16. Anaerobic bacteria

16.1. GENERAL FEATURES

Anaerobic bacteria obtain energy (ATP) exclusively by fermentation using small organic molecules (e.g. pyruvate) as final acceptors of electrons. They are members of the normal flora (e.g. in GIT, oral cavity) but because of their invasion and toxic properties they can cross the physiological barriers of the host and cause skin and soft tissue infections and toxinoses (e.g. genus *Clostridium*). Many of them are spore forming and hence can survive in environment for decades because they are able to resist to extreme conditions. Other anaerobic bacteria (e.g. genus *Bacteroides*) cause infections of various organs including also bacteremia.

16.2. VIRULENCE FACTORS & PATHOGENESIS

Virulence factors are genetic, biochemical, or structural features that enable an organism to cause disease.

Hydrolytic enzymes such as proteases, collagenases, hyaluronidase and DNases hydrolyze tissue and promote the spread of infection (*C. perfringens*). **Exotoxins:** Alpha toxin, a lecithinase produced by *C. perfringens,* causes degradation of host cell membranes, which leads to tissue destruction, increased vascular permeability and bleeding. Tetanospasmin is a bi-component A-B neurotoxin produced by C. *tetani*. The B component binds to neurons membranes which causes endocytosis of the toxin. The A component blocks release of inhibitory neurotransmitter, which causes unregulated excitatory stimulation and severe muscle spasm. Botulinum toxin is bi-component toxin produced by *C. botulinum*. It is composed of a light and a heavy chain. The light chain blocks the release of acetylcholine at peripheral synapses. This leads to flaccid paralysis. *C. perfringen's* enterotoxins alters of the membrane of enterocytes leading to loss of fluid and intracellular proteins. **Enterotoxin (toxin A),** potentiates fluid secretion and the inflammatory response (*C. difficile*). **Cytotoxin** (toxin B) disrupts protein synthesis and causes rearrangement of host cell cytoskeleton (*C. difficile*). The polysaccharide **capsule** is responsible for the resistance to phagocytosis (*Bacteroides fragilis*). **Fimbriae** are responsible for adhesion to host cells (*B. fragilis*, *Prevotella melanogenica*).

16.3. INFECTIONS & EPIDEMIOLOGY

Wound infections*.* Endospores of *Clostridia* spp. or vegetative cells of other anaerobic species (e.g. *Bacteroides*) can contaminate open wounds and cause severe infections (e.g. traumatic myonecrosis/gas gangrene, clostridial endometritis). Fermentation of tissue carbohydrates yield gas which can be felt on palpation. Tetanus has a variable incubation period (days-weeks), a shorter period occurs in more severe disease and when the wound is closer to the brain. The site of infection is the first region to be affect with spastic paralysis. This is followed by the characteristic spastic paralysis of the jaw muscles known as trismus or lockjaw. Gradually other muscles become involved and finally chest muscles paralysis leads to respiratory failure (mortality up to 60%).

Food poisoning caused by *C. perfringens* enterotoxin happens after cooking fails to inactivate spores, followed by keeping the meal for several hours in conditions that allow bacteria to germinate. The symptoms are nausea, abdominal cramps and diarrhoea and the infection is self-limited. Classic botulism starts from 12 – 36 hours after digestion of toxin-containing food, progressive paralysis develops and due to subsequent respiratory failure around 15% of the affected patient have fatal outcomes. Infant botulism is caused by consumption of honey contaminated with botulinum spores.

Antibiotic associated diarrheae (AAD) is caused by disruption of normal flora by propagation of *C. difficile* and their toxins.

16.4. TREATMENT, PREVENTION & CONTROL

Wound clostridial infections
Treatment: Start of the therapy should not be delayed! Combined therapy is applied to eliminate the agents by antibiotics (e.g. penicillin), to neutralize the exotoxins by applied antitoxins and to saturate the affected tissue with oxygen. Surgical debridement of necrotic tissue is also effective in large wounds. Once the toxin is bound to a cell surface receptor it is not able to be eliminated by the antitoxin. Prevention: Immunization with toxoid, usually administered in DTP triple vaccine (tetanus toxoid and pertussis antigen is also included).
Food poisoning. *C. perfringens* infections need not be treated with antibiotics as they are self- limiting. Botulism should be treated by ventilatory support, lavage of the organism from GIT and specific therapy by metronidazol or penicillin and antitoxin application.
Antibiotic associated diarrheae (AAD): discontinuation of suspected antibiotics, fluid replacement and metronidazol or vancomycin.

