Priorities for Tuberculosis Bacteriology Services in Low-Income Countries

Second edition
2007

International Union Against Tuberculosis and Lung Disease

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Foreword to the first edition

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
July 1998

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The authors are grateful to Adalbert Laszlo, who provided relevant input in the first edition, most of which has been included in this second edition.

Donald Enarson went meticulously through the second edition of our manuscript, made critical comments and asked pertinent questions that helped to greatly improve the content and flow of argumentation. In addition, he provided his expertise in the final editing.

The authors are indebted to Ivan Bastian and Richard Lumb who thoroughly and critically reviewed the manuscript, to Thuridur Arnadottir who suggested conceptual improvements, and to Abigail Wright who made critical annotations.

Preface to the second edition

The first edition of this monograph was entitled "The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network – Minimum Requirements and Operation in a Low-Income Country". The authors hope that the more concise "Priorities for tuberculosis bacteriology services in low-income countries" reflects the revisions made in this second edition of what we call more simply "The Red Book".

Helge Myking, co-author in our first edition, passed away before he could see the book in print. Helge had provided his skills as the architect to the national tuberculosis programme in Senegal in building the national tuberculosis reference laboratory. He shared his experiences with that project in this book. The chapter and the architectural plans written by Helge have been left virtually untouched, as they retain the relevance and novelty they had when they were written. We dedicate the second edition of this book to Helge Myking.

This monograph originates from the appreciation of the needs of our collaborators in numerous countries. Our gratitude goes first and foremost 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 intermediate level laboratories and the national tuberculosis reference laboratory. The monograph thus provides an outline of the responsibilities of the laboratory network and the national tuberculosis reference laboratory as the apex of the national laboratory network within the framework of the public health priorities of a national tuberculosis control programme. 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 simply to add another, but to complement those already available. It is limited in its scope and emphasis, as it does not attempt to cover the entire field of mycobacteriology; it rather focuses on the specific set of minimum required tasks at each level of the national laboratory network, with an emphasis on the responsibility of the national tuberculosis reference laboratory. Culture technique, for instance, is discussed solely as a prerequisite for drug susceptibility testing for surveillance of drug resistance. Its potential for increased sensitivity in the diagnosis of individual patients is addressed, but the practical impediments are such that, more often than not, it is not routinely used for diagnostic purposes in low-income countries within the context of a national tuberculosis control programme. Similarly, identification of mycobacteria is discussed only to differentiate the pathogenic species of the

Mycobacterium tuberculosis complex (henceforth interchangeably denoted as tubercle bacilli) with reasonable certainty from environmental mycobacteria, while no attempt is made to provide advice on how to identify species among the latter.

In writing this book, the authors paid particular attention to other publications by the World Health Organization and the International Union Against Tuberculosis and Lung Disease. Every effort was made to remain consistent with official recommendations of these organisations. However, science moves forward, and as new knowledge accumulates, it is only natural that some existing concepts are challenged. This was already the practice in the first edition, published in 1998, and we explicitly continue this line. However, we make it clear where we introduce new concepts, and we ensure that where we challenge, we do this scientifically, with supporting evidence.

While most of the material presented in this manual has been thoroughly tested in the field and has proven 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, Antwerp, Hong Kong, Seoul, Dar es Salaam,
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CHAPTER 1

Tasks and staffing of a national tuberculosis laboratory network

The structure of the health care system, and within it the structure of laboratory services, varies greatly among countries. Nevertheless, the laboratory network in a country is almost always a component of the general health services, providing the services at the peripheral, intermediate and national levels. At the national level, there is usually some specialisation within these laboratory services, one component of which may be tuberculosis laboratory services. National laboratory networks frequently have a system by which they ensure the training and proficiency of the work of laboratory workers throughout the network, be it general or specific, as in the case of tuberculosis laboratory services. The national tuberculosis programme must have an interest in collaborating with, and contributing its resources to, the laboratory network to maintain proficiency and to improve the quality of sputum smear microscopy at all levels of the laboratory network, as discussed in this chapter.

A. Tasks and staffing requirements at the peripheral level

The basic level of implementation of the national tuberculosis programme is the **unit of management**. The unit of management provides diagnostic and curative services for tuberculosis (an integral part of general health services) and collates and records the essential information required for the evaluation of programme performance, surveillance and planning of supplies. The unit typically serves a population of 50,000 to 150,000 and must be adapted according to the incidence of tuberculosis, population density, accessibility of the services and other factors that might be relevant in a particular setting.

The diagnostic component requires health care personnel to identify tuberculosis suspects from among the entire group of patients seeking health services (i.e., each and every service delivery point in the geographical area) and laboratory services capable of proficiently carrying out microscopic examination of sputum samples for acid-fast bacilli. Too much decentralisation of microscopy services may reduce proficiency and requires an unrealistically high input for quality assurance, provision of equipment and supplies. Too much centralisation effectively reduces the accessibility of the services and may result in loss of quality of microscopy

examination by overburdening the laboratory workers. Thus, the plans for the location and distribution of these services must take into account both resource requirements (especially supervision) and accessibility for the population.

The primary task of the basic level microscopy laboratory is examination of sputum specimens for acid-fast bacilli with the Ziehl-Neelsen method. This includes internal quality control, supplemented by support from the intermediate level through regular supervisory visits and external quality assessment. The laboratory worker is also responsible for keeping the microscope in good working order to prolong its useful life.

The results of sputum smear examination for acid-fast bacilli must be reported promptly to the health care worker requesting the examination and be recorded meticulously in a nationally standardised laboratory register. The tuberculosis laboratory register is the key source of information, and is designed to allow review of activities and to provide information for the evaluation of various indicators for assessment of performance. It is also the starting point for randomly selecting the slides for external quality assessment.

Usually only one, or perhaps two, workers staff the peripheral laboratory. A laboratory worker in these laboratories usually has other tasks to perform in addition to sputum smear microscopy. Consequently, great care must be taken not to overburden the worker, as this may result in smear microscopy of poor quality. A workload of 20 to 25 specimens per day is the maximum that can reasonably be expected of a single person. If this is regularly exceeded, it will almost certainly require additional staff.

B. Tasks and staffing requirements at the intermediate level

The intermediate level laboratory is, by definition, the critical link between the peripheral laboratory and the national reference laboratory. Often, this laboratory is located in a larger regional or provincial hospital, and usually has several multi-purpose laboratory workers.

In addition to the routine services provided for the catchment area of the intermediate level laboratory, its main task is the provision of support to the peripheral microscopy centres. This support includes the preparation and distribution of the solutions of reagents and regular supplies of slides, sputum containers, and other essential materials required by the peripheral laboratory to carry out its task without interruption.

Critically, the support to the peripheral units must include regular visits to review the activities and the problems encountered and to provide

feedback on results of external quality control. This will almost certainly increase the motivation of the laboratory workers in the peripheral facilities who are working in isolation with little opportunity to discuss technical and other problems with peers.

The intermediate level laboratory, in turn, is supported by the national reference laboratory, with which it coordinates all activities in the national tuberculosis laboratory network necessary to ensure its smooth functioning. The intermediate laboratory is the critical partner in the implementation of national policy and guidelines. It ensures that the procedures are applied consistently throughout the country.

The intermediate laboratory carries the responsibility of conducting external quality assurance for the peripheral laboratories, of providing feedback to these laboratories on the results and also of providing information on observed performance to the national level.

C. The tasks of the national tuberculosis reference laboratory

The core activities of a national tuberculosis control programme 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 prioritise its activities to support these objectives through the national laboratory network. Thus, the major tasks of the national tuberculosis reference laboratory are to:

- Maintain a high level of proficiency in routine smear microscopy carried out in peripheral health facilities
- Standardise techniques used in the network
- Organise and coordinate training of personnel in the network
- Organise quality assurance testing of smear microscopy in the national laboratory network
- Implement surveillance of anti-tuberculosis drug resistance
- Develop and carry out operational research.

Obviously, the national tuberculosis reference laboratory is rarely in a position to carry out all tasks related to training, supervision and proficiency testing of sputum smear microscopy throughout the entire country, nor would this be an efficient use of its resources. Nevertheless, it has overall responsibility for setting national standards and for overseeing the implementation of policies. Decentralisation of certain activities is highly desirable, and the national tuberculosis reference laboratory should take the lead in encouraging intermediate level (regional/provincial) laboratories to take part in carrying out the essential tasks of the national laboratory

network. In the long term, it will be desirable that effective decentralisation is implemented to make the support to the national tuberculosis programme increasingly more efficient.

1. Maintaining proficiency of routine smear microscopy

Sputum smear microscopy services can be made available throughout the country, as it relies primarily on the multi-purpose microscope integrated into the general health services.

Schematically, Figure I.1 shows the potential sensitivity of the various tools available to identify pulmonary tuberculosis. The relative frequencies are estimates and population-dependent. In industrialised countries, or in a context of active case-finding when relatively more 'early' (less advanced) cases are encountered, a far lower proportion of cases will be smear-positive. Among all tools in the diagnosis of pulmonary tuberculosis, clinical suspicion and chest radiography are the most sensitive tools, albeit at the cost of specificity.

In sophisticated and experienced laboratories, culture techniques might detect as few as 10 bacilli per millilitre of sputum. In many settings,

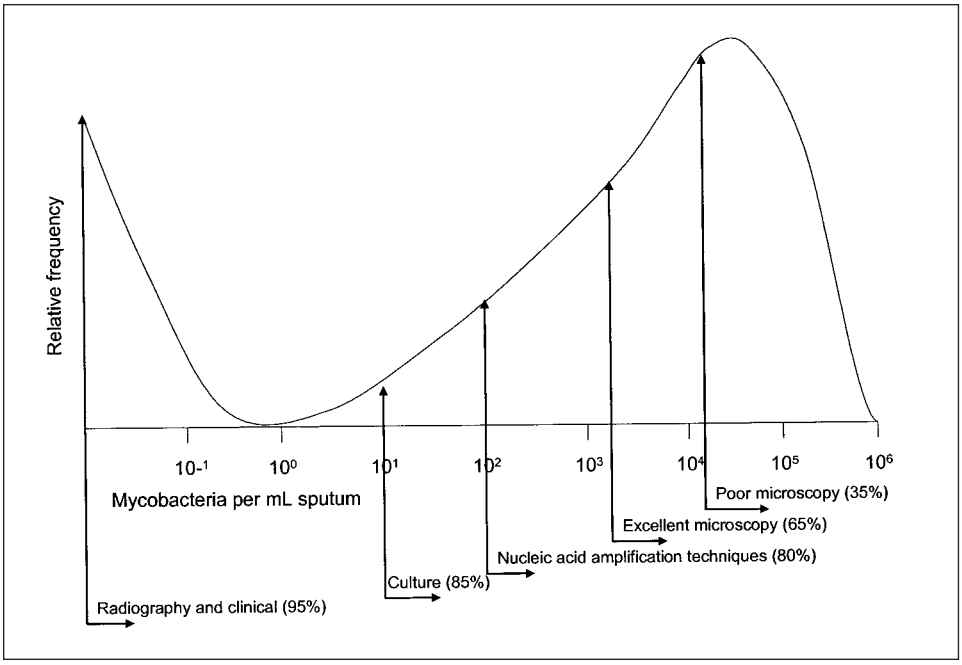

Figure I.1 Schematic presentation of the potential yield of different techniques in diagnosing pulmonary tuberculosis, by number of tubercle bacilli in sputum.

culture will not be that sensitive, as the technique is compromised by long specimen transport time, decontamination protocols, centrifugation and other necessary procedures. Losses in viability of tubercle bacilli are inevitable.

Nucleic acid amplification techniques may detect as few as 100 bacilli per millilitre of sputum. One advantage of the techniques is that living bacilli are not required, as dead bacilli still contain the nucleic acid target. To perform such tests successfully and reliably requires specialised infrastructure and equipment, highly trained staff that pay meticulous attention to detail, and substantially more financial resources than for microscopy and culture. Furthermore, a positive nucleic amplification technique result does not produce a culture that can be identified to species level and have drug susceptibility testing performed.

The technical limitation of sputum smear microscopy (with the recommended examination of up to 100 oil immersion fields) requires the presence of more than 1,000 bacilli per millilitre of sputum to have a 60% chance, and 10,000 or more to have a 95% chance, of identifying bacilli in a sputum smear examination. Even with poor microscopy, specimens containing 30,000 to 60,000 bacilli per millilitre sputum will not usually be missed. Relative to culture, sputum smear microscopy is insensitive in the diagnosis of pulmonary tuberculosis, while it is highly sensitive in identifying the most potent sources of transmission of tubercle bacilli, as determined by examination of contacts (Figure I.2). The public health purpose of sputum smear microscopy is to identify the greatest proportion of

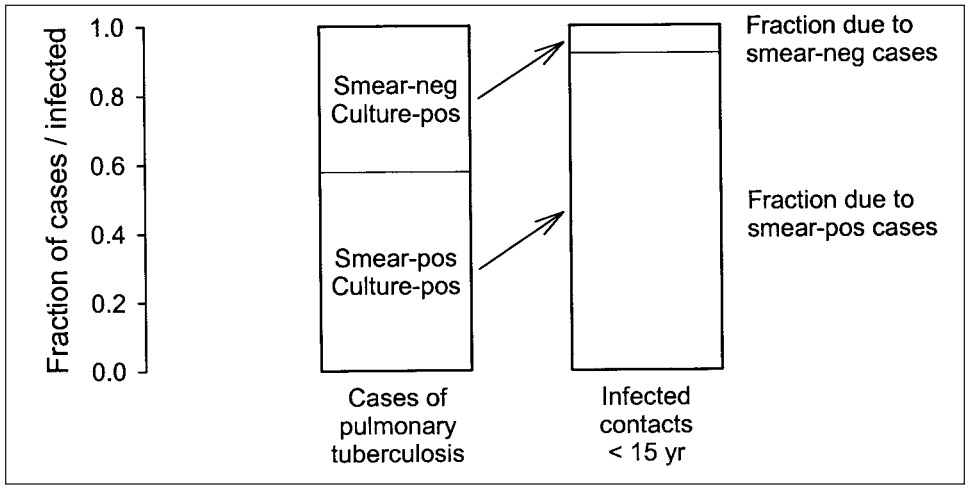

Figure I.2 Sensitivity of sputum smear microscopy in identifying culture-confirmed tuberculosis and transmitters of tubercle bacilli in the community (calculated from data in Grzybowski S, et al. Bull Int Union Tuberc 1975;50:90-106).

sources of transmission in the community and to ensure that the laboratory performs testing at the highest level of proficiency possible.

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

To maintain competence in mycobacteriology in the country, a minimum number of positive results must be obtained. Experience in collaborative programmes of The Union indicates that on average 15% of all new suspects examined will have sputum smear-positive tuberculosis, but the range in the prevalence of cases among suspects is wide, ranging 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. For each case, two smears would be positive (by definition) for diagnosis. At follow-up, one in ten cases would have positive smears at 2 months of treatment. This would be the equivalent of 2.1 positive smears per case. At a working capacity of 20 to 25 slides per day (see Section A above), a full-time job for smear microscopy would be equivalent to approximately 6,000 examinations in one year, including over 500 positive smears. Proficiency can still be maintained even if only one or two positive smears are identified per working week. However, there is no defined critical cut-off for a minimum of smear examinations for maintaining proficiency.

The national tuberculosis reference laboratory may run the risk of becoming overwhelmed with routine work and thus fail to complete its major responsibilities of quality assurance, training and supervision. Whenever the number of routine smear examinations exceeds 30 slides per day, fluorescence microscopy is recommended. While it is tempting to use only this method, it is not advisable to do so, as proficiency in the Ziehl-Neelsen method must be maintained. Whatever approach is chosen, the Ziehl-Neelsen technique must be in continuous use, even if the number of specimens per day is very large (in which case it will be used on a subset of the specimens). The services of the reference laboratory must be consistent with those provided in the general health services because training must be provided on an ongoing basis to laboratory technicians working at the peripheral health facilities. Such technicians must receive training that reflects the actual situation encountered in their daily work.

The national reference laboratory maintains its proficiency in the Ziehl-Neelsen technique by active participation in rechecking of slides in the national external quality assurance system, i.e., by assuring the second re-reading of discordant smears. If work overload is a problem, routine Ziehl-Neelsen examinations can then be handed over to health centres and hospitals in the area, so that the reference laboratory can concentrate on its priority tasks.

2. Training and quality assurance of sputum smear microscopy in peripheral laboratories

Quality assurance is a system that is designed to continuously improve the reliability and efficiency of laboratory services. It includes three components:

- *Quality control* (also called internal quality assurance): All means by which the tuberculosis smear microscopy laboratory controls operations, including instrument checks and checking new lots of staining solutions.
- *External quality assessment*: A process that allows participant laboratories to assess their capabilities by comparing their results with those in other laboratories in the network (intermediate and central laboratory) through panel testing and blinded re-checking. External quality assessment also includes on-site evaluation of the laboratory to review quality of performance, and should include on-site rereading of smears.
- *Quality improvement*: A process by which the components of smear microscopy diagnostic services are analysed with the aim of looking for ways to permanently remove obstacles to success. Data collection, data analysis, and creative problem-solving are the key components of this process. It involve continued monitoring, identifying defects, followed by remedial action, including retraining when needed, to prevent recurrence of problems. Quality improvement often relies on effective on-site evaluation visits.

Training of technicians in sputum smear microscopy must be relevant to daily work. The primary objective is to enhance proficiency of technicians in the technique they routinely use and, for this reason, theory is kept to a minimum; about two thirds of the training time (typically a five-day training course) should be reserved for practical work on microscopy.

It is the responsibility of a national tuberculosis reference laboratory to ensure that routine services of sputum smear microscopy in peripheral health facilities are provided in a standardised manner and at a high level of proficiency. This means that the head of the reference laboratory must elaborate an annual schedule of visits to the regions and to some peripheral

laboratories in the regions. These visits are preferably coordinated by, and carried out jointly with, the central team of the national tuberculosis programme. These full-day visits permit the supervision of daily work. While direct supervision of peripheral laboratories is of the utmost importance for quality assurance, more formal systems using external quality assessment of sputum smear microscopy must be established. The principal objective of such exercises is to maintain and continually improve microscopy services in the country.

To ensure nationwide coverage, active participation in external quality assessment by the intermediate level is critical as it will not be feasible, or desirable, for the national reference laboratories to be the direct link to all peripheral laboratories.

An external quality assessment scheme in a country can only be implemented correctly if the tasks are shared between the laboratory network and the national tuberculosis control programme. External quality assessment requires that peripheral laboratories are visited frequently to select and collect slides and to provide feedback on their performance. Without personnel from the national programme, an external quality assessment programme cannot be implemented programme-wide. Therefore, supervisory personnel from the national programme must be actively involved in the collection of slide samples and provision of feedback if such an external quality assessment programme is to succeed.

3. Surveillance of drug resistance

Drug resistance surveillance is among the key tasks of the national tuberculosis reference laboratory. Surveillance of drug resistance is a complex undertaking in any setting, and requires:

- comprehension of representative sampling and why it is critical if the results are to be meaningful;
- an infrastructure of the programme that allows close coordination between programme activities and the laboratory network;
- assurance that the specimens collected are of good quality and are transported in good condition;
- technical competence in performing specimen homogenisation, decontamination, inoculation, culture reading and identification;
- proficiency in drug susceptibility testing;
- a system of internal quality assurance and external quality assessment.

This monograph aims to promote collaboration between managers of the tuberculosis programme and laboratory specialists to obtain a valid assessment of resistance to anti-tuberculosis medications in their country.

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

In order to ensure that it is functioning properly, the national tuberculosis reference laboratory must be adequately staffed. For a small country, 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 maintenance (including autoclaving, glassware cleaning, and waste disposal)

Should the workload 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 programme to carry out the tasks essential for tuberculosis control in the country. In particular, the tasks of the reference laboratory must be clearly prioritised. 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 organising, coordinating and conducting all tasks at a very high level of competence.

The deputy head should be sufficiently qualified and competent to undertake the tasks of 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 seniority in the profession may be at a lower level.

Well-trained laboratory technicians knowledgeable in both bright-field and fluorescence microscopy are required 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 external quality assessment activities for sputum smear microscopy.

To ensure and maintain a high level of proficiency of standardised techniques for culture and drug susceptibility testing, two technicians must be available to assume these duties.

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

The rotation of personnel from the national tuberculosis reference laboratory to work in other national reference laboratories is rarely efficient and should be avoided.

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CHAPTER II

Sputum smear microscopy

When Robert Koch published his seminal paper in 1882 on the identification of the aetiological agent of tuberculosis, he presented methods for both staining and culturing. His staining method used a primary stain and a mordant (to facilitate diffusion of the stain into the cell through the lipid-rich cell wall), a decolourising agent, and a counterstain. These were required because the lipidity makes the cell wall hydrophobic and difficult for the stain to penetrate. Koch used methylene blue as the primary stain, and an alkaline potassium hydrate solution as the mordant. He skipped the decolourising agent and used vesuvium as both a decolourant and a counterstain. Ehrlich proposed (among other alternatives) the use of fuchsin as the primary stain, and used an alkaline aniline solution as the mordant. He introduced nitric acid as a decolourant and proposed a blue counterstain if the primary stain was red (as is the case with fuchsin). Ziehl challenged the use of the mordant and proposed phenol. Neelsen combined the best of all, proposing to use Ehrlich's approach to the primary stain, but using Neelsen's proposal of the mordant (phenol) and changing the decolourant from nitric acid to sulphuric acid, thus establishing the basis of the current Ziehl-Neelsen staining technique, which was born as early as 1882.

Sputum smear-positive patients are the most potent sources of transmission of *M. tuberculosis* 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, over two thirds of all adults with culture-confirmed pulmonary tuberculosis can be identified in this way in routine services in high-prevalence countries, but the proportion may be lower among patients co-infected with the human immunodeficiency virus (HIV), and is always substantially lower among young children. However, its sensitivity in identifying transmitters of tubercle bacilli is in excess of 80%, whatever the setting, as sputum smear-positive patients are much more likely to transmit *M. tuberculosis* than patients negative on sputum smear examination. Thus, sputum smear microscopy is one of the most effective and efficient tools for case finding in a national tuberculosis control programme: it identifies the cases that should have the highest priority in tuberculosis control. Moreover, sputum smear microscopy is highly specific in identifying acid-fast bacilli, which in high-prevalence countries are almost always diagnostic of tuberculosis.

Furthermore, it provides a very rapid diagnosis. It can be carried out by paramedical personnel using a multipurpose instrument at virtually any level of the health service of high-prevalence countries. For these reasons, it is the basic diagnostic tool in tuberculosis control.

A. Collection of specimens

The collection of sputum specimens for smear examination should be as efficient and as convenient as possible for both patients and laboratory workers. Numerous studies indicate that the incremental yield in detection of cases decreases with each succeeding specimen collected. Most countries have adopted the policy of collecting three specimens as an optimal means of identifying infectious cases of tuberculosis, following the recommendations of the WHO and The Union. Of patients ultimately positive on sputum smear examination, it has been reported that approximately 80% are positive on the first, another 15% on the second, and an additional 5% on the third specimen. However, several recent studies have suggested that the yield of the third specimen might be only in the range of 1% to 3%, thus constituting a high workload to identify the remaining additional cases. The number of slides required to identify one additional case depends on the product of the prevalence of cases among suspects and the incremental yield. For instance, if the prevalence of smear-positive cases is 10% among suspects and the incremental yield from the third specimen is 5%, then the number of slides that must be examined to find one additional case on a third consecutive examination following two negative ones is 1/(0.1*0.05) or 200 slides, i.e., approximately eight to ten full days of work. At an even lower yield of the third specimen, the amount of work is multiplied accordingly. Since the workload in smear microscopy laboratories has soared in countries with a high prevalence of HIV, adoption of a two-specimen policy for diagnostic examination has been considered appropriate in such settings.

The low sensitivity of sputum smear microscopy is being increasingly criticised, and more sensitive (but also more complex and labour-intensive) methods are proposed to be widely implemented. Countries must determine the best strategy depending on their setting, taking into account the results of an analysis of incremental yield:

– if adequate staffing or fluorescence microscopy permits accurate microscopy on large numbers of specimens, three (or even more) specimens should be examined to increase sensitivity. Where patients present early, this might be particularly rewarding, as specimens may be less uniformly rich in bacilli.
– where the yield of the third smear is low, examining series of two sputum specimens at intervals of one to two weeks will be more efficient for diagnosis (if symptoms persist despite the use of antimicrobials for

other common bacterial infections). It might also be the most sensitive strategy, particularly where a high workload results in poor quality.

The recommended collection of three sputum specimens (one at the time of the first consultation, the second produced in the early morning and the third at the time that the patient brings the second – the so-called 'spot-collection-spot' system) is a compromise between optimal yield, provided by the morning specimen, and convenience for the patient, provided by the on-the-spot specimens. The expected higher yield of three early morning specimens may not actually be achieved, since such a policy requires multiple (as many as four) visits by the patient. If one collects only morning specimens, the yield of the first smear can be as high as 95%, as the higher yield is explained by the fact that spot specimens are often of poor quality. Furthermore, excessive workload, as may sometimes be the case where HIV is highly prevalent among tuberculosis patients, carries the risk that only the first specimen is examined with sufficient care. Sputum from severely ill outpatients should always be collected on the spot and be examined immediately, while hospitalised patients who are suspected of having tuberculosis should have sputum collected on three consecutive mornings rather than by the less sensitive 'spot-collection-spot' method.

Current recommendations define a smear-positive case of pulmonary tuberculosis as a tuberculosis suspect with two positive sputum specimens. The principal argument for requiring a second (confirmatory) positive specimen has been to exclude false-positive results from laboratory errors caused by mixing up specimens. Little is known about the frequency of such errors in peripheral laboratories. However, the few published studies show that the frequency of false-positive results, including technical errors as well as misidentification, is in the range of only one to two per cent. An external quality assessment scheme that includes sampling of reported positive smears at the frequency they occur should identify such problems. Each such occurrence is of sufficient concern that it must be followed up, irrespective of statistical considerations.

The very high specificity of acid-fast bacilli for the *M. tuberculosis* complex in high-prevalence countries, compared to other standard diagnostic methods such as radiography or even culture (where cross-contamination is more frequent) is barely reduced by an occasional administrative error. A requirement that a positive result be confirmed by a second positive result may be more stringent than necessary, provided that correct technical execution is guaranteed (see Chapter III). In any event, initiation of treatment after a positive examination should not be delayed pending confirmation, as its purpose is not to guide treatment but to determine whether a case should be notified as "smear-positive" or as "smear-negative".

The quality of the sputum specimen submitted for examination is important. Patients must be encouraged to take their time to produce a specimen that comes from deep within the lungs. In spite of this, some studies show that even specimens that are predominantly saliva may demonstrate bacilli, albeit at a lower level. All specimens should therefore be accepted for examination, even if they look like saliva, if nothing better can be produced, e.g., by patients on treatment. It is a challenge to arrive at a definition of a specimen that is predominantly saliva that is both accurate and operationally feasible. To determine and record the quality of routine specimens in the laboratory, macroscopic classification is widely used. This may not be entirely accurate, but it is operationally acceptable. It classifies sputum specimens as "salivary", "mucous", "purulent", and "mucopurulent". In this classification, a specimen that is primarily saliva is characterised by its watery consistency, without any flecks of mucous or purulent material. The more accurate practice applied for sputum cytology (which may also be applicable to the Ziehl-Neelsen technique), is to count the number of leukocytes and epithelial cells per low power magnification field (approximately 100x magnification). The likelihood of a specimen containing mostly saliva increases if the ratio of leukocytes to epithelial cells drops below 1. However, as long as leukocytes are present, the chances of obtaining a positive result from smear or culture remain high.

B. Requesting a sputum smear examination

For microscopy of sputum specimens, the interval between collection and staining matters little. Consequently, specimens or smears can be sent from a collection point to a distant laboratory for examination without loss of accuracy. However, most countries have adopted the policy of sending the patient to the nearest microscopy centre, as the policy of sending the specimens or smears, although theoretically more convenient for the patient, is very difficult to carry out efficiently. The transport of fixed smears rather than sputum may be the easier of the two alternatives, but this requires additional investment in training and minor equipment for performing smears by health post staff. The logistics of transport remain significant problems and are, by and large, very hard to arrange in a reliable way in most poor countries.

The type of sputum container that is used is very important, and contributes to the quality of sputum collected. It should be of break-resistant transparent plastic, with a wide mouth, and a screw cap (with the screw threaded to one and a half times the circumference) to avoid leaks, desiccation and aerosol formation (Figure II.1). The container should have a capacity of at least 20 mL.

