

Gram positive, spore forming bacilli

Two genera

1- *Bacillus* spp. (aerobic)

2-*Clostridium* spp. (anaerobic)

Genus: *Bacillus* sp.

GENERAL CHARACTERISTICS

- ❖ They are spore forming, Gram positive and aerobic bacilli with more than 50 species
- ❖ The spores mainly central and oval in shapes but without bulging.
- ❖ Majority are non pathogenic species found as saprophytic in soil, water and vegetation. They are called anthracoids. Some of these like *B. cereus* can cause food poisoning
- ❖ Only one species is true pathogen is *Bacillus anthracis*, the causative agent of anthrax
- ❖ All are motile except *B.anthracis*
- ❖ Most are catalase positive
- ❖ Anthracoids are the main culture media contaminants
- ❖ Morphologically anthracoids are large bacilli with round ends while those of *B.anthracis* are larger bacilli with clear cut ends or square ends usually in chains giving bamboo like appearance.

Bacillus anthracis

General characteristics

- 1- Large clear cut ends, Gram positive bacilli in single or in chains (culture and in animal tissues) giving bamboo appearance)(Figure 1).
- 2- Have a capsule made of protein (D-glutamic acid) which is made in the animal tissue and in lab. With 2% CO₂
- 3- Mac Fadyean reaction used to detect capsule in tissue using polychrome methylene blue stain which stains capsule pink color while the body of bacilli deep blue.(common method used by veterinarian) (Figure 2).
- 4- Sporulation never occur within body but occurs when exposed to O₂ or D.Water, 2% NaCl, growth in oxalate agar.

Cultural characters

- 1- Grow on both simple and enriched media
- 2- Colonies are non-hemolytic on blood agar and look like tangles mass of long hair like curls (Medusa head) (Figure 3 and 4).
- 3- Give inverted fir like appearance in gelatin liquefaction test (Figure 5).

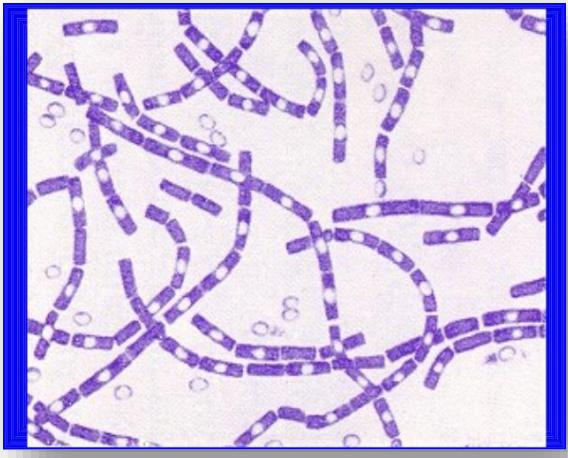


Fig.1. bamboo like appearance of bacilli chains



Fig.2. Mac Fadyean reaction for demonstration of capsule



Fig.3. frosted glass appearance of *B.anthracis* colonies

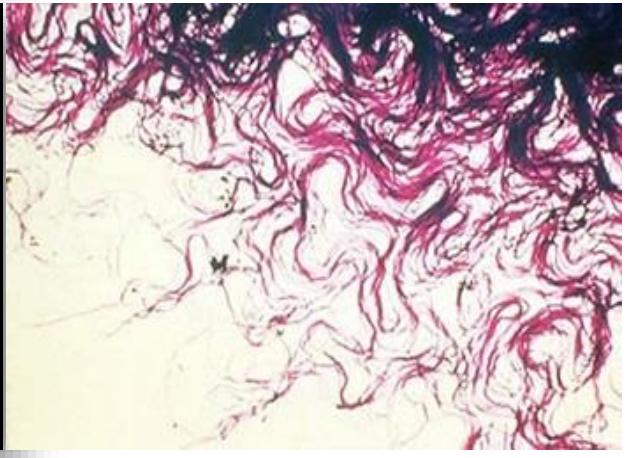


Fig.4. Medusa like colonies of *B.anthracis* after magnification

Gelatin liquefaction



Fig.5. Inverted fir like appearance of gelatin liquefaction

Biochemical reactions

1- Catalase +ve

2- Ferments glucose, maltose and sucrose with acid production only.

