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MEDICAL MICROBIOLOGY
PRACTICUM FOR MEDICAL STUDENTS

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MEDICINSKI FAKULTET

UNIVERSITY OF SPLIT
SCHOOL OF MEDICINE
DEPARTMENT OF MEDICAL MICROBIOLOGY AND PARASITOLOGY

Medical microbiology

Practicum for medical students

Marija Tonkić and associates

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PRACTICAL B1

Katarina Šiško Kraljević

Introduction to microbiological laboratory and the basics of safe laboratory work. Laboratory-acquired infections. Microscopic examination of principal bacterial shapes. Differential staining in bacteriology. Cultivation of bacteria. Media types.

1. Introduction to microbiology laboratory.

Medical microbiology laboratories are designed for study of microorganisms, the causative agents of human infectious diseases. Working in a microbiological laboratory carries certain health risks, therefore it is very important to strictly adhere to the principles of good laboratory practice.

GENERAL LABORATORY SAFETY RULES

- Appropriate protective clothing (work coats buttoned up) and closed shoes should be worn in the laboratory.
- Before entering the training room/laboratory, students should change into work clothes (protective coat), and all personal items (bag, books, mobile phones, coat, etc.) should be left in the dressing room. Only this practicum, pencils and crayons may be brought to the laboratory.
- The last student leaving the dressing room should lock the door and put the key in the designated place in the laboratory.
- If necessary, in agreement with the assistant, protect yourself additionally with gloves (when working with blood and other body fluids) or other personal protective equipment (safety glasses, masks, etc.).
- Do not work with exposed open wounds or cuts (cover them with a Band-Aid and gloves).
- Due to working with burners, it is necessary to tie back long hair to minimize its exposure to open flame.
- Do not leave personal items (eyeglasses, mobile phone) on the laboratory work surfaces.
- Do not touch your eyes, nose or mouth during work. Contact lenses must not be touched in the laboratory.
- Nothing should be put in the mouth in the laboratory. Smoking, eating, or drinking in the laboratory is not permitted!
- Glassware, needles and other sharp objects should be disposed of in special containers with a disinfectant intended for this purpose.
- Do not place contaminated instruments, such as inoculating loops (eze) on table tops.
- Microbiological loops should be flame sterilized before and after each use.
- Everything used (loops, cultures, stains, etc.) should be returned to its place.
- Any incident situation (spills of infectious material, etc.) should be reported to the assistant so that appropriate measures can be taken.

- At the end of the exercise, the microscope should be turned off and all plates with bacterial cultures and permanent preparations should be returned to the designated place (according to the instructor's instructions).
- It is forbidden to remove media, equipment or bacterial cultures out of the laboratory.
- Before leaving the laboratory, hands should be washed well with soap and running water (see instructions) and dried with a paper towel.

STATEMENT: I have read and understood the above laboratory safety rules and regulations and agree to abide by them.

Name: Date:

_____ _____

HAND WASHING INSTRUCTIONS:

- Wet your hands with warm running water and apply liquid soap.
- Rub hands palm to palm in circular motion.
- With the right hand palm, rub the back of the left hand and vice versa.
- Interlace the fingers and scrub the area between the fingers.
- Clean the back of your fingers by rubbing your interlocked fingers into your palm. Repeat with other hand.
- Using circular movements, rub the thumb of the left hand in the palm of the right hand and vice versa.
- Wash your hands under running warm water.
- Dry your hands with a paper towel.
- Close the faucet with a paper towel.

2. Microscopy of basic bacterial forms. Staining in bacteriology.

2. 1. Components of the microscope

OCULAR: The part of the microscope that you look through is a series of lenses that magnify 10x. The microscopes in the training room are binocular, i.e., they have two eyepieces, which enables microscopy with both eyes. The eyes should be relaxed as if we were observing an object 5-10 meters away. The mutual distance of the eyepieces should be adjusted according to the eyes distance. The left eyepiece is equipped with a diopter adjustment. It is best to set the ring of the left eyepiece to the neutral position (at "0") at the beginning of work.

OBJECTIVES: Part of the microscope located directly above the sample, a system of lenses that collect the light that has passed through the preparation and directs it through the tube into the eyepieces. Our microscopes have four objectives that have different magnifications: 4x, 10x (low power), 40x (high power) and 100x (oil immersion objective). Lower magnification objectives are used for microscopy of unstained, wet mounts. The immersion objective is used for microscopy of permanent stained slides by first placing a drop of immersion agent (cedar oil, anisole, etc.) on the slide, into which the objective is then immersed. Anisol does not need to be wiped off.

STAGE: A flat surface with an opening in the middle on which the slides are placed. The stage is movable with the help of knobs located on the lower right side of the platform. The slides can be moved vertically or horizontally, which facilitates a detailed search.

CONDENSER: A lens system under the microscope stage that collects light from the source (lamp) and focuses it on the sample. There is an iris diaphragm on the condenser that can be used to regulate the amount of light aimed at the slide. It is best to adjust the aperture to the magnification of the objective lens used.

CONDENSER KNOB: Under the microscope stage on the left side there is a small knob that can be used to move condenser vertically. During microscopy of wet mounts, the condenser should be placed in the lowest position, and for microscopy of stained slides, in the highest position.

LIGHT SOURCE: The lamp is turned on by the switch on the right, and the intensity of the light can be regulated with the help of a screw.

COARSE-ADJUSTMENT AND FINE-ADJUSTMENT KNOBS: Knobs for coarse and fine focusing of the image regulate the distance of the objective lenses from the preparation. The coarse-adjustment knob is used for searching, and the fine-adjustment knob for focusing the image.

PRACTICAL WORK

Task 1. Write the names of the microscope components on the diagram (Figure 1. 1.).

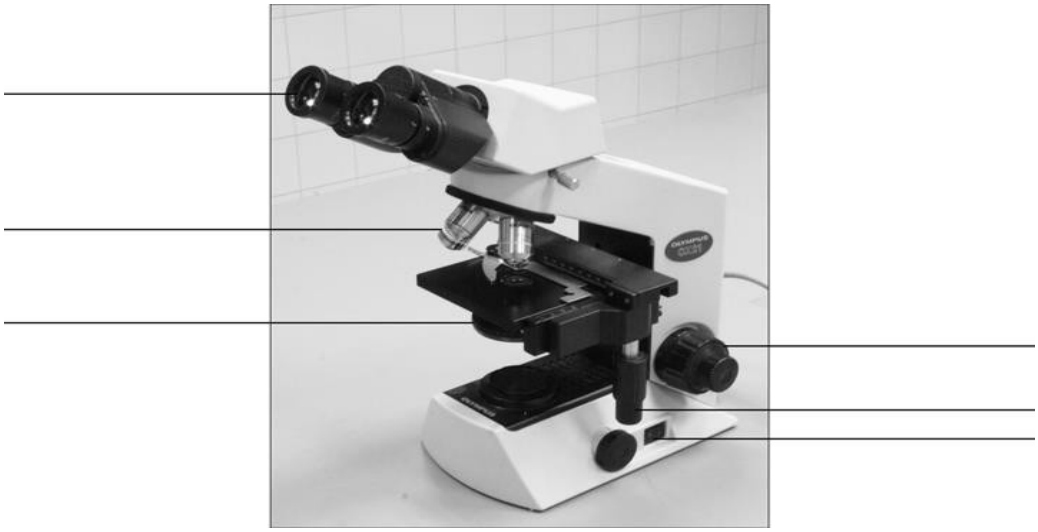


Figure 1. 1. Binocular light microscope

2. 2. Microscopy - procedure

Before microscopy:

- Turn on the light source.
- Check the left eyepiece and adjust it to the neutral position.
- Adjust the eyepieces distance according to your eyes distance.
- Place and secure the microscope slide on the microscope stage.

Microscopy of unstained (wet mount) preparations. Low power observations

- Lower the condenser using the knob.
- Close the condenser iris diaphragm as much as possible (set to 10x).
- Rotate the low power (yellow) objective lens (10x) into the position.
- Lower the objective with the coarse-adjustment knob to its lowest position (observe the movement with your naked eye, do not look through the ocular lens at this moment).
- While looking through the ocular lens, use the coarse-adjustment knob to slowly raise the stage until the specimen comes into focus.
- Use the fine-adjustment knob to bring the specimen into sharp focus.
- Rotate the blue objective lens (40x) into the position and adjust the iris opening accordingly.
- Use the fine-adjustment knob for optimal focus.
- Systematically inspect the entire surface of the preparation under the cover glass.

Microscopic examination of stained slides

- Examine entire surface of the smear under low magnification (10 or
- 40x). Select part of the slide in which the cells are distributed in a thin layer.
- Bring the condenser up and open the iris diaphragm (adjust it to 100x).
- Drop a small amount of anisole at the centre of the slide.
- Immerse black and white objective lens (100x) into the anisole and fine adjust the picture.

If you fail to find an image, proceed as follows:

- Use coarse-adjustment knob to bring the objective lens (100x) as close as possible to the preparation.
- The tip of the objective should be dipped in anisole and retracted. *IMPORTANT: Stop at the first sign of resistance, otherwise you will break the preparation and damage the objective lens!*
- Looking through the eyepieces, raise the eyepiece with the coarse-adjustment knob until the sample comes up in focus.
- Use the fine-adjustment knob for optimal focus.

3. Microscopy – interpretation

When interpreting microscopic finding, it is important to distinguish whether it is unstained or stained smear, and for stained preparations, to recognize the staining method.

With simple staining methods, all elements of the microscopic image will be stained in the same colour:

- Methylene blue: all bacteria and cellular elements are stained blue
- Carbol fuchsin: all bacteria and cellular elements are stained red

With complex staining, different elements of the image will take on a different colour depending on the properties of the cell wall:

- Gram stain:
  - Gram positive bacteria and yeasts are purple (P-PURPLE-POSITIVE).
  - Gram negative bacteria and different cell elements (epithelial, white blood cells) are pink or red.
- Ziehl-Neelsen stain:
  - Acid-fast bacteria are red.
  - Everything else, other bacteria and different cell elements, like epithelial and white blood cells, are blue.

Observing the individual isolated bacteria, one should notice the characteristic morphology and arrangement of microorganisms.

- Basic shape of bacteria:
  - spherical bacterium (coccus), coccobacillus, rod-shaped bacterium (bacillus), filament
- Nature of axis:
  - strait, curved, spiral
- Some gram-positive bacteria are spore formers:
  - spores may be narrower or wider than the body of the bacterium and located centrally, sub terminally and terminally
- Irregular staining:
  - e.g., metachromatic granules (i.e., granules that stain a colour different from the primary dye colour)
- Characteristic arrangements:
  - singles, pairs, chains, tetrads, clusters, palisades, “Chinese” letters, angular forms (V and L shapes), etc.

EXAMPLES OF DESCRIPTION OF A MICROSCOPIC IMAGE:

In the Gram-stained preparation, gram-positive cocci in pairs, consistent with Streptococcus pneumoniae; or gram-negative bacilli, consistent with enterobacteriae; or gram-positive spore forming bacilli in chains, consistent with Bacillus spp.

Task 2. Preparation and microscopy of the wet mount (demonstration microscope).

Materials:

- test tube with urine sediment
- microscope slides
- coverslips
- inoculating loop (eza)
- containers with disinfectant for discarding preparations

Procedure:

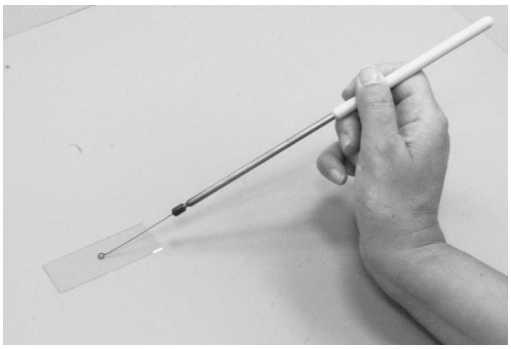
1. Using the sterile loop take one drop of the urine sediment and place it on the centre of the slide (Figure 1. 2. A and B). Sterilize the loop and return it to the rack.
2. Cover the urine drop with coverslip (Figure 1. 2. C).
3. Carefully place the slide on the microscope and view it under the low power objectives.
4. Observe and record results.
5. After finishing, discard the slide in a container with disinfectant.

(***Handle the preparations with care, it is an infectious material!***)

A



B



C

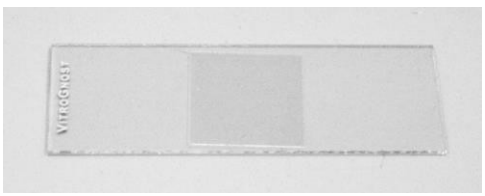
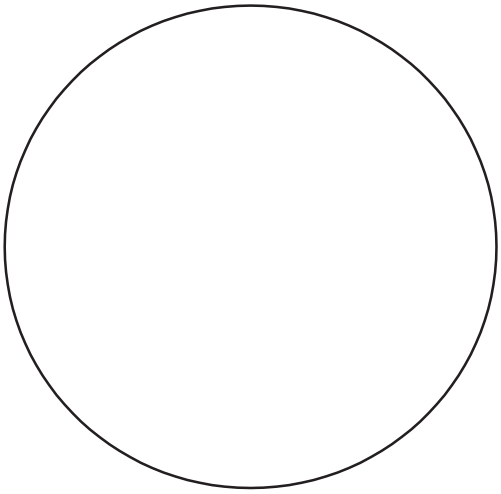


Figure 1. 2. (A) Sterilisation of inoculation loop by the flame. (B) Correct holding of the loop. (C) Wet mount.

Description:



Task 3. Preparation, staining and microscopy of permanent stained smears.

Material:

- test tube with urine sediment
- microscope slides
- coverslips
- inoculating loop
- stains: Carbol fuchsin and Methylene blue
- containers with disinfectant for discarding preparations

Procedure:

1. Using the sterile loop take one drop of the urine sediment and place it on the centre of the slide (Figure 1. 2. A and B).
2. With the circular movement of the loop spread the drop into a thin area approximately the size of a dime (Figure 1. 3. A).
3. Allow the smear to air-dry in the designated place.
4. Heat fix the air-dried smear by the rapid passage 2-3 times over the flame (Figure 1. 3.B).
5. Work in pairs: one student should stain the smear with Carbol fuchsin and the other with Methylene blue.
6. Place the air-dried and heat-fixed smear on the staining tray in the sink.
7. Using pipette flood the smear with the appropriate stain (Figure 1. 3. C).

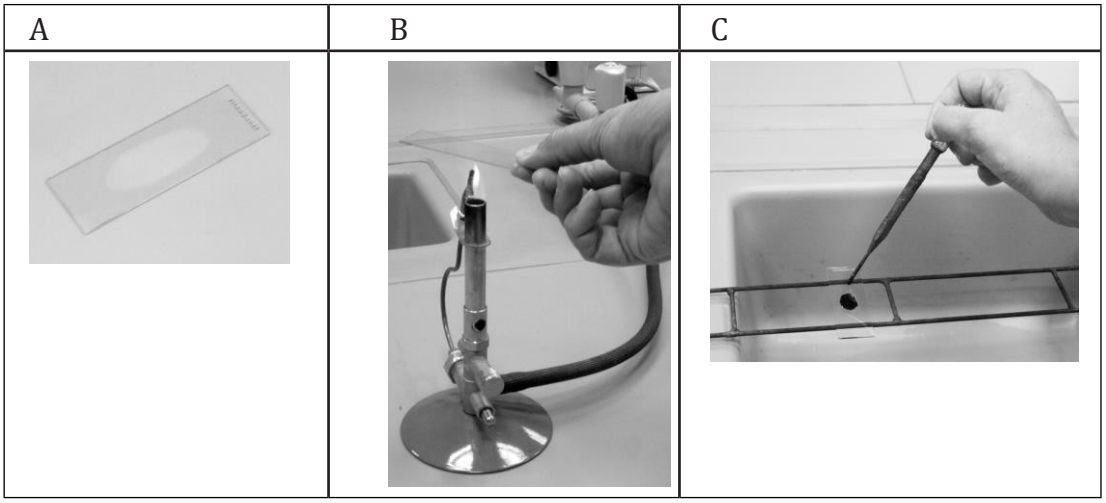


Figure 1. 3. (A) An example of a properly made smear. (B) Heat fixation of the smear. (C) Staining.

8. Leave it to stain for 15 minutes.
9. Wash the smear with tap water to remove excess stain (hold the slide parallel to the stream of water) and allow it to air-dry (Figure 1. 4. A and B).
10. It is very important to completely dry the freshly stained slide before microscopy, otherwise the picture will be blurry.
11. Examine all stained slides under oil immersion(100x).
12. Observe, draw and describe the microscopic picture of both smears.
13. After finishing, discard the slide in a container with disinfectant.

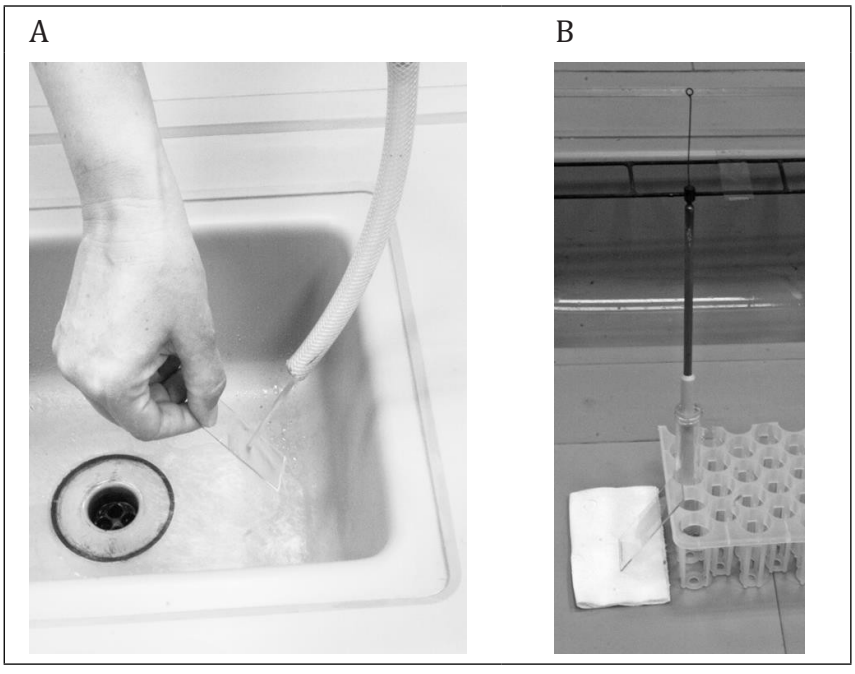
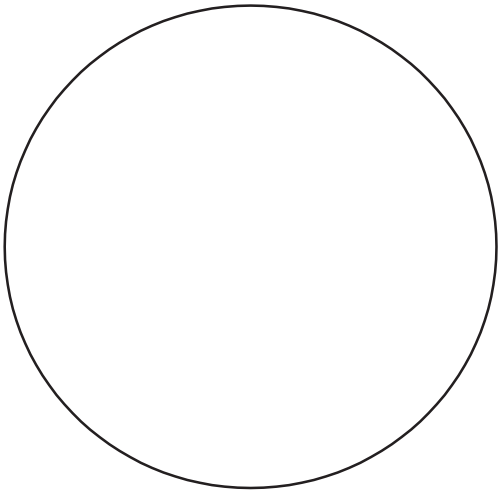
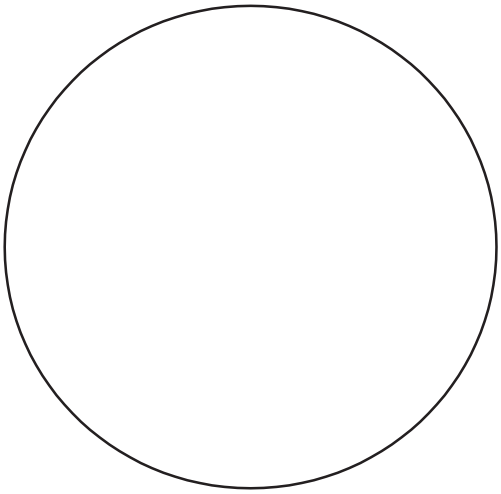


Figure 1. 4. (A) Washing of the slide. (B) Drying of the slide.

Description:



Description:

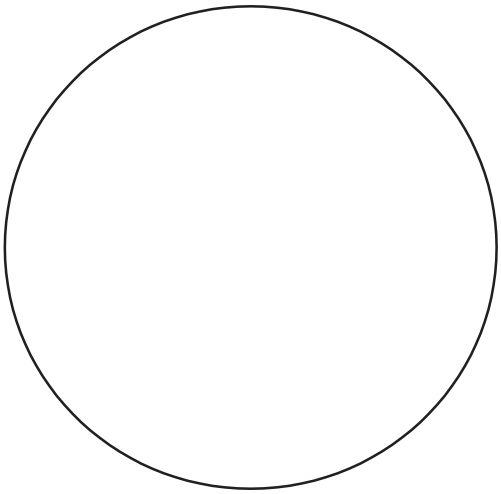


Task 4. Microscopy of Gram stained smear.

Describe the morphology of the microorganisms with reference to their shapes and arrangements. Draw a representative field for each microorganism.

NOTE: When finished, do NOT discard THIS slide into container with disinfectant.

Description:



3. Cultivation of bacteria: types of bacterial culture media, bacterial colonies morphology.

Many different artificial culture media can be used for cultivation of bacteria allowing identification of bacterial species involved.

3.1. Culture media

Based on the consistency, culture media can be divided into: liquid (broth), semi-solid and solid (agar). Based on functional use media can be divided into: simple (nutritious agar, NA) and enriched (blood agar, BA; chocolate agar). These are nonselective media that support the growth of most bacteria. The medium used for antimicrobial susceptibility testing (Mueller-Hinton agar, MH) is also non-selective.

Selective and differential culture media (e.g. CLED and SS agar) are used to grow microorganisms from non-sterile samples in which many bacterial species may be present.

- CLED (Cystine Lactose Electrolyte Deficient) agar is differential, non-selective culture medium used for the isolation of urinary microorganisms. It prevents excessive swarming of *Proteus* species. Lactose-positive bacteria form yellow colonies due to acid production during lactose fermentation (pH indicator bromothymol blue).
- Salmonella-Shigella (SS) agar is a selective and differential medium used for the recovery of *Salmonella* and *Shigella* spp. The growth of most gram-positive and many gram-negative bacteria is inhibited by the addition of bile salts, sodium citrate and brilliant green. Lactose fermenting bacteria form pink or red colonies (pH indicator neutral red) while non-fermenting bacteria form colourless colonies. Bacteria that produce hydrogen sulphide from the metabolism of sodium thiosulfate are detected by the black precipitate formed with ferric citrate.

