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How I do it?

Rapid Identification and Drug Susceptibility Testing of *Mycobacterium tuberculosis:* Standard Operating Procedure for Non-Commercial Assays: Part 2: Nitrate Reductase Assay v1.3.12

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ABSTRACT

In the previous part, we presented the standard operating procedure (SOP) of the microscopic observation drug susceptibility assay drug susceptibility testing (DST) for *Mycobacterium tuberculosis*. The present SOP is devoted to another non-commercial culture and DST method known as nitrate reductase assay (NRA). As the name implies, the NRA detects the ability of *M. tuberculosis* to reduce nitrate to nitrite. In the assay, the presence of nitrite is detected by the addition of *p*-nitrobenzoate into the growth yield. The reaction is detected by the naked eye. The incorporation of drugs in the medium allows to use the test for DST, which can be interpreted with naked eyes. The identification and drug susceptibility results can be obtained in 2-3 weeks. This SOP document has been developed through the culture and DST subgroup of the STOP tuberculosis (TB) Partnership New Diagnostic Working Group. It is intended for laboratories that would want to use or already using this rapid non-commercial method for culture identification and DST of *M. tuberculosis*, notably in resource-constraint settings in Asia and Africa.

Key words: Mycobacterium, tuberculosis, drugs, susceptibility, testing, training

INTRODUCTION

Scope

This standard operating procedure (SOP) document has been specially compiled for the implementation of non-commercial culture and drug susceptibility testing (DST) methods endorsed by New Diagnostic Working Group (NDWG)-STOP tuberculosis (TB) Partnership (WHO) for laboratory network performing the rapid non-commercial rapid culture identification and DST for *Mycobacterium tuberculosis* and intended for the use of TB Diagnostic Laboratories, located in various Asian countries. It is also intended as a companion to the TB laboratory training manuals.

This SOP describes the procedure for the nitrate reductase assay (NRA) that detects the ability of *M. tuberculosis* to reduce nitrate to nitrite, which is also used as the biochemical test for mycobacterial species. The presence of nitrite can be easily detected using specific reagents. The incorporation of drugs in the medium allows it to be used as DST. The rapid detection of *M. tuberculosis* and information about DST allows proper management of TB.

As suspensions with viable, infectious bacteria are handled, strict compliance with safety and protection measures are mandatory. The procedure must be

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carried out in a laboratory meeting the WHO standards for biosafety level 2 with access restricted to authorized personnel only.

Purpose

The purpose of this SOP is to rapidly provide clinicians with information about the patients suspected to have multidrug resistant *M. tuberculosis* for proper clinical management.

Personnel qualifications

The test performer should be having a diploma in laboratory technologies and preferably university graduate in biological sciences with sufficient experience.

Medical fitness

In accordance with the national laws and practices, arrangements should be made for appropriate health surveillance of TB laboratory workers:

- Before enrolment in the TB laboratory
- At regular intervals thereafter, annually or bi-annually
- After any biohazard incident
- In case of onset of TB symptoms

All cases of disease or death identified in accordance with national laws and/or practice as resulting from occupational exposure to biological agents shall be notified to the competent authority.

Education and training

Personnel are required to be knowledgeable about the procedures in this SOP. Documentation of training and familiarization with this SOP can be found in the training file for each employee.

The laboratory staff shall confirm (i.e., documentation in the training file of familiarization with the SOP) that they can properly perform the procedure before commencing work. Education and training must be given on the following topics:

- Potential risks to health (symptoms of TB disease and transmission)
- Precautions to be taken to minimize aerosol formation and prevent exposure
- Hygiene requirements
- Wearing and use of protective equipment and clothing;
- Handling of potentially infectious materials
- Laboratory design, including airflow conditions
- Use of biological safety cabinets (BSC) (operation, identification of malfunctions, maintenance)
- Use of autoclaves, incubators (operation, identification of malfunctions, maintenance)
- Prevention of incidents and steps to be taken by workers in the case of incidents (biohazard incidents, chemical, electrical, and fire hazards)
- Good laboratory practice and good microbiological techniques
- Organization of workflow
- Procedures
- Waste management
- Importance of laboratory results for patient management
- Importance of laboratory results for the national TB program
- Training shall be given before a staff member takes up his/her post
- Repeat training periodically, preferably every year

Bio-safety precautions in tuberculosis laboratory

TB laboratory has all the major facility requirements for handling *M. tuberculosis* safely, and involves minimum risk to the laboratory personnel if they take proper precautions, and employ proper techniques described in these SOPs. Laboratory safety involves all the procedures and methods one needs to follow to minimize the risks of laboratory acquired infections. Use of laboratory is limited to trained TB lab personnel.

