Gram-negative bacilli

The main Gram-negative bacilli significant for human pathology are represented by germs of the ***Enterobacteriaceae* family** and those of the **genus *Pseudomonas***.

Enterobacteriaceae (enterobacteria)

Many of the genera pertaining to the *Enterobacteriaceae* family are members of the indigenous intestinal microflora of humans and animals. Besides that, they are found in water and soil.

In terms of **clinical significance**, the *Enterobacteriaceae* family includes **pathogenic genera** such as *Salmonella, Shigella*, *Yersinia* and **facultatively pathogenic genera** such as *E.coli, Klebsiella, Enterobacter, Proteus, Serratia, Citrobacter*.

Depending on the **infection site**, enterobacteria may cause **intestinal** and/or **extraintestinal infections**.

Extraintestinal infections include wound infections, bloodstream infections, respiratory infections, urinary tract infections and CNS infections, which may be caused by *E.coli, Proteus, Klebsiella, and Enterobacter*

E.coli, *Shigella*, *Salmonella* and *Yersinia* cause the **intestinal infections**.

As may be observed in the examples above, some of these genera may be involved in intestinal as well as in extraintestinal infections (*E.coli, Klebsiella*), while others are only involved in intestinal infections (*Salmonella, Shigella*).

Common characters:

- Gram-negative bacilli, non-sporulating, non-fastidious
- Glucose fermenters
- Lactose is only fermented by some genera, making it a good differentiating criterion
- Catalase positive
- Oxidase negative
- Grow on MacConkey agar

Collection:

- Extraintestinal infections: urine, respiratory, digestive, blood or CSF samples, wound secretions
- Intestinal infections
    - Faeces in a transport medium (Stuart or Cary-Blair)

Isolation

- Extraintestinal specimens from normally sterile sites (e.g. Blood, CSF, Urine): Blood agar
- Extraintestinal specimens with moderate bacterial load (e.g. Pus, sputum): Blood agar and MacConkey
- Highly contaminated specimens (e.g. faeces): MacConkey (low selectivity), Hektoen agar (media selectivity), S-S agar (for *Shigella* and *Salmonella)* and Wilson-Blair agar (for *Salmonella)* both with high selectivity.

Identification

- **Biochemical tests:**
  - **TSI** (triple sugar iron) agar
  - **MIU** (motility, indol urea) agar
  - **Simmons agar** (use of citrate as an unique carbon source)
  - **PAD** (phenylalanine deaminase) test
  - **Fermentation of sugars**

- **Antigenic structure:** agglutination with antisera

Biochemical tests are based on testing for enzyme systems of the microorganisms tested

- **Method**: reinoculation of isolated colony from the primary culture into a series of tubes with culture media containing specific substrates and chemical indicators
- **Principle**: detection of
  - pH changes produced by utilisation of substrates
  - colour / other changes produced by specific by-products

There are commercial kits available (e.g. API 20E, BioMerieux; RapID ONE, Thermo Fischer Scientific) which provide the possibility to simultaneously perform around 20 biochemical tests by reinoculating bacterial colonies from primary cultures (low / medium selective culture media) into sets of culture media (galleries of microwells) offering the possibility to demonstrate biochemical characters e.g. fermentation of sugars, use of various substrates, production of H_2S, etc. The results of each test of the gallery, interpreted as “positive” or “negative”, are compared to known sets of results for each genus and species, thus offering the identification of the isolated colony.

Such commercial kits with sets of biochemical tests are also available for other bacterial genera such as *Staphylococcus, Streptococcus, Neisseria*, etc.

The following paragraphs will describe the main steps of the bacteriological diagnosis for some members of the *Enterobacteriaceae* family, starting with some of the pathogenic genera (*Salmonella* and *Shigella*) and continuing with some of the facultatively pathogenic genera (*E.coli, Klebsiella* and *Proteus*).

