Al-Mustaqbal University College
Dept. Medical Lab. Techniques, 4th. class
Diagnostic Microbiology 20/2021
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L. No. 8: Genus Clostridium

After reading and studying this lecture, the student should be able to:

- Describe species of **clostridia**.
- Discuss morphology, **cultural characteristics of *Cl. Perfringens*** (*welchii*).
- Discuss laboratory **diagnosis and prophylaxis of gas gangrene.**
- Discuss morphology, **cultural characteristics of *Cl. tetani*.**
- Discuss morphology and **cultural characteristics of *Cl. botulinum*.**

General features of Clostridium:

1. Morphology

The clostridia are: **1- all anaerobic**, **gram-positive**. **2- straight or slightly curved rods**. **3-** Most species of clostridia are **motile** with **peritrichous flagella except *Cl. perfringens* and *Cl. Tetani* type VI which are non-motile**. **4-** All clostridia are **non-capsulated except *Cl. Perfringens***. 5- All clostridia produce **endospores**. **Spores of clostridia are usually wider than the diameter of the rods in which they are formed**. **In the various species, the spore is placed centrally, sub-terminally, or terminally.**

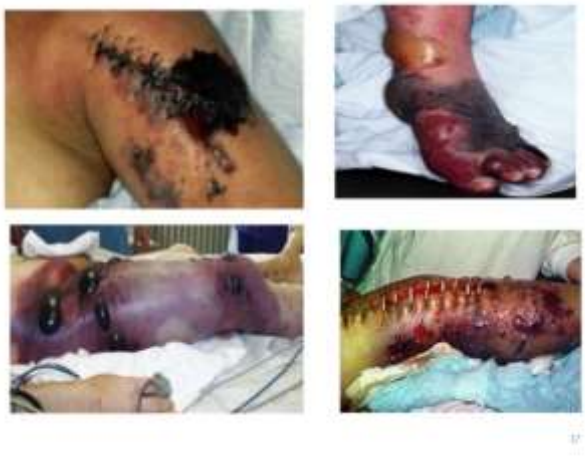
2. Culture

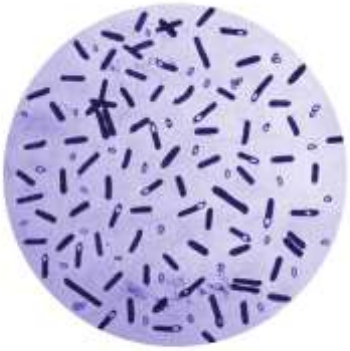
Most species are **obligate anaerobes**. **Clostridia grow on enriched media in the presence of reducing agent such as cysteine or thioglycollate** or in an O_2-free gaseous atmosphere provided by an air **evacuated glove box, sealed jar, or other device.**

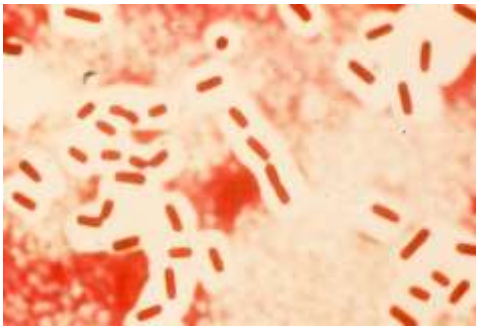
Liquid media like **cooked meat broth** (**CMB**) or **thioglycollate** media (containing reducing agent thioglycollste and 0.1% agar) are very useful for growing clostridia.

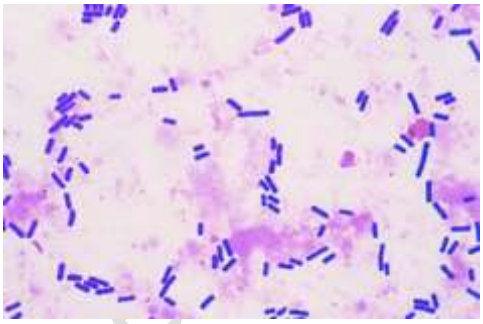
A very useful medium is **Robertson's cooked (RCM)** meat broth. It contains unsaturated fatty acids which take up oxygen.

