Chapter 1

INTRODUCTION

Tuberculosis is an infectious disease caused by the bacillus *Mycobacterium tuberculosis*. It commonly affects the lungs, although it can affect any other organ. Pulmonary tuberculosis cases that excrete tubercle bacilli in their sputum while coughing are the most infectious cases of tuberculosis and the most important source of transmission of infection in the community. Therefore the highest priority for tuberculosis control is the identification and cure of these infectious cases i.e. patients with sputum smears positive for TB bacilli.

Direct smear sputum examination is the most reliable and cost effective tool for diagnosing infectious tuberculosis cases and for monitoring of cases on treatment. Tuberculosis bacteriology is one of the most fundamental aspects of a national tuberculosis control programme and is a key component of DOTS strategy

Purpose of the manual

Sputum microscopy is the primary diagnostic tool for detecting the infectious cases of tuberculosis. Therefore it is essential to have clear guidelines on the standardized techniques and procedures for sputum microscopy to achieve reliable results.

This manual has been prepared for the use of laboratory technicians who are involved in tuberculosis control activities. The guidelines in this manual are based on the National policies for TB control. It consists of the technical aspects of sputum microscopy, procedure for culture and ABST, laboratory management including safety, and general information on tuberculosis and control activities. All personnel involved with tuberculosis control activities in the country should follow these guidelines.

Chapter 2

NATIONAL TUBERCULOSIS PROGRAMME

National Tuberculosis Programme (NTP) is a part of the national health services which functions under the Deputy Director General, Public Health Services (DDGPHS) within the Ministry of Health. National Tuberculosis Programme (NTP) is responsible for the TB control activities in the entire country. Its activities are closely integrated with the general health services.

Overall objectives of the NTP

- To reduce the mortality, morbidity and transmission of tuberculosis in the community until it is no longer a public health problem.
- To prevent the development of drug resistance.

How can we achieve these objectives?

By finding, treating and curing as many people with infectious pulmonary tuberculosis as possible.

What is our strategy?

Sri Lanka has adopted WHO recommended **DOTS** (**D**irectly **O**bserved **T**reatment, **S**hort course) as the main strategy to achieve these objectives.

What is DOTS?

This has five essential components.

- Government commitment to a National TB Programme
- Case detection by sputum smear microscopy of symptomatic patients attending general health facilities
- Short Course Chemotherapy (SCC) for all cases under direct observation of a health worker or a trained person

- Uninterrupted supply of all essential anti-TB drugs
- Monitoring system, which evaluates the treatment outcome of every patient on treatment

What is our target?

- To cure at least 85% of the detected sputum smear positive TB patients
- To find at least 70% of the existing sputum smear positive TB cases

Priority should be given to achieve high cure rates before increasing case finding.

Chapter 3

MICROSCOPY NETWORK IN THE NTP

Tuberculosis microscopy network consists of laboratory services organized at three levels, which are closely linked.

Peripheral level - Microscopy Centres based at health institutions other than District Chest Clinics
These belong to the general health care services and are closely Integrated with the NTP.

Intermediate level - Microscopy laboratories based at District Chest Clinics

Central Level - National TB Reference Laboratory at the centre

In addition to the above-mentioned laboratories, there are sputum collection centres at identified peripheral institutions in each district.

Functions at each level

Microscopy Centres

- Perform sputum smear microscopy using Zeihl-Neelsen method
- Receive, process, and report on sputum samples sent from the sputum collection centres.
- Ensure 3 sputa are examined for diagnosis and 2 during follow up

District Chest Clinic Laboratories

- Perform sputum smear microscopy
- Preparation and distribution of reagents and other laboratory materials to the microscopy centres
- Quality control of microscopy smears performed at the microscopy centers
- Supervision of the microscopy centres and sputum collection centre

- Ensure availability of reagents and slides to perform sputum microscopy
- Ensure that 3 sputa are collected for diagnosis and 2 for follow up
- Check that infected materials are disposed off properly
- Organization of training for laboratory staff at the microscopy centres

National TB Reference Laboratory

- Perform limited number of sputum smear microcopy
- Culture and species identification of Mycobacteria
- Drug susceptibility testing
- Quality control of microscopy smears performed at the District Chest Clinic laboratories
- Training of District Chest Clinic laboratory technicians and assisting them in carrying out the training of staff at the microscopy centres
- Supervision of District Chest Clinic laboratories regarding bacteriological methods and their support activities to the microscopy centres
- Organization of surveillance of primary and acquired mycobacterial drug resistance.

Chapter 4

GENERAL INFORMATION ON TUBERCULOSIS

What is Tuberculosis?

Tuberculosis is an infectious disease caused by the bacillus- ***Mycobacterium tuberculosis***. Tuberculosis commonly affects the lungs causing Pulmonary Tuberculosis but it can affect any other organ in the body.

Mycobacterium tuberculosis

Morphology

Tubercle bacillus is a long, slightly curved, slender beaded rod with rounded ends. It is 1-4μm long and 0.3-06μm thick (Fig.1). They are arranged singly, in pairs, or in small groups.

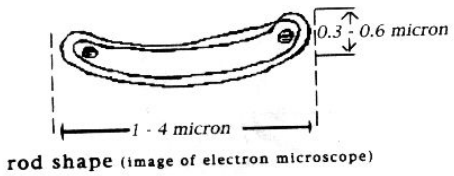


Fig. 1 Mycobacterium Tuberculosis

These bacteria have a cell wall with high lipid content and because of this; they are not easily stained by the usual staining methods. But once stained with stains like carbol fuchsin, they resist decolourisation with acid and alcohol. As such they are referred to as Acid and Alcohol Fast Bacilli (AAFB) often shortened as Acid Fast Bacilli (AFB). They appear microscopically as red or pink beaded rods by the Zeihl -Neelsen method of staining (Plate 1).

Growth

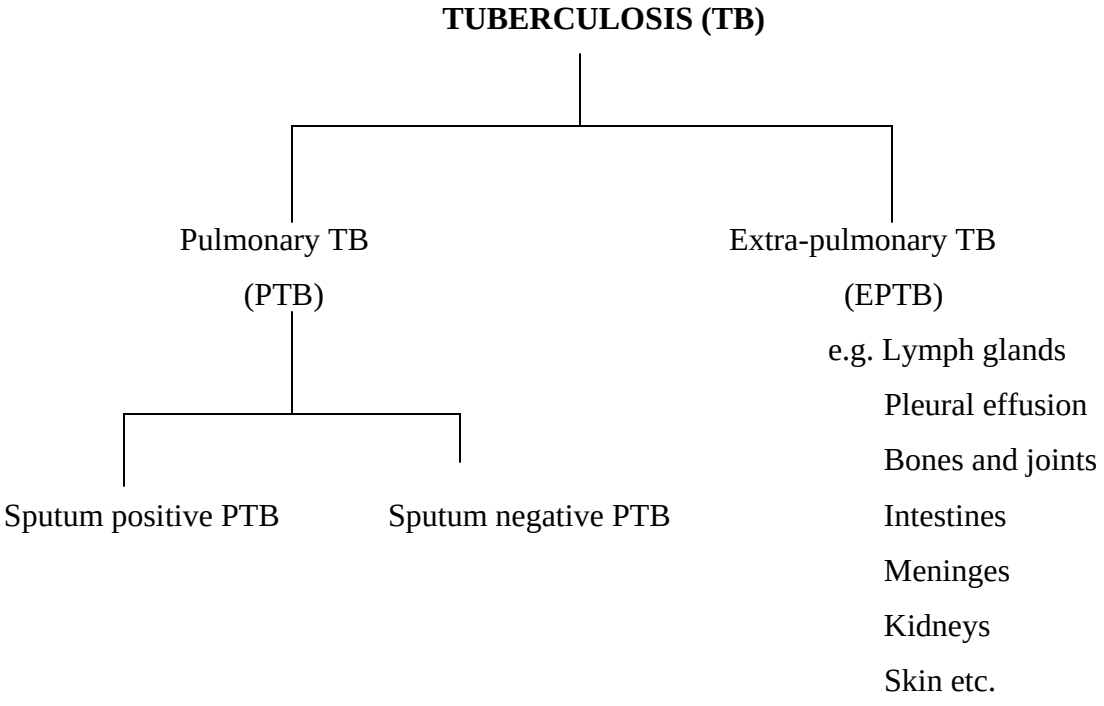
In the laboratory ***Mycobacterium tuberculosis*** grows only in enriched media and has a slow rate of growth. The optimum temperature for the growth of tubercle bacillus is 37°C. The growth is slow and it takes about 2-8 weeks for the colonies to appear.

