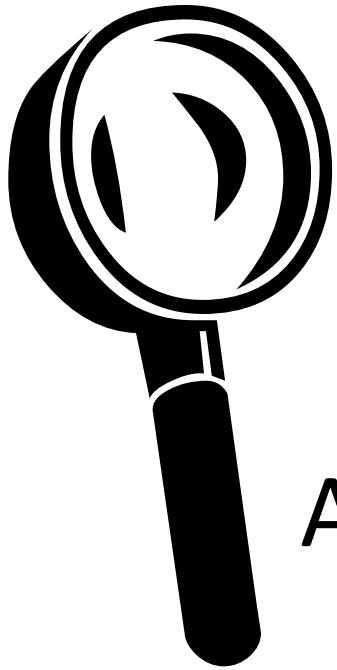


Institute for microbiology presents

# TRACING THE CRIMINAL

Part eight:  
Acidoresistant (acid-fast) criminals



# Intro: Spitoons in fight with TB

*In Czechoslovakia between World War I and World War II the a society was formed with personal engagement of **president Masaryk** and his „**League against TB**“. It showed a big effort in fight against this disease. The part of this was education for people not to spit to the floor, but to use spittoons.*



# Survey of individual parts

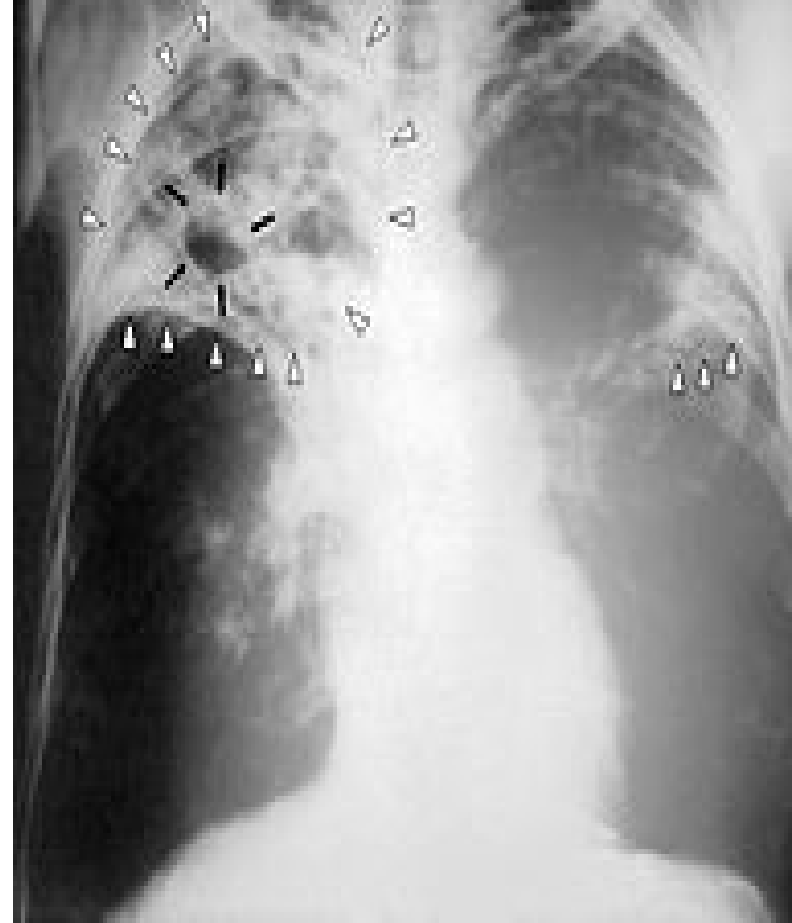
Clinical characteristics of acid-fast bacteria

Special properties of acid fast-bacteria

Diagnostics of acid-fast bacteria

# Clinical characteristics of acid-fast bacteria

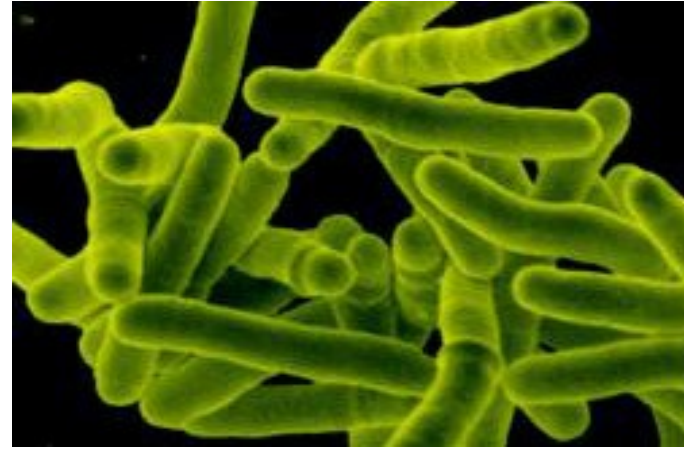
- **Johny** did know allready for many years that he is **HIV positive**. He knew pretty well that he is more vulnerable than other people and that each infection can get him more quickly than other people.
- Nevertheless he was surprised that **he started to cough recently**. His doctors tried various variants, but after roentgen, PCR examination and culture examination came to conclusion that it is a **miliar (granular) form of tuberculosis**.



<http://cs.wikipedia.org/wiki/Tuberkul%C3%B3za>

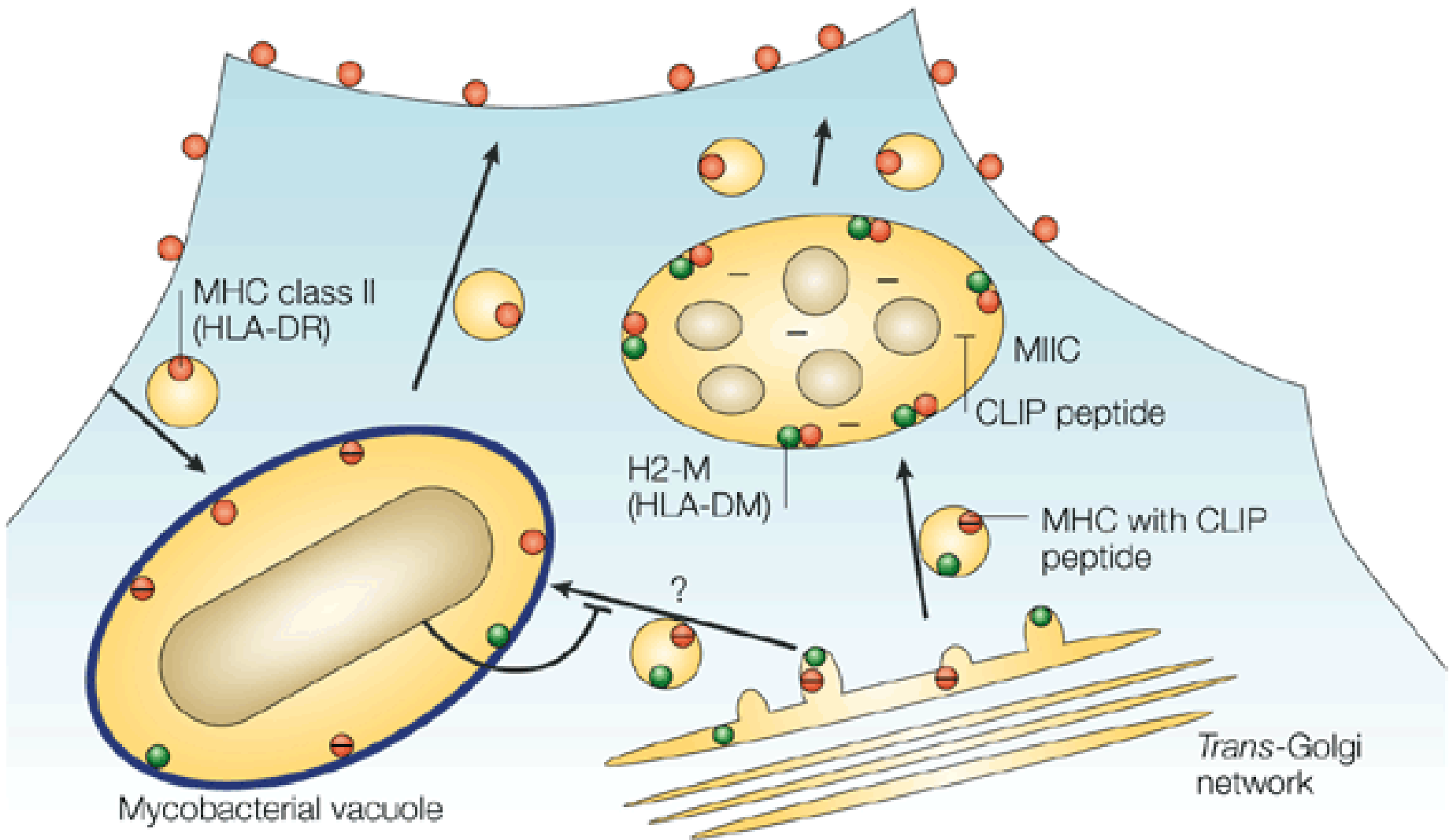
## Story One

# The criminal was...



- *Mycobacterium tuberculosis*, although TB may be caused by *Mycobacterium bovis*, too.
- Interesting for this microbe: it lives **inside cells**. This is also related with the fact that **antibody response is weak in tuberculosis** (so neither antigen nor antibodies are detected) and **cell immunity** is very important (in vaccination, too).
- As in HIV infection just cell immunity is damaged, TB is one of **oportune infections**.

