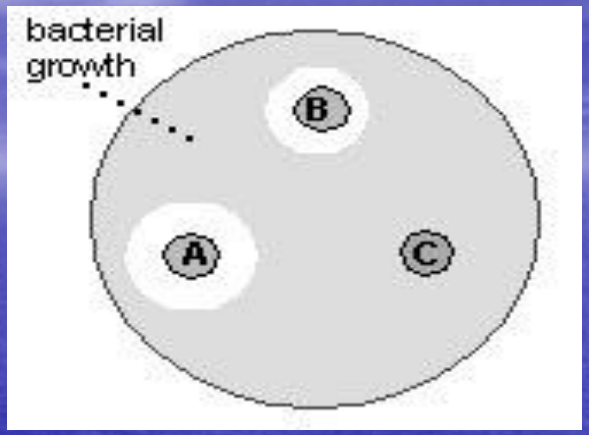
KIRBY-BAUER TEST for Antibiotic Susceptibility



- The basics are easy: The bacterium is swabbed on the agar and the antibiotic discs are placed on top.
- The antibiotic diffuses from the disc into the agar in decreasing amounts the further it is away from the disc.

- If the organism is killed or inhibited by the concentration of the antibiotic, there will be **NO growth** in the immediate area around the disc: This is called the **zone of inhibition**.
- The zone sizes are looked up on a standardized chart to give a result of sensititive, resistant, or intermediate. Many charts have a corresponding column that also gives the **MIC** (minimal inhibitory concentration) for that drug.

- You will need to subculture the isolated bacterium **(from ear, nose, or throat)** into a fresh TSB **medium** in order to have a young culture to run the antibiotic sensitivity test.
- Have you made sure that the culture is a G+ coccus, catalase +? Is it growing well in the TSB broth?
- The Mueller-Hinton medium being used for the K-B is very high in protein, in particular.

Swabbed correctly

Not swabbed correctly

THE PROCEDURES

1- Swab a Mueller-Hinton plate with each of the bacteria. Dip a sterile swab into the broth and express any excess moisture by pressing the swab against the side of the tube.

2- Swab the surface of the agar completely (**you do not want to leave any unswabbed agar areas at all**). In the pictures above and below, you can see what happens when the plate is not swabbed correctly with even coverage of the bacterium over the entire agar.

3- After completely swabbing the plate, turn it 90 degrees and repeat the swabbing process. (It is not necessary to re-moisten the swab.) Run the swab around the circumference of the plate before discarding it in the discard bag.

4- Allow the surface to dry for about 5 minutes before placing antibiotic disks on the agar.

5- **THE ANTIBIOTIC DISKS:**

- The antibiotic dispensers have 8 antibiotic cartridges in them. If you do not see 8 disks come out onto your agar plate, you will have to manually remove the antibiotic from a free cartridge.
- Each free antibiotic cartridge should have a little metal arm that allows you to dispense the disc right onto the agar. Lightly touch each disc with your sterile inoculating loop to make sure that it is in good contact with the agar surface. Incubate upside down and incubate at 37 ^{o} C.

INTERPRETATION:

- Place the metric ruler across the zone of inhibition, at the widest diameter, and measure from one edge of the zone to the other edge. HOLDING THE PLATE UP TO THE LIGHT MIGHT HELP.
- The disc diameter will actually be part of that number. If there is NO zone at all, report it as 0---even though the disc itself is around 7 mm.
- Zone diameter is reported in millimeters, looked up on the **chart,** and result reported as S (sensitive), R (resistant), or I (intermediate).


3
4
KIRBY-BAUER

Record the results for your sample in the table below.

Antibiotics	isolate 1		isolate 2	
	zone diameter	S, R, or I	zone diameter	S, R, or I

ZONE SIZE INTERPETIVE CHART FOR THE KIRBY-BAUER TEST					
ANTIMICROBIAL AGENT	**DISC CODE**	**R = mm or less**	**I = mm range**	**MS =**	**S = mm or more**
amoxicillin (Staph)	AMC	19			20
amoxicillin (other bacteria)	AMC	13	14-17		18
ampicillin (Staph)	AM	28			29
ampicillin (other bacteria)	AM	11	12-13		14
carbenicillin (Pseudomonas)	CB	13	14-16		17
carbenicillin (other bacteria)	CB	17	18-22		23
cefoxatime	CTX	14		15-22	23
cephalothin	CF	14	15-17		18
chloramphenicol	C	12	13-17		18
erythromycin	E	13	14-22		23
gentamycin	GM	12	13-14		15
methicillin (used for Staph only)	M (or DP)	9	10-13		14
penicillin	P	28			29
streptomycin	S	11	12-14		15
sulfamethoxazole-trimethoprim	SXT-TMP	10	11-15		16
tetracycline	TE	14	15-18		19

R = resistant I = intermediate S = sensitive
MS = moderate sensitive.

