**CHAPTER 11: ANTIMICROBIAL RESISTANCE DETECTION AND RAPID SUSCEPTIBILITY TESTING**

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**ANTIMICROBIAL RESISTANCE DETECTION**

1. **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).

1. **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 |

1. **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).

1. **Betalactamase 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 |

1. **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 3, 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 4 (2).

**Table 4** 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.

1. **Detection of Carbapenemase.**

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).

1. ***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 5** *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).

1. ***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).

1. **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.

1. ***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.

1. ***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).

1. **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).

1. **Coclusion**:

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 gives 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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**II RAPID SUSCEPTIBILITY TESTING**

**1. Introduction:**

In order to have rapid sensitivity results in Clinical Microbiology Laboratories, Microbiology Institute of Colombia IMICOL, did a research with many different samples to help physicians in their right treatment to improve patient’s health (1,2,3). The most useful result of a Clinical Microbiology Laboratory is to provide the most appropriate specific antibiotic for its treatment. In most laboratories a urine culture takes from 3 to 5 days to give the sensitivity tests, because it needs to have pure cultures to do the Antibiotic Sensitivity Tests (AST). This is a consequence in many cases a need to stay more days in the hospital and therefore, it increases the cost of their illness, plus the deterioration of their health. (4)

The new manual rapid techniques: Direct and Enrichment AST MOS. So, called MOS because they have been proposed and implemented by Margaret Ordonez Smith. These techniques are a modification of the standard disk diffusion technique described by Kirby-Bauer and Barry (5,6) and it was reconfirmed after 18 hours of incubation (7, 8, 9), as approved by CLSI (Clinical Laboratory Standards Institute) and EUCAST (European Committee on Antimicrobial Susceptibility Testing) (10, 11). These manual techniques are very useful for urgent cases, easy, economic, reliable and they don´t need special equipment’s only an incubator to put the Petri dishes in a 37°C temperature. Thefore, they can be standardized by any kind of laboratory in the world. (Figure 1).

**1.1. Standard Disk Diffusion Test by Kirby-Bauer**

This technique has been the golden standard disk diffusion test for many years (5). The sample needs to be a pure culture in order to be done. If the sample has two bacterias, each one should be isolated and incubation is done at 37°C. In the first day the sample is smeared in an enrichment media, example, a blood agar (BA), MacConkey (Mac) agar and/or Eosin Methylene Blue agar (EMB). In the second day, the Kirby-Bauer technique is done with the bacterium isolated in order to do the identification (ID) and AST test. To do the AST the microorganism is smeared onto an enrichment broth, such as Tryptone Soya Broth (TSB) and when the inoculum has a turbidity of the MacFarland No. 0.5 it is smeared onto a dried AST media Mueller Hinton Agar (MHA). The inoculum should streak back-and-forth motion, rotate the Petri dish three times every 60°, in order to have an even distribution of inoculum and with a confluent lawn of grown. After 18 hours of incubation the zones of inhibition are interpreted with the breakpoints tables as CLSI or EUCAST (10,11). If the sample has only one bacterium on the third day the result can be given. But, If the sample has more bacterias it can last even 5 days., the result of the AST is ready to have the interpretation of the discs **(Figure 1**).

**2.Procedures of the Novel Manual Techniques:**

**Specific Objectives:**

These techniques are a modification of Kirby-Bauer Disk Diffusion Test. They can be done with any sample and the great advantage is to have a reliable rapid result. The most important is to know just in hours which is the reliable antibiotic for the patient’s treatment. The principle of the AST MOS tests is for a suitable therapeutic agent to be used in vivo. AST discs can be used with filter paper discs impregnated with specified concentrations of antimicrobial agents placed on the surface of a suitable test medium. Direct AST MOS is done with direct samples and Enrichment AST MOS with an inoculum as MacFarland No.0.5 of the samples. Both techniques are inoculated onto the sensitivity test media and they are incubated at 37°C with AST discs. The discs diffuse into the agar in 16 to 18 hours. After incubation at human corporal temperature, the zones of inhibition around the discs are measured and compared against recognized zone diameter ranges for the specific antimicrobial agents/organisms with CLSI or EUCAST breakpoints tables. Example, if the sample has an Escherichia coli infection, AST test can be read in 4 to 8 hours as a preliminary test and after 16 to 18 hours the reconfirmation of the data.

