Lab. 1

There are many factors that determine the pathogenic bacterial virulence or ability to cause infection:

1. **1.** Toxin Production
2. **2.** Adherence factors
3. **3.** Invasion of host cells and tissues
4. **4.** Enzyme Production

Pathogenicity: Capability of Microorganisms to cause the disease or contributing to the events of natural or experimental methods in the host.

Toxicity: the ability of bacteria to cause harm or damage to the tissue.

Severity of disease (virulence): the number of Microorganisms or the number of micro grams of toxin (poison) sufficient to kill the host when entered it in different ways and are usually described Lethal dose 50 (LD 50) the dose of toxin, or pathogen is required to kill half the members of an animal tests such as laboratory mice.

TOXIN (poison): A metabolic materials produced from metabolic processes, whether beneficial or harmful to cells and tissues and are produced in the process of Idio phase which is located between the Logarithmic phase and Stationary phase.

These toxins are either excreted by the Microorganisms to the culture media or toxic substances (toxins) secreted by bacteria and released outside the cell called **Exotoxin**, or keep these toxins associated with the surface of pathological cells and are part of the components of the cell wall and then called **Endotoxin**.

Since the process of metabolism in bacteria are pathological and non-pathological So that the pathogenic bacteria possess additional specifications to enable them to cause the disease listed by the following:

1. The ability of bacteria to enter in host body (vivo).

2. Concentration of bacteria at the site of a permanent or temporary in host body (vivo).

3. Ability of bacteria to multiply.

4. The ability of bacteria to compete with the natural bacteria (normal flora) in the body.

5. Ability of bacteria to overcome the resistance of the host body (immune system).

The ability of bacteria to overcome these stages lead to the creation of the disease, and the most important of these factors is the ability of bacteria to produce substances known as toxins.

Microbial toxin: a secondary metabolic product produced by microorganisms and its harmful or fatal effect on cells and tissues.

Toxoid: is a bacterial toxin (usually an exotoxin) whose toxicity has been inactivated or suppressed either by chemical (formalin) or heat treatment, while other properties, typically immunogenicity, are maintained.

Thus, when used during vaccination, an immune response is mounted and immunological memory is formed against the molecular markers of the toxoid without resulting in toxin-induced illness international medical literature the preparation also is known as anatoxin or anatoxine. There are toxoids for prevention of diphtheria, tetanus and botulism. and can be accessed through the treatment of a number of substances, including toxins and formalin by specific temperature 37 and pH (6.9) for several weeks.

Toxigenicity: The ability of microorganisms to produce toxins.

Toxemia: The case of poisoning caused by the presence of the toxin in the blood.

Bacteremia: Is a disease caused by the presence bacteria in the blood circulation.

Septicemia: Is a presence of bacteria with their toxins in the bloodstream.

There are several types of toxins, which can be divided depending on several criteria:

1. According to chemical structure.

a- Protein toxin

b- Lipopolysaccharide (LPS).

2. According to mechanism.

A- Block protein synthesis Ex: Diphtheria toxin

B- Block nerve function Ex: Tetanus toxin

C- Toxin which help microorganism to separate in tissues Ex: Hyaluronidase

D- Toxin that lysis cells and killed them Ex: Lecithinase

3. According to site of action.

A- Enterotoxin Ex: Staph toxin

B- Neurotoxin Ex: tetanus toxin

C- Cytotoxin Ex: Shigella toxin

4. According to role of enter.

A- Toxin which pass through wound infection Ex: tetanus

B- By blood invasiveness Ex: Endotoxin

C- Initiating disease though intestinal tract Ex: Salmonella

Table 1: Comparison between Exotoxins & Endotoxins

Property	Exotoxin	Endotoxin
Bacterial source: **نوع البكتريا**	Mostly from Gram positivebacteria.	Mostly from Gram negative bacteria.
Relation to microorganism: **مصدر السم البكتيري**	Metabolic product of growing cell. هو منتوج ايضي من الخلية.	Present in LPS of outer membrane of cell wall & released by destruction of cell or cell division. ملتصق في الدار الخارجي ويتحلل عند موت او انقسام الخلية.
Chemistry: **التركيب الكيمياوي**	Protein usually with 2 parts(A&B). بروتيني	(Lipid-A) of LPS of outer membrane (Lipopolysaccharide).
Effect on body (Pharmacology): **التأثير الكيمياوي على جسم المضيف**	Specific for a particular structureor function in host, (Mainly affects cell function, nerves, & gastrointestinal tract GIT). خاص بتراكيب ووضائف معينة في المضيف غالبا يضعف الوضائف الخلويةوالأعصاب والأمعاء	General effect, such as; (fever, weakness, aches, & shock) all produce the same effects. تاثير اكثر عموما مثل الحمى، الوهن، الالم والصدمة.
Heat Stability **التأثر بالحرارة**	Unstable, (usually destroyed at60 – 80ºC) except for *Staphylococcal* enterotoxin.	Stable, can withstand autoclaving (121 ºC for 1 hour). لا يتأثر او يتلف بالحرارة
Toxicity (Ability to cause disease): **السمية**	High.	Low.
Fever producing:	No.	Yes.
Immunology (relation to antibody) **الخاصية المناعية**	Can be converted to Toxoids immunized against toxins, (Neutralized by antitoxin). يمكن تضعيغ السم وتحويله الى لقاح ضدالسم نفسه	Not easily neutralized by antitoxins, therefor effective toxoids cannot be made to immunize against toxins. لا يمكن عمل المضادات منه بسهولة ولذلك فان المصول المعمولة منه قد لا تعمل مناعة ضد السم نفسة
Lethal dose **الجرعة القاتلة**	Small.	Considerably Large.
Representative diseases **الامراض الناجمة عنه**	Gas gangrene, Tetanus, Botulism, Diphtheria, & Scarletfever.	Typhoid fever, Urinary tract infections, & Meningococcal meningitis.