It is important that laboratories obtain essential information on the person whose sputum they need to examine. This is best accomplished by

using a request form for sputum smear examination (Appendix 1) which should be widely available and facilitates the work of both the health care worker submitting the request and the laboratory technician carrying out the examination. Health care workers must clearly indicate on the request form whether the examination is for diagnosis or bacteriological follow-up of treatment. This information must be recorded in the laboratory register, both for management purposes (to allow the calculation of laboratory material requirements) and for epidemiological evaluation. For follow-up examinations, the patient's tuberculosis registration number and the month into treatment should be indicated; the same information should be provided to the laboratory after results have arrived for newly registered patients, and can then be added to the laboratory register.

C. Preparing and staining sputum smears

A direct smear is made from the best part of the sputum, dried, fixed and stained using the Ziehl-Neelsen technique of staining for examination in ordinary light (bright-field) microscopy. Sputum can be concentrated before smearing, by digestion with chemicals such as strong alkali or sodium hypochlorite (bleach) followed by centrifugation, sedimentation, flotation or even filtration.

The original staining technique used "saturated fuchsin" (Ehrlich) or a carbol-fuchsin solution containing 0.75% basic fuchsin (Neelsen). Shortly

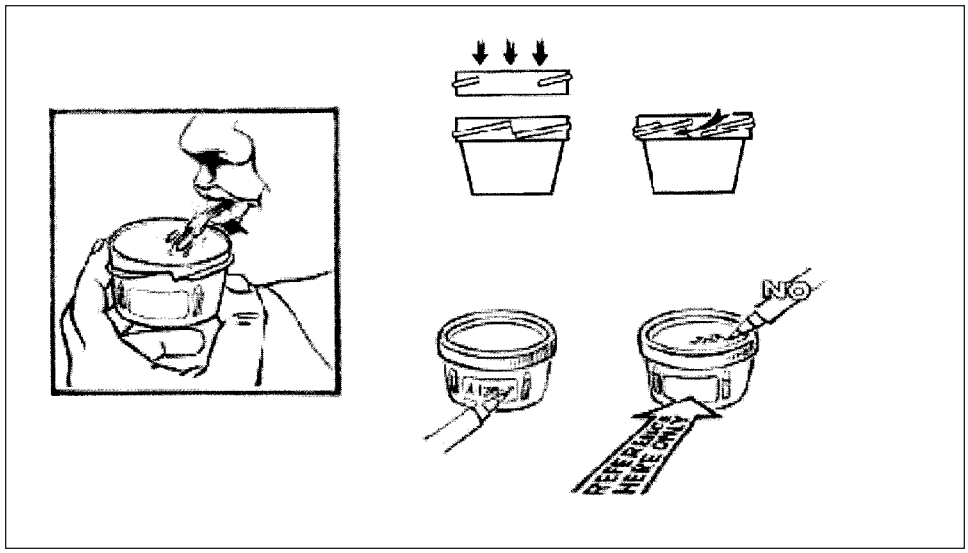

Figure II.1. Wide-mouthed, screw-cap, plastic sputum container for sputum collection in peripheral laboratories

after its development, Johne credited an oral communication by Neelsen for what became the established method: a solution with 1% fuchsin heated to steaming point, decolourised with 25% sulphuric acid, and counterstained with methylene blue. However, over time, many variations of these basic techniques have evolved and current practice uses 0.3% carbol-fuchsin. Stains other than basic fuchsin for bright-field microscopy have been used, but have never become popular; variations of the fuchsin technique, on the other hand, have. Cold staining methods using a saturated (3%) fuchsin stain are often referred to as Kinyoun staining, although its original description included a hypochlorite digestion/flotation step. The Gabbett modification combined the decolourising and counterstaining solutions; reducing the procedure to two steps results in a lower workload, which made the method popular particularly in countries with high labour costs. The Tan Thiam Hok staining technique combines the cold saturated primary stain with the Gabbett modification, and was also widely adopted. In a very thorough comparative study of various staining techniques with different concentrations of carbol-fuchsin, hot staining for 15 minutes using a 1% fuchsin stain produced the most consistent results with highest sensitivity. Decolourising with acid alcohol rather than sulphuric or other acids in water is now the rule in countries that can afford it, as it is easier to obtain a clean smear. In place of methylene blue, malachite green is sometimes routinely used. The disadvantage of the latter counterstain is the frequency of colour-blindness or -weakness among laboratory personnel (perhaps up to 10% of males have difficulties distinguishing red and green). Although comparative studies found picric acid to be the best counterstain, it is too difficult for routine use.

Bright-field microscopy has largely been replaced by fluorescence microscopy in low-prevalence countries where technician time costs far more than the instrument, and where its superiority for low positive specimens is important. The technique requires different staining solutions. Auramine O is virtually always used as the primary stain, alone or in combination with rhodamine for better differentiation. Several variations exist for the background of the smear in fluorescence microscopy: potassium permanganate is usually used to quench non-specific fluorescence, but methods without any counterstain or using acridine orange, thiazine red or methylene blue are standard practice in some countries. However, these counterstains may reduce the light/dark contrast too much. Recently, diluted blue ink has been found to be superior in the field, yielding good contrast without making the background so dark that maintaining focus is difficult (as is sometimes the case with permanganate). The addition of a little phenol satisfactorily reduces fluorescing artefacts (data courtesy, K Feldmann, Kuratorium Tuberkulose in der Welt, Gauting, Germany, 2 July 2004).

1. Preparing the direct smear

When preparing a smear from a sputum specimen, the technician must decide which part of the specimen to use. Sputum specimens are usually non-homogeneous except if left standing overnight (e.g., the first on-the-spot specimen), which may lead to partial autolysis. If the specimen contains obvious purulent parts, these should be selected. Thin disposable applicator sticks (made from bamboo or similar material) are preferable to wire loops, as they are better for picking up desired particles and provide a firmer tool for smearing. Moreover, such sticks are always destroyed after use, while wire loops have to be cleaned and disinfected before re-use, by rubbing off adherent sputum particles in a sand-alcohol flask (rarely available in laboratories in peripheral health facilities) and then flaming. The use of the sticks is clearly safer and wastes less time. The broken end of a bamboo stick is a fibrous brush of the right consistency. If held perpendicular to the slide it facilitates even spreading of the specimen over the slide. Sticks made of coconut palm leaves (broomsticks) are less fibrous, and wooden sticks tend to have a pointed end and are therefore less satisfactory.

Making a good smear is not as easy as it may seem and requires practice and patience. A good smear is neither too thick nor too thin, and is evenly spread, which may require prolonged smearing using circular movements. Smearing over the flame of a spirit lamp should be avoided as it will result in very uneven smears. Smears or areas of smears that are too thick will preclude proper reading of stained smears. Very thick smears also have a tendency to slough off the slide during staining because they are too difficult to dry and fix properly. As a rule, the thickness of a smear should be such that a newspaper can be read through the unstained smear if held under the slide, and the smear should be evenly spread on the slide. A well-stained smear shows a light blue colour from the methylene blue counterstain. If the smear is dark blue, it is too thick. The recommended size of the smear is 20 mm by 10 mm, because 20 mm in length corresponds to approximately 100 oil immersion fields. As the technician examining the smear should be able to concentrate on reading rather than on counting the fields, smears of such length are recommended.

Before heat fixation, the smear should be left to dry in air, not exposed to direct sunlight. Flame heating a wet smear is discouraged, as it can damage the acid-fast bacilli and may result in a cracked slide. In very humid climates, gentle heating on a glass pane mounted over an illuminated light bulb can be used to hasten the process.

Various devices such as slide holders and metal trays have been recommended for improved safety during smearing. They are not indispensable; readily available aids such as a newspaper or paper towel (changed daily) to cover the smearing area will serve the function just as well.

2. Concentrating the sputum

Concentration of sputum is a standard preparatory step for culture of *M. tuberculosis*, and the sediment from centrifugation is then usually examined microscopically. This procedure provides increased sensitivity. However, to be effective, powerful centrifuges are required, as acid-fast bacilli have a strong tendency to float. These are frequently not available in the microscopy units in low-income countries.

Other concentration techniques, based on flocculation followed by moderate centrifugation or flotation, have been used in the past, and have recently received renewed attention. Sputum concentration using sodium hypochlorite (ordinary household bleach) has been tried extensively, and results have been mostly encouraging. In this technique, sputum is mixed with an equal volume of commercial 5% bleach and left to liquefy for about 30 minutes. This is usually followed by dilution with water and a concentration step, although one recent report found additional positives in the sediment after 30 minutes, without concentration. Concentration is easy after bleach digestion of sputum, as a low centrifugation force is sufficient. Overnight sedimentation brings a more modest yield. This would make the technique applicable even in poorly equipped laboratories. The bleach technique kills tubercle bacilli, rendering the sputum safe but useless for culture. Other concentration techniques have been used after bleach digestion, such as flotation, where the tubercle bacilli come to the top of the liquid after shaking with organic solvents (i.e., petrol or chloroform), or filtration after treatment with alcohol.

Unfortunately, bleach concentration does not consistently produce a substantial improvement in sensitivity. In terms of specificity, there is a risk of false positive results because of carry-over of tubercle bacilli on re-used centrifugation tubes, or because of environmental mycobacteria that contaminate the water used for dilution. A few authors have reported little gain, or even a reduced yield of positives, from bleach concentrated smears, and the technique may not work for follow-up sputum specimens or thin watery specimens. It is likely that the sensitivity of the bleach concentration is mainly increased when the quality of direct microscopy is poor. With more highly positive specimens, or with an excellent direct Ziehl-Neelsen technique, the gain may be slight, and it becomes difficult to justify the additional workload or the increased risk of false positive results.

Bleach might improve the results of microscopy for acid-fast bacilli via several mechanisms. As suggested by the reported increase in positives simply upon standing for 30 minutes, the concentration effect may not be the most important component. Sputum digestion leads to homogenisation of the specimen, so that choosing the correct particle in the specimen is less important. The smear is easy to make and to read, because of its even

thickness and a background that cannot hide the acid-fast bacilli. As pre-treatment with strong oxidants makes it easier to stain tubercle bacilli, bleach may also compensate for the negative effect of a poor-quality carbol-fuchsin stain or a poor staining technique. It is obvious that a proper smearing and staining technique followed by careful reading has the same effect. In such a situation bleach concentration would increase the yield only from low-grade positive specimens, for example in settings with a high proportion of HIV co-infection among tuberculosis patients. So far, only one study has shown a greater yield for bleach treated specimens from HIV-positive compared to HIV-negative suspects. The technique should be further evaluated in settings with a high prevalence of HIV infection. The indication for when to use concentration techniques in peripheral smear microscopy laboratories remains unclear.

3. Staining acid-fast bacilli

Numerous methods have been developed for staining in microscopy for acid-fast bacilli, and not all of them may be suitable for use in a national laboratory network. Standardisation through the use of a single method throughout the country is crucial. The programme must insist on a single method and the appropriate formulation of stains for this method. The key decision for a programme with respect to technique for sputum smear examination is to select the one bright-field and the one fluorescence microscopy technique that will be standard throughout the system. Quality assurance is no longer feasible if each laboratory uses a different method. Ordering staining reagents becomes an outright impossibility, because different methods require different amounts/types of reagents. Moreover, mistakes will occur more frequently, e.g., due to the use of unsuitable stains meant for another technique. In the long run it is a good investment to train new laboratory personnel in a single technique and to convince those already trained to switch to the single recommended technique. Cold staining methods, even those using highly concentrated stains, such as the Kinyoun technique, are less reliable under field conditions. They tend to dete ct fewer positives unless the staining time is considerably prolonged. In practice, prolonged staining is often not carried out, with false negative results as a consequence. National tuberculosis programmes should therefore choose the hot staining method. In addition to staining time and heating, the concentration of fuchsin (and phenol) in the stain is a key factor in determining if acid-fast bacilli take a strong red colour, with a greater chance of detecting them. To obtain a sufficiently high concentration of fuchsin requires a high-quality fuchsin powder that is correctly dissolved. In addition, it is essential to ensure that the stain is not too old. These requirements are frequently not met under programme conditions. Furthermore, errors in the staining technique occur, especially in large, busy laboratories. For these reasons the authors prefer to use a

1% solution for 10 to 15 minutes, rather than the 0.3% for 5 minutes recommended in The Union and WHO guidelines. In their experience, this results in far better staining under routine conditions. Although a 0.3% concentration for 5 minutes can be shown to yield similar results in reference laboratories, it is too close to the minimum requirement, thus leaving little margin for error. Many programmes have used the original standard, the strong carbol-fuchsin 1% solution, with good results.

Filtration of the carbol-fuchsin stain is essential and is best done by pouring the stain on the slide through a funnel fitted with filter paper. Other techniques are no longer recommended. When the stain in a stock bottle reaches a low level, the remaining stain should be discarded as no filter paper will completely strain the mass of crystals it contains.

It is critical that the carbol-fuchsin covering the slide is heated until steam rises, and is kept at this state for at least five minutes. Repeated heating and longer staining time is not detrimental; on the contrary, they result in more strongly red acid-fast bacilli, provided the carbol-fuchsin does not dry out on the smear. To prevent drying, the slide should always be covered completely with the stain. To do this, staining racks are usually placed over a sink. In that case it is good practice to leave sufficient space (such as a finger-width) between the slides, to prevent the flow of solutions from one slide to the next.

4. Decolourising

Decolourisation is necessary to remove excess carbol-fuchsin from all but the acid-fast bacilli. This requires the use of strong acids. The addition of alcohol to the decolourising solution is not absolutely essential, but it makes it easier to obtain a clean smear. With some practice, and by wiping off the underside of the slide, satisfactory results can also be obtained using less expensive watery acids. Either sulphuric acid 20% to 25% in water or hydrochloric acid 3% in alcohol provide good decolourisation in most instances. It is almost impossible to decolourise acid-fast bacilli that have been well stained when using watery acids, so the decolourising time is not critical and the decolourising step can be repeated if too much red colour remains after a first attempt. Smears that are too thick cannot be decolourised properly. Acid alcohol has a stronger decolourising action than sulphuric acid and should not be used for too long. The choice between acid alcohol and watery acids depends mainly on local preferences and the availability and cost of alcohol, of which large quantities will be needed. There are other options for the choice of diluted acid, but their relative value needs to be carefully considered to make sure that practical advantages, such as cost and ease of use, are balanced by equivalent results.

5. Counterstain

The counterstain should give a good contrast and hide the remaining red of the background without hiding the acid-fast bacilli in thicker smears. It should give enough detail to enable the technician to keep the smear easily in focus while scanning, but not so much that the background diverts too much attention.

Methylene blue is not ideal in this respect. It gives a beautiful colour contrast, but it will hide acid-fast bacilli in thick smears when it is too concentrated or applied for too long. Consequently, some experts recommend a concentration of 0.1% (rather than the 0.3% recommended in Table II.1), and for not more than one minute. If this gives a result with too much red in the background, the smear is too thick. When these points are taken into consideration, it will be easy to detect a deficient smearing technique or under-decolourisation.

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 (but are not appropriate for the colour-blind), as it is less likely to conceal the red stained bacilli, even after prolonged counterstaining.

6. Rinsing water

The rinsing water must be clean, and, if re-staining is required for quality assessment, as free of environmental mycobacteria as possible. For this reason using rubber tubing on a tap to direct the jet is discouraged, as environmental mycobacteria from such a source may contaminate a smear. Clean water from a beaker that can be thoroughly cleaned should be used instead.

7. Preparation of stains for Ziehl-Neelsen

Programmes should pay considerable attention to the quality of fuchsin powder and the phenol they purchase, requesting a certified quality for both, while the other chemicals used for stains (acids, alcohol) can be of technical grade. Proper preparation of stains requires some equipment and a good technique. Procurement must be centralised at intermediate or central level, with distribution of ready-to-use stains to the microscopy centres. Minimum requirements for stain preparation are availability of pure, mycobacteria-free water (distilled or ultra-filtrated, not simply boiled or de-ionised), a measuring cylinder of appropriate capacity and some other glassware. Furthermore, the programme should ensure that precise instructions for making the stains are available at this level. A simple weighing scale with a sensitivity of 0.1 g is necessary. Phenol should be liquefied and measured by volume. If a 1% carbol-fuchsin stain is prepared, the dye content of the powder does not

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

Formula for 1 L of carbolfuchsin stain

Denatured ethanol or methanol ..... 100.0 mL

Phenol crystals ..... 50.0 g

* Basic fuchsin ..... 10.0 g

Water, distilled if possible ..... 850.0 mL

* In many recommendations, you may find 3.0 g basic fuchsin. The authors of this monograph prefer the stronger concentration to add a margin of safety.

Dissolve the phenol in the alcohol. Add the basic fuchsin and swirl the mixture until all crystals are dissolved. This may be difficult with some brands of fuchsin, in which case part of the water should be added followed by further swirling. Finally add the remaining water, mix again.

Transfer the prepared stain to a bottle (preferably dark) and label "Carbolfuchsin stain, date of preparation = ..."

Formula for 1 L of decolourising agent

Cold water, distilled if possible ..... 750.0 mL

Slowly add sulphuric acid (conc) ..... 250.0 mL

or

Ethanol 96% ..... 970.0 mL

Add hydrochloric acid ..... 30.0 mL

Formula for 1 L of counterstaining solution

Methylene blue ..... 3.0 g

Water, distilled if possible ..... 1000.0 mL

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

1) Cover the whole surface of the slides with Ziehl's carbol fuchsin, pouring the stain through a funnel with filter paper (e.g., Whatman no. 1). Heat gently until vapour rises. Do not bring the stain to boil or allow it to dry on the slide. Leave the warm stain for at least 5 minutes.

2) Rinse each slide gently with clean water until all macroscopically visible stain has been washed away. Cover each slide with the decolourising solution for 3-5 minutes and rinse with clean water. Tilt the slides using forceps to drain off rinsing water. Repeat this step once for smears that still look red. Tilt the slides using forceps to drain off rinsing water.

3) Flood smears with methylene blue solution and leave the stain for not more than 1 minute. Rinse each slide gently with clean water until all excess stain has been washed away, let the water drain off using forceps and place the slides on a drying rack. Allow to dry in open air but out of direct sunlight.

need to be taken into account, and the quantities used do not need to be so precise. The mode of preparation is much more important, especially for the carbol-fuchsin stain, since fuchsin solubility varies with its composition.

The laboratory preparing the staining solutions must also take responsibility for proper identification and quality control of the stains prior to distribution. As a minimum, the type of the stain and the date of preparation should be written on the label of each bottle at the time of preparation. The same will be used as a lot reference, with notes on its quality control kept in a stain logbook. Quality control of stains requires testing them on a few fixed but unstained smears, including known positives as well as negatives. The positives are best of a 1+ grade and should be subjected to a single staining cycle with subsequent appraisal of the number and intensity of staining of the acid-fast bacilli visualised. Fuchsin brands with an absorption maximum over 550 nm give strong red solidly stained acid-fast bacilli (particularly with a 1% fuchsin/5% phenol stain and sufficiently long staining time). Negative controls demonstrate absence of contaminants. For acids and methylene blue, they require a repeated staining cycle before microscopic examination. Freshly prepared stains do not need to be filtred if filtration is practised during carbol-fuchsin staining as described above. Stains should be stored out of direct sunlight, either in a dark bottle or in a closed cabinet. The shelf-life of the carbol-fuchsin stain varies according to the quality of reagents, their preparation, concentration and storage conditions. For this reason, stains should be prepared in quantities that will be consumed within twelve months. The shelf-life of acid and of methylene blue is not a problem.

8. Fluorescence microscopy with auramine O

If the number of daily specimens to be examined exceeds 30 per technician, and if electricity is continuously available, fluorescence microscopy is indicated. Additional requirements in training and other resources (capital investment and maintenance) must be taken into account before introducing fluorescence microscopy. Furthermore, mercury vapour lamps need to be replaced frequently (100 to 200 hours working time). They are frequently not kept in stock because they have to be imported and are very expensive. Consequently, they are often used longer than recommended and the intensity of the light they give may be inadequate. Such lamps may shatter, and may cause severe damage to the microscope. Adjustment of the lamps should be made only by someone who has experience; otherwise a faulty fluorescence microscope may go unrecognised.

The main advantage of fluorescence staining is that slides can be examined at a lower magnification, allowing the examination of a much larger area per unit of time. Fluorescence microscopy requires only two minutes to examine an area that requires ten minutes with bright-field

microscopy. Its main disadvantage is its cost: a fluorescence microscope costs at least five times as much as a bright-field microscope. The difference in running costs of fluorescence microscopy versus bright-field microscopy is mainly determined by the salaries of the technicians: they become increasingly efficient with increasing labour cost.

Fluorescence staining utilises the same approach as Ziehl-Neelsen staining, but carbol-fuchsin is replaced by a fluorescent dye, the acid for decolourisation is gentler, and the counterstain, although useful for quenching background fluorescence, is not essential (Table II.2). From a very large study of 23,000 specimens at the Statens Serum Institut in Copenhagen, fluorescence microscopy had a slightly better sensitivity and the same specificity as Ziehl-Neelsen staining, in detection of specimens with a positive culture. In practice this may not always be the case. More extensive training is required for fluorescence microscopy to attain the same specificity as with Ziehl-Neelsen, because of artefacts that are frequently present, including some that may superficially resemble acid-fast bacilli. On the other hand, the contrast is better, more fields can be screened, and the technician is less fatigued, often resulting in a higher sensitivity especially when the workload is high or the smears contain few acid-fast bacilli.

The national tuberculosis reference laboratory must be proficient in both Ziehl-Neelsen and fluorescence microscopy. The original staining technique with auramine O is recommended for fluorescence microscopy. With auramine O, acid-fast bacilli appear bright yellow against a dark background, and can be seen quite well at a magnification as low as 200x to 250x (objective 20x to 25x and oculars 10x). If there is uncertainty about the presence of a bacillus because of the lower magnification, a higher power objective (40x) can be used for confirmation of the suspected bacillus. Alternatively, auramine O stained slides containing rare acid-fast bacilli may be re-stained and confirmed by the Ziehl-Neelsen technique. The latter approach to confirm suspected bacilli has certain problems. It may have poor reproducibility after restaining because rare acid-fast bacilli may be washed away. This negates the increased sensitivity of fluorescence microscopy.

Grading of the density of acid-fast bacilli on a slide is not the same as with light microscopy because of the lower magnification and the resultant larger field of examination. 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, while this is ten to twelve times larger again at 200x to 250x magnification. Grading in this case must be adjusted according to the magnification used (see Table II.3 for a comparative grading with different magnifications).

Table II.2. Materials and reagents used 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, with a modification by Kuratorium Tuberkulose in der Welt, Gauting, Germany.

Auramine staining solution

Auramine O ..... 1.0 g
Ethanol ..... 100.0 mL
Phenol liquid ..... 30.0 mL
Distilled water ..... 870.0 mL

Dissolve the auramine O powder in ethanol, phenol liquid in water and mix both solutions. The working solution must be kept in dark bottles.

Liquid phenol is usually prepared as a stock solution by dissolving 9 parts (weight) of phenol crystals in 1 part (weight or volume) water by warming up. This hot solution will stay liquid at room temperature (melting point of phenol is 43°C, phenol with 6% water will melt at 20°C).

Decolourising solution

Hydrochloric acid (conc.) ..... 10.0 mL
Ethanol 95% or methanol ..... 990.0 mL

Always add hydrochloric acid to water **(do not add water to acid)**.

Background quenching solution

Potassium permanganate ..... 0.5 g
Distilled water ..... 100.0 mL

Alternative background staining and quenching solution

Ink blue ..... 100.0 mL
Distilled water ..... 900.0 mL
Phenol liquid ..... 5.0 mL

This background stain is better when permanganate stains the smear too dark, thus making focusing and maintaining focus very difficult. This may depend on the microscope system used.

Staining procedure

Place the slides on the staining rack over a sink, leaving some distance between individual slides

1) Pour freshly filtered auramine solution over the slides so that the smears are completely covered
2) Leave for 20 minutes
3) Cover the slides well with acid solution
4) Allow to act for 2 minutes
5) Rinse the slides with clean water until all macroscopically visible stain has been washed away
6) Flood smear with potassium permanganate or ink blue solution for 1 minute
7) Rinse with clean water
8) Stand the slide on edge to drain

The stained smear should show a light brown or blue colour (the latter from blue ink). If the colour is dark, it usually indicates that it is too thick.

Table II.3. Grading scales for bright field (Ziehl-Neelsen) and fluorescence microscopy

Union / WHO scale 1000x field=HPF Result	Bright field (1000x magnification; 1 length=2cm= 100 HPF)	Fluorescence (200-250x magnification; 1 length=30 fields=300 HPF)	Fluorescence (400x magnification; 1 length=40 fields= 200 HPF)
Negative	Zero AFB / 1 length	Zero AFB / 1 length	Zero AFB / 1 length
Scanty	1-9 AFB / 1 length or 100 HPF	1-29 AFB / 1 length	1-19 AFB / 1 length
1+	10-99 AFB / 1 length or 100 HPF	30-299 AFB / 1 length	20-199 AFB / 1 length
2+	1-10 AFB / 1 HPF on average	10-100 AFB / 1 field	5-50 AFB / 1 field
3+	> 10 AFB / 1 HPF on average	>100 AFB / 1 field	>50 AFB / 1 field

HPF = high-power field; AFB = acid-fast bacilli.

D. Examination of sputum smears

For examining the prepared smears, a binocular microscope with an oil immersion objective (magnification 100x) and eyepieces of moderate magnification (8x to 10x) should be used. In countries with an irregular supply of electricity, it is best to use microscopes with both electric and direct light sources. When using a mirror, a well-lit area should be chosen and the blue filter removed for better luminosity of the field.

Before examining the slide, one drop of immersion oil is 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 risk transferring acid-fast bacilli to the oil flask and 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, may have gaps, or may even appear granular. They occur singly or in small groups, and rarely in large clumps. The typical appearance of tubercle bacilli is of slender, slightly curved rods. If structures are seen that have the correct colour and a compatible morphology, they should be considered acid-fast bacilli and be reported as such. Environmental mycobacteria and some species of the genera *Nocardia* and *Corynebacterium* may have similar appearance. No attempt should be made to differentiate these microscopically, since it is very easy to make a mistake. *Nocardia* may show a sufficiently different morphology, e.g., branching filaments. Some bacterial and fungal spores may be acid-fast, but they can be distinguished easily from tubercle bacilli.

Several grading scales for the number of acid-fast bacilli found in a smear have been proposed over the years. The Union scale proposes five groups for reporting the results from reading a smear, and 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	+++

As a rule, it is sufficient to examine one length of a 20 mm long smear (corresponding to 100 fields examined at 1000x magnification) to report a negative result. However, if the scanning of the 20 mm length is carried out by fluorescence microscopy at 400x or 200x magnification, the area to be examined must be adjusted accordingly. If done carefully, the examination of 100 high-power fields is sufficient, as very few additional positives are detected by prolonging the search. Routinely doing so will result in fatigue that may more than offset any potential gain of checking a larger smear area.

Very scanty results (one to three bacilli per 100 fields) do not correlate well with culture. In such a case, examination of an additional specimen from the patient should be undertaken for confirmation of the result. On the other hand, for the results of four to nine bacilli per 100 fields, correlation with culture is good. In a well-functioning laboratory network such specimens are virtually always confirmed when another specimen is examined. Whether or not a finding of scanty bacilli is to be considered significant must be determined by the policy of the national programme and not by the individual laboratory technician. Such a result must be recorded as read and should never be changed after it has been recorded in the register or on the reporting form.

E. Recording and reporting results of sputum smear examinations

A specific register for tuberculosis laboratory examination has proved very useful (Appendix 2). Two essential features of the laboratory register recommended by The Union include the distinction made between tuberculosis suspects presenting for a diagnostic examination and

tuberculosis patients presenting for a bacteriological follow-up examination, and the single line allotted to each individual examined (rather than a single line for each examination). This facilitates the calculation of the proportion of cases among suspects, which in turn allows the calculation of requirements for laboratory materials based on the number of reported smear-positive cases in the quarterly case finding report. Moreover, the distinction of follow-up and suspect examinations and the clear presentation of results of the diagnostic series make it much easier to study various indicators of quality of the examinations, as described further on.

Results recorded in the laboratory register may not be reliable. Frequently, results of the only specimen examined are copied to the columns for the second and the third specimen. Sometimes all follow-up examinations are recorded as negative (an indication that due attention is not given to carefully examining such specimens, under the assumption that they should all be negative). In other instances, scanty results are absent or infrequent (again, indicating insufficient care in examination). Poor practice of this kind is bad for patient management, makes evaluation of programme performance difficult and renders quality assessment in the laboratory of no value. Fictitious recording may be the result of an exaggerated emphasis on programme guidelines and targets. For example, programme supervisors who insist on three smears for each and every suspect or on impossibly high two-month conversion proportions may encourage falsification. The laboratory technician should report and record what was observed, and supervisors should respect accurate and true recording as an important indicator of quality in the laboratory.

