Genus *Mycobacterium*

The family of *Mycobacteriaceae* includes more than 100 species. The medically important *Mycobacterium spp*. Include:

- *Mycobacterium tuberculosis, Mycobacterium bovis* (and *M. africanum, M.microti and M. canetti;* known as the *Mycobacterium tuberculosis* complex) – the causative agents for tuberculosis,
- *M. leprae* – the causative agent for leprosy and
- Non-tuberculous mycobacteria, causing mycobacterioses (infection of lungs, skin, bones, soft tissue, lymph nodes or systemic infections).

For the means of this compendium for dental medicine students, we will only focus on *Mycobacterium tuberculosis.*

Recall from the introduction to the medical microbiology laboratory, that only specially authorized mycobacterial laboratories are allowed to process suspected mycobacterial pathogens under strictly controlled safety precautions, which include biosafety cabinets, negatively pressurized rooms, protective clothing and ventilation. Personnel handling such specimens are subjected to annually conducted Mantoux tuberculin tests - to determine infection.

General characters of *Mycobacterium spp.* include:

- Filamentous, non-motile and non-sporulating bacilli
- Obligate aerobes
- Bacterial cell wall with high lipid content, especially mycolic acid

The high lipid content of *Mycobacterium spp.* has two important effects:

- Resistance to antiseptics
- Staining only possible with heating but discoloration with acids and alcohols is not possible - "**acid-alcohol resistant**" / "**acid fast**" bacilli
    - ➔ Gram-staining is not possible, or unreliable
    - ➔ Other staining techniques are required, e.g. **Ziehl-Neelsen acid fast stain**

Ziehl-Neelsen staining

Ziehl-Neelsen staining is a staining method to colour acid-fast bacilli, because these are impermeable to dyes due to their high lipid and wax content within their cell wall. In such cases, the usual Gram-staining technique is inconclusive. Acid-fast means that these bacteria cannot be decolourized by acids after staining with carbolfuchsin, e.g. they retain the red colour of the carbolfuchsin stain. Bacteria that are decolorized after addition of an acid decolorizing agent lose the red colour, thus they are termed non-acid fast bacteria. In order to help penetration of dye into acid-fast cells, heat and phenol are added. Ziehl-Neelsen staining is the gold standard for the diagnosis of tuberculosis and leprosy (+*Nocardia and Cryptosporidium)*

The reagents for the Ziehl-Neelsen stain include:

- Carbolfuchsin (red colour)

- Mordant (heat)
- 20% sulphuric acid (decolourizer) – acid-fast bacilli retain the red dye after decolourization
- Methylene blue (counter stain) – other elements of the smear including the background will be blue

Procedure:

1. Smear flooded with **carbolfuchsin**
2. **Heating** (for penetration of cell wall)
3. **Washing** with water
4. **Decolourizing** with 20% sulphuric acid
5. **Washing** with water
6. **Counterstain** with **methylene blue**
7. **Washing** with water

Under the microscope, the smear will reveal the following:

- red acid fast bacilli (could not be decolorized by the sulphuric acid)
- all the other elements (bacteria, epithelial cells, etc) stained in blue (they have been decolorized by the sulphuric acid and were then counterstained with methylene blue).

Mycobacterium tuberculosis

Clinical Significance

Mycobacterium tuberculosis is one of the causative agents of tuberculosis, a disease with pulmonary and extrapulmonary manifestations, e.g. ganglia, meninges, bones, joints, internal organs and urogenital areas.

The mode of transmission is via airborne aerosols, thus the lung poses the site of entry. In advanced stages, the microorganisms can haematogenously disseminate and cause systemic tuberculosis.

Bacteriological Diagnosis

Collection

Collection of specimens depends on the site of infection. Most commonly pulmonary specimens are submitted to the mycobacterial laboratories, as are bronchoalveolar fluids, e.g. sputum. Sputum samples are challenging to collect, because emphasis has to be given to avoid contamination with saliva and secretions from upper airways. The optimal moment for sputum collection is in the morning, because a higher amount of sputum is secreted during the night, which remains stagnant in the lower airways. An indirect method to collect such sputum samples is to instruct the patient to energically rinse their mouth with saline solution, after which the patient has to cough and expectorate into a sterile container. The direct method is performed via bronchoscopy or tracheal puncture. Other specimens include CSF, gastric lavage fluids, blood, urine, tissue, bone marrow aspirate or wound specimens.

Microscopic examination

In order to conduct a microscopic examination, preliminary treatment of the specimen has to be performed:

- Homogenization with 4% NaOH for 30 minutes at 37°C
- Centrifugation for 10-15 minutes at 3000rpm, after which the sediment is used for further examination

After application of Ziehl-Neelsen stain, the slide is examined for at least 10 minutes with a minimum of 100 microscopic fields.