Lab 2 Methods of investigations for toxins

The diseases caused by microorganisms are still problems that researchers must find a solutions to them, so that researchers engaged over the centuries to learn how the incidence of disease and the causative microorganism and the role of pathogenic agents lead to the creation of the disease, and perhaps that toxins produced by microorganisms are one of the virulence factors responsible for pathogenicity of microorganisms, so it was necessary to find an appropriate ways for the purpose of investigation:

Methods of investigations for toxins can be divided into four axes:

1. Culture method (bacteriological).

2. Biological method.

A- Tissue culture.
B- Rabbit legated loop assay method (intestine tethered to the rabbit).
C- Suckling mice assay.
D- Mice lethality assay.
E- Vascular permeability assay.

3. Immunological methods.

A- Enzyme linked immune-sorbent assay (ELISA).
B- Latex agglutination test.
C- Cold haemagglutination.
D- Counter immune - electrophoresis.

4. Molecular methods which includes the Polymerase chain reaction (PCR).

First: Culture method (bacteriological)

Using selective culture media depending on the type of microorganism and the poisons (toxins) are produced by them.

Advantages:

1. High sensitivity.
2. Specialty.
3. Cheap (Low cost).

Disadvantages:

1. Could not differentiate (discrimination) between the toxin-producing bacteria from non-productive.
2. Takes several days to isolate and diagnose the bacteria.

Second: Biological method

A- Tissue culture

This method is used for the detection of toxins to the cells and tissue, **advantages** of this method are:

1. High sensitivity.
2. Specialty.
3. Reveals about the disease and its causative organism.

But sometimes it may give false negative results due to dilution of samples significantly. False positive results may occur when the patient is infected with disease-causing.

Disadvantages

1. Laboratory method is expensive.
2. Takes a long time for to conduct the method about (24-72) hours depending on the type of toxin and pathogen.

B- Vascular permeability assay:

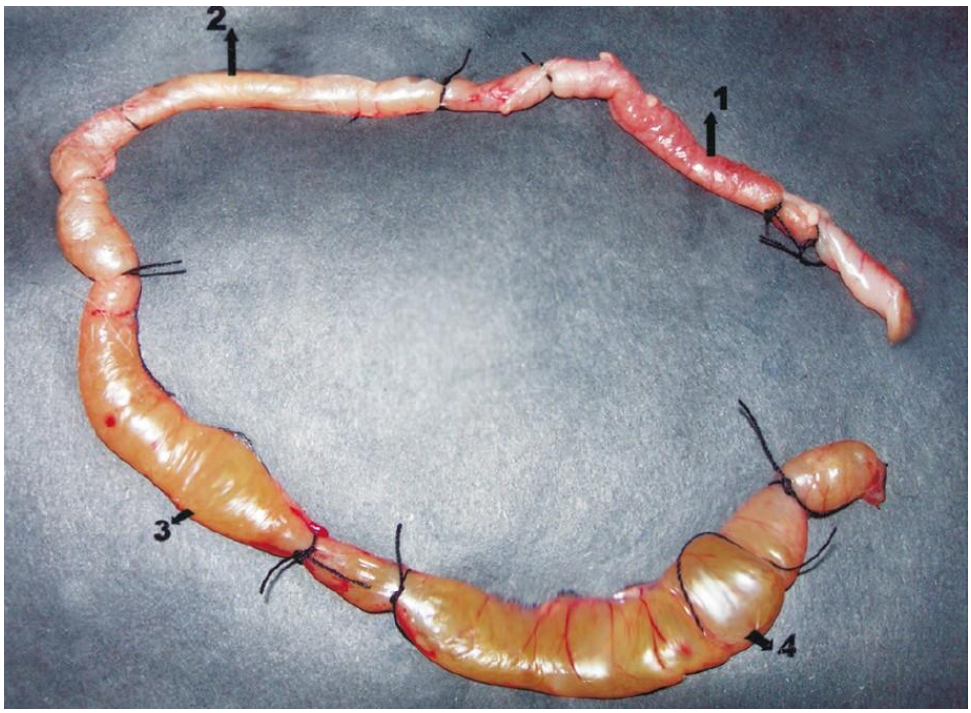
This method involves the following steps:

1. Injecting toxin in the Rabbit through the skin on either sides of the back of the rabbit (after shaving the back area).
2. Measure areas of skin reaction zone after 18 hours of injection.
3. Either vascular permeability estimated by Injection intravenous by colored dye (Evans blue).
4. Measure the diameter of blue areas after 3 hours of the start of the experiment.

C- Rabbit legated loop assay:

This method involves the following steps:

1. Knots (عقدة) are made in the small intestine of rabbit to make loops with length of (5-7 cm) each, and the number of loops are determined according to the experiment.
2. Toxin is injected into each loop.
3. After (10-18) hours remove the intestine of rabbit and estimate the proportion of the size / thickness (ml / cm) for each loop (as shown in figure below).