Clostridium perfringens: **Gas gangrene: Morphology:** It is a relatively **large gram-positive bacillus** with **straight, parallel sides and rounded**, occurring singly or in **chains** or **small bundles**. It is **capsulated** and **non-motile**. Spores are **oval**, **sub-terminal and not bulging.**





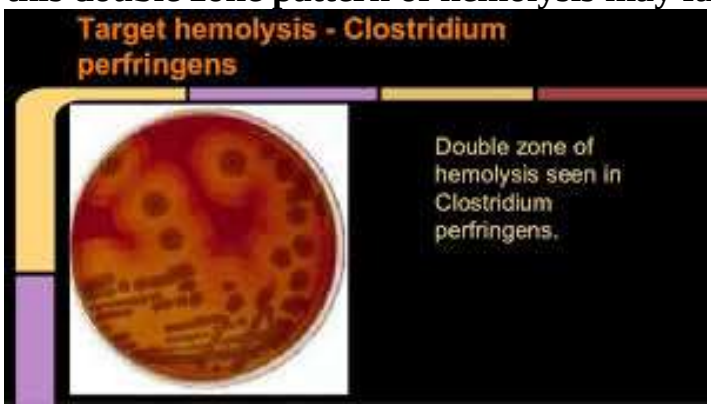




India ink preparation showing capsule **Gram-stain showing large, gram positive**

Characteristics: It is an **anaerobe.** It grows over a pH range of 5.5 to 8.0 and temperature range of 20°C to 50°C (**optimum temperature range 37-45°C**). **Good growth occurs in Robertson's cooked meat medium. The meat is turned pink but is not digested.**

It grows best on **carbohydrate-containing media** such as glucose blood agar. Surface colonies are large, smooth, regular, convex, slightly opaque disks. Colonies of most strains demonstrate a '**target hemolysis**, **double zone of hemolysis**' after overnight incubation on **rabbit**, **sheep**, or **human** blood agar. **It results from a narrow zone of complete hemolysis due to theta toxin and a much wider darker zone of incomplete hemolysis due to the α-toxin. On longer incubation this double zone pattern of hemolysis may fade.**



C. perfringens **also produces a characteristic pattern of synergistic Beta-hemolysis when streaked alongside *Streptococcus agalactiae*** (**CAMP** test).

Biochemical Reactions

It is actively **saccharolytic**. **1**- it ferments **Glucose**, **maltose**, **lactose** and **sucrose** are fermented with the **production of acid and gas.** **2-** It is **Indole negative**, **VP negative and MR positive**. **3-** Hydrogen sulfide (**H2S**) is produced abundantly; 4- most strains reduce **nitrates to nitrites.**

In litmus milk medium, fermentation of lactose leads to formation of acid, **which is indicated by the change in the color of litmus from blue to red.** The acid clots the milk "casein" and the clotted milk is disrupted due to the **vigorous gas production.** This is known as **'stormy fermentation or 'stormy clot' reaction** that is produced by all strains of C. *perfringens.*

Left to right:

a. **RCM:** Meat turned pink but not digested
b. **Litmus Milk:** Stormy fermentation & acid clot in Litmus milk
c. **BAM:** Target hemolysis

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Laboratory Diagnosis

1. Specimens:

- Edge of the affected muscles.
- Exudates from the wound.
- Necrotic tissue and muscle fragments.

