TECHNICAL GUIDE

Sputum Examination for Tuberculosis by Direct Microscopy in Low Income Countries

Fifth edition

2000

International Union Against Tuberculosis and Lung Disease

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PREFACE

A technical guide for sputum smear microscopy, based on one initiated in 1969 by Dr J Holm, the then Director of the International Union against Tuberculosis, was first published in 1978 by the IUAT as the *Technical Guide for Sputum Examination for Tuberculosis by Direct Microscopy*. The guide was included in the third and fourth editions of the IUATLD's *Tuberculosis Guide for Low Income Countries*. It was designed to be a simple reference standard for the collection, storage and transport of sputum specimens and for the examination of sputum smears by direct microscopy. It was meant to address the needs of health care workers in low income, high prevalence countries which represent the bulk of the global tuberculosis caseload.

More than twenty years have elapsed since its first publication, and the guide has remained unchanged throughout that time. Today, tuberculosis is one of the main causes of death from a single infectious agent among adults in low income countries, where it remains a major public health problem. The basic tool for TB diagnostic services, i.e., sputum smear microscopy, has not changed in its technical details in spite of major advances in modern diagnostic technologies. However, the context in which it is applied, the National Tuberculosis Programme, has been refined to a considerable extent in the last two decades.

The field use of the guide over the years has revealed omissions and inaccuracies that needed to be addressed. Furthermore, biosafety and quality assurance aspects of sputum smear microscopy were not sufficiently well covered in the previous edition. It was therefore felt that the IUATLD Technical Guide needed revision so it could better reflect its public health essence and keep up to date with modern TB control strategies. This document was carefully revised by members of the *Bacteriology and Immunology Section* of the IUATLD, by directors of the *WHO/IUATLD Supranational TB Reference Laboratory Network* and by other distinguished professionals in the field of tuberculosis control.

DR ADALBERT LASZLO

Ottawa 2000

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1. SPUTUM MICROSCOPY

1.1 Aims of tuberculosis laboratory diagnostic and follow-up services

In low income and high tuberculosis prevalence countries, sputum smear microscopy is, and is likely to remain for the foreseeable future, the only cost-effective tool for diagnosing patients with infectious tuberculosis and to monitor their progress in treatment. Sputum smear microscopy is a simple, inexpensive, appropriate technology method which is relatively easy to perform and to read. It yields timely results with a very high sensitivity of detection of tubercle bacilli transmitters, and provides most of the essential laboratory-epidemiological indicators needed for the evaluation of the National Tuberculosis Programme (NTP).

The aims of TB laboratory diagnostic services within the framework of an NTP are:
- diagnosis of cases
- monitoring of tuberculosis treatment

1.2 "Spot", "morning" and "spot" sputum specimens for diagnosis

Under NTP conditions, the IUATLD recommends collecting three sputum samples "on the SPOT – early MORNING – on the SPOT", preferably within two days, from each person presenting at health centres, out-patient clinics, etc., with respiratory symptoms of more than 3 weeks' duration. These samples are to be examined by smear microscopy in the nearest laboratory. Under these conditions, a case of sputum smear positive tuberculosis is usually defined as a person presenting with respiratory symptoms with at least two positive sputum smear microscopy examinations.

This approach, also known as passive case finding, detects about 80% of TB suspects ultimately positive on sputum smear examination with the first specimen, an additional 15% with the second and a final 5% with the third. "SPOT" specimens are obtained when the TB suspects present at the health centre; "MORNING" specimens consist of all sputum produced within one or two hours after rising.

1.3 "Morning specimens" for follow-up

There are two phases in the treatment of tuberculosis: the intensive phase, usually 2 to 3 months, and the continuation phase, which is 4 to 10 months, depending on the type of treatment. Regardless of the treatment regimen, one "MORNING" sputum specimen is collected for follow-up at the end of the intensive phase of treatment to determine whether the patient can proceed to the continuation phase if the smear is negative or, if the smear is positive, continue the intensive phase. Another sputum specimen must be taken during the continuation phase to check patient evolution and to detect possible treatment failure, and another upon completion of chemotherapy to verify cure. Sputum specimens at the end of treatment are often difficult to obtain, as many patients have stopped expectorating. The exact schedule of follow-up sputum examinations varies according to the drug regimen, and should be set out in the NTP Manual.

The patient is said to have completed treatment even if sputum specimens are not examined during and at the end of treatment. The cure rate is the proportion of initially sputum smear-positive patients who are declared cured based on negative sputum smear results on at least two occasions, including one at the end of treatment. The objective of the NTP is to achieve at least 85% cure rate among new sputum smear positive TB cases registered.

The IUATLD recommends:
- The examination of three sputum specimens – "SPOT" + "MORNING" + "SPOT" – for the diagnosis of tuberculosis cases.
- The examination of single "MORNING" sputum specimens on three occasions for follow-up of treatment: one at the end of the intensive phase, one during the continuation phase, and one at the end of treatment.

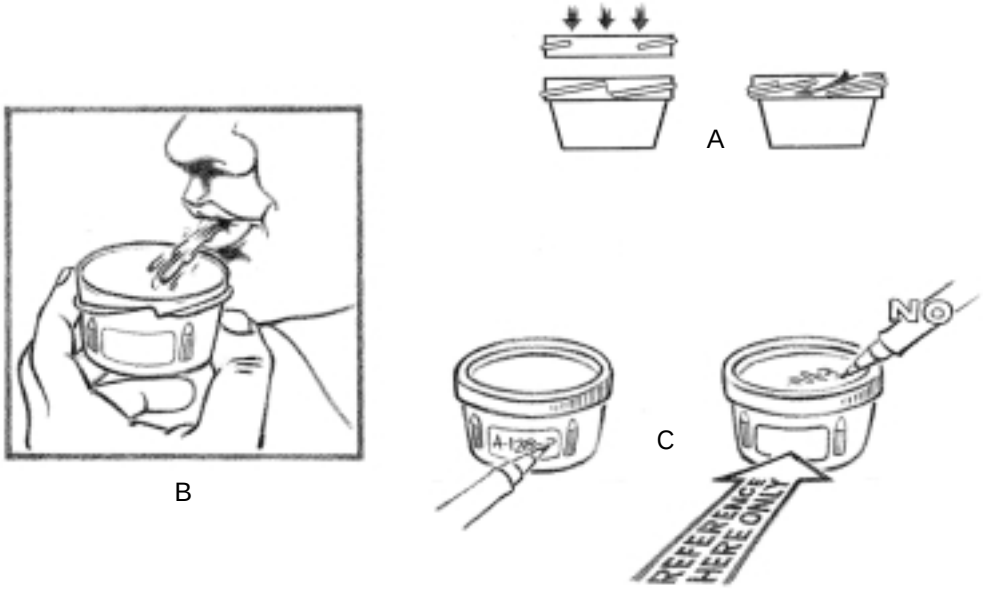

Figure 1

1.4 Sputum containers

The use of two kinds of sputum containers is recommended. One, available from UNICEF (Figure 1 A), is a rigid wide-mouthed screw-capped container made of unbreakable transparent plastic, readily disposable by burning, which is used for most routine diagnostic work. Its screw cap can be hermetically sealed to prevent desiccation of the sample and leakage.

The other, a screw-capped heavy glass container, such as the Universal bottle (Figure 2 A), is used for specimen transportation in custom-made boxes that can be made of metal, wood or styrofoam. A wooden box is a sensible compromise in terms of sturdiness and weight (Figure 2 B, C). The Universal bottle is reusable after disinfection by autoclaving for 30 minutes at 121°C and careful cleaning. If an autoclave is unavailable, a domestic pressure cooker is recommended.

1.5 Collection of sputum specimens

The risk of infection for health care workers is highest when TB suspects cough; sputum specimens should therefore be collected in the open air and as far away as possible from other people. Failing this, a separate, well-ventilated room should be used.

The health care worker should reassure persons suspected of having tuberculosis by explaining the reasons for the examination, and give instructions on how to cough so that the expectoration is produced from as deep down in the chest as possible. As an adjunct, written instructions can be handed out if the person is literate.

The health care worker should make sure that the specimen is of sufficient volume (3 to 5 ml) and that it contains solid or purulent material, the presence of which increases the sensitivity of detection, and not just saliva. However, if only saliva is obtained or, as frequently happens in "spot" sputum, volumes of less than 3 ml are produced, the specimen should nevertheless be processed, as it is sometimes likely to yield positive results. A sputum specimen can be classified by macroscopic examination as "salivary" when it consists mainly of saliva, "mucous" when it is mainly mucus, "purulent" when it appears yellow as pus, "muco-purulent" when there are visible yellowish particles in the mucus and "bloody" when it contains blood. The presence of blood should always be noted because it is indicative of severe disease and could interfere with the reading of the smear.

