CLOSTRIDIUM

Learning objectives

To know in detail about,

- Diseases caused by neurotoxic clostridia in domestic animals

- Morphology, cultural and biochemical characteristics of *Cl.tetani* and *Cl.botulinum*
- Classification of *Cl.botulinum'*
- Toxins and pathogenesis of *Cl.tetani* and *Cl.botulinum*
- Locked jaw, spinal typhus, Lamsiekte and Western duck sickness
- Distinguish toxins of *Cl.tetani* and *Cl.botulinum*
- General approaches used to demonstrate and identify neuro toxins of clostridium

SYSTEMATICS

Domain	Bacteria
Phylum	*Firmicutes*
Class	*Clostridia*
Order	*Clostridiales*
Family	*Clostridiaceae*
Genus	*Clostridium*

CLASSIFIACTION

- The clostridia can be divided into four major groups according to the kind of disease they produce. They are as follows.
- The Histotoxic clostridia causes a variety of tissue (often muscle) infections frequently following wounds or other trauma (eg).

C. chauvoei	Cattle, sheep (pigs)	Black quarter (Black leg)
C. septicum	Cattle Sheep Chicken	Malignant edema B raxy Necrotic dermatitis
C. novyi type A	Sheep Cattle and Sheep	Big head of rams Gas gangrene
Type B	Sheep (Cattle)	Black disease (necrotic hepatitis)
Type C	Water buffaloe	Osteomyelitis

- Hepatotoxic clostridia produces their toxins in the liver, thus resulting in the disease Bacillary haemoglobinuria and Black disease (Eg.).

C. haemolyticum (*C. novyi* type D)	Cattle, (sheep)	Bacilliary haemoglobinuria
C.sordellii	Cattle, Sheep, Horses	Gas gangrene
C. colinum	Birds	Quail disease,Ulcerative enteritis

C. piliforme	foals, laboratory animals Calves, dogs and cats	foals, laboratory animals Calves, dogs and cats

- The Enterotoxigenic clostridium produces mainly enterotoxaemia and food poisoning although they are occasionally histotoxic (Eg).

C. perfringens (C.welchii) Type A	Humans Lambs Broiler chickens	Food poisoning, gas gangrene Enterotoxaemic Jaundice (Yellow lambs disease) Necrotic enteritis
C. perfringens (C.welchii) Type B	Lambs (Under 3 weeks old)	Lamb dysentery
C. perfringens (C.welchii) Type C	Piglets, lambs, calves and foals Broiler chickens Adult sheep and goat	Haemorrhagic enterotoxaemia(Clostrid ial enteritis) Necrotic enteritis Struck
C. perfringens (C.welchii) Type D	Sheep(except neonates)	Pulpy kidney disease
C. perfringens (C.welchii) Type E	Calves and lambs	Enterotoxaemia

- The Neurotoxic clostridia cause the disease by the production of the potent exotoxins (Neurotoxins) (eg.)

C. tetani	Tetanus
C. botulinum	Botulism

FAMILY CHARACTERS

Classification

- They are pleomorphic, rod shaped; long filaments and involution forms are common.
- Spore formation occurs with varying frequency in different species.
- The shape and position of the spores vary in different species.
- The clostridia are motile with peritrichous flagella except *C.welchii* and *C.tetani* typeVI. *C.welchii* is capsulated, while others are not.
- Clostridia are anaerobic. *C.odematiens* are strict anaerobes and die on exposure tooxygen.
- *C.histolyticum* and *C.welchii* are aerotolerent and may even grow aerobically.
- The clostridia are fermentative, oxidase negative and catalase negative organisms.
- A very useful media for isolation of clostridia is Robertson's cooked meat broth.
- Clostridia grow in the medium, rendering the broth turbid most species produces gas.
- Saccharolytic species turn the meat pink – *C.odematiens, C.septicum, C.chauvoei* and *C.welchii*.

- Proteolytic species turn the meat black and produce foul and pervasive odour - *C.tetani, C. botulinum, C.haemolyticum.*
  In litmus milk medium, the production of acid, clot and gas can be detected

HISTORY

- Tetanus has been known from very early times, having been described byHippocrates.
- But the knowledge of the disease was achieved only in 1884.
- *Rosenbach –1886* - demonstrated a slender bacillus with round terminal spores in a case of tetanus.
- *Kitasato –1889* – isolated *C.tetani* in pure culture and reproduced the disease in animals by inoculation of pure culture.
- The Greek term -tetanus‖ which means ‗contracture' has been taken from the Latin medicine -rigor‖ .