Biological safety cabinets

- Switch ON the safety cabinets for at least 30 min before use. Note that the reading on the mini gauge pressure is satisfactory
- Wear double pair of gloves, every time you work inside the cabinet
- Biosafety cabinets need to be cleaned with 5% phenolic or 1% hypochlorite solution before work
- Keep disposal bin/vessel with 5% phenolic or 5% hypochlorite disinfect inside the cabinet at the right side corner
- Wipes of gauge-cloth soaked in 5% phenolic or 5% hypochlorite, should be readily available inside the cabinet
- Arrange all un-infected material required towards the left side
- All the processed samples need to be arranged right side
- Do not process more than six specimens at a time, inside the cabinet
- After completion of work, wipe off the surface

with 5% phenolic solution, and discard all wipes in biohazard bags, or in disposal container meant for infectious materials
- Discard off the outer glove, too, inside the bio-safety cabinet
- Wipe off the inner glove with disinfectant before touching anything else in the lab

Waste disposal and handling

All infectious waste should be discarded in the bio-safety disposal bin. All infectious solid waste-wipes, swabs, plastic, paper towels, gauze pads, gloves, etc., should be placed inside the double autoclave bags, sealed with autoclave tape and sterilized at 121°C for 30 min in the autoclave.

Liquid waste, in the steel discarding bins, should be disinfected in 5% phenol for at least 1 h, before sealing the caps and autoclaved at 121°C for 30 min.

Accidents and spillages

i. Spills inside biological safety cabinet

All workers using the bio-safety cabinets should keep absorbent materials (gauge cloth/adsorbent sheet) and 5% phenol within the cabinet.
- Alert all people in lab of immediate area of in the event of spill
- Spread 5% phenol soaked wipe immediately, while the BSC continues to operate. Wait for 15-20 min.
- Use paper towels to wipe the spill, working from the edges into the center
- Decontaminate equipment: Items that are not readily or easily surface decontaminated should be carefully placed into autoclave bags and removed for further treatment (e.g., decontamination by autoclaving)

Contaminated gloves and clothes (sleeves are most likely to be contaminated); remove and decontaminate the lab coat by autoclaving or soaking in decontaminant.

ii. Spills outside containment room in the BSC

Spills on equipment (such as vortex, centrifuge, incubator, refrigerator etc.), laboratory benches, walls, or floors:
- Immediately indicate to all the personnel working in the lab, and evacuate for 1 h to allow dissipation of aerosols created by the spill (negative air pressure system would clear the aerosols)
- Leave the BSC operating and cultures inside the cabinet
- Leave the containment facility following exit procedures
- Close laboratory doors and post warning signs to prevent others from entering the laboratory
- Thoroughly wash hands and other apparently contaminated areas with soap and water. Put on clean disposable gloves
- If personal clothing is contaminated, remove all outer clothing and place it in the autoclave or container for autoclaving. Put on clean garments
- Upon returning to the laboratory wear the N95 mask, fresh lab coat and double pair gloves to start decontamination, cover the spill area with paper towels soaked in 5% phenol solution or 1:10 dilution of 20% bleach (freshly prepared), or 70% ethanol solution (do not pour decontamination solution directly onto the spill in order to avoid additional release of aerosols)
- Let it stand for 20 min then wipe up with paper towels
- Wipe up the spill with the soaked paper towels and place the used towels in an autoclave bag and autoclave
- Place gloves and paper towels in autoclave bag and autoclave
- Spill inside the centrifuge bucket/tube: Always use the aerosol containment cups for centrifuging. Always open the centrifuge buckets inside the bio-safety cabinet. Autoclave the buckets
- Wash hands and other apparently contaminated areas again with soap and water

iii. Dont's
- Eating, drinking, smoking, applying cosmetics, use of mobile phones, or applying contact lenses in the TB laboratory
- Do not allow unauthorized personnel to enter the TB laboratory
- Mouth pipetting
- Crowding of lab with material that is not required inside

PROCEDURE

Principle

NRA on solid medium is based on the ability of *M. tuberculosis* to reduce nitrate to nitrite, the presence of which can be easily detected with specific reagents that produce a color change. It can be applied on solid medium either indirectly using strains or directly using samples resulting in a dramatic reduction of

time needed to obtain the results. NRA uses the detection of nitrite as an indication of growth when it is used as a DST.

