

**The Public Health Service National
Tuberculosis Reference Laboratory
and the
National Laboratory Network**

**Minimum Requirements, Role and Operation
in a Low-Income Country**

**International Union Against Tuberculosis and Lung Disease
1998**

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Foreword

At the time of the writing of this book, tuberculosis still poses an enormous health problem in many parts of the world, particularly in low-income countries. Progress has been made with the implementation of effective control strategies; many countries, however, still fail to sufficiently recognise the importance and priority of good tuberculosis control. Tuberculosis bacteriology for the detection of sources of infection, the diagnosis of clinical suspects, and the follow-up of the effect and results of treatment, are essential components of tuberculosis control. A well-organised network of laboratories performing the bacteriology of tuberculosis is indispensable for carrying out these functions. The laboratory network can provide valuable operational information which can be used to measure the performance and impact of the national tuberculosis programme, identify potential areas for improvement, and establish treatment policy. For these reasons, it is crucial to ensure that a credible, capable reference laboratory is in place to oversee not only the supervision of the laboratory network, but also the establishment of laboratory standards and policies.

The national tuberculosis reference laboratory, whether it exists as part of the central public health laboratory or as an upgraded laboratory of the country's principal tuberculosis institution, should be at the apex of the tuberculosis laboratory network in order to serve as a reference laboratory for the tuberculosis programme. The reference laboratory plays an essential role in the organisation and maintenance of the network in terms of developing guidelines, ensuring high quality and standardisation of smear microscopy, overseeing the training of laboratory staff, conducting surveillance of drug resistance, participating in epidemiological and operational research, and ensuring supplies and opportune reporting. In this capacity, it should have full facilities for culture and for testing of drug susceptibility.

This book provides useful guidance to national officers responsible for the reference laboratory on its role, main responsibilities and technical and organisational aspects of smear examination and surveillance of anti-tuberculosis drug resistance. It is important to note here that quality assurance and control, training, and monitoring are all general functions of the laboratory, and while this book addresses tuberculosis, the same principles are applicable to other diseases or functions. Accordingly, this book will be a welcome addition to the reference material for the professionals of both the national laboratory and the national tuberculosis control programmes. I congratulate the authors for this effort which will fill a real need in an essential area of tuberculosis control.

Dr Arata Kochi
Director, Global Tuberculosis Programme World Health Organization

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Fadila Boulahbal and Jacques Grosset have greatly contributed to the design and functionality of the national tuberculosis reference laboratory in Dakar, Senegal, which had an important influence on this monograph.

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We are particularly indebted to Chris Collins who made valuable comments on the manuscript and has helped edit this monograph.

Preface

This monograph originates from the appreciation of the needs of our collaborators in numerous countries. Our gratitude then goes primarily to those technicians in peripheral microscopy laboratories, largely in low-income countries, who have shared with us their problems and innovative solutions and who have repeatedly expressed their need for technical and moral support from the national tuberculosis reference laboratory. This monograph thus provides an outline of the responsibilities of the national tuberculosis reference laboratory as the apex of the national laboratory network, within the framework of public health priorities of a national tuberculosis control program. It is dedicated to the technicians who often work under difficult circumstances in remote rural areas of low-income countries and who contribute with their daily work to the success of tuberculosis control.

There are many superb books on mycobacteriology. The purpose of this monograph is not to simply add another one, but to be complementary. It is limited in scope and emphasis, as it does not attempt to cover the entire field of mycobacteriology; it rather focuses on the specific set of minimum tasks that a national tuberculosis reference laboratory has to accomplish as the apex of the national laboratory network. Culture technique, for instance, is solely discussed in the context as a prerequisite for drug susceptibility testing for surveillance of drug resistance, and its potential for increased sensitivity in diagnosis of individuals is not addressed. Similarly, identification of mycobacteria is discussed only to separate the pathogenic species of the *Mycobacterium tuberculosis* complex (including *M. tuberculosis*, *M. bovis*, and *M. africanum*, but excluding *M. bovis* BCG and *M. microti*) with reasonable certainty from environmental mycobacteria, while no attempt is made to provide advice on how to identify species among the latter.

While most of the material presented in this manual has been thoroughly tested in the field and proved to be appropriate and robust, some ideas need further field testing. The authors welcome comments from the field that might help to improve the document in any future edition.

Paris, Dar es Salaam, Oslo, Schiffweiler, Ottawa, Seoul, and Dhaka
September 1998
The authors

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CHAPTER I

Tasks and staffing of a national tuberculosis reference laboratory

I. The tasks of the national tuberculosis reference laboratory

The core activities of a national tuberculosis control program in a low-income country include case finding by sputum smear microscopy and the delivery of effective chemotherapy to patients. The national tuberculosis reference laboratory must concentrate its efforts to support these activities within the national laboratory network. Thus, the major tasks of the national tuberculosis reference laboratory are:

- Maintaining high proficiency in routine smear microscopy carried out in peripheral health facilities
- Training of personnel and quality assurance testing of smear microscopy in the national laboratory network
- Surveillance of anti-tuberculosis drug resistance

Obviously, the national tuberculosis reference laboratory is usually not in the position to carry out all tasks related to training, supervision, and proficiency testing of sputum smear microscopy in the entire country, nor would this be an efficient use of its resources. Nevertheless, the national tuberculosis reference laboratory has overall responsibility to set the standards in the country and to oversee the implementation of policies.

Decentralization of certain activities is highly desirable, and the national tuberculosis reference laboratory should take the leading role in encouraging regional/provincial laboratories to take part in carrying out the essential tasks of the national laboratory network. If in the following the national tuberculosis reference laboratory is the addressee, it is understood that depending on the country-specific situation, these tasks are shared by the intermediate level. In the long term, it will be desirable that effective decentralization is implemented to make the support to the national tuberculosis program increasingly more efficient.

Maintaining proficiency in routine smear microscopy

The primary task of a national tuberculosis reference laboratory is to ensure the quality of work in the national laboratory network. To remain credible, the national tuberculosis reference laboratory must be proficient in the same routine work that peripheral laboratories perform on a day-to-day basis. The national tuberculosis reference laboratory thus performs routine services in examining specimens by sputum smear microscopy from a limited number of designated centers which refer tuberculosis suspects.

To remain proficient and an accepted leader for mycobacteriology in the country, a minimum of positive results must be obtained. To maintain this proficiency in the national tuberculosis reference laboratory, it would be desirable that at least one case, on average, among newly examined suspects should be identified per working day. Experience in International Union Against Tuberculosis and Lung Disease (IUATLD) collaborative programs indicates that on average 15% of all new suspects examined will have sputum smear positive tuberculosis, but the range of positivity is wide, going from 5% to 30%. If it is recommended that each new suspect should have three specimens examined and each new case of tuberculosis must have three follow-up examinations, the numbers of slides to be examined per case of sputum smear positive tuberculosis is $(3/0.15)+3$, i.e., 23 slides per day, if one case is to be discovered on average per day. This amounts to approximately 115 slides per 5-day working week or 6,000 slides per 52 working weeks per year. It is important to emphasize here that the national tuberculosis reference laboratory may run the risk of becoming overwhelmed with routine work and thus failing to adhere to its major responsibilities of quality assurance, training and supervision. For this reason it is recommended that the health units for which routine diagnostic services are offered are carefully selected to ensure that the number of examined slides will not exceed 10,000 per year.

Whenever the number of daily routine smear examinations exceeds 30 slides, fluorescence microscopy is recommended for the excess slides. While it is tempting to take recourse solely to this method, it is not advisable to do so as proficiency in the Ziehl-Neelsen method should not be allowed to be jeopardized. For the practical organization of microscopy work in such a situation, the organization may be such that a portion of specimens are examined by the Ziehl-Neelsen technique, and another portion by fluorescence microscopy. Alternatively, the two techniques might be used on alternating days. Whatever approach works better might be chosen, but it must be ensured that the Ziehl-Neelsen technique remains in continuous use, even if the number of specimens per day is very large.

Integration of the work of the reference laboratory into the general health services is also essential because training must be provided on an ongoing basis to laboratory technicians working at the periphery. Such technicians must receive training reflecting the real life situation as encountered in their daily work. During the period of their training in the reference laboratory, they must participate directly in the routine microscopy services of the national tuberculosis reference laboratory.

Quality assurance of smear microscopy in peripheral laboratories

It is the responsibility of a national tuberculosis reference laboratory to ensure that routine services of sputum smear microscopy at the periphery are provided in a standardized manner and at a high proficiency level. This will require that the head of the reference laboratory, in collaboration with the deputy head, elaborate an annual schedule of visiting various regions and some peripheral laboratories in the selected region. These visits are preferably coordinated and jointly carried out as part of the activities of the central team of the national tuberculosis program. These full-day visits permit the supervision of the daily work. While direct supervision of peripheral laboratories is of utmost importance, more formal systems of proficiency testing and quality control of sputum smear microscopy must be established. The principal objective of such exercises is to maintain and continually improve microscopy services in the country.

Surveillance of drug resistance

One of the major tasks of the national tuberculosis reference laboratory is the surveillance of drug resistance.

Acquired drug resistance is defined as the acquisition of a resistant strain by selective multiplication of the spontaneously-resistant mutant fraction of the bacterial population as a result of inadequate chemotherapy. If a person becomes infected with a resistant strain without ever having been treated with anti-tuberculosis drugs, and develops disease with the drug-resistant strain, that person is said to have *primary resistant* tuberculosis. The term *initial resistance* has been coined to classify strains from patients obtained at diagnosis in whom it cannot be determined whether or not they had ever received prior treatment.

While these theoretical definitions are clear, it is rarely possible in practice to determine the exact nature of resistance. For instance, patients may choose not to disclose prior treatment for various reasons, thus leading to a potential over-estimation of primary resistance. On the other hand, patients who fail treatment

may do so because their disease-causing strain was initially primarily resistant and not because they acquired resistance during treatment. Acquired resistance is the only type that can be determined accurately, but only if both a pre-treatment and subsequent specimen are available and a change from susceptibility to resistance is documented. This is not usually feasible in low-income countries where drug susceptibility testing is not part of the routine diagnostic procedure in every newly diagnosed case of tuberculosis.

This monograph thus abstains from using the terms “primary” and “acquired” drug resistance in the context of drug resistance surveillance. Rather, strains of *M. tuberculosis* complex are stratified in the reporting according to the history of previous treatment given by the patient after careful interviewing. Patients who have never previously received as much as one month of anti-tuberculosis chemotherapy are classified as “never previously treated”, while patients with a history of at least one month of treatment (relapses, i.e., patients with recurrent disease after having been declared as cured in the past, patients returning with recurrent disease after interrupting treatment for more than 2 months, patients classified as treatment failures, and chronic excretors, i.e., patients failing on a full course of a second-line regimen) are classified as “previously treated”. This classification prevents the application of the well-defined terms “primary” and “acquired” drug resistance to situations where the exact nature of resistance must by necessity remain unclear. Furthermore, it follows generally recommended treatment strategies that provide a first-line regimen to previously untreated patients and a different second-line regimen to previously treated patients.

2. Staffing requirements and working days in the national tuberculosis reference laboratory

In order to function properly, the national tuberculosis reference laboratory must be adequately staffed. For optimum performance, the minimum number of employees required will include:

- 1 head of the laboratory
- 1 junior professional deputy head of the laboratory
- 2 laboratory technicians for microscopy
- 2 persons (rotating) responsible for culture services (preparing media, inoculation, drug susceptibility testing, reading)
- 1 person for cleaning and house maintenance (including autoclaving, glassware cleaning, and waste disposal)

Should the work load increase additional staff might be required.

The head of the laboratory is responsible for the overall operation of the reference laboratory and for maintaining close coordination with the national tuberculosis program to carry out the tasks essential for tuberculosis control in the country. In particular, the tasks of the reference laboratory must be clearly prioritized. The qualifications of the head of the laboratory ideally include a doctoral degree in microbiology, medicine or a related field. The head of the laboratory must be capable of organizing, coordinating, and conducting all tasks at a very high level of competence.

The deputy head should second the head in all laboratory-related activities and assume the acting role during the absence of the head of the laboratory. Qualifications should be similar to those of the head of the laboratory, but at the entry level.

Well-trained laboratory technicians knowledgeable in both light and fluorescence microscopy are important for the functioning of the reference laboratory. They are essential to guarantee a high quality of routine services, to teach trainees, and to assist in the quality assurance activities for sputum smear microscopy.

To ensure a continuous high level of proficiency of standardized culture and drug susceptibility testing techniques, two multi-purpose technicians to assume these duties should be available.

One full-time laboratory assistant is needed to assure the sterilization of contaminated material, the washing of glassware, and daily general cleaning of the laboratory.

Rotation of national tuberculosis reference laboratory personnel to work in other national reference laboratories is rarely a productive activity and should be avoided.

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CHAPTER II

Sputum smear microscopy

I. Collection of specimens

The collection of sputum specimens for smear examination should be efficient and as convenient as possible to both patients and laboratory workers. Numerous studies indicate that the incremental yield in detection of cases decreases with the number of serial specimens collected. Most countries have adopted the policy of collecting three specimens as an optimum means of identifying infectious cases of tuberculosis. With this approach, approximately 80% of patients ultimately positive on sputum smear examination will be positive on the first specimen, an additional 15% on the second, and a final 5% only on the third.

Because of the high yield among multi-bacillary cases, the IUATLD has recommended the “on-the-spot - early morning - on-the-spot” collection of sputum. In contrast to the policy of three early morning specimens, which requires four visits by the patient, the IUATLD approach requires only two visits. A first specimen is collected when the patient presents with relevant symptoms. At that time, the patient is given the second sputum container to collect an early morning specimen. The third specimen is collected the next morning when the early morning specimen is brought in. All three specimens can be examined (stained, smeared, and read) in the same morning. A disadvantage of examining all three slides on the second day, rather than examining the first specimen right away, is the potential loss of motivation of the patient to return the following morning, but this is perhaps offset by the convenience to the patient not to have to wait. Furthermore, patients may present for the first time at any time during the day, and it is not usually practical for a laboratory to prepare and examine smears throughout the day. This approach might ultimately yield more cases even if an early morning specimen is preferable to a spot specimen, because at least one specimen can be examined if the patient does not return. The fulfillment of the three-specimen requirement is only possible if the patient returns. If the laboratory register recommended by the IUATLD, which

assigns a single line for one tuberculosis suspect, is used (appendix 5), the implementation of this policy can be rapidly verified: among suspects with negative smears, the proportion of those with one and those with three examinations should approximate 100%, while the fraction of those with two should approach zero.

An evaluation of the policy of requesting three early morning specimens indicated that 26% of all those with negative examinations had only one specimen examined under routine conditions in Tanzania, 20% two specimens, and 54% had all three examined. Calculating the yield of cases that was expected if all suspects actually had three examinations, an estimated 5% of all potentially identifiable cases were not diagnosed because they had not completed three examinations.

The quality of the sputum specimen submitted for examination is important. In spite of this, some studies show that specimens which contain only saliva should be examined even though there may be a low yield. Patients must be encouraged to take their time to produce a specimen that comes from deep within the lungs.

The proportion of specimens containing only saliva among all specimens, which supposedly contain sputum, should be monitored continuously. The basic problem, however, is deciding on a definition of saliva that would be both accurate and operationally feasible.

In general bacteriology practice (which may be also estimated with the Ziehl-Neelsen technique), sputum cytology, i.e., counting the number of leukocytes and of squamous epithelial cells per low power magnification field (approximately 100x magnification), is sometimes used. If the ratio of squamous epithelial cells to leukocytes exceeds 1 to 10, then the likelihood of a salivary specimen increases. Nevertheless, the rule may not apply if the period between collection and examination exceeds 2 days, because leukocytes tend to disintegrate after this time. This method is therefore not recommended for routine practice in peripheral facilities.

The less accurate, but operationally acceptable macroscopic classification of sputum specimens into "salivary", "mucous", "purulent", and "mucopurulent" is widely used, with acceptable results.

A certain proportion of saliva specimens will always be submitted. The pragmatic approach is to select the lowest proportion encountered as the operational standard and to advise institutions obtaining higher proportions to revise their specimen collection systems.

2. Requesting a sputum smear examination

For microscopic examination the interval between collection and staining matters little. Because peripheral health services nearest to the residence of the patient do not usually have sputum containers, most countries have adopted the policy to send the patient to the nearest microscopy center. The policy of sending specimens rather than the patient, although theoretically more convenient for the patient, is very difficult to implement. It needs strong and permanent relations between microscopy center and health services, careful follow-up of the process and careful routine quality assurance and is, in fact, rarely feasible or efficient.

The type of sputum containers is also important. These should be made of break-resistant plastic, with a wide mouth, and a screw cap to avoid leaks, desiccation, and aerosol formation (figure II.1.). The container should have a fixed label for patient information on the side of the container. It has been possible, in practice, to obtain these containers mass-produced to order at a reasonable price.

It is important that laboratories obtain sufficient essential information on the person whose sputum they need to examine. This is best accomplished by wide distribution of a request form for sputum smear examination (appendix 3) which

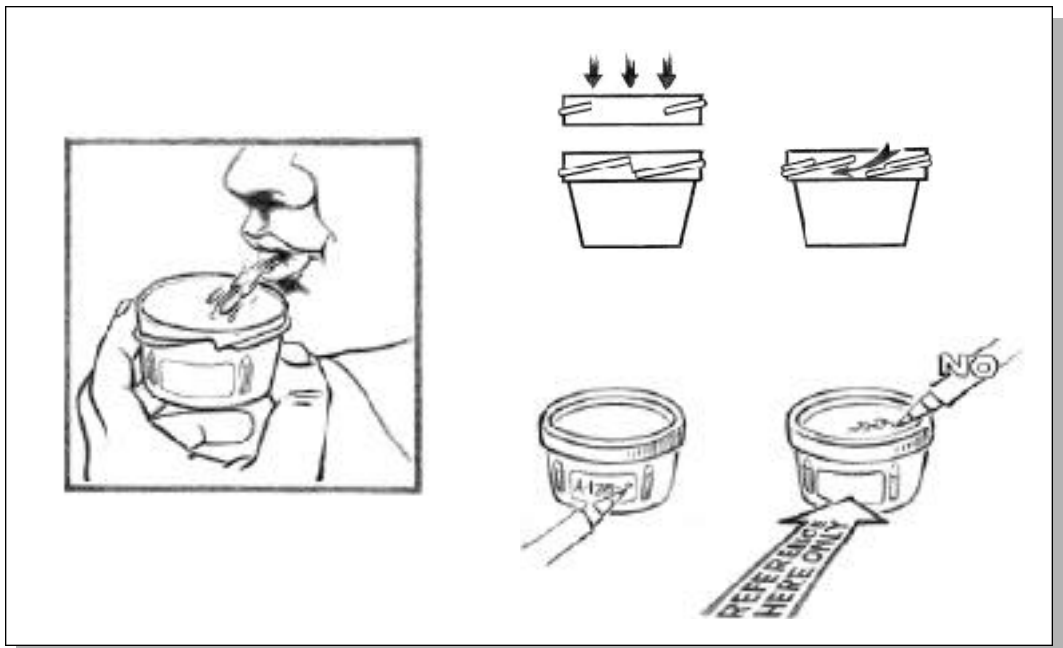


Figure II.1. Wide-mouthed, screw-cap, plastic sputum container for use of sputum collection in peripheral laboratories.

facilitates the work of both the clinician submitting the request and the laboratory technician carrying out the examination. Clinicians must clearly state on the request form whether the examination is for diagnosis or bacteriological follow-up on treatment. This information must be recorded in the laboratory register to allow calculation of laboratory material requirements and epidemiologic evaluations.

3. Preparing and staining of sputum smears

Sputum smear positive patients are those who are the most potent sources of transmission in the community. The sensitivity (proportion of actual cases identified) of sputum smear microscopy in the diagnosis of pulmonary tuberculosis is far from perfect. With a diligent technique, approximately 60% of all adults with pulmonary tuberculosis can be identified this way, and the proportion is perhaps even lower among patients co-infected with the human immunodeficiency virus, and very low among children. However, its sensitivity in identifying transmitters of tubercle bacilli is perhaps in excess of 90%, as sputum smear positive patients are up to 10 times as likely to transmit *M. tuberculosis* as patients negative on three consecutive examinations. Thus, sputum smear microscopy is one of the most efficient tools of case finding in a national tuberculosis control program because of its ability to identify and distinguish the cases with highest priority in tuberculosis control.

Preparing the smear

When preparing a smear from a sputum specimen, the technician must decide which part of the specimen is to be used for the smear. Sputum specimens are usually non-homogenous except if left standing over night (e.g., the first on-the-spot specimen) which may lead to some autolysis. If the specimen contains obvious purulent parts, these might be selected preferentially. Thin, disposable applicator sticks (e.g., made from bamboo) are much preferable to wire loops, as the former are better to pick up desired particles than the latter and can be disposed of easily, while wire loops need to be disinfected by heating before re-use.

Often smears are too thick, which will prevent proper reading of stained smears. As a rule of thumb, the thickness of a smear should be such that a newspaper can be read through the smear if held under the slide and the smear should be evenly spread out on the slide. A stained smear should show a light blue color from methylene blue. If the smear is dark blue it usually indicates that it is too thick. The size of the smear is often recommended to be 20 mm by 10 mm. A 20 mm wide smear will correspond to a length of approximately 100 oil immersion fields.

Before heat fixation, the smear should be left to dry in air.

Color of acid-fast bacilli

Numerous methods have been developed for acid-fast microscopy. All of these have some advantages and some disadvantages. Standardization of the method throughout the country is more important than which method is chosen. Quality assurance becomes extremely complicated if each laboratory uses a different method; ordering staining reagents becomes an outright impossibility, because different methods require different amounts of reagents. In the long run it is thus a good investment to train new laboratory personnel in a single technique and to convince those already trained to switch to a single recommended technique.

Acid-fast bacilli should be of a strong red color. If this is the case, a higher proportion will be visible, resulting in more positive results. This requires a high enough concentration of fuchsin. For this reason, some experts propose to use a 1% solution rather than the 0.3% recommended in table II.1. It is also very important that the stain on the slide is sufficiently heated, and that the stain remains sufficiently long on the smear. No harm is done as long as the stain does not dry up on the smear.

Decolorizing

Everything except acid-fast bacilli should be decolorized almost completely, to make reading easier. This is more easily accomplished by using sulfuric acid 20-25% than hydrochloric acid 3%, but it can be done with either acid (except when the smear was made too thick) by repeating the step several times. It is almost impossible to decolorize acid-fast bacilli that were well stained first using watery acids, so the decolorizing time is not that critical, while acid-alcohol has a strong decolorizing action. The choice of the acid (hydrochloric acid or sulfuric acid) is best ascertained by comparing the two methods to allow determination of the preference of the majority of experienced technicians before a countrywide policy is decided upon.

Counterstain

The counterstain should give a good contrast, and hide the remaining red of the background but without also hiding acid-fast bacilli in thicker smears. Furthermore, the counterstain should give enough detail for keeping the smear easily in focus while scanning, but not so much that the background takes too much attention.

Table II.1. Material and reagents to prepare solutions for Ziehl-Neelsen staining and method of staining.

Adapted from: International Union Against Tuberculosis. Technical guide for sputum examination for tuberculosis by direct microscopy. Bull Int Union Tuberc 1978; (Suppl 2): 4-16.

Solution A: Saturated alcoholic solution of fuchsin

Basic fuchsin 3 g
Ethanol 96%..... q.s. 100 mL

(q.s. = the quantity required to make up a certain volume. In the above formulation this means placing 3 g basic fuchsin in a volumetric flask or measuring cylinder and then adding enough ethyl alcohol or methylated spirit (q.s.) to obtain a total volume of 100 mL.

Solution B: Phenol solution, 50 g/L (5%), aqueous

Phenol crystals..... 10 g
Water, distilled if possible q.s. 200 mL

Then:

Solution A 10 mL
Solution B 90 mL

Formula for decolorizing agent

Water, distilled if possible 300 mL
Slowly add sulfuric acid, concentrated 100 mL

or

Ethanol 96% 970 mL
Hydrochloric acid 30 mL

Formula for counterstaining solution

Methylene blue 0.3 g
Water, distilled if possible q.s. 100 mL

For staining, the slides must be placed on a staining slide-rack over the sink with smeared side uppermost, their edges separated by 1 cm.

- 1) Cover the whole surface of the slides with Ziehl's carbol fuchsin after filtration. Heat gently until vapor rises. Do not bring the stain to boil or dry on the slide. Leave the warm stain for at least 5 minutes.
- 2) Rinse each slide gently under tap water until all macroscopically visible stain has been washed away. Replace the decolorized slides on the rack and cover each slide with the decolorizing solution for 3 minutes. Rinse each slide again gently under tap water until all macroscopically visible stain has been washed away.
- 3) Replace decolorized, rinsed slides on slide-rack and flood smear with methylene blue solution for 1 minute. Rinse each slide gently under tap water until all excess stain has been washed away and allow to dry in open air.

Methylene blue is rather difficult to use in this respect. It gives a beautiful color contrast, but it will hide acid-fast bacilli in thick smears or when it is too concentrated or for too long. Some experts would thus recommend using a maximum of 0.1% (rather than the 0.3% recommended in table II.1), and for only about half a minute.

Alternative counterstaining solutions have been used with good results. Light staining, e.g., with dilute malachite or brilliant green solutions, makes a better contrast with fuchsin in thicker parts of films. If provisions have to be made for the color blind, picric acid might be a good choice.

Rinsing water

The rinsing water must be clean, and, if re-staining is required for quality assurance, as free of environmental mycobacteria as possible. For this reason rubber tubing on a tap to direct the jet should not be used, as environmental mycobacteria may preferentially grow there. A beaker that can be thoroughly cleaned should be used instead.

Usually staining racks over a sink are used, and in that case it is good practice to leave sufficient space between the slides, to prevent the flow of solutions from one slide to the next.

Ziehl-Neelsen acid-fast staining as recommended by the IUATLD

The method of choice for sputum smear microscopy is the Ziehl-Neelsen technique. It is the only method providing consistently good results without need for special equipment, and the required binocular microscope can be used for other purposes as well. There are two methods of preparing Ziehl's carbol fuchsin; one requires a scale (table II.1), the other does not. Experience in the field has shown, however, that the method requiring no scale is difficult to use because basic fuchsin powder will tend to solidify into a paste if the preparation is not done precisely as recommended, thus making it impossible to process further. Whenever possible, arrangements should thus be made to have the solutions prepared at the nearest laboratory where a weighing scale is available, as it is preferable to prepare staining solutions in laboratories that are properly equipped to do it. Distilled water should preferentially be used whenever possible. Ideally, the reference laboratory or the laboratory at the intermediate level should be preparing and distributing staining solutions to the peripheral laboratories. Alternatively, it may distribute ready, exactly pre-weighted amounts of stain. Such a practice guarantees better standardization and makes quality assurance of this activity much easier.

Table II.2. Materials and reagents to prepare fluorescent dye and method of staining.

Adapted from: Hagemann P K H. Fluoreszenzfärbung von Tuberkelbakterien mit Auramin. Münch Med Wschr 1938;85: 1066-1068.

Solution A:

Auramine O	0.1 g
Ethanol 96%.....	10 mL

Solution B:

Phenol crystals	3.0 g
Water, distilled	87 mL

Then:

Mix solution A and solution B

Decolorizing solution:

Ethanol, 70%	100 mL
Slowly add hydrochloric acid, concentrated.....	0.5 mL

“Counterstaining” solution

Potassium permanganate	0.5 g
Water, distilled	100 mL

For staining, the slides must be placed on a staining slide-rack over the sink with the smeared side uppermost, their edges separated by 1 cm.