3- Reduce nitrate to nitrite

4- Give inverted fir like appearance in gelatin liquefaction test (Figure 5)

Virulence factors

1- Capsule of poly D-glutamic acid (major virulence factor) , antiphagocytic but Abs against it (hapten) are non protective.
Its plasmid encoded genes.

2-Anthrax toxin made of 3 proteins

A- Protective antigen or protein- binds to specific cell receptors

B- Edema factor

C- Lethal factor (B and C exert their toxic effect).

Anthrax toxin cannot act individually only when together and encoded by genes carried by plasmids.

Anthrax

It is a zoonotic disease of animals especially cattle and sheep but human can be infected when come into contact with infected animals meats, hides and fluids (occupational disease because infected special group of people like veterinarian, farmers and butchers)

It is one of the biological weapons

In animals its transmitted by ingestion of spores and can cause sudden death.

In human anthrax occurs in three forms

1- Cutaneous form or malignant carbuncle. This is the most common form 95% of infection. Transmitted by direct skin contact with animal tissues. This form occurs on exposed surfaces of the arms or hands followed by the face and neck. The lesion develops from papule to vesicles then ulcer surrounded by congested edema and black eschar covers the base (Figure 6)

CLINICAL FORMS OF ANTHRAX

- **Cutaneous form:**

- 7 days after exposure to infected hides or meat, a **painless or mildly pruritic papule** forms
- The lesion rapidly enlarges and ulcerates, often accompanied by **significant surrounding edema** and **regional lymphadenopathy**
- The case fatality rate of cutaneous anthrax is 20% without antibiotic treatment, and <1% with antibiotics.



Fig.6. Cutaneous form of anthrax

Pulmonary anthrax (Wool sorters disease) 5%

Due to the inhalation of spores and may develops to the hemorrhagic meningitis. This form is the most severe form of anthrax.

GIT form due to the ingestion of undercooked meat containing spores. This is very rare form.

Laboratory diagnosis

Direct microscopic examination of fluid and blood by Gram stain (Large Gram positive bacilli with square ends in chains) and Mac Fadyean reaction to demonstrate capsule.

Isolation of *B.anthracis*

Culture should be performed under biosafety level 2

If sample is blood of animals or swab from the base of cutaneous form, blood agar can be streaked .

If the samples from soil, selective media are needed like PLET medium (Polymyxin B, Lysozyme, EDTA and Thallus acetate).

The soil samples will mix with sterile distilled water and shake well then incubate overnight at room temperature (24 -26C).

The supernatant filter through 0.45 μ l pore filters and the deposit on the filer washed with sterile PBS and heated 90 C for 10 minutes. Centrifuge tube and discard the supernatant then inoculate the deposit on the selective media like PLET agar.

Incubate at 38 C for 48 hours. Look at growth of non hemolytic, frosted grass appearance colonies with medusa head. Gram stain to show Gram positive large bacilli with clear cut ends.

Animal inoculation like guinea pig : small amount of materials give S/C to guinea pig which die after 36 to 48 hrs then make slides from blood.

Ascoli test: serological test used to detect anthrax antigen in hides

PCR technique

Treatment

Ciprofloxacin is recommended for treatment; penicillin G, along with gentamicin or streptomycin,

There is a live attenuated vaccine for animals but for humans not available in our country

In USA there is only one vaccine made from protective antigen (acellular) and given to risk groups not for public use. Risk groups such farmers in endemic area, Veterinarian, Lab,workers with anthrax and military soldiers who serve in endemic area. The vaccination program differs between pre-exposure and post exposure. For prophylaxis usually 5 doses given in I.M 0,1,6,12,18 and booster dose fore every 12 months, while for post exposure cases, 3 doses 0, after 2 weeks, 3 after 3 weeks with antibiotics for 60 days.

