillin-tazobactam
Gentamicin	Amikacin
Cefepime	Imipenem
Ciprofloxacin	Meropenem
Ticarcillin-clavulanic acid	Colistin
Aztreonam	
Netilmicin	

Burkholderia pseudomallei

Amoxicillin – clavulanic acid	Doxycycline
Ceftazidime	Tetracycline
Imipenem	Trimethoprim – sulfamethoxazole

Burkholderia cepacia

Ceftazidime
Ticarcillin – clavulinic acid – recommended method in CLSI is MIC
Levofloxacin - recommended method in CLSI is MIC
Meropenem
Trimethoprim – sulfamethoxazole
Chloramphenicol - recommended method in CLSI is MIC

Stenotrophomonas maltophilia

Ceftazidime- recommended method in CLSI is MIC
Ticarcillin – clavulinic acid - recommended method in CLSI is MIC
Chloramphenicol - recommended method in CLSI is MIC
Levofloxacin
Trimethoprim – sulfamethoxazole

Other non – Enterobacteriaceae	
Ceftazidime	Levofloxacin
Gentamicin	Ticarcillin – clavulinic acid
Amikacin	Piperacillin-tazobactam
Aztreonam	Trimethoprim – sulfamethoxazole
Cefepime	Colistin
Ciprofloxacin	Polymyxim B
	Chloramphenicol

Important:
For all antibiotics for non – enterobacteriaceae recommended method of ABST in CLSI is MIC

Staphylococcus spp.	
1st line	**2nd line**
Penicillin	Ciprofloxacin
Cefoxitin /oxacillin / methicillin (preferably cefoxitin) - report as cloxacillin	Vancomycin (recommended method in CLSI is MIC)
	Teicoplanin
Erythromycin	Tetracycline
Clindamycin	Chloramphenicol
Trimethoprim-sulfamethoxazole	Gentamicin
	Linezolid
	Daptomycin – recommended method in CLSI is MIC

Important:

- **Screening tests for ß – lactamase production, detection of MRSA and inducible clindamycin resistance in *Staphylococcus spp.* and ß – haemolytic streptococcci are indicated**

Enterococcus spp.	
1st line	**2nd line**
Penicillin	Tetracycline
Ampicillin	Vancomycin
Gentamicin 120 µg – for high level resistance screening in endocarditis	Linezolid
	Teicoplanin
	Daptomycin – recommended method in CLSI is MIC

Important:

- **Testing ß lactamase production is indicated in selected cases**
- **Screening tests for high – level aminoglycoside resistance (HLAR) and vancomycin resistance may be indicated**

Streptococcus pneumoniae	
1st line	**2nd line**
Oxacillin (report as penicillin)	Vancomycin
Erythromycin	Clindamycin
Cefotaxime/Ceftriaxone	Tetracycline
Co-trimaxazole	Linezolid
Chloramphenicol	Levofloxacin
	Meropenem

Important:

- **Isolates of pneumococci with oxacillin zone sizes of ≥ 20 mm are susceptible to penicillin (MIC ≤ 0.06 µg/ml).**
- **Penicillin and cefotaxime or ceftriaxone or meropenem MICs should be determined for those isolates with oxacillin zone diameter ≤ 19 mm because zones of ≤ 19 mm diameter occurs with penicillin resistant, intermediate or certain susceptible strains.**

- **For isolates with oxacillin zones ≤ 19 mm, do not report penicillin as resistant without performing a penicillin MIC.**

β-haemolytic Streptococcus	
Penicillin	Clindamycin
Erythromycin	Cefotaxime or ceftrixone

Important:

- **Strains with penicillin MICs of greater than 0.12 µg / ml have not been observed yet. Therefore, if the isolate is resistant to penicillin reidentify, repeat ABST and inform Microbiologist.**

***Streptococcus* spp. viridans group**
Penicillin – recommended method in CLSI is MIC
Cefotaxime or ceftriaxone

Haemophilus influenzae* and *Haemophilus	***parainfluenzae***
1st line	**2nd line**
Ampicillin	Cefepime
Amoxicillin-clavulanic acid	Imipenem or Ertapenem
Cefuroxime	Meropenem
Cefotaxime or ceftriaxone	Tetracycline
Clarithromycin	Cefixime
Ciprofloxacin	Aztreonam
Trimethoprim-sulfamethoxazole	Chloramphenicol

Important:

- **ß lactamase test is useful in detecting ß lactamse producing strains. Direct ß – lactamse test can provide a rapid means of detecting ampicillin resistance.**

Chryseobacterium meningosepticum* (*Flavobacterium	**spp.)**
Ceftazidime	Netilmicin
Gentamicin	Piperacillin-tazobactam
Cefepime	Amikacin
Ciprofloxacin	Imipenem
Ticarcillin-clavulanic acid	Meropenem
Aztreonam	Vancomycin

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REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Safety Cabinet	for sub culturing
Microscope	
Bunsen Burners	
CO_2 jar	

CONSUMABLES

Glassware

Blood culture bottles - for adults	a) In house blood culture bottle with 60 ml of BHI (with SPS) in 100 ml medical flat bottle made up of clear glass with a hole at the centre of the screw cap with diaphragm b) Blood culture bottles for automated systems c) Other commercial blood culture bottles
Blood culture bottles for children	a) In house blood culture bottle with 30 ml of BHI (with SPS) in 50 ml medical flat bottle made up of clear glass with a hole at the centre of the screw cap with diaphragm b) Blood culture bottles for automated systems c) Other commercial blood culture bottles
Blood culture bottles for neonates	a) In house blood culture bottle(universal bottle) with 10ml of BHI b) Blood culture bottles for automated systems
Glass slides	

Media and reagents

BHI broth	
Liquoid (SPS)	Improves performance
Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity

Other items:

Disposable syringes / needles preferred for subculturing
Wire loops may be substituted if disposable syringes/needles are unavailable

Stains

Gram stain

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF INFECTIONS ASSOCIATED WITH INTRAVASCULAR (IV) CATHETERS

Types of specimens Central venous catheter lines
Intravenous alimentation lines
Intra-arterial lines

Introduction

Both local and systemic infections can result from contamination of IV devices. These include cellulitis, abscess formation, septic thrombophlebitis, device associated bacteraemia and rarely, endocarditis.

Colonization can occur as early as 24 hours after insertion of the catheter. The skin insertion site is the most common route of colonization and related infections.

A link has been demonstrated between the number of organisms on the catheter surface and the risk of infection/disease associated with these catheters.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Time of specimen collection	• While catheter is in-situ or • On removal of catheter
Correct specimen type and method of collection **While catheter is in-situ**	1. Need 2 specimens of blood. 2. Take the first sample of blood through the line (catheter) and another sample from a peripheral site at the same time. This is especially useful if automated blood cultures are available. 3. A similar blood volume from both sites is preferable. Comparison of above said cultures by using either quantitative cultures or time taken to signal positive cultures if processed in an automated instrument, may be useful for the diagnosis of catheter related sepsis. **Collection of blood for culture from intravascular catheters** 1. Label bottles with patient identification details. In addition include collection time, whether the collection was from a peripheral draw or a catheter and the initials of the person who drew the sample. 2. Wipe the top of the blood culture bottle with 70% alcohol and allow drying completely usually for 30 to 60 seconds as for a peripheral draw. 3. Clean the catheter hub with alcohol, povidone iodine or alcoholic chlorhexidine and allow adequate drying (usually 1-2 minutes). 4. Attach the syringe to the hub, draw and discard some blood (suggestive amounts are 3 ml for adults and 0.2 ml for paediatric patients) which is not used for culture.[4]

When specimen is collected on removal of catheter	Avoid drawing from lines within an hour of completion of administration of an antimicrobial agent. 5. Using a sterile syringe, collect recommended volume of blood for culture through hub taking aseptic precautions. Quickly reconnect tubing. 6. Inoculate into the blood culture bottles carefully, not more than the recommended volumes. 7. Thoroughly mix bottles to avoid clotting. **Tip of catheter** 1. Clean skin at insertion site using 70% alcohol. 2. Allow surface to dry. 3. Remove catheter aseptically using sterile forceps. 4. Avoid contact of tip of catheter with skin. 5. Using a sterile pair of scissors, cut the distal 5-6 cm (area under skin) of the catheter. 6. Place the cut portion in a dry sterile screw capped bottle.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	1. Specimen should be sent to lab immediately with accompanying request form. 2. If there is a delay in dispatch to the laboratory, blood culture bottles can be kept at room temperature. Note time of arrival in laboratory. 3. Process catheter tips immediately.

REJECTION CRITERIA

1. Catheter tips :
    a. Specimen sent in non sterile container
    b. Major delay in receipt of sample (> 1 day)
2. For blood cultures refer SOP on blood culture.

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality, where indicated.
2. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Culture	
Catheter tip	1. Using a sterile pair of forceps, roll the catheter tip 4-5 times on a blood agar plate using whole plate, without touching the edge.
Semiquantitative method (Maki method)	2. Incubate at 35°C overnight in 5-10 % CO_2.
	Reading 1. Note presence / absence of growth. 2. If growth present - count the number of colonies for each colony type. 3. < 15 colonies – not significant 4. Any species ≥15 colonies – significant 5. Mixed growth, <15 colonies of each type – not significant 6. Identify and perform ABST on all species with ≥ 15 colonies/plate
Blood culture	Refer SOP on blood culture. If automated blood cultures are available record the time of positivity of blood cultures.

INTERPRETATION & REPORTING

Catheter tip	
No growth	No growth.
<15 colonies	No significant growth.
≥15 colonies	……..isolated. Report ABST.
Interpretation of results	1. If blood cultures are negative or isolate different from those isolated from catheter tip comment - **Catheter colonization** 2. If blood culture positive with same species as that isolated from catheter tip comment - **Catheter related blood stream infection** 3. If blood culture positive with same species that was isolated from catheter tip but <15 colonies or different isolate from catheter tip comment **- Bacteraemia not associated with IV catheters**
Blood culture	1. If blood culture through the catheter becomes positive 2 or more hours before the peripheral blood culture with the same organism comment - **IV catheter associated blood stream infection** 2. If only blood through the line is positive and not the peripheral blood comment **- Catheter colonization** 3. If both blood cultures through the catheter and the peripheral blood become positive with the same organism but the time gap is less than 2 hours or peripheral blood culture becomes positive first. comment **- Bacteraemia not associated with IV catheters**

REQUIREMENTS

FOR COLLECTION OF -

catheter	**blood through catheter port**
70% alcohol	70% alcohol
Sterile forceps	Sterile disposable needle/syringe
Sterile pair of scissors	Blood culture bottle
Dry sterile screw capped bottle	

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen burners	
Sterile forceps	
Sterile pair of scissors	
CO_2 jar	

CONSUMABLES

Glassware

Sterile screw capped glass bottles
Glass slides/petri dishes

Media and reagents

Blood culture bottles with suitable medium

Blood Agar	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity testing

Other items:

Disposable syringes / needles preferred for sub culturing
Wire loops may be substituted if disposable syringes/needles are unavailable

Stains

Gram stain

References:

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. IDSA guideline 2009 on CVC infection control
3. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
4. Henry D. Isenberg .Clinical microbiology procedures hand book ,Volume 1, 2nd ed. 2004.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF CEREBROSPINAL FLUID (CSF)

Types of specimens	CSF	-	Obtained by lumbar puncture Ventricular tap From External Ventricular Drain (EVD) From CSF shunt (AV or VP)

Introduction

This SOP describes the examination of CSF for the presence of pathogens causing bacterial meningitis.

Likely pathogens

Neonates (< one month of age)
Group B Streptococcus
Escherichia coli
Listeria monocytogenes
Other coliforms
Pseudomonas spp.
Chryseobacterium meningosepticum

Childhood meningitis (1 month – 3 months)
Haemophilus influenzae
Streptococcus pneumoniae
Neisseria meningitidis
Neonatal pathogens

Childhood meningitis (3 months- 5yrs)
Haemophilus influenzae
Streptococcus pneumoniae
Neisseria meningitidis

Late childhood and adults
Streptococcus pneumoniae
Neisseria meningitidis

Adults over 65 years
Streptococcus pneumoniae
Listeria monocytogenes
Coliforms
Group B Streptococcus

Immunocompromised patients
Streptococcus pneumoniae
Coliforms
Staphylococcus aureus
Listeria monocytogenes
Amoebae
Pseudomonas spp.

Chronic meningitis
Mycobacterium tuberculosis
Cryptococcus neoformans

Trauma/ Neurosurgery
Streptococcus pneumoniae
Staphylococcus aureus
Pseudomonas spp.
Coliforms

Shunt infections
CNS
Staphylococcus aureus
Coliforms
Diphtheroids
Acinetobacter spp.
Candida spp.
Pseudomonas spp.
Propionbacterium acenes

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment
Correct specimen type And method of collection	**CSF obtained by lumbar puncture** 1. Clean skin with 70% alcohol and povidone iodine. 2. Collect CSF into 4 screw capped bottles: • Bottle 1 – 0.5 ml into a fluoride containing bottle for CSF sugar estimation • Bottle 2 – 1 ml into a sterile bottle for Microbiology • Bottle 3 – 0.5 ml for protein and cell counts since contamination with blood cells will be minimal at the end of CSF collection • Bottle 4 – 0.5 ml into a sterile bottle for any additional tests if needed. eg. virus studies, mycology, TB PCR or culture **CSF obtained from EVD or shunt** 1. Clean site of puncture with 70% alcohol. 2. Using sterile needle/syringe, aspirate CSF. 3. Collect into bottles as described above.
Additional specimens	1. 2 – 2.5 ml blood into a fluoride containing bottle for blood sugar 2. Blood for culture 3. Meningococcal meningitis – aspirate or biopsy from skin lesion

SPECIMEN TRANSPORT AND STORAGE

Sample should be accepted after signing the ward specimen book indicating time of receipt.

Accept all samples	1. Specimen should be sent to lab **immediately** with accompanying request form giving patient identification details. 2. Date & time of collection should be written on request form. 3. Note date & time of arrival in laboratory on request form. 4. Process CSF immediately. 5. **Do not refrigerate** CSF specimens sent for bacterial culture. 6. CSF samples sent for viral / mycobacterial studies or for bacterial antigen detection should be refrigerated. 7. Note following on report and inform responsible clinician a) if sample for bacteriology received in ice b) delayed sample c) received in an inappropriate container d) leaking sample

SPECIMEN PROCESSING

Test selection **CSF culture should be available in all hospitals with a paediatric and medical consultant service**	1. Cell count / Differential count →refer annexure at the end of this SOP. 2. Protein estimation 3. Sugar estimation 4. Microscopy (if culture is done from the same sample do culture before microscopy) a) Gram stain b) Ziehl-Neelsen → clinician request or if tests 1-3 abnormal c) Wet preperation → in compromised patients or if clinically indicated -examine for protozoa and spirochete d) Dark ground microscopy if clinically indicated e) India ink preparation 5. Culture a) Acute bacterial meningitis b) Tuberculous meningitis c) Cryptococcal meningitis 6. Antibiotic sensitivity 7. Antigen detection 8. Special tests a) Antibody detection - HSV/JE/rabies b) PCR - HSV/TB c) Culture for enteroviruses

Staining Gram stain	**Sample > 1 ml** a) Decant CSF into a sterile screw capped conical tube. b) Centrifuge at 1500 x g for 15 minutes. c) Carefully decant supernatant into a sterile container. d) Resuspend deposit in 5 drops of CSF. e) Using sterile Pasteur pipette, place a drop of CSF deposit on to a **new** (unused) slides. **Do not spread. Air dry.** f) Fix using gentle heat. g) Perform Gram stain on one slide. h) Examine stained smear using x100 magnification (oil immersion). i) Using battlement technique, scan whole smear (should take approximately 20 minutes). **Sample < 1 ml** a) Place one drop of uncentrifuged CSF onto slide. Allow to dry. b) Repeat with 2 more drops of CSF. c) Fix by gentle heat. d) Examine as given above.

	Clotted sample a) Use the clot to prepare a smear. b) Perform Gram stain.
Supplementary ZN stain	1. Ziehl-Neelsen stain for AFB a) Decant CSF into sterile screw capped conical tube. b) Centrifuge at 3000 x g for 15 minutes [(2)]. c) Decant supernatant into sterile container. d) Resuspend deposit in a few drops of CSF. e) Place one drop of resuspended deposit on a clean dry slide. f) Allow to dry. **Do not spread.** g) Place another drop over the dried first drop and allow to dry. h) Repeat the process once more. i) Fix using gentle heat. j) Stain. k) Examine the whole smear using the battlement technique. 2. India Ink stain for *C. neoformans* a) Steps (a) – (d) as above b) Place a drop of the resuspended deposit on a clean dry slide. c) Mix with one drop of India Ink. d) Mix gently. e) Examine with x10 and x40 magnification. 3. Dark ground microscopy Perform if spirochaetes are suspected
Culture **Standard**	**Sample > 1 ml** a) Follow steps (a) – (d) to obtain a centrifuged deposit. b) Inoculate one drop of resuspended deposit onto half plates and incubate as follows: *Blood agar – 35°C overnight in 5-10% CO_2 Chocolate agar – 35°C overnight in 5-10% CO_2 MacConkey agar. – 35°C overnight aerobically c) Inspect after overnight incubation. Reincubate for a further 24 hours if there is no growth. d) Enrichment in broth culture media is not necessary unless CSF is cultured from shunts or external reservoirs. These should be inoculated into aerobic blood culture bottles / BHI broth to enhance detection. Incubate for 5-7 days. Following overnight incubation subculture broth irrespective of its turbidity. *If Chocolate agar plate **NOT** quality controlled for growth of *H. influenza*e, make several stabs on the Blood agar plate with *Staphylococcus aureus.*
Supplementary	**Sample < 1 ml or clotted** Directly inoculate onto the 3 media – Follow steps (b) – (c) above Supernatant - Refrigerate for 1. antigen detection if required 2. viral antibody detection

Contact Reference Laboratory	Deposit - Retain at room temperature or incubator for further cultures / PCR 1. Mycobacterial culture and/or PCR – store in refrigerator (4 - 8^0C) 2. Culture for fungi (if clinically indicated)
Identification	**Refer SOP on identification of organisms.**
ABST **See SOP on antibiotics**	1. Choice of antibiotics in CNS infections dependent on sensitivity of organism and antibiotic concentrations in CSF. 2. If organisms seen on Gram stain– perform direct ABST. 3. ABST needs to be performed on all isolates.

Choice of antibiotics

Direct sensitivity test	
Gram positive diplococci	pneumococcal panel
Gram negative diplococci	Meningococcal panel
Gram negative cocco-bacilli	*H.influenzae* panel
Gram negative bacilli	Coliform panel

ABST

Pneumococcus	***H. influenzae***	**Meningococcus**	**Coliforms**
Oxacillin[*1] Chloramphenicol Cefotaxime[*2] Vancomycin	Chloramphenicol Cefotaxime[*2] Ciprofloxacin Meropenem	Penicillin[*3] Chloramphenicol Ciprofloxacin[*4] Cefotaxime[*2]	Ampicillin Chloramphenicol Cefotaxime[*2] Ciprofloxacin Gentamicin[*5] Meropenem

Streptococci eg: Group B	**Staphylococci**	**Pseudomonas**
Penicillin Chloramphenicol	Cefoxitin (oxacillin, methicillin) Ciprofloxacin Gentamicin Chloramphenicol Vancomycin	Ciprofloxacin Ceftazidime Cefepime Meropenem Ticarcillin-clavulanic acid Piparacillin-tazobactam

*****1 If sensitive report as penicillin. If resistant penicillin MIC should be done. CLSI advocates direct MIC testing for beta-lactams since disc diffusion is unreliable.
*****2 Cefotaxime can be substituted with ceftriaxone.
*****3 Check MIC. CLSI does not give zone diameters since disc diffusion is unreliable.
*****4 CLSI interpretive criteria do not apply to therapy of patients with invasive meningcoccal disease. May be appropriate only for prophylaxis of contacts.
*****5 Aminoglycosides penetrate into CSF very poorly.

REPORTING PROCEDURE

Macroscopy	Colour - Colourless / clear yellowish (xanthochromia) / clear red Turbidity- clear / turbid / Blood stained Clot + fibrin / blood
Microscopy **Turn around time: 1 hour**	Gram stain- Presence of organisms. Presence of pus cells. If test was done on a clotted sample indicate that on the report. **Microscopy results should be telephoned to ward as soon as available.** ∗ **Cell count, protein and sugar results should be phoned within 1 hour of receipt of specimen** ∗ **Inform microbiologist of positive CSF results -** a) bacteria seen on Gram stain b) cell count consistent with bacterial meningitis
Culture **Negative**	No growth at 48 hours. a) Send Final Report. b) If enrichment cultures are done include "Further report will follow if positive on enrichment". c) Consider need for virology / Mycobacterial culture / fungal culture (medical officer to contact ward for further clinical details if required) **If further cultures / studies being done – indicate on report**
Positive	Organism on list a) 24 hours –Inform positive result by telephone, with presumptive identification b) 48 hours - Final report with identification and ABST **If delays in identification or ABST, s**end preliminary report with available information with comment → Final report to follow Organism not on list a) Discuss with Microbiologist on possible significance before reporting

NORMAL VALUES

	Cell count	Predominant cells	Protein (g/l)	Glucose (% of blood sugar)
Neonates	<30 / mm^3	Neutrophils	0.4 – 1.2	60%
Children	<10/ mm^3	Lymphocytes	0.2 – 0.8	60%
Adults	<10/ mm^3	Lymphocytes	0.2 – 0.5	60%

Cellular response and biochemical properties in meningitis

	Bacterial meningitis	**Viral meningitis**	**TB / cryptococcal meningitis**
Cell count / mm^3	100-3000	10-500	100-500
Differential count	Mainly neutrophils	Mainly lymphocytes	Mainly lymphocytes
Protein g/l	0.5-3.0	0.5-1.0	1.0-6.0
Glucose % Blood sugar expected range	<60% (0.0-2.2mmol/l)	Normal	<60% (0.0-2.2mmol/l)

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Safety cabinet	
Centrifuge	Speed 3000 x g and 1500 x g
Microscope	
Bunsen Burner	
CO_2 jar / incubator	

CONSUMABLES

Glassware

Glass slides	New (unused)
Sterile 1-2 ml screw capped containers	For collection of CSF
Sterile 5 ml screw capped conical tubes	For centrifugation
Sterile pasteur pipettes	
Fuchs Rosenthal chamber (or Improved Neubauer chamber)	

Media and reagents

Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Sabouraud's agar	Refer SOP on media
Antibiotic discs	Refer SOP for antibiotic sensitivity testing

Stains

Gram stain
Ziehl-Neelsen stain
Leishman stains

MAINTENANCE OF RECORDS

Unique records register for CSF and sterile fluids

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
3. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; 21st Informational Supplement, M100-S21 Vol 31 No.1 January 2011.

ANNEXURE: PERFORMING CELL COUNTS IN CSF AND STERILE FLUIDS

Cell count

Reference:
Mackie & McCartney Practical Microbiology

1. Use specimen 3 of CSF. Gently mix well.
2. Clean and dry Fuchs-Rosenthal or Improved Neubauer Counting Chamber and place cover slip so that "Newton's rings" are visible.
3. Draw 1 drop of CSF into a sterile Pasteur pipette.
4. Carefully apply tip of Pasteur pipette to the edge of the counting chamber and allow the fluid to run into the chamber.
5. The chamber should be full with no air bubbles.
6. Allow to settle for a few minutes.

7. **a) Fuchs Rosenthal Chamber**
   Count leukocytes / red cells in 5 large squares (1,4,7,13,16) using the x 40 objective.

   Final count = number of cells / mm^3 (no calculation required)

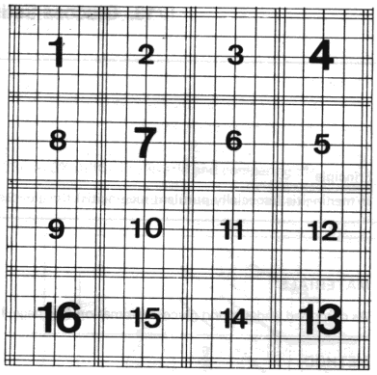
   b) Improved Neubauer chamber
   Count cells in 10 large squares (5 each on both sides)

   Final count = number of cells / mm^3 (no calculation required)

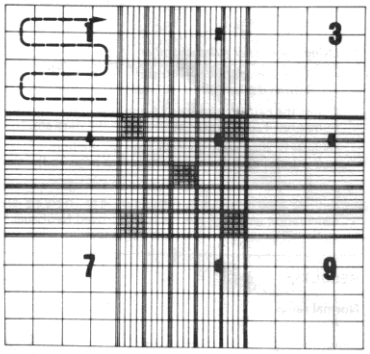
8. If a dye (Toludine blue / Methylene blue) is used, multiply the number of cells by the dilution factor to get the final cell count.

   Blood stained CSF
   Cell count of traumatic taps needs correction.

   1. Dilute CSF 1:10 with 2% acetic acid (1 drop CSF + 9 drops acetic acid).
   2. Final count = cells counted x 10 (dilution factor)



Fuchs Rosenthal Chamber



Improved Neubauer Chamber

Differential count	Perform if WBC (pus cells) > 5 / mm^3 using Leishman stain

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF STERILE FLUIDS OTHER THAN CSF

Introduction

This SOP describes the examination of fluids obtained from normally sterile sites.

Types of specimens

Ascitic fluid
Pleural fluid
Synovial fluid (joint fluid)
Peritoneal fluid and peritoneal dialysis fluid
Other fluids obtained by aspiration (cysts etc.)
Amniotic fluid
Pericardial fluid

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment
Correct specimen type And method of collection	Clean skin with 70% alcohol and povidone iodine. Aspirate with sterile needle and syringe. Transfer into sterile screw capped bottle.

SPECIMEN TRANSPORT AND STORAGE

Accept all samples	1. Specimen should be sent to lab **immediately** with accompanying request form giving patient identification and clinical details. 2. Date & time of collection should be written on request form. 3. Note date of arrival in laboratory on request form. 4. Process immediately. 5. **Do not refrigerate** specimens sent for bacterial culture. 6. Samples sent for viral/mycobacterial studies or antigen detection should be refrigerated. 7. Check with ward whether special cultures required (eg. Mycobacterial, fungal). 8. Save sample for one week (at room temperature/incubator) before discarding (in the event of further tests being required). 9. Note following on report and inform responsible clinician a) if sample for bacteriology received in ice b) delayed sample c) received in an inappropriate container d) leaking sample

SPECIMEN PROCESSING

Test selection	Cell count / Differential count – perform on all sterile fluids received → refer annexure page 24: Performing cell counts in CSF and sterile fluids 1. Microscopy a) Gram stain b) Ziehl-Neelsen stain c) Wet preparation - if indicated 2. Culture a) bacterial culture b) for *M. tuberculosis* if requested or indicated by cell count c) for fungi if requested or indicated by history/cell count 3. Antibiotic sensitivity on all isolated organisms 4. Antigen detection - if indicated 5. Special tests a) PCR (TB) b) Adenosine deaminase (pleural fluid for TB)
Staining **Gram stain**	**Sample > 1 ml** a) Decant fluid into a sterile screw capped conical tube. b) Centrifuge at 1500 x g for 15 minutes. c) Carefully decant supernatant into a sterile container. d) Resuspend deposit in 5 drops of fluid. e) Using sterile Pasteur pipette, place 1 drop of fluid deposit on to a **new** (unused) slides. **Do not spread. Air dry.** f) Fix using gentle heat. g) Perform Gram stain on one slide. h) Examine stained smear using x100 magnification (oil immersion). i) Using battlement technique, scan whole smear (should take approximately 20 minutes). **Sample < 1 ml** 1. Place one drop of uncentrifuged fluid onto a slide. Allow to dry. 2. Repeat with 2 more drops of fluid. 3. Fix by gentle heat. 4. Examine as given above. **Clotted sample** a) Use the clot to prepare a smear. b) Perform Gram stain.

Supplementary Ziehl-Neelsen stain	1. Ziehl-Neelsen stain for AFB a) Decant fluid into sterile screw capped conical tube. b) Centrifuge at 3000 x g for 15 minutes [(2)]. c) Decant supernatant into sterile container. d) Resuspend deposit in a few drops of fluid. e) Place one drop of resuspended deposit on a clean dry slide. f) **Do not spread. Air dry.** g) Place another drop over the dried first drop and allow to dry. h) Repeat the process once more. i) Fix using gentle heat. j) Stain. k) Examine the whole smear using the battlement technique. 2. Examination of the wet preparation for fungi and crystals a) Steps a) – d) as above b) Place a drop of the resuspended deposit on a clean dry slide. c) Mix with one drop of lacto-phenol cotton blue. d) Mix gently. e) Examine with x10 magnification. f) Report presence of crystals (only for joint fluid) & fungi.
Culture **Standard** **Supplementary**	**Sample > 1 ml** a) Follow steps a) –d) to obtain a centrifuged deposit. b) Inoculate one drop of resuspended deposit onto half plates and incubate as follows *Blood agar – 35°C overnight in 5-10% CO_2 Chocolate agar – 35°C overnight in 5-10% CO_2 MacConkey agar. – 35°C overnight in air a) Reincubate for a further 24 hours if there is no growth. b) Inoculate pericardial, pleural, synovial and peritoneal fluid into aerobic blood culture bottles / BHI broth to enhance detection (enrichment). Incubate for 5-7 days. Subculture this after overnight incubation irrespective of turbidity. * For joint fluids from children and pleural fluids , if chocolate agar plate is **not** quality controlled for growth of *H. influenzae*– make several stabs on the Blood agar plate with *Staphylococcus aureus.* **Sample < 1 ml or clotted** Directly inoculate onto the 3 media -steps b)-c) above Supernatant - Refrigerate Antigen detection if required a) *S. pneumoniae* (from pleural and pericardial fluids) b) *H. influenzae* (from joint fluids in children and pleural fluids)

Contact Reference Laboratory	Deposit - Retain at room temperature or incubator for further cultures / PCR 1. Mycobacterial culture and/or PCR 2. Culture for fungi (if clinically indicated) **Note:** Anaerobic culture facilities are not available in hospital based laboratories. If required - contact Anaerobic Reference Laboratory –MRI 0112693532 -4 ext -344/332
Identification	Any organism - proceed with identification and ABST
ABST **Choice of antibiotics**	1. If organisms seen on Gram stain - perform direct ABST. 2. ABST needs to be performed on all isolates. As in blood cultures.

REPORTING PROCEDURE

Microscopy	**Gram stain**- Presence of organisms. Presence of pus cells. **Microscopy results should be telephoned to ward as soon as available.** * **Gram stain results should be informed on same day.** * **Inform microbiologist of positive results.** Bacteria / fungi / pus cells seen
Culture **Negative**	**No growth at 48 hours** a) Send final Report → No growth after 48 hours b) If enrichment cultures are done include "Further report will follow if positive on enrichment". c) Consider antigen detection on supernatant (presence of pus cells). d) Consider need for Mycobacterial culture/fungal culture. e) Consider PCR. Medical officer to contact ward for further clinical details if required **If further cultures / studies being done – indicate on report**
Positive	**Positive culture** 24 hours –Inform positive result with presumptive identification. 48 hours - Final report with identification and ABST **If delays in identification or ABST** Send preliminary report with available information at 48 hours with comment "Final report to follow".

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burner	
CO_2 jar or incubator	
Centrifuge	Speed 3000 x g and 1500 x g

CONSUMABLES

Glassware

Glass slides	New (unused)
Fuchs Rosenthal chamber (or Neubauer chamber)	
Sterile screw capped 10 ml containers	For collection of samples
Sterile screw capped 5 ml conical tubes	For centrifugation
Sterile Pasteur pipettes	

Media and reagents

2% acetic acid	
Blood Agar	Refer SOP on media
Chocolate Agar	Refer SOP on media
MacConkey Agar	Refer SOP on media
Antibiotic discs	Refer SOP for antibiotics sensitivity testing

Stains

Gram stain
Ziehl-Neelsen stain
Leishman stains

MAINTENANCE OF RECORDS

Unique records register for Swabs and sterile fluids

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone
2. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF URINE

Types of specimens

- Midstream urine
- Clean catch urine
- Suprapubic aspirate
- Surgical sample
- Catheter specimen
- Nephrostomy urine

Possible pathogens

- **Acute, uncomplicated UTIs**

  - *Escherichia coli*- commonest
  - *Proteus mirabilis*- associated with renal tract abnormalities, particularly calculi
  - *Staphylococcus saprophyticus* - in young women
  - Enterococci
  - Group B streptococci

- **Complicated UTIs**

  Occur in case of structural abnormalities or instrumentation, is caused by a variety of organisms, many of them with increased antimicrobial resistance as a result of the prolonged use of antibiotics.

  - *E. coli* - commonest
  - *Klebsiella, enterobacter* - usually associated with instrumentation or catheterisation
  - *Pseudomonas aeruginosa* - associated with structural abnormality or long-term catheterisation
  - *S. aureus*- rarely causes UTI. Associated with renal abnormality or as a secondary infection due to bacteraemia, surgery or catheterisation. It is also seen as a contaminant due to perineal carriage.
  - Coagulase negative staphylococci - may cause complicated infections
  - *Candida* species - *Candida albicans* is the most frequently isolated species. Bladder colonisation is associated with indwelling catheters but may also be present as contamination from the genital tract.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment.
Correct specimen type and method of collection	• Correct instruction of patient on how to collect urine is essential. Written instructions in all 3 languages should be available in laboratory. • Screw capped wide mouth sterile bottle should be used for collection of urine for culture. • Midstream sample – Instruct patient to clean the genital area with soap and water and begin passing urine into the toilet bowl. Collect the mid part of the urine flow into a sterile container after the initial flow of the urine has been passed. • Clean catch sample (paediatric sample) – Following breast/ bottle feeding, baby should be kept without a nappy (perineal cleaning is recommended).Urine is collected straight from the stream into the container (preferably midstream). • Supra-pubic aspirate – Aseptic collection of urine directly from urinary bladder using a needle and syringe. Needs to be indicated on the request form. • Indwelling catheter – Clamp the tube and collect the urine aseptically using a needle and syringe. Insert the needle in head to toe direction. Collection of a satisfactory specimen can be very difficult in babies. Instruct mother to attempt collection soon after a feed. Sterile bags which can be attached to perineum are available but these are not satisfactory.

SPECIMEN TRANSPORT AND STORAGE

Specimen transportation and storage	• Specimen should be transported to the laboratory as soon as possible. • If there is a delay in transport keep the specimens at 4^0C. Refrigeration should be for less than 24 hours. • If transport take more than two hours transport in ice. • If boric acid containers are used (1.8% boric acid), only the amount of urine indicated on the bottle should be collected. Urine samples thus collected can be transported at room temperature.

REJECTION CRITERIA

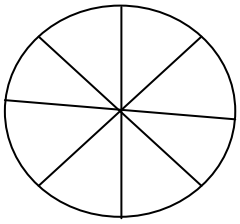
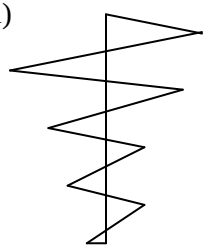
- Specimens kept at room temperature for more than two hours
- Specimens transported without ice if transport has taken more than two hours
- Specimens refrigerated for more than 24 hours
- Unlabelled specimens
- Specimens taken from a receptacle (catheter bag or bed pan)
- Catheter tips
- Unsterile container
- Leaking specimens

Cystoscopy, suprapubic and surgical samples should not be rejected.

SPECIMEN PROCESSING

Time of processing

Urine samples should ideally be processed on arrival to the laboratory. If a delay is unavoidable sample should be refrigerated until processed.

Appearance	Note appearance : clear / turbid / blood stained
Culture	Urine culture a) Mix urine sample well. b) Flame calibrated 0.001ml (1µl) wire loop and allow to cool. c) Insert the wire loop vertically into urine. Insert only the loop part. d) Inoculate CLED medium. e) Inoculate ¼ plate (9cm plate) or 1/8 plate 14 cm plate, in an inverted cone shaped manner. Incubate at 35^0C overnight. (i)  (ii) 
Microscopy	**Wet film examination of uncentrifuged urine[1]** **Assumption** If the field diameter of high power (X 40) is 0.44 mm (measured by a slide micrometer), the area of the HPF is 0.15 mm^3. If the size of cover slip is 22 X 22 mm and the depth of the film examined is 0.1 mm, the volume of urine observed in an HPF will be 0.015 mm^3. Under these conditions finding of 1 leucocyte per 7 HPF corresponds with 10^4 leucocytes per ml (10 pus cells / $mm^{3)}$. Finding clearly larger numbers than this indicate significant pyuria. **Method** 1. Mix the urine sample carefully. 2. Transfer 0.05 ml of urine on to the middle of a microscopic slide. 3. Immediately apply a cover slip (22 X 22 mm) avoiding trapped bubbles. (Film should show a small excess of fluid along the edges of the cover slip and then be about 0.1 mm in depth) 4. Examine under high power (X 40) and interpret as follows.

	Reporting **Observation** — **Interpretation** >1 pus cell / 7 HPF — > 10 pus cells / mm^3 Report as significant pyuria **Examination of centrifuged urine deposit[2]** This is a less reliable method than using a counting chamber. Urine is centrifuged for 5 minutes at 2000 rpm and then the sediment is examined under high power. With this method, 5 to 10 leukocytes/high-power field in the sediment is the upper limit of normal.

INTERPRETATION AND REPORTING PROCEDURE				
Colony count	**Interpretation**	**ABST**	**Report**	**Comment**
> 100	Pure growth of $\geq 10^5$ CFU/ml	Yes	*..... isolated Colony count $\geq 10^5$ CFU/ml	Not necessary
	Mixed growth of 2 organisms with predominance of one organism (organism $1 \geq 10^5$ CFU/ml, organism $2 < 10^4$ CFU/ml) Needs discussion with clinician	ABST for predominant organism when indicated only	Mixed growth with predominant growth of *…… isolated. Colony count $\geq 10^5$ CFU/ml	Interpret with clinical details
	Mixed growth of ≥2 organisms (each $>10^5$ CFU/ml).	No	Heavy mixed growth	Please repeat if clinically indicated
	Mixed growth with 2 or more types of organisms in catheter samples.	No	Heavy mixed growth.	Please repeat after removal/ replacement of catheter
Between 10-99	Pure growth of * 10^4 - 10^5 CFU/ml	Yes	10^4 - 10^5 CFU/ml of *…… Isolated.	Not necessary
	Mixed growth of ≥2 organisms	No	Mixed growth	Please repeat if clinically indicated
< 10	Any growth $< 10^4$ CFU/ml	No	No significant growth	Not necessary
None	No growth	NA	No growth	Not necessary
Any growth	Any growth taken as significant in supra-pubic aspirates, urine taken from the renal pelvis, ureter or bladder during surgery	Yes	Report the isolates with colony count	Not necessary
	* Identification of organisms may be important in recurrent infections. Discuss with Microbiologist.			

ABST

ABST

- **Select 5 or 6 antibiotics from list in consultation with Microbiologist/ clinicians.**
- **Do not report second line antibiotics unless clinically indicated.**

Choice of antibiotics

Coliform (LF & oxidase -ve NLF)

1st line	**2nd line**
Nitofurantoin (report as resistant for proteus)	Cefotaxime/Ceftriaxone
Nalidixic acid	Cefepime
Norfloxacin	Ciprofloxacin
Cephalexin	Amikacin
Gentamicin	Netilmicin
Cotrimoxazole /Trimethoprim	Meropenem/ Imipenem
Cefuroxime	Aztreonam
Co-amoxiclav	Piperacillin- tazobactam
Ampicillin/Amoxycillin	Ticarcillin- clavulanic acid
	Ampicillin- sulbactam
	Cefoperazone- sulbactam

***Pseudomonas* species**

1st line	**2nd line**
Ciprofloxacin	Amikacin
Gentamicin	Aztreonam
Ceftazidime	Meropenem/ Imipenem
Norfloxacin	Netilmicin
	Ticarcillin/Clavulanic acid
	Cefepime/ cefpirome
	Piperacillin tazobactam
	Cefoperazone- sulbactam

***Enterococcus* species**

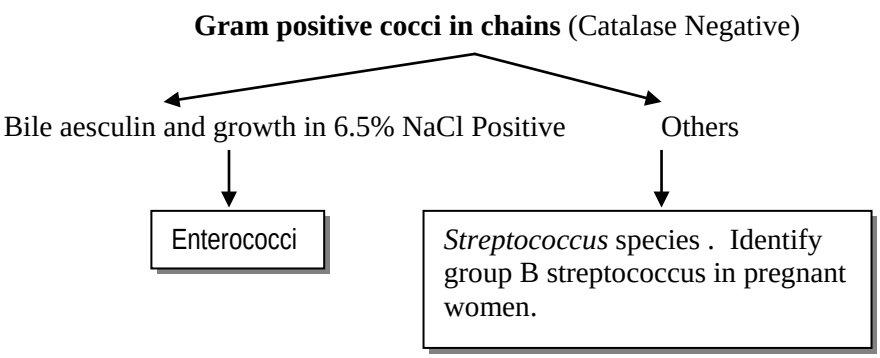
Ampicillin
Nitrofurantoin
Norfloxacin
Vancomycin (for resistant strains only and after consultation with Microbiologist)

***Staphylococcus* species**	**For MRSA**
Penicillin (report as Ampicillin)	(additional antibiotics)
*Cefoxitin (or oxacillin or methicillin)	Vancomycin
Co-trimoxazole	Ciprofloxacin
Nitrofurantoin	
Norfloxacin	
Gentamicin	

Novobiocin (For identification of coagulase negative Staphylococcus)
*Do not report as cloxacillin. If cefoxitin sensitive report cephelexin, cephredine, cefuroxime and co-amoxiclav as sensitive.
If cefoxitin resistant report as MRSA.

REPORTING PROCEDURE

Microscopy	**Uncentrifuged urine -** > 10 pus cells / mm^3 (significant pyuria) or < 10 pus cells/ mm^3 **centrifuged urine deposit -** ..... pus cells/high-power field
Culture **Organisms to be reported as**	Refer the table on interpretation and reporting. **Gram Negative Bacilli** Lactose fermenters Non lactose fermenters Oxidase Negative Oxidase Positive Urea Negative (KIA if suspecting Typhoid) Urea positive Coliform *Proteus spp.* *Pseudomonas spp.* **Gram positive cocci in clusters** (Catalase Positive) Coagualse Positive Coagulase Negative Novobiocin Resistant (≤15mm) Novobiocin Sensitive (>15mm) *Staphylococcus aureus* *S. saprophyticus* Coagulase negative staphylococcus



REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burner	

CONSUMABLES

Glassware (or disposables)

Screw capped wide mouthed sterile containers (10-20ml)	For collection of urine
Screw capped wide mouthed sterile containers (10-20ml) with 1.8% boric acid	For collection of urine
50 µl pipette, Sterile pipette tips	For microscopy
Glass slides 22 x 22 mm cover slips	For microscopy

Media and reagents

CLED agar	Refer SOP on media
Mueller Hinton agar	Refer SOP on media
Antibiotic discs	Refer SOP on ABST
Identification materials	Refer SOP for identification of organisms

Other items:

Calibrated wire loops – 0.001ml (1µl)

Stains

Gram stain

MAINTENANCE OF RECORDS

Unique records register for urine culture

References

1. 1.Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone

2. Mandell GL, Bennette J, Dolin R. **Mandell, Douglas and Bennett's Principles and Practice of Infectious diseases,** 7th Edition 2010. Churchill Livingstone

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF PUS & WOUND SWABS

Introduction

This SOP describes the examination of following types of specimens for the presence of bacterial pathogens.