- For the successful cultivation of bacteria, in addition to nutrients, it is necessary to provide the appropriate atmosphere (aerobic, anaerobic, microaerophilic conditions), temperature (incubators at 35 ± 2 °C, 42 °C) and incubation time (from 18 h to 12 weeks). Multiplication of one bacterial cell, on solid agar media, produces a visible mass of of genetically identical cells (a clone) called colony.

3.2. Bacterial Colony Morphology.

When describing the colonies on solid media, it is important to select well isolated colony, that has had optimal growth conditions and observe its characteristics using the interpretative key below.

Colony morphology characteristics:

- **Size** (diameter in millimeters):
  - large (> 1 mm)
  - medium (= 1 mm)
  - small (< 1 mm)
- **Form:**
  - pinpoint
  - circular
  - rhizoid
  - irregular
- **Margin**:
  - entire
  - undulate
  - serrate
- **Surface appearance:**
  - smooth
  - glistening
  - rough
  - wrinkled
  - spreading
  - mucoid

Pigment of the colony should be described exclusively on the indicator-free media, such as: nutrient agar (NA), blood agar (BA) and Mueller- Hinton agar (MH, agar used for antimicrobial susceptibility testing).

On **blood agar** the **haemolytic properties** must be described. There are three types of haemolysis:

- **Alpha haemolysis** – incomplete lysis of red blood cells. There is greenish halo around the colonies as a result of reduction of haemoglobin to methaemoglobin.
- **Beta haemolysis** – lysis of red blood cells with complete destruction and use of haemoglobin. There is a clear zone without red blood cells in the medium surrounding colony.
- **Gamma haemolysis** – there is no significant change in the appearance of the medium surrounding the colonies, no lysis of red blood cells.

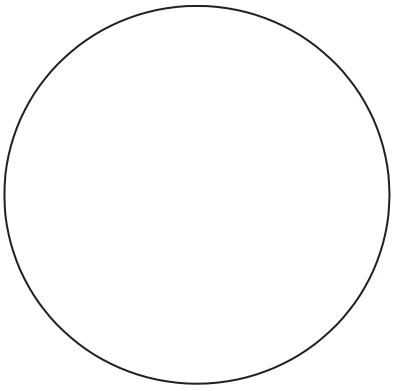
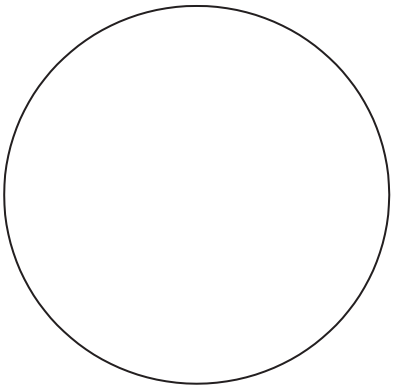
On differential, indicator media biochemical features should be observed:

- **CLED** (Cystine Lactose Electrolyte Deficient) **agar**
  - yellow colonies – Lactose-positive bacteria (L+)
  - colourless colonies – Lactose-negative bacteria (L-)
- Salmonella-Shigella **(SS) agar**
  - red colonies – Lactose-positive bacteria (L+)
  - colourless colonies – Lactose-negative bacteria (L-)
  - black dot in the centre of the colony - H_2S positive (H_2S +)
  - there is no black dot in the colony - H_2S negative (H_2S -)

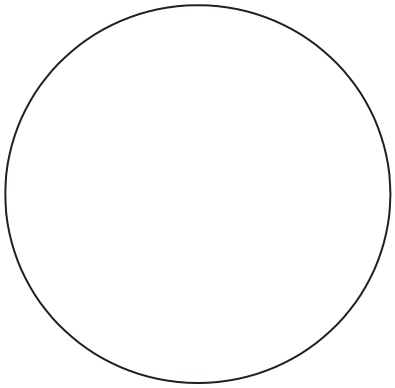
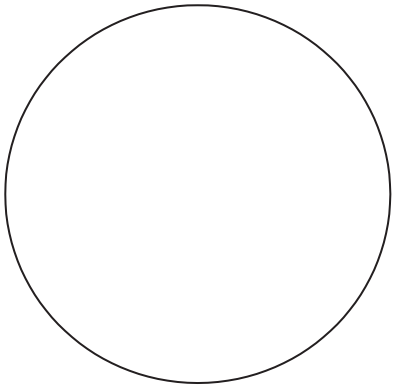
For each colony on SS agar, both properties should be read (eg *Citrobacter* spp. creates red colonies with a black dot, which we describe as L+ and H_2S +).

Task 5. Description of colonies.

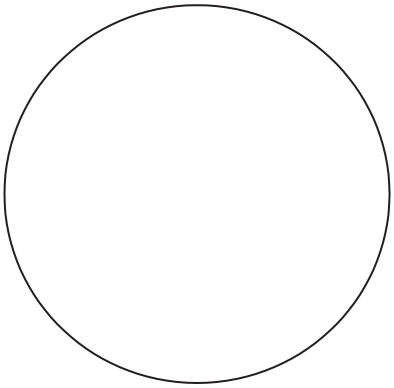
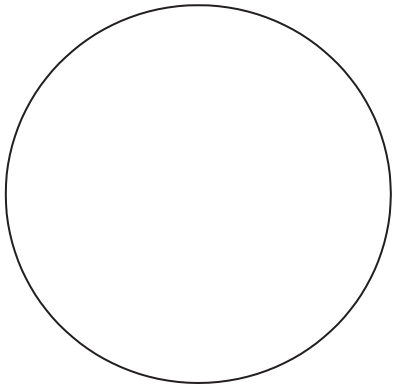
A Observe a single, well-isolated colony on the nutrient agar. Record your observations in the chart below:

Nutrient agar		
	Pseudomonas aeruginosa	*Serratia marcescens*
Size		
Form		
Margin		
Surface		
Pigment		
		

B Observe a single, well-isolated colony on blood agar. Record your observations in the chart below:

Blood agar		
	Streptococcus pyogenes	*Escherichia coli*
Size		
Form		
Margin		
Surface		
Haemolysis		
Pigment		
		

C. Observe a single, well-isolated colony on SS agar. Record your observations in the chart below:

SS agar		
	Escherichia coli	*Salmonella* spp.
Size		
Form		
Margin		
Surface		
Biochemical feature		
		

Task 6. Hand washing - cultivations of fingerprints.

- Mark a sterile plate of a blood agar with a permanent pen. Write on the reverse side of the agar, not on the lid. Enter your name, date and draw a line in the middle.
- Write “BEFORE” on one half of the plate, and “AFTER” on the other.
- Raise the lid and imprint the fingerprints of an unwashed dominant hand on the surface of the agar marked “BEFORE”.
- Return the lid on the plate and leave the agar on the desktop.
- Wash your hands according to instructions.
- Ensure that nothing is touched by the dominant hand after washing.
- Remove the lid from the plate with the non-dominant hand and leave the imprints of the fingertips of the dominant hand on the part of the agar marked with „ AFTER “.

Date: Teacher’s signature:

_____ _____

Notes:

Notes:

PRACTICAL B2

Zana Rubic

Principles of cultivation and identification of pyogenic cocci.

In order to successfully master this practical, the student should first study Chapters 13 and 14 from the book Jawetz, Melnick and Adelberg. Medical microbiology, 26th Edition. McGraw Hill Lange; 2013.

INTRODUCTION

Staphylococci

Staphylococci are widely distributed in nature, with natural habitat on the skin and mucous membranes of mammals. They are non-motile and non-spore-forming gram-positive cocci that mostly appear in irregular clusters. On blood agar, they grow in the form of smooth, shiny and convex colonies. *Staphylococcus aureus* may have a golden-yellow pigment. After 24 hours of incubation, the colonies have a diameter of 2-3 mm. Some of them show β-hemolysis, while others are without any hemolysis (which is sometimes called γ-hemolysis). They are mainly facultative anaerobes and have a fermentative metabolism.

Genus *Staphylococcus* is usually **positive in catalase test**, which is a distinguishing factor from the genus *Streptococcus*, which is catalase negative and have a different cell wall composition.

Among staphylococci, *Staphylococcus aureus* is more often associated with severe infections and it is important to distinguish it from opportunistic coagulase-negative staphylococci. In routine laboratory practice, the presence of **coagulase (bound or free)** is often used as a criterion to distinguish *S. aureus* from other staphylococci.

S. aureus can also be identified based on the production of **deoxyribonuclease (DNase)**. *S. aureus is DNase positive.*

Multiple resistance to antibiotics in staphylococci is most often associated with **methicillin-resistant *Staphylococcus aureus* (MRSA**).

Coagulase-negative staphylococci (CoNS) are common commensals on the skin and mucous membranes. For a long time, they were considered non-pathogenic, however, due to the increased use of various intravascular, prosthetic and other devices, and the increasing number of immunocompromised patients, CoNS have become important causes of some infections. *S. epidermidis* and *S. saprophyticus* are most commonly associated with infections, but more than 30 different species have been described and all can be potential causative agents.

Streptococci

The genus *Streptococcus* includes a large number of commensal and pathogenic species in humans and animals. Over 100 species have been described to date.

Under a microscope, streptococci are gram-positive cocci arranged in chains and/or pairs. They are non-motile and non-spore-forming facultative anaerobes, and some require CO_2 for growth. They metabolize carbohydrates fermentatively.

Streptococci are **catalase negative,** which distinguishes them from the genus *Staphylococcus*.

On blood agar, different species show different types of hemolysis (mostly **α or β**), which can be used as an early step in the identification of clinical isolates. Furthermore, β-hemolytic streptococci are characterized **by serotyping according to R. Lancefield**, based on group-specific carbohydrate antigens present on the bacterial cell wall. Clinically significant groups are A, B, C, D, F and G.

Streptococcus pyogenes or **group A β-hemolytic streptococcus (BHS-A)** is one of the most virulent species of the genus *Streptococcus*. These gram-positive cocci usually occur in chains. After 24 hours of incubation, it grows on blood agar in the form of small, smooth and convex colonies with a diameter of 0.5 to 1 mm, and with large zone of β-hemolysis. Some strains can form mucoid colonies. It differs from other β-hemolytic streptococci by its sensitivity to **bacitracin** and the presence of the enzyme pyrrolidonyl arylamidase (PYR).

Streptococcus agalactiae **(group B β-hemolytic streptococcus; BHS-B)** has a similar microscopic and macroscopic appearance as *S. pyogenes*. It is the cause of various infections, among which pneumonia, sepsis and meningitis in newborns are particularly important.

One way to distinguish *S. agalactiae* from *S. pyogenes* is the **CAMP test** (Christie, Atkinson, Munch, Peterson), which is based on the synergistic lysis of erythrocytes by hemolysin produced by *S. aureus* and the extracellular CAMP factor produced by *S. agalactiae* (triangular enhancement of hemolysis).

Streptococcus pneumoniae **("pneumococcus")** is a gram-positive elongated coccus that occurs in pairs or chains, sometimes with a visible capsule. Due to capsule, it can be sometimes stained gram-variably. On blood agar, colonies are small, flat, 1 to 2 mm in diameter, and surrounded by α-hemolysis. If the capsule is more pronounced, the colonies are mucoid. Over time, autolysis occurs, so the colonies collapse in the central part.

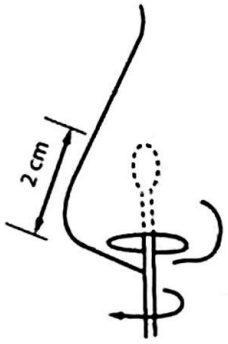
S. pneumoniae can be distinguished from other α-hemolytic streptococci by **optochin** sensitivity and bile solubility tests.

The viridans streptococci are large group of gram-positive cocci that occur in chains and are commensal flora of the mucous membranes of humans and animals, but under certain conditions they can cause endocarditis or other infections. The word "viridans" means "green" and refers to the color of α-hemolysis.

They are resistant to optochin.

PRACTICAL WORK

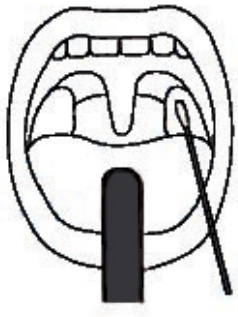
Nasal swab sampling and inoculation of agar plate



1. After hygienic hand washing, it is necessary to put on disposable protective gloves.
2. Write the patient's first and last name, date and sample type on the swab tube label.
3. Gently insert the swab into the nostril to a depth of about 2 cm and gently rotate it several times on the nasal mucosa.
4. Carefully put the swab back into the test tube and close it.

The sample should be delivered to the microbiological laboratory at room temperature within 2 hours of sampling. The sample can be stored for up to 24 hours at room temperature as well. After inoculation a blood agar plate, it should be incubated at 35-37°C/24 hours with an elevated CO_2 concentration.

Throat swab sampling and inoculation of agar plate



1. Write the patient's first and last name, date and sample type on the swab tube label.
2. The tongue is pushed down with a sterile stick (spatula), and the patient simultaneously pronounces the vowel 'A' to raise the uvula.
3. Firmly wipe the tonsils, palatal arches and the back wall of the pharynx with a sterile swab. Contact with the tongue, buccal mucosa and saliva should be avoided.
4. Carefully put the swab back into the test tube and close it.

The sample should be delivered to the microbiological laboratory at room temperature within 2 hours of sampling. It can be stored for up to 24 hours at room temperature as well. After inoculation a blood agar plate, it should be incubated at 35-37°C/18-24 hours with an elevated CO_2 concentration.

DEMONSTRATION

Hemolysis

Gram-positive cocci can produce different types of hemolysis on blood agar.

According to the degree of hemoglobin breakdown in blood agar, we distinguish incomplete or _____, with a greenish zone around the colonies, and complete or _____, with a transparent zone around the colonies, while the absence of hemolysis is called _____.

Catalase test

This test determines the ability of a microorganism to break down hydrogen peroxide into water and oxygen using the catalase enzyme.

Using sterile inoculating loop, transfer the culture sample to the slide and add 1-2 drops of 3% H_2O_2 solution. A positive reaction is manifested by foaming, that is, the formation of oxygen bubbles in the test drop.

Staphylococci are catalase _____, while streptococci are catalase _____.

Coagulase test

This test determines the ability of bacteria to synthesize enzymes that coagulate blood plasma. There are two types of enzyme that can be detected: bound and free coagulase.

a) Bound coagulase (clumping factor) is found on the surface of the bacteria and can be detected rapidly by a slide test. The tested culture is mixed with a drop of saline, and then rabbit plasma is added and mixed all together. In case of positive test, white clots will appear after 5-10 seconds.

b) Free coagulase is released from the cell to the medium, and can be detected by a test in tube. 0.5 mL of the suspension of the tested microorganism is added into the tube with 0.5 mL of diluted rabbit plasma and gently mixed using a circular motions. After incubation for 1-4 h at 37°C, a clot will be formed at the bottom of the test tube.

Staphylococcus aureus is _____ in both coagulase tests, which differs it from other staphylococci. The common name for other staphylococci is _____ _____ (CoNS), due to their property of not producing coagulase enzymes.

DNase test

This test determines the bacteria's ability to synthesize an enzyme that breaks down DNA. The strain is cultivated on a solid medium containing polymerized DNA.

After incubation at 35-37°C/24 hours, dilute hydrochloric acid is added to the medium, which causes the precipitation of undegraded DNA and clouding of the medium, while a transparent zone remains around the DNase producing strains. *S. aureus* is DNase _____.

Bacitracin susceptibility test

This test is used to distinguish *Streptococcus pyogenes* from other species of beta-hemolytic streptococci.

After inoculation of the tested strain on agar plate, a bacitracin disc is placed over it. After incubation for 18-24 h at 35-37°C, the zone of inhibition around the disc should be read.

If there is any zone of inhibition around the disc, the strain is considered sensitive to bacitracin, which means it is _____. If the strain is resistant to bacitracin, then it is some other type of _____ streptococcus.

CAMP test

This test is used to distinguish *Streptococcus agalactiae* from other species of beta-hemolytic streptococci. CAMP is an acronym created from the names of the test authors (Christie, Atkinson, Munch and Peterson).

A line of the *Staphylococcus aureus* is streaked in the middle of the blood agar, and lines with the tested strains are streaked perpendicular to this line, approaching each other to approximately 2 mm. After incubation for 18-24 h at 35-37°C, *Streptococcus agalactiae* will cause a triangular enhancement in beta-hemolysis in the vicinity of staphylococci.

Optochin susceptibility test

This test is used to distinguish *Streptococcus pneumoniae* from other species of alpha-hemolytic streptococci.

A disc of optochin is placed on the agar plate on which the tested strain is inoculated. After incubation for 18-24 h at 35-37°C, the zone of inhibition around the disc should be read. If the zone diameter is 14 mm or larger, the strain is considered susceptible to optochin, which means it is _____. If the strain is resistant to optochin, it belongs to another type of ___ _____ streptococcus.

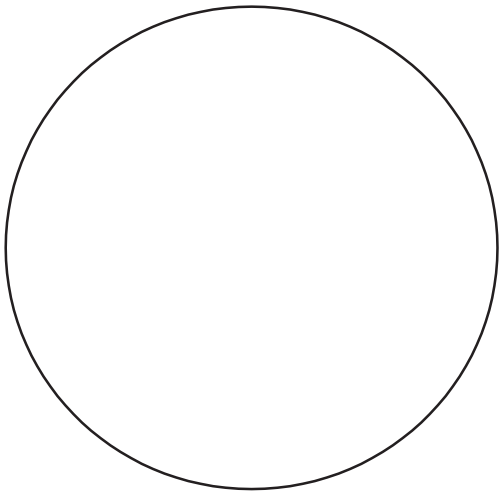
PRACTICAL WORK:

Gram staining procedure:

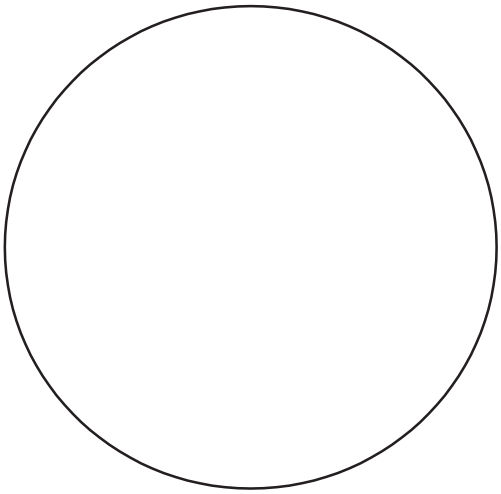
1. Make a thin smear of bacterial culture (or a biological sample) on the slide and leave it to dry in the air.
2. Heat fix the bacteria to the slide by passing it through the flame 3-4 times.
3. Place the slide on the grid for staining.
4. Cover the smear with crystal violet solution, leave for 1 min.
5. Pour off the excess crystal violet solution (without rinsing with water).
6. Cover the smear with Gram's iodine solution, leave for 1 min.
7. Rinse with water.
8. Holding the slide between the thumb and forefinger, rinse the smear using individual drops of acetone-alcohol as long as the excess colour is washed away.
9. Rinse with water.
10. Place the slide on the staining grid and cover with safranin solution, leave for 1 min.
11. Rinse with water.
12. Place the slide in the air-drying position.
13. Put a drop of anisole on the completely dry smear and look under high magnification (objective 100x).

Task 1. Preparation and interpretation of Gram stains: observation of micromorphological characteristics of bacterial species.

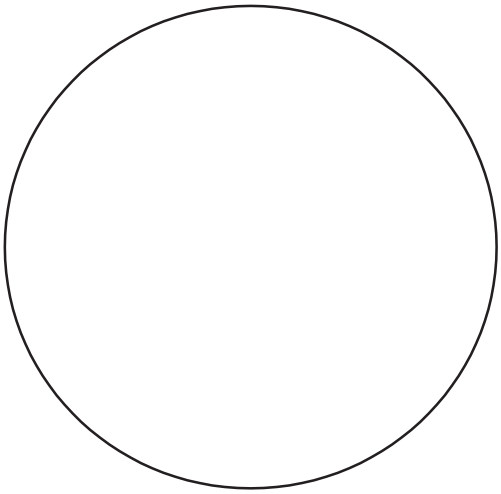
a) *Staphylococcus aureus* from blood agar culture



b) *Streptococcus pneumoniae* from blood agar culture

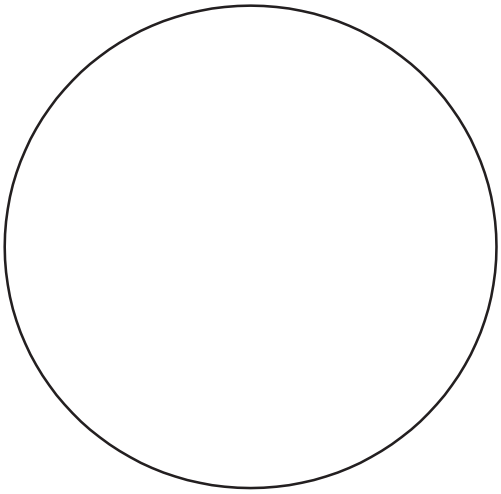


c) *Streptococcus pyogenes* from broth culture

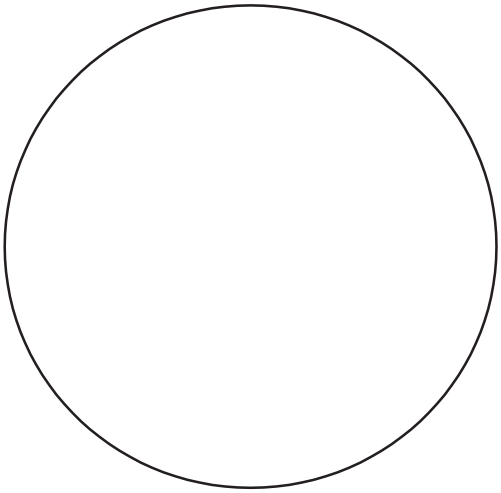


Task 2. Microscopy of methylene blue and Gram stains: observation of differences in staining and micromorphological characteristics of bacterial species, as well as intracellular or extracellular location of microorganisms

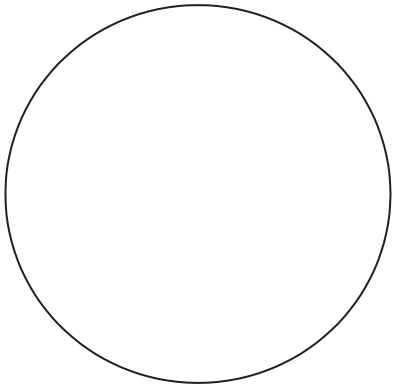
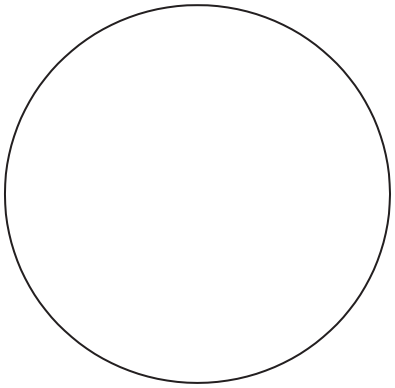
a) *Streptococcus pneumoniae* from mouse blood; methylene blue stain

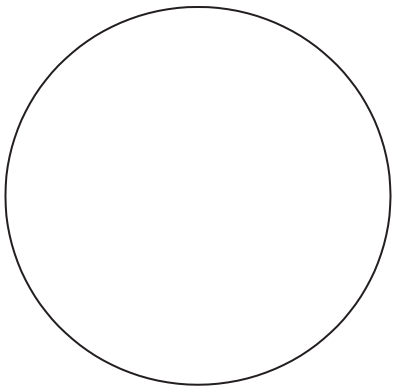
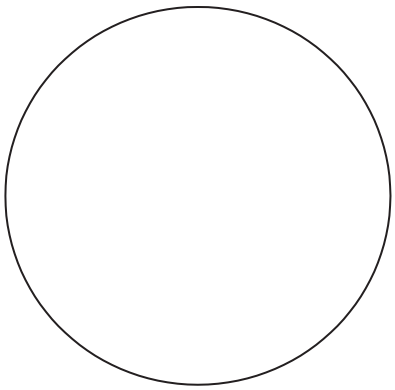


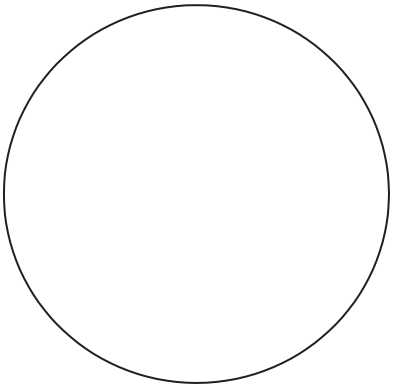
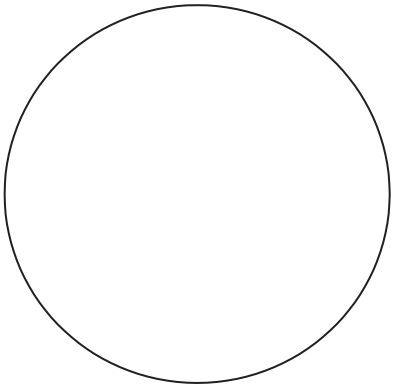
b) *Streptococcus pneumoniae* from cerebrospinal fluid, blood or sputum; Gram stain



Task 3. Description of colonies and interpretation of identification tests: identification of species according to observation of macromorphological characteristics and test results.

