Practical No. 8

Antibiotic susceptibility testing

Antibiotic sensitivity is a term used to describe the susceptibility of bacteria to antibiotics. Antibiotic susceptibility testing (AST) is usually carried out to determine which antibiotic will be most successful in treating a bacterial infection *in vivo*. Some antibiotics actually kill the bacteria (bactericidal), whereas others merely prevent the bacteria from multiplying (bacteriostatic).
Testing for antibiotic sensitivity is often done by;

1. **Diffusion methods**

Kirby-Bauer method or disk diffusion antibiotic sensitivity testing. Small filter paper disks containing antibiotics are placed onto a plate upon which bacteria are growing. The antibiotic diffuses from the disk into the agar . If the bacteria are sensitive to the antibiotic, a clear ring, or zone of inhibition, is seen around the disk indicating poor growth. Using special comparators that interpret the diameter of the zones of inhibition, consequently the organism can be described as resistant, intermediate, or sensitive. Tables are used to determine the breakpoint for each drug.

The zone of inhibition around the antibiotic disk is influenced by the: (1) type of growth medium, (2) its cation content, (3) the antimicrobial concentration in the disk, (4) inoculum concentration, (5) temperature of incubation, and (6) atmosphere of incubation .

Antibiotic disk may be single disks or strips of multiple antibiotic disks.

Other methods to test antimicrobial susceptibility include the E-test also based on antibiotic diffusion.

The Epsilometer test (usually abbreviated Etest) is a laboratory test used to determine whether or not a bacterium is susceptible to an antibiotic. The Etest is basically an agar diffusion method. The Etest utilises a rectangular strip that has been impregnated with the drug to be studied. A lawn of bacteria is inoculated onto the surface an agar plate and the Etest strip is laid on top; the drug diffuses out into the agar, producing an exponential gradient of the drug to be tested. There is an exponential scale printed on the strip. After 24 hours of incubation, an elliptical zone of inhibition is produced and the point at which the ellipse meets the strip gives a reading for the (MIC) of the drug.

Agar disk diffusion method procedure;

1- from a prepared bacterial suspension, dip a swab and seed the surface of an agar plate with the swab then rotate the plate through a 45º angle and streak the whole surface again, then rotate the plate another 90º and streak once more. Discard the swab in disinfectant.
2- Dip the tips of a forceps in 70% alcohol, flame rapidly and allow cooling.
3- Pick up an antibiotic disc with the forceps and place it on the agar surface, press the disk gently using the tips of the forceps.
4- Repeat with eight different antibiotic disks; make sure they are separated evenly from each other.
5- Invert plates and incubate at 37ºC overnight.
6- Using a ruler measure the diameter of any zones of inhibition and record your results, the results must be compared with values listed in standard charts as shown in the interpretative chart below

Antibiotic	Disk concentration	Diameter of zone of inhibition		
		Resistant	Intermediate	Susceptible
ampicillin	10 microgram	11 or less	12-13	14 or more
cephalothin	30 microgram	14 or less	15-17	18 or more
chloramphenicol	30 microgram	12 or less	13-17	18 or more
gentamicin	10 microgram	12 or less	13-14	15 or more
penicillin	10 U	20 or less	21-28	29 or more
Polymyxin B	300 U	8 or less	8-11	12 or more
sulphonamide	300 microgram	12 or less	13-16	17 or more
tetracycline	30 microgram	14 or less	15-18	19 or more

- ❖ Antibiotics maybe also placed in wells made in the agar medium by a cork borer.
- ❖ Or antibiotics may be incorporated with the melted agar and poured together in Petri dishes, in this case each dish will contain only on antibiotic.

when two antimicrobial agents act at the same time on the same microbial population, the effect may be either
indifference. 1+1= 1
addition. 1+1=2
synergism. 1+1= 3
antagonism 1+1= 1/2

2. <u>Dilution methods for Minimum Inhibitory Concentration determination.(MIC)</u>.

