

AIMS

The aims of this practical class are to introduce you to the growth, morphology and properties of some anaerobic bacteria

Bacteria exhibit a range of oxygen requirements. At one extreme are the strict aerobes such as *Corynebacterium diphtheriae* that have an absolute requirement for oxygen for growth. At the other extreme are the obligate (strict) anaerobes. These bacteria do not grow at all in the presence of oxygen, and some may even be killed by traces of it. *Clostridium spp.* and *Bacteroides fragilis* are examples of obligate anaerobes. Many bacteria may however grow in both the presence or absence of oxygen, and these are termed facultative anaerobes. Growth of facultative anaerobes is usually less luxuriant in the absence of oxygen.

REMEMBER TO TURN ON YOUR HOTPLATE

19.1 IDENTIFICATION OF CLOSTRIDIUM SPECIES

The Clostridia are Gram-positive spore-forming anaerobic bacteria. They are identified by the morphology of the bacilli and the spores that they form, their anaerobic growth requirement, and by their biochemical properties, particularly the volatile fatty acids and alcohols they produce. These can be detected by gas/liquid chromatography of culture filtrates (see Notes).

Work in pairs.

Materials:

Blood agar plates of:

- A. *Clostridium sporogenes*;
- B. *Clostridium tetani*

Clostridium perfringens, the causative agent of gas gangrene, is an important member of the Clostridia. It is **not** provided as class material but images are available on the Pathology Teaching web pages.

Procedure 1:

Make notes on the following aspects of the *Clostridia*:

- i) Colonial morphology of the species on blood agar.
- ii) Gram-staining property and morphology of the three species.

Catalogue Number	Small Image	Image Map	Large Image
M_BI_CL_26.jpg	Cl. sporogenes		Cl. sporogenes
M_BI_CL_25.jpg	Cl. perfringens- top and side lit		Cl. perfringens- top and side lit
M_BI_CL_23.jpg	Cl. tetani – shown lit from side		Cl. tetani – shown lit from side
M_BI_CL_20.jpg	Gram stain – Clostridia species		Gram stain – Clostridia species

Procedure 2:

Determine the morphology of the spores and vegetative cells of *Cl. sporogenes* and *Cl. tetani* **using the bacteria from the plates provided.**

Hot Malachite Green Stain for bacterial spores:

- Prepare smears by the usual method. Smears should be dried and fixed on the hotplate provided.
- Cover the smears with Malachite Green and heat gently on the hotplate for at least 5 minutes. Please ensure the stain does not run onto the hotplate. **Do not allow the stain to dry on the slide**; top-up as necessary.
- Wash well with tap water.
- Counterstain with safranin for 5 minutes.
- Wash. Blot dry. Examine under oil immersion.

Spores stain green and vegetative cells stain red.

Catalogue Number	Small Image	Image Map	Large Image
M_BI_CL_28.jpg	Cl. sporogenes – Malachite green		Cl. sporogenes – Malachite green
M_BI_CL_29.jpg	Cl. tetani – Malachite green		Cl. tetani – Malachite green

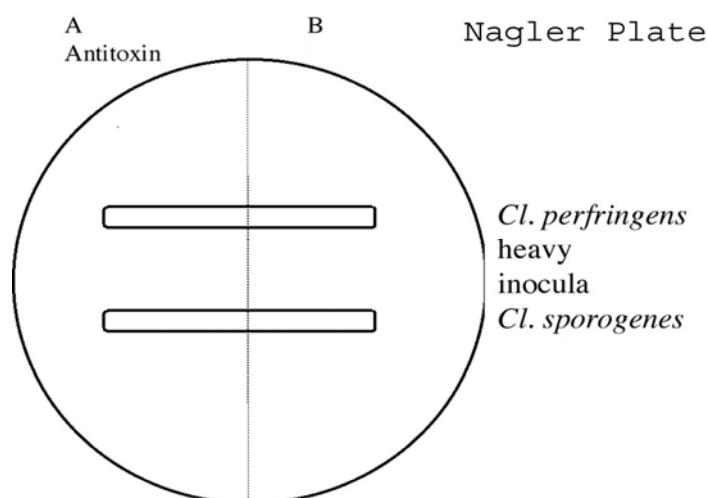
19.2 ACTION OF CLOSTRIDIUM PERFRINGENS α -TOXIN

At least 12 different toxins have been identified in strains of *Cl. perfringens*. One of these is the α -toxin, a lecithinase that hydrolyses the phospholipid lecithin (a component of cell membranes) to a diglyceride and phosphorylcholine. The activity of the α -toxin can be demonstrated by growth on agar containing egg yolk (as a source of lecithin): an opaque zone representing insoluble diglyceride, becomes evident around colonies of *Clostridium perfringens* (the Nagler reaction). The activity of α -toxin is inhibited by anti- α -toxin antibody (generated by vaccination with α -toxoid).