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CHAPTER III

Training and quality assurance in sputum smear microscopy

A. Training of technicians in sputum smear microscopy

One of the essential tasks of the national tuberculosis reference laboratory is to standardise the training of laboratory technicians. An overall plan of training needs should be developed taking account of staff turnover and the needs for programme development in the country. Overall responsibility for the level and frequency of training of laboratory technicians rests with the national tuberculosis reference laboratory. It should also regularly provide technicians with on-the-job training under the direct supervision of experienced laboratory technicians. It should engage intermediate level laboratories in the development of a standardised curriculum in training laboratory technicians, and in training and retraining activities, to allow progressive decentralisation of such activities. This includes a written text for the training curriculum and a manual of standard operating procedures for laboratory technicians.

Acid-fast microscopy is not difficult to learn. The challenge lies with maintaining high quality staining and reading. Thus, only a few hours in training need to be devoted to theory. More importantly, this must be followed by up to one week of practice, best followed by in-service supervision of work with systematic on-the-spot confirmation of positive findings. The ability to correctly identify negative results is checked by quality assurance sampling. To ensure that sufficient time is devoted to the practice of preparing and reading smears, targets might be set, such as requiring that 100 positive smears with various gradings and an equal number of negative slides have been read by the trainee.

The training content needs to include the comprehensive principles of the national tuberculosis programme and the DOTS strategy in order to assist the trainees to fully understand it and to enhance their motivation for the tuberculosis control programme and their role within it.

The trainee must acquire comprehensive skills and knowledge of the following technical aspects of acid-fast bacilli smear microscopy through lectures and extensive practice:

- Sputum collection, storage and transport for acid-fast bacilli microscopy and culture
- Smear preparation with mucopurulent particles of sputum
- Fixation, staining, destaining and counterstaining
- Proper use of the microscope and slide reading
- Reporting of results to clinics and recording the data in the tuberculosis laboratory registry
- Maintenance (and minor repair) of the microscope
- Storage of the slides for external quality assessment
- Disinfection, sterilisation and disposal of contaminated material
- Safety measures and practices in handling sputum specimens for microscopy
- Identification of problems occurring during sputum smear microscopy.

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

B. Quality assurance of sputum smear microscopy

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

- *Quality Control (QC)*: systematic internal monitoring of working practices, technical procedures, equipment and materials, including quality of staining solutions.
- *External Quality Assessment (EQA)*: a process to assess laboratory performance by an outside agency. EQA includes on-site evaluation of the laboratory to review QC, with on-site rereading of a few recent smears. EQA also allows the identification of problem laboratories by comparing the results with those obtained in other laboratories in the network (intermediate and central laboratory), through panel testing and rechecking.
- *Quality improvement (QI)*: a process by which the components of smear microscopy diagnostic services are analysed with the aim of looking for ways to permanently remove obstacles to success. Data collection, data analysis and creative problem solving are the key components of this process. It involves systematic monitoring, identification of defects, followed by remedial action to prevent recurrence of problems.

Problem-solving is done most efficiently during on-site supervisory visits.

1. Quality control of sputum smear microscopy

Maintenance of technical proficiency

To develop and maintain proficiency in the performance of sputum smear microscopy and its interpretation, a peripheral laboratory needs to cover a population of 50,000 to 150,000, depending on the incidence of tuberculosis suspects in the population and the use of health services. This can be done successfully, provided that continuous monitoring of quality allows early identification of laboratories with excessive errors.

Staining

Ideally, internal quality control includes monitoring of stain quality and staining procedure by including at least one slide known to be positive for acid-fast bacilli in each routine series of smears. Positive control smears will also help personnel to differentiate between acid-fast bacilli and artefacts. If possible, a second person should double-check all smears declared to be positive.

As a minimum requirement, each new batch of staining solution must be checked before it is dispensed or used, and records must be kept (date of preparation serving also as batch number, destination, results of control). Three positive and three negative smears should be used for such a control on each new batch of staining solutions. Positive controls should be 1+ positive smears, allowing precise counts of the acid-fast bacilli. Distilled water should be used for rinsing. This avoids contaminants (environmental mycobacteria) in the water and increases the likelihood that any abnormalities that are detected in negative control smears are due to defective (contaminated) stains. Negative control smears must undergo three full staining cycles before examination, to increase the probability that contaminants that might be present will stick to the smear and stain acid-fast, thus identifying defective (contaminated) stains.

Internal monitoring of case detection

A relatively simple tool for identifying the yield of laboratory examination is to plot the monthly proportion of positive cases among suspects (Figure III.1). Such a graph provides an immediate visual aid for all concerned—the technician, clinicians and the tuberculosis management team—even without estimating the standard deviation derived from an annual sample. 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 critical value (which might be defined as two standard errors above the annual mean), this might

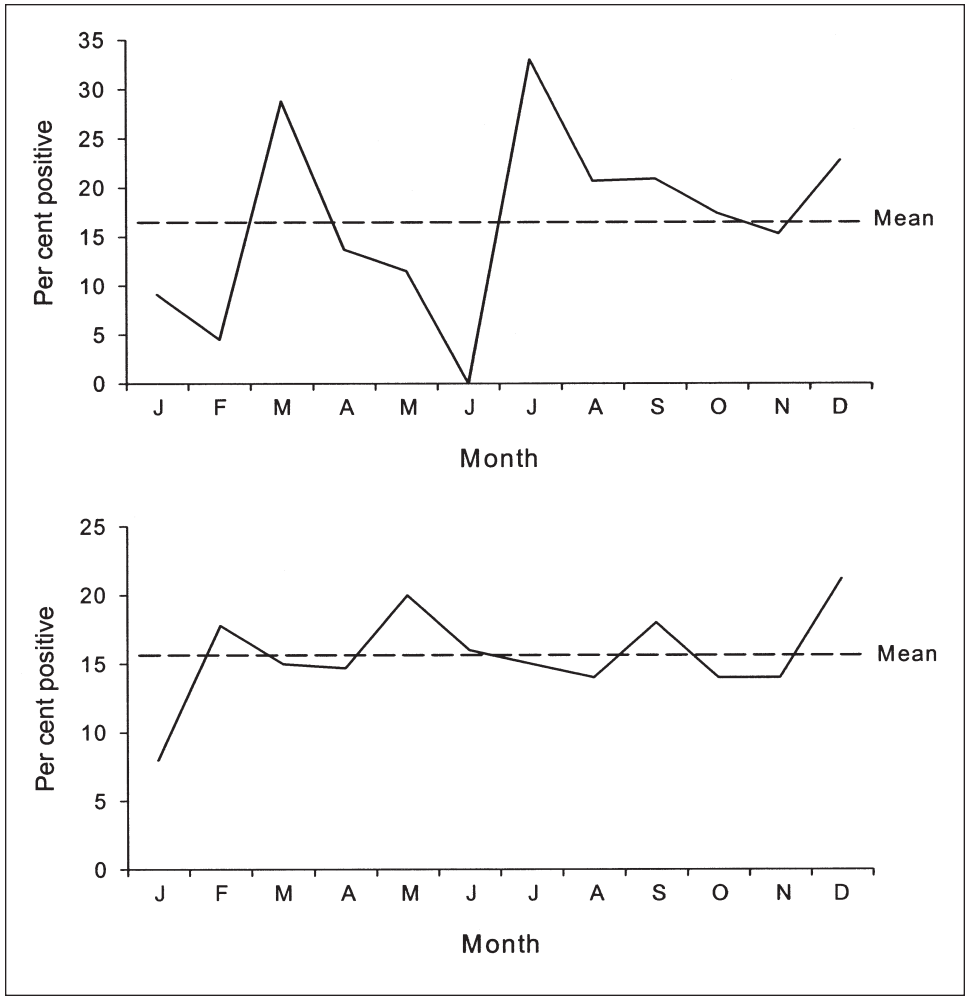

Figure III.1 Example of the frequency of cases among tuberculosis suspects, by month of examination in the laboratory. Data courtesy of Damien Foundation Project Bangladesh.

indicate an unacceptably restrictive policy for submitting specimens or impediments to patients' access to the health care facility. It may indicate that the laboratory checks specimens more carefully or reports acid-fast bacilli erroneously. On the other hand, if the frequency of cases among suspects falls below a certain critical value (which might be defined as two standard errors below the annual mean), clinicians may submit specimens from patients who do not qualify as tuberculosis suspects. Alternatively, the stain might have deteriorated, or the laboratory might have lessened its diligence in examining sputum smears and misses true cases.

2. External Quality Assessment (EQA) of sputum smear microscopy

There are three principal methods in EQA of smear microscopy results:

- Panel testing (centre to periphery)
- Monitoring the quality of sputum smear microscopy during supervisory visits in the field
- Blinded rechecking of slides (periphery to centre).

All three methods have distinct advantages and disadvantages, and consequently it is advisable to develop several methods in parallel, specifying the objectives of each (Table III.1). A detailed account of the indications, requirements and technical conduct for each of these methods can be found in reference 2.

In addition to these EQA methods, some aspects of external monitoring of laboratory performance are possible by means of regular reporting to the higher level. Although such reporting has not been general practice, it can be useful in addition to EQA, or in providing some basic information when efficient EQA (described later) is not (yet) possible. A recommended simple format that allows analysis of workload and some of the main quality indicators, besides providing information on essential supplies, is shown in Appendix 3. For maximum efficiency, this should be completed and sent by the laboratory technician, so that the national tuberculosis programme will have information on the performance of smear microscopy in the laboratory. Moreover, completing this report will simultaneously provide internal monitoring and facilitate random sampling for rechecking, simplifying the work of supervisors.

EQA by sending a panel of slides from the centre to the periphery

The simplest way of assessing the performance of peripheral laboratories is to conduct periodic panel testing of target laboratories. This is done by periodically collecting slides of good quality and keeping them in seven slide holding boxes. The boxes are kept in a cool, 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-monthly intervals, sets of six slides (three negative, one scanty, one 1+, and one 2+) are sent simultaneously to all target laboratories with a request to return the completed form with the recorded results of the labelled slides (Appendix 4) in an enclosed, pre-stamped, self-addressed return envelope.

This method does not address the quality of staining and it leaves some important gaps. The original negative results are not beyond doubt, and positives may have faded despite all precautions, so that it is necessary

to recheck slides for which errors were reported at the reference laboratory. For this reason, and to assess staining capacity, sending out a combination of stained and especially prepared unstained smears of known result is a more useful approach.

This, however, requires more sophisticated preparation by the reference laboratory using homogenised sputum specimens, with thorough validation of the batches manufactured, as described in reference 2.

The advantage of panel testing is the ability to efficiently obtain from both reference and peripheral laboratories a quick assessment of the technical ability to read smears in the target area. If important discrepancies

Table III.1. External quality assessment (EQA) in sputum smear microscopy

Method	Advantages	Disadvantages	Use
On-site supervision	• Direct contact • Observation of actual work • Motivating for staff • Identifying causes of errors • Permits verification of equipment and supplies	• Selective, not country-wide, if left solely to reference laboratory • Labour intensive • Costly	• Always during supervisory visits
Blinded rechecking (periphery to centre)	• Real life • Can be motivating • Low workload for periphery	• Heavy workload for centre • Unavoidable inaccuracies • Requires very careful technique to arrive at correct results • Personnel must be made available	• Standard for surveillance
Panel testing (centre to periphery)	• Low workload for central • Rapid response country-wide • May lead indirectly to identification of faults in equipment	• Ability testing, not necessarily routine performance • Good panels not easy to make	• After training • Rapid assessment of training and equipment needs • Investigation / confirmation of problems suspected from rechecking

from expected results are observed, the problem may not always lie with the capability of the examinee, but may indicate poor quality equipment (especially the microscope) or an insufficient light source. The method is routinely used after the training of new technicians or following refresher courses to assess optimal performance. It is the only reasonable method for EQA in affluent low-prevalence countries, where rechecking of routine smears becomes prohibitively expensive.

The major disadvantage of the method lies in the fact that technicians have unlimited time for the examination of control slides (unless the test is administered during supervision or in a training course), and that they are aware of being tested. It certainly does not provide an assessment of the quality of slide reading under routine conditions; it can only measure the ability of technicians to read (and stain) slides correctly. Despite these limitations, when well organised, it has been found to result in a remarkable improvement in quality.

The overall results can be tabulated at three levels:

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

Analysis by individual slide will allow exclusion of particular batch-slides for which too many participants reported aberrant results, due to lack of consistency of the batch produced. In the example shown in Table III.2, the performance of technicians was rather poor, as only half of them were capable of correctly identifying all positive and negative slides in the set of six. Such a large deviation from the expected result raises serious questions not about the capability of technicians, but about the quality of their equipment. It identifies the laboratories that need an urgent supervisory visit.

The evaluation of individual technicians requires a more elaborate scoring system, in which each slide is allotted an equal number of marks. If a serious error is reported, no marks are given, while a low-grade error will earn half of the marks. Total marks earned are then compared with a minimum target score, which can be pre-defined or derived from the results obtained (see reference 2).

A panel of stained and/or unstained slides can also be used to evaluate the reading accuracy or staining and reading skill of trainees before and after training.

Table III.2. Example of an analysis of results of panel tests using 3 negative and 3 variously graded positive slides sent from the reference laboratory to 42 laboratories. Analysis by slide and sets

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

Analysis by slide

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
Analysis by set of positive and negative slides	
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
Analysis of entire set of six slides	
Number correctly reading the set of six slides:	21 of 42
Fraction correctly reading the set of six slides:	0.500

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

Visits to peripheral laboratories by reference laboratory personnel are essential for several reasons. Only the field visit can convey a realistic picture of the conditions under which the technicians have to work. They allow the general layout of the laboratory, the condition of the equipment and the adequacy of supply logistics to be reviewed. Because it is the technician's home turf, concerns and problems encountered in the operation of the laboratory might be expressed more freely. However, in most countries the national reference laboratory can only visit a very limited number of peripheral laboratories. Routine visiting of the peripheral laboratories is mainly the task of the intermediate laboratories.

Ideally, a visit should be planned to allow observation of the work performed during one entire day. This includes collecting and receiving specimens, staining, examining, reporting and recording. Only such a full-day visit will allow an in-depth discussion of any observed deviations from recommended procedures. However, if a full day cannot be spent on a single laboratory, a supervisory visit is still useful to enquire about problems, to take a sample or give feedback for rechecking, to confirm the presence of sufficient supplies and to motivate the technicians by making them feel part of the tuberculosis programme. It is obvious that such a short visit can be carried out, and preferably so, by non-laboratory professionals, i.e., by tuberculosis programme supervisors. They must learn the principles of supervision of some aspects of acid-fast smear microscopy, such as registration and sampling for rechecking. However, solving observed problems will usually require a visit by a laboratory professional.

The issues to be addressed during a supervision visit are summarised under four headings:

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

Detailed checklists covering these areas are presented in reference 2. These can be used by general supervisors.

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 strenuous to do it day after day. For these reasons, it is not unusual to find that the performance of less qualified staff, such as laboratory auxiliaries, is better than that of highly qualified technicians, who are more interested in technically complex tests.

The requirements and management of laboratory personnel vary according to location. Most of the diagnostic work in the national tuberculosis programme in many low-income countries will take place in predominantly rural locations. In such settings, the work required for tuberculosis control is carried out by a multipurpose laboratory technician, only one of whose responsibilities has to do with tuberculosis.

Tuberculosis-related work is performed increasingly in urban settings where the workload may be very large 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. In

such a setting, 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 everything possible should be done subsequently to remedy overburdening the technicians. An average of over 30 slides per technician per working day means that on some days many more than 30 slides 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. Where the workload is excessive and the means and infrastructure permit (usually only in regional or large hospital laboratories), it is best to provide a fluorescence microscope, which will immediately increase capacity by about five-fold. After a careful introduction period, results will probably be more reliable compared to employing five technicians, each with an ordinary microscope (which will often not be feasible anyway).

Infrastructure

Visits are meant to assess if the facilities are adequate to permit good work performance. If visits are regular, infrastructure will not have to be checked each time unless major changes have occurred since the last visit. The room should offer sufficient space to make a division into at least two areas (one for registration and microscopy, one for handling sputum and smears) and have a provision for safe disposal of waste. The set-up should allow easy movement of all the persons working there. Good ventilation is essential for safety. Sufficient natural light will also improve protection of those working in the laboratory, apart from making microscopy possible when there is no electricity. A reliable supply of running water, good drainage and uninterrupted electricity are optimal, but are not always available despite all possible efforts. The tasks can be performed satisfactorily without running water and electricity if at least clean water can be obtained from a nearby well, and if microscope and light conditions permit slide examination without electricity.

Safety in the peripheral laboratory

Safety can be assessed 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 collecting a sputum specimen from the patient: this must always be done in the open air. Obtaining specimens by induction of sputum or by bronchoscopy requires particularly rigorous precautions to be taken.

Laboratory coats are a small cost in most countries, and there should be no problem in providing them. Although disposable applicators (the best

is bamboo) 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 investigated. Such material should certainly not be simply thrown into a shallow pit or dumped in a corner of the compound. Although relatively short-lived, a simple kind of burning drum may be the most appropriate device. Soaking or boiling in disinfectant would be easiest under most circumstances, but may be abandoned soon because of its unpleasantness. Autoclaving is only rarely possible. The presence of the necessary equipment should be confirmed along with evidence of recent use.

Safety cabinets are not recommended for smear microscopy work. They are not required because this work carries only a low risk for laboratory technicians, provided that good ventilation of the laboratory is assured and that standard safety procedures are followed. Commercial biological safety cabinets need to be checked and maintained regularly by certified technicians, which is often impossible. Neglecting such maintenance may then result in faulty operation of the cabinet and an increased risk of infection for the technicians. Locally made cabinets are often so poorly constructed that they are useless or even dangerous. If there is a serious concern, for instance because of insufficient ventilation of the laboratory space, a continuously operated extraction fan mounted in a wall may be the best solution. Ultra-violet lamps are much less effective than good ventilation, not only because of poor installation, replacement and maintenance, but also because of the limited protection they confer. They can only be used after the work has been done, or as shielded lamps, so that the air surrounding the technician at work is not directly sterilised.

For tuberculosis work with sputum, the use of gloves is discouraged (except for preparation of staining solutions). They make little sense, considering the transmission route, they are invariably changed too infrequently, resulting in contamination being carried around the laboratory, and the amount of money that has to be spent on good quality gloves is generally prohibitive. Surgical masks do not protect the laboratory worker, and respirators that reliably filter out particles of < 5 micrometres are expensive and rarely of any value in microscopy laboratories that are ventilated as recommended.

Equipment

A sufficient number of tables and chairs, a (plastic) basin for staining, and a large bucket with a lid for infectious waste must be available (see also Table V.1). It is the responsibility of the supervisor to obtain them for the laboratory. A candle-type water filter is the minimum requirement if staining solutions are to be prepared at the centre and no distilled water

is available. Some equipment for incineration or disinfection of infected waste should be available in or near the laboratory.

A good microscope is absolutely essential for reliable results. The chosen microscope must be robust and have high-quality optics. Every effort must be made by programme officials to obtain high-quality microscopes. They must ensure with those responsible for procurement that the choice is not primarily determined by costs. Spare bulbs are a must and should be kept at each microscopy unit, while other spare parts and 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 when a centre experiences problems with a microscope. Minimum requirements for a tuberculosis microscope include a mechanical stage, 8-10x magnification eyepieces and a good 100x objective. It may be a good idea to choose plan or semi-plan type 100x objectives, since this will not only facilitate the work but will also automatically assure higher quality. Microscopes should be ordered with an electric light source, but mirrors will still be needed in many places, even if electricity is available part of the time. While binocular microscopes are recommended, monocular microscopes may be adequate when very few smears have to be read, and are sometimes even preferable because of their higher luminosity when used with a mirror. In larger centres there may be a fluorescence microscope. In that case, it must be certain that at least one spare bulb for the excitation burner is available at all times, and that technicians know how to fit and adjust it correctly. Finally, as good microscopes are difficult to replace, provision has 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 or in their special carrying box when not in use. All openings meant to receive objectives or eyepieces should be closed (by one of the lenses, a plastic plug 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 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 another drying agent. However, this will need a large, 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 staining solutions are prepared at the unit, some essential equipment will be required. These include scales or a simple balance with a sensitivity of 0.1 g, as well as measuring cylinders. It is safest to use distilled water, in order to prevent contamination of staining solutions by environmental mycobacteria. Filtered water is a second choice and much care will be needed to prevent the filter from becoming colonised. De-ionised water is not recommended, as the installations for its preparation tend to become colonised by environmental mycobacteria.

Stocks of supplies and consumables

The stock situation must be checked during each visit. Stocks of laboratory items larger than those for drugs are advisable as some items are difficult to transport and they generally have a long shelf-life. Thus a six-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 staining solutions should not be excessive, i.e., covering not more than twelve months of consumption at any level. Larger centres need large volumes of staining solutions, 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 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 the availability of the necessary equipment.

Slide storage boxes are often sent from one place to another resulting in loss, and non-diamond slide-markers wear down, so both should be considered consumables to ensure continuous supply. 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 most laboratory errors result from faulty registration. Simply working carefully and systematically will go a long way to prevent such errors, and this is a fundamental requirement for any laboratory technician.

The sputum request form should be carefully reviewed to ensure that it is properly used. Each sputum container must be labelled immediately upon arrival to allow unambiguous identification of the patient. 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 be used. The number must be taken from the sequence in the laboratory register. By that time, the full identification of the patient must

be entered in the register, and the number written clearly on the request form. Subsequently, slides must be labelled to allow clear identification of the sputum specimen, using a pencil for frosted slides (or an indelible marker, engraving by diamond or substitute when frosted slides are not used). Writing with other markers will not work, since the writing comes off during staining. Identification of the slide must include the row (serial) as well as the column (slide) number of the register. A code for the centre 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 (Figure III.2). Careful attention should be taken to avoid making the smear on the wrong slide. Results must be entered in the laboratory register promptly after examination. During supervision it should be determined whether the results of the series examined the day before have actually been entered in the register. If all this is not done consistently, there is a definite risk of misidentification, resulting in the wrong patient being treated or not, and this may sometimes explain gross errors found during rechecking.

The request form and register need to show the correct address of the patient, and type of examination (diagnosis or follow-up). If not provided by the clinicians, the technicians should find this out by questioning the patient. This information is essential to trace patients who do 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 centres, reporting of results may pose problems. To find out if such problems exist, it is necessary to compare the individuals recorded 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 entered 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. All positive cases should have been put on treatment. The proportion not registered for treatment should be determined. Delayed receipt of results may result in patients seeking treatment elsewhere. Worse, the patient may have disappeared untreated back into the community. If such problems are identified, it must be ascertained whether information provided to patients is adequate. A policy requiring all sputum smear-positive cases identified to be automatically registered for treatment and to be counted as defaulters if treatment has never been commenced will help to reduce this deficiency.

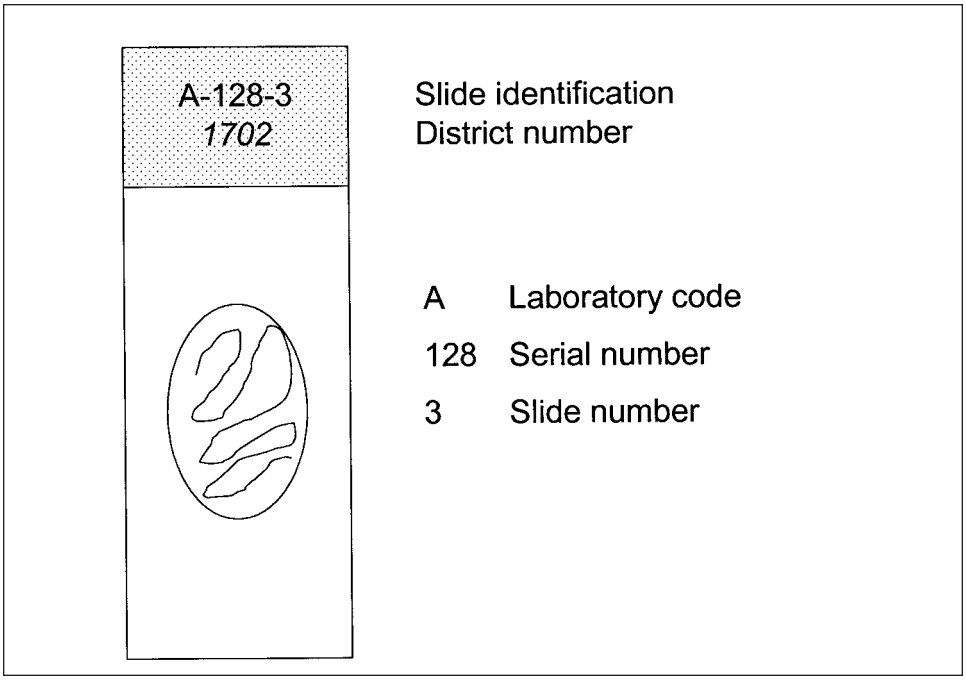

Figure III.2 Proper labelling of a slide for sputum smear examination. The "District number" is given in italics on the slide, because it needs to be entered only if the slide is selected for external quality assessment.

Laboratory technicians should take responsibility for clearly explaining the procedures to the patient at the time the first specimen is requested, and for encouraging the patient to submit all required specimens. The implementation of the policy is evaluated by calculating the proportion of suspects for which only a single diagnostic specimen was registered.

It is currently recommended that at least two positive smears are required to classify a patient as a sputum smear-positive tuberculosis case. The rationale for this rule is to prevent misidentification, most likely to occur in a busy centre. With regular quality assessment this should be a rare event. False-positive bacteriological results as a result of a laboratory mix-up or contamination are encountered more frequently when the more complex culture techniques are used.

The degree of variation in the quantification in a series of sputum smears with at least one positive result may be an indicator of the diligence in examination of serial smears. If all (two or) three serial results frequently show the same quantification, it may be assumed that only one specimen was examined adequately and its result copied. In a study conducted in four countries, suspects with at least one positive slide in a complete series of three slides were the denominator. Among these series, the frequency

of some variability was remarkably consistent in three of the countries, with around 60%, while in the fourth, the frequency of variability was only 14%.

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 is better 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 among countries and also depends on the kind of centre (e.g., first-line or referral institute). As a rule of thumb, the prevalence may be between 5% (frequently seen, e.g., in Latin America) and 20% (frequently seen in sub-Saharan Africa). Higher proportions are often encountered in situations where there is a large patient's and/or physician's delay or where access (distance, financial) to services is poor. Occasionally, a high proportion of positive cases among suspects may result from false positive results or rarely professional misconduct, such as selling positive results if there is a market for it. Unusually low proportions would occur in the reverse situations, i.e., over-permissive selection of suspects or extremely high frequency of chronic cough due to other causes, or with large numbers of false negative results. A strategy of active case detection also results in a low frequency of positivity. Only serious problems in the laboratory will cause this proportion to be clearly different from the average found in similar laboratories in the same area.

The proportion of positive (including scanty) results among all follow-up examinations leaves less doubt about the reasons for deviations. At the frequency and timing recommended by The Union and WHO, there must be some (low) positives among them, often just dead bacilli. Around 10 per cent of all follow-up examinations can be expected to be scanty or positive in countries where laboratory services are quality assured. Although the proportion of crude positivity in follow-up examinations may vary, depending especially on the grade of positivity of the average case at diagnosis (which may on average be lower, i.e., in HIV-positive patients), a total absence of positive results among follow-up cases is not possible. Very low frequencies, particularly at the two-month follow-up examination (where some 15% of sputum smear-positive cases might be expected to remain smear-positive), should raise the suspicion of superficial microscopy and/or poor staining, or again, misconduct.

Simultaneously checking the frequency of low positive results (scanty and 1+ positive) among all positive results of suspects, allows a better insight into the type of problem that may be present. Grading of positive suspect smears follows a normal distribution, with roughly one third of results being low positive in a setting without HIV and rather late case presentation. Detecting the high 3+ positive results obviously constitutes no problem at all, but the detection of low positive results requires sustained effort and high quality work, including good staining solutions and staining. On condition that quantification is already reasonably good, too few low positive suspect results may indicate superficial microscopy. This suspicion is further supported if there are virtually no positive results among follow-up examinations. A high proportion of low positive suspect smears might be real in some settings, but more often this indicates a staining problem, confirmed by their absence in follow-up smears. Rarely, such results will be false positive, caused by high levels of contamination of the staining solutions with environmental mycobacteria or inadequate proficiency in the identification of acid-fast bacilli.

Perusal of the register may give an impression of the plausibility of the results. It is rare that a series of three results of suspects contains only one positive or scanty result. If this is a common occurrence, it may indicate collection of poor specimens, erroneous slide identification, a deficient staining technique, lack of proficiency in reading or a contaminated staining solution.

