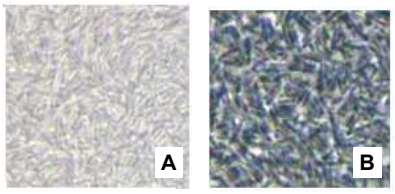
3. Direct detection & typing of infectious agent

DEFINITION OF DIRECT AGENT DETECTION & TYPING

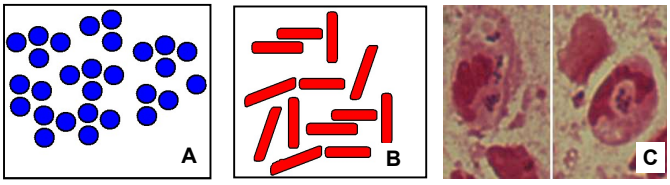
An infectious agent is detected in clinical material directly (confirmation of it or its structural part by microscopy, culture, DNA amplification, others) or indirectly (detection of specific antibodies).
Typing means a classifying of clinical isolates in particular types (e.g. serotypes, clones).

APPLICATION OF MICROSCOPY TECHNICS

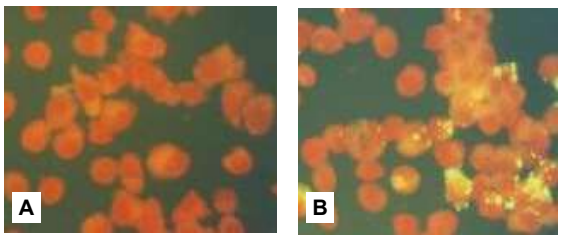
a) bright field microscopy: see in Diagnostic Principles (Course 1)
b) dark field microscopy: oblique light scattered by an object create an image, higher contrast
c) phase contrast microscopy: phase shifts in the light passing through a transparent specimen are converted into contrast changes in the image
d) fluorescence microscopy: structures of interest stained with fluorochromes emit fluorescence
e) transmission electron microscopy: electron beams passing through an object creating an image, higher resolution (0,05 nm)
f) scanning electron microscopy: visualizes details on the surfaces of cells and particles, nice 3D view



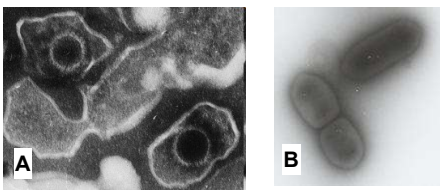
Bright-field (A) & phase contrast microscopy (B). Mesophilic bacteria in drinking water.



Schematic diagram of the most common bacterial shape and Gram properties as seen by a bright field microscopy: gram-positive cocci (A, Staphylococci), gram-negative rods (B, Enterobacteriae) and direct detection of intracellular *Staphylococcus aureus* in neutrophils of sputum from a pateint with cystic fibrosis.



Bacterial or viral agents could be detected directly after reaction with specific antibodies which are labeled by a fluorochrome. Emitted light is detected by an immunofluorescence microscope (1).



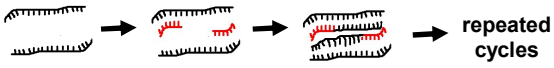
Electron microscopy is especially useful for detection of viral particles in clinical material. Dependig on their structure, symmetry and size, viruses can be sort at least into famillies (A, two Epstein Barr Virus virions loosely surrounded by the membrane envelope) (ref.2).
Bacteria are observed by electron microscopy usually only becuase of experimental reasons (*Listeria monocytogenes* from hemoculture of a febrile patient cultivated previously on a nutrient agar).

APPLICATION OF NUCLEIC ACID BASED METHODS

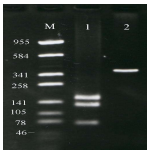
a) uncultured and fastidious agents: e.g. Bartonella sp., *Mycobacterium leprae*, some viral agents
b) too small number of agent in specimen: e.g. CF patient sputum – chronic pathogens – *Burkholderia cepacia*, *Pseudomonas aeruginosa*
c) non-viable agent: e.g. after antibiotic therapy
d) slow-growing agent: e.g. *Mycobacterium tuberculosis*
e) to determine resistance genes: also for therapeutical purpose
f) to determine structural genes of toxins: e.g. staphylococcal enterotoxins
g) agent typing: identification on subspecies level (outbreaks - especially nosocomial bacterial agents but also typing of isolates from various sources – food, animals etc. if they could be source of an infetction)

a) Principle of DNA amplification by PCR methods

Using specific primers (short DNA fragments complementary to detected DNA), polymerase (enzyme synthetizing DNA strands during repeating cycles) and magnesium cations. Amplicon with a known size is synthetized.



