Mycobacteria

Čermák P.
Ondráčková L.

Tuberculosis – big world health problem

M. tuberculosis

- estimate – one third of world population is infected *M. tuberculosis* (1)
- 2000 – 8,3 milion of new cases, 1,8 milion of death (2) 2018 – 9 milion
- COVID pandemia - increasing of mortality next years, problems in reporting (false low incidence)
- 6000 death on TBC by the day (3)

1. Pai M et al. New tools and emerging technologies for the diagnosis of tuberculosis: Part I. Latent tuberculosis. EXPERT REVIEW OF MOLECULAR DIAGNOSTICS 2006;6(3): 413-422.
2. The WHO/IUATLD Global Project on Anti-Tuberculosis Drug Resistance Surveillance: *Anti-Tuberculosis drug resistance in the world, report number 2 Volume* . Geneva; 2004:-.
3. Moore D et al. Microscopic Observation Drug Susceptibility Assay, a Rapid, Reliable Diagnostic Test for Multidrug-Resistant Tuberculosis Suitable for Use in Resource-Poor Settings. JOURNAL OF CLINICAL MICROBIOLOGY 2004;42(10):4432–4437.

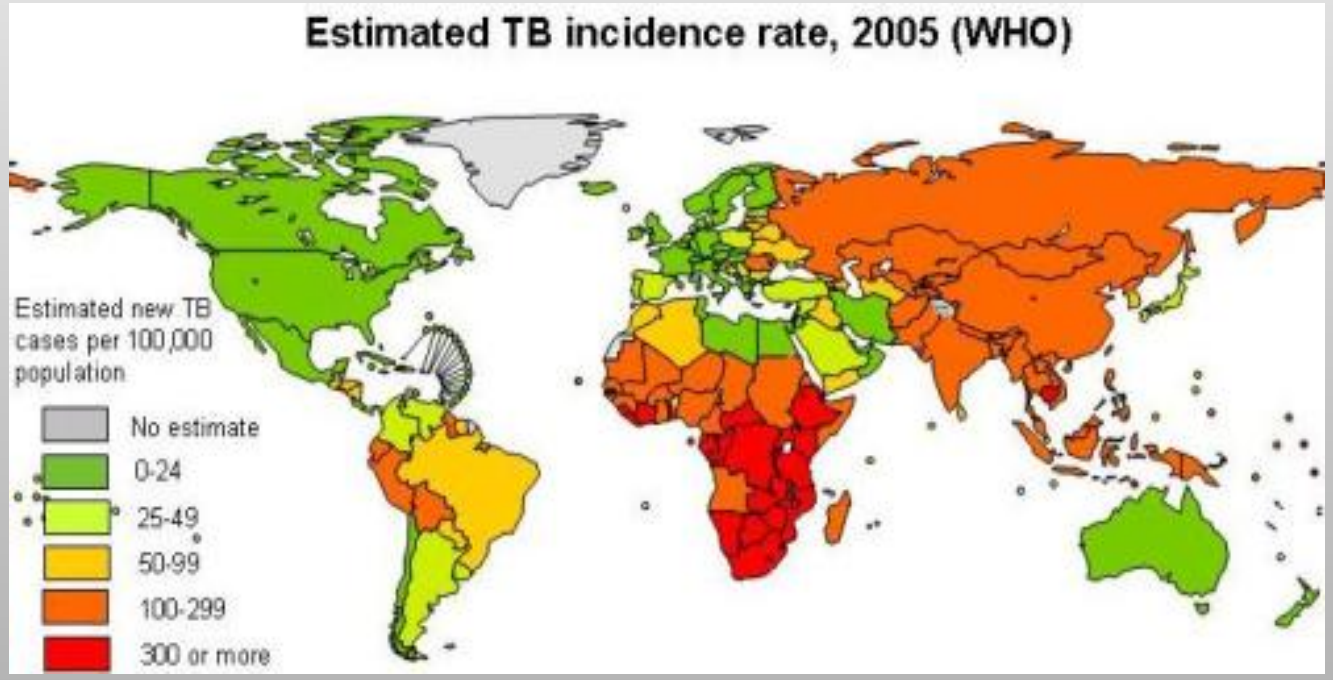
factors influenced TBC incidence

M. tuberculosis

- microbial – virulence factors, phylogenesis
- macroorganismus – genetic dispositions (american indians), acquired imunity
- health care
- targeted TBC controle programs
- social-economic factors

World TBC incidence

- subsaharian Africa – kombination with HIV/AIDS
- Russia and former republics of SSSR – 1990 – 2000 highest growth(3x more than in 1960) (1)
- China



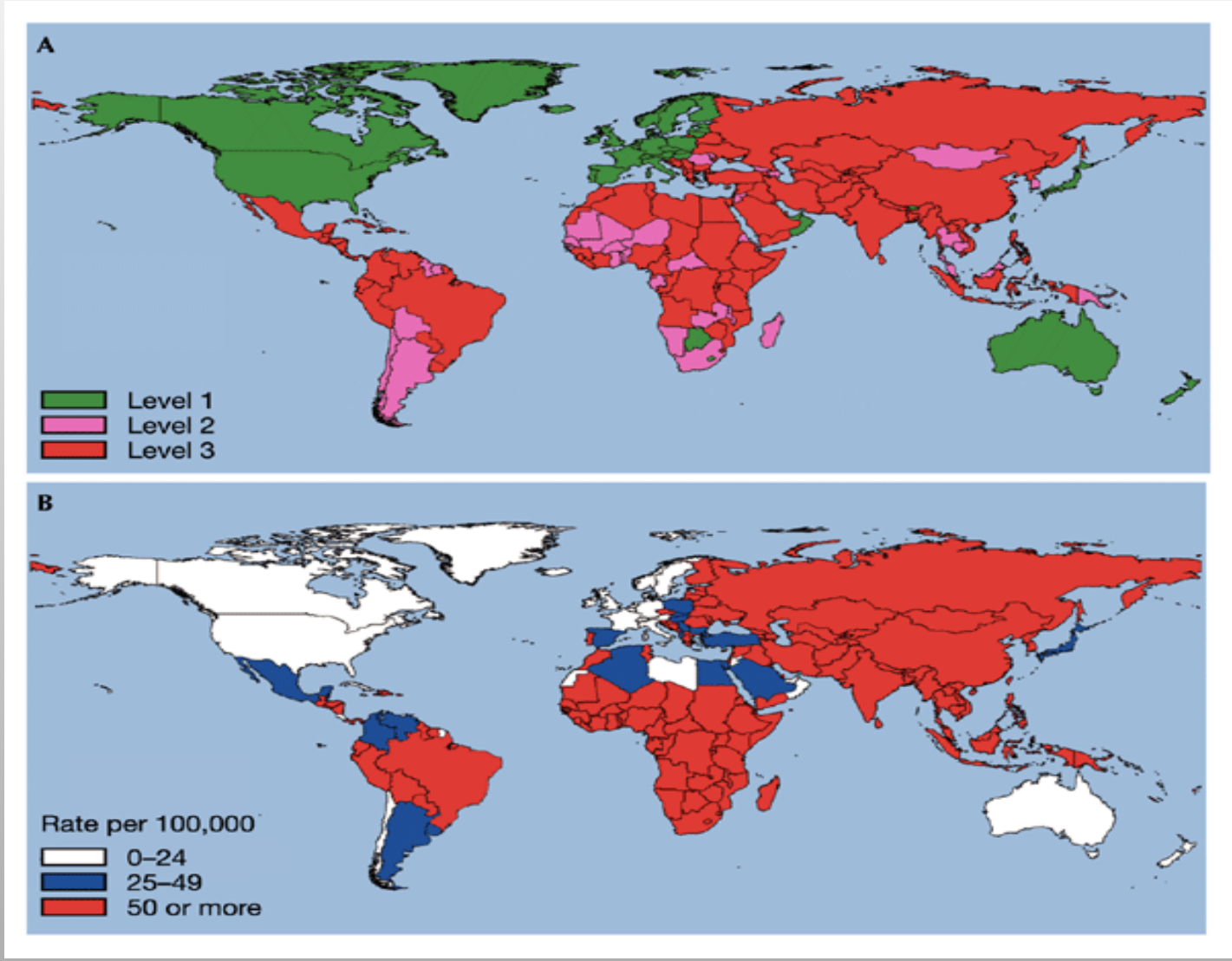
- WHO/International Union Against Tuberculosis and Lung Disease Global Project on Anti-tuberculosisDrug Resistance Surveillance
- Stop TB Partnership (2000)

M. tuberculosis

1. International Consortium on Tuberculosis.http://www2.ku.edu/~lba/Test/TB/ICTM.html

economical level a incidence of TBC

M. tuberculosis

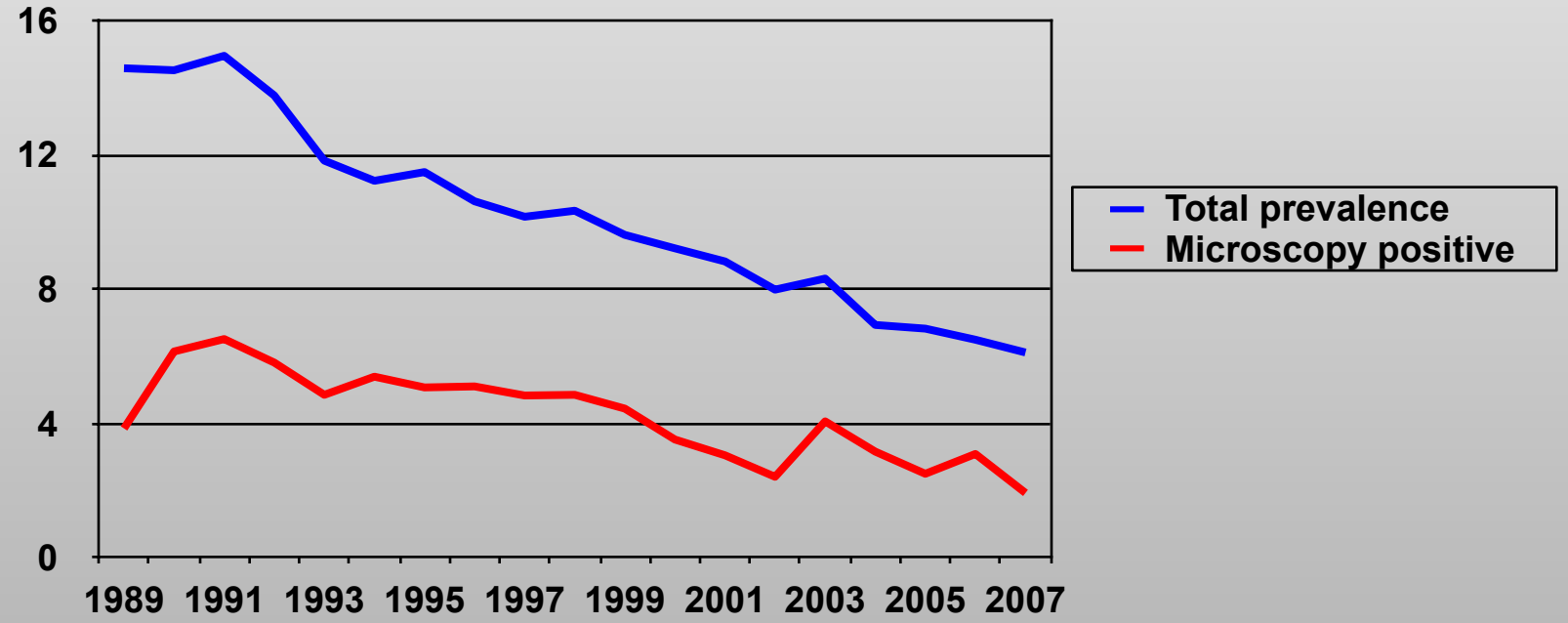


PD vnHelden. The economic divide and tuberculosis. ENBO reports 2003;4:S24-S28.