# Mycobacterium inside a cell

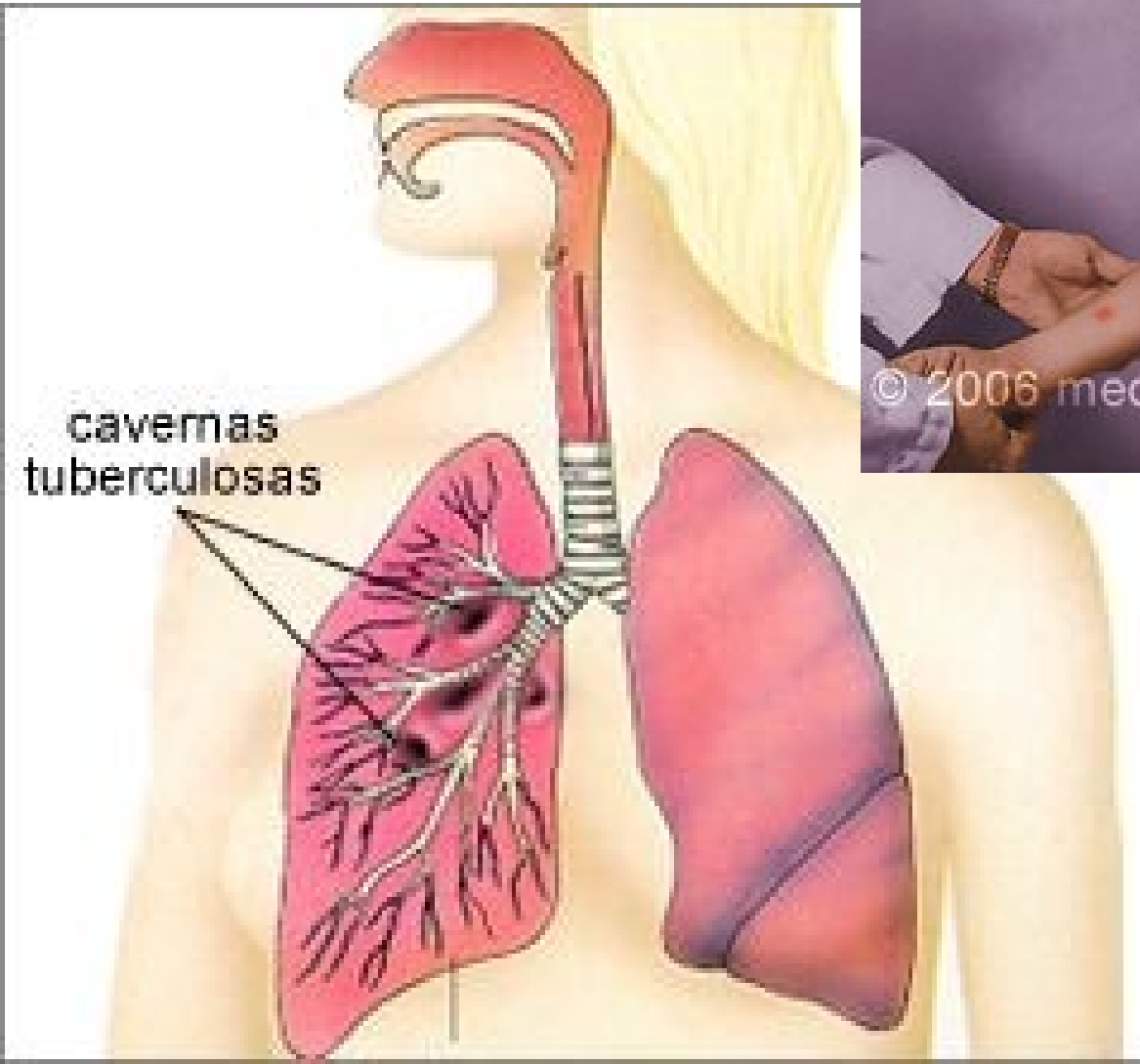


# Tuberculosis

- At the first contact with the infection is formed **primary complex**. It is a focus (usually localized in lungs) and corresponding regional lymphonode.
- During the next infection **post-primary TB** is formed. It is worse. Usually a granulomatous formation is formed, later it subdues caseification („becoming cheese-like“) and then it is not enlarged anymore. Paradoxically, majority of damage in the organism is caused by the host organism reaction (late hypersensitivity – in the matter of fact, a specific type of an allergy)
- After years the original focus may **re-activate**, mostly in old age, at immunodeficiency, or ethanol abuse. Such person may be very dangerous for his/her environment.



# Tuberculosis

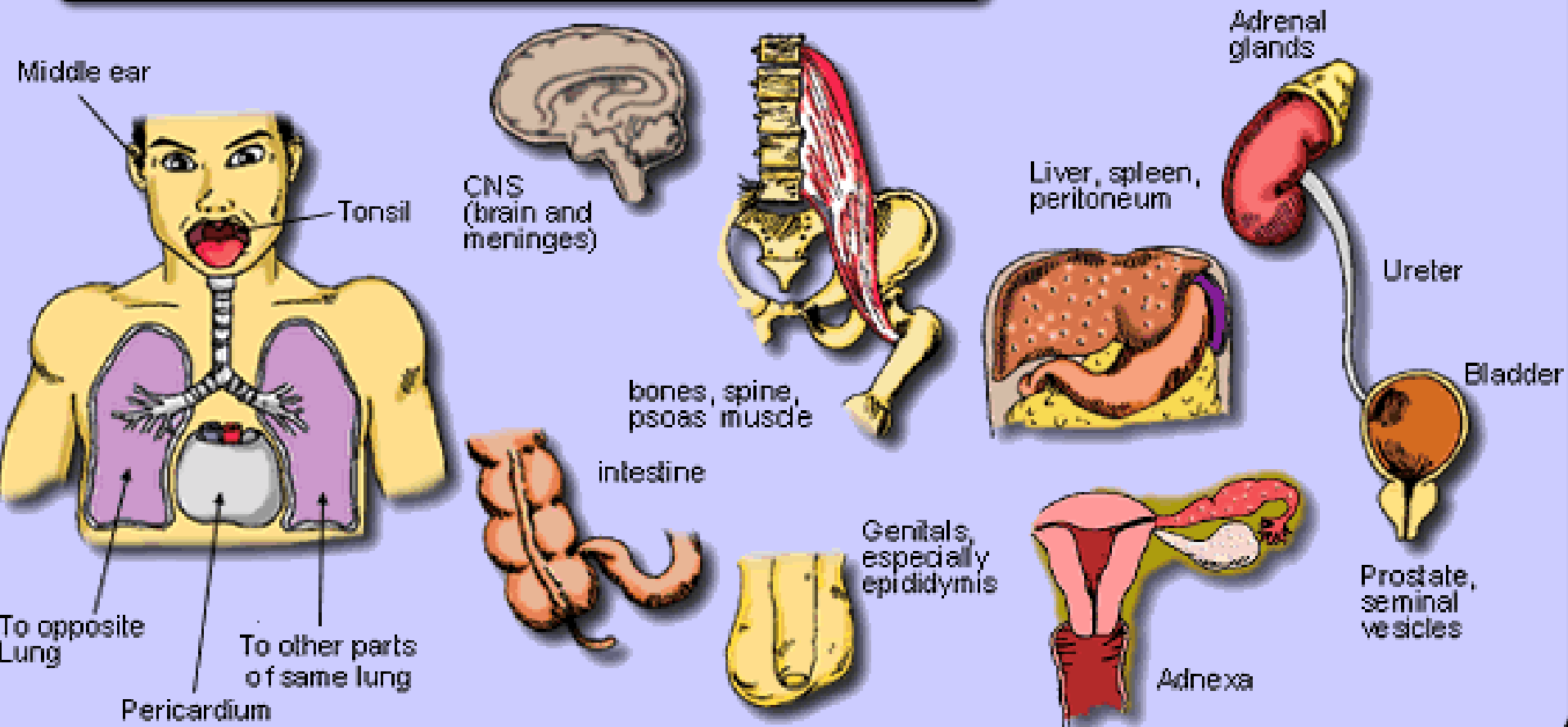


<http://www.stockmedicalart.com>

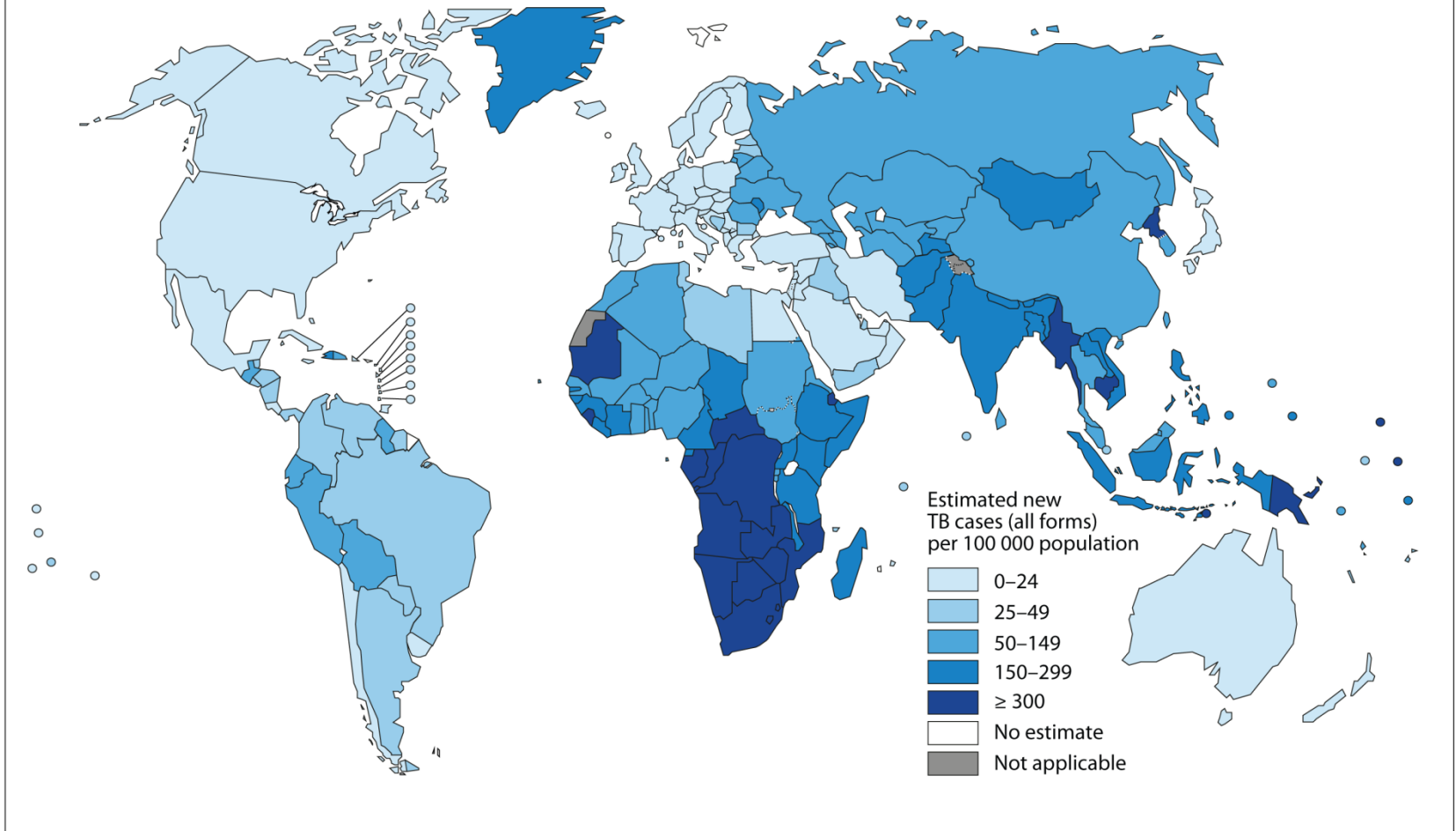
# Pulmonary form of TB is not the only one

sitemaker.umich.edu (2x)

## Tuberculosis Affects Many Parts of the Body



## Estimated tuberculosis (TB) incidence rates, 2011



The boundaries and names shown and the designations used on this map do not imply the expression of any opinion whatsoever on the part of the World Health Organization concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries.

