

**PLEASE BRING THESE NOTES TO ALL THE BACTERIOLOGY PRACTICAL CLASSES**

The bacteriology practical course is intended to give you the opportunity to examine several common pathogenic bacteria, and to learn how to isolate them in pure culture and how to identify them. Particular attention is paid to those features of pathogenic bacteria which enable them to cause disease. These introductory notes will be useful in all of the bacteriology classes.

**1. SAFETY**

Read the accompanying notes carefully. **Some of the organisms that you will handle are potentially dangerous pathogens.**

**2. IDENTIFICATION OF BACTERIA**

Bacterial Taxonomy: Bacterial species do not form discrete populations of inter-fertile individuals as mammalian species do. Although mating systems do exist among the bacteria, they are of little use as aids to classification, because bacterial DNA (in the form of plasmids) can quite frequently be transferred across species boundaries. Bacterial morphology is also often a poor guide to relatedness, as evolutionarily remote species may resemble one another e.g. micrococci and staphylococci.

Rational systems for *classifying* bacteria are based (a) on the systematic analysis and ranking of complex phenotypic characteristics (b) on the examination of bacterial DNA.

Methods for the examination of complex phenotypic characteristics include

- analysis of the chemical composition of bacteria
- immunochemical analysis of bacterial antigens
- metabolic comparison of different species i.e. finding out which biochemical pathways are present in a bacterium. These comparisons are sometimes based on substrate utilisation tests (e.g. sugar fermentation tests) and sometimes on end-product output tests (e.g. volatile fatty acid analyses of culture filtrates).

Methods for the examination of DNA include

- determination of nucleotide base-pair ratios (the GC:AT ratios)
- determination of the percentage similarity of nucleotide sequences within key genes such as those which code for ribosomal RNA.
- DNA hybridisation: determination of percentage sequence identity.

Bacterial identification: diagnostic bacteriology commonly proceeds along lines which may at first sight appear entirely arbitrary. A number of different tests are usually applied to bacteria derived from isolated bacterial colonies, and the results are interpreted by a skilled observer. However, one golden rule is

***Before any test can be applied reliably, an organism must first be isolated in pure culture, and its morphology, Gram's staining reaction and type of metabolism (e.g. aerobic, anaerobic or facultatively anaerobic) be determined.***

The apparently arbitrary nature of many bacterial identification procedures used in the clinical laboratory arises from the fact that they are designed as “short-cuts” to enable medically significant bacterial species to be identified as rapidly as possible. Much previous experience has revealed which key features of pathogenic bacteria are easiest to look for!

Many of the methods now in use are likely to be superseded within the next few years as fast procedures are developed for the recognition of specific DNA sequences e.g. by the use of DNA probes to detect species or strain-specific DNA sequences. These techniques can be made more sensitive with the polymerase chain reaction.

The most systematic of the diagnostic identification methods now in common use is **metabolic profile testing**. This method was first devised as a tool for taxonomists, and has now been simplified to make it suitable for routine microbial identification. One such system is the **API (Analytical Profile Index)** test system, which you will be shown later in the year.

### 3. HANDLING CULTURES

Agar plates are:

- always placed lid-downwards on the bench.
- always kept closed when not in use.
- never handed to others without their lids.

Tube cultures are:

- kept in a rack when not in use.
- discarded as contaminated if the cap is wet with medium by tilting the tube.

### 4. USE OF BACTERIOLOGICAL LOOPS AND NEEDLES: ASEPTIC TECHNIQUES

These provide a handy means of transferring bacteria from place to place. In these practical classes, sterile plastic loops are used to pick up bacteria from single colonies for transfer to a slide or to a liquid or solid medium in a tube.

