Clinical and Experimental Health Sciences

Which Phenotypic Method Is the Most Accurate for Detection of Extended – Spectrum β-Lactamases (ESBLs) in *Escherichia coli* ?

Montaser M.Y. Amro[1], Emrah Guler[2], Kaya Suer[3], Meryem Guvenir[4]

[1] Department of Medical and Clinical Microbiology, Faculty of Medicine, Near East University,Nicosia, Cyprus.
[2] Department of Medical and Clinical Microbiology, Faculty of Health Science, Near East University Nicosia, Cypus.
[3] Department of Infectious Diseases and Clinical Microbiology, Faculty of Medicine, Near East University Nicosia.
[4] Department of Medical and Clinical Microbiology, Vocational School of Health Sciences, Near East University, Nicosia, Cyprus.

Correspondence Author: Meryem Guvenir
E-mail: meryemguvenir@hotmail.com

Received: 27.08.2021 **Accepted:** 12.07.2022

ABSTRACT

Objective: The aim of the study is to determine the Extended-Spectrum β-Lactamases (ESBLs) by three different phenotypic methods of the *Escherichia coli (E. coli)* strains that isolated from various clinical samples.

Methods: A total of 93 *E. coli* samples were isolated from hospitalized patients. Antibiotic susceptibility tests were done by automated system Phoenix 100 (Becton Dickinson, Sparks, MD, USA). ESBL production was tested by double disc synergy test (DDST), combined disc test (CDT) and three-dimensional test (TDT). All statistical analyses were done using statistical packages SPSS Demo Ver 22 (SPSS Inc. Chicago, IL, USA).

Results: In the investigation of ESBL production among *E. coli* species, 87 (93.5%) strains were ESBL positive by DDST, 73 (78.5%) strains were ESBL positive by CDT, 71 (76.3%) strains were ESBL positive by TDT. According to statistical analysis: There were statistical differences between DDST-CDT (p=<0.001) and DDST-TDT (p=<0.001). However, there was no statistical difference between CDT-TDT (p=0.207)

Conclusion: According to our study results, DDST test was more advantageous than CDT and TDT such as was not require additional financial expenditure and time, and can be easily used in routine laboratories. Therefore, routine monitoring of ESBL with DDST should be determined because of the conspicuous prevalence of ESBL forming and multidrug-resistant of *E. coli.*

Keywords: *Escherichia coli*, ESBL, Disc Diffusion Test, *Enterobacteriaceae*

1. INTRODUCTION

Escherichia coli (*E. coli*) is a member of the normal flora which find in the gastrointestinal tract of humans. Since *E. coli* normally does not cause diseases, it is cause diseases when pass to a different tissue and organ. Although gastroenteritis is the main infection, they cause meningitis, peritonitis, septicemia and pneumonia (1). Extended-Spectrum β-Lactamases (ESBLs), a penicillin degrading penicillinase, was first discovered by Abraham and Chain in the late 1930s in a *E. coli* strain. ESBLs are enzymes that hydrolyze oxyimino cephalosporins, which can be inhibited by clavulonic acid (2). Until today, approximately 400 beta lactamase enzymes has been demonstrated, and nearly 150 of them ESBLs. ESBLs are responsible for resistance of broad spectrum cephalosporins and monobactams. ESBLs production and its rapid spread among bacteria has been causing serious problems in recent years (3)

Beta-lactam antibiotics inhibits bacterial cell wall formation by interfering with the protein necessary for cell wall formation where later bacteria are either killed or inhibited from growth. Penicillin-binding protein (PBP) considered as a specific role in the synthesis of peptidoglycan, and beta-lactam antibiotics bind to these PBP which later leads to lysis and death of cells. Beta-lactam antibiotics are primarily divided into four groups: penicillins, cephalosporins, monobactams, carbapenems (4).

Nosocomial infections caused by multidrug resistance of *E. coli* are associated with the highest mortality and the huge treatment costs in to the world (5). According to the WHO, at least 700,000 people were died each year, and it is estimated that drug-resistance could cause 10 million deaths each year by 2050, with a significant socioeconomic impact (6).

Although the antibiotic resistance detection is important, ESBLs producing bacteria are not detected by routinely antibiotic sensitivity tests (7,8). In ESBLs detection and verification tests are based on the demonstration of synergy between a third-generation cephalosporin and a beta-lactamase inhibitor (usually clavulanıc acid) (9).The following phenotypic methods can be used to determine the ESBLs such as double disc synergy test (DDST), combined disc

DOI: 10.33808/clinexphealthsci.987433

test (CDT), three-dimensional test (TDT), E-test, automatize systems and molecular techniques (10).