Inability of *M. tuberculosis* to grow in the presence of *p*-nitrobenzoate (PNB) is one of the key elements to differentiate tubercle bacilli from other mycobacterial species and is part of the identification process for *M. tuberculosis*. *M. tuberculosis* and other tubercle bacilli will not grow on culture medium containing PNB.

Samples

- Clinical sample for direct NRA
- Pure cultures of *M. tuberculosis* grown on solid/liquid medium for indirect NRA

Equipment and materials

- BSC, class II, annually certified
- Incubator set at 37°C
- Autoclave
- Balance
- Refrigerator/freezer
- Vortex
- Automatic micropipettes (1000 µl, 200 µl, and 20 µl)

Reagents and solutions preparation

- PNB solution (Sigma–Aldrich, ref. 227056): Prepare stock of (200 mg/ml). Weigh 2 g PNB (Sigma, ref. 72910) and dissolve in 10 ml dimethylformamide. Final concentration to be used is 500 µg/ml
- Potassium nitrate (Sigma, ref. P-8394): Prepare stock of 200 mg/ml. Weigh 2 g potassium nitrate and dissolve in 10 ml sterile DW. Final concentration to be used is 1000 µg/ml. (Note: Sodium nitrate can be used in place of potassium nitrate, depending on availability)
- Hydrochloric acid (HCl) (Merck, ref. HB9H590082): Prepare 50% (v/v) concentrated HCl. Add 10 ml concentrated HCl to 10 ml sterile distilled water (DW)
- Sulphanilamide (Merck, ref. 1.11799.0100): Prepare 0.2% (w/v) sulphanilamide. Weigh 0.1 g of sulphanilamide and dissolve in 50 ml sterile DW
- *n*-1-Naphthylethylenediamine dihydrochloride (Merck, ref. 1.06237.0005): Prepare 0.1% (w/v) *n*-1-naphthylethylenediamine dihydrochloride. Weigh 0.05 g of *n*-1-naphthylethylenediamine dihydrochloride and dissolve in 50 ml sterile DW
- Mineral salt solution for Löwenstein–Jensen (L-J): For 600 ml, potassium dihydrogen phosphate anhydrous (KH_2PO_4) (Merck, ref. 1048729025) - 2.4 g, magnesium sulphate ($MgSO_4 \cdot 7H_2O$) (Qualikems, ref. M009112) - 0.24 g, magnesium citrate - 0.6 g, asparagine (SRL, ref. 014035) - 3.6 g, glycerol (Merck, ref. 356350) - 12 ml, and DW – 600 ml
- Dissolve the ingredients in order in DW by heating. Autoclave at 121°C, 30 min to sterilize. Cool to room temperature. This solution can be kept indefinitely and may be stored in suitable amounts in the refrigerator
- Malachite green (oxalate) (Merck, ref. MB6M560027): Prepare 2% malachite green solution. Weigh 2.0 g of malachite green dye and using aseptic techniques dissolve the dye in 100 ml sterile DW by placing the solution in the incubator for 1-2 h. Filter before use

Drug solutions and dilutions

Drugs: Rifampicin (RIF) (Sigma, ref. R7382) and isoniazid (INH) (Sigma, ref. I3377).

For drug stock preparation refer to Table 1. For preparation of drug dilutions to be used in medium refer to Table 2.

Löwenstein–Jensen (L-J) Medium

- Fresh eggs
- Mineral salt solution

Table 1: Preparation of drug stock

Drugs	Stock concentration (µg/ml)	Stock prepared in	Critical concentration (µg/ml)
INH	1000	Sterile DW	0.2
RIF	4000	DMSO+sterile DW (1:1)	40

Note: Prepare drugs in required volume, filter using syringe filter, and store at–20°C. Calculate the weight of drug necessary according to the potency using the following formula: Concentration required (in µg/ml)/Potency (in mg/g)× total volume (in ml)= milligrams to weigh. INH: Isoniazid, RIF: Rifampicin, DW: Distilled water, DMSO: di-methyl sulfoxide

