Antimicrobial susceptibility testing (Antibiogram; AST)

An antibiogram is a laboratory technique designed to test the susceptibility of a bacterial strain to one or more known antibiotics. Antimicrobial susceptibility testing is the final step of the bacteriological diagnosis and it is performed in order to aid the clinicians in determining a proper antibiotic therapy for the patient.

There are several ways to perform antimicrobial susceptibility testing:

- Disk diffusion method
- Broth microdilution method
- Broth macrodilution method
- Antimicrobial concentration gradient method (E-test)

Disk diffusion method

Principle of the technique

Paper disks impregnated with a determined concentration of antimicrobial agent are deposited on the surface of a culture medium previously inoculated with an inoculum of pure culture of the bacterium to be tested. After incubation, the culture media are examined and the diameters of the **inhibition zones** (area in which no colonial growth has occurred due to inhibition of growth by the antibiotic) surrounding the disks are measured and compared to the critical values of various tested antimicrobial agents, in order to determine a clinical categorization (resistant, intermediate, sensitive). The diameter of the zone of inhibition is proportional to the sensitivity of the bacterium tested. The technique(s) are highly standardized in order to obtain consistent and comparable results from laboratories worldwide. The interpretation of AST results may be performed according to two systems (standards) i.e. the international CLSI (Clinical and Laboratory Standards Institute) of the CDC and/or the EUCAST (European Committee on Antimicrobial Susceptibility Testing). When an AST result is issued, the laboratory should always state which of these two standards was used.

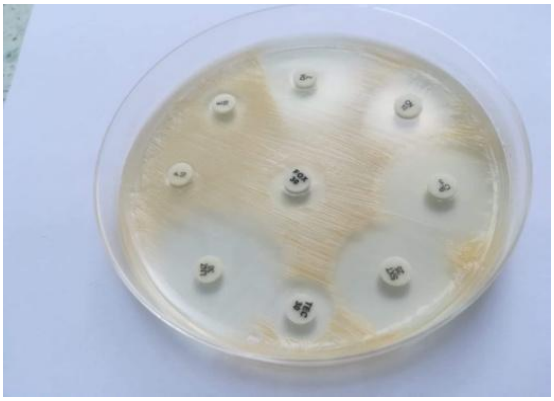

Figure 8: Diffusimetric method - Mueller-Hinton environment, methicillin-resistant staphylococcus - MRSA

Important parameters

The reliability of results of an antibiogram is influenced by many parameters that must be strictly controlled:

- The culture medium should allow the growth of many bacteria and should not contain antibiotic inhibitors. The medium selected for the majority of bacterial species is **Mueller-Hinton** (and 5% blood for fastidious germs).
- The antibiotic discs are made of high quality absorbent paper impregnated with antimicrobial agents at **precise concentrations**.
- The **density** of the bacterial inoculum is a crucial element. The cell suspension must be prepared in sterile physiological water from a young and pure culture on a suitable medium. The disk diffusion antibiogram is performed with a suspension calibrated at a turbidity of **0.5 McFarland units**, containing about 10^8 bacteria per ml.

Inoculation should be done within 15 minutes after the preparation of the inoculum. It is carried out by swabbing or flooding the plate in such a way as to have, after incubation, separate but joined colonies.

Reading the results

After incubation at recommended temperatures and atmospheres, the diameters of the inhibition zones will be measured accurately. The diameters of the zones of inhibition measured will be compared to the critical diameters given by the authorities in force (EUCAST or CLSI) - see above.

Clinical Categories

Three clinical categories were selected for the interpretation of in vitro sensitivity tests: **sensitive** (S), **intermediate** (I) and **resistant** (R):

- Strains categorized as **sensitive** are those for which the probability of **therapeutic success is high** in the case of a systemic treatment with the recommended dosage
- Strains categorized as **resistant** are those for which there is a **high probability of therapeutic failure** regardless of the type of treatment and the dose of antibiotic used
- Strains categorized as **intermediate** are those for which therapeutic success is **unpredictable**. These strains:
    - may have a resistance mechanism whose in vitro expression is low, with the consequent classification in the category S. However, in vivo, some of these strains appear resistant to treatment
    - may have a resistance mechanism whose expression is not sufficient to justify classification into category R, but sufficiently weak to hope for a therapeutic effect under certain conditions (high local concentrations or increased dosages)

Minimal inhibitory concentration

MIC is defined as the **minimum concentration** of an antibiotic that **inhibits** the **in vitro growth** of 99% of the bacterial population tested. In practice, the MIC is the lowest concentration of a half-to-half range of antibiotic dilutions that results in the inhibition of any visible bacterial growth.

