CHAPTER 11. ANTIMICROBIAL RESISTANCE DETECTION

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**DEFINITION OF RESISTANCE**

According to the CDC (Center of Control of Disease) of the United States the bacterial resistance occurs when bacteria can no longer be killed by the drugs that are normally used to treat them (1,2). When the bacteria become resistant, it produces enzymes, called beta-lactamases, fails to inhibit the growth or kill a microorganism. There are two types of resistance: acquired and clinic. The acquired is carried out in the laboratory through of mutations or by gene transfer, systems of conjugation, transduction and transformation. The clinical resistance is detected in everyday treatment in Medicine, where the physician can see that not all the microorganisms have the same sensibility or the same resistance to the antibiotics and there is the need to send the sample in order to use laboratory methods to use quantitative or qualitative techniques. Since 1948 the World Health Organization (WHO) has been working to improve the health in many countries, as a basic human right. Bacterial resistance is one of the major public problems for all nations in the world (3).

**MECHANISMS OF BACTERIAL RESISTANCE**

Bacterial resistance has been used to destroy or inactivate enzymatic, precursor alterations in cell walls, membranes and ribosomes (2).

Between the years 90 a series of antimicrobials such as beta-lactamases, aminoglycosides, macrolides, sulfonamides, quinolones, tetracyclines. The first antibiotic was the penicillin and the beta-lactamases were classified as natural (Penicillin G) then the semi-synthetics of wide spectrum as ampicillin, amoxicillin, carbenicillin. Nowadays the beta-lactamases classification is: A penicillin: methicillin, ampicillin, carbenicillins, mexclocillin, piperacillin. B Cephalosporins: first generation (cephalexin, cefradine, cephalothin), second generation (cefamandole, cefuroxime), third generation (cefotaxime, ceftriaxone, ceftazidime), fourth generation (cefepime), and fifth generation (ceftaroline, avibactam, relebactam, vaborbactam). C. Cephamycin, cefoxitin, cefotetan, cefmetazole. D. Carbapenems, imipenem, meropenem. E. Monobactams: Aztreonam (2).

When the bacteria are resistant, produces enzymes, called beta-lactamases, and they are named as (tem-1, tem-2, ampC, ampR, ampG, ampD, OmpF, OmpC, etc. For example, the bacteria that produce tem-3 and tem-5, are resistant to cephalosporins, penicillin and cefotaxime. When they have the oxa-1 and PSE2 are resistant to penicillin and cloxacillin. There are more than 250 enzymes in the literature (1,2).

Group 2 are cephalosporinases that hydrolyze extended-spectrum cephalosporins and to be inhibited by clavulanic acid or tazobactam. They are identified as ESBLs.

Group 3 MBLs (Metallo-beta-lactamases) is a unique group of β-lactamases both structurally and functionally. They are a combination with a second or third beta-lactamase in clinical isolates, they have a zinc ion at the active site. They are subdivided on base of their structure subclasses B1, B2 and B3 (4).

When the bacteria are resistant to macrolides, lincomisomides, the enzymes are called MLSb, against: erythromycin *erm*, tetracycline *mar*RAB, sulfonamides DHSP, quinolones: DNA gyrase, gyrA, norA, ofxA, cfxA, Chloramphenicol CAT, aminoglycosides: ant(2”) la, ant (3”) la, aac (3) la, aac (6) la. In Table 1 shows the Escherichia *coli* and some of the enzymes that produce antimicrobial resistance (5,6).

Table 1. *ESCHERICHIA COLI* ENZYMES THAT PRODUCE ANTIBIOTICS RESISTANCE (2)

|  |  |
| --- | --- |
| ***E COLI* ENZYMES** | **ENZYMES AGAINST ANTIBIOTICS** |
| AmpC, porines, OmpC, CTX-M, SHV, TEM | Β-LACTAMASES |
| MarRAB | TETRACYCLINES, CHLORANPHENICOL, β-LACTAMASES, QUINOLONES |
| Ant(2”)la, ant(3”)la | AMINOGLYCOSIDES |
| Erm | ERYTHROMYCIN |
| TET(A)/E | TETRACYCLINE |
| DPHS | SULFONAMIDES |
| DNA girax, GyrA gene | NALIDIXIC ACID, FLUORIQUINOLONE |
| Cat | CHLORANPHENICOL |
| DHFR | TRIMETOPRIM |
| RpsL | STREPTOMYCIN |

**METHODS IN A MICROBIAL LABORATORY TO DETECT BACTERIAL RESISTANCE**.

Nowadays there are manual and automated systems to detect bacterial resistance: *in vivo* we can detect the bacterial resistance because the antibiotic does not work and *in vitro* in the laboratory. The easiest one is a manual Sensitivity Antibiotic Test (AST) (7). In Europe EUCAST (European Committee on Antimicrobial Susceptibility Testing) and in United States CLSI (Clinical Laboratory Standards Institute) approves the methods to know the bacterial resistance (8,9).

