Institute for microbiology presents:

TRACING THE CRIMINAL

Part Five:

Gram-Negative Criminals II

Medically important G– rods

Story	Endo	Group
P04	grows	Enterobacterales (GLC +, OXI –)
P04	grows	*Vibrio* and *Aeromonas* (GLC +, OXI +)
P04	does not	*Campylobacter* and *Helicobacter*
3. + 4.	grows	G- non-fermenters (GLC –, OXI + or –)
1. + 2.	does not	*Pasteurellaceae*
P06	does not	*Legionella, Bordetella, Brucella* etc.

Summary

Clinical characteristics – *Pasteurellaceae*

Clinical characteristics – G– glucose non-fermenters

Diagnostics of *Pasteurellaceae*

Diagnostics of G– glucose non-fermenters

Clinical characteristics – *Pasteurellaceae*

Story One

- Four-years old Jimmy is a fine boy, but his parents are members of a strange religious society and so they do not wish get him vaccinated. They would like to keep him at home, but as they have to be at work, they sent him to a nursery.
- After a month Jimmy started to have a cold, difficult breathing, gasping for air, and it was so serious that emergency had to be called. Emergency even thought about coniotomy, but finally it was not necessary. It was epiglottitis – a disease not very common nowadays…

Who did this to Jimmy?

- Criminal: ***Haemophilus influenzae* ser. b (Hib)**
- Haemophili are **short Gram negative rods**.
- Haemophili belong to the family ***Pasteurellaceae***, together with *Pasteurella* (see later) and some more bacteria, like *Aggregatibacter actinomycetemcomitans*, important in some specific types of gingivitis, or *Aggregatibacter aphrophilus,* formerly classified as *Haemophilus aphrophilus* and having importance similar to that of *Haemophilus parainfluenzae* (see later)
- Genera *Haemophilus* and *Aggregatibacter* belong to so called „HACEK“ group

HACEK group

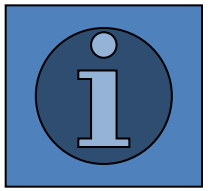
- The HACEK organisms are a group of **fastidious Gram-negative bacteria.** They are responsible for **some cases of infectious endocarditis,** especially those with negative result of cultivation.
- They are not common even as causative agents of endocarditis (they are told to be only responsible for less than 3 % of cases), but they are **dangerous by difficulties of the diagnostics**. They include:
  - ***H****aemophilus (H. haemolyticus, H. parainfluenzae*, but also *H. influenzae)*
  - ***A****ggregatibacter (A. actinomycetemcomitans, A. aphrophilus, A. segnis)*
  - ***C****ardiobacterium (C. hominis)*
  - ***E****ikenella (E. corrodens)*
  - ***K****ingella (K. denitrificans, K. kingae)*
- They are not taxonomically related. First two belong to *Pasteurellaceae* family, while *Cardiobacterium* is in family *Cardiobacteriaceae*, and last two belong to *Neisseriaceae* family.

Classification of haemophili

- ***Haemophilus influenzae***
  - **capsular type b (Hib)** – preventable (vaccine)
  - capsular types a, c, d, e, f
  - non-encapsulated strains
- ***Haemophilus parainfluenzae*** (much more common, much less pathogenic)
- ***Haemophilus ducreyi***, causative agent of a sexually transmitted disease **ulcus molle**

Pathogenicity of haemophili

- The most severe diseases caused by haemophili are **epiglottitis, meningitis and sepsis**. This is mostly typical for *Haemophilus influenzae*, serotype b.
- Other common diseases are **otitis media and sinuisitis** (after *Streptococcus pneumoniae* and together with *Moraxella catarrhalis*)
- Their **presence in throat is very common** and their pathogenic role is very questionable. Especially in a case of *Haemophilus parainfluenzae*, we usually do not suppose them to be pathogen.



Chancroid (ulcus molle)

It is a sexually transmitted disease found mostly in sub-tropical and tropical countries

Read more about chancroid

Pay attention:

Ulcus molle – chancroid – caused by *Haemophilus ducreyi*

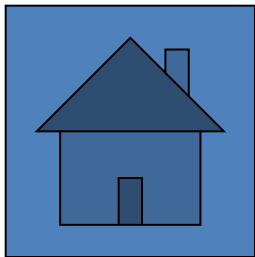
Ulcus durum – chancre – one of symptoms of syphilis, caused by *Treponema pallidum*

Story Two

- Joana was walking in gardens as usual. Unfortunately, one garden fence was too old and rotten and the dog behind too strong. The dog run out and Joana was bitten into her leg.
- The owners of the dog had proven that the dog has been vaccinated against rabies. Nevertheless, some pus soon occurred in the wound. The pus was sent to the laboratory. And the criminal was…

Pasteurella multocida

- *Pasteurella multocida* is normal respiratory microbiota in dogs.
- In humans, it causes mainly pyogene wound inflammations after being bitten by a dog or another animal.
- It smells similarly as *haemophilus* (some people say „like old rag“), but it grows on blood agar (not Endo agar).
- The morphology of colonies: something between *Streptococcus* and *Enterococcus*, but it is vancomycin resistant and this is suspicious to the microbiologist (especially with parallel susceptibility to penicillin)