How does Tuberculosis spread?

TB bacilli usually spread through the air. When a patient with infectious tuberculosis coughs, sneezes, talks or laughs, large numbers of bacilli are expelled into the air in the form of tiny droplets. A person can get infected by inhaling these droplets containing bacilli. These tiny droplets dry rapidly to form droplet nuclei 1-5 microns in size. These can remain suspended in the air for several hours. A person can get infected by inhaling these droplet nuclei containing the bacilli. The number of tubercle bacilli required to initiate infection is low, the infective dose being less than 10 bacilli. Ventilation removes droplet nuclei and direct sunlight quickly kills the bacilli, but they can survive in the dark for several days.

Classification of Tuberculosis

Classification by site of disease and sputum smear result



Pulmonary Tuberculosis

Pulmonary Tuberculosis refers to disease involving the lung parenchyma. This is the most common form of the disease occurring in over 80% of cases.

Sputum Smear-positive Pulmonary Tuberculosis

- A patient with at least two sputum smears positive for AFB by microscopy

OR

- A patient with at least one sputum smear positive for AFB by microscopy and X-ray abnormalities consistent with active pulmonary TB, as determined by a clinician.

OR

- A patient with one sputum smear positive for AFB by microscopy and sputum culture positive for *Mycobacterium tuberculosis.*

Sputum Smear-negative Pulmonary Tuberculosis

- A patient with symptoms suggestive of tuberculosis with at least three sputum smears negative for AFB by microscopy **and** having X-ray abnormalities consistent with active pulmonary tuberculosis **and** no response to a course of broad spectrum antibiotics **and** a decision by a clinician to treat with a full course of anti- tuberculosis therapy

OR

- A patient with at least three sputum smears negative for AFB, but whose sputum culture is positive for *Mycobacterium tuberculosis.*

Extra-pulmonary Tuberculosis

- Tuberculosis of any organ of the body other than the lung parenchyma, and the diagnosis based on clinical and/or histological evidence consistent with active tuberculosis or culture positive for M tuberculosis from a specimen from an extra-pulmonary site.

Classification by history of previous treatment for TB

New

A patient who has never taken treatment for TB

OR

Who has taken anti-TB treatment for less than one month and has not been registered.

Relapse

A smear positive patient who has taken the full course of treatment and declared 'CURED' and has again developed sputum smear positive TB.

Treatment after Failure

A patient who is still sputum smear positive, five months or later after starting treatment.

Treatment after default

A patient who interrupts treatment for two months or more and returns to health services for treatment.

Transfer in

A patient already registered in one district and transferred to another district for continuation of treatment.

Other

A patient who does not fit into any of the above categories, e.g. Smear-positive patient treated and declared cured, comes back with extrapulmonary TB

An extra-pulmonary patient or a smear negative patient completing treatment comes back with a positive sputum or extra-pulmonary TB at another site

Chronic

A patient remaining sputum smear positive after 5 months or moiré after a fully supervised re-treatment regimen

What are the symptoms of Pulmonary Tuberculosis?

- Cough –usually more than three (This is the most common symptom)
- Fever in the evenings
- Night sweats
- Loss of appetite
- Loss of weight
- Haemoptysis (blood stained sputum)
- Chest pain
- Tiredness

Symptoms of Extra pulmonary Tuberculosis

The symptoms depend on the organs involved.

Who is a Pulmonary TB suspect?

A Pulmonary TB suspect is a person who presents with symptoms or signs suggestive of TB, particularly cough of three weeks or more.

How are the infectious TB cases detected?

Detection of AFB in the sputum is the most reliable and cost effective method of confirming Pulmonary Tuberculosis. Any person having a cough of three weeks or more should get his sputum examined for Tuberculosis. When tuberculosis is suspected three samples of sputum should be collected for examination by microscopy.

The chances of finding tubercle bacilli are greater with three samples than with two or one sample. Several studies have shown that examination of two smears will on an average detect more than 90% of infectious tuberculosis cases. The incremental yield of acid-fast bacilli from serial smear examinations has been shown to be 80-82% from the first, 10-14% from the second and 5-8% from the third examination.

Since secretions build up in the airways overnight, an early morning sputum sample is more likely to contain tubercle bacilli than a sample later in the day.

A negative smear result does not exclude the diagnosis of tuberculosis as some patients harbour fewer bacilli than can be detected by microscopy. At least 5,000- 10,000 bacilli should be present in a milliliter of sputum to get a positive result.

It may be difficult for an outpatient to provide three early morning sputum samples. Usually a **spot** specimen on the first visit, an **early morning** sample the next day, and a second **spot** specimen when he comes with the morning specimen, is recommended.
In-patients can provide three early morning sputum samples under supervision in hospital.

Chapter 5

SPUTUM MICROSCOPY

Direct smear microscopy for AFB is the primary diagnostic tool for the detection and control of TB.

Aims of sputum microscopy

The aims of sputum microscopy are to:

1. Diagnose patients with infectious tuberculosis
2. Monitor progress of tuberculosis patients who are on treatment

Advantages of sputum microscopy

- Much more reliable diagnostic tool than X-ray for the diagnosis of infectious TB
- Simple to perform
- Easy to read
- Quick results
- Inexpensive
- Minimal infrastructure required to set up a microscopy centre
- High sensitivity and specificity for detecting **infectious cases.**

Acid Fast staining procedures for microscopy

Zeihl- Neelsen staining

The method of choice for sputum smear microscopy is the Zeihl- Neelsen technique. In this method, when stained with carbol fuchsin the acid-fast organisms appear red against a blue background.

Fluorochrome staining

For this method a fluorescent dye (auramine O, auramine-rhodamine) is used and when examined under a special fluorescent microscope the organisms appear bright yellow against a dark background. The advantage of fluorescent microscopy is that a low magnification objective is used to scan the smear allowing a much larger area of the smear to be seen. Therefore a smear can be scanned in a much shorter time than by the Zeihl Neelsen technique. If the number of smears to be examined exceeds 100, it may be more cost effective to use fluorescent microscopy. However the main disadvantage is the high initial cost of the fluorescent microscope and the running expenditure and the need for uninterrupted power supply.

When should the sputum specimens be collected?

For Diagnosis

Collection of three samples is recommended

(SPOT - MORNING – SPOT)

1. **SPOT** - Supervised spot specimen collected at the first visit
2. **EARLY MORNING SPECIMEN** - Patient is given sputum container to collect an early morning sample on the following morning
3. **SPOT** - Second supervised spot specimen is collected at the time patient comes with the morning sample

For follow up

Follow up sputum examinations are done during the treatment for monitoring the effectiveness of treatment and to declare them as **'CURED'** at the end of treatment. **Minimum of two samples (one collection and one spot specimen)** should be examined at the end of intensive phase, at 5-months and at the end of treatment.

