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Introduction
This guideline titled ‘Laboratory Guideline for Drug Resistance Surveillance for Tuberculosis’ is brought out by National Tuberculosis Reference Laboratory, Public Health Laboratory. This guideline is intended for laboratory personnel involved in collection of sputum samples from the patient diagnosed as smear positive Pulmonary TB for surveillance study purposes.
This guideline will guide laboratory personnel on how to collect sputum samples, storage, packaging and transport. It will also provide information, in brief, on documentation as well.
This guideline will be updated as and when deemed necessary in accordance with the standard operating procedure for revision of protocol guidelines which is in compliance with good clinical laboratory practices (GCLP).
We hope that this guideline will be helpful to all the laboratory staffs involved in this surveillance. Any suggestions on improving this guideline can be sent to ntcpphl@yahoo.com.

Content Overview
By reading this guideline the laboratory staff will be able to:
1. Know how to instruct patient on proper sample collection required for diagnosis of Tuberculosis.
2. Personal safety during and after sample collection.
3. Sample collection and storage.
4. Specimen packaging and transport.
5. Documentation
6. Use of preservative- Cetyl pyridinium chloride-Sodium chloride method.
1. Background

Tuberculosis is a major Public Health concern in the world predominantly caused by *Mycobacterium tuberculosis*. Tuberculosis is an air borne infection transmitted by droplet nuclei created through coughing by untreated persons suffering from pulmonary tuberculosis in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons. Pulmonary tuberculosis usually occurs in the apex of the lungs. These develop cavities which contain large populations of tubercle bacilli that can be detected in a sputum specimen. Pulmonary tuberculosis is suggested by persistent productive cough for three weeks or longer, weight loss, night sweats and chest pain. Comprehensive biosafety procedures are required in each laboratory, especially where there is direct contact with smear positive patient and where aerosol-producing procedures are being carried out on sputum samples.

2. Intake of patients-Inclusion criteria

A patient registered as a smear positive case (*new* or *previously treated*) according to WHO/IUATLD definition of smear positive should be included. Children under the age 15 years who meet the inclusion criteria should also be included.

Definition:

* New cases*: for the purpose of surveillance, resistance among new cases is defined as the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning denies having had prior anti-TB treatment (for more than 1 month), OR, has no evidence of such history from adequate documentation if available with patients or hospitals.

** Previously treated cases**: for the purpose of surveillance, resistance in a previously treated patient is defined as the presence of resistant strains of *M. tuberculosis* in a patient who, in response to direct questioning admits having been treated for tuberculosis for 1 month or more, OR, has evidence of such history from adequate documentation if available with patients or hospitals.

Case definition “New cases”

Patients with at least two sputum smears positive for AFB under direct microscopy who has no history of past tuberculosis infection.

OR

One sputum specimens positive for AFB and radiographic abnormalities consistent with PTB who in response to direct questioning denies having had any prior treatment (more than one month) or adequate document available for whom there is no evidence of previous TB infection of treatment history.

3. Specimen for Tuberculosis

Sputum specimen

Although M. tuberculosis is capable of causing disease in almost any organ of the body, more than 85% of tuberculosis disease in high prevalence area is pulmonary. Therefore, sputum is the specimen of choice in the investigation of tuberculosis and should always be collected. Sputum specimens are easier to collect and moreover a single good-quality sputum specimen may suffice for culture and isolation.
4. When to collect the samples?
For optimal results, obtain specimens under the following conditions:

- Collect sputum specimens before chemotherapy is started; even a few days of drug therapy may kill or inhibit sufficient numbers of bacilli which will reduce the chance of culture Positivity.

- Collect a series of two single, early morning sputum specimens on successive days. The single specimen is easier to handle in the laboratory and less likely to be contaminated during shipment.

5. Safety Considerations
Transmission of TB occurs through micro-aerosols that contain tubercle bacilli. The laboratory personnel collecting samples for tuberculosis must follow the universal precautions before and after collection of samples. Good laboratory methods aim to minimize formation of droplet nuclei. **Assume that all specimens are potentially infectious.**

In addition the laboratory personnel must follow the instructions below for keeping others and themselves safe.

