

TB



Bacteriology Examination to Stop



TB



Akiko FUJIKI



IRIT

The Research Institute
of Tuberculosis Japan

PREFACE

Tuberculosis (TB) is a serious problem globally both in developing countries and developed countries. WHO advocates DOTS (Directly Observed Treatment Short Course) strategy all over the world to control TB and TB bacteriology examination is a core component of DOTS. Since TB is a disease caused by TB bacilli, TB bacteriology examination is crucial for detecting TB bacilli and assessing the effect of the treatment. The success of TB control must be based on reliable results of bacteriology examination resulting from the use of quality techniques ensured by proper training.

This booklet has been developed for the overseas training participants conducted in our institute under the sponsorship of JICA (Japan International Cooperation Agency). It is focused on the fundamental procedures and technical methods of TB bacteriological examination, which are applicable in resource limited countries. It describes simply with step-by-step instructions and many illustrations, which can be utilized easily by any one who wants to learn these techniques. I hope this booklet will be useful not only for training course participants but also as a guide for field workers and their supervisors.

Detailed information on the procedures may be available in other references mentioned below. The booklet entitled "TB Microscopy", which has been developed few years ago covers sputum smear examination comprehensively and may be useful if it is applied together with this newly developed booklet.

Any comments and suggestions are highly welcome.

I wish to express my sincere thanks to Dr. Nobukatsu Ishikawa, Vice-director, RIT, for his support in making this booklet and Dr. Sang Jae Kim, Director, Korean Institute of Tuberculosis, KNTA for his valuable advice and suggestions.

Akiko Fujiki
Chief Medical Technologist
International Cooperation Department
The Research Institute of Tuberculosis, JATA
November 18, 2001

References:

- 1) Laboratory Services in Tuberculosis Control, Part II and III: Microscopy and Culture, WHO, 1998
- 2) The Public Health Service National Tuberculosis Reference Laboratory and the National Laboratory Network Minimum Requirements, Role and Operation in a Low-Income Country, IUATLD, 1998
- 3) Public Health Mycobacteriology, A Guide for the level III Laboratory, US Dept. of Health and Human Services, Public Health Service, CDC, 1985
- 4) TB Microscopy, The Research Institute of TB and Japan International Cooperation Agency, 1998

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Message

It is surprising to note how poorly doctors recognize the importance of quality in bacteriological examinations. This is sometimes also true for laboratory technicians themselves. This manual written by Ms. A. Fujiki was intended primarily to serve as a practical basis for those involved in laboratory work in the national tuberculosis control program, especially in developing countries, with special emphasis on the quality assurance aspect in laboratory work.

I would be most pleased if this document could help our colleagues in the front line of the fight against tuberculosis worldwide achieve a higher level of their work, resulting in high-quality diagnosis and treatment monitoring.

This book is a crystallization of the author's study and experience in teaching in the fields and in our Institute. Therefore, the partnership of the various countries' laboratory workers in publishing this is gratefully acknowledged.

Toru Mori, MD. PhD

Director,

Research Institute of Tuberculosis,

Japan Anti-Tuberculosis Association

TB BACTERIOLOGY EXAMINATION TO STOP TB

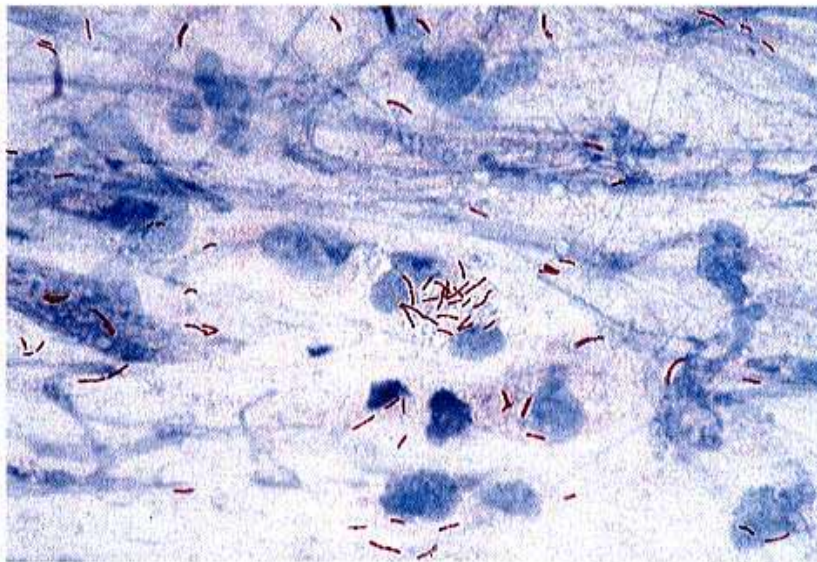
CONTENTS

DIRECT SMEAR EXAMINATION2 (Ziehl-Neelsen method)	2
PREPARATION OF EGG MEDIUM 8 (2% modified Ogawa medium)	8
CULTURE EXAMINATION FOR <i>M.tuberculosis</i>14 (NaOH modified Petroff method / NaOH Ogawa method)	14
2% MODIFIED OGAWA AND LOEWENSTEIN-JENSEN MEDIA	19
IDENTIFICATION OF <i>M.tuberculosis</i>20 (Niacin test / 68°C labile catalase test / Growth on PNB containing medium)	20
DRUG SUSCEPTIBILITY TEST26 (Proportion method)	26
<i>M.tuberculosis</i> and RUNYON GROUPS35	35
REAGENT FORMULAS36	36





DIRECT SMEAR EXAMINATION (Ziehl-Neelsen method)

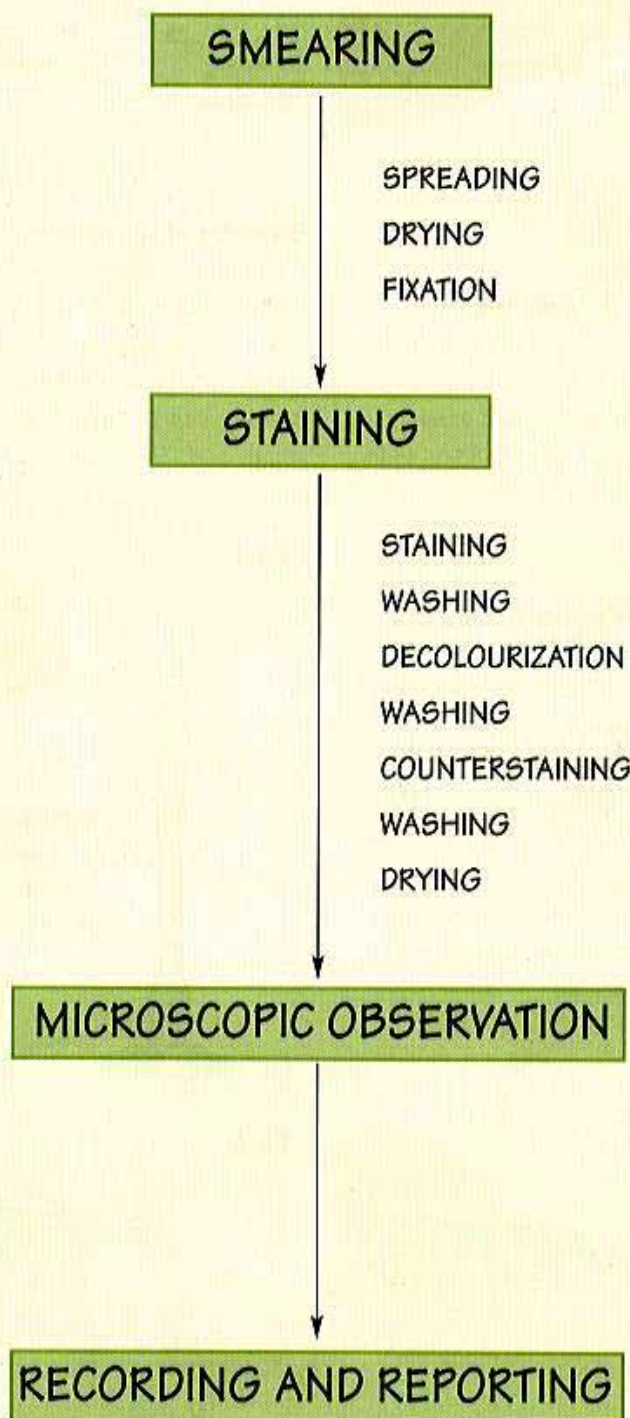


Tubercle bacilli in sputum observed as red rods by Ziehl-Neelsen staining under a microscopy at magnification of $\times 1,000$.

Microscopic examination of sputum is the most reliable method to detect the sources of TB infection in the community.

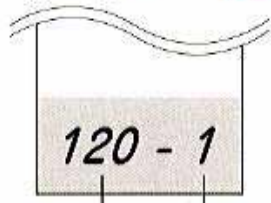
Before starting the examination, the date of examination, name, gender and age of the patient, reason for examination (diagnosis/follow-up), and sputum collection order (1st spot/morning/2nd spot) should be filled in laboratory note. Sputum to be examined is derived from the trachea and bronchi and not saliva from the buccal or nasal cavity.

FLOW CHART FOR DIRECT SMEAR EXAMINATION



DIRECT SMEAR PREPARATION

Fig.1

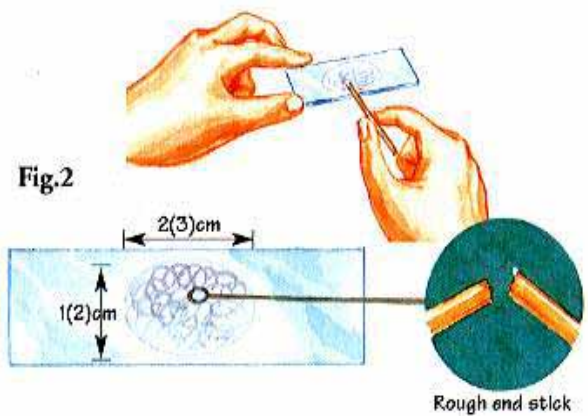


Yearly serial number Order number

Numbering the slide

Write down the yearly serial number and order number of sputum specimen on an end of the slide.

Fig.2



Smearing of the sputum

Fish out one loopful of yellowish particle of sputum. Spread the sputum evenly on a clean labeled glass slide, approximately 132cm or 233cm in size. The rough end bamboo splinter or coconut mid-rib stick can be used to take sputum to smear by using the rough end. The used sticks are burnt after day's work.

Fig.3



Removal of adherent sputum

Dip the wire loop in a washing bottle* and remove the excess sputum from the wire loop by moving it up and down in sand. (*Washed sand and 70% spirit or 5% cresol are contained in the bottle.)

Fig.4



Sterilization of the wire loop

Heat the wire loop in a flame till red hot. Adequate flame should be colorless or blue.

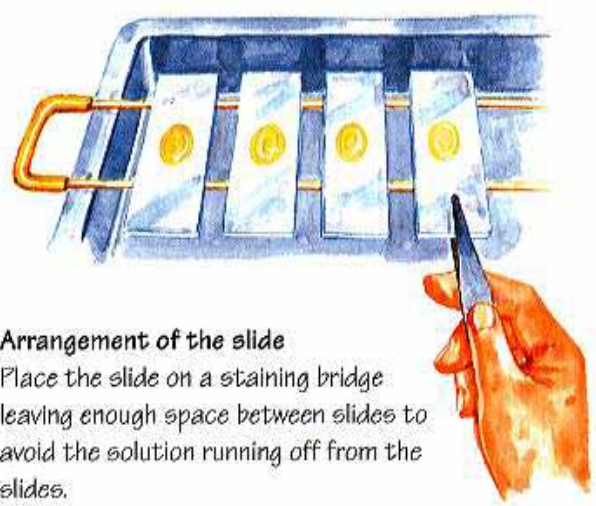
Fig.5



Drying and fixation of the smear

Allow the smear to dry completely at room temperature and fix it by passing through the flame 2 to 3 times, about 5 seconds each. Flame at the back of smeared surface of the slide.

Fig.6

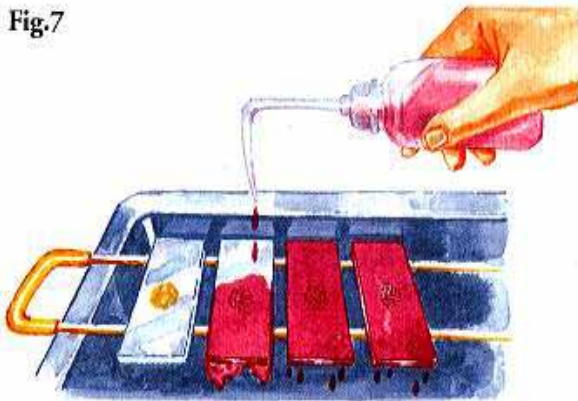


Arrangement of the slide

Place the slide on a staining bridge leaving enough space between slides to avoid the solution running off from the slides.

