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A Digital Manual for Medical Microbiology Laboratory.

By **Dr. Marion Chan, Ph.D.**

The manual is constructed as a collaborative effort of the Department of Microbiology and Immunology, School of Medicine and School of Podiatric Medicine, Temple University. Colored images of cultures and reactions are provided to allow students to prepare for the exercises as well as review them for exams.

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EXERCISE #1 MICROSCOPY, BACTERIAL ISOLATION AND STAINING.

FIRST SESSION

FOCI: Aseptic Technique, Staining, Microscopic Observation of Bacteria, and Bacterial Isolation Using Streak Plate Method.

A. Aseptic Technique and Isolation of Bacteria colonies

Man harbors a wide variety of microorganisms on the skin and in the oral cavity, nasopharynx and intestinal tract. In all these areas of the body, many different kinds of bacteria exist together. Thus, if they are to be studied individually, each species must be isolated and then sub-cultured to obtain a pure culture. This is particularly true in the study of disease where it is necessary to isolate and identify the specific etiologic agent. Each of the students is to develop lab skills that are collectively referred to as aseptic technique. This concept must be consistently kept in mind when handling bacterial cultures because the careless student will contaminate everything, including the pure cultures of bacteria provided for the study of general bacteriology. Without pure cultures this study becomes an effort in futility!

Aseptic technique will be demonstrated by the lab instructor and the student should become quite familiar with the procedure to aseptically transfer bacteria from pure cultures.

In general, pure cultures of isolated bacterial colonies are best obtained by using solid media and streak plates are primarily used for this purpose.

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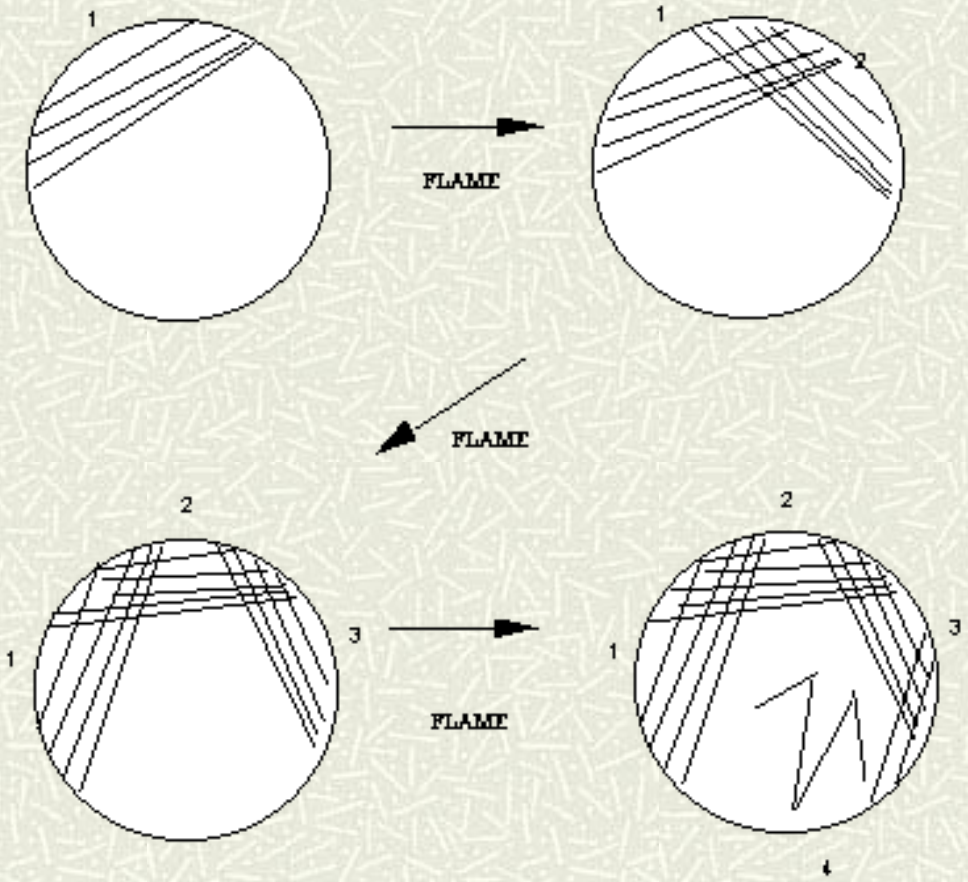
Each student will receive a tryptic soy agar plate and a tube of broth containing a mixture of bacteria. The "four-way" method will be used to streak the plate. This will be demonstrated by the instructor and is described below.

1. Never remove the top of the plate but lift the lid just high enough to make complete strokes.
2. Place a loop of organisms on one edge of the agar (area 1) and then streak back and forth, overlapping the streaks until you have covered about one fourth of the plate.
3. **Flame the loop before proceeding to each of the other steps**
4. Turn the plate approximately 90° and start streaking from one corner of the streaked area 1 and cover about one fourth of the surface (area 2). Turn again and streak surface area 3 by overlapping area 2, as shown below. Repeat this method and streak the surface of area 4 but do not run the final streaks back into any of the previous areas. You have now spread the

original inoculum over 90% of the surface of the agar plate.



5. Label the bottom of the plate (contains agar) using a wax marking pencil. Incubate all plates upside down, i. e., with the agar containing portion up. If they were incubated right side up, droplets of moisture would collect on the lid. These would then drop down on the agar causing the separate colonies to run together.

Isolated colonies

B. Differential Gram Strain

The differential gram stain is routinely used to stain microorganisms from culture or clinical specimens. In comparison to other microbiological tests, it is a relatively simple and quick test that yields valuable information to the clinician and microbiologist. A properly performed gram stain on a culture or specimen can provide important preliminary information concerning the type of microorganisms present, the techniques that should be pursued to characterize them, and the therapy to initiate while waiting for culture and antibiotic susceptibility test results. Please note that the gram stain should never be used as a substitute for culture and susceptibility studies!

The Gram stain method utilizes four reagents: Crystal violet as the primary stain, an iodine solution as the mordant, an acetone-alcohol mixture as the decolorizer, and safranin as the counterstain. The differential gram method outlined below divides microorganisms into two general categories, namely

gram-positive and gram-negative bacteria. The difference between these bacteria is that gram positives are not decolorized when alcohol or acetone is applied to the smear. They thus retain the primary stain and are colored purple when observed microscopically under oil immersion. Gram-negative bacteria, however, lose the primary stain upon decolorization and appear pink or red because of the safranin counterstain.

>>>>>

1. To prepare a smear from a broth culture (liquid suspension of bacterial cells), merely spread a loopful of the culture over a 2 cm square area of the microscope slide. Aseptic technique must be used to accomplish this transfer and remember to ALWAYS FLAME LOOP BEFORE AND AFTER USE!!
2. For colonies on agar plate, place a loop (or two) of water on a glass slide. Using aseptic technique, gently touch the bacterial surface growth with an inoculating needle or loop and remove a small part of the colony. Emulsify the bacteria on the loop into the drop of water until it just looks cloudy. Flame the loop to incinerate excess bacteria and allow it to cool. Continue to spread the smear with the loop until you obtain an even distribution of organisms over a 2 cm square area and remember to FLAME YOUR LOOP BEFORE PUTTING IT DOWN ON THE BENCH TOP.
3. Allow the smears to air dry (you may speed the drying by holding the slide high above the flame but do not allow them to become hot).
4. Heat fix the dried bacterial smears by passing the glass slides quickly through the flame two or three times.
5. Stain and observe the microscopic morphology and staining characteristics of each microorganism.

Gram stain

Place the slide on the staining rack and stain the films according to the following method:

(1) Crystal violet, 1-2 minutes
(2) Rinse in tap water and blot dry
(3) Gram's iodine, 1 minute
(4) Rinse in tap water and drain excess water
(5) Tilt the slide and add acetone-alcohol, drop by drop, until it flows colorlessly (about 5-10 seconds)
(6) Rinse in tap water and drain excess water
(7) Safranin, 30 seconds
(8) Rinse in tap water and blot dry
(9) Examine with the oil immersion lens and draw the microscopic morphology

microscopic morphology

C. Brightfield Microscopy

1. Compound microscope

Since all the living forms (microbes) with which we will be dealing are invisible to the naked eye, a microscope is an essential tool in any laboratory course in microbiology. The compound microscope employs two lens systems: the ocular or eyepiece and the objective lens. These two lens systems are separated so that the ocular lens can magnify the image formed by the objective lens. An object which is magnified 45 times by the high-dry objective lens (45x) is actually magnified 450 times when combined with the magnification provided by the ocular lens which is usually 10x. A bacterial cell with a linear diameter of 0.001 mm (1 u) can thus be magnified to the point of being visible in the microscope (0.001 mm times 450 equals 0.45 mm).

The resolving power of the compound microscope is approximately 0.2u (0.0002 mm). This means that objects which are smaller than 0.2 u cannot be distinguished clearly by the lenses and these will appear as small blurs in the field. Increased magnification will only make the blurs larger. Objects which are less than 0.2 u in diameter thus cannot be resolved by the optical microscope.

2. Use and Care of the Microscope

a. Never look through eyepiece when turning adjustment knobs downward. You should always begin focusing upward. Otherwise, the objective lens will go through the glass slide, thereby scratching and damaging the expensive lenses of the high-dry and oil-immersion objectives.

b. Lenses should be cleaned with a lens cleaning solution and lens paper each day before and after use. Xylene can be used to remove any oil film buildup on the lenses, but it should be used sparingly and must be removed by wiping with lens tissue (xylene is a solvent which can dissolve the cement that holds the lens in the objective).

3. Oil immersion Technique

a. Place a drop of immersion oil over the specimen that has been previously fixed onto a glass slide.

b. Using the course adjustment knob, move the oil-immersion objective (100x) into the oil and gently make contact with the slide (watch from side of scope during this procedure).

c. Focusing of specimen is accomplished by turning the fine adjustment knob in a direction which makes the objective travel upward!

d. If your microscope is parfocal, you can focus a specimen using the high dry objective, and then place a drop of oil on the specimen, switch to the

oil-immersion objective, and use the fine adjustment to bring specimen into proper focus.

e. To remove specimen, turn course adjustment knob in the direction that makes the objective travel away from the slide. Remove microscope slide and always clean lens with lens tissue before proceeding to examine the next specimen.

10X	40X	60X	Oil Immersion 100X

SECOND SESSION

FOCI: Gram Stain Technique, Characterization of Isolated Colonies

A. Colony Morphology of Isolated Colonies and Pure Cultures

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1. Check the TSA medium plates that were inoculated via the streak plate method for the presence of separate distinct colonies. Describe the cultural characteristics of these isolated colonies as to their size and shape.

2. Pure cultures of individual microorganisms are obtained by picking up a separated colony with a sterile inoculating needle or loop and streaking another plate of medium with this material. After 24 hours of incubation, one should have an entire plate of one specific microorganism, and such pure cultures are then used to perform various tests and inoculations so that the bacterium can be identified according to its enzymatic action on various substrates.

3. Prior to determining the metabolic activity of unknown bacteria, microorganisms are first identified based upon their morphology, namely their staining reaction, size, shape, pigment production, motility, and presence of capsules or spores. Gram stain several of the isolated colonies from the M-H streak plate and attempt to identify them morphologically.

4. Pure cultures of ***Staphylococcus epidermidis*** and ***Pseudomonas aeruginosa*** on M-H media will be available for student examination. Describe the cultural colony characteristics of these microorganisms and look for pigment production. Gram stain these bacteria and identify their microscopic morphology.

Staphylococcus epidermidis	*Pseudomonas aeruginosa*

B. Differential gram stain

>>>>>

Prepare a smear of *Escherichia coli* on one end of the slide and a smear of

Staphylococcus epidermidis on the other end of the slide. Preparing a smear from an agar culture (surface bacterial growth on solid medium) is a little more difficult and takes practice to obtain a good concentration (see SESSION 1: B.2).

Gram stain and examine under the microscope as in session one.

IMAGES:

10X mag

40X mag

60X mag

Oil immersion

Gram stain of mixed culture

S. epidermidis on M-H

P. aureginosa on M-H

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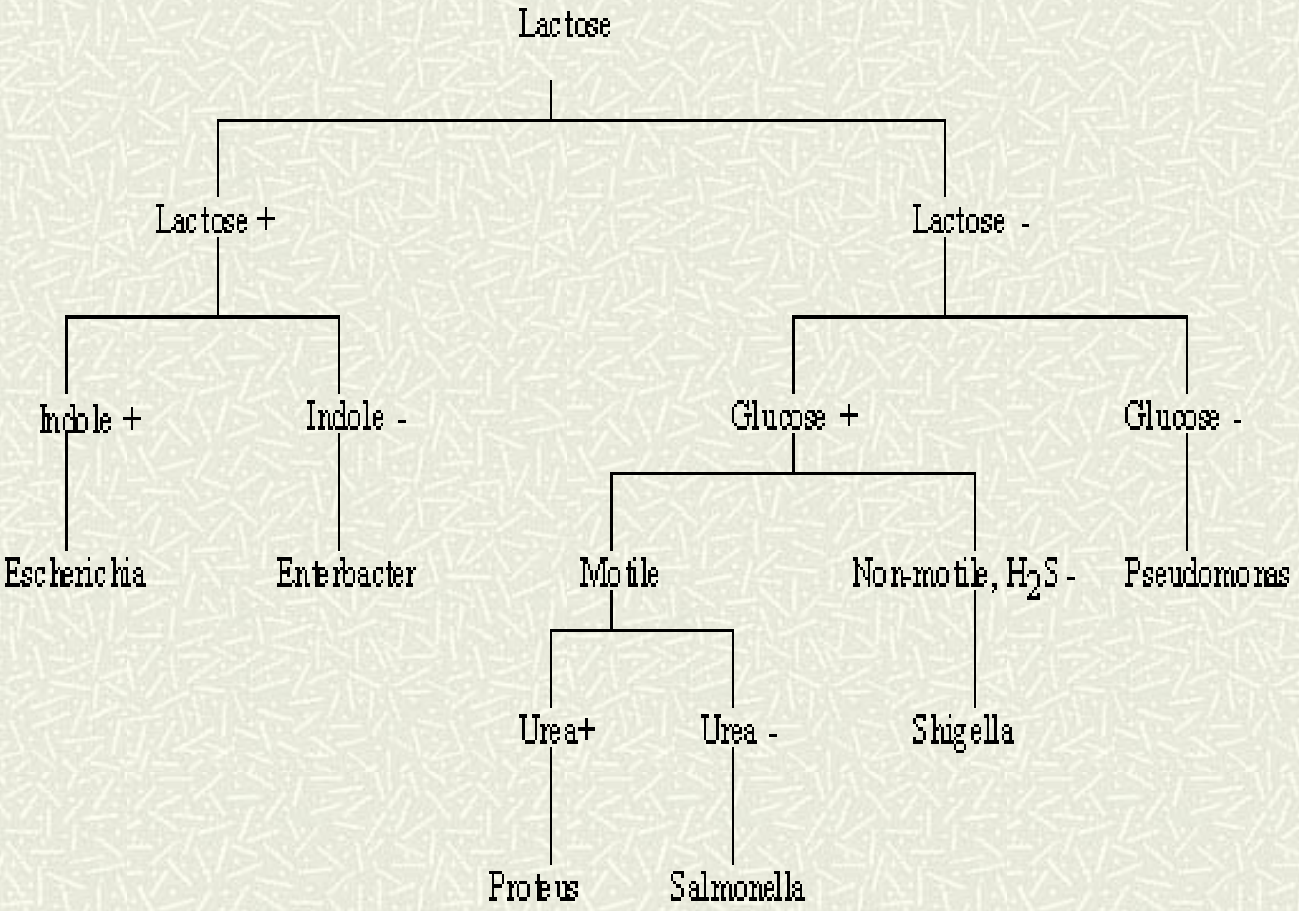
EXERCISE #2 GRAM-POSITIVE COCCI AND ANTIBIOTICS

FIRST SESSION :

FOCI

1. Identification *Streptococcus* by Macroscopic, Microscopic and Metabolic Characteristics.
2. Antibiotic susceptibility test

Identification of Bacterial Genera



The identification procedure almost always begins as a morphological study of the unknown clinical isolate. The microbiologist and the clinician initially require a determination so as to whether the organism is gram-positive or gram-negative, whether it is a rod or coccus, the predominant type of arrangement, and whether or not spores are formed. If the organism is a gram-positive, nonspore forming rod, then one needs to know whether or not it is acid- fast. Motility determinations are also significant especially with the gram-negative rods. These facts along with the cultural characteristics will enable the investigator to ultimately arrive at one of the nine groups of genera. A presumptive identification of the genus may also be possible at this time especially if symptoms of the clinical infection are known. Physiological tests and/or serologies are subsequently performed to confirm the genus as well as to determine the species of the etiologic agent.

A. STREPTOCOCCI

Streptococci are gram-positive cocci which typically occur in chains, clusters, and pairs. There are many members of the genus *Streptococcus* that form the dominant bacterial flora of the mouth and the pharynx. Some streptococcal species are normally found in the gastrointestinal tract.

Streptococcus pyogenes (Lancefield's GroupA, beta-hemolytic streptococci) is the most reknown pathogen in this genus. It causes infections such as acute pharyngitis (strep sore throat), impetigo, post-operative wound infections, cellulitis, erysipelas, post-streptococcal glomerulonephritis, and acute rheumatic fever. Asymptomatic colonization of *S. pyogenes* in the pharynx and on skin surfaces as well as anal carriage is not uncommon and may serve as a source of infection.

Streptococcus agalactiae (Lancefield's Group B, beta-hemolytic streptococci) can be recovered from the genital and intestinal tract of apparently healthy adults and children. It is primarily implicated in causing neonatal infections such as pneumonia, septicemia and/or meningitis. Nevertheless, it has been isolated from patients with urinary tract infections, wound infections and otitis media.

Enterococcus faecalis (Lancefield's Group D, nonhemolytic streptococci) is normally found in the intestinal tract along with other enterococci (E. faecium and E. durans). It causes infections such as subacute bacterial endocarditis (20%), urinary tract infections (10%), wound infections, and some other opportunistic infections. The resistance of enterococcal species to penicillin is an important fact to remember in treating patients infected with these streptococci.

Streptococcus pneumoniae can be isolated from the pharynx of 30-70% of apparently healthy individuals. It primarily causes a bacterial pneumonia especially in the debilitated or compromised patient. This diplococcus has also been isolated in patients with conjunctivitis, meningitis, otitis, pericarditis, empyema, endocarditis, and septicemia.

Viridans streptococci includes about 10 species (S. mutans, S. sanguis, S. salivarius) which are normally found in the mouth and pharynx. These streptococci are usually alpha or non-hemolytic on blood agar and cause about 50-70% of all subacute bacterial endocarditis. They have also been implicated in causing dental caries, bacteremia, and sepsis.

Blood agar plate cultures of each of the following microorganisms will be available for student examination:

a. *Streptococcus* (Group A Streptococci)

b. *Streptococcus agalactiae* (Group B Streptococci)

c. *Streptococcus pneumoniae* (Pneumococci)

d. *Enterococcus faecalis* (Group D Streptococci)

>>>>>
Each student should use one of these cultures to perform the following tests.

1. Describe the cultural characteristics of the various streptococci on **blood agar** medium. Note the type, size, and color of the colonies and describe the various types of **hemolysis** observed with these microorganisms.

Streptococcus pyogenes	***Streptococcus agalactiae***
Streptococcus pneumoniae	***Enterococcus faecalis***

1. Examine bacteria microscopically by preparing a gram stain.

Streptococcus pyogenes	*Streptococcus agalactiae*
Streptococcus pneumoniae	*Enterococcus faecalis*

2. **Catalase Test** -- Perform the slide test by emulsifying some visible growth material in a drop of hydrogen peroxide and note that all streptococci are catalase-negative (no rapid ebullition).

3. Rapid Enterococcus Identification -- The **bile esculin** (BE) test is the most accurate way for identifying Group D streptococci because they are all BE-positive and will cause a blackening of the agar medium after overnight incubation. All other streptococci are negative for this reaction. An enterococcus/Group A screen will be available for the rapid identification of Enterococcus faecalis from other streptococci. This specific medium contains esculin and also **PYR** (pyroglutamylbeta- naphthylamide) which will differentiate enterococci from other Group D streptococci.

a) Using a sterile loop, obtain a visible, heavy inoculum and stab into the medium in an area near one side next to the glass. Incubate the tube at 35°C for 30 minutes to 4 hours.

b) The esculin hydrolysis usually occurs in 30 minutes with a heavy inoculum and is indicated by development of a dark brown color. The PYR reaction is detected by adding 2 drops of the PYR reagent and observing a cherry red color within 2 minutes. No color change, light orange or light pink-orange is considered negative.

Positive and Negative Reactions of BE-PYR

c) From the foregoing table, you should realize that the screen test can also differentiate Group A streptococci from other beta-hemolytic streptococci by the PYR reaction. The identification of *Enterococcus faecalis* usually requires the inoculation of bile esculin agar and tube of broth containing 6.5% NaCl.

d) After overnight incubation, the black discoloration of bile esculin identifies Group D streptococci (*E. faecalis*, *S. bovis*, etc.) and growth in the presence of 6.5% NaCl (salt tolerance test) differentiates enterococci which are salt tolerant from other Group D streptococci (S. bovis) which will not grow in this broth.

	Group A	Groups B,C,F,G	Group D Enterococcus	Group D Non-Enterococcus
Esculin	-	-	+	+
PYR	+	-	-	-

5. **Rapid Streptococcal Grouping** Most streptococcal species possess group specific antigens which are usually carbohydrate structural components of the cell wall. These antigens can be extracted and identified by homologous antisera, and the bacterium can be assigned to one of the various **Lancefield groups**. The principal use of the test is to rapidly identify streptococci growing on agar plates (but satisfactory results have been reported with one hour extraction from blood cultures and from throat, skin, or wound swabs).

Extraction and Test Procedure:

a. To a small glass tube, 2 drops of reagent 1 and 2 drops of reagent 2 are added to generate

the nitrous acid solution.

b. With a wooden applicator, 3 to 4 colonies of the suspected streptococcal unknown are touched and emulsified into the nitrous acid solution. After some gentle agitation (to suspend the cells), 4 drops of reagent 3 (buffer solution) are added into the mixture and gently agitated again.

c. One drop of the above extract is dispense with a transfer pipette onto each of two circles of the card slide. Then, one drop of the Group A latex reagent is added to one circle and one drop of the Group B latex reagent is added to the other circle.

d. The reactants in each circle are mixed and spread out with a wooden applicator, the slide is rocked for 1 minute and the results are read. A positive result will appear as a passive agglutination reaction.

6. Demonstration - observe the susceptibility of Group A streptococci to **bacitracin** as evidenced by the zone of inhibition around the A disc. This bacitracin sensitivity distinguishes the beta-hemolytic Group A streptococci from all the other beta-hemolytic streptococci (Groups B, C, F, and G).

7. Demonstration - observe the susceptibility of pneumococci to **optochin** as evidenced by the zone of inhibition around the P disc. This optochin sensitivity distinguishes the pneumococci from all the other alpha-hemolytic streptococci.

	Bacitracin	Optochin	Sodium Hippurate	Bile Esculin	6.5% NaCl
Group A Strep. Beta-Hemolytic	+	-	-	-	-
Group B Strep. Beta-Hemolytic	-	-	+	-	+
Group D Strep. (S. bovis)	-	-	-	+	-
Group D Entero-cocci (E. faecalis)	-	-	-	+	-
Viridans Strep. Alpha-Hemolytic	+-	-	-	-	-
Strepto-coccus pneumoniae	+-	+	-	-	-

B. ANTIBIOTIC SUSCEPTIBILITY TEST

Variations in the susceptibility of microorganisms to antibiotics are recognized within bacterial species. While some bacterial strains may naturally possess such variable susceptibilities, others may develop resistance to these therapeutic agents in vitro as well as in vivo. Infections caused by these microorganisms (e.g., gram-negative enteric or gram-positive bacteria) thus cannot be effectively treated until information relative to their antibiotic susceptibility is obtained from the lab. Under these circumstances, and in cases where the infectious process may be fatal unless treated specifically (septicemia, meningitis, etc.), selection of the most effective antibiotic is extremely important. Once the causative organism has been isolated, it is a routine matter to determine the organism's degree of susceptibility to available antibiotics. The test is a simple one to perform but it should be kept in mind that

these in-vitro results are not always followed by clinical success. It is not uncommon that the lab's recommendation to a physician fails for various known and unknown reasons. Many variables, such as the size of the inoculum, thickness of the medium, rate of diffusion, deterioration of the antibiotic during incubation, etc., preclude precise quantitative evaluation. In spite of these limitations, however, the antibiotic susceptibility test is a necessary and useful one.

Many different systems have been proposed in attempts to improve the reliability of selecting the best antibiotic for the patient on the basis of test results. Currently, only two systems are primarily used in the clinical lab and these are the MIC dilution method and the Kirby-Bauer diffusion method.

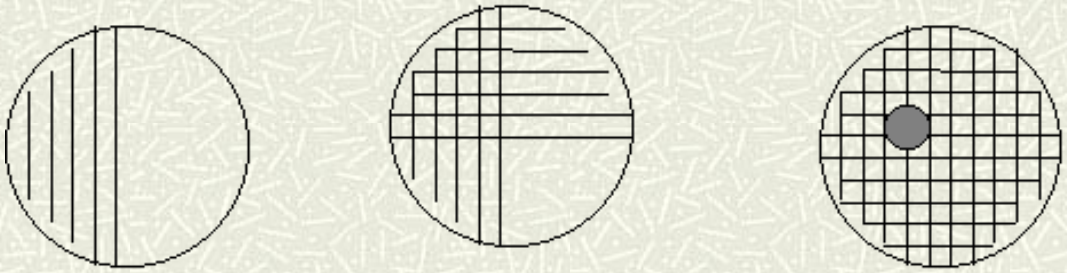
The Kirby-Bauer diffusion test involves using disks saturated with standard antibiotic concentrations and applying these to a large Mueller-Hinton agar plate which has been seeded with exponential phase cells of the clinical isolate. Results of this disk diffusion test are reported as the bacterium being susceptible or resistant to the various antibiotics.

>>>>>
Primary Specimen and Pure Culture: **Kirby-Bauer Diffusion Method**

1. The following exponential phase broth cultures will be available: *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, and *Proteus mirabilis*. Distribute these microorganisms so that each student at your table will use a different pure culture.

2. Insert a sterile swab into the broth using aseptic technique. Press the cotton against the side of the tube to remove some of the excess fluid.

3. Prepare a confluent lawn of bacteria on a Mueller-Hinton agar plate by streaking the swab from edge to edge, moving from the periphery to the center of plate until half the plate is covered.



4. Rotate the plate 90° and repeat the streaking with the swab (you may have to obtain some more specimen with another sterile swab). Continue rotating and streaking until the entire plate is covered. An evenly distributed population of microorganisms throughout the agar is desired.

5. Allow the plates to dry for 5 to 10 minutes and then apply 12 antibiotic discs with a Sensi-disc dispenser using aseptic technique. Press these discs gently with alcohol-flamed teasing needle and incubate overnight at 37°C.

6. Make sure you record name, code and concentration of each antibiotic.

SECOND SESSION:

FOCI:

1. Identification of Staphylococcus by Macroscopic, Microscopic and Metabolic Characteristics.
2. Antibiotic susceptibility test, reading of results

A. STAPHYLOCOCCUS

Staphylococci are among the most ubiquitous organisms associated with man and are common inhabitants of the skin, oral cavity, and nasopharynx. the two species usually encountered are *Staphylococcus aureus* (pathogen) and *Staphylococcus epidermidis* (opportunist, normal skin flora microorganism). Microscopically, both species appear as gram-positive cocci in grape-like clusters and therefore are morphologically indistinguishable.

Staphylococcus aureus disease in man includes wound infections, boils, carbuncles, impetigo, meningitis, endocarditis, osteomyelitis and food poisoning. This microorganism also plays an important role in hospital-acquired infections. Various strains of *S. epidermidis, S. haemolyticus*, and other species (referred to as coagulase-negative staphylococci) may also be implicated in causing infections especially in the presence of foreign bodies such as implants and catheters. In the laboratory, the production of hemolysin, fermentation of mannitol, and a positive coagulase test have been used as indicators of pathogenicity (for *S. aureus*) but coagulase production had the highest degree of correlation.

Staphylococcal diseases are characterized by suppuration although the mechanisms involved in pathogenicity are not well defined. Such factors may include antiphagocytic surface components (protein A), increased resistance to intracellular killing, production of alpha-toxin (hemolysin) which may promote necrosis, leucocidins, staphylokinase and hyaluronidase. *S. aureus* may also produce an enterotoxin which is the cause of staphylococcal food poisoning.

Staphylococci are notable for developing resistance to antimicrobial drugs including the penicillins. The determination of penicillinase (beta-lactamase) production and resistance to macrolide antibiotics are transferable by transduction and are believed to be controlled by a plasmid.

>>>>>

1. Each student will receive a TSA culture of either *Staphylococcus aureus* or *Staphylococcus epidermidis*. The following should be performed for identification and characterization of each microorganism.

2. Describe the cultural characteristics of the two staphylococcal species on **blood agar** medium. Note and record the type, size, and color of the colonies and describe the type of **hemolysis** produced by *S. aureus*.

Staphylococcus aureus	***Staphylococcus epidermidis***

3. Gram stain the bacteria and note the morphology and staining characteristics (the cocci average 0.8-1.0 micron in diameter).

Staphylococcus aureus	*Staphylococcus epidermidis*

4. Perform a **catalase slide test** by emulsifying some visible growth in a drop of hydrogen peroxide. This reaction is positive if there is a rapid ebullition of gas. *Micrococci* and *Staphylococci* are catalase-positive; Streptococci and Pneumococci are catalase-negative.

5. **Coagulase Test** - Perform the coagulase test for presence of coagulase by inoculating a tube containing 0.5 ml of rabbit plasma with your staphylococcal culture. Incubate this at 37oC and examine after 2-3 hours or after ovenight incubation. S. aureus will gel the plasma

(coagulase positive), whereas *S. epidermidis* will not.

