Compiled by: A.S. FUJIKI (JICA Expert on TB Laboratory Works)

(Illustration: T. KIKUCHI, JICA Expert on Malaria Vector Control)

August 1986

NORTH SUMATRA HEALTH PROMOTION PROJECT

JAPAN INTERNATIONAL COOPERATION AGENCY

ILLUSTRATIVE LABORATORY PROCEDURES FOR TB. DIAGNOSIS

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PREFACE

Science always pursues its frontier fields. Based on such scientific findings, technology been developed for progress of human welfare. It could be utilized to the maximum extent however, only when there exist battalions of technicians with sound knowledges.

In the field of public health, particularly in developing countries, the battalions are often in short in terms of number and/or of quality level, for executing full duty in response to the public needs.

Mrs. A.S. Fujiki has joined our Project three times as a JICA Expert on Tuberculosis Laboratory Works, most recently in 1986 for five months. During her latest assignment, she has done a lot of works, e.g. planning, organization and execution of the Microscopist Training Course for TB diagnosis, and studies on resistance in sputum samples collected from Kec. Bandar Pulau.

Her last but not least contribution is this booklet, in which she described laboratory procedures for TB diagnosis. Its constitution in due order, and the explanation is short and clear, which could be utilized easily by anyone who wants to mater the techniques. The illustrations were drawn by her comrade Expert, Mr. T. Kikuchi, who proved his capability in this field, in addition to his original job of malaria vector control.

This booklet is issued in two versions, Indonesian and English, so as to be utilized by anyone who is interested in.

On behalf of the JICA Expert Team of the Project, I would like to express my sincere gratitude to all the Indonesian staffs who kindly assisted in completion of this valuable booklet.

Medan, August 1986

T. Suzuki, Ph.D
Team Leader of JICA Expert
North Sumatra Health Promotion Project

CONTENTS

DEFEAT TB
NOW AND FOREVER

DIRECT SMEAR EXAMINATION
(Ziehl-Neelsen method)

Fig. 1

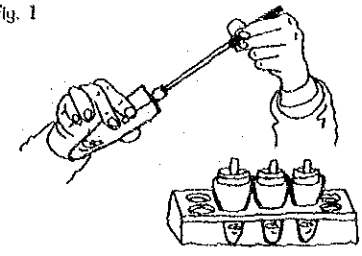


Fig. 2

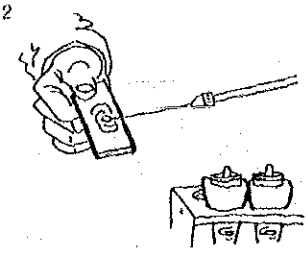


Fig. 3

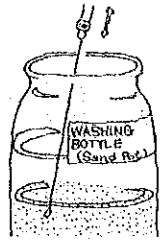


Fig. 4

Fig. 5

Fig. 6

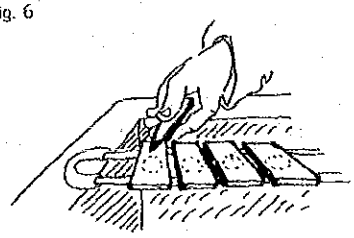


Fig. 7

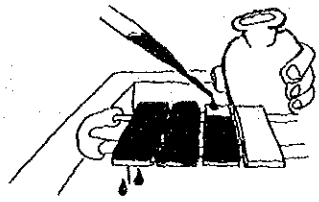


Fig. 8

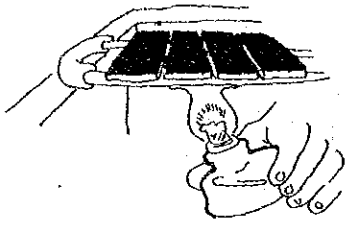


Fig. 9

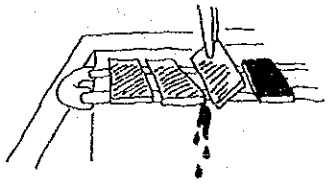


Fig. 10

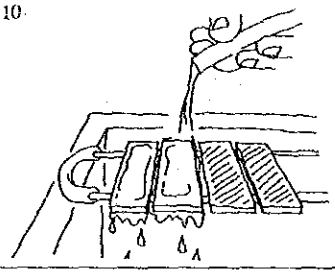


Fig. 11

Fig. 12

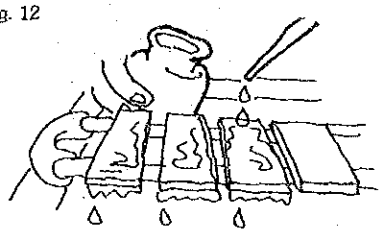


Fig. 13

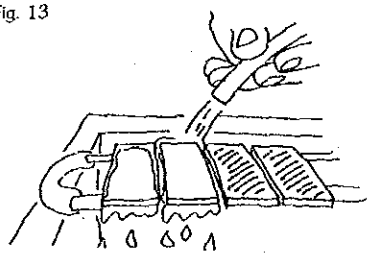


Fig. 14

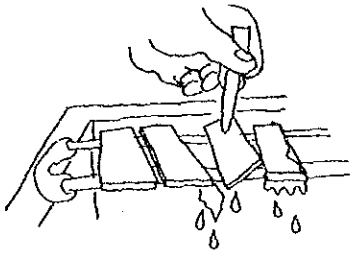


Fig. 15

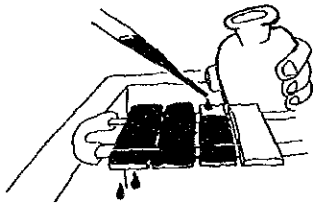


Fig. 16

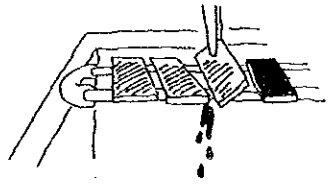
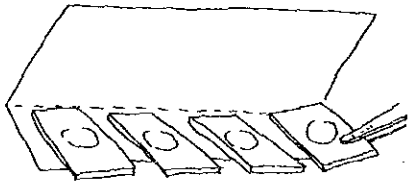


Fig. 17



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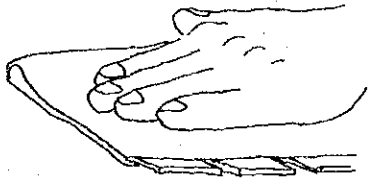


Fig. 19

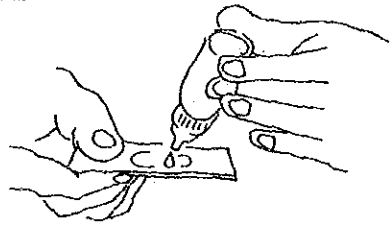
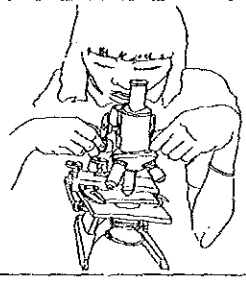


Fig. 20



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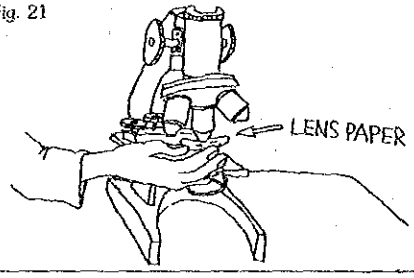
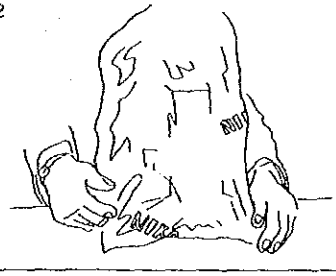


Fig. 22



I. DIRECT SMEAR EXAMINATION (Ziehl-Neelsen method)

Record the date, name of the patient and sex and age on the laboratory note. Arrange the equipments and specimens on the working desk before starting the examination.

I-1 PREPARATION OF DIRECT SMEAR (Fig.1 ~ Fig.6)

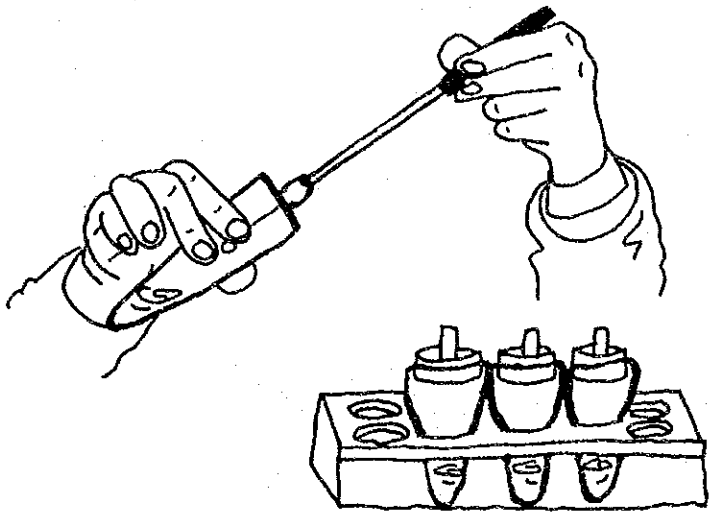


Fig.1 Selecting parts of the sputum

Write down the identification number of sputum specimen on an end of the slide. Fish out one loopful of yellowish particle of sputum.

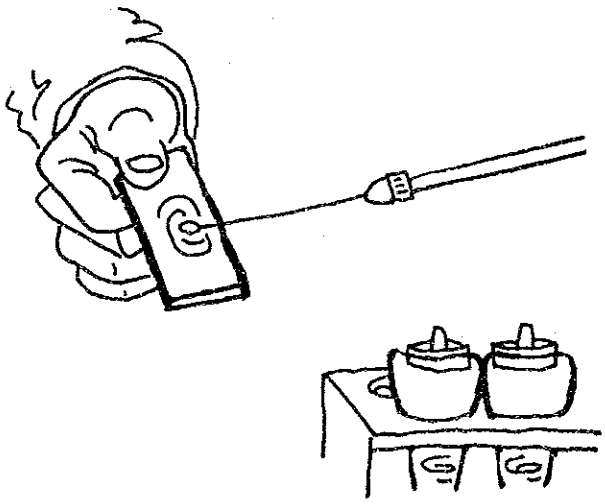


Fig.2 Smearing of the sputum

Spread one loopful of the sputum evenly on a clean labelled glass slide, approximately 2cm × 3cm in size.

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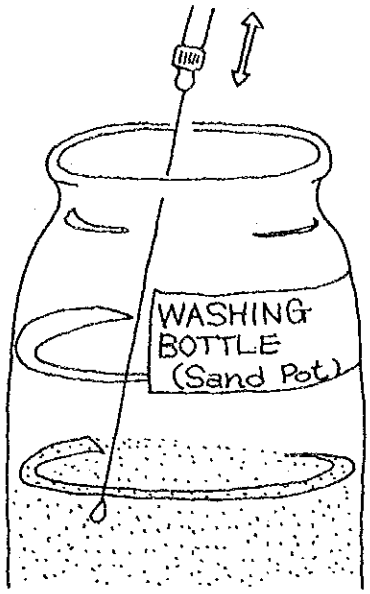

Fig. 3 Removal of adherent sputum

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.

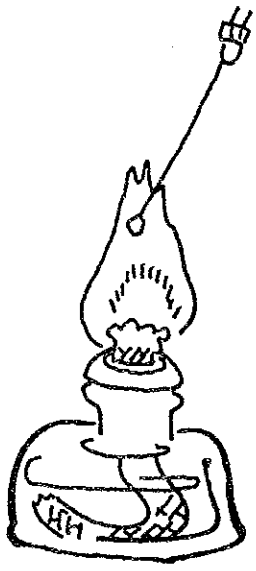

Fig. 4 Sterilization of the wire loop

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

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Fig. 5 Drying and fixation of the smear

Allow the smear dry completely at room temperature and fix it by passing through the flame 2-3 times, about 5 seconds each.

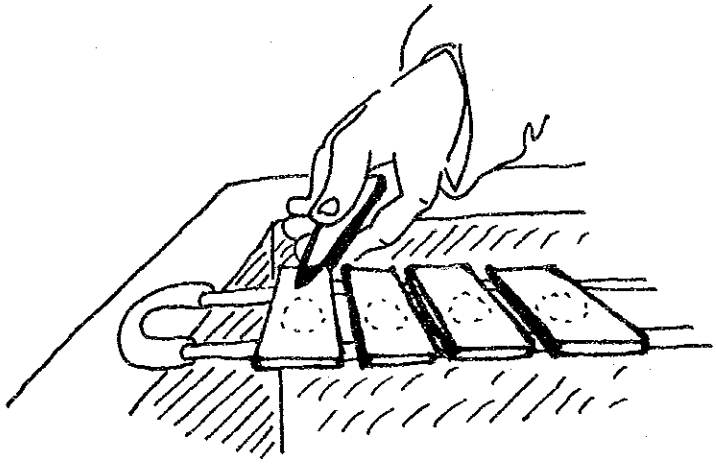

Fig. 6 Arrangement of the slide

Place the slide on a staining bridge.

MEMORANDUM

I-2 STAINING OF THE SMEAR (Fig. 7 ~ Fig. 11)

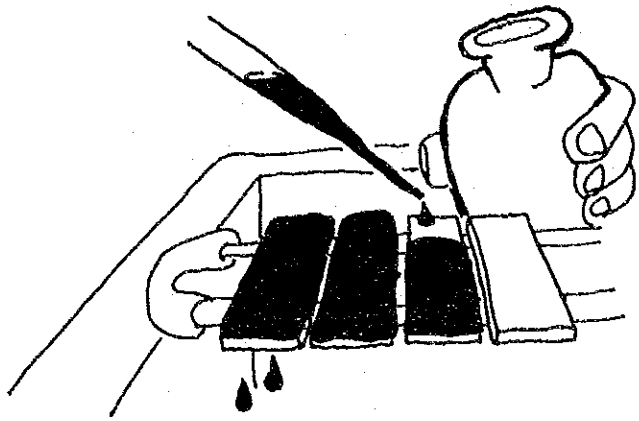

Fig. 7 Staining with Ziehl's solution

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

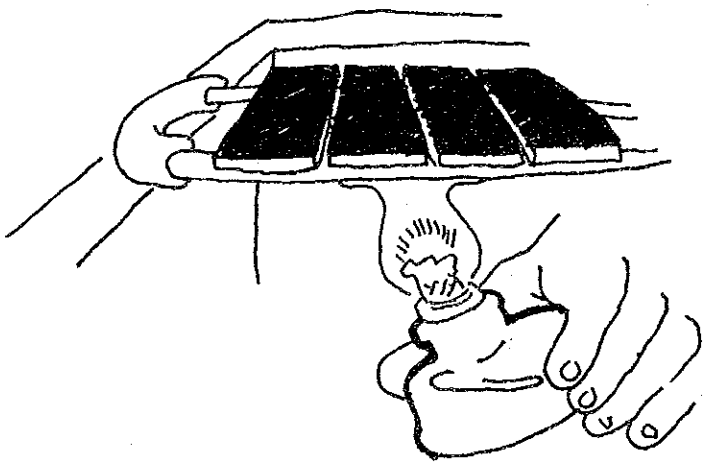

Fig. 8 Heating of the slide

Heat the slide till steam comes off from the stain. Do not boil and do not allow slide dry. Leave it for a few minutes.