	Staphyloccocus aureus	*Staphylococcus epidermidis*
Size		
Form		
Margin		
Surface		
Hemolysis		
Pigment		
		

	Streptococcus pyogenes	*Streptococcus agalactiae*
Size		
Form		
Margin		
Surface		
Hemolysis		
Pigment		
Bacitracin test		
		

	Streptococcus pneumoniae	Viridans group streptococci
Size		
Form		
Margin		
Surface		
Hemolysis		
Pigment		
Optochin test		
		

Review questions:

1. Nasal and throat swabs should be transported at _____ temperature.

2. Hemolysis occurs due to _____.

3. Group B beta-hemolytic streptococcus is _____.

4. The free or bound coagulase tests distinguish _____ from _____ _____.

5. *Streptococcus pneumoniae* can be confirmed using susceptibility test to _____.

6. When disk diffusion method is used, methicillin-resistant *Staphylococcus aureus* can be recognized by its resistance to _____ disk.

Notes:

Marina Radić Skelin

Performing, reading and interpreting of antibiogram (disk diffusion method, broth dilution, agar dilution, E-test)

Introduction

Antibiotic is a chemical substance that inhibits bacterial growth (bacteriostatic activity) or kills bacteria (bactericidal activity) causing minimal damage to the host. As there is no universal antibiotic that has an equal effect on all pathogens, it is necessary to choose the one that has maximal effect on bacteria causing infection.

In microbiological laboratory we routinely test bacterial susceptibility to different antibiotics using well standardized tests. The choice of antibiotics tested depends on bacterial species or genus and clinical specimen. In most cases, only one antibiotic from a specific group can be tested (e.g. erythromycin from macrolide group), as the susceptibility result can be extrapolated to the whole group.

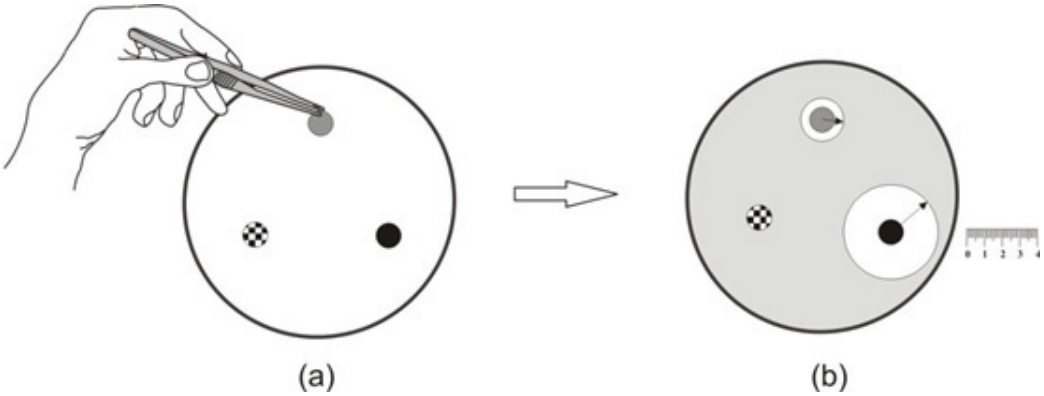
Test for antimicrobial susceptibility (antibiogram) determines the ability of antimicrobial substances to inhibit bacterial growth *in vitro.* Testing can be performed using diffusion or dilution methods. Therefore it is crucial to emphasize the importance of correct antibiogram performance and result interpretation, as it presents a clinical guideline in choosing the best antibiotic for treatment.

Disk diffusion (Kirby-Bauer method)

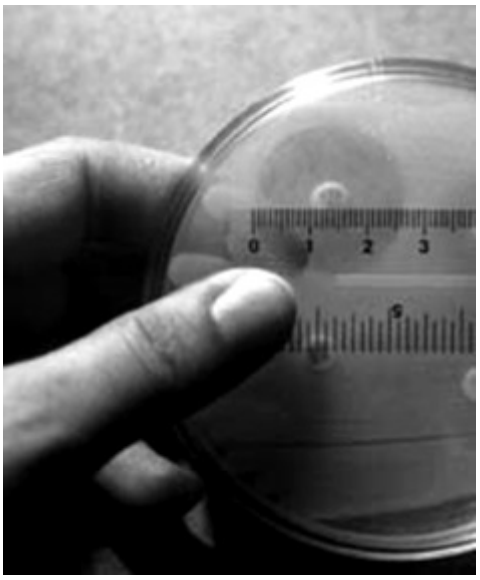
This method is the most commonly used for antimicrobial susceptibility testing.

Commercially available paper disks, impregnated with specific bacterial concentration, are placed on the solid surface of specialized standardized media for antimicrobial testing (Mueller-Hinton agar). Previously it is necessary to take pure bacterial culture using inoculating loop and make a standardized suspension, having density of 0.5 McFarland (McF). Within 15 minutes, sterile swab is placed in the suspension, drained on the walls of the vial and the suspension is spread on agar surface. Afterwards, antibiotic disks are placed on agar surface using dispenser (or sterile tweezer) at the distance of 20 mm. The agar with antibiotic disks is incubated in thermostat for 18-24 hours, on 35-37°C.

During incubation, antibiotic from the disk diffuses in the media, and if effective, inhibits bacteria in its growth and the bacteria grows at certain distance from the disk where the antibiotic concentration is rather small and, therefore, inefficient. That absence of bacterial growth is called **zone of inhibition**. We measure the **diameter** of the zone of inhibition and express it in **millimeters (mm)** (Pictures 1. and 2.).



Picture 1. a) Placing paper disks using sterile tweezer.
b) Bacterial growth is inhibited at certain distance from the disk (zone of inhibition).



Picture 2. Correct measuring of diameter of zone of inhibition (mm).

According to the size of the diameter of zone of inhibition, expressed in millimeters, the tested strain is placed in one of three categories, defined by the European standards (European Committee on Antimicrobial Susceptibility Testing - EUCAST):

- susceptible (**S**): the strain is subjected to usual or average administered antibiotic dosage in all organism;
- intermediate (**I**): the strain is subjected to usual administered antibiotic dosage only in places where the antibiotic is concentrated, or in maximum dosage otherwise;
- resistant (**R**): antibiotic administered in maximum dosage has no effect on bacterial strain.

Broth dilution

Broth dilution is a procedure where vials (macrodilution) or microtiter (microwell) plates (microdilution) containing Mueller-Hinton broth are used. An equal bacterial suspension (0.5 McF) and the antibiotic tested is added to the broth. The concentration of tested antibiotic is two-fold serially diluted in every subsequent vial or well. After incubation period (18-24 hours, 35-37°C) bacterial growth is visible if the broth is blurred. The aim of this procedure is to determine the lowest concentration that inhibits bacterial growth, minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC).

Minimum inhibitory concentration (MIC) is the lowest concentration in two-fold serial antibiotic dilutions (expressed as μg/ml or mg/l) that inhibits bacterial growth *in vitro*.

MIC represents the antibiotic concentration in first clear vial in the row, having no bacterial growth (no blurring).

When using dilution method, if after incubation period no blurring was observed, then this unblurred (clear) broth is subcultivated on solid agar without antibiotic (most commonly blood agar). The media are then incubated and the aim is to determine the first antibiotic concentration that completely inhibits bacterial growth on the media and is expressed as minimum bactericidal concentration (MBC). That value determines the lowest antibiotic concentration (expressed as μg/ml or mg/l) that kills 99.9% bacteria and is higher than MIC value. Determining MBC is routinely rarely performed, most commonly if there is suspicion in great difference between MIC and MBC, like in endocarditis, where it is necessary to achieve bactericidal antibiotic effect.

Agar diluton

Agar dilution is the same procedure as broth dilution, but the only difference is in use of Mueller-Hinton agar impregnated with certain antibiotic concentration instead of vials or wells. After adding bacterial strain and incubation of 18-24 hours on 35-37°C, the colony growth is observed on agar. MIC is interpreted as the concentration of antibiotic on the first agar without bacterial growth.

Gradient test (Epsilometer test)

Gradient test is a combination of both diffusion and dilution method.

Predefined gradient of antibiotic concentration is impregnated on a plastic test strip that is placed on Mueller-Hinton agar with bacterial suspension. After incubation (18-24 hours, 35-37°C) an elipse or tear-shaped zone of inhibition is observed. The lower edge of zone of inhibition „cuts" the graduated strip, and the MIC (minimum inhibitory concentration, expressed as μg/ml or mg/l) is read at the point of intersection. (Picture 3.).



Picture 3. Interpretation of gradient test. MIC is 0.75 μg/ml.

Additional tests used in performing antimicrobial susceptibility testing

Determining ESBL phenomenon

ESBL (extended spectrum beta-lactamase) presents extended spectrum beta-lactamases found most commonly in Enterobacterales (*E. coli*, *K. pneumoniae*, *P. mirabilis*). Those enzymes destroy penicillin and cephalosporin antibiotics, but carbapenems are preserved. If the bacteria produces ESBL, a synergism is observed on antibiogram between the beta-lactamase inhibitor combined with beta-lactam antibiotic (e.g. amoxicillin and clavulanic acid, AMC or AUG) and a cephalosporin antibiotic (e.g. ceftriaxone). That zone is commonly described as „ghost zone" or a zone looking like a „champagne cork".

Cefinase test

Cefinase disk is used for rapid detection of beta-lactamase produced by some bacteria, like *Neisseria gonorrhoeae*, *Moraxella catarrhalis*, *Staphylococcus* spp., *Haemophilus influenzae*, including some anaerobic bacteria. The disk is impregnated with the chromogenic cephalosporin,

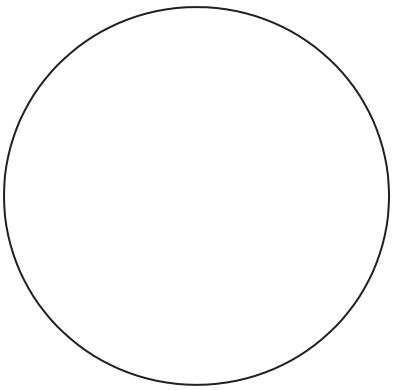
nitrocefin, that exhibits a very rapid colour change from yellow to red as the amide bond in the beta-lactam ring is hydrolysed by a beta-lactamase.

Procedure: paper disk is placed on a microscopic slide, soaked with saline and using inoculating loop, bacterial culture is smeared onto a disk surface. The disk is observed for colour change. A positive reaction will show a yellow to red colour change on the area where the culture was applied, meaning that the bacteria produces beta-lactamase.

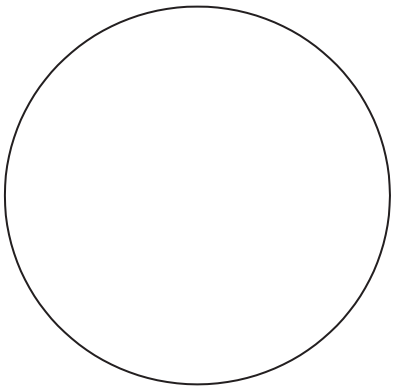
PRACTICAL WORK

Task 4. Interpretation of antimicrobial susceptibility tests using the disk diffusion method: practicing correct reading of inhibition zones and interpretation of susceptibility.

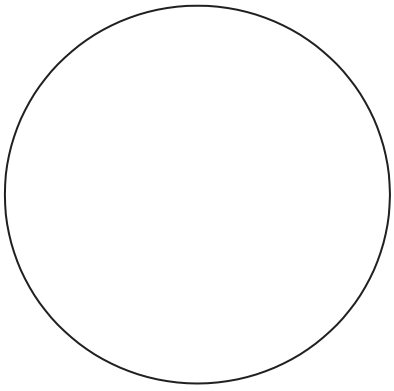
Methicillin-susceptible *Staphylococcus aureus* (MSSA)		
Antimicrobial agent	Zone (mm)	Interpretation (S, I, R*)



Methicillin-resistant *Staphylococcus aureus* (MRSA)		
Antimicrobial agent	Zone (mm)	Interpretation (S, I, R)

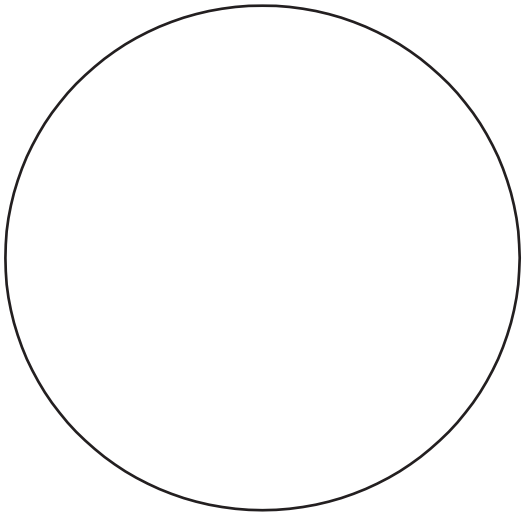


Streptococcus pneumoniae		
Antimicrobial agent	Zone (mm)	Interpretation (S, I, R)



*S - susceptible at standard dose
I - susceptible with increased exposure of the isolate to the antibiotic, by increasing the dose or concentrating at the site of infection
R – resistant

Task 5. Draw gradient test from routine work and interpret MIC value.



MIC:_____ μg/mL

Review questions:

1. _____ is standardized media for performing antibiogram.
2. What is the most commonly used method for antimicrobial susceptibility testing? _____ _____
3. The combination between diffusion and dilution method is:
   - disk dilution
   - Kirby-Bauer method
   - broth dilution
   - gradient test
   - agar dilution

4. When interpreting antibiogram, the radius of zone of inhibition is measured and expressed in millimeters.

Correct Not correct

5. ESBL - phenomenon can be found in all bacteria.

Correct Not correct

Date: Teacher's signature:

_____ _____

Notes:

Notes:

PRACTICAL B3

Anita Novak

Macroscopic, biochemical and serotyping identification of *Enterobacteria*ceae.

Widal's agglutination reaction. Main characteristics of genera *Bordetella* and *Brucella.*

In order to successfully master this practical, the student should first study Chapters 15 and 18 from the book Jawetz, Melnick and Adelberg. Medical microbiology, 26th Edition. McGraw Hill Lange; 2013.

INTRODUCTION

The family ***Enterobacteriaceae (Enterobacterales)*** includes heterogeneous group of gram-negative bacilli that are mostly part of a normal gastrointestinal microbiota of humans and animals.

Enterobacteria are aerobes or facultative anaerobes, ferment glucose and some other sugars and possess numerous virulence factors. They can cause various infections, from localized (e.g. gastrointestinal and urinary tract infections) to severe disseminated infections (e.g. sepsis).

Classic microbiological methods, like direct **light microscopy** and **cultivation** are most often used in the diagnosis of the infections they cause, but also new methods, such as **PCR** and **MALDI-TOF MS**, can be used.

For microscopic visualization of bacterial capsule, we use combined staining method (**India ink** and **Gram**).

Enterobacteria can grow on various **non-selective** (e.g. BA) and **selective and differential media** (e.g. SS agar, CLED agar, Selenit F broth, MacConkey agar). Grown colonies can be **identified** by a combination of several methods; describing their **morphology** on nutrient media, reading the results of biochemical tests (by the macro method in test tubes, the so-called **biochemical series** (BCS) or semi-automatic and automatic systems such as Vitek®, BD Phoenix™) and finally by **serotyping** (agglutination with specific sera).

By serotyping, we determine the existence of specific somatic or O antigens in the bacterial cell wall and, if the bacterium is motile, the existence of flagellar or H antigens. Encapsulated bacteria also possess a third type of antigen, the so-called capsular or K antigens (*Salmonella* Typhi has virulence antigen or Vi antigen).

In addition to direct methods, in the diagnosis of typhoid fever, **indirect microbiological diagnostics** can be used (the specific **Widal** agglutination reaction). Serum agglutinins increase sharply during the second and third week of infection caused by bacteria *Salmonella* Typhi.

Serial dilutions of paired serum samples taken from the same patient in the period of 7-10 days are tested using specific *S.* Typhi antigens. Antibody titer is calculated and compare in order to determine whether there is a diagnostically significant increase / decrease (2).

The agglutination reaction is also used in the diagnosis of brucellosis (**Wright** specific agglutination) and rickettsiosis (**Weil-Felix** non-specific agglutination).

***Bordetella* spp.** are tiny gram-negative coccobacilli. For humans, the most significant is *B. pertussis* that can be cultivated on specific nutrient media Bordet-Gengou and Regan Lowe.

***Brucella* spp.** are gram-negative coccobacilli, obligate intracellular bacteria of humans and animals. They can be cultivated on enriched media, such as trypticase-soy agar, brain heart infusion (BHI) and chocolate agar.

PRACTICAL

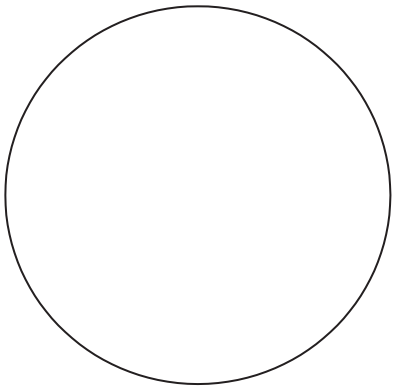
Task 1. Preparation and microscopic examination of *E. coli* and *P. mirabilis* (Gram staining from colonies grown on BA) - students work in pairs.

Objective: to observe different species of bacteria within the *Enterobacteriaceae* family

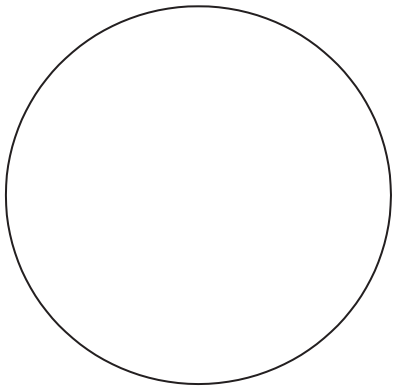
(there is no significant morphological differences for each species)

Procedure: one student prepares slide with the culture of *E. coli,* and the other one with *P. mirabilis.*

Place a drop of physiological solution (f.s.) on the slide. Touch the grown colony (about half of the colony) with an inoculation loop and make a homogeneous suspension in the prepared drop of f.s. Spread the suspension uniformly, in a thin layer, on the microscopic slide. Dry the preparation in the air, fix it on the burner and stain it according to Gram. Microscope the dried preparation under a high magnification (1000 x) of microscope, with an immersion objective (100 x) and draw what is seen (each student should look at, draw and describe both preparations).



E. coli – Gram staining

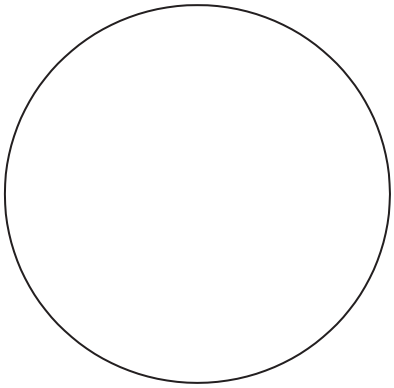


P. mirabilis – Gram staining

Task 2. Microscopy of *Klebsiella* sp. (India ink with Gram).

Objective: to observe the capsule around the bacterial cell.

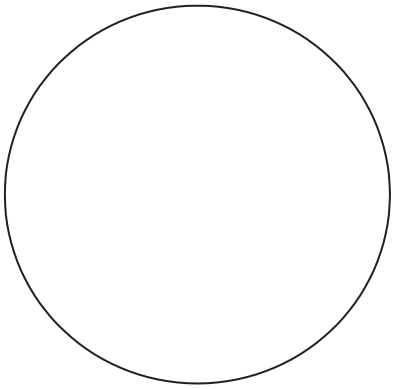
Procedure: microscope prepared slide under high magnification (1000 x) of the microscope, with an immersion objective (100 x) and draw what is seen.