- 1) Cover the whole surface of the slides with auramine O solution and stain for 15 minutes. Do not heat.
- 2) Rinse each slide gently with water until all macroscopically visible stain has been washed away. Replace the decolorized slides on the rack and cover each slide with the acid alcohol solution for 2 minutes. Rinse each slide again gently with water until all stain has been washed away.
- 3) Replace decolorized, rinsed slides on slide-rack and flood smear with potassium permanganate solution for 2 minutes. Time is critical with potassium permanganate because counterstaining for a longer time may suppress the fluorescence of the acid-fast bacilli. Rinse each slide gently with water until all stain has been washed away and allow to dry in open air.

There are also two options provided for the decolorizing agent: either hydrochloric acid or sulfuric acid. Hydrochloric acid is less expensive, less corrosive, and environmentally more acceptable. The advantage of using sulfuric acid, on the other hand, is its superior ability to destroy mucus and contaminant bacteria, thus rendering acid-fast bacilli more visible. While the overall cost of the decolorizing agent is of minor importance in the diagnosis (table V.5), the environmental impact as well as the corrosive nature of the acid need to be carefully considered before reaching a decision.

Fluorescence microscopy with auramine O

If the number of daily specimens to be examined exceeds 30, and if electricity is continuously available, fluorescence microscopy might become more cost-effective. Additional requirements in training and economic considerations (capital investment and maintenance) must be taken into account before introducing fluorescence microscopy. Fluorescence staining utilizes basically the same approach as Ziehl-Neelsen staining, but carbol fuchsin is replaced by the fluorescent dye, the acid for discoloration is gentler, and the counterstain is not essential although useful to quench background fluorescence (table II.2). Both sensitivity and specificity of fluorescence microscopy are comparable to the operating characteristics of the Ziehl-Neelsen technique. The most important advantage of fluorescence staining is that slides can be examined at a lower magnification, thus allowing the examination of a much larger area per unit of time. In fluorescence microscopy, the same area that needs examination for 10 minutes with light microscopy might be examined in 2 minutes. Its disadvantage lies with the cost for capital investment, as a fluorescence microscope costs approximately four to five times as much as a light microscope. Furthermore, the halogen lamps need frequent replacement (200 hours working time) and, because they have to be imported, they are often not kept in stock. The running costs of fluorescence microscopy versus bright-field microscopy are largely determined by the salaries of the technicians: the efficiency increases with increasing labor cost.

The national tuberculosis reference laboratory must be proficient in both Ziehl-Neelsen and fluorescence microscopy. Several fluorescent dyes have been utilized and all give very similar results. Here, the original staining technique with auramine O is recommended.

With auramine O, acid-fast bacilli appear bright yellow against a dark background. If there is uncertainty about the presence of a bacillus because of the lower magnification, the slide might be examined subsequently with oil immersion

where the bacilli appear larger than in light microscopy and with the granulation of the bacilli very clear. Alternatively, an auramine O stained slide can be re-stained by the Ziehl-Neelsen technique. Over-staining needs only prior de-oiling with xylene, but not de-staining.

Because of the lower magnification and consequently the larger field, the grading of a slide is not the same as with light microscopy. The area of examination grows with the square of the reciprocal value of the relative power of the objective, i.e., an objective of 40x with an eyepiece of 10x allows the examination of an area that is about five times larger than that seen through an objective of 100x and an eyepiece of 10x magnification. Any grading in this case must thus be adjusted by a factor of 5. To allow comparative grading of positive smears in fluorescence microscopy, the best approach is to re-stain the smear with the Ziehl-Neelsen method, and to re-read the slide, which will also permit confirmation of positivity.

Other methods

Other methods for light microscopy include cold staining techniques (such as with Kinyoun's or Gabett's solution, or Tam Tham Hok's method). It should be noted that the performance of cold staining might have been overestimated. A careful study has shown that the quantity of bacilli seen with a cold staining method is smaller than with Ziehl-Neelsen staining, which might pose problems in paucibacillary specimens. The Gabett solution has advantages only for experienced technicians who have to stain large numbers of smears, since it consists of only two steps (acid and methylene blue combined), but the background is often not satisfactory.

Other methods for fluorescence microscopy include staining with auramine/rhodamine, rhodamine, acridine orange, and others. These methods have proved reliable, but are not recommended here, because they are more expensive, use more toxic reagents, and offer no advantage in the operating characteristics of the test.

4. Examination of sputum smears

For the examination, a binocular microscope with an oil immersion objective (magnification 100x) and eyepieces of moderate magnification (8x to 10x) should be used. In countries with irregular electricity supplies, it is best to use microscopes that allow the power source to be changed from electric to sunlight through a collector mirror.

Before examining the slide, one or two drops of immersion oil are applied to the left edge of the stained smear. Attention should be taken not to touch the

slide with the oil applicator, to avoid potential contamination of the immersion oil and transferral of acid-fast bacilli to another slide. Acid-fast bacilli appear bright red against the background material counterstained in blue. Tubercle bacilli are quite variable in shape, from very short fragments to elongated types. They may be uniformly stained or with one or many gaps, or even granular. They occur singly or in small groups, and rarely in large clumps. The typical appearance is of bacilli that are rather long and slender, slightly curved rods. If structures are seen that have the correct color and a compatible morphology, they must be considered as acid-fast bacilli and be reported as such. They may occasionally not be tubercle bacilli, because environmental mycobacteria, some nocardia, and corynebacteria spores may also be acid-fast. Nevertheless, no attempt should be made to differentiate these in microscopy, since the margin of error is unacceptably high.

Several grading scales for the number of acid-fast bacilli found in a smear have been proposed over the years. The IUATLD scale proposes five groups for reporting the results of reading the smear; they should be recorded as follows:

Finding	Recording
No acid-fast bacilli found in at least 100 fields	negative
1 to 9 acid-fast bacilli per 100 fields	exact figure/100
10 to 99 acid-fast bacilli per 100 fields	+
1 to 10 acid-fast bacilli per field in at least 50 fields	++
More than 10 acid-fast bacilli per field in at least 20 fields	+++

Very scanty results (1 to 3 bacilli per 100 fields) do not correspond very well with culture, and a repeat examination of another specimen for confirmation of such a result is thus usually recommended. A decision as to whether a finding of 1 to 3 bacilli per 100 fields is to be considered as significant or not should be defined by the national program and not by the individual laboratory technician.

5. Recording and reporting results of sputum smear examinations

The utilization of a special register for tuberculosis laboratory examination has proved very useful (appendix 5). Two essential features of the IUATLD laboratory register are 1) that a distinction is made between patients presenting for diagnostic examination and patients presenting for a bacteriologic follow-up examination, and 2) that a single line is allotted to each examinee, not to each examination. This permits an evaluation of the proportion of cases among suspects, which in turn allows the calculation of requirements in laboratory materials based on the number of reported smear positive cases.

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CHAPTER III

Training and quality assurance in sputum smear microscopy

I. Training of technicians in sputum smear microscopy

One of the essential tasks of the national tuberculosis reference laboratory is to take responsibility for the training of laboratory technicians. It must take the responsibility to write a training curriculum and a manual of standard operating procedures for laboratory technicians. It should also regularly provide trainees with on-the-job training under the direct supervision of experienced laboratory technicians. Laboratory technicians also need refresher courses that should be offered by the national tuberculosis reference laboratory. Training activities and refresher courses must involve the participation of laboratories at the intermediate level (provincial/regional). The national tuberculosis reference laboratory should involve intermediate level laboratories in the development of a standardized curriculum in training laboratory technicians to allow progressive decentralization of such activities. Overall responsibility for the level and frequency of training of laboratory technicians resides in the national tuberculosis reference laboratory.

It should be stressed that acid-fast microscopy is not difficult to learn; its challenge lies with the perseverance of continued high quality reading. Thus, only a few hours in training need to be devoted to theory, followed by up to one week of practice, best followed by a period of in-service with systematic on-the-spot confirmation of positive findings, while the ability to correctly identify negative results is best checked by quality assurance sampling.

The value of refresher courses is more doubtful. They do have an essential place when policy changes have to be implemented (e.g., a change in the staining technique). Otherwise, technicians who perform acid-fast microscopy regularly with good supervision, including proficiency testing, are unlikely to be in need of such refresher courses. Occasionally, refresher courses may nevertheless be used as additional motivators.

2. Quality assurance of sputum smear microscopy

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

- *Quality control*: Quality control is a process of effective and systematic internal monitoring that allows the frequency of errors to be estimated against established limits of acceptable test performance. Although it will usually not be feasible to determine error frequencies accurately, it is nevertheless a mechanism by which tuberculosis laboratories can determine the competence of their diagnostic services.
- *Proficiency testing*: External proficiency testing is a program designed to allow participating laboratories to be visited and to assess their capabilities by comparing their results to those obtained in other laboratories, e.g., intermediate and national reference laboratories.
- *Quality improvement*: Quality improvement is a process by which the components of smear microscopy diagnostic services are analyzed with the aim of looking for ways to permanently remove obstacles to success. Data collection, data analysis, and creative problem solving are the key components of this process. It involves continued monitoring, identification of defects, followed by remedial action to prevent recurrence of problems. Often, problem solving can only be done efficiently during on-site supervisory visits.

Quality control of sputum smear microscopy

Maintenance of technical proficiency

To remain proficient in the performance of sputum smear microscopy and the interpretation of its results, a peripheral laboratory needs to examine at least 800 smears per year, which corresponds to a population of 50,000 to 150,000, depending on the incidence of tuberculosis suspects and the use of health services.

Staining

New batches of stains must be checked by including at least 1 slide known to be positive and 1 known to be negative for acid-fast bacilli in a series of smears to be stained with them. This monitors the staining capacity (known positive) and the eventual contamination of reagent solutions by acid-fast bacilli (checking the known negative slide), from, e.g., tap water.

A minimum requirement should be that each new batch of staining solution is checked before it is sent out or put into use, and that records are kept

(batch number, destination, results of control). Two to 3 positive and 2 to 3 negative smears should be used for such a control on each new batch of staining solutions. For this, distilled water should be used to exclude with certainty that eventually discovered contaminants originate from tap water.

Positive control smears will also help personnel to differentiate between acid-fast bacilli and artefacts. A second person should double-check all smears declared to be positive. After removing the immersion oil, the first slide of each examinee (see below) should be stored (in a dark and dry place, of particular importance for fluorochrome stained slides) until slide-holding boxes are full or until slides have been collected for external proficiency testing, whichever comes first.

A relatively simple tool observing laboratory yield is to plot the monthly proportion of positive cases among suspects (figure III.1). Even without plotting allowable departures from the mean by calculating standard deviations (on an annual sample), such a graph provides an immediate visual aid to both the

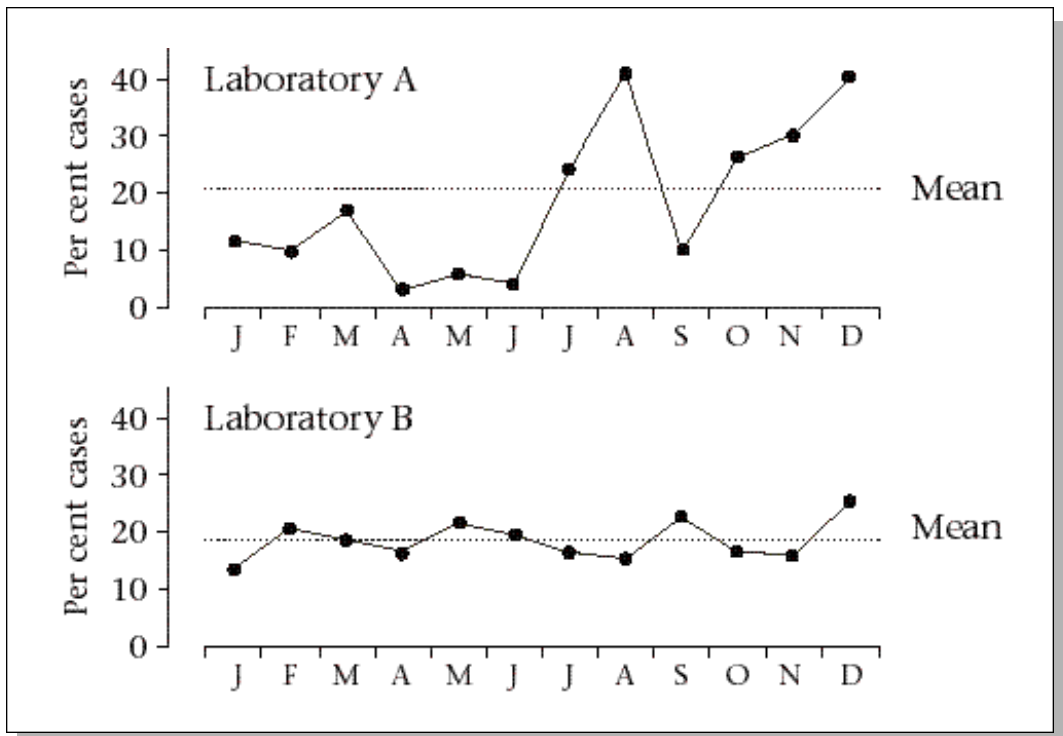


Figure III.1. Examples of the frequency of cases among tuberculosis suspects, by month of examination in the laboratory.
Data courtesy of Damien Foundation, Bangladesh.

technician, clinicians, and the tuberculosis management team. The results may point to a deficiency in case-finding procedures by the clinicians or deficiencies in the laboratory, or both: if the proportion of cases among suspects rises above a certain cut-off (e.g., two standard errors above the annual mean), this might indicate a lower index of suspicion by clinicians who request sputum smear examinations or impediments to patients to access the health care facility. Possibly, the laboratory checks specimens more carefully or erroneously finds acid-fast bacilli that are not tubercle bacilli. On the other hand, if the frequency of cases among suspects falls below a certain cut-off (e.g., two standard errors below the annual mean), clinicians may submit specimens from patients who do not qualify as tuberculosis suspects. Alternatively, the laboratory might have lessened its diligence in examining sputum smears, and misses true cases.

A more sophisticated approach that requires good skills in quantification would plot the proportions that are graded as scanty (1 to 9 bacilli per 100 fields), 1 +, 2+, or 3+ among all positive cases. Of particular interest here is the frequency of specimens among all positives that are graded as scanty as these are the most difficult to identify properly. If this is not done routinely, these proportions might be determined during supervisory visits.

Proficiency testing of sputum smear microscopy

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

- Sending slides from center to periphery
- Monitoring the quality of sputum smear microscopy during supervisory visits in the field
- Sending slides from periphery to center
- Sampling slides of registered patients.

All four methods have distinct advantages and disadvantages, and it is thus advisable to develop several methods in parallel, specifying the objectives of each (table III.1).

Proficiency testing by sending slides from center to periphery

The simplest way of assessing the performance of peripheral laboratories is to collect slides from the routine services provided by the regional/provincial or reference laboratory. This is done by collecting on an ongoing basis slides of good quality and keeping them in seven slide-holding boxes, keeping them in a cool and dry place to prevent fading of fuchsin.

Three holding boxes are for negative slides, one for slides graded scanty, one for slides graded 1+, one for slides graded 2+, and one for slides graded 3+. At six-month intervals, sets of 6 slides (3 negative, one 1+, one 2+, and one 3+) are sent simultaneously to all target laboratories with the request to return the completed form with the recorded results of labeled slides (appendix 7) in an enclosed, pre-stamped, and self-addressed return envelope.

The advantage of this method is the ability to obtain a quick assessment of the technical ability to read smears in the target area with very little effort from both reference and peripheral laboratories. If important discrepancies from

Table III.1. Proficiency testing in sputum smear microscopy

Method	Advantages	Disadvantages	Use
On supervision	<ul style="list-style-type: none"> • Direct contact • Observation of actual work • Motivating to staff • Identifies causes of errors • Permits verification of the quality of equipment 	<ul style="list-style-type: none"> • Selective, not country-wide, if left solely to reference laboratory • Labor intensive • Costly 	<ul style="list-style-type: none"> • Always during supervisory visits
Periphery to center	<ul style="list-style-type: none"> • Country-wide • Low workload for periphery 	<ul style="list-style-type: none"> • Heavy workload for center • Unavoidable inaccuracies • Biased, if technique is not careful • Personnel must be made available 	<ul style="list-style-type: none"> • Standard for surveillance
Center to periphery	<ul style="list-style-type: none"> • Low workload for center • Rapid response country-wide • May lead indirectly to identification of faults in equipment 	<ul style="list-style-type: none"> • Ability testing, not routine performance 	<ul style="list-style-type: none"> • After training • Rapid assessment of training needs
Tuberculosis register based	<ul style="list-style-type: none"> • Unbiased assessment of accuracy of classification of registered patients 	<ul style="list-style-type: none"> • Logistically difficult • Heavy workload for center 	<ul style="list-style-type: none"> • Consider for repeat spot checks in selected tuberculosis management units

expected results are observed, the problem may not lie with the capability of the examinee, but in poor equipment, such as poor condition of the microscope or an insufficient light source.

This method does not address the quality of staining. Sending unstained slides of known results can circumvent this. This approach, however, requires a more sophisticated preparation by the reference laboratory using homogenized sputum specimens or methyl cellulose spiked with quantities of bacilli known to result in the desired quantities per examination area. Mock sputum substitutes are difficult to prepare, have been tried repeatedly with little success, and are rightly considered as the fakes they are, i.e., not what is found in the field.

The major disadvantage of the method lies in the fact that technicians have an unlimited time for the examination of control slides and that they are aware of being tested. Therefore, this method does not allow the assessment of the quality of slide reading under routine conditions; it can only measure the ability of technicians to read slides correctly. It is routinely used after the training of new technicians or following refresher courses to assess optimal performance.

The results can be tabulated at three levels (table III.2):

- Analysis by slide
- Analysis of subsets of positive and negative slides
- Analysis by entire set of slides.

In the example shown in table III.2, the performance of the technicians was rather poor, as only half of them were capable of identifying correctly all positive and negative slides in the set of six. Such large deviations from the expected results raise serious questions not necessarily about the capability of the technicians, but the quality of their equipment, and quite obviously identify the laboratories that need an urgent supervisory visit.

Monitoring the quality of sputum smear microscopy during supervisory visits in the field

Visits to the peripheral laboratories by personnel of the reference laboratory are essential for several reasons. Only the field visit can convey a realistic picture of the conditions under which the technicians have to work. It allows review of the general layout of the laboratory with the technician, the condition of the equipment, and the adequacy of the supply line. Because it is on the technician's home-turf, concerns and problems encountered in the operation of the laboratory might be expressed more freely. However, the national reference

Table III.2. Example of an analysis of results of quality control using 3 negative, and 3 varyingly graded positive slides from the reference laboratory to 42 laboratories. Analysis by slide and sets.

Data courtesy of Dr. Fatoumata Ba, Programme National de lutte contre la Tuberculose, Sénégal.

<i>Analysis by slide</i>			
Peripheral laboratory	Reference laboratory result		
	Positive	Negative	Total
Positive	115	15	130
Negative	11	111	122
Total	126	126	252
Fraction discordant among positive slides (11/126):		0.087	
Fraction discordant among negative slides (15/126):		0.119	
Total fraction discordant ((11+15)/252):		0.103	
<i>Analysis by set of positive and negative slides</i>			
Number correctly reading a set of three positive slides:		32 of 42	
Fraction correctly reading a set of three positive slides:		0.762	
Number correctly reading a set of three negative slides:		29 of 42	
Fraction correctly reading a set of three negative slides:		0.690	
<i>Analysis of entire set of six slides</i>			
Number correctly reading set of six slides:		21 of 42	
Fraction correctly reading set of six slides:		0.500	

laboratory can only visit a very limited number of peripheral laboratories. Routinely visiting the peripheral laboratories is thus mainly a task of the intermediate laboratories.

A visit should be planned to allow observation of the work performed during one entire day. This includes specimen collection and reception, staining, examination, reporting, and recording. Only such a full-day visit will allow an in-depth discussion of any observed deviations from nationally recommended procedures.

The details to be looked into during a supervision visit can be considered under four headings:

- Personnel, infrastructure, safety, and equipment
- Stocks of supplies and consumables
- Registration and transmission of results
- Performance.

Personnel, infrastructure, safety, and equipment

Personnel

The main challenge with acid-fast microscopy is the human factor: it is not a difficult technique to master, but it is tough to do it day after day.

The requirements and management of laboratory personnel vary according to location. An important component of the diagnostic work in the national tuberculosis program in many low-income countries will take place in predominantly rural locations.

In such settings, the workload required for tuberculosis control is such that it is carried out by a multi-purpose laboratory technician, only one of whose responsibilities is to do with tuberculosis.

Alternatively, and increasingly, tuberculosis-related work is performed in large urban settings where the workload may be enormous and where personnel requirements are frequently underestimated. In such situations, it may happen (although frequently not) that the technician's sole task is related to tuberculosis. It is in such a setting that careful attention must be given to workload and consideration of the use of fluorescence microscopy for routine practice.

During supervision, an estimate of the average daily workload per technician should be made, and subsequently everything possible should be done to remedy overloading the technicians. It should be realized that the average tolerable load must not be set too high, especially when other tests are also being done in the laboratory. An average of over 20 slides per technician per working day means that on some days 40 slides or more may have to be processed. Although this may seem feasible simply calculating the time it would take, it is an illusion to think that reliable work can still be done with such a workload. In case of overload, if the means and the infrastructure permit (usually only in regional or large hospital laboratories), it is best to provide a fluorescence microscope which will increase the capacity immediately by about 5-fold. Results after a careful introduction period will probably be more reliable compared to installing 5 technicians, each with an ordinary microscope (which will often not be a realistic solution anyway).

Infrastructure

If visits are frequent, the infrastructure will not have to be checked unless major changes have occurred since the last visit. It is meant to indicate whether the facilities and personnel are adequate to permit good job performance. The room should offer enough space to make a division into at least 2 areas possible (one for registration and microscopy, one for handling sputum and smears). It should be observed whether the set up allows easy movement of all the persons working there. Good ventilation is essential for safety. Sufficient natural light will also aid health care worker protection, apart from making microscopy possible when there is no electricity. A reliable supply of running water and drainage as well as electricity are optimal, but difficult to provide when absent. The job can be performed satisfactorily without running water and electricity if at least clean water can be obtained from a nearby well, and if microscopes and light conditions (no trees in front of the window!) permit slide examination without electricity.

Safety in the peripheral laboratory

Safety can be checked by looking at the equipment that is present and by watching the handling of specimens. The most important aspect of safety is the method of obtaining a sputum specimen from a patient: this must always be done either in the open air or in a well-ventilated environment. Obtaining specimens by induction of sputum or by bronchoscopy requires particularly rigorous precautions.

Laboratory coats are a small cost in most countries, and there should thus be no problem in providing them. Although wooden (bamboo) applicators are recommended, if a bacteriological loop is used there should also be a jar with sand/alcohol to clean it before flaming. The methods used to dispose of infectious waste material, such as sputum containers and applicator sticks, should especially be checked. Such material should certainly not be simply thrown in a shallow pit or dumped in a corner of the compound. A simple kind of burning drum may be the most appropriate device, although it tends not to survive for very long. Soaking or boiling in disinfectant would be easiest under most circumstances, but may be abandoned soon because of its unpleasantness. Autoclaving may only be rarely possible. The presence of the necessary equipment should be confirmed along with evidence of recent use.

Equipment

As general equipment, at least a sufficient number of tables and chairs as well as a plastic basin for staining and a big bucket with a lid for infectious waste

should be available. If this is not available, the supervisor should do all possible to change this. A candle-type water filter is the minimum if stains have to be prepared at the center and no distilled water is available. Some equipment for incineration or disinfection of infected waste should be available in or near the laboratory.

Although some equipment might be dropped from the essential list, good microscopes are absolutely essential if reliable results are to be expected. Apart from the optical quality of the chosen microscope, it is essential that it is robust. The national program and all supervisors should do everything possible to provide for this. Reserve microscopes should be kept at the regional laboratory or the national tuberculosis reference laboratory in a safe, preferably air-conditioned store. Prompt reaction is required if a problem with the microscope emerges in one of the centers. Reliable performance can not be expected with a monocular microscope, or with one which does not have a mechanical stage. Appropriate mirrors will still be needed in many places, even if power is available part of the time. In larger centers there may be a fluorescence microscope. In that case, it must be checked that at least one spare bulb for the excitation burner is available at any time, and that technicians know how to fit it properly.

Finally, good microscopes are difficult to replace, so provisions have to be made to keep them in good condition. In many countries this means first of all taking precautions against theft, aided by a strong cupboard with a lock. In dry countries, microscopes should be kept under a dust-cover when not in use. All openings meant to receive objectives or eyepieces should be closed (by one of the lenses, a plastic cap, or a piece of tape). In humid climates, fungus threatens the optical system of the microscope. Prevention of fungus implies drying the lenses daily, which can be accomplished using ventilation and heat. If electricity is available during the night, the best solution is to mount a 20 to 40 watt bulb in the compartment of the cupboard where the microscope is kept. A few small holes near the bottom and others diagonally opposite to the first at the top of the compartment will allow air circulation. The dust cover should not be used in this case. Alternatively, if no electricity is available at all, one might rely on silica gel or an other drying agent. However, this will need a large and regular supply of the agent even when it is regenerated regularly by heating in an oven or pan. A small quantity is placed on the stage, in an open container, and the space to be dried is made as small as possible by keeping the microscope plus the silica under a well-sealed cover or in its box. Usually the silica will be saturated after only one night, and a new quantity will have to be used daily.

If stains have to be prepared at the center, some minimum equipment will have to be present. Scales or a balance with a sensitivity of at least 0.1 g are very much desirable, but most essential are measuring cylinders. It is safest to use distilled water, in order to prevent contamination of stains by environmental mycobacteria. Filtered water is a second choice and much care will be needed to prevent the filter from becoming colonized.

Stocks of supplies and consumables

The stock-situation must be checked during each visit. Stocks of laboratory items can be larger than for drugs, which is advisable also since some items are difficult to transport. Thus a 6-month reserve supply in addition to a three-month running supply is recommended as the norm, although much more (e.g. in the case of sputum containers, chemicals, methanol or immersion oil) may not be a problem at all. However, in the case of slides, conditions for storage may be a limiting factor as they must be kept dry. Stocks of ready-made stains should not be excessive, i.e., covering not more than 1 year of consumption, although it is not true that for a good result the stain has to be very recently prepared. It is clear that larger centers need large volumes of stains, and it may be more practical to provide them with all the chemicals for local preparation. However, before deciding on such a policy, it should be ensured that the technicians know perfectly well how to prepare a good stain. This includes knowledge on how to test new batches and keeping a logbook on these tests. It will also depend on availability of the necessary equipment.

Slide storage boxes are often moving around with losses as a result, and slide-markers (non-diamond) get worn, so both should be considered as consumables. Slide storage boxes should have well-fitting lids to prevent insects from entering the box and destroying the smears.

Registration and reporting of results

Registration is an essential part of the work in any laboratory, and in general most laboratory errors are known to result from faulty registration. Simply working carefully and systematically will go a long way to prevent such errors, and it is a fundamental exigency for any laboratory technician.

It should be checked if the sputum request form is properly used in all but the smallest centers (where the laboratory and consultation/treatment room may be one and the same, as well as the person attending to all these functions). Each sputum container must be labeled to allow non-ambiguous identification of the

patient, immediately upon arrival. The label should always be placed on the cup itself, never solely on the lid (figure II.1). Waterproof marker-pens, China-graph pencils or self-adhesive labels may all be used. The number must be taken from the laboratory register, and at that time the full identification of the patient should already be entered in the register, and the number also written clearly on the request form. Subsequently, slides must be labeled to allow clear identification of the sputum, using an indelible marker (engraving by diamond or substitute), or a pencil in case of frosted slides. Writing with other markers will not work, since the writing comes off during staining. Identification of the slide must include the serial (line) number as well as the column (slide) number of the register. A code for the center will be needed only for slides that are selected for cross-checking at a higher level, and might be added only if and when the slides are selected for control. An example of properly identified slides is shown in figure III.2. Results must be entered in the laboratory register promptly after examination. During supervision it should be checked if the results of the series examined the day before have already been entered in the register. If this is not consistently done, there may very well be misidentifications going on, resulting in the wrong patient being treated, and sometimes this may explain gross errors found during proficiency testing.

