

ANAEROBIC GRAM POSITIVE SPORE FORMING BACILLI

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This group represents the genus Clostridium , Clostridia are Gram positive spore forming ,motile except *Clostridium perfringens* grow anaerobically.

The Natural habitat is soil, dust , and the intestinal tract of animals and humans where they live as saprophytes.

- **Among pathogens are organisms causing botulism, tetanus and gas gangrene . Most clostridia decompose protein , produce toxin or do both .**

The most important species are :

Cl. perfringens causes gas gangrene

Cl. botulinum causes botulism

Cl. tetani causes tetanus.

Cl. defficile causes pseudomembranous colitis.

Morphology of anaerobic bacilli :

This group represents large gram positive bacilli , spores are usually wider than the diameter of the bacillus and they are bulging , some of them are located centrally , sub-terminal or terminal .

Cultivation And Growth

They grow anaerobically because:

1- They are unable to utilize oxygen as final acceptor.

2-They lack cytochrome and cytochrome oxidase, constituents of respiratory chain required for aerobic respiration.

3-They are unable to break down Hydrogen peroxide, as an end product for aerobic respiration because they are lacking peroxidase enzyme.

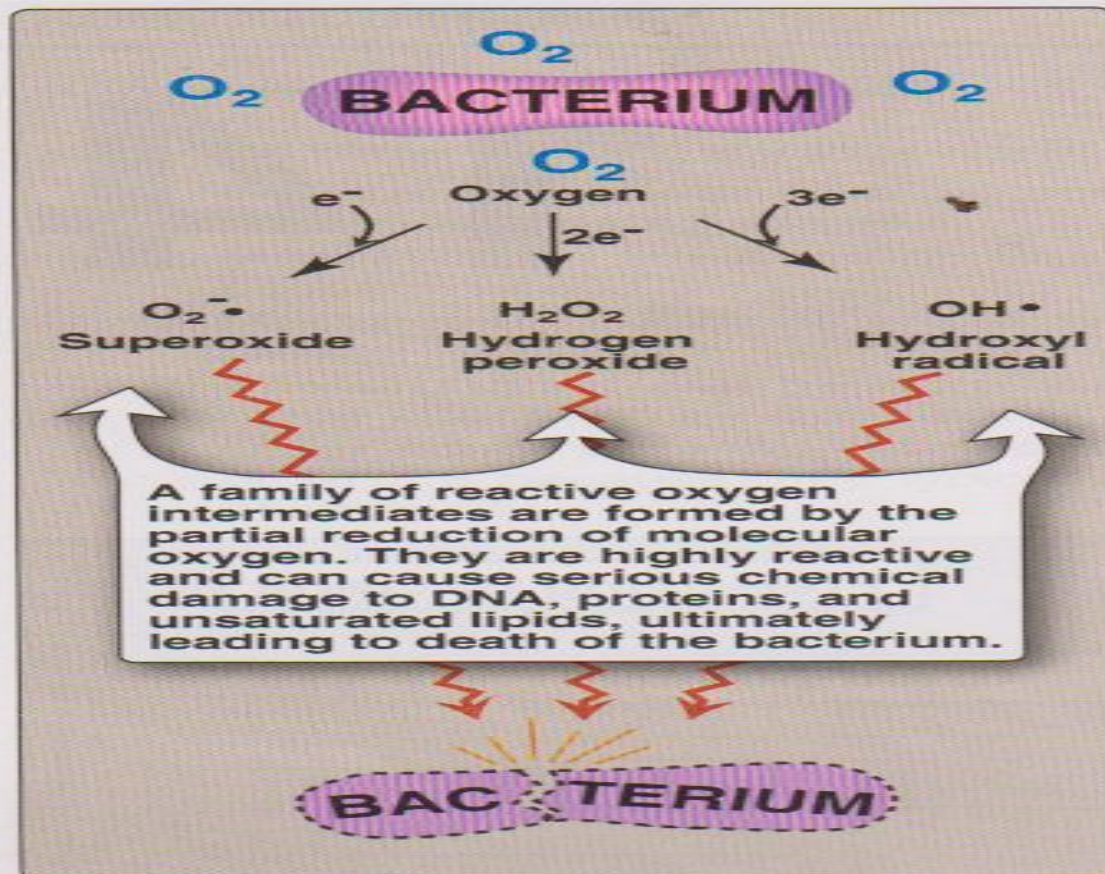


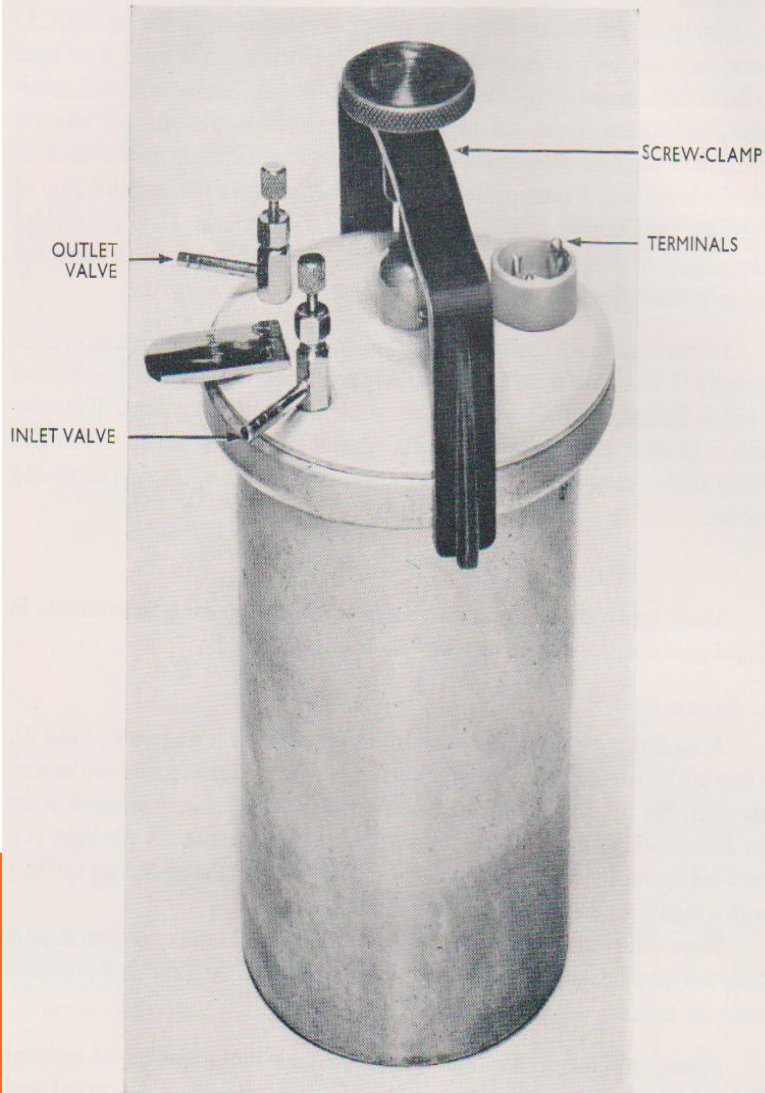
Figure 14.2

Toxic effects of reactive oxygen intermediates on anaerobic bacteria.

Methods Of Anaerobiasis:

1-Anaerobic Jar, agar or culture tubes are placed in empty tightly closed jars evacuated from air then carbon dioxide and nitrogen are pumped into the jar.

2-Gas pack kit, Co₂ gas generating kit through certain chemical reaction between chemicals can be used instead of the above mentioned method, this kit is employed with suitable gas pack jar .



Anaerobic jar with lid *in situ*.



The simplicity of the 'GasPak' system of generating hydrogen and carbon dioxide is shown here; the foil envelope is opened by peeling back the corner to a printed line and 10 ml. of water is injected. The envelope is immediately placed upright in the anaerobic jar, the lid replaced and screwed down and the jar is then ready for incubation.

3-Fluid media containing reducing substances like Thiol are used for the growth of anaerobes with no need for co₂ source.



Colony morphology and chemical activities:

Clostridial organisms are different in their colony morphology, some are showing large flat colonies while others are showing dome shape medium sized colonies, at the same time they are different in their hemolytic activities.

Majority of these bacteria has toxigenic and different enzymatic activities.

Clostridium perfringens

Previously it was known as *Cl. welchi*

It is widely found in nature, it is found in soil, water and intestine of human and animals as normal flora .

It is large Gram positive rod with sub-terminal non bulging spores non motile capsulated in the host tissue.

THE BASICS

Gram positive

Rod-shaped

Non-motile

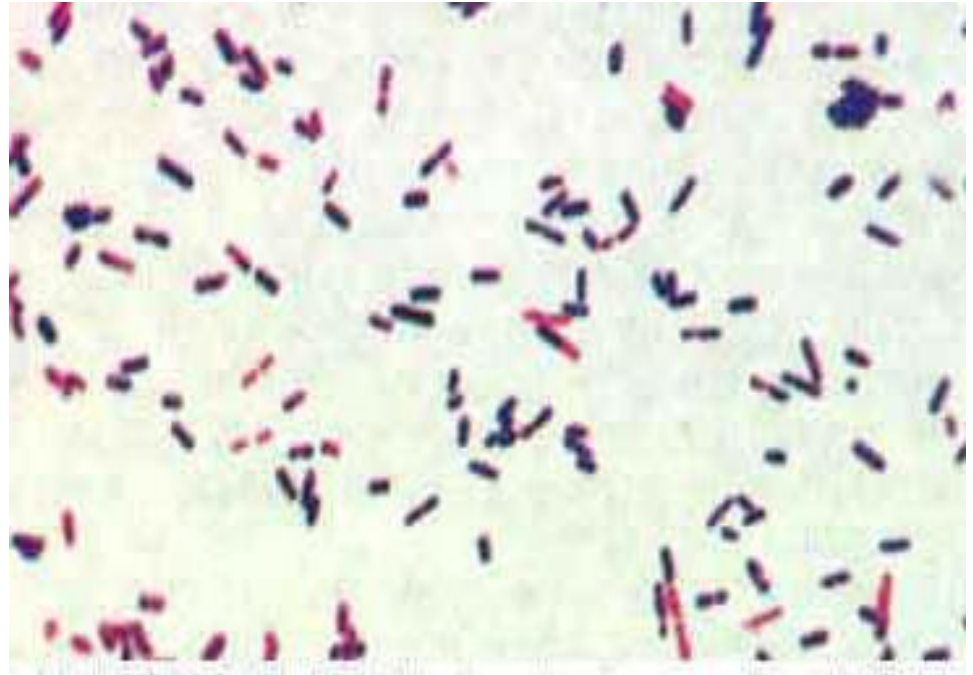
Anaerobic

Five types of strains

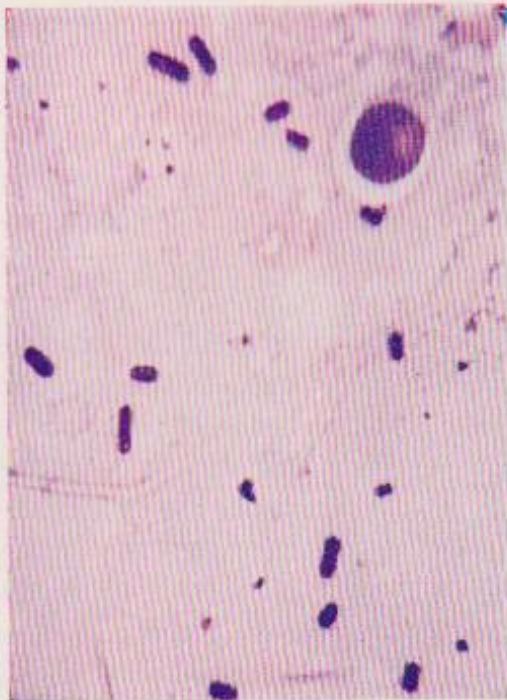
- **A - E**

Four lethal toxins

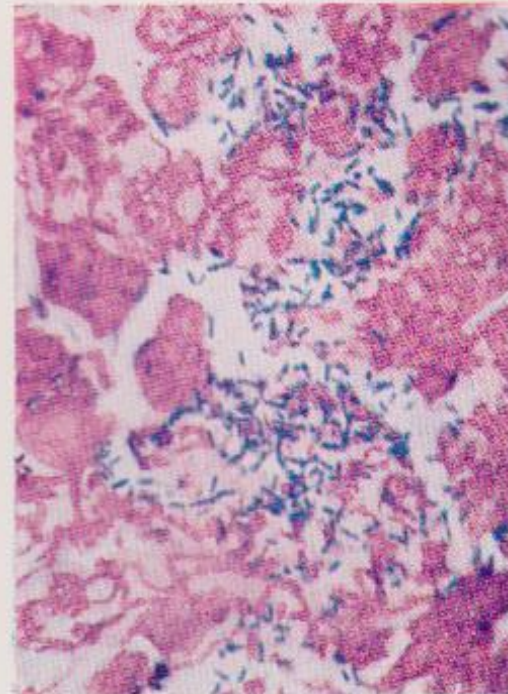
- **Alpha, Beta, Epsilon and Iota**



Microscopy. Relatively large bacilli, 5μ by 1μ , with square or rounded ends. Gram-positive, non-motile (all other *Clostridia* are motile), capsulate in animal tissues. Spores are oval, subterminal and non-projecting.



