

SECTION II

METHODOLOGICAL PROCEDURES

Chapter 1 - MINIMAL REQUIREMENTS FOR IDENTIFICATION

The correct identification of microorganisms depends on the availability of pure cultures. A mycobacterial culture contaminated by other microorganisms will lead to false positive or negative results in the identification tests.

The minimal requirements for identification (230) are:

- A pure culture
- A culture with at least 20 colonies; in the case of cultures with fewer colonies it is advisable to make a subculture, so that the amount of bacteria is enough for the performance of all the tests.

The first step for identification is, thus, to make a smear of the culture, stained by the acid fast stain (either Ziehl-Neelsen or Kinyoun methods).

The objectives of the smear are:

- to confirm that the culture is composed of acid-fast bacilli (AFB)
- to confirm that the culture is not contaminated with other bacterial species
- to verify the presence of cord formation, which suggests that the culture could be of *M. tuberculosis*

The second step is the observation of the culture aspect.

The objectives of this observation are:

- to confirm the purity of the culture by verifying the absence of contamination
- to evaluate the colony aspect: pigmentation and morphology.

1.1. Bacterial suspension preparation

- small screw-capped bottle
- glass beads, 3 mm in diameter
- distilled water or phosphate buffer pH 7.4

Place 10 glass beads in a bottle containing 1 ml of distilled water or phosphate buffer. Sterilize by autoclaving.

Suspension preparation: use a sterile swab to transfer certain amount of bacterial growth from the primary culture into the suspension bottle (a 10 µl loop can also be used instead of a swab but the swab gives a more homo-

geneous suspension). Make a homogeneous and heavy suspension with the help of the swab and the glass beads. Let stand for 5 minutes to allow the larger residual clumps of bacteria and aerosol generated to settle.

The suspension can be made with phosphate buffer or distilled water. In the case of mycobacteria the use of buffer is not important due to the particularity of the mycobacteria wall. The use of distilled water is more advisable because it is simpler and there is less risk of contamination than using the buffer.

This bacterial suspension is used to inoculate test media and biochemical tests in liquid media. For media inoculation, see below. For biochemical tests performed in liquid media, the inoculum will vary according to the test, and will be explained in the corresponding test description.

1.2. Inoculation of the test media

Using a pipette or a bacteriological loop (sterile), inoculate 10 µl of the suspension in test media. The inoculum should not be spread over the surface of the medium but streaked down the middle to facilitate the examination of growth, as the border between growth and medium is more easily observed.

1.3. Preservation of strains

There are several ways of preserving mycobacterial strains:

- a simple method of storing cultures grown on egg media, is to keep them in a freezer at -20°C . The viability of bacteria is maintained for more than one year
- to save space in the freezer, it is recommended to store the bacteria suspended in a small tube with 10% glycerol, 1% glutamate or 10% glucose in a freezer at -20°C . The viability is maintained for at least one year.
- Cultures in a freezer at -20°C , using 20% skim milk as a medium. The viability is maintained for more than two years.
- We recommend the glass embroidery beads method.

1.3.1. Conservation of mycobacteria using glass embroidery beads at -70°C

- Wash the glass beads with water and detergent.
- Dry the glass beads at 45°C

- Place 6-10 glass beads in small plastic flasks or microcentrifuge vials; sterilize by autoclaving.
- Put 0,5 ml of Sauton media with 10% glycerol in the microcentrifuge vial; be careful to avoid contamination.
- Identify the microcentrifuge vial with labels on the side and on the cap of the vial.
- Place a loopful of bacterial growth (it is important that the bacteria is in an exponential growth phase) and stir the medium with the loop to dissolve the clumps.
- Let the microcentrifuge vial settle for 15 minutes.
- Pipette out and discard all medium
- Place the vials in boxes and make a map of each box, with the numbers of the strains for quick localization.
- Freeze at -70°C. (viability is maintained for up to 10 years)

1.3.2. A modification of the method for conservation of mycobacteria in skim milk at -70°C (adding 10% glycerol)

- Use fresh cultures in Löwenstein-Jensen (2-3 weeks)
- Prepare skim milk (Difco ref 232100) according to manufacturer instructions and add 10% glycerol
- Aliquot 3 ml in 13x100 tubes and autoclave for 10 min, 121°C
- Use an sterile swab to make a heavy bacterial suspension
- Homogenize and aliquot 1 ml in 1.8 ml sterile cryovials
- Keep at -70°C (viability is maintained for up to 10 years)

1.4. Staining procedures

1.4.1. Ziehl-Neelsen

Materials

Basic fuchsin

Ethanol 95%

Phenol crystals

Hydrochloric acid, concentrated

Methylene blue chloride

Distilled water

Preparation

- Fuchsin: dissolve 3 g of basic fuchsin in 100 ml of 95% ethanol.
- Phenol: dissolve 5 g of phenol crystals in 100 ml of water (place it in a water-bath until it dissolves completely)

- Carbol fuchsin: mix 10 ml of fuchsin solution with 90 ml of phenol solution. Filter the carbol fuchsin prior to use.
- Acid alcohol: carefully add 30 ml of concentrated hydrochloric acid to 970 ml of 95% ethanol; mix gently.
- Methylene blue – dissolve 3 g of methylene blue chloride in 1000 ml of distilled water.

Procedure

- Prepare the smear and allow it to dry at room temperature
- Heat fix the smear passing the slide through a Bunsen burner flame as for other bacteriological smears
- Place the slides on a staining rack
- Cover the entire surface of each slide with carbol fuchsin
- Using a Bunsen burner or cotton and alcohol flame, gently heat the slides until vapour rises.

DO NOT ALLOW THEM TO BOIL OR DRY.

- Allow the stain to remain on the slides for 5 minutes. Maintain heat throughout this period in a way that vapour rises 3 times during the 5 minutes.
- Gently wash the stain from the slide.
- Cover the slides with acid alcohol; leave it on the slides for 3 minutes.
- Rinse the slides again.
- Counter stain with methylene blue for 1 minute, and rinse again.

1.4.2. Kinyoun (100)

Material

Basic fuchsin
Ethyl alcohol (95%)
Distilled water
Acid alcohol
Methylene blue
Phenol

Preparation

- Kinyoun carbofuchsin:
 - Basic fuchsin 4 g
 - Ethyl alcohol 20.0 ml
(dissolve and add slowly while shaking)
 - Distilled water 100.0 ml
 - Liquefied phenol (melted crystals) 8 g

- Acid-alcohol
 - Ethyl alcohol (95%) 97.0 ml
 - Concentrated hydrochloric acid 3.0 ml
- Counter stain:
 - Methylene blue chloride 0.3 g
 - Distilled water 100.0 ml

Procedure

- Heat-fix smears on slide warmer at 65°C to 75°C for 2 hours or overnight. An alternative is to use a Bunsen burner passing the slide through the flame without overheating.
- Cover smear with a 2x3 cm piece of filter paper (to hold the stain on the slide and to filter out any undissolved crystals of dye)
- Flood the paper strip with Kinyoun carbol fuchsin
- Stain for 5 minutes (no heat is necessary)
- Remove the paper strip with forceps
- Rinse off stain from the slide with tap water
- Decolorize with acid-alcohol for 2 minutes
- Rinse with water and drain
- Repeat decolorization for 1-2 minutes only if the smear remains red and rinse with water
- Flood slide for 3-4 minutes with methylene blue
- Rinse off with water
- Air dry (do not blot) and observe under the microscope

1.4.3. Auramine-rhodamine (57)

Material

Auramine O

Rhodamine

Glycerol

Phenol

Distilled water

Hydrochloric acid

Ethyl alcohol (70%)

Potassium permanganate (KMnO₄)

Preparation

Auramine-rhodamine

Solution 1: Dissolve 1.5 g of auramine O and 0.75 g of rhodamine B in 75 ml

of glycerol

Solution 2: Mix 10 ml of phenol with 50 ml of distilled water

Working solution: Combine solutions 1 and 2. Use magnetic stirring device and mix for 24 hours. Filter stain through glass wool and place it in a dark bottle at 4°C up to 3 months.

Decolorizing agent- 0.5% acid-alcohol: Add 0.5 ml of concentrated hydrochloric acid to 100 ml of 70% ethanol. Label and store at room temperature up to 3 months.

