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**REVIEW ARTICLE**

# Phenotypic and Molecular Strategies for Identifying Extended-Spectrum β-Lactamases: A Comprehensive Narrative Review

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## Abstract

Extended-Spectrum β-Lactamases (ESBLs) are enzymes that confer resistance to third-generation cephalosporins, penicillins, and aztreonam, posing a serious global threat to antimicrobial therapy and infection control. Accurate and timely detection of ESBL-producing organisms is essential for appropriate clinical management, epidemiological surveillance, and implementation of infection prevention measures. This narrative review comprehensively explores both phenotypic and molecular strategies employed for the identification of ESBLs in clinical microbiology laboratories. Phenotypic methods remain the cornerstone for initial screening and confirmation of ESBL production due to their cost-effectiveness and applicability in routine diagnostic setups. Screening tests, such as the disk diffusion method and automated susceptibility testing systems, are commonly used to identify potential ESBL producers. Confirmatory tests—such as the combined disk test, double-disk synergy test (DDST), and E-test—rely on the inhibition of β-lactamase activity by clavulanic acid and remain widely accepted for their simplicity and reliability. However, these methods may face limitations in detecting co-existing resistance mechanisms, such as AmpC β-lactamases or carbapenemases, which can mask ESBL activity. Molecular techniques have emerged as powerful tools for the precise detection and characterization of ESBL genes. Polymerase chain reaction (PCR) and multiplex PCR allow rapid identification of common ESBL gene families, including *bla*$_{TEM}$, *bla*$_{SHV}$, and *bla*$_{CTX-M}$. Advanced sequencing technologies, such as real-time PCR, DNA microarray, and whole-genome sequencing (WGS), enable in-depth analysis of gene variants, mobile genetic elements, and clonal dissemination patterns. Although molecular approaches offer superior specificity and sensitivity, their use is often constrained by high cost, technical expertise requirements, and limited availability in low-resource settings. This review emphasizes the complementary roles of phenotypic and molecular methods in ESBL detection. A combined diagnostic approach can enhance accuracy, guide appropriate antimicrobial therapy, and strengthen surveillance programs aimed at combating multidrug resistance. Future research should focus on developing rapid, affordable, and point-of-care diagnostic tools integrating both phenotypic and genotypic insights to support effective antimicrobial stewardship and global public health interventions. *[Journal of Current and Advance Medical Research, July 2024;11(2):115-122]*

**Keywords:** Phenotypic and Molecular Strategies; Extended-Spectrum β-Lactamases; ESBL producers

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## Introduction

Extended-Spectrum β-Lactamases (ESBLs) are a diverse group of enzymes produced by certain Gram-negative bacteria, notably members of the *Enterobacteriaceae* family such as *Escherichia coli* and *Klebsiella pneumoniae*[1]. These enzymes can hydrolyze and inactivate a wide range of β-lactam antibiotics, including penicillins, third-generation cephalosporins, and monobactams, while being inhibited by β-lactamase inhibitors such as clavulanic acid[2]. The global emergence and dissemination of ESBL-producing bacteria have become a major public health concern, leading to limited therapeutic options, treatment failures, and increased morbidity, mortality, and healthcare costs[3].

Detection of ESBL-producing organisms is crucial for effective patient management, infection control, and antimicrobial stewardship[4]. Several phenotypic and molecular tests have been developed to identify the presence of ESBLs in clinical isolates. Phenotypic methods, such as the disk diffusion screening test, double-disk synergy test (DDST), and combined disk test, are routinely used in clinical laboratories due to their simplicity, affordability, and ability to provide rapid results[5]. However, these methods may sometimes yield ambiguous interpretations, especially in the presence of other β-lactamases like AmpC or carbapenemases that can mask ESBL activity.

In contrast, molecular techniques such as polymerase chain reaction (PCR), real-time PCR, and sequencing-based approaches offer high sensitivity and specificity for the detection of ESBL genes, including *bla*TEM, *bla*SHV, and *bla*CTX-M[6]. These methods not only identify the presence of resistance genes but also help in understanding their genetic diversity and transmission dynamics. Despite their accuracy, molecular assays are often limited to well-equipped laboratories due to their higher cost and technical complexity[7].

This review provides a comprehensive overview of the various phenotypic and molecular strategies available for the detection of ESBLs, highlighting their principles, advantages, limitations, and relevance in current diagnostic microbiology.

