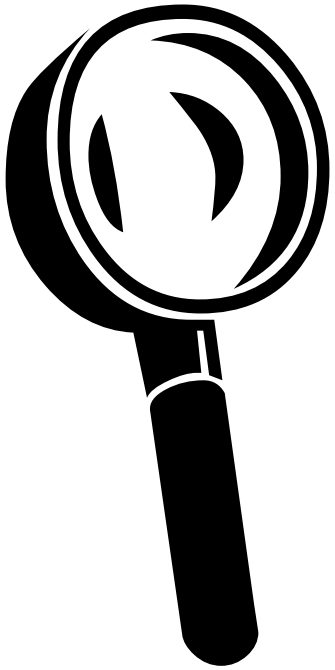
Institute for microbiology shows

TRACING THE CULPRIT



Part seven:
Anaerobic culprits

Anaerobes

Survey of topics

Pathogens with complicated diagnostics

Clostridia – clinical characteristics

Spore non forming anaerobes and lactobacilli – characteristics

Relation of bacteria to oxygen (repeating from spring term)

Diagnostics of anaerobic bacteria, anaerobiosis

Pathogens with complicated diagnostics

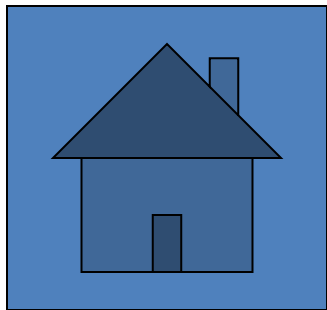
Before we start…

- …it is something to think about. Until now (P01 to P05, partially even P06) we spoke mostly about bacteria, that do not need special approach. *(but in P06 it already did not match for some bacteria: gonococcus, brucella, legionella etc.)*
- Clinical doctor simply sends a specimen „for bacteriological culture“, and something would grow out of it.
- Now, it is the END! Now, we are going to deal with bacteria that do not match to this system.

And so:

- If the clinical doctor wants his specimen to be examined for presence of anaerobes, mycobacteria or actinomycetes, it should be written on the request for examination. Special approaches have to be used.
- In other agents (e. g. mycoplasms or chlamydia) it is often necessary to take clotted blood (→ serum is examined) for indirect diagnostics

Remember this not just for clinical microbiology examination, but also – and even more – for your future practice!



Clostridia – clinical characteristics

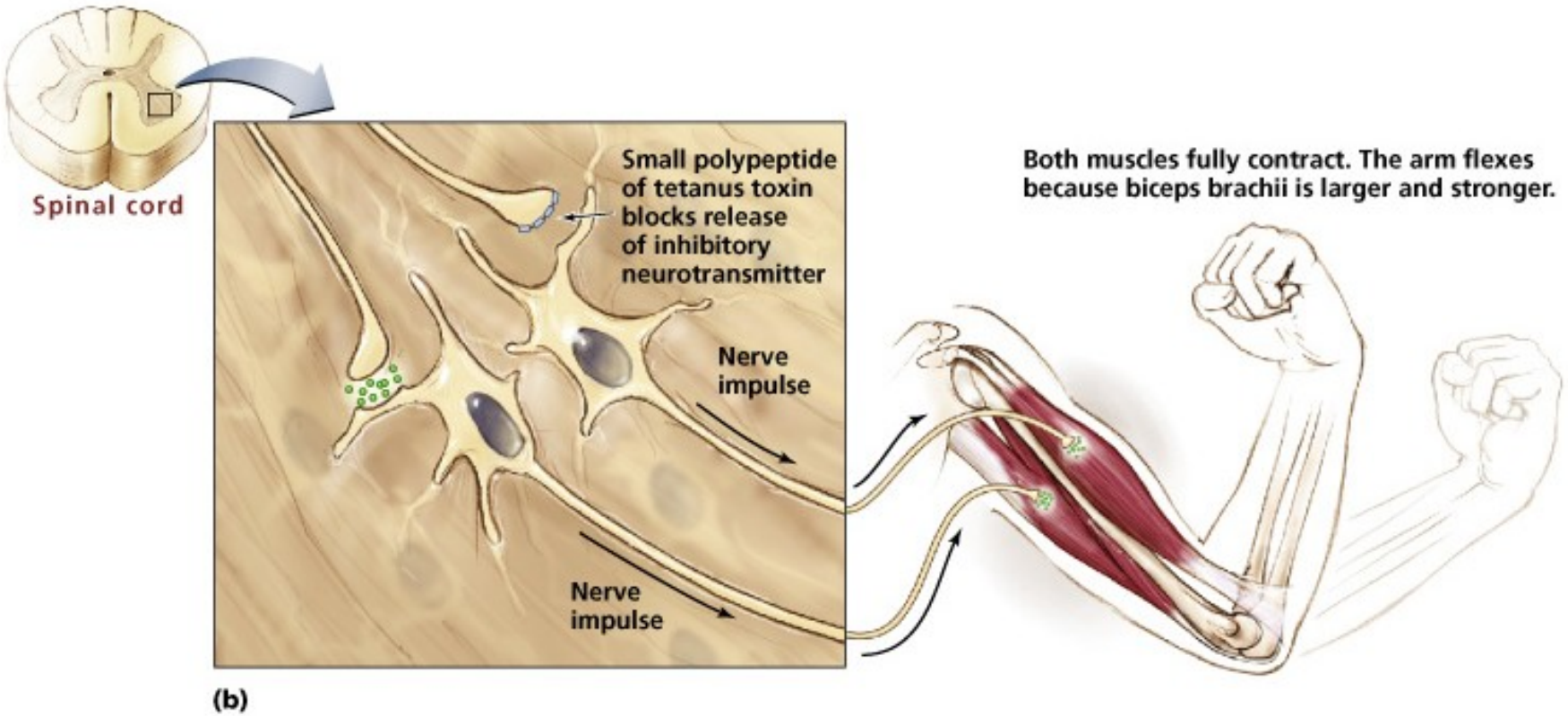
Story one

- Mrs. Cabbage was all the time seen working in the garden. It was her big hobby. Once she **injured** her hand, because a **pointed remainder of a plant was hidden in the soil**. She went to her general practitioner.
- The GP used local treatment for the wound, and then recommended **re-vaccination** against one serious disease
- If she would get the disease, it would be very dangerous, including **spasms** of her body

Neurotoxic clostridia

- The culprit that threatened Mrs. Cabbage was ***Clostridium tetani***, causative agent of **tetanus**. The disease is typical by a small, local inflammation, and toxin action throughout the whole body. The toxin leads to **spasms**.
- Another neurotoxic clostridium is ***Clostridium botulinum***, causative agent of **botulism**. Here the agent does not enter the body at all. Only its toxin comes to the body (usually from badly prepared conserved meat) acting again as neurotoxin, but here producing **pareses**.

Tetanus



Tetanus

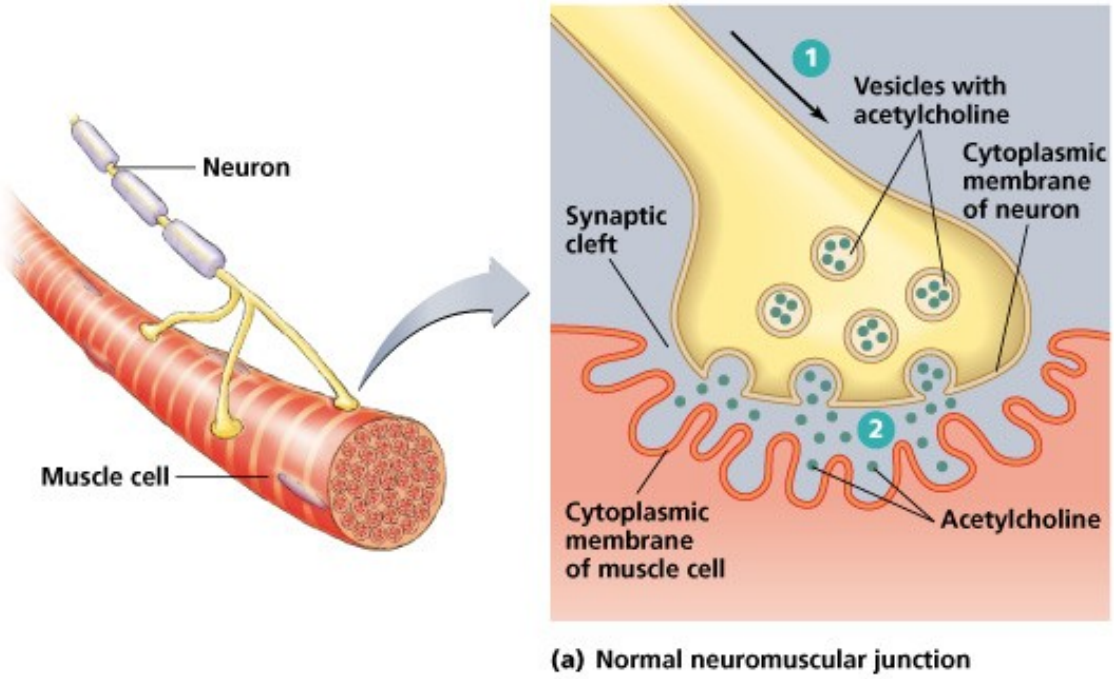
www2.bc.cc.ca.us


A tetanic man

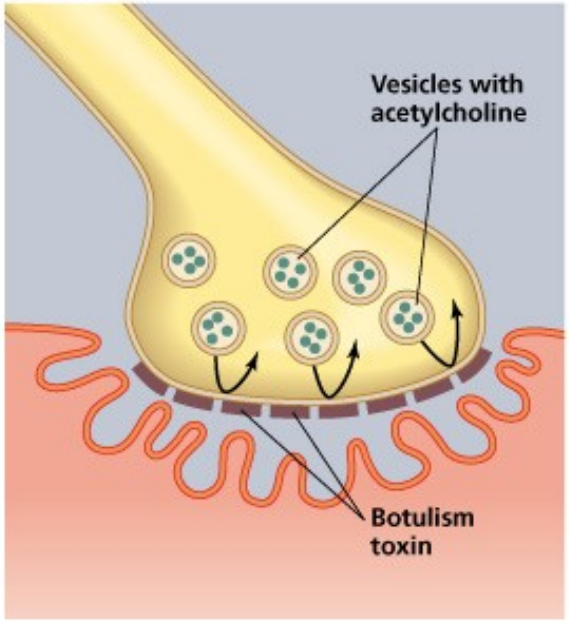
Once more tetanus

Trismus (spasm of chewing muscles)

