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LABORATORY SERVICES *in* TUBERCULOSIS CONTROL

PART III: CULTURE

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LABORATORY SERVICES IN TUBERCULOSIS CONTROL

CULTURE

PART III

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CONTENTS

ANNEXES

FIGURES

DIAGRAMS

PREFACE

Within the framework of National Tuberculosis Programmes the first purpose of bacteriological services is to detect infectious cases of pulmonary tuberculosis, monitor treatment progress and document cure at the end of treatment by means of microscopic examination. The second purpose of bacteriological services is to contribute to the diagnosis of cases of pulmonary and extra-pulmonary tuberculosis. Standardisation of the basic techniques for tuberculosis bacteriology has so many advantages that it has become an unavoidable need. The absence of standardised techniques complicates the activities of new laboratory services as well as the organisation of existing laboratories into an inter-related network. Standardisation makes it possible to obtain comparable results throughout a country; it facilitates staff training, delegation of responsibilities and the selection of equipment, materials and reagents to be purchased; it also facilitates the evaluation of performance and the establishment of suitable supervision in order to increase efficiency and reduce operational costs.

Standardised techniques and procedures are useful if they meet the needs of - and are prepared in accordance with - prevailing epidemiological conditions and different laboratory levels. These techniques should be simple (to obtain the widest coverage) and should be applicable by auxiliary laboratory workers. At the same time, their sensitivity and specificity must guarantee the reliability of results obtained.

While tuberculosis laboratory services form an essential component of the DOTS strategy for National Tuberculosis Programmes, it is often the most neglected component of these programmes. Furthermore, the escalation of tuberculosis world-wide, driven by the HIV epidemic and aggravated by the emergence of multidrug-resistance, has resulted in renewed concern about safety and quality assurance in tuberculosis laboratories.

The above considerations have led to the preparation of guidelines for laboratory services for the framework of National Tuberculosis Programmes. These guidelines are contained in a series of three manuals, two of which are focused on the technical aspects of tuberculosis microscopy and culture and a third which deals with laboratory management, including aspects such as laboratory safety and proficiency testing. These manuals have been developed for use in low-and middle-income countries with high tuberculosis prevalence and incidence rates. Not only are they targeted to everyday laboratory use, but also for incorporation in teaching and training of laboratory and other health care staff.

Finally, in order to adapt the functioning of bacteriological laboratories to the needs of integrated tuberculosis control programmes, information on control programme activities has been included. It is hoped that the series on laboratory services will enable National Tuberculosis Programmes to draw up national laboratory guidelines as one of their essential components.

1

INTRODUCTION

Tuberculosis is a disease of global importance. One-third of the world population is estimated to have been infected with *Mycobacterium tuberculosis* and eight million new cases of tuberculosis arise each year. The tuberculosis crisis is likely to escalate since the human immunodeficiency (HIV) epidemic has triggered an even greater increase in the number of tuberculosis cases. The majority of tuberculosis patients are 15 to 45 years of age, persons in their most productive years of life. Tuberculosis kills over two million people world-wide each year, more than any other single infectious disease, including AIDS and malaria.

Transmission of tuberculosis is virtually entirely by droplet nucleii created through coughing by untreated persons suffering from pulmonary tuberculosis (the most common form) in a confined environment. Infected droplets remain airborne for a considerable time, and may be inhaled by susceptible persons.

Pulmonary tuberculosis usually occurs in the apex of the lungs. These develop cavities which contain large populations of tubercle bacilli that can be detected in a sputum specimen. Pulmonary tuberculosis is suggested by persistent productive cough for three weeks or longer, weight loss, night sweats and chest pain. *The diagnosis can only be made reliably on demonstrating the presence of tubercle bacilli in the sputum by means of microscopy and/or culture in the laboratory*.

The cornerstone of the laboratory diagnosis of tuberculosis is direct microscopic examination of appropriately stained sputum specimens for tubercle bacilli. Between 5 000 and 10 000 tubercle bacilli per millilitre of sputum are required for direct microscopy to be positive and only a proportion of tuberculosis patients harbour large enough numbers of organisms to be detected in this way. It is also virtually impossible to distinguish different mycobacterial species by microscopy. Patients who have positive smears carry the greatest number of tubercle bacilli, are the most infectious and are therefore the most important patients to detect early because they are responsible for spreading tuberculosis disease.

Sputum examination by microscopy is relatively quick, easy and inexpensive and must be performed on cases suspected of having tuberculosis. Smear microscopy is also used to monitor treatment progress and control programme outcome.

Examination by bacteriological culture provides the definitive diagnosis of tuberculosis. Depending on the decontamination method and the type of culture medium used, as few as ten viable tubercle bacilli can be detected. However, the usual microbiological techniques of plating clinical material on selective or differential culture media and sub-culturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. Compared to other bacteria which typically reproduce within minutes, *M. tuberculosis* proliferate extremely slowly (generation time 18-24 hours). Furthermore, growth requirements are such that it will not grow on primary isolation on simple chemically defined media. The only media which allow abundant growth of *M. tuberculosis* are egg-enriched media containing glycerol and asparagine, and agar or liquid medium supplemented with serum or bovine albumin.
Culture increases the number of tuberculosis cases found, often by 30-50%, and

detects cases earlier, often before they become infectious. Since culture techniques can detect few bacilli, the efficiency of diagnosing failures at the end of treatment can be improved considerably. Culture also provides the necessary material for drug susceptibility testing. Culture of specimens is, however, much more costly than microscopy and requires facilities for media preparation as well as skilled staff.

Culture should be used selectively, in the following order of priority:

Selective use of culture

1. Surveillance of tuberculosis drug resistance as an integral part of the evaluation of control programme performance
2. Diagnosis of cases with clinical and radiological signs of pulmonary tuberculosis where smears are repeatedly negative
3. Diagnosis of extra-pulmonary and childhood tuberculosis
4. Follow-up of tuberculosis cases who fail a standardised course of treatment and why may be at risk of harbouring drug resistant organisms
5. Investigation of high-risk individuals who are symptomatic, eg. Laboratory workers, health care workers looking after multidrug resistant patients

2 LABORATORY LAYOUT AND EQUIPMENT

2.1 Plan of a culture laboratory

Persons who work with tubercle bacilli are at risk of laboratory-acquired infection, mainly by the airborne route, and it is well-known that sensible laboratory design may contribute much to the prevention of such infections. The detailed arrangement of a tuberculosis laboratory will vary according to the size and shape of the available room, the type of laboratory activity and whether other work is also done in the same room. Nevertheless, the most important aspect in tuberculosis laboratory design is to ensure a logical flow of specimens and activities, from clean to less clean areas.

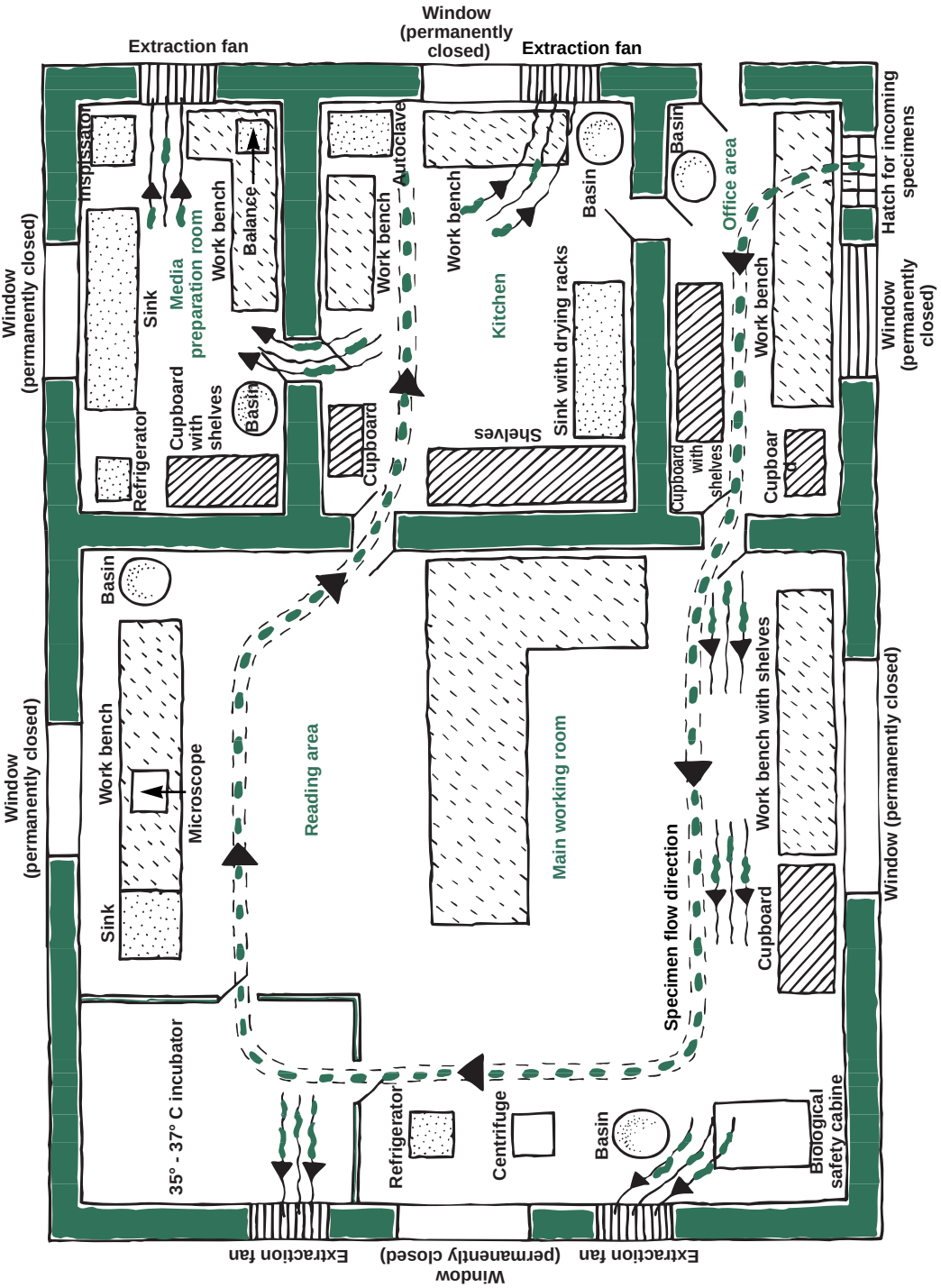
Mycobacterial cultures should always be performed in containment laboratories, physically separated from other laboratory areas. The objective is to reduce the infection risk, not only to tuberculosis technologists, but also to other individuals in the same building.

Contrary to common belief containment laboratories need not be overly sophisticated and expensive. Sophisticated and expensive air conditioning is not an essential requirement in tuberculosis culture laboratories. Rather, the principle should be that, during working hours, air is continuously extracted to the outside of the laboratory either through a biological safety cabinet or through simple extraction fans in walls or windows. Ventilation standards for air changes and pressure gradients should be considered in relation to the number of specimens processed per year and the prevalence of tuberculosis among these specimens. If bacteriological methods are performed with strict adherence to safety standards and high risk procedures are limited to the bio-safety cabinet, air-borne contamination will be minimised. Six to twelve room air changes per hour are sufficient to remove up to 99% of airborne particles within 30 to 45 minutes.

Supply and exhaust air devices should be located on opposite side walls, with supply air provided from clean areas and exhaust air taken from less clean areas. An excess of air supply of 50 cubic feet per minute (23.6 litres per second) or a similar negative pressure created by extracting air is sufficient to obtain the necessary pressure gradient. Air should be exhausted directly to the outside. Potentially contaminated air should be discharged at least 3m above ground level.

Figure 1 illustrates a floor plan for a tuberculosis culture laboratory with air flow in one direction, from clean to less clean areas.

Figure 1. Plan of a containment laboratory for tuberculosis culture



2.2 Arranging equipment and materials

Entry to the laboratory is via the *office* which contains the facilities necessary for administration and management of the laboratory. These include storage space for laboratory registers, laboratory reports, chemicals and reagents.

Specimens arriving at the laboratory are presented through a window/hatch to a separate *reception counter*. Here, specimen containers are checked for leakage and their surfaces decontaminated. Cross-checking of laboratory request forms against specimens is also done and the relevant details are entered into the laboratory register. On completion of these activities the specimens are passed into the *main laboratory area* for further processing.

The main laboratory area contains all the facilities necessary for smear preparation, for specimen decontamination and digestion, and for inoculation of media and incubation of cultures. This area houses work benches, a pH meter, a large domestic refrigerator, a wash basin with elbow-controlled taps and storage cabinets. The *isolation area* is situated at the most extreme end of the main laboratory and contains a biological safety cabinet and a centrifuge.

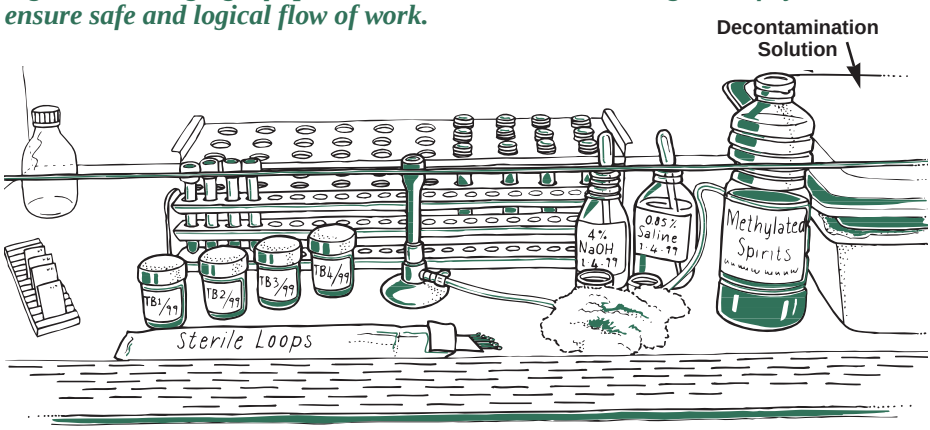
The *reading room* is reserved for performing microscopy on smears prepared in the main laboratory and for reading of cultures. This area contains work benches, a microscope and an elbow-operated wash basin. Laboratory reports may be completed here and then passed to the office for dissemination and entering of data into the laboratory register.

The *kitchen* functions as an area for the disposal of cultures and for subsequent cleaning and sterilisation of glassware. This area houses work benches, a large double-wash stainless steel sink and an autoclave.

In many countries, media preparation is performed at the central level only. If, however, media is prepared as part of the activities of the culture laboratory, a separate *media preparation area* is recommended. This area houses workbenches, an inspissator, a domestic refrigerator and a wash-basin with elbow-operated taps.

Before the processing of specimens and the preparation of cultures are started, equipment and materials should be arranged to ensure a logical and safe flow of work. All manipulations should be standardised and the arrangement of materials should always be the same to ensure maximum safety, as illustrated by Figure 2. For left-handed technologists it may be more convenient to arrange all or most items in the opposite direction, ie. in a mirror-image.

Figure 2. Arranging equipment and materials in the biological safety cabinet to ensure safe and logical flow of work.



2.3 Care and maintenance of essential equipment

Annex 1 contains a list of essential equipment and supplies for a culture laboratory using egg-based Löwenstein-Jensen culture medium, and 4% sodium hydroxide for specimen decontamination. Before purchasing new equipment and supplies it is worthwhile to obtain personal advice of laboratory persons who have had experience in their use. Do not rely entirely on advertisements, catalogues, extravagant claims of sales representatives and the opinion of purchasing officers.

2.3.1 *Biological safety cabinet*

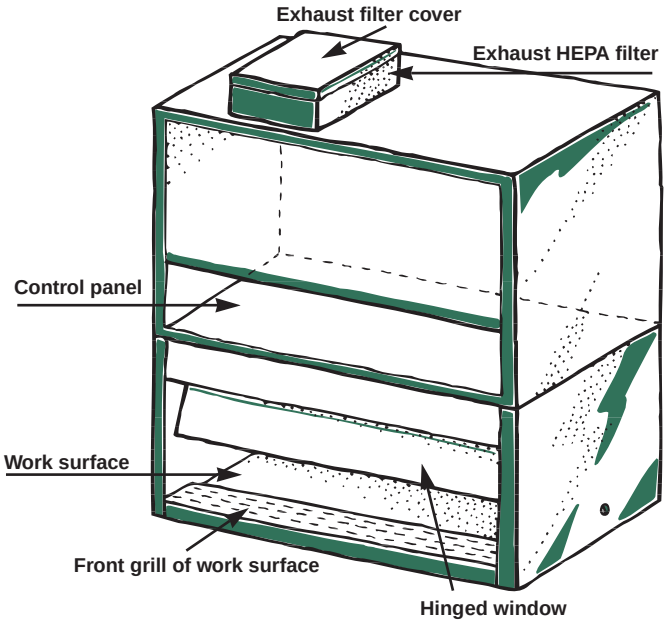
- ***Selecting the right model***

The single most important piece of laboratory equipment needed in a tuberculosis culture laboratory is a well-maintained, properly functioning biological safety cabinet (BSC). These cabinets have been designed to provide a combination of staff, environmental or product protection when appropriate practices and procedures are followed. BSCs use high efficiency particulate air (HEPA) filters in their exhaust and/or air supply systems. HEPA filters remove particles equal to and greater than 0.3µm (which essentially includes all bacteria, spores and viruses) with an efficiency of 99.97%.

Microbiological risks are assigned to biosafety levels I through IV. *Mycobacterium tuberculosis* is classified under Risk Group III. This group includes microorganisms that are particularly associated with infection by the airborne route. Precautions therefore involve measures to minimise the production and dispersal of aerosols and infected airborne particles and to prevent the laboratory worker from inhaling those that might be released, as well as measures intended to prevent infection by accidental ingestion and inoculation.

Of the three classes of biological safety cabinets, Class I and Class II are suitable for tuberculosis bacteriology. The Class I BSC provides staff and environmental protection, but no product protection (Figure 3).

