LABORATORY REPORT NO. 323

MYCOBACTERIOLOGY LABORATORY METHODS

Eugene B. Blair
Orman L. Weiser
Ann H. Tull

MICROBIOLOGY DIVISION

NOVEMBER 1969

U. S. ARMY MEDICAL RESEARCH & NUTRITION LABORATORY

FITZSIMONS GENERAL HOSPITAL
DENVER, COLORADO 80240

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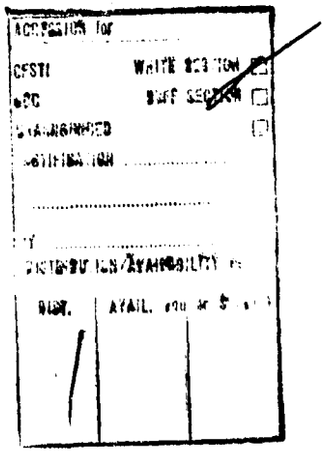

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MYCOBACTERIOLOGY LABORATORY METHODS

Eugene B. Blair, COL, MSC

Orman L. Weiser, M. S.

Ann H. Tull, B. S.

Microbiology Division

U. S. Army Medical Research and Nutrition Laboratory

ABSTRACT

This report presents in detail the latest methodology for isolation, culture, and identification of pathogenic mycobacteria. Methodology and equipment for blue light excitation of fluorochrome (auramine O)-stained mycobacteria and for blue light microscopy are discussed. Methods are described for direct and indirect drug susceptibility studies, serum drug levels, serum inhibition tests, and minimum inhibitory concentrations. Complete formulation and preparation of the modified Middlebrook and Cohn 7H10 OA agar medium and bioasssay media ar included.

FOREWORD

Revision of these previously published procedures on Mycobacteriology Laboratory Methods is intended for updating purposes for use by the investigator or technician with a basic background in mycobacteriology techniques.

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Work Unit Number: 065 Microbiological Research in Tuberculosis

INTRODUCTION

Definitive proof of mycobacterial infection is achieved only by isolating the causative agent. In the study of mycobacterial infections, the value of the laboratory is dependent on the adequacy of the decontamination methods, the isolation media employed, and valid drug susceptibility-resistance data.

The ideal methodology for isolation and study of mycobacteria should provide effective decontamination of specimens with minimal destruction of the bacilli. Culture media should stimulate rapid growth from small inocula to permit early detection of growth and ease of identification. Most of the media used since the discovery of mycobacteria are opaque, hindering the early detection of colonies, obscuring the identifying colonial characteristics, and delaying the laboratory confirmation of the clinical diagnosis. In addition, quantitative estimates of resistance and susceptibility are questionable when media are employed which must be sterilized by inspissation after incorporating heat-labile drugs.

Factors preventing isolation and complicating the collection of meaningful susceptibility data are:

1. Mycobacteria are lost from improperly collected and processed specimens.

2. Many strains of drug resistant mycobacteria may fail to grow or grow very slowly on some conventional isolation and drug-containing media.

3. Because of the excessively large inocula commonly used for inoculating drug-containing media for indirect susceptibility determinations, the data derived are of limited quantitative significance due to their failure to give a reliable picture of the resistant and susceptible bacterial populations being excreted by the patient.

GENERAL METHODOLOGY

The methods described in this report are currently in use in this laboratory. Preparation of reagents, media, etc. are listed in the APPENDIX.*

* The use of trade names is for the purpose of identification only and does not constitute an endorsement by the Department of Defense or USAMRNL.

The medium employed in our laboratory is Middlebrook's 7H10 OA medium with L-asparagine (1). This medium, in petri and quadrant plates, provides the following favorable characteristics:

1. Of primary importance is the uniformity obtained between batches of media. This is not possible using complex ingredients such as peptones, flour, eggs, or blood, which are used in other media.

2. The absence of these materials simplifies its preparation, sterilization, and dispensing.

3. Its transparency allows early detection of growth and facilitates the study of morphology. Differentiation between corded and non-corded colonies is readily determined using a stereoscopic microscope.

4. Mycobacteria other than M. tuberculosis also grow well, allowing colonial and chromogenic differences to be easily recognized.

5. The aseptic addition of antituberculous drugs to a sterile medium at dispensing temperature provides known amounts of these drugs and permits more accurate drug-susceptibility data.

6. Catalase tests can be performed directly on the plates, and other tests used to differentiate mycobacteria can be performed using colonies scraped from the plate.

The use of both isolation and primary drug susceptibility plates on admission specimens is amply justified. The isolation plate is needed, as the smaller inoculum used on susceptibility plates could result in a false negative report when only a few viable organisms are present in the specimen. When the number of viable organisms is sufficient, a direct susceptibility report can be given the clinician within 3-4 weeks of receipt of the specimen, allowing him to evaluate early in the course of therapy the efficacy of the regimen being used. Additionally, a direct susceptibility test is usually a more accurate representation of the drug characteristics of the patient's mycobacterial population than is an indirect study.

In our experience, clinical specimens containing large numbers of M. tuberculosis have given satisfactory susceptibility results, even though plated at only one dilution. Therefore, specimens are plated as soon as preparation of the inoculum is completed, and the fluorochrome-stained smear concentrates are read at a later time. However, all concentrates are held in the refrigerator until smears are examined; specimens with positive smears are inoculated to drug susceptibility plates, if these have not already been set up. While the rate of growth of a resistant strain

may be retarded by comparison with the control, a report of 100% resistance is made when the numbers of colonies on the control and drug-containing quadrants are approximately equal.

All cultures for primary isolation of mycobacteria are incubated in an atmosphere of 10% CO_2 (2), as some strains of M. tuberculosis require CO_2 for initiation of growth. Our experience, as well as that of others reported in the literature, has shown no strains to be inhibited in such an atmosphere. Carbon dioxide may be added to the incubator chamber by several means:

1. Use a compressed gas cylinder, 90% air-10% CO_2, to which has been attached a gas flow meter. Calculate the time necessary to fill the incubator at a given flow rate.

2. Place the amount of dry ice sufficient to achieve the desired CO_2 concentration on the top shelf of the incubator. Use a small fan in the incubator to disperse the CO_2 as it sublimes from the dry ice.

3. Include a culture of M. phlei with inoculated plates in CO_2-impermeable Mylar (a) plastic bags as described by Cohn and Middlebrook (3).

Fluorochrome Staining and Blue Light Excitation of Mycobacteria

This laboratory has found the staining method employing a fluorochrome stain, auramine O, to be more efficient for the rapid detection of mycobacteria than methods using phenol-fuchsin stains. The fluorescence method allows the technician to scan a larger area of the slide in much less time than is required for conventional oil immersion examination. The use of fluorescence microscopy for the detection of mycobacteria was described by Hagemann (4) in 1937 and subsequently used by many others (5, 6, 7). Advantages for its use are: staining is quicker, cleaner, and simpler; the examination time can be reduced as only low power (10X-25X) and high dry (63X) objectives are used, thus permitting a larger area of the preparation to be searched in a shorter period of time; the greater contrast between the stained organism and the background reduces eye strain. Weiser et al. (8) in a double blind comparison found the fluorochrome method reliable and equal to the Ziehl-Neelsen technique, but preferred the former because of the simplicity of staining and time saved.

(a) Sources of equipment and reagents, etc., indicated by a lower case letter, may be found in the APPENDIX.

Kubica and Dye (9) stated as a disadvantage of the fluorescence method that "organisms apparently dead or rendered noncultivatable by chemotherapy (and nonstainable by Ziehl-Neelsen stains) may still be fluorescence positive." Studies were undertaken in this laboratory to compare the efficacy of the auramine staining method with the Kinyoun cold stain and the xylene-phenol fuchsin method, described by Margineanu (10), to demonstrate INH-induced loss of acid-fastness in tubercle bacilli. Growth end points in serum drug levels and minimal inhibitory concentrations (MIC), as determined by loss of fluorescence in 50% of the cells, were within experimental error of end points determined by triphenyl tetrazolium chloride (TTC). Auramine-positive and fuchsin-positive cells, as well as many nonacid-fast cells, were seen in tubes wherein the INH concentrations were 6- to 8-fold greater than the MIC as determined by TTC. Subcultures of these tubes revealed growth of tubercle bacilli, indicating that the fuchsin- or auramine-fast cells were viable. Ethambutol, but not streptomycin, produced a similar effect with the three staining methods. The contention that the Ziehl-Neelsen stain will not stain "dead" or noncultivatable bacilli is highly speculative in the light of past reports, especially with regard to tissues, calcified nodes, the autoclave method of preparing smear concentrates, and staining reactions of nonviable cultures. Fusillo and Burns (11), Somlo et al. (12), and others advocate the fluorescence technique as being superior to conventional methods for reasons of increased numbers of positive smears found and/or because of time saved in examining smears.