**Dotted and dashed lines on maps represent approximate border lines for which there may not yet be full agreement.**

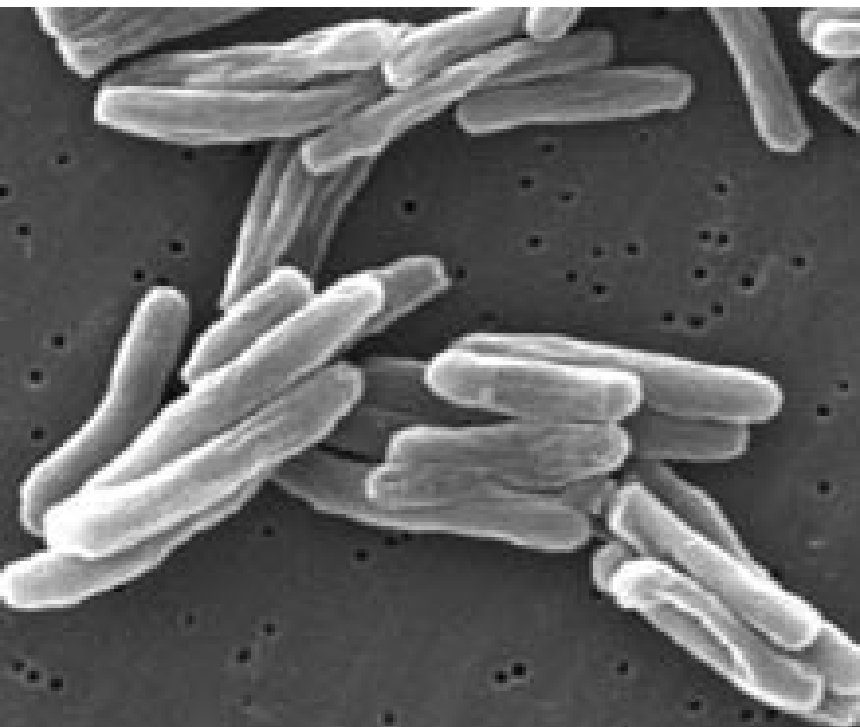
Source: *Global Tuberculosis Report 2012*. WHO, 2012.



# Once more TB

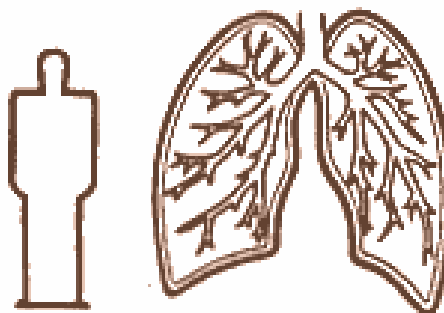
<http://www.lung.ca>

<http://www.cbc.ca>

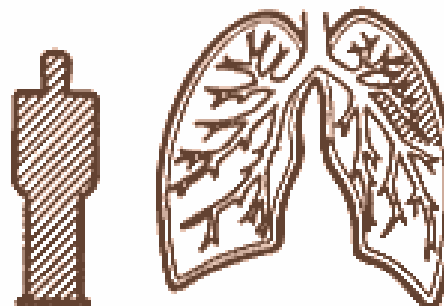


Det WD Exp |-----| 2.0µm  
SE 7.4 0 |hc

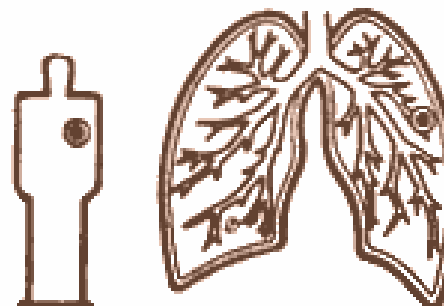
## Tuberculosis Develops by Stages



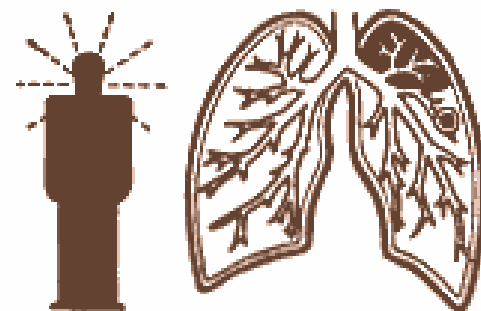
Every person is born with healthy lungs



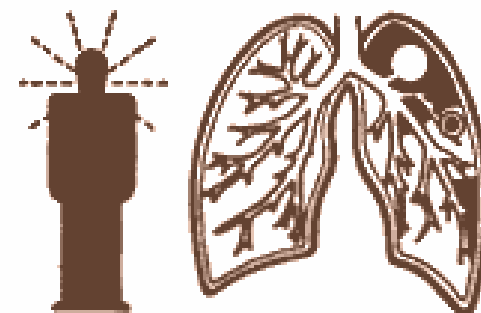
Many of us become infected from other people as we grow up.



The first infection usually heals. The person is well and no germs escape from his lungs. It is important to find this first infection in children.



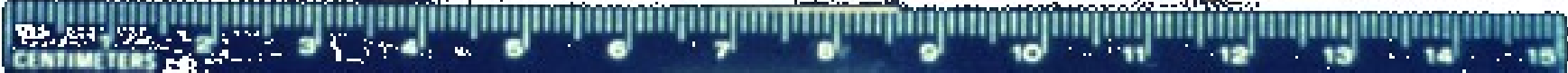
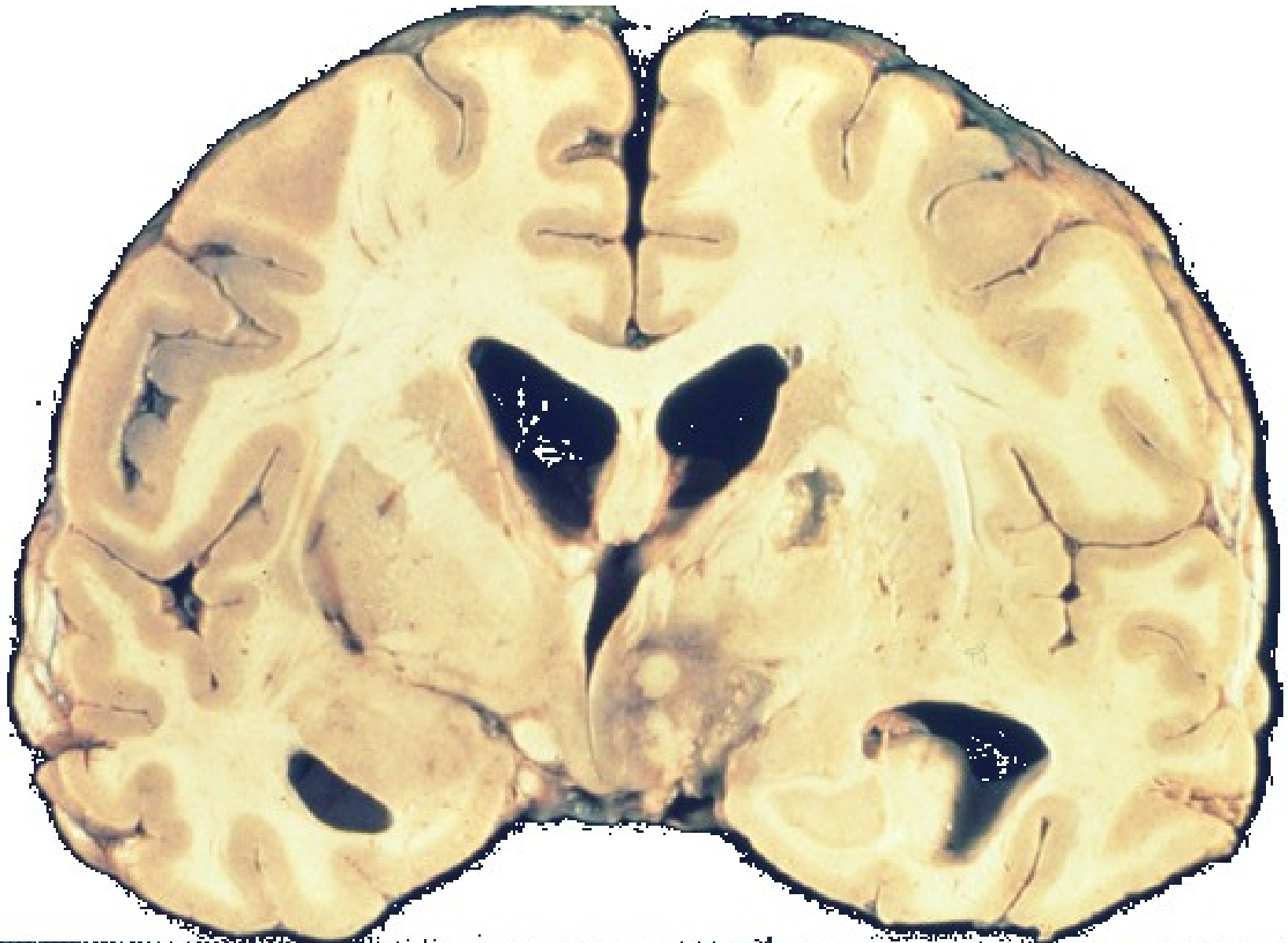
If later a person becomes infected again, the serious disease tuberculosis may begin. The germs that escape from the lungs are likely to infect others.



If tuberculosis is not discovered early, the disease spreads. A hole (cavity) may form in the lung which is an incubator of germs that escape and menace everybody who comes in contact with the sick person.

