International Journal of Recent Advances in Multidisciplinary Topics
Volume 3, Issue 5, May 2022
https://www.ijramt.com | ISSN (Online): 2582-7839

Extended Spectrum Beta Lactamase (ESBL) Confirmation Test using E-Test

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***Abstract*: Extended-Spectrum Beta-Lactamase (ESBL) is a β-Lactamase enzyme produced mainly by gram-negative bacteria *Enterobacteriaceae*, mostly found in *Klebsiella pneumonia* and *Escherichia coli.* Gram-negative bacteria produce ESBL enzymes that can hydrolyze β-lactam antibiotics and become resistant to almost all antibiotics, causing limited infection therapy options. This has an impact on difficult therapy, increased length of stay in the hospital, higher hospital costs incurred, and higher mortality rates. β-lactam antibiotics have a β-lactam ring structure that plays a role in inhibiting bacterial cell wall synthesis. The β-lactam ring bound to penicillin-binding proteins (PBPs) will stop the process of bacterial cell wall synthesis, causing bacterial cell death. Bacterial resistance to β-lactam antibiotics has 3 pathways, namely: the destruction of β-lactamase enzymes in antibiotics, changes in targets for antibiotics, and decreased intracellular uptake of antibiotics. β-lactamase-producing bacteria that destroy β-lactams are the main cause of resistance. Beta-lactamases cause antibiotic resistance by breaking down the structure of antibiotics. Beta-lactamases will open the β-lactam ring and change the structure of the drug and block the binding of penicillin-binding proteins (PBPs). Until now, no method has become the gold standard for detecting ESBL-producing *Enterobacteriaceae*. The Clinical and Laboratory Standards Institute recommends screening tests and confirmatory ESBL tests. E-Test (Epsilometer Test) is one of the confirmatory tests of ESBL, as an exponential gradient method to determine antimicrobial resistance, and also as a quantitative method to provide a quantity of antimicrobial sensitivity to microorganisms.**

***Keywords*: ESBL, confirmation test, E-Test.**

1. Introduction

Extended-Spectrum Beta-Lactamase (ESBL) is an enzyme Lactamase produced mainly by *Enterobacteriaceae* bacteria from gram-negative bacteria, mostly found in *Klebsiella pneumonia* and *Escherichia coli*. Gram-negative bacteria produce ESBL enzymes that can hydrolyze β-lactam antibiotics and have the ability to spread globally and become resistant to almost all antibiotics, causing infection therapy options to be limited. This has an impact on difficult therapy, increased length of stay in the hospital, higher hospital costs incurred, and higher mortality rates [1]-[4].

Extended-Spectrum Beta-Lactamase (ESBL) apart from being the most produced by *Enterobacteriaceae* bacteria, is also produced by other gram-negative bacteria such as *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, *Proteus mirabillis*, and *Shigella*. This strain of ESBL bacteria is widespread throughout the world and over the last two decades, there has been an increasing prevalence of resistance. In Asian countries, the prevalence of ESBL-producing *Enterobacteriaceae* strains varies from country to country and from species to species. For example, in *E. coli* the resistance rate varies from 5% in Korea to 23.3% in Indonesia. However, resistance caused by *Klebsiella spp.* was 48.8% in Korea and ranged from 20-40% throughout Southeast Asia, China and Japan. The results of the study on Antimicrobial Resistance in Indonesia: prevalence and prevention (AMRIN Study) in 2010-2011 found that the incidence of ESBL was quite high, namely 29% in *E. coli* and 36% in *K. pneumoniae* [1], [2], [5]-[10].