The most commonly employed methods are the tube dilution method and agar dilution methods. Agar dilution methods are the same as tube dilution methods except dilutions are plated on agar.

The tube dilution test is the standard method for determining levels of microbial resistance to an antimicrobial agent. Serial dilutions of the test agent are made in a liquid microbial growth medium which is inoculated with

a standardized number of organisms and incubated for a prescribed time. The lowest concentration (highest dilution) of test agent preventing appearance of turbidity (growth) is considered to be the minimal inhibitory concentration (MIC). At this dilution the test agent is bacteriostatic.

The minimal bactericidal concentration (MBC) or the minimum lethal concentration (MLC) of an antibacterial which is defined as the maximum dilution of the product that will kill a test organism can be determined by subculturing last clear MIC tube onto growth medium that does not contain antibiotic, and examining for bacterial growth. The antibiotic in the subculture diffuses into the agar, releasing any inhibitory effect it may have had on any living bacteria in the subculture. The MBC is identified as the smallest concentration of antibiotic that prevents any growth of the test bacterium (i.e., kills). MLC cannot be done without testing for MIC.

Procedure of (MIC):

1. Number sterile capped test tubes 1 through 9. All of the following steps are carried out using aseptic technique.
2. Add 2.0 ml of tetracycline solution (100 ug/ml) to the first tube. Add 1.0 ml of sterile broth to all other tubes.
3. Transfer 1.0 ml from the first tube to the second tube.
4. Using a separate pipette, mix the contents of this tube and transfer 1.0 ml to the third tube.
5. Continue dilutions in this manner to tube number 8, being certain to change pipettes between tubes to prevent carryover of antibiotic on the external surface of the pipette.
6. Remove 1.0 ml from tube 8 and discard it. The ninth tube, which serves as a control, receives no tetracycline.
7. Suspend to an appropriate turbidity several colonies of the culture to be tested in 5.0 ml of Mueller-Hinton broth to give a slightly turbid suspension.
8. Dilute this suspension by aseptically pipetting 0.2 ml of the suspension into 40 ml of Mueller-Hinton broth.
9. Add 1.0 ml of the diluted culture suspension to each of the tubes. The final concentration of tetracycline is now one-half of the original concentration in each tube.
10. Incubate all tubes at 35^{o}C overnight.
11. Examine tubes for visible signs of bacterial growth. The highest dilution without growth is the minimal inhibitory concentration (MIC).

Practical No.8
STAPHYLOCOCCI

Staphylococci are often found in the human nasal cavity (and on other mucous membranes) as well as on the skin. They are gram-positive cocci 0.5-1.0 μm in diameter and occur singly, in pairs, in short chains, and most commonly, in irregular grape-like clusters. The staphylococci are strongly catalase positive, reduce nitrates to nitrites, and generally tolerate relatively high concentrations of sodium chloride (7.5-10%). This ability is often employed in preparing media selective for staphylococci. Five species of staphylococci commonly associated with clinical infections: ***Staphylococcus aureus, S. epidermidis***, ***S. haemolyticus, S. hominis*** and ***S.saprophyticus***.

A. Coagulase-positive Staphylococci (*Staphylococcus aureus*)

Since most ***S. aureus*** strains produce the enzyme coagulase they are often referred to as coagulase-positive staphylococci.

Staphylococcus aureus is the most pathogenic species and is implicated in a variety of infections. Approximately 30% of adults and most children are healthy periodic nasopharyngeal carriers of ***S. aureus*****.** Around 15% of healthy adults are persistent nasopharyngeal carriers.

S. aureus causes**;** Pus-filled inflammatory lesions known as abscesses. Depending on the location and extent of tissue involvement, the abscess may be called: pustule**,** furuncle or boil, carbuncle , Impetigo, a superficial blister-like infection of the skin usually occurring on the face and limbs and seen mostly in young children.

