GENERAL MICROBIOLOGY PRACTICAL – week 11

Testing of Biochemical Properties ofBacteria (Part II)

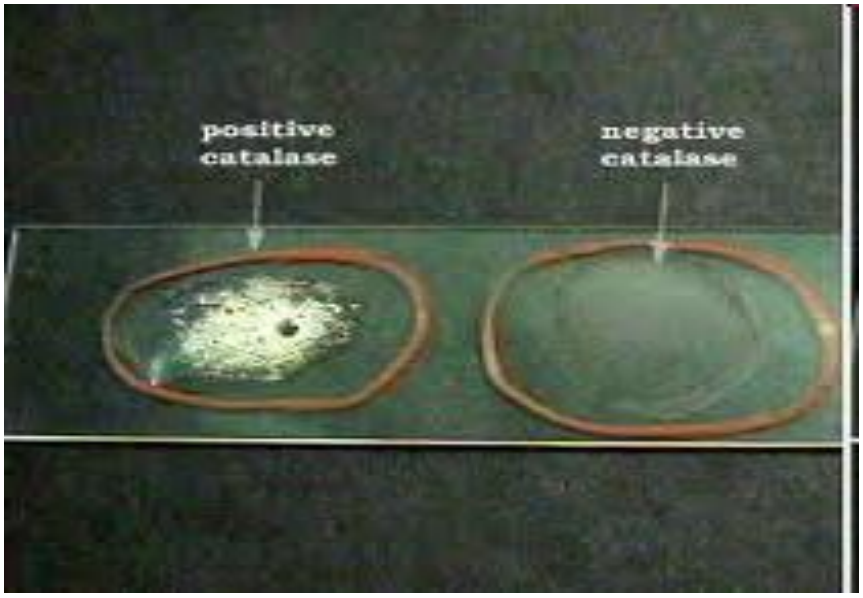
Bacterial Enyzmes and Toxins

1. Catalase test

Catalase Test

- This test is used to identify organisms that produce **the enzyme, catalase.**
- This enzyme detoxifies hydrogen peroxide by breaking it down into water and oxygen gas.
- **The bubbles resulting from production of oxygen gas clearly indicate a catalase positive result.**
- The sample on the left - catalase positive.
- **The *Staphylococcus*** spp. and the *Micrococcus* spp. **are catalase positive.**

The *Streptococcus* and *Enterococcus spp.* **are catalase negative.**



Some bacteria contain flavoproteins that reduce oxygen (O2), resulting in the production of hydrogen peroxide (H2O2) and, in some cases, an extremely toxic superoxide (O2–).

Accumulation of these substances will result in death of the organism as they are powerful oxidizing agents and destroy cellular constituents very rapidly unless they can be enzymatically degraded. These substances are produced when aerobes, facultative anaerobes, and microaerophiles use the aerobic respiratory pathway, in which oxygen is the final electron acceptor, during degradation of carbohydrates for energy production.A bacterium must be able to protect itself against such O2products or it will be killed. Many bacteria possess enzymes that afford protection against toxic O2products.

2. Oxidase test:

Oxidase test (cytochromoxidase test) determines the presence of cytochrome oxidase.

Kovac's oxidase reagent, tetramethyl-p-phenylenediamine dihydrochloride, is turned into a purple compound by organisms containing cytochrome c as part of their respiratory chain.

This test aids in the recognition of ***N. meningitidis***, but other members of the genus **Neisseria**,as well as unrelated bacterial species, may also give a positive reaction. Positive and negative quality control strains should be tested along with the unknown isolates to ensure that the oxidase reagent is working properly. Positive reactions will develop within 10 seconds in the form of a purple color. Negative reactions will not produce a color change.

Oxidase Test -Filter paper method

1.Grow the isolate to be tested for 18-24 hours on a blood agar plate (BAP)at 35-37°C with5% CO2(or in a candle-jar).

