12

ANTIBIOTIC SUSCEPTIBILITY TESTING

12.1 INTRODUCTION

Once we have identified the bacterium which is causing the infection we need to find out the antibiotics that would be effective against it. This is done by antibiotic sensitivity testing. there are various methods which can be employed for this purpose

OBJECTIVES

After reading this chapter, you will be able to :

- describe various terminologies related to Antibiotic susceptibility testing
- escribe principle for Antibiotic susceptibility testing.
- describe the procedure for performing Antibiotic susceptibility testing
- describe different methods used for Antibiotic susceptibility testing

12.2 TERMINOLOGY

Selectivity

All Clinically effective antimicrobial agents exhibit selective toxicity towards the bacterium rather than the host. It is this characteristic that distinguishes antibiotics from disinfectants. The basis for selectivity will vary depending on the particular antibiotic. When selectivity is high the antibiotics are normally non toxic. However, even highly selective antibiotics can have side effects.

Therapeutic Index

The therapeutic index is defined as the ratio of the dose toxic to the host to the effective therapeutic dose and the higher the therapeutic index the better the antibiotic.

Notes

Categories of Antibiotics

Antibiotics are categorized as bactericidal, if they kill the susceptible bacteria or bacteriostatic, if they reversibly inhibit the growth of bacteria. In general the use of bactericidal antibiotics is preferred but many factors may dictate the use of a bacteriostatic antibiotic. When a bacteriostatic antibiotic is used the duration of therapy must be sufficient to allow cellular and humoral defense mechanisms to eradicate the bacteria. If possible, bactericidal antibiotics should be used to treat infections of the endocardium or the meninges. Host defenses are relatively ineffective at these sites and the dangers imposed by such infections require prompt eradication of the organisms.

In vitro sensitivity tests

Bacterial pathogens are tested for their susceptibility to antibiotics to guide antibiotic treatment. Sensitivity tests are generally performed from single pure bacterial colonies on an agar plate. Direct sensitivity tests are set up directly from specimens or liquid cultures, producing quicker, but less standardized results.

Disk sensitivity tests

Antibiotic diffuses out of a disk placed on the surface of the agar. If bacteria are sensitive to the antibiotic, then a zone of growth inhibition forms around the disk after incubation. The zone size depends on several factors and two methods are available to control this process, comparative disk testing (where both a test and control organism are tested on the same plate), and standardized disk testing.

Breakpoint sensitivity tests

Antibiotic is incorporated into the agar at a uniform concentration and bacteria inoculated onto the agar surface. Only bacteria resistant to the antibiotic at the breakpoint concentration will then grow. Using multipoint inoculators, many bacterial strains can be tested simultaneously on each agar plate.

Minimum inhibitory concentration (MIC)

The MIC is the minimum (lowest) concentration of an antibiotic that will inhibit the growth of a bacterial strain. This can be determined by several methods including macro- and micro dilution tests, extended breakpoint sensitivity tests, and e-test strips. Determination of MIC is important in the management of certain infections (e.g. Endocarditis).

Minimum bactericidal concentration (MBC)

The MBC is the lowest concentration of the antibiotic that will kill a bacterial strain. The MBC is less clinically relevant than the MIC, as MBC tests are harder to standardize.

Detection of bacterial resistance mechanisms

Various bacterial resistance mechanisms (e.g. ß-lactamase production, antibiotic resistance genes) can be detected in the laboratory, providing a quick method of predicting *in vitro* sensitivity results.

Automated sensitivity tests

Automated systems can reduce the technical time required to perform sensitivity tests. These systems often utilize liquid culture, producing faster results than conventional agar based tests.

Clinical relevance of *in vitro* antibiotic sensitivity test

In vitro sensitivity test results should only be used as a guide to treatment, and the results do not always correlate with clinical response. The success of antibiotic treatment can be affected by many factors including immune responses, pharmacological factors and other biological variables, and the presence of biofilms.

In vitro sensitivity tests

In order to guide the appropriate antibiotic treatment of bacterial infections, bacterial pathogens isolated from clinical specimens are usually tested against a selection of antibiotics to assess their degree of susceptibility. This is usually done with bacteria that have been grown on solid media. Sensitivity tests are performed from **single pure colonies** and require a further 18–24 hrs of incubation. Thus while culture results may be available within 24 hrs of receipt of a specimen, sensitivity results usually take an additional day.