Counterstain - Potassium permanganate: Dissolve 0.5 g of potassium permanganate in 100 ml of distilled water. Label and store at room temperature for up to 3 months.

Procedure

- Flood the slide with fluorochrome stain
- Allow the smear to stain for 15 minutes (be sure the stain remains covering the smear, do not heat, do not use filter paper)
- Rinse the slide with water and drain excess water from the slide
- Flood the slide with 0.5% acid alcohol
- Allow the smear to decolorize for 2 minutes
- Rinse the slide with water, drain excess of water
- Flood the slide with counterstain
- Allow the smear to air dry (do not blot)
- Examine the smear with fluorescence microscope as soon as possible using 40X objective (to confirm morphology an objective of 100X may be used)
- This smears can be stained again with Ziehl Neelsen or Kinyoun after immersion oil is removed with xylene to confirm positive smears and morphology

1.5. Culture media preparation

1.5.1. Sauton Medium with 10% Glycerol

This medium is used for conservation of mycobacteria at -20°C or -70°C .

Material

L-asparagine	4 g
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.5 g
Dipotassium phosphate ($\text{K}_2\text{H}_2\text{PO}_4$)	0.5 g
Citric acid	2 g
Ferric ammonium citrate	0.05 g
Glycerol	100 ml
Distilled water	1000 ml
Final pH 7,2	

Preparation

Dissolve the salts in boiling water. To adjust pH, add NaOH 4% very carefully. Filtrate through filter paper. Autoclave at 121°C for 15 minutes.

1.5.2. Löwenstein-Jensen Medium

Löwenstein-Jensen (LJ) is the most common medium used for mycobacterial culture. LJ medium containing glycerol favours *M. tuberculosis* growth, while LJ medium with pyruvate instead of glycerol enhances *M. bovis* growth.

Material

Mineral salt solution	
Potassium dihydrogen phosphate anhydrous (KH_2PO_4)	2.4 g
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.24 g
Magnesium citrate	0.6 g
Asparagine	3.6 g
Glycerol	12 ml
Distilled water	600 ml

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

Malachite green solution 2%	
Malachite green dye	2.0 g
Sterile distilled water	100 ml

Using aseptic techniques dissolve the dye in sterile distilled water by placing the solution in the incubator for 1-2 hours. This solution may precipitate or change to a less-deeply coloured solution. In either case discard and prepare a fresh solution.

Homogenised whole eggs

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

Preparation

Put the ingredients aseptically in a large sterile flask and mix well

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

The complete egg medium is distributed in 6-8 ml volumes in sterile 14 ml or 28 ml bottles or in 20 ml volumes in 20 x 50mm screw-capped test tubes, and the caps are tightly closed. Inspissate the medium within 15 minutes of distribution to prevent sedimentation of the heavier ingredients.

Coagulation of medium

Before loading, heat the inspissator to 80°C. Place the bottles in a slanted position in the inspissator and coagulate the medium for 45 minutes at 80°C to 85°C (since the medium has been prepared with sterile precautions this heating is to solidify the medium, not to sterilize).

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

Sterility checking and storage

After coagulation, the whole batch or a representative sample of culture tubes should be incubated at 35°C to 37°C for 24 hours for sterility control. The medium should be dated and stored in the refrigerator or at room temperature, with caps tightly closed. The medium can be kept up to three months if it does not show drying aspect.

1.5.3. Löwenstein-Jensen Medium with pyruvate

This medium is useful for *M.bovis* cultivation.

Follow the same preparation of LJ medium, substituting glycerol for 8.0 g sodium pyruvate in the mineral solution preparation.

1.5.4. Ogawa Medium

This medium is cheaper than LJ because it is made without asparagine.

Material

Mineral salt solution

Potassium dihydrogen phosphate anhydrous (KH ₂ PO ₄)	3.0 g
Sodium glutamate	3.0 g
Glycerol	18 ml
Distilled water	300 ml

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

Preparation

Mineral salt solution	300 ml
Homogenized eggs (12-16 eggs, depending on size)	600 ml
Malachite green solution	18 ml

The final pH of the medium is 6.8.

The medium is mixed well and distributed in 6-8 ml volumes in sterile 14 ml or 28 ml bottles or in 20 ml volumes in 20 x 50mm screw-capped test tubes, and the caps are tightly closed. For coagulation and sterility control see LJ medium preparation.

1.5.5. Acid-Buffered Ogawa Medium

Material

Mineral salt solution	
Potassium dihydrogen phosphate anhydrous (KH ₂ PO ₄)	9.0 g
Sodium glutamate	3.0 g
Glycerol	18 ml
Distilled water	300 ml

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

Preparation

Mineral salt solution	300 ml
Homogenized eggs (12-16 eggs, depending on size)	600 ml
Malachite green solution 2%	18 ml

The final pH of the medium is 6.2.

The medium is mixed well and distributed in 6-8 ml volumes in sterile 14 ml or 28 ml bottles or in 20 ml volumes in 20 x 50mm screw-capped test tubes, and the caps are tightly closed. For coagulation and sterility control see LJ medium preparation.

1.5.6. Middlebrook media

Media prepared from dehydrated material should be prepared in accordance with directions given by manufacturer.

Middlebrook 7H9 Broth

Middlebrook and Cohn 7H10 Agar Base

Middlebrook and Cohn 7H11.

Add the following antimicrobial agents per litre of 7H11 agar:

- Carbenicillin 50 µg
- Polymyxin B 200.000 U
- Amphotericin B10 µg
- Trimethoprim lactate 20 µg

Oleic acid – Albumin – Dextrose – Catalase (OADC)

- Dissolve 50 g bovine albumin fraction V in 900 ml freshly prepared saline (0.85% NaCl)
- Add 30 ml sodium oleate prepared as follow:

0.05N NaOH	30.0 ml
Oleic acid	0.6 ml
- Warm to 56°C and swirl gently to dissolve
- Adjust to pH 7.0 with 4% NaOH
- Heat in water bath at 56°C for 1 hour
- Add 40 ml of sterile 50% solution of dextrose prepared as follows:
To 30 ml of boiling distilled water, add 25 g of dextrose (glucose).
Stir to dissolve.
Add distilled water to complete 50 ml total volume
- Autoclave at 121°C for 10 minutes

This constitutes the sterile OAD solution

- Prepare sterile catalase as follows: add 0.02 ml of catalase (technical grade) to 20 ml of 0.85% saline (this contains 1000 µg/ml) and sterilize by membrane filtration through a 0.2 µm membrane.
- Add 2 ml of the sterile catalase to each 100 ml of OAD solution.
- Sterilize the complete OADC solution by filtration through a 0.2 µm membrane. To facilitate this, use a pre-filter with a 0.45 µm membrane while the OAD solution is still warm.
- Dispense 20 ml volumes in sterile screw cap tubes
- Incubate at 37°C for 24 hours before use to check for sterility
- Store at 4°C in air-tight containers. DO NOT FREEZE.

1.5.7. Dubos broth

It is a commercial ready-to-use base to which sterile albumin or serum is added, according to manufacturer's recommendations.

Chapter 2 - PHENOTYPIC IDENTIFICATION

The Runyon classification of non-tuberculous mycobacteria can be a guideline when choosing what are the appropriate identification tests to be performed. It is based on the rate of growth, production of pigment in the dark or only after exposure to light. On the basis of this the non-tuberculous mycobacteria are divided into four groups:

- *Group I: slow growers – photochromogen*: actively growing cultures develop yellow pigment on exposure to light but fail to produce pigment in the dark. Cultures require 2-6 weeks of incubation before visible growth appears (example: *M. kansasii*, *M. marinum*)
- *Group II: slow growers – scotochromogen*: pigment is produced in the light or dark. Cultures require 2-6 weeks of incubation before visible growth appears. (*M. scrofulaceum*, *M. goodii*, *M. szulgai*)
- *Group III: slow growers – nonchromogen*: this group contains both potential pathogenic and non-pathogenic species. Most are non-pigmented and extremely slow growers (*M. avium-intracellulare*, *M. xenopi*, *M. terrae*)
- *Group IV: rapid growers* – characterized by their ability to grow rapidly, in 2 to 7 days. They may be pigmented or non-pigmented. Colonies are generally smooth but rough variants may occur (*M. fortuitum* complex, *M. peregrinum*, *M. abscessus*, *M. chelonae*)

Tests for identification of mycobacteria are depicted in ANNEX 2. Slow and rapid growers need different biochemical tests for identification. Flowcharts illustrated in ANNEX 1 suggest different approaches that can be used for identification.

