

# Searching for microbes Part VI.

## Testing of microbial susceptibility and their resistance factors

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To practical of VLLM0421c

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# Content of this slideshow

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Mechanisms of action of atb-s and mechanisms of resistance

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Methods of assessment of resistance factors

# Tale

- There was an **antibiotic**, let's call him ampicilline. And it decided to go out and to help people to fight with microbes.
- It was a brave antibiotic, decided to destroy any microbe, that would be found around. Nevertheless...

# Ampicilline meets microbes...

- The first microbe was called *Proteus mirabilis* and really it was destroyed by ampicilline. Ampicilline was pleased by that, and continued it's trip.
- But it met another microbe. *Klebsiella pneumoniae*, and it was disgusted. Ampicilline said: Oh, no, **Klebsiella, I cannot destroy you, you are primarilly resistant to me**, I can do nothing... I will continue my trip, and I will send you a more effective brother to destroy you...

## And the third meeting

- Ampicilline met a microbe called *Escherichia coli*. It is OK, it said to itself, I could destroy it. Nevertheless... *Escherichia* escaped, and giggled: Ha, ha, you thought you can destroy me! Well, some time ago, I was susceptible to you, but then I have got **secondary resistance**, and so you can never catch me!

## Ampicilline was very sad...

- But a microbiologist came and told him: don't worry, be happy, ampicillin, next time we'll into better. **Each microbe, that could be either susceptible or resistant to you, should be tested,** to see the situation. And if you will be no help? One of your bros will help us!
- So ampicilline dried its tears, and run to help people again.

## What to learn from our tbut

- Some microbes are primarily resistant to some antibiotics. It has no sense to test their antibiotic susceptibility – it is zero.
- Other microbes may (not necessarily) get **secondarily resistant**. Then
  - either we test **microbial antibiotic** in vitro **susceptibility** to the given antimicrobial agent
  - or we search for a certain **factor, responsible for the microbial resistance**

# Antimicrobial agents – overview



# Methods of „fight“ with microbes

- **Immunisation** – exploits natural mechanisms of a macroorganism
- **Decontamination methods** – crude physical and chemical influences, action outside the organism (see last practical)
- **Antimicrobial agents** – fine, targeted action inside the organism with aim of maximal effect of the microbe and minimal influence on the host macroorganism

# Types of antimicrobial agents

## Agents acting to the whole body:

- Antiparasital agents against parasites
- Antimycotics against yeasts and molds
- Antivirotics against viruses
- Antituberculotics against mykobacteria
- Antibiotics against bacteria (natural origin)
- Antibacterial chemoterapeutics also against bacteria, but syntetic

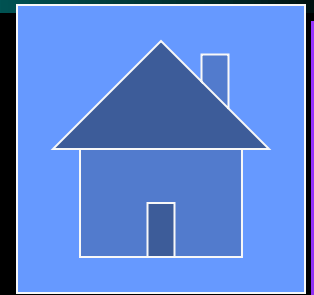
First antibiotic was penicillin, derived by A. Fleming



*In recent period, the last two groups are often put into one group called „antibiotics“*

## Locally acting agents: antiseptics

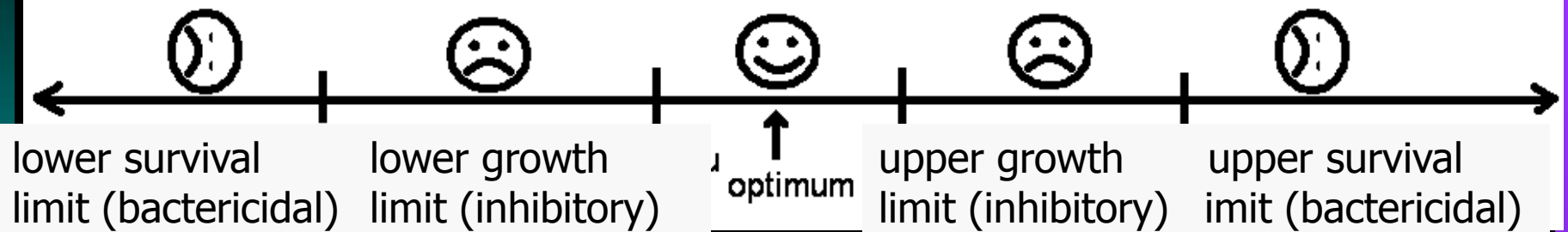
# Decontamination, or an antimicrobial agent?



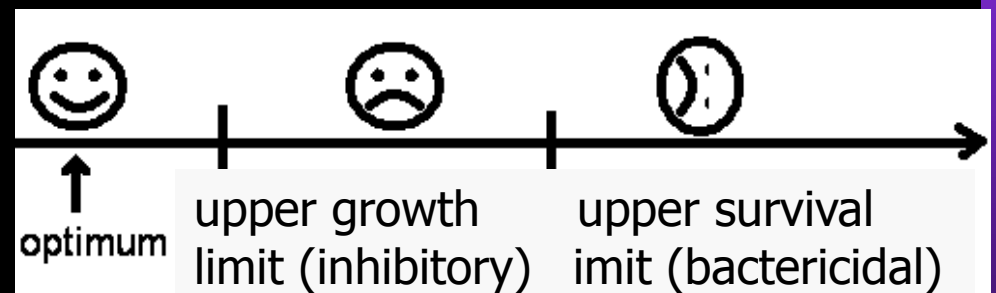
- The **borderline** between decontamination methods and antimicrobial agents is **not sharp**.
- Usually **action on intact skin** is still considered to be a decontamination.
- **Application into the wound** means already use of an antimicrobial agent (antiseptic)
- It has its **legal consequences**: decontamination agents have more simple system of certification than antimicrobial agents (and other drugs)

**MIC, MBC,  
bacteriostatic  
and bactericidal  
antibiotics**

# Action of certain influences on microbes I



- At action of an influence like pH the axe has both upper and lower extremes



- At action of antimicrobial agents only right part of the axe has a logical sense

## Action of influences on microbes II

- At **decontamination** we insist on **killing microbes** (microbicidal effect)
- At **use of antimicrobial agents** we can count with cooperation of patient's immunity, therefore **even microbistatic (inhibitory) effect** is usually sufficient
- This is not valid for **acute states or immunocompromised patients**, where we try to ensure **microbicidal action always**

# MIC and MBC

**MIC – minimal inhibitory concentration** is a term, that is used in antibiotics for growth (multiplication) limit of a microbe

**MBC – minimal bactericidal concentration** is a term used in antibiotics for survival limit of a microbe. (For simplicity, we talk about bacteria only. In viruses, we would use term „minimal virucidal concentration“ etc.)