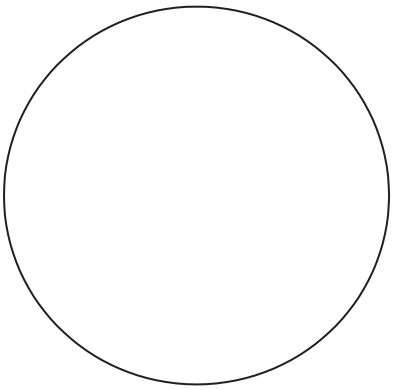
Klebsiella sp. – India ink with Gram

Task 3. Describe and draw the grown colonies (work in pairs).

3.1 selective medium: SS (Salmonella/Shigella agar) – differentiate bacteria upon lactose fermentation and H_2S production



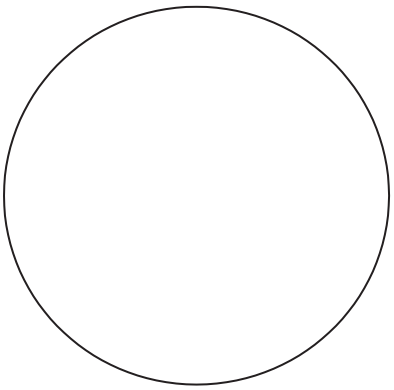
SS – *E. coli*



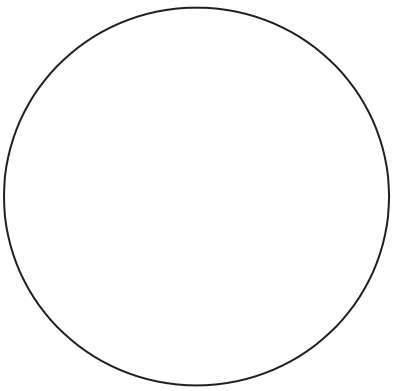
SS – *Salmonella* sp.

COLONIES	*E. coli*	*Salmonella sp.*
SIZE		
SHAPE		
EDGE		
SURFACE		
L, H_2S		

3.2 selective medium CLED – differentiate bacteria upon lactose fermentation

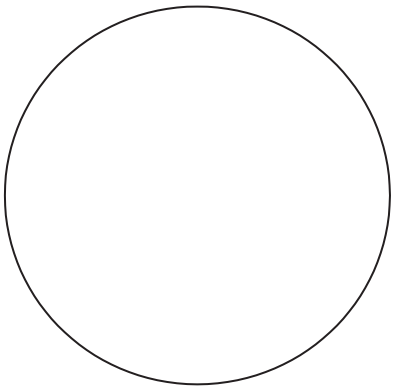


CLED – *E. coli*



CLED – *K. pneumoniae*

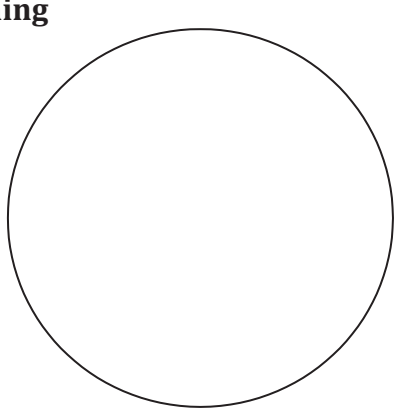
COLONIES	*E. coli*	*K. pneumoniae*
SIZE		
SHAPE		
EDGE		
SURFACE		
L		



CLED – *Proteus* sp.

COLONIES	*Proteus* sp.
SIZE	
SHAPE	
EDGE	
SURFACE	
L	

3.3 BA - Proteus sp. - swarming



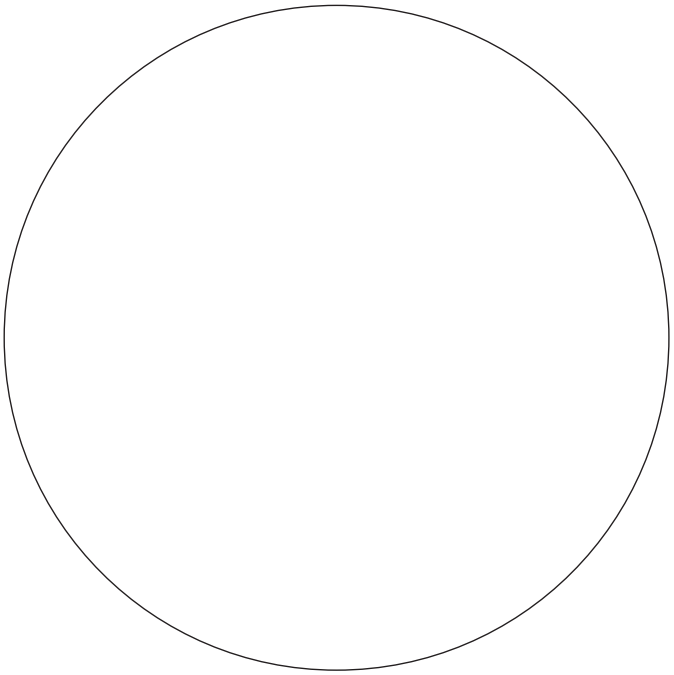
BA – *Proteus* sp.

COLONIES	*Proteus* sp.
SIZE	
SHAPE	
EDGE	
SURFACE	
HEMOLYSIS	

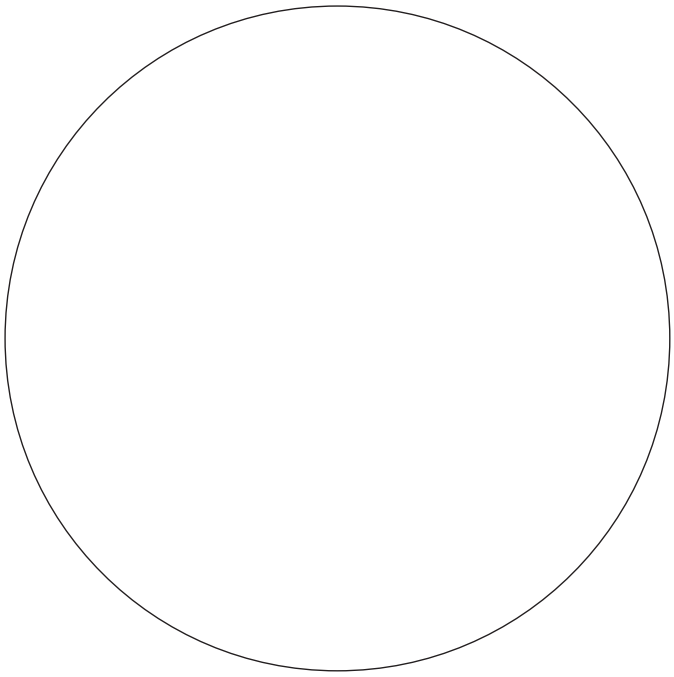
Task 4. Interpret the antimicrobial susceptibility of two different strains of *E. coli*, one that is highly sensitive to most of the tested antibiotics (ESBL -) and the other that produces extended spectrum beta lactamases (ESBL+ strain).

Measure inhibition zones for each listed antibiotic (in mm) and interpret the results according to the EUCAST standard (work in pairs). Antibiograms were made on MH-agar (Mueller-Hinton), using the disk diffusion method.

Objective: to observe the difference in the antibiograms of a highly susceptible *E. coli* strain from a multiresistant hospital strain that produces ESBL (extended spectrum β-lactamase). Recognize the falsely extended zone of growth inhibition between the amoxicillin clavulanic acid disc and the cephalosporin discs.



E. coli – susceptible strain

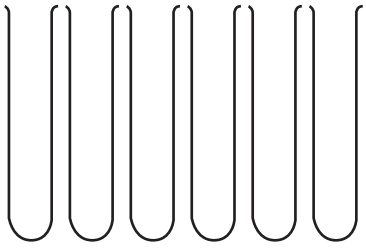


E. coli – ESBL +

	E. coli		*E. coli* ESBL+	
Antibiotic	(mm)	S / I / R	(mm)	S / I / R

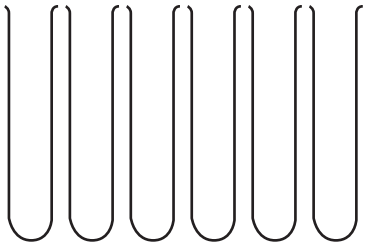
Task 5.

Calculate the results of serological agglutination reaction (Widal) - students work in pairs.



Initial dilution:

Titer of the first serum:

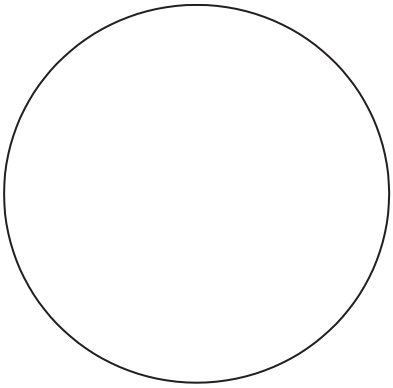


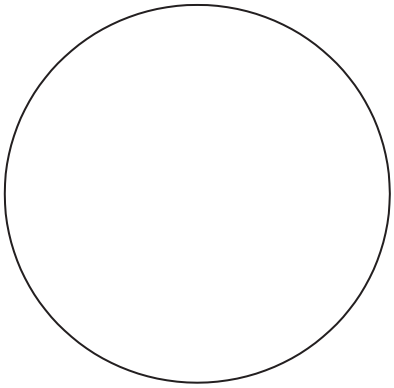
Initial dilution:

Titer of the second serum:

Interpretation of the serological reaction:

Task 6. Describe *Bordetella* and *Brucella* colonies grown on selective media.





COLONIES	*Bordetella*	*Brucella*
SIZE		
SHAPE		
EDGE		
SURFACE		
MEDIA	Bordet-Gengou agar	TSA

Review questions:

1. Why to use selective media for cultivation of stool and urine samples?
2. How to identify enterobacteria?
3. Why *K. pneumoniae* form extremely slimy colonies on solid media?
3. What does the abbreviation ESBL mean and how can we determine ESBL production on antibiogram?
4. Which antibiotics to use in the treatment of infections caused by ESBL-producing bacteria?
5. List the most important characteristics of *Bordetella*.
6. Describe the laboratory diagnosis of brucellosis.

Date: Teacher's signature:

_____ _____

Notes:

PRACTICAL B4

Merica Carev

Cultivation and identification of the genera *Pseudomonas, Campylobacter, Vibrio, Helicobacter, Neisseria,* and *Haemophilus*. Sampling and transport of the samples for the isolation of anaerobic bacteria. Principles of the anaerobic cultivation.

In order to successfully master this practical, the student should first study relevant Chapters from the book Jawetz, Melnick and Adelberg. Medical microbiology, 26th Edition, McGraw Hill Lange; 2013.

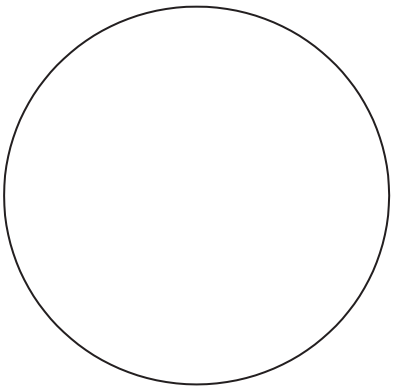
Genus *Pseudomonas*

Bacteria of the genus *Pseudomonas* are aerobic, mobile gram-negative bacilli that have enzymes cytochrome-oxidase and catalase and produce pigments. The oxidase test is important for distinguishing the *Pseudomonas* genus from the members of the family *Enterobacteriaceae* that do not possess this enzyme. Members of the *Pseudomonas* genus are present in the environment, but often also on wet hospital surfaces. They have a distinct ability to form a biofilm, which enables them to survive in adverse conditions and to be highly resistant to antimicrobials.

Pseudomonas aeruginosa causes severe infections in immunocompromised patients and is one of the most important causes of healthcare-associated infections.

PRACTICAL WORK

Task 1. Describe the colonies of *P. aeruginosa* on nutrient agar (NA).

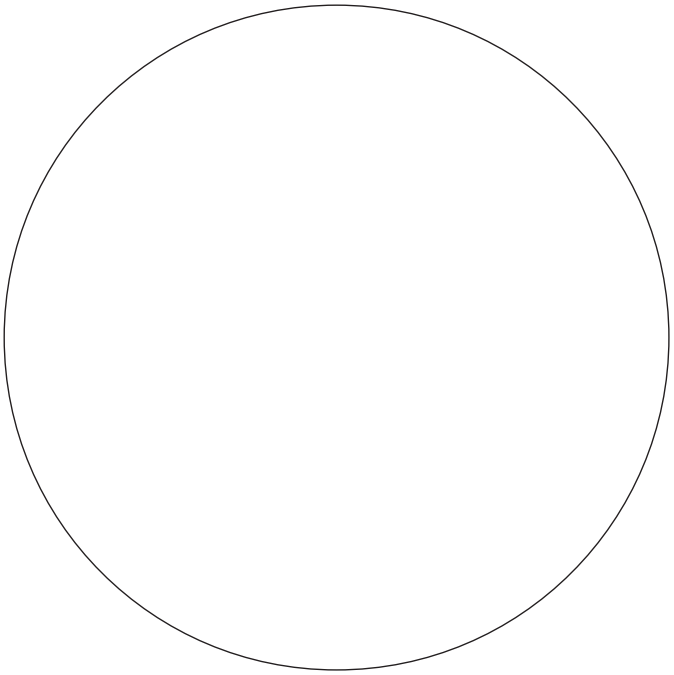


COLONY	*P. aeruginosa*
SIZE	
SHAPE	
MARGIN	
SURFACE	
PIGMENT	

NA – *Pseudomonas aeruginosa*

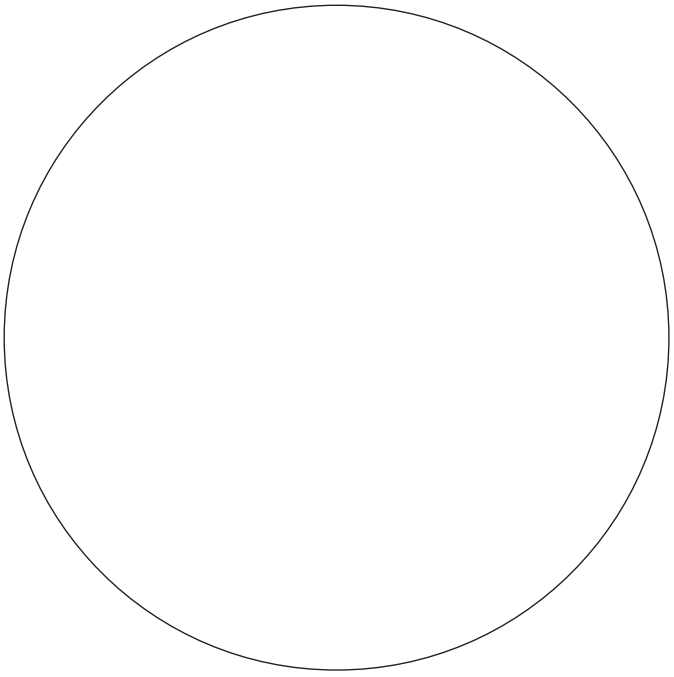
Task 2. Read antibiograms and observe the difference in the antibiograms of a highly susceptible, wild strain of *P. aeruginosa*, and carbapenem resistant, hospital strain.

MH agar, *P. aeruginosa* antibiograms: *P. aeruginosa* wild strain and *P. aeruginosa*, hospital, carbapenem resistant strain



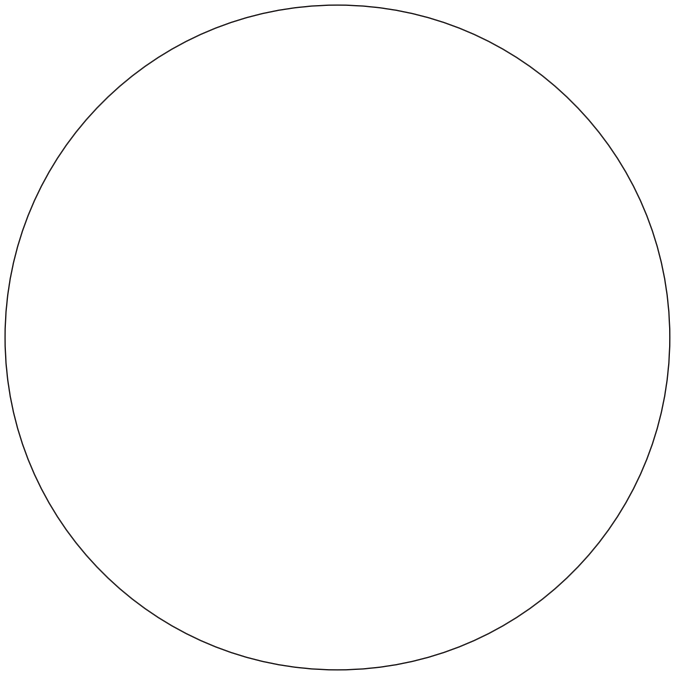
P. aeruginosa - susceptible to carbapenems

P. aeruginosa – resistant to carbapenemes



	P. aeruginosa susceptible strain	*P. aeruginosa* susceptible strain	*P. aeruginosa,* carbapenem resistant strain	
Antibiotic	(mm)	interpretation	(mm)	interpretation

Task 3. Draw an E-test of *P. aeruginosa* and determine the MIC (µg/mL).



MH agar

MIC = µg/ml

Genera *Campylobacter, Helicobacter,* and *Vibrio*

Bacteria belonging to the genera *Campylobacter, Helicobacter,* and *Vibrio* are tiny, curved, mobile, gram-negative bacilli. All of them are oxidase positive, they need special conditions for cultivation, and cause infections of the gastrointestinal system.

Campylobacter spp. is the most common bacterial cause of diarrhea in developed countries. For the cultivation of the genus *Campylobacter* from stool samples, selective nutrient media (Campylobacter Selective Agar, CSA, or Karmali agar) are used, which are incubated in the microaerophilic atmosphere at 42°C for 48 hours. *Campylobacter* spp. grows as milky-gray, moist colonies which are appropriate for making Gram smear. Small gram-negative rods in the shape of the letter S or seagull's wings can be seen on the smear.

Helicobacter pylori is a curved, gram-negative bacillus that can be isolated from the biopsy specimens of the gastric mucosa. It is associated with gastritis, peptic ulcer, gastric cancer, and MALT B-cell lymphoma. It can also be proven by detecting antigens in the stool.

Vibrio spp. is a gram-negative, comma-shaped rod. It is widespread in the aquatic environment and organisms. The most significant representative is *V. cholerae*, the causative agent of cholera. For stool cultivation, alkaline peptone water and TCBS (thiosulphate, citrate, bile salts, sucrose) medium are used on which, after incubation at 37°C for 24 hours, smooth, sucrose-positive (yellow) colonies are observed.

PRACTICAL WORK

Task 4. Draw a microscopic appearance of *Campylobacter* spp. culture.

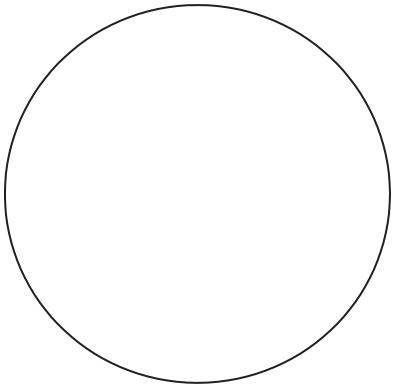
Describe the picture:

_____

_____

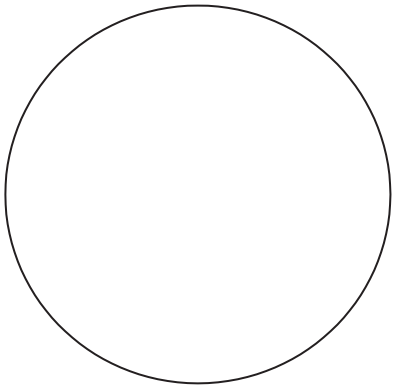
_____

_____



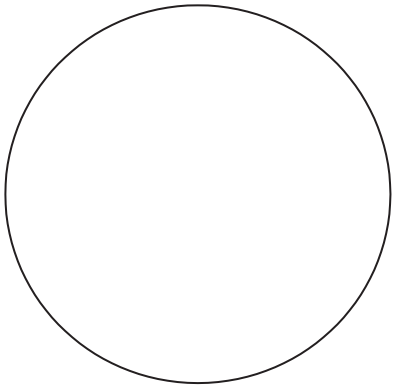
Task 5. Draw the appearance of *Campylobacter* spp. culture on the CSA medium.

	Campylobacter spp.
Size	
Shape	
Margin	
Surface	



Task 6. Draw the appearance of the *Vibrio cholerae* culture on the TCBS medium.

	Vibrio cholerae
Size	
Shape	
Margin	
Surface	
Saccharose	



Task 7. Read and draw *Helicobacter pylori* antigen detection test in the stool.

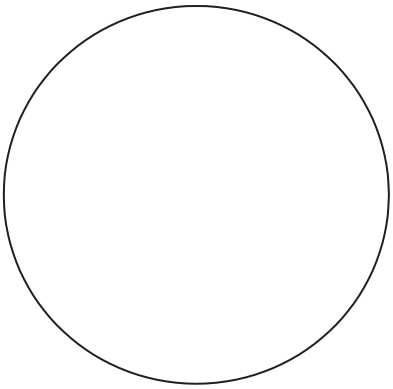
Describe the picture:

_____

_____

_____

_____



Genus *Neisseria*

Bacteria of the genus *Neisseria* are gram-negative diplococci in the form of coffee beans. Numerous species of this genus are apathogenic and they are part of the microbiota of the upper respiratory system. Pathogenic species are *N. meningitidis,* the causative agent of meningitis and sepsis, and *N. gonorrhoeae,* the causative agent of gonorrhea. For the cultivation of pathogenic species, enriched media (chocolate agar), the presence of $CO_{2,}$ and incubation at 37°C for 24-72h are required.

Oxidase test: serves to prove cytochrome-oxidase enzymes. The substrate is the so-called oxidase reagent, which is dripped on filter paper. A bacterial culture is added to this. A positive reaction is the appearance of dark purple to black color for 5-10 seconds.

PRACTICAL WORK

Task 8.

List some apathogenic species of the genus *Neisseria:*

_____

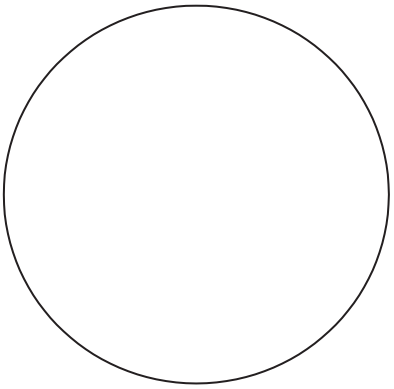
Specify pathogenic species of the genus *Neisseria:*

_____

The tests used in the identification of the genus *Neisseria* are:

_____

Task 9. Read the oxidase test with the prepared *Neisseria* spp. culture. Draw and describe the positive oxidase reaction.