Clinical laboratories should be able to differentiate *M. tuberculosis* from NTM and the most common mycobacteria usually present in clinical specimens, i.e. MAC, *M. kansasii*, *M. goodii* and rapidly growing mycobacteria.

A minimum set of phenotypic tests is necessary to identify these mycobacteria:

1. pigment
2. growth at 25 °C
3. growth at 45 °C
4. niacin production
5. nitrate reduction
6. growth in the presence of PNB

7. picric acid
8. peptone agar
9. arylsulfatase 3 and 14 days
10. Tween hydrolysis at 7 and 14 days
11. urease

Reference laboratories should be able to identify most mycobacterial species. For this purpose the following additional tests are recommended:

12. NaCl 5%
13. β -galactosidase
14. tellurite reduction
15. citrate
16. mannitol
17. inositol
18. hydroxylamine
19. isoniazid
20. acid phosphatase
21. iron uptake
22. pyrazinamidase
23. streptomycin
24. oxigen preference

2.1. Procedures

Test procedures described here are based on descriptions from selected references (36, 48, 100, 105, 106, 122, 132, 158, 230, 258)

It is very important to carry out all tests with active cultures of two weeks for the rapid growers and of 3-4 weeks for the slow growers. **Cultures older than 5 weeks are not appropriate.** It is essential always to make positive and negative controls for all tests. Whenever a specific negative control is mentioned, pure substrate can be used as negative control for tests based on substrates.

Bacterial suspensions: In a centrifuge tube put 3 ml of distilled water. With a swab pick up a good amount of bacterial growth from the LJ media and mix with water, making a heavy suspension. Dispense one drop of this suspension on LJ tubes to check for pigment production, growth at 25°C, 37°C and 45°C, and to check for growth with drugs. Especially for identification of mycobacteria from skin lesions, incubate all tests at 25°C. Always inoculate one tube of 7H10 or LJ to serve as control of growth for all tests.

2.1.1. Pigment production

Procedure

Inoculate 3 LJ tubes with the bacterial suspension

Tube 1 - incubate at 37°C

Tube 2 - incubate at 37°C wrapped in black paper or aluminum foil

Tube 3 - incubate at 25°C wrapped in black paper or aluminum foil

As an alternative to obtain darkness, tubes 2 and 3 could be put inside a box.

Reading

Compare growth in tube 1 with growth in tubes 2 and 3:

a - If colonies in tube 1 and 2 are pigmented = scotochromogenic

b - If colonies in tube 1 are not pigmented, uncover tubes 2 and 3, and place them under a 60 W lamp or a bulb of white light at a distance of 20-25 cm, for a period of 2 to 3 hours. Observe pigment production after incubation at 37°C for 24, 48 and 72 hours.

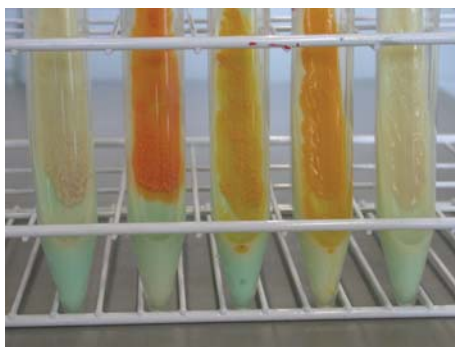
Interpretation

Growth of non-pigmented colonies in tubes 1, 2 and 3: non-chromogenic

Growth of pigmented colonies in tubes 1 and 2: scotochromogenic

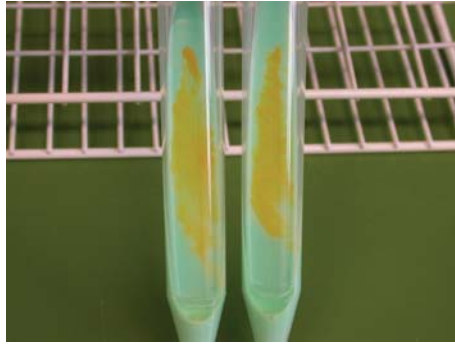
Growth of pigmented colonies in tube 2 after exposure to light: photochromogenic

Growth of pigmented colonies in tube 3 after exposure to light: photochromogenic at 25°C (*M. szulgai* is scotochromogenic at 37°C and photochromogenic at 25°C)



A. Martin

Figure 2 - The three central tubes show pigment production; the tubes at the left and right are non-pigmented. Some colonies are rough (first three tubes) and others are smooth (last two tubes)



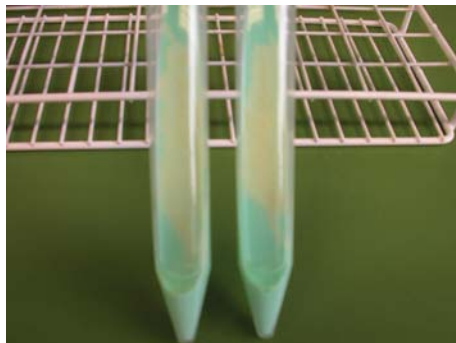
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Figure 3 - *M. gordonae*: scotochromogen. The culture on the left was incubated in the dark while the culture on the right was incubated under light. Both showed pigment.



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Figure 4 - *M. kansasii*: photochromogen. The culture on the left was incubated in the dark and did not produce pigment. The culture on the right produced pigment after exposure to light



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Figure 5 - *M. fortuitum*: non-chromogen. Pigment is not produced in cultures after being incubated in the dark or under light

2.1.2. Rate of growth

Procedure

Inoculate 1 LJ tube with a MacFarland 1 suspension and incubate at 37°C

Reading

Read in the 7th day - if it is negative incubate 7 more days. If there is no growth after 14 days, incubate for another week.

Interpretation

If growth appears in less than 7 days = rapid grower

If growth appears after 7 days = slow grower

Alternative

Use 1 peptone agar tube and 1 picric acid agar tube.

Prepare the peptone agar according to manufacturer's recommendation.

Dispense 4 ml in screw cap tubes (16x150). Autoclave for 15 minutes and coagulate with inclination.

Prepare one batch of Sauton or Middlebrook 7H10-OADC agar with picric acid at 0.2% dissolved in water. Dispense and coagulate: same as peptone agar.

Interpretation

- If growth appears in both media, peptone agar and picric acid = rapid grower

- If there is no growth in peptone agar and picric acid = slow grower

2.1.3. Growth in the presence of TCH

Procedure:

Prepare 5 µg/ml Thiophene carboxylic acid hydrazide (TCH) (weight 50 mg of TCH in 5 ml sterile distilled water). Transfer 1 ml of this solution to a tube with 9,0 ml of distilled water. Add 1 ml to 100 ml of LJ medium. Distribute in tubes and coagulate with inclination, at 80°C for 45 minutes.

Positive control: *M. tuberculosis*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth in both media (with and without TCH) = resistant to TCH

Growth only in the medium without TCH = sensitive to TCH

Alternative

Quadrant Petri dishes: prepare 2 quadrants with Middlebrook 7H10-OADC medium with TCH and two quadrants with medium without TCH. One quadrant with TCH and one without should be inoculated with the test mycobacterium. The same must be done with the positive control.

2.1.4. Growth in the presence of 5% NaCl

Procedure

Prepare one batch of LJ medium, add NaCl to final concentration of 5%, making sure of mixing well.

Positive control: *M. fortuitum*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Compare growth in both tubes (test and control).

If test tube shows at least 50% of the growth observed in the control tube:

Positive

If test tube shows less than 50% of the growth observed in the control tube:

Negative

2.1.5. Growth in the presence of PNB

Procedure

Prepare Middlebrook 7H10-OADC or LJ, adding PNB at 0.5 mg/mL before autoclaving or coagulating; dispense in Petri dishes or screw capped tubes. To prepare PNB, dissolve 1.25g in NaOH 1N with agitation and warming, complete to a volume of 10 mL of distilled water, aliquot and keep at -20°C for up to 3 months (stock solution concentration is 125 mg/mL, one mL is added to 250 mL of culture media).

