PHARMACEUTICAL MICROBIOLOGY

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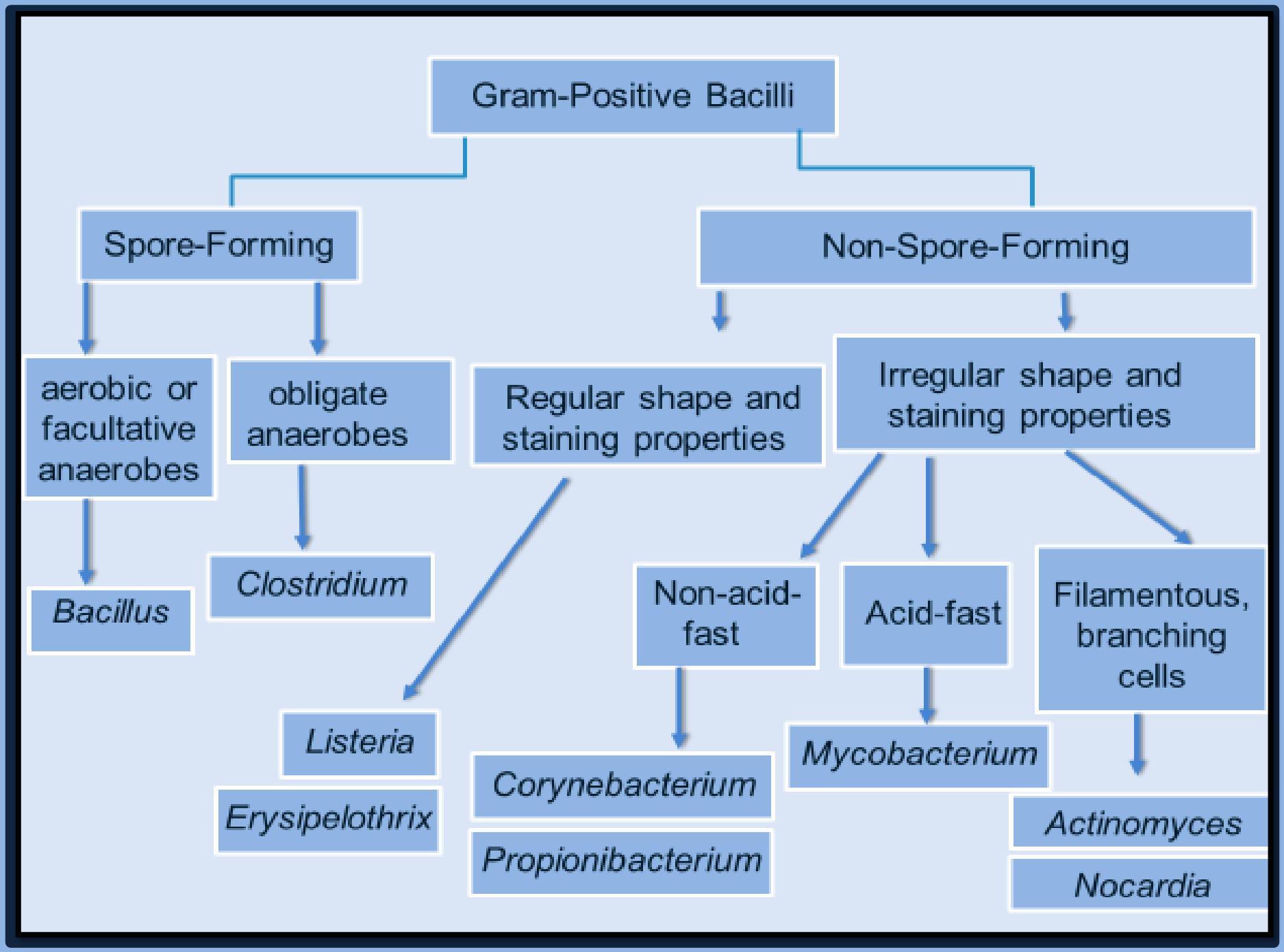