Techniques for the determination of the MIC: **dilution methods** and **E-test**

Dilution methods

The dilution methods may be carried out in a **liquid medium** or in a **solid medium**. They consist of putting a standardized bacterial inoculum in contact with increasing concentrations of antibiotics (in a geometric progression with the power of 2).

- In liquid medium, the bacterial inoculum is distributed in a series of tubes (macrodilution method) or 96-well microtiter trays (microdilution method) containing the antibiotic. After incubation, the tube or well that contains the lowest concentration of antibiotic where no growth is visible will indicate the MIC.
- In a solid medium, the antibiotic is added into a culture medium (agar). Agar dilution involves the incorporation of different concentrations of the antimicrobial substance into a nutrient agar medium followed by the application of a standardized number (0.5 McFarland units) of bacterial cells to the surface of the agar. The MIC is determined by the area of inhibition on the medium containing the lowest concentration of antibiotic.

The E-test

The E-test is performed on a solid culture medium and the MIC is determined through the use of strips impregnated with an exponential gradient of the antibiotic to be tested. The principle is based on the combination of the characteristics of diffusion and dilution methods on solid media. The strips (inert, hydrophobic supports, 5 mm wide and 50 mm long) are placed on the surface of an agar medium previously inoculated with an inoculum of the strain to be studied. After incubation, inhibition of growth results in an **inhibition ellipse** whose intersection points with the strip define the MIC.

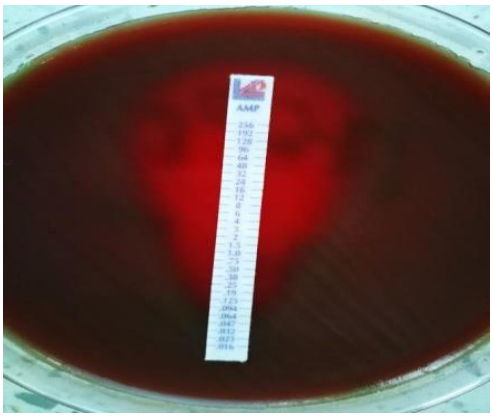

Figure 9. E- test

Combination of antibiotics

The interaction of two antibiotics can produce four main effects:

- **Synergistic** effect: the effect of the combination is greater than the sum of the effects produced by each of the antibiotics taken alone
- **Additive** effect: the effect the combination is slightly greater than the sum of the effects produced by each of the antibiotics taken alone
- **Indifferent** effect: the activity of one antibiotic has no influence on the activity of the other
- **Antagonistic** effect: the effect of the combination is less than the sum of the effects produced by each of the antibiotics taken alone.

Limitations in the interpretation of results

Uncertainty about the aetiology of the infection

The antibiogram can only be of use, if it is carried out on the bacterium that is responsible for the infection. Among the bacteria isolated from a sample, the laboratory must make a choice and perform the antibiogram only on the species likely to play an etiological role.

Absence of parallelism between in vitro and in vivo situations

The antibiogram cannot predict the behaviour of an antibiotic in vivo. This is a function of multiple factors:

- Choosing a dosage regimen
- Diffusion at the site of infection
- Penetration into cells, which is important to consider for infections caused by intracellular bacteria (antibiotics that penetrate cells well are **tetracyclines**)
- Influence of physiological or pathological factors on the pharmacokinetics of the antibiotic
- Transformation of the molecule in vivo
- Physiological state of the bacterium within the infectious focus (resting bacteria are insensitive to antibiotics that interfere with the biosynthesis of the peptidoglycan layer)
- Emergence of resistance during treatment
- Individual characteristics of the patient to be treated: age, associated pathologies, allergies, etc.

Given the above, the treating physician (the clinician) will decide on the most appropriate antibiotic/combination of antibiotics for his/her individual patient, based on the AST results corroborated to all the other aspects previously described.