16.5. LABORATORY DIAGNOSIS

The presumptive diagnosis a decision to treat anaerobic infections must be based on clinical observation and must never be delayed!
a) Specimens: pus, necrotic tissue, and other clinical material depending upon the localization of the infection.
b) Microscopy: gram-positive (usually spore-forming) or gramnegative bacteria (see fig. 1).

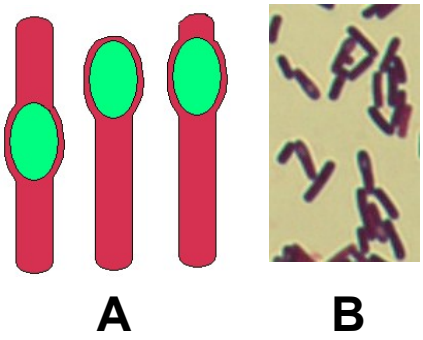
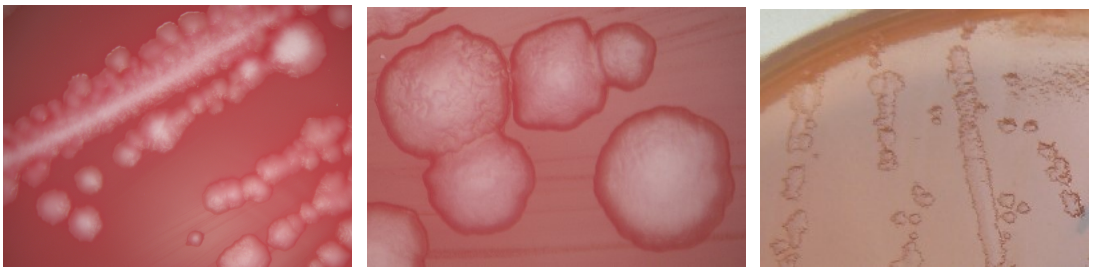


Fig. 1. Diagram of most frequent location of endospores (A, from the left to the right): central, terminal (C. tetani), subterminal (C. difficile). In some Gram-stained bacterial cells of C. difficile the subterminal spores can be seen (B).

c) Culture: Anaerobes grow on enriched culture media in atmosphere without oxygen (oxygen is toxic beacuse they do not synthetise catalase). Majority of anerobic infections are polymicrobic with facultative anaerobic species that complicate the isolation of a pure culture. So disks with antibiotics (e.g. aminoglycosides) are used to inhibit the other species or an agar media containing antibiotics can be used.



A B C

Fig. 2. Colonies of C. difficile growing on Schaedler agar (A), close of A (B) and colonies growing on yolk egg agar (C).

d) Phenotypical identification are focused on detection of specific biological properties (microscopy, bacterial colonies, spores, enzymes detection)(see also chapter 6 – Identification).
Screening tests (preliminary identification): detection of some phenotypic properties. Anaerobes are always catalase-negative.
Biochemical identification. To identify an isolate on species level multiple metabolic enzymes should be tested by the key dichotomous method or by numerical identification (see chapter 6 – Identification).

Fig. 3. Typical biochemical profile of C. difficile clinical isolate with substrates in a diagnostic kit.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry - (MALDI – TOF MS): analyses molecular structure (mainly proteins) of an unknown microbial isolate, as the mass spectral pattern consisting of a number of structurally related mass spectral peaks, and comparing with a known patterns analyzing the isolate. The mass spectrometer first ionizes, then mass separates and finally detects time of the ions flight, thus producing a mass spektrum and comparing it with known mass spektra analyse the studied microorganism (see also chapter 6 - Identification).

e) Genotypical identification. There are several ways to identify a strain or species using genotype. For instance, comparing electrophoresed fragments allows visualization of the specific fragments (amplicons) or restriction profile. Alternatively, homology of highly conserved regions, such as the 16S RNA gene, can assist identification of the species. (see also chapter 6 – Identification).
(fig. 4).