Schedule for follow up sputum examinations

Category	When to do
New smear-positive PTB cases	• End of 2nd month (End of 3rd month if smear is +ve at 2nd month) • End of 5th month • End of 6th month
New smear-negative PTB cases	• End of 2nd month • End of 6th month
Re-treatment cases - Relapse - Failures - Return after default	• End of 3rd month (End of 4th month if smear is +ve at 3rd month) • End of 5th month • End of 8th month

Value of smear examination for AFB on extra-pulmonary specimens

Because Mycobacterium Tuberculosis may infect almost any organ in the body, the laboratory may receive a variety of extra-pulmonary specimens, e.g. body fluids, tissues, pus, and urine. The benefit of microscopy on these specimens is limited because of their paucibacillary nature and due to the presence of mycobacteria other than tubercle bacilli in some of these specimens e.g. gastric aspirates and urine. Therefore extra-pulmonary specimens should be always referred for culture.

Chapter 6

MANAGEMENT OF A LABORATORY

Laboratory Layout

- A separate room or at least a separate area should be reserved for TB microscopy.
- Good ventilation is necessary for the protection of personnel from air borne infectious droplet nuclei.
- Lighting should be adequate.
- Walls, ceilings, floors and bench tops should be smooth, non-absorbent, easy to clean and disinfect and resistant to the chemicals used for microscopy.

A laboratory should have distinct sections for the following (Fig. 2).

- A bench space or a table (A) for incoming specimens (ideally near a window or latch)
- One well lit area for preparing and staining smears (B, C)
- One well lit area for microscopy reading (E)
- One area for recording and reporting (F).

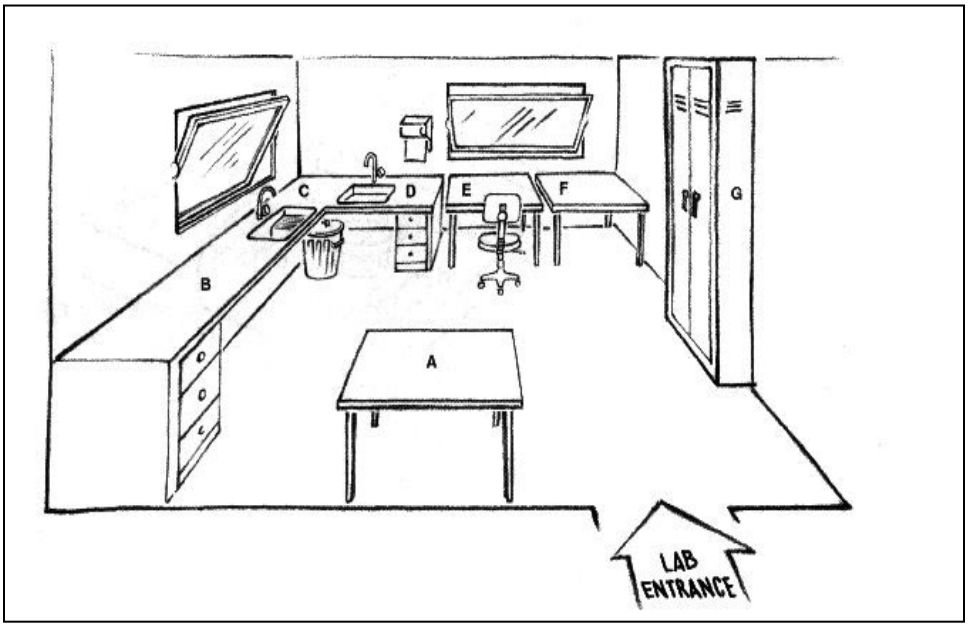


Fig. 2 Laboratory layout

Equipment and supplies for a peripheral microscopy laboratory

Equipment

Binocular microscope

Bunsen burner / spirit lamp

Gas cylinders

Supplies

Sputum containers

Glass slides

Slide holding boxes

Diamond pencil

Marking pens,

Grease pencils

Reagents and chemicals

Carbol fuchsin

Sulphuric acid /acid alcohol

Methylene blue or

Malachite green

Immersion oil

Xylene/ ether & alcohol 7: 3

Methylated spirit

Phenol 5%

Wire loops/wooden sticks

Wire loop holder

Forceps to hold smear slides

Foot operated bucket with lid

Staining rack to hold smears

Slide tray to place slides for drying

Amber reagent bottles (1 litre) - 03

Glass drop bottles (100 ml) - 03

Plastic wash bottle (500 ml) - 01

Plastic oil dropper bottle - 01

Plastic funnel - 03

Filter paper

Lens tissue

Cotton wool, paper towels

Laboratory over-coat, gloves

Timer

Laboratory Register

Laboratory Request Forms

Pens – red and blue/ black.

Additional equipment and supplies needed for a laboratory where reagents are prepared:

Equipment

Chemical balance

Apparatus for distilled water

Supplies

Measuring cylinder (1000 ml) - 01

(100 ml) - 02

Flat-bottomed flask (2 lt) - 03

Plastic funnel (90mm or125mm diameter) - 01

Funnel rack - 01

Reagents and chemicals

Basic fuchsin

Phenol crystals

Methylene blue

Absolute alcohol

HCL / Sulfuric acid

Malachite green

The Microscope

Parts of a binocular microscope

The main parts of a microscope are:

- Eye- pieces
- Microscope tube
- Nose piece
- Objective
- Mechanical stage
- Condenser
- Coarse and fine focusing knobs
- Light source