The health care worker should provide a sputum container with the health centre code and the TB suspect or patient's identification

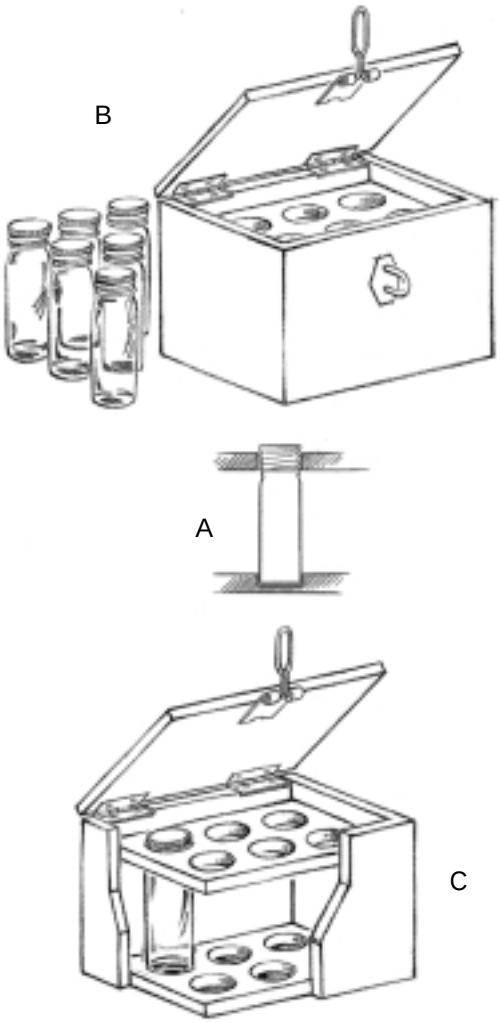

Figure 2

must be written on the side of the container, never on its lid. (Figure 1 C). The health care worker must ask the person being examined to bring it close to the mouth and expectorate into it (Figure 1 B). This specimen is called a "SPOT" specimen.

If expectoration is not produced, the sputum container must be considered as used and must be properly disposed of. Sputum containers must be closed securely and if they are to be sent to a nearby laboratory, they should be placed in the appropriate transport box. Collected specimens should be kept in a cool place, batched and transported without much delay, i.e., at least twice weekly, and processed as soon as possible. Sputum can also be processed in the health centre, and fixed smears can be sent to the nearest laboratory. This procedure is discouraged, however, because fixed smears are often of poor technical quality, having been prepared by untrained personnel, and tend to decompose quickly in warm and humid climates.

The health care worker should provide the TB suspect with a new, pre-labelled sputum container, explain how it should be used in the morning to collect the "MORNING" specimen and demonstrate how it should be securely closed before it is brought back to the health centre.

1.6 Transportation of sputum specimens

In countries lacking laboratory facilities that rely on specimen collecting units, transportation of specimens is required. Transportation is also required when operational research projects of interest to the NTP are undertaken, such as a survey of TB drug resistance, etc. If culturing of specimens is required, the specimens should reach the laboratory within 3-4 days and should be refrigerated while waiting for shipment. The most rapid and cost-effective means of transportation should be selected. Contaminating flora do not affect the acid-fastness of mycobacteria but may liquefy the sputum, making smear preparation difficult and reading of slides unreliable.

A list identifying the sputum specimens contained in the transport box and a completed *Request for Sputum Examination* Forms (Figure 3) for each specimen must accompany the shipment. Before the shipment from the health centre, the health care worker must verify for each transport box that:

– the total number of sputum containers in the box corresponds to that on the accompanying list and that on the *Request for Sputum Examination* Forms;

– the identification number on each sputum container corresponds to that on the accompanying list and that on the *Request for Sputum Examination* Forms;

– the accompanying *Request for Sputum Examination* Forms contain the requested information for each of the TB suspects.

REQUEST FOR SPUTUM EXAMINATION

Treatment Unit_____ Date_____

Patient's Name

Age_____ Sex (check one): M [ ] F [ ]

Address (precise) _____

_____

_____

Reason for examination (check one): diagnosis [ ] follow-up examination [ ]

Signature of person requesting examination

RESULTS (to be completed in laboratory)

Laboratory Serial No._____

Date	Specimen	Appearance*	Result (check one)				
			neg	1-9	+	+ +	+ + +
	1						
	2						
	3						

* visual appearance of sputum (blood-stained, muco-purulent, saliva)

Date_____ Examined by (Signature)_____

The completed form (with results) should be sent promptly to the treatment unit

Figure 3. Request for sputum examination form

When this verification is completed, the health care worker:

– dates the accompanying list;
– puts the list and the *Request for Sputum Examination* Forms in an envelope which will be attached to the outside of the transport box.

A laboratory code, a serial number and a specimen sequence identifier, i.e., 1 for first, 2 for second, 3 for third (Figure 12), will be assigned to each specimen by laboratory staff. Results of the examination will be entered on the bottom half of the *Request for Sputum Examination* Form. The Laboratory Serial Number begins with 1 on 1 January each year and increases by one with each patient until 31 December of the same year.

2. THE SPUTUM SMEAR MICROSCOPY LABORATORY

2.1 Role of the laboratory

In developing countries, most of the bacteriological diagnosis of tuberculosis is carried out in peripheral or local laboratories, whose major responsibility is to provide diagnostic microscopy for the NTP based on sputum smear examination by Ziehl-Neelsen (ZN) staining. These laboratories, located in health centres, health posts, hospitals, etc., usually have qualified technical personnel capable of performing – among other duties – sputum smear microscopy. They should be able to carry out the following functions:

– perform all sputum smear microscopy requested in their catchment area, usually a district (50,000-100,000 inhabitants);

– act as referral center for specimen collecting units;

– co-ordinate with Regional (intermediate) Laboratories the referral of specimens requiring culture and drug susceptibility testing;

– receive specimens during the opening hours of the health centre;

– send information to the Regional Laboratory;

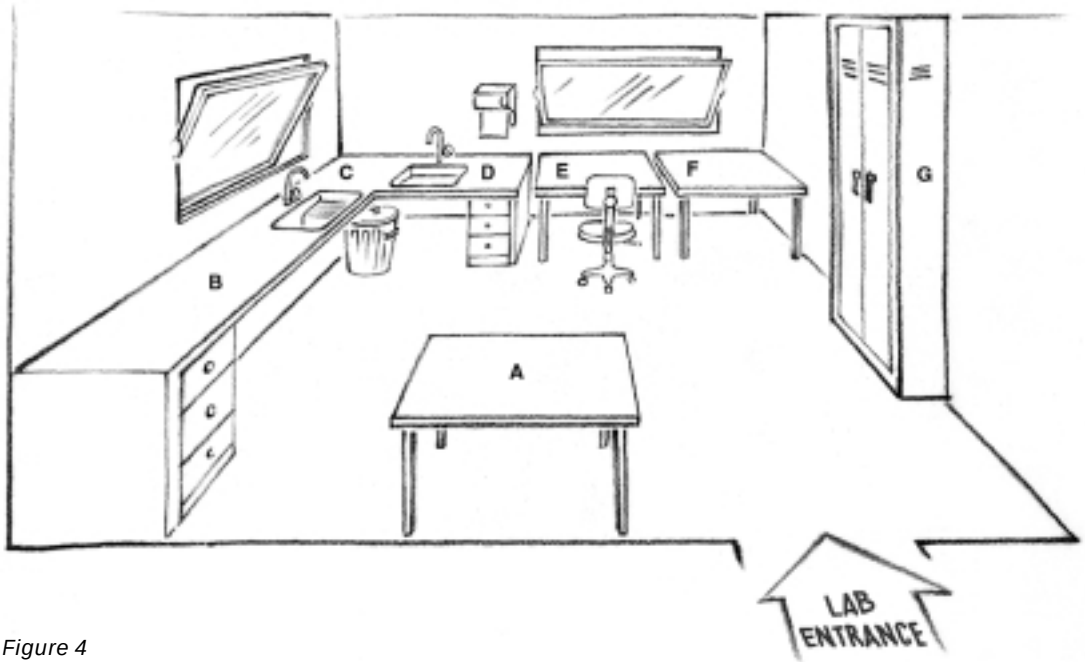

Figure 4

– comply with national quality assurance guidelines;

– order, manage and store laboratory supplies.