2.On a nonporous surface (Petri dish or glass plate), wet a strip of filter paper with a few drops of Kovac's oxidase reagent.

3.Let the filter paper strip air dry before use.

4.Use a disposable plastic loop, a platinum inoculating loop, or a wooden applicator stick to pick a portion of a colony from overnight growth on the BAP and rub itonto the treated filter paper.

5.Observe the filter paper for color change to purple

Positive Oxidase reactions: ***Pseudomonas aeruginosa, Moraxella catarrhalis, Neisseria meningitidis***

3.Coagulase test

- Coagulase – protein of unknown chemical structure with properties similar to prothrombin. It coverts fibrinogen on fibrin producing visible clot – coagulum.
- In vivo it is the cause of fibirne bariere – absces formation in Staphylococcus aureus.
- In vitro – used for dif.dg. of Staphylococcus aureus: PC+ and other staphylococci: PC –
- There is:

Free coagulase – present in filtrate of bacterial culture, tube method

Bound coagulase – on bacterial wall,clumping factor, slide method

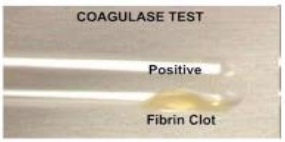
Coagulases are enzymes that clot blood plasma by a mechanism that is similar to normal clotting. The coagulase test identifies whether an organism produces this exoenzyme. This enzyme clots the plasma component of blood. The only significant disease causing bacteria of humans that produce coagulase enzyme are Staphylococcus aureus. Thus this enzyme is a good indicator of the pathogenic potential of ***S. aureus.***

Coagulase is described as virulence factor (disease-causing factor) of *Staphylococcus aureus.*

Most strains of *S.aureus* produce one or two types of coagulase; **free coagulase** and **bound coagulase.**

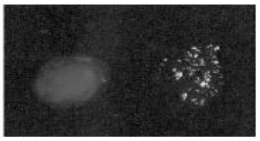
Free coagulase

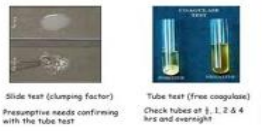
- Tube method – colony of tested strain is emulsified in 0,5 ml of plasma. Incubated for 6 h.at 37*C then at room temperature for 24 hrs.
- Reading after 1 hour, 2 hrs and 24 hrs.
- method – watching of coagulum. Formation of coagulum = pozit.,
- Because of possible presence of fibrinogen in plasma this can dissolve the coagulum. That is why we read it at 1,2 and 24 hrs. Fig.



Clumping factor -Bound coagulase

- Slide method
- In 2 drops of steril water or saline solution there is the suspension prepared from tested strain.
- 1 drop of plasma is added. Reading after 10-15´sec.
- White precipitate, agglutination = posit.
- Negative result must be confirmed by tube test
- Fig.





Bound coagulase is localized on the surface of the cell wall.

Free coagulase is an enzyme that is secreted extracellularly.

Free coagulase can be detected in tube coagulase test

Bound coagulase can be detected in slide coagulase test.

Slide coagulase test may beused to screen isolates of *S.aureus* and tube coagulase may be used for further confirmation. There are seven antigenic types of free coagulase,but only one antigenic type of bound coagulase exists. Free coagulase is always heat labile while bound coagulase is heat stable.In the test, the sample is added to rabbit plasma and held at 37° C for a specified period of time. Clot formation occurs within 4 hours is interpreted as a positive result and indicative of a virulent *Staphylococcus aureus* strain. The absence of coagulation after 24 hours of incubation is a negative result, indicative of an avirulent strain.

Detection of Bound Coagulase - Slide Test

This method measures bound coagulase. The bound coagulase is also known as clumping factor. It cross-links the α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus stick to each other and clumping is observed.