Positive control: *M. fortuitum*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth in the presence of PNB: Non tuberculous mycobacterium

2.1.6. Growth in the presence of SM

Procedure

Prepare 2 µg/ml Streptomycin (SM) (weight 2 mg in 2 ml sterile distilled water). Transfer 1 ml of this solution to a tube with 9,0 ml of distilled water. Add 1 ml of this solution to 100 ml of LJ.

Positive control: *M. avium*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth = resistant to SM

No growth = sensitive to SM

2.1.7. Growth in the presence of INH

Procedure

Prepare 10 µg/ml INH (dilute 100 mg of isoniazid in 10 ml of sterile distilled water). Dilute 1 ml of this solution in 9 ml of sterile distilled water. Add 1 ml of this solution to 100 ml of LJ.

Positive control: *M. avium*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth = resistant to INH

No growth = sensitive to INH

2.1.8. Growth in the presence of HA

Procedure

Prepare 500 µg/ml Hydroxylamine (HA) (dilute 0.5 g of hydroxylamine in 10 ml of sterile distilled water). Add 1 ml of this solution to 100 ml of LJ.

Positive control: *M. fortuitum*

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth = resistant to HA

No growth = sensitive to HA

2.1.9. Semi-quantitative catalase

Procedure

Prepare LJ and dispense in 5 ml tubes of 20x150 (long tubes), coagulate in vertical position. Inoculate one drop of bacterial suspension.

Positive control: *M. fortuitum*

Reading

After two weeks of incubation add 1 ml of a fresh solution of Tween-peroxide and wait for 5 minutes.

Tween-peroxide solution:

1 – H₂O₂ 30% (commercial 110 vol)

2 – Tween 80 10% (10 ml Tween + 90 ml distilled water), autoclave

3 – mix equal parts of the reagent 1 and 2 immediately before use

Interpretation

After 5 minutes, measure with a ruler the height of the produced column of foam > 45 mm: positive for semiquantitative catalase.

< 45 mm: negative for semiquantitative catalase



E. Roxo

Figure 6 – Semi-quantitative catalase test. The column of foam is above 45 mm yielding a positive semi-quantitative catalase test.

2.1.10. Nitrate Reduction test

Material

Substrate: Sodium nitrate 22 mM, pH 7

NaNO₃ 0.85 g

KH₃PO₄ 1.17 g

Na₂HPO₄·7H₂O 3.36 g

Distilled water 1000 ml

The final pH is 7

Dispense in several bottles, autoclave 15 minutes at 121°C and keep at 4°C

Reagent A: slowly add 50 ml of HCl to 50 ml of water (Attention: never add water to acid).

Reagent B: sulphanilamide (0.2 g in 100 ml of water)

Reagent C: N-naphthylethylene-diamide (0.1 g in 100ml of water).

Keep reagents A, B, and C in dark flasks under refrigeration. Discard if color changes, or if precipitation is observed.

Procedure:

Dispense 2 ml of the substrate in screw cap tubes (13x100) and inoculate with several colonies of mycobacteria. Incubate at 35 - 37°C for 2 hours.

After 2 hours add:

1 drop of reagent A

2 drops of reagent B

2 drops of reagent C

Positive control: *M. fortuitum*

Reading

Observe immediate appearance of clear pink to purple color.

Color observation:

Faint pink +/-

Clear pink 1+

Deep pink 2+

Red 3+

Deep red 4+

Purple red 5+

Interpretation

3+ to 5+: positive

no color formation, 1+ to 2+: negative

NOTE: Confirm true negatives by adding a small amount of zinc powder to all the negatives; if there is NaNO₃ in the suspension, when adding the Zinc, it will immediately turn to pink and it will be a true negative, if there is no color formation after addition of Zinc this means that the reaction occurred and went further than nitrite reduction and it is a positive reaction.

2.1.11. Acid phosphatase

Material

Substrate: 0.5 M phenolphthalein diphosphate

Solution A - 0.2 M acetic acid

Store at 4°C for no longer than 1 year.

Solution B - 0.2 M sodium acetate

Autoclave, store at 4°C for no longer than 1 year.

Mix 14.5 ml solution A + 85.5 ml solution B

Heat for 30 min

Cool down at room temperature and add 100 mg phenolphthalein diphosphate for 100 ml solution.

Store at 4°C for no longer than 6 weeks.

Na₂CO₃ 10%:

Na₂CO₃

20 g

Distilled water

200 ml

Autoclave, store at 4°C for no longer than 1 year.

Procedure

Use a fresh culture on LJ. Rinse the tube with approximately 1.5 ml sterile water and transfer the obtained bacterial suspension to another tube. Fill one tube with sterile water as control. Add 0.5 ml phenolphthalein diphosphate. Incubate the tubes for 2h at 37°C. Add 0.5 ml Na₂CO₃ (10%). Check the colour.

Positive control: *M. fortuitum*

Reading

Observe immediate appearance of the red color.

Interpretation

Red: positive

Colourless, faint pink: negative



A. Martin

Figure 7 - Acid phosphatase test. The tube on the left shows a negative result; the other three tubes show positive results.

2.1.12. Urease

Material

Substrate: urea-indole solution

L-tryptophan	3 g
KH ₂ PO ₄	1 g
K ₂ HPO ₄	1 g
NaCl	5 g
alcohol 95°	10 ml
0.2% phenol-red solution	12.5 ml
distilled water	900 ml

Dissolve by heating gently without boiling. After cooling down, adjust pH to 7.0. Filter with paper and autoclave for 15 minutes at 121°C. After cooling down, add 100 ml of sterile 20% urea solution (20g urea in 100 ml distilled water, sterilize by filtration through a 0.22 µ filter). Dispense 1.5 ml in sterile 13X100 mm screw cap tubes. Keep in refrigerator in the dark.

Procedure

Inoculate substrate in tubes with one loopful of bacteria

Positive control: *M. fortuitum*

Reading

Observe color after 2 and 18 hours.

Interpretation

change of color from red to pink: positive

no change of color: negative



A. Martin

Figure 8 - Urease test. The tube on the left is the negative control, the center tube is a positive control and the tube at the right is a positive test.

Alternative:

Disks of Urea (BBL TAXO Cod 231737)

Procedure

Place 1 disk in a screw cap tube (13x100) plus 0.5 ml of sterile distilled water, add several colonies of an active culture. Incubate at 35 - 37°C

Reading

Make readings after one hour and daily for 3 serial days.

Interpretation

color of the medium changes to purple or dark pink: positive

no change of color: negative

2.1.13. Pyrazinamidase 6 days (PZAse)

Material

Medium: Dissolve 6.5 g of Dubos broth base in 1000 ml of distilled water; add 0.1 g of pyrazinamide, 2.0 g pyruvic acid sodium salt and 15 g of agar. Heat to dissolve the agar and dispense in 5 ml amounts of 16x125 mm screw cap tubes. Autoclave for 15 minutes at 121°C. Allow the medium to solidify in an upright position. Store in the refrigerator for several months.

Reagent: 1% ferrous ammonium sulphate

Prepare the solution just before use: 0.1 g of ferrous ammonium sulphate in 10 ml of distilled water.

Procedure

Inoculate the surface of the medium with a heavy bacterial growth (take it with a 10 µl bacterial loop) from a new culture (2 to 3 weeks old). The inoculum should be heavy; insufficient bacterial growth may cause a false negative result. Incubate at 37° C.

Control + : *M. tuberculosis* H₃₇Ra

Control - : *M. avium* complex, *M. bovis* BCG

Reading

After 6 days, add 1 ml of 1% ferrous ammonium sulphate solution to each tube; refrigerate for 3 hours.

Interpretation

formation of a pink band in the subsurface of the agar that diffuses into the medium: positive, sensitive to PZA

no pink band in agar: negative, resistant to PZA

2.1.14. *Arylsulfatase 3 days (quick and slow growers) and 14 days (slow growers)*

Material

Reagent: 0.08 M phenolphthalein disulfate

Dissolve 2.6 g of phenolphthalein disulfate, tripotassium salt, in 50 ml of distilled water. Sterilize by membrane filtration, 0.22 μm pore size and store in the refrigerator.

Substrate: liquid medium Dubos broth (or Middlebrook 7H9)

Prepare two bottles of media with

180 ml of Dubos broth for the 3 days test

180 ml of Dubos broth for the 14 days test

Sterilize by autoclaving for 15 minutes at 121°C.