2. **Microscopy**: **If gas gangrene is present, predominate gram-positive rods are seen.**

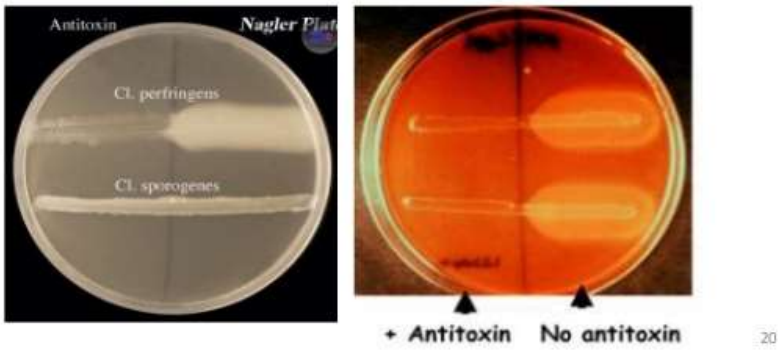
3. **Culture**: Fresh and heated blood agar are used for aerobic and anaerobic cultures. **To prevent swarming by some species of clostridia, the use of plates containing increased agar (5-6%) are used**. A plate of serum or egg-yolk agar, with *Cl. perfringens* antitoxin spread on one half is used for the **'Nagler's reaction'** (**see the diagram below, please).**

4.Nagler's Reaction

-Rapid detection of *Cl.perfringens* from clinical sample

-Done to detect the **lecithinase** activity of **alpha toxin**

-Characteristics **opalescence is produced** around colonies in **+ve test** due to breakdown of lipoprotein complex in the medium



Four tubes of cooked meat broth are inoculated and heated at 100°C for 5, 10, 15 and 20 minutes, incubated and sub-cultured on blood agar plates after 24 to 48 hours, to differentiate the organisms with heat resistant spores.

4. Identification

Examine plates for typical colonies. The isolates are identified based on their morphological, cultural, biochemical and toxigenic characters.

5. Animal Pathogenicity

A more specific diagnosis is possible by use of an enzyme-linked immunosorbent assay (**ELISA**) to detect *C. perfringens* enterotoxin in the feces of affected persons.

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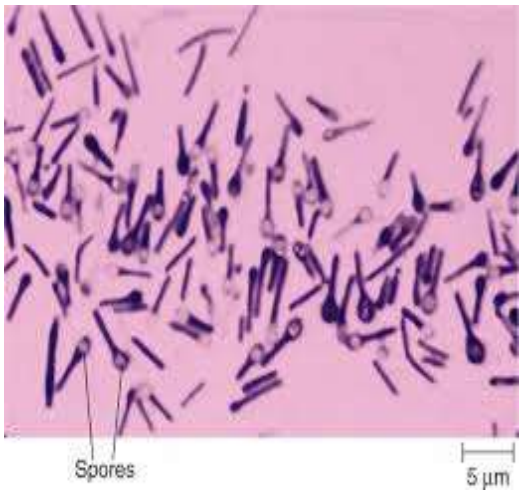
Morphology: **1**- It is **gram-positive**, bacillus with rounded ends. **2**- The **spores are spherical, terminal and twice the diameter of vegetative cells giving them typical drumstick appearance**. **3**- It is **non-capsulated but motile by peritrichous flagella**.



Cultural Characteristics

1- *Cl. tetani*is an **obligate anaerobe**. **2**- The optimal temperature for growth is 37°C, and the optimal pH is 7.4. **3**- It can grow well in cooked meat broth (CMB), thioglycollate broth, nutrient agar and blood agar. **4**- In cooked meat broth (CMB), growth occurs as turbidity with gas formation. **5**- On blood agar the bacilli produce a **swarming** (thin spreading film) growth. **6**- On horse blood agar, the colonies of *Cl. tetani*are surrounded by a zone of **α-hemolysis**, which subsequently develops into β-hemolysis, due to the production of hemolysin known as **tetanolysin. 7- In gelatin stab cultures a fir tree type of growth occurs, with slow liquefaction.**

Biochemical Reactions

1- *Cl. Tetani* has weak proteolytic and no saccharolytic. **2- It does not attack any sugar**. **3**- It is **indole positive** and negative for; **MR, VP, H2S and nitrate reduction.**