Botulism

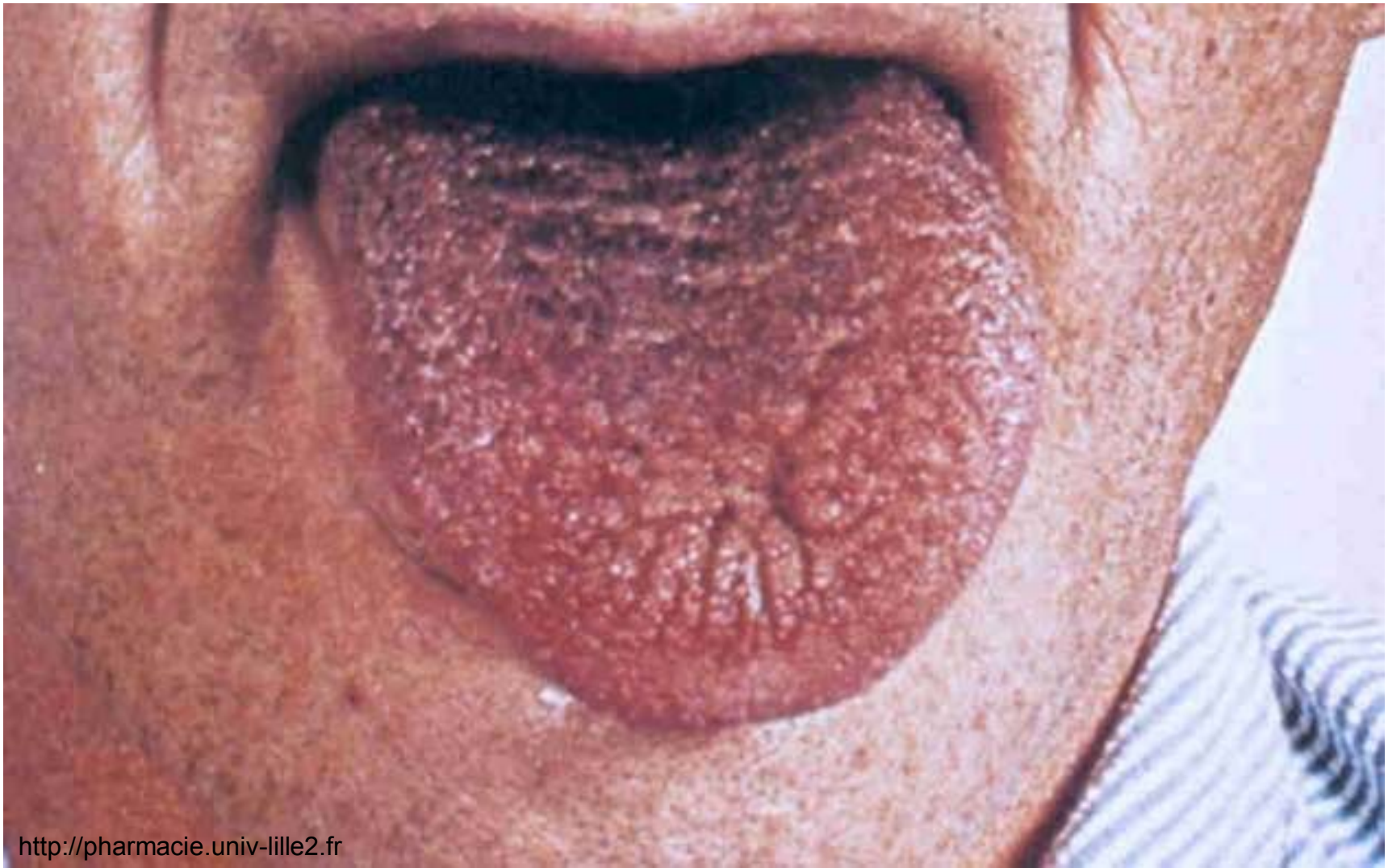


(a) Normal neuromuscular junction

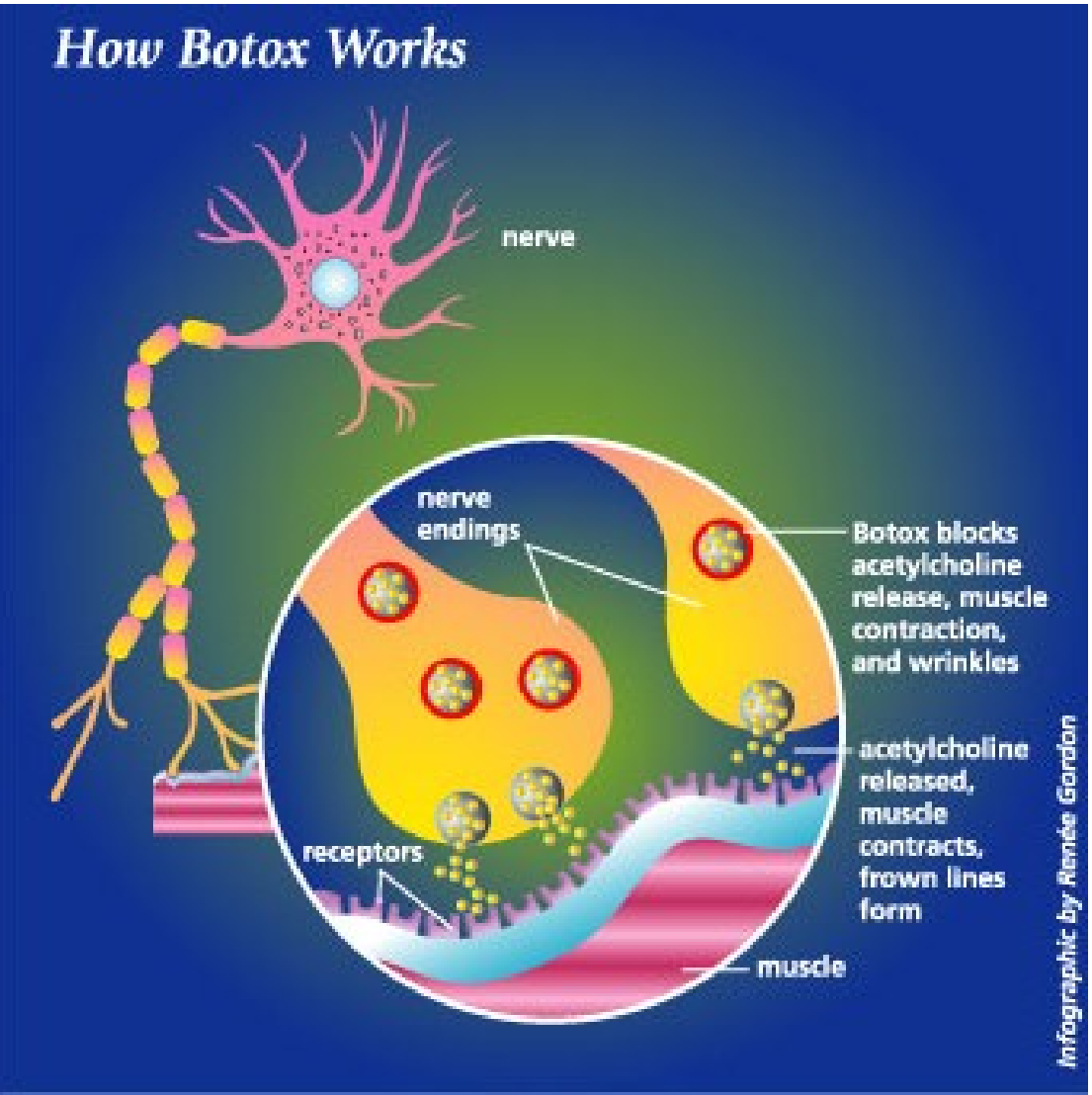


(b) Neuromuscular junction with botulism toxin present

Typical tongue appearance in case of botulism

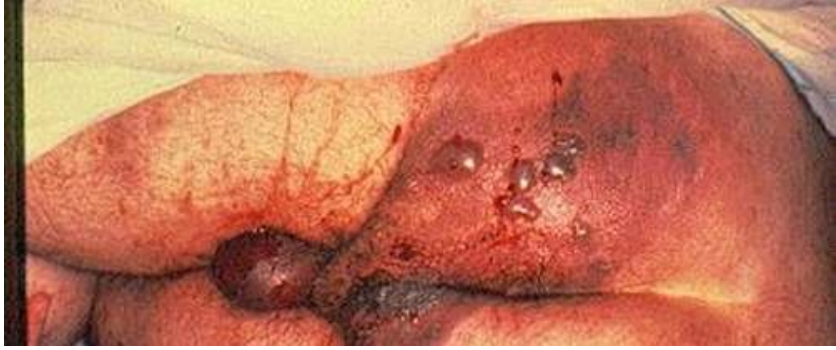


Botox: use of *Clostridium botulinum* toxin to became younger



www.fda.gov

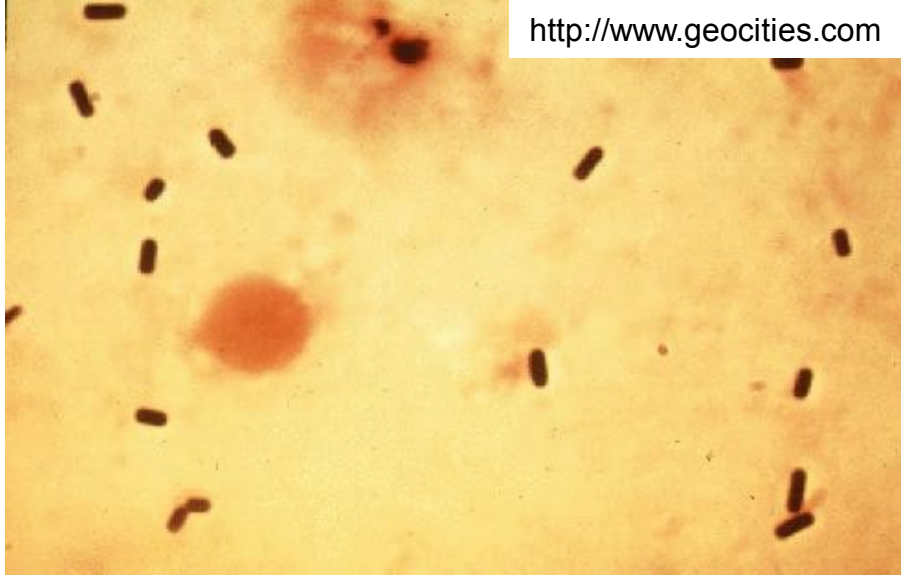
http://pharmacie.univ-lille2.fr



Story two

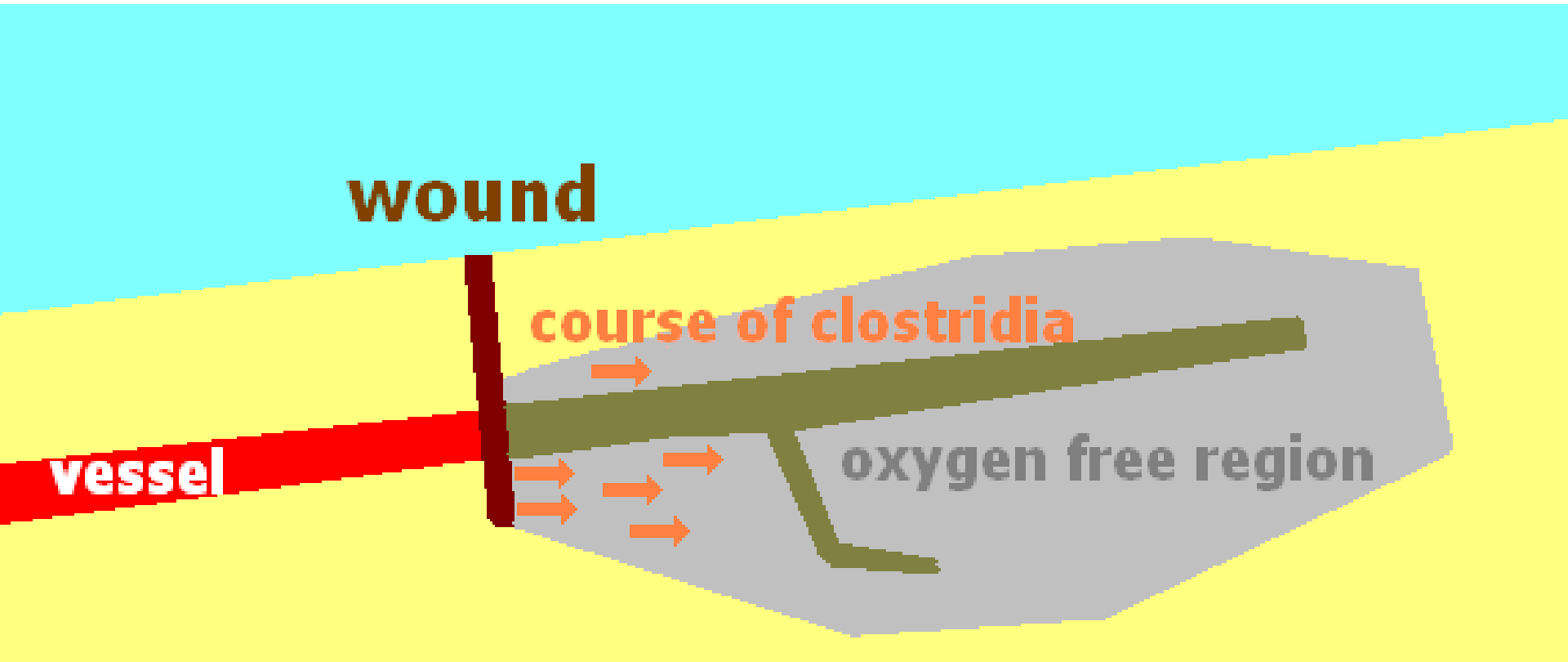
- Mustafa, Kosovo Albanian, decided to visit his cousin in neighbouring village. The field he went through had to be mines-free. Nevertheless, one mine was still present. A particle of the broken mine, dirty of mud, came deeply into Mustafa‘s thigh.
- Several days later, Mustafa came to one of field hospitals. His thigh was inflated and at knocking it was possible to hear breaking bubbles. Mustafa was operated immediately.

The culprit is now



- *Clostridium perfringens*, one of agents of gas gangrene (with *C. novyi*, *C. septicum,* etc.)
- Gas gangrene is a typical war disease. It is nevertheless possible to get it even during peace, e. g. in case of catastrophes
- Gas gangrene clostridia – or their enterotoxins – are intestinal pathogens, too

Gas gangrene formation



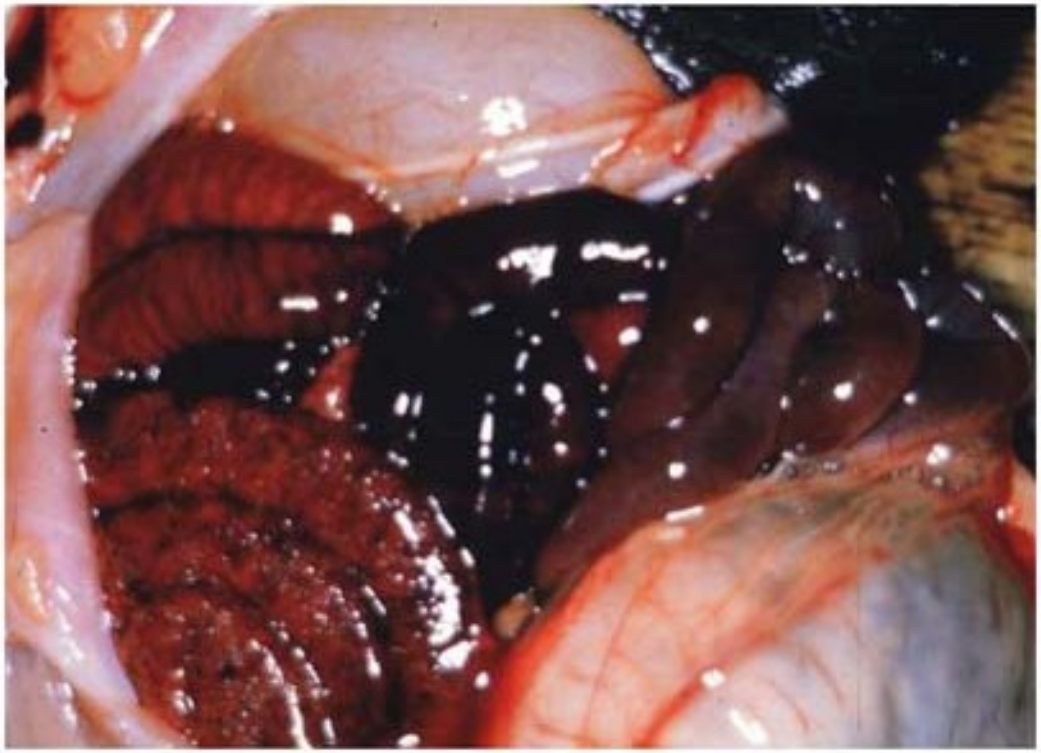
Necrotizing enterocolitis – this, too, may be caused by *C. perfringens*