Task 10. Make a catalase test for the *Neisseria* spp.

The catalase test is

Task 11. Tests for sugar degradation (glucose, maltose, lactose)

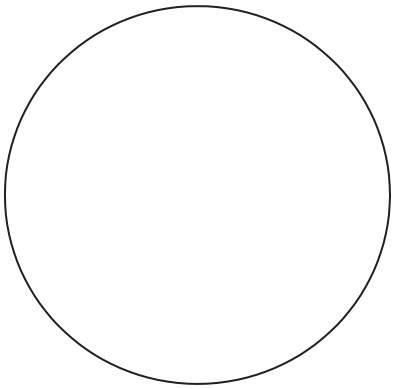
Read the sugar degradation test for the prepared bacterial culture of the *Neisseria* spp. (+/-). The red color represents a negative reaction, and the yellow color represents a positive fermentation reaction.

1. test tube: testing of glucose degradation + -

2. test tube: testing of maltose degradation + -

3. test tube: testing of lactose degradation + -

Task 10. Microscopic smear of *Neisseria* spp. culture stained by the Gram method. Draw and describe the microscopic appearance of the culture.

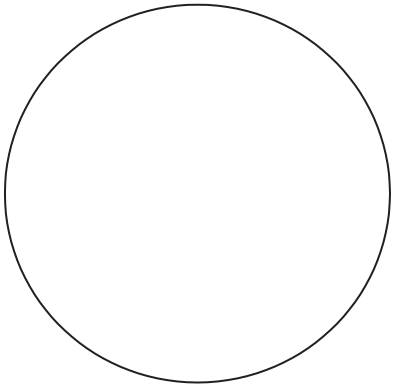


Micromorphology is _____

Task 13. Microscopy of prepared smears

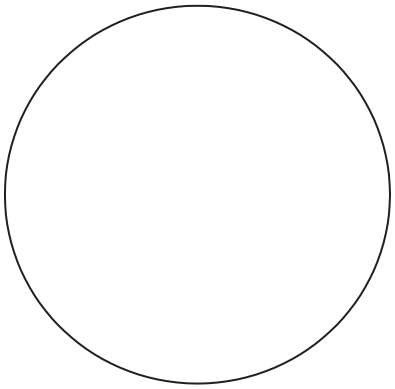
N. meningitidis - cerebrospinal fluid - Gram smear

Draw and describe the microscopic appearance of the culture.



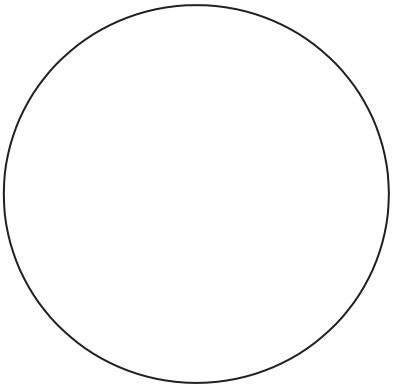
N. gonorrhoeae - urethral swab - Gram smear

Draw and describe the microscopic appearance of the culture.



N. gonorrhoeae - urethral swab – methylene blue smear

Draw and describe the microscopic appearance of the culture.



Genus *Haemophilus*

Haemophilus spp. are pleomorphic, gram-negative coccobacilli with special requirements for growth and cultivation. They partly belong to the microbiota of mucous membranes in humans, but they can also be the causative agents of meningitis, epiglottitis, and pneumonia, especially in children and immunocompromised patients. To ensure the special conditions of cultivation of this microorganism (hemin; X factor and NAD; V factor), respiratory samples are cultivated on blood agar (BA) with the addition of *Staphylococcus aureus* culture (the so-called staphylococcal line) or chocolate agar and incubated in an atmosphere enriched with CO_2. In testing the susceptibility of *Haemophilus* spp. to antibiotics, a cefinase test (nitrocefin test) is performed. The test proves the presence of β-lactamase in *Haemophilus influenzae*.

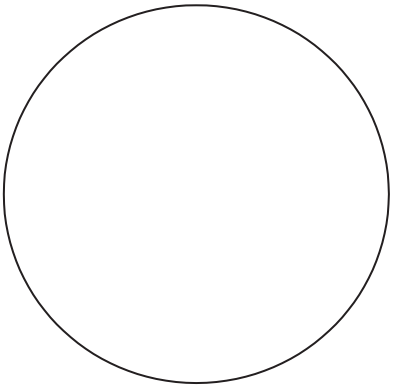
To perform the cefinase test, see the 2nd Practical. Disc color change is read within 5-30 seconds.

PRACTICAL WORK

Task 14. From the prepared bacterial culture make a Gram-smear.

Microscopy and draw a picture. The basic micromorphology of *Haemophilus* spp. is:

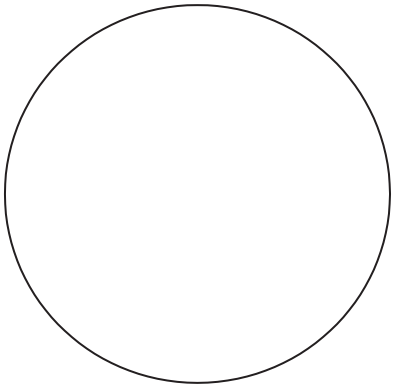
Describe the picture:



Task 15. Cultivation of *Haemophilus* spp. on blood agar requires additional growth factors.

List the necessary factors for the growth of *Haemophilus* spp. culture. Drow the satellite growth phenomenon on the blood agar.

Describe the picture:



COLONIES	*Haemophilius* spp.
SIZE	
SHAPE	
EDGE	
SURFACE	
HEMOLYSIS	

Anaerobic bacteria

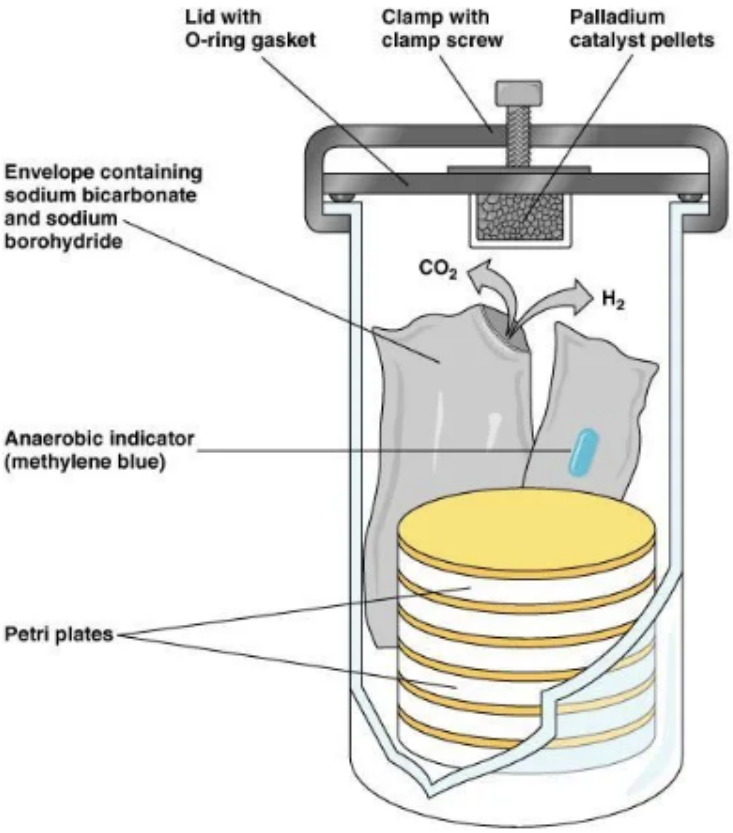
Microorganisms differ according to the ability to use_____for cellular respiration. Unlike _____microorganisms, which use O_2 as the final acceptor of electrons, anaerobic microorganisms use other molecules and have a different tolerance to oxygen. In strict or obligatory anaerobes, the presence of atmospheric oxygen leads to the formation_____so when they are exposed to atmospheric oxygen, they die. Aerotolerant anaerobes create_____ and/or which neutralize toxic radicals and thus can tolerate smaller amounts of oxygen_____ .

During sampling material for the isolation of anaerobic agents, it is important not to expose the sample to atmospheric oxygen. Therefore, the swab of the wound or the swab of the fistula is not suitable samples for the isolation of anaerobic pathogens. Suitable specimens for anaerobic cultivation are aspirate, punctate, or biopsy specimens from the site of infection. The sampling area should be disinfected, and then the contents or tissues are taken under anaerobic conditions and transported to the laboratory within 15 minutes. If the transport lasts longer (up to 24 hours), the samples should be aseptically transferred to the transport medium for anaerobes and stored at room temperature.

Anaerobic cultivation requires procedures that will create anaerobic conditions and media that contain chemical compounds with low redox potential. Examples of such reducing media are thioglycolate broth and Columbia agar.

DEMONSTRATION

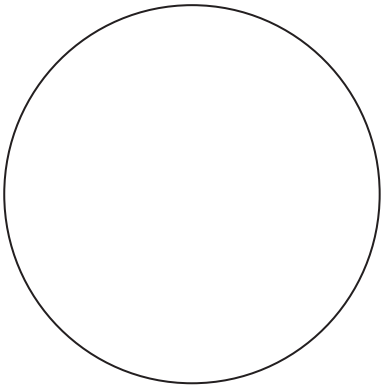
Medium	Mechanism	Principle of procedure
Fortner's plate	Biological	A facultative anaerobe is cultivated on half of the solid nutrient medium, and a strict anaerobe on the other half, and the lid of the medium is tightly closed with paraffin. When a facultative anaerobe uses oxygen,conditions for the growth of strict anaerobes are created.
		The procedure corresponds to the conditions present in mixed aerobic-anaerobic infections.
Tarozzi broth	Chemical	A piece of cooked meat immersed in broth creates a reducing medium suitable for growing anaerobes. A similar phenomenon occurs in deep wound pockets due to tissue destruction with trauma.
McIntosh Pot	Physical	O_2 is extracted from the container for the cultivation of anaerobic microorganisms, with the powerful vacuum pump and replaced with a mixture of other gases.
Thioglyolate broth	Chemical	The liquid substrate contains sodium-thioglycolate, which binds with O_2, whereby a reduced compound is formed.
GasPak system	Chemical	An open bag with chemical agents is placed in a container, which is moistened with several mL of water. The vessel is hermetically sealed. In the chemical reactions that follow, in a short period, oxygen is removed from the pots.*



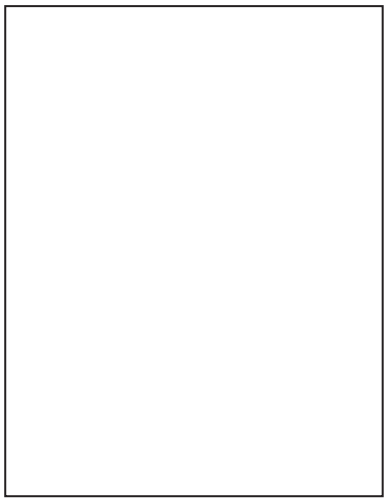
***Vessel for growing anaerobic bacteria GasPak procedure. When the contents of the bag are mixed with water, H_2 and CO_2 are formed, and then, with the help of a palladium catalyst, water is formed from the created H_2 and the atmospheric O_2.**

Task 16. Microscopy of prepared Gram-smears, observing the micromorphology; read and draw a quick detetction test of *Clostridioides difficile* GDH antigens and toxins A and B.

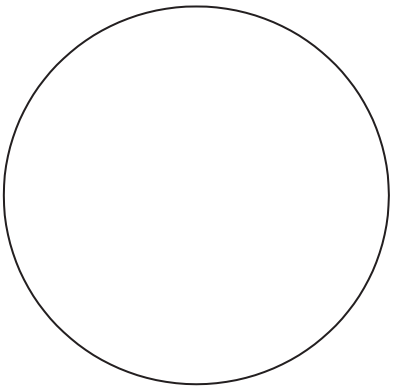
a) *Clostridium* spp. (without spores)



b) *GDH and TOXA, TOXB*

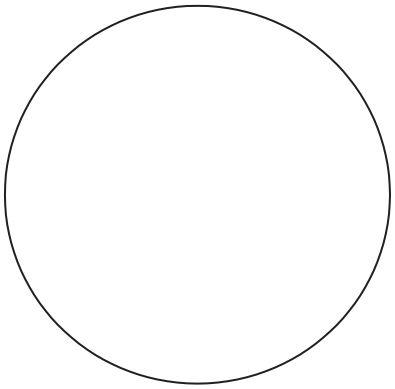


c) *Bacteroides* sp.

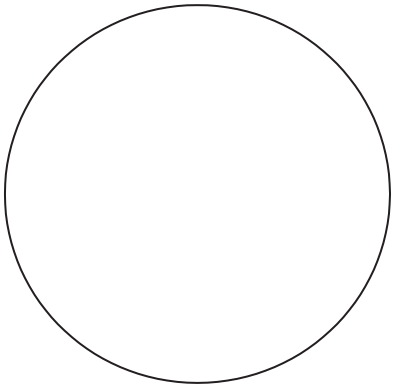


Task 17. Microscopy of prepared Gram-smears, observing the micromorphology on demonstration microscopes

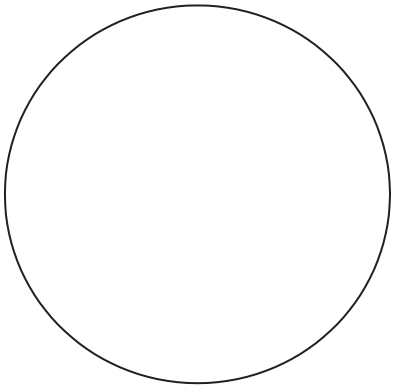
a) *Clostridium* spp. (with spores)



b) *Fusobacterium*

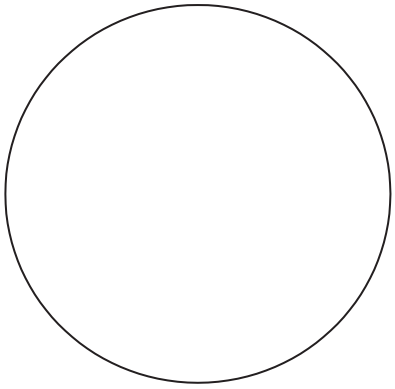


c) *Actinomyces* sp.



Task 18. Colonies description: BA - *Actinomyces* sp.

	Actinomyces sp.
Size	
Shape	
Margin	
Surface	
Hemolysis	



Review questions:

1. *Campylobacter* spp. , *Pseudomonas* spp., and *Neisseria* spp. are oxidase _____.

2. *Helicobacter pylori* can be isolated from _____.

3. Cultivation media for *Vibrio* spp. are_____.

4 Pathogenic *Neisseria* spp. are the causative agents of _____

5. Wound aspirate and tissue sample are _____ samples for bacteriological analysis in anaerobic infections .

6. If the transport of the sample to the laboratory takes more than 15 minutes, the sample should be placed in the anaerobic _____.

7. Strict anaerobes _____ exposure to small amounts of oxygen.

8. Anaerobic microorganisms generally grow_____, so it is necessary to cultivate them _____ h.

9. Susceptibility of anaerobic microorganisms to antimicrobial drugs is determined by _____.

Date: Teacher's signature:

_____ _____

Notes:

PRACTICAL B5

Ivana Goić Barišić, Vanja Kaliterna

***B. anthracis* - presentation of spores and capsule, *B. subtillis* - seeding of spores. *Corynebacterium* - cultivation, staining and microscopy. *Listeria* - culture, microscopy. Ziehl-Neelsen staining. Collection, sending and processing of samples for the isolation of mycobacteria. Cultivation of mycobacteria. Test of resistance to tuberculostatics. *Legionella* - diagnostic procedures. Serological tests for *T. pallidum*. *Mycoplasma*, *Ureaplasma* - culture, *Chlamydia* - diagnostics.**

To successfully master the exercise, it is necessary to study the 11th, 12th, 22nd, 23rd, 24th, 25th and 27th chapters of the textbook Brooks GF, Carroll KC, Butel JS, Morse SA, Mietzner TA, eds. Jawetz, Melnick and Adelbergs Medical Microbiology. 26th ed. New York: McGraw-Hill; 2013.

Genus *Bacillus*

Bacteria of genus *Bacillus* are gram-positive rods that form long chains. They have a spore located in the central part of the rod. They are present in air, water and soil. They do not cause disease, except *B. cereus* that causes food poisoning and *B. anthracis* causes anthrax.

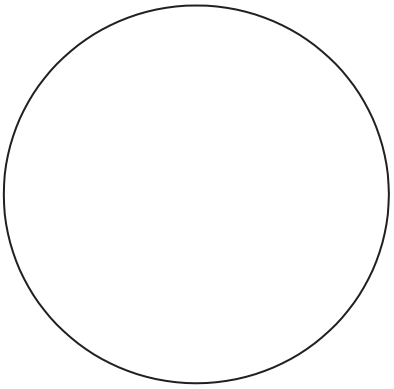
Anthrax is primarily an animal disease. Humans become infected by accidental contact with infected animals and their products or by spores (contact with wool or dust). The disease can develop as cutaneous, pulmonary or intestinal form of anthrax.

Bacterial spores of genus *Bacillus* (*B. subtillis*) are used for biological control of sterilization procedures.

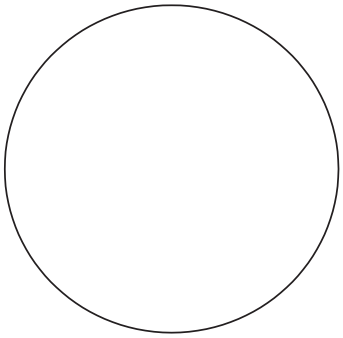
PRACTICAL WORK

Task 1. Make a Gram smear of anthracoid bacilli from BA

Smear description:



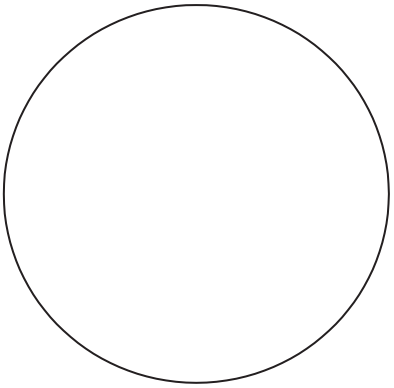
Task 2. Colonies description: BA – anthracoid

Size	
Form	
Margin	
Surface	
Hemolysis	
Pigment	
	

Task 3. Microscopy of prepared smears:

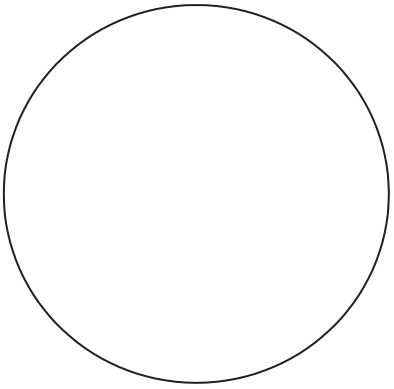
a) *B. anthracis* – metilen blue staining – presentation of bacteria with capsula in mouse blood

Smear description:



b) *B. anthracis* – Gram staining – spore presentation

Smear description:

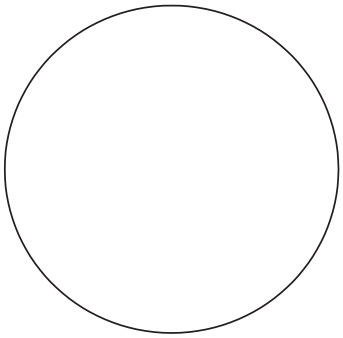


Genus *Corynebacterium*

Corynebacteria are gram-positive rods, thickened at the end, they are colored non-uniform because they contain the metachromatic granules. The majority of species from this genus does not cause diseases and are part of human's physiological flora. *Corynebacterium diphteriae* causes diphtheria in humans. Corynebacteria that do not cause diphtheria are called diphtheroids.

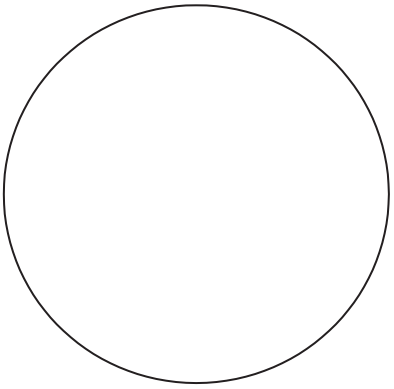
PRACTICAL WORK

Task 4. Colonies description: BA – corynebacteria

Size	
Form	
Margin	
Surface	
Hemolysis	
Pigment	
	

Task 5. Make a Gram smear of diphtheroids from BA.

Smear description:



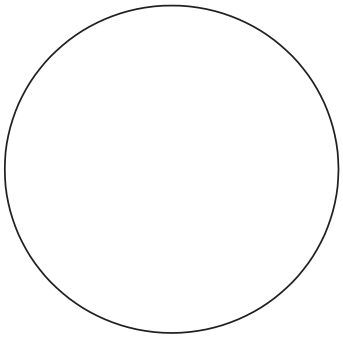
Task 6. On which way corynebacteria toxigenicity can be tested?

Genus *Listeria*

Genus *Listeria* are gram-positive non-sporogenous short bacilli with rounded ends. They are motile at temperature of 22 - 27 °C. The most important species is *Listeria monocytogenes*. It is transmitted through contaminated food (dairy products, vegetables). Risk groups for listeriosis development include pregnant women and newborns (perinatal listeriosis, neonatal sepsis and meningitis of newborns) and immunocompromised patients (bacteremia and meningitis).

PRACTICAL WORK

Task 7. Colonies description: BA – *Listeria monocytogenes*

Size	
Form	
Margin	
Surface	
Hemolysis	
Pigment	
	

Mycobacteria

Mycobacteria are aerobic, non-sporogenic bacilli. Due to the special composition of the cell wall, it is not possible to show them with ordinary staining techniques in microbiology (e.g. Gram staining or methylene blue), but to show mycobacteria special complex methods such as Ziehl-Neelsen (Z-N) staining or staining with fluorochrome dyes (auramine) are used. In Z-N staining, the basic red dye carbol-fuchsin is used, and due to the high content of lipids in the composition of the cell wall, mycobacteria retain their red color even after decolorization with acid alcohol. This is why they are described as acid-alcohol-resistant (acid-resistant) bacteria. As a rule, they grow slowly, their replication time (generation time) is 14-22 hours, so they are described as slow-growing bacteria. *Mycobacterium tuberculosis* causes tuberculosis and is an important human pathogen. *Mycobacterium leprae* causes leprosy. *Mycobacterium avium-intracellulare* (*M. avium complex* or MAC) is the most common representative of non-tuberculous (NTM) mycobacteria. It is an opportunistic pathogen of immunocompromised persons and often causes infections in patients suffering from AIDS.

