

Institute for microbiology shows:

# TRACING THE CRIMINAL



Part Five:

## Gram-Negative Criminals II

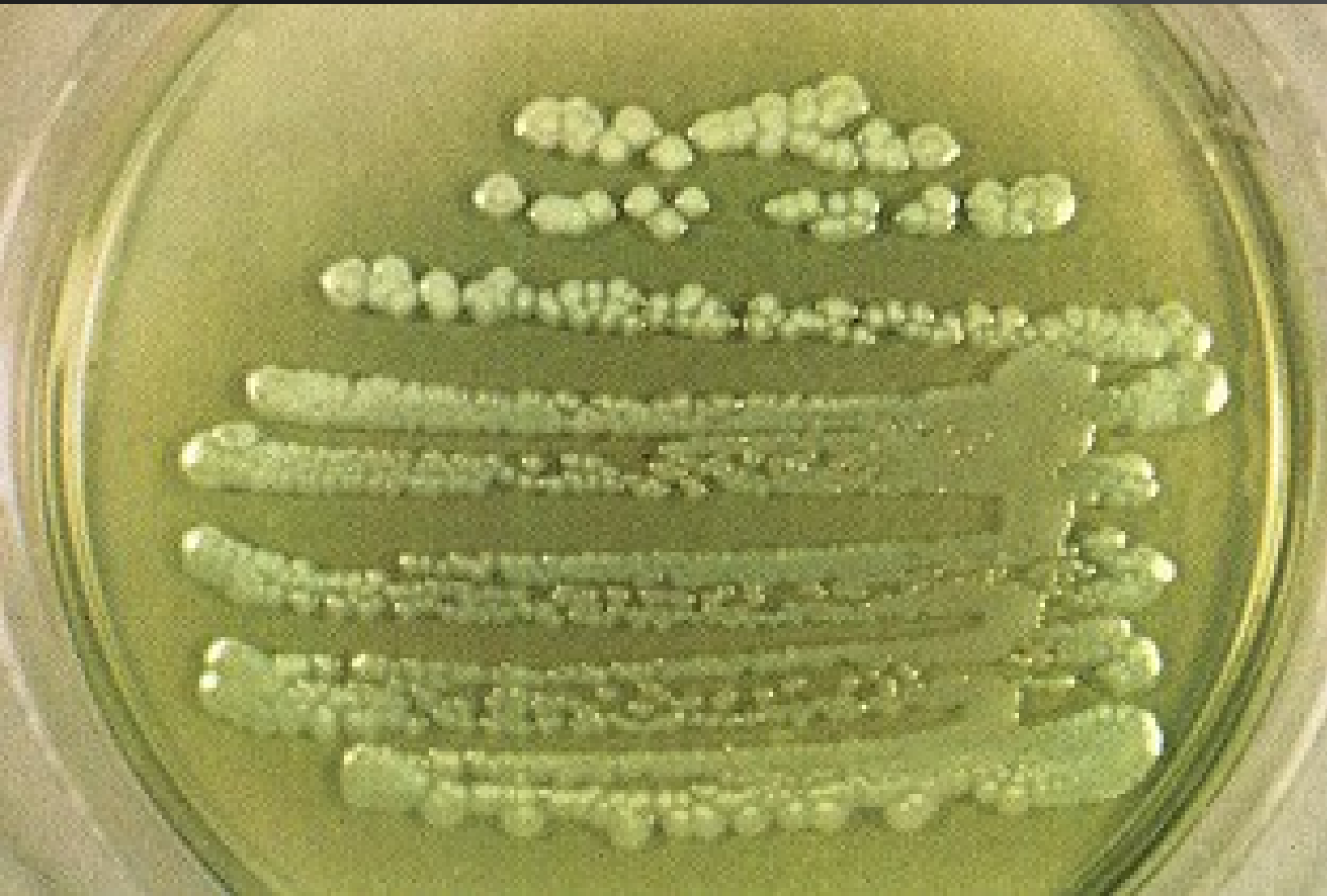
# Survey of medically important G- rods



Story	Endo	Group
P04	grows	<i>Enterobacteriaceae</i> (GLC +, OXI -)
P04	grows	<i>Vibrionaceae</i> (GLC +, OXI +)
P04	does not	<i>Campylobacter</i> and <i>Helicobacter</i>
3. + 4.	grows	G- non-fermenters (GLC -)
1. + 2.	does not	<i>Pasteurellaceae</i>
P06	does not	<i>Legionella</i> , <i>Bordetella</i> , <i>Brucella</i> etc.

# *Pseudomonas aeruginosa* – a microbiological everGREEN 😊

textbookofbacteriology.net



# Survey of topics

Clinical characteristics – *Pasteurellaceae*

Clinical characteristics – G– glucose non-fermenters

Diagnostics of *Pasteurellaceae*

Diagnostics of G– glucose non-fermenters

Clinical  
characteristics –  
*Pasteurellaceae*

# Story One

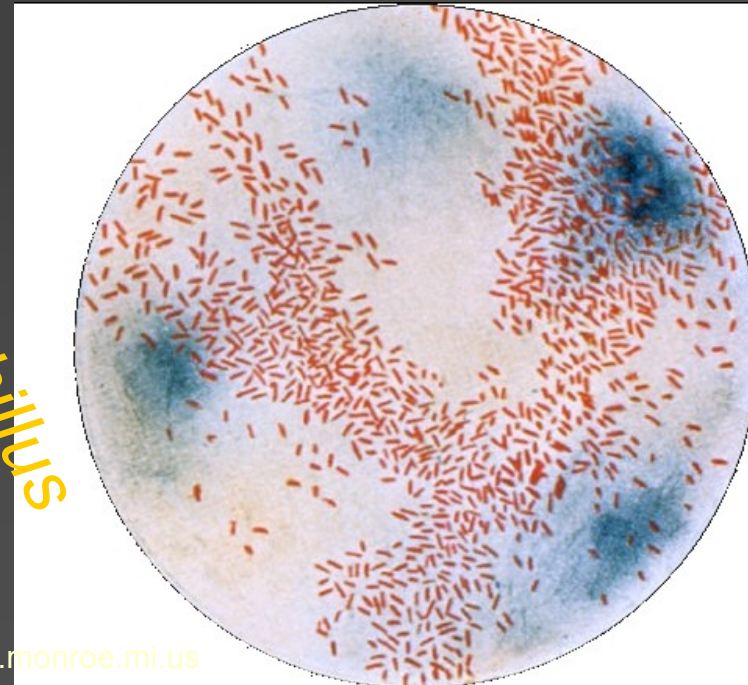


- **Four-years old Jimmy** is a fine boy, but his parents are members of a strange religious society and so they **do not wish let him get vaccinated**. They would like to keep him at home, but as they have to be at work, they sent him to a nursery.
- After a month **Jimmy started to have a cold**, difficult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

# 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)

Haemophilus




# 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 pathogenous)
- ***Haemophilus aphrophilus*** and many other species
- ***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 sinusitis** (after *Streptococcus pneumoniae* and together with *Moraxella catarrhalis*) 
- Their **presence in throat is very common** and their pathogenic role is very query. Especially in case of *Haemophilus parainfluenzae*, we usually do not suppose them to be pathogens.

# *A Haemophilus disease*

<http://www.immune.org.nz>





**H**

**I**

**B**

**מכ-הנאכ**

# Ulcus molle

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



[www.fmt.am.gov.br](http://www.fmt.am.gov.br)



[www.fmt.am.gov.br](http://www.fmt.am.gov.br)



<http://www.sexsgdom.dk>

**Ulcus molle – chancroid** – caused by *Haemophilus ducreyi*

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

# Story Two

- Joana did walking in gardens as usually. Unfortunately, one garden fence was too old and rotten and the dog inside too strong. The dog run out and just met Joana. So, **Joana was bitten into her leg.**
- The owners of the dog had proven that the dog was vaccinated against rabies. Nevertheless, some **pus was found soon in the wound.** So the pus was sent to the laboratory. And the criminal was...

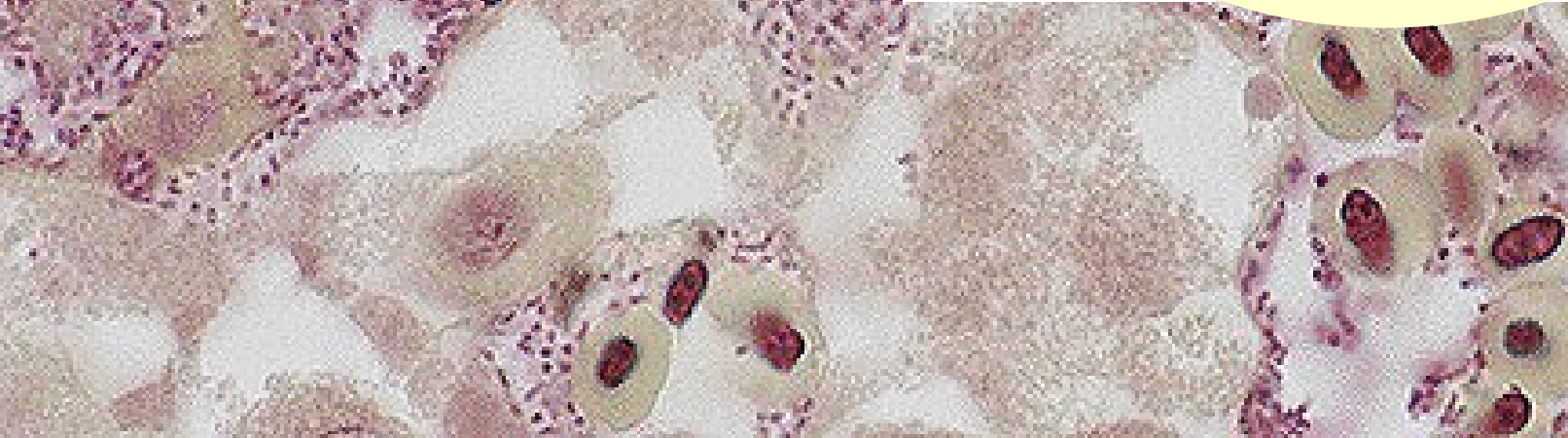
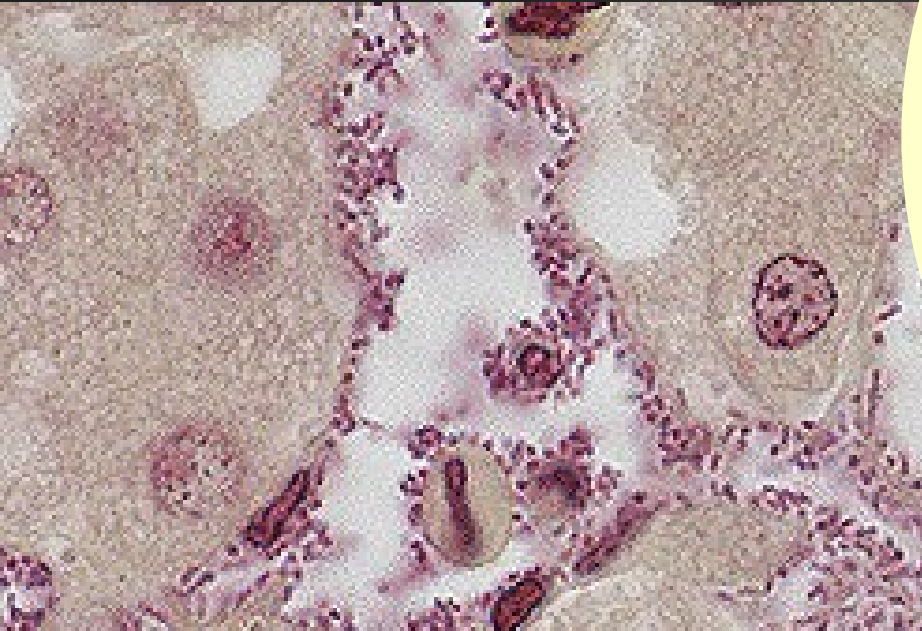
# *Pasteurella multocida*