Immunological reactions in the laboratory diagnosis of infections

Immunological reactions are based on the specificity of antibody (Ab) responses to antigens (Ag):
Ag + Ab = Ag-Ab complex

With this simplified formula, the microbiology laboratories diagnose infections by the detection of either antigens or antibodies. If such an Ag-Ab complex is formed, we conclude a positive test, whereas an absent Ag-Ab reaction indicates a negative test.

Immunological reactions used in microbiology may be classified in two main categories:

a. Bacteriological methods which are performed on bacterial cultures and/or biological samples and use specific antibodies for the identification of bacteria i.e. agglutination, immunofluorescence
b. Serological methods (e.g. ELISA) which are performed on biological samples (serum, CSF, urine) to detect the presence of:
    - (bacterial, viral, fungal, etc.) antigens or
    - antibodies against bacteria, viruses, fungi, etc.

Some of these techniques are briefly explained below.

Agglutination

- Direct bacterial agglutination into tubes
- Agglutination using Ag fixed on an inert particle carrier

When a reaction between the antigen (in tubes or on surface of particle carriers) and antibody has occurred, the Ag-Ab complex is formed and visible clumps are seen with bare eyesight.

Immunofluorescence

Immunofluorescence is a method that allows for the detection of an Ag-Ab complex by UV microscopy. The fluorescent substances most frequently used are *fluorescein isothiocyanate* (green fluorescence) and *rhodamine B* (orange fluorescence), which are conjugated to specific antibodies.

Direct immunofluorescence is one of the fastest, most sensitive and specific diagnostic methods used for the microbiological diagnosis. Through this method, microorganisms (i.e. antigens) can be identified directly from the biological product or from a bacterial culture. In addition, various antigens present in tissues can be demonstrated. The smear on which the antigen is searched on is stained with a fluorescent mixture of specific antibodies for about 30 minutes (to allow the labelled antibodies to bind to the antigen). After that, the slide is washed to remove excess non-fixed fluorescent antibodies, so that the slide can be examined under an UV microscope. If the antigen, to which the specific labelled antibody binds to, is present, observable fluorescent Ag-Ab complexes are visible on a dark background. Otherwise, if the antigen is absent, no fluorescence will be observable as the fluorescent Ab does not bind to the smear and is washed away.

Sandwich immunofluorescence is used to highlight anti-tissue antibodies. A solution containing the corresponding antigen is applied to the tissue preparation to be studied. Once it is united with the antibody, the preparation is covered with fluorescent antibodies (secondary antibodies) complementary to previously applied antigens.

Indirect immunofluorescence is generally used to detect antibodies in a biological specimen to which a secondary, labelled antibody attaches. Specific antigens for the antibodies to be investigated in the patient's serum are fixed on a slide. The slide is then covered with serum and incubated for a period of time. During this time, serum-specific antibodies will bind to the antigens on the slide. If the serum sample contains the antibodies to be investigated, labelled human anti-immunoglobulin antibodies (secondary antibodies) will bind to them and form a fluorescent Ag-Ab complex visible by UV microscopy. Indirect immunofluorescence is more sensitive than the direct method.

Enzyme-linked immunosorbent assay (ELISA)

The ELISA technology relies on Ag-Ab reactions bound (conjugated) to reactions between an enzyme and its specific substrate. The assays are performed on a solid support (usually a 96-well nitrocellulose plate).