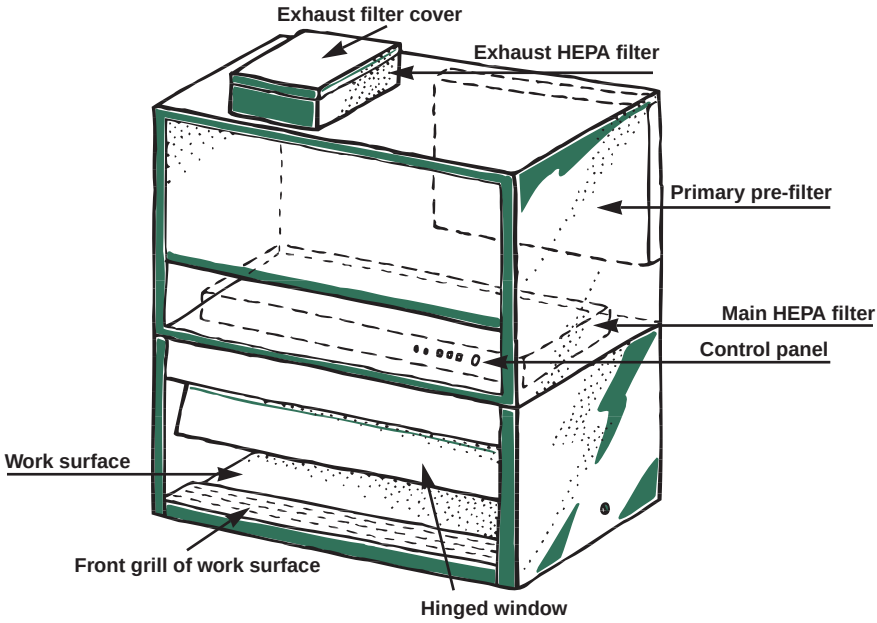
Figure 3. Class I biological safety cabinet



Unfiltered room air is drawn across the work surface. Staff protection is provided by this inward air flow as long as a minimum velocity of 75 linear feet per minute (22.8 meter per second) is maintained through the front opening. Any airborne bacteria are entrained and conveyed into the HEPA filter. The Class I BSC 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. Modern cabinets have airflow indicators and warning devices. The filters must be changed when the airflow falls below the minimum velocity level.

Class II BSCs provide staff, environmental and product protection. Air flow is drawn around the operator into the front grille of the cabinet, which provides staff protection. In addition, the downward laminar flow of HEPA-filtered air provides product protection by minimising the chance of cross-contamination along the work surface of the cabinet (Figure 4). Because cabinet air has passed through the exhaust HEPA filter, it is contaminant-free and may be circulated back into the laboratory (Type A BSC) or ducted out of the building (Type B BSC).

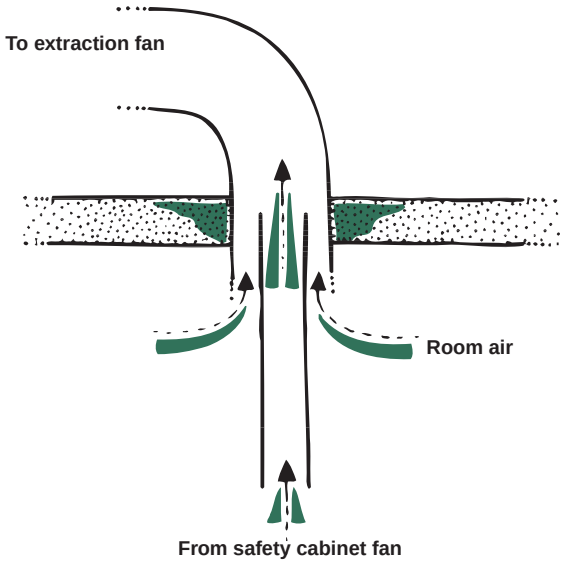
Figure 4. Class II biological safety cabinet



If a Class II BSC is preferred for tuberculosis bacteriology, the "thimble system" should be used in stead of an extract fan, as indicated by Figure 5. The thimble allows air to be extracted continuously from the room and from the safety cabinet when it is in use.

Figure 5. Thimble system recommended for air extraction in Class II biosafety cabinets.

(Adapted from: Collins CH, Lyne PM. Microbiological Methods. 5th ed. Butterworths, London, 1984)



- ***Placement of the BSC***

The ideal location for the BSC is remote from the entry to the laboratory (eg. the rear of the laboratory away from traffic) since people walking parallel to the BSC can disrupt the air curtain. This curtain is quite fragile, amounting to a nominal inward and downward velocity of 1 mile per hour (1.609 kilometer per hour). Open windows or laboratory equipment that create air movement (eg. centrifuges) should not be located near the BSC.

2.3.2 Centrifuge

Centrifuges are essential in laboratories where tubercle bacilli are cultured. Methods involving the use of a centrifuge are more efficient than simple decontamination and culture of sputum directly onto medium.

The recommended centrifuge for use in tuberculosis culture laboratories is a floor model with a lid and fixed angle rotor which contains sealed centrifuge buckets. Because of its great mass in relation to that of the centrifuge tubes, the fixed angle rotor permits centrifugation of tubes with small weight differences without causing vibration and possible tube breakage. For maximum safety, sealed centrifuge buckets should be fitted. These are usually made of stainless steel and are fitted with rubber buffers. They are paired and their weight engraved on them. Sealed buckets are always used in pairs, opposite one another and it is convenient to paint each pair with different colour patches to facilitate recognition. If the buckets fit in the centrifuge head on trunnions, these are also paired.

Centrifuges should preferably be fitted with an electrically operated safety catch which prevents the lid from being opened while the rotor is spinning.

Many reports and manuals describing sputum processing in tuberculosis laboratories record centrifuge speeds in revolutions per minute (rpm). However, revolutions per minute is a measure of speed for a particular centrifuge head and not a measure of sedimenting efficiency or relative centrifugal force (RCF). The amount of artificial gravity created by the spinning of a centrifuge is determined by the rate of spin (revolutions per minute) and by the distance from the centre of the spinning head to an outer point where the force is to be measured. This relative centrifugal force can be increased by either increasing the rate of spin or the distance from the centre and is expressed in multiples of g, eg. 3000 x g. The RCF may be calculated from the following formula:

$$RCF = 1.12 R_{max} (rpm/1000)^2$$

where R_{max} = radius (mm) from the center of the rotating head to the bottom of the spinning centrifuge tube

The required rpm to generate a desired RCF may be calculated as follows:

$$rpm = 1000 \sqrt{\frac{RCF}{1.12\ R_{max}}}$$

If the RCF is not high enough, many mycobacteria will remain in suspension following centrifugation and will be poured off with the discarded supernatant. A

95% sedimenting efficiency should be attained for optimal isolation of mycobacteria. This requires a RCF of 3 000 x g. Many of the old centrifuges still used in tuberculosis laboratories commonly spin at 2 300 to 3 000 rpm (only 1 500-2 000 RCF); most users of such equipment spin digested specimens for 15 minutes, thereby achieving sedimenting efficiencies ranging from 75%-84% and lower.

Depending on the type of rotor in a non-refrigerated centrifuge and the number of runs, the temperature in the specimen tube may increase by 4°C to 18°C. Tubes spun in a streamlined angle head are least affected by temperature rise even after several runs, but the contents of tubes in unprotected horizontal rotors may exceed 40°C if the centrifuge has been used for five or more successive runs. Even when centrifuge time remains consistent at 15 minutes, the percentage of organisms killed increase from 13% to 22% to 30% as the temperature rises from 20°C to 30°C to 40°C. It is, therefore, important to keep the spinning time low (15 minutes) and the RCF high (3 000 x g) to achieve 95% sedimentation. The use of angle head rotors minimises heat build-up due to air friction.

Glass tubes may break under the stress of centrifugation. If a centrifuge tube breaks, the liquid will splash or be blown out and aerosolised. Screw top centrifuge tubes should therefore be used for potentially infectious material.

The centrifuge head must be in balance while spinning. An out-of-balance head vibrates and may break. If a tube is added to one side of the head, an equivalent weight must be added to the opposite side. Tubes used in processing specimens will usually be balanced if matched pairs have matched levels of liquid in them. As an added safety precaution, matched tubes should contain 70% ethanol rather than water, which may limit the risk of infection should breakage occur.

Do not touch any centrifuge head while it is spinning. Touching it may not only cause injury, it may also cause rapid or erratic stops which stir and resuspend the sediment. Some centrifuges are equipped with a brake to gradually slow the spinning head.

2.3.3 35°-37°C incubator

Cultures are incubated at 35°-37°C for eight weeks. Incubators are available in various sizes. In general, it is best to obtain the largest possible model that can be accommodated and afforded. Small incubators suffer wide fluctuations in temperature when the doors are opened. Ensure a proper circulation of air by avoiding overloading and by using perforated trays. Maintain a constant temperature by not opening the incubator door unnecessarily.

Although incubators rarely develop faults, it is advisable, before choosing one, to ascertain that service facilities are available. The electrical circuits are not complex but require expert technical knowledge to repair. Transporting incubators back to the manufacturers is most inconvenient.

In a laboratory with a large volume of cultures it is of great advantage to incubate them in an incubator room. A hot room or walk-in incubator is not difficult to

adapt from a small room or corner of a large room: Windows must be blocked up. The walls need two layers of building paper on which is glued 48mm-thick slabs of expanded polystyrene or cork (more expensive) between battens at 600mm centres. The inner lining can be ordinary plaster or insulation board. The ceiling must be lagged in the same way and in rooms lower false ceilings are preferable. The floor can be covered with insulation board and hardboard and the doors lagged in their inner surfaces in the same way, and fitted in their jambs on sponge rubber draught-prevention strips.

Two methods of heating are possible. Tubular heaters around the walls are satisfactory and a power of 3kW is more than adequate for a room of 5-7m^3 to be maintained at 37°C. A large circulating fan to avoid hot and cold spots must be fitted on one wall and should operate constantly. An alternative arrangement is the greenhouse-or space-heater in which a 2.5-3.0 kW heater and a fan are built into a steel casing. The wiring must be altered so that the fan is always on and the heater connected to sensitive thermostats. (The thermostats build into ordinary greenhouse- or space heaters are too coarse for this purpose). Two thermostats are recommended, one normally operational at 37°C and the other set to turn off heating at around 39°C as a precaution against failure of the first thermostat and consequent over-heating of cultures. The two methods of heating may be combined. Smaller tubular heaters, permanently switched on, will supply background heat and the space heater will maintain the required temperature.

Wooden shelving and racks are undesirable. If a high humidity is maintained fungi may grow on the wood. Steel or aluminium racks are preferable and can be custom-made. Shelves should be free, ie. removed easily for cleaning and there should be space between the shelves and the walls to allow for circulation of air.

2.3.4 *Inspissator*

In the preparation of slopes of egg-based medium the amount of heating required to coagulate the protein must be carefully controlled. A steamer heats the medium too rapidly and raises the temperature too high.

Inspissators for the preparation of egg-based culture medium should be able to reach and maintain a constant temperature of 80°-85°C for 45 minutes. Modern inspissators are thermostatically controlled and fitted with a large internal circulating fan. Inside shelves on which the tubes are sloped should be made of wire mesh so that circulation is not impeded. It is convenient to have wire mesh or aluminium racks made which hold tubes or bottles at the correct angle (5°-10°) and which slide onto the shelf brackets. These facilitate rapid loading and unloading while the inspissator is hot.

A glass door that seals off the inside of the oven will contribute to maintaining the required temperature and is recommended. It is also recommended that the required temperature be raised first before batches of media are loaded for inspissation.

2.3.5 Autoclave

Tubercle bacilli are more readily killed by moist heat (saturated steam) than by dry heat. Steam kills tubercle bacilli by denaturing their protein. Air has an important influence on the efficiency of steam sterilisation because its presence changes the pressure-temperature relationship. For example, the temperature of saturated steam at 15 lb/in^2 is 121°C, provided that all of the air is first removed from the vessel. With only half of the air removed the temperature of the resulting air-steam mixture at the same pressure is only 112°C. In addition, the presence of air in mixed loads will prevent penetration by steam.

All of the air that surrounds and permeates the load must first be removed before steam sterilisation can commence. Materials to be sterilised should therefore be packed loosely. Contaminated material (eg. discarded cultures) should be in solid bottomed containers not more than 20cm deep. Large air spaces should be left around each container and none should be covered.

Only autoclaves designed for laboratory work and capable of dealing with a mixed load should be used. "Porous load" and "bottled fluid sterilisers" are not satisfactory for laboratory work. Two varieties of laboratory autoclaves are suitable:

- pressure cooker types
- gravity displacement models with automatic air and condensable discharge

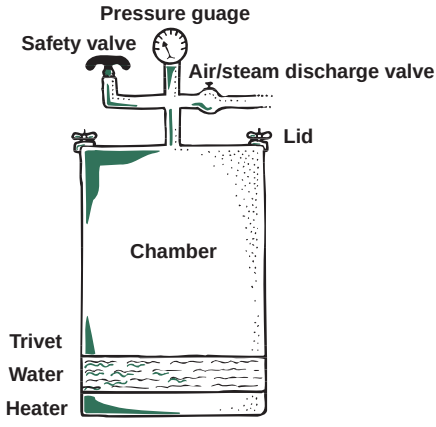
• *Pressure cooker autoclaves*

The most common type is a device for boiling water under pressure. It has a vertical metal chamber with a strong metal lid which can be fastened down and sealed with a rubber gasket. An air and steam discharge tap, pressure gauge and safety valve are fitted in the lid. Water in the bottom of the autoclave is heated by external gas burners, an electric immersion heater or a steam coil.

Figure 6 illustrates a typical pressure cooker autoclave.

Figure 6. Pressure cooker laboratory autoclave

(Adapted from: Collins CH, Lyne PM. Microbiological Methods. 5th ed. Butterworths, London, 1984)

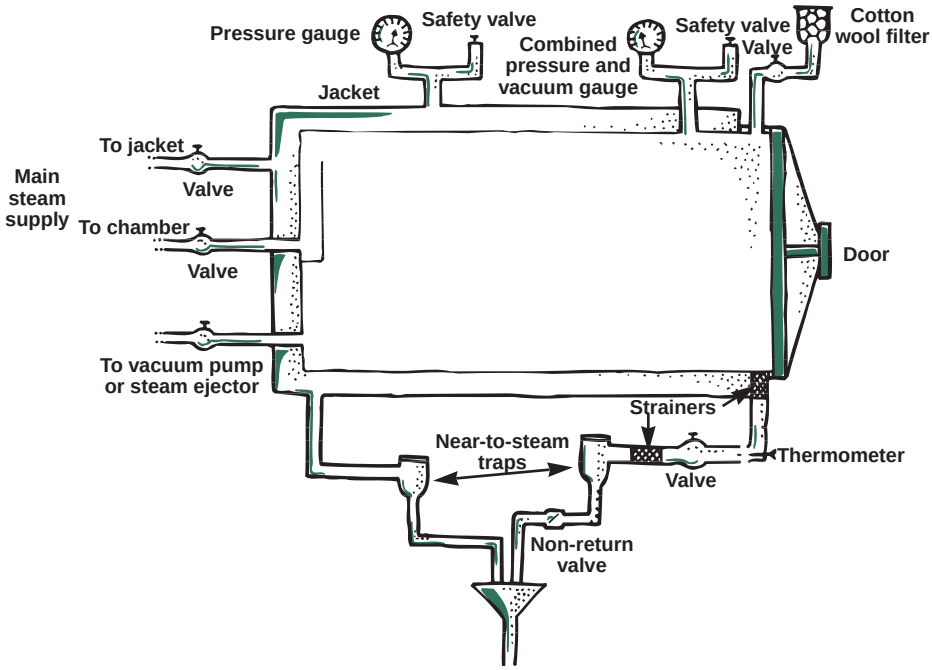


- ***Autoclaves with air discharge by gravity displacement***

These autoclaves are usually arranged horizontally and are rectangular in shape, thus making the chamber more convenient for loading. A palette and trolley system can be used. Figure 7 shows in diagrammatic form a jacketed gravity displacement type of autoclave. Similar autoclaves can be constructed without jackets. The door should have a safety device to ensure that it cannot be opened while the chamber is under pressure.

Figure 7. Autoclave with air discharge by gravity displacement

(Adapted from: Collins CH, Lyne PM. Microbiological Methods. 5th ed. Butterworths, London, 1984)



The jacket surrounding the autoclave consists of an outer wall enclosing a narrow space around the chamber, which is filled with steam under pressure to keep the chamber wall warm. The steam enters the jacket from the mains supply, which is at high pressure, through a valve that reduces this pressure to the working level. The working pressure is measured on a separate pressure gauge fitted to the jacket. This jacket also has a separate drain for air and condensate to pass through.

The steam enters the chamber from the same source which supplies steam to the jacket. It is introduced in such a way that it is deflected upwards and fills the chamber from the top downwards, thus forcing the air and condensate to flow out of the drain at the base of the chamber by gravity displacement. The drain is fitted with strainers to prevent blockage by debris. The drain is usually fitted with a thermometer for registering the temperature of the issuing steam. The temperature recorded by the drain thermometer is often lower than that in the chamber. A "near-to-steam" trap is also fitted.

The automatic steam trap or “near-to-steam” trap is designed to ensure that only saturated steam is retained inside the chamber, and that air and condensate, which are at a lower temperature than saturated steam, are automatically discharged. It is called a “near-to-steam” trap because it opens if the temperature fall to about 2°C below that of saturated steam and closes within 2°C to the saturated steam temperature.

The trap operates by the expansion and contraction of a metal bellows, which opens and closes a valve. The drain discharges into a tundish in such a way that there is a complete airbreak between the drain and the dish. This ensures that no contaminated water can flow back from the waste-pipe into the chamber.

2.3.6 Water bath

The contents of a test tube placed in a water bath are raised to the required temperature much more rapidly than in an incubator. Water baths are therefore useful for short term incubation required, for example, in some biochemical tests.