Highly pathogenic enterobacteria

Genus Salmonella

Clinical Significance

- Food poisoning = **salmonellosis =** intestinal infection with diarrhoea, abdominal cramps and fever
- Systemic infections **enteric fevers**

Transmission occurs via the **faecal-oral** route, e.g. contaminated water, foods (poultry, unpasteurized milk, eggs) and contact with humans.

Salmonellae are the most complex of all *Enterobacteriaceae;* over 2400 serotypes have been identified. The Kauffmann-White scheme classifies the genus based on bacterial antigens:

- O (somatic)
- H (flagellar)
- Vi (virulence) – derived from the K (capsular) antigen

Based upon the structure of the O (somatic) antigen, salmonellae are classified in several groups labelled as A – I; of these, four groups are especially important in human pathology:

- Group A: *S. paratyphi* A
- Group B: *S. paratyphi B*
- Group C: *S. paratyphi C*
- Group D: *S. typhi, S. enteritidis*

The habitat varies for the different species and subtypes of Salmonellae. *S. typhi* and *S. paratyphi A, B* and *C* are strictly human pathogens. The others are also found in animals.

Enteric fevers occur due to crossing of microorganisms through intestinal barrier into the lymphatic circulation and are caused by *S. typhi* (severe) and *S. paratyphi A, B* and *C* (less severe). A major type of enteric fever is the **typhoid fever**, a severe and serious disease with fever of 39-40°C, headache, vomit, diarrhoea, skin rashes (lenticular maculae = "rose spots"), muscle ache, mental confusion and hepatosplenomegaly after an incubation period of 14 days. Complications include internal bleeding, intestinal perforation and peritonitis. Typhoid fever may evolve fatally due to septic shock.

Septicaemia occurs if the germs pass into the cardiovascular system and can be caused by all species of *Salmonella.*

Bacteriological Diagnosis

Collection

Collection depends on two parameters: **Patient status** (diseased/chronic carrier) and **Clinical stage** (time from onset)

The specimens collected are:

- **Blood** for haemoculture, which is best collected during the 1st week
- **Faeces** for coproculture used in all cases of salmonellosis and best collected in the 4th week for the enteric fevers caused by *S. typhi* and *S. paratyphi*
- Urine

- Bone marrow in late stages with low patient compliance

Culture media and Identification

- **Haemoculture:** examined daily for 7-10 days - negative result only if haemoculture remained sterile for 10 days
- **Coproculture**: Inoculation performed into liquid enriched media, in order to favour the multiplication of salmonellae and inhibit other microorganisms:
    - **Leifson** (nutrient broth with acid sodium selenite)
    - **Müller-Kauffmann** (broth with tetrationate and bile)

For coprocultures, an initial incubation overnight at 37°C is performed on either of the two above mentioned liquid media. The overnight incubation is followed by reinoculation into **selective solid media** with nutrients, sugars, a pH indicator and substances that inhibit other germs.

Identification is based on the colonial characters on the selective solid media:

- **Wilson-Blair** (high selectivity; indicator: brilliant green)
    - Black, flat colonies, 1-2mm, metallic halo
- **S-S** (Salmonella-Shigella agar; selective for Salmonella and Shigella)
    - Fine, semitransparent colonies, with black centre (H_2S production)
- **Hektoen enteric agar**
    - Fine, green colonies with black centre (H_2S)
- **MacConkey** (medium selectivity)
    - Semi-transparent, lactose-negative (colourless) colonies

Further tests for identification include:

- Oxidase test (negative)
- Fermentation of sugars (glucose positive, lactose negative)
- Antigenic structure (slide agglutination with anti-O and anti-H sera based on the Kauffmann-White classification)

Antimicrobial susceptibility testing (Antibiogram)

It is essential to perform an antibiogram because of the resistance expressed by Salmonella and Shigella (beta-lactam resistance).

The most effective antibiotics currently are fluoroquinolones, and 3rd generation cephalosporins.