The request form and register need to show the correct address of the patient, and the indication of type of examination (diagnosis or follow-up detailed). If not provided by the clinicians, the technicians should find this out by questioning the patient. Both are absolutely necessary to trace patients who do

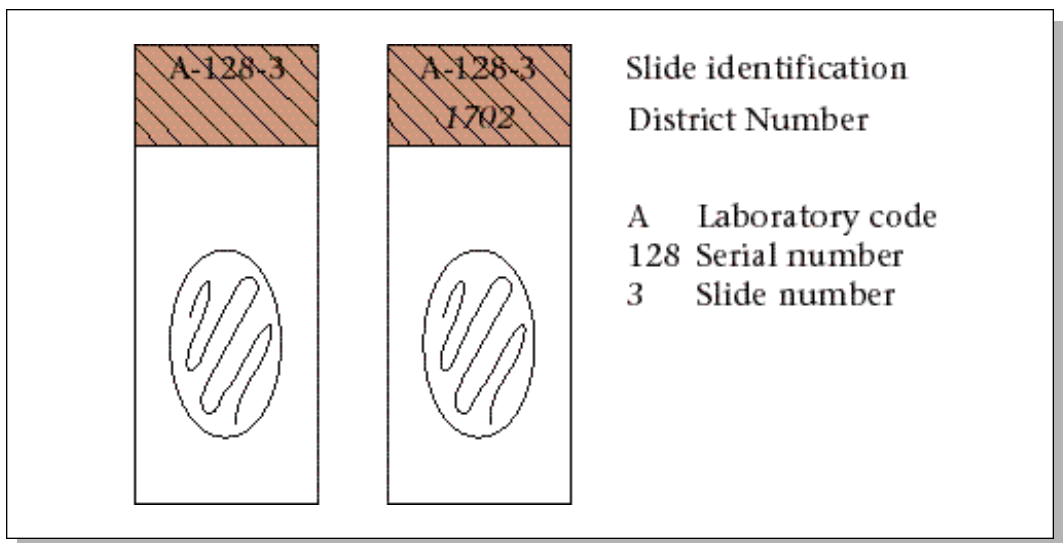


Figure III.2. Proper labeling of a slide.

not return to collect a positive diagnostic result, while the diagnosis/follow-up distinction is also necessary to enable more in-depth analyses of performance described further on.

In large centers, reporting of results may pose problems. To find out if such problems exist, it is necessary to compare the registrations in the laboratory register with those in the case register, in both directions. All positive cases identified by the laboratory should have been registered in the case register. Although not foreseen as a column, it helps in the identification of such problems to note the tuberculosis management unit case number permanently somewhere in the laboratory register (e.g., in the "Remarks" column) if the positive suspect was found in the case register. The other way around, the laboratory number of the diagnostic smear should be entered into the tuberculosis management unit register. Both are needed to obtain a complete picture. Ideally, all diagnosed positive cases should have been put on treatment. In some countries, few patients may refuse treatment, but if less than 95% of newly diagnosed cases are actually on treatment, then a serious problem exists. Under the best scenario, late arrival of results has led the patient to be treated elsewhere; under the worst, the patient has disappeared untreated back into the community, and must thus be traced immediately. If such problems are identified, it must be inquired whether patient information is adequate. The laboratory technicians should take responsibility for explaining the procedures clearly to the patients at the time the first specimen is delivered, and to encourage the patient to submit all required specimens. Estimating the proportion of cases for which only 1 positive diagnostic specimen was registered, a proportion, which should be less than 5% of all positive results, can evaluate the proper implementation of the policy. It is generally accepted that a patient should have at least 2 positive smears before being classified as a sputum smear-positive case of tuberculosis. Technicians may feel that this is a superfluous requirement, as they are certain to read positive slides correctly. It thus needs to be explained that the rationale of this rule lies in the prevention of misidentifications, which are most likely to occur in a busy center, and not because of erroneous readings. If all two or three results of suspects/cases identified show consistently the same quantification, it may be assumed that only one specimen was examined and its result copied.

Performance

Checking performance starts by looking around the laboratory. Reliable work cannot be expected in a laboratory that is untidy, dirty and/or in disarray. Making some simple calculations of frequencies (although this would better be

done regularly by the local technicians themselves as part of internal monitoring) provides a further orientation.

Reviewing the laboratory register

The laboratory register provides a wealth of information and should be reviewed extensively with the technician. The frequency of positivity among suspects varies considerably between countries and also depends on the kind of center (e.g., first-line or referral institute). As a rule of thumb, in countries where there is no other very frequent cause of chronic cough, the prevalence may be between 5% and 20%. Higher proportions are often encountered in situations where there is a large patient's and/or physician's delay. Although probably not easy to influence, and not usually the fault of the technicians, this should not be accepted as normal. Other possibilities for a high proportion of positive patients among suspects include a high proportion of false positive results or even professional mis-conduct, such as selling positive results. Too low proportions would occur in the reverse situations, i.e., too permissive selection of suspects or extremely high incidence of chronic cough, or with large numbers of false negative results for different possible reasons. A strategy of active case-detection would result in the same discouragingly low positivity frequencies.

The proportion of positive (including scanty positive) results among all follow-up examinations leaves fewer doubts about the reason for deviations. At the frequency and timing recommended by the IUATLD and WHO, there must be some scanty positives among them, often just dead bacilli. Ten per cent and more have been seen in several countries even with well supervised treatment and excellent final results. Although the proportion of positivity in follow-up examinations may vary, depending especially on the grade of positivity of the average case at diagnosis, a total absence of positive results among follow-up cases is not possible. Very low frequencies (e.g. < 5%) should raise the suspicion of superficial microscopy or poor staining, or again, mis-conduct.

Checking the frequency of scanty positive results among all positive results, among both suspects as well as follow-up patients, allows better fine-tuning. Grading of positive smears roughly follows a normal distribution, although it differs for suspect or follow-up examinations. Most results will be in the 1+ to 2+ groups, with fewer on both extremes of the scale. Detecting the high positive 3+ results obviously constitutes no problem at all, but the detection of the proportion of scanty positive results requires sustained efforts and high quality work. On condition that quantification is already reasonably good, the scanty

positive results reported may indicate superficial work, causing a low frequency of such results, or rarely high levels of contamination of the stains with environmental mycobacteria. The monthly frequency can also be shown graphically, thus constituting a tool for internal monitoring of quality. Scanning the register may give an impression of the plausibility of the results. The occurrence of positive follow-up examinations and the normal variation to be expected in a series of three specimens has already been discussed. On the other hand, it happens only rarely that a series of three results contains only one positive or scanty positive. If this is encountered regularly when leafing through the pages of the register, this may indicate a problem of poor staining or (rarely) of a low-grade contaminated stain.

Reviewing the preparation of smears

After the register has provided some clues about what to expect, further inspection can sometimes be more problem-oriented, with, e.g., in-depth evaluation of the staining practice. Otherwise, the next logical step is to make a macroscopic examination of already prepared smears. Stained smears allow an evaluation of both the macroscopic result of smearing and of staining at the same time, both of which are interdependent. Points to check include proper identification on the slide and the aspect of the smear. A correct size of smear is important only in relation to quantification. While its size can be allowed to vary as long as the technician understands how many fields are contained in one length, standardization is generally preferable. A standard length of approximately 2 cm is useful, as this corresponds to 100 oil immersion fields. The width should be such as to ensure that the smear does not reach the edge of the slide. The proportion of poor specimens (saliva) can be estimated to some extent from their aspect, which are very thin or hardly visible and often with small bubbles.

The most frequent problem, especially for beginners, is the preparation of too thick smears, or parts thereof. It should be possible to read newspaper print through the smear held at some distance above a newspaper. A smear that looks purple or red, or even black, usually indicates that it is too thick, and was impossible to decolorize properly. Another reason may be that technicians are afraid to use the acid long enough or repeatedly. They should be advised that it is hardly possible to decolorize the acid-fast bacilli using only watery acids. There is no point in looking for a strong color of the counterstain; on the contrary, smears should have only a light blue color. Especially in thick smears and when light is weak, a strong counterstain may hide the acid-fast bacilli so that even highly positive specimens may be missed completely.

Checking the microscope

Only the use of the microscope allows one to get an idea of the staining of the acid-fast bacilli themselves. At the same time, this step serves as a gross evaluation of correct knowledge of identification of the acid-fast bacilli by the center's technicians, and as verification of the condition of the microscope. For these reasons, it should never be omitted. It will need one or more positive (excluding scanty positive) smears, preferably from the same week to avoid a false impression because of possible fading of older smears. It must be done using the routine microscope(s).

Problems of a loose stage or stage-clamp will be obvious, especially when scanning through the smear. If the focus is difficult to maintain, but the slide is properly fixed, the fault may be a loose rack or stage-fixation. The luminosity of the field should be judged, especially if a mirror has to be used, and the technician may have to be advised about the best location for microscopy. If all is dark, or no absolutely clear view of the acid-fast bacilli can be obtained while the light is good and all parts are properly adjusted, the lenses and internal prisms should be inspected for dirt and/or for fungus. The 100x objective and the eyepieces must be taken off, and the empty objective-opening be aligned over the lighted field. By looking down through the tube, it is possible to check the prisms inside the tube for fungal masses or filaments or other dirt. If these are absolutely clean, the objective and eyepieces are inspected by holding them upside down against the light. If nothing is obvious, the objective is reinserted and another look down the tube may then show the dirt in it more clearly. External dirt can be cleaned away, if necessary by using a tissue dipped in xylene. If the internal surfaces are dirty, cleaning will have to be left to a microscope maintenance workshop. If spare parts are available, a new spare objective should be used to permit continued use of the microscope. In the case of scratches, or chipped lenses, replacement is the only possible solution.

Examining a stained positive smear

If the inspection shows no faults, attention should be given to the appearance of the acid-fast bacilli. This is necessary not only to ascertain that they are really acid-fast bacilli, but also to check whether they have a good strong red color, easily visible against the background. Anything that has the required basic morphology and color should be accepted as true acid-fast bacilli, even though the supervisor may be sure that it is not a tubercle bacillus. For optimal results, the red color should be strong enough not to be hidden too easily by the counterstain. If this is not the case, the problem lies with a too weak carbolfuchsin

stain or a staining method that does not use sufficient heat, and false negative results should be suspected. The effect can also be caused by alcohol decolorizing (if applied for hours, not minutes), but not by acids diluted in water.

The positive smears should also be inspected for deposits of fuchsin that may result from lack of (recent) filtration of the stain, which may result from using all carbolfuchsin from the bottle until the last drop, or if reagent bottles are never cleaned between refills.

It is not feasible and hence not recommended to try checking negatives and scanty positive slides during a supervision visit. Although re-examining negative slides is essential to obtain an accurate picture of the performance, too much time is needed since a large number of slides has to be included. This will have to be done in a proficiency testing program by re-examination of routine peripheral smears. As a supervision visit allows provision of feed-back on the proficiency testing as well as finding out why errors were made, the two procedures are complementary.

Establishing a representative slide collection

If a quality assurance system is in place, its reliability and functioning should be checked. The first requirement for such a system is that a representative sample of slides is being kept until a sample can be taken in a way that allows their easy retrieval as well as blinded re-examination of their results.

To keep all slides would ensure absolute representativeness. But this is not practically feasible. The most simple and feasible approach allowing a reasonably representative sample to be obtained is to keep one slide from each examinee, irrespective of whether the examinee is a suspect for diagnostic examination or a patient presenting for a follow-up examination. The very first slide of each examinee should be retained, irrespective of subsequent results. To simplify collection, it is proposed that each laboratory has five slide-holding boxes for negative, and two for positive slides. The slides are kept in the sequence of the laboratory register serial number. As only one slide is kept from each patient, keeping them in the same sequence as the laboratory serial number is easily accomplished. Slide-holding boxes are used sequentially until all five for negative slides (or both for positive slides) are full. If all five boxes for negative (both for positive) slides are full, the slides from the first respective holding box are discarded, to make space for uninterrupted, continuous collection. When slides are picked for proficiency testing, all slides can be discarded following selection. The only reason to keep slides is to allow proficiency testing on a representative sample.

Ensuring validity of proficiency testing

To ensure validity of proficiency testing, it must first be ensured that the slide collection is representative. To this end, it suffices to choose at random, e.g., 10 examinees from the laboratory register, comprising both examinees with positive and negative results, and both diagnostic and follow-up examinees, and to ask the technicians to select the corresponding slides from the respective slide holding boxes. If less than 9 of the 10 slides can be retrieved, doubts about the completeness of the collection must arise. Comparing the identification on the slides with the laboratory register must show if it allows unequivocal allocation of a registered result, and at the same time attention should be paid to the absence of any mark that may give away this result. Deficiencies on these points may very well indicate that the results of proficiency testing may not be valid.

Sending slides from the periphery to center

Proficiency testing by re-examining a sample of routine smears from the peripheral centers at a higher level of the service is the method of choice for evaluation and continuous motivation of the centers. This type of proficiency testing is based upon re-reading of smears at a higher level. Although this may seem to be simple and straightforward, it requires strict adherence to some technical principles in order to provide valid and interpretable results.

Sending slides from the periphery to a more central level requires a well-functioning network of laboratories and collaboration between the different levels. To be successful, the intermediate level laboratories have to play the major role as only they can relate directly with the peripheral laboratories on a routine basis.

Aim of proficiency testing

The aim of proficiency testing is to get a better idea of the performance level of the service in general, and to detect centers that may have an unacceptable level. In this way, it is complementary to supervision visits during which such an extensive control is not possible, but which will in turn provide an understanding of the causes of high error frequencies and ultimately will serve to remedy them. Technicians will know that they are being controlled, and at the same time, that the service takes an interest in their problems. Together this will mean a powerful motivation for those who are of good will.

It is sometimes stated that slides should be checked not only for errors in reading, but also for quality of smearing and staining. The point of proficiency testing of smear results is, however, simply to ascertain whether a slide is correctly

read, regardless of the cause of error if one is found. Furthermore, the potential of fading of fuchsin, particularly in hot and humid climates (figure III.3) makes it imperative to re-stain slides before re-examination. Should the error exceed the accepted critical value of 5% (proportion discordant among negative and positive slides, or overall discordance), then the entire procedure, including quality of smearing, reagents, and reading has to be reviewed by the supervisor and the technician concerned.

Technical requirements

Experience has shown that proficiency testing exercises are frequently invalid because the sampling was not representative, or the controls were not blind. All possible efforts should thus be taken to avoid these pitfalls. They comprise:

- A representative sample of slides examined at the periphery, whatever its result, must be kept until a random sample has been taken. A way to check on the completeness of the “population of slides” is described in the section on

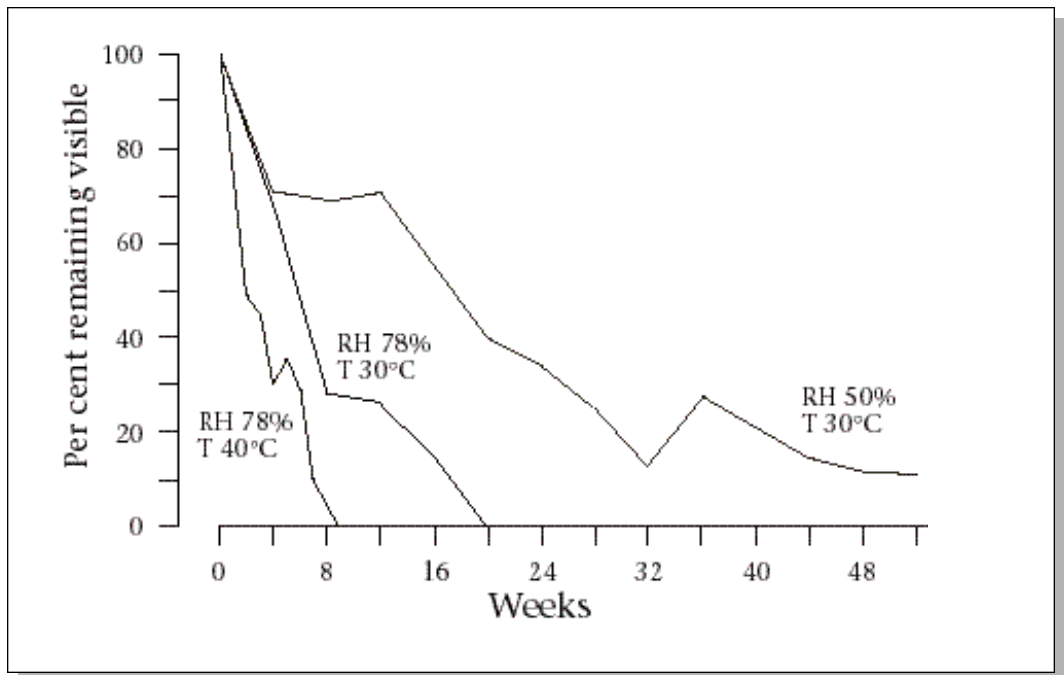


Figure III.3. Fading of fuchsin over time depending on relative humidity and ambient temperature.

Data courtesy of Damien Foundation, Bangladesh.

supervision. It requires discipline, a sufficient number of slide-holding boxes and something to remove (most of) the immersion oil. A xylene soak can be used, but even lightly wiping off most of the oil, using, e.g., toilet paper, is workable.

- Each slide must be identifiable unequivocally to allow it to be linked with only one result in the laboratory register. Under no circumstances should the result of the examination be marked on the slide itself, or the result be identifiable in another way. Before leaving the center, a code identifying the center must be added to the selected slides.
- The supervisor should make the selection. To avoid bias this must not be left to the technician of the peripheral laboratory. The required number of positive and negative slides (see below) is selected from the laboratory register (allowing selection by grading) or directly from the slide-holding box, if the collection is shown to be reasonably representative.
- Accurate listings of selected slides, mentioning date, center, slide numbers and their respective results must be made. This list should stay with a coordinator working near but not in the controlling laboratory. An example of a form is given in appendix 8.
- Only the slides themselves are brought to the laboratory, if necessary with a list of their identification numbers. First controllers should then make another list of slide identification numbers and their results, together with eventual remarks on the macroscopic aspect of the smears. Their results will afterwards be copied to the original list by the coordinator, who also identifies the slides showing significantly discordant results.

Limitations of the technique

The main problem is the lack of a reliable gold standard. Acid-fast microscopy is a technique which has inherent errors like any other technique, even when performed by the most experienced or hard-working technicians (table III.3). Thus, the result of the superior level cannot simply be assumed to be more correct than that of the technician at the peripheral first level, and indeed perhaps even more errors may be made than in the periphery. A second opinion by the next higher, third, level in the service will clear up most but not all of these uncertainties. This might be done by re-examining at the third level only slides with significantly different results between the first and second levels. The third level should not know the origin of the differing results.

If this approach, involving a third level to establish a gold standard, is used, the coordinator should send the slides with discordant results for

Table III.3. Example of a comparison of results obtained by different technicians, showing that the largest differences are obtained in paucibacillary smears. This example testifies to the inherent limitations of sputum smear microscopy.

From: Toman K. Tuberculosis case-finding and chemotherapy.

Questions and answers. Geneva: World Health Organization. 1979, I-239.

Report of one technician	Report of all three other technicians					Total
	Neg	+/-	1+	2+	3+	
Negative	233	25	8	2	0	268
+/-	24	5	1	7	4	41
1+	8	2	11	18	4	43
2+	2	8	16	39	50	115
3+	0	4	4	49	120	177
Total	267	44	40	115	178	644

re-examination at the third level. The coordinator should merge a list mentioning slide identification numbers and both results, but in a way that makes it impossible to determine which was the first level's and which the second level's result. Finally, the results of the third level are added to the original form, and these are taken as the "gold standard". Only then will it be possible to determine who made the errors, the first or second level, and of what type the errors are.

Even the third level will still, by necessity, miss some smears with very low or unevenly spread numbers of acid-fast bacilli. These rare "false false positives" can be accepted as an inherent limitation of the method. On the other hand, slides with concordant results between first and second level are not subjected to re-examination, although there are also errors to be expected in concordant results. Again, these are accepted as a limitation. There is no possible practical alternative for establishing a gold standard.

Overcoming staining and smearing problems

The re-examination of slides at a second level must be considered as a screening with the aim of detecting as many peripheral errors as possible. Knowing that a deficient staining method (or stain) is an important cause of false negative results, it is imperative that all smears are re-stained before reading them. If re-staining is not done, acid-fast bacilli that remained invisible at the first, peripheral, level, because of poor staining can not be seen by the second level either.

In hot and humid climates (above 30°C and 70% relative humidity), the possibility of rapid fading of the fuchsin-stained acid-fast bacilli is another reason for using re-staining routinely (figure III.3). However, the number of weeks it actually takes for complete fading depends on other factors as well, such as the consistency and thickness of individual smears. Thus, the graph is for demonstration only, and not for a decision for timing of sampling or the need for re-staining.

In comparison, an evaluation of the quality of original staining during controls is of secondary importance, and not even entirely possible. Quality of smearing is closely linked to it, however, and this can still be assessed. If, for instance, a high frequency of false negative results has been identified in this way, staining by the peripheral laboratory will have to be examined more closely as one of its possible causes.

Technique for re-examination

Re-examination should be done using the same technique as used in the peripheral laboratories to ensure that at least the technical sensitivity and specificity of the method are comparable, but the microscope used for re-examination should be of good quality and condition. For the same reasons, the second level should examine the same number of fields as specified in the guidelines for routine in the centers, and not more.

Acid-fast microscopy is not difficult, but tedious if large numbers of smears have to be examined. This should be kept in mind when deciding on the required sample size. Otherwise there is a real danger that technical accuracy is lost in trying to attain high statistical power and confidence. Technicians overburdened with re-examination should not be expected to perform better than the peripheral technicians, however experienced they may be. As a rule, they should not have to examine more slides per day than the centers. This means that there must be a sufficient number of staff for proficiency testing. Therefore, it will hardly ever be feasible to centralize all proficiency testing at the national tuberculosis reference laboratory. The other consequence is that sample size must be appropriate also in this regard, which means that the smallest possible number should be selected. Careful consideration of what needs to be known from examining the sample is thus necessary in order to calculate its minimum required size.

Issues related to sampling

The finding of a negative slide is the most frequent event in the laboratory. To determine that a slide is negative is more time consuming than

identifying a positive slide. The universe of negative slides is entirely different from that of positive slides and sampling must thus be done separately for both.

Statistically, one is concerned with two types of error. In this scenario, the first error (type I error) is defined as the failure to identify a laboratory which needs a review of its operation. The second type of error (type II error) is to classify a laboratory to be in need of review while the results are actually good. Because proficiency testing aims at improving the performance and enhancing motivation through regular visits to the laboratories, a type I error is considered much more serious than a type II error. To minimize the number of slides to be checked, the sample size required here is determined in such a way that the finding of a single positive slide among slides reported as negative will suffice for a decision. It must be possible to decide whether the laboratory procedures need review because the proportion of slides misclassified as negative is likely to exceed a critical value of 5%. The sample size required at various levels of type I error and the power to be able to exclude a type II error is summarized in table III.4 at different levels of true prevalence of errors, and is graphically depicted in figure III.4. Table and figure show that 60 negative slides already allow exclusion of a type I error with 95% certainty. Increasing the sample size will increase this certainty, but only marginally so. As power (ability to exclude a type II error) is of lesser concern, as mentioned above, a sample of 60 negative slides might be a reasonable starting point for external proficiency testing. Of these slides, none should be found to be positive.

Table III.4. Sample size, type I error (alpha level), power to exclude a type II error, and level of true prevalence (p).

Data courtesy of Dr. Beat Neuenschwander, Federal Office of Public Health, Switzerland.

	Sample size										
	50	60	70	80	90	100	110	120	130	140	150
	Alpha										
	0.077	0.046	0.028	0.017	0.01	0.006	0.004	0.002	0.001	0.001	<0.001
p	Power										
0.040 :	0.130	0.086	0.057	0.038	0.025	0.017	0.011	0.007	0.005	0.003	0.002
0.030 :	0.218	0.161	0.119	0.087	0.064	0.048	0.035	0.026	0.019	0.014	0.010
0.020 :	0.364	0.298	0.243	0.199	0.162	0.133	0.108	0.089	0.072	0.059	0.048
0.010 :	0.605	0.547	0.495	0.448	0.405	0.366	0.331	0.299	0.271	0.245	0.221

P: prevalence of true error
 For definitions of alpha error and power see text

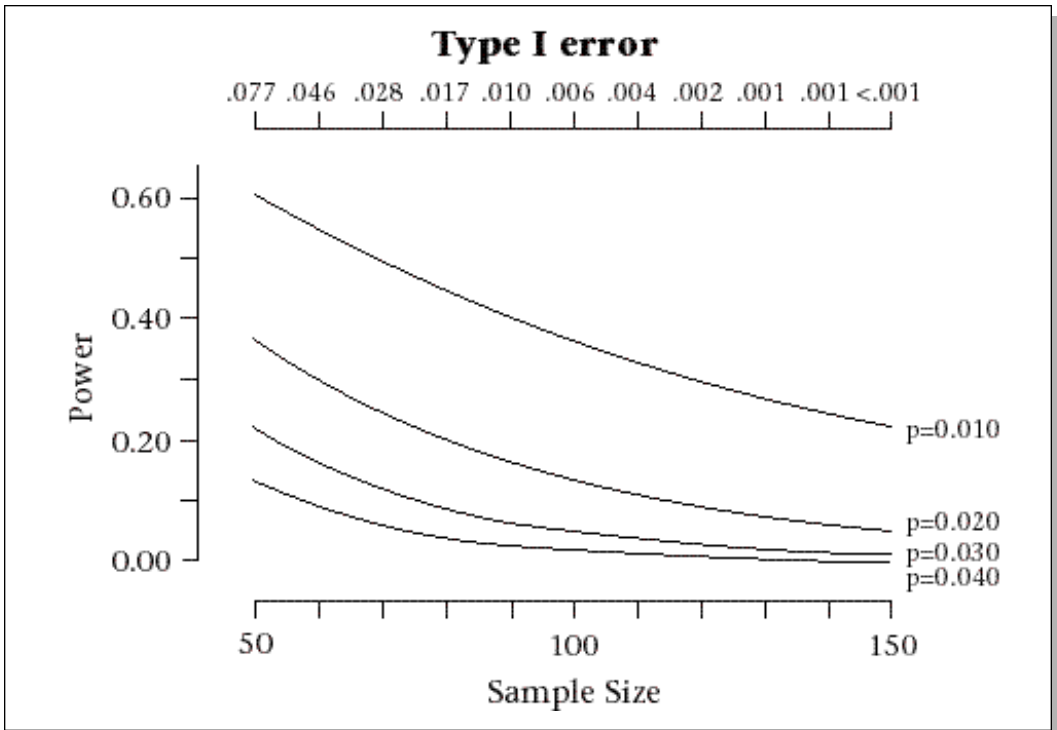


Figure III.4. Sampling for negative slides. Required sample size to have 96% certainty that finding no error indicates that the true error rate does not exceed 5%. Data courtesy of Dr. Beat Neuenschwander, Federal Office of Public Health, Switzerland.

An equal number of slides reported as positive (including scanty positive) should be checked, if that number is available; alternatively, all available positive slides should be re-examined. The sample of positives should include about 20% scanty-positives, which will serve as a check on the validity of the controls.