Clinical characteristics – Gram– glucose non-fermenting bacteria

Story Three

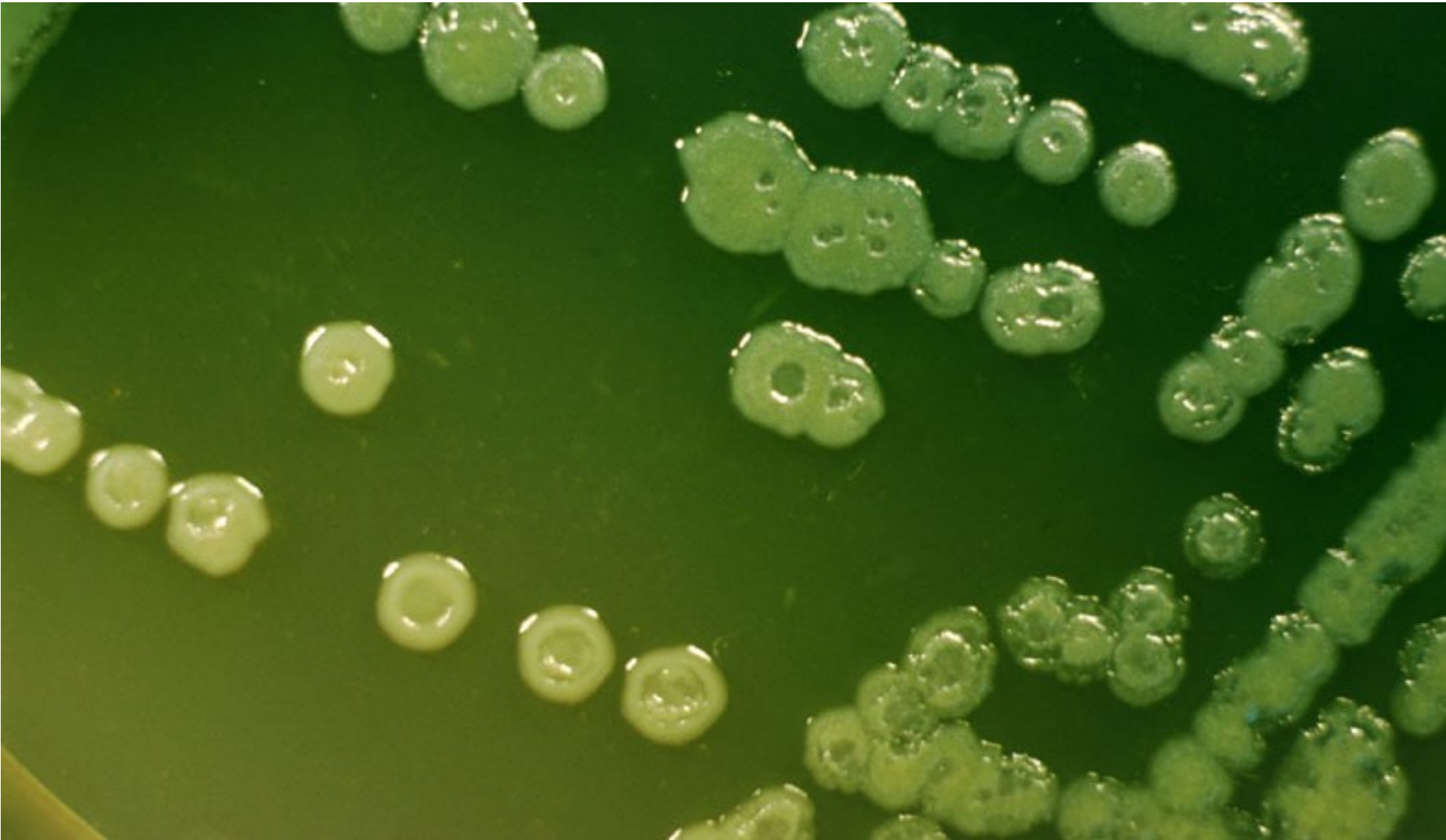
- Mr. Phosphoros is a pyromaniac. Several days ago, he burned himself and his burn was inflammated. He was hospitalised on a specialized centre and felt very badly. Doctors knew that it had no sense to try antibiotics accidentally, so they performed a swab from burn. Thanks to this, a targeted therapy was found, and Mr. P. was healed. Of course, sooner or later, he will probably play with his matches again *(like some students of these practical exercises)*.

Who is guilty this time?

- *Pseudomonas aeruginosa*, the most common of „Gram-negative non-fermenters“ (G– NF)
- On the other hand, the guilty one could be any of that group, e. g. *Acinetobacter*, *Burkholderia cepacia* or *Stenotrophomonas maltophilia*
- It is a group of unrelated bacteria (some of them are beta- and some gammaproteobacteria), but sharing some properties, especially the fact that they are not able to ferment glucose
- Those bacteria are mostly strict aerobes, instead of fermentation of sugars, they utilize them by aerobic respiration, and their adaptation to outer environment is obvious also in other properties – they have low temperature optimum and they are often pigmented, so they fight with sunlight in outer environment

Green pigmented strain of *Pseudomonas aeruginosa* on MH

Photo: Inst. for Microbiology, photo by prof. Skalka



Pathogenicity of G– NF

- Commonly: they are bacteria from outer environment, often plant pathogens, „not-brave-bacteria“, which are not able to infect a healthy person – such bacteria are known as *opportune pathogens*. Their aims are patients with burns, clients of emergency units, transplant centres, e. t. c.
- They often cause wound infections, can be found in respiratory ways, and even in the bloodstream of hospitalized persons.
- So they are important causative agents of nosocomial infections
- Sometimes it is difficult to differentiate between an infection and a colonisation – especially in superficial wounds it is often useless to use other than topical antibiotics

