S.2.C.4 Basic elements of applied microbiology in the intensive care unit

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Medically important microbes are generally divided into bacteria, fungi, viruses and parasites.

Bacteria

Bacteria are unicellular organisms (prokaryotes) consisting of a rigid or semi-rigid cell wall (cytoplasmic membrane not containing sterols, complex structure containing lipids, polysaccharides and proteins) both RNA and a single closed strand of DNA in the cytoplasm but no organized nucleus, no nuclear membrane, nucleic acids distributed in chromosomes and extrachromosomal packets (plasmid etc), no mitochondria, Golgi bodies or cytoplasmic reticulum and enzymes intracellularly (endoenzymes) as well as enzymes secreted into the environment (exoenzymes). They may excrete material (enzymes, proteins) which may be harmful (toxin) to the host [1].

They multiply asexually by binary fission, though some elementary sexual activity (conjugation) may be observed, and is playing an important role in the adaptation to the environment of the organisms (e.g. antibiotic resistance). Other genetic mechanisms are transformation, through which they are able to take up extraneous DNA, and transduction, a mechanism mediated by bacteriophages [2].

These mechanisms are used to induce new properties into bacteral cells which is the basis of genetic engineering, a process widely used in industry.

Bacterial metabolism is based on aerobic or anaerobic glycolysis. Some are able to multiply on simple or complex media outside of the host (the basis of tests in diagnostic laboratories), others are obligate intracellular parasites requiring live cells to multiply.

They are classified on the basis of morphology (cocci, bacilli, spiral organisms, etc), staining characteristics (Gram positive, Gram negative), and metabolic activities (e.g. lactose fermenters etc). Some have organs of motility (flagella) associated with the cell wall.

Some species develop under difficult environmental conditions (temperature, lack of nutrients, chemicals etc.) into spores, a life form with very low metabolism. Spores are extremely resistant to the changes in the environment (heat, lack of moisture, chemicals) and represent a form of survival. If placed under more favorable conditions, they germinate and form fully functional bacteria [3].

There are a great number of species in nature, in soil, air, and sea; a very small proportion of them being mammalian pathogens.

Fungi

Medically important fungi are either unicellular (yeasts) or multicellular organisms (filamentous fungi, thallophytes) containing a rigid cell wall and more organized nuclear material (nuclear membrane). They are eukaryots, similar to higher organisms. They have a cytoplasmic membrane containing sterols, mitochondria, Golgi bodies and endoplasmic reticulum in the cytoplasm. Their metabolic activities are more complex. Most of them are able to grow on artificial media in the laboratory which gives rise to diagnostic tests.

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Their classification is based on morphology (spore formation and arrangement) and metabolism.

Their morphology is not fixed; some of them (e.g. Histoplasma) can be dimorphic, having a yeast phase (in host tissue) or a mycelial phase (in the environment). In addition, Candida can form psedohyphae, filamentlike shapes *in vivo*. This represents an exaggerated form of budding.

Yeasts generally multiply by budding, the filamentous organisms by conidia (spore bearing organs).

They secrete a great number of extracellular products including enzymes used in industry, yeasts used in the fermentation industry and baking, products isolated and purified in the pharmaceutical industry (e.g. antibiotics,).

The pathogenic species (a small proportion) are divided into dermatophytes (organisms affecting the skin) and deep tissue mycoses.

Viruses

While the above two groups have sizes which can be seen under the light microscope (0.5–100 microns), viruses are invisible under the light microscope, their size measured in millimicrons. They are obligate, intracellular parasites not possessing enough organized nucleic acids to provide independent multiplication. Therefore; these organisms involve the host cell in their multiplication by replicating their own nucleic acids (RNA or DNA) inside the host cell. This results often in the destruction or permanent parasitization of the host.

They are classified by:

a. Size, shape, nucleic acid content (they have DNA or RNA but not both), envelope etc.
b. Mode of replication
c. Disease caused
d. Means of transmission (arboviruses [arthropod borne])
e. Host specificity
f. Organ tropisms

The structure of viruses generally consists of the nucleocapsid containing the single or double stranded DNA or RNA, which is either linear coiled or circular, coated with protein (capsid) and may have an envelope. Various projections may be present on the envelope containing enzymes etc. (Table 1).