Table 2: Preparation of drug dilutions to be used in medium

Drugs	Working solution			For 500 ml L-J	
Concentration of stock (µg/ml)	Volume of stock (ml)	Volume of sterile DW (ml)	Final conc. of working sol. (µg/ml)	Volume of working sol. (ml)	Final conc. in medium (µg/ml)
INH (1000)	0.5	4.5	100	1	0.2
RIF (4000)		Use stock solution		5	40

L-J: Löwenstein-Jensen, INH: Isoniazid, RIF: Rifampicin, conc.: Concentration, sol.: Solution

- Malachite green solution, 2%
- Stock of potassium nitrate
- Stock of PNB
- Drug solutions: INH and RIF
- Other material: 2 l Erlenmeyer with stopper; glass funnel; 1 l measuring cylinder; 25 ml measuring cylinder; sterile cotton gauze; sterile blunt end forceps; blender; inspissator

Preparation of the media

i. Homogenized whole eggs

Fresh hens' eggs, not more than 7 days old, are cleaned by scrubbing thoroughly with a hand brush in warm water and plain alkaline soap. Let the eggs soak for 30 min in the soap solution. Rinse eggs thoroughly in running water and soak them in 70% ethanol for 15 min. Before handling the clean dry eggs scrub the hands and wash them. Crack the eggs with a sterile forceps into a sterile flask and beat them with a sterile egg whisk or with a sterile blender.

ii. Complete medium

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

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

Divide medium in three different portions: Nitrate control, PNB control, and drug-containing medium.

Nitrate control: Potassium nitrate stock 0.2 g/ml - add 5 ml of stock to 1000 ml of medium.

PNB control: PNB stock 0.2 g/ml - add 2.5 ml of stock to 1,000 ml of medium.

iii. Drug-containing medium

Divide this portion again in two parts and add drugs to medium as per given in Table 2 as well as add potassium nitrate.

The complete egg medium is distributed in 6-8 ml volumes in sterile 28 ml McCartney bottles or in 20 ml volumes in 20 × 150 mm screw-capped test tubes and the tops are securely fastened. Inspissate the medium within 15 min of distribution to prevent sedimentation of the heavier ingredients.

iv. Coagulation of medium

Before loading, heat the inspissator to 80°C to quicken the build-up of the temperature. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 min at 80-85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilize it). Heating for a second or third time has a detrimental effect on the quality of the medium. The quality of egg media deteriorates when coagulation is done at too high a temperature or for too long. Discoloration of the coagulated medium may be due to excessive temperature. The appearance of little holes or bubbles on the surface of the medium also indicates faulty coagulation procedures. Poor quality media should be discarded.

Sterility check

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

Storage

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

Procedure for direct nitrate reductase assay: Using clinical sample

Samples not requiring decontamination

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

- Spinal or other internal body fluids but should be collected aseptically
- Bone marrow aspirate if collected aseptically
- Pus from closed cold abscesses such as fine needle aspiration cytology (FNAC) material
- Surgically resected specimens (excluding autopsy material)
- Material obtained from pleural, liver, and lymph nodes as well as biopsies (if not fistulised).

For samples requiring decontamination process the specimen Process the specimen with sodium hydroxide–N-acetyl-l-cysteine (NaOH–NALC) modified Petroff's method.

Reagents required

- 4% NaOH

- 2.9% sodium citrate dehydrate or 2.6% sodium citrate anhydrous
- NALC
- Anhydrous disodium hydrogen phosphate (Na_2HPO_4)
- Mono-potassium dihydrogen phosphate (KH_2PO_4)
- DW

Procedure for decontamination of sputum using sodium hydroxide–N-acetyl-l-cystein: Modified Petroff's method

i. Preparation of NaOH–NALC

- The mucolytic agent, NALC is used for rapid digestion of sputum and this enables the decontaminating agents like NaOH, to be used at a lower concentration (in sputum) of 1%
- The NaOH and sodium citrate may be mixed as given in Table 3, sterilized and stored in sterile screw cap bottle for use. After NALC has been added, the prepared volume of digestant must be used within 24 h as NALC loses mucolytic activity on standing for long. (Note: Acetyl-cysteine loses activity rapidly in solution, so should be made fresh daily. Sodium citrate is included in the digestant mixture to bind the heavy metal ions that might be present in the specimen and could inactivate the specimen and could inactivate the acetyl-cysteine.)

