Exercise 24-B
ANTIMICROBIAL SENSITIVITY TESTING
AGAR DIFFUSION METHOD OR KIRBY-BAUER TEST

Introduction

Antimicrobial drugs are a class of **chemotherapeutic agents** used in the treatment of infectious diseases. They can generally be divided into four groups as follows: 1) **synthetic antimicrobial drugs**, 2) **antibiotics** (substances produced at least originally by living organisms and used to control bacteria), 3) **antiviral drugs**, and 4) **anti-protozoan** and **anti-helminthic** drugs.

In clinical situations, once the causative agent of a specific disease has been isolated, the physician needs to know as quickly as possible, which antimicrobial drug will be most effective against it. The use of filter-paper disks impregnated with antimicrobial agents is one method that can readily provide this information.

Antimicrobial impregnated disks were introduced in the late 1940's when penicillin came into widespread use. Since then, a multitude of new antimicrobial drugs has been discovered, and much experimentation has been done to develop a method to accurately and reliably test their effectiveness.

In the **Agar Diffusion Method**, also known as the **Kirby-Bauer Test**, a Petri plate containing an agar medium is inoculated, "seeded", uniformly over the entire surface with a standardized amount of a test culture. The organism type being tested may be mixed with a small amount of liquefied agar and poured over the agar surface, or may be streaked onto the surface using a cotton swab dipped in broth culture.

Next, filter paper disks impregnated with known concentrations of chemotherapeutic agents are placed on the solidified agar surface. During incubation, the antimicrobial agents, or drugs, diffuse from the disks into the agar, and inhibit the growth of sensitive organisms in a zone immediately surrounding each disk (the **zone of inhibition**). Since the concentration of each drug decreases progressively the farther it diffuses from the disk, the concentration at the outside edge of the zone of inhibition represents the **minimum inhibitory concentration** (MIC) for that drug. The diameter of the zone of inhibition may be measured with a ruler; however, a wider zone does **not** necessarily indicate greater antimicrobial activity. The diameter of the zone of inhibition is influenced by the rate of diffusion for the antimicrobial agent being tested, the depth and type of agar medium being used, the incubation conditions, and many other factors. For this reason, Agar Diffusion tests are always performed under **standardized conditions**, and results are evaluated using **predetermined tables**.

For the Kirby-Bauer method as sanctioned by the FDA and the subcommittee on Antimicrobial Susceptibility Testing of the National Committee for Clinical Laboratory Standards, the recommended medium is **Mueller-Hinton Agar**, pH adjusted to 7.2 - 7.4 and poured to a uniform thickness of 4 mm in Petri plates. For certain fastidious microorganisms, 5% defibrinated animal blood (sheep, horse, or other) may be added. Inoculation is made from broth cultures with a specified turbidity. High potency disks are placed on the agar surface, and the plates are incubated for 16 - 18 hours at 37^{o} C. Zones of inhibition are measured to the nearest millimeter, and the results are evaluated.

The **minimum inhibitory concentration** (MIC) as described above is one example of a **breakpoint**, i.e., a discriminatory antimicrobial **concentration** used in the interpretation of the results obtained during susceptibility testing to define isolates as susceptible (sensitive), intermediate or resistant.

Data used to generate this type of breakpoint are obtained from multiple in vitro MIC tests. Bacterial strains are designated as "wild type" if they show no resistance to the drug being examined, or drugs with the same mechanism/site of action. **Clinical breakpoints** refer to those concentrations (MICs) that separate strains where there is a high likelihood of treatment success from those where treatment is more likely to fail, and are based on results obtained during multiple clinical trials. A third use of the term breakpoint can be applied to the **therapeutic dose**, i.e., the concentration of an antimicrobial drug expected to yield clinical control of a particular pathogen within the human body. This must integrate drug potency against specific populations of potential pathogens with the **pharmacokinetics** (**PK**)/**pharmacodynamics** (**PD**) of antimicrobial agents (what happens to drugs inside the body due to metabolic processes). The accurate determination of therapeutic dose must take into account **in vivo** complexities such as dosing schedule, the site of infection, likely PK/PD of the drug within a specific individual, adequacy of host defenses and a range of other factors. Typically PK/PD breakpoints are determined using data generated in animal models and then extrapolated to humans using mathematical or statistical techniques.