STAINING OF THE SMEAR

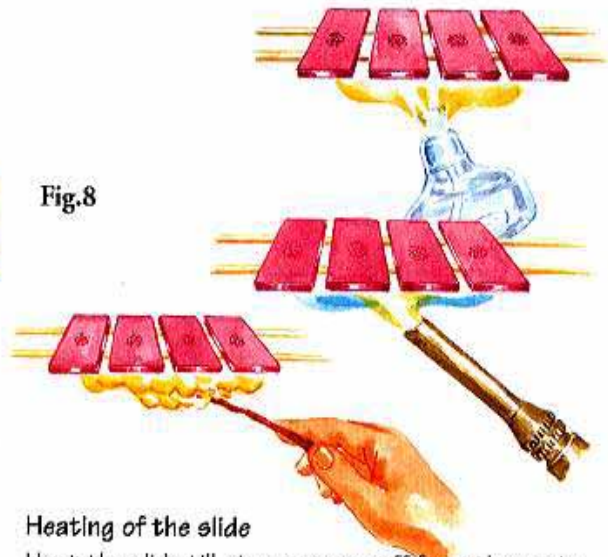
Fig.7



Staining with Ziehl's solution

Pour Ziehl's (carbol fuchsin) solution and cover the whole surface of the slide.

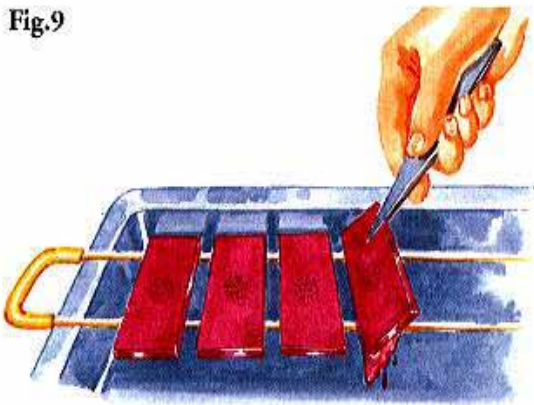
Fig.8



Heating of the slide

Heat the slide till steam comes off from the stain. Do not boil and do not allow the slide to dry. Leave it for 5 minutes. Repeat this procedure 2-3 times when alcohol lamp or spirit cotton are used for heating of the slide.

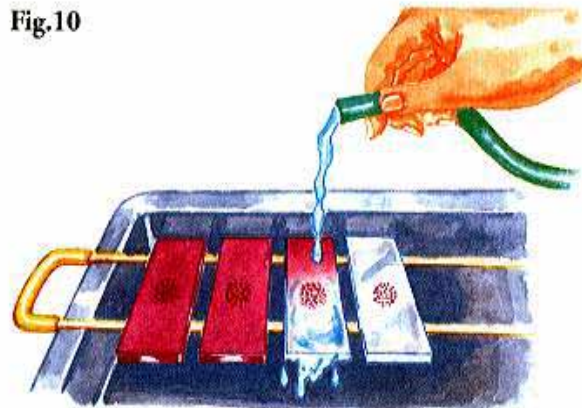
Fig.9



Removal of excess stain

Tilt the slide to drain off excess stain.

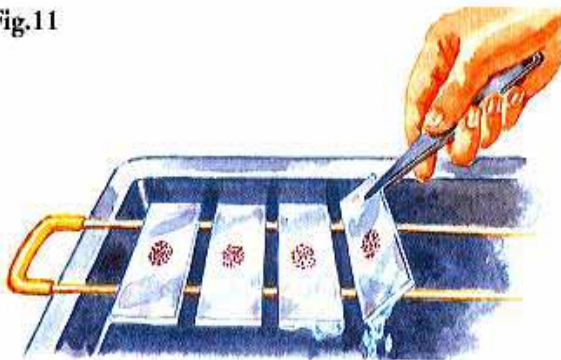
Fig.10



Washing of the slide

Wash the staining solution off with a gentle stream of running water.

Fig.11

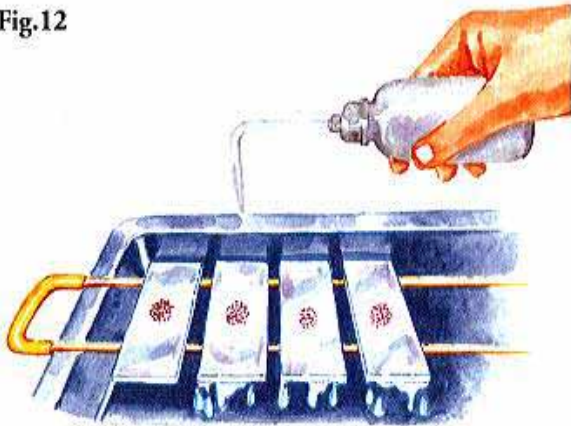


Draining off excess rinse water

Tilt the slide to drain off excess rinse water.

DECOLOURIZATION BY ACID-SOLUTION

Fig.12



Decolourization with 3% hydrochloric acid-ethanol
Decolourize with 3% hydrochloric acid-ethanol *until solution runs clear. (*25% H_2SO_4 water can be also used instead of 3% HCl-ethanol)

Fig.13



Washing of the slide
Wash the slide with a gentle stream of running water and tilt the slide to drain off excess rinse water.

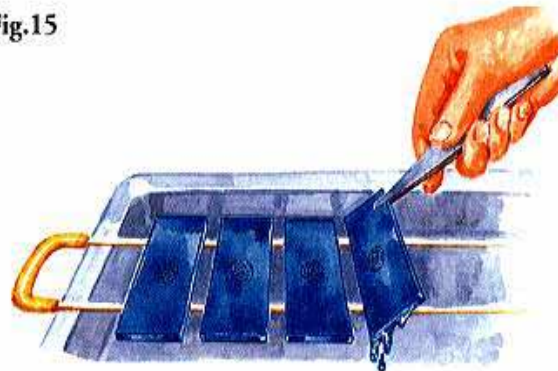
COUNTERSTAINING

Fig.14



Counterstaining with 0.1% methylene blue
Pour 0.1% methylene blue to cover the whole surface of the slide and leave for 2-3 seconds.

Fig.15



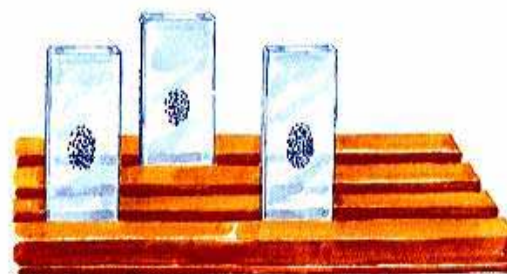
Removal of 0.1% methylene blue
Pour off 0.1% methylene blue.

Fig.16



Washing of the slide
Wash the slide with a gentle stream of running water.

Fig.17



Drying the stained slide
Tilt and place the slide on the slide rack to dry in the air. Do not place under the sun to dry.

MICROSCOPIC EXAMINATION AND MAINTENANCE OF MICROSCOPE

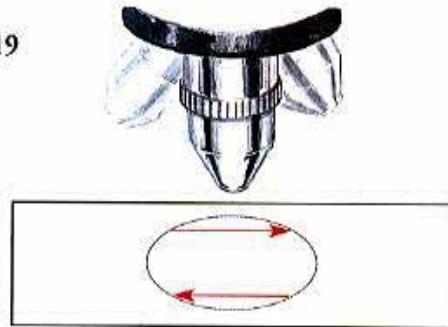
Fig.18



Application of immersion oil

Put one drop of immersion oil on the stained smear. Do not touch the smear with the tip of dropper to avoid transfer of AFB from one smear to another.

Fig.19



Scanning of the smear

Examine the smear under x100 objective with 10x eye piece lens. AFB appear red or pink coloured and the background is stained in blue colour. Read at least 100 visual fields to give a report as negative. Record the result on the laboratory notebook.

Fig.20



Storage of slides

Remove the immersion oil from the slides by dipping into the jar containing xylol. Keep them in the slide box for confirmation or for quality check.

Fig.21

Recording and reporting by WHO / IUATLD recommended method

Acid Fast Bacilli (AFB)* appear red or pink colored rod and the background is stained in blue colour.

(+++): More than 10 AFB per field in 20 fields

(++) : 1-10 AFB per field in 50 fields

(+) : 10-99 AFB per 100 fields

Exact figures : 1-9 AFB per 100 fields. Report number found and request repeat specimen(e.g.+,)

(-) : No acid-fast bacilli found in at least 100 fields

(*Never report as "M.tuberculosis" by smear result only.)

Fig.22



Cleaning of the microscope

Clean the objective lens with lenspaper moistened with petroleum benzene.

Fig.23

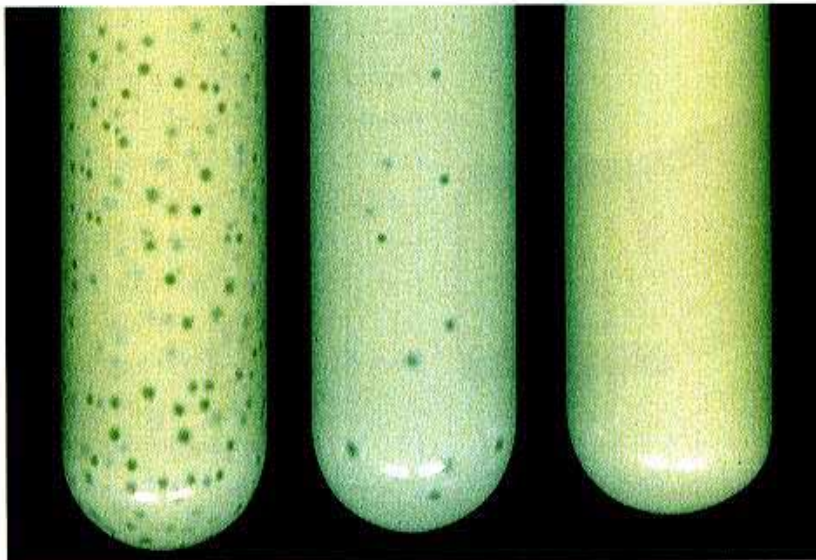


Storage of the microscope

Cover the microscope with vinyl cover and store it in a place free from moisture and dust.



PREPARATION OF EGG MEDIUM (2% modified Ogawa medium)

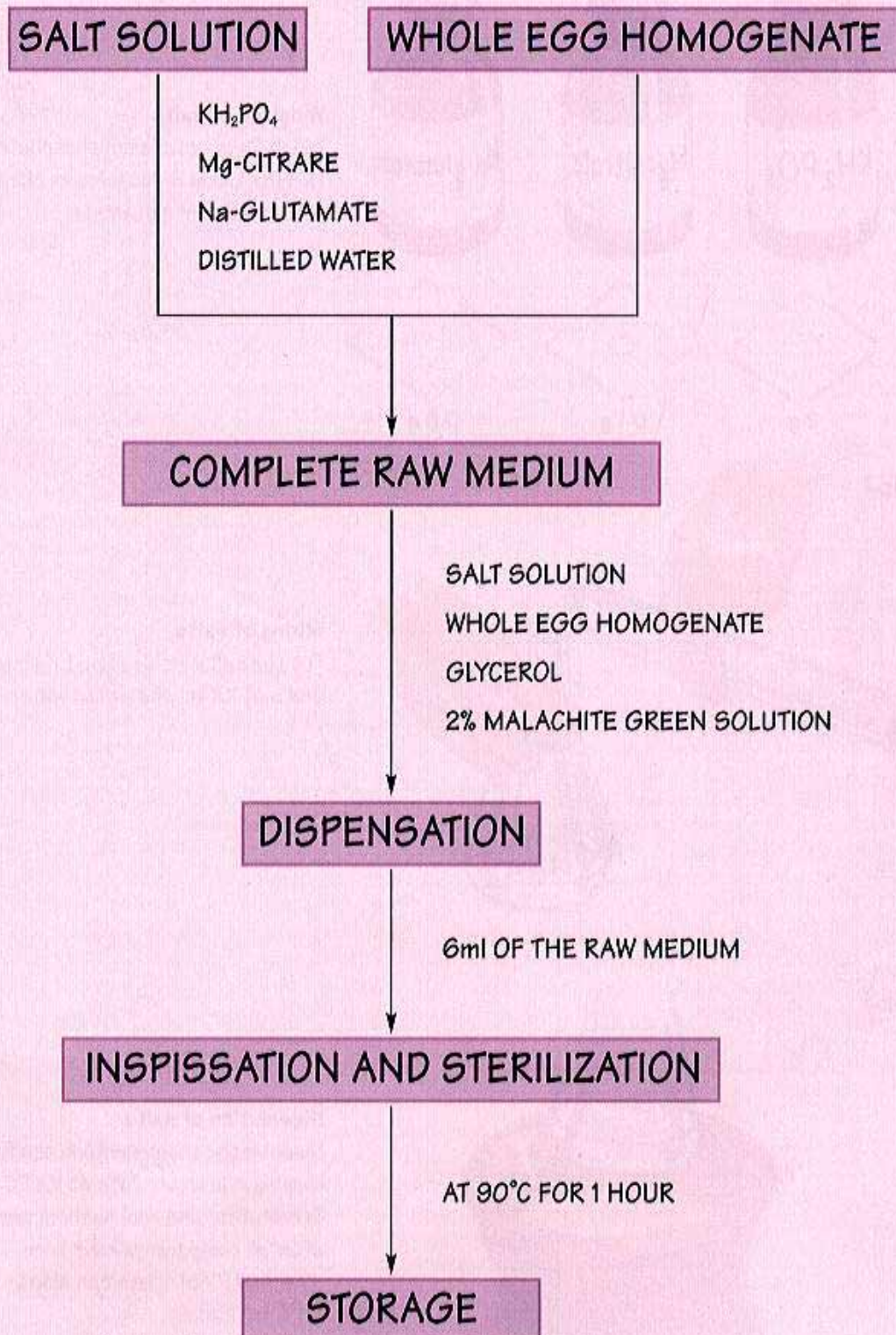


Adequacy of temperature for medium coagulation can be checked by the appearance of spots on the back of the culture slope (bubbles in the medium). From left, too high temperature, adequate temperature, adequate temperature.