Bacillus cereus

Cause food poisoning by producing enterotoxin that cause diarrhea

Two distinct forms

1- Emetic form associated with fried rice

2- Diarrheal type associated with meat dishes and soups.

The emetic form is manifested by nausea, vomiting, abdominal cramps, and occasionally diarrhea and is self-limiting, with recovery occurring within 24 hours.

The diarrheal form has an incubation period of 1–24 hours and is manifested by profuse diarrhea with abdominal pain and cramps; fever and vomiting are uncommon.

B cereus is an important cause of eye infections, such as severe keratitis, endophthalmitis, and panophthalmitis. The bacteria enter the eyes in association with foreign bodies during trauma

Laboratory diagnosis.

Food samples should be inoculated on the selective media such as MYP (mannitol-egg yolk-phenol red-polymyxin-agar) and PEMBA (polymyxin-pyruvate-egg yolk-mannitol-bromthymol blue-agar) and incubated at 30 °C for 24 hrs. Typical colonies are pink-orange and uniform and they are surrounded by a zone of precipitation indicating lecithinase production. PCR should be used for final diagnosis.

TABLE 28.1: Difference between *B. anthrax* and *B. anthracoid*

<i>B. anthrax</i>	<i>B. anthracoid</i>
1. Non-motile	Generally motile (by swarming, e.g. <i>B. cereus</i>)
2. Capsulated	Non-capsulated
3. Grows in long chain	Grows in short chain
4. Medusa head colony	Not present
5. Hemolysis of sheep RBC absent	Usually well marked
6. Inverted fir-tree growth in gelatin	Fir-tree growth absent
7. No turbidity in broth	Turbidity usually present
8. No growth in penicillin agar (10 unit/ml)	Grows usually
9. No growth at 45°C	Grows usually
10. Growth inhibited by chloral hydrate	Not inhibited
11. Susceptible to gamma phage	Not susceptible
12. Salicin fermentation negative	Positive
13. Pathogenic to man and laboratory animals	Not pathogenic
14. Methylene blue reduced weakly	Methylene blue generally reduced strongly
15. Liquefaction of gelatin slow	Liquefaction of gelatin rapid
16. Lecithinase reaction weakly positive	Strongly positive with <i>B. cereus</i>
17. Culture filtrates non-toxic to tissue culture cells	Culture filtrates (<i>B. cereus</i>) toxic to tissue culture cells
18. Produces toxin, neutralized by <i>B. anthrax</i> antitoxin	Any toxic substance produced not neutralized by <i>B. anthrax</i> antitoxin

Clostridium spp.

General characters

Gram-positive, anaerobic, spore forming, spindle shaped and highly pleomorphic bacilli

Spores are wider than bacillary bodies.

Some pathogens, e.g. *Clostridium welchii* now-a-days called *Clostridium perfringens* and *Clostridium tetani* are found normally in human and animal intestine as saprophytes.

Clostridia are motile except *Clostridium perfringens* (*Clostr.welchii*)

Most are strict anaerobic but few are aerotolerant

4 species are medically important

1-Clostr.perfringens causes gas gangrene and food poisoning

2- Clostr.tetani causes tetanus

3-Clostr.botulinum causes botulism (food poisoning)

4-Clostr.difficile (Pseudomembranous colitis following broad spectrum antibiotics)

C. tetani

General characters

It is widely distributed in **soil** and in **intestine** of man and animals.

It is slender, long, slightly curved, Gram-positive (rapidly becomes Gram negative) and occurring singly or in chain

Spores are spherical, terminal and bulging, giving the bacilli drumstick appearance (Figure 7)

It is non-capsulated and motile.