Laboratory Diagnosis

1. **Specimen**: **Wound exudate or tissue removed from the wound**.
2. **Microscopy**: **Microscopy is unreliable** It may not be possible to distinguish by microscopy between *Cl. Tetani* and similar bacilli such as *Cl. Tetanomorphum.* **Hence**, microscopy is unreliable.
3. **Culture**: The material is inoculated on one half of a blood agar plate. *Cl. Tetani* produces a **swarming growth** which may be detected on the opposite half of the plate after 1 to 2 days incubation anaerobically. The incorporation of **polymyxin B**, to which clostridia are resistant, makes the medium more selective. The material is also inoculated into **three tubes of cooked meat broth, one of which is heated to 80°C for 15 minutes, the second for five minutes, and the third left unheated. The purpose of heating for different periods is to kill vegetative bacteria, while leaving undamaged tetanus spores, which vary widely in heat resistance**. The cooked meat tubes are incubated at 37°C and sub-cultured on one-half of blood agar plates daily for up to four days. For identification and toxigenicity testing, blood agar plates (with 4% agar to inhibit swarming), having tetanus antitoxin (1500 units per ml) spread over one-half of the plate are used.
4. **Toxigenicity Test**
   Toxigenicity is best tested in animals. Control mice are protected with tetanus antitoxin. Two mice, one unprotected and other protected are used for each test. One animal is protected by giving 1,000 units of tetanus antitoxin intraperitoneally 1 hour before the test. Inject 0.1 ml of a 48 hour CMB culture supernatant of the organism intramuscularly into the hind limb of one mouse (the test) and the same amount in the another animal (control animals). The protected mouse remains well. Signs of ascending tetanus develop in the unprotected animal after several hours, they begin in the inoculated leg and extend to the tail, then the other hind limb is affected and then generalized signs appear. The animal dies within 2 days.

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Clostridium botulinum **L.10th.**

C. *botulinum* causes botulism. Botulism is a severe "fatal", form of food poisoning characterized by **neurotoxic** effects. The disease has been caused by a wide range of foods, usually preserved hams, sausages, home-preserved meats and vegetables, canned products such as fish, liver and other meat products contact.

Spores

colonies on egg yolk agar (EYA)

Morphology: *C. botulinum* is a **strictly anaerobic gram-positive bacillus**. It is **non-capsulated, motile with peritrichous flagella and produces spores which are oval, sub-terminal and bulging.**

Cultural Characteristics: It is a **strict anaerobe**. Optimum temperature is 35°C but some strains may grow even at 1 to 5°C. Good growth occurs on ordinary media. **Surface colonies are large, irregular, and semi-transparent, with fimbriate border**. **On horse blood agar, all strains except those of type G are beta-hemolytic. On egg-yolk agar (EYA) all types except G produce opalescence and a pearly effect (as in above photo)**

Resistance: Spores are heat and radiation resistant, surviving several hours at 100°C and for up to 10 minutes at 120°C.

Laboratory Diagnosis: Botulism confirmed by isolating the organism or detecting the toxin in food products or the patient's feces or serum.

1- **Specimens**: Feces, food, vomitus, gastric fluid, serum, environmental samples and some tine wound exudate.
2- Culture: For the isolation of *C. botulinum*, the specimen is inoculated on egg yolk agar (**EYA**), **blood agar and CMB**. Culture of the heated specimen on nutritionally enriched anaerobic media allows the heat resistant *C. botulinum* spores to germinate. The strains of *C. botulinum* associated with human botulism are characterized by **lipase production (appears as an iridescent film on colonies grown on egg-yolk agar) as well as the ability to digest milk proteins, hydrolyze gelatin and ferment glucose.**

Note; Presence of bacilli in food or feces in absence of toxin is of no significance. Hence, toxin in culture fluid must be demonstrated by toxigenicity test in mice.

Demonstration of Toxin:

Demonstration of toxin production must be done with a mouse bioassay. This procedure consists of the preparation of two parts of the suspension isolate, mixing of one part with antitoxin, and intraperitoneal inoculation of each part into mice. Toxin activity is confirmed if the antitoxin treatment protects the mice, control animals protected by antitoxin remain healthy. Samples of the implicated food, stool specimen and patient's serum should also be tested for toxin activity.

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