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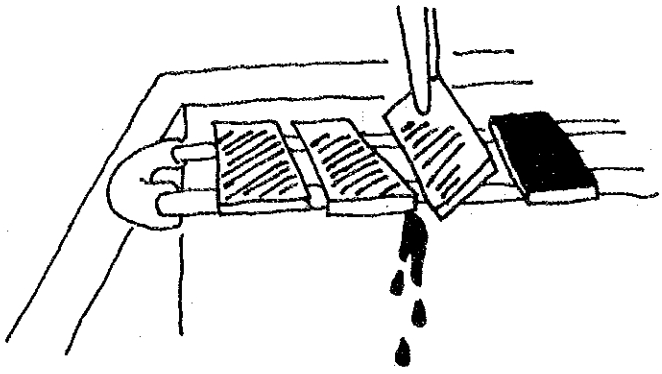

Fig. 9 Removal of excess stain

Tilt the slide to drain off excess stain.

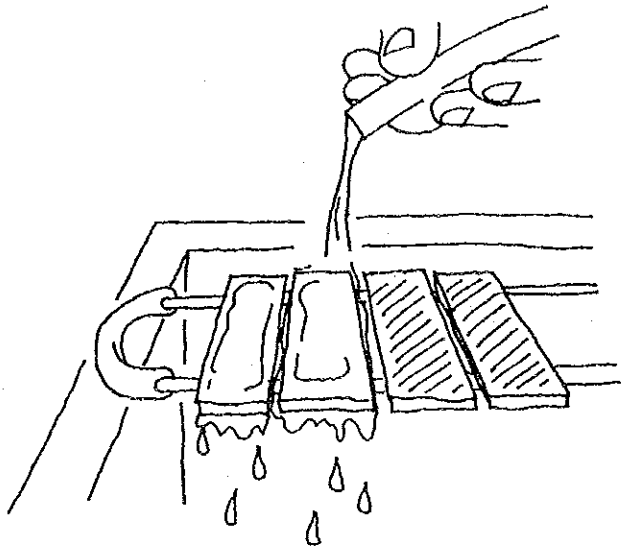

Fig. 10 Washing of the slide

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

MEMORANDUM

I-3 DECOLOURIZATION (Fig. 12 ~ Fig. 14)

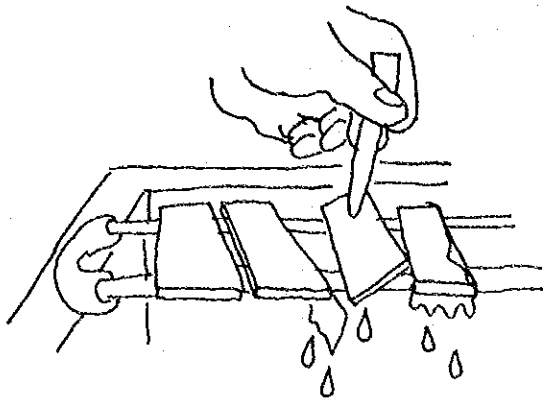

Fig. 11 Draining off excess rinse water

Tilt the slide to drain off excess rinse water

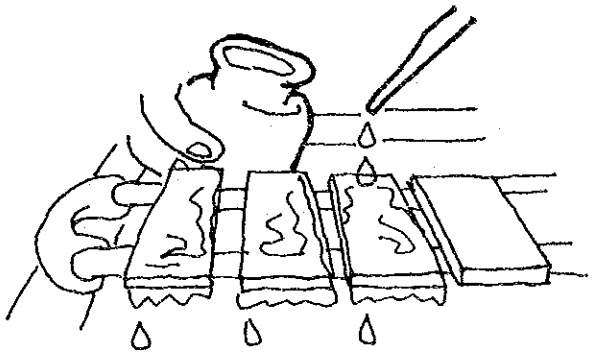

Fig. 12 Decolourization with 3% hydrochloric acid ethanol

Decolourization with 3% hydrochloric acid ethanol until solution runs clear.

MEMORANDUM

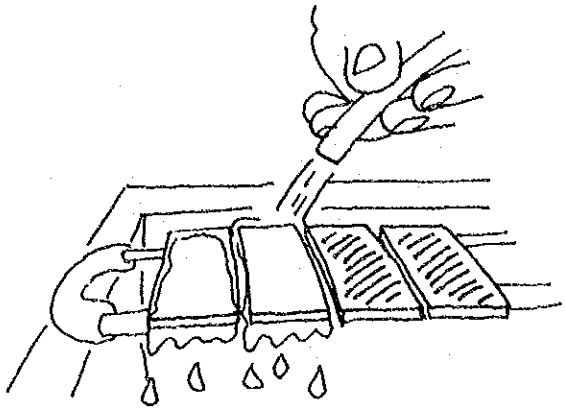

Fig. 13 Washing of the slide

Wash the slide with a gentle stream of running water.

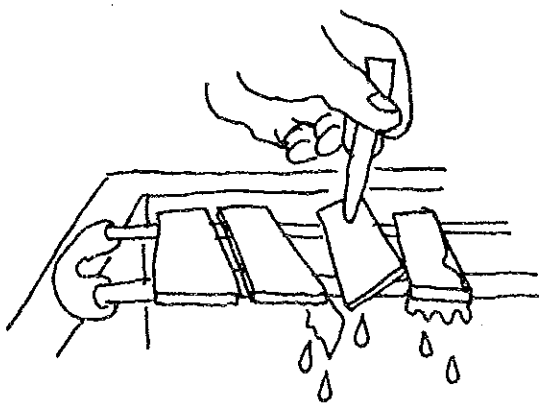

Fig. 14 Draining off excess rinse water

Tilt the slide to drain off excess rinse water.

MEMORANDUM

I-4 COUNTERSTAINING (Fig. 15 ~ Fig. 18)

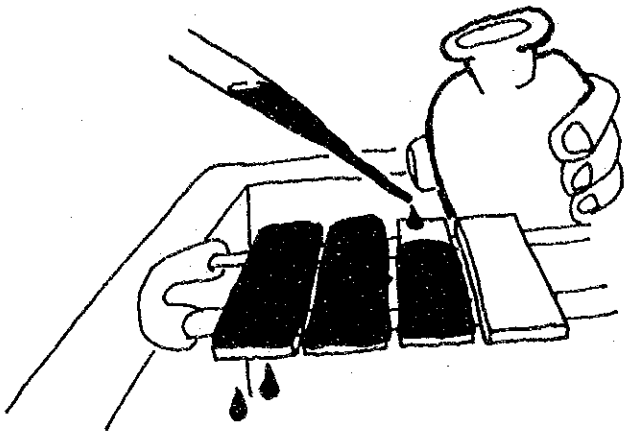

Fig. 15 Counterstaining with 0.1% methylene blue

Pour 0.1% methylene blue to cover the whole surface of the slide and leave for 10 ~ 20 seconds.

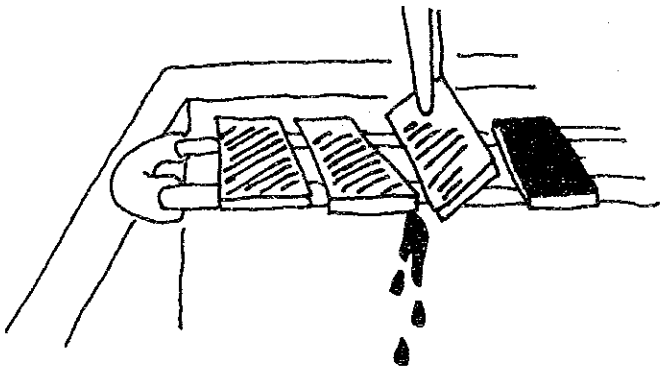

Fig. 16 Removal of 0.1% methylene blue

Pour off 0.1% methylene blue.

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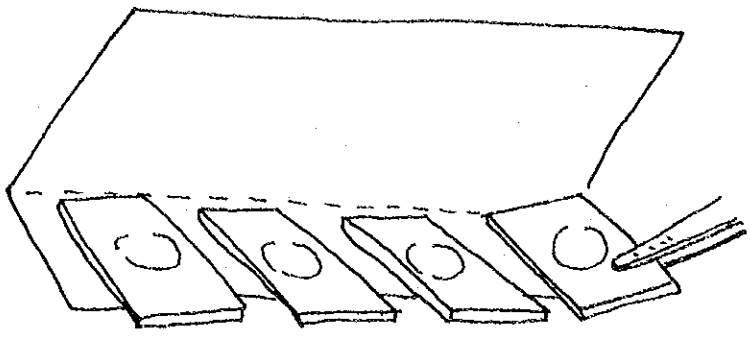


Fig. 17 Washing of the slide

Wash the slide with a gentle stream of running water. Tilt and place the slide on a piece of blotting paper.

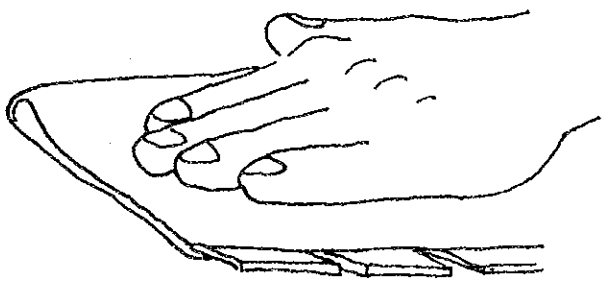


Fig. 18 Drying the stained slide

Dry the slide by gently pressing in between pieces of blotting paper.

MEMORANDUM

I-5 MICROSCOPIC EXAMINATION (Fig. 19 and Fig. 20)

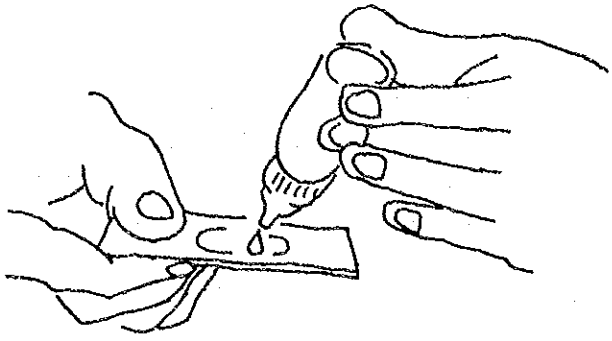


Fig. 19 Application of immersion oil

Put one drop of immersion oil on the stained smear.

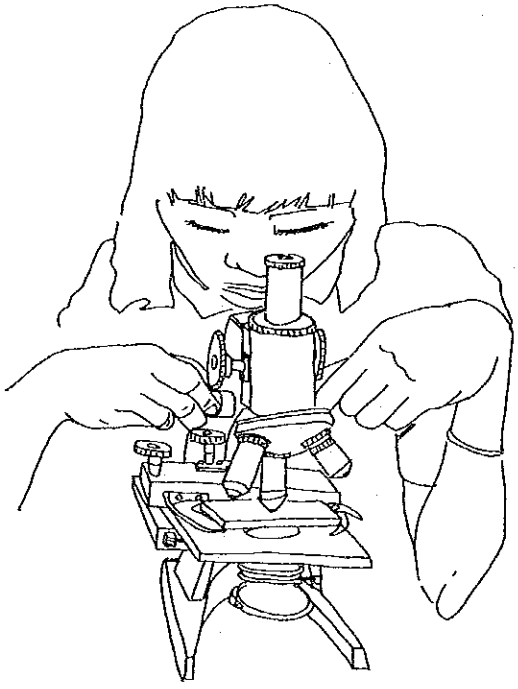


Fig. 20 Scanning of the smear

Examine the smear under ×100 objective with 10× eye piece lens.
Read at least 300 visual fields to give a report as negative.
Record the result on the laboratory note book.

MEMORANDUM

I-6 MAINTENANCE OF MICROSCOPE (Fig. 21 and Fig. 22)

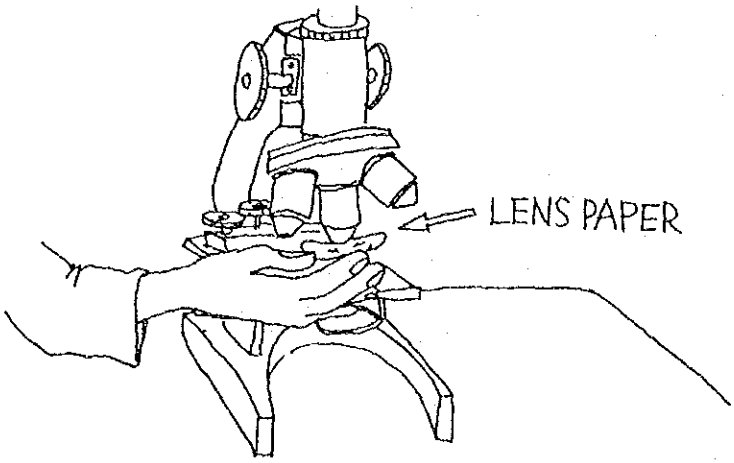


Fig. 21 Cleaning of the microscope

Clean the objective lens with lens paper moistened with petroleum benzine.