HABITAT

- Soil, especially that contaminated by animal faeces, is the natural habitat as *C.tetani* is often transient in the intestines of horses and other animals.
- It is ubiquitous and has been recovered from a wide variety of other sources, including street and hospital dust, cotton wool, bandages, catgut, plaster of paris, clothing etc. It may occur as an apparently harmless contaminant in wounds.

MORPHOLOGY

- *C.tetani* is a straight, slender, Gram positive rod that characteristically produces a terminal, spherical endospores that bulges the cell giving the characteristic drumstick appearance. (The young spore may be oval rather than spherical).
- It occurs singly and occasionally in chains.
- It is non-capsulated and motile by peritrichous flagella.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS

Cultural characters

- Very strict anaerobe, grows at an opt.temp. of 37^{o}C and pH7.4.
- It grows on ordinary media and the growth is improved by blood and serum and not by glucose.
- Surface colonies are very difficult to obtain as the growth has a marked tendency to swarm over the surface of the agar especially if the medium is moist.
- The swarming nature /spreading can be inhibited by increasing the concentration of agar upto 3% (stiff agar).
- In this stiff agar individual rhizoid colonies are formed.
- On blood agar, it develops partially translucent, grayish colonies with filamentous edges giving a fuzzy appearance.
- On horse blood agar, alpha haemolysis is produced, which later develops into beta haemolysis due to the production of haemolysin (tetanolysin).
- *C.tetani* grows well in Robertson's cooked meat broth, with turbidity.
- The meat is not digested, but is turned black on prolonged incubation.
- In gelatin stab cultures a fir tree type of growth occurs, with slow liquefaction.
- A greenish fluorescence is produced on media containing neutral red.

Biochemical properties

- *C.tetani* has feeble proteolytic property, so it does not ferment any sugars.
- It forms indole. It is MR and VP negative, nitrates not reduced.

RESISTANCE

- The endospores are highly resistant and while boiling kills the spores of most strains in 15mts.
- Autoclaving at 121°C for 15mts and dry heat temp of 150°C for more than one hour is completely sporicidal.
- Spores are able to survive in soil for years and they are resistant to most antiseptics.
- They are not destroyed by 5% phenol or 0.1% mercuric chloride solution in two weeks or more.
- Iodine (1% aqueous solution) and H2O2 kill the spores within a few hours.

ANTIGENICITY

- Ten serological types have been recognized based on the flagellar antigen (types I to X). Type VI contains non flagellated strains.
- All types produce the same neurotoxin- tetanospasmin. Which can be neutralized by one common antitoxin.
- They have a common heat stable somatic antigen shared by all types.
- A second somatic antigen is shared by type II, IV, V and X.

TOXINS

- *C.tetani* produces atleast two distinct toxins.
  - Tetanospasmin
  - Tetanolysin
- They are antigenically and pharmacologically distinct and their production is mutually independent.
- A third toxin – a nonspasmogenic peripherally active neurotoxin has been identified recently.
- It is not known whether this plays any role in the pathogenesis of tetanus.

Tetanolysin

- It is a heamolysin or cytotoxin causing lysis of rabbit and horse RBC'S.
- It is heat and oxygen labile, similar to those of streptolysin O, d toxin. (*C. oedematiens*) and j toxin (*C. welchii)*.

Tetanospamin

- It is a very potent neurotoxin responsible for the clinical manifestations of tetanus.
- It is oxygen stable, but relatively heat labile, being inactivated at 65°C in 5mts.
- The highly purified form gets toxoided spontaneously.
- It is a good antigen and is specifically neutralized by the antitoxin. The toxin exists in two forms.
- A monomer of MW 68000, which is toxic and a dimer is which is non toxic butantigenic.
- The purified toxin is active in extremely small amounts and it contains about $3x10^7$ minimum mouse lethal dose per mg protein.

- There is considerable variation in the susceptibility of different species of animals to the toxin.
- The horses and human are the most susceptible.
- Guinea pigs, mice, goats and rabbits are susceptible in that descendingorder . Birds and reptiles are highly resistant.

