

Procedure Manual

Thin Layer Agar (TLA)

Microcolony detection

Rapid culture of Mycobacterium tuberculosis

By

Anandi MARTIN, PhD amartin@itg.be

&

Juan Carlos PALOMINO, PhD palomino@itg.be

Institute of Tropical Medicine, Mycobacteriology Unit, Antwerp, Belgium

Version 04-2009

Procedure Manual

Thin Layer Agar (TLA)

Microcolony detection

Rapid culture of Mycobacterium tuberculosis

By

Anandi MARTIN, PhD

&

Juan Carlos PALOMINO, PhD

Institute of Tropical Medicine, Mycobacteriology Unit
Antwerp, Belgium

With the collaboration of

Jaime Robledo, MD, PhD

&

Lic. Gloria Isabel Mejia

Corporación para Investigaciones Biológicas
Medellin, Colombia

Version 04-2009

Table of contents

1. Principle of the test
 2. Reagents
 3. Preparation of the drug stock solution
 - 3.1 Trimethoprim
 - 3.2 Amphotericin B
 - 3.3 Piperacillin sodium salt
 4. Preparation p-nitro benzoic acid (PNB) 500 µg/ml
 5. Preparation of the medium Middlebrook 7H11 agar
 - 5.1 Procedure 7H11 agar
 - 5.2 Middlebrook 7H11+PNB (for 1000 ml of medium)
 6. Procedure for inoculation of plate
 7. Incubation
 8. Reading the results
 9. Discarding the plates
 10. Quality control
- References
- Pictures

Procedure of the Thin Layer Agar (TLA) for the rapid culture of *M. tuberculosis*

1. Principle of the test

Culture of clinical samples increase the detection of *Mycobacterium tuberculosis* in patients and are more sensitive than direct microscopic examination. Only 10 to 100 viable organisms are needed to have a positive culture, while a minimum of 5000 to 10.000 acid-fast bacilli per milliliter are required for detection by direct smear.

TLA use a solid medium and is based on the microscopic detection of early mycobacterial growth. This method is able to detect growth within 9–14 days and also allows the initial identification of *M. tuberculosis* on the basis of its colony morphology. The sample is inoculated on a plate containing Middlebrook 7H11 and Middlebrook 7H11 enriched with PNB (para-nitrobenzoic acid). The detection of growth and its comparison in the two media will help the identification of *M. tuberculosis* complex since it is expected to grow on 7H11 but not on 7H11+PNB where its growth will be inhibited.

2. Reagents

- Middlebrook 7H11 agar (Difco 0838-17)
- OADC: Oleic Acid Dextrose Catalase (ref.211886-10x20ml Becton Dickinson)
- Glycerol (ref.1.04094.1000-500 ml Merck)
- PBS tablet (ref BR0014G, 100 tablets Oxoid)
- PNB: p-nitro benzoic acid (ref.128460250- 25 g- Acros Organic)
- Drugs:
 - Trimethoprim (Sigma Chemical ref. T7883- 5 gr-soluble in DMSO)
 - Amphotericin (Sigma Chemical ref. A 4888 - 250 mg- in DMSO)
 - Piperacillin (Sigma Chemical ref P8396, 1 gr-in PBS)
- Dimethyl sulfoxide (DMSO, 1L, Merck)
- Petri dishes (divided in two compartments)
- NaOH
- HCl
- Analytic balance
- Incubator 37°C
- CO₂ incubator 37°C (5% CO₂)

3. Preparation of the drug stock solution

3.1 Trimethoprim (20 µg/L = 0.02 µg/ml)

Stock 200 µg/ml

- Weight 2 mg of the drug
- Dissolve in 0.5 ml HCl 0.05 N*
- Complete at 10 ml with distilled water (9.5 ml distilled water) (S.stock 200 µg/ml)
- Make aliquots in vials of 0.5 ml

- Add 0.1 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

* *HCl 0.05 N: with a HCl 1N make a 20x dilution; add 0.5 ml HCl 1N in 9.5 ml H₂O*

3.2 Amphotericin B (20 µg/L = 0.02 µg/ml)

Stock: 200 µg/ml

- Weight 2 mg of the drug
- Dissolve in 10 ml of distilled water (S. stock 200 µg/ml)
- Make aliquot in vials of 0.5 ml
- Add 0.1 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