2.2 Physical environment of the laboratory

The detailed arrangement of the microscopy laboratory varies greatly depending on local conditions. It is difficult to generalise about the design of such laboratories since TB diagnostic services have, over time, been integrated into existing general laboratory diagnostic services in many countries. Ideally, the TB microscopy laboratory should include the following distinct sections (Figure 4), adapted from Collins et al:[1]

– a bench space or a table (A) for incoming specimen (Figures 4 and 5);

– one well-lit work bench (B) for smear preparation (Figures 4 and 6);

– a staining sink (C) with running water (Figures 4 and 7);

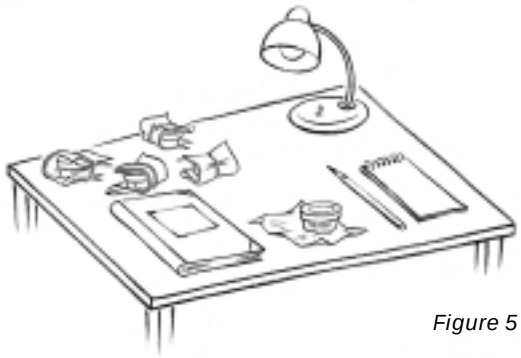

Figure 5

– a sink (D) with running water for washing hands;

– a bench area (E) for microscopy reading directly below a window (Figures 4 and 8);

– a bench area or a table (F) for the laboratory register books and slide storage space (Figures 4 and 9);

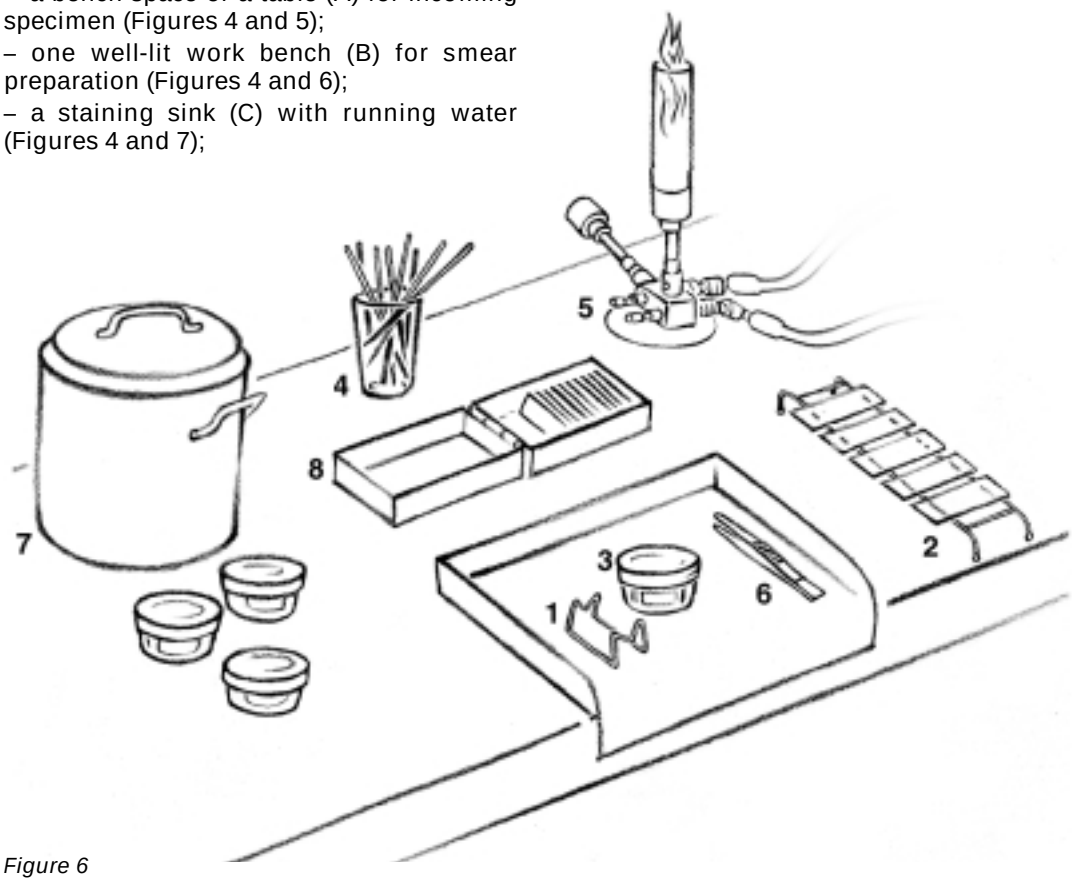

Figure 6

1. Slide-holder for the preparation of smears
2. Slide dryer
3. Sputum container placed as close as possible to the slide-holder on the right
4. Wooden applicators
5. Alcohol lamp / Bunsen burner
6. Forceps
7. Metal waste receptacle with lid to receive infectious material
8. Box of engraved slides for the smears

Figure 7

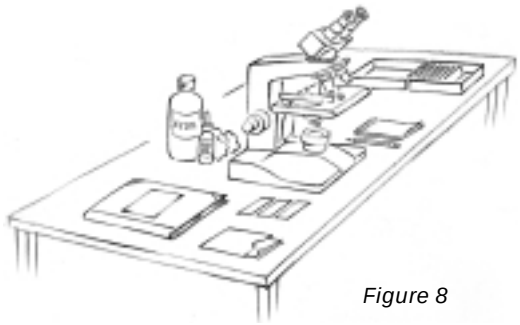

Figure 8

– a locker (G) for the technicians' clothing (Figure 4).

If the work bench is made of porous material, a non-porous surface plate, such as formica, a marble slab, or a galvanised metal or aluminum sheet, should cover the work bench: this plate should be about 80 cm wide, with borders 5 cm high. The front edge must be bent down at an angle of 90° to meet the edge of the table, thus facilitating manipulations (Figure 6). These must be conducted strictly over the surface plate, which should be decontaminated every day after use by soaking with a TB germicide (e.g., 5% phenol, 0.1% solution of sodium hypochlorite* [NaClO], also known as common household bleach, Chlorox, Javex, etc.).

Figure 9

NOTE: If the technician is left-handed, it may be more convenient to arrange all (or most) items in Figure 6 in exactly the opposite position on the table (i.e., in a mirror image).

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* Household bleach contains 5% of NaClO (50 g/litre); a 0.1% solution containing 1 g of NaOCl/litre is prepared by diluting 20 ml of household bleach in 1 litre of water. This solution is used as an all-purpose disinfectant for "clean conditions".[2]

3. THE STAINING METHOD

3.1 Preparation of Ziehl-Neelsen reagents

The method of choice for sputum smear microscopy is the Ziehl-Neelsen (ZN) staining technique because it is the only one that provides consistently good results without the need for special equipment. Preparing the necessary reagents requires a weighing scale that is not always available in a peripheral laboratory, and preparing the reagents in the National Reference Laboratory or in the nearest intermediate laboratory is therefore a frequently used option. The advantages of this option, i.e., better standardisation and quality assurance, outweigh the disadvantages of long term storage. Cold staining procedures such as the Kinyoun and Tan Thiam Hok methods are not recommended, as evidence shows that they have difficulty detecting acid-fast bacilli (AFB) in paucibacillary samples and the staining fades rapidly. Fluorescence microscopy, which is recommended when the daily workload exceeds 50 specimens, has no place in most peripheral laboratories of low-income countries.

3.1.1 Ziehl's carbol fuchsin

3% fuchsin alcoholic stock solution (solution A)

Basic fuchsin*. . . . . 3 g†
95% alcohol‡ . . . . . up to 100 ml

Place the required amount of fuchsin in a volumetric flask or measuring cylinder and, adding enough ethanol or methylated spirit to obtain a total volume of 100 ml, shake well until completely dissolved. Small quantities of this solution should be filtered prior to staining.

3.1.2 Aqueous phenol solution (solution B)

Phenol§ cristals . . . . . 5 g
Distilled water, if possible . . up to 90 ml

Before adding water, liquefy the phenol crystals in a flask by gentle heating.

To prepare the 0.3% Ziehl's carbol fuchsin working solution, mix 10 ml of Solution A with 90 ml of Solution B.

3.1.3 Decolourising agent solutions

– Acid-alcohol solution
Alcohol 95% . . . . . 970 ml
Concentrated (35%) hydrochloric acid** . . . . . 30 ml

Or, when alcohol is unavailable:

– 25% aqueous sulfuric acid solution
Distilled water if possible . . . . . 300 ml
Concentrated sulfuric acid†† . . . . . 100 ml

Pour 300 ml of water into a 1 litre Erlenmeyer flask. Slowly add 100 ml of concentrated sulfuric acid, allowing it to flow along the side of the flask. The mixture will heat up. Never pour water into concentrated sulfuric acid – explosive spills may occur.

3.1.4 Methylene blue counterstaining solution 0.3%

Methylene blue chloride‡‡ . . 0.3 g
Distilled water, if possible . . up to100 ml

3.2 Smear preparation

Sputum containers are arranged in sequential order. Laboratory serial numbers must match the corresponding information on the accompanying *Request for Sputum Examination* Form. The use of new slides is recommended; however, because they are often greasy, they tend to cling together and must be cleaned with alcohol and then carefully air-dried. When alcohol is not available the slides may be held over a flame to remove oils. Under weather conditions prevalent in most low income countries, the use of slides in tropical packaging (each slide separated from the next by a strip of impermeable paper) is recommended. Laboratory code, serial number and sequence identifier can be engraved with a diamond marker on the smear side and at one end of the slide. When diamond markers are not available, a discarded round-tipped dental drill inserted in the tapered end of a discarded plastic pen can be used.[3] An ordinary lead pencil can be used if frosted-end slides are available.

