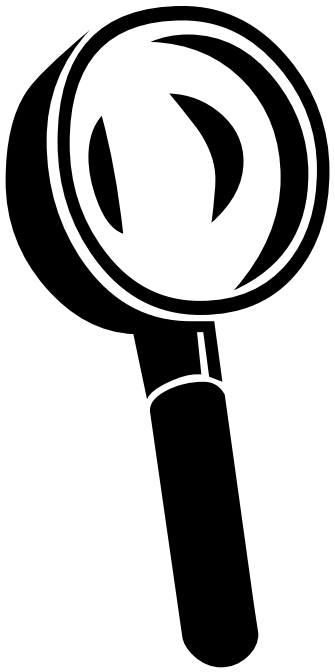
Institute for microbiology shows



TRACING THE CRIMINAL

Part fourteen: Repeating

Organisation of practical exam

- Usually student picks **one of 50–60 tasks**
- One topic of practical sessions corresponds to **2 to 4 tasks**; some tasks are related to more than one practical sessions (e. g. ASO – related to both neutralization and streptococci
- Some tasks are mostly practical (like Gram staining), some are rather discussion with practical parts. **Students may be asked also to topics not directly placed in the tasks**

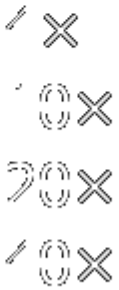
J01 Microscopy

- **Three tasks**
- **Wet mount** – for large and/or motile microbes (parasites, fungi, motile bacteria)
  - **To know also: Dark field wet mount** (mainly spirochetes)
- **Gram staining** – how to do it, + survey of other staining methods (Giemsa, Ziehl Neelsen, Burri…)
- **Observation of already stained preparations** – mainly interpretation

Wet mount – do it practically

- Do not forget to cover the preparation by a coverslip and to use **non immersion** objectives, magnifying e. g. 4×, 10× or 40×.
- We use no immersion oil
- After having it done, observe the objects in the microscope
- **Attention! Students that put into contact a non-immersion objective and immersion oil, finish immediately with F!!!**

Wet mount – procedure



Stained preparation

Gram, Lugol, Alcohol, Safranin
Tap water use
Drying with filtration paper

Gram staining – principle

Chemical	Gram-positive	Gram-negative
Crystaline violet	Staining violet	Staining violet
Lugol iodine	Confirmation	Less confirm.
Alkohol	Not decolorized	Decolorized
Safranin	Remain violet	Stain to red

Gram non staining bacteria do not stain in the first step, because of lack of any cell wall (*Mycoplasma*) or a very hydrophobic type of the cell wall (*Mycobacterium*).

Spirochetes would stain gram-negative, but they are very thin, so they, too, use to be often considered to be „Gram non-staining" and Gram staining is not used in diagnostic.

Gram staining – procedure

- **Gentian/crystalline violet = Sol. Gram-Nowy (20 –) 30 sec.**
- **Lugol (20 –) 30 sec.**
- **Alcohol 15 (– 20) sec.**
- **rinse by tap water!!! important!**
- **Safranin 60 – 120 sec.**
- rinse by tap water
- dry by filtration paper
- microscopy as in Task One

Specimen microscopy

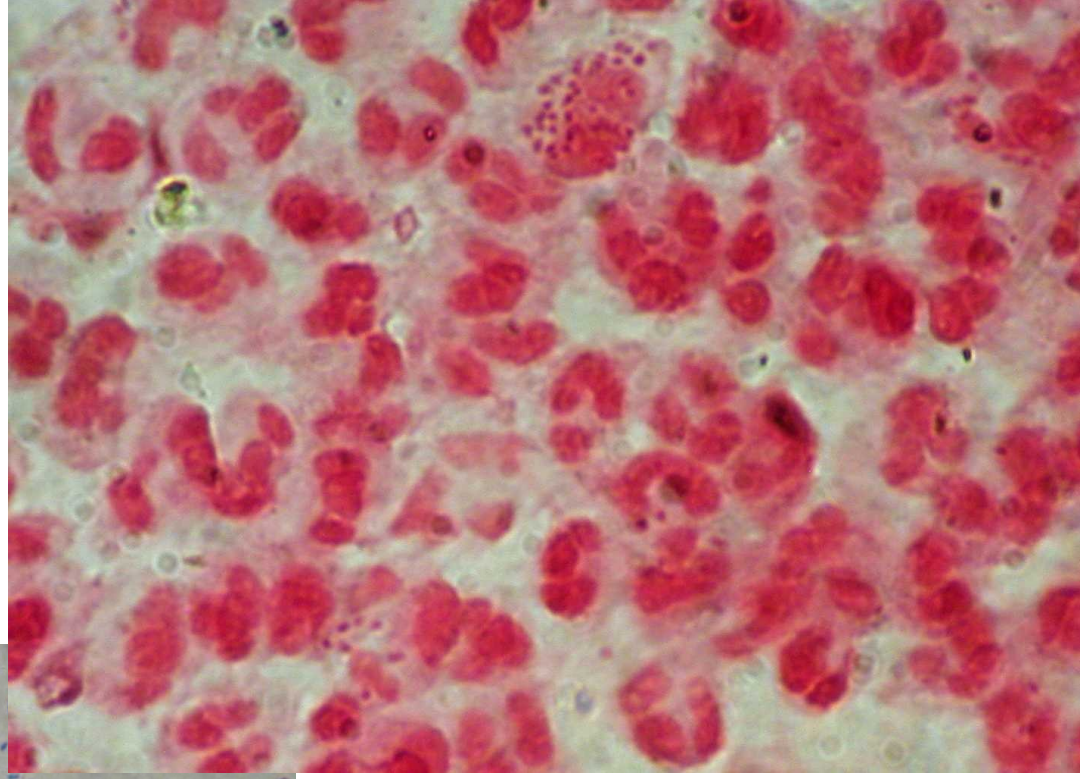
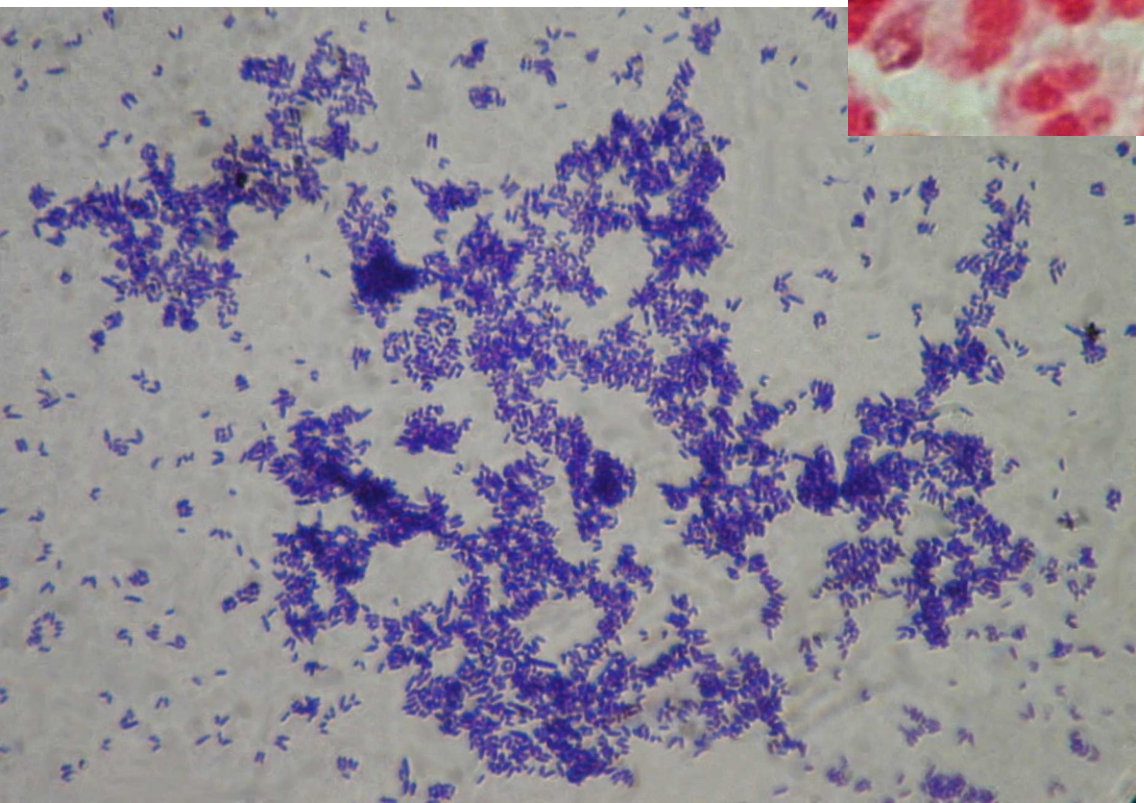


Photo O. Z.



Strain microscopy

In observation of slides…

- …it is important to have basic knowledge of size and morphology (yeast × staphylococci etc.) and to know something about interpretation (= knowledge of clinical microbiology, not just J01!)
- For example:
  - WBCs = inflammation
  - no WBCs in sputum = not properly taken specimen
  - G- diplococci inside WBC, urethral swab – suspicion for gonorrhoea

J02 – Culture. Two tasks:

Reinoculate a strain

Sterilize your loop

Take the strain

Inoculate first phase

Sterilize your loop

Do not take the strain again

Inoculate second phase

Sterilize your loop

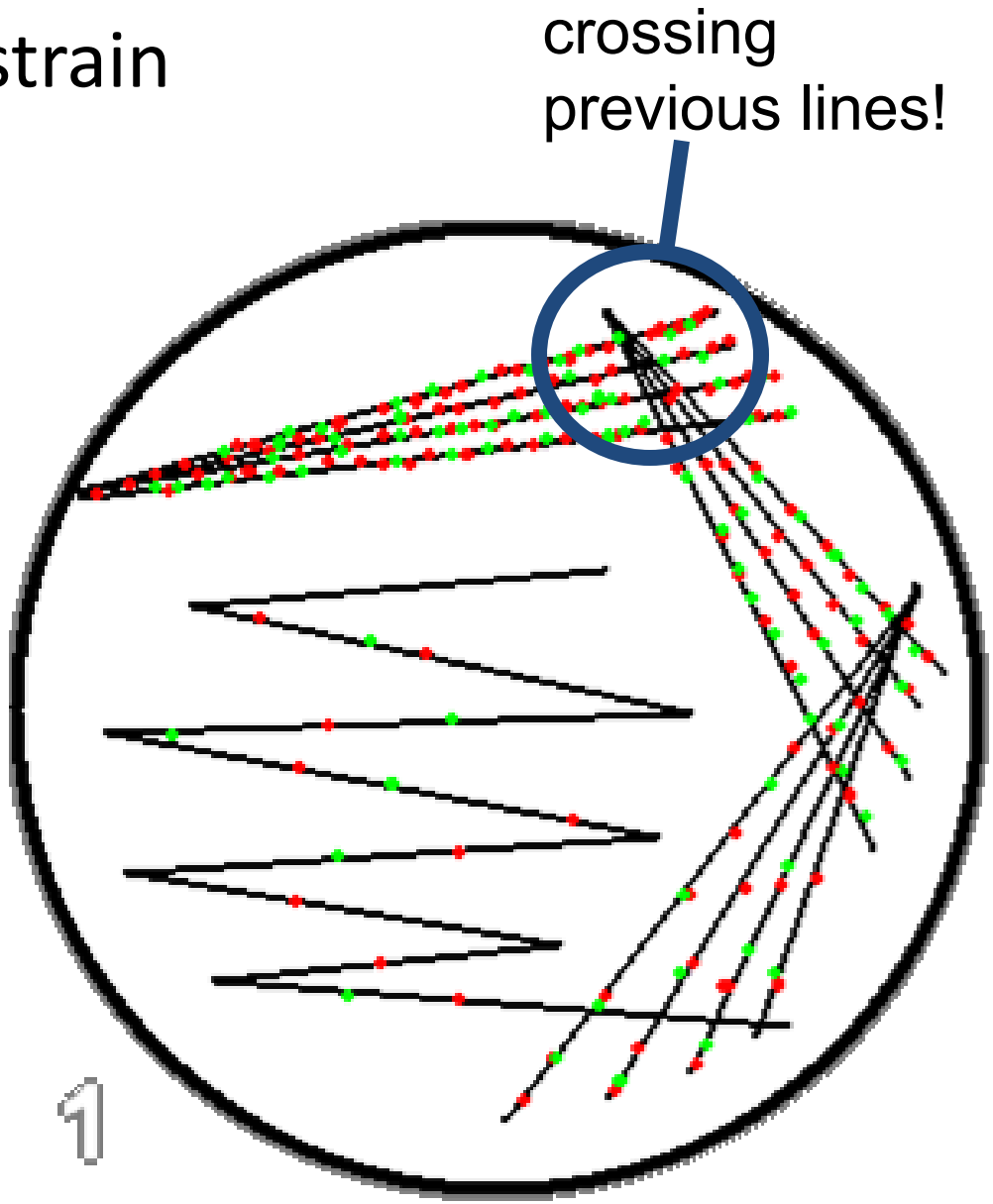
Do not take the strain again

Inoculate third phase

Sterilize your loop

Do not take the strain again

Inoculate the „serpent"



One more: Observe the media

1. Broth
2. VL-broth
3. selenite broth
4. Sabouraud
5. Löwenstein-Jenssen
6. Blood agar
7. Endo agar
8. MH
9. 10 % NaCl
10. VLA
11. XLD (+ MAL)
12. CHA
13. Levinthal
14. Slanetz-Bartley

Some more media at special bacteriology tasks.

J03 – biochemical identification tests

One only task, but other stuff is in other topics!

- In special bacteriology, you might meet:
  - Catalase test
  - Tests with diagnostic strips (oxidase, PYR, INAC)
  - Hajna medium (red = G-NF, other = ENT/VIB)
  - Eventually also MIU (not necessary to know details)
- The only „pure biochemical“ task
  - ENTEROtest 16 or something similar (STAPHYtest etc.)

Do not forget the ONPG test

it should be read before the other tests

	1 ONPG	2 H	3 G	4 F	5 E	6 D	7 C	8 B	9 A	10 H	11 G	12 F	13 E	14 D	15 C	16 B	17 A
		First row of the plate								2nd row of the plate							
+																	
—																	
?																	
?	+	—	+	+	+	—	—	—	—	—	—	—	—	+	+	+	+
	1	~~2~~	4	1	2	~~4~~	~~1~~	~~2~~	~~4~~	~~1~~	~~2~~	~~4~~	~~1~~	2	4	1	2
	5			3			0			0			6			3	

Decontamination tests – J04

- Important basic information:
- If we want to find **survival limit** of bacteria, we have to remove the tested extreme parameters to the conditions and to let them then in optimal conditions for a sufficient time.
  - In testing of disinfection effect, bacteria are treated by a disinfectant and then cultured on a medium without disinfectant
  - In testing of sterilisation, bacteria are placed to the sterilizer and then cultured in normal conditions

Microbes and outer influences

Sometimes the action of factor **combines**

The factor allways important is the **time**

A resistant, spore forming bacterium	160 °C	170 °C	180 °C
20 min	survives	survives	dies
30 min	survives	dies	dies
60 min	dies	dies	dies