-Cellulites.
-Accidental wound and postoperative wound infections
-Systemic infections include septicemia, septic arthritis, endocarditis, meningitis, and osteomyelitis, as well as abscesses in the lungs, spleen, liver, and kidneys. Pneumonia caused by ***S. aureus*** is considered as a secondary respiratory complication of viral infections such as measles and influenza. Finally, *S. aureus* is frequently introduced into food by way of abscesses or the nasal cavity of food handlers. If it is allowed to grow and produces an enterotoxin, it can cause staphylococcal food poisoning. It is also the causative agent of scalded skin syndrome and toxic shock syndrome.

B. Coagulase-Negative Staphylococci

Clinically common species of staphylococci other than *S. aureus* are often referred to as coagulase-negative staphylococci. These staphylococci are normal flora of the skin and, as such, frequently act as opportunistic pathogens, especially in the compromised host. *S. saprophyticus* is a relatively common cause of urinary tract infections, especially in healthy young women, but is seldom isolated from other sources. The great majority of infections caused by other coagulase-negative staphylococci, including *S. epidermidis, S. haemolyticus,*

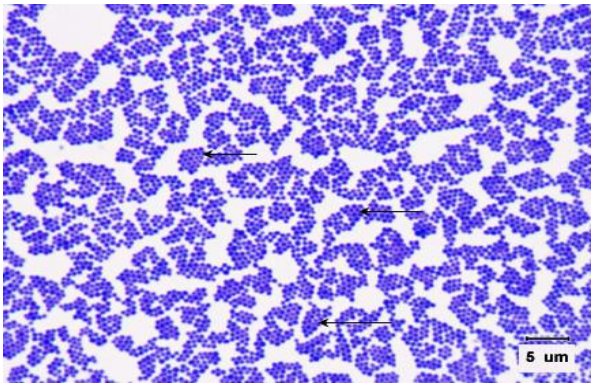
and *S. hominis,* are associated with intravascular devices (prosthetic heart valves and intra-arterial or intravenous lines) and shunts. Also quite common are infections of prosthetic joints, wound infections, osteomyelitis associated with foreign bodies, and endocarditis. Although certain reactions may vary from strain to strain, a series of biochemical tests will usually differentiate the most common clinically encountered species

of staphylococci. Today we will use a number of tests to isolate and identify ***S. aureus, S. epidermidis,*** and ***S. saprophyticus*****.**

Isolation and identification of Staphylococci

Specimen; blood, urine, stool, CSF, pus.

1- Gram stain



Note staphylococcus arrangement (cocci in irregular, often grape-like clusters).

2- Catalase test;

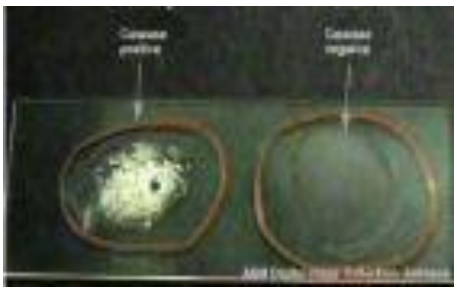
Although microscopic examinations of stained smears presumptively permit distinction between Staphylococci and streptococci, a definitive classification can be made on the basis of the presence or absence of the enzyme catalase. Staphylococci contain this enzyme, streptococci do not.

Procedure;

1. Place a drop of 3% hydrogen peroxide on a clean microscope slide.
2. Place a heavy loopful of cells from isolated colonies into the liquid (pick four to five colonies). Immediate generation of gas bubbles constitutes a positive test.
3. Avoid the inclusion of blood cells from blood agar plates as RBCs contain catalase. Lack of bubbles is a negative test.