Types of specimens	Pus obtained by aspiration of abscess or at surgery Swabs of pus Specimens obtained from wounds, sinuses or fistulae Biopsies /tissues Post operative drain fluid, blister fluid

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment
Correct specimen type	**Abscess** 1. Tissue or fluid is always superior to swabs. 2. If swabs are used collect two, one for culture and one for Gram Stain.
Method of collection	**Open** – 1. Remove surface exudate with sterile saline. 2. Aspirate if possible or pass a swab deep into the lesion. **Closed** – 1. Aspirate abscess with sterile needle and syringe 2. Transfer aspirated material into a sterile container.
*Contact Anaerobic Reference Laboratory, Medical Research Institute Tel: 0112 693532, Ext 344/332, 0112691350	**Anaerobic Culture** Transfer swabs or aspirate directly into anaerobic transport medium or send the sealed syringe itself.
Additional specimens	Blood culture should be taken when patients show systemic signs of sepsis.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	1. Specimens should be ideally processed within 2 hours (to prevent drying of swabs). 2. Maintain specimens at room temperature if there is a delay. 3. If a transport medium is used storage time can be up to 24 hours at room temperature. 4. Samples for anaerobic culture should reach the laboratory as early as possible.

REJECTION CRITERIA

General

1. Unlabelled specimen
2. Unsterile container
3. Leaking specimen
4. Improper transport time

For anaerobic culture

Superficial material from skin & wounds

Note:

1. If a specimen is unacceptable inform Microbiologist before rejecting.
2. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
3. Names of persons involved and action taken should be documented.
4. Do not discard samples which may be unrepeatable eg: pus from a brain abscess.

SPECIMEN PROCESSING

	Processing of tissue samples prior to microscopy and culture Grind under sterile conditions using sterile mortar and pestle or tissue grinder or stomacher (best method). or Cut into small pieces using a sterile scalpel. Specimens of pus/tissue should be processed promptly on arrival in lab.
Microscopy	1. Gram stain should be performed on all specimens. Perform AFB when necessary. Wound swabs – If two swabs are received prepare a smear for Gram stain using one swab. Use the other swab for culture. 2. Examine Gram stain smear under 10 x objectives. Record microscopy as follows: - Number of pus cells 0, occasional, <10, 10-24, ≥25
Culture	Specimens should be inoculated onto: - Blood agar Incubate overnight at 35°C in 5-10% CO_2. - MacConkey agar Incubate overnight at 35°C aerobically. - Chocolate agar Incubate overnight at 35°C in 5% - 10% CO_2.

	Enrichment culture - pus/tissue from 'inaccessible' sites such as brain abscess should be enriched in BHI broth up to 5-7 days. Subculture as above. If the clinical history is suggestive of actinomycosis or nocardia infection, get advice from Microbiologist regarding processing of samples (may require long duration of incubation). Aspirated pus samples should be stored for 48 hours at room temperature.

Supplementary Anaerobic Culture	Refer SOP on Anaerobic bacteria

REPORTING PROCEDURE

Microscopy

1. Pus cells

0	Nil
When pus cells are not seen in every field	Occasional
<10 / LPF	1+
10-24 / LPF	2+
≥25 / LPF	3+

2. Organisms -

Culture

Wound swabs

Report all primary and potential pathogens. Quantify growth as scanty 1+, 2+, 3+, 4+

Scanty – a few colonies only
1+ - growth in primary inoculum only
2+ - growth up to first streak
3+ - growth up to second streak
4+ - growth up to limits of streaking

a) Primary pathogens
   - *Staphylococcus aureus*
   - β-haemolytic streptococci (group A,C,G)

   Report with ABST.

b) Other organisms
   - Significance is considered depending on clinical details, site of infection and the Gram stain result.
   - If there is any doubt regarding the significance of an organism discuss with Microbiologist.

	*Pure growth 1. Report Gram negative organisms including pseudomonas which are moderate or heavy (2+,3+,4+) with ABST 2. Coagulase negative staphylococci, enterococci, diphtheroides and α-haemolytic streptococci are significant in certain circumstances only. *Mixed growth – probable colonizers. If there is a predominant growth consider doing ABST depending on clinical details and Gram stain results. Pseudomonas species from burns patients and plastic surgery patients–report with ABST.
Pus/tissue samples	Report and do ABST for all isolates from a single sample. Deep seated abscesses are generally of polymicrobial aetiology.

Identification	Refer SOP on identification of organisms
ABST **Choice of antibiotics** Select antibiotics depending on previous ABST profiles, availability and clinical use	***Staphylococcus aureus*** **1st line** # Cefoxitin / methicillin / oxacillin (Report as cloxacillin) Clindamycin –check for inducible resistance Erythromycin Co-trimoxazole Tetracycline/doxycycline **2nd line** Ciprofloxacin – selective reporting only. Discuss with Microbiologist. Fusidic acid *Vancomycin *Teicoplanin *Linezolid *Report only if cefoxitin/methicillin/oxacillin resistant. Do not report if resistant. Discuss with Microbiologist. **# If resistant to methicillin/ oxacillin / cefoxitin the isolate is also resistant to all currently available β-lactam antibiotics, β lactam - β lactamase inhibitor combinations, cephems, carbapenems, monobactams. DO NOT TEST / REPORT ANY OF THESE ANTIBIOTICS**

***Enterococcus* species**
Penicillin
Ampicillin/ Amoxycillin
Tetracycline

2nd line
*Vancomycin
*Linezolid
*Teicoplanin

*Report only if ampicillin is resistant

***Streptococcus* species**
1st line
Penicillin
Erythromycin
Clindamycin – check for inducible resistance
Tetracycline

2nd line
Vancomycin (Report if penicillin resistant)

- To detect **Inducible clindamycin resistence -** Erythromycin & Clindamycin discs should be placed adjacent to each other with 15mm-26mm gap for the Staphylococcus &12mm gap for the β- haemolytic Streptococci. Look for D zone.

Coliforms	**Resistance to all first line antibiotics**
1st line	**2nd line**
Ampicillin	Aztreonam
Amoxycillin- clavulanic acid	Amikacin
Cefotaxime/ceftriaxone	Ceftazidime
Cefuroxime	Cefepime
Co-trimoxazole	Netilmicin
Ciprofloxacin	Imipenem
Gentamicin	Meropenem
	Piperazillin-tazobactam
	Ticarcillin- clavulanic acid

Check for ESBL production

	***Pseudomonas* spp.**	
	1st line	**2nd line**
	Ceftazidime	Amikacin
	Ciprofloxacin	Aztreonam
	Gentamicin	Cefipime
	Ticarcillin-clavulanic acid	Cefeperazone-sulbactam
	Piperacillin-tazobactam	Meropenem
		Imipenem
		Colistin (MIC interpretive standards only)
		Polymixin
	***Acinetobacter* spp.**	
	1st line	**2nd line**
	Gentamicin	Amikacin
	Ceftazidime	Piperacillin-tazobactam
	Ciprofloxacin	Cefipime
	Ampicilln-sulbactam	Cefeperazone-sulbactam
	Ticarcillin-clavulanic acid	Meropenem
	Meropenem	Imipenem
		Doxycycline
		Colistin (MIC interpretive standards only)
		Polymixin B

References

1. Bowler P.G, Duerden B.I and Armstrong D.G 2001, Wound Microbiology and Associated Approaches to Wound Management. Clinical Microbiology Reviews 14.2 244-269

2. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; 18th Informational Supplement, M100-S18 Vol 28 No.1 January 2008.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burner	
CO_2 jar	
Anaerobic jar	Only if anaerobic cultures undertaken in laboratory

CONSUMABLES

Glassware

Glass slides

Media and reagents

Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Thioglycollate broth or Cooked meat broth	Refer SOP on media
Stuarts transport medium or Amies transport medium	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity testing
Identification materials	Refer SOPs for identification of organisms

Stains

Gram stain
Zeihl-Neelsen stain

Contact Reference Laboratory

Bacteriology Department, MRI for assistance with identification of significant isolates

Tel: 0112 693532/3/4

MAINTENANCE OF RECORDS

Unique records register for Swabs and Sterile fluids

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF EAR SWABS/ASPIRATES

Types of specimens Pus/aspirates obtained by tympanocentesis
Swabs of discharge from external auditory meatus
Ear swabs from neonatal unit (for investigation of neonatal sepsis/screening only)

Introduction

This SOP describes the examination of pus / exudate collected from the ear canal or fluid aspirated from the middle ear for the presence of aerobic bacterial pathogens causing otitis media or otitis externa.

Possible pathogens:

Acute Otitis Media	**Chronic Otitis Media**	**Otitis Externa**
S. pneumoniae	*S. pneumoniae*	*Pseudomonas spp.*
Haemophilus influenzae	*Haemophilus influenzae*	*Staphylococcus aureus*
Moraxella catarrhalis	*Moraxella catarrhalis*	Enterobacteriaceae
β-haemolytic streptococci	Enterobacteriaceae	β-haemolytic streptococci
Staphylococcus aureus	*Staphylococcus aureus*	*Vibrio alginolyticus*
	Pseudomonas spp.	Fungi
	Anaerobes	*Aspergillus spp., Fusarium spp.*
	Nocardia spp.	*Candida spp.*
	Mixed infection can occur	

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

Optimal time of specimen collection	Before starting antibiotic treatment (local/systemic)
Specimen & method of collection	**<u>Otitis media</u>** 1. Best specimen for otitis media would be the middle ear fluid aspirated by tympanocentesis. • Normally reserved for complicated, recurrent or chronic persistent otitis media. • Clean the external canal with mild detergent & aspirate fluid using a sterile syringe and a needle. 2. For perforated ear drum, collect the fluid using a sterile swab on flexible shaft inserted via an auditory speculum. Rotate swab & allow fluid to collect on swab. Obtain 2 swabs (one for Gram stain and the other for culture). **<u>Otitis Externa</u>** 1. Initially remove any debris using a moist swab. 2. Obtain sample by firmly rotating a fresh sterile swab in the outer canal.
Specimen transport & storage	• Specimens should be processed within 2 hours (to prevent drying of swabs). • Maintain **s**pecimen**s** at room temperature if there is a delay. If a transport medium is used storage time can be ≤ 24 hours at room temperature.

REJECTION CRITERIA

General

1. Unlabelled specimen
2. Unsterile container
3. Improper transport time

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.
3. Do not discard samples which may be unrepeatable eg. a sample obtained by tympanocentesis

SPECIMEN PROCESSING

Laboratory procedure	1. Perform Gram stain and a wet smear if an aspirate or a separate swab is available. If only one swab is sent prepare smears after inoculating plates. 2. Inoculate as follows: Blood agar plate - incubate overnight at 35^0C in 5-10 % CO_2 MacConkey agar plate - incubate overnight at 35^0C aerobically Chocolate agar plate - incubate overnight at 35^0C in 5-10 % CO_2 (Anaerobic cultures may be indicated for tympanocentesis fluid) 3. Inoculate two Sabouraud's dextrose agar plates if hyphae are present on wet smear. Incubate one plate at room temperature and one at 35^oC. (Refer SOP on Mycology- laboratory procedures.)
Reading & Interpretation	
Gram stain	Note the presence of pus cells, bacteria and yeast cells.
Wet smear	Note the presence of fungal hyphae.
Culture	Read after overnight incubation. If there is no growth incubate cultures for further 24 hours & tympanocentesis fluid up to 4 days, Note the colony appearances and identify. Tympanocentesis fluid**: Any growth is considered significant.**

Identification	Refer SOPs on identification of organisms. Fungal growths *Candida spp.* – Perform germ tube test. Refer SOP on Mycology- laboratory Procedures.
ABST **Choice of antibiotics** Discuss with ENT surgeon to include other frequently used antibiotics not on list	***S. aureus*** **Chloramphenicol** **Gentamicin** Cefoxitin*1 **Fusidic acid** Erythromycin **Norfloxacin** Ciprofloxacin **Streptococcus** **Chloramphenicol** Penicillin Erythromycin*3 **Coliforms** **Chloramphenicol** **Gentamicin** Co-amoxyclav Cefotaxime Ciprofloxacin **Neomycin** **Polymyxin B** Norfloxacin Cefuroxime **Pseudomonas** **Gentamicin** Ciprofloxacin Ceftazidime Ticarcillin-clavulanic acid Piperacillin-tazobactam **Norfloxacin** ***M.catarrhalis*** Ampicillin Trimethoprim-sulfamethoxazole Erythromycin Co- amoxiclav Cefuroxime ***S.pneumoniae*** **Chloramphenicol** Oxacillin*2 Erythromycin*3 Cefuroxime *****1 Can use methicillin or oxacillin discs as well. Report as cloxacillin. *2 Oxacillin- report as Penicillin *3 Erythromycin - If sensitive, report as erythromycin/azithromycin/clarithromycin • Consider second line antibiotics according to clinical need. • Some antibiotics are included in the ABST due to the availability of preparations for local therapy **(typed in bold).** This may be mentioned on report as topically used antibiotics can be used irrespective of ABST result.

REPORTING PROCEDURE

Gram stain	Pus cells, bacteria and yeast cells
Wet smear	fungal hyphae
Culture & ABST	**Acute Otitis Media** • Report the primary pathogens (*S. pneumoniae*, *S.aureus*, *Moraxella catarrhalis*, β-haemolytic streptococci, *H.influenzae*) with antibiotic sensitivity. • Tympanocentesis fluid- report any growth as significant with ABST. **Chronic Otitis Media** • Report pathogens with antibiotic sensitivity.

	Otitis Externa • *S. aureus*, Group A streptococci, *Pseudomonas spp*. – Report with Antibiotic sensitivity • Fungal isolates –Discuss with ENT surgeon. May need to send to reference laboratory. See SOP on mycology. • Candida spp. should be reported after a germ tube test. • In mixed cultures organisms of normal skin flora may be listed without ABST.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burner	
CO_2 jar	

C**ONSUMABLES**

Glassware

Glass slides

Media and reagents

Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Sabouraud's agar	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity testing
Identification materials	Refer SOP on identification

Stains

Gram stain

MAINTENANCE OF RECORDS

Unique records register for swabs

References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
2. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; 21st Informational Supplement, M100-S21 Vol 31 No.1 January 2011.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF EYE SWABS AND OTHER SPECIMENS FROM EYE

Types of specimens - Eye swabs
Scraping from eye
Pus/aspirate from a patient with endophthalmitis (vitreous or intraocular fluid)
Corneal buttons

Introduction

This SOP describes the examination of pus, discharge or aspirate from the eye for the presence of aerobic bacterial pathogens.

Possible pathogens

1. **Bacteria**
   - *Staphylococcus aureus*
   - *Streptococcus pneumoniae*
   - *Haemophilus spp.*
   - *Neisseria gonorrhoeae*
   - *Pseudomonas spp.*
   - Coagulase *Negative Staphylococci*
   - *Moraxella catarrhalis* (Acute conjunctivitis)
   - *Moraxella lacunata* (Chronic conjunctivitis)
   - β-haemolytic streptococci
   - Coliforms
2. **Fungi**
3. **Viruses**
4. ***Chlamydia spp.***
5. **Parasites**

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic /antifungal treatment
Correct specimen type & method of collection	**Conjunctival swabs** 1. Either attending clinician or nurse should collect the specimens. 2. Wash hands with soap and water or clean with alcohol hand rub. 3. Sample both eyes by rolling over each conjunctiva using separate swabs pre moistened with sterile normal saline. 4. Pass the swab firmly over lower lid fornix from nasal canthus to the lateral canthus. 5. Bed side inoculation is preferable as the amount of material is scanty and some of the pathogens are delicate. 6. Having inoculated the culture medium, make a smear on clean glass slide for Gram staining. (Preferable to collect 2 swabs for Gram stain and culture). 7. Swab the normal eye with a separate swab. This helps to compare the identified pathogen with the normal flora. **Corneal Scraping** 1. Always done by trained staff in eye clinic. 2. Instill 2 drops of local anaesthetic. 3. Use a sterile spatula to scrape the corneal ulcers or lesions and inoculate scrapings directly on to medium. 4. Apply remaining material to 2 sterile glass slides for further study. Corneal scrapings are collected (as given above) after anaesthetic drops are instilled. If swabs are collected specimens need to be collected prior to instillation of anaesthetic drops. **Intraocular fluids** 1. Always collected by an eye surgeon using a sterile needle & a syringe. 2. Bedside inoculation is ideal. If not transport the fluid in the syringe itself (as the amount of sample is scanty). **Corneal button** 1. Sample is collected by the attending surgeon at the time of surgery. 2. Send to the lab in a sterile screw capped container or directly inoculate into BHI at the bedside.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	Transport the specimen with minimum delay. Ideally eye swabs should be transported in Amies transport medium. All specimens should reach the laboratory within 24hrs. Plates should be incubated within 15 minutes.

REJECTION CRITERIA

General

1. Unlabelled samples
2. Unsterile container.
3. Dried out specimens and swabs.
4. Duplicate specimen on same day for same request.

Note:
Inform Microbiologist before rejecting the sample.

1. If a specimen is rejected, a responsible individual must be notified immediately.
2. Names and persons involved and action taken should be documented.
3. Do not discard corneal scrapings/aqueous or vitreous humour which may be unrepeatable. Discuss with Microbiologist and Clinician.

SPECIMEN PROCESSING

1. Inoculate/and incubate media according to the table given below.
2. After inoculation of the plates, smear the swab/ exudates on to a clean microscopic slide to prepare a smear for Gram stain.
3. Prepare a wet film for fungi in 10% KOH.

Clinical Details	**Standard media**	Incubation		
		Temp.	Atmosphere	Duration
Blepharitis Conjunctivitis Sticky eye	Chocolate agar	35 ^{0} C	5%-10% CO_2	18-24 hours
	Blood agar	35 ^{0} C	5%-10% CO_2	18-24 hours
	MacConkey agar	35 ^{0} C	Air	18-24 hours
Samples from immunocompromised patients Chronic blepharitis Corneal scrapings Cannaliculitis Post trauma Endopthalmitis	Sabourauds agar in addition to above media	30 ^{0} C	Air	24-48 hours
Venerology clinic samples Neonatal conjunctivitis	Selective GC medium eg. Thayer Martin Medium	35 ^{0} C	5%-10%CO_2	24-48 hours
Endopthalmitis Hypopyon Corneal button	Enrich in brain heart infusion broth and subculture appropriately			

Reading & Interpretation	Note the colony appearances and identify the possible pathogens as in SOP on identification. Corneal scraping/aspirate: Any growth is significant.

ABST

Choice of antibiotics

Discuss with eye surgeons and modify as required

Staphylococcus spp.	***Streptococcus spp.***	**Pneumococcus**	**Coliforms**
Chloramphenicol	**Chloramphenicol**	Oxacillin(report as penicillin)	**Chloramphenicol**
Gentamicin	Penicillin	**Chloramphenicol**	**Gentamicin**
Cefoxitin*		Co-trimaxazole	**Neomycin**
Ciprofloxacin		Cefuroxime	Cefuroxime
Fusidic acid		**Ciprofloxacin**	Coamoxyclav
Norfloxacin		**Levofloxacin**	Cefotaxime
		Moxifloxacin	

Pseudomonas spp.	***Haemophilus spp.***
Gentamicin	Amoxycilin
Ceftazidime	**Chloramphenicol**
Ciprofloxacin	Cefotaxime

***** Can also use oxacillin or methicillin. Report as cloxacillin.

- Some antibiotics are included in the ABST due to the availability of preparations for local therapy **(typed in bold).** This may be mentioned on report as topically used antibiotics can be used irrespective of ABST result.
- In **endophthalmitis -** second line antibiotics (see SOP for blood culture for choice of antibiotics))

REPORTING PROCEDURE

Report

When urgent microscopy is requested or when significant microscopic result found inform microbiologist/MO. Immediately inform the relevant ward by phone.

Microscopy

Report - Gram stain - Pus cells
- Organisms
Wet preparation - Yeast or fungal hyphae

Culture

Conjunctival swabs

- Report the possible pathogens with ABST.
- Mixed growth – report as "Probable colonizing flora"

- **Intraocular aspirate**
- **corneal scrapings**
- **corneal buttons**

Report any growth with ABST

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	
CO_2 jar	

CONSUMABLES

Glassware

Glass slides

Media and reagents

Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Sabouraud's agar	Refer SOP on media
BHI broth	
Antibiotic discs	Refer SOP on antibiotic sensitivity testing
Identification materials	Refer SOP for identification

Other items:

Wire loops
Swabs for collection of specimens
Sterile bottles

Stains

Gram stain

MAINTENANCE OF RECORDS

Unique records registers for Swabs and Sterile fluids

References

1. www.hpa-standardmethods.org.uk/documents/bsop/pdf/bsop2.pdf -

2. Mallett, J and Dougherty, L (2008). **The Royal Marsden Manual of clinical Nursing Procedures**. Oxford: Blackwell Science.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF LOWER RESPIRATORY TRACT SPECIMENS

Types of specimens
Expectorated sputum
Bronchial washings
Bronchoalveolar lavage (BAL)
Endotracheal aspirates (ET)
Transtracheal aspirates

Introduction

This SOP describes the examination of sputum and other specimens obtained from the lower respiratory tract, for common pathogens. Further discussion with microbiologist/clinician is required regarding BAL sent from immuno-compromised patients, as additional tests other than given in this SOP may need to be carried out.

Possible pathogens:

Streptococcus pneumoniae
Haemophilus influenzae
Staphylococcus aureus
Moraxella catarrhalis
Mycobacterium tuberculosis

Coliforms
Pseudomonas spp.
Acinetobacter spp.
Streptococcus pyogenes
*Mycoplasma pneumoniae**
Chlamydia spp.*
*Legionella pneumophila**

Possible contaminants from throat

Viridans streptococci
Coagulase negative staphylococci
Diphtheroids
Neisseria spp.

*will not grow on routine culture

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment.
Correct specimen type and method of collection	**Expectorated sputum** 1. Ask the patient to gargle throat & rinse mouth with water (without antiseptics) & then to collect at least 1ml of sputum by deep coughing. 2. Postural drainage with help from a physiotherapist would be helpful when expectoration is poor. **Induced sputum** 1. Rinse mouth of the patient with water after brushing gums and tongue. 2. With the aid of a nebulizer, let the patient inhale approximately 25ml of sterile normal saline. (Steam inhalation also can be used for this purpose). 3. Collect sputum in to a sterile container.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	The specimen should be transported and processed in the laboratory without delay. Ideally, specimens should be processed within 2 hours of collection. This maximizes recovery by reducing overgrowth of commensal oral flora and maintaining the viability of fastidious organisms. Store specimens at room temperature until transport and processing.

REJECTION CRITERIA

1. Salivary sample
2. Swabs of ET secretion
3. Tips of endotracheal tubes
4. Samples taken > 24hours ago
5. Sample in unsterile container
6. Repeat samples taken on same day

Note: Specimens should not be rejected solely on macroscopic appearance. Inform microbiologist before rejecting a specimen.

1. If a specimen is rejected, a responsible individual must be notified immediately.
2. Names and persons involved and action taken should be documented.

- Sputum of any quality from immuno-compromised patients should be accepted.
- When it is not possible to aspirate adequate volume of endotracheal or transtracheal aspirates, the tip of the suction catheter with the secretion inside can be accepted. **A new suction catheter has to be used for this purpose.**

SPECIMEN PROCESSING

All specimens should preferably be handled inside a class 1 or 2 biological safety cabinet (BSC).

Macroscopic appearance

mucoid / muco purulent/ muco salivary / salivary / blood stained/ purulent

Microscopy

Gram Stain

a) Select purulent or muco-purulent portion of the sample to do a Gram stain.
b) Observe under low power (x 10 magnification) for cells.
c) Under oil immersion, look for intra cellular bacteria and note the predominant micro organism.
d) Assess the quality of the specimen – refer Tables 1 & 2

Table 1: Murray and Washington's grading system for assessing the quality of sputum samples[2]

Grade	Epithelial cells per low power (x10) field	Pus cells per low power (x10) field
Group 1	≥25	<10
Group 2	≥25	10-25
Group 3	≥25	≥25
Group 4	10-25	≥25
Group 5	<10	≥25

- Accept the samples that belong to *Murray & Washington Group 3, 4 & 5 for processing.
- Reject the samples that belong to *Murray & Washington Group 1 & 2

Table 2: Screening ET & BAL specimens requested for routine bacterial culture to ensure quality[1]

Specimen	Results of screen	
	Good quality	Poor quality
Endotracheal aspirate	<10 SEC/average 10x field and bacteria detected in at least 1 of 20 fields (100x)	>10 SEC/average 10x field and no bacteria detected in 20 fields (100x)
Bronchoalveolar lavage fluid	<1% of cells present are SEC	>1% of cells present are SEC

SEC: Squamous epithelial cells

Note: Perform Ziehl-Neelsen stain for AFB when necessary.

Culture

SPUTUM

1. SPUTUM

Pre- treatment [3]

Ideally sputum should be treated as follows before inoculation.

a) Add equal volume of dithiothreitol (Sputolysin) solution.
b) With dithiothreitol, either mix rapidly on a vortex mixer for 15 seconds and stand for 15 min at ambient temperature or, preferably, mix gently and continuously on a machine that tilts to and fro placed for 30 min in an incubator at 35^0C.

Culture

Dilute the homogenized sputum a further 1 in 100 in sterile broth and inoculate a 0.005 ml loopful of the dilution on to each culture plate. The inoculums should be spread confluently over half of the plate and streaked out over the other half. The plates should be incubated as follows:

- Blood agar incubate 18-24 h at 35^0C in 5-10% CO_2
- Chocolate agar incubate 18-24 h at 35^0C in 5-10 % CO_2 *
- MacConkey agar incubate at 35^0C aerobically

  * (with a high potency Bacitracin(10 units) disc on the well)

- If the request form indicates that a candidal or fungal infection is suspected, the homogenizd sputum should be seeded on to a plate of Sabouraud's or malt extract agar with 50 units nystatin disc for aerobic incubation at 35^0C for 2 days for the culture of candida, and a slope of Sabouraud's or malt extract agar for aerobic incubation at 28^0C for 10 days for the culture of aspergillus.
- If lung abscess or bronchiectasis is suspected additional plates should be inoculated and incubated anaerobically for 2-4 days.

c) If colonies are still small and indistinct, re-incubate.

Reading and interpretation

The growth on the whole area of the plate of 25 or more colonies of the same potential pathogen will then indicate that 10^6 or more of that pathogen were present in each ml of the original sputum.

	• If $\geq 10^6$/ml of potential pathogen- report with ABST. • If $<10^6$ /ml potential pathogen- either ignore or report to the physicians as probably representing contamination of the specimen from the throat.
ENDOTRACHEAL ASPIRATE	**2. ENDOTRACHEAL ASPIRATE** Inoculate on blood agar, chocolate agar and MacConkey agar.
BAL & BB	**3. BRONCHOALVEOLAR LAVAGE FLUID AND BRONCHIAL BRUSH SPECIMENS** Perform quantitative cultures. **Bronchial brush specimens** which contain approximately 0.01 to 0.001 ml of secretions, should be placed in 1ml of sterile saline after collection. The specimen should be delivered to the laboratory immediately. In the laboratory, the specimen is agitated on a vortex mixer, a smear is prepared for Gram stain and 0.01 ml of specimen is inoculated to media mentioned for sputum culture by using a pipette or calibrated loop. Colony counts of more than 1000 CFU/ml of potential pathogens per ml (corresponding to 10^6 CFU/ml of original specimen) appear to correlate with disease. **Bronchoalveolar lavage** results in collection of 50 ml or more of saline. A smear is prepared after cytocentrifugation and Gram stained. The Gram stain report should include a comment about the presence of squamous epithelial cells and intracellular bacteria. Grossly contaminated fluid (>1% of all cells are squamous epithelial cells) may have falsely elevated counts of potential pathogens. A 0.01 or 0.001 ml aliquot of bronchoalveolar fluid should be inoculated to agar media. The recovery of <10,000 bacteria/ml suggests contamination. The recovery of >100,000 bacteria/ml suggests that the isolate is a potential pathogen. Detection of 10,000 to 100,000/ml represents a "gray" zone. Counts of pathogens may be reduced by prior antimicrobial therapy or variations in "return" of lavage fluid during the bronchoscopy procedure.
Identification of organisms	**Refer SOPs for identification of individual pathogen.**

REPORTING PROCEDURE

Distinction between tracheo-broncheal colonization & true pulmonary infection is difficult. Hence Gram stain & culture together with the clinical condition of the patient need to be considered.

Microscopy	**Gram stain** - Pus cells give number/LPF Epithelial cells give number/LPF Organisms
Culture	• Not likely to be significant – " No pathogen isolated" • Likely to be significant – ……..isolated – Report with ABST

ABST

Choice of antibiotics

Streptococcus pneumoniae

1st Line:	**2nd Line:**
Oxacillin (report as penicillin)	Vancomycin
Levofloxacin	Chloramphenicol
Trimethoprim-sulfamethoxazole	Tetracycline
Erythromycin	Linezolid
Clindamycin	

H. influenzae

1st Line:	**2nd Line:**
Ampicillin*1	Cefepime
Co-amoxiclav	Imipenem
Cefuroxime	Meropenem
Cefotaxime or ceftriaxone	Ertapenem
Erythromycin	Tetracycline
Trimethoprim-sulfamethoxazole	Cefixime

M.catarrhalis

1st Line	**2nd Line**
Ampicillin*1	Cefotaxime or ceftriaxone
Trimethoprim-sulfamethoxazole	Levofloxacin
Erythromycin	
Tetracycline	
Co-amoxiclav	
Cefuroxime	

*****1 β-lactamase test will be useful for *H. influenzae* & *M.catarrhalis*

***Acinetobacter* spp.**

1st Line:	**2nd Line:**
Cefotaxime or ceftriaxone	Ticarcillin-clavulanic acid
Gentamicin	Piperacillin-tazobactam
Ceftazidime	Amikacin
Cefepime	Imipenem
Levofloxacin	Meropenem
Ciprofloxacin	Netilmicin
Trimethoprim-sulfamethoxazole	Cefoperazone/sulbactam
Tetracycline	
Ampicillin-sulbactam	

Pseudomonas aeruginosa

1st line	**2nd line**
Ceftazidime	Imipenem
Gentamicin	Meropenem
Cefepime	Cefoperazone/sulbactam
Ciprofloxacin	
Ticarcillin-clavulanic acid	
Aztreonam	
Piperacillin-tazobactam	

Coliform

1st Line	**2nd Line**
Ampicillin	Cefepime
Gentamicin	Ticarcillin-clavulanic acid
Amoxicillin-clavulanic acid	Piperacillin-tazobactam
Cefuroxime	Imipenem
Ciprofloxacin	Meropenem
Netilmicin	Amikacin
Cefotaxime or ceftriaxone	Aztreonam
Ceftazidime	Ertapenem

Important:

- **Screening and confirmatory tests for ESBLs in *Escherichia coli, Klebsiella pneumoniae, Klebsiella oxytoca* and *Proteus mirabilis* are indicated.**
- **Screening and confirmatory tests for suspected carbapenemase production is indicated.**

S.aureus

1st line	**2nd line**
Cefoxitin / methicillin / oxacillin (Report as cloxacillin)	Ciprofloxacin*2
Clindamycin –check for inducible resistance	Fusidic acid
Erythromycin	Vancomycin*3
Fusidic acid	Teicoplanin
Co-trimoxazole	Linezolid
Tetracycline/doxycycline	

*****2 Selective reporting only. Discuss with Microbiologist.
*****3 Report only if cefoxitin / methicillin / oxacillin resistant. If vancomycin is resistant, do not report. Discuss with Microbiologist.

Stenotrophomonas maltophilia	***Burkholderia cepacia***
Trimethoprim-sulfamethoxazole	Trimethoprim-sulfamethoxazole
Levofloxacin	Ceftazidime
Minocycline	Meropenem
	Minocycline

References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
2. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
3. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Safety cabinet	Class 1 or 2 Biological safety cabinet
Microscope	
Bunsen Burners	
CO_2 jar	
Vortex mixer	
Calibrated loops	0.005ml/0.001 ml or 0.01 ml

CONSUMABLES

Sterile wide mouthed screw capped (leak proof) containers	For collection of samples
Glass slides	

MEDIA AND REAGENTS

Blood agar	Refer SOP on media
Chocolate agar	Refer SOP on media
MacConkey agar	Refer SOP on media
Dithiothreitol (Sputolysin)	
Sterile saline	
Sterile water	
Discs for identification (Optochin, X/V and XV disks	Refer SOP for identification
Antibiotic discs	Refer SOP for antibiotic sensitivity testing
Identification materials	Refer SOP for identification

Stains

Gram stain
Ziehl-Neelsen stain

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF THROAT SWABS

Types of specimens	Swabs from posterior pharynx or any other inflamed area in the throat, tonsils

Introduction

Most sore throats are caused by viruses. This SOP describes the examination of throat swabs for bacterial pathogens causing sore throat.

Possible bacterial pathogens:

Group A β-haemolytic streptococci (*Streptococcus pyogenes*)
Group C and G β-haemolytic streptococci

Less common pathogens

Corynebacterium diphtheriae (refer SOP on investigation of diphtheria)
Neisseria gonorrhoeae (refer SOP on investigation of uro-genital specimens)
Arcanobacterium haemolyticum

SPECIMEN COLLECTION AND TRANSPORT

Optimal time of specimen collection	Before starting antibiotic treatment and antiseptic mouth washes
Specimen Collection	1. Explain the procedure to the patient. 2. Ensure adequate lighting. 3. Ask the patient to extend the neck and open the mouth. 4. Swab tonsils, soft palate, uvula and finally the posterior pharyngeal wall. Use of a tongue depressor may be helpful. Care should be taken not to touch the tongue and other parts of the mouth.
Specimen transport	1. Swabs for *Neisseria gonorrhoeae* should be directly plated on selective media for gonococci or placed in to charcoal containing transport medium e.g. Amies transport medium. 2. Specimen should be sent to lab immediately with accompanying request form giving patient identification details and clinical details. 3. Process as soon as possible after receipt.

REJECTION CRITERIA

1. Unlabelled specimen
2. Delay in receipt of sample > 24 hours

In both instances, inform ward / responsible clinician to ensure that a second specimen is sent.

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Culture	1. Inoculate on ½ blood agar plate-incubate overnight at 35^0 in 5%-10% CO_2 and ½ Blood Tellurite (BT) agar plate to detect *C. diphtheriae.* 2. If suspecting *N. gonorrhoeae* inoculate Thayer Martin medium. 3. Make a stab while inoculating on blood agar plate (to provide an atmosphere with reduced O_2 content to enhance haemolysis). 4. *N. gonorrhoeae* is to be looked for only on specific request. (refer SOP on investigation of uro-genital specimens)
Reading & Identification	1. Pick colonies resembling β-haemolytic streptococci for further study. 2. Perform Gram stain. 3. If a streptococcal grouping kit is available, do grouping according to manufacturer's instructions after consulting the microbiologist. 4. If grouping kit is not available subculture on to another ½ blood agar plate and place a 0.04 IU bacitracin disc on the 2^{nd} streak and incubate overnight at 35^0C aerobically. 5. If there is any zone of inhibition around bacitracin disc, report as Group A β-haemolytic streptococcus (*Streptococcus pyogenes*). (5% - 10% of group B, C, G streptococci can also give a zone of inhibition).
ABST	Perform ABST with Penicillin and Erythromycin.

REPORTING PROCEDURE

Positive Report Group A, C and G β- haemolytic streptococci only	Report results as one of the following statements. • If grouping kit is available Group A (*S. pyogenes*)/Group C/Group G β-haemolytic streptococcus isolated (Report ABST) • If grouping kit is not available and bacitracin available Group A β-haemolytic streptococcus isolated or non Group A β-haemolytic streptococcus isolated (Report ABST) • If bacitracin or grouping kit not available β-haemolytic streptococcus isolated (Report ABST)

Negative	β- haemolytic streptococci not isolated **Note:** • Only Group A, C and G β-haemolytic streptococci are known to be causally related to sore throat. • The throat is often colonized with other organisms eg: coliforms, which should **NOT** be reported and for which ABST should **NOT** be done. • *S. aureus* may be significant in peritonsillar abscesses and pustular tonsillitis. • Interpretation of pathogens in throat swabs from immuno-compromised patients should be done in consultation with Microbiologist.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Microscope	
Bunsen Burners	

CONSUMABLES

Glassware

Glass slides

Media and reagents

Media	Blood agar, Blood Tellurite, Thayer Martin media	Refer SOP on media
Antibiotic discs	Penicillin and Erythromycin	Refer SOP for antibiotic sensitivity testing
Identification material	Bacitracin discs 0.04 IU	
	Streptococcal grouping kit	

Other items

Wire loops	
Swabs	For specimen collection
Tongue depressor	Only if specimens are collected in the laboratory

Stains

Gram stain

MAINTENANCE OF RECORDS

Unique records register for Swabs

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF DIPHTHERIA

If Blood Tellurite agar is not available send specimens to the Bacteriology Laboratory, MRI for culture and identification. Tel: 011 2691350

Introduction

This SOP describes the examination of specimens for the presence of *Corynebacterium diphtheriae.*
This may be carried out in the event of a patient presenting with suspected diphtheria (faucial, nasal, cutaneous) or for contacts of a proven case of diphtheria (usually throat swabs). Clinical infection in other sites such as vagina, ear or conjunctiva can be seen rarely.

Types of specimens

Throat swabs	Nasal swabs
Ulcer swabs	Vaginal, ear or eye swabs

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment
Correct specimen type and method of collection	**Faucial diphtheria and contacts of case** – throat swab Collect specimen as in SOP for throat swabs. Swab should be obtained from the inflamed areas surrounding the pseudomembrane [1]. Use of an electric light for proper visualization of the site is recommended. Swabbing a patient with an inflamed epiglottis is contraindicated. **Nasal diphtheria –** nasal swab Collect specimen as in SOP for nasal swabs. **Cutaneous diphtheria** – swab from ulcer Collect 2 swabs from the base of the ulcer. **Laboratory should be informed of suspected diphtheria as BT media are not prepared routinely.**

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	1. Specimen should be sent to the laboratory immediately with accompanying request form giving patient identification details & clinical details. 2. If swabs are to be transported from a distance, they should be sent in Amies or Stuart transport media within less than 24 hours. The swab should be retained inside the media bottle after breaking excess part of the wooden stick. 3. Process specimen immediately. Diagnosis of diphtheria is a laboratory emergency and should be given utmost priority. 4. Storage of specimen may be required if media are not available. Maximum acceptable storage time is 24hrs in the refrigerator, if sent in transport media. 5. Note, time of arrival of sample in laboratory. 6. If there had been >24 hours delay in transporting the specimen, continue processing but inform the clinician and request for a good quality sample.

SPECIMEN PROCESSING

Culture	1. Inoculate the following media using the swab. a) Blood Tellurite (BT) agar plate - incubate overnight at 35^0C in 5-10% CO2. b) Blood agar plate - incubate overnight at 35^0C in 5-10% CO2.
Reading & preliminary identification	1. Pick raised black or grey colonies on BT medium for further study. 2. Gram stain all the different colony types that appear on the BT agar plate. 3. Subculture any suspicious colony from BT agar plate on to a Loeffler's serum slope as soon as possible. 4. Incubate aerobically at 35^0C for 18 hours. 5. Stain smears (from Loeffler) as follows: a) Gram stain b) Methylene blue c) Albert's stain 6. Look for Gram positive pleomorphic club shaped rods with chinese letter arrangement on Gram stain and typical coryneform bacilli with metachromatic granules when stained with Methylene blue or Albert's stain. 7. If there are no typical colonies on BT / Blood agar after overnight incubation, re-incubate a further 24 hours and re-examine all the plates. 8. Send Loffler's serum slopes to MRI for further study.
Identification	Final identification of *C. diphtheriae* and detection of toxin production will be carried out at Reference Laboratory, Medical Research Institute, Colombo.
ABST	Penicillin Erythromycin

REPORTING PROCEDURE

Preliminary Report	1. Telephone report Inform Clinician the preliminary result if characteristic colony morphology together with microscopic morphology are present. 2. Written report → ***Corynebacterium spp.* isolated**. Isolate sent to Reference Laboratory, Medical Research Institute for confirmation and toxigenicity testing. Final report follows.
Final Report	
negative	If there are no typical colonies → ***Corynebacterium spp.* not isolated**
positive	Positive report → **Toxigenic / Non toxigenic *Corynebacterium diphtheriae* isolated**

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burner	

CONSUMABLES

Glassware

Glass slides

Blood agar	Refer SOP on media
Tellurite Blood agar	Refer SOP on media
Amies transport media	Refer SOP on media
Stuart transport media	Refer SOP on media

Other items:

Wire loops

Stains

Gram stain
Methylene blue stain
Albert stain

MAINTENANCE OF RECORDS

Unique records register for Swabs
Register for recording specimens / isolates sent to Reference Laboratory

References

1. Greenwood D, Slack RCB, Peutherer JF. **Medical Microbiology,** 16th Edition 2006. Churchill Livingstone
2. Mandell GL, Bennette J, Dolin R. **Mandell, Douglas and Bennett's Principles and Practice of Infectious diseases,** 7th Edition 2010. Churchill Livingstone
3. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF URO-GENITAL SPECIMENS

Specimens

Urethral swabs
Endocervical swabs
Fluid, scrapings or swabs from genital ulcers
Anorectal swabs
Throat swabs
Urine

Reference Laboratory
Central Laboratory for STI / AIDS programme
Microbiology Laboratory
Tel. 0112667163, 0112696433
Fax. 0115336873

Possible pathogens

- *Neisseria gonorrhoeae*
- *Chlamydia trachomatis*
- *Tryponema pallidum*
- Herpes simplex virus

Indications for microbiological investigation

- Urethral discharge
- Contact with a patient with proven gonococcal or chlamydial infection
- Vaginal discharge
- Genital ulcers

Rejection criteria

1. Unlabelled specimens
2. Specimens without accompanying request forms
3. Improper transport for isolation of *N. gonorrhoeae*
   - If collected into transport medium and kept for more than 48 hours
   - If collected into transport medium and refrigerated
   - If discharge sent on dry swab without transport medium
4. Duplicate specimen from patient for same investigation without justification

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.