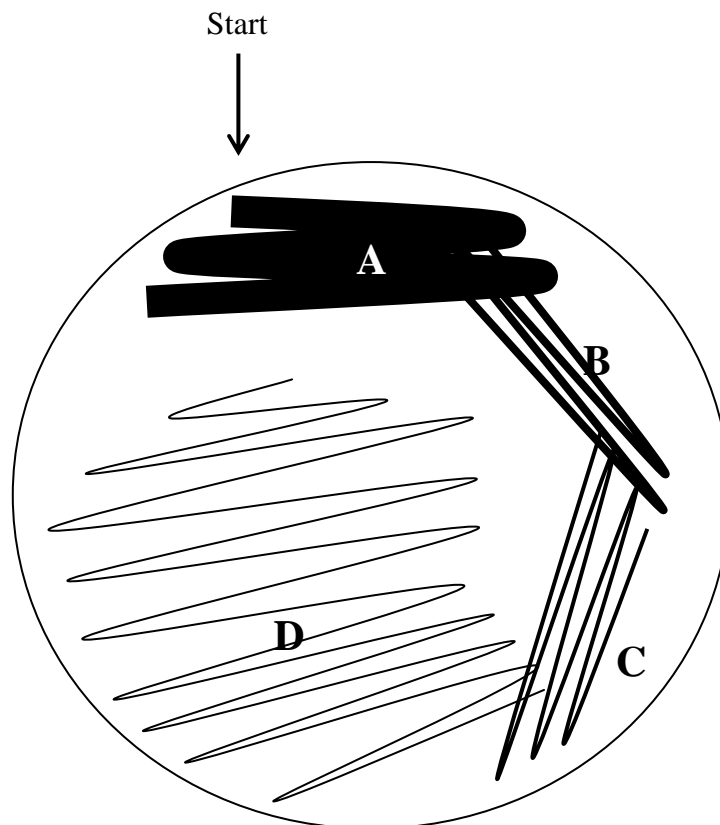
The loops are also used when making smears for staining, when suspending organisms in saline for agglutination tests, etc. and when inoculating agar plates.

### 5. AGAR PLATE CULTURES; OBTAINING ISOLATED BACTERIAL COLONIES I.E. PURE CULTURES

**A bacterium cannot be identified until it has been isolated in pure culture.** Bacterial mixtures can usually be resolved into their component species by spreading the organisms across the surface of a solidified nutrient medium in such a way that progressively fewer organisms are spread at each step, until finally individual colonies arise from individual organisms. Given a pure culture (i.e. of a single bacterial species) all the colonies present after sub-culture should look alike. However, differences in the sizes of colonies (due to crowding, competition for nutrients, or the production of inhibitory substances) are commonly seen on bacterial plate cultures. For this reason, you should only examine, and sample, bacterial colonies which are well separated from their neighbours.

Procedure: (see diagram below)

- a) Using a sterile plastic loop, remove a discrete, well separated colony from a plate culture or take an inoculum from a liquid culture,
- b) Lift a clean sterile Petri dish from its lid with your left hand (if right-handed).
- c) Spread the inoculum onto area "A" of the plate. Use the tip of your loop to cover the area thoroughly (as if lightly shading in an area when sketching with a pencil).  
Discard the loop into the sharps bin.
- d) Rotate the dish slightly in the palm of your hand.
- e) Using a new loop lightly draw out a portion of the inoculum from the end of the streaks in area 'A' into area 'B', Discard the loop, and using another clean loop continue streaking into area C,
- f) Make a final single wavy line, 'D' across the remaining surface of the centre of the plate without touching previous inoculation areas.
- g) Replace the dish in its lid.
- h) Discard your loop into the sharps bin.



Common Errors:

These include:

- a) Failure to spread the organisms across a large enough area with many sweeps of the loop.

b) Catching the edge of the loop in the agar.