Situation in the Czech republic

M. tuberculosis

- succesful monitoring systems
- total prevalence 2020 3,4/100 000
- ascending proportion of foreigners (Asia and east. Evropa)
- our east line = border of low TBC incidence (Ukraine 2020 44,6/100 000)



history

Robert Koch (1843 – 1910)
important discovery

1872 – 76 antrax
1882 – tuberculosis
1883 – cholera

handing of the Nobel Price (1905)
findigs related to TBC

Classification - total ≥179 species
(03.2018 - 179…)

order *Actinomycetales* rod *Mycobacterium*

mycobacteria

Obligatory pathogens

M.tuberculosis complex:
M.tuberculosis
***M.bovis, M.bovis* BCG**
M.africanum, M.microti, M.canetti,
M. pinnipedii, M. caprae,
M.suricattae, M. mungi, M. orygis

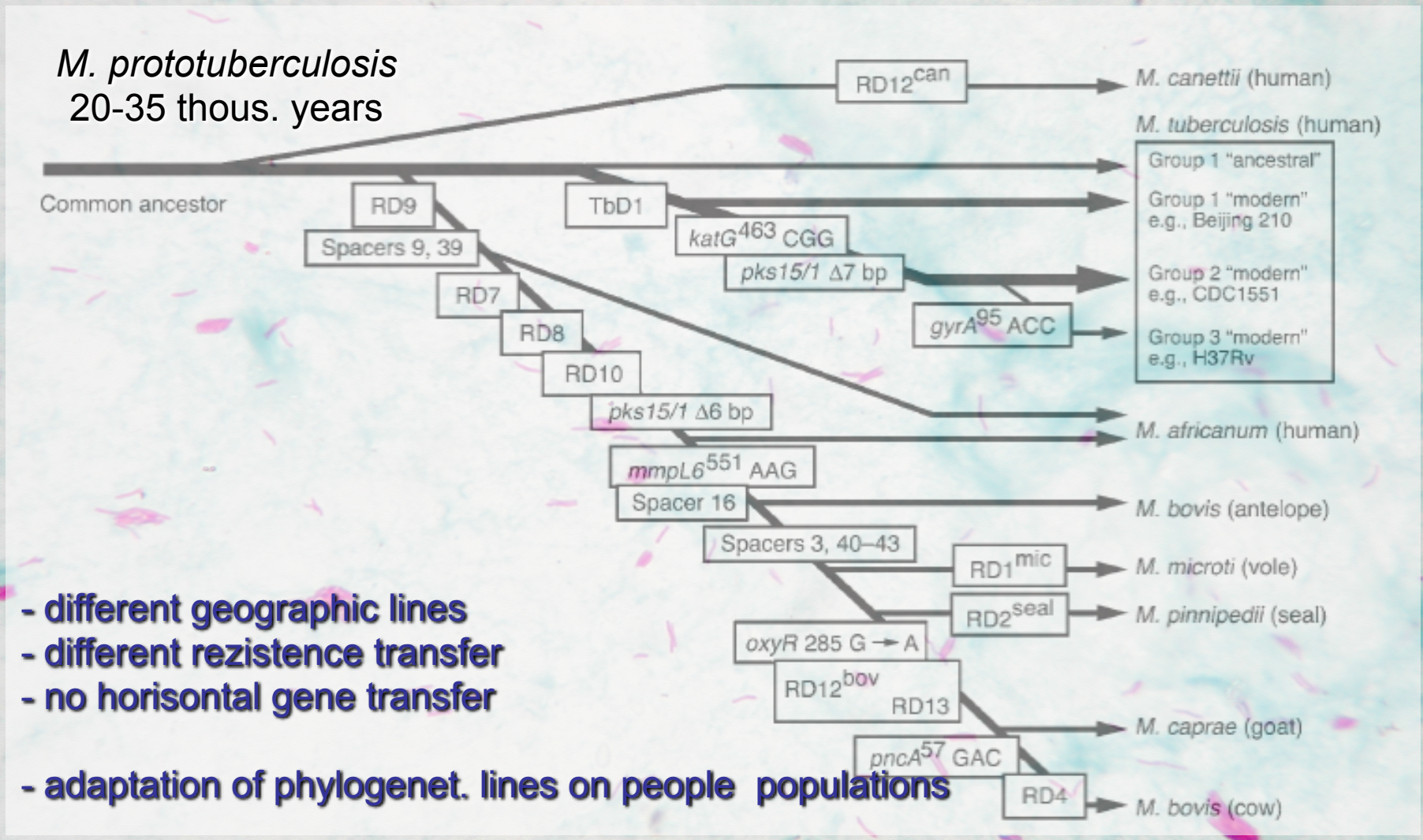
M.leprae

Opportunist patogens

M.avium
M.intracelulare
M.kansasii
M.malmonese, M.marinum
M.scrofulaceum, M.szulgai
M.xenopi and others…

M.fortuitum
M.chelonae

Evolution of *M. tuberculosis complex*

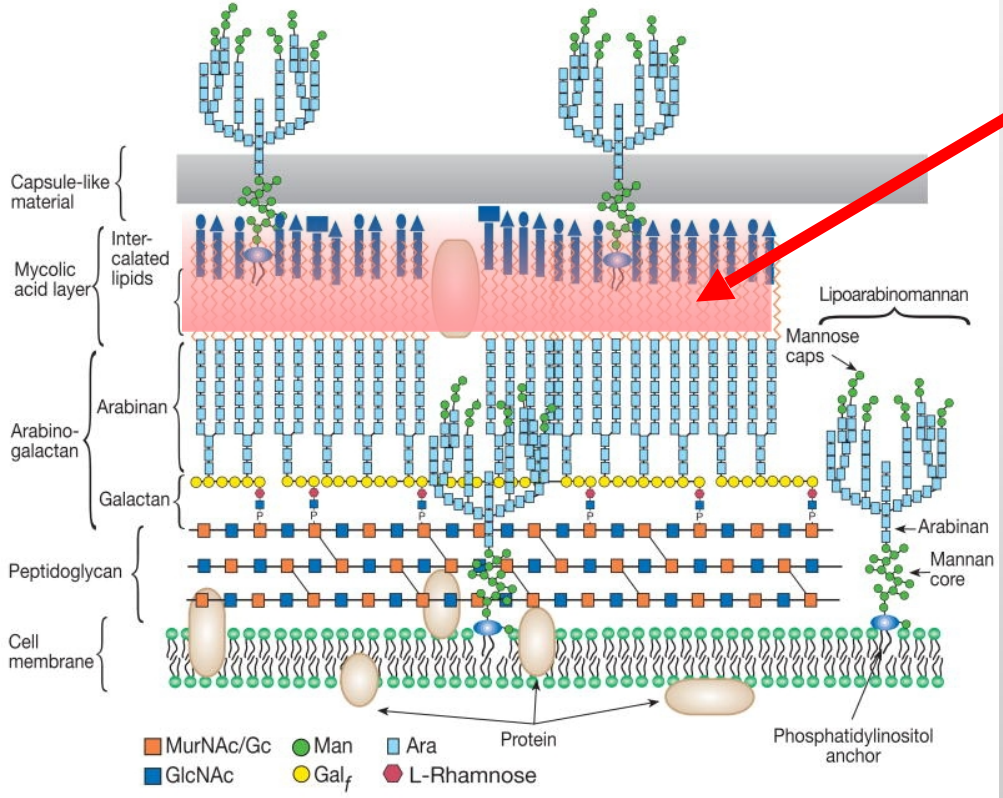


- different geographic lines
- different rezistence transfer
- no horisontal gene transfer

- adaptation of phylogenet. lines on people populations

Ernst JD, Trefeno-Nunez G, Banaiee N. Genomics and the evolution, pathogenesis, and diagnosis of tuberculosis. *J. Clin. Invest.*2007 **117**:1738–1745.

cell wall



complicated structure

mycolic acids
lipid bariere responsible for attributes of mycobacteria

role in pathogenesis, imunological response („tortois“ between bacteria)

virulence factors – components of cell wall
- dimycolate threalosis (TDM)
- = cord factor
- lipoarabinomanan (LAM)
- ftiocerodimykocerosat (PDIM)
- sulfolipids
- 17 kDa glykoprotein