Two Gram-stained preparations showing *Cl. welchii*. On the left is a smear made directly from muscle in a case of gas gangrene; on the right is a section of muscle from the same case. Separation of the muscle fibres, which are also oedematous, by gas production can be noted. $\times 1000$, $\times 500$.



Cultural appearances. Not as strictly anaerobic as *Cl. tetani*; grows very

Growth characters :

It grows anaerobically on blood agar and show a double zone of beta hemolysis on blood agar. Colonies are dome shape with entire borders.

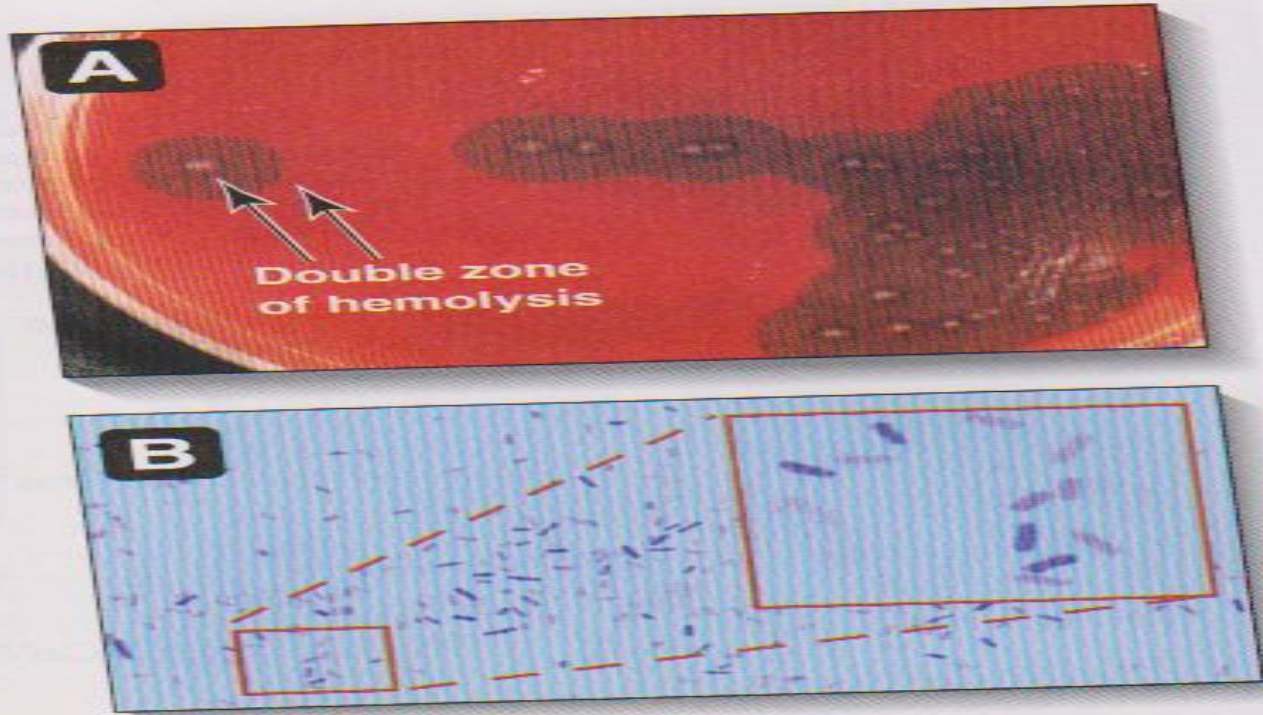


Figure 14.5
Clostridium perfringens. A. Colonies on blood agar showing double zone of hemolysis. B. Photomicrograph of Gram stain.

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Toxins And Enzymes Of Cl.perfringens

Alpha toxin acts as **lecithenase enzyme** and responsible for the severity and toxemia of **Cl. Perfringens**, **it causes RBC lyses and superimposed for Naglers reaction *in vitro*.**

Alpha-toxin and Beta-toxins
Gas gangrene – necrotizing cell membrane

The Lethal Toxins

Epsilon-toxin:

- **Increases intestinal permeability causing vascular damage and oedema in major organs**
- **Liver damage**
- **Higher blood pressure**

Iota-toxin

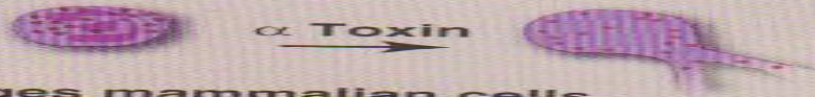
- **Food-borne illness**

Enzymes Of *Cl.perfringens*

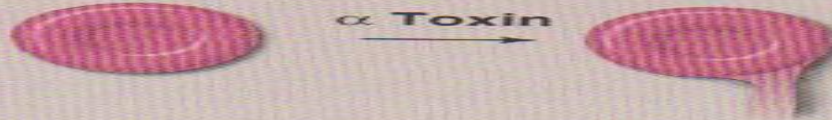
In addition to these toxins ,this organism produce different enzymes such as hyalouronidase , DNAase, collagenase

Clostridium perfringens

Exotoxins

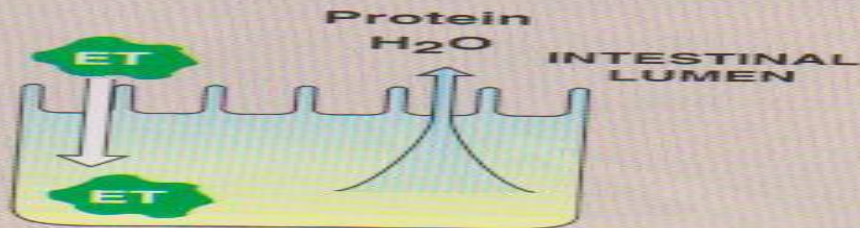


Damages mammalian cells, resulting in the release of lysosomal enzymes.



Lyses erythrocytes, leukocytes, and platelets.

Enterotoxin

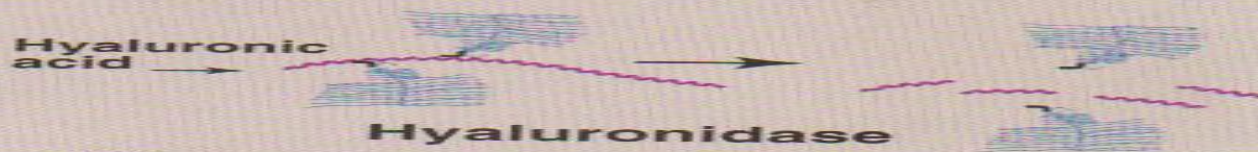


Toxin alters cell membrane, leading to loss of fluid and intracellular proteins.

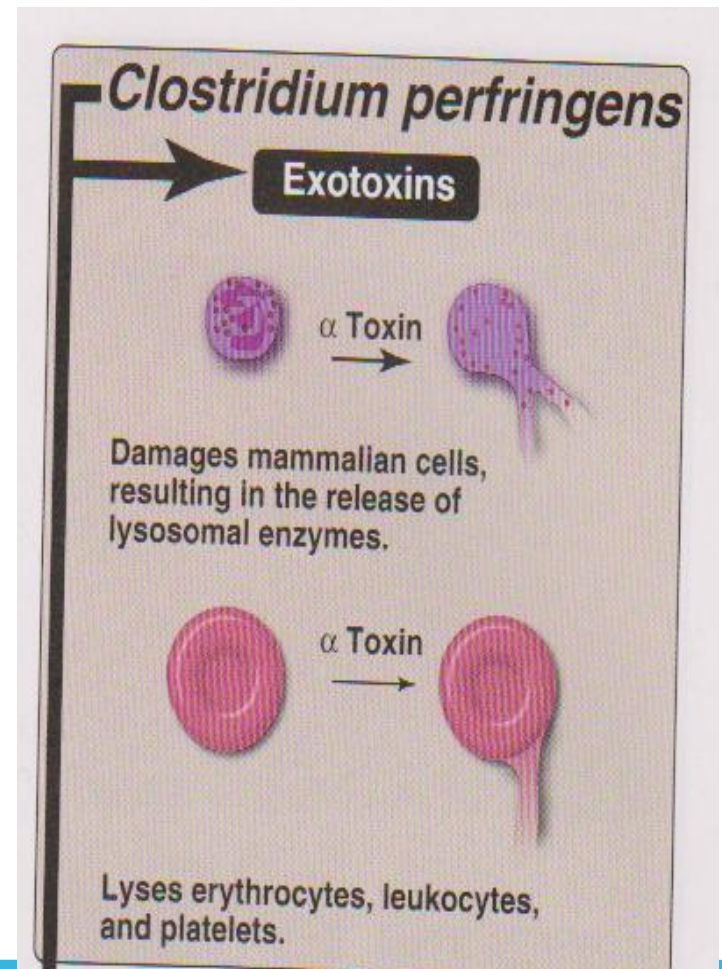
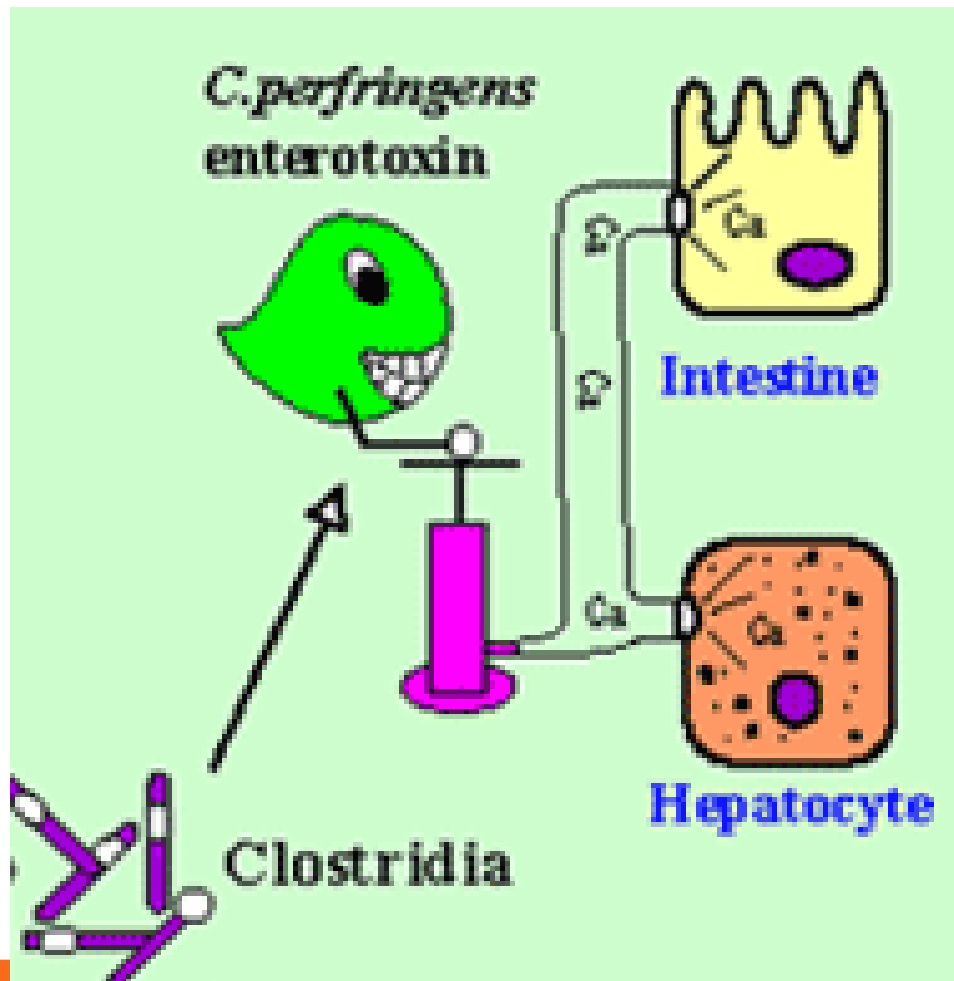
Other degradative enzymes

