المملكة العربية السعودية
جامعة الملك سعود
كلية العلوم
قسم الكيمياء الحيوية

المضادات الحيوية (BCH 476)

Antibiotics

Lecture 5,6

Methods of screening, identification and determination of antibiotics

Prepared by Dr. Farid S. Ataya

Lect No.	Topics
5,6	***Methods of antibiotics screening, identification and determination*** •Isolation of the microorganisms. •Isolation of antibiotics. •Classical tests & modern methods in primary screening. •Secondary screening techniques. **Fractionation of antibiotics.** •Chromatography & electrophoresis. •Characterization techniques, Hamill's scheme & Bostian computer system. •Quantitative determination of antibiotics: Diffusion methods and antibiotic sensitivity tests.

Isolation of microorganisms

- There are many types of microorganisms that can produce antibiotics; among them bacteria, fungi, actinomycetes, etc
- Each type needs specific conditions for appropriate growth.
- To grow and isolate certain type of microorganism we need to avoid the growth of other unwanted organisms.
- For example, to grow bacteria or actinomycetes we grow them in alkaline medium in which fungi cannot grow.
- In contrast, to grow fungi the pH of the medium must be acidic to prevent the growth of bacteria and actimomycetes.
- To grow actinomycetes, the media must be alkaline to inhibit the growth of fungi and we must add antibacterial agent to inhibit bacterial growth (like Bengal Red and/or streptomycin)

Primary screening of antibiotics

- There are two types of tests to screen the antibiotic activity of unknown bacterial strain. We well focus on the classical test.

Classical screening test:

In this assay two bacterial strains are used:

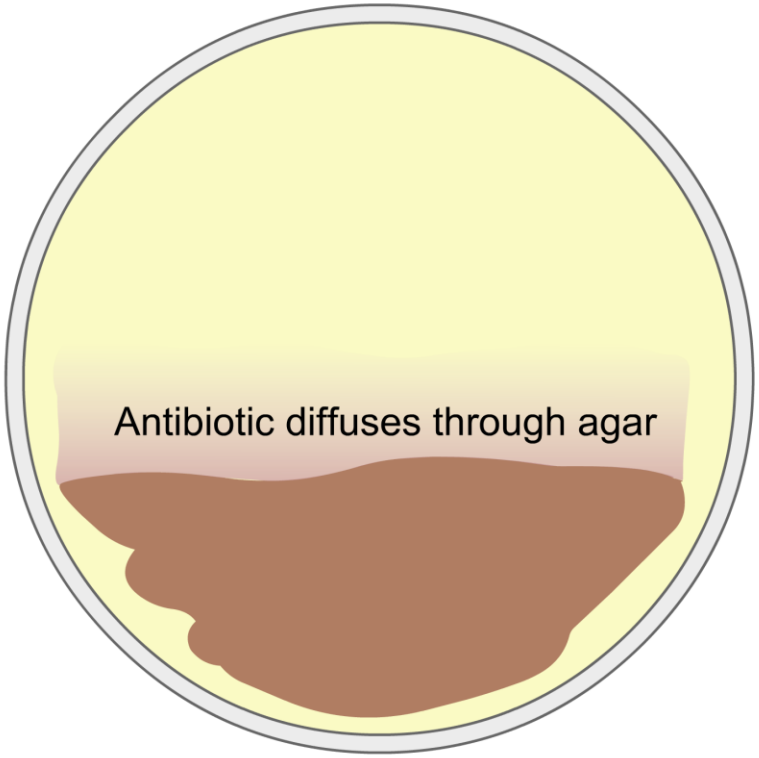
1- the unknown bacterial strain/s which is isolated from natural source.

2- the standard one which is available on a world-wide scale like gram positive or gram negative bacteria

(contain no mechanism for either antibiotic resistance or production).

Primary screening of antibiotics (classical diffusion method)

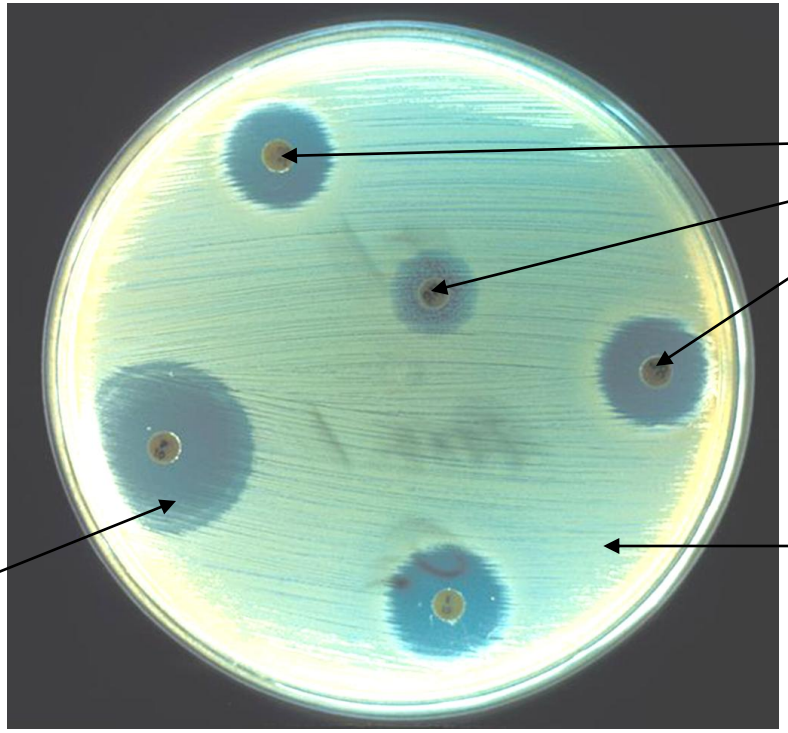
- Isolate the unknown microorganism from natural source, and dilute it to appropriate dilution.
- Inoculate the diluted microorganism on agarized medium in Petri dishes.
- Incubate under appropriate conditions for days, so that separated colonies could be seen.
- Add the standard bacteria to melted media (37 °C) and pour it on the growing colonies.
- After overnight incubation the poured bacteria will grow to form a cloudy agar surface containing some clear zones (circles) which called inhibition zones.
- Such zones were observed only on colonies that produce antibiotics.
- Colonies of such zones were picked, subcultured and isolated.