3.2.1 Smearing

– Verify that the numbers on slides and containers match.
– Take sputum container corresponding to the number on the slide.
– Open container carefully to avoid aerosol production.
– Break a wood or bamboo stick applicator (Figure 10), select yellow, purulent particle of sputum with jagged end of the broken wood or bamboo stick applicator. Use the broken

* Pararosaniline chloride, Minimum dye content 88% ($C_{19}H_{18}NCl$) Sigma P1528 or equivalent.
† Staining powders are seldom pure, so a corrected weight should be used to ensure proper staining. The percentage of available dye content is frequently listed on the original container label. The corrected weight is determined by dividing the desired amount of dye by the decimal equivalent of the available dye. So, if the desired amount of dye is 3 g and the per cent available dye is 75%, the actual amount of dye to be weighted is 3/0.75 = 4 g of impure dye. If the available dye content is 88% or more, there is no need to correct the weight.
‡ Ethanol 95% (C_2H_5OH) – United States Pharmacopeia XVIII, 20, 1067 (1970). Can be of industrial grade.
§ Phenol approx. 99% (C_6H_6O) – Sigma P 3653 or equivalent.
** Concentrated hydrochloric acid (HCl) – can be of industrial grade.
†† Concentrated sulfuric acid (H_2SO_4) – can be of industrial grade.
‡‡ Methylthionine chloride, minimum dye content 82% ($C_{16}H_{18}ClN_3S$) – Sigma M 9140 or equivalent.

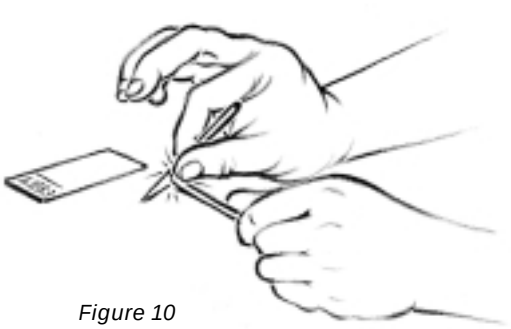

Figure 10

end of the two pieces of the applicator to break up larger particles.

– Spread the sputum evenly over the central area of the slide using a continuous rotational movement (Figure 11); the recommended

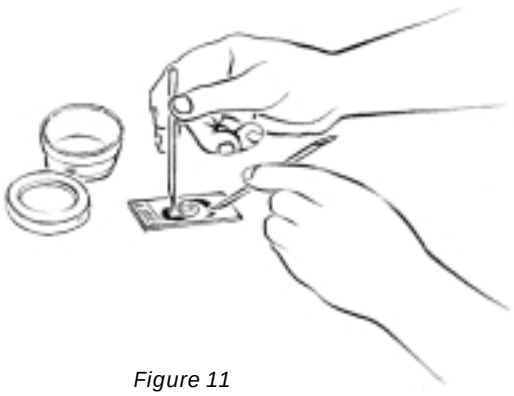

Figure 11

size of the smear is about 20 mm by 10 mm (Figure 12).

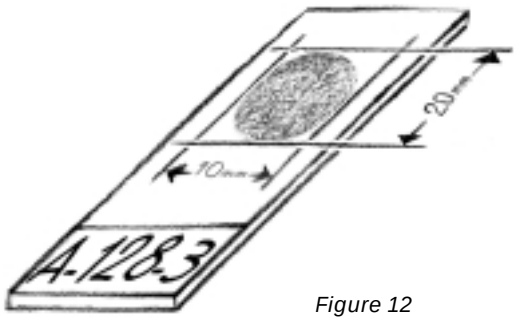

Figure 12

– Place slides on dryer with smeared surface upwards, and air dry for about 30 minutes.

– Re-cap sputum container, which should not be discarded before results are read and recorded.

Applicators are only used once. Discard by placing them in a waste receptacle containing 5% aqueous phenol solution or a 0.5% solution of sodium hypochlorite,* then autoclave or incinerate. CAUTION: Vapours are very toxic.

3.2.2 Fixing

Fix dried smears by holding them with forceps and passing them smear side up over the flame 5 times for about 4 seconds (Figure 13). Do not heat-fix moist slides, and do not overheat.

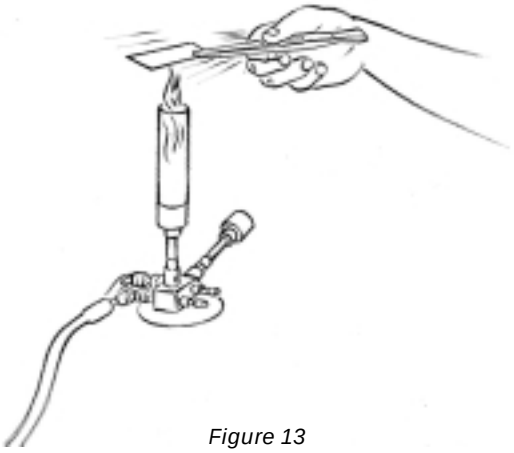

Figure 13

3.2.3 Staining

– Place fixed slides on the staining rack in serial order, smeared side up. Slides should be separated by a 1 cm gap, and should never touch one another.

– Cover slides individually with filtered 0.3% Ziehl's carbol fuchsin working solution (Figure 14). Placing a strip of absorbent paper such as filter or even newspaper will hold the staining solution and prevent deposits of fuchsin crystals on the smear.

– Heat slides from underneath with the flame of a Bunsen burner, an alcohol lamp or an alcohol soaked cotton swab until vapour starts to rise. Staining solution should never be allowed to boil. Do not allow the stain to dry (Figure 15).

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* Sodium hypochlorite is a strong oxidizing agent which is corrosive to metal. A 0.5% solution containing 5 g of NaClO/litre, prepared by diluting 100 ml of household bleach in 1 litre of water, is recommended for dealing with "dirty conditions".[2]

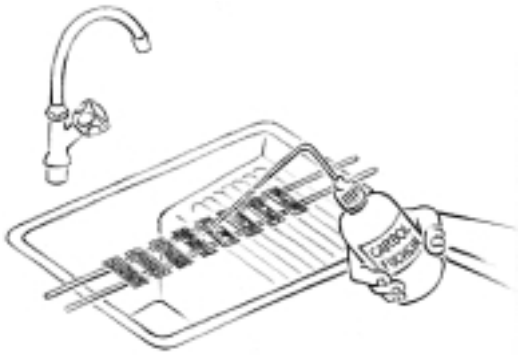

Figure 14

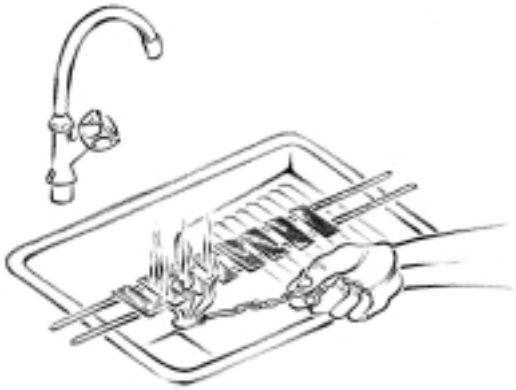

Figure 15

– Keep slides covered with hot, steaming carbolfuchsin for 5 minutes by re-flaming as needed.

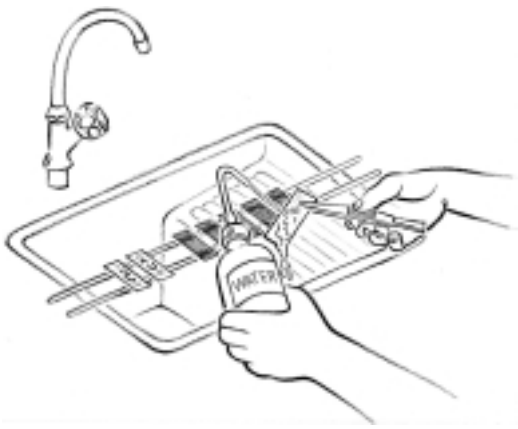

Figure 16

– Rinse slides gently with water to remove excess carbolfuchsin (Figure 16).

– Drain off excess rinsing water from slides (Figure 17). Sputum smears appear red in colour.

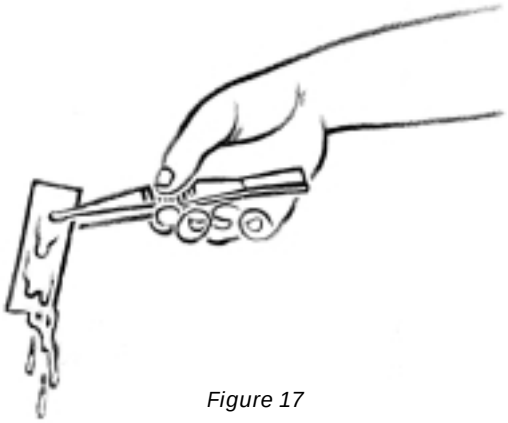

Figure 17

3.2.4 Decolourising

– Cover slides with 25% sulfuric acid or acid-alcohol solution and allow to stand for 3 minutes, after which the red colour should have almost completely disappeared (Figure 18). If needed, repeat sequence until the red colour disappears, but do not over-decolourise.