It is an obligatory anaerobe that grows only in absence of oxygen. Why?.

It grows well in cooked meat medium with turbidity and gas formation.

It produces swarming growth forming fine film over the medium (Figure 8)

A zone of α hemolysis is produced. It later on develops into beta hemolysis

It does not ferment any sugar and is slightly proteolytic. It forms indole. Milk is not coagulated.

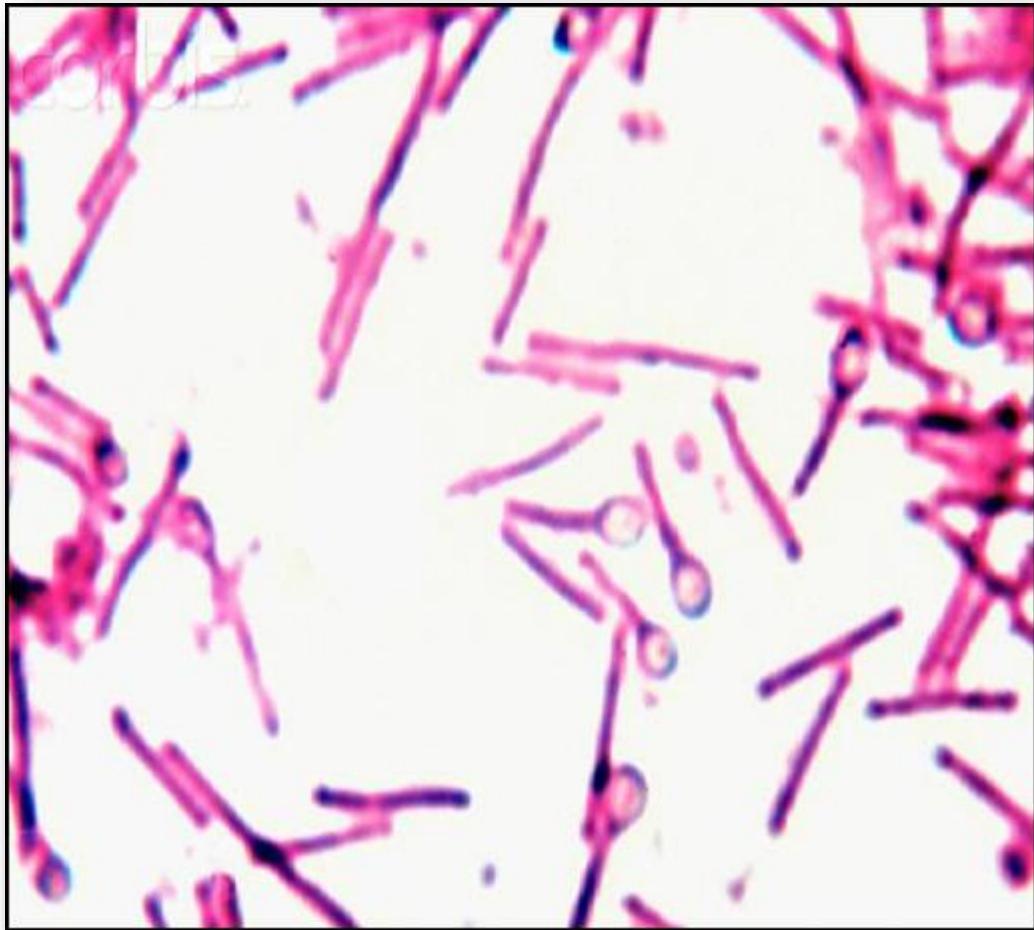


Fig.7. Drum stick appearance of *Clostridium tetani*



Fig.8. Swarming or thin growth of *Clostridium tetani* on blood agar.