In spite of these advantages, fluorescence microscopy has been used only on a limited scale. In the past the technique required costly equipment and a dark room for microscopic examination of slides. The use of intense blue light instead of ultraviolet light (critical illumination instead of Kohler illumination)* has made available comparatively inexpensive equipment which greatly simplifies the fluorescence method. Immersion oil is not used with the bright light condenser and planachromat objectives; a dark room is not required although subdued light is recommended. Richards and Miller (13) found that excitation light produced by "a low voltage, high amperage concentrated filament microscope lamp which emits sufficient ultraviolet radiation when used with a blue violet (ultraviolet transmitting) filter and a complementary yellow filter" imparted a bright-yellow fluorescence to auramine O-stained bacilli. The requirements for the use of blue light are: a simple incandescent lamp which is properly housed for

* Critical illumination is achieved by focusing the lamp filament light source on the plane of the specimen, rather than on the field diaphragm.

concentrating the light to greatest intensity, a KG 1 heat filter, a BG 12 excitation filter of either 3 or 4 mm thickness (peaking at a wavelength of 404 mμ), bright field substage condenser which does not require the use of immersion oil, and a barrier filter (No. 50 or 53 Zeiss barrier filter) between the objectives and eyepieces which transmits only wavelengths above 500-530 mμ. With proper conversion equipment most binocular microscopes can be converted for blue light fluorescence microscopy.

The combination of complementary excitation and barrier filters determines the color of the background: light green with the No. 50 (500 mμ)* barrier filter and the 3 mm BG 12 excitation filter, very dark green (almost black) with the No. 50 barrier filter and the 4 mm excitation filter, reddish-brown with the No. 53 (530 mμ) barrier filter and the 3 mm BG 12 excitation filter. The 3 mm BG 12 filter is preferred in this laboratory because the background is light enough to allow ease in focusing. Although all are good, the greater contrast afforded by the combination of the Zeiss No. 53 barrier filter and the 3 mm BG 12 filter results in slightly better visualization of fluorescing bacilli than do other combinations.

Excellent resolution of auramine O-stained mycobacteria and a few nocardia species is obtained through the use of planachromat objectives of high numerical aperture with compensating eyepieces and intense critical blue light. The low power planachromat objective (25/0.45) in combination with the compensating KPL 10X eyepieces gives 250 magnifications, a flat field of vision, and easy focusing. The high dry planachromat objective (63/0.9), without coverglass correction, in combination with the KPL 10X eyepieces develops 630 magnifications and excellent resolution of bacillary morphology, obviating the need for examination under oil immersion.

Equipment which has proved highly successful for microscopic examination of auramine-stained mycobacteria is available from Carl Zeiss, Inc. (b) and is appended for information purposes. Two lists are appended: the first for a complete unit and the second for the equipment necessary to convert most microscopes for blue light fluorescence microscopy. Certain items are included in both lists (as indicated), which enable the user to convert from fluorescence to white light microscopy.

* Transmits all wavelengths above approximately 500 mμ.

Collection of Specimens:

The order of preference for specimens from pulmonary tuberculosis patients is:

1. Ten ml of first morning voluntarily-raised sputum, not nasopharyngeal discharge or saliva. A mouthwash should not be used before collection.

2. Ten ml of induced sputum obtained by aerosolization.

3. Fasting gastric lavage.

Urine studies require that only the first morning, voided, midstream specimen be collected.

Twenty-four hour collection of sputum or urine is not acceptable, because processing sufficient for decontamination usually destroys the viable mycobacteria present.

Sputum specimens are collected in the sterile Falcon sputum collection system (c), and gastrics in sterile 4 oz. plastic cups to which has been added 1.0 ml of 10% $NaHCO_3$. All specimens should be collected early and forwarded immediately to the laboratory. Refrigerate specimens if immediate delivery to the laboratory is impossible or if processing in the laboratory must be delayed.

PROCEDURES FOR PROCESSING MYCOBACTERIAL SPECIMENS

ALL PROCESSING OF SPECIMENS OR CULTURES WILL BE PERFORMED UNDER THE SAFETY HOOD WITH THE EXHAUST FAN ON. All potentially infectious materials for discard will be placed in appropriate vessels containing disinfectant solution.

PRELIMINARY PREPARATION OF SPECIMENS

Sputum and Bronchial Washing

Remove the 50 ml plastic centrifuge tubes from their carriers. Number each tube with specimen number. The volume of sputum should never exceed one-fifth the volume (10 ml) of the container. If the specimen volume exceeds 10 ml, discard the excess. Proceed to "Decontamination Procedures."

Gastric Lavage

To 20-40 ml of gastric lavage in a 50 ml sterile Falcon plastic screw-capped centrifuge tube (c), add a pinch (50-100 mg) of powdered N-acetyl-L-cysteine (d). Mix on a Vortex mixer (e) to digest the mucoid material. Centrifuge the digested material at 2000 G for 30 minutes. (See Nomograph in the APPENDIX for determination of force relative to speed and the head size of centrifuges.) Decant all of the supernatant liquid. Resuspend the sediment in 5 ml of sterile saline. Proceed to "Decontamination Procedures."

Urine

Transfer the entire first morning specimen to 50 ml centrifuge tube(s). Centrifuge at 2000 G for 30 minutes. Pour off the supernate. Resuspend the sediment in 5 ml of sterile saline. (If more than one tube is used, the sediments may be pooled to the extent that the volume does not exceed 10 ml.) Proceed to "Decontamination Procedures."

Bone Marrow, Pleural Fluid, and "Sterile" Body Fluids

Prepare smears and inoculate the specimen directly (without decontamination) to isolation plates and drug susceptibility plates (all drugs). Inoculate a blood agar plate with one drop of the material to serve as a check for contaminants. Keep the remainder of the specimen refrigerated pending results of the check for contamination. If there is evidence of contamination, decontaminate the refrigerated specimen.

Tissues

Attempt to select portions of tissue most likely to yield mycobacteria. Grind a small portion of the tissue (tissues may be cut into smaller pieces if strict aseptic techniques are employed) in a sterile grinder (Teflon or TenBroek) containing 3-4 ml of sterile albumin-buffer (APPENDIX). If the tissue has been collected aseptically, prepare a smear and inoculate the ground tissue onto isolation plates, drug susceptibility plates, and liquid media (Tween-albumin broth). Check for contamination by inoculating the specimen to blood agar media. Retain excess specimen in the refrigerator pending results of the test for contaminants. Prepare a smear from the inoculated liquid medium for control purposes and place liquid media tubes on the revolving wheel used for stock strains (See "Serum Drug Levels"). Prepare and examine smears, and subculture from Tween-albumin broth to isolation plates at 2, 3, and 6 weeks. If AFB are seen in the smear, also subculture to drug susceptibility plates.

When the tissue is known or suspected to have been contaminated, treat the ground specimen with the NaOH--N-acetyl-L-cysteine (NaOH-NALC) decontaminating-digesting solution and proceed to "Decontamination Procedures," step 3.

DECONTAMINATION PROCEDURES

The sodium hydroxide-acetylcysteine (NaOH-NALC) (14, 15) method is described.

1. Add to the specimen in each tube an EQUAL volume of the NaOH-NALC decontamination-digestant solution. The volume of specimen should never exceed one-fifth the volume (10 ml) of the container. Replace and tighten caps, and invert the tube to wet the cap with the decontaminating solution.

2. Mix the specimen-digestant mixture for 10-30 seconds using a Vortex mixer. Especially viscid specimens may require longer periods; however, avoid excessive agitation (aeration), which may reverse the mucolytic activity of the NALC.

3. Allow the mixture to stand at room temperature (20-25°C) for 15 minutes. Avoid excessive exposure of the specimen to the NaOH solution; if excessive (>5%) contamination is encountered, it is preferable to increase the NaOH concentration rather than the decontamination time (9). If the contamination rate is less than 1%, the possibility exists that the decontamination procedure is too harsh, and that many mycobacterial cells are being destroyed (9).

4. Add to the digested specimen sufficient sterile M/15 phosphate buffer, pH 6.6, to fill the centrifuge tube to the 50 ml mark. Replace the cap and invert the tube to mix buffer and decontaminated specimen. (The buffer serves to neutralize the sodium hydroxide and to reduce the specific gravity.)

5. Centrifuge tubes at 2000 G for 30 minutes.

6. Using care to avoid disturbing the button of sediment, pour off ALL supernatant liquid.

7. Using 2 sterile applicator sticks, smear the sediment onto new glass slides for staining. (Gastrics and urines are not routinely examined microscopically, as these specimens sometimes contain saprophytic acid-fast bacilli.)

8. Resuspend the sediment by adding 3 ml of sterile M/15 phosphate buffer containing 0.2% albumin and use this suspension as the inoculum.

NOTE: The pH of this suspension should be between 6.6 and 7.6. The pH is checked by taking a drop of suspension on a sterile applicator stick and touching it to a strip of pH paper. If the suspension is excessively alkaline, add additional buffer, centrifuge, decant, and resuspend the sediment in 3 ml of fresh buffer.

PRIMARY ISOLATION AND DIRECT DRUG SUSCEPTIBILITY PROCEDURES

Each admission sputum specimen is plated (1) on an entire petri plate of 7H10 OA agar containing no drugs and (2) on 7H10 OA drug susceptibility media containing the following drugs: Isoniazid, streptomycin, p-aminosalicylic acid, Capreomycin, and Ethambutol. If smears from any specimens are positive for acid-fast bacilli, the concentrate is tested on all drug media, including Kanamycin and Ethionamide. Susceptibility tests for Rifampin are in an investigational status.

Sterile, cotton-plugged, disposable capillary pipettes are used for inoculation of plates. The isolation plate is inoculated with 6 drops of the suspension. Each of the 4 quadrants of the drug susceptibility plates is inoculated with 3 drops of suspension (quadrant I is always used as a control and contains no drugs). Rotate plates immediately after inoculation to insure that the specimen is evenly distributed over most of the agar surface. (Specimens from skin lesions or skin biopsies are inoculated to 2 sets of media: one set is incubated at room temperature in the dark, and the other set at 36-37°C under 10% CO_2.) Place

each plate in a CO_2-permeable, polyethylene plastic bag (6x8, .0015 gauge) (f). Seal bag by folding the open end and stapling. Incubate plates in an upright position at 36-37°C under 10% CO_2.