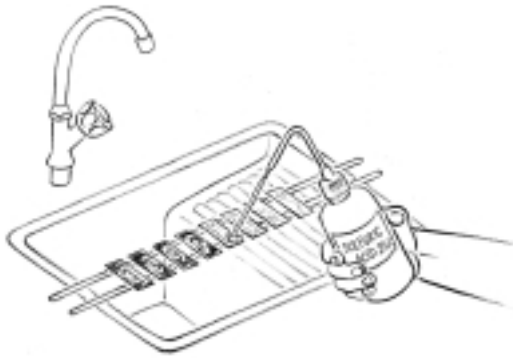

Figure 18

– Gently wash away the sulfuric acid or acid alcohol and the excess stain with water (Figure 19). Drain off excess rinsing water from slides (Figure 20).

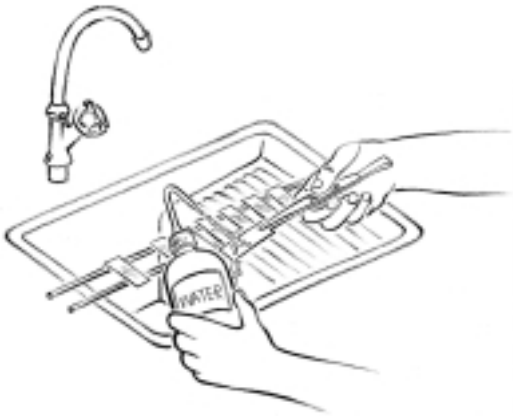

Figure 19

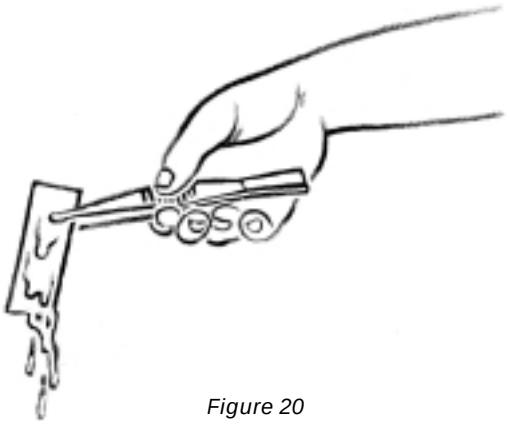

Figure 20

3.2.5 Counterstaining

– Cover slides individually with 0.3% methylene blue counterstaining solution and allow to stand for 1 minute (Figure 21).

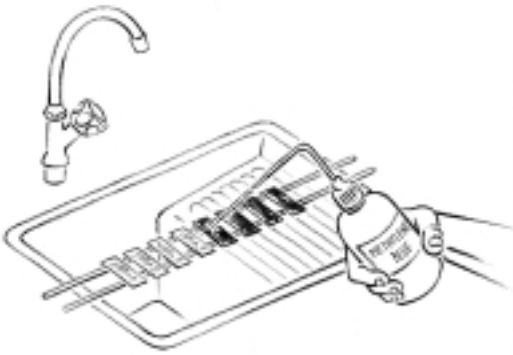

Figure 21

– Rinse slides individually with water (Figure 22).

Figure 22

– Drain water off the slides, which are then allowed to air dry (Figure 23).

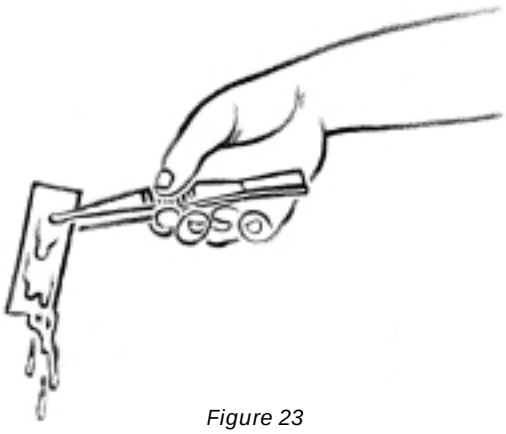

Figure 23

The Ziehl-Neelsen staining procedure requires:

– Staining for 5 minutes
– Decolourising for 3 minutes
– Counterstaining for 1 minute

3.2.6 Quality of smearing and staining

– A properly stained smear should show a light blue colour due to methylene blue. If dark blue, i.e., a newspaper cannot be read when held underneath the slide, the smear is too thick.

– Example of a good smear (Figure 24).

– Examples of bad smears (Figure 25)

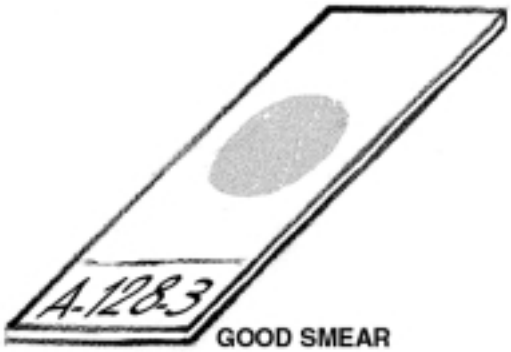

Figure 24

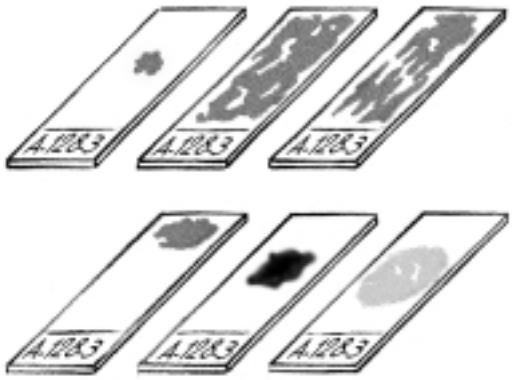

Figure 25

4. MICROSCOPIC EXAMINATION OF SPUTUM SMEARS

4.1 The microscope

A binocular microscope with two objectives – a regular 40 x magnification objective and an oil immersion 100 x magnification objective – and eyepieces of moderate magnification (8x or 10x) is required for the examination of smears (Figure 26).

Microscopes equipped with the light-collecting mirror option are strongly recommended, as they are useful in the event of power failures and necessary in laboratories that lack electricity. The mirror has one plane surface for artificial light and another concave surface for natural light. An illuminator is built into the base of the microscope; a halogen bulb provides good illumination. Halogen lamps have higher luminosity and longer life than tungsten lamps.

When not in use, microscopes should be kept in their case protected from dust, heat and humidity. Fungus growth is a constant threat to the microscope's optical system: it can be inhibited by fitting the storage case with a 20-40 watt lamp, which is kept lit during the storage of the microscope. The objective, eyepiece, condenser and light source are kept clean by wiping with lens paper on a daily basis.

4.2 Operating the microscope

– A drop of immersion oil is placed on a dry stained slide to increase the resolving power of the objective. To prevent cross contamination by AFB, the immersion applicator should not touch the slide. Cedarwood immersion oil should never be used, as it forms a thick paste upon drying that could damage the lenses of the microscope. "Makeshift" use of other oils, such as linseed, palm, olive, liquid paraffin, etc., is completely unsatisfactory. Some immersion oils can dissolve fuchsin stain,[4] a circumstance that might accelerate the fading of the ZN stain. Synthetic hydrocarbons and advanced polymers with a refractive index of 1.5, non-drying and non-hardening, with no solvent capability, are recommended.*

* Type A or B immersion oil (R. P. Cargille Labs, Inc. Cedar Grove, NJ. Catalogue No. 16484, or VWR brand Immersion Oil, Resolve, Catalogue No. 48218, or equivalent).

