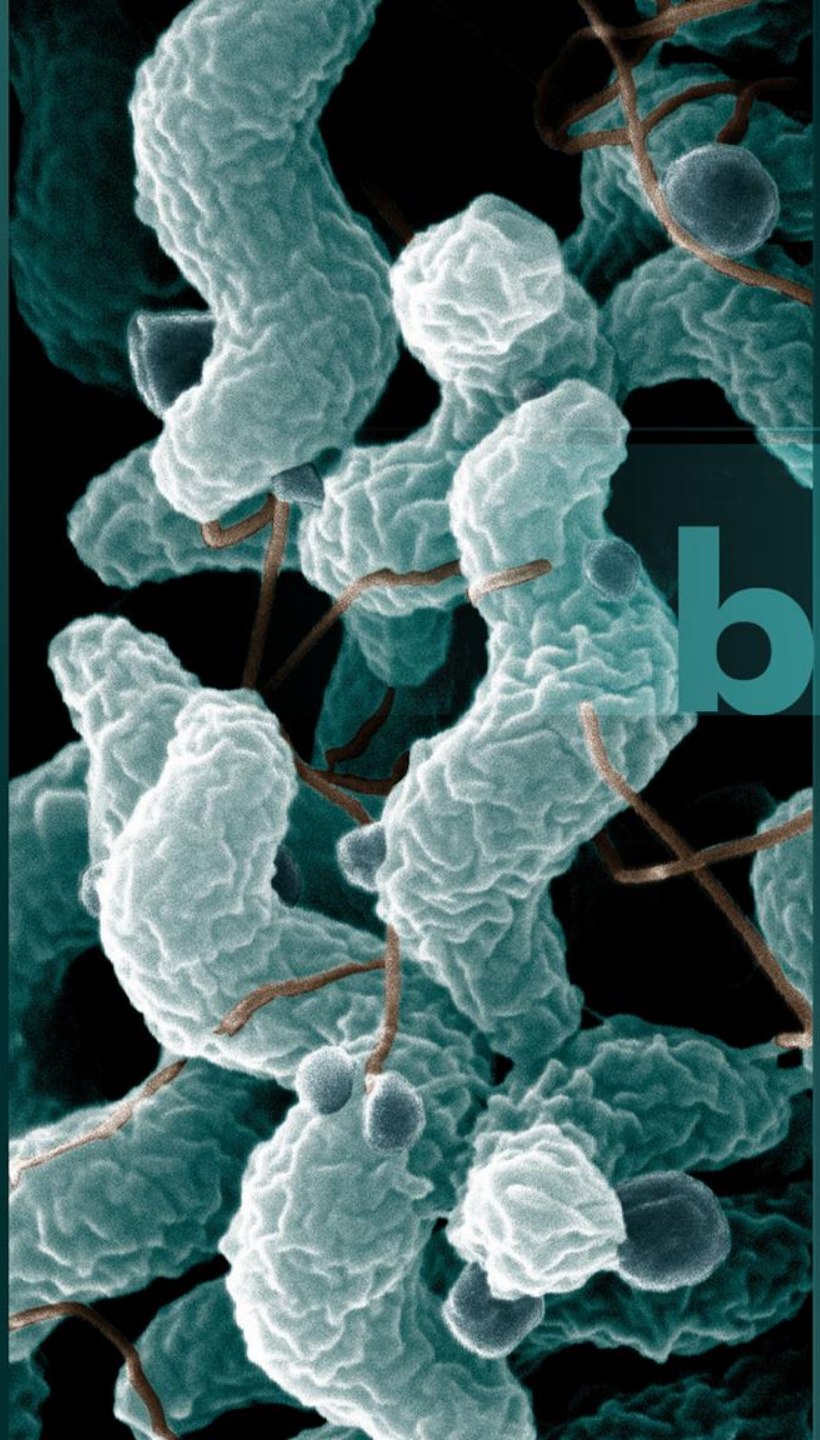
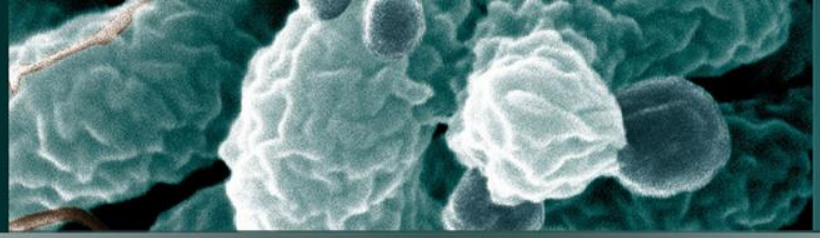


4rd Seminary from microbiology FaF VFU BRNO (theory for lab class no. 3,4)

bacteria

Diagnostic procedures (II)

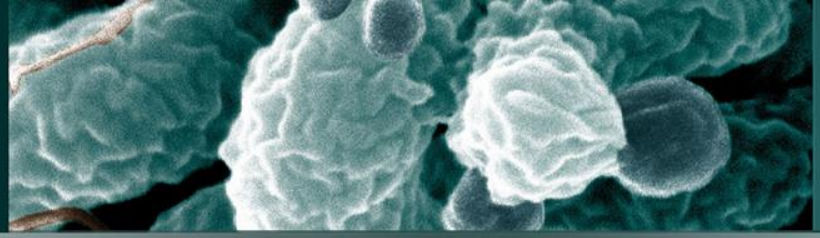


PROCEDURES

Direct methods (microb – part – product):

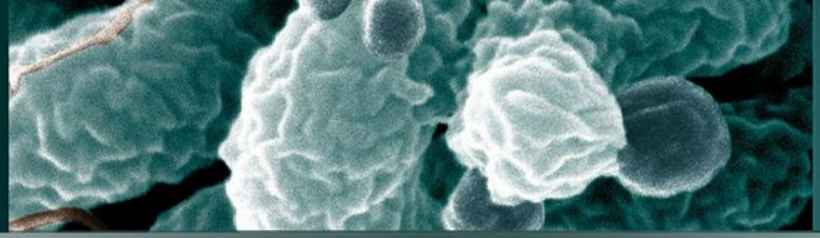
- Microscopy – evidence in sample and identification
- Cultivation – evidence in sample and identification
- Biochemical identification – only identification!
- Evidence of antigens – evidence in sample and identification
- Nucleic acids – mainly evidence in sample
- Experiment on animals – mainly evidence in sample

Indirect metods (antibodies)



BIOCHEMICAL REACTIONS

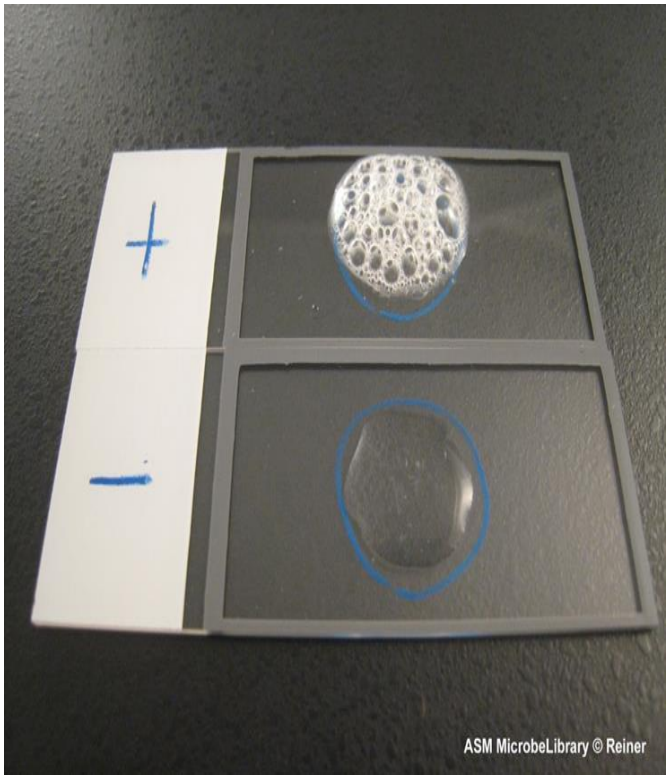
- Identification group/genus/species/intraspecific analysis
- Principle:
  - Substrate → reaction to product → detection of product/changes in environment
  - usually catabolic reactions
- Clean culture old max. 24 hours
- **Reactions in plastic microtitration plate („panel“)**
  - Principle: at the bottom of wells there are lyophilized substrates (or with indicator), bacterial suspension is then added and plaste is incubated.
  - change in colour – with naked eye or spectrophotometrically
  - result: some positive (+) and negative (-) reactions, which give you specific code number – there is code book to find the bacteria



Oxidoreductases

Catalase test

- Aerobic and fac. anaerobic bacteria equipped with cytochromoxidase
- Deactivation of peroxide (peroxidase)
- reaction releases oxygen (bubbles)
- $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$

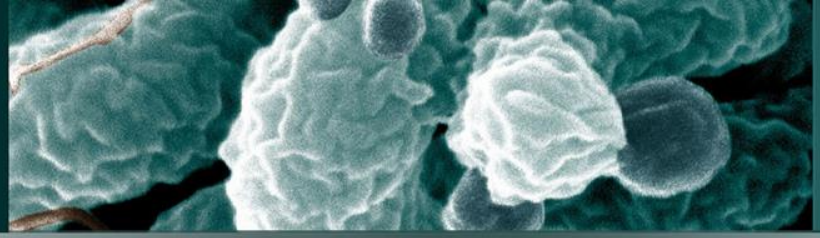


Positive

- *Staphylococcus*, *Corynebacterium*, *Neisseria, Listeria*

Negative

- *Streptococcus*, *Enterococcus*, *Arcanobacterium*



Oxidoreductases

Oxidases/cytochromoxidases

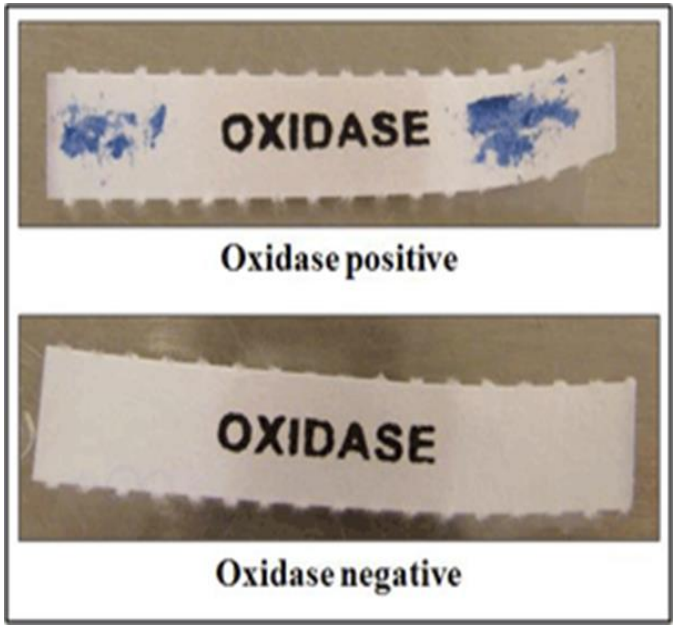
- enzymes involved in respiratory chain

Oxidase:

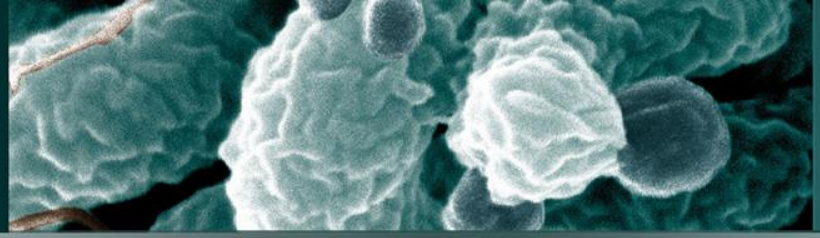
- tetramethyl-*p*-fenylendiamin chloride

Cytochromoxidase:

- dimethyl-*p*-fenylendiamin chloride



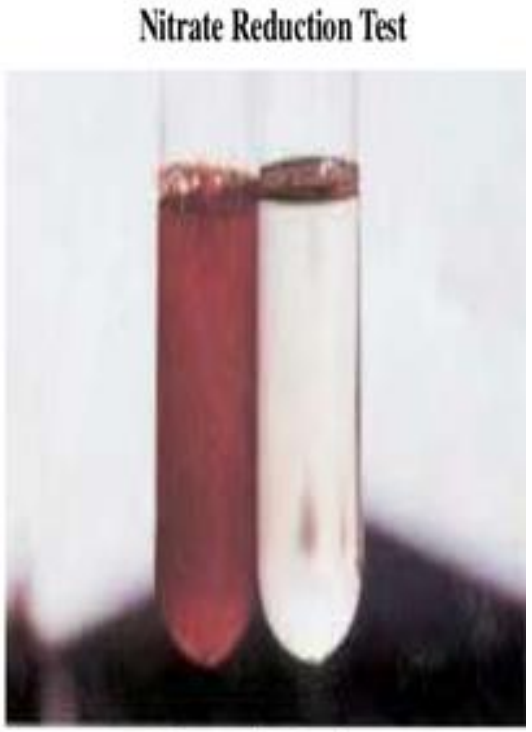
- on filtration paper
- in positive case we can see blueing.
- for differentiation of gramnegative bacteria
- Positive: *Neisseria*, *Moraxella*, *Pseudomonas, Vibrionaceae*
- Negative: *Enterobacteriaceae, Acinetobacter spp.*



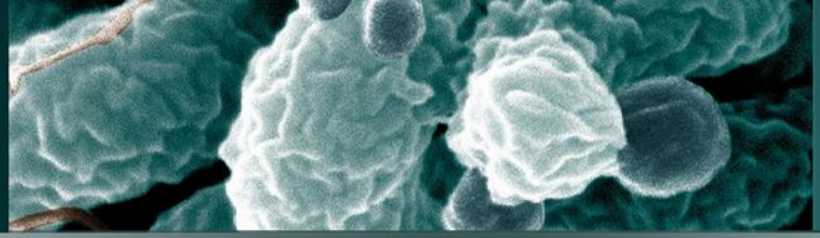
Oxidoreductases

Reduction of nitrates to nitrites

- evidence of presence of nitrate-reductase
- used in:
  - solid media with nitrate
  - nitrate liquid broth
  - detection of nitrites and NH_3
  - Gries-Ilosvay reagent (solution of naftylamin in acetic and sulfanilic acids)
- red colour shows positive result
- negative result (no colour change) must be verified with powdered zinc



Peptone nitrate broth
on left is positive (*E. coli*);
tube on right is negative.



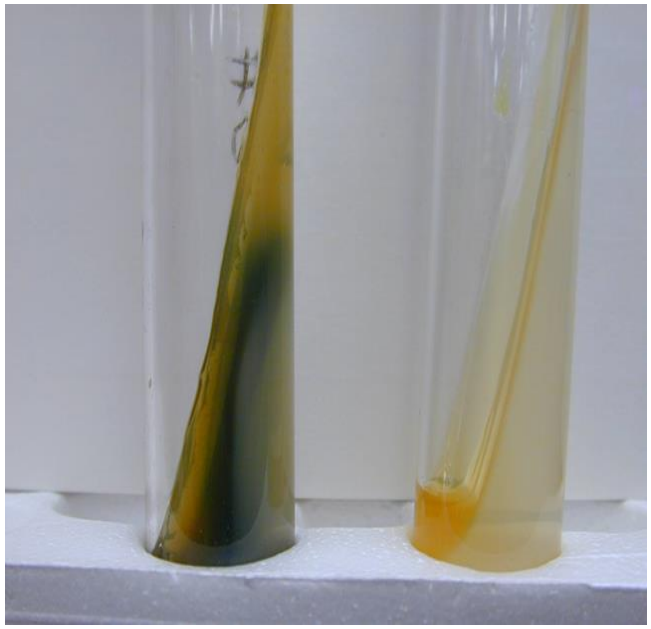
Oxidoreductases

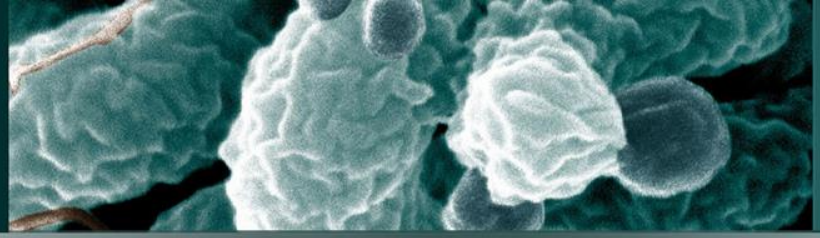
Oxidative deamination of phenylalanin

- evidence of presence of phenylalanindeaminase
- phenylalanin is deaminated to phenylpyruvate

- positive: reaction with $FeCl_3$ greening
- negative: yellow

- positive e.g.: *Proteus, Providentia, Morganella*

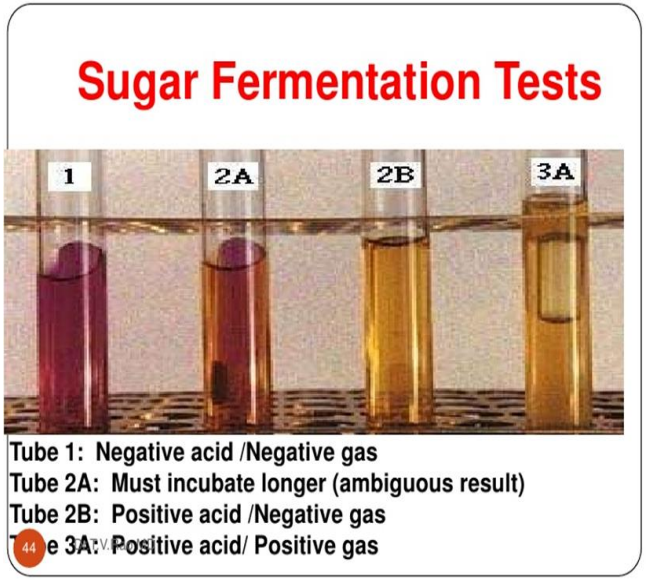




Sugar enzymes

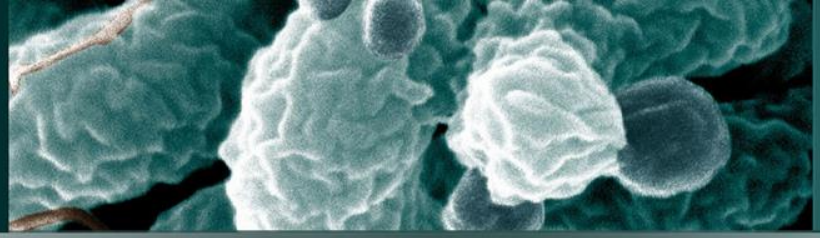
Evidence of fermentation of sugars

- pepton water with 1 % sugar (glucose, sucrose, lactose, manitol, xylose)
- bromthymol blue as indicator
- in positive case medium is acidified and turns yellow.



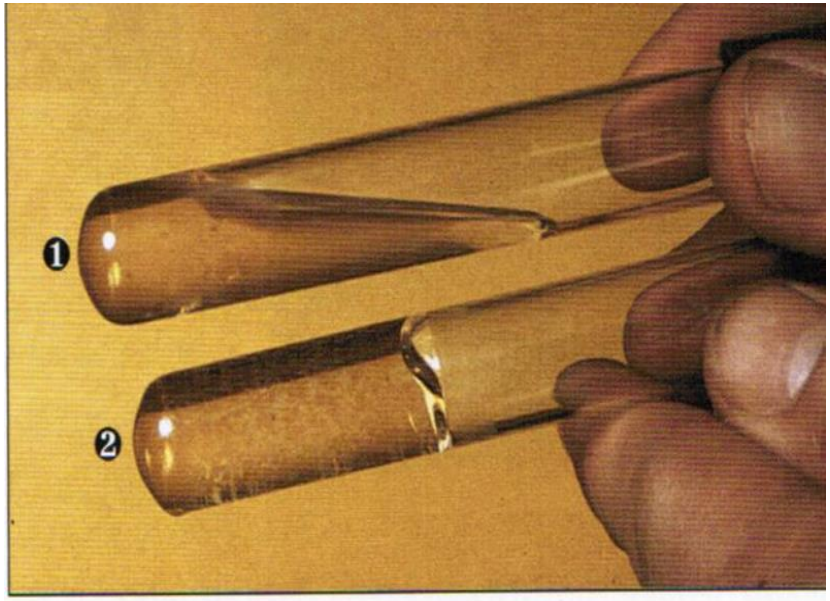
Reaction with *ortho*-nitrophenyl-β-D-galactopyranoside

- evidence of β-galactosidase
- cleavage of nitrophenol – yellowing
- distinction in *Enterobacteriaceae*

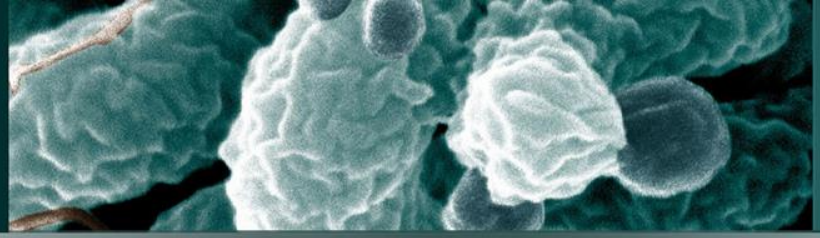


Proteases and peptidases

- enzymes released into environment
- proteolysis
- substrates are mainly casein and gelatin
- positive reaction leads to liqufaction
- positive e.g. *Pseudomonas aeruginosa*



III.6 Gelatin hydrolysis. After hydrolysis (**1**), gelatin remains liquid. **2** is unhydrolyzed gelatin (Exercise 15).