Antimicrobial Chemotherapy

- The modern use of antimicrobial chemotherapy started with the clinical use of **sulfonamide in 1936**- but "the golden age" of antibiotics began with the production of **penicillin in 1941**, when this compound was mass produced and first made available for limited clinical trail.
- Nowadays, at least 30% of all hospitalized patients receive one or more courses of antibacterial chemotherapy.

The ideal antimicrobial agent

- Is that agent which shows "selective toxicity" this means that the antimicrobial agent must produce toxic effect only on microbial cells but not on host cells. The feasibility of such selective toxicity depends primarily on the existence of biochemical and structural differences between the microbial cell and the host cell.

For example,

- Antibacterial agents that act via inhabiting the bacterial cell wall synthesis will show this selective toxicity where this cell wall absent in mammalian cells.

- The term antimicrobial chemotherapy includes not only antibiotics (which substances produced by the various species of living microorganisms such as bacteria, fungi, and actinomycetes e.g. penicillins, cephalosporins, tetracyclins, etc.) but also applied to the use of synthetic chemicals (such as saulfonamides, trimethoprim, and quinolones).

- The antibacterial agents are either bactericidal agent (destroy the bacterial cell) and/ or bacteriostatic (they only inhibit the growth of bacterial cells).

Why antimicrobial chemotherapy?

- Antimicrobial agents exhibit selective toxicity it interferes at a concentration tolerated by the host, with some metabolic or synthetic process that exist only in the infectious organism not in the cells of the host it acts.

- Inhibition of cell wall synthesis.
- Alteration of cell membrane permeability or inhibit active transport across cell membrane.
- Inhibition of protein synthesis, inhibit translation, transcription of genetic material.
- Inhibit nucleic acid synthesis.
- Competitive inhibition with PABA in sulfa.

Classification of Antimicrobial agents

1- According to the spectrum against bacteria:

- Agents acting mostly against Grame positive bacteria e.g., penicillin G, erythromycin, vancomycin.
- Agents acting mostly against Grame negative bacteria e.g., aminoglycosides and polymyxins antibiotics.
- Broad spectrum antibacterial agents e.g., chloramphenicl and tetracyclines. They act against both Grame positive and Grame negative bacteria.

2- According to the mechanism of action:

- **Which antibacterial agents affect bacterial cell membrane function?**
- **Antibiotics including the polymixins and gramicidin act by interfering with the functioning of the bacterial cell membrane by increasing its permeability. Gramicidin is one of a family of cyclic decapeptides active against Gram-positive bacteria.**
- **Polymixins have a smaller peptide ring attached to a peptide chain ending with a branched fatty acid. They act specifically against Gram-negative bacteria, although chemically modified derivatives do have a broader spectrum of activity. These antibiotics are toxic to humans and are now rarely used in clinical practice.**

What inhibits bacterial cell wall synthesis?

- Peptidoglycan is an exclusively bacterial polymer and so potentially should provide an excellent target for selective chemotherapy. Peptidoglycan is unique among biological polymers because it contains both L- and D- isomers of its constituent amino acids.
- Antibiotics may act at several stages during peptidoglycan synthesis. Some are valuable chemotherapeutic agents; others are too toxic for human use.

β- lactams

- The β-lactam group of antibiotics includes an enormous diversity of natural and semi-synthetic compounds that inhibit several enzymes associated with the final step of peptidoglycan synthesis. All of this enormous family is derived from a β-lactam structure:
- Clinically useful families of **β**- lactam compounds include the penicillins, cephalosporins, monobactams and carbapenems.

- The targets for β-lactam drugs are the **penicillin binding proteins (**PBP's**)**, so called because they bind radioactive penicillin and can be detected by autoradiography of gels on which bacterial proteins have been separated electrophoretically.
- The penicillin binding proteins have transpeptidase or carboxypeptidase activity and they act to regulate cell size and shape. They are also involved in septum formation and cell division.
- Bacteria have several individual penicillin binding proteins, each with a separate function. Conventionally these are numbered according to size, with PBP 1 as the largest protein. The PBP 1 of one bacterium will not necessarily have the same function as the PBP 1 of a different organism.