The concentrations of drugs employed in our laboratory at the present time are: (See APPENDIX for preparation of drug solutions.)

Isoniazid (INH)	1.0 γ/ml
Streptomycin (SM)	2.5 γ/ml
P-amino salicylic acid (PAS)	1.0 γ/ml
Capreomycin (CM)	2.5 γ/ml
Ethambutol (EMB)	5.0 γ/ml and 10.0 γ/ml
Kanamycin (KM)	2.5 γ/ml
Ethionamide (ETA)	5.0 γ/ml
Rifampin (RF)	0.5 γ/ml and 1.0 γ/ml

STAINING OF MYCOBACTERIA

Unstained slides should be treated as infectious. If slides are not to be stained immediately, they may be effectively sterilized and fixed by exposing them to formaldehyde vapor in a covered Coplin jar for 10 minutes.

To minimize the danger of cross-contamination of slides by detachment of smear material: fix sediments properly, adequately space slides on staining racks, avoid too forceful washing, and do not allow water or stain to run off one slide onto another.

Staining Procedure

1. Heat-fix films of sputum or body fluids using a flame or by heating for at least 2 hours on a covered slide warmer (g) set at 65°C. Tissue specimens are deparaffinized and made equivalent with water (16).

2. Flood slides with auramine O stain (see APPENDIX for preparation). Stain for 15 minutes at room temperature. Do not heat slides.

3. Rinse slides with tap water.

4. Decolorize for 2 minutes with acid-alcohol (0.5% HCl in 70% ethanol).

5. Rinse slides with tap water.

6. Counterstain 3 minutes with 0.5% aqueous potassium permanganate.

7. Rinse slides with tap water.

8. Allow slides to air-dry.

NOTE: Stain tissue sections for 30 minutes, decolorize for 20 minutes, and air-dry overnight. Mount in a low-fluorescing mounting medium (h). (Balsam, damar, and clarite fluoresce and are unsuitable.) Use 0.16 to 0.19 mm coverslips.

Microscopic Examination of Stained Smears

1. Scan slides under low power (250X) of the microscope. Examine fluorescent material with the high dry objective (630X) for morphology.

2. A minimum of 2 morphologically-characteristic fluorescent bacilli must be seen before calling the smear positive.

3. The numbers of acid-fast bacilli on smears from clinical specimens are reported based on recommendations similar to those of the National Tuberculosis Association:

Number of Organisms Seen	Report
2-9 per slide	Rare
10 or more per slide	Few
Greater than 2 per high power field (630X)	Numerous

INDIRECT DRUG SUSCEPTIBILITY STUDIES

Indirect drug susceptibility studies are performed when growth on the control quadrants of direct susceptibility plates is insufficient (less than 50 colonies) for valid comparison with drug-containing quadrants. The method described below is essentially the same as reported in the VA-Armed Forces Handbook of Tuberculosis Laboratory Methods (17).

1. Using a sterile cotton swab, take up several (not less than 5, if possible) colonies of the original culture and make a homogenous suspension in 2.5 ml of sterile saline in a 16 x 125 mm screw cap tube (insure that the cap has a good liner). Use the isolation plate and the CONTROL quadrants, NOT the drug-containing quadrants, of the drug susceptibility plates if it is necessary to obtain the minimum number of colonies. Avoid obvious contaminants when selecting colonies. Disperse cells by rubbing the bacterial mass against the bottom and side of the tube with the cotton swab. "Wring out" the swab against the side of the tube and discard it to a disinfectant solution.

2. Allow the suspension to stand one hour for settling of clumps.

3. Without disturbing the sediment use a sterile, plugged, 1 ml disposable capillary pipette and a propipette to carefully transfer the supernatant to a sterile 16 x 125 mm glass tube. Add sufficient sterile M/15 phosphate buffer to bring the suspension to a turbidity approximating that of a McFarland Barium Sulfate Standard No. 1, i.e., 65% light transmission at 629 mμ. Prepare and examine a smear of this dilution. Dilute this suspension 1:20 by mixing in a sterile tube 0.25 ml of the standardized suspension with 4.75 ml of sterile M/15 phosphate buffer. (Cultures which suspend readily should be diluted 1:100 or 1:200.) If there are too few colonies to prepare a suspension of the described turbidity, decrease the dilution factor accordingly.

4. Inoculate and incubate plates as described for "Primary Isolation and Direct Susceptibility Proceaures."

EXAMINATION AND IDENTIFICATION OF CULTURES

Examining Cultures

Cultures are examined weekly for 4 weeks with a final reading at 6 weeks. This method of examining cultures accomplishes four things:

1. Contaminated plates may be discarded and new specimens requested.

2. Growth rate characteristics of mycobacteria other than *M. tuberculosis* can be observed.

3. Preliminary results on positive cultures can be reported, although final-type reports are sent out only after 28 days have elapsed.

4. Cultures of mycobacterial strains whose growth rates are slower may be negative at 4 weeks, but positive at 6 weeks.

Test Procedures

Catalase. Growth which has developed should be tested for catalase activity by adding a solution, prepared daily by combining equal volumes of 10% aqueous Tween 80 and 30% hydrogen peroxide (Superoxol), dropwise to colonies of mycobacteria. The hydrogen peroxide and Tween solutions are stored in the refrigerator. The test solution is brought to room temperature to obviate the possible slow evolution of dissolved air from the cold solution. The reaction can be roughly quantitated (-, ±, +, ++, +++, ++++) by observing the amount of bubbling which occurs. Most positive reactions consist of the immediate, active evolution of grossly visible bubbles of gas (O_2) from the submerged colonies; however, weak catalase producers (±) often must be observed for longer periods using low power microscopy. Keep plates covered during observation. Most INH-susceptible M. tuberculosis produce + or ++ reactions, while INH-resistant colonies may be negative or only weakly positive. Runyon Groups I, II, and IV are predominantly strong (++++) catalase producers, but some members of Group III may show the same catalase activity as M. tuberculosis.

Niacin. (18, 19). Of the nonpigmented, slowly-growing (37°C) mycobacteria likely to be cultured from clinical material, only M. tuberculosis produces niacin in sufficient amounts to be detected under the conditions of the test employed. Correspondingly, a well-confirmed negative reaction indicates another mycobacterium species. The paper strip niacin test does not require a fume hood to carry off toxic reaction products, as they are formed in small amounts and confined to the reaction tube; however, a safety hood should be used during the niacin extraction procedure. Niacin production by M. tuberculosis on the Middlebrook-Cohn 7H10 agar medium is enhanced by the addition of L-asparagine to the medium (20, 21); therefore, this laboratory routinely adds asparagine to the 7H10 OA agar medium (APPENDIX). The niacin test procedure is as follows:

a. Use 7H10 OA liquid Tween medium or sterile distilled water to extract niacin from the agar medium. Seven ml are recommended to extract a petri plate and 3 ml to extract the control quadrant of a drug susceptibility plate. Pipette the liquid onto the surface of the solid medium and let stand at room temperature (20°-25°C) for 30-60 minutes.

b. Transfer 0.6 ml of the extract to a 12 x 75 mm tube. Drop a niacin test strip (see APPENDIX for preparation of test strips), Chloramine-T end up, into the tube. Allow capillary diffusion of the extract up the strip to mix the reagents. The acidified K-thiocyanate reacting with Chloramine-T forms cyanogen chloride which, in the presence of niacin, forms a yellow color when reacted with NaPAS. Cyanogen chloride is volatile; therefore, keep the tube tightly corked during the reaction.

c. Shake occasionally, but do not tilt. Observe at 10 and 15 minutes for color development. Any yellow color in the liquid (disregard color on the paper) constitutes a positive test. The test must be read at 10 and 15 minutes, because the color fades rapidly.

If the niacin test is negative, but other properties of the culture indicate M. tuberculosis, repeat the niacin test on another culture or subculture which has been permitted to incubate for as long as 8-10 weeks. A positive test should be confirmed by determining other characteristics of M. tuberculosis.

SOME CHARACTERISTICS OF M. tuberculosis

The most specific test for identification of M. tuberculosis is the niacin test. However, the following characteristics of M. tuberculosis have been found quite useful in its differentiation from other mycobacteria:

1. Rate of Growth - Slow (requires more than 10 days to appear on artificial media).

2. Growth Temperature - Optimum 37°C, no growth at 24°C or 45°C.

3. Colony Morphology - On 7H10 OA agar and using the stereoscopic microscope, "cording" of young colonies is usually seen. Vestal and Kubica (22) and Fregnan and Smith (23) have described in detail the growth characteriesics of various mycobacterial species on 7H10 media.

4. Pigment - M. tuberculosis colonies are nonpigmented or slightly buff colored. Exposure to light does not affect pigmentation.

5. Catalase activity - Drug-susceptible M. tuberculosis strains exhibit relatively weak (+ or ++) catalase activity as compared with many of the nontuberculosis strains. Isoniazid-resistant strains may be either negative or very weak (±) catalase producers. The 68°C catalase test (24) is negative.