- *Pasteurella multocida* is common flora in dog chaps.
- In humans, it causes mainly pyogene wound inflammations **after being bitten by a dog** or another animal.
- It **smells simillarly as *Haemophillus*** (some people say „like old rag“, but unlike *Haemophilus*, it grows on blood agar (but not Endo agar).
- The morphology is something between *Streptococcus* and *Enterococcus*, but it is **Vancomycin resistant** and this gives a suspicion to the microbiologist (especially at parallel susceptibility to penicillin)

# *Pasteurella multocida*

<http://library.thinkquest.org>

<http://www.biologico.sp.gov.br>



Clinical  
characteristics –  
Gram– glucose  
non-fermenters



# Story Three



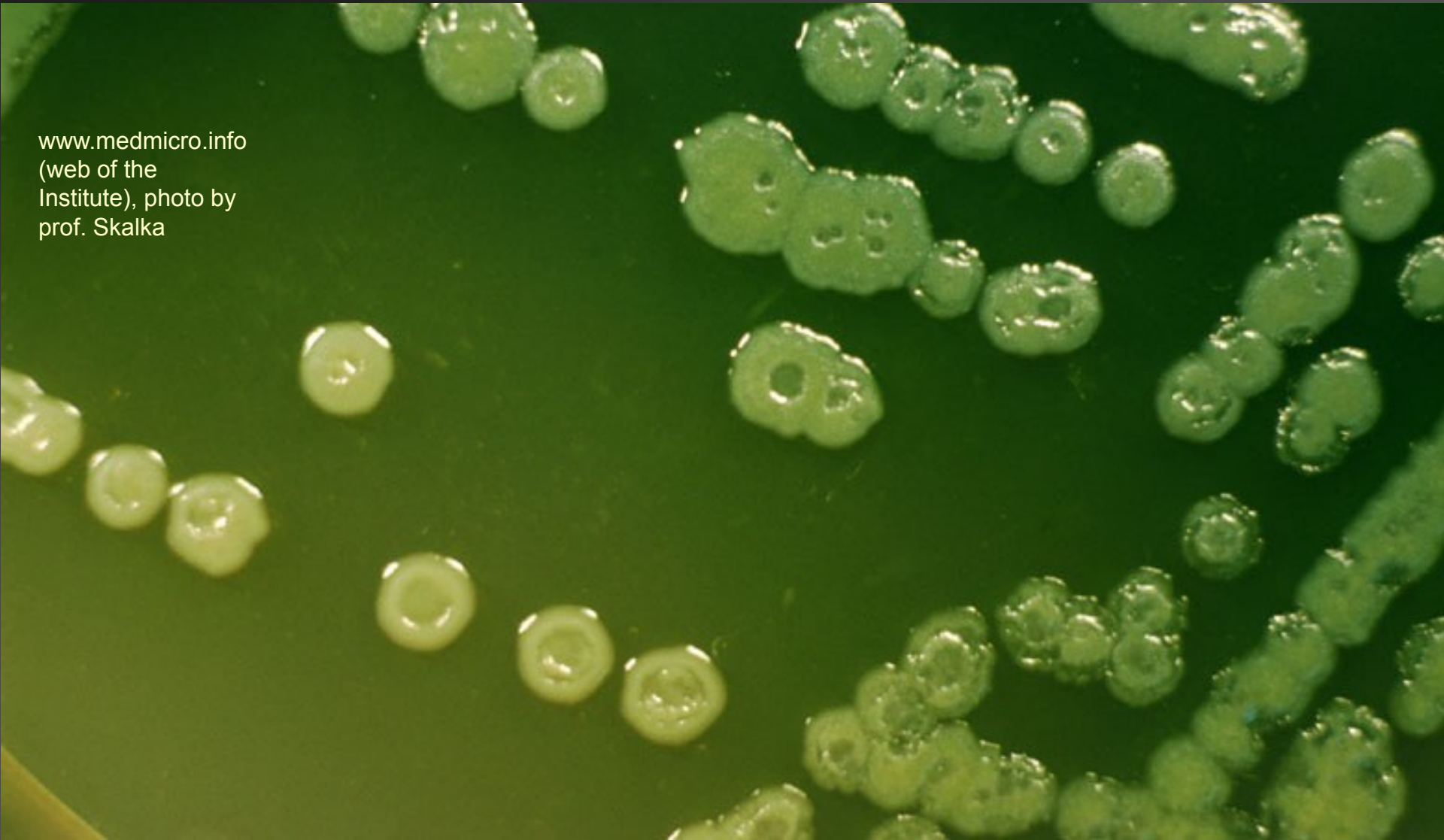
- **Mr. Phosphoros** is a pyroman. Several days ago, he burned himself. Now, his burn is **inflamated**. He is hospitalised on a specialized centre and feels very badly. Doctors knew that it has no sense to try antibiotics accidentally, so they **performed a swab**. Thanks to this, a **target therapy** was found, and Mr. Phosphoros healed. Of course, only temporarily: sooner or later, he will probably play with his matches again (*like some students of the practical*).

# Who is guilty this time?

- It is *Pseudomonas aeruginosa*, the most common so named „Gram– 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*
- Those bacteria are mostly **strict aerobes**, instead of fermentation sugars, they breakdown them by **aerobic respiration**, and their adaptation to outer environment is clear also of other properties – they use to have **low temperature optimum** and they are often **pigmented**, so they fight with sun in outer environment

# Green pigmented strain of *Pseudomonas aeruginosa* on MH

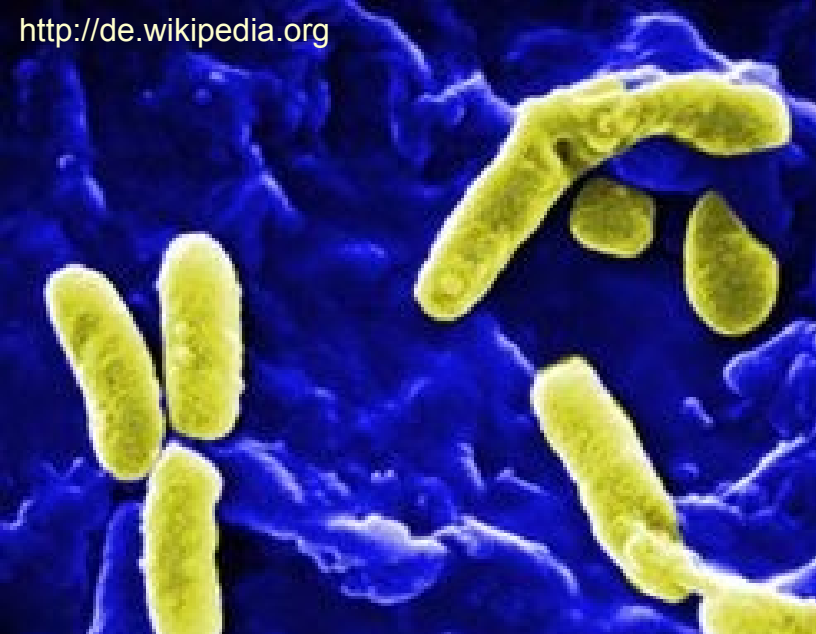
[www.medmicro.info](http://www.medmicro.info)  
(web of the  
Institute), photo by  
prof. Skalka



# Another picture of *Pseudomonas aeruginosa*







Exceptional  
*Pseudomonas*  
strain with blue  
pigmentation

# 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. Their target are patients with burns, clients of emergency units, transplant centers, e. t. c.
- In hospitalized persons they often cause not only wound infections, but we find them also in respiratory ways, and even in the bloodstream.
- So they are important 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 at finding of some of those bacteria

In disabled persons,  
they can  
cause even  
such  
problems as  
a nail  
inflammation



# Dr. Zahradníček's autocaustics

- Friday, 13th January 2006: **fall into a not-covered canalisation hole** in the city of Padang, West Sumatera, Indonesia. Quite large wound, reaching tibial periost
- Some three weeks later, **the wound started to smell like *Pseudomonas***, and really, this bacterium was successfully cultured from it.
- Dr. Zahradníček decided for **local treatment** (mixture of gentamicin and polymyxine)
- The therapy was **successful** (but it is not „lege artis and cannot be recommended for routine use)