The isolates that show evidence of antibiotic production are then tested for the antibiotic's spectrum of activity. Using a sterile loop, take a sample of a suspected antibiotic producer and streak it across a fresh plate. Incubate the plate to permit bacterial growth and antibiotic production. The antibiotic will diffuse through the agar medium.

http://www.sumanasinc.com/webcontent/animations/content/antibioticproducers.html

Classical diffusion method



Secondary screening techniques

- The secondary screening and identification of antibiotics include more advanced techniques which aims to detect, quantify, purify, characterize and examine the antimicrobial activity and ends at the introduction of the new antibiotic into the market.
- Various biological, chemical, physicochemical and pharmacological tests may be included to the secondary screening of antibiotics.

Detection of antibiotics

Three general standard methods are used for detecting and determining an *in vitro* antibiotic activities:

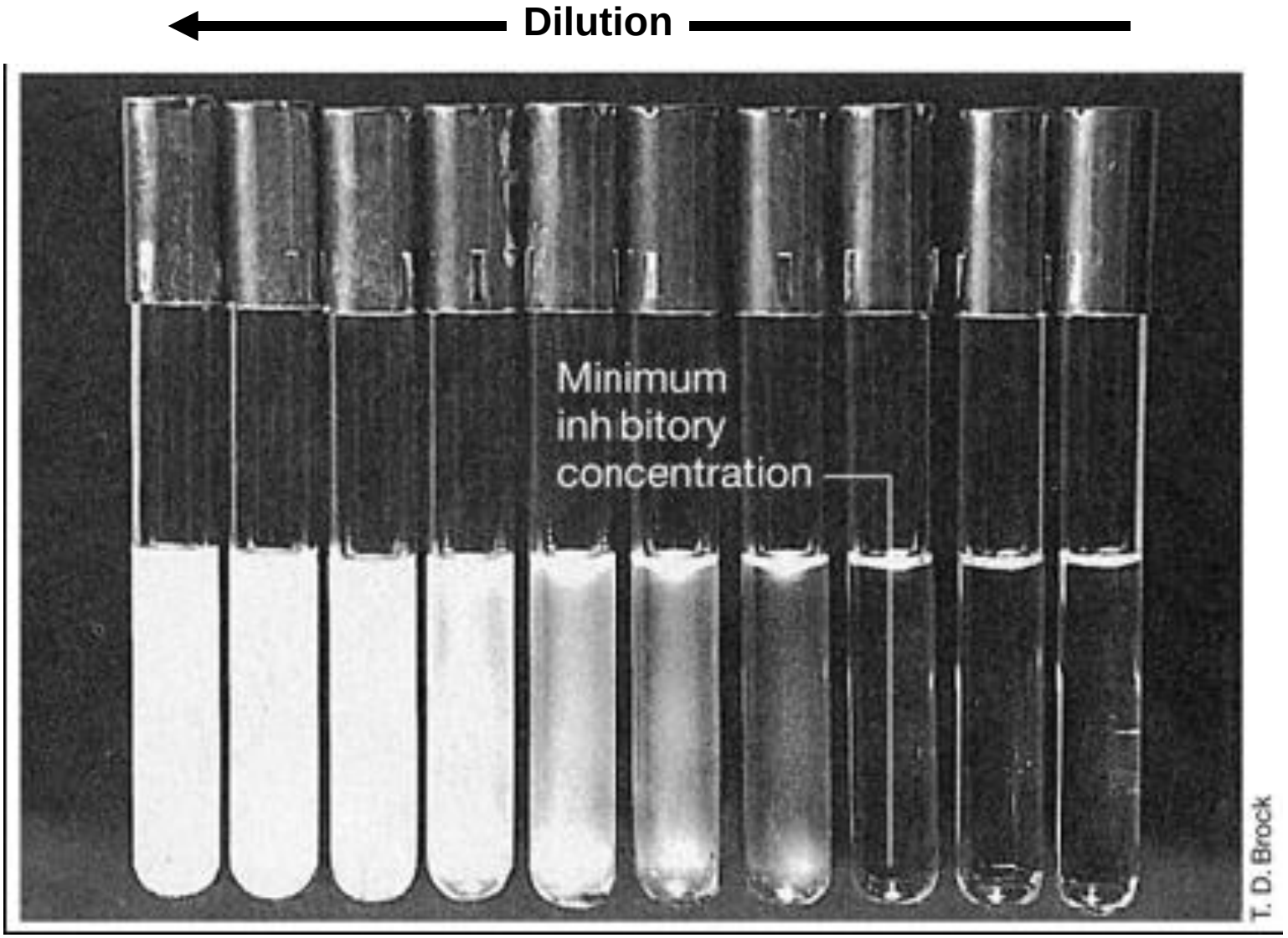
1. Serial dilution test
    a- broth dilution test
    b- agar dilution test.
2. Plate diffusion test
3. Streak test