Amidases

Ureases

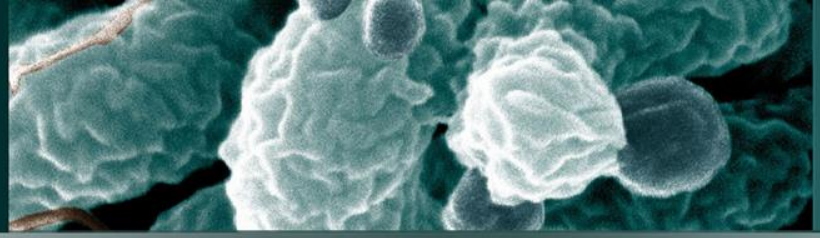
- substrates are media with 2% of urea
- production of urease – cleavage of urea to ammonium
- alkalisation leads to pink (indicator is phenol red)

Argininhydrolases

- cleaves arginin to ornithin, CO_2 and NH_3
- alkalisation – indicator: bromphenol red or bromcresol purpur)

PYRases

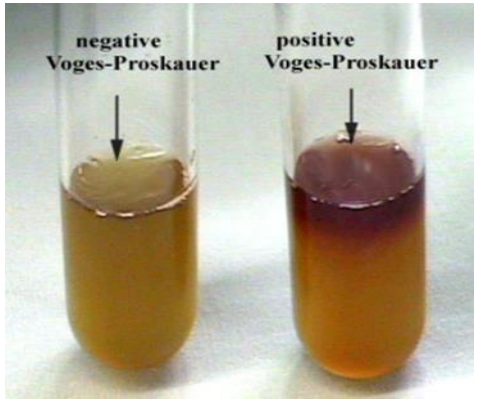
- cleavage of pyrrolidonyl-β-nafthylamide
- reaction with *N*,*N*-dimethylaminocinnamaldehydem
- red colour
- identification of group A β-hemolytic streptococci and enterococci (*Streptococcus pyogenes*)

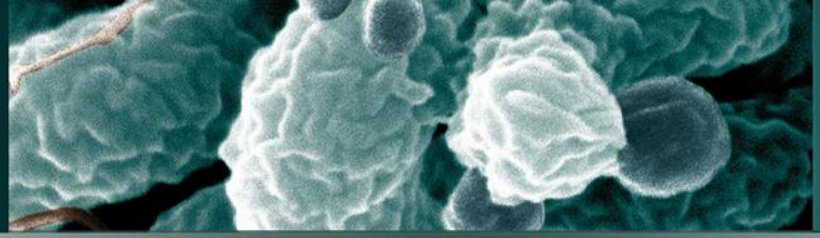


Lyases

Tryptophanase, cysteindesulphydrase a decarboxylases

- **Production of indole**
  - cleaved from tryptophan,
  - part of Hottinger broth
  - after incubation add drops of Ehrlich or Kovacs reagent
  - in positive case there is a red ring on borderline of the two liquids.
  - positive *E. coli*, negative *Salmonella* spp.
- **Production of hydrogen sulfide**
  - cleavage of sulfur-contaning amino acids (cystein)
  - in solid media with 1% lead acetate
  - in positive case, lead sulfide is formed and the media is blackening
  - negative *E. coli*, positive *Salmonella* spp.
- **VP (Voges-Proskauer's test for acetoin) test and MR (methyl red) test**
  - cleavage of pyruvate
  - change in pH
  - after 48 hrs
  - *Enterobacteriaceae*
- **Evidence of decarboxylation of lysin and ornithin**
  - Lysindecarboxylase – kadaverine + CO_2
  - Ornithindekarboxylase – putrescine + CO_2
  - change in pH (alkalisation)
  - longer cultivation (even 5 days)





Coagulase

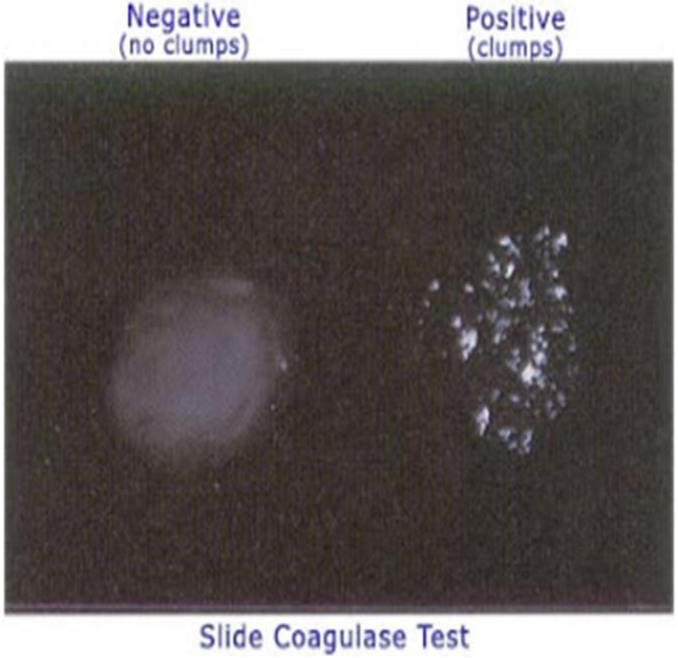
Detection of plasma-coagulase

- for staphylococci (mainly *S. aureus*)
- free form

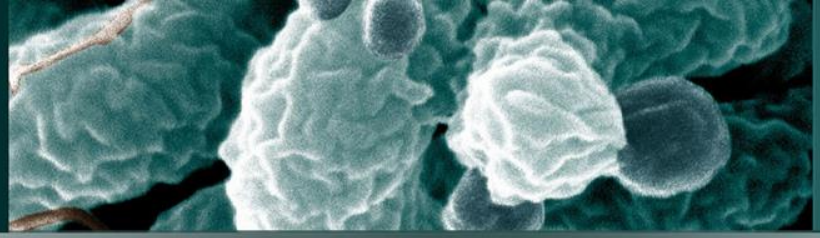
  adhesive factor
- bond form

  clumping factor
- to citrate rabbit plasma add tested colony and incubate

  4 - 12 hours at 37 °C.
- positive result = coagulated plasma

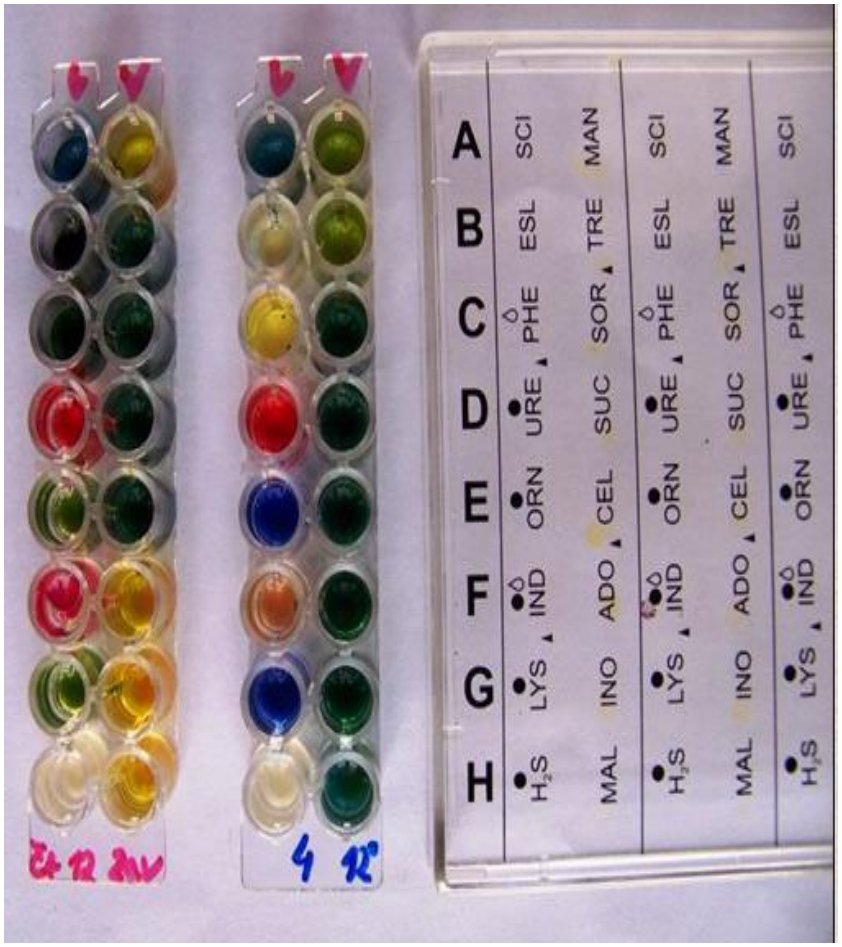


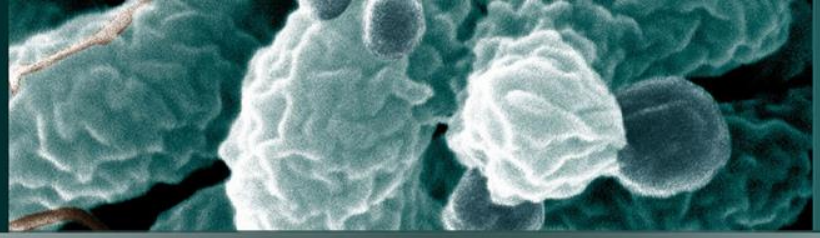
Slide Coagulase Test



Practical performance

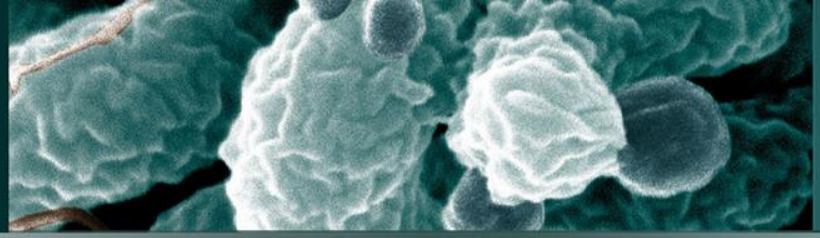
- manufacturer makes the test with dried substrates on hte bottom of wells
- clinical microbiologist prepares bacterial suspension in medium or phys. solution
- adds suspension to each well
- rest of suspension is used for other tests with diagnostic strips in test-tubes (ONPG, VPT)
- microplate and test-tubes are incubated in thermostat
- the result is determined with reading through transparent glass cover