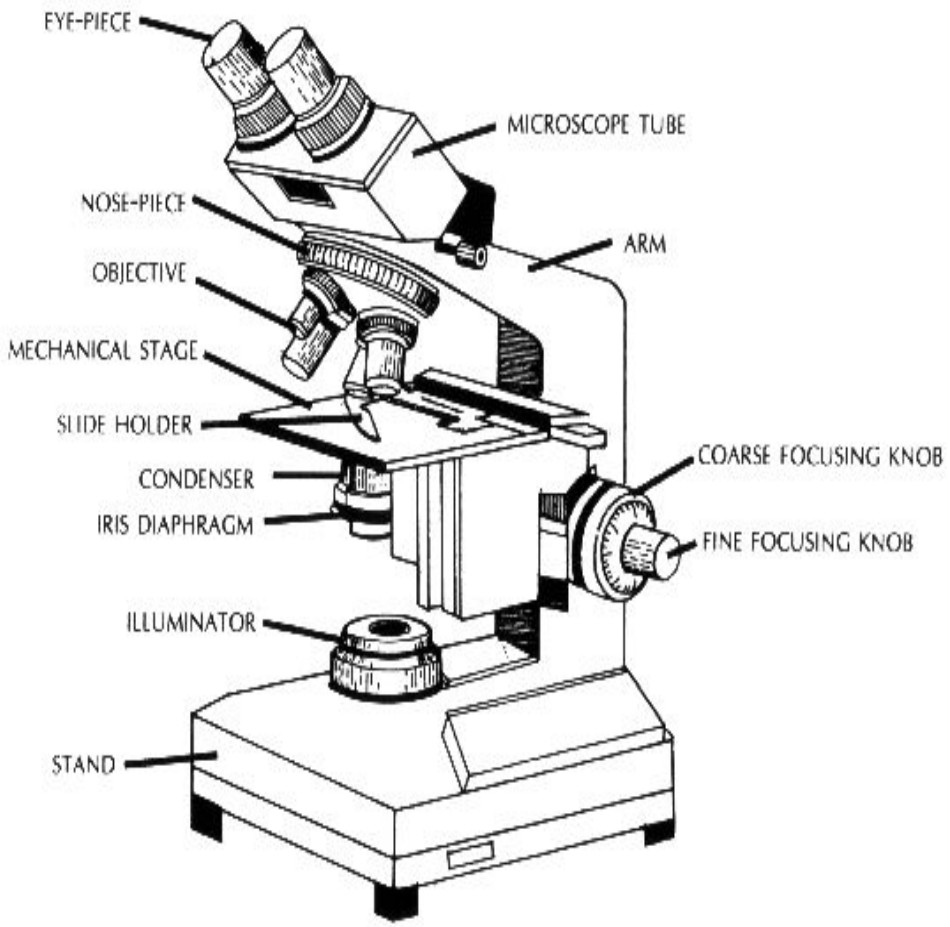


Fig. 3 Binocular microscope

Routine operation of the microscope

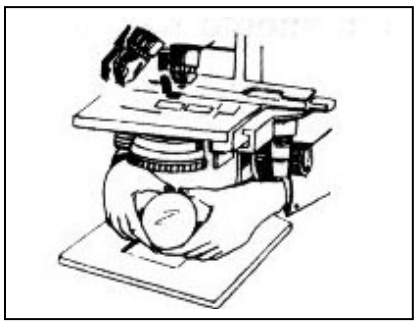


Fig 4.1

- Turn on the light source of the microscope or adjust the mirror if electric light source is not available (Fig. 4.1).

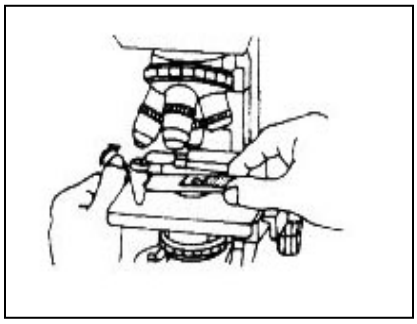


Fig. 4.2

- Place the specimen slide on the stage (Fig. 4.2).

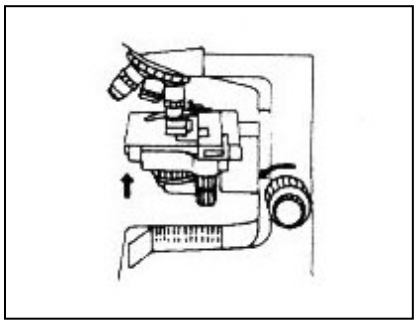


Fig 4.3

- Focus the specimen with 10x objective by turning the coarse focusing knob.
  Make sure the condenser is at its top position (Fig 4.3)

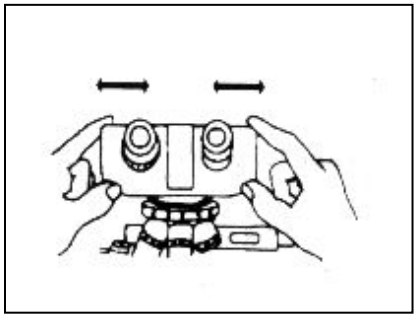


Fig. 4.4

- Adjust the distance between the eyepieces until both right and left images become one (Fig 4.4).

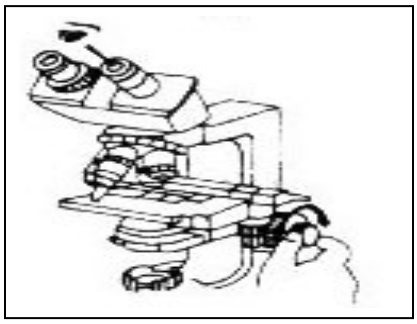


Fig. 4.5

- Focus the image with right eye by looking into the right eyepiece by turning the fine focusing knob (Fig. 4.5).

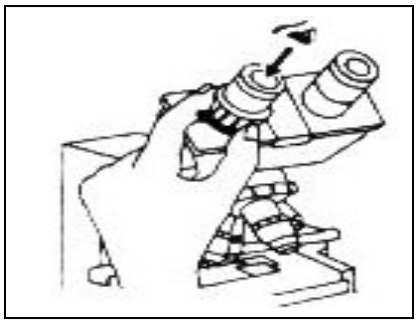
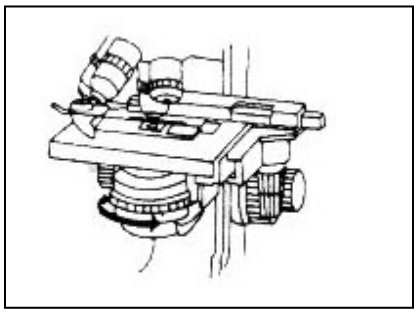


Fig. 4.6

- Focus the image with the left eye by looking into the left eyepiece by turning the dioptre ring (Fig. 4.6).



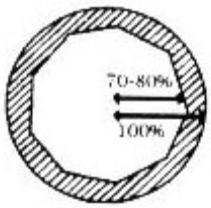


Fig. 4.7

- Open the condenser iris to 70-80 % of the aperture. (Fig. 4.7

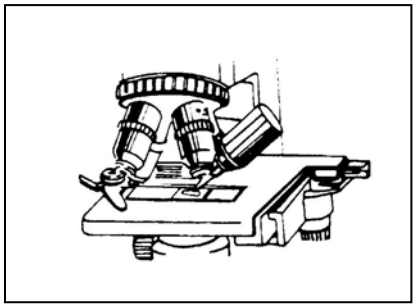


Fig 4.8

- Put one drop of immersion oil on the smear (Fig.4.8).

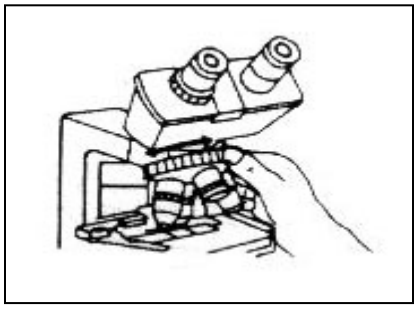


Fig. 4.9

- Change to 100x objective and focus the smear by turning the fine focusing knob (Fig 4.9).
- Screen the smear.

Common Problems with microscopy

Problem	Possible causes	Solution
1. Field dim	• Condenser may be too low • Condenser iris may be closed	Raise the condenser Open the diaphragm
2. Dark shadows in the field which move when eye piece is moved	• Eyepiece may be dirty. • Eye Piece or objective may be contaminated with fungus. • Surface of eyepiece may be scratched.	Clean the eye piece Eye piece may need repair A new eye piece may be needed
3. Image is not clear	• The smear may not be facing upwards • May be an air-bubble in the oil • There may be dirt on the objective • Oil may be too thick	Turn the slide over Move the x100 lens from side to side Clean the lens. Use only good quality immersion oil.
4.The image through low power is not clear	• There may be oil on the lens. • There may be dust on the upper surface of the lens. • The lens may be broken.	Clean the lens. Clean the lens New lens will be needed

Care and maintenance of the microscope

Proper handling and maintenance of the microscope is very important.

The following points should be observed.