Modern water baths are equipped with electrical stirrers and in some the heater, thermometer and stirrer are in one unit, easily detached from the batch for servicing. Water baths must also be lagged so as to prevent heat loss through the walls. A bath that has not been lagged by the manufacturers can be insulated with slabs of expanded polystyrene.

Water baths should be fitted with lids in order to prevent heat loss and evaporation. These lids must slope so that condensation water does not drip on the contents. To avoid chalky deposits on tubes and internal surfaces only distilled water should be used.

2.3.7 Bunsen burners

For material that may spatter or that is highly infectious a hooded Bunsen burner should be used. Electric burners are also available. These are tubular micro-incinerators in which the loop or wire is inserted, and is recommended for use in BSCs.

2.3.8 Glassware and plastics

Soda-glass or pyrex are satisfactory for tuberculosis culture and the use of more expensive resistance glass is not justified. New unwashed soda-glass should be soaked in hydrochloric acid overnight to partially neutralise the alkali content of the glass.

- ***Culture bottles***

Several sizes of culture bottles are useful for tuberculosis bacteriology. The most useful sizes are the small McCartney (14ml), the standard McCartney (28ml) and the Universal container (28ml), which has a larger neck than the others and is also used as a specimen container. These bottles usually have aluminium screw caps with rubber liners. The liners should be made of black rubber; some red rubbers are thought to give off bactericidal substances.

- ***Test tubes***

Rimless test tubes of heavy quality are most suitable for tuberculosis bacteriology. Thin glass, lipped chemical tubes should not be used. The most frequently used sizes of test tubes are 152x16mm, holding 5-10ml and 152x19mm, holding 10-15ml.

Cotton wool plugs have been used for many years to stopper test tubes but have largely been replaced by metal caps. Aluminium caps (Cap-O-Test) closures are recommended. They have a wide tolerance and fit most tubes, being held in place with a small spring. These caps are inexpensive, last a long time, are available in many colours and save a great deal of time and labour.

Temporary closures for bottles, flasks and tubes can be made from kitchen aluminium foil.

- ***Pasteur pipettes***

Pasteur pipettes are probably the most dangerous pieces of laboratory equipment in unskilled hands. Safer pasteur pipettes with integral teats and made of low density polypropylene (rather than glass) are available and are supplied pre-sterilised.

Pasteur pipettes are used once only.

- ***Graduated pipettes***

Straight side blow out pipettes, 1-10ml capacity are often used. They must be plugged with non-absorbent cotton wool at the suction end to prevent bacteria from entering from the teat and contaminating the material in the pipette. These plugs must be tight enough to stay in place during pipetting but not so tight that they cannot be removed during cleaning. About 25mm of non-absorbent cotton wool is pushed into the end with a piece of wire. The ends are then passed through a Bunsen flame to tidy them. (Wisps of cotton wool which get between the glass and the teat may permit air to enter and the contents to leak).

- ***Rubber teats***

Rubber teats provide a safe alternative to the highly dangerous practice of mouth pipetting. Teats with a capacity greater than that of the pipettes for which they are intended should be used, eg. a 1ml teat for pasteur pipettes, a 2ml teat for a 1ml pipette etc. (otherwise the teat must be fully compressed, which is tiring). Most novice laboratory workers compress the teat completely, then suck up the liquid and try to hold it at the mark while transferring it. This is unsatisfactory and leads to spilling and inaccuracy. Compress the teat just enough to suck the liquid a little way pass the mark of the pipette. Withdraw the pipette from the liquid, press the teat lightly to bring the fluid to the mark and then release it. The correct volume is now held in the pipette without tiring the thumb and without risking loss. To discharge the pipette, press the teat slowly and gently and then release it in the same way. Violent operation usually fails to eject all the liquid; bubbles are sucked back and aerosols are formed.

- ***Inoculating loops and wires***

These are usually made of 25 SWG Nicrome wire. They should be short (not more than 15cm long) in order to minimise vibration and therefore involuntary discharge of contents. Loops should be small (not more than 5mm in diameter). Large loops are inclined to empty spontaneously and scatter infected airborne particles. Loops should be completely closed. This can be achieved by twisting the end of the wire round the shank, or by taking a piece of wire 15cm long, bending the centre round a nail or rod of appropriate diameter and twisting the ends together in a drill chuck.

Loops and wires should not be fused into glass rods. Aluminium holders are available from most laboratory suppliers.

Disposable loops are excellent, albeit more expensive.

- ***Racks and baskets***

Test tube and culture bottle racks should preferably be made of polypropylene or nylon so that they can be autoclaved. This also minimises breakage, which is not uncommon when metal racks are used. Wooden racks are unhygienic.

Traditional wire baskets are unsafe for holding test tubes. They contribute to breakage hazards and do not retain spilled fluids. Autoclavable plastic boxes of various sizes are safer for use with cultures.

3 SPECIMEN COLLECTION

The presence of acid-fast bacilli in a clinical specimen may be confirmed either by microscopy or by culture. However, since individual mycobacterial species cannot be identified by smear examination, the definitive diagnosis of tuberculosis can only be made if *M. tuberculosis* is isolated from the clinical specimen.

In tuberculosis bacteriology attention tends to be focused on the problems of microscopy, culture and identification systems, while an often overlooked problem is that of obtaining adequate specimens. The advantages of subtle decontamination techniques, sensitive culture media and simple identification schemes will not be fully realised unless specimens are collected with the utmost care and promptly transported to the laboratory.

3.1 Containers

An essential prerequisite for the safe collection of a satisfactory specimen is a robust, leakproof and clean container. Containers must be rigid to avoid crushing in transit and must possess a water-tight wide-mouthed screw top to prevent leakage and contamination.

To facilitate the choice of a container the following specifications are recommended:

- Wide-mouthed (at least 35mm in diameter) so that the patient can expectorate easily inside the container without contaminating the outside
- Volume capacity of 50ml
- Made of translucent material in order to observe specimen volume and quality without opening the container
- Made of single-use combustible material to facilitate disposal
- Screw-capped to obtain an airtight seal and to reduce the risk of leakage during transport
- Easily-labelled walls that will allow permanent identification

An alternative container is the 28ml Universal bottle, which is a heavy glass, screw-capped bottle with a wide neck. This container is reusable after thorough cleaning and sterilisation in boiling water for at least 30 minutes.

3.2 Collection procedures

3.2.1 *Sputum specimens*

Although *M. tuberculosis* is capable of causing disease in almost any organ of the body, more than 85% of tuberculosis disease in high prevalence countries is pulmonary. Therefore, sputum is the specimen of choice in the investigation of tuberculosis and should always be collected. If extra-pulmonary disease is

suspected, sputum should be collected in addition to any extra-pulmonary specimens.

A good sputum specimen consists of recently-discharged material from the bronchial tree, with minimum amounts of oral or nasal material. Satisfactory quality implies the presence of mucoid or mucopurulent material and is of greater significance than volume. Ideally, a sputum specimen should have a volume of 3-5ml, although smaller quantities are acceptable if the quality is satisfactory.

Collecting a good sputum specimen requires that the patient be given clear instructions. Aerosols containing tubercle bacilli may be formed when the patient produces a sputum specimen. Patients should, therefore, produce specimens either outside in the open air or away from other people and not in confined spaces such as toilets.

In some countries, patients may present first to the laboratory for diagnosis. It is therefore appropriate that laboratory staff know the correct way of collecting sputum specimens. This procedure is described in Annex 2. It is best to obtain sputum early in the morning before the patient has eaten or taken medication (which may interfere with the growth of tubercle bacilli). If sputum specimens are collected for diagnostic purposes, tuberculosis chemotherapy should not be started until the specimens have been collected.

Because of the increased sensitivity of culture, a single good-quality sputum specimen may suffice. Some patients shed mycobacteria irregularly and in small numbers; for these patients the chance of obtaining a positive culture result will be improved if more specimens are cultured.

Specimens should be transported to the laboratory as soon as possible after collection. If a delay is unavoidable the specimens should be refrigerated to inhibit the growth of unwanted micro-organisms.

3.2.2 *Other specimens*

If a patient has a productive cough, obtaining a sputum specimen is a fairly straightforward procedure. However, if a patient finds it difficult to produce sputum, other methods may be used to obtain pulmonary secretions for diagnosis. Collection techniques fall outside the scope of this document and will not be discussed. However, induced sputum resemble saliva and it is important that these specimens be marked “induced” in order not to be discarded as unsuitable.

Because *M. tuberculosis* may infect almost any organ in the body, the laboratory should expect to receive a variety of extra-pulmonary specimens, eg. body fluids, tissues, pus and urine. These specimens may be divided into two groups, namely:

- aseptically collected specimens, usually free from other micro-organisms
- specimens known to contain contaminating normal flora or specimens not collected aseptically

Aseptically collected fluids

Body fluids (spinal, pleural, pericardial, synovial, ascitic, blood, pus, bone-marrow) should be aseptically collected in a sterile container by the physician using aspiration techniques or surgical procedures. For fluids that may clot, sterile potassium oxalate (0.01-0.02ml of 10% neutral oxalate per ml fluid) or heparin (0.2mg per ml) should be added. Specimens should be transported to the laboratory as quickly as possible.

Aseptically collected tissues

Aseptically collected tissue specimens should be placed in sterile containers *without fixatives or preservatives*. If the specimen is to be sent by mail it should be protected from drying by adding sterile saline and packing the container in dry ice or maintaining a temperature of 4-15°C. Specimens should be transported to the laboratory as quickly as possible.

Specimens expected to be contaminated

Urine is the most commonly encountered extra-pulmonary specimen that requires processing before culture. To minimise excessive contamination of urine specimens the external genitalia should be washed before the specimens are collected and the urine should be immediately processed or refrigerated. Three early morning, voided midstream specimen should be collected.

4

SPECIMEN STORAGE AND TRANSPORT

For successful culture of specimens the time between specimen collection and the culturing process should be kept to a minimum. Specimens should therefore be dispatched with the least possible delay. If sputum specimens can be kept refrigerated they could be sent to the laboratory once a week; extra-pulmonary specimens, however, should be submitted as soon as possible after collection.

If specimens have to be transported at ambient temperatures, chemical preservation may be used. Three methods provide reasonable results, *viz*:

- Mixing the fresh specimen with an equal volume of 1% cetyl pyridinium chloride in 2% sodium chloride. Tubercle bacilli will survive for up to a week, while the growth of unwanted organisms will be restricted
- Mixing the fresh specimen with anhydrous sodium carbonate in the proportion of 50mg reagent to 2ml specimen
- If the delay before cultural examination is to be less than 24 hours the specimens may be mixed with an equal volume of 23% trisodium phosphate

However, none of the abovementioned preservation methods is optimal and speedy transportation is essential for good results.

Requirements and recommendations for the safe transport of pathological specimens are given in various national and international codes of practice and guidelines. In addition, the postal and transport authorities of most countries as well as the International Air Transport Association (IATA) have regulations about conveying such materials.

As a general rule, diagnostic specimens must be packaged to withstand leakage of contents, shocks, pressure changes and other conditions incident to ordinary handling practices. Pathological material intended for postal or air transport should be in approved, robust, leak-proof primary containers which are packed into secondary containers made of metal, wood or strong cardboard with enough absorbent material so that if they are damaged or leak the fluids will be absorbed. For sending material across international or state boundaries this container may have to be packed in the same way in an outer container and special administrative arrangements with the postal authorities and airlines may be necessary.

Sputum specimens comprise the majority of specimens submitted to tuberculosis culture laboratories and special transport boxes of metal or wood should be provided. They should be made to hold between 20 and 30 specimen containers packed vertically to avoid leaking. The lid should be securely fastened and the box should preferably contain a locking mechanism. During transport it must be kept as cool as possible and protected from sunlight.

Request forms should be located separately from specimen containers. With each transport box an accompanying list must be prepared which identifies the specimens and the patients from whom the specimens were collected. Before

dispatch from the health centre the following must be verified:

- that the number of specimen containers in the box corresponds to that on the accompanying list
- that the identification number on each specimen container corresponds to the identification number on the accompanying list
- that the accompanying list contains the necessary data for each patient
- that the date of dispatch and the particulars of the health centre are on the accompanying list

A model laboratory request form is presented in Annex 3.

5 SPECIMEN HANDLING

5.1 Receipt of incoming specimens

Specimens should be received in the office area of the laboratory, preferably at a separate specimen delivery counter. Delivery boxes should be opened in the biosafety cabinet and the following procedures applied:

- Wear disposable gloves during receipt and inspection of incoming specimens
- Inspect the delivery box for signs of leakage. If mass leakage is evident discard the box by autoclaving or burning
- Disinfect the outside of the delivery box using cotton wool or paper towels saturated with a suitable disinfectant (eg. 5% phenol)
- Open the delivery box carefully and check for cracked or broken specimen containers. Autoclave or burn these without processing and request another specimen
- Check that specimens have been adequately labelled with individual identification numbers and that these correspond with the numbers on the accompanying list
- Disinfect the inside of the delivery box, discard gloves and wash hands after handling specimen containers (see part I, page 30) .

5.2 Safe handling of specimens

5.2.1 *Working within a BSC*

- Because of the increased risks of aerosol production during culture procedures, all manipulations should be carried out within the BSC. The cabinets are intended to protect the worker from airborne infection. *They will not, however, protect him/her from spillage and the consequences of poor techniques*
- BSCs are designed to be operated 24 hours per day and in order to maintain room air balance they should not be switched off. If electrical power has been interrupted or the cabinet has been switched off (eg. following replacement of filters), the cabinet blower should be operated for a least five minutes before work is started. The work surface, interior walls and interior window surface should be wiped with an appropriate disinfectant (eg. methylated spirits, see part I, page 30). This should be followed by a second wiping with *sterile* water
- Prepare a written checklist of materials necessary for tuberculosis culture. This will minimise the number of arm-movement disruptions across the fragile air barrier of the BSC, which may disrupt the air curtain and compromise the partial barrier
- Place only the materials and equipment required for immediate work in the BSC and store extra supplies (eg. additional culture media) outside the cabinet. Materials and equipment placed inside the BSC may cause

disruption to the airflow resulting in turbulence, cross-contamination or breach of containment

- Allow a delay of 60 seconds after placing hands/arms inside the cabinet, before manipulation of materials. This allows the BSC to stabilise and to remove surface microbial contaminants
- Ensure that the front grille is not blocked with laboratory notes, discarded plastic wrappers, pipetting devices, etc
- Perform all operations at least four inches from the front grille on the work surface. Raise arms slightly to allow room air to be drawn through the front grille
- Place absorbent paper towelling on the work surface (but not on the front or rear grille openings). This will facilitate routine cleanup and will reduce splatter and aerosol formation during an overt spill. Towelling can then be folded and placed in an autoclavable bag when work is completed
- Place all materials and aerosol-generating equipment (eg. vortex mixers) as far back in the cabinet as practical, towards the rear edge of the work surface and away from the front grille
- Place bulky items such as autoclavable bags, pipette trays and collection flasks to one side in the cabinet
- Arrange materials and equipment to allow work to flow from a clean to a contaminated area across the work surface. Place materials and supplies in such a way as to limit the movement of dirty items over clean ones
- Avoid the following common practices which may interfere with the operation of the BSC:
    - taping autoclavable disposal bags to the outside of the cabinet
    - placing pipette collection containers upright in the BSC or on the floor outside the cabinet

  The frequent inward/outward movement needed to place objects in these containers disrupts the integrity of the cabinet air barrier and can compromise both staff and product protection
- Use only horizontal pipette discard trays containing an appropriate disinfectant (eg. 5% phenol, see part I, page 30)
- Use proper microbiological techniques to avoid splatter and aerosols. This will minimise the potential for staff exposure to infectious materials manipulated within the cabinet. As a general rule, keeping clean materials at least 12cm away from aerosol-generating activities will minimise the potential for cross-contamination
- Do not hold opened tubes or bottles in a vertical position and recap or cover them as soon as possible. This will reduce the chance for cross-contamination
- Do not use large open flames in the BSC. This creates turbulence which disrupts the pattern of air supplied to the work surface. Special Bunsen burners for use in BSC's are recommended

- Use an appropriate liquid disinfectant (eg. 5% phenol, see part I, page 30) in a discard pan to decontaminate materials before removal from the BSC. Introduce items into the pan with the minimal splatter and allow sufficient contact time before removal. Alternatively, contaminated items may be placed into an autoclavable disposal bag within the BSC. Water should be added to the bag prior to autoclaving to ensure steam generation during the autoclave cycle
- Surface-decontaminate all containers and equipment before removal from the BSC (see part I, page 30)
- At the end of the work day, surface-decontaminate the work surface of the BSC, the sides and back and the interior of the glass window
- Handle small spills within the BSC immediately by removing the contaminated absorbent paper towelling and placing it into the autoclavable disposable bag. Wipe any splatter onto items within the cabinet or on its interior immediately with a paper towel saturated with a disinfectant solution (eg. 5% phenol)
- Spills large enough to result in liquids flowing through the front or rear grilles require more extensive decontamination. Surface-decontaminate and remove all items from the BSC. Ensure that the drain valve is closed and pour appropriate disinfectant (eg. 5% phenol) onto the work surface and through the grille(s) into the drain pan. Allow at least 30 minutes for decontamination. Empty the drain pan into a collection vessel containing disinfectant by attaching a flexible tube to the drain valve with the open end submerged in the disinfectant within the collection vessel. After decontamination, flush the drain pan with water and remove the drain tube
- Always decontaminate the BSC before HEPA filters are changed or internal repair work is done. The most common decontamination method uses formaldehyde gas and is described in the Management Series

5.2.2 *Using the centrifuge*

- Select two centrifuge tubes of identical length and thickness. Place the specimen to be centrifuged in one tube and an equal amount of 70% ethanol in the other. Ensure that the tubes are balanced
- Place the tubes in paired centrifuge buckets and place the paired buckets in diametrically opposite positions in the centrifuge head
- Close the centrifuge lid and ensure that the speed control is at zero before switching on the current. (Many centrifuges are fitted with a “no volt” release to prevent the machine starting unless this is done)
- Move the speed control slowly until the speed indicator shows the required rpm or g