3.3 Piperacillin sodium salt (50 µg/l = 0.05 µg/ml)

Stock: 500 µg/ml

- Weight 5 mg of the drug
- Dissolve in 10 ml of PBS buffer* pH 7.0 (S. stock: 500 µg/ml)
- Make aliquot in vials of 0.5 ml
- Add 0.1 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

PBS buffer: 1 tablet in 100 ml distilled water, autoclave at 115°C for 10 minutes*

4. Preparation p-nitro benzoic acid (PNB) 500 µg/ml

Mycobacterium tuberculosis complex is susceptible to PNB while other atypical mycobacteria will grow.

- Prepare a stock solution of 25 mg/ml PNB
 1. Dissolve 2,5 g of PNB in about 15 ml of 1N NaOH solution until complete dissolution
 2. Complete with sterile distilled water until 100 ml
 3. aliquot of 1ml and keep at -20°C for up to 3 months
- Alternative protocol
 1. Dissolve 2,5 g of PNB in about 15 ml of 1N NaOH solution until complete dissolution
 2. Complete with sterile distilled water until 80 ml
 3. Add 1 or 2 drops of 1% red phenol.
 4. Add, drop by drop 1N HCl solution until a change of colour it turns yellow
 5. If precipitation occurs due to excess of acid, add a bit more of NaOH; complete with sterile distilled water until 100 ml.
 6. aliquot and keep at -20°C for up to 3 months

Preparation red phenol 1%

- Add 1 gr of red phenol powder to 50 ml of distilled water
- Add NaOH 1N until the solution becomes purple (max 7 ml)
- Add distilled water to reach 100 ml store at 4°C for no longer than 1 year

Preparation 1N HCl solution

- Start with HCl 37% = 12 N
- To prepare 100 ml of 1N HCL add 91.7 ml distilled water to 8.5 ml HCl 37%
- To prepare 120 ml of 1N HCL add 110 ml distilled water to 10 ml HCl 37%
- Add indicator: 0.1 ml phenol red 1% for 100 ml HCl 1N
- Autoclave and store at 4°C for no longer then 1 year

Preparation 1 N NaOH

- Add 40 gr NaOH to 1000 ml of distilled water
- Autoclave and store at room temperature

5. Preparation of the medium Middlebrook 7H11 agar

Middlebrook 7H11 and Middlebrook 7H11 + PNB will be prepared and poured in petri dishes. Middlebrook 7H11 agar must be immediately poured in plates before it solidifies. Plates prepared are stored at 4°C for up to two months.

5.1 Procedure 7H11 agarFor 1000 ml of 7H11 medium

- Weight 21 gr Middlebrook 7H11 agar
- Add 900 ml distilled water
- Add 5 ml glycerol Autoclave at 121°C for 15 minutes
- Leave at room temperature until the medium is at 45°C-50°C
- Add 100 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 100 µl (200 µg/ml)
 - Piperacillin 100 µl (500 µg/ml)
 - Trimethoprim 100 µl (200 µg/ml)
- Pour petri plates

When the medium is kept out of the autoclave, keep it directly into a hot water bath (around 50°C) to avoid solidification.

For 200 ml of 7H11 media

It is better to prepare several small bottles of 200 ml to avoid contamination and it is easier to pool the plates with small volumes.

- Weight 4.2 gr Middlebrook 7H11 agar
- Add 179 ml distilled water
- Add 1 ml glycerol (Merck 1.04094.1000)
- Autoclave at 121°C for 15 minutes

- Leave at room temperature until the medium is at 45°C-50°C
- Add 20 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 20 µl (200 µg/L)
 - Piperacillin 20 µl (500 µg/L)
 - Trimethoprim 20 µl (200 µg/L)
- Pour petri dish plate

When the medium is kept out of the autoclave, keep it directly into a hot water bath (around 50°C) to avoid solidification.

5.2 Middlebrook 7H11+PNB

For 1000 ml of 7H11 media

- Weight 21 gr Middlebrook 7H11 agar
- Add 900 ml distilled water
- Add 5 ml glycerol
- **Add 20 ml PNB stock solution**
- Autoclave at 121°C for 15 minutes
- Leave at room temperature until the medium is at 45°C-50°C
- Add 100 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 100 µl (200 µg/L)
 - Piperacillin 100 µl (500 µg/L)
 - Trimethoprim 100 µl (200 µg/L)
- Pour Petri dish plate

When the medium is kept out of the autoclave, keep it in hot bath, to avoid solidification.