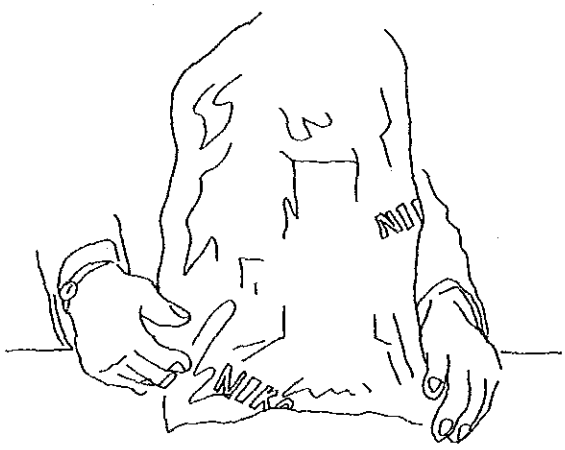


Fig. 22 Storage of the microscope

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

MEMORANDUM

PREPARATION OF EGG MEDIUM

(3% Ogawa medium)

Fig. 1

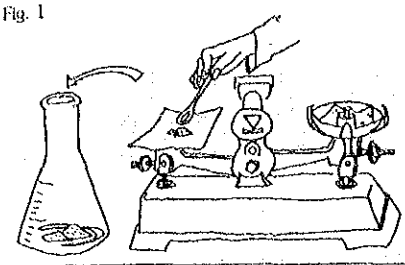
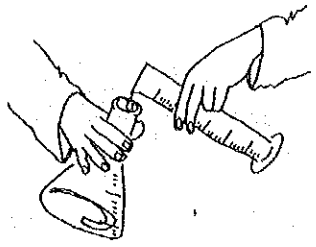


Fig. 2



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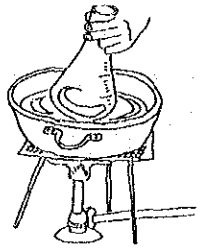


Fig. 4

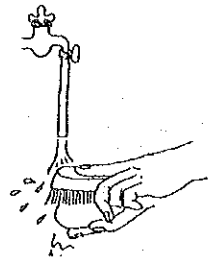


Fig. 5

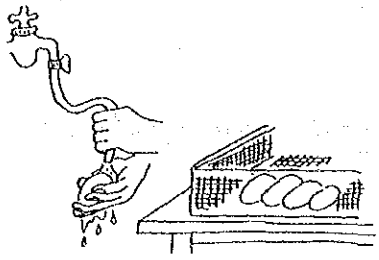


Fig. 6

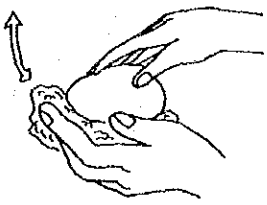


Fig. 7

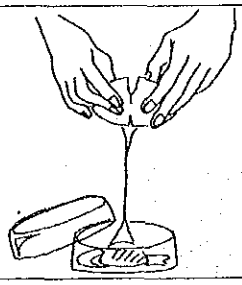


Fig. 8

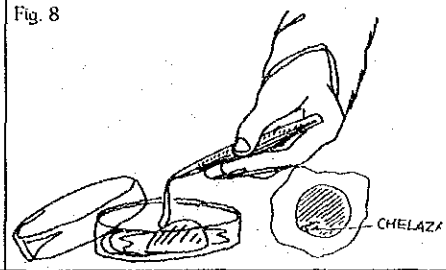


Fig. 9

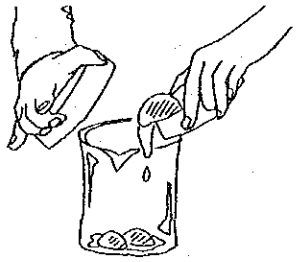


Fig. 10

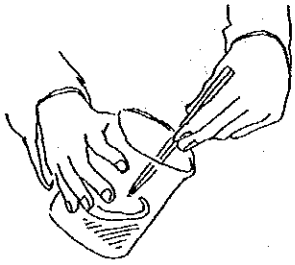


Fig. 11

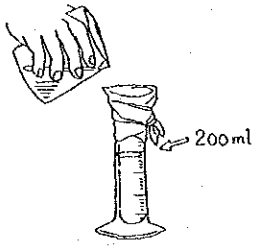


Fig. 12

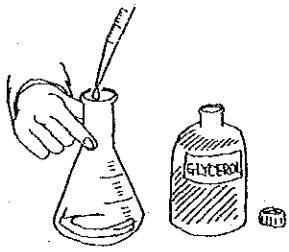


Fig. 13

Fig. 14

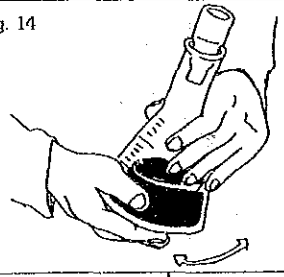


Fig. 15

Fig. 16

Fig. 17

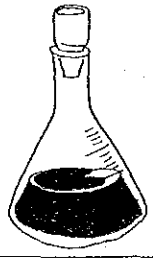


Fig. 18

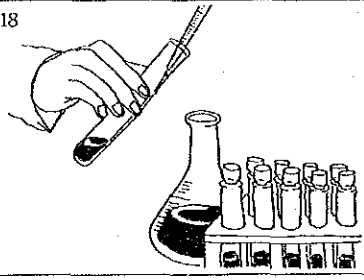


Fig. 19

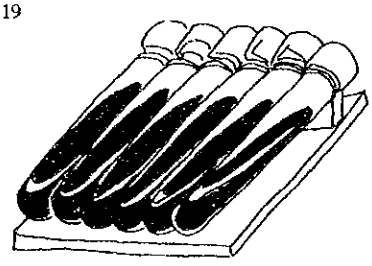


Fig. 20

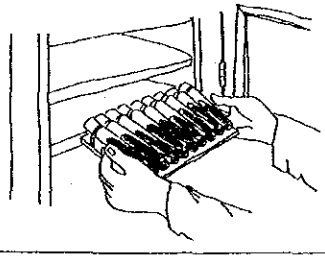
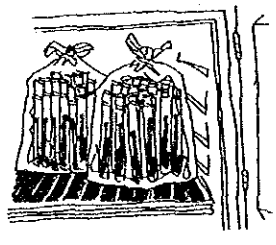


Fig. 21

Fig. 22



II. PREPARATION OF EGG MEDIUM (3% Ogawa medium)

Glasswares must be brushed and cleaned well. All utensils used in the preparation of the medium must be sterilized by autoclave or hot air oven.

II-1 PREPARATION OF SALT SOLUTION (Fig. 1 ~ Fig. 3)

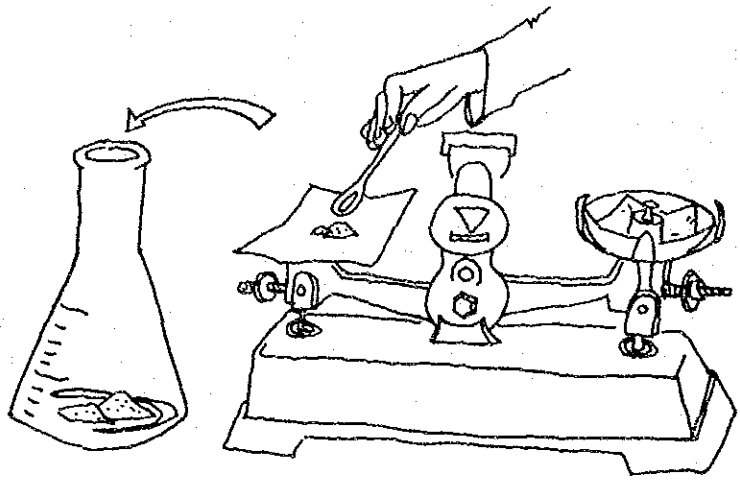


Fig. 1 Weighing of salts

Weigh 3g (1g*) of potassium phosphate and 1g of sodium glutamate.

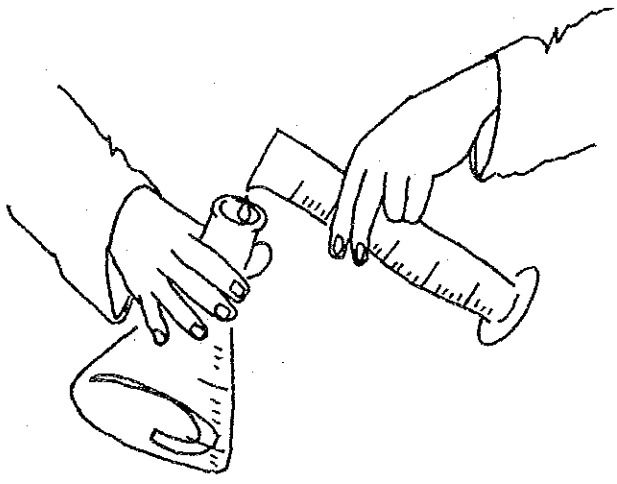


Fig. 2 Mixing of salts

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

*The amount of KH2PO4 for 1% Ogawa medium.

MEMORANDUM

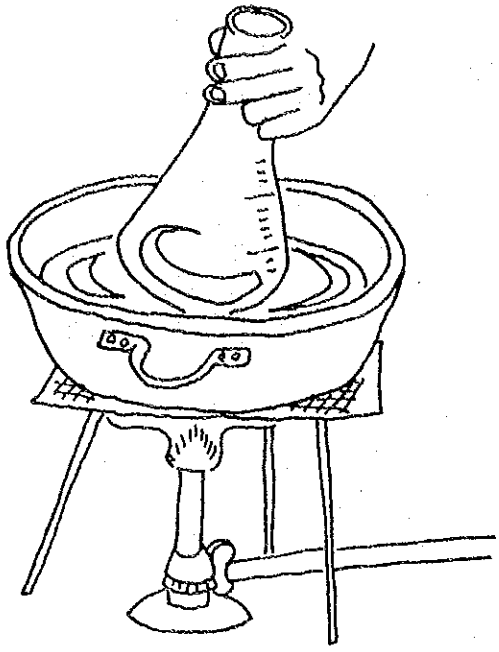


Fig. 3 Dissolution and sterilization of salts

Dissolve the components in the flask by keeping it in water bath at 100°C for 30 minutes.

II-2 PREPARATION OF WHOLE EGG HOMOGENATE (Fig. 4 ~ Fig. 11)

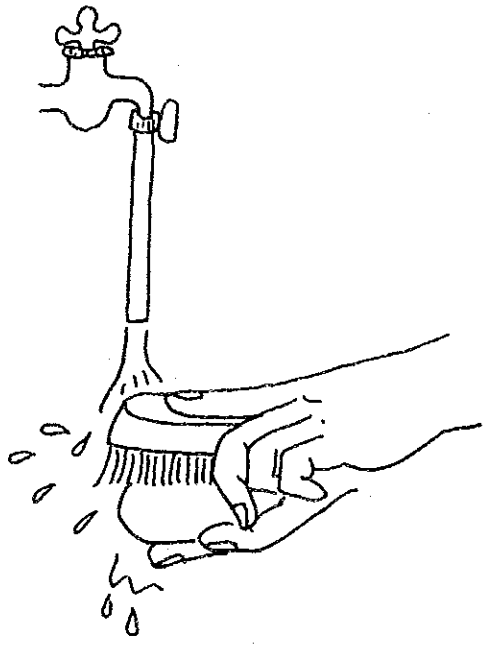


Fig. 4 Cleaning of outer egg shell

Clean the outer egg shell by means of brush and running tap water.

MEMORANDUM

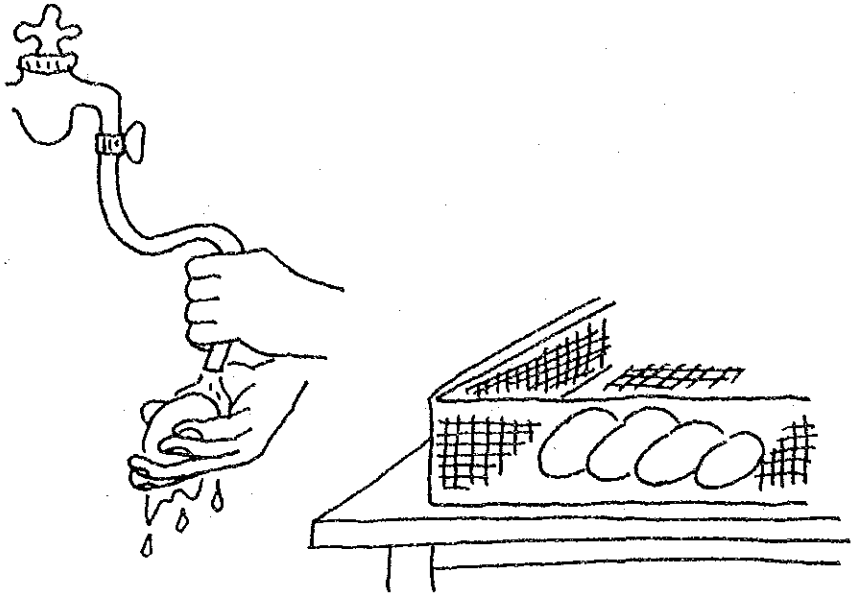


Fig. 5 Rinsing of the egg

Rinse the egg by running tap water and dry them up in a basket.

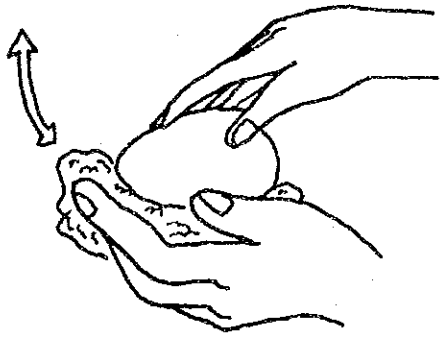


Fig. 6 Sterilization of outer shell

By means of spirit cotton, wipe the outer surface of eggs and allow alcohol to dry up before cracking the shell.

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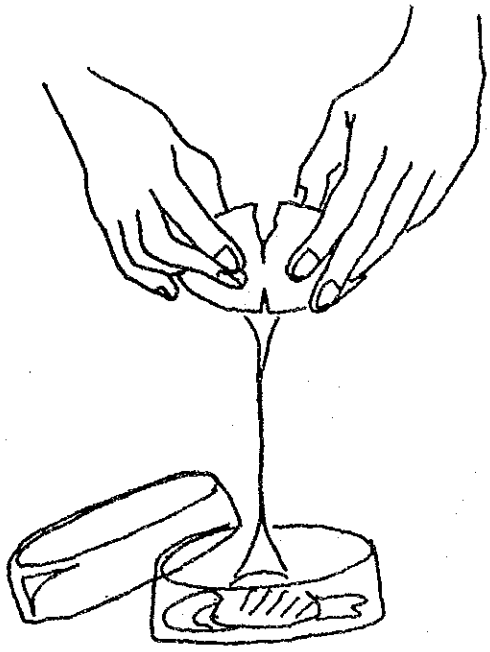

Fig. 7 Egg cracking

Crack the egg shell one by one and pour the contents into a petri dish to check the freshness of the egg. If not fresh, discard and change the petri dish to a new one.