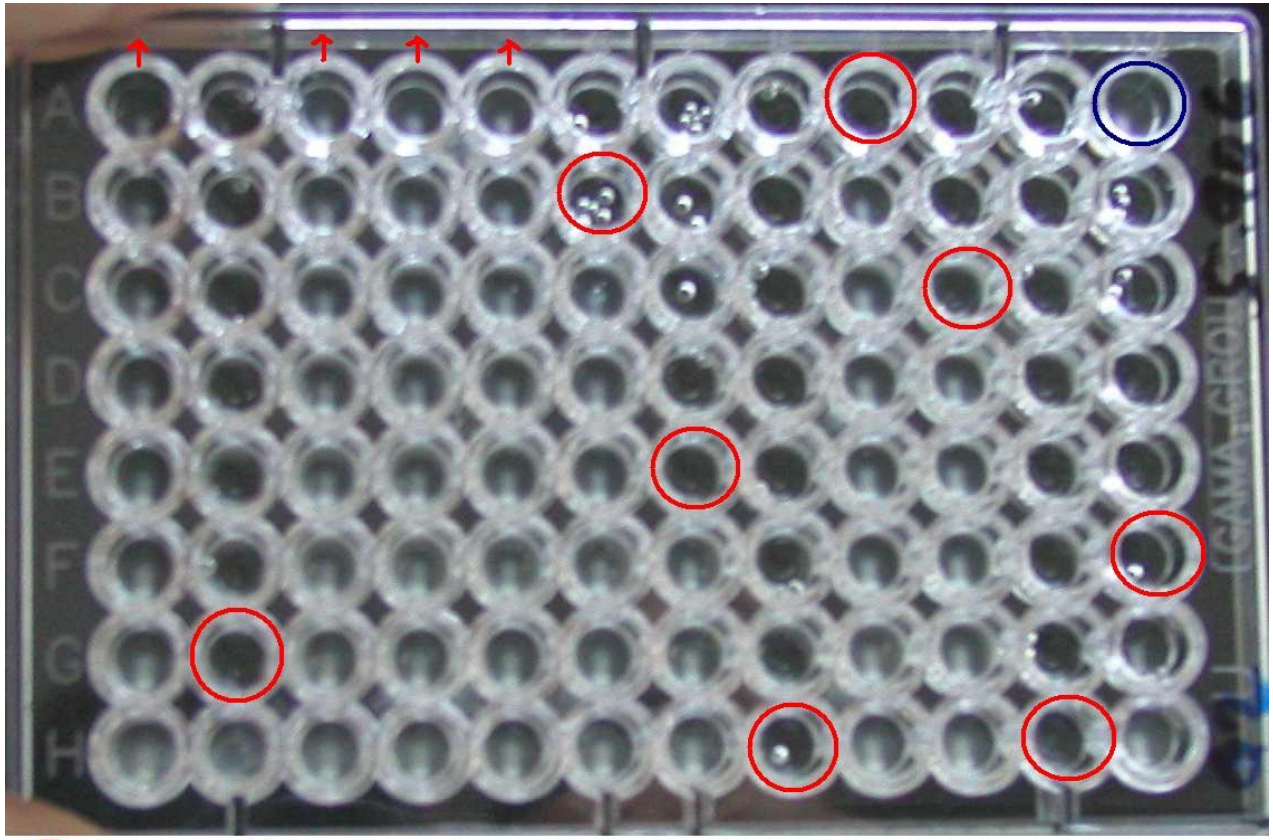
Plus (to both tasks):

- An extra sub-task to both tasks: Make pairs of cards with names of methods/disinfectants and cards of characterisation of methods
- The cards are on a working table, it is possible to see it

J05: Atb susceptibility
Diffusion disc test: to read it, to interpret it

www.medmicro.info

Microdilution test – reading



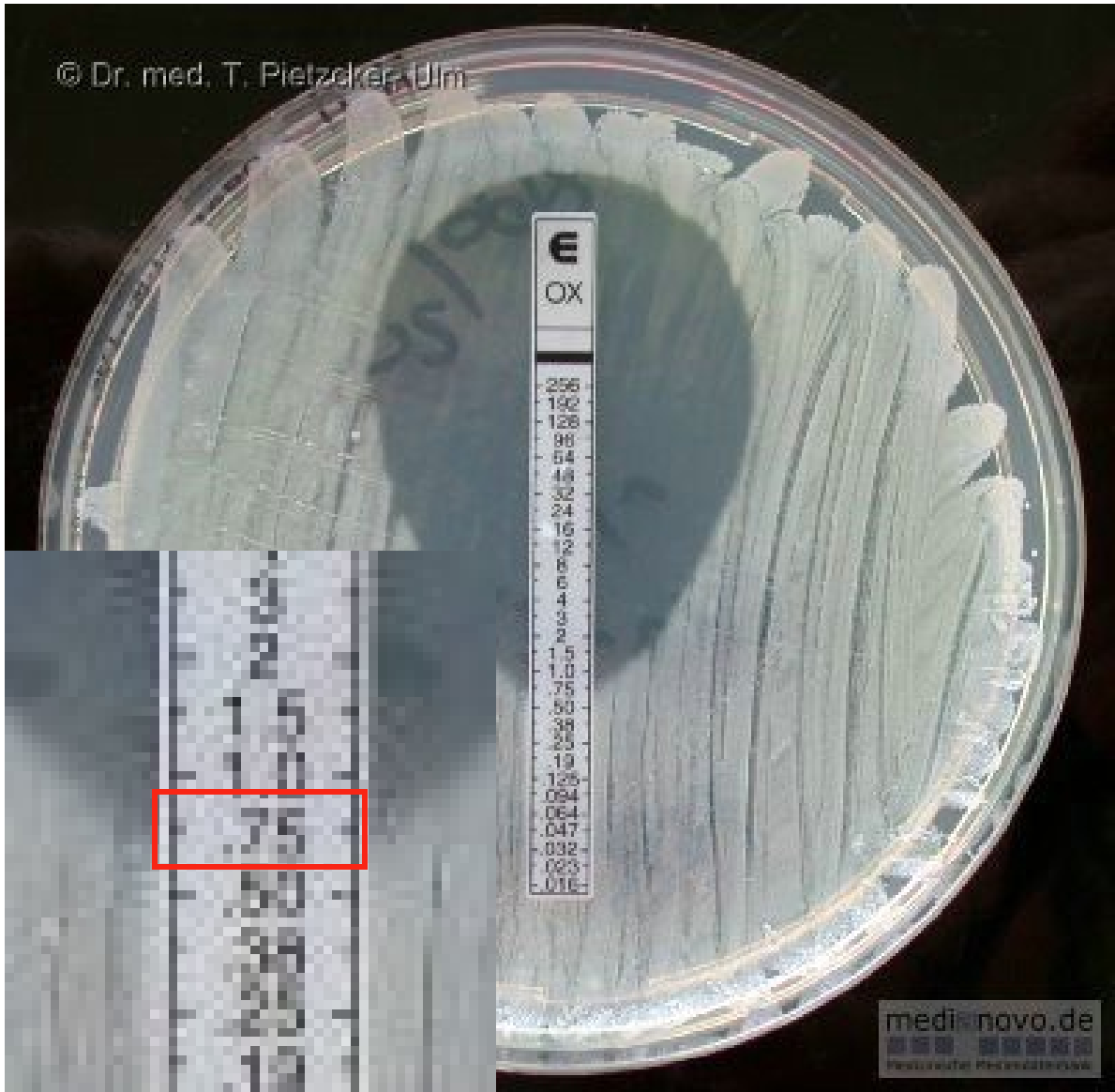
For interpretation, comparison with breakpoints is necessary

- In case of columns 1, 3, 4 & 5, MIC > the highest value
- *In wells 8 and 11, MIC ≤ the lowest value*

E-tests – reading

www.uniklinik-ulm.de

We can read the MIC value directly on the strip – in place, where the margins cross the strip



Beta-lactamases detection: limited knowledge is sufficient

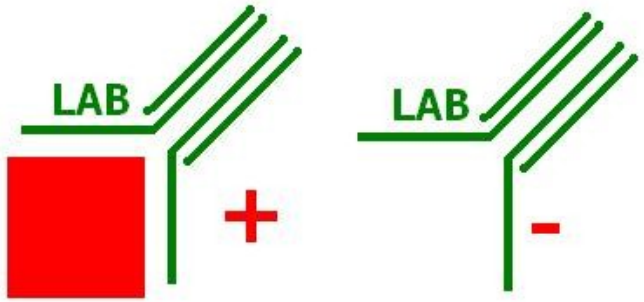
It is not directly in the tasks, but the examinator may ask you about it.

Photo O. Z.

J06–J08 (but also related with many others): serology

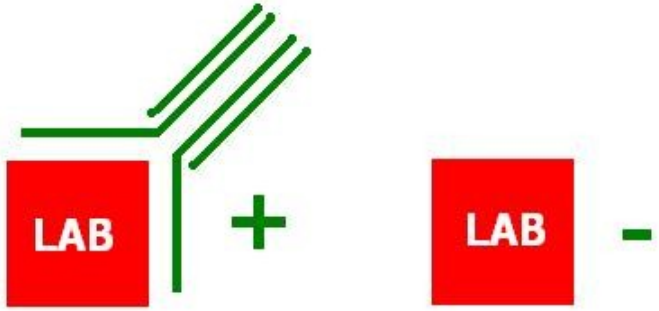
Antigen detection: laboratory (animal origin) antibodies + pacient‘s sample or microbial strain.

Direct method



Antibody detection: laboratory antigen (microbial) + pacient‘s serum (or saliva).

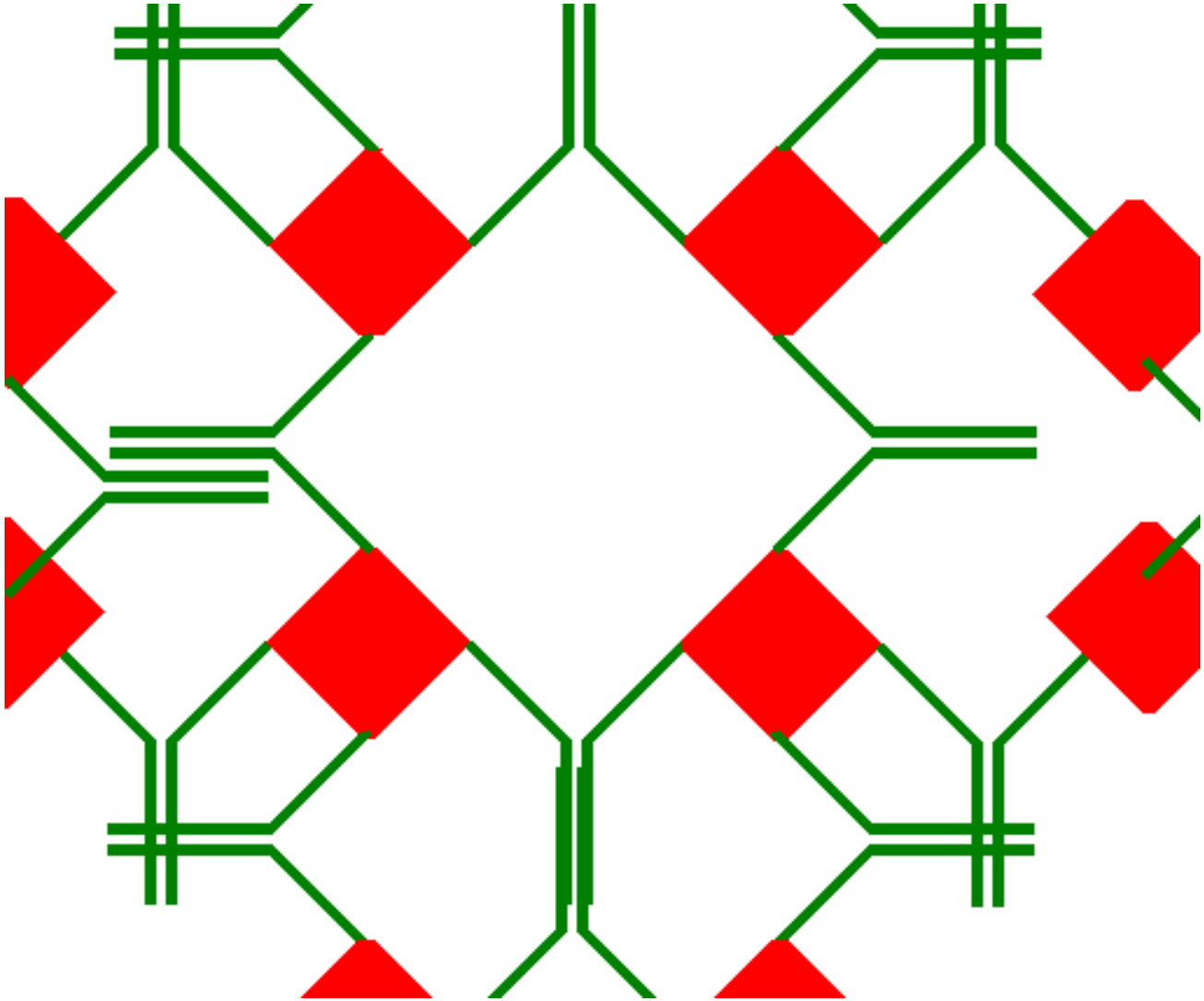
Indirect method



Interpretation

- Antigen detection (including antigen analysis): it is a direct method. Positive result means presence of the microbe in the patient's body
- Antibody detection: it is an indirect method. Nevertheless, there are some ways how to get the information – when the microbe met the body:
  - Amount of antibodies (relative – titre + titre dynamics; agglutination, CFT, neutralisation)
  - Class of antibodies: IgM/IgG (reactions with labelled components – mostly ELISA and immunoblotting)
  - *(Avidity of antibodies)*