5.2.3 *Precautions*

- Make sure that the rubber buffers are in the buckets, otherwise tubes will break

- Check the balancing carefully. Improperly balanced tubes will cause “head wobble”, spin-off accidents and wear out bearings
- Check that the balanced tubes are opposite one another in multi-bucket centrifuges
- Never start or stop the centrifuge with a jerk
- Observe the manufacturer instruction about the speed limits for various loads
- Open sealed centrifuge buckets in the BSC

5.2.4 Using a pressure cooker autoclave

- There must be sufficient water inside the chamber
- The autoclave is loaded and the lid is fastened down with the discharge tap open. The safety valve is then adjusted to the required temperature and the heat is turned on
- When the water boils, the steam will issue from the discharge tap and carry the air from the chamber with it. The steam and air should be allowed to escape freely until all of the air has been removed. This may be tested by attaching one end of a length of rubber tubing to the discharge tap and inserting the other end into a bucket or similar large container of water. Steam condenses in the water and the air rises as bubbles to the surface. When all of the air has been removed from the chamber, bubbling in the bucket will cease. When this stage has been reached, the air-steam discharge tap is closed and the rubber tubing removed. The steam pressure then rises in the chamber until the desired pressure, usually 15lb/in^2, is reached and steam issues from the safety valve
- When the load has reached the required temperature, the pressure is held for 30 minutes
- At the end of the sterilising period, the heater is turned off and the autoclave allowed to cool
- The air and steam discharge tap is opened very slowly after the pressure gauge has reached zero (atmospheric pressure). If the tap is opened too soon, while the autoclave is still under pressure, any fluid inside will boil explosively and bottles containing liquids may even burst
- The contents are allowed to cool. Depending on the nature of the materials being sterilised, the cooling (or “run-down”) period needed may be several hours

5.2.5 Using an autoclave with air discharge by gravity displacement

- If the autoclave is jacketed, the jacket must first be brought to the operating temperature
- The chamber is loaded, the door is closed and the steam-valve is opened, allowing steam to enter the top of the chamber. Air and condense flow out through the drain at the bottom

- When the drain thermometer reaches the required temperature a further period must be allowed for the load to reach that temperature. This should be determined initially and periodically for each autoclave. Unless this is done the load is unlikely to be sterilised
- The autoclave cycle is then continued for the holding time. When it is completed the steam valves are closed and the autoclave allowed to cool until the temperature dial reads less than 80°C. *Not until then is the autoclave safe to open*
- The autoclave door should first be "cracked" or opened very slightly and left in that position for several minutes to allow steam to escape and the load to cool further

Serious accidents, including burns and scalds to the face and hands have occurred when autoclaves have been opened, even when the temperature gauge reads below 80°C and the doors have been "cracked".
Liquids in bottles may still be over 100°C and under considerable pressure. The bottles may explode on contact with air at room temperature.

When autoclaves are being unloaded operators should wear full-face visors of the kind that cover the chin and throat. They should also wear thermal-protective gloves.

6

HOMOGENISATION AND DECONTAMINATION

The usual microbiological techniques of plating clinical material on selective or differential culture media and subculturing to obtain pure cultures cannot be applied to tuberculosis bacteriology. *M. tuberculosis* requires special media not used for other organisms and grows slowly, taking three to six weeks or longer to give visible colonies. Cultures are usually made in bottles rather than in petri dishes because tubercle bacilli are present in relatively small numbers in most specimens; this necessitates large inocula which are spread out over the surface of the media. Because of the long incubation time required, the bottles are tightly stoppered to prevent drying of the cultures (which would occur in petri dishes).

The majority of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by more rapidly growing normal flora organisms. These would rapidly overgrow the entire surface of the medium and digest it before the tubercle bacilli start to grow. Most specimens must, therefore, be subjected to a harsh digestion and decontamination procedure that liquefies the organic debris and eliminates the unwanted normal flora.

All currently available digesting/decontaminating agents are to some extent toxic to tubercle bacilli; therefore, to ensure the survival of the maximum number of bacilli in the specimen, the digestion/decontamination procedure must be precisely followed. In order for enough tubercle bacilli to survive to give a confirmatory diagnosis, it is inevitable that a proportion of cultures will be contaminated by other organisms. As a general rule, a contamination rate of 2%-3% is acceptable in laboratories that receive fresh specimens; if specimens (especially sputum) take several days to reach the laboratory then losses due to contamination may be as high as 5%-10%. It is also important to note that a laboratory which experiences no contamination is probably using a method that kills too many of the tubercle bacilli.

When culturing tubercle bacilli, three important aspects should be borne in mind:

- Specimens must be homogenised to free the bacilli from the mucus, cells or tissue in which they may be embedded. The milder this homogenisation the better the results
- Neither homogenisation nor decontamination should unnecessarily diminish the viability of tubercle bacilli
- The success of homogenisation and decontamination depends on:
    - the greater resistance of tubercle bacilli to strongly alkaline or acidic digesting solutions
    - the length of exposure time to these agents
    - the temperature build-up in the specimen during centrifugation
    - the efficiency of the centrifuge used to sediment the tubercle bacilli

Many different methods of homogenisation and decontamination of sputum specimens for culturing have been described but there is no universally recognised

best technique. The choice of a suitable method is to a large extent determined by the technical capability and the availability of staff in a laboratory, as well as the quality and type of equipment available. Each method has its limitations and advantages and it is recommended that regional/central laboratories standardise on one method only. Methods which consistently yield the highest percentage of positive cultures are those which require:

- well trained staff
- relatively expensive equipment (eg. centrifuges) and related supplies
- continued maintenance of equipment and of good staff performance

Any method which require the use of a centrifuge present some problems which must be considered:

- The centrifuge must be fast enough to attain a relative centrifugal force (RCF) of 3 000 x g. If the RCF is not high enough, many tubercle bacilli remain in suspension following centrifugation and are poured off with the discarded supernatant fluid. Recent studies have shown that 3 000 x g for 15 minutes would sediment 95% of mycobacteria in a digested sputum specimen. The specific gravity of tubercle bacilli ranges from 1.07 to 0.79, making centrifugal concentration of specimens ineffective if the RCF is not 3 000 x g
- Precautions must be taken to minimise the potential for staff infection in the event of tube breakage during centrifugation. These include:
  - using a floor model centrifuge with lid and a fixed angle rotor. The mass of the fixed angle rotor permits centrifugation of tubes with small weight differences without causing vibration and possible tube breakage
  - always ensuring that tubes in the centrifuge are balanced. The weight of centrifuge tubes can be balanced by adding sterile saline to specimens or by inserting tubes with sterile water or 70% ethanol among the tubes containing sputum (using 70% ethanol in stead of water in the tubes used as balances will reduce the risk in the event of breakage)
  - using aerosol-free safety cups if available
  - enclosing the centrifuge in a specially ventilated cabinet if possible

6.1 Digestion and decontamination procedures

6.1.1 *Sputum specimens*

Sputum specimens should not be pooled because of the risk of cross-contamination. Since the exposure time to digestants/decontaminants has to be strictly controlled it is best to work in sets equivalent to one centrifuge load (eg. eight specimens at a time).

Always digest/decontaminate the whole specimen, ie. do not attempt to select portions of the specimen as is done for direct microscopy. If the sputum will pour, it should be gently decanted from the specimen container into the centrifuge tube.

If the specimen is too viscuous to pour, an equal volume of digestant/decontaminant could be added to the sputum in the specimen container and the mixture poured carefully into the appropriate centrifuge tube.

Since sputum specimens are the most common clinical specimens submitted for tuberculosis culture, homogenisation and decontamination procedures have been largely targeted towards their processing. Specimens other than sputum demand even more care during processing because of the low numbers of tubercle bacilli present in positive specimens.

6.2 Sodium hydroxide (Modified Petroff) method

This method is used widely in developing countries because of its relative simplicity and the fact that the reagents are easy to obtain.

,NaOH is toxic, both for contaminants and for tubercle bacilli; therefore, strict adherence to the indicated timing is required

- ***Reagents***

  4% sodium hydroxide (NaOH) solution

Sodium hydroxide pellets (analytical grade)	4g
Distilled water	100ml

  Dissolve NaOH in distilled water and sterilise by autoclaving at 121°C for 15 minutes.

  Sterile saline

Sodium chloride pellets (analytical grade)	0.85g
Distilled water	100ml

  Dissolve NaCI in distilled water and sterilise by autoclaving at 121°C for 15 minutes.

  An alternative method for preparing 4% NaOH is as follows:

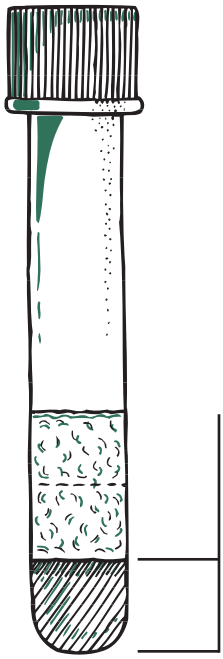
  Add the contents of a 250g bottle NaOH pellets to 500ml distilled water and fill water to the 625ml mark. Be careful since heat is released in this reaction. When needed, prepare a fresh 4% NaOH solution by adding 40% NaOH to sterile distilled water in the proportion 1:10.

- ***Procedure***

  Refer to Diagram 1 on page 40.

Diagram 1. Sodium hydroxide (NaOH) (Modified Petroff) method

PROCEDURE



To xml of sputum,
add 2xml of 4% NaOH

↓

Tighten cap of container
and shake to digest

↓

Let stand for 15 minutes
at room temperature with
occasional shaking

↓

Centrifuge at
3 000 x g for 15 minutes

↓

Pour off supernatant

↓

Add 15ml sterile saline or distilled water and resuspend sediment

↓

Centrifuge at 3 000 x g for 15 minutes

↓

Decant supernatant and inoculate onto culture medium immediately

6.2.1 Advantages

- The NaOH method is simple and inexpensive and provides fairly effective control of contaminants
- The time needed to process a single specimen is approximately one hour; 20 specimens would take approximately two hours, with centrifuge capacity being the limiting factor
- Sterilised NaOH solution will keep for several weeks. Store in plastic bottles

6.2.2 Limitations

- The specimen exposure times must be strictly followed to prevent over-kill of tubercle bacilli
- The NaOH procedure is very robust and may kill up to 60% of tubercle bacilli in clinical specimens. This initial kill is independent of additional contributory factors such as heat build-up in the centrifuge and centrifugal efficiency

A variety of more expensive and labour intensive homogenisation and decontamination methods[1] are available to countries with the required financial and human resources. Some of these will be discussed briefly.

OPTION

N-ACETYL-L-CYSTEINE-SODIUM HYDROXIDE (NALC-NaOH) METHOD

The mucolytic agent NALC (used for rapid digestion of sputum) enables the decontaminating agent (NaOH) to be used at a lower final concentration of 1%. Sodium citrate is included in the digestant mixture to bind the heavy metal ions which may be present in the specimen and could inactivate the acetyl-cysteine.

- Properly performed, this method provides more positive cultures than other methods, resulting in the killing of approximately 30% of tubercle bacilli
- The time needed to process a single specimen is approximately 40 minutes; 20 specimens would take approximately 60 minutes
- Acetyl-cysteine loses activity rapidly in solution, so the digestant should be made fresh daily
- The indicated specimen exposure time must be strictly adhered to and a 1:10 dilution of resuspended sediment must be made to decrease the concentration of any toxic components that may inhibit growth of tubercle bacilli
- Reagents such as bovine albumin and the required filters are expensive

1 Kent PT, Kubica GP. Public health mycobacteriology: Guide for the Level III Laboratory. US Department of Health and Human Services, Centres for Disease Control, USA, 1985.

OPTION

ZEPHIRAN-TRISODIUM PHOSPHATE (Z-TSP) METHOD

The use of trisodium phosphate and Zephiran (benzalkonium chloride) to homogenise and decontaminate specimens results in a more gentle digestion procedure.

- The procedure need not be as critically timed as NaOH digestion procedures
- The method results in the killing of approximately 30% of tubercle bacilli
- The time required for one specimen is nearly two hours; 20 specimens would require four hours

Excessive contamination is sometimes encountered in clinical material from certain patients, from certain areas or at certain times, and may present a difficult problem. For these problem specimens alternative decontamination methods such as 5% oxalic acid or 4% sulphuric acid may be used.[1]

OPTIONS

OXALIC ACID METHOD

This method is often helpful for specimens consistently contaminated with *Pseudomonas* species.

SULPHURIC ACID METHOD

This method is sometimes helpful for urine and other thin watery body fluids that consistently yield contaminated cultures when processed with one of the alkaline digestants.

6.2.3 Other specimens

Gastric lavage

These specimens should be processed within four hours of collection since their acidity is damaging to tubercle bacilli. Usually, gastric lavage does not need to be decontaminated, provided it has been collected aseptically in a sterile container. Centrifuge the total volume at 3 000 x g for 30 minutes. If contamination is suspected the sediment should be mixed with 2ml of 4% sulphuric acid and allowed to stand for 15 minutes, after which 15ml sterile saline is added. Centrifuge this mixture at 3 000 x g for 15 minutes and neutralise the sediment with 4% NaOH containing a phenol red indicator. Inoculate the sediment immediately onto culture medium.

[1] Kent PT, Kubica GP. Public health mycobacteriology: Guide for the Level III Laboratory. US Department of Health and Human Services, Centres for Disease Control, USA, 1985.

Urine

Centrifuge the total volume at 3 000 x g for 15 minutes. Discard the supernatant fluid and add 2ml of 4% sulphuric acid to the sediment. Let stand for 15 minutes, add 15ml sterile saline and centrifuge at 3 000 x g for 15 minutes. Neutralise the sediment with 4% NaOH containing a phenol red indicator. Inoculate the sediment immediately onto culture medium.

Laryngeal swabs

Cover the swab (in its original tube) with 5% oxalic acid and allow to act for 15 minutes. Remove the swab to another tube containing sterile saline. Lift after a few minutes, allow to drain and use to inoculate culture media.

For optimal results the oxalic acid (which might contain tubercle bacilli washed off from the swab) should be transferred to a centrifuge tube and centrifuged at 3 000 x g for 15 minutes. Wash the sediment once with sterile saline, centrifuge at 3 000 x g for 15 minutes and inoculate immediately onto culture medium.

Tissue

Lymph nodes, biopsies and other surgically resected tissue should be cut into small pieces with a sterile scalpel or scissors. Homogenise the specimen in a sterile porcelain mortar or tissue grinder, using 0.5-1ml sterile saline and a small quantity of sterilised sand (if necessary in mortar). This suspension can be directly inoculated onto culture media if the sterility measurements described before have been met; if not, decontaminate using 4% sulphuric acid as described for urine.

Mortars, pestles and tissue grinders must be cleaned and sterilised thoroughly to prevent false positive results or contamination due to organisms left over from previous specimens

Pus

This may be treated in the same way as aspirated fluids. If the material is very thick, it should be treated in the same way as sputum.

Cerebrospinal fluid

Cerebrospinal fluid should be concentrated by membrane filtration, by high speed centrifugation or by precipitation methods. Precipitation can be achieved by adding 0.1ml sterile rabbit serum or an equivalent albumin solution for every 10ml of cerebrospinal fluid. Mix until uniformly cloudy, centrifuge at 3 000 x g for 15 minutes and culture the sediment if contamination is not suspected.

If no sediment can be obtained by centrifugation, a sterile 20% solution of sulpho-salicylic acid may be added drop by drop until turbidity sets in. This precipitate is then more easily spun down to form a sediment.

If contamination of cerebrospinal fluid is likely, the sediment is mixed with 2ml of 4% sulphuric acid and allowed to stand for 15 minutes. Add 15 ml of sterile saline and centrifuge at 3 000 x g for 15 minutes. Inoculate sediment onto culture media.

Clots

In the case of specimens that form large clots, eg. pleural and ascitic fluids, it is recommended that clot formation be avoided by the addition of sodium citrate at the time of specimen collection. Add two drops of 20% sodium citrate for every 10ml fluid collected.

When clots are present, they can be digested with Petroff's NaOH method after homogenisation as described for tissue.

Other body fluids (including pleural fluid)

Mucopurulent fluid : Treat as for sputum when volume is 10ml or less.

Clear fluid : If collected aseptically centrifuge at 3 000 x g for 15 minutes and inoculate sediment directly onto culture media. If volume is more than 10ml treat as for gastric lavage.

Tubercle bacilli may adhere to glass or plastic surfaces. To optimise recovery, containers could be rinsed with sterile saline. Centrifuge the saline at 3 000 x g for 15 minutes and inoculate 2-3 drops onto culture media.

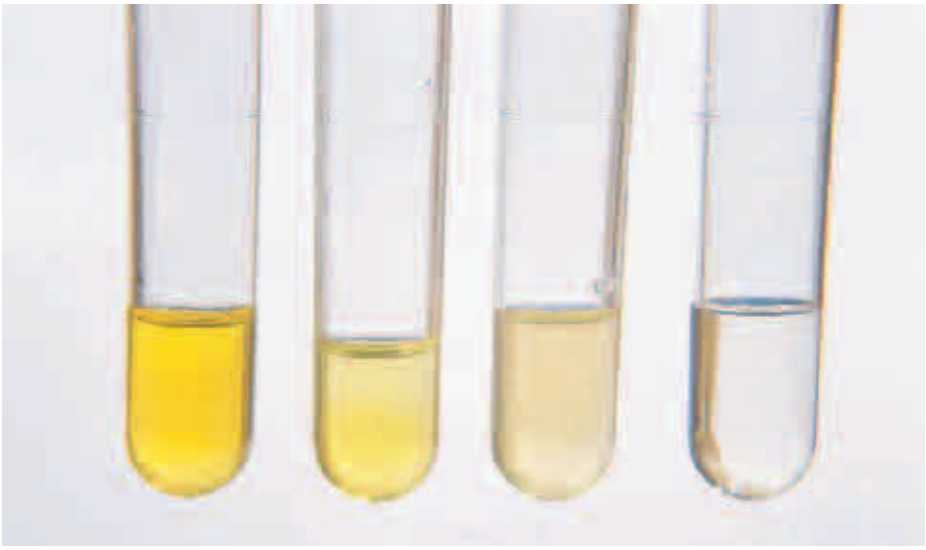
Specimens differ greatly in their degree of contamination and decontaminants should be selected to suit the nature of the specimens. The need for decontamination is also determined by the freshness of the specimen and by the efficiency of refrigeration before processing.