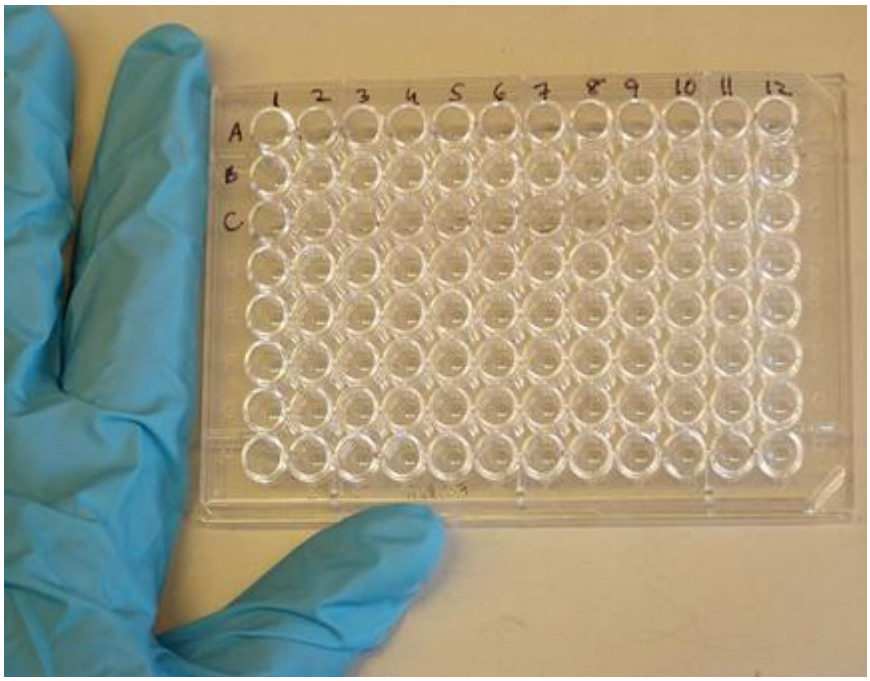

Figure 10. Solid support for ELISA: 96-well nitrocellulose plate
[https://www.thermofisher.com/Overview of ELISA]

These serological methods use two types of synthetic substances: the **conjugate** (antibodies conjugated to an enzyme) and the **substrate** (the substrate of the enzyme used in the synthesis of the conjugate).

When a certain antigen (bacterial, viral, etc.) is to be detected (target antigen) in the patient's serum the **conjugate** will contain specific antibodies against that antigen; these antibodies are bound (conjugated) to an enzyme.

For each serum sample, all the steps of the reaction take place in one of the wells on the plate.

If the patient's serum contains the target antigen, then, upon addition of the **conjugate**, the antibodies and its components will bind to the antigen, forming an Ag-Ab complex; this Ag-Ab complex will also contain the enzyme in the **conjugate**, so at this point a new structure is formed: **Ag-Ab-enzyme**. After an intermediate washing step, which is done in order to remove any unbound fragments/substances, the next step consists of adding the **substrate** of the enzyme; the enzyme will act upon its substrate producing a reaction visible with the naked eye (usually a colour change). This colour change will demonstrate that the patient serum contains the target antigen **= positive** result.

In case the patient serum does not contain the target antigen, the antibodies in the **conjugate** will have nothing to bind to, so there will be no Ag-Ab reaction and the above-mentioned new structure (Ag-Ab-enzyme) will not form. Consequently, the washing step will remove the unbound **conjugate** (Ab-enzyme). When the **substrate** is added there is no colour change because there is no enzyme left in the nitrocellulose well = **negative** result.

When the substance to be detected i.e. the target, is an antibody i.e. an immunoglobulin, then the enzyme in the **conjugate** should be conjugated to synthetic antigens or to synthetic monoclonal

antibodies specifically tailored to match and bind to the immunoglobulin in question i.e. the target antibody.

Radioimmunoassay

These tests use radioactively-labelled antigens to detect the presence of specific antibodies. The level of antibodies is assessed by measuring the radioactivity of the sample. Nowadays, the use of these tests in the microbiological diagnosis is being gradually replaced by ELISA and Western blotting/immunoblotting, as well as by molecular biology techniques (PCR, etc.).

Molecular techniques

The most used molecular diagnostic method is the **polymerase chain reaction (PCR)**, a diagnostic procedure that **detects** the **DNA** or **RNA** of microorganisms within biological specimens. The advantage of such method is that it does not necessitate the microorganisms to be alive and that only small amounts are sufficient, because the DNA or RNA is amplified, i.e. **PCR is a technique that can amplify a specific region of the genome**. The use of the enzyme DNA polymerase and DNA primers achieve the amplification of a specific genome region. Furthermore, PCR is **fast**, **sensitive** and **specific**.

PCR may be used for many purposes in microbiology e.g.:

- Infections due to bacteria with difficult or very slow growth. Even if the germ is present in the sample at very low concentrations, it can thus detect its presence
- Viral infections
- Detection of antibiotic resistance genes, etc.