*Later, you will meet also terms MBIC and MBEC, that are connected with action of antibiotic in biofilm*

Primarily bactericidal and  
primarily bacteriostatic antibiotics

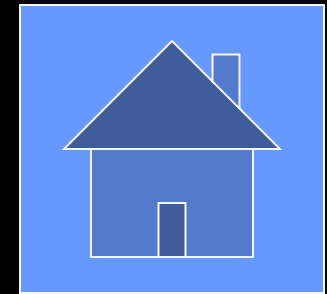
Primarily bactericidal are atb-s, where MIC  
and MBC are nearly equal

Primarily bacteriostatic are atb-s, where  
values over MIC, but not over MBC are  
exploited (they are inhibitory in substance)

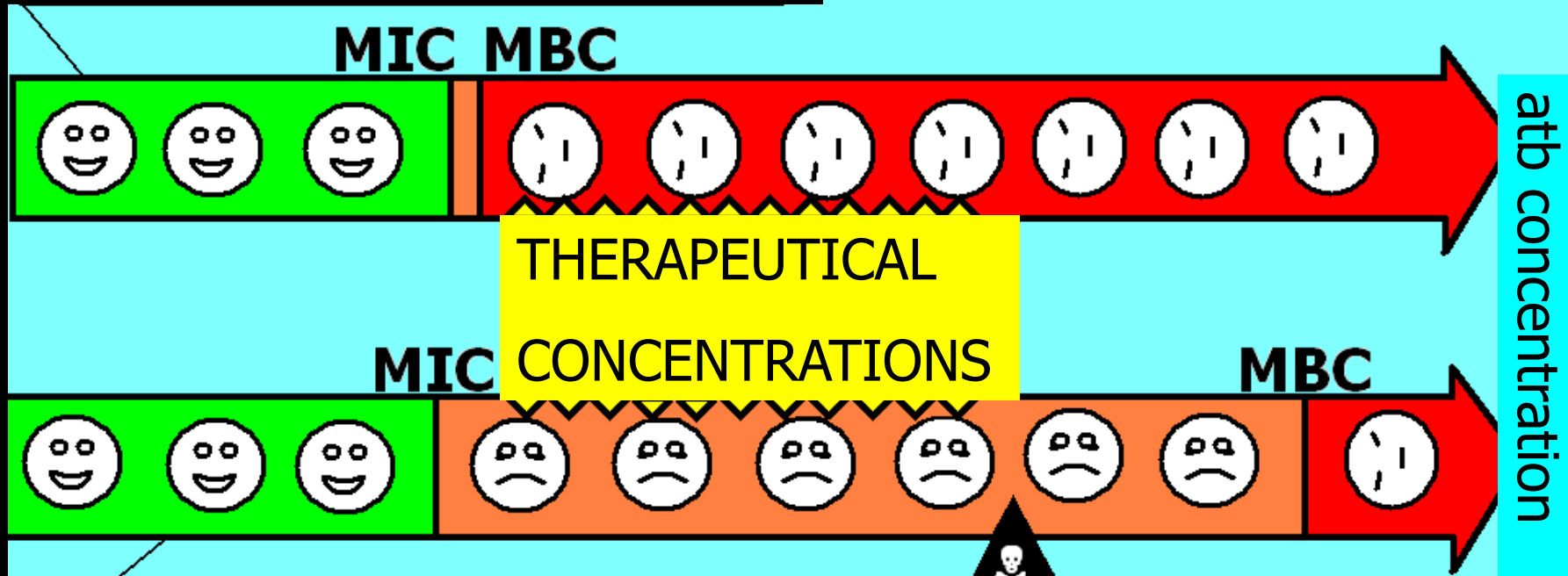
**In practice, never use bacteriostatic  
antibiotics in serious accute states,  
imunosupressed patients etc.**



# Primarily bactericidal and primarily bacteriostatic atb



Primarily bactericidal antibiotic



Primarily bacteriostatic antibiotic

**Mechanisms  
of action and  
mechanisms  
of resistance**

# Mechanisms of antibiotic action

- **To the cell wall (bactericidal)**
  - Betalactamic antibiotics
  - Glycopeptidic antibiotics (partially)
- **To cytoplasmic membrane** – polypeptids (bactericidal)
- **To nucleic acids** – quinolones (bactericidal)
- **To proteosynthesis:** aminoglykosides (bactericidal); makrolids, tetracyclins, linkosamids, amphenicols (bacteriostatic)
- **To metabolism** – sulfonamids, bacteriostatic

# Microbial resistance to antimicrobial agents

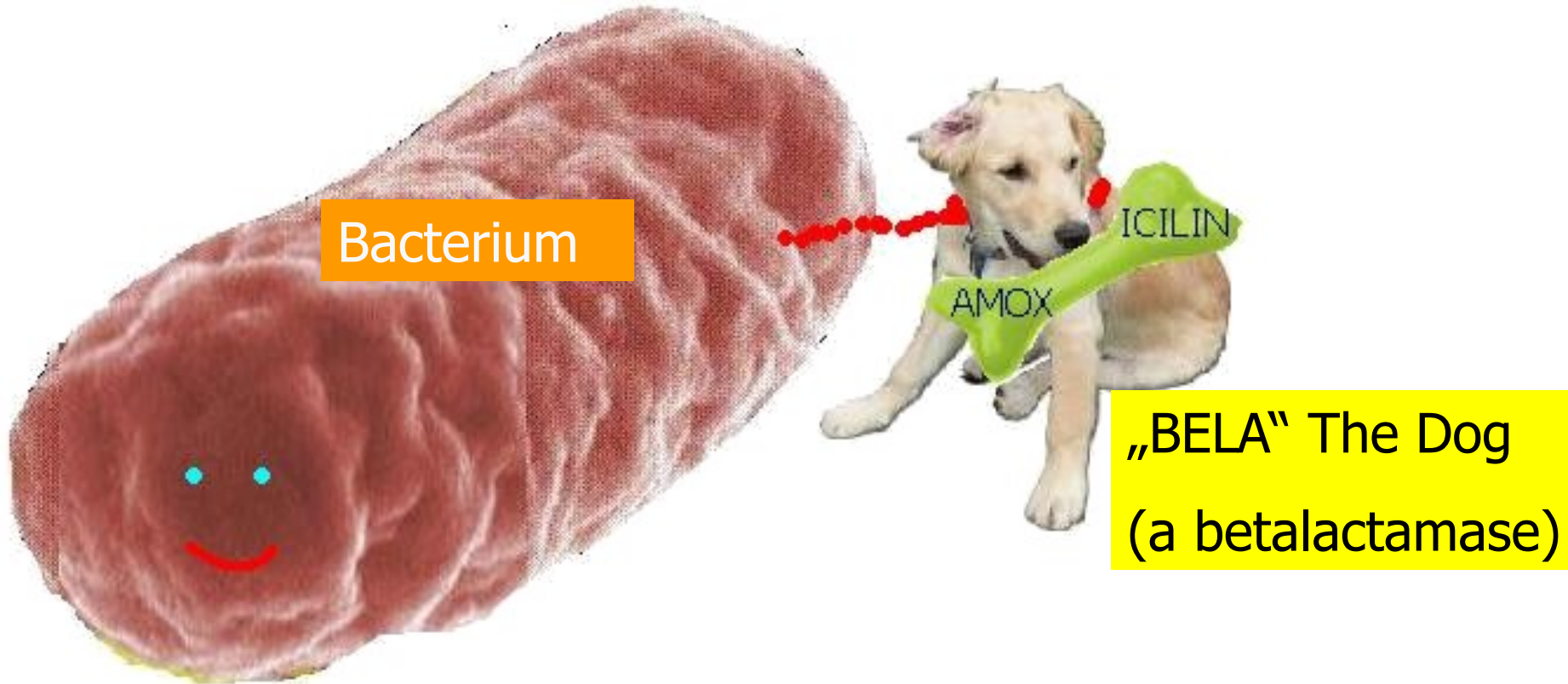
- **Primary resistance:** all strains of a given species are resistant. Example: betalactamic atb do not act on mycoplasmas, that do not have any cell wall at all.
- **Secondary resistance:** non-susceptible mutants raise, and under selection pressure of an antibiotic they start to be in majority. (*Escherichia* may be susceptible to ampicilline, although in recent period, resistant strains become very common)