Solid culture medium, such as egg medium containing glycerol and asparagine or glutamate, is the most commonly used medium to isolate mycobacteria. Modified Ogawa and L-J media do not differ in respect of the positivity and contamination rate. But the formula and preparation of modified Ogawa medium are fairly more economical and simpler.

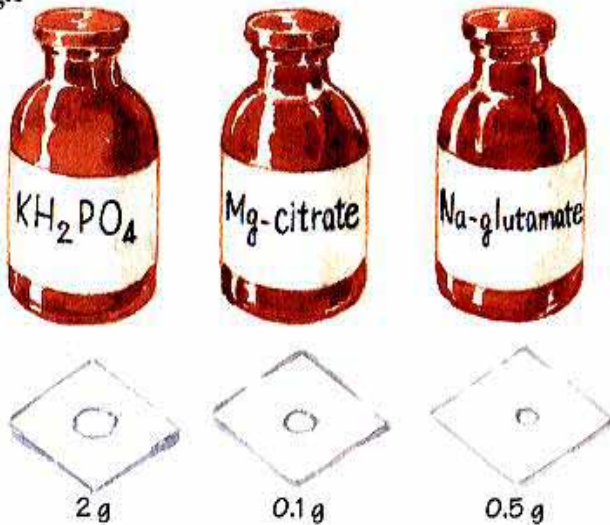
Glasswares must be brushed and cleaned well. All utensils used in the preparation of the medium must be sterilized with autoclave or hot air oven. It is recommended to set the temperature of incubator at 30°C beforehand.

FLOW CHART FOR PREPARATION OF EGG MEDIUM



PREPARATION OF SALT SOLUTION

Fig.1



Weighing of salts

Weigh 2g of potassium phosphate mono-basic (KH_2PO_4), 0.1g of magnesium citrate, and 0.5g of sodium glutamate.

Fig.2



Mixing of salts

Put the salts inside of an Erlenmeyer flask and add 100ml of distilled water.

Fig.3



Dissolution of salts

Dissolve the components in the flask by keeping it in water bath at 100°C for 30minutes* and cool to room temperature after all components have been dissolved. (*Autoclave can also be used at 121°C for 15min.)

PREPARATION OF WHOLE EGG HOMOGENATE

Fig.4



Cleaning of outside of egg shell

Clean outside of egg shell by means of brush and running tap water. Dry them up in a basket.

Fig.5



Sterilization of outer shell

Wipe the outer surface of eggs with spirit cotton and allow alcohol to dry up before cracking the shell.

Fig.6



Egg cracking

Crack the egg shell one by one and pour the contents into a Petri-dish to check the freshness of the egg (spoiled egg). If not fresh, discard and change the Petri-dish with a new one.

Fig.7



Removal of chalaza

Remove the chalaza attached to the egg yolk by means of a sterile forceps. Transfer the egg fluid to a beaker.

Fig.8



Beating of eggs

By means of chopsticks*, beat all eggs vigorously within the beaker till the egg fluid is no more sticking to the chopsticks. (*Electric mixer can be used for this purpose)

Fig.9



Filtration of eggs

Filter the whole egg homogenate through 2 layers of sterile gauze into a sterile cylinder until it reaches the 200ml mark.

PREPARATION OF COMPLETE RAW MEDIUM

Fig.10



Addition of glycerol

Add 4ml of glycerol to the previously prepared salt solution.

Fig.11



Addition of 2% malachite green

Add 4ml of malachite green on top.

Fig.12



Mixing of the contents

Mix contents of the flask gently.

Fig.13



Addition of egg fluid

Pour the filtered egg fluid gently to the solution along the side the wall of the flask to avoid the formation of air bubbles.

Fig.14



Mixing of whole egg homogenate

Mix the contents of the flask gently so as not create bubbles.

Fig.15

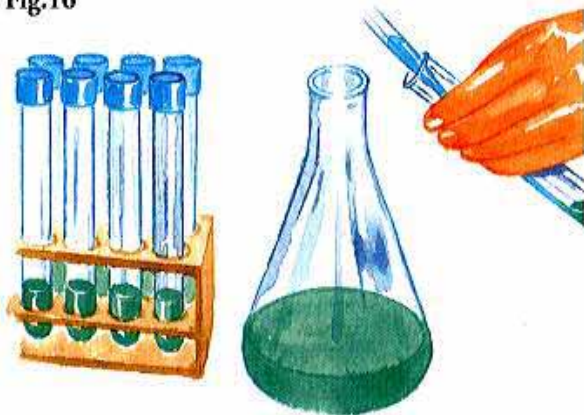


Riddance of air bubbles

Allow the flask to stand for 30minutes to float air bubbles to the surface. The colour of raw medium becomes light greenish colour from dark greenish colour.

DISPENSING OF RAW MEDIUM AND INSPISSATION OF MEDIUM

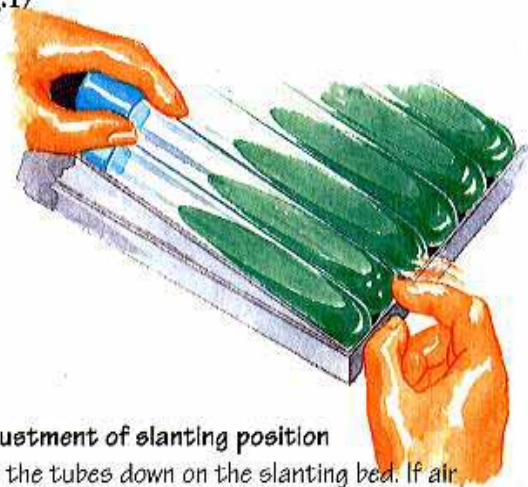
Fig.16



Distribution of medium

Distribute 6ml of raw medium along the side wall of each tube to avoid air bubble formation.

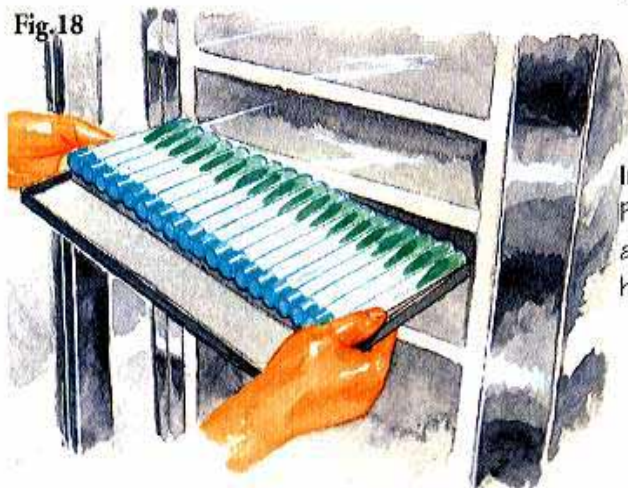
Fig.17



Adjustment of slanting position

Lay the tubes down on the slanting bed. If air bubbles are still observed, fillip the bottom towards the cap until the bubbles disappear.

Fig.18

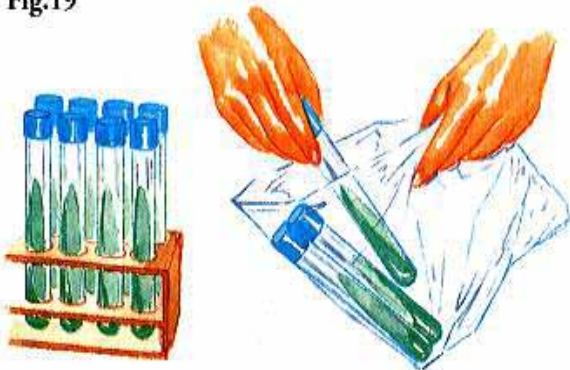


Inspissation

Place the beds in an inspissator which has been set at 90°C beforehand. Leave them at 90°C for one hour.

STORAGE

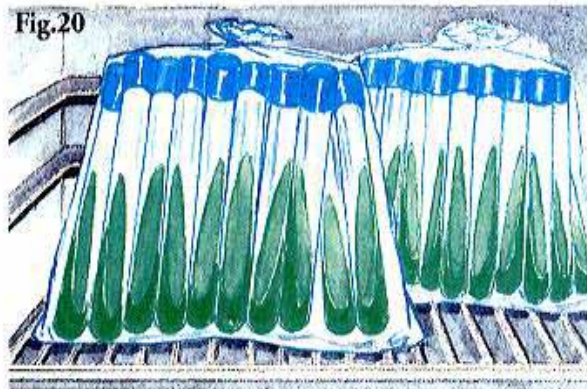
Fig.19



Preservation in plastic bag

After inspissation, leave the tubes on the bench to cool down to room temperature. Then keep them in a plastic bag, tighten its mouth with a rubber band.

Fig.20



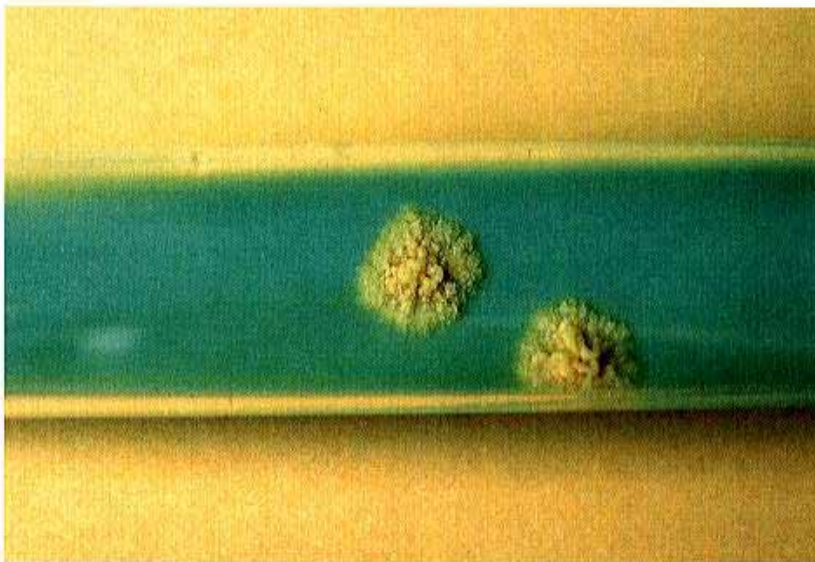
Storage in refrigerator

Note the date of preparation on the plastic bag and keep the tubes in upright position in a refrigerator till use.



CULTURE EXAMINATION FOR *M.tuberculosis*

(NaOH modified Petroff method / NaOH Ogawa method)

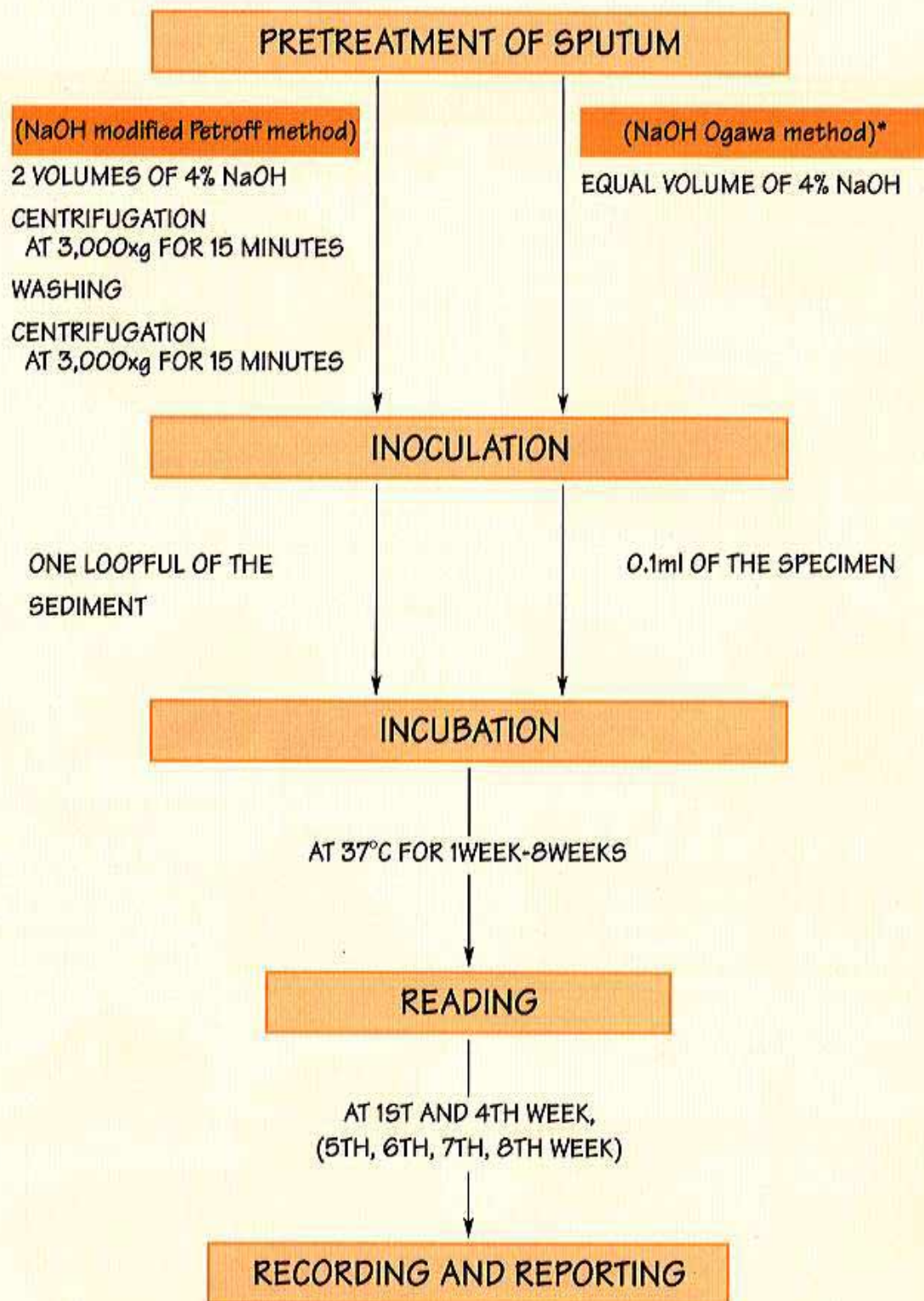


M.tuberculosis show eugonic growth on egg medium and form dry, friable colonies with irregular margin, a cauliflower center and buff color.