SPECIMEN COLLECTION

Optimal time of specimen collection	Before antibiotic treatment
Specimens	**For gonococcal infection** • Urethral swab from male and female patients • Endocervical swab from female patients • Anorectal swab • Throat swab • Urine In prepubertal girls, gonococcal vulvo-vaginitis is a possibility. Vaginal discharge can be collected with a swab without the use of a speculum. The best specimen to obtain should be discussed with the microbiologist. **For chlamydial infection** (Should be collected using the swab provided in the Chlamydia specimen collection kit.) • Urethral swab • Endocervical swab (should be collected from the squamo-columnar epithelial junction) • Urine
Method of collection	
Urethral discharge from males	**For investigation of gonococcal infection:** 1. Patient should not have passed urine for 1 hour before specimen is collected. 2. If discharge is evident collect pus directly. 3. If not, exert slight pressure on penis so that a drop of pus may appear at the meatus. 4. Collect pus with a sterile inoculating loop/swab or directly to a clean glass slide. 5. If no discharge appears, insert the sterile loop approximately 1 cm up the urethral canal to obtain a specimen. **For investigation of chlamydia infection:** 1. Clean the meatus with a swab moistened in normal saline or sterile water. 2. Insert a swab 2 - 3 cm into the urethra and rotate, making sure the swab is in contact with the urethral wall. 3. Remove the swab and place it in transport medium.
Endo-cervical swab	Preferably 2 swabs should be taken (for culture and preparation of a smear). 1. Cervical specimens should be collected by a medical officer. 2. Vaginal speculum may be moistened with sterile warm water if necessary. No lubricant cream or antiseptic should be used.

	3. Any cervical discharge, mucus or pus should be wiped away with a sterile swab. 4. Take endocervical swab by inserting the swab into the cervical canal for 1-2cm. 5. Rotate for 10 seconds in canal and withdraw swab without touching vaginal wall or secretions. 6. Inoculate the specimen directly on suitable media at collection site or place the swab in a suitable transport medium and send for culture. **Preparation of smear prior to transport to laboratory** 1. Prepare a thin smear on a clean slide. 2. To obtain a thin homogenous film, roll the swab unidirectionally on the glass slide. 3. Allow the smear to air-dry.
Ano-rectal swab and throat swab	These swabs should be collected if suspected of gonococcal infection in the throat or rectum in addition to the swabs from the primary sites as above. **Rectum** – Insert a cotton swab 3cm into the anal canal and rotate it for 10 seconds to collect exudates from the crypts just inside the anal ring. If faecal contamination occurs, discard the swab and use another to obtain the specimen. **Oropharynx** – Swab the region of the tonsillar crypts and the posterior pharynx.
Urine	**For Chlamydia** First part of urine (obtained at least 2 hours after last micturition) can be used after centrifugation for antigen detection and PCR test. **For *N. gonorrhoeae*** First voided urine sample can be used after centrifugation for culture.

SPECIMEN TRANSPORT AND STORAGE FOR N.GONORRHOEAE

Central STI Clinic in Colombo and some other clinics provide a culture service	1. Where culture facilities are not available locally, the specimens need to be transported to the reference laboratory. At present gonococcal culture services are available in the Central Laboratory of STI/AIDS Control Programme in Colombo and in the STI clinics in Kandy, Badulla, Ragama, Anuradapura and Kurunegala.
Transport media can be obtained from any STI clinic in the country	2. Ideally, the urethral or cervical discharge should be inoculated at the bedside directly onto a selective culture medium such as Thayer Martin medium for the isolation of *Neisseria gonorrhoeae*. Inoculated plates should be sent to the laboratory immediately. 3. If culture media are not available locally, transport medium can be used.

	4. Bottles of Amies transport medium can be obtained from any STI clinic in the country. However it should be remembered that even with the use of transport media, results are not satisfactory if transport takes more than 48 hours. 5. When inoculating, insert the swab into a container of Amies transport medium. Break off the swab stick aseptically and allow the bottle top to be replaced tightly. 6. These bottles should be transported at room temperature. 7. Isolation rate is approximately 100% if transported within 12 hours and about 90% within 24 hours, although the number of colonies decreases markedly. If selective media are not available, • Inoculate on to chocolate agar and incubate for 5-7 days for the colonies to appear. • Do a Gram stain and the oxidase test on typical colonies. • For further identification and confirmation of gonococci send the isolate to a STI laboratory. • Isolation rate is low in comparison to the use of selective media.

SPECIMEN TRANSPORT AND STORAGE FOR CHLAMYDIAE

• **Antigen detection**	Before collecting the specimen check with the reference laboratory whether the facility is available for chlamydia antigen detection.
• **Culture and PCR**	Use the special swab in the collection kit of chlamydia antigen detection test. Culture and PCR are not currently available.

SPECIMEN PROCESSING

Microscopy	**Staining the direct smear for examination** Direct microscopic examination is not recommended for rectal and pharyngeal infections. 1. Fix the smear by passing through the flame. 2. Stain with Gram stain using saffranin as the counterstain *(Neisseria gonorrhoeae* may not be well stained by carbol fuchsin). 3. Examine the stained smear under oil immersion by paying special attention to the edges of the smear, where the smear is very thin as it is easy to identify gonococci, if present. 4. Look for the following under the microscope a. Pus cells (polymorphs) – count number of polymorphs per oil immersion field b. Gonococci are oval, kidney shaped, Gram negative cocci that are arranged in pairs. They are present intracellularly in pus cells and/or extracellularly. c. Examine as many fields as possible (for at least 2 minutes) before declaring it negative for Gram negative intracellular diplococci.
Culture	1. A selective culture medium such as Thayer Martin medium gives best isolation rates for *Neisseria gonorrhoeae*. 2. If selective media are not available inoculate on to chocolate agar and incubate for 2 - 5 days for the colonies to appear. Do a Gram stain and the oxidase test on typical colonies. 3. For further identification and confirmation of gonococci send the isolate to a STI laboratory. 4. Isolation rate is low in comparison to the use of selective media.

SPECIMENS FROM GENITAL ULCERS

Investigation of Syphilis

Dark Ground Microscopy Darkfield microscopy is used to demonstrate *Treponema pallidum* Considerable expertise is required not only for the correct identification of the spirochetes but also for proper use of a darkfield microscope.	**Collection of specimen for darkground microscopy:** 1. Wearing gloves squeeze the base of the ulcer between two fingers and clean the ulcer surface with saline. 2. Remove any crusts, if present. 3. Wipe away the first few drops of fluid, especially if blood stained. 4. Collect the sample of serous exudate by touching the lesion with a clean glass slide (or cover slip). 5. Place a clean cover slip on the slide (or the cover slip on a clean slide). 6. Examine immediately under the dark-field microscope. 7. Alternatively the specimen may be aspirated from the lesion using a sterile syringe.

Investigation of Herpes simplex virus

Tzanck test Method used to demonstrate cellular changes caused by the herpes group of viruses	1. Samples should be taken from a fresh vesicle, rather than a crusted one, to ensure the yield of a number of virus infected cells. 2. The vesicle should be unroofed or the crust removed, and the base gently scraped with a swab. 3. The material thus obtained is smeared onto a microscopic slide, allowed to air dry, and stained with Giemsa stain. 4. The slide should be clean, since cells will not adhere to an unclean slide. 5. The stained nuclei may vary in colour from reddish blue to purple to pink. The cytoplasm stains bluish.
Culture	**Refer SOP on virology** At present HSV culture is performed only at MRI

REPORTING PROCEDURE

DIRECT SMEAR (Gram stain for *N. gonorrhoeae*)	Number of pus cells per oil immersion field : ▪ No pus cells - Not seen ▪ occasional (1) - Occasional ▪ few (1 – 4) - + ▪ Moderate (5 – 8) - ++ ▪ numerous (> 8) - +++
Positive smear	Intracellular Gram negative diplococci seen. (**or** Intracellular and extracelluar Gram negative diplococci seen.) Diagnostic of gonococal infection. **or** Extracellular Gram negative diplococci seen. Suggestive of gonococcal infection – suggest repeat sample.
Negative smear	Negative for Gram negative diplococci suggestive of gonococci. Please note in patients with chlamydial infection pus cells will be present with no organisms seen in the smear.

References

E. Van Dyck, A.Z Meheus, P.Piot**. Laboratory Diagnosis of Sexually Transmitted Diseases**, WHO Geneva1999.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	
CO_2 jar or preferably CO_2 incubator	

CONSUMABLES

Glassware

Glass slides
Cover slips

Media and reagents

Normal saline	
Thayer Martin medium with supplements	Available in reference laboratory
Amies transport medium	Can be obtained from the reference laboratory
Virus transport medium	Can be obtained from MRI
Collection kits of Chlamydia Ag detection tests.	

Other items:

Inoculating loop
Sterile swabs

Stains

Gram stain	Note that the counter stain for gonococci is saffranin
Giemsa stain	

MAINTENANCE OF RECORDS

Unique records register for Swabs.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF VAGINAL DISCHARGE

Type of specimen High vaginal swabs/vaginal swabs

Introduction

Specimens of vaginal discharge are sent for microbiological investigation in the following situations.

1. Investigation of vaginal discharge
2. Post-operative & post partum infections
3. Premature rupture of membranes (PROM)

1. Investigation of vaginal discharge (as a symptom of STI)

Likely pathogens in this clinical context:

- *Candida albicans*
- *Trichomonas vaginalis*
- *Gardnerella vaginalis* (as indicator of bacterial vaginosis)

Less commonly, gonococcal infection may present with a vaginal discharge in pre-pubertal girls. Refer SOP on urogenital specimens.

2. Investigation of post-operative & post partum infections - Refer SOP on Pus.

3. Investigation of PROM – Growth of any organism is considered as significant.

REJECTION CRITERIA

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.

SPECIMEN COLLECTION AND PROCESSING

Optimal time of specimen collection	Before starting antimicrobial treatment
Microscopy **Trichomonas infection** (Refer SOP on Parasitology)	• Insert speculum & collect the specimens from the posterior vaginal fornix. Require at least four swabs for different tests. If profuse discharge is present collect into a sterile container. • If cervicitis is suspected collect specimen from cervical canal. 1. Place a drop of saline on a clean slide. 2. Mix the discharge on the swab with the saline. 3. Examine immediately as a wet film under the microscope (low and high power). Detection of motile trichomonads is absolutely specific and confirms the diagnosis. Care should be taken for the specimen not to be contaminated with faecal matter.

Candida infection	Prepare a smear on a clean slide using a separate vaginal swab. 1. Stain with Gram stain. 2. Examine for yeast cells and pseudohyphae.
Bacterial vaginosis	1. Look for 'clue cells' in the Gram stain or wet smear - Clue cells are vaginal epithelial cells coated with 'Gram-variable' short bacilli which obscures the borders of the cells. 2. Note the absence of (or a decrease in) lactobacilli. 3. Note the absence of (scanty) pus cells.
Culture	Specimens should be inoculated onto, Blood agar Incubate overnight at 35°C in 5-10% CO_2. MacConkey agar Incubate overnight at 35°C aerobically. Chocolate agar Incubate overnight at 35°C in 5-10% CO_2. Sabouraud's Glucose agar Incubate 48 hours at 35°C. For processing, identification and ABST refer SOP for Pus.
Bacterial vaginosis	Not recommended as a diagnostic tool.
Trichomonas infection	Refer SOP on Parasitology.

REPORTING PROCEDURE

Report	
Gram stain	Pus cells - not seen / scanty / + / ++ / +++ Clue cells - seen / not seen Lactobacilli - seen / not seen Trichomonas - seen / not seen Yeasts / pseudohyphae - seen / not seen
Culture	1. Vaginal discharge – report candida and or other relevant isolates 2. Post partum/post op infection- Refer SOP on pus 3. PROM – Report all isolates with ABST

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	
CO_2 jar or preferably CO_2 incubator	

CONSUMABLES

Glassware

Glass slides
Cover slips

Media and reagents

Normal saline

Other items

Inoculating loop
Sterile swabs

Stains

Gram stain	Note that the counter stain for gonococci is saffranin
Giemsa stain	

MAINTENANCE OF RECORDS

Unique records register for Swabs.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF FAECES

Types of specimens	Faeces (watery or semi formed) Rectal swabs (for investigation of outbreaks)

Introduction

Diarrhoea is caused by a large number of organisms, which include bacteria, viruses and parasites. **This SOP describes the methods of detection of four frequently encountered bacterial pathogens**. The absence of these pathogens in watery or bloody faeces ***does not exclude*** an infective cause of diarrhoea.

Commonly encountered pathogens in a microbiology laboratory

- **Bacterial**
  - Enteropathogenic *E. coli*
  - *Salmonella* species
  - *Shigella* species
  - *Vibrio cholerae* (refer SOP on Cholera)
  - Campylobacter species – culture done at MRI (011-2691350)

- **Antibiotic associated colitis / Pseudomembranous colitis**
  Commonest cause of hospital acquired diarrhoea. May follow treatment with almost any antibiotic. The faeces may be watery or with blood and mucous.

  Laboratory diagnosis: detection of toxins A & B produced by *Clostridium difficile* and anaerobic cultures are done at Anaerobic laboratory, MRI (011-2693532/3/4 ext. 344). Specimen should be collected after 48hrs of diarrhoea into a sterile container and transported in ice.

- **Viral**
  Watery diarrhoea in children is most likely to be caused by rota virus.

  Laboratory diagnosis: Antigen detection is the preferred method but electron microscopy is also available (refer SOP on virology)

- **Parasites**
  (Refer SOP on Parasitology)

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Early in the course of diarrhoea, before starting antibiotic treatment
Correct specimen type (in order of preference) and Method of collection	1. **Faeces specimen** - If it is blood and mucous diarrhoea collect om a portion of faeces containing blood and mucous. Collect into a clean, wide mouthed, disinfectant free, screw capped container or leak proof container with a tight fitting lid (eg: Marmite bottle).

	2. **Faecal swab** to be taken if specimen is to be transported to a distant laboratory. 3. **Rectal swab** should be taken only when it is not possible to obtain faecal specimen or during a field investigation of an outbreak (eg: Cholera). Rectal swab needs to be visibly soiled with faecal matter. Specimen collection for virology and parasitology (Refer SOPs on Virology and Parasitology).

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	1. Process the specimen within two hours. 2. If unable to process immediately store at 2^0C - 8^0C. 3. If delay in transport for more than two hours is anticipated, inoculate the specimen in a transport medium such as Cary –Blair transport medium and transport preferably in ice. The same transport media can be used for cholera as well.

REJECTION CRITERIA

1. Containers with faecal contamination on the outside
2. Unlabelled specimen
3. Solid faeces except for the detection of salmonella carriage
4. Duplicate specimens from patient for same investigation without justification
5. Specimens without accompany request forms

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Macroscopy	Semisolid/ liquid ; Blood/mucous
Microscopy	Make a saline smear on a slide and examine microscopically (x 40 objective) for pus cells, red cells and parasites (amoebae, ova and cysts). (Refer SOP on Parasitology).
Culture	For 1. Salmonella 2. Shigella 3. Enteropathogenic *E. coli (*EPEC) –In children 2yrs and below
Laboratory processing	**Day 1** 1. Inoculate a loopful of specimen each on half a plate of: MacConkey agar (No.3) -incubate at 35°C aerobically overnight SS agar or / and XLD agar -incubate at 35°C aerobically overnight

Identification

2. Inoculate a loopful of faeces into Selenite F broth and incubate overnight at 35°C aerobically.

Day 2

1. Examine plates.
2. Look for typical colonies on these media (refer SOP on media).

	Salmonella	Shigella	EPEC
XLD	Red colonies with black center	red	yellow
SS	Pale colonies with black center	pale	pink
MacConkey	Pale colonies	pale	pink predominant growth

3. Inoculate typical colonies into Kligler iron agar (KIA) or TSI agar slants and urea slants. Incubate overnight at 35^0C.
4. Subculture from Selenite F broth on SS or XLD agar plate. Incubate at 35^0C overnight.

Day 3

1. Look for the typical Kligler patterns as given below.
2. Identify using serology as given below.
3. If no pathogens are isolated from the primary plate proceed with SS or XLD plates inoculated from Selenite F.

	Kligler pattern				**Urease**
	slant	butt	gas	H_2S	
Salmonella spp.	K	A	+	+	-
Shigella spp. (*Shigella flexneri* 6 may produce gas)	K	A	-	-	-
E. coli	A	A	+	-	-
Salmonella typhi	K	A	-	+ (little along the stab)	-
Salmonella paratyphi	K	A	+	-	-

K – alkali A –acid

(*S. typhi* and *S. paratyphi* A rarely present with diarrhoea. Usually requested in the diagnosis of enteric fever).

Isolates with a typical Kligler pattern which do not give agglutination with shigella antisera – perform oxidase test to exclude cholera

SERO TYPING

Idealy before Salmonella serotyping, isolate should be confirmed with lysine, indole and motility tests.

Polyvalent Salmonella antisera Poly O (A-S) } ***Salmonella spp.***
Poly H

	O	H (phase 1)	H (phase 2)
Salmonella typhi	9	d	- (Vi +)
Salmonella paratyphi A	2	a	-
Salmonella paratyphi B or S. java	4	b	1,2 (send isolates to reference lab for differentiation)
Salmonella paratyphi C	6,7	c	1,5
Salmonella typhimurium	4	i	1,2
Salmonella enteritidis	9	g,m	

Shigella dysenteriae	(polyvalent)	+ve - *Shigella dysenteriae*
Shigella flexneri	(polyvalent)	+ ve - *Shigella flexneri*
Shigella sonnei	(polyvalent phase 1 & 2)	+ve - *Shigella sonnei*
Shigella boydii	(polyvalent)	+ ve - *Shigella boydii*

ABST

Choose 4-5 antibiotics according to local use

Shigella spp.	***Salmonella spp.***	***Salmonella typhi***
Ampicillin	Ampicillin	Choramphenicol
Cotrimoxazole	Cotrimoxazole	Ciprofloxacin
Furazolidone	Furazolidone	Ceftriaxone / Cefotaxime
Ciprofloxacin	Ciprofloxacin	Ampicillin
Mecillinam	Mecillinam	Co-trimoxazole
Nalidixic acid	Nalidixic acid*	Azithromycin
Ceftriaxone		Nalidixic acid[*1]
		Cefixime

*If Nalidixic acid resistant, report ciprofloxacin also as resistant

REPORTING PROCEDURE

Wet prep

Direct smear - Pus cells – absent / scanty/ numerous
RBC -- absent / scanty/numerous
AOC --

Direct smear result should be sent on the same day.

Time frame for Culture report
Negative report – Day 3-4
Positive report – Day 3 – 5

Positive culture: Report as → ……. isolated (ABST will follow)

Report ABST when available

(Note: issue preliminary report as early as possible and final report later)

Negative culture: Report as → Shigella and Salmonella not isolated

When EPEC is looked for: Report as → Shigella , Salmonella and EPEC not isolated

In patients with HUS – contact Enteric Reference Laboratory, MRI Colombo 8 Tel. 0112 691350

Note:
***E. coli* can cause diarrhoea by several mechanisms**

a) Enterotoxigenic *E. coli* (ETEC) is the commonest. This cannot be identified in a routine laboratory.

b) Enterohaemorrhagic *E. coli* (EHEC) serotype O 157: H 7 may be associated with bloody diarrhoea and Haemolytic Uraemic Syndrome (HUS).
- Contact Enteric Reference Laboratory, MRI.

c) The definitive identification of Enteroinvasive *E. coli* requires cell cultures and can not be undertaken by a routine diagnostic laboratory.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	Maintained at 2°C - 8°C
Microscope	
Bunsen Burners	

CONSUMABLES

Glassware

Glass slides	
Wide mouthed sterile screw capped containers	For collection of samples
Bijou bottles	For Selenite F broth, Urea slant
Test tubes	For KIA / TSIA

Media and reagents

Cary Blair transport medium		
XLD medium		ReferSOP on media
SS agar		Refer SOP on media
MacConkey Agar		ReferSOP on media
Selenite F broth		ReferSOP on media
Kligler Iron Agar (KIA)		Refer SOP on media
Urea slope		Refer SOP on media
Antibiotic discs		Refer SOP on antibiotic sensitivity testing
Antisera	*Salmonella* *Shigella* EPEC	

MAINTENANCE OF RECORDS

Register for Faeces
Register for recording isolates sent to Reference Laboratory

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. **The Oxoid Manual**. 9th Edition 2006.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF CHOLERA

Types of specimens	Faeces Rectal swabs (investigation of outbreaks)

SPECIMEN COLLECTION/SPECIMEN CONTAINER

Optimal time of specimen collection	Before starting antibiotic treatment. Early after onset of diarrhoea.
Correct specimen type (in order of preference) and method of collection	1. Faecal sample – collect into a clean, leak proof wide- mouthed container (eg. Marmite bottle) 2. Swab of fresh faeces in suitable transport medium.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	1. Process the specimen within two hours. 2. Store at 2°C-8°C if there is delay in processing. 3. If delay in transport is anticipated, inoculate the specimen in a transport medium.
Transport medium	Cary-Blair medium. Can be obtained from Enteric Laboratory, MRI. If Cary-Blair medium is not available alkaline peptone water may be used but it is inferior to Cary-Blair medium.

REJECTION CRITERIA

1. Containers with faecal contamination on outside of the container
2. Unlabelled specimens
3. Specimens without accompanying request forms
4. Formed faeces
5. Duplicate specimens from patient for same investigation without justification.

Note:

1. If a specimen is unacceptable inform Microbiologist before rejecting.
2. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
3. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Macroscopy	Semisolid/ liquid
Culture	**Day 1** 1. Inoculate a loopful on TCBS medium (primary medium). 2. Inoculate a loopful in 5–10 ml of Alkaline peptone water (APW-enrichment medium). 3. Incubate the plates at 35^0C overnight. 4. Incubate alkaline peptone water for 5-8 hours at 35^0C. 5. Do not shake or mix APW before subculturing. 6. Streak a loopful (of the culture taken from the top most portion) of APW on TCBS. Incubate plates at 35^0C overnight. **Day 2** 1. Examine the TCBS plate for typical colonies. (*V.cholerae* – flat, golden yellow colonies. Gram stain –small Gram negative curved rods) 2. If characteristic colonies are seen – inform the ward. Inoculate Kligler and urea slopes. 3. If APW could not be subcultured after 5-8 hrs of incubation, after overnight incubation at 35^0C subculture to a fresh APW. Incubate for 5-8hrs and then subculture to TCBS. (Do not perform oxidase test or serotyping from growth on TCBS agar) **Day 3** 1. Kligler pattern → K/A, no gas, no H_2S. Urease negative. 2. Perform oxidase test and string test on isolates with typical kligler pattern. 3. String test - Make a suspension of growth from Kligler in 0.5% sodium deoxycholate on a glass slide and look for mucus string formation. If both tests are positive – Do serotyping if antisera are available. If not available – Contact Enteric Reference Laboratory (MRI) **SEROTYPING** 1. Mix one drop of organism suspension and one drop of antiserum O1 on a glass slide. (Usually equal volumes of antisera and growth suspension are mixed) 2. Rotate slide for one minute and observe agglutination. 3. If positive with O1 antiserum –send to MRI for further typing (subserotyping and biotyping). 4. If it is not agglutinating with O1 antiserum , test with O139 antiserum. 5. In an epidemic only initial few isolates of *V. cholerae* O1 need to be sent to MRI for biotyping/ subserotyping.
Send preliminary report	
Inform MOH	
Send first isolate to Reference Laboratory for confirmation	
ABST	Tetracycline Furazolidone Ampicillin Ciprofloxacin Erythromycin - (No CLSI zone diameters) Cotrimoxazole Doxycycline

REPORTING PROCEDURE

Preliminary report	Isolate suggestive of *Vibrio cholerae.* Confirmation of identity to follow. or Sent to reference laboratory for confirmation.
Final report **Negative cultures**	Negative for *Vibrio cholerae*.
Positive cultures	*V. cholerae* O1 or, O139 or non O1 isolated (depending on the serotyping). Report antibiotic sensitivity results.
Reference Laboratory	Reference laboratory for enteric pathogens, Medical Research Institute.

References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
2. CDC laboratory method for diagnosis of epidemic dysentery and cholera, 1999 WHO/CDS/CSR/EDE/99.8 http://www.cdc.gov/ncidod/DBMD/diseaseinfo/cholera_lab_manual.htm
3. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	

CONSUMABLES

Glassware

Clean **wide mouthed** screw capped bottles	for collection of specimen
Glass slides	

Media and reagents

TCBS agar		Refer SOP on media
Alkaline peptone water		Refer SOP on media
Cary Blair transport medium		Refer SOP on media
Antibiotic discs		Refer SOP on ABST
Kligler tubes		Refer SOP on media
Urea slopes		Refer SOP for media
Oxidase reagent		Refer SOP for identification procedures
0.5% sodium deoxycholate	for string test	
Vibrio cholerae antisera	For identification *V. cholerae* antiserum –O1 and O139 O1 subserotypes- Inaba, Ogawa	

Other items:

Sterile screw capped vials /containers with nutrient agar slopes for transport of isolates to reference laboratory
Wire loops

Stains

Gram stain

MAINTENANCE OF RECORDS

Specimen register for recording specimens / isolates sent to reference laboratory
Unique records register for faeces

STANDARD OPERATING PROCEDURE FOR SCREENING FOR GROUP B STREPTOCOCCAL CARRIAGE

Types of specimens Vaginal and rectal swabs

Introduction

Neonatal group B streptococcal disease has become the major infectious cause of illness and death among newborns. Up to 30% of normal women are colonized with group B streptococcus (GBS) in the vagina or rectum.

Studies have shown that the accuracy of prenatal screening for identification of GBS colonization can be enhanced by timing of cultures, the sites swabbed and the microbiological method used for culture. Collection of vaginal & rectal swabs between 35 and 37 weeks of gestation is recommended to improve the sensitivity and specificity of detection of colonization at the time of delivery.

Selective enrichment broth is recommended to increase the isolation of GBS and to avoid overgrowth of normal flora.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Collection of swabs between 35 and 37 weeks of gestation
Correct specimen type and method of collection	Sterile cotton swab should be provided by the laboratory. **Vaginal and rectal swabs:** Swab the lower vagina (vaginal introitus) and the rectum with the same swab or two different swabs. **Cervical swabs**: not recommended. Transport immediately or send swabs in Amies transport medium with charcoal. One combined vaginal/rectal swab or two separate swabs processed as one. If processing is delayed, refrigeration is preferable to storage at ambient temperature. Delays of over 48h are undesirable.

REJECTION CRITERIA

General:

1. Specimens without a label.
2. Unsterile container
3. Duplicate specimens.

Note:

1. If a specimen is unacceptable inform Microbiologist before rejecting.
2. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
3. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Test selection	**Laboratory procedure: Screening by enrichment**
GBS screening	**Enrichment Culture** 1. Remove the cap from the container and place the swab/s in the broth aseptically (LIM Broth -10 ml Todd-Hewitt broth supplemented with 10µg/ml colistin and 15µg/ml nalidixic acid). 2. Break off the swab sticks and replace the cap (The bottle should contain a volume of broth adequate to cover the swabs). Caps should be kept loose during incubation. Incubate the broth for 18-24 hrs in 5-10% CO_2. 3. After incubation, sub-culture with a sterile loop and inoculate on Blood agar and MacConkey agar. 4. Incubate blood agar for 18-24hrs in 5-10% CO_2 at 35^0C and MacConkey agar in ambient air at 35^0C. Culture plates are read at 18-24 hours and 40 hours.
Identification	Refer SOP for identification.
ABST	Penicillin, Erythromycin

REPORTING PROCEDURE

Report	
Positive	Group B streptococci isolated.
Negative	Group B streptococci not isolated.

References:

1. Health Protection Agency Guidelines; UK . 2006
2. Mandell GL, Bennette J, Dolin R. **Mandell, Douglas and Bennett's Principles and Practice of Infectious diseases,** 7th Edition 2010. Churchill Livingstone

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	
CO_2 jar	

Media and reagents

Blood agar	Refer SOP on media
LIM broth	Todd-Hewitt broth supplemented with 10µg/ml colistin and 15µg/ml nalidixic acid
Mueller Hinton agar	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity testing
Identification materials	Refer SOP for identification
Streptococcal grouping kit	

STANDARD OPERATING PROCEDURE FOR SCREENING FOR MRSA AND STAPHYLOCOCCUS AUREUS

Types of specimens Swabs sent for screening of MRSA/MSSA

Screening is only done in specific clinical situations listed below.

a) Screening of an individual patient in the event of recurrent staphylococcal sepsis (MSSA and MRSA are both reported).
b) If MRSA is isolated from one site in in-patients, for decolonization purposes.
c) Screening for MRSA carriage in the event of a hospital outbreak (eg.post operative sepsis) which may be performed on patients as well as on health care workers.
d) Screening carried out before high risk surgery such as cardiac, orthopaedic and neuro surgery (MSSA and MRSA are both reported).
e) Screening of patients from high risk units or hospitals on admission, depending on the local policy.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Correct specimen type and method of collection	Sterile cotton swabs should be provided by the laboratory. Skin and nasal swabs should be collected with swabs moistened with sterile normal saline. Either attending physician or nurse should collect the swab.
Nasal swab:	Swab both nostrils with a single swab in a circular motion.
Throat swab:	A swab of the back of the throat is taken by rotating a swab as it is moved gently back and forth across the throat.
Perineum or groin swab:	Swab from the perineum or groin area may be taken by the patients themselves following instructions given by the nursing staff about the technique, if the patient is able to. Again, the swab should be rotated whilst being brushed across the area.
Other sites e.g. axilla, skin lesions etc	Swab using the same technique. **Note:** Swabs could be collected into 7% NaCl nutrient broth or manitol salt broth.

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	Transport the specimen with minimum delay. In any delays in transport, for dry swabs use Amies medium.

The request form should clearly state that the swabs are for "**MRSA / *Staphylococcus aureus* screen**".
Note: A separate form is not required for each swab.

REJECTION CRITERIA

General:

1. Specimens without a label
2. Unsterile container
3. Dried out specimens and swabs
4. Duplicate specimens

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately. Request another sample of good quality.
2. Names of persons involved and action taken should be documented.

SPECIMEN PROCESSING

Test selection	**Laboratory procedure: Screening by enrichment:**
MRSA/ **MSSA screening**	Enrichment method 1. Inoculate swabs into selective enrichment broth (mannitol salt broth or 7% NaCl nutrient broth). 2. Incubate overnight at 35 ^{0}C. 3. Subculture onto mannitol salt agar. (if not available use blood agar) Selective media which can be used for MRSA screening are: • Blood agar with methicillin - Incubate for 24 hrs at 35^{0}C aerobically. • MRSA Chrome agar MRSA chrome agar should be incubated 48 hrs at 35^{0}C. Pick likely colonies of *S. aureus.* Identify according to the SOP on identification of *Staphylococcus spp.*
ABST Choice of antibiotics	Perform only for *S. aureus* isolates. • Cefoxitin (if not available oxacillin or methicillin) ▪ Methicillin/oxacillin sensitivity - Read after 24 hours of incubation at 35^{0}C. ▪ Cefoxitin sensitivity can be read after 18 hours. • Read mupirocin after 24 hours. For other antibiotics – discuss with Microbiologist.

REPORTING PROCEDURE

Report	
Positive culture	Methicillin sensitive *S. aureus* report as: → *S. aureus* isolated. If methicillin resistant report as: → MRSA isolated Report Mupirocin susceptibility results.
Negative culture	If no characteristic colonies, report as: → *S. aureus* not isolated

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	

CONSUMABLES

Glassware

Glass slides/petri dishes

Media and reagents

Mannitol salt agar / Blood agar Optional - Blood agar with methicillin MRSA Chrome agar	
Mannitol salt broth / 7% NaCl nutrient broth	
Amies Transport Medium	Refer SOP on media
Antibiotic discs	Refer SOP on antibiotic sensitivity testing
Identification materials	Refer SOP for identification
Mueller Hinton Agar	Refer SOP on media
0.5 % McFarland standard	

Other items:

Wire loops
Cotton swabs

Stains

Gram stain

Reference

Health Protection Agency Guidelines; UK

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF SPECIMENS FOR ANAEROBIC BACTERIA

Types of specimens

Aspirated material from abscesses, sinus tracts and pus from beneath skin flaps
Aspirated sterile fluids (peritoneal fluid, pleural fluid, joint aspirate)
Supra pubic bladder aspirate
Blood and bone marrow
CSF
Biopsy of tissue or bone
Material obtained by transtracheal aspiration
Material obtained by culdocentesis

Introduction

Proper selection of specimens for anaerobic culture is important in order to minimize contamination with endogenous micro flora. It is the task of the laboratory personnel to educate those responsible for selection, collection and transport of specimens for anaerobic bacteriology.

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

Optimal time of specimen collection	Before starting antibiotic treatment (local/systemic)
Method of collection	Specimen should be collected using aseptic techniques, and taking precautions to minimize contamination with endogenous microflora. The best specimens would be those collected with a needle and syringe and biopsies taken during surgery.
Specimen transport & storage	All specimens must be transported as rapidly as possible with minimum exposure to oxygen. Use anaerobic transport containers if available. If they are not available, transport pus in a sterile screw capped container. Biopsies in sterile saline in a screw capped container. Stools for *C.difficile* toxin and culture in a wide mouthed container with a lid. If a delay in transport is anticipated transport in ice.

REJECTION CRITERIA

1. Unlabelled specimen
2. Unsterile container
3. Unacceptable specimens
   - Throat , nasal or oral swabs
   - Expectorated sputum

- Stools, rectal swabs
  (Note: stool specimens will be accepted when specific pathogens eg. *C.difficile* are sought)
- Voided or catheterized urine
- Vaginal, cervical and urethral swabs
- Swabs from superficial wounds, burns or ulcers

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.
3. Do not discard samples which may be unrepeatable – eg. Pus from brain abscess

SPECIMEN PROCESSING

Laboratory procedure	**Processing a specimen of pus for anaerobic culture**
Macroscopic examination	Note : Colour Odour Consistency Presence of granules
Microscopic examination	1. Saline mount: x 40 Examine for pus cells and branching filaments 2. Gram Stain: The importance of the Gram stain in processing specimens for anaerobes cannot be overemphasized. Even in laboratories without culture facilities, the Gram stain findings may give useful information which will implicate an anaerobic infectious process. eg. a polymicrobial picture will indicate an anaerobic infection. It is sometimes possible to make **a presumptive identification** on the Gram stain findings alone. e.g:*Actinomyces* species, *Fusobacterium neucleatu*m, *Clostridium perfringens* .
Culture	Culture of specimens for anaerobes should be performed immediately after the specimen reaches the laboratory. A delay may result in death of obligate anaerobes. Specimen is inoculated into non selective and selective media. **Non selective media**: Blood agar(BA),Brucella Blood Agar with Hemin and Vit K (BBA), **Selective agar**: Neomycin Blood Agar(NBA), Bacteroides Bile Esculin Agar(BBE) 1. Incubate anaerobically for 48 hours or longer. 2. Examine plates with a hand lens and record the colony morphology of each colony. 3. Perform Gram stain. 4. Do aerotolerance testing. 5. Subculture each colony on BBA.

Aero tolerance Testing:	Aero tolerance testing is done to differentiate a true anaerobe from a facultative anaerobe. BA and a BBA plate are inoculated simultaneously from a single test colony, using a straight wire. Incubate the BA plate aerobically and the BBA plate anaerobically. After overnight incubation check the aerobically incubated plate for growth. If growth is present, the isolate is not a true anaerobe. Those isolates which show no growth when incubated in air will be considered as true anaerobes and be subjected to further testing.
Reading & Interpretation **Wet smear/Gram stain** **Culture**	 Note the presence of pus cells, branching filaments Read after 48 hrs of incubation. If there is no growth incubate cultures for a further 48 hours. If Actinomycosis is suspected, incubate for a total of 5-7 days. Note the colony appearances and identify.
Identification	If aero tolerance testing confirm that the isolate is a true anaerobe Identify the isolate by Rapid Identification kits(Rapid ANA II)

REPORTING PROCEDURE

Macroscopy **Wet smear Gram stain** **Culture**	 Pus cells, branching filaments bacteria …… isolated.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Microscope	
Bunsen Burners	
Anaerobic jars	

C**ONSUMABLES**

Glassware

Glass slides

Media and reagents

Blood agar	Refer SOP on media
Brucella Blood Agar with Hemin and Vit K (BBA)	
Neomycin Blood agar(NBA)	
Bacteroides Bile Esculin agar(BBE)	
Anaerobic gas packs	

Stains

Gram stain

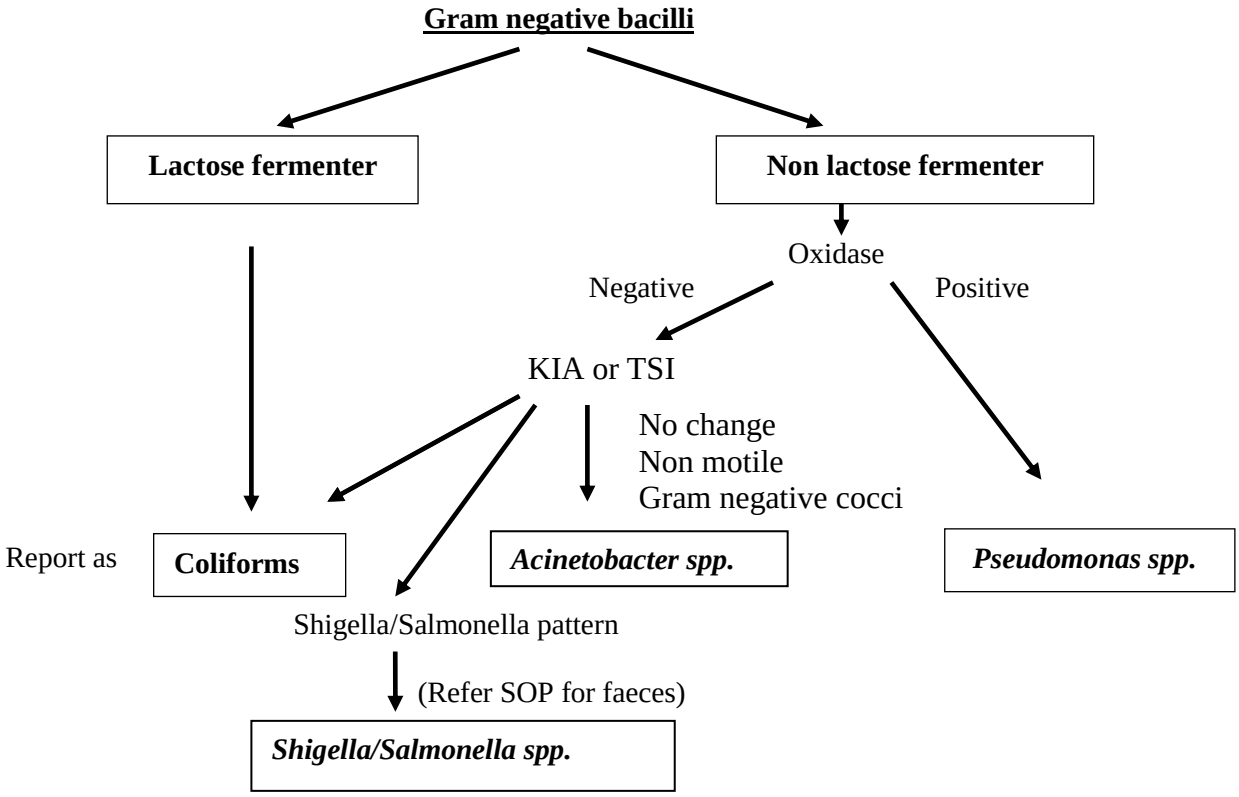
MAINTENANCE OF RECORDS

Unique records register for anaerobic cultures

IDENTIFICATION OF ORGANISMS

STANDARD OPERATING PROCEDURE FOR IDENTIFICATION OF ENTEROBACTERIACEAE

Procedure	**Appearance**	
Gram stain	Gram negative bacilli	
Culture	**Lactose fermenters**	**Non lactose fermenters**
MacConkey agar	Pink	Colourless/pale
Blood agar (not for faeces)	Grey, smooth & glossy	Grey, smooth & glossy
SS agar (for faeces)	Pink	Colourless/pale ± black centre
CLED medium (for urine)	Yellow	Blue/grey/green
XLD medium (for faeces)	**Xylose fermenters** Yellow	**Xylose non fermenters** Pink/Red ± black centre



Note:
This is not a very accurate identification but serves the purpose of deciding on antibiotic discs. Precise identification should be done if clinically required eg. multi-resistant organism from ICUs / isolates from blood, CSF and sterile sites and hospital cross infections etc. Discuss with the MO/ Microbiologist regarding the need for precise identification.

- If precise identification is required, use biochemical tests or commercial kits (eg. API).
- If these are not available store the isolates in nutrient agar slopes and send to MRI for identification. Record the date of dispatch of specimen to MRI and enter date of receipt of results and identification.
- However, oxidase negative Gram negative bacilli could be further identified with some degree of accuracy by the following tests. Citrate utilization, indole production, urease production, phenylalanine deaminase production, motility and Kligler or TSI reaction pattern.

Identification of Enterobacteriaceae

	Lac	KIA			Ind	Cit	Ure	PA	Motility (35^0C)
		S/B*	H_2S	Gas					
E. coli	+	A/A[#]	-	+	+	-	-	-	+
Shigella spp	-	K/A	-	-	-/+	-	-	-	-
S. sonnei	-	K/A	-	-	-	-	-	-	-
E. tarda	-	K/A	+	+	+	-	-	-	+
Salmonella spp	-	K/A	+	+	-	+	-	-	+
S. typhi	-	K/A	+	-	-	-	-	-	+
C. freundii	+/-	A/A	+	+	-	+	+/-	-	+
C. koseri	+/-	K/A	-	+	+	+	+/-	-	+
K. pneumoniae	+	A/A	-	+	-	+	+	-	-
K. oxytoca	+	A/A	-	+	+	+	+	-	-
E. aerogenes	+	A/A	-	+	-	+	-	-	+
E. cloacae	+	A/A	-	+	-	+	+/-	-	+
S. marcescense	-	K/A	-	+/-	-	+	-	-	+
P. mirabilis	-	K/A	+	+	-	+/-	+	+	+
P. vulgaris	-	K/A	+	+/-	+	-/+	+	+	+
Pv. rettgeri	-	K/A	-	-	+	+	+	+	+
Pv. stuartii	-	K/A	-	-	+	+	+/-	+	+
M. morganii	-	K/A	-	+	+	-	+	+	+
Y. enteocolitica	-	K/A	-	-	+/-	-	+/-	-	-

***Slant/Butt**
[#]**K – alkaline, A- Acid**
+ > 90% of strains positive, - > 90% of strains negative, +/- 50 – 90 % of strains positive, -/+ 50-90% of strains negative

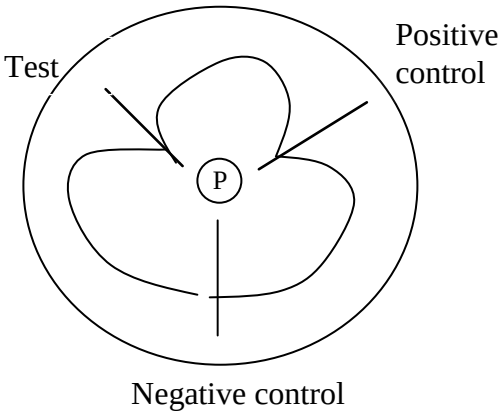
References

1. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Ed. 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
2. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

STANDARD OPERATING PROCEDURE FOR IDENTIFICATION OF HAEMOPHILUS SPECIES

Procedure	Result	Interpretation
Gram stain	Gram negative short bacilli with occasional long filaments (pleomorphic). Sometimes very poorly staining	Suggestive of *Haemophilus spp.*
Culture • Chocolate agar (5-10 % CO_2) • Blood agar with *S. aureus* stabs • MacConkey agar	Translucent small colonies with characteristic smell. Look for satellitism around colonies of *Staphylococcus aureus.* No growth.	Suggestive of *H.influenzae*.
Catalase test Oxidase test Refer SOP on identification tests and Cowan & Steel's table 7.10b	Positive Positive	
X & V factor requirement Requirements 1. Identification discs X, V, XV 2. Tripticase soy agar or BHI agar (NA/MHA can be substituted) 3. Media must have QC check before use	1. Plate heavily on Tripticase soy agar or BHI agar (if not available nutrient agar or MHA) 2. Place X, V, XV discs on plate 3. Read after 24 hours incubation at 35°C 4. Growth around XV disc only identifies *H. influenzae.* 5. Growth around V and XV discs - *H. parainfluenzae* **Quality control for X & V requirement** 1. Disk potency : With every batch of X, V and XV discs, check for potency using known *H. influenzae* and *H. parainfluenzae* strains at onset of use and at monthly intervals. 2. QC of medium used for test: Each batch of plates poured - innoculate *H. influenzae,* If growth + , medium contains these factors and cannot be used for this test. 3. In use QC: *H. influenzae* should grow only around the XV disc. Any growth around the X or V disc or on the entire plate invalidates the test. **Satellitism test** 1. Plate a blood agar plate heavily with the suspected colonies. 2. Stab/streak the plate with *Staphylococcus aureus.* 3. Incubate at 35°C overnight and look for colonies growing round *S. aureus* streaks/stabs.	

