General principles for the laboratory diagnosis of infections

Bacteriological diagnosis

Day 1:

- collection of biological product
- transport of samples
- macroscopic examination
- microscopic examination
- inoculation of specimens into culture media

Day 2:

Identification of bacteria

- morphological characteristics
- culture media characteristics
- biochemical properties
- identification of antigens

Sensitivity of bacteria on the culture medium to various antibiotics (antibiogram)

Day 3:

- completion of diagnosis
- publication of results

Serological diagnosis

= identification of microorganism-specific antibodies in the patients serum

What are the steps in the bacteriologic diagnosis?

- Collection of the biological product
- Transport of the samples
- Macroscopic examination
- Microscopic examination
- Isolation of the bacteria
- Identification of the bacteria by morphological characters, culture characteristics (colonial characters on solid culture media), biochemical properties, pathogenicity tests
- Sensitivity of the culture to antibiotics (antimicrobial sensitivity test / antibiogram)

Collection of specimens

Which are the specimens collected?

- Nasal secretions (nasal exudate)
- Pharyngeal secretions (pharyngeal exudate)
- Sputum
- Pus
- Faeces
- Urine
- Urethral secretions
- Blood

The choice of the specimen(s) to be collected depends on the clinical symptoms and on the suspected diagnosis.

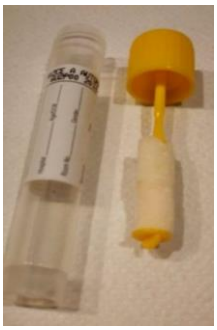
A   B  C  D 

Figure 7. Sterile containers for biological sample collection: a.System for collecting and transporting urine samples, b. Universal collection recipient, c. Faeces collection recipient, d. Sterile tampons in tubes

Nasal secretion

The sample is collected with a sterile nasal swab.

Collection technique: The patient's head is immobilised in a position of neck extension. The swab is removed from its protective cover and gently inserted into the nose along the nasal floor until it reaches the posterior wall of the nasopharynx. There, it is held for a few seconds, after which it is gently rotated in order to be soaked with exudate, followed by removal of the swab. The quantity of the sample/exudate is greater if the swab is removed and reinserted into the same place, because the primary insertion of the swab stimulates nasopharyngeal mucous secretions. The swab is then reintroduced into its protective tube and immediately sent to the laboratory or introduced into a preservative liquid for storage until analysis.

Pharyngeal secretions

The sample is collected with a sterile pharyngeal swab, preferably in the morning on an empty stomach and before washing the oral cavity, in order not to diminish the bacterial flora. Also, the patient should be instructed not to eat or drink before the procedure in order to avoid the mechanical removal

(swallowing) of germs from the pharynx. If this does not apply, it is carried out 3-4 hours after cleaning of the oral cavity or ingestion of foods.

Collection technique: The patient sits with the neck slightly extended, the mouth wide open, the pharynx enlightened and the base of the tongue (dorsal surface) fixed with a sterile spatula (lowering of the tongue). The patient is asked to hum "Ah". The swab is introduced without touching the tongue, cheeks or the spatula (to avoid contaminating the sample with the oral microbial flora) and the uvula (not to trigger the regurgitation reflex). It is firmly buffered by a circular motion on the surface of the tonsils, the posterior wall of the pharynx, and the inflamed and ulcerated areas (purulent secretions are rubbed). The swab is carefully removed, reintroduced into the protective tube and immediately sent to the laboratory or introduced into a preservative liquid for storage until analysis.

Sputum

The collection is preferably done in the **morning**, because in the course of the night the deposit of secretions is more abundant. There are two types of collection:

- **indirect**: the patient is asked to vigorously rinse the oral cavity with saline (do not use antiseptic solution), then to cough and expectorate the sputum into a sterile container
- **direct**: by bronchoscopy or tracheal puncture
- in children collection is performed by gastric lavage or gastric probing as they tend to swallow the specimen

Pus

Pus is a viscous liquid consisting of intact or altered **leucocytes**, **microorganisms**, **cell debris** and **fibrin**.

Collection technique: A surgeon drains a closed collection of pus (abscess) through an incision, because it is contraindicated to collect pus from the surface, as it contains dead microorganisms. After cleaning the wound, deep collections of pus are drained from within the wound.

