Microbiology lab 5
Gram-negative Rods
Enterobacteriaceae and *Pseudomonas*

Assis. lect.
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Learning objectives:

After this lab. You must be able to:

- Describe microscopic morphology and cultural biochemical characteristics of each member in this family.
- List infections caused by each of these members.
- Differentiate each member of this family from each other.
- Discuss principles of biochemical tests of each member in this family.
- Predict enterics causative agents causing clinical cases.
Gram-Negative Rods

Large group of diverse organisms, they are divided to:

- Gram-Negative Rods related to gastrointestinal tract.
- Gram-Negative Rods related to respiratory tract.
- Gram-Negative Rods related to animal source.
Gram-Negative Rods related to gastrointestinal tract

Enterobacteriaceae and Pseudomonas

- Enterobacteriaceae is a large family of bacteria commonly referred to as the fermentative, gram-negative, enteric bacilli, indicating that they are gram-negative rods which can ferment sugars.

- To differentiate them from non-fermentative, gram-negative rods such as *Pseudomonas*. 
Clinical Significance of Enterics

Enterics are ubiquitous in nature, some live in water, soil and sewage and most, except for few, are present in the intestinal tract of animals and humans as commensal flora; therefore, they are sometimes call “fecal coliforms”

Based on clinical infections produced, enterics are divided into two categories:

- **True pathogen** - *Salmonella, Shigella, Yersinia* sp and some strains of *E. coli*
- **Opportunistic pathogens** - normally part of the intestinal flora that may produce infection outside the intestine
## Family Enterobacteriaceae

<table>
<thead>
<tr>
<th>Genus</th>
<th>No. of species</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrobacter</td>
<td>4</td>
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<tr>
<td>Edwardsiella</td>
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<tr>
<td>Enterobacter</td>
<td>13</td>
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<tr>
<td>Escherichia</td>
<td>5</td>
</tr>
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<td>Shigella</td>
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<td>Ewingella</td>
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<td>Hafnia</td>
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<td>Klebsiella</td>
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<td>Kluyvera</td>
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<tr>
<td>Morganella</td>
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</tr>
<tr>
<td>Proteus</td>
<td>4</td>
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<tr>
<td>Providencia</td>
<td>5</td>
</tr>
<tr>
<td>Salmonella</td>
<td>7 subgroups</td>
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<tr>
<td>Serratia</td>
<td>10</td>
</tr>
<tr>
<td>Yersinia</td>
<td>11</td>
</tr>
</tbody>
</table>

Certain *E. coli* strains can be considered true pathogens.

True pathogen

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Important features

- Gram-negative nonspore forming rods.
- Most Enterobacteriaceae are motile, with the exception of the common isolates *Klebsiella*, *Shigella*, and *Yersinia*, the motile strains possess peritrichous flagella.
- Many Enterobacteriaceae also possess virulence factors: fimbriae, sex pilli, capsule, and endotoxin.
- Facultative anaerobes.
- All members ferment glucose.
- All members reduce nitrate to nitrite.
- All members are catalase positive and oxidase negative. (distinguish the Enterobacteriaceae from another medically significant group of organisms, the non fermenting gram-negative rods, the most important of which is *Pseudomonas aeruginosa*).
Colonial Morphology

 Ability to ferment lactose:

- lactose-fermenting strains *pink-purple colonies* (e.g., *Escherichia, Klebsiella, Enterobacter, Citrobacter*)
- Non lactose-fermenting strains *colorless colonies* (e.g., *Proteus, Salmonella, Shigella, and Yersinia spp*).
- Delayed lactose fermenter (DLF) (e.g., *Morganella, Providencia, Serratia, Edwardsiella, Erwinia, Hafnia*).

 Ability to grow on a large number of selective and differential media:

- Eosin-Methylene blue (EMB) agar: contains bile salt and dyes eosin and methylene blue, inhibit gram-positive bacteria.
- Hektoen Enteric agar (HE): contains high concentration of bile salt and dyes bromothymol blue and acid fuchsin.
- Xylose Lysine Deoxycholate (XLD) agar contains: sodium deoxycholate inhibits the growth of gram positive bacteria.