The following specimens usually do not need decontamination when aseptically collected into sterile containers:

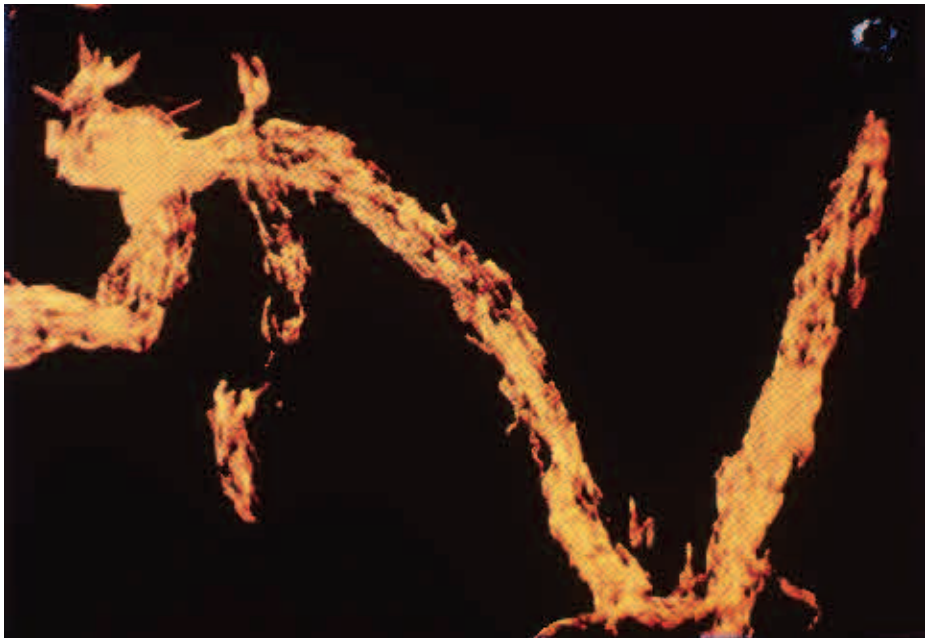
- Spinal, sinovial or other internal body fluids
- Bone marrow
- Pus from cold abscesses
- Surgically resected specimens (excluding autopsy material)
- Material obtained from pleural, liver and lymph nodes as well as biopsies (if not fistulised)

Whenever doubt exists about the contamination of specimens, an untreated portion may be inoculated onto nonselective bacteriological media, eg. nutrient agar, and incubated for 24 hours to check for the presence of fast-growing nonmycobacterial organisms. The remaining portion of the specimen is kept untreated and refrigerated until the absence of contaminants is confirmed. Should this not be the case the remaining specimen can then be appropriately decontaminated.

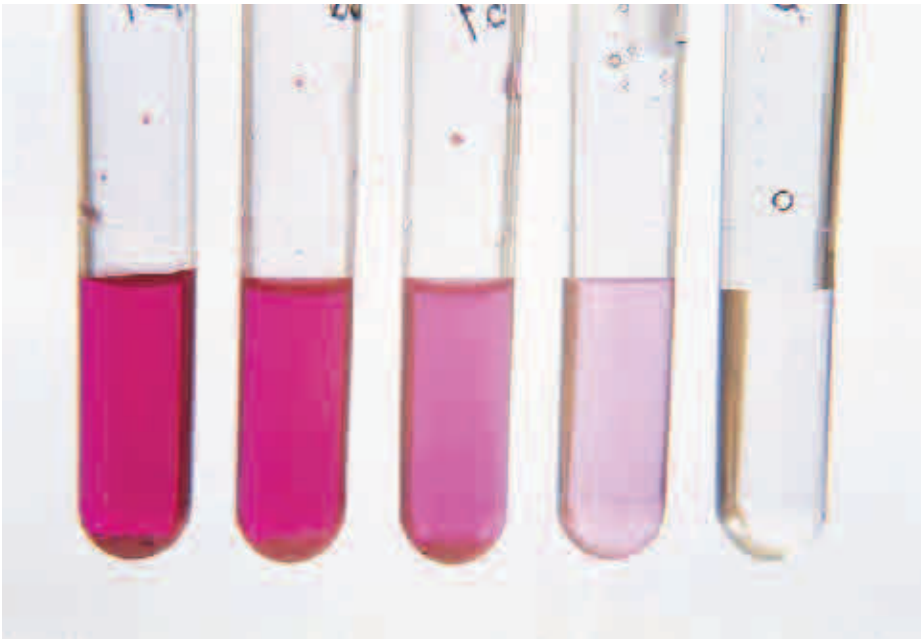
Culture of *Mycobacterium tuberculosis* on Löwenstein-Jensen medium. Organisms show typical cream-coloured, buff and rough colonies against the green egg-based medium.
(Courtesy of the South Africa Institute for Medical Research, Jahannesburg, South Africa).



Niacin production test to differentiate *Mycobaterium tuberculosis* from other mycobacterial species, with the formation of a yellow colour ondicating a positive reaction
(Courtesy of the National Tuberculosis Research Programme of the Medical Research Council, Pretoria, South Africa).



Fluorescent microscopy of a culture of *Mycobaterium tuberculosis* showing typical cord formation *(Courtesy of the National Tuberculosis Research Programme of the Medical Research Council, Pretoria, South Africa).*



Nitrate reduction test to differentiate *Mycobaterium tuberculosis* from other mycobacterial species, with the formation of an intense purple colour indicating a positive reaction.

7 CULTURE MEDIA

The definitive diagnosis of tuberculosis demands that *M. tuberculosis* be recovered on culture media and identified using differential *in vitro* tests. Many different media have been devised for cultivating tubercle bacilli and three main groups can be identified, *viz* egg-based media, agar-based media and liquid media.

The ideal medium for isolation of tubercle bacilli should (a) be economical and simple to prepare from readily available ingredients, (b) inhibit the growth of contaminants, (c) support luxuriant growth of small numbers of bacilli and (d) permit preliminary differentiation of isolates on the basis of colony morphology. For the culture of sputum specimens, egg-based media should be the first choice, since they meet all these requirements. There is increasing evidence that liquid media may give better results with other specimens. While cost prevents their routine use with sputum specimens, it is recommended that both egg-based and liquid medium be used for non-repeatable specimens, eg. cerebrospinal fluid and biopsy material.

It is recommended that all sputum specimens submitted for culture also undergo microscopic examination as outlined in the Technical Series on Microscopy.

7.1 Advantages and disadvantages of egg-based media

7.1.1 *Advantages*

- it is easy to prepare
- it is the least expensive of all media available and supports good growth of tubercle bacilli
- it may be stored in the refrigerator for several weeks provided it was made from fresh eggs and culture bottle caps are tightly closed to minimise drying by evaporation
- contamination during preparation is limited because it is inspissated after being placed in bottles. In addition, the malachite green added to the media suppresses the growth of nonmycobacterial organisms

7.1.2 *Disadvantages*

- it may take as long as eight weeks before cultures become positive, especially if specimens contain few bacilli or if decontamination procedures have been overly harsh
- when contamination does occur, it often involves the total surface of the medium and the culture is usually lost

7.2 Precautions during media preparation

For media of the best quality, chemicals of certified purity, clean glassware and freshly distilled and sterilised water should be used. Directions for preparing media must be followed precisely and without modification. A few general points to obtain good quality media and avoid contamination of reagents and media are as follows:

- Keep the environment as clean as possible. Swab the work surface with a suitable disinfectant (eg. 5% methylated spirits) before dispensing sterile reagents and media. Clean the floor with a wet mop to limit dust
- Use sterile glassware and equipment
- Use reagent grade chemicals and reagents unless otherwise specified
- Check the temperature of inspissators and hot air ovens
- Follow strict aseptic techniques when preparing media, eg. flaming flasks and tubes
- When preparing egg-based media, carefully clean egg shells before breaking
- Do not overheat medium during inspissation
- Do not leave prepared media exposed to light (including ultra-violet light), but store in the refrigerator in the dark when not in use
- Do not skimp on the volume of medium. Place 6-8ml of egg medium in each bottle or 20ml into each test tube

7.3 Preparation of egg-based media

1 LÖWENSTEIN-JENSEN MEDIUM

Löwenstein-Jensen (LJ) medium is most widely used for tuberculosis culture. The modification of the International Union Against Tuberculosis and Lung Disease (IUATLD) is recommended and will be described in detail. LJ medium containing glycerol favours the growth of *M. tuberculosis* while LJ medium without glycerol but containing pyruvate encourages the growth of *M. bovis.* Both should be used in countries or regions where patients may be infected with either organism.

- ***Ingredients***

Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4g
Magnesium sulphate ($MgSO_4.\ 7H_2O$)	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade)	12ml
Distilled water	600ml

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.

Malachite green solution, 2%

Malachite green dye	2.0g
Sterile distilled water	100ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will *not* store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

Homogenised whole eggs

Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. 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 complete medium***

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

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

The complete egg medium is distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20 x 150mm screw-capped test tubes, and the tops are securely fastened.

Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

- ***Coagulation of medium***

Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilise it). Heating for a second or third time has a detrimental effect on the quality of the medium.

The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discolouration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures.

Poor quality media should be discarded.

- ***Sterility check***

  After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 35°-37°C for 24 hours as a check of sterility.

- ***Storage***

  The LJ medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out of the medium. For optimal isolation from specimens, LJ medium should not be older than 4 weeks.

, *For the cultivation of M. bovis, LJ medium is enriched with 0,5% sodium pyruvate. Glycerol is omitted and 8.0g sodium pyruvate is added to the mineral solution.*

2 OGAWA MEDIUM

This medium is cheaper than Löwenstein-Jensen because it is made without asparagine.

- ***Ingredients***

  Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	3.0g
Sodium glutamate	3.0g
Distilled water	300ml

  Dissolve the ingredients in 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.

  Malachite green solution, 2%

Malachite green dye	2.0g
Sterile distilled water	100ml

  Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution will *not* store indefinitely and may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

  Homogenised whole eggs

  Fresh hens' eggs, not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and a plain alkaline soap. Let the eggs soak for 30 minutes in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub the hands and wash them. 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 complete medium***

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

Mineral salt solution	300ml
Malachite green solution	18ml
Whole hens' eggs (12-16 eggs, depending on size)	600ml
Glycerol	18ml

  The resulting pH of the medium is 6.8. The medium is mixed well and distributed in 6-8ml volumes in sterile 14ml or 28ml McCartney bottles or in 20ml volumes in 20x150mm screw-capped test tubes.

- ***Coagulation of medium***

  Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°-85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilise it). Heating for a second or third time has a detrimental effect on the quality of the medium.

  The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discolouration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures.

  Poor quality media should be discarded.

- ***Sterility check***

  After inspissation, the whole media batch or a representative sample of culture bottles should be incubated at 37°C for 24 hours as a check of sterility.

- ***Storage***

  The medium should be dated and stored in the refrigerator and can keep for several weeks if the caps are tightly closed to prevent drying out.

  In laboratories where centrifuges are not available, a simple culture technique could be employed as follows: Sputum specimens are decontaminated with equal volumes of 4% NaOH and inoculated directly onto modified or acid-buffered Ogawa medium. This technique shows a fairly comparable case yield when compared with concentrated culture techniques.

3 ACID-BUFFERED OGAWA MEDIUM

- ***Ingredients***

	Modified Ogawa	Acid-buffered Ogawa
Potassium dihydrogen phosphate	2g	3g
Magnesium citrate (KH_2PO_4)	0.1g	-
Sodium glutamate	0.5g	1.0g
Glycerol	4ml	6ml
Distilled water	100ml	100ml
Homogenised whole eggs	200ml	200ml
2% Malachite green solution	4ml	6ml
Final pH	6.4	6.2

- ***Preparation***

Dissolve the ingredients in the distilled water and boil for 30 minutes. Cool to room temperature and add the homogenised eggs and malachite green solution. Transfer 6-8ml volumes to suitable bottles and inspissate at 85°C for 45-60 minutes.

A variety of more expensive and labour intensive culture methods[1] are available to countries with the required financial and human resources. Some of these will be discussed briefly:

OPTIONS

HERMAN KIRCHNER LIQUID MEDIUM

This medium is most useful and least expensive of the liquid media for culture and tubercle bacilli. It has the additional advantage that it can support a large inoculum.

DUBOS OLEIC ACID-ALBUMIN LIQUID MEDIUM

This medium is recommended for the cultivation of tubercle bacilli from cerebrospinal, pleural and peritoneal fluid. It may be prepared from basic ingredients or may be obtained commercially as a ready-to-use base to which sterile albumin or serum is added.

1 Kent PT, Kubica GP. Public health mycobacteriology: Guide for the Level III Laboratory. US Department of Health and Human Services, Centres for Disease Control, USA, 1985.

MIDDLEBROOK 7H-10 AND 7H-11 AGAR MEDIUM

Middlebrook 7H-10 may be made from basic ingredients or may be prepared from commercially available 7H-10 agar-powdered base and Middlebrook oleic acid-albumin-dextrose-catalase (OADC) enrichment. 7H-11 is a 7H-10 agar enriched by the addition of enzymatic digest of casein. It is best to prepare 7H-10 and 7H-11 medium in small quantities of 200 to 400ml to minimise the amount of heat needed to melt the agar. Boiling the basal medium before autoclaving (either to solubilise the agar or to provide stocks of prepared base that may be stored and boiled for later use) should be avoided because the repeat heating produces medium of inferior quality.

When Middlebrook 7H-10 or 7H-11 medium is used for isolation cultures must be incubated in an atmosphere of 10% CO_2. Exposure of Middlebrook 7H-10 or 7H-11 agar to either daylight of heat results in the release of formaldehyde in sufficient concentration to inhibit the growth of mycobacteria.

OPTION

SELECTIVE MEDIUM

Specimens which are excessively contaminated may be inoculated onto selective antibiotic-containing media. Use may be made of antibiotics to which mycobacteria are not sensitive but which are capable of destroying the contaminants, eg. penicillin (50-100 units/ml), nalidixic acid (35µg/ml) or polymyxin (20-25µg/ml).

The antibiotics may be:

- Added to egg medium before inspissation
- Added to the surface of the medium slant or
- Mixed with the inoculum

Mycobactosel medium contains several antibiotics, eg. cycloheximide (0.4mg/ml), lincomycin (0.002mg/ml) and nalidixic acid (0.035mg/ml), while mycobactosel agar is commercially available. Antibiotic enriched medium should be stored in the refrigerator in the dark for a maximum of four weeks.

OPTION

RADIOMETRIC METHOD FOR TUBERCULOSIS CULTURE

Recent development in the diagnosis of tuberculosis include an automated system for detecting early growth of mycobacteria by a radiometric method (BACTEC: Beckton Dickinson). Sputum or other homogenates are decontaminated as necessary and added to vials containing Middlebrook 7H12 medium, an antibiotic mixture (to avoid the growth of other organisms) and ^{14}C-labelled palmitic acid. The medium is prepared commercially (BACTEC 12^{B}: Beckton Dickinson) in rubber-sealed bottles and inoculated with a syringe and hypodermic needle. If mycobacterial growth occurs, ^{14}C palmitic acid is utilised and $^{14}CO^{2}$ is produced. The air space above the medium in each bottle is sampled automatically by the BACTEC machine at fixed intervals and the amount of radioactive gas is estimated and recorded. Infectious aerosols are contained in the apparatus and captured in HEPA filters before the air is exhausted.

Growth of mycobacteria may be detected within 5-7 days, but positive results require further testing to distinguish between tubercle bacilli and other mycobacteria. In the BACTEC machine, p-nitro-a-acetylamino-b-priophenone (NAP) is used and tubercle bacilli can be differentiated within five days. NAP inhibits the growth of *M. tuberculosis* and usually does not affect the growth of MOTT bacilli.

Comparative tests have shown that the method is very successful and reliable and that confirmatory results for *M. tuberculosis* can be obtained within two weeks. However, the BACTEC machine is very expensive to purchase and to operate. In addition, two hazards must be considered if the machine is to be used for routine tuberculosis bacteriology: the use of hypodermic needles for the inoculation of media carries the risk of needle-stick injury, while the culture media is radioactive and presents a problem in terms of waste disposal.

In summary, the BACTEC method is invaluable for the detection of tubercle bacilli in material such as cerebrospinal fluid where rapid results are crucial in the management of the patient. However, the high cost of both the apparatus and the radio-labelled medium prohibits its routine use in most high tuberculosis prevalence countries.

8 INOCULATION AND INCUBATION PROCEDURES

8.1 Inoculation procedures

Condensed moisture is frequently observed at the bottom of culture medium slants. This should be removed before inoculation is attempted.

A common fault in inoculation is the use of too small an inoculum. Either loops (wire or disposable) or pipettes can be used for primary cultivation, although plastic Pasteur pipettes are recommended. Each slope should be inoculated with 0.2-0.4ml (2-4 drops or 2-4 loopfuls) of the centrifuged sediment, distributed over the surface. Fluid media can accommodate up to 1ml used for each specimen.

Two slopes of LJ medium should be inoculated per specimen. In areas where *M. bovis* may be a problem, an additional slope containing pyruvate should be added.

8.2 Incubation of cultures

All cultures should be incubated at 35°-37°C until growth is observed or discarded as negative after eight weeks.

Inoculated media should preferably be incubated in a slanted position for at least 24 hours to ensure even distribution of inoculum. Thereafter, if incubator space is needed, bottles could be placed upright. Tops should be tightened to minimise evaporation and drying of media.