D- Suckling mice assay:

This method involves the fallowing steps:

1. Toxins are given to suckling mice through the gastrointestinal tract.
2. Close the anus opining using a waxy substance (cyanoacrylate ester).
3. Conduct the experiment after 6 hours by killing mice to separate the neck area.
4. Measuring the accumulated liquid in the intestine by size / thickness (ml/cm) of bowel.

E- Mice lethality assay:

1. Toxin is injected in adult mice inside the Intra peritoneal layer or in the vein (Intra venous).
2. 16-24 hours after the injection, the diseased (مقتول) mice were observed (Mice lethality).

Third: Immunological methods:

A- Enzyme linked immuno-sorbent assay (ELISA).

This method relies on the use of Monoclonal antibodies to detect the specific antigens (toxins) to the medical staff or patients, this method detect effectively the toxins and the infective agent, while the previous methods were detecting toxins only.

B- Latex agglutination test:

The method is based on the detection of pathogen antigens and characterized by:

1. Less sensitivity and specificity.
2. Do not discriminate between toxin-producing isolates.

C- Cold haemagglutination.

1. (50 lμ) of toxins taken and undergo a decimal series dilution using (Tris pH = 7.5) and a concentration of 0.1 M, containing 0.05 M of Nacl and using the calibration micro titer plat.
2. Add to each dilution (50 lμ) of 1% of the rabbit RBCs suspension (محلول معلق).
3. Plate incubated in refrigerator at 4º C for three hours.
4. The results are observed is clumping of RBCs to be bloody.

Fourth: molecular methods which includes:

Polymerase chain reaction (PCR)

This method is used to investigate the microbial toxin-producing, by inflating the specified DNA (repeat) any production preparation endless sequences of DNA (gene responsible for toxins) in a chemical manner, and the used device for this test is small, this method is used in microbiology for the diagnosis of diseases such as:
Hepatitis B and tuberculosis, as well as the diagnosis of parasites such as *Toxoplasma gondii* as well as the detection of food pathogens

The advantage of this method is high in sensitivity.

Lab 3 Staphylococcus aureus toxin

Staphylococcus aureus (Also called pus-generating bacteria, Pyogenic bacteria).

Characteristics:

1. Aerobic & facultative anaerobic.
2. Gram Positive (G +ve.).
3. Cocci, grouped in Clusters.
4. Non motile.
5. Non spore forming.
6. Non capsulated.
7. Grow on Culture media easily and does not need complex nutritional requirements.
8. Catalase (+) and Oxidase (-).
9. Their ability to grow in Nacl up 0.01 and the temperature of 18-40^{o}C.
10. Found in water, air and can be isolated from milk and animal waste.

Coexistence

It is found naturally symbiotic bacteria on the skin, as well as in the mucous membranes of nose, and a large percentage of population carry these bacteria in the nasal cavity naturally (Nasal carriers).

Shape may be single or diploid or be a gathering in random arrangement because of the cell division axis are in different directions. A cell may split longitudinally (Vertically) becomes two cells then horizontally becomes four then divided laterally becomes more than four and after the split a physical thread remains draws cells together.

Advantage of these bacteria in its ability to bring the disease in addition to causing poisoning and by their ability of production of many toxins.

Production of Exotoxin:

1. Hemolysin:

This toxin lysis red blood cells (RBCs) and is considered as an important pathogenic agent and the bacteria are classified into several types according to their ability to decomposition of RBCS which α β γ δ.

β: Hemolytic of blood fully.

α, γ: partially blood Hemolytic.

The genetic determinants responsible for the production of hemolysin that affect humans mounted on the chromosome in strains, and that infect the animal is mounted on the plasmid.

A. Alpha toxin (a)

lysis a few types of cells with a lethal effect and cause skin necrosis, smashes Macrophage cells and Platelets for human, the Monocytes are resistant to the toxin lysis.

B. Beta toxin (β) or (B-hemolysin)

Affect the specificity of the Sphingomylin (a substance fall within the layers of fatty membranes of RBCs), and the effectiveness of this type has more against the red blood cells of sheep.

C. Delta toxin (δ) or (δ - hemolysin)

Lysis multiple types of cells where smashes Erythrocyte and Lymphocyte and Macrophages and Platelets and inhibits the absorption of water from the intestines.

D. Gamma toxin (γ) or (γ hemolysin)

Lysis a few types of cells and Agar material like cholesterol inhibits the toxin.

2. Leucocidin:

Kill the cells (Cytolysis) and lysis white blood cells (WBCS) especially macrophage, leading to the formation of pus, and its considered highly immunogenic protein can be converted into Toxoid.

3. Enterotoxin:

Affects the intestines, causing food poisoning and there are nine types of this poison which (A, B, C, C2, D, E, F, G, H), it is important in the case of food poisoning, if the food contains carbohydrate and proteins and the absence of competing bacteria (normal flora) and taking antibiotics so the bacteria is able to produce the poison in the intestine, as a result of certain diseases, which leads to the absence of digestive bacteria competition.

4. Exfoliatin:

Cause the separation the layers of skin surface and observed in newborns (Staphylococcal Scalded skin syndrome) (SSSS). The term syndrome is (A number of symptoms at one time).

5. Toxic shock syndrome toxin (TSST):

Produced by the Staphylococcus aureus bacteria by intervention the wounds in surgical operations and lead to death.

6. Coagulase toxin (clumping factor):

Coagulase is a protein enzyme produced by several microorganisms that enables the conversion of fibrinogen to fibrin. In the laboratory, it is used to distinguish between different types of Staphylococcus isolates. Importantly, S. aureus is generally coagulase-positive, meaning that **coagulase negative** usually excludes S. aureus. However, it is now known that not all S. aureus are **coagulase-positive**.