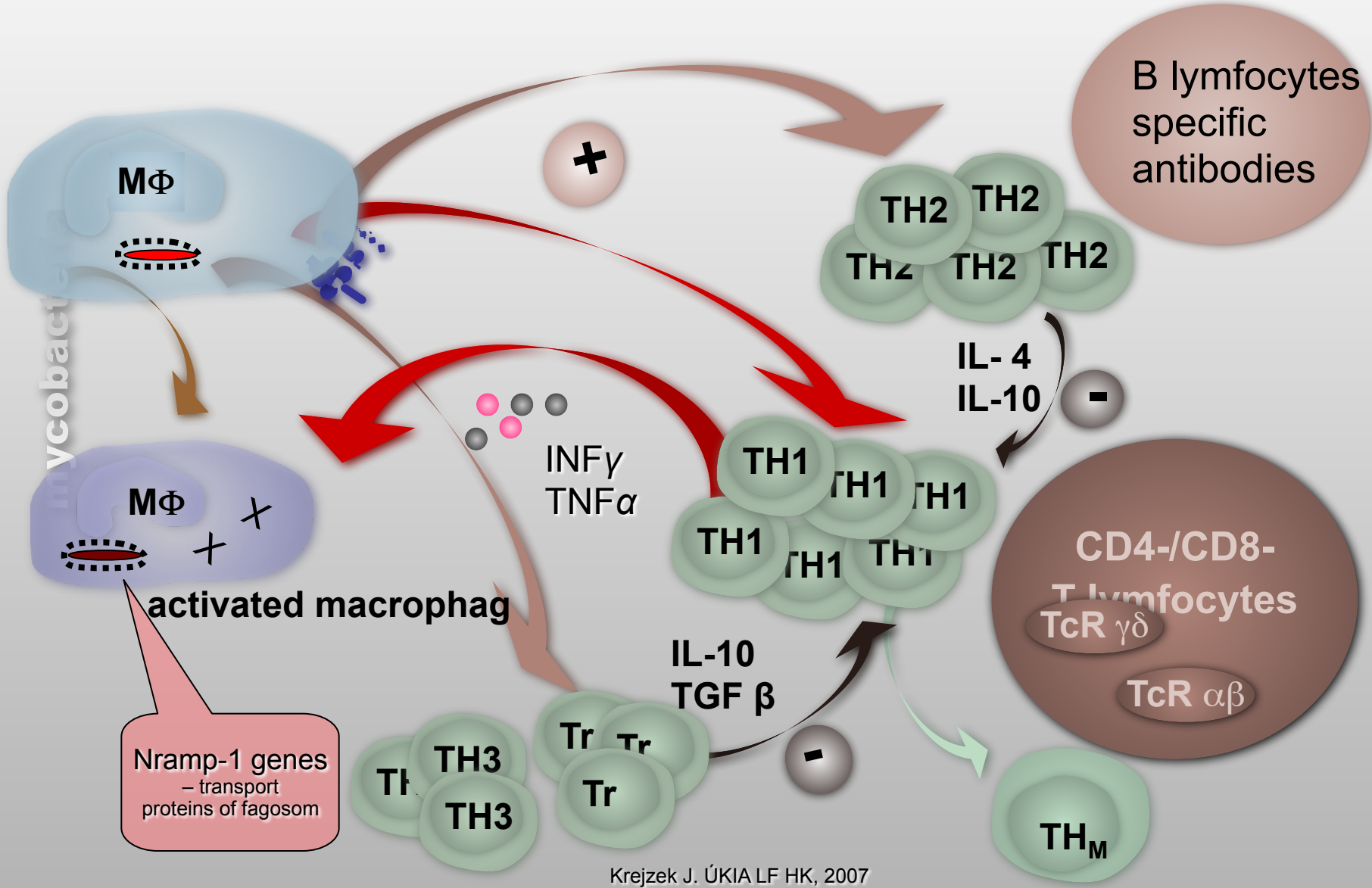
! no toxin estabilished to date!

Macela A. a kol. Infekční choroby a intracelulární parazitismus bakterií, Grada, Praha, 2006, 216.

mycobacteria x macroorganism

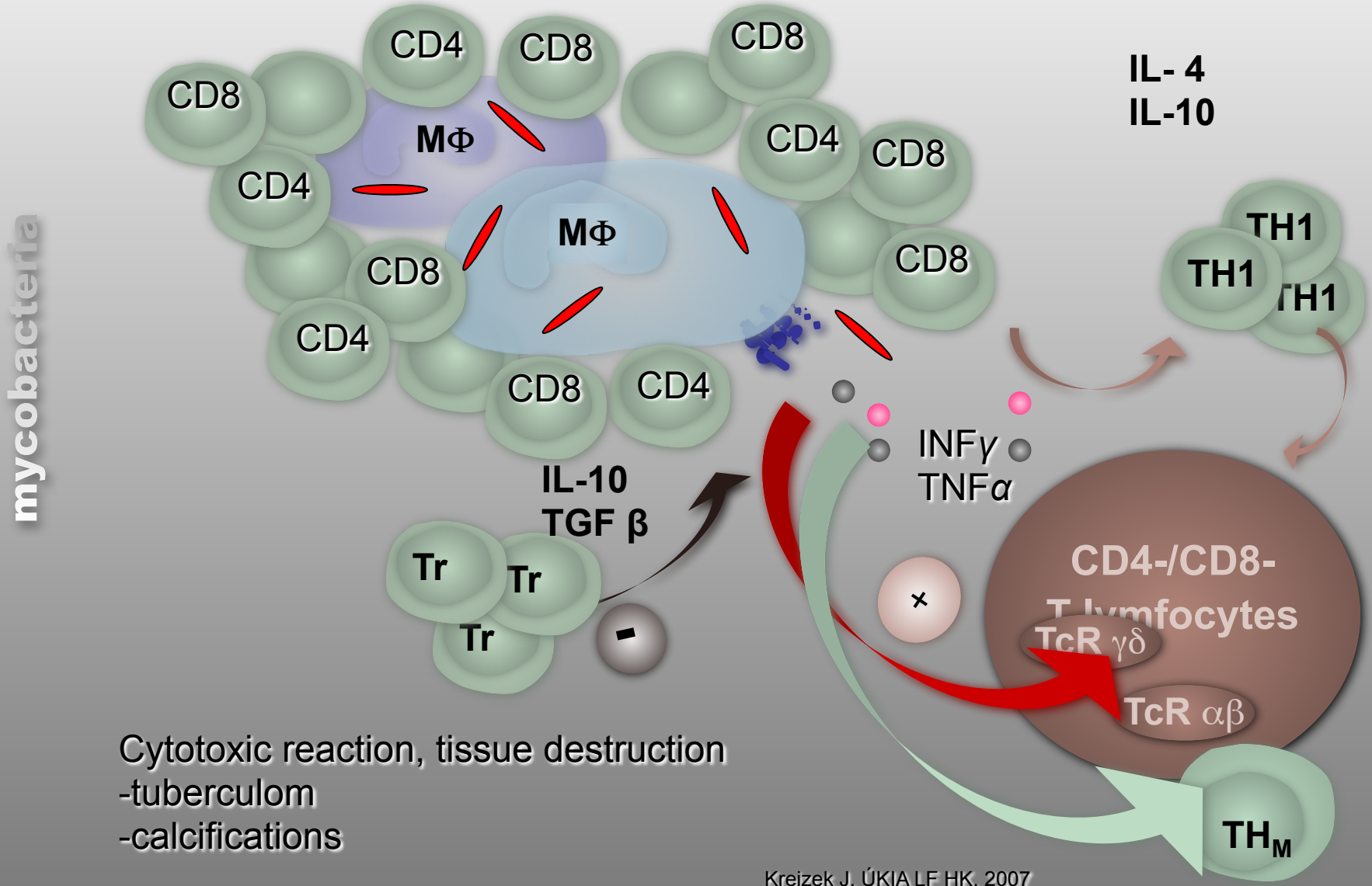
- typical intracelular parasite
- specific genes regulated mycobacterial answer on fagocytosis
- RD genes –virulence factors , secretory proteins (ESAT-6, CFP-10 atd…)
- cell immunity