Threshold for action

Table III.5 summarizes the possible errors in reading slides. Gross errors include “high false positive” and “high false negative” results, i.e., misclassifications that fundamentally change a decision about patient management. The main thrust of proficiency testing is to identify this type of error. The aim of this testing is to detect an unacceptable proportion of “high false negative” slides and to ascertain that there are no “high false positive” results. If the latter are found, each one of them must be followed up. Often it will be found that the error is administrative (mislabeling, erroneous sampling, etc).

Table III.5. Cross-tabulation and classification of errors found in quality control by re-examining slides from a peripheral laboratory by a superior level laboratory.

Examinee	Superior level control result				
	negative	1-9/100	1+	2+	3+
negative	correct	SFN	HFN	HFN	HFN
1-9/100	SFP	correct	QE	QE	QE
1+	HFP	QE	correct	QE	QE
2+	HFP	QE	QE	correct	QEΔ
3+	HFP	QE	QE	QE	correct

- Correct: No errors, including no quantification error
- QE: Quantification error, minor error with no influence on case management
- SFN: Scanty false negative, intermediate error, to be expected at a small rate
- SFP: Scanty false positive, intermediate error, to be expected at a small rate
- HFN: High false negative, gross error, missing a clearly positive slide
- HFP: High false positive, gross error, misclassifying a non-case as a case

“Scanty false positive” and “scanty false negative” errors are considered to be less serious, because these might represent patients with paucibacillary disease that might be over-diagnosed or missed. This type of error will occur by definition. As acid-fast bacilli are not homogeneously distributed in sputum, an examination of 100 fields by one technician may identify them by chance, while another technician examining the same slide but another 100 fields may not be able to find them. It might be advisable to analyze this type of error separately. The ratio of scanty false negative errors made by the peripheral level and the second level allows the validity of the second level controls to be checked. A 20% prevalence of such smears among the sample of positives is comparable to the frequency in the peripheral center’s routine. A good second level controller should not have missed more than the peripheral centers, and preferably much fewer.

“Quantification errors” are of minor importance, as they do not influence decision making in patient care. This type of error distinguishes only the good from the very good laboratory technician.

High false positive results are very rare if the basic requirements of knowledge and a good microscope are met. Such errors are intolerable and each one must be investigated as to its cause. An isolated occurrence will often be found to represent an administrative error. For these reasons, it is not even attempted to reach statistical significance by a properly calculated sample size.

A critical value is used for high false negative results, since few may occur because of unequal distribution of acid-fast bacilli in a smear. As described above, the hypothesis tested is that the number of high false negative results is not higher than the value chosen as the critical level. Centers with more errors are assumed to have an unacceptable false negative frequency, and again the cause has to be determined. If none is found, it may be that there has been a type II statistical error and that the center was actually performing well. Even when results were pooled over the course of an entire year, the sample size of negative slides will have been too small to allow the exact frequency of high false negative results to be determined for individual centers. However, if the proficiency testing system has covered a large number of centers, the total sample size will most probably be large enough to determine the global frequency within the system as a whole within narrow limits of confidence. The error frequency for individual centers nevertheless allows a rough scaling of centers. It is also possible to calculate the correlation between false negative results and workload, for example (total annual turnover of acid-fast smears), to see if this may be the cause of problems.

Even without these calculations (which are possible only after a long duration of sampling and checking), valid indications of problems will be obtained very soon after starting the exercise.

Examples of findings

A sample that shows nonsensical results (e.g., a large number of high false positive and high false negative results) must be attributable to a technician who doesn't know the acid-fast bacilli at all, or to a useless microscope (damaged, with fungus, or no light), or smears were not examined at all.

A single high false positive result will often be an administrative error and can be tolerated. If more than one occurs, administrative and identification procedures are obviously of poor quality.

A very low percentage of scanty false positive results should be ignored, as there are too many possible causes and most of them are linked with the limitations of the proficiency testing system itself, as elaborated above.

Regularly finding a high false positive result with more than just a few scanty false positive results may indicate that the technician is not completely clear about the recognition of acid-fast bacilli. Another possible explanation is contamination of solutions with environmental acid-fast bacilli, which were not fixed and may be washed off again before the control reading.

Many high false negative results together with many scanty false negative results are often caused by a poor quality lot of carbolfuchsin stain, or poor staining technique (insufficient heating). Other possibilities include a high degree of carelessness in reading, even to the extent of not looking at part of the smears at all on busy days, for instance. Extremely high frequencies of scanty false negative and some false negative 1+ smears are seen when smears get contaminated by tap water in the centers, as these acid-fast bacilli become visible only after restaining.

Lower frequencies of false negative results, mainly scanty false negatives, are usually attributable to superficial microscopy. This in turn may be due to work overload. Otherwise, there may be a problem with an unclear microscope or insufficient light and/or poor smearing.

Ascertaining the quality of bacteriologic classification of registered patients

All the above methods fail to provide an answer to a key question common to all proficiency testing methods of sputum smear microscopy, i.e., the accuracy of classification of patients registered and put on anti-tuberculosis chemotherapy.

An approach involving the laboratory in an overall assessment of the quality of patient classification is more complex. It is based on the tuberculosis case register and seeks a systematic sample based on it. This procedure is more complex and unlikely to be feasible as a routine. Nevertheless, periodic surveys might usefully be carried out using this procedure.

First, it requires the laboratory to label all slides properly (figure III.2, left side) and to keep the first of each examinee with the laboratory identification code, laboratory serial number, and specimen number as elaborated above (e.g., A-128-1 identifies the first slide of examinee no. 128 examined in laboratory A). The same system of slide collection as for any other method is thus used.

When reporting the result of examination, the laboratory must take care to report laboratory identification code and laboratory serial number as well (e.g., A-128).

For each newly registered case, the person responsible for registering patients in the tuberculosis case register must ensure that, in addition to the result, the laboratory code and serial number are also entered into the appropriate column (for the proposed tuberculosis case register form, see the IUATLD Tuberculosis Guide). Obviously, no patient should be registered without having

a sputum specimen examined. This includes patients primarily diagnosed with extrapulmonary tuberculosis to exclude concomitant sputum smear positive tuberculosis, which takes hierarchical precedence over extrapulmonary tuberculosis in recording and reporting.

Procedures of selection

The supervisor of proficiency testing (usually the tuberculosis regional coordinator) must be provided with the quality control form (appendix 9). During the visit of the supervisor to the treatment unit, the tuberculosis management unit coordinator must complete this form (with two additional copies), recording all cases as follows:

- tuberculosis management units reporting a total of 0 to 9 cases during the last complete quarter: all cases, to provide a 100% sample;
- tuberculosis management units reporting a total of 10 to 49 cases during the last complete quarter: cases with tuberculosis management unit number ending with even numbers (0, 2, 4, 6, 8), to provide a 50% sample;
- tuberculosis management units reporting a total of 50 and more cases during the last complete quarter: cases with tuberculosis management unit number ending with 0 or 5, to provide a 20% sample.

This will allow over-sampling of smaller tuberculosis management units, ensuring that specimens from these laboratories are included in proficiency testing.

The tuberculosis management unit number, the laboratory serial number, and the smear result for the case register must be recorded in the appropriate columns of the form. If no smear had been examined or recorded for a particular case, this should be recorded. Because samples are taken from all patients, irrespective of disease-type classification, the proportion of smear-positive slides among all sampled smears should closely correspond to the proportion of new smear-positive cases among all notified cases. The coordinator should mark in the case register the last case selected for proficiency testing during this round.

Checking the sample

The coordinator must then visit the laboratory and select the slides according to the list from the slide-holding box in the laboratory. All slides that are chosen should be checked for proper labeling and, at this point, additionally be labeled with the tuberculosis management unit number (figure III.2, right side). Unless explicitly stated otherwise, the laboratory can now discard all slides to make room for the next round.

The coordinator must now append the original form, which already contains tuberculosis management unit number and laboratory code, with smear results. The holding box collected by the coordinator and the original form and the two copies must now be brought to the next, second, level. This is usually the regional laboratory which is provided with the slides and a copy of the list containing all information except the results of the peripheral laboratory in order to allow the regional laboratory to examine the slides in an unbiased way. Results are recorded in the same way as at the periphery, using the appropriate column. The coordinator collects form and slides and forwards the slide box and an original form (without the results from peripheral and regional laboratory to the reference laboratory), while completing the results on a master form.

The reference laboratory may be given slides with discordant results only, without knowing the previous level's results, to establish a gold standard as described above.

At this point in time, results can be compared for discrepancies. Slides read concordantly by all levels are not further examined and their result is written into the last column. The reference laboratory is provided with the master copy and requested to re-examine all slides showing any discordance in reading. Equipped with the knowledge of discordance, the reference laboratory will now again read the discordant slides more carefully and establish a final result that will serve as the gold standard (recorded in the last column of the form).

Analysis can now evaluate the accuracy of patient classification, performance of the regional laboratory, and even the frequency of errors made at the reference laboratory.

Quality improvement

It is not sufficient in proficiency testing and quality control simply to identify errors or weaknesses in laboratory services; remedial action to permanently remove them must be taken. This implies continuous monitoring of performance by proficiency testing, a quality control program as well as a direct supervision program. Furthermore, the functioning of the laboratory is not to be regarded in isolation, but information gained from laboratory activities and results should aid in improving overall tuberculosis program performance.

It is important that any supervisory visit to a peripheral laboratory also aims at strengthening links and collaboration between diagnostic laboratory services and local program management. This implies that technical specialists in laboratory services are knowledgeable in national program policies, and conversely,

that technical specialists in program management take a strong interest in laboratory operation.

Should the monitoring reveal that the level of agreement in smear microscopy results is below expected levels, remedial action would include re-training of technicians in the technical aspects of smear microscopy. Should direct supervision show that there are serious deficiencies in the way that the laboratory register is kept, remedial action would include re-training of personnel in the administrative aspects of diagnostic services.

Should a large proportion of single negative smears be detected (as opposed to the required three examinations), remedial action would include discussion with clinical health care workers in charge of attending to patients with respiratory symptoms.

A simple method of ascertaining whether clinicians have a sufficiently high index of suspicion for tuberculosis is to utilize the laboratory register. For example, the proportion of cases with at least one positive smear result among the most recent 100 examinees presenting for diagnostic examination is easy to determine. If this proportion is in excess of what is expected in the country, then clinicians might not be requesting sputum smear examinations when indicated. Conversely, if the proportion of cases among suspects is smaller than expected, clinicians might overburden the laboratory with unnecessary requests in patients not fitting the definition of a tuberculosis suspect.

Supervision is useful also in detecting problems in the supply of laboratory reagents and materials.

While the above examples are not exhaustive, they show how a permanent monitoring system of laboratory services can help in continuously improving the quality of the national tuberculosis program.

In summary, quality assurance activities must be progressively implemented and then maintained. In a first step one may choose an approach from central to peripheral laboratories to identify those with insufficient knowledge or really poor quality equipment. This might be followed by a period of periphery to center quality assurance to identify poor performance and other problems. Plotting of frequencies of cases among suspects and proportional distribution of various grade levels over time is recommended, combined with occasional large samples upon suspicion of problems from the chart. Finally, periodic surveys to ascertain the quality of bacteriologic classification of registered patients may be undertaken.

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CHAPTER IV

Surveillance of anti-tuberculosis drug resistance

Surveillance of drug resistance requires that the laboratory is able to grow mycobacteria belonging to the *M. tuberculosis* complex in pure culture. Used in this context, the culture technique must thus satisfy population-based requirements. The superior sensitivity of the culture technique in comparison with smear microscopy is thus of much lesser importance in the surveillance of drug resistance than is the case where culture is used as a diagnostic tool in an individual.

In this manual, it is stressed that culture for diagnosis of individuals has a lower priority in the national tuberculosis reference laboratory than proficiency of the smear microscopy network, training of personnel, quality assurance of smear microscopy or surveillance of anti-tuberculosis drug resistance. Nevertheless, should diagnostic culture services be introduced into the activity spectrum of a national tuberculosis reference laboratory, then other aspects of the technique, not specifically addressed in this manual, must be taken into account:

- A high standard culture technique may increase the yield of bacteriologically confirmed cases of pulmonary tuberculosis (who are sputum smear negative) by 20% to 40%.
- Minimizing transit time between specimen collection and processing in the laboratory becomes crucial, since culture is expected to identify particularly paucibacillary cases not identified by microscopy, i.e., it concerns specimens whose probability of retaining viable bacilli is particularly low.

For surveillance of drug resistance, patients are classified as belonging to two categories: those who have never, and those who have received prior treatment for as much as one month. This has important practical consequences for patient management, as the recommended treatment for these two groups of patients differs.

Only strains from patients with sputum smear positive tuberculosis are eligible for assessment of drug susceptibility. There are several reasons for limiting the surveillance of drug resistance to such cases. First, knowledge of susceptibility

patterns among sputum smear positive cases will directly reflect the pattern of strains that are transmitted in the community, because patients with other than sputum smear positive disease transmit tubercle bacilli to a much lesser extent. Second, such cases can be identified rapidly in peripheral laboratories, and additional specimens can be obtained and submitted immediately following the diagnosis with the assurance that the specimens come from a verified case of tuberculosis. Thirdly, the number of bacilli in sputum smear positive cases is sufficiently high to tolerate some loss of viability during transport and, therefore, can give assurance of a high probability for recovery on culture.

Patients with newly discovered tuberculosis need therefore to be carefully interviewed to determine whether they have received prior treatment for tuberculosis. If enough attention is not paid to patient interview, misclassification will be frequent, and interpretation of the results will be flawed. There will always be patients who do not disclose prior treatment, either because they are unaware of it or because they have other reasons not to admit to it. The purpose of interviewing a patient aims at minimizing this classification error.

Resistance found in patients with a history of previous treatment reflects to a large extent inadequate treatment (true acquired drug resistance) administered in the program, while resistance in patients never previously treated reflects the extent to which cases with resistant strains have transmitted *M. tuberculosis* complex to susceptible members of the community. The frequency of drug resistance in both previously treated and untreated patients can thus be utilized to assess the quality of the program.

Surveillance of drug resistance should be a continuous activity of the national program. Epidemiologically it would suffice to repeat surveys at perhaps five-year intervals to obtain accurate trends. The reduced costs of repeat surveys compared with continuous surveillance must, however, be balanced against problems arising from interruption of established routines. Personnel turnover and the associated need for re-training may hamper surveys at regular intervals. The complexities in organizing a survey are considerable, and therefore, if feasible, continuous surveillance is preferred over repeat surveys.

In some tuberculosis control programs the monitoring of trends in anti-tuberculosis drug resistance, rather than the level at one point in time, may be the main objective of drug resistance surveillance.

Poly-resistance is defined here as resistance to any two or more anti-tuberculosis drugs. Resistance to both isoniazid and rifampicin is of particular

interest as a high prevalence may render otherwise powerful rifampicin-containing regimens obsolete. The subset among poly-resistant strains with resistance to both isoniazid and rifampicin is defined by common agreement as *multi-drug resistance*.

I. Representative sampling

The results of surveillance of drug resistance must reflect the situation in the country. It is thus essential to decide upon a sampling scheme which avoids bias. The three most commonly employed representative sampling methods are cluster sampling, systematic sampling, and random sampling. Because of their practicability, this manual gives preference to cluster sampling and systematic sampling methods.

The size of the population survey will depend on the required precision. In general 350 to 1,000 patients will suffice for the purpose of surveillance among previously never treated patients. Previously treated patients will be rarer the better the program is established. Although the intake period for such patients thus usually needs to be longer, the required sample size is also smaller, because the expected prevalence of resistance is higher.

Cluster sampling

Cluster sampling has been used extensively in assessing vaccination coverage within the Expanded Programme on Immunization, and much of its methodology has been developed in that context. Cluster sampling methods are particularly useful in situations where there are logistic difficulties to cover the entire area of the country, and where the number of tuberculosis cases is large. Rather than a single person, a "cluster" of persons is the sampling unit. Each cluster will consist of the same number of eligible sputum smear positive tuberculosis patients diagnosed consecutively at a health center. A minimum of 30 clusters is required to satisfy statistical considerations. Cluster sampling requires the availability of a sampling frame consisting of a complete list of all health centers which notify tuberculosis cases in the country, with the number of sputum smear positive patients diagnosed per year in each center. The selection of clusters is made from this list so that the possibility of selecting a cluster in a particular center is proportional to its number of eligible cases. Several clusters can thus belong to the same center if it is a particularly large one. A detailed procedure for cluster sampling is provided in the WHO/IUATLD Guidelines for Surveillance of Drug Resistance in Tuberculosis, which should be consulted before undertaking such a survey.

Systematic sampling

Every new sputum smear positive patient who denies ever having been treated in the past for as much as one month is potentially eligible for providing a specimen for assessment of the prevalence of drug resistance. If there are 6,000 or more new sputum smear positive cases per year in the country, it will generally suffice to collect a specimen for culture from only approximately 10% to 20% of these patients. This can be accomplished in two principal ways. One is to instruct each treatment unit to select every smear positive patient whose serial number ends with digit 0 and 5 (or any other pair of ending digits) for collection of the specimen.

Preferably, the central unit produces a list based on quarterly case reports by tuberculosis management units. The total number of cases reported by all these tuberculosis management units is then divided by twelve and as many tuberculosis management units as necessary are lumped together to give approximately this latter number of cases. This will give groups of tuberculosis management units that will be assigned to a specific period of time during the year during which they are requested to submit a specimen for culture and drug susceptibility testing from each newly diagnosed case during this period. Because of losses or inattention, the number of specimens actually received might be reduced considerably. Requiring the tuberculosis management units to submit specimens for a longer period to obtain the required minimum of drug susceptibility test results can control for anticipated losses.

An example, targeting a low level of resistance and the corresponding number of weeks required for sampling, is shown in table IV.1.

Because the number of patients in need of retreatment (failures, absconders, and patients returning after default with positive sputum smears) is usually considerably smaller than the number of new patients, the period of sampling amongst these must be much longer and might indeed require a 100% sample, i.e., sampling throughout the year. Experience has shown that obtaining a representative sample of strains from previously treated patients can be very difficult in practice.

Random sampling

A random sample is theoretically ideal, but often imposes insurmountable obstacles to implementation. The procedure is thus mentioned here only briefly.

The most appealing way is to produce a list of the number of reported tuberculosis cases in each tuberculosis management unit in one year. Epi Info or

Table IV.I. Systematic sampling for drug resistance. Required sample size depending on population at risk, prevalence of drug resistance, and upper level of confidence. The table was obtained using the software package Epi Info, United States Centers of Disease Control and Prevention.

z 90%	P point	P max	d	Population	Sample size	Weeks of sampling needed to obtain sample size
1.64	0.010	0.015	0.005	400	291	38
1.64	0.010	0.015	0.005	800	457	30
1.64	0.010	0.015	0.005	1600	639	21
1.64	0.010	0.015	0.005	3200	799	13
1.64	0.010	0.015	0.005	6400	913	7
1.64	0.015	0.023	0.008	400	255	33
1.64	0.015	0.023	0.008	800	375	24
1.64	0.015	0.023	0.008	1600	490	16
1.64	0.015	0.023	0.008	3200	579	9
1.64	0.015	0.023	0.008	6400	636	5
1.64	0.020	0.028	0.008	400	269	35
1.64	0.020	0.028	0.008	800	406	26
1.64	0.020	0.028	0.008	1600	544	18
1.64	0.020	0.028	0.008	3200	655	11
1.64	0.020	0.028	0.008	6400	730	6
1.64	0.025	0.035	0.010	400	248	32
1.64	0.025	0.035	0.010	800	360	23
1.64	0.025	0.035	0.010	1600	465	15
1.64	0.025	0.035	0.010	3200	544	9
1.64	0.025	0.035	0.010	6400	595	5
1.64	0.030	0.039	0.009	400	283	37
1.64	0.030	0.039	0.009	800	438	28
1.64	0.030	0.039	0.009	1600	602	20
1.64	0.030	0.039	0.009	3200	742	12
1.64	0.030	0.039	0.009	6400	840	7

z Value for 90% confidence
P point Point estimate
P max Maximum allowable prevalence given point estimate
d Maximum allowable upper difference from point estimate.

random tables are then used to produce a 20% or larger sample (depending on the required final sample size) of random numbers for each tuberculosis management unit between 1 and the number of notified cases in the previous year. These random numbers are then sent to each tuberculosis management unit with the instruction that one or preferably two specimens from cases having these tuberculosis management unit case numbers should be sent for drug susceptibility testing, if the patient is registered as having new sputum smear positive tuberculosis. Because only approximately half of all newly registered patients will have new sputum smear positive disease, the original 20% sample will be reduced by half. Again, it is important to note that in this case instructions should be given that specimens from all cases registered for retreatment have to be submitted for culture and drug susceptibility testing.

2. Collection of specimens

In the context of surveillance of drug resistance, the decision to obtain a specimen is usually made after the diagnosis of sputum smear positive tuberculosis is made. Tubercle bacilli do not lose their characteristic of acid-fastness over time, but they rapidly lose their viability. Specimens submitted for culturing must thus be dealt with differently from specimens submitted for microscopy only. Because viability of tubercle bacilli is rapidly diminished by a high ambient temperature and the time elapsed since collection, only specimens from sputum smear positive cases should be selected for surveillance of drug resistance. This will help to ensure that even after some transit time, viable bacilli may still be recovered. Patients identified as having sputum smear positive tuberculosis who are selected for culture and drug susceptibility testing should be requested to produce an early morning specimen as soon as possible after the diagnosis has been made. Two consecutive sputum specimens should be collected from each patient. The collected specimen should be kept in the coolest available place, and every effort should be made to ensure that the interval between collection of the specimen and processing in the reference laboratory does not exceed 4 days.

3. Transport of specimens

If sputum specimens are sent for culture, light plastic sputum containers are not appropriate. Universal containers are made of sturdy glass or plastic and are not easily broken, even if subjected to considerable strain as may happen during shipment. Specifically designed boxes to prevent breakage and desiccation and to hold universal containers are perhaps best made locally of plywood, but in a way

that reduces weight. An example of a suitable glass container and transport box is shown in figure IV.1. Alternatively, styrofoam boxes fitting the transport vials may be made. These will further reduce the weight and shipment costs, but are perhaps more vulnerable to breaking during transport.

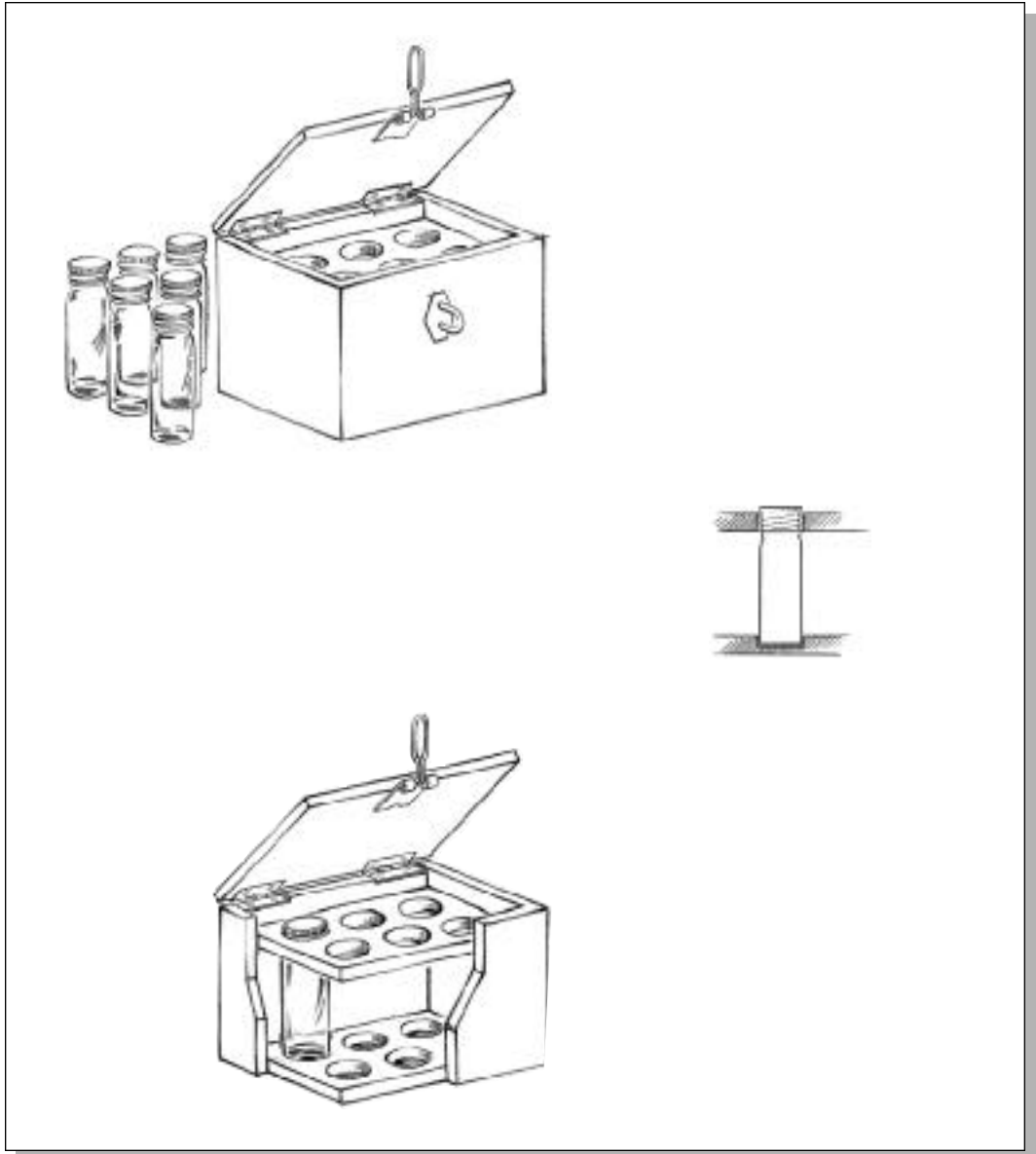


Figure IV.1. Example of sturdy glass containers (universal containers, 28mL) and transport box to ship sputum specimens for culture and drug susceptibility testing.

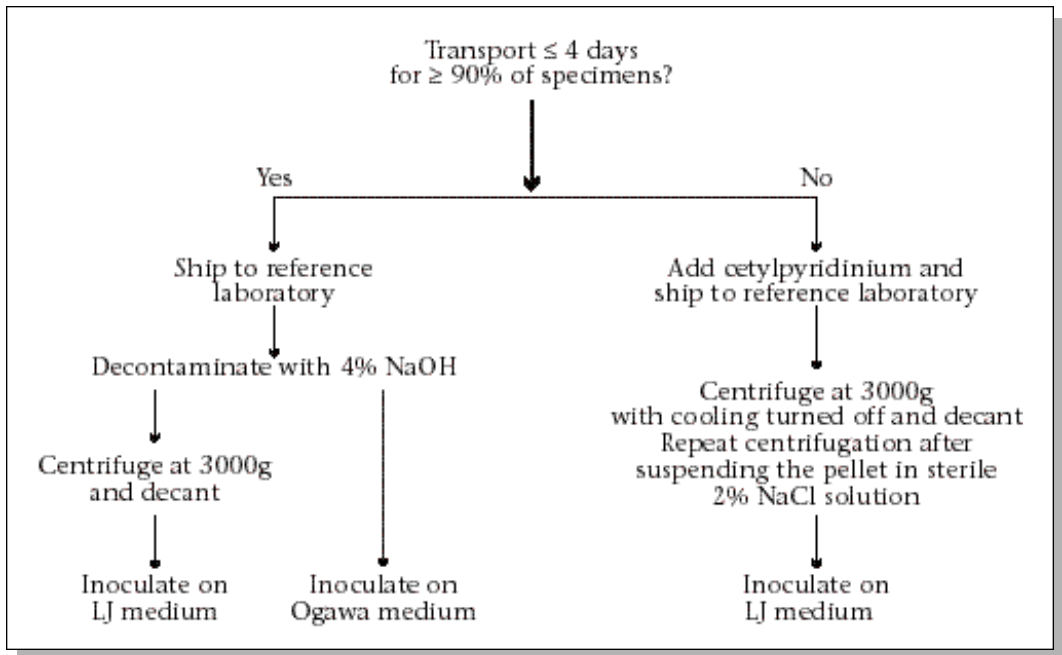


Figure IV.2. Decision tree for transport medium, decontamination, centrifugation, and choice of medium.