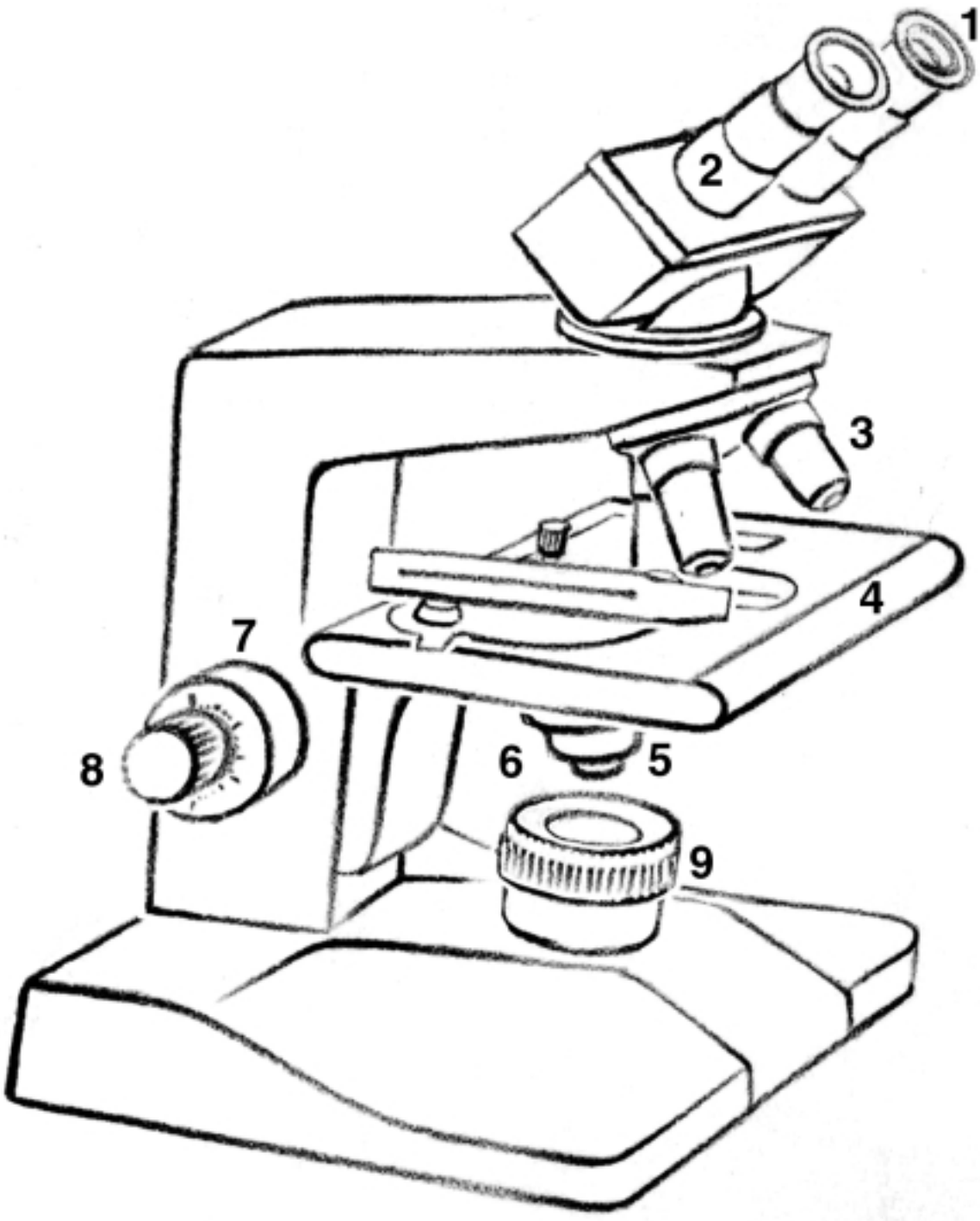

Figure 26

1) eye piece; 2) Diopter ring; 3) objective; 4) stage; 5) condenser; 6) diaphragm lever; 7) coarse focus knob; 8) fine focus knob; 9) light source.

– With the condenser raised to the uppermost position, the stained slide is placed on the stage and the light source is adjusted for optimal light by looking through the eyepiece and the regular 40 x objective.

– An area containing more leukocytes (pus cells) than epithelial cells (more frequent in saliva) is selected before placing the drop of immersion oil.

– By slowly changing to the immersion objective, a thin film of oil will form between the slide and the lens. The fine adjustment knob is used to focus the field; the lens should not be allowed to touch the slide.

For more on the use and operation of the microscope, see reference 5.

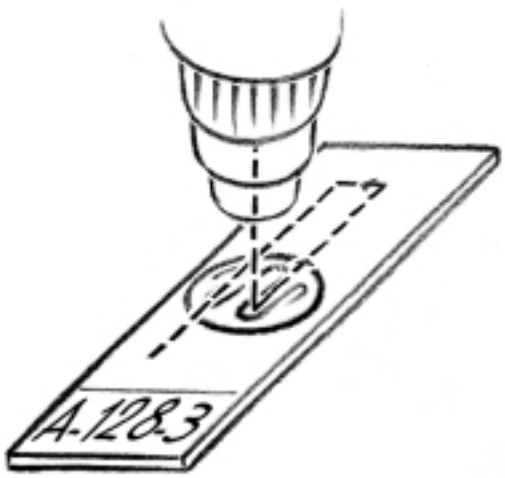

Figure 27

4.3 Microscopic examination of smears

– Acid-fast bacilli appear bright red or pink against the blue counterstained background. They vary greatly in shape, from short, coccoid to elongated filaments; they can be uniformly or unevenly stained, and can even appear granular. They occur singly or in variable sized clumps, and typically appear as long, slender curved rods.

– The microscopic examination must be systematic and standardised. It can start at the left end of the smear. The reading begins at the periphery of the field and ends at the centre (Figure 27). When the field is read, the slide is moved longitudinally to examine adjacent fields. The slide can be moved vertically so that a second length can be read from right to left. There are about 100 immersion fields in the 2 cm long axis of a smear.

4.4 Grading of sputum smear microscopy results

The information on the number of bacilli found is very important because it relates to the degree of infectivity of the patient as well as to the severity of the disease. For this reason, the report of the results of sputum smear microscopy must be not only qualitative but also semi-quantitative. The IUATLD recommends the following grading of results of smear microscopy (Table 1).

Table 1 IUATLD-recommended grading of sputum smear microscopy results

AFB counts	Recording/reporting
No AFB in at least 100 fields	0/negative
1 to 9 AFB in 100 fields*	Actual AFB counts†
10 to 99 AFB in 100 fields‡	+
1 to 10 AFB per fields in at least 50 fields†	++
> 10 AFB per field in at least 20 fields‡	+++

* A finding of 1 to 3 bacilli in 100 fields does not correlate well with culture positivity. The interpretation of the significance of this result should be left to the NTP and not to the microscopist. It is recommended that a new smear be prepared from the same sputum specimen and be re-examined.

† The reporting of actual AFB counts is recommended to allow a competent authority to determine whether the number fits the TB case definition of the NTP.

‡ In practice most microscopists read a few fields and confirm the finding by a quick visual scan of the remaining fields.

The microscopist should initial the smear result as well as other result entries in the laboratory register.

The microscopist should take at least 5 minutes to read 100 fields, and should never be expected to process and read more than 25 ZN-stained sputum specimens per day when working full time. No more than 10 to 12 specimens should be processed at one time. However, this situation seldom occurs even in the peripheral laboratories of high incidence countries. When TB sputum smear microscopy is fully integrated in to the general primary health care services, the real challenge is to reach a workload high enough to maintain testing proficiency.

4.5 The preservation of smears for quality assurance testing

Before storage of the slides, immersion oil must be washed from the smears. Cleaning the immersion oil from the smear by blotting with lens paper is discouraged because the smear might be scraped off the slide and the oil will never be thoroughly removed. Cleaning the slides of immersion oil by dipping them in xylene (xylol)* and drying them before storing them in slide boxes until the next supervision is recommended. Positive and negative slides should be kept in separate slide boxes. Filled slide boxes should be stored closed and as far removed from heat and humidity as possible until they are sampled for re-reading. Slides should not be dried and stored under direct UV light. The sampling and re-reading of slides should be done as soon as possible, because long term storage under tropical climatic conditions will cause fading of the ZN staining.

* Xylene, mixed ACS Reagent Sigma X 2377 or equivalent. A safer, less toxic, less flammable xylene substitute is available.[6]

5. RECORDING AND REPORTING OF SMEAR MICROSCOPY RESULTS

A positive sputum smear is like a document upon which the diagnosis of pulmonary tuberculosis is based. Results must be recorded and copies of these records must be kept in the laboratory. If possible, positive readings should be confirmed by a second reader. Examined slides should be kept in the laboratory for the period of time prescribed by the NTP for the purposes of supervision and proficiency testing (see chapter 6).

5.1 The laboratory register

The IUATLD laboratory register (Figure 28) has two essential and useful features: it distinguishes between diagnostic sputum smear examination and treatment follow-up microscopy, and allots a single line to each tuberculosis suspect examined, and not to each sputum specimen examined. This permits the rate of smear positive cases among suspects to be evaluated, which in turn allows laboratory supply requirements to be planned based on the number of reported smear positive cases.

– The laboratory code, serial and sequence number on the slide must be the same as that in the results section of the *Request for Sputum Examination* Form. The upper portion of the Form must be accurately completed. The results of the smear examination should be recorded according to the IUATLD grading scale (Table 1). The report form is then dated and signed by the laboratory's responsible officer.

– All information from the laboratory form should be entered in full in the appropriate spaces of the Laboratory Register. All the information requested in the laboratory register must be entered, i.e., a blank space is not a negative result but a missing record. Positive results are entered in red ink.

TUBERCULOSIS LABORATORY REGISTER

Year_____

Lab Serial No.	Date	Name	Sex M/F	Age	Name of treatment unit	Address - patient for diagnosis	Reason for examination*		Results of specimen			Signature	Remarks
							diagnosis	follow up	1	2	3		

* Check the appropriate category from the *Request for Sputum Examination*

Figure 28. Laboratory register for sputum smear microscopy

– Completed *Request for Sputum Examination* Forms must be sent back to the treatment centre or the treating physician within two working days. In case of a referral from another health unit, the patient should receive a copy of the completed form and the original must be sent to the treatment centre. Results should never be given to the patient only. If the patient fails to take the results to the treatment centre, he or she may not receive treatment.