Essential to remember:

- Gram-negative bacilli
- Non-lactose fermenting (colourless colonies on MacConkey and other lactose-containing culture media)

- Oxidase negative
- Sugar fermentation (glucose positive and lactose negative)
- Black centre (H_2S production) on S-S agar, Hektoen enteric agar and XLD
- Causative agents for salmonellosis and enteric fevers

Genus Shigella

Clinical Significance

Shigella is classified into 4 serological subgroups according to the antigenic structure and biochemical characters:

- Subgroup A: *Shigella dysenteriae*
  - Types: ***Shigella shiga*** (most severe disease with secretion of **neurotoxin**), *Shigella Schmitzi, Shigella Large-Sachs*
- Subgroup B: *Shigella flexneri*
- Subgroup C: *Shigella boydii*
- Subgroup D: *Shigella sonnei*

Shigella spp. are only infecting humans and primates and transmission occurs via the **fecal-oral** route.

The pathogenicity of *Shigella* spp. is marked by infections with several endotoxins favouring the pathogenesis of mucous and bloody or non-bloody diarrhoea, known as **shigellosis** (bacillary dysentery).

Bacteriological Diagnosis

Collection

Collection is performed from **faeces** (especially from portions with mucus and blood if present) and transported with a Cary Blair transport medium.

Culture media and Identification

Inoculation is performed on selective media, such as **S-S agar**, **Hektoen agar**, **XLD agar** and MacConkey.

Identification is based on the colonial characters on the selective media:

- Generally the colonies are small, 1-2mm, transparent, round, convex with irregular contour and lactose negative (colourless colonies). Due to the fact, that there is no H_2S production, no black centre will be seen on S-S and Hektoen agar.

Further identification tests:

- Oxidase test (negative)
- Fermentation of sugars (glucose positive and lactose negative)
- Antigenic structure (agglutination with sets of anti-sera)

Antimicrobial susceptibility testing (Antibiogram)

An antibiogram is not necessarily performed, as the genus is susceptible to the common antibiotics active on Enterobacteriaceae, such as fluoroquinolones and cephalosporins.

Essential to remember:

- Gram-negative bacilli
- Non-lactose fermenting
- Oxidase negative
- Sugar fermentation (glucose positive and lactose negative)
- Causative agents for shigellosis
- No black centre on S-S agar, Hektoen enteric agar and XLD

Facultatively pathogenic enterobacteria

Escherichia coli

Clinical Significance

Habitat: intestinal microflora and mucous membranes of humans and animals

Transmission: through direct contact with infected persons, animals or environment

Pathogenicity:

- **Extraintestinal infections**
  - **Urinary tract infections**
  - **Septicaemia**
  - **Meningitis** (rare; mostly in infants)
  - **Respiratory**
  - **Wounds and burns**

- **Intestinal infections –** caused by
  - **EPEC** (enteropathogenic *E.coli)*

- **ETEC** (enterotoxigenic *E.coli*)
- **EIEC** (enteroinvasive *E.coli*)
- **EHEC** (enterohemorrhagic *E.coli*) – produces **verotoxins** (Shiga-like toxins – action similar to toxins produced by *Shigella disenteriae* strains) with bloody severe diarrhoea (resembling bacillary dysentery/shigellosis)
- **EAEC** (enteroadherent *E.coli)*

Bacteriological Diagnosis

In urinary tract infections, the bacteriological diagnosis is based on:

- the **microscopic examination** of urine samples: Gram-stained smears reveal the cellular defence reactions against the infection (high number of polymorphonuclear cells) and Gram negative bacilli;
- the presence of a large number of *E. coli* colonies (≥100,000 CFU/ml) on **culture media** (quantitative urine culture).