6. The differentiation of *S. aureus* from *S. epidermidis* traditionally involves performing the clumping factor slide test, the coagulase tube test, the DNAse test, and at times, mannitol fermentation (often used for screening). Depending upon the strain and the quality of the reagents, these tests may infrequently present some difficulty in interpretation, may yield false results, and usually require overnight incubation. Consequently, the rapid identification of *S. aureus* strains in many laboratories is accomplished by utilizing commercial test kits.

The **Slidex™ Staph** kit will be available for student evaluation. This test specifically detects the presence of clumping factor and protein A on the bacterial surface and presumably eliminates problems in interpretation as well as false results. The procedure is outlined as follows:

a) Dispense one drop of positive reagent R1 in one circle of the disposable slide and one drop of the negative control (reagent R2) in another circle.

b) Pick colonies (1-2) from a blood agar culture and mix into each reagent using a different wooden applicator.

c) Rock the slide for 20 seconds and read the results. A positive reaction indicates the presence of clumping factor and/or protein A and is visualized by agglutination of the bacteria to the latex beads (that contain IgG) and/or to the stabilized red blood cells (that contain fibrinogen). A negative reaction remains homogeneous or turbid without any clumps forming as visualized in the circle with the R2 reagent. reaction indicates the presence of clumping factor and/or protein A and is visualized by agglutination of the bacteria to the latex beads (that contain IgG) and/or to the stabilized red blood cells (that contain fibrinogen). A negative reaction remains homogeneous or turbid without any clumps forming as visualized in the circle with the R2 reagent.

Positive and Negative Readings

B. ANTIBIOTIC SUSCEPTIBILITY RESULTS

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Record and discuss the result of the antibiotic susceptibility test that you performed. Compare results with students at your table and report the susceptibilities of each organism to the various antibiotics in the Kirby-Bauer Test (Use millimeter rulers to measure the various zones of inhibition).

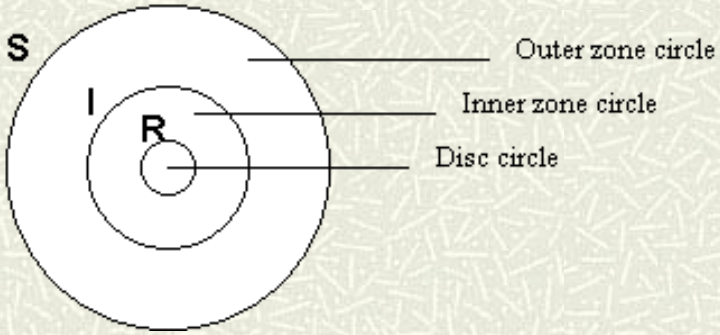
Antimicrobial Agent	Disc Content	Resistant mm or less	Intermediate mm or less	Susceptible mm or more
Ampicillin (when testing Gram-negative microorganism and Enterococci)	AM 10 mcg	11	12-13	14
Bacitracin	B 10 units	8	9-12	13
Cefoxitin	FOX 30 mcg	14		18
Cephalothin	CF 30 mcg	14	15-17	15

Erythromycin	E 15 mcg	13	14-17	18
Gentamicin	GM 10 mcg	12	13-14	15
Oxacillin	OX 1 mcg	10	11-12	13
Rifampin	RA 5 mcg	24		25
Tetracycline	Te 30 mcg	14	15-18	19

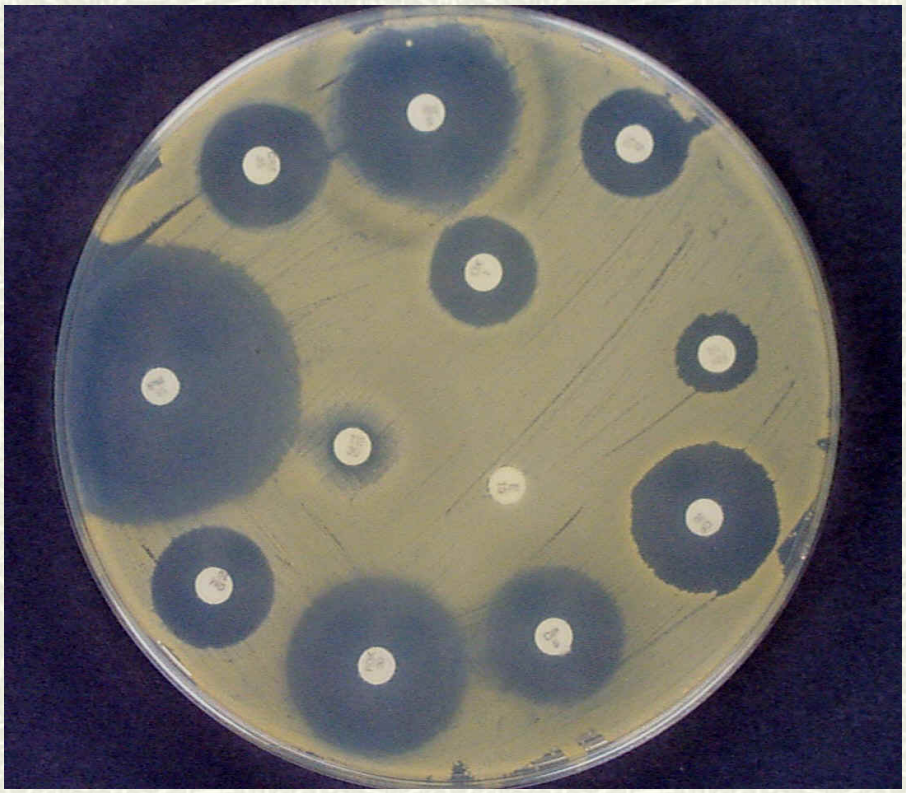
R-Resistant-Inhibitory zone equal to or less than inner zone circle. The organism is not inhibited by concentrations of the drug attainable in the blood (or urine in the case of Naladixic Acid, Nitrofurantoin and sulfonamides) on the usual dosage schedule.

I-Intermediate-Inhibitory zone between inner and outer zone circles.

S-Sensitive-Inhibitory zone equal to or greater than outer zone circle. The organism may be expected to be inhibited by concentrations of the drug attainable in the blood (or urine in the case of Nalidixic Acid Nitrofurantoin and sulfonamides) on the usual dosage schedule.

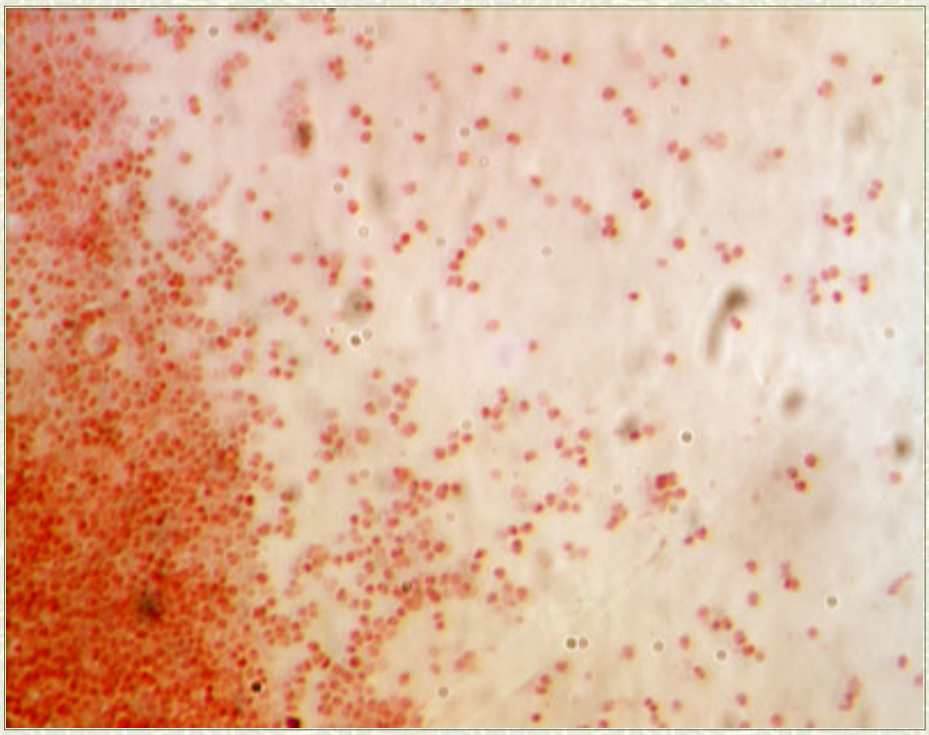


If the organisms used for the Kirby-Bauer were actual isolates causing septicemia or a postoperative abscess, which antibiotic would you prescribe for treatment?



Sample picture for Kirby-Bauer Antibiotic Susceptibility Tests

Challenge: What is the genus of the following gram (-) coccus?



Images:

Streptococcoccus pyogenes on blood agar

Streptococcus agalactiae on blood agar

Streptococcus pneumoniae on blood agar

Streptococococcus pyrogenes gram stain

Streptococococcus pneumoniae gram stain

BE-PYR Reactions

Staphylococcus aureus on M-H and Blood agar with beta-hemolysis

Staphylococcus epidermidis on M-H agar

Staphylococcus aureus gram stain

Staphylococci catalase tests

Staphylococcus coagulase tests in tube and on slide

Slidex agglutination for *Staphylococcus aureus*

Neisseria gonorrhoea gram negative cocci

Kirby-Bauer Antibiotic Susceptibility

EXERCISE #3. GRAM-POSITIVE AND GRAM-NEGATIVE RODS

FIRST SESSION

FOCI:

- Identification Gram positive rods (*Clostridium perfringens* and *Clostridium sporogenes*) by Macroscopic and Microscopic Metabolic Characteristics.
- Identification Gram negative rods (*Escherichia coli*, *Proteus mirabilis*, *Pseudomonas aeruginosa*) by Macroscopic Microscopic and Metabolic Characteristics.

A. GRAM-POSITIVE

Characteristics of *Clostridia*

All species of *Clostridium* are anaerobic spore-forming bacilli whose natural habitat is the soil and the intestinal tract of man and animals. In man, they may cause gas gangrene, tetanus, or botulism. In the first two diseases, cuts (abrasions) or puncture wounds are contaminated with soil or feces harboring these bacilli. In the case of botulism, preformed toxin is ingested with contaminated food. In all three, exotoxins are responsible for symptoms.

Clostridium perfringens is the most common species which causes gas gangrene (90%). After contamination of the wound, the organisms grow in devitalized tissue and produce a variety of toxins and enzymes. Many of these exotoxins have lethal, necrotizing, and hemolytic properties. Several important examples include the alpha toxin (lecithinase) which splits lecithin in mammalian cell membranes, theta toxin which has hemolytic and necrotizing effects, hyaluronidase which splits hyaluronic acid found in the substance that cements tissue cells together, collagenase which digests collagen on subcutaneous tissue and muscles, and DNase. The net result of such exotoxins is an invasive and spreading infection with death of surrounding tissue, crepitation, foul-smelling discharge, toxemia, shock, and ultimately death of the patient. As a general rule, gas gangrene is a mixed infection with proteolytic clostridia, various cocci, and gram-negative rods usually present. In some cases, clostridial infections may result only in anaerobic fasciitis or cellulitis without gas gangrene.

Clostridium tetani forms a distinctive terminal spore and is identified by toxin neutralization tests. Open wounds are contaminated with spores or vegetative cells and the organisms grow in the devitalized tissue. Exotoxin (tetanus toxin) which is produced and secreted reaches the anterior horn cells of the spinal cord (probably via the peripheral nerves) where it diminishes inhibitory synapses by blocking the normal function of the inhibitory transmitter. This produces convulsive tonic contractions of voluntary muscles which usually begin near the site of the infection (involvement of the jaw is called lockjaw). Tetanus neonatorium may result from infection of the umbilicus of the newborn. To successfully cultivate anaerobes from clinical material,

particular attention has to be paid to maintaining anaerobic conditions (low oxidation-reduction potential) from the time of collection of the specimen through cultivation. Immediately after collection, the specimen should be transferred to a holding medium which contains reducing reagents such as sodium thioglycollate or cysteine. For cultivation, pre-reduced media (or media stored under anaerobic conditions) are inoculated in an atmosphere of oxygen-free nitrogen (using a hood or stream of nitrogen). These media are then incubated anaerobically through the use of reducing reagents in the media, stoppering the tubes or placing them in an anaerobic jar.

>>>>>
Cultures of *Clostridium perfringens* and *Clostridium sporogenes* will be available for student examination.

a. Observe and describe the cultural characteristics of the clostridia on **blood agar** medium.

> ***C. perfringens*** produces large translucent colonies with peaked centers and also displays a double zone of hemolysis.
>
> ***C. sporogenes*** usually produces small to medium, rhizoid type of colonies on blood agar with a single zone of hemolysis (which may be difficult to observe).

b. Prepare a gram stain of one of these anaerobic rods (and your lab partner should gram stain the other Clostridia) and look for the presence of spores which should appear as unstained areas of the bacilli. Please note that spores are seldom observed with *C. perfringens* and also be aware that some clostridial species frequently stain gram-negative even though they are classified as gram positive microorganisms (especially after 20 hours of growth where almost all of the population is in the sporulation cycle).

Gram stains

C. perfringens	***C. sporogenes***

Summary Table

	C. perfringens	*C. sporogenes*
Colonies on blood agar	Round, smooth, opaque	Large rhizoid
Hemolyic zones	Double	Single
Spores	Rare, ovoid, eccentric	Ovoid, eccentric
Sporangia	Not swollen	Swollen
Motility	-	+
Cooked-meat medium	Gas, no digestion	Gas, blackening, digestion
Dextrose	+	+
Lactose	+	-

Sucrose	+	-
Salicin	Usually -	-
Indole	-	-
Nitrate production	+	+
Gelatin liquification	+	+

Litmus Milk Test: This assay has not been performed in the lab and will not be included in the lab quiz

Bacterial Endospores

Endospores are highly resistant resting forms that are produced within the cell. Of the several genera of bacteria able to produce endospores, only two are of medical importance and these are the aerobic Bacillus and the anaerobic Clostridium, both gram-positive rods. Most spore-forming bacteria are inhabitants of soil, but bacterial spores are found almost everywhere. Because many of the spore-formers can produce quite potent toxins, special precautions must be taken to destroy all spores. Endospores endow the microorganism with increased resistance to heat, irradiation, chemicals, and drying. Endospore formation is a type of cellular differentiation requiring synthesis of specific RNA, proteins, dipicolinic acid, and other components not found in the vegetative cell. Upon provision of appropriate environmental conditions that allow permeation of water, the spore germinates and differentiates to the vegetative cell.

Staining of Endospores: Spores produced by bacteria (*Bacillus anthracis, Clostridium tetani, Clostridium botulinum,* e.g) are extremely resistant to desiccation, heat, and chemicals. Ordinary dyes such as crystal violet, safranin, and methylene blue are unable to stain spores because they cannot penetrate the spore coats. These dyes can only stain the vegetative portion of these gram positive rods, while the endospore remains colorless. Students may perform gram stain on Bacillus species to observe unstained spores.

Several methods have been developed for staining spores. Prepared slides of bacteria with stained spores will be available for student examination (spores seen as red oval bodies)

DEMONSTRATION An aerobic spore-forming bacterium (*Bacillus Anthracis*) will be available for student examination. Please note that the presence of colorless oval areas within the bacterium should lead one to suspect that the microorganism is a spore-former.

B. GRAM-NEGATIVE RODS

Enterobacteriaceae Family

Species of the genera *Escherichia, Klebsiella, Proteus, Serratia, Enterobacter, Citrobacter, Edwardsiella, Providencia, Arizona, Salmonella, Shigella, and Yersinia* are all gram-negative rods that belong to the family Enterobacteriaceae. These microorganisms are found in the intestinal tract of man and animals under normal and disease conditions. They are transmitted to humans through fecal contamination of water, soil, food, or fomites and many species are pathogenic causing intestinal diseases, septicemia, urinary tract infections, and various opportunistic infections in hospitalized or compromised hosts (e.g., aged, diabetic, immunosuppressed). Treatment of such infections is often complicated by the fact that many of these gram-negative rods are resistant to various antibiotics and the patients are usually debilitated or in a compromised state.

>>>>>
Blood agar cultures of the following bacteria will be available for student examination: *Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa.* Each group of 4 students at the lab table should work together to become familiar with these microorganisms.

1. Observe and record the cultural characteristics (type of isolated colonies) of each microorganism growing on the **blood agar** medium. The student should note that most gram-negative rods are rather indistinguishable on this medium. They appear as large, smooth, gray colonies which are usually round and convex. Nevertheless, several distinctions can be made and should be recognized by each student.

Escherichia coli	***Pseudomonas aeruginosa***	***Proteus mirabilis***

a) The spreading, swarming type growth of *Proteus mirabilis* which usually covers the entire agar surface is very characteristic as is the unpleasant sewage or fishy type odor.

b) The very large, flat, and irregular colonies of *Pseudomonas aeruginosa* which also appear to have a metallic glistening sheen is quite characteristic as is the sweet or fruity odor.

Streak for isolated colonies on M-H agar. (Click on above table to see images.)

2. Prepare a **gram stain** of several microorganisms and examine microscopically under oil immersion.

Escherichia coli	*Pseudomonas aeruginosa*	*Proteus mirabilis*

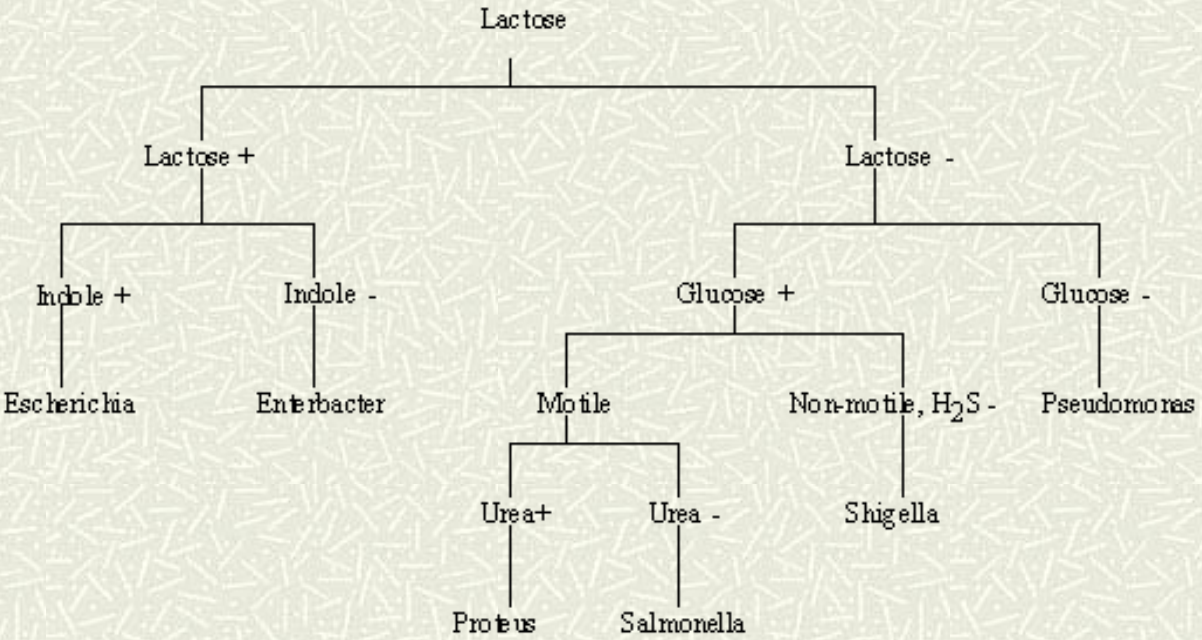
3. Perform an **oxidase test** by using the filter paper method:

a) Place some visible bacterial growth on filter paper with a wooden applicator stick.

b) Deliver a drop or two of oxidase reagent over the growth and spread it slightly using the same stick.

c) Wait several minutes to see whether the bacterial mass changes to dark purple or black (positive reaction).

Positive Oxidase Test of *Pseudomonas aeruginosa*



Demonstration of Streak *E. coli* (lactose fermentator) on MacConkey or EMB plates.

4. The identification of many gram-negative bacteria is accomplished by performing a variety of biochemical tests, and the subsequent reactions are used to classify the microorganisms. Instead of inoculating individual tubed media to perform these biochemical tests, the lab utilizes one of several commercially available micromethod systems such as the Enterotube or the

API test system.

The **API Enterobacteriaceae Test System** will be demonstrated. The procedure of this micro-method system is illustrated on the next page.

SECOND SESSION.

A. Enteric Gram-Negative Rods

1. Perform the **Indole Spot Test** with the various bacteria grown on M-H agar plate. The method requires that 2-3 drops of the reagent be placed on a piece of filter paper and a heavy bacterial mass be rubbed into this saturated area with an applicator stick. The appearance of a blue, blue-green, or red-violet color within 10 seconds-indicates indole production whereas negative reaction are colorless or light pink.

Results

2. Examine and note the growth of ***E. coli*** **on EMB agar** medium which will be available as a demonstration. Most, if not all, *E. coli* strains display a green metallic sheen which is rather characteristic.

Images:

Bacillis with endospores

Clostridium perfringens and

Clostridium perfringens on blood agar, with double zone of hemolysis

Clostridium sporogenes on blood agar, with single zone hemolysis

Clostridium perfringens gram stain

Clostridium sporogenes gram stain showing endospore

Litmus Milk test for *Clostridium perfringens*

Escherichia coli, Proteus mirabilis, Pseudomonas aeruginosa

Escherichia coli on blood agar

Escherichia coli on EMB agar

Escherichia coli gram stain

Indole test for *Escherichia coli*

Pseudomonas aeruginosa on blood agar

Pseudomonas aeruginosa on M-H agar

Pseudomonas aeruginosa gram stain

Indole test for *Pseudomonas aeruginosa*

Oxidase test for *Pseudomonas aeruginosa*

Proteus mirabilis on blood agar

Proteus mirabilis on M-H agar

Proteus mirabilis gram stain

API enterobacteriaceae test

Bacteriology Quiz Instructions

Unknown cultures will be distributed to students for examination and presumptive identification. Each student will receive two cultures (a gm+ cocci, a gm+ rod, gm- rod) and will be required to answer the following questionnaire after some preliminary characterization.

To obtain full credit for the identification of bacterial unknown, the answer sheet should be filled out using the following criteria:

1. Description of Specimen -- What type of specimen (sputum, exudate, skin scraping) was used to isolate the etiologic agent and what type of infection does the patient have?

2. Cultural Characteristics -- Describe type of colonies including such things as pigmentation and the medium on which it is growing. Is there any hemolysis around colonies and what type?

3. Microscopic Characteristics -- Describe gram reaction and morphological appearance of bacteria including arrangement (gram-positive rods in chains, for example).

4. Test Results -- Choose relevant tests, perform these tests and report results (catalase, coagulase, oxidase,
etc.).

5. Presumptive Identification -- All of the information above should enable one to presumptively identify the genus and perhaps the species of bacteria.

6. Tests Requested for Confirmation -- What test would one request to confirm the presumptive identification (coagulase tube test, bacitracin sensitivity, optochin sensitivity, growth on bile esculin and fermentation tests, etc.)?

Sample Answer Sheet

MICROBIOLOGY LAB PRACTICAL

Name_____

Date _____

1. Specimen Number _____

2. Description of specimen

_____
_____

3. Cultural Characteristics

_____
_____

4. Microscopic and Staining Characteristics

_____
_____

_____

5. Test Results (report results of relevant and applicable tests you chose to perform)

_____
_____

6. Presumptive identification (give genus and species)

_____

7. Tests requested for confirmation

_____
_____
_____

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EXERCISE #5 DERMATOPHYTES

Examination of Dermatophytes, Selective and Differential Media, and Clinical Specimens

Focus:

- Identification of dermatophytes by colony and microscopic morphology, and dermatophyte culture medium

Superficial Dermatophytoses

The dermatophytes comprise a special group of fungi (about 30 species) capable of degrading and utilizing keratin (nonliving tissue) found in skin, hair and nails. These fungi are classified into three genera based on their microscopic appearance and include the *Microsporum*, *Trichophyton*, and *Epidermophyton*. Infections with these microorganisms involve the colonization of the keratinized layers of the skin, hair, and nails without invasion of living tissue, and these are referred to as tinea capitis (scalp), tinea corporis (ringworm-like infections of trunk, legs, and arms), tinea cruris (groin infection), tinea unguium (nail infection), and tinea pedis (foot infection). The severity of these superficial diseases depends on the strain or species of dermatophyte and the sensitivity of the host to the particular fungus.

The majority of tinea pedis infections are caused by *Trichophyton mentagrophytes*, *Trichophyton rubrum*, and *Epidermophyton floccosum* which are anthropophilic species (spread from man to man). Other dermatophytes that are endemic to specific geographic areas (*Trichophyton tonsurans* and *Microsporum canis*, e.g.) may be involved in etiology of a small percentage of cases (<10%). The infection frequently starts in the toe webs and soles, and results in development of lesions that may be mild or chronic and scaling. Acute spreading lesions with vesicles and inflammation presenting as erythematous painful irritations (rash) may also occur as well as inapparent infection without lesions and inflammation. Transmission of tinea pedis is apparently enhanced under conditions of repeated exposures to desquamated skin contaminated with dermatophytes especially on floors of locker rooms and showers. In addition, moisture and warmth and anatomical problems that occur between the toes when shoes are worn predispose the individual to infections.

Tinea corporis is a cutaneous disease that can involve the legs, arms, chest, and back. The infection characteristically spreads in a circular fashion from the initial site and produces rings of inflammation. Spontaneous healing occurs at the center of the scaling lesions and the fungus is usually demonstrable in the advancing margin. *T. rubrum* and *T. mentagrophytes* are consistently and frequently isolated as etiologic agents but zoophilic species (contacted from animals) such as *M. canis* and *T. verrucosum* are occasionally implicated in such infections. *Microsporum gypseum* is an example of a geophilic species (contacted from soil) that can cause tinea corporis.

The dermatophytes may differ in their origin of infection (body area invaded) but they are similar in their mode of skin invasion. A microscopic examination of skin scrapings may reveal mycelium and arthrospores, thus indicating a fungus infection without differentiating between the organisms. For specific identification the lesion must be cultured and the fungus identified on the basis of the type of spore and pigmentation. Most of these infections respond to topical treatment but oral griseofulvin and ketoconazole are used for persistent cases.

Structure of Dermatophytes

The dermatophytes are rather ubiquitous microorganisms with some species being widespread throughout the world and others being limited to certain geographic and climatic areas. These fungi are usually found in soil or in association with animals and man. Different species may produce different clinical types of disease or diseases of different severity. Zoophilic and geophilic strains of dermatophytes generally produce more inflammation than anthropophilic strains.

The cultural characteristics of dermatophytes are quite similar with many species presenting as white and fluffy type molds on Sabouraud dextrose medium. Microscopically, the 3 genera can be distinguished based on the appearance of macroconidia which are rough, thick-walled, spindle-shaped, and abundant in the *Microsporum* genus. The infrequent to rare appearance of smooth, thin-walled, cigar-shaped or distorted macroconidia exemplifies the *Trichophyton* genus, whereas *Epidermophyton floccosum* produces thin walled, club-shaped macroconidia usually in small bunches. The absence of microconidia and production of abundant chlamydospores further characterized *E. floccosum*, whereas various amounts of round to rodlike microconidia aids in identification of *Trichophyton species*.

Further characterization includes the fact that *Microsporum* species do not infect nails (tinea unguium being primarily caused by *T. rubrum, T. mentagrophytes*, and *E. floccosum*) and *Epidermophyton* does not infect hair (tinea capitis being primarily caused by *M. audouinii, T. tonsurans,* and *M. canis* in the U.S.).

Examine and record the various macroscopic characteristics of each dermatophyte culture and prepare lactophenol cotton blue mounts for microscopic examination using the following tease prep procedure:

1. Aseptically remove some of the fungal growth with a teasing needle (dissecting pins) and place this sample on a clean microscope slide.

2. Add 1 to 2 drops of lactophenol cotton blue stain (LPCB) to the sample and tease or break it apart. Ideally, you should gently breakup one mycelial mat with the dissecting probes into several smaller pieces.

3. Place a cover slip over the preparation and examine it with your microscope using the high dry (40x) objective.

4. Please note that at times you may have to make more than one LPCB mount before you obtain a good representative slide of any particular fungus.

5. Since the student will be expected to identify each microorganism by its genus and species name, a record of the macroscopic and microscopic characteristics should be kept for every fungal culture. Permanent LPCB mounts can be made by encircling the cover slips with clear nail polish and allowing these to air dry. However, the student must be sure that their LPCB preparations are good representative slides. Therefore, each preparation should be examined microscopically prior to making permanent LPCB slides. The Medical Mycology Manual can be used as a reference for descriptions and photographs of the various fungal cultures.

Microsporum gypseum
Microsporum canis
Trichophyton rubrum
Trichophyton mentagrophytes
Trichophyton tonsurans
Epidermophyton floccosum

Identification of Dermatophytes on Special Media

Dermatophyte Test Medium

1. Dermatophyte Test Medium (DTM) is a color indicator medium for the selective growth of dermatophytes. DTM contains the antibiotics, cycloheximide, gentamicin sulfate, and chlortetracycline-HCl, which inhibit the growth of most saprophytic fungi and bacteria. Only dermatophytes should grow on DTM and produce alkaline by products which cause a change in medium color (from yellow to red).

2. The presence of a dermatophyte in clinical specimens is noted by a **color change in DTM** after 24 to 72 hours of growth. Full color change may require up to 7 days, but development of red color after 14 days of growth should not be considered significant.

3. Microorganisms such as *Candida albicans*, *Pseudomonas aeruginosa*, and *Pseudallescheria boydii* have been found to grow on DTM and produce a red color. Cultural and microscopic characterization of any growth on DTM is thus required for presumptive identification of the etiologic agent.