Culture examination can detect about twice more new cases of tuberculosis than direct smear examination, including the patients who are discharging not so large number of bacilli.

There is high possibility for making occurrence of aerosols throughout the procedure, especially opening the cap of sputum container, mixing of sputum and inoculating of sputum. Formation of aerosols must be avoided. Avoid contamination of the specimens as well as possible human infection.

FLOW CHART FOR PRIMARY CULTURE EXAMINATION



*Laboratories where centrifuges are not available, it could be employed.

PRETREATMENT AND INOCULATION OF SPUTUM SPECIMEN

The specimen exposure time to 4% NaOH must be strictly followed to prevent over kill of tubercle bacilli. Financial and human resources are available, NaOH modified Petroff method is recommended. If centrifuge is not available, NaOH Ogawa method can be used.

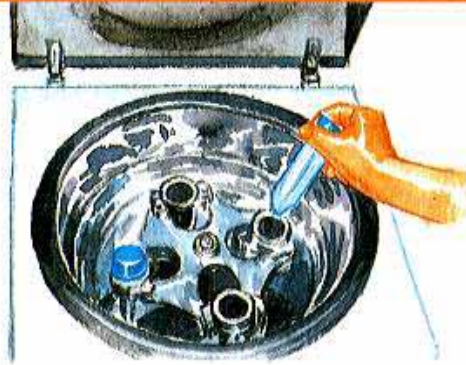
(NaOH modified Petroff method)

Fig.1



Addition of 4% NaOH
Add two volumes of 4% NaOH to one volume of sputum specimen. Then tighten cap of container and shake to digest and let stand for 15 minutes at room temperature.

Fig.2



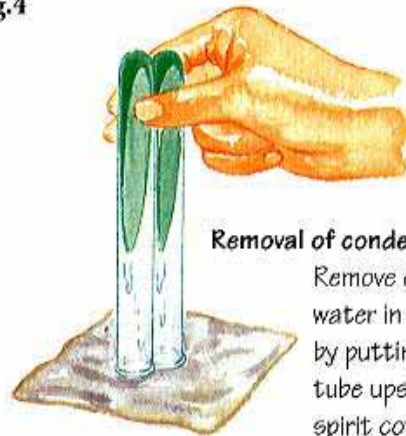
Centrifugation of the specimen.
Centrifuge at 3,000 xg for 15 minutes. Then leave it for 10 minutes and pour off supernatant.

Fig.3



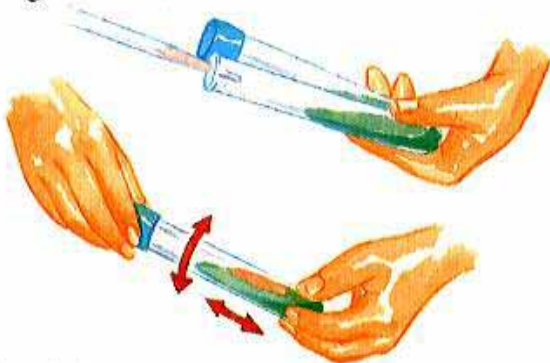
Washing of the specimen by centrifugation
Add 15ml of sterile distilled water and resuspend sediment. Then centrifuge at 3,000xg for 15 minutes again. Allow it for 10 minutes and decant supernatant.

Fig.4



Removal of condensed water
Remove condensed water in the medium by putting the media tube upside-down on spirit cotton.

Fig.5



Inoculation
Inoculate one drop of sediment onto each of two culture tubes.

Fig.6

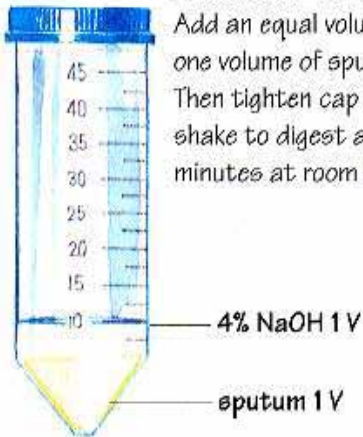


Loosening the caps
Loosen the caps of inoculated medium and lay the tubes on the slanting bed. Keep the slants face upward.

(Further procedures will be followed from Fig.7)

(NaOH Ogawa method)

Fig.1



Addition of 4% NaOH

Add an equal volume of 4% NaOH to one volume of sputum specimen. Then tighten cap of container and shake to digest and let stand for 15 minutes at room temperature.

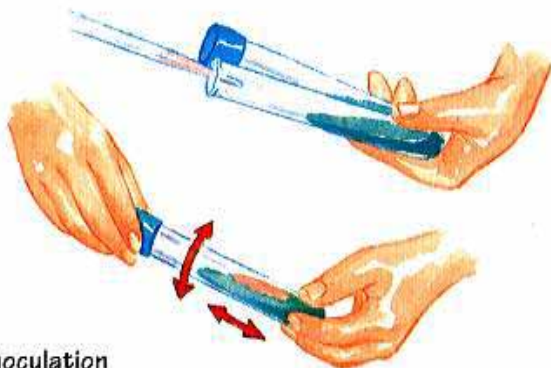
Fig.2



Removal of condensed water

Remove condensed water in the medium by putting the media tube upside-down on spirit cotton.

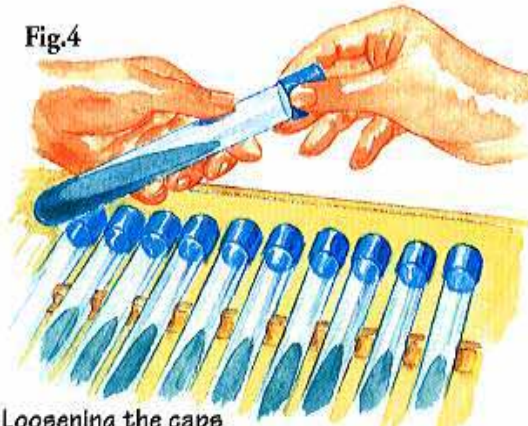
Fig.3



Inoculation

Inoculate 0.1ml of digested specimen onto each of two culture tubes. Then spread the inoculum evenly over the whole surface of each medium.

Fig.4



Loosening the caps

Loosen the caps of inoculated medium and lay the tubes on the slanting bed. Keep the slants face upward.

(Further procedures will be followed from Fig.7)

Decontamination in transit

- 1) Add the same volume of 1% CPC (Cetylpyridinium chloride) to the specimen, keep it at more than 20°C.
- 2) Add saline to the mixture as much as possible, then centrifuge 3,000 xg for 20min., and leave for 15min. Discard supernatant. Repeat the procedure.
- 3) Inoculate 0.1ml of the sediment to the media.

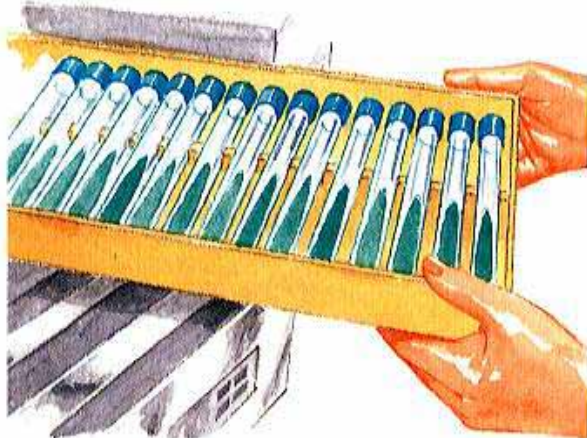
Decontamination for *Pseudomonas* species

- 1) Add equal volume of 5% oxalic acid to the specimen, then vortex to mix and leave for 30min.
- 2) Add 10 volumes of sterile saline to the mixture and centrifuge for 15 min. at 3,000 xg, then leave for 15min.
- 3) Decant the supernatant and add a few drops of phenol red indicator* to the sediment.
- 4) Add 4% NaOH until pale pink colour forms, then inoculate 0.1ml of sediment to the media.

*Phenol red 8mg+4% NaOH 20ml+H₂O 980ml

INCUBATION

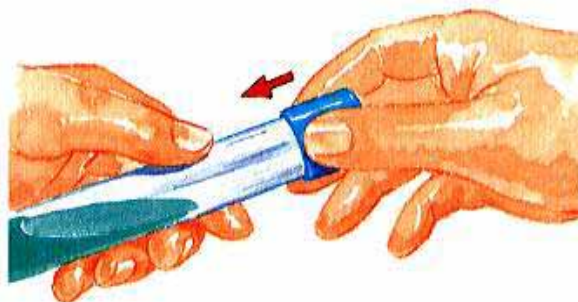
Fig.7



Incubation

Keep the inoculated slants in the incubator at 37°C.

Fig.8



Tightening the caps

After a few days or more of incubation, when the surface of media has been dried, tighten the caps of incubated medium, then continue the incubation.

RECORDING AND REPORTING

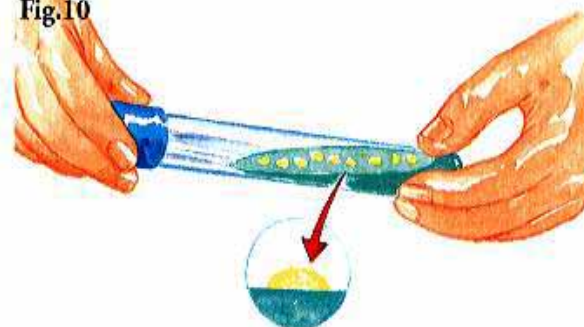
Fig.9



Observation at 1W and 4W

Observe the culture at one week for rapid growers and at 4 weeks for slow growers. If colonies do not appear at 4th week, observe weekly until 8 weeks before giving decision as NEGATIVE.

Fig.10



Reading the growth at 4W

Read the growth which appeared as colony of dry and irregular margin with buff color at 4 weeks of incubation.

Fig.11

Recording and reporting by WHO

- If the cultures have been contaminated, report immediately and a repeat specimen requested.
- If cultures are positive and growth has been identified as *M. tuberculosis*, a report should be sent out immediately.
- At 4 weeks an interim report (optional) could be sent out on all negative specimens.
- At 8 weeks a final report should be issued containing all the data previously reported so that earlier interim report can be destroyed and only the final report retained in the patients' file.

(-)	: No growth
Actual figures	: 1-19 colonies, record the actual number of colonies (e.g.+12)
(+)	: 20-100 colonies
(++)	: 100-200 colonies
(+++)	: 200-500 colonies, almost confluent growth
(++++)	: more than 500 colonies, confluent growth
contaminated	: contaminated (complete contamination)



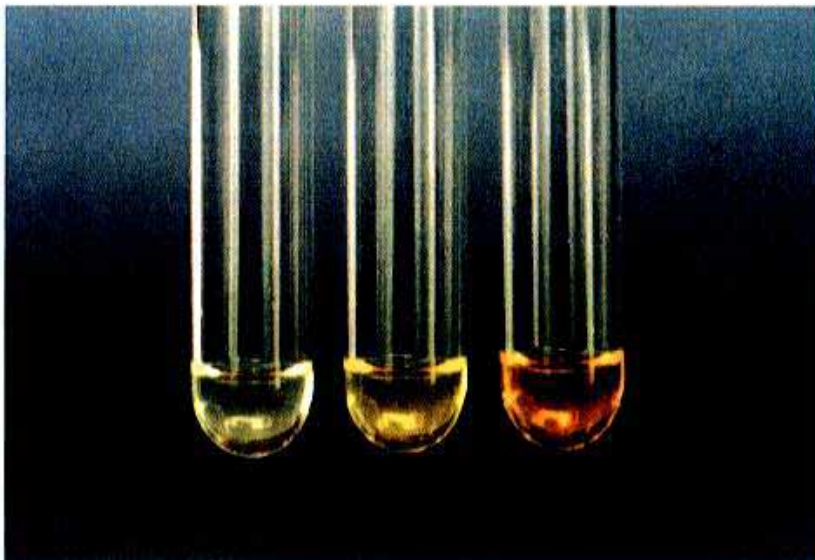
2% MODIFIED OGAWA AND LOEWENSTEIN-JENSEN MEDIA

Medium		2% modified Ogawa	L-J(without potato starch)
Ingredient & Preparation	Monopotassium phosphate(KH_2PO_4)	2.0g	2.4g
	Magnesium sulfate($\text{MgSO}_4 \cdot 12\text{H}_2\text{O}$)	-	0.24g
	Magnesium citrate	0.1g	0.6g
	Sodium glutamate	0.5g	-
	Asparagine	-	3.6g
	Glycerol	4ml	12ml
	Distilled water	100ml	600ml
	Egg homogenate	200ml	1000ml
	Malachite green (2%)	4ml	20ml
Inspissation	at 90°C for 60 min.	at 90°C for 50 min.	
pH of condensed water		6.4 - 6.6	6.8 - 7.0
Pretreatment		<ol style="list-style-type: none"> 1) Add an equal volume of 4% NaOH solution to one volume of the specimen. 2) Stir by mixing apparatus or pipette for 1-2min, and allow it for 15min. 	<ol style="list-style-type: none"> 1) Add two volumes of 4% NaOH solution to one volume of the specimen. 2) Shake vigorously by hand. 3) Allow it for 15min. at room temperature. 4) Centrifuge at 3,000 xg for 15 min. 5) Stand on the rack for 10min. 6) Decant the supernatant. 7) Add sterile distilled water and centrifuge at 3,000 xg for 15min. 8) Stand on the rack for 10min. and decant the supernatant.
Inoculation		Inoculate 0.1ml of mixture on the medium by the pipette.	Inoculate the sediment on the medium by the pipette or the loop.