The first decision to be made is whether adding a transport solution is required. The decision is based on actual transport time (figure IV.2). If 90% or more of the specimens are expected to arrive within 4 days in the reference laboratory, then no transport solution is needed. If the critical time exceeds 4 days in more than 10% of cases, then the most convenient approach is to utilize a 1% aqueous solution of cetylpyridinium chloride or 0.6% aqueous cetylpyridinium bromide. The accompanying documentation should indicate whether this agent was added.

4. Decontamination and homogenization

Decontamination of sputum specimens has two objectives:

- destruction of bacteria other than mycobacteria (“decontamination”), and
- homogenization of the specimen (usually sputum).

Theoretically, there are many different techniques available; none of them is ideal, i.e., none of them will selectively destroy only contaminating flora and achieve the complete liquefaction of the specimen. Indeed, what is needed is a reasonable compromise, i.e., to destroy as much of the contaminating flora as

possible while harming as few mycobacteria as possible. Furthermore, all reagents needed should be inexpensive and readily available.

For specimens not treated in the periphery with cetylpyridinium, decontamination is done with the specimen at arrival in the reference laboratory.

Method for specimens pretreated with cetylpyridinium chloride/bromide

The cetylpyridinium chloride/bromide, a quaternary ammonium compound, method was proposed as a means of digesting and decontaminating sputum in transit. When a digested/decontaminated specimen arrives in the laboratory, it is concentrated by centrifugation and the sediment is inoculated directly onto the medium, preferably after repeated centrifugation to remove cetylpyridinium chloride/bromide (figure IV.2). Centrifugation of cetylpyridinium treated specimens require that the cooling system of the centrifuge is turned off to prevent precipitation of the decontaminant.

The 4% NaOH method used in untreated specimens

The technique of Petroff to achieve decontamination with up to a final maximum concentration of 2% sodium hydroxide (using an equal amount of 4% NaOH stock solution and specimen) is given preference worldwide. A few general observations should be stressed:

- The 4% sodium hydroxide solution represents the upper limit of its concentration. (Petroff himself suggested 3% for sputum specimens). The decisive parameter is the culture media contamination frequency (see below): if it is below 5% with, e.g., 2% sodium hydroxide, then it is quite legitimate to use this concentration.
- The sodium hydroxide decontamination is harmful to mycobacteria: extending the indicated time of contact will kill an increasing proportion of tubercle bacilli in the specimen. Therefore, if necessary, always increase the sodium hydroxide concentration (up to 4% maximum), and never the exposure time.
- The Petroff technique requires centrifugation if a Löwenstein-Jensen medium is utilized. Alternatively, the Ogawa medium contains a strong buffer which may be used. This technique does not require centrifugation and is efficient for culture of smear positive sputum specimens collected for drug resistance surveillance (figure IV.2).

The Petroff decontamination procedure is as follows:

- Add an equal volume of 4% sodium hydroxide solution to about 4 mL of sputum in an appropriate container; this will reduce the initial concentration by half,

thus the final concentration will be 2% NaOH. Ensure that the sodium hydroxide container never comes in contact with the neck of the specimen container, to reduce the risk of cross-contamination.

- Homogenize by shaking on a shaker or by hand and decontaminate for 15 minutes at 37°C if possible.
- Dilute the homogenate with sterile distilled water to lessen the viscosity and specific gravity prior to centrifugation for 15 minutes. Mycobacteria have a low

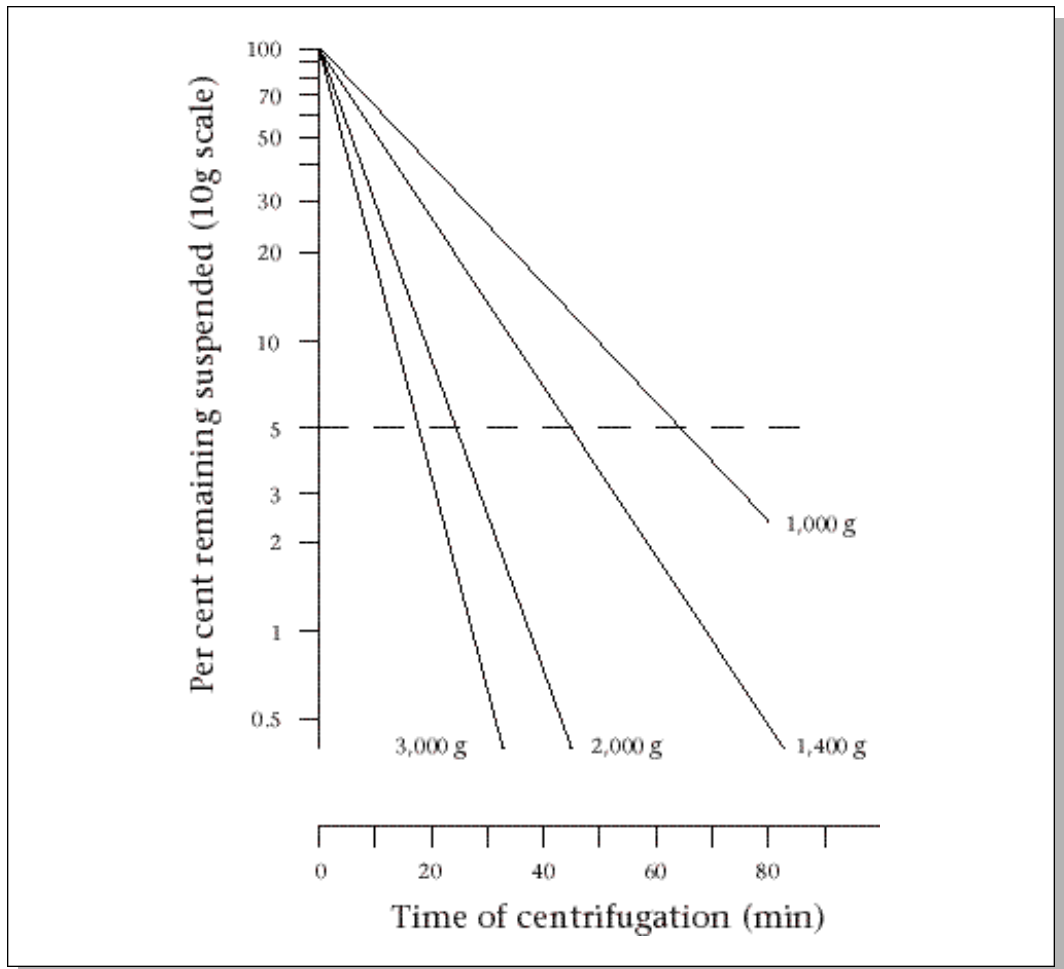


Figure IV.3. Efficiency of centrifugation in achieving sedimentation of mycobacteria as a function of relative centrifugal force (g) and time of centrifugation.

The crossing of the dashed line with the straight line depicting the relative centrifugal force indicates the time when only 5% of mycobacteria remain suspended.

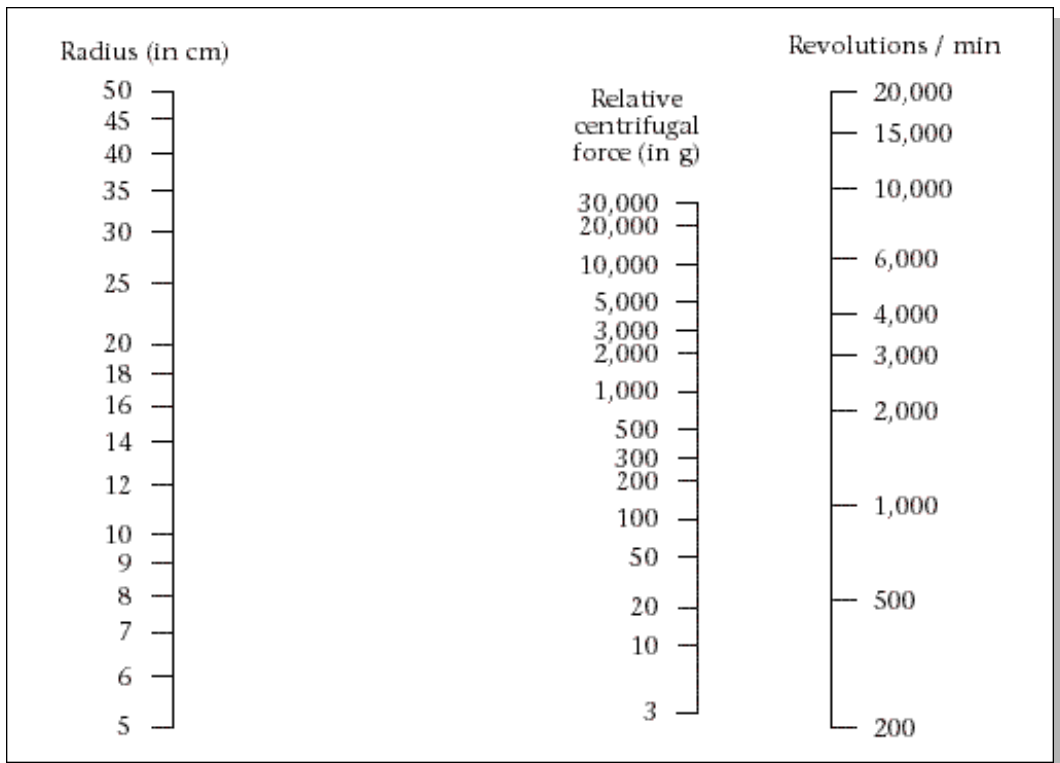


Figure IV.4. Nomogram showing relation between radius (r), relative centrifugal force (RCF in g), and revolutions per minute (RPM).

If the values of any of the two variables are connected, the value of the third variable is provided at the intersection of the respective line.

The formula of the relation is $g = 0.0001118 \times r \times \text{RPM}$.

specific gravity and may remain buoyant during centrifugation. On the whole, a relative centrifugal force of about 3,000 g appears to be adequate to precipitate mycobacteria. The rate at which mycobacteria sediment is critically dependent on time of centrifugation and the centrifugal force applied to the specimen (figure IV.3). While prolonging the time of centrifugation can offset a lower relative centrifugal force, increased centrifugation time increases the temperature of the specimen, which leads to additional killing of mycobacteria. A nomogram (figure IV.4) can be used to check the desired centrifugal force. It must be kept in mind that sputum containers must be capped securely and balanced before centrifugation and that a centrifuge should never be opened until it has come to a complete stop. Every possible effort must be made so that the total period of time of contact with the contaminating solution does not exceed 30 minutes.

- Pour off the supernatant fluid from the pellet into a splash-proof discard container with 5% phenol and remove any fluid on the outside of the container neck with absorbent paper. A funnel resting on top of a beaker or bottle, with its lower end dipping below the surface of the disinfectant, will prevent splashes.
- Add a few drops of 1 N hydrochloric acid with phenol red to neutralize the alkaline reaction. Alternatively, repeat centrifugation after re-suspending the sediment in sterile distilled water.
- Inoculate the media (see below) each with two large (5 mm) loopfuls of the pellet. Alternatively, use a Pasteur pipette to transfer the pellet to the media.
- It is desirable to store the leftover pellet or sediment, if any, for at least 2 days until it is confirmed that the inoculated media are not contaminated. If the media are found to be contaminated, the pellet is treated with a small amount of 5% oxalic acid for 20 to 30 minutes to remove alkaline resistant contaminants. It is subsequently centrifuged after diluting with sterile distilled water and inoculated onto media.

The sodium hydroxide concentration used depends on the contamination rate of the media. The contamination rate is determined by calculating the proportion of contaminated tubes out of all seeded tubes and not by determining the rate by using the number of specimens as the denominator.

5. Media preparation and inoculation

Basically, a simple analysis shows that the seeding of only one tube of medium is not cost-effective. Thus, at least two tubes must be seeded from each specimen.

For decades it has been known that different strains of tubercle bacilli prefer different media. A typical example includes the egg-based medium of Löwenstein-Jensen recommended by the IUATLD (designated as IUTM medium) without potato starch (table IV.2). Other commonly used egg-based media include the Ogawa medium (table IV.3) and the Stonebrink medium (table IV.4), where the replacement of glycerol by sodium pyruvate enhances the growth of *M. bovis*, of some strains of *M. africanum*, and of some drug-resistant strains of tubercle bacilli.

If culture is not used primarily for diagnostic purposes but for the surveillance of drug resistance of tubercle bacilli, two egg-based media are recommended. The first is either the IUTM medium or the Ogawa medium. The second is the Stonebrink medium.

Table IV.2. Preparation of the International Union Against Tuberculosis Löwenstein-Jensen medium (IUTM).

Adapted from: Jensen K A. Towards a standardisation of laboratory methods. Second report of the Sub-Committee of Laboratory Methods of the International Union Against Tuberculosis. Bull Int Union Tuberc 1955; 25 (1-2): 89-104.

a) IUTM base composition

L-Asparagine.....	2.25 g
Potassium phosphate, dibasic, anhydrous	1.50 g
Magnesium citrate	0.15 g
Magnesium sulfate 7H ₂ O	0.4 g
Malachite green	0.25 g

Malachite green. It is imperative that the malachite green crystals purchased are chosen with care: many of those which are commercially available are not suitable as they are bactericidal for mycobacteria. Ensure that those purchased have been tested for antimycobacterial activity.

The IUTM base is prepared in amounts sufficient for the preparation of 250 to 500 liters of Löwenstein-Jensen medium.

b) Preparation of Löwenstein-Jensen medium

IUTM base	4.5 g
Glycerol.....	7.5 mL
Distilled water	360.0 mL

Bring the solution to a boil, autoclave for 15 minutes at 121°C and let cool. For drug containing medium add the drug solution, for control medium add equivalent volume of distilled water.

The eggs should be fresh (2 days old), and antibiotic-free. Scrubbing with 70% ethanol cleans them. Eggs are broken, whites and yolk are homogenized in a sterile blender, then drained through sterile gauze. To the autoclaved solution 625.0 mL of homogenized whole eggs are added, the solution mixed and dispensed aseptically as needed.

Inspissate for 45 minutes at 85°C (80% humidity) or autoclave, all valves closed, for 50 minutes at 121°C.

Table IV.3. Preparation of Ogawa and modified Ogawa (Kudoh) medium.

Adapted from: Ogawa T, Saito N, Sawai T, Nakajima K, Toyama K, Honda A, et al. The comparative studies between NaOH method and neutralization method for isolation of tubercle bacilli. *Kitasato Arch Exp Med* 1960; 23:9-24; Kudoh S, Kudoh T. A simple technique for culturing tubercle bacilli. *Bull World Health Organ* 1974; 51: 71-82.

Component	Ogawa	Modified Ogawa (Kudoh)
Monopotassium phosphate	3 g	2 g
Magnesium citrate	--	0.1 g
Sodium glutamate	1 g	0.5 g
Glycero	16 mL	4 mL
Distilled water	100 mL	100 mL
Egg homogenate	200 mL	200 mL
Malachite green (2%)	6 mL	4 mL

Table IV.4. Preparation of Stonebrink medium.

Adapted from: Stonebrink B. Tubercle bacilli and pyruvic acid. *Proc Tub Res Council, The Hague* 1957; 44: 67-74.

Stonebrink medium	
Monopotassium phosphate	3.50 g
Disodium phosphate Na ₂ HPO ₄ anhydrous	1.59 g
Sodium pyruvate	6.25 g
Distilled water	500.0 mL

Sterilize in autoclave at 121°C for 15 minutes, let cool and add 1,000 mL homogenized whole eggs and 20.0 mL of a filtered sterilized 2% aqueous solution of malachite green. Dispense aseptically into the appropriate vials and inspissate for 45 minutes at 85°C.

6. Incubation and reading of cultures

Some countries have an unreliable power supply. Thus, continuous recording of the temperature in the incubator is advantageous. Temperatures recorded by the temperature gauge might be unreliable; it is therefore recommended that a thermometer is kept inside the incubator at all times to confirm that the recordings are accurate.

The seeded solid media should be incubated in a horizontal position overnight at 37°C to prevent the inoculum from sliding to the bottom of the tube. The subsequent incubation can be done in an upright position for a period of 9 weeks.

It is recommended that the inoculated media are examined every week. Most contaminated media and rapidly growing mycobacteria are detected within 1 week of incubation. The growth of *M. bovis* and some dysgonic strains of *M. tuberculosis* may appear after 5 to 9 weeks.

All positive cultures should be kept until a supranational laboratory (see section 12 in this chapter) has sampled the strains and reported the results of proficiency testing. They should be stored tightly stoppered in the refrigerator if possible, or at room temperature, and protected from light.

Long-term storage of cultures can be done by making heavy bacterial suspensions in skim-milk medium. Polyethylene vials (cryovials 2 mL) can be used; these vials can be stored in a freezer at -70°C for many years without significant loss of viability.

7. Identification of the *Mycobacterium tuberculosis* complex

Identification of mycobacteria can be rather complex and needs a multitude of biochemical tests ascertain to which species a mycobacterium belongs. In the context of surveillance of drug resistance, the process can be simplified to a considerable degree as it will only be necessary to decide whether the mycobacterium is a pathogenic species of the *Mycobacterium tuberculosis* complex (*M. tuberculosis*, *M. bovis*, or *M. africanum*) or not.

Usually, in the context of surveillance of drug resistance, it is only necessary to separate with reasonable certainty *Mycobacterium tuberculosis* complex from environmental mycobacteria, and there is no need to be certain about exact speciation within the complex. In this case a very simple identification procedure as proposed in figure IV.5. suffices.

If it is desirable to identify *M. tuberculosis* complex prior to processing for drug susceptibility testing, the culture, if affluently growing, can be subjected to biochemical tests, i.e., niacin, nitrate reduction, and 68°C labile catalase tests that permit identification of *M. tuberculosis* and *M. bovis*. It should be noted, however, that some strains of *M. tuberculosis* and *M. bovis* are niacin negative. Cultures with too scanty growth for biochemical tests are tested against the anti-tuberculosis drugs isoniazid, rifampicin, streptomycin, and ethambutol plus para-nitrobenzoic

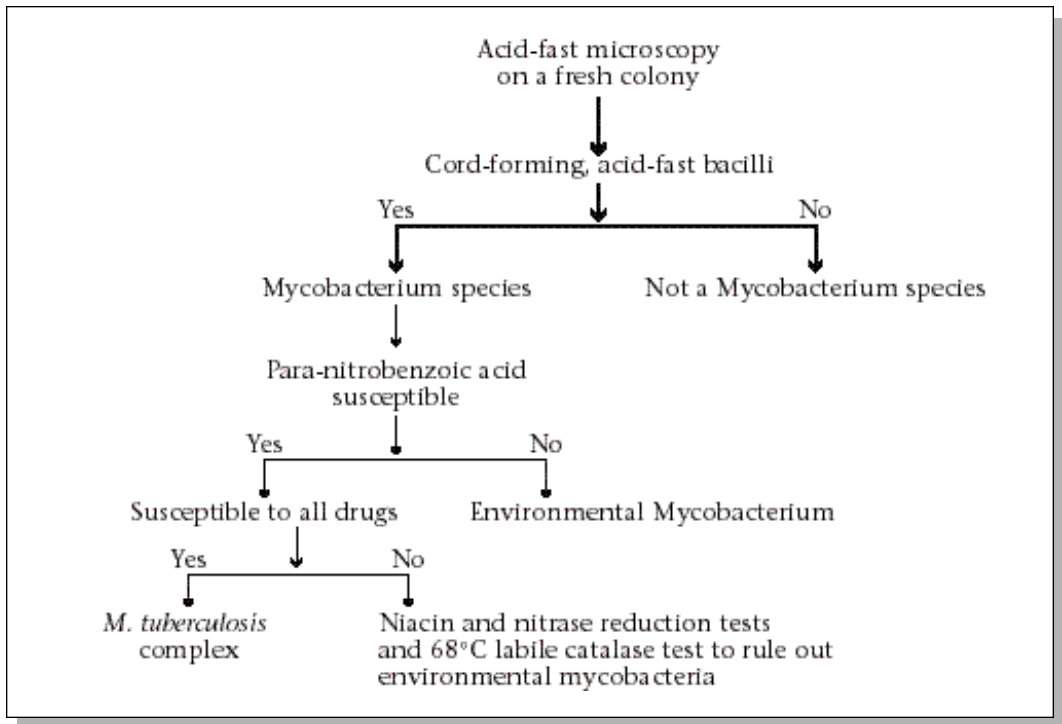


Figure IV.5. Identification of the *Mycobacterium tuberculosis* complex.

acid and thiophene-2-carboxylic acid hydrazide. Almost all strains of *M. tuberculosis* complex are susceptible to para-nitrobenzoic acid and *M. bovis* is susceptible to both para-nitrobenzoic acid and thiophene-2-carboxylic acid hydrazide. However, identification of *M. bovis* must be ensured by niacin and nitrate reduction tests. In areas of the world where *M. africanum* is prevalent, its proper identification is difficult and not recommended if pyrazinamide susceptibility is not being tested.

If it is considered to be more economical to perform identification and drug susceptibility testing simultaneously, the cultures grown on para-nitrobenzoic acid have to be submitted to biochemical tests.

8. Drug susceptibility testing

Accepting the hypothesis that drug resistance is the result of selection by a drug of pre-existing mutants in a population of tubercle bacilli by exposure to a drug, it is easy to understand why inoculum standardization is one of the most important issues in drug susceptibility testing. Decades ago inter- and intra-strain

variability in colony-forming units (CFU) of tubercle bacilli for a suspension defined as containing approximately 1 mg wet weight bacilli/mL was estimated to vary between $\geq 10^6$ and $\leq 10^8$ CFU. As a consequence, depending on the method used, with “over-seeding” a drug-containing medium, a susceptible strain may be misclassified as resistant.

The proportion method

Strains identified as belonging to the *M. tuberculosis* complex are routinely tested for their susceptibility to isoniazid, rifampicin, streptomycin, and ethambutol. The IUTM Löwenstein-Jensen medium in universal containers (28 mL) or in screw-capped tubes is used with and without incorporation of drugs at the recommended concentrations (tables IV.5 and IV.6).

A calibrated inoculating loop (made from 24-gauge wire) with an internal diameter of 3.0 mm will deliver 0.01 mL. The amount of growth in the primary culture should be recorded: in situations with 5 or fewer colonies the drug

Table IV.5. Critical drug concentrations and critical proportions for resistance (Löwenstein-Jensen medium).

Drug	Concentration	Critical proportion
Isoniazid	0.2 µg/mL	1%
Rifampicin	40. µg/mL	1%
Streptomycin	4.0 µg/mL	1%
Ethambutol	2.0 µg/mL	1%

Table IV.6. Quality control of drug containing media. Minimum, median, and maximum numbers of bacilli resistant to antituberculosis drugs for *M. tuberculosis* H₃₇Rv per 10⁶ bacilli. Readings formed at 6 weeks.

Drug	Concentration (µg / mL)	Minimum	Median	Maximum
Isoniazid	0.2	0	4	32
Rifampicin	40.0	0	0.02	--
Dihydrostreptomycin sulfate	4.0	0	7	300
Ethambutol	2.0	100	--	1,000

susceptibility test results may not be reliable. A representative sample of 5.0 mg to 10.0 mg from the primary culture or a sub-culture within 1 to 2 weeks after appearance of growth using an inoculating loop is placed into a sterile McCartney bottle (a 14 mL screw-capped bottle) containing 1.0 mL H₂O plus 10 glass beads (3.0 mm diameter). The mixture is homogenized on a Vortex mixer for up to 1 minute and then (if necessary) the opacity of the suspension is adjusted by the addition of sterile, distilled H₂O to that of a standard suspension of 1.0 mg/mL of BCG.

Two serial dilutions are made from the original suspension, 10⁻² and 10⁻⁴, using the calibrated inoculating loop and sterile McCartney vials containing 1.0 mL of H₂O. One slope of control media and two slopes of each drug-containing media are inoculated with a loopful (0.01 mL) of each dilution.

Drug-containing media

Slopes of the Löwenstein-Jensen medium in the IUATLD modification containing isoniazid 0.2 mg/L, dihydro-streptomycin 4 mg/L, rifampicin 40 mg/L, and ethambutol 2 mg/L are prepared and stored at 4°C for a maximum of 1 month.

The batch numbers of the substances should be registered (see chapter on quality control). It is necessary to convert the required amount of the substrate salt to the substance base: dihydro-streptomycin is usually supplied as sulfate, so that it is necessary to adjust the weight to obtain the desired amount of base (table IV.8). The base weight of rifampicin has to be adjusted similarly. Ethambutol is usually supplied as dihydrochloride, but in the original standardization this was not accounted for, so that a 1:1 ratio of salt to base is used. Isoniazid is always used as a 1:1 salt to base ratio.

**Table IV.7. Economic version of the proportion method.
Arrangement of vials with and without drugs in a 6 x 3 rack.**

Control 10 ⁻⁴ dilution	Control 10 ⁻⁴ dilution	Isoniazid 0.2 mg	Rifampicin 40.0 mg 4.0 mg	Dihydrostrepto- mycin sulfate	Ethambutol 2.0 mg
Control 10 ⁻² dilution	Control 10 ⁻² dilution	Isoniazid 0.2 mg	Rifampicin 40.0 mg 4.0 mg	Dihydrostrepto- mycin sulfate	Ethambutol 2.0 mg
PNB	TCH	Control 10 ⁰	Control 10 ⁰	X	X

PNB: para-nitrobenzoic acid
TCH: Thiophen-2-carboxylic acid hydrazide

Table IV.8. Drug preparation for proportion method of drug susceptibility testing.

Drug potencies

The true potency of the drug is the number of micrograms of active drug per milligram total weight of the product. Not all antimicrobial drugs have been isolated in pure form, and a portion of their weight may be due to impurities or to the sulfate or another radical component of the molecule.

Desired activity ($\mu\text{g}/\text{mL}$) = (weight of drug) x (potency) / (volume of solvent)

Isoniazid

Isoniazid potency is 1g to 1g substance

1. Dissolve 20.0 mg isoniazid in 40.0 mL of sterile, distilled water. This is a 500 $\mu\text{g}/\text{mL}$ solution.
2. Dilute 2.0 mL of this solution aseptically with sterile, distilled water using a 50.0 mL volumetric flask. This is the 20.0 $\mu\text{g}/\text{mL}$ stock solution.

Rifampicin

Rifampicin potency is generally > 980.0 $\mu\text{g}/\text{mg}$

1. Dissolve 80.0 mg/potency of rifampicin in 5.0 mL of absolute methanol. Further dilute the solution with 5.0 mL of 95% ethanol. This is the 8,000.0 $\mu\text{g}/\text{mL}$ stock solution (self-sterilizing).

Dihydro-streptomycin sulfate

Potency may vary from 667 $\mu\text{g}/\text{mg}$ to 800 $\mu\text{g}/\text{mg}$.

1. Dissolve 40.0 mg/potency of dihydro-streptomycin sulfate in a 50.0 mL volumetric flask with sterile, distilled water. This is the 800.0 $\mu\text{g}/\text{mL}$ stock solution.