Catalase

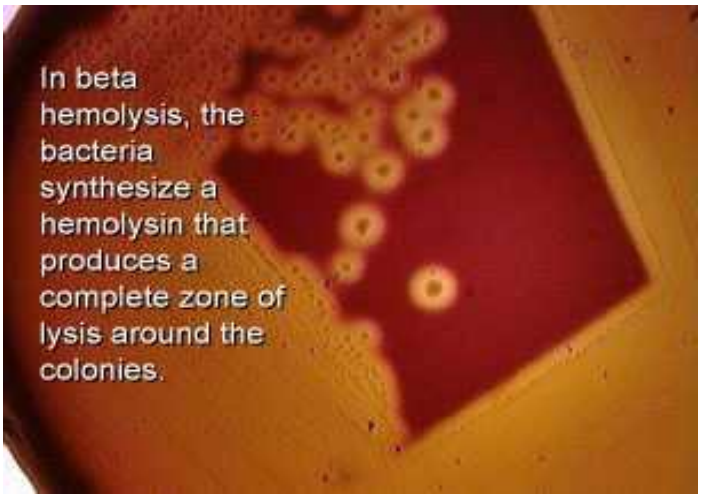
$2H_2O_2$ ----------> $2H_2O + O_2$ (bubbles)



3- Hemolysis on blood agar

Some bacteria produce hemolysins, exotoxins that cause red blood cells (RBC's) to burst open (hemolyse). When these bacteria are cultured on blood agar, this hemolysis is visible as an area of clearing around the colony (zone of hemolysis). If the organism produces enzymes that completely lyse the RBC's, this is termed beta hemolysis (β-hemolysis). Partial destruction of the RBC's produces a greenish color to the zone of hemolysis and is termed alpha hemolysis (α-hemolysis). Organisms lacking hemolysins cause no change in the color or opacity of the media and are termed gamma hemolytic or none hemolytic.

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Hemolysis	Usually beta	Usually none	Usually none



4- Agar plates with a novobiocin (NB) disc

Novobiocin is an antibiotic to which Staphylococcus sp. are either resistant or sensitive. The appearance of a zone of inhibition > 16 mm indicates sensitivity.

Filter paper discs impregnated with the appropriate chemical are placed on an agar surface. The chemical diffuses through the agar. Organisms that are susceptible to the chemical will not grow on the agar containing the chemical. The size of the zone of growth inhibition determines the organisms' susceptibility to the chemical.

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Novobiocin test	Sensitive	Sensitive	Resistant (at a concentration of 5 mg),

5- Production of staphyloxanthin (pigmentation)

Staphyloxanthin is an orange pigment produced by *Staphylococcus aureus* that contributes to its virulence. It is the main pigment of this pathogen. Milk agar provides a white background to visually observe the colonies of *Staphylococcus aureus*

Procedure

- Streaking colonies of staphylococci on milk agar plate
- incubating overnight,
- observing the golden yellow pigment of ***Staphylococcus aureus***

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Pigment	Often creamy gold	Usually white	Usually white

6- Mannitol fermentation on Mannitol Salt agar (MSA)

Staphylococci are able to tolerate the high salt concentration found in Mannitol Salt agar and thus grow readily. If mannitol is fermented, the acid produced turns the phenol red pH indicator from red (alkaline) to yellow (acid).

Procedure

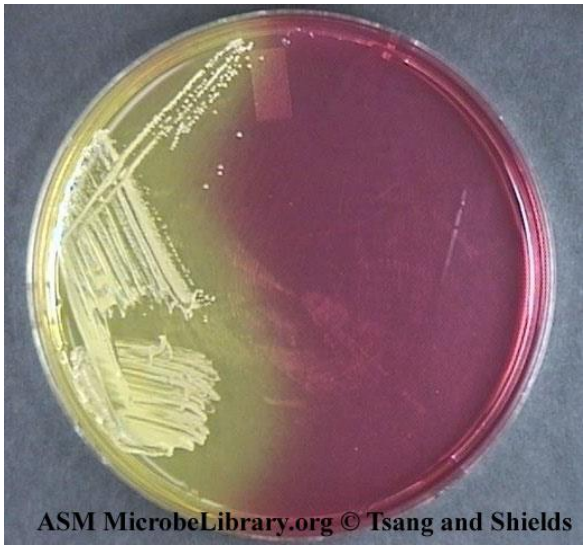
Mannitol-Salt Agar Tests;

Two separate tests are done with this plate. The plate contains 7.5% NaCl, mannitol, phenol red, and extracts of peptone and beef. If the bacteria in question grow on the plate they are osmotolerant and therefore NaCl growth positive. If surrounding the growth the media has turned from pink to yellow, the bacteria has fermented the mannitol and produced acid.