4. DTM agar slants may be available for student inoculation with one of the various dermatophytes.

Clinical Fungal Specimens and Cultures

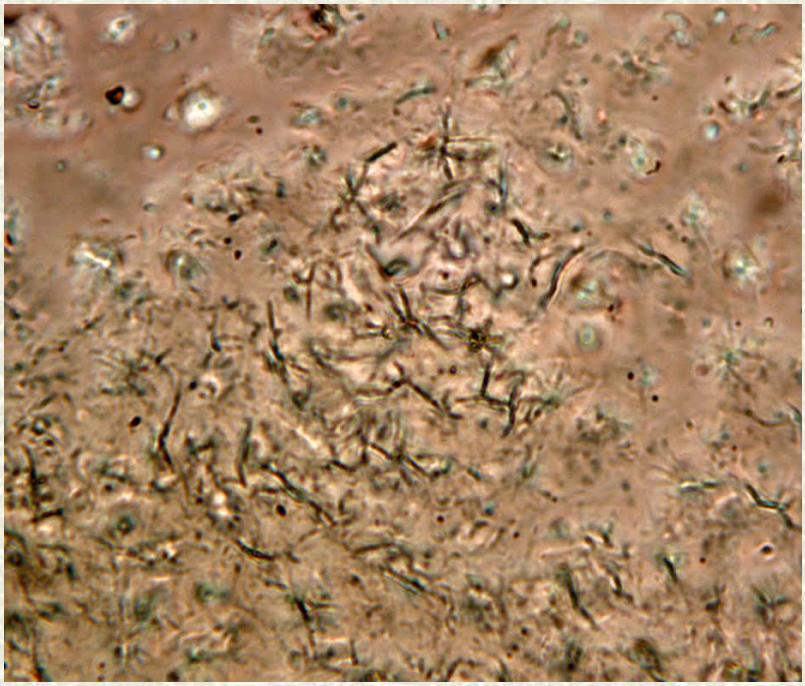
The following procedure should be used clinically for the microscopic examination of infected skin scrapings and nails (KOH procedure).

1. Prior to obtaining a specimen, thoroughly sponge the affected site with 70% alcohol to remove surface contamination and medication.

2. After the alcohol has evaporated, scrape the active edge of a lesion or the top of a vesicle with a sterile (flame-sterilized) scalpel. Skin scales or nail scrapings may be placed in a sterile paper envelope (or Petri dish) until microscopic mounts can be made.

3. Mount part of the specimen on a clean microscope slide in a drop of 10-20% potassium hydroxide (KOH). Apply a coverslip and allow to stand at room temperature for 5 to 10 minutes. The student may gently heat the preparation over a low flame but overheating may distort fungi (unnecessary for skin scrapings).

4. Examine the specimen microscopically using the low-dry and high-dry objectives. The KOH digests cellular components more rapidly than fungi and thus clears the specimen leaving fungal elements more clearly visible. Fungi should appear as fragments of mycelia with or without branches and septa (conidiospores may be present in some instances).



Perform the KOH procedure on clinical skin and nail scrapings, which may be available in the lab module, as described above.

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Clinical case studies

Suggestions

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EXERCISE #6. SAPROPHYTIC FUNGAL SPECIMENS AND YEASTS

Focus:

- Identification of three common saprophytic fungi
- Characterization and Identification of Yeasts, and slide culture technique

A. Saprophytic Fungi

Several saprophytic fungi can be mistakenly identified as dermatophytes. It is important to avoid such mistakes, especially since most clinical labs do not possess expertise in the area of mycology. Examine colony and microscopic (LPCB mount) morphology of the following fungi and determine why they can be confused with the dermatophytes.

a. *Acremonium* (*Cephalosporium*) species

b. *Fusarium* species

c. *Pseudallescheria boydii*

Acremonium species -- note cluster of elliptical conidia at tips of delicate, almost hair-like phialides

Fusarium species -- produces both macroconidia and microconidia, many of which are sickleform shaped (and resemble string beans).

Pseudallescheria boydii (*Scedosporium apiospermum*) -- note large, oval, single or small clustered conidia that are terminally produced on long and short conidiophores.

Acremonium species	Colony	LPCB mount
Fusarium species	Colony	LPCB mount
Pseudallescheria boydii	Colony	LPCB mount

Slide Culture Technique DEMONSTRATION

The basis for mold identification rests primarily on morphological characteristics -- the arrangement of various kinds of spores being particularly important. When one attempts to make a wet mount (LPCB) slide by transferring hyphae from a colony to the slide, the spore arrangement is generally completely destroyed so that all one encounters is a mass of dispersed spores and hyphae.

What is needed is a method of culturing the mold on a slide so that it can be

stained *in situ*. The slide culture technique described below permits the microscopic observation of the undisturbed relationship of spores to hyphae (one main criterion for the identification of saprophytic and pathogenic molds).

1. With a sterile scalpel, cut out agar blocks (about 1 cm square) of Sabouraud dextrose agar from a Petri dish containing a thin layer of this agar. Then, aseptically transfer this block to the sterile slide in a glass dish (sterile glass Petri dishes containing a piece of bent glass rod supporting a sterile slide are usually used).

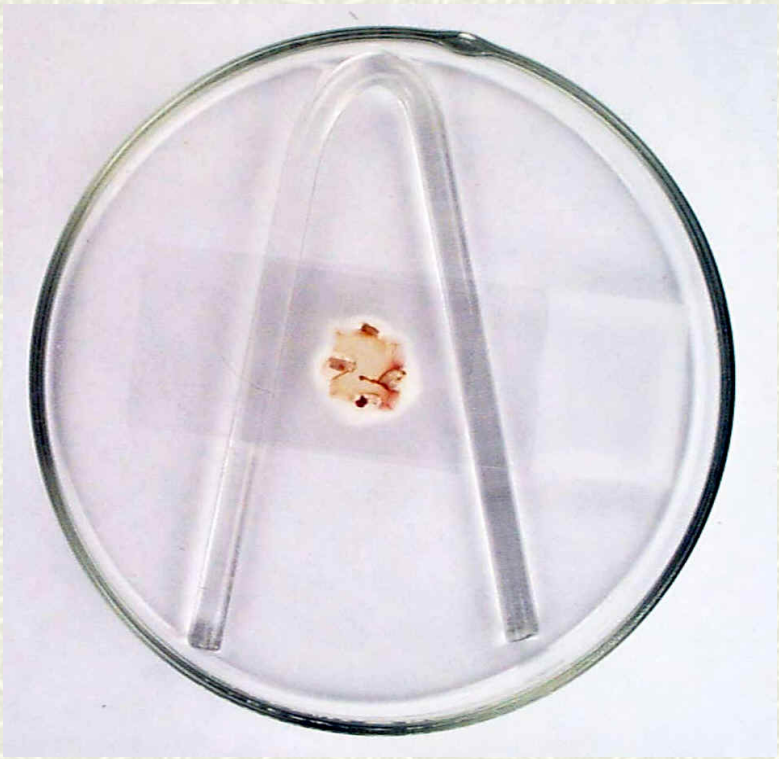
2. Inoculate the centers of the top and bottom sides of the agar block (or the center of the four sides) with the fungus under study, using a stiff teasing needle containing a minimal amount of growth from the culture.

3. Cover the block with a flamed cover glass, and then add about 8 ml of sterile water to the bottom of the Petri dish.

4. Incubate at room temperature and check periodically for growth and sporulation. This will require one to two weeks incubation.

5. Add sterile water as needed so that the culture does not dry out during the incubation period.

6. When sporulation occurs, carefully remove the cover slip from the agar block and place it on a drop of lactophenol cotton blue on a clean slide.Remove the agar block from the original slide, and discard it into a disinfectant jar. Then, place a drop of lactophenol cotton blue on the area mycelial growth remaining on the original slide and cover with a clean coverslip.



B. Yeasts

Yeast infections are among the most common fungal infections affecting humans, and their incidence has greatly increased since the advent of broad-spectrum antibiotics, corticosteroids, and anti-tumor agents. Their severity ranges from benign and transient through more severe, chronic, and recalcitrant. Although intertriginous infections are the most common, dissemination can occur to produce systemic infections which are sometimes fatal.

Yeasts exist in nature in a wide variety of organic substrates, including fruits, vegetables, and homemade fermented beverages. *Candida albicans*, the most frequent pathogen, is a normal inhabitant of the gastrointestinal tract of humans, various surveys showing an incidence of 20% to 40% in asymptomatic individuals. It is also found in small numbers of normal healthy individuals in the mouth, vagina and skin. Nevertheless, *C. albicans* can cause mild to severe or chronic superficial infections of skin, nails, and mucous membranes in individuals with normal immune defenses as well as serious systemic infections in debilitated patients.

Characteristic skin lesions of *Candida* infections have a red scalded appearance and a scalloped border. Satellite pustular lesions usually surround the primary lesion and this helps in differentiating Candida infection from a dermatophyte invasion. However, dry scaling lesions may occur in moist interdigital spaces which are indistinguishable from

dermatophytoses. Nail infections caused by *C. albicans* usually display a characteristic brownish color with striations of the nail and paronychia (erythematous swelling of the nail folds extending to tip of toe). Bacteria such as the gram-negative rods are often present as secondary invaders in *Candida* onychomycosis and may cause paronychia.

B. Identification of Yeasts

Once a yeast is isolated from a clinical specimen, the initial steps in its identification includes making and examining a wet mount prep, making and examining an India ink prep, performing a germ tube test, and inoculating corn meal agar to detect pseudohyphae and chlamydospores. Further steps include biochemical testing to determine the genus and species of the unknown isolate. Several commercial kits, e.g. Uni-Yeast-Tek system, are available for this purpose.

1. Note the color and consistency of the following colonies (yeasts) on Sabouraud dextrose agar.

2. Prepare LPCB mounts of cultures on Sabouraud dextrose agar and corn meal agar for microscopic examination:

   a. *Candida albicans* -- opportunist pathogen, flora

   b. *Candida parapsilosis* -- opportunist, skin flora

   c. *Rhodotorula rubra* -- moist skin inhabitant

Candida albicans	**Germ tube**	**Chlamydospores on corn meal agar**
Candida parapsilosis	-----	-----
Rhodotorula rubra	-----	-----

Comparison of size: bacteria versus yeast

Gram stain *Staphylococcus aureus* and *Candida albicans* smears prepared on the same microscope slide. Examine under oil immersion and note the difference in size between bacteria and yeasts. Save this slide for reference in Lab Practical!

Germ tube test

Suspend a small inoculum of the *Candida albcans* in a test tube of rabbit or human serum. Incubate the test tube at 37oC for no longer than 3 hours,

Placed a drop of the yeast-serum suspension on a microscope slide, overlaid with a cover slip and examined for the presence of germ tube.

Uni-Yeast-Tek system will be .

The Uni-Yeast-Tek system, similar to API test system for enterobacteriacea, combines in a single plate

- Sugar assimulation and fermentation (positive = red to yellow)
- Nitrate reduction (Positive = yellow to red)
- Urease tests (Positive = pink to yellow)
- Corn meal agar (colony and microscopic morphology)

The center well of the plate should be inoculated using a small amount of the yeast by scratching across the corn meal agar with an inoculating needle. This is then streaked with a sterilized loop across the scratch and a cover slip (not shown in photograph) is placed over this. The plate is incubated upright at room temperature for 2 to 6 days and the results are recorded and compared to standard reactions.

Uninoculated plate

Images:

Slide culture

Uni-Yeast-Tek system,

Acremonium (*Cephalosporium*) species colony and microscopic morphology

Fusarium species colony and microscopic morphology

Pseudallescheria boydii colony and microscopic morphology

Candida albicans in Sabouraud dextrose agar

Candida parapsilosis in Sabouraud dextrose agar

Rhodotorula rubra in Sabouraud dextrose agar

Candida albicans germ tube

C*andida albicans* chlamydiospores in corn meal agar

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Clinical case studies

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EXERCISE #7 MYCOLOGY LAB PRACTICAL EXAM

In this lab practical, each student will receive clinical case histories and unknown isolates presumed to be the etiologic agents. Cultures and corresponding case histories and cultures will be given to each student. **Check to confirm that the numbers match**.

The student is responsible for reading and evaluating each case history so as to presumptively identifying the causative agent. Make sure all results and impressions are recorded on the answer sheets that will be provided.

Since this is an exam, each student is required to work alone in determining the answers to each case! However, students may ask the lab instructor questions, consultation with other students will not be allowed!

Each student will be responsible for determining the following based upon the examination of the unknown culture provided:

Description of Specimen

What type of specimen (sputum, exudate, skin scraping) was used to isolate the etiologic agent and what type of infection does the patient have?

Culture Morphology

What is the colonial morphology of the unknown culture, pigment, and on what type of media is it growing? Is it a fungus? Is the microorganism is growing on selective differential media? Describe what type of reactions are evident and what this tells you about the unknown.

Microscopic Morphology

Is it a mold or yeast?. What are their microscopic characteristics, shapes, budding, present or absence of micro- and macroconidia, septated or non-septated hyphae, etc.?

Biochemical Characteristics

The appropriate tests will be available for your use. You should be able to perform and describe the results of the relevant tests that you chose to perform.

Presumptive Identification of Etiologic Agent

You should be able to identify the genus of your unknown microorganism and in many cases even the species. You will be required to record the suspected agent.

Tests Requested for Confirmation

Which tests would you choose to further identify the microorganis, e.g., dermatophyte test medium corn meal agar, germ tube test, urease, sugar assimilations, and/or Fermentations?

HAND IN ALL ANSWER SHEETS AND CASE HISTORIES TO THE LAB INSTRUCTOR AT THE END OF THIS SESSION.

Cover

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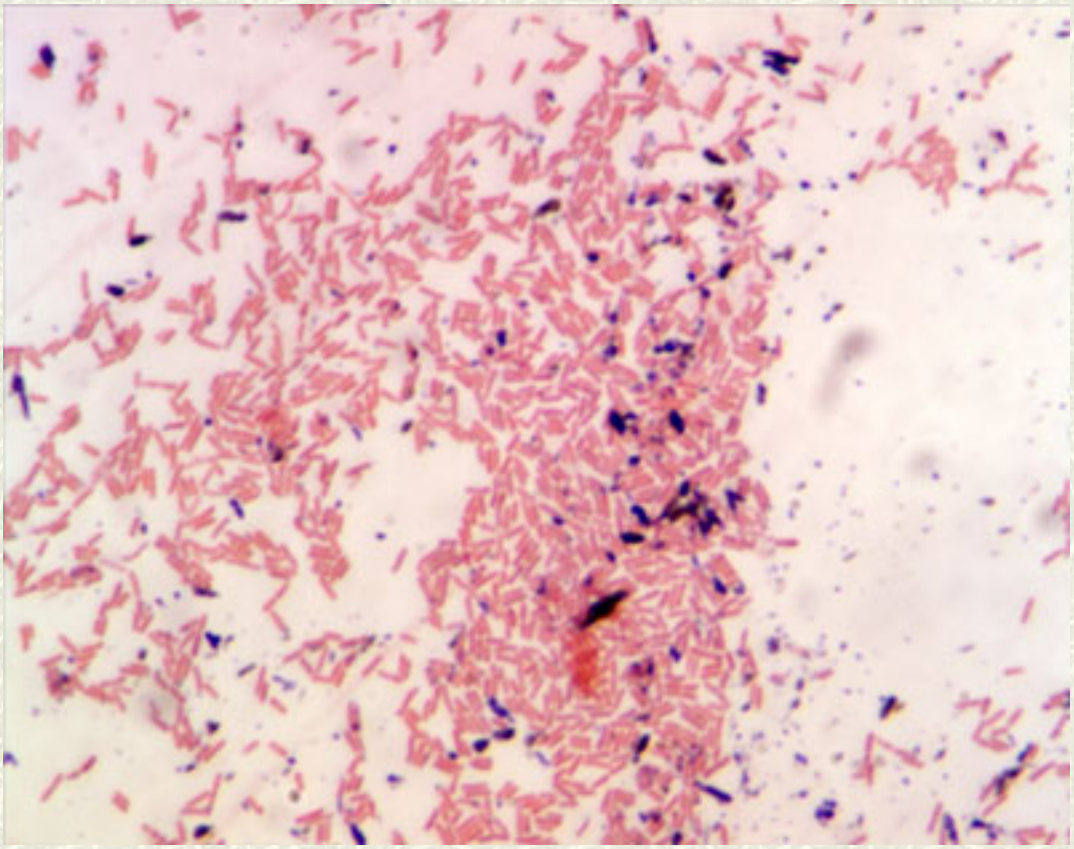

Gram stain of mixed culture

Presence of both gram (+) purple cocci and gram (-) pink rods.

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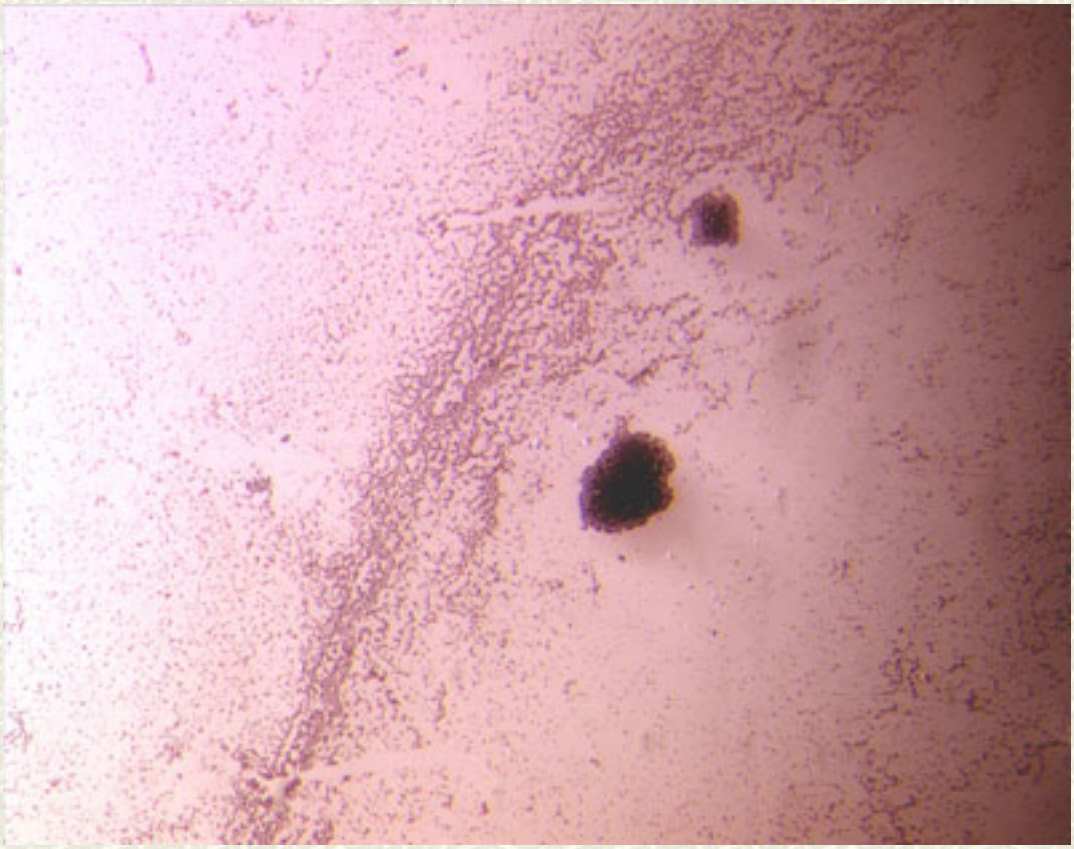

Observation of Gram stained Staphylococcus magnified with the 10X objective (100X)

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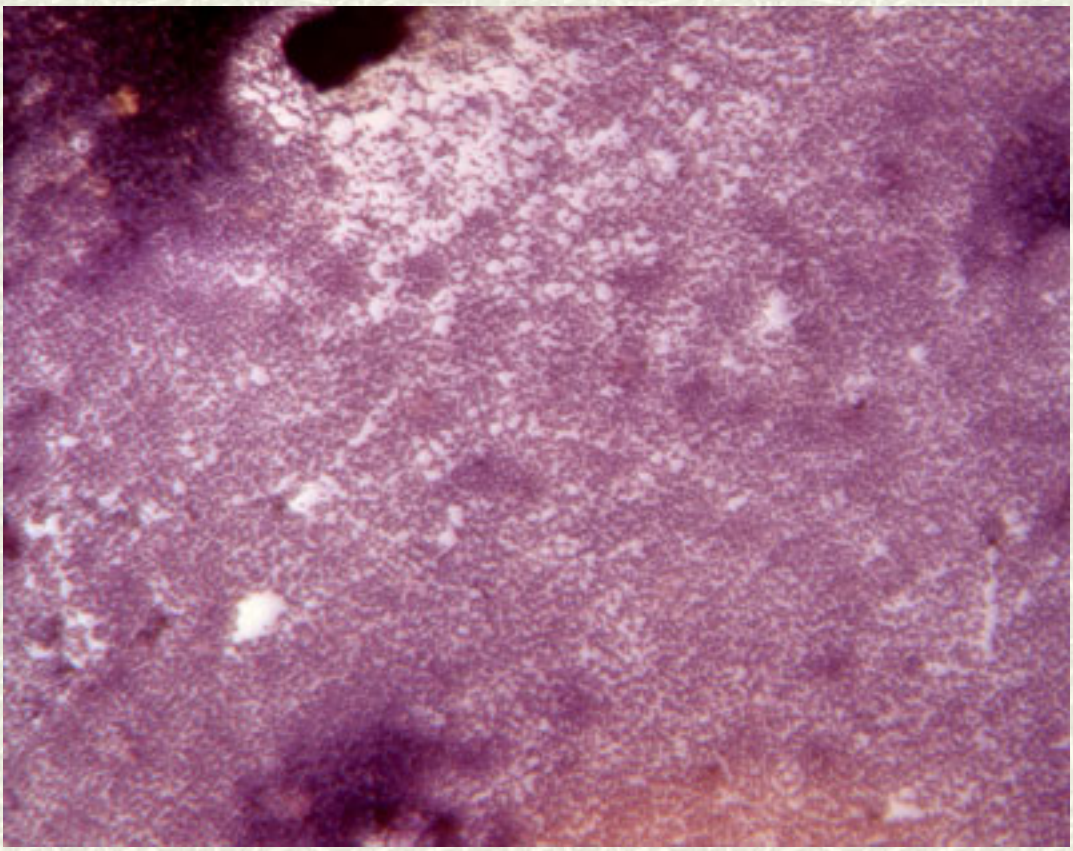

Observation of Gram stained Staphylococcus magnified with the 40X objective (400X)

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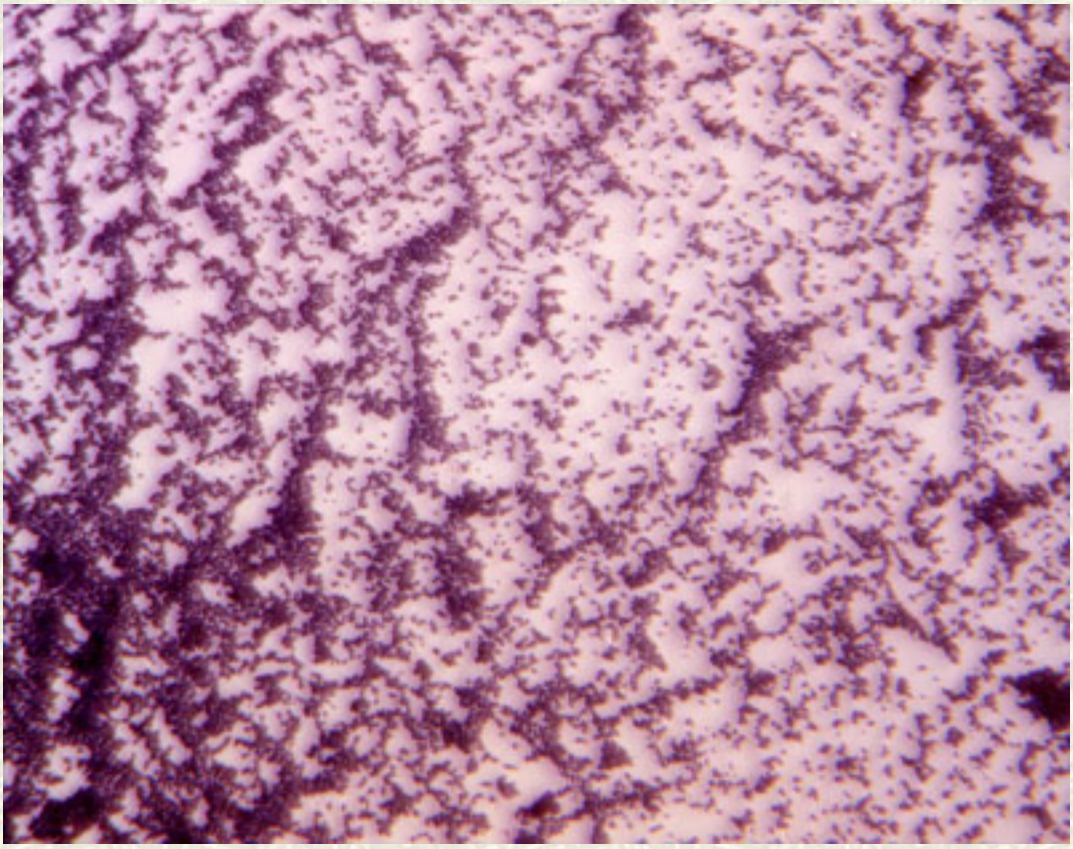

Observation of Gram stained Staphylococcus magnified with the 60X objective (600X)

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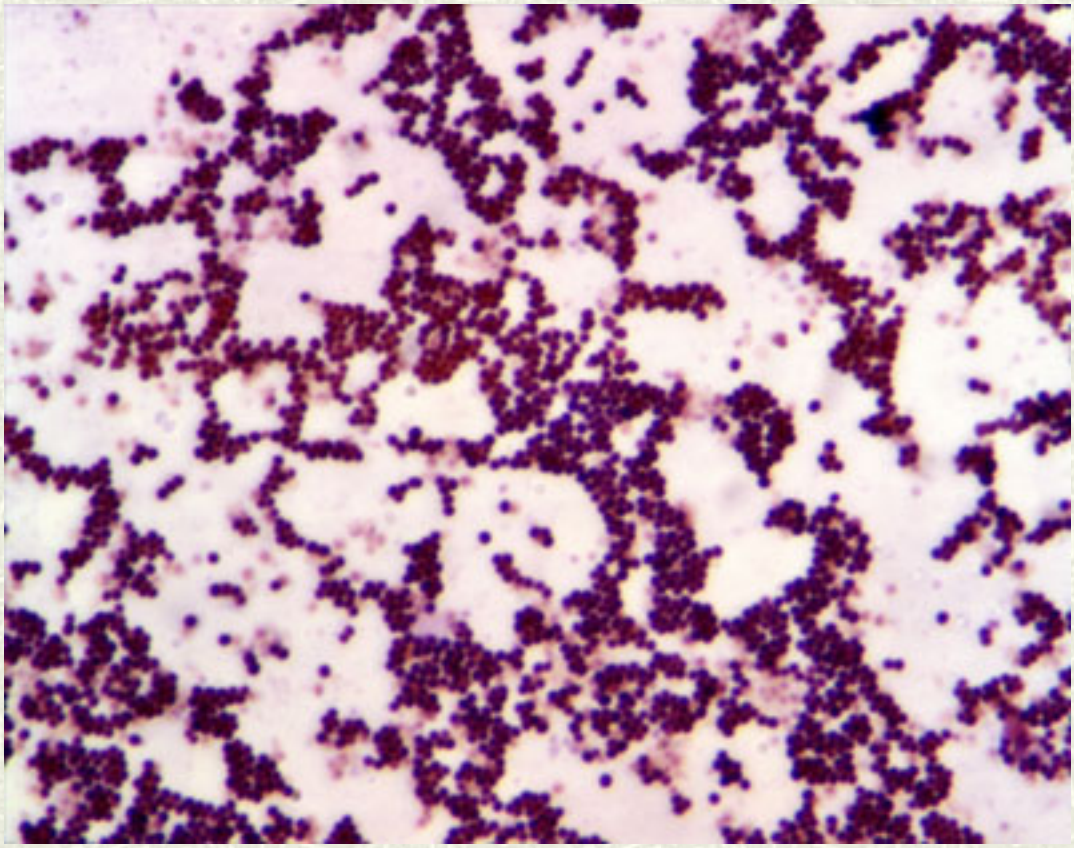

Observation of Gram stained *Staphylococcus* magnified with the oil immersion, 100X objective (1000X)

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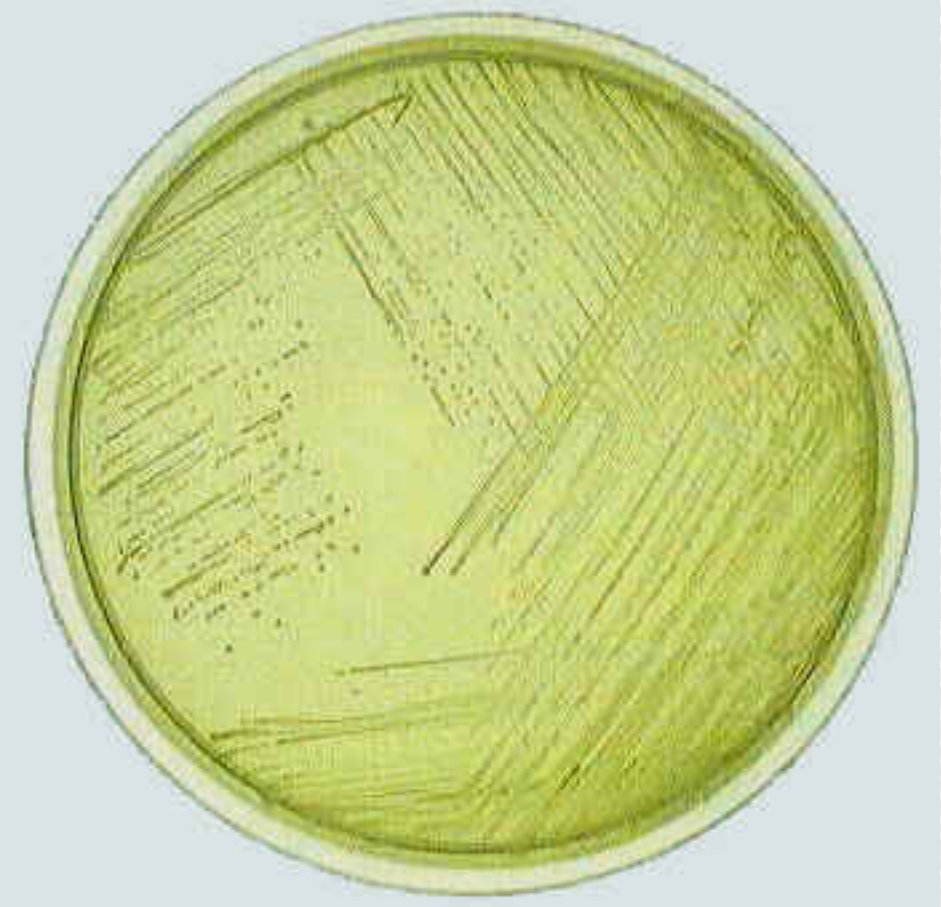