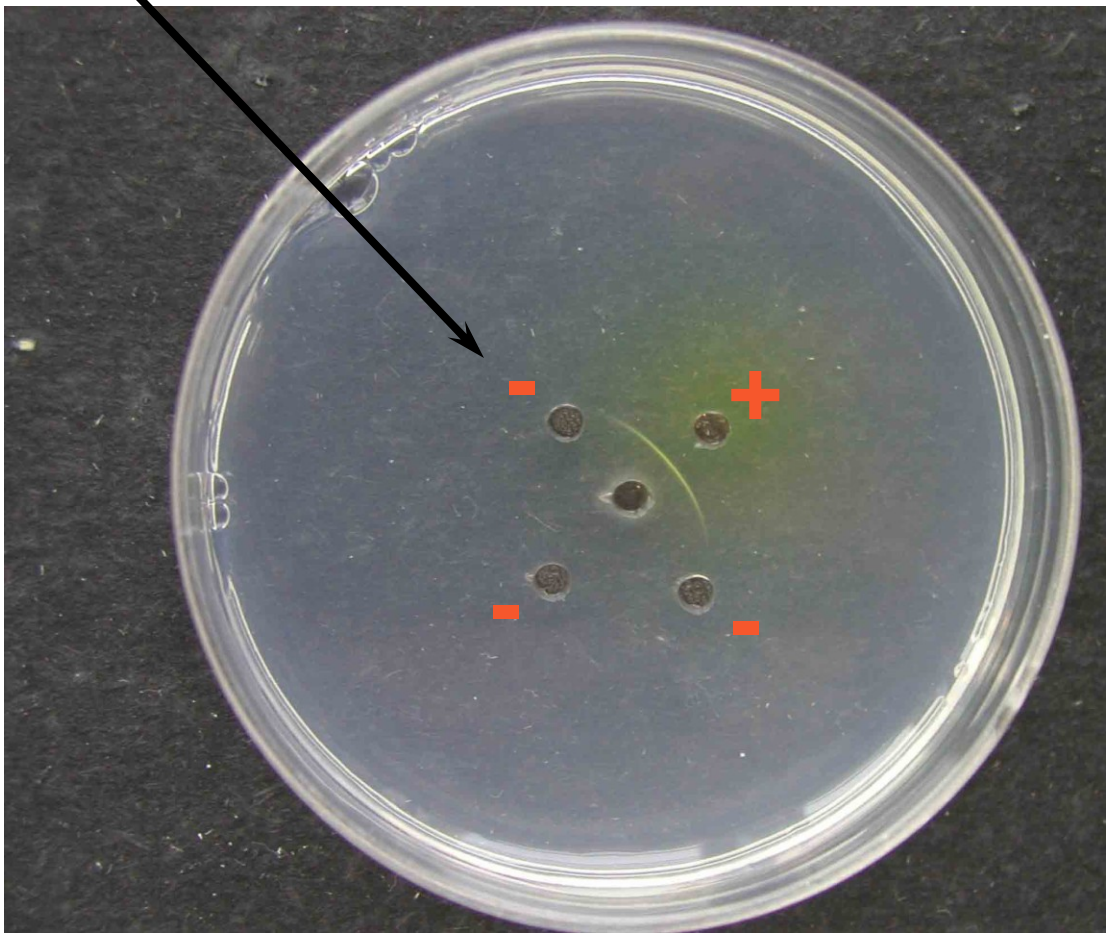
Precipitation



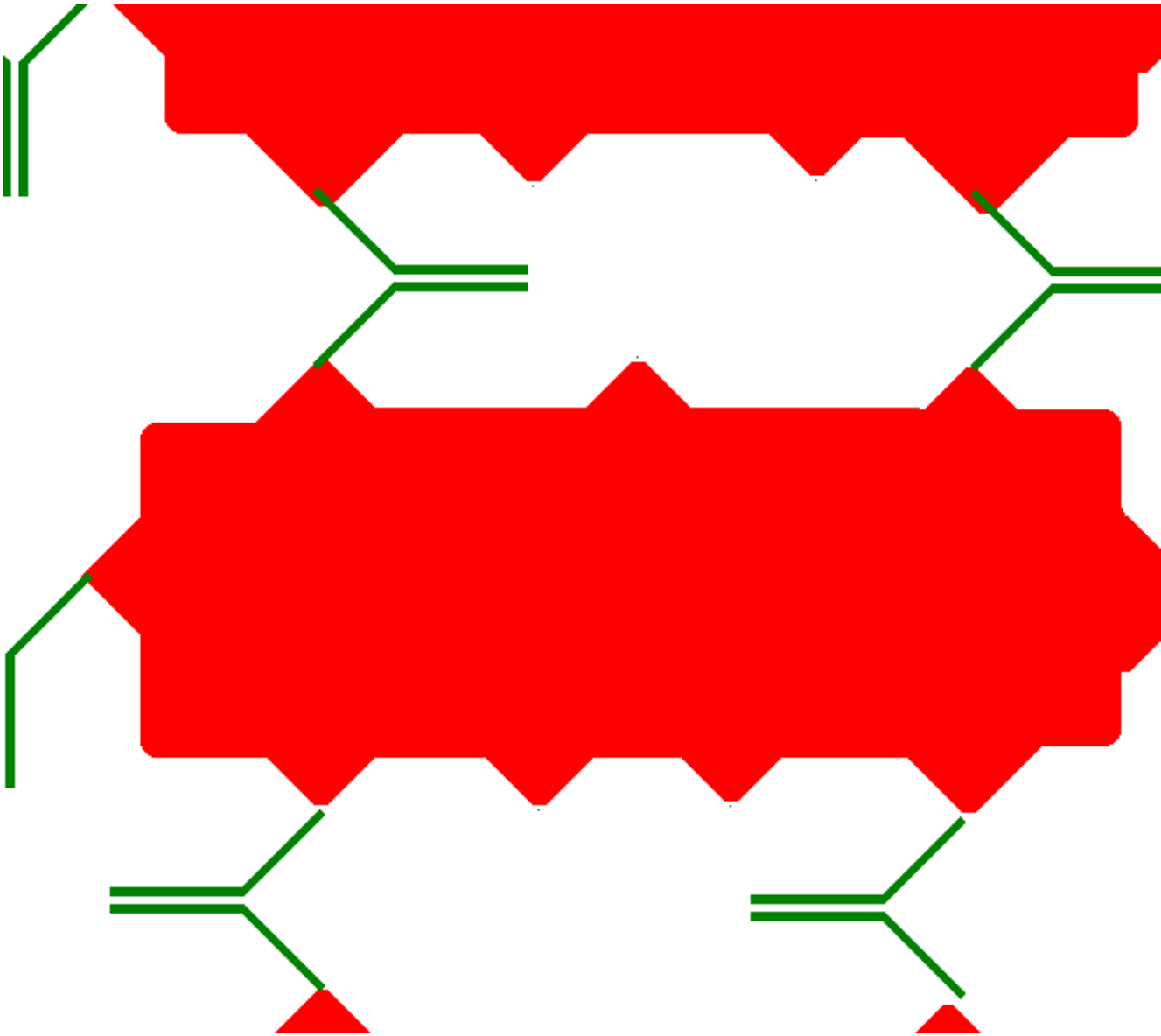
Two examples of precipitation

1) Microprecipitation in agar according to Ouchterlony

2) RRR/RPR reaction for syphilis diagnostics (flocculation)



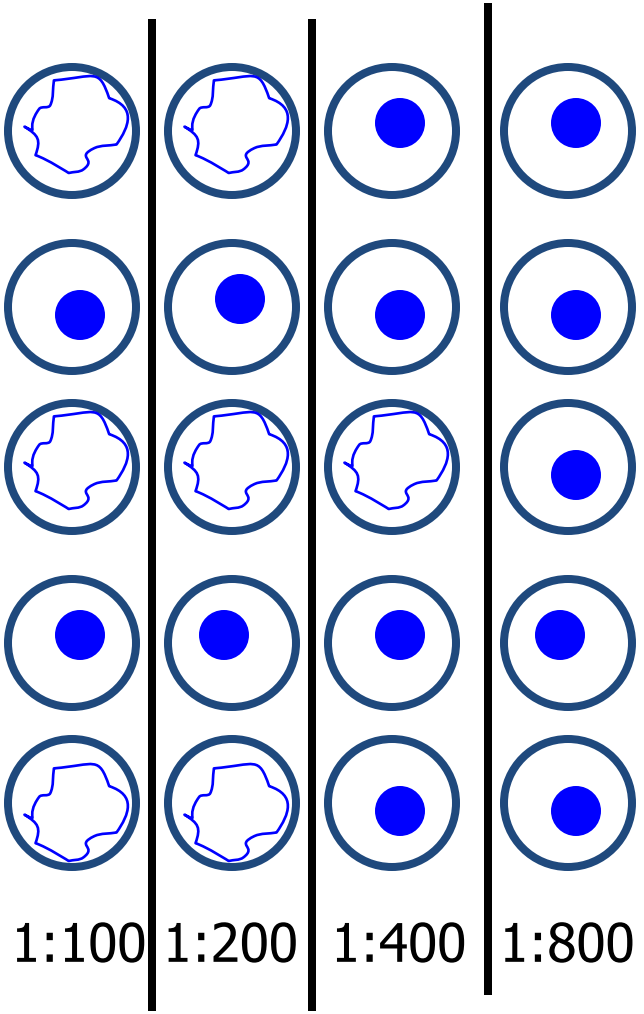
Agglutination



Agglutination for antigen detection or antigen analysis

- **EPEC detection:** in what situation we perform it, how it is performed… (Practically, you obtain a strain and you have to know what to do with it)
- **CSF agglutination** (task „Comment a video clip“). Important: besides microscopy this is the second way what to do as quick diagnostics of purulent meningitis