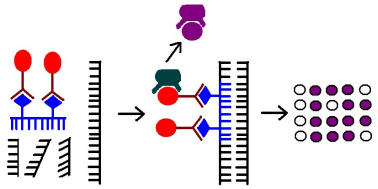
Schema of PCR reaction form the left to the right



Detection of PCR amplification:
Because of total negative charge of DNA it migrates in gel electrophoresis towards the anode. Speed of an amplicon migration depends on its size and could be detected be intercalating DNA dyes. The amplicon of *gltA* gen -379 bp(2) and its restriction profile (71, 137, 171 bp)(1) specific for *Bartonella henselae*.

b) Principle of DNA hybridization methods

A complemetary DNA probe (DNA labeled with e.g. an enzyme) is bound to the target DNA fixed on a carrier (e.g. nylon membrane). After repeated washing the prescense of labeled DNA is visualized by adding a substrate which react by the enzyme releasing a colour (see figure below).

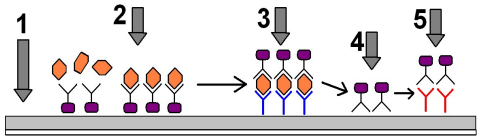


c) Principle of sequence based methods

Sequencing means to find the extact base sequence in the DNA. Target DNA fragment (amplicon) should be amplified first and then sequenced. Goal of the methods is that results are electronically portable comparing to band-based methods.

OTHER METHODS IN DIRECT ANTIGEN DETECTION

Immunochromatographic method: A specimen is processed and added into a well (1) on a solid chromatographic carrier. The Ag binds to the specific (rabbit) Ab (2) which is labeled e.g. by latex. This complex moves by capillary action to form a coloured line in position of immobilised anti-Ag antibody (3). Reaction control; unconjugated Ab (4) moves toward goat antirabbit Ab to create a coloured complex (5), indicating that the test has worked properly.



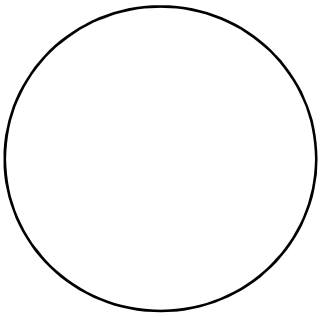
reference:
1. Melter o, Detection and molecular characterization of MRSA and Bartonella henselae in the Czech Republic, Thesis, 2003
2. http://biestmilch-seven.com/archives/2009/08/18/chronic-epstein-barr-virus-infection-in-athletes-or-dare-to-take-a-break.html

PRACTICAL PART – DIRECT DETECTION & TYPING OF INFECTIOUS AGENTS

1. NATIVE PREPARAT: Mix a bacterial biomass grown overnight on nutrient agar at 37˚C with physiological solution , drop on slide and put cover glass. Examine by magnification 40x and 100x with immersion oil.

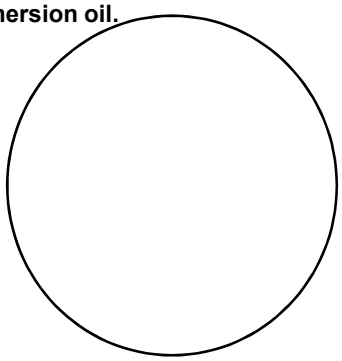
bacterial species:

magnification: 40X

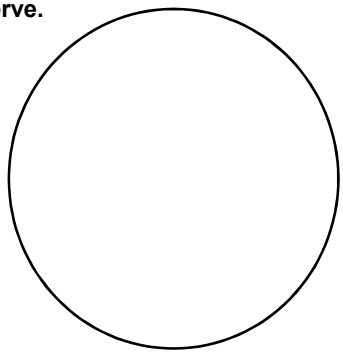


bacterial species:

magnification: 100X

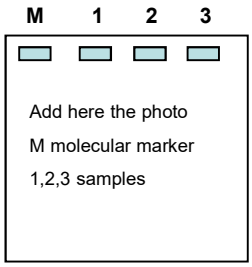


2. GRAM-STAINING: Mix a bacterial biomass from an agar with physiological solution on slide and dry it. Fix the preparat by drying the culture over the flame. Stain the preparat by Gram-staining procedure (Course 1). Examine it by 100x magnification and immersion oil. Specify which *morphology* the bacterial cells have and if they are *Gram-positive or Gram-negative* or other details you could observe.

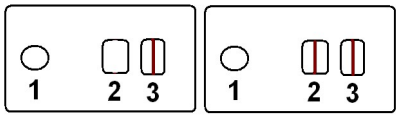


3. Isolate DNA from a patient specimens (e.g. sputum), prepare master mix for PCR amplification (using the protocol below) and using thermal cycler do PCR amplification of DNA. Prepare the gel for electrophoresis, load the wells with samples and a marker, run electrophoresis and detect specific amplicons using ethidium bromide staining. Take photo of the gel, label position of particular samples on it and stick below.

reaction	Primers (ul)	Water (ul)	PCR mix, ul
1	0,5	12	12,5
2	1	24	25
3	1,5	36	37,5



4. Read the results of immunochromatic tests for detection of *Streptococcus pneumoniae* or *Legionella pneumophilla* in patient samples.



LAB QUIZ

1.What branches of microscopy do you know?

2.Can we use light microscope for detection of viral agents? Yes No

3. Describe Gram staining procedure and what structures is responsible for differences in Gram staining?

4. Specify procedures for direct detection of infectious agent and describe their principles.

5. Specify limitations of DNA amplification methods in microbiological analysis.