Revised National TB Control Programme
Training Manual for
Mycobacterium tuberculosis
Culture & Drug susceptibility testing

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This training manual is companion volume for the Standard Operating Procedures. The Central TB Division acknowledges the contribution of Tuberculosis Research Centre, Chennai (Indian Council of Medical Research) and National Tuberculosis Institute, Bangalore (Directorate General of Health Services, Government of India) in the preparation of this document.

Printed: April 2009

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Training Manual for *Mycobacterium tuberculosis* Culture & Drug susceptibility testing

This manual provides the technical procedures for *Mycobacterium tuberculosis* Culture & Drug susceptibility testing (first-line anti-TB drugs) on conventional LJ media, under the Revised National Tuberculosis Control Programme.

BIOLOGY OF MYCOBCATERIA

Tuberculosis complex organisms are obligate aerobes growing most successfully in tissues with high oxygen content, such as the lungs.

They are facultative intracellular pathogens usually infecting mononuclear phagocytes (e.g. macrophages), slow-growing with a generation time of 12 to 18 hours (c.f. 20-30 minutes for *Escherichia coli*), hydrophobic with a high lipid content in the cell wall.

Because the cells are hydrophobic and tend to clump together, they are impermeable to the usual stains, e.g. Gram's stain.

Mycobacteria are "acid-fast bacilli" because of their lipid-rich cell walls, which are relatively impermeable to various basic dyes unless the dyes are combined with phenol. Once stained, the cells resist decolorization with acidified organic solvents and are therefore called "acid-fast" (other bacteria, which also contain mycolic acids, such as Nocardia, can also exhibit this feature.)

Tuberculosis complex organisms are *Mycobacterium tuberculosis, Mycobacterium bovis*, (the cause of tuberculosis in cattle and humans, as well as other carnivores); *M. bovis BCG* (a strain used as a vaccine against tuberculosis in many parts of the world); and *M. africanum* (the cause of human tuberculosis in tropical Africa).

In India, *Mycobacterium tuberculosis*, causes tuberculosis in almost 100% of patients and hence, this manual aims at diagnosis of tuberculosis by culture of this organism.

CULTURE OF SPUTUM SPECIMENS

In order to distinguish between different mycobacterial species as well to perform drug susceptibility and identification tests, culture examination becomes a necessity. Culture of sputum provides definitive diagnosis of tuberculosis by establishing the viability and identity of organisms. However, compared to other bacteria, which typically reproduce within minutes, ***M.tuberculosis*** proliferates 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 that allow abundant growth of ***M.tuberculosis*** are egg-enriched media with glycerol and asparagine (viz., Lowenstein-Jensen) or agar based media supplemented with bovine albumin (viz., Middlebrook, 7H10 or 7H11).

Culture increases the number of tuberculosis cases found, often by 30-50% and detects cases which are smear-negative. Since culture techniques detect fewer bacilli, the efficiency of diagnosing cases of failure at end of treatment can be improved considerably. Cultures also provide sufficient material for drug susceptibility and identification tests. However, culture methods are expensive and require considerable expertise.

Selective use of culture

1. Surveillance of drug resistance as part of Control Programme performance
2. Diagnosis of difficult extra-pulmonary/childhood tuberculosis cases and cases with clinical/ radiological symptoms where smears are negative and other non-mycobacterial diseases have been ruled out.
3. Follow-up of patients who fail on treatment regimens to check for drug resistance.
4. Investigation of high risk individuals who are symptomatic.

SPECIMEN COLLECTION

In tuberculosis bacteriology an often-overlooked problem is that of obtaining adequate specimens. The advantages of decontamination techniques, sensitive culture media and simple identification schemes will not be complete unless specimens are collected with care and promptly transported to the laboratory.

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 approximately 5ml,** although smaller quantities are acceptable if the quality is satisfactory.

Specimens should be transported to the laboratory as soon as possible after collection. If delay is unavoidable, the specimens should be refrigerated to inhibit the growth of unwanted micro-organisms. If refrigeration is not possible and a delay of more than 2 days is anticipated, a suitable preservative viz., an equal volume of a mixture of 1% cetyl pyridinium chloride (CPC) in 2 % sodium chloride solution is recommended.

HOMOGENISATION AND DECONTAMINATION

M. tuberculosis requires an enriched medium for culture. It grows slowly and takes three to six weeks or longer to give visible colonies. Cultures are usually made in bottles rather than in petri dishes because of the long incubation time required. The bottles are tightly stoppered to prevent drying of the cultures.

The majority of clinical specimens submitted to the tuberculosis culture laboratory are contaminated to varying degrees by more rapidly growing normal flora.. These would rapidly overgrow the entire surface of the medium before the tubercle bacilli start to grow. The 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. However,at any time 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 process fresh specimens. If processing delays are anticipated, beyond 3 days period culture 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.In such instances the culture negativity in the laboratory increases.

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

- Specimens must be homogenized to free the bacilli from the mucus, cells or tissue in which they may be embedded. **The milder the homogenisation process, the better the recovery of the tubercle bacilli**

- Homogenisation/ decontamination should not diminish the viability of tubercle bacilli

- The success of homogenisation and decontamination depends on:

    - The appropriate concentration of homogenization or decontamination solution

    - The length of exposure time to these agents

    - The centrifugation speed and time used to sediment the tubercle bacilli

    - The temperature build-up in the specimen during centrifugation

Many different methods of homogenisation and decontamination of sputum specimens for culturing have been described. The choice of a suitable method is to a large extent determined by the technical capability and the availability of skilled staff in the laboratory, as well as the quality and type of equipment available. Each method has its limitations and advantages .It is recommended that laboratories standardise on only one method Methods which consistently yield the highest percentage of positive cultures are those which require:

- Well trained staff
- Quality equipment (e.g. centrifuges) and related supplies
- Calibration of the equipments

DIGESTION AND DECONTAMINATION PROCEDURES

Since the exposure time to digesting/decontaminating has to be strictly controlled it is best to work in sets equivalent to one centrifuge load (eg., 12-16 specimens at a time).

Always digest/decontaminate the whole specimen, i.e., do not attempt to select portions of the specimen as is done for direct microscopy. This procedures are aimed at homogenisation and decontamination of sputum specimens.. Specimens other than sputum require even more care during processing because of the low numbers of tubercle bacilli present in positive specimens.

PROCESSING OF SPUTUM SPECIMENS CONTAINING CPC & NaCl:

If delay of more than 48-72 hours between collection of sputum samples and processing of the same by culture is anticipated, the sputum sample should be collected in a sterile container with 1% cetyl pyridinium chloride and 2% sodium chloride solution (CPC + NaCl). CPC, a quaternary ammonium compound, is used to decontaminate the specimen.

The use of this method not only decreases the number of cultures lost by contamination as a result of prolonged transit time but also decreases significantly the laboratory time required for processing the specimens.

Materials required:

Preparation of 1%CPC-NaCl :

One gm of cetylpyridinium chloride and two gms of sodium chloride are dissolved in 100ml of sterile distilled water and distributed in 5 ml aliquots in sterile McCartney bottles. The stock solution should be stored in dark, away from sunlight at room temperature. **This solution has a shelf life of one month**

Sputum specimen

Mucoid/mucopurulent sputum specimen consists of recently discharged material from the bronchial tree, with minimum amounts of oral or nasal material. The **sputum specimen should have a volume of approximately 5ml.**

Collection of specimen

Collect approximately 5 ml of sputum directly into the Mccartney bottles containing 5 ml of CPC. Tightly seal the cap of the bottle and wipe off the outer surface of the bottle with disinfectant solution in a cotton swab.

CPC containing specimen should be processed as described below:

To the specimen with CPC add 15-20 ml of sterile distilled water (to reduce viscosity)

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Tighten cap of the container and mix well by inversion

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Centrifuge at 3000g for 15 minutes

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Carefully pour off the supernatant

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Add approximately 20ml of sterile distilled water to re-suspend the sediment and fill up with sterile distilled water to the brim

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Centrifuge again at 3000g for 15 minutes

↓

Decant the supernatant and inoculate deposit on two slopes of LJ medium

↓

Incubate all the LJ media slopes at 37°C

↓

Check for growth weekly for 8 weeks

Precautions during processing of CPC containing samples:

- Sufficient time should be given for complete liquefaction of sputum with CPC. Process only those that have been homogenized by CPC. If homogenisation gets delayed beyond five days, as might happen when the ambient temperature is < 25^{o}C incubate the samples at 37^{o}C till the homogenisation is complete, before processing.
- The specimen should be processed according to the date of collection (older samples first)
- Processing should be done within 7 days from the date of the collection.
- While decanting supernatant, care should be taken to avoid discarding off the sediment

Final CPC-specimen deposit should be inoculated only on to egg media and not on to any other base media

PROCESSING OF SPUTUM SPECIMENS WITH SODIUM HYDROXIDE (NaOH) METHOD- MODIFIED PETROFF'S PROCEDURE:

Preparation of Sterile 4% NaOH solution:

Weigh 10 grams of Sodium Hydroxide pellets and dissolve in 250ml of distilled water in a conical flask (500 ml capacity); sterilize in solution cycle in an autoclave at 15 psi for 20 min.

Sputum Specimen

Mucoid or Mucopurulent sputum specimen consists of recently discharged material from the bronchial tree, with minimum amounts of oral or nasal material. The **sputum specimen should have a volume of approximately 5ml.**

Collection of specimen

Collect approximately 5ml of sputum directly into the sterile McCartney bottle. Tightly seal the cap of McCartney bottle and wipe off the outer surface of the bottle with disinfectant solution in a cotton swab. Transport the specimens with in 48 hours to the laboratory.

Specimen should be processed as described below:

1. Transfer 4 to 5 ml of sputum to centrifuge tube/Mc Cartney tubes and add double the volume of sterile 4% NaOH solution, aseptically.

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2. Tighten the caps of the Mc Cartney tubes and mix it well. Invert each bottle to ensure that NaOH solution contacts all the sides and inner portion of caps.

↓

3. The bottles are to be placed in shaker & kept in 37°C incubator for 15 minutes.

↓

4. At the end of 20 minutes, Mc Cartney tubes are to be removed from the incubator and 15ml of sterile distilled water should be added.

↓

5. Mix it well and centrifuge at 3000 x g for 15 minutes.

↓

6. Mc Cartney tubes are to be carefully removed from the centrifuge without shaking. The supernatant fluid should be discarded slowly into a container with 5% phenol solution.

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7. Wash the the pellet with sterile distilled water at 3000xg for 15min, and decant the supernatant

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8. From the sediment, inoculate two slopes of LJ medium (using a sterile cool 5 mm inoculatin loop made up of Nichrome wire (22 SWG). Individually wrapped, disposable 10mm loops can be used, if available.

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9. Label the caps with the lab serial number of the specimen and name them as 1 and 2. Use one loopful of sediment for each inoculation.

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10. Incubate all the LJ media slopes at 37°C.

↓

11. Check for growth weekly for eight weeks.

Precautions during processing:

- The specimen exposure times must be strictly followed to prevent over-kill of tubercle bacilli. The NaOH procedure may kill considerable 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
- Avoid splashing to minimize aerosols; and the whole process should be carried out in a bio-safety cabinet.

- The specimen should be processed according to the date of collection (older samples first)
- Processing should be done within 48-72 hours from the date of the collection.
- While decanting supernatant, care should be taken to avoid discarding off the sediment

INOCULATION AND INCUBATION PROCEDURES

INOCULATION PROCEDURES

Two slopes per specimen are inoculated each with one 5 mm loopful of the centrifuged sediment, distributed over the surface. An additional slope containing pyruvate may be used to identify *M. bovis* .Bottle caps should be tightened to minimize evaporation and drying of media. Care should be taken to avoid using red hot loop and loop should be cooled before inoculation.

INCUBATION OF CULTURES

All cultures should be incubated at 35-37^{o}C until growth is observed or discarded as negative after eight weeks. Contaminated slopes are also discarded.

CULTURE EXAMINATION AND IDENTIFICATION

EXAMINATION SCHEDULE

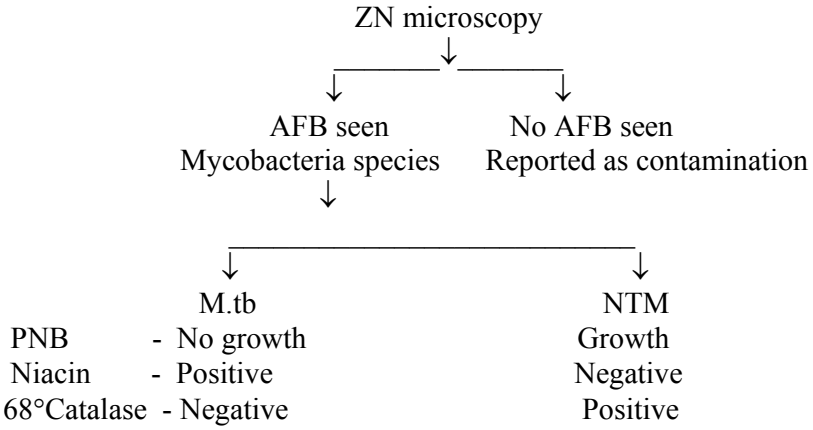
All cultures should be examined 48-72 hours after inoculation to detect gross contaminants. Thereafter cultures are examined weekly, up to 8 weeks on a specified day of the week. It is useful to label containers with cultures with the dates of inoculation and to place containers in the incubator in chronological order.