# Padang

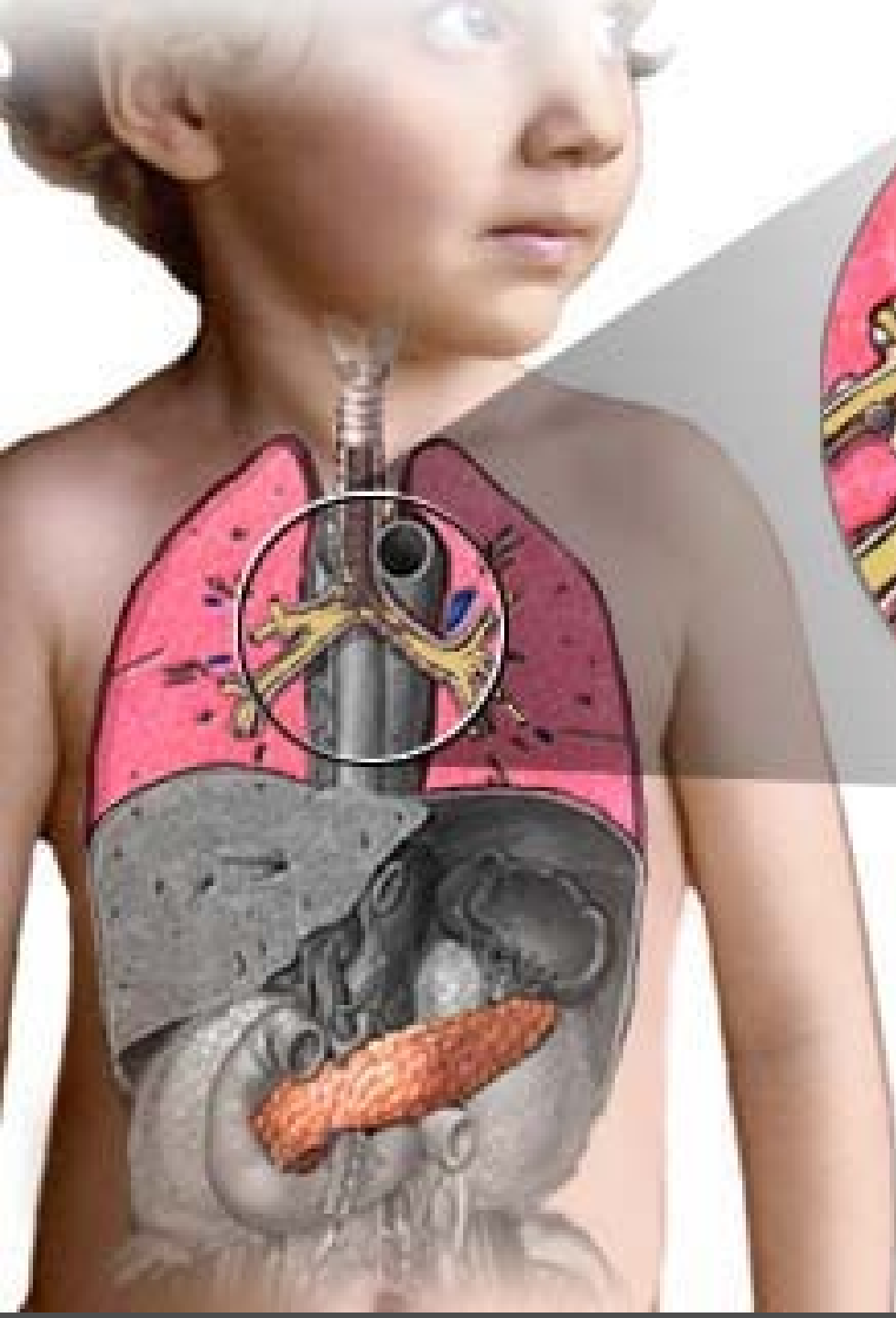


# Story Four

- Linda was a poor girl: she suffered because of **an inborne disease, cystic fibrosis.**
- Her **lung surfactant was different from surfactant of healthy people.** So, he was often infected.
- Last time it was *Staphylococcus aureus*. This time it was different: **the causative agent was *Burkholderia cepacia***, one of G– non-fermenters.

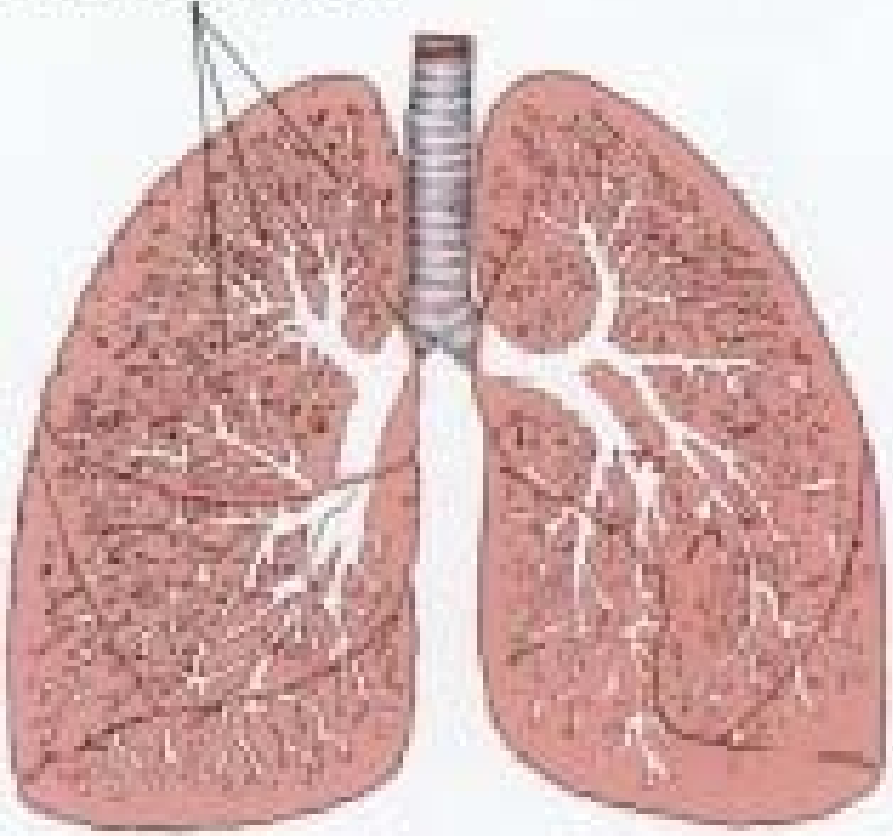
# Non-fermenters and Cystic fibrosis

- 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*, *Burkholderia cepacia* and *Staphylococcus aureus*. Strains often become **polyresistant** and many children with cystic fibrosis die very young.



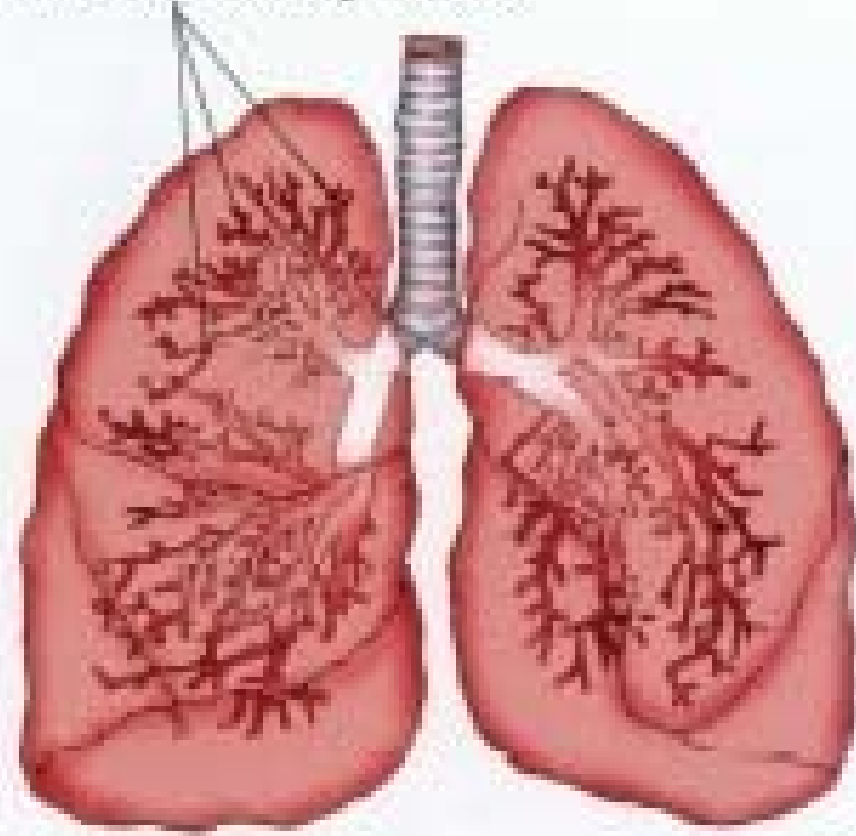
Cystic fibrosis is a hereditary disorder characterized by lung congestion and infection and malabsorption of nutrients by the pancreas

