MICROBIOLOGY LAB . 6

Gram- positive rods

Spore formers

Bacillus & Clostridium

Assis. lect.
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Learning Objectives:

After this lab. You must be able to:

- Distinguish between G +ve rods genera.
- Describe each species of Gram positive rods microscopically and culturally.
- Differentiate between *Bacillus anthracis* and other saprophytic species.
- Differentiate between *Clostridium* spp.
- List types of clinical infections these organisms produce.
- Predict G +ve causative agents causing clinical cases.
- Discuss the principles of identifying tests.
- Know prevention ways of some fatal organisms.
There are four medically important genera:

A. Spore forming
   1. Bacillus
   2. Clostridium

B. Non spore forming
   1. Corynebacterium (club-shaped)
   2. Listeria

   longer and more deeply staining than others.

   (Chinese letters).
Aerobic Spore-forming Gram-positive Rods

(Bacillus)
Table 19.1

Gram-Positive Bacilli

Endospore-formers
- Bacillus
- Clostridium

Gram-Positive Rods
- Regular shape and staining properties
  - Listeria
  - Erysipelothrix

Non-endospore-formers
- Irregular shape and staining properties
- Non-acid-fast
  - Corynebacterium
  - Propionibacterium
- Acid-fast
  - Mycobacterium

Filamentous, branching cells
*Actinomyces*
*Nocardia*
General Characteristics of Bacillus:

- Large gram-positive rods have square ends.
- They are frequently arranged in long chains.
- **Aerobic**, produce endospores located in the center of the bacilli.
- Most members are saprophytic organisms, primary habitat in soil.
- **Catalase positive** (most)
- Can flourish at extremes of acidity & alkalinity (pH 2 to 10)
<table>
<thead>
<tr>
<th>Organism</th>
<th>Diseases</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. anthracis</em></td>
<td>Anthrax (cutaneous, gastrointestinal, inhalation)</td>
</tr>
<tr>
<td><em>B. cereus</em></td>
<td>Gastroenteritis (emetic, diarrheal), ocular infections, catheter-related sepsis, opportunistic infections</td>
</tr>
<tr>
<td><em>B. mycoides</em></td>
<td>Gastroenteritis, opportunistic infections</td>
</tr>
<tr>
<td><em>B. thuringiensis</em></td>
<td>Gastroenteritis, opportunistic infections</td>
</tr>
<tr>
<td>Other <em>Bacillus</em> species</td>
<td>Opportunistic infections</td>
</tr>
</tbody>
</table>
Bacillus anthracis
1. *Bacillus anthracis*: **causative agent of anthrax**

1. Large, block-shaped or bamboo-shaped rods.
2. **Produce central spores** that develop under all conditions except in the living body.
3. **Virulence factors** – polypeptide capsule and exotoxins.
4. There are 3 types of anthrax:
   - **Cutaneous** *(malignant pustule)* – spores enter through skin, black sore- eschar; least dangerous.
   - **Pulmonary** *(wool sorter's disease)* – inhalation of spores, most deadly form.
   - **Gastrointestinal** – ingested spores, rare but commonly fatal disease.
Cutaneous anthrax

About 20% mortality
Lab Diagnosis:

- **Specimen:** aspirate or swab from cutaneous lesion, blood culture
- **Gram stain:** large, square-ended gram-positive rods; may appear end-to-end giving a "bamboo appearance".
- **Culture:** Nonhemolytic on blood agar; raised, large, grayish-white, irregular, fingerlike edges described as “Medusa head” or “beaten egg whites”.
- **Rapid methods:**
  - PCR: *Identification of toxin genes*
  - Direct fluorescent antibody test
  - ELISA
When bacteria grown on blood agar plate, the organism produce non-hemolytic gray to white colonies with a rough texture and ground-glass appearance. Comma shape outgrowth (medusa head, curled hair) may project from the colony.
**Key Characteristics to Distinguish between *B. anthracis* & Other Species of *Bacillus***

<table>
<thead>
<tr>
<th>Characteristic</th>
<th><em>Bacillus anthracis</em></th>
<th>Other <em>Bacillus</em> spp.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolysis</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>Motility</td>
<td>Neg</td>
<td>Pos (usually)</td>
</tr>
<tr>
<td>Gelatin hydrolysis</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>Salicin fermentation</td>
<td>Neg</td>
<td>Pos</td>
</tr>
</tbody>
</table>
Bacillus cereus
2- *Bacillus cereus*: causes food poisoning

- Large, *motile (swarming)*, saprophytic bacillus
- Produce two toxines:
  - ✓ heat and acid stable toxin (*Emetic syndrome*) Associated with fried rice
  - ✓ Heat labile enterotoxin (*Diarrhoeal disease*) Associated with meat, poultry, and soups
- Lab diagnosis – Demonstration of large number of bacilli in food. And it not usually done.
# Foodborne Diseases of *B. cereus*