IDENTIFICATION OF *M.tuberculosis*

(Niacin test / 68°C labile catalase test /
Growth on PNB containing medium)

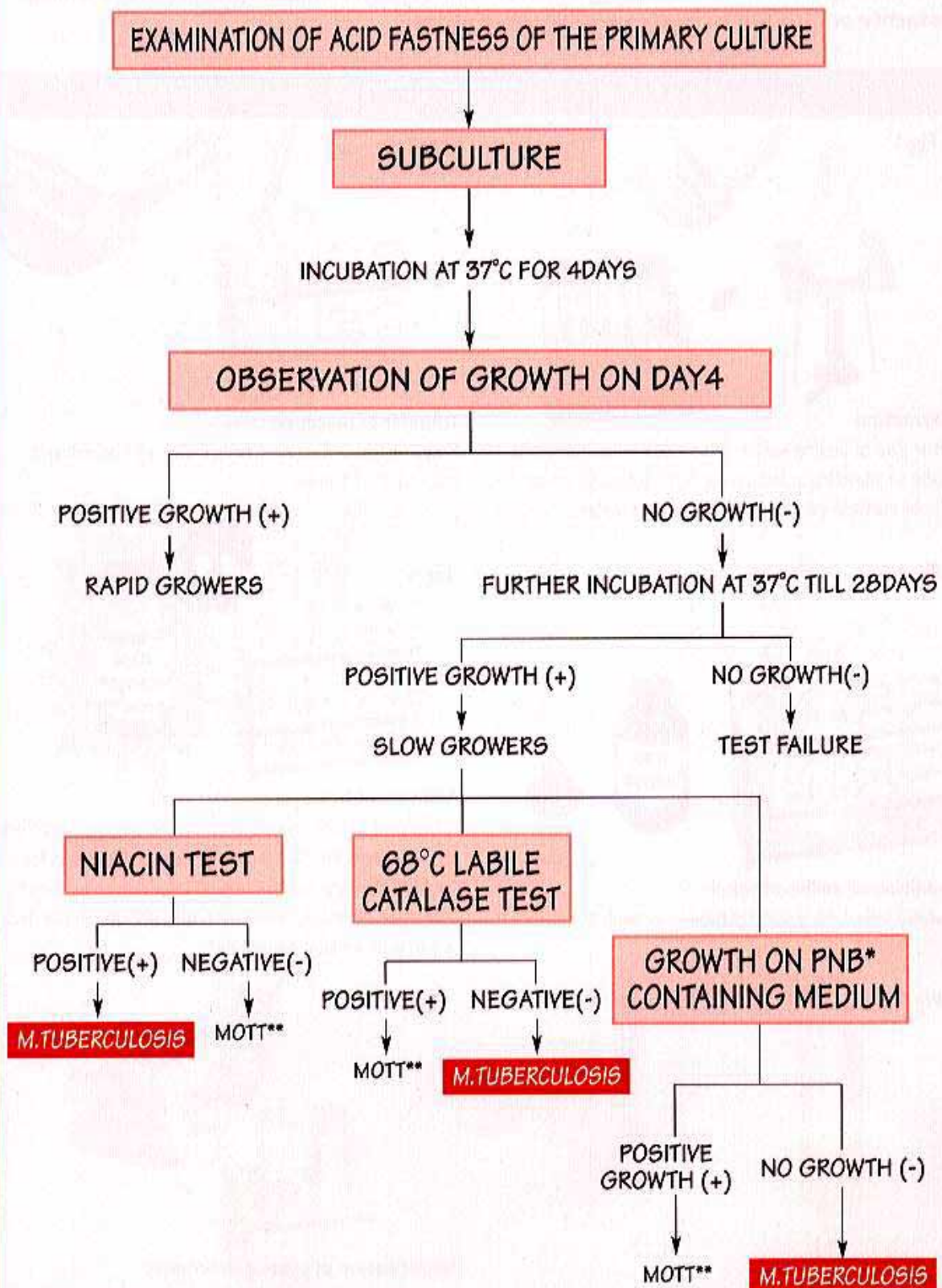


Yellow color indicates positive niacin test by aniline method. From left, (-), (+), (+++).

There is no completely reliable single test that will differentiate *M. tuberculosis* from other mycobacteria. Therefore at least one or more precise identification test must be applied besides morphological observation.

Identification tests must be performed with the subcultured strain, not with the primarily isolated strain. If culture is contaminated, decontamination is required.

FLOW CHART FOR IDENTIFICATION OF *M.tuberculosis*



* P-nitrobenzoic acid

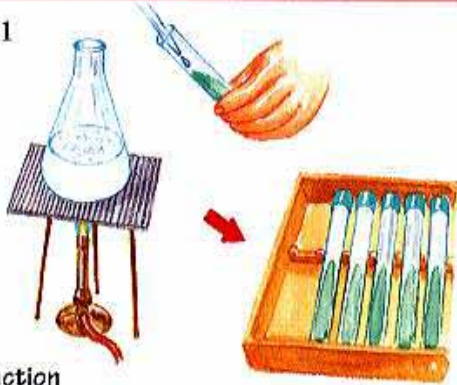
**Mycobacteria other than tuberculosis

NIACIN TEST

A culture must be at least 3 to 4 weeks old and must have without any contamination and sufficient growth of 100 or more colonies. Niacin test can be made by either with chemical reagents or with commercially available paper strips.

(Aniline method)

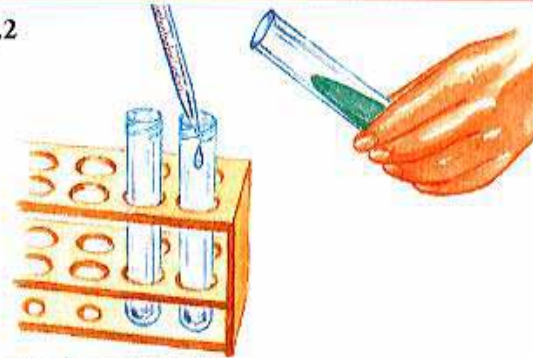
Fig.1



Extraction

Pour 2ml of boiling water onto the media. Keep the tube at slanting position for 10 minutes to cover whole surface of medium with boiling water.

Fig.2



Transfer of niacin extract

Prepare two tubes and take 0.2ml of the extract into each of tubes.

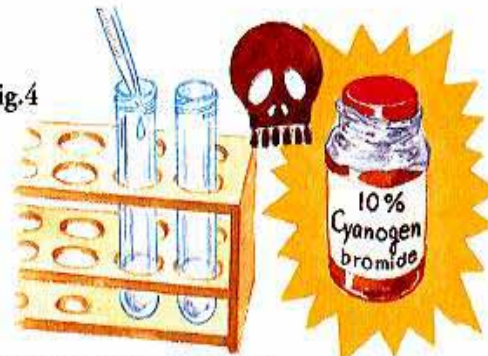
Fig.3



Addition of aniline ethanol

Add 0.1ml of 4% aniline ethanol in each tube.

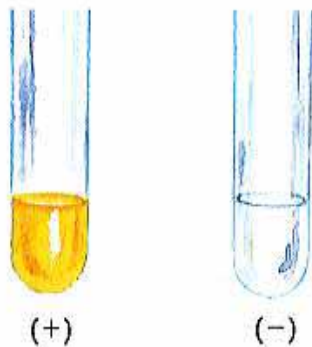
Fig.4



Addition of 10% cyanogen bromide

Add 0.1ml of 10% cyanogen bromide solution into one of the tubes. Mix them gently. The rest tube is for control. Be very careful not to inhale the vapour of cyanogen bromide. This procedure should be carried out in well-ventilated cabinet.

Fig.5



Result of niacin test

Yellow color indicates presence of niacin.

M.tuberculosis usually show niacin positive reaction.

Fig.6



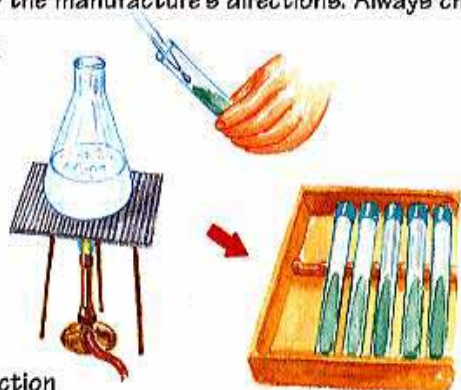
Detoxification of cyanogen bromide

Cyanogen bromide is POISON. Cyanogen bromide in the tube should be detoxified by adding 0.2ml of alkaline (4% NaOH) into the tube after the test, then autoclave and wash the tube.

(Paper strip method)

Follow the manufacturer's directions. Always check the expiration date of commercial paper strips.

Fig.1



Extraction

Pour 2ml of boiling water onto the media. Keep the tube at slanting position for 10 minutes to cover whole surface of medium with boiling water.

Fig.2



Transfer of niacin extract

Take 1ml of the liquid extract into a tube.

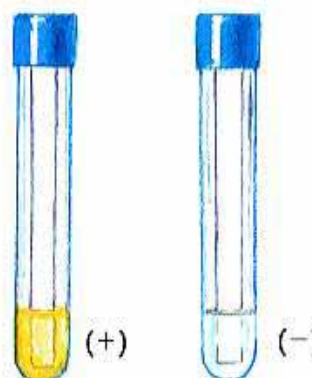
Fig.3



Introduction of Niacin test paper

Introduction the niacin test paper strip into the tube, then cap the tube. Leave it for 15 minutes.

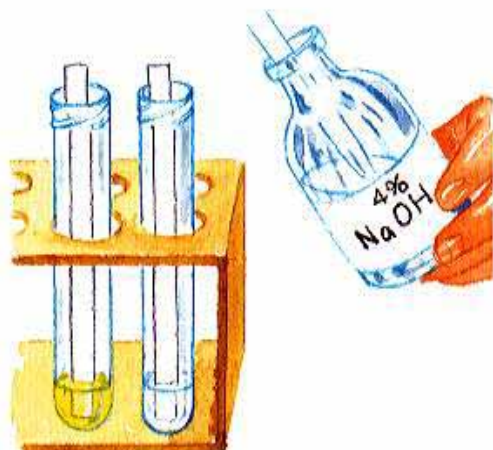
Fig.4



Result of niacin test

Observe the colour of the extract in the tube. Yellow colour indicates presence of niacin.

Fig.5



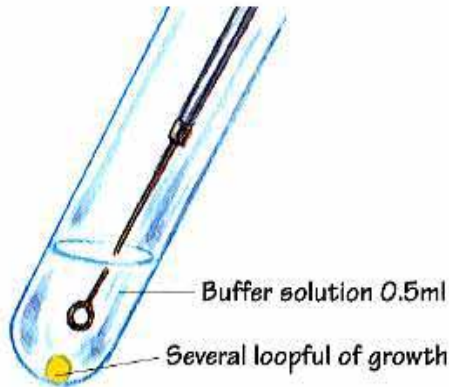
Detoxification of cyanogen bromide

Cyanogen bromide is POISON. Cyanogen bromide in the tube should be detoxified by adding 0.2ml of alkaline (4% NaOH) into the tube after the test, then autoclave and wash the tube.

68°C LABILE CATALASE TEST

If niacin test is not available, 68°C labile catalase test can be used to differentiate *M.tuberculosis* from other mycobacteria.

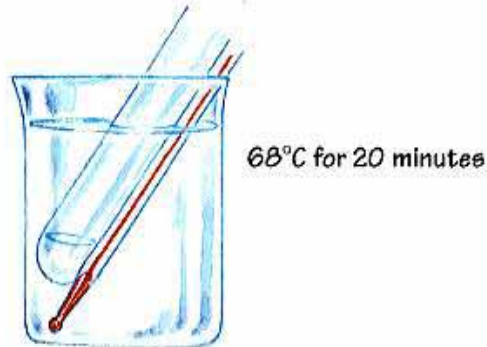
Fig.1



Suspension of the growth

Suspend several loopful of growth into the screw cap tube containing 0.5ml of buffer solution (pH7.0).