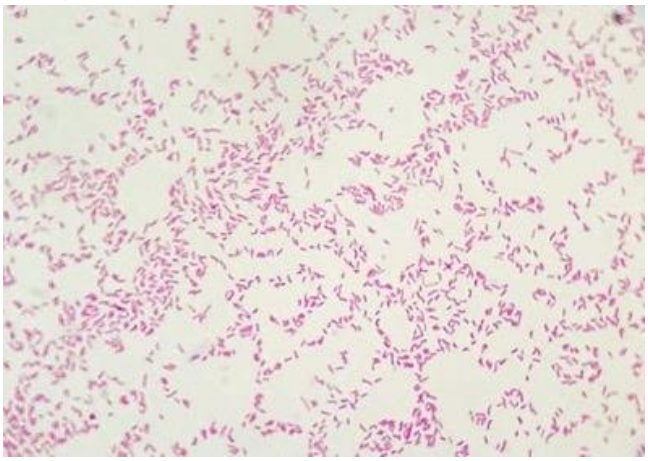


Figure 24. *E.coli*: Microscopic examination- colony - Gram staining

In local infections other than urinary (peritonitis, surgical wound infections, etc.), the diagnosis is made according to the usual procedures: aseptic samples, microscopic examination, culture, identification and antibiogram.

Collection

Intestinal infections**: stool**

Extraintestinal infections**:** depending on type of infection (urinary culture, coproculture, sputum, haemoculture)

Culture media and Identification

MacConkey: red colonies (lactose positive; due to the neutral red pH indicator), round, shiny, flat 2-3mm

Blood agar: smooth, grey, 2-3 mm

Identification is based on:

- Colonial characters
- Oxidase test (**negative**)
- Fermentation of sugars (**glucose** and **lactose positive**)
- Antigenic structure (slide agglutination with antisera)

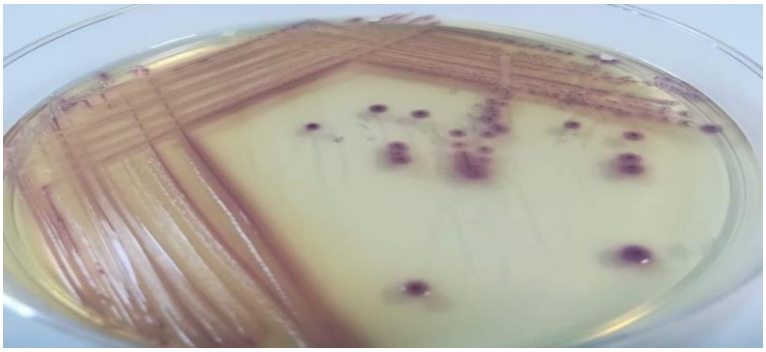


Figure 25. *E.coli* : Culturedifferential medium -CHROMagar UTI

Antimicrobial susceptibility testing (Antibiogram)

Wild type *E.coli* strains are sensitive to all beta-lactams, aminoglycosides, fluoroquinolones, sulphonamides, carbapenems and other antibiotics.

Other *E.coli* strains may produce penicillinases and cephalosporinases, making them resistant to aminopenicillins, carboxypenicillins, first, second and third generations of cephalosporins and ampicillin.

Some *E.coli* strains may carry extended spectrum beta-lactam resistant plasmids, making the success of therapy difficult for many beta-lactams, including third and fourth generations of cephalosporins.

Phenotypes resistant to carbapenems have been identified, carrying resistance to all carbapenems.

Essential to remember:

- Gram-negative bacillus
- Lactose fermenting (pink colonies on MacConkey)
- Oxidase negative
- Sugar fermentation (glucose and lactose positive)
- Most common causative agent in urinary tract infections

Genus *Klebsiella*

Clinical Significance

Klebsiella are commensal microorganisms, which colonize human respiratory and intestinal mucosa, thus the genus is considered facultatively pathogenic in immunocompromised patients with a potential for severe infections, such as pneumonia, sepsis and meningitis. Furthermore, this genus is involved in hospital acquired (=nosocomial) infections: such as surgical wound, urinary, respiratory and blood stream infections.