6. Niacin production - At present this is the most important single test for differentiating M. tuberculosis from M. bovis, M. avium and the nonphotochromogens. Only rarely encountered strains of the rapid growing M. abscessus (borstelense) produce niacin. M. tuberculosis produces substantially more niacin than any of the other mycobacteria seen in the clinical laboratory. The sensitivity of the paper strip method, described by Kilburn and Kubica (18) and modified in this laboratory (19), is such that only M. tuberculosis strains would be expected to elicit a positive reaction. Note: If 7H10 agar is used as the growth medium, then L-asparagine should be added (APPENDIX) to enhance niacin production.

7. Other tests - The nitrate reduction test (3+ to 5+), Tween 80 hydrolysis test (>10 days), and the tellurite reduction test (negative in 3 days) are more useful in identifying mycobacteria other than M. tuberculosis, but serve a useful purpose in characterizing the latter.

SOME CHARACTERISTICS OF THE MYCOBACTERIA OTHER THAN M. tuberculosis

Recognition of the role in disease of mycobacteria other than M. tuberculosis requires that an attempt be made to identify mycobacteria isolated from clinical specimens. Runyon (25) proposed a classification of "anonymous" mycobacteria based primarily on pigment formation and rate of growth. He divided these strains into 4 groups: Group I - Photochromogens; Group II - Scotochromogens; Group III - Nonphotochromogens; and Group IV - Rapid Growers. Stained smears cannot be relied upon for differentiation of mycobacterial species; however, direct smears from clinical specimens may be suggestive of M. kansasii if long, banded forms are seen, or of the "Battey bacillus" if very small cells are seen. In any event additional tests must be performed for identification.

Runyon Group I: Photochromogens - M. kansasii, M. marinum (balnei)

1. Rate of growth - Slow (requires more than 10 days to appear on artificial media.) Occasional strains of M. marinum grow rapidly at its optimum growth temperature.

2. Growth temperature -

a. M. kansasii. Faster growth at 37°C than 25°C, no growth at 45°C.

b. M. marinum. Rapid growth at 24°C and 31°C, grows much more slowly at 37°C. Causes lesions of extremities (27) and is not expected to be found in sputum.

3. Colony morphology - Colonies may be smooth and buttery when young and roughen with time, or may be intermediate between fully rough and fully smooth. Curving strands may be seen at the periphery of some colonies.

4. Pigment - Colonies are white to cream after incubation in the dark. A yellow color develops within 6-24 hours after short (60 minutes) exposure to light and subsequent reincubation in the dark. Color usually intensifies with time, ranging from yellow to yellow-orange. For optimal pigment production, cultures must be young, well-aerated (26), and have isolated colonies. Continuous exposure to light for 2 to 3 weeks will often result in the formation of orange crystals of carotene in the colonies.

NOTE: M. kansasii var. auranticum is scotochromogenic and produces carotene crystals readily, even in the dark; otherwise, it resembles the photochromogenic M. kansasii (28). M. kansasii var. album has been described as being similar to photochromogenic strains in all respects except lack of pigment formation.

5. Catalase activity - The catalase test is usually explosively positive with disease-related strains. Nondisease-related strains exhibit weaker catalase activity.

6. Other tests - The nitrate reduction test is positive for M. kansasii and negative for M. marinum, Tween 80 is hydrolyzed within 5 days, and tellurite is not reduced (3 days).

7. Drug susceptibility - Strains are usually resistant to PAS, often susceptible to low concentrations of INH, vary in susceptibility to other drugs, but are usually more resistant than M. tuberculosis.

Runyon Group II: Scotochromogens

1. Rate of growth - Slow (requires more than 10 days to appear on artificial media.)

2. Growth temperature - Optimum 37°C, some strains grow at lower or higher temperatures.

3. Colony morphology - Colonies are raised and smooth, edges vary from entire to scalloped.

4. Pigment - Colonies are definitely yellow to yellow-orange after 2-4 weeks incubation in the dark. Prolonged exposure to light will cause the pigment to deepen to orange or brick red. Pigment production is inhibited in old cultures grown in the dark, in poorly-aerated cultures, and in cultures wherein there is heavy or confluent growth.

5. Catalase activity - Most strains are strongly positive.

6. Other tests - Considerable variation, see reference No. 9.

7. Drug susceptibility - Strains vary in susceptibility to antituberculosis drugs, but are generally more resistant than M. tuberculosis.

Runyon Group III: Nonphotochromogens - M. intracellulare, M. avium (Battey-avium complex), M. gastri, M. terrae, "V" strains

1. Rate of growth - Slow (requires more than 10 days to appear on artificial media), M. ulcerans requires 6-9 weeks.

2. Growth temperature - Most grow well at 37°C, M. avium and some M. intracellulare grow at 24°C and 45°C. M. ulcerans grows slowly in a narrow temperature range around 32°C, but not at 24°C or 37°C, and is primarily found in skin lesions.

3. Colony morphology - Colonies are often smooth and suspend readily, although some species may produce dysgonic (thin) or rough colonies (strands or "cording" around the periphery of the colony). Colonies may be domed and hemispherical in shape, especially after subculture. The "V" group colonies often resemble M. tuberculosis.

4. Pigment - Colonies are nonpigmented to a very pale yellow, the latter not influenced by exposure to light. Prolonged (weeks) exposure of nonpigmented colonies may result in development of weak, yellow pigmentation.

5. Catalase activity - The catalase activity of M. avium and M. intracellulare is moderate, similar to M. tuberculosis. Other members of this group are strong catalase reactors. All except M. gastri are positive in the 68°C catalase test (24).

6. Other tests - M. avium and M. intracellulare reduce tellurite in 3 days and do not hydrolyze Tween 80. Other Group III species fail to reduce tellurite . .rolyze Tween 80 within 5 days.

7. Drug susceptibility - All demonstrate considerable primary resistance to antituberculosis drugs, but there is variation in this respect.

Runyon Group IV: Rapid Growers - M. fortuitum

1. Rate of growth - Rapid (fully matured colonies within one week or less on artificial media).

2. Growth temperature - Rapid growth at temperatures between 24°-37°C.

3. Colony morphology - M. fortuitum (the only recognized pathogen in Group IV) colonies may be either smooth, often mucoid, or rough resembling M. tuberculosis. Some patient strains are fully rough, some fully smooth; more commonly there is a mixture. Branching filamentous extensions around the colony are a prominent feature of smooth-colony M. fortuitum. (Colonies of M. xenopei, a scotochromogen, also may show branching, filamentous extensions; rough colonies may have aerial hyphae.)

4. Pigment - M. fortuitum colonies are nonpigmented. Colonies may resemble M. tuberculosis in appearance. Some strains take up malachite green from the medium.

5. Catalase activity - Strong catalase activity, the 68° catalase test is positive.

6. Other tests - The aryl sulfatase test (29) is positive in 3 days. M. fortuitum grows within 5 days on MacConkey's agar (light inoculum - streak out). Hydrolysis of Tween 80 is usually negative. The tellurite reduction test is positive within 3 days.

7. Drug susceptibility - Resistant to antituberculosis drugs.

SERUM DRUG LEVEL

Following assigned dosages, assays for antituberculosis drugs are performed to determine if the patient has sufficient drug in his serum to inhibit the mycobacterium causing his disease. In addition, the assay result serves to determine that toxic drug concentrations are not being reached.

The bioassay method (30) is used to determine serum levels of INH, SM, KM, EMB, CM, AND ETA. Chemical procedures are used for PAS (31), Cycloserine (32), and Pyrazinamide (33) drug levels. Routinely, drug levels are determined using serum obtained 2

and 6 hours after the patient has received his usual morning dosage of the drug. All drugs, with the exception of PAS and the drug being assayed, should be withheld for 24 hours prior to the time the blocd is drawn. The antimicrobial activity of PAS is blocked by adding 20 γ/ml of p-amino benzoic acid (PABA) to the medium. Liquid 7H10 OA medium without Tween 80 and containing 20 γ/ml of PABA (APPENDIX - Bioassay medium) is used for all drug level bioassays and Minimal Inhibitory Concentration (MIC) titrations.

Stock strains of M. tuberculosis H37Rv are maintained in the log phase of growth by transferring approximately 0.4 ml of the culture every 3-4 days to 9.0 ml of 7H10 OA liquid Tween medium (APPENDIX) in an 18 x 150 mm spectrophotometer tube. Cultures are incubated at 36°C in a Rollordrum (i) rotating at 18-20 RPM. The inoculum culture is standardized to contain approximately 12 x 10^7 viable cell units/ml (equivalent to a suspension grown to an OD of 0.33 at 690 mμ as read on the sensitivity scale 4 of a Beckman (j) Model B spectrophotometer). This laboratory maintains stock H37Rv strains: (1) susceptible to all drugs, (2) resistant to Streptomycin, and (3) resistant to INH. Using these strains plus PABA, drug levels can often be determined without interruption of the patient's therapy. Of course, the MIC of each drug for the strain used must be determined.

MIC Determination

The serum drug level is calculated by determining the MIC of the drug for the assay strain and multiplying this value by the reciprocal of the maximum inhibitory titer of the serum for the assay strain. MIC values are determined by preparing two standard drug solutions of equal concentrations and performing duplicate titrations on 5 initial dilutions from each solution. Titrations are performed monthly to insure that the MIC of assay strains has not changed. Frozen sera with known drug levels may also be used for control purposes. Whenever a new assay strain of H37Rv is used, the MIC titration must be repeated.