imunopathogeneis of infection



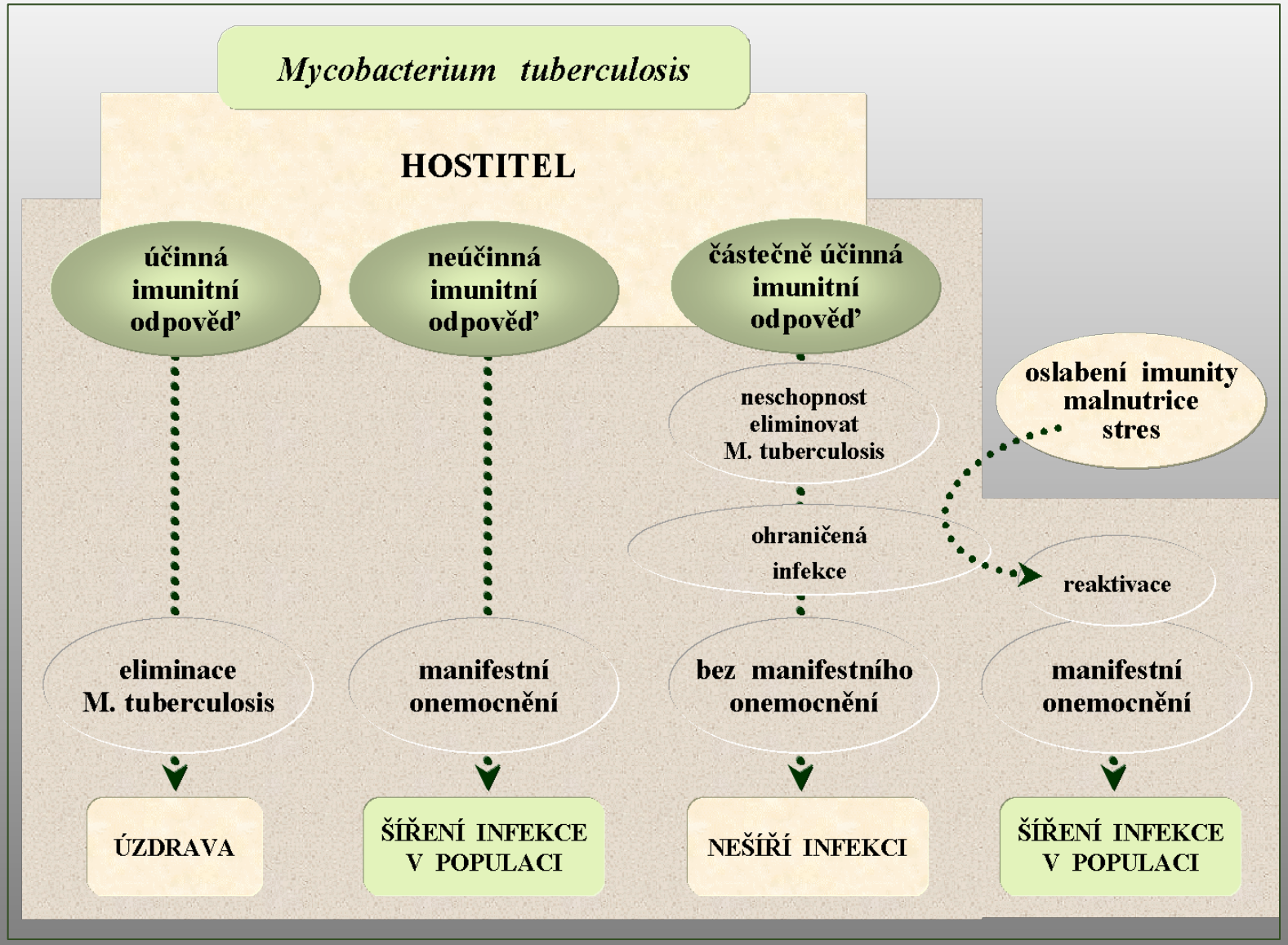
Krejzek J. ÚKIA LF HK, 2007

specific immunity



Cytotoxic reaction, tissue destruction
-tuberculom
-calcifications

M. tuberculosis



Clinical diseases – mycobact. infections

Spreeding – infectious aerosol, contact, infection doses <10 bact. cells

Entering – respitarory tract, other paths rare

time of incubaion – weeks to months

M. tuberculosis

- **Pulmonary TBC**
- **Extrapulmonary TBC**

Mortality with therapy 16%, without ter. - death

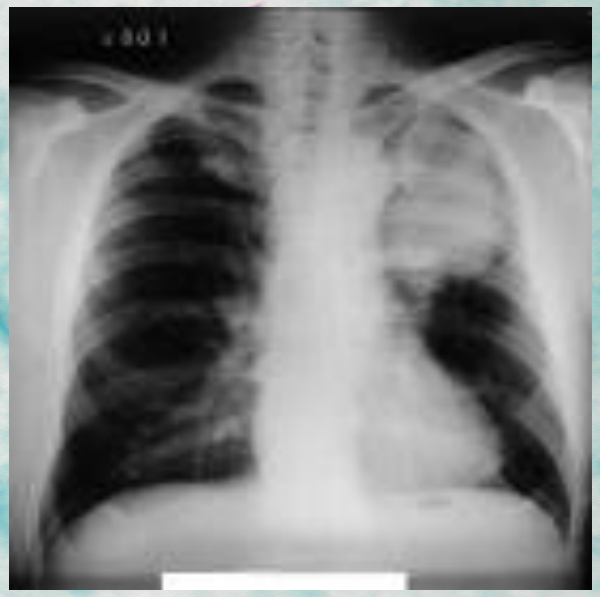
Mycobacteriosis = other mycobacteria then M. tuberculosis

Clinical desease - TBC

M. tuberculosis

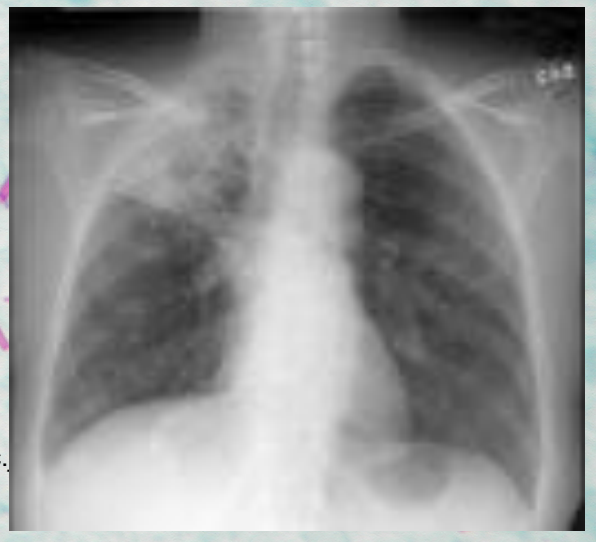
- **Pulmonary TBC**

  -TBC pneumonia
  -cavernosus TBC
  -miliary TBC (extrapulmonary)



- **extrapulmonary TBC**

  -lymphadenitis
  -genitourinaryTBC
  -TBC meningitis
  -skin TBC
  -bone TBC
  -GIT…



monka.hysteria.cz/.../images/tbc.
2. Lf UK

therapy

- historical development
- nutrition
- TB sanatorium – high mountains (ozone)
- surgical – pulmonary resection
- Chemotherapy - antituberculotics

M. tuberculosis

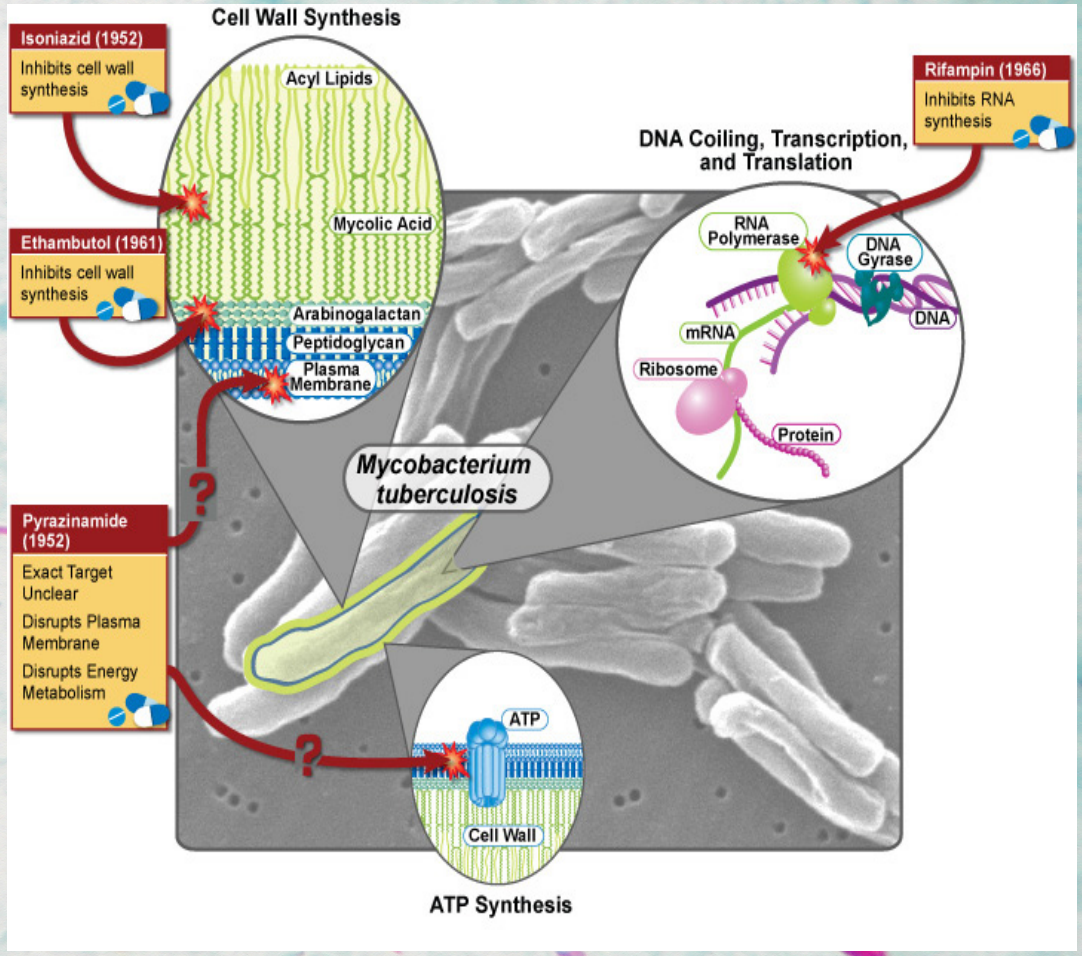
therapy – antituberculotics (AT)

M. tuberculosis

Basic tests (AT)

I. row
INH – isoniazid
EMB – ethambutol
RIF – rifampicin
PZA – pyrazinamid

II.row
STM – streptomycin



www.niaid.nih.gov/.../tuberculosis/tb1.jpg

therapy – antituberculotics (AT)

AT list:

I. row: INH, EMB, RIF, PZA
II. row: STM, kanamycin, amikacin, capreomycin, viomycin
III. row: fluorochinolons (ciprofloxacin, ofloxacin, moxiflofacin..)
IV. row: bacteriostatic AT (etionamid, PAS – paraaminosalicylic acid….)
V. row: clofazimin, linezolid and others

M. tuberculosis

Strong undesireble side effects !!!

Resistance issues

MDR-TB multidrug resistant tuberculosis = resistance min. on INH and RIF from basic row AT
XDR-TB extensively drug-resistant tuberc.= resistance on INH, RIF + fluorochin. + min. one inj. AT from 2. row (AMI, KAN, CAP)
TDR-TB = resistance on both row AT

- **2000 – 272 906 cases MDR-TB, 2004 – 424 903 cases MDR-TB = 4,3% of all treated cases. 62% in China, India a Russia (1)**
- **TBC resistance from 23 laboratories in 48 countries – 3520 (19,9%) MDR-TBC, 347 (9,9%) XDR-TB (2)**
- **USA – 80% MDR-TB foreigners (ilegal immigrants from Mexico) (3) CZ - east workers**
- **2020 MDR-TB CZ 10 patients, Ukraine - 3076 (24%)**

M. tuberculosis

1. Zignoli M et al. Global influence of multi-drug resistant tuberculosis. JOURNAL OF INFECTIOUS DISEASES 2006;194(4):479-485.
2. Shah NS et al. Worldwide emergence of extensively drug-resistant tuberculosis. EMERGING INFECTIOUS DISEASES 2008;13(3):380-387.
3. International Consortium on Tuberculosis.http://www2.ku.edu/~lba/Test/TB/ICTM.html

reasons of resistance

- **inadequate therapy**
  - ➢mistakes in dosages
  - ➢incontroled therapy
  - ➢intolerance AT
- **early ending of therapy**
- **bad patient cooperation**
- genetic mutation of mycobacteria
- **selection of resistant mutants**

mycobacteria

Therapy

- **Varies guidelines (new patient, recurrent infection)**
- **Obligatory hospitalisation**
  - ➢**to negative microscopy,** minimal 2-3 month
  - ➢4 combination of AT new patients
  - ➢5 combination of AT recurrent infection
- **Than 2-4months home therapy p.o.**
- **Childreen 2 or 3 combination of AT**

mycobacteria

vaccination

- atenuated strain M. bovis BCG „vaccinia“
- History – vaccination after birth, revaccination in 10 years
- Now – vaccination canceled as in others developed countries (complications after vaccination), vaccination only for risc population
- ??? time will show…