Unobstructed  
bronchial tubes



Healthy lungs

Bronchial tubes  
are blocked by mucus

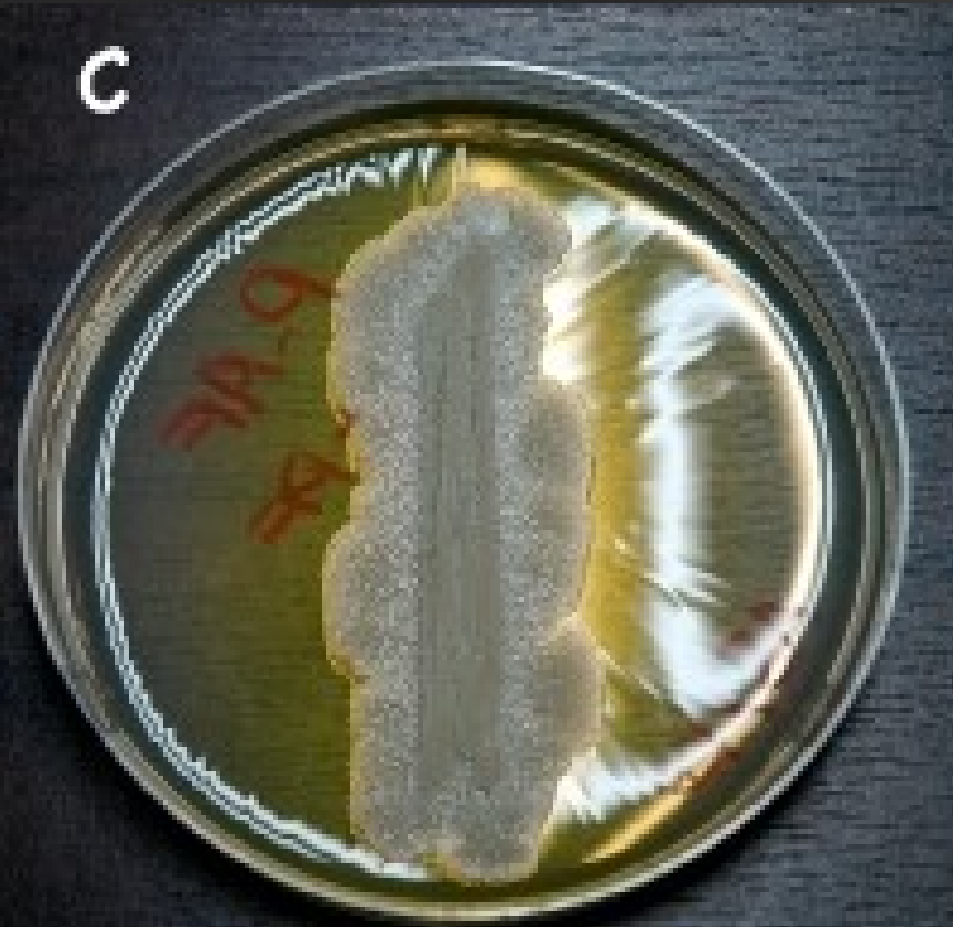


Lungs with cystic fibrosis

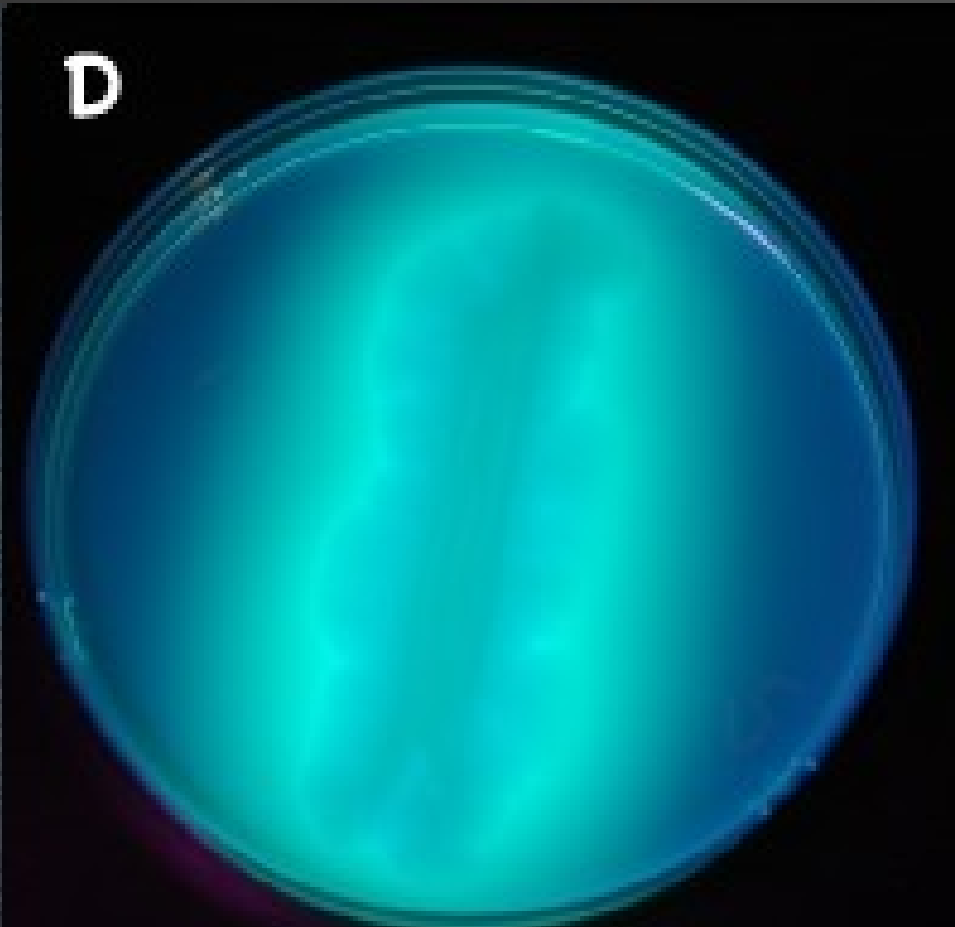


# More Gram non-fermenters: *Pseudomonas fluorescens*

C



D





# Burkholderia cepacia

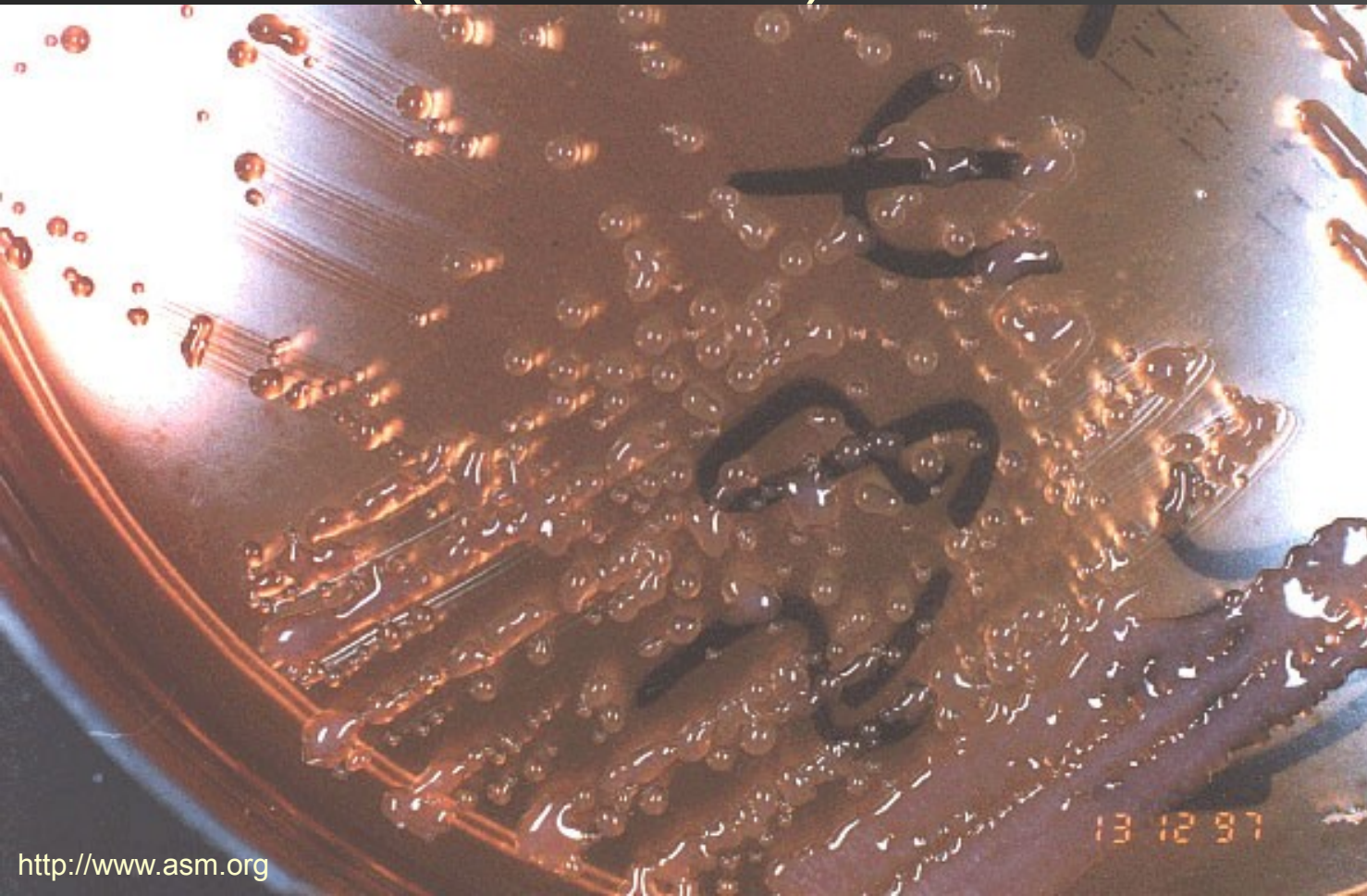
*Burkholderia cepacia* is responsible for rotten onions (*Allium cepa*), so it is really a typical plant pathogen





# *Burkholderia pseudomallei*

*Burkholderia pseudomallei* is causative agent of melioidosis. Related *B. mallei* is causative agent of malleus (a zoonosis)