**Intoxication**

<table>
<thead>
<tr>
<th>Emetic Form</th>
<th>Diarrheal Form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Implicated food</strong></td>
<td><strong>Meat, vegetables</strong></td>
</tr>
<tr>
<td>Rice</td>
<td></td>
</tr>
<tr>
<td><strong>Incubation period (hours)</strong></td>
<td>&gt;6 (mean, 9)</td>
</tr>
<tr>
<td>&lt;6 (mean, 2)</td>
<td></td>
</tr>
<tr>
<td><strong>Symptoms</strong></td>
<td><strong>Diarrhea, nausea, abdominal cramps</strong></td>
</tr>
<tr>
<td>Vomiting, nausea, abdominal cramps</td>
<td></td>
</tr>
<tr>
<td><strong>Duration (hours)</strong></td>
<td><strong>20–36 (mean, 24)</strong></td>
</tr>
<tr>
<td>8–10 (mean, 9)</td>
<td></td>
</tr>
<tr>
<td><strong>Enterotoxin</strong></td>
<td><strong>Heat-labile</strong></td>
</tr>
<tr>
<td>Heat-stable</td>
<td></td>
</tr>
</tbody>
</table>
Anaerobic Spore-forming Gram-positive Bacilli

( Clostridia)
General features:

All clostridia are:

- Gram positive, straight or slightly curved rods with slightly rounded ends.
- Anaerobes (acquire energy only by fermentation and grow well on the blood agar and other media).
- Spore forming (Spores of clostridia are usually wider than the diameter of the rods).
**Clostridia:**

There are four medically important species; *Cl. tetani*, *Cl. botulinum*, *Cl. perfringens*, and *Cl. Difficile*

<table>
<thead>
<tr>
<th>Species</th>
<th>Human Disease</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. difficile</em></td>
<td>Antibiotic-associated diarrhea, pseudomembranous colitis</td>
<td>Common</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>Soft tissue infections (i.e., cellulitis, suppurative myositis, myonecrosis or gas gangrene), food poisoning, enteritis necroticans, septicemia</td>
<td>Common</td>
</tr>
<tr>
<td><em>C. septicum</em></td>
<td>Gas gangrene, septicemia</td>
<td>Uncommon</td>
</tr>
<tr>
<td><em>C. tertium</em></td>
<td>Opportunistic infections</td>
<td>Uncommon</td>
</tr>
<tr>
<td><em>C. botulinum</em></td>
<td>Botulism</td>
<td>Uncommon</td>
</tr>
<tr>
<td><em>C. tetani</em></td>
<td>Tetanus</td>
<td>Uncommon</td>
</tr>
<tr>
<td><em>C. barati</em></td>
<td>Botulism</td>
<td>Rare</td>
</tr>
<tr>
<td><em>C. butyricum</em></td>
<td>Botulism</td>
<td>Rare</td>
</tr>
<tr>
<td><em>C. histolyticum</em></td>
<td>Gas gangrene</td>
<td>Rare</td>
</tr>
<tr>
<td><em>C. novyi</em></td>
<td>Gas gangrene</td>
<td>Rare</td>
</tr>
<tr>
<td><em>C. sordellii</em></td>
<td>Gas gangrene</td>
<td>Rare</td>
</tr>
</tbody>
</table>
Clostridioidium tetani
**Clostridium tetani:**

The causative agent of tetanus (lockjaw).

**Important features:**

- Form rounded or oval, terminal spores (2-4 times the diameter of bacillus, *drumstick appearance*).
- Motile with peritrichous flagella and produces a thin spreading film (swarming) when grown on enriched blood agar.
- Grows well in cooked meat broth.
- Produce very powerful exotoxins (*tetanolysin* - causes lysis of RBCs, *tetanospasmin* - neorotoxin).
- Proteolytic does not ferment sugars.
Clinical findings:

- Strong muscle spasm (spastic paralysis).
- Specific clinical features:
  - Lockjaw (trismus)
  - Risus sardonicus
  - Opisthotonos.

trismus, risus sardonicus, opisthotonos
Prevention: toxoid, 3% formaldehyde
Laboratory Diagnosis Of Tetanus

The diagnosis of tetanus depends primarily upon the clinical manifestation of tetanus including muscle spasm and rigidity.

- **Specimen:** Wound exudates using capillary tube
- **Gram stain** is a good method for identifying *Clostridium Cl. tetani* is Gram positive rod motile, with a round terminal spore giving a drumstick appearance

- **Culture:**
  - **On BA:** growth appears as a fine spreading film (swarming). The bacilli may produce hemolysis due to tetanolysin
  - **On NA:** after 24 – 72 hrs incubation are irregularly round, glistening grayish-yellow translucent colonies and the edge is filamentous
Treatment:
- Immune globulin to neutralize the toxin.
- Penicillin G or metronidazole to kill the toxin-producing bacteria.