Should contaminated cultures be found during examination,(those where the surface has been completely contaminated or where medium has been liquefied or discoloured) should be discarded.

READING OF CULTURES

Typical colonies of ***M. tuberculosis*** are rough, crumbly, waxy, non-pigmented (buff coloured) and slow-growers, i.e., only appearing two to three weeks after inoculation.

The colony with doubtful morphology, 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. If no AFB seen in the smear it is reported as contamination.



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 require two to four weeks to give visible growth from smear-positive specimens.
- The colonies are buff coloured and rough, having the appearance of breadcrumbs or cauliflower.
- They are not easily emulsified but give a granular suspensions
- Microscopically they are frequently arranged in serpentine cords of varying length or show linear clumping in liquid medium.

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.

Culture procedures for tuberculosis bacteriology are notoriously time-consuming, often taking 8-9 weeks to complete. For this reason, interim reports should be issued. The following schedule is recommended:

- If the cultures are contaminated, a report should be sent out immediately.
- At eight weeks a final report should be issued for culture negative specimens.
- If cultures are positive and growth has been identified as ***M. tuberculosis,*** a report should be sent out immediately to the referral authority
- If only a few colonies of non-tuberculous mycobacteria (NTM – often pigmented, with smooth morphology or PNB positive) are grown, a report should be given as "No growth".
- When the growth of NTM is more than 1+, a report should be given as "Negative for M. tb"..
- If the colony count is less than 20 or faint growth in the first or second week, incubation is continued upto 4^{th} week to obtain a colony count of at least more than 50 colonies or more than one loopful of growth (3mm).
- If the growth is still insufficient at the end of 4^{th} week, a subculture should be done on a fresh LJ by touching all the colonies. The exact number of colony count in primary growth should be recorded before doing subculture and incubated at 37° C degree not exceeding three weeks. During this period when sufficient growth is obtained for setting up DST.

Culture reports should be qualitative (i.e., positive or negative) as well as quantitative (i.e., number of colonies isolated). The following scheme is recommended:

Reading for primary culture and for DST	**Report**
No growth	Negative
1-100 colonies	Positive (actual number of colonies)
>100 discrete colonies	Positive (2+)
Confluent growth/innumerable colonies	Positive (3+)
Contaminated	Contaminated
< 20 colonies of only NTM colonies in one or both slopes	No growth
> 20 colonies of only NTM colonies in both slopes	Negative for *M. tb*.

Note :

If increased negative results observed in the lab the following might have occurred and should rectify them:

- Delays between sputum collection and processing
- The quality of the sputum specimen
- Increased concentration of malachite green used for LJ media preparation
    - Increased temperature of incubation of cultures
    - Increased time and temperature of inspissation during media preparation
    - Centrifugation speed is below 3000g(rcf) during the sedimentation and shorter times for centrifugation

If increased negative results along with the contamination is seen, the decontamination/ liquefaction was incomplete.

An illustration for the culture reading and action to be taken:

Date	Lab no	1 wk	2 wk	3 wk	4 wk	5 wk	6 wk	7 wk	8 wk	Final results	Remarks
29/8/2008	O445	–	*/–	–	–	–	–	–	–	Neg	
	0446	–	2 +/ –	–	1 + (D)					2+	
	0447	*/–	–	*/ *						Cont.	
	0448	–	2+/70							2+	
	0449	–	3 NTM/–	3 NTM/–	3 NTM/–	3 NTM/–	3 NTM/–	3 NTM/–	3 NTM/–	Neg	Since 3 NTM is insignificant
	0450	3+/3+ bothNTM								NTM	Confirm with PNB, Niacin & Catalase
	0451	25/45								70 cols	
	0452	–	–	–	–	–	–	–	–	Neg.	
	0453	4 / 5	4 / 5	5 / 5	5 / 5	5 / 6				11 col	S/C in LJ for DST in 5th WK

D= Duplicate, Cont = Contamination, */+ or – = contamination in one slope with or without growth of M. tb in the second slope.
NTM = Non-tuberculosis mycobacteria, S/C = Sub-culture

CULTURE MEDIA

The definite 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 are the first choice, since they meet all these requirements.

PREPARATION OF LOWENSTEIN-JENSEN MEDIUM

Lowenstein-Jensen (LJ) medium is most widely used for tuberculosis culture. LJ medium containing glycerol favours the growth of ***M.tuberculosis*** while LJ medium without glycerol but containing pyruvate encourages the growth of ***M. bovis***.

Ingredients

Mineral salt solution with malachite green

Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4g
Magnesium sulphate anhydrous	0.24g
Magnesium citrate	0.6g
Asparagine	3.6g
Glycerol (reagent grade)	12ml
Malachite green, 2% solution*	20ml

**Malachite green solution 2%*

Malachite green dye	2.0g
Distilled water	100ml

Dissolve the dye in distilled water completely. Filter and store in refrigerator.

Dissolve the ingredients in order in about 300ml distilled water by heating. Add glycerol, malachite green solution and make up 600ml with distilled water. This solution should be sterilized by autoclaving at 121^{o} C (15 psi) for 30 minutes. Cool to room temperature. If **required, this solution may be stored in the refrigerator.**

Homogenised whole eggs

Fresh country hen's eggs those are not more than seven days old, are cleaned by scrubbing thoroughly with a hand brush in water and soap. Let the eggs soak for 30 minutes in soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 minutes. Before handling the clean dry eggs scrub and wash the hands with a disinfectant. Crack the eggs with the edge of the beaker into a sterile flask and beat them in a sterile blender for 30 seconds to one minute.

Preparation of complete medium

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

Mineral salt solution with malachite green	600ml
Homogenised eggs (25-30 eggs, depending on size)	1000ml

The complete egg medium is distributed in 6-8ml volumes in sterile universal containers and the caps tightly closed and inspissated without delay to prevent sedimentation of heavier ingredients.

Coagulation of medium

Before loading, heat the inspissator to 85^{o} C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 50 minutes at 85^{o}C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilise it). 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.

Sterility check

After inspissation, the whole media batch of the media bottles should be incubated at 35^{o} C-37^{o}C for 24 hours as a check for bacterial sterility. After 24 hours 5% of the slopes should picked up randomly and continued for incubation for 14 days to check for fungal sterility. In both the cases the contamination rate should not be > 10 %.

Storage

The LJ medium should be dated and stored with the batch number in the refrigerator and can keep for upto 4 weeks if the caps are tightly closed to prevent drying of the medium.

Note :

- Prepare one batch of plain LJ media one week before the anticipated arrival of samples (one batch is 1620 ml of LJ fluid sufficient for 324 LJ slopes). The requirement is two LJ slopes for each specimen .

- One batch of media provides 20 sets for drug susceptibility testing and an additional 44 plain LJ slopes. One set of drug susceptibility testing media consists of 5 plain LJ slopes, 2 slopes each for four drugs and one PNB containing LJ media.

- In the initial period, it is recommended not to prepare more than one batch of media per day.

L-J Medium with sodium pyruvate

For the cultivation of ***M. bovis***, LJ medium is enriched with 0.5% sodium pyruvate. In the preparation of the mineral salt solution, glycerol is omitted and 8.0g sodium pyruvate is added for every 600 ml. This is added to 1 litre of egg fluid, mixed well and distributed.

DRUG SUSCEPTIBILITY TESTS

Drug susceptibility testing is one of the most difficult procedures to perform and standardize in the mycobacteriology laboratory. Proficiency in susceptibility tests demands an understanding of:

- The origin of drug resistance
- The variation in stability of drugs subjected to different conditions of filtration, heat or storage
- The alteration in the activity of certain drugs when incorporated into different kinds of media
- The type of susceptibility test performed
- The reading and reporting of test results
- The criteria of resistance

Drug susceptibility tests should be performed in the following instances:

- For relapse or re-treatment cases
- To change the drug regimen when drug resistance is suspected
- Undertaking drug resistance surveillance studies in a region/country

There are three general methods used for determining drug susceptibility of mycobacterium: the proportion method, absolute concentration method (MIC method) and the resistance ratio method, and When properly standardized and performed, all three methods have been shown to be equally satisfactory. In India, under RNTCP, proportion method is advised since large numbers of laboratories have standardized this method for DST

METHODS

THE PROPORTION METHOD:

It enables precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of inoculum are planted on to both control and drug –containing media; at least one dilution should yield isolated countable (50 -100) colonies. When these numbers are corrected by multiplying by the dilution of inoculum used, the total number of viable colonies

observed on the control medium, and the number of mutant colonies resistant to the drug concentrations tested may be determined. The proportion of bacilli resistant to a given drug is then determined by expressing the resistant portion as a percentage of the total population tested..

THE ABSOLUTE CONCENTRATION METHOD:

This method uses a standardized inoculum grown on drug-free media and media containing graded concentrations of the drug(s) to be tested. Several concentrations of each drug are tested, and resistance is expressed in terms of the lowest concentration of the drug that inhibits growth; i.e., minimal inhibitory concentration (MIC). This method is greatly affected by inoculum size and the viability of the organisms.

THE RESISTANCE RATIO METHOD:

It compares the resistance of unknown strains of tubercle bacilli with that of a standard laboratory strain. Parallel sets of media, containing twofold dilutions of the drug, are inoculated with a standard inoculum prepared from both the unknown and standard strains of tubercle bacilli. Resistance is expressed as the ratio of the minimal inhibitory concentration (MIC) of the test strain divided by the MIC for the standard strain in the same set.

The proportion method is currently the method of choice and the simple version of bacterial suspension and interpretation of results are given below.

The economic variant of proportion method used under RNTCP

PROCEDURE DETAILS FOR THE PROPORTION METHOD-STANDARD ECONOMIC VARIANT

BACTERIAL SUSPENSION

Inoculum

With a loop, a representative sample of approximately 4-5 mg is taken from the primary culture and placed in a McCartney bottle containing 1 ml sterile distilled water(SDW) and 6 glass beads of diameter 3 mm. The bottle is vortexed for 20–30 seconds; 4-5 ml of distilled water is added slowly under continuous shaking. Allow the coarse particles to settle down. Decant the mycobacteria carefully into another clear, sterile McCartney bottle. The opacity of the bacterial suspension is then adjusted by the addition of distilled water to obtain a concentration of 1 mg/ml of tubercle bacilli by matching with McFarland standard No.1

Preparation of suspensions for economic variant of proportion method:

Neat: 1 ml SDW with six 3 mm glass beads + 1 loopfull (3 mm internal diameter) of culture

↓

vortexed for 20 – 30 seconds

↓

Add 4 ml of SDW to the above

↓

Adjusted turbidity with McFarland 0.05 with SDW.

↓

S2-10 $^{-2}$ 2 loopfull of neat + 2ml of SDW

↓

S4-10^{-4} 2 loopful of S2 + 2ml of SDW

Precautions:

- Avoid touching the media while picking the colonies,
- Cool down the loop sufficiently before picking the colonies,
- Try to take loopful of colonies in one sweep, by touching all colonies on the LJ slope.
- Avoid touching the water of condensation while scrapping the colonies
- Emulsify the initial inoculums onto the walls of the McCartney bottle

PREPARATION OF McFarland NEPHELOMETER BARIUM SULFATE STANDARD No.1 (Paik, G. 1980)

1. Prepare 1% aqueous barium chloride and 1% sulphuric acid (AR) solutions. (100 mg of Barium chloride (anhydrous) in 10 ml of SDW and 0.1 ml of sulphuric acid (AR) in 10 ml of SDW).
2. Add 0.1 ml of 1% Barium Chloride to 9.9 ml of 1% Sulphuric acid to obtain the McFarland standard No.1, which matches with 1 mg/ ml of *M. tuberculosis*.
3. Seal the tube with parafilm and label as McFarland standard No.1 with date of preparation.

During preparation of neat bacterial suspension, the comparison is done against a white background.

After preparing the standard Neat suspension, either a loop or pipettes may be used for further processing and for inoculation. It is recommended to use loop method under RNTCP.

Inoculation for DST – Loop method

The loop should be of Nichrome wire (24 SWG) and should have an internal diameter of 3 mm, which delivers 0.01 ml.