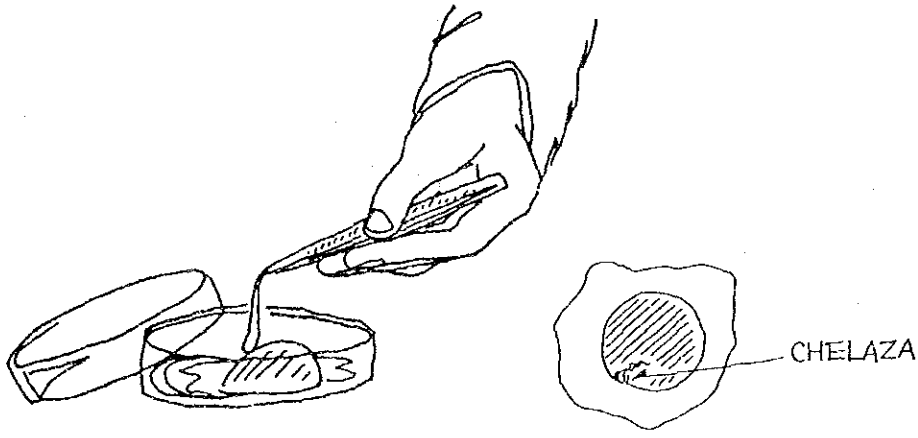

Fig. 8 Removal of chelaza

Remove the chelaza attached to the egg yolk by means of a sterile forceps.

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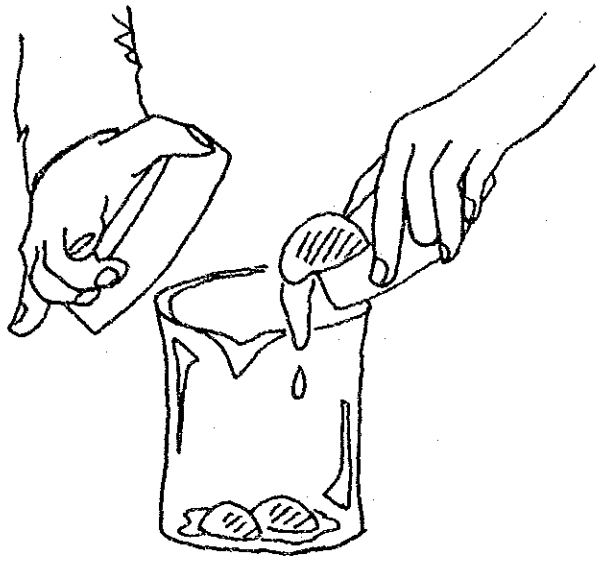

Fig. 9 Transfer of eggs

Pour some eggs content into a beaker.

Fig. 10 Beating of eggs

By means of chopsticks*, beat all eggs vigorously inside the beaker till the egg fluid is no more sticking to the chopsticks.

*Electric mixer can be used for this purpose.

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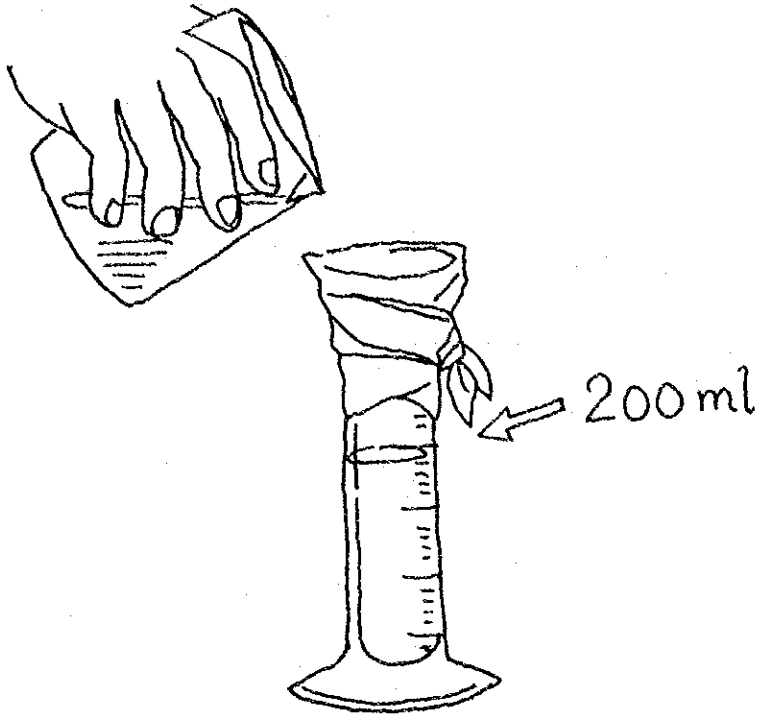

Fig. 11 Filtration of eggs

Filter the whole egg homogenate through 2 layers of sterile gauze into a sterile cylinder up 200mℓ of egg fluid.

II-3 PREPARATION OF COMPLETE RAW MEDIUM (Fig. 12~Fig. 17)

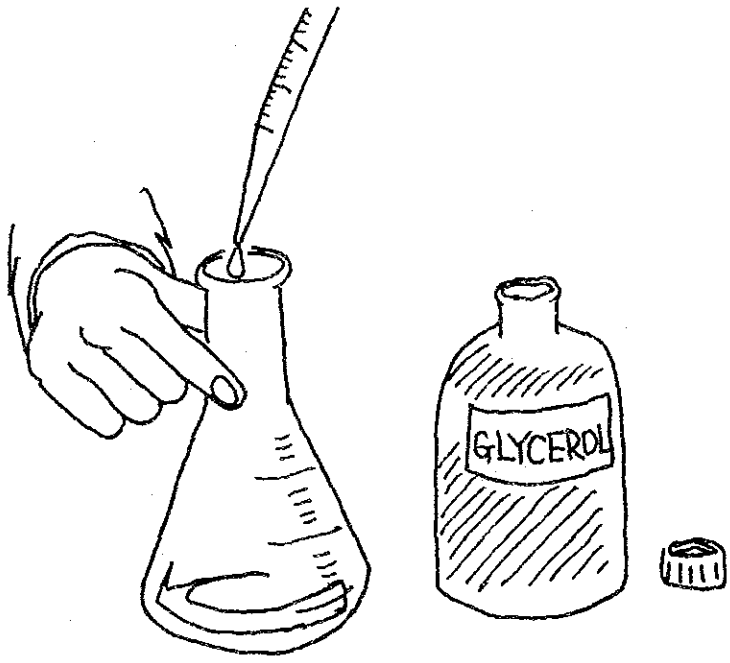

Fig. 12 Addition of glycerol

Add 6mℓ of glycerol to the previously prepared salt solution.

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Fig. 13 Addition of 2% malachite green

Add 6mℓ of malachite green on top.

Fig. 14 Mixing of the contents

Mix the flask contents gently.

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Fig. 15 Addition of egg fluid

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

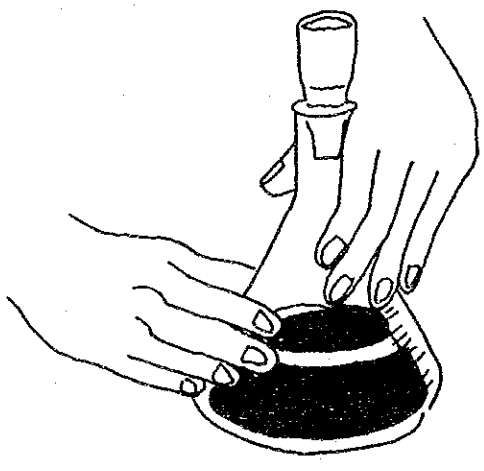


Fig. 16 Mixing of whole egg homogenate

Mix the contents of the flask gently.

MEMORANDUM

II-4 DISPENSING OF RAW MEDIUM AND INSPISSATION OF MEDIUM (Fig. 18 ~ Fig. 20)

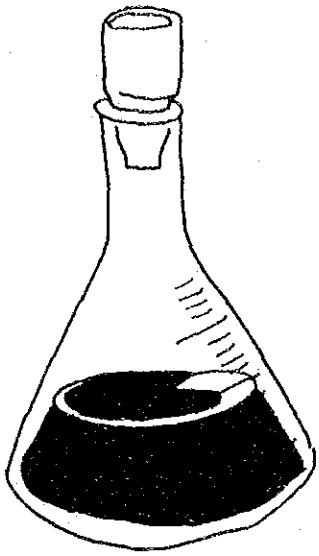


Fig. 17 Riddance of air bubbles

Allow it to stand for 30 minutes to float air bubbles up.

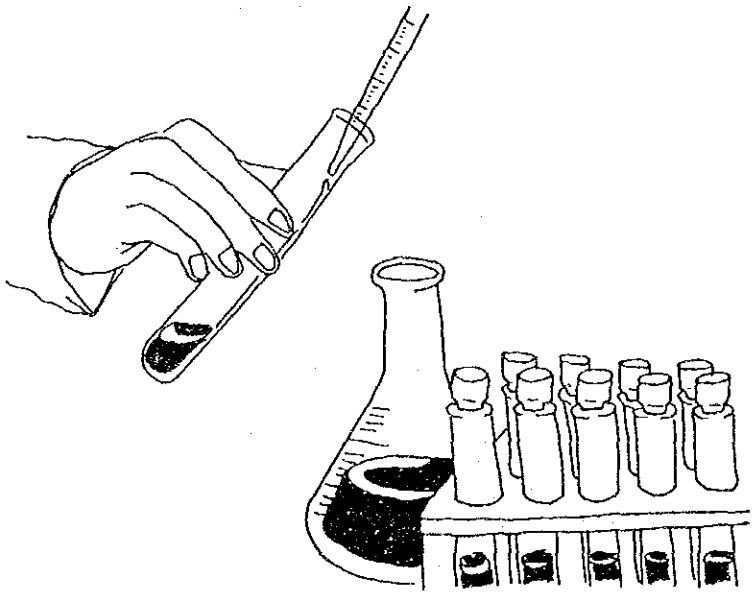


Fig. 18 Distribution of medium

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

MEMORANDUM

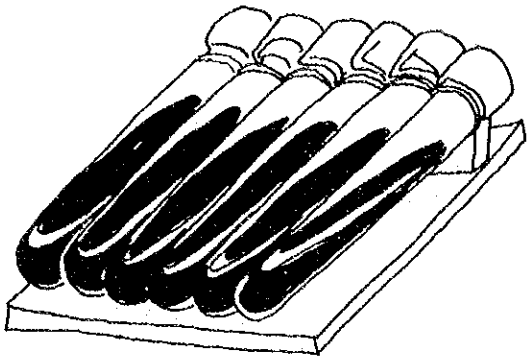


Fig. 19 Adjustment of slanting position

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

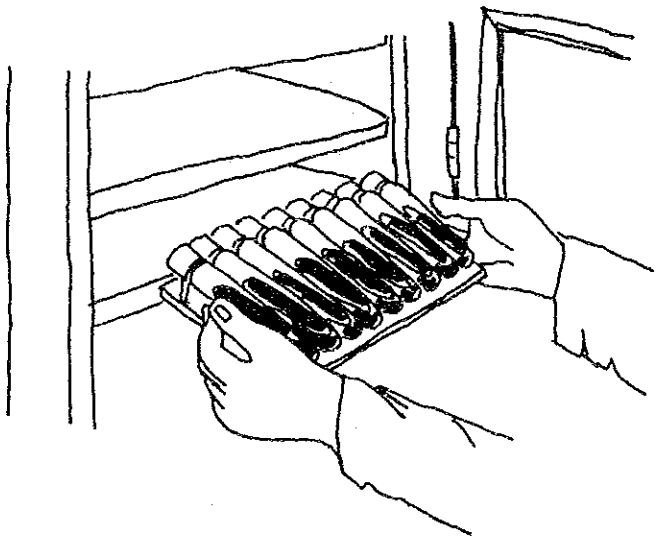


Fig. 20 Inspissation

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

MEMORANDUM

II-5 STORAGE (Fig. 21 ~ Fig. 22)

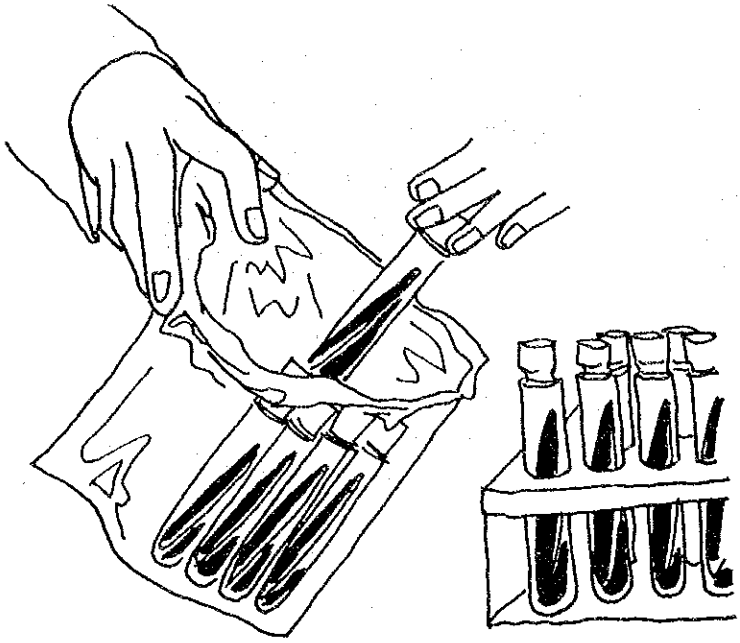


Fig. 21 Preservation in plastic bag

Leave the tubes on the bench to cool down after inspissation. Then keep them in a plastic bag, tighten its mouth with a rubber band.