For 200 ml of 7H11 media

It is easier to prepare several bottles of 200 ml of media to avoid contamination when pouring the media.

- Weight 4.2 gr Middlebrook 7H11 agar (Difco 0838-17)
- Add 175 ml distilled water
- Add 1 ml glycerol (Merck 1.04094.1000)
- **Add 4 ml PNB stock solution**
- Autoclave at 121°C for 15 minutes
- Leave at room temperature until the media become at 45°C-50°C
- Add 20 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 20 µl (200 µg/L)
 - Piperacillin 20 µl (500 µg/L)
 - Trimethoprim 20 µl (200 µg/L)
- Pour Petri dish

Update-TLA April 2008**New concentration of Trimethoprim, Amphotericin, Piperacillin**

Trimethoprim (4 µg/ml final conc) (T)
Amphotericin B (4 µg/ml final conc) (A)
Piperacillin sodium salt (4 µg/ml final conc) (P)

Trimethoprim (4 µg/ml final conc)

Stock: 1 mg/ml (=1000 µg/ml)

- Weight 10 mg of the drug
- Dissolve in 0.5 ml HCl 0.05 N*
- Complete at 10 ml with distilled water (9.5 ml distilled water) (S. stock 1mg/ml)
- Make aliquots in vials of 0.5 ml
- Add 4 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

* HCl 0.05 N: with a HCl 1N make a 20x dilution; add 0.5 ml HCl 1N in 9.5 ml H₂O

Amphotericin B (4 µg/ml final conc)

Stock: 1 mg/ml (=1000 µg/ml)

- Weight 10 mg of the drug
- Dissolve in 10 ml of distilled water (S. stock 1 mg/ml)
- Make aliquot in vials of 0.5 ml
- Add 4 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

Piperacillin sodium salt (4 µg/ml final conc)

Stock: 1 mg/ml (=1000 µg/ml)

- Weight 10 mg of the drug
- Dissolve in 10 ml of PBS buffer* pH 7.0 (S. stock: 1 mg/ml)
- Make aliquot in vials of 0.5 ml
- Add 4 ml of the stock solution for each 1000 ml of 7H11 agar medium
- Keep at -20°C for no more than 6 months.

PBS buffer: 1 tablet in 100 ml distilled water, autoclave at 115°C for 10 minutes*

Preparation of the medium Middlebrook 7H11 agar

Middlebrook 7H11 and Middlebrook 7H11 + PNB will be prepared and poured in petri dishes. Middlebrook 7H11 agar must be immediately poured in plates before it solidifies. Plates prepared are stored at 4°C for up to two months.

Procedure 7H11 agar

For 1000 ml of 7H11 medium

- Weight 21 gr Middlebrook 7H11 agar
- Add 900 ml distilled water
- Add 5 ml glycerol Autoclave at 121°C for 15 minutes
- Leave at room temperature until the medium is at 45°C-50°C
- Add 100 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 4 ml (1 mg/ml)
 - Piperacillin 4 ml (1 mg/ml)
 - Trimethoprim 4 ml (1 mg/ml)
- Pour petri plates

When the medium is kept out of the autoclave, keep it directly into a hot water bath (around 50°C) to avoid solidification.

For 200 ml of 7H11 media

It is better to prepare several small bottles of 200 ml to avoid contamination and it is easier to pool the plates with small volumes.

- Weight 4.2 gr Middlebrook 7H11 agar
- Add 179 ml distilled water
- Add 1 ml glycerol (Merck 1.04094.1000)
- Autoclave at 121°C for 15 minutes
- Leave at room temperature until the medium is at 45°C-50°C
- Add 20 ml OADC
- Add stock solution of the following drugs
 - Amphotericin B 800 µl (1 mg/ml)
 - Piperacillin 800 µl (1 mg/ml)
 - Trimethoprim 800 µl (1 mg/ml)
- Pour petri dish plate

When the medium is kept out of the autoclave, keep it directly into a hot water bath (around 50°C) to avoid solidification.