- Handle the microscope with care.
  - Always carry the microscope with both hands, one hand supporting the base and the other firmly grasping the arm.
  - Never carry the microscope with only one hand.
- Place and store the microscope in a dry, dust free and vibration free environment and away from chemicals.
  - Place the microscope on a sturdy vibration free surface. Never keep it on a surface where a centrifuge is placed.
    Also keep it away from refrigerators and air conditioners.
  - Avoid exposing the microscope to direct sunlight.
  - Avoid exposing the microscope to moisture. Humidity may allow fungus to grow on the lens and cause rusting of metal parts. To reduce moisture, keep dry blue silica gel or any other drying agent in the box, where the microscope is kept. Dry silica absorbs the moisture and when it is unable to absorb any more moisture, it changes colour from blue to pink. As soon as it changes colour the silica gel should be replaced or may be heated and re-used.
- When the microscope is not in use, it should be kept in the box or covered with a plastic or polythene cover so as to keep it free from dust.
- If the microscope is used everyday, do not remove it from the table and keep it covered with a plastic or polythene cover.
- During examination never let the immersion lens touch the slide. This can damage the lens and may break the slide.
- Use only the fine focusing knob while using the immersion lens.
- Keep the microscope and the lens clean.

After daily use

- Adjust the variable voltage regulator setting to the minimum before turning off the light. Turn off the microscope light source.
- Rotate the nosepiece to bring the lowest power objective into position before removing the slide.
- Gently wipe the immersion oil from the objective, condenser and mechanical stage with lens paper soaked in xylene
- Replace the microscope cover.

Monthly

- Use an airbrush to blow away dust.
- Clean the objectives, eye- pieces, and condenser with lens paper soaked in xylene.
- Remove the slide holder from the stage and clean.
- Wipe the dust off the body of the microscope and the window of the illuminator in the base of the unit with a tissue moistened with water.

Every six months

- Have the microscope inspected, cleaned and lubricated by professional service personnel.

Job responsibilities of Laboratory Personnel in the NTP

- Perform promptly and accurately all sputum smear examinations requested.
- Sputum collection
    - Explain, demonstrate and supervise the patient on collection of a good sample of sputum.
    - Label the sputum containers properly.
    - Assess the quality of sputum before the patient leaves the laboratory.
- Ensure patient submits 3 sputum for diagnosis and 2 for follow up
- Preparation of smears, staining and examination
- Recording and reporting
    - Enter the results in the Laboratory Request Form (TB 05).
    - Record the patient's details and enter the results in the TB Laboratory Register (TB 04).
    - Promptly send the Laboratory Request Form with the results to the medical officer or health institution from where it was sent. .
    - Send weekly a report of the case finding activities to the DTCO and in the case of microscopy centres a copy of the report should be sent to the medical officer in charge of the health institution.
- Preserve all the slides for quality control.
- Ensure safety measures
    - Keep the laboratory clean.
    - Disinfect and dispose all contaminated material.
- Management
    - Keep the microscope in good working condition.
    - Order the reagents and other laboratory supplies in advance to avoid shortages.

Preparation of Reagents

1% Carbol Fuchsin

Basic fuchsin	10 gms
Absolute alcohol	100 ml
Phenol	50 gms
Distlled water	900 ml

Dissolve Basic fuchsin in Absolute alcohol in a flask. Heat the phenol crystals to melt and add the melted phenol to the above solution. Then add distilled water to make up the final volume.

Filter the solution and store in an amber bottle. Label the bottle with the name of the reagent and the dates of preparation and expiry.

25% Sulphuric acid

Sulphuric acid	100 ml
Distilled water	300 ml

Add sulphuric acid slowly to the flask containing distilled water and mix.

***Never add water to sulphuric acid**

Store the solution in an amber bottle. Label the bottle with the name of the reagent and dates of preparation and expiry.

3% Acid alcohol solution

Alcohol 95%	970 ml
Concentrated hydrochloric acid	30 ml

Carefully add concentrated hydrochloric acid to 95% alcohol. Always add acid slowly to alcohol and not vice versa.

Store in an amber coloured bottle. Label the bottle with the name of the reagent and dates of preparation and expiry.

1% Methylene blue

Methylene blue	0.5 gms
Distilled water	500 ml

Dissolve the methylene blue in distilled water.

Store in an amber coloured bottle. Label the bottle with the name of the reagen and dates of preparation and expiry.

Each new batch of staining solutions must be checked by staining a known positive slide and a known negative slide as controls before being used or sent out.

These solutions can be used at room temperature for six months.

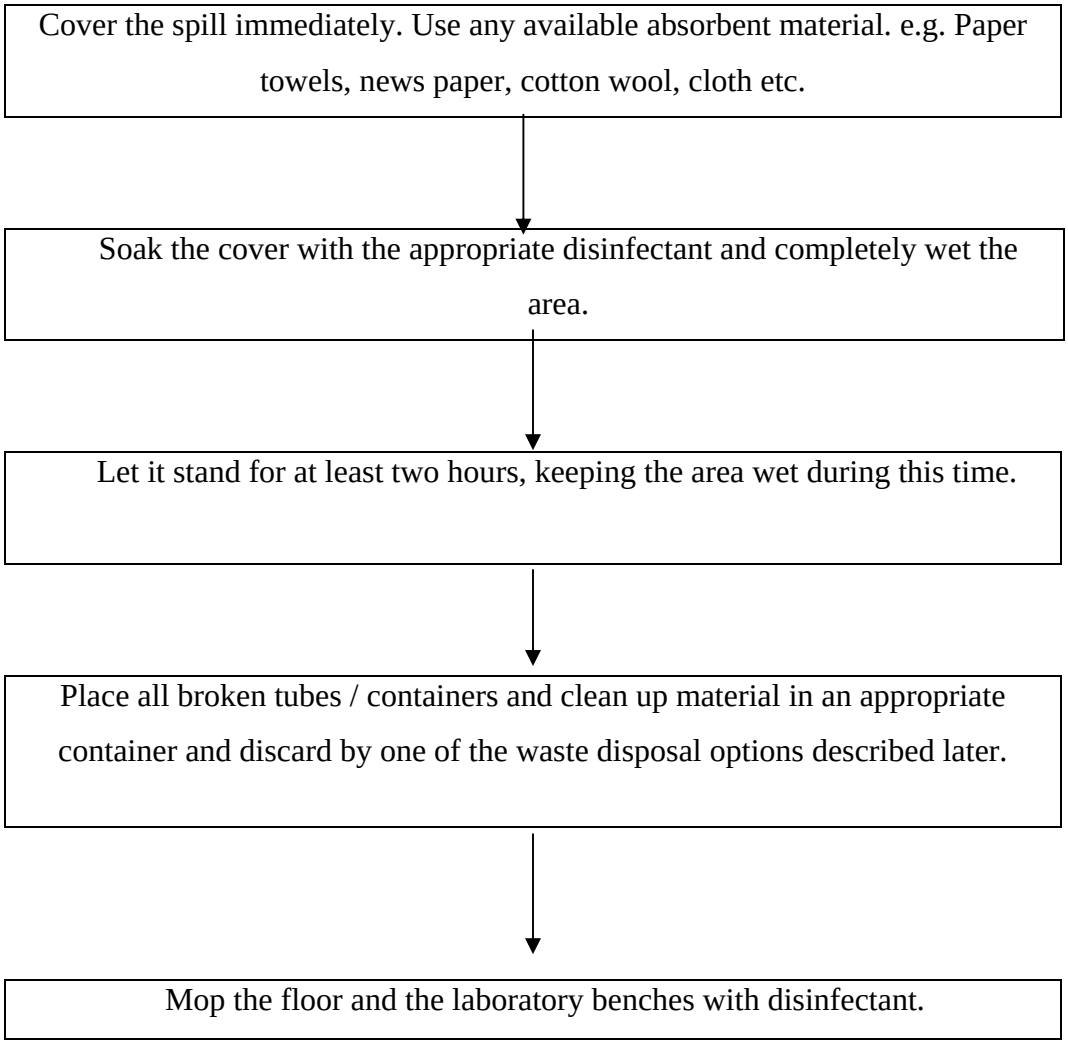
Safety Precautions and management of Laboratory accidents

Laboratory workers are responsible for their own safety and that of their co-workers. Therefore strict adherence to safety regulations in the laboratory is very important.