# Mechanisms of resistance

- Blocade of entrance of an atb into the cell
- Active efflux of an atb from a cell
- A false receptor is offered to an atb
- Microbes split antibiotics enzymatically (e. g. betalactamase split betalactamic antibiotic)

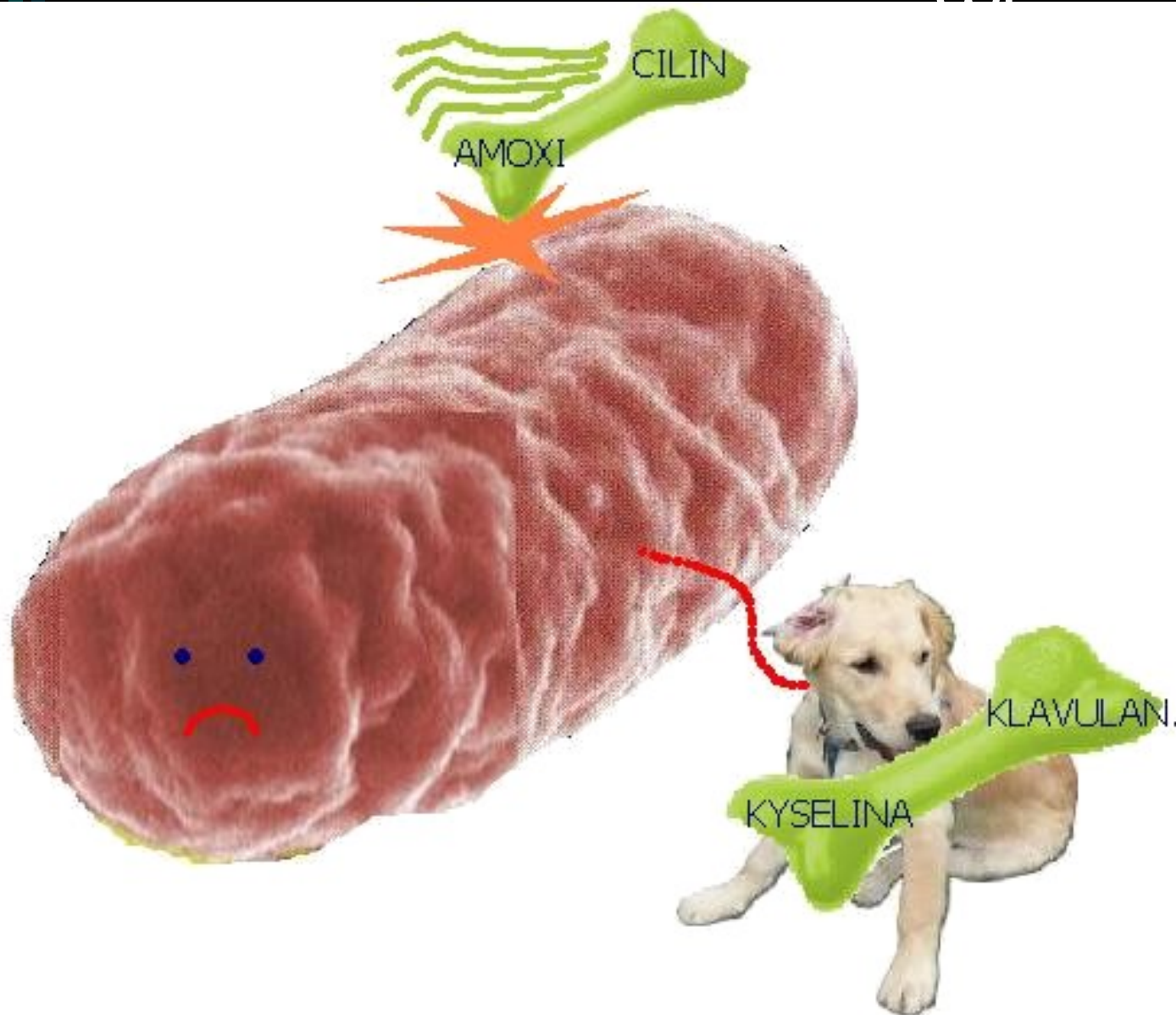
*Knowledge of the resistance mechanism enable us to try to defence ourselves against such a resistances.*

# Betalactamase inhibitors – 1



when we act by a single antibiotic, it is inactivated by a bacterial betalactamase.

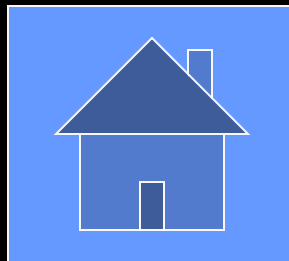
# Betalactamase inhibitors – 2



Beta-  
has a  
active  
to  
is used  
and the  
can act.