For preparation of NaOH–NALC solution refer to Table 3.

ii. Preparation of 0.067 M phosphate buffer (pH 6.8)

For stock solutions:

a. Disodium phosphate: Dissolve 9.47 g of anhydrous Na_2HPO_4 in 1 l of DW

b. Mono-potassium phosphate: Dissolve 9.07 g of KH_2PO_4 in 1 l of DW

- To prepare pH 6.8 buffer solution, mix 50 ml of (a) with 50 ml of (b) prepared above
- Check pH
- If final buffer requires pH adjustments, add solution (a) to raise the pH or solution (b) to lower it

Table 3: Preparation of sodium hydroxide-N-acetyl-l-cysteine solution

Volume of digestant needed	Mix indicated amounts (ml) of		Add NALC (g)
	4% NaOH	2.9% Na citrate $2H_2O$	
50	25	25	0.25
100	50	50	0.50
200	100	100	1.00
500	250	250	2.50
1000	500	500	5.00

Sample processing

- Transfer a maximum volume of 3-5 ml of specimen to a sterile 50 ml centrifuge tube (aerosol free and graduated)
- Add equal volume of NALC–NaOH–Na citrate solution aseptically
- Mix the control for approximately 20 s on vortex mixture. Be sure to invert the tube so that NALC–NaOH comes in contact with the entire surface of the tube
- Allow the mixture to stand at room temperature for 15 min to decontaminate the specimen with occasional gentle shaking of hand
- Dilute the mixture by adding sterile phosphate buffer (pH 6.8) upto the 50 ml mark on the tube and recap it
- Centrifuge the tube at 3000*g* for 15 min
- Decant the supernatant

Preparation of inoculum

- Re-suspend the pellet with 2 ml phosphate buffer (pH 6.8)
- Prepare 1:10 dilution of re-suspended pellet in 0.067 M phosphate buffer for inoculation of control tubes (add 0.2 ml of the re-suspended pellet in 0.8 ml phosphate buffer)

Inoculation of nitrate reductase assay tubes

- 0.2 ml of the 1:10 dilution is inoculated in three drugs free tubes (control tube containing potassium nitrate)
- 0.2 ml of the undiluted suspension is inoculated into the drug-containing tube
- 0.2 ml of the undiluted suspension is inoculated into the PNB containing tube
- Incubate at 37°C until 14 day

Reading of tubes

Reagent mixture: One part of 50% (v/v) concentrated HCl, two parts of 0.2% (w/v) sulfanilamide and two parts of 0.1% (w/v) *n*-1-naphthylethylenediamine dihydrochloride. These are mixed shortly before use.

After 14 days of incubation, 0.5 ml of a reagent mixture will be added to one drug free tube. If any color occurs, the corresponding antibiotic containing tube will be developed by the reagent mixture. If no color change occurs, the tube will be discarded and the other tubes will be re-incubated. The procedure will be repeated at day 21 using the second growth control tube, and if is

necessary, also at day 28 using the third growth control tube. Also observe tube with PNB.

Procedure for indirect nitrate reductase assay – Using pure cultures of acid-fast bacilli grown on solid/ liquid medium

Preparation of 7H9-S

Middle brook 7H9 broth supplemented with 10% oleic acid dextrose catalase and 0.2% glycerol.

Preparation of inoculum

Confirm the growth as *M. tuberculosis* (MTB) using either PNB or MPT-64 rapid test or other methods being practiced in the local laboratory.

i. Inoculum from growth on solid medium

It is very important to have fresh growth on a solid medium (21-28 days old). Older cultures may result in unreliable susceptibility test results.

- Take a loop full of bacterial growth with a sterile loop and put it in a sterile vial with glass beads just covering the bottom of the vial with 2.5 ml of 7H9-S broth (try not to take any medium when removing growth)
- Vortex the vial for at least 1 min to break the clumps until a fairly turbid suspension is obtained.
- Allow it to stand for 5 min
- Transfer the supernatant to a new sterile vial and leave it to sediment for 15 min
- Transfer the supernatant to a new sterile vial
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard
- Set the turbidity of inoculum as per McFarland 1.0 standard using 0.067 M phosphate buffer (pH 6.8)

ii. Inoculum from a liquid medium

It is very important to have fresh growth in liquid medium (21-28 days old). Older cultures may result in unreliable susceptibility test results.