The various Middlebrook agars require an atmosphere of 10% CO_2 and 90% air to ensure growth. CO_2 is not essential to initiate growth on egg-based medium but does stimulate earlier and more luxuriant growth. A separate CO_2 incubator is not necessary. Inlet and outlet petcocks can be attached to an airtight metal or plastic box, built to fit on a shelf of the incubator. This box, which contains the incubating cultures, should be flushed daily with a compressed mixture of 10% CO_2 and 90% air. Alternatively, agar plates can be placed in impermeable Mylar plastic bags and these charged three times a week with CO_2.

9 CULTURE EXAMINATION AND IDENTIFICATION

9.1 Examination schedule

All cultures should be examined 72 hours after inoculation to check that liquid has completely evaporated, to tighten caps in order to prevent drying out of media and to detect contaminants. Thereafter, cultures are examined weekly, or if this is not operationally feasible, on at least three occasions, *viz*

- after one week to detect rapidly growing mycobacteria which may be mistaken for *M. tuberculosis*
- after three to four weeks to detect positive cultures of *M. tuberculosis* as well as other slow-growing mycobacteria which may be either harmless saprophytes or potential pathogens
- after eight weeks to detect very slow-growing mycobacteria, including *M. tuberculosis,* before judging the culture to be negative

It is useful to label containers with cultures with the dates necessary for examination and to place containers in the incubator in chronological order.

Should contaminated cultures be found during the examination, those where the surface has been completely contaminated or where medium has been liquefied or discoloured should be sterilised and discarded. Certain contaminating organisms produce acid from constituents of the medium and the lowering of pH unbinds some of the malachite green from the egg (indicated by the medium changing to dark green). Tubercle bacilli will not grow under these conditions and cultures should be discarded. Cultures with partial contamination should be retained until the eighth week. Late contamination does not exclude the presence of *M. tuberculosis*; it is therefore advisable to prepare a smear from the surface of the medium. Should microscopy indicate the presence of acid-fast bacilli, an attempt could be made to re-decontaminate and re-inoculate the culture.

9.2 Reading of cultures

Typical colonies of *M. tuberculosis* are rough, crumbly, waxy, non-pigmented (cream coloured) and slow- growers, ie. only appearing three weeks after inoculation.

With doubtful cultures or when less experienced staff read cultures, the acid-fastness should be confirmed by Ziehl-Neelsen (ZN) staining. A very small amount of growth is removed from the culture using a loop and gently rubbed into one drop of sterile saline on a slide. At this point the ease with which the organisms emulsify in the liquid should be noted: Tubercle bacilli do not form smooth suspensions, unlike some other mycobacteria. The smear is allowed to dry, fixed by heat and stained by the ZN method.

For preliminary identification of tubercle bacilli the following characteristics apply:

- Tubercle bacilli do not grow in primary culture in less than one week and usually take three to four weeks to give visible growth
- The colonies are buff coloured (never yellow) and rough, having the appearance of bread crumbs or cauliflower
- They do not emulsify in the saline used for making smears but give a granular suspension
- Microscopically they are frequently arranged in serpentine cords of varying length or show district linear clumping. Individual cells are between 3µm and 4µm in length

9.3 Differentiation of *M. tuberculosis*

Although a presumptive diagnosis of tuberculosis may be made by an experienced laboratory technologist on the basis of the characteristics of tubercle bacilli described before, it is best to do confirmatory tests. Unfortunately there is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Nevertheless, the following tests, when used in combination with the characteristics described before will enable the precise identification of >95% of *M. tuberculosis* strains.

1 NIACIN TEST

Niacin (nicotinic acid) plays a vital role in the oxidation-reduction reactions that occur during metabolic processes in all mycobacteria. Although all mycobacteria produce niacin, comparative studies have shown that, because of a blocked metabolic pathway, *M. tuberculosis* accumulates the largest amount of nicotinic acid and its detection is useful for its definitive diagnosis. Niacin negative *M. tuberculosis* strains are very rare, while very few other mycobacterial species yield positive niacin tests.

Cultures grown on egg medium yield the most consistent results in the niacin test and LJ medium is therefore recommended. A culture must be at least three to four weeks old and must have sufficient growth of more than 50 colonies. Because *M. tuberculosis* excretes niacin into the growth medium, cultures with confluent growth may give a false-negative niacin reaction because the extracting fluid cannot come in contact with the culture medium. When this occurs, expose the underlying medium surface by either scraping away or puncturing through some of the culture growth.

Aeration of cultures intended for niacin testing is very important. Caps should be loose on slants throughout the entire incubation period and special Cap-o-Test stoppers are recommended.

9.3.1 Niacin test with chemical reagents

Controls

Control the reagents by testing the extract from an uninoculated tube of medium (*negative control*) and use an extract from a culture of *M. tuberculosis* H37Rv as *positive control*.

- ***Reagents***

  Aniline solution, 4%

  - *Aniline is oncogenic and penetrates through the skin. Work with gloves and be very careful*
  - *Aniline may change colour on exposure to air and light; prepare a fresh solution when necessary*

Fresh, clear colourless aniline	4ml
Ethanol 95%	96ml

  Mix aniline with ethanol in an amber bottle and store in the dark in the refrigerator. Discard if solution turns yellow.

  Cyanogen bromide solution, 10%

  - *Cyanogen bromide is a severe lacrimator and toxic if inhaled; work in a well-ventilated fume hood when preparing the solution and in a biological safety cabinet when testing cultures*
  - *Cyanogen bromide is oncogenic and penetrates through the skin. Work with gloves and be very careful*
  - *In acid solution, cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic. Discard all reaction tubes into a disinfectant solution made alkaline by the addition of sodium hydroxide*

Cyanogen bromide crystals	5g
Distilled water	50ml

  - Add cyanogen bromide crystals to distilled water in a glass beaker
  - Cover the beaker with foil and leave in the fume cupboard at room temperature. The crystals take approximately 24 hours to dissolve at room temperature
  - Do *not* heat the solution over a Bunsen flame
  - Pour into a tightly capped amber bottle and store in the refrigerator
  - Warm to room temperature to dissolve any precipitate formed upon cooling

- Prepare small amounts because cyanogen bromide is volatile and loses strength on storage. Weak solutions give false-negative results

To avoid unnecessary prolonged exposure to the cyanogen bromide while weighing the following procedure may be followed:

- Write down the weight of an empty beaker closed with a piece of aluminium foil
- Remove the approximate quantity (eg. approximately 1/10 of the contents of a 100g bottle for a 10% solution) of the white cyanogen bromide crystals into the beaker, cover it and record the weight
- Calculate the difference between the two readings to obtain the *exact* weight of crystals in the beaker
- Add the required amount of distilled water to give a final concentration of 10%

In some countries a 4% aqueous potassium cyanide solution containing bromide is used and is prepared as follows:

- *Bromine water is highly corrosive and volatile and should be stored away from other chemical reagents*
- *KCN is very poisonous and should be handled in a fume hood*
- Break an ampoule of bromine (50ml) in a 1 000ml capacity dark glass flask (with glass stopper) containing 150ml cold distilled water
- Prepare a 4% aqueous potassium cyanide solution by dissolving 4g KCN in 100ml distilled water. The KCN must be pure and not hydrated
- With a pipette, remove 1ml of the bromine layer beneath the surface of the bromine water and transfer it to the bottom of a 250ml Erleynmeyer flask. Rapidly add, drop by drop, the potassium cyanide solution, shaking by rotation until total decolorisation is obtained

- ***Procedure***

Refer to Diagram 2 on page 61.

Diagram 2. Niacin test for identification of M. tuberculosis

PROCEDURE

Add 1 ml of sterile water to the culture slant. If growth is confluent, puncture the medium with a Pasteur pipette to allow contact of the water with the medium

↓

Place the tube horizontally so the fluid covers the entire surface of the medium

↓

Allow 30 minutes for the extraction of niacin. The extraction time may be longer if the culture has few colonies

↓

Raise the slants upright for 5 minutes to allow the fluid to drain to the bottom

↓

Remove 0.5ml of the fluid extract to a clean screwcap tube

↓

Sequentially add 0.5ml of the 4% aniline solution and 0.5ml of 10% cyanogen bromide

↓

Close the tubes and observe the solution for the formation of a yellow colour (= positive result) within 5 minutes. The yellow colour appears as a ring at the interface of the two reagents, or if the tube is shaken, as a yellow column of liquid

↓

Add 2-3 ml of 4% NaOH to each tube and discard

- ***Results and interpretation***
  - Negative : No colour
  - Positive : Yellow colour appearing within 5 minutes. The colour appears as a ring at the interface of the two reagents, or if the tube is shaken, as a yellow column of liquid.

Niacin test with paper strips

Paper test strips for the detection of niacin are commercially available. They compare well to the chemical reagents in detecting niacin production. A paper-strip method obviates the need to prepare and store the unstable and toxic chemicals used to demonstrate the presence of niacin, but is much more expensive.

- ***Procedure***

  Refer to Diagram 3 on page 63.

- ***Results and interpretation***
  - Negative : No colour
  - Positive : Yellow liquid in the bottom of the tube. Discard any colour on the stip itself; this may occur because of oxidation of chemicals, especially at the top of the strip
- ***Precautions***
  - Always check the expiry date of commercial test strips
  - To prevent false-negative results promptly reseal tubes after inserting paper strip; if tubes are left unsealed the gas evolved as chemical mix on the strip may escape into the atmosphere

Diagram 3. Niacin paper strip test for the identification of M. tuberculosis

PROCEDURE

Add 1ml of sterile saline to the culture slant. If growth is confluent, puncture the medium with a Pasteur pipette to allow contact of the saline with the medium

↓

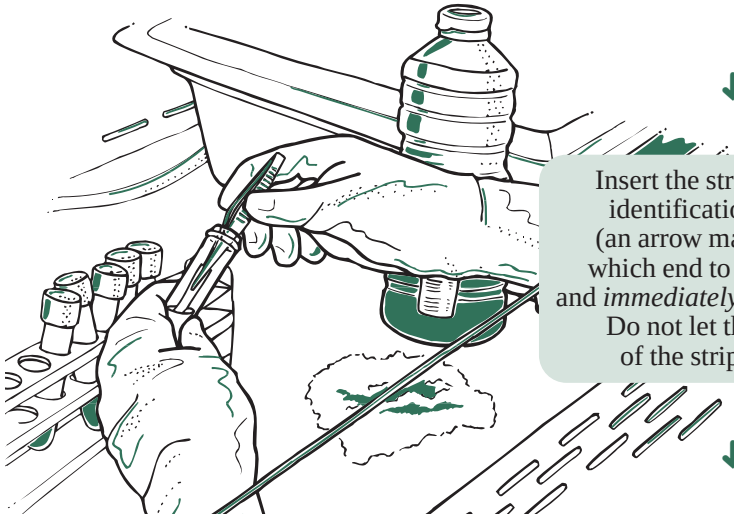
Place the tube horizontally so the fluid covers the entire surface of the medium

↓

Allow 30 minutes for the extraction of niacin. The extraction time may be longer if the culture has few colonies

↓

Raise the slant upright for 5 minutes to allow the fluid to drain to the bottom. Remove 0.5ml of the fluid extract to a clean screwcap tube



↓

Insert the strip with the identification end up (an arrow may indicate which end to insert first) and *immediately* seal the tube. Do not let the middle of the strip get wet

↓

Leave at room temperature for 15-20 minutes. Occasionally agitate the tube without tilting it

↓

Observe the colour of the liquid in the bottom of the tube against a white background (yellow = positive). Discard any colour on the strip itself; this may occur because of oxidation of chemicals, especially at the top of the strip

↓

Neutralise the strips with 10% sodium hydroxide or discard them into alkaline disinfectant

2 NITRATE REDUCTION TEST

M. tuberculosis is one of the strongest reducers of nitrate among the mycobacteria, which allows for this test to be used in combination with the niacin test in differentiating *M. tuberculosis* from the other mycobacteria.

Cultures to be tested for nitrate reduction should be four weeks old and have abundant growth Löwenstein Jensen egg medium is recommended.

Classical method with liquid reagents

- ***Reagents***

  Sodium nitrate substrate in buffer

  Prepare 0.01M sodium nitrate in 0.022M phosphate buffer, pH 7.0 as follows:

  KH_2PO_4 3.02g
  Distilled water 1000ml

  Dissolve potassium phosphate in distilled water
  to provide an 0.022M solution .....Solution 1

  Na_2HPO_4 3.16g
  Distilled water 1000ml

  Dissolve sodium phosphate in distilled water
  to provide an 0.022M solution .....Solution 2

  Add 611ml of solution 2 to 389ml of solution 1, and mix well.
  Check pH to be 7.0 .....Solution 3

  Complete sodium nitrate substrate buffer

  $NaNO_3$ 0.85g
  Solution 3 1000ml

  Dissolve the sodium nitrate in the buffer and dispense in 100ml aliquots. Sterilise by autoclaving at 121°C for 15 minutes. When needed, aliquots of the substrate solution are aseptically dispensed into sterile screw-capped tubes in 2ml quantities.

  Hydrochloric acid solution

  Concentrated HCI 10ml
  Distilled water 10ml

  Slowly add concentrated HCI to distilled water (*never the reverse*) to obtain a 1:1 dilution. Store in an amber bottle in the dark in the refrigerator.

Sulfanilamide solution, 0.2%

Sulfanilamide	0.2g
Distilled water	100ml

Dissolve sulfanilamide in distilled water and store in an amber bottle in the dark in a refrigerator.

N-naphthylethylene-diamine solution, 0.1%

N-naphthylethylene-diamine	0.1g
Distilled water	100ml

Dissolve naphthylethylene-diamine in distilled water and store in an amber bottle in the dark in a refrigerator.

- ***Controls***

  Control the reagents by testing the extract from an uninoculated tube of medium (*negative control*) and use an extract from a culture of *M. tuberculosis* H37Rv as *positive control*.

- ***Procedure***

  Refer to Diagram 4 .

Diagram 4.
Nitrate reduction test for identification of M. tuberculosis classical method

PROCEDURE

Add 0.2ml of sterile saline to a screw-cap tube

↓

Use a sterile loop/spade to emulsify two loopfuls/spadefuls of a 4-week old culture in the saline

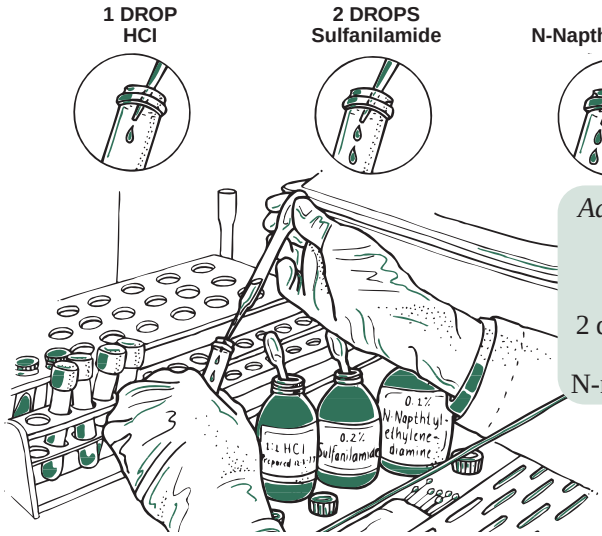
↓

Add 2ml of the $NaNo_3$ substrate

↓

Shake well and incubate upright in a 37°C water bath for 3 hours and remove

↓



Add in the following order:

1 drop diluted HCl
(shake tube well)
2 drops 0.2% sulfanilamide
2 drops 01.%
N-napthtylethylene-diamine

Examine immediately for a pink to red colour and compare to colour standard

- ***Results and interpretation***

  - Negative : No colour. If no colour develops, the test is either negative or the reduction has proceeded beyond nitrite. Add a small amount of powdered zinc to all negative tests by tipping the end of a slightly moistened applicator stick into dry zinc and shaking into the liquid.

    a) If nitrate is still present, it will be catalysed by the zinc and a red colour will develop, indicating a *true negative*

    b) If no colour develops the original reaction was positive but the nitrate was reduced beyond nitrite. *Repeat the test* to confirm the observation

  - Positive : Red colour, which vary from pink to very deep red-crimson:

    Faint pink = *+/-*
    Clear pink = *1+*
    Deep pink = *2+*
    Red = *3+*
    Deep red = *4+*
    Purplish red = *5+*

    Only 3+ to 5+ is considered positive.

Method with crystalline reagent

The dry crystalline reagent is easy to prepare, has a shelf-life of a least six months and has the added advantage that only one reagent is needed to detect nitrate rather than the three liquid reagents used in the conventional chemical test.

- ***Reagents***

  Sodium nitrate substrate in buffer

  Prepare as described in classical method, page 62.

  Crystalline reagent

Sulfanilic acid	1 part
N-(1-naphthyl)-etylenediamine dihydrochloride	1 part
L(+) - tartaric acid	10 parts

  Put the chemicals in an amber bottle and mix by vigorous manual shaking about 30 times. (Tartaric acid is much more crystalline than the other two chemicals and may have to be ground using a mortar and pestle to ensure good mixture of the reagents). The dry mixture has a heterogeneous crystalline appearance. Store in the amber bottle at room temperature.

- ***Controls***

  Control the reagents by testing the extract from an uninoculated tube of medium (*negative control*) and use an extract from a culture of *M. tuberculosis* H37Rv as *positive control*.

- ***Procedure***

Refer to Diagram 5 on page 68.

- ***Results and interpretation***

  - Negative : No colour. If no colour develops, the test is either negative or the reduction has proceeded beyond nitrite. Add a small amount of powdered zinc to all negative tests by tipping the end of a slightly moistened applicator stick into dry zinc and shaking into the liquid.

    a) If nitrate is still present, it will be catalysed by the zinc and a red colour will develop, indicating a *true negative*

    b) If no colour develops the original reaction was positive but the nitrate was reduced beyond nitrite. *Repeat the test* to confirm the observation

  - Positive : Red colour, which vary from pink to very deep red-crimson:

Faint pink	=	*+/-*
Clear pink	=	*1+*
Deep pink	=	*2+*
Red	=	*3+*
Deep red	=	*4+*
Purplish red	=	*5+*

  Only 3+ to 5+ is considered positive.