**Advantages of these novel techniques**

The most important and relevant issue is that the AST MOS techniques can be used in any laboratory, because they are very economic, easy and rapid tests. Both Direct MOS and Enrichment MOS can be read after 4 to 8 hours of reception of the sample as Barry (6) describes it, and it can be reconfirmed after 18 hours (7,8,9). The great advantage is to have the reliable antibiotic for the patient´s treatment, in order to reduce days of hospitalization, tests and/or medicines, plus the deterioration of their health (4). Any of the AST tests: manual or automated takes at least 3 to 8 days, to give the antibiotic information. The automated system needs a pure culture and the physician has to start an empiric treatment that maybe is helpful or not. This situation can increase the bacterial resistance to antibiotics.

**Samples:**

In the Direct AST MOS samples must have an inoculum with a high concentration, example, in urines with a concentration of 10ᵔ8; also, it is very helpful for vaginal (12), sputum, feces (13), abscess samples, the samples are smeared directly onto the Petri dish. In the Enrichment technique AST MOS, any sample can be used, because the sample is enriched in a tryptone soya broth (TSB) or any broth until it has a concentration of 10ᵔ8 (MacFarland No.0. 5). It can be done on eye or wound secretions, semen.

**Materials required:**

Petri dishes with special media to do the AST, such as Mueller Hinton Agar, ISO Sensitivity Agar, Diagnostic Sensitivity Test Agar can be used. An inoculum suspension with pure culture of the samples, sterile loops, swabs, sterile forceps, McFarland turbidity No.0.5 tube, incubator at 37°C, modified atmosphere environments. For quality control of the discs must be used strains with ATCC (American Type Culture Collection) or NCTC (National Collection of Type Culture), millimeter (mm) measuring ruler to read the zone sizes and interpretative criteria by Kirby-Bauer method of susceptibility testing with CLSI or EUCAST breakpoint tables.

**Storage and Handling:**

AST discs must be stored at -20°C to 8°C until required. Allow to reach room temperature when they are going to be used. Once opened, the discs should be stored within a dispenser in a container with desiccant to protect the discs from moisture. And the media in a refrigerator at approximately 8°C and the Petri dish should have 4 mm deep, usually each dish has 25 mL of media.

**Statistical studies:**

These novel techniques were compared with other laboratories and automated equipment. Giving a p>0.09 for Direct MOS and p>0.09 for the Enrichment MOS method, an accuracy of 99 %.

**2.1. Direct technique MOS.** It is very useful when the sample has a high concentration of bacteria, such as: feces, vaginal, throat, sputum, abdominal abscess, urine when it has abundant bacteria in a urine analysis with a urine culture of >100.000 CFU/mL**. Figure 1.** There is no problem to have a mixed inoculum, usually the bacteria causing the infection is in a higher amount. If there are two inhibition zones, a Gram stain and isolation must be done and compared with the cultures used to do the ID.

Urine samples are smeared onto the Petri dish with a sterile loop of 0.01 mL, twelve loops. Then three streaks are done with a sterile swab every 60° to have an even inoculum in a 90mm dish with any AST media, as, an Iso-Sensitest agar and incubated at 37°C. (1). The Direct AST MOS method is done as the gold standard technique is described by CLSI (10). In urgent cases, Barry (6) describes a preliminary reading in case the inhibition zone can be read after 4 to 8 hours. However, it is better to subtract 4 mm to have a reliable data, the interpretation is accurate between both techniques. There is no other method that can help the physician in 4 to 6 hours after reception of the sample.

**Table 1**

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  | | --- | | Inhibition zones in millimeters (mm) between Direct AST MOS and Standard Disk Diffusion Test | | | | | | | | | | |
| ANTIBIOTIC | FIM | SXT | AMC | SAM | ATM | CL | CIP | CAZ |
| Direct AST MOS | 20=S | 27=S | 21=S | 20=S | 29=S | 19=S | 0=R | 26=S |
| Standard DF | 19=S | 21=S | 19=S | 18=S | 26=S | 17=I | 0=R | 22=S |
| Interpretation | S | S | S | S | S | Subtract 4mm = I | R | S |

FIM= Nitrofurantoin, SXT= Sulphamethoxazole/Trimethoprim, AMC= Amoxycillin/Clavulanic Acid, SAM= Ampicillin/Sulbactam, ATM= Aztreonam, CL= Cephalexin, CIP= Ciprofloxacin, CAZ= Ceftazidime, TSB =Tryptone Soya Broth, McF = MacFarland Scale No. 0.5, S=Susceptible, I= intermediate, R= Resistant

The sample of feces are inoculated onto EMB agar and MacConkey Agar for the identification (ID) of the microorganisms, and at the same time the sample is inoculated to the MHA with the discs. All the Petri dishes must be incubated at 37°C. For the ID the incubation is for 1 to 3 days (if necessary), the AST can be read in 4 to 8 hours in order to give a preliminary AST result for the patient´s treatment and in 18 hours of incubation after reception of the sample the AST result can be given. If the zones of Inhibition have a mixed culture do a Gram stain and isolate the bacteria or fungus.