# Examples of antibiotics potentiated by betalactamase inhibitors



From FN USA intranet





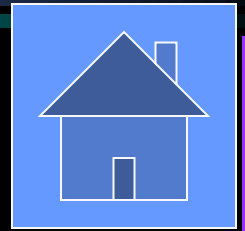
Epidemio-  
logically  
important  
resistances

## Epidemiologically important resistances – 1

- **MRSA** – methicillin resistant staphylococci. Oxacilin or other beta lactams are not able to enter their cells. Many MRSA are also resistant to more antibiotics (macrolids, lincosamids). Glycopeptids (vancomycine, teicoplanin) remain effective.
- **VISA, VRSA** – staphylococci partially or fully resistant to glycopeptids, too
- **VRE** – vancomycin resistant enterococci. They sperad easily – many people have enterococci in their intestine

## Epidemiologically important resistances – 2

- **ESBL** (Extended Spectrum Beta Lactamase) **producers**. G- bacteria (*Klebsiella*, but also *E. coli* and other) may produce extended spectre betalactamase, where even inhibitor effect is not sufficient. Only carbapenems and sometimes certain non-betalactam atb-s are effective.
- Similar are **ampC betalactamases**. Besides carbapenems also 4th generation cephalosporins remain effective.
- **MLS resistance** is a resistance to macrolids and lincosamids (and streptogramins) in streptocoi and enterococci. In *S. aureus* by good luck still rare.



## „Antibiotic politivity“, atb centres

- Use of broad spectre antibiotics performs a **selection pressure** – resistant strains of bacteria survive.
- In countries, where atbs are used freely, there is usually **high high ratio of atb resistance**
- In Czechia there exist „**free atb-s**“, that can be prescribed freely, and „**special atb-s**“; their use should be approved by antibiotic centre.
- **Atb centre** is usually part of microbiological labs in big hospitals. They do advisor work, too.

# Methods of susceptibility

## I: Diffusion

### disc test

# In vitro susceptibility observation methods

- Assessment of susceptibility **in vitro = in the laboratory**
- No guarantee of 100% treatment effect
- Nevertheless, useful in majority of findings of culturable bacteria
- **In common cases, qualitative tests** (susceptible – resistant)
- **In indicated cases, quantitative tests** (assessment of MIC). *Usually risky strains at risky patients.*

## When „in vitro“ does not correspond with „in vivo“

- **At urinary infections** we should use a breakpoint derived from urinary concentrations, not serum concentrations. (In majority of UTI infections, MIC is not measured)
- **In abscessi, processi in bones and mostly in meningitis:** breakpoints are derived from serum concentrations, but in various parts of body the concentration may be much lower
- It is necessary to count that microbes may exist in a **biofilm form** – so, MBIC and MBEC values (biofilm inhibitory/eradication concentrations) should be assessed rather than MIC values

# Diffusion disc test – 1

- To MH agar (or another one) a bacterium is inoculated from a saline suspension using a cotton swab
- After that, antibiotic discs are added – round bits of papers with antibiotic
- Atb diffunds from disc through the agar
- In a standard Petri dish we use mostly six discs, sometimes the seventh to the middle



# How to prepare a diffusion disc test

- Prepare a bacterial suspension in physiological saline with glucose
- Suspension should be distributed regularly onto the MH agar surface
- After drying of the suspension, place carefully and uniformly the atb discs onto the agar surface

## Diffusion disc test – 2

- Concentration of atb (and its inhibitory properties) decrease with distance from the disc
- When a microbe growth to a disc, or its inhibition zone is very small, it is resistant (not susceptible, not sensitive)
- When a zone large enough is present around the disc (more than a limit for the given antibiotic), it is susceptible (sensitive).

# Results of the diffusion disc test

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REZISTENTNÍ



- 1 Bacteria are affraid of antibiotics. Large zone (sometimes so large that it is impossible to measure it)
- 2 Bacteria are not affraid of antibiotics, they are resistant. A small zone around the disc, or no zone.

# Sets of discs

- Usually we use sets of atb discs. Some sets are suitable for G+ or G- bacteria, but it is also possible to construct a set of relatively „universal“ antibiotics (but because of polyresistant strains, no „universal“ antibiotics are really universal)

	<b>G+ microbe</b>	<b>G- microbe</b>
<b>Set for G+</b>	usually susceptible	usually resistant
<b>Set for G-</b>	usually resistant	usually susceptible
<b>Broad specter set</b>	usually susceptible	usually susceptible

# Diffusion disc test in practice: zones are measured and compared with reference zones



# Sometimes the zones are too large



When zones are so large that it is impossible to measure them, do not measure them and just write, that the strain is susceptible to a given atb.

***In green, you have theoretical margins of zones – as you can see, majority of them are confluent or they go through the edge of the dish***

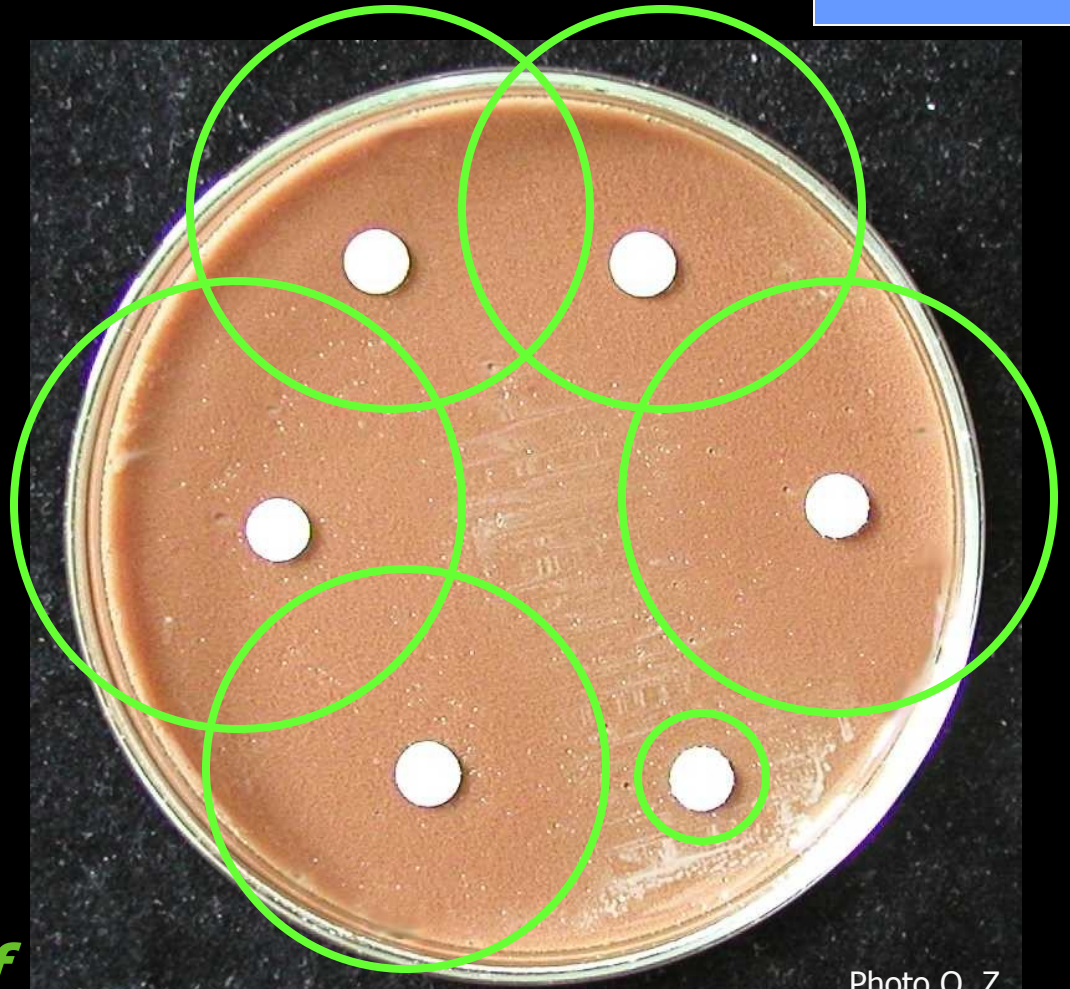


Photo O. Z.