Diagram 5. Nitrate reduction test for identification of M. tuberculosis Crystalline reagent method

PROCEDURE

Add 0.2ml of sterile saline to a screw-cap tube

↓

Use a sterile loop/spade to emulsify two loopfuls/spadefuls of a 4-week old culture in the saline

↓

Add 2ml of the $NaNo_3$ substrate

↓

Shake well and incubate upright in a 37°C water bath for 3 hours and remove

↓

Use the tip of a spatula to add a small amount of crystalline reagent to the test solution. The quantity of reagent is not critical

↓

Examine immediately for a pink to red colour and compare to colour standard

Nitrate test with paper strips

Paper test strips for the detection of nitrate following nitrate reduction are commercially available. The paper strip test method yields most consistent results with mycobacteria that vigorously reduce nitrate, such as *M. tuberculosis*. It therefore provides reliable results and is much less labour-intensive than the chemical method, but is much more expensive.

- ***Procedure***

  Refer to Diagram 6 on page 69.

- ***Results and interpretation***
  - Negative : No colour change
  - Positive : Top portion of the strip changes to light or dark blue

- ***Precautions***
  - Always check the expiry date of commercial test strips
  - Because the strips are sensitive to sunlight, excess heat and moisture, they should be stored between 2°C and 8°C in the original container, tightly capped
  - Discard strips if they become discoloured, for this indicates deterioration of the reagent
  - Do not rely on results of test strips if the positive control culture gives weak or negative reactions

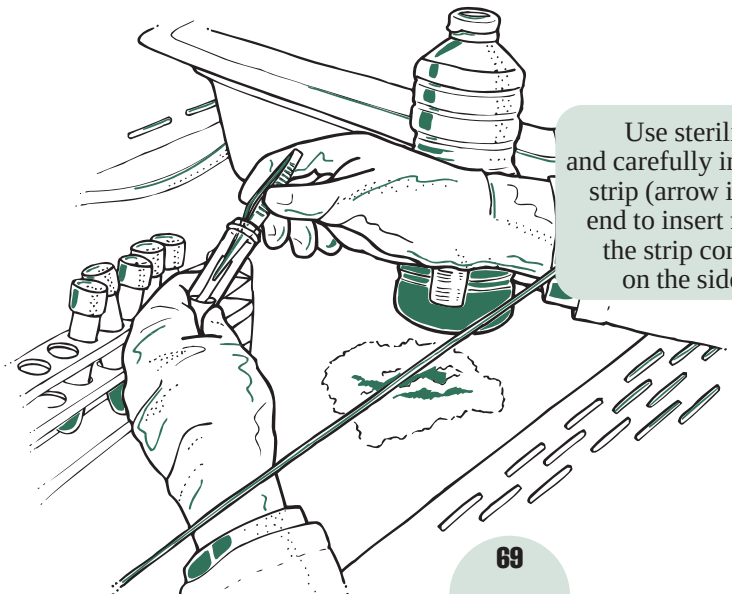
Diagram 6. Nitrate paper strip test for confirmation of M. tuberculosis

PROCEDURE

Add 1ml sterile saline to a sterile screwcap test tube

↓

Use a sterile spade/applicator stick to emulsify in the saline two spadefuls of growth from a four-week-old culture



↓

Use sterilised forceps and carefully insert a nitrate test strip (arrow indicates which end to insert first); do not let the strip contact any fluid on the side of the tube

↓

Cap the tube tightly and incubate in a vertical position at 37°C for two hours

↓

After one hour of incubation, shake the tube gently without tilting

↓

After two hours of incubation, tilt the tube six times to wet the entire strip

↓

Allow the tube to remain slanted for 10 minutes with the liquid covering the strip

↓

Examine the top portion of the strip for changes to light or dark blue (= positive)

9.4 Nitrate reduction standards

In order to ensure consistency in interpreting nitrate reduction reactions it is recommended that a series of standards depicting the colour intensity from +/- to 5+ be prepared. These keep indefinitely and should be used whenever nitrate tests are done.

- ***Reagents***

  Stock solution

  1. 0.067M disodium phosphate (9,47g of anydrous Na_2HPO_4 per 1 000ml)
  2. 0.067M monopotassium phosphate (9.07g of KH_2PO_4 per 1 000ml)
  3. 0.067M trisodium phosphate (25.47g of $Na_3PO_4C12H_2O$ per 1 000ml)
  4. 1% phenolphthalein (1g in 100ml 95% ethyl alcohol)
  5. 1% bromthymol blue (1g in 100ml 95% ethyl alcohol)
  6. 0.01% bromthymol blue: prepare by mixing 1.0ml of no.“ above in 100ml of distilled water.

  Working buffer solution
  Mix 35ml of stock solution 1, 5ml of stock solution 2 and 100ml of solution 3.

- ***Procedure***

  - Place eight clean test tubes (number 1-8) in a rack. Use the same size tubes as used to perform the nitrate reduction test.
  - Put 2ml of working buffer solution into tubes 2 through 8.

- To 10ml of working buffer solution,
  add 0.1ml of 4 and 0.2ml of 6 ..... Solution 7
- Add 2ml of solution 7 to the tube numbered 1. This is the 5+ colour standard.
- To the tube number 2 in the series, add 2ml of solution 7. Mix well and transfer 2ml to the next tube (number 3). Continue to make serial dilutions of 2ml, discarding 2ml from the 8th tube.
- The colour standards:

  tube 1 = 5+
  tube 2 = 4+
  tube 3 = 3+
  tube 5 = 2+
  tube 6 = 1+
  tube 8 = +/-

- Autoclave tubes, seal and store at 5°C.

3 CATALASE TEST

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen, ie. $2H_2O_2 \rightarrow 2H_2O + O_2$. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all mycobacteria passes catalase enzymes, except for certain isoniazid-resistant mutants of *M. tuberculosis* and *M. bovis*.

Mycobacteria posses several kinds of catalase that vary in heat stability. Quantitative differences in catalase activity can be demonstrated by one or more of the following tests:

- Room temperature or drop method (indicates the presence of catalyse)
- Semiquantitative test (indicates level of catalyse production)
- 68°C test at pH7 (indicates loss of catalyse activity due to heat)

Drug susceptible strains of *M. tuberculosis* do form catalyse as indicated by the drop method, produce less than 45mm of bubbles in the semiquantitative test and lose catalase activity when heated to 68°C for 20 minutes. For these tests 14 day-old cultures on LJ butts should be used, ie. the media tubes should be inspissated in an upright position to provide a butt and should not be slanted. The tubes must have stoppers which permit exchange of air, eg. Cap-o-Test stoppers. The cultures should be incubated in a well-humidified incubator at 35°-37°C, with loose caps, for 14 days.

- ***Reagents***

0.067M phosphate buffer solution, pH 7.0

Na_2HPO_4 anhydrous	9.47g
Distilled water	1000ml

Dissolve disodium phosphate in distilled water to provide an 0.067M solution .....Solution 1

KH_2PO_4	9.07g
Distilled water	1000ml

Dissolve monopotassium phosphate in distilled water to provide an 0.067M solution .....Solution 2

Hydrogen peroxide, 30%

30% hydrogen peroxide (H_2O_2), also known as Superoxol (Merck) is stored in the refrigerator.

- Ensure that the H_2O_2 used is 30% and not the 3% kind obtained from pharmacies
- Wear rubber or plastic gloves and a protective eye shield when handling superoxol

Tween 80, 10%

Tween 80	10ml
Distilled water	90ml

Mix Tween 80 with distilled water and autoclave at 121°C for 10 minutes. The Tween may settle during autoclaving and may be resuspended by swirling immediately after autoclaving and during cooling. Store in the refrigerator.

Complete catalase reagent (Tween-peroxide mixture)

Immediately before use, mix equal parts of 10% Tween 80 and 30% hydrogen peroxide. Allow 0.5ml reagent for each strain to be tested.

- ***Controls***
  - *Drop method*

Use an uninoculated tube of medium as negative control and an LJ butt of *M. tuberculosis* H37Rv as positive control

- *Semiquantitative and 68°C tests*

Use an uninoculated tube of medium as negative control and an LJ butt of *M. terrae* as positive control.

- ***Procedures***

  - *Drop method*

  Examine the 14 day-old LJ slant to ascertain that growth has occurred. Add one to two drops of the freshly-prepared Tween-peroxide mixture to the slant with the culture growth. Observe for a period of 5 minutes for the formation of bubbles.

  Results and interpretation

Negative	:	No bubbles formed
Positive (slow)	:	Few slowly forming bubbles
Positive (rapid)	:	Immediate copious formation of bubbles

  - *Semiquantitative test*

  Examine the 14 day-old LJ butt to ascertain that growth has occurred. Add 1ml of the freshly-prepared Tween-peroxide mixture, replace caps loosely and allow to stand at room temperature for 5 minutes. A column of foam will form.

  Measure the height of the foam column (ie. from the top of the liquid on the LJ medium to the top of the foam).

  Results and interpretation

Low or no catalase activity	:	Less than 31mm of foam
Inconclusive result	:	Between 31 and 45mm of foam
High catalase activity	:	More than 45mm of foam

  - *68°C, pH7.0 test*

  Refer to Diagram 7 on page 74.

  Results and interpretation

  - Positive : Bubbles
  - Negative : No bubbles

  On rare occasions, bubbles may be seen rising from the sedimental cells in such small quantity that foam does not form at the surface of the fluid. *This is still recorded as a positive reaction.*

Diagram 7. Heat labile catalase test (68°C, pH 7.0) for identification of M. tuberculosis

PROCEDURE

With a sterile pipette, aseptically add 0.5ml of 0.067M phosphate buffer, pH 7.0 to 16x125mm screw-cap tubes

↓

Suspend several loopfulls of test cultures in the buffer solution, using sterile loops

↓

Place the tubes containing the emulsified cultures in a previously heated water bath at 68°C for 20 minutes. *Time and temperature are critical*

↓

Remove the tubes from heat and allow to cool to room temperature

↓

Add 0.5ml of the freshly-prepared Tween-peroxide mixture to each tube and replace caps loosely

↓

Observe the formation of bubbles appearing on the surface of the liquid. *Do not shake the tubes* because Tween 80 also may form bubbles when shaken, resulting in false positive results

↓

Hold negative tubes for 20 minutes before discarding

4 GROWTH ON MEDIUM CONTAINING p-NITROBENZOIC ACID (PNB)

In laboratories where facilities and reagents for niacin and nitrate testing are not available, identification of tubercle bacilli may be done by a combination of one or more of the catalase tests described previously together with growth at 25°C on LJ medium and growth on LJ medium containing p-nitrobenzoic acid at 37°C. Problems with incubation at 25°C may be encountered in tropical regions. A refrigerated incubator should be used where available; as an alternative, a water bath within a refrigerator or cold room should be used.

- ***Procedure***
  - Inoculate two slopes of LJ medium containing glycerol and one tube of LJ medium containing p-nitrobenzoic acid (PNB) at a concentration of 500mg/litre
  - Incubate one LJ slope and the PNB slope at 37°C in an internally illuminated incubator and examine at 3, 7, 14 and 21 days. When growth is evident on the LJ slope examine it for pigment. If an internally illuminated incubator is not available, remove slopes from the dark incubator as soon as growth is evident, loosen the caps to admit some oxygen and expose them to daylight (but not direct sunlight) or place 1m from a laboratory bench lamp for 1 hour. Reincubate and examine for pigment the following day
  - Incubate the other LJ slope at 25°C and examine at 3, 7, 14 and 21 days

- ***Results and interpretation***

  M. tuberculosis does not grow within three days at 37°C and does not grow at all at 25°C or on PNB medium. It also does not produce yellow or orange pigment in the dark or after exposure to light.

SUMMARY

IDENTIFICATION OF M. tuberculosis

- Growth rate slow
- Growth temperature 35°-37°C only
- No pigmentation
- Niacin positive
- Nitrate positive
- Catalase negative at 68°C
- No growth on LJ medium containing p-nitrobenzoic acid

10

RECORDING AND REPORTING OF LABORATORY RESULTS

Tuberculosis laboratories must establish a uniform procedure for reporting culture results. If laboratory findings are to be useful, they must be communicated in ways that make sense to the different authorities: Health care workers use the findings for the diagnosis and management of tuberculosis; public health authorities use them for statistical and epidemiological purposes, while tuberculosis managers use the information to ensure that bacteriologically proven patients are receiving appropriate chemotherapy.

Culture procedures for tuberculosis bacteriology are notoriously time-consuming, often taking weeks or months to complete. For this reason, interim reports should be issued. The following schedule is recommended:

- If the cultures have been contaminated, a report should be sent out *immediately* and a repeat specimen requested
- If cultures are positive and growth has been identified as *M. tuberculosis* a report should be sent out *immediately*
- At *four weeks* an interim report (optional) could be sent out on all negative specimens, stating that another report will be issued in the event of the specimen becoming positive later on
- At *eight weeks* a final report should be issued containing all the data previously reported so that earlier interim reports can be destroyed and only the final report retained in the patients' file

Culture reports should be qualitative (ie. positive or negative) as well as quantitative (ie. number of colonies isolated). The *average number* of colonies on all the bottles/tubes per specimen should be reported. The following scheme is recommended:

Reading	Report
No growth	Negative
1-19 colonies	Positive (number of colonies)
20-100 colonies	Positive (1+)
100-200 colonies	Positive (2 +)
200-500 colonies (almost confluent growth)	Positive (3 +)
>500 colonies (confluent growth)	Positive (4 +)
Contaminated	Contaminated

In high tuberculosis prevalence countries more than 85% of disease is due to *M. tuberculosis* and other mycobacteria rarely are responsible for clinical disease. Also, the robust decontamination techniques used favour the isolation of *M. tuberculosis* while killing some of the more fragile mycobacteria. A positive culture with the characteristics as described before can, therefore, fairly safely be labelled as "tubercle bacilli" and the recommended way of reporting is as follows:

"Cultivation yielded _____ growth of mycobacteria with the characteristics of tubercle bacilli"

Model tuberculosis culture reports are presented in Annex 4.

Obviously, all the technical details of each laboratory test cannot be recorded on the reports, but they should be precisely outlined in the laboratory manual and all the results must be noted in the laboratory register. A copy of the completed final report should be retained in the laboratory. When reading cultures the following should be recorded in the laboratory register:

- Growth rate (slow / rapid)
- Number of colonies isolated
- Pigment production in the colonies (none / present and colour)
- Colony morphology (rough / smooth / shiny / flat)
- Results of differential tests

A model laboratory register is presented in Annex 5.

11

QUALITY CONTROL (see part I, page 41)

Quality assurance with regard to tuberculosis culture is a system designed to continuously improve the reliability, efficiency and use of culture as diagnostic and monitoring option. The purpose of a quality assurance programme is to improve the efficiency and reliability of culture services. The components of a quality assurance programme are:

- quality control (see part I, page 41),
- quality improvement (see part I, page 41),
- proficiency testing (see part I, page 43).

The following section will focus on aspects of *quality control* in the culture laboratory. For a discussion on quality improvement and proficiency testing please refer to the Management Series.

Quality control of culture is a process of effective and systematic *internal* monitoring of the performance of bench work in the culture laboratory. Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible. This is accomplished by assessing - against acceptable established limits - the quality of specimens, the performance of decontamination, digestion and culture procedures, the quality of reagents, media and equipment, by reviewing culture results and by documenting the validity of culture methods.

Quality control should be performed on a regular basis in the culture laboratory to ensure reliability and reproducibility of laboratory results. For a quality control programme to be of value, it must be practical and workable.

Quality control is the responsibility of all laboratory workers

Quality control must be applied to:

- laboratory arrangement
- equipment
- collection and transport of specimens
- handling of specimens
- reagents and media
- culture methods
- reporting of results

The keys to successful quality control are:

- adequately trained, interested and committed staff
- common-sense use of practical procedures
- a willingness to admit and rectify mistakes
- effective communication

Quality control measures which must be in place in all tuberculosis culture laboratories include:

Laboratory arrangement and administration

- Ensure that doors in the laboratory are always closed. Work areas, equipment and supplies should be arranged for logical and efficient work flow. Work areas should be kept free of dust. Benches should be swabbed at least once a day with an appropriate disinfectant (eg. 5% phenol)
- Every procedure performed in the laboratory must be written out exactly as carried out and be kept in the laboratory for easy reference. Any changes must be dated and initialised by the laboratory supervisor
- All records should be retained for two years
- Laboratory procedures used routinely should be those that have been published in reputable microbiological books, manuals or journals

Laboratory equipment

- Equipment should meet the manufacturers claims and specifications
- Written operating and cleaning instructions must be kept in a file for all equipment
- Dated service records must be kept for all equipment
- Equipment must be monitored regularly to ensure the constant accuracy and precision necessary.
    - *Biological safety cabinet:* The BSC is the primary containment device that protects the worker, product and/or environment from exposure to tuberculosis and its performance needs to be verified at the time of installation and annually thereafter. The purpose and acceptance level of the performance tests are to ensure the balance of inflow and exhaust air, the distribution of air onto the work surface and the integrity of the cabinet. Other tests check electrical and physical features of the BSC.

      Daily checks of the BSC include the following:

        - Ensure that the rate of airflow across the front opening is 75 linear feet/minute (22.86 meter/second) for Class I and 75 to 100 linear feet/minute (22.86 to 30.48 meter/second) for Class II cabinets.

- Check the magnetic gauge in the exhaust duct for any pressure drop across the filters and replace the filters when the gauge indicates that the airflow across the front opening has dropped below optimal levels.

The following tests should be performed *annually* on Class I and Class II cabinets:

- *Downflow velocity and volume test*

This test is performed to measure the velocity of air moving through the cabinet workspace.

- *Inflow velocity test*

This test is performed to determine the calculated or directly measured velocity through the work access opening, to verify the nominal set point average inflow velocity and to calculate the exhaust airflow volume rate.