Detection of hemolysin enzyme in bacteria:

1. Plate method:

A- Prepare the Culture media (Blood agar pour in sterile plates) then incubated at 37^{o}C for 24 hours to ensure the purity of the agar and has no contamination.

B- Staphylococcus aureus bacteria are cultured on the plates and incubated at 37 ^{o}C for 18-24 hours.

C- Read the results of blood decomposed areas (clear zone) that surround bacterial colonies, by measuring the size of the decomposition zone.

NOTE:

- The Components of the Culture media affect the lysis capability of RBCs since the presence of serum cholesterol which inhibition of decomposition leading to disappear of Hemolysin.

- The proportion of bacteria rely analyst blood on several variables, most notably:

  a- Source of used blood. **b-** Source of bacterial isolates. **c-** Test method used.
  d- Type of Hemolysin.

2. Tube method:

a. Staphylococcus aureus bacteria are cultured in test tubes containing Nutrient broth incubated at 37 **º**C for 18-24 hours.

b. Transfer 0.1 ml (100 lμ) of the previous culture(a) into a test tube containing 10 ml of Brain heart infusion to ensure the purity of grown for 24 hours at 37**º**C in shaker incubator.

c. Transfer 0.05ml (50 lμ) of the previous culture(b) into a test tube containing (1 ml) of human RBCs mixed with 0.02 % of Normal slain.

d. Incubate in a water bath for 15 minutes at 37**º**C.

e. Centrifuge tubes quickly at 3000 rpm for 5 minutes.

f. Decomposition of RBCs seen with naked eye and compared with a control tube, (No bacteria added), to make sure of no contamination in test tubes.

Lab 4 *Streptococcus pyogenes* toxins

Toxins secreted by the bacteria *Streptococcus pyogenes:*

It is one of the pyogenic species bacteria, , gram positive, cocci, gather in short or long chains depends on type of culture media, (in liquid media formed much longer than in the solid media), facultative anaerobic, Negative to oxidase and for catalase, non-capsulated, the bacteria is (Fastidious) demanding complex media contain nutritional requirements, such as blood culture.

Colonial morphology:

Characterized by small-sized, convex colonies, semi-transparent with few space in between, able to cause disease by the production of Exotoxin (do not produce endotoxin), causing inflammation of the larynx, Tonsillitis, scarlet fever, and rheumatic fever.

This bacteria decompose the blood completely (type ß hemolysis) production of bacterial toxins dissolving the blood in a large transparent area (clear zone). The bacteria Is sensitive to the Bacitracin more than other type of hemolytic *Streptococcus.*

The most important toxins produced:

1. **Streptolysin - 0 (SLO): Features**
   - **A.** Lysis red blood cells (RBCs), and lysis the cells, (cytolytic).
   - **B.** Sensitive to oxygen works in anaerobic conditions.
   - **C.** Toxin is a protein substance molecular weight 70,000 Dalton, which makes it a good antigenic stimulates the immune system to form antibodies called Anti-Streptolysin-O (ASL-O Ab.), these antibodies used to determine the severity of infection by measuring the antibody titer in patients serum, the highest dilution indicates the acute infection.
   - **D.** Normal Titer limit is up to (200 international units) and any titer above this indicates a severe infection.

E. Plays a role in the incidence of rheumatic fever as it leads to the destruction of joint tissue and the heart tissue called (Cardiotoxin),this toxin attack heart and also toxic to white blood cells (WBCS).

F. Toxin detection made by two methods :

Serological method: (Latex agglutination) Using latex granules for the detection of Anti - (SLO) antibodies appeared as visible agglutination, if titer more than (200 IU.) then it shows high toxicity of infection.

Culture method: Through cutler of toxin-producing bacteria in test tube contain (glucose free Blood agar base media). Stabbing bacteria inside agar to the bottom of the Blood agar media and incubated at 37**º**C for 24 hours, the colonies are growing in tube surrounded by areas of decomposition of blood.

2. Streptolysin -S (SLS)

a. This toxin is responsible for the fully decomposition of blood (ß-hemolysis) appears transparent regions at the center of the culture in Blood agar.

b. Not affected by the presence of oxygen, and it is active under aerobic and anaerobic conditions.

c. Heat-resistant.

d. The toxin is a protein with low molecular weight (28000 Dalton), its non-antigenic material which does not stimulate the formation of Antibody in serum.

e. Toxin is white blood cells killer causing cell death and lysis, the toxin is dissolved in serum. Toxin is responsible for decomposition zones (clear zone around the colonies growing on blood agar media.

3. Erythrogenic toxin:

a. Protein with low molecular weight (29000 Dalton) it stimulate antibody formation in small amounts.

b. Heat stable does not damage easily in high temperatures.

c. Toxin is responsible for the so-called (Skin rash: is a change of the skin which affects its color, appearance, or texture), this occurs with pharyngitis and tonsillitis, and may cause scarlet fever as well as named (Dick toxin) and examination called Dick test.

Dick test:

Is depending on the Erythrogenic toxin done by injecting (0.2 ml) of diluted toxin (1000 ml water /1 ml toxin) Intradermaly in human skin, the toxin is extracted from bacterial growth and diluted as above.

A positive result is the emergence of red swollen area with a diameter about (10 mm) within (6-24 hours) after injection.