Remedy: more practice at using the loop at the correct angle on the surface of the agar!

c) Using too large an inoculum: the smallest visible quantity of a concentrated bacterial suspension will contain hundreds of thousands of bacteria: only one viable organism is needed to start a new colony.

d) Moving the loop carelessly back into one of the more concentrated areas e.g. into "A" while spreading "D"

**6. A CHECK-LIST OF THE BACTERIA YOU WILL ENCOUNTER IN THE PRACTICAL CLASSES - (GENERIC NAMES ONLY)**

<p><b><u>Gram-Positive Cocci</u></b></p> <p><i>Staphylococcus spp</i> (FAN*)</p> <p><i>Streptococcus spp</i> (FAN)</p>	<p><b><u>Gram-Negative Cocci</u></b></p> <p><i>Neisseria spp.</i> (Pathogenic species are grown aerobically in 5-10% CO<sub>2</sub>)</p>
<p><b><u>Gram-Positive Bacilli</u></b></p> <p><i>Corynebacterium sp.</i> (OA)</p> <p><i>Clostridium spp.</i> (AN§)</p>	<p><b><u>Gram-Negative Bacilli</u></b></p> <p><i>Escherichia sp.</i> (FAN)</p> <p><i>Salmonella spp.</i> (FAN)</p> <p><i>Pseudomonas sp.</i> (OA†)</p> <p><i>Bacteroides sp.</i> (AN)</p>
<p><b><u>Acid-Fast Bacilli</u></b></p> <p><i>Mycobacterium sp.</i> (OA)</p>	

In addition you will encounter the yeast, *Candida albicans* (OA) which stains Gram-positive

\* (FAN) = Facultative Anaerobe

§ (AN) = Obligate Anaerobes

† (OA) = Obligate Aerobes

## 7. A BRIEF GUIDE TO THE CLASSIFICATION OF SOME BACTERIA OF MEDICAL IMPORTANCE

The organisms listed here are those that you will encounter most frequently in the Part IB Practical classes. The characteristics shown are not intended to be exhaustive.

### Gram positive cocci

#### **Spherical: grape-like clusters from broth;**

#### **catalase positive**

Coagulase positive; often orange yellow

*Staphylococcus aureus*

Coagulase negative; usually off-white

*Staphylococcus epidermidis*

N.B. ageing cultures of staphylococci will often also contain some Gram-negative organisms, due to autolysis.

#### **Spherical/ovoid, often diplococci; chains from broth;** **catalase negative**

Haemolytic, tiny 0.5mm diameter colonies

*viridans Streptococcus*

Haemolytic, 2.0mm diameter, water-clear colonies, often flat topped or with sunken rings, yielding lancet-shaped diplococci; capsular polysaccharides typed with antisera

*Streptococcus pneumoniae*

Haemolytic;

Lancefield test for cell-wall polysaccharide;

*Streptococcus pyogenes*

Usually non-haemolytic, uncommonly haemolytic; usually oval diplococci; Lancefield Group D

*Enterococcus faecalis*

### Gram negative cocci

Usually diplococci but can be tetrads; oxidase positive; the pathogenic species grow only in increased CO<sub>2</sub> on medium containing blood and serum and need nitrite for anaerobic growth

*Neisseria gonorrhoeae*  
(the gonococcus)

*Neisseria meningitidis*  
(the meningococcus)

*Neisseria pharyngis*  
(commensal species)

## **Gram positive bacilli**

Curved or club-shaped non-spore-forming rods which contain polymerized polyphosphate granules seen with Albert's stain; obligate aerobes; toxigenic if appropriate bacteriophage present. Non-toxigenic related strains, called diphtheroids, are found as commensals in throat.