To prevent laboratory staff from acquiring infection, the staff must:
- Wear personal protective equipment which includes apron, mask and disposable gloves.
- Use appropriate respiratory protection, while in direct contact with patient and during procedures in which aerosols may be produced.
- It is better not to perform all the paper works and sample collection in the same place.

6. Specimen collection

6.1. Requirements

1. Sputum container
2. 1% CPC (Cetylpyridinium chloride)
3. Gloves
4. Mask

6.2. The Procedure

6.2.1. Pre-collection procedure-Patient Instruction

- Give the patient confidence be explaining him/her the reason for sputum collection.

- Instruct the patient to rinse mouth with water before producing the specimen. This helps to minimize residual food particles, mouthwash and oral drugs that might contaminate the specimens or inhibit growth of any acid-fast bacilli present.

- Remember that saliva and nasopharyngeal discharge are not sputum. Collect only the exudative material brought up from the lungs after a deep, productive cough.

- Specimen to be collected in laboratory-approved containers only that is clean, sterile, one-use, plastic, disposable containers. Put a mark at the 5 ml level on the sputum container (if
not already marked) to show the patient the minimum amount of sputum needed. (Consider 5 to 10 ml an adequate amount.)

- Provide the following guidance to patient on how to collect the sputum sample.
  - Instruct the patient to take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Ask him/her to breathe in a third time and then forcefully blow the air out.
  - Place open container close to the mouth. Ask him/her to breathe in again and then cough during exhalation & to spit the sputum gently into the container.
  - Encourage patient to cough again until a satisfactory specimen is obtained. Remember that many patients cannot produce sputum from deep in the respiratory track in a few minutes. Give him/her sufficient time to produce an expectoration which s/he feels is produced by a deep cough.
  - Demonstrate to patient how the container should be securely closed after collection.
  - Make sure that patient understands that a specimen must be produced as soon as s/he wakes up in the morning.
  - Instruct the patient to bring the specimen back to laboratory. Make sure that two samples on consecutive days are collected per patient for shipment.

6.2.2. Receiving Sample in Laboratory

- Wear disposable gloves during receipt and inspection of incoming specimens.
- Inspect the container for signs of leakage and check for any cracks or broken container.
- Make sure that specimen collected is of correct type.
- Label the side of container with patient name, age/sex, Lab ID number, specimen type & date of collection.
- Re-check that specimens have been adequately labelled with individual identification numbers & that correspond with the number on the accompanying form.
- Sample should be kept in refrigerator at 4°C after collection.

7. Microscopic Examination (Using Ziehl Neelsen staining technique)

Prepare smears from the two samples collected from a single patient and examine using ZN staining technique. Record the results of the smear in the Sputum Shipment Form.

8. Storage

All samples for culture and Drug sensitivity testing should be stored at 4°C after collection and shipped to Public Health Laboratory within 48 hours. Make sure that the caps of sputum containers are secure tightly and place the individual sputum container in a self sealing plastic bag during storage. It should not be placed along with the reagents and other chemical meant for other diagnostic tests. Arrange specimens in serial order based on Lab ID number in storage tray.

Hospitals far from PHL who are not able to ship the samples within 48 hours must store samples below 0°C till they are transported and they could be sent to PHL once a week. Add equal amount of 1% Cetylpyridinium chloride (CPC) to the sputum just before they are packed for transport. Refer Annexure 1 for detail on Cetylpyridinium chloride.
9. Sample packaging
Sputum samples for diagnosis of Tuberculosis falls under category A and must be packaged appropriately in accordance with category A (UN2814). The samples should be transported using cold chain (without preservatives).
This provides the required containment of the sample which can minimize risk of infection during transport and laboratory personnel handling the samples package. Moreover appropriately packed samples are more likely to arrive at its destination intact, uncontaminated, and sample in good condition which ultimately yields a good culture Positivity.