Toxins

C_{lostridium} tetani produce two types of toxins

1- Hemolysin (tetanolysin) lysis RBCs

2- Tetanospasmin responsible tetanus

Route of infection by wound contaminated by soil with spores. Germination and production of tetanospasmin. The toxin travels to the spinal cord and brain via retrograde along motor nerve fibers and prevent releasing of acetyl choline inhibitors at neuromuscular junction causing spastic paralysis.

Laboratory Diagnosis

The diagnosis is always clinical and bacteriological findings confirm the diagnosis.

1- Microscopic examination: Smears from wound material after Gram's staining show Gram-positive bacilli with typical drumstick appearance.

2- Culture: Diagnosis by culture is more dependable. Excised bits of tissue from necrotic depth of wound is inoculated into cooked meat broth, blood agar and lactose egg yolk medium. The addition of polymyxin B to which clostridia resist, make the medium more selective.

Tetanus is a preventable disease which achieved by both passive and active immunization. Passive immunization give to those who injured , while active immunization is given to children either alone or in triple (DPT vaccine). Care should be paid for every wound with proper cleaning combined with anti-tetanus antibodies.

Cl.botulinum

General characters

Clostridium botulinum spores are widely distributed in soil, animal manure, sea mud, vegetables, etc. It causes botulism, a severe form of food poisoning.

It is strict anaerobic. There are 6 different types (A to F) which identified on the basis of immunological difference in toxin production.

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.

Single colony is difficult to get because of tendency to spread. Colonies develops after 48 hours.

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 which prevent release of acetyl choline and cause flaccid paralysis.

C botulinum toxins are among the most toxic substances known: The lethal dose for a human is probably about 1–2 µg/kg.

During the growth of *C botulinum* and during autolysis of the bacteria, toxin is liberated into the environment.

Most cases of botulism represent an **intoxication** resulting from the ingestion of food in which *C botulinum* has grown and produced toxin. The most common offenders are spiced, smoked, vacuum packed, or canned alkaline foods that are eaten without cooking.

In **infant botulism**, honey is the most frequent vehicle of infection. The pathogenesis differs from the way that adults acquire infection. The infant ingests the spores of *C botulinum* and the spores germinate within the intestinal tract. The vegetative cells produce toxin as they multiply; the neurotoxin then gets absorbed into the bloodstream.

Wound botulism results from wound infection by *C. botulinum* is very rare.

Botulinum toxin is considered to be a major agent for **bioterrorism** and biologic warfare

Laboratory Diagnosis

Diagnosis is based on demonstration of bacillus or toxin in food or feces. In early stages toxin may be detected from patient's blood.

Culture: Isolation of organism (toxigenic strain) from vomit, food or feces in absence of toxin is of no significance.

Demonstration of *Clostridium botulinum* toxin: using experimental animals (animal; bioassay) like guinea pig with type specific antiserum is the most commonly method for detecting A, B and E toxins from serum, stool and foods.

Treatment

Trivalent (A, B, E) antitoxin must be promptly administered intravenously

Adequate respiration must be maintained by mechanical ventilation if necessary

Cl.difficile

Clostridium difficile is a commensal bacterium of the human intestine found in 2-5% of the population.

Two types of toxins are produced by *Clostridium difficile*, an enterotoxin (toxin A) and a cytotoxin (toxin B). Toxin A usually results in diarrhea and toxin B produces cytopathogenic effects in several tissue culture cell lines