Agglutination reaction for detection of antibodies



K+ positive, titre = 1 : 200

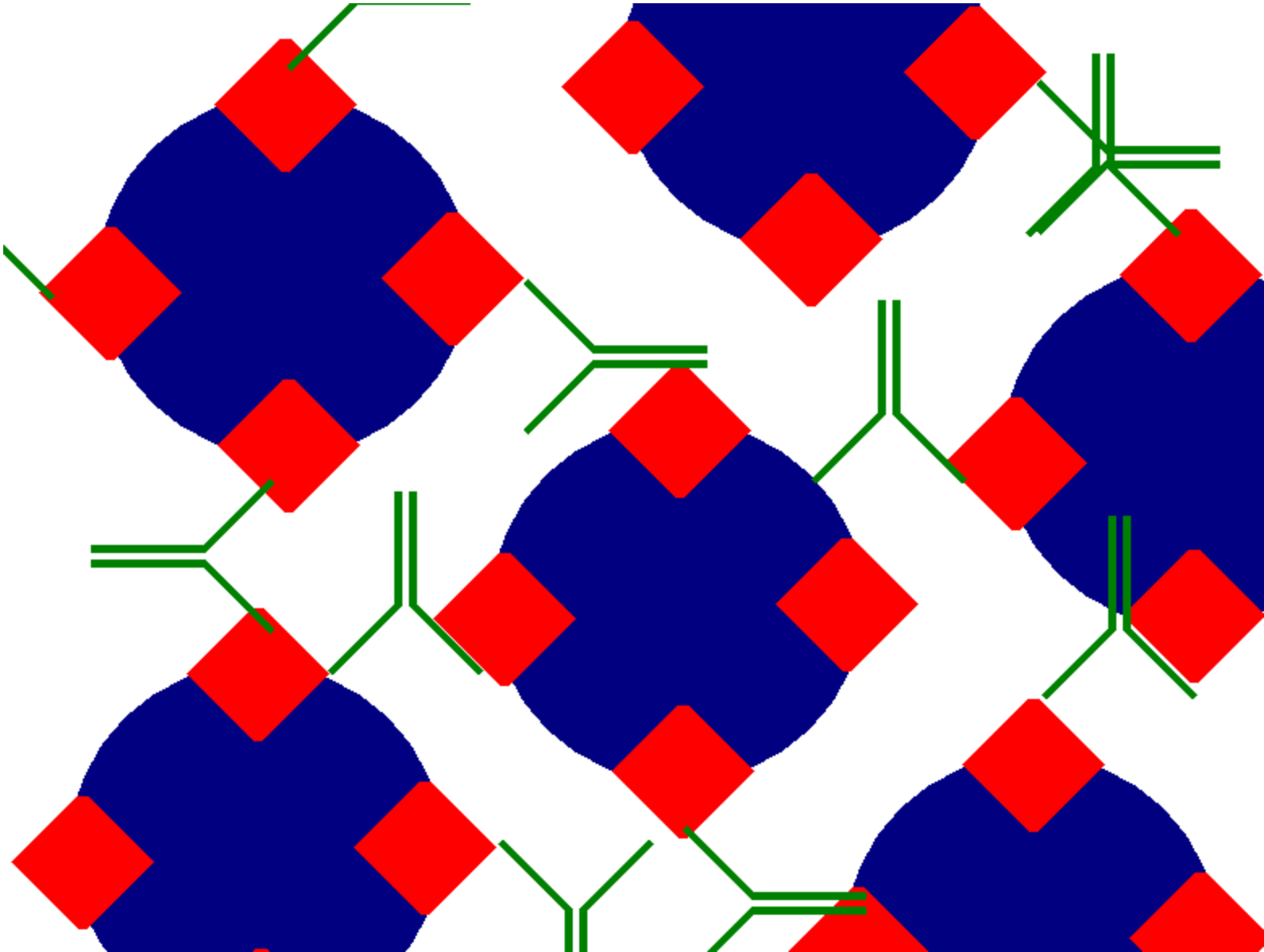
No 1 negative

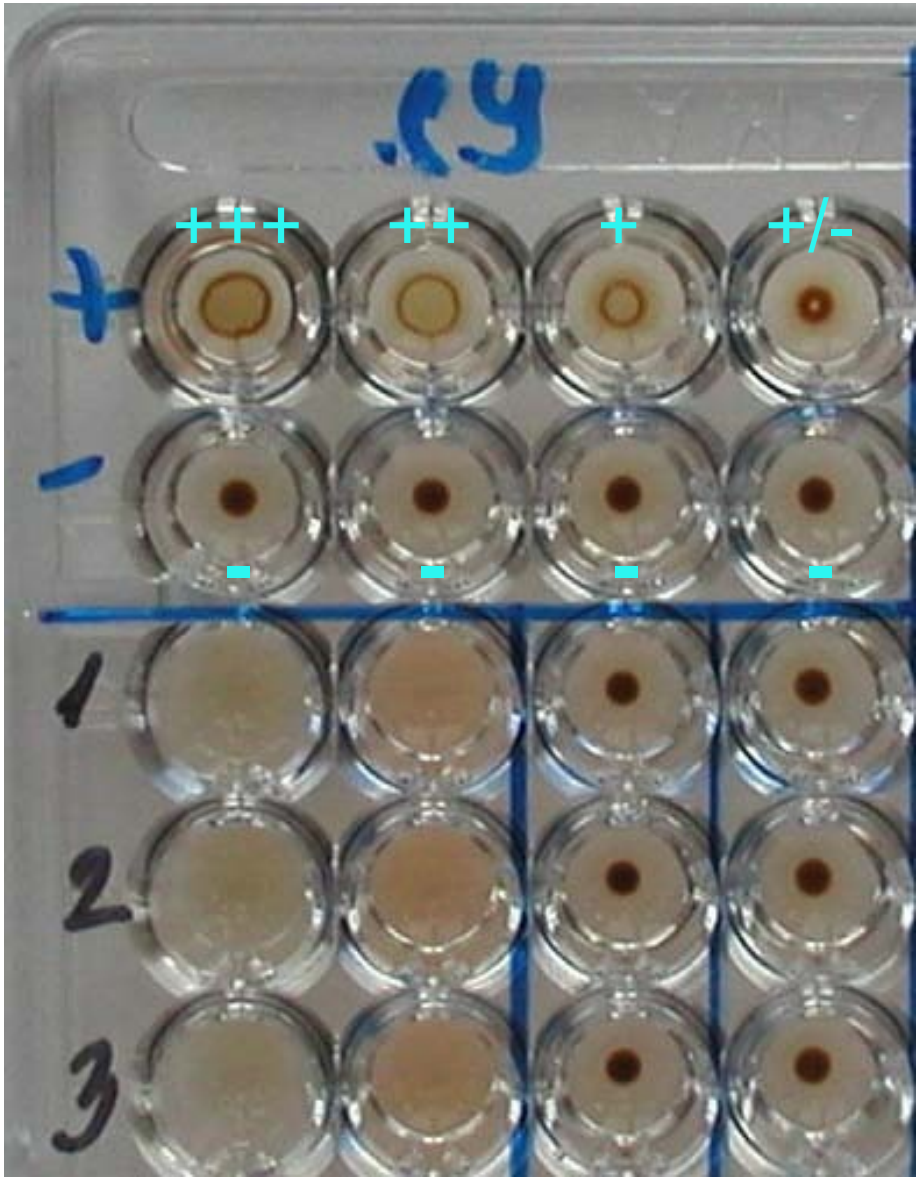
No 2 posit., titre = 1 : 400

No 3 negative

No 4 posit., titre = 1 : 200

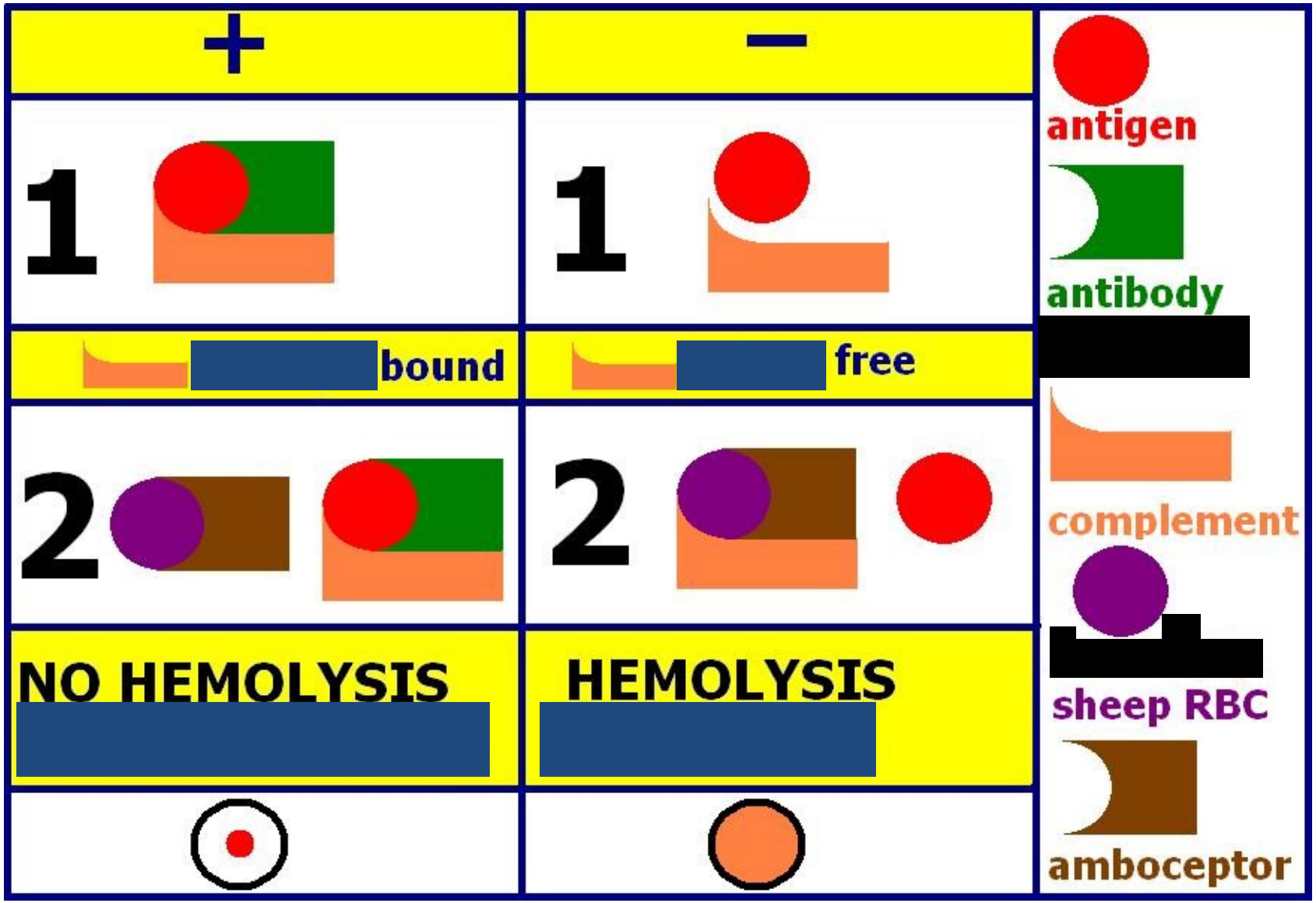
Agglutination on carriers



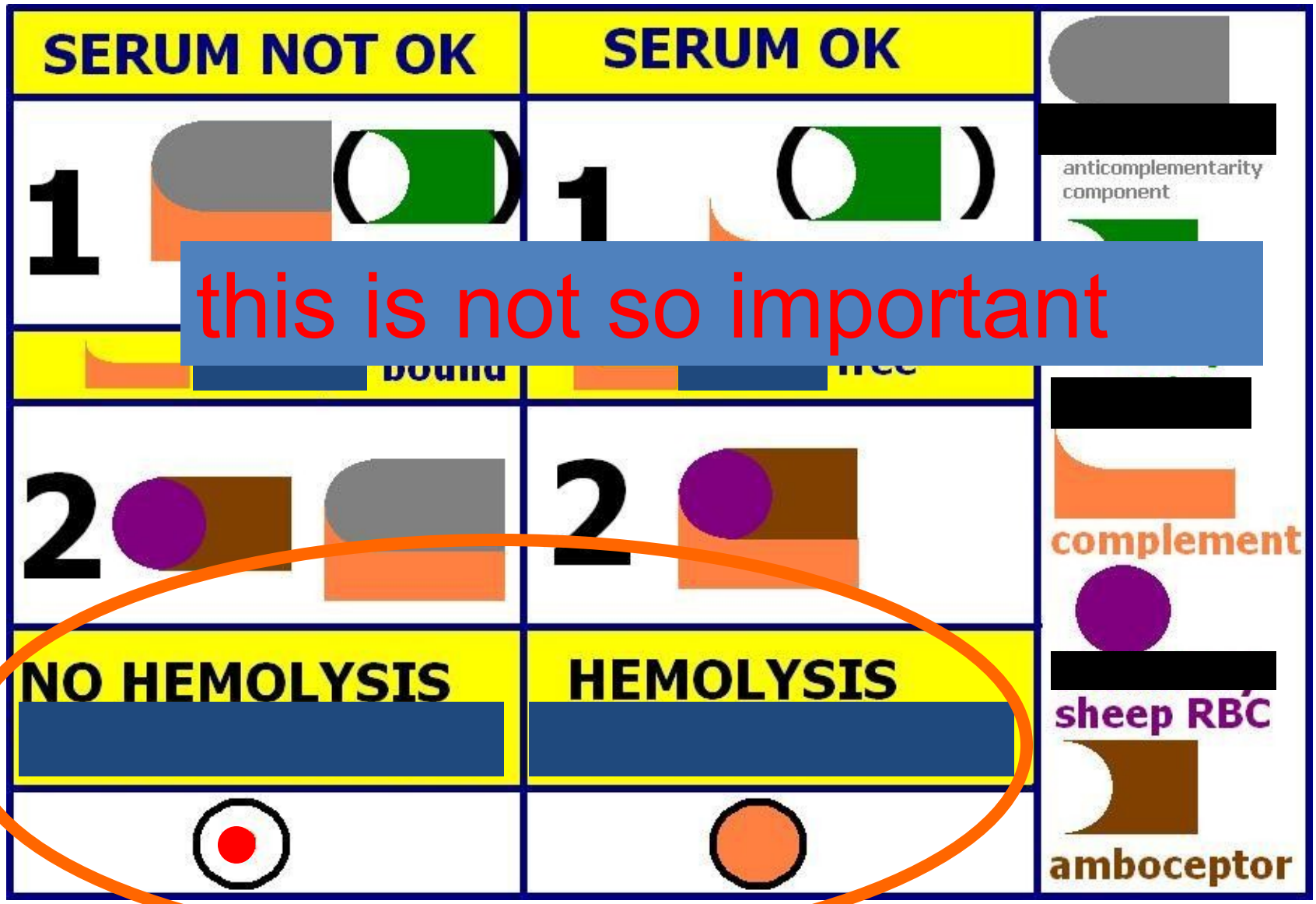


Example
MHA-TP
(www.medmicro.info)

CFT – principle



Anticomplementarity test



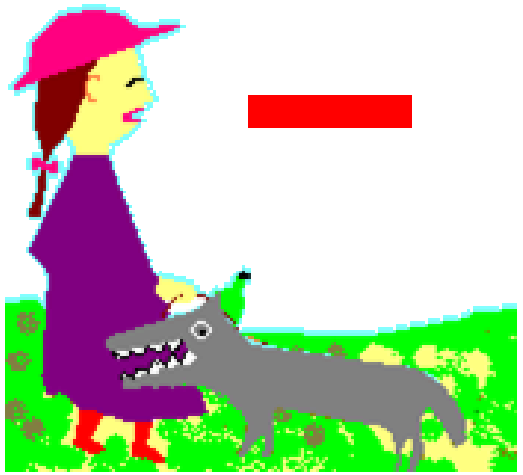
Neutralisation schematically

- Antibody (Ig) prevents an effect of a toxin/virus to a cell / red blood cell

Cell in a tissue culture or a red blood cell

Antibody

Toxin or virus



Cell in a tissue culture or a red blood cell

Toxin or virus

Examples of neutralisation reactions

	Neutralised	Object	Reaction
	Bacterial toxin (haemolysin)	RBC haemolysis	ASO
	Virus	RBC agglutination	HIT
	Virus	*Cell metabolic effect*	*VNT*

Important: What is the antistreptolyzin O

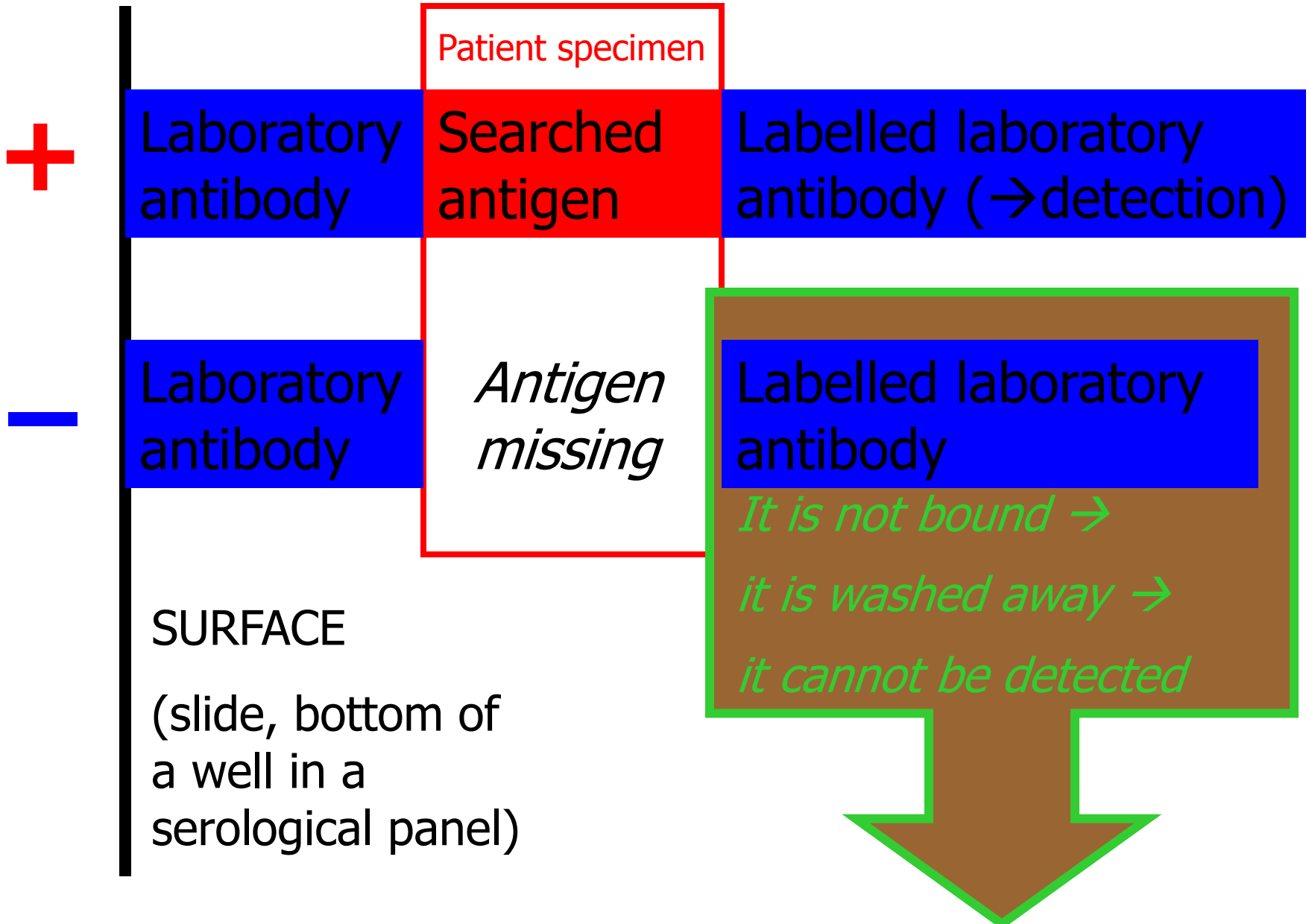
and why we attempt to detect it

- After every streptococcal infection antibodies are produced, often including antibodies against streptococcal toxin – streptolysin O.
- Nevertheless, sometimes after infection the antibodies increase instead of decreasing. Antibodies are bound to some structures of the host organism (autoimmunity), so a „circulus vitiosus“ starts to run
- In such a situation, paradoxically the antibodies are worse than the pathogen that challenged the antibody response to protect us.

HIT

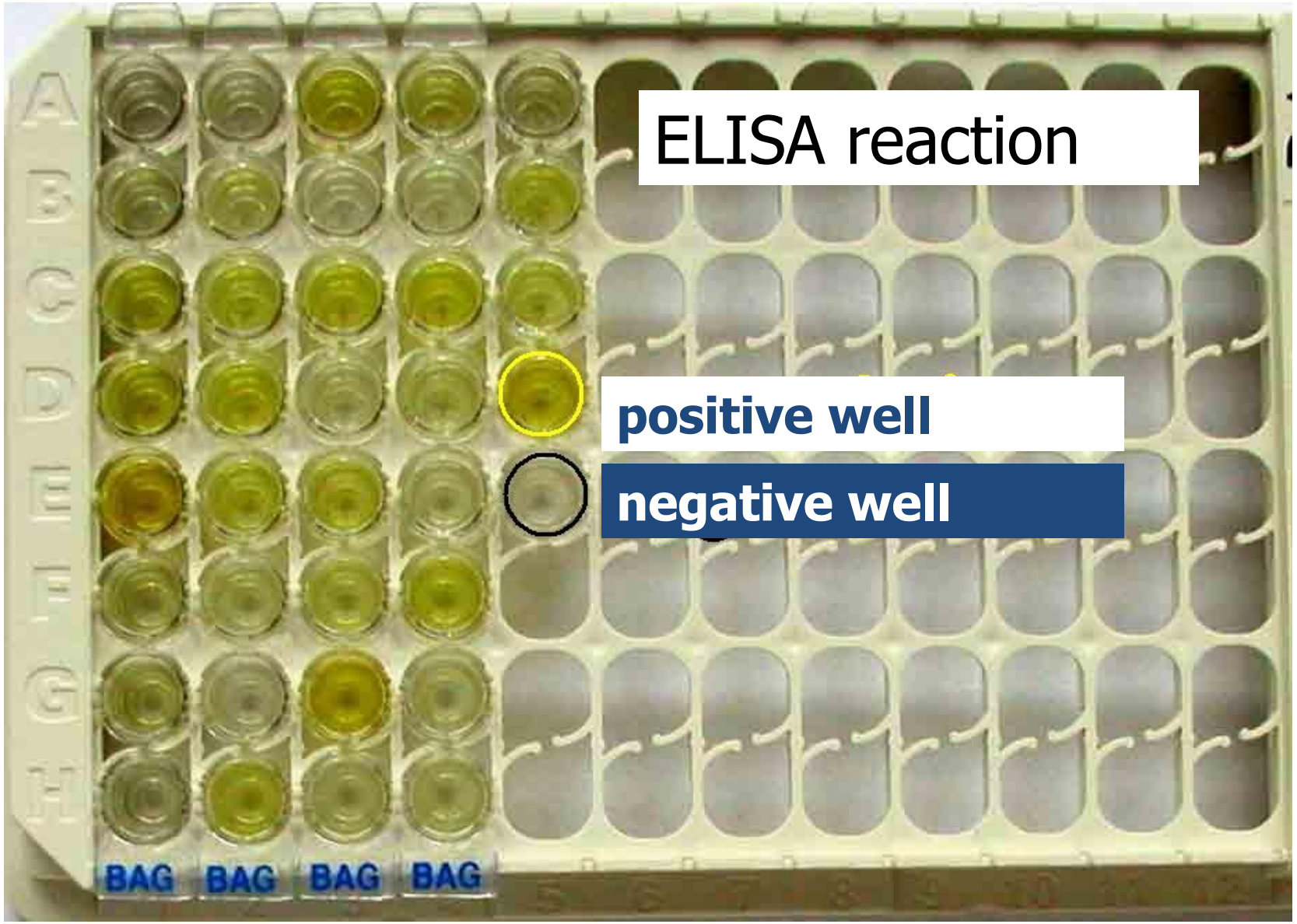
- Haemagglutination Inhibition Test: Pay attention, it is NOT an agglutination reaction, it is a neutralisation! Antibody neutralises the aggregation of RBCs due to viruses.
- **So: Potato-like shape = negative response. Dense round target = positive response**
- HIT differs from ASO reaction mostly by the fact, that the RBCs are not haemolysed, but agglutination. But the fact, that a specific antibody blocates the reaction is valid in both of the

Reactions with labelled components



ELISA – an example

(www.medmicro.info)



One task: HBsAg / anti-HBS puzzle

- HBsAg testing – positive
- HBsAg testing – negative
- anti-HBs testing – positive
- anti-HBs testing – negative

Reading of ELISA

BL	4
K-	5
K-	6
K+	7
K+	8
1	9
2	10
3	11
IgA	

BL	4
K-	5
K-	6
K+	7
K+	8
1	9
2	10
3	11
IgG	

c. o. (IgA) = (0.107 + 0.137)/2 + 0.320
c. o. (IgA) = 0.122 + 0.320 = 0.442
90 % c. o. = 0.398 110 % c. o. = 0.486
all values bellow 0.398 are negative
all values above 0.486 are positive

c. o. (IgG) = (0.034 + 0.029)/2 + 0.320
c. o. (IgG) = 0.032 + 0.320 = 0.352
90 % c. o. = 0.317 110 % c. o. = 0.387
all values below 0.317 are negative
all values above 0.387 are positive

FIND POSITIVE AND BORDERLINE WELLS FOR BOTH IgA and IgG!