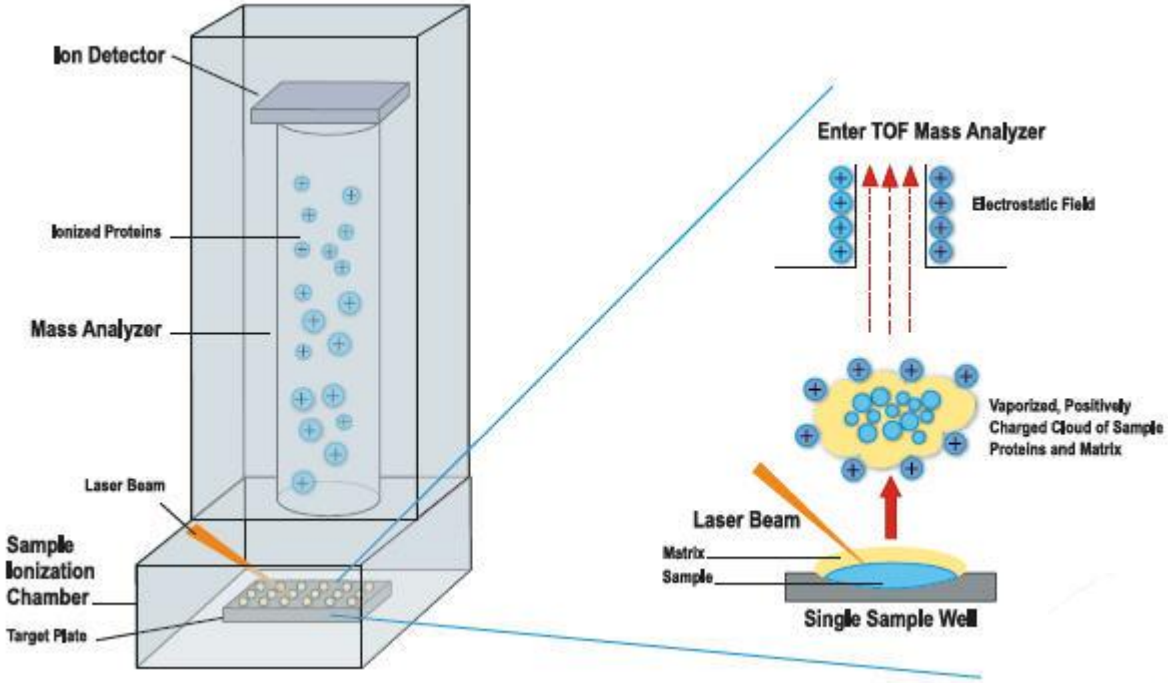


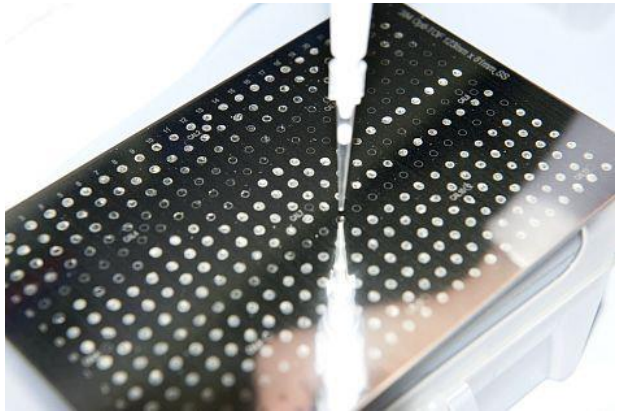
MALDI-TOF

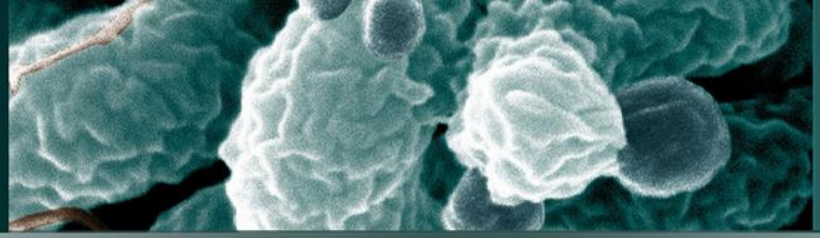
- separation of ionized particles according to their molecular mass
- due to laser ionisation we are able to detect also large molecules specific for different species of bacteria and yeasts
- MALDI, *matrix assisted laser desorption/ionization* in combation with TOF detector, *time-of-flight*
- speed of particle (inversely proportional to its mass, or weight) can be calculated from time of flight in detector
- mixture of matrix (e.g. 4-hydroxycinnamic acid) and tested strain on stainless plate is impacted with nanosecond pulse of laser
- matrix absorbs energy of the pulse and molecules of sample are ionised by its decay
- the method then „finds“ specific proteins of tested strain and compares them with database



MALDI-TOF







MIC/MBC

MIC – Minimal inhibition concentration

= lowest concentration of antimicrobial compound, which prevents visible growth of bacterial culture in 24 hours.

- Preparation of stock solution of antibiotic
- Preparation of its dilution series
- Preparation of bacterial inoculum
- inoculation
- Incubation
- evaluation

MIC_{50} a MIC_{90}

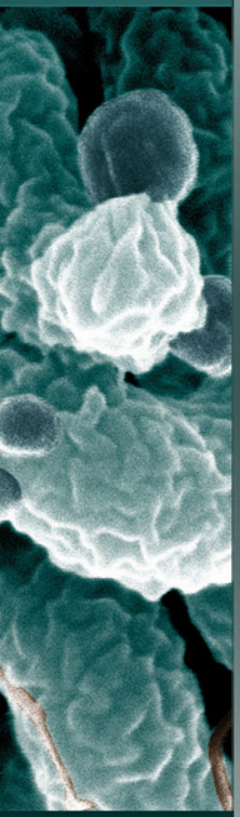
MBC – minimal bactericidal concentration

= lowest concentration of antimicrobial compound needed to kill a microorganism. For right evaluation at least 99,9% of cells must be dead.

McFarland scale of optical density

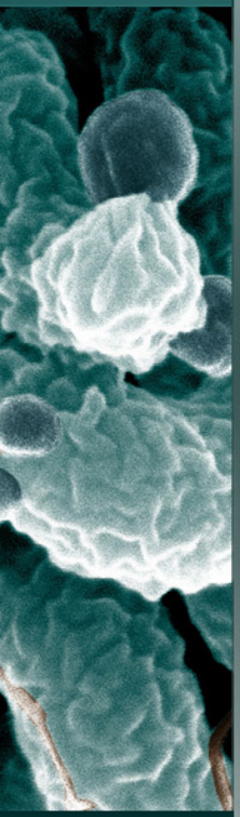
- suspension of bacteria in phys. solution or medium must be standardized
- there is recommended level of optical density (i.e. level of transparency of that suspension)
- we use densitometer: device (simple photometer) able to measure directly optical density

- ***application***: biochemical identification tests, preparation of microdilute test when testing sensitivity to antibiotics



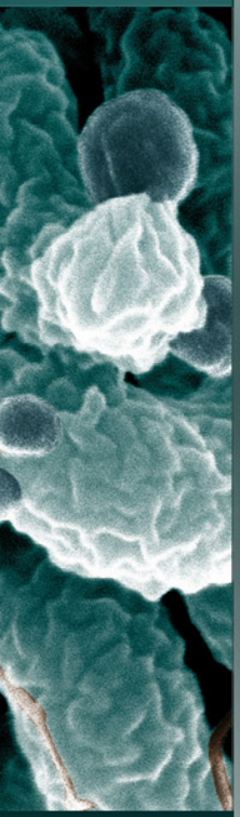
McFarland scale of optical density

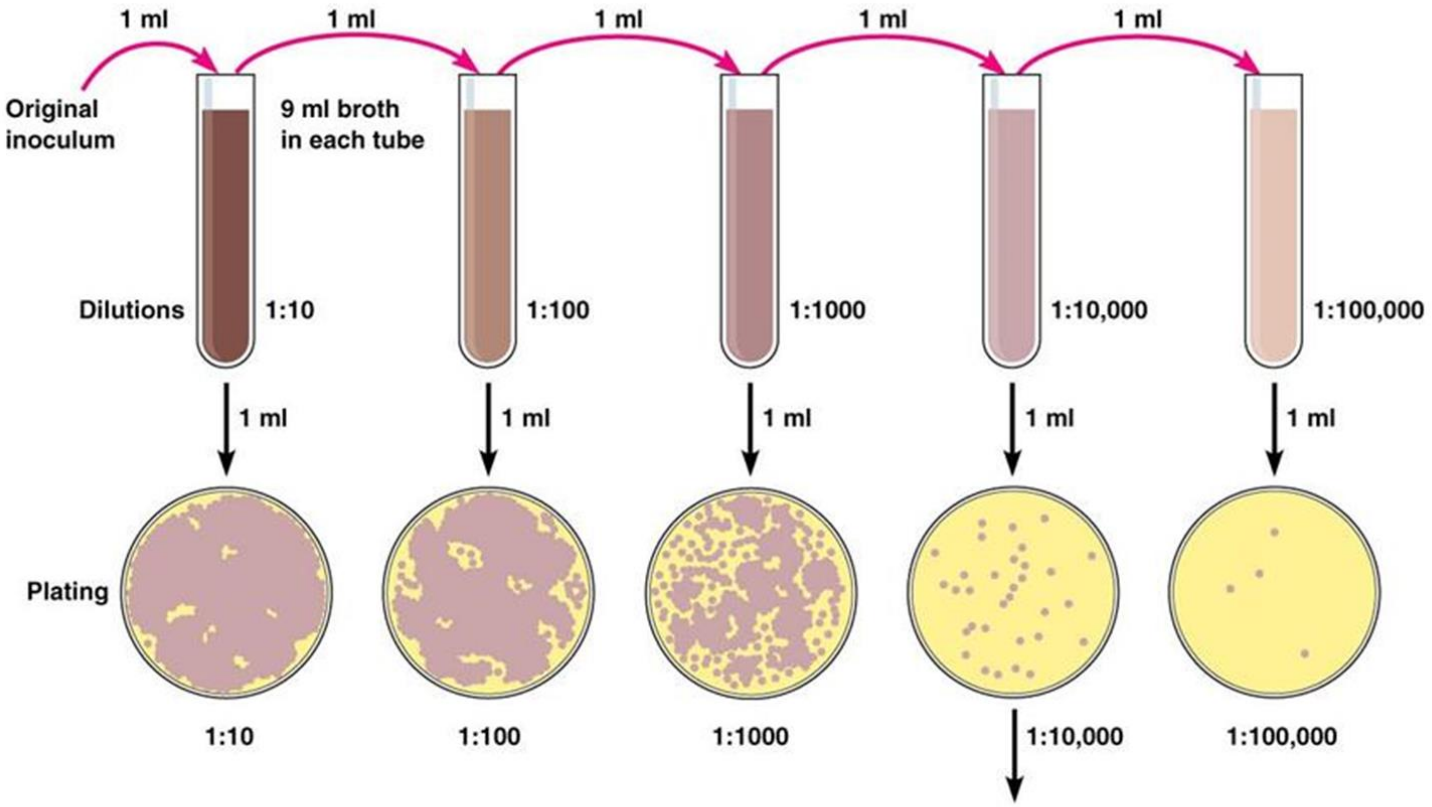
Mixing solution of barium chloride and sulfuric acid → insoluble precipitate of barium sulfide (basis for preparation of turbidity scale, by which one can assume concentration of bacterial suspension



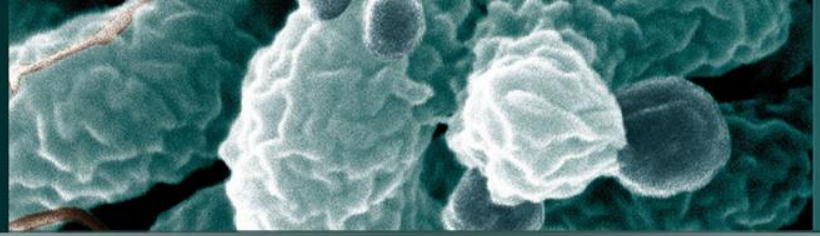
	Stupeň číslo										
	0,5	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
1,175% roztok chloridu barnatého (ml)	0,05	0,1	0,2	0,3	0,4	0,5	0,6	0,7	0,8	0,9	1,0
1% roztok kyseliny sírové (ml)	9,95	9,9	9,8	9,7	9,6	9,5	9,4	9,3	9,2	9,1	9,0
Přibližná koncentrace bakterií (x 10^8/ml)	1,5	3	6	9	12	15	18	21	24	27	30