 Most have similar colonial morphology in blood agar plate.

 moist, smooth, gray colonies and some strains are beta hemolytic.
**Escherichia coli**

- One of the most predominant intestinal flora, causes wide range of infections including: UTI, gastrointestinal infection, meningitis, wound infection and bacteremia.
- **Ferments lactose.** (pink colony on MacConkey’s agar)
- Have characteristic metallic sheen on EMB.
- Positive in indole and methyl red tests
- Negative in Voges-Proskauer and Simmons citrate tests
- Does NOT produce H₂S
- Usually motile.
- **IMViC test:** + + - -
- **TSI test:** A/A/ -
Klebsiella pneumoniae

- Usually found in intestinal tract and frequent cause of nosocomial pneumonia.
- **Ferments lactose.** (pink colony on MacConkey’s agar)
- Possess a polysaccharide capsule makes the colonies mucoid and moist.
- **Negative** in indole and methyl red tests
- **Positive** in Voges-Proskauer and Simmons citrate tests
- Does NOT produce H$_2$S
- Non motile.
- **IMViC test:** - - ++
- **TSI test:** A/A/ -
Enterobacter

- The most clinically important species are: *E. cloacae* and *E. aerogenes*
- Isolated from wounds, urine, blood and CSF
- Ferments lactose. (Colonies resemble *Klebsiella*)
- Motile (differ from *Klebsiella*)
- Negative in indole and methyl red tests
- Positive in Voges-Proskauer and Simmons citrate tests
- Urease test positive
- IMViC test: - - + +
- TSI test: A/A/ -
**Serratia marcescens**

- Causes nosocomial UTI, and respiratory tract infections.
- Ferments lactose slowly.
- Motile
- Produce characteristic reddish-pink color on nutrient agar when cultured on room temp.

*S. marcescens* on nutrient agar →
Proteus

- The most clinically important species are: *P. mirabilis* and *P. vulgaris*
- Isolated from urine, wound, ear and bacteremic infections.
- Do not ferment lactose. (colorless on MacConkey’s agar)
- Motile (produce swarming on non selective media).
- Negative in indole and Voges-Proskauer tests
- Positive in methyl red and Simmons citrate tests
- Produce H₂S
- Urease test positive
- IMViC test: - + - +
- TSI test: Alk/A/ +
Pseudomonas aeruginosa

- Non-fermenter gram-negative bacilli.
- Strict aerobes (acquire energy by oxidation not by fermentation)
- Oxidase-positive.
- Some Pseudomonads are motile by means of polar flagella.
- Produces a characteristic fruity or sweety grape juice-like aroma.

Oxidase test
- Commonly habitat soil and water and found in small numbers in human feces.

- Have the ability to grow in lower nutrient environment, and have the ability to grow in disinfectant, so they persist in hospital environment.

- It is especially dangerous to the debilitated or compromised patient (burn and cystic fibrosis), it cause a nosocomial UTIs, wound infections, pneumonia, and septicemia.
- Produce two characteristic pigments, diffused in agar:

✓ **Pyocyanin**: color the pus in wound (blue)

✓ **Pyoviridin** (fluorescein): fluoresces under UV light (yellow green), help in early diagnosis of skin infection.
Laboratory diagnosis:

- **Specimen:** site of origin must be considered
- **Gram-stain:** not of value
- **Culture:** blood agar and a selective-differential medium such as MacConkey’s agar
- **Lactose-fermenter:** pink colonies
- **Non lactose fermenter:** colorless colonies.
- **For stool:** highly selective media such as Hekton-enteric, SS agar is used along with Mac.
- **Biochemical tests:**
  - IMViC test
  - TSI tests
  - API test
  - Urease test
  - Oxidase test
  - Serological test
IMViC Test

- **Indole, Methyl red, Voges-Proskauer, Citrate tests**
  - **Indole test:**
    - **Principle**
      - Certain microorganisms can metabolize tryptophan by tryptophanase to pyruvic acid, indole and ammonia.
      - The presence of indole is detected by addition of Kovac's reagent.