# Methods of susceptibility

## II: E-test

# E-tests

- Principally similar to diffusion disc test
- Instead of a disc, a **strip** is used
- The strip has **raising atb concentration** from one end to another ( grace to a special technology – that is why they are expensive)
- The zone is **not round, but egg-shaped**
- The test is **quantitative**
- The strip has a **scale** – sipmle reeding (*see image on the next screen*)

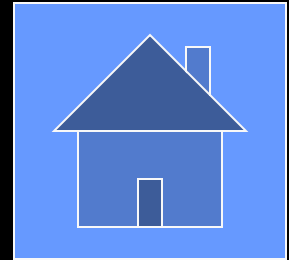
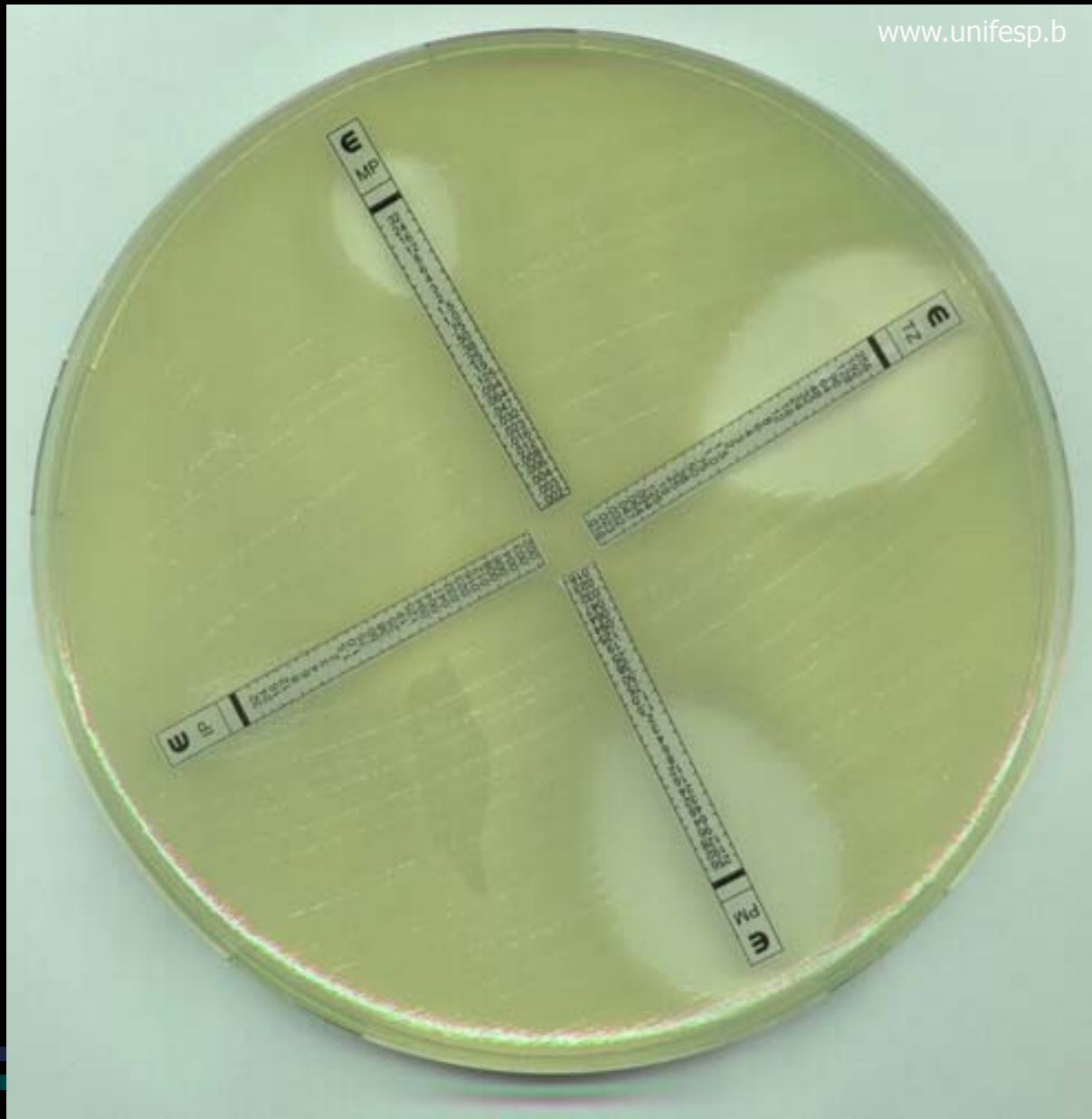


# E-tests – reading

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



# Somewhere, special large dishes are used



# Methods of susceptibility

## III: Microdi- lution test

## Microdilution test

- Atb-s are in a **row of wells** in a plastic microtitration plate, concentration decreases
- **The lowest concentration, that inhibits the growth, is the MIC value**
- For interpretation, we need **breakpoint** values for each antibiotic.  $MIC < \text{breakpoint} \Rightarrow$  the strain is susceptible.  $MIC > \text{breakpoint} \Rightarrow$  resistance
- One plate is usually used for one strain, e. g. **12 antibiotics**, each in 8 concentrations

# Practical reading a microdilution test

- Find and write MIC values for twelve tested antibiotics
- **Turbid well = it grows**
- **Clear well = no growth**
- **No growth = it is inhibited**
- The lowest inhibitory concentration = **minimal inhibitory concentration**
- **MIC  $\leq$  breakpoint  $\rightarrow$  strain susceptible**
- **MIC  $>$  breakpoint  $\rightarrow$  strain resistant**