Ethambutol

Ethambutol potency is 1g to 1g substance

1. Dissolve 20.0 mg of ethambutol powder in 100.0 mL sterile, distilled water. This is the 200.0 $\mu\text{g}/\text{mL}$ stock solution.

Table IV.9. Preparation of antituberculosis drug media.

Critical drug concentration			To volume media	add	of stock drug solution
Isoniazid	1	0.2 µg / mL	1,000.0 mL	10.0 mL	20.0 µg / mL
Rifampicin	1	40.0 µg / mL	1,000.0 mL	5.0 mL	8,000 µg / mL
Streptomycin	1	8.0 µg / mL	1,000.0 mL	10.0 mL	800 µg / mL
Ethambutol	1	2.0 µg / mL	500.0 mL	5.0 mL	200 µg / mL

Stock solutions of drugs (table IV.9) remain stable for at least 6 months at -20°C, and for 1 week at 4°C. As a consequence it is reasonable to prepare new stock solutions for each batch of drug-containing media should no freezer (-20°C) be available.

The preparation of drug-containing media is done as summarized in table IV.7.

It is essential during inspissation of media to maintain the temperature at exactly 85°C and for exactly 45 minutes.

Reading, interpreting, and reporting

Examine the seeded media for contamination after 1 week. The first reading of drug susceptibility test results is done at 4 weeks (28 days) of incubation at 37°C. At that time all strains showing drug resistance can be reported as drug resistant. Strains showing drug susceptibility at 28 days usually need further reading at 6 weeks (42 days) before reporting susceptibility.

Resistance is expressed as the percentage of colonies on drug-containing media in comparison to the growth on drug-free medium at the critical concentrations of the substances, i.e., 0.2 mg/L for isoniazid, 40 mg/L for rifampicin, 4 mg/L for dihydro-streptomycin, and 2 mg/L for ethambutol (table IV.7). The usual criterion for resistance is 1% of growth for all these four drugs (table IV.5). The use of the term “borderline resistance” is discouraged because it leads only to confusion. Should a clear interpretation not be possible, the test should be repeated.

Other methods

The resistance ratio method

This method compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain H₃₇Rv, or preferably three wild strains,

taking their modal resistance as control (Chris Collins, written communication, August 1998). Parallel sets of media containing two-fold dilutions of the drug are inoculated with a standard inoculum prepared from both the unknown and the standard strain of tubercle bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test strain to the MIC of the standard strain in the same test.

The absolute concentration method

The test was used originally to determine the MIC of isoniazid and streptomycin by adding a carefully controlled inoculum of *M. tuberculosis* to the control and drug-containing media. Media containing several sequential dilutions of each drug are used, and resistance is indicated by the lowest concentration of the drug which will inhibit growth, i.e., less than 20 colonies by the end of 4 weeks.

9. Recording of drug susceptibility test results

A distinction must be made between information essential for public health surveillance and information essential for monitoring progress in the laboratory work. The latter will vary according to the preferences of the individual laboratory, the number of different tests performed, the frequency of reading, and other factors. The focus here will be on the minimum requirements for a register that allows exploitation of information for surveillance of drug resistance and a system that will allow future linkage between patient characteristics and drug susceptibility test results.

Reported results from drug susceptibility testing must be exhaustive, that is, only cultures on which all four drugs were tested should be reported, and all possible combinations should be listed. This will facilitate future comparability. Results should also be reported by type of patient to ascertain the estimate of the frequency of primary and acquired drug resistance. The format of reporting is shown in appendix 10.

The tuberculosis reference laboratory register for cultures

The tuberculosis reference laboratory register for cultures must allow linkage of information concerning individual patients from the tuberculosis management unit's case register with the tuberculosis reference laboratory register. Thus, the service unit submitting a specimen for culture must provide information about the patient on a specifically designed request form (appendix 4). The information is limited to essentials and entered in the tuberculosis reference

laboratory register for cultures (appendix 6) to which reference laboratory results are added. This information will allow, among other things, differentiation of drug susceptibility test results in patients with and those without prior treatment history, and a description of the prevalence of resistance by demographic characteristics. If desired, the register allows a retrospective analysis of treatment outcome by drug susceptibility test result by linking reference laboratory information to the patients by the tuberculosis management unit's tuberculosis registration number.

Choice of computer hardware and software

Basic information recorded in the tuberculosis reference laboratory register for cultures should be computerized on a regular basis. A desktop computer of the newest generation with appropriate software is essential for efficient analysis of data. Minimally required software includes the Epi Info package and one of the office suites for word processing and spreadsheet utilization. The Epi Info package (jointly supported by the United States Centers for Disease Control and Prevention and the World Health Organization) is in the public domain, and thus may be freely copied and distributed.

10. Analysis of drug susceptibility test results

It is very important to know the population from which strains of tubercle bacilli have come. Ideally, they should be representative of the entire tuberculosis patient population as outlined above. If such representativeness has not been achieved, the bias must be specified clearly.

Patients with or without a history of previous treatment must be distinguished very clearly and be reported separately. There are two reasons for this. First, sampling is often different for patients with a history of previous treatment compared with those without, as over-sampling is often recommended for the former. A simple mixing would thus greatly distort the picture. Secondly, patients with a history of prior treatment and a resistant strain often represent very recent errors of the program, while the emergence of resistance in never previously treated patients may be delayed, depending on the varying incubation period of latent tuberculous infection progressing to overt clinical disease.

The level of resistance in previously treated patients gives a rather good picture of current treatment practices, if the total number of such cases can be reasonably estimated for the entire country. The prevalence of drug resistance in patients without prior treatment is much more difficult to interpret. It is essential

to collect information on the age of the patients and to calculate age-specific prevalence of drug resistance. To get information on the actual type of strains currently circulating, it would be ideal to obtain cultures for drug susceptibility testing from children under the age of 5 years, as these reflect by definition strains circulating in the recent past and most likely from patients without prior treatment. Because this is not usually feasible as children very rarely have sputum smear-positive disease, the shape of the age-specific distribution of drug resistance among patients without prior treatment can provide a rough proxy measurement of trends. If a decline in the prevalence of drug resistance in never previously treated patients is observed with increasing age, then drug resistance is likely to have increased over time. Conversely, if an increase with age is observed, the picture may suggest that the situation is improving, or that misclassification of patients (assignment of previously treated cases to never treated cases) has occurred.

To determine the level of resistance in previously untreated patients, calculation of the proportion of resistant strains suffices. The proportion of resistant strains is, however, less meaningful for resistance among patients with prior treatment. In this particular case, the absolute number of cases with drug resistance identified during one year is much more informative. It will thus be important to know as closely as possible what proportion of patients with prior treatment eligible for surveillance of drug resistance in the country actually had drug susceptibility testing performed. The more complete the assessment, the less extrapolation will be needed to estimate the absolute number of such cases with resistant strains.

11. Internal quality control of culturing and drug susceptibility testing

Internal quality control covers all aspects of management by the reference laboratory in order to monitor the accuracy and reproducibility of the results it obtains.

A manual must be available to all laboratory workers concerned with the specific tasks, along with a flow chart describing step by step what should be done when significant deviations occur in the course of monitoring quality control. The production of graphs by plotting critical performance indicators, such as culture contamination frequencies, culture positivity frequencies, and annual prevalence of drug resistance, is helpful for such monitoring.

The technical personnel should undergo continuing education. Senior staff members should participate in an annual meeting of a major laboratory

organization (or, alternatively, in a meeting organized by the central reference laboratory of the country). Their absence from work during training should be considered working time, and their expenses reimbursed.

Quality control of drug susceptibility tests is best performed by titrating the standard strain H₃₇Rv of *M. tuberculosis* for each newly produced batch of drug susceptibility testing media. Minimum, median, and maximum number of resistant bacilli by 10⁶ CFU are given in table IV.5. If these values are exceeded then the batch is considered to be inadequate. This circumstance invalidates all results obtained with that particular batch. An alternative method of quality control consists of including in each series of testing the standard strain H₃₇Rv as well as two strains of *M. tuberculosis* containing drug resistance markers, which together express all four drug resistance markers.

12. Proficiency testing of drug susceptibility tests

To ensure that results of drug susceptibility testing are reliable and internationally comparable, the WHO and the IUATLD established a network of supranational reference laboratories in 1994.

The first purpose of this network was to standardize *M. tuberculosis* drug susceptibility testing throughout the world. Today the supranational network provides proficiency testing of drug susceptibility to national laboratories engaged in anti-tuberculosis drug resistance surveillance. This proficiency test consists of exchanging samples of *M. tuberculosis* strains between the corresponding supranational laboratory and the national tuberculosis reference laboratory, and comparing the results with the gold standard which has been defined as the consensus result of the supranational laboratory network.

The second step in proficiency testing of results from national surveys consists of random sampling by the corresponding supranational laboratory of the strains included in the national surveys and the comparison of results in a double-blinded manner.

It is recommended that each national tuberculosis reference laboratory initiating surveillance of anti-tuberculosis drug resistance establishes a link with the most appropriate supranational tuberculosis reference laboratory to ensure its integration into the global network.

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CHAPTER V

Means, materials, equipment, and physical environment in the national tuberculosis laboratory network

I. The design of the peripheral microscopy laboratory

The detailed arrangement for the peripheral laboratory will greatly vary depending on: whether other work is also done; the size and shape of the available room; whether electricity or daylight is used for microscopic examination. For example, the laboratory can be arranged to include three separate sections (appendix 1):

- one well-lighted area for preparing and staining smears,
- one well-lighted area for microscopy,
- one area for registration and storage.

The multi-purpose laboratory, which includes mycobacteriology, should contain at least a sink and three tables:

- a table to prepare the smears,
- a sink to stain the smears,
- a table to examine the smears,
- a table for paperwork.

The laboratory should also have chairs, or preferably adjustable height stools. Some of the equipment necessary in the peripheral laboratory is listed in table V.1.

Good ventilation is necessary for the protection of the personnel from airborne infectious droplet nuclei. The best way to ensure ventilation is by use of windows and doors, ensuring that expelled air flows to the outside environment. To ensure proper ventilation in closed-off rooms an extraction fan will be required.

The laboratory should also have access to a local technology incinerator for safe disposal of contaminated materials.

Table V.I. Equipment needed in the peripheral laboratory.

Item	Units	Cost	
		US\$	FF
Binocular microscope, electric and mirror	1	1,528	9,170
Sink with drain, or plastic bowl	1	local variation	
Water tap, or large bucket with lid	1	local variation	
Waste burning drum with lid and lock	1	local variation	
Metal rack for staining	1	20	120
Drying rack, wood	1	local variation	
Spirit lamp, metal	1	25	150
Slide-holding boxes	5	145	871
Conical flask 1 L, borosilicate glass	2	21	124
Measuring cylinder glass, 250 mL	1	14	81
Measuring spoon	1	3	18
Reagent bottles 1 L, safe-break Winchester, amber	4	20	122
Staining bottles, 250 mL, plastic with jet	3	5	33
Oil dropper bottle, plastic	1	2	13
Beaker, 1 L, plastic, with handgrip for rinsing	1	16	94
Small funnel, plastic	3	<1	2
Table, small	3	local variation	
Chair	1	local variation	
Laboratory stool	1	local variation	
Shelf for bottles, etc	1	local variation	
Cupboard for microscope	1	local variation	

2. The design of the national tuberculosis reference laboratory

Location of the laboratory

The decision concerning the location of the laboratory must be determined at the highest level of the country's public health authorities in consultation with the national tuberculosis program. There are many considerations to take into account. As a national facility it should logically be located in the country's capital, where the required infrastructure for operating it in a satisfactory manner is likely to be found.

It is important, from an organizational point of view, that the laboratory is under the administrative control of the public health administration. It is usually convenient to have it located in the close vicinity to other public health institutions.

Physical infrastructure such as roads, water supply, sanitation, electricity, and possibly gas, should be available, but may be replaced or supplemented with supplies from internal installations in the building. In many places an emergency power supply generator is necessary. Furthermore, a supply of bottled gas (butane is recommended) would be expedient, and the building should have its own water tank or water purification plant.

When a particular site is selected for the laboratory, an architect should evaluate its suitability. It is important to assess the size of the plot in relation to the predetermined space requirements. Additional parameters such as the terrain, accessibility, view, traffic noise, exposure to sun, wind, and airborne dust, will be factors that will influence the design of the building.

Should the site chosen be unsuitable for the purpose, an alternative site must be selected before planning is commenced. When the definitive site has been identified, assurances must be obtained from the authorities that all encumbrance relating to the plot has been clarified (ownership, conflicts in relation to other possible builders, technical limitations, etc.), and that the choice of site is not liable to alteration after the planning has been commenced. Such alterations are not unusual and would cause delays and conflicts with regard to responsibility for the additional costs incurred.

Space planning criteria

Table V.2 shows the required rooms and their approximate dimensions in the reference laboratory, with a brief explanation of the purpose of each room. The assessment of size should be regarded as approximate and will vary in relation to the shape and to the furnishing/equipping of the room. The indicated dimensions represent minimum requirements.

The functions should not be divided between more than two floors. All technical functions should be on the same level.

Space planning relationships

Table V.3 indicates the proximity/relations between the building's various functions. It is important that one does not get too concerned with the precise details of the room/space plan. The description of functions should be seen as "an

Table V.2. Space planning criteria

Technical laboratory functions		
Description of area	m²	Work done in the area
Specimen reception	8	Unpacking and registration of specimens
Media processing section	12	Production of media and distilled water
Storage section	15	Storage of glassware, disposable equipment, etc
Gas storage room	3	Storage of gas containers (needs access from outside)
Microscopy section	25	Light microscopy
Culture processing section	30	Identification and drug susceptibility testing
Sterilization section	12	Cleaning and sterilizing of material
Room for fluorescence microscopy	4	Dark room for fluorescence microscopy
Cooler room (4°C)	6	Storage of chemicals and culture media in a cool room or refrigerators
Incubation room (37°C)	8	Incubating
Administrative functions		
Secretariat	20	Record-keeping for general administrative functions
Conference room	25	Meetings, breaks, cafeteria, training courses
Manager's office	12	Office of Head of Reference Laboratory
Archives	12	
Storeroom	12	
Cloakroom	10	Cloakroom and shower for male employees
Cloakroom	10	Cloakroom and shower for female employees

area designated for a specific purpose/function", and not necessarily a space confined within walls and a closed door. Related functions may often be advantageously located inside the same four walls. There are, however, a few functions which have to be totally isolated from others, for example the media processing section, which has quite specific criteria for cleanliness and, if feasible, a dark room for fluorescence microscopy.

The reference laboratory in Dakar (Senegal), as shown in the drawings in appendix 2, has been designed to take account of the fact that all laboratory functions are to constitute a compact unit in which the employees can move

Table V.3. The proximity/reasons between the building's various functions

	Specimen reception	Microscopy section	Culture processing section	Sterilization section	Media processing section	Fluorescence microscopy	Cooler room 4°C	Incubator 37°C	Storage room	Gas storage room
Specimen reception		DA	SF		SF					
Microscopy section	DA		DA	CL		DA		CL		CL
Culture processing section	SF	DA		DA	DA	DA	CL	DA	CL	CL
Sterilization section		CL	DA		DA			CL	DA	CL
Media processing section	SF		DA	DA			DA		CL	CL
Room for fluorescence microscopy		DA	DA							
Cooler room 4°C			CL		DA					
Incubator 37°C		CL	DA	CL						
Storage room			CL	DA	CL					
Gas storage room		CL	CL	CL	CL					

DA = Direct access
 CL = Close to
 SF = Separated from

between the various parts of the laboratory without having to pass through doors. In this manner the staff who work in various parts of the laboratory can also see and communicate with one another. Hygienic considerations should be taken care of through standard operating procedures. It is the standard operating procedures that determine whether contamination is likely to occur or not.

The laboratory installations represent a sub-division of the laboratory so that the functions have their allotted places. The furnishing/equipping is, however, general, and if certain functions or activities at certain times demand a larger space than originally allocated, they can expand within the same four walls. This facilitates great flexibility and the possibility of immediate contact between colleagues.

The laboratory's supporting functions are located in an ancillary room, with direct access from the laboratory. Thus a corridor is not needed, the distances are shorter and the construction costs lower. The sterilization section has direct access to the storeroom. In this way, all materials can be washed and/or sterilized on their way from the store to the laboratory. A second autoclave is highly desirable in close vicinity to the biological safety cabinet, so that the risk of accidental spills is reduced. The media processing section has been separated on account of the requirements for cleanliness. Connected with this room is a storeroom containing refrigerators and a freezer for the storage of chemicals and other materials. This room could be transformed into a 4°C cold-room. Should one choose to have such a cold-room, one should take necessary precautions to prevent condensation within the wall structure, particularly in warm and humid climates. Only prefabricated cold-rooms placed inside and free from the structural walls should be used. A cold-room also requires the availability of rapid repair services. The availability of several refrigerators provides a useful backup capability.

The weakness in this particular plan (appendix 2) is that in order to gain access to the cold-room one has to pass through the media processing section. In this case, the media processing section has been separated from other rooms. This section can also serve as a storeroom for sterilized glassware and other sterilized items. A window from the sterilization section is convenient for transferring equipment after sterilization. The main storeroom adjacent to the sterilization section is used for non-sterile equipment and packed sterile equipment delivered from external suppliers. Items that need to be sterilized before use pass through the sterilization section. Sterilized supplies are to be unwrapped in the laboratory.

The laboratory can also profit from an extra storeroom or spare room, which is available for future developments. It may also be an advantage to have a small bucket-and-mop closet to facilitate cleaning, while preventing the laboratory cleaning equipment from being used for other parts of the building. Space should allow for a small wash/change room at the entrance to the laboratory so that personnel can change clothing and wash themselves when entering and leaving. A wash basin, a hand dryer, and locker should be provided.

Ventilation

In order to limit problems resulting from high ambient temperature, the Dakar laboratory has been designed so that the ancillary rooms, such as the store rooms etc., project from the facade and have only small windows, while all the work rooms and rooms in which staff are located have been drawn into the building and

provided with large windows. This provides shade for the glass surfaces. If the windows are being exposed to direct sun, the rooms will become uncomfortably hot. A double roof construction (outer roof plus ceiling) provides a layer for ventilation and thereby also prevents overheating. Air-conditioning has nevertheless been introduced to provide staff with maximum comfort, but this represents an additional cost which can be eliminated should it be unaffordable. In a fully air-flow controlled laboratory, air-conditioning needs to be controlled to allow flow from the clean to the contaminated areas.

An optimal environment is created with a balanced ventilation system, which supplies clean areas with fresh air from the outside, and an equal flow is extracted from the safety cabinets (or from other contaminated areas of the laboratory). This will create a constant air flow from the clean to the contaminated area.

However, this is a sophisticated technical solution and will in many countries be difficult to obtain. It requires the constant running of the safety cabinets and in hot climates it will be expensive to cool a constant stream of fresh air from the outside. Conversely, in cold climates, heating of the air would be a major expenditure. A system with filtration of the contaminated air through a safety cabinet with a recycling system may be a good solution for cutting costs, but it increases the risk of contamination of the atmosphere in the laboratory, thus the air of the biological safety cabinet should be exhausted preferably through the roof. If cooling is necessary, the air-conditioning fans should be situated so that the air stream does not influence sensitive working areas. Above the autoclaves it may be convenient to hang glass or steel exhaust canopies from the ceiling to collect the steam and odors when opening the autoclaves.

Installation and equipment requirements

At an early stage the architect must have an overview of which equipment the building is to house, and whether this equipment requires a water supply, drainage, and electricity. Because some equipment is bulky (e.g., biological safety cabinets, autoclaves, etc.) the architect must be informed about the dimension of all equipment, so that the building can accommodate it. Thus suppliers must be contacted to obtain dimensions, power requirements and other important specifications in good time.

Suppliers with local representatives who can provide maintenance services should be given preference. Before purchasing a piece of equipment, it should be verified that warranties are available.

Table V.4. Minimal technical requirements in the reference laboratory.

Section	Minimal technical requirements
Specimen reception	Laboratory bench, running water, sink, several 220V/1-phase
Microscopy section	Laboratory bench, running water, sink, several 220V/1-phase, gas-butane
Culture section	Laboratory bench, running water, sink, several 220V/1-phase, gas-butane
Sterilization section	Laboratory bench, running water, large double sink, draining board, several 220V/1-phase, 380V/3-phase (autoclave), gas-butane
Media processing section	Laboratory bench, running water, sink, several 220V/1-phase, gas-butane, stove-top
Room for fluorescence microscopy	Possibility to shut out light, laboratory bench, 220V/1-phase
Cooler room (+4°C)	Several 220V/1-phase for refrigerators or isolated room with temperature gauge, shelves
Incubation room (+37°C)	Temperature control, temperature recorder with printer outside, shelves
Storage section	Shelves
Gas storage room	Access from outside

Table V.4. provides an overview of the construction measures which need to be carried out in order to facilitate the installation of necessary equipment in the most important sections of the laboratory.

3.The construction of the national tuberculosis reference laboratory

Working with public authorities

It is important that the reference laboratory be under the administrative control of the national public health authorities that will be responsible for the project. Contact should be established as high up the hierarchy as possible. A senior official of the public health services provides the liaison with the top of the hierarchy to get the project back on track in the event of major problems arising.

It is important that the political authorities responsible for public health understand the necessity for a reference laboratory, and that all those involved have discussed and agreed on the tasks to be performed by such a laboratory at an early stage. There should also be awareness that the establishment of the basic premises for the laboratory may be marked by professional rivalry. In this connection one should not lose sight of the primary objectives of the reference laboratory.

When the project has been allotted a specific location, and before detailed planning commences, written assurances should be obtained at the highest level of authority that this plot has been set aside for this purpose, and also that this decision will not be changed once the construction has begun.

Similarly it should be clearly established what regulations are to be applied with respect to import duties, taxes and other fees. Should the project be exempted from such charges, it is important to have this confirmed in writing. The same applies to the importation of technical equipment from outside the country. Should there be a tax exemption agreement under which alternative methods of paying the tax are adopted (for example, interdepartmental transfers of funds) the mechanism and responsibility for accomplishing this must be clearly established. It is frequently simpler and more efficient to include the tax in the original budget.

Establishing the project team

Establishing the project team is decisive for the organization of the project, whether it is to be a purely national project or a project with input from an external donor. Nevertheless, it is difficult to lay down general guidelines for the organization and composition of the project team. This has to be done on the basis of specific local conditions. In any event it has to be decided what expertise is to be hired locally, and what expertise is to be brought in from outside. It will also be appropriate in some cases to involve one or several international organizations. If it is a project with external donor support, it may be useful for the project to have expert advice from a building expert from that donor country.

In the case of the reference laboratory in Dakar, an architect from the donor country, in cooperation with experts from the IUATLD, drew up the building plans. Additional expertise was provided by the Senegalese authorities as well as by local private companies. This worked well in this instance, but once more it is important to stress that the framework conditions of the project have to be taken into consideration.

A key person in the building phase is the local construction manager. A key person in the building phase is the local supervisor who is a technically skilled person (architect, engineer, or experienced construction worker) paid by the builder. The construction supervisor's role is to supervise the entire work and to ensure that the building corresponds to the drawings.

Project documentation

Formulation of the various documents should start by consulting local professional builders on what documentation is normally required. Documentation includes building plans, technical descriptions, and quantity surveying. To a considerable extent the scope of the documentation will depend on the contractual form and entrepreneurial strategy chosen.

Whether one elects to put out a project to open or closed bidding, soliciting quotations or other models, a general requirement as to documentation will often be:

Site/situation plan	scale 1: 200
Drawing showing floors	scale 1: 50 (1:100)
Cross sections	scale 1: 50 (1:100)
Elevations	scale 1: 50 (1:100)
Important details	scale 1: 20 (or other suitable scale)

It is important that the scale should not be too small, especially if the follow-up is sporadic. It may generally be said that the greater the distance, geographically and culturally, between those who draw up the plans and the building site, the more detailed the drawings and descriptions need to be.

The technical description should be detailed, and the separate operations in the building process should be described in writing. As far as preliminary price estimates and the contract itself are concerned, it is important to use quantifiable amounts for every aspect of the construction or delivery of supplies. Should it be available, a computer-based spread sheet is an excellent tool as a basis for the technical description. This makes it easy to relate quantities to the items, and facilitates calculations. Naturally there are specific programs for working out technical descriptions, but these are often based on national standards and are not universal.

What is of great significance is that the builder discusses the project material in detail with the local building manager and with the building foreman at the site, so that both parties can agree on what has been specified, thus preventing misunderstandings.

Tendering and contracting

It is necessary to select the contractual form and entrepreneurial strategy in cooperation with local building expertise. In countries with a market economy, a form of tendering should be used, either an open round which is publicly advertised and in which firms declare their interest, or a closed competition in which quotations are solicited from certain selected firms. In countries without market economies, contact may be made with a reputed firm to agree on a price for the specified tasks. In some countries the authorities have their own public or parastatal organizations which carry out building operations of this kind. In such cases the costs are often of less importance. However, it is still necessary to have the various requirements incorporated in the contract as to quality and progress, as is the case in negotiations with private entrepreneurs.

It is important to check the entrepreneur's written credentials before the contract is signed. This applies both to the technical quality of the building and to economic dependability. This is of special importance with open bidding. As far as solicited quotations are concerned, experienced persons who have intimate knowledge of the local situation should draw up the list of firms invited to participate.

Binding plans showing expected progress should be worked out before the contract is signed. Clear requirements as to progress, and the responsibility incumbent on each of the participants, should be spelled out. Departures from the agreed progress plans should be subject to daily based fines. Progress should not only be tied to a final date of completion, but also to clearly defined markers en route.

Payments for work should not exceed 85% of the value of the work completed at any time. Alternatively, the entrepreneur may present a bank guarantee for a corresponding amount.

Project management

The importance of choosing a knowledgeable local building entrepreneur has already been emphasized, but it is equally important that there be open communication between this person and the builder/project-leader. Regular written reports should account for what has been carried out in the period, and the degree of progress is to be stated as a per cent value. Reports should also be supplemented with photographs at least once a month. It is also advisable for the builder/project-leader to undertake several inspections during the construction period. Informal direct telephone contact with the builder/project-leader can also be very useful.

Meetings at which all the involved parties are present should be held every 14 days in order to discuss progress at the building site and any problems. It is important that the minutes of such meetings include conclusions and that copies are distributed to all parties.

The local builder should also keep a simple, signed record of the payments made as the building progresses. This should be followed up by periodic reports.

Important decisions or major problems are to be taken up with the builder/project-leader immediately.

All additional work or alterations are to be cleared with the builder before they are ordered, and the cost should be agreed upon in advance.

Completion and hand-over

As the hand-over date nears, the entrepreneur must arrange a final inspection in good time. A preliminary inspection at which all faults and defects are registered and recorded must be carried out. Thereafter, agreement is reached on a deadline for improvements of defects, which may be, for instance, 14 days hence. Then the final hand-over of the building is performed on condition that all the faults and defects have been remedied. It is important that the building not be brought into use before formal hand-over has taken place. Defects which for some reason or other cannot be remedied must be compensated financially.

The builder must take out insurance for the building from the date of takeover.

A guarantee period should be included in the contract with the entrepreneur, should the country's laws not cater for this. Such a guarantee period should be for a minimum of 1 year.

While construction costs will vary greatly in different countries, an idea of the magnitude of the costs is provided in table V.5 for the free-standing reference laboratory and two administrative buildings constructed in Dakar, Senegal, in 1994. The costs for the laboratory alone can be estimated to be approximately 33% of the prices shown in the table.

4. Materials management and costs for peripheral sputum smear microscopy

The national tuberculosis reference laboratory must closely cooperate with management of the national program in providing input to ensure that at all times

Table V.5. Construction costs for the reference laboratory and two administrative buildings in Dakar, Senegal, in 1994. The costs for the laboratory alone are estimated to be about 33% of the total cost.