Gram-Positive Bacilli
Spore-Forming
Non-Spore-Forming
aerobic or facultative anaerobes
obligate anaerobes
Regular shape and staining properties
Irregular shape and staining properties
Bacillus
Clostridium
Listeria
Erysipelothrix
Non-acid-fast
Acid-fast
Filamentous, branching cells
Corynebacterium
Propionibacterium
Mycobacterium
Actinomyces
Nocardia

OBJECTIVES

Spore-Forming Gram-Positive Bacilli:

- **Clostridium**
  - *Clostridium perfringens*
  - *Clostridium tetani*
  - *Clostridium botulinum*
  - *Clostridium difficile*

Clostridium

- The genus Clostridium is extremely heterogeneous, and more than 200 species have been described.

- Most clostridia are harmless saprophytes, but some are well-recognized human pathogens.

- They are **obligate anaerobes** capable of producing endospores. Most species grow only in the complete absence of oxygen.

Clostridium

- Clostridium are found in soil, water, and the intestinal tracts of humans and other animals.

- They cause several important **toxin-mediated diseases**.

- The major toxins produced by the species are **neurotoxins** affecting nervous tissue, **histotoxins** affecting soft tissue and **enterotoxins** affecting the gut.

Clostridium

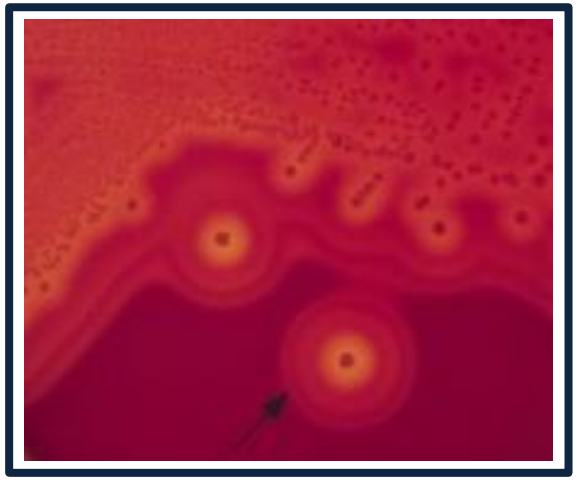
- **Clostridium grows in anaerobic conditions**; Bacillus grows in aerobic conditions.

- **Clostridium forms bottle-shaped endospores**; Bacillus forms oblong endospores.

- **Clostridium does not form the enzyme catalase**; Bacillus secretes catalase to destroy toxic by-products of oxygen metabolism.

Clostridium

- Clostridia can ferment a variety of sugars; many can digest proteins. These metabolic characteristics are used to divide the Clostridia into groups, **saccharolytic** or **proteolytic.**
- Many clostridia produce a zone of **β-hemolysis** on blood agar.
- ***C. perfringens*** characteristically produces **a double zone of β-hemolysis around colonies.**



Anaerobic culture of *Clostridium perfringens* on blood agar

https://www.pinterest.es/pin/291326669631450707/

Clostridium

- ***Clostridium tetani*** is the cause of **tetanus**
- ***C. botulinum*** is the cause of **botulism**
- ***C. perfringens***, ***C. septicum***, ***C. histolyticum*** and ***C. novyi*** are the causes of **gas gangrene** and other infections.
- ***C. perfringens*** is also associated with a form of **food poisoning.**
- ***C. difficile*** is the cause of **pseudomembranous colitis** and **antibiotic-associated diarrhea**.

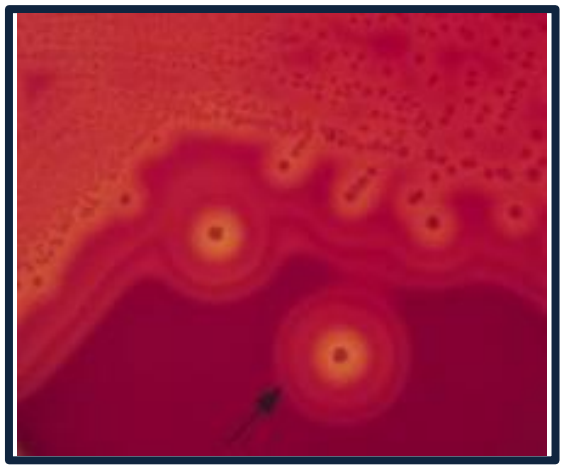
Clostridium perfringens

- *Clostridium perfringens* is a Gram-positive, rod-shaped, **anaerobic, spore-forming** pathogenic bacterium that is normally found in the intestines of humans and animals.