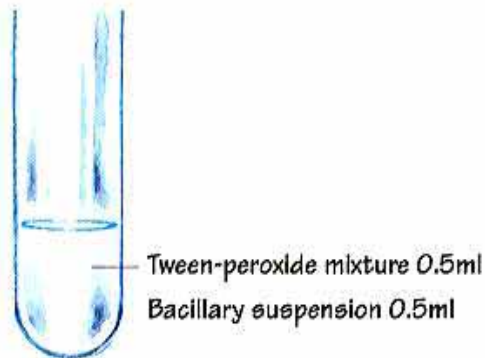
Fig.2



Incubation of the suspension

Incubate the suspension in a 68°C water bath for 20 minutes. Then cool the suspension to room temperature.

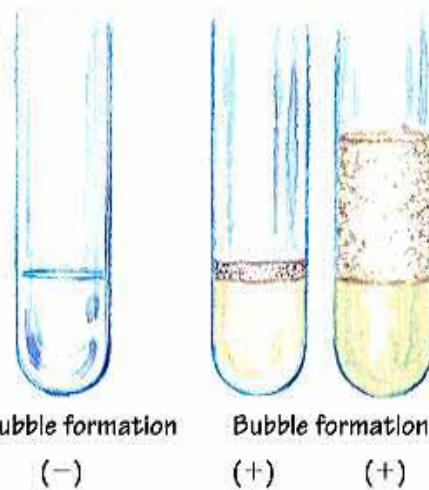
Fig.3



Addition of Tween-peroxide mixture

Add 0.5ml of the freshly-prepared Tween-peroxide mixture and recap tubes loosely.

Fig.4



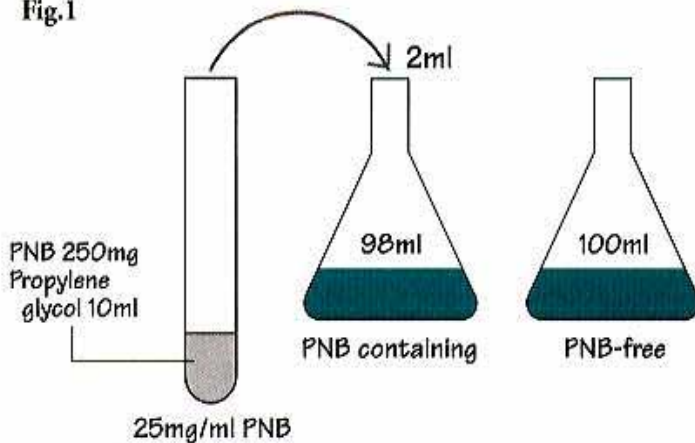
Result of the test

Observe the formation of bubbles appearing on the surface of the liquid. Do not shake the tube. Tween 80 also may form bubbles when shaken. Hold negative tubes for 20 minutes before discarding.



GROWTH ON PNB CONTAINING MEDIUM

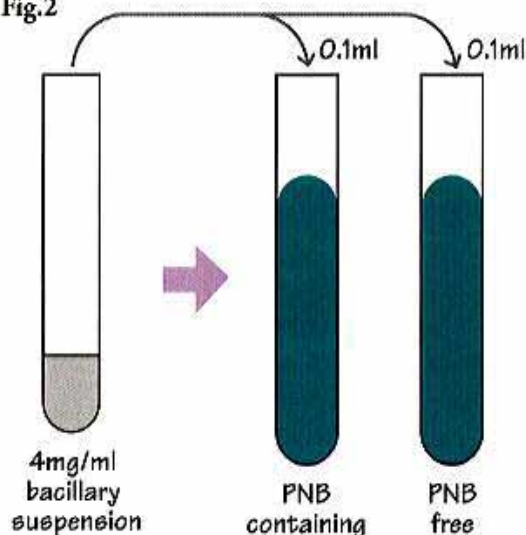
Fig.1



Preparation of 0.5mg/ml-PNB containing medium

Dissolve 250mg of PNB into 10ml of propylene glycol. Add 2ml of PNB solution into 98ml of L-J or 2% modified Ogawa raw medium. PNB free medium is prepared with the same batch of the medium as well. Dispense 6ml of PNB free or 6ml of PNB containing medium into each tube, then inspissate them at 90°C for 50minutes (L-J) or 60minutes (2% modified Ogawa)

Fig.2



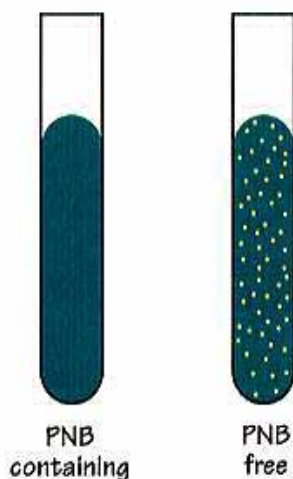
Inoculation and incubation

Inoculate 0.1ml or one loopfull of the suspension* onto two slants of PNB containing and PNB free media. Incubate the slants at 37°C for 4days to 4weeks.

*One loopful of growth with a 3mm diameter-loop is mixed with 5ml of sterile distilled water (4mg/ml-suspension).

Fig.3

No growth(-) Positive growth(+)



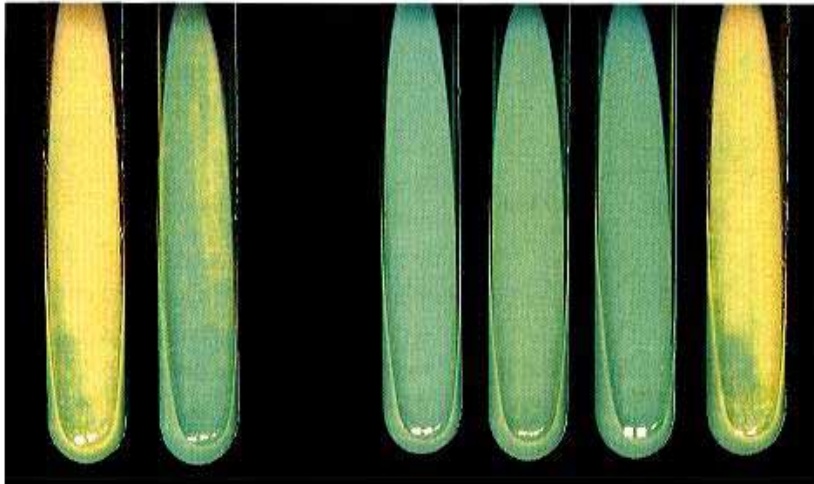
Reading of the result

Observe the culture at 4th day and 4th week of incubation. *M.tuberculosis* does not grow on PNB containing medium even at 4weeks of incubation. Though few colonies are observed on PNB containing medium, it is regarded as negative.



DRUG SUSCEPTIBILITY TEST

(Proportion method)

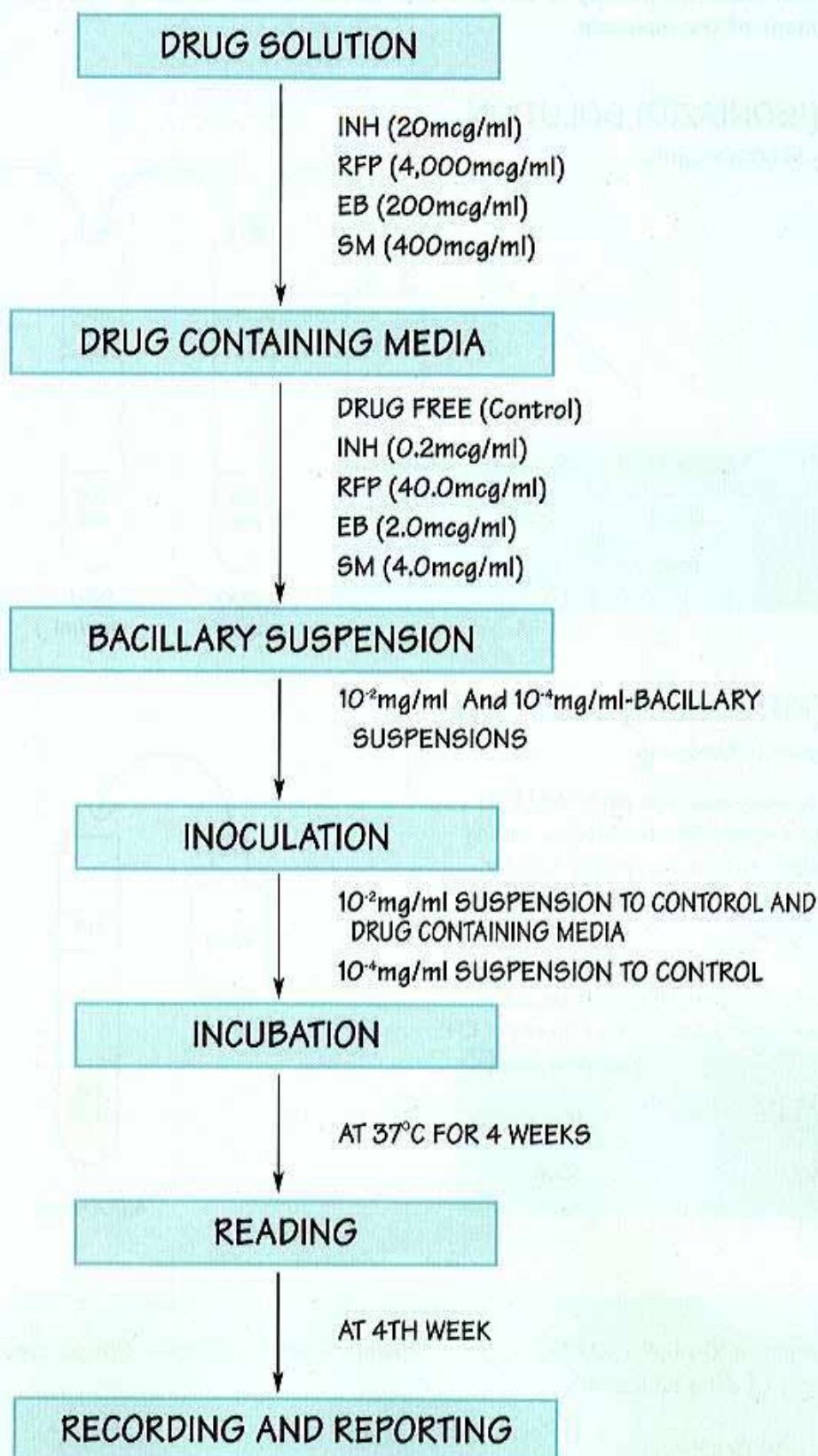


Growth of *M.tuberculosis* on drug free / containing media. From left, drug free medium at 10^{-2} dilution of bacillary suspension (++++), drug free medium at 10^{-4} dilution of bacillary suspension (+++), INH 0.2mcg (-/ Sensitive), RFP 40mcg (-/ Sensitive), EB 2.0mcg (-/ Sensitive), SM 4.0mcg (+++ / Resistant).

Drug susceptibility test is very important not only for the treatment of individual patients to choose most effective drug regimen but also for epidemiological purpose to assess the efficiency of the treatment service in national tuberculosis control program. The test should be conducted only at national or central level of reference laboratory.

If colonies are less than 5 colonies in the primary culture, the drug susceptibility test should not be made. Drugs used for the test must be as pure as possible and never use the tablets or capsules to be used for clinical purpose. Drug-free medium and drug containing media should be prepared with the same batch of medium. All glasswares and distilled water must be sterilized before the test and every procedures should be strictly aseptic. The test must be performed with enough growth of subculture within 1 to 2 weeks.

FLOW CHART FOR DRUG SUSCEPTIBILITY TEST



PREPARATION OF DRUG SOLUTIONS

The true potency of the drug is the number of micrograms of active drug per milligram total weight of the product. Not all antimicrobial drugs have been isolated in pure form, and a portion of their weight may be due to impurities or to the sulfate or another radical component of the molecule.

INH (ISONIAZID) SOLUTION

Potency is 1,000mcg/mg

INH	2,000mcg/ml	200mcg/ml	20mcg/ml
INH	20mg	1ml	1ml
Dis.water	10ml	9ml	9ml

2,000 mcg/ml 200 mcg/ml 20 mcg/ml

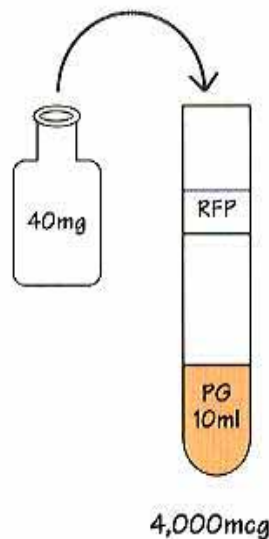
RFP(RIFAMPICIN) SOLUTION

Potency is 1,000mcg/mg

RFP is scarcely soluble in water. Add 10ml of propylene glycol into the tube containing RFP while shaking and keep it at 70°C for about 5 minutes in water bath to dissolve RFP completely.