Staphylococcus epidermidis on Muller Hinton agar

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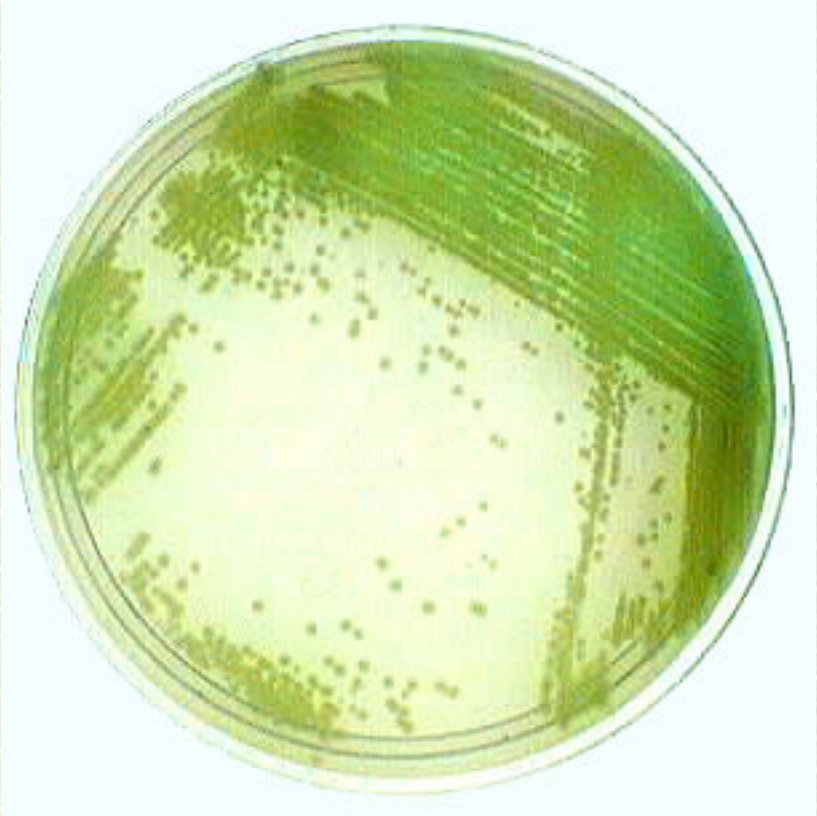

***Pseudomonas aeruginosa* on M-H media**

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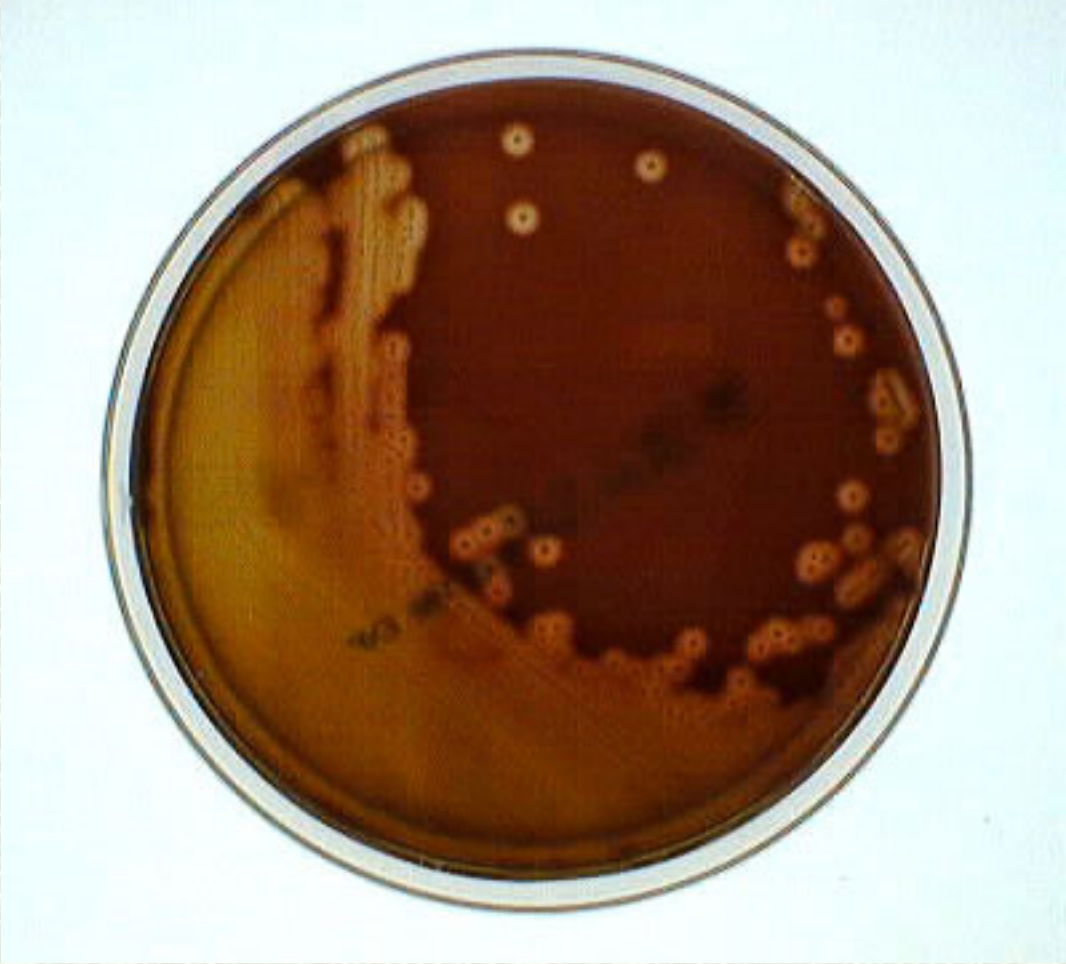

Streptococcus pyrogenes **(group A) on blood agar plate**

Note beta-hemolysis, clearing of red blood cells around colony

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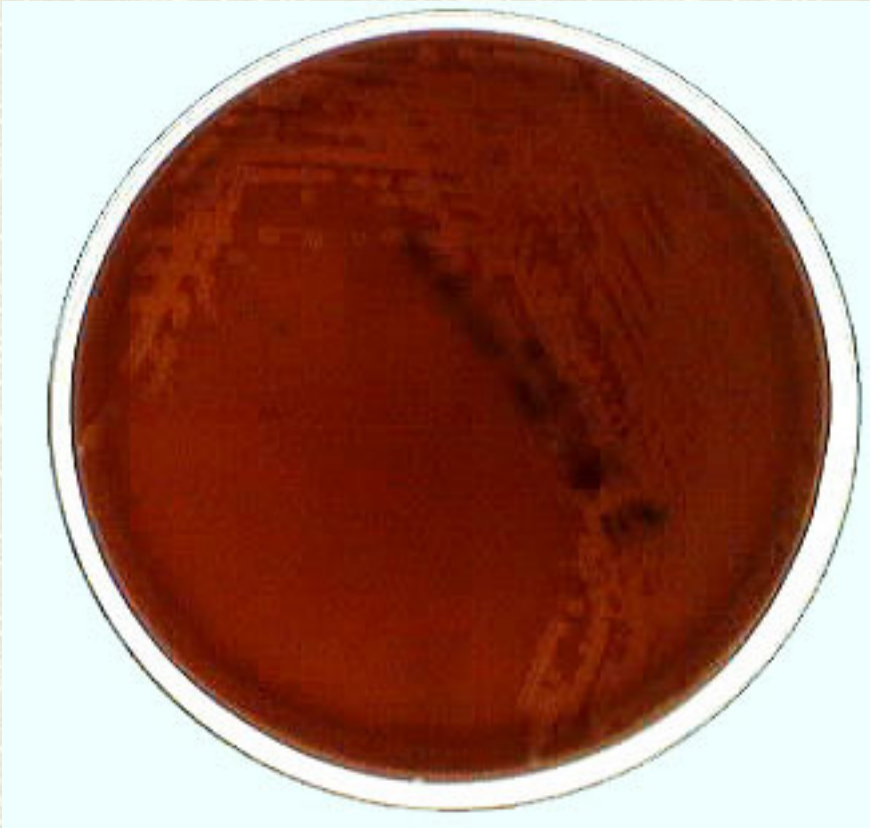

Stretopococcus agalactiae on blood agar.

Note beta-hemolysis around colony.

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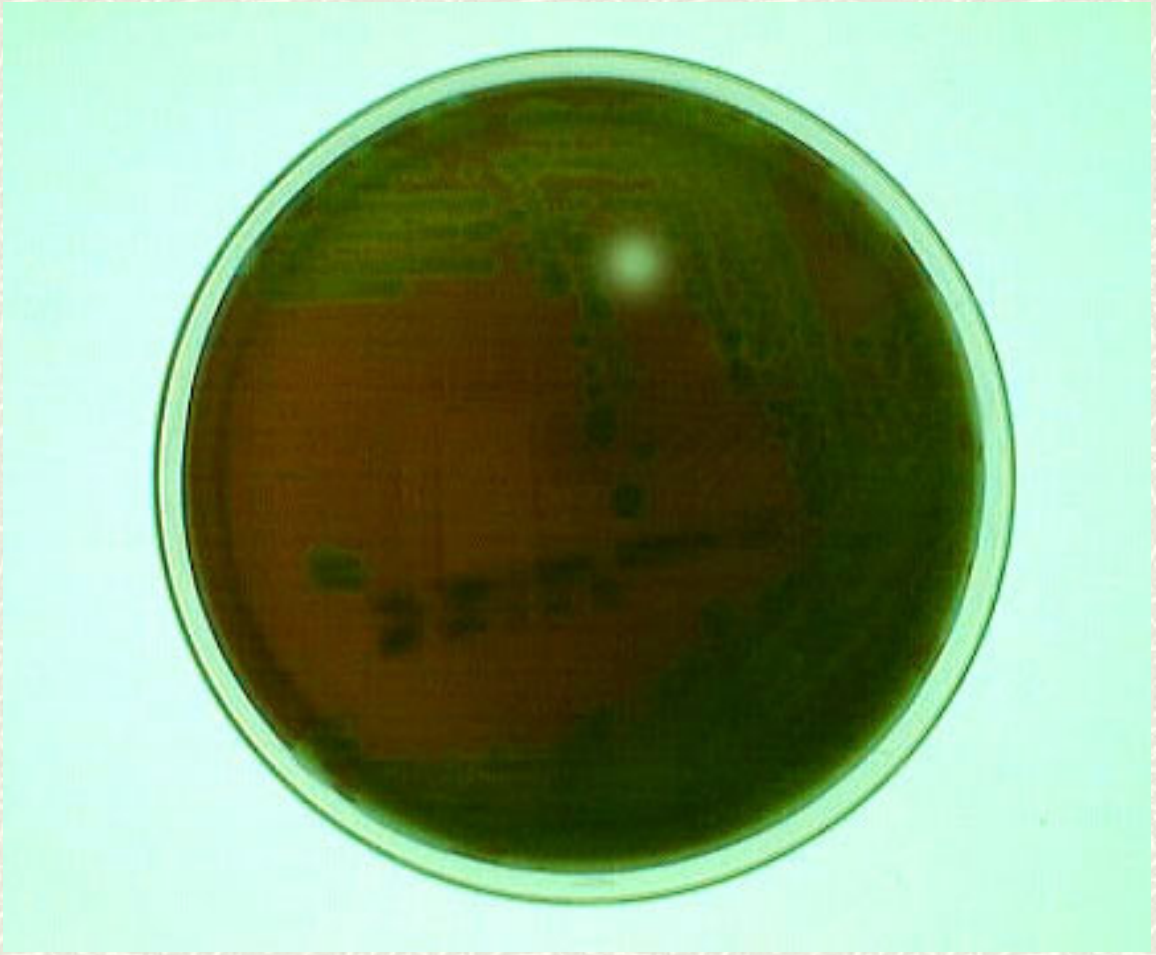

***Streptococcus pneumoniae* on blood agar plate**

Note alpha-hemolysis with green tint.

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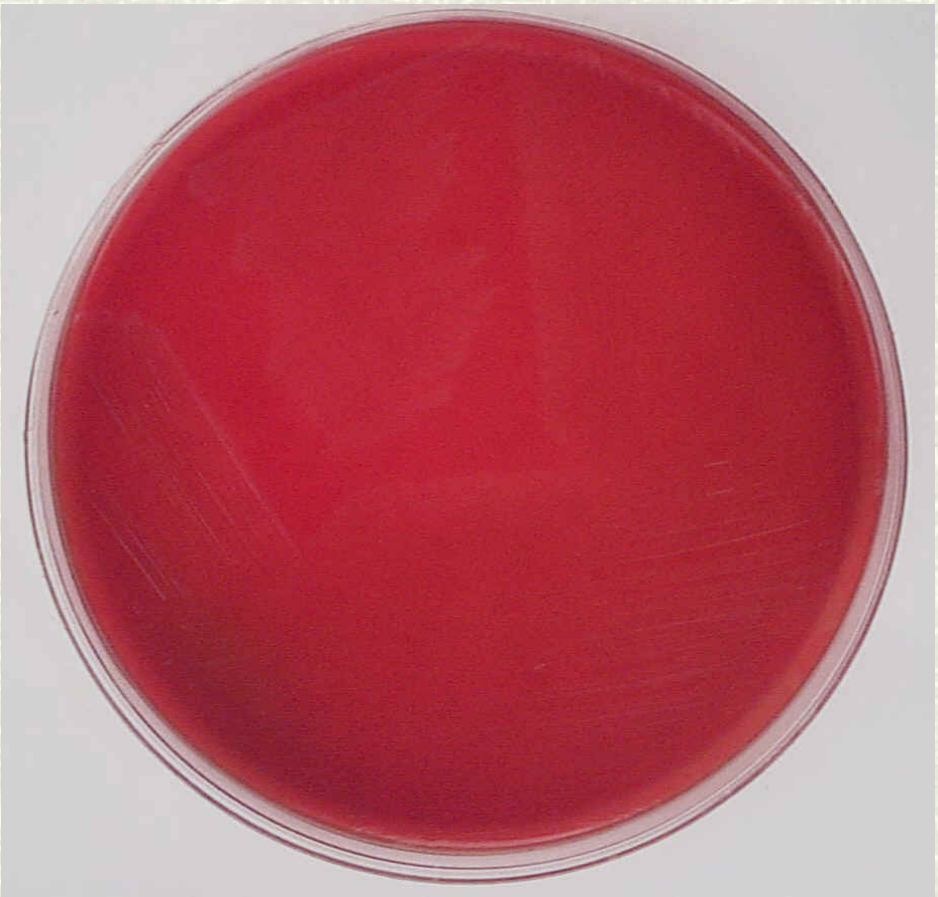

Enterococcus faecalis **on blood agar plate, no hemolysis occurred.**

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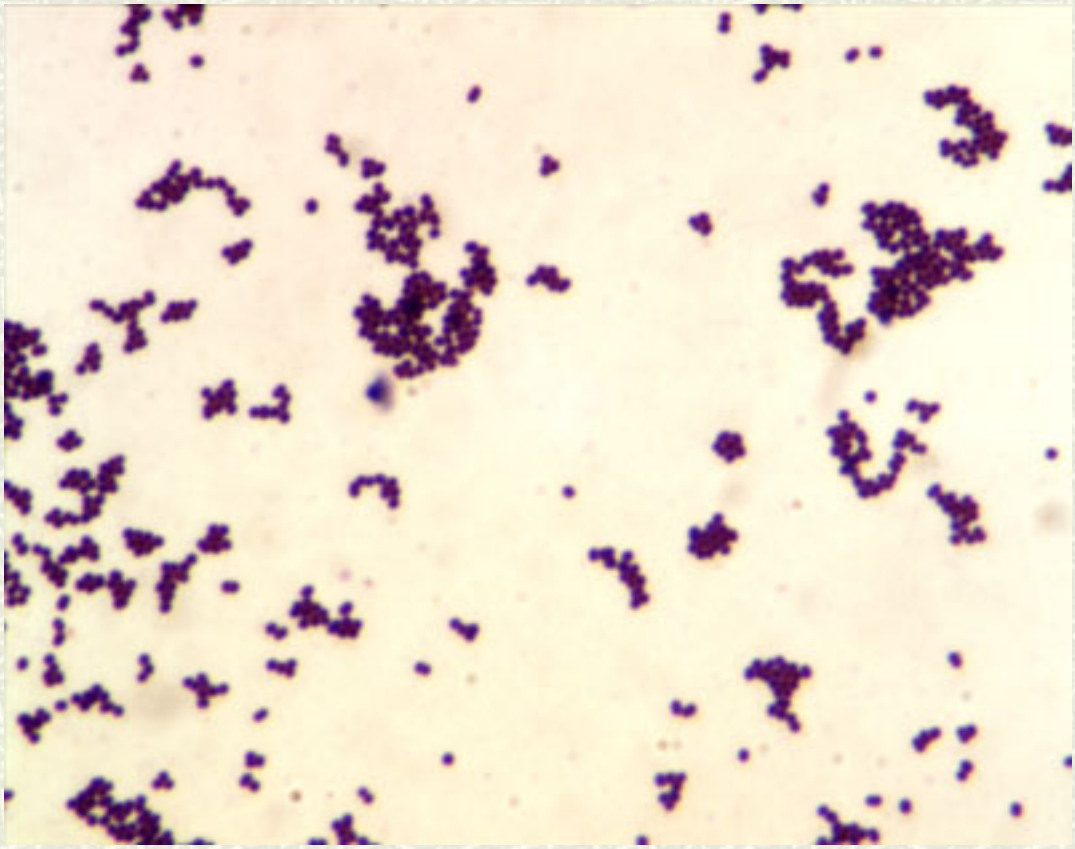

Streptococcus pyrogenes: gram +, cocci, in chains and clusters

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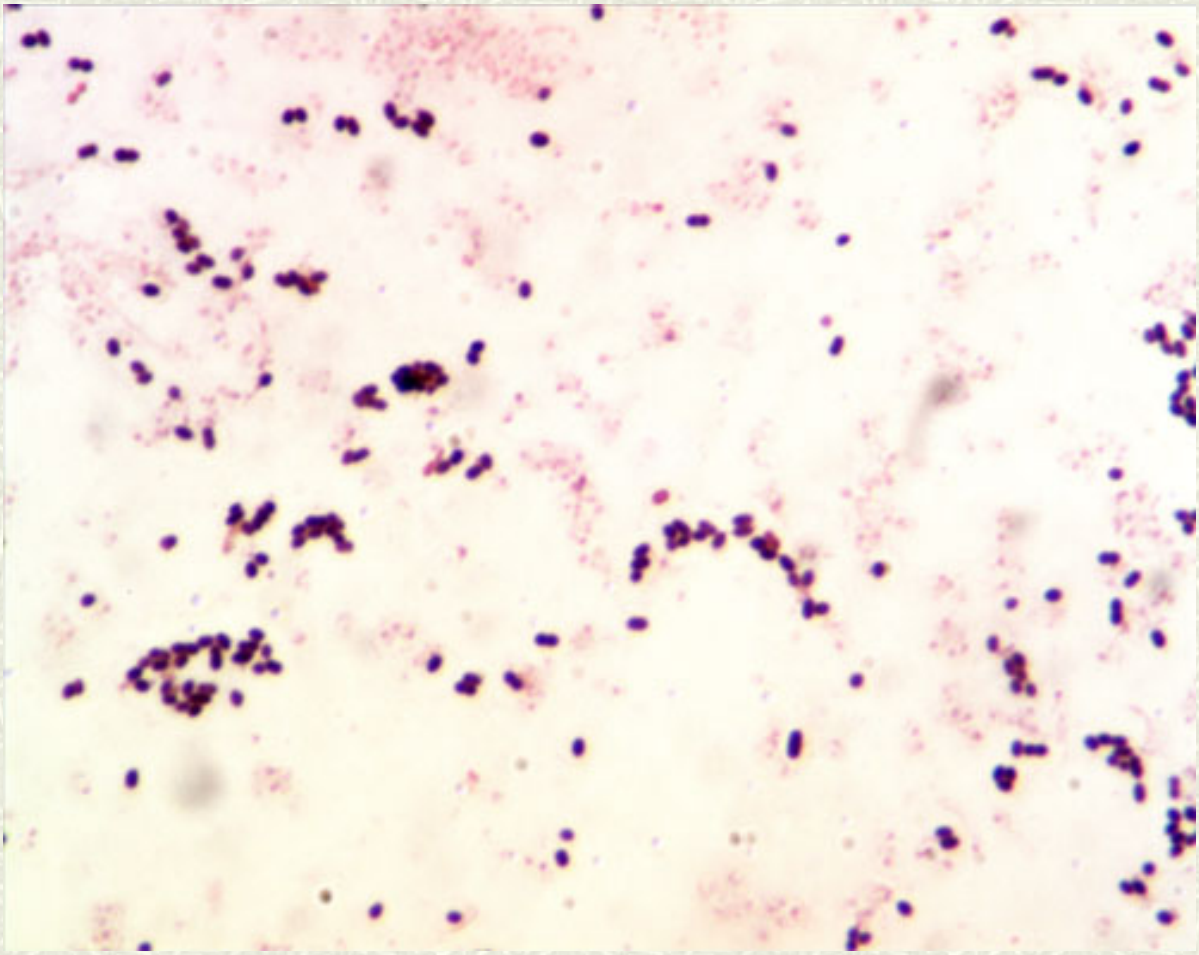

Streptococcus pneumoniae: gram +, cocci, in pairs

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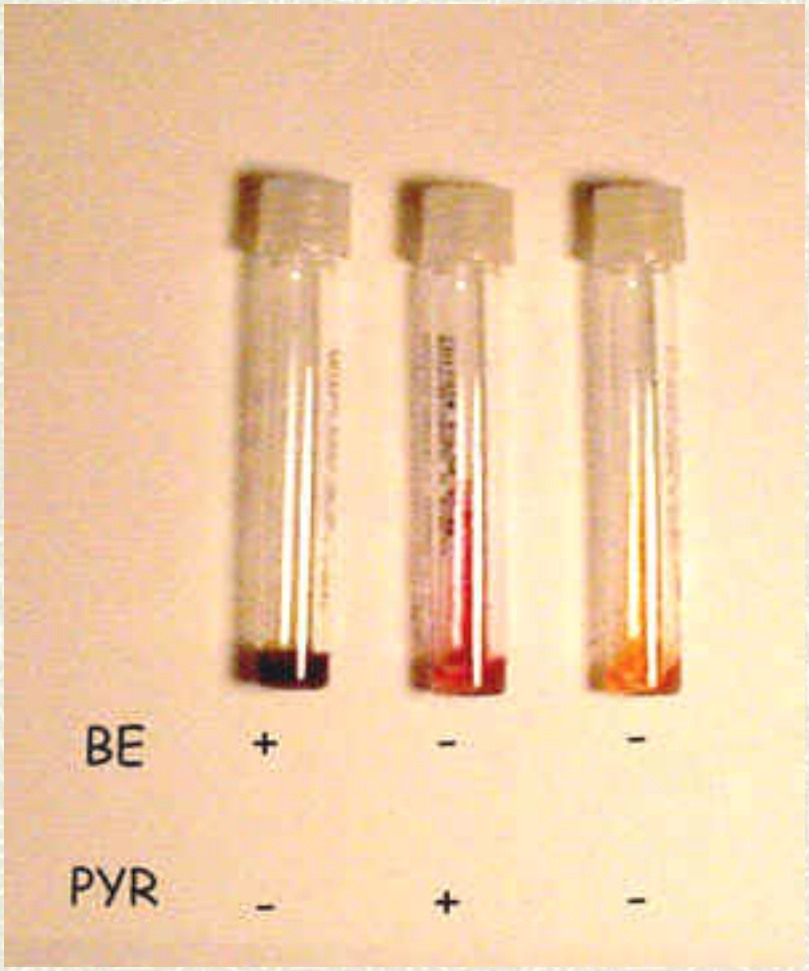

Appearances after 24 hour of incubation

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Clinical case studies

Suggestions

Identification of *Streptococcus pyrogenes* by agglutination, clumping, of latex particles that have been coated with antibodies to group A carbohydrates (1).

Particles that are coated with antibodies to group B carbohydrates are not agglutinated, homogenous suspension (2).

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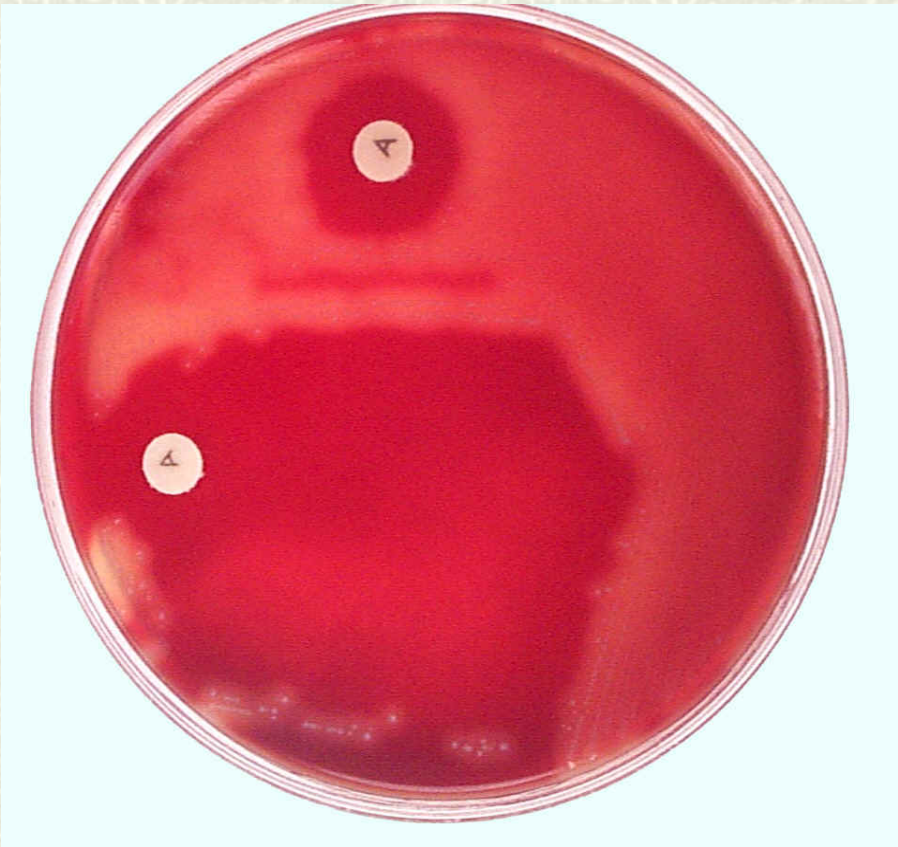

Note lack of growth and beta-hemolysis around bacitracin antibiotic disc.

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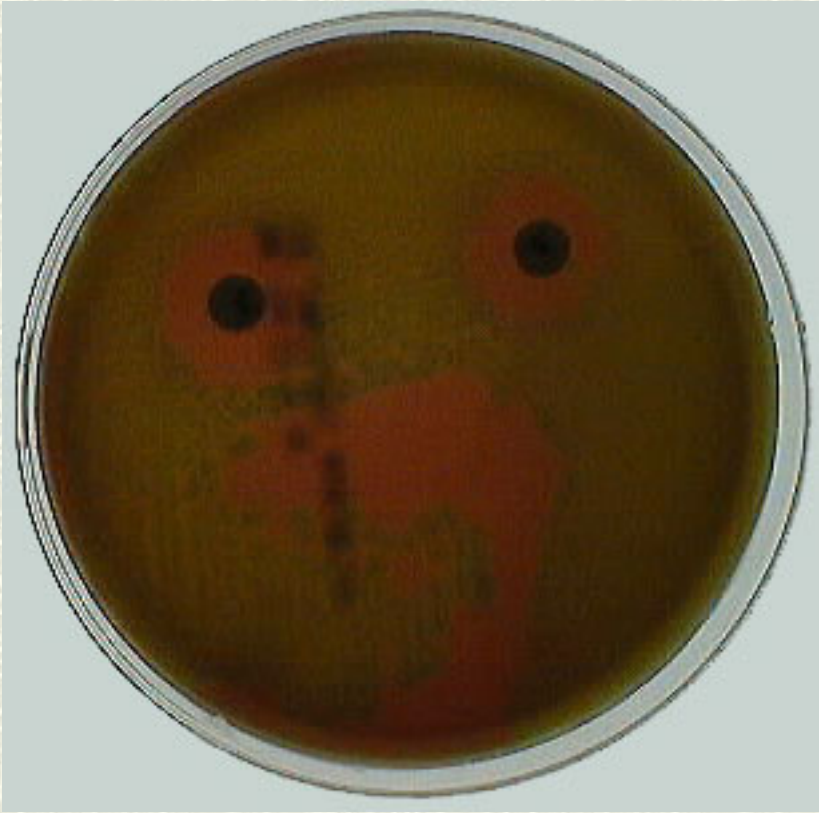

Note lack of growth and alpha-hemolysisaround antibiotic disc.

