Standard Operative Procedure for collection, transport and processing and inoculation of Extra-pulmonary specimens

1. Introduction:

Mycobacteria may not be suspected as the causative agent of an extra pulmonary disease because the chest X-ray or the tuberculin test is negative or both. However, based on clinical symptoms and because mycobacteria can infect almost any organ in the body, the laboratory should expect to receive a variety of extra pulmonary specimens such as body fluids, surgically excised tissues, aspirates or draining pus and urine.

Extra pulmonary specimens are divided in to two groups based on the site and mode of collection and the extent of contamination.

- Aseptically collected specimens, usually free from other microorganisms (sterile) – fluids like spinal, pleural, pericardial, synovial, ascitic, blood, bone marrow, tissues (lymph node, tissue biopsies) and fine needle aspirates (FNAs)
- Specimens contaminated by normal flora or specimens not collected aseptically (not sterile) – gastric lavage, bronchial washings, urine , pus and stool (in case of disseminated TB in HIV infected patients and infants)

2. Collection of extra pulmonary specimens

Body fluids (spinal, pleural, pericardial, synovial, ascitic, bone-marrow) should be aseptically collected in a sterile container by the physician using aspiration techniques or surgical procedures. Specimens should be transported to the laboratory as quickly as possible.

2.1 Pleural fluid

Considered a suboptimal specimen as tubercle bacilli are mainly in the pleural wall and not within the fluid. The minimum volume for pleural fluid required for processing for culture is 20–50ml. The fluid is collected using pleural tap or thoracocentesis.

2.2 Pericardial fluid

Should be collected using ultra sonogram

2.3 Blood

Blood as a specimen for isolating *M. tuberculosis* should be generally discouraged for the low diagnostic yield and high possibility of contamination with respect to the technique required for its culture. However, if there are specific indications when a physician suspects disseminated TB in a HIV infected patient, blood can be collected provided, the culture systems for recovery of

mycobacteria is available in that laboratory (BacTAlert, MB Bact or MycolyticF medium on BACTEC 9050 systems)

2.4 Tissues

The aseptically collected tissues are placed by the physician in sterile containers preferably without fixatives or preservatives. If the specimen is to be shipped, it should be protected from drying by adding sterile saline or ideally in selective Kirchner's liquid medium and maintaining a temperature of 4- 15^0C. Specimens should be transported to the laboratory as quickly as possible.

2.5 Swabs

Swabs are always sub optimal specimens and not recommended because of risk of infection for specimen collector. They may be useful in children and patients who cannot produce sputum or may swallow it. A sterile absorbent cotton swab should be used for collection. The best time for the collection is early morning before food and drinks are taken. The swab should be placed in a screw capped container containing normal (0.9%) saline to prevent drying. Swabs except for laryngeal swabs or from discharging sinus should be avoided.

2.6 Urine

Among specimens expected to be contaminated, urine is the most common. To minimize excessive contamination of urine specimens, special instructions for collecting urine with adequate cleansing of external genitalia to prevent contamination by commensals should be given. Early morning sample should be collected in 500 ml screw capped sterile containers. Once received in the laboratory, urine must be immediately processed or centrifuged and the pellet refrigerated for further processing. As excretion of tubercle bacilli in urine is intermittent, three early morning specimens must be collected on different days.

2.7 Bronchial secretions

Other respiratory specimens that can be submitted to the laboratory for mycobacteria culture are bronchial secretions (minimum volume: 2- 5ml) and bronchial alveolar lavage (BAL) (minimum volume of 20 – 50 ml). Trans-bronchial and other biopsies should be collected under sterile conditions and placed in 0.5-1.0 ml of sterile normal (0.9%) saline to prevent drying during transportation to the laboratory.

2.8 Gastric Lavage

In children, who rarely produce sputum, the aspiration of the early morning (gastric content) may be used for TB diagnosis. This is done as an inpatient procedure. This should be transported immediately to the lab and processed (nor more than 4 hours) to prevent the killing action of the acid content in the gastric lavage on the tubercle bacilli. In the event of delay, the sample can be neutralised using 1-2 ml of sterile 10 % sodium bicarbonate solution depending on the volume of gastric aspirate. Trisodium phosphate at a final concentration of

25% can be used but it may affect the viability of tubercle bacilli with prolonged storage.