Story Four

- Linda was a poor girl: she suffered from **an inborn disease, cystic fibrosis.**
- Her **lung surfactant was different from surfactant of healthy people**. So, it was infected very often.
- It was *Staphylococcus aureus* last time . This time it was different: **the causative agent was *Burkholderia cepacia***, one of G– non-fermenting bacteria.
- ***Burkholderia cepacia*** is responsible for rotten onions (***Allium cepa***), so it is really a typical plant pathogen
- Genus *Burkholderia* also includes *B. mallei*, causative agent of glanders, and *B. pseudomallei*, causative agent of melioidosis

More Non-fermenters

- Apart from *Burkholderia* and *Pseudomonas*, important G- non-fermenters are also *Stenotrophomonas maltophilia, Acinetobacter baumannii* complex, *Acinetobacter lwoffii* and others
- ***Stenotrophomonas maltophilia*** is a long name, but it is possible to learn it easily: it is narrow-nutrition-unit maltose-loving, so it is a „bacterial panda“, chewing maltose instead of bamboo ☺.
- ***Acinetobacter*** has its name derived from Greek (a-kineto- = not moving)

Non-fermenters and Cystic fibrosis

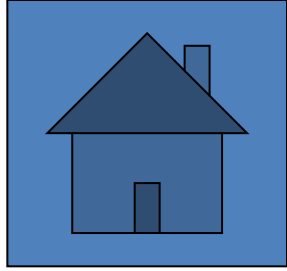
- Cystic fibrosis is a severe, **inborn lung disease**, with failure of production of normal lung surfactant. This leads to changed characteristics of lungs, including many times increased risk of infection
- **Most common causative agents** are *Pseudomonas aeruginosa, Burkolderia cepacia* and *Staphylococcus aureus*. Strains often become **polyresistant** and many children with cystic fibrosis die very young.

Bacterial metabolism and relation of bacteria to oxygen

We know already that G– non-fermenters are bacteria that do not ferment sugars, but they perform aerobic respiration. Let‘s compare two bacteria:

- *Escherichia coli* lives in the intestine. It has enough nutrients, but not enough oxygen (unlike other gases ☺), so it prefers glucose (and other substrates) fermentation. *Escherichia coli* is a **facultative anaerobe**. Some other intestinal bacteria are **strict anaerobes**.
- On the other hand, *Pseudomonas* has oxygen enough, but nutrients not enough. It uses aerobic respiration: enables better exploitation of nutrients. *Pseudomonas* is a **strict aerobe**.

Pseudomonas as a strict aerobe (unlike other bacteria)



- Unlike strain I (*Escherichia coli*) and strain II (*Bacterioides fragilis*, a strict anaerobe), *Pseudomonas aeruginosa* (strain III) is a strictly aerobic bacterium (more about *Bacteroides fragilis* in P07)

Strain	Broth	VL-broth	Result
III	growth	clear	Strictly aerobic bacterium
II	clear	growth	Strict anaerobe
I	growth	growth	Facultative anaerobe

Diagnostics of *Pasteurellaceae*

Methods in *Pasteurellaceae* diagnostics

- Direct methods
  - Microscopy – short G– rods
  - Culture – *Pasteurellaceae* do not grow on Endo agar, *Haemophilus* even does not grow on Blood agar (except being co-cultivated with another microbe)
  - Biochemical identification – it is possible to use it
  - Antigen analysis – used in haemophili (Hib)
  - Nucleic acid detection – not used routinely

Indirect methods used rarely

Differentiation of *Pasteurellaceae* (differential diagnostics)

- Gram staining: Gram– rods × other bacteria
- Endo medium: as we now, among clinically important bacteria, only *Enterobacteriaceae*, *Vibrionaceae* and Gram– non-fermenters are able to grow. ***Pasteurellaceae* do not grow**.
- *Pasteurellaceae* are detected by typical smell, biochemical properties, growth on individual media, typical antibiotic susceptibility etc.

Haemophilus and *Pasteurella* diagnostic

- *Pasteurella* is able to grow on blood agar
- Haemophili are not able to grow on blood agar, they are not able to „open the RBC“. So, they grow only on chocolate agar or Levinthal agar (filtrated chocolate agar)
- On BA, they are able to grow in presence of a bacterium that „opens the RBC“ (satellite phenomenon). Such bacterium is e. g. *Staphylococcus aureus.*
- They grow in tiny colonies, so we use a disc to inhibit the growth of other bacteria (bacitracin, but in higher concentration than in bacitracin test)

Satellite phenomenon

- As we already know, haemophili need factors from RBC, but they are not able to break an RBC. They need the RBCs to be broken
  - by heating – chocolate agar
  - by presence of another microbe
- Satellite phenomenon is an example of the second way how to make haemophili be able to exploit blood factors. That means the growth of *Haemophilus* around *Staphylococcus* line only.
- Presence of satellite phenomenon is a confirmation, that our bacterium is really a *Haemophilus*