Mycobacteria mostly cause respiratory infections (pulmonary tuberculosis), and the diagnosis of tuberculosis is made by examining respiratory samples, sputum or respiratory aspirates

and bronchoalveolar lavate. To establish a diagnosis, it is necessary to process such samples in a biosafety cabinet/chest and inoculate them on a special solid medium (Löwenstein-Jensen) and liquid commercial medium, Mycobacteria Growth Indicator Tube (MGIT), in which mycobacteria grow faster than on a solid medium. A direct microscopic smear is made from all respiratory samples, stained according to Ziehl-Neelsen and microscopy under immersion to prove the presence of acid-resistant bacilli. In a liquid medium for the cultivation of mycobacteria, virulent strains create intertwined serpentine cord-like forms and are easily observed in a microscopic preparation by Z-N.

Preparation of sputum smear and Ziehl-Neelsen staining

Smear preparation is done in a **biosafety cabinet/chest!**

Take a small amount of sputum from the sample container with sterile loop, spread it on a glass slide, dry it and fix it on a flame.

1. apply carbol fuchsin to the smear and heat it so that the color slowly evaporates over 5 minutes
2. rinse with tap water
3. rinse the smear with 3% sulfuric acid mixed with 95% alcohol
4. rinse with tap water
5. apply methylene blue and leave for 5 minutes
6. rinse with tap water
7. dry and microscope under immersion

Antituberculosis sensitivity testing (resistance test)

The antituberculosis sensitivity test determines sensitivity/resistance to the most commonly used drugs in the treatment of tuberculosis (rifampicin, isoniazid, ethambutol and pyrazinamide). *M. tuberculosis* sensitivity testing to antituberculosis drugs is performed using the **Canetti proportion method** and molecular methods (proof of resistance genes). The Canetti proportion method is still the standard method for testing the susceptibility of *M. tuberculosis* to the most commonly used antituberculosis drugs. The resistance test is read after four weeks of incubation on a solid L-J medium to which a precisely determined concentration of antituberculosis was added. If the growth of mycobacteria on the medium with the drug is greater than 1% compared to the control medium without antituberculosis, the isolate is resistant to the tested drug.

PRACTICAL WORK

Task 8. State the composition of the cell wall of mycobacteria and emphasize the difference compared to other bacteria**.**

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Task 9. Microscope and draw a Ziehl-Neelsen-stained sputum smear. Acid-resistant bacteria should be observed**.**

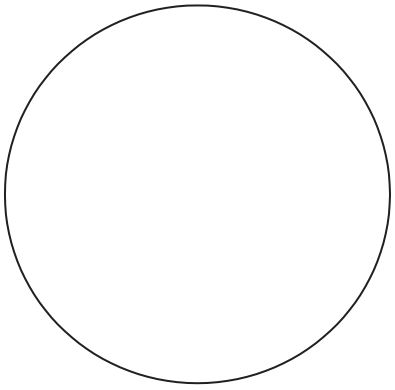
Image description:

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Task 10. Microscope and draw a Ziehl-Neelsen-stained preparation from a liquid medium (MGIT). Specific clusters should be observed (serpentin cord).

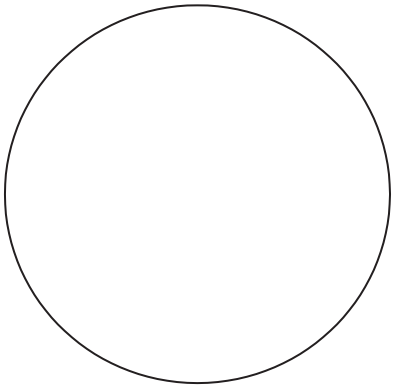
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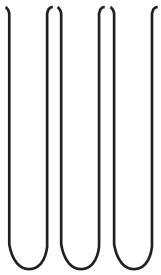


Task 11. Describe the appearance of *Mycobacterium tuberculosis* colonies on Löwenstein-Jensen medium.

Image description:



Task 12. Read the previously prepared *M. tuberculosis* resistance test to antituberculosis drugs. It is necessary to notice the difference in the number of colonies on the media with antituberculotic drugs. The tested antituberculosis drugs are isoniazid, rifampicin and ethambutol.



Genus *Legionella*

Gram-negative bacilli, ubiquitous in warm, moist environment. Immunocompromised people become infected after inhaling bacteria trough aerosol from contaminated showers, air conditioning devices and similar sources, and then develop pulmonary infiltration.

Task 13. Describe the test for legionella antigen detection in urine.

Genus *Treponema*

Treponemeae are slender spiral bacteria, very motile, rotating along their axis. They are exclusively human pathogen. The most important species is *Treponema pallidum*. It is transmitted sexually and conatally. It causes acquired (primary, secondary, tertiary) and conatal syphilis.

Diagnosis is made using serological tests:

a) non-treponemal tests - screening tests for syphilis, flocculation reaction after mixing the patient's serum with cardiolipin antigen (VDRL, *Venereal Disease Research Laboratory*; RPR, *Rapid Plasma Reagin*) and

b) treponemal test - hemagglutination reaction after mixing the patient's serum with treponema antigen adsorbed on the surface of erythrocytes (TPHA, *Treponema Pallidum Hemagglutination*). It is used to confirm positive non-treponemal tests.

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Genus *Mycoplasma*, genus *Ureaplasma*

Mycoplasmas are the smallest free-living bacteria. They do not have a cell wall and do not stain according to Gram staining procedure. The species that cause disease in humans are *Mycoplasma pneumoniae*, *Mycoplasma genitalium*, *Mycoplasma hominis* and *Ureaplasma urealyticum*. They cause diseases of the respiratory and urogenital system. Diagnostic methods for proving these bacteria are different. They are proven by molecular methods (*M. genitalium*), serological methods (*M. pneumoniae*) or commercial tests (*M. hominis* and *U. urealyticum)*.

Task 14. Reading of commercial tests for *M. hominis* and *U. urealyticum*.

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Chlamydiae

Chlamydiae are nonmotile gram-negative bacteria, obligate intracellular pathogens. The most important species is *Chlamydia trachomatis*. It causes diseases of genital system and inclusion conjunctivitis. Diagnostics is done with nucleic acid amplification tests (NAATs).

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_____

_____

Review questions:

1. What types of spores are used during the biological control of the sterilization process?
2. What are the microbiological characteristics of the causative agent of diphtheria?
3. For which groups of people is *Listeria* important as a cause of disease?
4. Describe the appearance of *M. tuberculosis* bacilli after Ziehl-Neelsen staining.
5. List the drugs being tested and used in the treatment of infection caused by *M. tuberculosis*.
6. How long does the cultivation of the causative agent of tuberculosis last?
7. List the media used to isolate the causative agent of tuberculosis.
8. How is legionella transmitted?
9. What is the confirmatory test for evidence of the infection with *Treponema pallidum*?
10. Why can't urogenital mycoplasmas be stained by Gram?
11. Which test is the "gold standard" for proof of chlamydial infection?

Date: Teacher's signature:

_____ _____

Notes:

PRACTICAL M

Anita Novak

Yeast and molds – macro- and micro- morphology. Cultivation and identification of medically important fungi. Diagnostics of dermatomycosis.

In order to successfully master this practical, the student should first study
Chapter 45 from the book Jawetz, Melnick and Adelberg. Medical microbiology, 26th Edition. McGraw Hill Lange; 2013.

INTRODUCTION

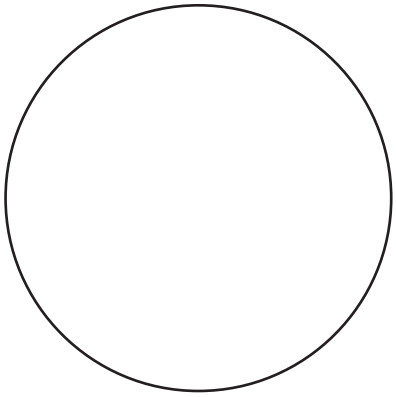
Unlike bacteria, fungi are **eukaryotic** organisms. They are obligate or facultative aerobes.
In general, fungi are divided into two basic groups based on their shape – **yeasts** (unicellular fungi) and **molds** (multicellular fungi).
Infections caused by fungi are called **mycoses**.
The natural habitat of most pathogenic fungi is water and soil, so in these cases, **exogenous** human infections occure (e.g. cryptococcosis, aspergillosis and dermatophytosis).
However, the most common fungal infections in humans are of **endogenous** origin, i.e. they are caused by fungi that are part of the microbiota of various human organ systems (e.g. *Candida*). They are considered **opportunistic** pathogens because they cause infections in high-risk groups of people (immunocompromised, patients with chronic and malignant diseases, people receiving antibiotics, chemotherapy or immunosuppressive therapy).
Endemic mycoses are caused by dimorphic fungi, which are the primary pathogens and are associated with certain geographical areas.
Dermatophytoses are skin mycoses caused by fungi (molds) that can infect only keratinized tissues (skin, hair and nails). These fungi are divided into three genera, ***Microsporum***, ***Trichophyton*** and ***Epidermophyton***. They differ according to their natural habitat (zoophilic, anthropophilic and geophilic species) and according to the appearance of grown colonies on selective media (macro morphology) and microscopic morphology.
Most fungi that cause infections in humans do not require special cultivation conditions. Although they can grow on media for the cultivation of bacteria (e.g. BA), we most often use selective media for the isolation of fungi (solid and liquid), in order to speed up the time of isolation and identification. The most commonly used nutrient media are **Sabouraud agar** (SA) and **Sabouraud broth** (with or without the addition of inhibitors for the growth of saprophytic molds).
All *Candida* species grow very similarly on SA and we cannot distinguish between colonies of different species. For the identification of *Candida* species, it is necessary to do a germination test, sugar assimilation and fermentation tests, subcultivation to a nutrients deficient agar (e.g. corn agar) or use new identification methods, such as MALDI-TOF MS.
Molds (including dermatophytes) form characteristic colonies on Sabouraud agar, so their identification is based on the description of the macro (appearance of the front and back of the colony) and micromorphology (characteristic structures, e.g. micro and macro conidia in a microscopic preparation) of the grown colonies.

PRACTICAL WORK

Task 1. Microscopy of the prepared germination tube test (native preparation of the *C. albicans* culture mixed with serum).

Objective: to recognize the blastoconidial germ tube.

Procedure: microscope under low (10 x) and medium (40 x) magnification of the objective, observe the germinal tubules and draw what is seen.

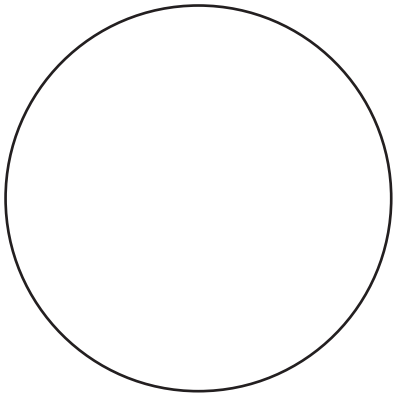


C. albicans – positive germ tube test

Task 2. Microscopy of the prepared mixed culture (*Candida albicans* and *Staphylococcus aureus*) stained with Gram staining.

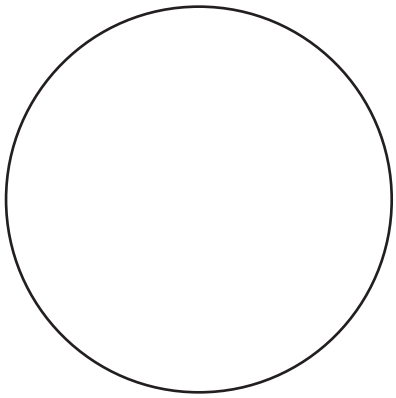
Objective: to observe the difference in the morphology and size of gram-positive cocci and blastoconidia.

Procedure: microscope the preparation under high magnification (1000 x) of the microscope, with an immersion objective (100 x) and draw what is seen.



Gram preparation of mixed culture *S. aureus* and *C. albicans*

Task 3. Microscopy of *Cryptococcus neoformans* stained with India ink and Gram.

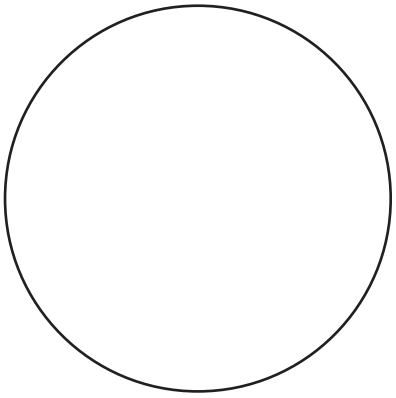


Cryptococcus neoformans

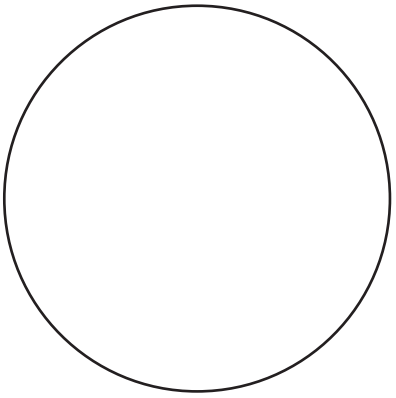
Task 4. a. Microscopy of *Penicillium* sp. and *Aspergillus* sp. – native preparations made with lactophenol cotton blue.

Objective: to recognize the characteristic structures of these genera of molds.

Procedure: microscope under low (10 x) and medium (40 x) magnification of the objective and draw what is seen.

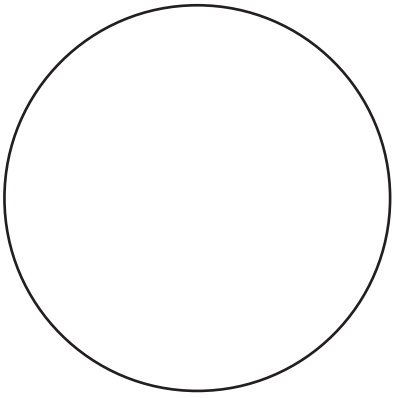


Penicillium sp.

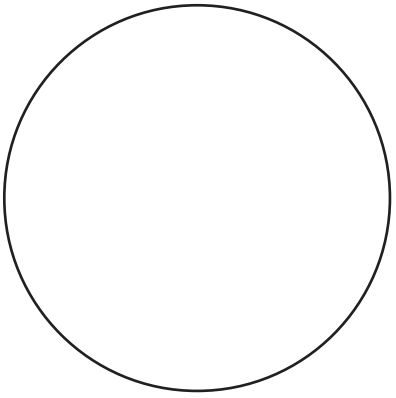


Aspergillus sp.

Task 4. b. Describe and draw the colonies of *Penicillium* sp. and *Apergillus* sp. on Sabouraud agar.



Penicillium sp.



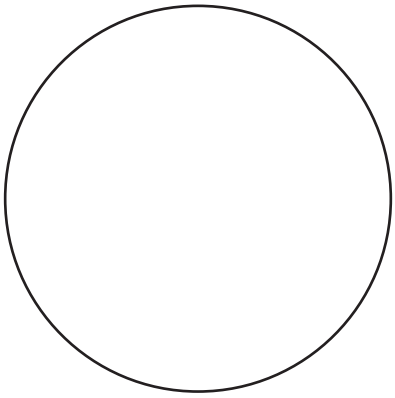
Aspergillus sp.

COLONIES	*Penicillium* sp	*Aspergillus* sp.
SIZE		
SHAPE		
EDGE		
SURFACE		

Task 5. Microscopy of the direct preparation of sputum (Gram staining).

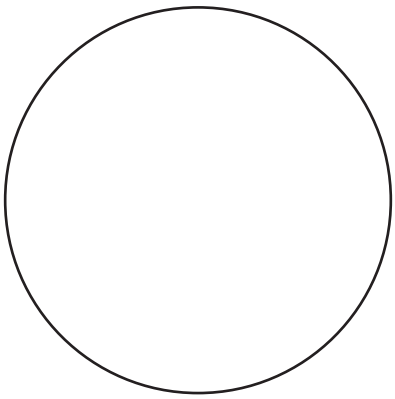
Objective: to recognize blastoconidia, pseudohyphae and hyphae of yeasts in a direct microscopic preparation stained by Gram.

Procedure: microscope and draw what is seen.



C. albicans, sputum, Gram staining

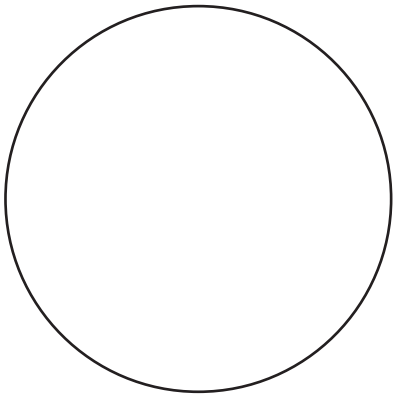
Task 6. Describe and draw the colonies of *C. albicans* on BA.



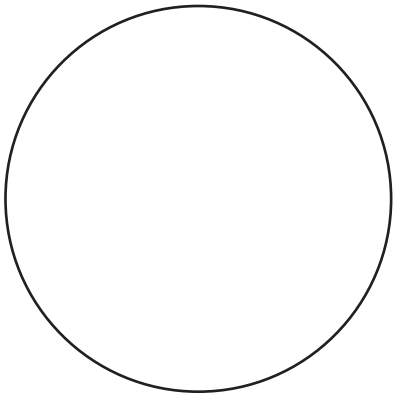
C. albicans

COLONIES	*C. albicans*
SIZE	
SHAPE	
EDGE	
SURFACE	
PIGMENT	

Task 7. Describe and draw the colonies of *C. albicans* and *Cryptococcus neoformans* on Sabouraud agar



C. albicans



Cryptococcus neoformans

COLONIES	*C. albicans*	*Cryptococcus neoformans*
SIZE		
SHAPE		
EDGE		
SURFACE		

Task 8. Observe dermatophyte colonies on Sabouraud agar with cycloheximide and *C. albicans* (green) and *C. parapsilosis* (pink) on a chromogenic medium.

Review questions

1. Describe the basic structure of yeasts and molds.
2. Which clinical samples are suitable for mycology diagnosis?
3. How to cultivate fungi (atmosphere, temperature and incubation time)?
4. What tests do we use to identify yeasts?
5. How to identify molds?

Date: Teacher's signature:

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Notes:

Notes:

PRACTICAL P1

Katarina Šiško Kraljević

Diagnosis of toxoplasmosis, leishmaniasis and malaria. Giemsa-Romanowsky-stained biopsy specimen, thick and thin blood films. Preparing, staining and microscopy of specimen (recognition of parasitic forms). Serological diagnostic testing.

To successfully master the exercise, you need to consult 46th chapter of the textbook Brooks GF, Carroll KC, Butel JS, Morse SA, Mietzner TA, eds. Jawetz, Melnick and Adelbergs Medical Microbiology. 26th ed. New York: McGraw-Hill; 2013.

INTRODUCTION

Life cycle, pathogenicity and epidemiology of blood-tissue parasites: *Toxoplasma gondii, Plasmodium* spp. and *Leishmania* spp.

Giemsa-Romanowski staining: the working dye solution is prepared immediately before use, by adding 2 drops of the Giemsa stock solution to a test tube with 2 ml of pH-neutral, distilled water and allowed to diffuse in it. The test tubes must not be shaken, because the stain is unstable, it easily deposits and loses its properties. The diluted Giemsa stain is poured over the air-dried and fixed slides and left for 20-25 minutes. Rinsing, drying and microscopy is the same as for other stained slides. Microscopically, in Giemsa-Romanowski stained slides, the cytoplasm of blood protozoa stains blue, and the nuclear material (nuclei, kinetoplasts) stains red to purple-red.

1. Diagnostics of toxoplasmosis

Task 1.1 Complete the sentences and answer the questions.

The only known definitive hosts for *Toxoplasma gondii* are_____, and humans serve as _____ hosts.

Slender, crescentic, rapidly multiplying types of parasites called _____ are responsible for acute phase of disease. Later, in chronic infections slow-growing, shorter forms of parasites called _____ develop, and form cysts in different tissues (e.g. brain, eye, and hearth).

Humans can become infected by_____

_____.

What is the clinical picture of toxoplasmosis? _____

_____.

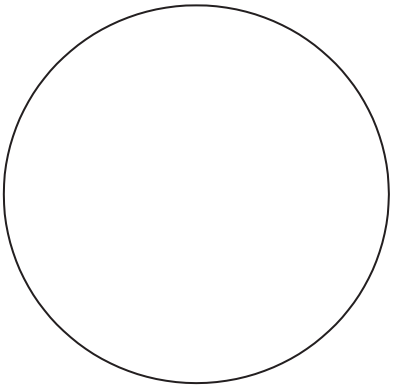
Which method is most often used to diagnose toxoplasmosis? _____

_____.

List all the methods that may be used in the diagnostics of toxoplasmosis: _____

_____.

Task 1.2. Draw and describe *Toxoplasma* tachyzoites in Giemsa-Romanowski stained smears of mouse ascites.



Task 1.3. Interpret the toxoplasmosis ELISA test results.

IgM	IgG	Interpretation
-	-	
+	-	
+	+	
-	+	

Task 1.4. Read and interpret the toxoplasmosis ELISA test results.

Patient No	Test result	Interpretation

2. Diagnostics of leishmaniasis

Task 2.1. Complete the sentences and answer the questions.

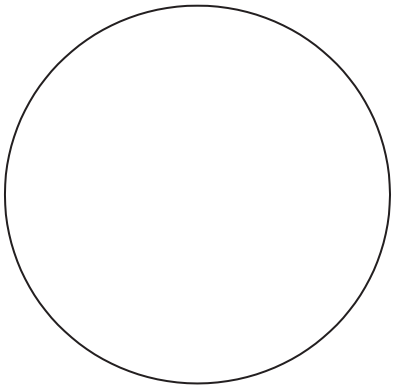
Leishmaniasis is a vector-borne disease, transmitted by the bites of infected female _____.

Zoophilic species *Leishmania* _____ is endemically present on the Croatian coast. It is responsible for _____ and _____ leismaniasis. Reservoir hosts for this Mediterranean species are _____.