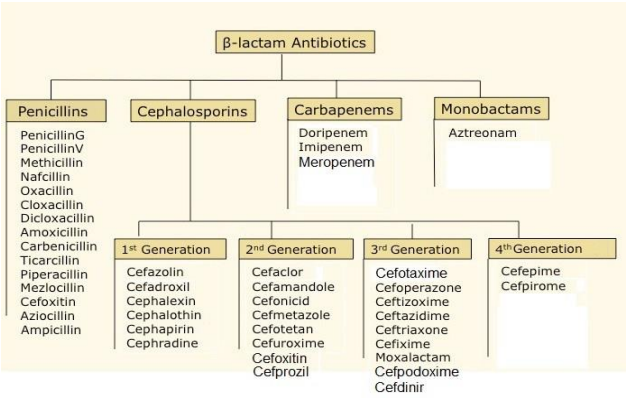

Fig. 1. The four main classes of β-lactam antibiotics

β-lactam antibiotics (divided into 4 main groups, namely: Penicillins, Cephalosporins, Carbapenems, and Monobactams), have an β-lactam ring structure that plays a role in inhibiting bacterial cell wall synthesis. The β-lactam ring bound to penicillin-binding proteins (PBPs) will stop the process of bacterial cell wall synthesis. The process of cell wall synthesis that stops will cause bacterial cell death. This occurs due to an osmotic imbalance caused by a failure of synthesis. Bacterial resistance to β-lactam antibiotics has 3 pathways, namely: the destruction of β-lactamase enzymes in antibiotics, changes in

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targets for antibiotics, and decreased intracellular uptake of antibiotics. All of these pathways have an important role in antibiotic resistance. However, β-lactamase-producing bacteria that destroy β-lactams are the main cause of resistance. Beta-lactamases cause antibiotic resistance by breaking down the structure of antibiotics. Beta-lactamases will open the β-lactam ring and change the structure of the drug and block the binding of penicillin-binding proteins (PBPs). This process will cause cell wall synthesis to continue. Changes in the structure of the drug will cause the inactivation of the drug (Figure 2) [11]-[13].

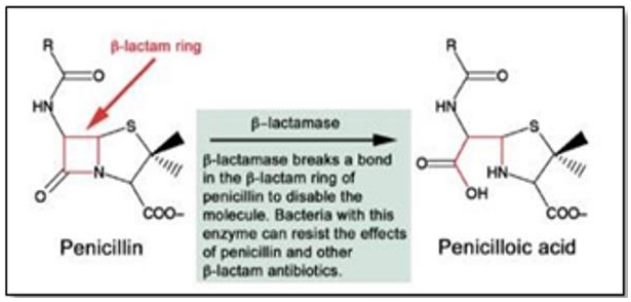

Fig. 2. Mechanism of β-Lactamase in the cleavage of beta lactam ring

Antibiotic resistance that occurs has an impact on therapy difficulties, the longer the length of stay in the hospital, the more hospital costs incurred, and the higher the mortality rate [3], [4].

Until now no method has become the gold standard for detecting ESBL-producing *Enterobacteriaceae*. It is a challenge for laboratories to detect ESBL-producing gram-negative bacilli. The National Committee for Clinical Laboratory Standards (NCCLS) which later changed its name to the Clinical and Laboratory Standards Institute (CLSI) recommends: [10]

1. ESBL Screening Test:
   a. Disc Diffusion Methods
   b. Screening by Dilution Antimicrobial Susceptibility Test

There is a ChromID ESBL screening test which is a chromogenic agar medium with a rapid culture method to detect bacteria [14].

2. ESBL Confirmation Test:

Phenotypic Confirmatory Methods:

   a. Double disc synergy/ Disc approximation method
   b. E-Test (Epsilometer Test) ESBL strips
   c. Three-dimensional test
   d. Vitek system

Several Molecular Methods, including Isoelectric point, DNA probes, PCR, Oligotyping method, PCR-RFLP, PCR-SSCP, LCR, Nucleotide sequencing, Pulsed Field Gel Electrophoresis (PFGE).

If the results of the screening test indicate the presence of ESBL production, a confirmatory test is necessary to establish the diagnosis. The disadvantage of screening tests is that the required process requires additional costs and takes a long time to produce results. The test sensitivity is high with low specificity because a positive test not only indicates ESBL production but other β-lactamase production can also give a positive result [15], [16].