Four species are important for human pathology:

- ***Klebsiella pneumoniae***: commensal of human airways and intestinal mucosa and facultatively pathogenic; involved in nosocomial infections: urinary tract, respiratory, wound and blood infections.
- ***Klebsiella oxytoca***: same as for *Klebsiella pneumoniae*
- ***Klebsiella ozenae***: causative agent of ozena, a chronic inflammatory infection of the nasal mucosa with atrophy, crusts and purulent secretions.
- ***Klebsiella rhinoscleromatis***: causative agent for rhinoscleroma, a chronic granulomatous disease with chronic hypertrophic rhinitis.

Bacteriological Diagnosis

Collection

The collection of specimens depends on the site of infection, i.e. respiratory, urinary or reproductive tracts and blood.

Microscopic examination

Klebsiella are Gram-negative, short, nonsporulating, **encapsulated** bacilli arranged **in diplo** on the long axis.

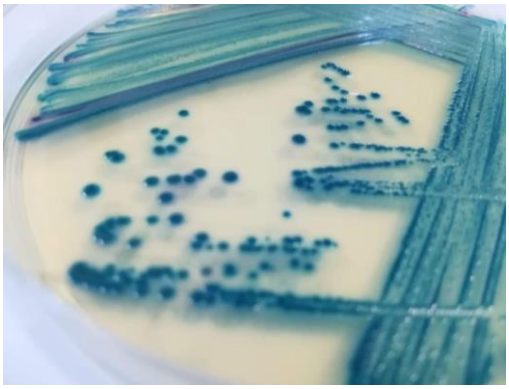
Culture media and Identification

Isolation of the genus *Klebsiella* depends on the site of infection:

- Specimens from normally sterile sites (blood, CSF)
    - Nutrient broth + reinoculation on blood agar
- Specimens from highly contaminated sites (faeces)
    - MacConkey, XLD

Identification is based on colonial characters on:

- **Blood agar**: large, white-grey, mucoid colonies
- **MacConkey**: large, pink to red (**lactose positive**), mucoid colonies with chameleoning phenomenon (colour changes to yellow in time, as if they were lactose negative, due to alkalinisation of the media)

A 

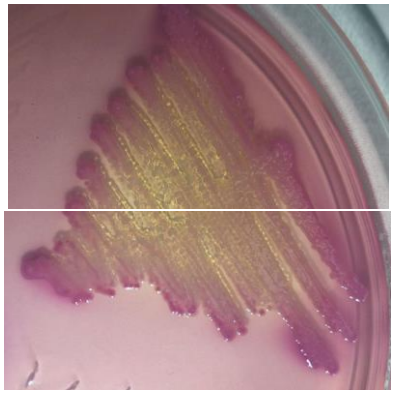
B 

Figure 26. *Klebsiella sp*. A. - on differential medium-CHROMagar, B.- chameleon phenomenon on Mac Conkey

Antimicrobial susceptibility testing (Antibiogram)

An antibiogram is performed for the genus *Klebsiella*, because phenotypes with low level of resistance to amino-, benzyl- and carboxypenicillin have been identified. Resistance is surpassed by addition of beta-lactamase inhibitors

Other antibiotics to be tested are aminoglycosides, fluoroquinolones, trimethoprim and sulfamethoxazole

Furthermore, phenotypes expressing extended-spectrum beta-lactamase have been identified, accounting for resistance to penicillins, cephalosporins of 1st, 2nd, 3rd and 4th generations and aztreonam.

Klebsiella spp. are sensitive to cefoxitin, cefotetan, latamoxef and carbapenems.

Essential to remember:

- Gram-negative, **encapsulated** bacilli arranged in diplo

- Lactose fermenting (pink colonies on MacConkey with chameleoning effect)
- Produce mucous colonies due to capsule
- Causative agents for several nosocomial infections, facultative pathogenic