In some situations, **direct sensitivity tests** are performed, either from the specimen itself (e.g. Urine) or from a liquid broth with bacterial growth (e.g. Blood culture bottle). In this case, sensitivity tests are setup at the same time as the specimen is subcultured to agar plates. Although this speeds up the process, there are several disadvantages:

(i) it is difficult to ensure the correct **inoculum** (the number of bacteria spread onto the agar surface)

(ii) the inoculum may be **mixed** (more than one type of bacteria), making the results difficult to interpret and requiring the test to be repeated

(iii) the selection of antibiotics tested may be inappropriate for the bacterium subsequently grown.

INTEXT QUESTIONS 12.1

Match the following

1. Selectivity	(a) Kills bacteria
2. Therapeutic index	(b) Minimum concentration for inhibiting bacterial growth
3. Bacteriocidal	(c) Selective toxicity to antimicrobial agents
4. Bacteriostatic	(d) Minimum concentration that kills bacteria
5. Minimum Inhibitory Concentration	(e) Ratio of toxic and effective dose
6. Minimum Bactericidal Concentration	(f) Inhibits bacterial growth

Several different methods are available for assessing the susceptibility of bacteria to antibiotics.

Disk sensitivity tests

Disk sensitivity tests are performed on agar plates. A small disk of filter paper, pre-impregnated with a defined quantity of antibiotic, is placed on the surface of an agar plate that has already been inoculated with a suspension of bacteria. The antibiotic diffuses out of the disk into the agar, along a concentration gradient, as the plates are incubated (for 18–24 h). If the bacterial strain is sensitive to the antibiotic, then a **zone of inhibition** (no growth) occurs around the disk (Fig. 12.1).

The diameter of the zone depends on a number of factors including

(i) the quantity of antibiotic within the disk

(ii) the degree of susceptibility of the bacteria to the antibiotic

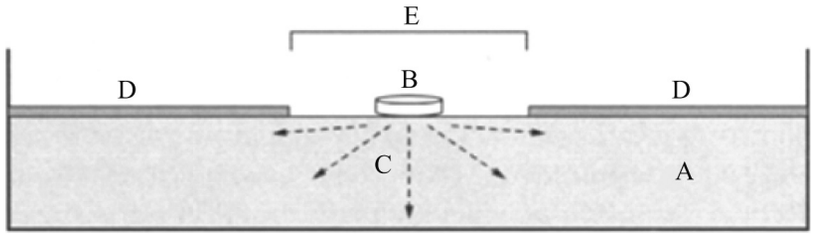


Fig. 12.1: Disk sensitivity test. A – agar; B – antibiotic disc; C – antibiotic diffuses into agar along concentration gradient; D – bacterial growth on surface of agar after 18 hours of incubation; E – zone (diameter) of inhibition.

(iii) the physicochemical properties of the antibiotic;

(iv) the depth (in mm) of the agar plate;

(v) the concentration of bacteria in the inoculum (semiconfluent growth is required).

There are two methods employed to determine the sensitivity pattern. The **comparative disk test (stokes' method)** uses both a test organism and a control organism on the same plate *(fig. 12.2)*. The control organism is of defined sensitivity to the antibiotics being tested, and this method allows a direct comparison of the diameter of the zones of inhibition between the test and control organisms.

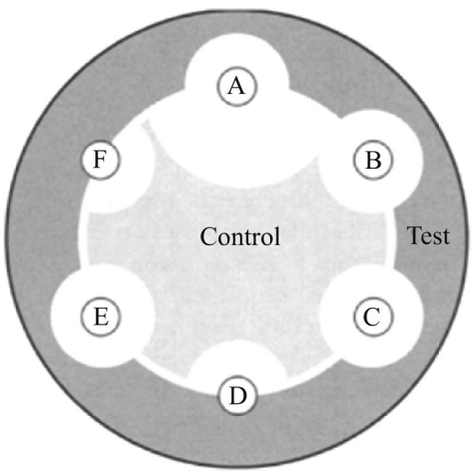


Fig. 12.2: Schematic representation of comparative disk sensitivity test (stokes′ method). Control—control bacterial strain (known sensitivity to antibiotics);test—bacterial strain under test; A-F- six different antibiotic disks. In this figure, the test organism is sensitive to antibiotics B, C & E, but resistant to antibiotics A (>3 mm reduction in zone diameter compared to control), D & F.