NOTE:

- Samples for culture should **never be** collected in formalin.
- If histo pathological examination is required, two samples should be collected
- No preservative should be used for any extra-pulmonary specimen for culture. Necessary instructions are to be given to the concerned staff for sending the biopsy specimen in normal saline for culture and NOT IN FORMALIN as it will kill the bacilli.
- Extra pulmonary specimens should never be collected or transported in CPC.

3. TRANSPORTATION OF EXTRA PULMONARY SPECIMENS

As for pulmonary samples, extra pulmonary specimens will need to be transported in cool boxes which maintain temperatures below 20^0C for specimens to be compatible for solid, liquid culture systems as well as molecular methods. Triple packing system should be utilised for transportation. All precautions that are followed for transporting pulmonary samples should be followed. For sending material across international or state boundaries this container may have to be packed in the same way with an additional outer container; in such cases, special administrative arrangements with postal authorities and/or airlines may be necessary.
When sending out specimens or when receiving them, check that:

- Request forms are located separately from the specimen containers
- Containers are labelled not on the cap but on the wall of the container
- Each transport box has an accompanying list which identifies the specimens and the patients; the information on the specimen containers should correspond to that on the accompanying list.
- Accompanying list contains the necessary data for each patient
- Date of dispatch and particulars of the health centre are on the accompanying list.

3.1 Specimens and request forms

All specimen transported to the laboratory must be accompanied by the request form for C & DST in hard and soft copy formats (See C & DST request form). For quality control reasons, the tests must be performed only upon written request of authorized persons and oral requests without follow up written instructions should not be allowed. It is also important that specimen request forms are kept separate from the specimens themselves. Forms that have been contaminated by specimens should be sterilized by autoclaving. If mistakes in filling request forms and labelling of specimens are found, reject specimens and do not register them. Document the arrival of specimens in the laboratory and note any delays in

delivery in the remarks column of the specimen register and on the report form, particularly for negative/contaminated results. The packaging material should be autoclaved before discarding.

4. REGISTRATION OF SAMPLES

4.1 Receipt of incoming specimens

For safety and work-flow reasons, specimens should be received in the office area of the laboratory and delivery boxes should be opened using all the applicable biosafety procedures inside the lab.

To minimize risk of infection, the following procedures should be applied:

1. The specimen package received should be opened only in a biosafety cabinet which may be located in a small area within the reception or in the culture room, as they could potentially be MDR or XDR Tuberculosis. (DO NOT OPEN ON AN OPEN BENCH AT THE LAB RECEPTION)
2. Before opening the packet, inspect the delivery box for signs of leakage; if there is gross leakage evident, discard the box by autoclaving or burning; do not try to open and retrieve any specimen.
3. If on gross inspection there is no leakage, disinfect the outside of the delivery box using cotton wool or paper towels saturated with a suitable disinfectant (5% phenol)
4. Open carefully and check for cracked or broken specimen containers or leakage within the packaged container. If there is minimal leakage without any gross loss of specimen, they may be processed with an asterix that leakage was noted on receipt. (This will assist in identifying reasons for contamination used in lab performance indicators). In case of gross leakage, with only very little sample being available, accept the sample and process after carefully making a note of the same – as extrapulmonary specimens are precious and repeat collection may not be possible.
5. Check labelling of specimens with individual identification numbers and correspondence with numbers on the accompanying list or Clinical information forms (CIF) that are accompanying the specimens.
6. Disinfect the inside of the delivery box, wash hands after handling specimen containers
7. Autoclave the packaging material before discarding.
8. Assign unique lab serial number to each patient.
9. Evaluate the quality of specimens and make a note as to volume (in case of fluids), leakage, blood mixed etc. Always register the incoming specimen in the laboratory register; each specimen receives a serial number that should be used to label every test for the specimen. Other data that should be reported on the laboratory register are: the date of the receipt of the specimen, patients name, age, sex and address, the name of the referring health centre, the reason for DST. The signature (with the name in capitals) of the person requesting the examination should always be present.