9.1. Triple Packaging System
All the sputum samples must be packaged using triple packaging. This packaging method provides three layers of containment to protect the substance being shipped. It consists of primary receptacle, secondary receptacle and outer containers (see figure below)

9.2. Requirements
1. Primary receptacle (sputum containers)
2. Secondary receptacle
3. Absorbent paper ( tissue paper)
4. Outer box
5. Samples

9.3. The Procedure
1. Make sure that the sputum samples placed in a primary container are secured tightly and labeled with proper identification numbers.
2. Wrap the container with absorbent material (e.g. absorbent cotton or tissue paper) to absorb the accidental leakage, place in a suitably sized self sealing plastic bag and seal the bag. Two specimens
from the same source may be placed in a larger plastic bag in batches and sealed. Specimen from a different source must not be placed in the same bag.

3. Place the sealed primary containers inside a secondary self-sealing plastic container and seal it. Specimens from several sources may be packed inside the same secondary plastic container.

4. Place additional absorbent material inside the secondary container to cushion and to absorb any leakage that may occur.

5. Put ice packs around the primary containers inside secondary container and close the secondary container. *(Do not use ice packs if 1% Cetylpyridinium chloride is added to sputum samples as preservative).*

6. Place the Sputum shipment form & Clinical Information Form sealed in a plastic bag to the outside of this secondary container and put into TB mailing container.

7. Close the out container and seal it with cello tapes.

8. Ensure the box is correctly labeled with the delivery address and contact numbers.

**10. Transport of samples**

Upon packing the samples appropriately in accordance with the instruction provided above, the laboratory technician should arrange to ship the samples to PHL. Specimens should be transported to PHL as soon as possible after collection. If a delay is unavoidable the specimens should be refrigerated (below 0°C) to inhibit the growth of unwanted micro-organisms. If the sputum specimens can be kept refrigerated they could be sent to PHL once a week. Make sure that the transport temperature is maintained at 2-8°C and the samples are shipped to PHL with proper cold chain within 48 hours.

If the shipment is delayed beyond 48 hours, specimens can be transported at ambient temperatures, chemical preservation may be used. The following method provides reasonable results:

> Mixing the fresh specimen with an equal volume of 1% cetyl pyridinium chloride in 2% sodium chloride just before packaging and transport the sample at ambient temperature [20°C to 25°C] without adding ice packs. Tubercle bacilli will survive for up to a week, while the growth of unwanted organisms will be restricted.

However, speedy transportation is essential for good results.

Clinical Information Forms and Sputum Shipment forms should be located separately from specimen containers. With each transport box an accompanying forms must be prepared which identifies the specimens and the patients from whom the specimens were collected. Before dispatch from the health centre the following must be verified:

1. That the number of specimen containers in the box corresponds to the accompanying forms.

2. That the identification number on each specimen container corresponds to the identification number on the accompanying form (sputum shipment form).

3. That the accompanying forms contain the necessary data for each patient.

4. That the date of shipment and the particulars of the health centre are on the accompanying form.
11. Documentation
While collecting the samples the Laboratory personnel should appropriately fill in all the required information. Along with the Clinical Information Form (To be completed by TB Unit Incharge), Sputum Shipment form (To be completed by Laboratory personnel) must also be filled. The examples of both forms are given below. It is the duty of laboratory technician to cross check Clinical Information form whether they are filled completely or not. If the information is not completed by the TB Unit Incharge, the laboratory should inform the concerned TB Incharge about the same.

A photocopy of Sputum shipment Form must be retained and filed in the respective hospital laboratories.
CLINICAL INFORMATION FORM

District: .................................................................

Hospital: ............................................................... Code: ..................................................

A. IDENTIFICATION OF THE PATIENT

Name: .................................................................................................................................

TB district number: ................. Date registered (DD/MM/YY): .......... / ........ / ..........

Sex: Male / Female Age: ........... years

Date of sputum collection: A: ...... / ...... / .......... B: ...... / ...... / ..........

B. HISTORY GIVEN BY THE PATIENT

B1. Previously treated for TB? No [___] Yes [___]

If the answer is no, go to B2, if yes, go to B3.

B2. Standardized history

• For how long have you been sick? .................................................................