Most of the open pus-containing wounds can be collected in the laboratory. The skin is disinfected with an iodine solution around the pus collections. The sample is taken with a sterile swab (the same as for nasal secretions), or with a bacteriological loop after removal of the superficial layer of pus.

Fistulised pus collections: the skin is disinfected with an iodine solution. A Pasteur pipette is introduced into the path of the fistula and the pus is aspired.

Faeces

After the defecation, there are certain morphologically **distinguishable pathological products** that can be picked up with a spatula that is attached to the screw cap of the **faecal container**, if a pathological process is taking place. Generally, one should take a precise look at the **colour** (usually dark brown), **texture** (usually with a mucus coating) and **consistency** (usually semisolid) of the faecal matter:

- mucosal fragments
- blood-stained fragments

- purulent fragments
- or fragments of faecal matter taken from different location if the stool is homogenous but an infection is suspected

After removal, 1 µl of pathological product is suspended into the transport medium, which is already contained in the faecal transport container. The collected sample is immediately sent to the laboratory for processing.

Urine

In healthy persons, the urine in the **urinary bladder is sterile** but it becomes contaminated during urination due to its passage through the distal urethra. In order to avoid such contamination, the patient should be instructed to first clean the urogenital area with water and soap; then, without using a towel, release the first stream of urine into the toilet, briefly stop and then continue with the mid-stream of urine into the sterile container. By this technique, the first stream of urine will wash away most of the microbes which contaminate the anterior urethra. It is best to collect the first morning urine or at least 3 hours after the last urination.

- Patient urine:
  - ~ 10 mL for quantitative screening of pathogenic microorganisms.
  - ~ 30-50 mL for specific pathogenic microorganisms (e.g. Koch bacillus).

The collected sample is immediately sent to the laboratory or is stored at 4 ° C until analysis.

Urethral secretions

The collection is performed in the morning **before urination**. In acute urethritis, the spontaneous urethral discharge is collected. In addition, it is obligatory to collect the secretion within the interior of the urethra. In order to do this, a thin pad is introduced into the urethra at a distance of 1-2 cm, which is rotated for a few seconds (painful for the patient). Through this manoeuvre, one obtains urethral epithelial cells, in which C*hlamydia* might multiply, a frequent causative agent of urethritis. The cotton pad can be replaced by a platinum loop, which has been sterilized, warmed and subsequently introduced into the urethra following the same indications as for the cotton pad. The collected sample has to be processed immediately. In patients with chronic urethritis, the secretion is reduced and appears as morning drops.

Blood

Under normal conditions, **blood is sterile** because it has the ability to eliminate microbes. As a pathological product, it can be used for bacteriological or serological examination. For bacteriological examination, a blood culture is performed which reveals the presence of bacteria in the blood by inoculating a blood sample into a suitable culture medium.

The collection of blood is preferably performed as soon as the first chills occur (chills and fever appear 1-2 hours after the introduction of the bacteria into the blood, at which point they begin to multiply).

10-20 mL of blood (average 15mL) is extracted in the acute phase of the disease; in the chronic phase, more is taken, on average 30mL.

In infants, 1-3 mL of blood is collected.

The blood is immediately inoculated into a liquid culture medium.

The methods of collection vary according to the site of the infection and germs found. It is invariably necessary to use properly sterilized equipment, preferably sterile disposable equipment and to conduct the procedures through a sterile technique. The most opportune time to practice collection sometimes varies with the clinical setting. In general, early collection increases the chances of discovering the causative microorganism.

Transport of samples must be done according to precise rules that are adjusted to the characters of the suspected microbes, such as their survival time outside the infected host, resistance/sensitivity to environmental factors (oxygen, temperature, humidity, light, etc.).

Macroscopic examination: all the bacterial infections are accompanied, in addition to the presence of bacteria, by biological signs related to the inflammation with the possible presence of leucocytes, in particular polymorphonuclear cells. These elements can lead, beyond a certain threshold, to a visually observable modification, clearly perceptible to the naked eye.

Microscopic examination

Preparation of slides:

Wet mounts

• From a liquid (puncture, drainage): using the pipette, a drop of specimen is taken and added to the slide, after which a coverslip is placed over the added drop.

• From colonies: first, a drop of saline solution or water is added onto a slide. With a bacteriological loop, the bacterial colony is withdrawn from the culture medium and emulsified into the drop of water. Then, a coverslip is placed on top of the drop of water.