Detection of antibiotics

1- Serial dilution test

a- broth dilution

- A series of tubes of liquid media was prepared and supplied with decreasing concentrations of antibiotics.
- An equal amount of test bacteria are inoculated into the tubes and incubated for 12-16 hours at 37 °C with shaking.
- The tubes are examined either visually or turbidimetrically to measure the bacterial growth.
- Results are recorded as **M**inimum **I**nhibitory **C**oncentrations (MIC).



Broth dilution test

Detection of antibiotics

1- Serial dilution test

b- Plate dilution test

- Several different microorganisms can be tested on the same agar plate.
- A series of agar plates are prepared containing two to ten-fold serial dilution of antibiotic to be tested and control plate without antibiotics.
- The microbial strains are then inoculated on these plates in streaks and incubated for 18-24 hours at 37 °C.
- The lowest concentration which has prevented the growth is considered to be the minimal inhibiting concentration (MIC).

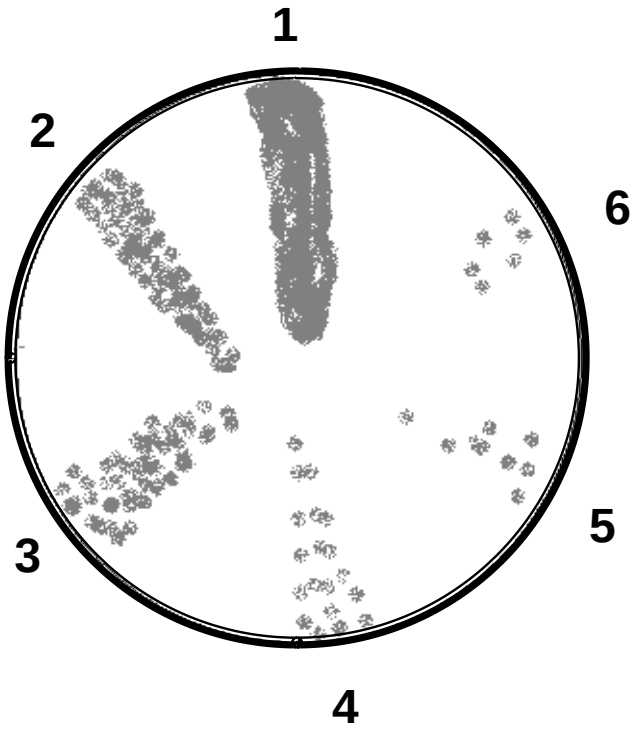


Plate 1 dilution 1:100

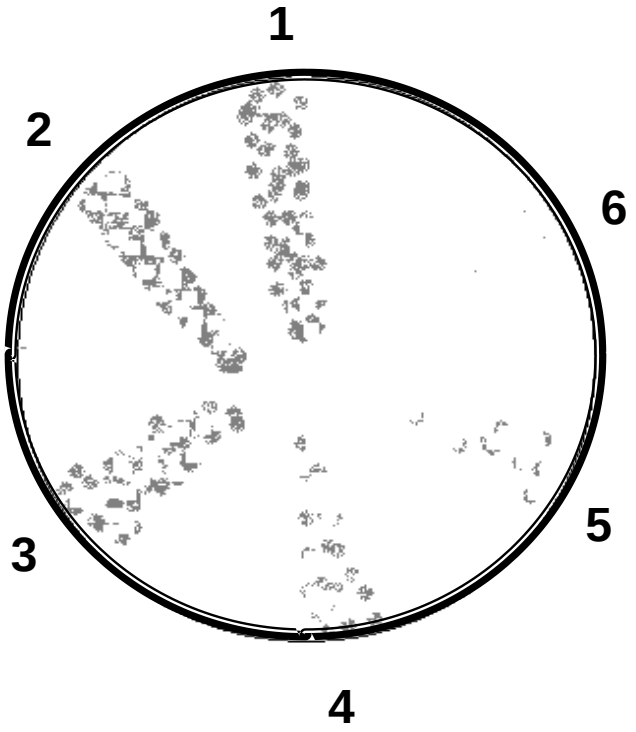


Plate 2 dilution 1:10

1- Serial dilution test - b- Plate dilution test

Detection of antibiotics

2- Plate diffusion test

(Kirby Bauer disk diffusion test)

- The surface of an agar nutrient medium is uniformly inoculated with the test bacterial culture.
- The tested antibiotics are added in the form of holes on the agar and filled with antibiotics or as circular discs of filter paper placed and impregnated with the antibiotic.
- The culture plates are incubated at 37 °C for 18-24 hours and inhibition zones are determined.
- The zone diameter (zone of inhibition) is measured and reference tables are used to determine if the bacteria are **S**ensitive (S), **I**ntermediate (I) or **R**esistant (R) to the antimicrobial drugs.