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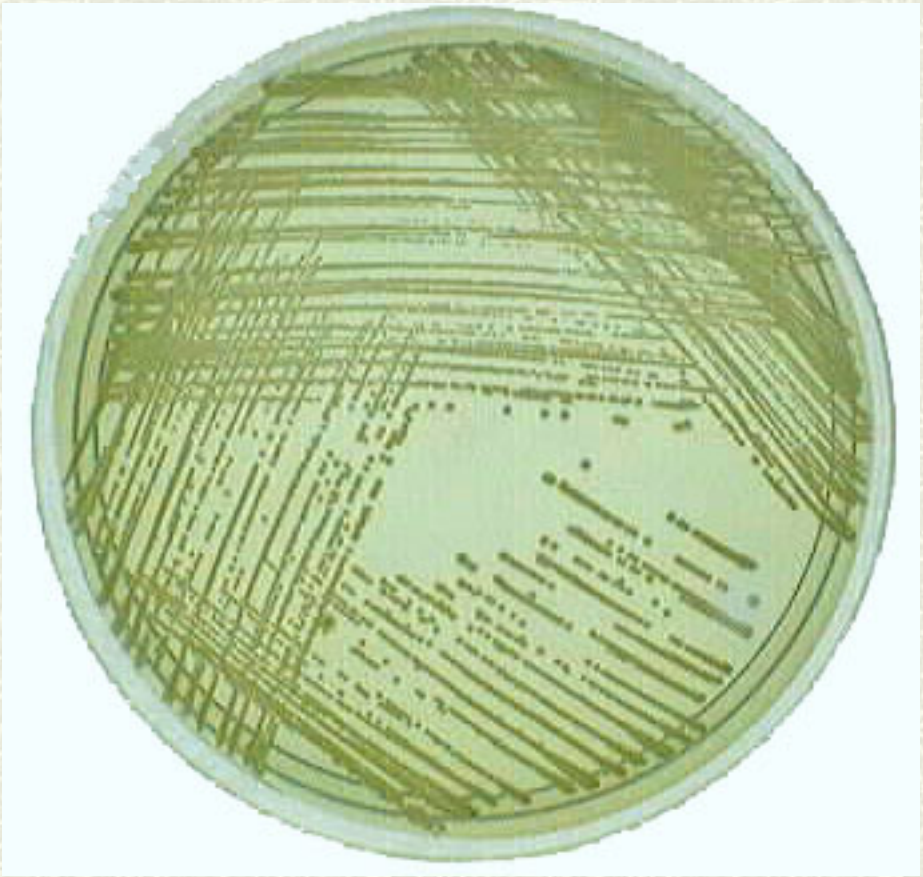
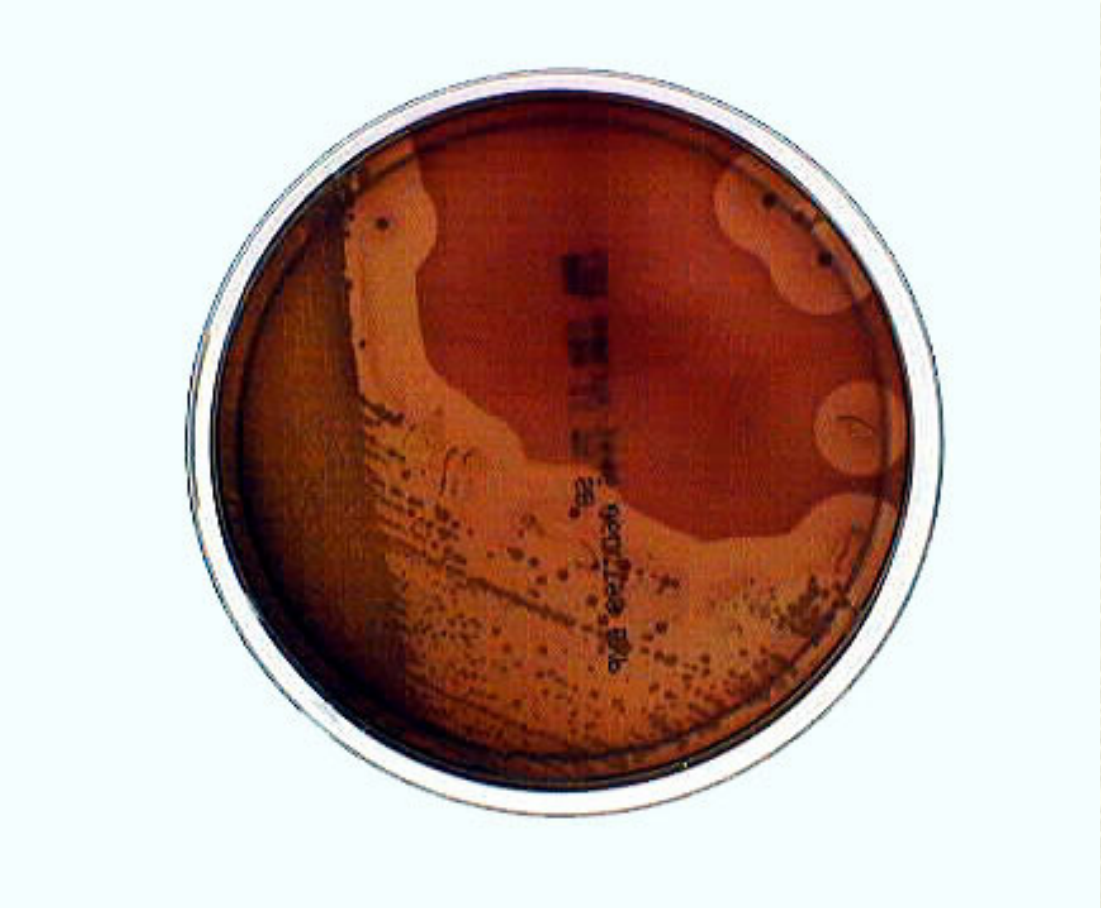


***Staphylococcus aureus* on M-H agar:**

Colonies are round, smooth, yellowish and larger than *S. epidermidis*



***Staphylococcus aureus* on blood agar agar: Note extansive beta-hemolysis.**

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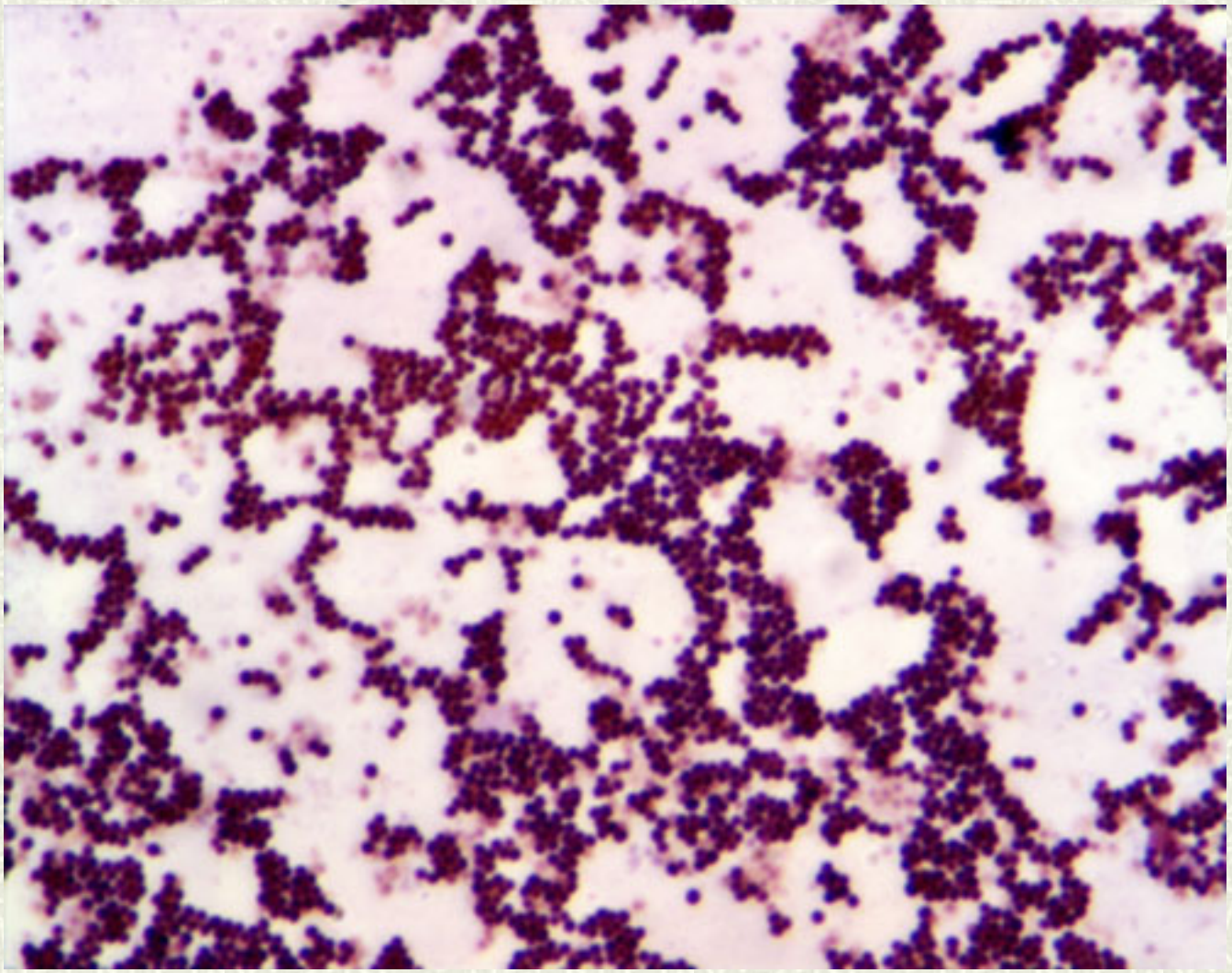

Gram (+) cocci in grape-like clusters

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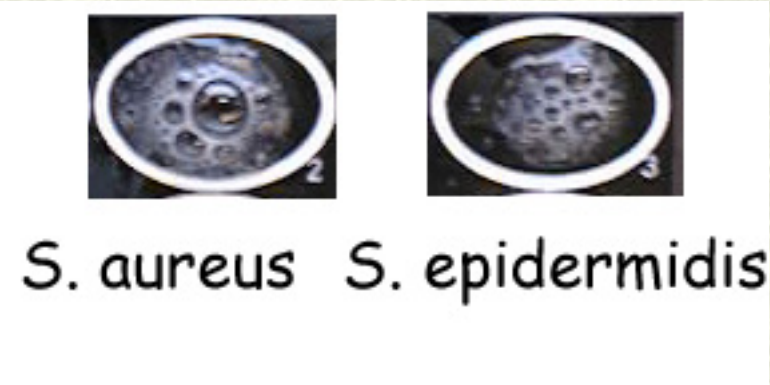

Catalase test (+)

Note lack of generation of oxygen bubble with the Catalase test (-) *Stretptococci* in previous sections

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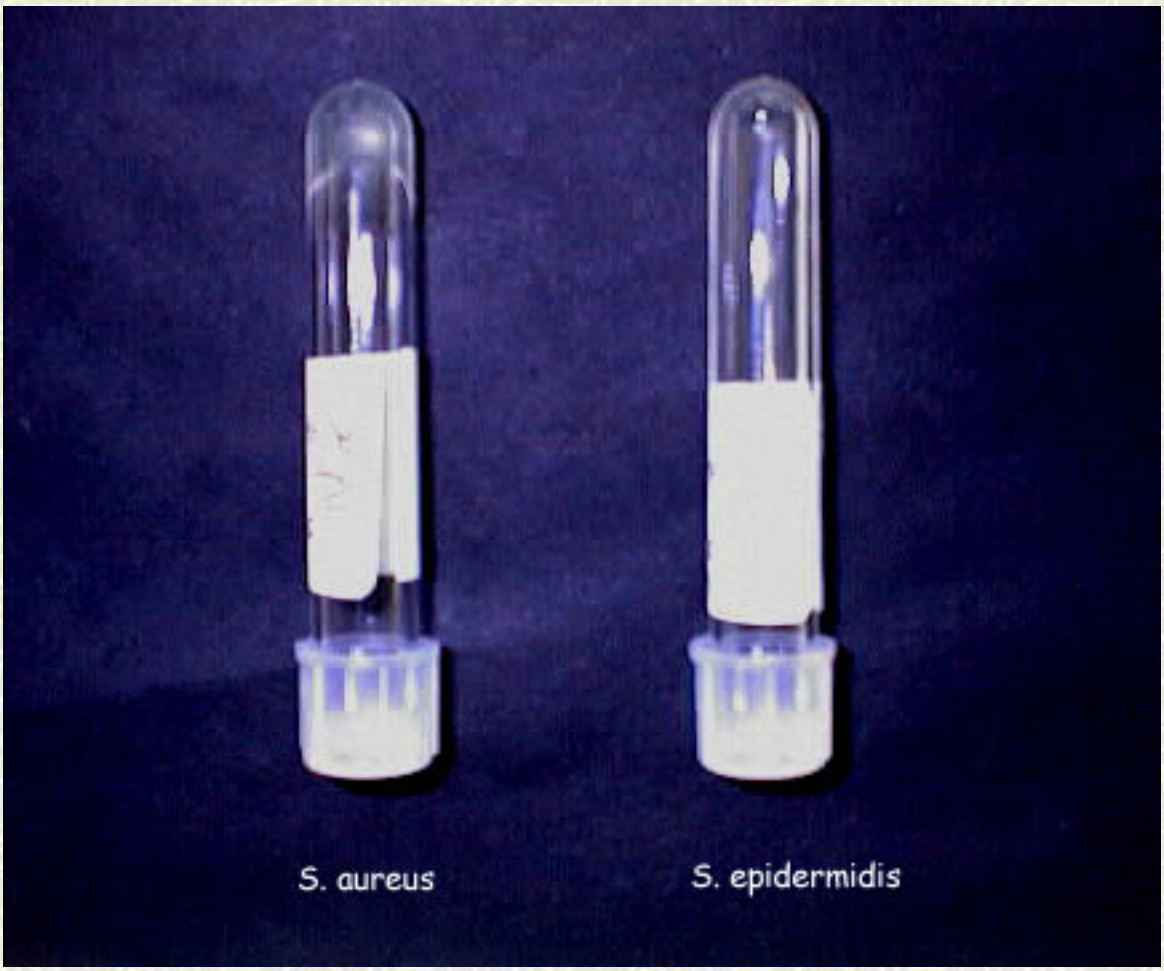

Note, in 4-6 hours, rabbit plasma in the tube with coagulatase

(+) *S. aureus* is gelled and does not run

A quicker assay may be performed on slides

Note gelling of plasma in the left chamber.

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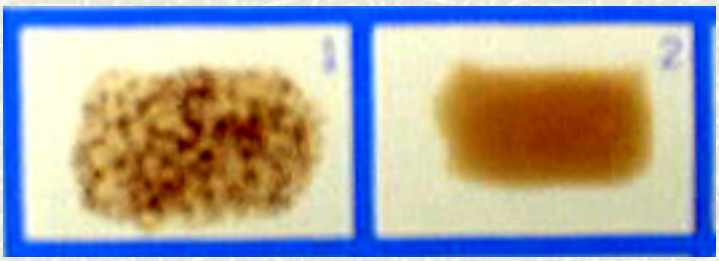

R

Positive (clumping) from *S. aureus* on the left

Negative (homogenous milky suspension) from *S. epidermidis* on the right

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Clostridium perfringens **with double zone of hemolysis on blood agar plate. The arrow to the far left points to the outer zone and the other points to the inner zone**

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Suggestions

***C. sporogenes* with a single zone of hemolysis on blood agar. Observing from the top, one would see small to medium, rhizoid type colonies.**

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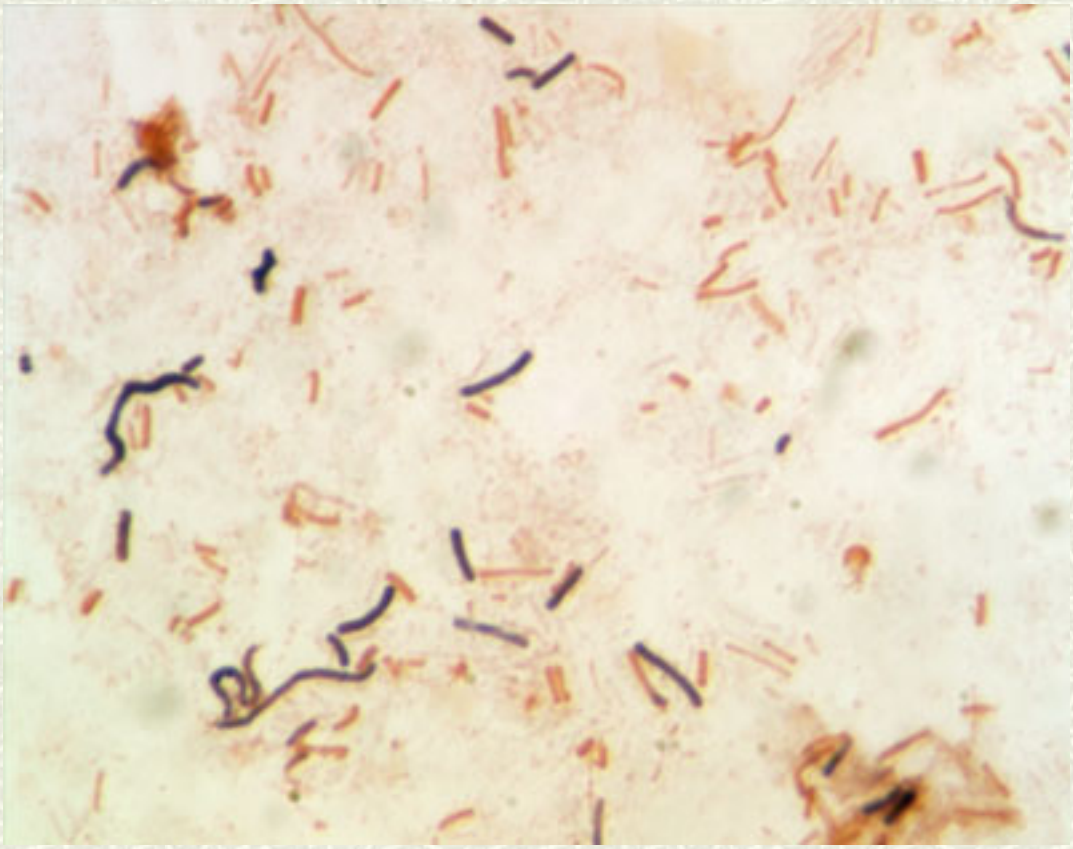

Gram stain of *Clostridia perfringens*

Note presence of (+) and (-) stained bacteria

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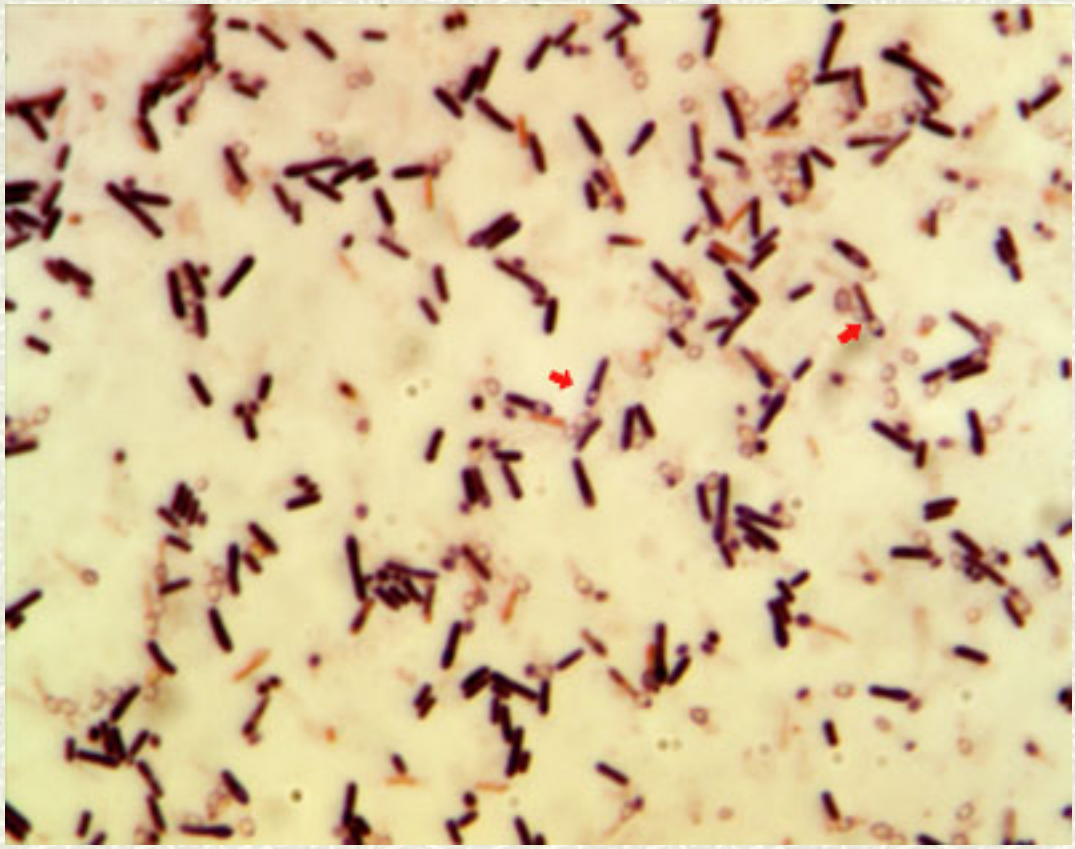

Gram stain of *Clostridia sporogenes*

Red arrows point to spores that appear as unstained areas

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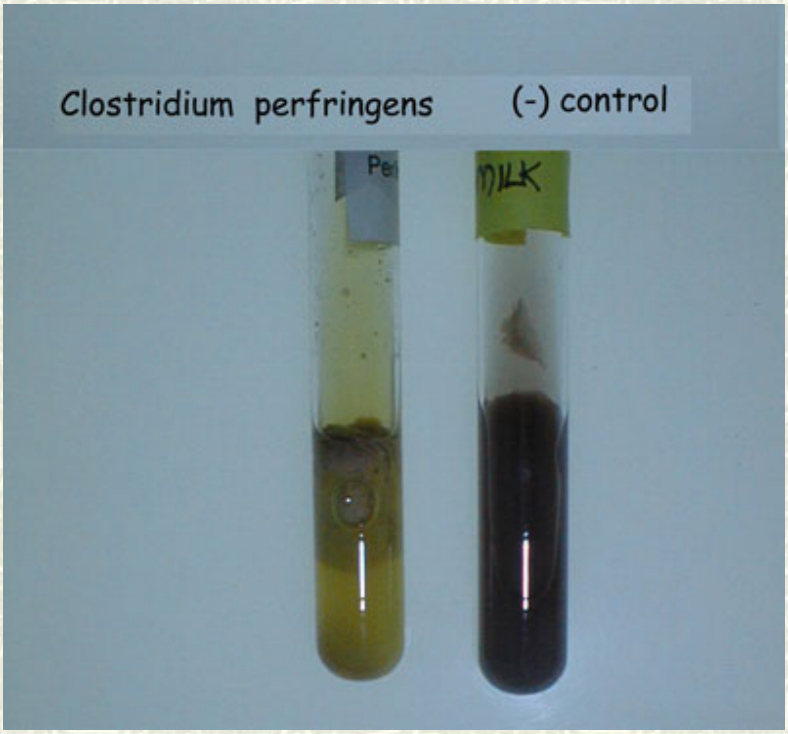

Litmus milk test

Positive reactions are seen in *C prefringens* Fermentation leads to production of acid turning the color of the medium from purple to yellow. The acidity also leads to clotting of the milk and production of gas that breaks the clots

This assay has not been performed in the lab and will not be included in the lab exam

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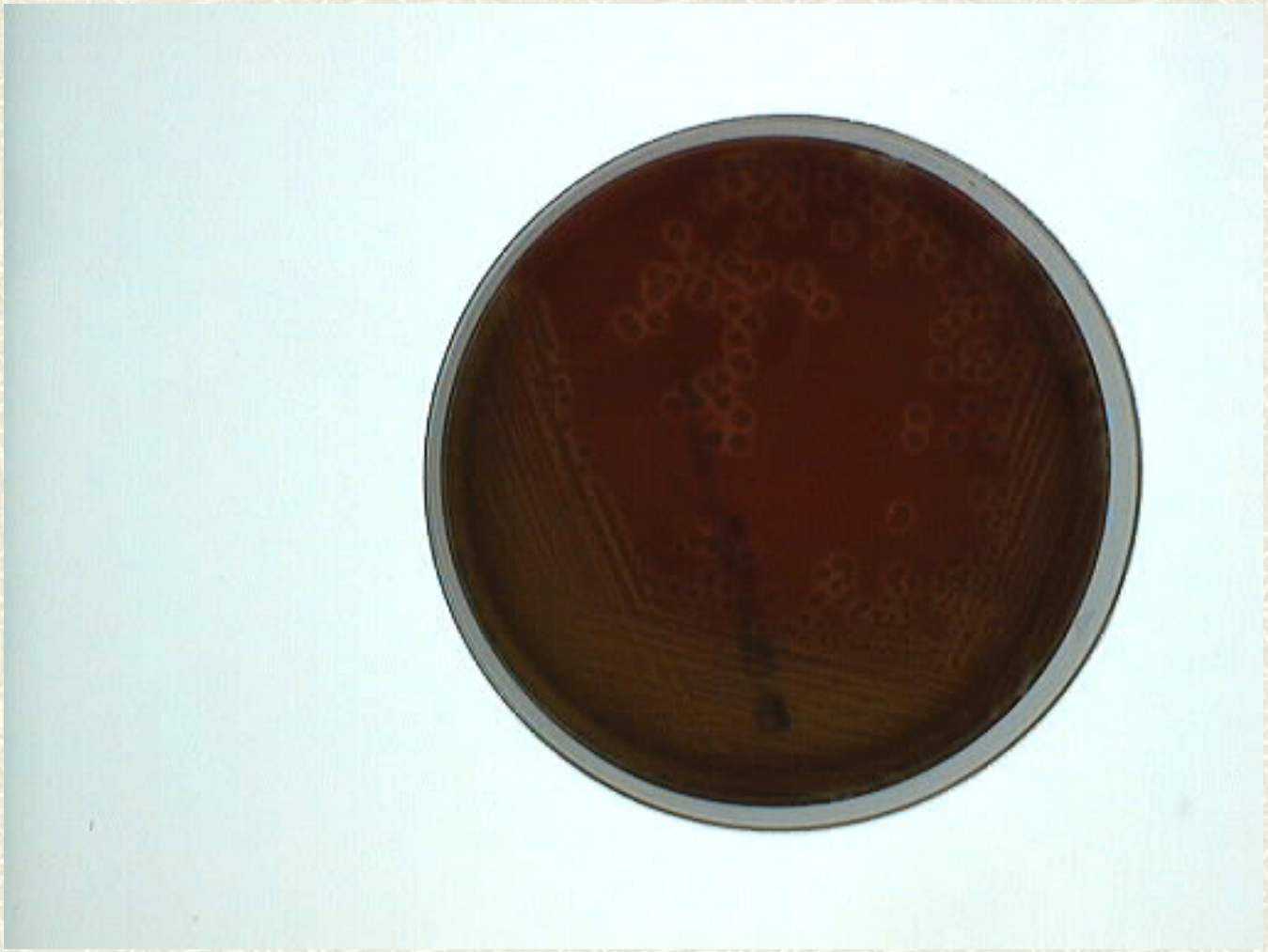

Escherichia coli on blood agar

Note beta-hemolysis

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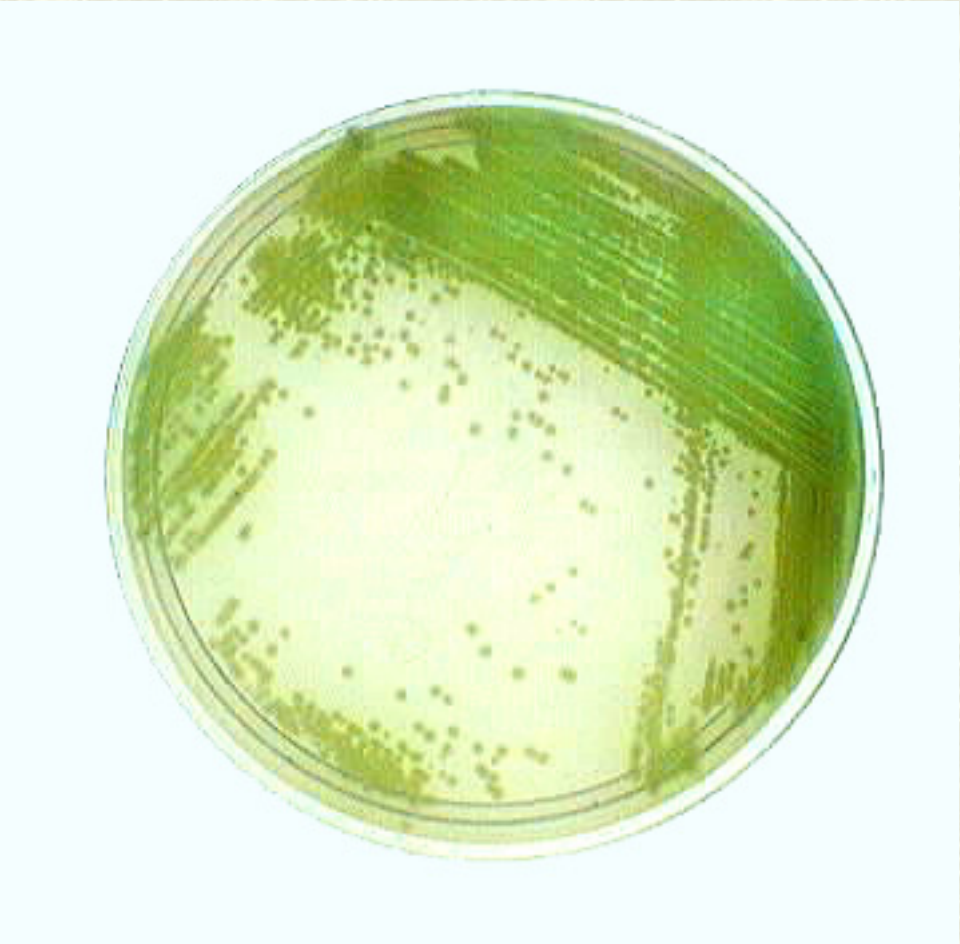
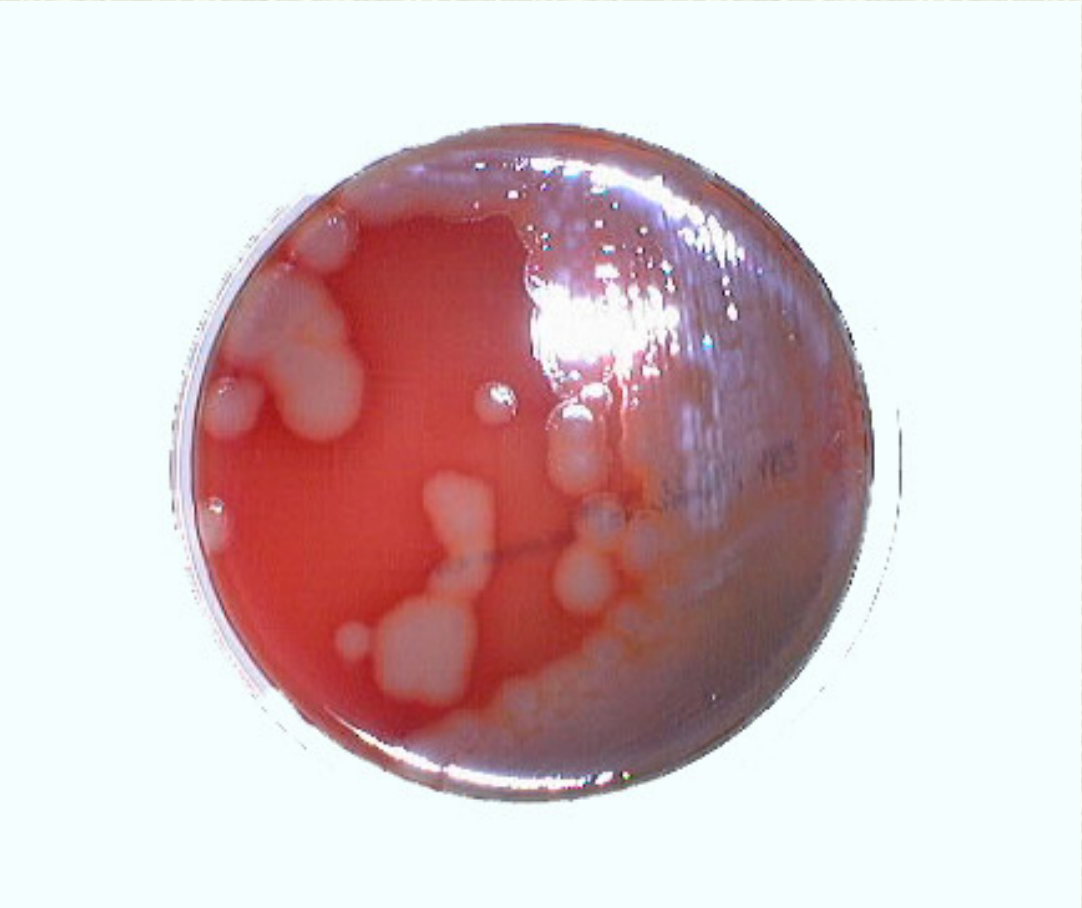


Green colored *Pseudomonas aeruginosa* on M-H agar



Pseudomonas aeruginosa on blood agar

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***Proteus mirabilis* on blood agar plate.**

Notice spreading of the organism from the center where it was inoculated. [The organism does not have to be inoculated in the center to see spreading.]