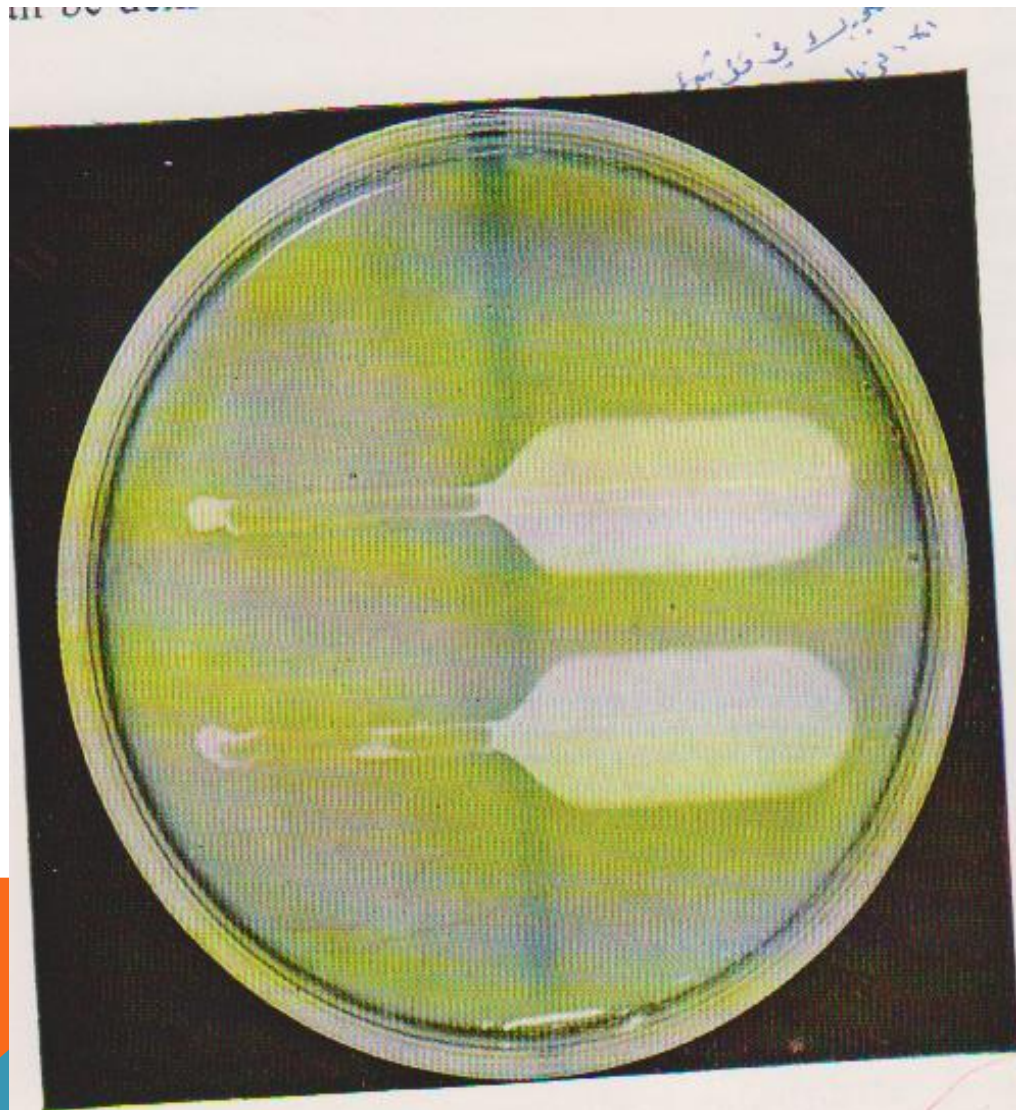


DNase degrades the viscous DNA in necrotizing tissue or exudates, aiding the spread of infection.



Disrupts the organization of ground substance, facilitating spread of infection.





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الکلی



N. wj
2 5% of egg
3 drops of a

Nagler's reaction. The medium comprises nutrient agar with 5 per cent. egg yolk. Three drops of *Cl. welchii* type-A antitoxin were smeared over the left half of the plate as marked by the blue line; a culture of *Cl. welchii* was streaked in two parallel lines across the surface of the medium and the plate then incubated anaerobically at 37°C for 20 hr. *Lecithinase activity produces zones of opacity on the antitoxin-free half of the plate and this activity has been inhibited in the presence of antitoxin on the left half.

(all types) and

Pathogenesis :

The spores reach the traumatized area from soil or intestine, then it will germinate to vegetative cells .These cells multiply and ferment carbohydrates present in tissue producing gas.

Distension of tissue and interference with blood supply, together with the necrotizing toxin and hyalouronidase favor spread of infection and tissue necrosis.

This necrosis increase bacterial growth, hemolytic anemia and severe toxemia and death.

***Cl. perfringens* occurs in the genital tract of 5% of women, clostridial uterine infections may follow instrumental abortion.**

THE LETHAL TOXINS

Epsilon-toxin

- **Increases intestinal permeability causing vascular damage and oedema in major organs**
- **Liver damage**
- **Higher blood pressure**

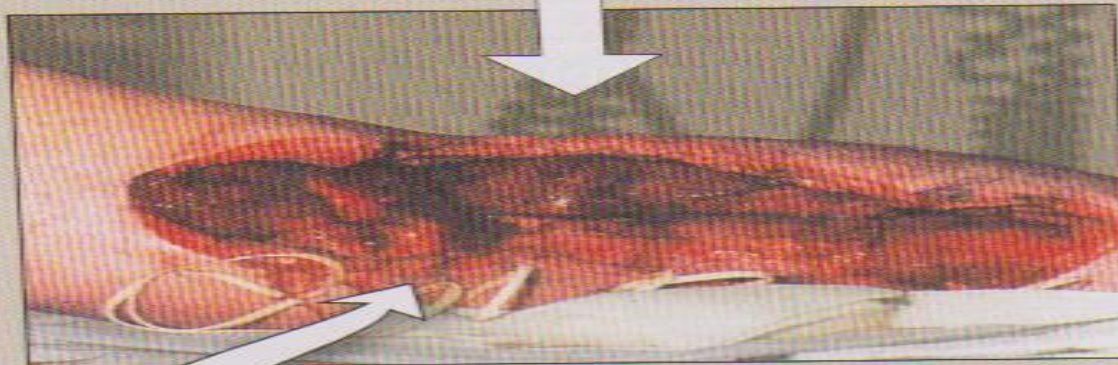
Iota-toxin

- **Food-borne illness**

Alpha-toxin and Beta-toxins

- **Gas gangrene – necrotizing cell membranes**
- **Food-borne illness**

Gas gangrene of 5 cm superficial laceration across the left antecubital fossa (depression in front of the elbow) on presentation 3 days after injury.



Following surgical debridement.

Figure 14.4
Gas gangrene of arm.

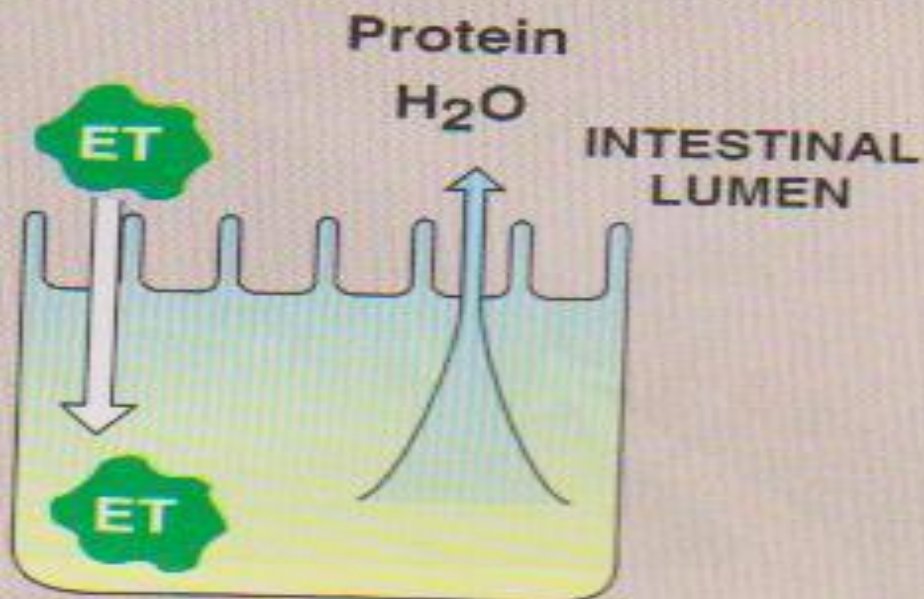
**Clostridial infection is frequent in patients with neoplasms.
Type C involved in necrotizing enteritis in children.**



***Cl .perfringens* in adults causes food poisoning due to enterotoxin causes fluid hypersecretion in intestine.**

This food poisoning usually follows ingestion of in warmed meat dish containing high number of this organisms.

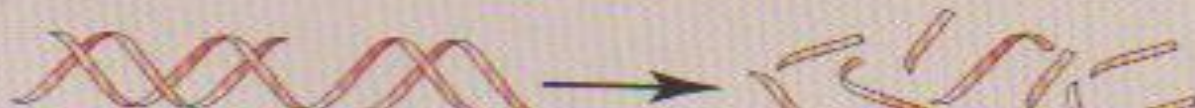
Enterotoxin



Toxin alters cell membrane, leading to loss of fluid and intracellular proteins.

Other degradative enzymes

DNA



Enterotoxin

Most common mediator for food-borne illnesses

Can tolerate >100°F temperatures for more than 1 hour

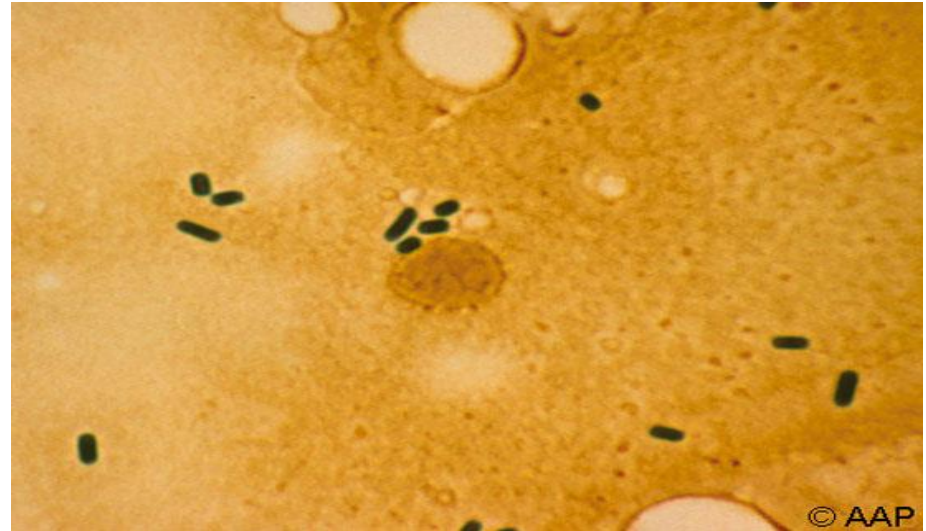
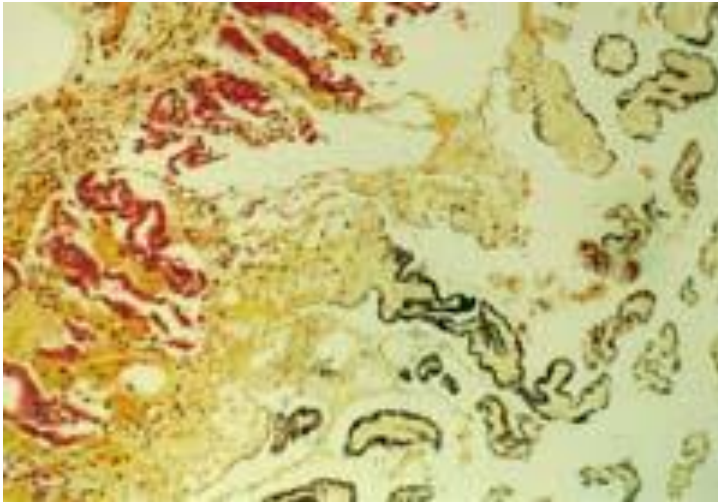
Can persist and multiply in animal intestinal tracts