1. Divide the slide into two sections with grease pencil. One should be labeled as „test" and the other as „control".
2. Place a small drop of distilled water on each area.
3. Emulsify one or two colonies of Staphylococcus on blood agar plate on each drop to make a smooth suspension.
4. The test suspension is treated with a drop of citrated plasma and mixed well with a needle.
5. Do not put anything in the other drop that serves as control. The control suspension serves to rule out false positivity due to auto agglutination.
6. Clumping of cocci within 5-10 seconds is taken as positive (Fig. 9).

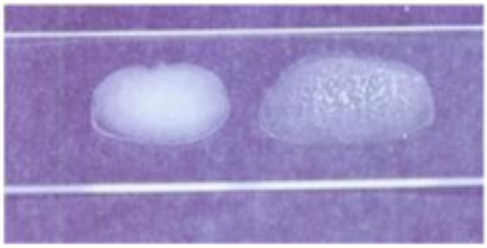


Fig. 9 Slide Coagulase Test.

Some strains of *S.aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test

Detection of Free Coagulase - *Tube Coagulase Test*

Most strains of *S.aureus* produce one or two types of coagulase; free coagulase and bound coagulase. Free coagulase is an extracellular enzyme which reacts with prothrombin and its derivatives. This method helps to measure free coagulase. The free coagulase secreted by *S.aureus* reacts with coagulase reacting factor (CRF) in plasma to form a complex, which is thrombin. This converts fibrinogen to fibrin resulting in clotting of plasma (Fig. 10).

1. Three test tubes are taken and labeled "test", "negative control" and "positive control".
2. Each tube is filled with 1 ml of 1 in 10 diluted rabbit plasma.
3. To the tube labeled test, 0.2 ml of overnight broth culture of test bacteria is added.
4. To the tube labeled positive control, 0.2 ml of overnight broth culture of known *S. aureus* is added.
5. To the tube labeled negative control, 0.2 ml of sterile broth is added.
6. All the tubes are incubated at 37°C.
7. Positive result is indicated by gelling of the plasma, which remains in place even after inverting the tube.

8. If the test remains negative until four hours at 37°C, the tube is kept at room temperature for overnight incubation.

Fig. 10 Tube Coagulase Test.

The coagulase test is used to distinguish between pathogenic and nonpathogenic members of the genus Staphylococcus. All pathogenic strains of *S. aureus* are coagulase positive whereas the nonpathogenic species (*S. epidermidis*) are coagulase negative.
While slide coagulase test is useful in screening, tube coagulase test is useful in confirmation of coagulase test. Samples must be observed for clotting within 24 hours. This is because some strains that produce coagulase also produce an enzyme called fibrinolysin, which can dissolve the clot. Therefore, the absence of a clot after 24 hours is no guarantee that a clot never formed. The formation of a clot by 12 hours and the subsequent disappearance of the clot by 24 hours could produce a so-called false negative if the test were only observed at the 24-hour time.

4.Proteolytic properties testing:

Proteolytic activity testing

- Some strains of certain bacteria can produce proteolytical enzymes that are able to dissolve proteins in tissues.Such strains are more pathogenic
- in vitro we use dissolvation of gelatinose to demonstrate proteolytical activities of a strain
- Demonstration of *E. coli* – negat.,
- *Ps.aeruginosa* – posit.,
- *Proteus mirabilis* – posit. with method of gelatinose disks, dishes method and in figures

Proteolytic activity

- Tube method – agar medium in tube is innoculated by tested strain and incubated at 37*C. 1 hr before reading the tube is placed to refrigerator. Strain with proteolytical enzymes dissolves gelatine and this will remain liquid even in cool.
- Dishes method – agar dish with gelatine is innoculated with tested strain and read . The clearing and transparency closed to the line of innoculation indicate the proteolysis (fig. And demonstration)
- Method of gelatinous disks – in liquid media the tested strain is innoculated and a gelatinous carbon disk is applicated. When proteolytical activity is present the disk will be dissolved(demonstration)

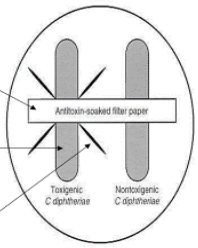
5. Detection of toxin production (*Corynebacterium diphtheriae*): in vitro (Elek´s test)

Elek´s method for demostration of C. diphterie

- Immunediffusion in gel:
- suspension of tested strain is apllicated to the pre-formed wells in the gel
- and to the well situated in the middle – the antitoxin is apllicated.
- The liquid materials diffuse from wells and in the meeting point, the precipitation line is formed.