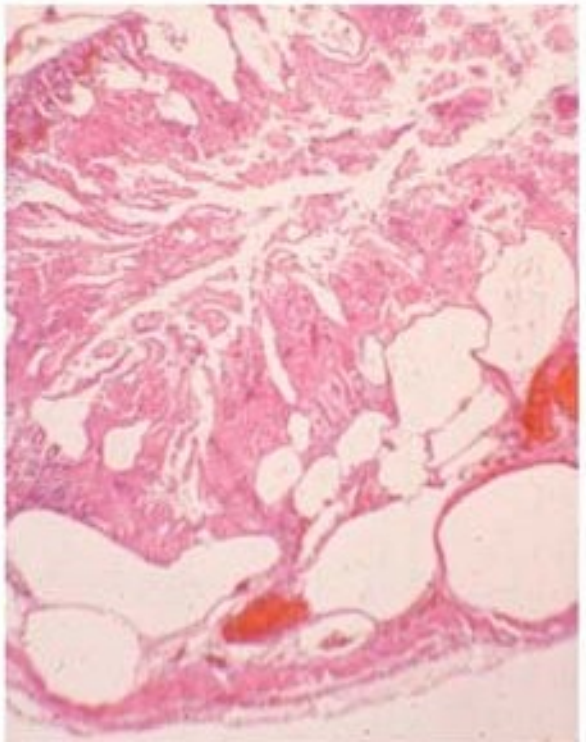
豚の壊死性腸炎 (Necrotic enteritis)

左：小腸は出血しており,結腸には菌の産生したガスによる嚢胞が見られる。

右：空腸の組織像。絨毛は壊死に陥り，固有層にはガスによる空胞が見られる。

http://www.niah.affrc.go.jp





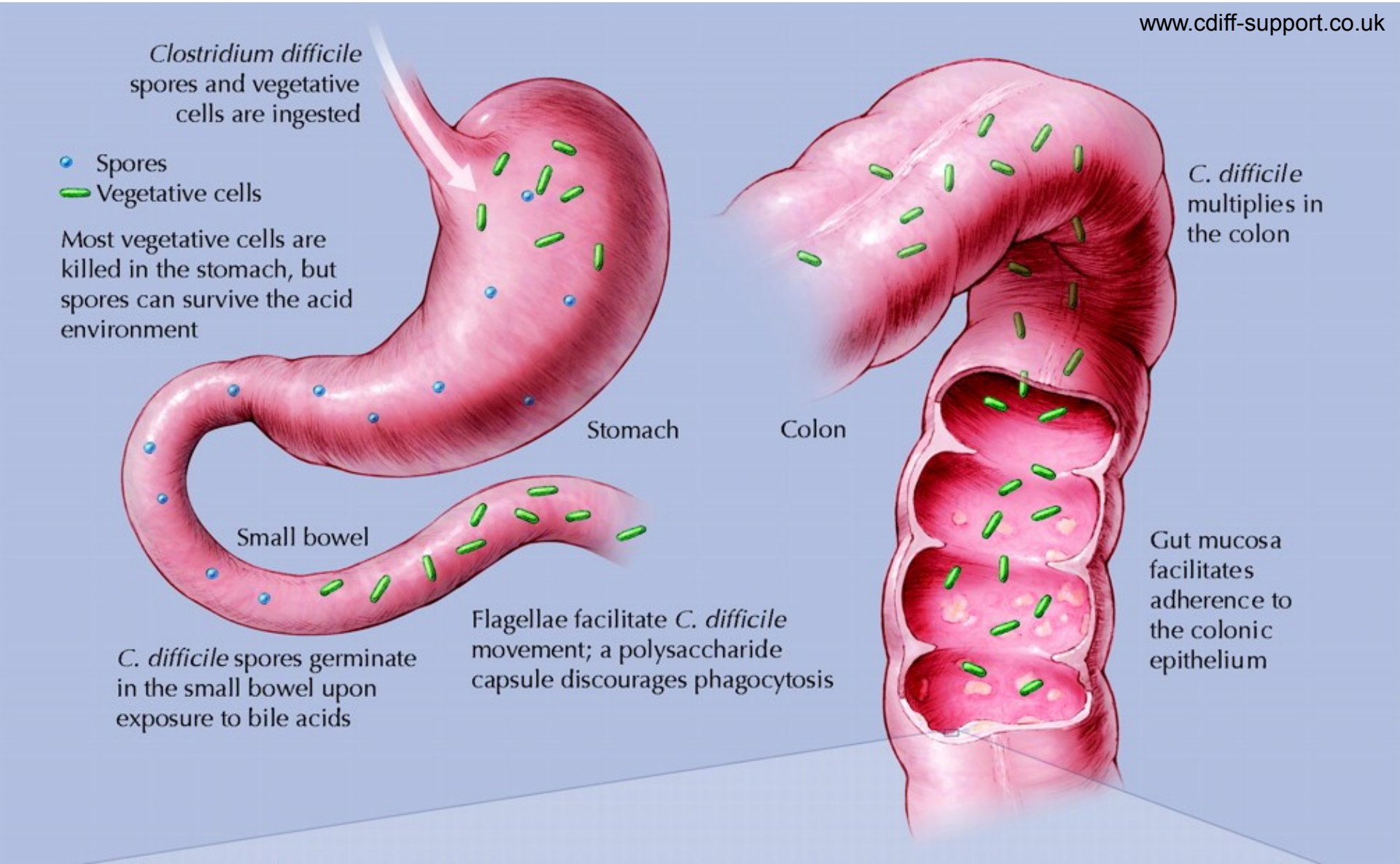
Story three

- Mr. Bones was third week in the hospital because of bacterial inflammation of bone marrow. The inflammation was treated by clindamycine (lincosamidic antibiotic). Suddenly, Mr. Bones started to have heavy diarrhoea. The department did not have metronidazol, and so they used the old method: Mr. Bones had to drink an ampoule of vancomycin – an antibiotic, that is normally administered only administratively.

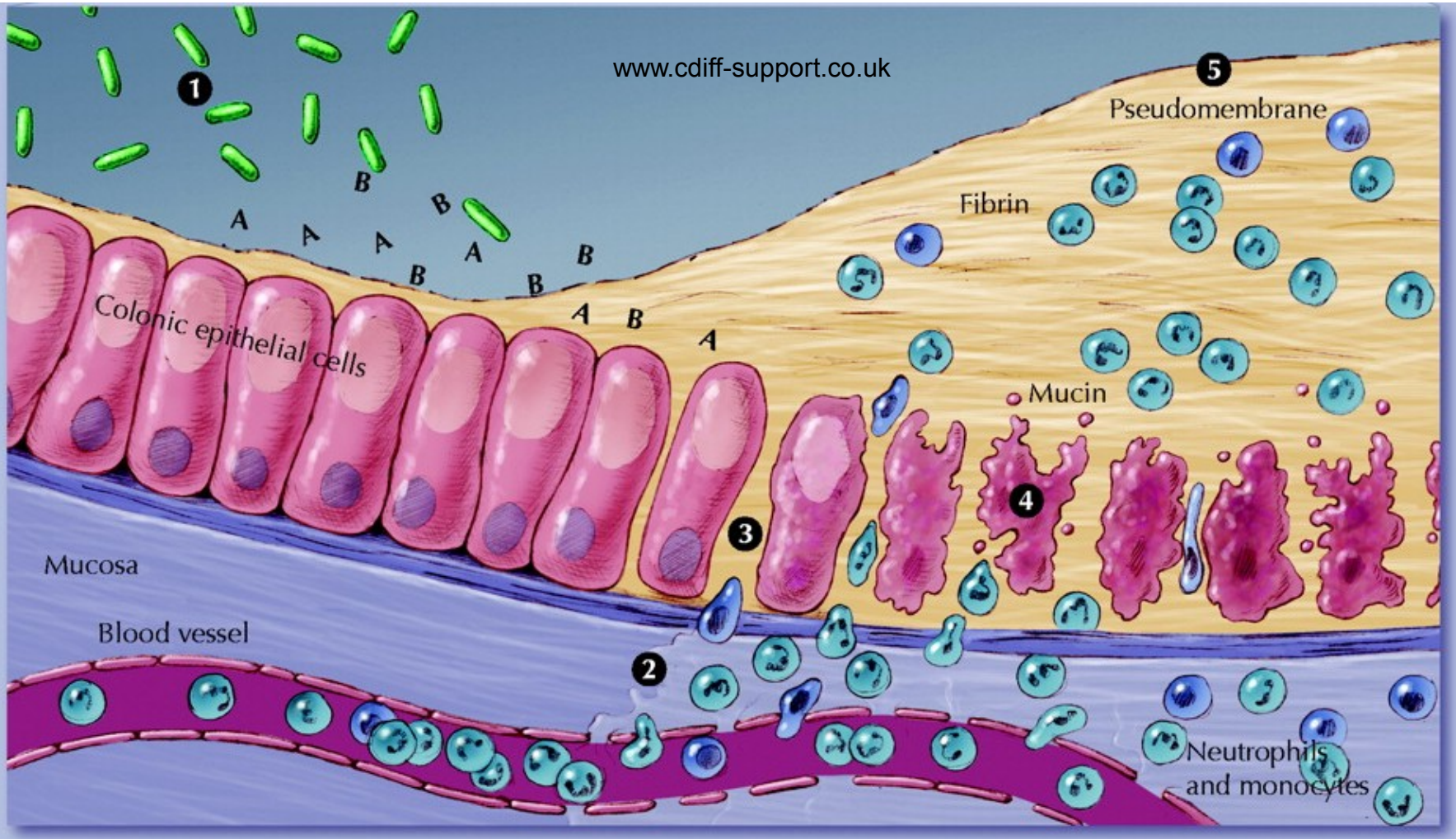
The culprit was

- *Clostridium difficile*, or its toxin
- The microbe is present commonly in the intestine; a problem appears when the toxin starts to be produced, and mostly when its concurrence is destroyed and it over-multiplies.
- Destroying of concurrence is mostly due to treatment by some antibiotics, formerly mostly lincosamids, but unfortunately, now also aminopenicillins and other drugs. Lincosamids are effective against majority of strictly anaerobic bacteria, but not *C. difficile*.
- Treatment is performed mostly using antibacterial chemoterapeutic metronidazole now. There exist another method, so called „stool transplantation“.