## Screening for ESBL

Screening for Extended-Spectrum β-Lactamase (ESBL) production is a critical first step in detecting resistant bacterial isolates in clinical microbiology laboratories. The goal of screening is to identify potential ESBL producers among *Enterobacteriaceae* and other Gram-negative bacilli before confirmatory testing. Early and accurate screening is essential for guiding antimicrobial therapy, preventing nosocomial transmission, and supporting antimicrobial stewardship programs[8].

Screening for ESBLs is primarily based on susceptibility testing using third-generation cephalosporins such as cefotaxime, ceftazidime, ceftriaxone, and aztreonam. The Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommend that bacterial isolates showing reduced susceptibility to one or more of these antibiotics should be considered potential ESBL producers[9]. Disk diffusion and automated systems like VITEK 2, MicroScan, or BD Phoenix are commonly used for this purpose. The use of specific screening breakpoints helps to identify isolates that may harbor ESBLs for further confirmatory testing[10].

In the disk diffusion method, screening is performed using antibiotic-impregnated disks (e.g., cefotaxime 30 µg and ceftazidime 30 µg) placed on Mueller–Hinton agar inoculated with the test organism. A reduced inhibition zone diameter—typically ≤27 mm for cefotaxime or ≤22 mm for ceftazidime—indicates possible ESBL production[11]. Broth microdilution methods measuring minimum inhibitory concentrations (MICs) can also be used, with elevated MICs suggesting ESBL presence.

Automated systems offer the advantage of standardization, rapid turnaround time, and integration with laboratory information systems. However, they may occasionally misclassify isolates due to overlapping resistance mechanisms[12]. Therefore, isolates that screen positive for ESBL should always undergo confirmatory testing to differentiate ESBL activity from other β-lactamase-mediated resistance, such as AmpC enzymes or carbapenemases.

Screening results play a pivotal role in infection control measures, including patient isolation and antibiotic policy adjustments. Despite being relatively simple and cost-effective, phenotypic screening tests may sometimes fail to detect low-level or masked ESBL expression. Thus, continuous monitoring of performance standards and integration of molecular surveillance in high-prevalence settings are essential for accurate

detection and containment of ESBL-producing organisms[13].

The Clinical and Laboratory Standards Institute (CLSI) has been developed broth microdilution and disc diffusion screening tests using selected antimicrobial agents. Each *Klebsiella pneumoniae, Klebsiella oxytoca,* or *Esch. coli* isolate should be considered a potential ESBL producer if the test results are as follows:

**Table 1: Test Results of Screening**

| Antibiotics | Disc Diffusion | MIC |
|---|---|---|
| Cefpodoxime | 22 mm | 2µg/ml |
| Ceftazidime | 22 mm | 2µg/ml |
| Azteonam | 27 mm | 2µg/ml |
| Cefotaxime | 27 mm | 2µg/ml |
| Ceftriaxone | 25 mm | 2µg/ml |

The sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are tested. The use of more than one of the five antimicrobial agents suggested for screening will improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection.

**Double Disc Synergy Test**

The Double Disc Synergy Test (DDST) is one of the most widely used phenotypic methods for the detection of Extended-Spectrum β-Lactamase (ESBL) production in clinical isolates, particularly among *Enterobacteriaceae*[14]. The principle of this test is based on the inhibition of ESBL enzymes by β-lactamase inhibitors such as clavulanic acid, which restores the activity of extended-spectrum cephalosporins when used in combination. In the DDST, a standardized inoculum of the test organism (equivalent to 0.5 McFarland turbidity) is evenly spread over the surface of a Mueller–Hinton agar plate. A disk containing amoxicillin–clavulanic acid (20/10 µg) is placed at the center of the plate. Around this disk, third-generation cephalosporin disks—such as cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), or aztreonam (30 µg)—are positioned 20–30 mm apart (center to center). The plate is then incubated at 35–37°C for 18–24 hours.

After incubation, the presence of a "keyhole" or enhanced zone of inhibition between the clavulanic acid disk and any of the cephalosporin disks indicates synergy, confirming ESBL production. This occurs because clavulanic acid inhibits the ESBL enzyme, thereby restoring the antibiotic's activity and expanding the inhibition zone[15]. The DDST is simple, cost-effective, and reliable for routine laboratory use, especially in resource-limited settings. However, its sensitivity may vary depending on the distance between the disks, inoculum density, and the type of ESBL enzyme present. It may also fail to detect certain ESBLs when co-produced with AmpC β-lactamases or in isolates with high-level resistance[16].