4.2 Decontamination of extra pulmonary samples

Most of the extra pulmonary specimens are paucibacillary in nature. Hence, they require milder decontamination. When using solid culture for primary isolation of tubercle bacilli from these specimens, it is preferable to use multiple media including one liquid medium made selective by the use of specific antibiotics that inhibit the growth of other micro organisms. The media include, LJ, LJ with sodium pyruvate (LJ–P) and selective liquid Kirchner's medium (SK). Sodium pyruvate facilitates the growth of *M. bovis*. Antibiotics incorporated in the liquid medium include polymixinB, amphotericin B, carbenicillin and trimethoprim (PACT) and vancomycin.

Preparation of media

LJ MEDIUM WITH SODIUM PYRUVATE

LJ medium is enriched with 0.5% sodium pyruvate. In the preparation of the mineral salt solution, glycerol is omitted and 8.0g sodium pyruvate is added for every 600 ml. This is added to 1 litre of egg fluid, mixed well and distributed.

SELECTIVE KIRCHNER'S MEDIUM (For culture of extra-pulmonary specimens**)**

Disodium hydrogen phosphate, $Na_2HPO_4.12H_2O$, A.R.	19.0 g (7.5g of anhydrous salt)
Potassium dihydrogen phosphate, KH_2PO_4, A.R.	2.0 g
Magnesium sulphate, $MgSO_4.7H_2O$, A.R.	0.6 g
Sodium citrate	2.5 g
L-asparagine	5.0 g
Casein hydrolysate (Bactocasitone)	0.5 g
Glycerol	20.0ml
Phenol Red, 0.4% solution	3.0 ml
Distilled water, to	1 litre

Check pH to 6.9 – 7.2

Autoclave at 15 lbs/15 minutes

Then add aseptically the following:

Polymyxin B (20,000 units)	31 mg
Carbenicillin	100 mg
Trimethoprim	10 mg
Amphotericin B, solubilised	10 mg

Dissolve the above in 5 ml sterile distilled water before addition

Also, add sterile calf serum 100 ml

Mix the above carefully and distribute, under sterile conditions, in 10 ml amounts. Check sterility by overnight incubation at 37°C and store in the cold.

5. CULTURE BY SOLID CULTURE METHODS

5.1 CSF and pericardial fluid

Smear:

1. Label a clean dry slide with the lab number and place the slide and the sample container inside the cabinet
2. Mix well and aseptically remove one loopful of the fluid and place in the centre of the slide; close the container and allow the drop to air-dry
3. Place one more drop of the CSF on the same spot and let dry.
4. Place the third drop after processing the sample as below:

Culture:

Culture of CSF is done in two steps:

1. Direct inoculation in media 2. Inoculation after decontamination

Direct

1. Place one loopful of CSF on to one slope each of LJ and LJ-P
2. Add 0.2 ml of CSF in to one bottle containing SK medium
3. Label the set as 'A'

Decontamination

1. Add 1ml of 5% H_2SO_4 to CSF
2. Mix well and let stand for 15 minutes
3. Fill the container with sterile distilled water and centrifuge at 3000 x g for 15 minutes
4. Aspirate the supernatant carefully without disturbing the deposit or discard carefully in to a disinfectant bin containing 5% phenol or any other mycobactericidal solution
5. Inoculate one slope each of LJ and LJ-P with one loopful of deposit for each slope
6. Transfer the remaining deposit in to one bottle of SK
7. Label the set as 'B'
8. Incubate both set A and B at 37°C

5.2 BAL

1. Make a direct smear
2. Process using 5% H_2SO_4 as in CSF
3. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit using 5mm twisted wireloop
4. Transfer the remaining deposit in to one bottle of SK
5. Incubate the slopes and SK medium at 37°C