- It is **nonmotile**, but rapidly spreading growth on laboratory media (resembling the growth of motile organisms) is characteristic.

Clostridium perfringens

- On blood agar, it produces a characheristic **double zone of β-hemolysis** around colonies (a narrow transparent zone and a wide shadowy zone)
- It is mainly **saccharolytic** and **produces acid and gas from milk**, producing a '**stormy clot**' in a test tube (**Litmus Milk Test**)



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https://www.muhadharaty.com/lecture/16482/

Clostridium perfringens

- *C. perfringens* produces a number of **potent toxins** (at least 12 toxins) and **enzymes**.

- *C. perfringens* is categorized into five serotypes (**A, B, C, D,** and **E**) depending on the types of extracellular toxins (**alpha, beta, epsilon**, and **iota toxins**) they make.

Lethal Toxins				
	Alpha	**Beta**	**Epsilon**	**Iota**
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

Distribution of Lethal Toxins in *C. perfringens* Types A to E

Clostridium perfringens

- **Alpha (α) toxin** is the most important toxin which is produced by all five types of *C. perfringens*, is a **lecithinase (phospholipase C)** that lysis erythrocytes, platelets, leukocytes and endothelial cells.

- This toxin mediates massive **hemolysis, increased vascular permeability** and **bleeding** (augmented by destruction of platelets), **tissue destruction** (as found in myonecrosis), **hepatic toxicity** and **myocardial dysfunction** (bradycardia, hypotension).

Clostridium perfringens

- The largest quantities of **Alpha toxin** are produced by *C. perfringens* type A.
- **Beta (β) toxin** is responsible for intestinal statis, loss of mucosa with formation of the necrotic lesions, and progression to **necrotizing enteritis.**
- **Epsilon (ε) toxin** is a protoxin. It is **activated by trypsin** and increases the vascular permeability of the gastrointestinal wall.
- **Iota (ι) toxin** has **necrotic activity** and increases vascular permeability.

Clostridium perfringens

- The *C. perfringens* **enterotoxin** is produced primarily by type A strains. The **heat-labile toxin** is **susceptible** to **pronase**. Exposure to trypsin enhances toxin activity threefold.

- The enterotoxin also acts as a **superantigen*** simulating T lymphocyte activity.

(**Superantigens (SAgs**) are a class of antigens that cause non-specific activation of T-cells resulting in polyclonal T cell activation and massive cytokine release. SAgs are produced by some pathogenic viruses and bacteria most likely as a defense mechanism against the immune system)

Clostridium perfringens

Virulence factors associated with *C. perfringens*

Virulence Factors	Biologic Activity
Alpha	Lethal toxin; **phospholipase C (lecithinase)**; increases vascular permeability; hemolysin; produces necrotizing activity, as seen in myonecrosis
Beta	Lethal toxin; necrotizing activity
Epsilon	Lethal toxin; permease
Iota	Lethal binary toxin; necrotizing activity; adenosine diphosphate (ADP) ribosylating
Lambda	Protease
Mu	Hyaluronidase
Enterotoxin	Alters membrane permeability in ileum (cytotoxic, enterotoxic), superantigen
Neuraminidase	Alters cell surface ganglioside receptors; promotes capillary thrombosis

Clostridium perfringens

- Type A *C. perfringens* commonly inhabits the intestinal tract of humans and animals and is widely distributed in nature, particularly in soil and water contaminated with feces.

- **Type A** *C. perfringens* is responsible for most human infections, including **soft-tissue infections, food poisoning and primary septicemia.**

- **Type C** *C. perfringens* is responsible for one other important infection in humans - **enteritis necroticans**.

- Spores are formed under adverse environmental conditions and can survive for prolonged periods.