Category of work	US\$	US\$/m²	FF	FF/m²
Builders' work	184,300	273	950,000	1,407
Incubator room	6,790		35,000	
Electrical installations	81,480	121	420,000	622
Air-conditioning/cooling installations	19,400			
Sanitation and plumbing	5,820	9	30,000	44
External works: fence, gate, walls	15,520		80,000	
External works: roads, hardstandings, services	79,540		410,000	
Telephone/electricity connections	5,820		30,000	
Structural engineering	12,610	19	65,000	96
Global cost	411,280	609	2,120,000	3,141

there are sufficient supplies of laboratory material to allow uninterrupted microscopy services in peripheral laboratories. Supplies from overseas are usually shipped by sea freight after an order has been accepted, assembled, and made ready for dispatch. The time elapsed between placing the order and receiving the supplies may be up to six months, and this must be kept in mind when placing orders. It must be assured that not only running requirements are met, but that a sufficient reserve is always available at all levels.

In addition to laboratory material for peripheral microscopy services, the national tuberculosis reference laboratory needs specific materials for culture and drug susceptibility testing.

Supplies for peripheral sputum smear microscopy

To ensure a continuous flow of materials, programs must budget rationally for requirements. The only quantifiable basis for determining requirements is the number of patients. The recording and reporting system must provide this information in a timely manner.

The material that is required is based on empirical observations of the proportion of cases found among tuberculosis suspects. This proportion may vary

considerably between countries. The fraction of suspects identified as cases is ascertained relatively easily from the Tuberculosis Laboratory Register. If the proportion is not yet known, the IUATLD has recommended using 10% as a rule of thumb. Studies in Benin (32%), Malawi (17%), Nicaragua (5%), Senegal (19%), and Tanzania (19%), show that the proposed rule of thumb must be used with caution, as it would seriously underestimate the requirements in, e.g., Nicaragua.

To demonstrate an example of the procedures to calculate requirements in laboratory material and reagents, the fraction of sputum smear positive cases among tuberculosis suspects examined in the microscopy laboratory will be assumed to be 15% (table V.6). An additional assumption is that each suspect requires three sputum examinations, and that each case among these has an additional three follow-up examinations during treatment. The number of slides that need to be examined per sputum smear positive case is thus $(1/0.15) \times 3 + 3 = 23$ slides. The amount of material and reagents required for each notified case of sputum smear positive tuberculosis is thus equal to the amount of material estimated to be required per one slide times 23. For capital investments (slide-holding boxes, wire loop holders, nickel-chromium wire, and microscopes), the number of slides examined per year in the laboratory needs to be known. It is estimated that 20 slides can be examined per working day, and that the laboratory works for 250 days per year, thus one fully dedicated microscopy laboratory technician can examine 5,000 slides in one year. It may be re-emphasized here that locally available wooden applicators are generally preferable to wire-loop holders.

For each notified smear positive case, 23 slides and 23 sputum containers must be ordered.

The amount of basic fuchsin, methylene blue, methanol, and phenol is calculated from the IUATLD recommended method for Ziehl-Neelsen staining, assuming additionally that 5 mL of each of the respective solutions are needed for each slide. For sulfuric acid and alcohol, technical grade purity gives satisfactory results.

The calculation for immersion oil assumes here that 2 drops (1 drop \approx 1/20 mL) are used for one slide.

One third of a box of filter papers is assumed to be consumed per six-month period, and a half box of lens tissue.

Among capital investments, a laboratory is estimated to require 7 slide-holding boxes, 2 wire loop holders, 1 reel of nickel-chromium wire, and 1 microscope. As these materials depreciate over time, a factor to account for the amount to be replaced in each order needs to be taken into account (column C).

Table V.6. Calculating the requirements for peripheral laboratories and cost of material per identified case.

Item	Unit	Require- ment per slide A	Fraction of cases among suspects B	Annual depre- ciation C	Require- ment per identified case $D=(1/Bx3+3)$ xAXC	No of cases E	Running require- ment F=DxE	Reserve require- ment G=2xF	In stock H	Total order I=F+G-H	Cost per case (FOB) identified US\$	Per cent of cost FF
Slides	1	1	0.15	1.00	23	1000	23,000	46,000	19,550	49,450	0.667767	30.5
Sputum containers	1	1	0.15	1.00	23	1000	23,000	46,000	19,550	49,450	0.909092	41.6
Methylene blue	1 g	0.01500	0.15	1.00	0.34500	1000	345	690	293	742	0.002216	0.1
Basic fuchsin	1 g	0.01500	0.15	1.00	0.34500	1000	345	690	293	742	0.002625	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	0.7
Sulfuric acid	1 mL	1.25000	0.15	1.00	28.75000	1000	28,750	57,500	24,438	61,813	0.213038	9.7
Phenol	1 g	0.25000	0.15	1.00	5.75000	1000	5,750	11,500	4,888	12,363	0.036216	1.7
Xylene	1 mL	1.00000	0.15	1.00	23.00000	1000	23,000	46,000	19,550	49,450	0.315561	14.4
Methanol	1 mL	0.50000	0.15	1.00	11.50000	1000	11,500	23,000	9,775	24,725	0.024731	1.1
Filter paper sheets	1 box	0.00007	0.15	1.00	0.00153	1000	2	3	1	3	0.000005	<0.1
Lens tissue	1 pack	0.00010	0.15	1.00	0.00230	1000	2	5	2	5	0.000007	<0.1
Slide storage box	1	0.00140	0.15	0.10	0.00322	1000	3	6	3	7	0.000125	<0.1
Wire loop holder	1	0.00040	0.15	0.10	0.00092	1000	1	2	1	2	0.000004	<0.1
Ni-Cr wire	1 reel	0.00020	0.15	0.15	0.00069	1000	1	1	1	1	0.000003	<0.1
Microscope	1	0.00020	0.15	0.10	0.00046	1000	0	1	0	1	0.000138	<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

Based on the amount of material required per slide, the fraction of cases expected to be found among suspects, and the depreciation, the amount of material per identified and notified case can then be calculated (column D). In this example, it was assumed that 1,000 smear positive cases were notified during the most recent six-month period (column E), thus the running requirement for a six-month period can be calculated (column F). The reserve requirement for laboratory material should be double that of the running requirement (column G). This is chosen because experience has shown that delivery of laboratory material from many companies offering such supplies can be a lengthy process. It was assumed that 85% of the running requirement was still in stock (column H; in reality, an inventory must be made before preparing the order), thus the total order equals the running requirement plus the reserve stock minus what is still in stock (column I). The cost of material per notified case is given in US dollars and French Francs, according to suppliers costs and currency exchange rates in June 1998.

To the total cost of laboratory material for peripheral smear microscopy, one must add approximately 30% to the Free On Board (FOB) value to account for costs such as insurance, freight, storage, and distribution to the periphery. It may be noted that the most expensive item, making up half of the final cost, is sputum containers, while capital investments rank fairly low in the overall costs.

5. Supplies for the national tuberculosis reference laboratory

The reference laboratory can be thought of as being an assembly of five major sections. These sections do not necessarily need to be physically separated in distinct rooms, but planning the tasks of the reference laboratory should be based on such a concept.

The reference laboratory cannot become functional without a minimum amount of equipment.

The necessary equipment is briefly described. A complete list of minimum requirements in hardware equipment and the approximate costs is provided in table V.7.

Microscopy section

At least three binocular microscopes for light microscopy need to be available, preferably of the same brand as used in the national network of microscopy laboratories. This is important, to familiarize trainees with the equipment they will be using in the field, and to allow the technicians in the

Table V.7. Equipment for a national tuberculosis reference laboratory

Although equipment costs vary from supplier to supplier and over time, the following table provides a rough estimate of what might have to be invested in a national tuberculosis reference laboratory for capital investment.

Section	Item	US\$	FF
Microscopy	1 fluorescence microscope	9,700	58,200
	1 teaching microscope	6,200	37,200
	3 light microscopes	4,575	27,450
	3 bunsen burners	280	1,680
	1 slide drying plate	90	540
Media processing	1 2-speed blender	920	5,520
	2 inspissators, optional	6,800	40,800
	1 laminar flow hood	6,000	36,000
	1 top loading electronic balance	800	4,800
	1 magnetic stirrer hot plate	320	1,920
	1 vortex mixer	180	1,080
	1 water still	1,100	6,600
	5 pipetting syringes	1,350	8,100
Storage	1 deep freezer	12,000	72,000
	3 refrigerators	2,100	12,600
	20 trays for McCartney bottles	420	2,520
	30 trays for universal containers	1,500	9,000
	3 bench tidies	130	780
	2 desiccators	175	1,050
Culture processing	2 biological safety cabinet Class I	24,000	144,000
	2 centrifuges	18,200	109,200
	3 bunsen burners	280	1,680
	1 twin beam balance, 210 g	200	1,200
	1 slide drying plate	90	540
	2 vortex mixers	360	2,160
Sterilization	2 vertical loading autoclaves	18,600	111,600
	1 oven	2,100	12,600
	10 buckets	460	2,760
	5 pipette cases	100	600
	1 vortex mixer	180	1,080
	1 glass brushing machine	1,300	7,800
	1 hot air oven 110 L	1,500	9,000
	Total equipment		122,010

reference laboratory to be thoroughly familiar with the equipment used in the field. It is also psychologically important that students see that the reference laboratory uses the same equipment as everybody else.

One additional microscope should be equipped with a sidearm to allow a student to follow scanning and interpretation of findings by a teacher and to allow the teacher to follow the student's capacity to scan and interpret findings on a slide.

A fluorescence microscope should also be available to efficiently examine large numbers of slides from routine laboratory requests.

Media processing section

Media can be processed in a laminar flow work station (which is not a biological safety cabinet to protect the operator) to reduce the risk of media contamination.

A blender is necessary for homogenizing the whole egg media base and added reagents.

A top loading electronic balance (capacity 300 g, with a sensitivity of 1 mg) is desirable to measure ingredients which are used in very small quantities.

Continuous pipetting syringe outfits (5) to dispense set amounts of media into universal containers are recommended. To increase the lifetime of such systems it is necessary to take extreme care in cleansing after use.

A magnetic stirrer hot plate and magnetic stirring bars of various sizes are needed in the preparation of reagents for the culture medium.

Distilled water, essential in the laboratory, is best produced by a glass water still that produces about 4 L to 8 L single distilled water per hour. Two to three polyethylene water reservoirs (carboy) with a capacity of 50 L to 60 L should be available for storing distilled water to be used in case of water shortage or power breakdown.

An inspissator to allow the coagulation of the media in the universal containers, slanted, completes the list of equipment necessary for the media processing section. Alternatively to an inspissator, a fan-operated hot air oven, with the temperature adjusted, can be used.

Storage section

Necessary equipment should include refrigerators, a freezer, trays, and wire racks. Most chemicals can be stored without refrigeration, but freshly prepared

media need to be refrigerated, and seed and standard strains need to be kept frozen at at least -40°C.

Racks that fit McCartney bottles (7 mL and 14 mL), and universal containers (28 mL) should be available.

Some chemicals and antimicrobial agents need to be stored in desiccators using silica gel as a recyclable drying agent.

Culture processing section

The probability of transmission of tubercle bacilli in the reference laboratory is increased when handling cultures of *M. tuberculosis*, particularly in aqueous suspensions, and the potential of their aerosolization during processing. The processing of cultures should always be carried out in a biological safety cabinet.

A high-quality centrifuge is essential in the preparation of specimens for culture. The centrifuge buckets should be of the safety type with individual lids.

A mechanical twin beam balance is needed to measure the ingredients for media preparation. Because large quantities are required, the balance should have a capacity of 200 g, but a sensitivity of 0.1 g is sufficient.

There are two approaches to the incubation of cultures. One option is to utilize batteries of incubators. This is useful if different temperatures for incubation are desired. Because the interest of the national tuberculosis reference laboratory is primarily the isolation of *M. tuberculosis* complex, incubation at a constant temperature of 37°C is sufficient. Should there be an exceptional interest in identifying mycobacteria that grow preferentially at temperatures other than 37°C, a low temperature and high temperature incubator are needed with a capacity of approximately 200 L. Thus, the most convenient and practical solution to incubation at a constant temperature is a dedicated room with shelves around all three walls away from the entrance. It facilitates work tremendously because cultures can easily be moved according to the number of weeks they have been incubated.

Sterilization section

Before cleaning and removing media, it is essential that mycobacteria, bacteria and yeasts grown on the media are destroyed by autoclaving. Because autoclaves are essential pieces of equipment, two should be available which might be used on alternative weeks. This will allow sterilization of “dirty” and “clean” material. Dirty material includes cultures of mycobacteria that need to be destroyed.

Clean material includes glassware, reagents, and material similarly not grossly contaminated. Using spores that are autoclaved and subsequently incubated on appropriate culture media to ensure that the autoclaves properly sterilize the material should regularly check the efficiency of autoclaves.

A glass brushing machine with appropriate brushes greatly facilitates the work of cleaning glassware.

A hot air oven for sterilizing clean glassware proves very useful.

Glassware in the reference laboratory

Glassware is widely used in medical laboratories. It is therefore essential to become familiar with the common and essential glassware used in a national tuberculosis reference laboratory.

Laboratory glassware made from borosilicate glass (hard glass) is recommended for routine work because it is resistant to chemicals and heat and can withstand repeated autoclaving.

Glassware is occasionally obtainable from local medical stores - but often it is purchased from overseas laboratory supply companies. Under these circumstances, the person in charge of ordering laboratory materials should:

- plan and order materials correctly;
- prepare regular orders every six months. This is essential for maintaining reliability and smooth running of the laboratory;
- use the correct catalogue number and provide the relevant specifications and descriptions for the items ordered;
- make sure that the glassware ordered is indeed relevant and that it can be used in a tuberculosis reference laboratory;
- ensure that the cost of purchasing the items is within the available resources.

A list of essential laboratory glassware is summarized in table V.8.

Supplies for culture and drug susceptibility testing in the reference laboratory

The requirements for the reference laboratory cannot be simply calculated on the basis of the number of reported cases, because the number of specimens processed will vary greatly from laboratory to laboratory. Similarly, requirements in supplies for drug susceptibility testing can not be calculated directly from the number of reported cases. A summary of material and reagents and their approximate costs is provided in table V.9.

Table V.8. Recommended glassware, description and use in the national tuberculosis reference laboratory.

Bottles, universal. These are very strong glass bottles with a wide mouth, fitted with an aluminum screw cap and rubber liner, capacity 28 mL. Universal bottles are used for collection and transport of sputum specimens and for media preparation.

Bottle, McCartney. Glass, with aluminum screw cap and 3 mm rubber liner, capacity 7 mL and 14 mL. Bijou bottles are useful for homogenization of bacterial suspensions and storage of mycobacterial stock cultures when the bottle contains Löwenstein-Jensen medium, because of their convenient, space-saving size for long-term storage.

Bottle, Winchester. Glass, narrow mouth, with aluminum screw cap and rubber liner for stains and chemicals, capacity 150 mL to 2,500 mL. Winchester bottles are mainly used for Löwenstein-Jensen mineral salts, and for other chemicals.

Beakers, borosilicate, heavy duty. Borosilicate heavy duty beaker with spout, heavy banded rim, for general laboratory use.

Cylinders, measuring, with spout. Borosilicate glass, graduated capacities 10 mL to 2,000 mL, for general laboratory use.

Flasks, borosilicate, heavy duty. Heavy duty flask, Erlenmeyer, with narrow neck, capacities 50 mL to 5,000 mL, for general laboratory use.

Flasks, volumetric. Graduated 25 mL, 100 mL, 500 mL, for general laboratory use.

Funnels. Funnels for filtering, graduated 50 mL to 2,000 mL, used for filtering stains like Auramine O, carbol fuchsin, and methylene blue.

Pipettes, volumetric. 1 mL graduated in 1/ 100 mL, 5 mL graduated in 1/ 10 mL, and 10 mL graduated 1/ 10 mL. These pipettes are useful for titration of drug-containing solutions in preparing for drug susceptibility testing and for inoculating bacillary suspensions.

Slides for microscopy. Twin frosted (roughed on both sides at one end) super-premium 27 x 75 mm, thickness 1.0 mm to 1.2 mm, tropical packing. While other slides exist (and may be less expensive), these are the preferred slides because a graphite pencil can be used for marking the slides instead of diamond markers which are very expensive. Tropical packing (a paper tissue between each slides) prevents slides from sticking together.

Table V.9. Reagents used in the reference laboratory

Item	Unit size	Unit	Cost in	
			\$US	FF
<i>Fluorescence microscopy</i>				
Auramine O	50	g	14	85
Phenol crystals	1000	g	24	146
Ethanol 96%	1000	mL	7	43
Potassium permanganate	100	g	7	40
Hydrochloric acid, conc 37%	1000	mL	8	49
<i>Concentration - Petroff method</i>				
Potassium hydroxide	1000	g	11	64
<i>IUTM base</i>				
Potassium dihydrogen phosphate	1000	g	13	77
Magnesium sulfate	1000	g	80	482
L-Asparagine	100	g	7	40
Magnesium citrate	2500	g	58	346
Malachite green	100	g	30	181
Glycerol	1000	mL	47	281
<i>Addition in Ogawa medium</i>				
Monopotassium phosphate	250	g	11	64
Sodium glutamate	1000	g	19	112
<i>Addition in Stonebrink medium</i>				
Sodium pyruvate	250	g	77	459
<i>Addition in para-nitrobenzoic medium</i>				
Para-nitrobenzoic acid	500	g	16	96
Hydrochloric acid 1 N solution	1000	mL	8	50
NaOH 1 N solution	1000	mL	7	45
<i>Drugs for susceptibility testing</i>				
Rifampicin	25	g	634	3802
Dihydrostreptomycin sesquisulfate salt	25	g	25	152
Isoniazid	50	g	10	63
Ethambutol dihydrochloride	25	g	38	228
Ethylene glycol	1000	mL	27	160

Table V.9 (cont'd)

Item	Unit		Cost in	
	size	Unit	\$US	FF
<i>Nitrate reduction test</i>				
di-Sodium hydrogen phosphate	500	g	18	106
di-Potassium hydrogen phosphate	1000	g	35	210
Sodium nitrate	500	g	13	75
Hydrochloric acid, conc 37%	1000	mL	11	68
Sulfanilamide	100	g	44	264
N-(1-naphthyl)-ethylenediamine dihydrochloride	5	g	21	124
Zinc dust	100	g	9	52
<i>Niacin test</i>				
Isoniazid test strips	25	strips	64	385
<i>TCH test</i>				
Thiophen-2-carboloxic acid hydrazide	50	g	17	105

6. Special biosafety considerations in the laboratory

Transmission of *M. tuberculosis* results essentially from micro-aerosols, that is, droplet nuclei containing tubercle bacilli of $\geq 1 \mu\text{m}$ and less than $10 \mu\text{m}$ diameter, i.e., sufficiently small to reach the alveoli, and sufficiently large to be capable of adhering to the alveolar cell lining rather than remaining suspended ineffectively in the alveolar air.

Infection control in the laboratory must aim at reducing airborne transmission, taking into account that different procedures entail considerably different risks of airborne transmission, depending on the extent of aerosolization and the number of infectious particles that can be produced by the procedure.

Specimen collection

In many countries, patients suspected of tuberculosis are referred directly to the laboratory for instructions to produce sputum and its subsequent collection. This is a rational and efficient approach because it limits the number of those giving instructions to a minimum, and to those who know best what kind of specimen they need. At the same time, it exposes the laboratory technicians to a potentially higher risk of acquiring tuberculous infection than many other health

care workers, as they need to communicate often on a daily basis with untreated infectious cases of tuberculosis. Nevertheless, because laboratory technicians know that the probability of a tuberculosis suspect's actually having sputum smear positive tuberculosis is very high (actual values can be calculated from the laboratory register), precautions can be taken that can substantially reduce the risk. First, laboratory technicians must instruct patients to cover their mouths when coughing before proceeding to teach the patients how to produce sputum. Secondly, the production of the sputum specimen must take place outside the laboratory, preferably, if climatic conditions allow, completely outside the building, to allow aerosols to be diluted and exposed to the ultraviolet radiation of direct sunlight.

Smear preparation

While opening sputum containers and making a smear may produce micro-aerosols, the risk of transmission from such procedures is negligible in comparison to that generated by aerosols produced by a single unprotected cough. There is no epidemiologic evidence that solely preparing smears is associated with any measurable excess risk of acquiring tuberculous infection. Biological safety cabinets are thus not necessary in peripheral laboratories performing only smear microscopy.

Dealing with aqueous suspensions of *M. tuberculosis*

The reference laboratory must work with aqueous suspensions of *M. tuberculosis* in identifying mycobacteria from cultures and in drug susceptibility testing. The number of *M. tuberculosis* organisms grown in pure culture is huge, and any aerosol created while handling such suspensions is likely to contain large numbers of tubercle bacilli. Such work must therefore be done inside a biological safety cabinet. Biological safety cabinets are classified in three groups, classes I, II, and III.

Class I biological safety cabinets provide personnel and environmental protection, but no product protection. Unfiltered room air is drawn across the work surface. Personnel protection is provided by this inward airflow as long as a minimum velocity of 25 linear meters per minute is maintained through the front opening. The class I biological safety cabinet is hard-ducted to the building exhaust system, and the building exhaust fan provides the negative pressure necessary to draw room air into the cabinet. Cabinet air is drawn through a high efficiency particulate air (HEPA) filter as it enters the exhaust plenum. HEPA filters are effective at trapping particulate and infectious agents, but not volatile chemicals or gases. A second HEPA filter may be installed at the terminal end of the exhaust. Basically, class I biological safety cabinets are adequate for work with aqueous suspensions of *M. tuberculosis* in the reference laboratory.

Disposal of infectious waste material

Every laboratory, be it peripheral or national, should have the capability to incinerate infectious waste material (used sputum containers, applicator sticks, etc.). In the reference laboratory, used culture bottles should be autoclaved before cleaning. Infectious waste from the reference laboratory should always be autoclaved before incineration.

7. International requirements for safe shipment of cultures of *M. tuberculosis* complex

For external quality control of the drug susceptibility testing in the reference tuberculosis laboratory, cultures have to be exchanged with a supranational reference laboratory. Cultures of *M. tuberculosis* are enriched infectious materials containing great numbers of viable organisms that can cause disease in humans. The hazard risk is compounded when cultures of resistant strains are transported.

Some international organizations, such as the Universal Postal Union, the International Civil Aviation Organization and the International Air Transport Association, have developed guidelines and procedures designed to facilitate the safe and expeditious shipment of infectious substances, while at the same time ensuring the safety of transport personnel and the general public. These organizations have also developed agreed common definitions, and packaging and labeling requirements. Information on the documentation requirements should be obtained from the appropriate national authorities of the country where the cultures are sent.

Infectious substances, and diagnostic specimens likely to contain infectious substances, require triple packaging in accordance with the recommendations of the United Nations. Cultures of mycobacteria should be shipped on solid medium in screwcap tubes or freeze dried in vials as primary watertight containers. Petri dish cultures should not be shipped. The most convenient method for shipping is to mail mycobacterial suspensions in skim milk medium in 1.5 mL amounts kept in 2 mL cryovials. The primary container should be entirely surrounded by at least two cm of absorbent material and enclosed in a second, durable watertight container (figure V.1). The tissue paper or cellulose wadding in the secondary container must be sufficient to absorb all of the fluid in the specimen in case of leakage of the primary container. Several primary containers may be enclosed in a single secondary container, if the total volume of all the primary containers does not exceed 50 mL and there is no contact between them. Each set of primary and secondary containers

should be enclosed in an outer shipping container made of corrugated fiber board, cardboard, wood or other material of equivalent strength.

One copy of the request forms, letters and other information that identifies or describes the specimen should be taped to the outside of the secondary container. Another copy should be sent by air mail to the receiving laboratory and a third retained by the sender. The outer container must bear the infectious substance (biohazard) label. The label should be about 10 cm wide and printed in red on a white background. In addition to the sender's and recipient's addresses, the telephone numbers should also be put on the outside of the package.

Compliance with the shipment requirements is the responsibility of the shipper, who must be familiar with the regulations. Failure to comply may result in fines and other penalties. Hand carriage of infectious substances is strictly prohibited by international air carriers, as is the use of diplomatic pouches.

References:

1. Centers for Disease Control and Prevention, National Institutes of Health. U.S. Department of Health and Human Services, editor. Primary containment for biohazards: selection, installation and use of biological safety cabinets. Washington: U.S. Government Printing Office 1995, 1-51.
2. Collins CH, Grange JM, Yates MD. Tuberculosis bacteriology. Organization and practice. 2nd ed. Oxford: Butterworth-Heinemann, 1997.
3. Committee on Bacteriology and Immunology. Basic safety measures in tuberculosis laboratories in developing countries. Bull Int Union Tuberc 1985; 60 (1-2): 66-67.
4. International Air Transport Association. Dangerous Goods Regulations. 37th ed., effective 1 January 1996. IATA Montreal - Geneva 1996; 1-676.
5. International Union Against Tuberculosis. Technical guide for sputum examination for tuberculosis by direct microscopy. Bull Int Union Tuberc 1978; (Suppl 2): 4-16.
6. World Health Organization, International Union Against Tuberculosis and Lung Disease. Guidelines for surveillance of drug resistance in tuberculosis. World Health Organization Document 1996; WHO/TB/96.216: 1-35.

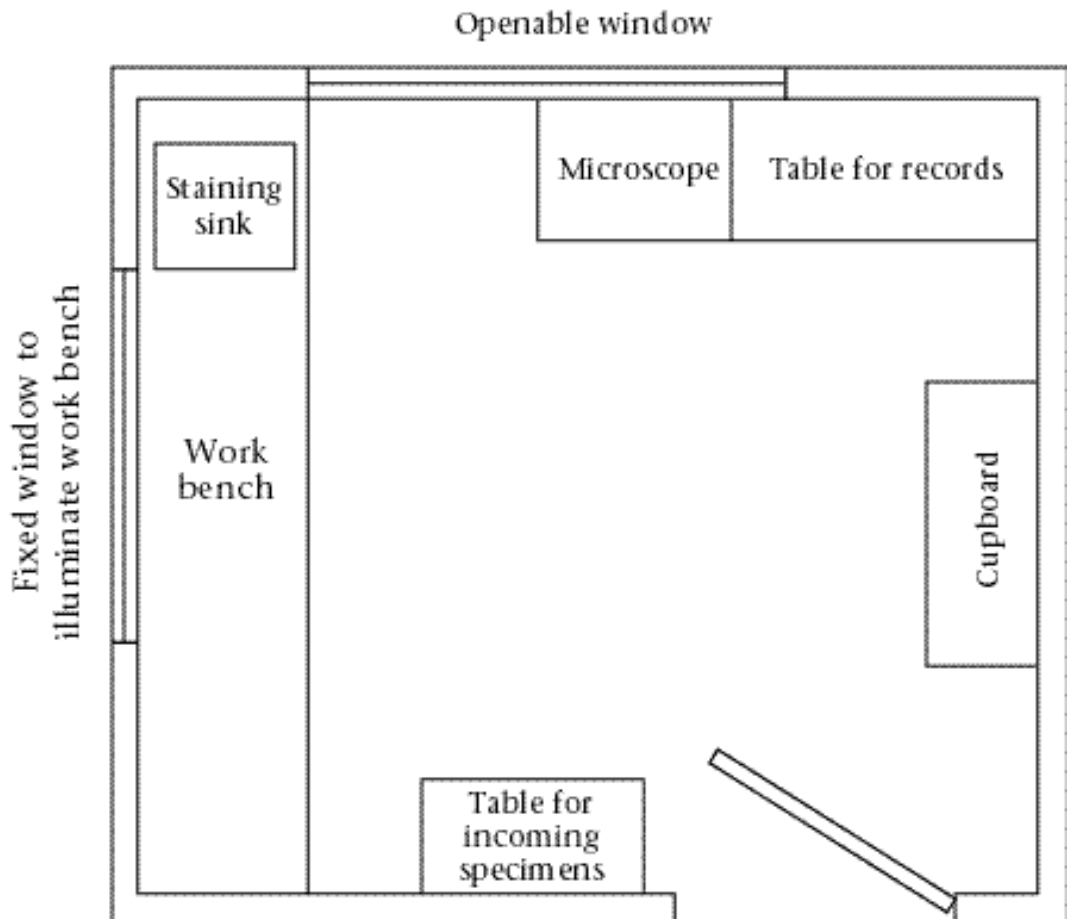
CHAPTER VI

Appendices

- Appendix 1.** Design of the peripheral microscopy laboratory
- Appendix 2.** Design and plans of the national tuberculosis reference laboratory
- Appendix 3.** Request form for sputum smear examination
- Appendix 4.** Request form for culture examination of sputum
- Appendix 5.** Laboratory register for sputum smear microscopy
- Appendix 6.** Laboratory register for cultures of mycobacteria
- Appendix 7.** Form for proficiency testing of sputum smear microscopy using slides sent from the reference laboratory to the periphery
- Appendix 8.** Form for proficiency testing of slides sent from the periphery to the reference laboratory
- Appendix 9.** Form for quality control of classification of registered tuberculosis patients
- Appendix 10.** Form for reporting results of drug susceptibility testing

Appendix 1. Design of the peripheral microscopy laboratory

Adapted from: Collins CH, Grange JM, Yates MD. Tuberculosis bacteriology. Organization and practice. 2nd ed. Oxford: Butterworth-Heinemann, 1997.