# Brain tuberculoma

<http://pathology.mc.duke.edu>

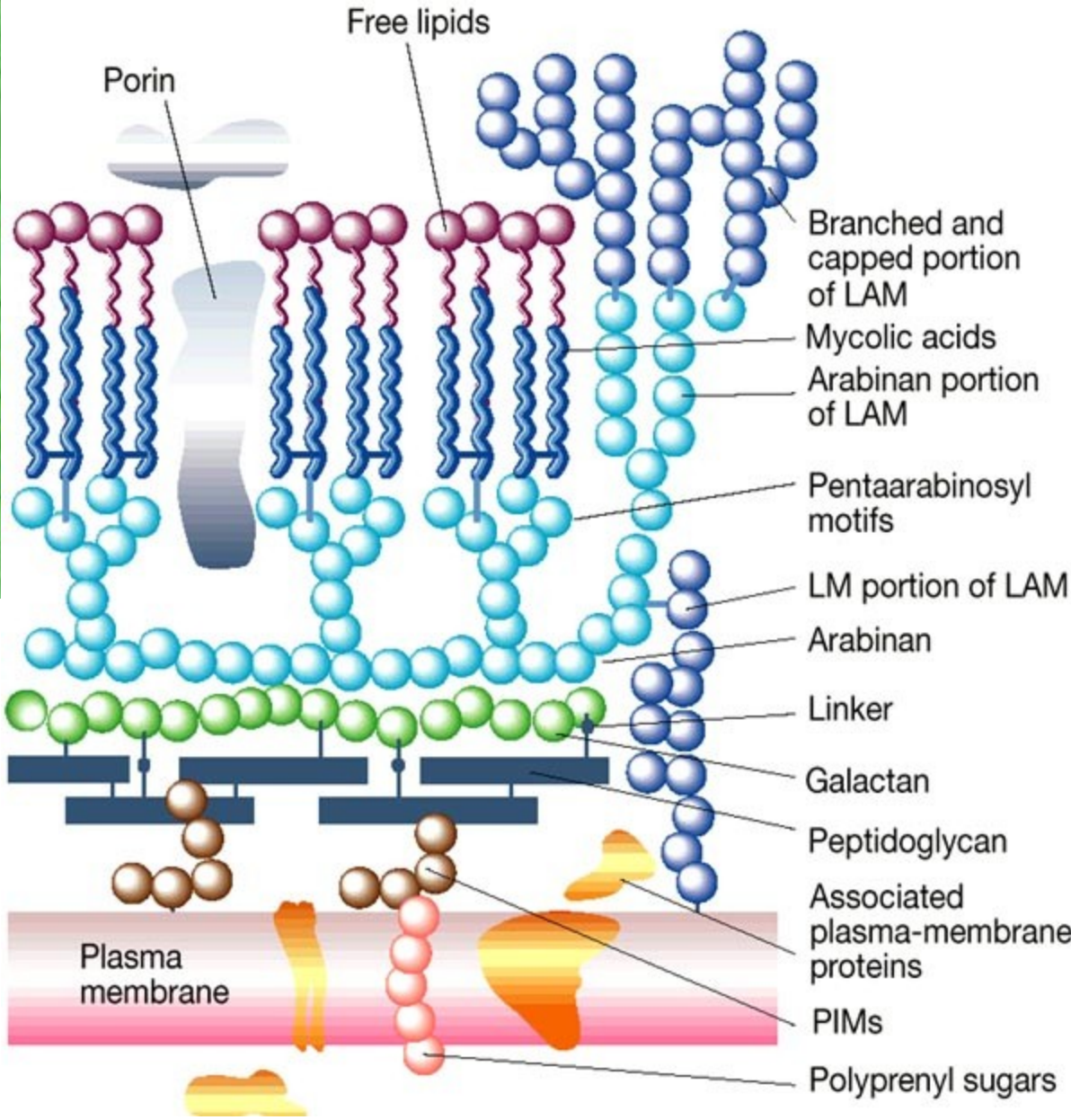


# More special things in TB

- Their cell wall is highly hydrophobic, it contains, mycolic acids. They nearly do not Gram stain, special staining methods are needed.
- It grows slowly, its generation period is long, so special media are needed
- They are very resistant to disinfectants. It is impossible to use „A“ class disinfectants against common bacteria, you need „T“ (against TB), eventually „M“ (against atypical mycobacteria)
- They are also resistant to antimicrobial drugs.



www.primer.ru



<http://oregonstate.edu>

# Cell wall of mycobacteria

# Story two

- Mr H. lived in **desert part of Sudan**, where wars and unrests were very common.
- Recently even friends that still did neither run away nor die started to dislike contacts with mr. H. **Mutilation of face** of mr. H. was a clear mark, that mr. H. suffers the disease still **too common in this part of world**.
- Good luck that H. met members of a **non-governement organisation**, that where sure about diagnose. Using **dapson** it was possible to help Mr. H.



# Causative agent is

- *Mycobacterium leprae*, a microbe even stranger than TB mycobacterium.
- Its generation period is much longer than in TB mycobacteria. In vitro culture was successful only recently and it lasted the whole year.
- Basic treatment of leprosy is not expensive, nevertheless in countries where leprosy is endemic it is still too much.
- Therefore leprosy still requires help of foundations, non-government and charitable groups.

# Leprosy



<http://blanicti-zoldneri.tym.cz/clanky/lepra.html>

- Long incubation period – 2, 3, 7 ... yrs

- **Tuberculoid leprosy**

- Depigmented spots on skin, swollen peripheral nerves
- Active cellular immunity
- Spontaneous consolidation



<http://abicko.avcr.cz/archiv/2002/10/obsah/9-gallery.html>

- **Lepromatous leprosy**

- Insufficient cellular immunity
- Confluent and swollen skin lesions, eyebrows fall out, nose is in depression, fingers are lost, people become blind

# Leprosy

[www2.bc.cc.ca.us](http://www2.bc.cc.ca.us) (2x)

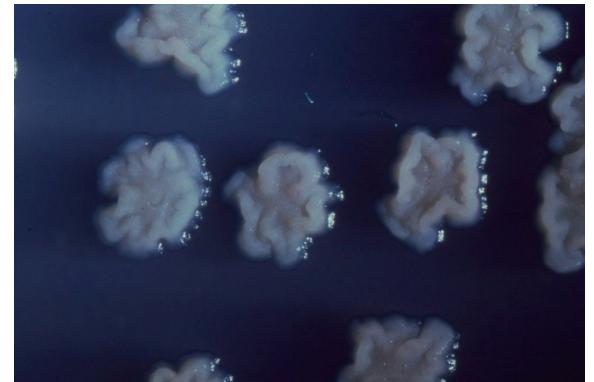


# Story three

- Mr. P. was a furious aquarist. Last month he had a problem: he had to use his left hand only to do anything inside the fish tank, as he had an ulcer on his right hand.
- After examination, his case was closed as so named **fish tank granuloma**, common in aquarists. A similar disease in swimmers is called **swimming pool granuloma**.
- Causative agents are...

# Atypical mycobacteria

- Besides *M. tuberculosis* and *M. leprae* there exist also plenty of **other mycobacteria**. Some of them, e. g. *Mycobacterium marinum*, are so named **atypical mycobacteria**, sometimes causing wound infections and other problems.
- **Some mycobacteria are non-pathogenous** and they are normal part of human microflora, e. g. *M. smegmatis*.



# Infection of *M. marinum*



# Story four

- Mrs. F. found several months ago some **small formations in her neck**.
- Recently one of them **opened to skin** and dense, yellow, unpleasantly smelling pus appeared in the orifice. So, Mrs. F. visited her doctor.
- The doctor sent the **pus for microbiological examination**. Examination durated **very long time** – mrs. F. was already angry that microbiologists are lazy and don't want release the results. Finally the criminal was found...

# ...it was actinomycosis

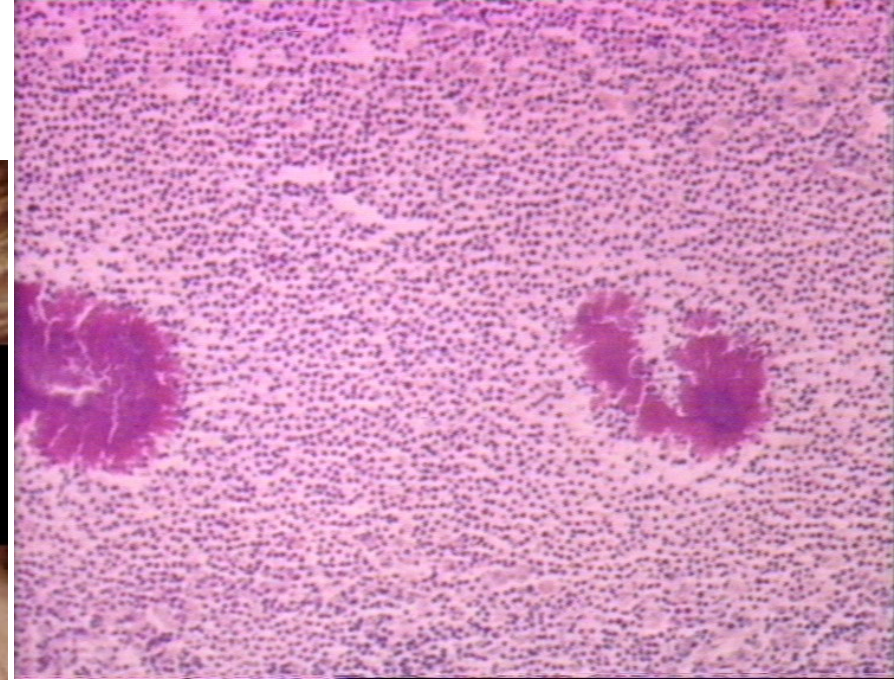


<http://en.wikipedia.org>

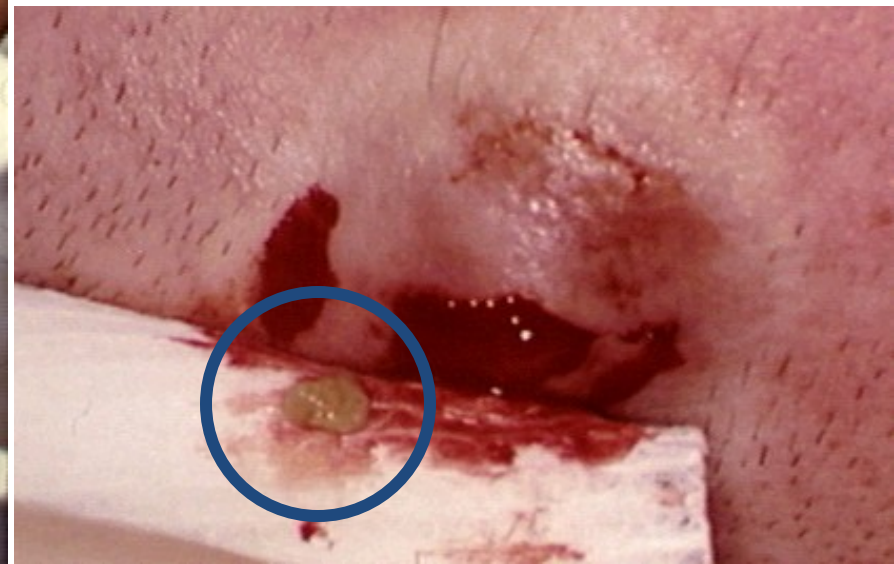
- So the criminal was *Actinomyces* sp.
- Actinomycetes are filamentous bacteria, in fact Gram-positive, but they do not Gram stain very well, because their cell wall is hydrophobic and contains many mycolic acids.
- Actinomycetes are commonly found in oral cavity of healthy persons. From here they might commonly get to soft tissues of neck, face or thorax. They are anaerobic bacteria (or at least they grow the best at anaerobic conditions).
- Similar to actinomycetes are nocardiae, but they are strictly aerobic. Otherwise they are similar in many ways.



