Topic P07+8: Laboratory diagnostics of anaerobes, tuberculosis, actinomycetes and nocardiae

Task 1: Microscopy of acid-fast and filamentous microorganisms

While entirely acid-fast microorganisms (*Mycobacterium*) cannot be stained at all according to Gram, only partially acid-fast ones (*Nocardia*) or not at all (*Actinomyces*) can be Gram-stained. Typical morphology of *Nocardia* and *Actinomyces* is that of branched filaments, but sometimes they might be shorter, or even coccoid.

a) Staining of (negative) clinical sample using Ziehl-Neelsen staining method

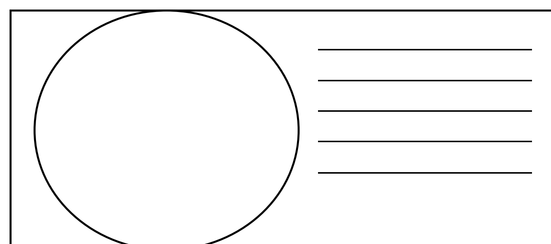
Ziehl-Neelsen staining is used for mycobacteria (*M. tuberculosis, M. leprae*), but also for some parasites (*Cryptosporidium parvum*, *Cyclospora cayetanensis*). The acid-fast organisms are stained only when heated during staining or in the „cold“ variant (according to Kiyoun) at use of concentrated carbolfuchsin and concentrated phenol. On the other hand, after that, they are not decolorized even by so called „acid alcohol“ (solution of alcohol with HCl or H_2SO_4). Decolorized background is then counterstained by a blue or green contrast dye.

In the double dental practical session just describe the staining procedure – fill in the following table with names of used reagents.

1.	During the staining the preparation is until	
2.	This reagent is made of	and
3.	Instead of this reagent, it is also possible to use	

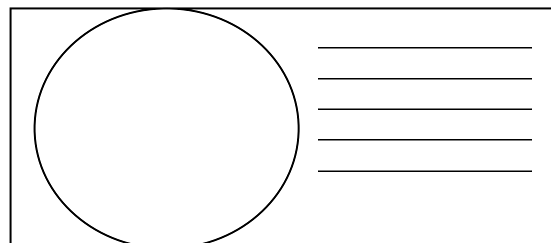
b) Microscopy of a mycobacterial culture

Examine microscopically (immersion oil, immersion 100× objective) the preparation of mycobacterial culture stained by Ziehl-Neelsen staining method.
Evaluate presence of red acid-fast rods.
Draw observed structures.
Do not forget do **describe** your picture (use lines)!



c) Microscopic examination of actinomycetes and nocardia strains

Examine microscopically the slide stained by Gram. Describe and draw observed formations. Observe high polymorphism of the microorganisms (from coccal shape, through rods to fibre/strings, often branched; Gram-positive, but often staining half Gram-negative).
Do not forget do **describe** your picture (use lines)!



Task 2: Culture of mycobacteria, *Actinomyces* and *Nocardia*.

The culture requests of acid fast and partialy bacteria are very different.

- For ***Mycobacterium tuberculosis*** we use special media: liquid media (Šula, Banič) and solid media (Ogawa, Löwenstein-Jenssen). The solid media are different from majority of other solid media used in medical microbiology; they do not contain agar, they are „solid“ because of coagulated egg proteins. Before culturing, the examined specimens should be rid of other microbes, usually by NaOH (“pickling”)
- For ***Nocardia*** a current blood agar is sufficient.
- For ***Actinomyces*** we need VL-agar and culture in anaerostat/anaerobic jar (see P07), as this organism is microaerophilic with so low need for oxygen that anaerobic conditions are optimal for it.

a) Describe media for mycobacterial cultivation

Medium name	liquid/solid	colour	notes
Löwenstein-Jensen	solid	pale green	
Šula	liquid	colourless	

b) Describe and draw the growth of *Mycobacterium*, *Actinomyces* and *Nocardia* on (in) given media

Bacterium	Medium name	Presence/absence of growth, eventually growth character (use your own words to characterize the growth)
Mycobacterium	Löwenstein-Jensen	
	Šula	
Actinomyces	blood agar (labelled "KA")	
	anaerobic WCHA agar (labelled "WCHA")	
Nocardia	blood agar (labelled "KA")	
	anaerobic WCHA agar (labelled "WCHA")	

Task 3: Assessment of antimicrobial drugs susceptibility

For treatment of mycobacterial infections, it is necessary to use special drugs, called antituberculotics (and not standard antibiotics). The way of testing is different from other bacteria, too: antituberculotics are added directly to the culture media. On the other hand, *Actinomyces* and *Nocardia* are treated by „normal" antibiotics and also „normal" diffusion disc test is used for testing.

a) Assessment of mycobacterial susceptibility to antituberculotics

By comparing with a control test-tube, read the results of antituberculotic susceptibility tests of *Mycobacterium tuberculosis* strain.