- Transfer 2.5 ml of the culture pellet to a sterile vial containing glass beads
- Vortex for at least 1 min until a fairly turbid suspension is obtained
- Allow it to stand for 5 min
- Transfer the supernatant to a new sterile vial and leave it to sediment for 15 min
- Transfer the s upernatant to a new sterile vial
- Compare the turbidity of the suspension using the scale McFarland 1.0 standard
- Set the turbidity of inoculum as per McFarland 1.0 standard using 0.067 M phosphate buffer (pH 6.8)

Dilution of the inoculum

The inoculum turbidity (from liquid or solid culture) is adjusted to a McFarland tube no. 1 and further diluted to 1:10 in 0.067 M phosphate buffer (pH 6.8).

Inoculation of nitrate reductase assay tubes

- 0.2 ml of the 1:10 dilution is inoculated in three drugs free tubes (control tubes containing potassium nitrate)
- 0.2 ml of the undiluted suspension will be inoculated into the drug-containing tube. Incubate at 37°C until 7 days. (Note: Inoculation onto PNB tube is not required in case of Indirect NRA.)

Reading of tubes

Reagents mixture: One part of 50% (v/v) concentrated HCl, two parts of 0.2% (w/v) sulfanilamide, and two parts of 0.1% (w/v) *n*-1-naphthylethylenediamine dihydrochloride. They will be mixed shortly before use.

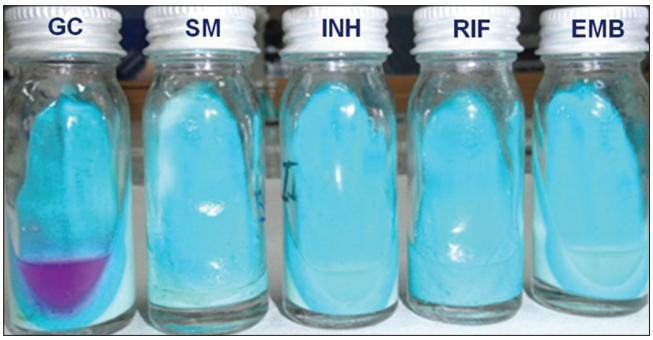

Figure 1: Demonstration of nitrate reductase assay results: Sensitive to all tested drugs. GC: Growth control tube without any drug, SM: Growth sensitive to streptomycin, INH: Growth sensitive to isoniazid, RIF: Growth sensitive to rifampicin, EMB: Growth sensitive to ethambutol

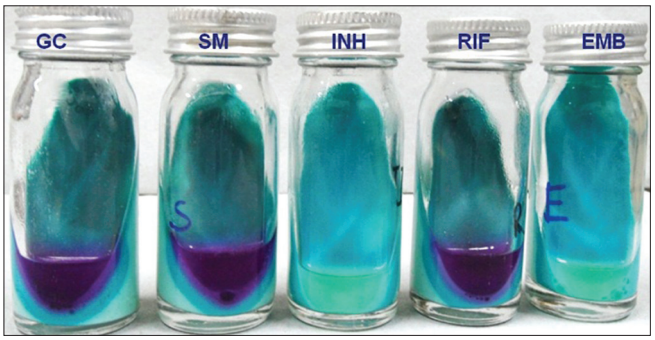

Figure 2: Demonstration of nitrate reductase assay results: resistant to two drugs. GC: growth control tube without any drug, SM: growth resistant to streptomycin, INH: growth sensitive to isoniazid, RIF: growth resistant to rifampicin, EMB: growth sensitive to ethambutol

After 7 days of incubation, 0.5 ml of a reagent mixture will be added to one drug free tube. If any color occurs, the corresponding antibiotic containing tube will be developed by the reagent mixture. If no color change occurs, the tube will be discarded and the other tubes will be re-incubated. The procedure will be repeated at day 10 using the second growth control tube, and if necessary, also at day 14 using the third growth control tube.

Quality control

It is important to perform a quality control (QC) of drug susceptibility testing for drugs testing. Add the standard isolate of *M. tuberculosis* ($H_{37}Rv$). Preferably a drug resistant strain should also be included into the QC. If the susceptible $H_{37}Rv$ shows some resistance, then all the results obtained during the experiment become invalid and the test should be repeated.

Result interpretation

For direct NRA, if growth appears on PNB containing medium growth is positive for mycobacterial strain other than tubercle bacilli. If there is no growth on PNB medium growth is of *M. tuberculosis* complex.

A strain will be considered resistant if a color change in the drug-containing tube is greater or the same as in 1:10-diluted growth control tube [Figures 1 and 2].

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