Mechanical work of Dick test:

This toxin effects on human mononuclear cells where enhance the production of (Tumor necrosis factor) (TNF), which is responsible for the rise in body temperature and leading to toxic shock.

Lab 5

Shigella toxin

Causative bacterium: *Shigella dysenteriae*

Characteristics:

1- Bacteria belong to the family Enterobacteriacae and produce intestinal toxins (Exotoxins) that cause bloody diarrhea.
2- Negative to gram stain G-ve.
3- Rod shaped, coccobacilli bacteria, non-casuals.
4- Possess genes help to the invasion of epithelial cells in the intestine but does not have the genes helps them to spread into the bloodstream.
5- Acidity resistant, which can resist the acidity of stomach.

Types of toxins produced:

1. **Endotoxin (**Lipopolysaccharide toxins); all types of bacteria have the ability to produce this type of toxins, which are responsible for intestinal wall scratching causing injury associated with bleeding.
2. **Exotoxin** is consists of two parts:

   Part (A): enzymatically responsible for inhibit the process of protein synthesis.

   Part (B): It is responsible for the association of bacterial cells to receptors present on the special epithelial cells (target).

This type of toxins is protein, part (B) is associated with glycolipid to a host cell in the gastrointestinal tract and then part (A) prevents making of the protein lead to cell death of the epithelial and sabotage cells of capillary blood vessels of the intestine causing mild bleeding that comes with stool, losing carbohydrate and bicarbonates cause mild acidity of the blood and lead to the death of a patient.

Extraction of Shigella toxin

1. *Shigella dysenteriae* cultured on (Trypticase soy agar) media at 37**º**C for 24 hours.
2. Transfer bacterial colony to the Brain heart infusion broth and incubated at 37**º**C for 24 hours.
3. Centrifugation the broth where sediment is (bacterial content) and supernatant is (fluid) and then re-suspend the deposit (bacterial cells) in normal saline solution.
4. Washing the bacterial suspension (from step 3) by centrifuge it to obtain the sediment and drop the supernatant, this step is repeated 3 times.
5. Breaking the bacterial cells using sonication for 3 minutes, to extract the toxin out of the cell (in the sediment).
6. Centrifuge to precipitate broken bacteria which is neglected, where supernatant is taken and filtrate using precise filters (pore diameter 0.45 μ) to sterilize fluid toxin and get rid of broken bacterial cells remnants, if any.

Structure

The toxin has two subunits-designated A (mol. wt/32000 D) and B (mol. wt. 7700 D)-and is one of the AB5 toxins. The B subunit is a pentamer that binds to specific glycolipids on the host cell, specifically globotriaosylceramide (Gb3 Following this, the A subunit is internalized and cleaved into two parts. The A1 component then binds to the ribosome, disrupting protein synthesis. Stx-2 has been found to be about 400 times more toxic (as quantified by LD50 in mice) than Stx-1.

Gb3 is, for unknown reasons, present in greater amounts in renal epithelial tissues, to which the renal toxicity of Shiga toxin may be attributed. Gb3 is also found in central nervous system neurons and endothelium, which may lead to neurotoxicity Stx-2 is also known to increase the expression of its receptor GB3 and cause neuronal dysfunctions.

The toxin requires highly specific receptors on the cells' surface to attach and enter the cell; species such as cattle, swine, and deer which do not carry these receptors may harbor toxigenic bacteria without any ill effect, shedding them in their feces, from where they may be spread to humans.

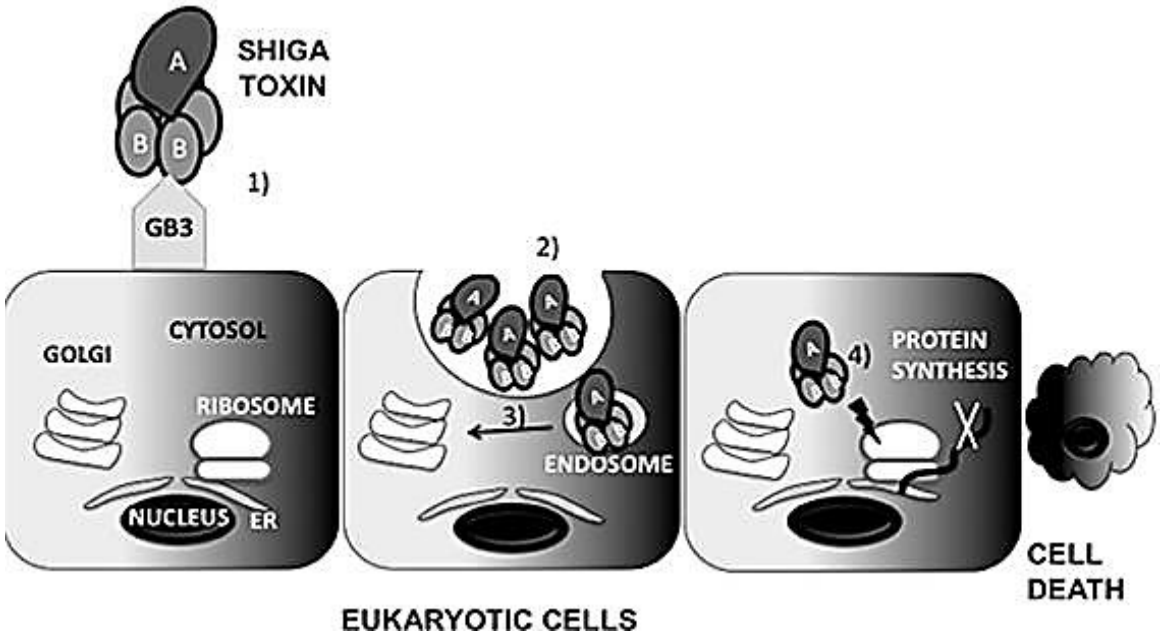
Mechanism

Shiga toxins act to inhibit protein synthesis within target cells by a mechanism similar to that of ricin. After entering a cell via a macropinosome, the protein cleaves a specific e adenine nucteobase from the 28S RNA of the 60S subunit of the ribosome, thereby halting protein synthesis.