Standardized disk testing

This uses carefully standardized agar plates and inocula. A standardized inoculum of the test organism is plated out across the whole surface of the agar plate (control organisms are tested on a separate plate). The diameter of the zones of inhibition are measured in mm, and the organism

Reported as sensitive or resistant based on defined cut-off points (for example <18 mm=resistant).

One disadvantage of disk testing is that it is usually only possible to have a maximum of six different antibiotic disks on a standard agar plate.

Breakpoint sensitivity tests

Breakpoint sensitivity tests use a different principle to disk testing. A defined concentration of antibiotic (the 'breakpoint') is incorporated into the agar during production of the agar plates. Bacteria are then inoculated onto a small part of the surface of the agar (usually with 'multipoint' inoculators that allows many different stains to be tested on the same plate) and the agar plate incubated for 18–24 hrs. Bacteria that are resistant to the antibiotic (at the breakpoint concentration) will grow, whilst those that are sensitive will not. A control agar plate with no added antibiotic is used to check for viable bacterial growth (Fig. 12.3).

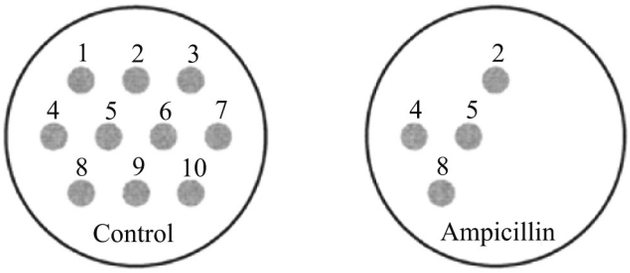


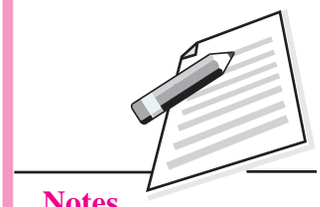
Fig. 12.3: Breakpoint sensitivity tests. Control – sensitivity agar plate with no added antibiotic; Ampicillin—sensitivity agar plate incorporating Ampicillin at a defined (uniform) concentration 1 to 10- different bacterial strains inoculated on to the surface of the agar plates with a multipoint inoculator. In this example, all 10 strains have grown on the control plate. Strains 1, 3, 6, 7, 9 & 10 are sensitive to Ampicillin. Strains 2, 4, 5 & 8 are resistant.

Using a multipoint inoculator, >30 different strains of bacteria can be tested against a wide range of different antibiotics in one batch. This process is less technically time-consuming than the equivalent number of disk tests.

Sometimes the same antibiotic is used at two different concentrations in separate agar plates (e.g. 1 and 4 mg/1). Using these low and high breakpoint concentrations, bacteria can be classified as 'sensitive', 'intermediate' or 'resistant'. By including a whole range of concentrations of the same antibiotic in separate plates, the minimum inhibitory concentration of the antibiotic can be determined for each of the strains being tested (see below).

Minimum inhibitory concentration (MIC)

The MIC is the minimum (lowest) concentration of an antibiotic that will inhibit the growth of a bacterial strain. Conventionally, this is determined using a series of doubling dilutions of the antibiotic in liquid culture medium, to produce a range of concentrations in test tubes (macrodilution) or in a microtiter tray (microdilution). After inoculation of the test strain into each antibiotic concentration, bacterial growth is determined by visible turbidity after 18–24 h of incubation (Fig. 12.4). The MIC is the lowest concentration of antibiotic with no visible bacterial growth.