Clostridium difficile and its action I

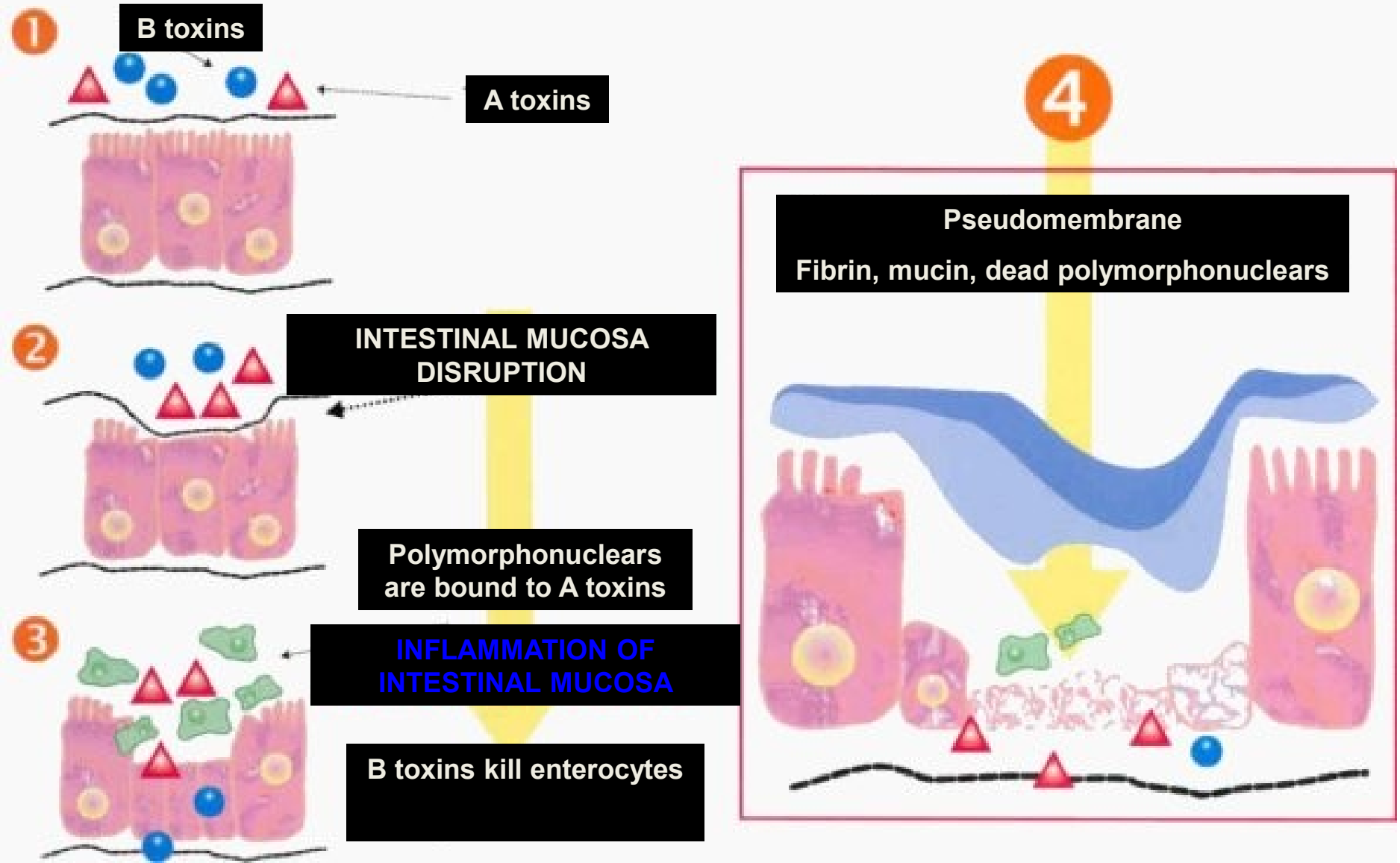


Clostridium difficile and its action II



C. difficile vegetative cells produce toxins A and B and hydrolytic enzymes (**1**). Local production of toxins A and B leads to production of tumour necrosis factor-alpha and proinflammatory interleukins, increased vascular permeability, neutrophil and monocyte recruitment (**2**), opening of epithelial cell junctions (**3**) and epithelial cell apoptosis (**4**). Local production of hydrolytic enzymes leads to connective tissue degradation, leading to colitis, pseudomembrane formation (**5**) and watery diarrhea.

Toxins of *Clostridium difficile*



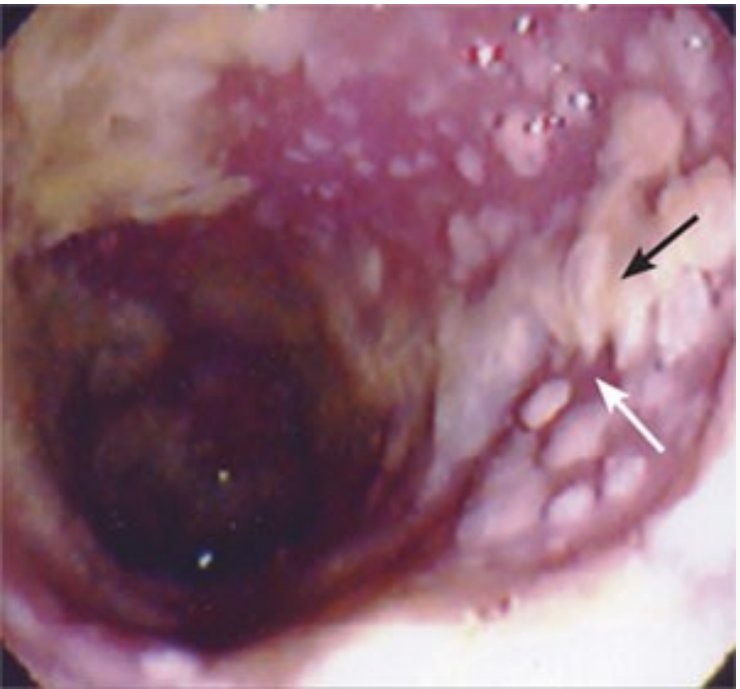
Pseudomembranous colitis

www.zuova.cz

Figure 2. Colon Specimen Obtained during a Colectomy in a Patient with Pseudomembranous Colitis.

Characteristic raised, adherent yellow plaques that vary in size from 2 to 10 mm are visible on the colonic mucosa. The intervening mucosa is hyperemic but not ulcerated.

sitemaker.umich.edu



Clostridia – survey

C. tetani	Causes tetanus
C. botulinum	Produces botulotoxin
Clostridium perfringens, C. septicum, C. welchii a aj.	Gas gangrene clostridia (+ intestinal pathogenicity)
C. difficile	Enteropathogenous

It is necessary to know that even clostridia take normally part on common intestinal microflora. Problems start in over-multiplication, in cases of coming to places that are not normal for them, appearance of a strain, producing big amounts of a toxin etc.

Spore non forming anaerobes (and lactobacilli) – clinical characteristics

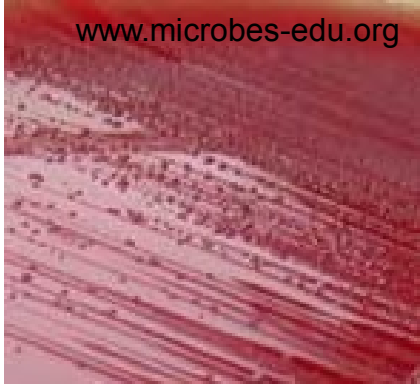
Story four

- Mrs. Cancer was hospitalized because of intensive abdominal pain
- Description methods found an abscess of pelvic region. It showed, though, a tumour cervicis – later described as a carcinoma
- In Mrs. Cancer a surgical treatment of the abscess and than also a cancer was possible, although hysterectomy was necessary. Fortunately, no metastases was found.

The disease is formed by

- A mixture of strictly anaerobic, but also facultative anaerobic bacteria
- It is likely, that the mixture was previously present in Mrs. Cancer‘s vagina, without making any problems
- The cancer broke the anatomical barrier, and so microbes came to other places, causing the abscess
- Spore-not-forming anaerobic bacteria have limited ways of transmission because of their characteristics
- Majority of infections are endogenous

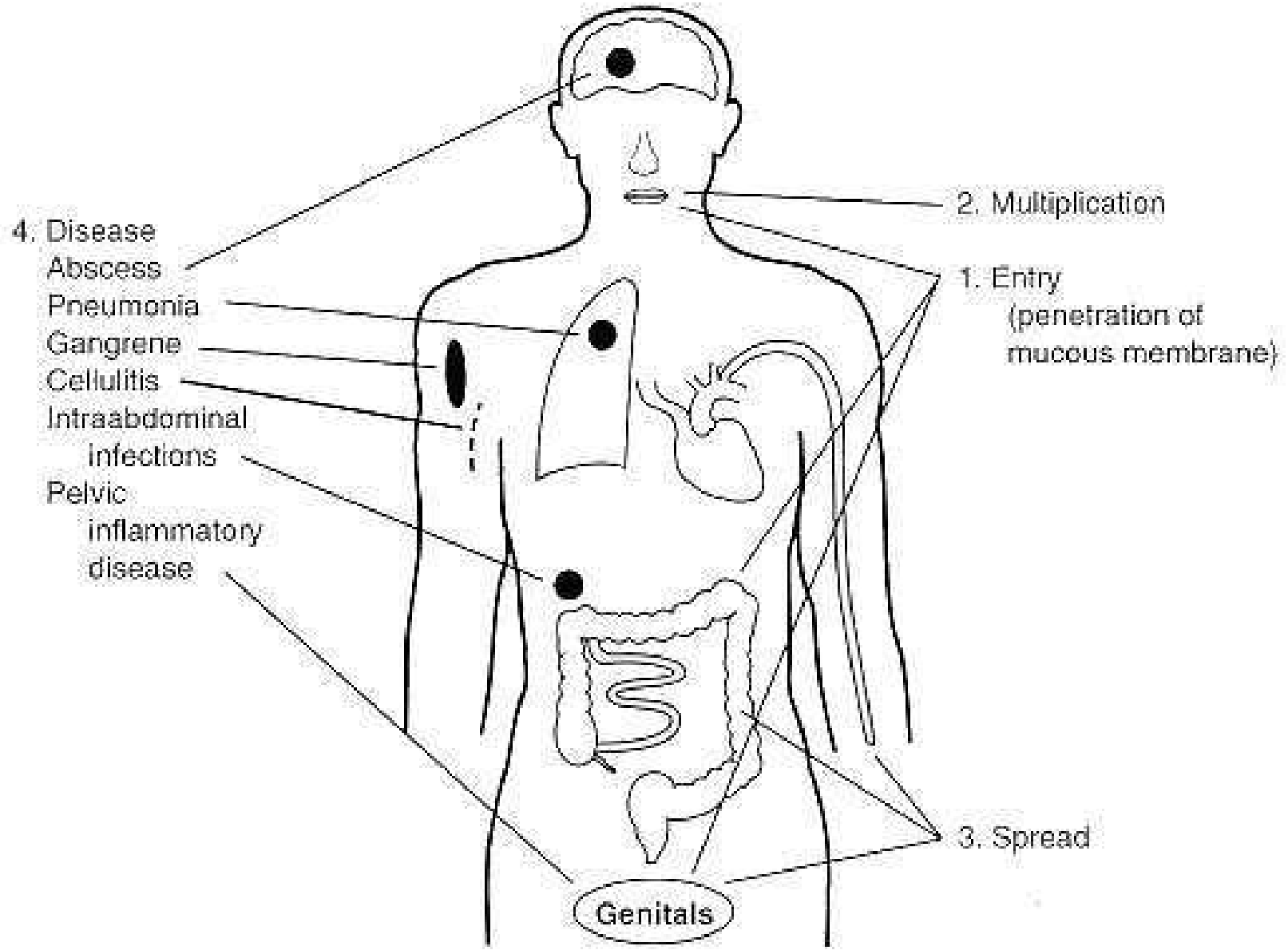
Common characteristics of spore-not-forming anaerobes



- They are present as a part of common microflora:
  - in the large bowel they form 99 % of the total amount of microorganisms, up to one kilogram of them
  - in oral cavity they live thanks to biofilm – they are inside and so they have no access to the air that would be harmful for them
  - in vagina they are not present in all females, but about 70 % of women have some anaerobes in vagina; in case of over-multiplication, it is a dysmicrobia, requiring treatment
- In inflammation usually there is no single pathogen, but rather a mixture, „Veillon microflora“

Anaerobes in the body

gsbs.utmb.edu



Anaerobic infection from oral cavity

aapredbook.aappublications.org

Newborn anaerobic pneumonia

aapredbook.aappublications.org

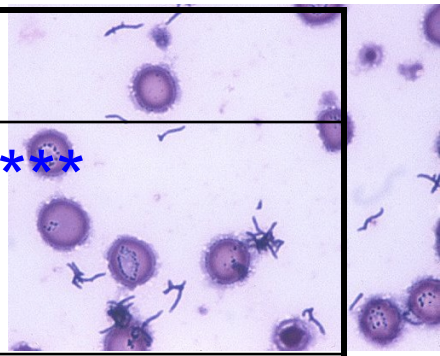
Bacteroides fragilis pneumonia in newborn (*B. fragilis* isolated from the placenta and blood culture from the newborn). Anaerobic cultures were obtained because of a faecal smell in the amniotic fluid

Gingivostomatitis:
Prevotella gingivalis

www.mamagums.com

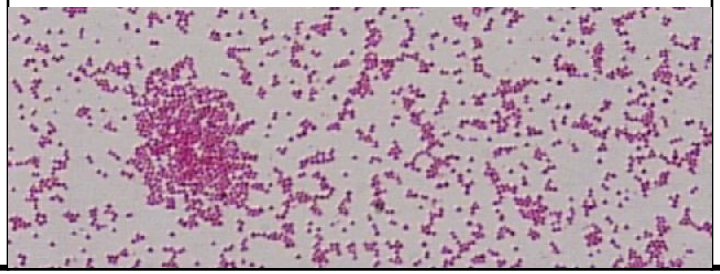
Spore not-forming anaerobes (most common species in humans)

http://www.geocities.com



	Cocci	Bacilli
G+	*Peptococcus* *Peptostreptococcus*	*Propionibacterium**** *Eubacterium*
G-	*Veillonella*	*Fusobacterium,* *Leptotrichia** *Bacteroides, Prevotella,* *Porphyromonas***

http://www.geocities.com



*pointed ends of the rod

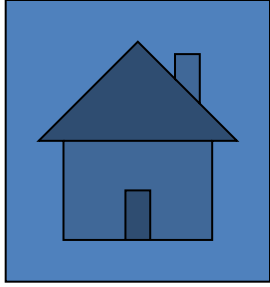
**round ends of the rod

***it is not a full anaerobe

Story five

- Miss Clark had chronic problems with her **vaginal infections**
- Topic antibiotics in form of vaginal globules of cream with applicator gave her only partial help, pathogens often came back again
- Finally, her gynaecologist advised to use a **probiotic drug** with some „good“ bacteria, that would **bring back the original vaginal microflora** and not allow the pathogens to multiply again
- The main „good bacterium“ was…

Lactobacillus acidophilus, „Döderlein‘s bacillus“



- Lactobacilli are quite robust Gram-positive rods. They are called lactobacilli, because they ferment various substrates (mostly glucose and lactose) to lactate
- Lactobacilli are the most important part of normal vaginal microflora, and also important part of intestinal microflora
- Lactobacilli are **not** anaerobic bacteria. Nevertheless, as they are often microaerophilic, they are usually not able to grow at the normal atmosphere. On the contrary, the imperfect anaerobiose of our common anaerobic jars and anaerostats enable them to grow better.