- **Method:**
  - Inoculate *tryptone water* with the tested microorganism,
    - Incubate at 37°C for 24 hours
  - After incubation interval, add 1 ml *Kovac’s reagent*, shake the tube gently and read immediately
Methyl Red-Voges Proskauer (MR-VP) Tests

**Principle:** depend on the patterns of glucose metabolism

- **Acidic pathway:**
  - Mixed acids $\downarrow$ pH less than 4.4
  - Methyl Red indicator
  - Red color $\rightarrow$ MR positive
  - $E. coli$

- **Neutral pathway:**
  - Acety methyl carbinol (ACETONE)
  - VP positive
  - Klebsiella
  - Barrit’s A
  - Barrit’s B
  - Pink color

Glucose
**Methods:**

- Inoculate the tested organism into tubes of MRVP broth, incubate the tubes at 37°C for 24 hours
- **AFTER INCUBATION:**
  - For methyl red: Add 6-8 drops of methyl red reagent.
  - For Voges-Proskauer: Add 6 drops of Barritt’s A (α-naphthol), and 2 drops of Barritt’s B (40% KOH), mix.
  - Let, undisturbed, for at least 1 hour, then note color change.
**Citrate Utilization Test**

- **Principle:** citrate utilized by bacteria produce citrase enzyme.

Citrate $\rightarrow$ Pyruvate $\rightarrow$ $CO_2 + Na + H_2O$ $\rightarrow$ $Na_2CO_3$

Alkaline, ↑pH

Simmon’s Citrate media
Contains Citrate as a sole of C source

Blue color

Positive test

Bromothymol blue
**Method:**

Streak a **Simmon's citrate agar** slant with the organism and incubate at 37°C for 24 hours. Growth on the medium is accompanied by a rise in pH to change the medium (**containing bromothymol blue indicator**) from its initial green color to deep blue.

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**Citrate utilization test**

- **Positive Klebsiella**
- **Negative E. coli**
Urease Test

- **Principle:**
  - Urea agar contains urea and phenol red, Urease is an enzyme that catalyzes the conversion of urea to $CO_2$ and $NH_3$.
  - Ammonia combines with water to produce ammonium hydroxide, a strong base which ↑ pH of the medium.
  - ↑ in the pH causes phenol red to turn a deep pink.

  ![Urea conversion diagram]

- **Method:**
  - Streak a *urea agar* tube with the organism incubate at 37°C for 24 h.
  - If color of medium turns from yellow to pink indicates positive test.
Triple Sugar Iron (TSI) Agar

- **TSI contains:**
  - Three different types of sugars
    - Glucose, Lactose, Sucrose
    - Phenol red (acidic: Yellow)

- **Principle:**
  - To determine the ability of an organism to utilize a specific carbohydrate incorporated into a basal growth medium, with or without the production of gas, along with the determination of possible hydrogen sulphide production.

- **Method:**
  - Inoculate TSI medium with an organism by inoculating needle by stabbing butt and streaking the slant incubate at 37°C for 24 hours.
**H₂S Production**

- **Principle:**
  - Bacteria use the enzyme cysteine disulfurase to hydrolyze the amino acid cysteine, forming hydrogen sulfide as end product.
  - cysteine disulfurase
  - Cysteine $\rightarrow$ NH₃ + pyruvic acid + H₂S
  - H₂S + FeSO₄ $\rightarrow$ FeS + H₂SO₄
<table>
<thead>
<tr>
<th>Slant color</th>
<th>Butt color</th>
<th>H₂S</th>
<th>Result</th>
<th>Example</th>
</tr>
</thead>
<tbody>
<tr>
<td>Red</td>
<td>Red</td>
<td>Negative</td>
<td>Alk/Alk/- (No action on sugars)</td>
<td>Non fermenter e.g. <em>Pseudomonas</em></td>
</tr>
<tr>
<td>Red</td>
<td>yellow</td>
<td>Negative</td>
<td>Alk/A/- (Glucose fermented without H₂S)</td>
<td>LNF e.g. <em>Shigella</em></td>
</tr>
<tr>
<td>Red</td>
<td>yellow</td>
<td>Positive</td>
<td>A/Alk/+ (Glucose fermented with H₂S)</td>
<td>LNF e.g. <em>Salmonella &amp; Proteus</em></td>
</tr>
<tr>
<td>Yellow</td>
<td>Yellow</td>
<td>Negative</td>
<td>A/A/- (three sugars are fermented)</td>
<td>LF e.g. <em>E. coli, Klebsiella, Enterobacter</em></td>
</tr>
</tbody>
</table>