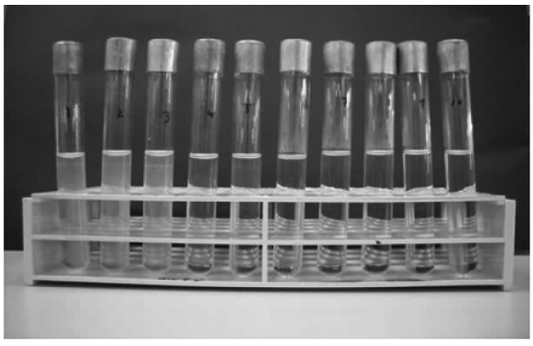


Fig. 12.4: Broth Dilution test

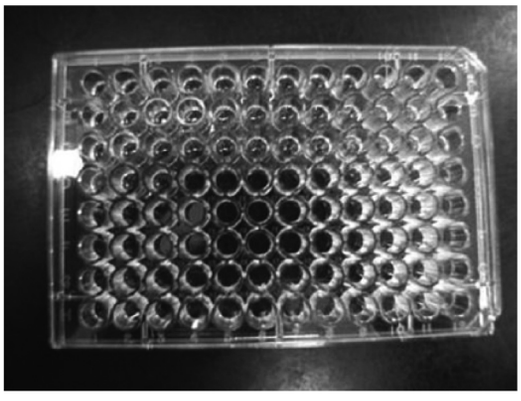


Fig. 12.5: Microbroth dilution Test

MIC tests can also be done by **extended breakpoint sensitivity tests** (see above). These methods are technically time-consuming and relatively expensive. An alternative method is by use of commercially available **E-test** strips. These are specialized antibiotic-impregnated strips which, like disk testing, are placed on the surface of inoculated agar plates. During incubation, antibiotic diffuses into the agar forming a zone of inhibition. There is a manufactured concentration gradient within the strip, and numerical gradations are marked along the edge of the strip to reflect this. The MIC is determined by measuring the point at which the edge of the zone of inhibition crosses the e-test strip *(fig. 12.5)*.

Antibiotic MIC tests are usually performed only in certain situations in a clinical bacteriology laboratory. They are most commonly used when a very precise assessment of the *in vitro* susceptibility of a bacterial strain is required, for instance in the treatment of pneumococcal meningitis (topic f3) or Streptococcal endocarditis. MIC tests are also used to assess the overall degree of activity of antibiotics against different strains of the same bacterial species, particularly when evaluating or developing new antimicrobial agents. A simple way of describing the relative activity of an antibiotic against a group of organisms, is by using the terms mic50 and mic90. These are the lowest concentrations of the antibiotic that inhibit 50 and 90% of the bacterial strains tested, respectively.