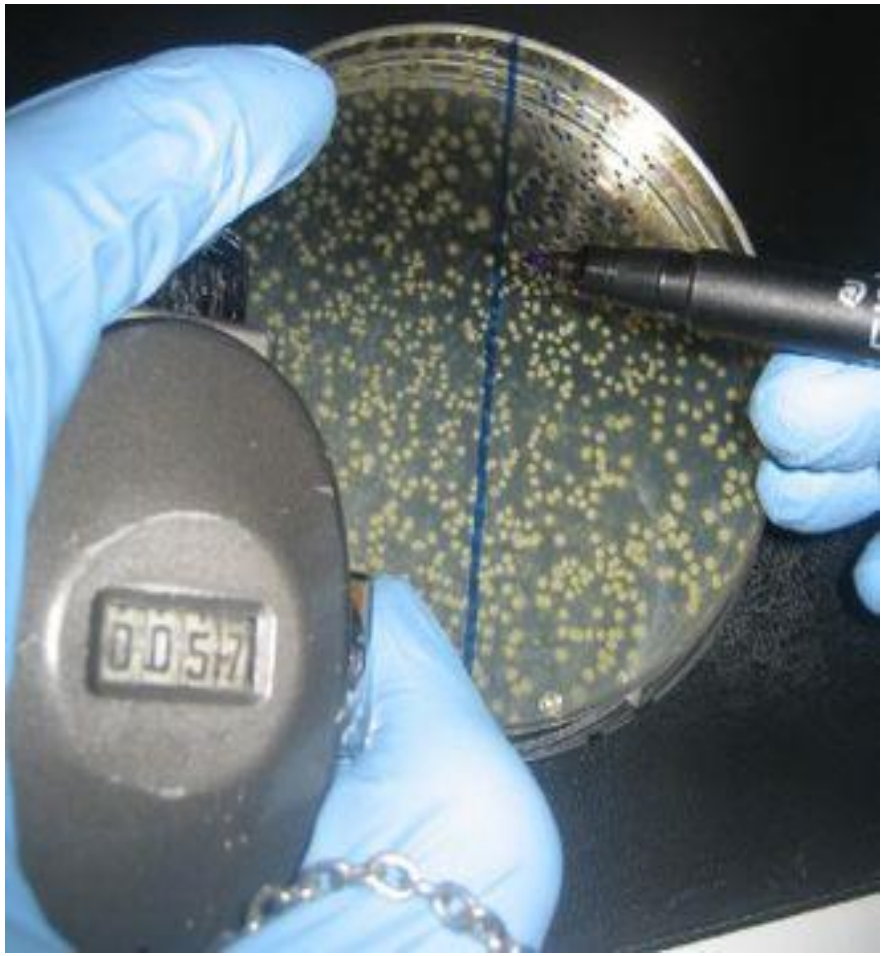
How else can we determine concentration of bacterial suspension?



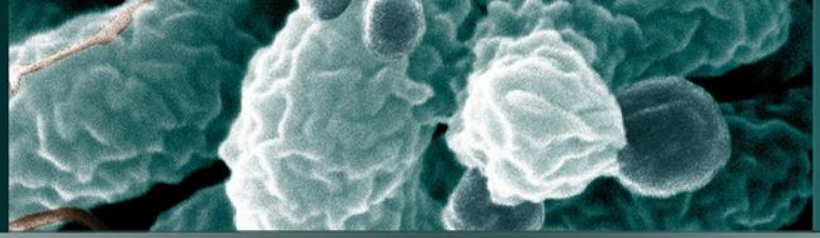


Calculation: Number of colonies on plate × reciprocal of dilution of sample = number of bacteria/ml
(For example, if 32 colonies are on a plate of $^{1}/_{10,000}$ dilution, then the count is 32 × 10,000 = 320,000 bacteria/ml in sample.)

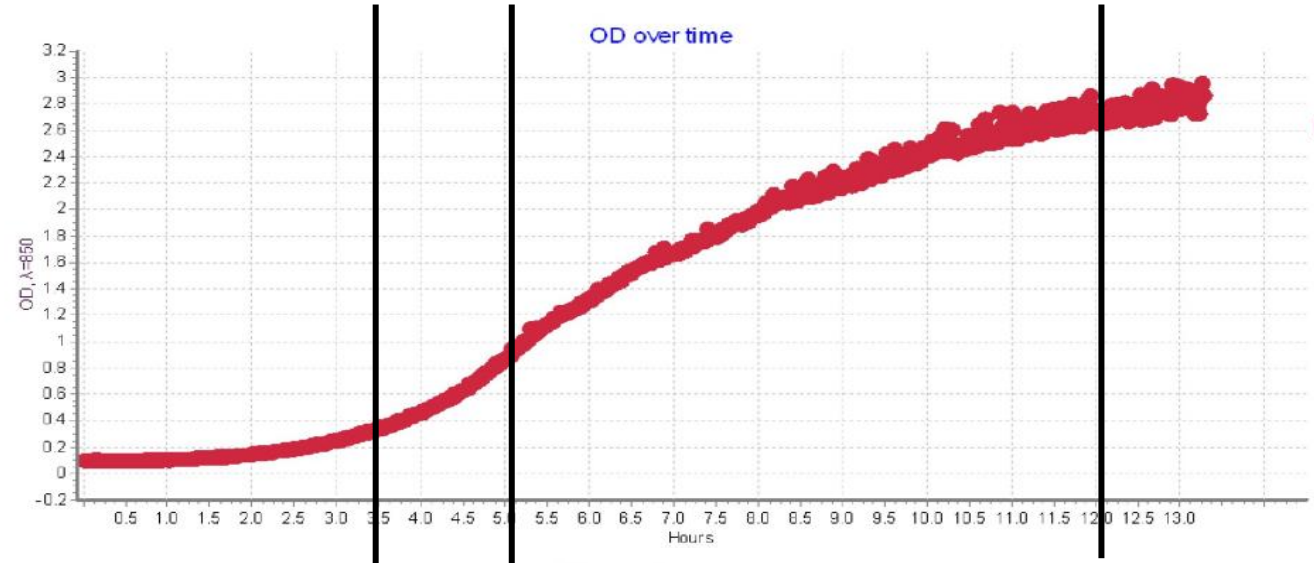




RTS-1 Personal Bioreactor (BioSan Ltd.)

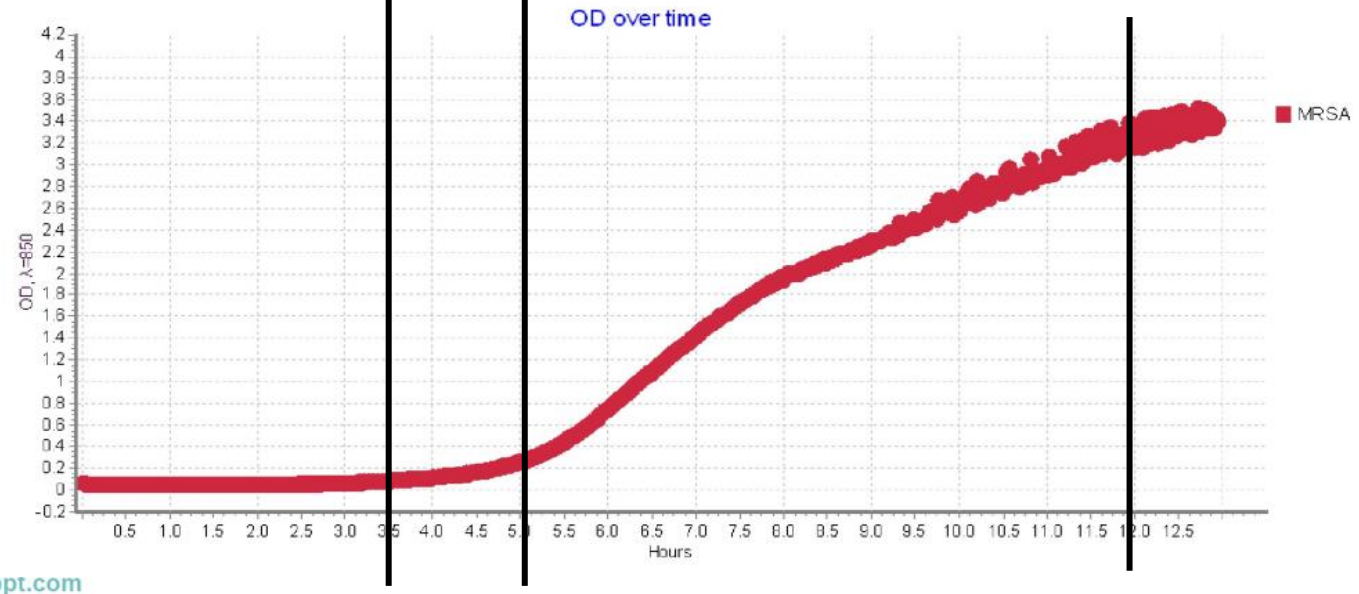


Determination of growth curve of suspension of *S. aureus*

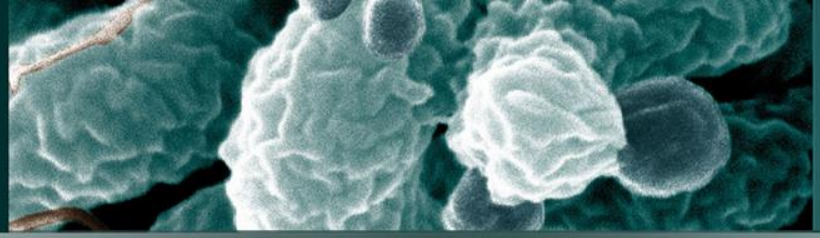


Opt. density after 12 hrs

- MSSA 2,8



- MRSA 3,8



Diffuse methods

Procedure:

1) Take colony of particular bacterial strain and add to physiological solution

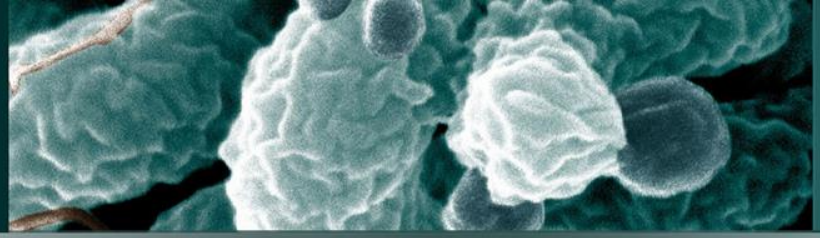
   (it is necessary to determine optical density according to McFarland; recommended value is 0,5)

2) Suspension of bacteria inoculate with sterile cotton swab onto agar plate (e.g. Müller-Hinton medium)

3) Apply ATB discs

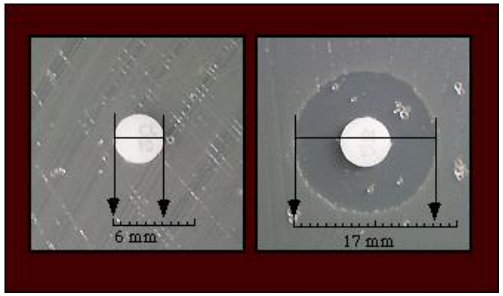
4) Cultivation; mostly 24 hrs. at 37 °C

5) Evaluation – measuring diameters of inhibition zones



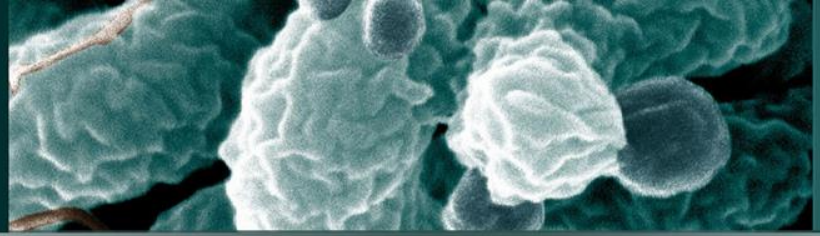
Diffuse methods

- **qualitative (semiquantitative)**
- determination of either sensitivity or resistance of bacteria to given ATB
- diffusion of ATB to agar plate
- in case of effectivity of ATB there is formed so called inhibition zone



Evaluation:

- measurement of inhibiton zones and comparison with reference values



Diffuse methods

Enterobacteriaceae

EUCAST Clinical Breakpoint Tables v. 6.0, valid from 2016-01-01

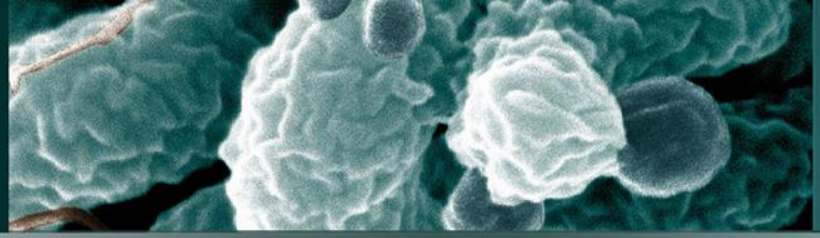
Disk diffusion (EUCAST standardised disk diffusion method)
Medium: Mueller-Hinton agar
Inoculum: McFarland 0.5
Incubation: Air, 35±1°C, 18±2h
Reading: Read zone edges as the point showing no growth viewed from the back of the plate against a dark background illuminated with reflected light.
Quality control: *Escherichia coli* ATCC 25922. For control of the inhibitor component of beta-lactam inhibitor-combination disks, use either *Escherichia coli* ATCC 35218 or *Klebsiella pneumoniae* ATCC 700603.