Prevention:
- Immunization with tetanus toxoid (formaldehyde-treated toxin) which is usually given in combination with Diphtheria and Pertussis vaccines (DTaP)
Clostridium botulinum
Cl. botulinum:
The causative agent of botulism

Important features:
- Form oval, bulging sub-terminal spores wider than the bacilli.
- Motile
- Producing the most powerful exotoxin.
- Result in flaccid paralysis can lead to respiratory failure.
- Associated with canned food, the highest food are:
  1- alkaline vegetables
  2- smoked fish.
Laboratory Diagnosis Of Botulism

Botulinum confirmed by isolating the organism or detecting the toxin in food products or the patient feces or serum.

* microscopic detection or culture are often unsuccessful (few organism and slow growing)

** toxin detected and typed in lab via toxicity and antitoxin neutralization test in mice or ELISA.
TREATMENT AND PREVENTION

• Prompt antitoxin can be life-saving  
  (mortality 100% ➔ 25%)
• Airway protection and respiratory support
• There is no vaccine
• Prevention relies on regulated food manufacturing
Clostridia perfringens
**Cl. Perfringens**: the causative agent of gas gangrene (myonecrosis) and food poisoning

**Important features:**

1. Form oval, not bulging, **central or sub-terminal** spores.
2. Capsulated in animal tissues.
3. Non motile, but **rapid spreading growth** mimics motile organism.
4. Produce variety of toxins, but the most important is alpha toxin (**lecithinase**) responsible for histotoxic and enterotoxigenic infection in humans.
5. Produce **cellulitis in the wound area**, Crepitation indicates gas in tissue.
6. Produce **two zone of hemolysis** around the colonies, first zone is due to theta toxin and the second is due to alpha toxin.
LABORATORY DIAGNOSIS

- **Specimen**: Histological specimen or wound exudates, food and feces
- **Microscopical examination**: Large gram-positive bacilli, spores are rarely observed.
- **Culture**: Anaerobically at 37°C
  - On Robertson's cooked meat medium → blackening of meat will observed with the production of $\text{H}_2\text{S}$ and $\text{NH}_3$
  - On blood agar double zone of $\beta$-hemolytic colonies.
  - Thioglycollate broth (contains sodium thioglycollate)
- **Biochemical test**:
  - It ferments many carbohydrates with acid & gas
  - It acidified litmus milk with stormy clot production
  - Nagler’s reaction is positive
LITMUS MILK REACTION:

- **Principle:** milk contains lactose with three main proteins; casein, lactoalbomin, and lactoglobuline. Therefore, an organism may exhibit one or more of the following metabolic properties:
  
  - Lactose fermentation
  - Litmus reduction
  - Clot formation
  - Peptization (digestion)
  - Gas formation
REACTION ON LITMUS MILK

1- Acidic Reaction

Lactose (Milk Sugar) \(\rightarrow\) Fermentation \(\rightarrow\) Acid \(\rightarrow\) Litmus Indicator \(\rightarrow\) Pink Color

2- Basic Reaction

Casein (Milk Protein) \(\rightarrow\) Digestion \(\rightarrow\) Alkaline amines \(\rightarrow\) Litmus Indicator \(\rightarrow\) Blue Color
REACTION ON LITMUS MILK

[Images showing two test tubes labeled 'base' and 'acid', and a close-up of a curd labeled 'acid']
Nagler’s Reaction:

- Presumptive identification of *Clostridium perfringens*
- Alpha toxin (lecithinase) breakdown of phospholipids (lecithin) in egg yolk agar to insoluble diglycerides resulting in an opaque halo (becomes turbid)
- This activity is specifically blocked by antitoxin
hemolysis on blood agar
NAGLER REACTION

Procedure of Nagler Reaction

Positive Nagler Reaction
GROWTH ON FLUID THIOGLYCOLATE

Clostridium sporogenes Growing in Thioglycolate Medium

Reducing agents in the medium absorb oxygen and allow obligate anaerobes to grow.
TREATMENT:

- Early diagnosis and aggressive treatment essential
  - Removal of necrotic tissue (surgical debridement)
  - Penicillin G in high doses if more serious infection
Clostridia difficile
**Cl. Difficile**: the causative agent of antibiotic associated pseudomembranous colitis.

- **Important features:**
  - GIT normal flora in 3-30% of population
  - Extremely oxygen sensitive

- **Laboratory diagnosis:**
  - Isolating the organism or detecting the cytotoxin or enterotoxin in patient’s feces by ELISA.