Elek´s method – fig.

- Sterile filter paper impregnated with diphtheria antitoxin is imbedded in agar culture medium.
- Isolates of *C. diphtheriae* are then streaked across the plate at an angle of 90° to the antitoxin strip.
- Toxigenic *C diphtheriae* is detected because secreted toxin diffuses from the area of growth and reacts with antitoxin to form lines of precipitin.



The **Elek culture plate precipitin test** is routinely used for the detection of exotoxin from toxigenic strains of *Corynebacterium diphtheriae*. The test for toxigenicity, which detects the potent exotoxin, a phage-encoded protein, is the most important test and should be done without delay on any suspect isolate that is found by routine screening or while investigating a possible case of diphtheria. The toxigenic species *C. diphtheriae* acquire this characteristic when infected by the family of β-phages or other families of corynephages. The Elek test was

first described in 1949 and replaced the in vivo virulence test in guinea pigs, a test that was used by many countries at that time.

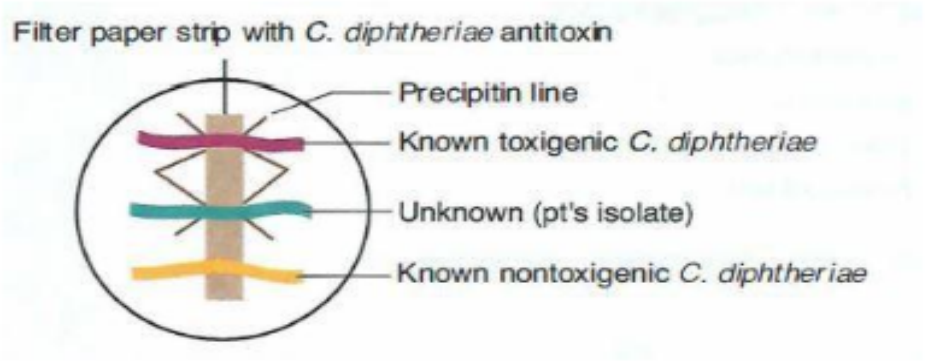


Fig. 75 Elek´s Test.

The Elek Test Principle

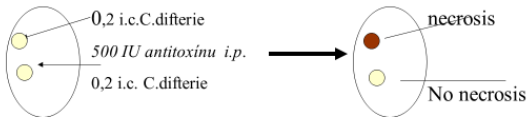
A filter paper strip impregnated with diphtheria antitoxin is buried just beneath the surface of a special agar plate before the agar hardens. Strains to be tested, known positive and negative toxigenic strains are streaked on the agar's surface in a line across the plate, and at a right angle to the antitoxin paper strip. After 24 hours of incubation at 37° C, plates are observed for the presence of fine precipitin lines at a 45-degree angle to the streaks (Fig. 75). The presence of precipitin lines indicated that the strain produced toxin that react with the antitoxin.

Diphteria Toxin – Mode of Action

Diphtheria toxin (DT) is an extracellular protein of *Corynebacterium diphtheriae* that inhibits protein synthesis and kills susceptible cells. Diphtheria toxin is a single polypeptide chain consisting of two subunits linked by disulfide bridges, known as an A-B toxin. Binding to the cell surface of the B subunit (the less stable of the two subunits) allows the A subunit (the more stable part of the protein) to penetrate the host cell. The diphtheria toxin catalyzes the transfer of NAD^+ to a diphthamide residue in eukaryotic elongation factor-2 (eEF2), inactivating this protein. It does so by ADP-ribosylating the unusual amino acid diphthamide. In this way, it acts as a RNA translational inhibitor. The acceptor is diphthamide, a unique modification of a histidine residue in the elongation factor found in archaebacteria and all eukaryotes, but not in eubacteria.