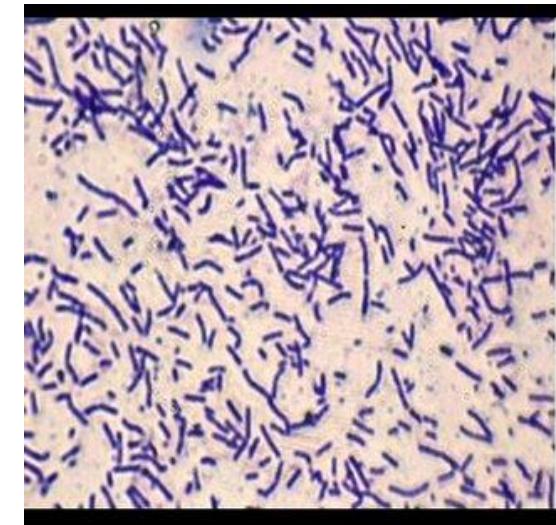
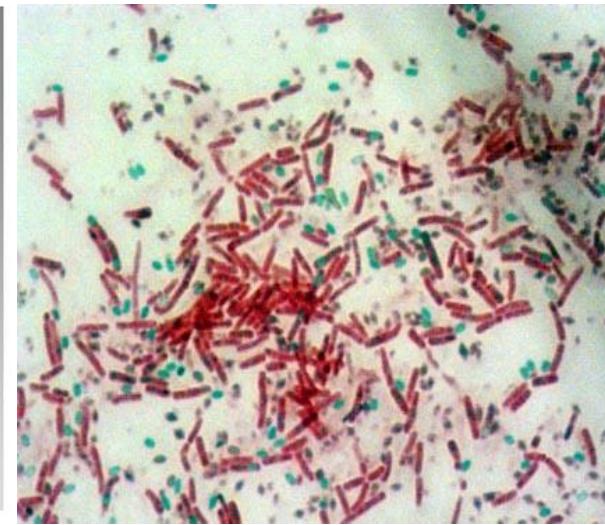
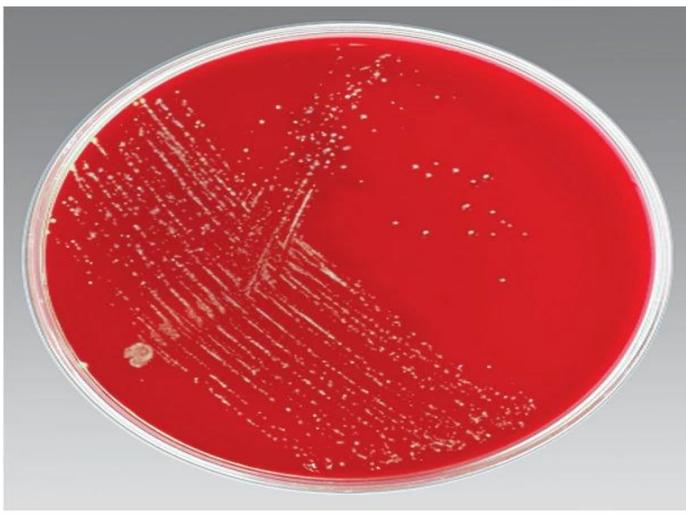
Prolonged use of antibiotics, especially those with a broad spectrum (ampicillin, clindamycin and fluoroquinolone) of activity can result in disruption of normal intestinal flora, leading to an overgrowth of *Clostridium difficile*, which results in either antibiotic associated diarrhea (mild) or acute colitis with pseudomembranous colitis or without membrane formation.

Laboratory diagnosis

Detection of glutamate dehydrogenase enzyme in stool. All strains of CD produce this enzyme and can be used as a screening test followed by detection of toxin A and B. There is a strip method test which can detect both GDH and toxins simultaneously or by ELISA technique. Before isolation, the stool sample should be treated with absolute alcohol with shaking for 1 hr to kill all vegetative bacilli and remain spores then cultured on selective media like cycloserine cefixitin fructose (CCF agar) containing supplements and incubated for 48 hrs at 37 C under anaerobic conditions. After 48 hrs C. difficile colonies grow circular, raised, opaque grey-white, sometimes with irregular borders, and 2-4 mm in diameter. In addition ,under UV light they produce a characteristic fluorescence. but with no signs of hemolysis. Well-grown cultures have a characteristic “horse-stable-like” odour. Gram stain: positive bacillus cell shape with spore formation.