Middlebrook 7H11+PNB

For 1000 ml of 7H11 media

- Weight 21 gr Middlebrook 7H11 agar
- Add 900 ml distilled water

- Add 5 ml glycerol
- **Add 20 ml PNB stock solution**
- Autoclave at 121°C for 15 minutes
- Leave at room temperature until the medium is at 45°C-50°C
- Add 100 ml OADC
- Add stock solution of the following drugs

Amphotericin B	4 ml (1 mg/ml)
-Piperacillin	4 ml (1 mg/ml)
-Trimethoprim	4 ml (1 mg/ml)
- Pour Petri dish plate

When the medium is kept out of the autoclave, keep it in hot bath, to avoid solidification.

For 200 ml of 7H11 media

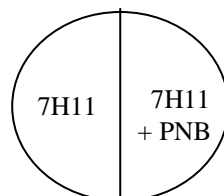
It is easier to prepare several bottles of 200 ml of media to avoid contamination when pouring the media.

- Weight 4.2 gr Middlebrook 7H11 agar (Difco 0838-17)
- Add 175 ml distilled water
- Add 1 ml glycerol (Merck 1.04094.1000)
- **Add 4 ml PNB stock solution**
- Autoclave at 121°C for 15 minutes
- Leave at room temperature until the media become at 45°C-50°C
- Add 20 ml OADC
- Add stock solution of the following drugs

-Amphotericin B	800 µl (1 mg/ml)
-Piperacillin	800 µl (1 mg/ml)
-Trimethoprim	800 µl (1 mg/ml)
- Pour Petri dish

6. Procedure for inoculation of plate

Use a Petri plate (100 mm x 15 mm) divided in 2 compartments. Half of the plate will contain 7H11 and the other half 7H11+PNB. Pour around not more than 10 ml (by hand) in each compartment.



Samples will be inoculated on Middlebrook 7H11 and Middlebrook 7H11+PNB

- Decontamination and centrifugation according to the recommended procedures (NALC-NaOH or Petroff method).
- Label the plates with date and specimen code on the border and NOT on the middle of the plate (later you will need to read the plate under the microscope)

- Take 100 µl of the sample and inoculate one 7H11 compartment.
- Repeat on the other half of the plate: take 100 µl of the sample and inoculate on TLA+PNB medium.
- The Petri dishes are closed with parafilm, leaving a space of about 1 or 2 cm to allow the exchange of CO₂.
- Put the plates in the incubator until the inoculum on the surface of the medium is absorbed (overnight). Plates can be incubated upside down the day after if the inoculum is absorbed otherwise incubate one day more, to avoid condensation on the lid of the plates.
- Plates will be incubated in 5-10% CO₂ (CO₂ incubator or hermetically closed candle jar) at 37 °C.

7. Incubation

Plates must be sealed with a parafilm, leaving a space about 1 or 2 cm to allow the exchange of CO₂, and then incubated at 37°C in atmosphere of 5% of CO₂ for 4 weeks.

8. Reading of the plates

Plates must be read after 48 hours to check for contamination. The plates should be observed twice a week to detect growth using a conventional microscope (objective 10x) for up to 6 weeks, identifying microcolonies as *M. tuberculosis* by their appearance and morphology. Any observation of growth or contamination should be registered in a record sheet.

The growth on TLA medium is compared with the growth on TLA+PNB, as PNB inhibits the growth of *M. tuberculosis* complex. Growth of *M. tuberculosis* complex on TLA in the first few days looks like small cords. After a few days the colony of *M. tuberculosis* complex on TLA assumes its typical appearance: colonies are bigger in size and are constituted by cords (see pictures at the end of this manual). At the same time the compartment TLA+PNB does not show any growth or cord formation. Both compartment TLA and TLA+PNB should be compared.