Haemophili on chocolate agar (left) and as a satellite on blood agar

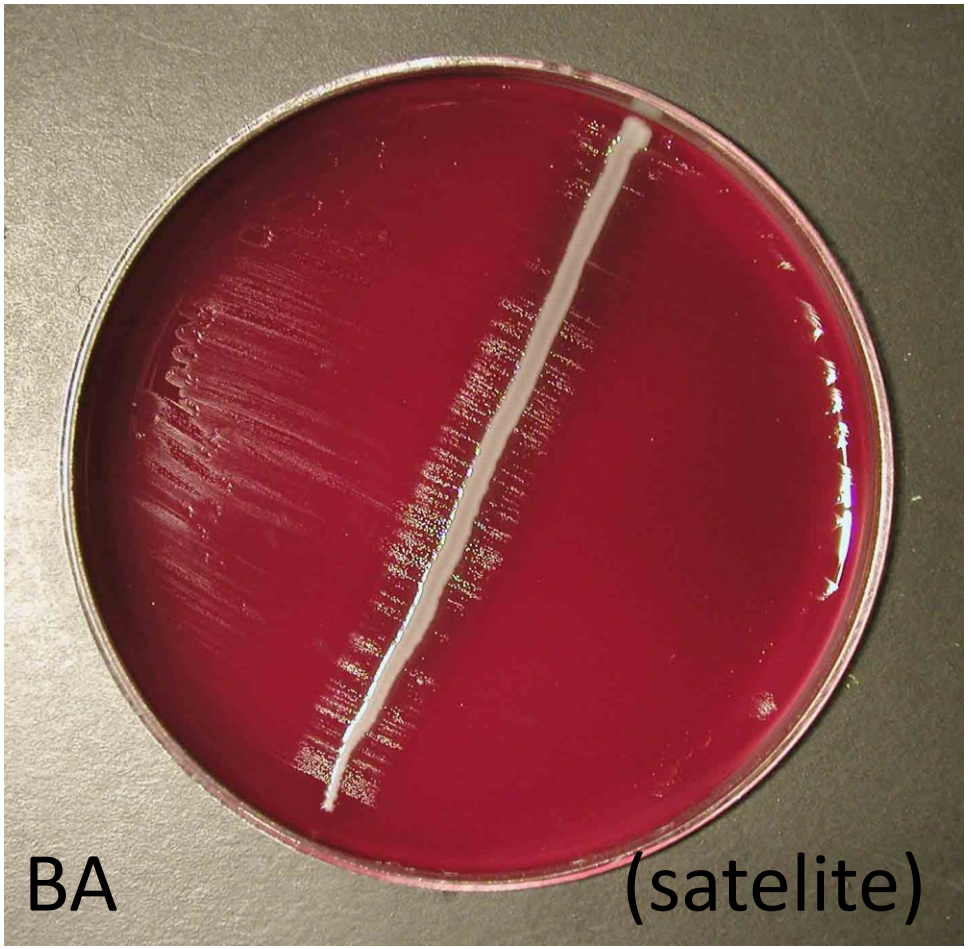
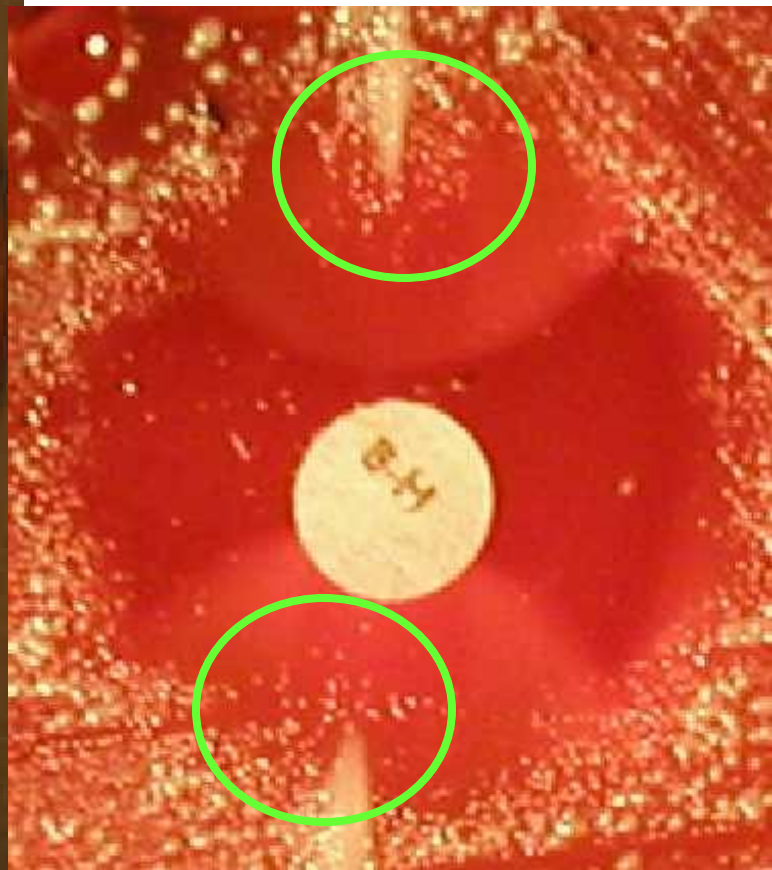


Photo: Inst. for Microbiology (web of the Institute)

Detection of haemophili

Haemophili are more resistant than the bacteria of the common flora, so they grow inside the zone, but only near to staphylococcus line (satellite phenomenon)

Photo: Inst. for



Growth factors of Haemophili

(the test also includes *Aggregatibacter aphrophilus*, formerly *Haemophilus aphrophilus*)

- The tested bacteria need factors from blood, but the need of individual factors is species specific.
  - *H. parainfluenzae* needs factor V (= NAD)
  - *A. aphrophilus* needs factor X (= hemin)
  - *H. influenzae* needs both factors.
- We use discs with these factors: one with X, another with V, and the third with a mixture of both of them.