In this test, discs of third-generation cephalosporins and augmcntin are kept 20 to 30 mm apart from center to center on inoculated Mueller-Hinton Agar (MHA). A clear extension of the edge of the inhibition zone of cephalosporin towards the augmenting disc is interpreted as positive for ESBL production.

**Figure I:** Double disc synergy test positive ESBL strain shows enhancement of the zone of inhibition of the oxyimino β- lactam caused by the synergy of the clavulanic acid in augmentin disc

**Three-Dimensional Test**

This test provides the advantage of simultaneous determination of antibiotic susceptibility and β-lactamase substrate profile. Inoculum produced in this method contains between $10^9$ and $10^{10}$ CFU/mL of cells that actively produce β-Iactamase[16]. Two types of inocula are prepared one disc diffusion test inoculum optical density equal to that of 0.5 McFarland standard and a three-dimensional inoculum containing between $10^9$ and $10^{10}$ CFU of cells. The plate is inoculated disc diffusion procedure. A circular slit is cut on the agar 4mm inside the position at which the antibiotic discs are placed.

Conventional (two-dimensional) disc diffusion susceptibility test results are measured according to the recommendations of NCCLS. Distortion or discontinuity in the circular inhibition zone is interpreted as positive for ESBL production. Inhibitor-potentiated disc diffusion test[17]. Cephalosporin disc is plated on clavulanate-containing and non-clavulanate-containing MHA plates. More than 10 mm increase in the zone of inhibition on the cefavulanate-containing MHA plate indicates ESBL production.

**Disc Approximation Test**

Cefoxitin (inducer) disc is placed at a distance of 2.5 cm from the cephalosporin disc. Production of inducible β-lactamase is indicated by flattening of the zone of inhibition of the cephalosporin disc towards the inducer disc by more than I mm.

**MIC Reduction Test**

By this method MIC of cefotaxime or ceftazidime with and without clavulanic acid (4μg/ml) is determined. An 8-fold reduction in the MIC of cephalosporin in the presence of clavulanic acid compared with cephalosporin alone indicates production of ESBL[18].

**Vitek ESBL Test**

This is an automated microbial susceptibility test system that utilizes ceftazidime or cefotaxime alone and in combination with clavulanic acid[19]. Cards containing four wells are inoculated. Presence of ESBL was indicated if there is a reduction in growth of cephalosporin well containing clavulanic acid compared with the level of growth in a well with cephalosporin alone[20].

**E-Test ESBL Method**

The E-test ESBL strip carries two gradients, on the one end, ceftazidime and on the opposite end ceftazidime plus clavulanic acid. MIC is interpreted as the point of intersection of the inhibition ellipse with the E-test strip edge[21]. Ratio of ceftazidime MIC and ceftazidime plus clavulanic acid MIC equal to or greater than 8 indicates the presence of ESBL.