For coprocultures the container should be sterile to receive the feces, most common study of enteropathogens are, *Shigella, Salmonella, Yersinia, Campylobacter, Proteus* (13). Usually in a diarrhea, there is only one bacterium and this Direct AST MOS test can give the same day of recollection the reliable antibiotic for the treatment.



**Figure 2.** It shows a coproculture with *Proteus mirabilis* and *Escherichia coli.* This method also helps to isolate the bacteria and between 8 to 18 hours the laboratory can give the AST reliable result. (3,7).

This Direct AST MOS method can help in vaginal secretions, specially it is useful with fastidious bacteria as *Mobiluncus* and *Gardnerella vaginalis.* In sputum with pneumonia can give a preliminary result with Gram negative bacterias as *Klebsiella pneumoniae.*

**2.2.** **Enrichment technique MOS**.

The procedure is to smear the sample onto a tube with TSB, and must be incubated at 37°C and every hour, the tube has to be checked until it has the turbidity of MacFarland No.0.5. Immediately it can be smeared onto the MHA media or the sensitivity test media that the laboratory uses. Put the antimicrobial susceptibility test discs and incube at 37 ˚C for 4 to 18 hours then read the zones of inhibition and give the reliable AST result. The AST inhibition zones can be read as Barry described after 4 to 8 hours of incubation (1,2,3,6,7,8,9).

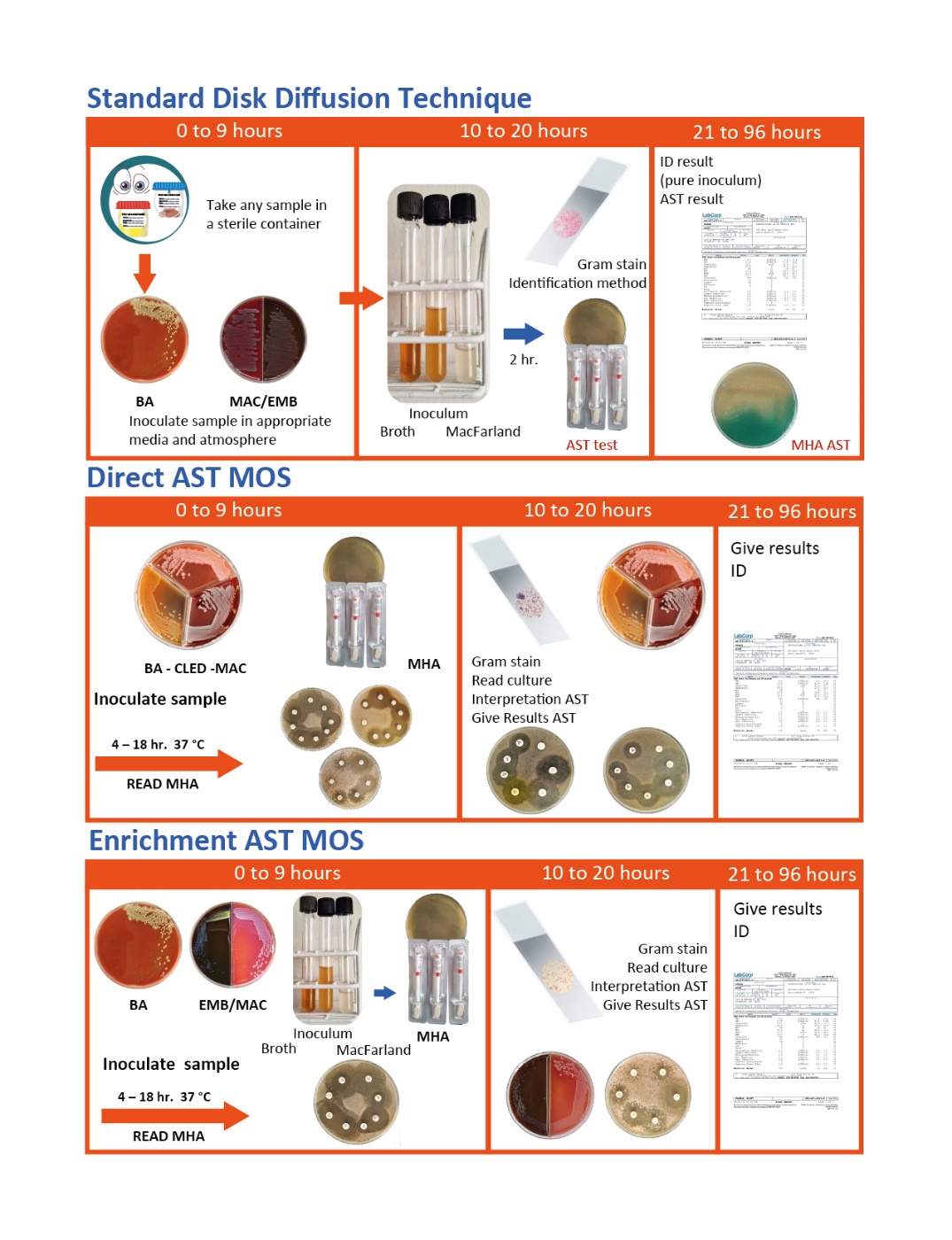
The great advantage of this Enrichment AST MOS technique is that it can be used **with any sample**, for example, eyes secretions that are very difficult to grow, because the inoculum is very tiny. Its interpretation is as accurate as the standard disk diffusion of Kirby Bauer (5,3,7,8). The procedure with urine sample: 1 mL (one milliliter) smeared onto 2 mL (two milliliter) of broth (TSB) and every hour the turbidity must be checked until it has the turbidity of MacFarland No.0.5. In table 2 all the zones were the same as the standard disk diffusion test. Gram positive bacteria grow slower than Gram negative. Figure No. 1