The ESBL confirmatory test, which has high sensitivity and high specificity, is indispensable for establishing the diagnosis. E-Test is one of the ESBL confirmation tests, therefore in this paper, we will discuss the ESBL confirmation test using the E-Test.

2. Aim

The aim of this test is to find out the method and principle of examining the ESBL confirmation test for the detection of ESBL-producing bacteria using the E-Test.

3. Method

A. Pre-analytic [16]-[18]

1) Patient preparation

No special preparation is needed.

2) Sample preparation

The sample is ESBL bacterial isolate.

3) Instruments

1. Petri dish
2. Incubator
3. Ose wire
4. Sterile swab
5. Bunsen
6. McFarland standard 0,5 and Wickerman card
7. MacFarland Densitometer
8. E-Test strips (containing the desired antibiotic)
9. Inoculum tube
10. Normal sterile saline

4) Media material

Mueller-Hinton Agar Media containing acid hydrolysate of casein, beef extract, starch, and agar.

Fig. 3. Instruments

B. Analytic

1) Working principle

E-Test (Epsilometer Test) is an exponential gradient method for determining antimicrobial resistance. E-Test is also a quantitative method to provide a quantity of antimicrobial sensitivity to microorganisms. The antibiotic concentration gradient is contained along the E-Test strip. When the E-Test strip was placed on agar media that had been inoculated with ESBL bacterial isolates, the release of antibiotics occurred, and the formation of an antimicrobial concentration gradient on the

agar medium in the form of an inhibition zone. After overnight incubation, the test was read by looking at the strip from the top of the agar medium, an elliptical inhibition zone was formed. The intersection of the lower part of the inhibition zone with the E-test strip shows the Minimum Inhibitory Concentration (MIC) value of the antibiotic [17].

2) Method [17], [18]

1. Inoculum Preparation:

a. Remove the E-Test package from the freezer (-20°C) at least 30 minutes before use.
b. Using a sterile wire loop that has been heated over a Bunsen, remove 3 or 4 bacterial colonies from the culture medium and transfer them to a 4 ml saline tube used as an inoculum tube.
c. Compare and adjust the turbidity of the inoculum tube with the 0.5 McFarland Standard using the Wickerman Card background. Add bacterial colonies if the density/turbidity of the suspension is less than 0.5 McFarland Standard or add more normal saline if the density is more than 0.5 McFarland Standard. Use this suspension within 15 minutes of preparation.

Measurement of density/turbidity, apart from being visual, can also be measured digitally using a McFarland densitometer.

The standard most commonly used in clinical microbiology laboratories is the 0.5 McFarland Standard (equivalent to 1.5 x 108 bacterial suspensions) which is used for antimicrobial susceptibility testing and performance testing of culture media. The advantage of using the McFarland Standard is that it does not require sufficient incubation time to obtain the desired amount of bacterial density. While the disadvantage, there will be different views to assess the level of turbidity of bacterial cells.

Fig. 4. Collection of bacterial colonies from the culture medium to the inoculum tube

Table 1
McFarland turbidity standard

McFarland turbidity standard no.	0.5	1	2	3	4
1% barium chloride (ml)	0.05	0.1	0.2	0.3	0.4
1% sulfuric acid (ml)	9.95	9.9	9.8	9.7	9.6
Approx. cell density (1×1^8 CFU/ml)	1.5	3	6	9	12

Fig. 5. 0.5 McFarland Standard turbidity ratio using Wickerman card background

Fig. 6. McFarland Densitometer

2. Inoculation on Muller Hinton Agar:

a. Dip a sterile cotton swab into the inoculum tube and pull it out slightly, rubbing it around the inside of the tube several times to remove excess fluid.
b. Apply the cotton to the surface of the agar media by rotating the petri dish at 60° and spreading it around the edges of the agar media.
c. Leave the petri dish lid open for 5 minutes (no more than 15 minutes) to allow excess moisture to be absorbed before applying the E-Test strip.