1. Disk Diffusion Test (DDT) describe by Kirby Bauer (1,2,7), since 1961.
2. MIC (Minimal Inhibition Concentration) since (1,2,8,9) are automated and many equipment are used nowadays.
3. E test since 1991, Glupezynski et al (2,10) is a combination of both DDT and MIC. It is the epsilometer system.
4. Automated systems. Since 1963 Gerlach et al (1,2,11,12) verified a correlation of 97.7% between the microdilution technique and automated systems. There are many equipments as MS2 AutoSCAN, Vitek, API, Unisept, Conbas, Vitek 2AST-YS06, MALDI TOF.
5. Sensititre system is a MIC plate testing supports for a mix of manual, semi- and fully automated solutions to have AST results. It uses a nephelometry and automated equipment. In this link you can see the way it works. (13)

<https://www.thermofisher.com/ie/en/home/clinical/clinical-microbiology/antimicrobial-susceptibility-testing/sensititre-ast.html>

1. The molecular technique can determine the genetic material, both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA). Polymerase chain reaction (PCR) is the molecular technique that has acquired the greatest diagnostic value, this helps to know the identification of the infectious agent plus the resistance and virulence genotypes. Conventional PCR takes more or less 12 hours to do the method in 3 steps: 1. Extraction of genetic material, 2. Thermocycler a DNA amplification and 3 Detection of the amplicons (14,15,16).
2. There are other microarrays, colorimetric, flow cytometry, chemiluminescence, bioluminescence (12,17).
3. Nowadays the MALDI-TOF mass spectrometry is the most used in United States to have the ID and AST of the samples in a Clinical Microbiology Laboratory. It needs pure cultures to give a result. (12,18,19,20,21,22). The MALDI TOF system can detect the enzymes produced by the bacteria that hydrolyze the antibiotics, such as carbapenems and ESBLs, in less than 3 hours. The method is to incubate the microorganism for a while with the antibiotic, then centrifugate and the supernatant obtained is analyzed using MALDI-TOF. If the bacterium does not hydrolyze the antibiotic, the only peak that shows the system is of the antibiotic.

These techniques are used for bacteria that grow fast. For anaerobe bacteria it is used a dilution broth or agar, test E for anaerobes (1,2,6,11,12).

**BETALACTAMESES DETECTION**

Many techniques have been described. In 1972 Rosen searched an acidimetric method can be used disk or strip. In 1975 Catlin used an iodometric system in paper with starch and penicillin in order to see the color change from iodine to iodide, in 1977 Slack used the paper with penicillin and a pH indicator, when the beta lactamase structure is broken to produce an acid penicilloic and in 1980 other researcher used sticks with nitrocefin and a chromogenic cephalosporin, a positive reaction changes to red and negative no color occurs. These sticks change color to pink when a beta-lactamase is in the bacteria, it used for *Neisseria, Haemophilus, Staphylococcus or Bacteroides* (1,2,11).

Table 2. Beta-lactamase methods (11).

|  |  |  |  |
| --- | --- | --- | --- |
| **TEST DETAIL** | **NITROCEFIN** | **ACIDIMETRIC** | **IODOMETRIC** |
| Substrate | Nitrocefin (chromogenic cephalosporin) | Citrate-buffered penicillin plus phenol red | Phosphate-buffered penicillin plus starch-iodine complex |
| Reaction | Color change when beta-lactam ring opens | Penicilloic acid produces pH decrease | Penicilloic acid reduces iodine and prevents it from combining with starch |
| Result Positive Negative | Red  No color change | Yellow  Red | Colorless  Blue/purple |

**DETECTION OF BETALACTAMASE BROAD SPECTRUM**

ESBLs: is a beta lactamase of broad spectrum, as TEM, SHV, CTX-M, OXA and now there is more than 150 different types of enzymes. (1,2,4,6,11,12). In 1983 the cephalosporins were detected in Germany, and in 1985 in France. This type of ESBLs has been found in *Klebsiella pneumoniae* (23,24)*, Escherichia coli, Enterobacter, Salmonella, Proteus, Aeromonas* and *Pseudomonas.* They can be detected by broth dilution with MIC with Vitek automated system or Microscan, E test or doble disk. CLSI suggest for *Klebsiella* and *E coli* to make a first discard if the zones of inhibition in an AST is as Table 2, then to do the ESBLs technique to reconfirm data.

Also, there is anaerobic bacteria with resistance to beta lactamase as *Bacteroides* thetaiotaomicron (2).

