

Institute for microbiology shows

# TRACING THE CRIMINAL

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Part seven:  
Anaerobic criminals

# Anaerobes



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# Survey of topics

Pathogens with complicated diagnostics

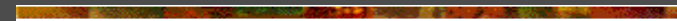
Clostridia – clinical characteristics

Spore non forming anaerobes – clinical characteristics

Relation of bacteria to oxygen (repeating from spring term)

Diagnostics of anaerobic bacteria, anaerobiosis

Pictures of anaerobic bacteria



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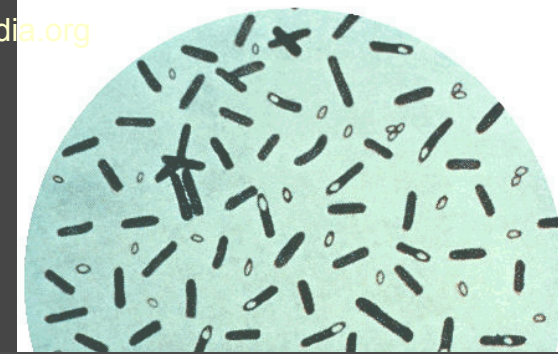
# Pathogens with complicated diagnostics

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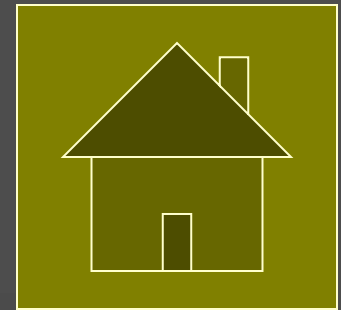
# Before we start...

- ...it is something to think about. **Until now (P1 to P6) we spoke mostly about bacteria, that do not need special approach.** (*Although, some bacteria from P06 did not match: 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 will have 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. mycoplasmas or chlamydia) it is often necessary to take **serum and to perform indirect diagnostics**
- Remember especially this for your practice!



- You may pass the examination – but *this* should be clear for you even after 20 years.

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# Clostridia – clinical characteristics

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# Story one

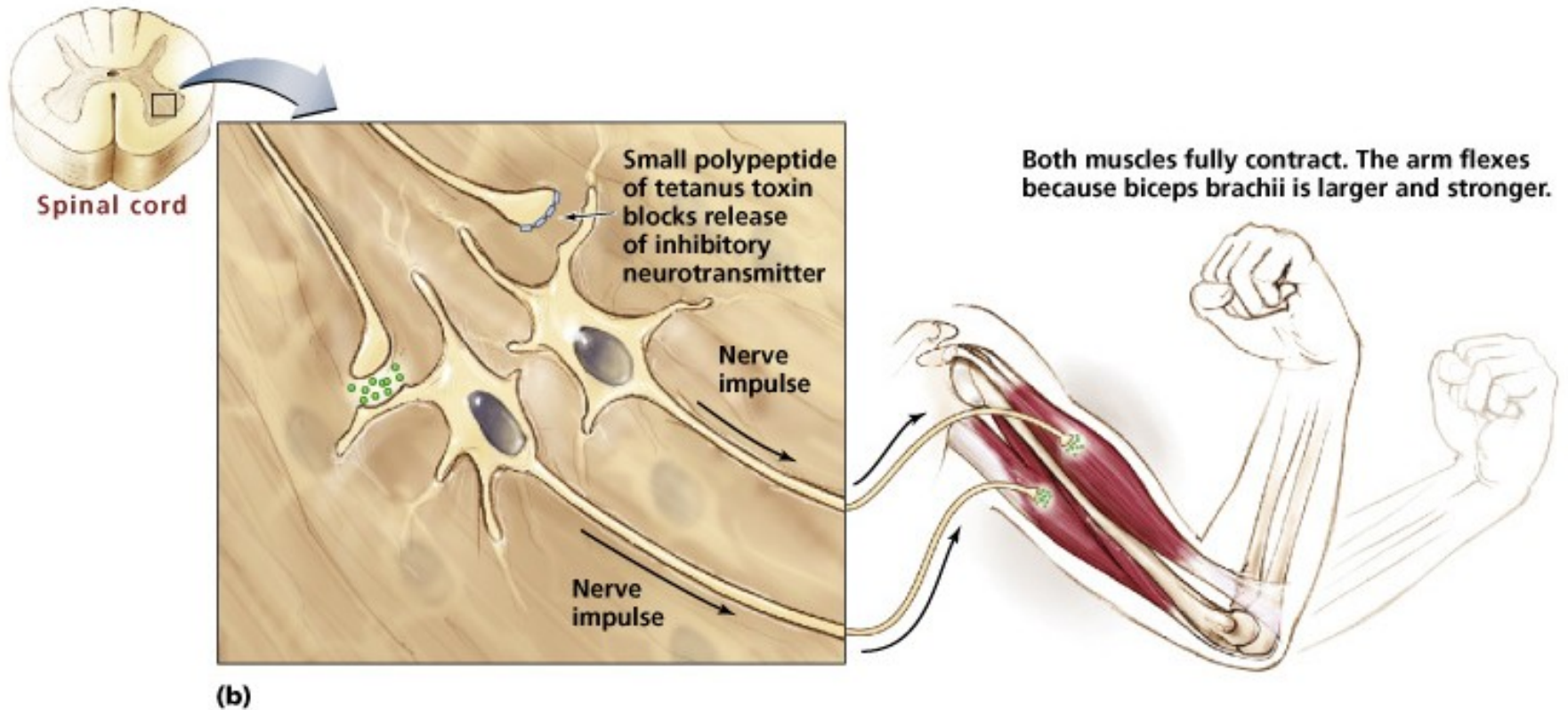
- Mrs. Cabbage was all the time seen working in the garden. It was her big hobby. Once she **injured** her hand, because a **pointed remainder of a plant was hidden in the soil**. She went to her general practitioner.
- The GP used local treatment for the wound, and then recommended **re-vaccination**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



# Neurotoxic clostridia

- The criminal that threatened Mr. 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](http://www2.bc.cc.ca.us)



# A tetanic man



# Once more tetanus

<http://pharmacie.univ-lille2.fr>

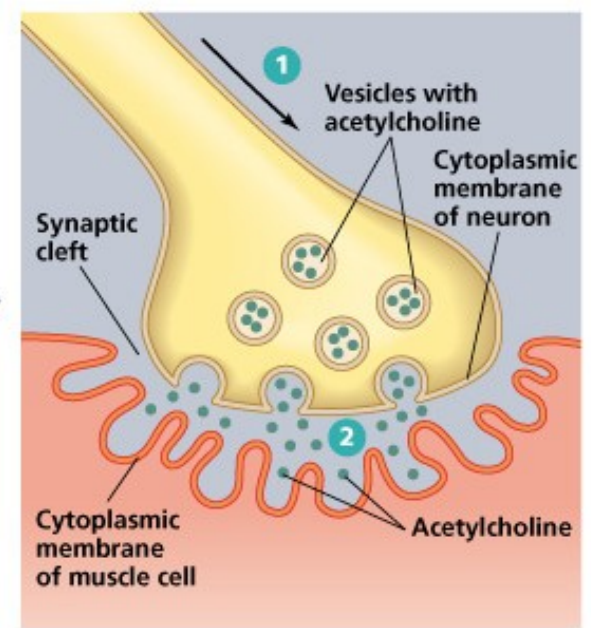
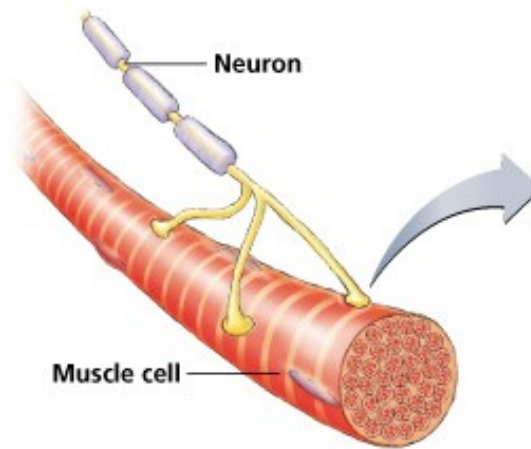


# Trismus (spasm of chewing muscles)

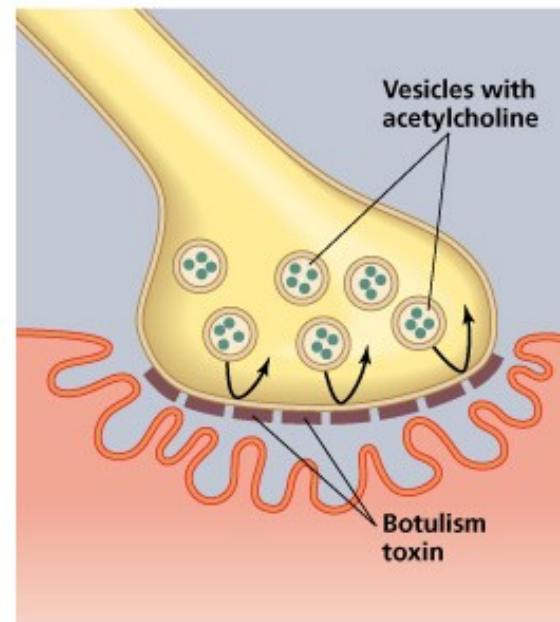
<http://pharmacie.univ-lille2.fr>



# Botulism



(a) Normal neuromuscular junction



(b) Neuromuscular junction with botulism toxin present



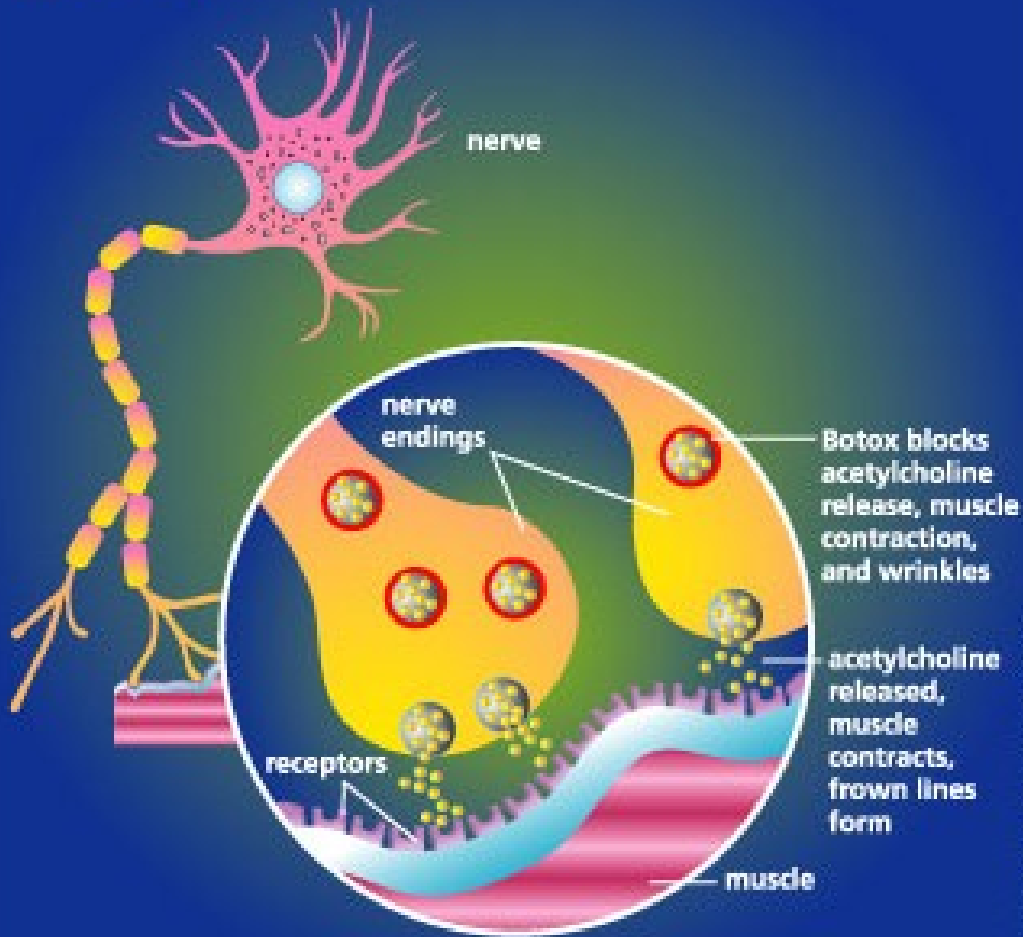
# Typical tongue appearance in case of botulism





# Botox: use of *Clostridium botulinum* toxin to become younger

## How Botox Works



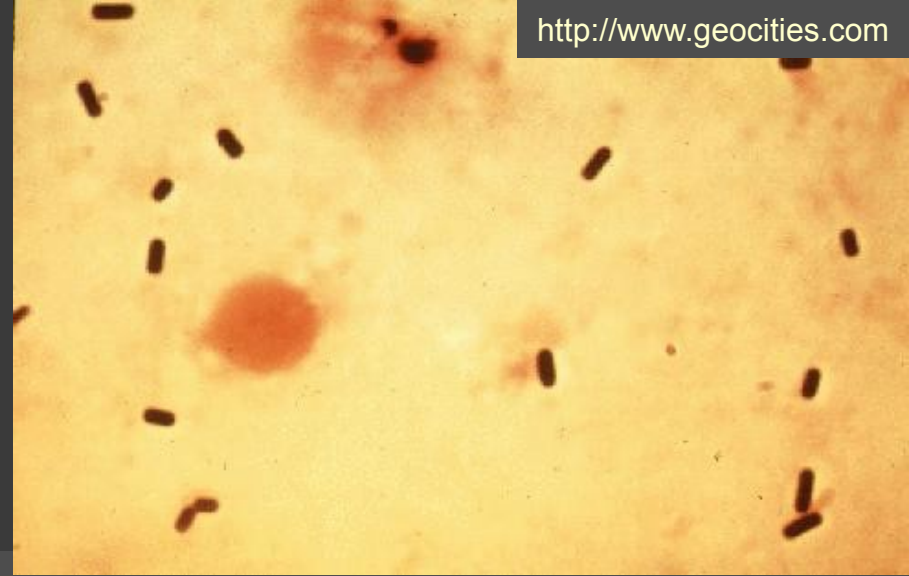
Infographic by Renée Gordon

## 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 mood**, 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 criminal is now