The toxin acts on the lining of the blood vessels, the vascular endothelium. The B subunits of the toxin bind to a component of the cell membrane known asGb3 and the complex enters the cell. When the protein is inside the cell, the A subunit interacts with the ribosomes to inactivate them. The A subunit of Shiga toxin is an N-glycosidase that modifies the RNA.

Component of the ribosome to inactivate it and so bring a halt to protein synthesis leading to the death of the cell. The vascular endothelium has to continually renew itself, so this killing of cells leads to a breakdown of the lining and to hemorrhage. The first response is commonly a bloody diarrhea This is because Shiga toxin is usually taken in with contaminated food or water.

Interestingly, the bacterial Shiga toxin can be used for targeted therapy of gastric cancer, because this tumor entity expresses the receptor of the Shiga toxin. For this purpose, an unspecific chemotherapeutical is conjugated to the B-subunit to make it specific. In this way only the tumor cells, but not healthy cells, are destroyed during therapy.



Lab 6 Diphtheria toxin

Corynebacterium diphtheriae

Is a pathogenic bacterium that cause Diphtheria. It is also known as the **Klebs–Löffler bacillus**, because it was discovered in 1884 by German bacteriologists Edwin Klebs and Friedrich Löffler.

Characteristics:

Corynebacterium diphtheriae is a rod-shaped, Gram positive, non-spore forming, and non-motile bacterium, the ends of the bacilli either pointed or bulbous (مكور), catalase positive, no gas production due to sugars fermentation, acid resistant.

The virulence of bacteria is the production of Diphtheria exotoxin and the toxin is the only factor that causes the disease (Diphtheria: A thick gray coating accumulates in the nasopharyngeal region, making it difficult for the individual to breathe and swallow).

Colonial morphology:

Small, granular, shiny(برلق), gleaming (لامع) and irregular edges.

Bacteria do not have the ability to invasion, but remain in location such as in the nasopharyngeal passageways (ممرات البلعوم الأنفي), (localized infection) where secrete diphtheria toxin that spreads to distant places, causing the disease.

Features of diphtheria toxin:

1. A protein with a molecular weight of 63,000 Dalton

2. Toxin consists of two Fragments (A & B), Fragment A catalyzes the NAD^+-dependent ADP-ribosylation of elongation factor 2, thereby inhibiting protein synthesis in eukaryotic cells. Fragment B binds to the cell surface receptor and facilitates the delivery of fragment A to the cytosol.

3. All strains of *Corynebacterium* secrete Monotypic toxin thus any toxin of bacteria facilitates the formation of antibodies against toxin.

4. The toxin is sensitive to heat and formalin, it is stable for (4-6) weeks after formalin or heat treatment.

5. Toxin production in culture media requires some important factors, including:

a) **ph** limits between (7.8 -8).
b) Good Oxygen supply and peptone.
c) Iron is considered an important factor in toxin production, provided in concentration of (0.1 mg./ml.),and if increased to (0.5 mg. /ml) will inhibit toxin production.

Mechanical work of toxin: inhibiting protein synthesis in eukaryotic cells, thus it damaging the myocardial tissues and Nerve endings.

Pathogenicity (disease caused by poison): The incubation period of disease about (3-4) days, and it's considered a local infection in mucous membranes of throat, pharynx and nose. Infection process begins with removal of the surface layers of epithelial cells lining of the mucous membranes by secreting a little amounts of toxin to adjacent cells which leads to cells lysis within a few hours to make it suitable for the growth of more bacteria and more toxin production and spread of infection.

Features of infection:

The presence of fibrous abscess (تقيح ليفي) forming a thick gray membrane consists of fibers, WBCS and dead tissues of containing a large number of bacilli bacteria this membrane quickly turns into thick white opaque membrane called **pseudo membrane**, which cover the entire infected region leading to block the nasopharyngeal passageways and tonsils and cut air and oxygen O2 to the lungs causing patient death from suffocation.

Prevention:

Taking DPT vaccine: (DPT) Diphtheria, Pertussis, Tetanus

Differential diagnosis of diphtheria infection:

Must distinguish Diphtheria from the other diseases that give the same Clinical symptoms such as **tonsillitis**, **mumps**, salivary glands inflammation and **pseudomembrane** caused by Candida infection, ❶ this membrane could be removed easily by using forceps (ملقط) while Diphtheria cannot be removed, ❷ also to differentiate between them by taking swab and staining with Methylene blue which reveals In microscopic examination the (Blue granulated bacilli).

Toxigenicity tests:

Is an **in vitro immunoprecipitation (immunodiffusion) test** to determine whether or not a strain of ***Corynebacterium diphtheriae*** is toxigenic by conducting the following tests:

1. In vivo tests (inside the body)

These tests are conducted on laboratory animals and is used for this purpose, rabbit or guinea pig in two ways:

a- Intra dermal.