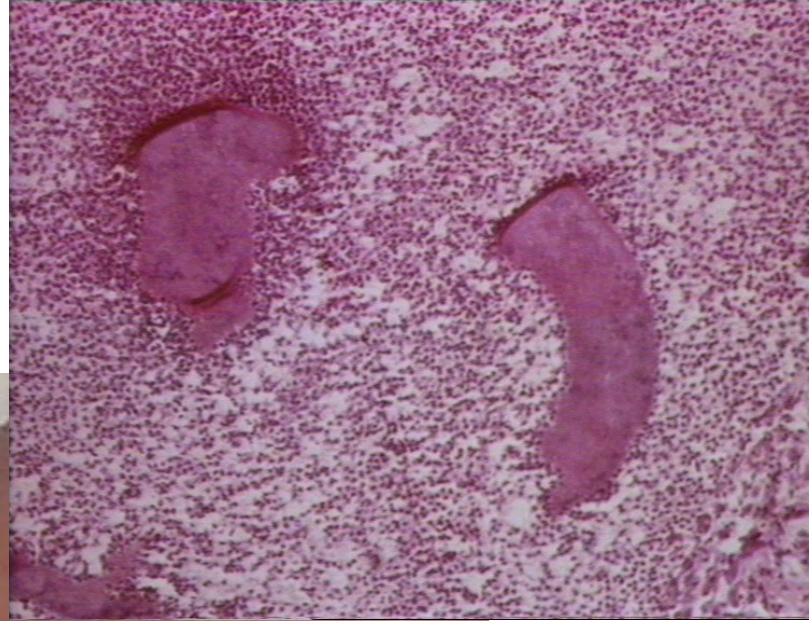
# Actinomycrosis



pathmicro.med.sc.edu (3x)



# Nocardiosis

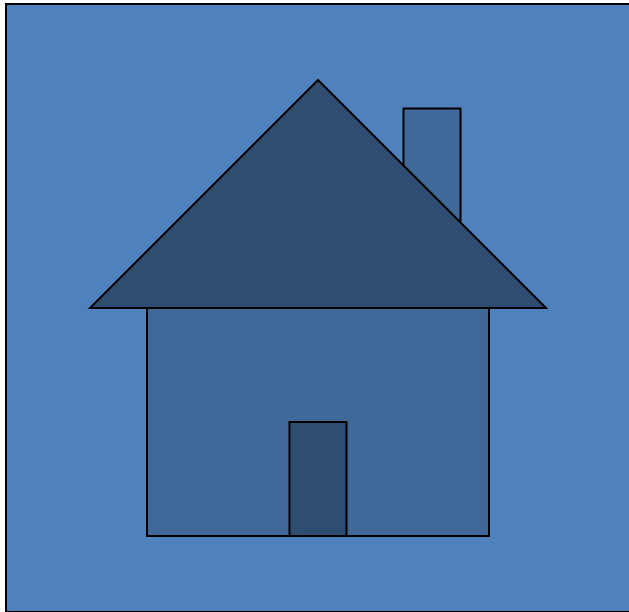


# Nocardia pneumonia with septicaemia



A previously well 57-year-old man ... 3-day history of severe dyspnoea. We conclude that *N. asteroides* infection can present as a fulminant community-acquired pneumonia with bacteraemia in the absence of immunosuppression or coexistent infection. (From the article related to the picture)

# Brain nocardiose on CT

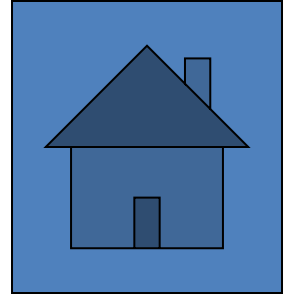


# Special properties of acid-fast bacteria

# Acidoresistance + alkaliresistance

- Acids and alkali act only to hydrophilic components, communication to water environment. In mycobacteria this is not fulfilled.
- So acids and alkali have weak effect only to them.
- Acids are also not able to decolorise them, when somehow it was possible to stain them.
- Majority of dyes is hydrophilic, too, and so mycobacteria stain poorly, usually it is necessary to stain them at hot temperature, to stain them at all.
- Nocardia and Actinomycetes, unlike mycobacteria, are only partially acidoresistant. So, we stain them by Gram, but we have to know, that they stain poorly and inconstantly.

# Consequences for clinical doctors



- Clinical doctor, sending sample (sputum, urine, pus or anything) „for bacteriological culture“, cannot hope in getting reference of eventual TB infection.
- To get info about TB, it is necessary to send sample separately and to mark it so that it should be examined for TB (TB-culture or TB-PCR). If so, the laboratory can perform the needed procedures.

# Diagnositics of acid- fast bacteria



# How to search for criminals

- **Microscopy:** We use Ziehl-Neelsen stain and fluorescence stain.
- **Culture:** We use special media, and before the culture the specimen should be **treated by a hydroxide**. The aim is to **kill other bacteria**, that would be more successful as they grow more quickly. Alkaliresistant mycobacteria survive that easily.
- **Automatic culture:** Various types of culture automats are used: they are able to detect culture positivity much sooner than classic culture.
- **Biochemical differentiation** is possible in specialized laboratories.
- **Animal experiment:** guinea pig is used sometimes.
- **PCR diagnostics** is more and more important.

# *Mycobacterium tuberculosis*

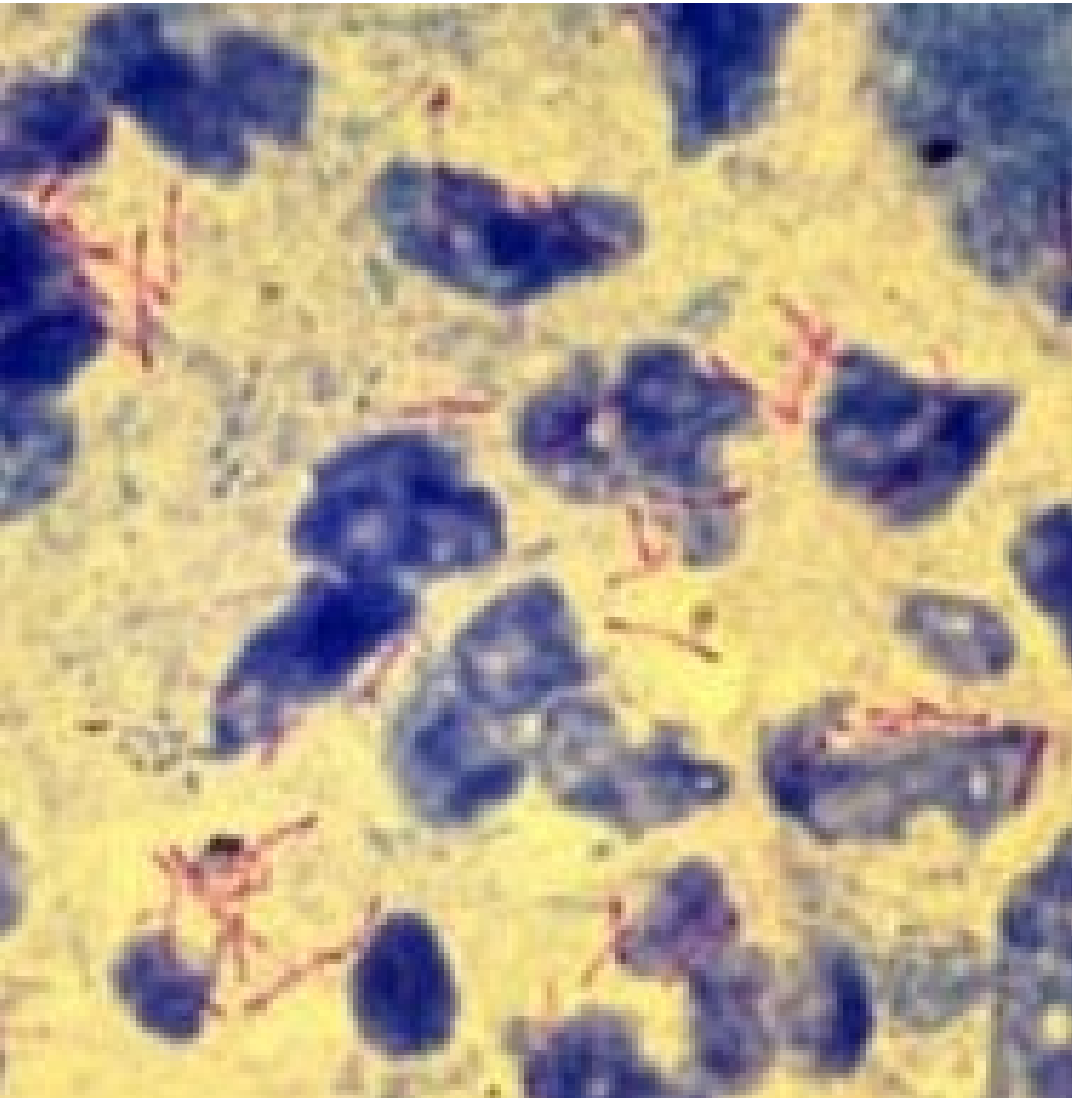
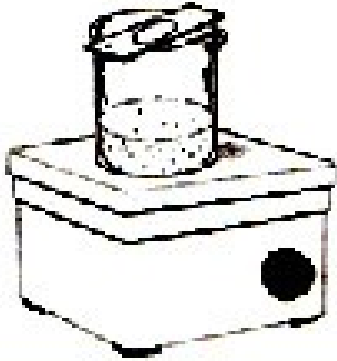


Photo O. Z.

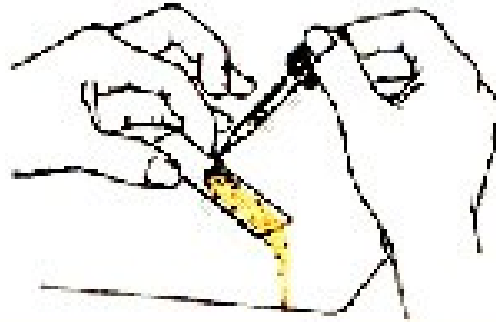
# Ziehl-Neelsen staining

- **In step 1** we stain by **carbolfuchsin** (Gabbet) in hot until steam rises. Without heating mycobacteria could not be stained, except use of more concentrated carbolfuchsin.
- **In step 2** we decolorize (cca 15 s) by „acid alcohol“, what is mixture of alcohol with a mineral acid, most commonly HCl. After that we rinse the slide with water.
- **In step 3** we counterstain the background, so everything decolorized in Step 2. We counterstain by **methylene blue**. cca 30 s (it would be also possible to use **malachit green**) and we rinse the slide with water, we dry it and we observe it with immersion objective.
- **Result: red acidoresistant rods on blue or green background.**

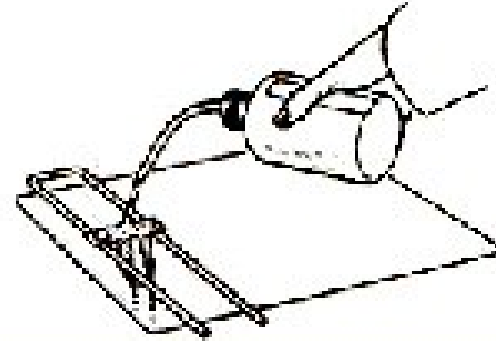
# Ziehl-Neelsen stain



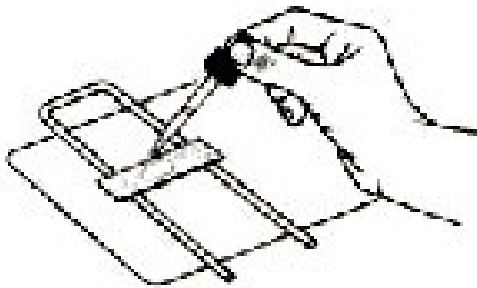
**1** Cover smear with carbolfuchsin. Steam over boiling water for 8 minutes. Add additional stain if stain boils off.