Microscopic examination reveals thin red, encurved bacilli arranged in groups, angulated pairs or isolated

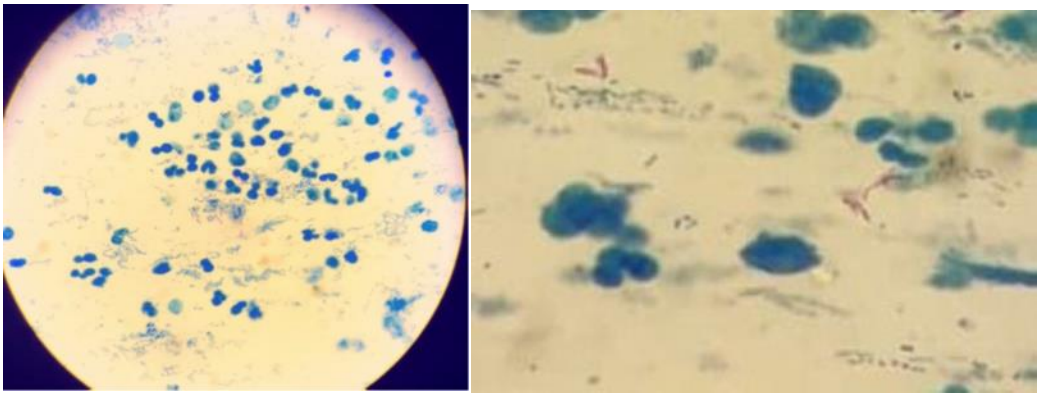

Figure 29. *M.tuberculosis* : microscopic examination –Ziehl-Nielsen coloration

Culture media and Identification

The sediment obtained from centrifugation with prior homogenization is inoculated into solid media. The media used is the **Löwenstein-Jensen** media that contains egg, glycerine and asparagin. The tubes are then incubated at 37°C for **2-4 weeks**, because mycobacteria are slow growing germs.

Colonial characters include prominent, rough, irregular colonies that are slightly yellowish.

Besides the identification by colonial characters and microscopic examination, specific biochemical tests are to be performed, in order to diagnose *M. tuberculosis*:

- Niacin test
  - ➔ Production of niacin by *M. tuberculosis* in egg-containing media - canary yellow colour (negative in other mycobacteria)
- Peroxidase test: positive
- Catalase test: weakly positive
- Tuberculostatic susceptibility tests

Antimicrobial susceptibility testing (Antibiogram)

Multidrug resistant species have been identified and pose a major public health problem. Treatment of tuberculosis is standardised and employed according to a treatment scheme. The curative treatment of tuberculosis lasts 6-12 months with association of at least three antituberculous agents. The first-line antituberculous drugs are isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. The second-line antituberculous drugs are used only for the treatment of drug-resistant tuberculosis and include kanamycin, amikacin, ethionamide, cycloserine and paraaminosalicylic acid.

Essential to remember:

- Thin, red, encurved acid-fast bacilli
- Slow growing
- Niacin positive
- Major causative agent for tuberculosis
- Gram-staining not possible - Ziehl-Neelsen staining

Spirochaetales

Spirochetes are bacteria that are spiral in shape and coiled. They include two medically important families i.e. *Spirochaetaceae,* to which the genera *Treponema* and *Borrelia pertain, and Leptospiraceae* to which the genus *Leptospira* pertains.

General characters of the *Spirochaetales* include that they are highly motile due to flagella and axial filaments, as well as a long (up to 250µm) and thin (0.1-0.6µm) spirally or helically coiled morphology.

For the means of this compendium, we will only focus on the genus *Treponema*, more specifically *Treponema pallidum*.

Treponema pallidum

Clinical Significance

T. pallidum is divided in to three pathogenic subspecies:

- *T. pallidum spp. pallidum* – the causative agent of **syphilis**
- *T. pallidum spp. endemicum* – the causative agent of **endemic syphilis**, also known as **bejel** (Africa, Middle east, Australia)
- *T. pallidum spp. pertenue* – the causative agent of **yaws**, a granulomatous skin lesion with degenerative lesions in lymph nodes, bones and joints

T. pallidum spp. pallidum is the causative agent of **syphilis**, which is a sexually transmitted disease (STD) via sexual intercourse. Another route of transmission is from mother to child (transplacental or intra-partum), also known as **congenital syphilis**

Syphilis has three evolutionary stages if **left untreated**:

- **Primary** syphilis: ~15 days after infecting contact with the manifestation of a **chancre** – a painless ulceration at the entry site (penis, vagina, oral or anal mucosa)
- **Secondary** syphilis: follows ~45 days after onset of primary syphilis through haematogenous dissemination with the manifestation of reddish papules on trunk and extremities (**skin rash)**.
- **Tertiary** syphilis: after a latency of 5-15 years with destructive lesions of CNS, cardiovascular system, muscles, bones.
    - ➔ 3 forms of tertiary syphilis:
        - **gummatous** (15%): gummas are soft, tumour-like granulomas of the skin, bones, soft tissue and liver
        - **cardiovascular** (10%): aortic aneurisms
        - **neurosyphilis** (6.5%): early meningitis with later development to general paresis, tabes dorsalis and dementia

Bacteriological Diagnosis

Collection

Collection of specimen depends on the stage of infection. In primary syphilis, the chancre secretions are collected. For secondary syphilis, secretions from skin lesions are collected; the clinician should choose the most recent lesion, remove the crust and press the lesion in order to cause bleeding to collect the serous exudate. Blood for serological examination can be collected in **all stages** of disease.

The specimens must be transported and examined as soon as possible, as treponemae are not viable for a long time outside the body.

Microscopic examination

T. pallidum cannot be visualized through brightfield microscopy, because of its very thin morphology. Thus, microscopic examination is achieved through darkfield and phase contrast microscopes.

When examining a wet mount, the treponemae have shining, corkscrew-like appearance with high motility. Silver staining can be used to stain *T. pallidum.*

Keep in mind, that oral specimens might contain commensal treponemae with no diagnostic relevance.

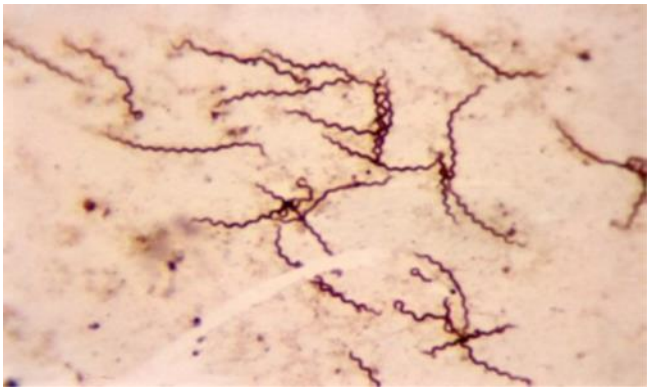

Figure 30. *T. palidum* - microscopic examination, Fontana-Tribondeau staining

Culture media and Identification

T. pallidum are extremely fastidious and cannot grow on culture media.

Thus, identification is based on microscopic examination of the chancre/tissue samples combined with serological tests (detection of antibodies against various Treponema antigens)

- Cardiolipinic Ag present in all treponemae and in other bacteria
- Proteic Ag (Reiter), genus specific and present in all treponemae
- *Treponema pallidum* specific Ag, only present in *Treponema pallidum*

The diagnostic tests include:

- Nonspecific (nontreponemic):
  - VDRL (flocculation)
  - Bordet-Wassermann reaction (complement fixation)
- Specific (treponemic)
  - TPI (*Treponema pallidum* immobilization) test
  - Passive hemagglutination

VDRL

Principle: antibodies (**anti-cardiolipin Ab**) produced by a patient with syphilis react with an extract of ox heart. The reaction is visualized through foaming of the test tube fluid, known as **flocculation**.

Bordet-Wassermann test

Principle: Ab in patient serum will inactivate a serum complement in the presence of “reagines”, which are produced by infected tissue in response to a bacterial infection.

TPI

Principle: specific **anti-*Treponema pallidum* Ab** in the patients serum, in the presence of a serum complement, immobilize actively motile *T. pallidum* obtained from testes of syphilis infected rabbits.

Passive hemagglutination

Principle: specific **anti-*Treponema pallidum* Ab** in the patient's serum cause agglutination of treponemic Ag adsorbed on the surface of RBCs.

Figure 31. *T. pallidum*-serological tests: (a) RPR, (b) Sandwich immunocromatographic test

Antimicrobial susceptibility testing (Antibiogram)

T. pallidum is universally susceptible to penicillins. Alternatively, in the case of allergy to penicillins, Doxycycline, tetracycline or azithromycin can be administered. Pregnant women with syphilis are obliged to receive penicillin in order to prevent congenital syphilis.

Essential to remember:

- Very thin, coiled and motile microorganisms (only visible through darkfield or phase contrast microscopy)
- Causative agents for syphilis, bejel and yaws
- Three stages of syphilis, if left untreated
- Cannot grow on culture media