Treatment

Metronidazole is the drug of choice. Other antibiotics that may be effective against *Clostridium difficile* include vancomycin and linezolid. Drugs traditionally used to stop diarrhea should not be used as they frequently worsen the course of *Clostridium difficile* related pseudomembranous colitis.



Clostridia that produce invasive infections

About 30 species of clostridia can produce invasive infection (including myonecrosis and gas gangrene) if introduced into damaged tissue, but the most common in invasive disease is *C perfringens* (90%). An enterotoxin of *C perfringens* is a common cause of food poisoning.

C. perfringens (*C. welchii*)

It is a normal inhabitant of the large intestine of man and animals.

It is pleomorphic. It is capsulated and non motile

Produces double zone of hemolysis (alpha prime) (Figure 10)

In litmus milk it produces acid with gas (Fig. 9. Milk is disrupted due to vigorous production of gas. This is called stormy clot.

Clostridium perfringens are differentiated into 6 types (A, B, C, D, E, F) on the basis of toxin produced by the strains. Toxins are antigenic and antitoxic sera are used for routine typing of strain.

The 4 major toxins, alpha, beta, epsilon and iota are responsible for pathogenicity

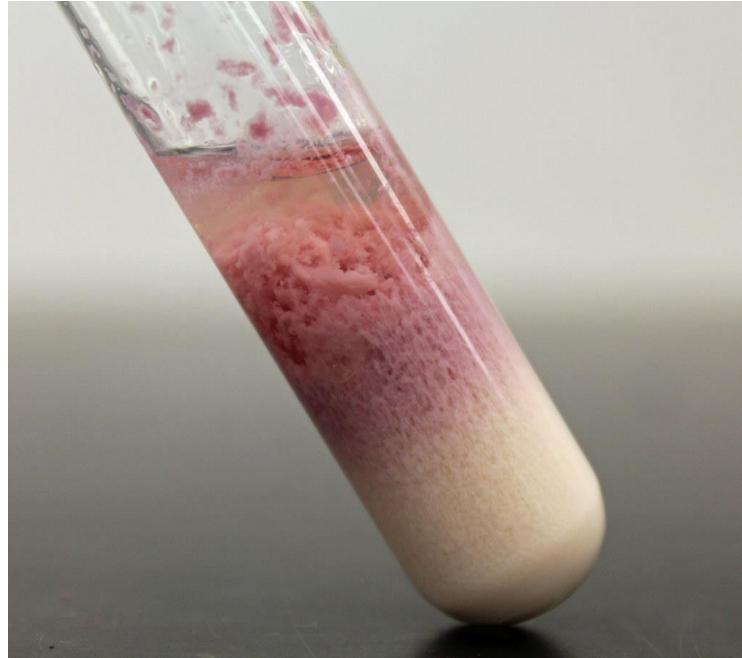


Fig. 9. Stormy formation (coagulation of casein with gas production)



Fig.10. Double zone of hemolysis,a narrow zone beta-hemolysis surrounded by large zone of alpha hemolysis

Only type A and F are pathogenic for man. Type A is responsible for gas gangrene and food poisoning.

Gas gangrene (anaerobic myonecrosis): *Clostridium perfringens* type A is the predominant agent causing gas gangrene

Mere presence of clostridium in wound does not constitute gas gangrene.

Gas gangrene is most serious and is associated with abundant formation of exotoxins. The clostridia multiply and elaborate toxins which cause further damage. The **lecithinase** (toxin) damages cell membranes, muscle fibers and increases capillary permeability. **Hemolytic anemia and hemoglobinuria** are due to lysis of RBC by a toxin. The **collagenase** destroys collagen barriers in tissue. **Hyaluronidases** break down intercellular substances. Abundant production of **gas** reduces blood supply. The incubation period is 7 hours to 6 weeks. The disease develops with increasing pain, tenderness, edema of affected part with systemic signs of toxemia. Profound toxemia and prostration develops and death occurs due to circulatory failure.