Relation of bacteria to oxygen (repeating)

Remember, what condition enable bacterial growth

Conditions	Normal	↓ O_2	↑ CO_2	No O_2
Strict aerobes	yes	yes	yes	no*
Facultative anaerobes	yes	yes	yes	yes
Aerotolorant bact.				
Microaerofilic bact.	no	yes	(yes)	no*
Capnofilic bacteria	no	(yes)	yes	no*
Strict anaerobes	no	no	no	yes**

*In practice often growing – common anaerobiose is not ideal

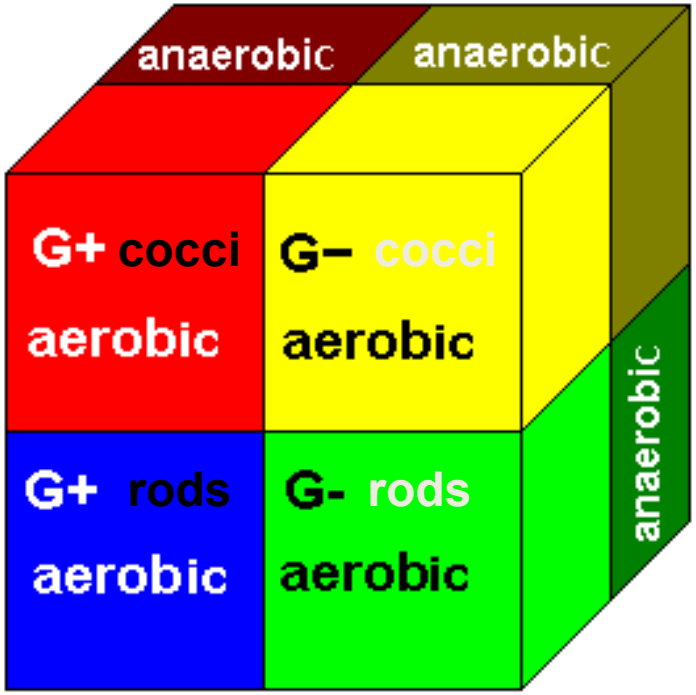
**In practice, sometimes not growing – common anaerobiose is not ideal. Such bacteria (EOS – Extremely oxygen sensitive) are not commonly culturable

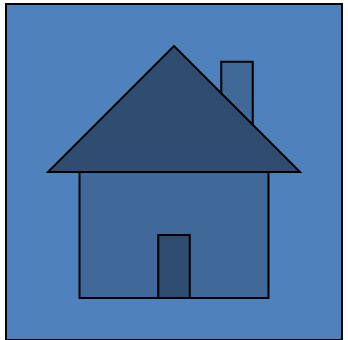
What we know until now

- In the practices P1 to P6 we made acquaintance with four groups of microbes growing at aerobic conditions – some of them strictly aerobic as e. g. pseudomonades, some facultative anaerobic as e. g. *Escherichia coli.*

Now, we add four more groups

- Each of the four groups have anaerobic „brothers“. Their characteristics differ considerably from aerobic bacteria and have some common characteristics. Only genus *Clostridium*, spore forming, is different





Diagnostics of anaerobic bacteria, how to obtain anaerobiosis

How to search for the anaerobic bacteria – I

- Microscopy: More important than in aerobic bacteria, because of morphological diversity
- Culture: It is necessary to get anerobiosis using anaerobic jars or boxes. In liquid media it is sufficient to pour paraffin oil over the medium. VL (viande levure) broth, VL blood agar and various special media are used
- Biochemical identification: catalase and oxidase usually negative, mutual differentiation possible biochemically, and chromatographical gas analysis (they are biochemically active)
- Antigen analysis and indirect diagnostics are rarely used in diagnostics

Sampling and material transport in anaerobic cultivation

- Priority has liquid specimen, e. g. pus, the best is to send it in syringe with a cap after elimination of oxygen*
- When a swab is sent, it is necessary to send it in a transport medium; on the other hand, common, e. g. Amies medium, is sufficient
- It is also possible to talk with laboratory and to inoculate the specimen directly to media, e. g. during surgery.

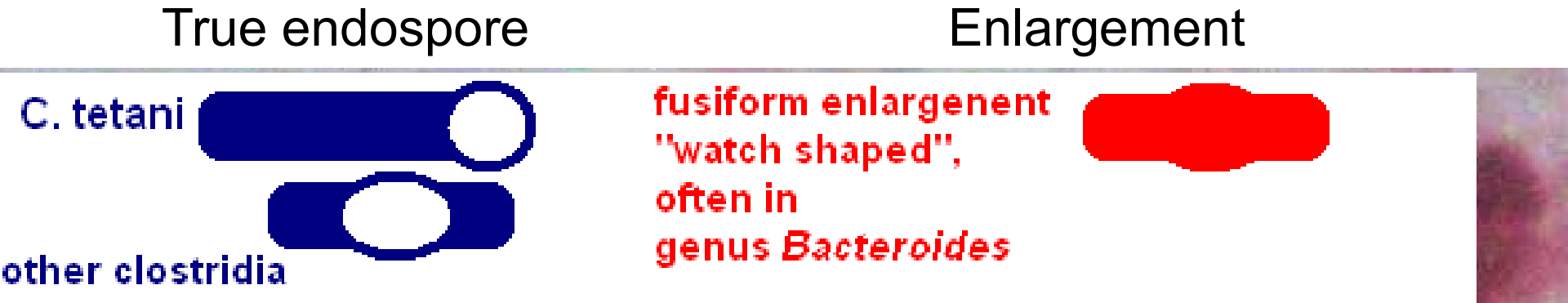
**for safety reasons, unlike in older recommendations, it is no more recommended to use a syringe with needle*

Microscopy of anaerobic bacteria

- We perform normal Gram staining. We differentiate bacteria according to shape and cell wall type into cocci and bacilli, G+ and G–.
- Anaerobic rods vary in shape very much – one preparation contains various formations from filamentous to nearly coccoid ones
- In Gram-negative rods, it is useful to differentiate between those with rounded ends (*Bacteroides*, *Prevotella*, *Porphyromonas*) and those with pointed ends, often spindle-shaped (*Fusobacterium*, *Leptotrichia*)

Note to microscopy of anaerobes: various shapes of anaerobes

- Students sometimes confuse an endospore (unstained formation, only its margins are visible) and enlargements of rods (visible in some non spore-forming and Gram negative rods).
- In real spore-forming microbes it is useful to follow position of the spore. In *Clostridium tetani* the spore is terminal (at the end of the cell)



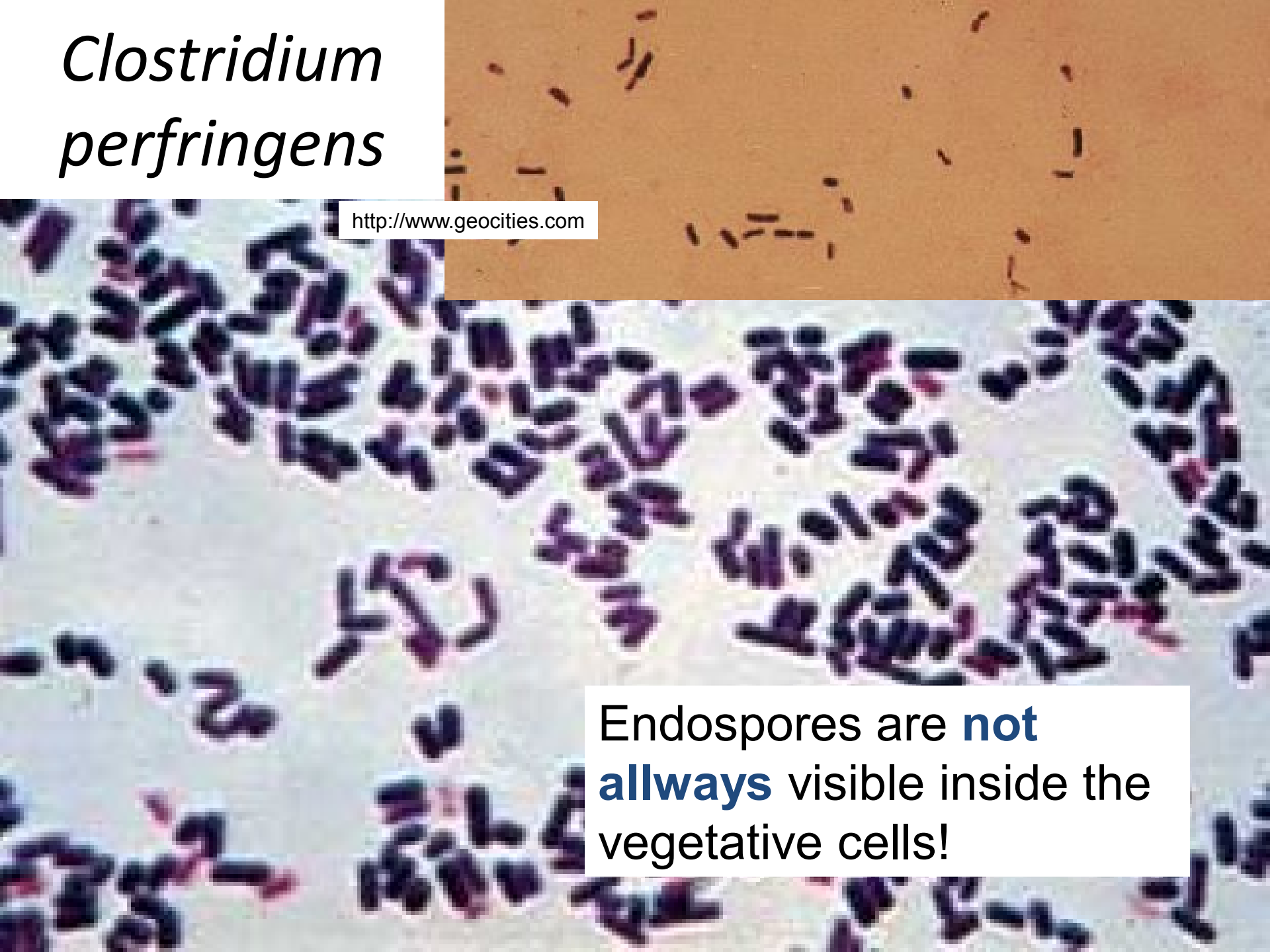
Clostridium tetani

C. botulinum


acpcommunity.acp.edu/

www2.bc.cc.ca.us
Vegetative cells
Endospore-bearing cells
10 µm

Clostridium perfringens



Endospores are **not allways** visible inside the vegetative cells!