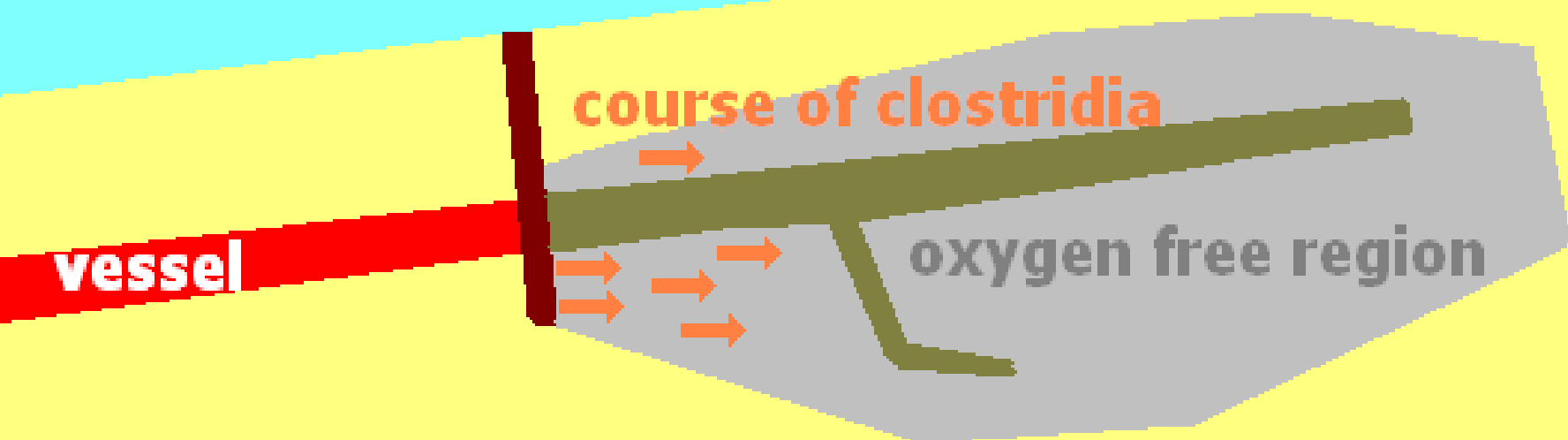
- *Clostridium perfringens*, one of agents of gas gangrene (with *C. novyi*, *C. septicum*, etc.)
- Gas gangrene is a typical **war disease**. It is nevertheless possible to get it even during peace, e. g. in case of catastrophes
- **Gas gangrene clostridia** – or their enterotoxins – are intestinal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

# Gas gangrene formation



<http://pharmacie.univ-lille2.fr>

**wound**



**vessel**

**course of clostridia**

**oxygen free region**

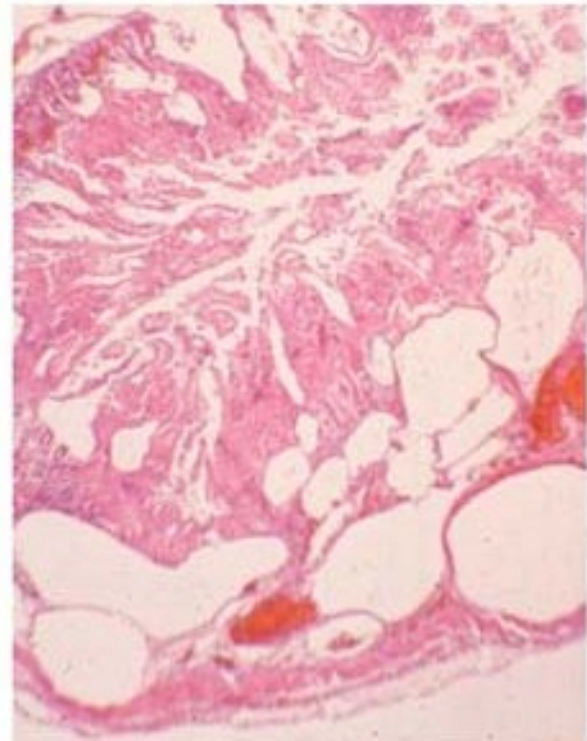
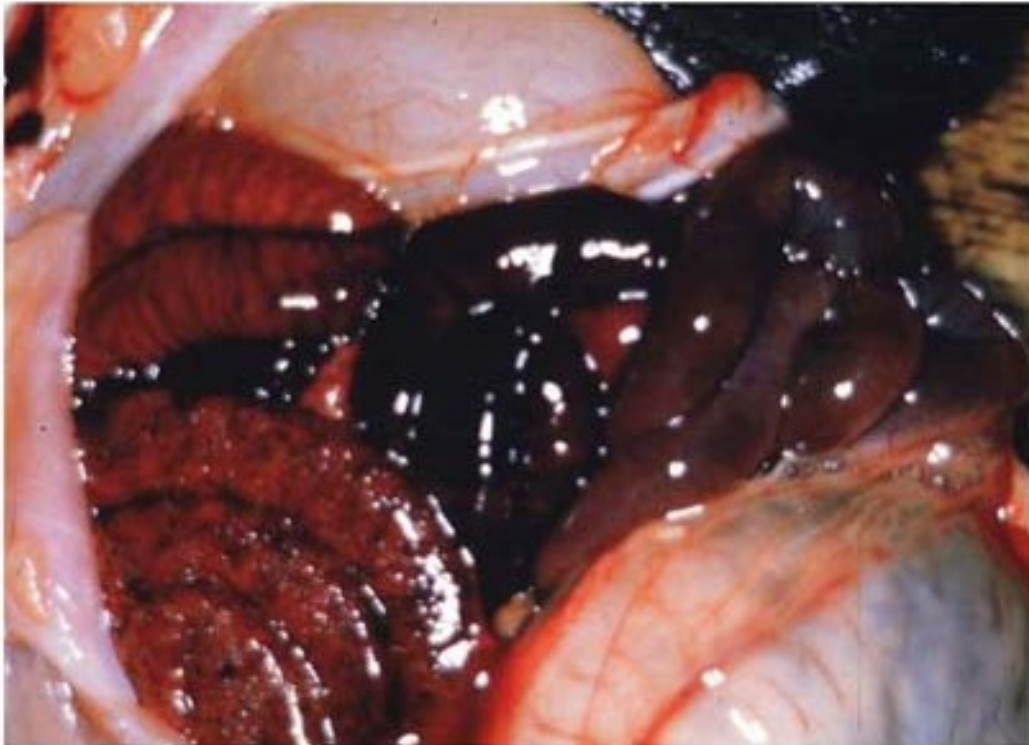
# Necrotizing enterocolitis – this, too, may be caused by *C. perfringens*

## 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>





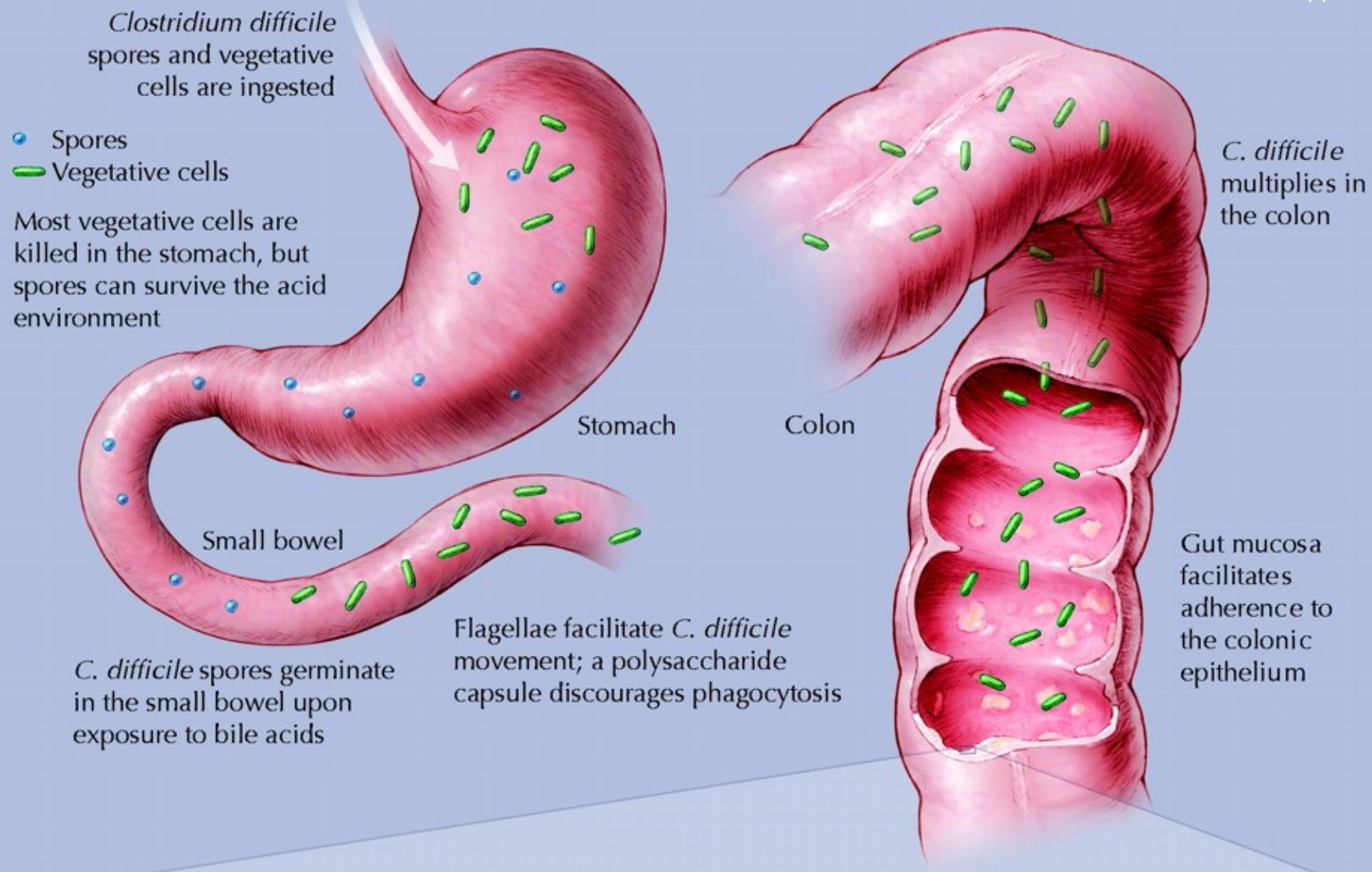
# Story three

- 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 methode: Mr. Bones **had to drink an ampule of vancomycine** – an antibiotic, that is normally administered only administratively.

# The agent

- *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 overmultiplies.
- Destroying of concurrence is mostly due to **treatment by some antibiotics**, mostly lincosamids. Lincosamids are effective against majority of strictly anaerobic bacteria, but not *C. difficile*.
- Treatment is performed mostly using antibacterial chemotherapeutic **metronidazol** now

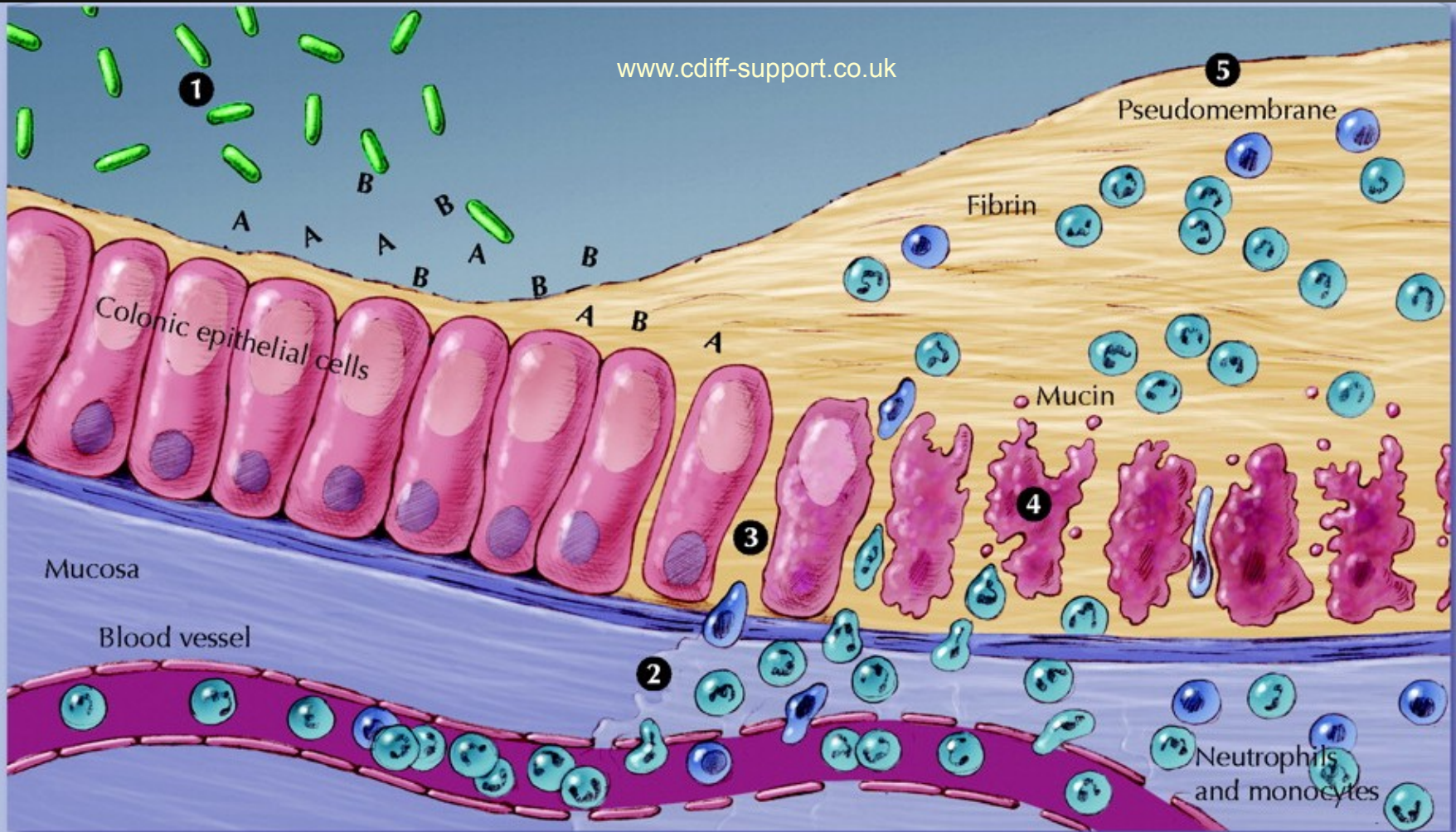
# Clostridium difficile and its action I