Clostridium perfringens

Soft tissue infections caused by *C. perfringens* are subdivided into:

- **cellulitis**
- **fasciitis (suppurative myositis)**
- **myonecrosis (gas gangrene)**

Clostridium perfringens

- Clostridial **gas gangrene** is a highly lethal necrotizing soft tissue infection of skeletal muscle caused by **toxin- and gas-producing** Clostridium species.

- Toxins of *C. perfringens* kill human muscle cells, causing necrosis (**myonecrosis**). Death of these cells creates an even more suitable anaerobic condition where the organism can grow rapidly and release gas, hence the disease is known as '**gas gangrene**'. Infected and discolored blood inside the wound and under the skin turns the infected area to black.

Clostridium perfringens

- Gas gangrene can be caused by other, less common clostridia, including *C. novyi, C. septicum, C. histolyticum.*

- Gas gangrene is treated by surgical debridement of infected areas and the use of high doses of penicillin given intravenously.

Clostridium perfringens

- Some strains of *C. perfringens* produce **enterotoxins** which cause **food poisoning**.

- Clostridial food poisoning is characterized by;
  - a short incubation period (8 to 24 hours)
  - a clinical presentation that includes abdominal cramps and watery diarrhea but no fever, nausea or vomiting
  - a clinical course lasting 24 to 48 hours

- Disease results from the ingestion of meat products (e.g. beef, chicken, turkey) contaminated with large numbers (10^8 to 10^9) of enterotoxin-containing type A *C. perfringens*.

Clostridium perfringens

Holding contaminated foods at temperatures below 60 ^{0}C (46 ^{0}C is optimal) allows spores that survived the cooking process to germinate and multiply to high numbers.

The refrigeration of food after preparation prevents this bacterial growth. Alternatively, reheating of the food can destroy the heat-labile enterotoxin.

Clostridium perfringens

- The microscopic detection of gram-positive rods in clinical specimens, usually in the **absence of leukocytes**, can be a very useful finding because these organisms have a characteristic morphology.

- It is also relatively simple to culture these anaerobes.

- *C. perfringens* can be detected on simple media after incubation for 1 day or less.

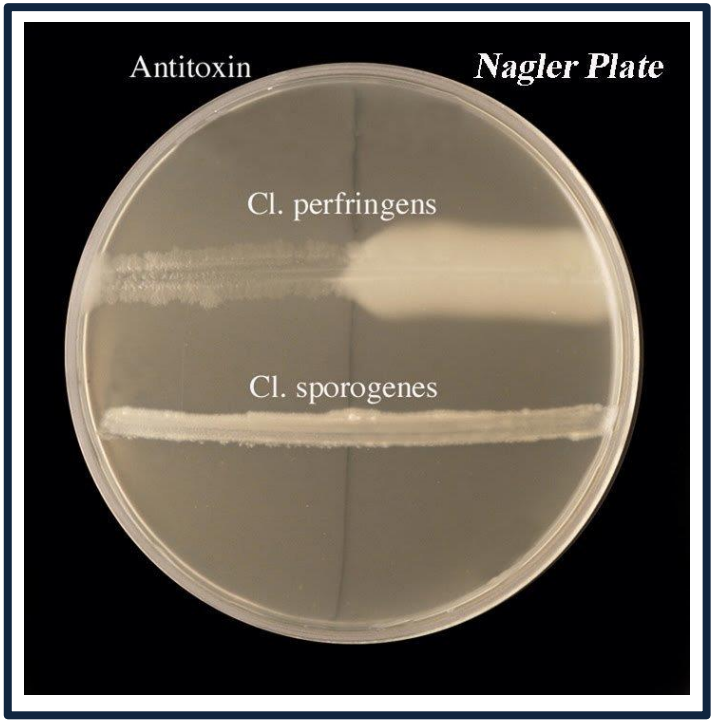
Clostridium perfringens

- The role of *C. perfringens* in food poisoning is documented by recovery of **more than 10^5 bacteria per gram of food** or **more than 10^6 bacteria per gram of feces** collected within 1 day of the onset of disease.

- Immunoassays have also been developed for detection of the **enterotoxin in fecal specimens**; however, clostridial food poisoning is a clinical diagnosis, and culture or immunoassays are not commonly used for this diagnosis.