Western blotting – principle

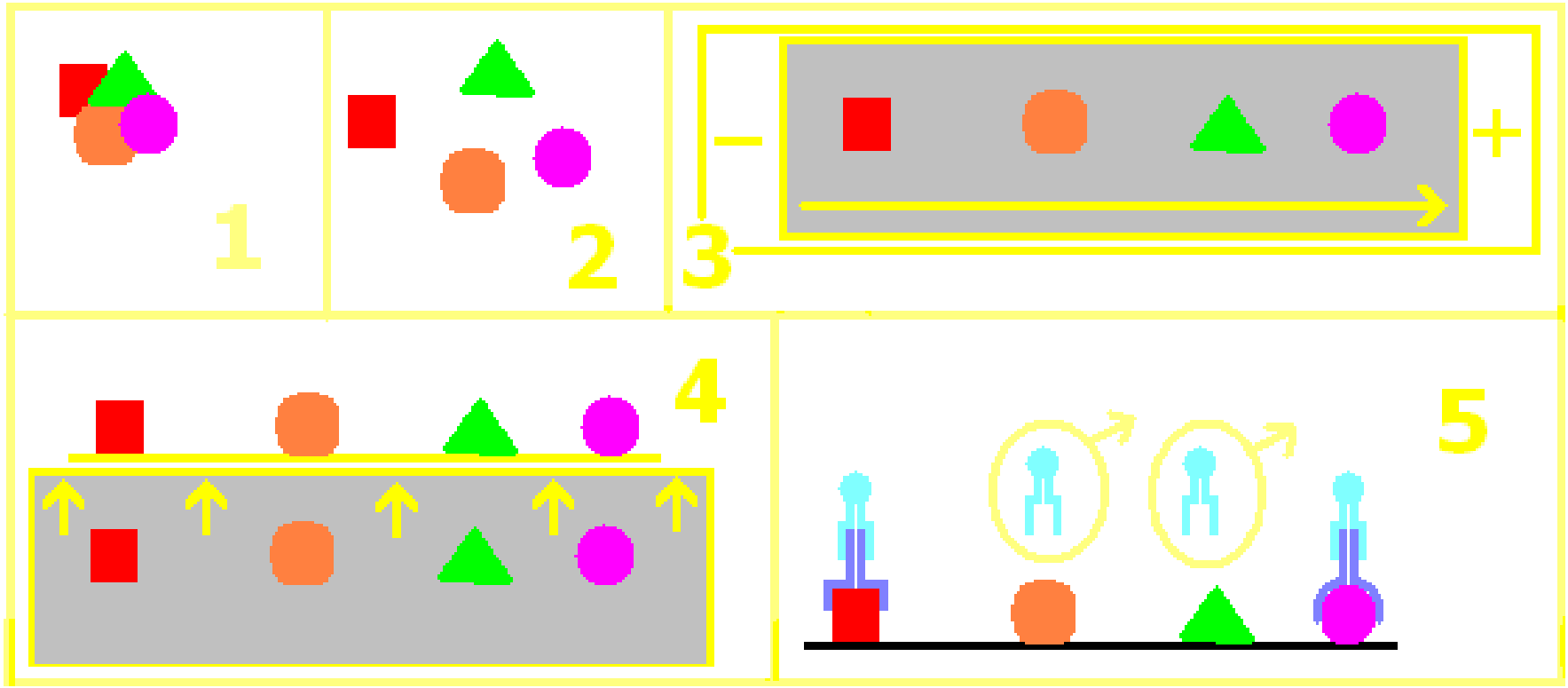
1: original antigen (mixed)

2: decomposition of antigen by a detergent

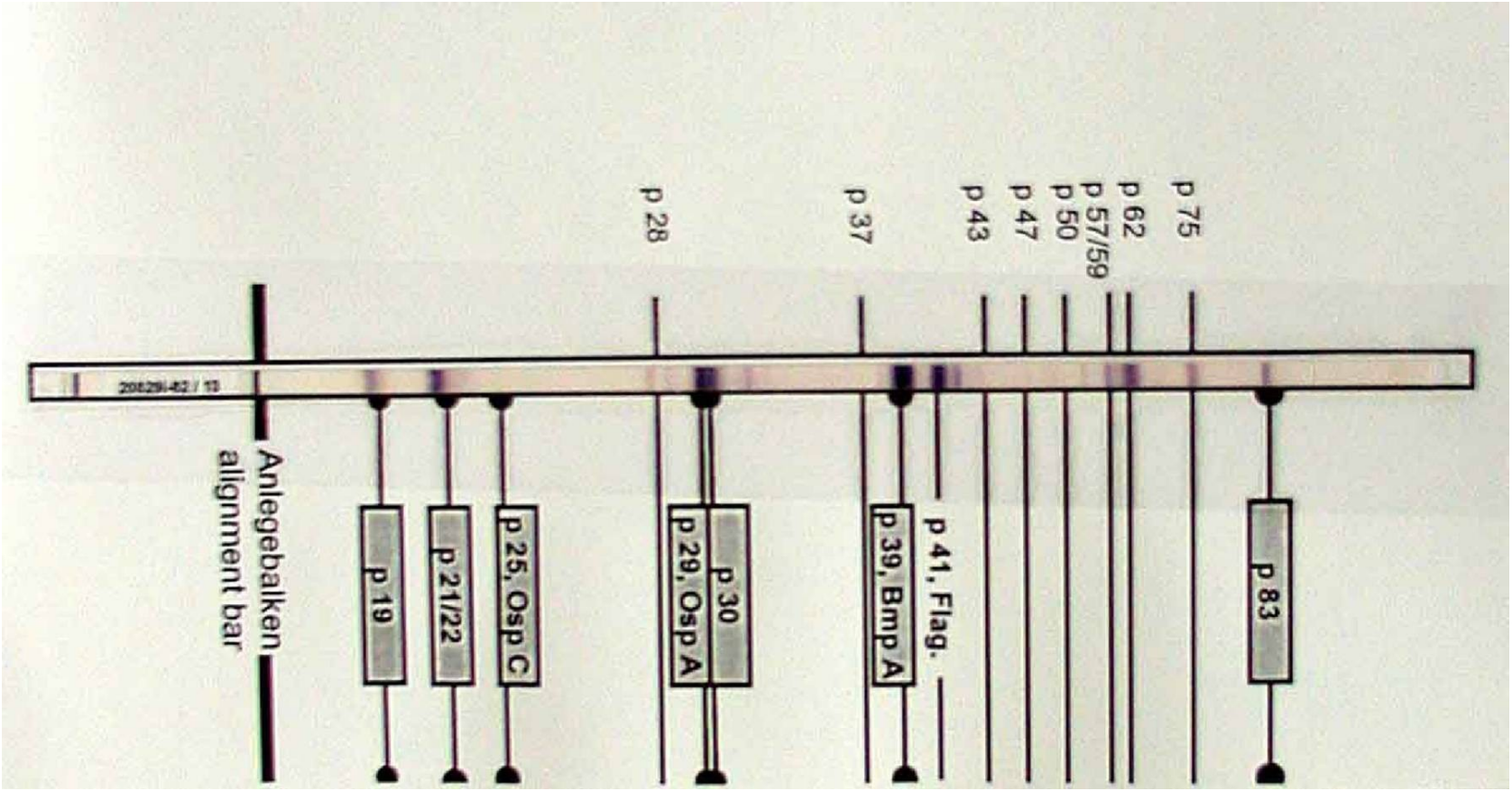
3: electroforetic division of antigen

4: „blotting“ of divided antigen to a nitrocellulose membrane

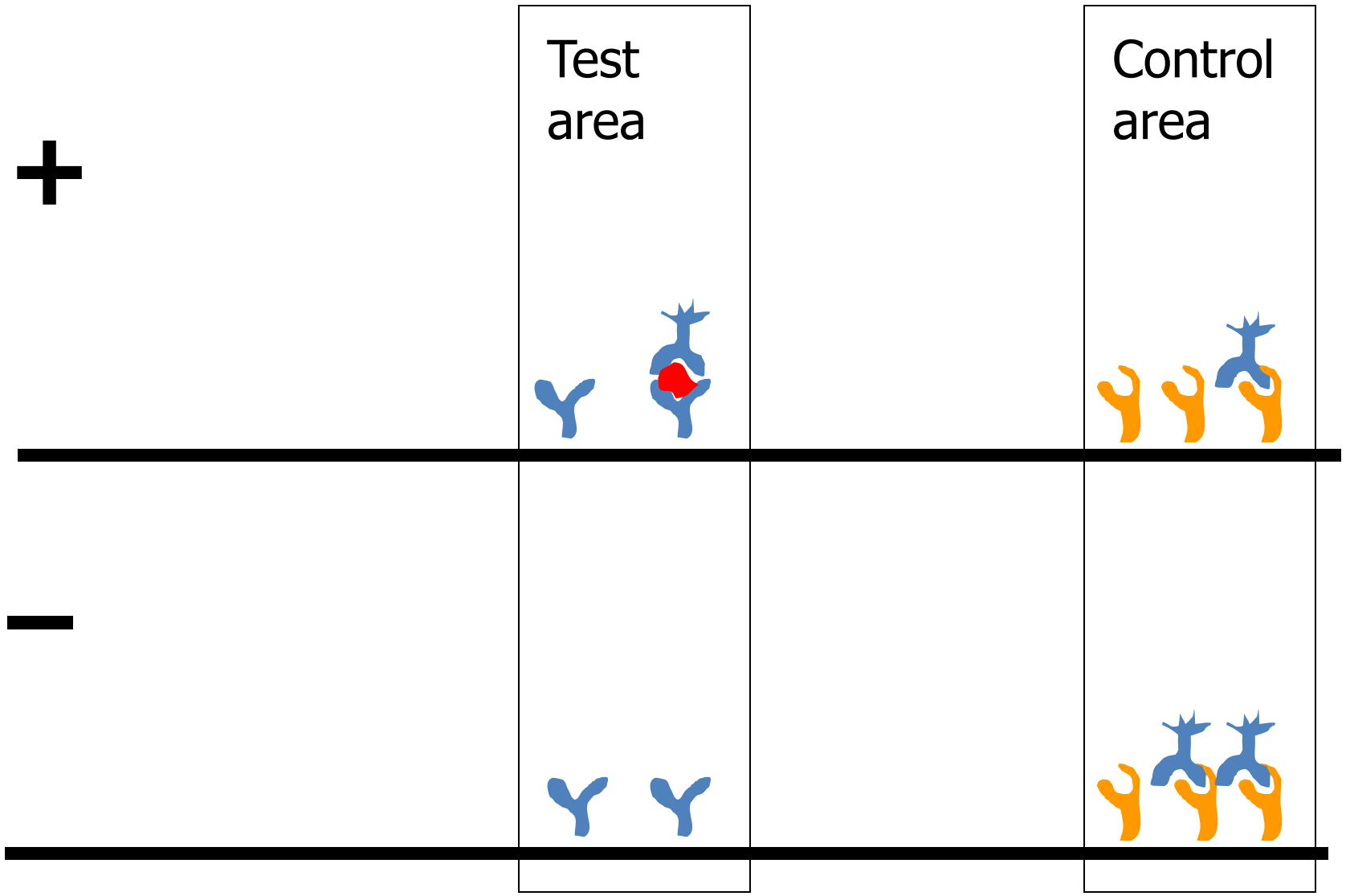
5: ELISA reaction (only some antibodies present)



Western blot – example
(picture from www.medmicro.info)



Immunochromatography



Important!

- It is necessary to know not only how to read the test, but also how to interpret it.
- There are two special tasks (concerning toxoplasmosis and Lyme disease) based on complex interpretation of all results including anamnesis!
  - E. g. pregnant woman with IgG anti-toxoplasmosis is NOT ill, but protected!

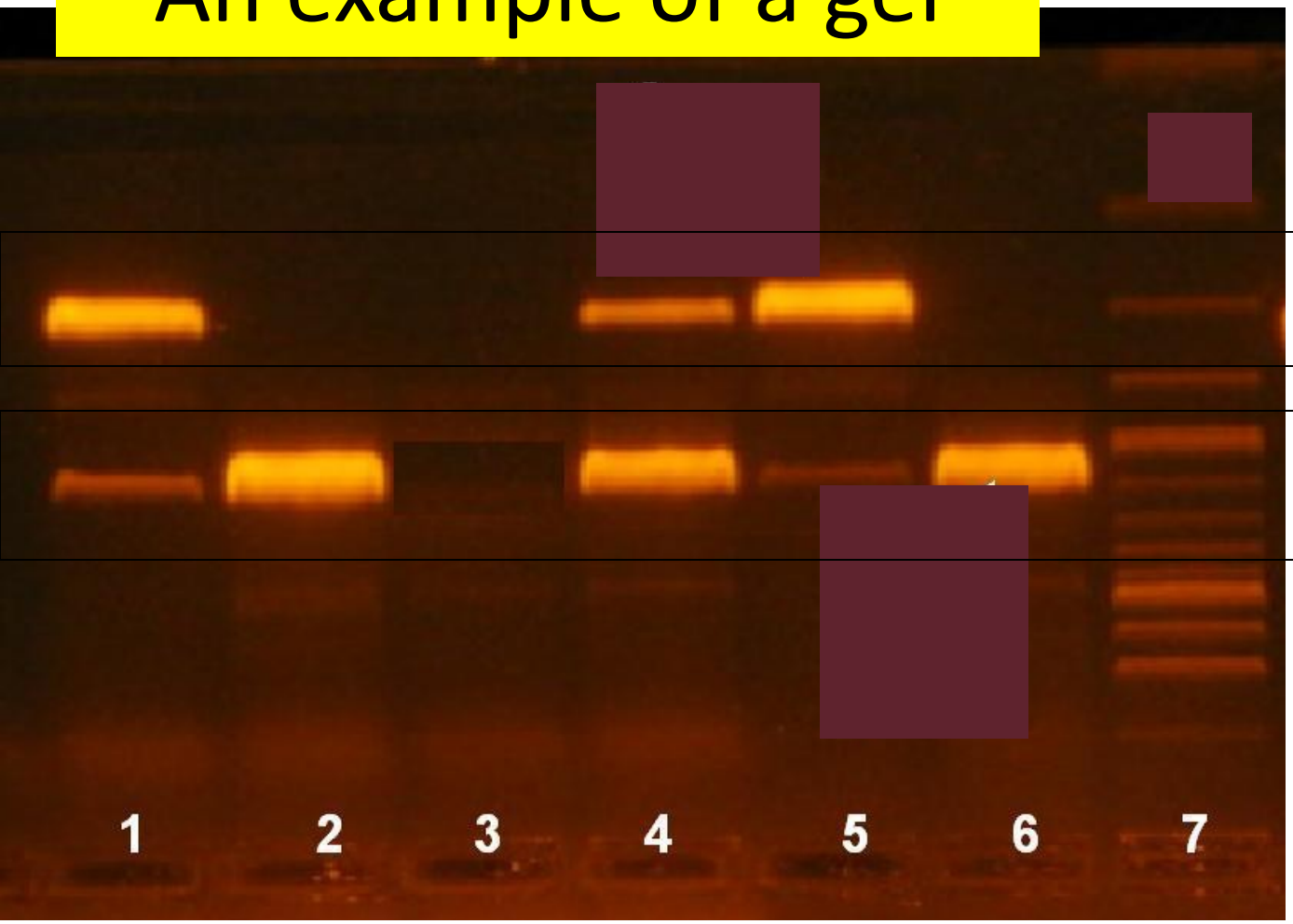
J09: DNA detection (PCR)
Mostly use of PCR in medical microbiology

- The methods are used mostly in situations, where microscopic and culture diagnostic is difficult or impossible
- It is not very useful for common, ubiquitous pathogens. Because of its sensitivity they would detect accidental molecules coming from environment
- The methods are neither useless, as some people think, nor all-problems-solving, as some other people suppose.