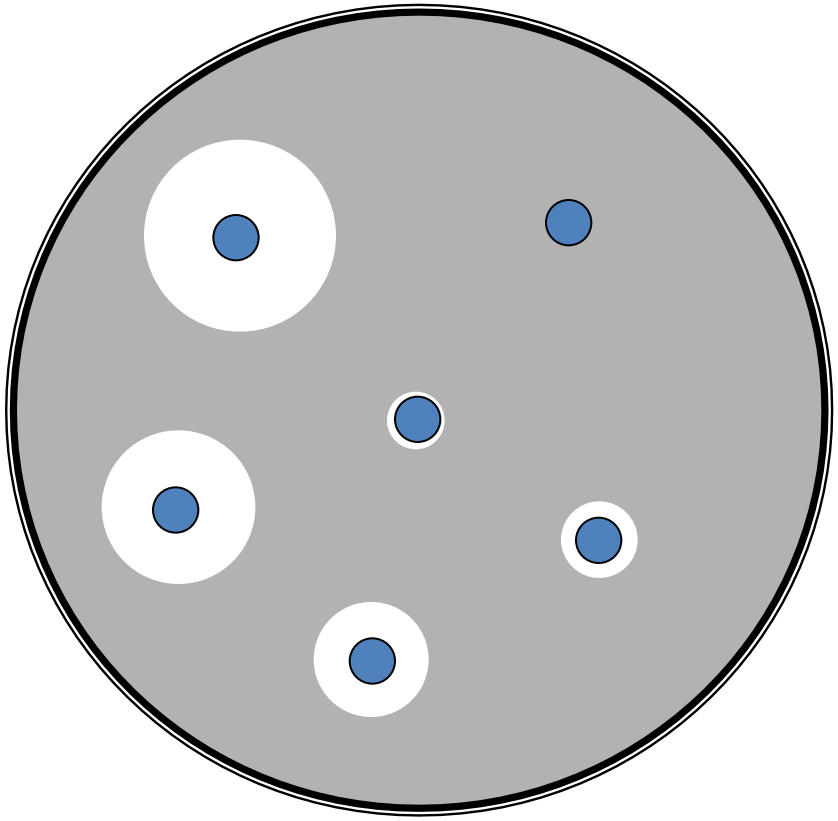
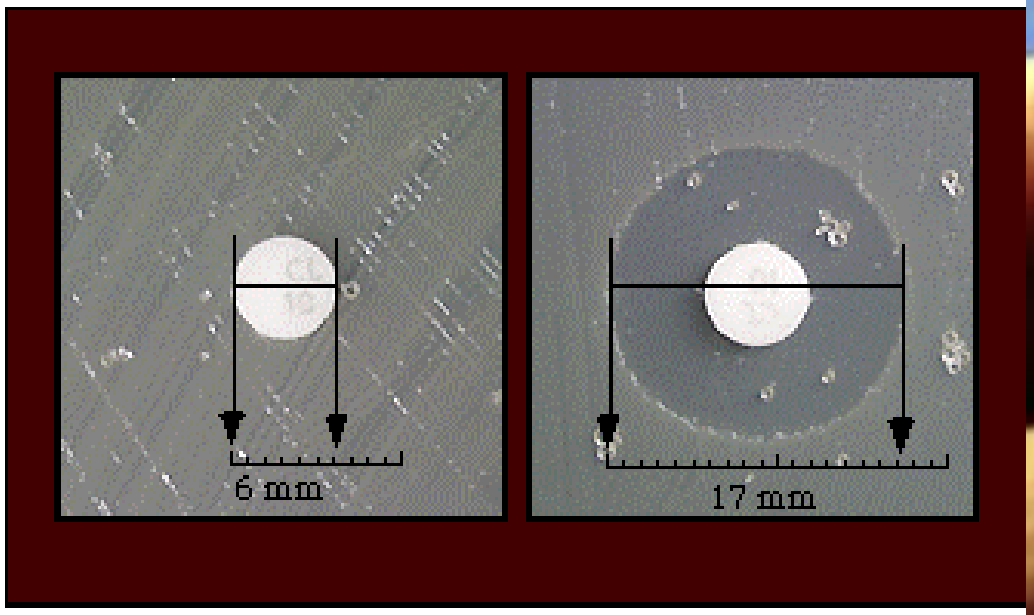
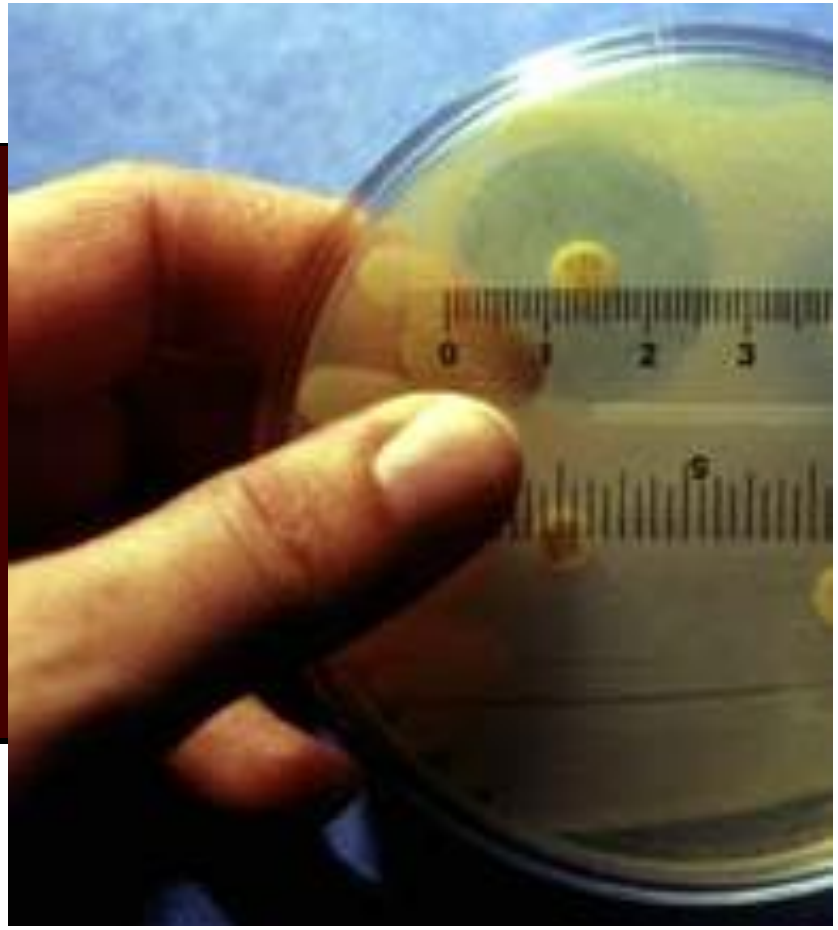