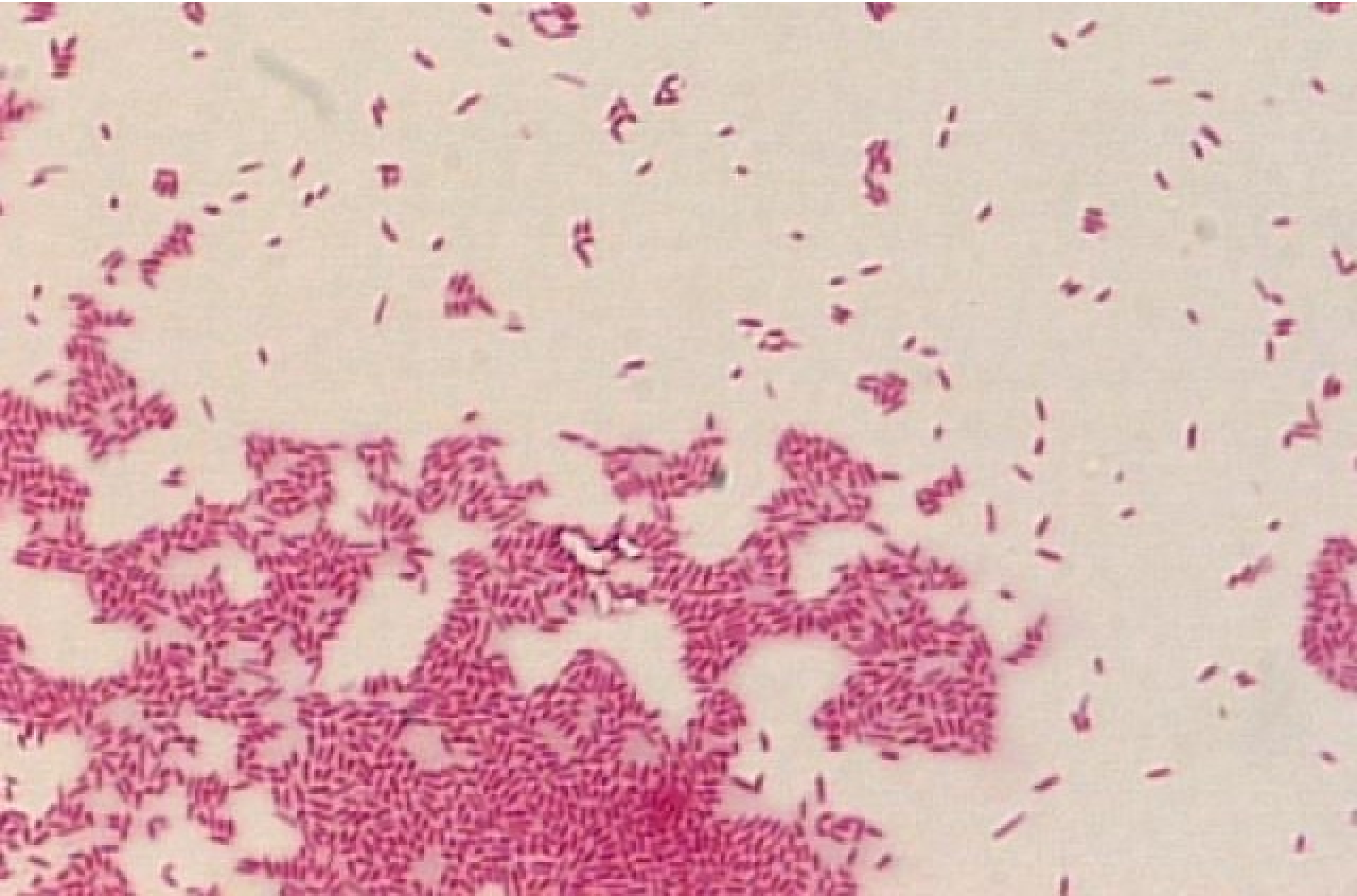
Clostridium difficile

http://medecinepharmacie.univ-fcomte.fr

Clostridium difficile

Bacteroides fragilis

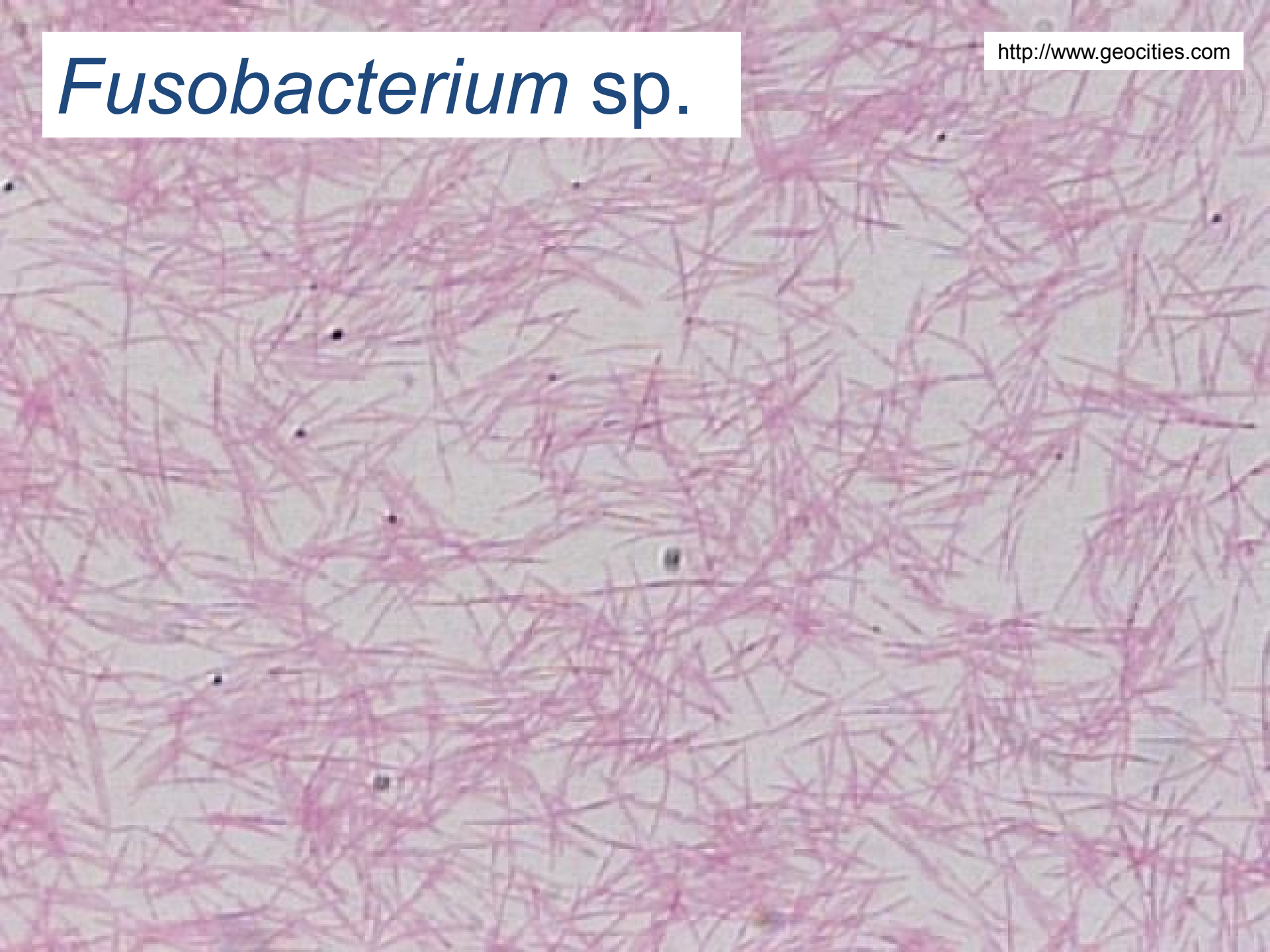
http://www.geocities.com
Bacterloides fragilis



Bacteroides sp.

www.medmicro.info, photo O. Z.

Fusobacterium sp.



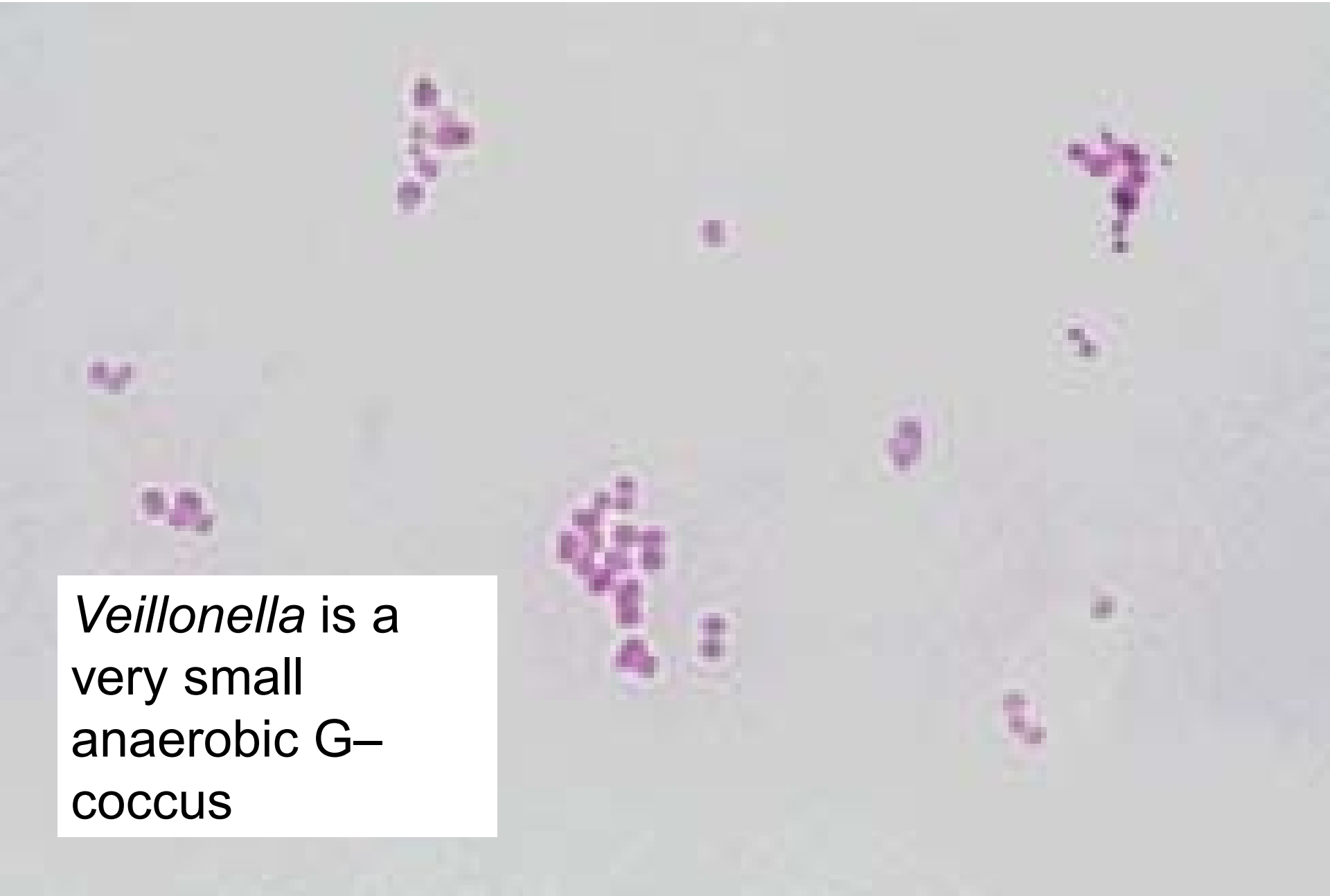
Peptostreptococcus sp.

http://www.geocities.com

Peptostreptococci are anaerobic G+ cocci in chains, while peptococci are anaerobic G+ cocci in clusters.

Veillonella sp.

http://www.primer.ru



Veillonella is a very small anaerobic G– coccus

Culture of anaerobic bacteria

- Anaerobic bacteria grow often in tiny, irregular colonies, that may have tails on margins. It smells typically.
- Aerobic culture on blood agar enables only growth of strictly aerobic and facultative anaerobic bacteria. So, if a bacterium does not grow here, but does grow in anaerobic conditions, it is a strictly aerobic bacterium. To culture anaerobes, we use VL blood agar (in practice we say simply „VL agar“).

To anaerobic culture: How to get the anaerobiose

- Mechanically – VL broth is covered by paraffin oil
- Physically – in the anaerobic box, air is replaced by a mixture of anaerobic gases from a bomb
- Chemically – in the anaerobic jar
  - organic acids → H_2 and CO_2
  - in the second phase on palladium catalyser hydrogen reacts with oxygen, and water is formed, so oxygen is consumed

Covering of VL-broths by paraffin oil

www.medmicro.info, photo O. Z.

Anaerobic box

www.medmicro.info, photo O. Z.

Anaerobic box (detailed description)

www.medmicro.info, photo O. Z.

Anaerobic jar
(principle)

Palladium catalyser (beneath the lid) necessary for the second phase

Generator of anaerobiose (packet with chemicals) necessary for the whole reaction

www.medmicro.info, photo O. Z.

Anaerobic jar (detailed description)

air-proof lid

palladium catalyser (beneath the lid)

construction for placing of Petri dishes

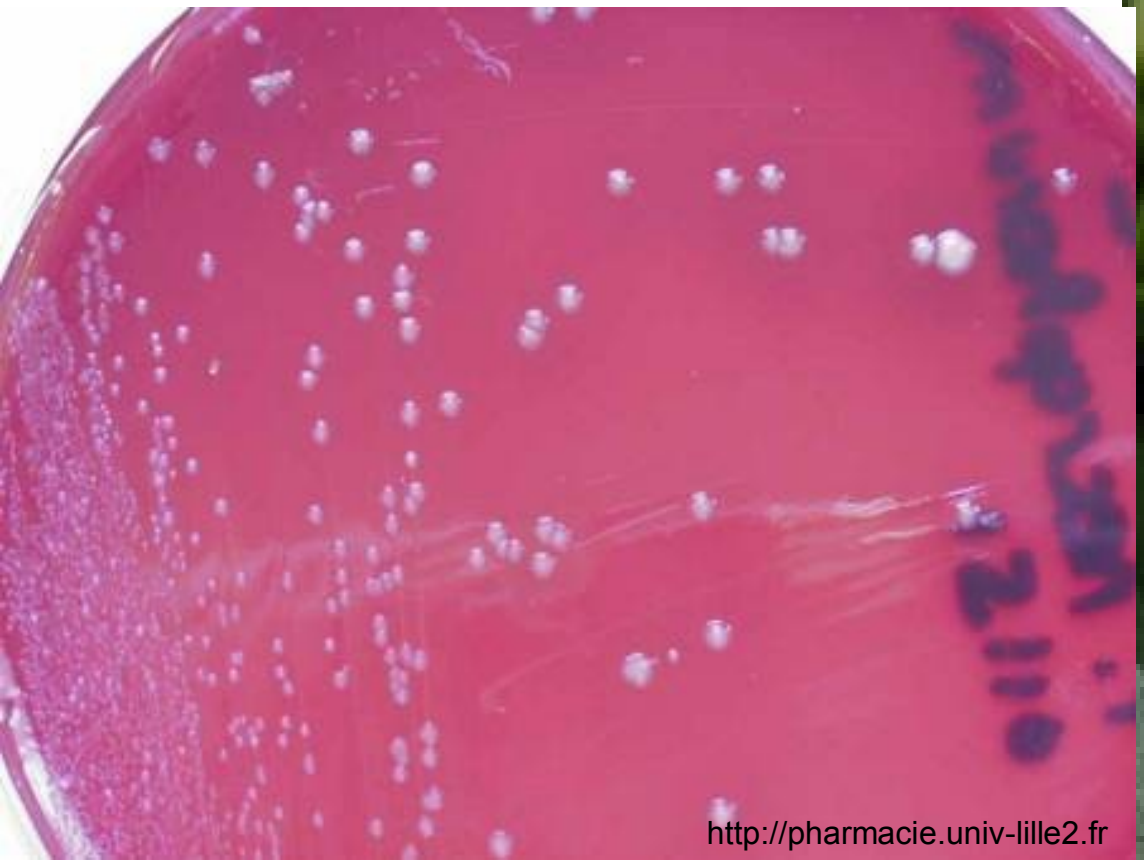
Anaerobiose generator (packet with chemicals)

www.medmicro.info, photo O. Z.

gold.aecom.yu.edu

Another anaerobic jar

Fusobacterium sp.