Naked capsid viruses are more resistant to the environment, enveloped viruses need moisture.

The multiplication of viruses consists of:

a. Recognition of target cell
b. Attachment
c. Penetration
d. Uncoating
e. Synthesis of nucleic acid and protein
f. Assembly of virus
g. Budding, or release of virus

Table 1. Families and examples of viruses

Family	DNA viruses examples	Family	RNA viruses examples
Poxviruses	Smallpox, vaccinia	Paramyxoviruses	Measles, mumps
Herpesviruses	Herpes simplex, Varicella zooster, EBvirus, cytomegalovirus, HSV-6, HSV-8	Orthomyxoviruses	Influenza A, B, C
Adenoviruses	Adenovirus	Coronaviruses	Coronavirus
Hepadnaviruses	Hepatitis B	Arenaviruses	Lassa fever, LCM
Papovaviruses	polyomaviruses	Rhabdoviruse	Rabies
Parviviruses	Parvovirus B19	Filoviruses	Marburg, Ebola
		Bunyaviruses	Crimean hemorrhagic fever, California encephalitis
		Retroviruses	HIV, HTLV
		Reoviruses	Rotavirus
		Picornaviruses	Poliovirus
		Togaviruses	Rubella, equine encephalitises
		Flaviviruses	Yellow fewer, dengue
		Calciviruses	Norwalk agent

Parasites

Medically important parasites belong to two subgroups: Protozoa and Metazoa.

Protozoa are simple, unicellular organisms with no cell wall but surrounded by a cell membrane, the protoplasm containing several organelles, endoplasmic reticulum food granules, contractile and digestive vacuoles, a nucleus limited by a nuclear membrane containing dispersed or clumped chromatin and a central karyosome. The are able to move by amoeboid movement, extruding pseudopodia, or the may contain organs of locomotion (*Balantidium coli*).

They generally multiply by binary fission, some of them alternate with multiple fission (schisogony) and sexual reproduction (gametogony or sporogony e.g. malaria).

Many of them can survive under harsh conditions by developing into a cyst, a form which has reduced metabolism.

They are, mostly, highly host specific, sometimes requiring for part of their development an intermediary host (arthropod), which will in turn play a role in their penetration into a human host [6].

Metazoa are all animals that are not protozoa. Two groups have microbiological importance: Helmints (''worms'') and Arthropodes (crabs, insects, ticks etc.).

Microbial pathogenicity

Microbes can be divided into two groups:

a. Obligate parasites. These organisms cause infection on penetration, which may develop into disease, depending upon the host's defense (e.g. viruses).
b. Facultative parasites. These organisms may live in/on the host without necessarily causing infection; usually they will start infection if they can penetrate into an unusual site in the host, or if the defense of the host weakens (e.g. E. coli in the intestine).

Some organisms are normal inhabitants of the human body (normal flora), living in symbiosis with the host. Occasionally, when these organisms enter an unusual site (e.g. skin organisms in wound), or become unusually large in numbers (e.g. yeast in stool during oral antibiotic therapy), they may cause disease.

Organisms are present normally in the respiratory tract down to the large bronchi, the gastrointestinal tract in its full length, outer ear, eye, anterior urethra, vagina and the skin.

Closed body cavities do not contain organisms and therefore are called normally sterile sites (e.g. cerebrospinal space, pleura, peritoneum).

Microbes exert their disease causing ability by entering the human body through penetration sites (portals of entry).

Portals of entry are:

a. Ingestion
b. Inhalation
c. Direct penetration
    - Trauma
    - Needle sites
    - Arthropode bites
    - Sexual
    - Transplacental
    - Organism directed (e.g. Schistosoma)

Some pathogenic organisms may colonize certain individuals, or certain sites, creating the carrier state, thus representing danger for other individuals or for themselves.

To establish a disease state, bacteria usually have to penetrate the host, defend themselves from immune mechanisms, multiply and exert pathogenic activity by enzymes, toxins or overwhelming destruction of host cells.

Mechanisms of microbial defenses against the host. These may act singly or in concert:

- Capsule
- Antigenic mimicry
- Antigenic shift
- Antigenic masking
- Production of anti-immunoglobulin proteases
- Inhibition of chemotaxis
- Inhibition of phagocytosis
- Phagocytic destruction
- Inhibition of phagolysosome fusion
- Resistance against lysosomal enxymes
- Adaptation to intracellular reproduction
- Toxin production

Microorganisms can rapidly rearrange their defense mechanisms by derepressing genetic traits or by selection and rapid multiplication of survivors. The main area where selection or genetic rearrangement is important, is antibiotic resistance.