**Table 2**

|  |  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| |  | | --- | | Inhibition zones in millimeters (mm) between Enrichment AST MOS and Standard Disk Diffusion Test | | | | | | | | | | |
| ANTIBIOTIC | FIM | SXT | AMC | SAM | ATM | CL | CIP | CAZ |
| Enrichment AST MOS    TSB= McF | 20=S | 22=S | 18=S | 19=S | 27=S | 17=I | 0=R | 24=S |
| Enrichment AST MOS with urine at 37C | 21=S | 24=S | 19=S | 20=S | 27=S | 17=I | 0=R | 22=S |
| Standard D F | 19=S | 21=S | 19=S | 18=S | 26=S | 17=I | 0=R | 22=S |
| Interpretation | S | S | S | S | S | I | R | S |

FIM= Nitrofurantoin, SXT= Sulphamethoxazole/Trimethoprim, AMC= Amoxycillin/Clavulanic Acid, SAM= Ampicillin/Sulbactam, ATM= Aztreonam,  CL= Cephalexin, CIP= Ciprofloxacin, CAZ= Ceftazidime, TSB =Tryptone Soya Broth, McF = MacFarland Scale No. 0.5, S=Susceptible, I= intermediate, R= Resistant

**Figure No.1**. Procedures in: Standard Diffusion Disk Test, Direct AST MOS, Enrichment AST MOS techniques



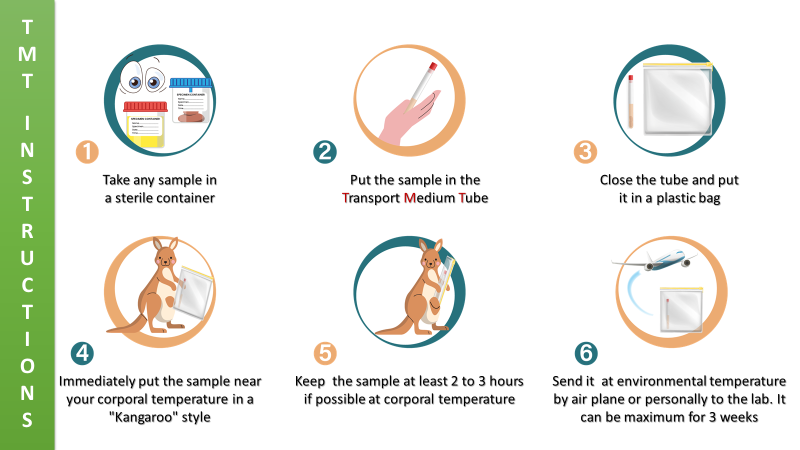
BA= Blood Agar, EMB= Eosin Methylene Blue Agar, CLED = Cysteine Lactose Electrolyte Deficient medium with Andrade Indicator, MHA= Mueller-Hinton Agar, Broth= Tryptone Soy Broth, MacFarland No. 0.5, AST= Antibiotic Susceptibility Test, ID= Identification

<https://drive.google.com/file/d/1s3hl0_y5EnWzB4ObM1hotdrChqSULHA/view?usp=sharing>

**2.3. New transport media tube**

This method is a modification of the Enrichment AST MOS technique, it is a new way to recover the bacteria from any infectious disease and can be done in any place of the world. The only requirement is to have the Transport Media Tube (TMT) MOS when the sample is taken.