# *Clostridium difficile* and its action II

www.cdiff-support.co.uk



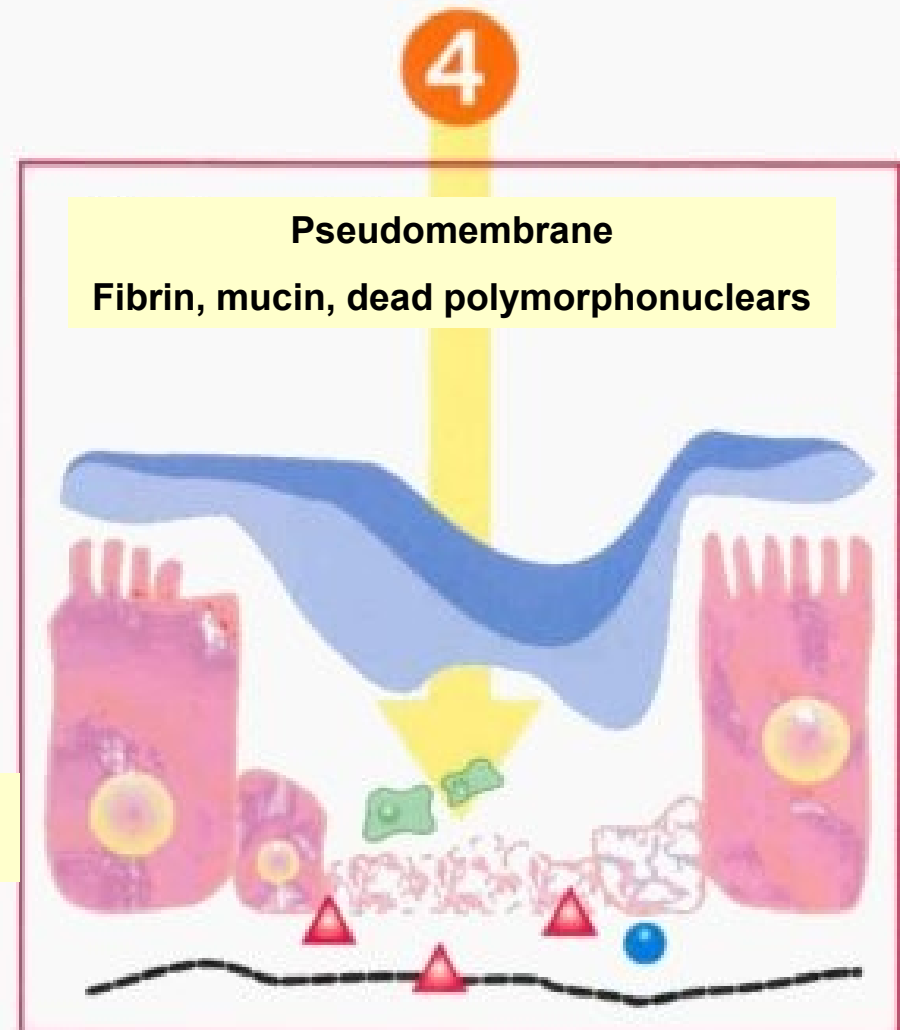
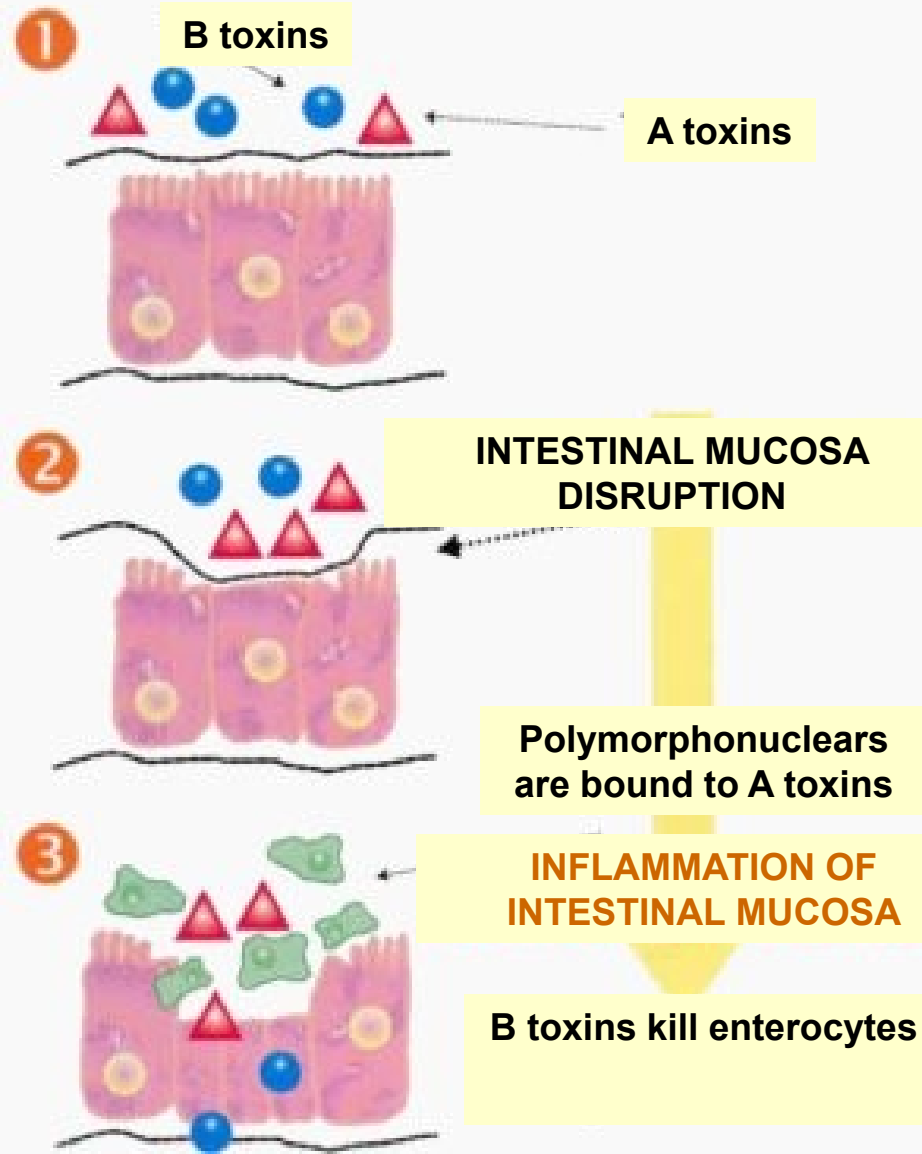
*C. difficile* vegetative cells produce toxins A and B and hydrolytic enzymes (1). Local production of toxins A and B leads to production of tumour necrosis factor-alpha and proinflammatory interleukins, increased vascular permeability, neutrophil and monocyte recruitment (2),

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*

## Toxins of *Clostridium difficile*

www.zuova.cz





# Pseudomembranous colitis

www.zuova.cz

sitemaker.umich.edu

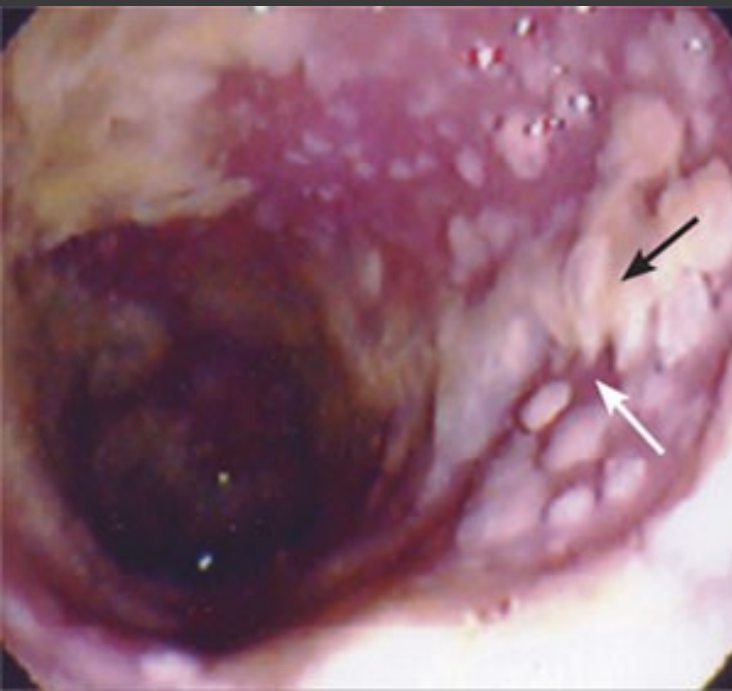


Figure 2. Colon Specimen Obtained during a Colectomy in a Patient with Pseudomembranous Colitis.

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.

# Clostridia – survey



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

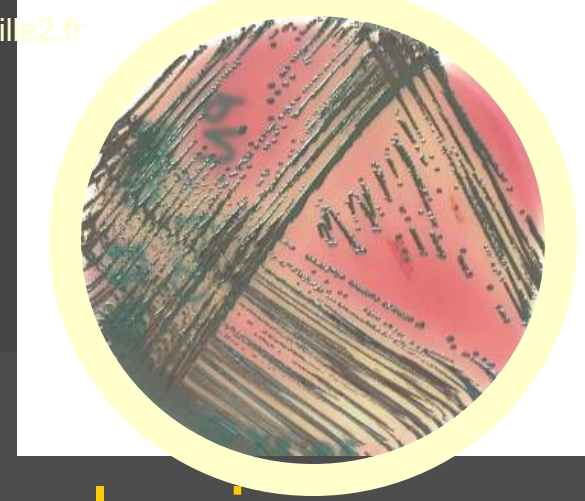
It is necessary to know that **even clostridia take normally part on common intestinal microflora.**

Problems start in overmultiplication, in cases of coming to places that are not normal for them, appearance of a strain, producing big amounts of a toxin etc.