Detection of β-lactamase activity in *H. influenzae*

1. Chromogenic cephalosporin method (nitrocefin test) **Positive Result** **Negative Result**	1. Dispense nitrocefin disk/strip onto a clean glass slide. 2. Moisten disk/strip with a drop of sterile distilled water. 3. Using a sterile loop, remove several well isolated colonies of similar morphology from the agar plate and smear onto the disk. 4. Observe disk for colour change within 5 minutes. Yellow ⟶ Red 5. No colour change.
2. Apple plate 1. Spread *S. aureus* (NCTC 6571) on a blood agar plate to create a lawn. 2. Streak *H. influenzae* strain as shown (Test) with positive and negative controls. 3. Place a 10μg penicillin (P) disc at centre as shown. 4. Incubate overnight at 35°C. 5. Examine 6. Inoculum of *S. aureus* growing towards penicillin disc adjacent to the test streak indicates β lactamase activity.	
3. Quality Control	Maintain positive and negative control organisms
Positive control	*Staphylococcus aureus* ATCC 29213
Negative control	*Haemophilus influenzae* ATCC 10211

If an isolate with characteristic colony appearance, smell and Gram stain appearance is both catalase and oxidase tests positive and gives a positive satellitism test, a probable identification of *Haemophilus influenzae* could be made.

X factor dependence

This is best tested with the porphyrin test.[1]

Reference

1. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Ed. 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
2. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.

STANDARD OPERATING PROCEDURE FOR IDENTIFICATION OF STAPHYLOCOCCI

Procedure (Method)	**Identification / Result**	**Remarks**
Gram stain	Gram positive cocci in clusters Sometimes seen as single cocci in addition to clumps	Direct smears from relevant samples and colonies on solid culture media and broth media and blood culture
Culture Blood agar	Usually white to cream some strains yellow to orange, with or without haemolysis	
Chocolate agar	Usually white to cream while some strains yellow to orange	Clinical samples except urine.
MacConkey agar	Pink colonies	
CLED	Yellow to white colonies	urine
MRSA Chrome agar	MRSA – denim-blue colonies or deep green colonies	

FURTHER IDENTIFICATION OF ISOLATES:

Test procedure	Result	Remarks
Catalase	Positive	Preferably done from a colony on chocolate agar plate. If chocolate agar plate is not available from a colony on MacConkey agar plate.
Sensitivity to Furazolidone 100 µg and Bacitracin 0.04 unit disc on MHA	Furazolidone sensitive (≥15mm) Bacitracin resistant (< 10 mm)	Micrococcus < 9 mm Micrococcus > 10 mm

Coagulase test 1. Slide Coagulase test 2. Staphylococcus latex agglutination test a. Slidex Staph kit b. Staphylase 3. Tube Coagulase test	For all staphylococcal isolates, slide and tube coagulase tests should be performed. These are results of slide & tube tests. Slide tube *S.aureus* + + *S. saprophyticus* - - *S. epidermidis* - - *S. lugdunensis* + - *S. schleiferi* + - Other CNS - - Some strains of *S. aureus* may be slide test negative but positive by tube test.	
DNase test	*S. aureus* gives positive results but some other staphylococci (*S. schleiferi*) can also give a positive result.	
PBP2 latex test	*mec*A gene positive staphylococci including MSRA isolates gives a positive result	Detects PBP2, the product of *mec*A gene
Further identification of clinically significant isolates of Coagulase negative Staphylococci	Bio**c**chemical tests (including API staph) Vitek	
Novobiocin sensitivity	Resistance to novobiocin (diameter of ≤16mm) indicates *Staphylococcus saphrophyticus*.	Used for urinary isolates of Coagulase negative Staphylococci

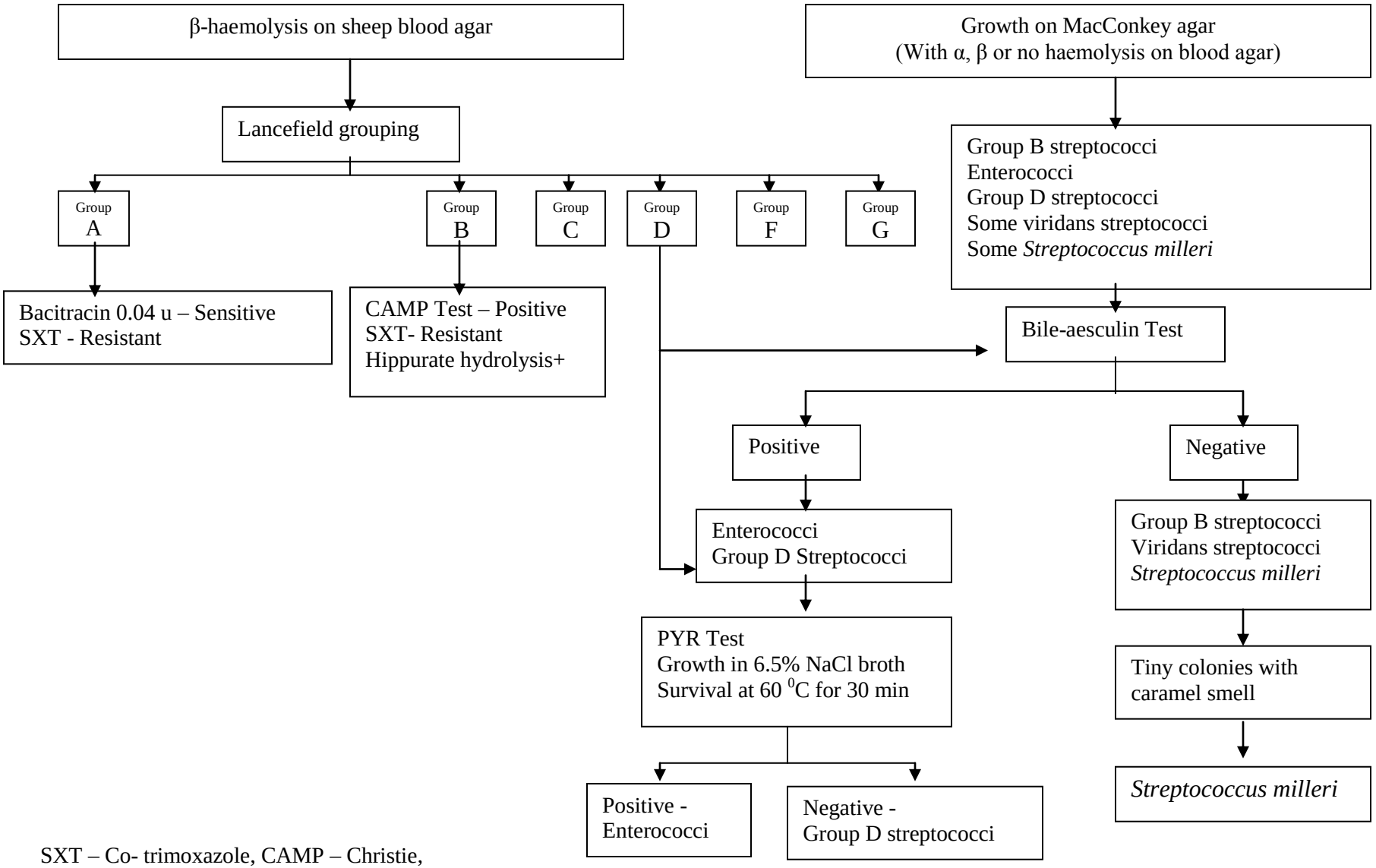
References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
2. Henry D. Isenberg 2004, **Clinical Microbiology Procedure Hand book**, p.1.0.1 – 4.12.6, Second Ed. American Society for Microbiology, Washington, D.C.

STANDARD OPERATING PROCEDURE FOR IDENTIFICATION OF STREPTOCOCCI (EXCLUDING S.PNEUMONIAE) AND ENTEROCOCCI

Procedure	Appearance or result	Interpretation
Gram stain	Gram positive cocci in chains. If uncertain, subculture to a broth (preferably thioglycolate) and repeat Gram stain after overnight incubation	Possibly streptococci/ enterococci
Culture • Blood agar (preferably sheep blood agar)	Small translucent colonies with haemolysis ➢ β- haemolysis - complete haemolysis ➢ α- haemolysis -partial haemolysis ➢ no haemolysis	β-haemolytic streptococci α-haemolytic streptococci Non-haemolytic streptococci
• Chocolate agar	Small translucent colonies	
• MacConkey agar	Positive growth (pink colonies)	Enterococci/Group D Streptococci/Group B streptococci
• Selective agar (used for isolation from heavily colonized sites. eg. Neomycin / Gentamicin Blood agar)		
Catalase test Refer SOP on identification tests	Negative	Differentiates streptococci/ Enterococci from most other organisms (esp. staphylococci)
Bacitracin 0.04 U Refer SOP on identification tests	Any zone of sensitivity	*S. pyogenes* (Group A) Note: 5% - 10% of group B,C,G streptococci can also give a zone of inhibition.
CAMP test Refer SOP on identification tests	Positive	*S. agalactiae* (Group B)
Very small colonies with characteristic caramel smell		Possibly *S. milleri* (Group F, A, C, G or non groupable)
Lancefield grouping	Use according to instructions available with kit	
Bile-aesculin	Positive	Enterococci & Group D streptococci
Growth in 6.5% NaCl	Positive	Enterococci
Survival at 60^0C for 30 min	Positive	Enterococci

Schematic diagram for the presumptive identification of Streptococci



SXT – Co- trimoxazole, CAMP – Christie, Atkins & Munch-Petersen, PYR – Pyrrolidonyl arylamidase

Notes on antibiotic susceptibility testing:

1. Inducible resistance of clindamycin should be checked & reported.

2. Penicillin resistance has not been reported in Group A streptococci. Re-check identity, repeat sensitivity test & inform Microbiologist.

3. Occasional isolates of group C & G showing intermediate susceptibility to penicillin has been reported. All these are susceptible to 3rd & 4th generation cephalosporins and vancomycin.

4. All enterococcal isolates from blood & CSF should have a direct beta-lactamase test. Strains testing positive should have penicillin & ampicillin reported resistant, irrespective of disc diffusion readings.

5. All viridans streptococci isolated from sterile body sites (blood, CSF and bone) should have penicillin MIC done.

6. Enterococci with intermediate zones to vancomycin should have MIC done to exclude VRE.

7. Enterococci resistant to vancomycin/teicoplanin should be saved and forwarded to the reference laboratory, after informing the Microbiologist.

8. All enterococcal isolates from patients with infective endocarditis should have high level gentamicin resistance (HLAR) assessed with gentamicin 120 µg disc.

References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.

2. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.

3. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; Eighteenth Informational Supplement, M100-S18 Vol 28 No.1 January 2008.

4. Stokes EJ, Ridgway GL, Wren MWD. **Clinical Microbiology,** 7th edition. 1993.

STANDARD OPERATING PROCEDURE
FOR IDENTIFICATION OF
STREPTOCOCCUS PNEUMONIAE
(PNEUMOCOCCI)

Procedure	Appearance or result	Interpretation
Gram stain	Gram positive diplococci, ovoid or lanceolate (with distal end narrowed) or short chains. If uncertain, subculture to a broth (preferably thioglycolate) and repeat Gram stain after overnight incubation	Possibly *S. pneumoniae*
Culture Incubated in 5 -10% CO_2 or candle jar • Blood agar (preferably sheep blood agar) • Chocolate agar • MacConkey agar	Flat gray colonies. Greening of media around the colony (α-haemolysis). Draughtsman colonies may be seen in 48 hours No growth	Possibly *S. pneumoniae*
Catalase test Refer SOP on identification tests	Negative	Differentiates streptococci/enterococci from most other organisms (esp. staphylococci)
Optochin sensitivity Refer SOP on identification tests	Sensitive	Differentiates *S. pneumoniae* from viridans group
Bile solubility Refer SOP on identification tests	Soluble	Differentiates *S. pneumoniae* from viridans group
Direct antigen detection using agglutination	CSF antigen detection kit or polyvalent pneumococcal antibody can be used according to manufacturer's instructions.	

Notes on antibiotic sensitivity testing of *Streptococcus pneumoniae*

1. Penicillin sensitive strains are sensitive to all other β-lactams.

2. CLSI does not recommend disc diffusion testing for β-lactams other than penicillin.

3. CLSI advocates direct performance of MIC for penicillin, ceftriaxone etc for CSF & blood isolates of patients with meningitis.

4. Isolates that are oxacillin resistant (zone diameter ≤19 mm) should have penicillin MIC done. Reporting other β-lactams (ceftriaxone) in such cases should be based on MIC.

References

1. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.
2. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
3. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; 21st Informational Supplement, M100-S21 Vol 31 No.1 January 2011.

BACTERIAL IDENTIFICATION TESTS

BILE SOLUBILITY TEST

PURPOSE

Helps differentiate *S. pneumoniae*, which is soluble in bile and bile salts, from viridans streptococci which are insoluble. The addition of bile salts activates the autolysins and accelerates the natural lytic reaction observed with cultures of pneumococci.

REQUIREMENTS

- 10% sodium deoxycholate[3]
- 2% Sodium deoxycholate
- Sterile saline
- Positive control – *S. pneumoniae*
- Negative control – *S. agalactiae*[1]

A. PLATE METHOD

Method

1. Touch a suspected colony on the primary plate with a loop charged with 10% sodium deoxycholate solution.
2. Incubate the plate for 15 min at 35ºC.

Interpretation

Colonies of pneumococci disappear leaving an area of α-haemolysis on blood agar

Note: The use of 2% sodium deoxycholate in this method has been documented in some literature.

B. TUBE TEST

Method

1. Prepare a milky suspension (McFarland No. 1) from an overnight culture in 1 ml of saline.
2. Divide the suspension in two tubes (test and control) of 0.5 ml.
3. Add 0.5 ml of 2% sodium deoxycholate (bile salts) to the test tube and 0.5ml saline to the control tube.
4. Vortex and incubate at 35ºC up to 2 hours (inspect after 10 – 30 minutes initially).

Interpretation

- If it is *S. pneumoniae*, suspension will be completely transparent without any turbidity.
- Any other α-haemolytic streptococci will remain turbid after 2 hrs of incubation.

Note:

- In some reports 10% sodium deoxycholate has been used in this procedure.
- Bile solubility test should be preformed only for optochin resistant isolates.
- The test should not be performed on old cultures, as the active enzyme may not be present.

References

1. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the identification of medical bacteria**, 3rd Edition 1993. Cambridge University Press.
2. Collee J. G, Fraser A. G., Marmion B. P., Simmons A**. Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
3. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.

CAMP TEST

PURPOSE

Used in the identification of *Streptococcus agalactiae* (Group B streptococcus) which produces a factor called the CAMP factor which enhances the effect of the β-lysin produced by *S. aureus*. This phenomenon is seen with both haemolytic and non-haemolytic isolates of group B streptococci.

REQUIREMENTS

- β-lysin producing *Staphylococcus aureus* (NCTC 7428)[2]
- Blood agar plate containing 5% sheep blood
- Positive control – *S. agalactiae*
- Negative control – *E. faecalis*[2]

METHOD

1. Streak a β-lysin producing *Staphylococcus aureus* down the center on the 5% sheep blood agar plate.
2. Make a single streak of the test isolate perpendicular to the staphylococcal streak.
3. Leave about a 1cm space between the two inoculation lines.
4. Negative and positive controls should be inoculated on the same plate.
5. Incubate the inoculated plates for 24 hours at 35ºC.

INTERPRETATION

An area of enhanced haemolysis occurs where the β lysin produced by the *Staphylococcus aureus* and the CAMP factor produced by the group B streptococcus intersect and may appear in the shape of an arrow head.

Note:
Some group A streptococci will be CAMP test positive if incubated in a candle jar or in a CO_2 atmosphere.

References

1. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania
2. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the identification of medical bacteria**, 3rd Edition 1993. Cambridge University Press.
3. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

CATALASE TEST

PURPOSE

Used to differentiate bacteria which produce catalase from non-catalase producing bacteria.

REQUIREMENTS

- Sterile wooden sticks or glass rods
- Hydrogen peroxide - 3% H_2O_2 stored in a brown bottle under refrigeration
- An 18 -24 hr culture of the organism to be tested
- Positive control – Staphylococcus species
- Negative control – Streptococcus species

Note: Hydrogen peroxide reagent must be tested with positive and negative control organisms immediately before unknown bacteria are tested. Shaking the reagent before use will help expel any dissolved oxygen. False positive results may occur if the H_2O_2 contains dissolved oxygen.

METHOD

1. Pour 2-3ml of the H_2O_2 solution into a test tube.
2. Using a sterile wooden stick or glass rod, remove a good growth of the test organism and immerse in the H_2O_2
3. Look for immediate bubbling (within 20 seconds).

If the organism is on an agar slope, 1 ml of H_2O_2 can be poured onto the slope. Release of bubbles indicates a positive result.

INTERPRETATION

Positive result – active bubbling
Negative result – no bubbling

Some bacteria possess enzymes other than catalase that can decompose H_2O_2. Therefore, a few tiny bubbles forming after 20 – 30 seconds is not considered a positive test

Note:

- Performing the test on a slide is not recommended for safety reasons (production of aerosols).
- A nichrome wire should not be used as it may give a false positive result.
- Blood agar and other blood containing media are unsuitable for this test. When test is done from colonies on blood agar, false positive results can occur. Catalase is present in red blood cells and care must be taken to prevent the carryover of red blood cells with the colony material if the test is done from colonies on blood containing media.

References

1. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
2. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the identification of medical bacteria**, 3rd Edition 1993. Cambridge University Press.

COAGULASE TEST

PURPOSE

Used to identify coagulase positive staphylococci (*Staphylococcus aureus*).

- Test should be done on fresh isolates (less than 24 hrs old) from a non selective medium. Test cannot be performed from growth on mannitol salt agar.
- Reagent is either rabbit or human plasma. Use EDTA as anticoagulant (citrated blood can give false positive results). Rabbit plasma is preferable as it gives better clotting, is free from inhibitors and is safe.[1]
- Store the plasma in small aliquots at -20^0C and keep a stock of in-use plasma at 4^0C, bringing into room temperature before use.
- Quality control (QC) of plasma
  - Do not use plasma that has not been stored refrigerated or frozen or that appears turbid.
  - Perform QC on new lots of plasma prior to putting them into use.
- QC Organisms
  Staphylococcus aureus ATCC 25923 – coagulase positive
  Staphylococcus epidermidis ATCC 12228 or ATCC 14990 – coagulase negative

A. SLIDE COAGULASE TEST

Slide coagulase test detects protein A/ clumping factor by agglutination method.

Method

1. Emulsify a staphylococcal colony in a drop of water or saline on a microscope slide with a minimum of spreading to make a smooth milky suspension. If clumps occur and organism does not suspend in water do not proceed with the test.
2. Make similar suspensions of positive and negative control strains to confirm the proper reactivity of the plasma.
3. Dip a flamed and cooled straight inoculating wire into the undiluted plasma at room temperature, withdraw and stir the adhering traces of plasma into the staphylococcal suspension (not a loopful of plasma).

Reading and interpretation

- Positive - a coarse clumping visible to the naked eye within 10 seconds. *Staphylococcus aureus* gives positive results.
- Negative – absence of clumping or any reaction taking more than 10 seconds to develop. Coagulase negative staphylococci give negative results.

Note: *S. lugdunensis* and *S. schleiferi* can also give positive results.

STAPHYLOCOCCUS LATEX AGGLUTINATION TEST USING COMMERCIAL KITS (Slidex Staph kit, Staphylase kit)

Perform according to manufacturer's instructions.

B. TUBE COAGULASE TEST

Tube method 1[1]

1. Prepare a 1 in 6 dilution of the plasma in saline (0.85% NaCl). (e.g. 2 drops of plasma and 10 drops of saline).
2. Emulsify a colony of staphylococcus under test.
3. With each batch of tests include tubes with positive and negative controls and a tube of uninoculated diluted plasma to confirm that it does not clot spontaneously.
4. Incubate at 35ºC and examine at 1, 2 and 4 hrs for clot formation by gently tilting the tube through 90^0.
5. Leave negative tubes at room temperature overnight and re-examine.

Reading and interpretation

Positive – any degree of clot formation
Negative – plasma remains wholly liquid or shows only a flocculent or ropy precipitate

Tube method 2[1, 2]

1. Grow the staphylococcus to be tested in brain-heart infusion broth overnight at 35^0C.
2. Add 0.1ml of this to 0.5ml of undiluted plasma in a small tube.
3. Incubate the tubes at 35ºC preferably in a water bath up to 4 hrs.
4. Examine at 1, 2 and 4 hrs for clot formation by tilting the tube through 90^0.
5. Leave negative tubes at room temperature overnight and re-examine.

Note:

- Do not leave the tube coagulase test at 35ºC for more than 4 hrs.
- If 4 hrs of incubation is inconvenient the test is most sensitive when incubated at 25^0C for the entire time, but the clot may take longer to form.[2]
- This method can be used for rapid detection of *S.aureus* from positive blood cultures containing Gram positive cocci in clusters.

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. Henry D. Isenberg, **Clinical Microbiology Procedure Hand book**, 2nd Edition. 2004. American Society for Microbiology, Washington, D.C.

DEOXYRIBONUCLEASE (DNase) TEST

PURPOSE

Used to differentiate *Staphylococcus aureus* which produces the enzyme DNase from other staphylococci which do not produce this enzyme. It is particularly useful if plasma is not available or when results of the coagulase test are difficult to interpret. When HCl is added to the DNase agar plate unhydrolyzed DNA is precipitated and produces a white opacity or cloudiness in the agar. Positives are surrounded by a clear zone.

REQUIREMENTS

- DNase agar plate (up to 6 organisms may be tested on one plate)
- Hydrochloric acid 1 mol/L (HCl)
- Positive control - *Staphylococcus aureus*
- Negative control - *Staphylococcus epidermidis*

METHOD

1. Divide the DNase agar plate into the required number of sections by marking the underside of the plate with a marker pen.
2. Using a sterile loop or swab, spot inoculate the test and control organisms.
3. Make sure each test area is clearly labeled.
4. Incubate the plates at 35ºC overnight.
5. Flood the surface of the plate with 1 mol/L HCl solution to precipitate unhydrolyzed DNA.
6. Tip off excess acid.
7. Look for clearing around the colonies within 5 minutes of adding the acid.
8. Positive result – clearing around colonies.
9. Negative result – no clearing around colonies/inoculation line.

LIMITATIONS

Some MRSA strains do not give positive reactions and some coagulase-negative staphylococci *(S. schleiferi*) give weak reactions.

Reference

Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

OPTOCHIN TEST

PURPOSE

Used to differentiate *Streptococcus pneumoniae* from viridans streptococci. Ethylhydrocupreine hydrochloride (optochin) selectively inhibits the growth of *Streptococcus pneumoniae* at very low concentrations (5μg /ml or less).

REQUIREMENTS

- Optochin (ethylhydrocuprein chloride) 5μg discs
- Blood agar plate
- Positive control – *S. pneumoniae*
- Negative control – *E. faecalis*

METHOD

1. Inoculate a blood agar plate with light broth suspension (or a heavy inoculum) of the test organism.
2. Place an optochin disc in the inoculated area and gently press down the disc so that it adheres firmly to the agar surface.
3. Incubate at 35ºC overnight in 5-10% CO_2

INTERPRETATION

- Zone of inhibition of 14mm or more around a 6 mm disc or 16 mm or more around a 10 mm disc presumptively identifies the test organism as *Streptococcus pneumoniae.*
- Zones smaller than these- Perform bile solubility test. If bile soluble, organism is identified as *Streptococcus pneumoniae.*
- No zone of inhibition - viridians streptococci.

Reference

Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.

OXIDASE TEST

PURPOSE

This test is used to differentiate bacteria that produce intracellular oxidase enzymes from those that do not produce these enzymes. It is an important test in differentiating members of the genus pseudomonas from other non fermentative Gram negative bacteria.

REQUIREMENTS

- Freshly prepared oxidase reagent (1% solution of tetramethyl-p-phenylene-diamine dihydrochloride)
- Positive control - *P. aeruginosa*
- Negative control - *E. coli*[2]

METHOD

A. WET FILTER PAPER METHOD

1. Soak a small strip of filter paper with freshly prepared 1% solution of the oxidase reagent.
2. Rub a speck of culture on it with a platinum loop, glass rod or wooden stick.

Interpretation

- Positive result – deep purple colour developing within 5-10 seconds
- Delayed positive result – colour development in 10- 60 seconds
- Negative result – no colour or colour developing after 60 seconds

B. PLATE METHOD

1. Cultures are made on suitable solid growth medium (a medium free from glucose and nitrate).
2. Pour a freshly prepared 1% solution of the oxidase reagent on the plate to cover the surface.
3. Decant excess reagent.

Interpretation

- Positive result – purple colonies
- Negative result – no change in colour

Important

- The oxidase reagent is unstable. Fresh preparations must be used for reliable results.
- Test isolate must be transferred to filter paper using a clean platinum loop, wooden stick or a glass rod. Traces of iron can catalyze the reaction and give false positive results.

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the identification of medical bacteria,** 3rd Edition 1993. Cambridge University Press.

UREASE TEST

PURPOSE

Urease is an enzyme possessed by many species of microorganisms that can hydrolyze urea producing ammonia and CO_2. This is an important test in differentiating enterobacteria. Proteus species are strong urease producers. Salmonella and shigella do not produce urease.

REQUIREMENTS

- Urea Medium
- Positive control – *Proteus vulgaris*
- Negative control – *Escherichia coli*

METHOD

1. Using a sterile straight wire, inoculate a tube of sterile urea medium with the test organism.
2. Incubate at 35ºC

INTERPRETATION

- Examine for development of pink colour in the medium after 4h and after overnight incubation[2].
- Positive result – pink colour
- Negative result – no change in colour (yellow)

Note:

No tube should be reported negative until after 4 days of incubation.

References

1. Koneman EW, Allen SD, Janda WM, Schreckenberger PC, Winn WC. **Color Atlas and Textbook of Diagnostic Microbiology**, 6th Edition 2006. Lippincott-Raven Publishers. Philadelphia, Pennsylvania.
2. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.

STANDARD OPERATING PROCEDURE FOR ANTIBIOTIC SENSITIVITY TESTING BY DISC DIFFUSION

CLINICAL LABORATORY STANDARDS INSTITUTE (CLSI, FORMERLY NCCLS) METHOD

Introduction

Standardized methods for disc sensitivity testing have been developed in several countries, including the USA. UK, Sweden etc. The most popular method is the Kirby Bauer technique, originally described by Bauer and associates in 1966 and subsequently modified and updated by the NCCLS (National Committee for Clinical Laboratory Standards, USA). It is now named as Clinical and Laboratory Standards Institute (CLSI) method.

The CLSI method is a disc diffusion test which is stringently standardized. The zone diameters obtained by this method have an approximately linear relationship with the log of MIC.

The test uses materials which are easily available. All steps of the technique are strictly standardized. The correct use of this method allows inter-laboratory comparison of results. It also enables the user to become part of surveillance programmes which use CLSI method.

Standardized antibiotic sensitivity testing is the goal. Laboratories which aim to use this test method must ensure that all test requirements are met. The validity of the results cannot be assured if there is deviation from the described method.

REQUIREMENTS FOR TEST

Media	1. Mueller Hinton Agar (MHA). 2. Mueller Hinton Blood Agar – Add 5% defibrinated sheep blood for fastidious organisms (*Streptococcus spp. & N. meningitidis.)* 3. Haemophilus Test Medium (HTM) should be used for *Haemophilus spp.* Dry plates with lids ajar until there are no drops of moisture on the agar surface (incubator set at 35°C for 15 minutes could be used for this purpose). Air drying is the best. Caution: over drying inhibit growth of organisms.

INOCULUM

Preparation of inoculum	It is essential that an approximately standard number of bacteria are tested (1-2 x 10^8 bacteria/ml).
Method A	1. Use an overnight plate culture of test organism. 2. Touch the tops of 3-5 colonies with a sterile loop. 3. Transfer this to a tube of 4-5 ml sterile normal saline. 4. Compare the tube with the 0.5 McFarland turbidity standard against thick black lines on a white background. 5. Adjust density of test suspension to that of the standard by adding more bacteria or more sterile saline. 6. It is essential to adjust turbidity of inoculum to ensure that the resulting lawn of growth is confluent. Caution: vortex both MacFarland turbidity standard and test suspension prior to use.

Method B	1. Transfer 3-5 colonies with a sterile loop or swab into Brain Heart Infusion (BHI) or Tryptic Soy Broth (TSB). 2. Incubate at 35°C for 1 – 8 hours until 0.5 MacFarland turbidity is obtained. 3. Adjust turbidity by dilution with the broth or normal saline as required.
Method of inoculation	1. Dip a sterile swab into the inoculum within 15 minutes of preparation. 2. Remove excess inoculum by pressing and rotating the swab firmly against the side of the tube above the level of the liquid. 3. Streak the swab all over the surface of the medium three (3) times, rotating the plate through the angle of 60° after each application. 4. Finally, pass the swab round the edge of the agar surface. 5. Leave the inoculum to dry for a few minutes at room temperature with the lid closed.
Disc application • Do NOT place more than 7 discs per plate • Antibiotic diffusion begins immediately.	1. Place antibiotic discs on the inoculated plates using a pair of sterile forceps or needle tip. 2. Use a template to place the discs uniformly. 3. Place only 5 discs on a 9cm plate. 4. Press each disc gently to ensure even contact with the agar surface. 5. Do not move displaced discs on the agar surface.
Incubation • Temperatures >35°C invalidate methicillin sensitivity results	1. Place the plates (inverted) in an incubator at 35°C within 15 minutes of preparation. 2. For β-haemolytic streptococci, *Streptococcus pneumoniae*, *Streptococcus spp.*, *Neisseria spp.* & enterococci – incubate 20-24 hours in 5-10% CO_2 at 35°C.

READING AND MEASUREMENT OF ZONES OF INHIBITION

	Read after overnight incubation (16-18 hours). 1. Measure the diameter of each zone (including diameter of disc). 2. Make measurements with a ruler on the undersurface of the plate without opening the lid. 3. End-point of inhibition is generally judged by the naked eye at the edge where growth starts. Exceptions: a) Sulphonamides and trimethoprim –ignore slight growth within zone of inhibition. b) Proteus may swarm into area of inhibition. Measure clear outline of zone of inhibition ignoring swarming. c) Colonies within zones of inhibition represent either contaminants or resistant mutants. Gram stain and if necessary repeat sensitivity testing with these colonies. With *S. aureus*, if such colonies present in cefoxitin or oxacillin zones, report as MRSA. 4. Record results.

QUALITY CONTROL

1. Maintenance of QC strains

a) Maintain permanent stock cultures in brucella broth (or validated alternative such as glycerol blood broth or skimmed milk) at -70°C for up to 3 years. Subculture twice to Blood agar prior to testing.
b) Maintain working stock culture on Trypticase Soy Agar slants (or validated alternative) at 2°C-8°C for up to 2 weeks. Subculture once to Blood Agar plate prior to testing.

2. Frequency of QC testing

a) Perform QC daily or each time a patient test is requested.
b) Frequency of performing QC testing can be reduced from daily to weekly if a laboratory can document proficiency in performing this test as follows:
    i. perform QC testing daily (or each day patient tests are performed) until results from 30 consecutive days of testing have been obtained
    ii. proficiency in performing QC tests is confirmed, if for **each** drug, no more than 3 of 30 results are outside the accuracy limits
c) Document proficiency each time a new drug is added to the testing protocols.
d) Perform QC testing prior to or concurrent with testing of patient isolates each time a new lot or new shipment of materials (MHA, discs) is put into use.

Save records of documenting proficiency indefinitely.

3. QC controls for reporting

a) with daily QC testing – no more than 1 in 20 results in outside accuracy limits
b) No zone is more than 4 standard deviations from the midpoint between stated limits
c) With weekly testing – all zones are within specified accuracy limits
d) Lawn of growth of test isolates is confluent and not mixed
e) Zones appear reasonable (not overlapping, oval etc).
f) Antibiogram is appropriate for isolate tests.

4. Corrective action

Inform supervisor and proceed with corrective action as follows when out of range results are observed with QC strains.

a) Review records.
b) Check test materials (including reference strains).
c) Check equipment (refrigerators, incubators etc.).
d) Review technical aspects of test performance with individuals performing the tests.

CONTROL ORGANISMS

- *Escherichia coli* ATCC 25922
- *Pseudomonas aeruginosa* ATCC 27853
- *Haemophilus influenzae* ATCC 49247
- *Haemophilus influenzae* ATCC 49766
- *Staphylococcus aureus* ATCC 25923
- *Enterococcus faecalis* ATCC 29212
- *Escherichia coli* ATCC 35218
- *S. aureus* ATCC BAA-977 *and S. aureus* ATCC BAA-976

Source of control organisms
Department of Bacteriology, MRI.
Colombo 8
Tel. 0112 691350

Refer CLSI performance standards M100 – S21 for antibiotics to be tested and Q ranges.

Disc diffusion testing- acceptable limits (mm) for quality control strains

Antimicrobial agent	***Escherichia coli* ATCC 25922**	***Staphylococcus aureus* ATCC 25923**	***Pseudomonas aeruginosa* ATCC 27853**	***Escherichia coli* ATCC 35218**
Amikacin 30 µg	19-26	20-26	18-26	
Amoxicillin-clavulanic acid 20/10 µg		28-36		17-22
Ampicillin 10 µg	16-22	27-35		
Aztreonam 30 µg	28-36		23-29	
Cefepime 30 µg	31-37	23-29	24-30	
Cefixime 5 µg	23-27			
Cefotaxime 30 µg	29-35	25-31	18-22	
Cefoxitin 30 µg	23-29	23-29		
Ceftazidime 30 µg	25-32	16-20	22-29	
Ceftriaxone 30 µg	29-35	22-28		
Chloramphenicol 30 µg	21-27	19-26		
Ciprofloxacin 5 µg	30-40	22-30	25-33	
Clarithromycin 15 µg		26-32		
Clindamycin 2 µg		24-30		
Colistin 10 µg	11-17		11-17	
Daptomycin 30 µg		18-23		
Erythromycin 15 µg		22-30		
Ertapenem 10 µg	29-36	24-31	13-21	
Gentamicin 10 µg	19-26	19-27	16-21	
Imipenem 10 µg	26-32		20-28	
Levofloxacin 5 µg	29-37	25-30	19-26	
Linezolid 30 µg		25-32		
Meropenem 10 µg	28-34	29-37	27-33	
Minocycline 30 µg	19-25	25-30		
Netilmicin 30 µg	22-30	22-31	17-23	
Nitrofurantoin 300 µg	20-25	18-22		
Norfloxacin 10 µg	28-35	17-28	22-29	
Oxacillin 1 µg		18-24		
Penicillin 10 units		26-37		
Piperacillin-tazobactam 100/10 µg		27-36	25-33	24-30
Polymyxin B	13-19		14-18	
Rifampicin 5 µg		26-34		
Teicoplanin 30 µg		15-21		
Tetracycline 30 µg	18-25	24-30		
Ticarcillin-clavulanic acid 75/10 µg		29-37	20-28	21-25
Tigecycline 15 µg	20-27	20-25	9 -13	
Trimethoprim-sulfamethoxazole 1.25/23.75 µg	23-29	24-32		
Vancomycin 30 µg		17-21		

Zone diameter interpretive standards for gram positive organisms

Antimicrobial disc	*Staphylococcus* species			*Streptococcus pneumoniae*			β- haemolytic streptococcus			*Enterococcus spp.*			*Streptococcus spp.* Viridans group		
	R	I	S	R	I	S	R	I	S	R	I	S	R	I	S
Amikacin 30 µg	≤14	15 – 16	≥17												
Ampicillin 10 µg	≤28	-	≥29						≥24	≤16	-	≥17			
Cefepime 30 µg	≤14	15 – 17	≥18						≥24				≤21	22 - 23	≥24
Cefotaxime 30 µg	≤14	15 – 22	≥23						≥24				≤25	26 - 27	≥28
Cefoxitin 30 µg	≤24		≥25												
Ceftriaxone 30 µg	≤13	14 – 20	≥21						≥24				≤24	25 - 26	≥27
Chloramphenicol 30 µg	≤12	13 – 17	≥18	≤20	-	≥21	≤17	18 - 20	≥21	≤12	13 - 17	≥18	≤17	18 - 20	≥21
Ciprofloxacin 5 µg	≤15	16 – 20	≥21							≤15	16 - 20	≥21			
Clarithromycin 15 µg	≤13	14 – 17	≥18	≤16	17 – 20	≥21	≤16	17 - 20	≥21				≤16	17 - 20	≥21
Clindamycin 2 µg	≤14	15 – 20	≥21	≤15	16 – 18	≥19	≤15	16 - 18	≥19				≤15	16 - 18	≥19
Erythromycin 15 µg	≤13	14 – 22	≥23	≤15	16 – 20	≥21	≤15	16 - 20	≥21	≤13	14 - 22	≥23	≤15	16 - 20	≥21
Gentamicin 10 µg	≤12	13 – 14	≥15												
Levofloxacin 5 µg	≤15	16 – 18	≥19	≤13	14 – 16	≥17	≤13	14 - 16	≥17	≤13	14 - 16	≥17	≤13	14 - 16	≥17
Linezolid 30 µg	≤20	-	≥21	-	-	≥21	-	-	≥21	≤20	21 - 22	≥23	-	-	≥21
Minocycline 30 µg	≤14	15 – 18	≥19							≤14	15 - 18	≥19			
Netilmicin 30 µg	≤12	13 – 14	≥15												
Nitrofurantoin 300 µg	≤14	15 – 16	≥17							≤14	15 - 16	≥17			
Norfloxacin 10 µg	≤12	13 – 16	≥17							≤12	13 - 16	≥17			
Oxacillin 1 µg	≤10	11 – 12	≥13	-	-	≥20									
Penicillin 10 units	≤28	-	≥29						≥24	≤14	-	≥15			
Rifampicin 5 µg	≤16	17 – 19	≥20	≤16	17 - 18	≥19				≤16	17 - 19	≥20			
Teicoplanin 30 µg	≤10	11 - 13	≥14							≤10	11 - 13	≥14			
Tetracycline 30 µg	≤14	15 - 18	≥19	≤18	19 - 22	≥23	≤18	19 - 22	≥23	≤14	15 - 18	≥19	≤18	19 - 22	≥23
Vancomycin 30 µg	-	-	-	-	-	≥17	-	-	≥17	≤14	15 - 16	≥17	-	-	≥17

Zone diameter interpretive standards for gram negative organisms

Antimicrobial disc	Enterobacteriaceae			*Pseudomonas spp.*			*Acinetobacter spp.*			*Haemophilus app.*			*Burkholderia cepacia*			*Stenotrophomonas maltophilia*		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Amikacin 30 µg	≤14	15 – 16	≥17	≤14	15 – 16	≥17	≤14	15 - 16	≥17									
Amoxicillin-clavulanic acid 20/10 µg	≤13	14 – 17	≥18							≤19	-	≥20						
Ampicillin 10 µg	≤13	14 – 16	≥17							≤18	19 - 21	≥22						
Aztreonam 30 µg	≤17	18 – 20	≥21	≤15	16 – 21	≥22				-	-	≥26						
Cefepime 30 µg	≤14	15 – 17	≥18	≤14	15 – 17	≥18	≤14	15 - 17	≥18	-	-	≥26						
Cefixime 5 µg	≤15	16 – 18	≥19															
Cefotaxime 30 µg	≤22	23 – 25	≥26	≤14	15 – 22	≥23	≤14	15 - 22	≥23	-	-	≥26						
Cefoxitin 30 µg	≤14	15 – 17	≥18															
Ceftazidime 30 µg	≤17	18 – 20	≥21	≤14	15 – 17	≥18	≤14	15 - 17	≥18	-	-	≥26	≤17	18 - 20	≥21			
Ceftriaxone 30 µg	≤19	20 – 22	≥23	≤13	14 – 20	≥21	≤13	14 - 20	≥21	-	-	≥26						
Cefuroxime 30 µg (oral)	≤14	15 – 22	≥23							≤16	17 - 19	≥20						
Cefuroxime 30 µg (parenteral)	≤14	15 – 17	≥18							≤16	17 - 19	≥20						
Cephalothin 30 µg	≤14	15 – 17	≥18															
Chloramphenicol 30 µg	≤12	13 – 17	≥18							≤25	26 - 28	≥29						
Ciprofloxacin 5 µg	≤15	16 – 20	≥21	≤15	16 – 20	≥21	≤15	16 - 20	≥21	-	-	≥21						
Clarithromycin 15 µg										≤10	11 - 12	≥13						
Colistin 10 µg				≤10	-	≥11												
Ertapenem 10 µg	≤15	16 – 18	≥19							-	-	≥19						

Antimicrobial disc	Enterobacteriaceae			*Pseudomonas spp.*			*Acinetobacter spp.*			*Haemophilus app.*			*Burkholderia cepacia*			*Stenotrophomonas maltophilia*		
	R	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**	**R**	**I**	**S**
Gentamicin 10 µg	≤12	13 – 14	≥15	≤12	13 – 14	≥15	≤12	13 - 14	≥15									
Imipenem 10 µg	≤13	14 – 15	≥16	≤13	14 – 15	≥16	≤13	14 - 15	≥16	-	-	≥16						
Levofloxacin 5 µg	≤13	14 – 16	≥17	≤13	14 – 16	≥17	≤13	14 - 16	≥17			≥17				≤13	14 - 16	≥17
Meropenem 10 µg	≤13	14 – 15	≥16	≤13	14 – 15	≥16	≤13	14 - 15	≥16	-	-	≥20	≤15	16 - 19	≥20			
Minocycline 30 µg	≤12	13 – 15	≥16				≤12	13 - 15	≥16				≤14	15 - 18	≥19	≤14	15 - 18	≥19
Nalidixic acid 30 µg	≤13	14 – 18	≥19															
Netilmicin 30 µg	≤12	13 – 14	≥15	≤12	13 – 14	≥15												
Nitrofurantoin 300 µg	≤14	15 – 16	≥17															
Norfloxacin 10 µg	≤12	13 – 16	≥17	≤12	13 – 16	≥17												
Piperacillin-tazobactam 100/10 µg	≤17	18 – 20	≥21	≤17	-	≥18	≤17	18 - 20	≥21	-	-	≥21						
Polymyxin B				≤11	-	≥12												
Rifampicin 5 µg										≤16	17 - 19	≥20						
Tetracycline 30 µg	≤11	12 - 14	≥15				≤11	12 - 14	≥15	≤25	26 - 28	≥29						
Ticarcillin-clavulanic acid 75/10 µg	≤14	15 – 19	≥20	≤14		≥15	≤14	15 - 19	≥20									
Trimethoprim-sulfamethoxazole 1.25/23.75 µg	≤10	11 – 15	≥16				≤10	11 - 15	≥16	≤10	11 - 15	≥16	≤10	11 - 15	≥16	≤10	11 - 15	≥16

Note : Please refer the latest CLSI performance standards for zone diameters.