The quality of the work in the laboratory is also reflected in the consistency of results within positive case series. At least 80% of subsequently positive cases should be positive on the first examination using a spot-morning-spot collection strategy. A lower proportion suggests inadequate reading and/or staining or may point to poor quality of specimens collected, all of which will affect the spot specimens (lower bacillary concentration to begin with) more than the morning specimen.

After the register has provided some indication of what to expect, further investigation may be more problem-oriented, with, for example, in-depth evaluation of the staining solutions and staining practice. Otherwise the next logical step is to make a macroscopic examination of already-prepared smears. The slides kept for rechecking should be reviewed, so that an impression of the average smearing and staining quality is obtained. Stained smears allow an evaluation of both the macroscopic result of smearing and of staining at the same time, which are interdependent. Points to check include proper identification on the slide and the aspect of the smear. The size of the 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, standardisation is generally preferable.

A standard length of approximately 20 mm 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 a macroscopic examination of the smear, which is often very thin or hardly visible and often contains small bubbles. The most frequent problem, particularly for beginners, is the preparation of smears that are too thick overall, or in 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 that it was impossible to decolourise it properly. Another reason may be that technicians are afraid to use the acid long enough or repeatedly. They should be advised that it is virtually impossible to decolourise the acid-fast bacilli using only watery acids. There is no point in looking for a strong colour of the counterstain; on the contrary, smears should have only a light blue colour. 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 entirely.

Checking the microscope

Looking at slides under the microscope allows the reviewer to assess i) the condition of the microscope, ii) the general ability of the centre's technician to identify acid-fast bacilli, and iii) the quality of staining. Examination of available slides should always be performed. It will need one or more positive smears, preferably from the same week, to avoid a false impression because of possible fuchsin fading on older smears. A good number of recent scanty slides may have to be checked if their frequency is abnormally high. All this must be done using the routine microscope(s).

Problems such as a loose stage or stage-clamp will be obvious, especially when scanning 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 binotube should be inspected for dirt and/or 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 reversed and against the light. If nothing is obvious, the objective is reinserted and another look down the tube then may show the dirt in it more clearly. External dirt can be cleaned

away, if necessary by using a tissue dipped in xylene, followed by thorough drying. If the internal surfaces are dirty, i.e., with dust that got inside through an uncovered objective hole or because of poor quality immersion oil or xylene with damage to the lens-cement, cleaning will have to be left to a microscope maintenance workshop. If spare parts are available, a new objective should be used to permit continued use of the microscope. In the case of scratched or chipped lenses, or damaged lens-cement, replacement is the only possible permanent 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 really are acid-fast bacilli, but also to examine whether they have a good strong red colour and are easily discernible against the background. Anything that has the required basic morphology and colour should be accepted as true acid-fast bacilli, even though the supervisor may be sure that it is a *Mycobacterium* species other than *M. tuberculosis*. For optimum results, the red colour 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 carbol-fuchsin stain or a staining method that does not use sufficient heat and/or staining time, and false negative results should be suspected. The effect can also be caused by excessive alcohol decolourising, but not by acids diluted in water. The positive smears should also be inspected for crystals of fuchsin that may result from lack of (recent) filtration of the stain, from using all carbol-fuchsin from the bottle until the last drop, or from reagent bottles that are never cleaned between refills.

Smears with scanty acid-fast bacilli are not easily confirmed. They may have to be re-examined during a supervisory visit if their frequency seems to be too high, suggesting confusion with artefacts. In that case, several recent scanty smears will need to be checked to overcome their low reproducibility as well as the possible effect of fading of the fuchsin stain.

It is not feasible and hence not recommended to try extensive re-examination of negative slides during a supervisory 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 have to be included. This will have to be done by a systematic rechecking programme for routine peripheral smears. As a supervisory visit allows provision of feed-back on the rechecking as well as finding out why errors were made, the two procedures are complementary.

Establishing a representative slide collection

If a rechecking quality assessment system is in place, its reliability and performance should be checked. The first requirement for such a system is

that all slides examined for diagnosis and follow-up are kept in a way that allows their easy retrieval as well as blinded re-examination of their results. This is not as easy as it sounds, as technicians might be tempted to keep only "beautiful" positive slides. Nevertheless, it is a critical and essential component for representative sampling.

Representativeness can only be ensured when all slides are kept. This may be difficult in high-volume laboratories, in which case the collection could be allowed to cover only part of the period, i.e., one week per month. After removing the immersion oil by letting it soak into old newspaper, toilet paper or the like, all slides should be stored in slide-holding boxes as soon as possible (a dark, dry place, of particular importance for fluorochrome-stained slides). The slides are kept in the sequence of the laboratory register serial number, irrespective of their result, to facilitate blinded rechecking. Slide boxes are used sequentially until all are full, then the slides from the first box are discarded, to make space for uninterrupted, continuous collection. Following selection of slides for re-checking, all remaining slides should be discarded.

Ensuring the validity of re-checking EQA

The representativeness of the slide collection must be assured, as the validity of re-checking depends on it. Selection for rechecking, starting from the laboratory register, will automatically ensure that this is the case. It is sufficient to choose at random, for example, ten 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 fewer than nine of the ten slides can be retrieved, the completeness of the collection is in doubt. Details of the labelling of the slides must be checked against the laboratory register to ensure accuracy. The slide itself should contain no indication of the examination result. Deficiencies on these points may very well indicate that the results of rechecking may not be valid.

Blinded rechecking of slides (periphery to centre)

External quality assessment by re-examining a sample of routine smears from the peripheral centres at a higher level of the service is the method of choice for evaluation and continuous motivation of the peripheral centres. Although this may seem simple and straightforward, it requires strict adherence to some technical principles in order to provide valid and interpretable results. Moreover, it cannot be done reliably and profitably unless the national tuberculosis programme is already well established and prepared to allocate the necessary resources to make this demanding system work. In intermediate- or low-prevalence countries with a highly decentralised microscopy network, a very low prevalence of positives

among suspects and higher salary costs, the resources required will often be excessive, and another method must be used. The logistics of 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 rechecking EQA

The principal aim of re-checking EQA is to improve the quality of work through identification of centres that may have unacceptable performance. In this way, it is complementary to supervisory visits which, while unable to systematically detect them, provide an understanding of the causes of high error frequencies and ultimately will serve to remedy them. Technicians will know that they are being checked, and at the same time that the service takes an interest in their problems. Together this will mean a powerful inducement for those who are well-motivated.

It is usually not feasible to correct diagnoses and patient management through rechecking, and this is not the primary aim of the exercise. The cycle of work requires too much time to be useful for the individual patient, and the system should be used rather for maintenance of quality work. Furthermore, the sample represents only a small fraction of all examined specimens. Appraisal of individual technicians is also not feasible: because of, among other things, the small sample size, short-term results are not always precise and should be interpreted with caution.

Overview of rechecking EQA

A representative sample of routinely examined slides is collected from each of the microscopy units for re-reading at an intermediate level laboratory (first controller). The size of this sample has been pre-determined by the national tuberculosis programme and the national reference laboratory, so that the sample can be collected by any supervisor. Blinding of this first screening is assured by the supervisor who keeps the list with the original results (Appendix 5 shows the recommended format of a rechecking sampling form that can also be used to give routine feedback on the results). The supervisor will compare these with the re-reading results in order to identify slides with a discordant result (positive/negative or serious quantification differences). Only those are subsequently forwarded to a third reader, preferably at a higher level laboratory, for another, more thorough examination (second controller). This last result should then be considered the gold standard (although not strictly error-free) for these discordant slides, allowing the definition of errors and their allocation either to the peripheral microscopy centre or to the first controller. Rechecking with

coverage of all possible sources of error requires restaining of all smears prior to first screening in some settings, while restaining before second controls is always indicated. The validity of the controls must be assured: first controllers should on average make fewer false negative errors than their microscopy units, and the second controller should only very rarely miss a clearly positive smear. Feedback of provisional results should be regular, but the final analysis is done only after completion of a full cycle (one year). All units with at least one clear-cut false positive result, or with a number of false negative results higher than the acceptable number for the chosen sample size, fail the screening test and may have problems with microscopes, staining solutions, smearing or staining technique, superficial reading or lack in proficiency. However, failing the screening may also be due to chance (sampling of slides with an error although the permissible level is not exceeded for the total collection) or to an administrative error (copying results). True problems and their source have to be identified during a supervisory visit by a laboratory professional, while chance findings will be confirmed by their absence in later samples. Qualitative elements such as the aspect of smears and staining are also covered by the controllers. However, as they occur frequently in the absence of error, they will receive particular attention only for identification of the source of error if one is identified. Otherwise, just a brief appraisal of these elements for the total sample is given on the routine feedback sheet. If restaining was not practised, more attention will need to be given to subjective appraisal of colour of the acid-fast bacilli.

Technical requirements

Technical requirements and possible pitfalls are described in detail elsewhere (see bibliography). The technical difficulty of rechecking should not be under-estimated, as simple as the exercise may seem to be. A major error in many national tuberculosis programmes has been that too many slides were sent for rechecking, striving for statistical precision. This usually results in overburdened controllers unable to re-examine the slides carefully, and sometimes in simple copying of results when precautions for blinding were inadequate. In most situations it will not be possible to attain statistical as well as technical precision, and in general the smallest possible sample size should be chosen. This is done using the Lot Quality Assurance Sampling System (LQAS) for negative slides (discussed further on), while positive and scanty smears are automatically included at their frequency of occurrence by selecting a single sample, irrespective of results. This arbitrary sample of positive and scanty slides is sufficient (except maybe in the smallest units) to detect the true false positive problems, which occur rarely but then usually systematically, and will thus be apparent from re-examining just a few positives. At the same time, the almost equal proportion of positives in controllers' samples and units' routine work allows a direct comparison

of false negatives identified in the samples for the controller and his/her units, to validate rechecking. If in doubt, i.e., because of slightly too many false low positives for a unit, a special sample targeting positive and scanty smears can easily be taken for confirmation. In the smallest units with only few positive identified annually, all positive and scanty slides should be added to the random sample.

Technically accurate controls covering all possible sources of serious error may require restaining of all smears prior to first screening to detect gross deficiencies of staining since, without restaining, the acid-fast bacilli may remain invisible even to the controllers. This will be imperative whenever the national tuberculosis programme cannot control procurement of high quality reagents and a correct preparation of carbol-fuchsin stain, or when a suboptimal staining technique (e.g., cold staining) is used. Under these conditions, complete fading of the weakly stained acid-fast bacilli will occur so rapidly that it becomes impossible to recognise most of the false negatives, resulting in gross underestimation of this problem. The same will happen to well-stained acid-fast bacilli in hot and humid climates (Figure III.3). A combination of both may cause fading within just one week. Fading may also explain a perceived problem of false positives, and it is generally recommended to attempt restaining before declaring a false positive to avoid false accusations of such a serious deficiency. However, this still does not guarantee that the level of false negatives has been determined correctly. Moreover, restaining of discordant slides only makes it impossible to evaluate the work of the first controllers, who may then have missed many faded true positives. In case restaining of a discordant series systematically makes positives re-appear (so confirming the peripheral result, with many false negatives for the first controller), the rechecking exercise should be redone after restaining even the smears with concordant results, to have a fair chance of detecting false negatives from the periphery.

Limitations of the technique

Even the second controller will miss some smears with very low or unevenly spread numbers of acid-fast bacilli. These rare "low false positives" can be accepted as an inherent limitation of the method as long as they occur at a low and similar frequency among the first controller and the controlled microscopy units. 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. These are accepted, as the first controller serves only as a comparison of what can be attained under optimal conditions, respecting the technical guidelines. For this reason, the first controller should not even try to detect each and every error that may be there by reading more fields than the units are supposed to read, contrary to the second controller, who has to confirm as

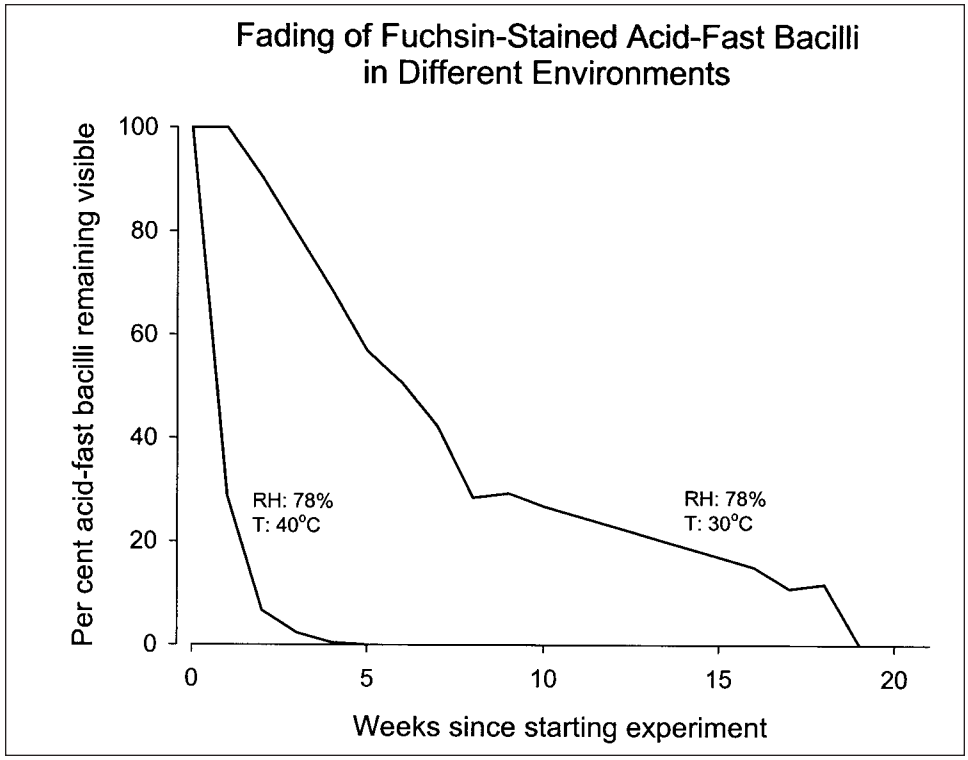

Figure III.3 Fading of fuchsin over time in function of relative humidity (RH) and temperature (T). Data courtesy of Damien Foundation Project Bangladesh.

many positive results as possible. There is no possible practical alternative for establishing a gold standard, and the one proposed is adequate as long as optimum (not error-free) performance of the controllers is guaranteed. This requires regular validation of the controls by comparison of error levels as defined before, and by returning slides for which serious errors were found to the technician who made the error. This person should be given the opportunity to show acid-fast bacilli eventually missed by the second controller to a supervisor, so that the value of the gold standard is continuously validated.

The sample size required for false negatives is meant to ensure that units with not more than their accepted number found in the sample operate at or above the minimally required level of sensitivity. However, this does not automatically mean that units for which too many false negative errors were found in the sample do not reach this minimum level, but only that there is no statistical assurance of good performance. Beyond this point, interpretation based on the number and seriousness of the errors is required, followed by a supervisory visit targeting first of all the microscopy units

where the existence of true problems seems most likely. Other units that fail the screening test can wait to be visited until further samples have confirmed the presence of unacceptable error levels. For a reasonably well-functioning service, this will mean that the great majority of units do not need an urgent visit by supervisory level laboratory staff.

Issues related to sampling

The WHO-defined LQAS sampling scheme has been chosen as the most appropriate sampling method as it will give an answer to the only question relevant during the screening stage, i.e., whether there is assurance that sensitivity (or level of false negatives) is acceptable for the smallest possible sample size. This will make a technically correct execution of re-checking feasible. It is only applied to negatives, as the technique has inherent errors only in this group. As shown in Table III.3, any reader will unavoidably miss a low positive from time to time, however good he/she may be. This results in a certain level of discrepancy between two readers checking the same slides, even when they are working at the same high level of sensitivity and specificity. A statistical system is thus needed to differentiate this "normal variation" from higher levels of false negatives possibly due to underlying problems.

As the system is rather difficult to understand, determination of sample sizes should not be left to the people in the field. Instead, the national level will need to collect the required information, define appropriate sample size, and tell the people in the field how many slides need to be collected on a monthly or quarterly basis. The information needed per microscopy unit comprises the number of negative and positive smears read annually. The prevalence of positive results in the laboratories then needs to be calculated, since this factor is directly related to the proportion of false negatives allowed for a given sensitivity. While the target minimum sensitivity must be chosen arbitrarily by the national tuberculosis programme, the permissible rate of false negatives corresponding to this minimum sensitivity level is directly proportional to the prevalence of positives. This also means that performance of microscopy units, districts or national tuberculosis programmes is not directly comparable unless their prevalence of positives is the same. For example, in a country with an average 25% of positives among suspects reported by the laboratories, the frequency of false negatives for the same sensitivity will be over six times as large as that for a country with only 5% positives in the laboratories. The LQAS sample size tables provided in the reference do not show the accepted frequency of false negatives, and it has been replaced by the prevalence of positives for a given target sensitivity. It is recommended to simplify the work in the field by choosing a single sample size adequate for the averages of these parameters (prevalence of positive and negative turn-over), as long as their variation in the region or country is not extremely large.

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 clearly illustrates the inherent limitations of sputum smear microscopy

From: Toman K. Toman's tuberculosis. Case detection, treatment, and monitoring. Questions and answers. Geneva, Switzerland: World Health Organization, 2004.

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

It is best to choose a modest minimum sensitivity target, such as 75% or 80%. In fact, with a 75% target sensitivity, one is reassured that units performing below this level will almost always fail the test, but some units reaching a slightly higher sensitivity, i.e., 80% to 85%, will also fail. Those with less than adequate performance which do not fail during the first year will probably fail the next one, so that opting for relatively low target sensitivity will force the focus on the centres needing most urgent attention. The recommended acceptance number of false negatives in the sample should equal zero. This does not mean that in routine work not a single false negative is allowed, but just that the sample size is kept so small that there is not much chance that these rare false negative errors will be detected. Opting for a higher acceptance number, i.e., allowing a single false negative, will almost double the required sample size. The probability of finding these rare admissible false negative results by chance also increases considerably without gaining much additional statistical precision, while the technically correct execution of the controls will certainly suffer. National tuberculosis programmes tend to opt for far too large sample sizes, thus overloading the controllers, with invalid results as a consequence. For high-prevalence settings, an annual total sample of 40 to 60 slides per microscopy unit is virtually always sufficient to detect the real problems.

Threshold for action

Table III.4 summarises 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 and indicate a serious problem in the laboratory. The main thrust of rechecking is to detect an unacceptable proportion of "high false negative" slides and to ascertain that there are no "high false positive" results. High false positive results are very rare if the basic requirements of knowledge and a good microscope are met. Such errors are not acceptable, and each one must be investigated. An isolated occurrence will often be identified as an administrative error. As described above, for false negatives the hypothesis is tested that the total number of false negative results is not higher than the critical level, corresponding to a chosen minimum sensitivity. Centres with more errors may have an unacceptable, false negative frequency, and this will be more likely when it concerns high false negatives or higher numbers of false negatives (high plus low). In the absence of high level errors, rare "low false positive" and "low false negative" errors are considered to be less serious, as they are more likely to be found even when there is no serious laboratory problem. Low false positives are most often due to low reproducibility of this type of result: the controllers will regularly fail to find rare acid-fast bacilli that were identified by chance in the microscopy units. Low false negatives are often found because of "overshooting" of the method.

Table III.4. 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	*LFN*	**HFN**	**HFN**	**HFN**
1-9 / 100	*LFP*	correct	correct	QE	QE
1+	**HFP**	correct	correct	correct	QE
2 +	**HFP**	QE	correct	correct	correct
3 +	**HFP**	QE	QE	correct	correct

Correct:	No errors, including no quantification error
QE:	Quantification error, minor error but may indicate important problems
LFN:	Low false negative, minor error, to be expected at a small frequency
LFP:	Low false positive, minor error, to be expected at a small frequency
HFN:	High false negative, gross error, missing a clearly positive slide
HFP:	High false positive, gross error, misclassifying a non-case as a case

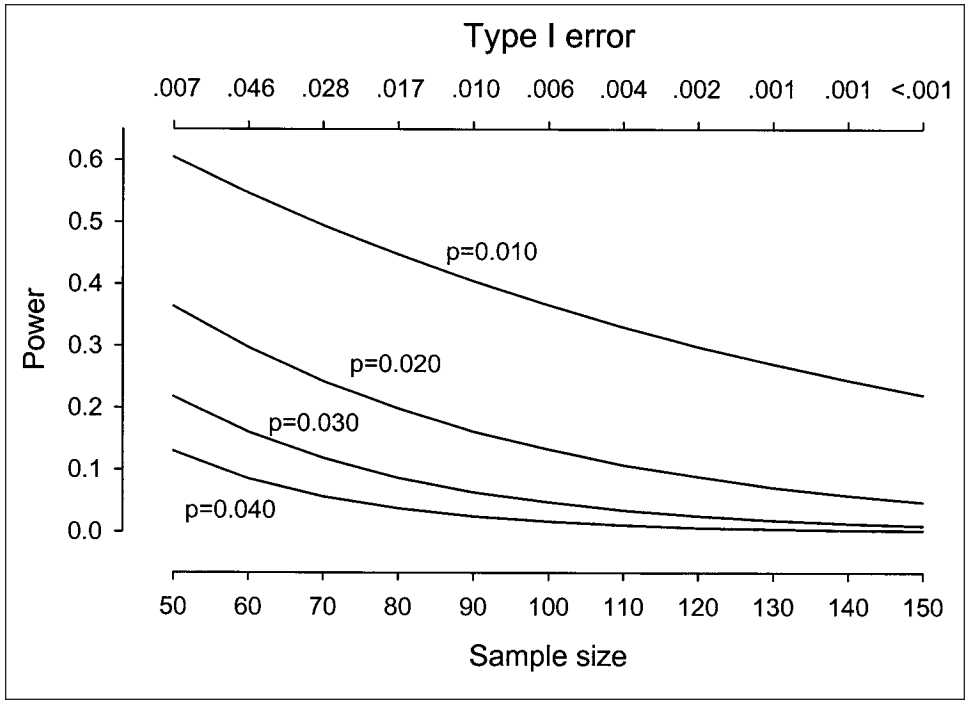

Figure III.4 Sampling for negative slides. Required sample size to have 96% certainty that finding no error indicates that the true error frequency does not exceed 5%. Data courtesy of Dr. Beat Neuenschwander.

"Quantification errors" are as such of minor importance, as they do not influence decision making in patient care. However, they will help to identify problems of staining solutions and staining. Provided that restaining of all slides was practised prior to rechecking, poor staining by the microscopy unit will show as a systematic shift toward values that are too low. Conversely, without restaining, fading of positives is suggested by (falsely) finding a shift toward too high quantification in addition to finding false positive results.

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 does not know the appearance of acid-fast bacilli at all, or to a defunct microscope (seriously damaged, with fungus, or no light), or the smears were not examined at all.

A single high false positive result will often be a clerical error and can be tolerated. If more than one such error occurs, administrative and identification procedures are obviously of poor quality and this should not be tolerated.

A very low number of low false positive results should be ignored, as the possible causes are mostly linked to the limitations of the rechecking system itself, as elaborated above. In general they will then occur at levels comparable with those found in other centres and with the first controllers.

Regularly finding a high false positive result with more than just a few low false positive results may indicate that the technician is not entirely 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.

The finding of high false negative results coupled with many low false negative results is often caused by poor quality of carbol-fuchsin staining solution, or poor staining technique (provided that restaining was practised, otherwise this problem may only be suggested by finding weakly stained acid-fast bacilli in positives). Other possibilities include carelessness in reading, even to the extent of not examining specimens at all on busy days. A very high frequency of low false negative and some false negative 1+ positive smears is occasionally seen when smears are contaminated by tap water in the centres, revealing such acid-fast bacilli only after restaining.

Lower frequencies of false negative results, mainly low 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.

Analysis and reporting of rechecking results

Appendix 6 shows a suggested format for a rechecking report, meant to be sent to the higher or national level. It summarises the numbers of slides rechecked per laboratory and the numbers of different errors found, allowing an analysis of rechecking coverage, average performance and the number/identity of laboratories failing the screening. Moreover, the performance of the first rechecker(s) should also be shown, so that a comparison of their total false negative errors with the total for the peripheral centres allows an assessment of the quality of the controls.

If a uniform sample size was used, and the total number of slides routinely examined was also reported, a more objective analysis becomes possible. Converting the numbers of false negatives into sensitivity relative to the controllers for each centre will then allow a more accurate identification of laboratories with possibly unsatisfactory performance.

Ascertaining the quality of bacteriological classification of registered patients

The EQA methods of sputum smear microscopy described so far only address the quality of the work in the laboratory. They fail to provide an answer to a key question, 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 based on the tuberculosis case register. This procedure is more complex and unlikely to be feasible as a routine, and not at all until ordinary laboratory register based rechecking has been sufficiently streamlined. Periodic surveys might then usefully be carried out using this procedure.

First, it requires the laboratory to properly label all slides (Figure III.2) and to keep them according to the system already described. When reporting the examination result, the laboratory must take care to report the laboratory identification code, the laboratory serial number and the slide number as well (e.g., A-128-2). 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 form for the tuberculosis case register, see the Tuberculosis Guide of The Union). 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.

This system, while desirable from a programmatic point of view, has not yet proved feasible in the field and is thus not discussed further.

3. Quality improvement

It is not sufficient in EQA and quality control simply to identify errors or weaknesses in laboratory services; remedial action must be taken to permanently remove them. This implies continuous monitoring of performance by EQA, a quality control programme as well as a direct supervision programme. 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 programme 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 programme management. This implies that

technical specialists in laboratory services are knowledgeable in national programme policies, and conversely, that technical specialists in programme 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 might include retraining of technicians in the technical aspects of smear microscopy if poor technique seems to be the cause. In other cases, the microscope or staining solutions might have to be replaced. If direct supervision shows that there are serious deficiencies in the way that the laboratory register is kept, remedial action would include retraining of personnel in the administrative aspects of diagnostic services.

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

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

In summary, quality assurance including external quality assessment 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 the standard periphery-to-centre method of EQA to identify poor performance and other problems. Plotting of frequencies of cases among suspects over time is recommended. Finally, periodic surveys to ascertain the quality of bacteriological classification of registered patients may be undertaken.

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CHAPTER IV

Surveillance of anti-tuberculosis drug resistance

This chapter is divided into four parts:

A Purpose of the surveillance system and the role of culture: general considerations on culturing and the purpose of the surveillance system

B. General considerations on laboratory-specific issues: recommendations for practical aspects of carrying out drug resistance surveillance

C. Specific technical procedures in the surveillance of drug resistance: elaborated in sufficient detail to assist in appropriate sampling and to allow implementation of the procedures

D. Ethical considerations in the surveillance of drug resistance.

A. Purpose of the surveillance system and the role of culture

1. Purpose of surveillance

Surveillance of drug resistance provides i) information on the susceptibility pattern of currently circulating strains and ii) an objective measure of programme quality. The prevalence of drug resistance among patients who have never previously been treated quantifies the extent to which secondary cases have already emerged as a result of transmission of drug-resistant bacilli. Drug resistance among previously treated patients is a cumulative measure of errors in the implementation of the programme. The purpose of surveillance of drug resistance is to quantify these two indicators in as unbiased a manner as possible.

Success in carrying out drug resistance surveillance is crucially dependent on three factors: accurate determination of the history of prior treatment (with a minimum of misclassifications), successful isolation of *M. tuberculosis* complex from specimens (with a low proportion of contamination or failure of cultures), and efficient performance of drug susceptibility testing (with acceptable reproducibility and validity).

For surveillance of drug resistance, patients are classified as belonging to one of two categories: those who have, and those who have never 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. Consequently, surveillance of drug resistance must provide quantitative information on the frequency of resistance among these two groups.

Only strains from patients with sputum smear-positive tuberculosis are eligible for assessment of drug susceptibility for the purpose of drug resistance surveillance. 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 other than those with sputum smear-positive disease transmit tubercle bacilli at a much lower level of efficiency. 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. Third, the number of bacilli in sputum smear-positive cases is sufficiently high to tolerate some loss of viability during transport and can therefore ensure a high probability of recovery on culture.

Patients with newly-discovered tuberculosis must therefore be interviewed carefully to determine whether they have received prior treatment for tuberculosis. If insufficient attention is paid to patient interview, misclassification will occur, and interpretation of the results will be flawed (biased). 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 interview of a patient aims to minimise this classification error.

Resistance found in patients with a history of previous treatment is, to a large extent, that already existing prior to treatment and reflects, to a lesser extent, inadequate treatment (true *acquired drug resistance*) if a programme operates well. Resistance in patients never previously treated (*primary drug resistance*) reflects the extent to which cases with resistant strains have transmitted *M. tuberculosis* complex to other members of the community, leading to secondary cases of resistance. Considering together a representative sample of those who have and those who have not previously been treated (*combined drug resistance*) also takes into account the relative proportions of both groups, and the figure indicates the magnitude of drug resistance among the patient population in the community surveyed. The frequency of drug resistance in both those who have and those who have not been previously treated can thus be utilised to assess the quality of the programme.