The aim of the study was determined the ESBLs using three different phenotypic methods (DDST, CDT, and TDT) in *E.co*li strains.

2. METHODS

2.1. Design of Study

The study was conducted in the Microbiology Laboratory at the Department of Medical and Clinical Microbiology, in the Faculty of Medicine, Near East University. Total of 93 *E. coli* strains were included in the study. This study was approved by Near East University Ethics committee of 25.02.2021 / 2021/88.

2.2. Identification and Antibiotic Susceptibility Test (AST)

Bacterial identification and ASTs were performed by full automated system Phoenix 100 (Becton Dickinson, Sparks, MD, USA) in line with the manufacturer's recommendations. AST results were evaluated according to the EUCAST (European Committee on Antimicrobial Susceptibility Testing) criteria. *E. coli* ATCC 25922 used as negative control.

2.3. Double-Disc Synergy Test (DDST)

The bacterial suspension was prepared in accordance to the manufacturer's standard density (0.45-0.55 McFarland) then spread on Mueller-Hinton Agar (MHA) (Merck, KgaA, Germany) plate. Ceftazidime (CAZ; 30 µg) , ceftriaxone (CRO; 30 µg) (Cat. NO: ASD02300), cefotaxime (CTX; 30 µg), and aztreonam (ATM; 30 µg) (Cat. NO: ASD00700) were placed to plates to a disk contain amoxicillin-clavulanic acid (AMC; 20/10 µg) in the center, positioned at a distance of 20 mm (center to center). After incubation at 35°C overnight, the expansion of the inhibition zone around the cephalosporins or ATM towards the AMC disc, or the presence of a synergy area in which bacteria grows indicate the presence of ESBLs (Fig. 1). DDST has been done according to the EUCAST guidelines (11).

2.4. Combined Disc Test (CDT)

The bacterial suspension was prepared in accordance to the manufacturer's standard density (0.45-0.55 McFarland) then spread on MHA (Merck, KgaA, Germany) plate. CTX (30 µg) discs with cefotaxime/clavulanic acid (CTC; 40 µg) were placed on MHA plates. Plates were incubated at 35°C overnight. Inhibition zones around the discs with and without CTX/ clavulanic acid were measured. If the difference between the discs containing and without CTX/clavulanic acid was greater than or equal to 5 mm, ESBLs was considered positive. CDT has been done according to the EUCAST guidelines (Fig. 1).

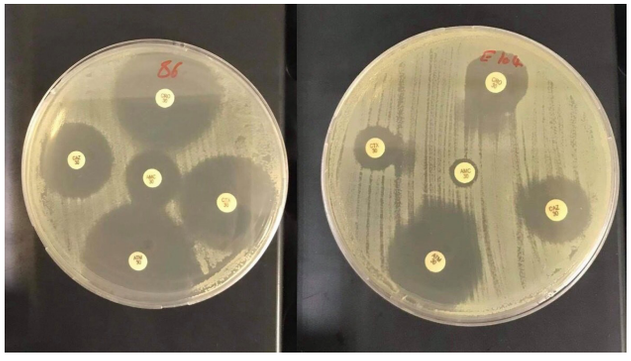

Figure 1. *ESBLs positive by combined disc test*

2.5. Three-Dimensional Test (TDT)

The prepared bacteria suspension with a density of 0.5 McFarland and spread on the agar plate. The medium was cut in a circle, close to the center of the petri dish, and 3 mm away from the antibiotic discs used. The formed medium line was filled with a liquid medium in which the microorganism to be tested was growing. After the inoculations were performed, CAZ (30 µg), CTX (30 µg), CRO (30 µg), and ATM (30 µg) discs were placed. The plate was then incubated at 35°C for 16-18 hours. Heart-shaped distortion of the zone of inhibition around the antibiotic disc was indicated ESBLs production (Fig. 2).

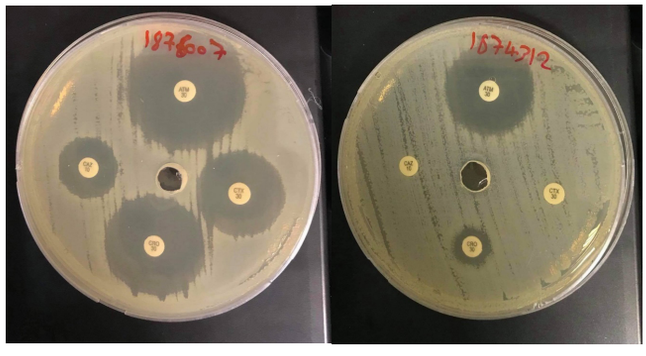

Figure 2. *: ESBLs positive by three dimensional test*

2.6. Statistical Analysis

SPSS (Statistical Package of the Social Sciences) Demo Ver 22 (SPSS Inc., Chicago, IL, USA) program was used for all statistical analysis of the data. Additionally, binary logistic regression was performed for comparison between methods. Relative risks was calculated as odds ratios (ORs) and 95% confidence intervals (CIs) by the use of binary logistic regression.