Penicillins[1]	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S ≤	R >		S ≥	R <
Benzylpenicillin	-	-		-	-
Ampicillin	8[1]	8	10	14[A,B]	14[B]
Ampicillin-sulbactam	8[1,2]	8[2]	10-10	14[A,B]	14[B]
Amoxicillin	8[1]	8	-	Note[C]	Note[C]
Amoxicillin-clavulanic acid	8[1,3]	8[3]	20-10	19[A,B]	19[B]
Amoxicillin-clavulanic acid (uncomplicated UTI only)	32[1,3]	32[3]	20-10	16[A,B]	16[B]
Piperacillin	8	16	30	20	17
Piperacillin-tazobactam	8[4]	16[4]	30-6	20	17
Ticarcillin	8	16	75	23	23
Ticarcillin-clavulanic acid	8[3]	16[3]	75-10	23	23
Phenoxymethylpenicillin	-	-		-	-
Oxacillin	-	-		-	-
Cloxacillin	-	-		-	-
Dicloxacillin	-	-		-	-
Flucloxacillin	-	-		-	-
Mecillinam (uncomplicated UTI only) *E. coli*, *Klebsiella* spp. and *P. mirabilis*	8	8	10	15[D]	15[D]

Notes
Numbered notes relate to general comments and/or MIC breakpoints.
Lettered notes relate to the disk diffusion method.

1/A. Wild type Enterobacteriaceae are categorised as susceptible to aminopenicillins.
Some countries prefer to categorise wild type isolates of *E. coli* and *P. mirabilis* as intermediate. When this is the case, use the MIC breakpoint S ≤ 0.5 mg/L and the corresponding zone diameter breakpoint S ≥ 50 mm.
2. For susceptibility testing purposes, the concentration of sulbactam is fixed at 4 mg/L.
3. For susceptibility testing purposes, the concentration of clavulanic acid is fixed at 2 mg/L.
4. For susceptibility testing purposes, the concentration of tazobactam is fixed at 4 mg/L.
~~**5/D.** Mecillinam (pivmecillinam) breakpoints relate to *E. coli*, *Klebsiella* spp. and *P. mirabilis* only.~~

B. Ignore growth that may appear as a thin inner zone on some batches of Mueller-Hinton agars.
C. Susceptibility inferred from ampicillin.
D. Ignore isolated colonies within the inhibition zone for *E. coli*.



Disc diffuse methods

Results:

Tested strain is **resistant** to given ATB:

ATB diffusing from disc was not able to stop growth of bacteria and there is no inhibition zone

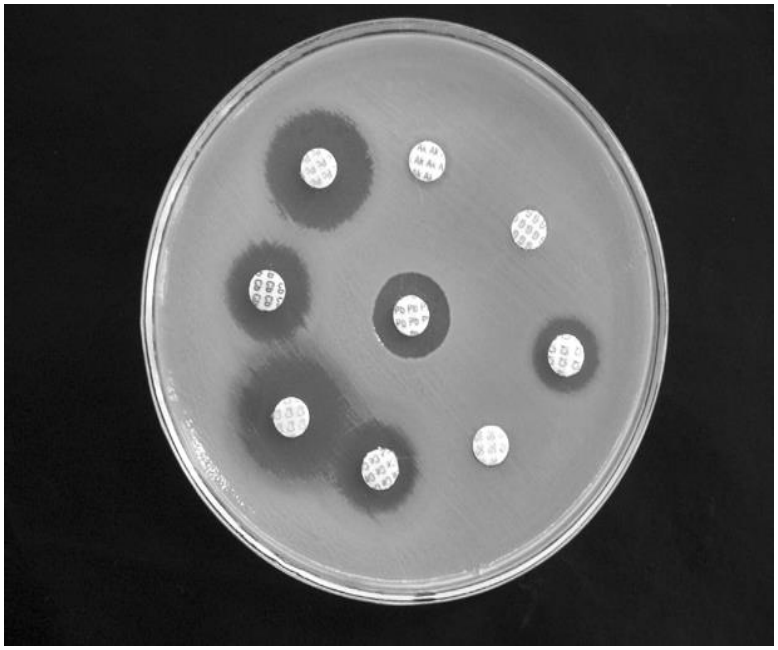
Tested strain is **sensitive** to given ATB:

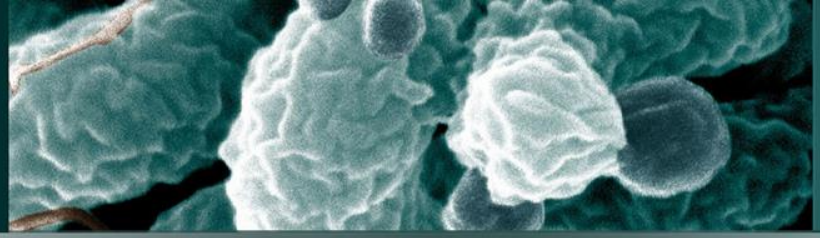
ATB diffusing from disc stops gowth of bacteria and inhibition zone is formed

- inhibition zone is smaller than reference value = bacteria is resistant to given ATB

- inhibition zone is larger than reference value = bacteria is sensitive to given ATB

Disc diffuse methods





E-test
Gradient method

- test for determination of MIC of an antibiotic

Principle:

- diagnostic strip, in which concentration of ATB decrease in logaritmic grandient – when laid on medium, similar concentration gradient is formed in medium
- after incubation inhibition zone is formed in shape of a drop – in spot where the drop touches diagnostic strip, we see the MIC value

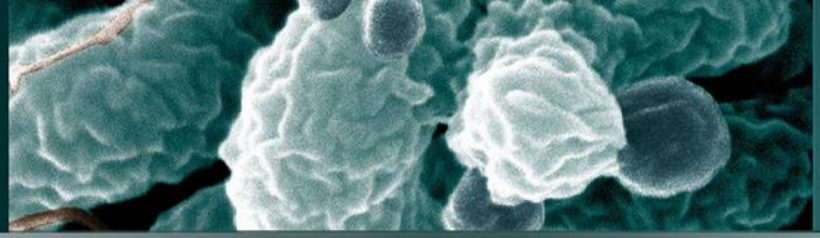
Advantage:

- this method combines advantages of disc method (easy manipulation) with ability to determine MIC directly

Disadvantage:

- costly

E-test
Gradient method



Dilution methods

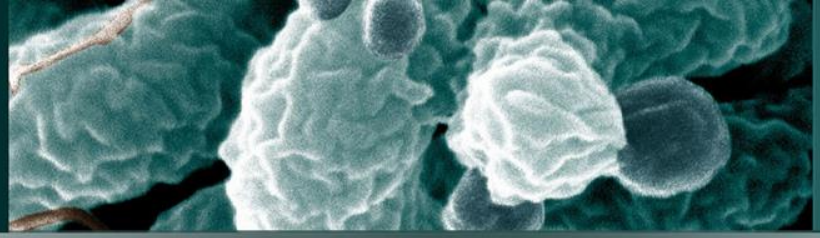
- **quantitative** tests

Principle:

- inhibition of bacterial growth due to given concentration of ATB
- ATB diluted in geometric series (two-fold)
- diluted ATB are mixed with liquid medium
- mixtured is added to 96-well microtitration plate
- growth of bacetria is demonstrated as **turbidity**
- in case of **inhibition of bacteria with ATB the turbidity disappears**

Results:

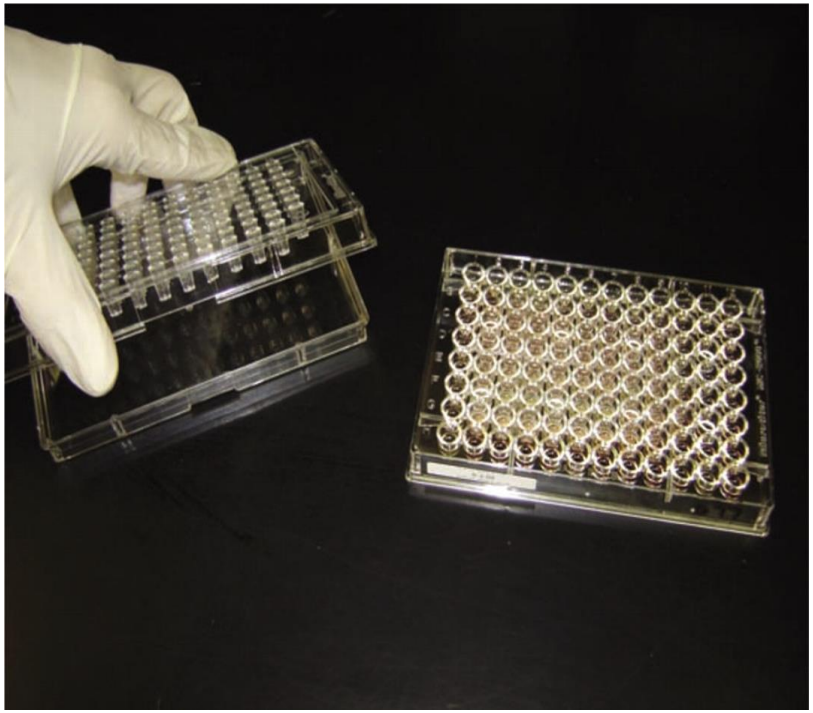
- the well in which the growth is stopped is evaluated as **minimal inhibition concentration = MIC** (more accurated than disc diffusion methods)