# Microdilution test – an example

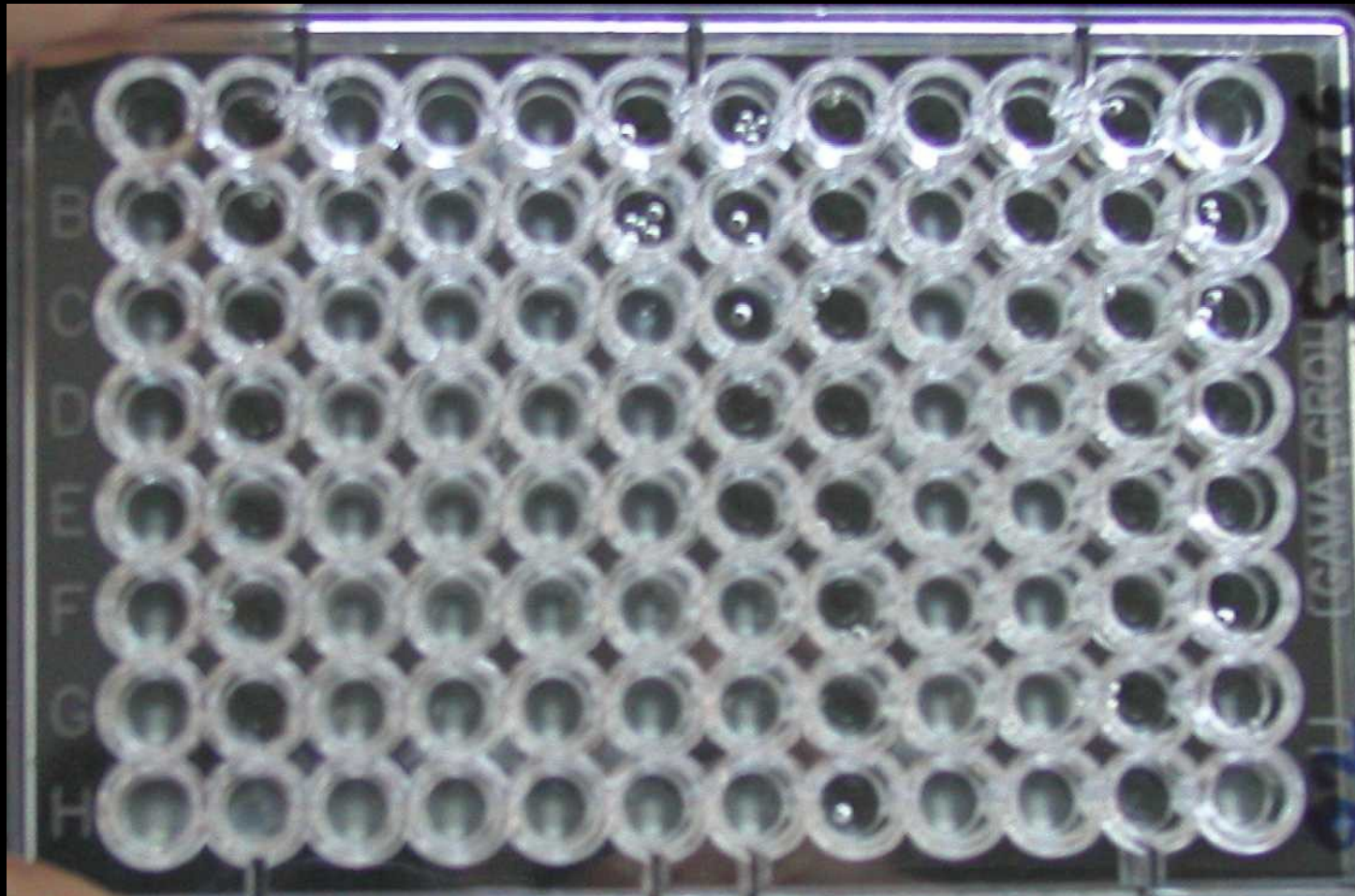
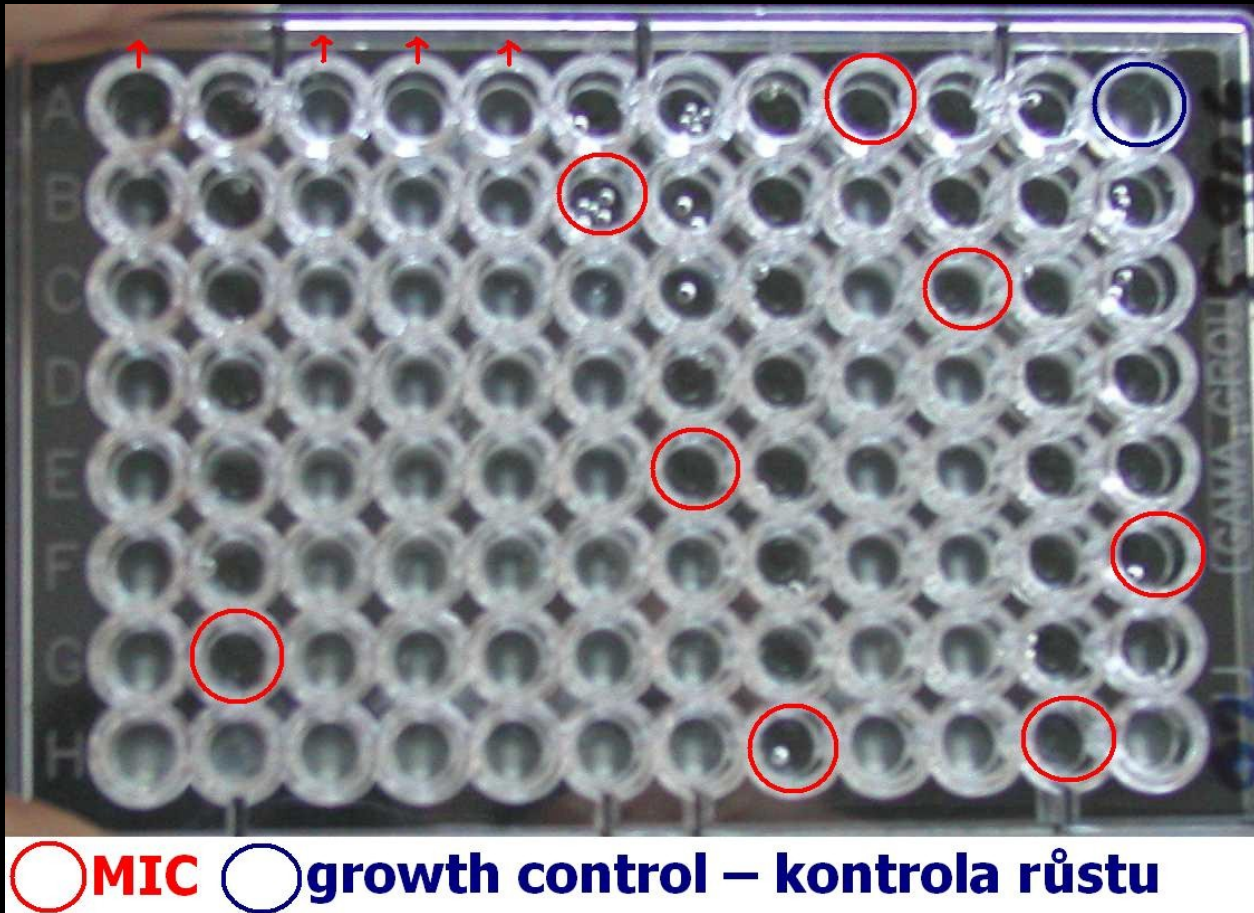


Photo: O. Z.