This method is termed **wet mount** and it is usually easier and faster to perform. It is mostly used to differentiate between bacteria and yeasts, or to determine motility of microorganisms.

Gram-staining

Is the most commonly used staining technique and was discovered by Hans Christian Gram in 1883. By using this method, bacteria can be distinguished based on the way they are coloured: **blue to purple** (Gram-positive) from those in **pink to red** (Gram-negative). The difference in colour arises due to differences in structure, more precisely cell wall permeability, within different types of bacteria. Thus, the cell wall structure of bacteria determines the colour and consequently separates bacteria into the groups: **Gram-positive** and **Gram-negative**.

The staining principle consists of first staining the bacteria with an acridine dye, such as **crystal violet** (primary stain) and fixing with a solution of **Lugol** (potassium iodine solution; mordant). This process is termed "**colourization**" and the result is that any bacterial cell (Gram-positive and Gram-negative) subjected to this process is coloured **blue** to **purple**. Here, the primary stain, i.e. crystal violet is bound to the cell wall. Fixing with a Lugol solution will result in the formation of a complex between iodine and crystal violet, making it more difficult to be removed from the cell wall layer. The differentiating step consists of a process termed "decolourization", meaning that a 95% ethanol solution or a 50/50 ethanol-acetone mixture is applied, with the intention of removing the crystal-violet iodine complex from the cell wall. This step will bear the differentiating result between Gram-positive and Gram-negative bacteria. Recall from theory that Gram-positive microorganisms have a thicker peptidoglycan layer with a different composition than Gram-negative microorganisms. Applying this knowledge into practice, this leads to the conclusion that it makes the crystal violet-iodine complex more difficult to be removed from the cell wall of Gram-positive bacteria by the decolourization step. While the decolourization step in Gram-negative bacteria removes the crystal violet-iodine complex, the crystal violet-iodine complex in Gram-positive microorganisms is maintained. Thus, at this point, Gram-negative bacteria are colourless, while Gram-positive microorganisms remain coloured blue/purple. In order to colour the Gram-negative bacteria, a counterstain called **safranin** or **fuchsine** is applied, staining Gram-negative bacteria pink/red, a feature arising because after decolorizing there is no more crystal violet-iodine complex lipopolysaccharides within the thin peptidoglycan layer of Gram-negative bacteria. The Gram-positive bacteria are already stained with the crystal violet dye which they did not lose during the decolorizing step, so they will not absorb the pink/red dye and remain purple.

To remember:

- Gram-staining is the most commonly used staining method in microbiology laboratories
- Gram-positive bacteria are coloured in blue to purple, Gram-negative bacteria are coloured in pink to red
- Microorganisms that stain Gram-positive have a thicker peptidoglycan layer, which makes the cell wall difficult to decolourize (i.e. to remove the primary crystal violet stain)
- The difference between Gram-positive and Gram-negative bacteria is a difference in cell wall permeability and structure for the primary stain after decolourization with alcohol-acetone or pure alcohol
- Gram-positive bacteria maintain the purple complex, while Gram-negative bacteria lose it and become colourless. Gram-negative bacteria will turn red after counterstaining with either fuchsine or safranin
- Note that Gram-positive bacteria retain the purple complex only if the wall structure is intact. If the bacterial cell has an altered cell wall, it will lose the crystal violet-iodine complex
- Not all bacteria can be stained with the Gram technique; for instance, the genus *Mycobacterium* is impermeable to staining agents due to the presence of lipid substances (mycolic acids) in the structure of the cell walls which resist staining by ordinary methods such as a Gram-stain; consequently, mycobacteria are stained by another technique e.g. the Ziehl-Neelsen staining

Steps of coloration:

1. Application of **primary stain**: crystal violet - all bacteria stained blue

2. Rinse with water to remove excess crystal violet

3. Application of **mordant** or "**trapping agent**": Lugols iodine solution - forms a complex with crystal violet

4. Rinse with water to remove excess iodine

5. **Decolourization**: application of 95% ethanol or 50/50 ethanol-acetone mixture - removes crystal violet-iodine complex from Gram-negative bacteria

6. Rinse with water to remove the decolourizing agent & stop decolourizing process

7. **Counterstain**: safranin or fuchsine - stains Gram-negative bacteria

8. Rinse with water to remove excess safranin or fuchsine

Isolation of microorganisms

In order to isolate microorganisms, the collected biological product has to be **inoculated** into a **favourable culture medium**: simple, enriched or optionally selective media when a particular germ is suspected and subsequently incubated into **aerobic** or **anaerobic** conditions (depending on the microorganism). In the microbiology laboratory and in clinical practice mainly solid media are used to isolate microorganisms.