– Upon completion of the examination of each batch of submitted specimens, the date of examination is recorded on the dispatch list which is returned along with the transport box to the originating health centre as soon as possible. Transport boxes are cleaned with a cloth wet with a TB germicide (5% phenol or 0.1% sodium hypochorite) and also returned to the health centre. Caution: both of these solutions are extremely corrosive – protective gloves should be used.

6. QUALITY ASSURANCE OF SPUTUM SMEAR MICROSCOPY

6.1 Definitions

Quality assurance of sputum microscopy is an indispensable part of an effective TB Control Programme. It encompasses the whole process of sputum collection, smear preparation, smear staining, microscopy, recording and reporting.

The purpose of quality assurance programmes is the improvement of the efficiency and reliability of smear microscopy services. A quality assurance programme has three main components:

– Quality control: Quality control is a process of effective and systematic internal monitoring which aims to detect the frequency of errors against established limits of acceptable test performance. Although it is not usually feasible to determine error frequencies accurately, it is nevertheless a mechanism by which tuberculosis laboratories can at least validate the competency of their diagnostic services.

– Proficiency testing: Also known as External Quality Assessment, this is a programme designed to allow participant laboratories to assess their capabilities by comparing their results with those obtained with the same specimens in other laboratories of the network, e.g., Regional and National Reference Laboratories.

– Quality improvement: Quality improvement is a process by which the components of smear microscopy diagnostic services are analysed with the aim of looking for ways to permanently remove obstacles to success. Data collection, data analysis, identification of problems and creative problem solving are key components of this process. It involves continued monitoring and identification of defects, followed by remedial action to prevent recurrence of problems.

6.2 Procedures

Internal quality control of staining is mandatory. New lots of staining solutions need to be tested. This usually involves the staining of known, unstained, positive and negative smears. The inclusion of known, unstained smears each time staining is carried out in the laboratory is also strongly recommended. The re-reading of positive smears by another technologist is highly desirable; in practice however, very few peripheral laboratories employ two TB microscopists. Direct observation by an experienced observer of laboratory technicians performing their routine tasks at all stages is an essential aspect of quality assurance.

There are four principal methods of proficiency testing of smear microscopy:

– Sending smears from the Reference Laboratory to the peripheral laboratory for checking reading and reporting.

– Monitoring the quality of sputum smear microscopy in all its stages during supervisory visits in the field.

– Sending smears from the peripheral laboratory to the Reference Laboratory for re-reading.

– Sampling smears of registered patients found in the District Tuberculosis Register

All four methods have distinct advantages and disadvantages; it is therefore advisable to implement them according to the needs and the circumstances of each NTP.

In the present context, quality improvement consists of correcting deficiencies in smear microscopy performance and reading by taking appropriate remedial action. It is the responsibility of the higher level laboratories of the network, i.e., Regional and Central Reference Laboratories, to retrain technologists who demonstrate less than optimal performance. For a more detailed discussion of quality assurance programmes in TB microbiology, see references 7 and 8.

7. DISINFECTION, STERILISATION AND DISPOSAL OF CONTAMINATED MATERIALS

After the smears are examined, the lids of all used sputum containers are removed. Used containers, lids and applicators are placed in a waste receptacle containing 5% phenol or 0.5% sodium hypochlorite solution and are fully submerged. Thereafter, these materials can be disposed of by autoclaving. If an autoclave is not available, all materials should be burned in an incinerator, an open pit or an empty oil drum (Figure 29). NB: If large numbers of plastic containers are being burnt, the fumes produced are toxic.

In the event that both burnable materials and glass sputum bottles are used, the latter should be discarded into a separate container so they may be boiled and washed for re-use.

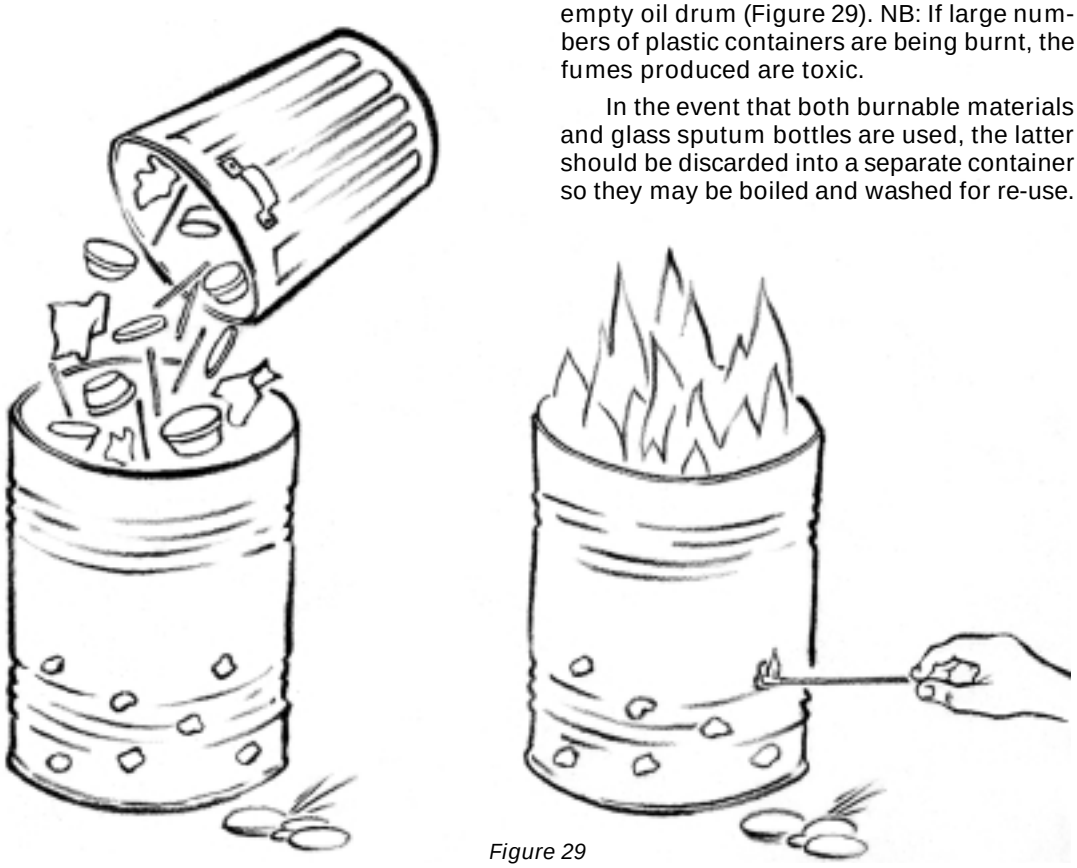

Figure 29

Other items such as slide holders, the dryer and the work surface should be soaked in 5% phenol or 0.5% sodium hypochlorite solution.

After the slides have been quality controlled, the positive slides should be broken and disposed of like other "sharps". Negative slides can either be disposed of, or if necessary, washed clean and re-used for non-TB work (e.g., malaria, haematology).

Slides with negative smears are boiled for half an hour in soap or detergent solution (dishwashing liquid), washed under running water, wiped with cotton or cloth, air dried, examined to confirm absence of scratches, cleaned with an alcohol soaked cotton swab and stored for re-use.

TB slides, whether negative or positive, should never be re-used for TB work.

8. BIOSAFETY IN THE TB MICROSCOPY LABORATORY

8.1 General aspects

Laboratory workers are responsible for their own safety and that of their co-workers. Transmission of *Mycobacterium tuberculosis* results essentially from micro-aerosols, i.e., tubercle bacilli contained in droplet nuclei, 1 to 5 microns in diameter, which are sufficiently small to reach lung alveoli, yet sufficient large to adhere to the lining of the lung alveoli.

Infection control in the laboratory must aim at reducing the production of aerosols. Good ventilation is necessary for the protection of the laboratory staff from airborne infectious droplet nuclei. An easy way to ensure ventilation and directional airflow is by judiciously locating windows and doors so that airborne particles are blown away from the laboratory worker (see Figure 4). Where electricity is available, extractor fans can be used to remove air from the laboratory.

Each time the technologists enter or leave the laboratory they must wash their hands. Staff should wear protective clothing such as laboratory coats while exercising their duties, returning them to the lockers before leaving the laboratory. Access to the laboratory should be restricted to laboratory staff only.

The wearing of disposable gloves for smearing and staining is desirable; however, because they are meant to be discarded after each laboratory manipulation, their use represents a major expense for peripheral labs. Disposable gloves are for single use only, but in many laboratories they tend to be re-used until torn. This improper use affords a sense of false security and carelessness that often impacts negatively on the biosafety conditions of the laboratory – contaminated gloves are used to handle or to operate laboratory equipment that would otherwise never become contaminated. As the use of gloves is impractical in most settings where this guide will be used, soaking hands in 70% alcohol followed by washing with a detergent solution, rinsing with water and drying with paper, is highly recommended.

Wearing conventional surgical masks does not significantly reduce the risk of infection by aerosol inhalation. The emphasis again is to be placed on the reduction of aerosols produced during laboratory procedures by adopting and strictly enforcing Good Laboratory Practices.[8]

Eating, drinking and smoking are not permitted in the laboratory.