Growth factor test of *Hemophili*

One disk is with factor X, second with factor V, third a mixture

Aggregatibacter

H. influenzae (left), *H. parainfluenzae* (right)

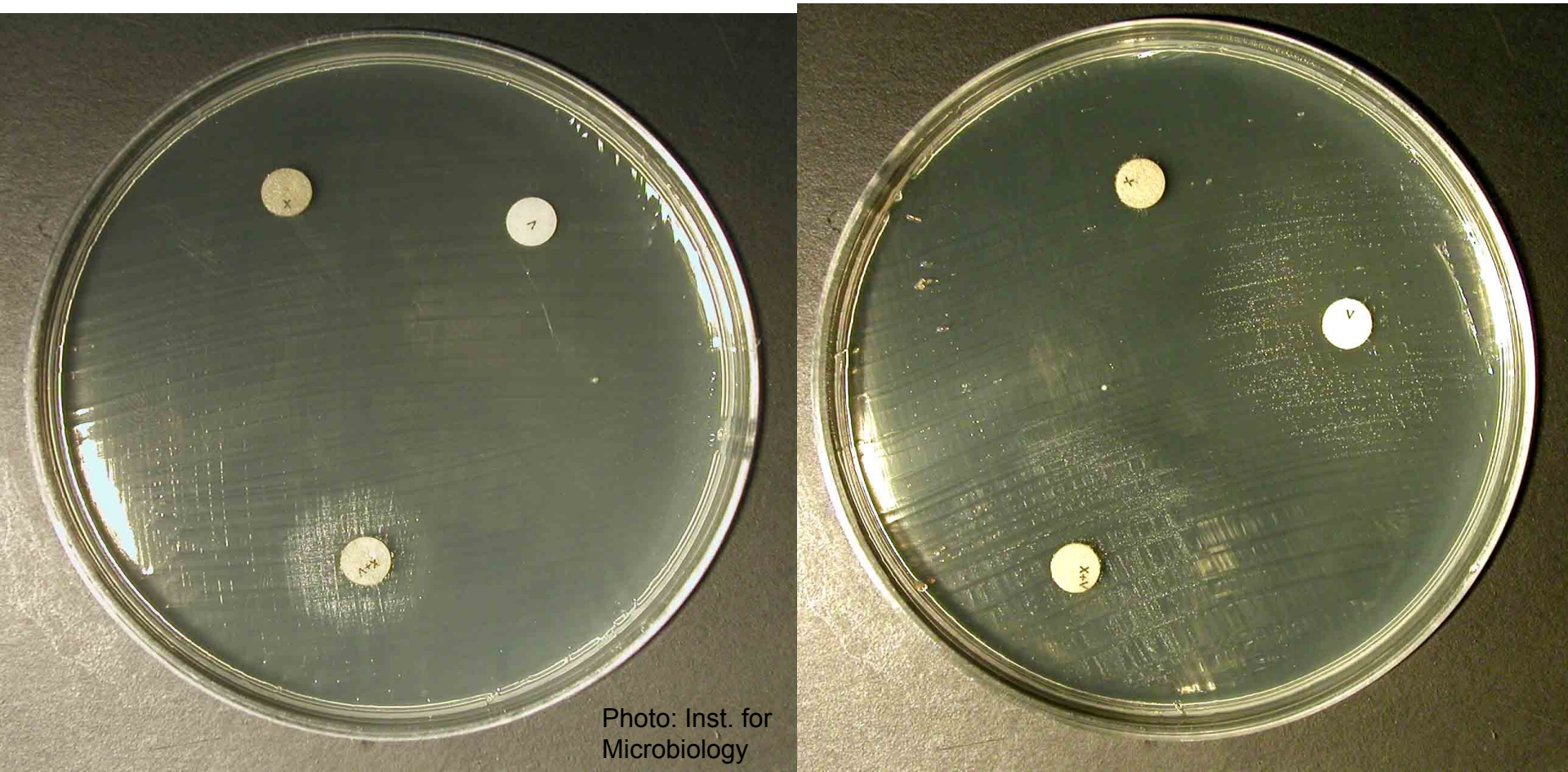


Photo: Inst. for Microbiology

Haemophilus influenzae: antigen analysis (intra-species diagnostics

- Antigen analysis in *Haemophilus influenzae* is performed like in other bacteria. The main goal is differentiation of Hib. Today, we have commercially available sets, containing e. g. latex particles. We try to assess the capsular type of *H. influenzae* (a, b, c, d, e, or f). When the strain does not agglutinate with any sera, it is probably an non-encapsulated strain
- Formerly, so named co-agglutination with *Staphylococcus* strain was used: agglutinate was more dense because of *Staphylococcus* binding the Fc-end of anti-haemophilus antibody

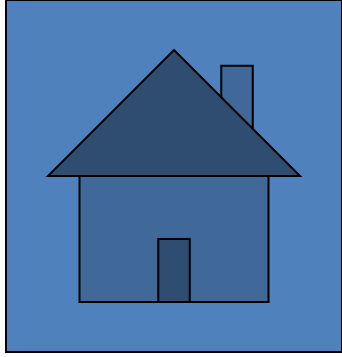
Detection of *Pasteurella* using typical susceptibility pattern

- **No Gram-negative bacterium is susceptible to vancomycin.** Vancomycin can be used for Gram-positive bacteria only, it is very strong; all streptococci and majority of staphylococci and enterococci are susceptible
- On the other hand, **very little bacteria are susceptible to penicillin, especially among G– bacilli.**
- **So, susceptibility to penicillin and resistance to vancomycin is quite typical for *Pasteurella*.**

Tests of atb susceptibility

- haemophili do not grow on MH agar
- Usually Levinthal agar (filtrated chocolate agar) is used for diffusion disc test – for this purpose, this agar is better than classical chocolate agar
- Our laboratory uses „Haemophilus agar“, similar to Levinthal agar
- Reading of the zones is the same as for any other bacteria

Antibiotic susceptibility testing: An example of *Pasteurellaceae* antibiotic set