Plate dilution (Kirby Bauer disk diffusion test)

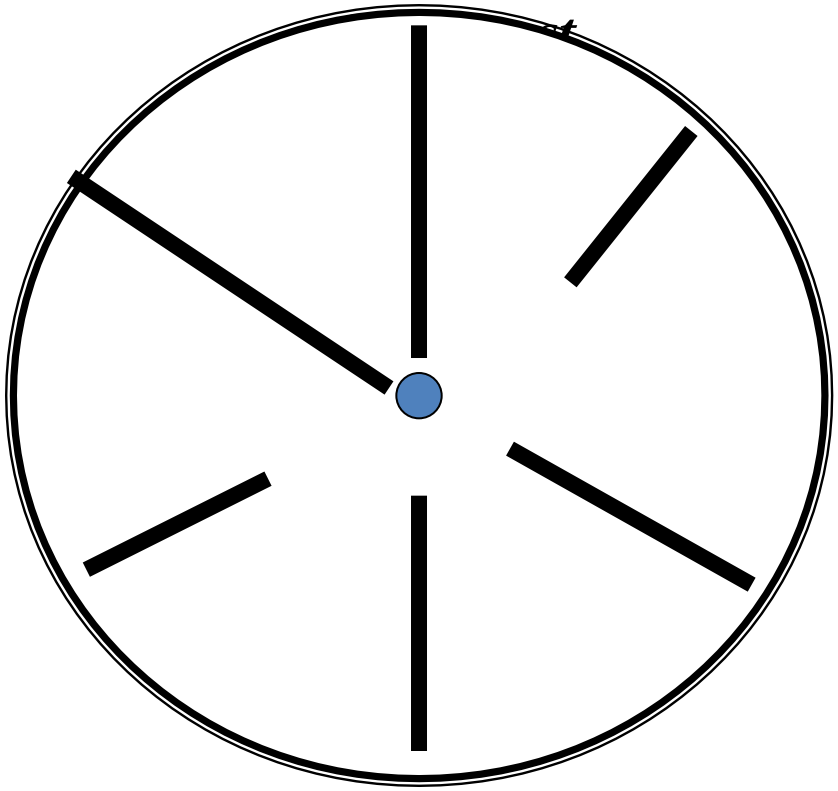

6 mm
17 mm

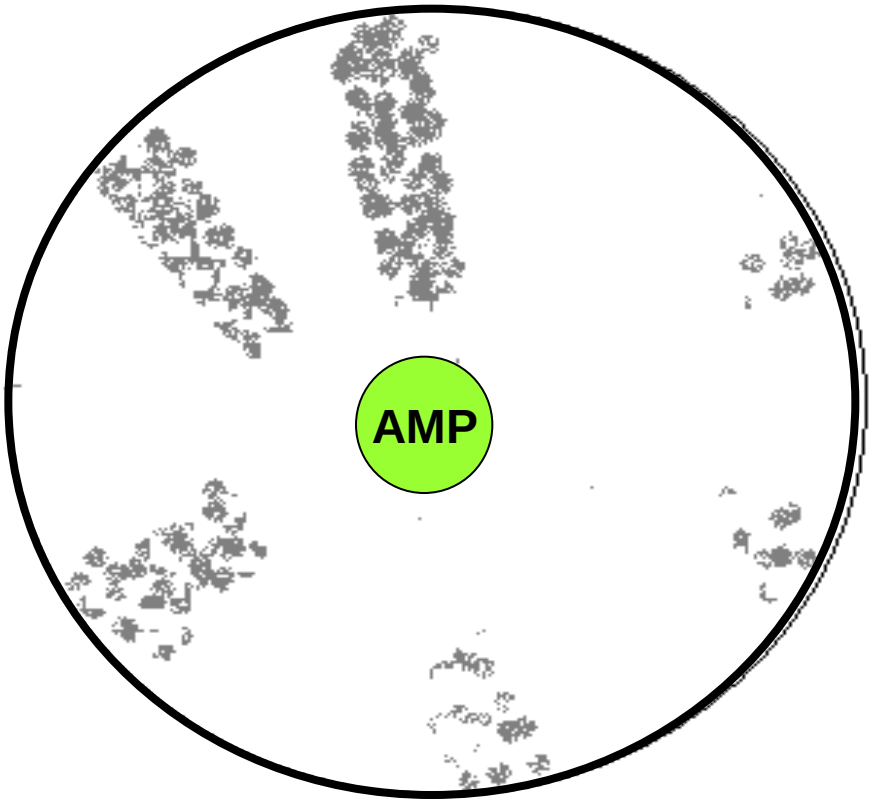


Detection of antibiotics

3- The streak test

- This assay permit testing the effect of single dilution of antibiotic against different microbial strains.
- A filter paper disc is placed in the middle of agar plate and impregnated with the tested concentration of certain antibiotic.
- A suspension of the test microorganisms are inoculated radially as streaks.
- The plates are incubated at 37 °C for 18-24 hours and inhibition zones are determined.