REPORTING PROCEDURE:

ABST report **Turn around time** Normal – 48 hours Unusual circumstances – longer (always inform ward / clinician by telephone if results are delayed**)**	1. Test and report appropriate antibiotics (as in SOP for specimens / identification). 2. Test and report second line antibiotics only if there is resistance to first line antibiotics or if patient is on a particular antibiotic (as stated in the request). 3. Do not report antibiotic sensitivity of colonizing flora. 4. If antibiotic disc not available –indicate on report. If clinically important, consider sending the isolate to Reference laboratory for testing.

Special interpretive rules:

1. Cephalothin can be used to interpret sensitivities to cephalexin and cephradine for Enterobacteriaceae.

2. Inducible clindamycin resistance can be detected in β haemolytic streptococci and staphylococci by D-zone test using Erythromycin (15 µg) and clindamycin (2 µg) discs together as follows:
   a) For streptococci : discs 12 mm apart
   b) For staphylococci : discs 15-26 mm apart

3. Salmonella isolates from extra-intestinal sites should be tested for nalidixic acid and if it is resistant to nalidixic acid, even though sensitive to ciprofloxacin, physicians should be informed that ciprofloxacin may be associated with clinical failure or delayed response

4. Extended Spectrum β lactamase producers (ESBL)
   a) Screening tests:
      i. Place cefotaxime / ceftriaxone or ceftazidime disc and amoxycillin-clavulanic acid / ticarcillin-clavulanic acid disc with their centers 20 mm apart. Presence of ESBL is indicated by an enhanced clear inhibitory zone resembling a "key hole" or a clear elliptical area between the discs. [1]
      ii. cefpodoxime 10 µg ≤ 17mm , ceftazidime 30 µg ≤ 22mm, cefotaxime 30 µg ≤ 27mm or ceftriaxone 30 µg disc ≤ 25mm or aztreonam 30 µg ≤ 27mm
   b) confirmatory test:
      ceftazidime 30 µg and ceftazidime/clavulanic acid 30/10 µg
      or cefotaxime 30 µg and cefotaxime/clavulanic acid 30/10 µg discs-
      a ≥ 5 mm increase in diameter with the combined disc confirms ESBL.

Antimicrobial agents that must not be reported as susceptible:

Organism	**Antimicrobial agents that must not be reported as susceptible**
ESBL producing organisms	Penicillins, cephalosporins and aztreonam
Salmonella spp., Shigella spp.	1st and 2nd gen cephalosporins, aminoglycosides
Oxacillin resistant *Staphylococcal spp.*	Penicillins, β- lactam/β-lactamase inhibitor combinations, cephalosporins, carbapenems
Enterococcus spp.	Aminoglycosides (except high level resistance), cephalosporins, clindamycin and co-trimoxazole
Yersinia pestis	β- lactam agents

Unusual results that should be verified:

Organism or organism group	**Unusual result**
Coliform	Carbapenem -R or I
Staphylococcus spp, Enterococcus spp	Vancomycin- R, Linezolid- R
Stenotrophomonas maltophilia	Carbapenem -S
Klebsiella/ Proteus	Ampicillin -S
β- haemolytic streptococci	Penicillin- R, Vancomycin -R
Streptococcus , including *Streptococcus pneumoniae*	Vancomycin -R

References

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone.
2. Clinical and Laboratory Standards Institute**. CLSI (formerly NCCLS) Performance Standards for Antimicrobial Susceptibility Testing**; 19th Informational Supplement, M100-S18 Vol 29 No.1 January 2009.

REQUIREMENTS

EQUIPMENT

Incubator	35 °C
Refrigerator	2°C - 8°C
Vortex mixer	
Bunsen Burners	

CONSUMABLES

Media and reagents

MHA	See SOP on media
Antibiotic discs	• Use validated discs from a single manufacturer • Note expiry date and use within this date • Bulk stock of discs should be stored at –20°C • In use discs may be stored up to a week at 2°C-8°C • In use discs should be removed from the refrigerator and left at room temperature for ½ hour before the container is opened minimize deterioration due to condensation of moisture

Other items

Wire loops
Straight wire
Calipers, dividers or millimeter ruler
Sterile swabs
Sterile Pasteur pipettes
MacFarland 0.5 turbidity standard
Sterile saline suspension in bijou bottles
Control organisms

MAINTENANCE OF RECORDS

1. ABST results are usually maintained with specimen results
2. Always record zone diameters
3. Keep a record of zone sizes of control organisms
4. Always record antibiotics in the same order. This minimizes transcription errors.
5. Keep QC records as indicated in SOP

STANDARD OPERATING PROCEDURE FOR ANTIBIOTIC SENSITIVITY TESTING BY DISC DIFFUSION

STOKES COMPARATIVE METHOD

Introduction

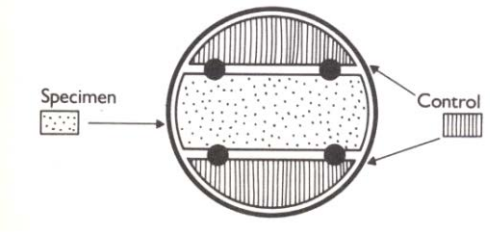
The Stokes method is a disc diffusion method, where comparison of the inhibition zone of the test isolate with the inhibition zone of a control organism forms the basis for interpretation of sensitivity. Variations in the test are controlled by inoculation of the test organism and the control strain at the same time on the same plate. It is assumed that any variation in the test affects the test and control strains equally and thereby cancels them out. It is therefore termed a "comparative" approach.

This method allows more flexibility in the use of media, disc strengths and inoculum than standardized methods. However, lack of standardization makes inter laboratory comparison difficult and the reliability of results obtained by this method has been questioned. Information on the reliability of this method in testing newer antibiotics is hard to find. Where control organisms are very sensitive to the newer antibiotics, incorrect reports of resistance or intermediate resistance may be issued by laboratories using this method. Newer mechanisms of resistance such as ESBL production cannot be detected using this method. Therefore many laboratories worldwide have shifted to standardized methods. Most of the recent published literature on antibiotic sensitivity testing and resistance patterns is based on standardized ABST testing methods.

Therefore it is recommended that, wherever possible, standardized methods of ABST are used. However, where the precise requirements of the standardized methods cannot be followed because of constraints in the supply of the required media / discs, this method may still be useful.

REQUIREMENTS FOR TEST

Medium	1. Any antibiotic sensitivity test medium that sustains the growth of the test organism can be used. MHA, diagnostic sensitivity test (DST) medium, Iso-Sensitest agar are commonly used. 2. Lysed blood can be added for organisms which are fastidious. a) prepare and sterilize medium as per manufacturer's instructions (refer SOP on media) b) cool the medium to 50°C c) add blood to a concentration of 5% 3. Pour medium into petridishes placed on a flat horizontal surface to a depth of 3-4 mm. 4. Allow to set. 5. Store poured plates at 2°C - 8°C and use within a week of preparation. 6. Dry plates with lids ajar until there are no drops of moisture on the agar surface (eg drying in an incubator set at 35°C for 15- 30 minutes).

Preparation of inoculum & Method of inoculation • Several methods can be used for the preparation of the inoculum • Inoculum should give a semi-confluent growth after overnight incubation	1. Touch 4-5 colonies lightly with a straight wire. 2. Inoculate plate at centre. 3. Use a sterile swab moistened with sterile normal saline (or distilled water) to spread the inoculum through area in two directions perpendicular to each other. 4. Number of colonies touched will vary with isolate (enterobacteria and pseudomonas may produce a confluent growth if a large number of colonies are touched. Streptococci may produce too light a growth unless sufficient number of colonies are touched). 5. If semi-confluent growth is not obtained, the test should be repeated. 6. Up to 4 discs per 9cm plate may be applied. 7. Place discs at least 3cm apart using a sterile pair of forceps. Press lightly into agar. 
Incubation	Incubate the plate 18 – 24 hours at 35°C.

CONTROL ORGANISMS

Control strain	**Test organism**
Escherichia coli NCTC 10418	Coliform organisms
Pseudomonas aeruginosa NCTC 10662	Pseudomonads
Haemophilus influenzae NCTC 11931	Haemophilus species
Staphylococcus aureus NCTC 6571 (Oxford staphyloccus)	Other organisms that will grow aerobically
Escherichia coli NCTC 11560	β lactam / β lactamase inhibitor combinations(co-amoxiclav)

Source of control organisms

- Department of Bacteriology, MRI, Colombo 8. Tel.0112691350
- Antibiotic Laboratory, Department of Microbiology, Faculty of Medicine, Peradeniya.
  Tel . 081 2396534/081 2396540

DISC CONTENTS FOR ABST (STOKES METHOD)

Antimicrobial	Organisms from sites other than urine	Organisms from urine
Amikacin	30	30
Ampicillin/Amoxycillin		
a) Enterobacteriaceae /enterococci	10	25
b) Haemophilus,Moraxella and Staphylococcus species	2	2
Amoxycillin-clavulanic acid		
a) Enterobacteriaceae /enterococci	20/10	20/10
b) Haemophilus,Moraxella and Staphylococcus species	2/1	2/1
Azithromycin	15	-
Cefuroxime	30	30
Cefotaxime	30	30
Ceftazidime	30	30
Cephradine[1]	30	30
Cephalexin[1]	30	30
Cephaloridine	5	30
Chloramphenicol		
a) Enterobacteriaceae	30	-
b) Haemophilus, Pneumococcus meningococcus	10	-
Ciprofloxacin (Ofloxacin)	1	1
Clindamycin	2	-
Co-trimoxazole[1]	1.2/23.8	1.2/23.8
Erythromycin (Clarithromycin)	10	-
Fusidic acid	10	-
Gentamicin	10	10
Imipenem	10	10
Kanamycin (Neomycin)	30	30
Methicillin	5	-
Metronidazole	5	-
Mupirocin	5	-
Nalidixic acid	-	30
Netilmicin	10	10
Nitrofurantoin	-	50
Oxacillin[2] (for testing of pneumococci)	1	
Penicillin		
a) Staphylococcus spp	*2	*2
b) meningococcus gonococcus	*0.25	*0.25
Piperacillin	30	30
Rifampicin	5	-
Tetracycline	10	30
Ticarcillin	75	75
Trimethoprim	1.25	5
Vancomycin	30	30

*International Units

READING AND MEASUREMENT OF ZONES OF INHIBITION

• It is useful to record control zone diameters daily as a QC exercise. • Deterioration of discs may be detected early.	1. Use a measuring instrument for reading - calipers, dividers or millimeter ruler. 2. Read plates only if both test and control show semi-confluent growth. 3. When the inoculum is optimal control zones sizes should be 8 – 15 mm radius. 4. If test zone is larger than the control or gives no zone at all, measurement is not required. 5. Measure wherever there is a zone of inhibition which appears equal to or smaller than the control zone.
Interpretation	6. Measure zones of inhibition from the edge of the disc to the edge of the zone (referred to as zone radius).
Sensitive	Zone radius equal to, wider than or not more than 3 mm smaller than the control
Intermediate	Zone radius greater than 2mm but smaller than the control by more than 3mm
Resistant	Zone radius 2mm or less
Exceptions	1. Penicillinase producing staphylococci show heaped up, clearly defined zone edges and should be reported as resistant irrespective of zone sizes. 2. Ciprofloxacin gives large zones with control strains. Interpretation criteria differ (as given below). *S. aureus* or pseudomonas used as a control - difference 7 mm *E. coli* or *H. influenzae* used as a control - difference 10 mm 3. With organisms that swarm, the zone edge should be measured as for other organisms and swarming should be disregarded.

REPORTING PROCEDURE

ABST report **Turn around time** Normal – 48 hours Unusual circumstances – longer (always inform ward / clinician by telephone if results are delayed**)**	1. Test and report appropriate antibiotics (as in SOP for specimens). 2. Test and report second line antibiotics only if resistant to first line antibiotics or if patient is on a particular antibiotic as stated in the request. 3. Do not report antibiotic sensitivity of colonizing flora. 4. If antibiotic discs not available –indicate on report. If clinically important, consider sending the isolate to a Reference laboratory for testing.

REQUIREMENTS

EQUIPMENT

Incubator	35°C
Refrigerator	2°C - 8°C
Bunsen Burners	

CONSUMABLES

Media and reagents

Media	MHA, DST or AST
Antibiotic discs	• Use validated discs from a single manufacturer • Bulk stock of discs should be stored at -20°C • In use discs may be stored up to a week at 2°C-8°C • In use discs should be removed from the refrigerator and left at room temperature for ½ hour before the container is opened to minimize deterioration due to condensation of moisture.

Other items:

Wire loops
Sterile swabs
Straight wire
Calipers, dividers or millimeter ruler
Control organisms

MAINTENANCE OF RECORDS

1. ABST results are usually recorded with specimen results.
2. Always record zone radii.
3. Keep a record of zone sizes of control organisms.
4. Always record antibiotics in the same order. This minimizes transcription errors.

Reference

1. **Journal of Antimicrobial Chemotherapy.** A guide to sensitivity testing Supplement D Vol 27 June 1991
2. Gosden PE, Andrews JM, Bowker KE et al. **Journal of Antimicrobial Chemotherapy** (1998) 42 161-169
3. Stokes, Ridgeway and Wren. **Clinical Microbiology**. 7th Edition 1993 pp 242

SEROLOGY

STANDARD OPERATING PROCEDURE FOR WIDAL TEST (STANDARD AGGLUTINATION TEST – SAT)

Type of specimen Blood collected in to a plain, clean bottle or serum

Introduction

This SOP describes method of performing the Widal test (SAT) using antigens provided by the Medical Research Institute (MRI). Though Widal test is done for the diagnosis of enteric fever it is not a very reliable diagnostic test. False positives and false negatives and interpretation problems are frequently encountered. Therefore encourage requesting clinicians to send blood for culture as isolation of *Salmonella typhi* & *Salmonella paratyphi A* is the most reliable method of diagnosing typhoid fever.

Commercial kits (slide agglutination and tube agglutination) are available. The use of only a slide agglutination test is not recommended. Commercial agglutination kits should be used according to the manufacturer's instructions.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	Acute and convalescent stages of the disease. (10 – 14 days apart)

SPECIMEN TRANSPORT AND STORAGE

Refrigerate until dispatched to laboratory.

REJECTION CRITERIA

1. Inadequate serum for the test
2. Decomposed sample
3. Broken Specimen container or spillage of blood in transit
4. Unlabelled specimen

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.
3. Do not discard samples which may be unrepeatable.

Required antigens

Salmonella typhi O
Salmonella typhi H
Salmonella typhi Vi
Salmonella paratyphi A H

Preparation of antigens

1. Dilute the antigens with normal saline according to the instructions given with each batch.
2. Prepare weekly requirement of antigen to minimize contamination of stock suspension (referred to as 'in use' antigen) .
3. Maintain stock antigen and diluted 'in use' preparations at 4^0C.

Preparation of serum dilutions

Preparation of initial serum dilution

a) Measure 0.1ml serum into a clean dry tube (eg Wasserman tube).
b) Add 0.3 ml N saline to make a 1:4 dilution.

Test method

O and Vi antigens

1. Place 3 Kahn tubes in a rack for O antigen and 1 Kahn tube for Vi antigen.

O antigen Tubes 1-3

2. Add 1.25 ml of O antigen to each of the 3 tubes and 1.25 ml of Vi antigen to the remaining tube.

Vi antigen Tube 4

3. Add serum as indicated in the following:

	1	2	3	4
Antigen	1.25(O)	1. 25(O)	1. 25(O)	1.25(Vi)
Serum	neat	1:4 dilution	1:4 dilution	neat
	0.025ml	0.05ml	0.025ml	0.05 ml
Final dilutions	**1:50**	**1:100**	**1:200**	**1:25**

1. Incubate all tubes at 37^0C for 2-4 hours (water bath preferable if available).
2. Remove from water bath and leave at 4^0C overnight.
3. Raise tube and inspect the bottom (without disturbing the sediment).
4. Control tube should have a clear button.

Reading

5. **The presence of granular deposit indicates a positive result .**
6. **If all 3 tubes positive, repeat test with further dilutions up to 1: 1600 as follows (a) – (h)**

a) Number 4 Wasserman tubes as 1, 2, 3, 4.
b) Add 0.6 ml N.Saline to tube 1 and 0.4 ml each to tubes 2, 3, 4.
c) Add 0.2 ml of serum to tube 1 and mix well (1:4 dilution).
d) Transfer 0.4 ml of 1:4 dilution (tube 1) to tube 2 (to make 1:8 dilution).
e) Transfer 0.4 ml of 1:8 dilution (tube 2) to tube 3 (to make 1:16 dilution)**.**
f) Transfer 0.4 ml of 1:16 dilution (tube 3) to tube 4 (to make 1:32 dilution)**.**
g) Follow the test method using 2 sets of 6 Kahn tubes.
h) Add one drop of diluted serum to the relevant tubes as follows:

Antigen	1.25 ml	1.25 ml	1.25 ml
Serum	1:8 dilution	1:16 dilution	1:32 dilution
	0.025ml	0.025ml	0.025ml
Final dilution	**1:400**	**1:800**	**1:1600**

i) Incubate the tubes at 37^0C and follow the same steps as in the earlier test.(4-9).
j) Run a positive control and a negative control along with the test samples.

Test method for H antigen

Arrange 2 sets each of 3 dryers tubes each in a metal rack.

1. Label one set for *S. typhi* (set 1) and the second set for *S. paratyphi* A H (set 2) antigens
2. Add 1.25ml of 'in use' *S. typhi* H antigens to each dryer's tubes in set 1 and *S. paratyphi* A H antigen to each tube in set 2.
3. Add serum as indicated to each of the 2 sets:
   a) 0.025ml neat serum to tube 1
   b) 0.05ml of 1:4 serum dilution to tube 2
   c) 0.025ml of 1:4 serum dilution to tube 3

Final dilutions

	1	**2**	**3**
antigen	1.25	1. 25	1. 25
serum	neat 0.025ml	1:4 dilution -0.05ml	1:4 dilution-0.025ml
	1:50	**1:100**	**1:200**

Reading

1. Incubate in a water bath at 56^0C for 2-4 hours.
2. Remove from water bath and leave tubes at room temperature for 30 minutes .
3. **Examine tubes for the presence of flocculation (seen as uneven clumps / feathery appearance /cotton wooly appearance).**
4. If floccules in all 2 tubes of a set, make serial dilutions of serum up to 1:1600 and re do test from D 2-9 .
5. Titre: highest dilution at which flocculation present

Quality control

1. Maintain positive serum controls of known titre at -20^0C.
2. Run positive and negative controls weekly and record results.

It is useful to store all sera at 4^0C and if a convalescent serum sample is received both sera should be tested on the same run.

REPORTING

S. typhi O antibody titre
H antibody titre
Vi antibody titre
S. paratyphi H antibody titre

Interpretation

4 fold rise between acute and convalescent samples is indicative of active infection

REQUIREMENTS

.

EQUIPMENT

Water bath	37^0C
Water bath	56^0C
Refrigerator	2^0C - 8^0C
Freezer	-20^0C

CONSUMABLES

Glassware

Sterile screw capped 5 ml containers-	For collection of samples
Graduated pipettes/ Microtitre pippette	
Kahn tubes	
Dryers tubes	
Test tube racks	
Wasserman tubes	

Antigens and reagents

Antigens obtained from MRI	O, H, Para A- H and Vi
Antigens obtained from Faculty of Medicine, Peradeniya	O and H
Normal saline	

Control sera

Positive and negative sera	maintained at -20^0C

MAINTENANCE OF RECORDS

Unique records register for SAT

STANDARD OPERATING PROCEDURE FOR DETERMINATION OF ANTI STREPTOLYSIN O TITRE (ASOT)

Type of specimen Blood collected into a plain, clean bottle or serum

Introduction

The ASOT is based on the immunochemical reaction between anti-streptolysin O antibodies and streptolysin O coated latex particles. Presence of anti streptolysin O antibodies results in visible agglutination.

Commercial kits are readily available for this test. Perform the test in accordance with the manufacturer's instructions.

SPECIMEN COLLECTION / SPECIMEN CONTAINER

Optimal time of specimen collection	1. Patients with acute rheumatic fever 2. Patients with acute glomerulonephritis 3. Patients in whom evidence of recent Group A streptococcal infection is being sought Previous antibiotic use does not usually interfere with test results.

REJECTION CRITERIA

1. Inadequate serum for the test
2. Decomposed sample (when sent by post)
3. Unlabelled specimen

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.

TEST METHOD

Reagents	1. Record details of kit used a) Manufacturer b) Lot number c) Expiry date 2. Store reagents at 2-8°C (do not freeze). 3. Allow reagents to reach room temperature before use.
Processing	Follow manufactures instructions.

REPORTING PROCEDURE

ASOT	Upper limit of normal is 200 Todd units. A 4 fold or greater rise in anti-streptolysin titre is significant in all age groups

REQUIREMENTS

EQUIPMENT

Incubator	35°C - 37°C
Refrigerator	2°C - 8°C
Centrifuge	for lipaemic specimens
Shaker	Optional

CONSUMABLES

Glassware

Sterile screw capped 5 ml containers	For collection of samples

Reagents

ASOT kits
Template

MAINTENANCE OF RECORDS

Unique records register for ASOT

OTHER SEROLOGY TESTS

Reference Laboratory
MRI, Colombo 8
Tel No: (011) 2693532, 2693533, 2693534

Tests available in Reference Laboratory

Test	Specimen required
Brucella antibody test	2 ml of clotted blood or serum
Legionella urinary antigen test	urine
Leptospira Microscopic agglutination test (MAT)	2 ml of clotted blood or serum
Leptospira IgM and IgG ELISA	2 ml of clotted blood or serum
Mycoplasma antibody test	2 ml of clotted blood or serum
Weil Felix test	2 ml of clotted blood or serum
Brucella agglutination test	2 ml of clotted blood or serum
H.pylori antibody test	2 ml of clotted blood or serum
Monospot test (IMN)	2 ml of clotted blood or serum

Collection of blood samples for serology

1. Clean skin with 70% alcohol.
2. Draw 2-3 ml of blood using a sterile, disposable syringe and needle (or vacutainer system).
3. Collect blood in to a dry sterile screw capped bottle.
4. Label bottle with patient identification data.
5. Send blood directly to the Reference Laboratory in an ice box along with a properly filled request form.

Separation of serum

1. Keep the blood sample in room temperature for 2 hours undisturbed for the formation of a clot.
2. Centrifuge at 1500 x g for 10 min to separate the serum.
3. Transfer the separated serum in to a new tube under aseptic conditions.
4. Separated serum need to be sent in an ice box.
5. If transport of this specimen is needed and if sterile conditions are not available, do not separate the serum. Send blood directly to the Reference Laboratory in an ice box.
6. Acute specimen should be processed on receipt.
7. On receipt of the convalescent sample acute and convalescent specimens to need to be run in parallel.

Test	Specimen required	Optimal time of specimen collection	Time taken for results to be available	Other remarks (supplementary tests etc...)
Mycoplasma antibody test	**Serum or 2ml blood**	**Acute and convalescent**	**2 days**	
Legionella urinary antigen test	**Urine sent in sterile container at 4^0 C**	**Acute sample**	**Please check with lab as tested in batches**	
Leptospira Microscopic agglutination test (MAT)	**Serum or 2ml blood**	**Acute and convalescent**	**Same day**	**For culture contact lab.**
Leptospira IgM and IgG ELISA	**Serum or 2ml blood**	**Acute and convalescent**	**Please check with lab as tested in batches**	
ASOT	2ml clotted blood or serum		1 day	
Brucella antibody test	2ml clotted blood or serum		3 days	
Helicobacter pylori antibody test	2ml clotted blood or serum		1 day	
Latex agglutination test for Infectious mononucleosis	2ml clotted blood or serum		1 day	EBV antibody test
SAT	3ml whole blood		3 days	Salmonella culture
Weil-Felix test	2ml clotted blood or serum		3 days	

BACTERIOLOGICAL MEDIA

BRAIN HEART INFUSION BROTH (BHI)

1. DESCRIPTION

Nutritious infusion medium recommended for the cultivation of fastidious and nonfastidious microorganisms, including aerobic and anaerobic bacteria, from a variety of clinical materials. The medium is recommended for blood culture work.

2. SUPPLEMENTS

Addition of sodium polyanethol sulfonate (SPS or Liquoid) 2.5 gram per Litre will improve isolation rate.

3. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water. Mix well and distribute the reconstituted broth into final containers (for blood culture use bottles with perforated screw caps and rubber liners).
b) Autoclave with caps closed tight at 121°C for 20 minutes.
c) Cover exposed area of the liner in the perforation with a foil cap before autoclaving.
d) Brain heart infusion broth which is not used on the day that it is sterilised should be placed in a boiling water bath for several minutes to remove absorbed oxygen, and cooled rapidly without shaking just before use.

4. QUALITY CONTROL [1]

Positive controls:	**Expected results**
Streptococcus pneumoniae ATCC 6303	Turbid growth
Candida albicans ATCC 10231	Turbid growth
Negative control:	
Uninoculated medium	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

AMIES MEDIUM

1. DESCRIPTION

This medium is used to preserve the viability of anaerobes, *Neisseria gonorrhoeae* and other pathogens.

2. METHOD

1. Suspend 20g of medium in 1litre of distilled water. Bring to boil to dissolve the agar completely.
2. Distribute into small screw capped bottles stirring to keep charcoal suspended. Completely fill the bottles. Tighten the screw-caps.
3. Autoclave at 121°C for 15 minutes.
4. Allow to cool.
5. During cooling, invert the bottles to ensure an even distribution of charcoal.
6. Date the medium and give it a batch number. Record the expiry date (9 months from preparation, provided there is no change in volume or appearance of the medium) on each bottle.
7. Store in a cool place away from direct light.

3. QUALITY CONTROL [1]

Positive controls:	**Expected results**
Staphylococcus aureus ATCC 25923	Good growth
Escherichia coli ATCC 25922	Good growth
Negative control:	
Uninoculated medium	No change

Reference

1. Monica Cheesbrough. **Medical Laboratory Manual for Tropical Countries - Volume 11** Microbiology, 1984 (reprinted 1994). Butterworth-Heinemann
2. **The Oxoid Manual**. 9th Edition 2006

ALKALINE PEPTONE WATER (APW)

1. DESCRIPTION

APW is a broth medium used as the enrichment medium for *Vibrio cholerae* and other vibrio species. Sodium chloride in the medium favours the multiplication of vibrios while the alkalinity (pH 8.6-9.0) inhibit the growth of faecal commensals. The vibrios grow on/ just below the surface of the medium. It may be used as a transport medium providing the transportation time does not exceed 10 hours.

2. METHOD

Formula and preparation (for about 100 bottles)

Peptone	10g	
Sodium chloride	20g	
Distilled water	1 litre	pH 8.6 +/- 0.2

a) Dissolve the peptone and sodium chloride in the water.
b) Adjust the reaction of the medium to pH 8.6-9.0
c) Dispense the medium in 10 ml amounts in screw-capped bottles.
d) Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes.
e) Tighten the bottle caps after the medium has cooled.
f) Date the medium and give it a batch number.
g) Label the bottles and record the expiry date of the medium (30 days from date of preparation).
h) Store in a cool dark place with bottle caps screwed tightly to prevent a change in pH.

3. STORAGE CONDITIONS AND SHELF LIFE

Prepared medium – Clear, straw coloured solution without any precipitate. Can keep up to one month at room temperature.

4. QUALITY CONTROL

Positive controls:	**Expected results**
Vibrio parahaemolyticus ATCC 17802	Turbid
Vibrio vulnificfus ATCC 27562	Turbid
Vibrio furnissii ATCC 11218	Turbid
Negative control:	
Uninoculated medium	No change

References

1. Monica Cheesbrough. **Medical Laboratory Manual for Tropical Countries - Volume 11** Microbiology, 1984 (reprinted 1994). Butterworth-Heinemann.
2. **The Oxoid Manual**. 9th Edition 2006.

BLOOD AGAR

1. DESCRIPTION

This is a general purpose medium capable of growing a range of microorganisms of clinical significance. The medium gives good colonial appearances, haemolysis patterns and pigment production. The typical haemolytic reactions are important diagnostic criteria for streptococci and staphylococci.

2. SUPPLEMENT

5% blood (preferably sheep blood)

3. METHOD

a) Suspend the weight of blood agar base as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0c for 15 minutes.
d) Cool the base to 50^0c and add sterile sheep blood to make a concentration of 7%.
e) Mix with gentle rotation and pour into petri dishes. Pour about 20ml per plate.
f) Move a flame over the surface of the agar to remove air bubbles as a final step.

Note: Human blood should be used only if sheep blood is not available. (Citrated human blood from blood banks is unsuitable as citrate inhibits the growth of β-haemolytic streptococci. The presence of even small amounts of glucose may alter the haemolytic reaction).

4. QUALITY CONTROL[1]

Positive controls*:*	**Expected results**
Staphylococcus aureus ATCC 25923	Good growth; grey white coloured colonies
Streptococcus pyogenes ATCC 19615	Good growth; pale colonies; beta haemolysis
Streptococcus pneumoniae ATCC 6303	Good growth; green grey coloured colonies; alpha haemolysis
Negative control:	
Uninoculated plate	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

BLOOD TELLURITE AGAR

1. DESCRIPTION

This medium is used for isolation, differentiation and identification of *Corynebacterium diphtheriae*. Other organisms are generally inhibited. The medium is enriched by the addition of blood and made selective by the addition of potassium tellurite which inhibit the growth of Gram negative organisms, Staphylococcus and Streptococcus.

2. FORMULATION

K_2TeO_3 (Pottasium tellurite), 2% aq. Solution - 16 ml
Sterile blood - 50 ml
* Infusion Agar[1] - 1000 ml
* MRI use blood agar base instead of infusion agar.

3. METHOD[1]

a) Melt the Infusion Agar medium, cool to 50^0C and aseptically add the blood and sterile tellurite solution.
b) Medium must not be heated after addition of the tellurite.
c) Mix and pour into petridishes.

Note: Sterilize the tellurite solution by filtration, not by heat.
Tubes of base medium can be stored and melted to make the complete medium when culture plates are needed

Organism		**Size of colony (mm)**	**Colour and appearance of colony**
C. diphtheriae	gravis	1.5-2.5	convex, crenated, dull, grey-black
	mitis	1.5-2.0	convex, entire, glossy, grey
	intermedius	0.5-1.0	convex, entire, glossy, grey
Corynebacterium ulcerans		1.0-1.5	convex, entire, glossy, grey

Note:
Some *Staphylococcus spp*. Gram negative bacilli and yeasts may overcome inhibition and grow on this medium

4. MINIMUM RECOMMENDED QC ORGANISMS

C. diphtheriae var mitis NCTC 10356 (non-toxigenic)

References

1. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.
2. PHLS MEDIA SPECIFICATION - Technical Services, PHLS ,UK

CARY- BLAIR MEDIUM

1. DESCRIPTION

This is a transport medium used for the collection and transport of clinical specimens. The low nutrient content of the medium and utilisation of phosphate as a buffering agent prevents bacterial overgrowth of several enterobacteria. The low oxidation-reduction potential of the medium ensures bacterial survival over long periods. This medium is used to preserve the viability of enteric pathogens in faecal specimens.

2. METHOD

a) Follow manufacturer's instructions to prepare 1 litre of the medium.
b) pH of medium should be within 8.3-8.5 at room temperature.
c) Distribute into small screw capped bottles and sterilise by immersing in free steam for 15 minutes (refer manufacturer's instructions).
d) Allow to cool and tighten the screw caps to prevent water loss.
e) Date the medium and give it a batch number.
f) Record the expiry date (6 months from preparation) on each bottle.

3. STORAGE CONDITIONS AND SHELF LIFE

Prepared medium - Store in a cool dark place. Shelf life is 6 months provided there is no change in volume or appearance of the medium.

4. QUALITY CONTROL

Positive control:	**Expected results**
Shigella sonnei ATCC 25931	Good growth on subculture
V.parahaemolyticus NCTC 11344	Good growth on subculture
Negative control:	
Uninoculated medium	No change

References

1. Monica Cheesbrough. **Medical Laboratory Manual for Tropical Countries - Volume 11** Microbiology, 1984 (reprinted 1994). Butterworth-Heinemann
2. **The Oxoid Manual**. 9th Edition 2006.

CHOCOLATE AGAR (HEATED BLOOD AGAR)

1. DESCRIPTION

This medium is suitable for the growth of fastidious organisms such as *Haemophilus influenzae, Pneumococci and Neisseriae.* During heating the red cells are ruptured and nutrients are liberated.

2. SUPPLEMENT

7% blood (preferably sheep blood)

3. METHOD

a) Suspend the weight of blood agar base as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0C for 15 minutes.
d) Cool in water bath up to 50^0C and add the blood to make a final concentration of 7%.
e) Heat it in a water bath to 80^0C with frequent mixing until the medium has a chocolate colour.
f) Pour into petri dishes. Pour about 20ml per plate.
g) Move a flame over the surface of the agar to remove air bubbles as a final step.

4. QUALITY CONTROL[1]

Positive control:	**Expected results**
Haemophilus influenzae NCTC 8143 or 11931	Good growth
Negative control:	
Uninoculated plate	No change

Reference

1. E. Joan Stokes, G.L. Ridgway, M.W.D. Wren. **Clinical Microbiology**, 7th edition 1993. Edward Arnold Publishers, UK.

CYSTINE LACTOSE ELECTROLYTE DEFICIENT (CLED) AGAR

1. DESCRIPTION

CLED medium is a valuable non inhibitory diagnostic medium for urinary bacteria as it supports the growth of all urinary pathogens. It is recommended for diagnostic urinary bacteria. It gives good colonial differentiation and clear diagnostic characteristics. The absence of electrolytes inhibits the swarming of *Proteus* species. Cystine is added for growth of organisms that require it. Differentiation of lactose fermenters and non lactose fermenters is achieved by using Bromothymol blue as a pH indicator.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0C for 15 minutes.
d) Pour into petri dishes (about 20ml per plate).
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

3. COLONIAL CHARACTERISTICS

Organism	Colour of colony	Comments
Escherichia coli	Yellow	Non lactose fermenting strains – blue colonies
Klebsiella spp.	Yellow	Extremely mucoid colonies colour may vary from yellow to whitish blue.
Pseudomonas aeruginosa	Green	Green pigment and odour
Salmonella spp.	Blue	
Staphylococcus aureus	Deep Yellow	
Other staphylococci	Pale yellow or white	
Enterococcus spp.	Yellow	
Proteus spp.	Blue	

4. QUALITY CONTROL[1]

Positive controls:	**Expected results**
Proteus mirabilis ATCC 10975	Good growth; blue colonies; no swarming
Staphylococcus aureus ATCC 25923	Good growth; yellow colonies
Negative control:	
Uninoculated medium	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

COOKED MEAT MEDIUM

1. DESCRIPTION

Cooked Meat Medium is used for the cultivation of anaerobic microorganisms. It initiates growth from small inoculums and maintains viability of micro-organisms for long periods of time. In mixed cultures, slower growing organisms survive without being displaced. Products of growth do not rapidly destroy inoculated organisms. Therefore it is an excellent medium for storage.

This medium can be used to differentiate saccharolytic from proteolytic *Clostridium spp.* Saccharolytic species rapidly form acid and gas without digesting meat. Proteolytic species break down meat to amino acids and form sulphur compounds (blackening and putrid smell).

2. METHOD

a) Place 1 g of medium in screw capped tubes/bottles and add10 mL of purified water
b) Allow to stand 15 minutes until meat particles are wetted.
c) Autoclave at 121°C for 15 minutes.
d) Do not cool rapidly as meat particles can get expelled from the container.
e) Prepared medium is clear solution over brown pellets, medium gold/amber solution.

3. QUALITY CONTROL

Positive controls:	**Expected Result**
Clostridium histolyticum	Turbid growth, proteolysis
Clostridium perfringens ATCC 13124	Turbid growth, proteolysis, saccharolysis
Negative control:	
Uninoculated medium	No change

Inoculate specimen deep into meat particles (bottom of the tube). Tissue specimens should be ground prior to inoculation. Typically growth is visually observed in media by turbidity and/or presence of gas bubbles.

Reference

1. Robertson, M. Notes upon certain anaerobes isolated from wounds. **J. Pathol. Bacteriol.** 1916 ;20:327.
2. **The Oxoid Manual**. 9th Edition 2006.

KLIGLER IRON AGAR (KIA)

1. DESCRIPTION

A differential slope medium used to assist in the identification of Salmonella, Shigella and other enteric bacteria. This is based on double sugar (glucose & lactose) fermentation and H_2S production.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Mix well and distribute into containers.
c) Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes.
d) Allow to set as slopes with 1 inch butts and about 1 inch long slopes.
e) Store in a cool dark place or at 2°C - 8°C. Shelf life – approximately 3 weeks.

3. KLIGLER REACTIONS

butt	**slope**	**Cracks in medium/bubbles**	**Black discolouration**	**Explanation**
yellow	Pink-red			Fermentation of glucose only
yellow	yellow			Fermentation of lactose ± glucose
		Cracks/ bubbles		Gas production
			Black discolouration	H_2S production

4. QUALITY CONTROL [1]

Positive controls:	**Expected results**			
	Slope	**Butt**	**Gas**	**H_2S**
Citrobacter freundii ATCC 8090	Yellow	Yellow	+	+
Shigella sonnei ATCC 25931	Red	Yellow	-	-
Alcaligenes faecalis ATCC 19018	Red	Red	-	-
Negative control:				
Uninoculated medium – No change				

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

MacCONKEY AGAR

1. DESCRIPTION

This is a useful selective, differential primary plating medium for the cultivation of enterobacteria. It contains bile salt to inhibit non intestinal bacteria and lactose with neutral red to distinguish lactose fermenting from non-lactose fermenting organisms. The omission of NaCl from the medium prevents the spreading of *Proteus* species.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0C for 15 minutes.
d) Pour into petri dishes (about 20ml per plate).
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

Note: Different formulations for separate purposes are available. It is important to select the correct formulation for the required purpose.
For faeces - MacConkey agar with added bile salts/crystal violet
- Medium is inhibitory to Gram positive cocci

3. COLONIAL CHARACTERISTICS

Organism	**Colour of colony**	**Comments**
Escherichia coli	Dark pink	Non mucoid
Enterococcus spp.	Pink (magenta)	Very small round colonies
Staphylococci spp.	Pale pink	Opaque colonies
Pseudomonas spp.	Pale /translucent	-

4. QUALITY CONTROL [1]

Positive controls:	**Expected results**
Enterococcus faecalis ATCC 29212	Good growth; red coloured colonies
Staphylococcus aureus ATCC 25923	Good growth; pale pink coloured colonies
Negative control:	
Uninoculated medium	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

MANNITOL SALT AGAR

1. DESCRIPTION

Selective and differential primary culture medium for the isolation of Staphylococcus from specimens containing mixed flora (detection of nasal and skin Staphylococcus carriage / food). The high sodium chloride level (7.5%) inhibits most organisms except *Staphylococcus spp.* and some halophilic organisms such as *Vibrio* species.

Addition of egg yolk emulsion enables the lipase activity of Staphylococci to be detected. The high salt concentration in the medium clears the egg yolk emulsion and lipase production is detected as a yellow opaque zone around colonies that produce the enzyme.

2. SUPPLELMENT (optional)

5% egg yolk emulsion

3. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0c for 15 minutes.
d) Pour into petri dishes (about 20ml per plate).
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

4. COLONY CHARACTERISTICS

Organism	**Colour of colony**	**Comments**
Staphylococcus aureus	Bright yellow / with yellow halo	
Other Staphylococci / Micrococci	Reddish colonies with red or purple surrounding zones	Some ferment mannitol
Enterobacteriaceae	No growth	
Vibrio species and other halophiles	Usually pink	Yellow if ferment mannitol

Note:
Confirmation of the identity of *Staphylococcu. aureus* must be performed.
If supplemented with egg yolk emulsion, *Staphylococcus aureus* colonies will show an opaque halo due to lipase activity.
After inoculation plates should not be discarded until 48 hrs of incubation as *Staphylococcus aureus* may be slow in mannitol fermentation.

5. QUALITY CONTROL[1]

Positive controls:	**Expected results**
Staphylococcus aureus ATCC 25923	Good growth; yellow colonies with yellow halo
Staphylococcus epidermidis ATCC 12228	Good growth; pink colonies with pink medium
Negative control:	
Escherichia coli ATCC 8739	No growth

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

MUELLER – HINTON AGAR (MHA)

1. DESCRIPTION

Muller-Hinton agar is a transparent medium that is recommended for antimicrobial susceptibility testing. The composition of the medium may vary from batch to batch which could affect zones of inhibition. Quality control of MHA is essential if CLSI method of antimicrobial susceptibility testing is carried out.

2. SUPPLEMENTS

5% defibrinated sheep blood for testing of *Haemophilus influenzae, Streptococcus pneumoniae* and *Neisseria* species.

3. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Bring to the boil to dissolve completely.
c) Sterilize by autoclaving at 121^0C for 15 minutes.
d) Pour into petri dishes (about 25-30 ml per 90mm plate). The depth of the medium should be 4mm.
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

4. QUALITY CONTROL[1]

Positive controls:	**Expected results**
Escherichia coli ATCC 25922	Good growth; pale straw coloured colonies
Pseudomonas aeruginosa ATCC 27853	Good growth; straw coloured colonies
Staphylococcus aureus ATCC 25923	Good growth; cream coloured colonies
Negative control:	
Uninoculated medium	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

7% NaCl NUTRIENT BROTH

1. DESCRIPTION

Nutrient broth is an economical medium for cultivation of non fastidious organisms. The addition of 7% NaCl (analytical grade) makes it a suitable enrichment and selective medium for *Staphylococcus aureus.*

2. METHOD:

Prepare nutrient broth from a good quality commercial base. Add NaCl to make a 7% broth. The sodium chloride concentration should be reduced if local prevalent strains are known to be inhibited by 7% sodium chloride.

3. QUALITY CONTROL[1]

S. aureus NCTC 8331 (known MRSA)
S. aureus NCTC 6571 (known MSSA)

Reference

1. **Microbiology Laboratory Manual** - Sri Lanka College of Microbiologists. 1st Edition. 2001

PEPTONE WATER

1. DESCRIPTION

Peptone water is used as a growth medium or as the basal medium for carbohydrate fermentation media. It is also used to obtain overnight cultures for biochemical testing or antibiotic sensitivity testing.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Mix well and distribute into final containers.
c) Sterilize by autoclaving at 121^0C for 15 minutes.

3. QUALITY CONTROL[1]

Positive control:	**Expected results**
Escherichia coli ATCC 25922	Turbid growth
Negative control:	
Uninoculated medium	No change

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

PEPTONE WATER SUGARS

1. DESCRIPTION

Peptone water is used as the basal medium for carbohydrate fermentation media. It may be modified for use in fermentation tests by the addition of Andrade's indicator.

2. METHOD

Formula and preparation

Peptone water with indicator (To make 95 ml of peptone water with indicator)

Peptone	- 1 g	(Final concentration 1%)
Sodium chloride	- 0.5g	(Final concentration 0.5%)
Andrade indicator	- 1 ml	
Distilled water	- 90ml	

a) Add peptone powder and NaCl to 94 ml of distilled water.
b) Add 1 ml of Andrade indicator.
c) Mix well and sterilize by autoclaving at 121°C for 15 minutes.

To prepare 25 bottles of 1% peptone water sugar:

Sterile peptone water with indicator	- 90 ml	
*Sterile 10% w/v sugar solution	- 10 ml	(Final concentration 1%)

* Prepare 10% sugar stock solution by dissolving 2.5 grams of the required sugar in 25 ml of distilled water. Sterilize by filtration or tyndallisation. (Tyndallisation is steaming for 30 minutes for 3 days).

a) Add the sterile sugar solutions aseptically to the sterile peptone water and mix well. (At MRI, unsterilized sugar is added to sterilized peptone water with indicator and then the whole solution is sterilized by tyndallisation).
b) Dispense aseptically 4 ml amounts into sterile Bijou bottles (containing an inverted Durham tube which was sterilized with the bottle, to look for gas production in glucose tube).
c) Date the medium and code the sugars to identify them.
d) Store in a cool dark place.

3. QUALITY CONTROL

Test for performance using control micro-organisms of known positive and negative fermentation reactions.

4. INSTRUCTIONS FOR USE

a) Use a sterile Pasteur pipette to inoculate the medium.
b) Make sure the Durham tube is filled with medium.
c) If an air bubble is present, invert bottle to expel the bubble.
d) Read after overnight incubation at 35°C-37°C.

Reference

1. Collee J. G, Fraser A. G., Marmion B. P., Simmons A. **Mackie & McCartney Practical Medical Microbiology**, 14th Edition 1996. Churchill Livingstone
2. **The Oxoid Manual**. 9th Edition 2006

SELENITE F BROTH

1. DESCRIPTION

This medium is used for selective enrichment of *Salmonella* species. Sodium biselenite is used as a selective agent. Selenite inhibits coliforms and other organisms present in faeces such as faecal streptococci. Inhibited strains may eventually overgrow pathogens.
Lactose in the medium maintains a uniform pH. As selenite is reduced by the growth of bacteria alkali is produced. An increase in pH would reduce the toxicity of selenite and result in overgrowth of organisms. As lactose is fermented acid is produced which serves to maintain a neutral or slightly decreased pH. Phosphate serves to maintain a stable pH and lessen the toxicity of the selenite.

2. FORMULATION

Selenite broth base (Lactose)	- 19g
Sodium biselenite	- 4 g
Distilled water	- 1 litre

3. METHOD

a) Dissolve 4 g of sodium biselenite in 1 litre of distilled water.
b) Then add 19 g of Selenite broth base.
c) Warm to dissolve, mix well and fill into containers. Give a depth of 25mm.
d) Sterilise in a boiling water bath , or in free flowing steam, for 10 minutes. **DO NOT AUTOCLAVE.**
e) Caution: Sodium hydrogen selenite is a toxic chemical with possible teratogenic properties. It must therefore be handled with care, avoiding hand to mouth contact. Whenever possible, the medium should not be prepared by a woman of child-bearing age
f) Tighten the caps after sterilizing. A small amount of red precipitate may form. This will not interfere with the performance of the medium
g) Store in a dark cool place.
h) Discard the prepared medium if there is a large amount of red precipitate in the bottom of the bottle.
i) See SOP for faeces. Subcultures should be performed after no more than 24h incubation as there is an increasing loss of selectivity if incubation is prolonged.

4. QUALITY CONTROL[1]

Positive controls:	**Expected results**
Salmonella typhimurium ATCC 14028	Good growth
Negative control:	
Escherichia coli ATCC 29222 Subculture to MacConkey agar	Inhibited or No growth

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

SALMONELLA-SHIGELLA AGAR

1. DESCRIPTION

Salmonella-Shigella (SS) agar is a differential selective medium used to isolate salmonella and some strains of shigella species from faecal specimens. Gram positive and coliform organisms are inhibited by the action of a specially prepared bile salts mixture. Thiosulphide in combination with iron act as an indicator for sulphide production which is indicated by blackening in the centre of the colonies**.**The medium is inhibitory and it is important to ensure by quality control that the expected pathogens can be isolated by the medium used in the laboratory.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Bring to boil to dissolve completely.
c) Sterilize by autoclaving at 121^0c for 15 minutes.
d) Pour into petri dishes (about 20ml per plate).
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

3. COLONIAL CHARACTERISTICS

Organism	**Colour of colony**
Non lactose fermenters	Transparent ,colourless colonies
Lactose fermenters	Pink colonies
Salmonella species	Transparent, colourless colonies with blackening of centre
Proteus / Citrobacter spp.	Transparent colonies with grey-black centres

Note : A heavy inoculum of stools should be plated on SS agar as the formulation is inhibitory to some strains..

4. QUALITY CONTROL[1]

Positive controls:	***Expected results***
Salmonella enteritidis ATCC 13076	Good growth; straw coloured colonies with black centers
Shigella sonnei ATCC 25931	Good growth; straw coloured colonies
Negative control:	
Enterococcus faecalis ATCC 29212	No growth

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

THIOSULPHATE CITRATE BILE SALT SUCROSE (TCBS) AGAR

1. DESCRIPTION

A selective isolation medium for *Vibrio* species. TCBS agar can also be used to differentiate various *Vibrio* species depending on their ability to ferment sucrose. The high pH of TCBS encourages the growth of *Vibrio* species while inhibiting other Gram negatives. Most enterobacteriaceae other than *Vibrio* species are suppressed for at least 24 hrs. Bile salts inhibit Gram-positive organisms. When sucrose is fermented it produces acid which changes the pH. This is indicated by bromothymol blue and thymol blue. The medium is alkaline which enhances the recovery of *Vibrio cholerae*.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Boil to dissolve the medium completely.
c) **Do not autoclave.**
d) Pour plates without further heating. Pour about 20 ml per 90mm plate.
e) Move a flame over the surface of the agar to remove air bubbles as a final step.

3. COLONIAL CHARACTERISTICS

Organism	**Colour of colony**	**Diameter of colony (mm)**
Vibrio cholerae	Yellow	2-3
Vibrio parahaemolyticus	Blue-green	3-5
Vibrio alginolyticus	Yellow	3-5
Vibrio metschnikovii	Yellow	3-4
Vibrio fluvialis	Yellow	2-3
Vibrio vulnificus	Blue-green	2-3
Vibrio mimicus	Blue-green	2-3
Enterococcus species	Yellow	1
Proteus species	Yellow-green	1
Pseudomonas species *Aeromonas* species *Plesiomonas* species	Blue-green Light yellow Light yellow	1 2-3 2-3

Note:

When using TCBS a heavy inoculum should be applied as *Vibrio* species die quickly. A fresh specimen is best as organisms are sensitive to drying, sunlight and acid pH.

4. QUALITY CONTROL[1]

Positive controls:	**Expected results**
Vibrio furnissii NCTC 11218	Good growth; yellow colonies
Vibrio parahaemolytics NCTC 10885	Good growth; green colonies
Negative control:	
Eschericia coli ATCC 25922	No growth

Reference:

1. **The Oxoid Manual**. 9th Edition 2006.

THIOGLYCOLLATE BROTH

1. DESCRIPTION

The use of thioglycollate broth permits growth of anaerobic and aerobic bacteria. No paraffin or special seal is required. An anerobic jar is also not required for the growth of anaerobes in this medium. It is well buffered so that acid or alkaline inocula produce negligible alteration in the reaction of the medium.

Thioglycollate broth contains sodium thioglycollate, a reducing agent that creates anaerobic conditions when it reduces molecular oxygen to water. Dyes such as resazurin or methylene blue are usually added to the broth to provide a visual indication of the presence of oxygen. Resazurin is pink when oxidized and colorless when reduced. Methylene blue is blue when oxidized and colorless when reduced. A pink/blue/blue band near the top of the broth results when oxygen diffuses in. Strict aerobes will grow only in the pink/blue band, microaerophiles will grow near the bottom of the band where the concentration of oxygen is lower. The absence of pink/blue in the rest of the tube indicates the absence of oxygen and a suitable environment for strict anaerobes. Both facultative anaerobes and aerotolerant anaerobes will grow throughout the tube; however, facultative anaerobes will grow most densely where oxygen is present.

2. METHOD

a) Best and most economically prepared using commercially available dehydrated medium.
b) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
c) Dispense the well mixed medium in 50 ml amounts in screw capped bottles with a central hole and rubber liner.
d) Sterilize by autoclaving (with caps loosened) at 121°C for 15 minutes.
e) When cool, tighten bottle caps.
f) Cover each bottle with a foil cap or other protective covering (previously soaked in 70% v/v ethanol).
g) Label the bottles.
h) Store in a cool dark place, preferably between 20^0C and 30^0C.
i) If at any time, more than a narrow band at the surface appears pink, this indicates oxidation and the medium should not be used. Reuse after carrying out procedure given below.
    - Place the bottle in a container of boiling water (with caps loosened) for 15 minutes to expel dissolved oxygen.

3. QUALITY CONTROL[2]

Positive controls*:*	**Expected results**
Bacteroides vulgatus ATCC 8482	Turbid growth; and/or colonies
Clostridium sporogenes ATCC 19404	Turbid growth; and/or colonies
Negative control:	
Uninoculated plate	No change

4. INSTRUCTIONS FOR USE

1. When used for inoculation of blood, ensure a 1/10 dilution.
2. **DO NOT SHAKE THESE TUBES.**
3. Gently label each Thioglycollate broth being careful not to tip the tube horizontally.
4. Using your inoculating loop, inoculate each Thioglycolate broth with assigned species.
5. Incubate the Thioglycollate broth at 37C for 24 - 48 hours.

References

1. Monica Cheesbrough. **Medical Laboratory Manual for Tropical Countries - Volume 11** Microbiology, 1984 (reprinted 1994). Butterworth-Heinemann
2. **The Oxoid Manual**. 9th Edition 2006.

CHRISTENSEN'S UREA BROTH

1. DESCRIPTION

Christensen's modified urea broth is a biochemical fluid medium used to test microorganisms for urease production. It can be prepared from a urea broth base obtained in dehydrated form from a commercial source. To this base, a sterile solution of urea is added.

If preferred, urea agar (prepared from urea agar base) can be used as a slope or stab. When used as a stab, only 1 ml of medium is needed, providing a narrow bore tube is used. A stab technique gives more rapid results.

2. METHOD AND PREPERATION

Formula and preparation (to make about 33 bottles)

Urea broth base 95ml
Sterile urea solution 40% w/v 5 ml
Use Membrane filters (pore size 0.45µm), placed in a Swinnex filter holder attached to a syringe to sterilize the urea solution .

a) Prepare urea broth base according to manufacturers instructions and sterilize by autoclaving.
b) Cool urea broth base to 50°C - 55°C and aseptically introduce 5 ml of sterile 40% urea solution.
c) Mix well and dispense aseptically, 3 ml amounts into sterile bijou bottles or screw capped tubes. Store in a cool dark place or at 2°C - 8°C.
d) Shelf life: up to 6 months providing there is no change in the volume or appearance of the medium to suggest contamination or alteration of pH (pH 6.6-7.0 at room temperature).

3. QUALITY CONTROL [1]:

Positive control:	**Expected results**
Proteus mirabilis ATCC 29906	Pink broth; urease positive
Negative control:	
Escherichia coli ATCC 25922	No colour change; urease negative

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

XYLOSE LYSINE DESOXYCHOLATE AGAR (XLD)

1. DESCRIPTION

A selective and differential medium for the recovery of *Salmonella* and *Shigella* species. It is low in nutrients and contains a small amount of sodium desoxycholate for selectivity. Relies on Xylose fermentation, lysine decarboxylation and production of H_2S for the primary differentiation of *Salmonellae* and *Shigellae* from non pathogenic bacteria. Most enteric organisms except *Shigella, Providencia* and *Edwardsiella* ferment xylose to produce acid. *Salmonella* also decarboxylate lysine which keeps the pH neutral or slightly alkaline. At this pH *Salmonella* species can produce hydrogen sulphide from the reduction of thiosulphate. This is indicated by ferric ammonium citrate producing black or black-centred colonies. Some organisms, such as *Citrobacter*, can also decarboxylate lysine. However, they ferment lactose and sucrose which keeps the pH too acid for hydrogen sulphide to be produced.

2. METHOD

a) Suspend the weight of the medium as specified by the manufacturer in 1 litre of distilled water.
b) Heat with frequent agitation until the medium boils.
c) Do not overheat.
d) Transfer immediately to water bath at 50^0C.
e) Pour into petri dishes as soon as medium has cooled. Pour about 20ml per plate.

3. COLONIAL CHARACTERISTICS

Organism	**Appearance**
Salmonella, Edwardsiella	Red colonies with black centres
Shigella, Providencia, H_2S negative *Salmonella*	Red colonies
Escherichia, Enterobacter, Klebsiella, Citrobacter *Proteus , Serratia*	Yellow, opaque colonies

Note : To enhance blackening of salmonella colonies,XLD agar should be incubated at 35^0C for 24 hours and in ambient air up to 48 hours.
Shigella dysenteriae and S*higella flexneri* may occasionally be inhibited on XLD agar.

4. QUALITY CONTROL[1]:

Positive control:	**Expected results**
Salmonella typhimurium ATCC 14028	Good growth; red colonies with black centre
Negative control:	
Escherichia coli ATCC 25922	No growth

Reference

1. **The Oxoid Manual**. 9th Edition 2006.

STAINS

ALBERT'S STAIN

1. DESCRIPTION

Used for staining metachromatic (volutin) granules of *C diphtheriae.* Laybourn's modification in which malachite green is substituted for methylene blue is described.
Make a smear from a culture on Loeffler Serum medium.

2. METHOD

Formula and preparation of reagents

a) Albert's staining solution

Malachite green	0.2 g
Toluidine blue	0.15 g
Ethanol (95%)	2 ml
Glacial acetic acid	1 ml
Distilled water	100 ml

Dissolve the dyes in the ethanol. Mix the acid with the water and add to the dye solution. Allow to stand for 24 hours and then filter.

b) Lugol's iodine

Iodine	5 g
Potassium iodide	10 g
Distilled water	100 ml

Dissolve the iodide and iodine in some of the water, and adjust to 100 ml with distilled water. For use dilute 1/5 with distilled water.

3. PROCEDURE

1. Fix the air dried smear by passing slowly through the flame three times.
2. Satin with Albert's stain for 3-5 minutes.
3. Wash with water and blot to dry.
4. Stain with Lugol's iodine solution for 1 minute.
5. Wash with water and drain or blot to dry.

4. INTERPRETATION

- Granules stain blue-black
- Cytoplasm stains green
- Other organisms stain light green

Reference

Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.

GRAM STAIN

1. DESCRIPTION

The Gram stain is used to classify bacteria on the basis of their forms, sizes, cellular morphology and Gram reactions. In a clinical microbiology laboratory, it is a critical test for the rapid presumptive diagnosis of infective agents and serves to assess the quality of clinical specimens. Several modifications of the original Gram stain have been proposed. The Preston and Morrell modification is given below.

2. METHOD

Formula and preparation of reagents

a) Ammonium oxalate-crystal violet

Solution A

Crystal violet	10 g
Ethanol (95%)	100 ml

Mix and dissolve

Solution B

Ammonium oxalate	1% aq. soln

For use, mix 20 ml of solution A and 80 ml of solution B. Filter the solution before use.

b) Lugol's Iodine

Iodine	5 g
Potassium iodide	10 g
Distilled water	100 ml

Dissolve the iodide and iodine in some of the water, and adjust to 100 ml with distilled water. For use dilute 1/5 with distilled water.

c) Acetone-iodine solution

Strong iodine solution

Iodine	10 g
Potassium iodide	6 g
Distilled water	10 ml
Ethanol (90%)	to 100 ml

Dissolve the iodine and potassium iodide in the water and adjust to volume with ethanol.

Acetone-iodine mixture

Strong iodine solution	3.5 ml
Acetone	96.5 ml

Mix well before use.

d) Dilute carbol fuchsin

Strong carbol fuchsin	50 ml
Distilled water	950 ml

(Strong carbol fuchsin –

Phenol	85g
Basic fuchsin	15g
Ethanol	250ml
Distilled water	1250ml

Mix the phenol and fuchsin and if necessary heat gently to dissolve the phenol. Add the ethanol and distilled water and filter into a stoppered bottle. Propanol may be substituted for ethanol.)

3. PROCEDURE

Preston and Morrells (1962) modified Lillies method

1. Fix the air dried smear by passing slowly through the flame three times.
2. Apply ammonium oxalate-crystal violet stain for 30 seconds
3. Wash off thoroughly with Lugol's iodine solution*
4. Apply Lugol's iodine for 30 seconds
5. Wash thoroughly with Iodine-acetone*
6. Apply fresh iodine acetone for 30 seconds
7. Wash thoroughly with water
8. Counter stain with dilute carbol fuchsin for 30 seconds**
9. Wash thoroughly with water and drain or blot dry

*Water can be used as a substitute for reagents in routine laboratories for washing purposes.

**Safranin is used as a counter stain for specific organisms. (Eg: *Neisseria gonorrhoeae*)

It is important to flood the whole slide with each reagent in turn and that previous reagent is thoroughly removed at each step.

4. INTERPRETATION

- Gram positive organisms are blue or purple
- Gram negative organisms are red or pink.

References

1. Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.
2. Murray PR, Baron EJ, Jorgensen JH, Pfaller AM, Yolken RH. **Manual of Clinical Microbiology**, 9th Edition 2007. ASM Press. Washington, DC.

METHYLENE BLUE STAIN

1. DESCRIPTION

This stain is useful to demonstrate beading and metachromatic granules of *Corynebacterium spp.* when grown on Loffler serum slopes. With sporing organisms spores appear as unstained bodies within blue cells.

2. METHOD

Formula and preparation of reagents

Loffler's methylene blue
Saturated ethanolic solution

Methylene Blue	1 g
Ethanol (95%)	100 ml

Staining solution

KOH 1% aq. soln	1 ml
Distilled water	99 ml
Ethanolic methylene blue	30 ml

Mix in order; this reagent must be ripened by oxidation, a process taking several months to complete, but ripening can be hastened by aeration. Bottles should not be more than half-full, the stopper replaced by a light cotton-wool plug and the bottle shaken frequently. The stain improves with keeping and batches sufficiently large to last for 5-10 years may be prepared. This ripened stain is called Polychrome methylene blue.

3. PROCEDURE

1. Fix the air dried smear by passing slowly through the flame three times.
2. Cover the slide with stain solution for 1minute.
3. Wash thoroughly with water.

Reference

Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.

ZIEHL-NEELSEN STAIN

1. DESCRIPTION

This method is a modification of Ehrlich's (1882) original method for the differential staining of tubercle bacilli and other acid fast bacilli. It incorporates modifications suggested successively by Ziehl and Neelsen. Ordinary aniline dyes do not readily penetrate the substance of the tubercle bacillus and are therefore unsuitable for staining. However, by the use of a powerful staining solution that contains phenol, and the application of heat, the dye can be made to penetrate the bacillus. Once stained, the tubercle bacillus will withstand the action of powerful decolourizing agents

2. METHOD

Formula and preparation of reagents

a) Strong carbol fuchsin

Phenol	85 g
Basic fuchsin	15 ml
Ethanol	250 ml
Distilled water	1250 ml

Mix the phenol and fuchsin and if necessary heat gently to dissolve the phenol. Add the ethanol and distilled water and filter into a stoppered bottle. Propanol may be substituted for ethanol.

b) Acid-alcohol

Conc. HCl	3 ml
Ethanol (95%)	97 ml

Mix well before use.

c) Loeffler's methylene blue

Saturated ethanolic solution

Methylene Blue	1 g
Ethanol (95%)	100 ml

Staining solution

KOH 1% aq. Soln	1 ml
Distilled water	99 ml
Ethanolic methylene blue	30 ml

Mix in order; this reagent must be ripened by oxidation, a process taking several months to complete, but ripening can be hastened by aeration. Bottles should not be more than half-full, the stopper replaced by a light cotton-wool plug and the bottle shaken frequently. The stain improves with keeping and batches sufficiently large to last for 5-10 years may be prepared.

3. PROCEDURE

1. Fix the air dried smear by passing slowly through the flame three times.
2. Flood the slide with strong carbol fuchsin and heat until steam rises (DO NOT BOIL).
3. After 3-4 minutes apply more heat until steam rises again; do not let the stain dry on the slide.
4. About 5-7 minutes after the first application of heat wash the slide thoroughly under running tap water.
5. Decolourize in acid-alcohol until all traces of red have disappeared from the film. Decolourization should not be attempted in one stage; there should be intermittent washings in water and re-application of acid-alcohol.
6. Wash well in water when decolourization is complete.
7. Counter stain with Loeffler's methylene blue or 0.5% malachite green for 1 minute.
8. Wash and stand on end to drain; DO NOT BLOT.

- Acid fast organisms are red; other organisms are blue or green depending on the counter stain.
- Do not use staining jars as tubercle bacilli from positive samples may become detached and float about in the staining fluid or decolourizing agent. This could cause false positive results.

Reference

Barrow GI, Feltham RKA. **Cowan and Steel's Manual for the Identification of Medical Bacteria**, 3rd Edition 1993. Cambridge University Press.

VIROLOGY

GUIDELINES FOR COLLECTION AND TRANSPORT OF BLOOD AND CSF FOR SEROLOGY

National Virus Reference Laboratory
Medical Research Institute, Colombo 8
Tel. 011 269 7280, 011 269 3532-4

1. Collection of blood

a) Detection of IgG
- Paired blood samples (acute & convalescent) 7-14 days apart are required to demonstrate a four-fold antibody rise/seroconversion.
- The acute serum sample should be obtained as soon as possible during the course of illness, and no later than 5-7 days after onset of illness.

b) Detection of IgM
- A single sample of serum should be collected after the 4^{th} day of illness.

General guidelines

a) 2-5 ml of venous blood should be collected into a labelled, clean (preferably sterile), dry, screw capped container.
b) The sample should be kept at room temperature for 30 min to 1 hour for the clot to form and retract.
c) The sample should then be sent to the hospital laboratory to be transported to the national reference laboratory.
d) All specimens should be accompanied with a duly filled request form. (Date of onset of illness, date of collection of sample should be included).
e) If a delay in transport for >48 hours is anticipated, serum should be separated aseptically, and stored at $+4^0$C.

2. Collection of CSF

- Collect 0.5-1 ml of CSF in a sterile screw capped container.
- Sample should be stored at $+4^0$C until transport.

3. Rejection criteria

- Leaking sample
- Contaminated sample
- No or improperly labelled sample
- Decomposed sample
- Haemolysed sample (blood)
- Samples unaccompanied by a request form
- Unmatching patient details on request form and label

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.
3. Do not discard samples which may be unrepeatable eg. CSF

4. Transport of specimens

- Blood/serum and CSF can be transported at room temperature if there is no delay in transport.
- If the sample has been stored at $+4^0$C, this temperature should be maintained during transport.

Note: The Medical Research Institute is open 24 hours for receipt of samples.

References

1. Steven Specter, Richard L Hodinka, Stephen A Young**. Clinical Virology Manual**, 3rd Edition 2002.
2. Douglas D Richman, Richard J Whitley, Fredrick G Hayden **Clinical Virology**, 2nd edition 2003.
3. Sirimali Fernando. **A Handbook on Collection and Transport of Specimens for Microbiological Investigations.** 2001.

GENERAL GUIDELINES FOR SAMPLE COLLECTION AND TRANSPORT FOR VIRUS DETECTION, ANTIGEN DETECTION, VIRUS ISOLATION AND MOLECULAR DIAGNOSIS

National Virus Reference Laboratory
Medical Research Institute, Colombo 8
Tel. 011 269 7280, 011 269 3532-4

1. **Virus detection by Electron Microscopy**

   - Rotavirus and other gastroenteritis viruses
   - Pox viruses

2. **Antigen detection**

   - Respiratory viruses [Adenovirus, Influenza virus A & B, Parainfluenza virus, Respiratory Syncitial Virus (RSV)]*
   - Herpes simplex virus*
   - Cytomegalovirus*
   - Rabies virus

3. **Virus isolation**

1) Respiratory*	**2) Ocular***	**3) Stools**
i) Influenza	i) Adenovirus	i) Eneterovirus
ii) Other resp. viruses	ii) Enterovirus	
iii) Rubella	iii) HSV	
iv) Measles		

4) CSF*	**5) Vesicle fluid***	**6) Blood***
i) Enterovirus	i) HSV	i) Dengue
ii) HSV	ii) Enterovirus	ii) Chikungunya

4. **Molecular diagnosis***

1) Respiratory	**2) Ocular**	**3) Stools**
j) Influenza	i) Adenovirus	i) Eneterovirus
ii) Other resp. viruses	ii) Enterovirus	
iii) Rubella	iii) HSV	
iv) Measles		

4) CSF	**5) Vesicle fluid**	**6) Blood**
i) Enterovirus	i) HSV	i) Dengue
ii) HSV	ii) Enterovirus	ii) Chikungunya
		iii) Enterovirus

*** Special tests**

General guidelines

- Samples should be collected within the first 4 days of illness .(Exception: AFP surveillance – Refer SOP for AFP surveillance)
- Samples should be collected into dry, sterile, screw capped containers. (Exception: AFP surveillance – Refer SOP for AFP surveillance)
- All samples should be collected into Viral Transport Medium (VTM). (Exception: Blood, CSF, Stools)
- VTM can be collected from Department of Virology, Medical Research Institute, Colombo 8.
- The samples should be transported within 48 hours of collection.
- Samples should be stored and transported at $+4^0$C in reverse cold chain box with frozen ice packs.
- The samples should be properly labelled with date of collection.
- The samples should be accompanied with duly filled request form.

Rejection criteria

- Leaking sample
- Visible contamination of sample
- Improperly collected sample
- Unlabelled samples
- Samples sent by post
- Specimen not transported in VTM (Viral Transport Media) & in ice
- Samples not collected during the acute stage (within 4-5 days of onset)
- Samples unaccompanied by a request form
- Unmatching patient details on request form and label

Note: If a specimen is unacceptable inform Microbiologist before rejecting.

1. If a specimen is rejected, a responsible individual must be notified immediately and request another sample of good quality.
2. Names of persons involved and action taken should be documented.
3. Do not discard samples which may be unrepeatable – eg. CSF

SPECIMEN COLLECTION

Respiratory specimens	**Nasal swabs** Insert flexible, fine-shafted swab into the post-nasal space. Rotate swab and let swab rest in place for several seconds to absorb secretions. Use separate swabs for each nostril. Place both swabs in the bottle of viral transport medium (VTM). **Throat swabs** Vigorously swab both tonsil areas and place swab in VTM. Use tongue depressor to depress tongue so that contamination of swab with saliva is prevented. **Note: Both nasal swabs and one throat swab from the same patient should be transported in one bottle of VTM**
	Nasopharyngeal aspirate (NPA) Using a mucous collection device (eg: disposable mucous extractor), insert an NG tube (size 8) into the posterior naso-pharynx. Apply suction, using intermittent suction as catheter is withdrawn. Wash aspirate through tubing using 2 ml of VTM. **Broncho alveolar lavage (BAL) and Tracheal aspirate** Place 3-5 ml in a sterile container with or without VTM. **Lung Biopsy (PM)** Obtain through intercostal space using a true cut biopsy needle as early as possible after death. For virus isolation - Collect in to 5-10 ml VTM For PCR - Collect in to 70 % alcohol
Ocular specimens	**Conjunctival swab** Swab lower conjunctiva with flexible, fine-shafted swab pre-moistened with saline. Place swab in VTM using aseptic method. **Corneal / conjunctival scrapings** Only obtained by Ophthalmologist or trained individual. Place scraping in VTM.
Vesicular fluid and skin scrapings	Prior cleaning of the site with disinfectants may inactivate the viruses. Aspirate vesicular fluid with a fine needle attached to a 2 ml syringe or a heparinized capillary tube. Rinse/aspirate with 2 ml of viral transport medium (VTM) in to sterile container. Swab open lesions to obtain both fluid and cells from the base of the lesion.
CSF	Collect 0.5-1.0 ml of CSF in to a sterile, screw capped bottle.

Note:
The Medical Research Institute is open 24 hours for receipt of samples.

ACUTE FLACCID PARALYSIS SURVEILLANCE AND ENTEROVIRUS ISOLATION

Tests available

1. Enterovirus isolation for CSF, stools, ocular swabs vesicular fluids, respiratory secretions

Polio Regional Reference Laboratory
Medical Research Institute, Colombo 8.
Tel. 011 269 3532-4 (Ext. 435),
011 2697280

2. Polio virus isolation from stools of AFP patients and typing and intra - typic differentiation by Real Time PCR and Real Time VDPV screening PCR.

SPECIMEN COLLECTION FOR AFP SURVEILLANCE

Required Specimens and optimal time for collection	• Collect 8-10g of stools (size of 2 tamarind seeds) into a wide mouthed, leak-proof, externally threaded, screw capped, clean, dry container (Containers are available at the Infection control units of hospitals). • 2 stool samples should be collected at least 24 hours apart within 14 days of onset of paralysis. • The samples should be labelled with dates and times of collection in addition to the relevant patient information. A repeat sample will be required if the original sample is not suitable for processing.

SPECIMEN COLLECTION FOR ENTEROVIRUS ISOLATION

Required Specimens and optimal time for collection	• The sample should be collected within 4 days of onset. • All samples except stools and CSF should be collected into VTM.

REJECTION CRITERIA

Refer to general guidelines for sample collection and transport for virus detection.

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	• The sample should be stored at $+4^0$C until transport. • Send to Regional Reference Laboratory packed inside the reverse cold chain box with frozen ice packs, within 3 days of collection

REPORTING

AFP surveillance	Reports will be available from 21 days after arrival of sample at the laboratory.
Enterovirus isolation	Reports will be available 14 days after receipt of sample.

DIAGNOSIS OF CMV / EBV / MEASLES / MUMPS / VARICELLA ZOSTER VIRUS INFECTIONS

National Virus Reference Laboratory
Medical Research Institute, Colombo 8
Tel. 011 269 7280, 011 269 3532-4

Tests available

1. CMV - IgM & IgG antibody (EIA)
2. EBV - IgM & IgG antibody (EIA)
3. Measles - IgM & IgG antibody (EIA)
4. Mumps - IgM & IgG antibody (EIA)
5. VZV - IgM & IgG antibody (EIA)
6. CMV antigenaemia test* - (DFT)

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	• 3-5 ml of blood in a dry, sterile container or 2-3 ml of serum in a sterile container after 3-5 days of onset of symptoms • Congenital CMV infection – cord blood or blood sample within 3-5 days of birth

SPECIMEN TRANSPORT AND STORAGE

Time between specimen collection and processing	Refer to guidelines for collection and transport of blood and CSF for serology. **Full Clinical details essential.** • Immunization history should be mentioned in requests for IgG (VZV, Measles, Mumps) • Immunodeficient or immunosuppression state (especially in post-transplant patients) – VZV, CMV

REJECTION CRITERIA

Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Test		Time for results
CMV	IgG/IgM	Up to 7 days
EBV	IgG/IgM	Up to 7 days
Measles	IgG/IgM	Up to 7 days
Mumps	IgG/IgM	Up to 7 days

Tests will be performed depending on the availability of test kits.

*CMV antigenaemia test (Special test)
The virologist should be contacted for a date for testing. The blood sample should be collected in to a container of lysis buffer which is available at the Department of Virology, MRI. The test is performed only for immunocompromised patients.

DIAGNOSIS OF DENGUE INFECTION

National Dengue Reference Laboratory
Medical Research Institute, Colombo 8
Tel. 011 269 3532- 4 (Ext. 462),
011 2697280

Tests available

1. IgM detection (EIA)
2. IgG detection (Haemagglutination Inhibition test HAI)
3. Antigen detection
4. Virus isolation*
5. Genome detection by PCR*

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	3-5ml of blood or 2-3 ml of serum in a sterile container. 1. Serum for antibody tests • Single sample for IgM / IgG – collected after the 5th day of illness • Paired serum samples for IgM / IgG – acute and convalescent samples collected 10 - 14 days apart 2. Antigen detection/virus isolation/PCR* • Acute samples collected within 4 days of illness

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	Refer to general guidelines for sample collection and transport for virus isolation & PCR. Refer to guidelines for collection and transport of blood and CSF for serology.

REJECTION CRITERIA

Refer to general guidelines for sample collection and transport for virus isoaltion.
Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Test	Time for results
IgG** (HAI test)	Up to 7 days
IgM** (EIA test)	Up to 7 days

* Special tests – The virologist has to be contacted before sending samples.

** Both are batch tests carried out once a week.

DIAGNOSIS OF VIRAL HEPATITIS

National Hepatitis Reference Laboratory
Medical Research Institute, Colombo 8
Tel. 011 269 7280, 011 269 3532-4

Tests available

Hepatitis A	1) IgM antibody (EIA)		
	2) IgG antibody (EIA)		
Hepatitis B	1) Hepatitis B surface antigen	–	HBsAg (RPHA, EIA)
	2) Hepatitis B surface antibody	–	HBsAb (PHA, EIA)
	3) Hepatitis B core (total) antibody	–	HBcAb (EIA)
	4) Hepatitis B core (IgM) antibody	–	HBcAb (EIA)
	5) Hepatitis B e antigen	–	HBeAg (EIA)
	6) Hepatitis B e antibody	–	HBeAb (EIA)
Hepatitis C	1) Hepatitis C antibody (PA, EIA)		
Hepatitis E	1) IgM antibody (EIA)		
	2) IgG antibody (EIA)		

All these serological tests are performed using commercial test kits. The manufacturer's instructions should be followed when performing the test.

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	3-5 ml of blood in a dry, sterile container or 2-3 ml of serum in a sterile container

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	• Refer to general guidelines • **High Risk label should be affixed on to container and request forms of known HIV or hepatitis B positive patients**

REJECTION CRITERIA

Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Test	Time for results
HAV IgM	Up to 7 days
HBsAg	Up to 7 days
HbsAb	Up to 7 days
HCV antibody	Up to 7 days
Hepatitis B core (total, IgM) antibody	Varies depending on availability of test kits
HBeAg, HBeAb	Varies depending on availability of test kits
Hepatitis E IgM and IgG	Varies depending on availability of test kits
Hepatitis A IgG	Varies depending on availability of test kits

* All tests are batch tests and are carried out once a week.

DIAGNOSIS OF HIV 1 AND HIV 2 INFECTION

Tests available

1. HIV 1 & 2 antibody (EIA, PA, rapid tests)
2. HIV-1 antigen detection*
3. CD4/CD8 counts*
4. Viral load assay*

Reference Laboratory for STD & HIV
National STD/AIDS Control Programme,
Colombo 10.
Tel. 011 266 7163

Validated EIA is the preferred method for detection of HIV-1 & 2 antibody. These tests are performed using common test kits. The manufacturer's instructions should be followed when performing the test.

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	3-5 ml of blood in a dry, sterile, screw capped container (preferably in a vacuum tube) Sample should be collected 6 months after a suspected exposure.

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	• Refer to general guidelines • **High Risk label should be affixed on to container and request forms of known HIV or hepatitis B positive patients.**

REJECTION CRITERIA

Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Screening tests are performed daily. Reports will be available 1-2 days after receipt of sample.

CONFIRMATORY TESTING

- When screening test is positive a second sample should be sent to the National Reference Laboratory with duly completed Health 1214 form for confirmation of results.
- Confirmatory tests will be performed once a week and the report will be available on the same day.

Special tests

- Other special tests such as antigen detection tests, CD4 counts and viral load assay will be performed only with prior arrangement with the reference laboratory.

* According to availability of test kits.

DIAGNOSIS OF JAPANESE ENCEPHALITIS INFECTION

National JE Reference Laboratory
Medical Research Institute, Colombo 8.
Tel. 011 269 7280, 011 269 3532-4

Tests available

1. CSF/serum for IgM antibody

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	1. Serum for antibody test (IgM) – after 5th day of illness 2. CSF for antibody test (IgM) – after 5th day of illness

SPECIMEN TRANSPORT AND STORAGE

Storage and transport	**Refer to general guidelines** collection and transport of blood and CSF for serology. **Relevant clinical details and details of JE vaccination is necessary.**

REJECTION CRITERIA

Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Test	Time for results
JE - IgM*	Up to 10 days

* IgM assay is a batch test carried out once a week.

DIAGNOSIS OF RESPIRATORY VIRAL INFECTIONS

Tests available

1. Direct fluorescent test (DFT) for respiratory viral antigen detection (Influenza A&B, Adeno virus, Parainfluenza & Respiratory Syncitial Virus)
2. Virus Isolation, typing & subtyping
3. Molecular testing, typing & subtyping

National Respiratory Virus Reference Laboratory
Medical Research Institute, Colombo 8
Tel: 011 269 3532-4 (Ext. 462),

SPECIMEN COLLECTION

Required specimens and optimal time for collection	**Refer to general guidelines on virus isolation** Nasal swabs Throat swabs Nasopharyngeal aspirate (NPA) Broncho-alveolar lavage (BAL) Tracheal aspirate Lung Biopsy (PM) **Samples should be collected within first 4 days of illness**

REJECTION CRITERIA

Refer to general guidelines for sample collection and transport.

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	Refer to general guidelines

REPORTING

Respiratory viral Ag detection by DFT	Within 1-2 days of arrival of sample at the laboratory
Virus Isolation, typing & sub typing	2-3 weeks
Molecular typing	Carried out as a batch test. Results available in 1-5 days

DIAGNOSIS OF FOR RUBELLA INFECTION

Tests available

1) IgM antibody (EIA)
2) IgG antibody (HAI)
3) Virus isolation

National Rubella Reference Laboratory
Medical Research Institute, Colombo 8.
Tel: 011 269 3532-4 (Ext. 462),
011 269 7280

SPECIMEN COLLECTION

Required Specimens and optimal time for collection	1. Fever and rash (Acute infection) • IgM detection – blood sample collected 4 days after onset of rash • IgG detection – paired blood samples collected 10-14 days apart 2. CRS (Always tested with maternal serum) – immediately after delivery • Baby's serum (or cord blood) – IgM • Mother's and baby's serum – IgG Virus isolation – throat swab, naso-pharyngeal aspirate, blood sample – collected within 4 days of onset of illness.

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	**Full Clinical details essential – eg. pregnancy** **A) Rash in a pregnant woman / pregnant woman in contact with person with suspected rubella** • Period of gestation • Date of onset of rash / date of exposure to rubella • Date sample collected • Vaccination history **B) Congenital Rubella Syndrome (CRS)** • H/O fever and rash in mother • H/O rubella vaccination in mother • Clinical features suggestive of CRS

REJECTION CRITERIA

Refer to general guidelines for sample collection and transport for virus detection.
Refer to guidelines for collection and transport of blood and CSF for serology.

REPORTING

Test	Time for report
IgG (HAI)	Up to 7 days
IgM **(**EIA)	Up to 7 days

DIAGNOSIS OF RABIES INFECTION

Tests available

National Rabies Reference Laboratory
Medical Research Institute, Colombo 8.
Tel. 011 269 8660, 011 269 3532-4
Ext 131, 141

1. Direct smear (DS) - Sellar's stain
2. Fluorescent antibody test (FAT)
3. RT/PCR ,immunochromatography test (ICT) and mouse inoculation test (MIT) done when indicated
4. Anti-rabies antibody in serum /CSF (only with prior arrangement)

SPECIMEN COLLECTION

Optimal time of specimen collection	1 antemortem 2-3 post mortem
Specimen	1. Serum / CSF – anti-rabies antibody 2. Post mortem brain (human) – Sellar's stain, FAT, RT/PCR, ICT,MIT 3. Head of suspect animal – Sellar's stain / FAT, RT/PCR, ICT, MIT

SPECIMEN TRANSPORT AND STORAGE

Transport and storage	
Human brain	Fresh brain as a whole without preservatives to be transported. The specimen should be packed in ice in a leak proof container and sent with the request form. Relevant clinical history is essential.
Animal head	If transported more than 8 hours after death, the specimen should be packed in ice and transported in a leak proof container. A short history would be obtained at the time of receipt of specimen.

Note:
MRI is open 24 hours, for receipt of specimens.

REJECTION CRITERIA
Refer to general guidelines for sample collection and transport.
Full animal carcass

REPORTING.