laboratory diagnostic

mycobacteria

direct

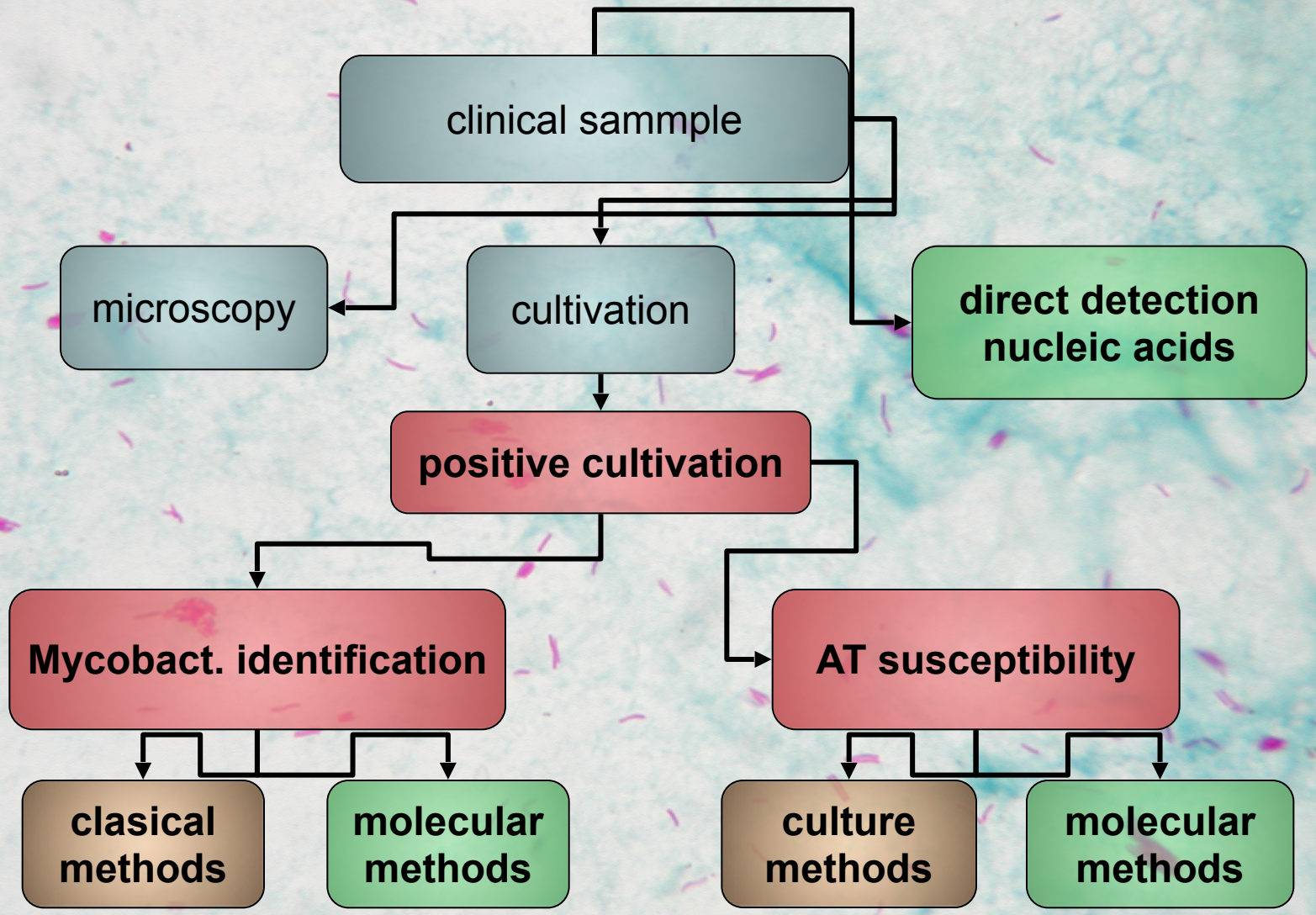
3 sputum (90%) in various days

- microscopy of clinical sample
- clasical cultivation on solid and liquid medium
- cultivation in automatic system
- nucleid acids detection

indirect

- **activation of immunity system**

direct detection



mycobacteria

microscopy

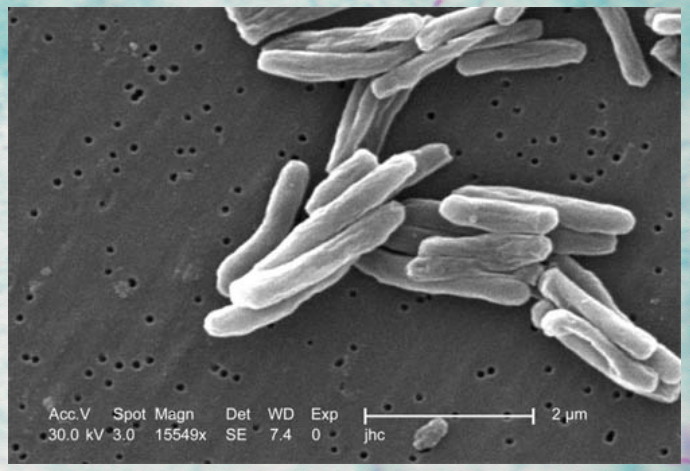
mycobacteria

- **Ziehl-Neelsen staining** – resistant again discolour of acids-alcohol = acid-alcohol fast bacilli /AAFBs, AFBs/
- **Fluorescent staining = auramine-phenol**
- „light emiting diode“

reporting

0	AFBs nof founded
+	10 - 20 AFBs
++	21 - 100 AFBs
+++	více než 100 AFBs

- fast but very low sensitive (>1000 bact.)



http://www.allamericanpatriots.com/news_topics/pandemics

www.asm.org/Division/c/photo/tb1.jpg

Marais BJ et al. Use of light-emitting diode fluorescence microscopy to detect acid-fast bacilli in sputum. *Clin Infect Dis* 2008;47(2):203-207.

basic cultivation problem – slowly growth of mycobacteria

Special culture medium: solid egg

mycobacteria

Prolongated cultivation

growth	1 cycle	40 cycles
Enterobacteria	30 minutes	1 day
M. tuberculosis	1 day	40 days (6 weeks)

Mycobacteria - cultivation

- **Decontamination of sample =** destroying other bacterial cells (long time cultivation
  - ➢ **N-acetylcystein** (Kubica) method, Laurynylsulphate method, Petroff method, acid (HCL) method
  - ➢ **Princip** - lytic effect of NaOH or acid combined with mucolysis and neutralization
- **Destroying a lot of mycobacterial cells**
- **Complicated proces with centrifugations**
- ***M. tuberculosis* is in III. group of dangerous infection = special laboratory** (separated, climatization with hepa filtr, work in laminar flow box, personal protection….)

Mycobacteria - cultivation

- **Innoculation of sample to minimal 3 solid and liquid media in bottles !!!**

- **Cultivation media**
  - ➢Solid - eggs (**Loewenstein-Jensen**, Ogawa), Middlebrook
  - ➢Liquid protein (Šula, Middlebrook)

- **Thermobox - 37^0 C, humidity 85%, air circulation**

metabolic methods

„fast cultivation“

mykobakterie

Growth of mycobacteria in closed system
fast detection – bacterial metabolism

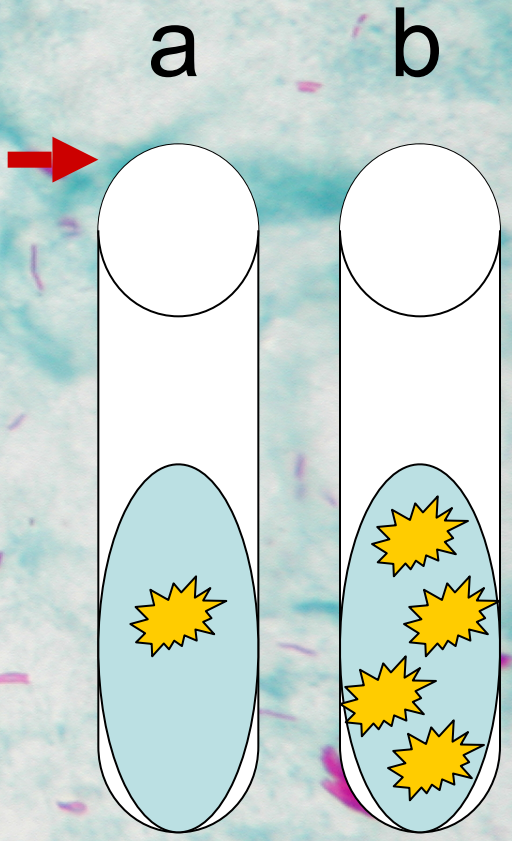
- CO_2 detecion - radioactive (Bactec 460TB), colorimetric (MB/BacT), fluorescent Bactec 9240 TB
- O_2 consumption - **MGIT 960**
- manual or automatised systems
- average time to detection **MGIT – 13 days**

Negative samples - cultivation 42 days !!!

Antimicrobial susceptibility testing

mycobacteria

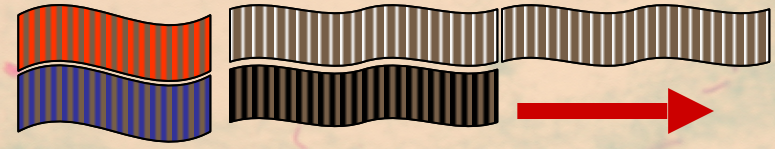
- **Proportial Canetti method** on solid medium with AT(a) and control witout AT(b) – tolerance of 1% resistant cells
- **Metabolic methods** – SIRE, Bact/Alert 3D, Versa TREK
- **MIC**, MODS, MABA, E-test, NRA and others
- **Molecular methods**



molecular methods – basic principles

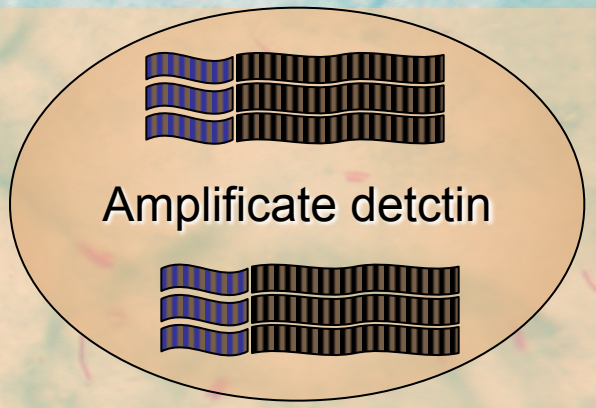
mycobacteria

PCR DNA, RNA



spec. probe

End point, RT PCR,
multiplex, nested….



elektroforesis, fluorescence, hybridisation

16S rRNA (např. NASBA)
16S DNA

1. genome amplification (multiplex)