5.3 Gastric Lavage

1. Gastric Lavage should be processed immediately upon arrival in the lab to prevent the killing action of the gastric pH (due to HCl) on the tubercle bacilli
2. Make a direct smear and process by modified Petroff's method
3. Place one drop of the final pellet on the direct smear
4. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
5. Transfer the remaining deposit in to one bottle of SK
6. Incubate the slopes and SK medium at 37^{o}C

5.4 Tissue / Biopsy

1. Ideally, biopsy specimens should be collected and transported in SK medium
2. Carefully place the tissue inside a sterile petriplate inside the BSC
3. Using sterile scissors and forceps, cut the tissue in to tiny pieces
4. Transfer to a sterile tissue grinding tube – add a little water to the petriplate to facilitate transferring
5. Add sterile distilled water to the tube (not more than 5 ml)
6. Homogenise using a sterile Teflon grinding rod using a foot operated tissue grinder
7. Make a direct smear from the homogenate
8. Centrifuge the homogenate at 3000 x g for 15 minutes
9. Decant the supernatant carefully in to the disinfectant bath
10. To the deposit add 1 ml of sterile distilled water
11. Add one drop to the direct smear, air dry, fix and stain
12. To the remaining pellet, add 1ml of 5% H_2SO_4
13. Proceed as for CSF
14. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
15. Transfer the remaining deposit in to one bottle of SK
16. Incubate the slopes and SK medium at 37^{o}C, along with the SK medium used for transporting

5.5 Fine Needle Biopsy specimen

1. Fine needle specimens should be collected and transported only in SK medium or any other liquid medium
2. The medium is incubated as such at 37^{o}C, since only a very tiny piece of the tissue is obtained as sample

If the sample is received without SK

1. Add the contents of two SK medium bottles to the specimen
2. Shake vigorously and let stand for 10 minutes
3. Divide the medium in to two aliquots and incubate both at 37^{o}C

5.6 Pus

1. Make a direct smear, air dry, fix and stain

2. If the pus is thick or purulent, process by modified Petroff's method using 4% NaOH
3. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
4. Transfer the remaining deposit in to one bottle of SK
5. Incubate the slopes and SK medium at 37°C
6. If the pus is thin or dilute, proceed with decontamination using 5% H_2SO_4
7. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
8. Transfer the remaining deposit in to one bottle of SK

5.7 Urine / Ascitic fluid

1. Distribute the entire specimen in to 20 or 40 ml volumes in Universal containers / Falcon tubes inside a BSC
2. Centrifuge at 3000 x g for 15 minutes

Process the supernatant and deposit independently as follows:

Supernatant:

3. Aspirate carefully 1ml of the top layer from each tube and pool
4. Process by 5% H_2SO_4 as for CSF
5. Transfer 1ml of the final supernatant on to two bottles of SK each – Label the set as DSS (Decontaminated Supernatant Supernatant)
6. Decant the supernatant carefully in to the disinfectant bath
7. From the deposit transfer about 0.2 ml and the remaining in to 2 bottles of SK respectively – Label as DSD (Decontaminated Supernatant Deposit)

Deposit:

8. Pool all the deposit in to one tube
9. Process using 5% H_2SO_4 as for CSF
10. Inoculate two slopes each of LJ and LJ-P with one loopful of deposit for each slope
11. Transfer the remaining deposit in to one bottle of SK

5.8 Swabs:

If two swabs are available, use one for smear and one for culture; if only one is available do only culture

1. Immerse the swab in 5 ml of 4% H_2SO_4 for 1 minute
2. Transfer the swab to another tube containing 5 ml of 4% NaOH
3. Directly inoculate two slopes each of LJ, LJ-P
4. Transfer the swab finally to a tube containing SK medium
5. Incubate all tubes at 37°C

5.9 Culture Reading

1. Read all cultures used for isolating *M. tuberculosis* from extrapulmonary specimens every week for up to 8 weeks using the same methodology used for pulmonary samples
2. If the solid media show typical growth report immediately after confirmation