Delivery volume must be verified by weighing 10 loopfuls of distilled water deposited on a filter paper.

The two bacterial dilutions required for inoculation with the loop are 10^{-2} and 10^{-4} from the neat prepared as above. The dilutions 10^{-2} is produced by discharging two loopfuls of the bacterial suspension, standardized at 1 mg/ml, into a Bijou bottle

containing 2 ml of distilled water. Mix the contents by shaking. Similarly, the dilution 10^{-4} is produced by discharging two loopfuls of the 10^{-2} dilution into a small tube containing 2 ml of distilled water. Mix the contents by shaking. Two slopes of medium without drug and one slope of medium with drug for each of the four drugs are inoculated with a loopful of each dilution.

Precautions:

- Avoid touching the water of condensation while inoculation. Don't allow water condensation to spread over the media surface

- Inoculate uniform suspension in all the slopes

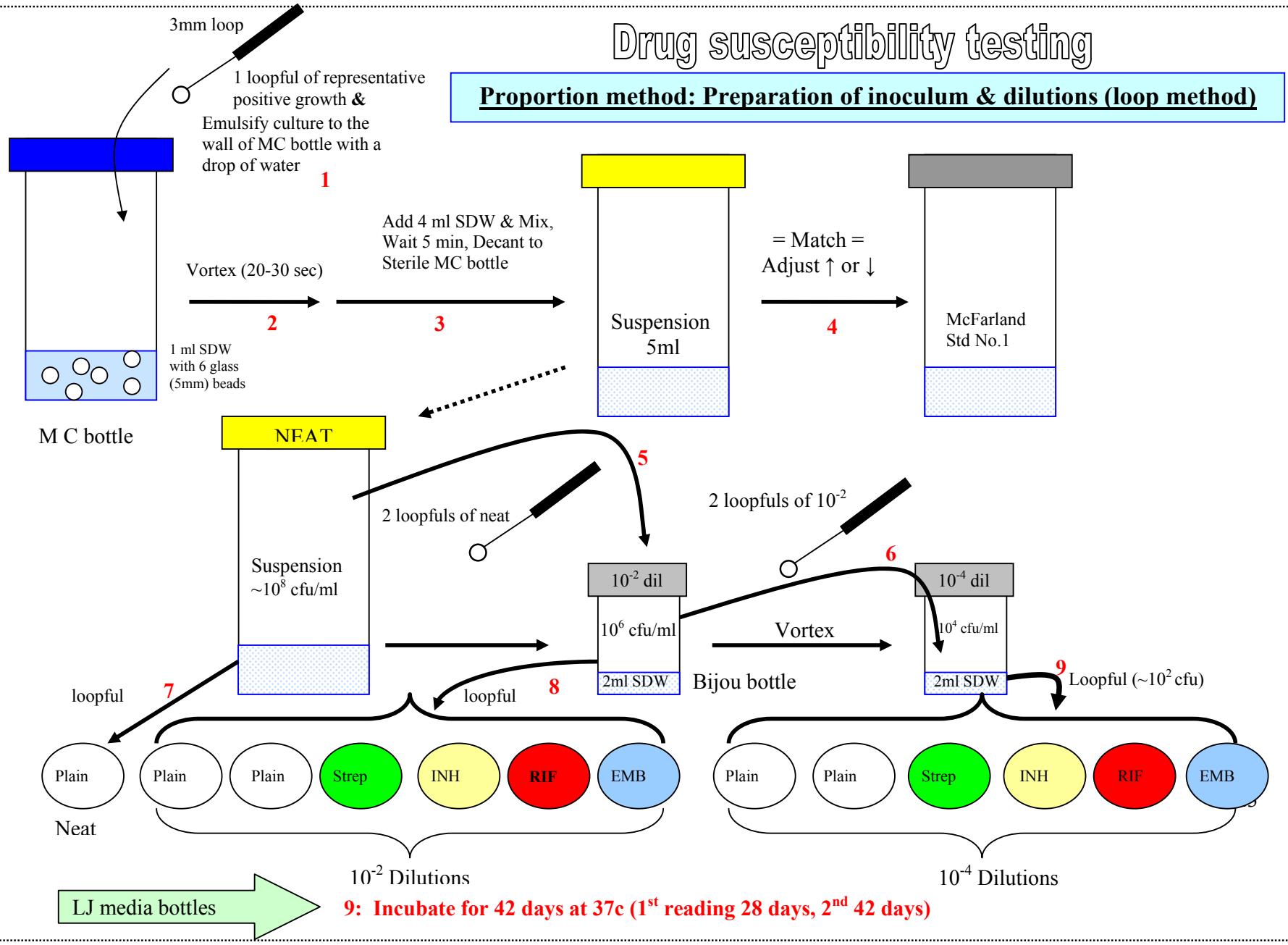
The standard strain ***M. tuberculosis***, $H_{37}Rv$ is tested with each new batch of medium. The recommended drug concentrations are 4 mg/l for streptomycin, 0.2 mg/l for isoniazid, 40 mg/l for rifampicin and 2 mg/l for ethambutol.

Additional recommendation:

The inoculum indicated above usually provides satisfactory countable colonies for the test, i.e., more than 10 and less than 100 colonies per control slope seeded with smaller inoculum. However, in some laboratories, the yield may prove smaller, owing to different growth conditions. In such cases, initially, the labs should seed 10^{-2}, 10^{-3} and 10^{-4} dilutions.

Drug susceptibility testing

Proportion method: Preparation of inoculum & dilutions (loop method)



INCUBATION AND READING

Incubate the slopes at 37^{o}C.

Read the proportion tests at 28 days and again at 42 days.

Record growth as

3+	confluent growth
2+	more than 100 colonies
01-99 cols.	the actual number of colonies

When the number of colonies on a given dilution is less than 15, count the number of colonies with the next larger inoculum, or estimate if more than 100. (Make no attempt to estimate the number of colonies if the growth is 3+)

INTERPRETATION OF TESTS

1. First reading is taken at 28th day after inoculation.

2. Count the colonies only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). This inoculum may be the same for the control slopes and the drug-containing slopes, or it may be the low inoculum (10^{-4} dilution) for the control slopes and the high inoculum (10^{-2} dilution) for the drug-containing slopes.

3. The average number of colonies obtained for the drug-containing slopes indicates the number of resistant bacilli contained in the inoculum.

4. Dividing the number of colonies in drug containing slopes by that in drug free slopes gives the proportion of resistant bacilli existing in the strain. Below a certain value – the critical proportion – the strain is classified as sensitive; above that value, it is classified as resistant. The proportions are reported as percentages.

5. If, according to the criteria indicated below, the result of the reading made on the 28th day is "resistant", no further reading of the test for that drug is required: the strain is classified as resistant. If the result at the 28th day is "sensitive", a second reading is made on the 42nd day only for the sensitive strain. The final definitive results for all the four drugs should be reported on 42nd day. If the strain is resistant for all the four drugs on 28th day, then the report can be given on the same day. Otherwise, incomplete reports should not be given before 42nd day.

6. In case growth on the control media is poor even after six weeks (i.e., few or no colonies on the 10^{-4} bacterial dilution), the test should be repeated.

7. Repeat the test: Test showing an average of less than 10 colonies in the control slopes seeded with the smaller inoculum.

DEFINITIONS OF RESISTANCE- PROPORTION METHOD

Drug	Concentration (mg/l)	Proportion
Isoniazid	0.2	1% or more
Ethambutol	2	1% or more
Streptomycin	4	1% or more
Rifampicin	40	1% or more

CRITERIA OF RESISTANCE

Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – rifampicin, isoniazid, ethambultol, and streptomycin – is classified as resistant to that drug. For calculating the proportion of resistant bacilli, the highest count obtained on the drugfree and on the drug-containing medium should be taken (*regardless of whether both counts are obtained on the 28th day, both on the 42nd day, or one on the 28th day and the other on the 42nd day.)*

Calculation of proportions – An illustration

Suspension	**Dug-free medium**		**Drug concentration (mg/l)**	
			INH $_{0.2}$	**Rif $_{40}$**
Neat	3+	(50000)	2+	26
S_2	2+, 2+	(5000)	62	1
S_4	46, 54	50	3	0
Result			Resistant	Sensitive

PREPARATION OF DRUG CONTAINING MEDIA FOR ECONOMIC VARIANT PROPORTION METHOD DST

Only one concentration per drug is used. The concentrations are as follows:

Isoniazid 0.2 µg/ml

Ethambutol 2 µg/ml

Streptomycin 4 µg/m, (dihydrostreptomycin sulfate, at a concentration corresponding to 4 mg/ml base)

Rifampicin 40 µg/ml

Preparation of drug stock solution is done along with drug free LJ media and PNB media, as a set for each wild strain being tested. One set consist of one LJ slope each for neat, two 10^{-2} drug free slopes, two 10^{-4} drug free slopes, eight LJ drug containing slopes, two each for drugs H, R, E & S (one each for 10^{-2} and 10^{-4} suspensions) and one for PNB slope, total 14 LJ slopes. Each LJ slope requires approximately 5 ml of LJ fluid and hence each set would require 70 ml of LJ fluid to be prepared. The following table gives the number sets to be prepared is calculated in the following manner;

The number of positive cultures isolated in the previous week should be noted. Two positive culture slopes are expected from each patient. One positive culture showing more than 1+ growth in LJ slope should be selected for DST. The other positive culture from same patient should be stored in cold till the DST tests are finalised.

Number of sets required to be prepared	Total volume of LJ solution required for each set for proportion method (ml)	Minimum number of batches of LJ media to be prepared (one batch = 1620 ml of LJ fluid)
10	700	1
20	1400	1
30	2100	2
40	2800	2
50	3500	3
60	4200	3
70	4900	3
80	5600	4
90	6300	4
100	7000	5

Drug-containing L-J slopes are made by adding appropriate amounts of drugs aseptically to L-J fluid before inspissation. A stock solution of the drugs is prepared based on the potency of the drug in sterile distilled water for streptomycin, isoniazid and ethambutol and rifampicin is dissolved in absolute methanol. The solutions of isoniazid and ethambutol are sterilized by membrane filtration. Suitable working dilutions are made in sterile distilled water and added to the L-J fluid, dispensed in 5-ml amounts and inspissated once at 85^{o}C for 50 minutes.

The above quantities would yield sufficient number of slopes to test approximately 40 cultures. The medium can be stored in the cold for 3-4 weeks.

Drug stock solutions should be prepared and used fresh on the day of drug media preparation

Isoniazid (H):

Preferred substance is Sigma I-3377

Drug potency = 1g to 1g substance.

Stock solution preparation:

Weigh out 20mg of isoniazid in 40ml of sterile distilled water (500µg/ml). Label with date of preparation, drug (H)

Working solution:

Prepare the working solution on the day of drug media preparation (Sterilise by filtering through a 0.22 µ membrane filter.)

2 ml of stock solution (500µg/ml) + 48ml of sterile distilled water (=50ml of 20µg/ml). **Do not store this solution**.

Number of sets required to be prepared	Number of bottles of 'H' drug media required (each bottle with ~5ml of LJ fluid)	Ml of working solution of H (20µg/ml)	Amount of LJ fluid to be added (ml)	Final concentration of H in LJ (µg/ml)
5	10	0.5	49.5	0.2
10	20	1	99	0.2
15	30	1.5	148.5	0.2
20	40	2	198	0.2
25	50	2.5	247.5	0.2
30	60	3	297	0.2

Ethambutol (E):

Preferred substance is Ethabutol Dihydrochloride Sigma E-4630

Drug potency = 1g to 1g substance

Working solution preparation:

Weigh out 20mg of ethambutol and dissolve in 100ml of sterile distilled water to get 200µg/ml of stock solution. Sterilise by filtering through a 0.22 µ membrane filter. **Stock solution of E should not be stored.**

Number of sets required to be prepared	Number of bottles of 'E' drug media required (each bottle with ~5m of LJ fluid)	Ml of stock solution (200µg/ml)	Amount of LJ fluid to be added	Final concentration of E in LJ (µg/ml)
5	10	0.5	49.5	2
10	20	1	99	2
15	30	1.5	148.5	2
20	40	2	198	2
25	50	2.5	247.5	2
30	60	3	297	2

Dihydro Sterptomycin sulphate (S):

Preferred substance is Sigma S - 7253

Correction for potency required.

Working solution preparation:

Weigh out 20mg / Potency of dihydro streptomycin sulphate and dissolve in 50 ml SDW to obtain 400µg/ml of stock solution. For example, if the potency is 0.731, the required amount of active drug is 40/0.731 = 27.35 mg. 27.35 mg is dissolved in 50 ml of SDW to obtain 20 mg of active drug. Always look for the potency mentioned on the drug bottle. Sterilise by filtering through a 0.22 µ membrane filter.