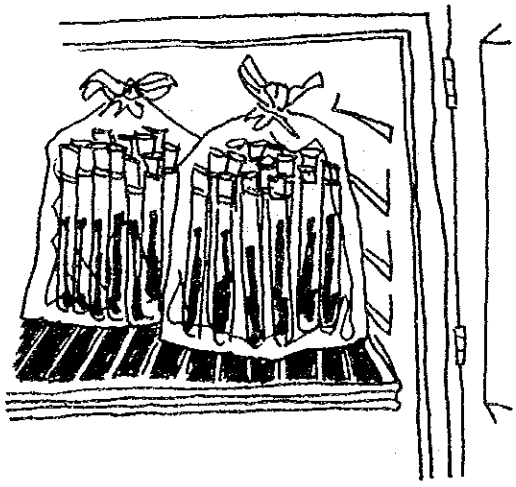


Fig. 22 Storage in refrigerator

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

MEMORANDUM

CULTURE EXAMINATION FOR M. TB
(Ogawa method)

Fig. 1

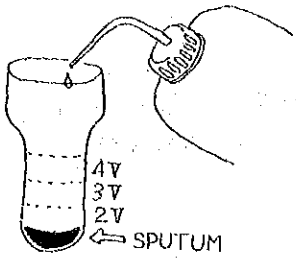


Fig. 2

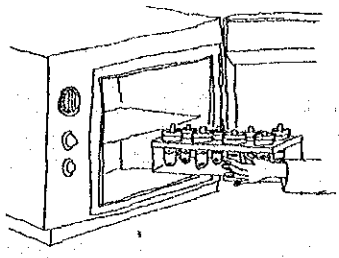


Fig. 3

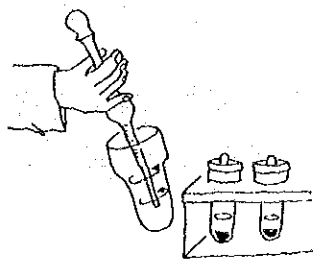


Fig. 4

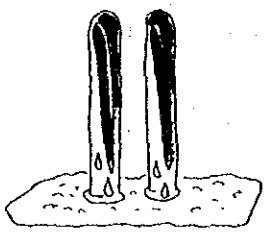


Fig. 5

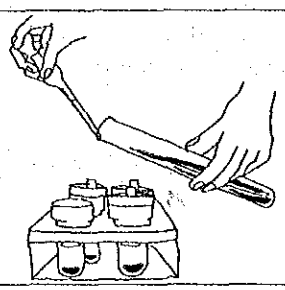


Fig. 6

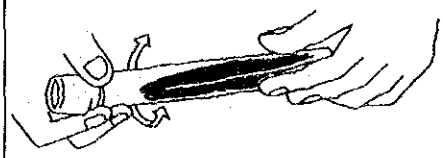


Fig. 7

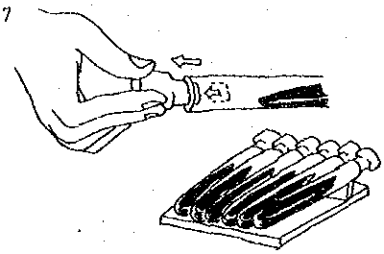


Fig. 8

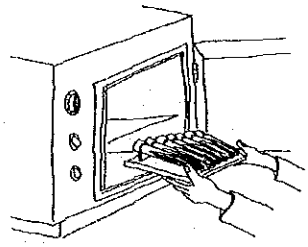


Fig. 9

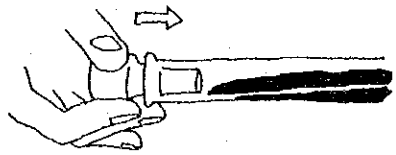


Fig. 10

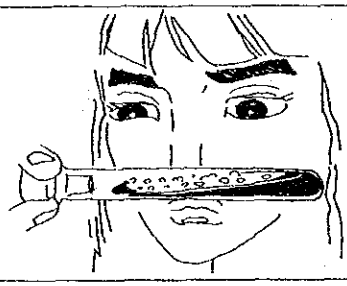


Fig. 11

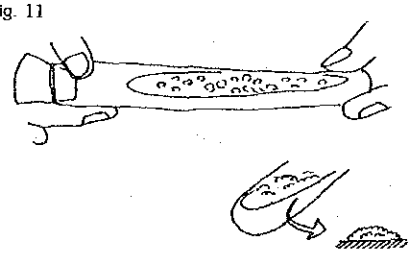
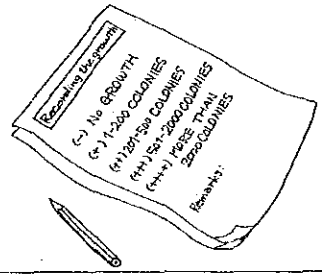


Fig. 12



III. CULTURE EXAMINATION FOR M. TUBERCULOSIS FROM SPUTUM SPECIMEN (Ogawa method)

The date examined, name of the patients, sex and age, serial number of specimens, and result of sputum smear examination should be recorded on the laboratory note before starting the examination.

III-1 PRETREATMENT (Fig. 1 ~ Fig. 3)

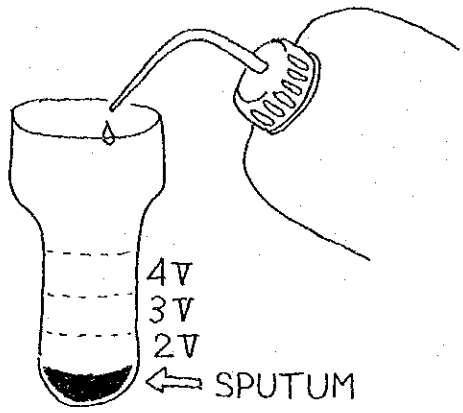


Fig. 1 Addition of 4% NaOH

Pour about 4 volumes of 4% NaOH into one volume of sputum specimen.

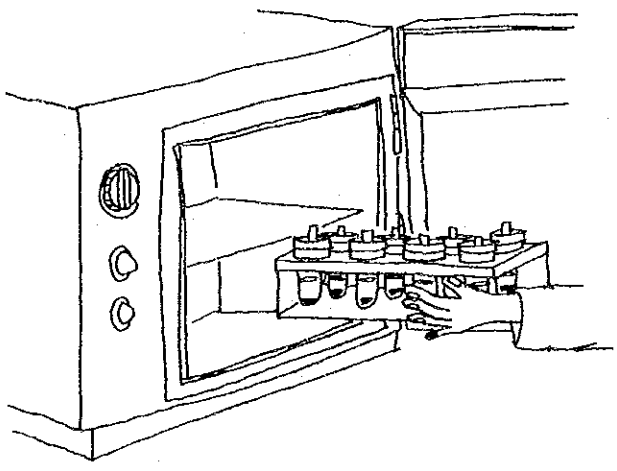


Fig. 2 Digestion of the specimen

Keep it in an incubator at 37°C for 15 minutes to digest the specimen.

MEMORANDUM

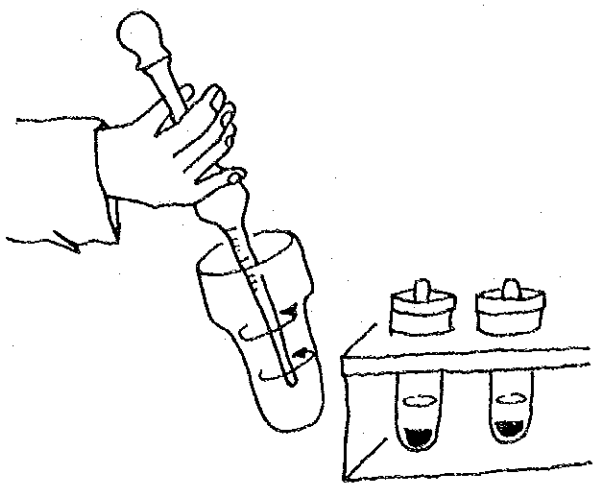


Fig. 3 Homogenization of the specimen

Take it out from the incubator and stir gently the contents to homogenize it.

III-2 INOCULATION (Fig. 4 ~ Fig. 7)

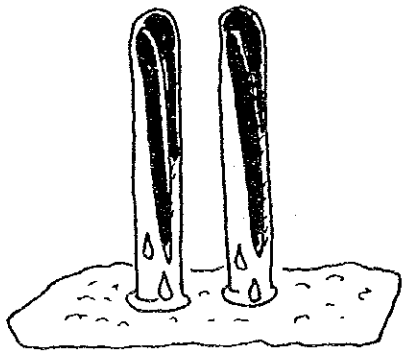


Fig. 4 Removal of condensed water

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

MEMORANDUM

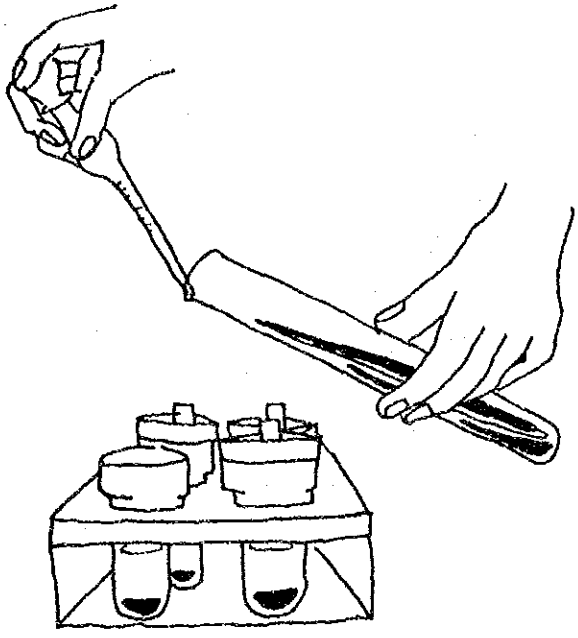


Fig. 5 Inoculation

Inoculate 0.1mℓ of sputum specimen onto each of 2 culture tubes of 3% Ogawa medium.

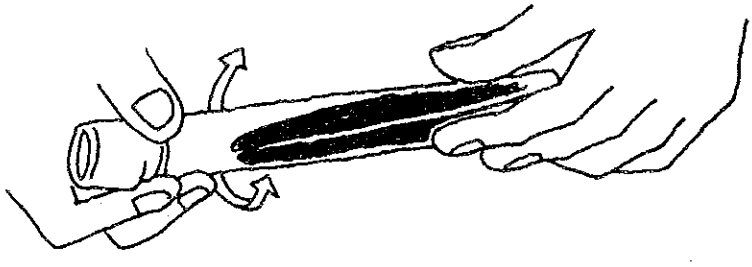


Fig. 6 Spreading of the inoculum

Spread over the inoculum evenly on the whole surface of each medium.

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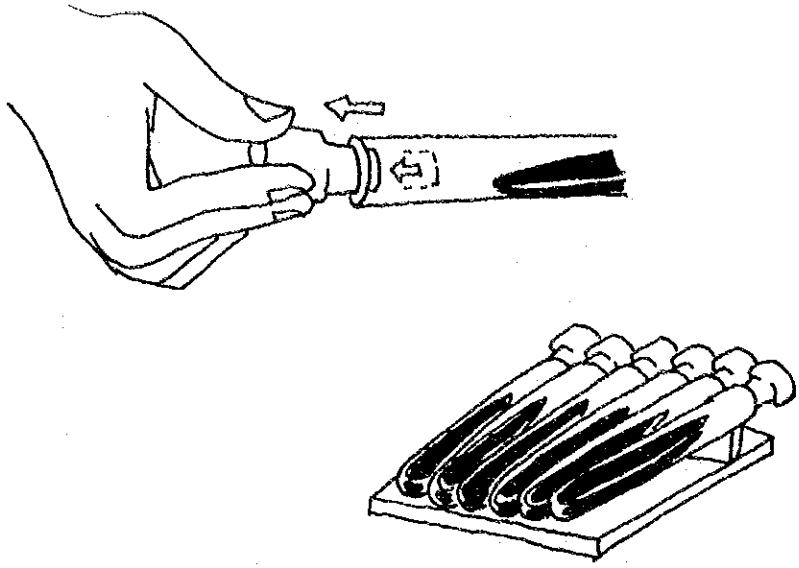

Fig. 7 Loosening the caps

Loosen the caps of inoculated media and lay the tubes on the slanting bed.

III-3 INCUBATION (Fig. 8~Fig. 10)

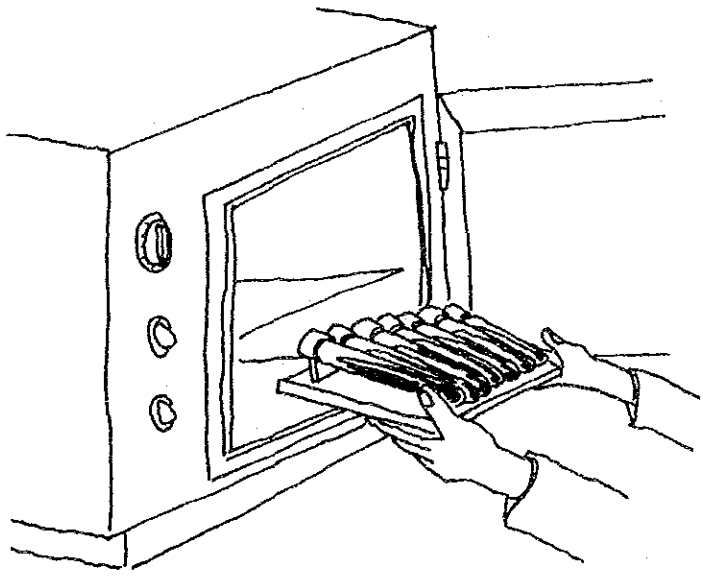

Fig. 8 Incubation

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

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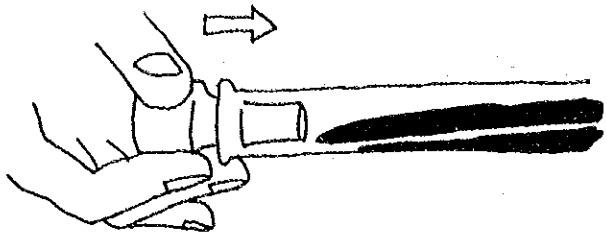


Fig. 9 Tightening the caps

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

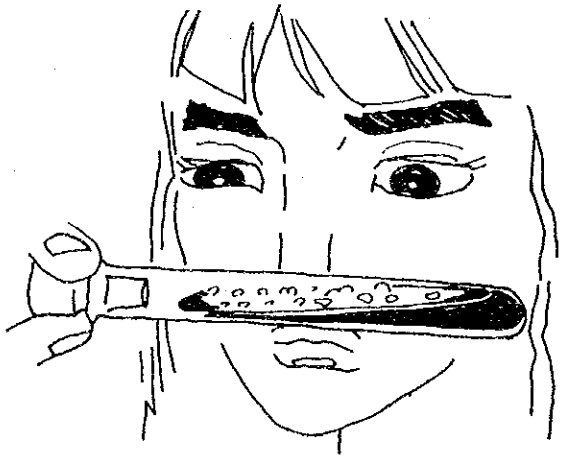


Fig. 10 Observation at 1W and 4W

Observe the culture at one week for rapid growers and at 4 weeks for slow growers.