Antibiotic	Abbr.	Reference zone
Penicillin (penicillin)	P	S ≥ 12 / R < 12
Ko-amoxicillin (penicillin)	AMC	S ≥ 15 / R < 15
Cefuroxime (CS II. gen.)	CXM	S ≥ 26 / R < 25
Nalidixic acid (quinolone)	NA	S ≥ 23 / R < 23
Tetracyclin (TE)*	TE	S ≥ 25 / R < 22
Co-trimoxazol (SXT)	SXT	S ≥ 23 / R < 20
*valid also for doxycyclin **valid for all quinolones		

Diagnostics of Gram– non-fermenters

Methods for G– non-fermenters

- Direct methods
  - Microscopy – mostly G– rods, but *Acinetobacter* is a G– coccus
  - Culture – non-fermenters grow on majority of media, including Endo agar. As glucose-non fermenters, they are mostly also lactose-non fermenters, but their colonies are sometimes quite dark, because of pigmentation
  - Biochemical identification – possible, but tests checking aerobic respiration (not fermentation) should be used. We also use mostly decreased temperature and prolonged incubation
  - antigen analysis, nucleic acid detection – not used routinely

Indirect methods used rarely

Differentiation of G– non-fermenters (differential diagnostics)

- Gram staining: **Gram– rods** × other bacteria
- Endo agar: **they grow**. As glucose-non fermenters, they are mostly also lactose-non fermenters, but their colonies are sometimes quite dark, because of pigmentation
- Non-fermenters are differentiated from enterobacteria/vibria by no fermentation of glucose (e. g. **Hajna medium remains completely red** after culture, no colour change; but **eventual light brown colour does not matter, it is due to presence of pigments**)

Further diagnostics of individual genera and species of G– NFs

- *Pseudomonas* is usually detected by:
  - Presence of typical odour (young cultures)
  - Pigments, mostly **green**, sometimes blue or maroon. Best visible on MH, worse on BA and Endo agar
  - Positive oxidase
- Other non-fermenters, or not-sure *Pseudomonas*, should be differenciated biochemically, e. g. by NEFERMtest 24

Pseudomonas on MH agar and other media

- Remember, that MH agar itself is nearly colourless (or slightly yellowish).
- All green colour you see is product of *Pseudomonas*, or more precisely, of its pigment pyoverdin
- On BA and Endo, pigment production is not so strong, but partially visible, too. Nevertheless, something more visible on these media is the typical pearl smooth surface of the colonies

Oxidase test in non-fermenters

- Among the most common G– non-fermenters, ***Pseudomonas*** **is oxidase positive,** ***Burkholderia*** **usually too; on the contrary,** ***Stenotrophomonas*** **and** ***Acinetobacter*** **are usually negative .**

NEFERMtest 24

- For precise biochemical identification of G– non-fermenters we use mostly NEFERMtest 24 (or a similar test).
- It is a triple-strip (not double as last week)
- There is a different way of code-formation than for (for example) ENTEROtest 16:
  - first number is 0 (oxidase –) or 1 (oxidase +)
  - next 6 numbers come from columns H to C
  - columns B and A are not counted (they are eventually used for more detailed determination)

NEFERMtest 24

- One frame is used for four triple-strips (for four strains). Each strain is detected using 24 reactions.
- Requires 30 °C, 48 h