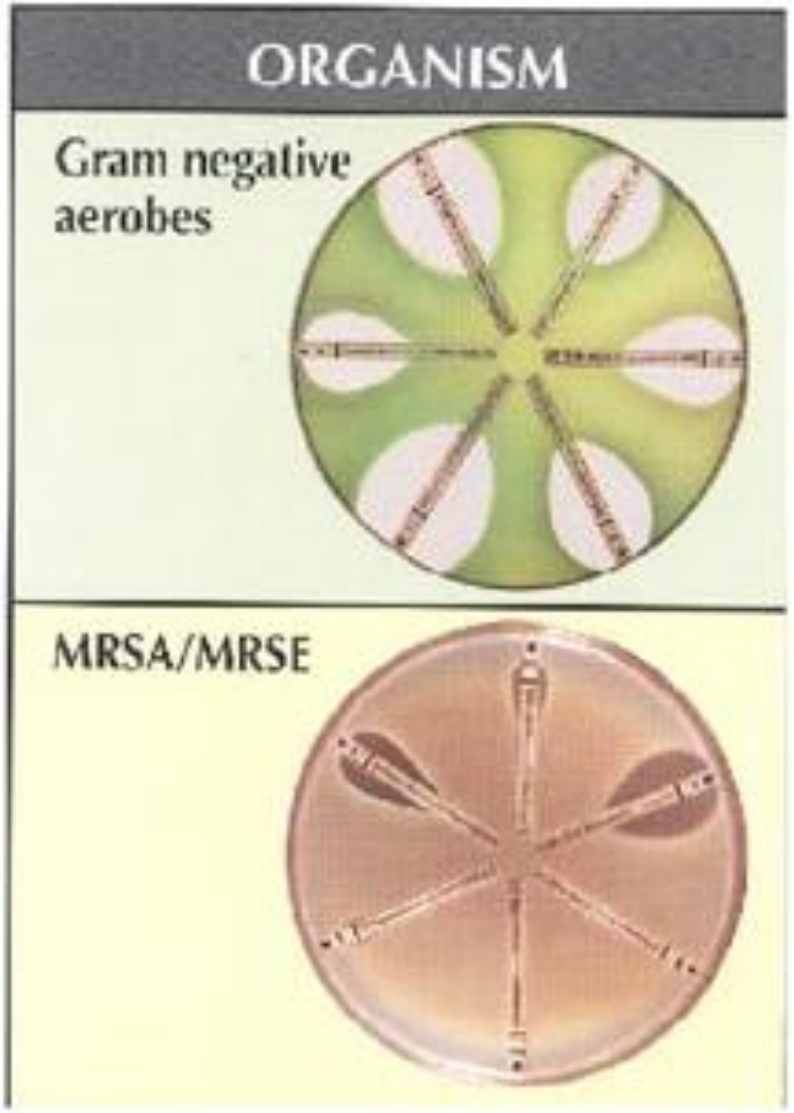


Detection of antibiotics

3- The streak test

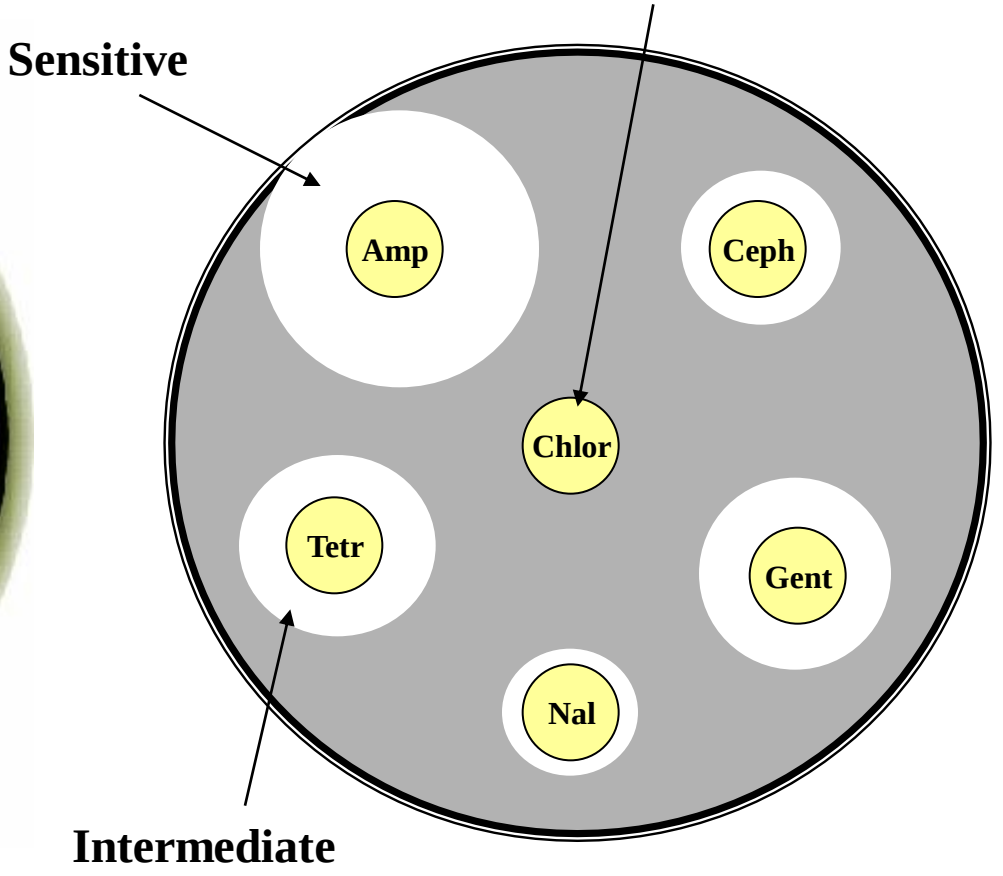
Susceptibility Testing

E-Test



Antibiotic sensitivity test

- The surface of an agar nutrient medium is uniformly inoculated with the test bacterial suspension (e.g. urine sample).
- Discs containing antibiotics are placed on the top of the inoculated nutrient agar medium.
- The culture plates are incubated at 37 °C for 18-24 hours and inhibition zones are determined.
- The widest inhibition zone indicates that this antibiotic is the most effective one so that it inhibit the growth on the infecting microorganism (existing in the urine sample).



Antibiotic sensitivity test

What is the MIC?

- The MIC is the lowest concentration (highest dilution) of antimicrobial drug that completely inhibits bacterial growth.
- The MIC value is reported as recommended by the **C**linical and **L**aboratory **S**tandards **I**nstitute (CLSI) with interpretation guidelines as follow:
  - Sensitive (S),
  - Intermediate (I) or
  - Resistant (R)

Methods for antibiotic identification

Chromatographic methods

1.Paper chromatography

2.Thin layer chromatograph

3- Liquid chromatography

4.High performance liquid chromatograph (HPLC)

Electrophoretic techniques