Appendix 2. Design and plans of the national tuberculosis reference laboratory

While a reference laboratory will usually have to be integrated into an existing building, and space planning criteria thus may vary considerably depending on the specific situation, the case of the National Center for Tuberculosis Control and the National Tuberculosis Reference Laboratory in Dakar, Senegal, provided the opportunity to construct free-standing buildings on space allocated by the Ministry of Health and Social Affairs. The building complex had to incorporate a reference laboratory, two administrative buildings for the central tuberculosis unit, and sufficient space for storage for drugs and laboratory material, because such space was at that time not available elsewhere. The budget ceiling for construction of the entire complex was pre-determined not to exceed US\$ 420,000. The reference laboratory was built to provide what was minimally required as determined by consultation with international experts in mycobacteriology and public health.

The original plans reproduced here in part are available upon written request from the International Union Against Tuberculosis and Lung Disease.

Figure 1:

Plan A Site and situation plan.

Figure 2:

Plan B Details of the tuberculosis reference laboratory.

Figure 3:

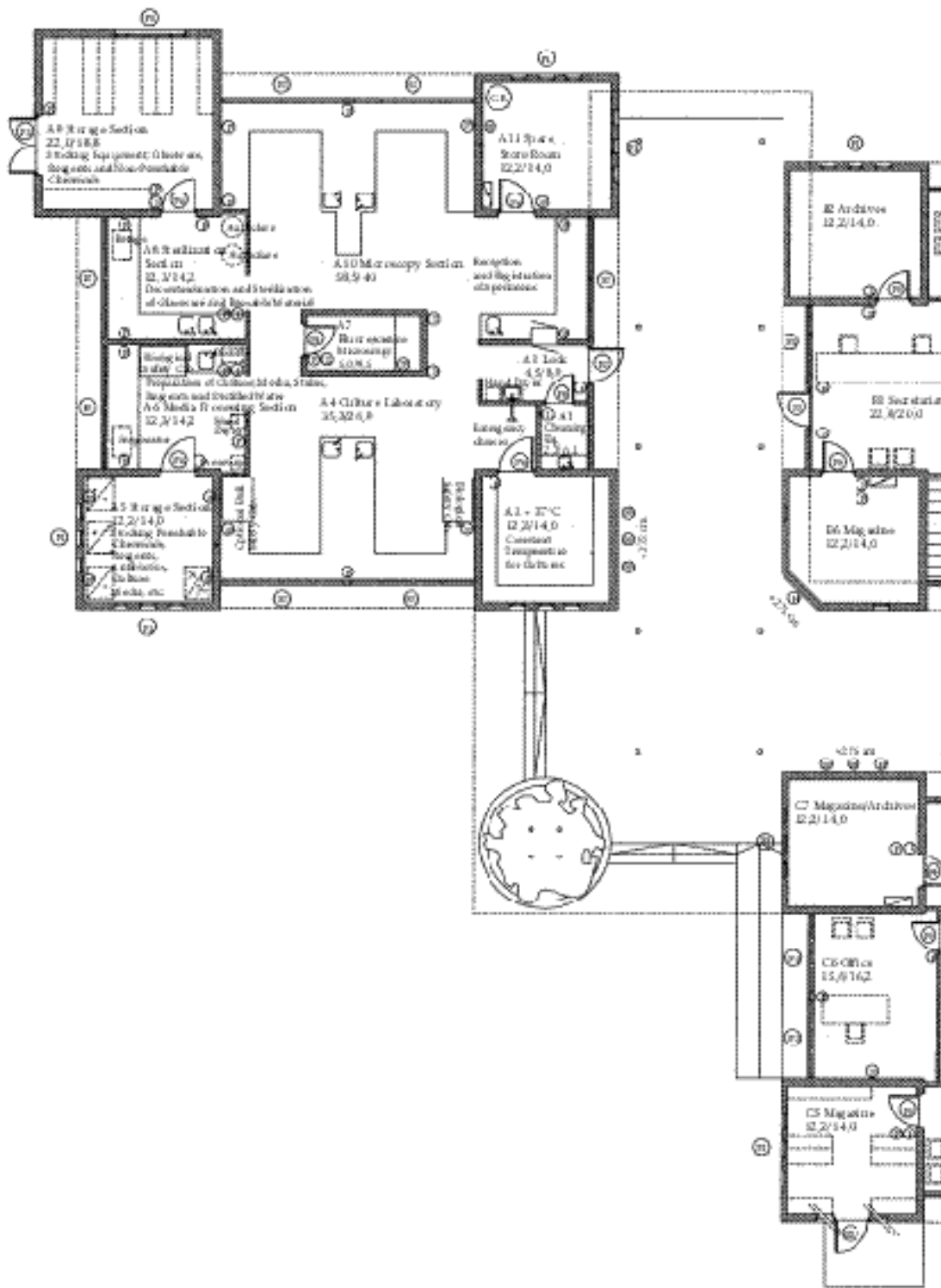
Plan C Elevations north, west, south, and east of the tuberculosis reference laboratory.

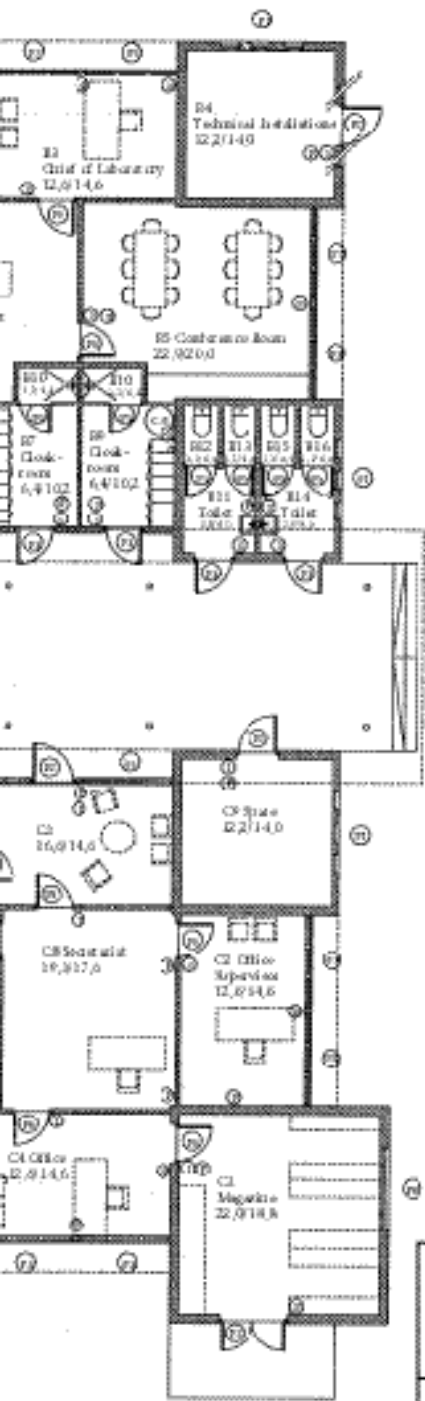
Figure 4:

Depending on available resources, a tuberculosis reference laboratory may be constructed in such a way that the air flow is controlled in the entire laboratory. The establishment of direction of air flow requires careful planning and construction.

The sketch of a laboratory with directional air flow illustrates two basic concepts in bio-containment of class III organisms, i.e., directional air flow and the concept of interlocking doors. Interlocking doors are doors that cannot be opened simultaneously: while one door (door 1) is open, the other one (door 2) is prevented from being opened by a locking mechanism.

The sketch for this laboratory was kindly provided by Isabel N. de Kantor, Sc.D, Buenos Aires, Argentina.





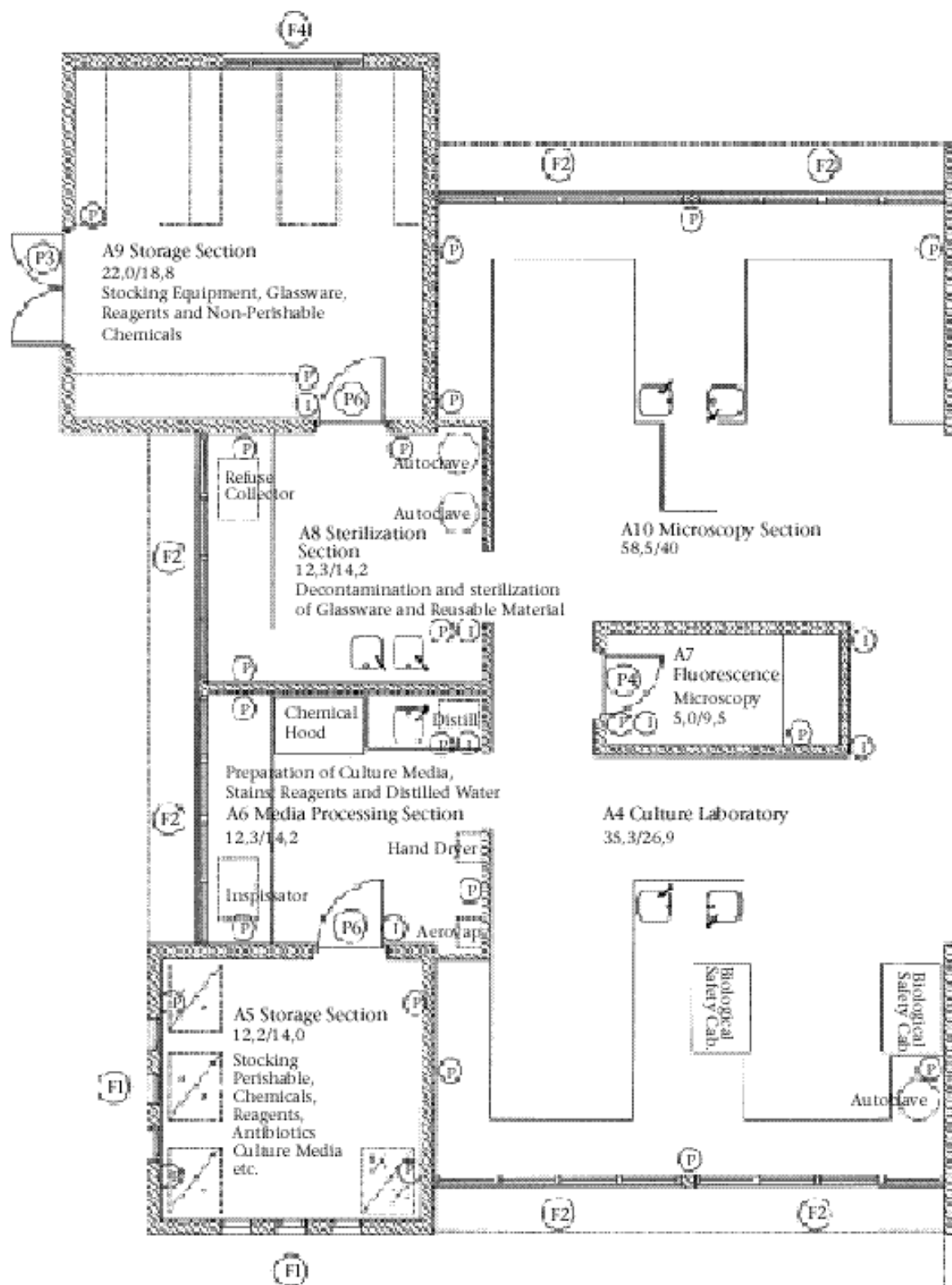
- (S) Switch
- (P) EL. Outlet
- (L) Wall Light
- (C.E.) Hot Water
- (N) EL. Fuses

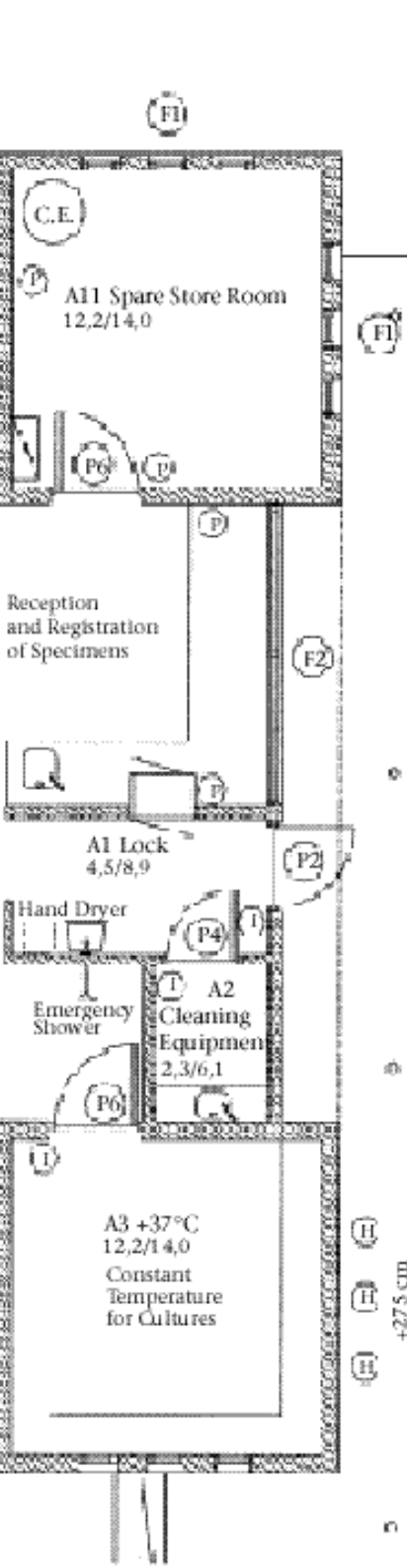
- (F1) Window Aluminium 40x40 CM
- (F2) Window Aluminium 335x190 CM
- (F3) Window Aluminium 215x190 CM
- (F4) Window Aluminium 190x60 CM

- (P1) Steel Door 90x120 CM
- (P2) Steel Door 100x210 CM
- (P3) Steel Door 160x210 CM
- (P4) Door isoplane 90x210 CM
- (P5) Door isoplane 80x210 CM
- (P6) Door isoplane 100x210 CM



NATIONAL REFERENCE LABORATORY - SENEGAL PLAN	SCALE	DATE: 1984
		DRAWING: H.M.
		ARCHITECT: H.M.
MADE BY: I.H.E. FOR IUATLD	REV:	PLAN No:
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- Ⓚ Switch
- Ⓚ El. Outlet
- Ⓚ Wall Light
- Ⓚ C.E. Hot Water
- Ⓚ El. Fuses

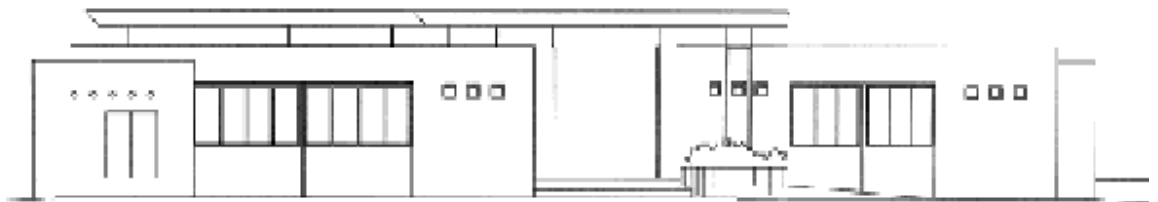
- Ⓚ F1 Window Aluminium 40x40 CM
- Ⓚ F2 Window Aluminium 335x190 CM
- Ⓚ F3 Window Aluminium 215x190 CM
- Ⓚ F4 Window Aluminium 190x60 CM

- Ⓚ P1 Steel Door 90x120 CM
- Ⓚ P2 Steel Door 100x210 CM
- Ⓚ P3 Steel Door 160x210 CM
- Ⓚ P4 Door Isoplane 90x210 CM
- Ⓚ P5 Door Isoplane 80x210 CM
- Ⓚ P6 Door Isoplane 100x210 CM

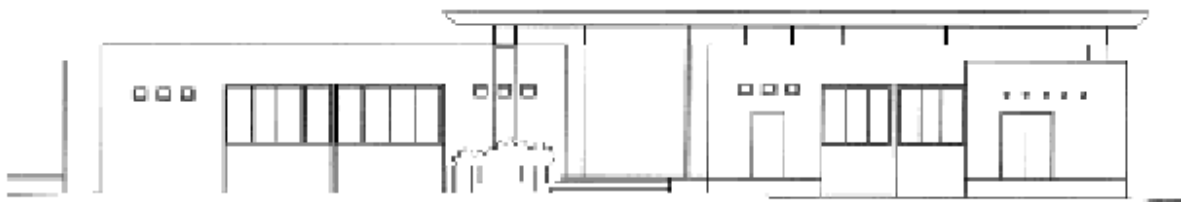
NATIONAL REFERENCE LABORATORY - SENEGAL PLAN OF LABORATORY	SCALE	DATE:
	1:100	1964
MADE BY I.I.B. FOR I.U.A.T.L.D.	REV:	ARCHITECT:
	—	H.M.
	PLAN No:	
	—	

Appendix 2.

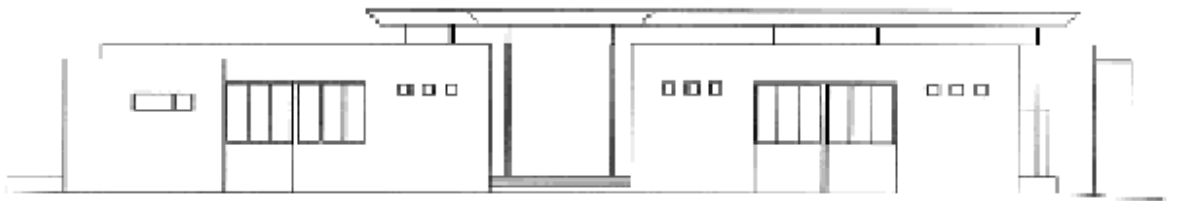
Figure 3 - Plan C



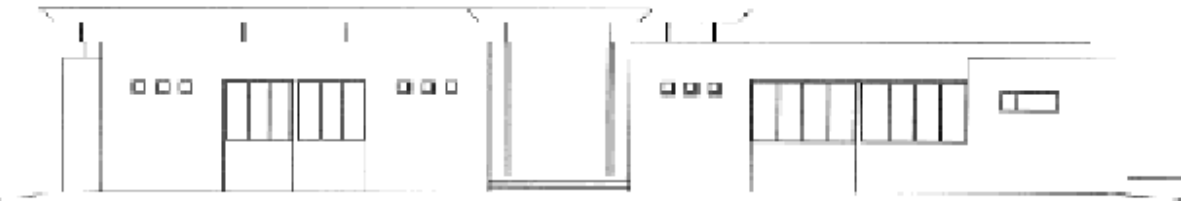
Elevation North



Elevation West



Elevation South



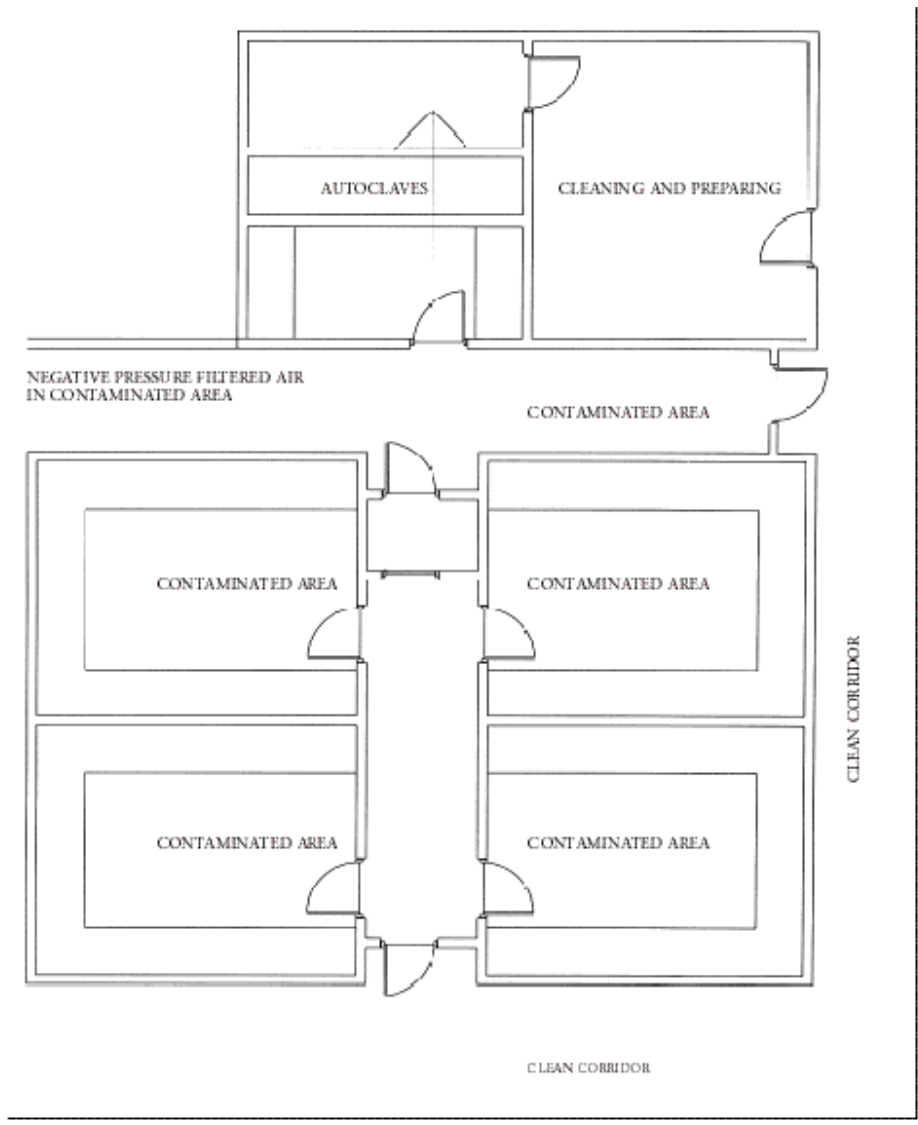
Elevation East



NATIONAL REFERENCE LABORATORY - SENEGAL	SCALE	DATE
	1:200	19/04/2006
ELEVATIONS	DESIGNED BY	H.M.
MADE BY IFL FOR IUATLD	APPROVED BY	H.M.
	REV.	PLANNED BY
	—	—

Appendix 2.

Figure 4 - Sketch of laboratory with controlled air flow



LABORATORY OUTLINE OF LAB. WITH AIR FLOW CONTROL.	SCALE	DATE
	1:100	10/08
		DRAWN BY: H.M.
		ARCHITECT:
IUATLD	REV.	REVISION
	—	—

Appendix 3. Request form for sputum smear examination

Treatment Unit..... Date.....

Patient's name:

Age..... Sex (M/F)

Address (precise)

.....

.....

Reason for examination: diagnosis..... follow-up examination

Signature of person requesting examination:

Results (to be completed in the laboratory)

Laboratory serial No.

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

* visual appearance of sputum (blood-stained, purulent, mucous, mucopurulent, salivary)

Date Examined by (Signature):

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

Appendix 4. Request form for culture examination of sputum

Origin of request:

District: Region: Local laboratory identification:

Date specimen was collected:/...../..... Local laboratory serial number:

Person requesting examination: Name:..... Position:.....

Patient identification:

Surname and first name of patient: Age (yrs): Sex:

Patient TB register number:

Type of patient and site of disease:

- new (never treated before for ≥ 1 month) Site: pulmonary
 relapse extrapulmonary (specify):.....
 failure
 return after default
 chronic excretor

Specimen type:

sputum Local laboratory smear result: 1st..... 2nd..... 3rd..... specimen

other (specify):

Reference laboratory results:

Reference laboratory serial number:

Microscopic examination

Specimen	Neg	1-9	1+	2+	3+
1					
2					

Culture result

Specimen	Neg	≤ 50 colonies actual count	$>50 - 100$ col 1+	$>100 - 200$ col 2+	>200 col 3+
1					
2					

Results of drug susceptibility testing

ISONIAZID	RIFAMPICIN	ETHAMBUTOL	STREPTOMYCIN

Date:/...../.....

Signature:

Appendix 6.
Laboratory register for cultures of mycobacteria

Year

	Ref lab serial number	Name and first name of patient	Age	Sex	District name / code	Reg
a						
b						
c						
d						
e						
f						
g						
h						
i						
j						
k						
l						
m						
n						
o						
p						
q						
r						
s						
t						

	Site of disease	Type of patient	Date specimen received in ref lab	Ref lab microscopy result	Date specimen inoculated for culture	Culture result	Date of culture result
a							
b							
c							
d							
e							
f							
g							
h							
i							
j							
k							
l							
m							
n							
o							
p							
q							
r							
s							
t							

Appendix 7. Form for proficiency testing of sputum smear microscopy using slides sent from the reference laboratory to the periphery

Region: District: Laboratory code:.....

Name of the laboratory technician examining the slides:

Date slides were dispatched from the reference laboratory:/...../.....

Date slides were examined in the peripheral laboratory:/...../.....

Result of examination:

Slide code	Result in reference laboratory	Result in peripheral laboratory
A		
B		
C		
D		
E		
F		

How to report the result:

Examine slides for acid-fast bacilli and report the result as follows:

Finding	Report (in table above)	
No acid-fast bacilli	per 100 immersion fields	Neg
1 to 9 acid-fast bacilli	per 100 immersion fields	report exact figure, e.g., 4/100
10 to 99 acid-fast bacilli	per 100 immersion fields	1+
1 to 9 acid-fast bacilli	per field in 50 fields	2+
>10 acid-fast bacilli	per field in 20 fields	3+

Appendix 9. Form for quality control of classification of registered tuberculosis patients

Results from Quality Control Assessment of Sputum Smear Microscopy.

Region: Coordinator:

Date compiled:/...../.....

No.	Case register number	Local laboratory serial number	Local laboratory smear result	Regional laboratory smear result	Reference laboratory smear result	Reference laboratory final smear result
1						
2						
3						
4						
5						
6						
7						
8						
9						
10						
11						
12						
13						
14						
15						
16						
17						
18						
19						
20						