Antituberculotic				Growth control
Growth Y/N				
Interpretation				

b) Antibiotic susceptibility of *Nocardia* and *Actinomyces*

Diffusion disc test would be used as usually. The task is not performed in the double practical session.

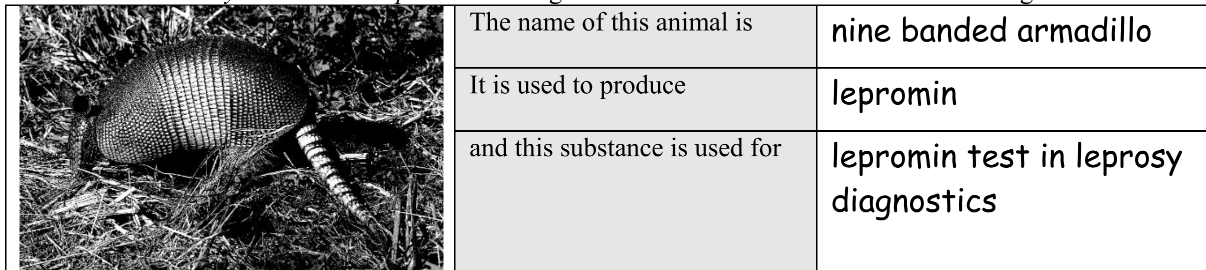
Task 4: PCR in diagnostics of TB

As the culture of mycobacteria is complicated, PCR becomes a very important method in its diagnostics.
Read a result of PCR TB diagnostics (from slideshow), write the results and interpret them.

Patient No.	Sample band	Control band	Interpretation
1			
2			
3			
4			

Task 5: Diagnostics of leprosy

Leprosy is a disease that still affects millions of people in underdeveloped countries. Its laboratory diagnostics is difficult because *Mycobacterium leprae* does not grow on artificial media. Fill in the following table.



	The name of this animal is	nine banded armadillo
	It is used to produce	lepromin
	and this substance is used for	lepromin test in leprosy diagnostics

By Hans Stieglitz - Own work, CC BY-SA 3.0, https://commons.wikimedia.org/w/index.php?curid=11037077. Adapted.

Task 6: Indirect TB detection by means of QUANTIFERON©-TB Gold test

It is a test of induced interferon gamma release checking and by means of this, checking of the cell-mediated immunity. **Test principle:** It was proven that in TB, including latent TB, tuberculosis antigens activate T-lymphocytes and they produce big amounts of interferon gamma. Similarly those T-lymphocytes may be activated non-specifically by so called mitogene; that is why mitogene is used as a positive control (MIT). As a negative control we use a test tube containing nothing (NIL). The test tube with proper TB antigen is labelled "TB". Interferon itself is detected by ELISA reaction.
Interpret the Quantiferon-TB Gold examination in four patients with use of interpretation table.

Anna: MIT = 4.8 TB = 1.2 NIL = 1.1 Your interpretation: ____________________

Berta: MIT = 5.3 TB = 4.8 NIL = 2.1 Your interpretation: ____________________

Cecil: MIT = 0.9 TB = 0.9 NIL = 0.8 Your interpretation: ____________________

Dimos: MIT = 8.4 TB = 8.3 NIL = 8.2 Your interpretation: ____________________

(all values are in IU/ml)

Interpretation table (according to test recommendations; simplified!)

NIL	TB minus NIL	MIT minus NIL	Final test interpretation	Presence of *M. tuberculosis* infection
≤ 8,0	< 0.35	≥ 0.5	negative	Not likely
	≥ 0.35	any value	positive	Likely
	< 0.35	< 0.5	unsure	Cannot be determined
> 8,0	any value	any value		
> 8,0	any value	any value		

Note: *Updated variant of QUANTIFERON test contains four (and not three) test tubes, as "TB" is replaced by two types of antigens. Nevertheless, for simplification, we count here with the classic variant of the test.*

Table for major results of Task 11 to Task 14 (to be filled step by step):

Strain		K	L	M	N
Gram stain of a strain – Task 11b (including information concerning possible spore formation)					
Culture – task 13	Blood agar ("KA") growth Y/N				
	VL agar ("VLA") growth Y/N				
	VL broth growth Y/N				
	Description of colonies on BA/VLA*				
FINAL CONCLUSION (morphology + relation to the oxygen)					

*Use VLA (VL agar) for microbes not growing on BA (blood agar)

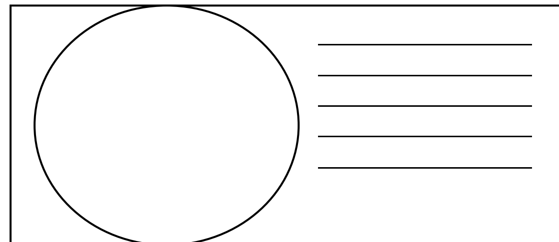
Task 11: Microscopy of the clinical specimen and microscopy of the strain

a) Observation of a clinical specimen

Observe a Gram-stained smear.