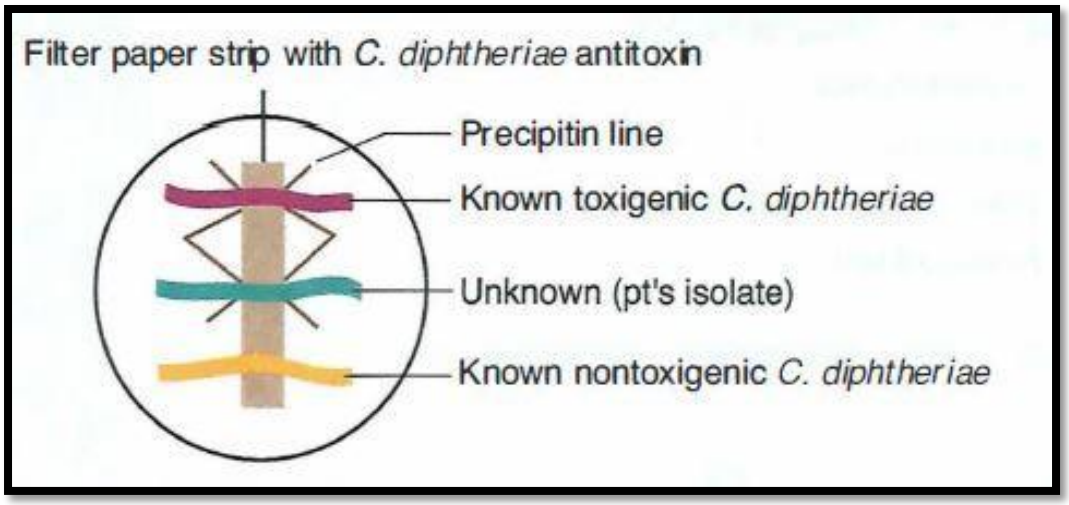
b- Subcutaneous.

This is done by injecting poison in small quantities in (a or b) areas, which leads to the appearance of the Necrosis at the injection site due to the effect of the poison and the appearance of symptoms and death of the animal.

2. In vitro tests (outside body) Elek's test screening:

Elek test is an **in vitro immunoprecipitation (immunodiffusion) test** to determine whether or not a strain of ***Corynebacterium diphtheriae*** is **toxigenic**. A test strip of filter paper containing diphtheria antitoxin is placed in the center of the agar plate. Strains to be tested (patient's isolate), known positive and negative toxigenic strains are also streaked on the agar's surface in a line across the plate and at a right angle to the antitoxin paper strip.

Antitoxin diffuses away from the strip of filter paper whereas toxin produced by toxin-producing strains diffuse away from growth. At the zone of equivalence a precipitin line is formed as illustrated in figure below.



Procedure:

1. Mix a tube of melted **nutrient agar** with 2 ml of sterile horse serum.
2. Rotate the tube to mix the serum and agar. Do not shake the tube.
3. Pour the mixture into a sterile petri dish.
4. Using lightly flamed forceps, lay the strip of anti-toxin impregnated filter paper across the centre of the petri dish allowing it to sink beneath the agar surface.
5. Allow the agar to set, then lift one corner of the lid and let the plate dry for 30-45 min in the incubator.
6. When dry inoculate with a toxigenic strain of *C. diphtheriae* by streaking a single line of inoculum across the plate and paper strip at right angles to the strip.
7. Repeat this about 1 inch away from the C. *diphtheriae* inoculum with a test strain.
8. Incubate the plate for 24 hrs and observe the results.

Result:

After 24 hours of incubation at 37°C, plate is examined with transmitted light for the presence of fine precipitin lines **at 45 degree angle to the streaks.**

Positive Test: Precipitin lines form at zone of equivalence, test organism is toxigenic (produce toxin).

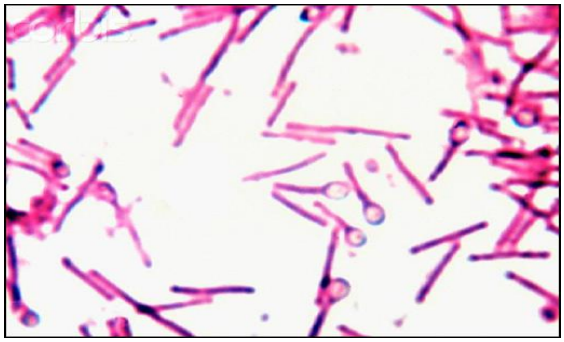
Lab 7 Tetanus toxin (Tetanospasmin)

Toxin-producing bacteria: *Clostridium tetani*

Characteristics:

1. Gram positive +ve, bacilli.

2. Drum stick appearance bacterium due to terminal spore diameter greater than the cell diameter.



3. Obligate anaerobic.

4. Motile, proteolytic (decompose protein), produces very effective exotoxin causes tetanus.

5. Existing in soil and feces and transmitted to the wounds through spores or any object contaminated with spores of *C.tetani* during surgical operations and then move to other areas of the body.

Toxins produced by the bacteria:

C.tetani produces two types of exotoxins only:

A- Tetanolysin:

❶Is sensitive to O2 and heat, ❷it is effective on many types of animal`s RBCs and decompose rabbit and horse RBCS ❸its role in pathogenicity is done by killing WBCs called (Leucotoxin). ❹ As well as a necroting agent and toxic to heart muscles (cardio toxin).