Table 3. MIC zone inhibition to detect ESBLs in *K pneumoniae* and *E coli* (2)

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibiotic** | **Inhibition zones for sensible strains** | **Inhibition zones with possible production of ESBLS** | **Sensible strains for MIC** | **Possible strains produce ESBLs with MIC** |
| Aztreonam | ≥ 22 mm | ≤ 27 mm | ≤ 8 mg/L | ≥ 2 mg/L |
| Cefotaxime | ≥ 23 mm | ≤ 27 mm | ≤ 8 mg/L | ≥ 2mg/L |
| Cefpodoxime | ≥ 21 mm | ≤ 22 mm | ≤ 8 mg/L | ≥ 2mg/L |
| Ceftazidime | ≥ 18 mm | ≤ 22 mm | ≤ 8 mg/L | ≥ 2 mg/L |
| Ceftriaxone | ≥ 21 mm | ≤ 25 mm | ≤ 8 mg/L | ≥ 2 mg/L |

The ESBLs as CLSI recommends it, to do an inoculum as MacFarland 0.5. This inoculum is smeared onto Mueller Hinton Agar and put the sensidiscs of ceftazidime, cefotaxime with the other shown in Table 3 (2).

Table 3 Combined discs to detect ESBLs

|  |  |
| --- | --- |
| **COMBINED DISCS** | **CONCENTRATION IN** **µg** |
| Cefpodoxime/Clavulanic Acid | 10/1 |
| Cefpriome/Clavulanic Acid | 30/7.5 |
| Cefotaxime/Clavulanic Acid | 30/10 |
| Ceftazidime/Clavulanic Acid | 30/10 |

When there is a difference greater than 5 mm from the discs combined in relation with the discs with only one antibiotic, the ESBLs is confirmed o in automated when it gives >2mg/L. For example, with a combined disc in the DDT technique has a zone of inhibition of 20mm and the disc with only one antibiotic and has 14mm, that means that the bacteria is a ESBLs producer.

The CDC states that there is ESBLs when cephalexin with Augmentin and the elliptic form of the cefotaxime with imipenem in the DDT. And if the ampicillin has a mayor zone of inhibition to cefotaxime (1).

*Pseudomonas aeruginosa, Acinetobacer baumanii and Stenotrophomonas maltophila* have major clinical implications and can cause outbreaks in hospitals (25). These bacilli are difficult to treat because they have a great capacity to acquire new resistance mechanisms during treatment, and therefore often have multiresistance.

**DETECTION OF CARBAPENEMENS.**

Doctor Vikas Gautam, Ana Elizabeth Markelz and Mischka Moodley verify that to detect the carbapenems resistance the manual methods are accurate than the automated systems (26,27,28) and the automated and PCR methods are very similar (26,29,30,31,32,33,34,35,36). The GeneXpert (Cepheid) MTB/RIF (*Mycobacterium tuberculosis* with Rifampicin resistance or not. This method can give in two hour the PCR result stead of the culture that takes time to give the result of bacterial resistance to the antibiotic, it detects the gen rpoβ (37).

Vading et al studies showed that the DDT that is manual are better than E test or MIC automated system to see the carbapenems in *Klebsiella pneumoniae* (31)*.* In Argentina in 2019 Maria Ines Lespada et al (35) found that the KPC (Kp-KPC) the *Klebsiella pneumoniae* that produces the carbapenemase KPC causes in bacteremia a 50% of mortality.

The BD MAX (Becton Dickenson) check-points CPO assay provides direct detection of carbapenemase producing CPO for improved patient management and infection control. A large proportion of carbapenem-resistant *Enterobacteriaceae* (CRE) infections currently lack effective and relatively safe antibiotic treatment options. CRE patients usually receive empiric therapy and are 3 times more likely to receive inappropriate antibiotic treatment than non-CRE persons (46.5% vs 11.8%) and this system leads to a deterioration in health plus in a longer hospital stays and increased costs. Carbapenemase genes KPC and OXA-48 were found in most of *Klebsiella pneumoniae* and *Escherichia coli* (31,34,35,36).

**STAPHYLOCOCCUS SPECIES**

There are many species of *Staphylococcus aureus* that shows more bacterial resistance and is a frequent cause of community- and healthcare-associated in different parts of the body. In 2011 in France in an intensive care unit was found by Brunel AS, et al 2014 (38) that methicillin-sensitive *S aureus* complex 398 was the most common clone (29/125, 23.2%) and in Netherlands (39,40) this hyper-virulent lineage originated in the Asia-Pacific Region and could become acquired in Europe. In 1944 there were 5% of penicillin resistance, in 1949 a 50% and in 1995 a 90% (1,2).