Physiochemical methods

1.Ultraviolet spectroscopy

2.Infrared spectrum

3.Mass spectroscopy

4.X-ray diffraction

5.Titration

6.^{13}C and ^{1}H nuclear magnetic resonance spectroscopy

Computerization of overall data

Fractionation of antibiotics

1- Chromatography of antibiotics

There are many types of chromatographic techniques that are used in fractionating antibiotics:

- Paper chromatography
- Thin layer chromatography
- Liquid column chromatography
- Gas liquid chromatography

1- Chromatography of antibiotics

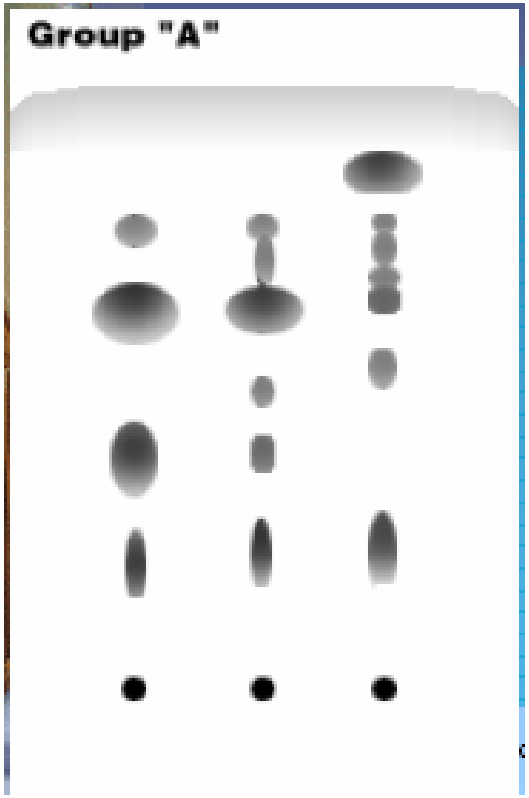
a- Paper chromatography

- **Preparation of sample.**
  - Samples may have different degrees of purity. It may come from filtrate of liquid media, extract from filtrate or cell extract or other form of sample preparation.
- **Development of chromatogram.**
  - Three forms of chromatograms are used routinely, strips, sheets or circular papers. The samples are separated either by descending, ascending, horizontal or radial separation.
- **Selection of solvent systems.**
  - Because the differences in antibiotic structures, different solvent systems may be used depending on the nature of antibiotic to be separated.
- **Detection.**
  - Physical, chemical, radiochemical or bioautographic detection can be used.