TEST	RESULT	TIME FOR RESULT
Human brain (FAT)	Positive /negative for rabies antigen	Within 72 hours
Animal head	Direct smear (DS): Negri bodies positive / Inconclusive If DS inconclusive – FAT done - Positive /Negative for rabies antigen	Within 24 hours Within 72 hours
CSF /serum antibody	Antibody titre	Discuss with Virologist/Dept. of rabies

References

1. Meslin FX, Kaplan MM, Koprowski H. **Laboratory techniques in rabies**. WHO 1996. 4th edition. page 271 – 77

2. WHO Expert committee on Rabies. **WHO Technical Report Series**. 8th report. 1992

3. WHO first report of the expert consultation on rabies 2004. **WHO Technical Report Series.** 931, 2005

MYCOLOGY

STANDARD OPERATING PROCEDURES FOR THE INVESTIGATION OF FUNGAL INFECTIONS

COLLECTION AND TRANSPORT OF SPECIMENS

Mycology Reference Laboratory
MRI, Colombo 8
Tel: 0112 698725

SUPERFICIAL MYCOSES

a) Skin	• Clean area with 70% alcohol, sterile saline or distilled water. • Scrape the edge of the lesion, with blunt scalpel and collect material onto clean paper or slide. • When there is minimal scaling as in Pityriasis, clear sticky tape (cellotape) is used to remove material by pressing the sticky side onto lesion and then placing it sticky side down on a slide containing a drop of lactophenol cotton blue or Parker's stain. • In moist lesions, a swab or pieces of skin can be taken with forceps.
b) Hair	• Scalp should be scraped with a blunt scalpel to obtain hair stubs and skin scales. Hairs should also be plucked with intact roots using forceps and transported in paper or between two slides. • Piedra – pieces of hair with nodules should be collected.
c) Nails	• Clippings should be taken from discoloured, brittle or dystrophic nails, by cutting as far back as possible. Scrapings of nails and sub ungual debris should be collected.
d) Mucous membranes	• Take scrapings with a plastic / wooden spatula from epithelial surfaces of vagina or mouth or a high vaginal swab. • Take scrapings from cornea with a sterile plastic or wooden spatula.
e) Eye specimens	• Immediately transfer material at the bed side, to two Sabouraud's glucose agar (SGA) slants or plates and to a glass slide for microscopy. • Corneal buttons removed during keratoplasty can be sent in a sterile bottle without additives. • Intra ocular fluid, vitreous humour when collected should be sent in a sterile bottle without additives.
f) Ear specimens	• Scrapings from external auditory meatus are preferred to a swab. • Transfer material onto a slide or paper. • May be transported by post, if necessary.

Transport - Skin, hair and nail specimens can be sent by post.

SUBCUTANEOUS MYCOSES

g) Pus	• Collect dried crusts into a folded square paper, sterile petri dish or sterile bottle. Pus from undrained subcutaneous abscesses or sinus tracts or grains is collected aseptically with a sterile needle and a syringe.
h) Biopsy specimens	• Biopsies from chronic ulcers, sinuses and subcutaneous lesions are preferred to swabs containing pus. • Specimens should be taken from deep within the lesion as close as possible to healthy tissue. • Place one tissue sample in a sterile bottle containing sterile normal saline and the other in formol saline and send to the laboratory. • Refrigerate the sample in sterile normal saline if there is a possible delay in transport, to prevent decomposition.

SYSTEMIC MYCOSES

i) Abscesses, ulcers (skin)	• Pus from undrained abscesses or expressed pus is collected aseptically with a sterile needle and syringe and transported as such. • Biopsy specimens are taken from ulcerated lesions of skin and mucosa. For collection and transport see "h" above.
j) Blood • Requires biphasic blood culture medium • Contact Mycology Reference Laboratory	• 5-10 ml of venous blood is collected. • Inoculate the blood into biphasic blood culture bottle under aseptic conditions. The lid with a hole in the center should be wiped with 70% alcohol before inserting the needle to inoculate the blood. • Mix well but do not shake vigorously and keep at room temperature. • Smaller volumes of blood from neonates are collected into paediatric bottles (0.5-1ml).
k) Bone marrow	• 2-3 ml of bone marrow aspirate is placed in a sterile container with 0.5 ml of 1:1000 sterile heparin. • Send within 24 hours to the laboratory.
l) CSF	• 0.5-1ml of CSF is collected into a sterile screw capped bottle.
m) Fluids	• Pleural, abdominal and synovial fluids are collected aseptically into a sterile bottle containing 1:1000 sterile heparin in a ratio of 10:1. • Drain fluid from patients on continuous peritoneal dialysis should be collected into a sterile screw capped container **without heparin.**

n) Urine **catheterised patients**	• Urine is collected into a sterile bottle supplied by the laboratory. • Procedure of collection is the same as for bacterial cultures- patient is advised to collect a mid stream sample. • Aspirate urine via a sterile needle and a syringe after clamping the catheter for 2-3 minutes. • Transport as quickly as possible to the laboratory or refrigerate at 2°C - 8°C.
o) Broncho alveolar lavage fluid (BAL) & bronchial brush specimens	• More suitable than sputum. • Collect into sterile screw capped bottles supplied by the laboratory.
p) Sputum (Only to be processed if BAL is not available)	• Three consecutive early morning expectorated samples of sputum or a specimen taken with the aid of physiotherapy. • Collect into sterile bottle and transport with minimum delay.
q) Blood for serology	• 2 ml of blood is collected into a plain bottle.

SPECIMEN RECEIPT AND MAINTAINING RECORDS

Receiving of specimens	1. A designated area outside the laboratory should be allocated for receipt of all specimens.
Maintenance of specimen records	2. All specimens received for fungal studies are accepted by a MLT/MLA, and should be recorded in a separate register with details such as date, type of specimen, patient details, clinical diagnosis and test required. 3. Those that can be processed in the laboratory should be identified, a special number given and sent to the laboratory. The same number should be exhibited clearly on the sample as well. 4. Those that have to be referred to another laboratory should be forwarded to a separate area and such details should be entered in the register**.**

Example of the register entries

Serial no.	Date of receipt	Specimen	Spec. no.	Test requested	Patient details			Processed in house or referred	Hospital referred to & date of dispatch
					Age /sex	Name BHT no.	Clinical history		

Storage of specimens	• Biopsy specimens in sterile normal saline should be stored at 2^0C-8^0C till processing is done to prevent decay. • Scrapings and specimens from superficial sites can be kept at room temperature till processed.

References

1. **Guidelines on Standard Operating Procedures for Microbiology**, Mycological Techniques - Chapter 21, <http://www.who.com, > (10/12/2009).
2. **Mycology sample collection**, Leeds & Bradford Pathology Partnership <http://www.nhs.com> (last updated on 10/12/2009).

LABORATORY PROCEDURE OF TESTS THAT CAN BE UNDERTAKEN AT TEACHING, PROVINCIAL AND GENERAL HOSPITALS

SUPERFICIAL MYCOSES

MICROSCOPY

Skin	• Transfer a portion of scrapings in the paper packet on to a clean glass slide. • Add 1-2 drops of 10% potassium hydroxide (KOH) * (appendix I) to scrapings on the slide and apply a cover slip. • Keep for 15 minutes at room temperature (on the bench), and press down on the cover slip with the help of a filter paper to make a thin layer of the specimen. • Remove the extra KOH on the slide by blotting with filter paper. • Examine initially under x10 magnification with reduced illumination. • Any suspicious area is re-examined under x40 magnification. • Look for fungal filaments, spores and budding yeasts.
Hair	• Keep the specimen on a glass slide. • Add 1-2 drops of 10% potassium hydroxide (KOH) * (appendix II) on to the specimen on the slide and apply a cover slip. • Keep for 2-3 minutes at room temperature (on the bench) and without pressing down the cover slip, remove extra KOH on the slide by blotting with filter paper. • Examine the hair follicle initially under x10 without pressing down the cover slip. • If fungal spores are not seen, examine after pressing down very lightly on the cover slip and re-examine to look for spores and hyphae.
Nails	• The nails should be cut into very small pieces under sterile conditions. • After addition of 10% KOH to small pieces of nail and debris, place the slide in incubator at 35^0C-37^0C for 30 minutes or keep on bench for 1-2 hours. • Blot excess KOH with a filter paper. • Firmly press down on the cover slip and examine as for skin scrapings. **Note:** For nails and skin 30% KOH can be used with less exposure time.

CULTURE

Skin, hair and nail specimens	• Inoculate Sabouraud's glucose agar (SGA) with chloramphenicol and gentamicin (appendix II) and SGA with chloramphenicol, gentamicin and cyclohexamide (actidion) (appendix II) incorporated into it. • Incubate at 26^0C or room temperature for up to 2 weeks.

IDENTIFICATION

Candida and dermatophytes	• Examine for growth from day 2 onwards to detect Candida. • To detect dermatophytes examine cultures from day 5 onwards, every other day. • If a contaminant is seen and likely to overgrow the pathogen, sub-culture the likely pathogen, onto another appropriate culture media. 1. When a filamentous fungal growth is detected, the culture is identified as follows: a. Appearance of the colony eg. colour of obverse and reverse sides, texture, grooves and furrows, adherence to the medium etc. b. A teased mount preparation is done by picking up a small portion from the mid area of the colony, and teasing it in a drop of Lacto-phenol cotton blue (appendix II) on a slide. Apply cover slip and examine under microscope x10 and x 40 for morphology. c. Slide culture only for moulds (appendix III). 2. Cultures of Candida are identified by performing following tests a. Lacto phenol cotton blue mount. b. Germ tube test (appendix III). c. If negative, the microscopic morphology is visualized by growing the culture on corn meal or rice agar plates (appendix IV). d. Sugar assimilation and fermentation tests (in the reference laboratory-MRI).

REPORTING

PRELIMINARY REPORT

MICROSCOPY:	**KOH mount**	
	Fungal filaments / spores	seen / not seen
	Budding yeasts / pseudohyphae	seen / not seen
CULTURE:	**Report will be available in two weeks**	

FINAL REPORT

MICROSCOPY: **KOH mount**

Fungal filaments / spores	seen / not seen
Budding yeasts / pseudohyphae	seen / not seen

CULTURE: Dermatophyte isolated / not isolated
Species: ……..

Yeast isolated / not isolated
Species: *Candida albicans* / Non albicans Candida species

TESTS UNDERTAKEN AT REFERENCE LABORATORY

Mycology Section
MRI, Colombo 8
Tel: 0112 698725
	• Identification of fungal isolates referred from other laboratories.
	• Microscopy and culture of swabs, tissues, biopsy specimens & body fluids from,
	  o Fungal keratitis, Otomycosis
	  o Mycetoma , Sporotrichosis, Chromoblastomycosis , Rhinosporiodiosis etc.
	  o Aspergillosis, Mucormycosis, Invasive candidiasis, Cryptococcosis, Histoplasmosis etc.
	• Identification of isolates and determining their significance in relevance to the clinical syndrome.
	• Histo-pathology for identification of fungi.
	• Serological studies of Candida, Aspergillus, Histoplasma, Blastomyces and Coccidioides infections.
	• Immunological studies of fungal infections.
	• Molecular studies on Candida species.

APPENDIX I – EQUIPMENT

Minor	• Banana scalpels • Inoculating needles
Major	• Incubators– low temperature 26 ^{0}C (optional) & 37^{0}C

APPENDIX II – STAINS, REAGENTS AND MEDIA

STAINS

1. Lactophenol cotton blue

- Lactophenol: 20 g of phenol crystals, 20 ml lactic acid, 40 ml of glycerol and 20 ml of distilled water. Heat gently to dissolve. Keep in a dark bottle away from direct light.
- Lactophenol cotton blue: 100 ml of lactophenol and 0.075 g of cotton blue. Allow to stand for a few days and filter.

2. Parkers stain

- Equal volumes of Parkers Quink (blue black) and KOH reagents are mixed together.

REAGENTS

1. Potassium hydroxide

- Potassium hydroxide in concentrations varying from 10 – 30% is normally used.
- (For 10% KOH -Potassium Hydroxide crystals 20 g and distilled water 200 ml, dissolve and use).

MEDIA

1. Sabouraud's Glucose agar (SGA) with antibiotics

In house method:

Peptone (mycological)	10 g
Glucose	40 g
Agar	20 g
Distilled water	1 litre

Chloramphenicol 50 mg is added to media prior to autoclaving[1].

SGA is also available in dehydrated form commercially.

SGA (Oxoid)	65 g
Chloramphenicol	250 mg
Gentamicin (40 mg/ml)	0.65 ml
Distilled water	1000 ml

- Dissolve all ingredients in 100 ml of water. Once dissolve add remaining water and bring to boil while stirring. Autoclave at 121^0 C for 10 minutes and dispense to slopes or plates.

2. Sabouraud's Glucose agar (SGA) with cyclohexamide

- Add 500 mg of Cyclohexamide (Actidion) to 1 litre of medium prepared as above before autoclaving.

APPENDIX III – STANDARD OPERATING PROCEDURES FOR TESTS UNDERTAKEN IN THE LABORATORY.

1. Slide culture	• A Petri dish containing a microscope slide, two cover slips and a bent glass rod support and small filter paper is autoclaved. • At the time of preparing the slide culture, the slide is placed on the glass rod support which is kept on the filter paper. • With a sterile needle or scalpel blade cut a small square of solid Sabouraud's glucose agar medium, measuring 0.75 - 1cm. • Transfer aseptically the agar block on to the center of the slide in the Petri dish. • Inoculate the center of each side of the agar block with the fungus by means of a sterile needle. • Place the sterile cover slip on top of the inoculated agar block. • Add adequate sterile water to soak the filter paper in the Petri dish, to ensure that the agar block does not dry out. • Incubate at 25^0C – 30^0C for two-weeks (for dermatophytes). If mycetoma is suspected incubate for 3-4 weeks. • Add more sterile distilled water if required. • When sufficient growth has occurred, remove the cover slip, and place it on the bench with the growth upwards. Add a drop of absolute alcohol to the center and allow it to spread outwards to dissipate air from the fungal growth and allowed to evaporate. • Add a drop of lacto phenol cotton blue to the center of cover slip and place a new clean glass slide over it and turn over. • Examine microscopically x10 and x40. • The agar block can be removed from the original slide, and this slide can be used to make a second preparation (as above).
2. Germ tube test	• This test provides a rapid and presumptive identification test of 95% – 97% of *Candida albicans*. • It is carried out on primary isolates or purified cultures. • Place 0.5 ml of serum (human, fetal calf or horse) in a small test tube. • Emulsify a small portion of the yeast colony to be tested in the serum. • Incubate at 37 ^{0}C, for 2 hours. • Remove a drop of serum onto a slide, place a cover slip and examine (x10 or x40) microscopically for germ tubes, which appear as cylindrical filaments originating from blastospores, without any constriction at point of origin and without obvious swelling along the length of the filament.

APPENDIX IV – PREPARATION OF CORN MEAL AGAR AND RICE AGAR

1.Cornmeal agar	Commercially available. Prepare according to manufacturer's instructions.
2.Rice agar	Rice 20 g Tween 80 (40 or 20) 10 g Agar 10 g Distilled water 1000 ml • Boil rice in a beaker for 45 minutes and filter the solution with a piece of gauze. • Pour it to a measuring cylinder and top up with distilled water up to the 1000 ml mark. • Weigh Tween 80 and agar and put into a 2000 ml flask. • Add the above mixture to the same flask and boil until everything is dissolved. • Pour into tubes with screw cap lids and sterilize by autoclaving at 121^0C for 15 minutes. • Store at 4-8^0C. • When required re-heat in beaker with water and pour to petri dish.

Reference

Evans EGV, Richardson MD, **Medical Microbiology a practical approach.** 1989 . page 50. Oxford University press England.

PARASITOLOGY

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF FAECES FOR PROTOZOAN INFECTIONS

Type of specimen: Faeces

Introduction

This SOP describes the examination of faeces for the presence of cysts and trophozoites of *Entamoeba histolytica, Giardia intestinalis* and oocysts of *Cryptosporidium* species. Trophozoite stages are most often found in diarrhoeic faecal specimens and cysts are typically found in formed faecal specimens. Several other protozoans that are generally considered as non pathogens can also be seen in faeces. If any unusual protozoans are seen please contact a reference laboratory.

SPECIMEN COLLECTION

1. Patient should be provided with a suitable container for specimen collection such as an 80 or 100 ml wide-mouthed plastic cup with tight fitting, leak-proof lid and spoon (such as those used to sell ice-cream or yoghurt).
2. Patient should be instructed to defecate onto a paper or a large leaf such as a banana leaf (that may be disposed of later). A small quantity of faeces (at least half a teaspoon) should be transferred *immediately* into the container using the spoon. Remaining faeces should be disposed of in a sanitary latrine.
3. Faeces should not be mixed with urine or dirt.
4. The container should be labeled clearly with the patient's name, date of collection and the time the patient passed faeces.

SPECIMEN TRANSPORT AND STORAGE

1. Specimens should be transported as quickly as possible to the laboratory, especially if patient has diarrhoeic faeces.
2. Transport specimens at room temperature. Refrigeration may cause changes in appearance of protozoan cysts and trophozoites.
3. If protozoan cysts or trophozoites are seen in wet smears of a faecal sample, confirmation of diagnosis may be obtained from a reference laboratory after examination of permanent, stained smears. In order to do so, a fresh specimen of faeces should be preserved in two vials, one containing polyvinyl alcohol (PVA) and the other containing 10% formalin. [Vials containing PVA can be obtained from a reference laboratory on request.]

SPECIMEN PROCESSING

Specimens should be examined as soon as possible after defaecation. If a number of specimens are received at the same time, first examine specimens containing mucous or blood as they may contain motile trophozoites of *Entamoeba histolytica*. Motile trophozoites are seen only in wet saline smears of specimens examined within 30 minutes of passing stools. [Wet films can be warmed slightly to stimulate motility of non-motile trophozoites.]

A. Wet smears in saline and iodine

1. The specimen should be examined microscopically using saline & Lugol's iodine wet mounts prepared directly from the faecal sample. Write the patient's name or number on one end of the microscope slide, using a marker pen or wax pencil

2. Place a drop of saline in the centre of the left half of the slide and place a drop of Lugol's iodine solution in the centre of the right half of the slide.
3. With an applicator stick, pick up a small portion of the faecal specimen and FIRST mix with the drop of saline. Pick up another small portion of faeces and mix with the drop of iodine.
   In patients with dysentery, make smears from 'suspicious looking' areas of the sample containing blood or mucous.
4. Cover each drop with a coverslip. Hold the coverslip at an angle, touch the end of the drop and lower gently onto the slide, to reduce the chance of including air bubbles in the mount.
5. Examine the slide initially using the low power (x10) objective, with reduced illumination. Any suspicious object should be re-examined using the high power (x40) objective.
6. Look for cysts and trophozoites of *Entamoeba histolytica/ Entamoeba dispar* and *Giardia intestinalis*. Focus the objective on the top left-hand corner of the coverslip and move the slide systematically backwards and forwards or up and down so that the entire coverslip area is examined.

IDENTIFICATION

For identification of protozoa, especially cysts, size is very important as otherwise the morphology could be similar (Refer Figure 1- page 220). Ideally the microscope should have a calibrated eyepiece. If this is not available make an assessment of the size based on the size of a red cell (7.5µm) under the same magnification or a common faecal finding such as a hookworm or round worm ova.

Trophozoites- Saline smear is useful for identification of trophozoites. Apart from size and shape the characteristic motility helps in identification. Amoebic trophozoites, unlike those of flagellates, are fragile and as such *E. histolytica* trophozoites can be identified with certainty only in a fresh faecal sample in saline when the characteristic directional motility with rapidly thrusting, clear, finger-like pseudopodia is seen. The nucleus is small and ingested red cells may be seen as refractile bodies. The trophozoites of *G intestinalis* have a tumbling down motility like falling leaves. (Refer Figure 2- page 220) – Trophozoites in saline smears

Note: Macrophages also can show sluggish 'amoeboid' movement but their nucleus is proportionately much larger.

REPORTING

The report should identify the parasite(s) seen, by scientific name, indicating whether it is the cyst, trophozoite or oocyst stage which was identified.

<u>*Entamoeba* species</u>

Cysts of *E. histolytica* and *E. dispar* are indistinguishable. Therefore *Entamoeba* cysts and trophozoites without ingested red blood cells should be reported as *E. histolytica* / *E.dispar*. Only trophozoites with ingested red blood cells, should be reported as *E. histolytica.*

<u>*Blastocystis hominis*</u>

Report should indicate whether the numbers seen are light/moderate/ heavy.

<u>Non-pathogenic protozoa</u>

Protozoa considered as nonpathogenic such as *Entamoeba coli, Entamoeba hartmanii, Endolimax nana* and *Iodamoeba butschlii* should also be reported as their presence in stool indicates fecal contamination of food or water.

B. Modified Ziehl-Neelsen stain for identification of Cryptosporidium, Cyclospora and other coccidian oocysts

1. Prepare a thin smear of faeces [3cm x 1cm] by rolling a wooden applicator stick [ekel] smeared in faeces lengthwise along a glass slide to give thin and thick areas.
2. Air dry
3. Fix in methanol for 3 minutes.
4. Stain with cold strong carbol-fuchsin for 15 minutes.
5. Differentiate in 1% HCl – 95% ethanol until colour ceases to flow out of the smear. [about 20 seconds]
6. Rinse in tap water.
7. Counterstain with 0.5 % malachite green [or Loeffler's methylene blue] for 1 minute.
8. Rinse in tap water.
9. Air dry and examine under oil immersion lens (x100)

Cryptosporidium oocysts are oval to round (diameter 4-6µm) and are acid–fast, irregularly staining pink to red. *Cyclospora cayetanensis* oocysts are larger (diameter 8-10µm). (Refer Figures 8 & 9 page 223)

Note: About half of all *Cyclospora* oocysts do not take the stain.

C. ***Formol-ether sedimentation technique***

1. Using a rod or stick, emulsify about 1 g (pea size) faeces in 4ml of 10% formalin in a screw capped bottle or tube.
   Note: include in the sample, faeces from the surface and several places in the specimen.
2. Add a further 3-4ml of 10% formalin, cap the bottle/tube and mix well by shaking.
3. Sieve the emulsified faeces using a nylon tea strainer/ 2 layers of gauze and collect the sieved suspension in a beaker.
4. Transfer the suspension to a conical centrifuge tube.
5. Add 3-4ml of diethyl ether or ethyl acetate.
   Caution: Ether is highly flammable and ethyl acetate is also flammable, therefore use well away from an open flame. Ether vapour is anaesthetic therefore make sure the laboratory is well-ventilated.
6. Vortex for 15 seconds without closing the tube. If no vortex is available, stopper the tube with a cotton wool plug/screw cap and mix well for 1 minute.
   Note: Do not use a rubber bung or a cap with a rubber liner as ether attacks rubber.
7. Loosen the stopper and centrifuge at 750-1000 g (approximately 3000 rpm) for 1 minute.
8. Using a stick (ekel) loosen the layer of faecal debris from the side of the tube and *invert* the tube to discard the ether, faecal debris and formalin (Refer Figure 3 page 221). The sediment will remain.
9. Return the tube to its upright position and allow the fluid from the side of the tube to drain to the bottom. Tap the bottom of the tube to re-suspend and mix the sediment.
10. Add a drop of Lugol's iodine to a slide and mix a drop of sediment with the iodine.
11. Cover with a coverslip and examine under low power (x10) and high power (x40).

D. Formol ether oocyst concentration technique

Follow steps 1-6 of the above method.

7. Centrifuge immediately at low speed RCF 300-400 g (1000 rpm) for 1 minute.
8. Using a plastic bulb pipette or Pasteur pipette, carefully remove the entire column of fluid below the faecal debris and ether, and transfer this to another centrifuge tube. (Refer Figure 3 page 221)
9. Add 10% formalin to make the volume up to 10-15ml. Centrifuge at 750-1000 g (approximately 3000 rpm) for 5-10 minutes.
10. Remove the supernatant. Tap the bottom of the tube to re-suspend and mix the sediment.
11. Transfer the sediment to a slide, stain with modified Ziehl-Neelsen method and examine under high power (x40) and oil; immersion (x100) objectives.

DISPOSAL OF SPECIMENS

1. Add enough 10% formalin to the container to cover the faeces left in the container. This will kill any parasites that may be present. Allow to stand for 1 h or more before discarding or washing.
2. Slides and coverslips should be put into separate jars of disinfectant (e.g. sodium hypochlorite), because coverslips break easily, especially if they are discarded together with slides. An applicator stick can be used to push to coverslip off the slide into the disinfectant

References

1. District laboratory practice in tropical countries - Monica Cheesebrough 1998
2. Manual of basic techniques for a health laboratory (2nd edition) WHO 2003
3. Practical Medical Microbiology – Mackie and McCartney 1989
4. Diagnostic Techniques in Medical Parasitology – Fleck & Moody. Butterworth-Heinemann 1988

EQUIPMENT AND MATERIALS REQUIRED

For wet smears

1. Microscope slides
2. Coverslips – 22 x 22 mm
3. Dropping bottles containing normal saline and Lugol's iodine solutions.
4. Marker pens or wax pencils for labeling slides
5. Applicator sticks (10 cm length ekel sticks)
6. Compound microscope with x10 and x40 objectives and adjustable condensor

For air-dried smears stained with Ziehl-Neelsen stain

Equipment

1. Microscope slides
2. Marker pens or wax pencil for labeling slides
3. Applicator sticks (10cm ekel sticks)
4. Compound microscope with x10, x40 and x100 objectives and adjustable condenser

Reagents and stains

5. Methanol
6. Cold strong carbol-fuchsin
7. 1% HCl-95% Ethanol
8. Malachite green 0.5% or Loeffler's Methylene blue
9. Immersion oil

For formol-ether sedimentation technique

Equipment

1. Microscope slides
2. Coverslips
3. Marker pens or wax pencil for labeling slides
4. Applicator sticks (10cm ekel sticks)
5. Plastic tea strainer or gauze
6. Screw- capped bottles/tubes
7. Conical centrifuge tubes
8. Vortex mixer (optional)
9. Centrifuge
10. Plastic bulb pipettes or Pasteur pipettes
11. Compound microscope with x10, x40 and x100 objectives and adjustable condenser

Reagents

1. 10% formalin
2. Diethyl ether or ethyl acetate
3. Immersion oil

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF FAECES FOR HELMINTH OVA

Type of specimens Faeces

Introduction

This SOP describes the examination of faeces for the presence of helminth eggs (ova of nematodes, cestodes and trematodes). Examination of a wet mount in saline is sufficient to diagnose a clinically significant infection with any of the intestinal helminths.

SPECIMEN COLLECTION

1. Patient should be provided with a suitable container for specimen collection such as an 80 or 100 ml wide-mouthed plastic cup with tight fitting, leak-proof lid and spoon (such as those used to sell ice-cream or yoghurt)
2. Patient should be instructed to defecate onto a paper or a large leaf such as a banana leaf (that may be disposed of later). A small quantity of faeces (at least half a teaspoon) should be transferred *immediately* into the container using the spoon. Remaining faeces should be disposed of in a sanitary latrine.
3. Faeces should not be mixed with urine or dirt
4. The container should be labeled clearly with the patient's name, date of collection and the time the patient passed faeces

SPECIMEN TRANSPORT AND STORAGE

1. Specimen should be brought to the lab for examination within 6 hours of collection
2. Specimen can be transported and stored at room temperature
3. If it cannot be examined within 6 hours, it may be refrigerated in order to prevent hookworm eggs from hatching

SPECIMEN PROCESSING

1. The specimen should be examined microscopically using a saline wet mount prepared directly from the faecal sample.
2. Write the patient's name or number on one end of the microscope slide, using a marker pen or wax pencil
3. Place a drop of saline in the centre of the slide
4. With an applicator stick, pick up a small portion of the faecal specimen and mix with the drop of saline
5. Cover the drop of saline with a coverslip. Hold the coverslip at an angle, touch the end of the drop and lower gently onto the slide, to reduce the chance of including air bubbles in the mount.
6. Examine the slide initially using the low power (x10) objective, with reduced illumination. Any suspicious object should be re-examined using the high power (x40) objective.
7. Look for ova of intestinal nematodes, cestodes and trematodes, as well as larvae of hookworms or *Strongyloides* spp., while examining the entire coverslip area. Focus the objective on the top left-hand corner of the coverslip and move the slide systematically backwards and forwards or up and down.
8. Quantification of infection should not be attempted on examination of direct wet smears.

REPORTING

The report should identify the parasite(s) seen, by scientific name, except in the case of hookworm (which cannot be identified at species level by the examining the morphology of eggs or L1 larvae). Other helminth eggs that are commonly seen in Sri Lanka include those of *Ascaris lumbricoides*, *Trichuris trichiura* and *Enterobius vermicularis.*

Hookworm larvae are not usually present if the faecal sample is fresh, but it may be necessary to distinguish these from the larvae of *Strongyloides stercoralis* if an old sample is examined.

Cestode and trematode infections are uncommon. If such infections are suggested, send a sample preserved in formol-saline to any of the reference laboratories listed in the section on identification of adult worms. Refer Figure 4 (page 221).

DISPOSAL OF SPECIMENS

1. Add enough 10% formalin to the container to cover the faeces left in the container. This will kill any parasites that may be present. Allow to stand for 1 h or more before discarding or washing.
2. Slides and coverslips should be put into separate jars of disinfectant (e.g. sodium hypochlorite), because coverslips break easily, especially if they are discarded together with slides. An applicator stick can be used to push to coverslip off the slide into the disinfectant

EQUIPMENT AND MATERIALS REQUIRED

1. Microscope slides
2. Coverslips – 22 x 22 mm
3. Dropping bottle containing normal saline solution
4. Marker pens or wax pencils for labeling slides
5. Applicator sticks (10 cm length of ekel sticks)
6. Compound microscope with x10 and x40 objectives and adjustable condensor

References

1. Manual of basic techniques for a health laboratory (2nd edition) WHO 2003
2. Basic laboratory methods in medical parasitology. WHO, 1991.

STANDARD OPERATING PROCEDURE FOR DIAGNOSIS OF ENTEROBIASIS

Type of specimen Perianal swab

Introduction

This SOP describes the procedure for examination of perianal swabs for the detection of the eggs of *Enterobius vermicularis.* Although these eggs may be occasionally seen in wet smears of faeces, examination of a peri-anal swab is the more sensitive technique when enterobiasis is suspected.

SPECIMEN COLLECTION

1. Fold a strip of transparent adhesive tape over the end of a glass slide so that the sticky surface is outermost.
2. Wearing disposable gloves, separate the patient's buttocks with one hand and press the end of the slide covered with the tape against the skin around the anus in several places.
3. Fold back the adhesive tape so that the sticky surface now rests on the glass slide.
4. Label the slide using a marker pen or wax pencil, with the patient's name or number
5. The swab should be taken early in the morning before the patient defaecates or bathes, in order to increase the chances of picking up eggs. At least 2 swabs should be taken on 2 consecutive days before excluding a diagnosis of enterobiasis.

SPECIMEN TRANSPORT AND STORAGE

1. Peri-anal swabs can be transported and stored at room temperature until they are examined.
2. Persons handling the swabs should be careful to wash their hands after handling the slides, as eggs become infective within a few hours.

SPECIMEN PROCESSING

1. Add a drop of toluene under cellotape to aid visualization (this is not essential)
2. Examine the slide microscopically, initially using the low power (x10) objective, with reduced illumination. Any suspicious object should be re-examined using the high power (x40) objective.
3. The entire slide should be examined systematically, starting at the top left-hand corner and moving systematically backwards and forwards, or up and down.

DISPOSAL OF SPECIMENS

1. The tape-slide should be discarded into a disinfectant and left immersed for at least 1 h before removing the tape and washing the slide for re-use.

EQUIPMENT AND MATERIALS REQUIRED

1. Microscope slides
2. Transparent adhesive tape (such as Cellotape) of 25 mm width
3. Toluene
4. Marker pens or wax pencils for labeling slides
5. Compound microscope with x10 and x40 objectives and adjustable condensor

References

1. Manual of basic techniques for a health laboratory (2nd edition) WHO 2003
2. Basic laboratory methods in medical parasitology, WHO, 1991.

STANDARD OPERATING PROCEDURE FOR IDENTIFICATION OF ADULT HELMINTHS

Type of specimen Adult worms (whole worm or segments)

Introduction

Adult worms and segments can be seen macroscopically in faeces or on passage from the anus. Adult or immature worms can also be seen macroscopically in surgical specimens of lymph nodes (lymphatic filarial parasites) or in subcutaneous nodules (zoonotic filarial worms). This SOP describes how to send such worms that may be passed out from the anus, or found in tissues, to a reference laboratory, for full identification.

SPECIMEN COLLECTION AND TRANSPORT

1. If still alive, adult nematodes should be placed in a dish containing tap water and refrigerated for 2 – 4 h. This will induce relaxation of the worms and prevent them from contracting and curling up (which makes visual identification of some of the key morphological characteristics difficult).
2. Nematodes can be killed by hot water (60 - 63° C) and then transferred to a preservative. The best preservative is alcohol-glycerin.
3. Hot (60 - 63° C) buffered formalin is recommended for the fixation of cestodes. All tapeworms and proglottids should be handled with care since the eggs are often infectious for humans.
4. Specimens should be placed in a screw-capped bottle, in an appropriate fixative of sufficient quantity to immerse the worm, and sent to a reference laboratory.

Adult worms should not be put directly into 10% formalin since this will cause the worm to contract and curl up. If no other preservative is available, put the worm in normal saline and send to reference laboratory without delay.

In Sri Lanka, the most commonly seen intestinal worms are nematodes*: Enterobius vermicularis*, *Ascaris lumbricoides*, *Necator americanus* (during upper GI endoscopy) and *Trichuris trichiura* (during sigmoidoscopy or colonoscopy). Cestode proglottids are also seen occasionally. Intestinal trematode infections are rare.

Adult worms of lymphatic filarial worms (*Wuchereria bancrofti*) or zoonotic filarial worms (*Dirofilaria repens*) are seen in surgical specimens.

Reagents required

Alcohol glycerin
Buffered formalin

Reference laboratories: Departments of Parasitology in

1. The Medical Research Institute, Colombo 8
2. The Faculty of Medicine, University of Colombo, Kynsey Road, Colombo 8
3. The Faculty of Medicine, University of Peradeniya at Peradeniya.
4. The Faculty of Medicine, University of Ruhuna, at Karapitiya, Galle.
5. The Faculty of Medicine, University of Kelaniya at Ragama.
6. The Faculty of Medical Sciences, University of Sri Jayewardenepura, at Gangodawila, Nugegoda.

References

Garcia LS and Bruckner DA. Chapter 33. Fixation and special preparation of fecal parasite specimens and arthropods In: *Diagnostic Medical Parasitology*, 3rd ed. Washington DC, ASM Press, 1997.

STANDARD OPERATING PROCEDURE FOR THE INVESTIGATION OF BLOOD FOR MALARIAL PARASITES

Type of specimen Blood
Capillary blood – finger-prick, ear lobe, heel
Venous blood
Cord blood
Blood from donor pack

Introduction
This SOP describes the examination of blood for the presence of malarial parasites. Prompt and accurate diagnosis of malaria is critical for effective patient management. Microscopic examination of thick/thin blood smears remains the operational gold standard for diagnosis of malaria infections but rapid diagnostic tests (RDTs) could be used as an alternative when microscopy is unavailable or is impractical.

SPECIMEN COLLECTION

1. Collection of capillary blood:
    - Wear gloves and follow safety procedures.
    - With the patient's left hand palm upwards, select the middle finger (big toe can be used for children)
    - Clean the finger with a swab soaked in alcohol
    - Puncture the ball of the finger with a sterile lancet using a quick rolling action
    - Apply gentle pressure to the finger, express the first drop of blood and wipe it away with a swab of cotton wool. Re-apply pressure, express blood and collect a single small drop of blood on to the middle of the slide. This is for the thin film. Apply further pressure to express more blood and collect two or three larger drops on to the slide about 1 cm from the drop intended for the thin film.
2. Timing: The sample of blood for identification of malarial parasites should be collected as soon as possible and before starting chemotherapy. In febrile patients timing of blood collection in relation to febrile episode is irrelevant. If the result is negative in the first instance, the test should be repeated a few hours later, and daily at least for 2 or 3 days.
3. If the preparation of slides is done immediately, use of anticoagulants is not necessary. Otherwise, a clean, leak-proof container with sufficient anticoagulant (dipotassium salt EDTA 1.5 mg/mL blood or heparin 15-30 mg/mL blood) should be used for collection of blood.
4. The container or slides should be labeled clearly with the patient's name, code/identification number and date of collection. The dry thin film could be labeled with a soft lead pencil by writing the patient's name, number and date across the thicker portion of the thin film.

Blood smear preparation

1. Preparation of thin smear: A small drop of blood is placed on the middle of the slide. Another clean slide is used to spread the blood. Touch the drop of blood with the short edge of the slide with the slide held at an angle of 45^{o}. Let the blood spread along the edge and then with a sweeping movement spread the blood along the slide.
2. Preparation of thick smear: 2-3 drops of blood are placed on the slide and mixed gently together with the corner of another slide to make a smear of about 1 cm in diameter.

SPECIMEN TRANSPORT AND STORAGE

1. Smears on slides should be air-dried thoroughly and packed in an insect-proof container. Request form with patient details (patient's identification (name, age, sex), ward, BHT number, date and time of specimen collection, a brief clinical history, area of residence for the last 3

months, travel history (if from non-endemic areas), treatment given and antimalarial prophylaxis) should be enclosed.
2. Blood can be transported and stored at room temperature, thick/thin blood smears should be made as soon as possible, preferably within 2 hours of sample collection.

SPECIMEN PROCESSING

1. **Fixing**: Fix the thin film by dipping it in a container of pure methanol for 30 seconds. The thick film should not be fixed in order to permit dehaemoglobinization. Therefore, avoid methanol or methanol vapour touching the thick film.

2. **Staining with Giemsa**:
   a) 5% Giemsa stain used as a fresh solution each day. Gently pour the stain on to the slide (a pipette can be used for this purpose).
   b) Stain for 20-30 minutes (in emergencies, 10 minutes in 10% Giemsa solution can be used). Gently flush the stain off the slide by adding drops of clean water or flushing with a gentle stream of tap water. Do not tip off the stain and then wash, as this will leave deposits of scum over the smear.
   c) Place the slide in an upright position to drain and dry.

3. If Giemsa is not available, **Leishman's stain** may be used:
   a) Place the slide on a staining rack over a sink, taking care that the film side is uppermost.
   b) Cover the film with about 10 drops of Leishman's stain and leave for 30 seconds.
   c) Add double the quantity (20) drops of distilled water which has already been buffered to pH 7.2.
   d) Mix the solution thoroughly but gently by rocking the slide and taking care not to spill the stain.
   e) Allow to stain for 10 minutes.
   f) Flush for a second or less in a gentle flow of tap water.
   g) Stand the slide in an upright position to drain and dry.

4. **Light microscopic examination**: Examine 200 microscopic fields at a magnification of 10 x 100 (oil-immersion lens) before reporting as 'negative'. Examine the whole smear of the stained thick film. Examine the tail end of the thin smear, which has a single layer of cells. Number of parasites in 200 consecutive microscopic fields of the thin smear are counted and expressed as a percentage of the total red blood cells (% parasitaemia)

REPORTING

Species, stages (asexual/sexual) should be indicated together with the parasite count or parasitaemia (number of parasites per micro liter of blood or percentage of infected red cells).

Calculation of % parasitaemia:

$$\frac{\text{Number of parasites counted}}{\text{Number of fields examined (200)}} \times \frac{100\ (\%)}{\text{Number of RBC per field (200)}}$$

$$\text{Number of parasites per micro liter of blood} = \frac{\%\ \text{parasitaemia}}{100} \times \text{red cell count}$$

[Refer Figure 10 (10A – 10E), pages 224 - 226 for different stages of vivax and falciparum parasites in thin and thick smears]

Further investigations:

1. Rapid diagnostic tests for malaria antigen detection. If a kit is available in hospital laboratory, this should be performed according to the manufacturer's instructions

2. Molecular biological methods for species typing, genotyping, bar coding etc.
   Detailed information could be obtained from:
   Malaria Research Unit, Department of Parasitology in the Faculty of Medicine, University of Colombo, Kynsey Road, Colombo 8. Tel. 0112699284

EQUIPMENT AND MATERIALS REQUIRED

1. Clean and grease-free slides
2. Sterile lancets
3. Pure methanol
4. Cotton wool
5. Slide box or cover to protect the blood films
6. Soft lead pencil
7. Register or record form and pen
8. Giemsa stain
9. Leishman stain
10. Compound microscope with x10 and x100 objectives and adjustable condenser

STANDARD OPERATING PROCEDURE FOR DIAGNOSIS OF LYMPHATIC FILARIAL INFECTIONS

Specimens: Capillary or venous blood

Introduction

This SOP describes examination of a thick smear made of peripheral blood for the presence of microfilariae of lymphatic filarial parasites prevalent in Sri Lanka, *Wuchereria bancrofti* & *Brugia malayi.* This is applicable for investigation of patients with clinical features suggestive of lymphatic filariasis, as well as for screening of asymptomatic individuals resident in endemic areas.

SPECIMEN COLLECTION, TRANSPORT AND STORAGE

1. Optimal time of collection is between 10 pm - 2 am, in accordance with the nocturnal periodicity of the endemic strains.
2. To obtain finger prick blood, clean the finger to be pricked (3^{rd} or 4^{th} finger of left hand) with ethanol. Prick the finger rapidly and firmly at the side (which is less sensitive than the tip) and collect the first 2-3 drops of blood directly onto the center of the slide.
3. Spread the blood in a circular manner with the corner of the second slide to produce a coin shaped smear of an even thickness. Label the slide and leave to dry.
4. Dried blood films can be transported easily and stored under cool, dry conditions for several months.

SPECIMEN PROCESSING

1. Place the slides vertically in the staining trough filled with clean water and leave for 10 minutes. The haemoglobin sinks to the bottom.
2. Take the slides and drain them
3. The dehaemoglobinised smear may be stained with Giemsa or Delafield's haematoxylin

A. Staining thick smears for microfilaria with Giemsa stain

1. Place the slide to be stained in a horizontal position on a staining rack with smear on top
2. Cover the smear with 10% Giemsa stain and leave for 30 minutes
3. Wash off the stain with buffered water. Do not tip off the stain and then wash as this will leave a deposit of stain over the smear
4. Drain off the water and place the slides in a rack to dry

B. Staining thick films for microfilaria with Delafield's haematoxylin stain

1. Place the slide to be stained in a horizontal position on a staining rack with smear on top
2. Pour methyl alcohol on to the slide to cover the smear
3. Allow the alcohol to fix the smear for 1 minute and then pour off methyl alcohol
4. Pour enough Delafield's Haematoxylin stain onto the slide to cover the smear
5. Carefully heat the slide with the flame from a Bunsen burner until steam rises from the stain. Do not allow it to boil
6. Leave the stain to act on the smear for 10 minutes
7. Wash away the stain in a gentle stream of running water until the smear is blue in colour

Microscopic examination

- Examine the smear under low power using the x 10 objective
- When a microfilaria is found, centre it in the field, cover the smear with a thin film of immersion oil and examine using x10 x100 magnification (oil immersion objective) for species identification.

REPORTING
The report should identify the species of the microfilaria seen, by scientific name.

Further investigations:

1. Rapid diagnostic tests for filarial antigen detection. If a kit is available in hospital laboratory, this should be performed according to the manufacturer's instructions
2. Filarial Fluorescent Antibody Test (FFAT): antibodies are detected using the indirect fluorescent antibody technique at the Dept of Parasitology, Medical Research Institute, Colombo 8.

EQUIPMENT AND MATERIALS REQUIRED

Sterile blood lancets
Cotton wool
Slides 2
Ethanol
Giemsa stain
Delafield's haematoxylin stain
Compound microscope with x10, x40 and x100 objectives and adjustable condensor

References

1. World Health Organization. Part II A. Parasitology. In: *Manual of basic techniques for a health laboratory*, Geneva 1980.