Clostridium perfringens

- C. perfringens can be diagnosed by **Nagler's Reaction** where the suspect organism is cultured on an egg yolk media plate. One side of the plate contains anti-alpha-toxin, while the other side does not.
- A streak of suspect organism is placed through both sides. An area of turbidity will form around the side that does not have the **anti-alpha-toxin**, indicating uninhibited **lecithinase activity**.



https://microbeonline.com/nagler-reaction-lecithinsae-test-principle-procedure-results-limitations/

Clostridium perfringens

- *C. perfringens* soft-tissue infections, such as suppurative myositis and myonecrosis, must be treated aggressively with **surgical and high-dose penicillin therapy**.

- Antibiotic therapy for clostridial food poisoning is unnecessary because this is a self-limiting disease (i.e. the diarrhea washes the bacteria out of the intestines, and the normal intestinal flora reestablishes itself). **Food poisoning caused by *C. perfringens* enterotoxin usually requires only symptomatic care.**

Clostridium perfringens

- Exposure to *C. perfringens* is difficult to avoid because the organisms are ubiquitous.

- Disease requires introduction of the organism into **devitalized tissues** and **maintenance of an anaerobic environment favorable for bacterial growth.**

- Thus proper wound care and the judicious use of prophylactic antibiotics can do much to prevent most infections.

Clostridium tetani

- *Clostridium tetani* is a Gram-positive, **rod-shaped, anaerobic, motile, spore-forming** pathogenic bacterium.

- *C. tetani* which causes **tetanus**, is worldwide in distribution in the soil and in the feces of horses and other animals.

- These bacteria produce round, **terminal spores** that give it the appearance of a **drumstick**.

Clostridium tetani

- Unlike *C. perfringens*, ***C. tetani*** **is difficult to grow** because the organism is **extremely sensitive to oxygen toxicity**, and when growth is detected on agar media, it typically appears as a film over the surface of the agar rather than discrete colonies.

Clostridium tetani

- Although the vegetative cells of *C. tetani* die rapidly when exposed to oxygen, spore formation allows the organism to survive in the most adverse conditions.

- Several types of *C. tetani* can be distinguished by specific **flagellar antigens**. All share **a common O (somatic) antigen**, which may be masked, and all produce the same antigenic type of **neurotoxin, tetanospasmin**.

Clostridium tetani

- *C. tetani* produces two toxins, an **oxygen-labile hemolysin (tetanolysin)** and a **plasmid-encoded, heat-labile neurotoxin (tetanospasmin).**

- The **plasmid carrying the gene** for **tetanospasmin** is **nonconjugative**, so a **non-toxic *C. tetani* strain cannot be converted to a toxigenic strain.**

Clostridium tetani

- **Tetanolysin** is serologically related to **streptolysin O** and ***C. perfringens*** and ***Listeria monocytogenes*** **hemolysins**.

- The clinical significance of this enzyme is unknown, however, **because it is inhibited by oxygen and serum cholesterol.**

- **Tetanospasmin** is produced during the stationary phase of growth, is released when the cell is lysed, and is responsible for the clinical manifestations of tetanus.

Clostridium tetani

- **Tetanospasmin** is a protein and synthesized as a single polypeptide chain. On release from the bacterium, the peptide is split by an endogenous protease into two chains:

  - a light chain (A) and **a heavy chain (B)**.

- The two chains are joined by noncovalent forces of a **disulfide bond**.

- **The purified toxin is extremely potent.**

Clostridium tetani

- The **toxin acts by preventing the release of neurotransmitters,** such as **gamma-aminobutyric acid (GABA), glycine** etc., thereby specifically **blocking synaptic inhibition in the spinal cord.** This leads to unregulated spread of impulses, inhibited anywhere in the central nervous system.

- **Tetanospasmin** inactivates proteins that regulate release of the inhibitory neurotransmitters glycine and GABA. This leads to unregulated excitatory synaptic activity in the motor neurons resulting in **spastic paralysis.**

Clostridium tetani

- *C. tetani* is ubiquitous. It is found in soil and transiently colonizes the gastrointestinal tracts of many animals, including humans.