- The β-lactam antibiotics may bind preferentially to different penicillin binding proteins and at sub-lethal concentrations may cause alterations in cell morphology.
- For example, mecillinam binds preferentially to *Escherichia coli* PBP 2 and causes spherical cells to form, whereas cephalexin causes *Escherichia coli* to grow as filaments as a result of its preferential binding to PBP 3. This indicates that PBP 2 in *Escherichia coli* is involved in cell elongation whereas its PBP 3 is has a role in the cell division of this bacterium.

- The β-lactam antibiotics also stimulate the activity of **autolysins**. These are enzymes that are responsible for the natural turnover of cell wall polymers to permit growth of the cells. Under normal conditions, these enzymes produce controlled weak points within the peptidoglycan structure to allow for expansion of the cell wall structure.

- This activity is stimulated by β-lactams, causing a breakdown of peptidoglycan and leading to osmotic fragility of the cell and ultimately to cell lysis.

Vancomycin

- The molecule of **vancomycin** is relatively large. The drug acts to prevent peptidoglycan subunits from being added to the growing cell wall polymer. This is accomplished by vancomycin binding to the D-alanyl D-alanine residue of the lipid-bound precursor.
- Its primary activity is against Gram-positive bacteria. It is particularly useful in the treatment of serious staphylococcal infections. In these cases, it is given either intramuscularly or intravenously since it is not absorbed from the gut. It is also used for the treatment of pseudomembranous colitis caused by *Clostridium difficile* when it is administered orally.

Cycloserine

- The simple, cyclic molecule **cycloserine** is an analogue of alanine that interferes with two steps in peptidoglycan synthesis. It is a competitive inhibitor of the racemase that converts L-alanine to D-alanine and it also prevents the action of the D-alanyl D-alanine synthetase.
- The stable ring structure of cycloserine holds the molecule in a sterically favourable position, permitting preferential binding of this compound both to the racemase and to the synthetase, rather than their natural substrates.

- This results in competitive inhibition of these enzymes. Cycloserine is a neurotoxin and is not used clinically except for the treatment of drug-resistant *Mycobacterium tuberculosis*, or in other life-threatening infections where alternative therapies have failed.

Which antibacterial agents are inhibitors of protein synthesis?

- **Aminoglycosides**

- The aminoglycosides are a clinically important group of antibiotics that have a broad-spectrum of activity and that are bactericidal in action. The family includes **streptomycin**, **gentamicin**, **tobramycin**, **kanamycin**, **amikacin** and **netilmicin**. The aminocyclitols such as **spectinomycin** are closely related and have a similar mode of action. Aminoglycosides have a variety of effects within the bacterial cell but principally they inhibit protein synthesis by binding to the 30S ribosomal subunit to prevent the formation of an initiation complex with messenger RNA.

- They also cause misreading of the messenger RNA message, leading to the production of nonsense peptides. Another important function of the aminoglycosides is that they increase membrane leakage.
- Antibiotics such as **gentamicin** and **kanamycin** exist as mixtures of several closely related structural compounds; those like **netilmicin** and **amikacin** have a single molecular structure.

- Aminoglycosides are toxic to humans, causing problems with kidney function and damage to the eighth cranial nerve. This leads to hearing loss and balance difficulties.
- The therapeutic use of the aminoglycosides requires careful monitoring to ensure adequate therapeutic levels are maintained, without the accumulation of the drug to toxic levels.

Tetracyclines

- The **tetracyclines** are a family of antibiotics that have a four-ring structure. They are broad-spectrum agents that inhibit binding of the aminoacyl tRNA to the 30S ribosomal subunit in bacteria.
- The action is bacteriostatic and can therefore be reversed upon removal of the drug. The clinical use of tetracyclines is generally confined to adults.
- This is because tetracyclines affect bone development and can cause staining of teeth in children.

Chloramphenicol

- The broad-spectrum bacteriostatic agent **chloramphenicol** is toxic to humans. It has been recognised as a cause of aplastic anaemia and so its use is confined to life-threatening infections where no alternative therapy is available.
- It acts by binding to the 50S ribosomal subunit and blocking the formation of the peptide bond by inhibiting peptidyl transferase activity. It is a potent inhibitor of mitochondrial protein synthesis in eukaryotic cells.