MIC titrations are performed as follows:

a. Prepare a "standard drug solution," by dissolving the drug in sterile distilled water or an appropriate solvent, to contain the desired concentrations: INH - 0.5 γ/ml, SM - 5.0 γ/ml, CM -10.0 γ/ml, EMB - 10.0 γ/ml, KM - 5.0 γ/ml, and ETA - 10.0 γ/ml.

b. Initial dilutions of the standard drug solution, i.e., 10 γ CM/ml, are made in a total volume of 5.0 ml according to the following table:

Initial Dilution	Ml of Std. Drug Soln. First Tube	Ml of Medium First Tube	Drug Concentration First Tube γ/ml
1:5	1.00	4.00	2.00
1:6	0.83	4.17	1.67
1:7	0.71	4.29	1.43
1:8	0.62	4.38	1.25
1:9	0.55	4.45	1.11

c. Serially dilute through 7 tubes, each containing 2.5 ml of medium, by transferring 2.5 ml. Discard 2.5 ml from the last tube.

d. Inoculate each tube with 0.05 ml of the standardized H37Rv suspension (1 drop from the tip of a vertically-held 5 ml serologic pipette as shown in Figure 2, APPENDIX).

e. Incubate the tubes at 36°C for 5 days.

f. To determine growth endpoints for MIC titrations and serum drug levels, one drop of sterile (5%) 2, 3, 5-triphenyl tetrazolium chloride (TTC) solution (k) is added to each tube in the series. Two hours' incubation at 36°C results in a red precipitate of the reduced formosan dye in tubes where there are metabolizing cells. This reaction is not specific for M. tuberculosis; therefore, it cannot be used to determine an endpoint, if there is contamination present.

MIC Calculation

The MIC is determined using 5 starting dilutions of the standard drug solution. The midpoint between the highest inhibiting concentration (TTC not reduced) and the lowest noninhibiting concentration (TTC reduced) is arbitrarily taken as the MIC. The following examples illustrate this calculation:

Example 1

Concentration of standard drug solution = 5.0 γ/ml

Initial Dilution	Maximum Inhibiting Dilution	Minimum Noninhibiting Dilution
1:5	1:10	/1:20/
1:6	1:12	1:24
1:7	1:14	1:28
1:8	1:16	1:32
1:9	/1:18/	1:36

Maximum inhibiting conc. = 5.0 γ/ml x 1/18 = 0.278 γ/ml

Minimum noninhibiting conc. = 5.0 γ/ml x 1/20 = 0.250 γ/ml

$$\text{MIC} = \frac{0.278 + 0.250}{2} = 0.264 \text{ γ/ml} \pm 0.14 \text{ γ/ml}$$

Example 2

Concentration of standard drug solution = 10.0 γ/ml

Initial Dilution	Maximum Inhibiting Dilution	Minimum Noninhibiting Dilution
1:5	1:10	1:20
1:6	1:12	1:24
1:7	/1:14/	1:28
1:8	1:8	/1:16/
1:9	1:9	1:18

Maximum inhibiting conc. = 10.0 γ/ml x 1/14 = 0.71 γ/ml

Minimum noninhibiting conc. = 10.0 γ/ml x 1/16 = 0.62 γ/ml

$$\text{MIC} = \frac{0.71 + 0.62}{2} = 0.66 \text{ γ/ml} \pm 0.04 \text{ γ/ml}$$

Bioassay Procedure for Serum Drug Level

Serum drug levels are determined in much the same manner as the MIC. Routinely, two initial dilutions (1:5 and 1:7, or 1:2 and 1:3) of the patient's serum are prepared in the bioassay medium in a total volume of 5.0 ml. Initial dilutions for the serum drug levels are made according to the following table:

Initial Dilution	Ml of Serum First Tube	Ml of Medium First Tube
1:2	2.50	2.50
1:3	1.67	3.33
1:5	1.00	4.00
1:7	0.71	4.29

Serial two-fold dilutions are carried out as in the MIC titration. Tubes are inoculated, incubated, and growth endpoints determined as for the MIC titration.

Serum Drug Level Calculation

The midpoint between the highest dilution inhibiting growth and the lowest noninhibiting dilution is arbitrarily taken as the bioassay endpoint. To calculate the serum drug level, multiply the reciprocal of the bioassay endpoint by the MIC value of the assay strain. A more accurate endpoint is achieved by using more than two initial serum dilutions.

The following examples illustrate the serum drug calculation:

Example 1

MIC = 0.04 γ/ml

Initial Dilution	Inverse of Maximum Inhibiting Dilution	Inverse of Minimum Noninhibiting Dilution
1:5	/20/	40
1:7	14	/28/

Midpoint between Maximum Inhibiting Dilution and Minimum Noninhibiting Dilution = $\frac{20 + 28}{2}$ = 24

Serum Drug Level = 24 x 0.04 γ/ml = 0.96 γ/ml ± 0.16 γ/ml

Example 2

Had 3 initial dilutions been used and resulted in the following growth endpoint:

Initial Dilution	Inverse of Serum Dilutions		
1:5	10	/20/ [a]	40
1:6	12	/24/ [b]	48
1:7	14	28	56

a = maximum inhibiting dilution

b = minimum noninhibiting dilution

Then the serum drug level would be:

$\frac{20 + 24}{2}$ = 0.04 γ/ml = 0.88 γ/ml ± 0.88 γ/ml

SERUM INHIBITION TITER

Serum inhibition titers are performed to determine the total inhibitory effect of the patient's therapy regimen on his mycobacterial strain. Titers are determined on sera drawn 2 and 6 hours following the administration of ALL drugs in the patient's current regimen. Inhibition titers are determined with both the patient's strain and the H37Rv sensitive strain, each grown in Tween-albumin broth and standardized as for other drug level determinations. The contribution of PAS to the inhibitory activity of the serum is assessed by performing the test with and without PABA (20 γ/ml) in the bioassay medium. Initial serum dilutions, prepared in a total volume of 5 ml of bioassay medium and diluted through a total of 10 tubes, are incubated and the endpoints determined as for MIC and serum drug level titrations. The serum inhibition titer is expressed as the highest dilution of serum which inhibits growth of the test organism, as determined with TTC.

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GLOSSARY OF ABBREVIATIONS

γ	Gamma (microgram)
CM	Capreomycin
EMB	Ethambutol
ETA	Ethionamide
INH	Isoniazid
KM	Kanamycin
MIC	Minimal inhibitory concentration
NALC	N-acetyl-L-cysteine
NaPAS	Sodium p-amino salicylate
PABA	p-amino benzoic acid
PAS	p-amino salicylic acid
RF	Rifampin
SM	Streptomycin
TTC	2,3,5-Triphenyltetrazoliumchloride

APPENDIX

MEDIA AND REAGENTS

A. Middlebrook - Cohn 7H10 OA Agar

The preparation of 7H10 OA Agar or liquid medium, as originally described, was somewhat tedious. Its preparation is simplified using a combination of 6 stock solutions which may be prepared in advance and will remain stable for at least a month. If precipitates form either in stock solutions or in the final medium, the stock solution or medium is unsatisfactory and a fresh solution or medium must be prepared. The stock solutions are prepared as follows:

Solution #1 (Maintain sterile at room temperature to keep in solution.)

Monopotassium phosphate, ACS	15.0	g
Disodium phosphate, ACS (anhydrous)	15.0	g
Distilled water to	250.0	ml

Solution #2 (Maintain sterile at 4°C. Prepare monthly.)*

Ammonium sulfate, ACS	5.0	g
Monosodium glutamate	5.0	g
Sodium citrate·2 H_2O, USP	4.0	g
Ferric ammonium citrate (green scales)	0.4	g
Magnesium sulfate·7H_2O, ACS	0.5	g
Biotin (made soluble in 2 ml of 10% ammonium hydroxide)	5.0	mg
Distilled water to	250.0	ml

Solution #3 (Maintain sterile at 4°C. Prepare monthly.)

Calcium chloride·2H_2O, ACS	50.0	mg
Zinc sulfate·7H_2O, ACS	100.0	mg
Copper sulfate·5H_2O, ACS	100.0	mg
Pyridoxine HCl	100.0	mg
Calcium pantothenate	100.0	mg
Distilled water to	100.0	ml

* The addition to Solution #2 of 10 ml casein hydrolysate (enzymatic) (k) will permit the growth of certain drug-resistant bacilli which otherwise may fail to multiply on 7H10 OA medium at primary isolation. This medium has been described as 7H11 (34).

Solution #4 (Maintain at 4°C.)

Reagent grade glycerol

Solution #5 (Maintain at room temperature.)

Malachite green, 0.01% aqueous solution

Solution #6 OADC Enrichment (Maintain sterile at 4°C.)

Distilled-deionized water	900	ml
Sodium Chloride, ACS	7.65	g
Bovine Albumin, Fraction V (1)	50.0	g

Dissolve in large flask using magnetic stirrer.
Add sodium oleate, prepared as follows:

Distilled-deionized water	30.0	ml
6N NaOH	0.6	ml
Oleic Acid (k)	0.6	ml

Adjust pH to 7.0 with 6N HCl.

Add:

Glucose, 50% aqueous solution	40.0	ml
Catalase, Technical (k)	0.02	ml

Clarify and sterilize the enrichment mixture using Millipore filter equipment (m). To facilitate filtration procedures, the enrichment mixture should be maintained at a temperature of 50-56°C.