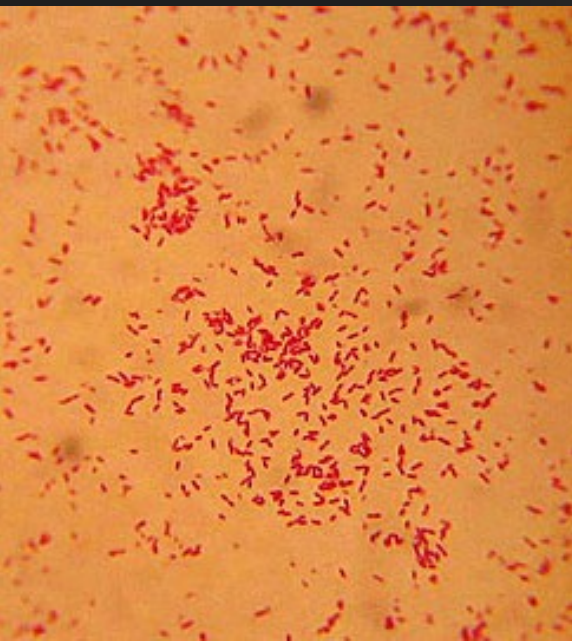


# *Stenotrophomonas maltophilia*

<http://www.scielo.cl>

<http://clinicalmicrobiology.stanford.edu>

<http://www.microbelibrary.org>

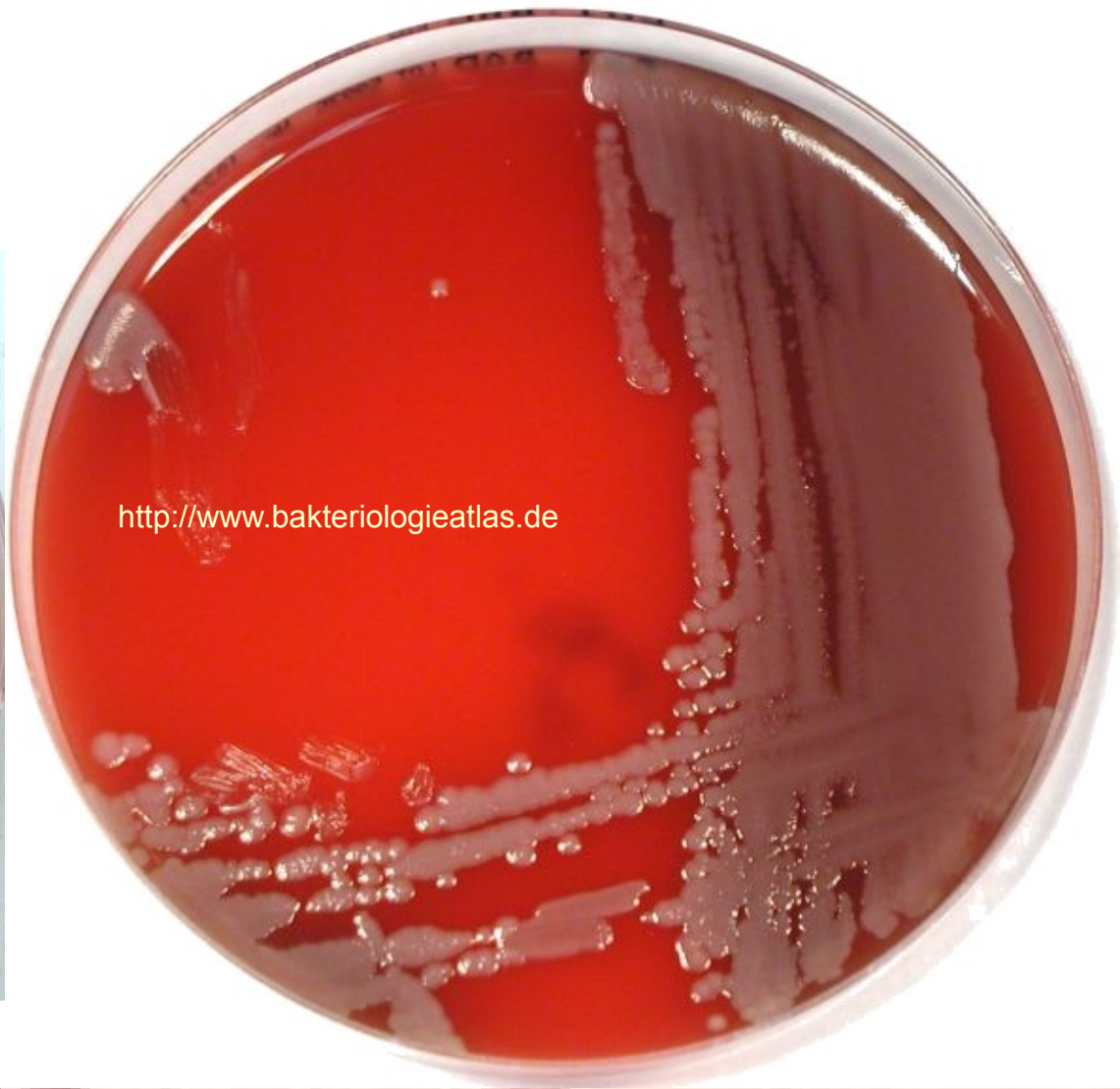
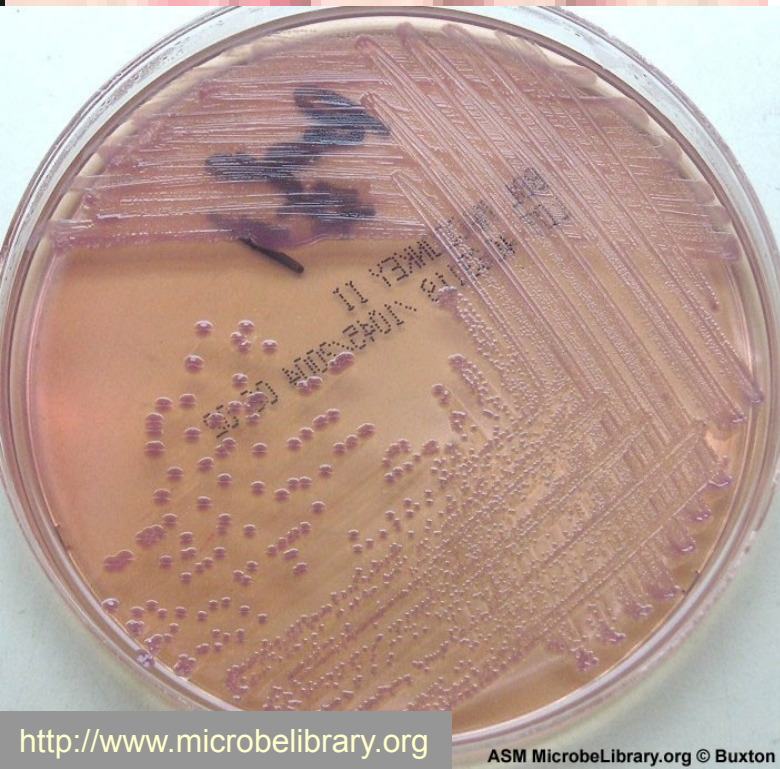


ASM MicrobelLibrary.org © Buxton

*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



<http://www.buddycom.com>

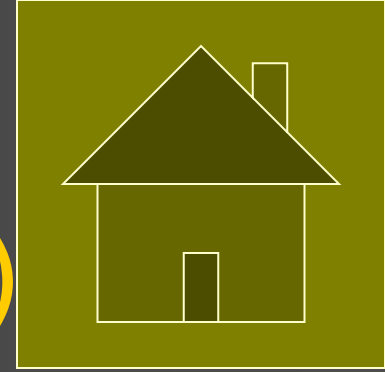
**Greek: a-kineto- = „non motile“**

# Bacterial metabolism and relation of bacteria to oxygen

We know already that G<sup>-</sup> non-fermenters are bacteria that do not ferment sugars, but performs aerobic respiration. Let's compare now two bacteria:

- *Escherichia coli* lives in the intestine. It has enough nutrients, but not enough oxygen (unlike other gases 😊) 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 (*Bacterioides fragilis*, more in P07)