After cooling at room temperature, aseptically add 20 ml of commercial Bacto Dubos Medium Albumin (or ADC) to each bottle.

For the 3-day test medium, aseptically add 2.5 ml of 0.08 M phenolphthalein disulfate.

For the 14-day test medium, aseptically add 7.5 ml of 0.08 M phenolphthalein disulfate.

Dispense 2 ml of each substrate, aseptically, into sterile 12x120 screw cap tubes, and store in the refrigerator. Mark the tubes with color codes or numbers 3 and 14, to identify the 3- and 14-day substrates.

Developer: 2 N sodium carbonate

Dissolve 10.6 g of anhydrous Na_2CO_3 in 100 ml of distilled water. Sterilize by 0.22 μm pore membrane filtration. Keep at room temperature.

Procedure

For each strain, inoculate 2 drops of bacterial suspension in one tube of 3-day and one of 14-day substrate. Incubate at 37°C.

Positive control: *M. fortuitum*

Reading

After 3 days of incubation add 4 drops of 2N sodium carbonate to the 3-day substrate tube.

After 14 days of incubation add 4 drops of 2N sodium carbonate to the 14-day substrate tube.

Interpretation

red or pink color: positive. Positive result may be compared with a set of color standards and the intensity of the color recorded in number of +.

no color change: negative

2.1.15. β -Galactosidase

Material

Medium: Modified Dubos Broth Medium

KH ₂ PO ₄	1.0 g
Na ₂ SO ₄	2.48 g
MgSO ₄ ·7H ₂ O	0.6 g
C ₆ H ₅ Na ₃ O ₇ ·2H ₂ O	1.5 g
C ₄ H ₈ N ₂ O ₃ ·H ₂ O	2.0 g
10% Tween 80	5.0 ml

Dissolve in 100 ml of distilled water and complete to 1000 ml of distilled water.

Autoclave.

Substrate

Dissolve 0.1 g of 2-nitrophenyl- β -d-galactopyranoside in a small volume of Modified Dubos Medium, filter and complete to 100ml. Add 7.5 ml of OADC enrichment. Distribute 2 ml in screw cap tubes (16x160). Keep in the refrigerator.

Procedure

Inoculate 1 tube of substrate with a heavy inoculum (from solid or liquid culture). Incubate at 35-37°C for 7 days.

Positive control: *M. chelonae*

Reading

At day 7 just check the color

Interpretation

bright yellow color: positive

no color: negative

2.1.16. Niacin production

Materials

Niacin strips (BBL-Difco)

Procedure

Use a 4-5 week old culture having at least 50 to 100 colonies on solid egg medium.

Add 1.5 ml of sterile distilled water to the culture. Using a pipette or a loop, gently scrape off surface growth and stab the medium to permit extraction of the niacin. Put the tubes in a horizontal position so that the liquid covers the surface of the medium. Allow to remain in this position for 30 minutes. Carefully remove approximately 0.6 ml of the liquid with a pipette and transfer to a sterile 13x100 mm screw cap tube.

Place one niacin strip with the arrow downward into each tube (positive control, negative control and tests tubes), and immediately seal the tubes. Leave at room temperature and occasionally agitate the tube.

Positive control: *M. tuberculosis* H₃₇Ra

Reading

After 15 minutes observe the color of the liquid in the bottom of the tube against a white background.

Interpretation

yellow color: positive. Color only on the strip is not considered positive, this may be due to oxidation of chemicals, especially at the top of the strip.

no color change: negative.



A. Martin

Figure 9 - Niacin test. The tubes on the left show a negative and positive result using the niacin chemical test. The tubes on the right show negative and positive results using the niacin test strips.

2.1.17. Iron uptake

Procedure

Add 5 g of ferric ammonium citrate on 200 ml of LJ medium. Distribute in screw cap tubes 16x150 mm; coagulate at 80°C for 45 minutes.

Inoculate with one drop of the bacterial suspension. Incubate at 37°C for two weeks or until the culture grows.

Positive control: *M. fortuitum*

Negative control: *M. chelonae*

Reading

Observe after 14 days.

Interpretation

Colonies of brown color or brown orange: Positive for iron uptake

Colonies of the same color as colonies in LJ: Negative for iron uptake

2.1.18. Tween 80 hydrolysis

Procedure

phosphate buffer 67 mM pH 7.0

Solution A: Dissolve 9.47 g of anhydrous Na_2HPO_4 in 1000 ml distilled water.

Solution B: Dissolve 9.07 g of KH_2PO_4 in 1000 ml distilled water.

To prepare 100 ml of phosphate buffer mix 61.1 ml of solution A with 38.9 of solution B. Check the pH of final solution.

Add the following, in order, to 100 ml of phosphate buffer: 0.5 ml of Tween 80 and 2 ml of a 0.1% aqueous solution of neutral red. Dispense 2 ml into 13x100 mm screw cap tubes. Autoclave for 15 minutes at 121°C. The substrate should be amber after autoclaving. Store in the dark in the refrigerator for no more than two weeks.

Inoculate the tubes with the organisms from a culture 2-3 weeks old. Make a heavy inoculum picking up the colonies with a swab. Incubate at 37°C for two weeks.

Positive control: *M. kansasii*

Reading

Observe after 5 and 10 days.

Interpretation

change of color from amber to pink or red: positive

no change of color: negative

NOTE: it is necessary that the medium changes color. If colonies are red, but the medium remains amber, the test is reported as negative.



A. Martin

Figure 10 - Tween hydrolysis test. The tube on the left shows a negative result while the tube on the right is a positive test.

2.1.19. Tellurite reduction

Material

Reagents:

Potassium tellurite solution 0.2% (Potassium tellurite 0.1 g in 50 ml distilled water)

Dispense 2 ml in small tubes or vials and autoclave. Store at 4°C.

Procedure

Prepare 7H9 medium and supplement it with 0.5 ml of Tween 80 for each 1000 ml of medium (do not use glycerol), autoclave and then supplement with ADC. Dispense 2.5 ml in screw cap tubes. Inoculate 1 tube with one drop of bacterial suspension. Incubate at 37°C for 7 days (shake to stimulate growth). After day 7 add 2 drops of tellurite solution. Re-incubate at 37°C for 3 more days (do not shake the tubes during this re-incubation)

Positive control: MAC

Negative control: *M. terrae* complex

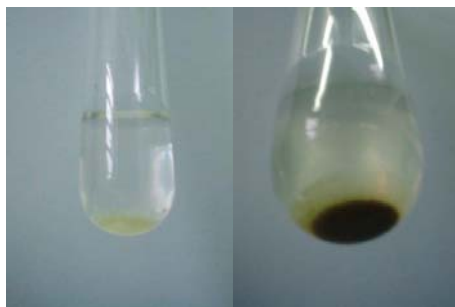
Reading

Observe after 3 days the formation of a black metallic precipitate. If it is negative, incubate for 6 more days and read again.

Interpretation

Formation of black metallic precipitate: positive

No formation of precipitate black: negative. Some species produce a light brown or gray precipitate that must be considered as negative.



E. Roxo

Figure 11 - Tellurite test. The tube on the left shows a negative result. The tube on the right shows a black metallic precipitate indicating a positive test.