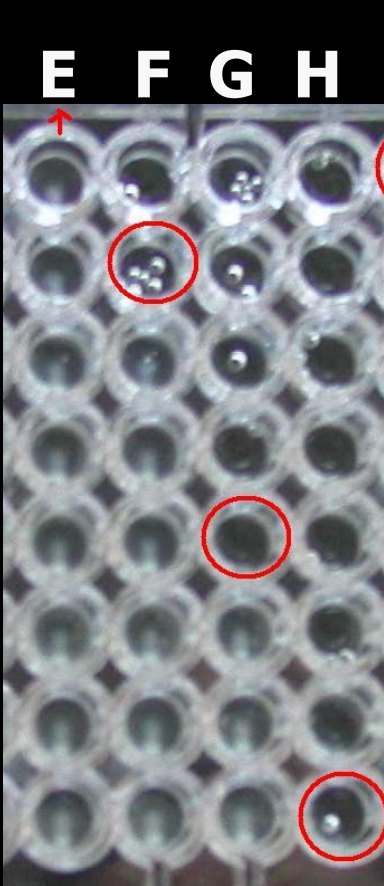


# Microdilution test – reading



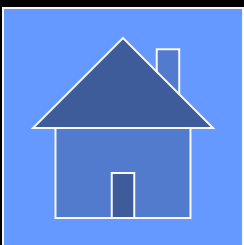
- In case of collumns 1, 3, 4 & 5, MIC value is too high and cannot be measured using this test.

# Example of reading



E	F	G	H
32	64	128	64
16	32	64	32
8	16	32	16
4	8	16	8
2	4	8	4
1	2	4	2
0.5	1	2	1
0.25	0.5	1	0.5

- E: MIC > 32, breakpoint = 16, conclusion: resistant
- F: MIC = 32, breakpoint = 16, conclusion: resistant
- G: MIC = 8, breakpoint = 32 conclusion: susceptible
- H: MIC ≤ 1, breakpoint = 8, conclusion: susceptible





# Methods of assessment of resistance factors

# Assessment of resistance factors

- Sometimes, instead of susceptibility testing, we should rather **assess the presence of individual resistance factors** by special methods, e. g. betalactamases
- The reason for this may be following:
  - **susceptibility testing does not give sure results** (bad diffusion in diffusion test, antibiotic does not work directly, but as a metabolite...)
  - we want to know, whether the **resistance belongs to a specific type** (ESBL, ampC)

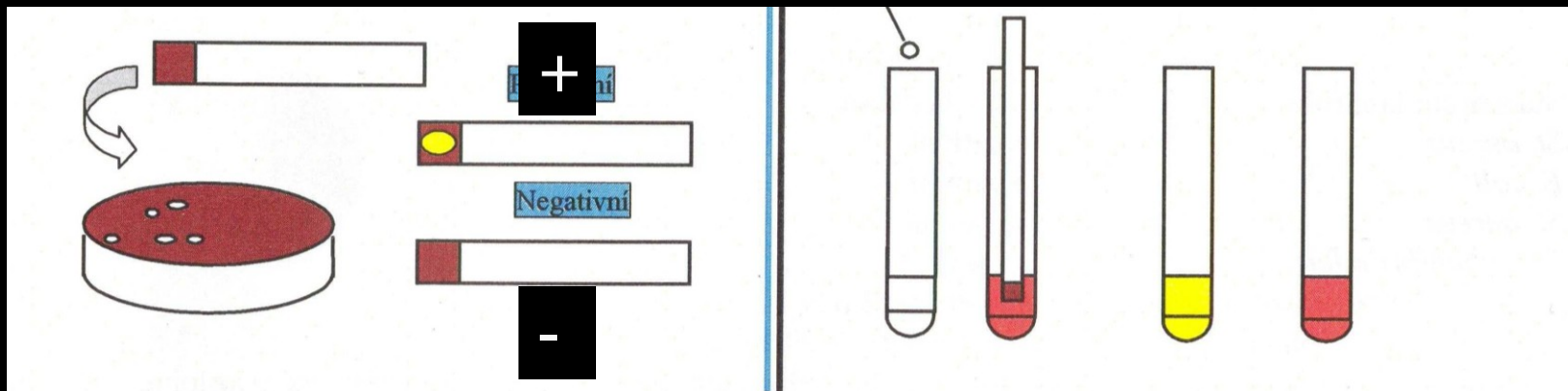
# Nitrocephine test for presence of common betalactamase

- It is used in situations, where **the result of a diffusion disc test**, but even microdilution test, **is not clear**
- Mostly we speak about
  - neisseria (instead of penicilline susceptibility testing)
  - *Moraxella catarrhalis* (replaces ampicilline)
  - *Haemophilus influenzae* (replaces ampicilline)
- **In practice**, it is a strip test similar to biochemical identification tests (oxidase test)
- It gives **good results for fresh strains only**, therefore it is not performed practically in the practical sessions (results were not allways good)

# Two ways of nitrocephin test use

Testing by touching the colonies

Testing in a liquid



+

-

# Testing for production of broad specter betalactamase type ESBL

- In **ESBL testing** we use two variants tests. The principle of both of them is potentiation of antibiotic effect due to clavulanic acid. *(The effect would not be sufficient for being used practically, but it is sufficient to be used in diagnostics)*
- In „**double synergy test**“ we study deformation of zone of a betalactamic antibiotic on a side close to a disc with co-amoxicilin
- In „**CLSI test**“ we compare effect of discs of the same antibiotics with / without clavulanic acid

## Detection of broad specter betalactamases (ESBL) in two ways

- Both tests are based on comparison of an effect of the same antibiotic (cefotaxime, ceftazidime) without / with clavulanic acid
- In **synergism test**, diffusion of clavulanic acid from co-amoxicilin disc (amoxicilin + clavulanic acid) is used
- In **the second test** we have directly discs of the same antibiotic (1) alone and (2) in combination with clavulanic acid

# Double synergy test

On a picture, the result is positive – the zone is enlarged to the left side (presence of co-amoxicillin disc)