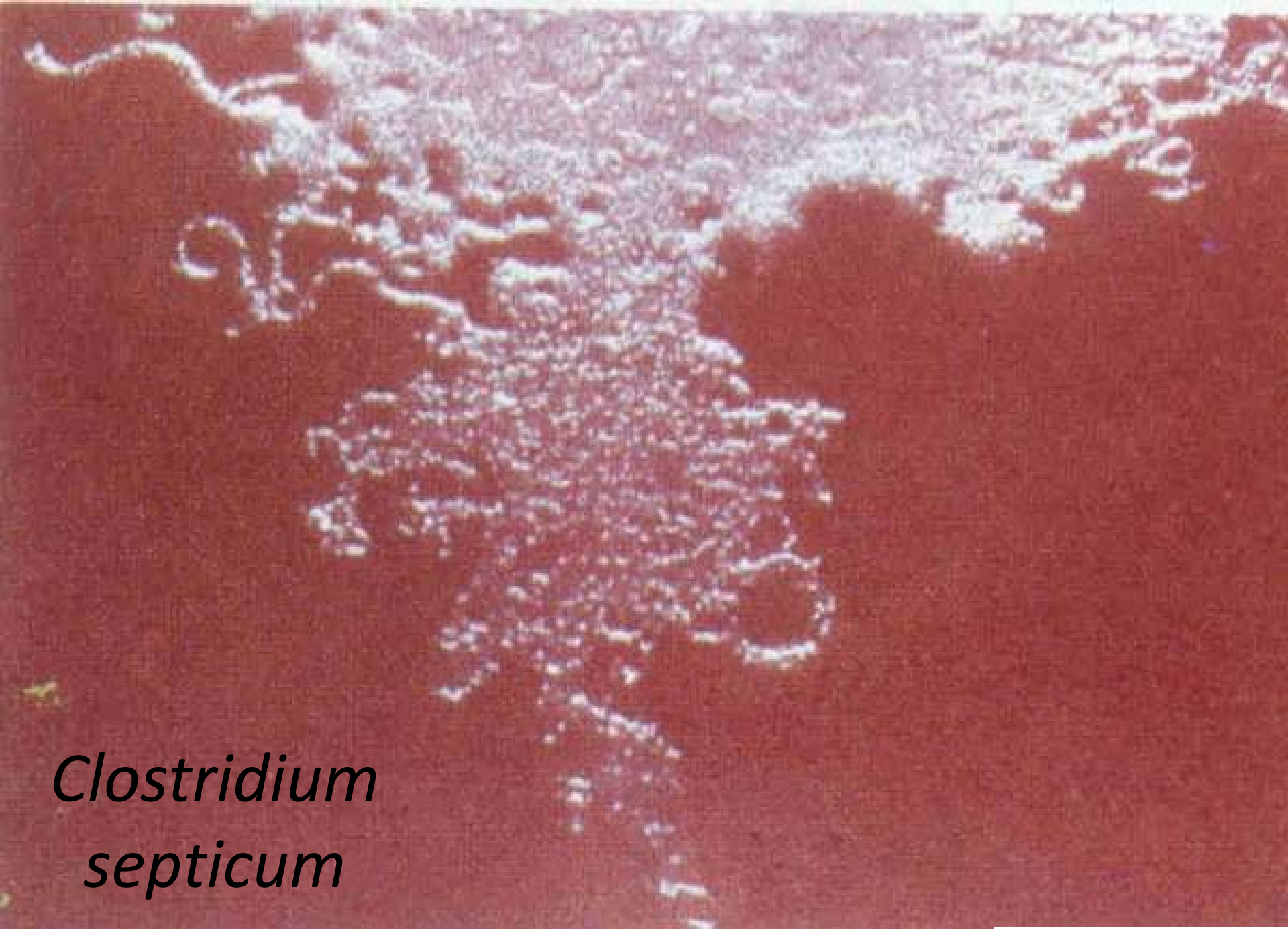


http://pharmacie.univ-lille2.fr

Morphology of colonies of anaerobic bacteria

- Clostridia use to have quite large, irregular, badly smelling colonies
- Other anaerobic bacteria have rather small colonies
- Some anaerobic bacteria (*Prevotella melaninogenica*) have pigmented colonies

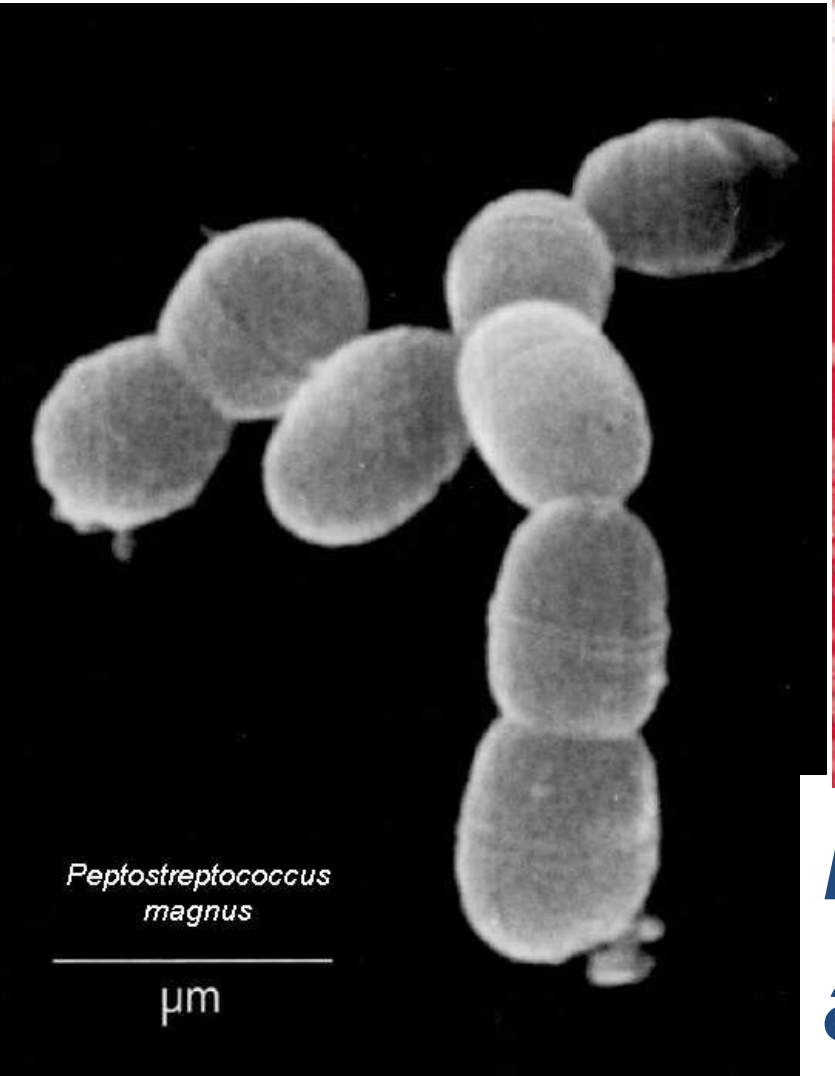
Clostridium perfringens



Prevotella melaninogenica (black pigmentation)


Leptotrichia buccalis
http://pharmacie.univ-lille2.fr

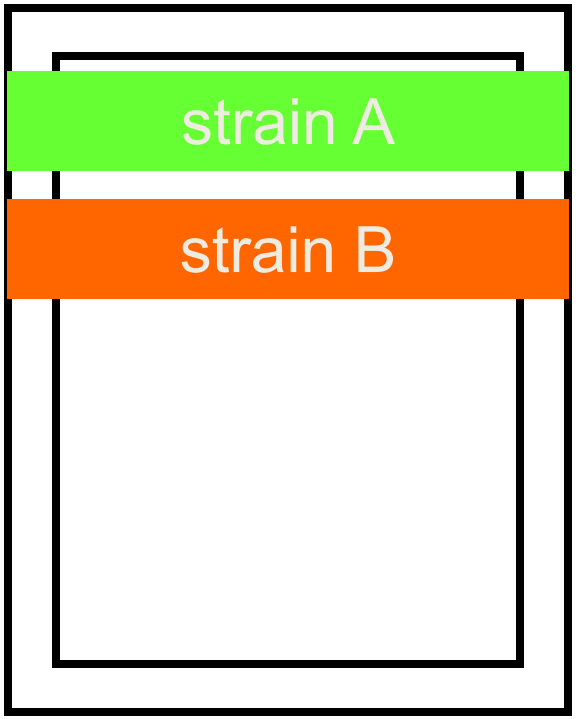
Peptostreptococcus magnus



Peptostreptococcus anaerobius

Biochemical differentiation

- Different tests are used, in Czech conditions mostly ANAEROtest 23 Lachema.



We write results of the strains („+“ or „-“) and count the octal code

We assess the result according to the codebook

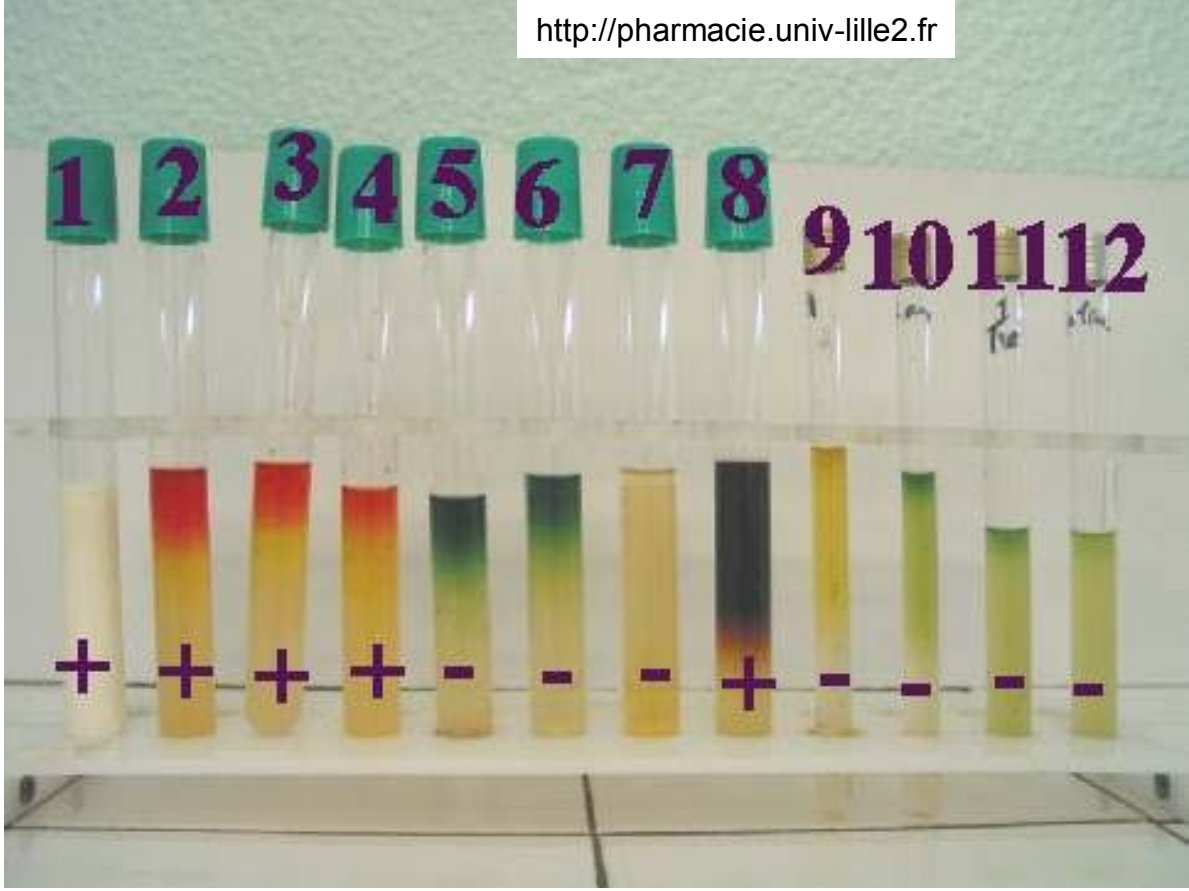
ATTENTION – the codebook is divided into several parts according to morphology of anaerobic bacteria. It is necessary to search in the proper part of the codebook

ANAEROtest 23 – example

NEC = negative control (that is why it is ANAEROtest 23 and not 24

+	-	+	+	-	-	-	+
1	1	1	1	1	1		
-	+	+	-	+	-	-	-
2	2	2	2	2	2		
+	-	+	+	+	-	+	NEC
4	4	4	4	4	4		
5	2	7	5	6	0		

Other sets for diagnostics of anaerobes



Antibiotic susceptibility tests

- Antibiotic susceptibility in anaerobic bacteria is tested on media enabling their growth, so not MH agar, but usually VL blood agar
- The most classical therapy used to be performed by means of diffusion disc test (not on MH agar, but VL blood agar). Now it is mostly replaced by E-test (the MIC value is counted at crossing of the zone and the strip

Detection of toxin I: lecithinase

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

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

Positive
Negative I
Negative II

Half with antitoxin

Detection of toxin II: animal experiment for tetanic/botulinic toxin

- Animal experiment is used in tetanus and botulism. In tetanus mouse is spastic, in botulism we can see pareses.