Surveillance of drug resistance should be a continuous activity of the national programme. For epidemiological purposes, it is sufficient to repeat surveys at intervals of five years to obtain accurate trends. The reduced costs of repeat surveys as 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 are negative aspects of choosing periodic surveys as the selected approach. The complexities of organising a survey are considerable, and therefore, if feasible, continuous surveillance is preferred over periodic surveys. Where resources permit, maintaining a few key centres functioning in between repeat surveys will help minimise costs and disruption. The addition of more clusters after each survey is a good means of laboratory capacity building.

The main objective of this effort is monitoring of trends in anti-tuberculosis drug resistance, rather than the level at one point in time.

Polydrug resistance is defined here as resistance to any two or more anti-tuberculosis drugs, while *monoresistance* is defined as resistance to only one drug. Resistance to both isoniazid and rifampicin is of particular interest, as a high prevalence may render otherwise powerful rifampicin-containing regimens ineffective. The subset among polydrug-resistant strains with resistance to both isoniazid and rifampicin is defined, by common agreement, as *multidrug resistance*.

2. Culture for surveillance of drug resistance

Culturing specimens to isolate *M. tuberculosis* is more sensitive than sputum smear microscopy to identify persons with pulmonary tuberculosis: to have a 50 per cent chance of finding acid-fast bacilli on sputum smear microscopy requires the presence of some 1,000 bacilli per 1 mL of sputum, while a carefully performed high-quality culture technique may be able to detect as few as 10 to 100 cultivable bacilli per 1 mL of sputum.

For the diagnosis of tuberculosis, this increased sensitivity over smear microscopy is critical in defining the role of cultures in case management: if the marginal gain in discriminating between patients who do and those who do not require treatment is modest, it cannot be considered efficient. In contrast, for the surveillance of drug resistance alone, only multibacillary specimens from known sputum smear-positive cases are processed, and thus requirements on test sensitivity are much less critical.

In the following pages, the focus will be on the role of culture and susceptibility testing for the purpose of surveillance of drug resistance. The purpose of culture to increase the sensitivity of diagnosis is not discussed in this text; it is left to others to address the complexities of the issues involved.

Indiscriminate promotion of culture to increase tuberculosis detection is problematic. Such a scheme is most likely to fail if the high requirements regarding general infrastructure and skilled personnel are not fulfilled. It will then be impossible to set up or sustain a service with adequate coverage and quality. Even if basic requirements are met, the expectation that cultures will have a yield similar to that observed in industrialised countries may not be substantiated because of differences between high- and low-income countries in the characteristics of managing tuberculosis patients. Tuberculosis patients in low-income countries frequently do not present with early disease, so that only a minor fraction is found smear-negative but culture-positive, given correct execution of both techniques. Delays in arriving at a definitive diagnosis often make it impossible to trace such a patient, so that even rapid culture techniques (requiring a minimum of two to three weeks for a result in sputum smear-negative patients) are too slow. Moreover, tuberculosis is high on the list in the differential diagnosis of physicians in such countries, even more so where diagnosis and treatment are free of charge. Tuberculosis diagnosis is made (correctly or incorrectly) with much less delay when based either on clinical opinion or on chest radiography (if available and affordable). The role of culture in such a setting is to subsequently validate this diagnosis, but only if the laboratory is able to inspire confidence in its results. The tuberculosis programme can seek to change this by adopting a restrictive approach, requiring bacteriological proof of diagnosis, as is the case in some countries. This is not justifiable from the point of view of individual patient care (to the point of being unethical), and most probably not from that of tuberculosis control.

B. General considerations on laboratory-specific issues

In conducting surveillance of resistance for *M. tuberculosis*, four laboratory-specific issues need to be considered:

- Specimen collection
- Specimen transportation
- Specimen processing and culturing
- Identification of mycobacteria and drug susceptibility testing

1. Specimen collection

Tuberculosis is an infectious disease that most frequently affects the lungs. Specimens from the lower respiratory tract provide the best opportunity for detection and recovery of *M. tuberculosis*. To produce a good quality specimen, patients need to be instructed on how to produce a sputum specimen from the lungs. Once collected, the specimen should be processed rapidly, as tubercle bacilli will die over time, even if the specimen is kept refrigerated.

2. Specimen transportation

Every effort should be made to immediately transport a freshly collected specimen to the laboratory. This is not always practicable, and delays between specimen collection and processing do occur. Such delays create the opportunity for contaminating micro-organisms to multiply and overgrow the tubercle bacilli.

One means of reducing the emergence of contaminants is to use a primary decontaminating agent or preservative. The most widely used is cetylpyridinium chloride (or bromide), a quaternary ammonium compound. The laboratory uses centrifugation to remove the cetylpyridinium agent from the specimen before processing for culture.

3. Specimen processing and culturing

Sputum specimens also contain other micro-organisms that multiply much more rapidly than tubercle bacilli. Specimen processing is designed to kill contaminating micro-organisms whilst maintaining, as much as is possible, the viability of the tubercle bacilli.

A variety of decontamination agents and procedures have been developed. Selection of the specific decontaminating agent depends on the purpose (targeted contaminants, desired exposure time, etc). The most widely used is the alkaline sodium hydroxide method originally proposed by Petroff and which carries his name.

Culture media might be egg-based, agar-based or broth-based. Each has advantages and disadvantages. Laboratories in affluent countries commonly use at least two types of media, as the main objective of the culture in such settings is a high sensitivity for diagnosis. In the following discussion, only egg-based media are considered; while they have a somewhat reduced sensitivity (when used for diagnosis), for surveillance purposes this is negligible, particularly when only multi-bacillary smear-positive specimens are selected.

To prevent death of tubercle bacilli due to continued exposure to the decontaminant, the alkaline must be removed either by repeated addition of water and centrifugation, or by neutralisation. Neutralisation can be achieved by inoculation on a buffered medium, or by use of acids before inoculation onto a non-buffered medium. This underlines the distinction between buffered media (such as the modified Ogawa medium) and non-buffered media (such as Löwenstein-Jensen medium or the International Union Against Tuberculosis Medium [IUTM]).

4. Identification of mycobacteria and drug susceptibility testing

Environmental mycobacteria might be isolated from sputum specimens. As their drug susceptibility pattern may differ from that of *M. tuberculosis* and has no epidemiological significance, the species grown in culture must be identified as belonging to the *M. tuberculosis* complex. The pattern of susceptibility to the main drugs isoniazid, rifampicin, streptomycin and ethambutol is determined for *M. tuberculosis* isolates. Susceptibility to pyrazinamide cannot be reliably determined on these media, and it is therefore omitted from surveillance.

C. Specific technical procedures in the surveillance of drug resistance

In the following section, technical information is provided to assist national tuberculosis control programmes to participate in the WHO/IUATLD (Union) Global Project on Anti-Tuberculosis Drug Resistance Surveillance.

1. Representative sampling

The results of surveillance of drug resistance must reflect the situation in the country or jurisdiction surveyed. Consequently, it is essential to decide upon a sampling scheme that is representative of the population for which conclusions are to be drawn (avoiding bias). The three most frequently employed methods to obtain a representative sample are cluster sampling, systematic sampling and random sampling. Because of their practicability, this manual gives preference to the cluster sampling and systematic sampling methods.

The size of the sample to be surveyed will depend on the precision required. Commonly, 350 to 1,000 patients will suffice for the purpose of surveillance among patients who have never previously been treated. Previously treated patients become increasingly less frequent as the performance of the programme improves. However, the required sample size for such patients is also smaller, because the expected prevalence of resistance is higher. Moreover, this group is of particular interest for continuous surveillance of trends, extending beyond the survey period.

Adjustment in sample size should be made, taking account of losses due to sputum culture failures resulting from contamination or no growth and of eligible patients who decline to participate. Expected culture failure frequencies may vary depending on transport delay and the sputum culture proficiency of the participating laboratory, and the sample size must be increased accordingly.

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 logistical difficulties in covering the entire area of the country, where there is no reliable census of the population to be surveyed (sampling frame) 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 roughly the same number of eligible sputum smear-positive tuberculosis patients diagnosed consecutively at a health centre. 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 centres that notify tuberculosis cases in the country, with the number of sputum smear-positive patients diagnosed per year in each centre. The selection of clusters is made from this list so that the possibility of selecting a cluster in a particular centre is proportional to its number of eligible cases. Several clusters can thus belong to the same centre if it is a particularly large one. A detailed procedure for cluster sampling is provided in the WHO/Union Guidelines for Surveillance of Drug Resistance in Tuberculosis, which should be consulted before undertaking such a survey.

Systematic sampling

Every newly diagnosed sputum smear-positive patient is potentially eligible to provide 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 approximately 5% to 15% of these patients.

Preferably, the central unit produces a list based on annual case reports by tuberculosis management units. Assuming a stable calendar trend of patients over the year (no seasonal variation), 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 number of cases. This will give groups of tuberculosis management units that will be assigned to a specific month 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 considerably reduced. Requiring the tuberculosis management units to submit specimens for a longer period to obtain the required minimum of drug susceptibility test results can adjust for anticipated losses.

Because the number of patients in need of retreatment (failures, relapses 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. It is recommended to move toward continuous monitoring of resistance among previously treated cases from as many of those patients and from as many diagnostic units as possible in between surveys, which allows more efficient monitoring.

Although previously treated cases are a complex group, consisting of failures, relapses, re-infection disease, returning after varying time periods of interruption with positive sputum smears, and with resistance that may have been acquired during treatment, or may have predated the treatment, they constitute a useful group for trend analysis. Drug resistance levels are higher and may reflect changes more clearly and earlier, especially in case of a rise due to acquisition of resistance as a result of inadequate treatment. But it will be difficult to obtain relative frequencies of those patients reflecting the actual figure of the prevalent cases, especially in those settings where a considerable number of cases are managed by the health sector outside the national tuberculosis programme, so that point prevalence in this group may not be precise.

Random sampling

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

The most appealing method is to produce a list of the number of reported tuberculosis cases in each tuberculosis management unit in one year. OpenEpi (http://www.openepi.com), as an example, is 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. The 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 need to be submitted for culture and drug susceptibility testing.

2. Collection of specimens

The required representative sample of *M. tuberculosis* strains is obtainable only when sputum culture failures due to contamination or no growth do not exceed the expected range. For this reason, the greatest effort should be made to prevent sputum culture failures.

In the context of surveillance of drug resistance, the decision to obtain a specimen is usually taken after the diagnosis of sputum smear-positive tuberculosis is made. Tubercle bacilli do not lose their characteristic of acid-fastness over time, but they do lose their viability rapidly. Specimens submitted for culturing must therefore be dealt with differently from specimens submitted for microscopy only (for instance, bleach-treated specimens cannot be used at all). The viability of tubercle bacilli rapidly diminishes in high ambient temperatures and with increasing time since collection. Therefore, only specimens from sputum smear-positive cases should be taken for surveillance of drug resistance. This will increase the likelihood that even after some transit time, viable bacilli may still be recovered. Patients with sputum smear-positive tuberculosis 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. Except when cetylpyridinium was added as a preservative, the collected specimen should be kept in the coolest available place and the interval between collection of the specimen and processing in the reference laboratory should not exceed three days.

3. Transport of specimens

It is desirable to process sputum specimens in laboratories at the location where they have been collected as it is more preferable by far to process a fresh specimen. However, species identification and anti-tuberculosis drug susceptibility testing must be performed only at the national tuberculosis reference laboratory. The only exception is a laboratory where drug susceptibility is a routine activity with all necessary safety precautions and supplies. Pilot runs with involvement of both specimen collection centres and central level are useful for gathering experience before starting.

If sputum specimens are sent to the reference laboratory for culture, simple plastic sputum containers are not appropriate. For this purpose, universal containers are required. They have leak-proof caps (thread with one and a half circumference) and are made of sturdy glass or plastic that is 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 best made locally of plywood, but should be light weight. Where layers of containers are carried within one box, the containers must be of uniform size and sturdy enough

to bear the weight of upper layers. An example of a suitable glass container and transport box is shown in Figure IV.1. Alternatively, styrofoam boxes into which the transport vials are fitted may be used. These will further reduce the weight and shipment costs, but they are more vulnerable to breaking during transport.

A decision must be made as to whether 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 reach the reference laboratory within three days, then no transport solution is needed. If the time exceeds three

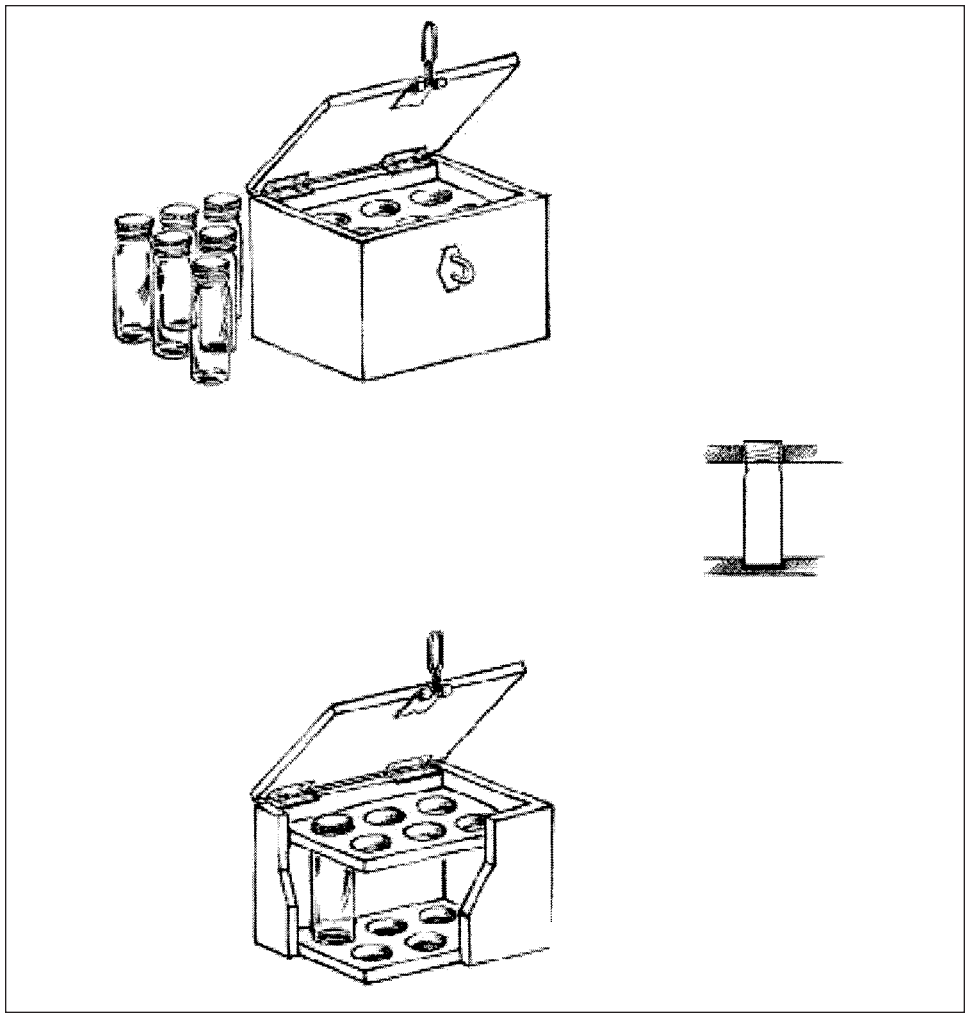

Figure IV.1 Example of sturdy glass containers (universal containers, 28 mL) and transport box to ship sputum specimens for culture and drug susceptibility testing.

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 (neither is soluble in pure water but in 2% sodium chloride solution). The accompanying documentation must indicate whether this agent was added (as centrifugation is always necessary). Where centrifugation cannot be done effectively at the central laboratory, this agent should not be added.

4. Decontamination and homogenisation

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. A reasonable compromise is needed 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.

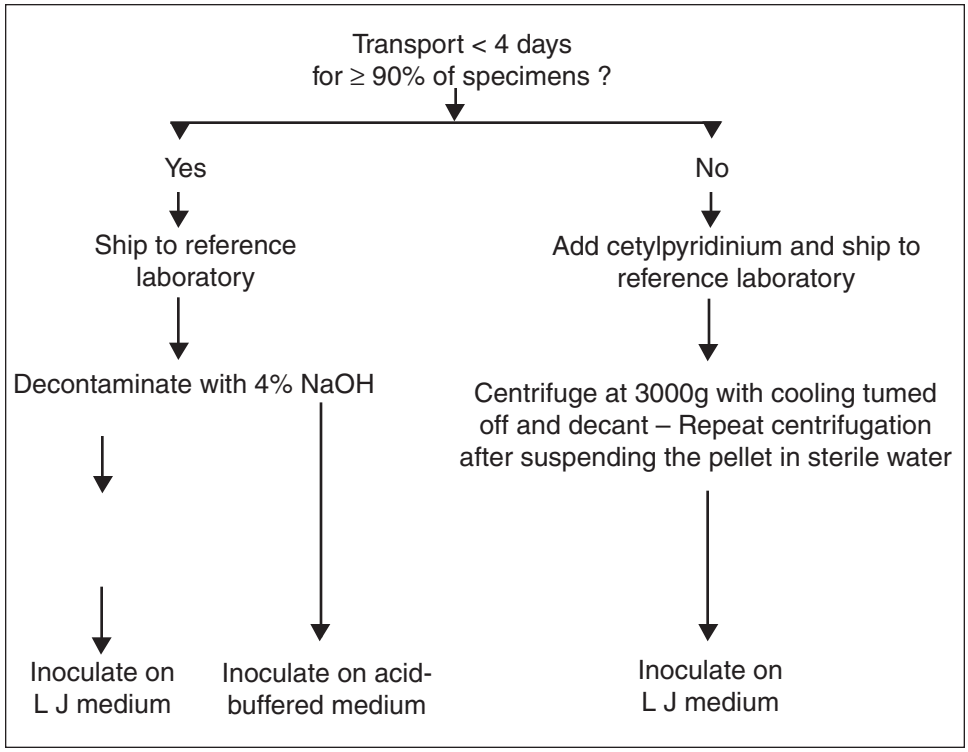

Figure IV.2 Decision tree for transport medium, decontamination, centrifugation and choice of medium.

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

Contamination of media usually results from insufficient decontamination (exposure time and/or concentration), processing specimens spoiled by improper and prolonged storage, and inadvertent re-contamination of a decontaminated specimen or culture medium. No growth of *M. tuberculosis* in the specimen usually results from a decontamination process that is too harsh, delayed culturing, inadequate centrifugation (centrifugal force and time), or inappropriate processing of the specimen decontaminated with cetylpyridinium chloride (or bromide).

Method for specimens pre-treated 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 centrifuged to concentrate tubercle bacilli and to remove cetylpyridinium chloride/bromide. This can be done by topping up with water before centrifugation, followed by a second centrifugation cycle after decanting and adding fresh water (Figure IV.2). The sediment is then inoculated directly onto the medium. Cetylpyridinium treated sputum should not be stored in a refrigerator or a cold environment, where it may re-crystallise and will therefore not protect the specimen from contamination and may inhibit the growth of *M. tuberculosis* when transferred onto medium. For this reason centrifugation of cetylpyridinium treated specimens requires that the cooling system of the centrifuge is turned off to prevent precipitation of re-crystallised cetylpyridinium in the sediment.

Should this treatment result in a high contamination frequency because of excessively long transit time or incorrect use, a shortened decontamination (i.e., five to ten minutes until neutralisation) with 4% NaOH, rather than simple washing, may be indicated.

The 4% NaOH method used in untreated specimens

The technique of Petroff to achieve decontamination with a final maximum concentration of up to 2% sodium hydroxide (using an equal amount of 4% NaOH solution and specimen) for 15 to 30 minutes is given preference worldwide for specimens that cannot be inoculated promptly. 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 contamination frequency of the culture media. If it is below 5% with, e.g., 2% sodium hydroxide, then it is quite legitimate to use this concentration. The sodium hydroxide concentration used depends on the proportion of contaminated media. This frequency is determined by dividing the number of contaminated tubes by all inoculated tubes and not by determining the proportion by using the number of specimens as the denominator.

To 3 mL of sputum in a universal container, 3 mL of 4% sodium hydroxide solution is added. The concentration will thus be 2% NaOH. Great care must be taken to ensure that the sodium hydroxide container and the transfer pipette never come in contact with the neck of the specimen container, to reduce the risk of cross-contamination.

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 neutralisation or repeated washing with water followed by centrifugation, if a Löwenstein-Jensen medium is used. Neutralization involves bringing the alkaline mix to a neutral pH. There are several methods to achieve this. Many laboratories use a pH indicator (such as phenol red) with acid, but when not done accurately this may end in over-acidification, especially when done on a centrifuged sediment. Alternatively, a sufficient volume of buffer (i.e., phosphate buffered saline pH 6.8) is added. A third possibility is to use only a single washing step with water after 15 to 30 minutes decontamination, followed by a single centrifugation. While this theoretically leaves some alkalinity, it might not be practically relevant.

As another option, an acid-buffered medium can be used. This technique does not require centrifugation because the NaOH decontaminated specimen is directly inoculated onto medium and is efficient for culture of smear-positive sputum specimens collected for drug resistance surveillance (Figure IV.2). The procedure is so simple as to be easily used and prevents exogenous contamination and additional killing of *M. tuberculosis* by reducing manipulation time and the number of steps of decontamination.

5. Media preparation and inoculation

Conventional growth-based methods utilise egg-based media, agar-based media and liquid media.

For diagnostic purposes, the speed of reporting a result ("turnover time") is critical. Newer techniques therefore utilise liquid media and an approach for *early growth detection*. These techniques are based on carbon dioxide production by mycobacteria, oxygen consumption, adenosine triphosphatase production, or lysis by mycobacteriophages.

As speed is not critical for the purpose of drug resistance surveillance, the recommendation is to use *conventional growth-based methods*. Additional advantages include lower contamination frequencies and lower costs. Among the three media types, the most commonly used for the purpose of surveillance of drug resistance is the egg-based medium.

Different strains of tubercle bacilli prefer different media. A typical example includes the egg-based Löwenstein-Jensen medium recommended by the Union (designated IUTM) without potato starch (Table IV.1). In settings where *M. bovis* or some strains of *M. africanum* are frequent pathogens among the *M. tuberculosis* complex, the IUTM medium might be used with 4.5 g sodium pyruvate replacing the 7.5 mL glycerol without any other modification.

If culture is used primarily for the surveillance of drug resistance of tubercle bacilli rather than for diagnosis, the simple technique is recommended for primary isolation (Table IV.2). For drug susceptibility testing, the IUTM medium should always be used.

Simple technique using acid-buffered medium

The so-called "simple technique" uses direct inoculation onto a medium, without centrifugation. For the purpose of surveillance of drug resistance which analyses specimens from patients with sputum smear-positive tuberculosis, this is the preferred method.

Many different "simple culture" methods have been introduced, including methods proposed by Marks, Ogawa, and Kudoh, to name only the most prominent ones (see bibliography). While all are used by different laboratories, the so-called modified Ogawa medium proposed by Kudoh is often given preference (Table IV.2). Basically, the simple technique utilises a medium containing a buffer to neutralise the alkaline decontaminant. It is therefore best referred to as "acid-buffered medium".

The sputum/decontaminant specimen (about 6 mL, with close to 2% to 3% NaOH concentration) is shaken on a vortex mixer for 20 seconds. The subsequent action time of the decontaminant is 15 minutes.

The decontaminated sputum is directly inoculated on the acid-buffered medium. The usual inoculum size is two drops (approximately 0.1 mL) of the decontaminated (but not neutralised) specimen, using a disposable

Table IV.1 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 (No 1-2): 89-104.

a) Mineral salt solution

L-Asparagine	3.6	g
KH_2PO_4, Potassium phosphate, dibasic, anhydrous	2.4	g
Magnesium citrate	0.6	g
$MgSO_4$ $7H_20$, magnesium sulphate	0.24	g
Glycerol	12.0	mL
Distilled water	600.0	mL

Dissolve the ingredients, in order, in the distilled water by heating. Autoclave at 121°C for 30 minutes to sterilise. Cool to room temperature. This solution keeps indefinitely and may be stored in suitable amounts in the refrigerator.

b) Malachite green solution

Malachite green. It is imperative that the malachite green crystals purchased are chosen with care. Use only commercially available malachite green which has passed antimycobacterial activity testing, as many batches of this stain are bactericidal for mycobacteria.

Malachite green dye	2.0	g
Sterile distilled water	100.0	mL

Using aseptic techniques, dissolve the dye in sterile distilled water by placing in the incubator for 1–2 hours. This solution will not store indefinitely. If precipitation occurs or the solution becomes less deeply coloured, discard and prepare a fresh solution.

c) Homogenised whole eggs

Fresh hens' eggs (not more than 7 days old), from hens that have not been fed antibiotic containing feed, are cleaned by scrubbing thoroughly with a brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution, then rinse them thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs, scrub and wash hands. Crack the eggs with a sterile knife into a sterile flask and beat them with a sterile egg whisk or in a sterile blender.

Preparation of medium

The following ingredients are aseptically pooled in a large, sterile flask and mixed well:

Mineral salt solution	600	mL
Malachite green solution	20	mL
Homogenised eggs (20–25 eggs, depending on size)	1000	mL

Inspissate for 45 minutes at 85° C (80% humidity).

Table IV.2. Composition of acid-buffered egg-based medium (modified Ogawa medium)

Kudoh S, Kudoh T. A simple technique for culturing tubercle bacilli. Bull World Health Organ 1974; 51: 71-82.

Component	Amount
Potassium phosphate, dibasic, anhydrous (KH_2PO_4)	2 g
Magnesium citrate	0.1 g
Sodium glutamate	0.5 g
Distilled water	100 mL
Glycerol	4 mL
Egg homogenate	200 mL
Malachite green (2%)	4 mL

The proposed preparation is as follows:

Recommended medium and its preparation

Modified Ogawa medium (Kudoh)

Salt solution

Monopotassium phosphate (KH_2PO_4)	2.0 g
Magnesium citrate	0.1 g
Sodium glutamate	0.5 g

These compounds are dissolved in 100 mL of distilled water. The solution is heated to dissolve the salts.

Glycerol	4.0 mL

Add the glycerol to the above salt solution.

Egg homogenate

The eggs should be fresh (2 days old), and antibiotic-free. Thoroughly scrub the eggs with 70% ethanol. After breaking the eggs, the whites and yolks are homogenised using a sterile blender and drained through sterile gauze. For each 100 mL of salt solution, add 200 mL of egg homogenate.

Malachite green

Use only commercially available malachite green that has passed antimycobacterial activity testing, as many batches of this stain are bactericidal for mycobacteria.

Malachite green (2% solution)	4.0 mL

Malachite green is added to the egg- and glycerol-containing salt solution

Dispension

The solution mixed and dispensed as required by the type of culture bottles. Inspissate for 45 minutes at 85° C (80% humidity).

Pasteur pipette. When these are not available, two large loopfuls can be applied.

At least two tubes must be inoculated from each specimen. Excess culture water must first be removed.

For the primary isolation of tubercle bacilli for the purpose of drug resistance surveillance, this is the preferred method. The inoculated culture medium is now incubated.

Concentration of tubercle bacilli by centrifugation

The sensitivity of culturing mycobacteria can be increased by using centrifugation. However, this is of lesser concern for the surveillance of drug resistance among sputum smear-positive cases than it is for diagnosis of tuberculosis which raises many more issues that are not the subject of this monograph. Centrifugation will also be required to remove toxic substances such as cetylpyridinium chloride/bromide, used for transport and/or decontamination. Used repeatedly for washing out the decontaminant, it helps to reduce contamination further.

The centrifugation-based method requires washing the decontaminating agent out through cycles of adding distilled water (almost filling up the container or tube) and centrifugation until the decontaminating agent is removed, or to neutralise the decontaminating agent. Neutralisation can be performed before or after centrifugation, but since the decontamination time must be strictly limited, it is better to neutralise first. The advantage of the centrifugation-based method is a concentration of tubercle bacilli in the sediment. Its disadvantages include extended exposure to the decontaminating agent, an additional step (centrifugation), and higher equipment costs (high gravitational force centrifuge equipped with closed system to prevent aerosol release into the environment).