MEMORANDUM

III-4 READING AND RECORDING (Fig. 11 and Fig. 12)

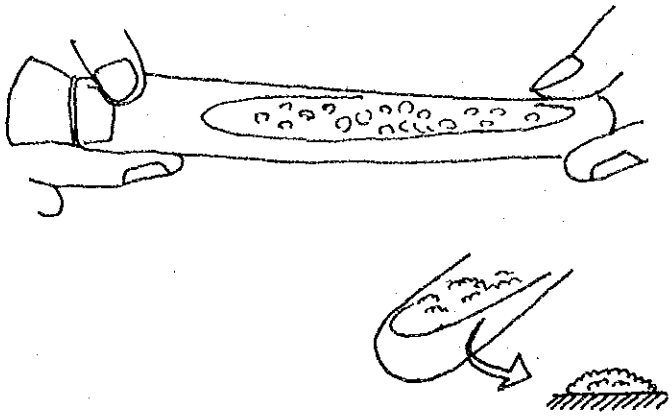


Fig. 11 Reading the growth at 4W

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

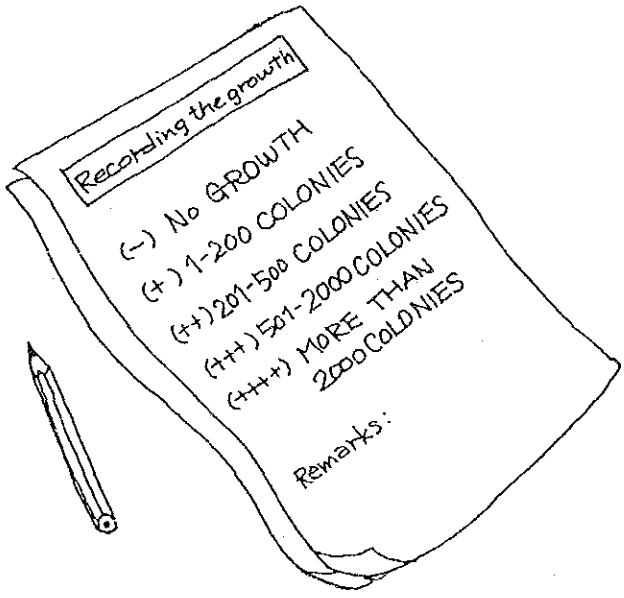


Fig. 12 Recording the growth

Record the growth as above.

MEMORANDUM

IDENTIFICATION OF M. TB

Fig. 1

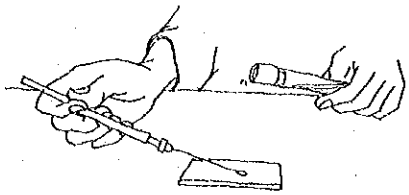


Fig. 2

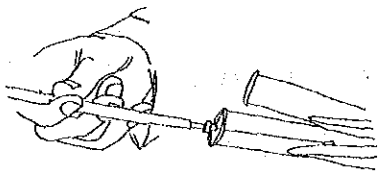


Fig. 3

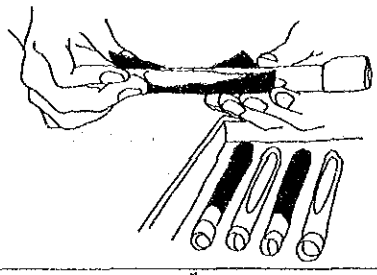


Fig. 4

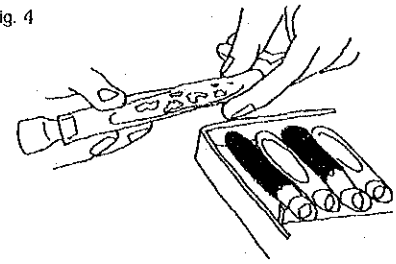


Fig. 5

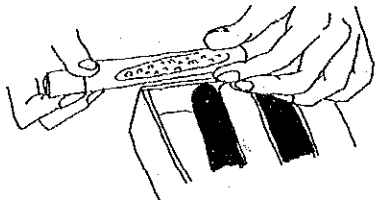


Fig. 6

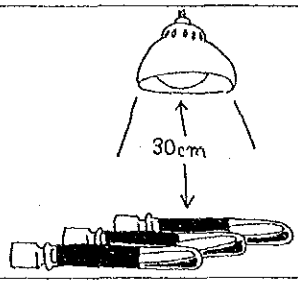


Fig. 7

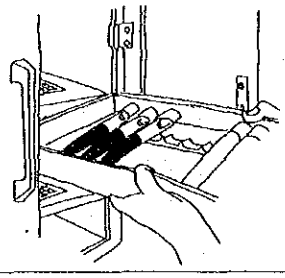


Fig. 8

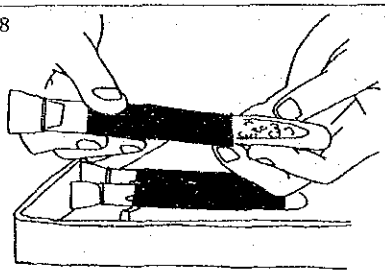


Fig. 9

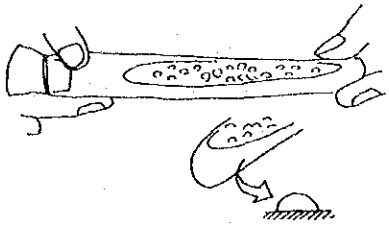


Fig. 10

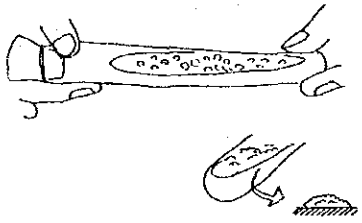


Fig. 11

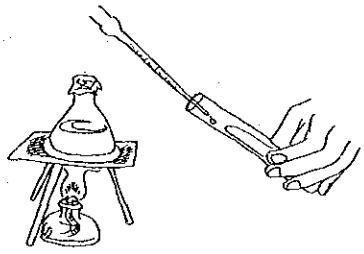


Fig. 12

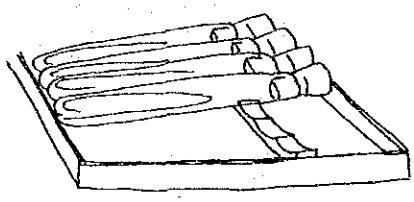


Fig. 13

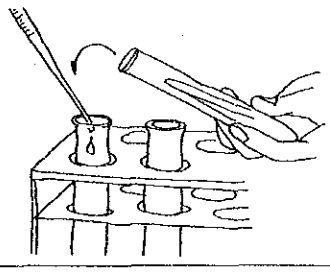


Fig. 14

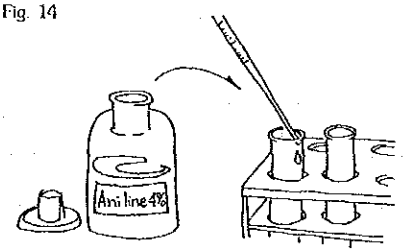


Fig. 15

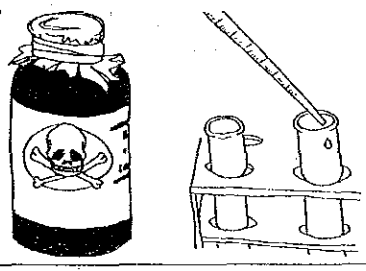
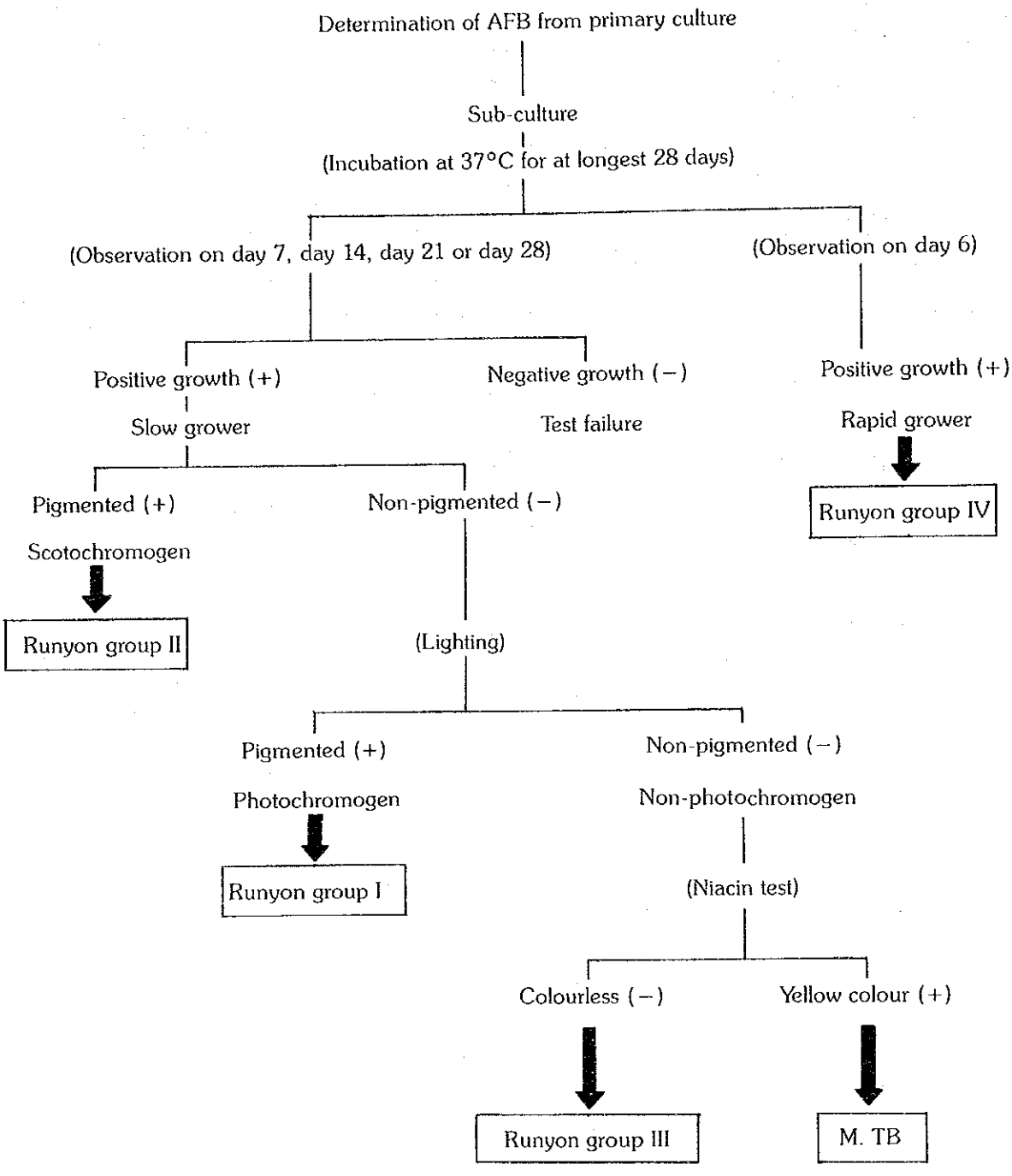


Fig. 16

FLOW CHART FOR IDENTIFICATION OF M. TB



MEMORANDUM

IV. IDENTIFICATION OF M. TUBERCULOSIS

Identification test must be performed with the subcultured strain, not/with the primarily isolated strain. If culture contaminated, decontaminate it.

IV-1 DETERMINATION OF ACID-FASTNESS OF BACILLI (Fig. 1)

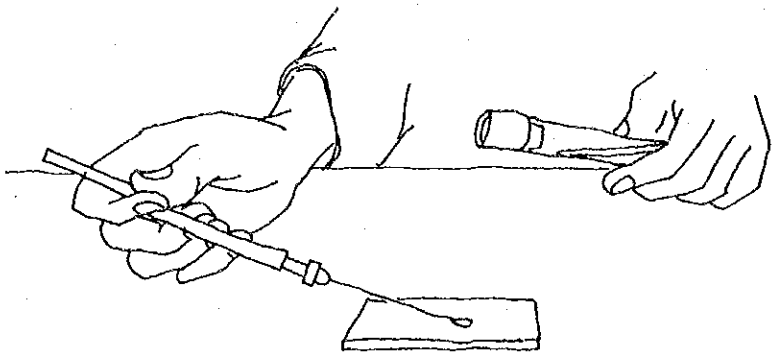


Fig. 1 Staining of the isolated bacilli

Stain the bacilli isolated from the primary culture by acid-fast staining. Acid-fast bacilli are stained in red colour.

IV-2 SUBCULTURE (Fig. 2 and Fig. 3)

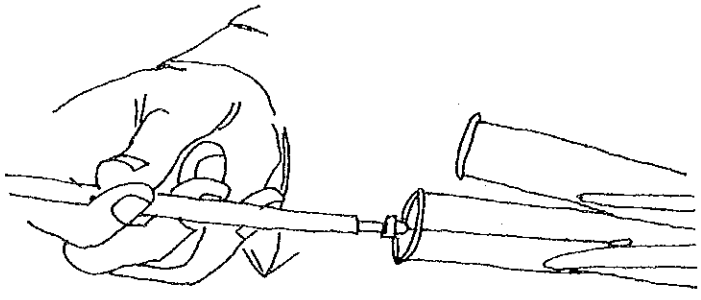


Fig. 2 Subculture

Make two subcultures for each case with a small inoculum to obtain isolated and pure colonies.

MEMORANDUM

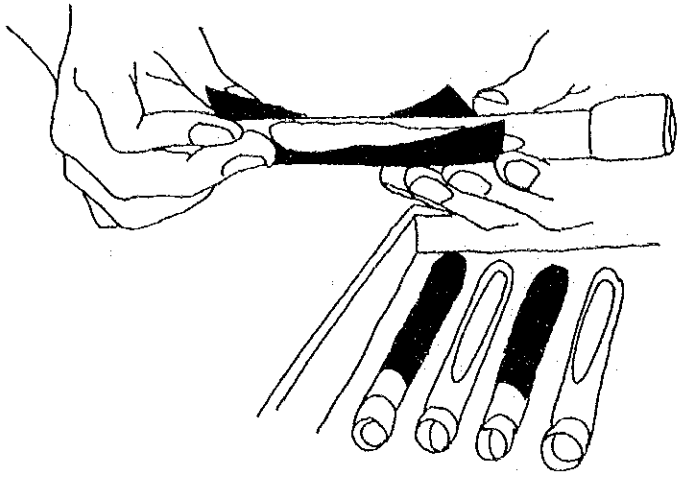


Fig. 3 Wrapping of the tubes

Wrap one of the culture tubes with black paper for pigmentation test and keep both unwrapped and wrapped tubes in an incubator at 37°C for 1~4 weeks.