Toxins are proteins, glycoproteins, or glycolypids, some of them are enzymes, biologically active in very small concentrations.

Destruction of microorganisms. Sterilization, disinfection and antisepsis

It is essential to remove or destroy infectious organisms from materials (instruments) in contact with host tissues. Similarly, removal of potentially infectious agents from tissue surfaces, utensil, and generally from the environment, is an important and continous task.

Sterilization is the application of physical or chemical agents to destroy *all* microbial forms, including spores.

Disinfection is the application of physical or chemical agents to destroy most microbial forms. Some spores or resistant organisms may survive. Disinfecting agents are divided into high, intermediate, or low level disinfectants.

Antisepsis is the application of chemical agents on the skin or tissues to inhibit multiplication, or eliminate microbial forms.

Germicide is a chemical agent capable of killing microbial forms.

Sporicide is a chemical agent capable of killing bacterial or fungal spores.

Sometimes, these definitions are erroneously interchanged.

a. Sterilization. Always a high level procedure. Has to assure the complete lack of microorganisms. In addition, handling of material prior to sterilization should reduce the possible number of organisms on the instrument (clean processing). For hospital departments (ICU), equipment is purchased already sterilized (usually for single use). Equipment or draping which is re-used is usually prepared and packaged in a central area specializing in this work (CSD, central supply department). Sterile equipment is always packaged and has a stamped preparation and expiration date. This should be always observed.

Sterilization can be achieved by:
Moist heat: steam under pressure at 121°C (250°F) or 132°C (271°F).
Dry heat: 171°C for 1 hr, 160°C for 2 hours.
Gas: Ethylene oxide 450-500 mg/lit at 60°C
Liquid (for instruments unable to withstand heat): Glutaraldehyde, or peracetic acid.

Nonionizing radiation
Disinfection. High level: Moist heat (including hot water) at 75–100°C. This is sometimes called sanitization.

Intermediate level: Chlorine compounds with 500–5000 mg free or available chlorine.
Peracetic acid or glutaraldehyde.
Formaldehyde 5–8%.
Intermediate level
Alcohol (ethyl, isopropyl) 70%
Phenolic compounds 0.5–3%
Iodophores (betadyne etc) 10–10,000 mg of available iodine per liter
Low level
Quaternary ammonium compounds 0.1–02%

Antisepsis
Chlorhexidine 1–4%
Hexachlorophene 1–3%

Removal of soiled material. (Garbage removal)

To protect the environment, soiled material has to be removed from the ICU for disposal. Special procedures have to be observed to achieve this.

Biohazardous material. This is all material which is *significantly* soiled with blood or other body fluids. All such material (excluding those which are recycled) has to be collected into specially marked containers (bags) and removed by a licensed collector of biohazardous material. This material is usually incinerated in industrial incinerators. If the hospital has its own incinerator, safe removal, storage and disposal procedures have to be introduced.

Biohazardous material to be recycled (instruments, cloth drapes etc.), has to be collected in a safe way and sterilized before cleaning and re-use, to protect the operators.

All sharp disposable material (needles etc.) has to be collected in specially designed containers and disposed of without opening, through the local biohazard disposal procedure.

All other materials can be disposed of through the usual household disposal to garbage dumps.

Principles of antibiotic action and development of resistance

To discuss mechanisms of resistance, one has to understand the basic action of antibiotics on bacteria.

The following pharmacological actions are most important in the understanding of development of resistance:

Inhibition of cell wall synthesis
- Penicillins
- Cephalosporins
- Cephamycins
- Carbapenems
- Monobactams
- beta-lactamase inhibitors
- Vancomycin
- Isoniazide
- Cycloserine
- Ethionamide

Inhibition of protein synthesis
- Aminoglycosides
- Tetracyclins
- Macrolides
- Clindamycin

Inhibition of nucleic acid synthesis
- Rifampin
- Quinolones
- Metronidazole

Antimetabolites
- Sulfonamides
- Trimethoprim

For the so-called beta-lactam group (penicillins, cephalosporins etc.) there are three general mechanisms of resistance:

1. Failure to penetrate the outer cell membrane of the bacteria
2. Failure to bind to the target site (penicillin binding proteins) [17]
3. Breakdown of the antibiotic by beta-lactamases. [15]

The penetration of beta-lactam antibiotics through the cell membrane of gram negative organisms requires passage through porins (micro channels through the outer cell membrane). Mutations on the porin sites may lead to resistance (e.g. Pseudomonas resistance to Imipenem). [16]

For the action of beta-lactam antibiotics on cell wall synthesis (the main mechanism of antibiotic action), the presence of penicillin binding proteins is essential. These proteins are specific in their action of binding antibiotics. Mutations in the structure, or presence, of penicillin binding proteins will lead to resistance to certain antibiotic(s).

The presence of beta-lactamase (cephalosporinase), enzyme(s) capable of breaking the beta-lactam ring in the structure of all penicillin, or penicillin like (cephalosporins, etc), antibiotics will inactivate the action of the antibiotics before they can reach the bacterium in effective concentrations (extracellular beta-lactamase), or inside of the cell (intracellular beta-lactamase).

Beta-lactamases may be constitutive (present from the beginning due to genetic instruction) or acquired (adaptive). The development of beta-lactamase positive bacteria have been observed ever since the introduction of penicillin.

Penicillin became widely available commercially in the second half of 1945. By 1948 about 90% of *S. aureus* strains became resistant to penicillin by possessing beta-lactamase.

To prolong the usefulness of penicillins (cheap and effective with low toxicity), various approaches were taken.

a. Development of beta-lactamase resistant penicillins.

Methicillin, oxacillin, nafcillin etc were developed by modifying the beta-lactam ring protecting it from enzyme action. This approach, still used with some success, led to the development of the so called methicillin resistant *S. aureus* (MRSA) which resulted by 1980 in 10–30% resistance among *S. aureus* strains.

b. New beta-lactam antibiotics (cephalosporins, cephamycins etc).

Many of these antibiotics are resistant to basic beta-lactam enzymes, though cephalosporinases were observed rapidly after introduction of large scale cephalosporin use. Unfortunately the effectiveness of these antibiotics against gram positive cocci is diminished. [22]

c. Beta-lactamase inhibitors.

Such substances were introduced to inhibit enzyme action by giving it simultaneously with the antibiotics. They had limited success.

Resistance to Vancomycin.

It develops by development of alterations on the D-alanyl-D-alanine terminal site of the side-chain of the peptidoglycan molecule, the precursor of the gram positive cell wall, the main site of action of Vancomycin. Recently this mechanism became important

because of the increase of vancomycin resistance among enterococci (VRE). [13, 19]

Resistance to protein synthesis inhibitors

Aminoglycosides primarily inhibit bacterial multiplication by penetrating the cell and binding to the bacteral ribosomes. The mechanisms of resistance are modifications of the ribosomal binding site, decreased uptake into the cell, but mainly by increased intracellular acetylation and phosphorylation [14] of the antibiotic, thus making it inactive.

Resistance to quinolones

Quinolones inhibit bacterial DNA gyrases which are required to supercoil bacterial DNA by binding to the alpha subunit. Modifications in the structure of the alpha subunit is the mechanism of resistance together with some changes in the bacterial porin structure, thus inhibiting the uptake of the antibiotic.

The development of resistance to antibiotics in nature is controlled by selection and by genetic mechanisms.

Bacterial populations have varying degrees of constitutive resistance to antibiotics. On widespread use of antibiotics, selective pressure will develop, which gives preference to the antibiotic surviving subpopulations. As these (sub)populations become more widespread, antibiotic resistance appears. A good example of this is the rapid development of penicillin resistance of *S. aureus*, after the introduction and uncontrolled use of penicillin. Reversal of this selective pressure can sometimes (rarely) be reversed by suspending the use of the offending antibiotic in a community (hospital) for some period [21]. In the long run, only strict control measures [11, 12, 18] can reduce the speed of development of resistance (see below).

Genetic control of antibiotic resistance can be chromosomal and extrachromosomal. In chromosomal mechanisms, the information for resistance is an integral part of genomes, and multiplies with the organism. Extrachromosomal control is through plasmids. These are usually circular, doublestranded DNA molecules inside the cytoplasm with autonomous replicating capacity, sometimes called replicons.