• Did you have the same symptoms prior to this episode? .........................

• Did you have other symptoms of lung disease prior to this episode (haemoptysis, chest pain, cough)? .......

• Did you have X-ray examinations prior to this episode? .........................

• Did you have sputum examinations prior to this episode? .................

• Did you ever take tuberculosis drugs for more than one month? ............

If yes, what was the name? .............................................................................................
• Did you ever have injections for more than one month? …………………

• Have you ever been incarcerated? ……………………………………………

• Did you ever test yourself for HIV? ……………………………………………

If yes, what was the test result? ………………………………………………………

Did the patient remember previous treatment for TB after these questions?

No [ ] Yes [ ]. If the answer is yes, continue with B3

B3. Information about previous treatment

• Where was the patient treated? …………………………………………………

• When was the patient treated? …………………………………………………

• How many times was the patient treated? ………………………………………

• Which drugs were used for treatment? …………………………………………

• By whom was the patient treated? ……………………………………………

• Outcome of the last treatment according to the patient: Cured [ ] Not cured [ ] Unknown [ ]

C. MEDICAL RECORDS

After extensive checking through the medical files and other documents available in the health centre, have you
discovered that the patient has been registered for tuberculosis treatment before? No [ ] Yes [ ]

If the answer is yes, what was the outcome of the last course of chemotherapy: Cured [ ] Treatment

Completed [ ] Defaulted [ ] Failed [ ] Transferred out [ ] Unknown [ ]

Did you find out that the patient has had HIV testing according to the record? No [ ] Yes [ ]

If yes, when was the test done according to the record: (DD/MM/YY). _____ / _____ / _____; and what was the outcome
according to the record? Negative [ ] Positive [ ]
D. FINAL DECISION

D1. Patient has been previously treated for TB for more than a month:

Yes [____] (answer to question B1 or B2 and/or C was 'yes')

No [____] (answer to B1 and B2 and/or C was 'no')

Doubtful [____]

D2. If yes, what was the outcome of previous treatment?

Cured/treatment completed [____]

Failed [____]

Defaulted [____]

Chronic [____]

Relapse/defaulter not distinguishable [____]

Unknown [____]

Responsible Medical Officer: .................................................................
## SPUTUM SHIPMENT FORM

District: ............................................

Hospital ........................................... Code: ............................................

### IDENTIFICATION OF THE PATIENT

District Laboratory Serial Number: ........................................................................

Name: .........................................................................................................................

TB district number: ....................... Date registered: (DD/MM/YY) ........ / ........ / ........

Sex: Male/ Female Age: .......... years

Date of sputum collection: Sample A: ...... / ...... / ........ Sample B: ........ / ........ / ........

Result of smear: Sample A: .......................................................... Sample B: ..........................................................

CPB/CPC added: Yes / No

Shipment prepared by: ...................................................... Date Sample shipped: _____ / _____ / _____

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### Public Health Laboratory Use Only

Shipment received by: ...................................................... Date samples received: ...... / ...... / ........

Date of culture: ...... / ...... / ........

Laboratory Specimen Number: DRS- .................
Preservative: Cetyl pyridinium chloride-Sodium chloride Method

Requirements:

1. Cetylpyridinium chloride
2. Sodium chloride
3. Distilled water

Preparation:

1% Cetyl pyridinium chloride in 2% Sodium chloride:

Dissolve 1 gram of Cetyl pyridinium chloride and 2 gram of Sodium chloride in 100 ml of distilled water. This is the working solution which contain 1% CPC (wt. / vol.) and 2% Sodium chloride (wt. / vol.).

Dissolve with gentle heat, if any crystals are formed in the working solution.

This solution is self-sterilizing and remains stable for an extended period if kept tightly capped and away from excessive heat and light.