Temperature-abuse in cooked or raw food causes food contamination

Dangerous Amount

$\sim 10^5$ spores/g can cause illness

$\sim 10^6$ spores/g is considered food contaminated



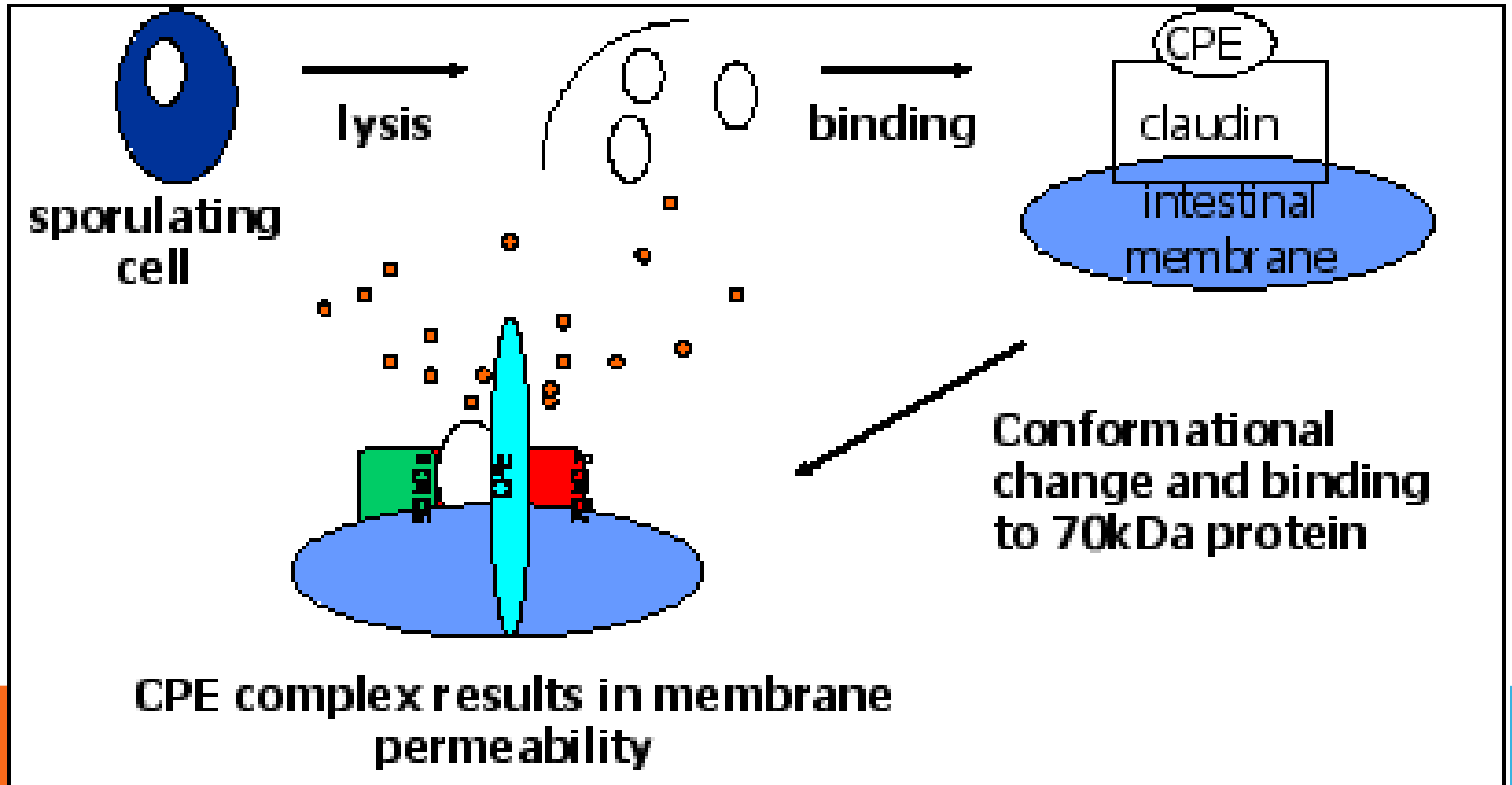
Attack Method For Enterotoxin

Stomach acids initiate spore germination

When the cell lyses, it releases mature endospores

Spores bind to intestinal epithelial cells and induce intestinal tissue damage

Usually symptoms occur within 6-24 hours of ingestion and can last ~24 hours



Results of enteric infection:

Can cause stomach aches, diarrhea and vomiting

Rarely fatal in humans

Very rapid death occurs in animals

Mistaken for the “24 hour” flu



Lab Diagnosis :

Specimens :

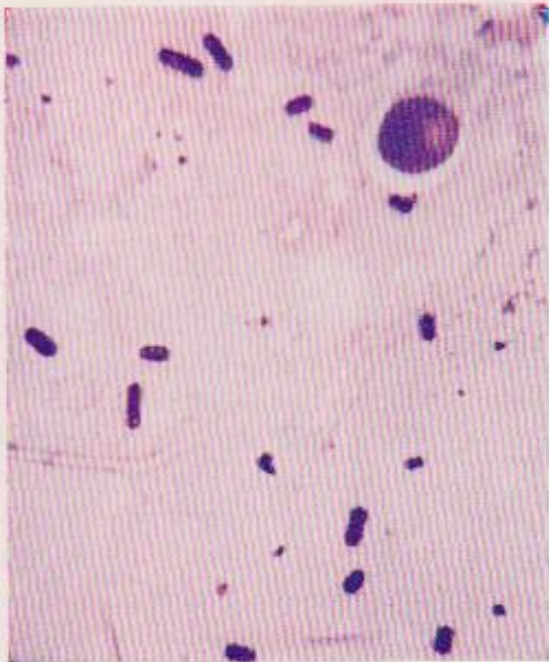
Wound materials, pus, food samples, and uterine swabs.

Direct tests:

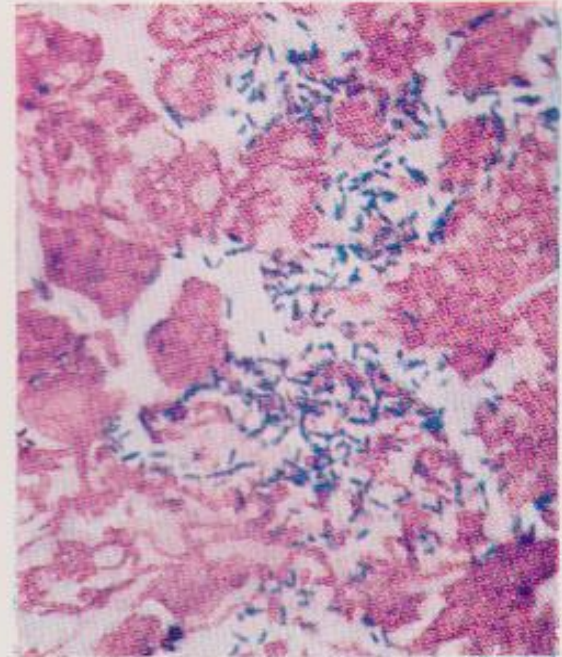
Gram stain shows infiltration of high count of this organism in specimen.

Motility test reveals non-motile organism.

Microscopy. Relatively large bacilli, 5μ by 1μ , with square or rounded ends. Gram-positive, non-motile (all other *Clostridia* are motile), capsulate in animal tissues. Spores are oval, subterminal and non-projecting.



Two Gram-stained preparations showing *Cl. welchii*. On the left is a smear made directly from muscle in a case of gas gangrene; on the right is a section of muscle from the same case. Separation of the muscle fibres, which are also oedematous, by gas production can be noted. $\times 1000$, $\times 500$.



Cultural appearances. Not as strictly anaerobic as *Cl. tetani*; grows very

Culture :

Culture on cooked meat broth, thioglycolate broth .

On blood agar reveal beta hemolytic dome shaped colonies surrounded by double zone of beta hemolysis

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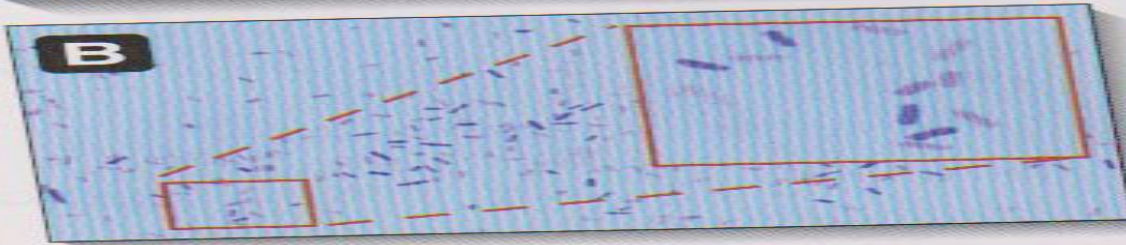
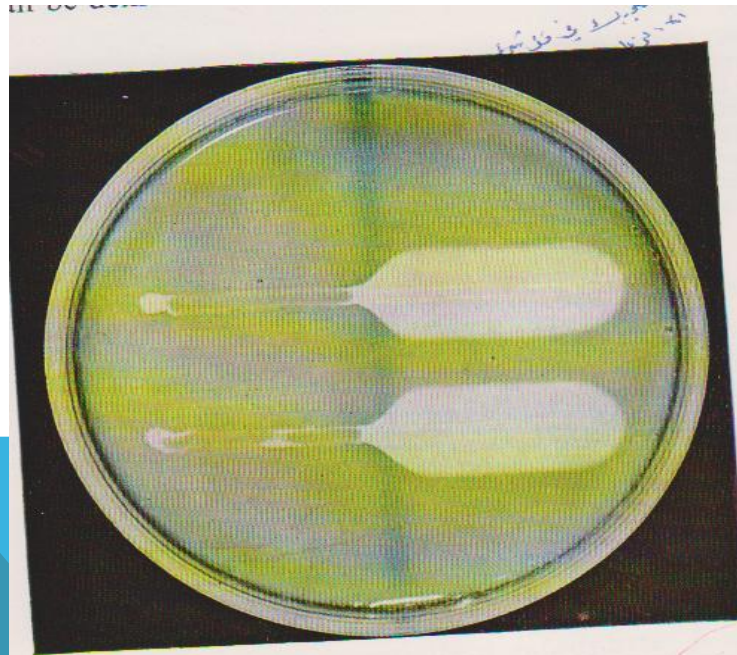


Figure 14.5
Clostridium perfringens. A. Colonies on blood agar showing double zone of hemolysis. B. Photomicrograph of Gram stain.

Naglers reaction:

Lecithenase activity of alpha toxin of this organism reveals opalescence around colonies on egg yolk agar in case of positive test .



Nagler's reaction. The medium comprises nutrient agar with 5 per cent egg yolk. Three drops of *Cl. welchii* type-A antitoxin were smeared over the left half of the plate as marked by the blue line; a culture of *Cl. welchii* was streaked in two parallel lines across the surface of the medium and the plate then incubated anaerobically at 37°C for 20 hr. *Lecithinase activity produces zones of opacity on the antitoxin-free half of the plate and this activity has been inhibited in the presence of antitoxin on the left half.

(all types) and

Treatment and prophylaxis :

Depends on toxicity and type of strain ingested

For Animals (Polyvalent spore vaccine is available for animal vaccination).

- **Not much can be done once spores are ingested**

Treatment :

Polyvalent antitoxin

Antimicrobial therapy , Amoxicillin , Cloxacillin , Penicillin, Flagyl

Are suitable for treatment and prophylaxis

- **Surgery is used for cases in which severe tissue damages occur**
- **Keep hydrated**

CONCLUSION: *CL. PERFRINGENS*

***C. perfringens* is an anaerobic bacteria found in soils and sediments, especially in areas of pollution**

Persists in fecal matter and intestinal tracts of animals and humans,

Four lethal toxins are produced during spore germination,

Enterotoxin is most common cause for food-borne illnesses.

In humans, the illness only lasts ~24 hours

Using the correct temperatures in cooking, cooling, or reheating food is crucial to inhibit bacteria growth in food.