2.1.20. Utilization of carbon sources

Materials

Reagents

(NH ₄) ₂ SO ₄	2.4 g
KH ₂ PO ₄	0.5 g
MgSO ₄ ·7H ₂ O	0.5 g
Agar 2%	20 g
Distilled water	950 ml
Sodium citrate	5.6 g
Mannitol	5 g
Inositol	5 g

Procedure

Basal medium

- Dissolve in distilled water all reagents except citrate, inositol or mannitol
- Adjust the basal medium pH at 7.0
- Autoclave for 20 minutes at 121°C

Citrate medium

- Allow cooling at 56°C in a water bath
- Dissolve 5.6 g of sodium citrate in 50 ml in distilled water
- Sterilize using membrane filtration
- Aseptically add to the basal medium
- Aliquot 8 ml per tube
- Allow solidifying in a slant position

Mannitol medium

- Adjust the basal medium pH at 7.2 before autoclavation
- Allow cooling at 56°C in a water bath
- Dissolve 5.0 g of mannitol in 50 ml in distilled water
- Sterilize using membrane filtration
- Aseptically add to the basal medium
- Aliquot 8 ml per tube
- Allow solidifying in a slant position

Inositol medium

- Adjust the basal medium pH at 7.2 before autoclavation
- Allow cooling at 56°C in a water bath
- Dissolve 5.0 g of inositol in 50 ml in distilled water

Sterilize using membrane filtration
Aseptically add to the basal medium
Aliquot 8 ml per tube
Allow solidifying in a slant position

Procedure

Using a 7-day-old 7H9-ADC broth or a suspension from LJ culture, make serial tenfold dilutions in sterile saline until no turbidity is visible. Use this last suspension to inoculate 0.1 ml onto each of the carbon source media and one control slant. Incubate all slants at 37°C

Positive control: *M. smegmatis* for the three media

Reading

Observe growth after 14 days

Interpretation

growth on the test medium (citrate, inositol or mannitol): positive

no growth on test media: negative

2.1.21. Oxygen preference

Procedure

Add pure agar to make a 0.1% semisolid Middlebrook 7H9 medium. Dispense in 10 ml amounts in screw-capped bottles. Pipette 0.2 ml of bacterial suspension about 1 cm below the medium surface and mix carefully, avoiding bubbles and aeration.

Reading

Observe after 14 days. If there is no growth after 14 days, incubate for another week.

Interpretation

Growth at or near the surface = aerobic

Growth as a band 10-20 mm below the surface (sometimes extending upwards) = microaerophilic.

Chapter 3 - MOLECULAR IDENTIFICATION

3.1. Equipment

Thermal Cycler
Microcentrifuge
Water baths or dry baths at 37°C, 65°C, and 80°C
Vortex mixer
Submarine gel electrophoresis system and power supply
Gel documentation (Polaroid camera + UV transilluminator or Gel Documentation System)
Micropipettes of different volumes
Tips and microcentrifuge tubes (autoclaved)

3.2. DNA extraction

VERY IMPORTANT!

It is very important to use separate rooms for DNA extraction, DNA amplification and detection. It is especially important also to have separate individual micropipettes, tubes, etc for each of these steps. Tips and microcentrifuge tubes need to be sterilized by autoclave before use.

Contamination of reagents and DNA with amplification products from previous reactions can be a problem and has to be strictly avoided.

Commercial tests have mechanisms to overcome contamination with previous amplicons, but special care must be taken when in-house PCR protocols are used. Commercial tests have protocols for DNA extraction that have been standardized for these kits.

3.2.1. Solutions

1x TE buffer

(10 mM Tris/HCl, pH 8.0, 1 mM EDTA)

1.211 g Tris

2 ml 0.5 M EDTA.

Adjust pH to 8.0 with concentrated HCl

Add distilled water to a final volume of 1 L.

Autoclave.

Store at room temperature for no longer than one year.

Lysozyme solution

10 mg lysozyme/ml distilled water.

Store in small aliquots at -20°C for no longer than one year.

Proteinase K

10 mg proteinase K/ml distilled water.

Store in small aliquots at -20°C for no longer than one year.

10% SDS

10 g SDS/100 ml distilled water.

Dissolve by heating at 65°C. Do not autoclave.

Store at room temperature for no longer than 1 month.

CTAB/NaCl solution

Dissolve 4.1 g NaCl in 80 ml distilled water. While stirring, add 10 g CTAB (N-cetyl- N,N,N,-trimethyl ammonium bromide). If necessary, heat the solution to 65°C. Adjust the volume to 100 ml with distilled water.

Store at room temperature for no longer than 6 months.

24:1 chloroform/isoamyl alcohol

Mix 24 volumes of chloroform with 1 volume of isoamyl alcohol.

Store at room temperature for no longer than one year.

25:24:1 phenol/chloroform/isoamyl alcohol

Mix 25 volumes of phenol with 24 volumes of chloroform and 1 volume of isoamyl alcohol.

Store at room temperature for no longer than one year.

70 % Ethanol

Mix 7 volumes absolute ethanol with 3 volumes distilled water.

Store at room temperature for no longer than one year.

3.2.2. DNA extraction from clinical samples

1. collect samples in 1.5mL microcentrifuge tubes
2. centrifuge at 10,000 x g for 5 min
3. discard supernatant and wash the pellet twice with TE buffer, each time centrifuging at 10,000 x g for 5 min
4. discard supernatant without disturbing the pellet

5. resuspend in 75 μL of TE 1X and 25 μL of lysozyme solution (final concentration of 2.5mg/mL) and incubate for 30 min at 37°C
6. add 3 μL of proteinase K (final concentration of 150 $\mu\text{g}/\text{mL}$), plus 20 μL of 10% SDS (final concentration of 1%) and complete with TE to a final volume of 200 μL
7. incubate at 65°C with occasional agitation
8. extract DNA with 300 μL of phenol/chloroform/isoamyl alcohol (25:24:1), centrifuge at 14,000 x g for 5 min, and transfer the aqueous phase to a clean microcentrifuge tube
9. add 30 μL of sodium acetate 3M pH 4.8
10. precipitate DNA with 1 vol isopropanol (300 μL), agitate manually, and centrifuge at 14,000 x g for 15 min.
11. discard supernatant and add cold ethanol 70% (300 μL)
12. centrifuge at 14,000 x g for 5 min
13. discard the supernatant and let dry at room temperature
14. resuspend in 10 μL TE
15. use 2 μL for PCR

3.2.3. DNA extraction from pure cultures

Be sure to work with **pure cultures** before starting (see chapter 1)!

Protocol 1 (complete) (234)

1. Resuspend several loopful of bacteria in 400 μL of TE 1X
2. Inactivate the bacteria at 80°C for 20 min
3. Cool at room temperature and add 50 mL of lysozyme solution
4. Vortex and incubate at 37°C for at least 1 h under agitation or for 12 h standstill.
5. Add 70 mL of SDS 10% and 5 mL of proteinase K
6. Vortex and incubate at 65°C for 10min
7. Add 100 mL of NaCl 5M and 100 mL of CTAB/NaCl solution
8. Vortex until the liquid content becomes white (“milky”) and incubate for 10 min at 65°C
9. Add 750 mL of chloroform/isoamylic alcohol (24:1), vortex for 10 sec and centrifuge at room temperature for 5 min at 14,000 x g.
10. Transfer supernatant to a clean microcentrifuge tube and add 0.6 volumes (~ 450 mL) of isopropanol
11. Incubate the mixture at -20°C for 30 min and centrifuge for 15 min at 14,000 x g.

12. Discard the supernatant, wash the pellet with 1 mL of ethanol 70% and centrifuge for 5 min at 14,000 x g
13. Add to precipitate the DNA 20-30 mL of TE and conserve at 4°C for immediate use or at -20°C for future use.

Protocol 2 (simple)

1. Transfer a loopful of culture in solid medium to a microcentrifuge tube and add 100 µL of distilled water. If liquid cultures are used, transfer 1 mL to a microcentrifuge tube, centrifuge at 14,000 x g for 5 min and resuspend in 100 µL of distilled water.
2. Inactivate the bacteria at 80°C for 20 min
3. Centrifuge at 14,000 x g for 5 min and wash the pellet with saline by centrifugation
4. Resuspend bacteria in 0.2 – 1 mL (depending on bacterial mass) of TET buffer (Triton X-100 1% in TE)
5. Boil once for 10 minutes and freeze at -20°C overnight.
6. Alternatively, perform 3 freeze-and-boil cycles of 10 minutes each
7. Store at -20°C until use
8. Thaw and centrifuge samples briefly and use 5-10 mL of supernatant for amplification

Protocol 3 (simple) (260)

1. Follow steps 1 and 2 of the Protocol 2
2. Let it cool to room temperature, add 100 µL of chloroform, and vortex briefly (10 sec)
3. Incubate for 20 min at 80°C.
4. Incubate for 20 min at -20°C
5. Thaw and centrifuge for 3 min at 14,000 x g.
6. Collect the aqueous phase supernatant in a clean microcentrifuge tube
7. Store at -20°C until use

3.3. Molecular identification of *M. tuberculosis* complex

Prepare 48 µL of the reaction mix and add 2 µL of DNA.