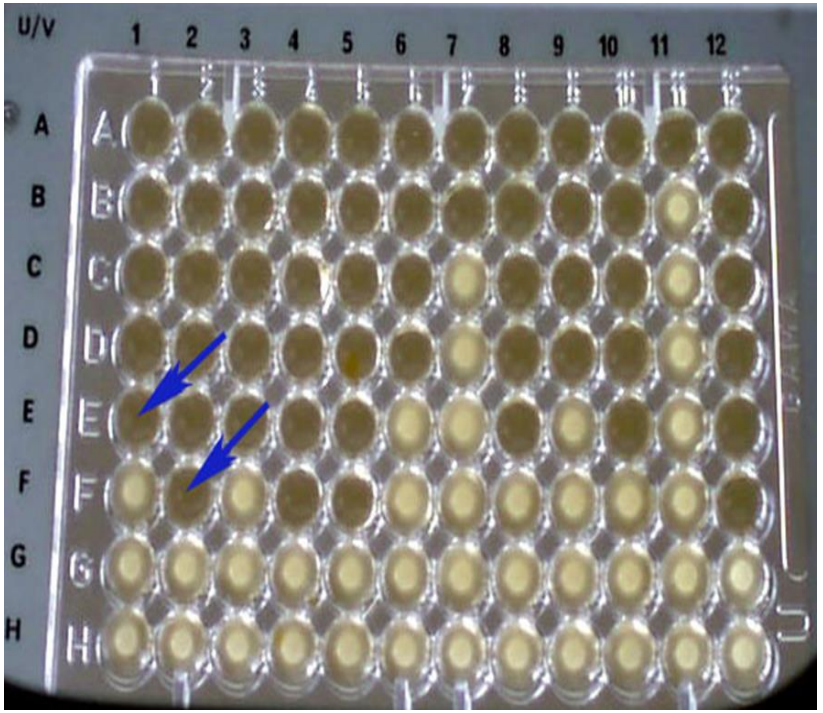


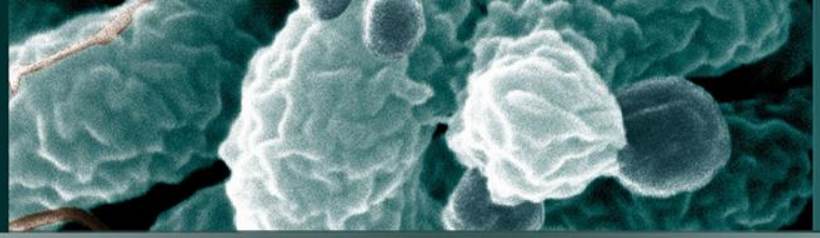
Dilution methods

Performance:

After application of mixture of bacteria and ATB, plate is incubated 24 hrs/ 37 °C and MIC is read.







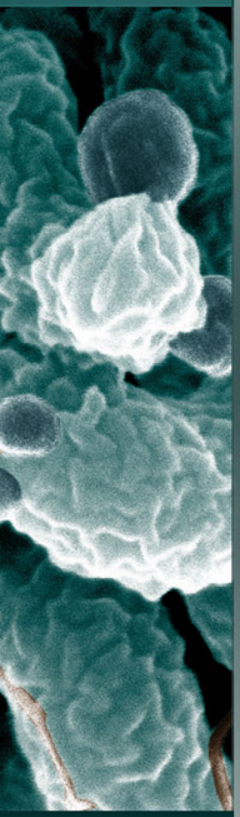
Antigen analysis of strains

- we need to know the specific antigen
- and have antibody to that antigen
  - many antigens
  - many antibodies
- in sample
  - precipitation
  - aglutination on carriers
  - immunoenzymatic reactions
  - immunofluorescence

Determination of bacterial toxin

- Not very often (small amounts of toxin in samples)
- ELISA
- Experiment on animal

- Antigen – antibody
  - ELISA
  - Toxins of *Clostridium difficile*
- Experiment on animal
  - Botulotoxin
  - Staphylococci enterotoxin
- Limulus test
  - Endotoxin of gramnegative bacteria



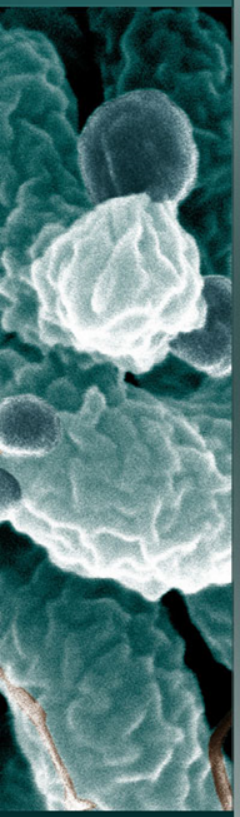
Determination of bacterial nucleic acids

- Without amplification

  - Gene probe
    - DNA complementary to wanted DNA
    - Chemically labeled
    - *Neisseria gonorhoae* and *Chlamydia trachomatis* in samples from urogenital tract

- With amplification

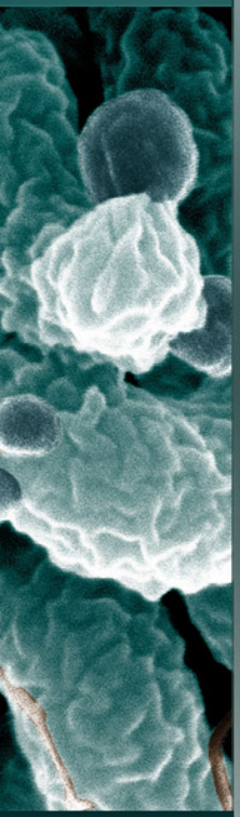
  - PCR (*polymerase chain reaction*)
  - Duplication of wanted sequence
  - Electrophoresis in gel

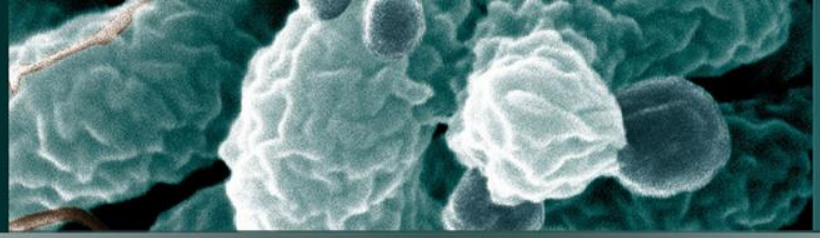


Indirect methods

- Determination of antibodies in plasma
  - Precipitation
  - Aglutination on carriers
  - Complement binding
  - Neutralisation
  - Methods with labels: Western blot, ELISA, immunofluorescence

- Determination of specific parts of acquired immunity



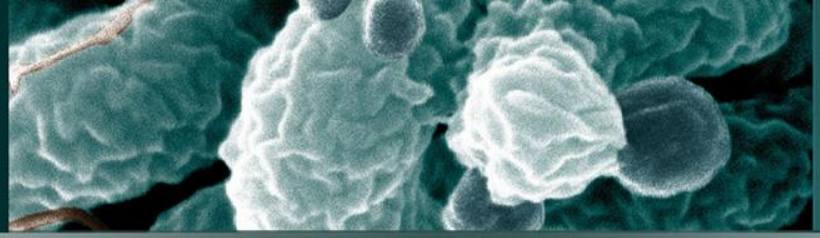


Lab class no. 3

Evaluation of microorganism sensitivity to antimicrobial compounds

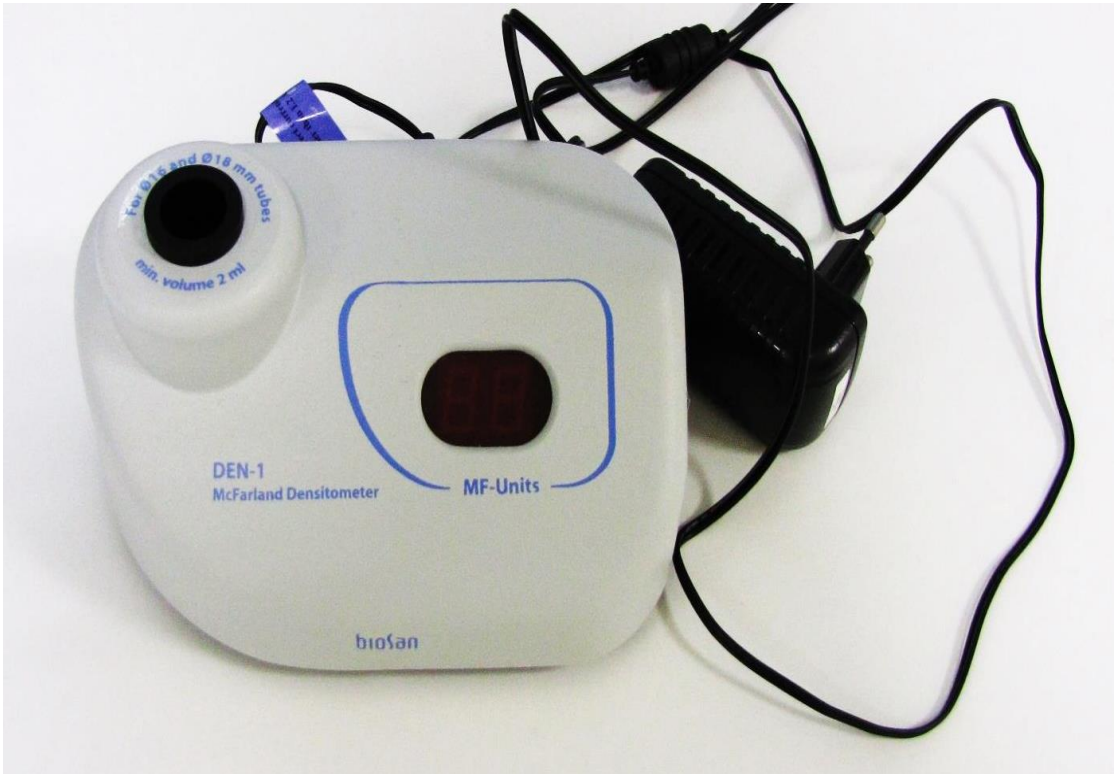
Aims:

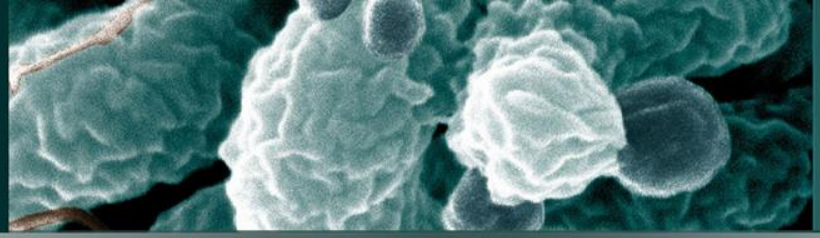
- ***Disc diffusion test***
- ***Microdilution method – determination of MIC***