Clostridium tetani

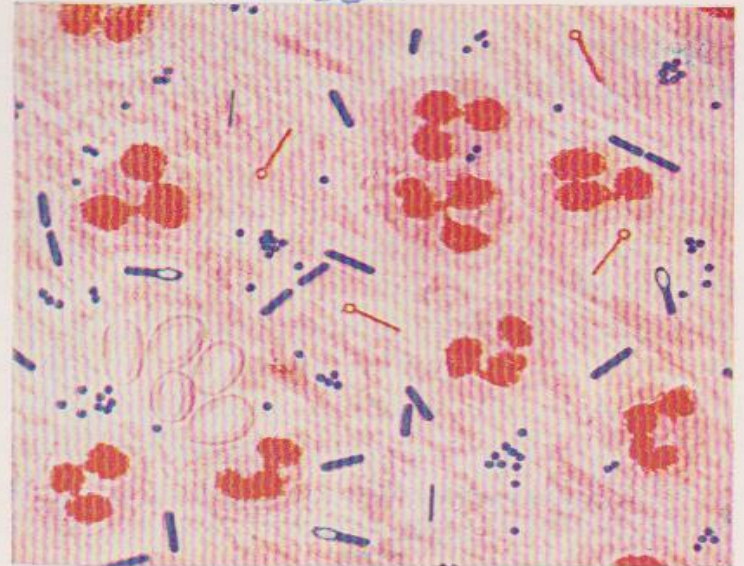
It is Gram positive spore forming, spores are located terminally giving the organism the drum stick appearance and motile distributed in soil and feces of animals.

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GAS GANGRENE
تفكرين كغاز



This Gram-stained film of material from a wound shows pus cells (degenerate polymorphonuclear leucocytes), a few necrotic muscle fibres and clumps of Gram-positive cocci which on culture proved to be coagulase positive staphylococci.

Two species of *Clostridia* were also isolated, *Cl. tetani* which in the film is represented by the slender Gram-

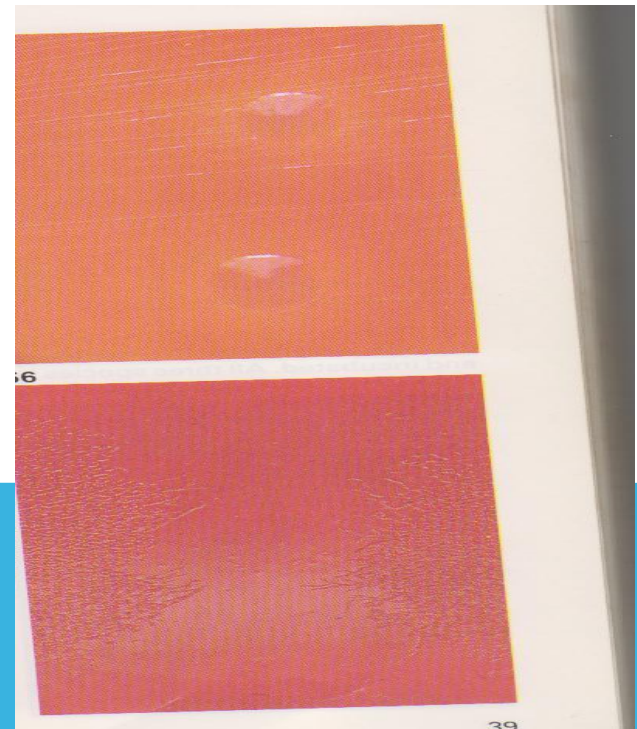
Clostridium tetani

General Characteristics

- **Obligate anaerobes**
- **Heat sensitive**
- **Limited flagella-mediated motility**
- **Formation of terminal spore gives drumstick-like shape and appearance**
- **11 strains, differing in capability of producing toxins**

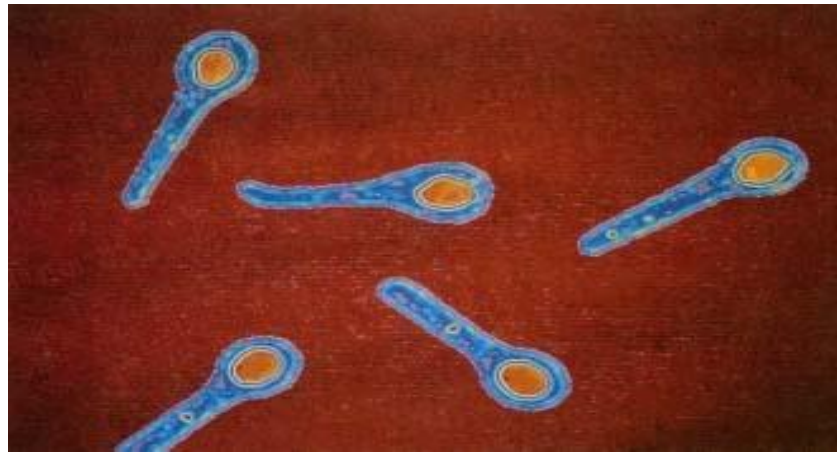
Growth Characters

***Cl. tetani* grow on blood agar and show filamentous with meshwork colony, fresh colony show alpha hemolysis which shifts later into beta hemolysis.**



C.tetani: the endospore

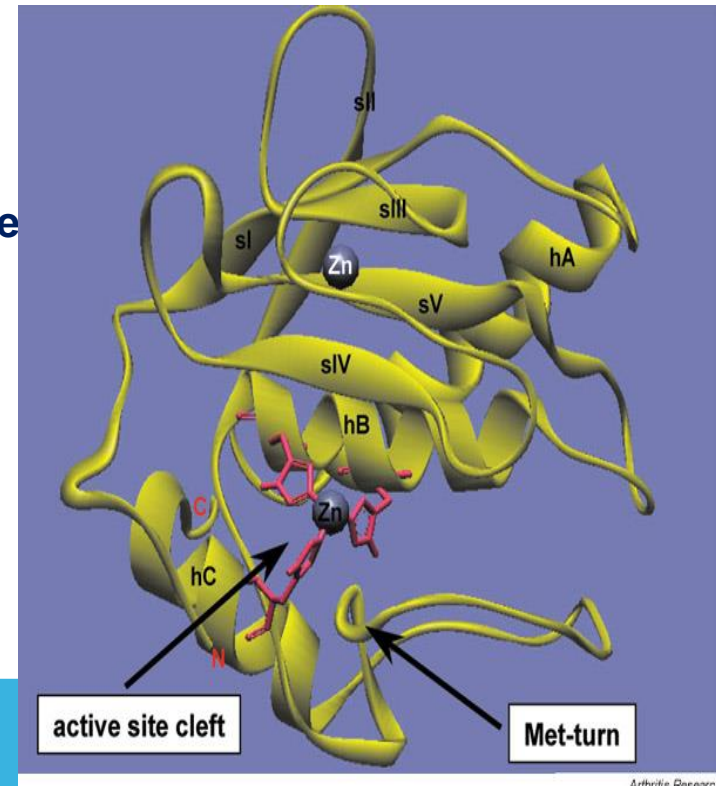
- Heat resistant (can survive autoclaving at 121 C for 10-15 min)
- Antiseptic resistant
- Chemical agent resistant (ie: phenol)



MECHANISM OF PATHOGENICITY

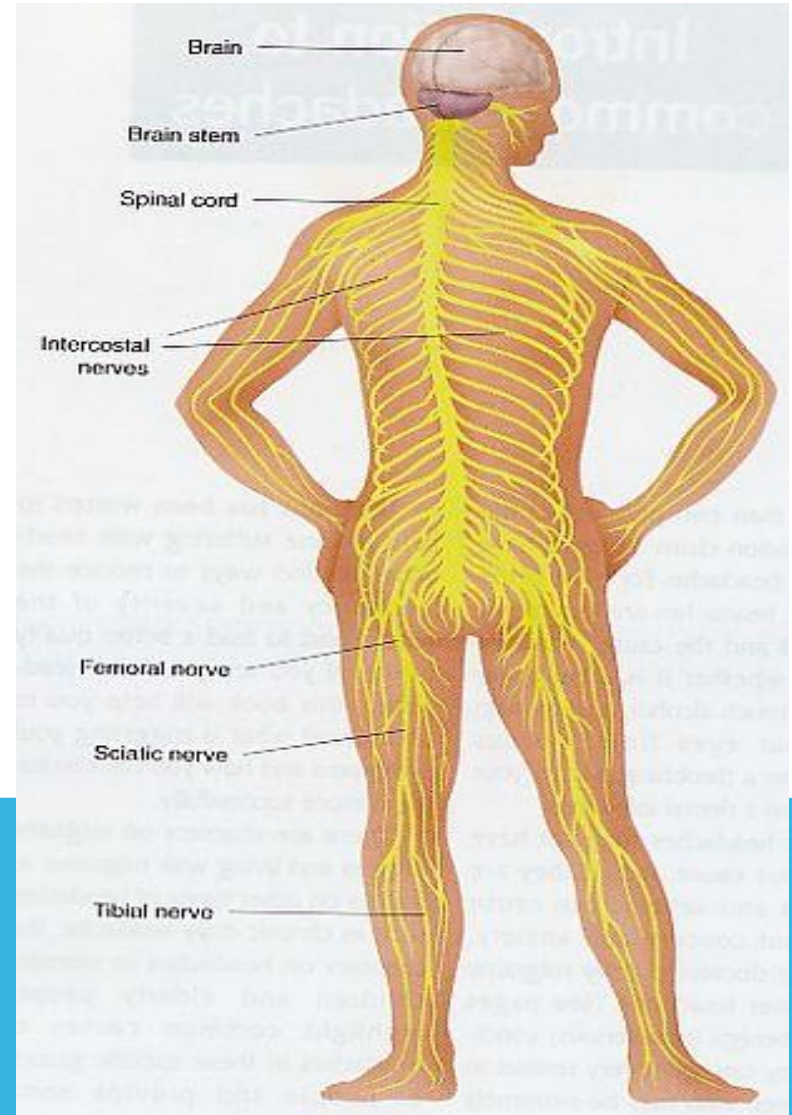
Production of two exotoxins

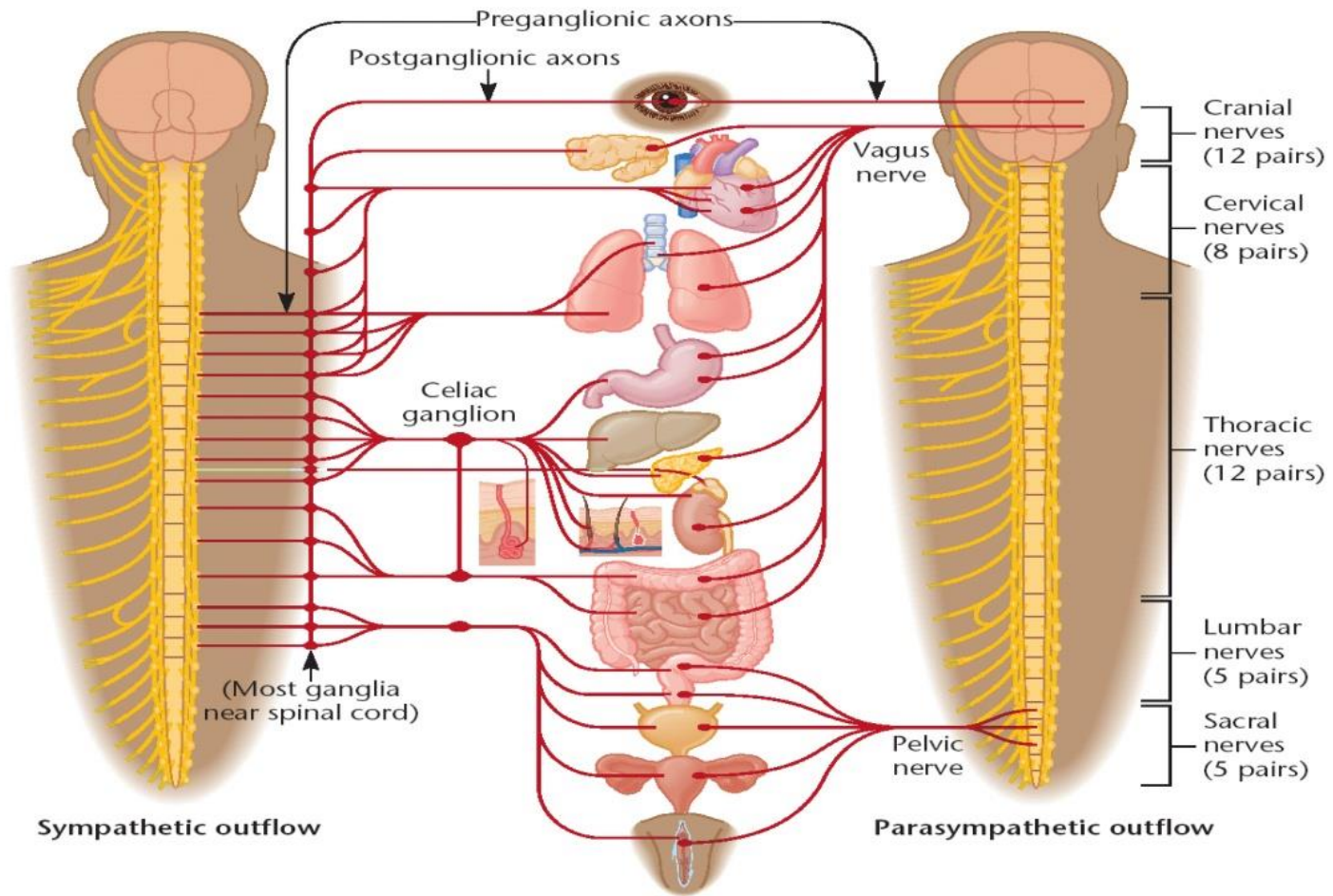
- Tetanolysin: function not determined
- Tetanospasim
 - Neurotoxin
 - One of most potent toxins known:
 - minimum human lethal dose: 2.5 ng/kg body weight
 - 175 nanograms for 154 lb. person
 - Zinc-dependent metalloproteinase