Survey of interpretation

Proper reaction	Internal control	Interpretation
negative	positive	negative
negative	negative	inhibition of reaction
positive	positive	positive
positive	negative	(highly) positive

An example of a gel



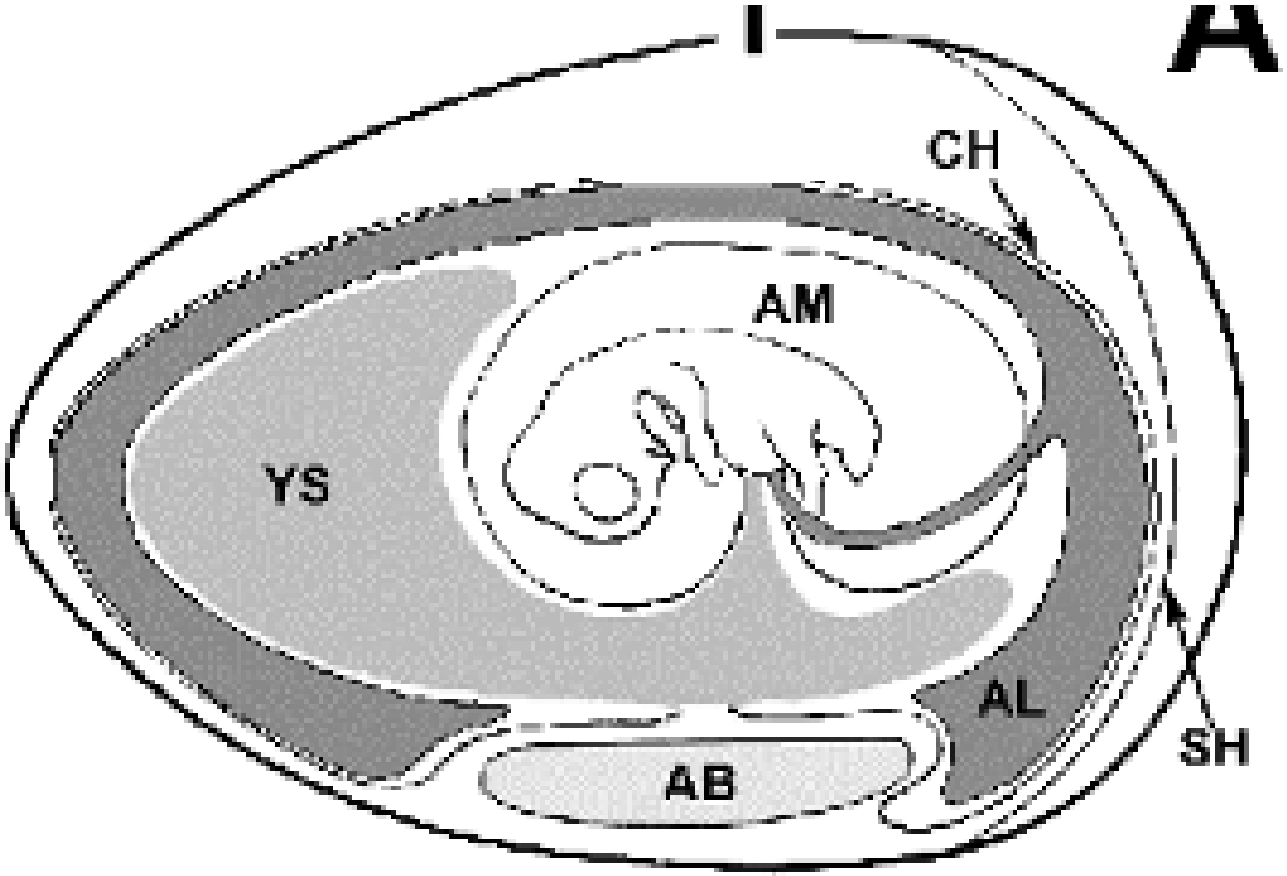
Patients 1 and 4 – positive, patient 2 – negative, patient 3 – inhibition of reaction. 5 – positive control, 6 – negative control, 7 – ladder

J10+11: viruses

- Majority of viral tasks are serological examinations (HBsAg, anti-HBs)
- Two extra virology tasks concern isolation of viruses
  - Fertilized egg – parts of fertilised egg, used for isolation
  - Cytopathic effect – what is it, how to find it

Fertilized egg

(+ how to get the info that virus is there)



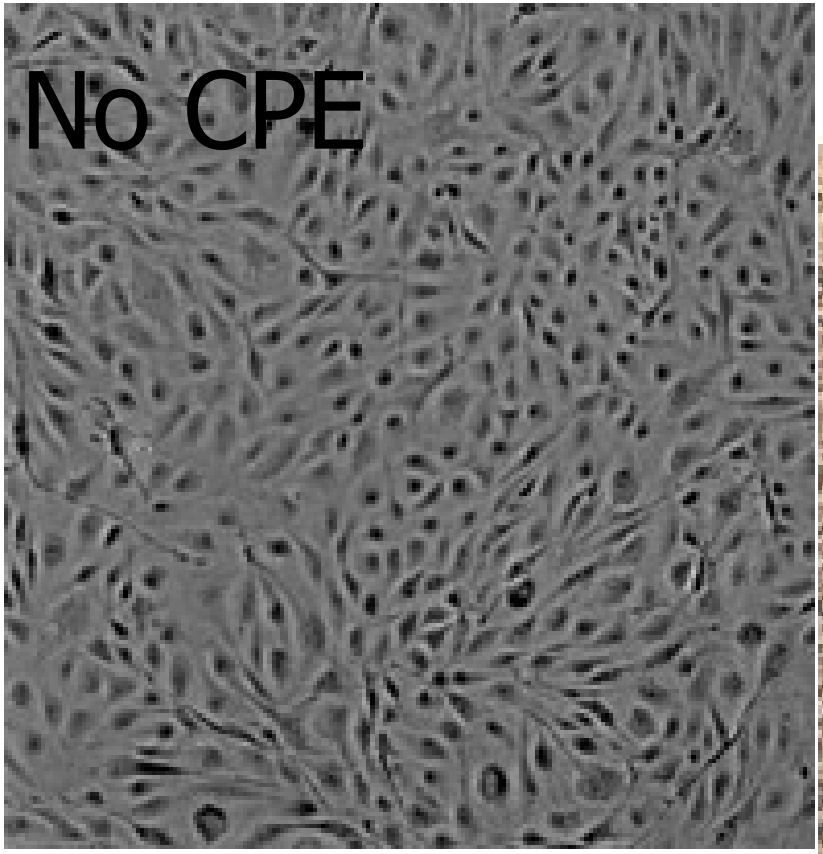
SH – shell membrane (paper membrane)

AB – albumen

http://www.scielo.cl/fbpe/img/bres/v38n4/fig02.gif

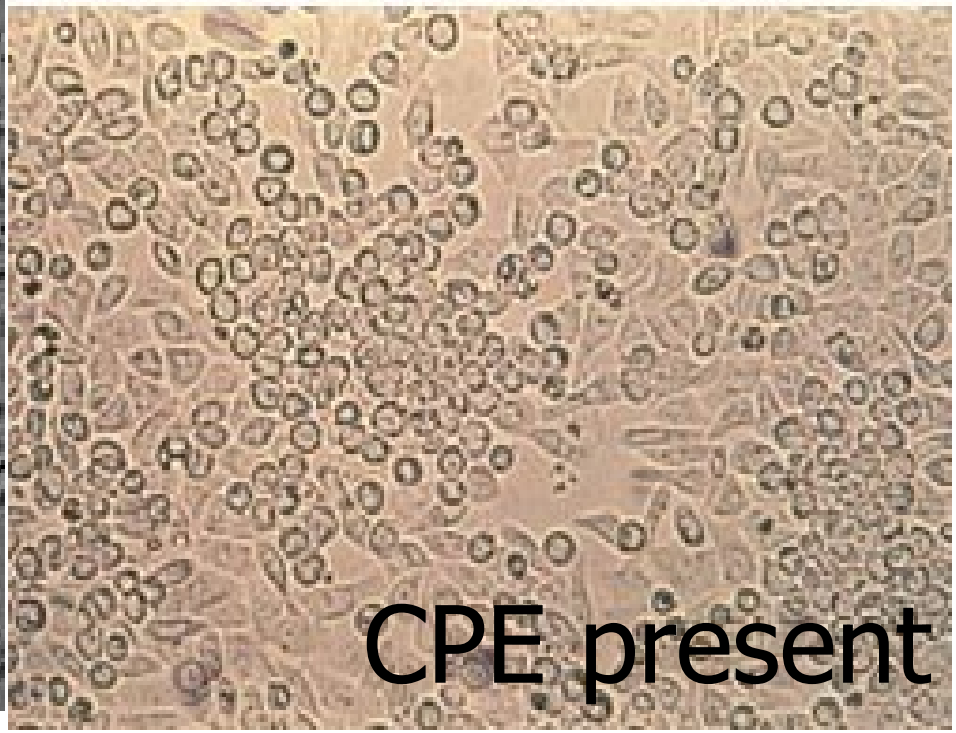
AM – amniotic sac, YS – yolk sac, AL – allantois

CH – chorioallantoic membrane (CAM)



http://cmir.mgh.harvard.edu/cellbio/cellculture.php?menuID_=122

HSV Growing in Tissue Culture



www.herpesdiagnosis.com/diagnose.html

(HSV is Herpes Simplex virus – HSV 1 causing mostly herpes labialis, HSV 2 herpes genitalis)

J12 Parasitology

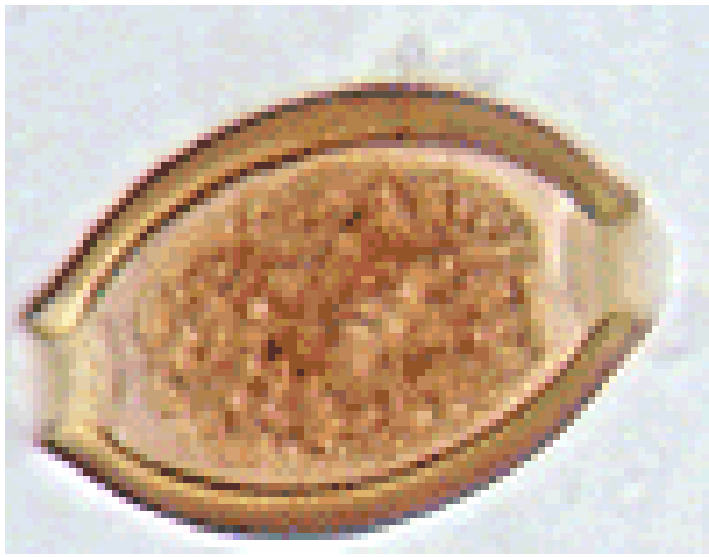
- As a basis, we use methods based on **modified wet mount:**
  - In **Kato method** counterstain with malachite green is used, to make parasites better visible
  - **Faust method** is a concentration one (see later)
- **Graham method** is used in pinworms only (and as one task you can do it practically!)
- *Wet mount „sensu stricto“ and stained preparations (e. g. trichrom) are used in increased suspicion for intestinal protozoa (either primarily, or after seeing Faust and Kato)*

Morphology of eggs of intestinal parasites

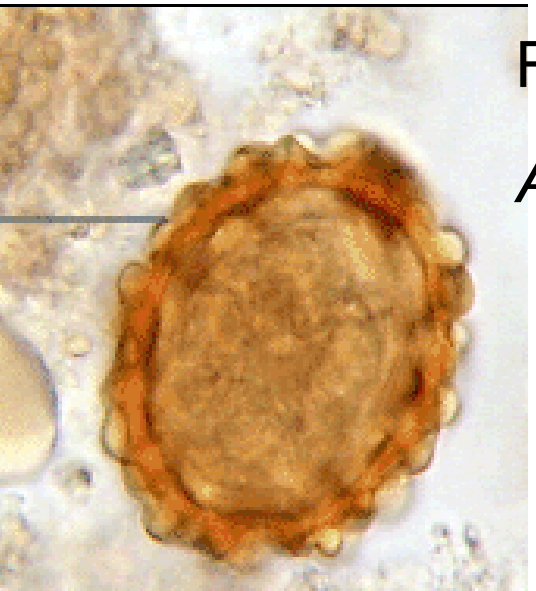
You should know at least these shapes to the examination – another task

Pinworm

Enterobius



Trichuris

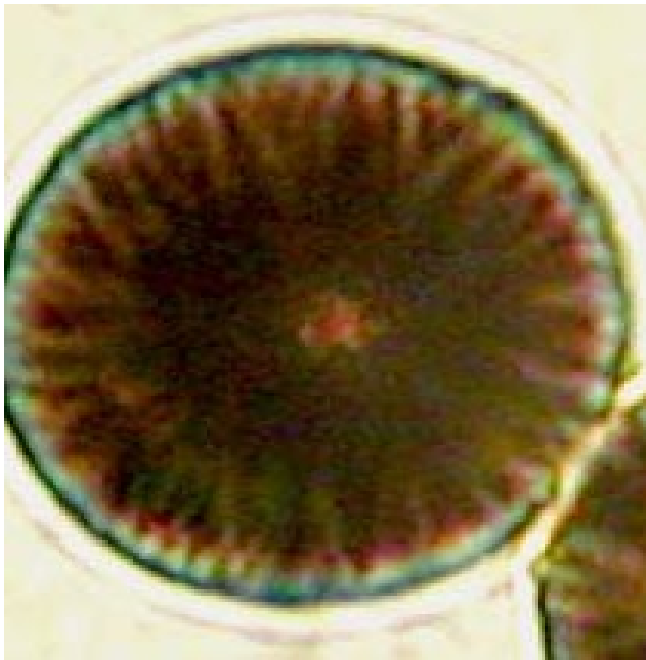


Roundworm

Ascaris

Tapeworm

Taenia



Pictures taken from CD-ROM „Parasite-Tutor“ – Department of Laboratory Medicine, University of Washington, Seatle, WA

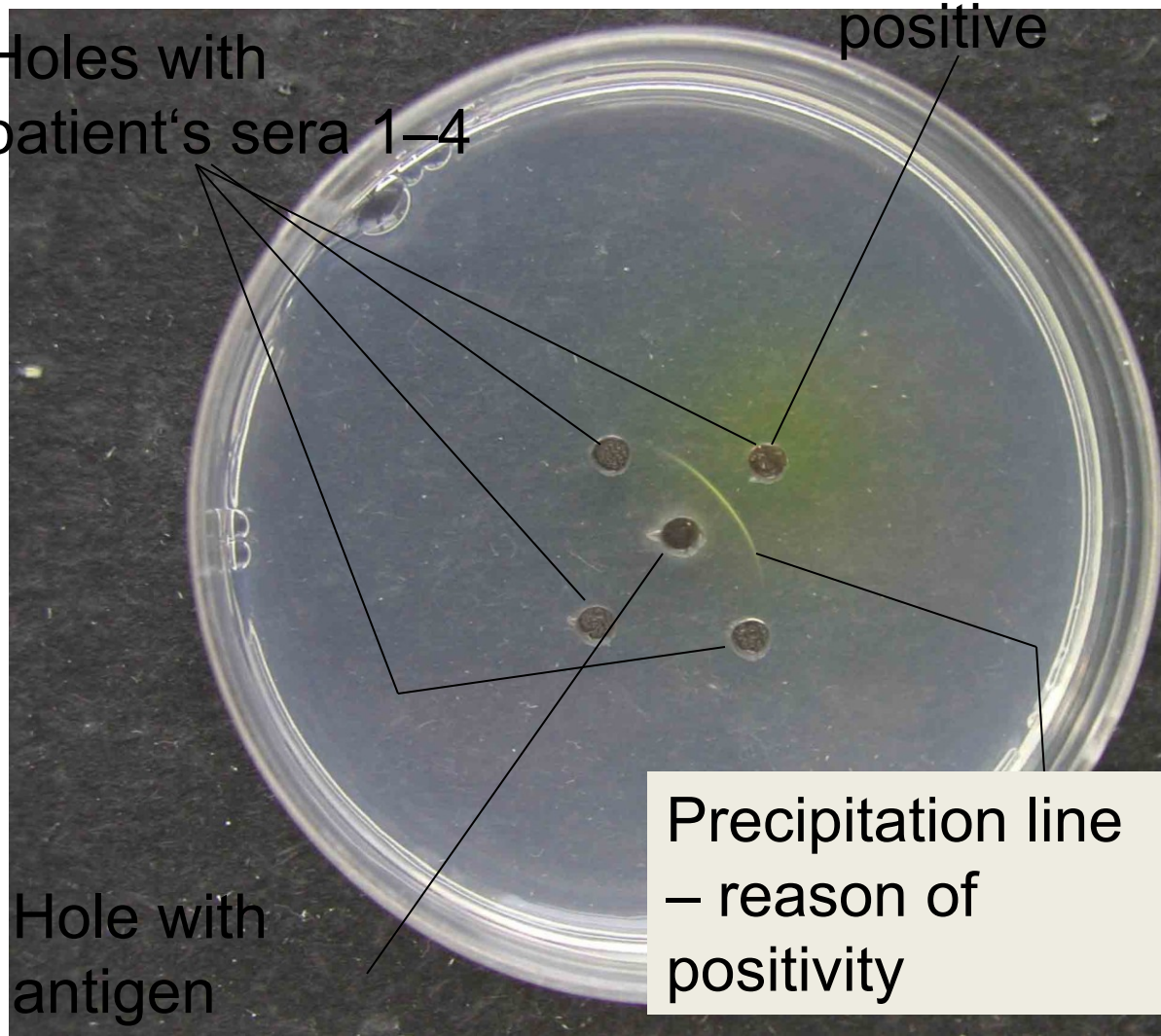
Toxoplasmosis – another task, including definition of patients

- P: healthy pregnant woman, cats at home
- Q: another healthy pregnant woman, no cats
- R: a young lady trekking in forest; no cats, but contact with objects contaminated by faeces of wild animals
- S: a senior, working in garden, cats use to walk through the garden, symptoms of retinitis + enlarged lymph nodes

J13 – mycology: two tasks

One is like P01–P06 (see later), another is this one

One of many ways, how to perform it, is microprecipitation in agar. It was already in J06. Precipitation line is formed between the hole with antigen and the hole with antibody