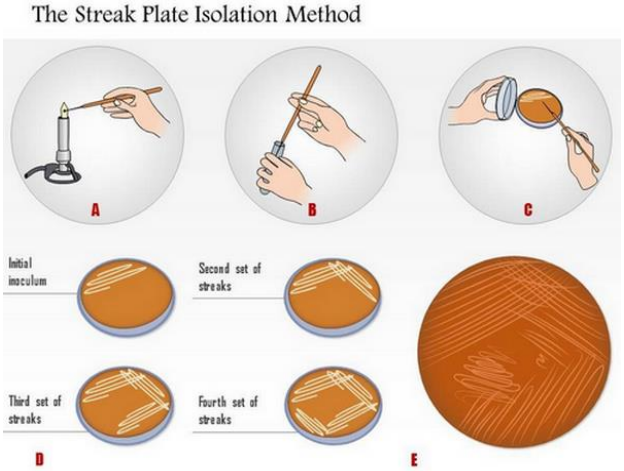

Fig. 7. Inoculation of bacteria on the media

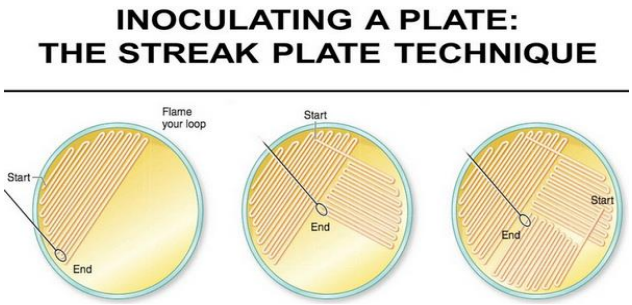

Fig. 8. Bacterial inoculation technique on media

3. Application of E-Test strip:

a. Open the E-Test package by cutting the package along the dotted line on the package wrapper. Place the strip on the surface of the agar plate using forceps (or the E-Test applicator if available).
b. Place the strip in such a way that the part of the strip marked with the letter "E" on the strip is placed on the edge of the agar plate and make sure it is visible.
c. Position 4 to 6 E-Test strips each containing the desired antibiotic on a 150 mm agar plate or 1 (sometimes 2) strip on a 90 mm agar plate. Do not move or swap strips after touching agar media. If the strips stick together, pull & separate them from each other by holding the part of the strip marked with the letter "E". Do not touch other parts of the strip. Each E-Test strip that contains different antibiotics can also be combined by placing the E-Test strips across each other.
d. Incubate the agar plate at 37°C for 18-24 hours.

Fig. 9. Opening the E-Test package and placing it on the youth plate media

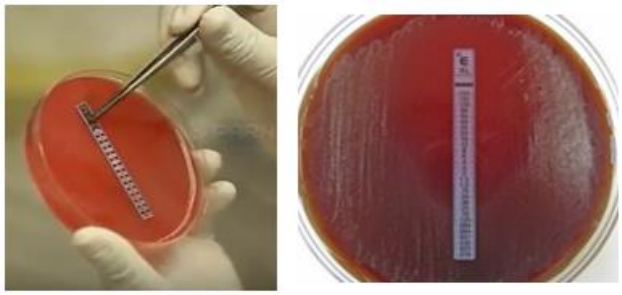

Fig. 10. Placing the E-Test strip on the youth plate media

C. Post-analytic

1) Interpretation

Looking at the E-Test strip from the top of the agar medium, an elliptical inhibition zone was formed. The intersection of the lowest part of the inhibition zone with the E-Test strip shows the Minimum Inhibitory Concentration (MIC) value of the antibiotic contained in each E-Test strip [17], [18].

Furthermore, the MIC value obtained is compared with the breakpoint value of each antibiotic according to the criteria recommended by the Clinical and Laboratory Standards Institute (CLSI), then interpreted as S (Susceptible), I (Intermediate), or R (Resistant).