Penicillin disk diffusion zone edge method for *Staphylococcus aureus.* The method is with a penicillin 10U disc, if the inhibition zone is ≥29 mm and the inoculum have a lawn of growth is susceptible and if it is lower or equal to 28 is interpreted as resistant. (8,9)

Table 4 *Staphylococcus aureus* CLASSIFICATION (2,9)

|  |  |
| --- | --- |
| ***Staphylococcus aureus* type** | **CHARACTERISTICS** |
| MRSS | Susceptible to methicillin |
| MRSA | Resistant to methicillin, cephalosporins, carbapenem, monobactam |
| SCV´s | Small variable colonies |
| GISA | Glycopeptides Intermediate |
| VISA | Vancomycin intermediate |
| VRSA | Vancomycin resistance |
| hVISA | Vancomicin-Heteroresistant |

The MRSA (Methicillin Resistance *Staphylococcus aureus)* can be detected by different techniques: disk diffusion test, agar dilution, agglutination by latex or Vitek a systemic or automated system (30,38,39,40).

**ENTEROCOCCI**

Enterococcal species has over 60 different kinds of species, they are Gram positive *cocci*. The most common ones are *Entrerococcus faecalis* and *Enterococcus faecium*, both are in the human microbiome, and nowadays leading causes of multidrug resistant hospital associated infections. It can be found in endocarditis, prostatitis, cellulitis, intra-abdominal, bacteremia, urinary and wound infections (41,42). There are different types HLRs are highly resistant to aminoglycosides and VRE (Vancomycin resistant). Can be detected by DDT or automated with different systems (2).

**PNEUMOCOCCUS: STREPTOCOCCUS PNEUMONIAE**

In South Africa in 1978 was the first time to discover a *Streptococcus pneumoniae* resistant to penicillin, then in 1992 in United States and in Colombia in 1999 (43,44). The vaccine has been applied to prevent pneumococcal infection in adults (45,46). They found with penicillin there are 6 PBPs (bound proteins: 1a,1b,2a,2x,2b,3) and with MIC automated systems it is susceptibility to penicillin when it is ≤0.06 µg/ml, intermediate between 0.12 to 1 µg/ml and ≥2.0 µg/ml resistant.

**STREPTOCOCCUS PYOGENES**

*Streptococcus pyogenes* has been observed an increase after COVID-19, since November of 2023 a rare *emm* type 3.93 has increased in England up to 20% and in Netherlands a 60% of incidence. This type has been associated with children of 6 to 17 years old in pneumonia or pleural empyema and meningitis in both countries. (47,48). For many years *S pyogenes* had always susceptibility to penicillin, erythromycin or fluoroquinolones (47,48,49). The following genes have been described: ermTR (50) gyrA, parC (2). If the Direct or Enrichment ATS MOS tests are used the throat samples can have the result of an antibiotic resistance in less than 16 hours. This will help a lot to avoid bacterial resistance.

**NEISSERIA GONORRHOEAE**

Regarding *Neisseria gonorrhoeae*, Veal (2,51) found the enzymes MtrC-MtrD´-MtrE caused due to the mtrR mutation at the chromosomal level giving resistance to penicillin, Ropp also detected ponA mutation and penCo Olesky analyzed the mutation porin Ib giving intermediate resistance to tetracycline, macrolides (azithromycin), ceftriaxone and penicillin.

Antimicrobial resistance in gonorrhea has increased rapidly in recent years and has reduce treatment options. Most of the patients have between 15 and 49 years old and most cases are in Africa and West Pacific regions (51).

**SOLUTION TO THE RESISTANCE?**

Sara Hernando-Amado in USA in 2023 studied a collateral sensitivity (CS). They have been tackling antibiotic resistance by inducing transient and robust collateral sensitivity. CS is an evolutionary trade-off traditionally linked to the mutational acquisition of antibiotic resistance. *Pseudomonas aeruginosa* presents a low intrinsic susceptibility to different antibiotics. Their results support that “the identification of new compounds able to induce CS patterns might be valuable for the design of evolution-based strategies to tackle antibiotic -resistant infections“ (52).

There is a need to prevent infections with immunization, to wash our hands frequently and thoroughly, use the acute antibiotic if possible with the *in vitro* analysis result not an empiric treatment (53).

**CONCLUSION**:

It is very expensive to treat patients with these multi-resistant bacteria, therefore, the best way to give an antibiotic to a patient is knowing the reliable antimicrobial. In public health it is a big problem, because nowadays there are no vaccines for every microorganism that is in the world. The Direct MOS AST (1) techniques helps to reduce costs and to use narrow spectrum antibiotics and to do more cultures to give an accurate antibiotic not an empiric treatment. While with an empiric treatment the physician usually give broad spectrum antibiotics (54). CDC published in 2024 that six bacterial antimicrobial-resistant hospital-onset infections increased by a combined 20% during COVID-19 pandemic compared with the pre-pandemic 2021 and 2022 (55).

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