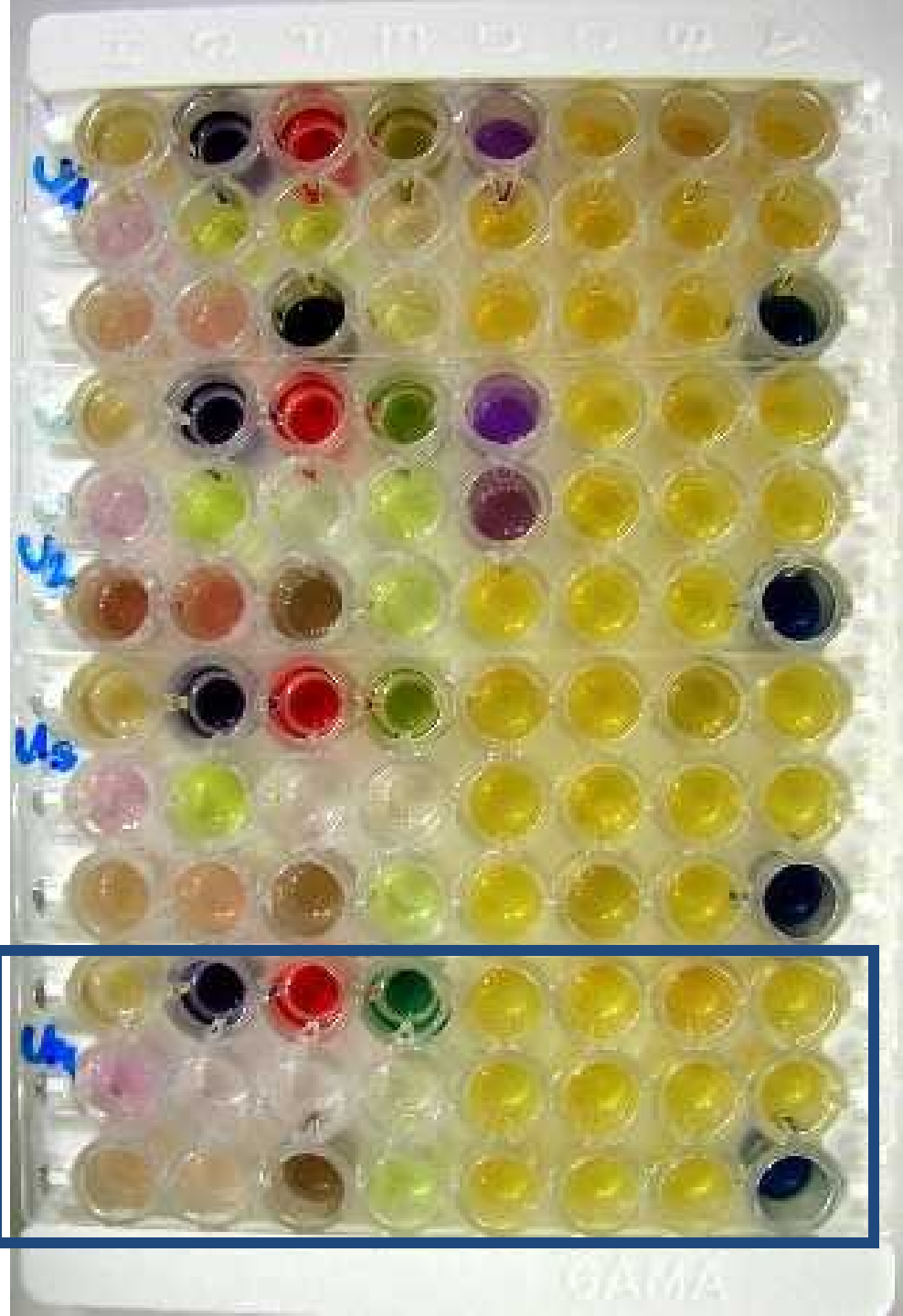


Photo: Inst. for Microbiology (web of the Institute), photo O. Z.

Antibiotics susceptibility of G– NF

- G– non-fermenters may be tested on common media.
- We use strong antibiotics, that should not be used for other infections
- We use:
  - 3rd generation cephalosporins* (but only some of them – „anti-pseudomonad“ ones, like ceftazidime)
  - Anti-pseudomonad penicillins, monobactams and carbapenems* (imipenem, piperacillin/tazobactam)
  - aminoglycosides (gentamicin, amikacin)
  - fluoroquinolones (ciprofloxacin, ofloxacin)
  - polypeptides (colistine)

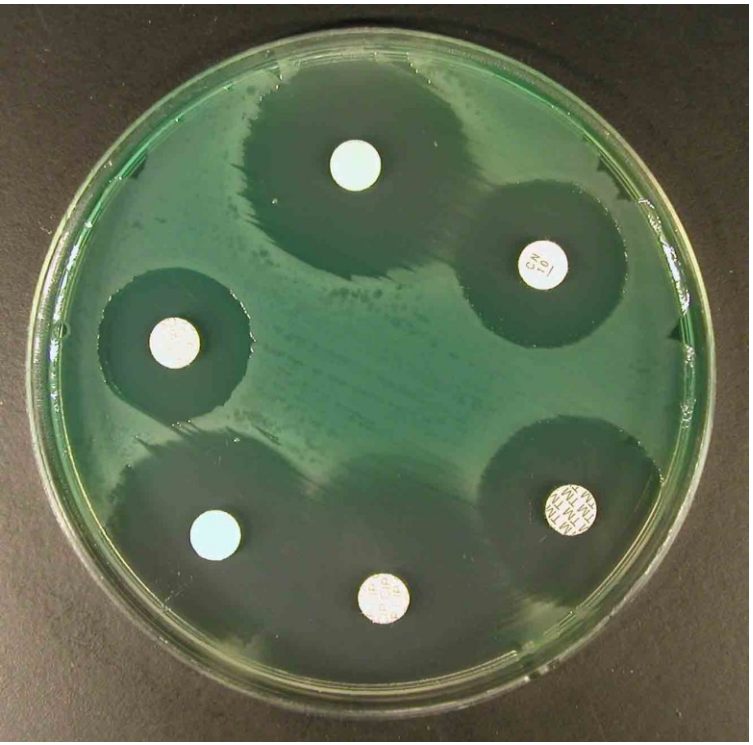
**or combinations with beta-lactamase inhibitors*

An example of *Pseudomonas* atb set

Antibiotic	Abbrev.	Reference zone
Piperacilin+tazobactam*	TZP	C ≥ 18 / R < 18
gentamicin (aminoglycoside)	CN	C ≥ 15 / R < 15
ofloxacin (quinolone)	OFL	C ≥ 16 / R < 13
ciprofloxacin (quinolone)	CIP	C ≥ 26 / R < 26
ceftazidime (CS III G)	CAZ	C ≥ 17 / R < 17
colistin (polypeptide)	CT	C ≥ 11 / R < 11
*antipseudomon. penicilin + β-lactamase inhibitor		

Pseudomonas aeruginosa susceptibility

Photo: Inst. for Microbiology



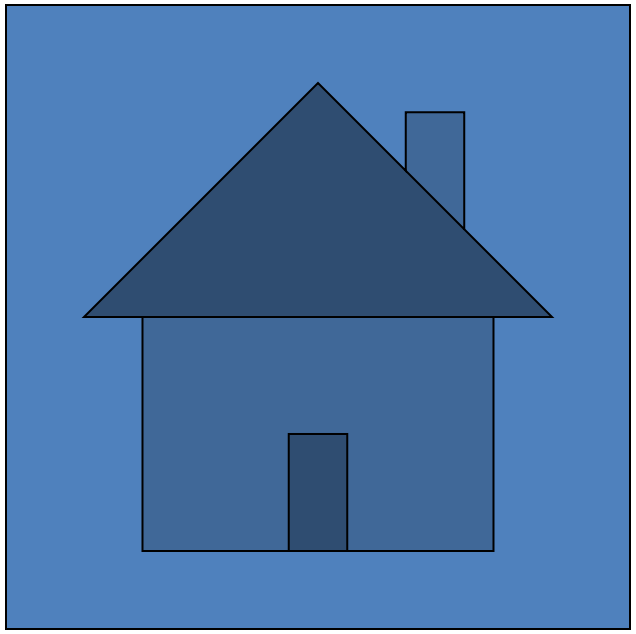
On this picture, *Pseudomonas aeruginosa* is probably susceptible to all tested antibiotics, but it is possible set containing only discs with special anti-pseudomonad drugs. There exist poly-resistant strains that have secondary resistances even to such antibiotics.