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

# Diagnosics of *Pasteurellaceae*

# Survey of 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.



# To *Haemophilus* and *Pasteurella* diagnostics



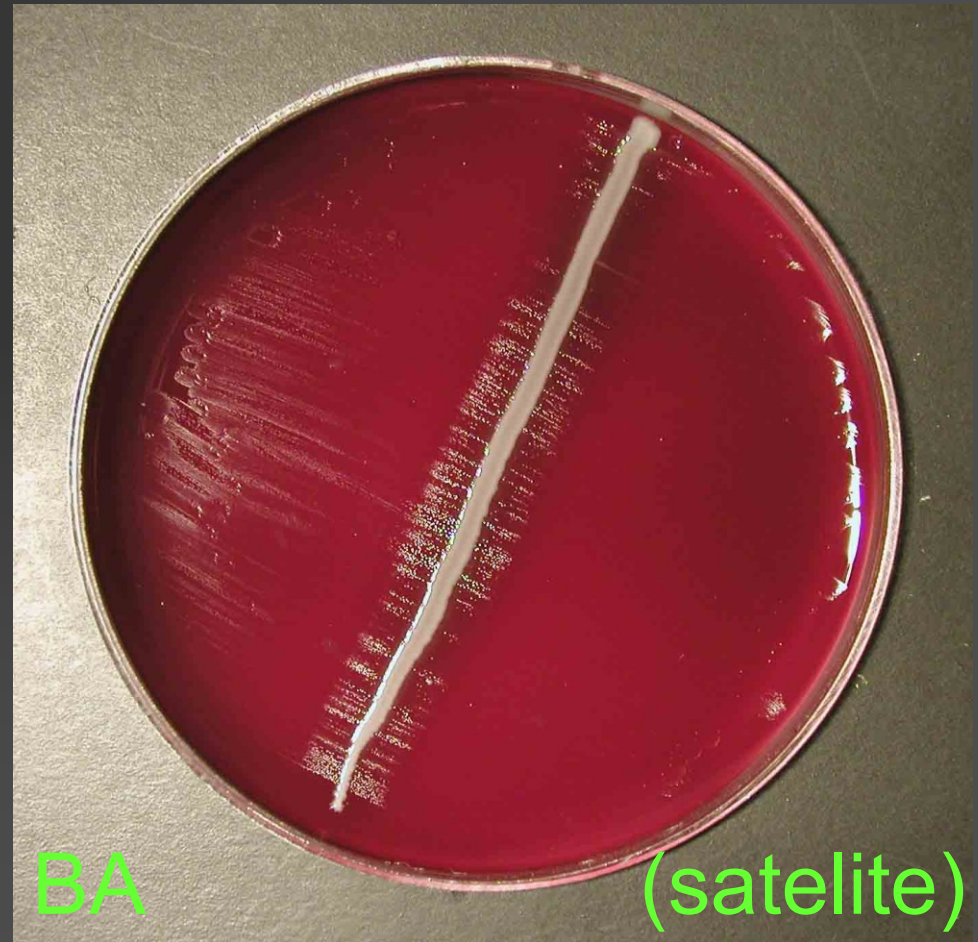
<http://www.uni-ulm.de>

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

# Satelite phenomenon

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

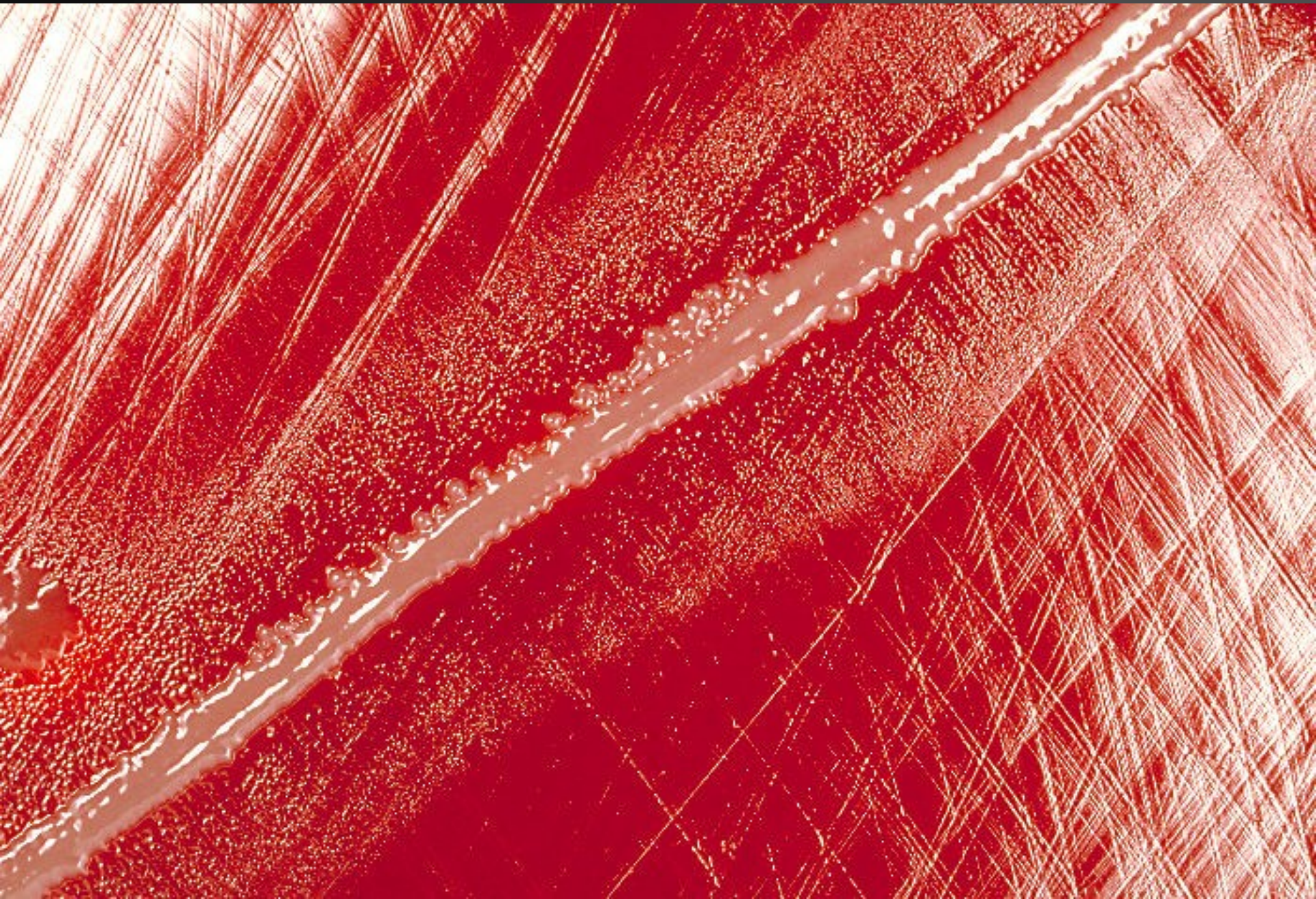
# Haemophili on chocolate agar (left) and as a satellite on blood agar





# Satellite once more

<http://phil.cdc.gov>





# Detection of haemophili

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



# Growth factors of Hemophili

(= determination of individual species)

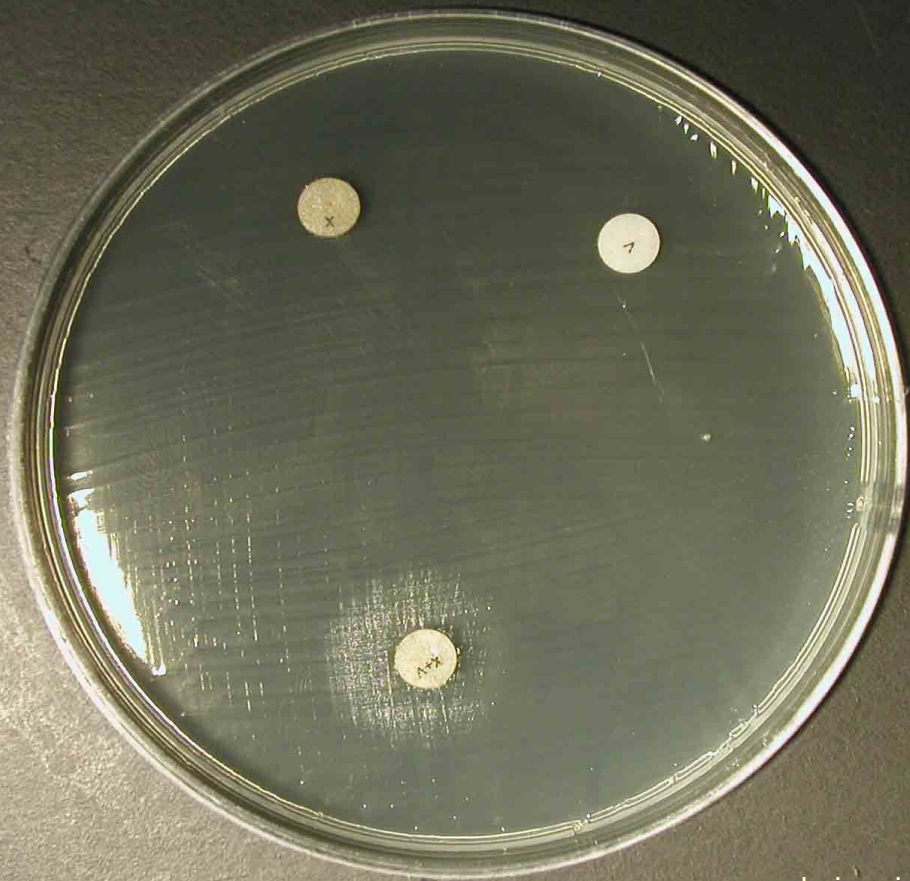
- Haemophilli need factors from blood, but the need of individual factors is species specific.
  - *H. parainfluenzae* needs factor **V** (= NAD)
  - *H. aphrophilus* needs factor **X** (= hemin)
  - *H. influenzae* needs **both factors**.
- We use discs with these factors: one with **X**, another with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

# Growth factor test of *Hemophilii*

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



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

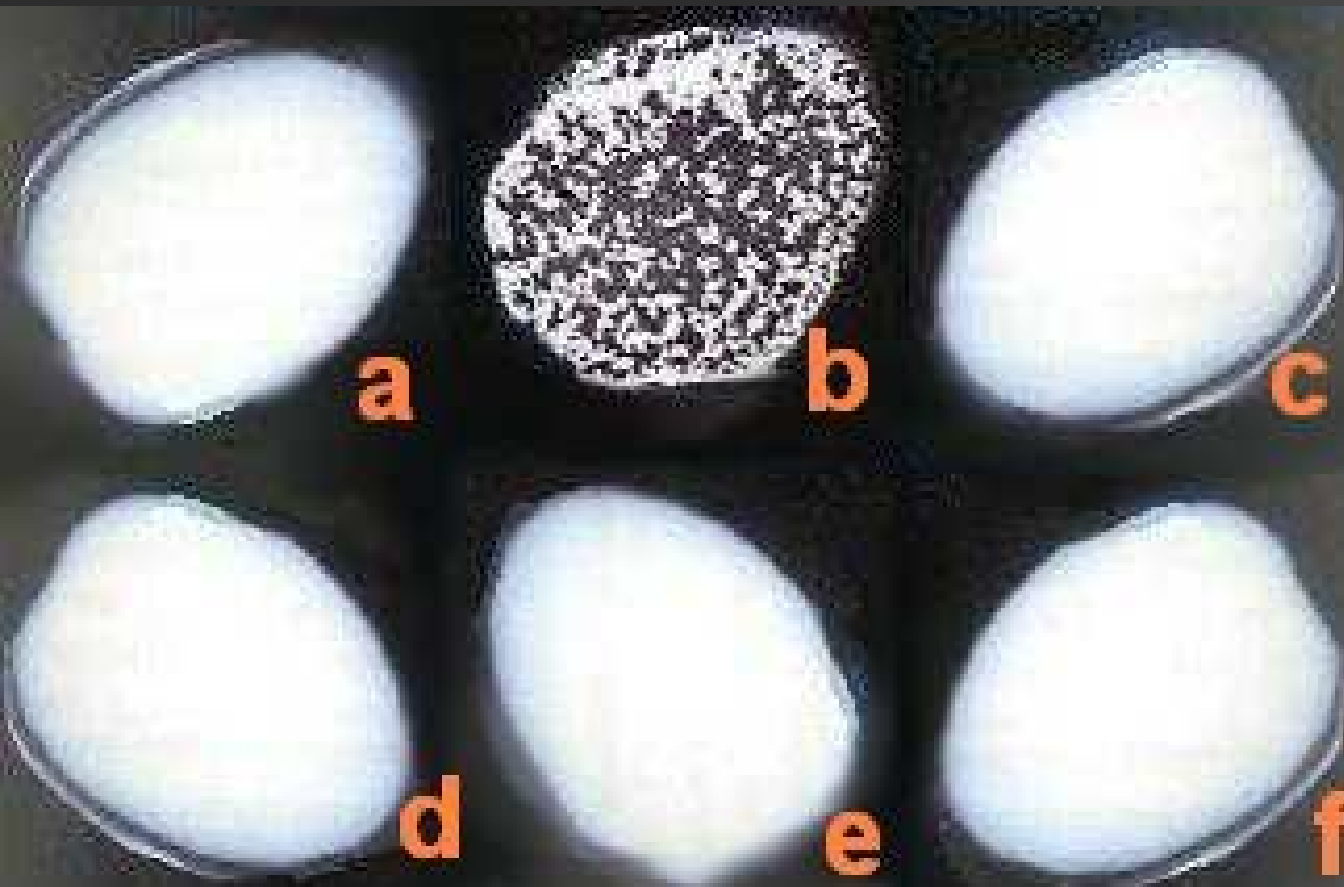


# *Haemophilus influenzae*: antigen analysis (intra-species diagnostics)

- Antigen analysis in *Hemophilus influenzae* is performed like in other bacteria. The main goal is diferenciation of Hib. Today, we have **comercionally 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 un-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

# Antigen analysis of *H. influenzae*: an example of the result

The agglutination results for haemophili are observed  
similarly as other agglutination reactions



# Detection of *Pasteurella* using typical susceptibility pattern

- **No Gram-negative bacterium is susceptible to vancomycin.** Vancomycin can be used for Gram-positive bacteria only, but here 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

- Haemophilli 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



Antibiotikum	Abbrev.	Reference zone
Ampicilin (aminopeniciline)	AMP	22 mm
Co-amoxicilin (am.+inhib.)	AMC	18 mm
Chloramphenicol	C	29 mm
Doxycycline (tetracycline)	DO	29 mm
Co-trimoxazol (mixture)	SXT	16 mm
Azithromycin (macrolid)	AZM	12 mm



# Diagnostics of Gram–non- fermenters

# Survey of 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 non-fermenting 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)
  - They form 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 differentiated 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 pyoverdinin
- 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<sup>-</sup> non-fermenters, *Pseudomonas* is oxidase positive, *Burkholderia* usually too; on the contrary, *Stenotrophomonas* is usually negative and *Acinetobacter* too.