Toxin production
Corynebacterium diphteriae

- **In vivo** – in annimal model – neutralisation test. 0,2 ml of tested suspension is applicated i.c. in guinea pig. After 5 hrs. 500IU of antitoxin is apllicated by intra peritoneal way and then again 0,2 ml of tested suspension is applicated to another place of the same guinea pig.



6. Detection of tetanospasmin (*Clostridium tetani*) –annimal ň model

Clostridium tetani (TETANUS)

Clostridium tetani, a Gram-positive rod that forms a terminal spore is commonly found in the soil, dust and animal feces. Contamination of wounds, which provide anaerobic conditions, can lead to spore germination and tetanus, a relatively rare, but frequently fatal disease. Tetanus is also know as lockjaw because of the patient's inability to open the mouth as a result of muscle paralysis.

Infection usually occurs when spores (in dirt, feces or saliva) enter wounds and scratches where they germinate and produce tetanus toxin. The organism is non-invasive and thus remains in the local wound.

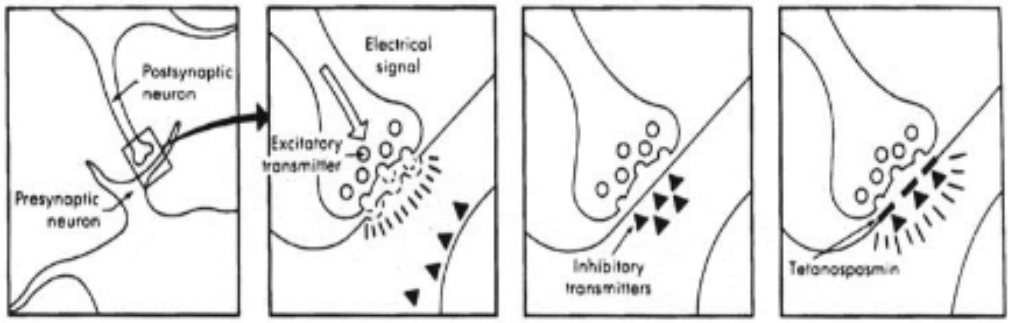


Fig. 83 Tetanospasmin – mode of action.

The exotoxin (tetanospasmin) binds to ganglioside receptors on inhibitory neurones in central nervous system. The effect of the toxin - to block the release of inhibitory neurotransmitters (glycine and gamma-amino butyric acid) - it produces the generalized muscular spasms characteristic of tetanus. This stops nerve impulse transmission to muscle leading to spastic paralysis. The toxin can act at peripheral motor nerve end plates, the brain, spinal cord and also in the sympathetic nervous system. It is transported within the axon and across synaptic junctions until it reaches the central nervous system. Because inhibitory neurons are involved, the result is unopposed muscle contraction.

In generalized tetanus, the most common form, the patient typically experiences lockjaw (trismus). This is a stiffness of the jaw muscles that results in inability to open the mouth or swallow leading to the appearance of a sardonic smile (*risus sardonicus*). Cephalic tetanus is a rare infection involving the middle ear. It can affect cranial nerves. Local tetanus is also rare and manifests itself as localized muscle contractions in the area of infection.

Clostridium tetani toxin

- Cultivation in anaerobic conditions
- Microscopy: G positive rods with spores located at the end of rod (fig.)

- Annimal trial (fig..)
  thest for neutralisationt)

- generalised tetanus :
  tetanus in a mouse showing body curvature (opisthotonos)

Local tetanus – erected tail