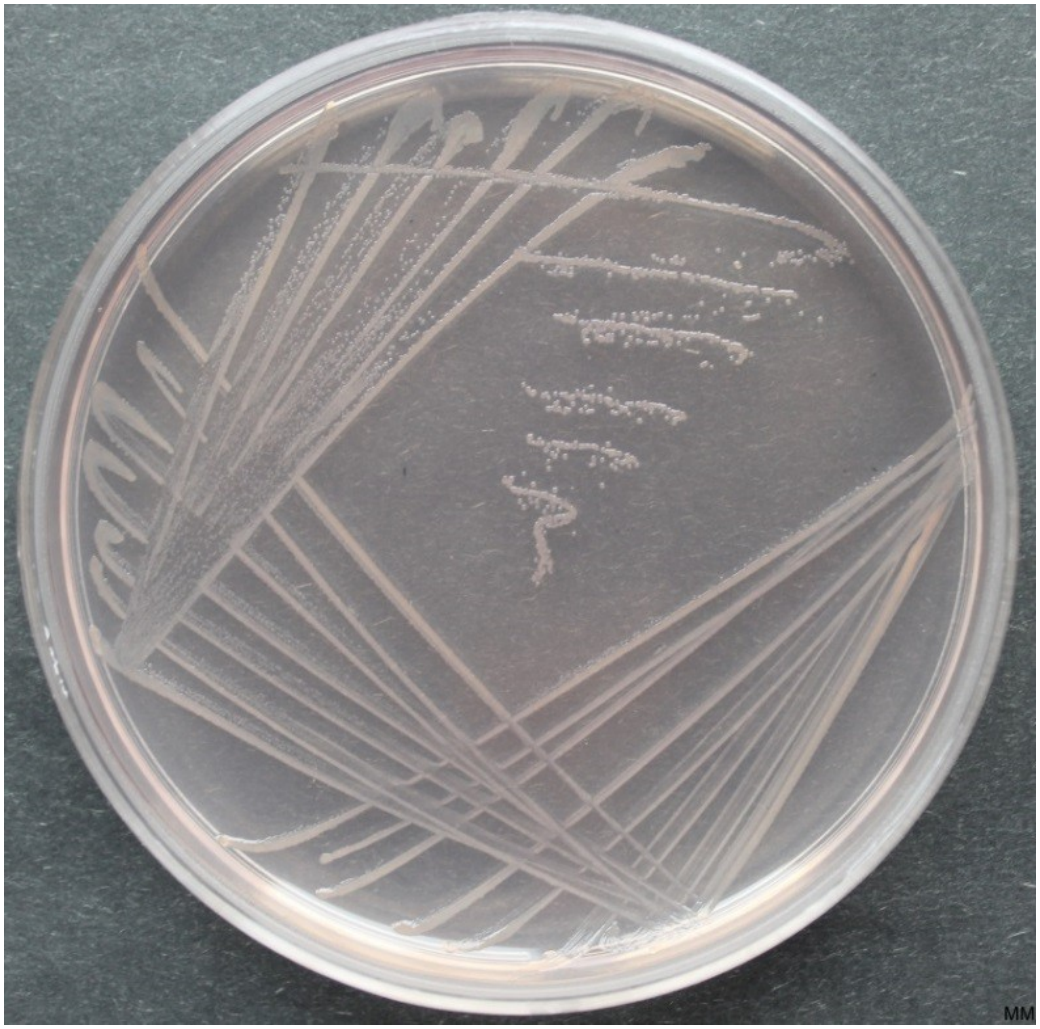
Producers of so called metalo-betalactamases (MBL) use to be only susceptible to amikacin and colistin.

It is also possible to use E-test (here) or microdilution test

Photo: Inst. for Microbiology

The End





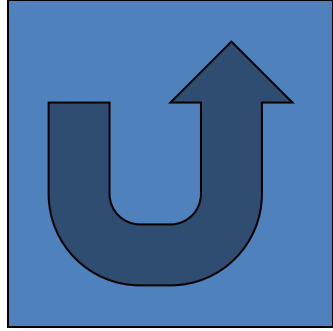
Burkholderia cepacia, photo: Inst. for Microbiology

Inflammation of external ear – otitis media (bonus)

- **Common in children** (short horizontal Eustach tube)
- **Caused by:** *Streptococcus pneumoniae, Haemophilus influenzae, Moraxella catarrhalis*
- **In chronical cases** also some G– rods

It is necessary to differentiate otitis externa: here Staphylococcus aureus is the main pathogen (as in other skin inflammations), local therapy, e. g. Framycoin drops

Examination and treatment of otitis media



- **Therapy** is indicated in case of a real inflammation (pain, redness, fever) and it does not react to anti-inflammatory treatment
- **Drug of choice** is amoxicilin (e. g. AMOCLEN), an alternative is co-trimoxazol
- **Ear swab** examination is meaningful only after paracentesis
- Otherwise it is also possible to examinate **pyogenic liquid** taken during paracenthesis