2. identification

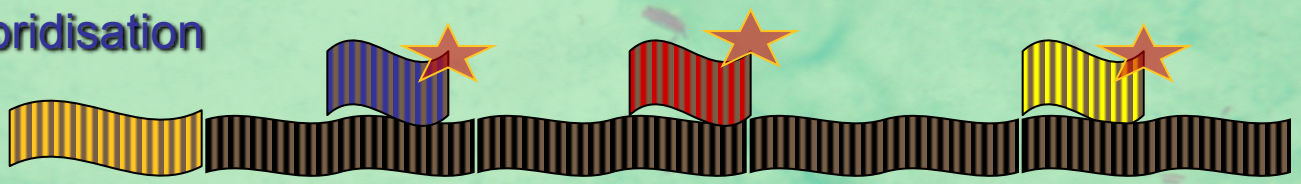
hybridisation

microarray
restriction
sequenation

molecular methods
16S rRNA, 16S rDNA

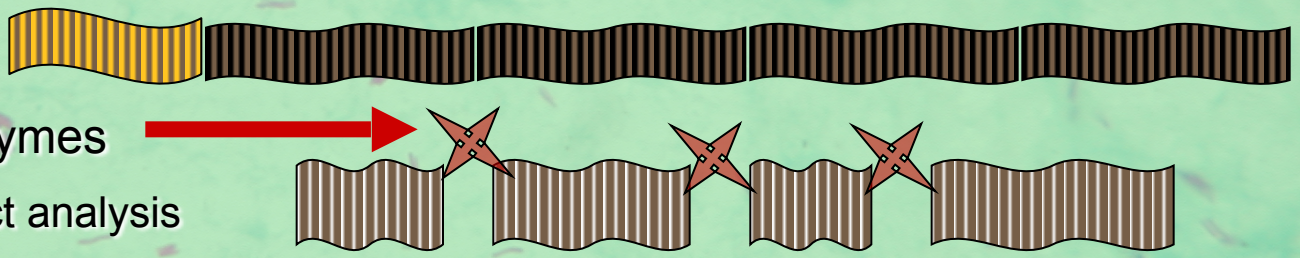
mykobakterie

hybridisation



STRIP = probes bounded on paper strip
Microarray = probes bounded on spec. porter – „chip, biochip“

restricted methods



restrict analysis

Sequence of bases

sequence analysis, pyrosequenation

mol. methods –resistance detection

INH	***katG, inhA, ndh, ahpC***	**Amikacin**	***rrs***
RIF	***rpoB***	**Capreomycin**	***tlyA***
PZA	***pncA***	**fluorochinolons**	***gyrA, gyrB***
EMB	***embCAB***	**Ethionamid**	***etaA/ethA, inhA***
STM	***rpsL, rrs***		

mykobakterie

- **strip metodhods – Inno-LiPA, GenoType**
- Multiplex Real-time PCR
- DNA chip
- PCR-DNA sequenation

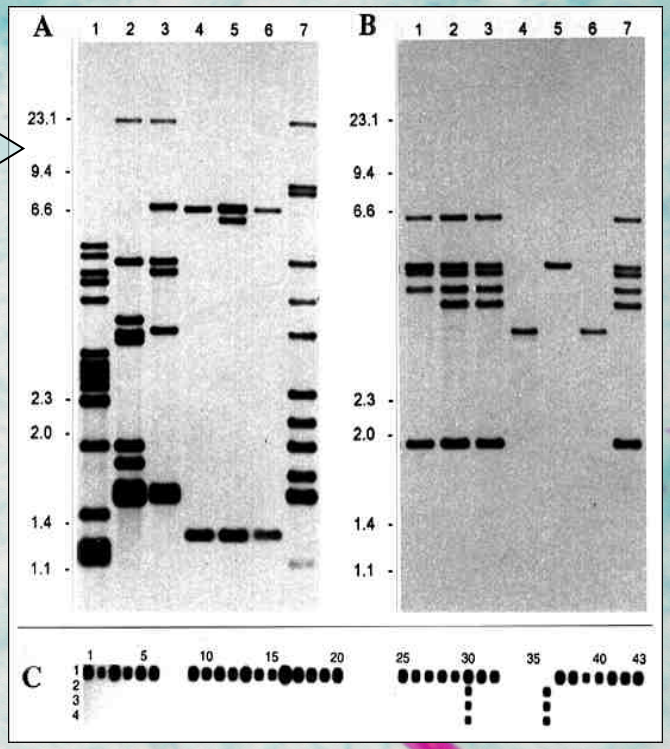
Methods of molecular epidemiology

fingerprinting methods

for epidemiological findings – identifaication to subspecies

M. tuberculosis

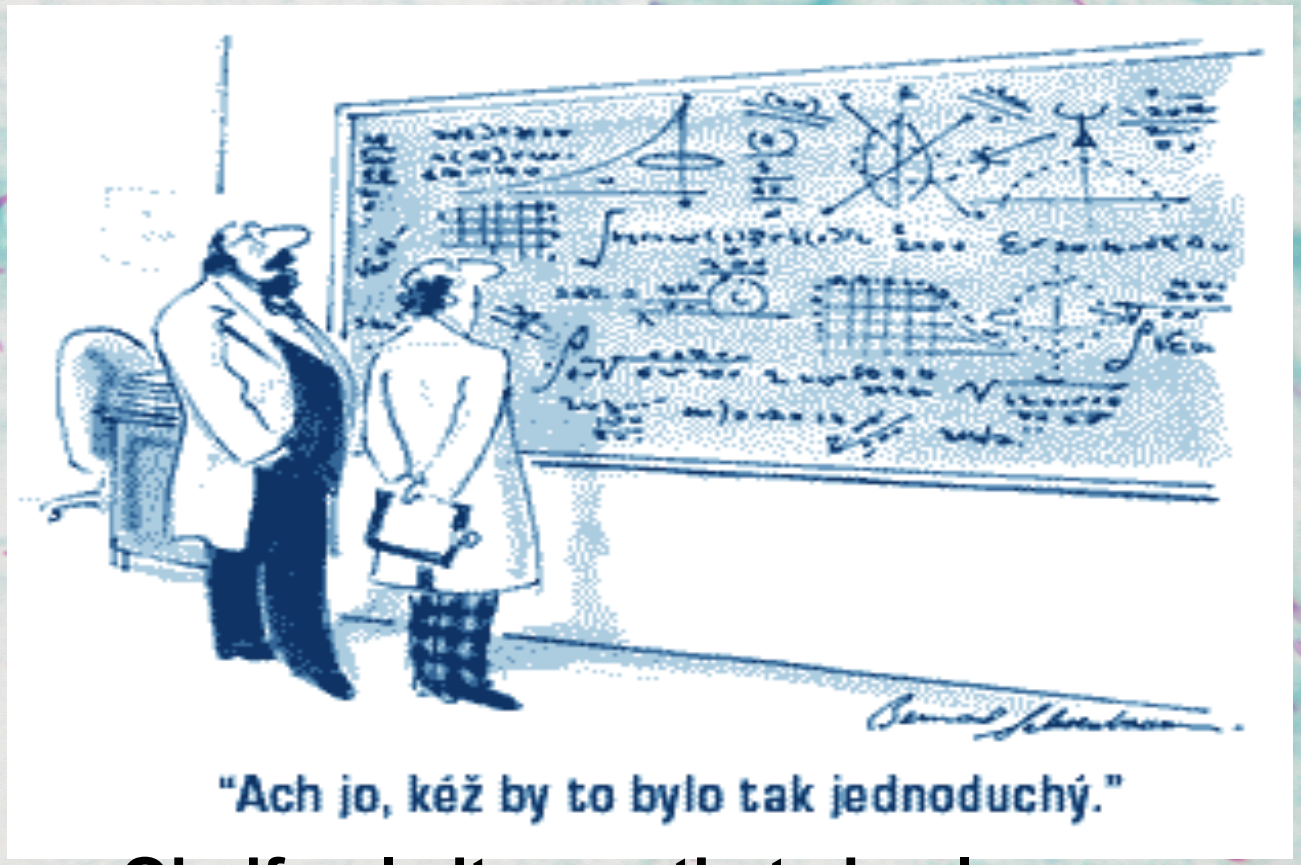
- IS1610-RFLP – restrict analysis
- methods detected repeated sequences – PCR, spoligotypisation, MIRU-VNTR
- **sequenation**



www.cdc.gov/ncidod/eid/vol4no4/pfyffer2b.jpg

Molecular methods in praxis…

M. tuberculosis



Oh, if only it were that simple

Technology of molecular methods

1 NA isolation → 2 amplification → 3 product detection

washing
centrifugation
repeatly, 1-2 h.

cycler
1-2 h.

on metod
0,5 – 3 h.

varies step of automatisation
- NA isolators
- Pipetors
- Full automatic devices

M. tuberculosis

Technology of molecular methods

M. tuberculosis

Problem

Detection of genes common for MTBC complex

Most of tests are not able to different species – problem is using of probe common for all species of MTBC complex

next tests

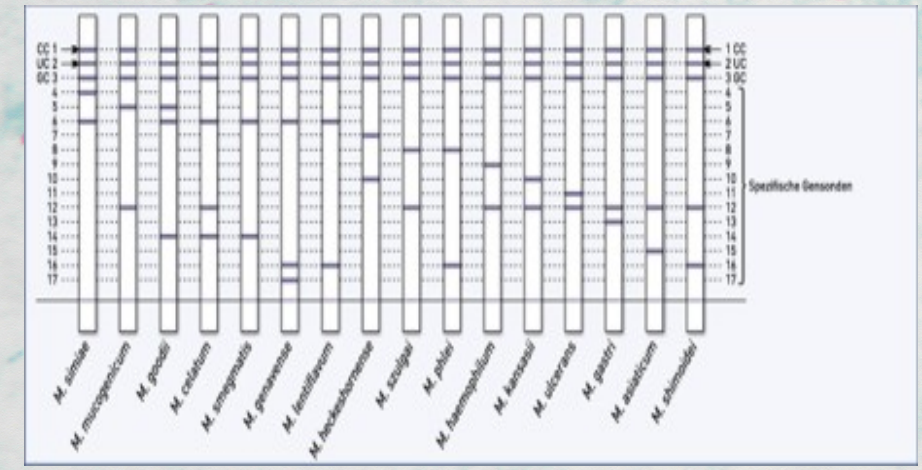
Low sensitivity – best - GeneXpert 112 CFU/ml

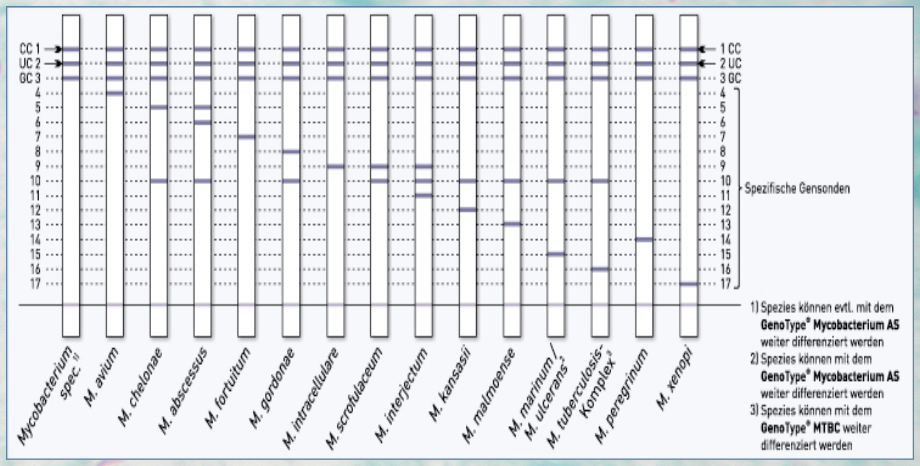
(Jones M., 2009)