1. Tuberculosis is transmitted through air. Therefore every effort must be made to avoid or reduce the production of aerosols in the laboratory.
    - Never collect sputum specimens inside the laboratory.
    - Instruct the patients to cover their mouths while coughing and collect sputum specimens outdoors.
    - Handle the sputum specimens carefully when you open the sputum containers and during smear preparation.
    - Do not flame the slides before the smear is completely dry.
2. Wear an apron inside the laboratory.
3. Wash hands with soap and water frequently and always before and after performing any procedure.
4. Entry to the laboratory should be restricted to only the laboratory staff.
5. Refrain from eating, drinking, smoking and applying make up inside the laboratory.
6. Do not use the same desk for smear making and microscopy work.
7. Sterilize all contaminated material by boiling, burning or soaking in disinfectant.
8. All working surfaces should be cleaned with disinfectant (5% phenol) at the end of the day.
9. All floors should be wet-mopped daily. (Sweeping should be avoided).

Laboratory accidents

Plan of action for a limited aerosol accident



Laboratory accident book

This book should be kept by the Laboratory supervisor and should contain details about laboratory accidents and the necessary measures taken. Each laboratory accident should be reported to the person in charge and all details entered in the book-

- Date of accident
- Name of person concerned

- Description of accident
- Laboratory number of specimen / strain involved.
- Extent of injury
- Containment and follow up measures taken

Both the Laboratory supervisor and the person who was involved with the accident should sign the statement.

Disposal of contaminated material

All sputum specimens examined in the laboratory and the contaminated materials are potentially infectious and should be disinfected or sterilized before disposal or re-use, so that the risk of infection is avoided.

Sterilization means the complete destruction of all organisms while disinfection implies the destruction of organisms causing disease. Sterilization is usually accomplished by heat and disinfection by treating with chemicals.

All disposable containers must be used only once.

After the sputum smears are examined, the lids of all used sputum cups are removed. All sputum cups and lids should be placed in a bucket containing 0.5% sodium hypochlorite or 5% phenol solution and should be fully submerged in the solution. Similarly, used wooden sticks should be put into the same bucket. This bin/bucket should have a lid which is foot operated.

Thereafter these materials can be disposed of by any of the following methods –

Sputum containers

- Plastic sputum containers and wooden sticks may be disposed of by burning in an incinerator, open pit or in an empty drum.
- Glass or metal sputum containers, which are re-usable, may be boiled in a barrel of water for 20 minutes and washed thoroughly and re-used.
- Or preferably autoclaved at 121˚ C for 15 minutes, washed, and cleaned for re-use.

Glass pipettes, wire loops, slide holders.

These. should be soaked in 5% phenol or 0.5% hypochlorite solution for two hours, after which they can be washed and sterilized before re-use. Contaminated fluids should not be poured down drains but discarded into autoclavable containers.

Used glass slides.

Positive slides-

All positive slides should be kept for quality control. After that, they should be broken and destroyed and disposed by burning or burying.

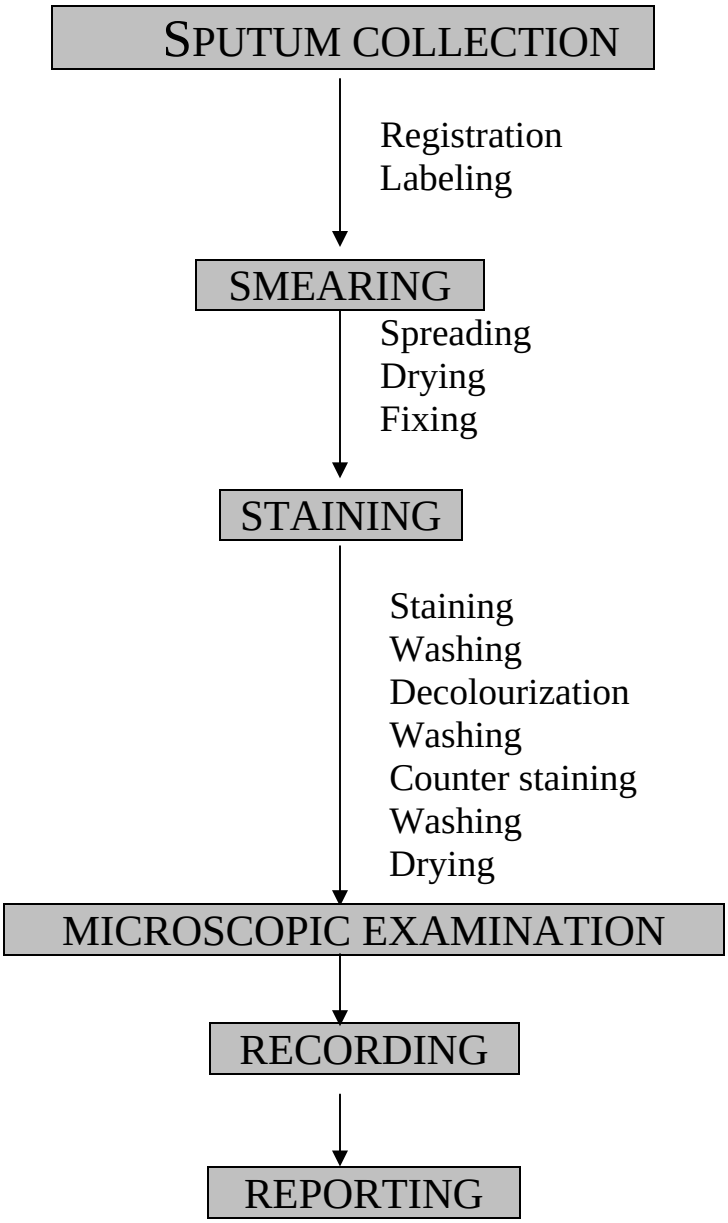
Negative slides

Negative slides can be either disposed of or if necessary washed, cleaned and re-used for non-TB work. If re-used, negative slides should be boiled for 30 minutes in soap or detergent solution, washed under running water, air dried and cleaned with alcohol soaked cotton swab

Chapter 7

PROCEDURE FOR SPUTUM EXAMINATION

Flow chart for smear



Sputum Collection, Storage and Transport

Sputum collection

When a new patient is referred to the laboratory for sputum examination, three sputum samples should be collected for examinaton.

1. Supervised **spot** specimen at first visit
2. **Early morning** sample on the next day
3. Supervised **spot** specimen when he comes with the morning sample

Place of sputum collection

The risk of infection is very high when the patient coughs. Therefore sputum should be collected in the open air and as far away as possible from other people. If conditions do not permit collection of sputum outdoor, use a separate, well-ventilated room.

Sputum Containers

A good sputum container should be:

- Wide mouthed
- Leak proof
- Provided with a tight fitting lid (preferably screw-capped)
- Easily disposable by burning
- Clean
- Transparent
- Unbreakable.

Recording information before sputum collection

- Receive the patient and the Laboratory Request Form (TB 05).
- Make sure the Laboratory Request Form is complete including the patient's name, address, reason for examination, and the District TB Number if the sputum is for follow up.