Dimethyl sulfoxide (DMSO) and absolute methanol can be used for the solvent of RFP

RFP	4,000mcg/ml
RFP	40mg
Propylene glycol	10ml

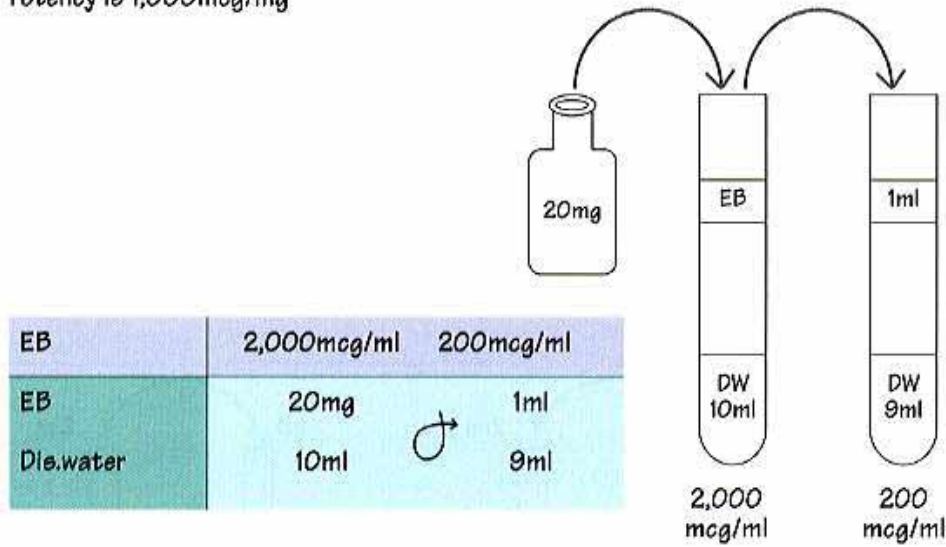


To prepare 10ml of a 10,000mcg/ml solution, following formula can be used to calculate the weight of drug necessary.

$$\frac{10,000(\text{mcg/ml})}{\text{potency}(\text{mcg/ml})} \times 10(\text{ml}) = \text{mg to weigh}$$

EB (ETHAMBUTOL) SOLUTION

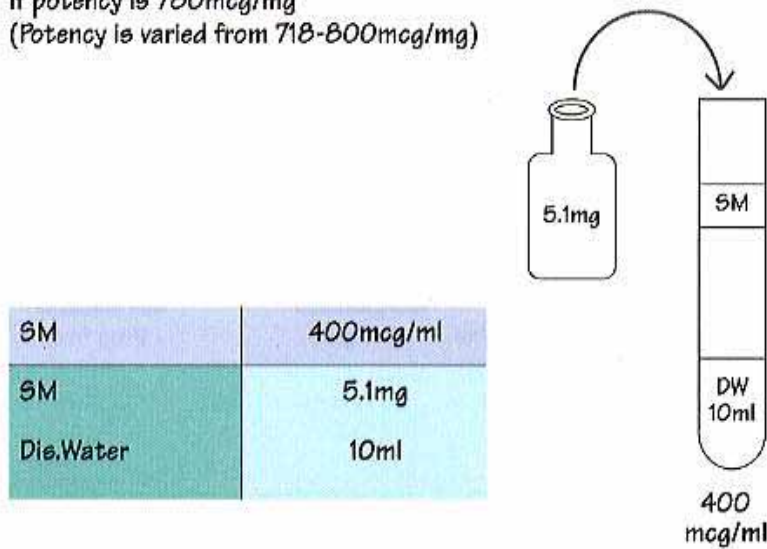
Potency is 1,000mcg/mg



SM (STREPTOMYCIN) SOLUTION

If potency is 780mcg/mg

(Potency is varied from 718-800mcg/mg)



Example : potency of SM is 780mcg/mg

$$\frac{10,000}{780} \times 10 = 128\text{mg}$$

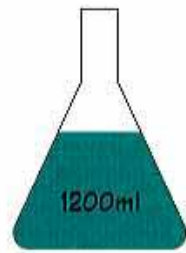
128mg of SM is dissolved in 10ml of distilled water for 10,000mcg-SM per milliliter

PREPARATION OF DRUG CONTAINING MEDIA

Fig.1

Preparation of L-J medium

Prepare 1200ml of raw L-J medium. Then divide it into 5 portions as below.



Preparation of drug free (control) medium

Keep one of the 5 portions; 400ml of raw L-J medium is used as drug-free or control medium.

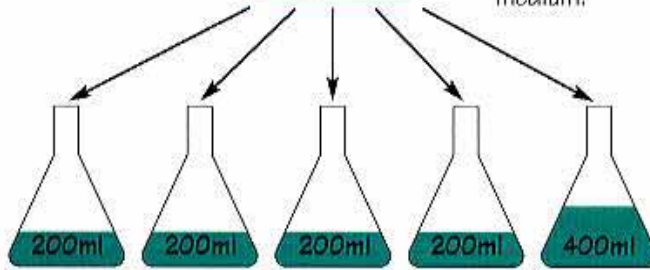
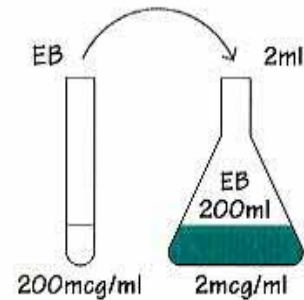
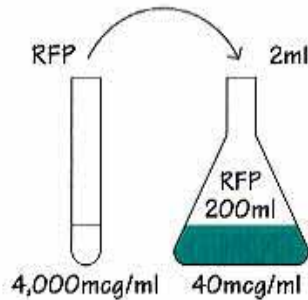
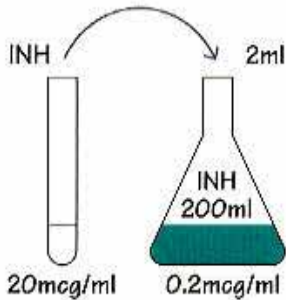


Fig.2



Addition of drugs to medium

Add 2ml of the working solution of each drug to 200ml of remaining 4 portions of medium. Mix them well avoiding bubble formation.

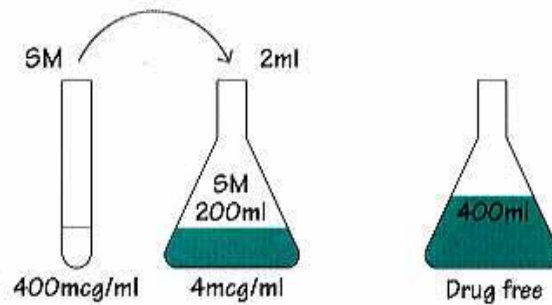
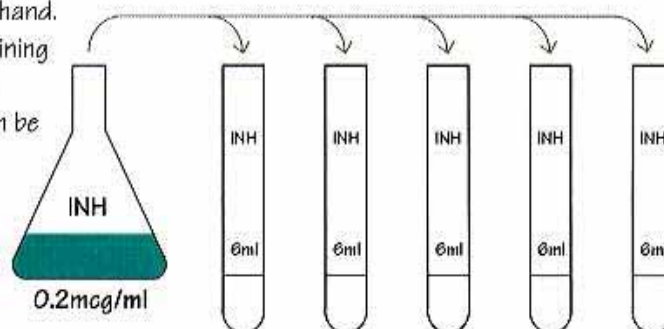


Fig.3

Distribution and Inoculation of media

Label the name of the drug on each tube beforehand. Dispense 6ml of drug free or 6ml of drug containing L-J medium into each tube. Inoculate them at 90°C for 50 minutes. Drug containing media can be stored at 4°C for a maximum of one month.



PREPARATION OF BACILLARY SUSPENSION

Fig.4

Harvest of the growth

Put one to two drops of sterile distilled water into the screw-capped homogenizer*. Harvest one loopful of growth with a 3mm diameter-loop from the wide spread surface of the growth. Transfer to the homogenizer.

*Tube, a 14ml screw-capped bottle, containing several 5-6mm sized glass or plastic beads.

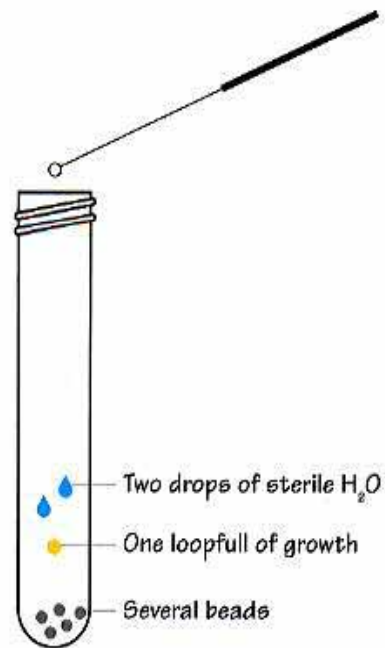


Fig.5

1mg/ml-bacillary suspension

Homogenize on vortex mixer for few minutes and leave for 10minutes. Add 7ml of sterile distilled water. Allow large particles to settle. Transfer supernatant suspension to sterile tube and adjust density to that of a MacFarland No.1 with sterile distilled water. This is 1mg/ml-bacillary suspension.

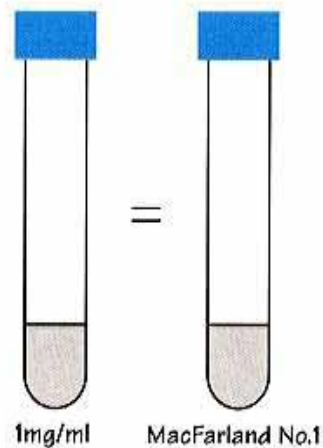


Fig.6

Dilution of bacillary suspension for inoculation

Dilute 1mg/ml-bacillary suspension to 10^{-2} (0.01mg/ml) and 10^{-4} (0.0001mg/ml) by making serial 10 fold dilutions with sterile distilled water.

	10^{-1}	10^{-2}	10^{-3}	10^{-4}
1mg/ml-bacillary suspension	0.5ml	0.5ml	0.5ml	0.5ml
Sterile distilled water	4.5ml	4.5ml	4.5ml	4.5ml

INOCULATION, INCUBATION AND READING

Fig.7

Inoculation of 10^{-2} (0.01mg/ml) and 10^{-4} (0.0001mg/ml) of bacillary suspensions

Firstly inoculate 0.1ml of 10^{-2} and 10^{-4} suspensions onto 2 slants of drug free (control) medium. Then inoculate 10^{-2} dilution of the suspension onto a series of drug containing media.

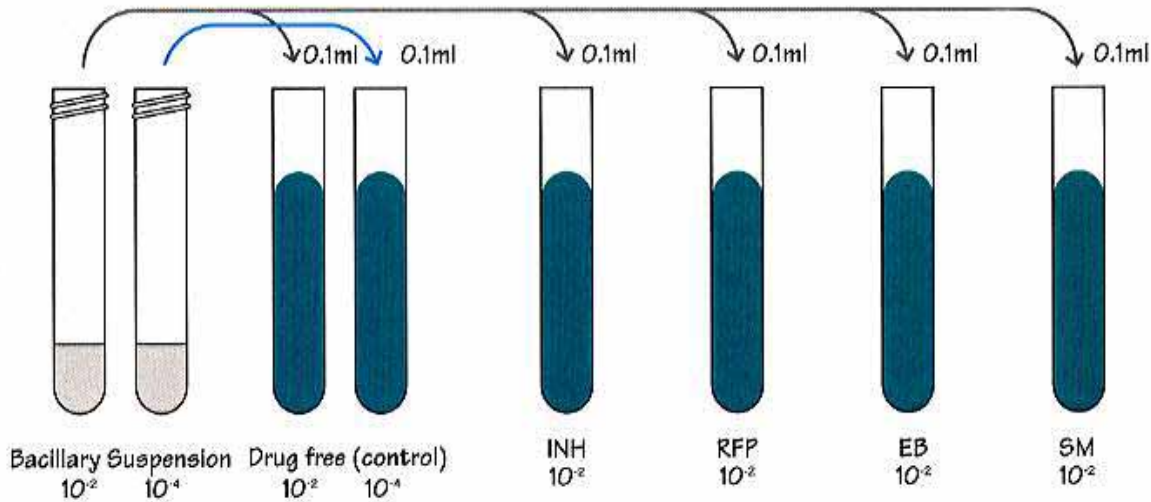
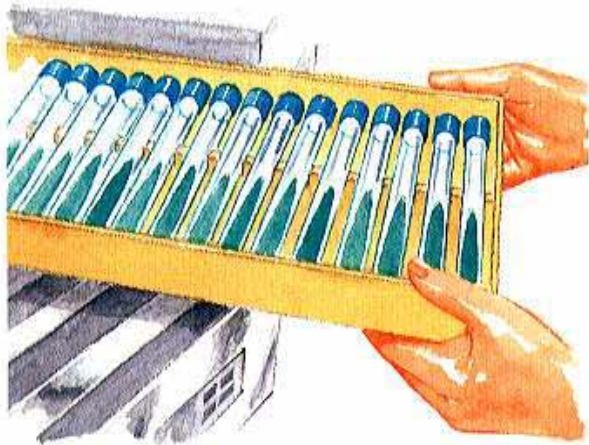


Fig.8



Incubation

Spread over the suspension on the whole surface of medium, then keep them at slanting position with loosen caps.

Keep the slants in the incubator at 37°C. When the growth appears on the control media, tighten the cap and continue the incubation for 4-6 weeks.

Fig.9



Reading

When enough growth, more than 100 colonies for 10^{-2} suspension and more than 50 colonies for 10^{-4} suspension, was observed on the drug free medium at 3 or 4 weeks of incubation, read the growth on all media. If strains showing "drug susceptibility" at 4 weeks, further reading at 4 weeks is required before reporting susceptibility.

INTERPRETING, RECORDING AND REPORTING

Fig.10

Interpreting of the results

10^{-4} dilution represents 1/100th (or 1%) of the 10^{-2} dilution. Therefore any colonies growing on drug containing medium inoculated with the 10^{-2} dilution that equal or more the number of colonies growing on the drug free (control) medium inoculated with the 10^{-4} dilution represent 1% or more of the test population.