Isolation NA - during the destruction of a bacterial cell (cell wall) is destroyed also detected NA.

strip methods GenoType

- GenoType Mycobecteria Direct – ***M.tuberculosis*** **komplex, M. avium, M. intracelullare, M. kansasii, M. malmoensae**
- GenoType MTBC – **differentiation *M. tuberculosis* complex from cultured strain**
- GenoType Mycobacterium AS/CM – **resistance on AT**



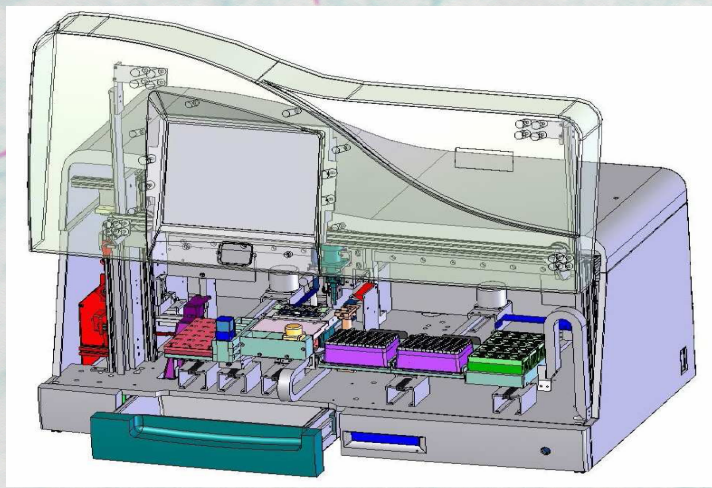


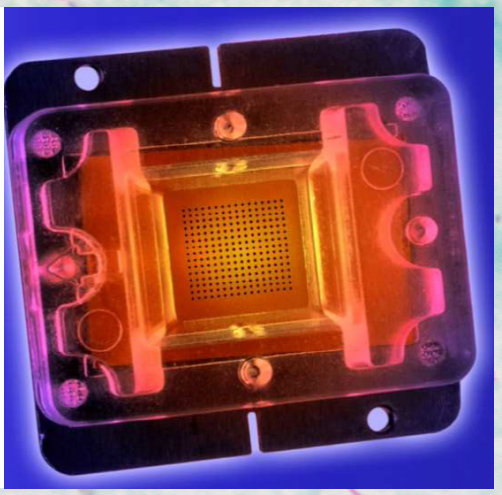
GeneXpert

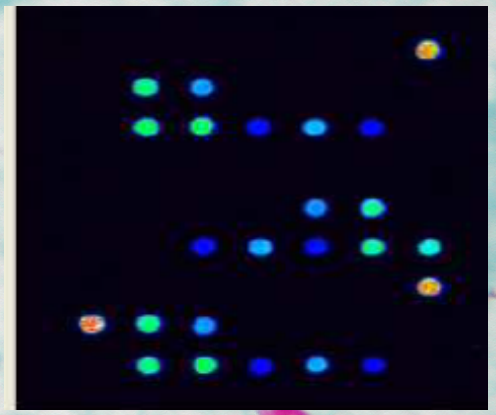
- full automatic system multiplex RT PCR
- NA isolation + RT PCR
- result in 1,5 hours
- MTBC complex + resistance on RIF
- High susceptibility

INFINITI -microarray

- NA isolation
- end point PCR
- microarray hybridisation – 240 probes
- viral respiratory infection, MTBC + RIF, INH, PYR







reporting results of direct microbiological methods

Positive microscopy – pacient spread AFBs (mycobacteria) = TB infection

Positive cultivation (solid m., metab. met.) – pacient spread alive mycobactera = TB infection

genetic methods

Cultured strain – 100% good result

Direct from clinical sample

positive
- alive bacteria = dg TB infection
- NA of death bacteral cell
- false positive

negative
- it is not mycobacterial infection
- false negative

mydobadteria

M. tuberculosis 2001

62 MTBC findings

	positive	negative
Microscopy	**23(39%)**	**36(61%)**
LJ	**61(91,4%)**	**1(1,6%) MGIT +**
MGIT	**57(98,3%)**	**1(1,7%) LJ +**
PCR	**23(92%)**	**2(8%) MGIT, LJ +**

Average time of cultivation:

LJ	**27,9 days**
MGIT 960	**12,4 days**

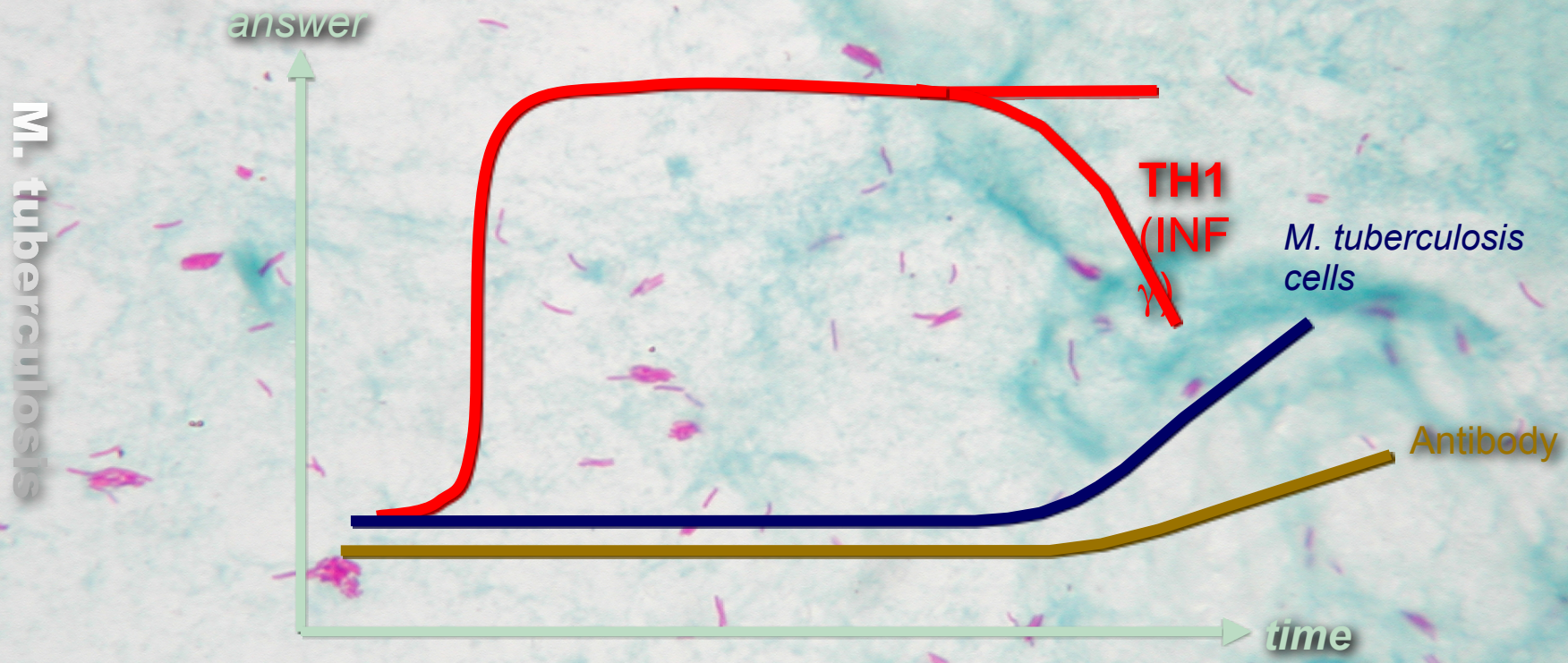
undirect diagnosis (latent TBC)