8.2 Specific aspects

Laboratory procedures differ considerably in their potential to create aerosols:

– Specimen collection

Sputum from tuberculosis suspects is often collected in the laboratory for sputum collection. This practice exposes laboratory workers to a high risk of contagion by aerosols and should not be allowed under any circumstance. As mentioned in Chapter 1, precautions to lower this risk can be taken by instructing the tuberculosis suspects to cover their mouths while coughing and by having

them produce the specimen outdoors, where the aerosols will be diluted and sterilised by the UV light of direct sunlight.

– Smear preparation

While opening sputum containers and the smearing of slides may produce aerosols, these manipulations entail less risk of transmission than the unprotected coughing of a smear positive patient. There is little evidence that preparing sputum smears is correlated with an increased risk of tuberculosis infection. However, absence of evidence is not evidence of absence, and laboratory workers must be careful and remain vigilant at all times.

Expensive and sophisticated equipment is no substitute for good microbiology laboratory practice. Moreover, commercial type biosafety cabinets (BSCs) require expert and extensive yearly maintenance, an expense that is seldom considered at the time the equipment is purchased. Commercial type BSCs which are not properly maintained give a false sense of protection, and the same applies to the home made variety. Twenty years of field experience in low income countries have demonstrated the impracticality of the design proposed in the first edition of this Guide. Therefore, BSCs are not mandatory in peripheral laboratories that perform smear microscopy only.

9. MATERIALS MANAGEMENT

To ensure the continuous flow of laboratory supplies, programmes must budget rationally for requirements. The only quantifiable basis for planning is the number of patients recorded and reported. The number and percentage of smear positive patients can be determined from the Laboratory Register.

Assuming that the smear positivity rate is 15%, that each tuberculosis suspect requires three sputum examinations and that each case of smear positive tuberculosis has three follow-up examinations, the number of microscope slides and sputum containers needed for each sputum smear positive case detected is (1 / 0.15) x 3 + 3 = 23.

Laboratory material requirements are relatively small and for this reason are ordered every 6 months rather than every 3 months and the reserve requirement is estimated at one year's supply (Figure 30).

The amounts of basic fuchsin, methylene blue, ethanol and phenol are calculated from the IUATLD recommended method for ZN staining, assuming that 5 ml of each of the solutions are needed for each slide. It is further assumed that 2 drops or 1/10 ml of oil are used for each slide.

The calculation is performed as follows:

– the total number of smear positive patients (new patients and retreatment cases) recorded on the previous two Quarterly Reports on Case-finding is entered under the column headed "No. of patients";

– the requirements for the next half year (A) are calculated by multiplying the number of patients by a predetermined factor, based on the assumption that 10 suspects of TB need to be examined for each smear positive case;

– the reserve stock requirements (B) are equal to twice the amount requirements for 6 months (A x 2);

– the amount of materials inventoried (C) in the district store;

– the total order (D) is the sum of the amount required for the next semester (A) plus the amount required for "reserve" stock (B) minus the inventoried amount (C) at the time the order form is completed.

CALCULATING THE REQUIREMENTS FOR PERIPHERAL LABORATORIES AND COST OF MATERIAL PER IDENTIFIED CASE.

Item	Unit	Requirement per slide	Fraction of cases among suspects	Annual depreciation	Requirement per identified case	No of cases	Running requirement	Reserve requirement	In stock	Total order	Cost per case (FOB) identified		Per cent of cost
		A	B	C	D=(1/Bx3+3) xAxC	E	F=DxE	G=2xF	H	I=F+G–H	US$	FF	
Slides	1	1	0.15	1.00	23	1000	23,000	46,000	19,550	49,450	0.667767	4.006600	30.5
Sputum containers	1	1	0.15	1.00	23	1000	23,000	46,000	19,550	49,450	0.909092	5.454554	41.6
Methylene blue	1 g	0.01500	0.15	1.00	0.34500	1000	345	690	293	742	0.002216	0.013294	0.1
Basic fuchsin	1 g	0.01500	0.15	1.00	0.34500	1000	345	690	293	742	0.002625	0.015748	0.1
Immersion oil	1 mL	0.10000	0.15	1.00	2.30000	1000	2,300	4,600	1,955	4,945	0.015859	0.095157	0.7
Sulfuric acid	1 mL	1.25000	0.15	1.00	28.75000	1000	28,750	57,500	24,438	61,813	0.213038	1.278225	9.7
Phenol	1 g	0.25000	0.15	1.00	5.75000	1000	5,750	11,500	4,888	12,363	0.036216	0.217298	1.7
Xylene	1 mL	1.00000	0.15	1.00	23.00000	1000	23,000	46,000	19,550	49,450	0.315561	1.893364	14.4
Methanol	1 mL	0.50000	0.15	1.00	11.50000	1000	11,500	23,000	9,775	24,725	0.024731	0.148388	1.1
Filter paper sheets	1 box	0.00007	0.15	1.00	0.00153	1000	2	3	1	3	0.000005	0.000029	<0.1
Lens tissue	1 pack	0.00010	0.15	1.00	0.00230	1000	2	5	2	5	0.000007	0.000040	<0.1
Slide storage box	1	0.00140	0.15	0.10	0.00322	1000	3	6	3	7	0.000125	0.000748	<0.1
Wire loop holder	1	0.00040	0.15	0.10	0.00092	1000	1	2	1	2	0.000004	0.000025	<0.1
Ni-Cr wire	1 reel	0.00020	0.15	0.15	0.00069	1000	1	1	1	1	0.000003	0.000019	<0.1
Microscope	1	0.00020	0.15	0.10	0.00046	1000	0	1	0	1	0.000138	0.000829	<0.1
Total cost FOB											2.19	13.12	
Insurance, freight, storage, and distribution (30%)											0.66	3.94	
Total cost, including product, insurance, freight, storage, and distribution											2.84	17.06	

Figure 30. Taken from Reference 7 (Table V.6)

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ANNEX 1

PREVENTION OF FALSE-POSITIVE SPUTUM RESULTS

- Use new slides
- Use a new applicator stick for each sample
- Use filtered carbolfuchsin
- Keep slides separate from one another while staining
- Do not use staining jars
- Do not allow carbolfuchsin to dry on the slide
- Do not allow oil immersion applicator to touch the smear
- Do not allow oil immersion lens to touch the smear
- Label sputum containers, slides and laboratory forms completely and accurately
- Cross check the number on the *Request for Sputum Examination* Form and sputum container before recording
- Record and report results accurately

CONSEQUENCES OF FALSE POSITIVITY

- Unnecessary treatment - wastage of drugs
- Loss of confidence in the NTP

PREVENTION OF FALSE-NEGATIVE SPUTUM RESULTS

- Make sure sample contains sputum, not just saliva
- Make sure there is at least 3 ml of sputum
- Select thick, mucopurulent particles for smearing
- Smears should not be too thick nor too thin
- Stain smears for 5 minutes
- Decolourise smears for 3 minutes
- Counterstain for 1 minute
- Read all 100 fields before declaring the slide to be negative
- Known positive control smears should show well stained AFB
- Label sputum containers, slides and laboratory forms carefully
- Cross check the number on the *Request for Sputum Examination* Form and on the sputum container before recording
- Record and report results accurately

CONSEQUENCES OF FALSE-NEGATIVE RESULTS

- Patient remains untreated, resulting in suffering, spread of TB and death
- Intensive phase treatment may not be extended, leading to inadequate treatment
- Loss of confidence in the NTP

ANNEX 2

CARE OF THE MICROSCOPE

The microscope is the centrepiece of the TB diagnostic services of the NTP. Proper handling and maintenance of the microscope by laboratory staff is essential to prolong its useful life. The following points should be observed:

- When not in use the microscope should be stored in a dry, dust and vibration free environment
- Avoid exposing the microscope to direct sunlight and moisture and humidity
- Use silica gel in the microscope storage box; restore by heating when silica gel becomes pink
- Clean the microscope with lens paper before and after use
- Wipe the surface of immersion lens with a piece of clean cotton before and after use. Do not use alcohol for cleaning lenses
- Oil immersion lens should never touch the smear
- Use fine focusing knob only while using the oil immersion lens

ANNEX 3

TROUBLE-SHOOTING GUIDE FOR MICROSCOPY

PROBLEM	POSSIBLE CAUSES	SOLUTION
Field is dim	Condenser too low Diaphragm closed	Raise condenser Open diaphragm
Dark shadows in the field that move with eye piece when it is rotated	Eye piece dirty Eyepiece or objective contaminated with fungus Surface of eyepiece scratched	Clean eyepiece A new eyepiece may be needed A new eyepiece may be needed
The image is not clear	Smear portion of slide upside down Air bubble in the oil Poor quality oil Lens dirty	Turn slide over Move immersion oil objective from side to side Change oil Clean lens
Low power image not clear	Oil on the lens Dust on upper surface of lens Broken lens	Clean lens Clean lens A new lens is needed

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