3. Read SK medium up to 6 weeks
4. MTB appears as whitish granular or flaky growth that settles down at the bottom
5. If the SK medium shows growth or contamination (in the form of turbidity) within 6 weeks, decontaminate as sputum by modified Petroff's method and inoculate deposit on LJ medium alone and read up to 8 weeks
6. Even if the SK medium shows no growth within 6 weeks, proceed with decontamination using modified Petroff's method and inoculate deposit on LJ medium alone and read up to 8 weeks
7. If LJ shows typical MTB growth within 8 weeks, report immediately after confirmation
8. Report as negative only after LJ completes 8 weeks (a total of 14 weeks)

6. Processing of extra pulmonary samples for MGIT960

Isolation of M. tuberculosis by MGIT system requires the final inoculum to be in an ideal condition that will not interfere with the fluorescence.

6.1 Pus and other muco-purulent specimens

1. Thick pus of volume >10 ml is decontaminated using the NALC – NaOH method as sputum
2. If the volume is < 10 ml, either aliquot and process only 10 ml by NALC – NaOH method or concentrate the initial volume by centrifugation for 15 – 20 minutes and resuspend the pellet in 5 ml of sterile distilled water. If the pus is too thick, add about 50-100 mg of NALC powder; mix well and decontaminate using NaOH. Resuspend the final pellet in buffer to reduce the pH
3. If the pus is not thick, decontaminate using 2-4% NaOH. The concentration of NaOH can be changed based on the expected level of contamination in the specimen which depends on the site of collection

6.2 Gastric aspirates

1. Distribute the volume in smaller aliquots and centrifuge the tubes at 3000 x g
2. Pool the deposits, add 5ml distilled water and decontaminate it using NALC-NaOH or 2-4% NaOH

6.3 Bronchial washings

1. Process using NALC-NaOH like sputum
2. If the specimen is >10 ml in volume, process the whole specimen.
3. If <10ml, concentrate the specimen by centrifugation (3000x g, 15-20 minutes)
4. Add 5 ml sterile water to the pellet and decontaminate as for sputum

6.4 Laryngeal swabs

1. Transfer the swab into a sterile centrifuge tube and add 2 ml sterile water.
2. Add 2 ml of NaOH-NALC solution and mix well in a vortex mixer.

3. Let stand for 15 minutes. Remove the swab with forceps, squeezing the liquid out of the swab and discarding it.
4. Fill the tube with phosphate buffer and mix
5. Centrifuge at 3000xg for 15-20 minutes.
6. Discard the supernatant fluid and resuspend the sediment in 1-2 ml sterile buffer. Use this suspension for smear and culture.

6.5 Tissue

1. Homogenize the tissue in a tissue grinder with a small quantity of sterile saline or water (2-4 ml).
2. Decontaminate the homogenized specimen using NALC-NaOH procedure as in sputum.
3. Resuspend the sediment with phosphate buffer
4. If the tissue grinder is not available, use a mortar and pestle.
5. Tissue may also be placed in a Petri dish with sterile water (2-4 ml) and be torn apart with the help of two sterile needles.

6.6 Urine

Isolation of mycobacteria from urine specimens using MGIT has not been validated.

1. Aliquot the entire volume in several centrifuge tubes
2. Concentrate the specimen by centrifugation for at least 20-25 minutes
3. Resuspend the pellet in each tube with 1-2 ml of sterile water and pool together
4. Decontaminate using 4% NaOH as for sputum

6.7 Other body fluids (CSF, synovial fluid and pleural fluid)

As these fluids are collected usually under aseptic conditions, they require only milder decontamination

1. If the specimen volume is more than 10 ml, concentrate by centrifugation at about 3000x g for 15-20 minutes
2. Liquefy thick or mucoid specimens prior to centrifugation by adding NALC powder (50-100 mg).
3. Resuspend the sediment in about 5 ml of saline
4. Mix and decontaminate as for sputum

6.8 Blood

Isolation of mycobacteria from blood specimens by MGIT 960 has not been evaluated thoroughly. A few published studies have used blood after lysis centrifugation. Ideally BACTEC Myco/F Lytic medium is recommended for isolation of mycobacteria from blood samples.