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# Spore non forming anaerobes – clinical characteristics

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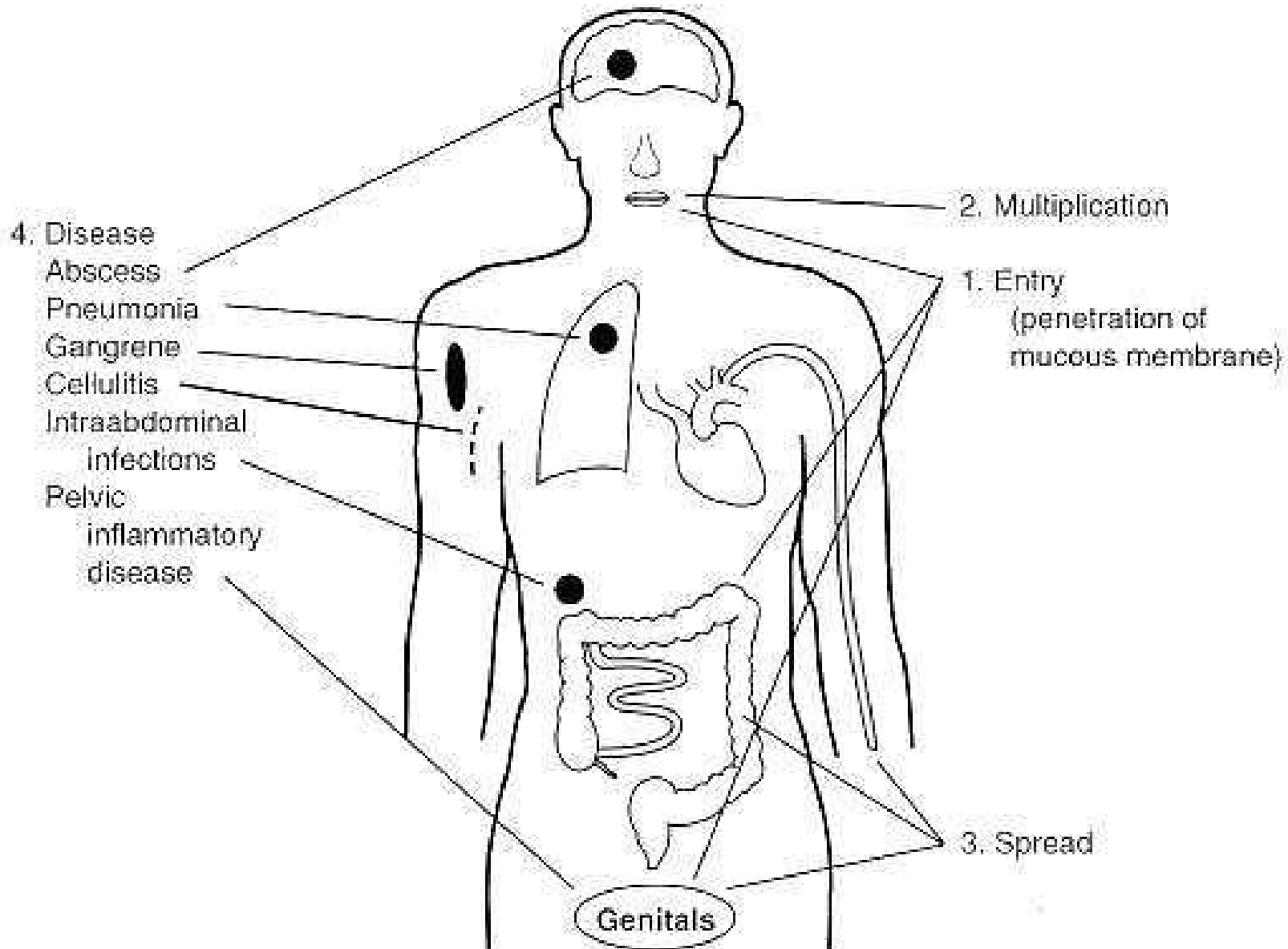
# Story four

- Mrs. Cancer was hospitalized because of **intensive abdominal pain**
- Description methods found an **abscessus of pelvic region**. It showed, though, a **tumor cervicis** – later described as a **carcinoma**
- In Mrs. Cancer a **surgical treatment** of the abscessus 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 abscessus
- Non-sporulating anaerobic bacteria have limited ways of transmission because of their characteristics
- Majority of infections are endogenous

# Anaerobes in the body





# Anaerobic infection from oral cavity



# 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 fecal odor in the amniotic fluid



# Gingivostomatitis: *Prevotella gingivalis*

[www.mamagums.com](http://www.mamagums.com)

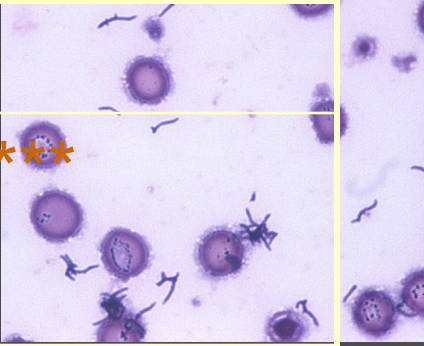


# Common characteristics of spore-non-forming anerobes

- They are present as **a part of common microflora**:
  - **in the large bowel** they form 99.9 % of the total amount of microorganisms, about one kilogram of them
  - **in oral cavity** they live thanks to biofilm – they are inside and so they have no acces 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 overmultiplication, it is a dysmicrobia, requiring treatment
- In inflammation **usually there is no single pathogen**, but rather a mixture, „Veillon microflora“

# Spore non-forming anaerobes (most common species in humans)

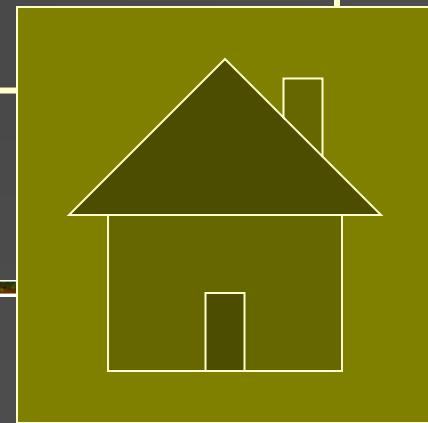
<http://www.geocities.com>

	Cocci	Bacilli	
G+	<i>Peptococcus</i> <i>Peptostreptococcus</i>	<i>Propionibacterium</i> *** <i>Eubacterium</i>	
G-	<i>Veillonella</i> <a href="http://www.geocities.com">http://www.geocities.com</a>	<i>Fusobacterium</i> , <i>Leptotrichia</i> * <i>Bacteroides</i> , <i>Prevotella</i> , <i>Porphyromonas</i> **	

\*pointed ends of the rod

\*\*round ends of the rod

\*\*\*it is not a full anaerobe





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# Relation of bacteria to oxygen (repeating)

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# Remember, what condition enable bacterial growth

Conditions	Normal	↓ O <sub>2</sub>	↑ CO <sub>2</sub>	No O <sub>2</sub>
Strict aerobes	yes	yes	yes	no*
Facultative anaerobes	yes	yes	yes	yes
Aerotolerant 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

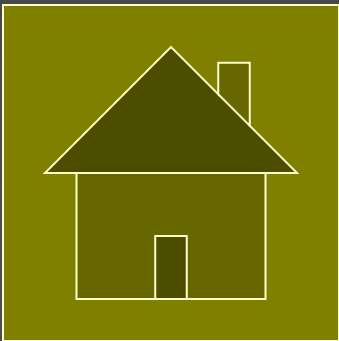
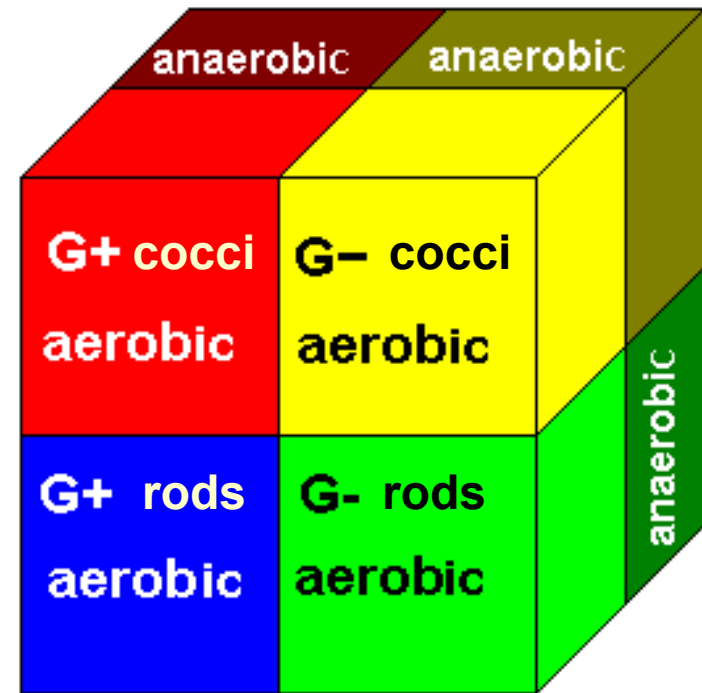
- In practicals P1 to P6 we made acquaintance with four groups of microbes growing at aerobic conditions – some of them strictly aerobic as e. g. pseudomonads, some facultative anaerobic as e. g. *Escherichia coli*.

G+ cocci aerobic	G- cocci aerobic
G+ rods aerobic	G- rods aerobic



# 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



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Diagnositics of  
anaerobic bacteria,  
how to obtain  
anaerobiosis

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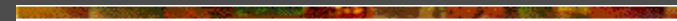
# How to search for the anaerobic bacteria – I

- **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 parafin 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

# Microscopy of anaerobic bacteria

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- 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 coccal ones



# Note to microscopy of anaerobes: various shapes of anaerobes

- Students sometimes confuse a spore (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)



**fusiform enlargement  
"watch shaped",  
often in  
genus *Bacteroides***



# Culture of anaerobic bacteria

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- 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 facultatively 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 generate anaerobiosis

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

# Covering of VL-broths by parafin oil



# Anaerobic box



# Anaerobic box (detailed description)



- source of anaerobic gases
- space for entering culture plates
- entrances for hands of personel

# Anaerobic jar

(principle)

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

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



# Anaerobic jar (detailed description)

air-proof lid

palladium calalyser  
(beneath the lid)

construction for placing  
of Petri dishes

Anaerobiose generator  
(packet with chemicals)

screw closer  
pressure  
ventile





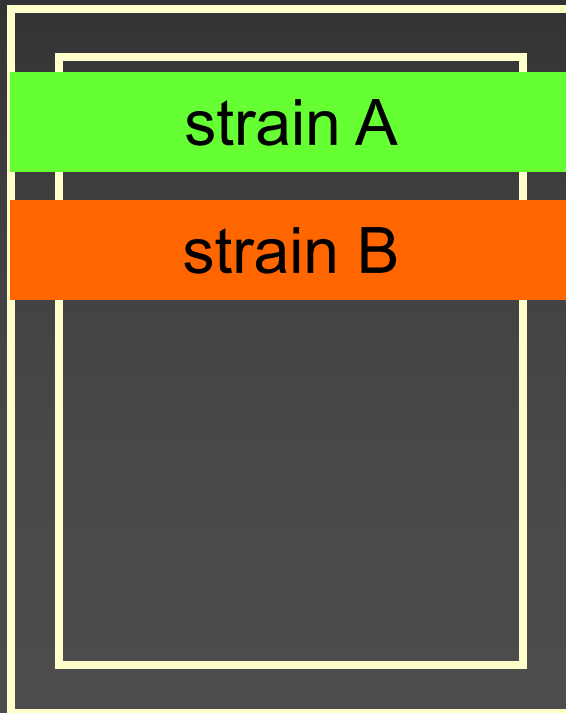
# Another anaerobic jar

*Fusobacterium* sp.



# 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**

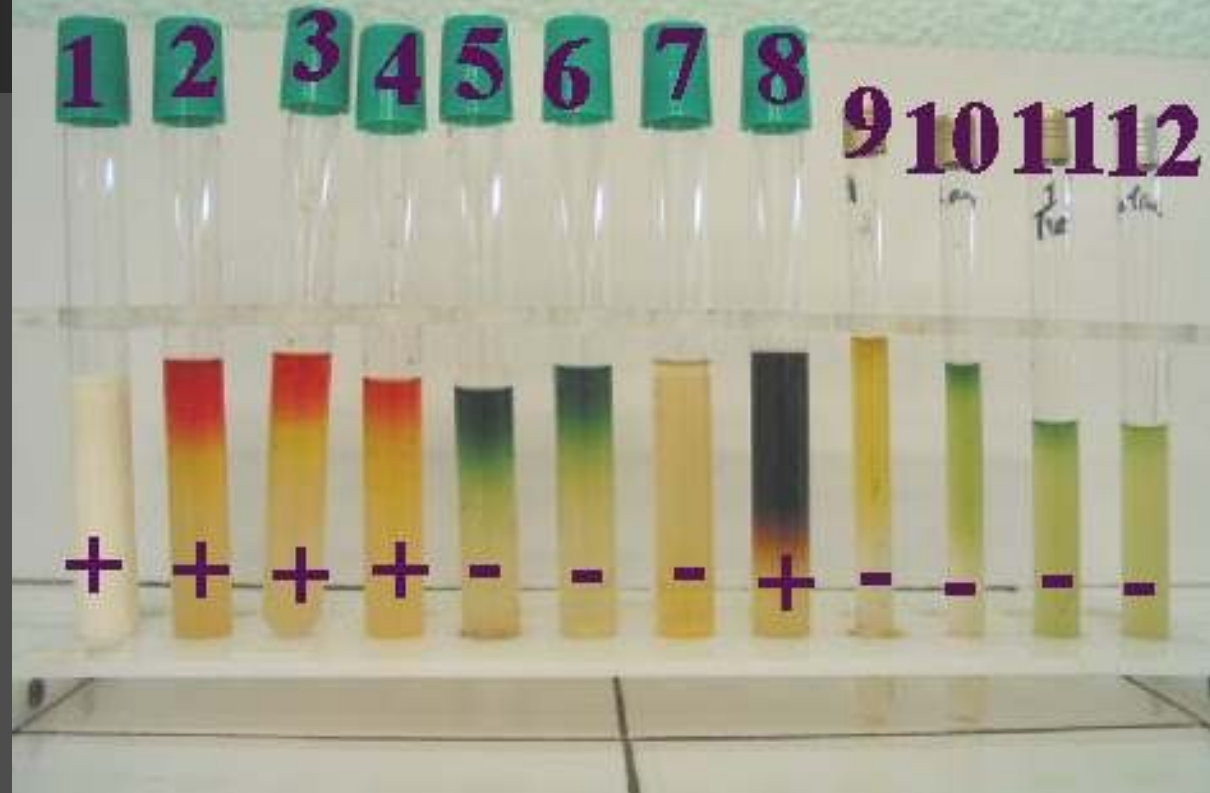
**ATTENTION** – the codebook is divided into several parts **according to morphology of anaerobic bacteria**. It is necessary to search in the proper part of the codebook

# Anaerotest – example of reading

NEC = Negative control

<b>+</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>-</b>	<b>-</b>	<b>-</b>	<b>+</b>
<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>	<b>1</b>		
<b>-</b>	<b>+</b>	<b>+</b>	<b>-</b>	<b>+</b>	<b>-</b>	<b>-</b>	<b>-</b>
<b>2</b>	<b>2</b>	<b>2</b>	<b>2</b>	<b>2</b>	<b>2</b>		
<b>+</b>	<b>-</b>	<b>+</b>	<b>+</b>	<b>+</b>	<b>-</b>	<b>+</b>	<b>NEC</b>
<b>4</b>	<b>4</b>	<b>4</b>	<b>4</b>	<b>4</b>	<b>4</b>		
<b>5</b>	<b>2</b>	<b>7</b>	<b>5</b>	<b>6</b>	<b>0</b>		