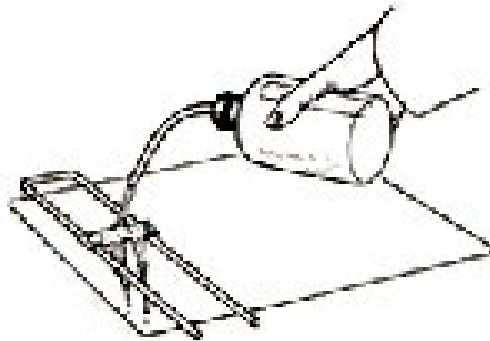
**2** After slide has cooled decolorize with acid-alcohol for 15 to 20 seconds.



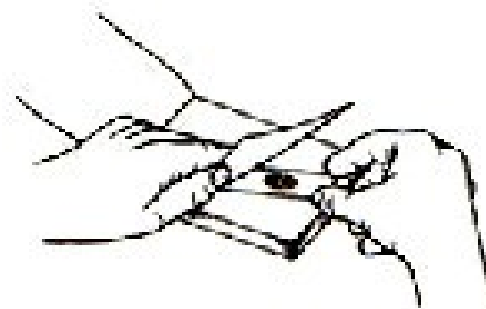
**3** Stop decolorization action of acid-rinsing briefly with water.



**4** Counterstain with methylene blue for 30 seconds.



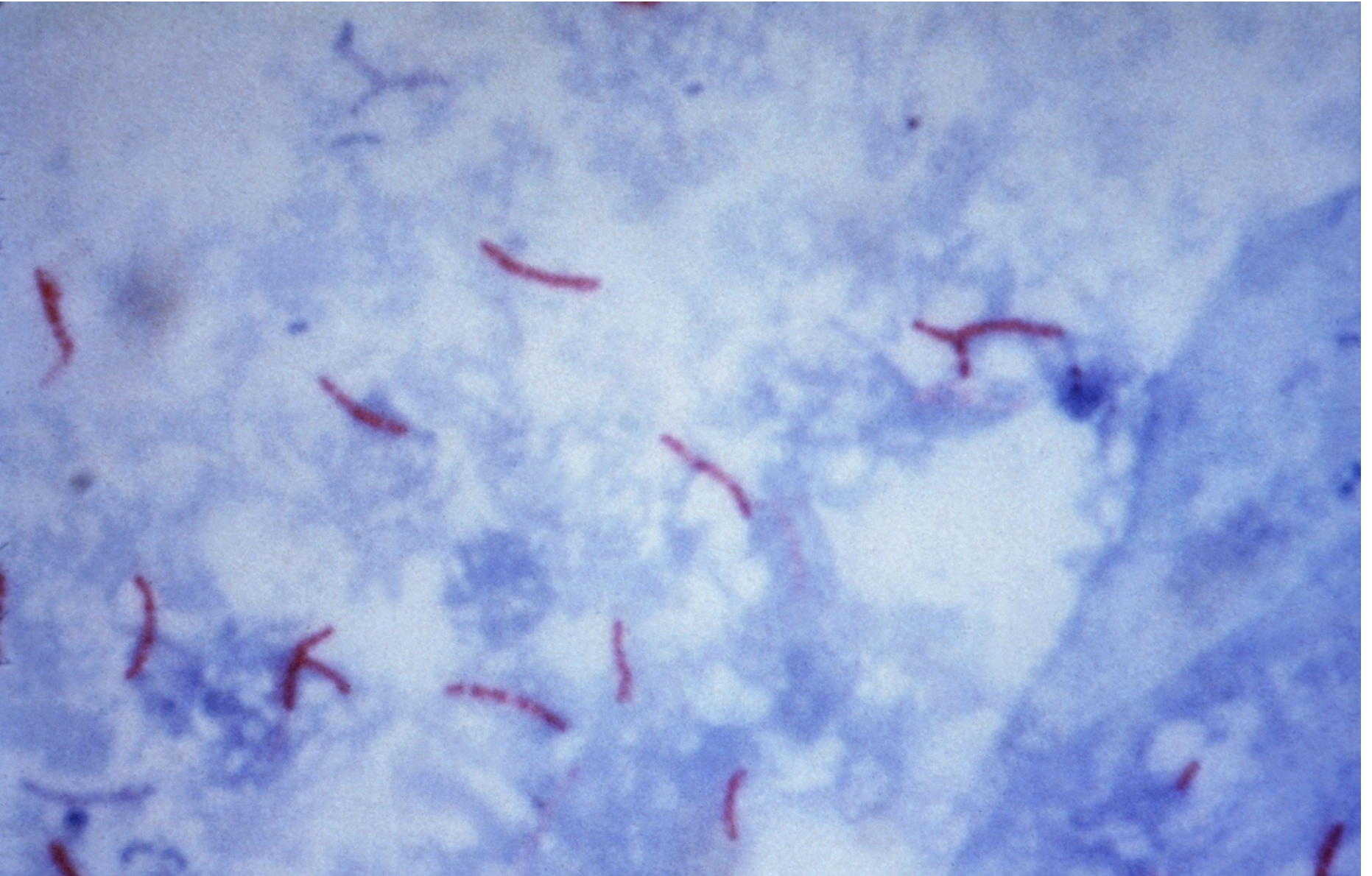
**5** Rinse briefly with water to remove excess methylene blue.



**6** Blot dry with bibulous paper. Examine directly under oil immersion.

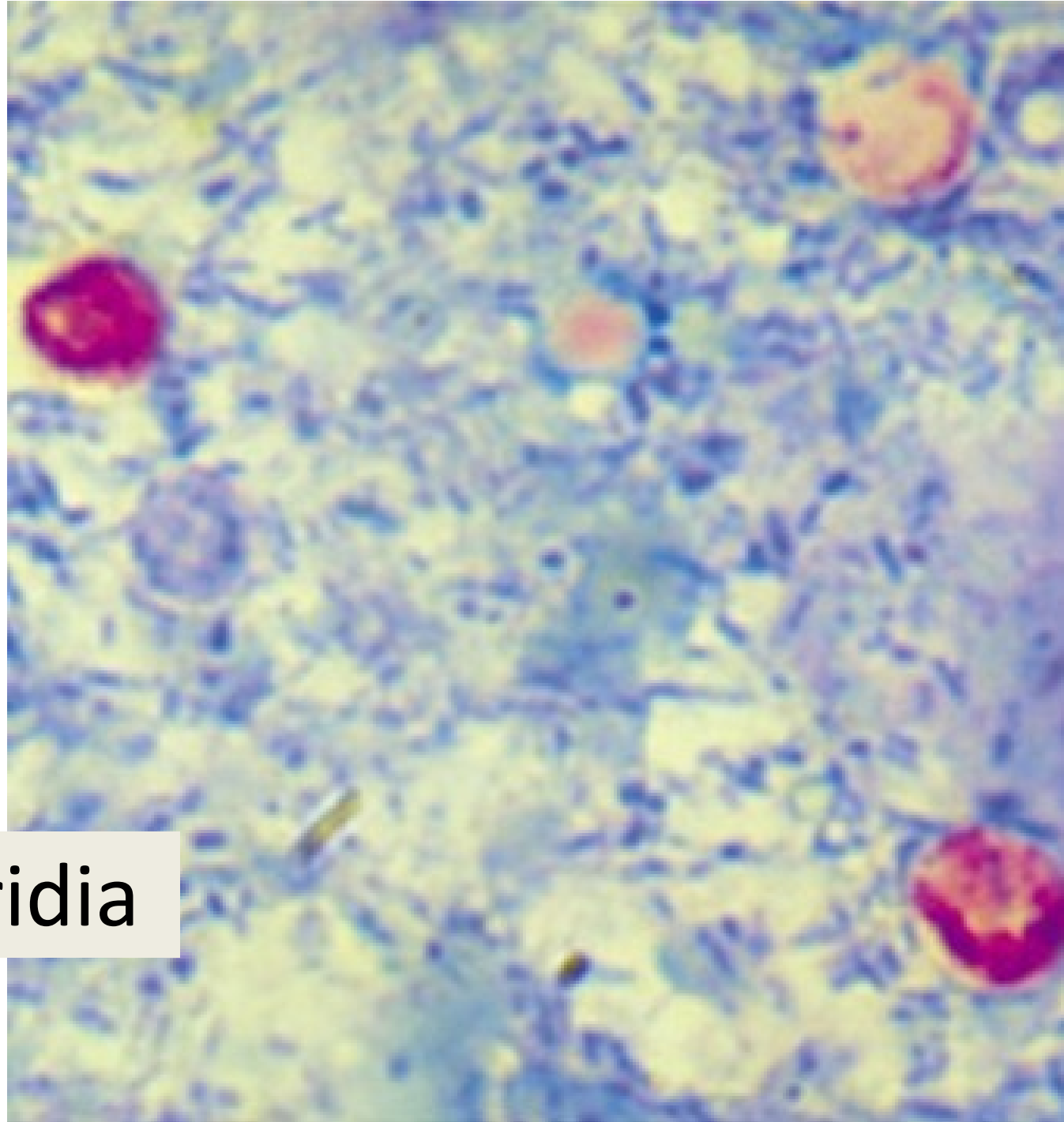
Ziehl-Neelsen acid-fast staining procedure

# Ziehl-Neelsen stain



It is interesting, that Ziehl-Neelsen staining may be used also for a group of parasites, so called intestinal coccidia (cryptosporidia and cyclospores)

## Cryptosporidia



# Culture of mycobacteria

- Hydroxide should be used before culture.
- We use liquid Šula or Banić media and egg Ogawa or Löwenstein-Jenssen media. Egg media are solid because of egg white coagulation, they do not contain agar.
- Even solid media are in test tube and closed firmly. This is not only because personel would be endangered, but also as media would dry.
- Results are read after 1 (check for contamination), 3, 6 and for sure after 9 weeks of culture. (Positive results are mostly found after 6 weeks of culture.)