Mycobacterium species have complex lipids that may contribute up to 60% of the total weight of the cell wall. The lipids make the tubercle bacilli difficult to sediment by standard centrifuges and much more powerful equipment is required to produce and maintain a relative centrifugal force (RCF, expressed as *g*) of at least 3,000 *g*. Such centrifuges are expensive and may need refrigeration to prevent heat build-up killing the tubercle bacilli. Laboratories using the centrifugation method should constantly check if an adequate centrifugal force of 3,000 *g* is reached in their centrifuges. A centrifuge maintenance plan as well as contingency plan for tube breakage must also be in place. The RCF is determined by the number of revolutions per minute (RPM), the radius of the centrifuge rotor, and a constant. The relation is as follows:

$$RCF = 1.118 \times 10^{-5} \times radius_{max\ (cm)} \times RPM^2$$

By convention, the farthest distance is used (the maximum radius), measuring from the rotor axis to the bottom of the tubes when the rotor is turning. The determination of the number of rotations required to reach 3,000 *g*, the minimum required, is thus:

$$RPM = SQRT \{3{,}000 / (1.118 \times 10^{-5} \times radius_{max\ (cm)})\},$$

where "SQRT" stands for the square root. The rate at which mycobacteria sediment is critically dependent on centrifugation time and the centrifugal force. 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. Sputum containers must be capped securely and balanced before centrifugation, and a centrifuge should never be opened until it has come to a complete stop. The total period of time of contact with the decontaminating solution should not exceed 30 minutes, i.e., 15 minutes prior to centrifugation and 15 minutes during the first centrifugation cycle.

The supernatant is poured into a splash-proof discard container with 5% phenol and any fluid on the outside of the container neck removed 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. After complete centrifugation, the pellet is re-suspended adding 1 mL of distilled water, with brief vortexing, followed by inoculation.

It is desirable to store the leftover pellet or sediment, if any, for at least two 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.

6. Incubation and reading of cultures

Not all countries have a reliable power supply. Thus, continuous monitoring of the temperature in the incubator is required. Temperatures recorded by the temperature gauge might be unreliable. A thermometer should be kept inside the incubator at all times to confirm that the recordings are accurate.

The pipetted inoculum should be spread on the surface of the medium by tilting the culture bottle. The bottle is then closed but the lid is not too tightly screwed on to allow evaporation of any excess water. For the first night the culture bottle is incubated in a horizontal position at 37° C. On the day after incubation, the bottle is checked. If all excess water

has not evaporated, the bottle cap should be left unscrewed for some time. If all water has evaporated, the cap is tightly screwed on and never opened again until drug susceptibility testing is done. After screwing on the cap, subsequent incubation can be done in an upright position for a period of nine weeks. In laboratories using plastic bottles or tubes for culture, excess water may or may not evaporate during incubation. This is dependent on the quality of the plastic used, and on the type of caps. When cultures are transported to another laboratory for identification and drug susceptibility tests, the presence of excess water will lead to problems of contamination.

The inoculated media should be examined weekly. Most contaminated media and rapidly growing mycobacteria are detected within one week of incubation. The growth of *M. bovis* and some dysgonic strains of *M. tuberculosis* may appear after five to nine weeks.

Most peripheral culture laboratories limit their activities to these tasks. Such laboratories should not attempt any identification or drug susceptibility testing, but send the culture bottles when growth appears to a more central laboratory *without ever opening the culture bottles for any purpose*. If they adhere to this strict rule, they will not need biological safety cabinets. Such cabinets are mandatory whenever culture bottles containing colonies with *M. tuberculosis* complex are opened for any purpose. Strictly following this rule does not seem to allow removal of excess water before shipping. To address this problem, the safest and most practical way to remove excess water is to leave caps slightly loose immediately after preparation to let the media dry in the incubator for a couple of days, after which they should be secured and never opened again.

At the laboratory processing positive cultures, any culture positive for *M. tuberculosis* complex should be kept until a supranational laboratory has sampled the strains and reported the results of proficiency testing. They should be stored tightly stoppered in the refrigerator if possible and protected from light.

Long-term storage of cultures can be done by making heavy bacterial suspensions in skim-milk or other liquid media. Alternatively, a polyethylene vial (cryovials 2 mL) culture of Löwenstein-Jensen medium can be used, which will preserve the strain better at -20°C. If stored in a freezer at -70°C, strains can be preserved in a wide variety of media for many years without significant loss of viability.

7. Identification of the *Mycobacterium tuberculosis* complex

Procedures for identification of mycobacteria are complex and need a

multitude of biochemical tests (or expensive molecular tests) to ascertain the *Mycobacterium* species to which they belong. In the context of surveillance of drug resistance, the process can be simplified as it will only be necessary to decide, to a reasonable degree of certainty, whether or not the *Mycobacterium* is a pathogenic species of the *M. tuberculosis* complex. A very simple identification procedure integrated in the process of susceptibility testing (Figure IV.3) is recommended. Contaminated cultures are immediately discarded, while growth looking like mycobacteria (with compatible morphology, colour and timing of appearance) is confirmed and checked for purity by Ziehl-Neelsen staining after smearing in a drop of water. Pure cultures of mycobacteria are subjected to drug susceptibility testing and subcultured on para-nitrobenzoic acid medium (PNB; Table IV.3). Only cultures grown on PNB and drug-resistant strains have to be subjected to biochemical tests. What looks like *M. tuberculosis*, grows like *M. tuberculosis*, and has the susceptibility of most *M. tuberculosis* strains doesn't need further confirmation, particularly in high-prevalence settings.

For further identification, the culture can be subjected to biochemical tests, if it contains abundant growth. The 68°C labile catalase test (described in Table IV.3) allows distinction between the *M. tuberculosis* complex and other mycobacteria except *M. gastri, M. haemophilum* and *M. marinum* (extremely rare in sputum specimens). The niacin, nitrate reduction and thiophene-2-carboxylic acid hydrazide tests allow distinction between *M. tuberculosis* and *M. bovis* as well as many other mycobacteria. It should be noted, however, that like *M. bovis* some strains of *M. tuberculosis* are niacin-negative. Moreover, niacin testing is now more difficult due to transport restrictions on the required reagents due to the fact that they are very toxic. Niacin test strips are expensive and regularly yield doubtful results, and have been abandoned by many laboratories. 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 (TCH). However, identification of *M. bovis* must be confirmed by negative nitrate reduction tests. In areas of the world where *M. africanum* or the thiophene-2-carboxylic acid susceptible variant of *M. tuberculosis* is prevalent (in large parts of Asia for instance), its proper identification is difficult and not recommended. For these reasons, the niacin, TCH and nitratase tests are not further described here.

8. Drug susceptibility testing

Drug resistance is the result of selection, by exposure to a drug, of a small number of pre-existing mutants in a population of tubercle bacilli. Therefore, inoculum standardisation 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 modified Ogawa (Kudoh) medium is recommended for primary isolation only. For drug susceptibility testing the IUTM Löwenstein-Jensen medium must be used.

Drug susceptibility testing methods, advantages and disadvantages

Three methods have gained wide acceptance among mycobacteriologists. These are the *resistance ratio method*, the *absolute concentration method*,

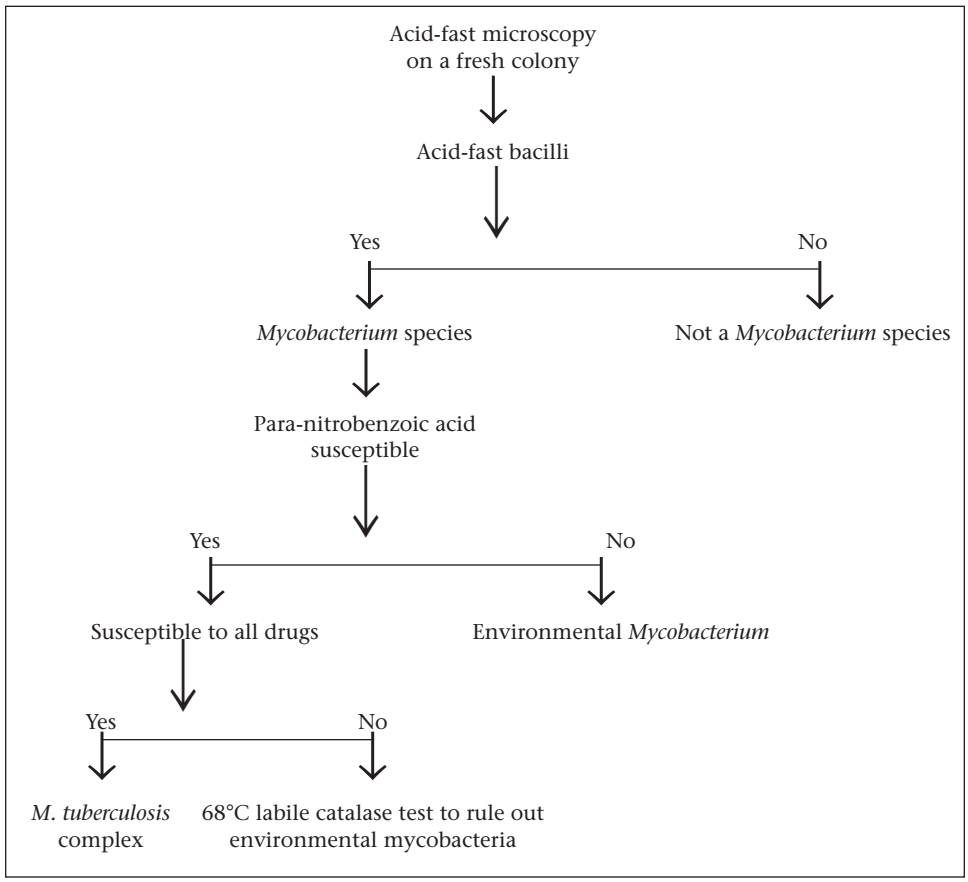

Figure IV.3 Identification of the *Mycobacterium tuberculosis* complex.

Table IV.3 Essential identification tests: para-nitrobenzoic acid (PNB) medium and the 68°C labile catalase test

Growth on para-nitrobenzoic acid (PNB) medium

Bacilli of the *M. tuberculosis* complex do not grow on PNB medium, while most other members of the genus do. This allows identification of environmental mycobacteria, and also of mixtures of environmental mycobacteria and *M. tuberculosis*. The test is set up at the same time as the drug susceptibility test, adding one tube of PNB medium 500 µg/mL. This tube is inoculated with about 1000 bacilli, i.e., 10 µl of the 10-2 dilution used to inoculate one set of control and drug-containing media.

To prepare 500 µg/mL of PNB medium, 250 mg of PNB is dissolved in 10 mL of dimethylsulfoxide or methanol. From this stock, 2 mL is added per 100 mL of Löwenstein-Jensen medium prior to dispensing. The media are coagulated by inspissation at 85°C for exactly 45 minutes.

Heat labile catalase test (at pH 7.0 and 68°C)

Method:

(1) Suspend 2-3 loopfuls of growth from culture bottles in 0.5 mL of 0.067M phosphate buffer (pH 7.0) in a 16x125 mm screw-cap tube. Two tubes are prepared.

(2) Incubate one tube in a 68°C water bath for 20 minutes; keep the other tube at room temperature.

(3) After cooling the heated tube to room temperature, 0.5 mL of freshly prepared Tween peroxide mixture is added into both heated and unheated tubes with a loosened cap.

(4) If the unheated tube forms air bubbles and the heated tube does not, the test strain produces heat-labile catalase and can then be identified as *M. tuberculosis* complex, if isolated from sputum. Allow 20 minutes before declaring negative.

Most environmental mycobacteria produce heat stable catalase, forming bubbles in both heated and unheated tubes. If no bubbles form in any of the tubes, the test has failed or the strain may be catalase-negative (i.e., some strains of isoniazid-resistant *M. tuberculosis)*.

Reagents:

Phosphate buffer pH 7.0

– Stock solution 1: disodium phosphate 0.067 M

  dissolve 9.47 g anhydrous Na_2HPO_4 in distilled water to make one litre

– Stock solution 2: monopotassium phosphate 0.067 M

  dissolve 9.07 g of KH_2PO_4 in distilled water to make one litre

– mix 61.1 mL of stock 1 with 38.9 mL of stock 2. Check on pH metre.

Tween 80 at 10%

Mix 10 mL of Tween 80 with 90 mL of distilled water. Autoclave at 121°C for 10 minutes. Mix by swirling after autoclaving and during cooling to assure good dissolution of the reagent. Store in a refrigerator.

Tween – peroxide mixture

30% hydrogen peroxide is mixed with 10% Tween 80 at 1:1 ratio just before testing.

and the *proportion method*. These methods address different issues: ease of application, errors in drug concentration, and uncertainties about inoculum size. Put simply, the resistance ratio method controls for errors in drug concentration. It has limited usefulness where the difference between the minimum inhibitory concentration of susceptible and resistant strains is very narrow. The absolute concentration method is technically the easiest of the three, but it does not address the problem of inoculum size, poor or slow growth of some strains on drug-containing media, nor potential errors in drug concentrations. The proportion method does not address the problem of drug concentrations, but it is superior for addressing problems with inoculum size. As the latter is the most serious problem in the view of many mycobacteriologists, the proportion method has gained the widest acceptance. However, clumping effect can introduce serious errors in the proportion method, especially when dry colonies are used. In order to overcome possible errors in calculating the proportion of resistant organisms due to clumping effects, a well dispersed inoculum must be made, especially for colonies in dry cultures. More time is required to disperse organisms of colonies in dry cultures by extending homogenisation time or to subculture them prior to drug susceptibility testing. In this respect, the absolute concentration method has an advantage as it uses the wet weight as inoculum standard.

Strains appearing to belong to the *M. tuberculosis* complex (because of their appearance and slow growth) are routinely tested for susceptibility to isoniazid, rifampicin, streptomycin and ethambutol. The IUTM Löwenstein-Jensen medium in universal containers (28 mL) or in tubes is used with and without incorporation of drugs at the recommended concentrations (Tables IV.4 and IV.5).

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: if there is growth of only five or fewer colonies, the drug susceptibility test results may not be reliable and it is recommended that no drug susceptibility testing be done on such cultures. A representative sample of 5.0 mg to 10.0 mg from the primary culture or a sub-culture within one to two 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 H_2O plus ten glass beads (3.0 mm diameter). The mixture is homogenised on a Vortex mixer for approximately one minute with brief interruptions and then (if necessary) the opacity of the suspension is adjusted by the addition of sterile, distilled H_2O to that of a standard suspension of 1.0 mg/mL of BCG or McFarland standard No. 1 (for the exact preparation refer to: Kent P T, Kubica G P. Public Health Mycobacteriology. A guide for the level III laboratory, US Department of Health & Human Services, Center for Disease Control, Georgia, USA, 1985: p 168).

Table IV.4. Critical drug concentrations and critical proportions for resistance (Löwenstein-Jensen medium)

Drug	Concentration	Critical proportion
Isoniazid	0.2 mg/L	1 %
Rifampicin	40.0 mg/L	1 %
Dihydro-streptomycin	4.0 mg/L	1 %
Ethambutol	2.0 mg/L	1 %

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 H_2O. The original suspension sediment should not be disturbed in order to avoid large clumps into serial dilution. One slope of control and of each drug-containing medium are inoculated with a loopful (0.01 mL) of each dilution.

The proportion method minimises unreliable drug susceptibility test results due to variation of inoculum size if performed appropriately.

Drug-containing media

Slopes of the Löwenstein-Jensen medium in the IUTM 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 one month.

The batch numbers of the substances should be recorded (see chapter on quality control). For some drugs it is necessary to convert the required amount of the substrate salt to the active substance, taking into account the salt moiety and purity by using a potency factor that must be specified by the manufacturer (Table IV.5). This is the case for dihydro-streptomycin (usually supplied as sulphate) and for rifampicin. Ethambutol is usually supplied as dihydrochloride and most laboratories correct for potency. Isoniazid is always used as a 1:1 salt to base ratio.

It is essential during inspissation of media to maintain the temperature at exactly 85°C for 50 minutes or when the egg is just set, and to maintain equal heating for all tubes or bottles. This requires special types of inspissators, using steam or a water bath. Excessive temperature and duration diminishes the potency of drugs in the medium.

Table IV.5. Drug preparation for proportion method of drug susceptibility testing

1. Weighing and preparing stock solutions of anti-tuberculosis drugs
2. Preparing dilutions of the stock solutions for incorporation in the medium
3. Storage of drugs and stock solutions

1. Weighing and preparing stock solutions of anti-tuberculosis drugs

Only purified drug powders from a reputed manufacturer should be used. They are best kept in a desiccator to prevent absorption of moisture from the environment. These must be replaced regularly, based on information on batch activity that can be obtained by consulting the manufacturer's website. If this is impossible, it may be best to purchase fresh powders every one or two years. Except perhaps for rifampicin, most laboratories will thus need to buy only one small vial at any time.

The potency of the powders purchased must be taken into account, as it varies from one manufacturer to the other and from batch to batch. It depends on 1) purity of the drug, 2) the water content, and 3) the salt / counter-ion fraction:

Potency = (assay purity) x (active fraction) x (1 – water content)

Example: If the assay purity of rifampicin is 0.998 (made up as an example, check the actual purity provided by the manufacturer), the active fraction is 0.950 (made up as an example, check the actual fraction provided by the manufacturer) and the water content is 0, then:

Potency = 0.998 x 0.950 x (1 – 0)

= 0.948

The potency is thus not marked on the drug vial. It can be calculated as above or sometimes obtained from the manufacturer's website. If this is not possible, it is acceptable to use average potency to calculate the fraction of active product contained in the amount weighed by multiplication: these are 1.00 for isoniazid, 0.95 for rifampicin, 0.80 for dihydrostreptomycin and 0.75 for ethambutol.

Stock solutions of the drugs keep better when more concentrated. For any drug, a stock solution of 100 mg active drug in 10 mL solvent (10 mg/mL) should be prepared and frozen in properly identified aliquots of 1 mL. This is done most easily by weighing accurately an amount close to 100 mg divided by the potency (or its average as above), i.e., about 100 mg (100/1) of isoniazid, but about 133 mg (100/0.75) of ethambutol dihydrochloride. The exact weight in mg is noted, and the exact content of active product is then calculated by multiplying the weighted amount with the potency. This amount divided by 10 gives the exact volume of solvent needed to make a 10 mg/mL stock.

Example: about 133 mg ethambutol hydrochloride has to be weighed, but the amount taken out was only 121 mg. This gives 121 x 0.75 = 90.75 mg of active product. We need to dissolve this in 9.075 (rounded 9.1) mL of solvent to obtain the stock of 10 mg/mL.

The solvent used for the stocks is sterile distilled water, except for rifampicin, where it should be dimethylsulfoxide (DMSO) or pure methanol.

Drug solutions are considered self-sterilising and should not be autoclaved or ultra-filtered.

Table IV. 5 (contd... . .)

2. Preparing dilutions of the stock solutions for incorporation in the medium

Rifampicin final concentration in the medium is 40 µg/mL, so that for 100 mL of medium 4000 µg or 4 mg are needed. The stock solution contains 10 mg/mL and does not need to be diluted further. Just add 0.4 mL stock per 100 mL of Löwenstein-Jensen medium and mix well.

The other drugs need one or more dilution steps in sterile distilled water, as shown in the table below.

Drug and stock solution concentration	Final active product concentration in the medium	Dilution factor Amount needed per 100 mL of medium	needed from stock for 100 mL of medium	Dilution steps	Volume of the last dilution needed for 250 mL medium
Isoniazid 10 mg/mL	0.2 µg/mL	20 µg = 0.02 mg	10/0.02 = 500	10 x/10 x/5 x	2.5 mL
Ethambutol 10 mg/mL	2 µg/mL	200 µg = 0.2 mg	10/0.2 = 50	10 x/5 x	2.5 mL
Dihydrostreptomycin 10 mg/mL	4 µg/mL	400 µg = 0.4 mg	10/0.4 = 25	10 x/ 2.5 x	2.5 mL

All pipettes and tubes used must be sterile, and accurate volumetric pipettes should be used. For the 10 x dilutions, add exactly 0.5 mL of stock solution to exactly 4.5 mL of sterile distilled water and mix. For the subsequent dilutions, add 1 mL of the preceding well mixed dilution to 9 mL (isoniazid second dilution) or 4 mL (isoniazid third dilution, ethambutol second dilution) or 1.5 mL (dihydrostreptomycin second dilution) sterile distilled water, and mix well.

From the last dilution, add 1 mL per 100 mL medium and mix well. For large volumes of medium, the last dilution step may have to be omitted. To prepare, for example, 500 mL of dihydrostreptomycin medium at 4 µg/mL, it is sufficient to dilute 10 x the stock at 10 mg/mL, and to add 2 mL of this dilution to the 500 mL of medium (it contains 2 mg = 500 x 4 µg active product).

3. Storage of drugs and stock solutions

Drug powders can be stored for one to two years if temperature requirements are respected:

- isoniazid and ethambutol are kept at room temperature, best with some silica gel in a dessicator (ethambutol dihydrochloride is hygroscopic)
- dihydrostreptomycin must be kept in a refrigerator at 4°C
- rifampicin must be kept frozen at -20°C.

Drug and PNB stock solutions can be kept frozen as aliquots of 1 mL in well closed and well labelled tubes for a maximum of 12 months. What is left after thawing and diluting must be discarded. However, most often the content of a vial of an antibiotic is far too much to be consumed within one or two years; in case freezing is not reliable because of power cuts, stock solutions should thus not be kept.

What is left of the other dilutions should never be kept.

Drug-containing media should be kept refrigerated, with tubes well closed, and are best used within three months.

Reading, interpreting and reporting

The seeded media are examined for contamination after one week of incubation at 37°C. The first reading of drug susceptibility test results is done at four weeks (28 days) of incubation. At that time all strains showing drug resistance can be reported as drug-resistant. Because some multidrug-resistant strains grow very slowly, it is preferable to await a second reading at six weeks (42 days) before reporting susceptibility, even if the growth on drug-free medium is profuse at 28 days. In case growth on the control media is poor even after six weeks (i.e., few or no colonies on the 10^{-4} bacterial dilution), the test should be repeated. A more correct result may then be obtained using more concentrated inocula (10^{-1} and 10^{-3}), on condition that this does not result in an excessive number of colonies. The test should also be repeated if the 10^{-4} (or 10^{-3}) bacterial dilution control slope shows profuse growth and resistance to some drugs seems to be present (risk of false resistance because of too dense inoculum).

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. The usual criterion for resistance is 1% of growth for all these four drugs (Table IV.4). If no clear interpretation is possible the test must be repeated.

9. Recording of culture and drug susceptibility testing results

A distinction must be made between information essential for public health surveillance and information essential for monitoring the process of laboratory work.

To monitor laboratory work, information is best recorded on individual cards or in a special register for each specimen with detailed information on reading results allowing accurate identification of problems with culture and drug susceptibility testing (Appendices 7 and 8 show examples). Such registers will vary according to the preferences of the individual laboratory, the number of different tests performed, the frequency of reading, and other factors.

The service unit submitting a specimen for culture must provide information about the patient on a specifically designed request form (Appendix 9).

Reported results from drug susceptibility testing must be exhaustive, i.e., 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 drug resistance of strains from new versus

those from previously treated cases. The format of reporting is shown in Appendix 10.

10. 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, and 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 among smear-positive specimens from untreated cases, and frequencies and profiles of drug resistance, is helpful for such monitoring.

The quality of drug-free culture media can be tested most simply by using a rapid growing *Mycobacterium* such as *M. fortuitum*. If no growth is observed within five days after inoculation, the medium does not have the properties required.

Quality control of drug susceptibility tests is best performed by titrating the standard strain H37Ra of *M. tuberculosis* for each newly produced batch of drug susceptibility testing media. An alternative method of quality control consists of including in each series of testing the standard strain H37Ra as well as two strains of *M. tuberculosis* with moderate levels of drug resistance to some of the drugs, which together express all four drug resistance markers.

11. Proficiency testing of drug susceptibility testing

To ensure that results of drug susceptibility testing are reliable and internationally comparable, the WHO and The Union established a network of supranational reference laboratories in 1994. The first purpose of this network was to standardise *M. tuberculosis* drug susceptibility testing throughout the world. Today the supranational network provides proficiency testing of drug susceptibility testing results for 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 ("judicial") result of the supranational laboratory network.

The second step in proficiency testing of results from national surveys consists of systematic (for resistant strains) or random (susceptible strains) 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.

12. 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 potential for bias must be clearly specified.

Patients with or without a history of previous treatment must be distinguished very clearly and reported separately. There are two reasons for this. First, sampling may have been 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 then greatly distort the picture. However, analysis of resistance combined for both groups is justified for a sufficiently large sample in which they are each represented proportionally to their frequency of occurrence, for example, the results of a population survey with systematic sampling of all newly registered cases. This may give a fair idea of the total pool of resistant strains circulating in the population at that point in time. Results from continuous sampling of previously treated cases cannot be used to study prevalence, although they should be similar to point survey results for the same group, provided that their sampling was sufficiently comprehensive and representative. They will rather be used to study trends, for which the successive year samples should be representative of all types of incident previously treated cases and their relative proportions.

In a good programme, a resistant strain from patients with a history of prior treatment most often represents transmitted primary resistance: the strain was already resistant at start of treatment and this is the reason for failure or relapse. Levels of resistance are higher because of the selection mechanism during treatment. While this has nothing to do with programme deficiencies, a subset of resistance observed in this group will be acquired due to treatment errors. However, the latter can only be distinguished by comparison with the resistance profile prior to treatment. Since this is generally impossible, only monitoring of trends in the previously treated group will allow conclusions to be drawn regarding programme performance. In poor programmes, this resistance will (rapidly) increase due to a considerable proportion of acquired resistance, while it will slowly decrease with adequate programme performance.

The previously treated group may bring to light very recent errors in the programme; the emergence of resistance in never previously treated patients is often delayed, depending on the varying incubation period of latent tuberculous infection progressing to overt clinical disease. The prevalence of drug resistance in patients without prior treatment is consequently a much less efficient means of monitoring programme performance. It is essential to collect information on the age of the patients and to calculate the 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 five 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 are very much less likely to 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. This is the case because the older the tuberculosis patient, the more likely it is that the average time elapsed since acquisition of the strain leading to the current tuberculosis is longer than in younger patients. This is particularly true where tuberculosis is declining and the risk of re-infection is relatively low. Where risk of re-infection is high, it may be less reliable.

To determine the level of resistance in previously untreated patients, calculation of the proportion of resistant strains is sufficient. The proportion of resistant strains is less meaningful for resistance among patients with prior treatment. In this case, the absolute number of cases with drug resistance identified during one year is much more informative. It is important to know as precisely 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.

Basic information recorded in the tuberculosis reference laboratory register for cultures should be computerised on a regular basis. A computer with appropriate software is essential for efficient analysis of data. Minimally required software includes EpiData and an open source office suite (such as OpenOffice) for word processing and spreadsheet utilisation. Both EpiData and OpenOffice are in the public domain and may be downloaded for free from the Internet (www.epidata.dk and www.openoffice.org).

13. Future prospects

While much has been achieved in terms of standardisation of techniques within the Global Project on Anti-Tuberculosis Drug Resistance Surveillance, the overall picture is still far from clear: only a minute fraction of the global tuberculosis patient population is covered by the project. This failure to cover entire countries through representative sampling might be at least partially attributable to the technically demanding centralised culture and susceptibility testing services and associated problems in obtaining viable bacilli in countries with difficulties in timely transport.

The complexities associated with the implementation of accurate surveillance of anti-tuberculosis drug resistance by national reference laboratories have frequently been underestimated. What is primarily needed from a surveillance point of view is simply a system that monitors rifampicin resistance, an excellent predictor of failure of regimens based solely on the six essential drugs. Molecular techniques identify mutations in the *rpoB* gene. The commercially available technique approaches the sensitivity in discovering rifampicin resistance with 95%, using the standard phenotypic method as the gold standard. Depending on the prevalence of specific *rpoB* mutation sites, its specificity is possibly on par with it. The test can be made from a sputum smear-positive specimen involving a DNA amplification step which does not require viable bacilli, followed by DNA hybridisation or sequencing to identify the possible mutations.

Specimens for molecular testing could be collected in the periphery and be transported to the central level without concern for viability. Depending on the qualification of the national reference or research laboratories, determination of rifampicin susceptibility or resistance may be carried out locally or, alternatively, the sterilised specimen sent to a supranational reference laboratory paid for by the international community to offer this service. Such a perspective may offer a possible way out of the current state of affairs which has by and large been disappointing.

D. Ethical considerations in the surveillance of drug resistance

In accordance with the World Medical Association Declaration of Helsinki, research subjects should be volunteers and informed participants in medical research which should be undertaken only if there is a reasonable likelihood that the populations in which the research is carried out stand to benefit from the research. At the conclusion of the study, every patient entered into the study should be assured of access to the best proven prophylactic, diagnostic and therapeutic methods identified by the study. It is necessary during the study planning process to identify post-trial access by study participants to prophylactic, diagnostic and therapeutic

procedures identified as beneficial in the study or access to other appropriate care. Post-trial access arrangements or other care must be described in the study protocol so that the ethical review committee may consider such arrangements during its review.