Do not store this solution.

Number of sets required to be prepared	Number of bottles of 'S' drug media required (each bottle with ~5ml of LJ fluid)	Ml of stock solution (400µg/ml)	Amount of LJ fluid to be added	Final concentration of S in LJ (µg/ml)
5	10	0.5	49.5	4
10	20	1	99	4
15	30	1.5	148.5	4
20	40	2	198	4
25	50	2.5	247.5	4
30	60	3	297	4

Rifampicin (R):

Preferred substance is Sigma R-3501

Correction for potency required. Check the SOPs.

Working solution preparation:

Weigh out 40mg / Potency of rifampicin and dissolve in 5 ml of absolute methanol, followed by addition of 5 ml of 99% ethanol to get 4000µg/ml of stock solution. **Do not store this solution**

Number of sets required to be prepared	Number of bottles of 'R' drug media required (each bottle with ~5m of LJ fluid)	Ml of stock solution (4000µg/ml)	Amount of LJ fluid to be added in ml	Final concentration of R in LJ (µg/ml)
5	10	0.5	49.5	40
10	20	1	99	40
15	30	1.5	148.5	40
20	40	2	198	40
25	50	2.5	247.5	40
30	60	3	297	40

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IDENTIFICATION TESTS

Although a presumptive diagnosis of tuberculosis may be made by an experienced laboratory technologist on the basis of the morphological 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 below will enable the precise identification of >95% of ***M.tuberculosis*** strains:

1) Susceptibility to *p*-nitrobenzoic acid (PNB)
2) Niacin test
3) catalase activity at 68^0C/pH 7.

Of these, the PNB test can be included along with the drug susceptibility test.

GROWTH ON MEDIUM CONTAINING p-NITROBENZOIC ACID

Procedure: Weigh out 0.5 gm PNB and dissolve in the minimum amount of dimethylformamide (~15ml). Add to 1 litre of L-J fluid, distribute and inspissate once for 50 minutes at 85 degrees. Store in cold room .

Inoculate with the **neat** bacterial suspension one slope of LJ medium and one slope of p-nitrobenzioc acid (PNB) at a concentration of 500 μg/ml and incubate at 37^0C for each set. Read on 28^{th} day. PNB should not be kept for reading at 42 day.

It is critical to inoculate with neat suspension prepared for DST and reading should be only on 28^{th} day.

Results and interpretation

M. tuberculosis does not grow on PNB medium. All other mycobacteria are resistant to PNB.

NIACIN TEST

Although all mycobacteria produce niacin, comparative studies have shown that, ***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 containing Asparagine 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 at least 100 colonies.

Controls

Check the reagents by testing extract from an uninoculated tube of medium (negative control) and use an extract from a culture of ***M.tuberculosis*** H_{37}Rv as positive control.

Reagents

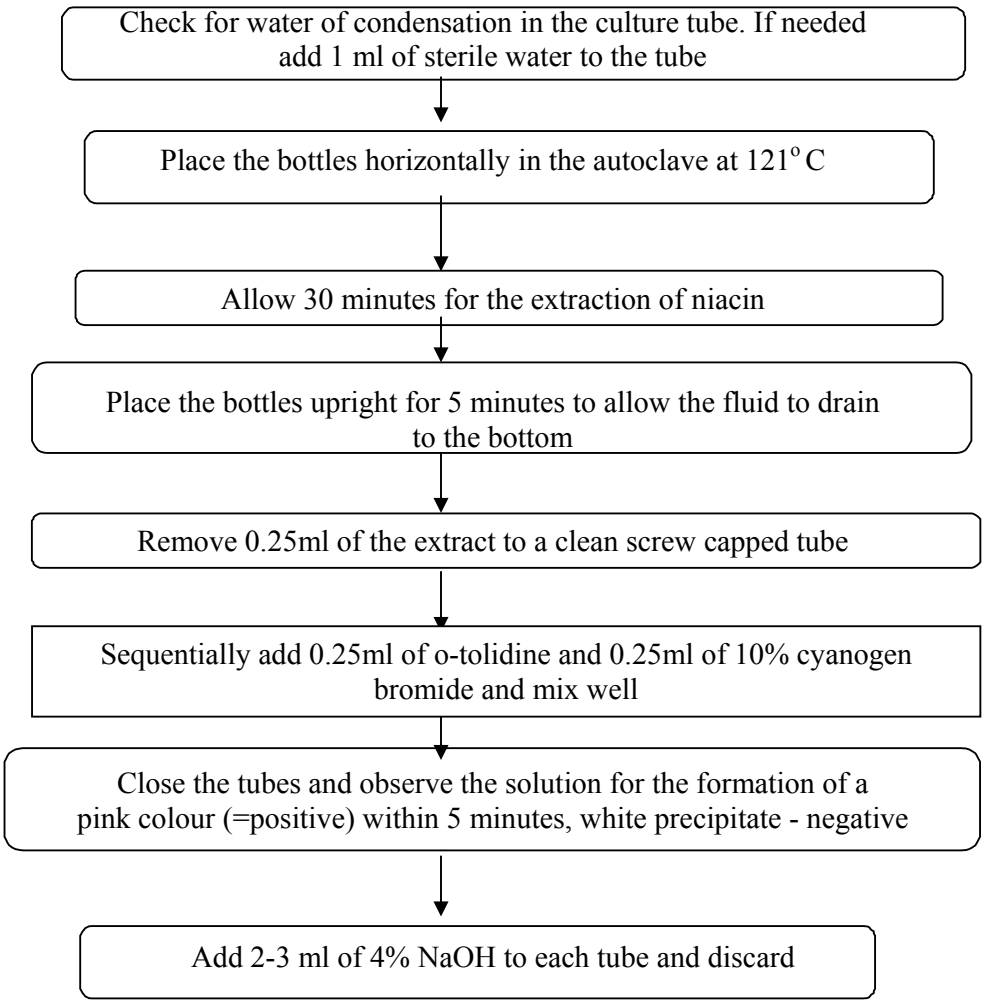
1) O-tolidine - 1.5%
   O-tolidine - 1.5 g
   Ethanol -100ml

Mix in an amber bottle and store in the dark in the refrigerator, prepare fresh weekly.

2) Cyanogen bromide solution, approx. 10%.

A saturated aqueous solution of cyanogen bromide is approx 10%. Store at 4^{o}C in the refrigerator.

PROCEDURE



Precautions:

- 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 while testing cultures. Alternatively, commercially available niacin strips could also be used**

- In acid solutions, cyanogen bromide hydrolyses to hydrocyanic acid, which is extremely toxic. Discard all reaction tubes into a disinfectant solution made alkaline by addition of sodium hydroxide.

CATALASE TEST

Catalase is an intracellular, soluble enzyme capable of splitting hydrogen peroxide into water and oxygen. The oxygen bubbles into the reaction mixture to indicate catalase activity. Virtually all mycobacteria possess 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 68^0C test at pH7 (indicates loss of catalase activity due to heat). Drug susceptible strains of ***M.tuberculosis*** lose catalase activity when heated to 68^0C for 20 minutes. For these tests cultures on LJ should be used.

Controls- Check reagents by testing extract from an uninoculated tube of medium (negative control).

Reagents

(1) 0.067M phosphate buffer solution, pH 7.0

Na_2HPO_4, anhydrous ..... 9.47 g
Distilled water ..... 1 litre
Dissolve disodium phosphate in water to provide 0.067 M solution Solution 1

KH_2PO_4 ..... 9.07 g
Distilled water ..... 1 litre
Dissolve in water to give 0.067 M KH_2PO_4 solution Solution 2

Mix 61.1 ml of solution 1 with 38.9 ml of solution 2. Adjust the pH to 7.

(2) ***Hydrogen peroxide , 30% solution***. Store in the refrigerator.

(3) ***Tween-80, 10%***

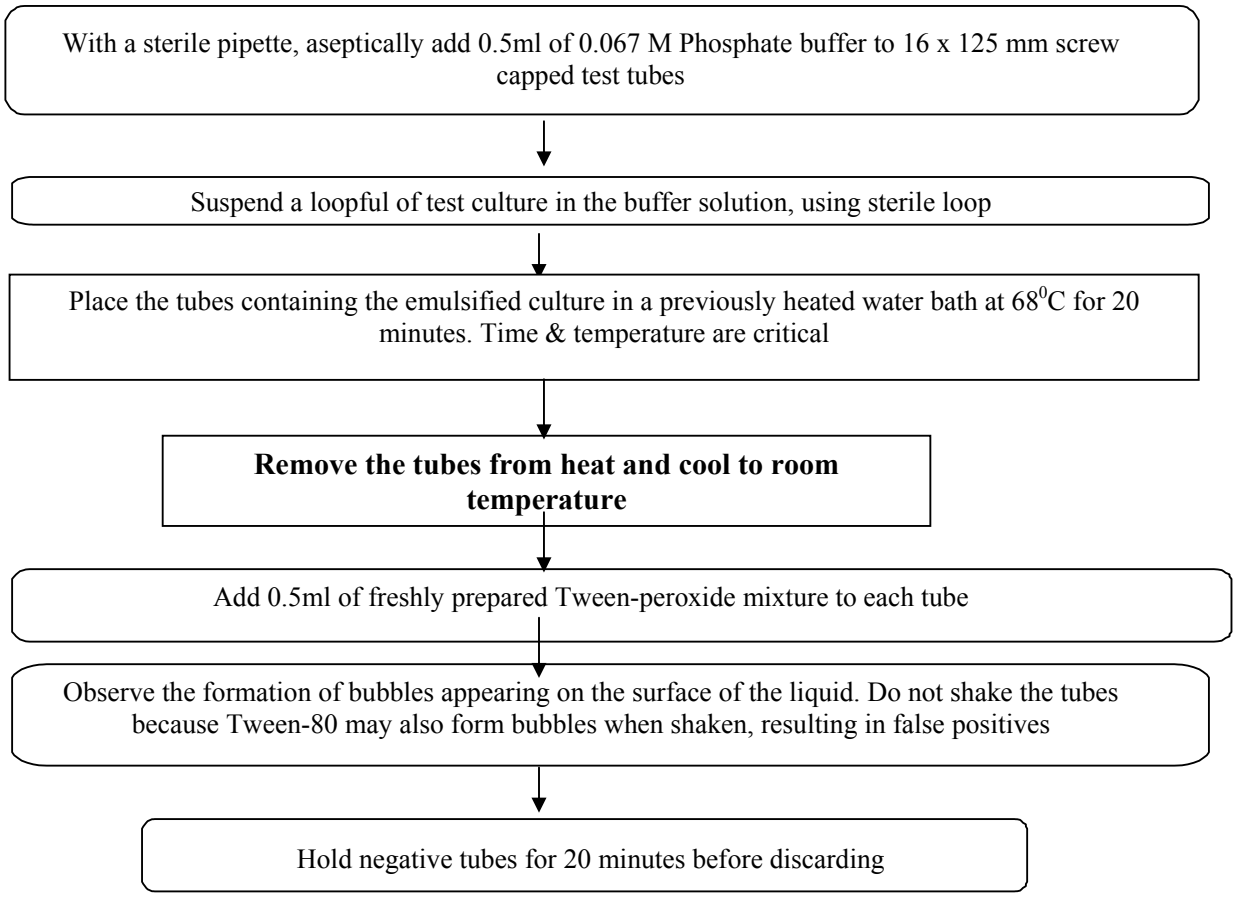
Tween-80 ..... 10 ml
Distilled water ..... 90 ml

Mix Tween-80 with distilled water and autoclave at 121^oC for 10 minutes. Allow to cool.. Store in the refrigerator.

(4) Complete catalase reagent (Tween-peroxide mixture):

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

Procedure



SUMMARY

Identification of M. tuberculosis

- Growth rate slow
- No growth on LJ medium containing p-nitrobenzoic acid
- Optimal growth temperature 35^0-37^0C only
- No pigmentation
- Niacin positive
- Catalase negative at 68^0C

STORAGE OF THE CULTURE ISOLATES

LIQUID MEDIA

Liquid media are used in the mycobacteriology laboratory for sub-culturing stock strains for storing strains at - 80°C and other in-vitro tests.
Mycobacteria have a tendency to clump in liquid medium; Tween 80 is a wetting agent that encourages more homogenous growth is to be included in the basal medium.