3.3.1. Protocol for amplification of the 123 bp fragment from IS6110

PRIMERS	IS1: 5' CCTGCGAGCGTAGGGCTCGG IS2: 5' CTCGTCCAGCGCCGCTTCGG	
REACTION MIX	5 µL of 10 x Taq polymerase buffer 1.5 µL of 50 mM MgCl ₂ 5 µL of 1 mM dNTP 1 µL of each primer at 25 pmoles/µL 0.2 µL Taq DNA Polymerase 5 U/µL add water and DNA up to 50 µL	Final concentration KCl 50 mM, Tris-HCl 10 mM pH8 1.5 mM 100 mM 0.5 mM 1 U
AMPLIFICATION	1 cycle 95°C 5 min { 94°C 2 min 40 cycles { 60°C 2 min { 72°C 2 min 1 cycle 72°C 7 min	
PRODUCT	123 bp	

3.3.2. Protocol for amplification of the 245 bp fragment from IS6110

PRIMERS	INS1: 5' CGTGAGGGCATCGAGGTGGC INS2: 5' GCGTAGGCGTCGGTGACAAA	
REACTION MIX	5 µL of 10 x Taq polymerase buffer 1.5 µL of 50 mM MgCl ₂ 5 µL of 1 mM dNTP 1 µL of each primer at 25 pmoles/µL 0.2 µL Taq DNA Polymerase 5 U/µL add water and DNA up to 50 µL	Final concentration KCl 50 mM, Tris-HCl 10 mM pH8 1.5 mM 100 mM 0.5 mM 1 U
AMPLIFICATION	1 cycle 95°C 5 min { 94°C 2 min 40 cycles { 60°C 2 min { 72°C 2 min 1 cycle 72°C 7 min	
PRODUCT	245 bp	

3.3.3. Protocol for amplification of the *mtp40* fragment

PRIMERS	PT1: 5' CAACGCGCCGTCGGTGG PT2: 5' CCCCCACGGCACCGC	
REACTION MIX	5 μ L of 10 x Taq polymerase buffer 2.5 μ L of 50 mM MgCl ₂ 10 μ L of 1 mM dNTP 1 μ L of each primer at 20 pmoles/ μ L 0.2 μ L Taq DNA Polymerase 5 U/ μ L add water and DNA up to 50 μ L	Final concentration KCl 50 mM, Tris-HCl 10 mM pH8 2.5 mM 200 mM 0.4 mM 1 U
AMPLIFICATION	1 cycle 95°C 10 min 35 cycles { 94°C 1 min 74°C 2min 1 cycle 72°C 7 min	
PRODUCT	396 bp	

3.3.4. Protocol for *gyrB-RFLP*

PCR		
PRIMERS	Mtubf: 5'TCGGACGCGTATGCGATATC Mtubr: 5' ACATACAGTTCGGACTTGCG	
REACTION MIX	5 μ L of 10 x Taq polymerase buffer 1.5 μ L of 50 mM MgCl ₂ 5 μ L of 1 mM dNTPs 5 μ L of each primer at 20 pmoles/ μ L 0.25 μ L Taq DNA Polymerase at 5 U/ μ L add water and DNA up to 50 μ L	Final concentration KCl 50 mM, Tris-HCl 10 mM pH8 1.5 mM 100 mM 2 mM 1.25 U
AMPLIFICATION	1 cycle 95°C 5 min 35 cycles { 94°C 1 min 65°C 1 min 72°C 1.5min 1 cycle 72°C 7 min	
PRODUCT	1020 bp	

Visualize 10 μ L of the reaction in 0.8 - 1% agarose gel to verify amplification.

Enzymatic digestion

Use 5µL of the PCR product separately for digestion with *Rsa*I and *Taq*I.

Reaction condition

5 µL of PCR product

1µL buffer specific for each enzyme

1µL enzyme

3 µL water

total volume = 10µL

Incubate digestions at 37°C for 1 h.

Agarose gel

5 x TBE

(Tris 445 mM, boric acid 445 mM, EDTA 10 mM, pH 8.2)

54 g Tris

27.5 g boric acid

3.72 g EDTA or 20 mL 0.5 M EDTA pH 8.0.

do not adjust pH

Add distilled water to a final volume of 1 L

Autoclave

Store at room temperature for no longer than one year.

Prepare 2% agarose gel in 0.5 x TBE. Ethidium bromide 0.5 µg/mL final concentration can be added directly to the gel or the gel can be stained after electrophoresis. Subject digestion products to electrophoresis at 5V/cm (distance between electrodes). In 2 slots at both extreme of the gel run 50bp ladder for calculation of band sizes.

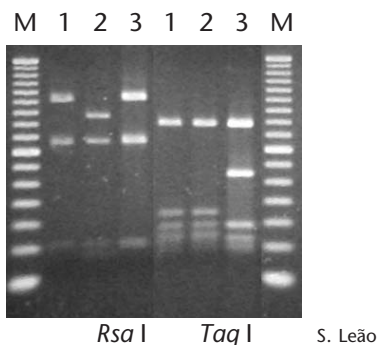


Figure 12 - 2% agarose gel showing *gyrB*-RFLP patterns of *M. tuberculosis* complex members. M = 50bp ladder, 1 = *M. africanum* I, 2 = *M. bovis* / *M. bovis* BCG, 3 = *M. tuberculosis* / *M. africanum* II .

<i>Rsa</i> I		<i>Taq</i> I	
←————→		←————→	
660-385	440-160-130-105-100-80		<i>M. microti</i>
560-385-100	{ 440-270-130-100-80		<i>M. tuberculosis</i> / <i>M. africanum</i> II / <i>M. canettii</i>
	{ 440-160-130-105-100-80		<i>M. africanum</i> I / <i>M. pinnipedii</i>
480-385-100	440-160-130-105-100-80		<i>M. bovis</i> / <i>M. bovis</i> BCG

Figure 13 – Algorithm of *gyrB*-RFLP patterns.

3.4. Molecular identification of NTM

3.4.1. PRA (PCR-Restriction Enzyme Analysis)

PCR

PRIMERS	Tb11: 5' ACCAACGATGGTGTGTCCAT Tb12: 5' CTTGTGCAACCGCATACCCT	
REACTION MIX	5 µL of 10 x Taq polymerase buffer 1.5 µL of 50 mM MgCl ₂ 10 µL of 1 mM dNTPs 5 µL of glycerol 1 µL of each primer at 25 pmoles/µL 0.2 µL Taq DNA Polymerase 5 U/µL add water and DNA up to 50 µL	Final concentration KCl 50 mM, Tris-HCl 10 mM pH8 1.5 mM 200 mM 10% 0.5 mM 1 U
AMPLIFICATION	1 cycle 95°C 5 min 45 cycles { 94°C 1 min 65°C 1 min 72°C 1 min 1 cycle 72°C 7 min	
PRODUCT	441 bp	

Visualize 10 µL of the reaction in 1% agarose gel to verify amplification.

Enzymatic digestion

Use 10-15µL of the PCR product separately for digestion with *Bst*E II and *Hae* III.

Reaction conditions

15 µL of PCR product
2.5µL buffer specific for each enzyme
1µL enzyme
6.5 µL water
total volume = 25µL

Incubate digestion with *BstE* II at 60°C and with *Hae* III at 37°C for at least 2-3 hours to avoid partial digestion.

Agarose gel

Prepare the gel in TBE using 3% NuSieve 3:1 agarose (FMC Bioproducts, Rockland, Maine), 3% MethaPhor agarose (FMC Bioproducts), or 4% Agarose 1000 (Invitrogen) or regular agarose (Invitrogen or SeaKem LE). Ethidium bromide 0.5 µg/mL final concentration can be added directly to the gel or the gel can be stained after electrophoresis. Subject digestion products to electrophoresis at 5V/cm (distance between electrodes). In 2 slots at the extreme and in one slot in the middle of the gel run 50bp ladder to allow calculation of band sizes.

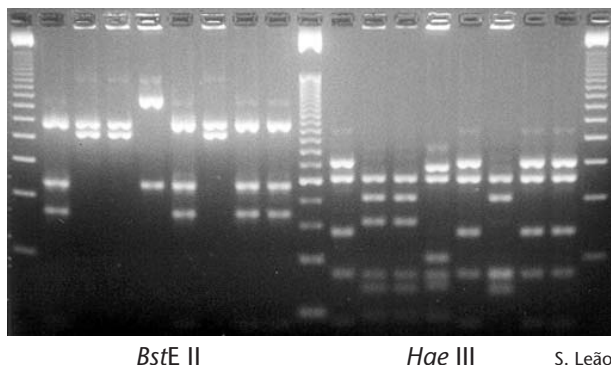


Figure 14 - Example of gel showing PRA patterns after digestion with *BstE* II and *Hae* III. M1 = 50bp ladder, M2 = 25bp ladder, 1 – 8 = samples digested with *BstE* II (left) and *Hae* III (right).