While the identification of resistance other than multidrug resistance is not usually associated with an adverse ultimate treatment outcome using combinations of the available essential drugs, cure of tuberculosis due to organisms resistant to both isoniazid and rifampicin is much less likely with any of these standardised regimens and will usually require the use of so-called second-line drugs. Not all countries have access to these second-line drugs or have services able to organise the complex treatment of patients with multidrug-resistant tuberculosis.

Where treatment of multidrug-resistant tuberculosis is available, every effort should be made to trace the patient from whom the strain was isolated, to enquire about the clinical condition, and, if the patient is not responding to treatment, to provide access to drugs that offer a chance of curative treatment. This should be described in the protocol, taking into consideration the difficulties linked with the delay to have the drug susceptibility test results available.

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CHAPTER V

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

A. The design of the peripheral microscopy laboratory

The detailed arrangement for the peripheral laboratory will vary greatly, depending on i) whether other work is also done, ii) the size and shape of the available room, and iii) whether electricity or daylight is used for microscopic examination. For example, the laboratory can be arranged to include three separate sections (Appendix 11):

- One well-lit area for preparing and staining smears
- One well-lit area for microscopy
- One area for registration and storage.

The multi-purpose laboratory, which includes mycobacteriology, should contain at least a sink and three benches or 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 stools. Some of the equipment necessary in the peripheral laboratory is listed in Table V.1.

Good ventilation is essential for the protection of the personnel from airborne infectious droplet nuclei. The best way to ensure ventilation is by using windows and doors to direct air flow to the outside environment (and not vice versa). To ensure proper ventilation in closed 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.1. Equipment needed in a peripheral laboratory.

Item	Units	Cost in Euro	
		Unit	Total
Binocular microscope, electric/mirror, spare objective, bulbs	1	800	800
Sink with drain, or plastic bowl	1	Local variation	
Watertap or large bucket with lid	1	Local variation	
Waste burning drum with lid and lock	1	Local variation	
Metal rack for staining	1	Local variation	
Drying rack, wood	1	Local variation	
Spirit lamp, metal	1	20	20
Slide-holding boxes	5	4	20
Reagent bottles 1 L, Safe-break Winchester, amber	4	15	60
Staining bottles, 250 mL, plastic with jet	3	4	12
Oil dropper bottle, plastic	1	2	2
Beaker, 1 L, plastic, with handgrip for rinsing	1	10	10
Small funnel, plastic	3	<1	2
Table, small	3	Local variation	
Chair	1	Local variation	
Laboratory stool	2	Local variation	
Shelf for bottles, etc	1	Local variation	
Cupboard for microscope	1	Local variation	

B. The design of the national tuberculosis reference laboratory

1. Location of the laboratory

The decision concerning the location of the laboratory must be taken at the highest level of the country's public health authorities, in consultation with the national tuberculosis programme. There are many considerations to take into account. National facilities are frequently located in the country's capital, where the required infrastructure for operating them in a satisfactory manner is likely to be found.

It is important, from an organisational 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 close proximity to other public health institutions, and usually together with other public health laboratories.

Physical infrastructure such as roads, water supply, sanitation, electricity and possibly gas, should be available. The latter services may be obtained 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) is expedient, and the building should have its own water tank or water purification plant.

When a particular building 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 like the terrain, accessibility, view, traffic noise, exposure to sun, wind, and airborne dust, are factors that will influence the design of the building.

Should the site 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 encumbrances relating to the plot have 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 can cause delays and conflicts with respect to responsibility for the additional costs incurred.

2. Space planning criteria

Table V.2 shows the required rooms and approximate dimensions of 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.

3. Space planning relationships

Table V.3 indicates the proximity/relations between the building's various functions. It is not necessary to get too concerned with the precise details of the room/space plan. The description of functions should be seen as "an area designated for a specific purpose/function", and not necessarily a space confined within walls and a closed door. Related functions are best located inside the same four walls. There are, however, a few functions that have to be totally isolated from others, for example the media processing section, which has quite specific criteria for cleanliness and a darkroom for fluorescence microscopy.

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
Sterilisation section	12	Cleaning and sterilising of material
Room for fluorescence microscopy	4	Dark room for fluorescence microscopy
Cold room (4°C)	6	Storage of chemicals and culture media in a cold room or refrigerators
Incubation room (37°C)	8	Incubating room
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

The reference laboratory in Dakar, Senegal, as shown in the drawings in Appendix 12, 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 between the various parts of the laboratory without having to pass through doors. In this manner the staff working in various parts of the laboratory can also see and communicate with one another. Hygiene is best assured through standard operating procedures; it is the standard operating procedures that determine whether or not contamination is likely to occur.

Table V.3. Proximity/relations between the building's various functions

	Specimen reception	Microscopy	Culture	Sterilisation	Media processing	Fluorescence microscopy	Cold room	Incubator room	Gas storage	Storage
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
Sterilisation section		CL	DA		DA			CL	DA	CL
Media processing section	SF		DA	DA			DA		CL	CL
Room for fluorescence microscopy		DA	DA							
Cold 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

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 sterilisation section has direct access to the storeroom. In this way, all materials can be washed and/or sterilised 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 to 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. Several refrigerators should be available to provide a useful backup capability.

The weakness of this particular plan (Appendix 12) 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 sterilised glassware and other sterilised items. A window from the sterilisation section is convenient for transferring equipment after sterilisation. The main storeroom adjacent to the sterilisation section is used for non-sterile equipment and packed sterile equipment delivered from external suppliers. Items that need to be sterilised before use pass through the sterilisation section. Sterilised supplies are unwrapped in the laboratory.

Where structural conditions allow, it is desirable to use sturdy but transparent panels to form the upper parts of walls or partitions between rooms. This enables easier visualisation amongst workers and monitoring of activities, including accidents, that may require immediate action.

The laboratory can also benefit from an extra store room or spare room, which is available for future developments. It may also be useful 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 be made available for a small wash/changing room at the entrance to the laboratory so that personnel can change their clothing and wash when entering and leaving. A wash basin, a hand dryer and wardrobe should be provided.

4. 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 storerooms, 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 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 that can be eliminated should it be unaffordable. In a laboratory that is fully air-flow controlled, air-conditioning needs to be controlled to ensure flow is from the clean to the contaminated areas.

An optimum 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 directional air flow from the clean to the contaminated area.

However, this is a sophisticated technical solution that will be difficult to achieve in many countries. It requires constant operation 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-conditioner fans should be situated so that the air stream does not influence sensitive working areas, such as the safety cabinets. Above the autoclaves it may be convenient to hang glass or steel exhaust canopies from the ceiling to collect the steam and odours when opening the autoclaves.

5. 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 dimensions 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 provides an overview of the construction measures that need to be carried out in order to facilitate the installation of necessary equipment in the most important sections of the laboratory.

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

Section	Minimum 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
Sterilisation 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
Cold-room (+4°C)	Several 220V/1-phase for refrigerators or isolated room with temperature gauge, shelves
Incubation room (+37°C)	Temperature control, temperature reading and registration outside, shelves
Storage section	Shelves
Gas storage room	Access from outside

C. The construction of the national tuberculosis reference laboratory

1. Working with public authorities

It is important that the reference laboratory be under the administrative control of the national public health authorities who will be responsible for the project. Contact should be established at the highest possible level of administration. A senior official of the public health services provides the liaison with the director of public services 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 site has been set aside for this purpose, and also that this decision will not be changed once construction has begun.

Similarly, the relevant regulations with respect to import duties, taxes and other fees must be clearly understood. 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 often simpler and more efficient to include the tax in the original budget.

2. Establishing the project team

Establishing the project team is decisive for the organisation 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 organisation and composition of the project team. This depends on the specific local conditions. In any event it must be clear 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 organisations. 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 Union, drew up the building plans. Additional expertise was provided by the Senegalese authorities as well as by local private companies. In this instance, it worked well, but once more it is important to stress that the framework conditions of the project have to be taken into consideration.

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

3. 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 chooses to put a project out 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 follow-up is sporadic. It is a general rule 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. A spreadsheet 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 programmes for working out technical descriptions, but these are often based on national standards and are not universal.

It is crucial 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 agree on what has been specified, thus preventing misunderstandings.

4. 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, the contract 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 organisations that 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 as to quality and progress incorporated in the contract, 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 with regard 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 specified daily fines. Progress should be tied not only 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.

5. Project management

The importance of choosing a knowledgeable local building entrepreneur has already been emphasised, but it is equally important that there be open communication between this person and the builder/project leader. Monitoring progress is carried out through regular written reports of what has been achieved 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 that the builder/project leader undertake several inspections during the construction period. Informal direct telephone contact with the builder/project leader can also be very useful.

Meetings of all parties involved in the project should be held every 14 days in order to verify progress at the building site and to discuss 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 should be taken up with the builder/project leader immediately.

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

6. Completion and hand-over

As the hand-over date nears, the entrepreneur must arrange a timely inspection process. 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 the correction 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 that for whatever reason 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 one 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.

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 one third of the total cost

Category of work	Euro	Euro / m²
Builders' work	147,500	218
Incubator room	5,430	
Electrical installations	65,200	97
Air-conditioning/cooling installations	15,500	
Sanitation and plumbing	4,660	7
External works: fence, gate, walls	12,420	
External works: roads, hardstandings, services	63,630	
Telephone/electricity connections	5,660	
Structural engineering	10,090	15
Global cost	**330,090**	**487**

D. Materials management for peripheral sputum smear microscopy

The national tuberculosis reference laboratory must cooperate closely with management of the national programme in providing input to ensure that there are sufficient supplies of laboratory materials at all times 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 more than 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. These items in particular will often need expert input for correct specifications.

1. Supplies for peripheral sputum smear microscopy

To ensure a continuous flow of materials, programmes must budget rationally for requirements. Unless comprehensive data on the number of sputum smear examinations performed are available, 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.

If recent data are available on the number of sputum smear examinations for acid-fast bacilli performed, this will be used directly as the basis for calculation of requirements. Otherwise, the material that is required is based on empirical observations of the proportion of cases found among tuberculosis suspects. This proportion may vary considerably among countries. The fraction of suspects identified as cases is relatively easily ascertained from the Tuberculosis Laboratory Register. If the proportion is not yet known, The Union 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 Nicaragua, for example.

To demonstrate an example of the procedures for calculating 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%. An additional assumption is that each suspect requires three sputum examinations, and that each case among them 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)*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 required for one slide, multiplied by 23 slides. Needs for items of equipment, such as microscopes, spirit lamps, wire loop holders and wire and slide boxes, are better estimated from the number of functioning microscopy centres, taking into account their expected lifespan and the fact that they are also used for other purposes. For the same reason, the quantity of good quality immersion oil ordered should be two to three times more than what is strictly needed for tuberculosis. This will ensure that tuberculosis programme microscopes are always operated with good oil, thus prolonging their useful life. It should be re-emphasised here that locally available wooden (or preferably bamboo) stick applicators are preferable to wire-loop holders.

In the above example, 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 must be calculated from the amount actually used with the method for staining, assuming in addition that 5 mL of each of the respective solutions are needed for each slide. For sulphuric acid and alcohol, technical grade purity gives satisfactory results. However, basic fuchsin and phenol should be of high quality.

From the most peripheral levels of the health service, it is most efficient to simply request a report on the balance in stock and the number of smears done. This information can be used by those responsible for dispatching supplies to calculate the quantities required, using a standard spreadsheet. If these calculations are made by those requesting the supplies, they have to be rechecked at the dispatch level in any case, as request forms that require calculations are often incorrectly completed. Appendix 3 shows a peripheral laboratory report form, including, in addition to the number of smears examined, the amounts of ready-made stains and other essential items left in stock. At the intermediate level, where stains are prepared, a more elaborate request form is needed, showing the various chemicals needed to prepare stains. An example of an intermediate level supply request form is given in Appendix 13. This form is best used as a standard computer spreadsheet, prepared for and distributed by the national tuberculosis programme. To ensure reserve stocks at all levels, it is important to enter as balance in stock only the remaining quantities physically counted in the regional level store, without adding quantities reported in stock at more peripheral levels.

E. Supplies for the national tuberculosis reference laboratory

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

The reference laboratory can only function if it has the required minimum amount of equipment.

The necessary equipment is described briefly. A complete list of minimum requirements for hardware and the approximate costs is provided in Table V.6.

1. Microscopy section

At least three binocular microscopes for bright-field microscopy are needed, of the same brand as used in the national network of microscopy laboratories. This is important for familiarising trainees with the equipment they will be using in the field, and for allowing the technicians in the reference laboratory to be thoroughly familiar with the equipment used in the field. It is also psychologically important for students to see that the reference laboratory uses the same equipment as everybody else.

Teaching microscopes with side arms are of limited usefulness. Initial demonstration of acid-fast bacilli in a highly positive slide does not require them, while further self-practice on a mix of positive and negative slides will be too time-consuming for the facilitators to follow closely. A more practical method is to require on-the-spot confirmation by the facilitator of any positive specimen identified by the trainee, and verification of declared negative results from a list of known results. A fluorescence microscope should also be available to efficiently examine large numbers of slides from routine laboratory requests.

2. 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 can be used to homogenise the whole egg media base, but because of the bubbles and foam created, it is more practical to prepare the material by shaking with large glass pearls or marbles.

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

Table V.6. 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 (all costs are in Euro).

Section	Item	Units	Unit cost	Total cost
Microscopy	Fluorescence microscope	1	5,714	5,714
	Teaching microscopes	1	2,571	2,571
	Light microscopes	3	1,905	5,714
	Bunsen burners, gas lighters	3	12	37
	60 L drying oven	2	1,219	2,438
	Mercury lamp	2	114	229
Media processing	2-speed blender	1	381	381
	Inspissator, optional	2	3,048	6,095
	Laminar flow hood	1	6,571	6,571
	Top-loading electronic balance	1	286	286
	Magnetic stirrer hot-plate	1	286	286
	Vortex mixer	1	190	190
	Water still, 8L/hr	1	3,810	3,810
	Pipetting syringes	5	194	969
	Siphon tubes with clamp for dispensing media	3	5	14
Storage	Deep freezer (2.0 m*0.6 m*0.6 m)	2	3,619	7,238
	Refrigerator, 3 doors	1	2,571	2,571
	Tray for McCartney bottles	20	29	571
	Tray for Universal containers	30	29	857
	Bench tidy to hold wire loops, markers	3	50	150
	Desiccator	2	139	278
	Thermometers	2	5	10
Culture processing	Biological safety cabinet Class IIB, type B2, spare HEPA filters, air flow metres	2	10,476	20,952
	Centrifuge, refrigerated, 4 buckets, each with lid	2	4,762	9,524
	Bunsen burner	3	12	37
	Twin beam balance, 210g	1	171	171
	60 L drying oven	1	1,219	1,219
	Vortex mixer	2	190	381
	Timer	2	20	40
	Dispenser	2	190	381
	Roll-in trolley	3	381	1,143
Sterilisation	Vertical loading autoclave	2	23,810	47,619
	Oven	1	1,714	1,714
	Buckets, stainless steel	10	67	667
	Pipette case	5	20	100
	Stainless steel discard jug with funnel on top	4	5	19
	Vortex mixer	1	190	190
	Glass brushing machine	1	11,429	11,429
	Hot air oven 110 L	1	1,181	1,181
Total			88,585	143,750

Continuous pipetting syringe outfits are recommended for dispensing set amounts of media into universal containers. To increase the lifespan of such systems it is necessary to take extreme care in cleaning after use. Some laboratories use easy-to-clean wide-bore burettes, graduated per 5 mL, with flexible tubing and a Mohr clamp to dispense the medium. If the laboratory produces media on a large scale, it is better to use the electric dispenser.

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 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. De-ionised water should never be used, as saprophytic mycobacteria tend to colonise these installations.

An inspissator for coagulation of the media in the universal containers, slanted, completes the list of equipment necessary for the media processing section. When ordering the inspissator, the type of culture vials for which it is meant should be specified, as some models can only be used with universal or McCartney bottles, others with culture tubes. As an alternative to an inspissator, a fan-operated hot air oven with accurate temperature control can be used, although this may result in less homogeneous batches.

3. 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.

4. Culture processing section

The risk of transmission of tubercle bacilli in the reference laboratory is increased when handling cultures of *M. tuberculosis*, particularly in aqueous suspensions, as is the potential of their aerosolisation during processing. The processing of cultures should always be carried out in a biological safety cabinet (see further on for specifications).

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 that are opened inside the safety cabinet.

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 at least 200 g, but a sensitivity of 0.1 g is sufficient.

There are two approaches to the incubation of cultures. One option is to utilise 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. As an alternative to shelves, roll-in trolleys made to fit the size of the racks, and with a capacity sufficient for all vials seeded during one week, constitute a practical alternative for high volume laboratories.

5. Sterilisation 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, at least two should be available. This will allow sterilisation of "dirty" and "clean" material. Dirty material includes cultures of mycobacteria that need to be destroyed. Clean material includes reagents and material not grossly contaminated. Using spores that are autoclaved and subsequently incubated on appropriate culture media provides a regular check on the efficiency of autoclaves. As standby equipment in case of autoclave breakdown or power failure, large pressure-cooker type autoclaves heated over a gas or kerosene burner are recommended.

A glass brushing machine with appropriate brushes greatly facilitates the work of cleaning glassware. Immersion in a strong detergent for three to four days has been used to effectively remove the Löwenstein-Jensen media from universal bottles before washing.

A hot air oven for sterilising 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 put 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 summarised in Table V.7.

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 cannot be calculated directly from the number of reported cases. A summary of material and reagents and their approximate costs is provided in Table V.8.

F. Special biosafety considerations in the laboratory

Transmission of *M. tuberculosis* results essentially from micro-aerosols, droplet nuclei containing tubercle bacilli of a size greater than 1 µm and less than 10 mm 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. Different procedures entail considerably different risks of airborne transmission, depending on the extent of aerosolisation and the number of infectious particles that can be produced by the procedure.

1. Specimen collection

In many countries, patients suspected of tuberculosis are referred directly to the laboratory for instruction on how to produce sputum and its subsequent

Table V.7. Recommended glassware and consumables, description and use in the national tuberculosis reference laboratory

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

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

Glass beads. 3 mm glass beads 1 kg per pack, useful for homogenisation of bacterial suspensions.

Bottles, Winchester. Glass, narrow mouth, with aluminium screw cap and rubber liner for stains and chemical, capacity 150 mL to 2,500 mL. Winchester bottles are mainly used for Löwenstein-Jensen mineral salts and for other chemicals, and are useful for storing 4% NaOH for decontamination of clinical specimens.

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.

Test tubes. Glass, capacity 5 to 10 mL for performing biochemical identification tests. Sterile disposable, capacity 10 mL for titration of bacterial suspensions for drug susceptibility tests.

Funnels. Funnels for filtering, graduated 50 mL to 2,000 mL, are used for filtering stains such as 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.

Pipettes, disposable. 1 mL disposable plastic pipettes for dispensing chemicals for biochemical tests and inoculating into Löwenstein-Jensen media. Eppendorf pipettes and tips 1 mL – 5 mL are useful for making accurate titrations of anti-tuberculosis drug solutions while preparing drug-containing media.

Loops, disposable. 10 µL sterile disposable plastic loops for specimen transfer, making direct smears and inoculating into Löwenstein-Jensen media.

Slides for microscopy. Twin frosted (roughed on both sides at one end) superpremium 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 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. 7 (contd.)

Item	Packing	Cost in Euro
Bottles, universal.	144/pk	110
Bottles, McCartney: 7 mL	144/pk	101
14 mL	144/pk	103
Glass beads	1Kg	21
Bottles, Winchester		
Beakers	1 No	10
Cylinders	1 No	20
Flasks, heavy duty	1 No	10
Flasks, volumetric	1 No	5
Test tubes	500/pk	48
Funnel	1 No	3
Pipettes, volumetric: 1 mL	50/pk	16
5 mL	50/pk	25
10 mL	50/pk	26
Pipettes, disposable	500/pk	12
Loops, disposable	4,000/pk	63
Slides for microscopy	72/box	7
Total		579

collection. This is a rational and efficient approach because it limits the number of those giving instructions to those who know best what kind of specimen they need. At the same time, it exposes the laboratory technicians to a 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 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 them how to produce sputum. Second, the production of the sputum specimen must take place well outside the building, to allow aerosols to be diluted and exposed to the ultraviolet radiation of direct sunlight.

2. Smear preparation

While opening sputum containers and making a smear may produce micro-aerosols, the risk of transmission from such procedures is negligible (due to the small physical force on a highly viscous material) in comparison to that generated by aerosols produced by a single unprotected cough. There is no epidemiological evidence that solely preparing smears is associated with any measurable excess risk of acquiring tuberculous infection. Consequently, biological safety cabinets are not necessary in peripheral laboratories that perform only smear microscopy.

Table V.8. Reagents used in the reference laboratory

Item	Unit size	Cost in Euro
Fluorescence microscopy		
Auramine	50 g	11
Phenol crystals	1000 mL	38
Ethanol 95%	1000 mL	2
Potassium permanganate	100 g	33
Hydrochloric acid (conc 37%)	1000 mL	10
Sodium chloride	5000 g	42
Ziehl-Neelsen acid-fast stain		
Basic fuchsin	500 g	267
Ethanol 95%	1000 mL	2
Phenol crystals	1000 mL	38
Sulphuric acid, at least 95%	2500 mL	55
Methylene blue	500 g	225
Catalase test		
Hydrogen peroxide 30% p.a.	1000 mL	50
Tween 80	500 mL	30
Decontamination - Petroff method		
Sodium hydroxide	2000 g	18
Phosphate buffer 0.067 M pH 6.8 or 7.0		
Disodium phosphate anhydrous	2500 g	90
Monopotassium phosphate	1000 g	60
IUTM base		
Potassium dihydrogen phosphate	2500 g	43
Magnesium sulphate	1000 g	23
L-asparagine	1000 g	167
Magnesium citrate	100 g	15
Malachite green	2500 g	786
Glycerol	100 mL	2
Addition in acid-buffered medium		
Monopotassium phosphate	250 g	Variable
Sodium glutamate	1000 g	Variable
Addition in Stonebrink medium		
Sodium pyruvate	250 g	131
Addition in para-nitrobenzoic medium		
Para-nitrobenzoic acid	500 g	38
Hydrochloric acid 1 N solution	1000 mL	1
NaOH 1 N solution	1000 mL	1
Drugs for susceptibility testing		
Rifampicin	5 g	217
Dihydrostreptomycin sesquisulphate salt	25 g	19
Isoniazid	100 g	33

Table V. 8 (contd.)

Item	Unit size	Cost in Euro
Ethambutol dihydrochloride	25 g	33
Dimethylsulfoxide	1000 mL	40
Nitrate reduction test		
di-Sodium hydrogen phosphate	500 g	8
di-Potassium hydrogen phosphate	1000 g	19
Sodium nitrate	500 g	29
Hydrochloric acid conc 37%	1000 mL	6
Sulfanilamide	100 g	14
N-(1-naphthyl)-ethylenediamine dihydrochloride	5 g	15
Zinc dust	100 g	17
Detergent for washing bottles	200 kg	71
Disinfectant for *M. tuberculosis*		
5% hycolin (phenolic disinfectant)	5 L	15

3. 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 during 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 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 metres 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. 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.

If, in addition to environment and personnel protection, product protection is sought, then the Class II B is required. Type B of Class II biological safety cabinets provides exhaust out of the building and is the preferred type in this class.

Regular certification and maintenance, with replacement of filters as needed, is a major challenge in low-income countries. Without this, the filters will become clogged and sooner or later tubercle bacilli may be blown into the face of the operator. For this reason, a Class I cabinet without filters but with the exhaust high above the ground outside may constitute the safest option whenever good maintenance can not be guaranteed.

4. Disposal of infectious waste material

Each 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.

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

For external quality control of 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 is compounded when cultures of resistant strains are transported.

Some international organisations, 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 organisations have also developed agreed common definitions, and packaging and labelling requirements. Information on the documentation requirements should be obtained from the appropriate national authorities from both sending and recipient countries.

Infectious substances and diagnostic specimens likely to contain infectious substances require triple packaging, in accordance with the recommendations of the United Nations (UN), using standard UN type 6-2/02 packing material. Petri dish cultures should not be shipped. Cultures of mycobacteria might be shipped on solid medium in screw-cap tubes or freeze-dried in vials as primary watertight containers. Alternatively, the

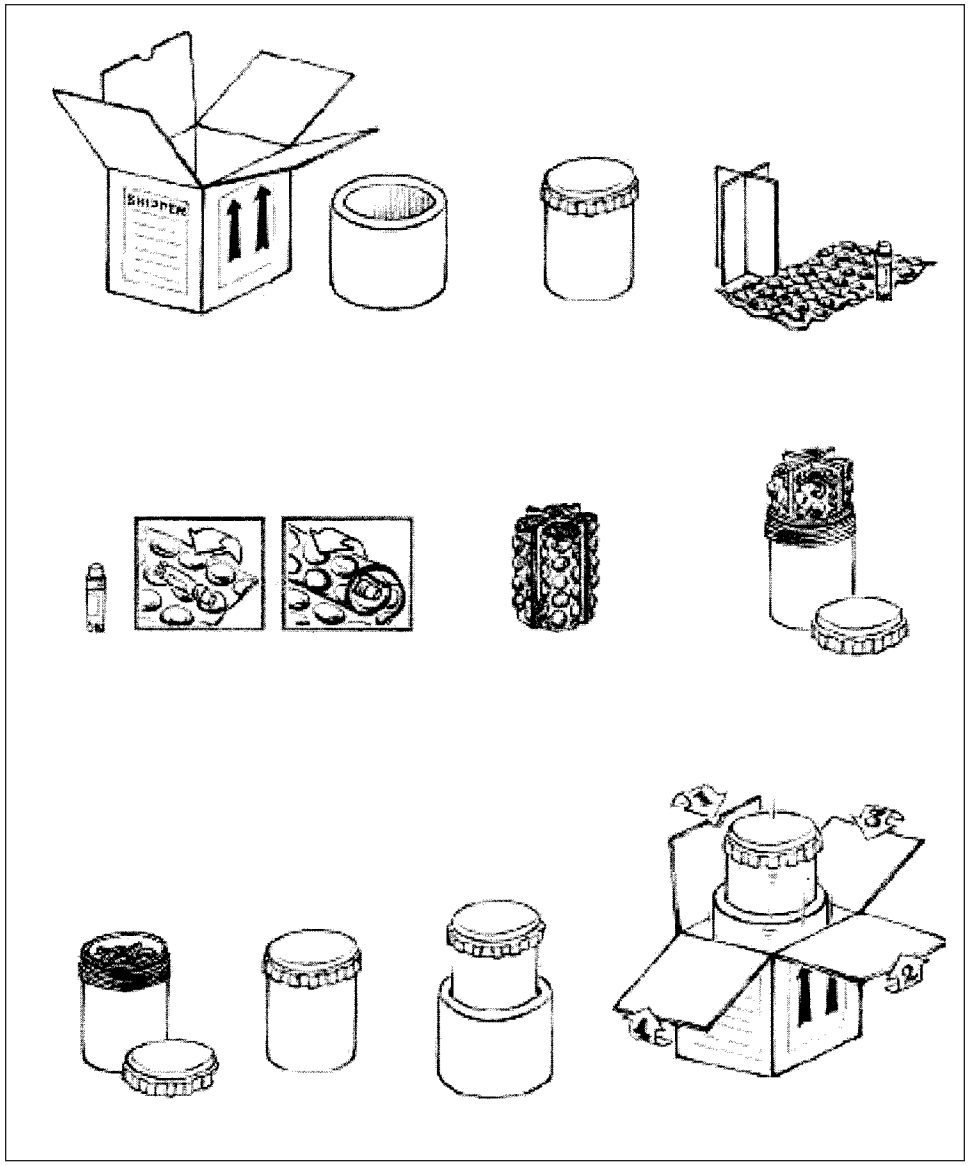

Figure V.1 Standard packaging for safe international shipment of *Mycobacterium tuberculosis*.

most convenient method for shipping is to mail mycobacterial suspensions in liquid medium in 1.5 mL amounts kept in 2 mL cryovials. The primary container should be entirely surrounded by at least 2 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 fibre 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. In addition to the sender's and recipient's addresses, the telephone numbers should be put on the outside of the package, taking care that the biohazard label remains intact and well visible.

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.