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**Summary of morphology, cultural characteristics, and biochemical reactions of *Enterobacteriaceae***

<table>
<thead>
<tr>
<th></th>
<th>Gram stain</th>
<th>Oxidase</th>
<th>Nitrate reductase</th>
<th>O/F</th>
<th>Mac</th>
<th>SS</th>
<th>EMB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em></td>
<td>-ve rod</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>LF</td>
<td>LF</td>
<td>Metallic sheen</td>
</tr>
<tr>
<td><em>Citrobacter</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>LF</td>
<td>LF</td>
<td>Dark</td>
</tr>
<tr>
<td><em>Klebsiella</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>LF</td>
<td>LF</td>
<td>Dark</td>
</tr>
<tr>
<td><em>Enterobacter</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>LF</td>
<td>LF</td>
<td>Dark</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>NLF</td>
<td>NLF/H₂S</td>
<td>Colorless</td>
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<tr>
<td><em>Shigella</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>NLF</td>
<td>NLF</td>
<td>Colorless</td>
</tr>
<tr>
<td><em>Proteus</em></td>
<td>-ve rods</td>
<td>-ve</td>
<td>+ve</td>
<td>O+/F+</td>
<td>NLF</td>
<td>NLF/H₂S</td>
<td>Colorless</td>
</tr>
<tr>
<td></td>
<td>TSI</td>
<td>Indole</td>
<td>MR</td>
<td>VP</td>
<td>Citrate</td>
<td>Urease</td>
<td>Motility</td>
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<td>----------------</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td>A/A/-</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Motile</td>
</tr>
<tr>
<td><strong>Citrobacter freundii</strong></td>
<td>A/A/-</td>
<td>+ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>Motile</td>
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<td><strong>Klebsiella pneumoniae</strong></td>
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<td>Non motile</td>
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<tr>
<td><strong>Enterobacter cloacae</strong></td>
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<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>+ve</td>
<td>Motile</td>
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<td><strong>Salmonella typhi</strong></td>
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<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>Motile</td>
</tr>
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<td><strong>Shigella boydii</strong></td>
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<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>-ve</td>
<td>-ve</td>
<td>Non motile</td>
</tr>
<tr>
<td><strong>Proteus mirabilis</strong></td>
<td>Alk/A/+</td>
<td>-ve</td>
<td>+ve</td>
<td>-ve</td>
<td>+ve</td>
<td>+ve</td>
<td>Motile</td>
</tr>
</tbody>
</table>

Summary of morphology, cultural characteristics, and biochemical reactions of *Enterobacteriaceae*
Identification of Gram’s -ve rods

Oxidase Test

Positive

Pseudomonas

✓ O/F test: O⁺/F⁻
✓ Nitrate test: +ve further reduction to N₂
✓ Growth on cetrimide agar: Pale colonies with green pigmentation

Negative

Enterobacteriaceae

MacConkey’s agar & TSI

Nitrate test: +ve

Growth on cetrimide agar:
- Pale colonies with green pigmentation
- Colorless colonies with black centers on SS agar

Lactose fermenter

IMV/C test & EMB

E. coli

Klebsiella

Enterobacter

Proteus

Salmonella

Lactose non-fermenter

IMV/C

Shigella

Motility

Not motile

Motile

O/F test:
- O⁺
- F⁻

Motility:
- Not motile
- Motile

Nitrate test:
- +ve further reduction to N₂
THANK YOU FOR ATTENTION