- Antibody – negative in 30% of microscopy positive TBC
- **Skin test**

- **Cytokine tests (IGRA tests)**

M. tuberculosis

imunity answer – latent TB



Infection, recurrence

nfection, recurence

latent TB

deseas

undirect diagnostic - princip

M. tuberculosis

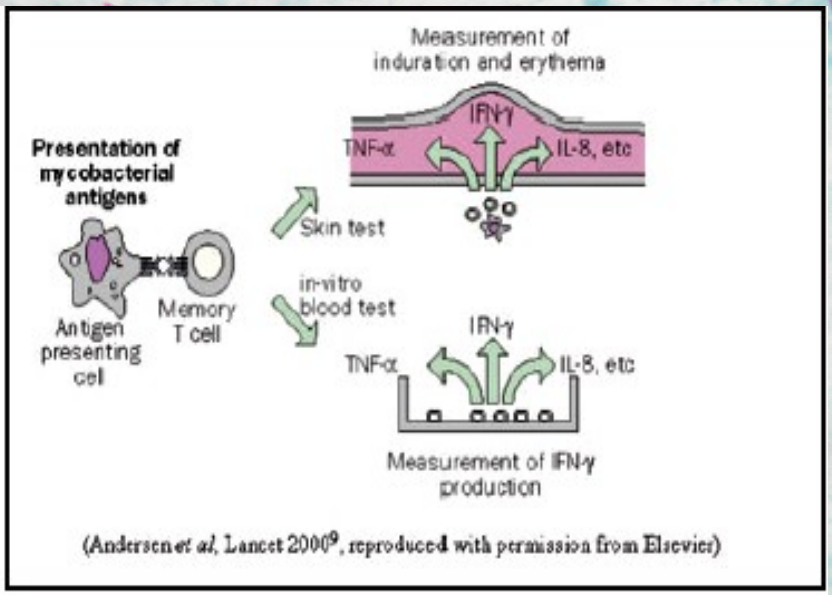


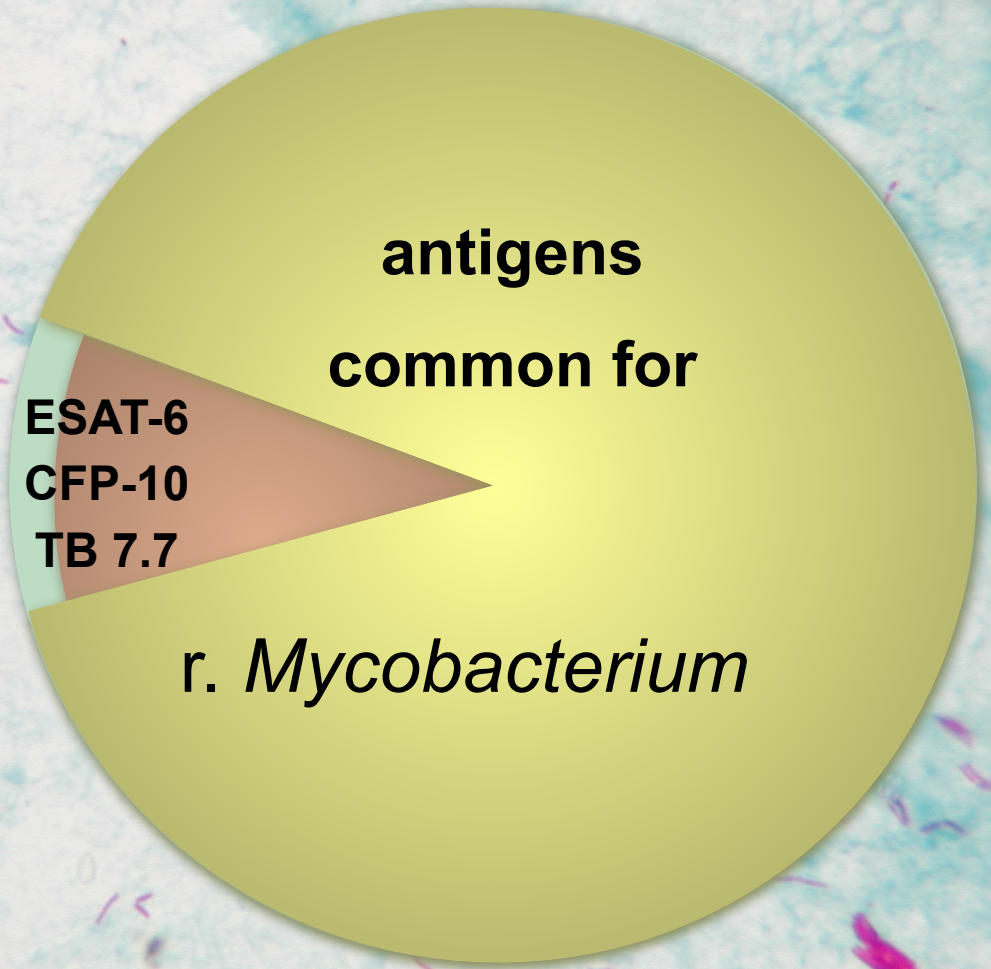
Figure 1: Biological basis of cellular immune based tests[9]

- **Stimulation of mycobacterial antigens**
- **Accumulation of monocyto-makrophag cells and T lymfocytes**
- **INF*γ* production**

Mycobacterial antigens

***M. tuberculosis Complex* specific antigens**

Encoded of genes Regions of Difference (RD)



future

- Molecular methods – detection, identification, resistance, epidemiology
- culture methods

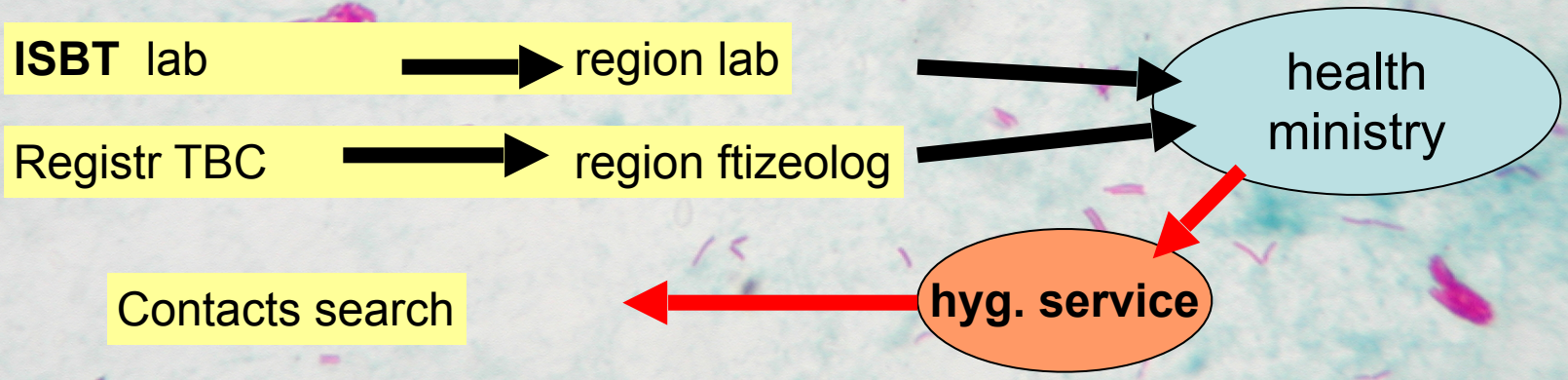
  gold standard
- Indirect methods

  INF γ

M. tuberculosis

Systems for TBC control

- **Registr TBC** = duty for every doctor report new case TBC – „region ftizeolog“ – hygienic service
- **ISBT** = information system of bacilar TBC. Duty of every lab doing TBC detection report all positivne findings. Electronic form.
- Both systems are linked.
- Goal – TBC control .



Examples from practise

Woman 38 years
Labor technician (TBC lab.), personal problems,
Autumn 2003 – pneumonia, positive tests on chlamydia
control X-ray – determined dg lung TBC.
All microbiological invetigetions negative incl. PCR.
6 month therapy, reparation – industrial disease.

Problematical work placement.
It was not tuberculosis, but they had taken money for professional ilness…...

Casuistics - my experiences

Woman 75 years
Very „good looking“, without health problems.
Long time cough, sputum - culture on TB negative,
PCR M.tbc complex positive – closed as TBC.

All others microbiological investigations negative!

TB therapy – after therapy without problems.

6 month in sanatorium
mental trauma also for husband
…most likely she never had TBC …

Casuistics - my experiences

Man 50 years, artist (painter)
In 1980-1989 treated for tuberculosis
Living alternately by 3 woman (Prag, Brno, Liberec)
Refused medical (TB) controls.

Accidet in car by Hradec Kralove – hospitalised in University hospital.
Chest contusion, broken leg.
Worsing respirátory function, arteficial respiration,
after 3 weeks diagnosed TBC (microscopy – masive expectoration)

Chenging clinics (pulmonary clinic, infection clinic).
After 1 month death.
…3 EPV devices contanonated, many workers i contact …

mystery

	sputum A	sputum B
Date of investigation	3.5.2002	3.5.2002
Microscopy	neg.	neg.
L-J cultivation	neg.	neg.
MGIT 960	positive	positive
PCR	-	-
	M.TBC	M.TBC
INH	R	R
STM	R	R
RIF	R	R
PZA	R	R
EMB	R	R

= XDR TBC !!!

Pacient A (V.J.)
80 years, man, HK (town)
polymorbid (ICHS,OCHPN…)
Repeatedly hospitalised

repeatedly investigated
on TB, all negat.

Pacient B (J.J.)
51 years, man, village
repeatedly investigated
on TB, all negat.

...in historyTBC...

…in next course both pacients repeatedly investigated on TB – all negative incl.PCR….
From TB wiew – both evidently healthy…..

possibilities

1. Laboratory misteak

Contaminaion of samples

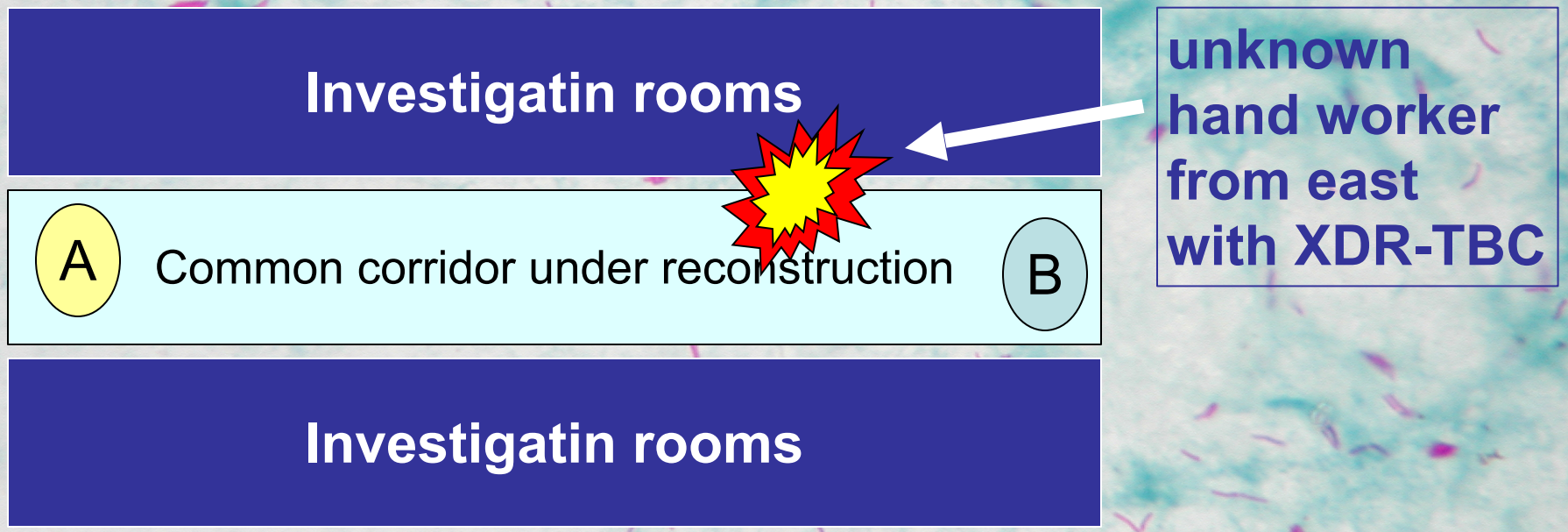
- **Both of another sample** — **excluded**
- **Bad susceptibility on AT** — **repeately excluded**

2. Pacients were in contact

3. Pacients were in contact with third person

Possibile solving

Both patiens were in the same day on special investigetion included sputum taking on TB cultivation...



Bohouš a beer bunch

Man 48 years

- „homeles“, daily abusus
- death of body chilling + alkohol
- autopsy – developed lung TBC

Did he infect his friends in pub? **YES**

Will bee his friends ill? **NO (body mass > 100 kg)**

…fortunatelly nobody was investigetad of new methods or PCR

…Bohouš „job“ was **occupated in 14 days !!!**

construction

Heat – I drink beer in pub – I become cough – sputum on TBC + PCR – it is fuck…….


…děkuji za pozornost