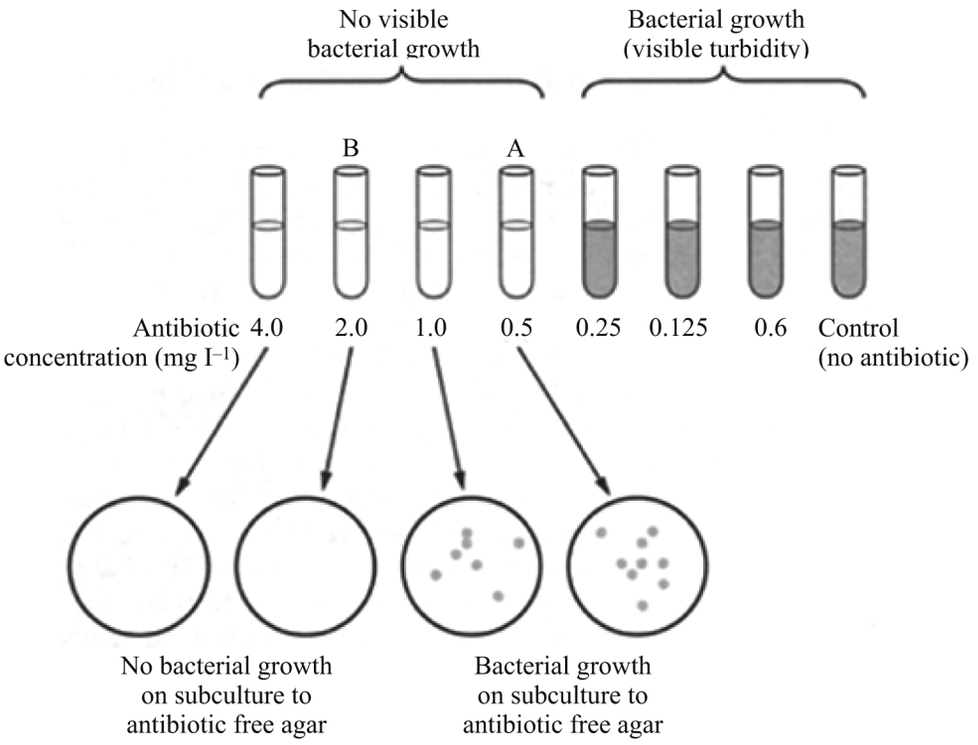


Fig. 12.6: MIC and MBC testing. In this example, the minimum inhibitory concentration (MIC) of the antibiotic is 0.5 mg/l (tube a). The Minimum Bactericidal Concentration (MBC) is 2.0 mg/l (tube B).

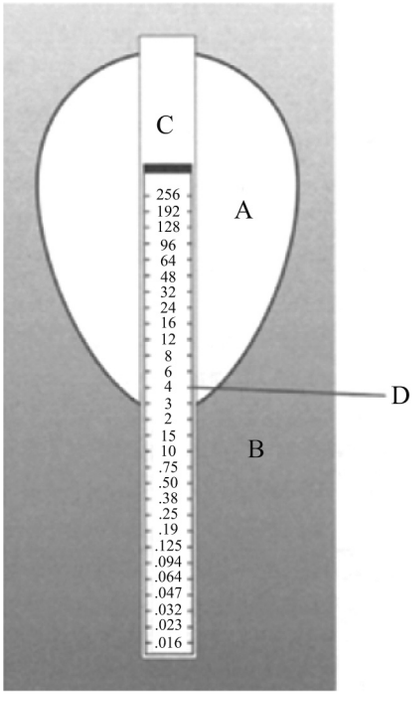


Fig. 12.6: Determination of MIC by E-test. A – Zone of inhibition; B – Bacterial growth; C – E-test strip; D – the MIC is the point at which the edge of the zone crosses the E – test strip – in this example it is 3 mg/l.

Minimum bactericidal concentration (MBC)

The MBC is the lowest concentration of the antibiotic that will 'kill' a bacterial strain. The definition of 'killing' is a 99.9% (3 Iog10) reduction in viable bacteria. The MBC test is an extension of an MIC test *(fig. 12.6)*. The simplest method for determining the MBC is to perform a subculture from antibiotic concentrations with no visible growth in the MIC test on to antibiotic-free agar. This will determine whether the bacteria have been inhibited from growing but are still viable, or whether they have been killed.

Some antibiotics are highly **bactericidal**. In this case the MIC and MBC are usually very similar. B**acteristatic** antibiotics on the other hand have much higher MBC than MIC. Occasionally a bacterial strain may have a high MBC but low MIC with a normally bactericidal antibiotic (e.g. Penicillin). This is described as bacterial **'tolerance'** to the antibiotic.

MBC tests are very difficult to standardize and are often not entirely reproducible. The clinical relevance of MBC tests and the demonstration of tolerance is less clear than with MIC determinations, but they are occasionally performed to guide antibiotic therapy in some difficult cases of infection.

Detection of bacterial resistance mechanisms

An alternative method for guiding appropriate antibiotic therapy is through the detection of bacterial resistance mechanisms. These can be used to predict the results of conventional sensitivity tests, especially when a specific resistance mechanism is detected. Often these tests do not require overnight incubation, and thus the results may be available at an earlier stage to guide treatment.

Some common examples of bacterial resistance detection used in clinical laboratories are given in table 12.1. This type of approach is likely to become increasingly used, especially as molecular techniques become more widely available.