- The vegetative forms of *C. tetani* are extremely susceptible to oxygen toxicity, but the organisms sporulate readily and can survive in nature for a long time.

- Disease is relatively rare in developed countries because of the high incidence of **vaccine-induced immunity**.

Clostridium tetani

- The incubation period for tetanus varies from a few days to weeks.
- The duration of the incubation period is directly related to the **distance of the primary wound infection from the central nervous system**.
- *C. tetani* causes tetanus, which can be of the following types:
  - **Generalized Tetanus**
  - **Localized Tetanus**
  - **Neonatal Tetanus**

Clostridium tetani

- **Generalized Tetanus**; is the most common form. Involvement of the masseter muscles (trismus or lockjaw) is the presenting sign in most patients.

- The characteristic sardonic smile that results from the sustained contraction of the facial muscles is known as **risus sardonicus**.

- Other early signs are drooling, sweating, irritability and persistent back spasm (opisthotonos).

Clostridium tetani

- Another form of *C. tetani* disease is **Localized Tetanus**; in which the disease remains confined to the musculature at the site of primary infection.

- A variant is **cephalic tetanus**, in which the primary site of infection is the head.

Clostridium tetani

Neonatal Tetanus is typically associated with an initial infection of the umbilical stump that progresses to become generalized.

The mortality in infants exceeds 90% and developmental defects are present in survivors.

Clostridium tetani

- The **diagnosis of tetanus**, as with that of most other clostridial diseases, is **made on the basis of the clinical presentation.**
- The microscopic detection of *C. tetani* or recovery in culture is useful but frequently unsuccessful.
- Culture results are positive in only approximately 30% of patients with tetanus.
- **Neither tetanus toxin nor antibodies to the toxin are detectable in the patient** because the toxin is rapidly bound to motor neurons and internalized.

Clostridium tetani

- The highest mortality is in newborns and in patients in whom the incubation period is shorter than one week.

- Treatment of tetanus requires debridement of the primary wound, use of metronidazole, passive immunization with human tetanus immunoglobulin and vaccination with tetanus toxoid.

- Wound care and metronidazole therapy eliminate the vegetative bacteria that produce toxin, and the antitoxin antibodies work by binding free tetanospasmin molecules.

Clostridium tetani

Vaccination with a series of three doses of tetanus toxoid, followed by booster doses every 10 years, is highly effective in preventing tetanus.

https://www.who.int/immunization/programmes_systems/interventions/TT_and_VMMC/en/

Clostridium botulinum

- *Clostridium botulinum*, the etiologic agent of **botulism**, is a **fastidious, spore-forming, anaerobic rods**.
- These bacteria are **subdivided into four groups** based on phenotypic and genetic properties.
- **Seven antigenically distinct botulinum toxins (A to G)** have been described. **Human disease is associated with types A, B, E and F.**

Group	Neurotoxin Type	Phenotypic Properties
I	A, B, F	Proteolytic, saccharolytic
II	B, E, F	Nonproteolytic, saccharolytic
III	C, D	Weakly proteolytic, saccharolytic
IV	G	Weakly proteolytic, asaccharolytic

Clostridium botulinum

- Other species of clostridia produce botulinum toxins, including ***Clostridium butyricum*** **(type E toxin),** ***C. baratii*** **(type F toxin)** and ***C. argentinense*** **(type G toxin).**

- *C. botulinum* is worldwide in distribution; it is found in soil and occasionally in animal feces.

- Spores of the organism are highly resistant to heat, withstanding 100°C for several hours. Heat resistance is diminished at acid pH or high salt concentration.

Clostridium botulinum

- During the growth of C. botulinum and during autolysis of the bacteria, toxin is liberated into the environment.
- Types A, B, E, and F are the principal causes of human illness.
- Types A and B have been associated with a variety of foods and type E predominantly with fish products.
- Botulinum toxin (Botox) is a **neurotoxic protein**. It **prevents the release of the neurotransmitter acetylcholine** from axon endings at the neuromuscular junction and thus causes **flaccid paralysis**.

Clostridium botulinum

- Botulinum toxin is absorbed from the gut and binds to receptors of presynaptic membranes of motor neurons of the peripheral nervous system and cranial nerves.