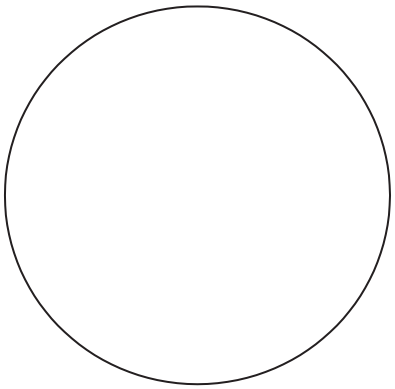
Spherical to ovoid formes of parasites called _____ may be found within macrophages in Giemsa stained smears of biopsy samples (i.e. bone marrow, lymph node or spleen aspirates) and specimens of cutaneous lesions.

Leishmania parasites may be cultivated on rich biphasic (blood agar and liquid phase) media (_____). After few days of incubation at 25-27°C _____ forms of parasite will start to appear in the liquid phase.

Task 2.2. Draw and describe *Leishmania* parasites in the Giemsa-stained bone marrow smear. Observe the intracellular location of the parasite, pay attention to the nuclei and kinetoplasts.



Task 2.3. Stain the air-dried and methanol fixed smears of the liquid phase of medium for cultivation of *Leishmania,* with diluted Giemsa stain. Examine using oil-immersion (1000 x). Observe, draw and describe.



3. Malaria diagnostics

Task 3.1. Complete the sentences and answer the questions.

There are more then 150 different species of *Plasmodium* which infect various species of vertebrates. Four species are responsible for human malaria: *Plasmodium*_____, *Plasmodium*_____, *Plasmodium*_____ and *Plasmodium*_____.

Definitive host for *Plasmodium* parasites are _____.

The first phase of *Plasmodium* growth in humans (exoerythrocytic phase) takes place in

_____. Some species (e.g., *P.* _____, *P.*_____) can establish a dormant hepatic phase (called hypnozoites, or sleeping forms). This viable plasmodia can lead to the relapse of infections (relapsing malaria).

Clinical manifestations of the disease are result of asexual replication of the plasmodia in the ___ _____. The intracellular parasite initialy appears as a _____ shaped trophozoite, which enlarges and becomes irregular in outline (_____ stage). After a few hours, nuclear division occurs producing the immature _____. Multinucleated cells subsequently rupture releasing merozoites that invade other RBCs. Cycles of infection, replication

and cell lysis develop periodically every _____ h (*P.*_____, *P.*_____, *P.*_____) or _____h (*P.*_____).

Some parasites differentiate into sexual erythrocytic stages called _____.

What is a 'gold standard' method in malaria diagnostics?_____.

List other procedures useful in the diagnosis of malaria:_____

_____.

Task 3.2. Briefly describe the thick and thin blood smear procedure.

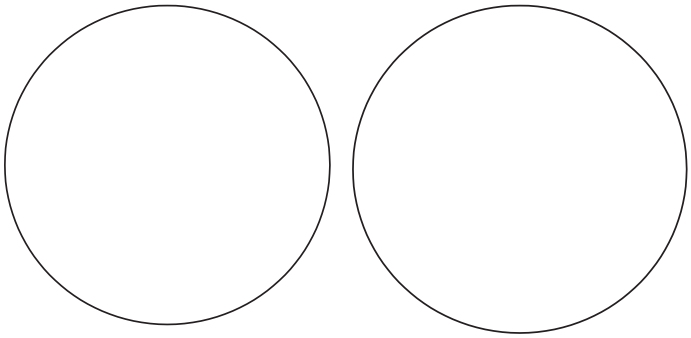
_____

_____

_____

_____

_____



Task 3.3. Complete the table with text and drawing. Observe the characteristic morphology of different stages of *Plasmodium* parasites.

	P. falciparum	*P. vivax*	*P. ovale*	*P. malariae*
Size and shape of infected RBC				
Ring-form stage				
Young trophozoite (amoeboid) stage				
Mature schizonts				
Gametocytes				

Task 3.4. Thin film microscopy. Draw the characteristic stage of the parasite and identify which species of *Plasmodium* is involved.

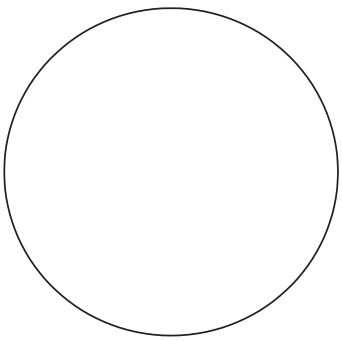
_____

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_____

_____



4. Medically important arthropods

Task 4.1. Using a magnifying glass and a microscope, study some medically significant arthropods.

- *Sarcoptes scabiei* - adult
- *Demodex folliculorum*
- *Pediculus humanus* var. *capitis* – egg and adult
- *Pediculus humanus* var. *vestimenti* – adult
- *Phthirius pubis* – adult
- *Phlebotomus* sp.
- *Anopheles* sp.

Review questions:

1. How do we interpret high avidity anti-toxoplasma IgG test result?
2. How is congenital toxoplasmosis diagnosed?
3. Which diagnostic methods are used for cutaneous, and which for visceral leishmaniasis diagnosis?
4. Which *Plasmodium* species is most dangerous, and why?
5. Are there vectors for malaria present in Croatia, Italy and other Mediterranean European countries?

Date: Teacher's signature:

_____ _____

Notes:

PRACTICAL P2

Katarina Šiško Kraljević

Diagnostics of intestinal parasitosis. Microscopy of direct wet mount, Lugol's iodine and concentration method (MIFC). Micromorphology of protozoan cysts, eggs and larval helmints. Recognition of adult forms. Graham's anal swab. Diagnostics of echinococcosis and trichinellosis.

INTRODUCTION:

To successfully master the exercise, you need to consult 46th chapter of the textbook Brooks GF, Carroll KC, Butel JS, Morse SA, Mietzner TA, eds. Jawetz, Melnick and Adelbergs Medical Microbiology. 26th ed. New York: McGraw-Hill; 2013.

Required prior knowledge:

Life cycle, pathogenicity and epidemiology of the most common protozoan (*Trichomonas vaginalis, Giardia duodenalis, Entamoeba histolytica, Cryptosporidium* spp.) and helminth parasites (*Echinococcus granulosus, Trichinella spiralis, Enterobius vermicularis, Ascaris lumbricoides, Trichuris trichiura, Taenia* spp.).

PRACTICAL WORK

1. Intestinal parasite diagnostics

Task 1.1 Name the specimens that are appropriate for the diagnosis of intestinal parasites.

_____

_____

_____

_____

Faecal specimens



Faecal specimens should be collected in a sterile leak proof container with a spoon or spatula fixed on lid (Figure to the right). It is optimal to fill in 1/4-1/2 of the receptacle with a sample. The samples should not be mixed with soil, water or urine. Free living organisms can be found in water and soil, and urine destroys protozoan trophozoites and may encourage the release of larvae from helminth eggs. Presence of certain substances (i.e. barium or bismuth, antimicrobial agents, and gallbladder dyes) will make the stool specimens unsatisfactory for microscopic examination. The specimens should be collected before administration, or collection must be delayed (1-3 weeks). Due to the fact that parasitic organisms are passed intermittently and in fluctuating numbers examination of minimum three stool specimens collected over a 7 to 10 day period is recommended. Liquid (diarrheic) stool specimens (which are more likely to contain trophozoites) should be examined within 30 minutes of passage, soft specimens should be examined within 2h and formed stool specimens may be stored at 4°C for up to one day.

Task 1.2. Name the procedures used in the laboratory diagnosis of parasitic infections of the intestinal tract.

1. _____

2. _____

3. _____

4. _____

The first step in diagnostics is to macroscopically examine stool specimens for consistency and for the presence of blood, mucus, adult worms and proglottids.

Task 1.3. Study and briefly describe the adult forms of intestinal worms. Indicate the site of parasitism and infective stage for humans.

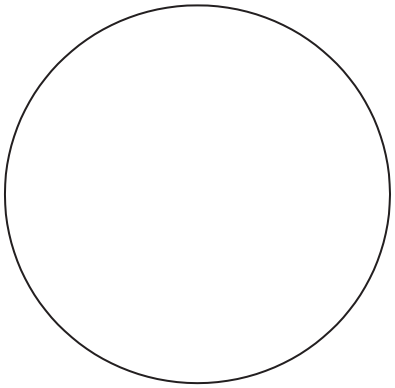
Organism	Description	Site of parasitism	Infective stage
1.			
2.			
3.			

Microscopic examination of faecal specimens is the fundamental test in the diagnostics of intestinal parasites. Complete ova and parasite examination consists of direct saline and iodine

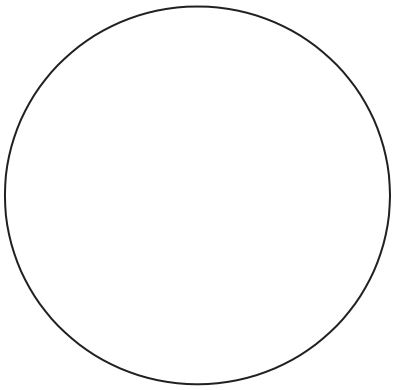
(Lugol's solution) wet mounts, and preparations obtained by concentration methods, such as MIFC (Merthiolate Iodine Formaldehyde Concentration), FEAS (Formalin- Ethyl Acetate Sedimentation) or flotation with 33 % zinc sulphate solution. Permanently stained smears (trichrome or iron hematoxylin) are prepared for a more detailed study of cysts and trophozoites morphology, and an accurate identification of intestinal protozoa. Modified acid-fast staining methods (Ziehl-Neelsen) are used for detection of acid-fast *Cryptosporidium* oocysts.

Task 1.4. Draw representative sketches of the intestinal parasites in the examined microscopic preparations. Describe the key distinguishing microscopic characteristic.

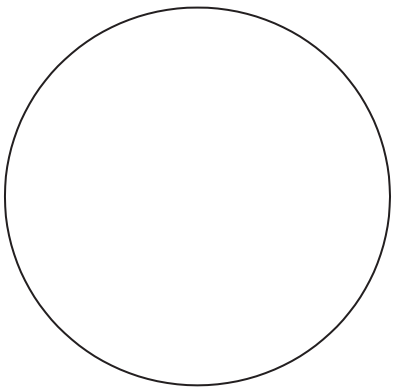
Ascaris lumbricoides – eggs (MIFC)



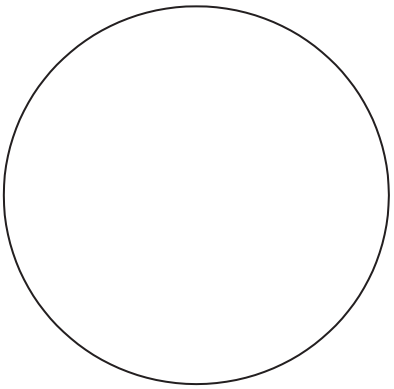
Trichuris trichiura – egg (MIFC)



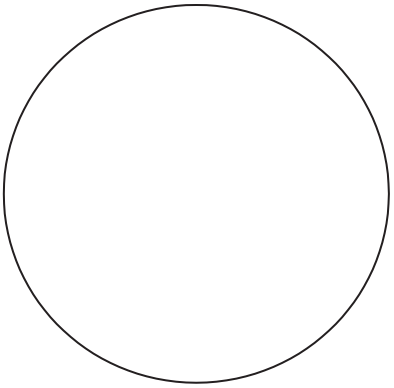
Taenia spp. – egg (MIFC)



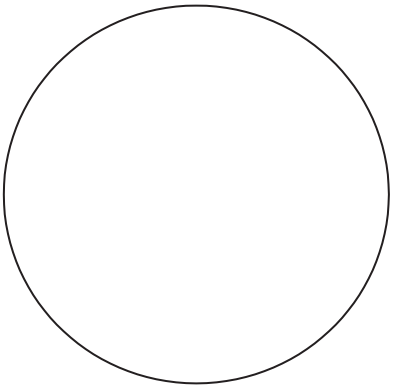
Giardia duodenalis – cyst (MIFC)



Entamoeba histolytica/dispar, Entamoeba coli – cyst (MIFC)



Cryptosporidium spp. – oocyst (Ziehl-Neelsen)



For a limited number of most common intestinal parasites, antigen detection tests are also available: enzymatic immunoassays (EIA) to distinguish *Entamoeba histolytica* from non-pathogenic amoebas, immunochromatographic tests (ICT) for the detection of *Cryptosporidium* spp., *Giardia duodenalis* and *Entamoeba histolytica*/*dispar*, and the direct immunofluorescent tests for the detection of *Cryptosporidium* spp. and *Giardia duodenalis* antigens.

Molecular techniques can be used for research as well as for routine diagnosis of some parasites. PCR tests for the detection of *Cryptosporidium* spp., *Giardia duodenalis*, *Entamoeba histolytica*, *Dientamoeba fragilis* and *Blastocystis* spp. are available.

Task 1.5. Specify the advantages and the disadvantages of EIA, ICT and PCR in comparison with the microscopic examination of the stool.

Task 1.6. Complete sentences.

The diagnostic test of choice for demonstrating pinworm eggs is _____, since _____ eggs are only occasionally recovered in stool samples (5 % infected). The sample is best collected _____, before the patient takes a shower or goes to the bathroom. Material required for the procedure: microscopic slides, 5-7 cm of transparent adhesive cellulose tape ('Scotch tape') and protective gloves. The adhesive tape is applied sticky side down to the skin around _____. The strip is then carefully transferred to a glass slide (its surface should be as flat as possible). The slides are examined _____ _____.

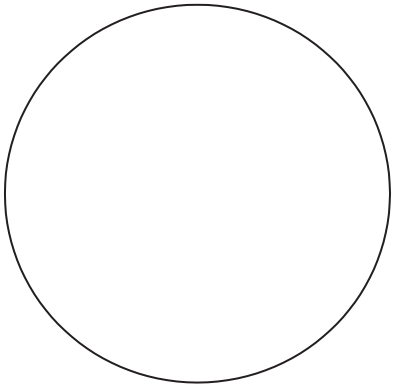
Enterobius vermicularis – egg

_____

_____

_____

_____



2. Parasitological examination of urogenital samples.

Task 2.1. Complete sentences.

Trichomonas vaginalis is_____ that possess only a _____ stage. It is usually transmitted by _____. Appropriate specimens for the diagnosis are _____ _____. The specimens should be transported at _____temperature. *Trichomonas* can be grown on _____. Beside microscopy and culture other diagnostic tests like _____ can also be used.

Task 2.2. Draw and describe.

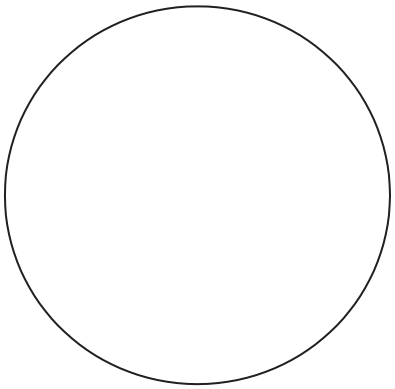
Trichomonas vaginalis – Giemsa

_____

_____

_____

_____



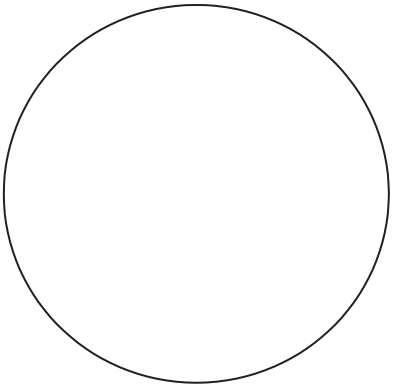
3. Trihinelosis, ehinococcosis and cysticercosis diagnostics

Task 3.1. Fill in the table.

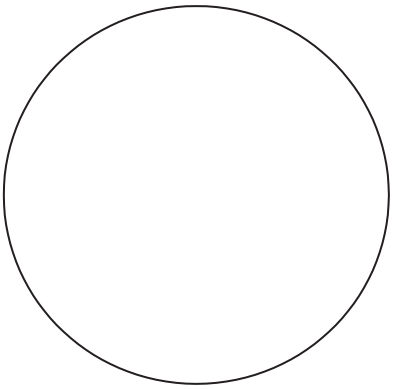
Disease	Etiologic agent	Infective stage	Diagnostics
Trihinelosis			
Ehinococcosis			
Cysticercosis			

Task 3.2. Observe, draw and describe.

Hydatid sand - protoscolex



Trihinoscopy - larvae *T. spiralis*



Review questions:

1. For which tapeworm species humans can serve as a final and an intermediate host?
2. What are distinguishing morphologic characteristics of *Entamoeba histolytica* compared to non-pathogenic amoebas?
3. Name the protozoan species that can cause outbreaks linked to contamination of the municipal water supply.
4. For which disease cultivation of the parasite on enriched media is routinely used?
5. Describe the structure of hydatid cyst.

Date: Teacher's signature:

Notes:

Notes:

PRACTICAL V1

Irena Tabain

Laboratory diagnostics of viral infections-direct methods. Clinical specimens for direct laboratory diagnostics, transport and storage. Virus isolation methods.

INTRODUCTION

Samples for direct detection methods in laboratory diagnostics

In general, samples for direct methods for laboratory diagnostics should be collected from the portal of entry and/or the primary site of viral replication. Specimen should be collected as soon as possible after the onset of illness, before the virus ceased to shed, preferably within 3-7 days after the symptoms occur.

Clinical samples for virus detection could be primary sterile samples (e.g. cerebrospinal fluid, blood) or nonsterile samples (e.g. nasopharyngeal swab or aspirate, stool).

Clinical samples: stool, urine, cerebrospinal fluid, nasopharyngeal swab or aspirate, skin or mucosal lesion swab, blood, tissue. All specimens should be placed in sterile container/vial. Swabs for virus laboratory diagnostics should be placed in viral transport media. Deliver all specimens to the laboratory as soon as possible after collection, since loss of infectivity occurs over time. If immediate delivery is not possible, refrigerate specimens, place them on wet ice or use a cold pack to keep them cool. Loss of viability is slower at low temperatures (2-8°C). In case the sample will not be processed within 48-72 hours, the sample should be stored at -20°C, or -70°C.

Direct methods for laboratory diagnostics of virus diseases

a. Virus detection

i. Electron microscopy or immunoelectron microscopy-essential tool to analyse virus structure. This method allows detection of viruses which are difficult to grow in cell culture, but can be detected directly in clinical sample.

ii. Antigen detection

1. Immunoflourescence assay-antigen detection directly in the clinical sample by using monoclonal fluorescently labelled antibodies.
2. Immunochromatography -immunological reaction carried out on the chromatographic paper intended to detect the presence (or absence) of a target antigen. The antigen in the sample forms an immunocomplex with the labelled antibody which generates a coloured line.
3. Latex agglutination - agglutination is observed when a sample containing the specific antigen is mixed with an antibody which is coated on the surface of latex particles. Agglutination is the reaction between a particulate antigen and an antibody which results in visible clumping.
4. *Enzyme ImmunoAssay (EIA)* - general principle is based on the binding of conjugated enzyme molecule with specific antibodies to detect and quantify the presence of antigens in the test sample. Reaction produce a visible coloured product which can be determined by using spectrophotometer.

iii. Molecular detection (RNA or DNA)

1. Nucleic acid amplification test methods (polymerase chain reaction/PCR/ reverse transcription PCR /RT-PCR/ loop-mediated isothermal amplification/LAMP/)
2. Genome sequencing - is the process of determining the nucleic acid **sequence** – the order of nucleotides in DNA. Methods can be used to analyse whole genome (Whole Genome Sequencing) or focus on regions of interest.

   DNA sequencing methods have different characteristics based on read-length, speed, accuracy, throughput, cost, as well as the automation of sample handling and preparation. DNA sequencing methods can be divided in several generations: first generation is based on enzymic analysis (e.g. Sanger method) and other chemical methods; second generation is based on pyrosequencing method-DNA sequencing by the detection of released pyrophosphateor without electrophoresis (e.g. Illumina system) and the third generation is based on methods that in real time sequence single molecules using nanopore.

b. Virus isolation

i. Laboratory animals

ii. Embryonated eggs- virus isolation, antigen and vaccine production.

Virus can be cultivated on different sites of an embryonated egg like chorioallantoic membrane, amniotic or allantoic cavity and yolk sac. Candling eggs should be perform before inoculation to check if egg is fertilized and the embryo alive. After inoculation and incubation, collect the inoculated parts for the virus identification. Several methods can be used for virus identification like haemadsorption, haemagglutination, haemagglutination inhibition, antigen detection or molecular methods.

iii. Cell culture-virus isolation

In vitro system for virus isolation contains cell carrier (flask or tube), cell culture media for growth and maintenance of cells and cells (e.g. epithelial or fibroblasts). Cell cultures can be primary which are capable to grow for limited time or continuous which can be serially cultured indefinitely (e.g. HeLa cells).

The cells may be of human or animal origin.

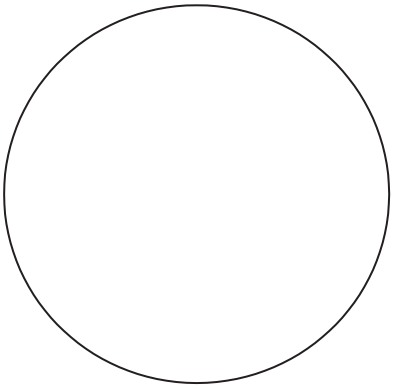
During the viral replication in cell culture the viruses may cause distinguish signs which implicates the presence of virus in the cells like cytopathic effect (CPE) or inclusion bodies.

Different methods can be used for detection and identification of the virus in the cells e.g. antigen detection or molecular methods.