Table 2 shows the results of SDD (Susceptible-Dose Dependent) interpretation which is the latest category of interpretation for antimicrobial susceptibility testing. The results of this interpretation of SDD have been applied to antifungal susceptibility testing for several years. This SDD interpretation category is a breakpoint category for the sensitivity of bacterial isolates depending on the regimen dose used. The higher regimen dose will reduce mortality due to the sensitivity of bacterial isolates, while the lower regimen dose will increase mortality due to resistant bacterial isolates. Therefore, the sensitivity of bacterial isolates depends on the dosage of the regimen used.

Fig. 11. Position 4 to 6 E-Test strips on the youth plate media

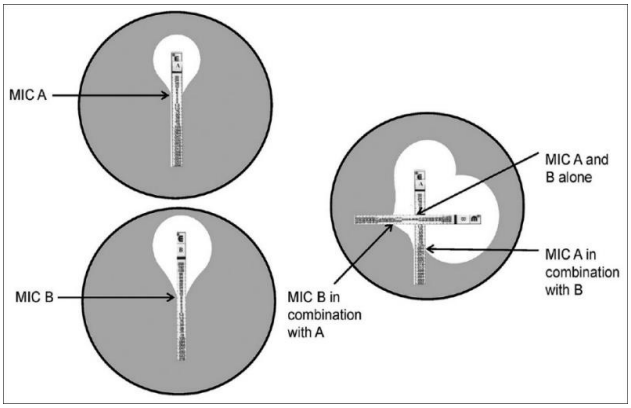

Fig. 12. Position of the E-Test strip for the combination of antibiotics on the surface of the media plate

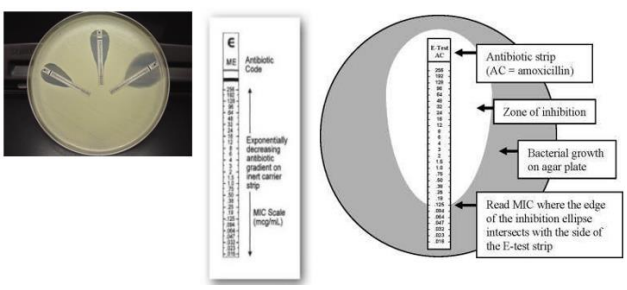

Fig. 13. Determining the MIC value of antibiotics

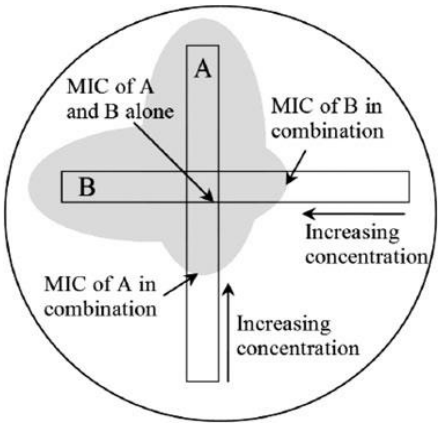

Fig. 14. Determine the MIC value of 2 synergistic antibiotics

Table 2
CLSI 2018-revised MIC breakpoints for certain antibiotic agents and organisms

AGENT	ORGANISM	MIC (MG/L)			
		SUSCEPTIBLE	SUSCEPTIBLE-DOSE DEPENDENT	INTER-MEDIATE	RESISTANT
Ceftaroline	*Staphylococcus*				
• Previous*	*aureus*	≤1	-	2	≥4
• Newly approved		≤1	2-4	-	≥8
Ciprofloxacin	Enterobacteriaceae				
• Previous*		≤1	-	2	≥4
• Newly approved		≤0.25	-	0.5	≥1
Ciprofloxacin	*Pseudomonas*				
• Previous*	*aeruginosa*	≤1	-	2	≥4
• Newly approved		≤0.5	-	1	≥2
Daptomycin	*Enterococcus* spp.				
• Previous*		≤4	-	-	-
• Newly approved		≤1	2-4	-	≥8
Levofloxacin	Enterobacteriaceae				
• Previous*		≤2	-	4	≥8
• Newly approved		≤0.5	-	1	≥2
Levofloxacin	*Pseudomonas*				
• Previous*	*aeruginosa*	≤2	-	4	≥8
• Newly approved		≤1	-	2	≥4