Breakpoints must be continuously monitored and modified because mutations and gene transfer mechanisms allow potential pathogens to survive the selective pressures exerted by human application of antimicrobial agents (evolution happens). A list of organizations involved in setting breakpoints can be found at: http://cmr.asm.org/content/20/3/391/T1.expansion.html

Note – Antimicrobial drugs are sometimes used in the identification of microorganisms, since certain species are more or less susceptible to particular drugs. For example, *Streptococcus pyogenes* is sensitive to Bacitracin (0.04 units per disc) while other pathogenic streptococci are not. In addition, certain drugs may be added to media to inhibit the growth of unwanted contaminants or to insure that the only cells growing are carrying specific "marker" genes.

Procedure:

1. Select the organism type you are to work with from the following table:

Organism types:	Student #'s:					
Staphylococcus aureus	1,	7,	13,	17,	21,	25,
Escherichia coli	2,	8,	14,	18,	22,	26,
Proteus mirabilis	3,	9,	15,	19,	23,	27,
Pseudomonas aeruginosa	4,	10,	16,	20,	24,	28,
Bacillus cereus	*5,*	*11,*				
Serratia marcescens	*6,*	*12,*				

2. Obtain a plate of Mueller-Hinton agar and label it with the name of the culture being tested (as indicated in the table above).

3. Inoculate the surface of the medium using a sterile cotton swab that has been dipped into the appropriate broth culture. Be careful to squeeze the excess fluid from the swab by pressing and rotating it against the inside of the tube above the fluid level. Cover the entire surface of the agar evenly by swabbing in 3 directions. Make a final sweep around the inner rim of the plate to insure coverage near the edge. **Do not** create a "checker-board" pattern.

4. Place the inoculated plate agar-side down on a **clean surface**, remove the plate lid and place the automatic disc dispenser over the plate.

5. Apply disks from the automatic dispenser by pushing down firmly on the plunger (do not hit it). This will release the discs, allowing them to drop onto the agar surface in desired locations. A tamping device should secure each disc in place.

6. Return the lid to the plate and incubate it in an inverted position at 37^{o} C for 16-18 hours. Following incubation, observe the results obtained.

7. Measure the **diameter** of each zone of inhibition to the nearest whole millimeter. If the zone diameter is too great, you can also measure the **radius** (distance from the outermost zone edge to the disc center), and then double this value. **Note** – The zone of complete inhibition is determined without magnification. Ignore faint growth or very tiny colonies that may be detected by very close scrutiny. Large colonies within the clear zone may represent resistant variants or a mixed culture, and may require re-identification and retesting in clinical situations. Ignore the "Swarming" characteristic of *Proteus*, measuring to the margin of heavy growth.

8. Record the zone measurements and then refer to the charts provided to determine the degree to which your organisms are susceptible (sensitive) to each of the drugs being tested. You should record your organisms as being **sensitive** or **susceptible (S)**, **intermediate (I)**, or **resistant (R)** to each drug. Record this information on the blackboard and observe trends indicated by the class data. Determine which drugs are **broad spectrum** and which appear to be **narrow spectrum** drugs, as well as which of the microorganisms being tested are most and least sensitive to the drugs used.

Questions:

1. What are antimicrobial agents? Which of these tested would be effective for controlling *Staphylococcus aureus, Escherichia coli, Proteus mirabilis,* or *Pseudomonas aeruginosa*?

2. What is a zone of inhibition, and what is meant by minimum inhibitory concentration (MIC)?

3. What are antibiotics? How do broad and a narrow spectrum drugs differ?

4. The concentration of an antimicrobial drug that is the minimum required to inhibit the growth of the microbe in question, i.e., the Minimum Inhibitory Concentration (MIC) is found where on the test plate?

5. What are breakpoints and how do they relate to therapeutic dose?

ANTIMICROBIC ZONE OF INHIBITION EVALUATION

Kirby-Bauer Method

(Significance of Zone Diameters When Using High Potency Antimicrobial Sensitivity Disks)