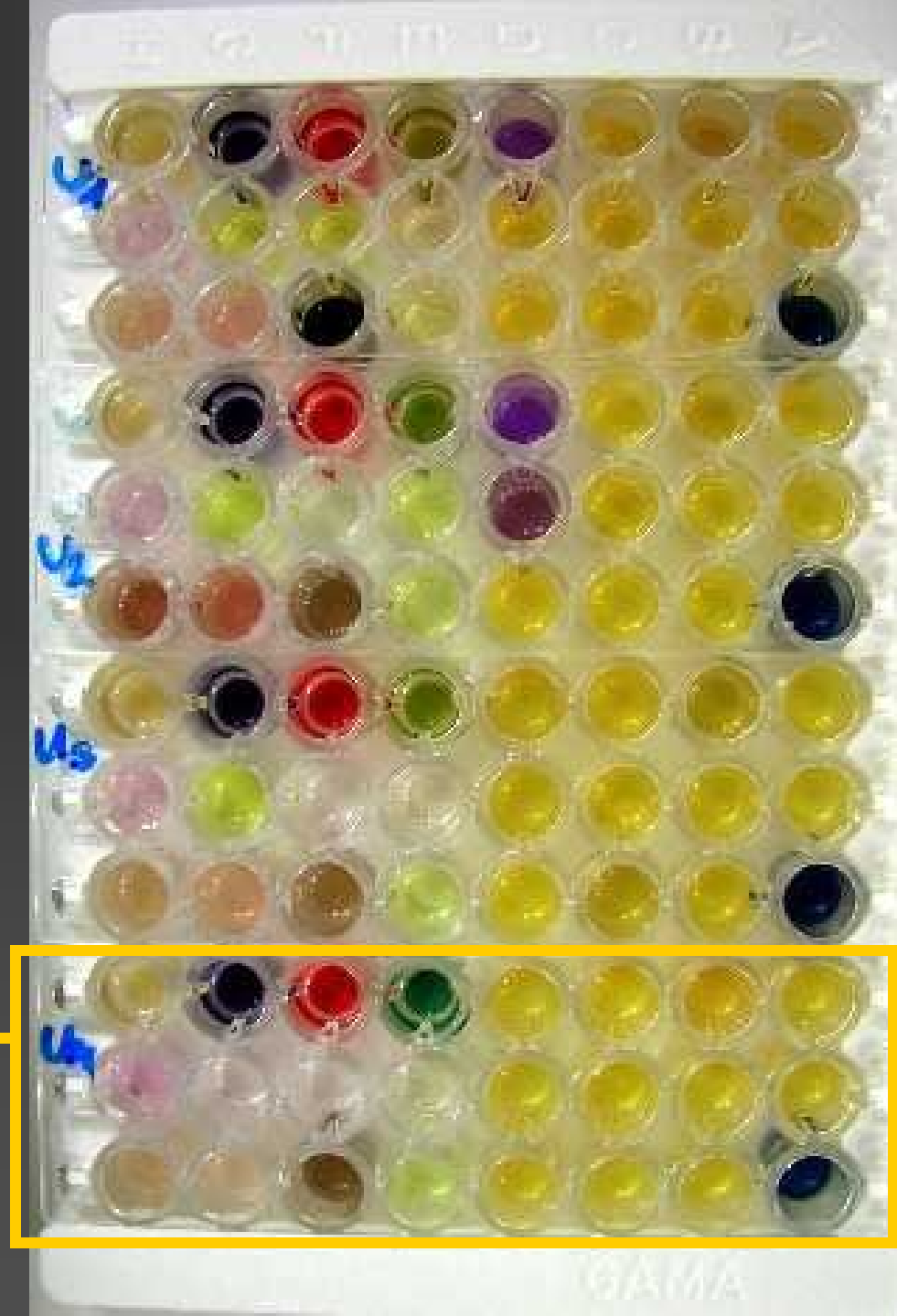


# NEFERMtest 24

- For precise biochemical identification of G–non-fermenters we use mostly **NEFERMtest 24** (or a similar test of other provenience).
- 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



# 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 here
  - **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 NF atb set

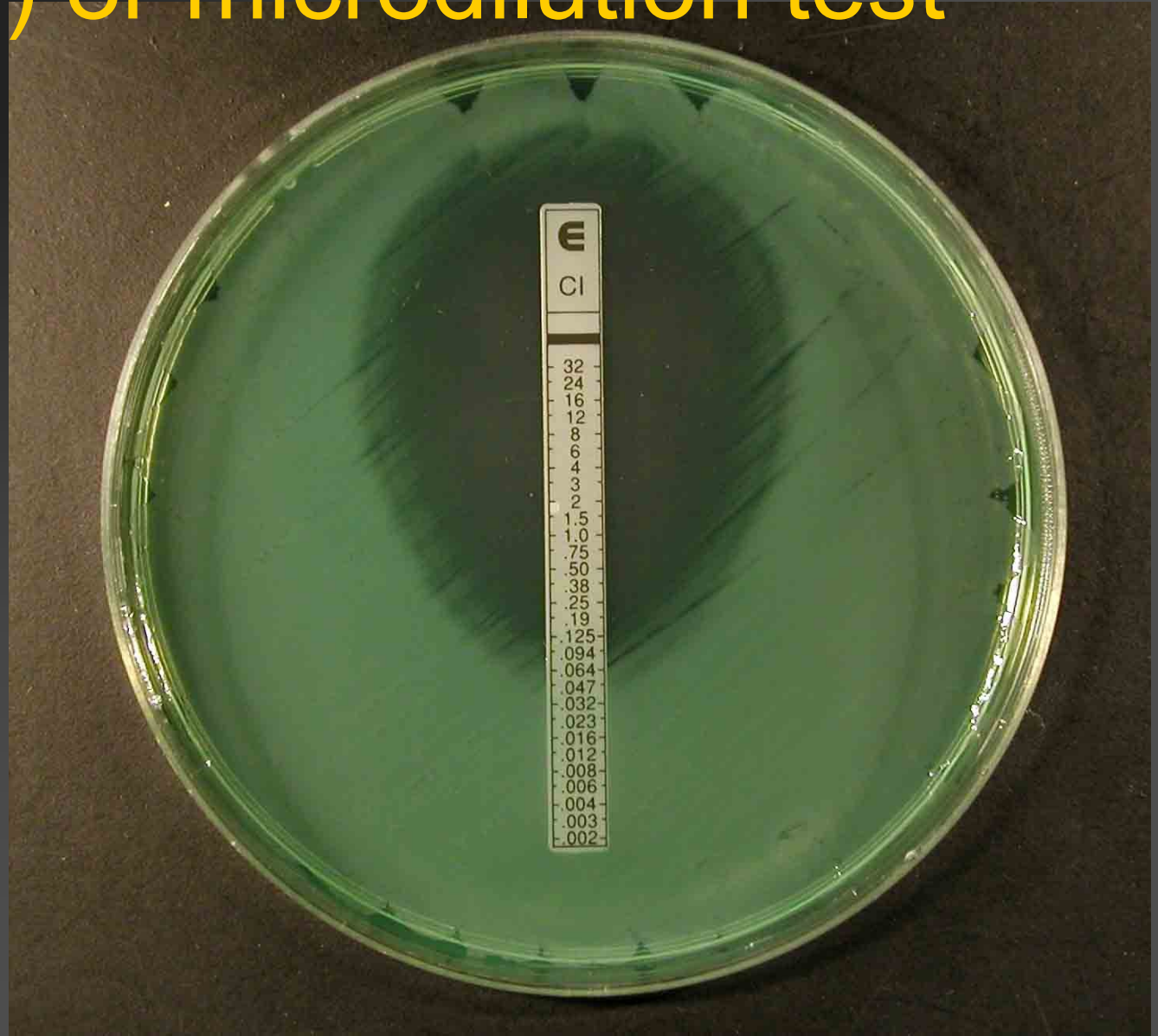
Antibiotic	Abbrev.	Reference zone
Piperacillin + tazobactam*	TZP	22 mm
Gentamicin (aminoglykos.)	CN	18 mm
Imipenem (karbapenem)	IMP/IMI	22 mm
Ciprofloxacin (quin 3 gen)	CIP	29 mm
Ceftazidime (CS 3 gen)	CAZ	16 mm
Colistin (polypeptide)	CT	12 mm
*antipseudomon. peniciline + $\beta$ -lactamase inhibitor		