B- Tetanospasmin

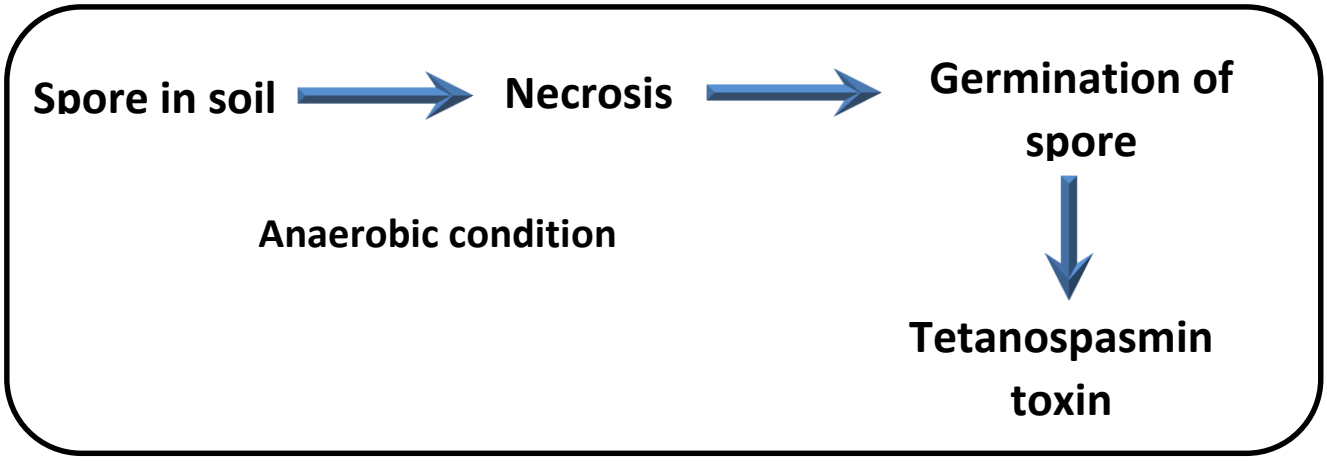
This toxin is responsible for pathogenicity, which is ❶ Heat labile protein, ❷Stable in oxygen, ❸ It's molecular weight 160,000 Dalton which makes it a good immunogenic, ❹ It is effective on CNS cells (brain and spinal cord) and less effective on peripheral nerves so that it is (Neurotoxin) affect the nerves system, ❺ its damaged by decomposing enzymes so its destroyed by gastric enzymes when orally intake.

Mechanism (mode of action):

The tetanus toxin produced as one protein which is subsequently cleaved into two parts: ❶ part(B) heavy or B-chain which bind to the target cell and deliver part(A) to the cell, ❷ and part(A) light or A-chain which is absorbed by the blood and goes to the central nervous system and in particular the spinal cord where bound to nerve and prevents the transmission of nerve signals from cell to another, and this prevent the brain from giving signals to muscles and vice versa, and cause muscle contraction.

Disease initiation:

spore in soil inters the body After a wound when anaerobic conditions are available in wounded area, spores germinate and form vegetative cells producing toxin causing necrosis in that region.



Symptoms of the disease:

Incubation period of tetanus is about 6-10 days. Shorter the incubation period, graver اخطر is the disease prognosis (the symptoms of disease). Muscles of the face and jaw are often affected first (due to shorter distances for the toxin to reach the presynaptic terminals).

Patients have prolonged muscle spasms of both **flexor** and **extensor muscles**. Patients with tetanus have spastic muscle contractions, difficulty opening the jaw (called lockjaw, "**trismus**"), a characteristic smile called "**risus sardonicus**" and contractions of back muscles resulting in backward arching (**Opisthotonos position)**. Patients are extremely irritable سريع الانفعال, and tetanic seizures develop, brought about by violent, painful muscle contractions following some minor stimulus, such as noise.

Flaccid paralysis vs. Spastic paralysis

Flaccid paralysis شلل مترهل occurs when the muscle cannot contract at all. The muscle stays weak and floppy.

Spastic paralysis شلل تشننجي occurs when the muscle stays in contraction. The muscle is too rigid and the patient cannot move the muscle properly. It causes muscles to twitch uncontrollably or spasm.

Laboratory Diagnosis made by two methods:

A-Culture

Culture is more reliable than microscopy.

Robertson cooked meat (RCM) broth – C. tetani being proteolytic turns the meat particles black and produces a foul odor.

Blood agar with polymyxin B: C. tetani produce characteristic swarming growth when incubated at 37°C for 24-48 hours under anaerobic conditions.

B-Toxigenicity Test

As pathogenesis of tetanus is toxin mediated, the association of the isolated organism can only be established when its toxin production is demonstrated. Toxigenicity can be detected by both in vitro and in vivo methods.

i. **In vitro hemolysis inhibition test:** *C. tetani* produces hemolysis on blood agar which is inhibited by adding antitoxin. This test indicates the production of tetanolysin only but not tetanospasmin.

ii. **In vivo mouse inoculation test:** RCM broth with black turbid growth is injected into the root of the tail of a test mouse. The test animal develops stiffness which begins with the tail and progresses to involve the hind limbs on the inoculated side- the other limb-trunk-forelimbs. Death occurs within two days. This test indicates the production of tetanospasmin.

Note:

The diagnosis of tetanus is clinical and does not require a demonstration of C.tetani, treatment should be started immediately based on clinical diagnosis. Laboratory diagnosis provides supportive evidence for confirmation.