The acid lowers the pH of the media and the phenol red turns yellow. If yellow, the bacteria are positive for the ability to ferment mannitol.

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Mannitol fermentation	Positive	Negative	Usually positive

Positive = acid end products turn the phenol red pH indicator from red to yellow
negative = phenol red remains red



Staphylococcus aureus turning the indicator from red to yellow

7- Production of deoxyribonuclease (DNase) on DNase agar

DNase agar contains 0.2% DNA. To detect DNase production, the plate is inoculated and incubated. After growth, the plate is flooded with 1N hydrochloric acid (HCl). DNase positive cultures show a distinct clear zone around the streaked area, where the DNA in the agar was broken down by the bacterial DNase. DNase negative cultures appear cloudy around the growth where the acid caused the DNA in the agar to precipitate out of solution.

Procedure

1- Inoculate by making a single streak line using inoculum from an agar slant or plate.

2- Incubate at 35 ± 2°C for 24-48 hours. Plates should be incubated in an inverted position.

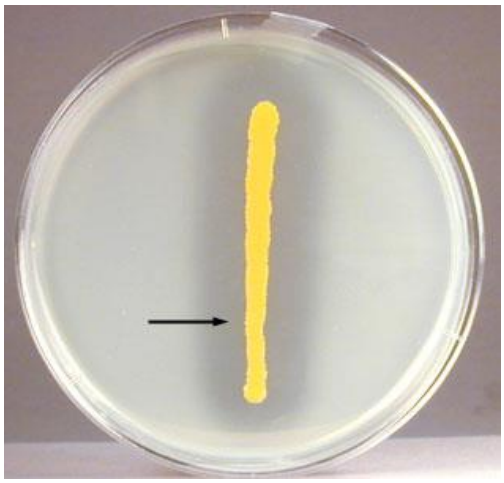
3-Following incubation, flood DNase Test Agar plates with 1N HCl reagent and observe for reaction.

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
DNase production	Positive	Negative	Negative

Positive = clear zone around growth after adding 1N HCl (no DNA remaining in the agar)

negative = cloudy around growth after adding 1N HCl (DNA remains in the agar forming a precipitate)

A Positive DNase Test



Note there is breakdown of the DNA in the agar. There is a clear zone (arrow) around the bacterial growth where there is no longer any DNA left in the agar to precipitate out of solution after the HCl was added.

8- Production of coagulase

The staphylococcal enzyme coagulase will cause inoculated citrated rabbit plasma to gel or coagulate. The coagulase converts soluble fibrinogen in the plasma into insoluble fibrin. . In the laboratory, it is used to distinguish between different types of *Staphylococcus* isolates.

Procedure:

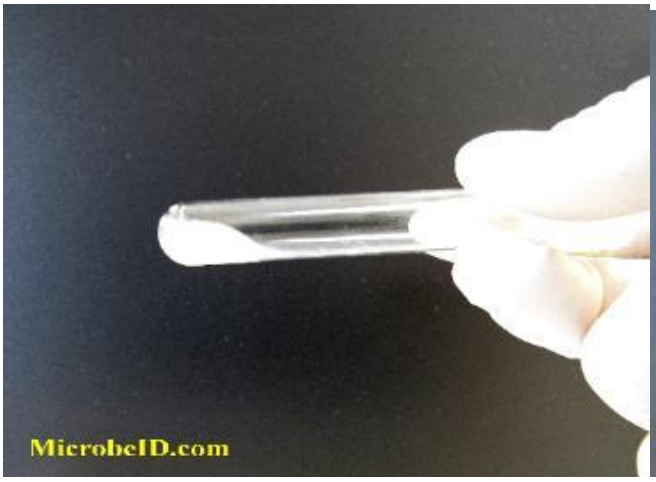
- Add 0.5 mL reconstituted lyophilized rabbit coagulase plasma with EDTA to a sterile 13 x 100mm tube
- Touch an isolated colony with an inoculating loop
- Place the loop, carrying some of the isolate, into the tube containing the rabbit plasma, and mix thoroughly
- Incubate the tube at 35º C for 6 hours
- Observe the tube for the presence of clotting.
- If no clotting is observed, reincubate for 24 hours and observe again.