Macrolides and lincosamides

- The macrolides are a group of antibiotics that have a large, lactone ring structure. These may be 14- or 16-membered rings. The most widely used macrolides are erythromycin and clarithromycin. These are relatively non-toxic antibiotics, most active against Gram-positive bacteria. Erythromycin is, however, the treatment of choice for Legionnaire's disease caused by the Gram-negative bacillus *Legionella pneumophila* and it is also active against *Haemophilus influenzae*, another Gram-negative bacillus.

- Erythromycin binds to the 50S ribosomal subunit and inhibits either peptidyl transferase activity or translocation of the growing peptide.

Fusidic acid

- The steroid antibiotic **fusidic acid** is used to treat Gram-positive infections. It acts by preventing translocation of peptidyl tRNA. Resistant mutants may easily be selected, even during therapy and therefore fusidic acid is usually administered in combination with another antibiotic.

Streptogramins

- The streptogramins fall into two groups, A and B. Streptogramins belonging to Group A have a large non-peptide ring, which is polyunsaturated.
- Streptogramins related to streptogramin B are cyclic peptides. They differ in their modes of action although both inhibit bacterial protein synthesis. Group A streptogramins distort the ribosome to prevent binding of the t-RNA; Group B streptogramins are thought to block translocation of the growing peptide.

Which antibacterial agents are inhibitors of nucleic acid metabolism?

- Nucleic acid metabolism may be interrupted at many steps. Antibacterial agents show selective toxicity either because humans lack the metabolic processes that act as targets, or because the bacterial targets are much more susceptible to particular chemicals than their eukaryotic counterparts.

Sulphonamides and trimethoprim

- Humans are unable to make folic acid, a precursor of purine synthesis. We require an exogenous supply of this metabolite obtained from our diet. Many bacteria are, however, able to generate folic acid from *para*-amino benzoic acid (PABA) and this pathway provides a target for synthetic antimicrobial agents like the **sulphonamides** and **trimethoprim**. **Sulphonamides** act by inhibition of dihydropteroate synthetase because it acts as a structural analogue of the normal substrate, PABA.
- Trimethoprim inhibits dihydrofolate reductase, the next step in the folic acid biosynthetic pathway.

- **Trimethoprim** was first introduced to be used in combination with sulphonamides to potentiate their activity. Studies of the combination *in vitro* show that the combination is synergistic. This means that the combined activity of the drugs is more effective than the additive action of the individual components. The synergism observed *in vitro*, however depends upon maintaining a critical ratio of the two antimicrobials. Because of pharmacological constraints, this cannot be achieved in the body, raising doubts about the synergism *in vivo*.
- Furthermore, using two agents for chemotherapy significantly increases the risk of the patient developing an adverse reaction to the treatment. Such arguments led to the introduction and successful use of **trimethoprim** as a **single agent**.

Quinolones

- Bacterial DNA exists in a supercoiled form and the enzyme DNA gyrase, a topoisomerase, is responsible for introducing negative supercoils into the structure. Quinolone antibacterial drugs such as **nalidixic acid**, **norfloxacin**, **ofloxacin** and **ciprofloxacin** act by inhibiting the activity of the bacterial DNA gyrase, preventing the normal functioning of DNA.

- Humans do possess DNA gyrase but it is structurally distinct from the bacterial enzyme and remains unaffected by the activity of quinolones.
- These are broad-spectrum agents that rapidly kill bacteria and are well absorbed after oral administration.
- Overuse of these drugs in certain situations is selecting quinolone resistant mutants and these may threaten the long term use of such compounds.

Which antibacterial agents are inhibitors of RNA metabolism?

- The bacterial DNA-dependent RNA polymerase is inhibited by **rifampicin** but this drug has little effect on eukaryotic cells. It is active against the mitochondrial RNA polymerase but its penetration into mitochondria is so poor that it displays very little activity in intact eukaryotic cells. The action of rifampicin prevents production of messenger RNA and thus ultimately stops protein synthesis.

- Clinically, rifampicin is used in treating tuberculosis and for prophylaxis against meningococcal meningitis. In such cases, it is offered to close contacts of people with the disease. The synthetic antibacterial **nitrofuran compounds** also act by preventing messenger RNA production.