The photograph provided (19.2), shows a nutrient agar plate enriched with egg yolk. Anti α -toxin was spread over half of the plate and a heavy inocula of *Cl. perfringens* and *Cl. sporogenes* were streaked across the plate at right angles to the anti α -toxin boundary.

Examine the photo and interpret the result.

Q1 Do both bacterial species produce α toxin?



Catalogue Number	Small Image	Image Map	Large Image
M_BI_CL_27.jpg	Nagler reaction		Nagler reaction

19.3 VOLUNTARY MUSCLE:GAS GANGRENE 50-457

Voluntary muscle from the foot of a 36-year-old man who suffered a compound fracture of the tibia and fibula while digging potatoes. His foot became gangrenous; he had a mid-thigh amputation of the leg. You can easily find the haematoxylin-stained bacilli and nearby the dead voluntary muscle fibres. There are a few nuclei visible in the lesion and the cells are very eosinophilic.

Catalogue Number	Small Image	Image Map	Large Image
A_IN_BI_MC_08.jpg	Gas gangrene		Gas gangrene
A_IN_BI_MC_10.jpg	Gas gangrene		Gas gangrene

19.4 BACTEROIDES FRAGILIS

This organism is a component of the normal flora of the large intestine, where it is found in vast numbers far out-numbering *E. coli*. It is a Gram-negative obligate anaerobe and is medically important as it may cause a variety of infections, including wound infections or septicaemia after injury, surgery or appendicitis.

Examine the pure culture of *B. fragilis* grown anaerobically noting the effects of the metronidazole disc and prepare a Gram stain.

Q2. Are the bacteria gram positive or negative and are they bacilli or cocci?

Catalogue Number	Small Image	Image Map	Large Image
M_BI_BT_06.jpg	B. fragilis		B. fragilis
M_BI_BT_05.jpg	B. fragilis – Gram stain		B. fragilis – Gram stain

19.5 IDENTIFICATION OF BACTERIA CULTURED FROM TWO CASES OF DEEP WOUNDS

In this exercise, you are asked to identify the bacteria cultured from two infected wounds.

Plates E & F (Look at Photographs E & F)

Plates **E** and **F** were inoculated with a swab from a surgical abdominal wound that became infected post-operatively. Plate **E** was incubated aerobically. Plate **F** was incubated anaerobically and a metronidazole disc was placed on the second segment. (See notes for practical 19).

Using the following procedure determine the identity of the bacterial species present.

Procedure:

1. Examine the colonies on the aerobic plate first. Prepare & examine a Gram-stain(s).
2. Examine the colonies on the anaerobic plate next. Prepare & examine Gram-stain(s) Are these bacteria obligate aerobes or facultative anaerobes? (Compare both plates).
3. Identify the colonies which represent the facultative anaerobes. (These grow less vigorously on the anaerobic plate, but otherwise their Gram-stain reactions & cell morphology are identical).
4. The remaining colonies on the anaerobic plate should be obligate anaerobes. Identify these.

Catalogue Number	Small Image	Image Map	Large Image
M_BI_MX_27.jpg	Plate E		Plate E
M_BI_MX_26.jpg	Plate F		Plate F

Look at Photographs G & H

Plates **G & H** were inoculated with a swab from a compound fracture sustained by a rugby player. Plate **G** was incubated aerobically. Plate **H** was incubated anaerobically. Images of these plates are available on the Pathology Teaching website.

Catalogue Number	Small Image	Image Map	Large Image
M_BI_MX_16.jpg	Plate G		Plate G
M_BI_MX_25.jpg	Plate H		Plate H

Please consult your demonstrator for additional guidance on how to tackle the investigation of a pair of plates, one incubated aerobically, the other anaerobically.

Please cover your microscope and put it away.

Disinfect your work area.

Turn off the hotplates.

Thank you.