Gel documentation

Photograph gels in Polaroid camera or documentation system coupled to computer. Calculate band sizes by comparison with the DNA marker.

Identification table

A table for interpretation of PRA results was especially designed for this Manual.

Band sizes were calculated using GelCompar II v. 2.5 software (Applied Maths, St- Martens-Latem, Belgium) from gel images obtained using isolates from different laboratories from RELACTB: Institute of Tropical Medicine (Antwerp, Belgium), Universidade Federal de São Paulo (São Paulo, Bra-

zil), Instituto Adolfo Lutz (São Paulo, Brazil), Fundação Oswaldo Cruz (Rio de Janeiro, Brazil), Instituto Malbrán (Buenos Aires, Argentina), Pasteur Institute (Guadelupe), and Instituto Pedro Kourí (Havana, Cuba).

Consensus *Bst*EI and *Hae*III band sizes were obtained by comparison of sizes calculated from gel images (empirical data) with patterns described by Telenti et al. (226), Devallois et al. (53), Brunello et al. (26), da Silva Rocha et al. (46), and available at PRASITE (<http://app.chuv.ch/prasite>) (published data). PRA patterns from frequently encountered and clinically important species were selected. For interpretation of patterns not included in this handbook the reader is referred to published tables and to patterns available on the Internet (PRASITE <http://app.chuv.ch/prasite>, PRAONLINE <http://www.ioc.fiocruz.br/praoonline>).

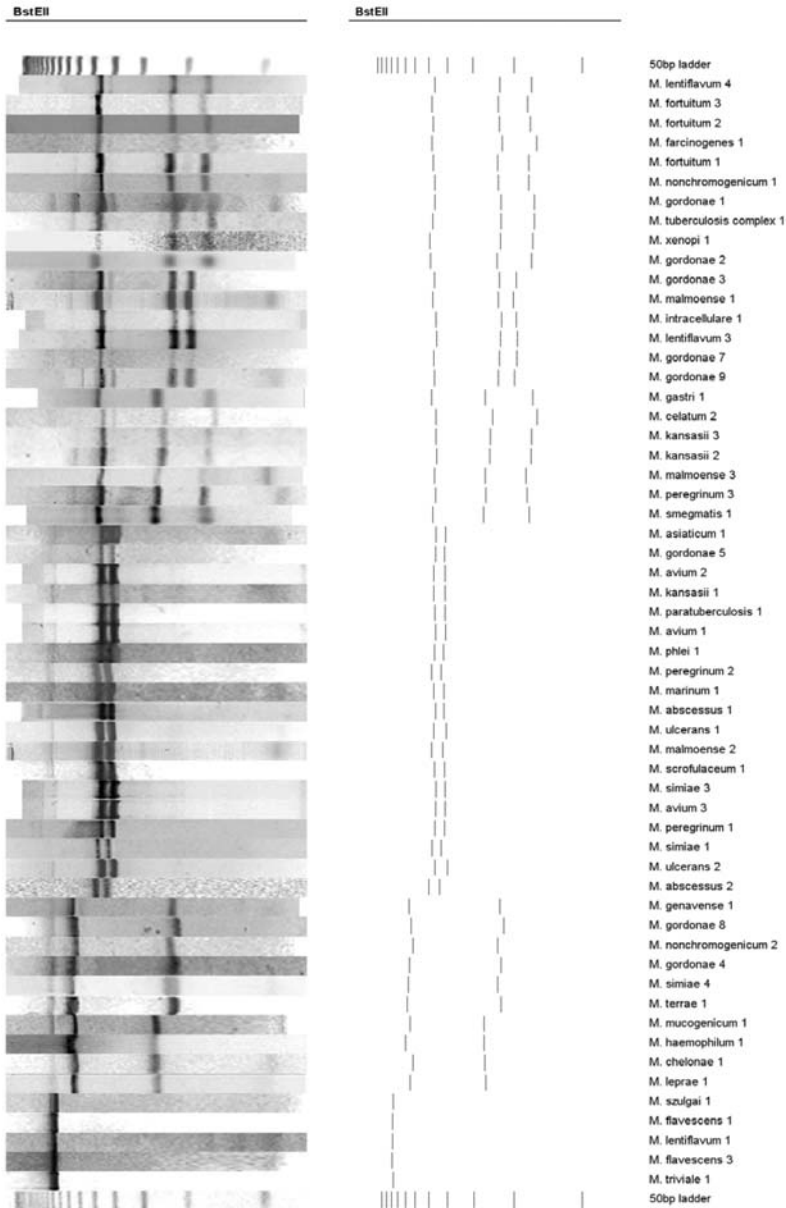


Figure 16 – PRA patterns after digestion with *BstEII*. Upper image = gel images, lower image = bands after analysis by GelCompar II.

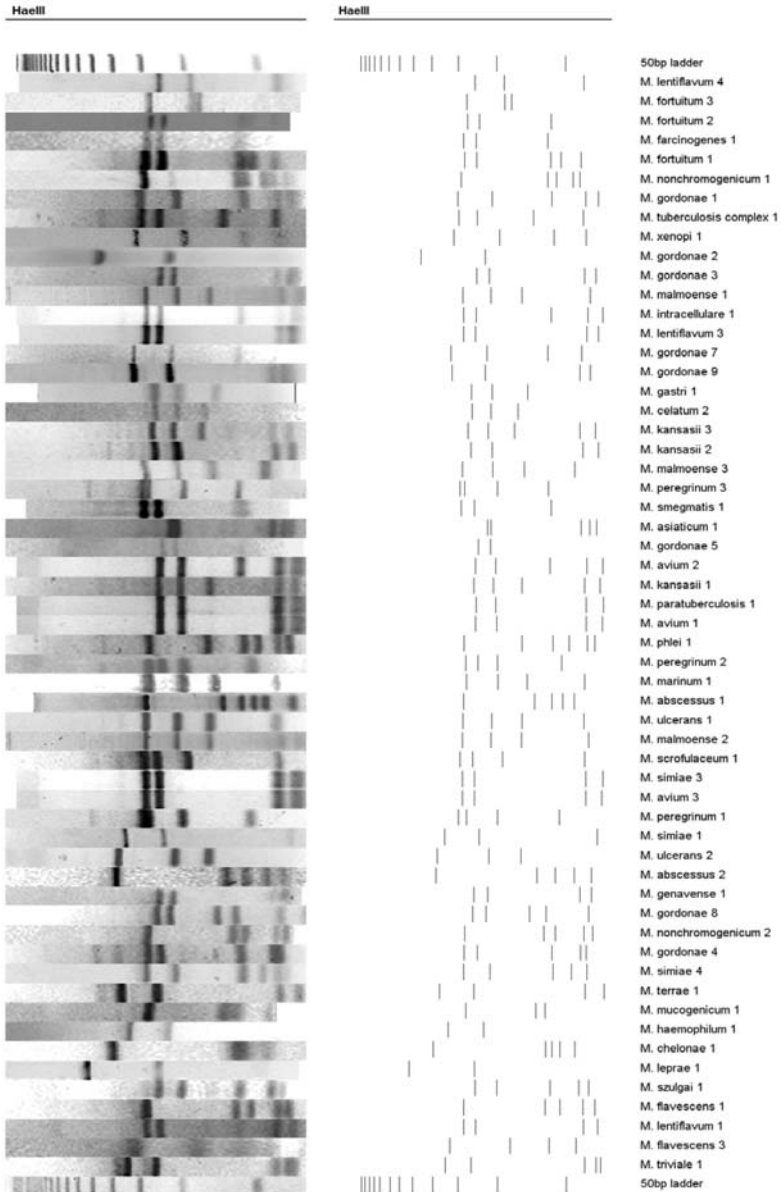


Figure 17 – PRA patterns after digestion with *Hae* III. Upper image = gel images, lower image = bands after analysis by GelCompar II.