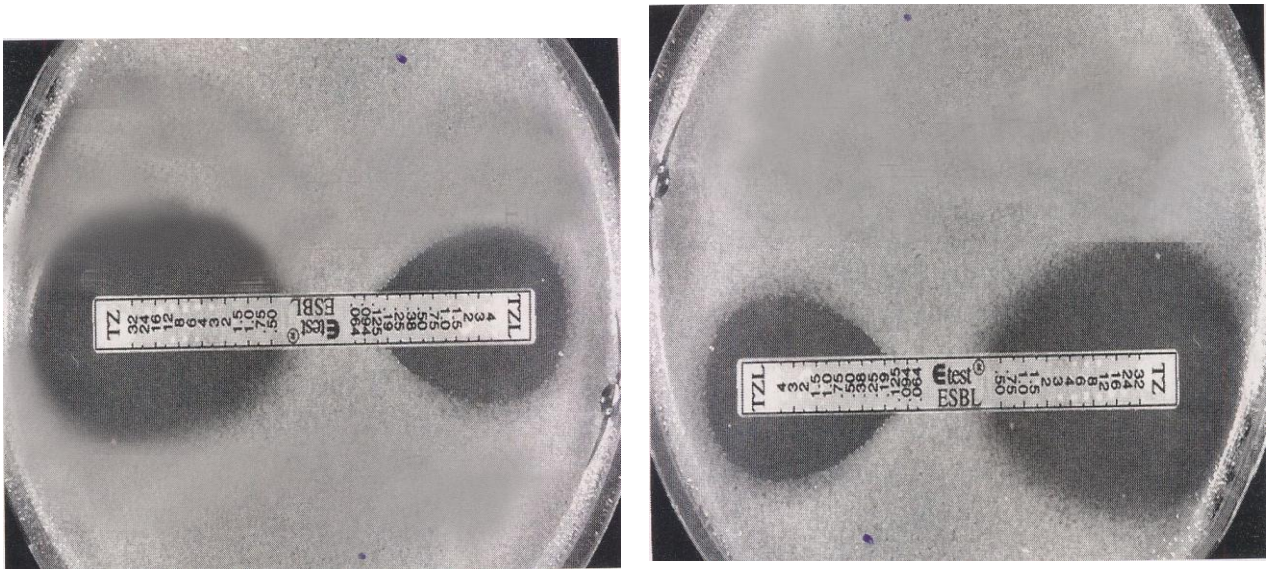


**Figure II:** E-test ESBL strips test positive strain shows MIC ratio of ceftazidime vs ceftazidime/clavulanic acid more than (>) 8 indicates ESBLs

**Table 2: Comparison between Vitek system and the DD test**[21]

| Parameter | Result by indicated test | Compared with standard |
|---|---|---|
| | Vitek system | DD test |
| Number Of Strains with Positive Standard and Test Result | 205 | 202 |
| Number Of Strains with Negative Standard and Test Result | 159 | 157 |
| Number Of Strains With Positive Standard And Negative Test Result | 07 | 04 |
| Number Of Strains With Negative Standard And Positive Test Result | 0 | 1 |
| Sensitivity (%) | 99.57 | 98.06 |
| Specificity (%) | 100 | 99.37 |

Note: DD test= double disc test

Result of the indicated test compared with the standard result. Standard in these comparisons was the identity of the β-lactamase that had been established by standard biochemical and molecular methods. A total of 365 tests were performed with Vitek ESBL test, while 364 were performed with the double disc (D D) test. One test result by the double disc test could not be interpreted at one site.

**Molecular Detection Methods**

Double disc test, three-dimensional test, MIC reduction test, The Cica Beta test, and E test presumptively identify the presence of an ESBL. Whereas the following molecular methods can identify specific (TEM, SHV) ESBLs is present in a clinical isolate.

**Isoelectric Point**

This was used to identify ESBL in the early days. However, with more than 90 TEM-type β-lactamases, many of which possess identical isoelectric points, determination of the ESBL by isoelectric point is no longer possible. A similar situation is found in the SHV, CTX-M, and OXA families of ESBLs[22].

**DNA Probe**

Early detection of β-lactamase genes was performed using DNA probes that Were specific for TEM and SHV enzymes. However, using DNA probes can Sometimes be rather labor intensive[23].

**PCR**

The easiest and most common molecular method used to detect the presence of a β-lactamase belonging to a family of enzymes is PCR with oligonucleotide primers that are specific for a β-lactanlase gene. These primers are usually chosen to anneal to regions where various point mutations are not known to occur. However, PCR will not discriminate among different variants of TEM or SHV[24].

**Oligotyping Method**

The first molecular method for the identification of β-lactamase was the oligotyping method, which was used to discriminate between TEM-I and TEM-2. This method used oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions[25]. Subsequently, additional oligonucleotide probes to detect mutations at six positions within the *bla*TEM gene was developed. Using this method, several new TEM variants were identified within a set of clinical isolates. The probes used in oligotyping tests for TEM β–lactamases have been labeled either with a radioisotope or with biotin[26].

**PCR-RFLP**

Another approach for molecular characterization of the TEM β-lactamase gene was to add restriction fragment length polymorphism analysis to PCR (PCR-RFLP). In this test, amplified PCR products were subjected to digestion with several restriction endonucleases, and the subsequent fragments were separated by electrophoresis[27]. The sizes of the fragments generated by each restriction enzyme indicate point mutations within the *bla*TEM structural gene. A number of different tests have been proposed for the detection and identification of SHV derivatives[28].

The simplest of these was suggested by and employs PCR-RFLP. Following PCR, the amplified

DNA is digested with restriction enzyme *NheI,* which detects the G-to-A nucleotide change that gives rise to the glycine-to-serine substitution at position 238 that is common to many of the early SHV type ESBLs. Although this method cannot determine which SHV type ESBL is present, it can detect the specific mutation at position 238.