IV-3 RUNYON GROUP (Fig. 4~Fig. 9)

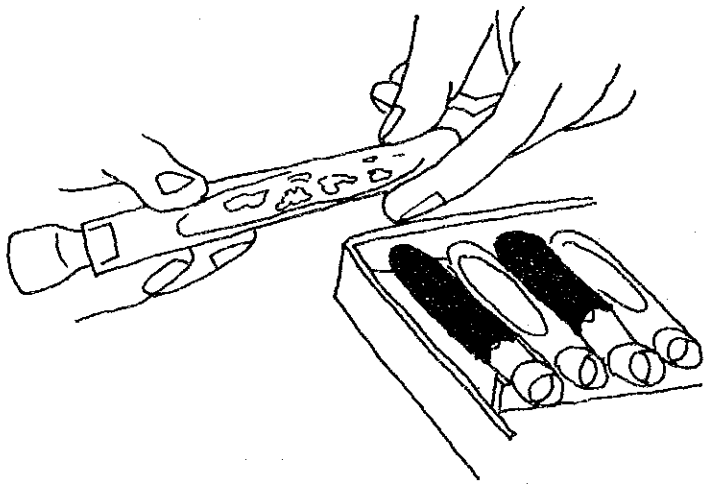


Fig. 4 Runyon group IV

Observe the unwrapped tube whether visible growth is present or not after 1 week of incubation.
If mature colonies are visible in less than 7 days, it is classified as Runyon group IV.

MEMORANDUM

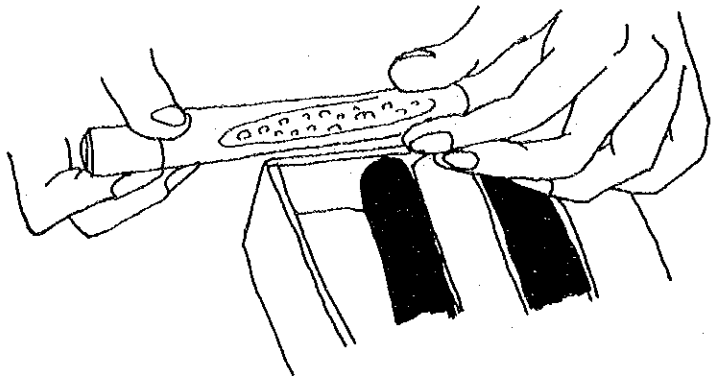


Fig. 5 Runyon group II

Mature colonies are not visible on day 7, continue the incubation. If mature colonies are pigmented in orange colour after 1 week of incubation, it is classified as Runyon group II.

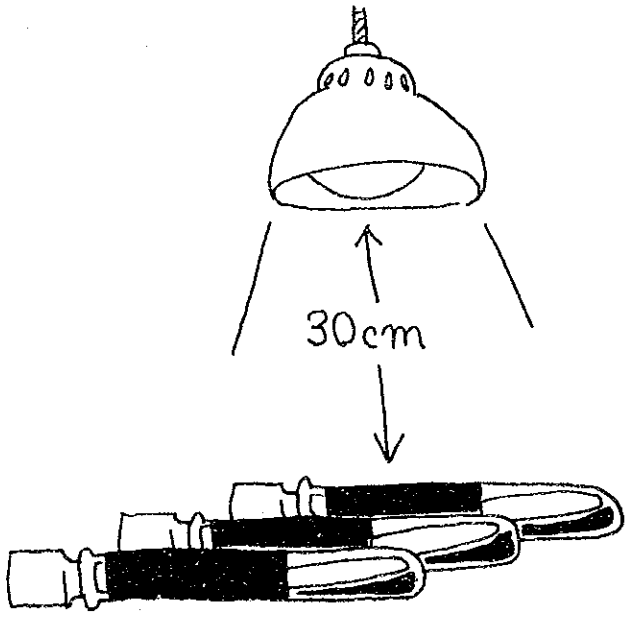


Fig. 6 Exposure to the light

If the colonies are not pigmented, open half the wrapped paper over the tube and expose the growth to a 60W tungsten lamp for 60 minutes at the height of 30cm.

MEMORANDUM

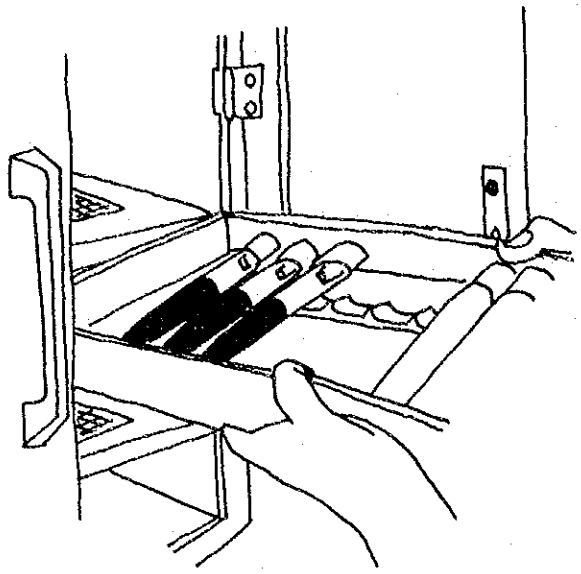


Fig. 7 Reincubation

Cover the whole tube again, then reincubate it overnight.

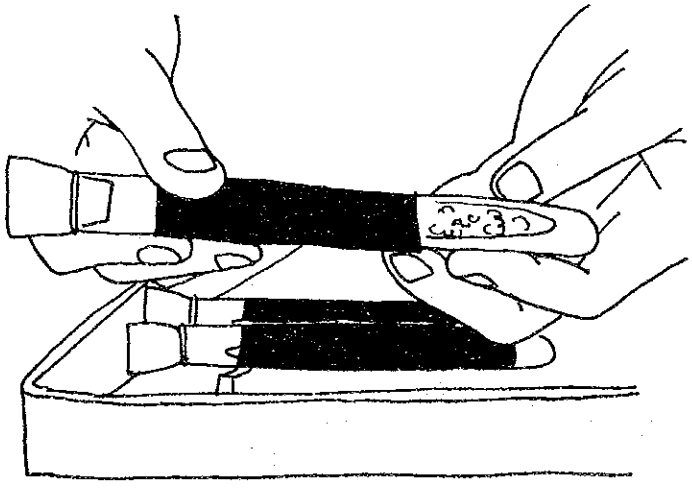


Fig. 8 Runyon group I

If the light-exposed part of colonies is pigmented in lemon yellow, it is classified as Runyon group I.

MEMORANDUM

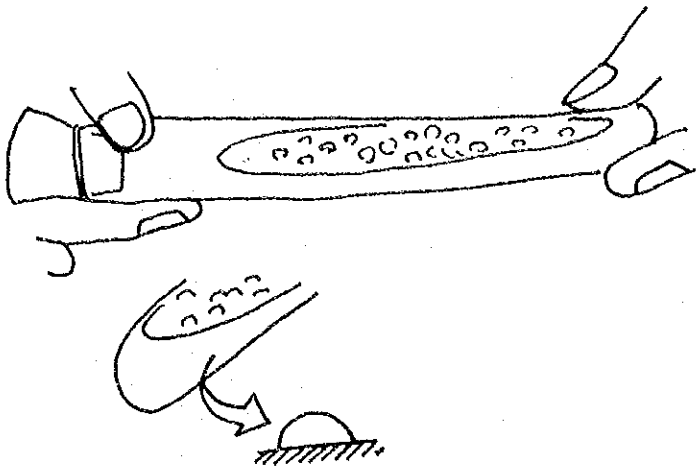


Fig. 9 Runyon group III

If the colonies are not pigmented with smooth type either in wrapped paper or after the exposure to light, it is classified as Runyon group III.

IV-4 NIACIN TEST (Fig. 10~Fig. 16)

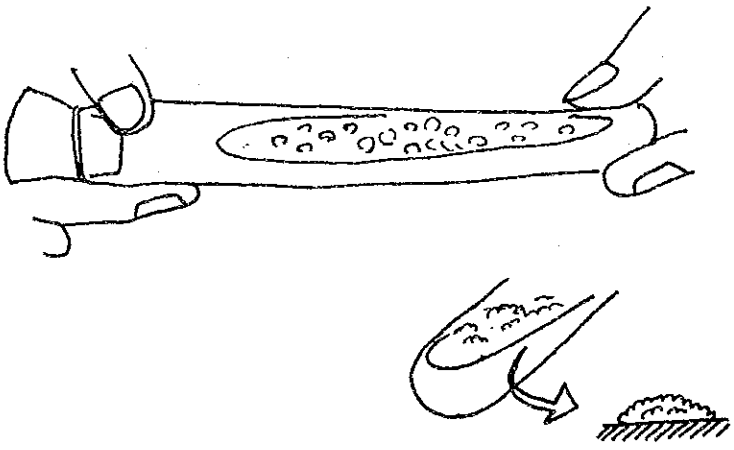


Fig. 10 Colony of M. tuberculosis

If not pigmented, perform Niacin test for identification of M. tuberculosis.

MEMORANDUM

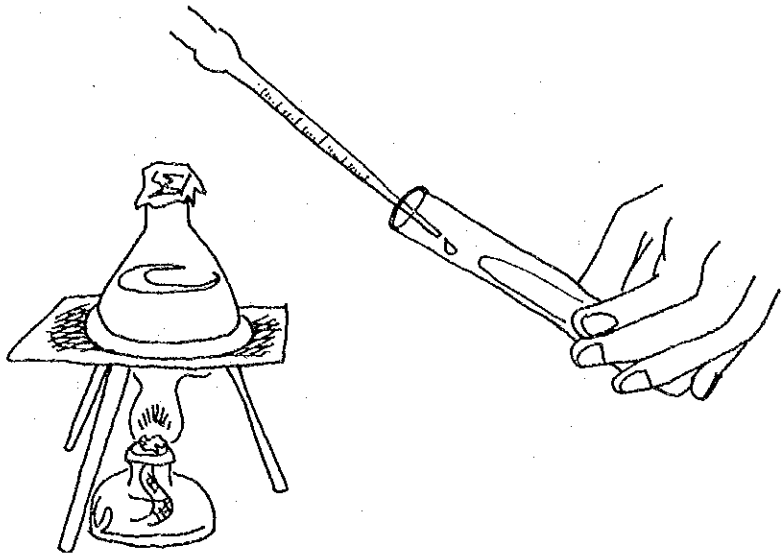


Fig. 11 Addition of boiling water

Pour 2mℓ of boiling water on the enough growth (at least more than 100 colonies) of medium.

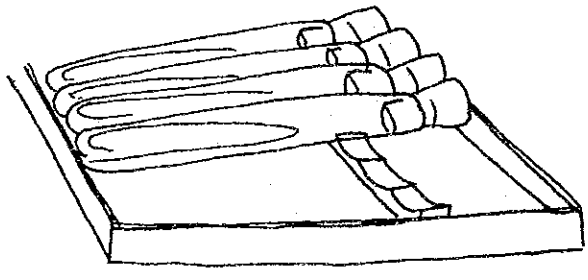


Fig. 12 Extraction of Niacin

Keep the tube at slanting position for 10 minutes.

MEMORANDUM

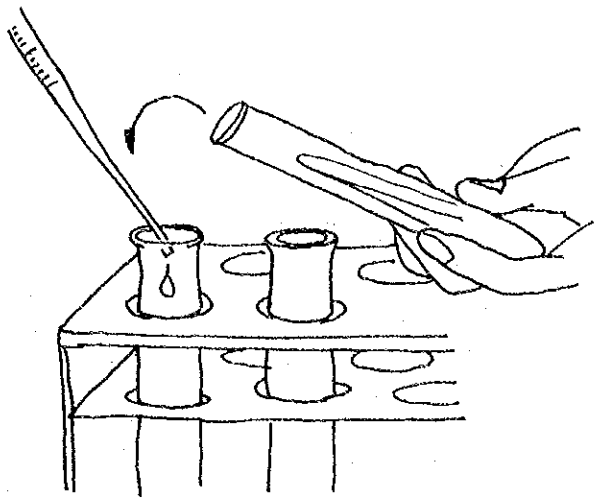


Fig. 13 Transfer of Niacin extract

Prepare 2 tubes and pour 0,2mℓ of the liquid extract into each of tubes.

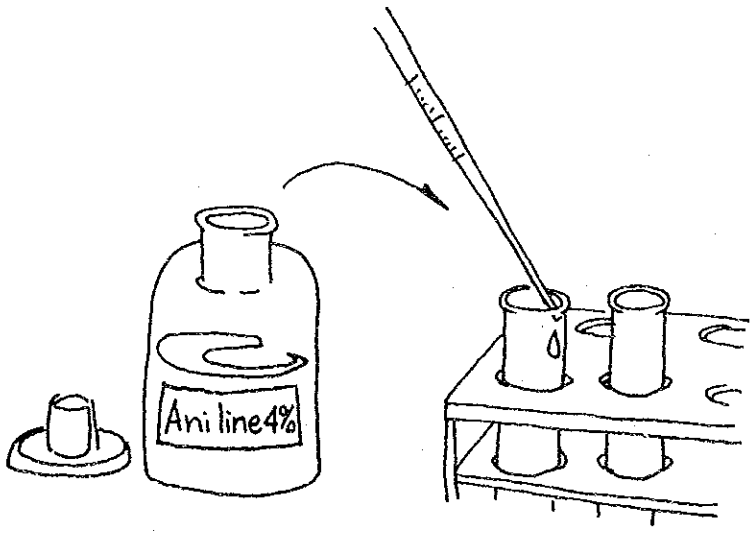


Fig. 14 Addition of aniline ethanol

Add 0,1ml of 4% Aniline Ethanol in each tube.

MEMORANDUM

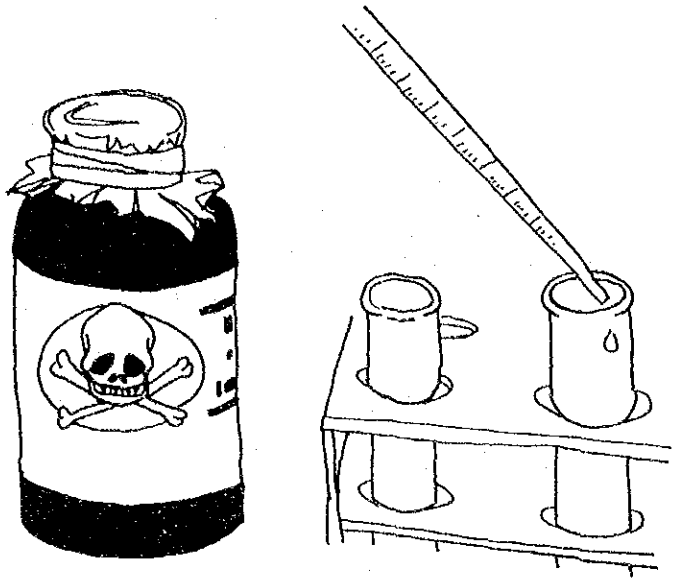


Fig. 15 Addition of 10% cyanogen bromide

Add 0,1mℓ of 10% cyanogen bromide solution into one of the tubes.
Mix them gently.
The rest tube is for control.