3. RESULTS

Antibiotic susceptibility test results of 93 *E. coli* strains were the resistance of amikacin were 0%, the resistant of amoxicillin/clavulanic acid were 63.4%, the resistant of ampicillin were 91.4%, the resistant of cefixime were 77.4%, the resistant of ceftazidime were 82.8%, the resistance of ceftriaxone were 81.7%, the resistance of cefuroxime were

63.4% , resistance of ciprofloxacin were 65.6%, the resistance of ertapenem were 0%, the resistance of fosfomycin were 4.3%, the resistance of gentamicin were 23.7%, the resistance of imipenem were 0%, the resistance of meropenem were 0%, the resistance of nitrofurantoin were 2.2%, the resistance of piperacillin/tazobactam were 30.1%, and the resistance of trimethoprim/sulfamethoxazole were 71.0% (Table 1).

Table 1. *Antimicrobial susceptibility results of E. coli strains.*

Antibiotic Name	Susceptible /Resistance	n (%)
Amikacin	Susceptible	93 (100%)
	Resistant	0 (0%)
Amoxicillin Clavulanic Acid	Susceptible	34 (36.6%)
	Resistant	59 (63.4%)
Ampicillin	Susceptible	8 (8.6%)
	Resistant	85 (91.4%)
Cefixime	Susceptible	21 (22.6%)
	Resistant	72 (77.4%)
Ceftazidime	Susceptible	16 (17.2%)
	Resistant	77 (82.8%)
Ceftriaxone	Susceptible	17 (18.3%)
	Resistant	76 (81.7%)
Cefuroxime	Susceptible	34 (36.6%)
	Resistant	59 (63.4%)
Ciprofloxacin	Susceptible	32 (34.4%)
	Resistant	61 (65.6%)
Ertapenem	Susceptible	93 (100%)
	Resistant	0 (0%)
Fosfomycin	Susceptible	89 (95.7%)
	Resistant	4 (4.3%)
Gentamicin	Susceptible	71 (76.3%)
	Resistant	22 (23.7%)
Imipenem	Susceptible	93 (100%)
	Resistant	0 (0%)
Meropenem	Susceptible	93 (100%)
	Resistant	0 (0%)
Nitrofurantoin	Susceptible	91 (97.8%)
	Resistant	2 (2.2%)
Piperacillin/ Tazobactam	Susceptible	65 (69.9%)
	Resistant	28 (30.1%)
Trimethoprim/ Sulfamethoxazole	Susceptible	27 (29.0%)
	Resistant	66 (71.0%)

According to the three different phenotypic test; 87 (93.5%) *E. coli* strains were ESBLs positive by DDST, 73 (78.5%) *E. coli* strains were ESBLs positive by CDT and 71 (76.3%) *E. coli* strains were positive by TDT. The comparative information of all three tests was shown in Table 2.

According to statistical analysis; comparing the DDST with other methods for highest accuracy rate, it was found that there was a statistically significant difference in the results. Accordingly, the difference between DDST-CDR and DDST-TDT was statistically significant (p=<0.001; p=<0.001, respectively), but the difference between CDT-TDT was not significant (p=0.207).

Table 2. *The results of double disc synergy test ,combined disc test, and three dimensional test.*

Method to Detect ESBLs			ESBLs in *E. coli*
DDTS	**Positive**	Number	87
		Percent (%)	93.5%
	Negative	Number	6
		Percent (%)	6.5%
	Total		93 (100%)
CDT	**Positive**	Number	73
		Percent (%)	78.5%
	Negative	Number	20
		Percent (%)	21.5%
	Total		93 (100%)
TDT	**Positive**	Number	71
		Percent (%)	76.3%
	Negative	Number	22
		Percent (%)	23.7%
	Total		93 (100%)

DDT: Double Disc Synergy Test (DDT); CDT: Combined Disc Test (CDT); TDT: Three-dimentional Test; ESBLs: Extended-Spectrum Beta Lactamases

4. DISCUSSION

Beta-lactamase activity is the most important for resistance to beta-lactam antibiotics. The effectiveness of broad-spectrum cephalosporins against to the *Enterobacteriaceae* family is challenged by newly emerging enzymes especially in the enzyme group called ESBLs. ESBLs is responsible for the development of resistance to beta-lactams such as aztreonam, ceftazidime, ceftriaxone, and cefotaxime. In Europe, these enzymes were first identified, then it is appeared in different countries, such as the United States of America and Japan. These spread of resistance were based on the using overuse and unnecessary of the third-generation cephalosporins (12,13). Epidemiological studies of the ESBLs in *E. coli* strains are important informations for public and hospital infection (14) .