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CHAPTER VI

Appendices

Appendix 1 Request and reporting form for sputum smear examination

Appendix 2 Laboratory register for sputum smear microscopy

Appendix 3 Reporting form for peripheral sputum smear laboratory performance and stock situation

Appendix 4 Form for panel testing of sputum smear microscopy using slides sent from the reference laboratory to the periphery

Appendix 5 Form for blinded rechecking of slides sent from the periphery to the reference laboratory

Appendix 6 Reporting form for regional summary results of rechecking quality assessment

Appendix 7 Laboratory register for primary cultures

Appendix 8 Laboratory register for bacterial identification and susceptibility testing

Appendix 9 Request and reporting form for culture examination of sputum

Appendix 10 Format for reporting summary results of drug susceptibility testing

Appendix 11 Design of the peripheral microscopy laboratory

Appendix 12 Design and plans of the national tuberculosis reference laboratory

- Figure 1: Plan A Site and situation plan
- Figure 2: Plan B Elevations north, west, south and east of the tuberculosis reference laboratory
- Figure 3: Plan C Details of the tuberculosis reference laboratory
- Figure 4: Sketch of laboratory with controlled air flow

Appendix 13 Reporting and request form for regional sputum smear laboratory supplies

Appendix 1. Request and reporting form for sputum smear examination

Request for sputum examination

The completed form with results should be sent promptly to the referring facility (originator)

Referring facility (originator):* _____
Basic Management Unit: _____
Date: _____ Referring facility (recipient):_____

Name of patient: _____ Age:____ Sex: M ___ F ___

Complete patient's address: _____

_____

Reason for examination:

Diagnosis ☐ OR Follow-up ☐

Number of month of treatment: _____ BMU TB Register No:† _____

Disease site: Pulmonary ☐ Extrapulmonary ☐ (specify): _____

Number of sputum samples sent with this form: _____
Date of collection of first sample: _____

Name and signature of specimen collector: _____

* Including all public and private health facility/providers

† Be sure to enter the patient's BMU TB Register No. for follow-up of patients on chemotherapy

---

RESULTS (to be completed by laboratory)

Lab. Serial No.: _____

DATE collected	SPECIMEN	Visual appearance‡	RESULTS				
			Neg	Low (+) (1-9)	(+)	(++)	(+++)
	1						
	2						
	3						

‡ (B): blood-stained; (M): muco-purulent; (S): saliva

Date _____ Examined by _____ Signature _____

Appendix 2. Laboratory register for sputum smear microscopy

Lab. Serial No.	Date specimen received	BMU TB Register No.*	Name (in full)	Sex M/F	Age years	Complete address (for new patients)	Name of referring health facility†	Reason for examination		Microscopy results¶			Signature Remarks
								Diagnosis‡	Follow-up§	1	2	3	

* Only for confirmed TB case registered in the BMU TB register.

† Facility that referred (sent) the patient (or specimen or slides) for sputum smear examination. Use standardised type of referring facility according to block 2 of the yearly report. Referring facility/provider type is defined as health structure or health providers working in health structure in any of the following TB control functions (DOTS): referring TB suspects/cases, laboratory diagnosis, TB treatment support

‡ Tick or indicate if TB suspect is re-examined just after antibiotics.

§ Indicate month of treatment at which follow-up examination is performed.

¶ (NEG): 0 AFB/100 fields; exact number if 1-9 AFB/100 fields; (+): 10-99 AFB/100 fields; (++): 1-10 AFB/field; (+++): > 10 AFB/field

Appendix 3. Reporting form for peripheral sputum smear laboratory performance and stock situation

Quarterly report Quarter / Year: .....

AFB laboratory performance and stocks

Centre:..... District:..... Region:.....

Smears examined	Positives	Negatives	1-9/100 fields	Total
Number of suspect smears examined during the quarter				
Number of follow-up smears examined during the quarter				
Total smears examined				

Stocks left at the end of the quarter

Carbolfuchsin solution .....millilitres

Methylene blue solution.....millilitres

Immersion oil.....millilitres

.....

.....

Destaining solution.....millilitres

Burning spirit .....millilitres

Slides ..... pieces

Sputum containers..... pieces

.....

Appendix 4. Form for panel 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		
G		
H		
I		
K		

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 10 acid-fast bacilli	per field in 50 fields	2+
>10 acid-fast bacilli	per field in 20 fields	3+

Appendix 5. Form for blinded rechecking of slides sent from the periphery to the reference laboratory

Blinded rechecking of sputum smear examinations for acid-fast bacilli

Peripheral laboratory: _____ Second level technician: _____

Local technician(s): _____ Laboratory: _____

Date sampled: _____ Third level technician: _____

Period in lab. register checked: _____ Laboratory: _____

Peripheral lab		Results of		Specimen	Size	Thickness	Staining	Comment
Slide No.	Result	second level	third level					

Peripheral lab		Results of		Specimen	Size	Thickness	Staining	Comment
Slide No.	Result	second level	third level					

Specimen, size, thickness and staining appropriate: M = marginal, P = poor

Peripheral results	Final countercheck results					
	Negative	1-9 AFB	1+	2+	3+	Total
Negative						
1-9 AFB						
1 +						
2 +						
3 +						
Total						

Summary of errors identified (nos.)				
Major errors		Minor errors		
HFP	HFN	LFP	LFN	QE
Total major errors:		Total minor errors:		

HFP = high false positive; HFN = high false negative; LFP = low false positive; LFN = low false negative; QE = quantification error

Second level results	Final countercheck results					
	Negative	1-9 AFB	1+	2+	3+	Total
Negative						
1-9 AFB						
1 +						
2 +						
3 +						
Total						

Summary of errors identified (nos.)				
Major errors		Minor errors		
HFP	HFN	LFP	LFN	QE
Total major errors:		Total minor errors:		

Goal met Yes: _____ No: _____

Recommendations: _____

_____

_____

Appendix 6. Reporting form for regional summary results of rechecking quality assessment

Re checking of sputum smears for AFB

Quarterly / Annual Report Form

Region:_____ First controller: _____

Quarter: _____ Second controller:_____

Year: _____ QA coordinator: _____

Name of the controlled labs	Numbers of smears routinely examined during the period			Numbers of smears rechecked			Numbers of errors made				
	Pos	1-9	Neg.	Pos.	1-9	Neg.	HFP	LFP	HFN	LFN	QE
TOTAL REGION											

First controller evaluation	Number of positive control results	Number of 1-9 control results	Number of negative control results	Numbers of errors made				
				HFP	LFP	HFN	LFN	QE

Appendix 7. Laboratory register for primary culture

	Specimen					Patient		AFB smear		Quantified growth readings									
Primary culture serial number	Date received	Date sampled	Type	Specimen ID number	Centre of origin	Patient identification	New / follow-up	Local result	Culture lab result	Media inoculated	Week 1	Week 2	Week 3	Week 4	Week 5	Week 6	Week 7	Week 8	(Provisional result)
										LJ 1									
										LJ 2									
										Pyruvate									
										LJ 1									
										LJ 2									
										Pyruvate									
										LJ 1									
										LJ 2									
										Pyruvate									
										LJ 1									
										LJ 2									
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										Pyruvate									
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										LJ 2									
										Pyruvate									
										LJ 1									
										LJ 2									
										Pyruvate									

Appendix 8. Laboratory register for bacterial identification and drug susceptibility testing

				Quantified results of growth / reactions																	Final results	
Drug susceptibility test serial number	Primary culture serial number	Data culture declared positive	Date tests set up	Reading time	Control - 2	Control - 4	Isoniazid - 2	Isoniazid - 4	Rifampicin - 2	Rifampicin - 4	Streptomycin - 2	Streptomycin - 4	Ethambutol - 2	Ethambutol - 4	PNB	Catalase	.....	.....	.....		Identification	Resistance profile
				4 weeks																		
				6 weeks																		
				4 weeks																		
				6 weeks																		
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				4 weeks																		
				6 weeks																		

Appendix 9. Request and reporting form for culture examination of sputum

Origin of request:

District:_____ Region:_____ Local laboratory identification:_____

Date specimen was collected: ____/____/20____ Local laboratory serial number:_____

Person requesting examination: Name:_____ Position:_____

Patient identification:

Surname and given 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	Contaminated	Neg	1-9 colonies actual count	10 - 100 col 1+	>100 - 200 col 2+	>200 col 3+
1						
2						

Results of drug susceptibility testing

Isoniazid	Rifampicin	Ethambutol	Streptomycin

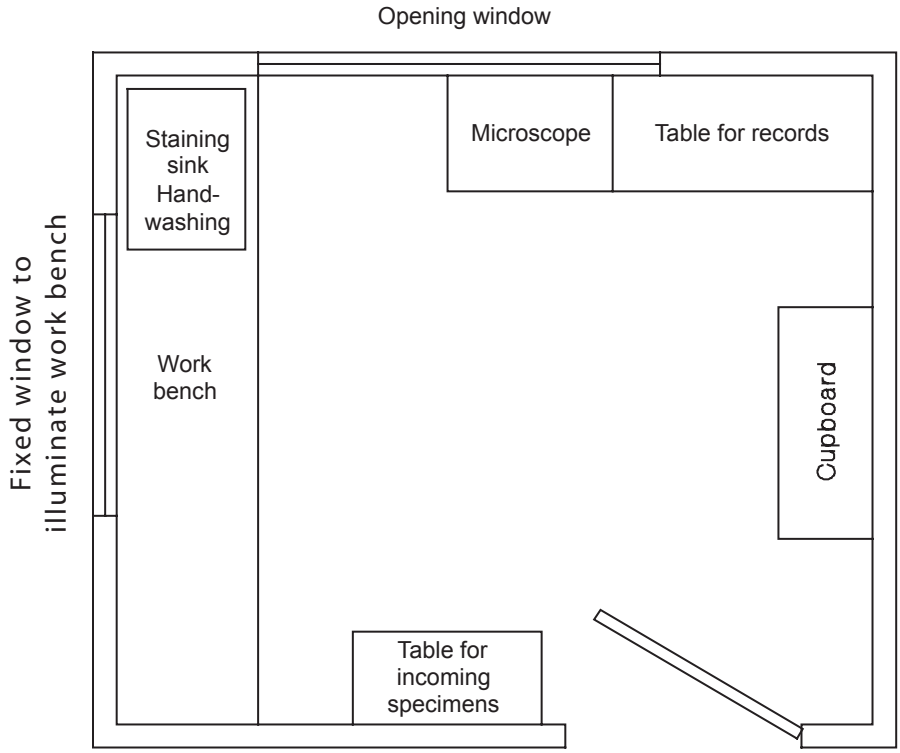
Date: _____/_____/20_____ Signature:_____

Appendix 10. Format for reporting summary results of drug susceptibility testing

Pattern of resistance / susceptibility	Type of patient								
	No previous treatment (or < 1 month)	Previously treated (≥ 1 month)						Unclassified treatment status	Total
		Relapse	Failure	Return after default	Chronic excretor	Unclassified	Total		
Total tested									
Fully susceptible									
Any resistance									
Monoresistance									
Isoniazid (H)									
Rifampicin (R)									
Ethambutol (E)									
Streptomycin (S)									
H + R resistance									
HR									
HRE									
HRS									
HERS									
H + other resistance									
HE									
HS									
HES									
R + other resistance									
RS									
RE									
RES									
Other polyresistance									
ES									
Any R resistance									
Any H resistance									
Any HR resistance									

Appendix 11. Design of the peripheral microscopy laboratory

Adapted from: Collins C H, Grange J M, Yates M D. Tuberculosis bacteriology. Organization and practice. 2nd ed. Oxford, UK: Butterworth-Heinemann, 1997.



Appendix 12. Design and plans of the national tuberculosis reference laboratory

While a reference laboratory will usually be integrated into an existing building and space, and planning criteria may vary considerably depending on the specific situation, the case of the National Centre 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.

Figure 1:

Plan A Site and situation plan

Figure 2:

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

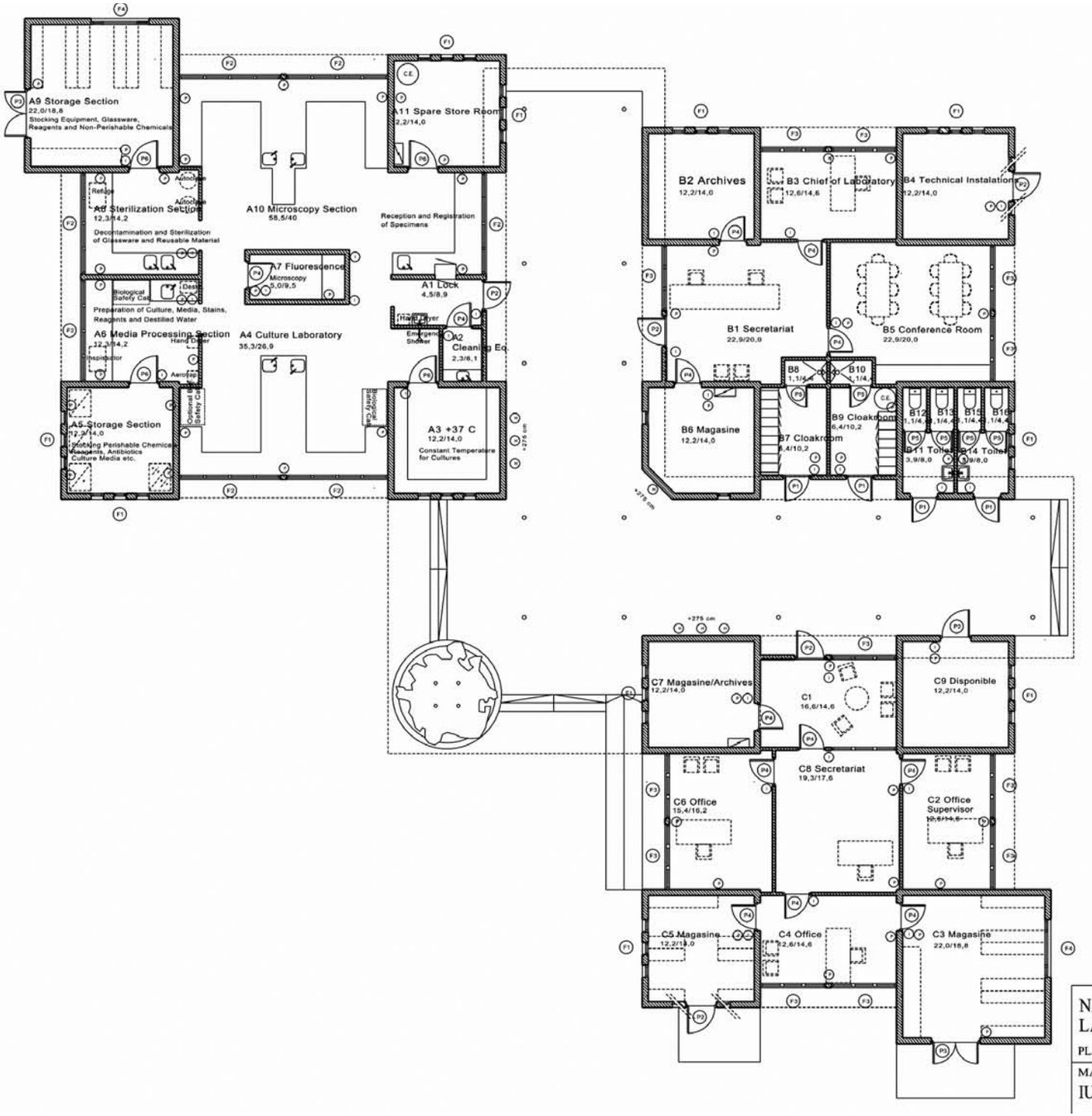
Figure 3:

Plan C Details of the tuberculosis reference laboratory

Figure 4: Sketch of laboratory with controlled air flow

Appendix 12.

Figure 1 – Plan A



- Switch
- El. outlet
- Wall Light
- 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 90X210 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

0 1 2 3 4 5 6 7 8 9 10 meters

NATIONAL REFERENCE LABORATORY - SENEGAL

PLAN

MADE BY LHL FOR

IUATLD

SCALE:

DATE: 1994

DRAWING: H.M.

ARCHITECT: H.M.

REV.:

PLAN No:

Figure 2:

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

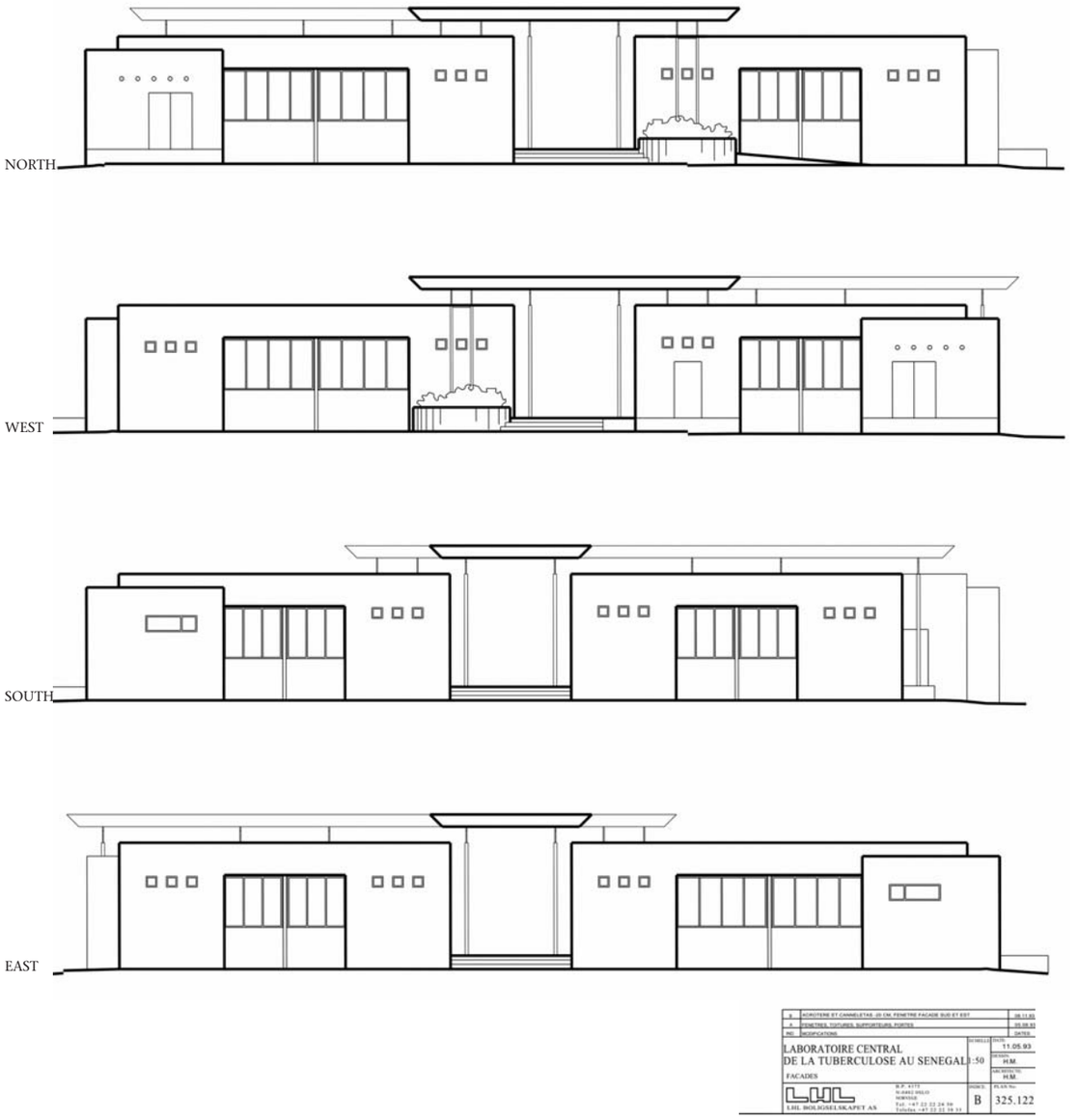
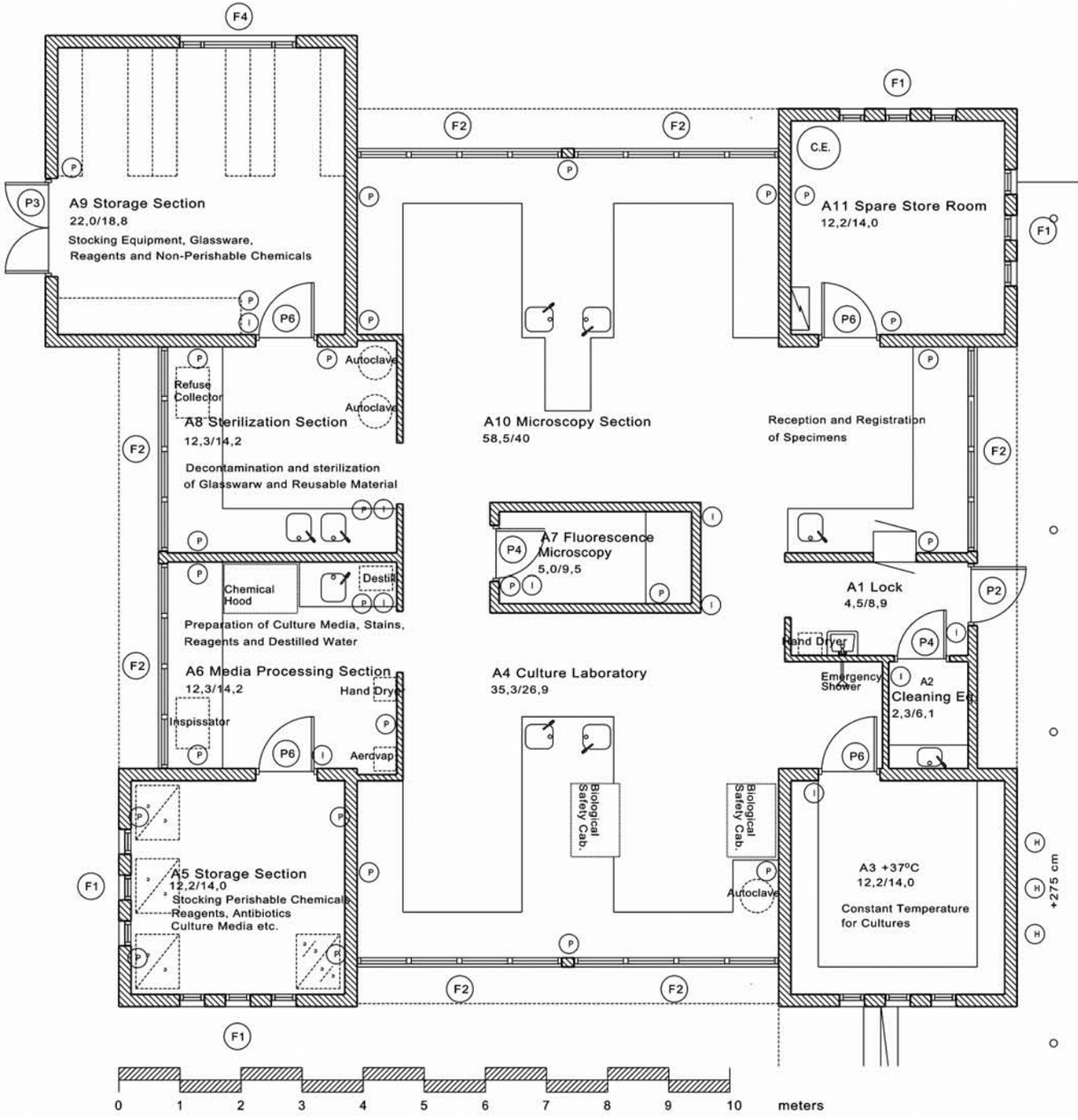


Figure 3:

Plan C Details of the tuberculosis reference laboratory



- I — Switch
- P — El. Outlet
- H — 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 90X210 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

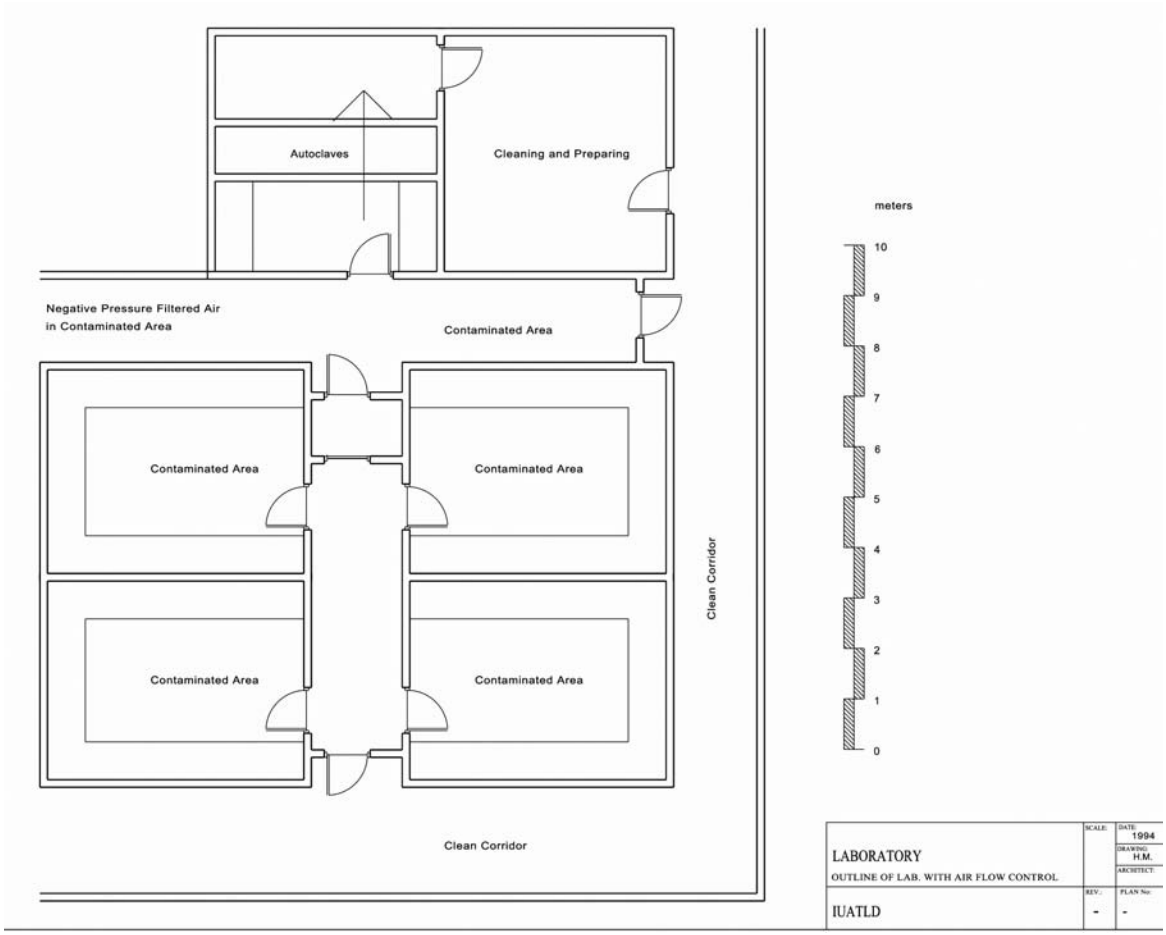
NATIONAL REFERENCE LABORATORY - SENEGAL	SCALE:	DATE: 1994
PLAN OF LABORATORY		DRAWING: H.M.
		ARCHITECT: H.M.
MADE BY LHL FOR IUATLD	REV.: -	PLAN No: -

Figure 4. Depending on available resources, a tuberculosis reference laboratory may be constructed in 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, ScD, Buenos Aires, Argentina.

Figure 4: Sketch of laboratory with controlled air flow



Appendix 13. Reporting and request form for regional sputum smear laboratory supplies

Request for AFB microscopy supplies

Region: ..... Semester:..... Year: .....

**A Number of smears done in the region during the previous semester_____*

Item	Quantity of stain or other item used per smear B	Stain formulation factors (amount of chemical per litre of stain)† C	6 months consumption requirements (= A x B x C) D	3 months reserve (= D/2) E	Consumption plus reserve (=D+E) F	Quantities left in stock‡ G	Calculated amount requested (=F-G) H	Request rounded off to number of complete packing units I	Packing unit
Sputum containers	1 piece								carton of _____ pieces
Slides	1 piece								carton of _____ pieces
Basic fuchsin powder	0.003 litre	10							bottle of _____ grams
Phenol crystals	0.003 litre	50							bottle of _____ litres
Sulphuric acid conc.	0.005 litre	0.25							bottle of _____ grams
Methylene blue powder	0.003 litre	3							bottle of _____ litres
Denatured alcohol	0.003 litre	0.1							bottle of _____ litres
Burning spirit	0.001 litre								bottle of _____ litres
Immersion oil	0.0001 litre								bottle of _____ litres

* Enter here the number of smears examined in all centres, if reported; otherwise use a factor based on smear-positive cases detected and average prevalence of positives in the laboratory registers

† Adapt factors if stain formulation used is different (here carbolfuchsin contains 1.0% basic fuchsin and 5% phenol; methylene blue is at 0.3%; sulphuric acid at 25%)

‡ Enter here only the balance left in stock at the regional headquarters, without counting what laboratories have left in stock

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