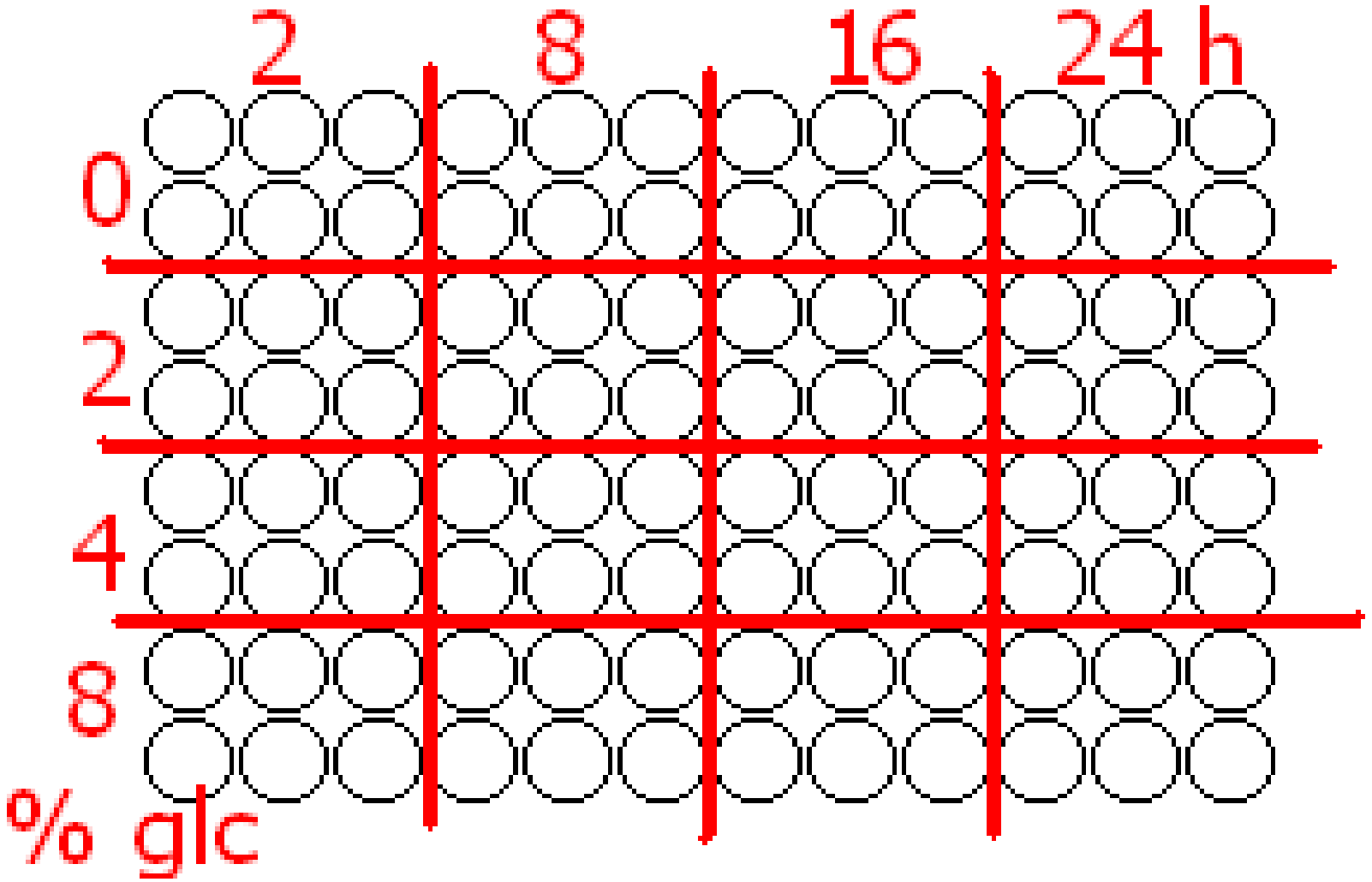


J14: biophilm
Influence of saccharides presence to dental plaque formation: like in practical, including 3D-graph making

- Assess the influence of uptake of various amounts of saccharides in food on rate of biofilm formation in a cariogenic *Streptococcus mutans.*

What are the conclusions of this experiment, as to amounts of saccharides in food, how long they stay in oral cavity etc.?

Wells in the panel



Another task: MBEC assessment

MBEC … minimal biofilm eradicating concentration

Photo: Archive of Veronika Holá

P01 to P06, J13

- There is a universal task to those topics:

„Among given strains, find strain(s) of Xxxxx, perform more detailed diagnostics (and perform antibiotic susceptibility testing).

- It is necessary to follow the logical algoritm, for example like this:

Gram staining → catalase test → plasmacoagulase test → STAPHYtest 16 etc.

Exceptions:

- ASO is examined as other serological tasks (but important to know the meaning of the test for clinical practice)
- G+ rods are not examined in this algorithmic way, but you get pictures and you have to say „this looks like Corynebacterium, this does not look like Corynebacterium, because it is spore forming“ etc.
- Very similar is also a task to *Clostridium tetani* (to P07)

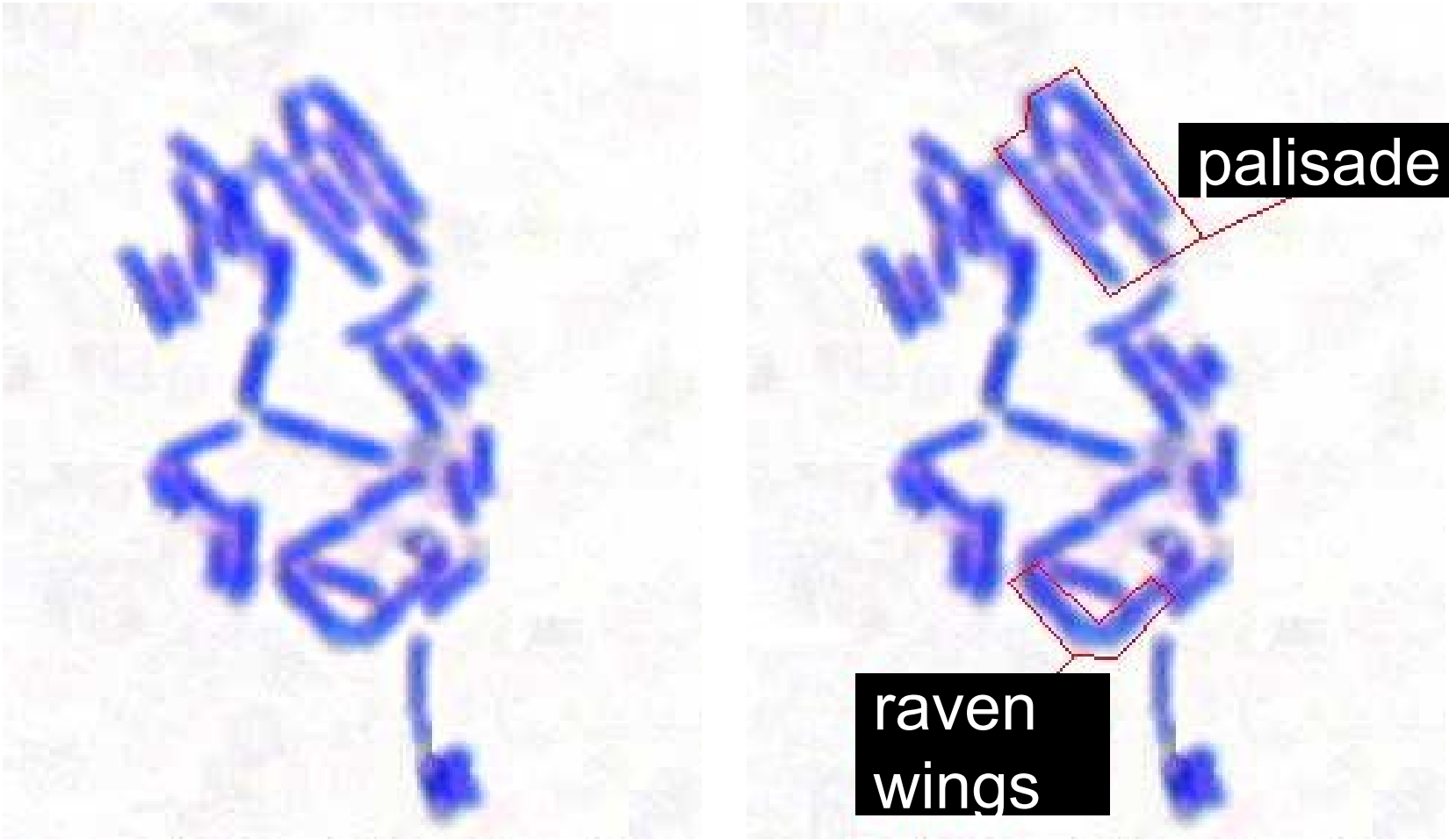
Survey of diagnostics (simplified)

(or other tests)

Enterococcus or

http://www.ratsteachmicro.com/Staphylococci_Notes/HCOE_CAI_Review_Notes_Staphylococci.htm

Corynebacteria, forms



www.medmicro.info, Photo O. Z.

J07 Anaerobic jar

description, explaining function

air-proof lid

palladium catalyser
(beneath the lid)

construction for placing
of Petri dishes

Anaerobiose generator
(packet with chemicals)

Anaerobic box (again)

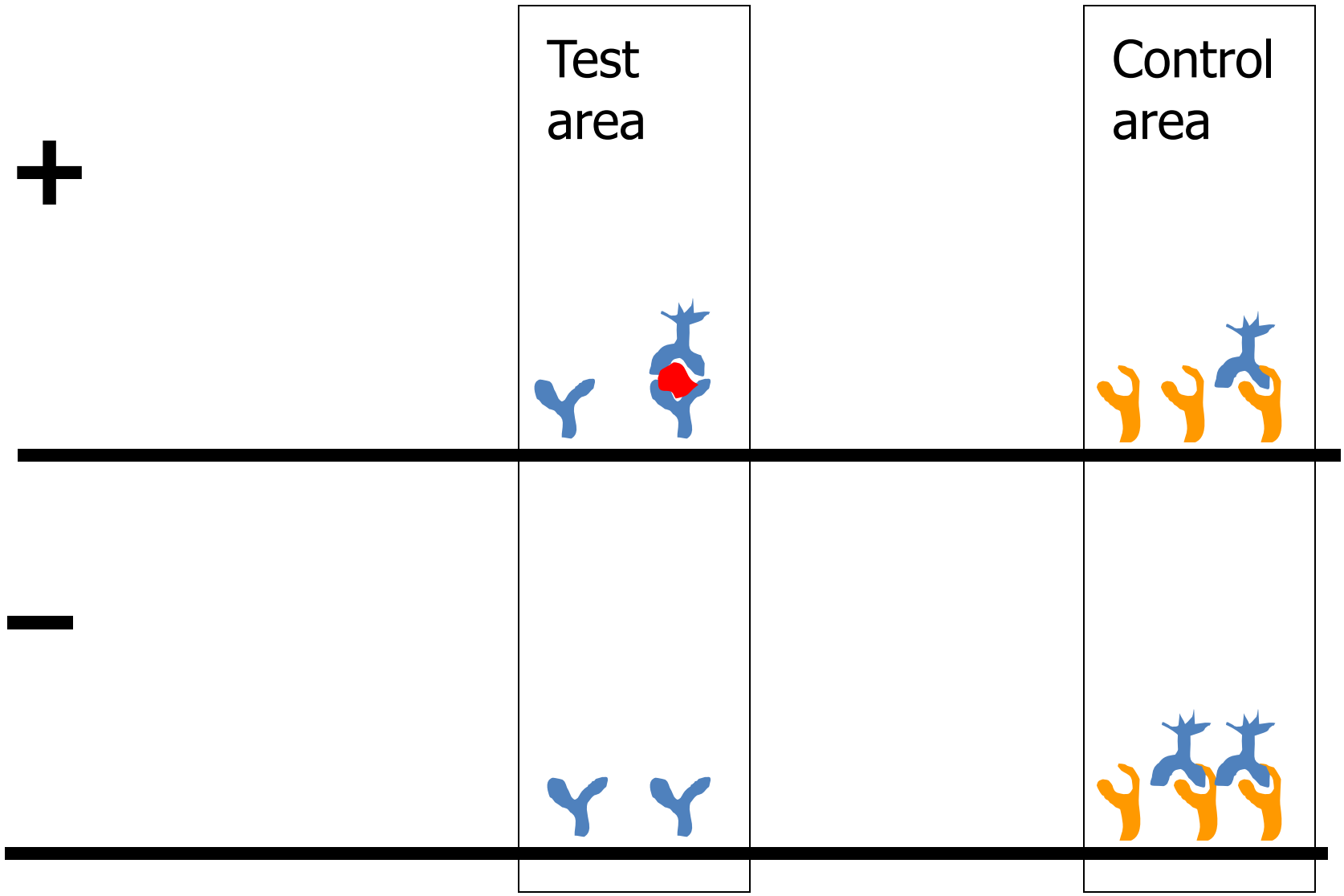
www.medmicro.info, photo O. Z.

Detection of lecitinase

- Lecitinase production is detected as strain precipitation on the yolk agar. Nevertheless, there are many lecitinases, and one only, that of *Clostridium perfringens* is interesting for us, we have to test, whether the lecitinase bay be inhibited by a specific antitoxin.

„Negative I“ no lecitinase production.
„Negative II“ a lecitinase is produced, but not the tested one

Detection of *C. difficile* toxin



Survey of other tests, e. g. animal experiment

- Look at the picture of tetanic mouse

Drawing by Petr Ondrovčík (1959–2007)

Graphically adapted.

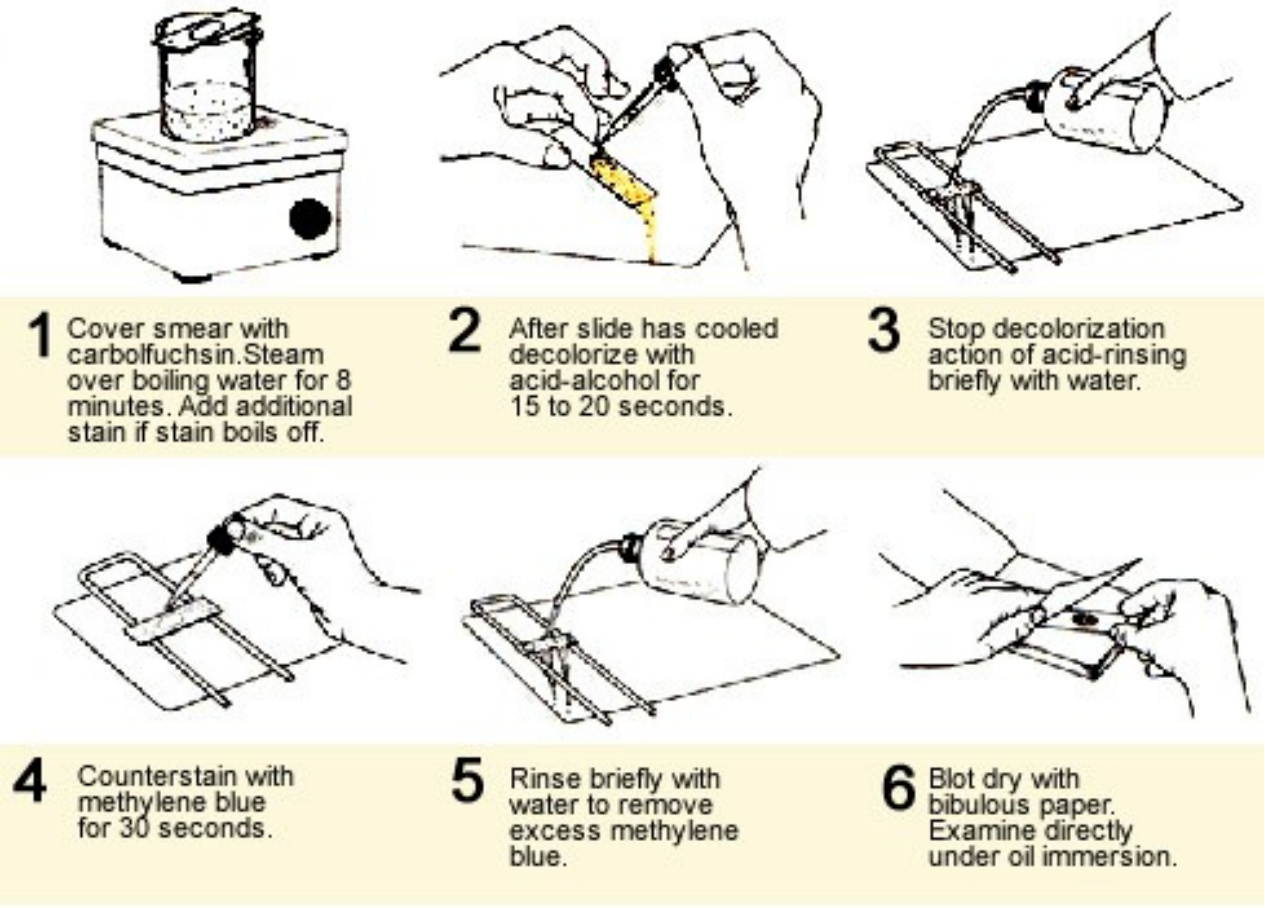
Backgound counterstained using not malachit green, but „Paint“ programme by Microsoft

www.biotox.cz

Opistotonus is typical both for mice and humans

P08: Ziehl-Neelsen stain:

principle and knowledge about results (red bacteria on blue or green background)