Storage:

Store the working solution in amber colored bottle, screw cap tightly and keep it away from heat and light. Label with following detail on the container:

<table>
<thead>
<tr>
<th>Reagent Name: Cetyl pyridinium chloride-Sodium chloride</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration: 1% CPC in 2% NaCl</td>
</tr>
<tr>
<td>Date of preparation: ________________________________</td>
</tr>
<tr>
<td>Expiry Date: _________________________________________</td>
</tr>
<tr>
<td>Storage condition: room temperature (15ºC to 20ºC)</td>
</tr>
</tbody>
</table>

Procedure:

- Collect the sputum specimens into the screw-cap sputum container and add equal volume of CPC-NaCl working solution to each specimen. The final concentration of CPC and NaCl becomes 0.5% and 1% respectively.
- Screw cap the sputum container and shake gently until the sputum appeared liquid.
- Pack the specimen container for shipment using the triple packaging system and transport the specimens at ambient temperature (20ºC to 25ºC).

Advantage:
• It acts as preservative for transport of sputum where Tubercle bacilli remains viable for up to a week, while the growth of unwanted organisms are restricted.

• It also acts as a means of digesting and decontaminating sputa during transit. Cetylpyridinium chloride, a quaternary ammonium compound, is used to decontaminate the specimen while sodium chloride effects liquefaction.

• It allows specimens to be transported at ambient temperature (20ºC to 25ºC) for long duration i.e. up to a week after adding CPC to specimens.

• Sputum specimens treated with 1% CPC-2% NaOH are digested-decontaminated during transit where by need only to be concentrated by centrifugation and the sediment inoculated directly onto LJ media.

• The contamination rates are lower when specimens are treated by the CPC-NaCl method. Furthermore, more chance of isolating other mycobacteria from the specimens.

• Both CPC and NaCl are inexpensive.

Disadvantage:

• In cold places especially in winter where temperature falls below <10ºC, 1% CPC forms crystal where by it is ineffective as preservative and cannot maintain the tubercle bacilli viable and cannot protect samples from contamination during transit.

• Samples with added CPC cannot be transported in Cold chain with ice packs which is used for transporting samples without any added preservative.

• CPC acts as bacteriostatic for mycobacteria inoculated onto agar base medium, therefore the sediments from specimens with CPC can be inoculated only onto egg base medium.

• CPC-NaCl working solution tends to form crystals during storage. (Dissolve with gentle heat if any crystals are formed in the working solution before adding it to specimens).
### List of Laboratory Sites and their code

<table>
<thead>
<tr>
<th>Sl. No.</th>
<th>Name of Hospital Laboratory</th>
<th>District</th>
<th>Code</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Bhajo BHU Gr. I</td>
<td>Wangdiphodrang</td>
<td>LAB-01</td>
</tr>
<tr>
<td>2.</td>
<td>Bali BHU Gr. I</td>
<td>Haa</td>
<td>LAB-06</td>
</tr>
<tr>
<td>3.</td>
<td>Wangdicholing Hospital</td>
<td>Bumthang</td>
<td>LAB-02</td>
</tr>
<tr>
<td>4.</td>
<td>Dagana BHU Gr. I</td>
<td>Dagana</td>
<td>LAB-28</td>
</tr>
<tr>
<td>6.</td>
<td>Damphu Hospital</td>
<td>Tsirang</td>
<td>LAB-25</td>
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<tr>
<td>7.</td>
<td>Dewathang Military Hospital</td>
<td>Samdrupjongkhar</td>
<td>LAB-03</td>
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<tr>
<td>8.</td>
<td>Gedu Hospital</td>
<td>Chhukha</td>
<td>LAB-04</td>
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<tr>
<td>9.</td>
<td>Gelephu R. R. Hospital</td>
<td>Sarpang</td>
<td>LAB-05</td>
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<td>Samtse</td>
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<td>20.</td>
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<td>Sipsoo Hospital</td>
<td>Samtse</td>
<td>LAB-20</td>
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<td>25.</td>
<td>Tashigang Hospital</td>
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<td>LAB-22</td>
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<td>26.</td>
<td>Tashiyan Tse Hospital</td>
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<td>Tencholing Military Hospital</td>
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<td>Trongsas Hospital</td>
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<td>Tsimalakha Hospital</td>
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<td>LAB-24</td>
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<td>31.</td>
<td>Gasa BHU Gr. I</td>
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