# Other sets for diagnostics of anaerobes



[www.microbes-edu.org](http://www.microbes-edu.org)



# Antibiotic susceptibility tests

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- 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 is usually classic penicillin. But *Bacteroides* genus is resistant (unlike genera *Prevotella* and *Porphyromonas*, formerly also part of old genus *Bacteroides*, that are susceptible)
-

# Susceptibility zones – a set that can be used for anaerobes

Antibiotic	Abbrev.	Reference zone
Penicilin (basic penicilin)	P	20 mm
Amoxicilin + klavulanate (protected aminopenicilin)	AMC	20 mm
Chloramfenikol	C	21 mm
Klindamycin (lincosamid)	DA	21 mm
Imipenem (karbapenem)	IPM	16 mm
Metronidazol (imidazol)	MTZ	16 mm



# Illustration photo



*B. fragilis* ATCC 25285

# How to get out facultative anaerobes

- Many anaerobes are resistant to vancomycin and/or amikacin. (*In the same time they are susceptible to „not so strong“ antibiotics, as penicillin or metronidazol; see the picture*)



# 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



# 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.

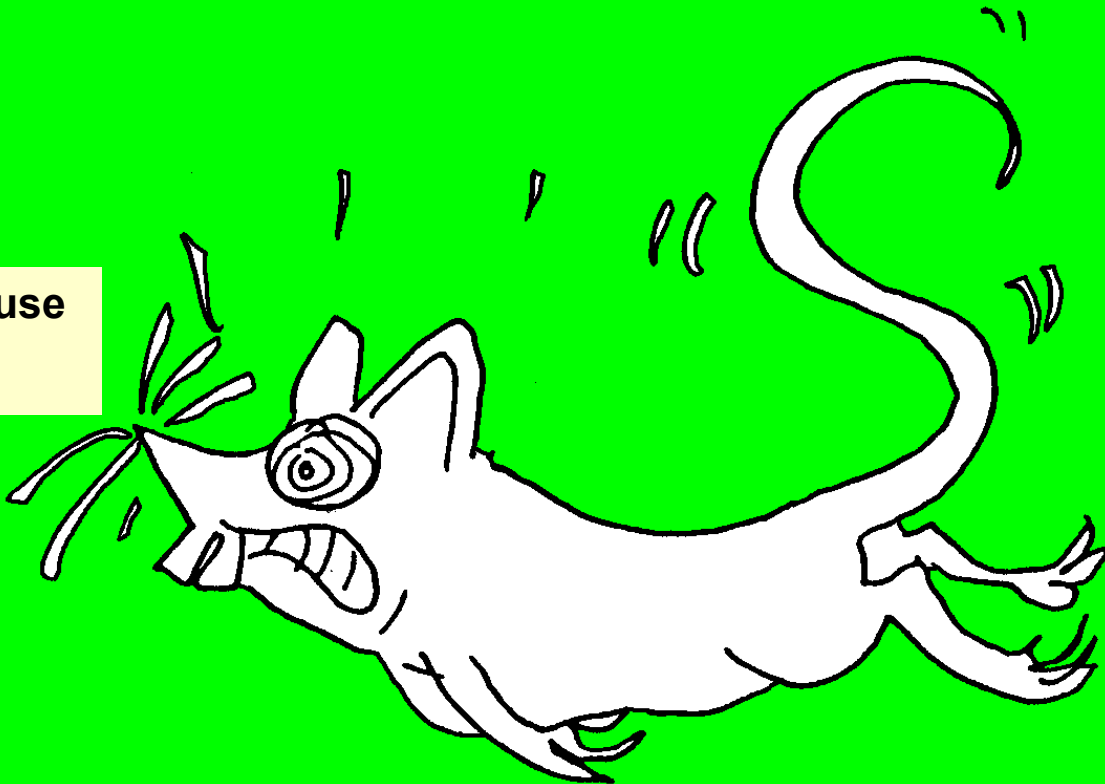


Tetanic mouse

# Toxin detection using animal experiment

- Look at the picture of tetanic mouse

Tetanic mouse



Drawing by Petr Ondrovčik  
(1959–2007)

Graphically adapted.

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

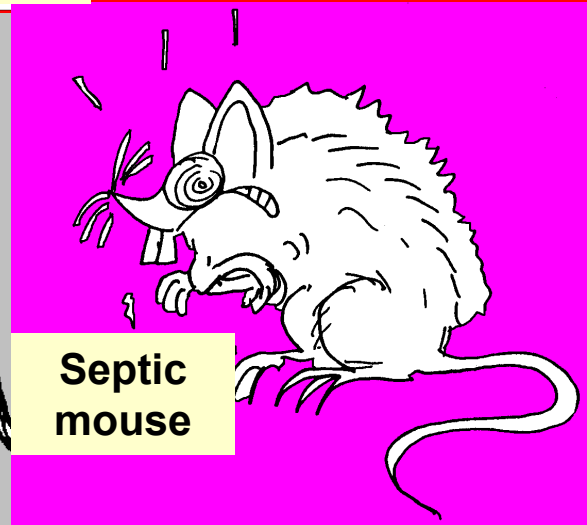
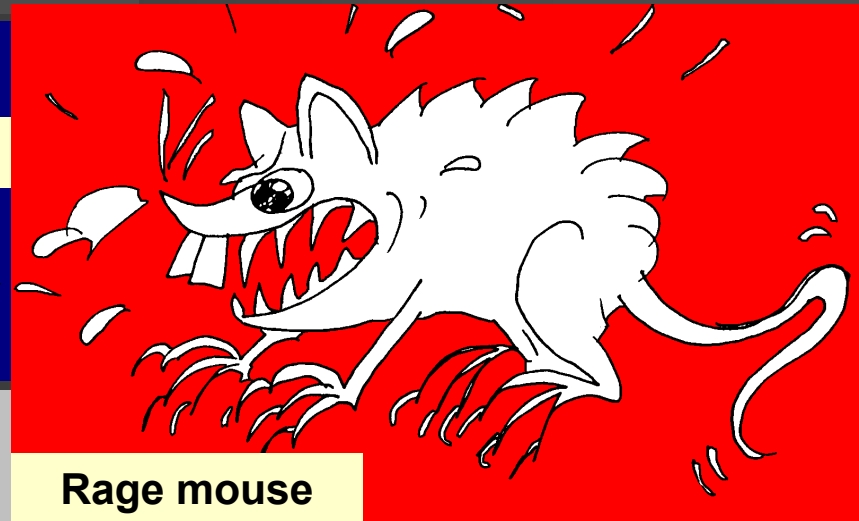
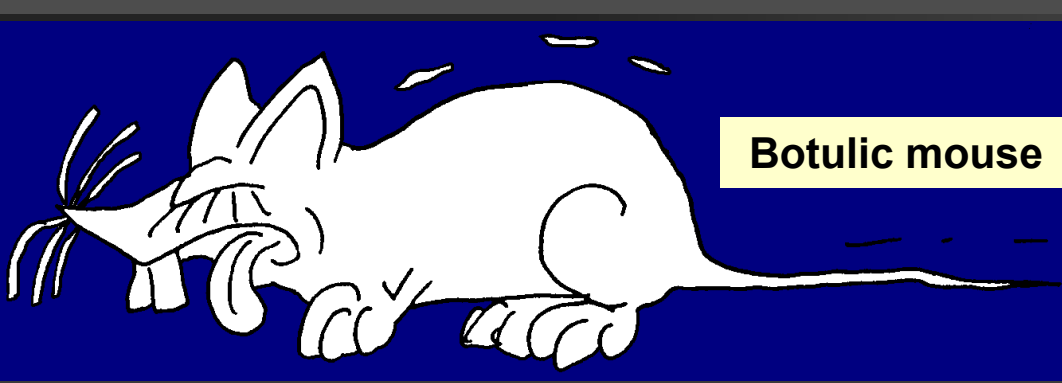
[www.biotox.cz](http://www.biotox.cz)



Opisthotonus is typical both for mice and humans

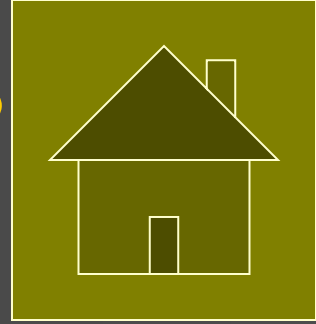
# Appearance of an experimental animal is observed also in other situations, e. g. botulism.

- In botulism, we can see pareses, not spasms





# 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**, microaerophilic bacteria commonly found in vaginal swabs (and rather rarely present in normal aerobic culture)
- Our imperfect anaerobiosis enables growth of microaerophilic bacteria, as you can see.

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# Pictures of anaerobic bacteria

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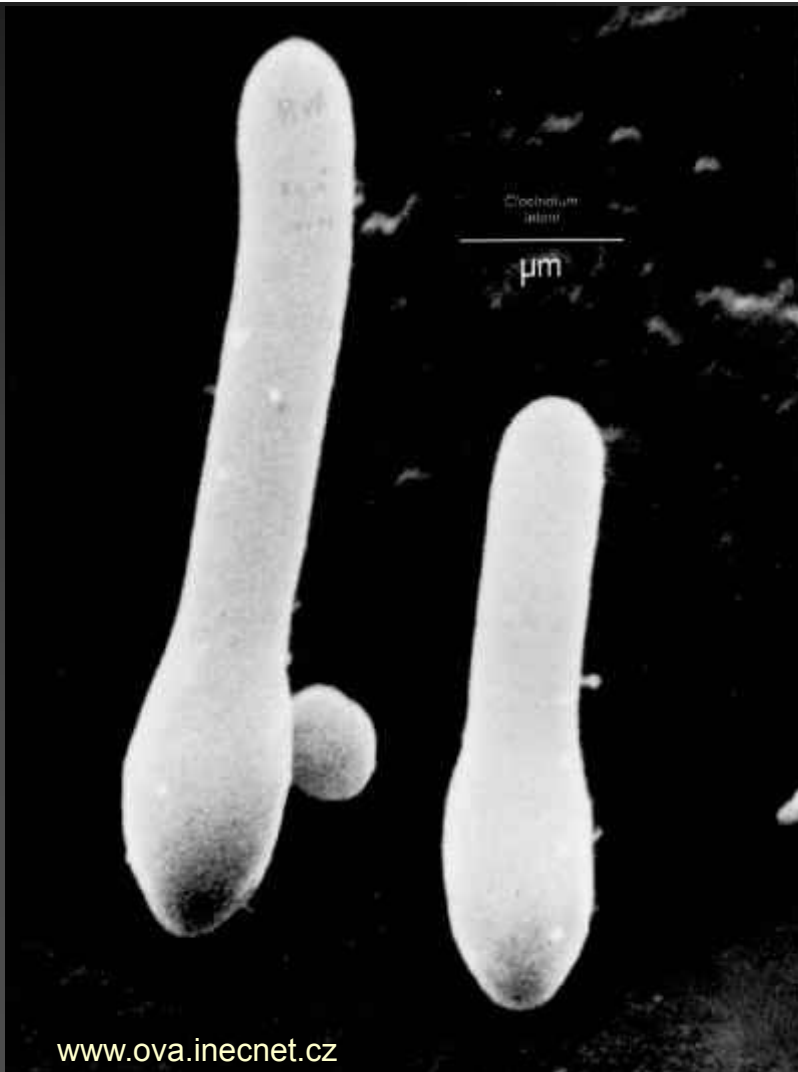
# Criminal survey: *Clostridium tetani*