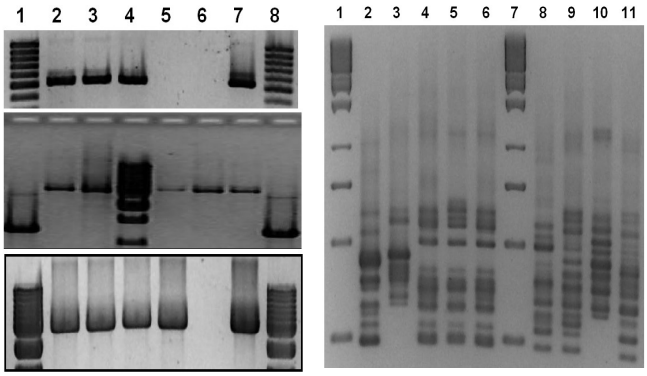
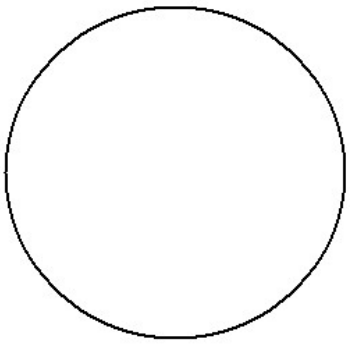


Fig. 4. Detection of the genes of binary, A and B toxin of C. difficile (from the top to the bottom) (A). As well as being virulence factors they can also be used as species specific markers. Clonal analysis (typing) of C.difficile on subspecies level by ribotyping (B).

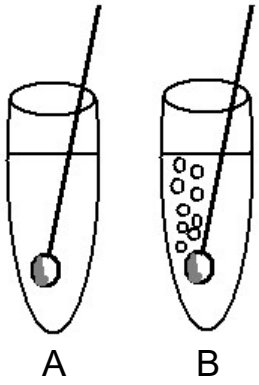
f) Susceptibility testing. *Qualitative (disk diffusion method), quantitative methods or their combination (Etest) are used to test the susceptibility in atmosphere without oxygen but the analysis is not as well standardised as it is in aerobic bacteria (see chapter 5 – Antibiotic susceptibility testing).*

16.6. PRACTICAL PART – ANAEROBIC BACTERIA

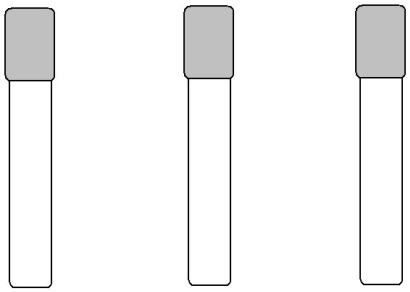
Excercise 1: *MICROSCOPY OF CLOSTRIDIUM CULTURE: Dry, fix and stain a clostridial culture by the Wirtz-Conklin staining procedure (stain spores with 5% malachite, heat over flame for 3 minutes until steam appears – don't heat it too much, the sample must remain as a solution!, repeat 3 times, wash with water between the steps, counterstain cells with carbolfuchsin (or safranin) for two minutes). Draw the objects morphology you can see in microscope view including also the localization of endospores).*



Excercise 2: *CULTURE: Describe the colony morphology of the anaerobic species you are analysing. Pick up a sample of the colony with a sterile loop and re-suspend it in 3% hydrogen peroxide to perform catalase test. Compare the test with that of enterobacteria and staphylococci. Indicate which results you find: A or B.*



Excercise 3: *LITMUS MILK TEST. The test aids in species differentiation primarily for the genus Clostridium. Lactose fermentation leads to acid and clot formation. Read the results and interpret them.*



Excercise 4: *READ AND INTERPRET THE RESULT OF SUSCEPTIBILITY TESTING OF VARIOUS ANAEROBES. Determine the minimal inhibitory concentration of antibiotics using Etest and interpret the result.*

14.8. LAB QUIZ

1. Specify the virulence factors of clostridia.
2. Specify infections caused by clostridia. How should the infections be diagnosed?
3. Describe the principles of combined treatment of clostridial infections.
4. Specify microbiological methods to diagnose anaerobic infections.
5. Specify prevention and control of clostridial infections.