You will probably find a mix of various bacteria, as it is typical for anaerobic infections, in which usually not one particular microbe, but a combination of them is responsible for an infection. Besides bacteria, you might see leucocytes (mostly polymorphonuclears), possibly epithelial cells or tissue detritus and so on.

Do not forget to **describe** your picture (use the arrows)!



b) Microscopy of suspicious strains

Anaerobic bacteria can be cocci, bacilli or spirals, Gram-positive or Gram-negative, so in their shape, they are not different from other bacteria. On the other hand, anaerobes tend to be much more pleomorphic. In the *Clostridium* genus, the shape, dimension and localization of endospores are used as an important diagnostic sign. Try to find endospores in one of your strains (robust G+ rods).

Task 12: Anaerobic jar and anaerobic box

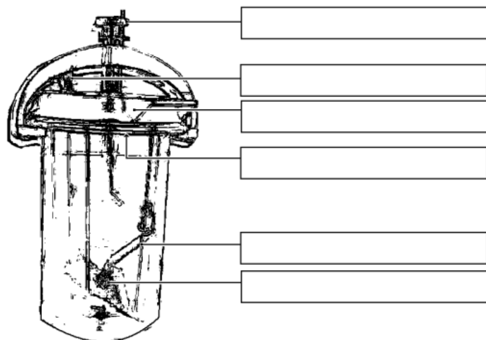
Anaerobiosis can be obtained using three ways in the laboratory:

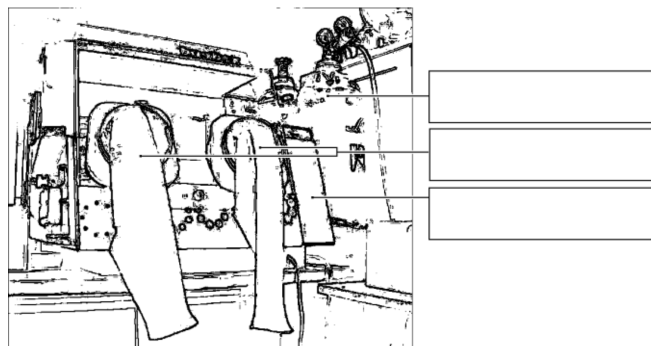
a) For liquid media, **paraffin oil** is used as a barrier between the medium and the atmosphere.

b) Solid media are placed into an **anaerobic jar**, where oxygen is chemically replaced by a mixture of other gases.

c) Solid media may also be placed into an **anaerobic box**; the mixture of other gases comes from a pressure cylinder (bomb).

Add your description to the pictures of an anaerobic jar and an anaerobic box (you will see a real anaerobic jar and pictures of both an anaerobic jar and an anaerobic box in the slideshow).





Task 13: Cultivation on agar media

Describe cultivation results of the presented strains on both aerobic and anaerobic media.

a) Aerobic culture on blood agar (BA)

Write down whether the bacteria grow on it or do not grow, and possibly describe the colonies.

b) Anaerobic culture on VL agar (VL blood agar)
VL (blood) agar is similar to blood agar, but it has a decreased reduction-oxidation potential and it is cultured either in the anaerobic jar or anaerobic box. Write down which strains are able to grow on it and describe those not growing on BA

c) Multiplication of anaerobic bacteria in VL broth
VL broth is used especially for the multiplication of rare anaerobic bacteria. Check the presence of turbidity (i.e. the growth) in VL broth, write it in the table and compare with the results of Part b)

Task 14: Species diagnostics of anaerobic bacteria using biochemical tests

In the strains found to be anaerobes we can read the biochemical microtest (ANAEROtest 23 Erba-Lachema) inoculated two days prior. The reading scheme is similar to that of NEFERMtest 24. The test is not performed in the double practical session.

Task 15: Susceptibility tests of anaerobic bacteria to antibiotics

Anaerobic bacteria were tested using diffusion disc test, but it was proven that diffusion disc test is not sufficiently reliable for anaerobic bacteria. Recently, according to EUCAST recommendation, infections caused by anaerobic bacteria are either treated without in vitro testing, or, especially for serious infections, E-tests are used for in vitro testing.
Evaluate E-test for both strains. Make conclusion (S, I or R).
Do not forget that, although principally similar to the diffusion disc test, E-test is a quantitative test. The concentration values are written directly on the strip. The site where the margin of the zone crosses the strip shows us the MIC (minimal inhibitory concentration) value.