- ***preparation of inoculum (suspension) of E. coli:***

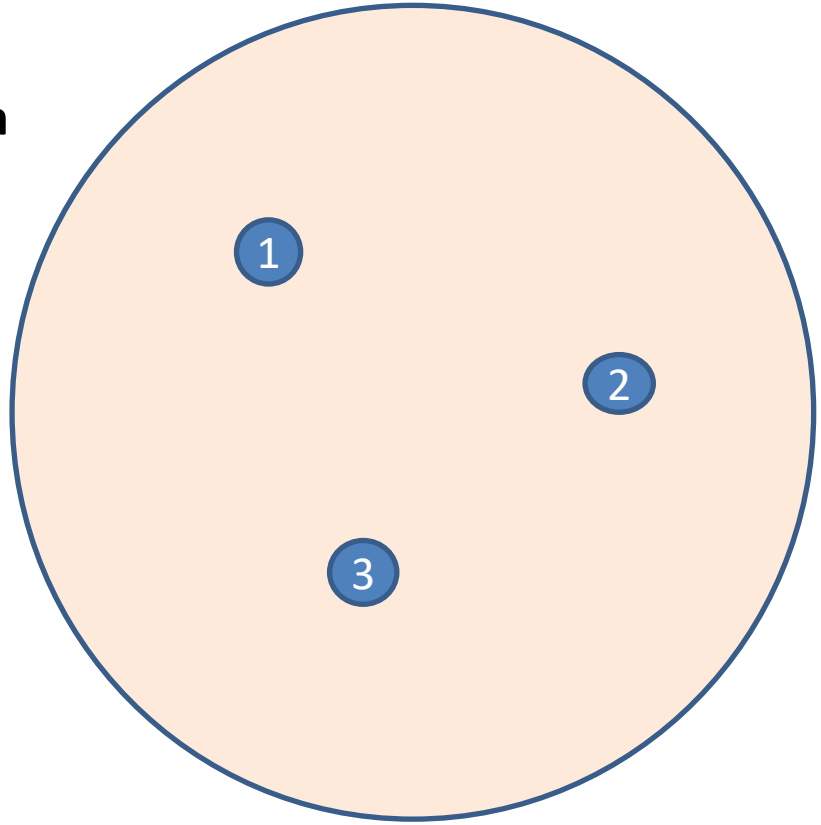
use one colony from cross scattering; suitable density is 0,5 McFarland, measure with densitometer



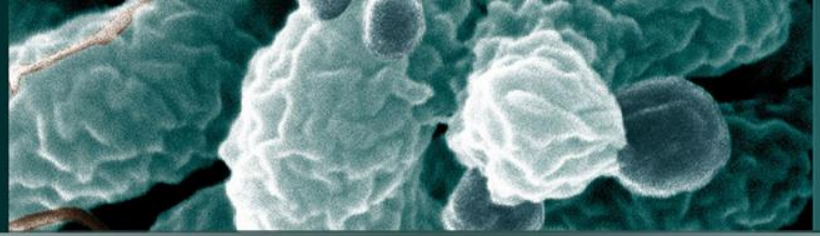


- *disc diffusion test*

transfer 0,5 ml of bacterial suspension to Nutrient broth agar with pipette; aspirate surplus liquid



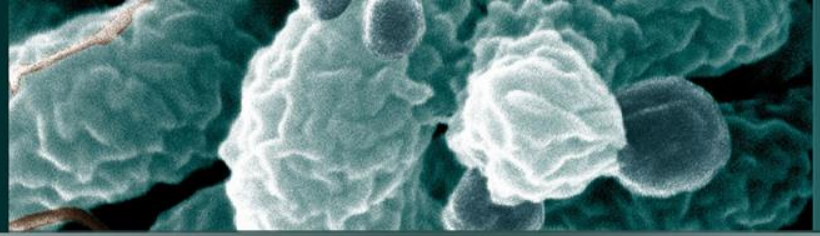
1st disc 3000 µg/mL sol. chloramphenicol (amount in disc is 30 ug)
2nd disc 1500 µg/mL sol. chloramphenicol (amount in disc 15 ug)
3rd disc 750 µg/mL sol. chloramphenicol (amount in disc 7,5 ug)

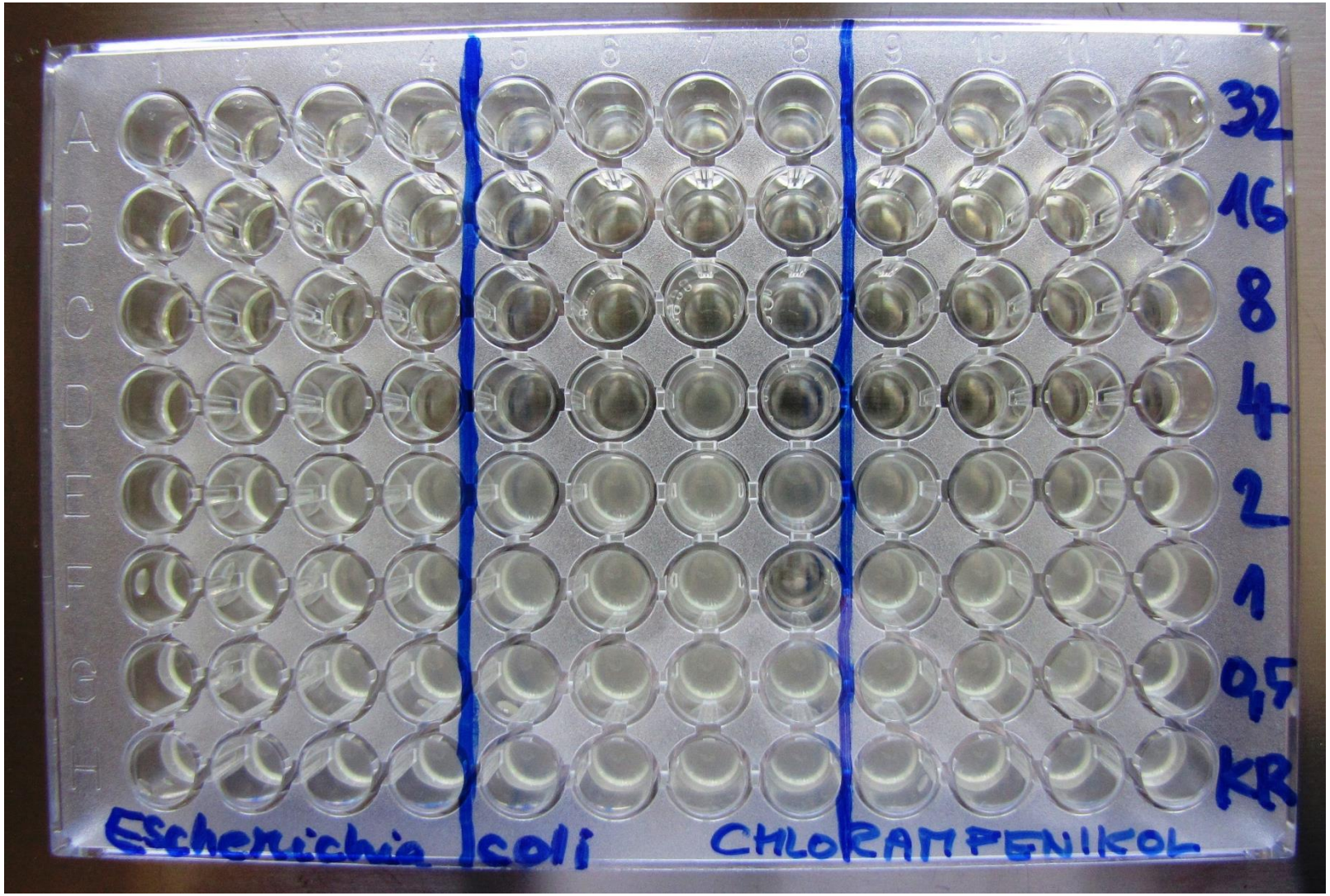


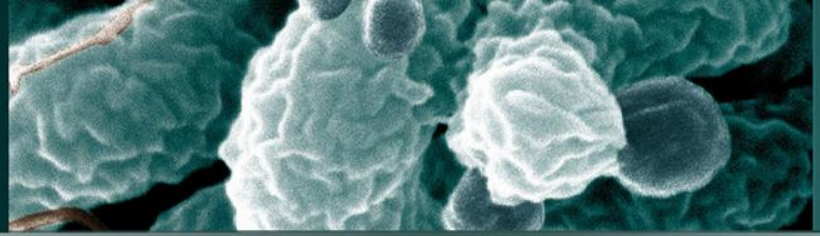
- ***microdilution method – determination of MIC***

chloramphenicol 320 µg/ml

	1. dvojice studentů				2. dvojice studentů				3. dvojice studentů				
	1	2	3	4	5	6	7	8	9	10	11	12	výsledná koncentrace chloramfenikolu:
A													32 µg/ml
B													16 µg/ml
C													8 µg/ml
D													4 µg/ml
E													2 µg/ml
F													1 µg/ml
G													0.5 µg/ml
H													KONTROLA




1 2 3 4 5 6 7 8 9 10 11 12
A B C D E F G H
32
16
8
4
2
1
0,5
KK
Escherichia coli
CHLORAMPENIKOL



Enterobacteriaceae

EUCAST Clinical Breakpoint Tables v. 7.1, valid from 2017-03-10

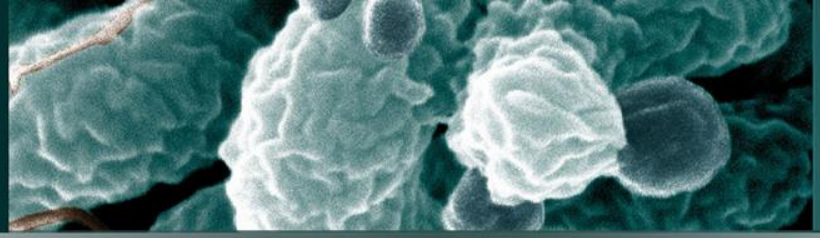
Miscellaneous agents	MIC breakpoint (mg/L)		Disk content (µg)	Zone diameter breakpoint (mm)	
	S ≤	R >		S ≥	R <
Chloramphenicol	8	8	30	17	17
Colistin[1]	2	2		Note[A]	Note[A]
Daptomycin	-	-		-	-
Fosfomycin iv	32[2]	32[2]	200[B]	24[C,D]	24[C,D]
Fosfomycin oral (uncomplicated UTI only)	32[2]	32[2]	200[B]	24[C,D]	24[C,D]
Fusidic acid	-	-		-	-
Metronidazole	-	-		-	-
Mupirocin					
Nitrofurantoin (uncomplicated UTI only), *E. coli*	64	64	100	11	11
Nitroxoline (uncomplicated UTI only), *E. coli*	16	16	30	15	15
Rifampicin	-	-		-	-
Spectinomycin	-	-		-	-
Trimethoprim (uncomplicated UTI only)	2	4	5	18	15
Trimethoprim-sulfamethoxazole[3]	2	4	1.25-23.75	14	11

Notes

Numbered notes relate to general comments and/or MIC breakpoints.
Lettered notes relate to the disk diffusion method.

1. Quality control of colistin must be performed with both a susceptible QC strain (*E. coli* ATCC 25922 or *P. aeruginosa* ATCC 27853) and the colistin resistant *E. coli* NCTC 13846 (*mcr-1* positive).
2. Agar dilution is the reference method for fosfomycin. MICs must be determined in the presence of glucose-6-phosphate (25 mg/L in the medium). Follow the manufacturers' instructions for commercial systems.
3. Trimethoprim:sulfamethoxazole in the ratio 1:19. Breakpoints are expressed as the trimethoprim concentration.

A. Use an MIC method.
B. Fosfomycin 200 µg disks must contain 50 µg glucose-6-phosphate.
C. Zone diameter breakpoints apply to *E. coli* only. For other Enterobacteriaceae, use an MIC method.
D. Ignore isolated colonies within the inhibition zone (see pictures below).



Questions for the last test:

- **Name basic fast biochemical tests**
- **Which test can discern *Staphylococcus aureus* from other staphylococci?**
- **Define MIC and MBC**
- **How can we measure concentration of bacteria in unknown sample and what are the units?**
- **What is E-test?**
- **Describe microdilution method, what is it for and where can we find reference values?**
- **Describe principle of biochemical tests**
- **What is MALDI-TOF and what is it used for?**
- **What is the use of McFarland scale**
- **Describe disc diffusion method, what is it for and where can we find reference values?**