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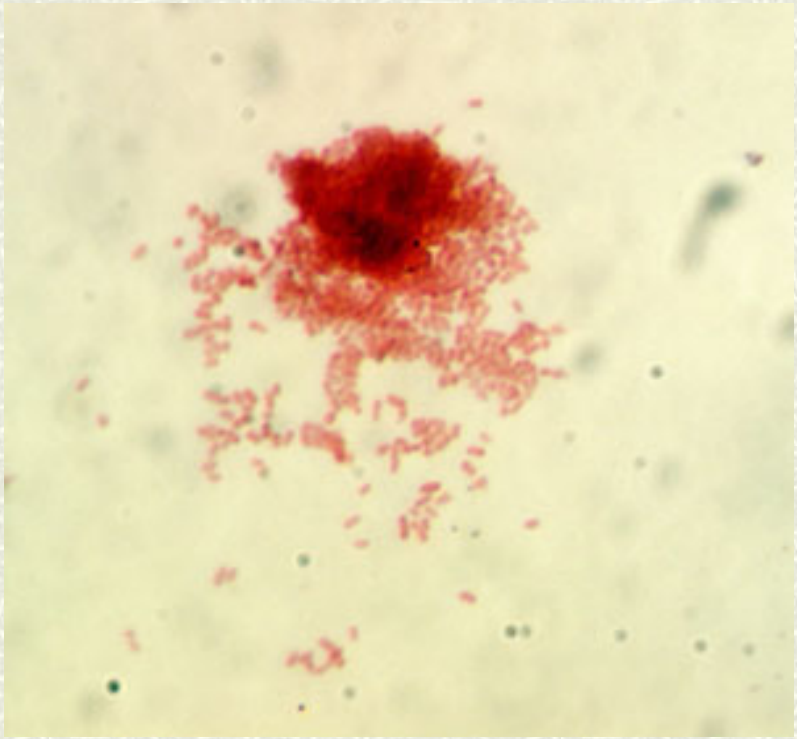
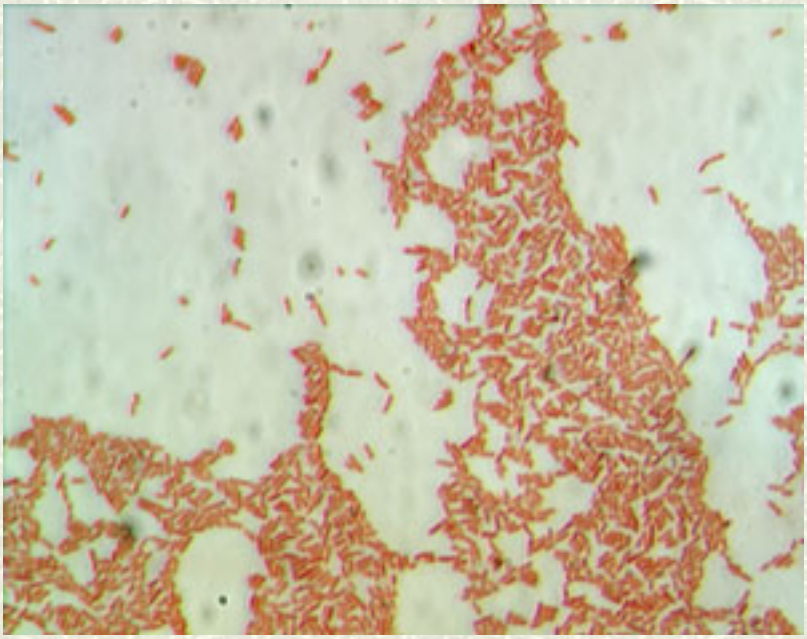
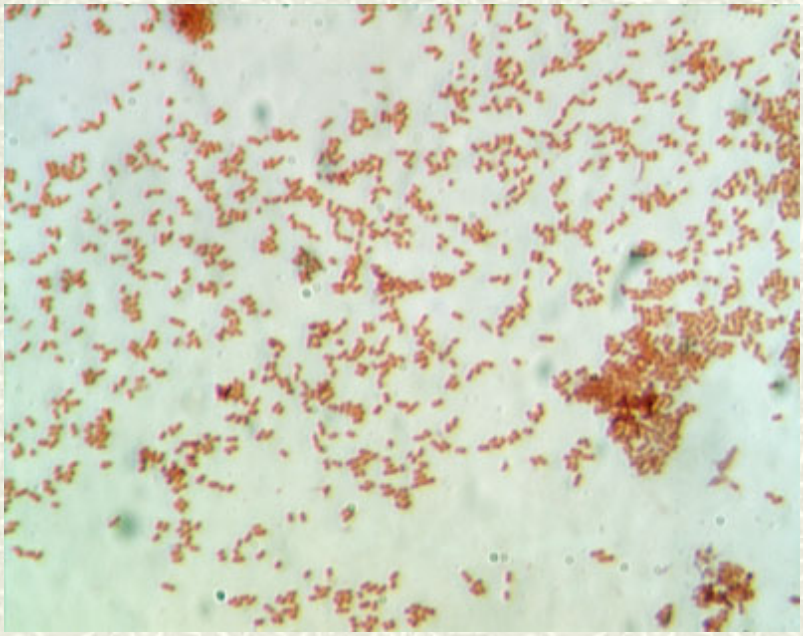


Escherichia coli



Pseudomonas aeruginosa



Proteus mirabilis

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Suggestions

Positive: purple-black

Negative: no color development

Note: Be sure to apply bacteria heavily

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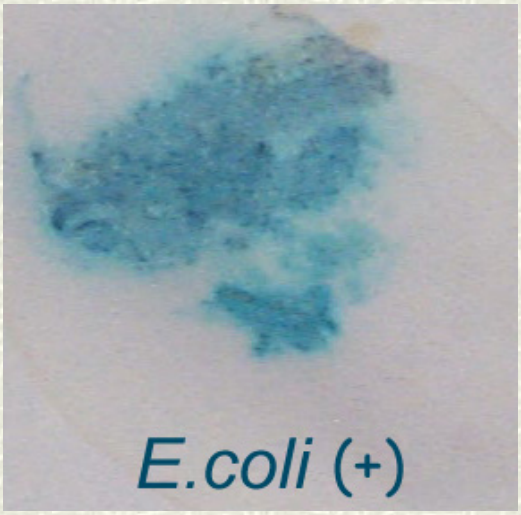

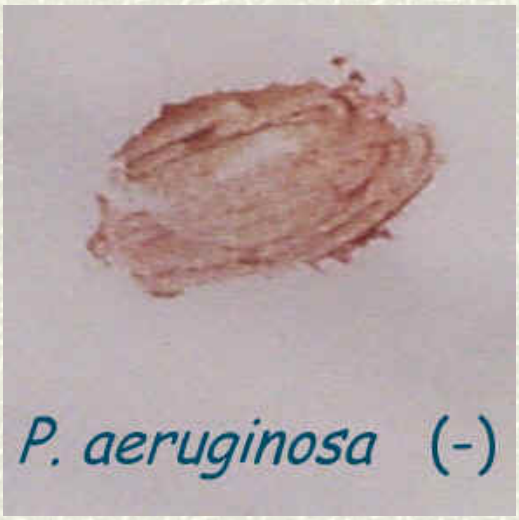

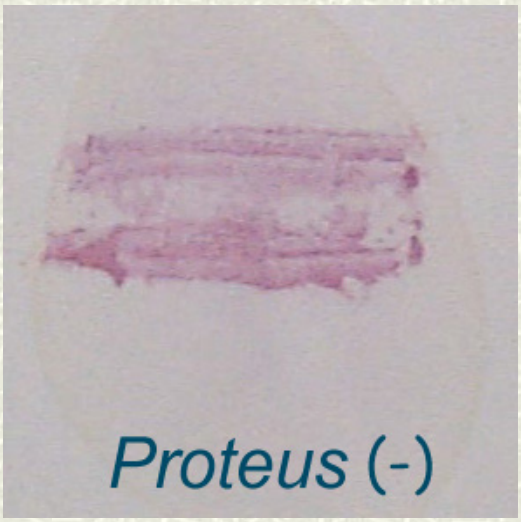

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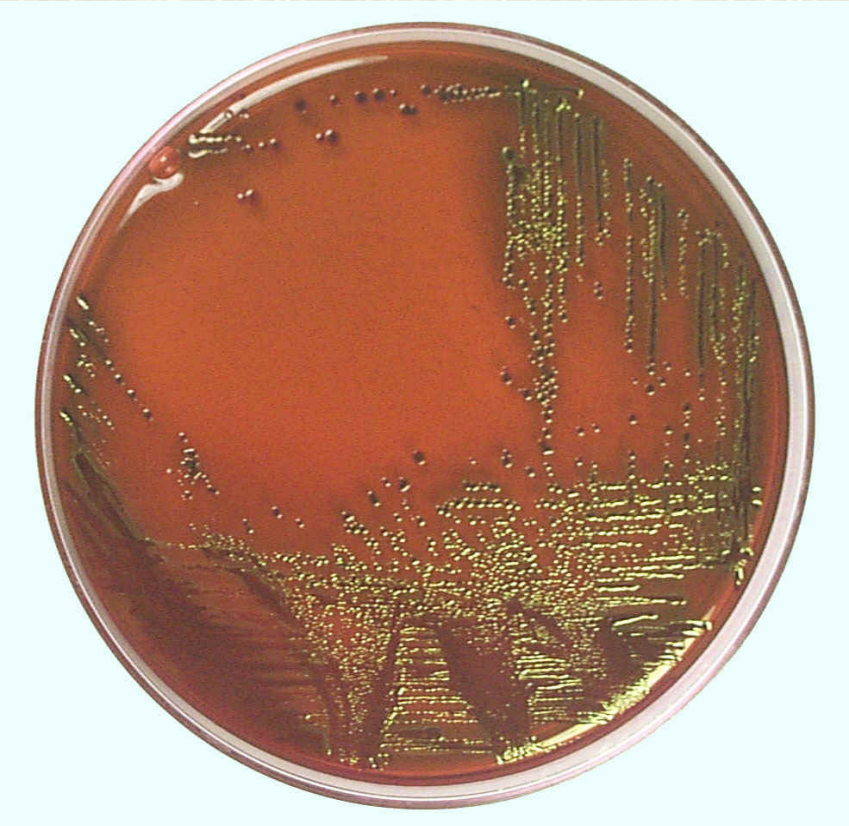

***E. coli*, a strong lactose fermentor, on EMB agar plate**

Note metallic sheen

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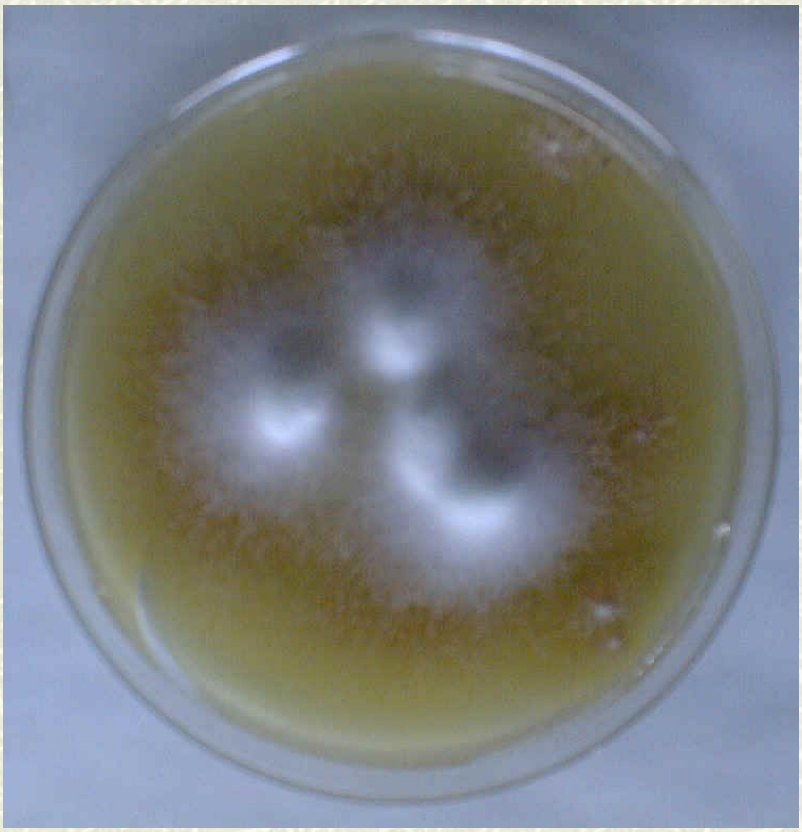
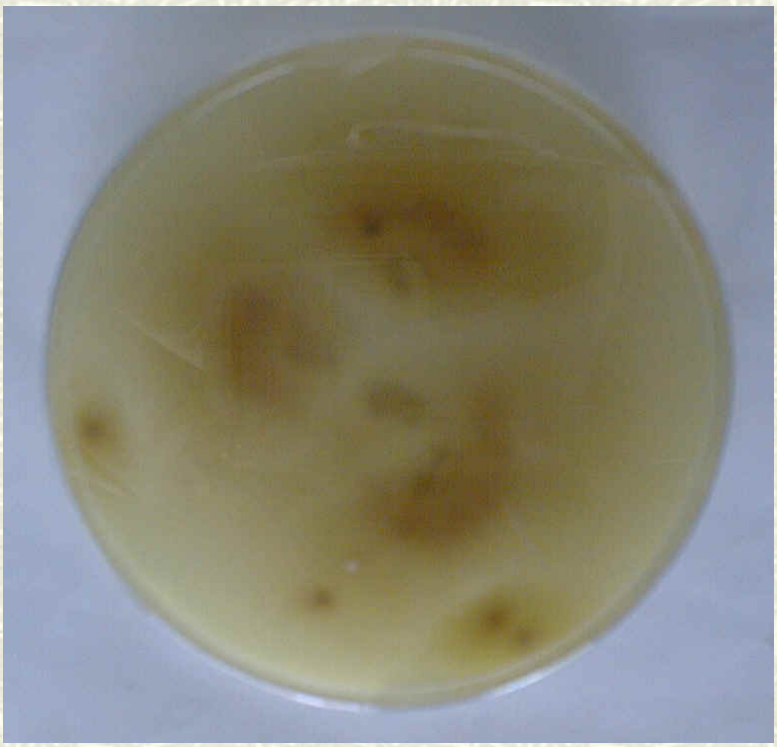
Microsporium gypseum

Colony morphology:



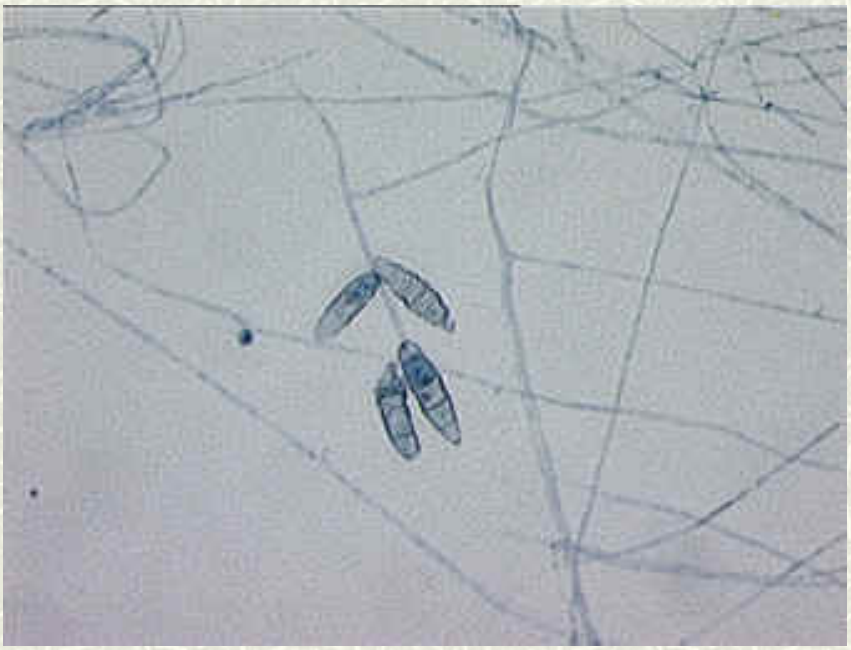
Front: Granular (due to numerous macroconidia)

Often cinnamon colored



Reverse: Light tan

Microscopic morphology:



Macroconidia: Large, thick walled, many (4-5)celled

Spindle-shaped

Rounded tip that do not tend to curve

Microconidia: Few or absent

Hypae: Septated

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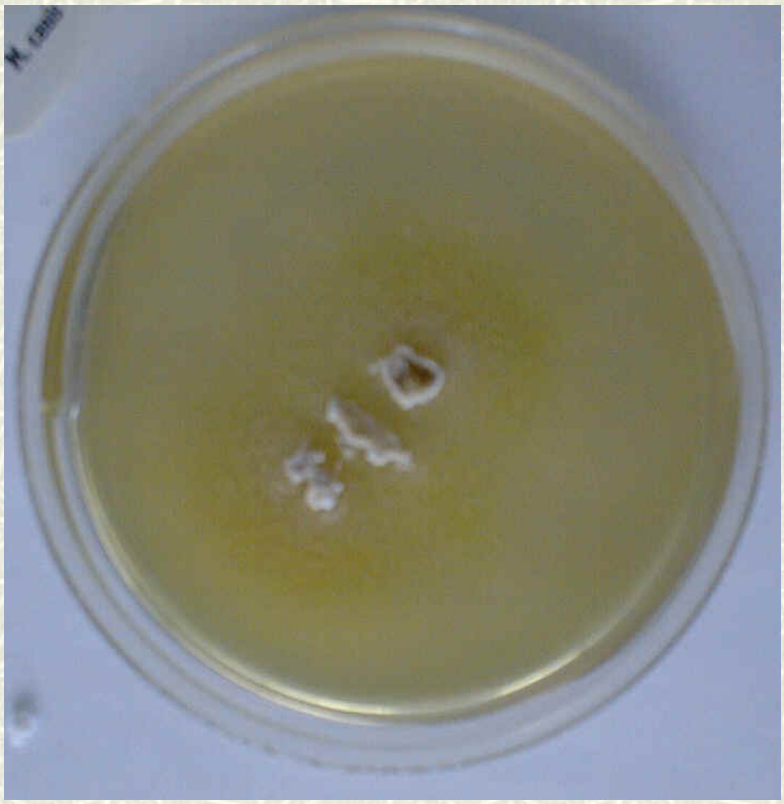
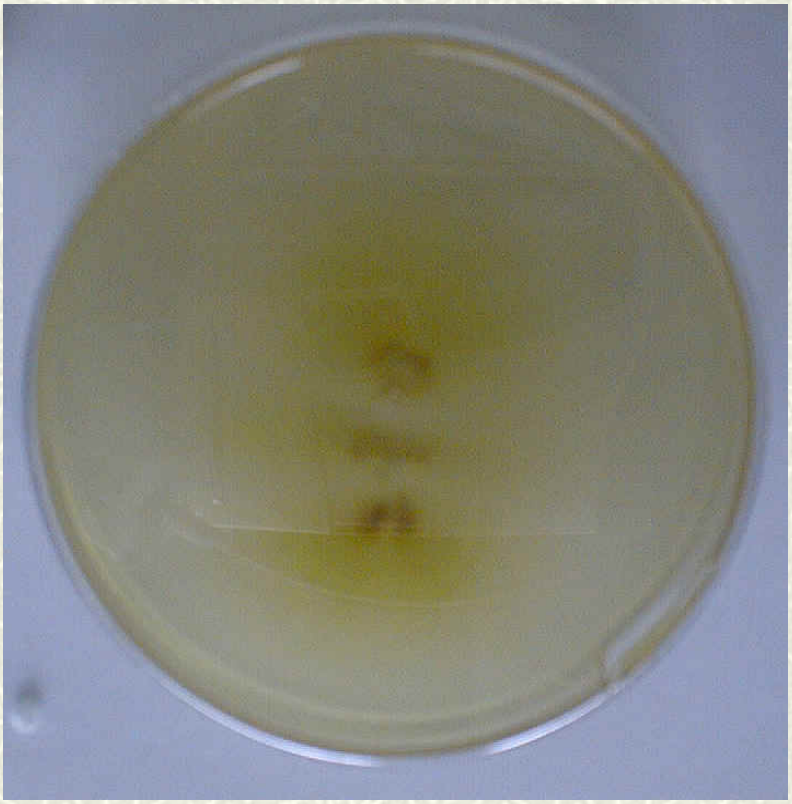
Microsporum canis

Colony morphology:



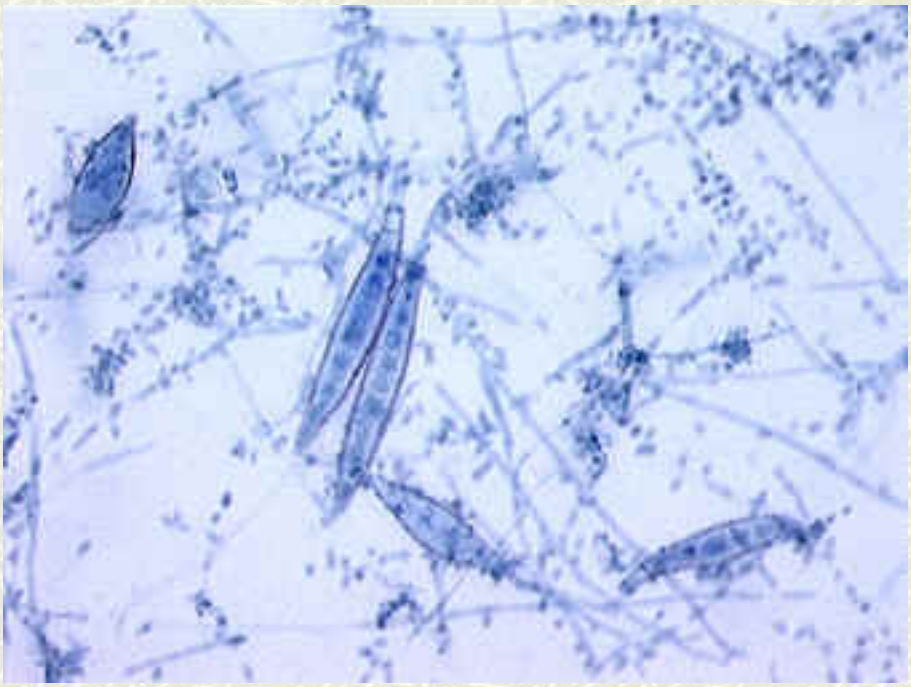
Front: Fluffy white surface



Reverse: Orange or yellow

Lemon yellow apron at peripheral margin

Microscopic morphology



Macroconidia: Large, thick walled,

Many (7-11)celled

Spindle-shaped, curve tip

Microconidia: Few or absent

Hypae: Septated

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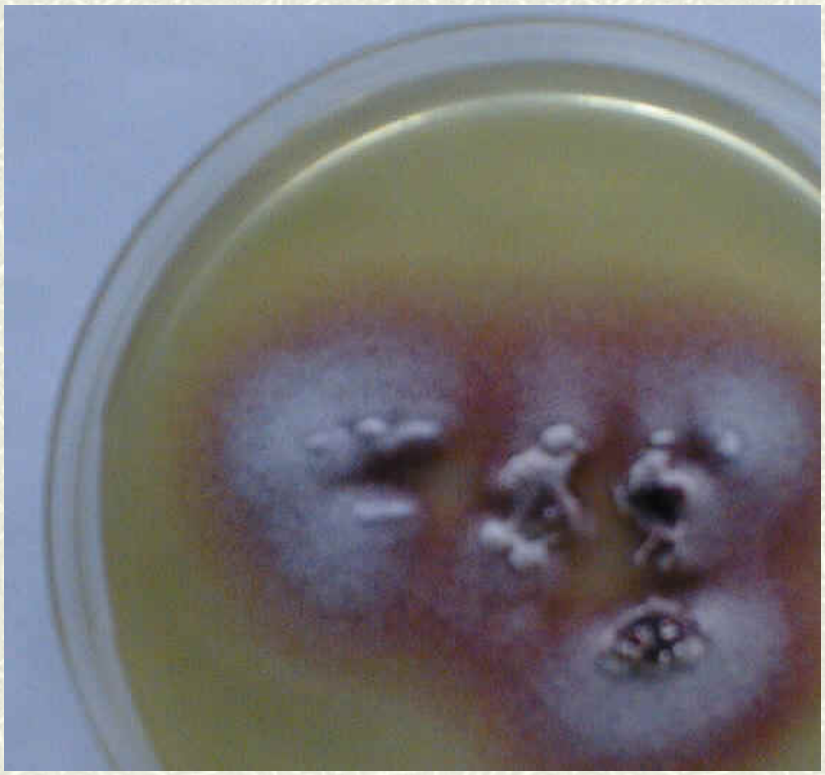
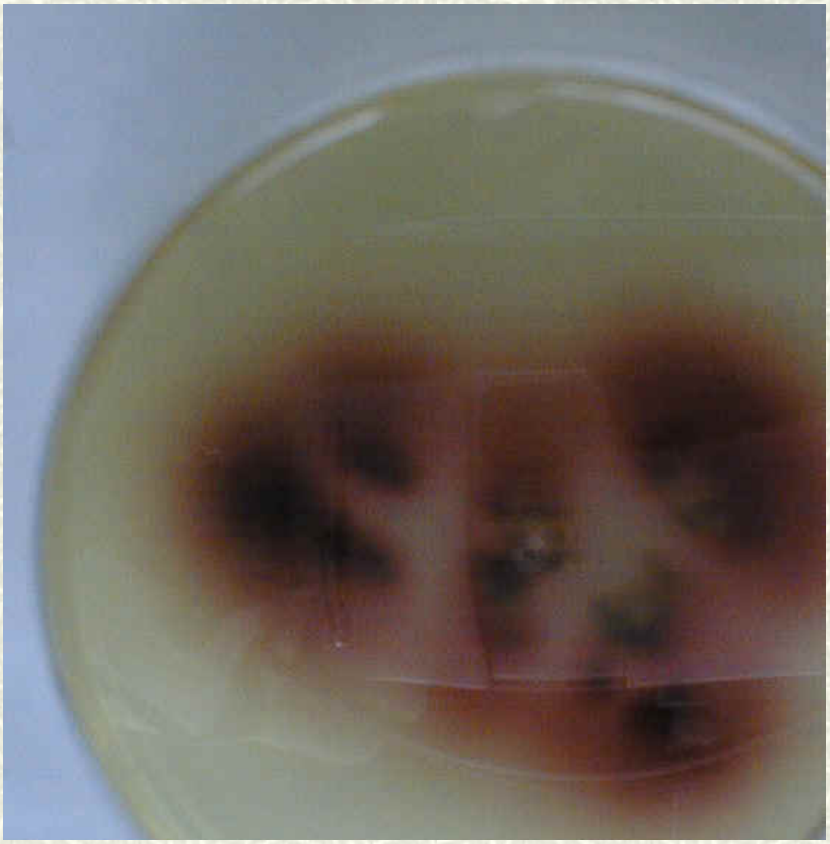
Trichophyton rubrum