Commercially available Middlebrook -7 H9 media along with growth supplement OADC can be utilized for this purpose

In house preparation of SELECTIVE Middlebrook - 7H9 LIQUID MEDIUM

Potassium dihydrogen phosphate, KH_2PO_4	: 1.0 g
Disodium hydrogen phosphate, anhydrous, Na_2HPO_4	: 2.5 g
L-sodium glutamate	: 0.5 g
Ammonium sulphate	: 0.5 g
Tri-Sodium citrate (2H20)	: 0.1 g
Ferric ammonium citrate (green)	: 1.0 ml of 8% aq. Solution
Magnesium sulphate ($7H_2O$)	: 1.0 ml of 5% aq. Solution
Calcium chloride ($2H_2O$)	: 1.0 ml of 0.05% aq. Solution
Zinc sulphate ($7H_2O$)	: 1.0 ml of 0.1% aq. Solution
Cupric sulphate ($5H_2O$)	: 1.0 ml of 0.1% aq. Solution
Pyridoxine hydrochloride	: 1.0 ml of 0.1% aq. Solution
Biotin	: 1.0 ml of 0.05% aq. Solution
Tween 80, 10% solution	: 5.0 ml
Distilled water, to	: 1 litre

Dissolve the ingredients (base)and autoclave.
Add, with sterile precautions, the following:

a) Bovine albumin-dextrose complex : 100 ml

i) Bovine albumin, Fraction V	: 5 g
Glucose	: 2 g
Sodium chloride, 0.85% solution	: 75 ml

Make up to 95 ml with water

Mix well till dissolved by leaving it in 4°C. Filter by 0.22 µ membrane filter.

Before use for every 900 ml base 100 ml bovine albumin dextrose solution should be added

Distribute in 5 ml aliquots in sterile McCartney bottles.

If Readymade Middlebrook- 7H9 base is available:

Solution a :

Middlebrook- 7H9 base	: 4.7 g
Tween 80	: 0.5 ml
Sterile D.water	: 900 ml
Dissolved and autoclaved.	

Solution b

Bovine albumin-dextrose complex	: 100 ml
Bovine albumin, Fraction V	: 5 g

Glucose	: 2 g
Sodium chloride, 0.85% solution	: 75 ml

Make up to 95 ml with water

Mix well till dissolved by leaving it in 4°C. Filter by 0.22 µ membrane filter.

Before use for every 900 ml base 100 ml bovine albumin dextrose solution added

Check sterility by incubation for 24 hours at 37°C and store in the cold.
If turbidity observed in any bottle, discard.

Inoculation:

Prepare a culture suspension in a bijou bottle and inoculate aseptically 2 – 3 loopful of culture suspension and incubated at 37°C.

Sterility:

After 10th day a loopful of the 7H9 culture is spotted on a nutrient agar/MHA, incubated for overnight at 37°C.

If any colonies (contamination) appear the culture would have contaminated, it should be discarded and repeated.

Storage of culture

For final storing the 2 ml of the 7H9 culture is transferred aseptically into sterile 2 ml cryo (nunc screw cap) vials, labelled with lab numbers and kept frozen in -80°C.

Revival of the frozen cultures

Take the cryo vial with culture from – 80°C freezer and place it in an ice bucket. Wipe off the outer surface with 70% ethanol cotton swab and allow thawing on ice. Inoculate a loopful of culture aseptically onto LJ media slopes. Incubate the inoculated LJ slopes for 4 weeks at 37°C. Store the cryo vial with culture back in -80°C

Note: Repeated freeze and thaw will affect the viability of the stored cultures

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ESSENTIAL EQUIPMENTS

BIOLOGICAL SAFETY CABINET

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.

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, the Class II, BSC provides staff and environmental protection and protection.

Bio-safety cabinets need to be certified, annually, for accurate airflow measurements and HEPA filter leak testing. Inappropriate airflow measurements and breach in HEPA filter integrity leads to harmful exposure from biohazard substances.

CENTRIFUGE

Centrifuges are essential in laboratories where tubercle bacilli are cultured by concentration methods. Methods involving the use of 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 or table model with a lid and rotor which contains sealed centrifuge buckets. At least an RCF of 3000g is required for TB culture. Sealed buckets are always used in pairs, opposite one another. If the buckets fit in the centrifuge head on trunnions, these are also paired.

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

35^{o}C-37^{o} C INCUBATOR

Cultures are incubated at 35^{o}C -37^{o} 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.

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

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 $85^{o}C$ for 50 minutes. Modern inspissators are thermostatically controlled and fitted with large internal circulating fan.

AUTOCLAVE

Tubercle bacilli are more readily killed by moist heat (saturated steam) than by dry heat. Steam kills tubercle bacilli by denaturing protein. Air has an important influence on the efficiency of steam sterilization because its presence changes the pressure-temperature relationship. For example, the temperature of saturated steam at 15lb/sq.inch is 121^{o} 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 is only 112 ^{o} C at the same pressure. 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 sterilized should therefore be packed loosely.

Only autoclaves designed for laboratory work and capable of dealing with a mixed load should be used.

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 bath for servicing. Water baths must also be lagged so as to prevent heat loss through the walls.

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.

BUNSEN BURNERS

For materials that may splatter or that is highly infectious a hooded Bunsen burner should be used. Electric burners are also available, in which the loop or wire is inserted, and is recommended for use in BSCs.

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.

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 past 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. Manual or electric pipetting devices are now available and these are recommended for pipetting. However, these items are expensive.

SAFE HANDLING OF SPECIMENS

- 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.

- The work surface, interior walls and interior window surface of the should be wiped with an appropriate disinfectant every day.

- 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 (e.g.,. 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 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.

- 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.

- Place bulky items such as pipette trays and discard bins at one side in the cabinet

- Use only horizontal pipette discard trays containing an appropriate disinfectant (eg. 5% phenol)

- Use proper microbiological techniques to avoid splatter and aerosols. This will minimise the potential for staff exposure to infectious materials manipulated within the cabinet.

- Recap or cover opened tubes 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 (e.g. 5% phenol) 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

- At the end of the workday, surface-decontaminate the work surface of the BSC, the sides and back and the interior of the glass window.

- Always decontaminate the BSC before HEPA filters are changed or internal repair work is done. The most common decontamination method uses formaldehyde gas.

USE OF CENTRIFUGE

- Select two centrifuge tubes of identical length and thickness. Place the specimen to be centrifuged in one tube and an equal amount of water 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.
- Move the speed control slowly until the speed indicator shows the required 'rpm' or RCF

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.
- Open sealed centrifuge buckets in the BSC.

ESSENTIAL EQUIPMENT AND SUPPLIES

Essential Equipment and Supplies for a Culture Laboratory Using Modified Petroff Decontamination and Lowenstein-Jensen Culture Medium

Sl. No	Item / Equipment	Quantity/ volume required
1.	Autoclave, laboratory type, mixed load pressure cooker type or gravity displacement model with automatic air and condensate discharge	1
2.	Balance, top pan	1
3.	Biological safety cabinets, class I, complying with international standards or a prefabricated exhaust hood with UV lamp	1
4.	Bunsen burner for use with gas	3
5.	Centrifuge, with lid, 3000xg RCF capacity, rotor, sealed buckets, safety catch	
6.	Culture bottle washer	1

7.	Homogenizer, for eggs	1
8.	Incinerator	1
9.	Inspissator 240 litre capacity, thermostatically controlled at 85^{o}C	1
10.	Microscope, binocular, oil immersion lens (100x), eye pieces (8x /10x) spare bulbs	1
11.	Refrigerator, Vortex mixer, Aluminium racks, Incubator, large, 37 ^{o} C	1 each
12.	Wire loops, with holders, for inoculation	20

A more detailed list is given in Annex VII.

SELECTED REFERENCES

1. Laboratory Services in Tuberculosis Control. Part III – culture /WHO/TB/98. 258, 1998

2. IUATLD. The Public Health Service National Tuberculosis reference laboratory and The National Laboratory Network, 1998

3. PAHO/WHO Advisory Committee on Tuberculosis Bacteriology. Manual of technical standards and procedures for tuberculosis bacteriology. Part II: The Culture of ***Mycobacterium Tuberculosis***. PAHO, Marlinez, Argentina,1998

4. Jensen, K.A. Towards a standardisation of Laboratory methods. Second report of the Sub-Committee of Laboratory Methods of the IUAT. Bull Int. Union Tuberc. 1995, 25 (1-2): 89-104

QUALITY ASSURANCE PROGRAMME

Quality assurance with regard to tuberculosis bacteriology is a system designed to continuously improve the reliability, efficiency and use of the tuberculosis laboratory services In order to achieve the required technical quality in laboratory diagnosis, a continuous system of quality assurance needs to be established. The reference laboratory should supervise the laboratory network.

The components of a quality assurance programme are:

- Quality control
- Quality improvement
- Proficiency testing

QUALITY CONTROL

Quality control is a process of effective and systematic monitoring of the performance of bench work in the tuberculosis laboratory against established limits of test performance. Quality control ensures that the information generated by the laboratory is accurate, reliable and reproducible and serves as a mechanism by which tuberculosis laboratories can validate the competency of their diagnostic services.

The specific aspects of quality control for microscopy, culture, sensitivity and other procedures are discussed below briefly.

Quality control must be applied to:

- Laboratory arrangement
- Equipment
- Collection and transport of specimens
- Handling of specimens
- Stains, reagents and media
- All bacteriological methods
- Performance of decontamination and digestion methods in culture
- Drug susceptibility/identification tests
- Reporting of results

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

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 laboratories, include:

Laboratory arrangement and administration

- Work areas, equipment and supplies should be arranged for logical and efficient workflow. Work areas should be kept free of dust. Benches should be swabbed at least once a day with an appropriate disinfectant(e.g.,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.
- All records should be retained.
- 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 constant accuracy and precision.
- This includes checking the safety cabinets periodically, checking efficiency of centrifuges, and daily check of temperatures in incubators, water baths, refrigerators, freezers, inspissators and autoclaves.

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%

Culture media

- Use fresh eggs(<seven days) for preparation of Lowenstein-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 35^0-37^0C 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 of cultures with few colonies that follow a heavily positive culture. This could be due to carry over of the positive due to improper sterilisation of the inoculating loop.

Biochemical tests

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

Drug susceptibility /identification tests

Include a standard strain with every batch of medium as a check on drug concentration. Include known positive and negative control in all biochemical tests for identification. As an internal quality control (IQC), use a strain of ***M.tuberculosis*** with known resistance pattern to different drugs with every batch of tests as a check on procedures.

QUALITY IMPROVEMENT

Quality improvement is a process by which the components of tuberculosis laboratory service are analysed continuously to improve their reliability, efficiency and utilisation. It has been shown that the most effective and long-lasting improvements are achieved by anticipating and preventing problems rather than by identifying and correcting defects after they have occurred. Data collection, data analysis and creative problem-solving are the key components of this process. It involves continuous monitoring to prevent recurrence of problems. Often, problem-solving can be done efficiently only during on-site supervisory visits. These are the quickest and most effective forms of quality improvement because of the personal contact and permits on the spot corrective action. Supervision should always be

done by a more experienced laboratory technician. Activities during these visits should include the following:

- Observing general laboratory hygiene and safety practice
- Checking that written standard procedure for equipment and laboratory methods are in place and easily accessible
- Checking that service and maintenance records of equipment are up to date
- Evaluating the proportion of unsatisfactory specimens. Should a particular health facility be identified as a source of the problem , the necessary consultation with health care staff should take place
- Checking that stains and reagents and culture media contain the necessary information on preparation dates and that old stock is not in use
- Checking that positive and negative controls are used as necessary during microscopy, culture or identification procedures
- Analysing the monthly proportion of positive smear or culture results for deviations from the normal
- Collecting a selection of positive and negative slides for re-checking
- Evaluating the monthly culture contamination rate in terms of the proportion of specimens contaminated as well as the proportion of cultures contaminated
- Checking the performance of the standard strains in drug susceptibility tests
- Checking the results of controls used in identification tests

PROFICIENCY TESTING

Proficiency testing, which is called External Quality Assessment by WHO standards refers to a system of retrospectively and objectively compared results from different laboratories by means of programs organized by external agency, such as a reference laboratory. The main objective is to establish between - laboratory comparability, in agreement with a reference standard. For this purpose, material for testing is prepared by a reference laboratory and distributed to lower level laboratories. The recipients perform the necessary procedures and report their results to the reference laboratory which can be then assess proficiency. Detection of deficiency through this indirect system will then determine the need for quality improvement.

Proficiency testing is highly desirable but not easy to achieve. In order to be successful they must run in the form of continuous assessments and they require skilled and dedicated staff.

Although quality improvement is the quickest and most effective form of (external) quality assurance, it is often difficult to perform on a regular basis owing to limitation of time and

travel. Indirect technical and administrative control through proficiency testing programs (also called "external quality assessment" or "inter-laboratory test comparison") should therefore become an essential component of quality assurance.

Participation in proficiency testing programs must be compulsory. The following minimum activities are recommended preferably every six months.