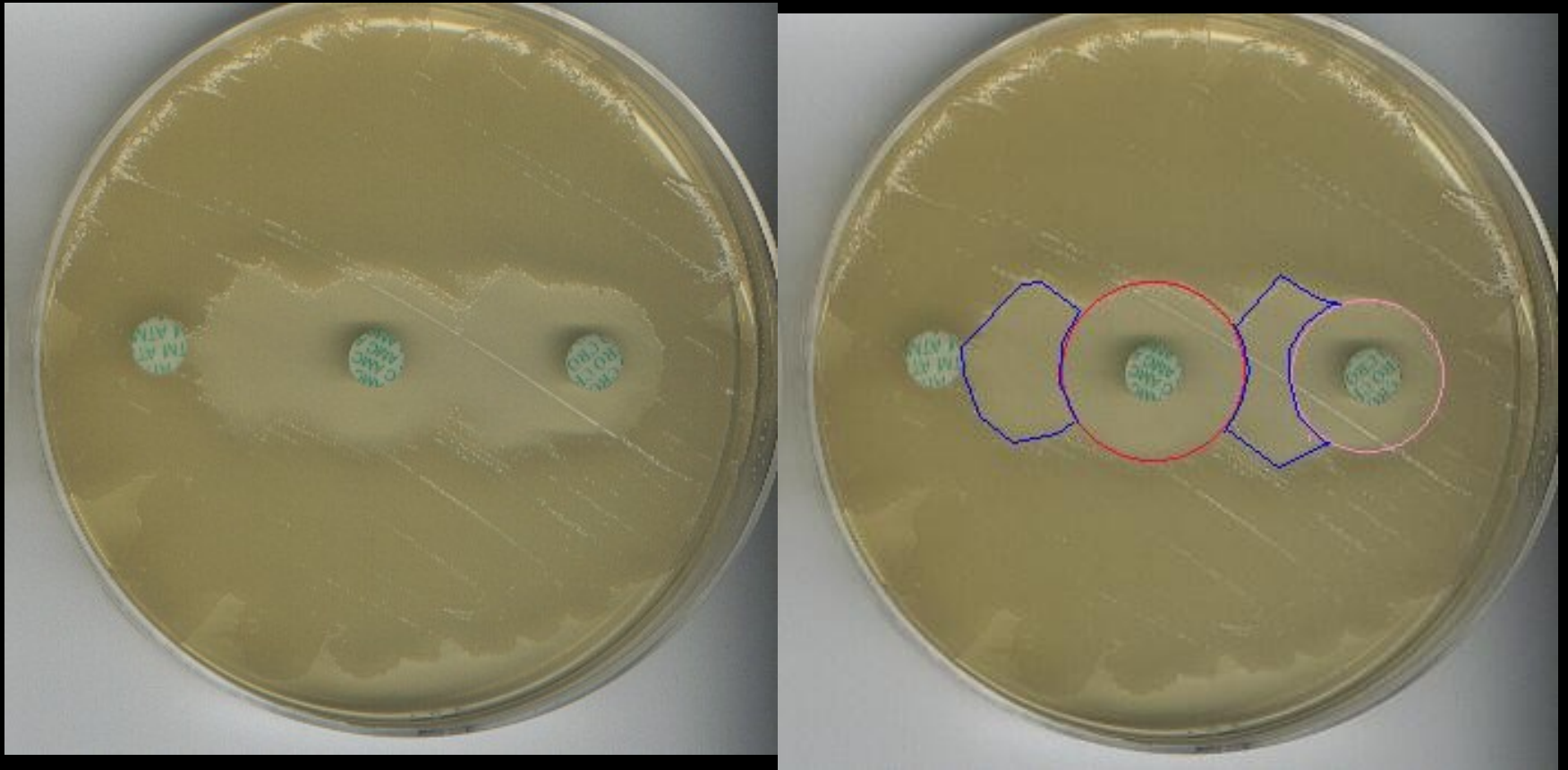
Cefotaxime is enhanced by clavulanate diffused from AMC disc (large zone)



Cefotaxime works alone (narrow zone)

# An example of a positive result

The area labelled blue is the important one





# CLSI test

When the **difference** of zone diameter of **cefotaxime with inhibitor** : **cefotaxime without inhibitor** is **more than five millimeters**, the strain is considered to be a (broad specter)  $\beta$ -laktamase producer. The same for ceftazidime.

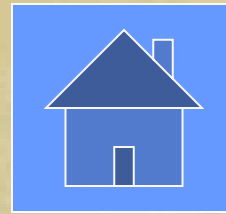


# Testing for constitutive ampC betalactamase production

- In **constitutive ampC detection** we compare sizes of susceptibility zones of four betalactamic antibiotics on common MH medium and medium with oxacilin
- This test enables us to know that ampC producers are important group of resistant enterobacterial strains of today
- It still does not take part in the practical sessions (maybe next year 😊)

*Besides constitutive ampC betalactamase also induced ampC betalactamase exists. For time reasons we do not speak about it more profoundly; it is possible to find more facts about it in textbooks and internet sources.*

The end



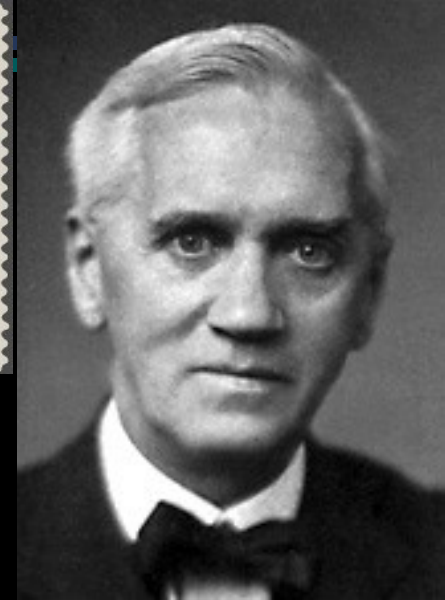
# Sir Alexander Fleming

- Sir Alexander Fleming was born at Lochfield near Darvel in Ayrshire, Scotland on August 6th, 1881.
- In 1921, he discovered in «tissues and secretions» an important bacteriolytic substance which he named Lysozyme. In 1928, while working on influenza virus, he observed that mould had developed accidentally on a staphylococcus culture plate and that the mould had created a bacteria-free circle around itself. Isolation was only possible in 1940 by other scientists. Dr Fleming died on March 11th in 1955 and is buried in St. Paul's Cathedral.



[http://cs.wikipedia.org/wiki/Alexander\\_Fleming](http://cs.wikipedia.org/wiki/Alexander_Fleming)

[http://nobelprize.org/nobel\\_prizes/medicine/laureates/1945/fleming-bio.html](http://nobelprize.org/nobel_prizes/medicine/laureates/1945/fleming-bio.html)



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