1- Chromatography of antibiotics

b- Thin layer chromatography (TLC)

- The antibiotics are loaded on thin layer of matrix (like silica) spread on a glass plate and separated by migration with a certain solvent.
- The steps are similar to that of paper chromatography.
- TLC is greatly simplified by the use of precoated plates e.g Eastman silica gel chromatogram sheets is used
  - Isolation of antibiotics
  - Separation of a mixture of antibiotics
  - Study of chemical modification
  - Purity control



b- Thin layer chromatography (TLC)

1- Chromatography of antibiotics

c- Gas -Liquid chromatography (GLC)

- It is old technique and is largely replaced by high-performance liquid chromatography (HPLC).
- It is used in studying of aminocyclitol, aminoglycosides, macrolide, aromatic, penicillin and other miscellaeous antibiotics

1- Chromatography of antibiotics

d- Liquid chromatography (LC)

This method is used mainly for isolation, separation, and purification of antibiotics.

Many types of LC are used like ion exchange, gel filtration, affinity, hydrophobic chromatography, etc.

The most advanced technique is HPLC which uses small quantity of sample and small column and gives very good separation profile.

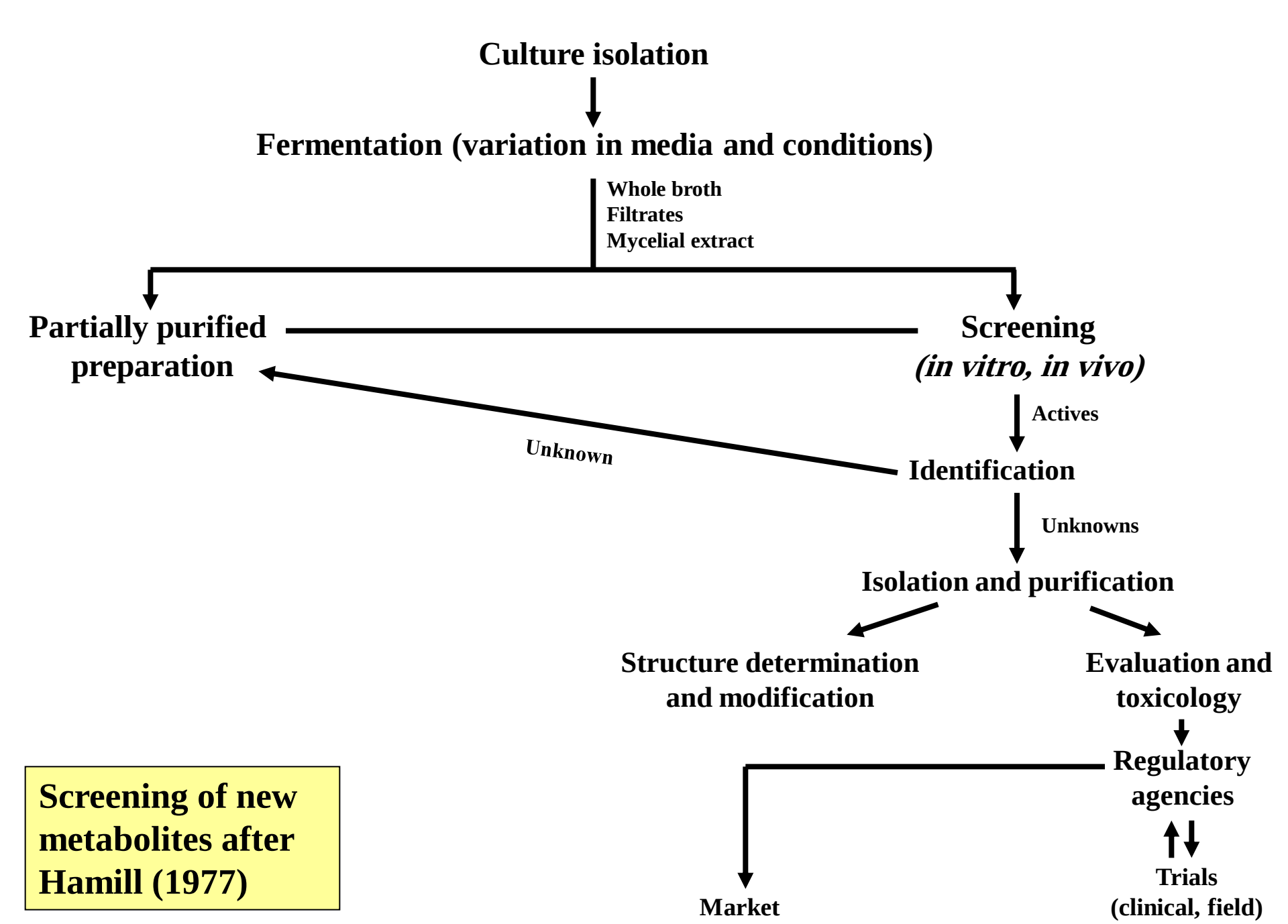
The separated fractions can be monitored by measuring the absorbance of UV light or by assaying the content of certain chemical.

2- Electrophoresis of Antibiotics

- The principle of this technique is the separation of the antibiotics depending on the charge it carries or depending on its size when subjected to electric field.
- Many types of electrophoritic techniques are used, including paper electrophoresis, agarose, polyacrylamide, etc.
- After electrophoresis antibiotics are detected by bioautography, UV light or with reagent special for functional group.

Identification of structural structure using computers

- Recently computerized data is available about the whole gene sequence of bacteria.
- Computer –assisted elucidation of structure is possible now. Computer programs are available



Screening of new metabolites after Hamill (1977)