# Appearance of mycobacterial colonies

<http://www.stockmedicalart.com/>





# To liquid Šula medium

- Even positive test tube is clear by first view, as the growth of mycobacteria is visible only at the bottom („*blue mess*“, as student J. H. called it 😊)



# Tests of antituberculo**t**ic susceptibility (not antibiotic!)

- Antituberculo**t**ics are strange chemicals, different from antibiotics (with exceptions).
- Always we combine 3 or 4 of them: resistances appear quickly, and some have only intra- of only extracellular effect.
- We cannot use diffusion disk tests.
- Antituberculo**t**ics are added directly into culture media, growth control is added.
- **Growth present → mycobacteria resistant.**
- **Growth absent → mycobacteria susceptible.**

# Survey of commonly used antituberculotics

Antituberculotic	Abbrev.
Isoniazid	H, INH
<b>Ethambutol</b>	<b>E</b>
<b>Rifampicin</b>	<b>R</b>
Pyrazinamid	Z
<b>Streptomycin</b>	<b>S, STM</b>

Tuberculous liver of an experimental guinea pig

Courtesy of dr. Jana Svobodová and  
dr. Lev Mezenský



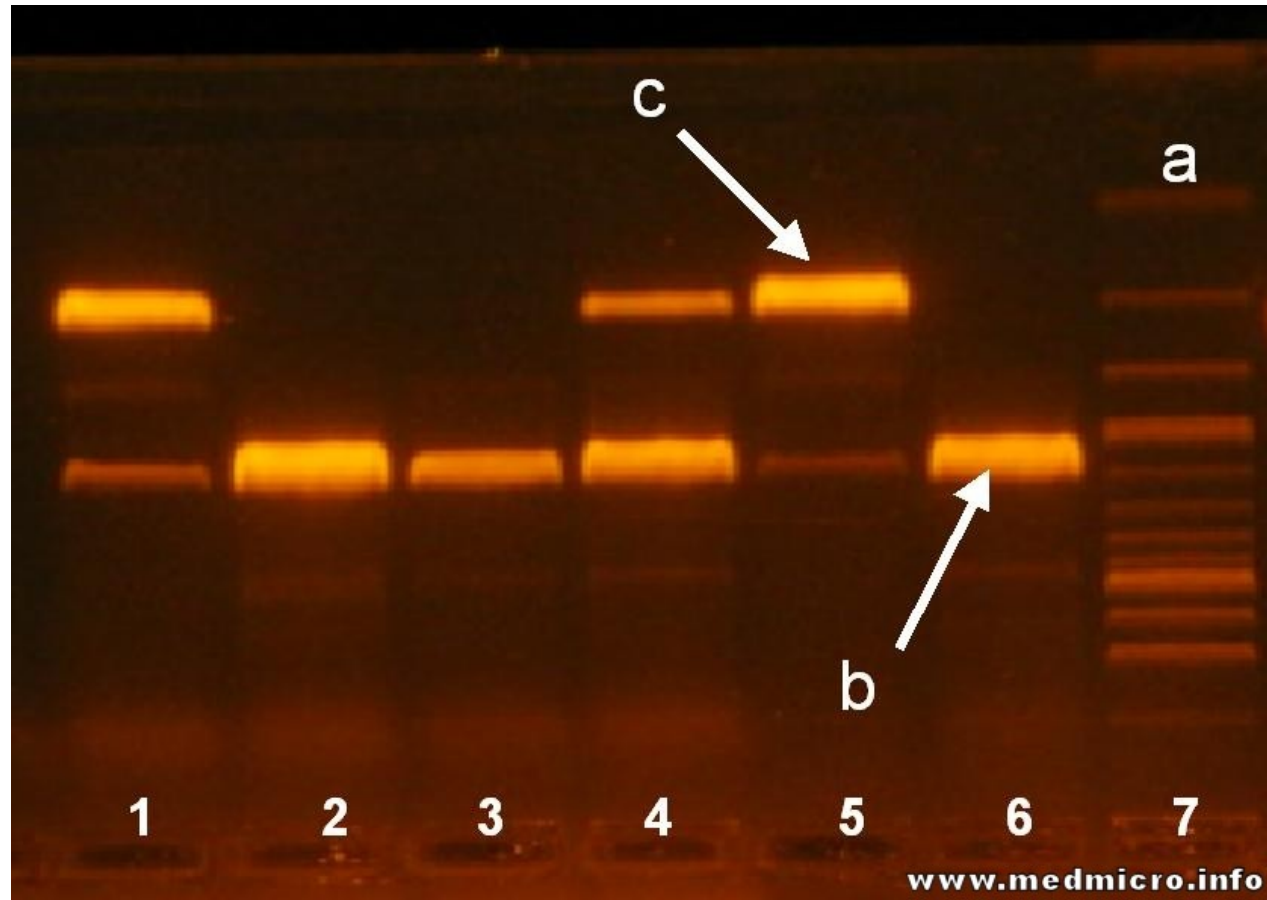
# PCR for TB

1, 2, 3, 4 = patients No.  
1, 2, 3, 4

5 = positive control 6 =  
negative control

7 = ladder (to measure  
position of a band)

upper row (c) = sample  
strip, lower row (b) = IC



PCR is a method used in TB diagnostics more than diagnostics of other bacteria. The reason is that it makes the diagnostics much faster and the risk of environmental contamination is not so serious.

# PCR kit for TB diagnostics



# Indirect diagnostics of tuberculosis

- The most important type of immunity in TB is cell-mediated immunity.
- Formation of **antibodies** occurs, but measurable levels of antibodies are present only in some cases. So positive finding of anti-TB antibodies is a sign of infection, but negative finding has very low information value.
- **Cell-mediated immunity** may be tested
  - by **skin test (tuberculin test)**, especially after vaccination
  - by **INF-gamma release test** (reaction of patient cells to antigen exposition is tested).

# Skin test (Mantoux)

- It is used for **checking of vaccination effect**, but also for proof of an eventual latent infection.
- The **complete living patient** is needed for the test, so it is not a laboratory test. Test is performed by dermatovenerology or other specialized departments. Recently they are replaced by next type tests
- The tests are **positive** in case of activation of cell-mediated immunity; in the matter of fact, it is a specific type of delayed allergy.

# Test of interferon gamma release (Quantiferon<sup>©</sup> TB-GOLD)

- A modern way of checking the cell-mediated immunity is examination of induced interferon gamma release; in practice, the only really used test is Quantiferon TB-GOLD, that is why only this test would be mentioned later.
- It was proven that in TB, including latent TB, tuberculosis antigens activate T-lymphocytes and they produce big amounts of interferon gama.
- Similarly those T-lymphocytes may be activated non-specifically by mitogene, that is why mitogene is used as a positive control.



# Quantiferon – three test tubes

- We need non-clotted (heparinized) blood to three test tubes (we need lymphocytes!).
- First test-tube contains the **mitogene (MIT)** – here, in normal circumstances, **always** stimulation of IFN-gamma should be observed.
- Second test-tube contains **TB antigens (TB)** – here IFN-gamma formation stimulation should be observed **in TB infection only**.
- Third test-tube **does not contain anything (NIL)** – here we should (normally) **never** see IFN-gamma stimulation.

# Quantiferon – results

- Interferon concentration is measured by ELISA
- As **positive** we consider a result, where T-lymphocytes react to stimulation of mycobacterium antigen, but in test-tube with „nothing“ the INF-gamma is not formed.
- As **negative** we consider a result, where T-lymphocytes react to mitogen stimulation, but they do not react to mycobacterial antigen stimulation.
- **Unsure result** is seen (1) if T-lymphocytes are not activated by the mitogen or (2) IFN-gamma is formed even in the test-tube where no stimulator was present.

# Results–example\*

\*the result may be different in subtypes of the test

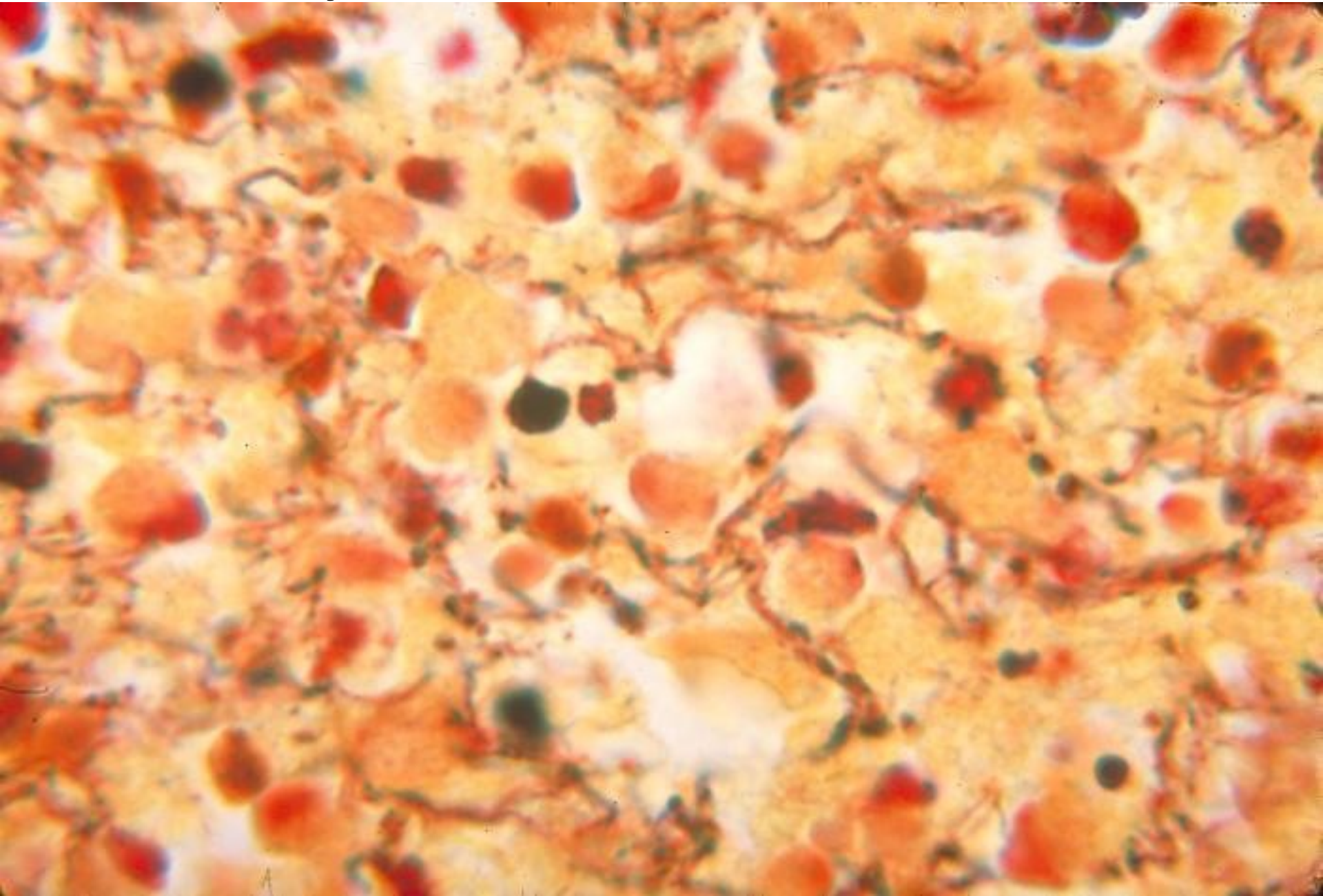
NIL [IU/ml]	TB minus NIL [IU/ml]	MIT minus NIL [IU/ml]	Final test interpretation	Presence of <i>M. tuberculosis</i> infection
<b>≤ 8,0</b>	<b>&lt; 0,35</b>	<b>≥ 0,5</b>	<b>Negative</b>	<b>Not likely</b>
	<b>≥ 0,35 and &lt; 25% of NIL value</b>	<b>≥ 0,5</b>		
	<b>≥ 0,35 and ≥ 25% of NIL value</b>	<b>Any value</b>	<b>Positive</b>	<b>Likely</b>
	<b>&lt; 0,35</b>	<b>&lt; 0,5</b>	<b>Unsure</b>	<b>Cannot by determined</b>
<b>≥ 0,35 and &lt; 25% of NIL value</b>	<b>&lt; 0,5</b>			
<b>&gt; 8,0</b>	<b>Any value</b>	<b>Any value</b>		