Food poisoning: caused by some strains of type A characterized by diarrhea usually without vomiting and fever. Enterotoxin is released when more than 10^8 bacteria in contaminated meat are ingested and sporulate under alkaline conditions in the small intestine.

Clostridial food poisoning is similar to that produced by *B. cereus* and tends to be self-limited.

Other infections of *Clostridium perfringens* are

- 1- Gangrenous appendicitis
- 2- Necrotizing enteritis
- 3- Biliary tract infection
- 6- Brain abscess

Laboratory diagnosis

Specimens: They are collected from:

1. Muscles at the edge of affected area.
2. Exudate from area where infection appears more active.
3. Necrotic tissue and muscle fragment

Microscopic Examination

Gram-stained smear shows Gram-positive, long and thick bacilli. Gram-positive bacilli without spore are suggestive of *Clostridium perfringens*.

Culture: Material is inoculated on fresh blood agar and cooked meat media. Surface culture is incubated for 48 to 72 hours. Look at double zone of hemolysis

Further identification is done by:

1. Nagler's reaction.
2. Biochemical reaction.
3. Animal pathogenicity.

NAGLER'S REACTION

Clostridium perfringens are cultured on plates containing 20 percent of human serum or egg yolk. The organism produces opalescence in media containing human serum and egg yolk. The opalescence is due to lecithinase activity of alpha toxin. Alpha toxin splits lipoproteins and liberates lipids. The lipid deposits around the colony to give opalescence (Fig. 11). The reaction is specific and is inhibited by alpha toxin antitoxin sera

Treatment

The most important aspect of treatment is prompt and extensive surgical debridement of the involved area and excision of all devitalized tissue, in which the organisms are prone to grow. Administration of antimicrobial drugs, particularly penicillin, is begun at the same time. Hyperbaric oxygen may be of help in the medical management of clostridial tissue infections.

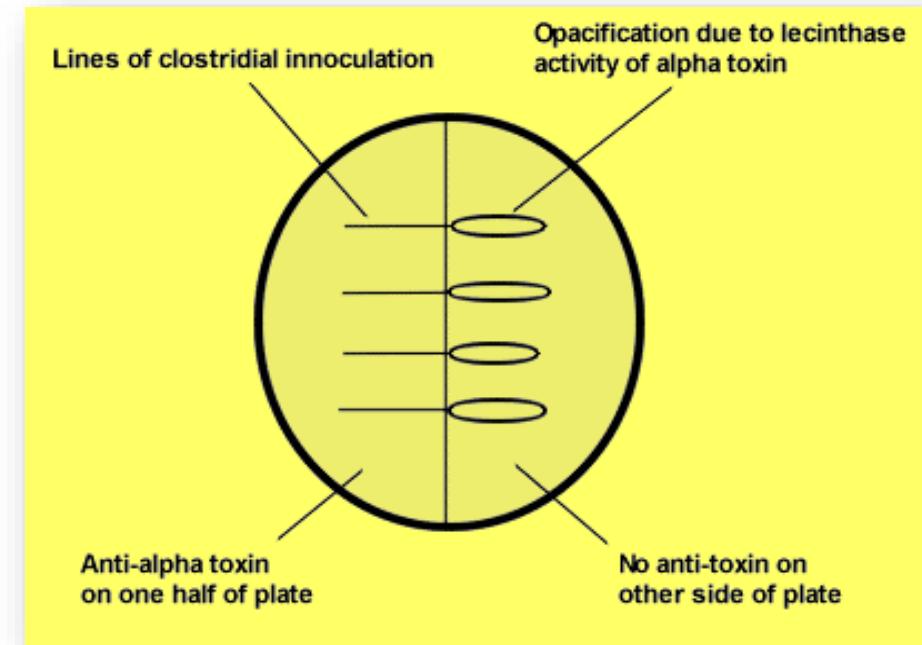


Fig.11. Nagler's reaction