The criterion for resistance is 1% of growth for all drugs.

0.01mg/ml (10^{-2}) and 0.0001mg/ml(10^{-4})
bacillary suspensions

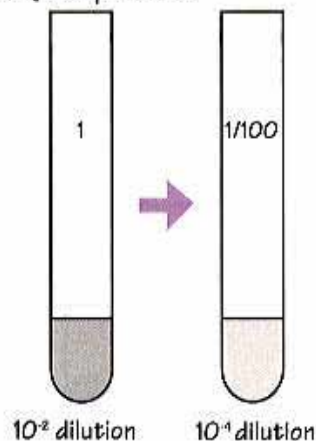
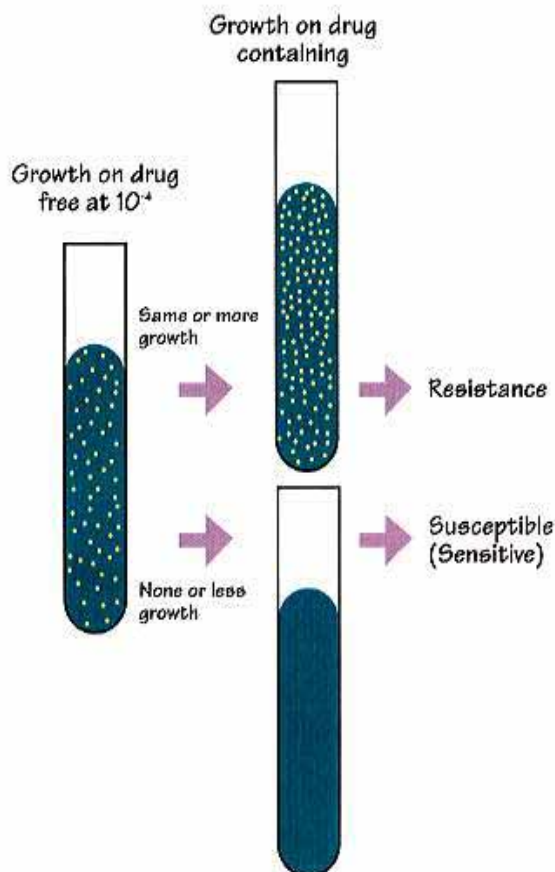


Fig.11

Recording and reporting

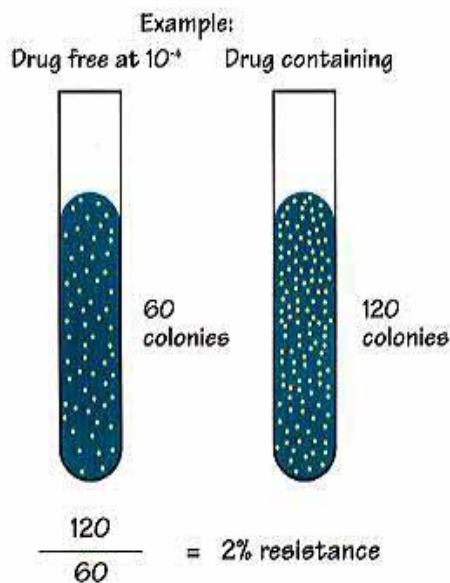
Compare the growth on the drug containing medium with the growth on the drug free medium at 10^{-4} dilution. When the growth on the drug containing medium is same or more to that of a drug free medium at 10^{-4} dilution, it is classified as "Resistant".

When the growth on the drug containing medium is none or less that of a drug free medium at 10^{-4} dilution, it is classified as "Susceptible / Sensitive".



Following formula can be also used to calculate the % of resistant;

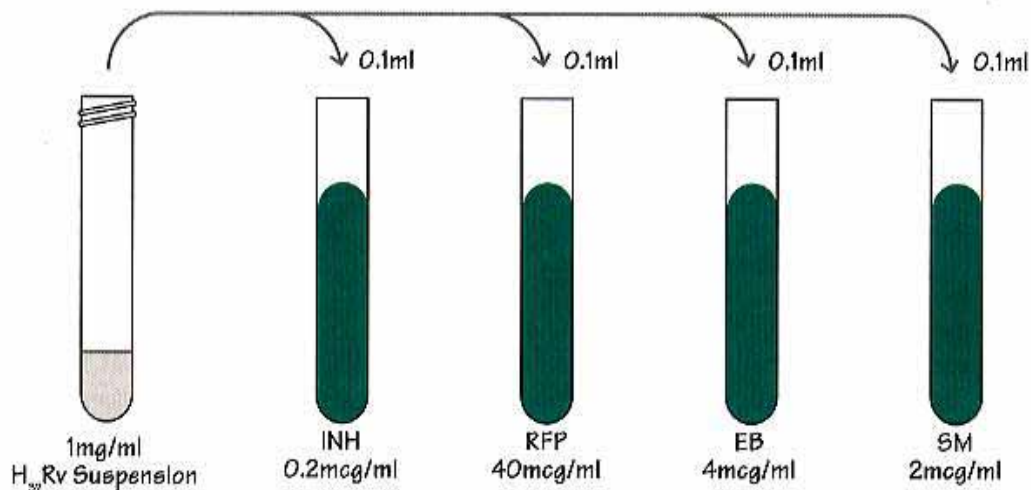
$$\frac{\text{Number of colonies on the drug containing medium}}{\text{Number of colonies on the drug free medium at } 10^{-4} \text{ dilution}} = \% \text{ resistance}$$



QUALITY CHECK OF MEDIA FOR DRUG SUSCEPTIBILITY TEST

When each newly batch of media are prepared, the quality of media should be checked by inoculating the standard strain H₃₇Rv of *M.tuberculosis* suspension onto a series of drug containing media.

0.1ml of 1mg/ml H₃₇Rv suspension, approximately 10⁸ bacilli, are inoculated onto each drug containing medium. Then incubate at 37°C for 6 weeks. Observe the number of colonies for natural resistant mutants.



Expected results are shown below. Number of colonies are far more than maximum, media, may contain lower concentration than it indicates.

Minimum, median, and maximum numbers of bacilli resistant to antituberculosis drugs for *M.tuberculosis* H₃₇Rv per 10⁸ bacilli.

Drug	Concentration (mcg/ml)	Minimum	Median	Maximum
INH	0.2	0	4	32
RFP	40.0	0	0.02	--
SM	4.0	0	7	300
EB	2.0	100	--	1,000



M.tuberculosis AND RUNYON GROUPS

Colonies of mycobacteria on Ogawa medium.

M.tuberculosis



M.tuberculosis(H₃₇R_v)

Typical colonies of *M.tuberculosis* on Ogawa medium show dry, friable, irregular margin, eugonic growth and buff color.

Runyon Group I
(Photochromogens)



M.kansasii

Colonies are cream colored when grown in the dark (left) and become a bright lemon yellow after exposure to light (right).

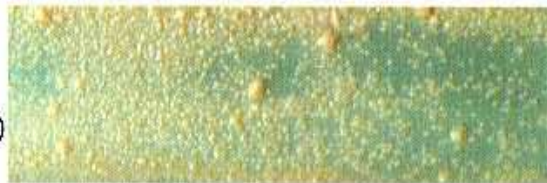
Runyon Group II
(Scotochromogens)



M.gordonae

Colonies are pigmented to orange in both the light and dark.

Runyon Group III
(Nonphotochromogens)



M.intracellulare

Colony pigment is not developed by exposure to light.

Runyon Group IV
(Rapid growers)



M.chelonae

Colonies are visible within 7 days at primary culture. Some old colonies appear greenish by absorbing malachite green egg medium.

REAGENT FORMULAS

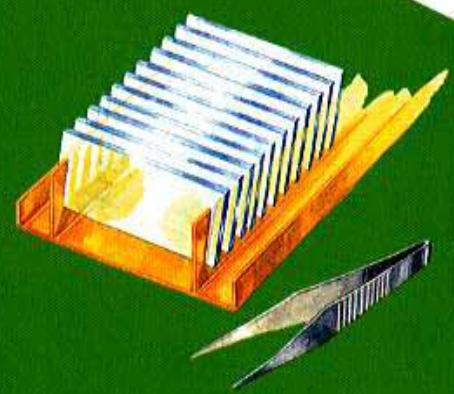
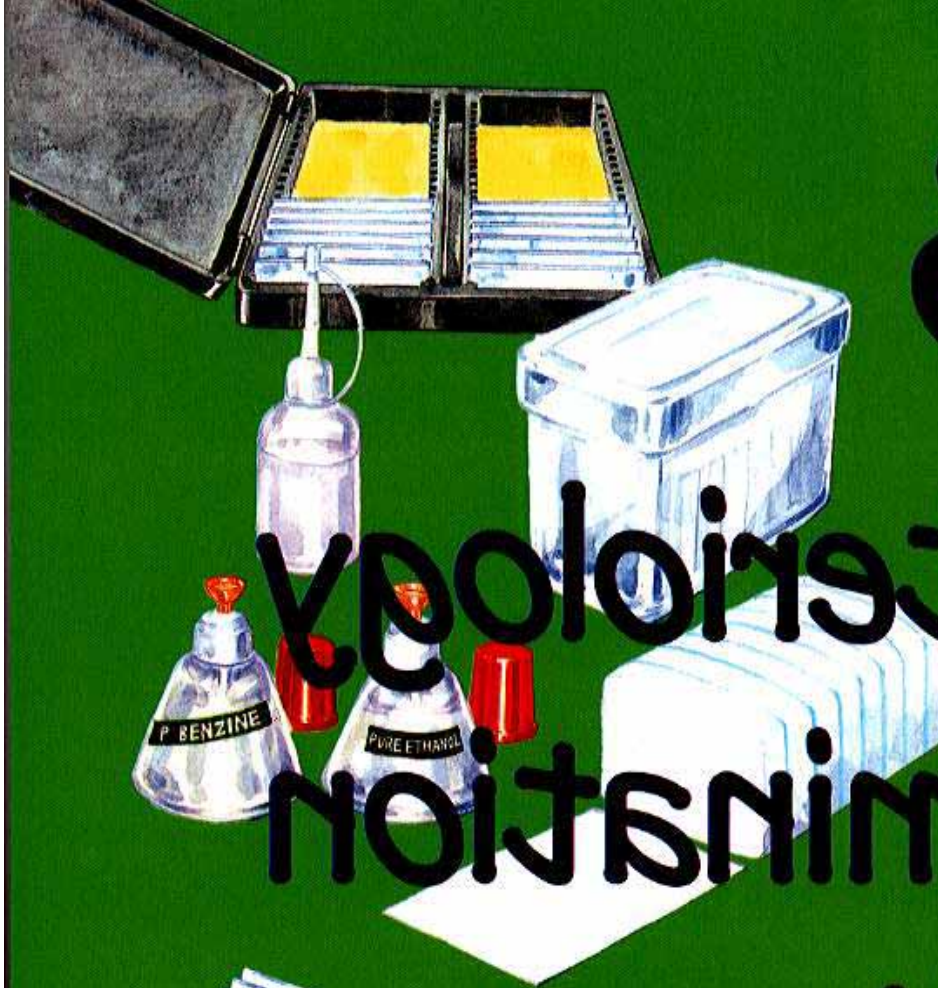
- 1) Saturated alcoholic fuchsin
 - Fuchsin (basic)3g
 - Ethanol (95%)100ml
- 2) 5% Phenol solution
 - Phenol melted5ml
 - Distilled water95ml
- 3) Ziehl's solution (Phenol fuchsin solution)
 - Saturated alcoholic fuchsin10ml
 - 5% Phenol solution90ml
- 4) Acid alcohol (3% HCL-ethanol)
 - Hydrochloric acid (conc.HCl)3ml
 - Ethanol (95%)97ml
- 5) 25% Sulfuric acid water (25% H_2SO_4 solution)
 - Sulfuric acid (H_2SO_4)25ml
 - Distilled water75ml
- 6) 0.1% Methylene blue solution
 - Methylene blue0.1g
 - Distilled water100ml
- 7) 2% Malachite green
 - Malachite green2g
 - Distilled water100ml
- 8) 4% NaOH
 - Sodium hydroxide (NaOH)4g
 - Distilled water100ml
- 9) 4% Aniline ethanol
 - Aniline4ml
 - Ethanol (95%)96ml
- 10) 10% Cyanogen bromide (Saturated cyanogen bromide)
 - Cyanogen bromide5g
 - Distilled water50ml
- 11) Buffer solution(pH7.0)
 - Stock solution (a) Anhydrous Na_2HPO_4 9.47g
 - Distilled water 1000ml
 - (b) KH_2PO_4 9.07g
 - Distilled water 1000ml
 - pH7.0-buffer solution (a) solution61.1ml
 - (b) solution38.9ml
 - Check pH. If pH is lower, add (a)solution. If pH is high, add (b)solution
- 12) Tween-peroxide mixture
 - (a) 10% Tween 80 solution1V
 - (autoclaved at 121°C for 10min. store at 5°C)
 - (b) 30% Hydrogen peroxide1V
 - (store at 5°C)
 - Just before use, mix same volume of (a) and (b)



TB Bacteriology Examination

ପ୍ରତି ରୋଗ

କାର୍ଯ୍ୟ



TB Bacteriology Examination to Stop TB
Author / Akiko FUJIKI
Designer / Shoji MAJIMA
Illustrator / Nobuyoshi MATSUI
Publisher / Tori MORI

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