# *Nocardia* and *Actinomyces* microscopy

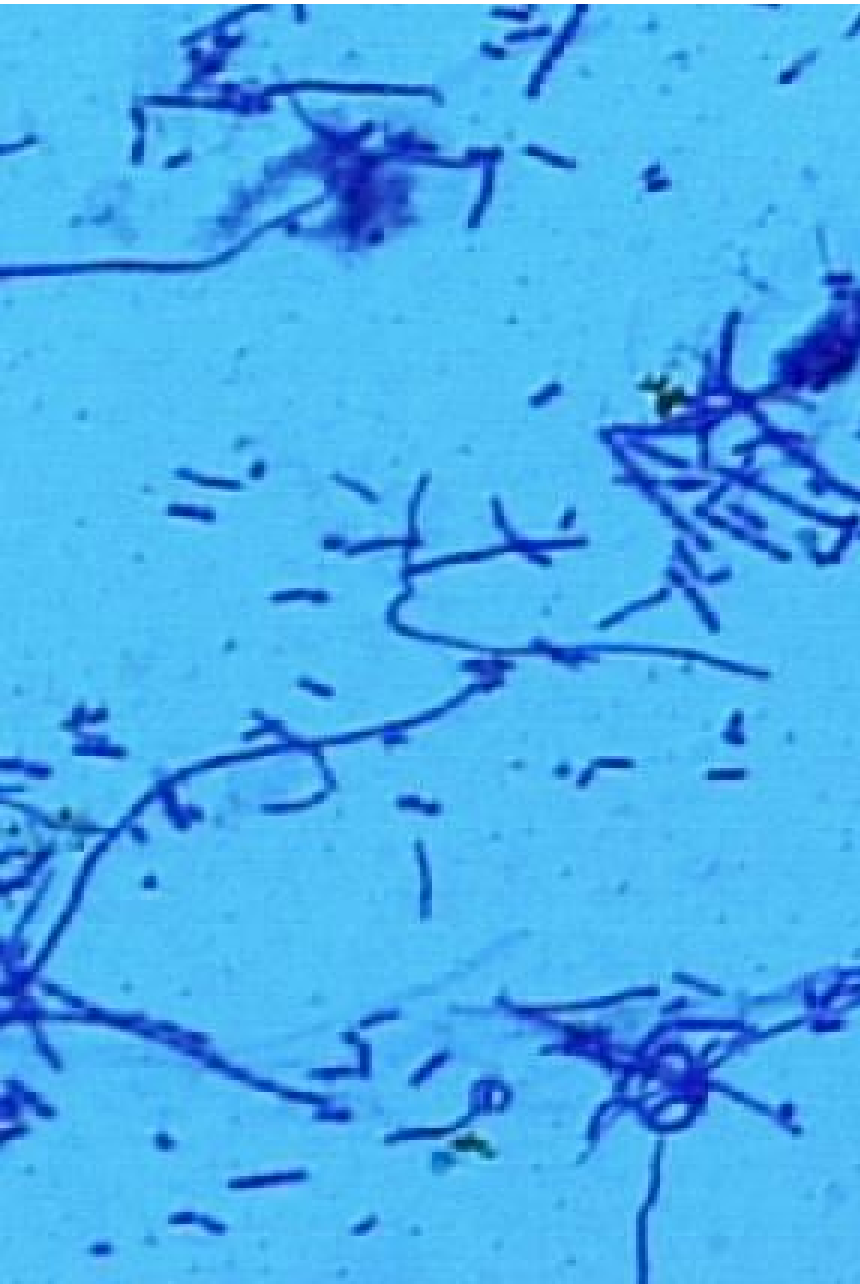
- These bacteria are Gram staining, although they staining poorly and they are very pleomorphous.
- Both of them are typical by their **branched filaments**, staining **Gram-positive**, although some parts of the filament may stain Gram-negative or they might remain unstained at all.
- Sometimes, short (coccoid) forms may also occur in microscopy.

# *Actinomyces israeli*

pathmicro.med.sc.edu



# *Nocardia asteroides*

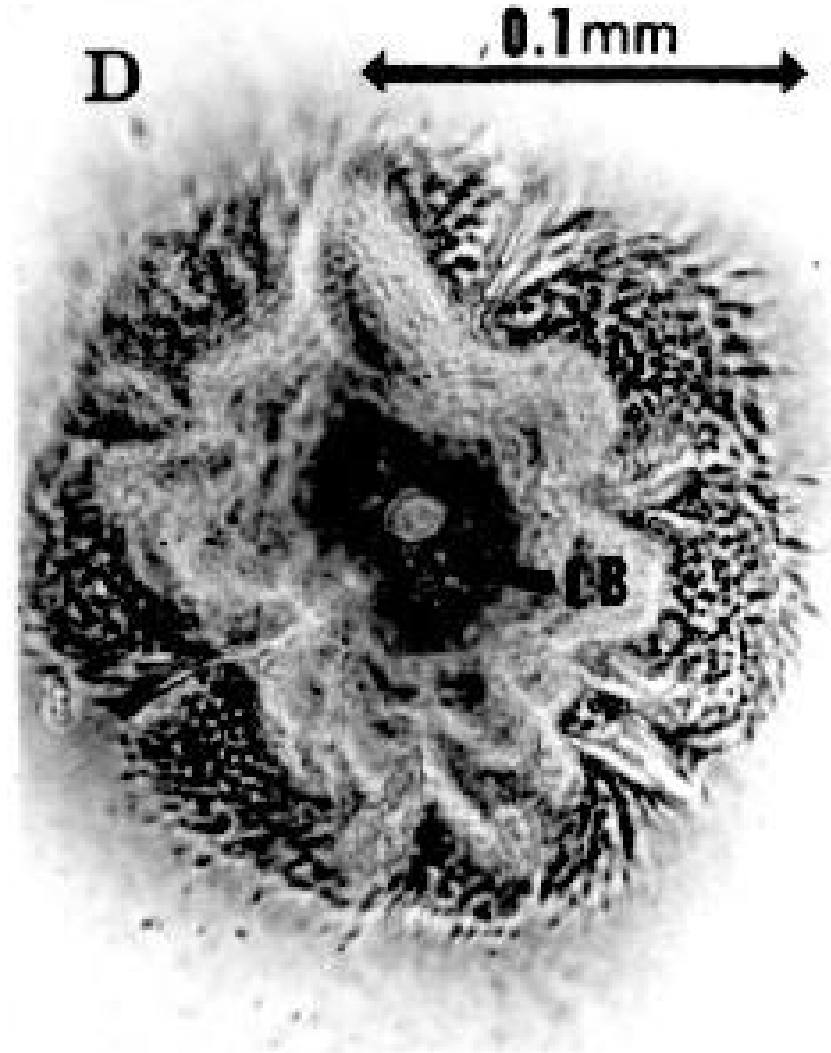


**NOCARDIA**

# *Nocardia* and *Actinomyces* culture

<http://filebox.vt.edu>

- Although both genera are similar in many properties, one is different: *Nocardia* is strictly aerobic, while *Actinomyces* grows in anaerobic conditions.



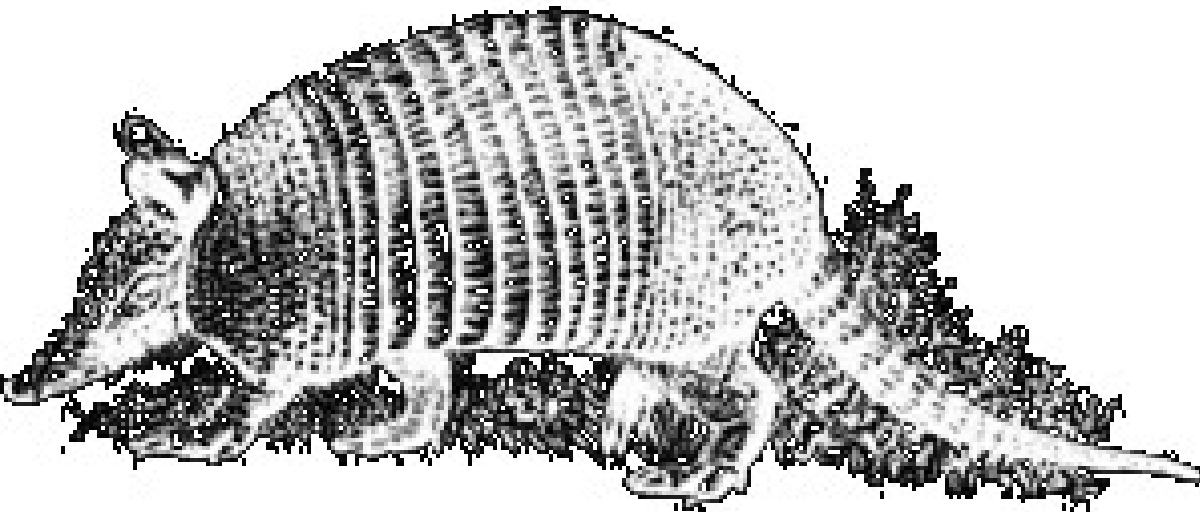
# Antibiotic susceptibility of *Actinomyces* and *Nocardia*

- Unlike mycobacteria, in nocardiae and actinomycetes antibiotic susceptibility can be tested using diffusion disc test. We have to know, that they grow slowly and badly.
- For nocardiosis we use co-trimoxazol for therapy, eventually ampicillin or macrolides.
- In actinomycosis we use penicillin, eventually doxycyklin and more antibiotics.

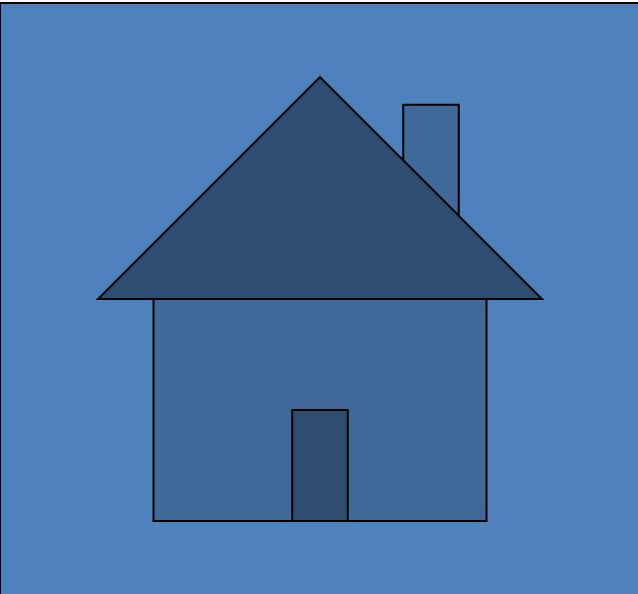


# Lepromin test in leprosy diagnostics

- There is an animal. Its name is **nine banded armadillo**.
- It is necessary for production of **lepromin**.
- This substance is used in **lepromin test**, the equivalent of tuberculin test for TB.



Logo of a TB congress



The End