Colony morphology:

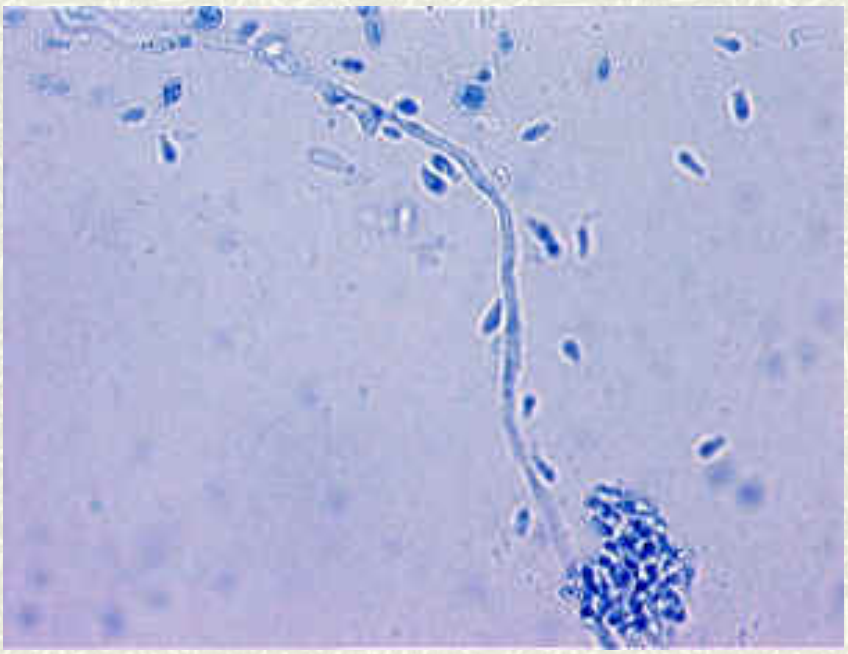


Front: White and downy. May be pink or red



Reverse: wine-red to red yellow

Microscopic morphology:



Macroconidia: usually absent; thin-walled or pencil-shaped

Microconidi: tear shaped produced in profusion,

borne laterally and singularly from the hypae

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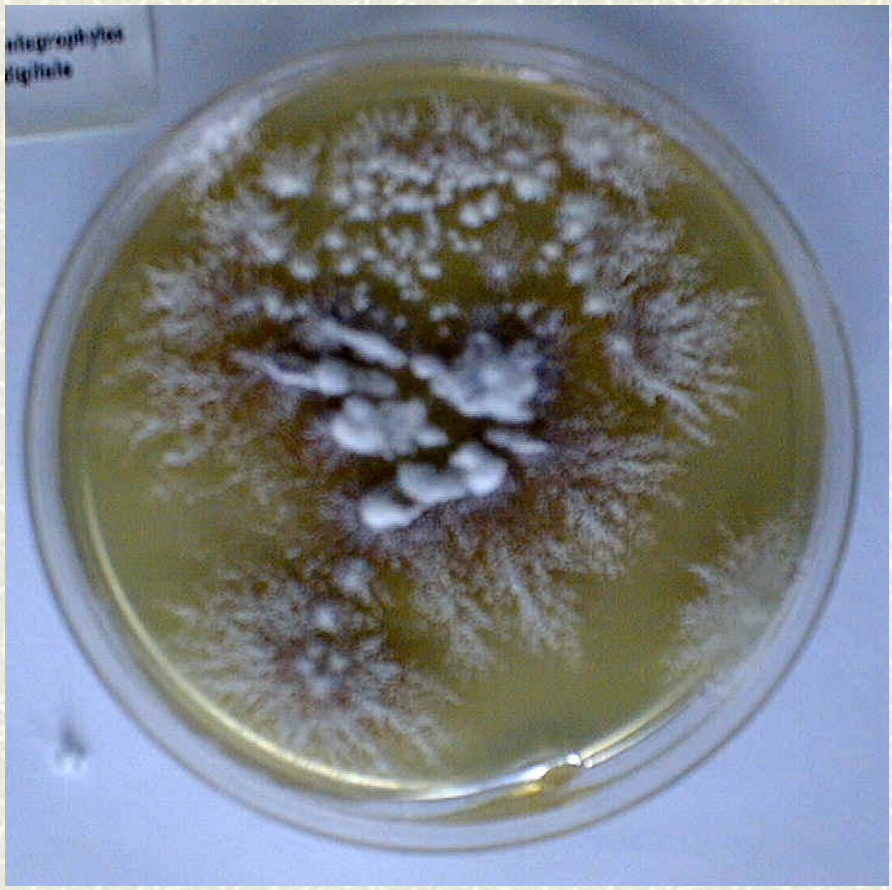
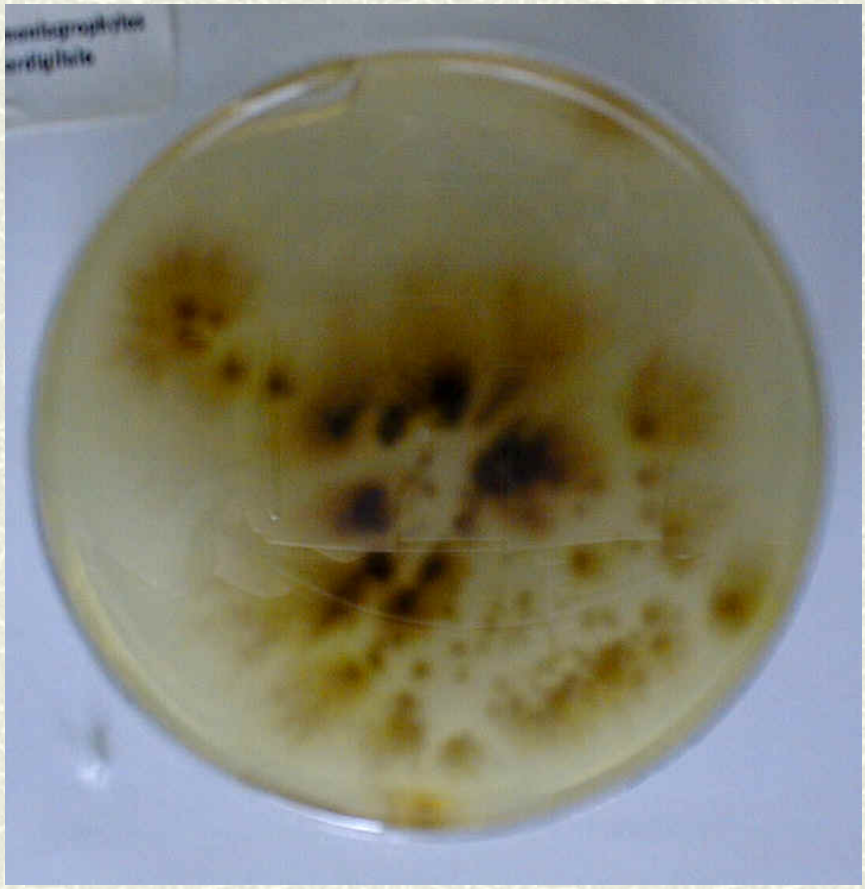
Hit Counter

Trichophyton mentagrophytes

Colony morphology:

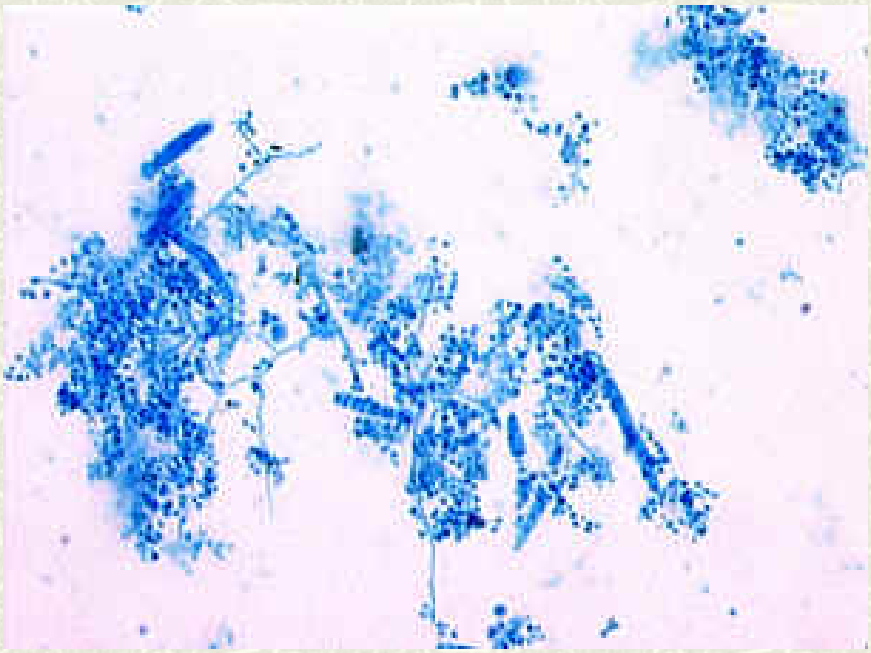


Front: White and fluffy to pink and granular



Reverse: Buff and reddish brown

Microscopic morphology:



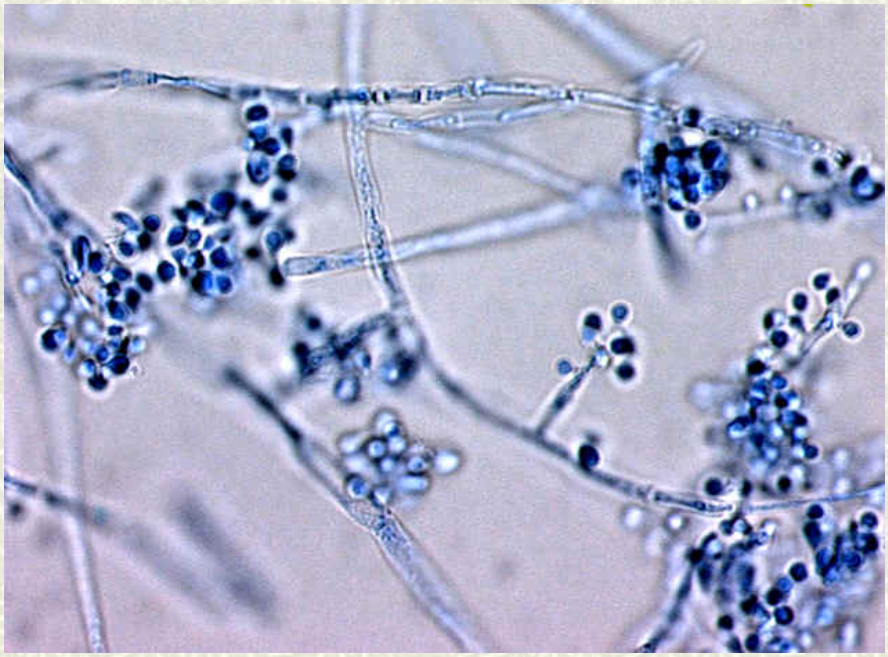
Macroconidia: Thin-walled or pencil-shaped

Usually absent

Microconidi: Many, tear shaped produced

In grape-like clusters

Hyphae: Sometime may see a few spiraled hyphae.



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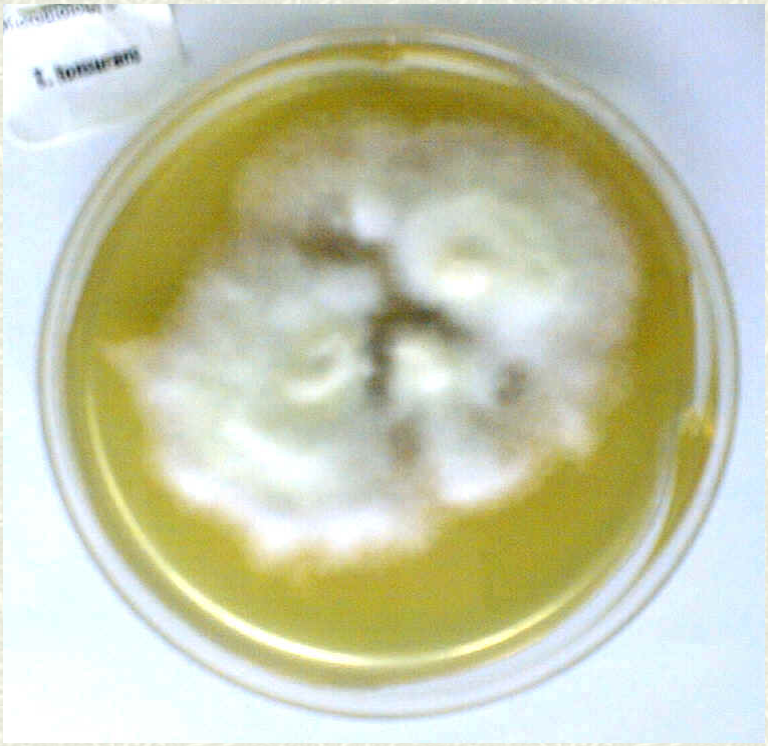
Hit Counter

Trichophyton tonsurans

Colony morphology:

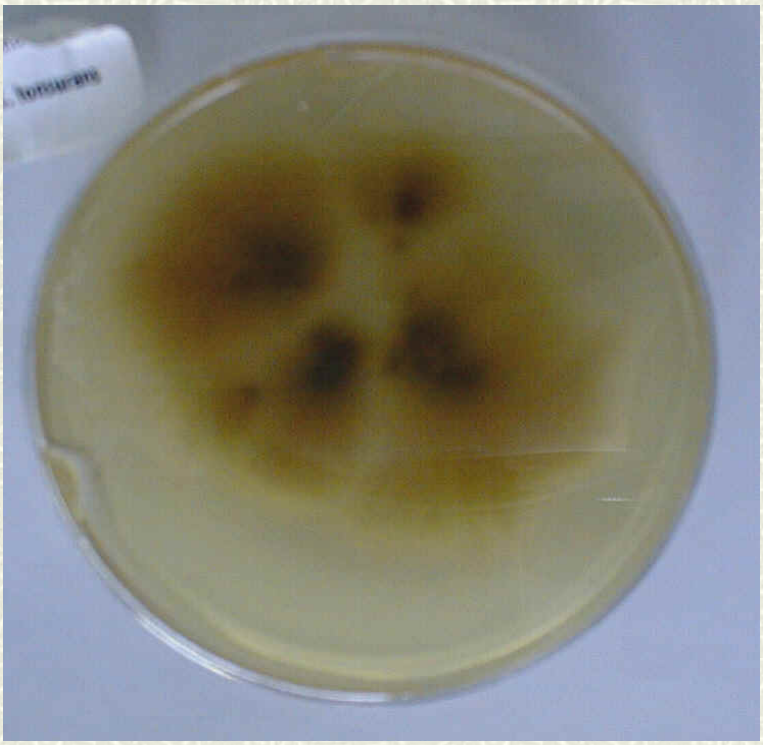


Front: Whitish, tan, brown;

Mycelium is low producing a

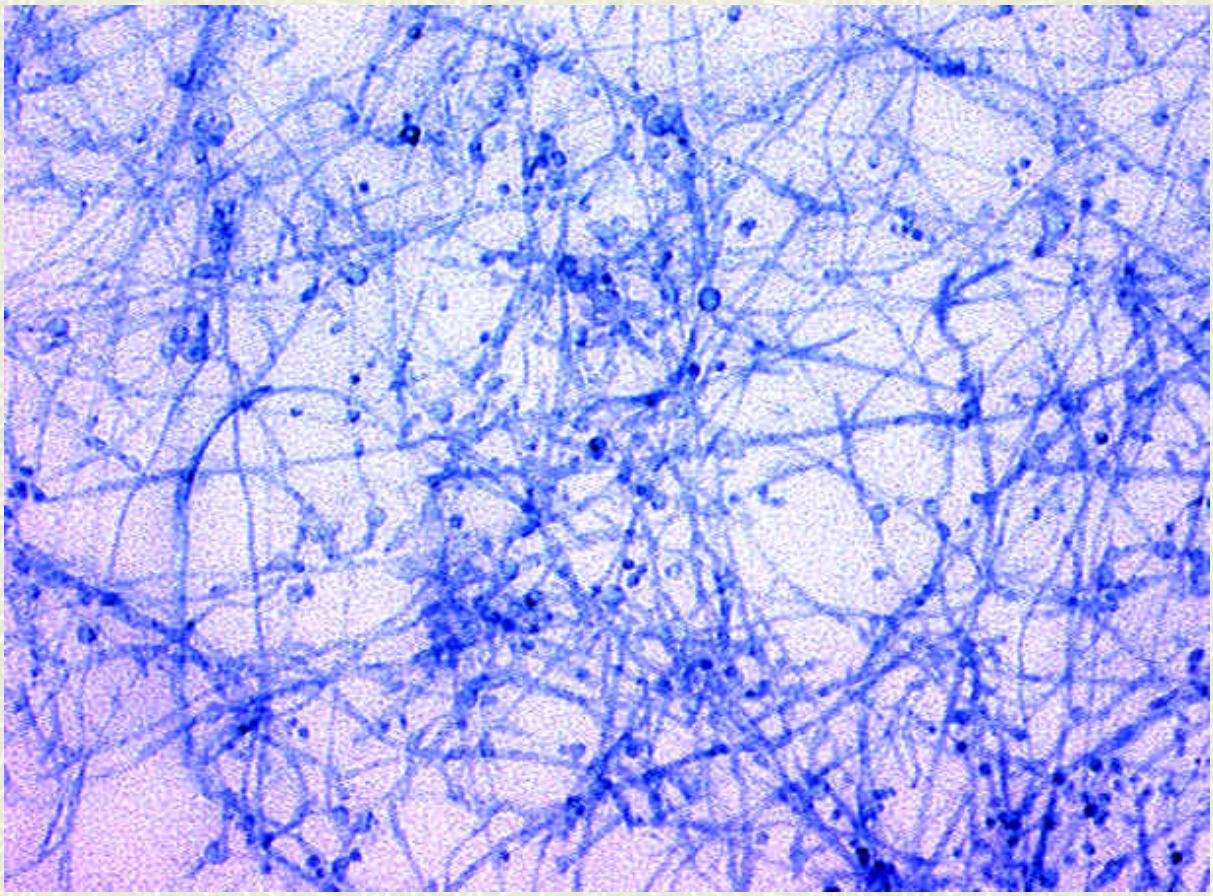
Velvety surface with

Folds and centers are common.



Reverse: yellow to tan

Microscopic morphology:



Macroconidia: Rare

Microconidi: Tear shaped with flat bottom and

Larger than the those in the other species.

Occasionally see ballon forms

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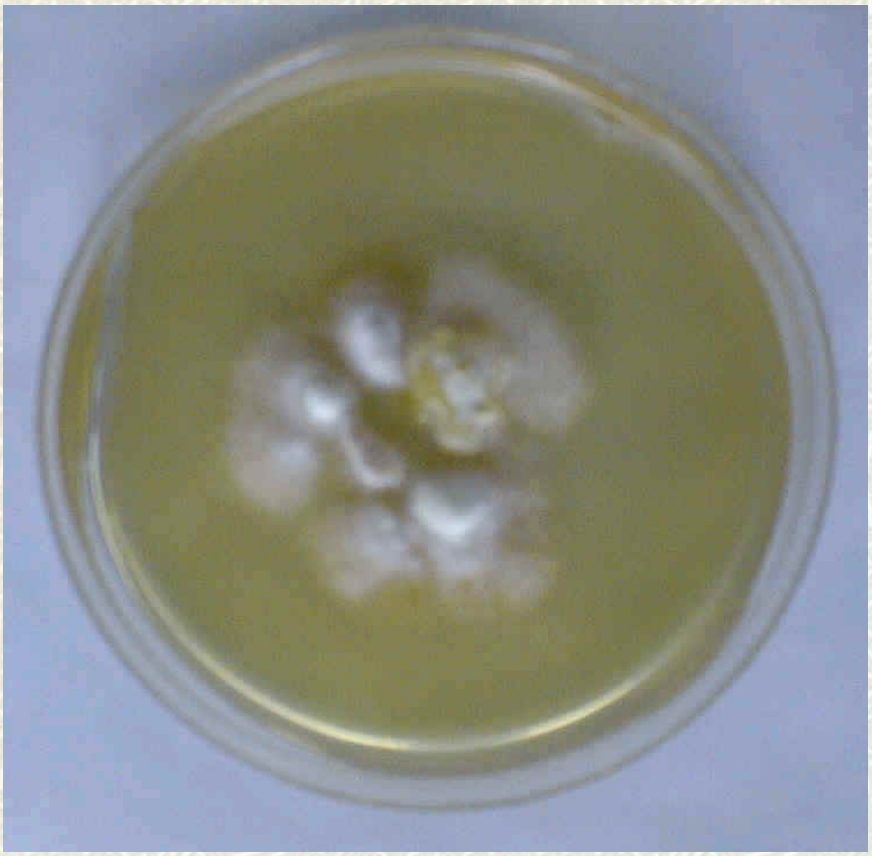
Epidermophyton floccosum

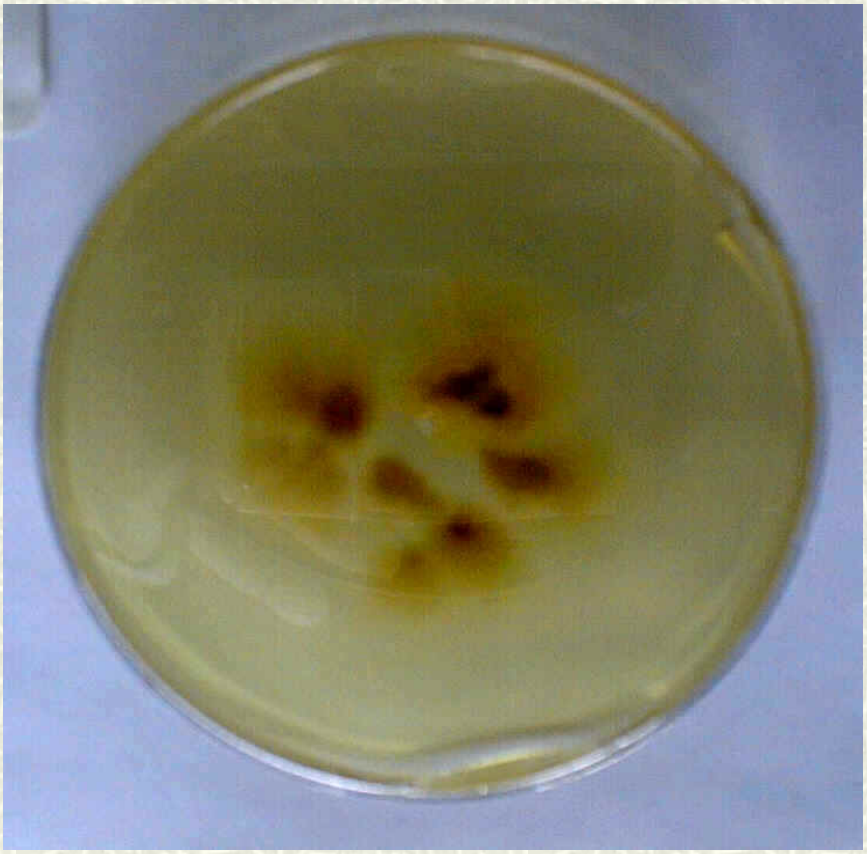
Colony morphology:



Front: White and floccose

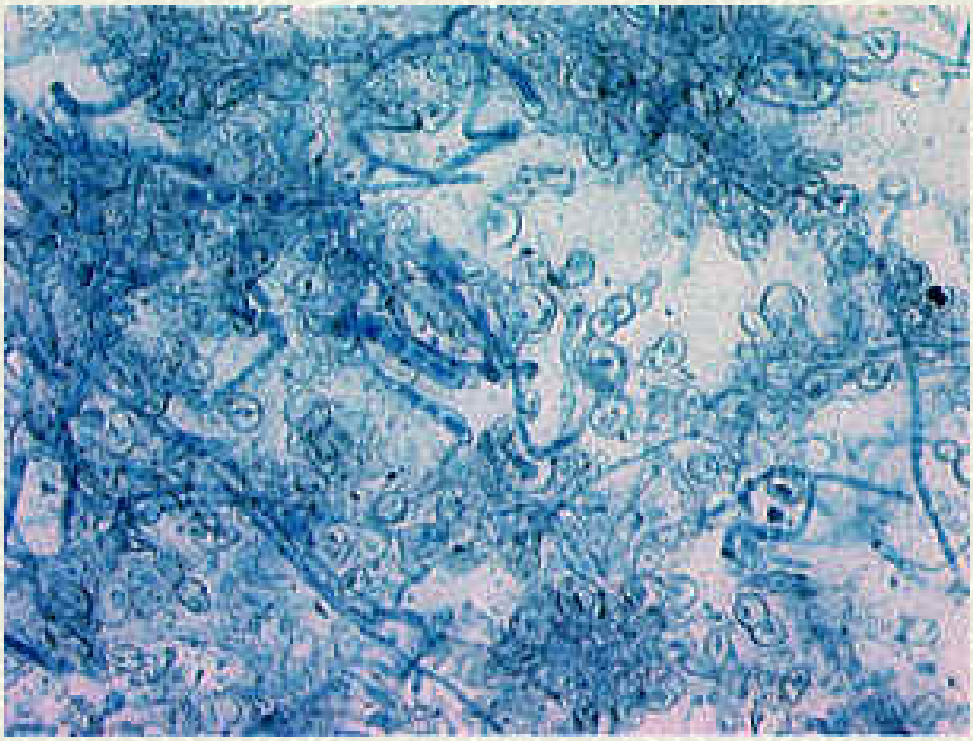
May become greenish with age

Folded center



Reverse: Yellow to brown with folds

Microscopic morphology:



Macroconidia: Thin-walled or pencil-shaped

Usually absent

Microconidi: Many, tear shaped

Produced in grape-like clusters

Hyphae: Sometime may see a few spiraled hyphae

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Dermatophyte test medium

Uninoculated **Dermatophyte:**

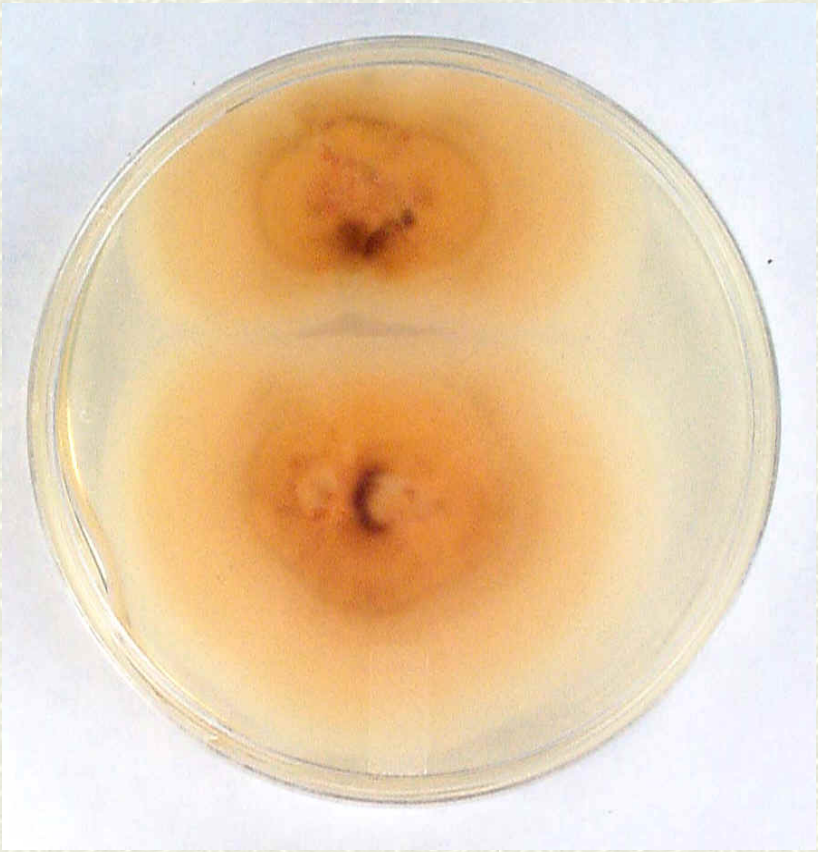
Orange red

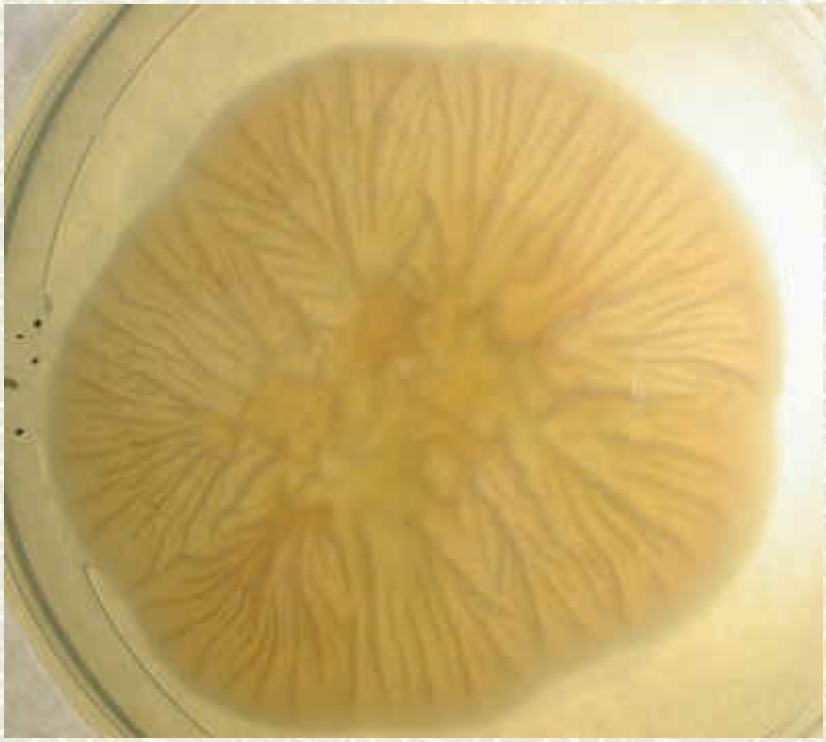
Acremonium (Cephalosporium) species

Colony morphology:

Front: White, pink or gray yellow variants

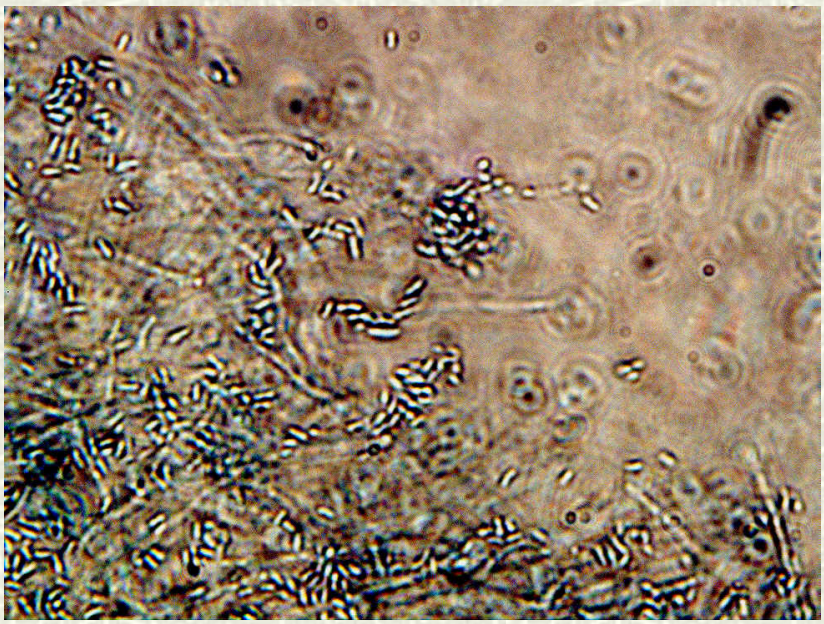
Almost yeast-like because of delicate mycelium





Reverse

Microscopic morphology:



Long, delicate, hair like conidiophores

One cell elliptical conidia in irregular clusters

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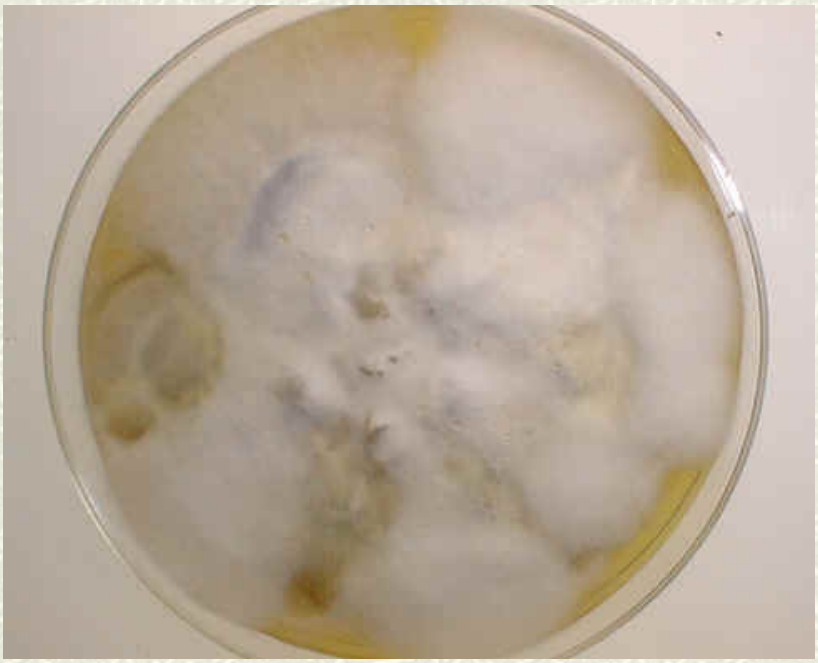
Fusarium species

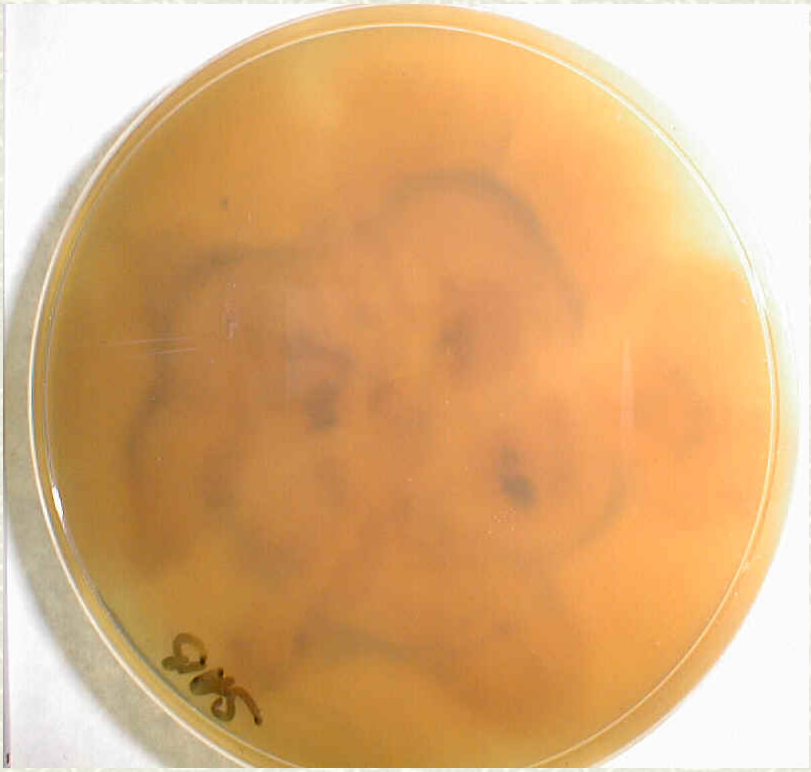
Colony morphology

Front: Yellow, pink or purple variants

Cotto-like, woolly or granular



Back:



Microscopic morphology

Microconidia are sickleform shaped macroconidia

Delicate conidiosphores

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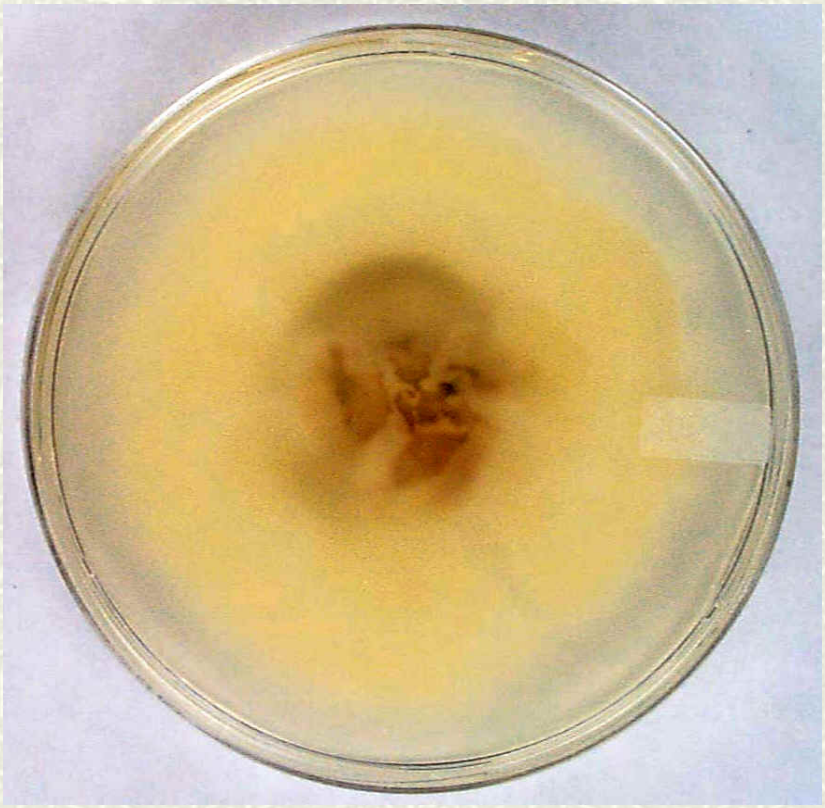
Pseudallescheria boydii

Colony morphology:

Front: Grayish

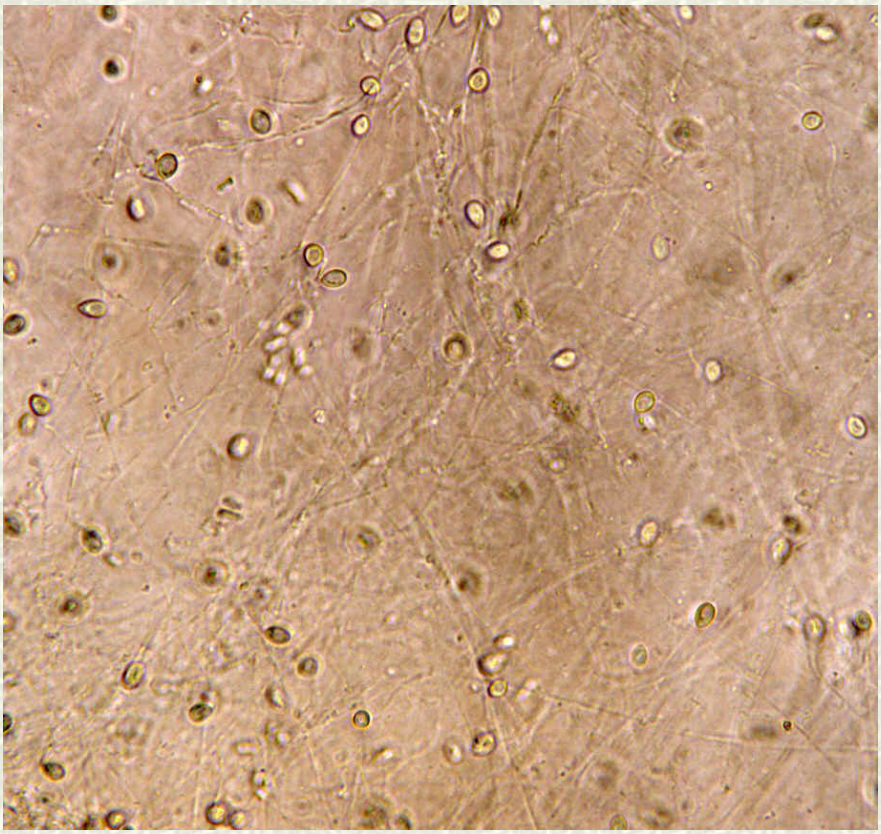
Back: Dark-brown or brown black



Microscopic morphology:

Oval, single-celled conidia

Clustered on terminal of conidiophores.



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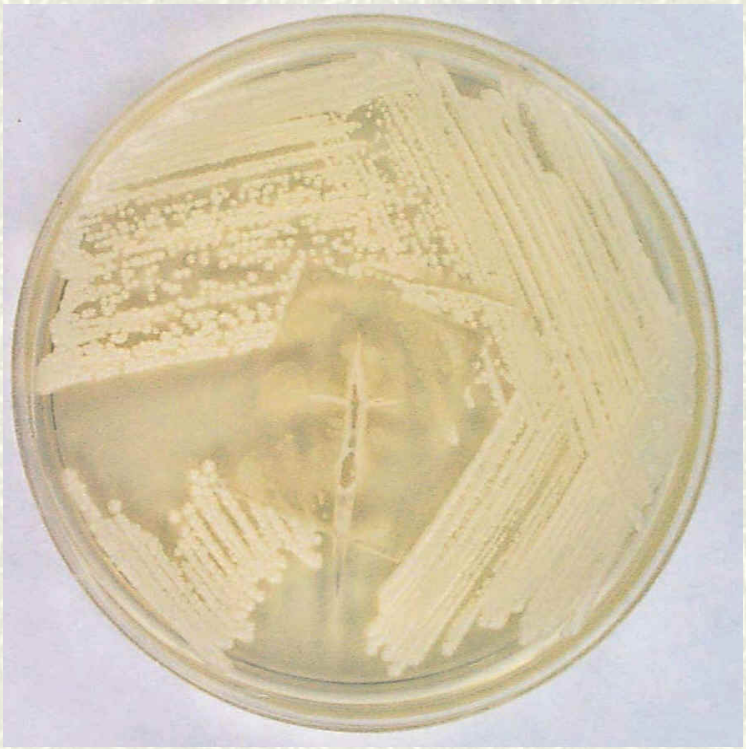
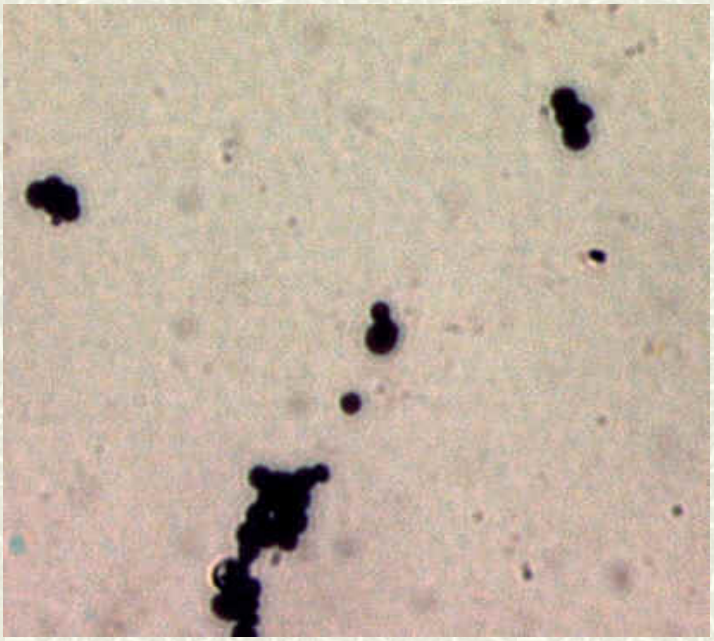
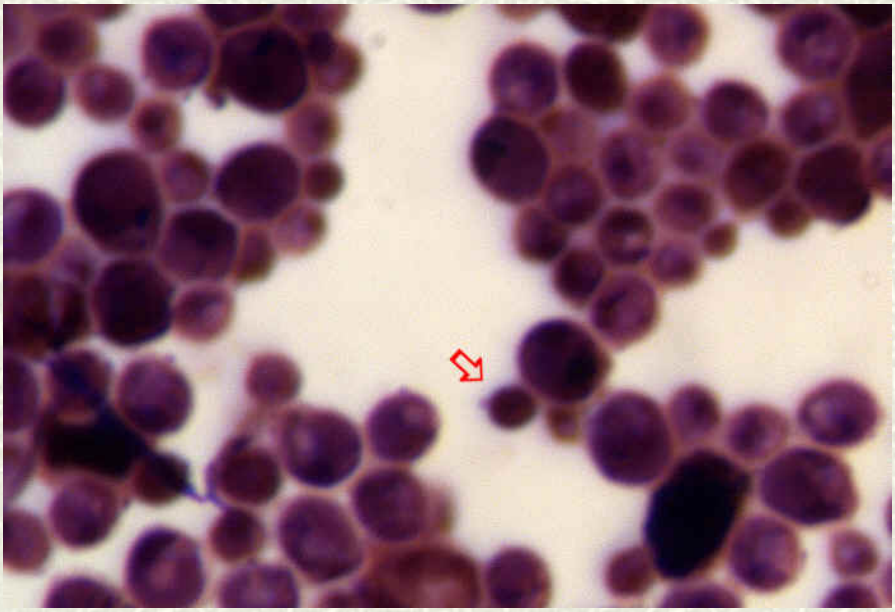
Candida albicans on **Sabouraud dextrose agar**



Gram stained (400X magnification)



Gram positive, oval shaped, with budding (allow)



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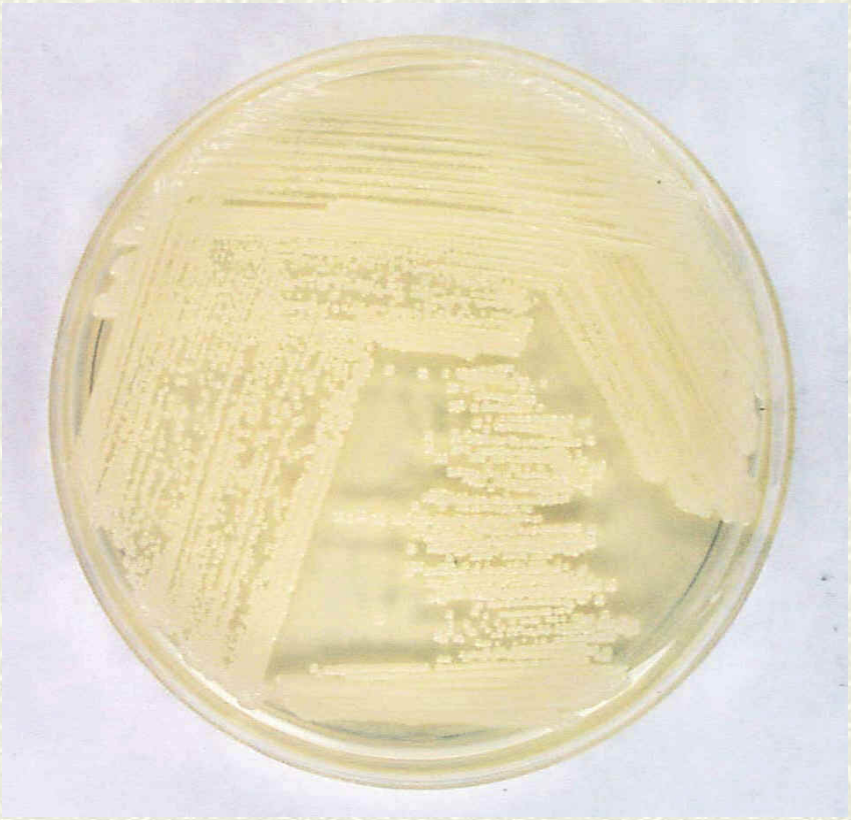
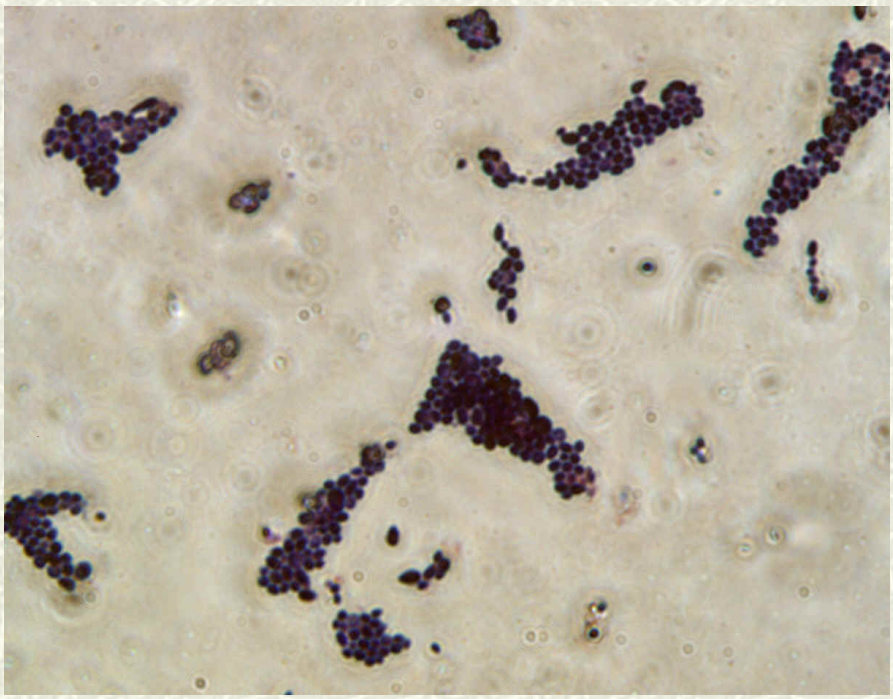
Candida parapsilosis on **Sabouraud dextrose agar**



Gram stained



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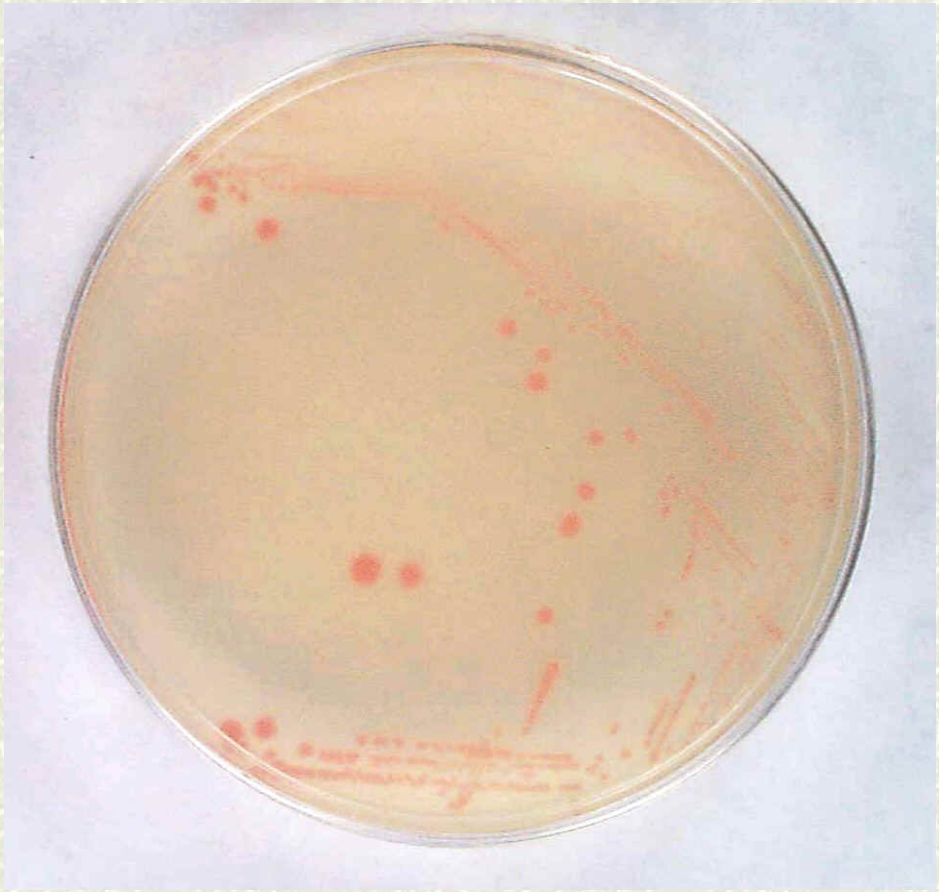
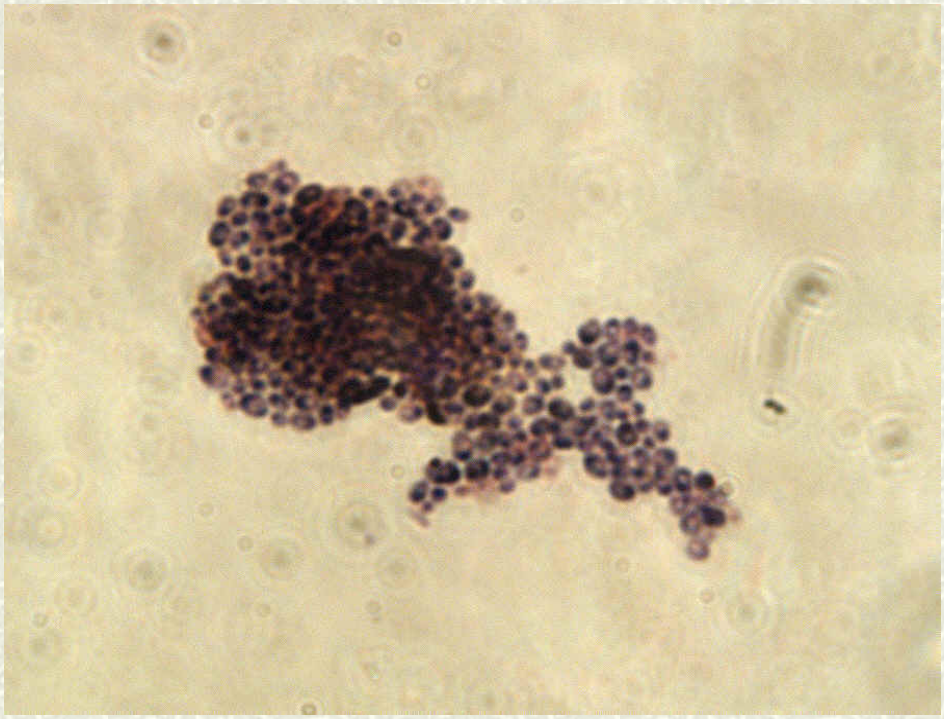
Rhodotorula rubra on Sabouraud dextrose agar



Gram stain



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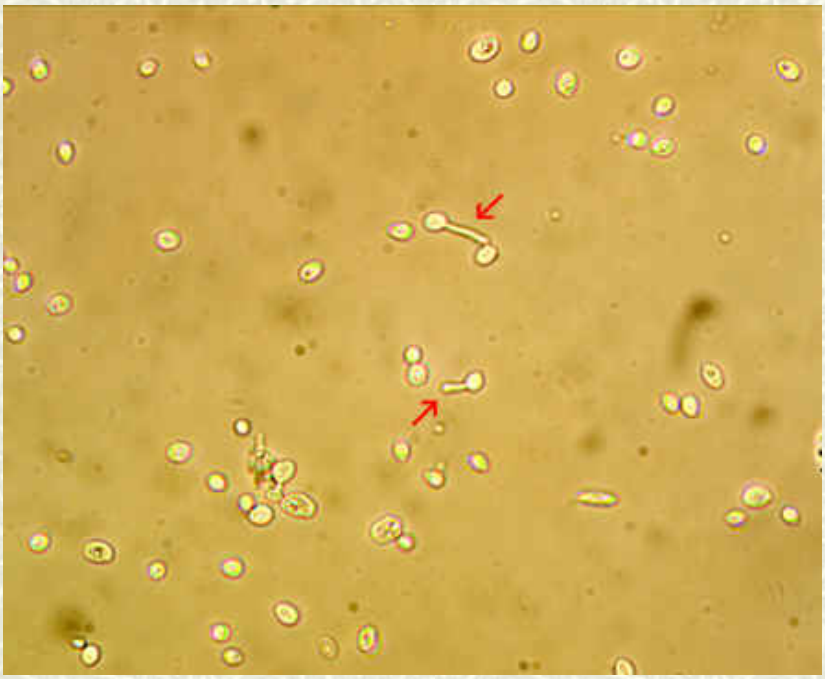
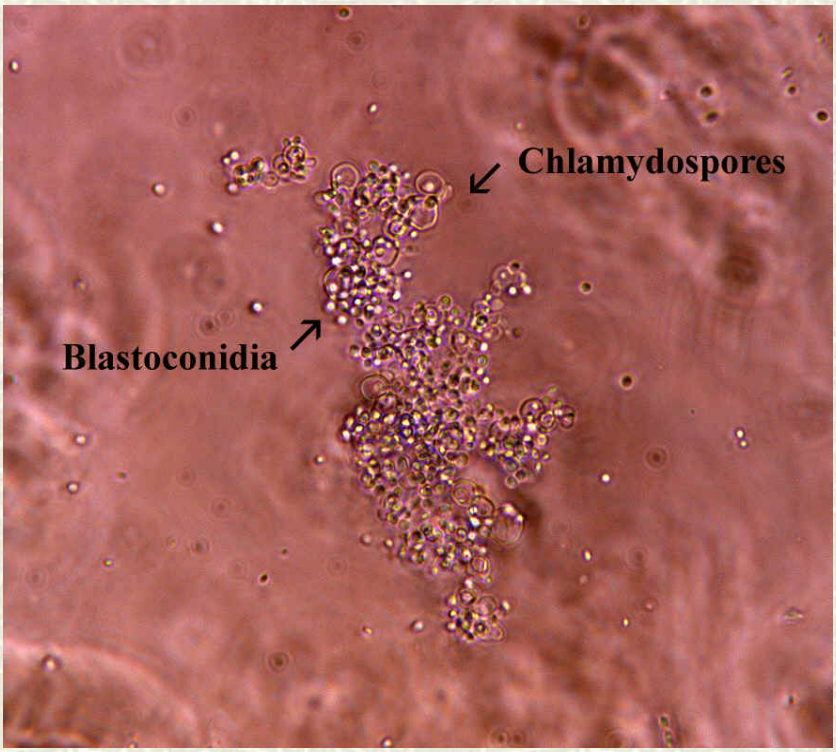
Germ tube in sheep serum



Chlamydospores in corn meal agar culture

LPCB mount



Gram stain