Table 12.1: Examples of the detection of bacterial resistance mechanisms

Resistance mechanism	Organism	Method of detection	Comment
ß-Lactamase production	Haemophilus influenza neisseria gonorrhoeae	Rapid 'stick' test (hydrolysis of nitrocefin)Rapid 'stick' test (hydrolysis of nitrocefin)	Predicts resistance to ampicillin and amoxicillin predicts high level resistance to penicillin
Methicillin resistance (altered pbp2?)	Staphylococcus aureus	Latex agglutination for pbp2?detection of mec*a* gene by pcr	Predicts resistance to ?-Lactam antibiotics (mrsa)
Rifampicin resistance	Mycobacterium tuberculosis	Detection of *rpo b* gene mutations by pcr	Detects 95% of rifampicin-resistant *m. Tuberculosis* strains

PBP- Penicillin Binding Protein; MRSA - Methicillin Resistant Staphylococcus *aureus.*

Notes

Automated sensitivity tests

There are a variety of commercially available automated systems available to help reduce the technical time required to perform and record routine sensitivity tests. For example, the results of disk sensitivity tests and breakpoint sensitivity tests can be read using a camera interfaced to a computer system. Other

Systems utilize **liquid cultures,** and detect the effect of antibiotics on the rate of bacterial growth through measurement of turbidity **(nephelometry)** or the production of co2. These automated systems can significantly shorten the necessary incubation time, with the possibility of some results being available within the same working day. They can also significantly reduce the time taken to produce sensitivity results for slow-growing organisms, notably *mycobacterium tuberculosis*.

Clinical relevance of *in vitro* antibiotic sensitivity tests

It must be remembered that *in vitro* sensitivity tests are only a **guide** to the appropriate antibiotic treatment. A laboratory report indicating that organism A, is resistant to antibiotic B does not necessarily mean that antibiotic B will not work, and *vice versa*. Whilst *in vitro* tests are designed to try and reflect the *in vivo* situation (e.g. through utilization of appropriate breakpoints to reflect antibiotic pharmacokinetic parameters), they can never take account of all the human and bacterial biological variables.

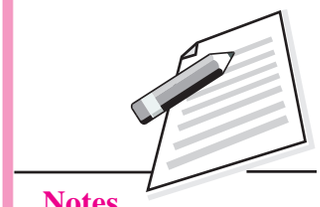
There are a number of factors to consider when interpreting laboratory reports that include sensitivity test results:

(i) many infections will resolve spontaneously (assuming a normal immune system). If a patient has already responded clinically to a certain antibiotic treatment, then it is not always necessary to change the antibiotic if the laboratory report indicates that the organism isolated is 'resistant'.

(ii) the organism identified on the laboratory report may not be the primary pathogen.

(iii) an organism reported with sensitivity results does not always require treatment. A good example of this is with catheter-specimens of urine. Treatment is generally required only if the patient is symptomatic (i.e. Treat the patient not the result!).

(iv) an antibiotic with apparent *in vitro* activity may not work clinically, as there are many pharmacokinetic and other factors to consider in choosing the most appropriate antibiotic therapy



INTEXT QUESTIONS 12.2

1. ß lactamase production is detected by ..... test
2. Methcillin resistance is detected by ..... test
3. Rifampicin resistance is seen in ..... infection
4. Bacterial stains having high MBC with low mic is described as ..... to antibiotics

WHAT HAVE YOU LEARNT

- Clinically effective antimicrobial agents exhibit selective toxicity towards bacterium
- Therapeutic index is the ratio of dose toxic to host to the effective therapeutic dose.
- Antibiotics are bactericidal, if they kill the susceptible bacteria or bacteriostatic, if they inhibit the growth of bacteria
- Antibiotics diffuses out of a disc placed on the surface of agar
- Bacteria resistant to antibiotic at breakpoint concentration will then grow
- Minimum inhibitory concentration is the lowest concentration of antibiotic that will inhibit the growth of bacterial strain.
- Minimum bactericidal concentration is the lowest concentration of antibiotic that will kill a bacterial strain.

TERMINAL QUESTIONS

1. Describe the difference between MIC and MBC.
2. What are various culture media that can be used for Antibiotic Susceptibility Test
3. Describe in brief various methods for antibiotic susceptibility testing.

ANSWERS TO INTEXT QUESTIONS

12.1

1. (c)
2. (e)
3. (a)
4. (f)
5. (b)
6. (d)

12.2

1. Rapid stick
2. Latex agglutination
3. Mycobacterium tuberculosis
4. Tolerance