- Register the patient in the TB Laboratory Register (TB 04) and assign a Laboratory Serial Number to the patient. Write the Laboratory Serial Number on the Laboratory Request Form and on the side of the sputum container.
- The following data from the Laboratory Request Form should be entered in the TB laboratory Register:
    - Name of the patient
    - Age, and sex
    - Address of patient
    - Name of referring health unit
    - Reason for examination

Laboratory Serial Number

- When a new patient comes for sputum examination for diagnosis all of his three sputum samples are given one **Laboratory Serial Number.**
- When the same patient comes for sputum examination for follow up at 2nd month, a new Laboratory Serial Number is given for the follow up sample he submits.
- When the patient comes for subsequent follow up sputum examinations, his sputum samples are given a new Laboratory Serial Number at each visit.
- The Laboratory Serial Number begins with 1 on 1st of January each year and continues serially with each patient until 31st of December of the same year.

Write the Laboratory Serial Number on the Laboratory Request Form and on **the side of the sputum container** using a permanent marker and never on the lid. This is because the lid from one container may be placed on another container causing incorrect labelling of specimens.

Fig. 1 Sputum Container

Procedure for sputum collection

- Explain to the patient, the reason for sputum examination.
- Explain how many samples are needed.
- Give the patient the labelled sputum container and demonstrate to the patient how to open and close the container and explain the importance of not rubbing off the number written on the container.
- Explain the difference between sputum and saliva and the importance of bringing out sputum deep from the lungs.

Spot Specimen

Give the patient the labelled container and bring him to the nearby sputum collecting area and then instruct him by demonstrating with actual actions:

- inhale deeply 2-3 times
- cough out deeply from the chest
- open the container, bring it close to the mouth and bring the sputum out into it
- avoid contaminating the outside of the container
- not give saliva or nasal secretions
- close the container firmly.

Before the patient leaves the laboratory, visually examine the sputum sample for quality. If the sample is not good, ask the patient to cough again until a good sample is obtained.

Early Morning Sample

- Give the patient another container with the same Laboratory Serial Number written on its side for the collection of the morning sample.
- Ask the patient to rinse his mouth with plain water before collecting the early morning sample. This will remove any food particles in mouth.
- Drinking a glass of warm water may help to bring out the sputum.
- Then repeat the above instructions for bringing out the sputum.
- Close the container firmly and ask him to bring it back to the microscopy centre / laboratory.

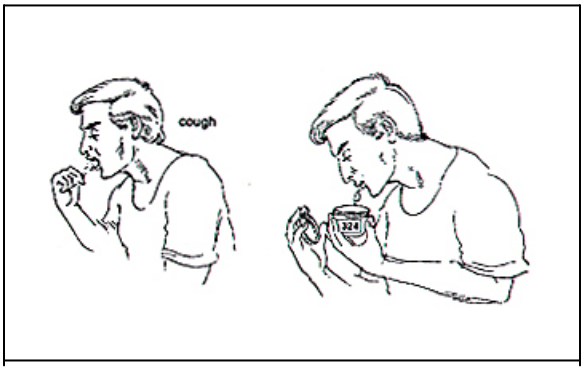


Fig. 2 Correct procedure of collecting sputum

A good sample of sputum should be:

- Thick sputum coughed out deeply from the lungs
- Purulent (yellowish mucoid)
- Sufficient in amount (at least 3-5ml)
- Not saliva or nasal secretions.

Sputum storage and transport

If the health centre has no microscopy facilities, sputum should be collected and transported to the nearest designated microscopy centre for examination.

Storage

- If the sputum containers are not transported immediately to the laboratory, they should be stored in a cool place or in a refrigerator until transported.
- If the sputum is intended for culture, the specimen should be kept in a refrigerator (approx. 4° C). If facilities are not available, add 5ml of CPC to tne bottle
- Sputum specimens should be protected from excessive heat and direct sunlight.
- Should be transported as soon as possible, but definitely within one week. If there is likely to be a delay of more than 3-days for the specimen to reach the referral laboratory, add a preservative (CPC)

Transport

Sputum containers should be placed carefully in a transport box. For this purpose, a wooden box with space to lodge the sputum containers tightly can be made locally (Fig. 3).

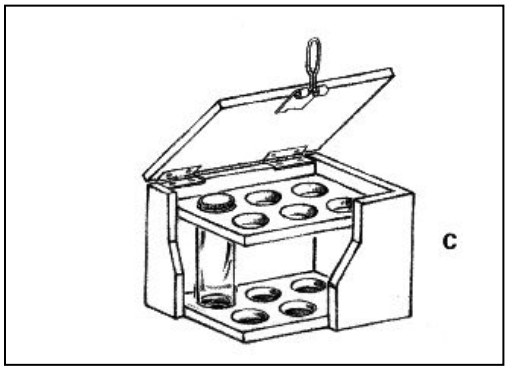


Fig. 3 Wooden transport box

One Laboratory Request Form should accompany each patient's sputum specimens. A **Specimen Identification Number** should be written on the Laboratory Request Form and the sputum container. The **Specimen Identification Number** should be the Clinic No, OPD No, BHT No or the District TB No.

With each transport box, an accompanying dispatch list must be prepared. This list should identify the sputum specimens it contains and the data regarding the patients from whom the specimens were collected (Fig 4). A copy of the dispatch list should be retained at the health centre.

Health unit:				**Received on:**		
Microscopy Laboratory:				**Examined on:**		
Sent on:				**Sent back on:**		
Specimen Identification No.	**Name**	**Age**	**Sex**	**Address**	**Date of collection**	**AFB Result**

Fig. 4 Dispatch list

Before dispatch verify:

- That the total number of sputum containers corresponds to the total number on the accompanying dispatch list
- That the Specimen Identification Numbers on the sputum containers corresponds to the Identification Numbers on the accompanying dispatch list and that on the Laboratory Request Forms
- The accompanying Laboratory Request Forms contain the necessary data for each patient
- As far as possible collect all three samples from a new patient before dispatch

After checking:

- Mark the date of dispatch on the accompanying list
- Put the list in an envelope and attach it to the outside of the transport box.

Results of examination will be reported from the microscopy centre to the health centre on both the Laboratory Request Form and the accompanying list, which the health centre sent with the transport box.

Receipt of incoming specimens

Specimens should be received at a separate specimen delivery counter and the following procedures applied:

- Wear disposable gloves during receipt and inspection of incoming specimens.
- Inspect delivery box for signs of leakage.
- Disinfect outside of the box using cotton wool or paper towels saturated with a suitable disinfectant (e.g. 5% phenol).
- Open the delivery box carefully and check for cracked or broken specimen containers. Autoclave or burn these without processing and request another specimen.

- Check that specimens have been adequately labelled with individual identification numbers and that these correspond with the numbers on the accompanying list.
- Enter the relevant patient and specimen details into the TB Laboratory Register and assign a Laboratory Serial Number.
- Disinfect the inside of the delivery box; discard gloves and wash hands after handling specimen containers.

Preparation and Staining of Sputum Smears

- Assess and record the visual appearance of the sample. Enter the information on the Laboratory Request Form by ticking the appropriate box.
- Make sure the Laboratory Serial Number on the Form matches the Laboratory Serial Number on the container.

Arrange the specimen containers in serial order.