PATHOGENESIS

- *C.tetani* has little invasive power. The endospores enter traumatized tissue or surgical wounds, especially after castration or docking, via the umbilicus or into the uterus, following dystocia in cattle and sheep.
- The spore implanted in a wound can germinate and multiply only if the conditions are favourable.
- Destruction and necrosis of tissue, lack of drainage in the area, presence of extraneous matter especially of soil, all create anaerobic conditions and favour germination of *C.tetani* spores.
- The resultant vegetative cells multiply at the site and produce the potenttetanospasmin.
- This travels via peripheral nerves or blood stream to ganglioside receptors of the motor nerve terminals and eventually to cells of the ventral horn of the spinal cord, thus affecting many groups of muscles at various levels.
- The toxin acts presynaptically on motor neurons, blocking synaptic inhibition and causing a spastic paralysis and the characteristic tetanic spasms.
- Tetanospasmin binds specifically to gangliosides in nerve tissue and once bound cannot be neutralized by antitoxin.
- When toxin travels up to a regional motor nerve in a limb, tetanus first develops in the muscles of that limb, then spreads to the opposite limb and moves upwards.
- This is known as ascending tetanus and is usually seen only in the less susceptible animals such as dogs and cats.
- Descending (generalized) tetanus is the common form in susceptible species such as human and horses.
- In this form toxin circulating in the blood stream affects the susceptible motor nerve centers that serve the head and neck first and later the limbs. Once established, signs of tetanus are similar in all animals.

PATHOGENICITY

Symptoms

- It is influenced by several factors, such as the site and nature of the wound, the dose and toxigenicity of the contaminating organism.
- The incubation period is variable from 2 days to several weeks but is commonly 6-12days.
- Initial symptoms include mild stiffness and unwillingness to move. This may proceed to with head, neck and tail becoming rigid.
- Mild twitching of muscles develops into obvious spasms of muscles, which can occur in response to sudden noises, animal fall over to one side and unable to rise.
- In the terminal stages the rigidity of muscles extend from the limbs to the trunk, nostrils get dilated, earserect , nictitating membrane protruded and mastication becomes impossible because the mouth cannot be opened – hence called Locked Jaw**.**
- Respiration becomes shallow and rapid before final respiratory failure.

Lesions::No characteristic lesion for this disease but there may be a superficial wound which has developed from accidental injury or from surgery.

DIAGNOSIS

Direct microscopy

- Demonstration of characteristic drumstick spores of *C.tetani* by Gram stained smears of material from a wound (but it is not confirmative,

because *C.tetanomorphum* and *C.tetanoides* also produce drumstick spores).

Isolation

- Necrotic tissue from a wound or wound exudates can be heated to 80°C for 20mts and used to inoculate a blood agar plate and another blood agar plate containing stiffagar.
- A tube of thioglycollate medium or cooked meat broth could also be inoculated and sub cultured into blood agar.
- The plates are to be incubated anaerobically for 2-3 days. Growth is noticed using a hand lens as a filamentous growth spreading through out the medium.
- The edges of this growth give pure culture on sub cultivation.
- Confirmation is done by identification of toxin.
- The toxin present in animal's serum or in filtrate from cooked meat broth or thioglycolate medium can be inoculated in to mice S/C or I/M-ly and identified by neutralization or protection tests using specific antitoxin.

CONTROL AND PREVENTION

- The disease is due to the action of the toxin, and hence, the obvious and most dependable method of prevention is to build up antitoxic immunity by activeimmunization.
- Theavailable methods of prophylaxis are
  - Surgical attention
  - Antibiotics
  - Immunization – passive, active or combined.
- Surgical attention aims at removal of foreign bodies, necrotic tissue and blood clots, inorder that an anerobic environment favourable for the tetanus bacillus is not provided.
- Flushing with hydrogen peroxide in the wound area is produces aerobic conditions.
- Tetanus can be prevented by antibiotics (Large doses of penicillin) when administered 4hrs after infection but not after eight hrs. Hence, prompt administration is essential.
- Bacitracin or neomycin may be applied locally.
- Penicillin can be given as both injections and orally till healing is established. Antibiotics have no action on the toxin.
- Antitoxin should be administered promptly, either i/vly or into the sub arachnoid space, on three consecutive days to neutralize unbound toxin.
- Toxoid may be given subcutaneously topromote an active immune response even on those animals, which have received antitoxin.