<http://www.geocities.com>



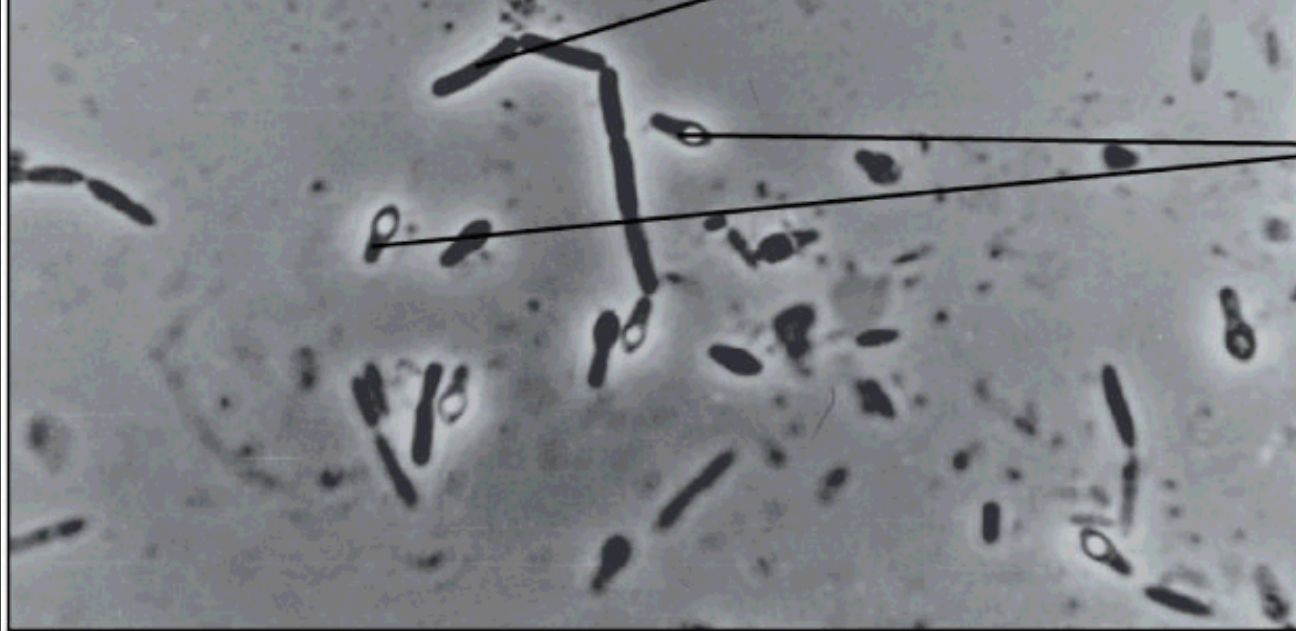
# *Clostridium tetani*

www.zuova.cz



# *C. botulinum*

www2.bc.cc.ca.us



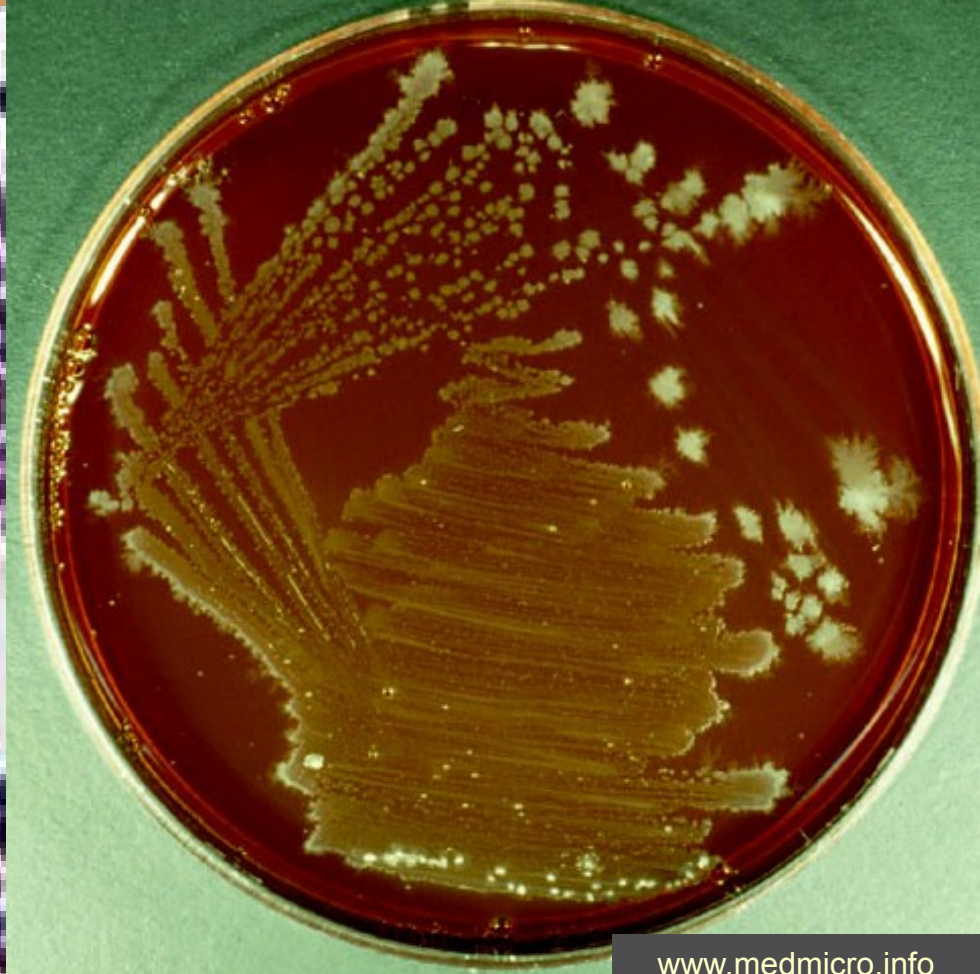
Vegetative cells

Endospore-bearing cells

10  $\mu$ m

# *Clostridium perfringens*

<http://www.geocities.com>





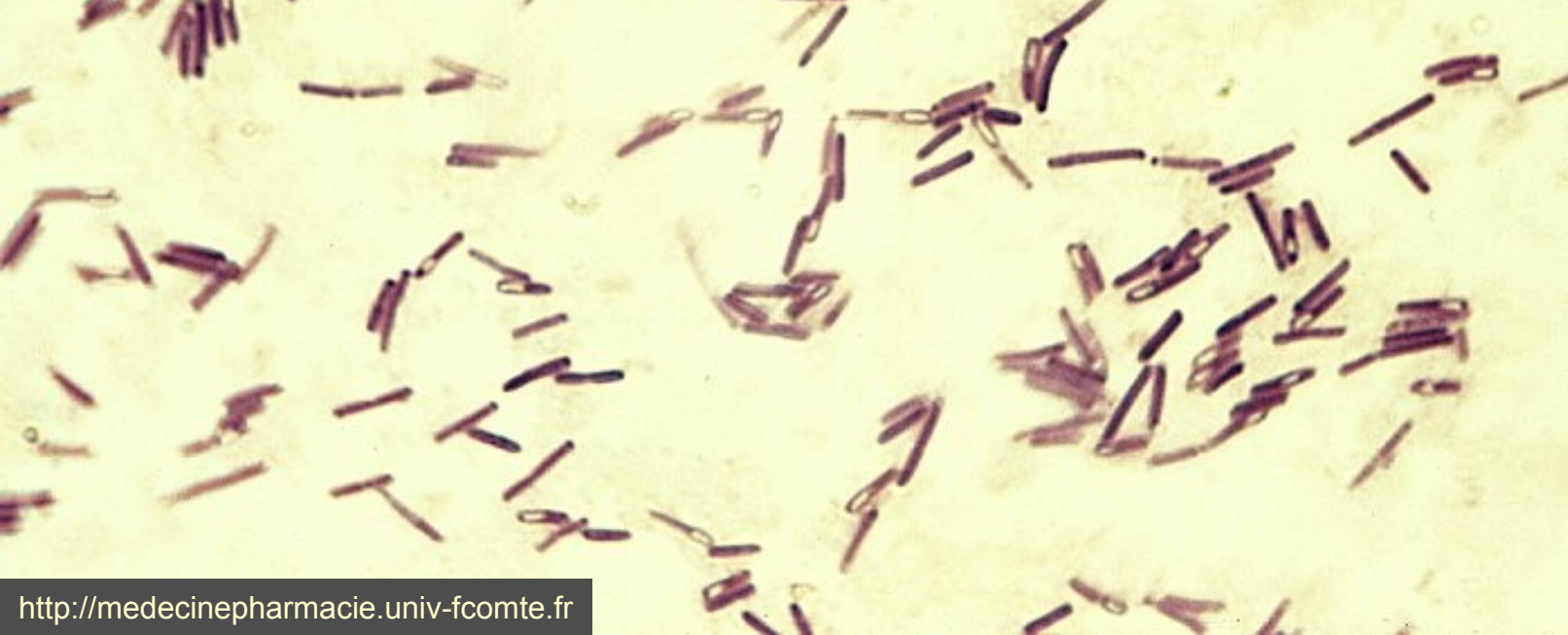
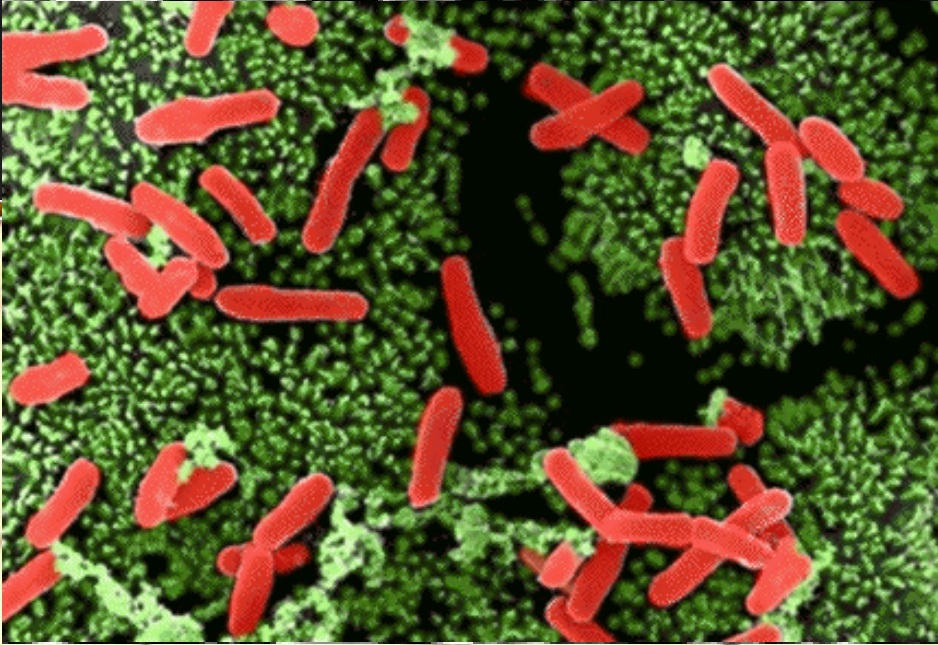
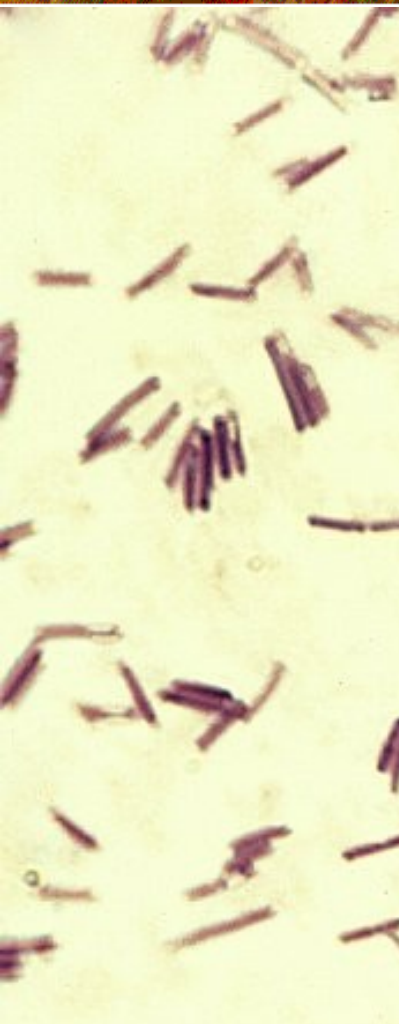
A microscopic image showing a central cluster of spores, characteristic of Clostridium septicum. The spores are arranged in a roughly circular pattern, with some individual spores visible at the periphery. The background is a dark, reddish-brown color, likely due to the staining used in the preparation.

*Clostridium  
septicum*

# *Clostridium difficile*

sitemaker.umich.edu

<http://www.health.qld.gov.au>



<http://medecinepharmacie.univ-fcomte.fr>

# *Clostridium difficile*





*Clostridium  
scindens*



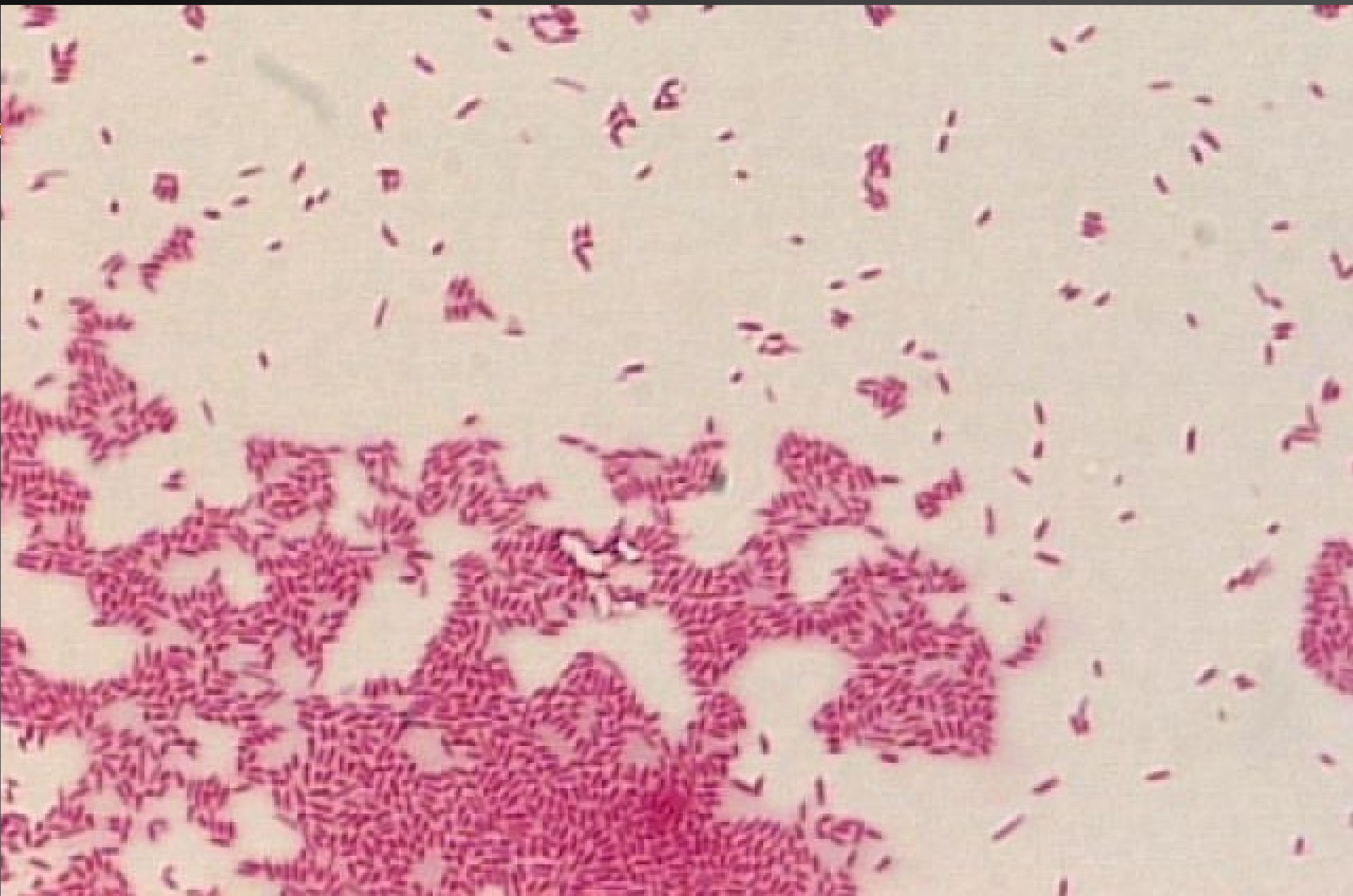
# *Clostridium innocuum*

<http://pharmacie.univ-lille2.fr>



# *Bacterioides fragilis*

<http://www.geocities.com>  
Bacterioides fragilis





# *Bacteroides* sp.

www.medmicro.info, photo O. Z.