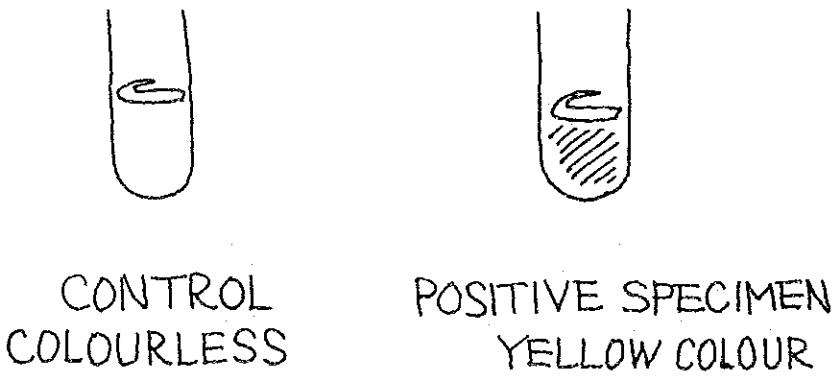
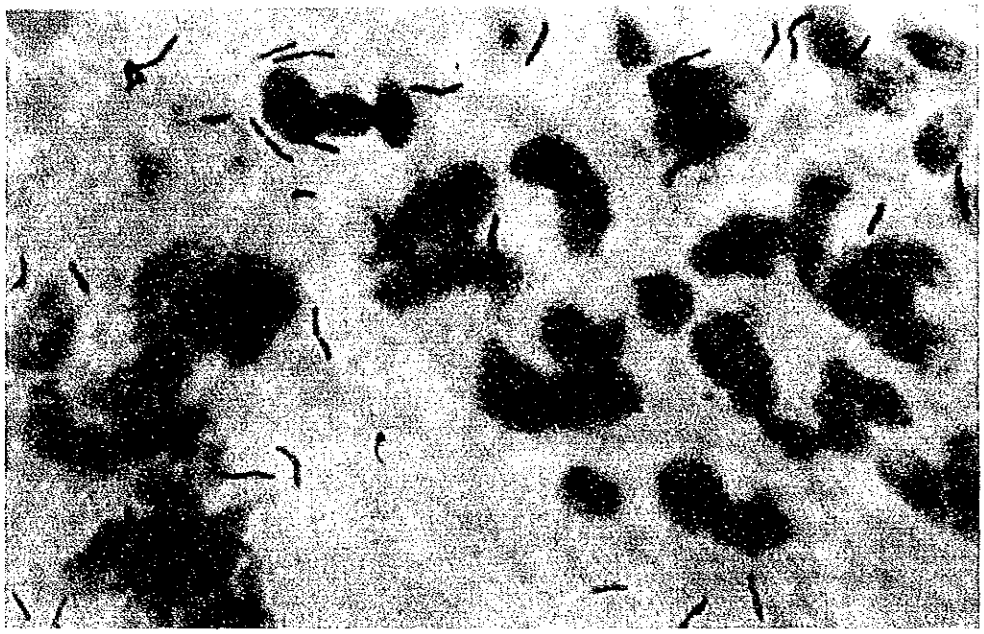


Fig. 16 Result of Niacin test

Yellow colour indicates presence of Niacin. M. tuberculosis usually show niacin positive reaction.

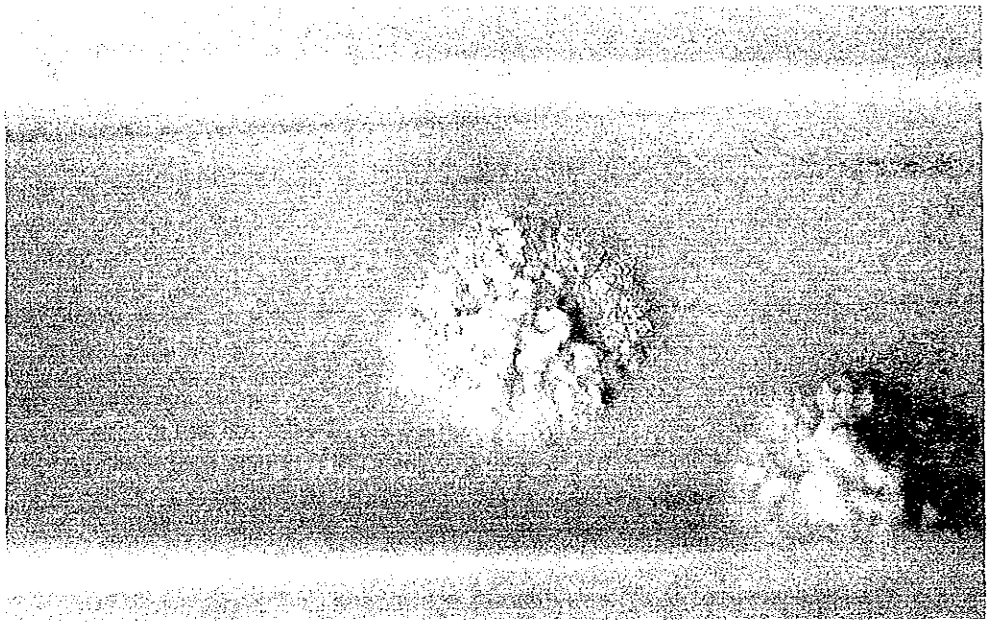
MEMORANDUM

TUBERCLE BACILLI



Mycobacterium tuberculosis in sputum stained by Ziehl-Neelsen method at magnification of $\times 1000$.
Tubercle bacilli are stained red and the background material is stained blue.

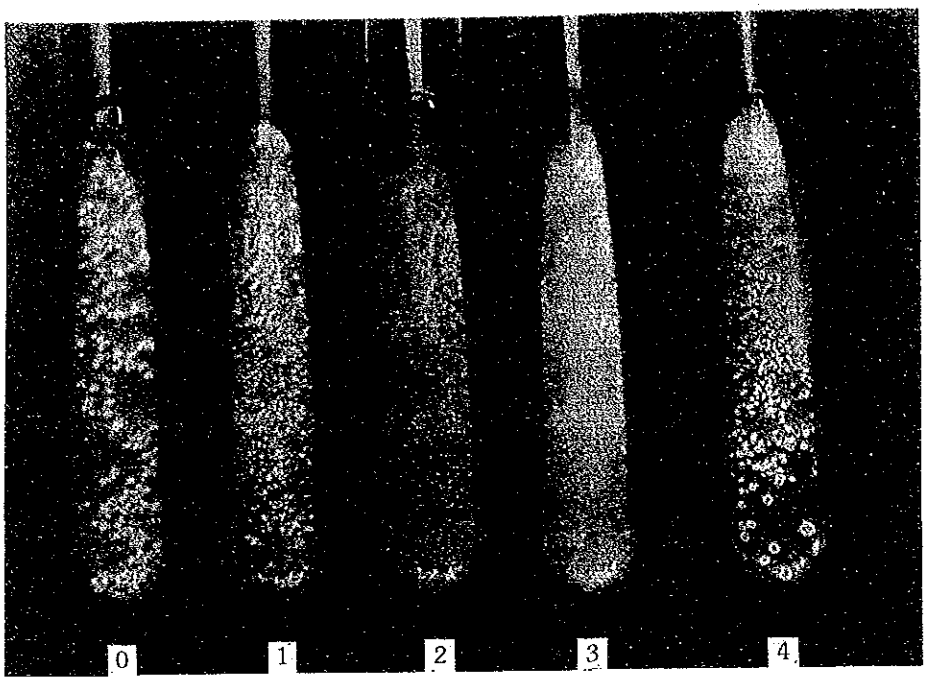
COLONIES OF M. TUBERCULOSIS



M. tuberculosis on Ogawa medium.
Dry, friable, irregular margin, eugonic growth with a cauliflower center and buff colour.

M. TUBERCULOSIS AND RUNYON GROUPS

Colonies of mycobacteria on Ogawa medium



from left to right; M. tuberculosis ($H_{37}R_V$), M. kansasii, M. gordonae. M. intracellulare. M. chelonei.

0 : M. Tuberculosis
Typical colonies of M. turberculosis on Ogawa medium show dry, friable, irregular margin, eugonic growth and buff colour.

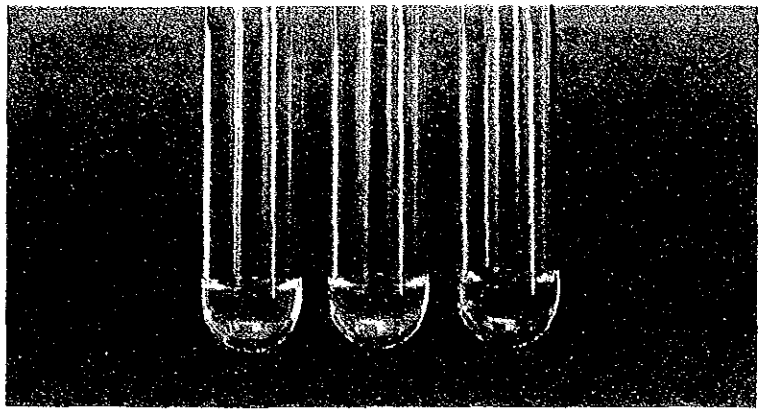
1 : Runyon Group I
Colonies are cream coloured when grown in the dark (upper part) and become a bright lemon yellow after exposure to light (lower part).

2 : Runyon Group II
Colonies are pigmented to orange in both the light and dark.

3 : Runyon Group III
Colony pigment is not developed by exposure to light.

4 : Runyon Group IV
Colonies are visible within 7 days. Some old colonies appear greenish by absorbing malachite green from egg medium.

NIACIN TEST (Aniline method)



Yellow colour indicates the presence of Niacin, from the left (−), (+), (卌).

Procedure : (1) Specimen — more than 100 colonies of growth

+

(2) Bioling water — 2mℓ

(3) 10 minutes

(4) Niacin extract — 0.2mℓ (for specimen) | 0.2mℓ (for control)

+ | +

(5) 4% Aniline ethanol — 0.1mℓ | 0.1ml

+

(6) 10% Cyanogen bromide — 0.1ml

↓ | ↓

Yellow (positive) | Colourless

Cyanogen bromide is POISON. Cyanogen bromide in the tube should be detoxified by alkaline just after the test.

+

Detoxification: (7) 4% Sodium hydroxide — 0.2mℓ

REPORTING AND RECORDING

The expression of the results may differ from a laboratory to another but it should be unified and the implication of each expression must be well understood.

Examples:

for Smear Examination

AI.A scale (by the American Lung Association in U.S.A.)

(−) No acid-fast bacilli found
(±) 1 ~ 2 AFB/300VF
Report number found and request repeat specimen
(+) 3 ~ 9 AFB/300VF
(＋) 10 ~ 299 AFB/300VF
(＋＋) More than 300 AFB/300VF

for Culture Examination

Growth rate (by Japanese national standard)

(−) No growth
(+) 1 ~ 200 colonies.
Record the actual number, if possible (e.g. $+_{15}$).
(＋) 200 ~ 500 colonies or partially confluent growth.
Record the approximate number, if possible (e.g. $+_{205}$).
(＋＋) 500 ~ 2000 colonies or almost confluent growth.
(＋＋＋) More than 2000 colonies or confluent growth.

LABORATORY RECORD NOTE

For Direct smear examination

Date of exam.	Name	Reg. no.	Age	Sex	Sl. no.	Specimen	Smear	Remarks
1-Sep-'86	Jane Doe	9001	20	F	11	sputum	+	
〃	John Doe	9002	35	M	12	〃	+++	
〃	Hanako Honda	9003	65	F	13	〃	—	
〃	Taro Suzuki	9004	19	M	14	〃	++	

For Culture examination

Date of exam.	Name	Reg. no.	Age	Sex.	Sl. no.	Specimen	Smear	Culutre			
								1W		4W	
1-Sep-'86	Jane Doe	9001	20	F	8	sputum	+	—	—	++	++
	John Doe	9002	35	M	9	〃	+++	—	—	++	+++
	Hanako Honda	9003	65	F	10	〃	—	—	—	—	—
	Taro Suzuki	9004	19	M	11	〃	++	—	—	+++	+++

For Identification tests

Date of exam.	Name	Reg. no.	Age	Sex	Sl. no.	Acid-fastness	Growth rate		Colony type	Pigmentation			Niacin	Remarks
							1W<	<4W		Photo	Scoto	non photo		
20-oct-'86	Jane Doe	9001	20	F	5	pos.		+++	R	—	—		+++	M.+b
	John Doe	9002	35	M	6	〃		〃	〃	—	—		〃	〃
	Taro Suzuki	9004	19	〃	7	〃		〃	〃	—	—		〃	〃

FORMULATION OF REAGENTS

1) Saturated alcoholic fuchsin
   - Fuchsin (basic) 3g
   - Ethanol (95%) 100mℓ

2) 5% Phenol solution
   - *Phenol melted* 5mℓ
   - Distilled water 95mℓ

3) Ziehl's solution (Phenol fuchsin solution)
   - Saturated alcoholic fuchsin 10mℓ
   - 5% Phenol solution 90mℓ

4) Acid alcohol
   - *Hydrochloric acid (conc. HCℓ)* 3mℓ
   - Ethanol (95%) 97mℓ

5) 0.1% Methylene blue solution
   - Methylene blue 0.1g
   - Distilled water 100mℓ

6) 2% Malachite green
   - *Malachite green* 2g
   - Distilled water 100mℓ

7) 4% NaOH
   - Sodium hydroxide (NaOH) 4g
   - Distilled water 100mℓ

8) 4% Aniline ethanol 4mℓ
   - *Aniline* 4mℓ
   - Ethanol (95%) 96mℓ

9) 10% Cyanogen bromide (Saturated cyanogen bromide)
   - Cyanogen bromide 5g
   - Distilled water 50mℓ