For prevention, the farm animals should be vaccinated routinely with tetanus toxoid.

HISTORY

- The name botulism is derived from sausage (botulus, latin for sausage), an article of food that used to be associated with the type of food poisoning.

- *C.botulinum* was first isolated by Van Ermengam (1896) from a piece of ham that caused an outbreak of botulism.
- *C. botulinum* denotes a group of bacteria that produce extremely potent neurotoxins.
- These toxins cause botulism , a disease characterized by flaccid paralysis in many animals and humans.
- Botulism is most common in water birds, ruminants, horses, mink and poultry.
- Botulism in animals has been called by a variety of names,
    - Horses: Spinal typhus / Shaker foal syndrome
    - Cattle: Lamsiekte, loin disease and contagious bulbar paralysis
    - Water fowl: Limber neck, alkali poisoning and western duck sickness
- Botulism is rare in domestic cats. Pigs and dogs are relatively resistant.

HABITAT

- The endospores are widely distributed in soils and aquatic environment through out the world.
- The disease botulism is mainly due to the ingestion of preformed toxin.
- Germination of the endospores, with growth of vegetative cells and production of toxin, occurs in anaerobic situations such as contaminated cans of meat, fish or vegetables, carcases of invertebrate and vertebrate animals, rotting vegetation and baled silage.

MORPHOLOGY

- Gram +ve, straight rods, occur in single, pairs or occasionally in chains.
- Spores are oval, wider than the bacilli, situated centrally, terminally or subterminally.
- Non-capsulated, motile by peritrichous flagella.

CULTURAL AND BIOCHEMICAL CHARACTERISTICS

- It is a strict anaerobe, opt.temp is 30-37^{o} C and pH is 7-7.6. Good growth occurs on ordinary media.
- Surface colonies are large, irregular, and semitransparent with fimbriate border.
- Spores are produced consistently when grown in alkaline glucose gelatin media at 20 to 25 ^{o}C.
- In horse blood agar, large transparent colonies with irregular edges are developed with a narrow zone of haemolysis.
- On sheep blood agar *C.botulinum* produce beta-haemolysis, the colonies are slightly domed with a ragged edge.
- In cooked meat medium, the proteolytic strains type A, B and F produces blackening of meat and non-proteolytic strains C, D and E do not blacken meat.
- Gelatin liquefied rapidly by type A&B and slowly or not at all by type C, D and E.

RESISTANCE

- *Clostridium botulinum* spores are highly resistant, surviving several hours at 100^{o}C and for upto 10mts at 120^{o}C.
- But spores can be killed at 121^{o}C for 15mts, while the toxins are destroyed at 100^{o}C for 20mts.

ANTIGENS AND TOXINS

C.botulinum possesses a number of H, O and spore antigens

- Eight types of *C.botulinum* have been identified (Types A to G) based on the immunological difference in the toxins produced by them.
- These neurotoxins are identical in pharmacological action but differ in potency, distribution and antigenicity.
- They are neutralized only by the homologus antiserum.

Type	Toxin produced	Most susceptible animals	Sources of toxin	Disease
A	A	Humans, chickens, pigs	Vegetables, fruits, meat and fish	Food borne botulism
Cα	C1	Waterfowl	Invertebrate carcases, rotting vegetation and material on refuse dumps	Limberneck in long necked birds
Cβ	C2	Cattle, horses, mink, dogs	Carcases, baled silage, chicken manure as feed supplement	Forage poisoning
D	D	Cattle, sheep	Eating contaminated bones and carcases of small mammals (Phosphorus deficiency-Pica)	Lamsiekte

- The toxins differ from other exotoxins in that it is not released during the life of the organism.
- It is appears in the medium only on the death and autolysis of the cell.
- It is believed to be synthesized initially as a nontoxic protoxin or progenitor toxin.
- Trypsin and other enzymes activate progenitor toxin to active toxin.
- The toxin is heat labile and its mol.wt. is 70,000.
- One mg of neurotoxins contains more than 120 million mouse lethal doses.
- The lethal dose for human is 1-2µg. This toxin acts slowly taking several hours to kill.