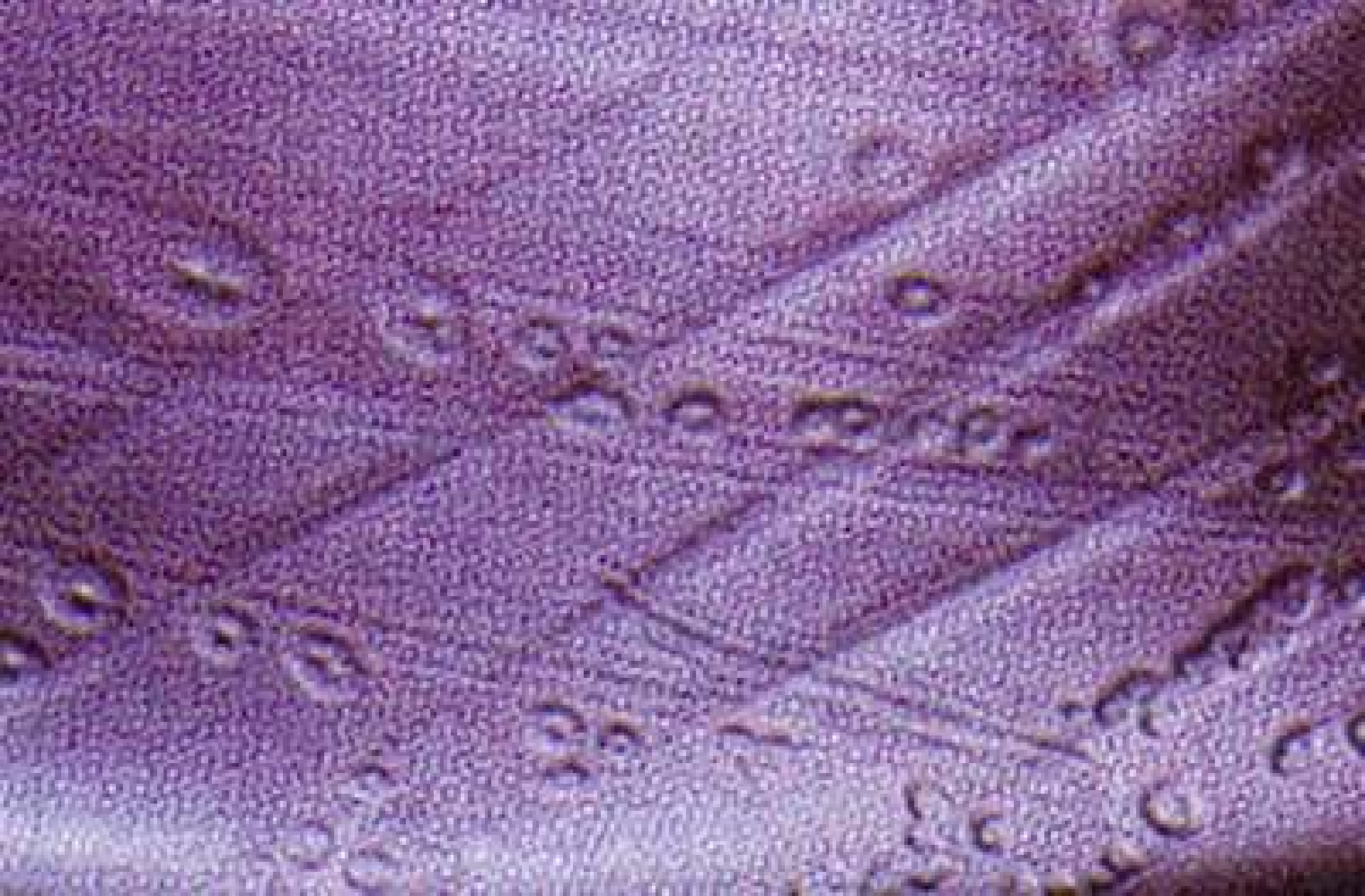
Sooner these objects were usually called „Sphaerophorus necrophorus“  
= „globe and death bearing  
bacterium“