Procedure: As the Enrichment AST MOS, the great advantage is that the patient puts the sample immediately onto the TMT MOS (figure 3). The tube is placed in a "kangaroo" style for 2 to 3 hours or more, in order, to keep the sample at a corporal temperature. Then the TMT MOS is sent to the laboratory by airplane or personally. This method has a recovery of 100%, compared with Cary Blair transport media of 67.9%. (14,15,16)



**Figure 3**. Transport Medium Tube MOS instruction for the patient to take the sample.

**3. Automated AST test compared with novel manual MOS techniques**

62 samples were analyzed with urines >100.000CFU/mL with the Direct MOS AST with the automated system MIC (Minimum Inhibitory Concentration) and statistically comparative there was no difference p>0.09. The sensitivity was 97.9%, specificity 81.8%, positive predictive value (PPV) 99.5% and negative predictive value (NVP) 44.4% (2). Therefore, the Direct MOS AST is of great value because can give in urgent cases the reliable antibiotic in 4 to 18 hours after the recollection of the sample, and it can be confirmed by Disk Diffusion Test or automated MIC system as CLSI or EUCAST approvals (2).

**4. Blood Direct Standard**

Blood cultures are among the most delicate tests to be done. A rapid AST result is always needed, because sepsis or bacteremia are critical health conditions. With this modification the hospitalization time was shortened between 28 to 40 hours. It is a simple, reliable, economic and very useful method (2,10,17,18).

Procedure: the blood is inoculated into an enrichment broth or a blood culture bottle, when the systematized equipment indicates bacterial growth, 0.3 mL is immediately taken to inoculate on a Mueller Hinton Agar Petri dish with the discs with an even inoculum as described in the Standard Disk Diffusion Test and then incubated at 37°C for 18 hours. This technique can be performed in any Clinical Microbiology Laboratory (2,17,18,19).

CONCLUSION:

There are many automated systems to know which bacteria is causing the infectious disease as Maldi Tof MS System and other equipments can give the bacteria identification, but not the same day of sample reception is given the AST information (20). In a health service the most important is to know the reliable treatment, not the name of the bacteria, in order to give as soon as possible the antibiotic for the patient´s treatment. Most of the cases the physician has to start with an empirical medicine.

All these rapid MOS methods reduces the hospitalization time, costs on tests or medicine unnecessary. It is the aim to publish so that many laboratories in the whole world can start to process them. Since they are very rapid, simple, economic, and **reliable.**

**5. Gram Stain with Blood Sample**

This technique is not used with blood sample, but it is very useful in just only 4 to 6 minutes the laboratory can give the physician the cause of an infectious disease: if it is a bacteria Gram negative or Gram positive or a fungus. In the Microbiology Institute of Colombia IMICOL is used for all the samples, but blood is not use as a standard technique and it is very useful (2). A drop of blood is just what you need; it is very economic, easy and rapid to give guidance in a treatment. Since many years ago, a sepsis is caused mostly by bacteria Gram positive or negative and fungi as *Candida* (21,22,23)*.*

Procedure: Put a drop of the blood in a slide, dry the sample and stained it with Gram. Dry it and read with immersion oil and see it at the microscope at 100x. The laboratory can give a preliminary result and it helps to the physician for a reliable treatment. This improves the way of life of the patients and avoids unnecessary medicines and days of hospitalization. Usually when a patient has sepsis or bacteremia a deterioration is done; therefore, knowing the type of bacteria or fungus the treatment is faster, because the hemocultures takes from 2 to 5 days to have a result.

In 2013, Stoneking et al (24) studied that 55.7% of the treatments can be changed to narrower spectrum antimicrobials if the AST result is done in the same day. Some places or laboratories don’t have expensive equipments so we suggest to use a direct smeared of the blood sample onto a blood agar or chocolate agar to have the bacterium or a Sabouraud agar to recover fungi.

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