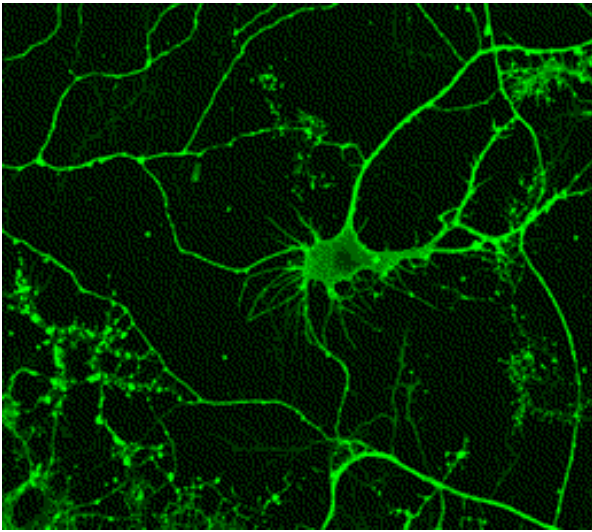


TETANOSPASIM

- **Targets:**
 - **Several sites within the CNS, including spinal cord and brain**






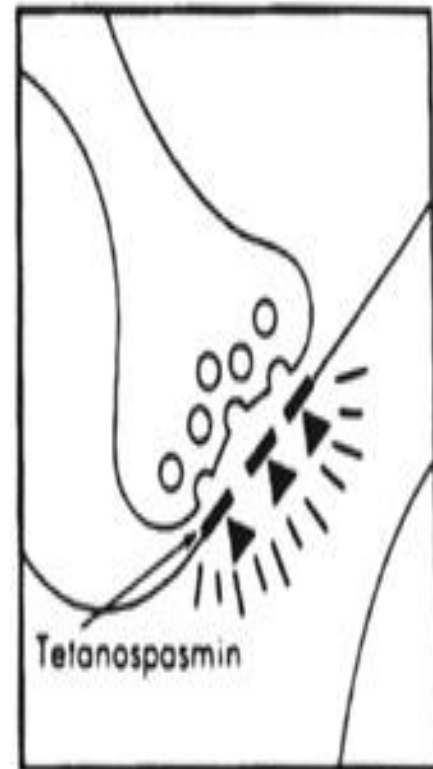
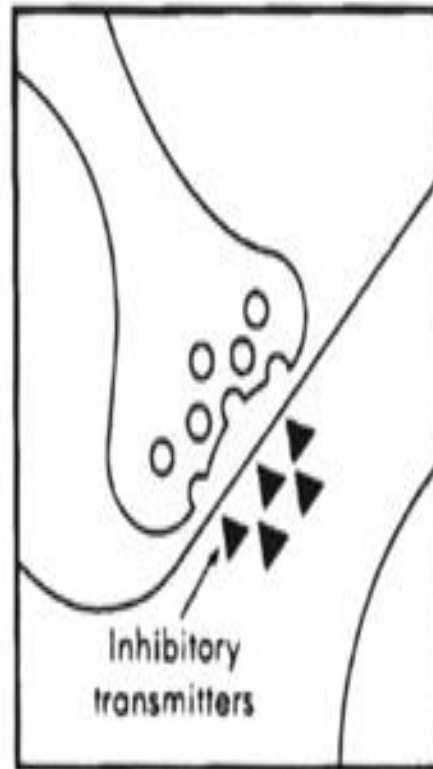
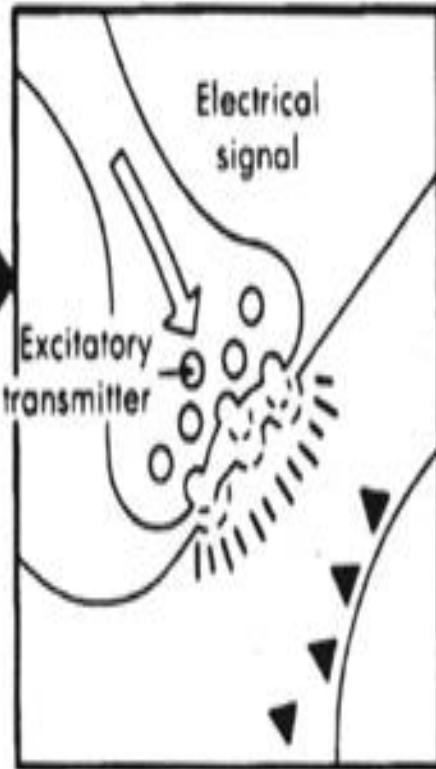
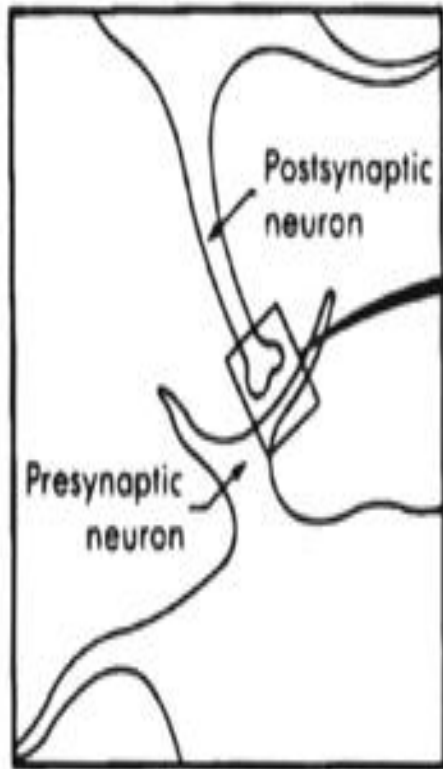


Tetanospasim

- **Absorption into CNS**
 - Absorbed into axon and transported across synaptic junctions until reaches CNS
 - Circulates through circulatory and lymphatic systems
 - Rapidly fixed to gangliosides at the presynaptic junction of inhibitory motor nerve endings

Mode of Action:

- **Blocks inhibitory impulses via interference with neurotransmitter release, including that of glycine and gamma-amino butyric acid,**
 - **Prevents neurotransmitters by cleaving synaptobrevin II, component of synaptic vesicles,**
 - **Causes unopposed muscle contraction and spasm and seizures.**
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Common Classifications Of Tetanus

Common types:

- **Local tetanus: persistent muscle contraction in region of injury.**
- **Cephalic tetanus: concurrent with otitis media, associated with head injuries and cranial nerves,**

- **Generalized tetanus:(80% prevalence) lockjaw other symptoms include elevated blood pressure, sweating, elevated temperature, rapid episodic heart rate, spasms continue for 3-4 weeks.**
- **Neonatal tetanus: born without passive immunity, usually through infection of unhealed umbilical stump**

Lab Diagnosis :

Anaerobic cultivation for the pathogen diagnosis followed by toxicity test in lab animals (Mice or Guinea pigs) to confirm toxin release by the isolate organism .

203 Intramuscular injection. Intramuscular injection is done in the thigh. Anaesthesia is unnecessary. Fix the mouse as shown, pulling the skin of the back between thumb and fingers. Always disinfect the skin with iodine to prevent abscesses.



API SYSTEM (Analytical Profile Index)is available for anaerobic bacteria diagnosis Particularly Clostridium types.



Treatment:

- **Treatment includes three foci:**

1-Control of muscle spasms

2-Ceasing of toxin production

- **Metronidazole and intramuscular penicillin G**

3-Neutralization of toxin effects

- **TIG: binds to and eliminates unbound toxins from body but cannot affect already bound toxins, as this is an irreversible event**

Prevention

- **Rigorous hygienic response to injury**
- **Vaccination**
 - **First 4 immunization shots (DTP: diphtheria-tetanus-pertussis) given within 2 years**
 - **Every 10 years: booster shot**

Conclusion: *Cl. tetani*

Obligately anaerobic pathogenic bacteria

Pathogenicity based on production of neurotoxin tetanospasim

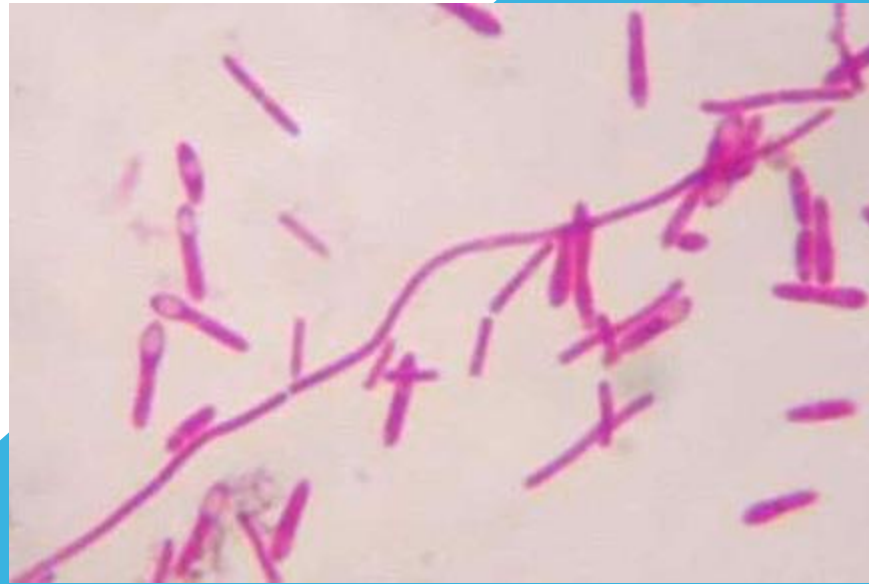
**Affects developing countries and tropical-type regions
preferentially**

Vaccination is the most efficient way of battling this pathogen





C. BOTULINUM



Characteristics:

Anaerobic bacillus that forms **sub-terminal endospores**

Heat resistant

Found in soil, sediments of lakes, ponds, coastal waters, decaying vegetation.

Intestinal tracts of birds, mammals and fish

Neutral or low acid environments,

Seven toxigenic subtypes of the organism:

- **A, B, C, D, E, F and G**
- **Differ by pre-synaptic proteins bound at exocytosis stage**

Clinical Syndromes :

Food-borne: ingested from foods that spores have germinated and grown in, considered an intoxication – most common form.

Wound: infects a wound and then produces toxins that spread through the bloodstream – very rare.

Infant: infection, establishes itself in the bowels of infants, colonizes and produces the toxin – common source is honey.

Unidentified: source is unknown, usually from intestinal colonization with *in vivo* production of toxin – usually from surgeries.