- indicates absence of MIC breakpoint; CLSI, Clinical and Laboratory Standards Institute; MIC, minimal inhibitory concentration.
*All previous breakpoints are cited from the 28th edition (January 2018) of the CLSI M100 document.

2) Strengths and Weaknesses

Strengths: [19], [20]

a. High sensitivity, can detect ESBL even in small amounts. More sensitive than the Double disc synergy/ Disc approximation method in detecting ESBL in clinical isolates.
b. Easy to do.
c. Can be used for clinical isolates in small quantities.
d. Contamination is easy to spot.
e. Resistant strains can be easily measured according to the antibiotic concentration gradient on the E-Test strip.

Weaknesses:

a. The E-Test strip is sensitive to changes in pH level.
b. The expensive price of E-Test strips.
c. Requires proper storage of strips, laboratory settings for proper inoculation, and plate incubation.

4. ESBL Examination Scheme

First, a screening test is carried out aimed at detecting resistance to one or more 3rd generation oxymino cephalosporines such as cefotaxime, ceftazidime, ceftriaxone, cefpodoxime, or aztreonam using two sensitivity test methods, namely Disc Diffusion and MIC Determination (Dilution Antimicrobial Susceptibility Test). Both of these methods use third-generation cephalosporin antibiotics. The National Committee for Clinical Laboratory Standards (NCCLS) has developed the Disc Diffusion and Dilution Antimicrobial Susceptibility Test for screening tests using selective antimicrobial agents. In vitro sensitivity testing using the approved NCCLS procedure was performed with ceftazidime (30 mg), cefotaxime (30 mg), ceftriaxone (30 mg), aztreonam (30 mg), and cefpodoxime (10 mg). Any zone diameter within the "grey zone" should be considered as an ESBL generator which may require phenotypic confirmatory testing. The interpretation of the screening test using disc diffusion and MIC determination methods was based on the NCCLS guidelines [21]-[23].

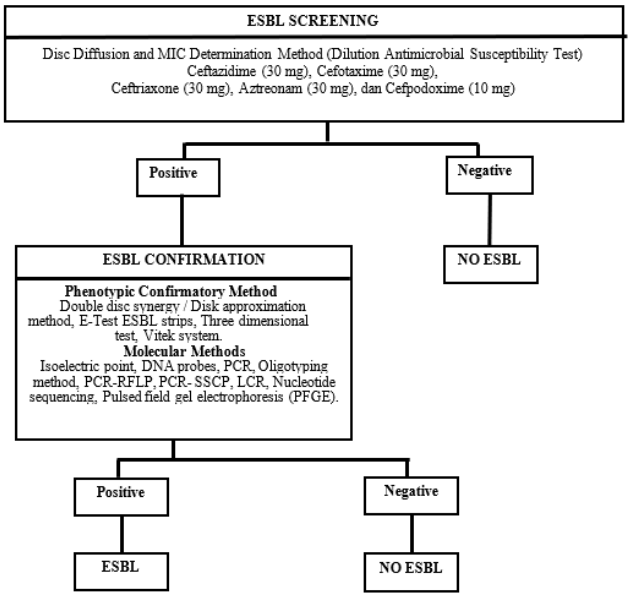

Fig. 15. ESBL examination scheme

5. Conclusion

E-Test (Epsilometer Test) is one of the confirmatory tests of ESBL, as an exponential gradient method to determine antimicrobial resistance, and also as a quantitative method to provide a quantity of antimicrobial sensitivity to microorganisms.

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