Comparison of the toxins of *C.tetani* and *C.botulinum*

	C.tetani	*C.botulinum*
Site of toxin production	Wounds	Carcases, decaying vegetation and occasionally wounds and intestine

Mode of action	Centrally by blocking synaptic inhibition	Peripherally by blocking neuromusculartransmission
Type of paralysis	Spastic paralysis	Flaccid paralysis
Antigenic types of toxin	Tetanospasmin (one antigenic type)	Eight different toxins produced by types A-G

PATHOGENESIS

- *C.botulinum* is non invasive and virtually non infectious. Botulism is of three types.

Food borne botulism

- It is due to the ingestion of preformed toxin in foodstuffs.
- The toxin is adsorbed from the intestinal tract and is transported via the blood stream to peripheral nerve cells, where it binds to susceptible cells and suppresses the release of acetylcholine at the myoneural junctions.
- This result in flaccid paralysis , death being caused by circulatory failure and respiratory paralysis.

Wound botulism

- The spores are introduced into wounds where they germinate.
- Toxin is formed at this localized site and spreads through the body.
- The shaker foal syndrome in horse is thought to be caused in this way.

Infant botulism

- It occurs when spores are ingested in food and get germinate in the intestines when the normal flora has not been fully established.
- This form is seen in human infants (Floppy baby syndrome) and as rare epidemics of type C in broiler chickens and turkey poults.

PATHOGENICITY

Symptoms

- In cattle, the incubation period varies between 2-10 days depending upon the dose of toxin ingested.
- Initially there will be excitation, followed by incoordination and paralysis of the hind limbs.
- There will be paralysis of muscles in the mouth, pharynx and neck, resulting in the animal being unable to swallow and the tongue protruding from the mouth. This is followed by death.
- In South Africa this condition is termed as lamsiekte in cattle caused by type D, especially in the phosphorus deficient animals.

- In poultry it results with the ingestion of type C toxin and the disease is known as duck sickness or western duck disease and Limberneck in chicken.
- The symptoms include paralysis of the wings, legs and neck, protrusion of nictitating membrane, diarrhoea and comatase before recovering in 5-6 days time.

Lesions

- Pathological changes are noticed in the CNS, especially the brain stem and 3rd ventricle, catarrhal gastroenteritis, hepatitis and nephrosis

DIAGNOSIS

- The diagnosis of botulism is based on history, clinical signs and demonstration and identification of toxin in serum of moribund or recently dead animals as well as the detection of toxin and /or *C.botulinum* in the suspected foodstuff.

Toxin demonstration

- Serum or centrifiuged serum exudates from animals can be directly inoculated i/v ly (0.3ml) or i/p ly (0.5ml) into mice.
- If toxin is present the characteristic wasp waist appearance in the mice will be seen in a few hrs or upto 5 days.
- The appearance is due to abdominal breathing because of paralysis of respiratory muscles.
- Extraction of toxin in foodstuffs is accomplished by grinding the material in saline.
- The suspension is centrifuged and the supernatant is filtered through a 0.45µm filter.
- As the toxin can be in a protoxin form 9 parts of filtrate are treated with one part of 1% trypsin solution and incubated at 37°C for 45mts.
- Mice or guinea pigs are inoculated intra-peritoneally.

Toxin identification

- Mouse (or guinea pig) neutralization tests using a polyvalent antitoxin initially, followed by monovalent antitoxin.

Isolation of *C.botulinum* from foodstuffs

- Several samples of the foodstuffs are macerated in a small amount of physiological saline.
- The suspension is heated at 65-80°C for 30mts to kill most of the contaminating organism and to induce the *C.botulinum* spores to germinate.
- Blood agar plates are inoculated with the suspension and incubated under Co_2 at 35°C for up to 5 days.
- To determine whether the isolate is a toxin producing strain, a cooked meat broth is inoculated and incubated at 30°C for 5-10 days.
- Filtrates are prepared and lab. animals can be used for demonstration and identification of the toxin.

CONTROL AND PREVENTION

- Neutralization by polyvalent antiserum is effective.

- Therapeutic agents such as tetraethylamide and guanidine hydrochloride, whichenhance transmitter release at neuromuscular junctions, may be of valuewhen given intravenously.
- Immunization is not followed. In South Africa attempts were made by giving twoinjections of types C and D toxoid at an interval of several weeks.
- Bivalent & Trivalent antitoxins areavailable .