1. Clarify the mixture by passing it through a series of filters:

   a. Millipore microfiber glass prefilter

   b. Millipore filter #DA 0.65 micron

   c. Millipore filter #HA 0.45 micron

The first 2 filters are used together, a being placed above b in the filter holder. (Filter equipment is not sterilized for the clarification process.)

2. For final sterilization of the clarified mixture, sterile equipment is used throughout.*

a. Sterilize as many millipore filter holders, fitted with #GS 0.22 micron millipore filters, as needed. To insure an adequate seal, filter holders must be tightened with the wrench supplied. (Filtering equipment is autoclaved at a temperature no higher than 121°C and no longer than 30 minutes. The autoclave must be exhausted slowly, because rapid changes of pressure will burst the filter discs.) Also, sterilize glass filter flasks, rubber tubing with connections, stainless steel reservoir pressure vessel, glass dispenser, and small containers for the sterilized OADC enrichment.

b. Check all connections. Set the compressed air gauge to 1.5-2 pounds of pressure until all the material has been filtered. Positive pressure filtration is more efficient than negative pressure filtration, as the negative pressure requires continuous pressure adjustments to avoid bubbles.

c. Aseptically dispense the filtered OADC mixture into small sterile containers. The volume dispensed is governed by the amount to be used.

3. Inactivate and pasteurize the OADC mixture as follows:

a. Heat for 1 hour in a 56°C water bath.

b. Incubate for 24 hours at 36°C.

c. Heat for 1 hour in a 56° C water bath.

4. Perform an additional sterility check by culturing a 0.5 ml aliquot from each container. These sterility checks are incubated at least 3 days before the mixture is cleared for use. The OADC enrichment can be stored indefinitely at refrigerator temperatures in airtight containers, but should not be allowed to freeze.

* Prepackaged 0.45μ and 0.22μ filters in sterile holders are commercially available (c).

B. Preparation of Media from Stock Solutions:

1. 7H10 OA AGAR MEDIUM* WITH ASPARAGINE

	1000 ml	500 ml
Distilled water	875.0 ml	437.5 ml
L-asparagine** (completely dissolve)	2.5 g	1.25 g
Solution #1	25.0 ml	12.5 ml
Solution #2	25.0 ml	12.5 ml
Solution #3	1.0 ml	0.5 ml
Solution #4 (warm to room temperature)	5.0 ml	2.5 ml
Adjust to pH 6.6 with 6 N HCl, approximately	0.5 ml	0.25 ml
Solution #5	2.5 ml	1.25 ml
Agar (n) or (o)	15.0 g	7.5 g
OR		
Ion Agar #2 (p)	8.5 g	4.25 g
Autoclave for 15 minutes at 121°C.		
Cool to 56°C, then add Solution #6	100.0 ml	50.0 ml

Immediately dispense the medium after the addition of solution #6. The medium must not be exposed to direct light during or after dispensing. Store in the dark at room temperature; drug-containing media should be stored in the dark in the refrigerator.

2. BIOASSAY MEDIUM

A 7H10 OA liquid medium (without asparagine) is prepared as for the agar medium except that agar and solution #5 (malachite green) are omitted, and p-amino benzoic acid (PABA) is added to a final concentration of 20 γ/ml of medium. PABA will block the activity of p-amino salicylic acid (PAS) in serum.

3. LIQUID TWEEN-ALBUMIN MEDIUM FOR CLINICAL SPECIMENS AND BIOASSAY INOCULA

A 7H1C OA-Tween 80 liquid medium (without asparagine) is prepared as for the agar medium except that agar, solution #4 (glycerol), and malachite green are omitted. Prior to autoclaving, add 5.0 ml of a 10% aqueous Tween 80 solution per liter of medium. Tween 80 allows dispersed growth of M. tuberculosis.

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*Laboratories which require only small volumes of the medium described may purchase it commercially (n) (o).

**The addition of 0.25% L-asparagine to 7H10 OA agar medium will enhance the production of niacin by M. tuberculosis (20).

4. AGAR MEDIUM FOR DRUG SUSCEPTIBILITY STUDIES

Prepare the agar medium as described in 1., above. Just prior to dispensing, sterile stock solutions of the drugs are added to the sterile, cooled medium (50°C), to give the desired final concentration. The preparation and storage of standard drug solutions is outlined in the Laboratory Methods for Clinical and Public Health Mycobacteriology (9) and the Handbook of Tuberculosis Laboratory Methods (17). Drug susceptibility plates should be stored in the dark at 4°C-10°C.

C. NaOH--N-Acetyl-L-Cysteine (NaOH-NALC) Decontamination-Digestant Solution (9)

	Volume of Digestant Required (ml)				
Stock Solutions and Reagents	50	100	200	400	500
2.5N(10%) NaOH (ml)*	25	50	100	200	250
M/10 Trisodium citrate·$2H_2O$ (ml) (58.89 g/liter)	25	50	100	200	250
N-acetyl-L-cysteine powder (g)	0.25	0.50	1.0	2.0	2.5

D. M/15 Phosphate Buffer Solutions, pH 6.6 to 7.2

1. Stock Solutions:

Solution A	g/liter
KH_2PO_4	9.08
Solution B	
Na_2HPO_4	9.47
or	
$Na_2HPO_4 \cdot 2H_2O$	11.87
or	
$Na_2HPO_4 \cdot 12H_2O$	23.88

*The final concentration of NaOH, after addition to the specimen, should be 2.5%. If 10% NaOH fails to suppress contamination, the concentration should be raised to 12% (3N NaOH).

2. To prepare 100 ml of buffer use:

pH	Solution A (ml)	Solution B (ml)
6.6	62.5	37.5
6.8	50.4	49.6
7.0	38.9	61.1
7.2	28.0	72.0

Sterilize at 121°C for 15 minutes. Use a pH meter to check the final pH.

E. Albumin-Buffer Solution (0.2%)

1. Stock albumin solution: 5% aqueous, Fraction V bovine albumin solution (filter sterilized).

2. Add 1 volume of sterile 5% albumin solution to 24 volumes of sterile M/15 phosphate buffer, pH 6.6.

F. Niacin Test Strips*

1. Materials:

a. White blotting paper (q) cut into strips - 6 cm x 8 mm.

b. 10% aqueous NaPAS (freshly prepared).

c. 60% K-thiocyanate in 8% citric acid.

d. 50% aqueous Chloramine-T (heat in a 56°C water bath to dissolve).

2. Preparation of Paper Strips:

a. To the strip, add one drop of K-thiocyanate-citric acid to the middle, one drop of Chloramine-T to the top, and one drop of NaPAS to the bottom. Do not permit drops to merge.

b. Air dry strips in the dark at 36°C.

c. Store in bulk at 4°C in tightly-closed containers.

* Available from (n)

G. Auramine O Stain

This stain is similar to the one described by Richards and Miller (13). The staining solution is easier to prepare, less expensive, and equivalent in staining properties to other auramine stains. In our experience, no advantage is derived from the addition of rhodamine to the staining solution, as described by Truant et al (6).

Procedure:

1. Dissolve completely 0.1 g of auramine O in 10 ml of 95% ethyl alcohol.

2. Mix 3 ml of liquified phenol with 87 ml of distilled water.

3. Mix the alcoholic-auramine O solution with the phenol-water. It is not necessary to filter the stain.

4. Store in amber bottles.

NOTE: Turbidity develops upon standing, but does not affect the staining reaction.

DRUGS FOR SUSCEPTIBILITY TESTING*

1. Sources of Drugs for Laboratory Use: When requesting drugs, specify that drugs are for laboratory use only.

a. Lyophilized Isoniazid 'Panray' (Sterile) - For Laboratory Use Only.

Panray Corporation
223 South Dean Street
Englewood, New Jersey 07631

b. Lyophilized Sodium p-Aminosalicylate 'Panray' (Sterile) - For Laboratory Use Only.

Panray Corporation
223 South Dean Street
Englewood, New Jersey 07631

c. Diagnostic Streptomycin Sulfate (Sterile) - For Laboratory Use Only.

Hospital Laboratory Advisory Service
Chas. Pfizer & Co.
235 East 42nd Street
New York, New York 10017

d. Capreomycin Disulfate - For Investigational Use Only.

Eli Lilly & Co.
Indianapolis
Indiana

e. d-Ethambutol (Sterile)

Lederle Laboratories
Pearl River
New York 10965

f. Kanamycin Sulfate (Sterile)

Bristol Laboratories
Syracuse
New York

* Information obtained from Veterans Administration - Armed Forces Handbook of Tuberculosis Laboratory Methods (17).

g. Ethionamide Base (Sterile)

Ives Laboratories
685 Third Avenue
New York, New York 10017

h. Rifampin (Sterile) - For Investigational Use Only.

Dr. Robert Nolan
Director of Clinical Pharmacology
Dow Chemical Company
P. O. Box 10
Zionsville, Indiana 46077

2. Stock Solutions:

In preparing drug solutions, concentrations must be based upon the weight of the active drug, excluding salts and other impurities. For example, the hydrochloride or sulfate radical may contribute significantly to the total weight of the molecule. When impurities are present with the active drug, the potency may vary from lot to lot; the label may reflect active drug per gram, or this information should be obtained from the manufacturer (specify lot number).