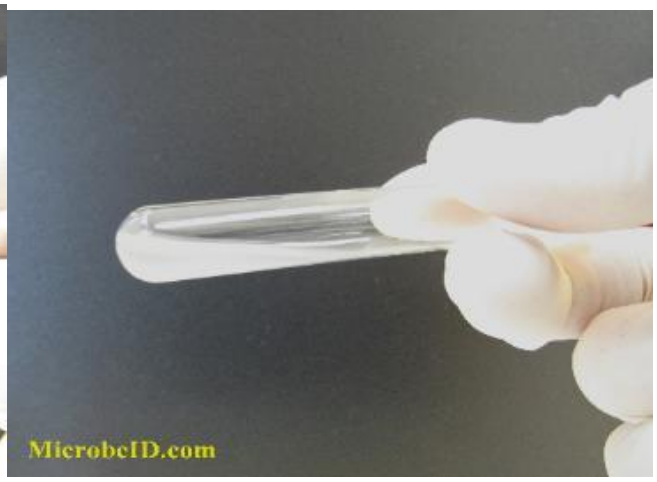
The reaction is positive if clotting is present. (The plasma will gel to a viscous form, where it will not flow down the tube when tilted at a 45º angle.)

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Coagulase production	Positive	Negative	Negative

Positive = plasma will gel or coagulate

negative = plasma will not gel





Coagulase negative coagulase positive

The Staphyloslide Latex Test for cell-bound coagulase (clumping factor) and/or Protein A:

The Staphyloslide Latex Test is an agglutination test that detects cell-bound coagulase (clumping factor) and/or Protein A. This test uses blue latex particles coated with human fibrinogen and the human antibody IgG. Mixing of the latex reagent with colonies of the suspected ***S. aureus*** having coagulase and/or Protein A bound to their surface causes agglutination of the latex particles.

Procedure:

1. Gently mix the Staph latex reagent bottle (make sure latex is resuspended and warmed to room temperature) and place 1 drop of the latex reagent (by holding dropper bottle vertically) into one circle of the reaction card.
2. Spread 1 colony (using either an applicator stick or an inoculating loop) onto the circle and then mix into the drop of latex reagent. Slowly blend the staphylococci into the reagent.
   Spread the mixture over much of the circle. Discard the stick into biohazard waste or flame the loop.
3. Rotate the slide with circular motion for up to 60 sec. Aggregation of the black latex suspension with subsequent loss of black background represents a positive reaction for agglutination. For ***S. aureus***, this usually occurs within 15 sec. A negative reaction is reported as little or no agglutination within 60 sec.

Test	***S. aureus***	***S. epidermidis***	***S. saprophyticus***
Cell-bound coagulase (clumping factor) and/or Protein A	Positive	Negative	Negative

Positive = clumping of latex particles

negative = no clumping of latex particles

9- Gelatinase test.

Nutrient gelatin is a differential medium that tests the ability of an organism to produce an exoenzyme, called gelatinase that hydrolyzes gelatin. The gelatinase test can be used to differentiate between *Staphylococcus aureus* and *Staphylococcus epidermidis.*

Test	***S. aureus***	***S. epidermidis***
Gelatinase	Positive	Negative