*Corynebacterium diphtheriae*

### **Relatively long, narrow rods; strictly anaerobic; often form spores; widely present in soil:**

Non-motile;  $\alpha$ -toxin detected by action as a phospholipase;  $\alpha$ -toxin also causes haemolysis; rough-edged leaf-like colonies (not used in Class)

*Clostridium perfringens*

Motile, non-toxigenic; highly proteolytic (the plates smell foul); oval bulging sub-terminal spores in profusion; non-pathogenic by itself

*Clostridium sporogenes*

Motile, producing 'wispy colonies' or swarms over plate; haemolytic; toxigenic; round bulging, terminal ('drumstick') spores

*Clostridium tetani*

## **Gram negative bacilli**

Ferments lactose; motile; commensal strains always present in gut; invasive strains may be haemolytic; pathogenic strains are commonly toxigenic

*Escherichia coli*

Non-fermenter of lactose; motile; species identification by antisera against surface and flagellar antigens

*Salmonella* spp

Very large colonies; green diffusible pigment on nutrient agar; acrid smell

*Pseudomonas aeruginosa*

Strictly anaerobic; non-sporeforming; pleomorphic; smell of faeces

*Bacteroides fragilis*

## **8. ANALYSING AN UNKNOWN SPECIMEN**

Bacterial samples are usually collected by swabbing an infected site. Anaerobic bacteria (which are frequently killed by atmospheric oxygen) survive better if the swab is then pushed into a vial of sterile transport medium. Swabs should be sent to the laboratory to be plated out as soon as possible onto growth media.

The important questions that need to be answered when examining bacterial colonies grown from an infected swab are

- which pathogen(s) is/are present, and
- how should the infection be treated?

The first question is answered by identifying the organism(s), the second by determining the antibiotic sensitivity of the isolate(s).

The identification of pathogenic bacteria is complicated by the presence of commensal organisms on the external surfaces of the body, the enteric tract and parts of the upper respiratory and

urogenital tracts. These commensals are frequently co-cultured with the pathogenic organisms, and need to be distinguished from them.

Because there are so many different types of commensal organisms we simplify most of the cultures we show you. This means that microbial cultures seen in “real life” will include more species and strains of bacteria than you see in these classes. You should be able to learn how to spot the pathogenic bacteria studied in these classes if you pay attention to the following questions, and develop your practical skills at handling, staining and observing the organisms correctly.

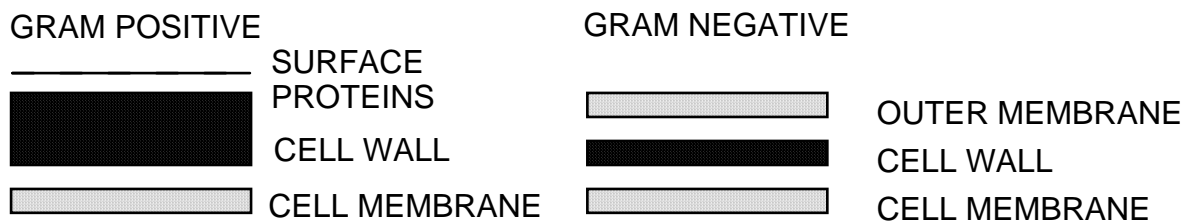
a) **WHAT WAS THE SOURCE OF THE INFECTED MATERIAL?** Micro-organisms occupy distinctive habitats, and some bacteria are more commonly associated with infections in these sites than others e.g. *Staphylococcus aureus* is commonly associated with skin infections.

b) **DO THE ORGANISMS STAIN GRAM-POSITIVE OR GRAM-NEGATIVE?** Gram’s stain reflects a fundamental difference in the structure of the cell walls of Gram-positive and Gram-negative bacteria which is used as a key point in bacterial classification and identification. Gram-positive cells stain blue/violet; Gram-negative cells stain red.

The difference between the Gram-positive and Gram-negative staining reactions arises because the thick peptidoglycan of the Gram-positive cell prevents the violet coloured dye-iodine complex formed inside the cells in the first stage of the reaction being washed away easily by ethanol. The thinner peptido-glycan of Gram-negative cells allows the stain to be rapidly eluted, and the unstained cells then stain red with the safranin counter stain.