Antimicrobial Agent	Theoretical Potency of Pure Drug (of Activity/mg Total Weight)
Isoniazid	1,000
Sodium p-Aminosalicylate	877
Streptomycin sulfate	798
Kanamycin sulfate	950
Ethionamide hydrochloride	820
d-Ethambutol	1,000
Capreomycin disulfate	920
Cycloserine	1,000
Pyrazinamide	1,000
Viomycin sulfate	911

At times vials will be labelled with information concerning the quantity of solvent required to give a solution containing a specific concentration of the drug. When such information is not provided, the necessary volume of solvent may be calculated using the following formula:

$$\frac{a \times b}{c} = d$$

where, a = number of milligrams total weight of drug, weighed to the 4th decimal place.

b = the potency of the specific drug under test, expressed in micrograms per milligram of drug.

c = the desired number of micrograms of <u>active</u> drug per milliliter of solution.

d = the volume of solvent, in milliliters, required.

<u>NOTE</u>: When only partially purified drugs are used, each lot will vary from previous ones. The lot number is clearly shown on the label of the vial, and must be used when requesting information concerning potency of the drug. This formula does not take into account the displacement of volume due to the drug itself. When concentrated solutions are used, this is often considerable. Therefore, it is advisable to use the prescribed volume of solvent indicated on the label of the drug vial.

When stock solutions are prepared, it is advisable to use approximately 1 gram of drug, determining the weight to the 4th decimal place. Using the above formula, prepare a stock solution containing 10,000 γ per ml of <u>active</u> drug. Use analytical (not serological) pipets and quantitative procedures. <u>Sterile</u> distilled water is used as a solvent for all drugs, except ethionamide (use ethylene glycol) and rifampin (N, N-dimethylformamide), and as a diluent for all drugs except rifampin (phosphate buffer, pH 7.4). If other solvents are used, controls must be run to insure that antimicrobial activity is not due to the solvent.

Drug solutions must be kept sterile. If necessary, filter solutions using membrane or sintered glass filters. Filter highly concentrated solutions in volumes in excess of 10 ml. Avoid evaporation of solvent when filtering under negative pressure and use small containers.

Routinely, use freshly-prepared drug solutions to avoid possible evaporation, loss of potency, or contamination. (Cycloserine and rifampin solutions must always be freshly prepared.) Drug solutions which may be stored should be concentrated (not less than 10,000 γ/ml), as stability is better at these concentrations. Storage at 4°C should not exceed 1 month. Storage in the frozen state must not exceed 2 to 3 months. Do not thaw and refreeze solutions. Rapid freezing, at least -20°C, is required. The freezing compartment of a refrigerator does not freeze rapidly enough to insure stability of all drugs.

3. Drug Dilutions for Incorporation into Culture Media:

a. Use stock solutions containing 10,000 γ/ml of active drug.

b. Serially dilute stock solution, in the appropriate sterile diluent, using aseptic and quantitative techniques.

c. Use a separate, clean, sterile pipet for each dilution: do not use the same pipet for preparation of all dilutions of a given drug.

DRUG(S)	CONCENTRATION (γ/ml)	DILUTION	RESULTANT CONCENTRATION (γ/ml)
INH & PAS	10,000	1:20	500
	500	1:5	100
SM, KM, CM & Cycloserine	10,000	1:10	1,000
	1,000	1:2	500
	500	1:2	250
ETA*	5,000	1:10	500
EMB	10,000	1:10	1,000
	1,000	1:2	500
RF**	10,000	1:10**	1,000
	1,000	1:10	100
	100	1:2	50

* Dissolve 100 mg of ethionamide in 20 ml of ethylene glycol (analytical grade) to obtain a stock solution of 5,000 γ/ml. Place in incubator at 37°C for about 24 hours, after which time the drug should be in solution and sterile. If dissolution is not complete, place the flask in a steam bath for a few minutes to completely dissolve the drug. Store stock solution at 4°C. Dilute with sterile distilled water.

** Weigh out rifampin and dissolve in N, N-dimethylformamide (Eastman Chemical Company, Rochester, New York) to give a final concentration of 10,000 γ/ml of rifampin. This is a toxic solvent, handle with care and do NOT mouth-pipette.

*** Dilute the rifampin solution in phosphate buffer, pH 7.4, (Fisher Buffer Solution, Cat. No. So-B-110, pH 7.4, or the equivalent), to give final concentrations of 1,000, 100, and 50 γ/ml, respectively. Do not autoclave or filter rifampin solutions, as this drug is highly unstable. Do not store rifampin solutions.

4. Preparation of Drug-Containing Media:

a. Prepare working solutions of drugs, from the stock solutions described above, on the day that media is to be prepared.

b. Insure that agar-base media has been thoroughly melted and cooled to 50°C before adding drugs. (Inspissation for sterilization of egg-base media decreases activity of some drugs (e.g., SM, RF).

c. Measure both media and drug solutions carefully, taking care to maintain sterility.

d. Be sure that the volume of drug solution is small (in relation to volume of medium used), so that it does not alter the final concentration of salts and other substances in the medium.

Working Solution of Drug*		Volume Needed for 500 ml of	Final Drug Concentration in Medium
Drug	(γ/ml)	Medium (ml)	(γ/ml)**
INH	100	5	1
PAS	100	5	1
SM	250	5	2.5
ETA	500	5	5
EMB+	1,000	5	10
EMB	500	5	5
CM	250	5	2.5
RF#	100	5	1
RF#	50	5	0.5

* Make dilutions in sterile distilled water, except for rifampin

** Concentrations used at USAMRNL.

\+ Never autoclave or filter. Unopened stock solutions (10,000 γ/ml) of EMB received from the manufacturer can be stored for about 6 months at 4°C. Use only the Middlebrook 7H10 agar medium for EMB susceptibility tests.

\# Use solvent and diluent described in paragraph 3, above. Store drug-containing media at 4°C.

EQUIPMENT FOR FLUORESCENCE MICROSCOPY

Minimal Equipment Requirements for Blue Light Excitation and Fluorescence Microscopy of Auramine O-Stained Mycobacteria

The U. S. Army Medical Research and Nutrition Laboratory does not endorse the purchase of equipment from a sole manufacturer. Unfortunately, our laboratory is aware of only two manufacturers (b) (r) producing all necessary equipment for proper, complete blue light microscopy as described earlier in this report. At present, we have had the opportunity to work only with the equipment described below. It is known that other American manufacturers are currently endeavoring to develop similar equipment. Prior to request for purchase of equipment listed below, domestic manufacturers should be canvassed to determine the availability of systems to provide complete blue light excitation; this would be based on a satisfactory demonstration of the equipment.

Carl Zeiss, Inc.		Description	Price*
1.	47 09 34	Stand RA 34, Complete	$268.00
2.	46 70 41	Connecting Tube	21.00
3.	46 70 58	Diaphragm Insert	98.00
4.	46 80 15	Lamp Socket 60/1	52.00
5.	47 34 20	Fixed Square Stage, No. 20	8.00
6.	47 33 22	Attachable Mechanical Stage	69.00
7.	47 30 10	Inclined Binocular Body, 2	249.00
8.	46 72 57	Lamp Housing, 50 watts	79.00
9.	46 72 86	Elevating Plate	33.00
10.	47 93 00	Plastic Dust Cover	3.00
11.	46 78 26**	Neutral Filter, NG9	6.00
12.	46 78 35**	Blue Glass, Clear	3.00
13.	39 25 63	Regulating Transformer, 3-15 volts, with ammeter	95.00
14.	38 02 16	Bulbs, 12 volts, 60 watts, 3 ea. @$6.[illegible]	18.00

Carl Zeiss, Inc.		Description	Price
15.	46 52 53	Condenser, 1.3Z	82.00
16.	46 06 10	Planachromat 25/0.45 (0.4mm)	148.00
17.	46 08 60***	Planachromat 63/0.90 OD (0.9mm)	252.00
18.	46 19 00**	Achromat 100/1.25, Oil	98.00
19.	46 29 81	Nosepiece Plug	nc
20.	46 40 20	Complan Eyepiece, KPL 10X 2 ea. @$32.00	64.00
21.	46 78 89****	Filter, BG 12 (3 mm) light green background	6.00
		OR	
22.	46 78 84	Filter, BG 12 (4 mm) dark green background	6.00
23.	46 78 75	Barrier Filter, No. 50, 18 mm, in screw-in mount	19.00
		AND/OR	
24.	46 78 66	Barrier Filter, No. 53, 18 mm, without screw-in mount	6.00
25.	46 78 96	Screw-in Mount for 18 mm Filter	2.00

Minimal Equipment Required to Convert Microscopes for Fluorescence Microscopy of Auramine-O-Stained Mycobacteria

	46 40 20	Complan Eyepiece, KPL 10X 2 ea. @ $32.00	64.00
	46 06 10	Planachromat 25/0.45 (0.4 mm)	148.00
	46 08 60***	Planachromat 63/0.90 OD (0.9 mm)	252.00
	46 19 00**	Achromat 100/1.25 Oil	98.00
	46 78 26**	Neutral Density Filter, NG 9	6.00
	46 72 51	Lamp Holder with Clamp, Filter Receptacle and Iris	22.00
	46 72 87	Short Connecting Bar (for Zeiss microscopes only)	2.00
	46 70 42	Connecting Tube	17.00

Carl Zeiss, Inc.	Description	Price*
46 72 52	Filter Holder, 2 ea. @ $2.00	4.00
46 80 15	Lamp Socket 60/1	52.00
46 72 57	Lamp Housing, 60 watts	79.00
46 72 80	Stand for Lamp Housing	6.00
38 02 16	Bulb, 12 volts, 60 watts	6.00
39 25 63	Regulating Transformer, 3-15 volts with ammeter	95.00
46 78 89****	Filter, BG 12 (3 mm) light green background	6.00
	OR	
46 78 84	Filter, BG12 (4 mm) dark green background	6.00
46 78 75+	Barrier Filter, No. 50, 18 mm, in screw-in mount	19.00
	AND/OR	
46 78 66	Barrier Filter, No. 53, 18 mm, without screw-in mount	6.00
46 78 96	Screw-in Mount for 18 mm Filter	2.00
46 29 81	Nosepiece Plugs	nc
46 78 30‡	Heat absorption Filter, KG 1, 32 mm	9.00

* Prices at the time of this report.
** For white light microscopy. Not used for fluorescence microscopy.
*** When a cover slip is used, the high dry objective must be:

46 08 22	Neoflur 63/0.80, in correction mount	271.00

**** Used in this laboratory.

+ Barrier Filters. If there is no means for inserting a single filter between the objective lens and both eyepieces, then two barrier filters should be ordered. A filter should be screwed into each eyepiece.