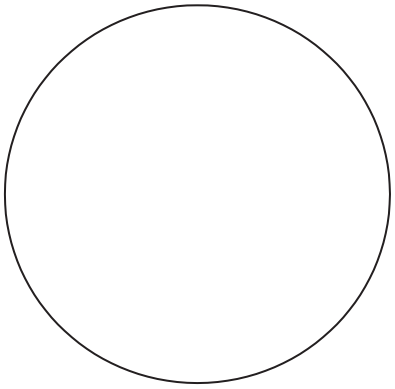
PRACTICAL WORK

Task 1. Draw and describe cytopathic effect (CPE):

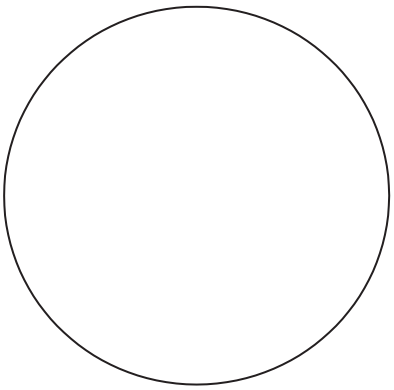
1. Uninoculated cell culture



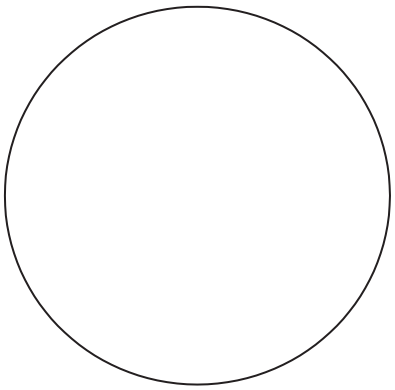
2. Enterovirus induced CPE

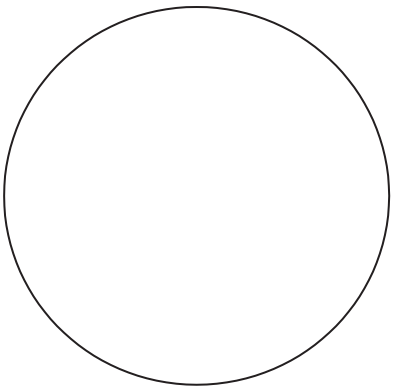


3. Respiratory syncytial virus CPE

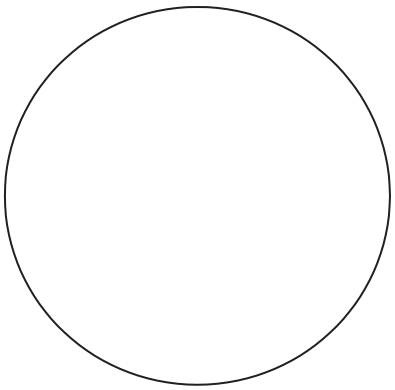


4. Adenovirus CPE

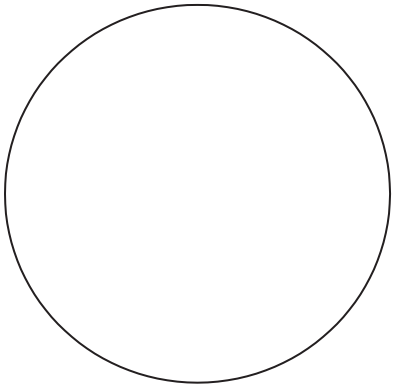




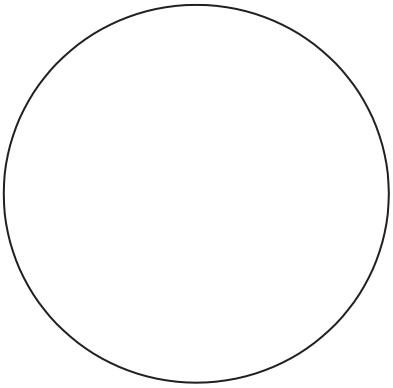
5. Herpes simplex virus CPE



6. Varicella-zoster virus CPE

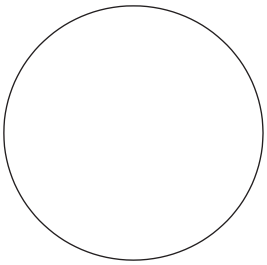


7. Inclusion bodies of cytomegalovirus

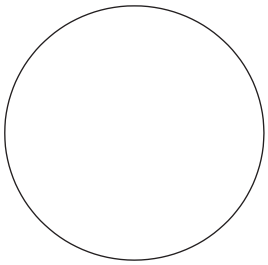


8. Inclusion bodies of rabies virus

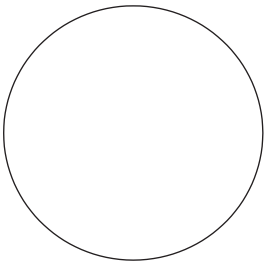
9. Cervical smear



10. Morbili



11. Parotitis



Task 2. Rapid antigen test – antigen detection in the sample

Put the sample (stool or swab) into the sampling tube with the extraction solution, secure tightly. Put sample (swab or stool) into the extraction tube. Close the extraction tube with the cap. Add few drops of mixed liquid from the extraction tube to the sample well on the test cassette.

Draw and describe the result:

Review questions:

1. How should the samples for the direct virus laboratory diagnostics be stored and transported?
2. Which methods are used for the virus isolation?
3. What types of cell cultures are used for the virus isolation?
4. What types of changes the virus produce during the replication in the cell culture?
5. Describe the typical cytomegalovirus CPE.

Date: Teacher's signature:

_____ _____

Notes:

PRACTICAL V2

Marija Tonkić

Methods of indirect diagnostics of infectious diseases - serology.

INTRODUCTION

The use of serological methods in the diagnostics of infectious diseases usually involves the determination of the host's humoral immune response, i.e. the production of specific antibodies of different classes (IgA, IgM, IgG) to the presence of a specific infectious agent. Demonstration of antibodies is an indirect diagnostic method, in contrast to, for example, cultivation, which belongs to direct diagnostic methods. However, it is not possible to isolate all the causative agents of infectious diseases. For those that can be cultivated, isolation is usually possible in the acute phase of the disease and unsuccessful in the subacute or convalescent phase. The conditions for the isolation of the causative agent can vary greatly, and the isolation and identification of the causative agent itself requires a certain amount of time. The result of the cultivation of the causative agent (except viruses) may be falsely negative after taking antibiotic therapy. Cultivation is not a method that can retrospectively establish a diagnosis in the case of complications occurring a few weeks or later after the onset of the disease. On the other hand, the advantage of cultivation is that it allows determining the antimicrobial sensitivity of bacterial agents.

Serological tests are usually negative in the early acute phase of the disease and positive in the subacute or convalescent phase. Most serological tests can be performed relatively easily in one laboratory, the result is quickly available and does not depend on previous antibiotic treatment. One serum sample can be tested for multiple pathogens, and serological tests also enable retrospective diagnosis.

Serological methods enable the proof of infection (acute, chronic,...), based on the determination of the dynamics of antibody formation in the serum. In addition, they are used to determine the immune status of, e.g., patients who need a transplant or pregnant women, and those people who, due to the nature of their work, are more exposed to the risk of getting a certain infection (healthcare workers).

The sample for serological testing is serum extracted from the patient's blood sample. The blood sample is taken on an empty stomach, by venipuncture of the cubital vein, in aseptic conditions, in a test tube without added anticoagulant. The amount of blood for adult patients is 8 to 10 ml, and 3 to 4 ml for children. After collection, the tube with the blood sample is left for 20 to 30 minutes at room temperature to separate the coagulum (clot), which is then removed, and the remaining part is centrifuged (3000 rpm/5-10 minutes) to separate the serum. The separated serum is placed in a new test tube and stored at 2-8°C if the test is performed within a few days. If testing is delayed, the serum is frozen, i.e. stored at temp. -20°C. The serum must not be thawed and then re-frozen, as this affects the level of antibodies that may be present. It is not acceptable to test haemolysed, icteric or lipemic serum.

Some serological reactions can be used to determine total antibodies (e.g. neutralization test, complement fixation test -CFT), while using ELISA (*Enzyme Linked Immunosorbent Assay*) or indirect immunofluorescence test (IFA, *Indirect Fluorescent Antibody Assay*) it is possible to determine certain classes of antibodies.

The most commonly used serological reactions and their basic principle are:

- **agglutination** - antigens are formed, insoluble particles
- **hemagglutination** – agglutination of erythrocytes occurs
- **precipitation** – soluble antigen is used
- **complement fixation test** (CFT) - erythrocytes serve as an indicator
- **neutralization** - inactivation of toxins or viruses
- **fluorescent techniques** - indicator antibodies are labeled with a fluorescent dye
- **ELISA** - indicator antibodies are labeled with an enzyme

How long it will be possible to demonstrate antibodies in the serum after infection depends on the characteristics of the microorganism itself, its possible persistence in the body, and the sensitivity of the serological test used. The rule is that, in the case of primary infection, when there is the first contact with an unknown antigen, IgM class antibodies first appear and are present in the serum for up to several months. IgG antibodies appear somewhat later than IgM antibodies, but remain present in low titers lifelong. Reinfection with the same agent enhances and accelerates the immune response and leads to an increase in the titer of IgG antibodies.

For serological testing, it would be ideal to sample two sera from each patient, **1st** or **acute serum** and **2nd**. or **convalescent (paired) serum**. The first serum should be taken as early as possible at the beginning of the disease, no later than the end of the first week. The second serum should be sampled 2-3 weeks after taking the first serum. Both serums should be tested simultaneously, with the same serological test, and determine the antibody titer in each serum and the dynamics of the titer when the titers of the first and secnd serum are compared. Serial double dilutions are made from the serum samples and at the end, as a result of the serological test, the so-called antibody titer is obtained.

Antibody titer is the reciprocal value of the highest dilution of the patient's serum tested in which the serological reaction is positive, i.e. it has been determined that a reaction (binding) between antigen and antibody has occurred. It is important to compare the titer of the first and second serum and to establish whether this dynamic has diagnostic significance.

The dynamics of the formation of antibodies is not the same in all types of infections. It also depends on the time of incubation, the place of entry of the causative agent, whether it is a localized or generalized infection, etc. Thus, in example, in the case of pneumonia caused by *M. pneumoniae*, due to the long incubation time, significant amounts of antibodies can be found in the patient's serum already at the beginning of the disease. On the other hand, antibodies are produced slowly and in small quantities in the case of superficial infections of the mucous membranes (eg. acute gonococcal or chlamydial urethritis). However, in most cases, specific antibodies are created at the end of the first or the beginning of the second week after the onset of the disease. The antibody level (titer) will rise as a reflection of the response to the infection. However, any increase in antibody titer is not clinically significant. When tests are performed in which total antibodies are determined, and not individual classes, an increase in the antibody titer by at least 4 or more times is characteristic of an acute infection. A drop in the antibody titer is characteristic of a recent infection (recovery). An acute infection is also indicated by the so-called **seroconversion** when antibodies are not demonstrated in the patient's first serum, and antibodies are demonstrated in any titer in the second serum. The advantage of tests such as ELISA and IFA is that individual classes of antibodies can be determined in one serum sample (Table 1). These are sensitive tests and require small amounts of serum and other reagents to perform them. In these tests, primary infection is proven by the finding of IgM antibodies in one serum sample and a fourfold or higher increase in the titer of IgG antibodies when comparing acute and convalescent serum. Reinfection (repeated infection with the same pathogen) will be indicated by a four-fold or higher increase in the titer of IgG or total antibodies when testing acute and convalescent serum and the absence or slight increase of IgM antibodies.

Table 1. Possible results and interpretation of tests in which IgM and IgG antibodies are determined

IgM	IgG	Stage of infection	Remark
-	-	No infection	The serum may have been taken too soon, repeat the test
+	-	Early stage of acute infection	
+	+	Acute infection, later stage	
-	+	Past infection	

In addition to proving the presence of certain classes of antibodies in the serum, it is also possible to serologically prove the avidity of IgG antibodies (e. g. for CMV or *Toxoplasma gondii*). Avidity represents the strength of the affinity of specific antibodies to the complementary antigen, i.e. how strong is the bond between the antigen and antigen specific antibody. The rule is that the avidity is low at the beginning of the infection and high a few months after the beginning of the infection.

The results of serological tests should be interpreted taking into account information about anamnesis and clinical findings, information about previous immunizations, and whether it is, in example, a pregnant woman, an immunosuppressed person, a recipient of a transfusion, and information about a possible epidemic, hospital infection, etc. When interpreting serological findings, it is important take into account the time of serum sampling because, e.g., antibodies cannot be detected in the first serum taken too soon. Also, in some patients, there is a non-specific stimulation of the immune response (polyclonal activation of B lymphocytes). The sera of some patients who have, e.g., systemic lupus, rheumatoid arthritis, etc. react (usually in low titers) with many antigens used in serological reactions. Cross-reactions to certain causative agents may also occur due to common antigenic determinants.

Serological methods are used in the diagnosis of infections caused by different types of microorganisms, most often viral (hepatitis viruses, EBV, CMV, HIV). In the diagnosis of bacterial infections, serological methods are used most often to prove the causative agent of atypical pneumonia, syphilis and infections caused by *Streptococcus pyogenes*, and more recently also to prove latent and active tuberculosis (Quantiferon test).

Complement fixation test (CFT)

The test is based on the fact that complement binds to the antigen-antibody complex and is thereby removed from the reaction mixture. This test can be used to determine antigen or antibody, depending on what is known.

The test is performed in two phases.

1. phase:

- the patient's serum is mixed with a known antigen in a test tube, and if there are antibodies for that antigen in the serum, antigen-antibody complexes are formed that irreversibly bind the complement (with overnight incubation at 4-8 °C).

  The complement that is added to the antigen-antibody mixture is in the form of guinea pig serum. Since the amount of complement added to the test mixture must be known exactly and human serum also contains complement, the complement is removed from the patient's serum before testing by inactivation at 56 °C for 30 minutes (so-called thermoinactivation).

2. phase:

- an indicator system is added (at a temperature of 37 °C, for 30 minutes)to the test mixture that was incubated overnight, which enables the reading of the test and the interpretation of the results. The indicator system consists of sheep erythrocytes that were previously sensitized with hemolysins, antibodies that lyse sheep erythrocytes. Lysis of sheep erythrocytes will occur only if there is free complement in the test mixture, i.e. if there were no antibodies to the antigen in the patient's serum, therefore no antigen-antibody complexes were formed and the complement remained free. Therefore, the appearance of hemolysis in the CFT test means a negative reaction.
- if there were antibodies to the tested antigen in the patient's serum, the complement will bind to the antigen-antibody complex and erythrocyte hemolysis will not occur. Therefore, the absence of hemolysis, i.e. the appearance of precipitated erythrocytes at the bottom of the test tube, means a positive reaction in the CFT test

Direct agglutination

This test is used most often in the diagnosis of bacterial infections, to prove antibodies to salmonella (Widal test), brucella (Wright test), etc., where the antigen is a whole bacterial cell that has been treated with formalin, phenol, cooking and similar procedures. The test is performed in a test tube or microtiter plate, using different, always double, serum dilutions.

Binding of antibodies to the antigen results in a visible accumulation (agglutination) of bacteria that is visible to the naked eye.

Hemagglutination (passive or indirect)

In this test, erythrocytes are antigen carriers. When erythrocytes are mixed with serum containing specific antibodies, erythrocyte agglutination occurs.

Inhibition of hemagglutination (IHA)

This test is based on the fact that numerous viruses agglutinate erythrocytes of humans and various animals. This phenomenon is called hemagglutination, and infection with some of these viruses causes the appearance of antibodies in the patient's serum that can inhibit the ability of the virus to cause hemagglutination, which is the basis for the hemagglutination inhibition test. The test is performed in microtiter plates. The antigen is viral hemagglutinin, i.e. the supernatant of the cell culture fluid in which the virus was grown. After adding the patient's serum

and incubation, erythrocytes are added as an indicator. If there were antibodies in the patient's serum, erythrocyte agglutination will not occur, but the erythrocytes will settle at the bottom of the well of the microtiter plate. Therefore, the absence of hemagglutination means a positive reaction in IHA. The appearance of hemagglutination means a negative test result.

Neutralization test (NT)

The test is based on the fact that the virus loses its infectivity if it is previously mixed with the appropriate antibodies, i.e. the antibodies block ("neutralize") it.

The test can be used to determine antibodies or to identify viruses. It is most often performed on cell culture where the virus causes the so-called cytopathic effect (CPU). If the virus is blocked by antibodies, it will not cause a cytopathic effect on cell culture. The test is performed by mixing a certain amount of virus with serial, two-fold dilutions of the patient's serum and inoculating the cell culture with this mixture. The absence of CPU means that there are antibodies for that virus in the patient's serum.

This procedure is rarely used to prove antibodies because it is demanding, time-consuming and expensive.

Paul-Bunnell test

It is used to diagnose infectious mononucleosis (IM) based on evidence of heterophilic (non-specific) antibodies (non-specific agglutinins) that are not specific for the infectious agent (EBV) but are characteristic of IM.

Serum from a patient with MI agglutinates ram erythrocytes. Since agglutination can also occur when testing the serum of a healthy person, a modification of the Davidshon test is performed, i.e. adsorption of the patient's serum with guinea pig kidney and bovine erythrocytes.

The serum of a patient with IM, after adsorption with guinea pig kidney, will agglutinate ram erythrocytes, and it does not agglutinate ram erythrocytes.

after adsorption with bovine erythrocytes.

Mason hemolysis test

The serum of a patient with infectious mononucleosis contains hemolysins for bull erythrocytes. Bull erythrocytes and complement are added to the serum and, in case of a positive reaction, hemolysis occurs.

ELISA (*Enzyme Linked Immunosorbent Assay*)

An indirect ELISA test is used to prove antibodies of the IgM, IgG or IgA class.

The test is based on the fact that one enzyme molecule can degrade many substrate molecules and the amount of degraded substrate can be measured.

Principle of the test

1. stage: the antigen is attached to the support, usually at the bottom of the well of the microtiter plate. The patient's serum is added and, if the serum contains antibodies to that antigen, antigen-antibody complexes are formed
2. stage: an enzyme-labeled antibody for human IgM or IgG or IgA is added, which binds to the previously formed antigen-antibody complex. In order to determine the amount of complexes formed in this way, substrate for the enzyme is added to the wells. In the case of a positive reaction, i.e. the presence of antibodies in the patient's serum, there will be a change in the color of the reaction mixture, which is the result of the accumulation of the end products of enzymatic degradation of the substrate. The test is read spectrophotometrically to determine the optical density (OD), which correlates with the amount of antibodies bound to the antigen.

Indirect Fluorescent Antibody Test, IFA

The test is based on the fact that some dyes (fluorochromes) become "excited" when are exposed to short-wave light from the violet-blue part of the spectrum.

This test is used to detect antibodies of different classes (IgM, IgG or IgA).

The antigen (cells infected with a virus or intracellular bacteria, bacteria, fungi or parasites) is attached to a solid surface, most often to a glass slide. The patient's serum is added and, if there are antibodies in it, they will bind to the antigen. After that, an antibody specific for human IgM or IgG or IgA labeled with a fluorescent dye (fluorochrome) such as fluorescein isothiocyanate (FITC) is added. The test is read using a fluorescent microscope.

Western blot

The test is used to determine antibodies for certain specific antigens of the microorganism. The microorganism is destroyed chemically or mechanically and the dissolved antigens are placed on a polyacrylamide gel and separated by size by electrophoresis (smaller proteins travel faster). The protein bands thus obtained are transferred to strips of nitrocellulose paper. The patient's serum is added, and during the incubation the antibodies from the patient's serum, if any, bind to the antigens (proteins on the strip). Antibodies are then demonstrated by an enzymatic procedure similar to EIA (enzyme immunoassay). Western blot is most often used to prove antibodies to HIV.

PRACTICAL WORK

1. Complement fixation test (CFT)

Complete the sentences:

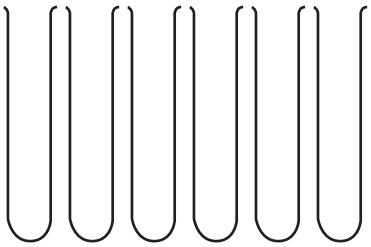
1. CFT is a serological technique used to determine _____ antibody titer.
2. A positive reaction in CFT is manifested as _____.
3. A negative reaction in CFT is manifests as_____.
4. CFT requires testing_____ serum to monitor _____ antibody titer.

Task 1. Read the CFT in paired sera of the patient and determine the dynamics of the titer of the antibodies to the mumps virus. Show the result schematically in the test tubes shown below.

1. serum

Initial serum dilution:

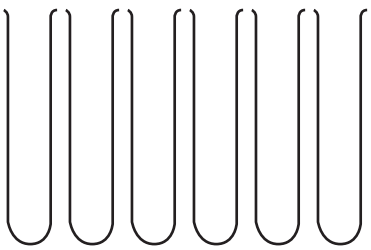
Antibody titer in serum:



2. serum

Initial serum dilution:

Antibody titer in serum:



Interpretation of antibody titer dynamics:_____.

2. ELISA test

Complete the sentences:

1. ELISA test can be used to determine individual_____ of immunoglobulins.
2. In ELISA test anti-human antibodies are labeled with _____.
3. In the ELISA test, the color_____ is measured, which is changed by the action of _____.
4. 4. ELISA test can be qualitative or _____.

Task 2.1. Interpret the stage of EBV infection based on the given values.

ELISA test result			Interpretation of the results
EA-IgM	EA-IgG	EBNA-IgG	
-	-	-	
+	+	-	
-	+	-	
+	+/-	+	
-	+	+	

Task 2.2. Read the ELISA test in a microtiter plate and interpret the stage of EBV infection based on the results.

Serum	EA-IgM	EA-IgG	EBNA-IgG	Interpretation of the results
1.				
2.				
3.				
4.				
5.				

Task 2.3. Read the ELISA test for CMV and write an interpret the results.

Serum	CMV-IgM	CMV-IgG	Interpretation of the results
1.			
2.			
3.			
4.			
5.			

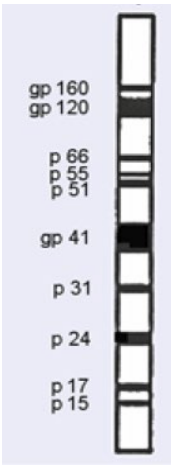
3. Western blot (WB)

Complete the sentences:

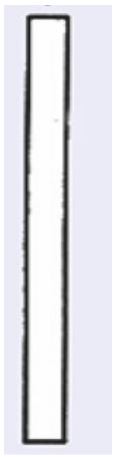
The serological technique Western blot is used to determine _____ _____.

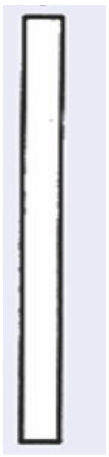
WB serves as a _____ test in the diagnosis of HIV infection.

Task 3.1. According to the attached scheme, read and draw the WB test for two patients assigned to the exercise and interpret the findings.



Pac. 1.





Pac. 2.

Task 4. Put the following options at right places in the table:

Acute infection; infection that happened before in life and immunity; result of vaccination; an old infection or diagnostic window; chronic infection; early stage of acute infection; sensitive.

Diagnostic findings				Interpretation
HBsAg	**Anti-HBs**	**Anti-HBc**	**Anti-HBc IgM**	
+	-	+	-	
-	-	+	-	
-	-	-	-	
+	-	+	+	
-	+	-	-	
-	+	+	-	
+	-	-	-	

Review questions:

1. Antibody titer is _____
   _____.
2. Seroconversion is _____.
3. What serologically indicates a primary infection?_____
   _____.
4. Serological reactions that can determine the titers of individual classes of immunoglobulins are _____.
5. What is the advantage of the ELISA test compared to CFT?
   _____
6. Describe CFT _____
   _____
   _____
   _____
7. How is reinfection manifested serologically?_____
   _____
8. Give examples of agglutination tests:_____

Date: Teacher's signature:

_____ _____

Notes:

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