- The reading is made using a conventional microscope (10x objective).
- Plates should be read:
 - After 48h (identification of rapid growing species)
 - Regularly 2 times a week to detect growth
- If growth on TLA is observed, compare it with the growth on TLA with PNB
- Confirm with Ziehl-Nielsen staining technique
- If growth is seen on PNB and has the same appearance that TLA without PNB, it could represent the presence of atypical mycobacteria
- Report results

9. Discarding the plates

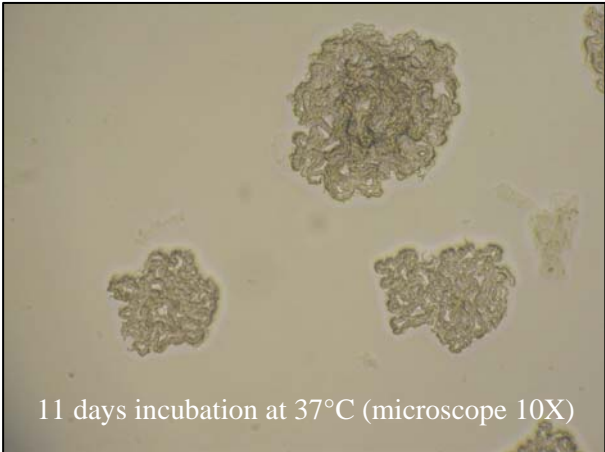
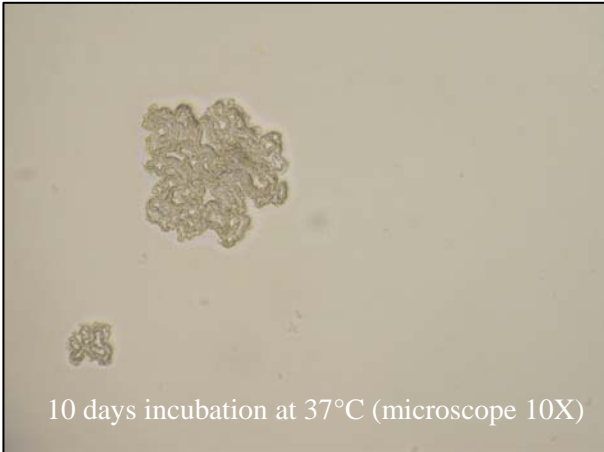
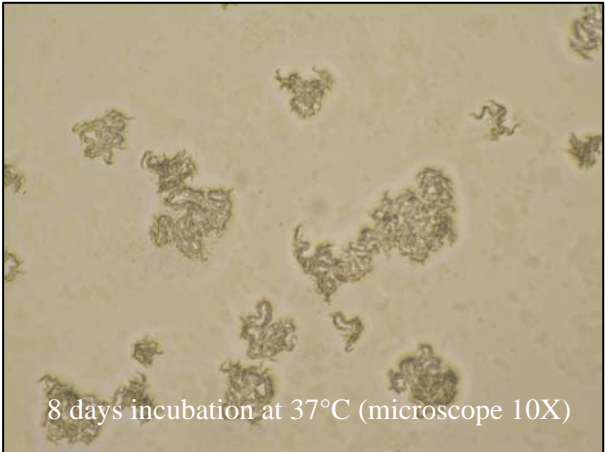
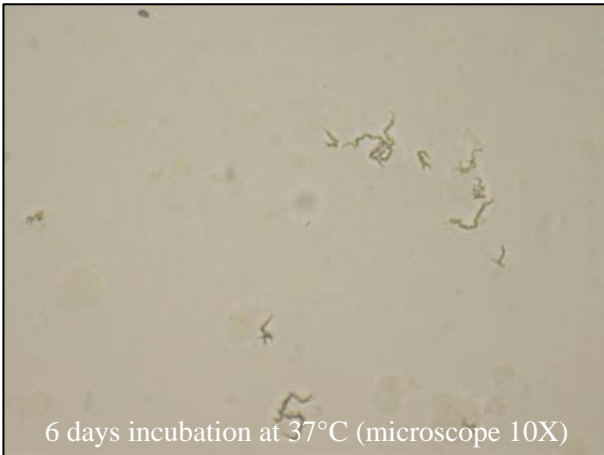
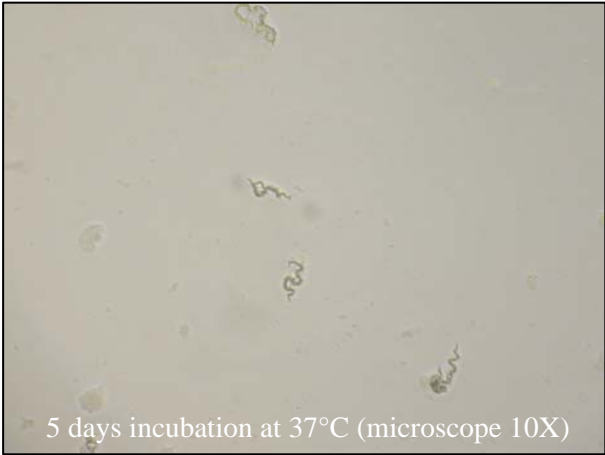
Plates, before being incinerated, must be autoclaved for 30 minutes at 121°C.