DRUG SUSCEPTIBILITY TESTS

The NRL should send strains of known susceptibility pattern to the participating STDCs. Similarly the results obtained at the lower laboratories can be checked by requesting for a set of cultures, which should be retested at the NRLs. This exercise can be undertaken at six monthly intervals. The NRLs should supply the drugs required for susceptibility tests to ensure proper standardization.

It should be realized that results from proficiency testing programmes may be biased, since laboratory staff are aware of the origin of proficiency smears and cultures and may dedicate more time and attention to their correct processing and examination.

Irrespective of the methods of proficiency testing, the most important aspect is regular feed back and corrective measures taken in a spirit of mutual trust and agreement.

In summary, well designed and properly managed quality assurance programmes are an asset to any laboratory. Positive aspects of such programs for tuberculosis bacteriology include the following:

- Potential problems in the isolation and identification of ***M.tuberculosis*** can be greatly reduced by monitoring media and reagents before using them on clinical specimens
- Serious and costly breakdowns of equipment may be minimised by routine monitoring and maintenance
- Laboratory reports can be more accurate and expeditious as the use of inadequate media, equipment and techniques are minimized.
- The quality assurance program can serve a learning exercise, enabling the recognition and identification of problem areas that might otherwise have been over looked
- A good quality assurance program will enhance the credibility of the laboratory to out side clients and patients.

Intermediate Reference Laboratory, or Culture & DST laboratory Proficiency testing is conducted by the designated National Reference Laboroary (NRL) once in a year. The IRL, or culture & DST laboratory should send a list of all cultures to NRLs, who would randomly select cultures for proficiency testing. These cultures would be then sent to NRLs by IRL, or culture & DST laboratory and the result of NRLs will be communicated to sending lab with corrective actions, if required.

In addition, NRLs will send a set of 20 cultures to Intermediate Reference Laboratory, or Culture & DST laboratory for testing and the results will be compared for concordance and suggestions for improvement would be provided, if required.

REFERENCES

1. Laboratory services in tuberculosis control. Part I, II and III. WHO/ TB/98. 258, 1998.
2. The public health service national tuberculosis reference laboratory and the national laboratory network. IUATLD, 1998
3. Quality assurance in bacteriology and immunology: WHO regional publication, SEA series No. 28, 1998.

Calculation of the chemicals required for 100 patients for DRS pilot study or 200 samples:

Mineral salt solution with malachite green

Potassium dihydrogen phosphate : 26.4 g (2.4g x 11 batches)

Anhydrous (KH_2PO_4)

Magnesium sulphate anhydrous : 2.64 g (0.24g x 11 batches)

Magnesium citrate : 6.6 g (0.6g x 11 batches)

Asparagine : 39.6 g (3.6g x 11 batches)

Glycerol (reagent grade) : 132 ml (12ml x 11 batches

**Malachite green solution 2%*

Malachite green dye : 22 g(2.0g x 11 batches)

PNB : 750 mg (75mg x 10 batches)

4 % NaOH : 10 ml (1 ml x10 batches)

0.1 % Phenol red : 1 ml (0.1 ml x 10 batches)

N/10 HCL : 1000 ml (100 ml x 10 batches)

Membrane filters : 30 nos. (3 x 10 batches)

Syringes 5 ml : 30 nos (3 x 10 batches)

Eggs (country eggs) : 30 eggs x 11 batches = 330 eggs

Isoniazid (H) : 100 mg (10mg x 10 batches)

Ethambutol (E): : 100 mg (10mg x10 batches)

Dihydro Sterptomycin sulphate (S) : 274 mg (27.35mg x 10 batches)

Rifampicin (R) : 200 mg (20mg x 10 batches)

Methanol : 25 ml (2.5 ml x 10 batches)

95 % ethanol : 25 ml (2.5 ml x 10 batches)

CPC : 10 g (for 200 bottles)

NaCl : 20 g (for 200 bottles)

McFarlands tube 0.5 : 1 tube

McCartney bottles : 4000 bottles (app.)

Bijou bottles : 200 bottles

Distilled water (Double sterile) : 10 liters

Pipette 10 ml, 5 ml, 1 ml : 20 each

Conical flasks 2 litre : 5 nos.

Conical flasks 1 litre : 20 nos.

Conical flasks 500 ml : 20 nos..

Measuring jar 50 ml, 100 ml, 500ml, 1 litre : 5 each

Steel funnel big (8 cm dia.) : 4 nos.

Steel beaker 1 litre : 4 nos.

Round bottom flask 2 litres : 3 nos.

Mortar and pestle medium size : 2 nos.

Retort stand 12 cms., 6cms. : 4 nos.each

Bossheads : 4 nos.

Corks for 1 litre : 15 nos. each

Clamps : 10 nos.

Egg beater :

Balance (digital balance) : 1 no.

Inspissator : 1 no.

Wire racks 150 holes (for McCartney): 20 nos.

Autoclave : 2 nos.

Refrigerator 235 L : 1 nos.

Wire baskets : 5 nos.

Steel drums 30 Litre capacity : 2 nos.

5-litre capacity Discard bins : 6 nos.

2 litre capacity discard bins with lid : 6 nos.

Hot air oven : 1 no.

Hair dryer : 1 no.

Fan head brush : 5 nos.

Small brush (7 cm for McCarteney bottles): 5 nos.

Surgical bottle washer : 1 no.

Surgical bottle washer brush : 50 nos.

10 litre water can : 5 nos.

Distillation plant : 1 no.

20 litre water can : 5 nos.

Plastic buckets : 5 nos.

Plastic mugs : 5 nos.

Incubator of large size : 1no.

Geyser large size : 1 no.

Centrifuge Swing head : 2 no

Domestic balance : 1 no.

BSC cabinets (class1 / 2) : 1 no.

Bunsen burne (hooded/ with chimneys): 1 no.

Loop holder : 12 nos.

24 SWG,27 SWG nichrome wire : 10 metres.

12 holes rack for McCartney bottles : 10 nos.

Metal pipette case	: 4 nos.
Bijou bottle racks	: 4 nos.
Glass beads 3mm	: 500 gms.
Pipette aid	: 2nos.
Water bath	: 1 no.
Screw cap tubes 10 cm x 1 cm	: half-gross.
Test tube racks	: 5 nos.
Thermometer	: 2 no.
Reagent bottle 100ml	: 5 nos.
Porceline tile with cavity	: 5 nos./Niacin tubes 5cm x 1 cm: half gross.
Disposible droppers	: 2 packs.
Concentrated HCL	: 500ml
Sodium carbonate	: 5 k.g.
Soap solution	: 10 litres.
Lysol	: 10 x 5 litre cans
Acetone	: 1 litres
Cotton absorbent	: 10 rolls
Cotton Non-absorbent	: 10 rolls
Gauge	: 10 meters
Brown papers	: 10 sheets
Aluminum foils	: 5 rolls
Hydrogen peroxide	: 500 ml
Tween 80	: 500ml
Potassium hydrogen phosphate	: 500g
Sodium dihydrogen phospate	: 500 g

Cynogen bromide : 5 g

Benzidine : 10 g

Washing of glasswares :

Already used ones:

- Immerse them in 1 litre of 5%HCl - acetone mixture (1 part of acid, 2 part of acetone) for 1 hour,
- Wash in running tap water,
- Rinse in soap water with fan head brush
- Wash in running tap water
- Rinse them in distilled water.

Media containing McCartney bottles:

- Autoclave them at 18 lb./sq. inch for 45 minutes,
- Remove the caps,
- Using surgical bottle washing machine or by vigorously using the brush remove the media,
- Place the bottles in a wire basket and immerse them in boiling water containing handful of sodium carbonate powder and leave it for 45 minutes,
- Using soap water and test tube brush clean the bottles and wash them in running tap water,
- Immerse them in 5 % HCl for at least 2 hours other wise overnight,
- Wash again running tap water and keep immersed in distilled water for 5 to 10 minutes.

Metal caps:

- Remove the liners,
- Remove the numbers written on the caps with acetone,
- Check the liners, wash in soap water and running tap water before replacing them in the caps if they are in good condition,

Bijou bottles are treated the same way as it is mentioned above.

Steps to be followed while starting the DST:

1. Switch on the biological safety cabinet and ensure if its airflow is in order. It is preferable to keep the airflow on 24 hours a day and seven days a week.
2. Ensure the availability of sufficient media sets for DST.
3. Ensure that sufficient growth (1 loopfull / 5 mg) can be obtained from primary isolate.
4. Arrange the media for the selected sets.
5. Arrange the isolates in ascending order.
6. Arrange the bottles with beads and bottles for dilution and label them with the isolates number along with the corresponding dilution.
7. Distribute 1 ml of sterile distilled water (SDW) in bottles containing beads and 2ml of SDW in to bottles for dilution.

8. Take 1 loopfull of the colonies with sterile loop (24swg of 3 mm internal diameter) by touching different areas of the culture, not picking up the media while doing so.
9. Vortex well to get uniform suspension.
10. Add approximately 4ml of SDW with individual sterile pipette into each bottles.
11. Leave the bottles undisturbed for 15 minutes and transfer the supernatant into another bottle carefully without disturbing the sediment.
12. While waiting for 15 minutes write the lab numbers on to the LJ slopes including the drug containing slope.
13. Compare the supernatant transferred with Mcfarland tube no.0.05 and adjust the turbidity by adding SDW drop by drop.
14. With the help of calibrated loop, which will deliver 0.01 ml, transfer 2 loopful into bijou bottle containing 2ml of SDW which is labelled as S2
15. Mix well and transfer 2 loopful from S2 into another bijou bottle labelled S4 and mix well.
16. Now loosen the caps of the LJ slopes, just one thread.
17. With the help of 3mm internal diameter 27 SWG wire loop inoculate uniform drop size into the correspondingly labelled slopes, starting from S4, S2 and Neat.
18. After inoculation before transferring the rack into incubator cross check the isolate number with the primary isolates and label the rack with the date

Points to remember while taking the sensitivity reading:

- ◊ Proceed with reading if there is no growth in the PNB, if growth is noticed in PNB, ignore that isolate for reading and subject the culture for identification tests with Niacin and rate of growth.
- ◊ Repeat the test if;
    - If there is less than 100 colonies in the neat suspension
    - If the growth in the S4 control slope (plain LJ) is confluent and counting cannot be done
    - If no countable colonies are obtained in any of the control slope
    - If all the slopes are grossly contaminated
- ◊ During counting fused colonies should be counted as single colony
- ◊ Peaks of the fused colonies should not be counted as individual colonies.
- ◊ The confluent bottom growth is counted as single colony.
- ◊ If growth along with other contamination the colony counts should be recorded with the star mark (*). In such cases sensitive results cannot be accepted and the test should be repeated

Performance Indicators for C&DST laboratories

Laboratory Performance Indicators for Mycobacterial Culture and Drug Susceptibility Testing

Introduction

Under RNTCP, a large number of culture and drug susceptibility testing (DST) laboratories will eventually be available, and maintenance of this laboratory network will be a growing challenge. National reference laboratories (NRLs) and intermediate reference laboratories (IRLs) are also expected to provide culture and DST quality assurance for accredited culture and DST laboratories in public sector, private sector, and in medical colleges. This quality assurance is an ongoing activity. Systematic assessment of laboratory quality should be built into the system, that can guide supervisors and facilitate monitoring by reference laboratories. Culture and DST lab staff should understand that their service quality will be subject to ongoing scrutiny, in the form of quarterly reports and an annual round of proficiency evaluation.

These Standard Laboratory Performance Indicators for Mycobacterial Culture and DST can help NRL's maintain organized and standardized measures to assess laboratory quality and changes in quality over time, and allow them to focus and guide their supervision activities.

How to use these indicators

- Each accredited laboratory is responsible for reporting on a **quarterly** basis.
- Laboratories should **report numbers** for numerators and denominators, and percents where indicated.
- Most indicators refer to **all specimens processed in a quarter**,
- Suggested **data sources are listed** for each indicator. Standard RNTCP registers and formats should be used for day to day work, and the monthly abstracts used to summarize activities. These can be found in the "Manual of Standard Operating Procedures for Intermediate Reference Laboratory for Tuberculosis". Otherwise, existing registers & forms may have to be modified to collect the information.
- **Final data collection for reporting should begin 2 months after the end of the quarter**, to allow for diagnostic cultures inoculated from the previous quarter to be finalized.
- If the laboratory cannot "diagnostic" and "follow-up" specimens from existing records then, then revised registers and forms are required to allow that distinction. Till those are implemented, specimens submitted for culture and DST can be considered as "diagnostic" and those submitted for culture only can be considered as "follow-up".
- Those laboratories performing culture only can ignore DST specific indicators.
- **Reporting format should be submitted to NRL & CTD by the end of the following quarter**.
    - Example: 1st quarter report should be started on 1st June, and submitted by 30st June.
    - Softcopies should be emailed to: labreport@rntcp.org
    - Hardcopies should be maintained for NRL/CTD visits.