Genetic mechanisms are responsible for the development of resistance by operating through transduction or through conjugation, rarely by other transfer modes.

In transduction the resistant (donor) bacterium will transfer information for resistance by having such DNA transferred to sensitive organisms with the help of phage particles.

In conjugation the resistant (donor) organism will form a physical bond with the sensitive (host) organisms and will transfer plasmids into the host through pili (bacterial sex organs). The information becomes incorporated into the host nucleic acid and, on multiplication, will result in a resistant subpopulation. Many resistant plasmids have been described in the literature [20].

Both mechanisms (while described mainly under laboratory conditions) can be operational in nature. Conjugation with plasmid mediated resistance is probably the more frequent occurrence.

Prevention of the development of resistance is dependent on the following precautions:

Restriction of antibiotics to effective preparations, and placing duplicate or redundant antibiotics on the reserve list. The development of carefully planned and executed antibiotic prescription policy cannot be overemphasized. Good prescription habits will reduce resistance problems. The following points are critical:

- The use of specific antibiotics (secondary to antibiotic sensitivity information), rather than wide spectrum (antibiotics active against different types of bacteria) antibiotics.
- The use of antibiotics for the shortest necessary time and using them in effective concentrations. The use of suboptimal doses of antibiotic lead to rapid development of resistance. Antibiotic dosages should always be adjusted to the degree of renal failure.
- The use of antibiotic combinations rather than wide spectrum antibiotics when initial, presumptive therapy has to be introduced.
- The restriction of certain antibiotics for use in cases of resistance to more frequently used antibiotics.
- Continued monitoring of antibiotic resistance in the ICU and rapid response by changing antibiotic prescription policies.

Principles of diagnostic microbiology

Specimen collection

Material for bacterial cultures should be collected in sterile containers which are leakproof. They should also be packaged in a plastic ziplocked outer bag (biohazard protection). All body fluids and tissue specimens are suitable for bacterial culture.

Blood cultures should be collected from sterile venopuncture into special blood culture media according to local instrumentation. Each blood culture should be taken from a separate site. A minimum of 2 blood cultures should be collected, the maximum is not definite but it appears that more than 3 pairs in 6 hours do not increase the detection rate.

Blood for antibiotic levels should be taken immediately before a dose (trough level) and 30 minutes after a dose (peak level).

Specimens should be clearly labelled (at least two identifying properties, e.g. name and hospital number) dated and timed as to the taking of the specimen. All other identification (biopsy site etc.) should also appear on the label.

Viral specimens. Most virus laboratories use special transport media for viral specimens. Such containers should be stored in the refrigerator of the ICU, ready for use. Consult the virus laboratory for suitable viral specimens in different diseases.

Fungal specimens should be taken similarly to the bacteriological specimens. They should be clearly labelled, indicating that they are for fungal cultures, since they require special media. Skin scrapings, or nail or hair clippings, are sometimes required.

Parasitic specimens are blood, stool and, in case of arthropodes, the intact specimen. In parasitology special fixatives are used for certain specimens; consult the parasitology laboratory.

Smears for blood parasites should be made either directly at the bedside (malaria, filaria etc), or from heparinized blood in the laboratory.

Serum specimens are used for serological identification of causative agents. Tests for antibodies usually require paired specimens 3–6 weeks apart; providing only retrospective diagnosis. Recently, new methods became available to diagnose the presence of the nucleic acids of the causative agents (PCR polymerase chain reaction, direct DNA test etc). While these tests are rapid, giving results in a few hours, they are expensive and complex. Consult the laboratory for availability.

Transportation of specimens. Specimens should be transported immediately to the appropriate laboratory.

Availability of results

Bacteriological results.

PCR, DNA test usually a few hours.

Antibiotic levels 2–6 hours.

Gram stained smears 1–3 hours (very preliminary test only helpful if it is positive).

Bacteriological cultures preliminary results 24 hours, confirmation 48–72 hours.

Antibiotic sensitivity tests 48-72 hours.

Viral results

Usually several days.

Fungal results

As in bacteriological cultures. Some specimens may require several days or sometimes weeks.

Parasitic results

Direct examination (for ova and parasites), a few hours.

Definitive diagnosis a few days.

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