- **Proteolysis**—by the light chain of botulinum toxin—of the **target SNARE proteins in the neurons inhibits the release of acetylcholine at the synapse**, resulting in **lack of muscle contraction and paralysis.**

- The SNARE proteins are synaptobrevin, SNAP 25, and syntaxin.

Clostridium botulinum

- ***C. botulinum* toxins are among the most toxic substances known.**
- The lethal dose for a human is probably about 1–2 µg/kg.
- The toxins are destroyed by heating for 20 minutes at 100°C.
- **The toxin is also used commercially in medicine, cosmetics and research.**
- *C. botulinum* is commonly isolated in soil and water samples throughout the world.

Clostridium botulinum

Four forms of botulism have been identified:

- **Foodborne Botulism:** Most are associated with the consumption of **home-canned foods (types A and B toxins)** and occasionally with the consumption of preserved **fish (type E toxin)**. The food may not appear spoiled, but even a small taste can cause full-blown clinical disease.

  Patients with foodborne botulism typically become weak and dizzy 1 to 2 days after consuming the contaminated food. The initial signs include blurred vision with fixed, dilated pupils, dry mouth, constipation and abdominal pain.

Clostridium botulinum

- **Foodborne Botulism:** Fever is absent. Bilateral descending weakness of the peripheral muscles develops in patients with progressive disease (**flaccid paralysis**), and death is most commonly attributed to respiratory paralysis.

Clostridium botulinum

- **Infant Botulism:** This type of botulism has been associated with the consumption of foods (**particularly honey**) contaminated with botulinum spores.

  In contrast with foodborne botulism, the disease is caused by neurotoxin produced in vivo by *C. botulinum* colonizing the gastrointestinal tracts of infants.

  Although adults are exposed to the organism in their diet, *C. botulinum* can not survive and proliferate in their intestines. In the absence of competitive bowel microbes, however, the organism can become established in the gastrointestinal tracts of infants.

Clostridium botulinum

- **Infant Botulism:** The disease typically affects infants younger than 1 year (most between 1 and 6 months), and the symptoms are initially nonspecific (e.g. constipation, weak cry or failure to thrive).

Progressive disease with flaccid paralysis and respiratory arrest can develop; however, mortality in documented cases of infant botulism is very low (1% to 2%).

Clostridium botulinum

- **Wound Botulism:** This type of botulism is rarely seen. It develops from toxin production by C. botulinum in contaminated wounds. Although the symptoms of disease are identical to those of food borne disease, the incubation period is generally longer (4 days or more), and the gastrointestinal tract symptoms are less prominent.

- **Inhalation Botulism:** This type of botulism is important in terms of bioterrorism. Botulinum toxin has been concentrated for purposes of aerosolization as a biologic weapon.

Clostridium botulinum

- **Botulism** confirmed by isolating the organism or detecting the toxin in food products or the patient's feces or serum.

- Isolation of *C. botulinum* from specimens contaminated with other organisms can be improved by heating the specimen for 10 minutes at 80 ^{0}C to kill all non-clostridial cells.

- Culture of the heated specimen on nutritionally enriched anaerobic media allows the heat-resistant *C. botulinum* spores to germinate.

Clostridium botulinum

Patients with botulism require the following treatment measures:

- adequate ventilator support
- elimination of the organism from the gastrointestinal tract, through the judicious use of gastric lavage and metronidazole or penicillin therapy
- the use of trivalent botulinum antitoxin versus toxins A, B and E to bind toxin circulating in the bloodstream.
- ventilator support is extremely important in reducing mortality.

Clostridium botulinum

- Disease is prevented by destroying the spores in food, preventing spore germination (by maintaining the food in an acid pH or storage at 4 ^{0}C or colder), or destroying the performed toxin (all botulinum toxins are inactivated by heating at 60 ^{0}C to 100 ^{0}C for 10 minutes).