10. Quality control

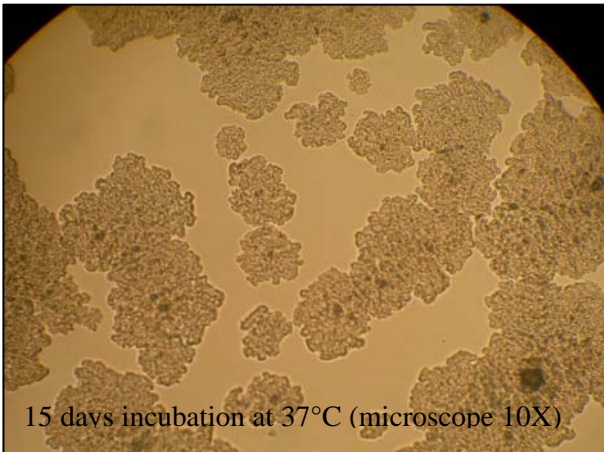
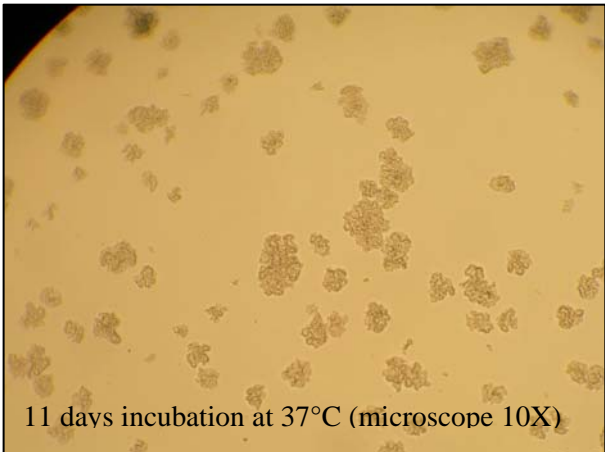
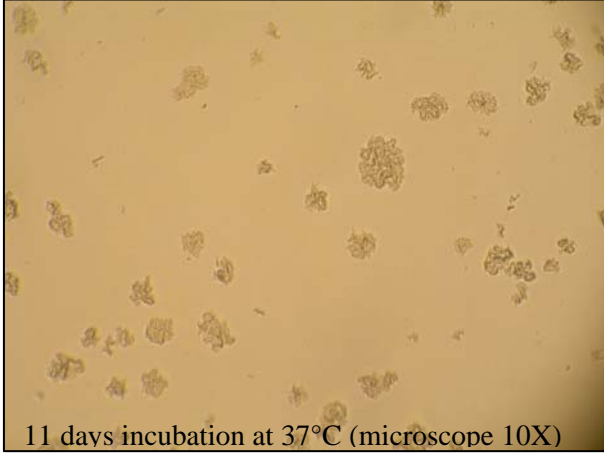
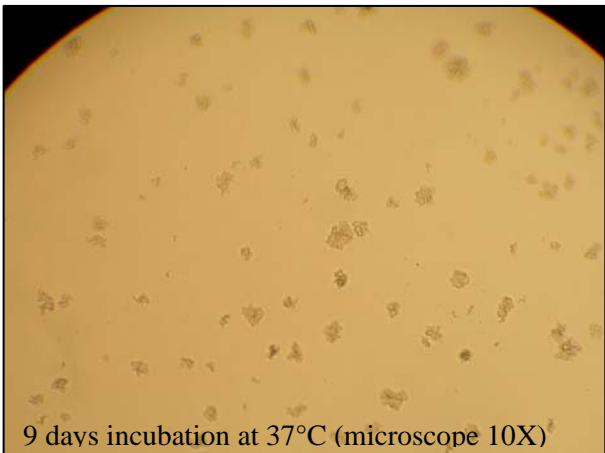
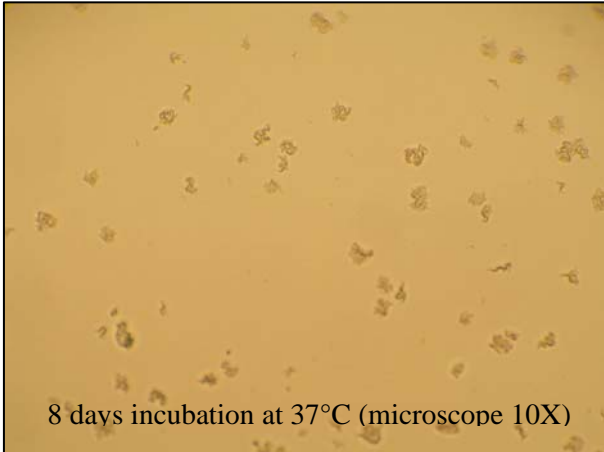
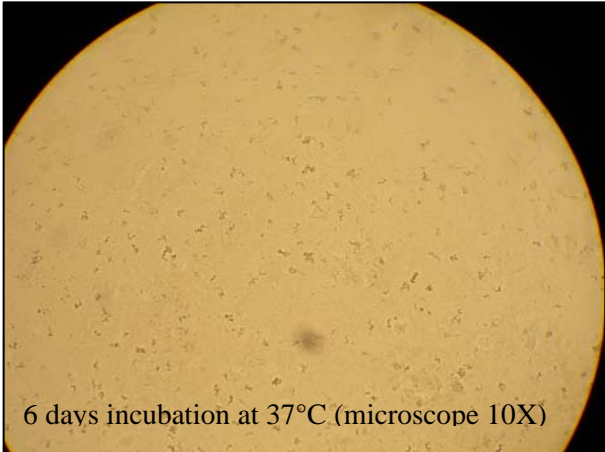
Quality control must be performed on:

- Media preparation: after the preparation of each lot of TLA, one plate will be incubated overnight at 37°C. The plate will be checked for any contamination.
- Decontamination procedures
- Sterilization procedures

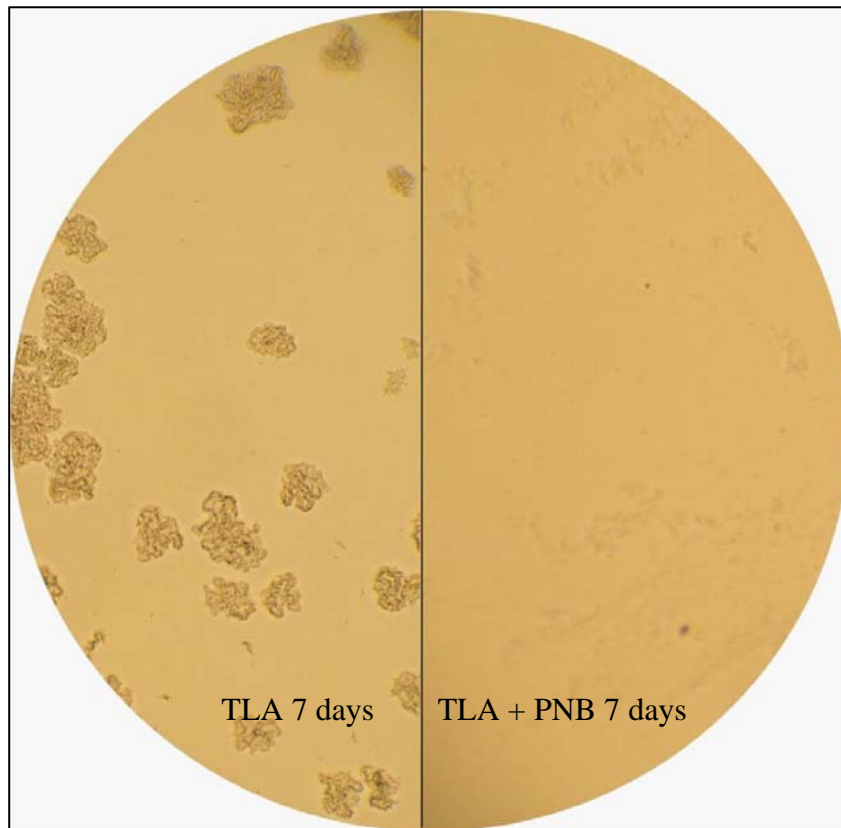
Pictures: cord formation of *M. tuberculosis*