An electronic vane type anemometer should be used to measure airflow. The airflow into a Class I cabinet should be measured in at least five places in the plane of the working face and an average calculated. At no place should there be a reading that is 20 linear feet/minute (0.1meter/second) more or less than any of the others. If there is such a difference there will be turbulence within the cabinet.

In Class II cabinets the airflow is greater at the bottom than at the top of the working face. The average inward flow is calculated by measuring the velocity of air leaving the exhaust and the area of the exhaust vent. From this the volume per minute is calculated, which is also the amount entering the cabinet. Divided by the area of the working face it gives the average velocity. The downward velocity of air should be measured at 18 points in the horizontal place, 10cm above the top edge of the working face. No reading should differ from the mean by more than 20%.

- *Airflow smoke patterns tests*

This test is performed to determine if the airflow along the entire perimeter of the work access opening is inward, if airflow within the work area is downward with no dead spots or refluxing, if ambient air passes onto or over the work surface, and if there is refluxing to the outside at the window wiper gasket and side seals. The smoke test is an indicator of airflow direction, not of velocity.

Commercial airflow testers are recommended. They are small glass tubes, sealed at each end. Both ends are broken off with the gadget provided and a rubber bulb fitted to one end. Pressing the bulb to pass air through the tube causes it to emit white smoke.

- *HEPA filter leak test*

This test is performed to determine the integrity of supply and exhaust HEPA filters, filter housing, and after-mounting frames while the cabinet is operated at the nominal set point velocities. An aerosol in the form of generated particulates of dioctylphthalate (DOP) or an accepted alternative is required for leak-testing HEPA filters and their seals. Although DOP has been identified as a potential carcinogen, competent service personnel are trained to use this chemical in a safe manner. The aerosol is generated on the intake side of the filter, and particles passing through the filter or around the seal are measured with a photometer on the discharge side. This test is suitable for ascertaining the integrity of all HEPA filters.

- *Cabinet leak test*

The pressure holding test is performed to determine if exterior surfaces of all plenums, welds, gaskets, and plenum penetrations or seals are free of leaks. It is performed just prior to initial installation when the BSC is in a free-standing position in the room in which it will be used, after a cabinet has been relocated to a new location, and again after removal of access panels to plenums for repairs or a filter change. This test may also be performed on fully installed cabinets.

- *Electrical leakage and ground circuit resistance and polarity tests*

These safety tests are performed to determine if a potential shock hazard exists by measuring the electrical leakage, polarity ground fault interrupter function, and ground circuit resistance to the cabinet connection. The polarity of electrical outlets are checked using a polarity tester. The ground fault circuit interrupter should trip when approximately 5 milliampere (ma) is applied.

- *Lighting intensity test*

This test is performed to measure the light intensity on the work surface of the cabinet as an aid in minimising cabinet operator's fatigue.

- *Vibration test*

This test is performed to determine the amount of vibration in an operating cabinet as a guide to satisfactory mechanical performance, as an aid in minimising cabinet operator's fatigue, and to prevent damage to delicate tissue culture specimens.

- *Noise level test*

This test is performed to measure the noise levels produced by the cabinet, as a guide to satisfactory mechanical performance and an aid in minimising cabinet operator's fatigue.

- *Centrifuge:* Check brushes and bearings every 6 months
- *Incubator 35°-37°C:* Record the temperature daily, preferably in the morning. Test the temperature at several sites within the incubator by placing a thermometer in a water reservoir (eg. Erlenmeyer flask). Control the light within the incubator by covering the glass front of the incubator door and by restricting the use of any lights inside the incubator
- *Inspissator:* Check temperature daily. Clean after each batch of culture media prepared
- *pH meter*: Compensate for temperature with each run. Date buffer solutions and discard when unsatisfactory. Standardise with pH 4.0 and 7.0 buffers before each test or series of tests
- *Water baths*: Check temperature before and during use. Clean monthly
- *Refrigerator 2°-8°C*: Check temperature daily. Clean monthly. Defrost or check refrigerator and freezer compartment every 3 months
- *Freezers:* Check daily. Clean every 6 months
- *Glassware:* Discard chipped or etched glassware. Ensure that glassware are free of detergents. Do not store sterile glassware for more than three weeks before it is used

Specimens and request forms

- Perform tests only upon written request of authorised persons and do not allow oral requests without follow-up written instructions
- Insist on specimen request forms being kept separate from the specimens themselves. Forms that have been contaminated by specimens should be sterilised by autoclaving
- Insist on adequately completed request forms and proper labelling of specimens to ensure positive identification of patients. Reject specimens that cannot be properly identified
- Evaluate the quality of sputum specimens and make a note if a specimen resembles saliva. The report should state “specimen resembled saliva - treat a negative result with caution” (to facilitate reporting a rubber stamp of the comment can be made)
- Discard leaking and broken specimen containers by autoclaving and request a repeat specimen
- Document the arrival time of specimens in the laboratory and note any delays in delivery on the report form, particularly with negative/contaminated results

Reagents and stains

- All containers of stains and reagents should show the date received and the date first opened. Any material found to be unsatisfactory should be recorded as such and removed from the laboratory immediately. Stocks should be limited to six months' supply and regular stock rotation should take place to avoid unnecessary expiry.

Digestion and decontamination

- Process sputum specimens in batches according to centrifuge capacity
- Keep a monthly record of the percentage of clinical specimens contaminated: the acceptable range is 2-5%. Contamination rates <2% indicates overly harsh decontamination, which means that too many tubercle bacilli are killed. If the laboratory is experiencing delays in delivery of specimens the contamination rate may be greater than 5%. If a rate of >5% persists, ensure that specimens are completely digested, since partially digested specimens may not be completely decontaminated. Thoroughly mix the contents of centrifuge tubes to ensure that the inside surfaces have been well decontaminated

Culture media

- Use fresh eggs (< seven days) for preparation of Löwenstein-Jensen media
- Control coagulation time and temperature for egg-based medium. Discard media that are discoloured or have bubbles following inspissation
- Check all batches of media for sterility by incubation at 35°-37°C for 24 hours
- Keep all media in the dark in the refrigerator and discard unused media after four weeks

Culture procedures

- Avoid cross-contamination of cultures by using individual pipettes or loops and strict aseptic techniques
- Be suspicious of several successively positive specimens or of cultures with few colonies that follow a heavily positive culture

Biochemical tests

Prepare reagents as indicated and check the expected biochemical test response by using appropriate positive and negative controls

Water

Check both distilled and tap water regularly for the presence of acid-fast contaminants. If water appears cloudy or dirty, centrifuge 200-250ml in multiple tubes and make a smear of the combined sediment. Alternatively, filter 1 000ml of water through a sterile 0.22µm pore size membrane filter, cut the filter into strips with a sterile scissor and place on Löwenstein-Jensen culture medium

12

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ANNEX 1

ESSENTIAL EQUIPMENT AND SUPPLIES FOR A CULTURE LABORATORY USING MODIFIED PETROFF DECONTAMINATION AND LÖWENSTEIN-JENSEN CULTURE MEDIUM (6000 SPECIMENS PER YEAR)

Item	Quantity/volume required
Equipment	
Autoclave, laboratory type, mixed load pressure cooker type or gravity displacement model with automatic air and condensate discharge	1
Balance, top pan	1
Biological safety cabinets, Class I or Class II, complying with international standards	1
Bunsen burner for use with butane gas	3
Centrifuge, with lid, 3 000 x g RCF capacity, fixed angle rotor, sealed buckets, safety catch	1
Culture bottle washer	1
Fogging device	1
Homogenizer, for eggs	1
Incinerator	1
Inspissator 240 litre capacity, thermostatically controlled at 80-85°C	1
Microscope, binocular, oil immersion lens (100x), eye pieces (8x or 10 x), spare bulbs	1
Refrigerator	1
Steam pots	3
Vortex mixer	1
Walk-in incubator, walk-in or shelf, heater and circulating fan, steel or aluminium racks	1
Supplies	
Aluminium foil, heavy duty	8 rolls
Adhesive labels for sputum containers	7000
Aspirator flask, with outlet for distilled water 5 to 10 litre capacity	1
Autoclave tape, 1cm width	12 rolls
Beakers, glass, 100ml, 250ml, 500ml, 1 000ml	5
Bowl, plastic, 50x30cm	4
Canisters for sterilising pipettes	5
Centrifuge tubes, glass or plastic, screw cap, round bottom	7000
Cotton wool, white absorbent	2kg
Cultures boxes, 50 culture capacity	10
Culture tube racks,48 tube capacity, polypropylene or metal covered with polypropylene or nylon	5
Diamond pens	2
Discard bottles, splashproof	2
Discard dishes, stainless steel or polypropylene	3
Disinfectant, bactericidal eg.5% phenol, 5% sodium hypochlorite	40 liters
Filter funnels, glass, 45mm or 60mm diameter / 90mm or 125mm diameter	2 each
Filter paper, 15cm diameter, No	4 boxes

Forceps , stainless steel, 15cm 2
Glassware, 250ml and 500ml Erleynmeyer flasks, 250ml, 500ml .....2 each
Gloves, disposable 400
Hand lens 1
Inoculation loops,25 SWG nichrome wire, 15cm long, 5mm diameter *or* 500
Inoculation loops, disposable 7000
Laboratory registers for culture.....2-4
Laboratory request forms 7000
Laboratory report forms, preliminary and final .....7000 each
Lens tissue, for cleaning objectives and eye-pieces .....1 box
Loop holders.....10
Masks, industrial, disposable 400
McCartney bottles, 14ml or 28ml, wideneck.....9 000
Measuring cylinders, glass, 25ml, 100ml, 250ml, 1 000ml.....2 each
Microscope slides, 25mmx75mm, 1.1-1.3mm thick.....600
Nichrome wire, for loops .....20 metres
Overalls, laboratory coats.....2 per person
Paper towels, disposable.....2 boxes
Pasteur pipettes, low-density polypropylene, integral teats 500
Pens, ball point, red ink 12
ball point, black or blue 24
Pipette washer 1
Pipettes, blow-out 1ml, 5ml, 10ml.....5 each
Racks, for inspissation of bottles containing media 10
Reagent bottles, different capacities varying from 50ml to 1 000ml.....25
Rubber teats, for pipettes, 2ml, 5ml.....5 each
Scissors, stainless steel, 25cm, ...4
Self-filling syringes, 5ml and 10ml capacity .....1 each
Slide rack, plastic, 12-25 slide capacity.....4
Slide storage box, cardboard or metal, 12-25 slide capacity 20
Spatulas, stainless steel.....4
Specimen containers, plastic, disposable, 50ml capacity, wide mouthed, screw-capped, combustible.....3
Stain bottles, amber glass, 100ml capacity.....3
plastic, 10ml capacity 3
Staining racks 2
Stainless steel buckets, large, with lids 4
Stirring rods 5
Test tubes, glass, rimless, 16mm x 152mm.....200
glass, rimless, 19mm x 152mm.....200
Test tube caps, aluminium, Cap-o-Tests.....600
Thermometers.....2
Timers, 0-60 minutes with alarm 2
Tripod stand with wire mat 2
Volumetric flasks, glass, 500ml capacity 4
Wash bottles, plastic, 500ml capacity.....2
Waterproof markers 12
Waterbath, with lid, thermostat and heater.....2

Reagents

Acid-alcohol, for Ziehl-Neelsen staining .....6 liters
Aniline 125ml
Aqueous methylene blue.....4 liters
Basic fuchsin.....125g
Carbolfuchsin, for Ziehl-Neelsen staining .....12 liters
Cyanogen bromide (BrCN).....250g
Dry zink dust.....2g
Eggs.....10 doz per month
Ethyl alcohol (70%).....2.5 litre x 2
Glycerol .....2.5litre x 1
Hydrochloric acid concentrated (reagent grade).....2.5 litre x 1
Hydrogen peroxide 30% (superoxol) 250ml
Immersion oil 100ml
L-asparagine 250g
Magnesium sulphate $MgSO_4.7H_2O$.....250g
Magnesium citrate 250g
Malachite green.....25g
Methylated spirit.....750ml x 5
N-naphthylethylene-diamine-dihydrochloride.....2.5g
Na_2HPO_4 (anhydrous) 250g
p-Nitrobenzoic acid (PNB) 200g
Phenol crystals 125g
Potassium permanganate $KMNO_4$.....250g
Potassium dihydrogen phosphate KH_2PO_4 (anhydrous) 500g
Sodium hydroxide (NaOH) 500g
Sodium chloride 250g
Sodium pyruvate.....100g
Sodium nitrate.....125g
Sulfanilamide 15g
Sulfuric acid (reagent grade) .....2.5 litre x 1
Tween 80 .....100ml
Xylene 400ml

ANNEX 2

SPUTUM COLLECTION

PROCEDURE

- Give the patient confidence by explaining to him/her the reason for sputum collection
- Instruct the patient to rinse his/her mouth with water before producing the specimen. This will help to remove food and any contaminating bacteria in the mouth
- Instruct the patient to take two deep breaths, holding the breath for a few seconds after each inhalation and then exhaling slowly. Ask him/her to breathe in a third time and then forcefully blow the air out. Ask him/her to breathe in again and then cough. This should produce a specimen from deep in the lungs. Ask the patient to hold the sputum container close to the lips and to spit into it gently after a productive cough. Sputum is frequently thick and mucoid, but it may be fluid, with chunks of dead tissue from a lesion in the lung. The colour may be a dull white or a dull light green. Bloody specimens will be red or brown. Thin, clear saliva or nasopharyngeal discharge is not sputum and is of little diagnostic value for tuberculosis
- If the sputum is insufficient encourage the patient to cough again until a satisfactory specimen is obtained. Remember that many patients cannot produce sputum from deep in the respiratory track in a few minutes. Give him/her sufficient time to produce an expectoration which s/he feels is produced by a deep cough
- If there is no expectoration, consider the container used and dispose of it in the appropriate manner
- Check that the container is securely closed and label the *container* (not the lid) clearly
- Wash hands with soap and water
- Give the patient a new sputum container and make sure that s/he understands that a specimen must be produced as soon as s/he wakes up in the morning
- Demonstrate to the patient how the container should be securely closed
- Instruct the patient to bring the specimen back to the health centre or laboratory

ANNEX 3

LABORATORY REQUEST FORM

Name of Health Centre _____ Date _____

Name of patient _____ Age _____ Sex M ❒ F ❒

Complete address:_____

_____

Patient's register number* _____

Source of specimen ❒ Pulmonary
❒ Extra-pulmonary Site _____

Reason for examination ❒ Diagnosis
❒ Follow-up of chemotherapy

Specimen identification number _____ Date _____
Signature of person requesting examination _____

** Be sure to enter the District TB Register number for the follow-up of patients on chemotherapy*

_____

ANNEX 4a

CULTURE RESULTS: PRELIMINARY REPORT

_____

Laboratory serial number _____ Date specimen received _____

Culture results

Culture method _____

No growth	❒	3+	❒
1-19 colonies	❒	4+	❒
1+	❒	Contaminated	❒
2+	❒		

Cultivation yielded _____ growth of mycobacteria with the characteristics of tubercle bacilli.

A final report will be issued within the next four weeks.

Date _____ Signature _____

ANNEX 4b

CULTURE RESULTS: FINAL REPORT

Laboratory serial number _____ Date specimen received _____

Microscopy results

Staining method ❒ Ziehl-Neelsen ❒ Fluorochrome

Negative	❒	1+	❒
Not done	❒	2+	❒
1-9 AFB	❒	3+	❒

Culture results

Culture method _____

No growth	❒	1-19 colonies	❒
Contaminated	❒	1+	❒
Not done	❒	2+	❒
		3+	❒
		4+	❒

Culture identification

Growth rate_____ Colony morphology _____

Niacin production	❒ positive	❒ negative
Nitrate production	❒ positive	❒ negative
Other, list_____	❒ positive	❒ negative
_____	❒ positive	❒ negative

Culture identified as *Mycobacterium tuberculosis* ❒
MOTT ❒

Date _____ Signature _____

LABORATORY REGISTER FOR CULTURE

Lab Serial no.	Name (in full)	Sex M/F	Age	Complete adress (for new patients)	Name of referring Health Center (for new patients)	Microscopy results	Culture results	Colony morphology	Growth rate	Niacin / Nitrate	Catalase / PNB	Ziehl-Neelsen confirmation	Signature	Remarks

ANNEX 6

UNIT CONVERSION FACTORS

LENGTH

1mm	=	0.0394 inch	1 inch	=	25.4mm
1m	=	39.37 inches	1 inch	=	0.0254m
1m	=	3.28 feet	1 foot	=	0.305m
1m	=	1.09 yard	1 yard	=	0.91m

TEMPERATURE

0°C	=	(°F-32) x 5/9
EF	=	(°C x 9/5) + 32
100°C	=	212°F

WEIGHT

1g	=	0.035 ounce	1 ounce	=	28g
1kg	=	2.2lb	1lb	=	0.454kg

AREA

$1cm^2$ = $0.155in^2$ $1m^2 = 10^4\ cm^2 = 10.76ft^2$

$1in^2$ = $6.452cm^2$ $1ft^2 = 144in^2 = 0.0929m^2$

VOLUME

$1\ litre = 1000cm^3 = 10^{-3}\ m^3 = 0.0351ft^3 = 61.02in^3$

$1ft^3 = 0.02832m^3 = 28.32$ liters = 7.477 gallons

1 US gallon = 3.78 liters

UK gallon = 0.26 US gallon

1ml = 1cc = 0.034fl oz

500ml = 16fl oz = 1pint

1 000ml = 1 liter = 33.8fl oz = 1 quart

1UK gallon = 4.55 liters 1 000ml = 0.22

VELOCITY

1cm / s = 0.03281 ft/s

1km/h = 0.2778 m/s

1ft/s = 30.48cm/s

PRESSURE

$1Pa = 1N/m^2 = 1.451 \times 10^{-4}\ lb/in^2 = 0.209\ lb/ft^2$

$1lb/in^2 = 6\ 891Pa$

$1atm = 1.013 \times 10^5 Pa = 14.7lb/in^2 = 2117\ lb/ft^2$

$1lb/ft^2 = 47.85Pa$

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