Laboratory methods for screening and confirmation of ESBLs should be accurate, simple and rapid. There are several phenotype and molecular tests have been used for determine the ESBL (8). However, there are some limitations for the molecular methods such as experienced staff, costs and the complexity (15). This study aimed to investigate the most accurate phenotype methods for the detection of ESBL positive *E.coli.*

Shaikh et al. indicated that 77.78% of *E. coli* isolates were ESBLs positive by DDST method (16); Mehrgan H. et al. (17) reported that 212 isolates of *E. coli* (49%) were ESBLs positive by DDST. In the study performed by Al-Muhtaseb et. al (18) ESBLs was studied with 59 *E. coli* isolates and ESBLs was positive in 20 samples (34%). Güzel M et al. (19) were found 81 ESBLs *E. coli* strains by CDT methods. Öztürk et. al (20) were reported that DDST and E-test (p=0.187) were found similar but, screening test was significantly effective than DDST (p<0.05) for *E. coli* strains. According to our results; DDST (93.5%) was more accurate than other test for determeing ESBLs.

All phenotyping methods have advantages and disadvantages. The advantage of the TDT is simultaneous determination of antibiotic susceptibility and β-lactamase detection but not specific for determination of the ESBLs (21). For the CDT, the sensitivity is 79-97% and the specificity is 94-100%. Its advantages are easy, routine and cheap. The disadvantage of the CDT is that there is no standardization of the distance between the discs (22).

In our study, there were statistical differences between DDST-CDT (p=<0.001) and DDST-TDT (p=<0.001). However, there was no statistical difference between CDT-TDT (p=0.207). Although, the EUCAST recommends the CDT test for detection of ESBLs by phenotyping methods, our results indicated that DDST had the highest percentage (93.5%) to determine the ESBLs. Limitation of the study were the moleculer techniques might be used for the determination of the ESBLs and then determine the source of the ESBLs either nosocomial nor community.

Infections caused by ESBL positive bacteria often constitute a problem for the therapeutic options and cause treatment failures. Therefore, detection of ESBL positive bacteria should be performed routinely in microbiology laboratories for the appropriate antimicrobial therapy can be instituted and the dissemination of ESBL positive bacteria may be prevented by employing appropriate infection control measures. Although, the molecular methods may provide accurate results in the identification of ESBL genes, their accessibility is often limited, and they are expensive. However, phenotypic methods are easy to perform and interpret. In our study, DDST was found to be a superior method than CDT and TDT for detection of ESBL positive bacteria.

According to studies resullts, we said that there is a missing laboratory knowledge and testing have generated several unresolved issues. The first step is a screening for reduced susceptibility to any of the screening agents such as cefotaxime, ceftriaxone, ceftazidime, cefpodoxime, or aztreonam. Therefore, confirmatory testing which is doing after a positive screening result, is based on tests with combinations of screening agents and the beta-lactamase inhibitor clavulanate. Confirmatory testing may need to one more day to deternine the ESBLs. If the laboratory reports a positive ESBL screening result and the isolate subsequently proves to be ESBL negative, the report could lead to unnecessary use of a carbapenem. On the other hand, if the laboratory withholds the positive screening result and the isolate is subsequently confirmed as ESBL positive, appropriate therapy may have been delayed for a day (22).

Unfortunately, a reporting rule cannot cover all situations. Rather, the need to report a positive screening result should be determined on a case-by-case basis using common sense and experience as guides, taking into account the patient's status, infection control considerations, and the likelihood of a positive confirmatory test (based on prior experience with isolates from the same patient population). Using a reliable, rapid confirmatory test could minimize the time required for the second-step test and lessen this reporting dilemma. Another solution would be including ESBL confirmation testing in the routine susceptibility test.

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How to cite this article: Amro MMY, Guler E, Suer K, Guvenir M. Which Phenotypic Method Is the Most Accurate for Detection of Extended–Spectrum β-Lactamases (ESBLs) in *Escherichia coli*? Clin Exp Health Sci 2022; 12: 624-628. DOI: 10.33808/clinexphealthsci.987433