ACTION OF TOXIN:

Structure: Synthesized as a polypeptide chain that cleaves into two chains, a light and heavy linked by disulfide bonds

Binding occurs at the carboxy terminal

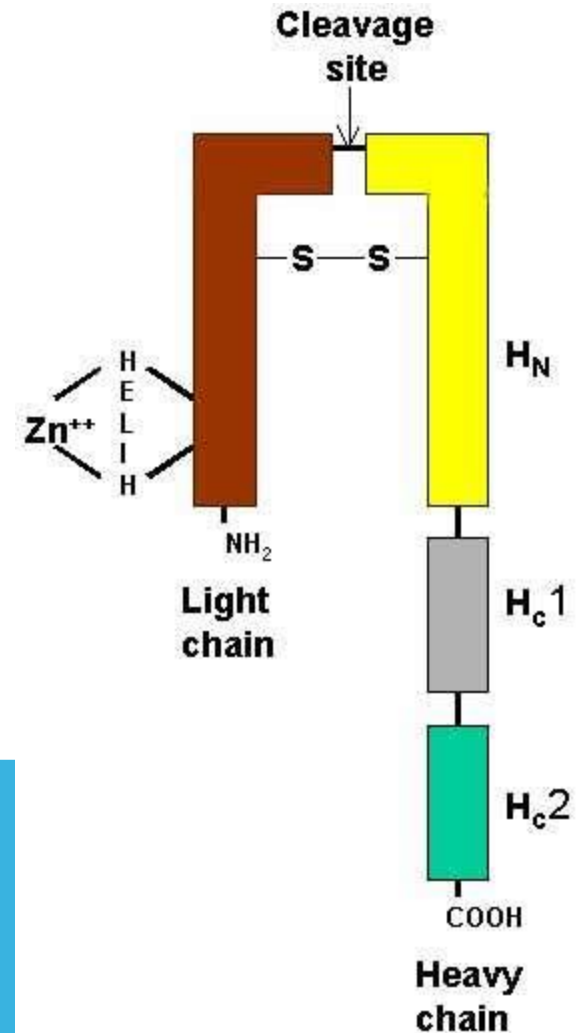
Enters receptors via endocytosis

Blocks release of Ach = failure to release neurotransmitter

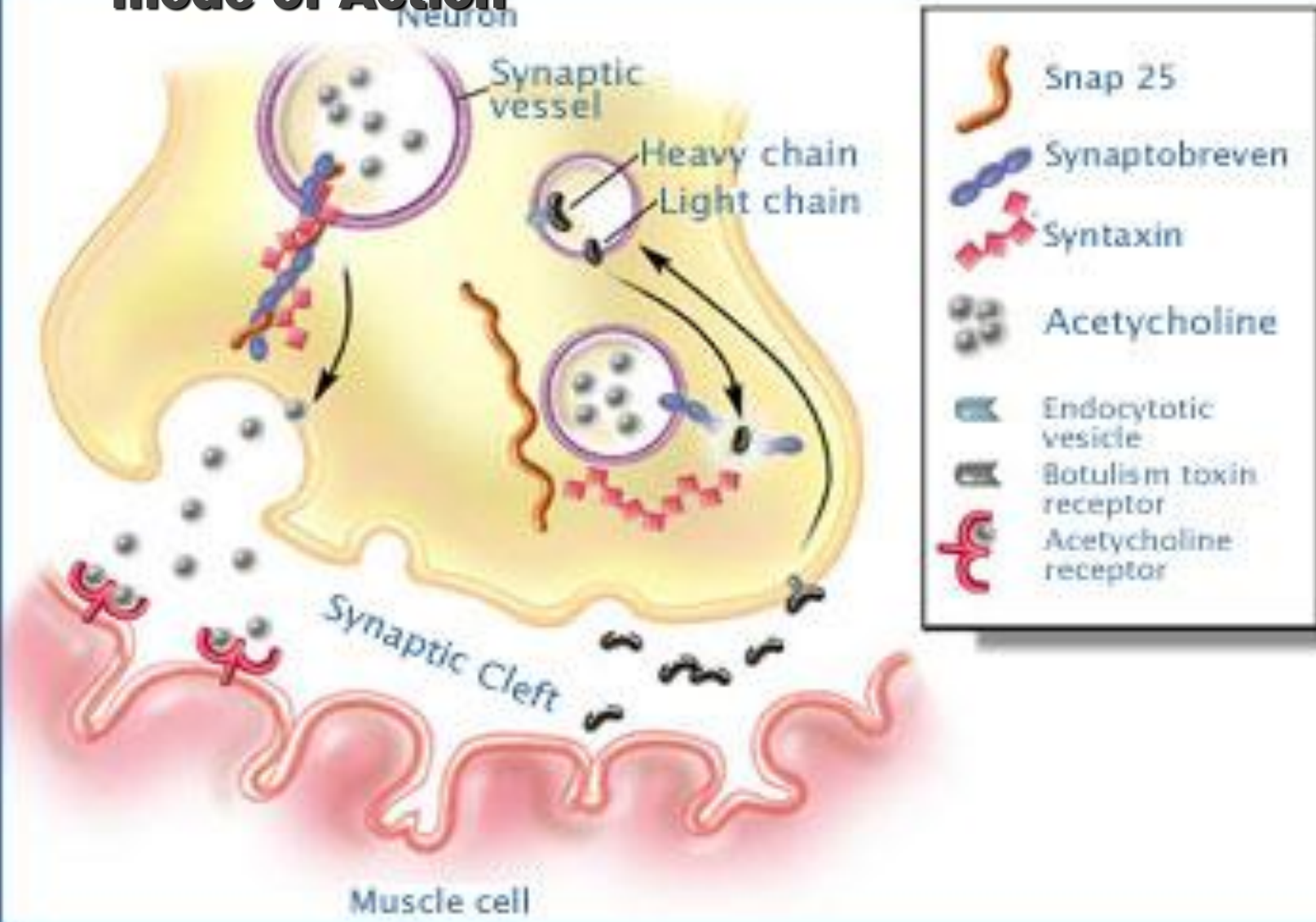
- **Zinc-dependent endopeptidase that cleaves synaptobrevins**

Flaccid Paralysis

Permanent damage



mode of Action



Symptoms:

Begin 8-36 hours after ingestion

Length: 2 hours to 14 days after entering circulation

Preliminary symptoms: weakness, dizziness, dryness mouth, nausea, vomiting

After Neurological disturbance: blurred vision, inability to swallow, difficulty in speech, descending weakness of skeletal muscles and respiratory paralysis.

Diagnosis And Treatment:

Electrodiagnostic testing = repetitive nerve stimulation

Test serum or feces of the patient for the toxin

Mouse neutralization test

- **48 hours to complete**
- **5-7 days to culture specimens**

Neutralized by an antitoxin - only in circulation

Prevention:

Proper food handling and preparation

- **80°C for 10minutes or longer.**

Manufacturers use thermal processes designed to destroy spores

Processors add salt or nitrites to reduce growth,



Conclusion: *Cl. botulinum*

***Cl. botulinum*:**

Seven toxigenic subtypes

Four clinical syndromes, all have similar symptoms but the mode of infection is different

Action of toxin = blocks release of neurotransmitter

Becoming more common (olives and wild fish)

Always finding new ways to incorporate botulism into the medical field.

Clostridium difficile

General Characteristics:

- **Gram-positive**
- **Anaerobic**
- **Spore-forming**
- **Cell Morphology: rods rod shaped Bacillus**
- **Pathogen disease causing Pseudomembranous Colitis**
- **Commensal Bacterium**
- **Resistant to most antibiotics**
- **Produces Cytotoxin and Enterotoxin**

Habitats

- **Gastrointestinal tract of Humans and other animals**
- **Soils**
- **Marine Sediments**





***Cl. difficile* Toxins Produced**

Enterotoxin:

- **Toxin A**
- **Protein toxin released by *C. difficile* in the lower Intestine**
- **Frequently cytotoxic**
- **Alters the permeability of the epithelial cells of the intestinal wall.**
- **Pore forming toxins, secreted by bacteria, form pores in cell membranes causing cells death.**

■ **Cytotoxin:**

- **Toxin B**
- **Associated with Inflammation**

DISEASE: PSEUDOMEMBRANOUS COLITIS


Target Area of Infection: Colon


***Cl. difficile* infection is responsible for approximately 3 million cases of diarrhea and colitis annually in the United States.**

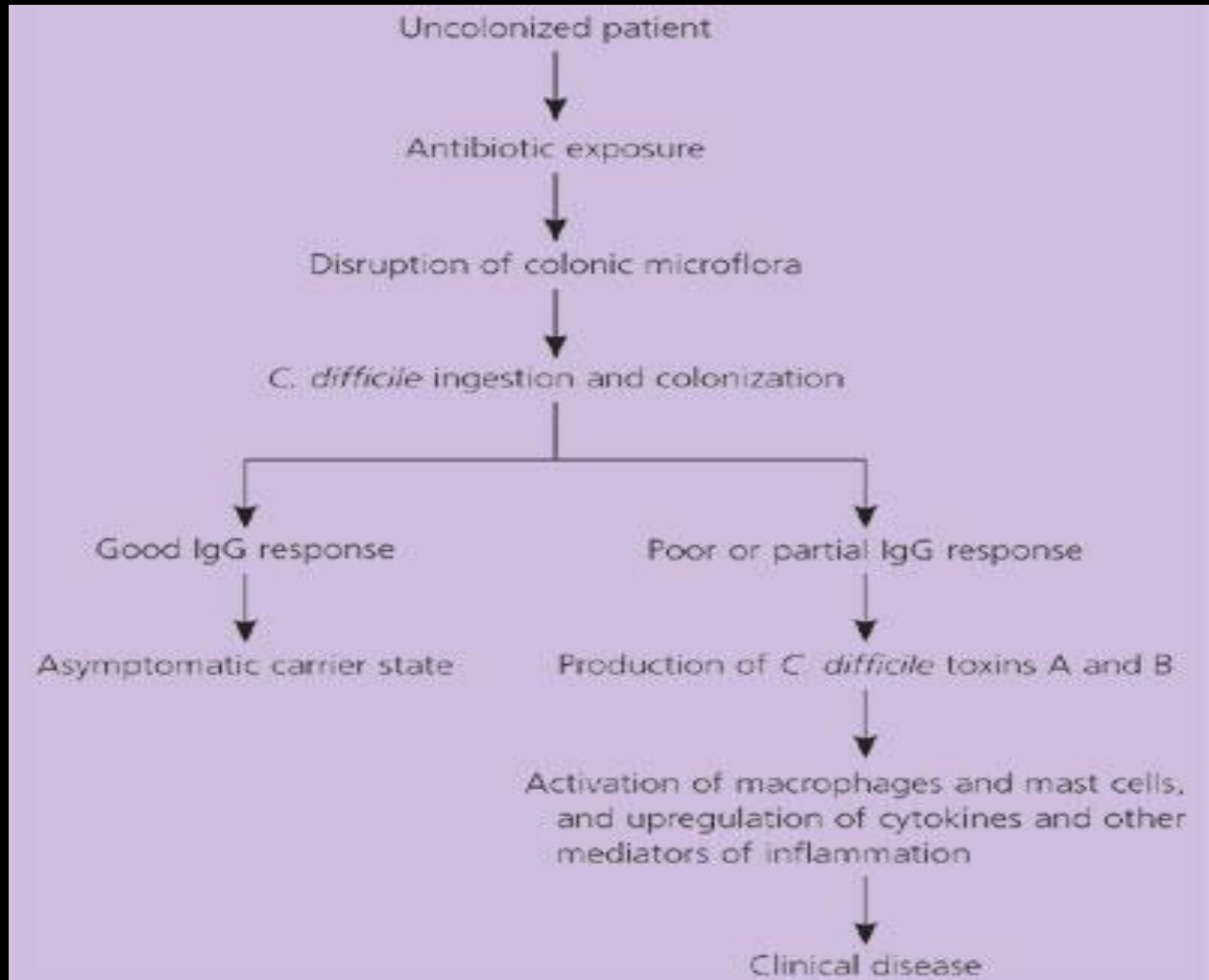
The mortality rate is 1 to 2.5 percent.