Pictures: A. Martin



Pictures: A. Martin

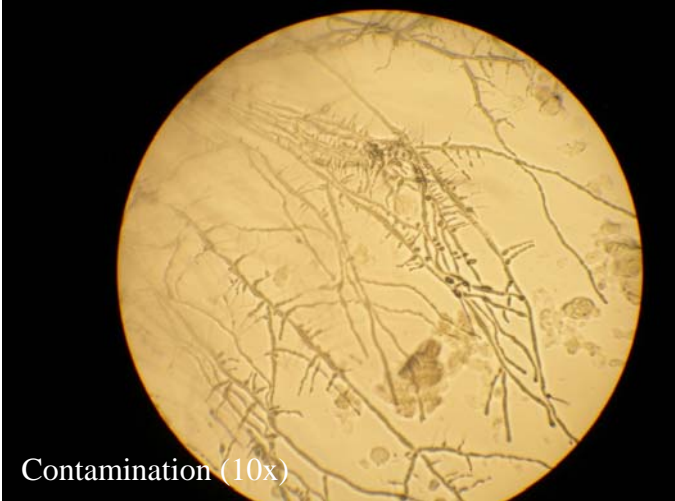


Pictures: A. Martin

Comparison compartments with 7H11 (TLA 7 days) and with 7H11+PNB (TLA+PNB 7 days)-(microscope objective 10x).

On TLA+PNB: there is no growth; no cord formation compared to 7H11, it means that is *M. tuberculosis* complex.

Contaminations



Pictures: A. Martin

References:

1. **WHO: Toman's Tuberculosis.** 2004. Case detection, treatment, and monitoring questions and answers Edited by T. Frieden WHO/HTM/TB/2004.334
2. **WHO.** 1998. Laboratory services in tuberculosis control. Part III: culture. WHO/TB/98.258
3. **Mejia GI, Castrillon L, Trujillo H, Robledo JA.** Microcolony detection in 7H11 thin layer culture is an alternative for rapid diagnosis of Mycobacterium tuberculosis infection Int J Tuberc Lung Dis. 1999 Feb;3(2):138-42
4. **Heifets L, Lindholm-Levy P.** Dilemmas and realities in the laboratory diagnosis of tuberculosis in low income countries. Int J Tuberc Lung Dis. 1999 Feb;3(2):88-9.
5. **Mejía GI, A Guzmán , CA Agudelo , H Trujillo, J Robledo.** Cinco años de experiencia con el agar de capa delgada para el diagnóstico rápido de tuberculosis. Biomedica 2004, 24 :52-9
6. **Robledo JA, Mejia GI, Morcillo N, Chacon L, Camacho M, Luna J, Zurita J Bodon A, Velasco M, Palomino JC, Martin A, Portaels F.** Evaluation of a rapid culture method for tuberculosis diagnosis: a Latin American multi-center study. Int J Tuberc Lung Dis. 2006 Jun;10(6):613-9
7. **Irfan Seema, Hasan Rumina, Kanij Akber, Hassan Qaiser and Azam Iqbal.** Evaluation of a microcolony detection method and phage assay for rapid detection of Mycobacterium tuberculosis in sputum samples. Southeast Asian J Trop Med Public Health. Vol 37 (6):1187-1195. 2006
8. **Robledo J, Mejia GI., Paniagua L, Martin A, Guzmán A.** Rapid detection of rifampicin and isoniazid resistance in *Mycobacterium tuberculosis* by the direct thin-layer agar method. Int J Tuberc Lung Dis. 2008, 12: 1482-1484.
9. **Martin A, Munga Waweru P, Babu Okatch F, Amondi N, Bonte L, Varaine F, Portaels F.** Implementation of the thin layer agar for the diagnosis of smear-negative pulmonary tuberculosis in high HIV prevalence settings in Homa Bay, Kenya. JCM, Aug 2009; p2632-2634