Antimicrobial Agent	Disk Potency	R Resistant mm.	I Intermediate mm.	S Sensitive mm.
Amikacin	10 mcg	<12	12-13	>13
Ampicillin				
Gram-negative and enterococci	**10 mcg**	**<12**	**12-13**	**>13**
Staphylococci & pcn G susceptibles	10 mcg	<21	21-28	>28
Bacitracin	10 units	< 9	9-12	>12
Carbenicillin				
Proteus sp. and E. coli	50 mcg	<18	18-22	>22
Pseudomonas aeruginosa	50 mcg	<13	13-14	>14
Cephalothin				
For cephaloglycin only	30 mcg	<15		>14
For other cephalosporins	30 mcg	<15	15-17	>17
Chloramphenicol	30 mcg	<13	13-17	>17
Clindamycin	2 mcg	<15	15-16	>16
Colistin	10 mcg	< 9	9-10	>10
Erythromycin	**15 mcg**	**<14**	**14-17**	**>17**
Gentamicin for P. aeruginosa	10 mcg	<13		>12
Kanamycin	30 mcg	<14	14-17	>17
Lincomycin (Clindamycin)	2 mcg	<17	17-20	>20
Methicillin (Penicillinase-resistant penicillin class)	5 mcg	<10	10-13	>13
Nafcillin	1 mcg	<11	11-12	>12
Nalidixic Acid	30 mcg	<14	14-18	>18
Neomycin	30 mcg	<13	13-16	>16
Nitrofurantoin	300 mcg	<15	15-16	>16
Novobiocin	30 mcg	<18	18-21	>21
Oleandomycin	15 mcg	<21	18-21	>16
Oxolinic Acid	2 mcg	<11		>10
Penicillin G for staphylococci	**10 units**	**<21**	**21-28**	**>28**
For other organisms	10 units	<12	12-21	>21
Polymyxin B	300 units	< 9	9-11	>11
Rifampin for **S. pneumoniae**	**5 mcg**	**<16**	**17-18**	**>19**
For other organisms	5 mcg	<16	17-19	>20
Streptomycin	10 mcg	<12	12-14	>14
Tetracycline	**30 mcg**	**<15**	**15-18**	**>18**
Tobramycin	10 mcg	<12	12-13	>13
Triple Sulfa	350 mcg	<12	13-16	>17
Vancomycin	30 mcg	<10	10-11	>11

AM-10	Ampicillin	E-15	Erythromycin	OA-2	Oxolinic Acid
AN-10	Amikacin	FM-300	Nitrofurantoin	OL-15	Oleandomycin
B-10	Bacitracin	GM-10	Gentamycin	P-10	Penicillin G
C-30	Chloramphenicol	K-30	Kanamycin	PB-300	Polymyxin B
CB-50	Carbenicillin	L-2	Lincomycin	RA-5	Rifampin
CB-100	Carbenicillin	N-30	Neomycin	S-10	Streptomycin
CC-2	Clindamycin	NA-30	Nalidixic Acid	SSS-25	Triple Sulfonamides
CF-30	Cephalothin	NB-30	Novobiocin	Te-30	Tetracycline
CL-10	Colistin	NF-1	Nafcillin	Va-30	Vancomycin
DP-5	Methicillin	NN-10	Tobramycin		

A	Aureomycin	GM	Gentamycin	PB	Polymyxin B
Am	Ampicillin	K	Kanamycin	RA	Rifampin
An	Amikacin	L	Lincomycin	S	Streptomycin
B	Bacitracin	LR	Cephaloridine	SD	Sufadiazine
C	Chloromycetin	ME	Methicillin	SSS	Triple Sulfa
CB	Carbenicillin	N	Neomycin	SM	Sulfamerazine
CC	Clindamycin	NA	Nalidixic Acid	ST	Sulfathiazole
CL	Colistin	NB	Novobiocin	T	Terramycin
CR	Cephalothin	NF	Nafcillin	TE	Tetracycline
CX	Cloxacillin	OA	Oxolinic Acid	TM	Tobramycin
E	Erythromycin	OL	Oleandomycin	V	Viomycin
FD	Nitrofurantoin	P	Penicillin G	VA	Vancomycin

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION

Name _____ **Lab Section** _____

WORKSHEET
Exercise 24B
Antimicrobial Sensitivity Testing

Goals: _____
_____
_____

Materials & Methods:
The Kirby-Bauer antimicrobial sensitivity procedure was followed.

Organism type used: _____
Medium used: _____
Incubation temperature: _____ Duration of incubation: _____

Data & Results:

Antimicrobial Agent	Zone of Inhibition	R, I, or S?

Conclusions:
_____
_____
_____
_____
_____
_____
_____

NOTES, OBSERVATIONS & ADDITIONAL INFORMATION