A tetanic mouse

microvet.arizona.edu

Toxin detection using animal experiment

- Look at the picture of tetanic mouse

Drawing by Petr Ondrovčík (1959–2007)

Graphically adapted.

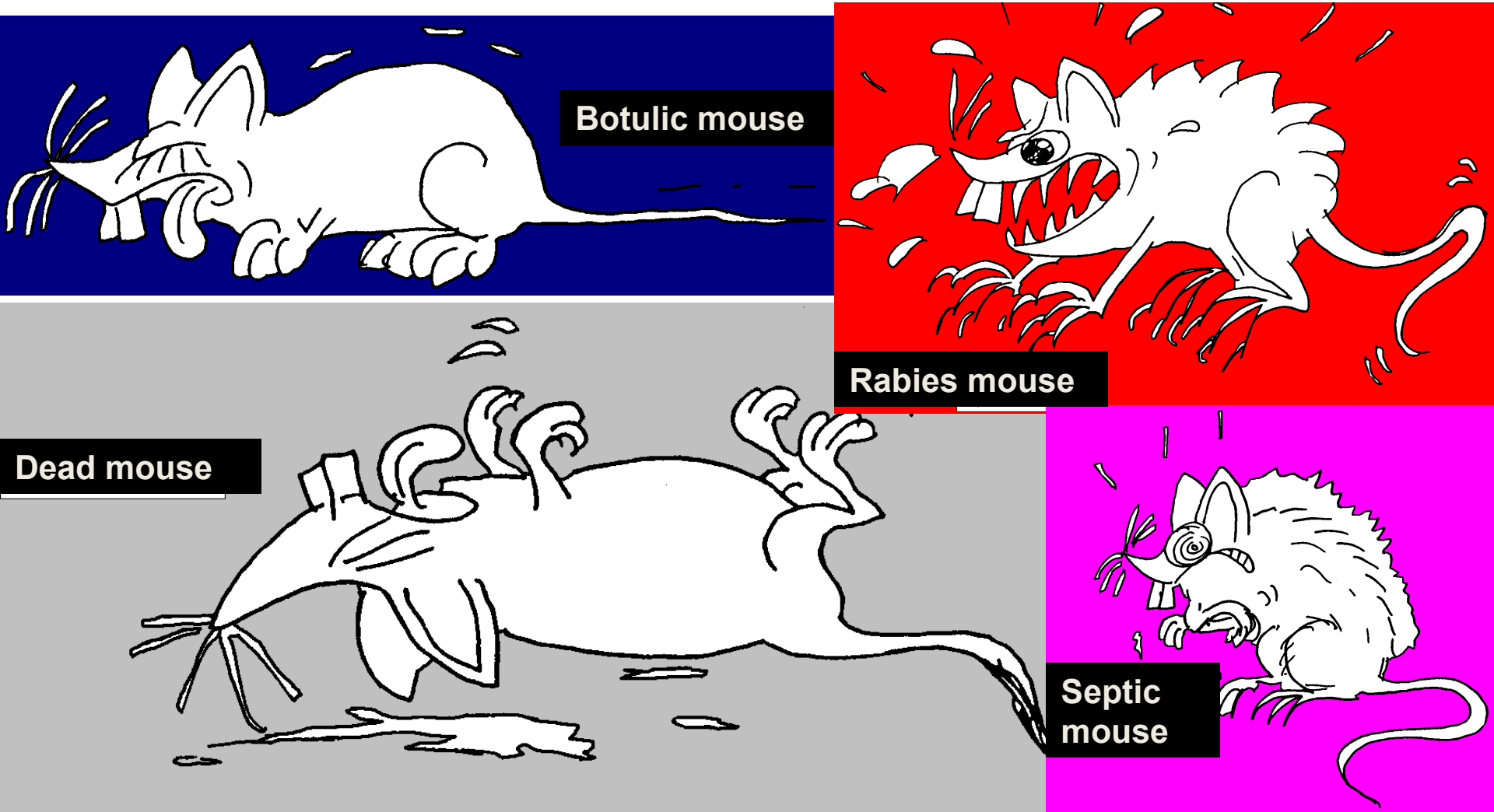
Background counterstained using not malachite green, but „Paint“ programme by Microsoft

www.biotox.cz

Opistotonus is typical both for mice and humans

Appearance of an experimental animal is observed also in other situations, e. g. botulism.

- In botulism, we can se pareses, not spasms

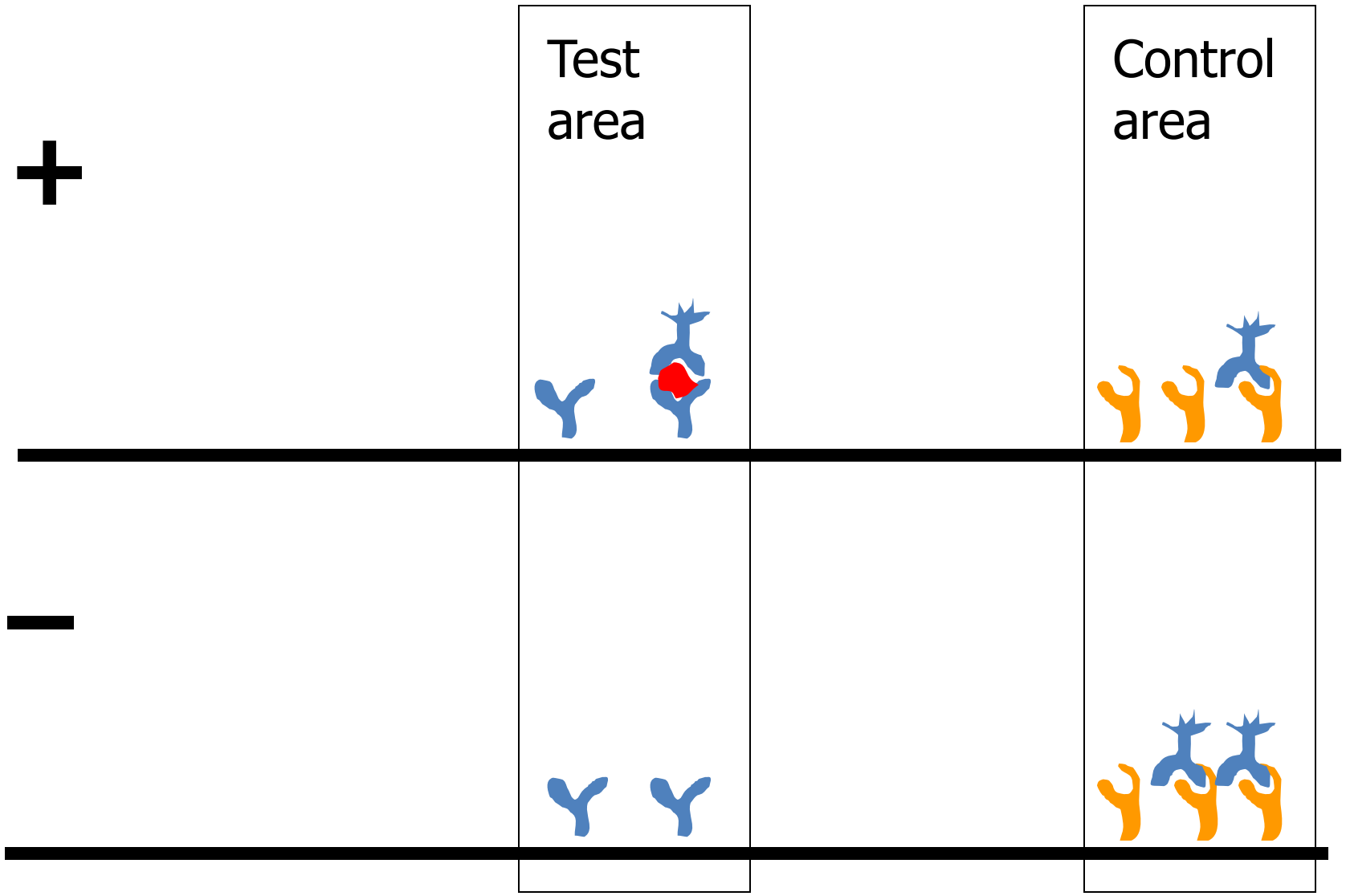


Detection of toxins III: Immunochromatographic tests

- Immunochromatographic tests are based on **binding of individual components**, similarly as ELISA or immunofluorescence
- The most typical example is **pregnancy test**
- The principle was explained in **J08** practical session.
- In case of ***Clostridium difficile*** **toxin producing strains, unlikely other cases, we make a parallel testing of both antigen and A and B toxins**

At a test positive for both toxin(s) and antigen we can see two lines and dots. One line and dots means positivity of antigen, but not toxin. „Dots only“ mean a completely negative, but valid, test.

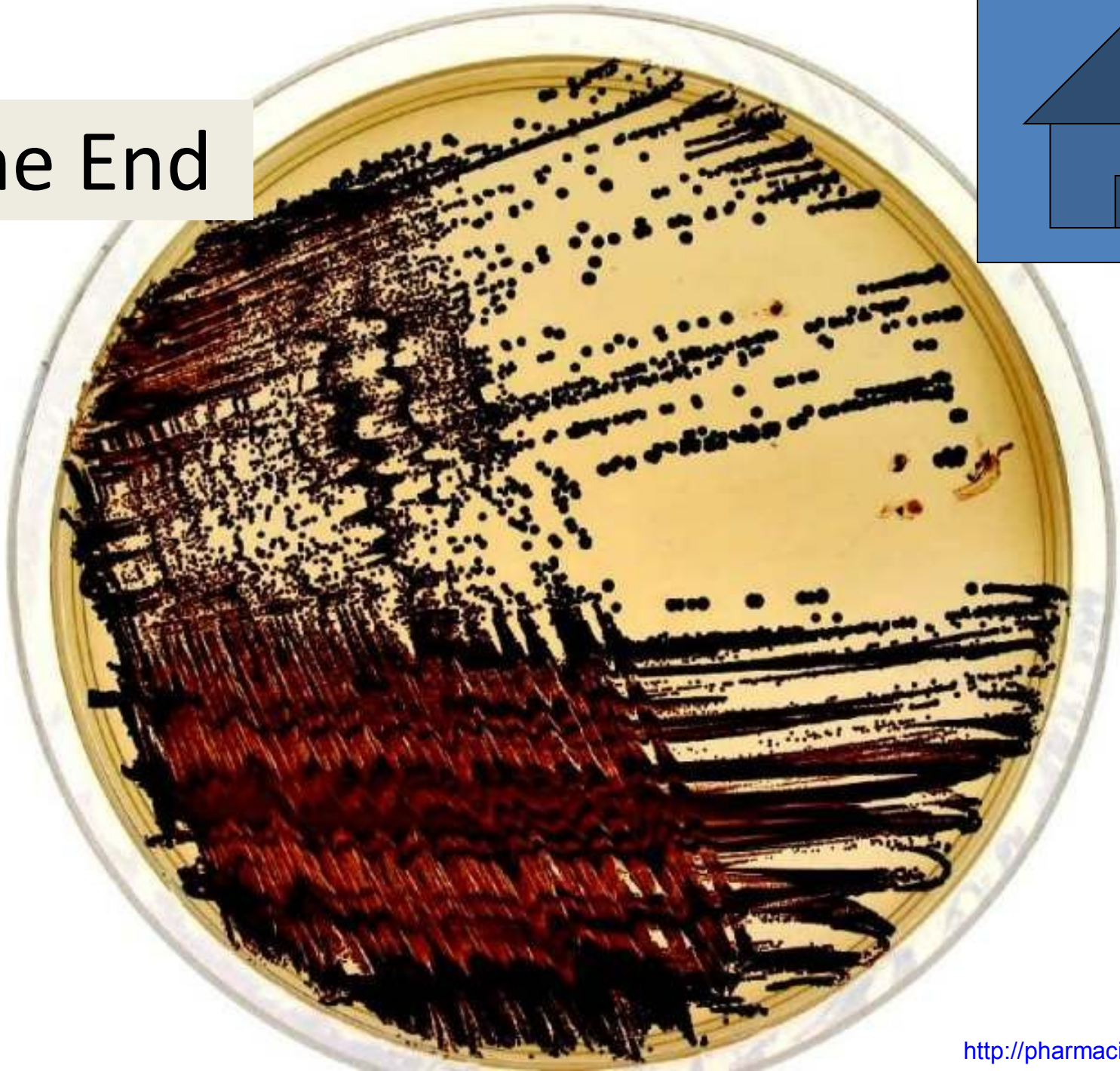
Principle (only for illustration)



Practical search for anaerobes (example in vaginal microbes)

- For vaginal swabs where anaerobic culture is requested we use VL agar with disks of vancomycin and amikacin. Usually, anaerobic bacteria grow between these two disks.
- Besides eventually present anaerobic flora, we can see a lot of **vaginal lactobacilli**, microaerofilic bacteria commonly found in vaginal swabs (and rather rarely present in normal aerobic culture)
- Our imperfect anaerobiosis enables growth of microaerofilic bacteria, as you can see.

The End



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