**Ligase Chain Reaction (LCR)**

LCR (ligase chain reaction) method was proposed for the identification of SHV genes is the use of ligase chain reaction LCR allows the discrimination of DNA sequences that differ by a single base pair by the use of a thermostable ligase with four oligonucleotide primers that are complementary to the target sequence and hybridize adjacent to each other[29]. A single base mismatch in the oligonucleotide junction will not be ligated and subsequently amplified. In this LCR test, the target DNA containing the *bla*SHV gene is denatured in a thermocycler and annealed with biotinylated oligonucleotide primers that detect mutations at four positions. The LCR product is detected by an enzymatic reaction using NADPH-alkaline phosphatase. This method was able to detect seven of the SHV variants. For OXA-10-derived ESBLs, the presence of an OXA-type gene in clinical isolates of *Pseudomonas aeruginosa* was first detected using a colony hybridization technique. Subsequently, positive isolates were subjected to PCR with specific OXA primers and then digested with restriction endonucleases that would distinguish several groups of related OXA enzymes based on the sizes of the restriction fragments. While this technique does not completely identify which OXA gene is present in a strain, it can distinguish the ESBL OXA type β-lactamases from non-ESBLs that are also related to OXA-10.

**Quality control organisms used for ESBL detection**

*Klebsiella pneumoniae* ATCC 700603 (positive control) and *Escherichia coli* ATCC 25922 (negative control) should be used for quality control of ESBL test.

**Problems in Detection**

Identifying organisms that are ESBL producers is a major challenge for the clinical microbiology laboratory. Due to the variable affinity of these enzymes for different substrates and the inoculum effect, some ESBL isolates may appear susceptible to a third-generation cephalosporin in vitro. However, treatment of infections due to an ESBL producing organism with third-generation cephalosporins may result in clinical failure if infection is outside the urinary tract[15]. Cefpodoxime and ceftazidime (3rd generation cephalosporin) have been proposed as indicators of ESBL production as compared to cefotaxime and ceftriaxone. Hence, an institution where only cefotaxime and ceftriaxone are used in the routine sensitivity testing panel may have difficulty in detecting ESBLs[19]. These enzymes can be induced by certain antibiotics, amino acids or by body fluids. Organisms possessing genes for inducible β-lactamases show false susceptibility if tested in the uninduced state[11]. For ESBL-producing bacteria, there is a dramatic rise of MIC for extended-spectrum cephalosporins as the inoculum is increased beyond that used in routine susceptibility[13]. Therefore, they may be reported as false sensitive if tested by routine methods[18]. The latest guidelines by NCCLS recommend screening *Klebsiella species* and *Escherichia coli* isolate with a MIC greater than or equal to 2 μg/mL against cefpodoxime, ceftazidime, aztreonam, cefotaxime, or ceftriaxone as potential ESBL producers. Two indicators of ESBLs are a 8-fold reduction in MIC in the presence of clavulanic acid when using the broth dilution and the potentiation of the inhibitor zone by clavulanic acid more than 5 mm increase in diameter of inhibition zone when using disc diffusion method. These methods, though useful, may not detect those ESBLs that are poorly inhibited by β-Iactamase inhibitors[18]. Two studies[21,23] evaluated the ability of clinical laboratories to detect and report the presence of ESBLs. A survey in Connecticut found that 21.0% of laboratories fail to detect ESBL-producing isolates. A proficiency testing project for clinical laboratories participating in the National Nosocomial Infections Surveillance System indicated that as many as 58.0% laboratories failed to detect and report ESBL isolates correctly. These data suggest that improvements in the ability of clinical laboratories to detect ESBL are needed[22].

**Conclusion**

The presence of an ESBL-producing organism in a clinical infection can result in treatment failure if one of the above classes of drugs is used. ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins. Thus, the choice of which antimicrobial agents to test is critical. If an isolate is confirmed as an ESBL producer by the CLSI-recommended phenotypic confirmatory test procedure, all penicillins, cephalosporins, and aztreonan should be reported as

resistant. This list does not include the cephamycins (cefotetan and cefoxitin), which should be reported according to their routine test results. If an isolate is not confirmed as an ESBL producer, current recommendations suggest reporting results as for routine testing. Interpretations of penicillins, cephalosporins, and aztreonam for isolates not confirmed as ESBLs should not be changed.

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None

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Any inquiries regarding supporting data availability of this study should be directed to the corresponding author and are available from the corresponding author on reasonable request.

**Ethics Approval and Consent to Participate**
Not Applicable



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