STANDARD OPERATING PROCEDURE FOR DIAGNOSIS OF CUTANEOUS LEISHMANIASIS

Specimens Lesion aspirates
Slit skin scrapings
Punch biopsies

Leishmaniasis reference laboratories:
1. Department of Parasitology, Faculty of Medicine, University of Colombo, Colombo 8. Tel. 011 2695300, Ext 179 and 180
2. Department of Parasitology, Faculty of Medicine, University of Peradeniya. Tel. 081 2396510

Introduction

This SOP describes light microscopic examination of specimens for the presence of amastigotes in specimens obtained from patients clinically suspected of having cutaneous leishmaniasis. *In vitro* cultivation of *Leishmania* parasites can also be carried out to increase sensitivity, but specimens should be sent to the reference laboratory for this purpose.

SPECIMEN COLLECTION

1. If there are any skin lesions, they should be cleaned with soap and clean tap water. Any scab/crust should be gently removed during the procedure. Then gently wipe off the lesion with 70% alcohol.
2. ***Lesion aspiration:*** aspirate the active edge of the lesion or prominent nodule using 0.2 to 0.3 ml of sterile saline injected to the site using a 22 or 23 gauge sterile needle. Place one drop of the aspirate on a clean glass slide. Make a smear by spreading the aspirate drop using the needle.
3. ***Slit-skin scrapings:*** make a slit over the active edge of the cleaned wound using a sterile no. 10 scalpel. Take tissue scrapings from both edges of the slit to the scalpel by gently moving the scalpel blade along the slit. Immediately smear the scrapings on to a clean glass slide using the scalpel.
4. Both lesion aspirates and slit skin scrapings can be smeared on to one slide as shown in Figure 5 (Refer page 222).
5. ***For PCR****:* Two millimeter (2mm) punch biopsies should be taken from the active edge of the ulcers or the prominent and active sites of nodules, using a sterile 2 mm sterile punch biopsy needle, according to standard dermatological procedures. Put the biopsy in to a sterile Eppendorf tube (usually provided by the reference lab) **without formalin, containing 1ml of sterile normal saline** so that the sample can be used for PCR and culture

SPECIMEN STORAGE AND TRANSPORT FOR FURTHER INVESTIGATIONS

1. Unstained smears or fixed smears should be clearly labeled, individually wrapped in clean tissue or packed in a suitable container before dispatch.
2. Lesion aspirates/biopsies/organ aspirates need to be put in sterile tubes (preferably tubes that can be frozen, e.g. Eppendorf tubes). **Formalin is not recommended if the sample is to be used for culture or PCR**. Samples for PCR can be sent at room temperature without any transport media or in normal saline if a delay is expected.

SPECIMEN PROCESSING

1. All slides should be clearly labeled with patient ID and the sample type (especially if two smears are made on a single slide) before staining.
2. Smears should be fixed in methyl alcohol for 30 seconds.
3. Fixed smears should be stained with 10% Giemsa in buffered water. Single slides can be stained face downwards in a staining plate for 20-30 minutes. If large numbers of slides are used, a staining trough can be used.

4. Stain should be gently washed off using water and air dried by placing them upright at an angle.

Giemsa stained smears are examined under x10 x100 with the oil-immersion lens. *Leishmania* amastigotes may be found inside macrophages or extra-cellularly. The whole smear should be examined systematically before issuing a negative report as low parasitaemias can be easily missed by light microscopy. Species identification is not possible with light microscopy.

REPORTING

The report should identify the parasites by the scientific name up to the genus level. (i.e., *Leishmania* sp. parasites seen/not seen). Please note that it is not possible to establish species identification by light microscopy, culture or routine PCR. (Refer Figure 11 page 226)

EQUIPMENT AND MATERIALS REQUIRED

1. Soap, clean tap water
2. 70% alcohol
3. Cotton wool
4. Surgical plasters
5. 1cc syringes
6. 22-23G needles
7. No. 10 scalpels
8. Microscope slides
9. Cover slips
10. Marker pens or wax pencils
11. Normal saline (preferably aliquoted in to small containers)
12. Giemsa stain
13. Compound microscope with x10, x40 and x100 objectives and adjustable condenser

References:

National Action Plan for *Leishmania* control. Proceedings of the Leishmaniasis Colloquium, 2009

STANDARD OPERATING PROCEDURE FOR DIAGNOSIS OF TRICHOMONIASIS

Specimen: In the female – a high vaginal swab from the posterior fornix
In the male - urethral discharge/ a sample of urine sediment

Introduction:
This SOP describes the 'wet mount' preparation and direct microscopy for diagnosis of trichomonal infection, a sexually transmitted parasitic disease caused by a flagellated protozoan *Trichomonas vaginalis*. Female patients (50-75%) will present with a frothy greenish-yellow vaginal discharge with a characteristic fishy odour. Males may present with a urethral discharge, but majority will be asymptomatic. The wet mount is rapid, easy to perform, accurate, and costs little to carry out.

SPECIMEN COLLECTION

In the female:

1. A **high vaginal swab** should be collected by a medical officer, using a cotton-tipped applicator stick, under a speculum examination, from secretions in the posterior fornix.

2. Vaginal discharge will be collected with a swab if the hymen is intact.

In the male:

1. Urethral discharge can be collected with an applicator stick similarly, at the urethra, or

2. Urine: collect the early morning **first catch** fresh urine sample.

SPECIMEN TRANSPORT AND STORAGE

The wet mount should be prepared with the **fresh sample**.

1. The specimen should be examined within **20 minutes** of collection. Delays will make the parasite immotile and die and the diagnosis will be impossible. **Do not refrigerate**.

2. This is preferably performed as a **bed side test** in the laboratory and then the sample can be directly applied on the slide with a **drop of saline**.

3. If the sample is to be dispatched to the laboratory, the applicator stick has to be inserted into a test tube containing 0.5-1 ml of 0.9% N. saline and be sent to the lab as quickly as possible.

SPECIMEN PROCESSING

Wet mount for direct microscopy

1. Keep the slide on a flat surface
2. Put a drop of N. saline onto a glass slide
3. Mix the sample well with the drop of saline using the sample containing applicator stick
4. Cover with a cover slip
5. Look under the light microscope (x 40)
6. If it is urine, centrifuge the urine (~2000 rpm for 2-3 mins), discard the supernatant, take a drop from sediment, mix well with a drop of saline on a glass slide and examined as described above.

Diagnosis

In a wet mount, *T. vaginalis* trophozoites will appear as motile (with a rapid rolling movement), pear-shaped, 10 µm by 7 µm organisms, with visible flagellae (5) and a posterior axostyle (Refer Figures 6 & 7 page 222) (in *T. vaginalis* life cycle only trophozoite form has been identified). The field will also have many polymorphonuclear neutrophils (PMNs) and epithelial cells. If the specimen dries up the parasites can be confused with PMNs.

REPORTING

The report should be given as "wet mount positive/negative for *Trichomonas vaginalis".* A minimum of 10 fields should be examined before giving a negative report.

DISPOSAL OF SPECIMENS

Specimen and the applicator stick have to be considered as potentially infectious waste.

EQUIPMENT AND MATERIALS REQUIRED

1. Cotton tipped applicator stick
2. Normal saline
3. Glass slides
4. Coverslips
5. A binocular light microscope

References

1. Cook G.C (2003) Trichomonal infections In: **Manson's Tropical Diseases** (ed G.C. Cook & A. Zumla) 21st edition, 1427-29pp, Saunders, Elsevier Science, Jamestown Road, London.

2. Arlene C. Senã AC, Miller WM, Hobbs MM, Schwebke JR, Leone PA *et al* (2007) *Trichomonas vaginalis* Infection in Male Sexual Partners: Implications for Diagnosis, Treatment, and Prevention. *Clinical Infectious Disease, 44:13*–22

STANDARD OPERATING PROCEDURES FOR DETECTION OF *TOXOPLASMA* ANTIBODIES

Type of Specimen: Blood or serum

Toxoplasmosis reference laboratory:
Department of Parasitology, MRI, Colombo 8
Tel. 011 2695102

Introduction

Toxoplasmosis is caused by the intracellular parasite *Toxoplasma gondii*. Due to the non-specific nature of symptoms and the presence of asymptomatic /sub-clinical infections in the community, diagnosis is frequently based on serology. This SOP describes how to send specimens for the detection of *Toxoplasma* antibodies by ELISA, which is the commonly used technique in Sri Lanka.

SPECIMEN COLLECTION

1. 3ml of blood should be collected into a plain dry sterile screw capped container
2. Send to Reference Laboratory packed in ice.
3. If specimen is to be posted to the reference laboratory, it is preferable to send serum.

SPECIMEN TRANSPORT AND STORAGE

1. Blood should be sent to the reference laboratory preferably within two hours with accompanying request form giving patient identification details and clinical details.
2. Serum sample - if it is not possible to post on the same day, store it in a refrigerator in the freezer compartment and transport the labeled specimen packed in ice.

Specimens may be rejected for the following reasons:

1. Insufficient serum for test
2. Decomposed sample (when sent by post)
3. Specimen container broken
4. Inadequate patient details

Limitations of the Procedure

1. There is a possibility of getting false negative and false positive IgM results in specimens with extremely high Rheumatoid factor and high autoimmune antibodies.
2. Lipemic, hemolyzed, icteric or heat inactivated sera may cause erroneous results.
3. As with other serological assays, the results of these assays should be used in conjunction with information available from clinical evaluation and other diagnostic procedures.

STANDARD OPERATING PROCEDURE FOR DETECTION OF *TOXOCARA* ANTIBODIES

Type of Specimen: Whole blood or serum

Toxocariasis Reference Laboratories

1. Department of Parasitology, Faculty of Medicine, University of Peradeniya. Tel. 081 2396510
2. Department of Parasitology, MRI, Colombo 8. Tel. 011 2695102

Introduction

Toxocariasis (also known as Visceral Larva Migrans) is caused by the larval stages of *Toxocara* spp. Since parasitological diagnosis is usually not possible, diagnosis is frequently based on serology. This SOP describes how to send specimens for the detection of *Toxocara* antibodies using Excretory – Secretory Antigen of second stage larva of T*oxocara* spp. (TES-ELISA)

SPECIMEN COLLECTION

1. 5ml of blood should be collected into a plain dry sterile screw capped container
2. Send to Reference Laboratory packed in ice.
3. If specimen is to be posted to the reference laboratory, it is preferable to separate serum.

SPECIMEN TRANSPORT AND STORAGE

1. Blood should be sent to the laboratory preferably within two hours with accompanying request form giving patient identification details: Name/age/ward/BHT Number/clinical details or
2. Serum sample - if it is not possible to post on the same day, store it in a refrigerator in the freezer compartment and transport the labelled specimen packed in ice

Specimens may be rejected for the following reasons:

1. Inadequate serum for test
2. Decomposed sample (when sent by post)
3. Specimen container broken or spillage of blood in transit
4. Unlabeled specimens

REAGENTS AND STAINS FOR PARASITOLOGICAL DIAGNOSIS

ALCOHOL-GLYCERIN

95% ethyl alcohol	70 ml
Distilled water	25 ml
Glycerin	5 ml

BUFFERED DISTILLED WATER OR PHOSPHATE BUFFER (PH 7.2)

KH_2PO_4	0.6 g
$NaHPO_4$	3.0 g

Bring to 1L in distilled water and adjust pH to 7.2

BUFFERED FORMOL-SALINE SOLUTION

Formaldehyde	400 ml
Normal saline	7,600 ml
Na_2HPO_4	6.10 g
NaH_2PO_4	0.15 g

Formaldehyde is normally purchased as a 37% HCHO solution; however, for dilution, it should be considered to be 100%.

DELAFIELD'S HAEMATOXYLIN STAIN

Haematoxylin crystals	1g
Ethanol absolute	10ml
Saturated solution of aluminium ammonium sulfate ($NH_4Al(SO_4)$) in distilled water	100ml
Glycerol ($C_3H_3(OH)$)	25ml
Methanol absolute	25ml

Dissolve the haematoxylin crystals in absolute ethanol. Add a few drops at a time to the saturated aluminium ammonium sulfate solution. Leave this solution unstoppered in direct sunlight or in a 37◦C incubator for 3-4 months to oxidize the haematoxylin to haematin.
Stopper and label the bottle and write the date. When reopened, filter and add the glycerol and methyl alcohol, and the stain is ready for use. Keep stoppered to prevent evaporation. The stain will remain good for 18 months.

GIEMSA STAIN (STOCK SOLUTION)

Giemsa powder	3.8 g
Methanol	250 ml
Glycerol	250 ml
Solid glass beads 5 mm diameter	50 nos

Use a chemically clean and dry dark glass or polyethylene bottle of suitable size.

1. Put the glass beads in the bottle; pour in the measured amount of methanol and add the stain powder.
2. Tightly stopper the bottle. Allow the stain powder to sink slowly through the methanol until it settles to the bottom. Shake the bottle in a circular motion for 2-3 minutes.

3. Add the measured amount of glycerol and repeat the shaking process. Continue to shake for 2-3 minutes at half-hourly intervals for at least six times.
4. Leave the bottle for 2-3 days, shaking it 3-4 times each day until the stain is thoroughly mixed.
5. Filter.

Each newly prepared batch of stain should be properly labelled, including date of preparation and should be tested for stain quality prior to routine use. Always keep the bottle tightly stoppered, in a cool place, away from sunlight.

GIEMSA STAIN WORKING SOLUTIONS

- **5% solution:** Take 9.5 ml of buffered distilled water (pH 7.2) and 0.5 ml of stain and mix gently with a glass rod.
- **10% solution**: Take 9 ml of buffered distilled water (pH 7.2) and 1 ml of stain and mix gently.
- Diluted Giemsa stain is good for use for about 8 hours.

1% HCL – 95% ETHANOL

Conc. HCl (100%)	1ml
95% ethanol	99ml

Add 1ml conc. HCl to 99ml of 95% ethanol

LEISHMAN'S STAIN

1. Add 1.5 g of dry Leishman's powder to 1000 ml of absolute methanol in a glass bottle.
2. Add a few glass beads.
3. Keep the bottle lightly capped and shake well occasionally throughout the day.
4. The stain is ready for use after 24 hours.

LOEFFLER'S METHYLENE BLUE

Saturated solution of methylene blue in alcohol	300ml (1g in 100ml 95% ethanol)
Potassium hydroxide (KOH 0.01% in water)	1 litre (1ml to 99ml distilled water)

Mix for 30 minutes

LUGOL'S IODINE

Iodine	1g
Potassium iodide (KI)	2g
Distilled water	100ml

Weigh the iodine in a porcelain dish or watch glass. Grind the dry iodine and potassium iodide in a mortar. Add water a few milliliters at a time, and grind thoroughly after each addition until the iodine and iodide dissolve. Put the solution into **an amber glass bottle** with the remainder of the distilled water. Sunlight will decolourize the stain.

MALACHITE GREEN 0.5 %

Malachite green	5g
Distilled water	1 litre

Add about a ¼ of the water to the dye and mix until the dye is fully dissolved. Then add the remainder of the water and mix well. The stain is stable for several months.

NORMAL SALINE

Sodium chloride (NaCl)	8.5 g
Distilled water	1000 ml

Weigh out the NaCl. Measure the distilled water into a clean, glass-stoppered bottle. Dissolve the NaCl in the water and mix thoroughly. Label the bottle 'NORMAL SALINE' and write the date. Put a piece of string or a narrow strip of paper between the glass stopper and the neck of the bottle to keep the stopper from sticking. Pour some saline into a dropping bottle or dispensing bottle for daily use.

STRONG CARBOL-FUCHSIN

Solution A

Basic fuchsin	10g
Absolute Ethanol (100%)	100ml

Mix and dissolve in a stoppered bottle and keep at 35^0C-37^0C overnight

Solution B

Phenol	5g
Distilled water	100ml

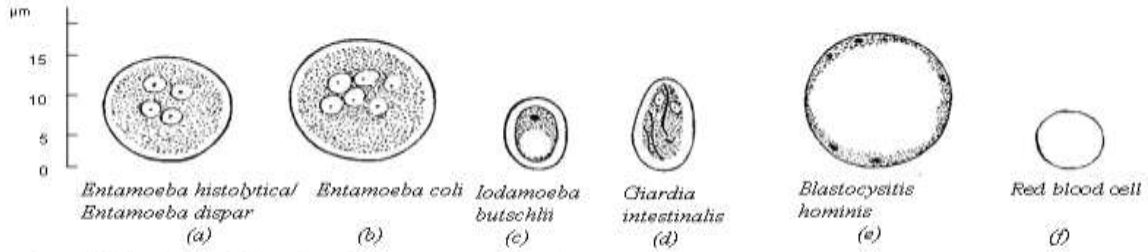
Mix and dissolve

Add solution A to solution B

References

1. World Health Organization. Annex 2. Reagents and solutions and their preparation. In: *Basic Laboratory methods in Medical Parasitology* Geneva, WHO, 1991.

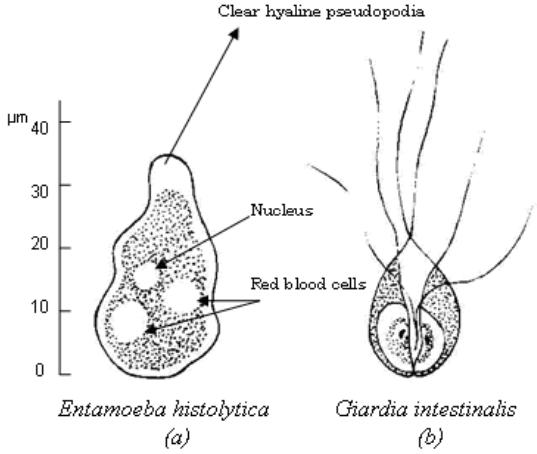
Figure 1 - Protozoan cysts in iodine smear



(a) Cysts with identical morphology but <10µm are non-pathogenic *Entamoeba hartmanii*
(e) Central body form commonly seen in faeces. Size varies widely from 6-40µm.

Drawn by Dhilma Atapattu

Figure 2 - Trophozoites in saline smear



Drawn by Dhilma Atapattu

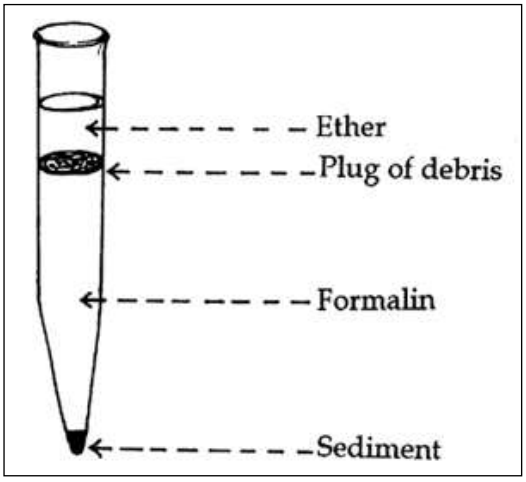


Figure 3. Test tube after centrifugation in formol-ether concentration technique

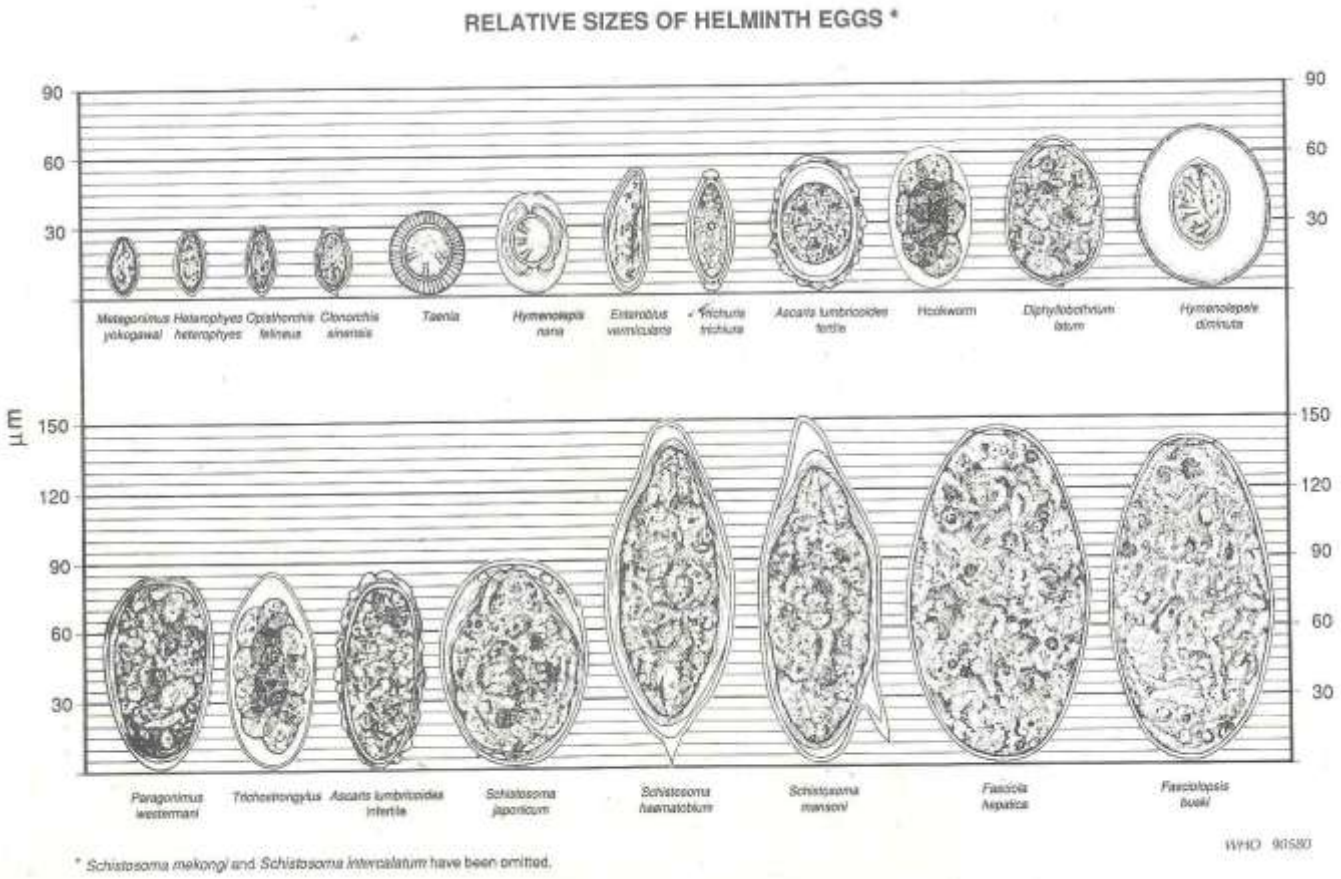


Figure 4. Comparative sizes and morphology of helminth eggs found in human faeces

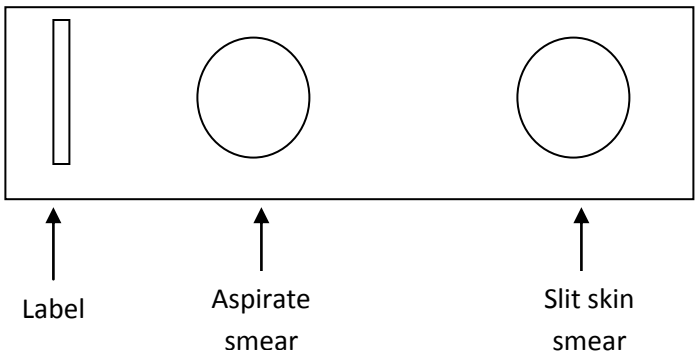


Figure 5. Preparing skin smears for examination for *Leishmania* parasites

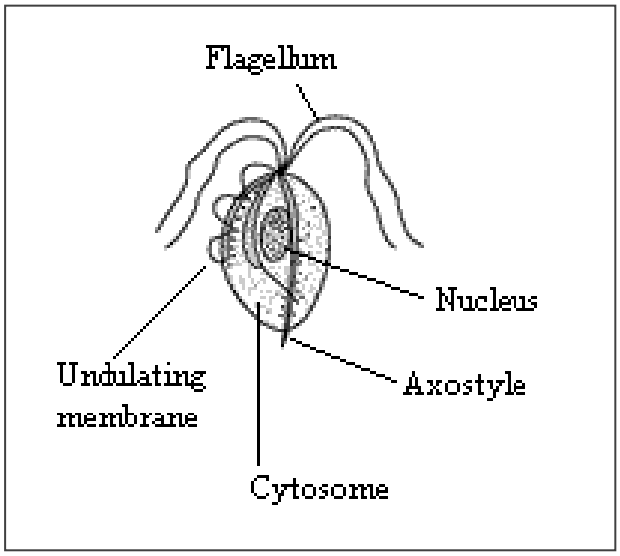


Figure 6. *Trichomonas vaginalis* trophozoite

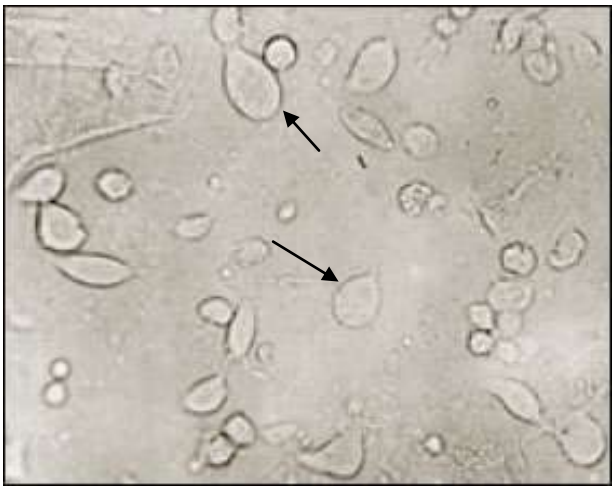


Figure 7. Photomicrograph of *T. vaginalis* trophozoites in a wet mount

Oocysts in faecal smears stained with Ziehl-Neelsen stain

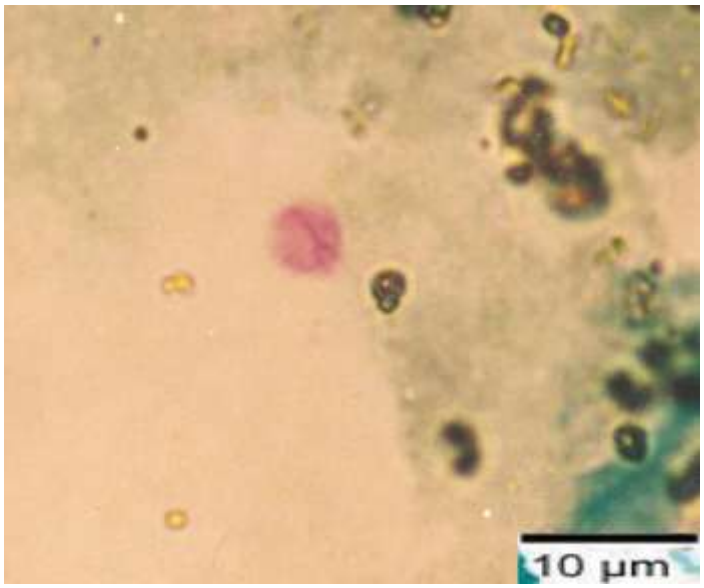


Figure 8. *Cryptosporidium* oocyst (4-6µm) - Ziehl-Neelsen stain

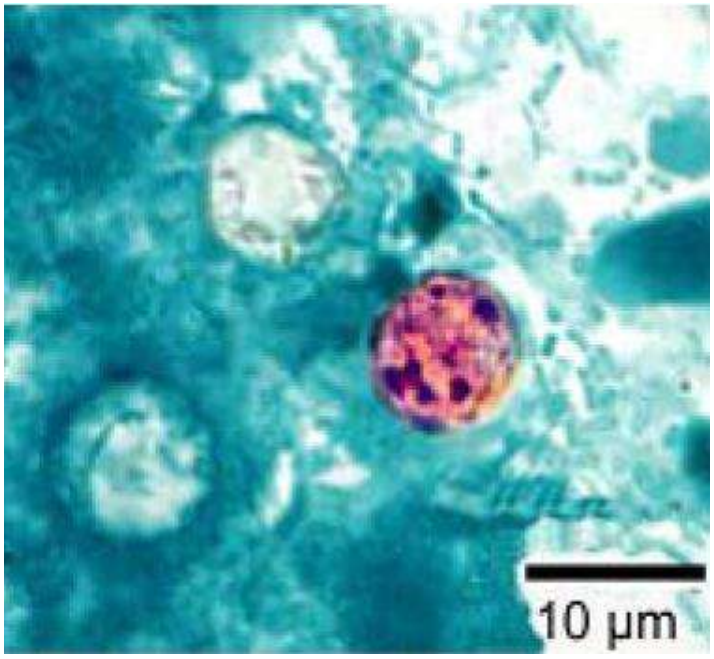


Figure 9. *Cyclospora cayetanensis* oocysts (8-10µm) - Ziehl-Neelsen stain

Figure 10 (10 A- 10 E) - . Malaria and *Leishmania* parasites

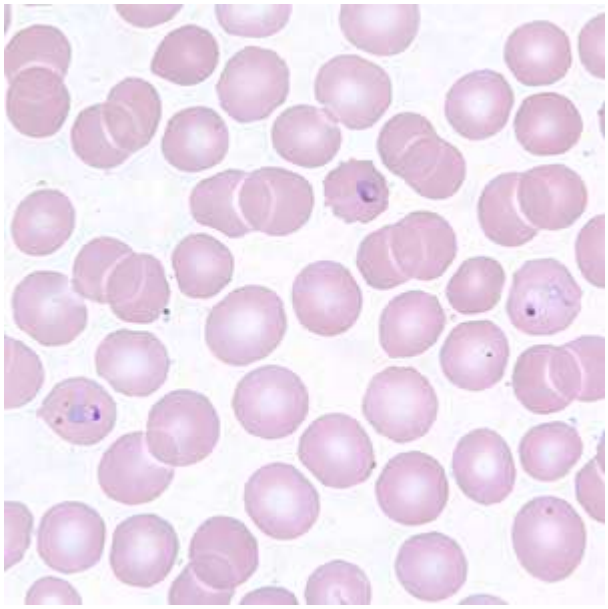


Figure 10 A. *Plasmodium vivax* ring trophozoites in thin blood film stained with Giemsa (x1000 magnification)

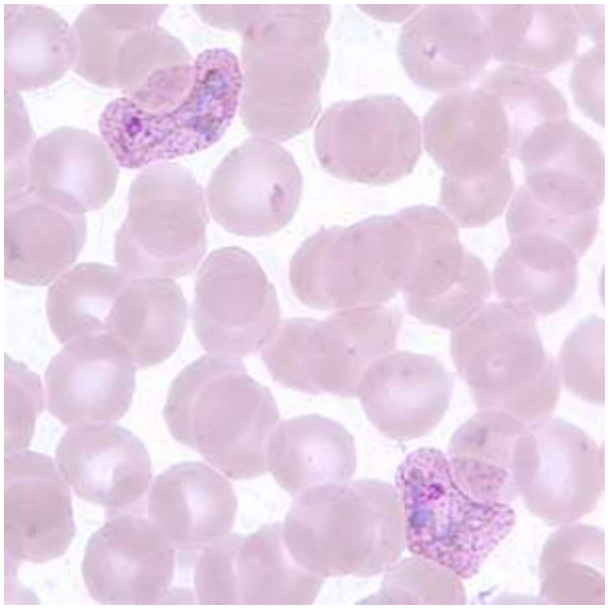


Figure 10 B. *Plasmodium vivax* amoeboid trophozoites in thin blood film stained with Giemsa (x1000 magnification)

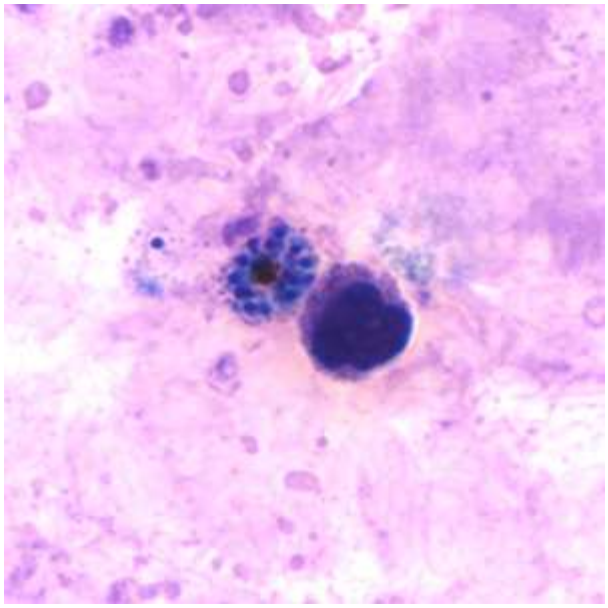


Figure 10 C. *Plasmodium vivax* schizont in thick blood film stained with Giemsa (x1000 magnification)

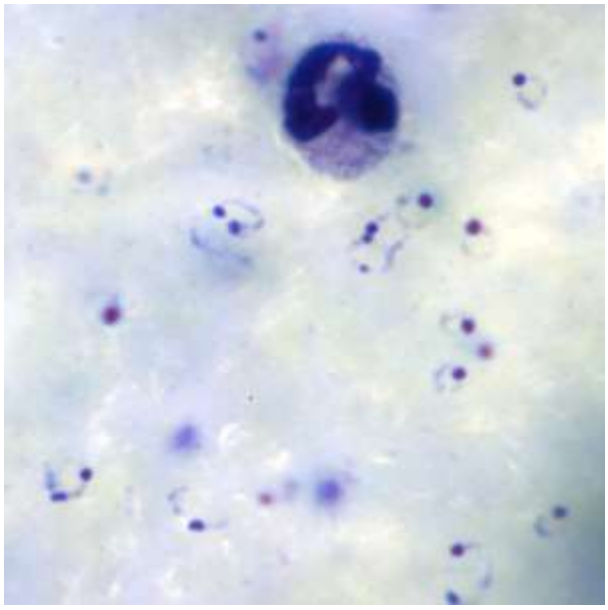


Figure 10 D. *Plasmodium falciparum* ring trophozoites in thick blood film stained with Giemsa (x1000 magnification)

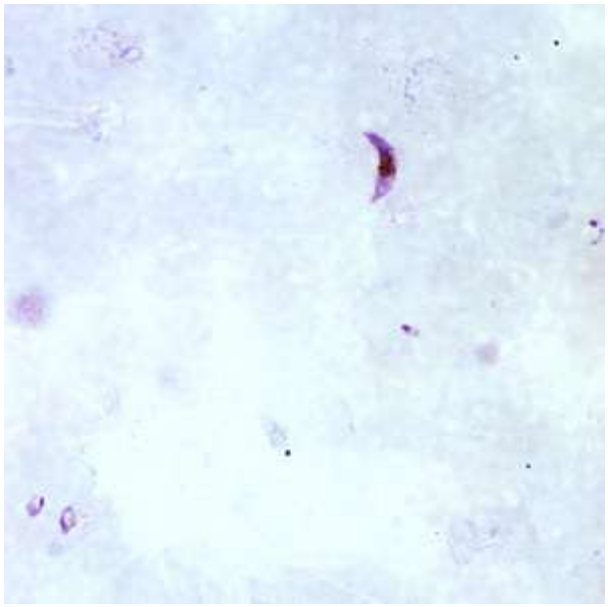


Figure 10 E. *Plasmodium falciparum* gametocyte in thick blood film stained with Giemsa (x1000 magnification)

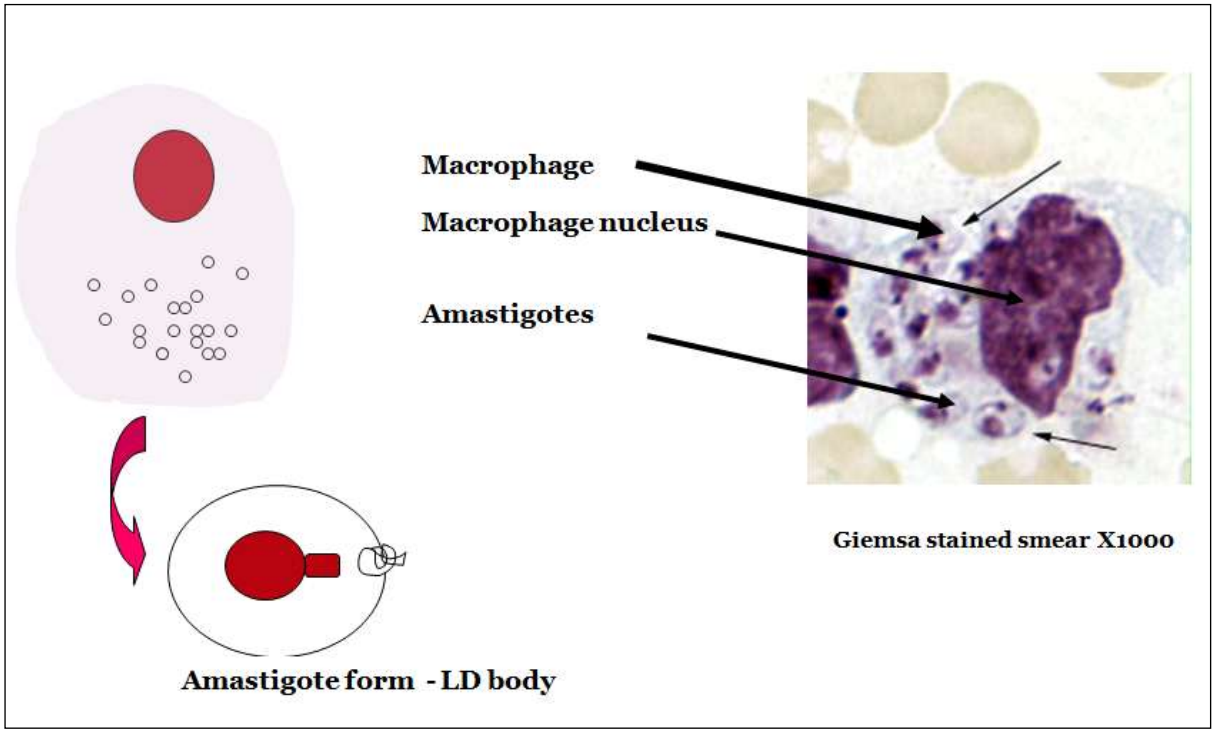


Figure 11. *Leishmania* amastigotes in macrophage

IMMUNOLOGY

IMMUNOLOGICAL DIAGNOSTIC TESTS

INTRODUCTION

This section lists investigations suitable for local laboratories, and tests which are available at the Immunology Reference Laboratory, MRI.

A. The following tests should be made available at local laboratories.

i. WBC/DC
ii. C- reactive protein (CRP)
iii. Rheumatoid factor (RF)

CRP and RF levels are measured using commercial assays (slide agglutination).
Please follow the manufacturers' instructions on performing the tests.

Immunology Reference Laboratory
Medical Research Institute
Colombo 8

Tel. 011-2693532 ext. 352 / 354 / 355
011-2691819

In case of any queries please contact
Consultant Immunologist, MRI

B. Tests available at Reference Laboratories

I. ANTIBODIES

TEST	By appoint-ment	Patient to be sent	Specimen details	Points to note	Availability of report
Cryoglobulins	NO	YES (Mon-Fri Morning)	Blood	To be drawn in the Immunology laboratory	7 days
Simple electrophoresis Serum / Urine	NO	NO	Blood - 2ml in a plain bottle Urine - 25ml	All urine samples should be accompanied by a sample of blood to comment on selectivity of proteinuria	3-4 days
Quantitative estimation of serum Immunoglobulins (Ig) (IgG, IgA, IgM)	NO	NO	2ml of blood in a plain bottle State **age** of patient & a brief history	Postpone if blood or plasma transfusion given within 1month. If plasma/ blood transfusion is given before 1month, state the date	Test commences on Mondays and the report issued on the following Monday
Anti-nuclear antibodies (ANA)	NO	NO	2ml of blood in a plain bottle		3-4 days
DS-DNA	NO	NO	Blood sample sent for ANA can be used for DS-DNA	DS-DNA will be done **only for ANA positive samples performed at MRI**	
Anti-Cardiolipin IgG	NO	NO	2ml of blood in a plain bottle	Proper history is mandatory	

II. COMPLEMENT

TEST	By appointment	Patient to be sent	Specimen details	Points to note	Availability of report
Complement components C3,C4	NO	NO	2ml of blood in a plain bottle		Test commences on Tuesdays and report issued on Fridays of same week

III. PHAGOCYTES – QUALITATIVE

TEST	By Appointment	Patient to be sent	Specimen details	Points to note	Availability of report
Nitro blue tetrazolium (NBT) test	YES	YES Before 10 am	Blood to be drawn in the Immunology laboratory	For neutrophil oxidative function to diagnose chronic granulomatous disease (CGD)	Same day after 3.00pm

IV. LYMPHOCYTES

a) Quantitative assay

TEST	By Appoint-ment	Patient to be sent	Specimen details	Points to note	Availability of report
Lymphocyte subsets by flowcytometry	YES	YES before 10 am	**If patient cannot be sent** 2 ml of EDTA blood before 10.00am	Quantification of lymphocyte subsets ▪ CD3 ▪ CD4 ▪ CD8 ▪ CD19 ▪ CD56/CD16 *for evaluation of immunodeficiencies including HIV/AIDS Other relevant CD markers for classification of leukaemias **Refer rejection criteria**	Next day

b) Qualitative assay

TEST	By Appointment	Patient to be sent	Specimen details	Points to note	Availability of report
Lymphocyte function test	YES	YES before 10 am	**If patient cannot be sent** 3 ml of heparinised blood collected in to a sterile container under sterile conditions.	**Refer rejection criteria**	3 days

v. DIAGNOSIS OF BULLOUS DISEASES OF THE SKIN BY DIRECT IMMUNOFLORESCENCE (IF)

Skin biopsy sample needs to be sent in Michel's medium. Medium can be obtained from Department of Immunology, MRI.
Samples will be accepted only if the clinical history is written in the form provided by the Department.

GENERAL INSTRUCTIONS FOR COLLECTION AND TRANSPORT OF SPECIMENS

1. It is IMPORTANT to note that all specimens should be accompanied by a duly filled request form with the patient's **age** etc. and a **brief history**. (**Age** is important because normal range of immunoglobulins vary in different age groups)
   Note: History is essential for interpretation of results.

2. Note that appointments are required for some tests. If an appointment cannot be kept, please ensure that the laboratory is informed.

REJECTION CRITERIA

1. Specimen leaking from container (likely contamination of sample)
2. Decomposed specimens
3. Clotted blood in the case of lymphocyte subset analysis/ lymphocyte function test
4. Specimens sent without obtaining an appointment for tests which require appointments.

Reference

Rose N. R., de Macario E C., Folds J. D., Lane H. C., Nakamura. R. N. **Manual of Clinical Laboratory Immunology**, 5th Edition 1997, American Society of Microbiology