- **Infant botulism** has been associated with the consumption of honey contaminated with *C. botulinum* spores, so **children younger than 1 year should not eat honey.**

Clostridium difficile

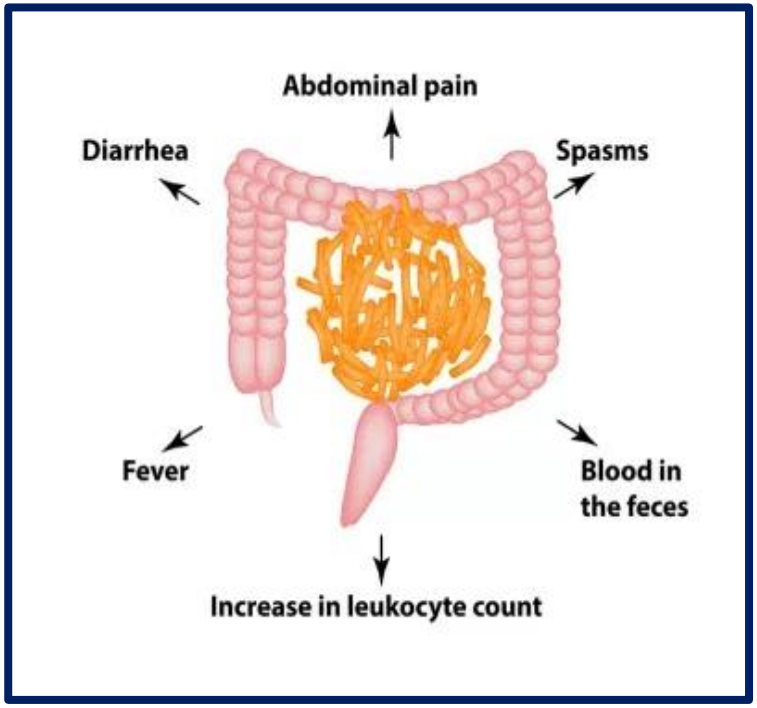
- *Clostridium difficile* are spore-forming, Gram-positive, **strict anaerobe** (vegetative cells are extremely oxygen sensitive) rods.

- *C. difficile* is naturally present in the gut, or intestinal tract. Healthy people are not usually affected by *C. difficile*.

- However, some antibiotics may alter the balance of good bacteria in the gut, allowing this bacteria to multiply. Then it can cause **diarrhea** and possibly more serious illness.

Clostridium difficile

C. difficile is responsible for **antibiotic-associated gastro intestinal diseases** ranging from a relatively benign, **self-limited diarrhea** to severe, life-threatening **pseudo membranous colitis.**



https://www.davidwolfe.com/c-diff/

Clostridium difficile

Clostridium difficile produces two toxins, an **enterotoxin (toxin A)** and a **cytotoxin (toxin B)**.

- The **enterotoxin** binds to the brush border membranes of the gut at receptor sites. It is **chemotactic for neutrophils**, stimulating the infiltration of polymorphonuclear neutrophils into the ileum with release of cytokines. It also produces a **cytopathic effect**, resulting in disruption of the tight cell-cell junction, increased permeability of the intestinal wall, and subsequent **diarrhea**.

Clostridium difficile

- **Toxin B is a potent cytotoxin.**

- Both toxins are usually found in the stools of patients with pseudomembranous colitis. However, toxin A–negative, toxin B–positive infections have been described.

Main clinical symptoms of *C. difficile* infection include; watery diarrhea, fever, loss of appetite, nausea and abdominal pain/tenderness.

Clostridium difficile

- The diagnosis of *C. difficile* infection is confirmed by **demonstration of the enterotoxin or cytotoxin in a stool specimen** from a patient with compatible clinical symptoms.
- The enterotoxin and cytotoxin can be detected with a number of commercial immunoassays.
- Isolation of the organism in stool culture documents colonization but not disease.
- Discontinuation of the implicated antibiotic (e.g. ampicillin, clindamycin) is generally sufficient to alleviate mild disease.

Clostridium difficile

- However specific therapy with metronidazole or vancomycin is necessary for the management of severe diarrhea or colitis.

- Relapses may occur in as many as 20% to 30% of patients after the completion of therapy, because only the vegetative forms of *C. difficile* are killed by the antibiotics; the spores are resistant.

- A second course of treatment with the same antibiotic is frequently successful.