Ziehl-Neelsen acid-fast staining procedure

Ziehl-Neelsen stain

Another task: Culture of mycobacteria

- Hydroxide should be used before culture
- We use liquid Šula or Banić media and egg Ogawa or Löwenstein-Jenssen media. Egg media are solid because of egg white coagulation, they do not contain agar
- Results are read after 1 (check for contamination) 3, 6 and for sure after 9 weeks of culture. (Positive results are mostly found after 6 weeks of culture.)
- *+ knowledge of more methods (PCR etc.)*

Appearance of mycobaterial colonies

http://www.stockmedicalart.com/

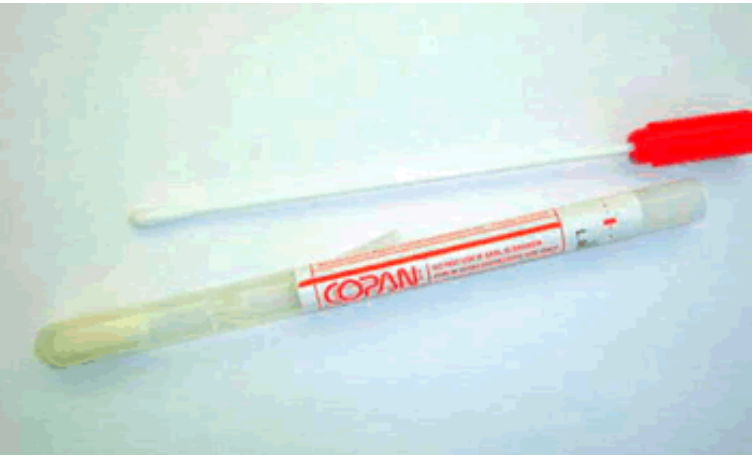
P09: complete serology, plus knowledge of screening vs. confirmation tests

Historical	BWR – Bordet Wassermann	Nontr.
Screening	RRR – Rapid Reagin Test or RPR or VDRL test	Nontr.
	MHA-TP (TPHA)	Treponema
Confirmatory	ELISA	Treponema
	FTA-ABS (indir. imunofluor.)	Treponema
	Western Blotting	Treponema
Historical, or superconfirmation	*TPIT (Treponema Pallidum Imobilisation Test) = Nelson*	Treponema

P10–P13: Clinical microbiology I-IV

- Several tasks, all of them the same:
- „For three minicasuistics, find suitable sampling methods and vessels/swabs for sampling“
- Knowledge of swabs and vessels necessary

Some types of swabs



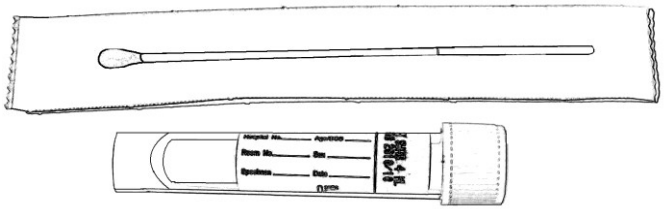
Plain (dry) swab
www.calgarylabservices.com

Today its use is for PCR and antigene detection only, not for culture!

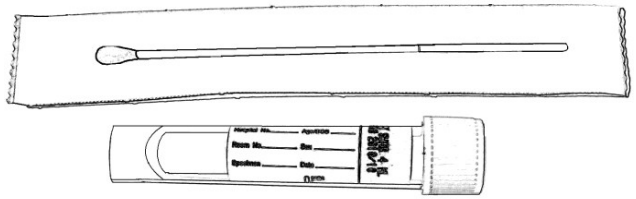
Amies medium with charcoal www.herenz.de

Universal transport medium for bacteriology (all types of swabs). The wire variant important, if we want to go „behind the corner“

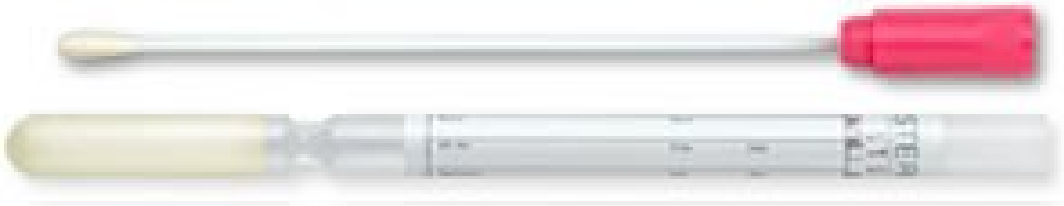
More swabs



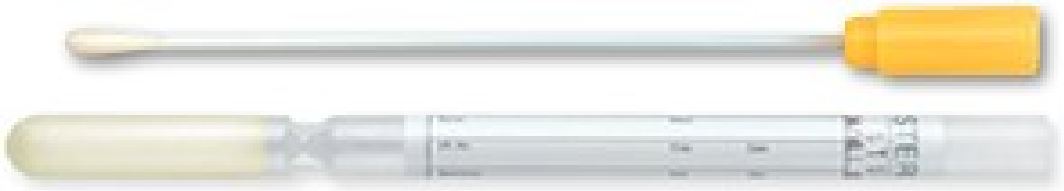
Fungi Quick (for yeast and molds)
www.copanswabs.com



C. A. T. swab (for Candida And Trichomonas, from genitals only www.copanswabs.com

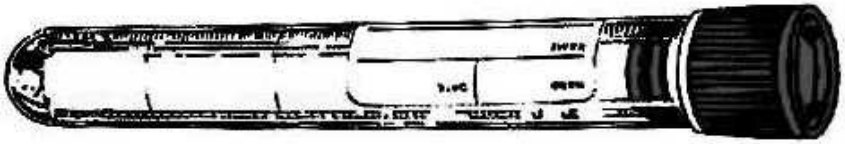


Virus swab
www.copanswabs.com

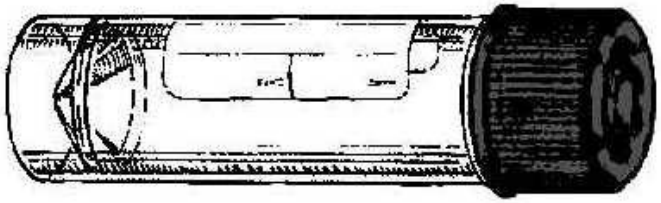


Chlamydia swab
www.copanswabs.com

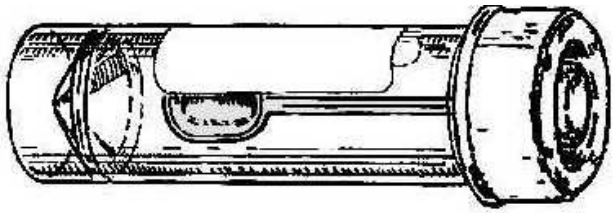
Vessels



Common test tube. Universal use: clotted blood (serology), urine, CSF, pus, punctate etc.; blood and urinary cathethers, parts of tisue…



Sputum vessel. Not only for sputum, but also larger parts of tissue etc.



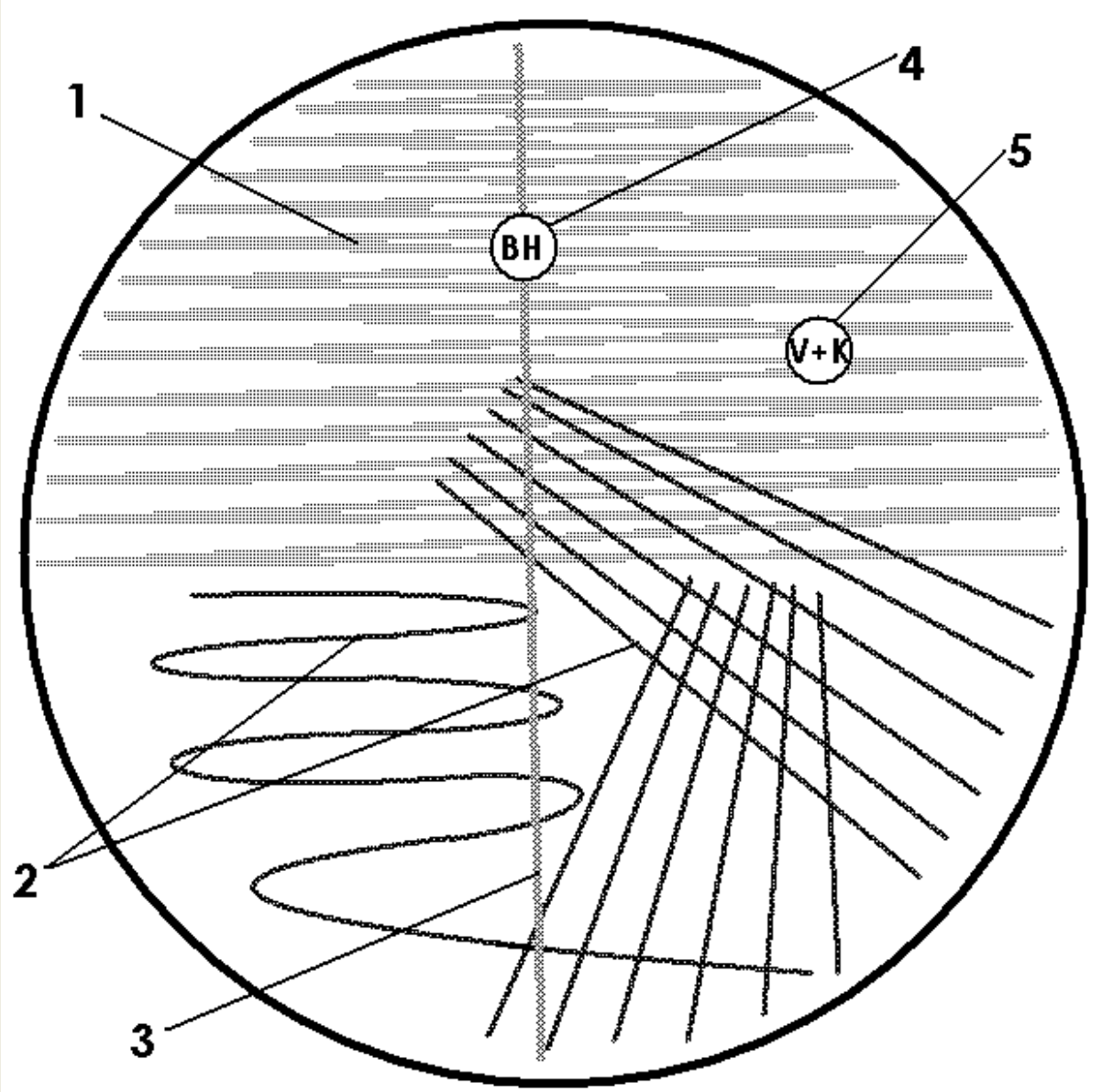
Stool vessel, for parasitology. Only this one does not have to be sterile!

Vessel for urine sampling. It is better, if the patient urinates just into a test-tube, but especially for women it is difficult (except if they are in shower). So they can urinate into this vessel, and then a nurse removes the urine into a test-tube.

Clinical microbiology (continuing)

- Several more tasks. One: Find a pathogen in oropharyngeal flora
- Normal flora consists of greyish, viridating colonies (oral streptococci) and yellowish, usually non-haemolytic colonies (oral Neisserias). Possible pathogens are:
  - Haemolytic streptococci (and also *Staphylococcus aureus*) are visible by a strong haemolysis on blood agar
  - For haemophili detection we use antibiotic disc with bacitracin – higher concentrations than in bacitracin test (to decline the normal micro flora) – plus *Staphylococcus* line
  - For meningococcal detection we use another disk, with mixture of vankomycin and colistin

Detection of pathogen in throat/sputum



1 swab inoculation

2 loop inoculation

3 staphylococcus line

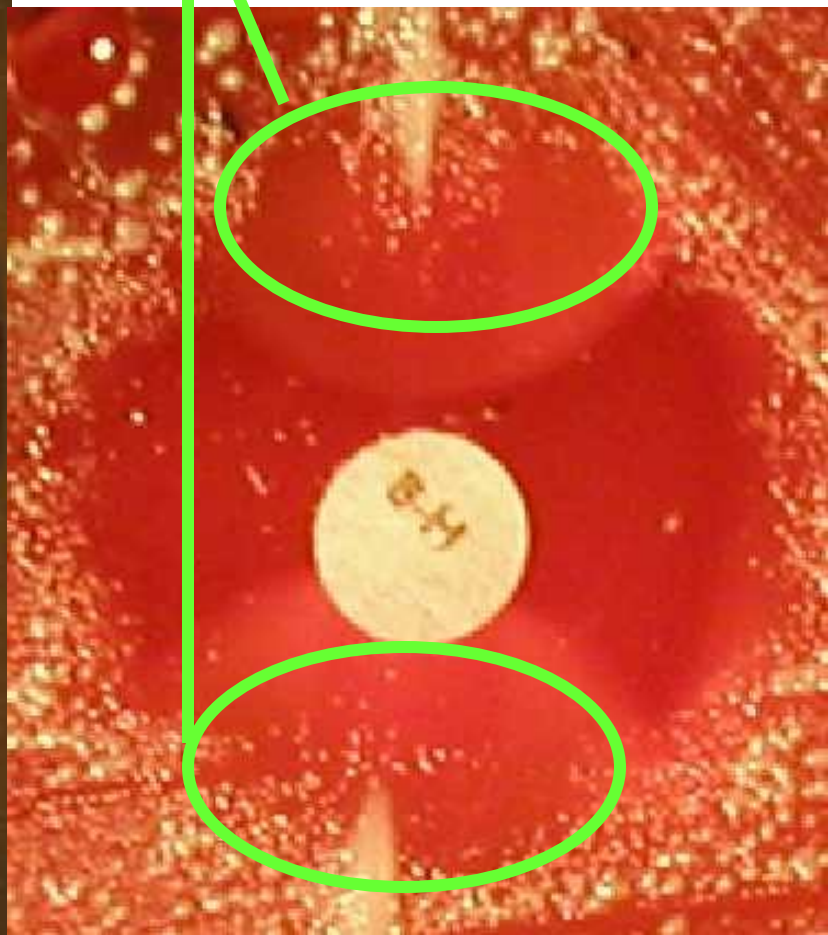
4 bacitracin disc (for hemophili)

5 V + K disc (colistine and vancomycine) for meningococci

In all parts of inoculated area we search for colonies with haemolysis. They could be streptococci (rather colourless) or goldish)

Cultivation result of throat swab with common flora

In these sites we search for haemophili



Another task: Urine

- **Task: Perform semi quantitative and qualitative examination of urine**
- As likely contamination (or accidental finding) is counted everything below 10^4 / ml, everything below 10^5 / ml in finding of two various bacteria and everything in three/more bacterial strains
- Among pathogens, the most common are enterobakterie, Enterococcus, *S. agalactiae*, staphylococci etc.

Semiquantitative processing

- A **plastic loop** is used – the „eye“ of the loop catches always 1 µl of urine
- This microlitre is inoculated to one **half of blood agar plate** (you have it on a **total plate**)
- Further **we inoculate Endo agar or URIchrom**, here we assess it only qualitativelly
- Of course, besides quantity examination we also examine genus and species of the bacterium as usually
- **In our case, we would recommend nitrofurantoin for treatment.**

Semiquantitative urine evaluation

Number of colonies	Number of CFU (bacteria) in 1 µl of urine	Number of CFU (bacteria) in 1 ml of urine	Evaluation (valid for 1 bacterium)
Less than 10	Less than 10	Less than 10^4	Contamination
10 – 100	10 – 100	10^4 - 10^5	Borderline
More than 100	More than 100	More than 10^5	Infection

Some more tasks

- Examination of stool (observation of result on various media)
- Examination of vaginal smear, Nugent score counting
- Examination of vaginal swab (culture)
- Examination of wound swab (sampling, moulage-imprint method)
- Examination of blood cultures (microscopy + culture)

See you at the examination!

www.medmicro.info