The major structural differences are illustrated in the following diagram:

### SIMPLIFIED, SCHEMATIC VIEW OF THE CELL WALLS OF GRAM POSITIVE AND GRAM NEGATIVE BACTERIA



c) **WHAT SHAPE ARE THE INDIVIDUAL BACTERIA?** Stained preparations will reveal overall bacterial morphology (see separate notes on Staining Methods; Bacterial Morphology).

d) **WHAT WERE THE CONDITIONS OF CULTURE?** unless otherwise stated, assume that the cultures you have been given have been grown aerobically at 37°C. Notice, however, that the check list above shows that several of the organisms you will examine can also grow anaerobically, because they are facultative anaerobes. You will also encounter obligate anaerobes (e.g. *Clostridium* sp.; *Bacteroides* sp). When you are given mixed cultures which contain anaerobic organisms you will be shown aerobic plates for comparison, so that the differences in the colony types present on the two types of plate can be taken to indicate which colonies on the anaerobic plates represent obligate rather than facultative anaerobes.

N.B. Anaerobic growth always produces less biomass than aerobic growth. Colonies of facultative anaerobes grown anaerobically will therefore always be smaller than colonies of the same species grown aerobically. They may also be slower to form pigment. You will need to be very observant at first to spot that a smaller, paler colony of say, *Staphylococcus aureus* growing on an anaerobic

plate represents the same species as the larger, brightly pigmented colony you will get used to seeing on an aerobic plate. These differences in microbial phenotypes are unfortunately a little confusing for the beginner; all we can do is warn you that they occur!

Some organisms will only grow at temperatures below 37°C (e.g. some *Mycobacteria*).

e) *WHAT IS THE MEDIUM USED TO GROW THE ORGANISMS?* Some organisms are more fastidious than others, and will only grow on enriched media, e.g. *Streptococcus pyogenes* grows on blood agar, but not on nutrient agar.

f) *HOW MANY DIFFERENT TYPES OF COLONY ARE PRESENT?* You must base your answer to this question only on the characteristics of **ISOLATED** colonies growing on the least densely colonised segments of the plate. Crowded colonies are reduced in size by having to compete with one another for the available nutrients. They may also show abnormal features. On blood agar, colony crowding can result in haemolysis due to acid production.

To decide how many different colony types are present on a plate, try to make a systematic catalogue of the size, shape, elevation, type of margin, consistency, surface quality, colour, etc., of the *isolated* colonies present.

g) *WHAT OTHER PROPERTIES DO THE BACTERIA POSSESS?* Later on in these classes you will be told about sugar fermentation and other metabolic tests, antibody-mediated agglutination, bacteriophage typing. You will also learn that the chemical composition of bacteria and their products can act as aids in their identification and possible pathogenicity.

## 9. ISSUES THAT CAN ARISE WHEN INTERPRETING PLATE CULTURES

a) Pathogenic and commensal bacteria can represent different strains of the same species:

*E. coli* is normally commensal in the human gut, so that cultures of faeces samples will always contain *E. coli*. Some *E. coli* serotypes, however, may cause severe diarrhoea. In such cases the organisms will be present in greatly increased numbers, and the strains involved will be capable of forming specific adhesins and toxins. Other *E. coli* serotypes are known which are capsulated and resistant to the lethal effects of serum complement components; these may be invasive, and cause meningitis in children. Recovery of *E. coli* from wounds often indicates faecal contamination.

b) Bacteria can live as commensal organisms in one site in the body, but be pathogenic elsewhere:

*Viridans* or  $\alpha$ -haemolytic streptococci are commensal in the human throat, and seem to live there without detriment to the host. After dental treatment or sepsis in the mouth they may spread in the blood, and form septic foci or colonise heart valves, especially if these have already been damaged e.g. by rheumatic fever. Therefore their presence on throat swab cultures is normal; finding them in cultured blood samples is highly significant.