Steps in the preparation of smears

Step 1 - Labelling the slides

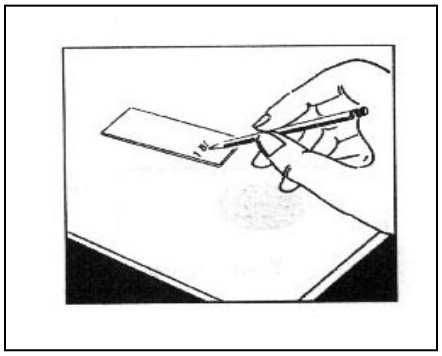


Fig. 5

- Select a new clean unscratched slide and write the Laboratory Serial Number and the Sample Number A, B or C using a diamond pencil on one end of the slide. (A, B and C indicate the 1st 2nd and the 3rd sample) E.g.325/A (Fig.5)

Step 2 - Smearing

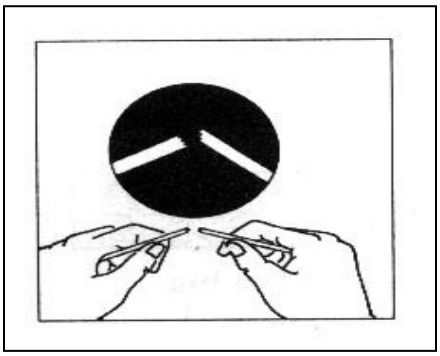


Fig. 6

- Break a wooden stick into two pieces with rough ends (Fig 6)
- A wire loop may be used. When a wire loop is used, it should be flamed until red-hot and allowed to cool.

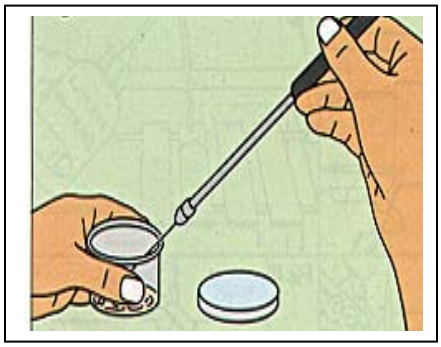


Fig. 7

- Ensure the number on the slide corresponds to the number on the sputum container.
- Using the jagged ends of the broken stick or wire loop, select and pick up a small portion of purulent particles and transfer on to the slide (Fig.7)
- Use a separate stick for each slide.

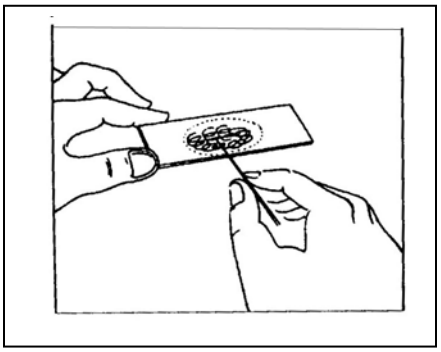


Fig. 8

- With the stick, spread the sputum evenly to cover 2/3 of the central portion of the slide, using a continuous movement. (Fig 8)

Fig. 9

- Place the used wooden sticks in a container with disinfectant (e.g.5%phenol).

- If a wire loop is used, sterilize the loop between successive specimens by first dipping it in a flask containing disinfectant and sand and then holding it to the Bunsen burner and flame until it is red hot (Fig. 9).

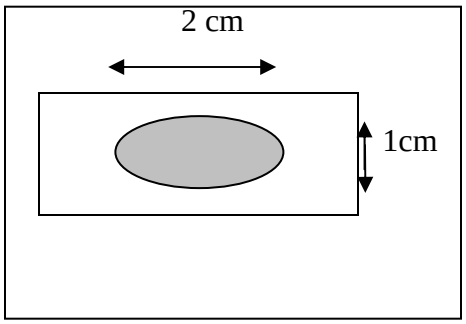


Fig. 10

- The size of the smear should be approximately 2 x 1 cm (Fig 10).

- The smear should be spread evenly and not too thick nor too thin.

- It should be thin enough to read newsprint through.

Step 3 - Drying

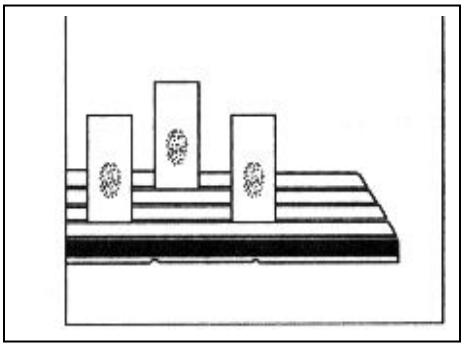


Fig. 11

- Place the smeared slides on the drying rack (Fig 11).
- Let the slides dry in the air for about 15 – 30 minute.
- Do not use the flame for drying.
- Replace the lid of the sputum container, but do not dispose of the specimens until the smears have been examined and results recorded.

Step 4 - Fixation

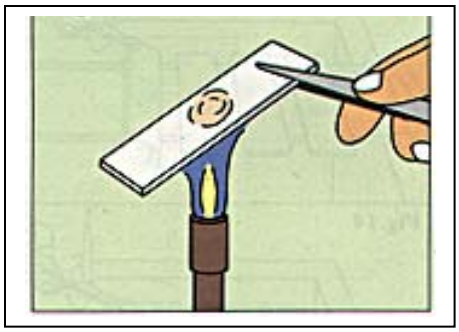


Fig. 12

- Make sure the slide is completely dry. If the slide is put over the flame before the smear is completely dry,
- Hold the dry slide using forceps with the smeared side facing upwards (Fig.12)
- Pass the slide 2-3 times over the flame of the Bunsen burner for about 3-5 seconds each time. Fixation ensures that the sputum will stick to the glass slide. If not heated sufficiently, the AFB may be washed of during staining.
- Do not heat the slide for too long or keep it stationary over the flame .It could damage the bacilli.

Step 5 - Staining

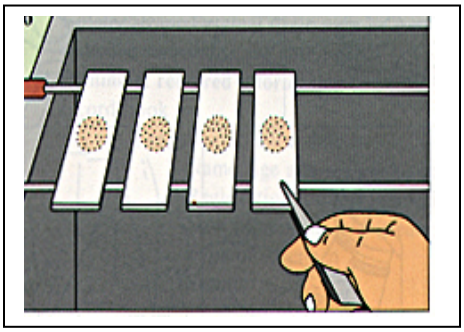


Fig 13

- Place the slides in serial order on the staining rack with the smeared side facing upwards (Fig 13).
- Leave space between the slides so that they do not touch each other.
- Never stain more than 12 slide at a time

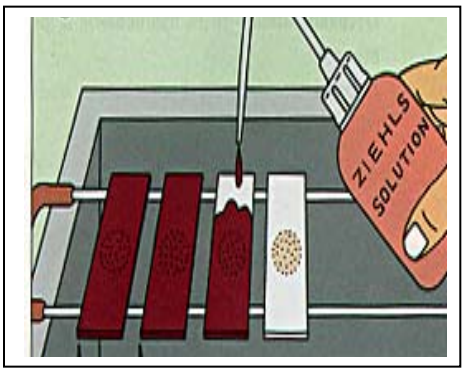


Fig. 14

- Include positive and negative controls with each day's reading specially if the number of slides to be read is less than 10.
- Pour filtered 1% carbol fuchsin to cover the entire surface of the slide (Fig.14).

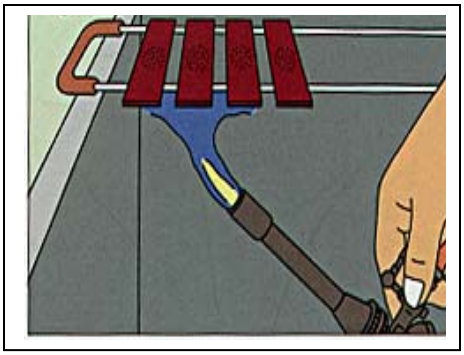


Fig. 15

- Heat the slide gently by passing the flame underneath the slide, until vapour rises (Fig 15).

- When the slide is heated, carbol fuchsin on the slide penetrates the wall of the TB bacilli to stain the bacilli red.

- Leave the carbol fuchsin on the slide for 5 minutes and maintain the heat by flaming intermittently.

- Do not allow the carbol fuchsin to boil or dry on the slide. Boiling will alter the shape of TB bacilli and could result in a false negative reading.