Inoculation is usually performed by **spreading** the biological product by means of a **sterile platinum** or **plastic loop**, in tight streaks on the surface of a culture medium – a process called **streaking** (shown to you during the laboratory class).

In order to allow the growth of the inoculated microorganisms, the culture medium has to be incubated into an **appropriate atmosphere** with an **appropriate temperature**. Most germs are incubated into an incubator that resembles the conditions that microorganisms meet in the human body, i.e. a temperature of approximately 37°C and a CO_2 concentration of approximately 5%. Anaerobic germs are incubated in incubators with atmospheres devoid of oxygen.

Keep in mind that the culture media have to be placed into the incubator in an **inverted position** in order to **prevent dripping of water vapour** caused by the metabolism of the microorganism, which would make some microorganisms incapable of surviving or motile organisms to move across the water surface and prevent distinctive colony formation.

After incubation, bacterial colonies should be visible, if inoculation and incubation were correctly performed. These colonies can be further extracted and inoculated (reinoculated) into another culture medium in order to grow pure cultures that consist of only one bacterial species. The reinoculation consists of choosing 3-5 colonies which are picked up with a sterile bacteriological loop and reinoculated in the second culture medium. The selection of these colonies relies on their aspect (colonial characters

i.e. shape, colour, contour, presence of haemolysis on blood containing media etc. which could suggest certain aetiologies).

Identification of microorganisms

Identification is done from a pure culture and depends mostly on morphological and biochemical characters:

- **Morphological characters: shape** (cocci, bacilli, spiral, curved, filamentous), **grouping** (pairs, tetrads, chains, clusters), **motility**, type of **ciliature**, **capsule** (presence or absence), location of **spores**, **dye affinity** (Gram-positive or Gram-negative). All these morphological characters are observable by microscopic examination of the cultured bacteria: 2-3 drops of sterile saline solution are placed on a microscopic slide and then a sterile bacteriological loop is used to pick up a small portion of a pure culture which is resuspended (emulsified) in the saline solution and carefully smeared onto the microscopic slide; after the smear is air dried, staining techniques may be applied i.e. Gram-staining
- **Culture media characters**: **atmospheric** and **nutritional requirements**, **colonial morphology** (general shape, colour, margins), haemolysis on blood agar, specific odour
- **Biochemical and metabolic properties** that define the enzymatic equipment of the bacteria: carbohydrate, lipid, protein metabolism and presence of absence of various enzymes (oxidase, urease, coagulase, catalase, API20 kit)

While for certain bacteria the identification might stop at this stage, for others it is important to specify the antigenic constituents by means of specific agglutinating or precipitating sera (*Salmonella*, *Shigella*, enteropathogenic *E. coli*, streptococci).

Colonial characters: Morphology of colonies on the surface of culture media

Blood agar

- ***Staphylococcus aureus***: round, yellowish-white, opaque, smooth, 1-2 mm in diameter, with haemolysis
- ***Streptococcus***: fine ("pinpoint") colonies, diameter from 0.5-1 mm, with complete (beta) /incomplete (alpha) haemolysis or non-haemolytic (depending on species/strain)
- ***Pseudomonas***: round, smooth, transparent colonies, shiny, 1-3 mm in diameter
- ***Enterobacteriaceae***: round, grey, opaque, smooth colonies, 3-4 mm in diameter

Mac Conkey

- **Lactose-positive bacteria** = pink or red colonies (due to a pH indicator that changes colour after lactose fermentation): *Escherichia coli*, *Klebsiella*
- **Lactose-negative bacteria** = colourless colonies (colour of culture medium): *Proteus*, *Salmonella*, *Shigella*

Chapman medium

- ***Staphylococcus aureus*** = bacterial growth changes the colour of the medium from pink to yellow due to mannitol fermentation (acidification)

Sabouraud medium

- **Yeasts**: colonies of 1-2 mm in diameter after 24 hours, matt white