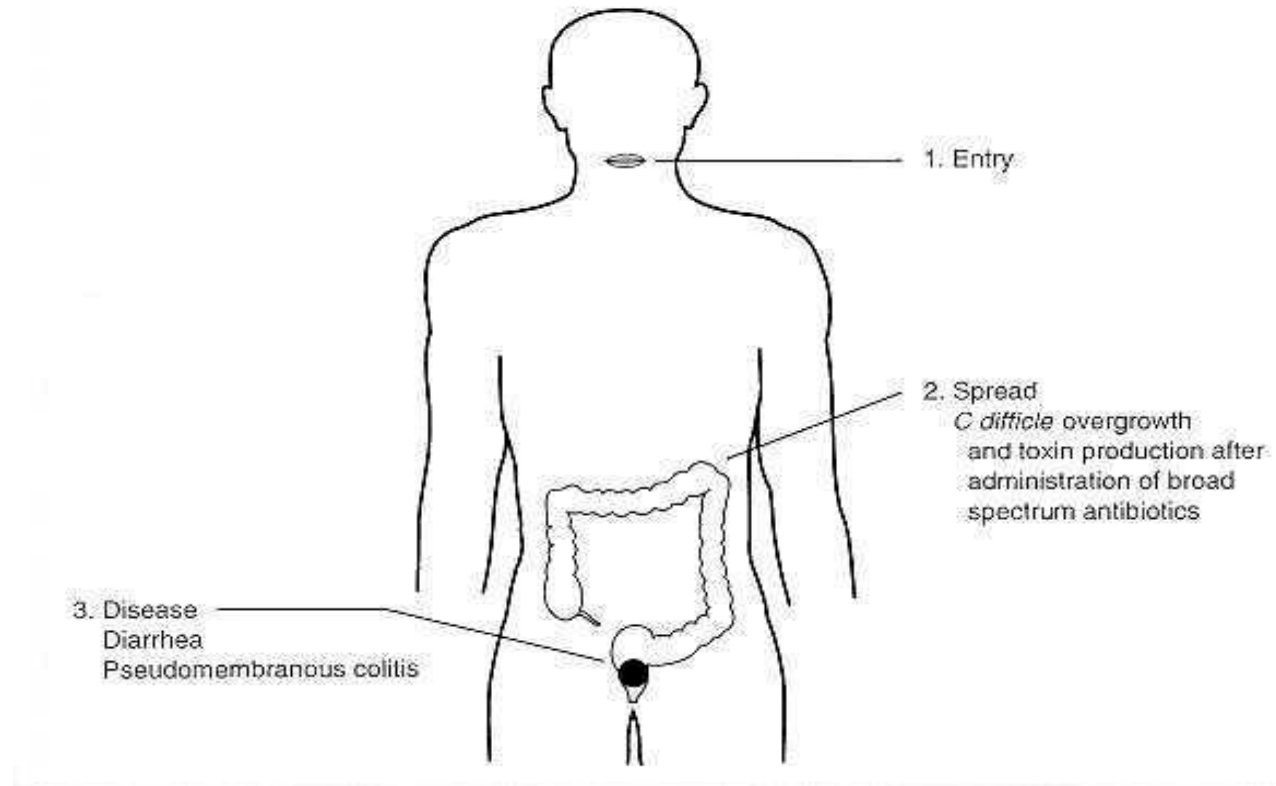
Those most susceptible to disease:

- **Antibiotic therapy such as cephalosporins and clindamycin, which are frequently used in hospital settings.**
 - **Advanced age over 65. (80% reported cases)**
 - **Multiple, severe underlying diseases.**
 - **Faulty immune response to toxins produced by *Cl. difficile* toxins.**
- 

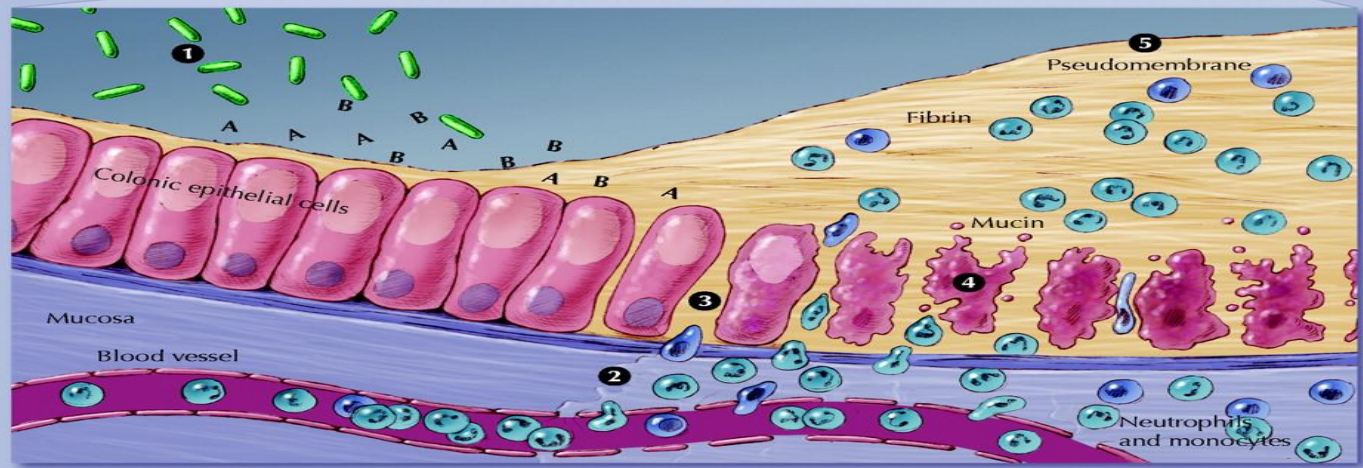
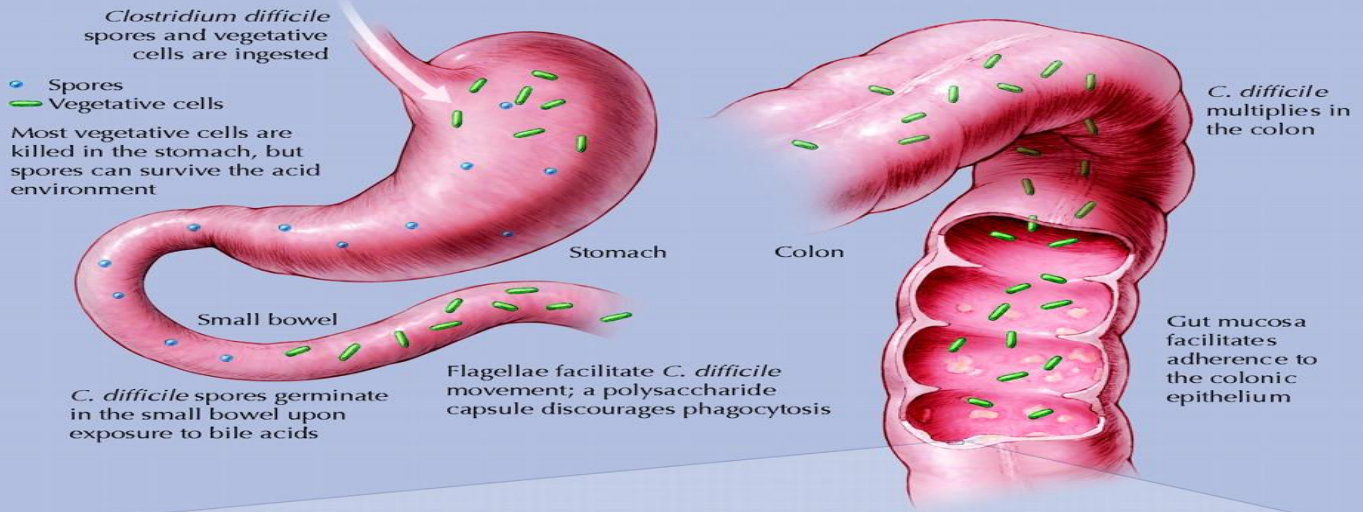
- **The rate of *Clostridium difficile* acquisition is estimated to be 13% percent in patients with hospital stays of up to two weeks and 50 percent in those with hospital stays longer than four weeks.**
 - **Those taking medications to suppress gastric acid production:**
 - **H2-receptor antagonists increased the risk twofold,**
 - **Proton pump inhibitors increased the risk threefold, mainly in the elderly. It is presumed that increased gastric pH leads to decreased destruction of spores.**
- 



***Cl. difficile* Mode Of Infection:**



Cl. difficile Mode Of Infection:



C. difficile vegetative cells produce toxins A and B and hydrolytic enzymes (1). Local production of toxins A and B leads to production of tumour necrosis factor-alpha and proinflammatory interleukins, increased vascular permeability, neutrophil and monocyte recruitment (2), opening of epithelial cell junctions (3) and epithelial cell apoptosis (4). Local production of hydrolytic enzymes leads to connective tissue degradation, leading to colitis, pseudomembrane formation (5) and watery diarrhea.

SYMPTOMS

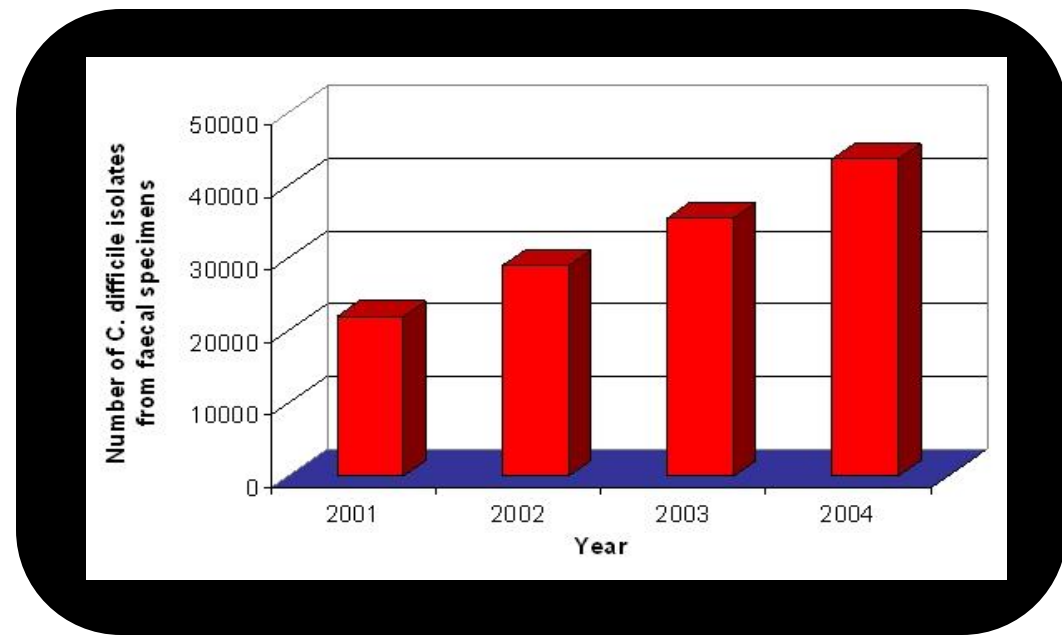
Diarrhea

Fever

Abdominal pain

Toxic mega-colon

- Increased abdominal pain
- Abdominal bloating
- Abdominal tenderness
- Fever
- Tachycardia (rapid heart rate)
- Dehydration



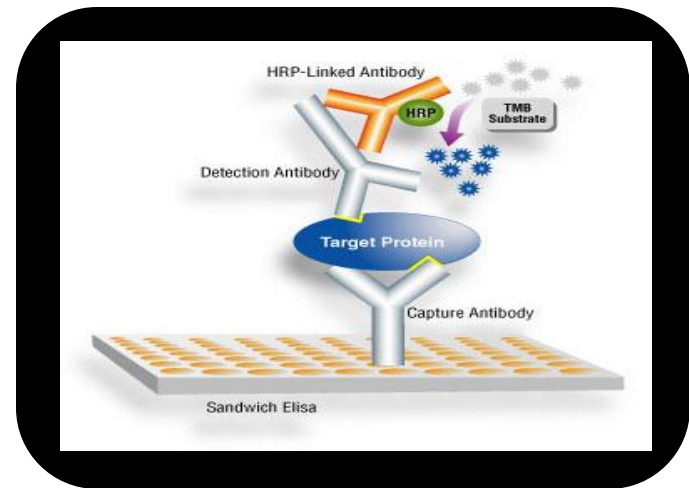
How To Diagnose Pseudomembranous Colitis

Enzyme-Linked Immunosorbent Assay (ELISA)

- **Colorimetric assay for toxin A and toxin B**
- **Sensitivity of 63-99% and a specificity of 93-100%**

CT scan:

- **88% probability of testing positive on stool assay**



Treatment

Fecal Bacteriotherapy:

*** Procedure related to probiotic research, has been suggested as a potential cure for the disease.**

Involves infusion of bacterial flora acquired from the feces of a healthy donor in an attempt to reverse bacterial imbalance responsible for the recurring nature of the infection.

Has a success rate of nearly 95% according to some sources.

Two Specific Antibiotics can be used as treatment are **Metronidazole and **Vancomycin****

20-30% of patients treated with this method experience a relapse.