Acknowledgements:
These indicators have been generally inspired by McCarthy K, Metchok B, Kanphukiew A, et al. Monitoring the performance of mycobacterial laboratories: a proposal for standardized indicators. Int J Tuberc Lung Dis 2008: 12(9):1015-1020.

Summary of Standard Laboratory Performance Indicators for Mycobacterial Culture & DST

Indicator	Numerator	Denominator	Expected Data sources	Frequency	Relevant activity evaluated by indicator
(1) The percentage of specimens received within 7 days of sputum collection	Number of sputum specimens received within 7 days of collection	Total number of sputum specimens received	Specimen register	Quarterly	Specimen transportation
(2) The percentage of all cultures reported as *Mtb*. complex	Number of specimens (all) inoculated in one quarter reported as *Mtb* complex	Total number of specimens (all) inoculated in one quarter	Culture register	Quarterly	Workload
(3) The correlation between positive smears and positive cultures in smear-positive diagnostic specimens	No. of smear-positive diagnostic specimens inoculated in one quarter that were culture-positive for MTB or NTM	No. of smear positive diagnostic specimens submitted for culture inoculated in one quarter	Culture register	Quarterly	Specimen quality, processing, decontaminatio n
(4) The percentage of all cultures contaminated (If liquid culture or other culture system used, reported separately for each culture system)	Number of specimens (all) inoculated in one quarter discarded due to contamination (both slants cont)	Total number of specimens (all) inoculated in one quarter:	Culture register	Quarterly	Specimens quality and decontaminatio n
(5) The percentage of all cultures reported as non-tuberculous mycobacterium (NTM)	Number of specimens (all) inoculated in one quarter reported as NTM	Total number of specimens (all) inoculated in the reporting quarter	Culture register	Quarterly	Specimen preparation
(6) The percentage of drug susceptibility results available within 84 days.	Total number of DST processed in reporting quarter with results available within 84 days	Total number of DST processed in the reporting quarter	Specimen register	Quarterly	Timeliness, quality of service
(7) The percentage of final culture/DST results[1] reported within 3 days	Total number of culture/DST with final results reported to referring provider within 3 days of final result being available	Total number of cultures inoculated in the reporting quarter	DST register, DOTS-plus IRL register	Quarterly	Timeliness, quality of service
(8) Performance on Drug Susceptibility Testing (DST).	Total number of correct responses	Total number of specimens provided for DST re-testing	Proficiency testing report	Annual	DST quality
(9) Annual on-site evaluation conducted	Not applicable	Not applicable		Annual	On Site Evaluation

[1] A "final culture/DST result" refers to final DST result if available, or final culture result (culture+ M. Tb complex, culture-negative, contaminated, NTM) if DST was not performed for that specimen.

Reporting Format	Quarterly / Annual report on Laboratory Performance
Laboratory:	
Quarter / Year of reporting	
Microbiologist:	
Contact Phone Number:	

Workload and DST results

	Culture workload (from culture register)		DST workload and results (from DST register)				
Month	Diagnostic Sputum Specimens inoculated	Follow-Up Specimens inoculated	DST Processed	Total H+R Sens	Total H+R Res	Total H only Res	Total R only Res

Performance indicators

	Numerator (No.)	Denominator (No.)	Percent
(1) The percentage of sputum specimens received within 7 days of sputum collection			
(2) The percentage of all cultures reported as *Mtb.* Complex			
(3) The correlation between positive smears and positive cultures in smear-positive diagnostic specimens			
(4) The percentage of all cultures contaminated (reported by culture system)			
(5) The percentage of all cultures reported as non-tuberculous mycobacterium			
(6) The percentage of drug susceptibility results available within 84 days			
(7) The percentage of final culture/DST results reported to provider within 3 days of result being available			
INDICATORS (7 – 8) EXPECTED ON ANNUAL BASIS ONLY			
(8) Performance on Proficiency Testing for DST **(Report Date _____)** **(Reference lab_____)**	**Sensitivity (%)**	**Specificity (%)**	
	H:		
	R:		
	E (opt):		
	S (opt):		
(9) Annual On-Site Evaluation	Date: Ref. Laboratory: Evaluator:		

Indicator 1: The percentage of sputum specimens received within 7 days of sputum collection

Numerator / Denominator

$$\frac{\text{Number of sputum specimens received in one quarter within 7 days of sputum collection}}{\text{Number of sputum specimens received in one quarter}}$$

What it measures

- This indicator measures the time lag between sputum collection and receipt by the culture laboratory, which strongly affects specimen viability and risk of contamination.
- Although laboratories are not usually responsible for specimen handling themselves, laboratories are the only place that can detect and indicate problems in the pre-laboratory specimen handling chain.
- At a minimum, 80% of specimens should be received within 7 days of sputum collection. This may vary if special activities such as drug resistance surveillance are ongoing, and if special transport media is being used.
- This indicator will assist in the interpretation of laboratory contamination rates.
- This indicator is dependent upon the reporting of date of specimen collection.
- The indicator does not address the quality of the sputum collection, nor the loss of viability during transportation. The benchmark may be exceeded during special surveys with the use of transportation media.

How to measure it

The numerator can be determined by calculating the time difference between date of collection and receipt, usually available in the specimen receipt or culture register, and determining the proportion that met the 7-day benchmark during the quarter of interest. The denominator is the total number of specimens received during that quarter.

Data Sources

- Specimen receipt register
- Laboratory information systems

Indicator 2: The percentage of all cultures inoculated in one quarter reported as *Mtb* complex

Numerator / Denominator

$$\frac{\text{Number of cultures inoculated in one quarter reported as Mtb complex}}{\text{Total number of all specimens inoculated for culture in one quarter (regardless of result, i.e. including } \textit{Mtb}\text{, NTM, negative, and contaminated cultures)}}$$

What it measures

- This indicator is useful for establishing an acceptable recovery rate of *Mtb* complex within a given population/province
- This indicator represents the percentage of mycobacterial cultures inoculated in one quarter reported as *Mtb* complex. This indicator is directly related to the population from which specimens are received, and may vary between different populations.
- Approximately 12-14 months of data should be evaluated before determining an acceptable recovery rate for the population, and before responding to major fluctuations in the established trend
- A dramatic decrease (>20%) in this indicator may reflect a change in the population, but should also prompt a sputum collection, handling, and laboratory investigation. Sputum collection may have changed or become inadequate. Sputum specimens collected may have been inappropriately delayed, transport medium used may have been improperly prepared. Excessive decontamination during specimen processing, the use of expired reagents and/or reagents of suboptimal quality, and the use of equipment performing outside of the expected limits may have a negative impact on the recovery rate. A systematic review of processing procedures, reagents used, instrumentation, and laboratory records should be performed.
- A dramatic increase (>20%) in this indicator may represent a change in the population, or may suggest the reporting of false-positive cultures due to cross contamination. Molecular techniques may be used to detect cultures that are cross contaminated.

How to measure it

The numerator is the total number of specimens reported as *Mtb* complex within one quarter. The denominator is the total number of specimens inoculated in one quarter regardless of result, including NTM, *Mtb* complex, culture-negative, and contaminated.

Data Sources

- TB laboratory culture register
- Laboratory information systems

Indicator 3: The correlation between positive smears and positive cultures in one quarter in smear-positive diagnostic specimens

Numerator / Denominator

$$\frac{\text{No. of smear-positive diagnostic specimens inoculated for both culture that were culture-positive for MTB or NTM in one quarter}}{\text{No. of smear-positive diagnostic specimens inoculated for both culture in one quarter}}$$

What it measures

- This indicator is useful in evaluating technical proficiency in microscopy and specimen processing.
- This indicator represents the percentage of positive smears from diagnostic specimens that yield positive cultures reported in one quarter.
- Smear-positive diagnostic specimens are chosen as the measure of specimen processing because a high yield is generally expected. This would include specimens from patients on treatment but with suspected MDR TB.
- Diagnostic specimens for this purpose are defined as those specimens submitted for both culture and DST.
- If “diagnostic” and “follow up” specimens are not clearly indicated, than those specimens submitted for both culture and DST should be taken as diagnostic specimens.
- Approximately 90% of all positive smears performed and reported should result in positive cultures, though this may vary by laboratory depending on the characteristics of the population tested. Less than 90% for this indicator should prompt an investigation by the laboratory. This investigation should include a review of: patient history including previous positive cultures and/or smears, accuracy of classification of type of specimen received {diagnostic vs. follow up}, anti-TB treatment at time of specimen collection, specimen handling and transportation, quality of smear microscopy in the culture laboratory, the decontamination methods used for specimen processing, and the adherence to decontamination procedures.
- Loss of bacterial viability between specimen collection and receipt by the culture laboratory and the effect of decontaminating agents used in transporting sputum may result in a difference in smear results between collection and receipt. Hence to maintain focus on culture laboratory specimen processing, the smear-result from the culture laboratory of the actual specimen inoculated should be used when calculating this indicator.

How to measure it

The numerator is the total number of smear-positive specimens (submitted for both culture and DST) inoculated with a positive MTB or NTM culture result in one quarter. This can be obtained from the TB culture registers, culture worksheets or other data management system. The denominator is the total number of smear-positive diagnostic specimens inoculated for culture within one quarter.

Data Sources

- TB laboratory culture register (which should also record microscopy results of inoculated specimens for ease of preparation of this indicator)
- TB laboratory smear-microscopy register (if necessary)
- Laboratory information systems

Indicator 4: The percentage of all cultures contaminated in one quarter [Culture system specific]

Numerator / Denominator

$$\frac{\text{Number of cultures inoculated in one quarter discarded due to contamination}}{\text{Total number of all cultures inoculated in one quarter}}$$

What it measures

- This indicator is useful for monitoring specimen collection and transport procedures, and evaluating processing procedures.
- This indicator represents the percentage of cultures documented as discarded due to contamination in one quarter.
- This indicator may be related to the patient population sampled; however in most cases it is directly related to the collection, storage, and transport of specimens; and/or the media, reagents, and procedures used in the laboratory for specimen processing.
- Separate indicators should be calculated for each culture system, if more than one is used.
- Lowenstein Jensen (LJ)
    - An acceptable range for this indicator is 2–4%.
    - Contamination rates of 1–2% suggest the use of harsh decontamination reagents and/or excessive decontamination. The laboratory should review the specimen processing procedures and the stringency of reagents used, specifically the final concentration of NaOH if 1–2% contamination rates are observed. NaOH is sensitive to storage conditions, and the concentration can vary over time.
    - A contamination rate of >4% suggests incomplete digestion/decontamination, the use of suboptimal reagents or contaminated media for specimen processing and inoculation, or problems with specimen collection, storage and/or transport. LJ media is intrinsically quite resistant to contamination, and high contamination rates usually indicate a significant problem. If contamination rates exceed 4%, a laboratory investigation should be initiated.
- Liquid cultures
    - The acceptable range for contamination established by the manufacturers is 8–9%.
    - If the liquid culture contamination rate >10%, and the contamination rate of the solid media (LJ) exceeds 5%, an evaluation of all procedures relating to specimen collection and processing should be evaluated as above.
    - If liquid culture contamination rate >10%, but solid media contamination rates are <5%, then procedures to troubleshoot liquid culture contamination should be prioritized. Attention should be given to the preparation of liquid culture (reconstitution and addition of antimicrobials [e.g. PANTA]) prior to specimen inoculation, and the handling of tubes. However, given the relative sensitivity of liquid culture systems to contamination due to specimen processing compared to LJ, it is possible that specimen processing procedures are suboptimal. Hence the investigation should include review of these procedures.
- Contamination beyond thresholds for any culture system should also prompt consideration of the timing of contamination. Early contamination (within the first few days of inoculation) suggests gross contamination of the media, bottle, or specimen. Late contamination suggests inadequate decontamination processing of specimens.

How to measure it

The numerator is the total number of cultures in that system inoculated in one quarter that were documented discarded due to contamination, and can be obtained from the TB laboratory culture register or other information management system. The denominator is the total number of all specimens inoculated for culture in one quarter in that system, and can also be obtained from the same data source.

Data Sources

- TB laboratory culture register
- Laboratory information systems

Indicator 5: The percentage of all cultures reported as nontuberculous mycobacterium (NTM) in one quarter [Culture system specific].