## **Notes on Sterilisation and Disinfection**

The observance of simple hygienic principles in everyday life from the late 19th century onwards has had a greatly beneficial effect on public health. The provision of clean food and water, and the safe disposal of sewage have been key factors in reducing exposure to infection. With the introduction first of antiseptic and later of aseptic techniques into medical, surgical & obstetric practice post-operative and post-natal mortality rates fell dramatically during the last decades of the 19th century & the first decades of the 20th century.

It is now routine practice to ensure that instruments, surgical wraps and dressings used in medical and surgical practice are sterilised before they are used. The general hospital environment is kept as free as possible from infection by cleansing with disinfectants. Within operating theatres the microbial load is further reduced by positive pressure ventilation with filter-sterilised air. However, emergency disinfection can be required if a surgical suite becomes contaminated with antibiotic-resistant organisms, such as methicillin-resistant *S. aureus* (MRSA) brought in by a patient.

The need to sterilise or disinfect objects of varying size, composed of a range of different materials means that different physical and chemical agents are in common use to kill off micro-organisms. Some of the commonest antimicrobial agents and procedures are outlined here.

### **PHYSICAL AGENTS**

#### **A. Heat:**

1. DRY      i)      160°C in oven for 1-2 hr. For glassware and metal objects only.
2. WET      i)      High-pressure steam in an autoclave. 121°C for 15 minutes, or 134°C for 3 minutes. For surgical instruments, dressings, wraps, blankets and some media.  
  
              ii)      Pasteurisation: 62°C for 30 minutes: kills the common milk-borne pathogens (e.g. *Mycobacterium tuberculosis*, *Salmonella* spp. *Streptococcus* spp., *Brucella* spp.).

#### **B. Radiation**

1. Ultraviolet light. Wavelengths below 270 nm. Used to diminish airborne cross-infection in laboratories, operating theatres, tissue culture hoods. Poorly penetrating.
2. X-rays and g-rays. Ionising radiation is used to sterilise disposable syringes, needles, plastic Petri dishes etc.

#### **C. Filtration:**

Used to sterilise heat-sensitive fluids, e.g. sera, some culture media, vaccines, etc.  
Also to filter air: laminar flow hoods are used in laboratories.

## CHEMICAL AGENTS

When used, the quantities and/or concentrations should be adjusted to take account of the extent of microbial contamination, and the amount of other organic material present.

### **A. Biocides**

(Kill all microbes including spores, *Mycobacterium tuberculosis* and *Pseudomonas* spp. that are sometimes resistant to other chemical agents).

1. Chlorine                      Used for water. Also as hypochlorite or compounds liberating Cl<sup>-</sup> in solution as the active component of cleansing and scouring powders (Vim). Useful for sinks, lavatories, baths, etc.
2. Formaldehyde              Used as a vapour to fumigate rooms; high humidity required concurrently.
3. Glutaraldehyde            A liquid used for the cold sterilisation of small objects.
4. Iodine                        With alcohol (methyl, ethyl or propyl), used on skin when all microbes, including spores, must be killed e.g. before lumbar puncture, or before the withdrawal of blood that is to be tested for the presence of infectious agents ('blood culture').

### **B. Disinfectants**

(Kill most infectious agents, but not spores).

Phenolic compounds like 'Dettol', 'Clearsol' and 'Lysol'.

### **C. Antiseptics**

(Biocides which are sufficiently non-toxic to be applied directly to the tissues).

Favoured compound now is chlorhexidine, used in:

1. Water to irrigate wounds.
2. Alcohol for pre-operative skin preparation.
3. Soap for 'Scrubbing-up' before surgery.
4. Detergent for cleansing infected wounds.

Points you may wish to discuss with your Demonstrator:

How would you:

1. Dispose of infected Petri dishes?
2. Dispose of blood from a hepatitis patient?
3. Check that an autoclave is reaching the temperatures shown on the external thermometer?
4. Clean and sterilise a newly-built operating theatre?
5. Provide a sterile environment in which to build a computer to be sent to Mars?