‡ Place the Heat Absorption Filter KG 1 in the holder between the light source and the Excitation Filter BG 12.

NOTE 1. Cleaning of the 63X objective external lens is best accomplished using a cotton-tipped applicator stick and chloroform (not xylene or alcohol), as the lens is inset in its housing and cannot be adequately cleaned by wiping with lens paper.

NOTE 2: Optional Equipment to Facilitate Conversion from Blue Light to White Light Microscopy (Zeiss Microscope Only). The addition of an intermediate tube and an insert with barrier filters enables the user to quickly convert from blue light to white light microscopy. The excitation and barrier filters are swung out of the light path, and the neutral density filter is placed in the light path. The insert with barrier filters contains 6 filters, 2 of which are satisfactory for viewing fluorochrome-stained mycobacteria (Nos. 50 and 53).

Carl Zeiss, Inc.	Description	Price
47 30 59	Intermediate Tube	$210.00
47 25 47	Insert with Barrier Filters	131.00

If these items are ordered, then items 23, 24, and 25 (above) are not required.

RADIUS
cm
RELATIVE CENTRIFUGAL FORCE
G
RPM
EXAMPLE:
1. RADIUS OF CENTRIFUGE HEAD = 14 cm
2. FORCE 'G' REQUIRED = 2000
3. PLACE STRAIGHT EDGE FROM RADIUS READING (14 cm), THROUGH 'G' REQUIRED (2000), AND READ RPM REQUIRED TO ATTAIN DESIRED CENTRIFUGAL FORCE.
4. CENTRIFUGE TIME = 30 MINUTES

FIGURE 1.

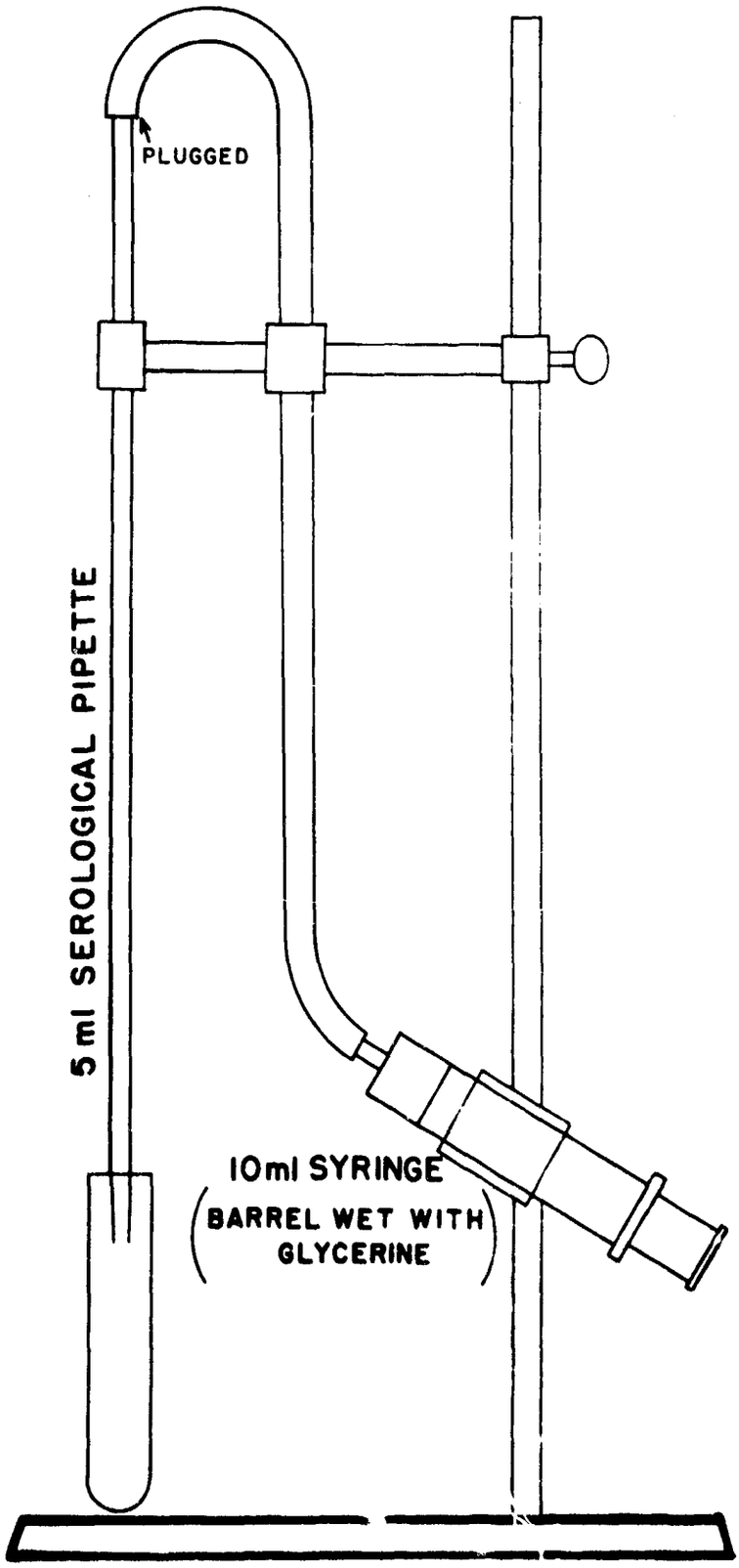
PLUGGED
5 ml SEROLOGICAL PIPETTE
10ml SYRINGE
(BARREL WET WITH GLYCERINE)

FIG 2

SOURCES OF EQUIPMENT AND REAGENTS*

(a). Vac Pac, Inc., 150 West Ostend Street, Baltimore, Maryland 21230.

(b). Carl Zeiss, Inc., 444 Fifth Avenue, New York, New York 10018.

(c). Falcon Plastics, Division of B-D Laboratories, Inc., 5500 West 83rd Street, Los Angeles, California 90045.

(d). Sigma Chemical Co., 3500 DeKalb Street, St. Louis, Missouri 63118.

(e). Scientific Industries, Inc., 15 Park Street, Springfield, Massachusetts 01103.

(f). Mehl Manufacturing Co., 2055 Reading Road, Cincinnati, Ohio.

(g). E. H. Sargent and Co., 10558 Metropolitan Avenue, Kensington, Maryland 20795.

(h). Hartman-Leddon Co., Inc., 60th and Woodland Avenue, Philadelphia, Pennsylvania 19143.

(i). New Brunswick Scientific Co., Inc., 1130 Somerset Street, New Brunswick, New Jersey 08903.

(j). Beckman Instruments, Inc., 1117 California Avenue, Palo Alto, California 94304.

(k). Nutritional Biochemicals Corp., 26201 Miles Road, Cleveland, Ohio 44128.

(l). Pentex, Inc., 195 West Birch Street, Kankakee, Illinois 60901.

(m). Millipore Corp., Bedford, Massachusetts 01730.

(n). Difco Laboratories, Detroit, Michigan 48201.

(o). Baltimore Biological Laboratories, P.O. Box 175, Cockeysville, Maryland 21030.

(p). Colab Laboratories, Inc., Chicago Heights, Illinois.

(q). Paper, Blotting, white, Grade A, 3 x 9 1/2 in., Bermingham and Prosser Co., New York, New York (GSA No. 7530-663-2734).

(r). American Optical Corp., Scientific Instrument Div., Buffalo, New York, 14215

*This list is presented to aid in the identification of equipment and reagents used in this laboratory and is not to be construed that all of the manufacturers listed are sole source suppliers. Nor does this constitute an endorsement by the Department of Defense or USAMRNL.

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13. ABSTRACT

This report presents in detail the latest methodology for isolation, culture, and identification of pathogenic mycobacteria. Methodology and equipment for blue light excitation of fluorochrome (auramine O)-stained mycobacteria and for blue light microscopy are discussed. Methods are described for direct and indirect drug susceptibility studies, serum drug levels, serum inhibition tests, and minimum inhibitory concentrations. Complete formulation and preparation of the modified Middlebrook and Cohn 7H10 OA agar medium and bioassay media are included.

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Identification						
Microscopy, fluorescence						
Mycobacterium						
Niacin						
Nicotinic acid						
Specimen handling						
Stains and staining						
Susceptibility						
Tuberculosis						

END