Numerator / Denominator

$$\frac{\text{Number of final isolates reported as NTM in one quarter}}{\text{Total number of all cultures inoculated in one quarter}}$$

What it measures

- This indicator is useful for establishing an acceptable recovery rate NTM within a given population and/or province; and will help laboratories monitor and troubleshoot contamination due to NTM.
- This indicator represents the percentage of mycobacterial cultures reported in one quarter as NTM. This indicator is related to the frequency of infection caused by mycobacterium other than tuberculosis within a given population, and the distribution of environmental mycobacterium.
- Laboratories using a liquid based culture system have frequently reported a marked increase in the proportion of NTM isolates recovered when compared to solid media. The significance of that observation has not been established, and requires clinical correlation.
- There is no specific target that can be applied across laboratories, but the trend within a laboratory should be followed. Approximately 12-14 months of data should be collected and evaluated before determining an acceptable recovery rate and responding to major fluctuations in the established trend.
- A dramatic increase (20%) in the same culture system (solid or liquid) in this statistic may suggest environmental contamination.
- Contamination can be introduced during the pre-analytical phase of testing (the use of contaminated water and/or equipment during specimen collection), or may be introduced during testing (through contaminated buffers and/or water). If the laboratory observes the above noted increase, an investigation to determine the possibility and cause of contamination should be initiated. Speciation of the NTM recovered should be considered as relevant to the investigation, if laboratory capacity for speciation exists.

How to measure it

The numerator is the total number of cultures reported within one quarter as NTM, which can be obtained from the TB laboratory notebook or other data management system. The denominator is the total number of all specimens inoculated for culture in one quarter in the same culture system, and can also be obtained from the same data source.

Data Sources

- TB laboratory culture register
- Laboratory information systems

Indicator 6: The percentage of drug susceptibility results available within benchmark turn arount time [culture system specific]

Numerator / Denominator

$$\frac{\text{Total number of DST processed in reporting quarter with results available within target turn-around time}}{\text{Total number of DST processed in the reporting quarter}}$$

What it measures

- This indicator is useful in identifying problems with the timeliness and quality of service, which usually indicates weaknesses in laboratory administrative and technical procedures.
- This indicator measures the number of days from laboratory receipt to the availability of drug susceptibility test results.
- The turn around time is specific for the culture and DST system used.
- The standard benchmark is that turn-around times should be reached for 90% of all DST processed.
- The following turn around times (TAT) from specimen receipt to DST results are recommended:
    - Solid media: within 84 days from receipt of specimen
    - Liquid media: within 42 days from receipt of specimen
    - Line-probe assay: within 5 days of receipt of specimen

How to measure it

- Turn around time should be routinely documented in the TB laboratory registers.
- Calculation of turn around time days:
    - Culture TAT: [date of culture result report – date of specimen receipt]
    - DST TAT: [date of DST result report – date of specimen receipt]
- A minimum of 90% of specimens should meet turn around times, though this may vary if a laboratory is heavily involved in reference or research functions. Turn-around times that exceed these standards for more than 10% of isolates tested should prompt an investigation of timeliness of laboratory procedures for specimen receipt, smear preparation and processing, inoculation, and recording in laboratory.
- Delays in reporting to providers due to 'certification' of results by senior laboratory officials have been observed, and should be minimized. One solution to this common administrative delay is to deputize a senior laboratory technician to certify results.

Data Sources

- The RNTCP culture and DST register has two columns that should be used to calculate the turn-around time: [Date of result] – [Date of receipt]
- One technician can be assigned to calculate the turn-around time achieved for each specimen

Indicator 7: The percentage of final culture/DST results reported to provider within 3 days

Numerator / Denominator

$$\frac{\text{Total number of final culture/DST results reported to provider within 3 days}}{\text{Total number of all cultures inoculated in one quarter}}$$

What it measures

- This indicator is useful in identifying problems with the timeliness and quality of service, which usually indicates weaknesses in laboratory administrative and technical procedures.
- This indicator measures the number of days from the availability of laboratory results to the time when those results are communicated back to the referring provider. Quick communication of results is required for proper clinical management of patients.
- This reporting turn-around time should be reached for **100% of all specimens received.**
- In an effort to reduce turn around time due to courier delivery of paper results, all positive results should also be reported by email, telephone, or fax no later than 2 days from the availability of results in the laboratory.

How to measure it

- "Final culture/DST results" are defined as the DST result if available. If DST was not performed for that specimen, then the final culture result (culture+ *M. Tb* complex, culture-negative, contaminated, or NTM) should be taken.
- Date of results and date of report to providers should both be recorded in routine laboratory registers.

Data Sources

- The RNTCP culture and DST register has two columns that should be used to calculate the turn-around time: [Date of result] – [Date of receipt]
- One technician can be assigned to calculate the turn-around time achieved for each specimen

Indicator 8: Performance on Proficiency Testing (PT) in Drug Susceptibility Testing (DST).

Numerator / Denominator

$$\frac{\text{Total number of correct responses}}{\text{Total number of specimens provided for DST proficiency testing}}$$

What it measures

This indicator is a measure of the laboratory's proficiency to perform and interpret first and/or second-line (if applicable) drug susceptibility tests. This indicator is dependent upon a commitment to a proficiency testing programme.

- This indicator is useful in measuring laboratory proficiency and identifying training needs. It does not measure routine performance, but only whether or not a laboratory is capable of conducting drug susceptibility testing.
- The laboratory should enroll in a proficiency testing program (e.g. those provided by a WHO Supranational Reference Laboratory, the National Reference Laboratory, or other internationally recognized program providing panels for proficiency testing).
- Ideally, laboratories should have acceptable performance before reporting patient results.
- The same staff members who will routinely do the work should conduct the PT, and not supervisors or senior microbiologists from the laboratory.
- Though not a measure of routine performance, this indicator lends some integrity to the patient test results reported.
- A laboratory's performance on DST PT should be equal or greater than the following:
    - Isoniazid: 90% accuracy
    - Rifampicin: 90% accuracy
    - (optional) Streptomycin: 80% accuracy
    - (optional) Ethambutol: 80% accuracy

How to measure it

The laboratory should annually receive a panel of (20) cultures for drug susceptibility testing from a reference laboratory. The number of specimens is determined by National Guidelines. Performance can be measured by dividing the total number of points associated with correct responses by the total number of available points, or by comparing laboratory results with expected responses.

Data Sources

PT results certified by Reference Laboratory or external testing organization.

The reporting and recording registers

Specimen Registration, Culture, and DST Results Register Month_____ Year_____ (part 1-Left half)

S. No.	Lab No.	Name (in full) & Address	Sex (M/F) & Age	Name of referring site (DMC/DOTS-plus site) & District	Reason for Testing (mark one)				Specimen	Date Specimen Collected from Patient	Date Specimen Received in culture lab	Specimen Condition (CPC or [MP, BLD, SAL, Contam]) †	Culture lab concentrated smear result ‡
					Diagnosis		Follow-up						
					TB No.	TB Type*	DOTS-Plus No.	Month of F/U					
									A				
									B				
									A				
									B				
									A				
									B				
									A				
									B				
									A				
									B				
									A				
									B				
									A				
									B				
									A				
									B				

***** Using standard RNTCP definitions for TB type: **NSP, NSN, NEP, Relapse, TAD, Failure,** or **Other**

† CPC=specimen contains CPC. If CPC present then no further description needed. For all other specimens with no CPC, describe condition: **MP**=mucopurulent specimen, **BLD**=gross blood in specimen, **SAL=**Salivary specimen, **Contam** if gross bacterial overgrowth is suggested by visual examination.

‡ Smear results for specimen deposit after concentration in culture laboratory, using standards definitions: **3+, 2+, 1+ , Sc, Neg.**

Specimen Registration, Culture, and DST Results Register Month_____ Year_____ (part 2-right half)

Culture Results				Standard DST Results							Date Sending Report to DOTS-Plus Site & DTO		
Date Inoculated	Type (Solid / Liquid)	Culture Results §	Date Result Reported	Date Inoculated	Type (Solid / Liquid)	RIF (R/S)	INH (R/S)	SM (R/S)	EMB (R/S)	Date Result Reported	Culture	DST	Remarks

‡ **R**=Resistant, **S**=Sensitive, **NA**=no result.

§ **Negative**=no growth, **Contam**=contaminated, **NTM**=Non-Tuberculosis Mycobacteria/rapid grower, **3+**=confluent growth, **2+**= >100 colonies, **1+**=10-100 colonies; **Sc(number)**=Scanty<10 colonies (indicate number of colonies). Positive culture results should only be reported after identity for *M. tuberculosis* is confirmed with PNB, Niacin, Catalase, Rapid Immunoassay, or other methods.

Primary Culture Register

Lab Number	Specimen					Patient		AFB smear		Quantified growth readings*									Final result**
	Date received	Date sputum collected	Date processed	Type of specimen	DMC Name	TB number (RNTCP 1st line drugs)	Diagnosis / follow-up & month	DMC result	IRL results	Media inoculated *	Wk 1	Wk 2	Wk 3	Wk 4	Wk 5	Wk 6	Wk 7	Wk 8	
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									
										LJ1									
										LJ2									

* specify the media, if not LJ.

** Indicate positivity in terms of growth. No growth= Negative; Fewer than 10 colonies=Report exact number of colonies; 10-100 colonies= 1+ Positive; More than 100 colonies 2+ Positive; confluent growth= 3+ Positive. Contaminated=C; Non-Tuberculosis *Mycobacteria*/fast grower= NTM.

IDENTITY & DRUG SUSCEPTIBILITY RESISTER

Date of DST	Lab number	Primary culture Positive date	Reading time*	**Neat** (Drug free-media)	**10^{-2} dilution**						**10^{-4} dilution**						Identity: PNB /& Niacin/ others (neat inoculums)	Remarks/ colony morphology
					Control (Drug free-media)	Control (Drug free-media)	Strep	INH	RIF	EMB	Control (Drug free-control)	Control (Drug free-control)	Strep	INH	RIF	ETB		
			4 weeks															
			6 weeks															
			4 weeks															
			6 weeks															
			4 weeks															
			6 weeks															
			4 weeks															
			6 weeks															
			4 weeks															
			6 weeks															
			4 weeks															
			6 weeks															

* Results are read for the first time on 28th day and second time on 42nd day. No growth= Negative; Fewer than 10 colonies=Report exact number of colonies; 10-100 colonies= 1+ Positive; More than 100 colonies 2+ Positive; confluent growth= 3+ Positive. Contaminated=C; Non-Tuberculosis *Mycobacteria*/fast grower= NTM. The colonies are counted only on the slopes seeded with the inoculum that has produced exact readable counts or actual counts (up to 100 colonies on the slope). Any strain with 1% (the critical proportion) of bacilli resistant to any of the four drugs – Rifampicin, Isoniazid, Ethambultol, and Streptomycin – is classified as resistant to that drug. Refer the manual/SOPs for details.

MEDIA REGISTER (SOLID EGG based MEDIA)

Date	Batch No	Total bottles	Inspissator		Sterility Test					Type of media prepared								Drug stock solution date	Remarks/ prepared by
						48 Hours*		14 days**											
			Started	Closed	Setup date	Read on	Result	Read on	Result	Plain LJ	PNB	SM	INH	RIF	EMB	Nut. Agar	Nut. Broth		

* Bacterial sterility check for two days (37c); ** Fungal sterility check for up to 14 days (25c)

AUTOCLAVE REGISTER*

Sl.No	Date	Article(s) for sterilization	Pressure and duration for autoclaving	Starting time	Time pressure reached	Time Switched off	Whether dried & duration	Time of opening	Remarks/ Person sterilizing

* Follow the SOPs or manufacturers operation instructions.

EGG STOCK REGISTER**

Date	Opening Balance	No. of eggs received	Total	Eggs broken/ cracked	Eggs Used	Balance	No. of batches	Media Batch No.	Remarks

**Eggs should be fresh; should be stored at 4c, and should be used within 7 days.

DRUG STOCK REGISTER*

Sl. No.	Name of drug	Date of Preparation	Amount weighed (mgs)	Solvent name & Volume (ml)	Date of expiry*	Prepared by	Assisted by	Date discarded	Remarks

* Check the SOPs for solvents, never store the working stock.

Monthly Laboratory abstract of *M. tuberculosis* Primary *C*ulture & DST activities

Month	Sputum smear status	Patient Type	Culture results								Total Primary culture	DST processed*				
			Positives		Negative		Contaminated		NTM/ fast growers							
			Nos.	%	Nos.	%	Nos.	%	Nos.	%		Total DSTs	All suscept ible	MDR	Only H resistan ce	Only Rif resist ant
Jan	Smear +ve	Diagnostic														
		Follow-up														
	Smear -ve	Diagnostic														
		Follow-up														
		Total														
Feb	Smear +ve	Diagnostic														
		Follow-up														
	Smear - ve	Diagnostic														
		Follow-up														
		Total														
Mar	Smear +ve	Diagnostic														
		Follow-up														
	Smear - ve	Diagnostic														
		Follow-up														
		Total														

* DST is not advised for treatment follow-up cultures.