Strain →	**Strain ___ determined as ______________**			**Strain ___ determined as ______________**		
Antibiotic ↓	**Criteria**	**MIC value**	**Conclusion**	**Criteria**	**MIC value**	**Conclusion**
Clindamycin (DA)	S if MIC ≤ 4 R if MIC > 4			S if MIC ≤ 4 R if MIC > 4		
Amoxicillin/ clavulanate (AMC)	S if MIC ≤ 4 I if MIC = 8 R if MIC > 8			S if MIC ≤ 4 I if MIC = 8 R if MIC > 8		
Metronidazole (MTZ)	S if MIC ≤ 4 R if MIC > 4			S if MIC ≤ 4 R if MIC > 4		

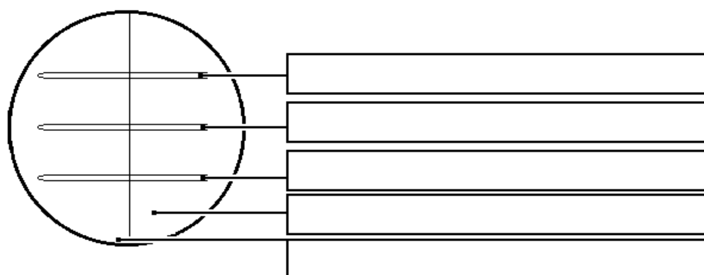
All values are in mg/L. S = susceptible, I = intermediary, R = resistant

Task 16: Detection of *Clostridium* toxins

In clostridia, for toxin detection we use various tests

a) Demonstration of the *Clostridium perfringens* toxin (lecithinase)

C. perfringens lecithinase is a toxin that can be neutralized by a specific antibody. One half of your yolk agar plate has been treated with the antiserum (anti-lecithinase), the other has not. The toxic effect of the lecithinase can be seen as a precipitation area around the examined strain; the particular toxin is neutralized by the antitoxin, other lecithinases are not. Draw the effect into the picture and add description.



b) Demonstration of the *Clostridium tetani* toxin

Look at a picture of a mouse suffering from tetanus (from your slideshow). Remark the position of the extremities and of the tail. (Normally students draw it, but this is not performed in this double practical session.)

c) Detection of the *Clostridium difficile* A and B toxins and it's structural antigen

Pseudomembraneous colitis due to *Clostridium difficile* toxins is very serious, especially in hospitalized patients.

Cultivation of the pathogen may be performed using special cultivation media, but it is rather recommended to perform testing for toxins and structural antigen instead. It is important, that a specimen of stool in a container (e. g. **not** rectal swab) is sent to the laboratory for testing.
The testing is performed by means of an immunochromatographic test similar to those performed in the J08 practical, but more complex: it checks both production of clostridium of clostridium antigen and toxins. It is essential in practice to send a genuine piece of stool (NOT rectal swab) to the laboratory.
The tests consists of two parts, in both cases the positivity is marked by the presence of a blue line:
(1) testing of a structural antigen (a part of the cell)
(2) testing of both A and B toxins TOGETHER (the positive line means presence of A *or* B *or* both toxins).
Interpretation of the test:

Toxin positive, antigen positive **(Situation 1)**	In case of corresponding symptoms, *Clostridium difficile* infection (CDI) may be considered proven and treatment necessary. After treatment re-testing is not needed; clinical course predicts better the effect of treatment.
Toxin negative, antigen positive **(Situation 2)**	In case of corresponding symptoms, (CDI) may be still considered possible or even likely, as toxin result is not sure enough. So treatment may be considered useful according to individual consideration of clinical status*
Toxin negative, antigen negative **(Situation 3)**	CDI is considered very unlikely
Toxin positive, antigen negative	*Laboratory mistake*
Absence of three dots(control)	*Invalid test*

**It is also recommended to try to cultivate the strain of Clostridium difficile (from the same stool specimen) and then to repeat the test with the strain used instead of the stool specimen.*

Observe the result of the *Clostridium difficile* (CD) antigen and *Clostridium difficile* A + B toxins detection in stool specimens X, Y and Z and write down the results:

Patient	Controls	CD A + B toxins	CD antigen	No. of situation (1/2/3)
X	OK – not OK	positive – negative	positive – negative	
Y	OK – not OK	positive – negative	positive – negative	
Z	OK – not OK	positive – negative	positive – negative	