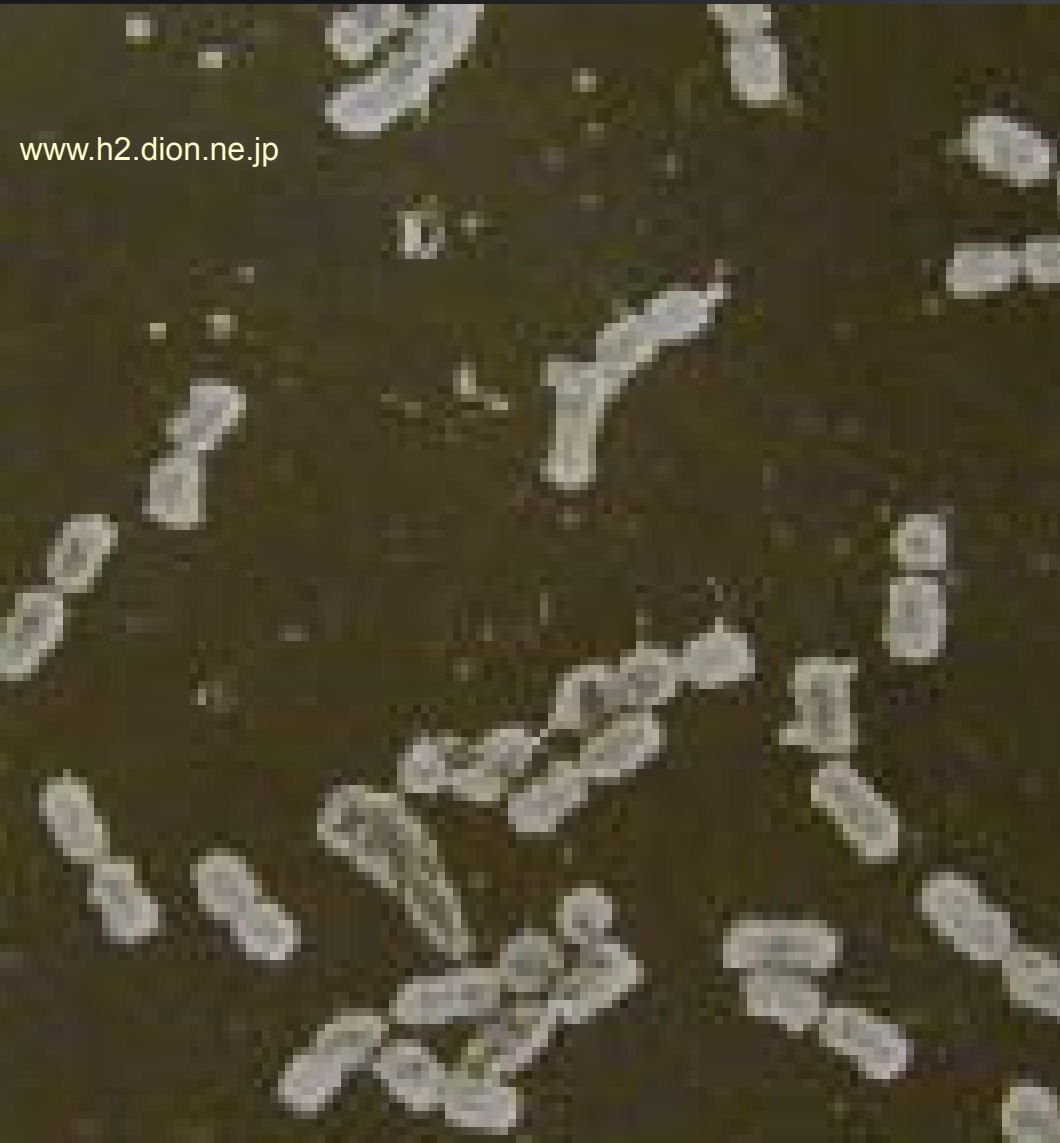


# *Bacterioides ureolyticus*

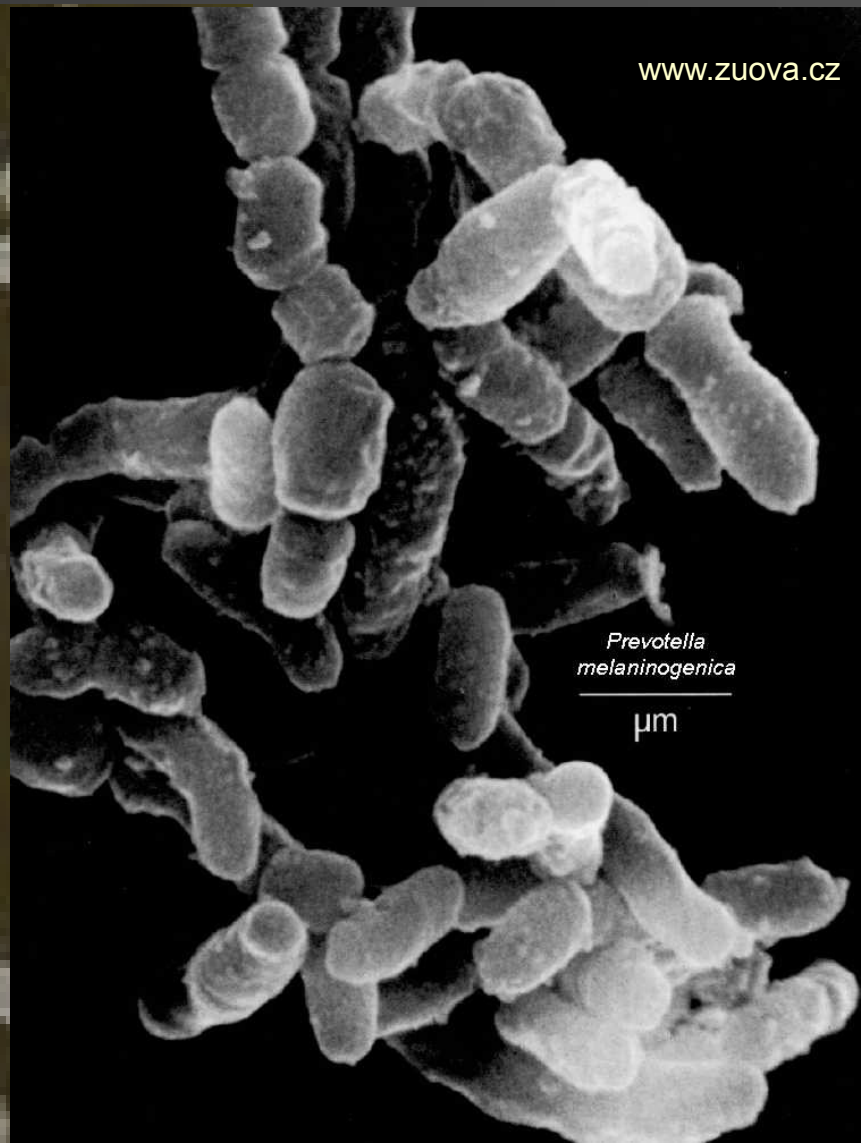
<http://pharmacie.univ-lille2.fr>



# *Porphyromonas gingivalis*



# *Prevotella melaninogenica*



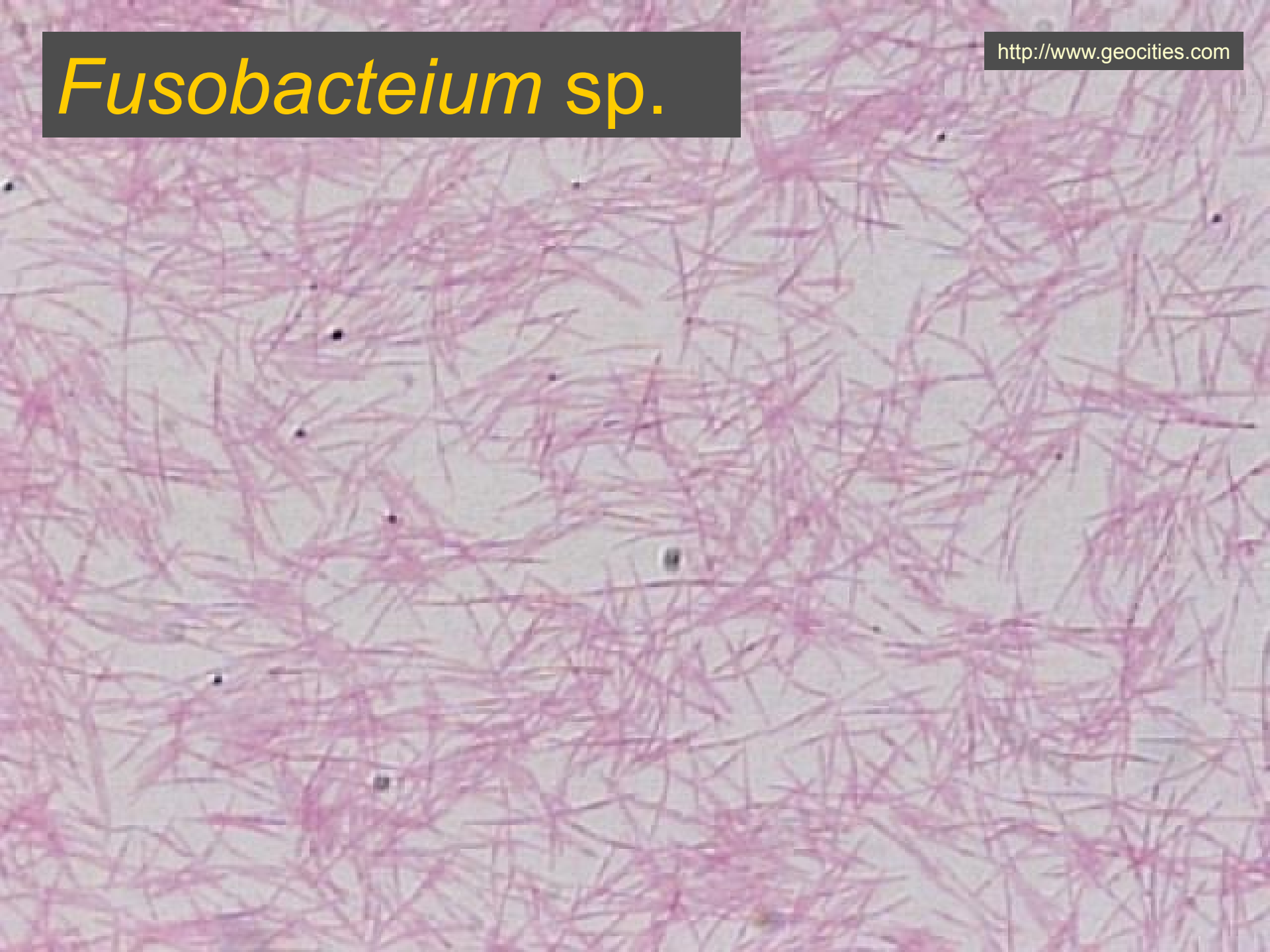
# *Prevotella melaninogenica* (black pigmentation)

<http://pharmacie.univ-lille2.fr>



# *Fusobacterium* sp.

<http://www.geocities.com>





# *Fusobacterium nucleatum*

<http://www.geocities.com>





A petri dish containing a red agar medium. The surface of the agar is covered with numerous small, dark, pinpoint-sized spots, which are characteristic of Leptotrichia buccalis. The spots are distributed across the entire surface, with some areas appearing more densely populated than others. The petri dish is placed on a light-colored surface.

*Leptotrichia  
buccalis*

<http://pharmacie.univ-lille2.fr>

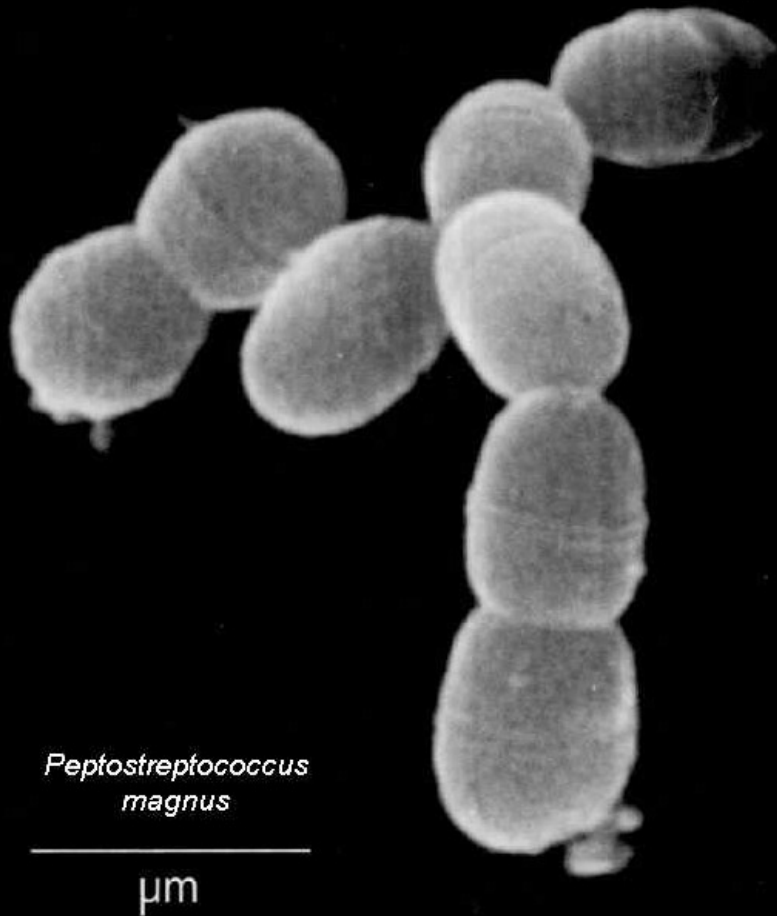
*Peptostreptococcus* sp.





# *Peptostreptococcus magnus*

www.zuova.cz

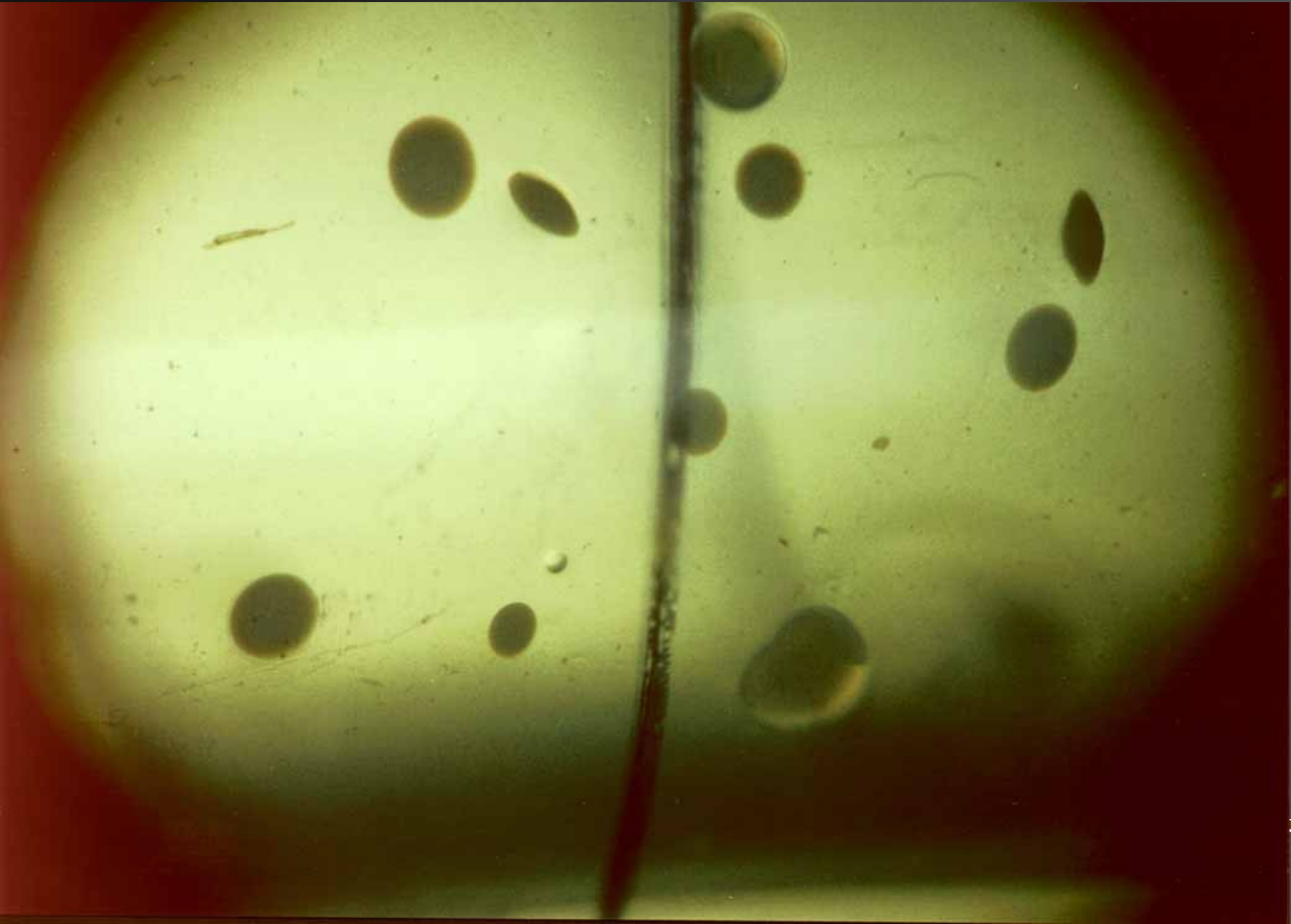


www.szu.cz

# *Peptostreptococcus anaerobius*

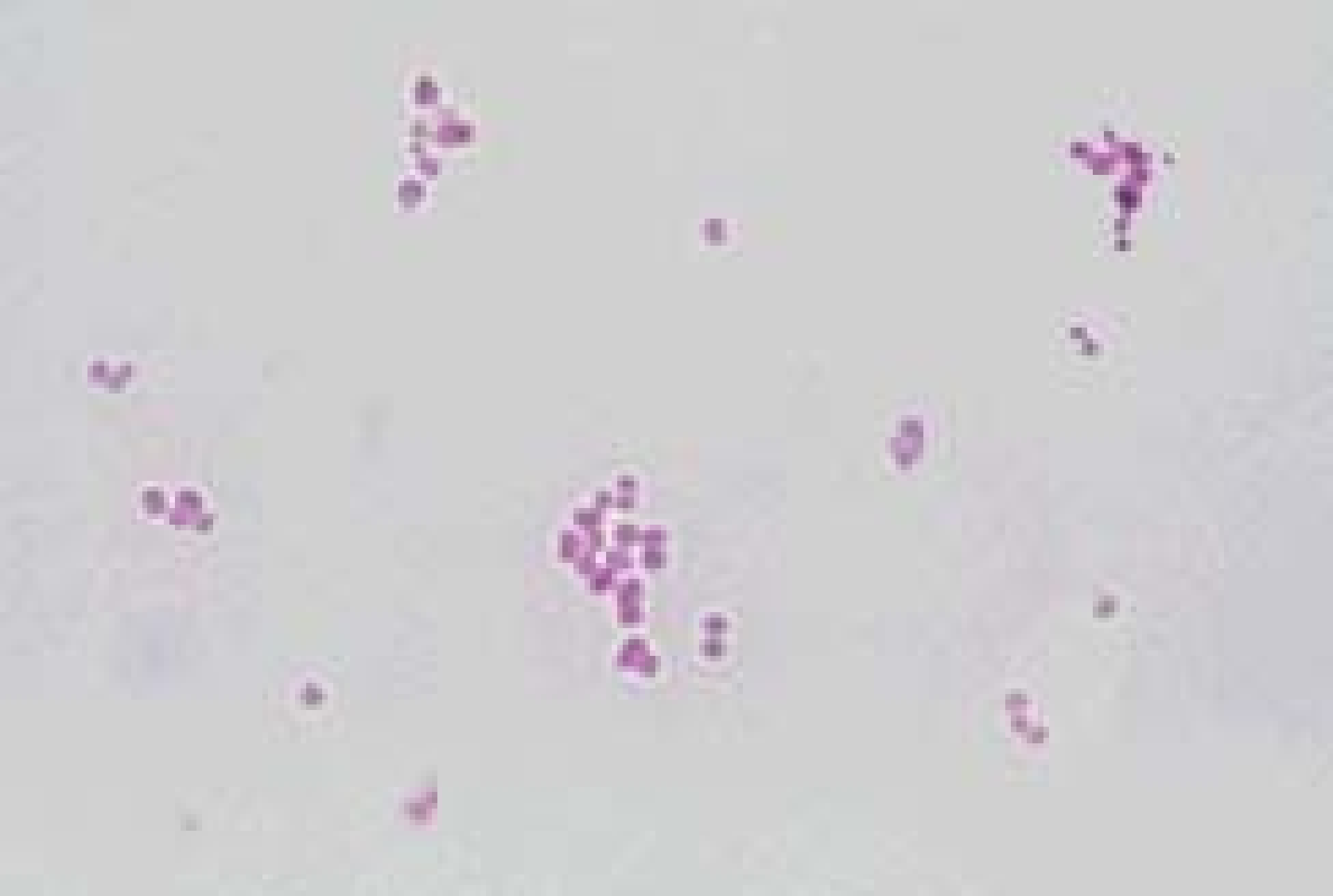
# *Peptococcus constellatus*

[www.cebtechservices.com](http://www.cebtechservices.com)



# *Veillonella* sp.

<http://www.primer.ru>





The End