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



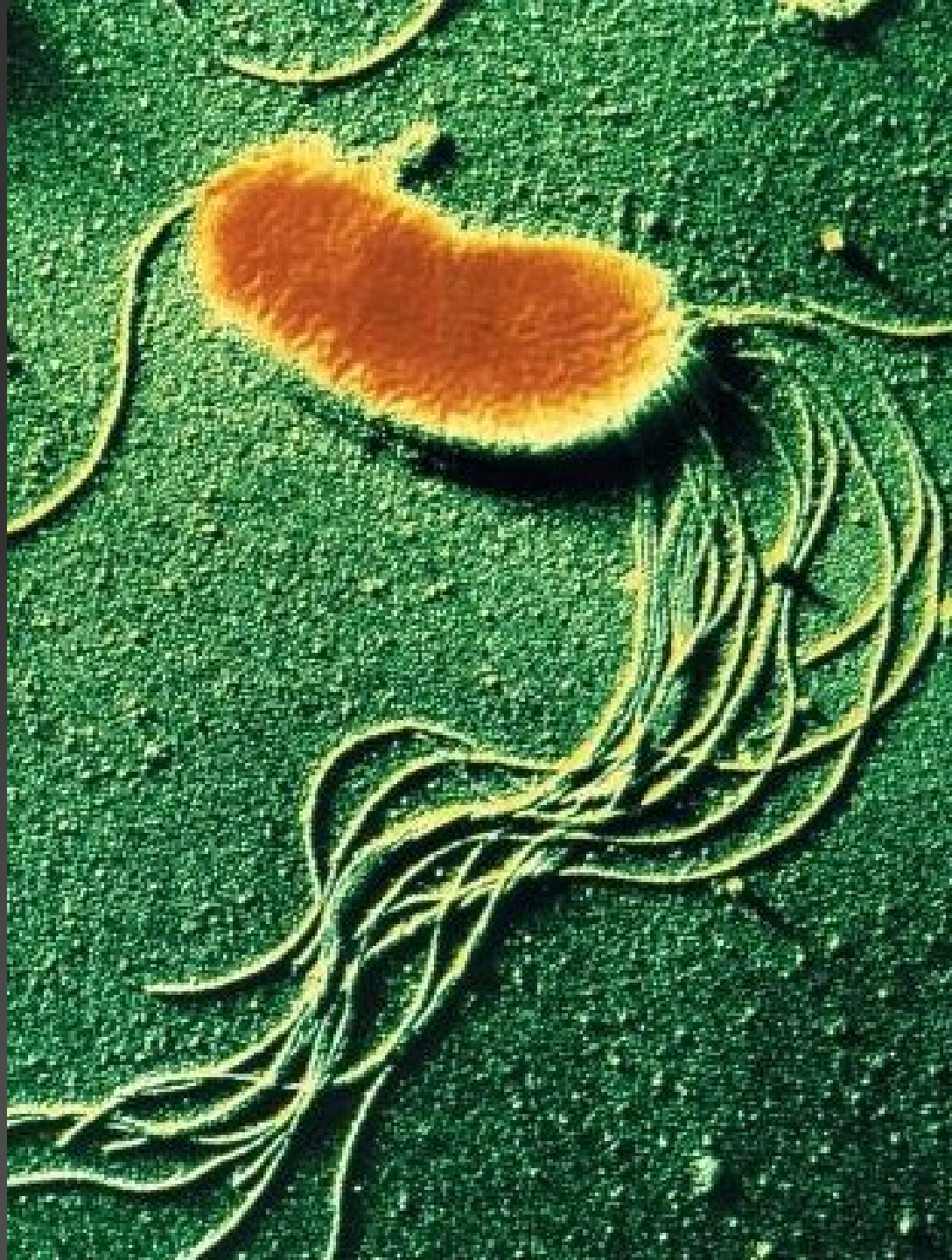
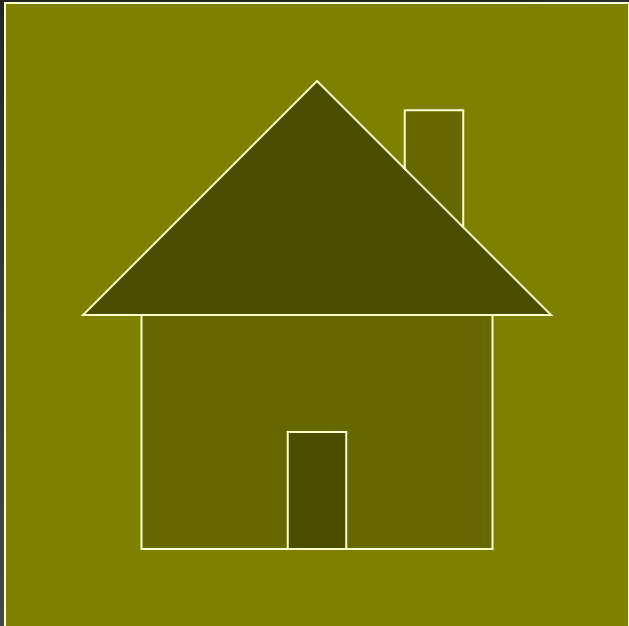
Especially producers of so called metallo-beta-lactamases (MBL) use to be only susceptible to amikacin and colistin.

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

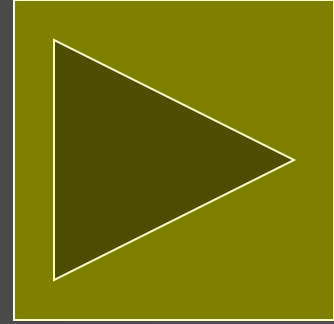




# The End



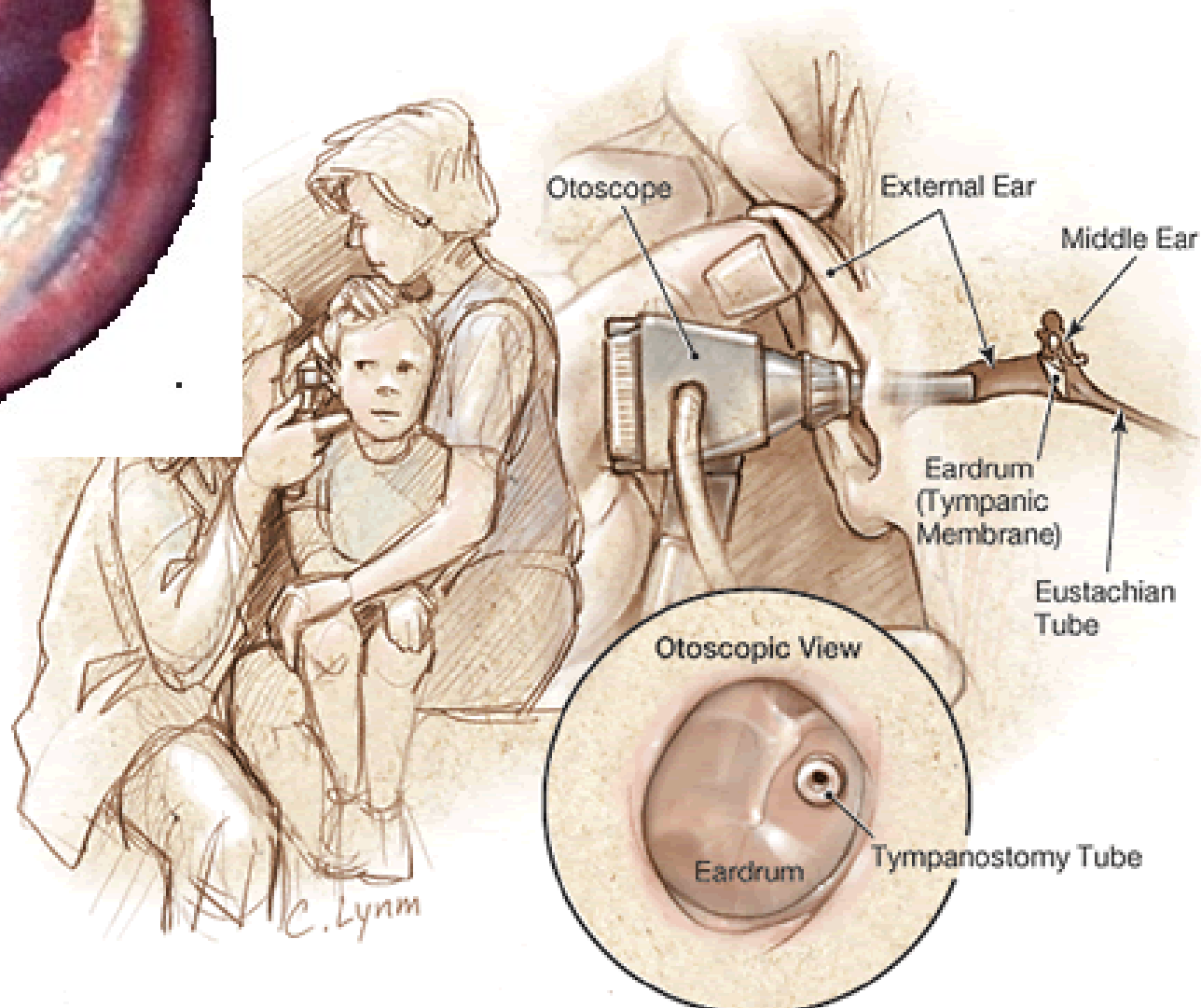
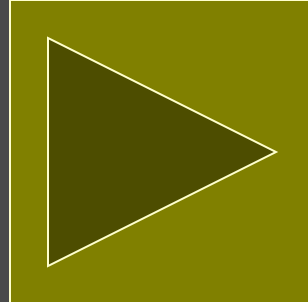
# Inflammation of external ear – otitis media (bonus)



- **Common in children** (short horizontal Eustach tube)
- **Caused by:** *Streptococcus pneumoniae*, *Haemophilus influenzae*, *Moraxella catarrhalis*
- **In chronic 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*

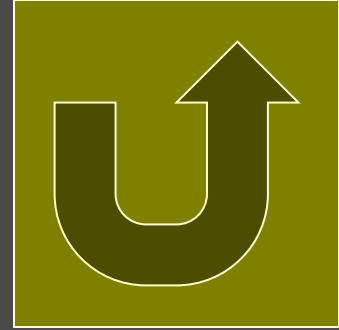
# Otitis media



<http://www.otol.uic.edu/research/microtology/Microtology/acute1.htm>

[http://www.medem.com/MedLB/article\\_detailb.cfm?article\\_ID=ZZZPMV6D1AC&sub\\_cat=544](http://www.medem.com/MedLB/article_detailb.cfm?article_ID=ZZZPMV6D1AC&sub_cat=544)

# 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 meaningfull only after paracentesis
- Otherwise it is also possible to examine **pyogene liquid** taken during paracentesis