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Clinical Laboratory and Molecular Detection of Extended Spectrum beta lactamases: A Review Update

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Abstract

Extended Spectrum β-lactamases (ESBLs) can confer resistance to all extended spectrum cephalosporins, all penicillins and monobactam. Being plasmid and transposon mediated has facilitated the spread of these enzymes to other species of bacteria. This is a challenge for the laboratory to detect ESBL-containing Gram-negative bacilli because they can appear susceptible in vitro to certain beta-lactam antimicrobial agents yet result in clinical treatment failure. Till now there is no gold standard test for detection of ESBLs. CLSI recommended the phenotypic method as confirmatory test. However, the traditional methods need much labor and time for cultivation and require at least overnight incubation after isolated colonies are available from primary culture. Therefore, about 48 hours is required for ESBLs reporting by traditional methods. Molecular characterization of the isolated ESBL was also not possible in maximum laboratories due to lack of facilities. But rapid detection of ESBLs from the patient with severe infection is urgently required; otherwise it may be fatal. In this present review this diagnostic dilemma of ESBL has been discussed. [*Bangladesh J Infect Dis 2014;1(1):12-17*]

Keywords: ESBL, beta lactum antibiotics, beta lactamse

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1. INTRODUCTION

Emergence of resistance to Beta lactam antibiotics began even before the first Beta lactam, penicillin, was developed[1]. Many genera of Gram negative bacteria possess a naturally occurring, chromosomally mediated β-lactamase[2-4]. Within few years after its first isolation, the extended spectrum β-lactamase spread worldwide and is now found in many different species of members of the family *Enterobacteriaceae*, *Pseudomonas aeruginosa*, *Haemophilus influenzae* and *Neisseria gonorrhoeae*[5-7].

This is a challenge for the laboratory to detect ESBL-containing Gram-negative bacilli because they can appear susceptible in vitro to certain beta-lactam antimicrobial agents yet result in clinical

treatment failure[8-11]. Several ESBL detection tests that have been proposed are based on Clinical Microbiology Techniques are Screening for ESBL, NCCLS phenotypic confirmatory method, Double disc synergy / Disk approximation method, Etest ESBL strips, Three dimensional test, Vitek system, The Cica Beta Test 1. Several Molecular Methods also have been proposed for detection of ESBLs including Isoelectric point, DNA probes, PCR, Oligotyping method, PCR-RFLP, PCR-SSCP, LCR, Nucleotide sequencing, Pulsed field gel electrophoresis (PFGE) etc. Till now there is no gold standard test for detection of ESBLs. NCCLS recommended the phenotypic method as confirmatory test[11]. Molecular characterization of the isolated ESBL was also not possible in maximum laboratories due to lack of facilities[12-14]. But rapid detection of ESBLs from the patient with severe infection like septicemia, meningitis with gram negative rods is urgently required; otherwise it may be fatal. The Cica Beta Test 1/ HMRZ-86/ Chromogenic cephalosporin can rapidly detect ESBLs in Gram negative rods within 15 minutes directly with isolated colonies from primary culture[3]. The great advantage of the kit remains its rapid turnaround time, which facilitates reporting of clinically relevant information 24 hours earlier then phenotypic confirmatory test and other tests. Handling the kit is very simple and can be used without any complications[13].

2. ESBL DETECTION METHODS

The increased prevalence of *Enterobacteriaceae* producing ESBLs creates a great need for laboratory testing methods that will accurately identify the presence of these enzymes in clinical isolates[15]. In the years since ESBLs were first described, a number of different testing methods have been suggested[16]. No gold standard method is available like molecular test[3]. National Committee for Clinical Laboratory Standards recommendations exist for detecting ESBL producing isolates of *Escherichia* coli and *Klebsiella* spp., no recommendations exist for detecting ESBLs in other organisms[17].

2.1 Clinical Microbiology Techniques

Several ESBL detection tests that have been proposed are based on the Kirby-Bauer disk diffusion test methodology. Clinical microbiology tests employ a β-lactamase inhibitor, usually clavulanate, in combination with an oxyimino-cephalosporin such as ceftazidime or cefotaxime. In these tests, the clavulanate inhibits the ESBL, thereby reducing the level of resistance to the cephalosporin.

2.1.1 Screening for ESBL[10]

The National Committee for Clinical Laboratory Standards (NCCLS) has developed broth microdilution and disc diffusion screening tests using selected antimicrobial agents. In-vitro sensitivity testing using established NCCLS procedure is carried out with ceftazidime (30mg), cefotaxime (30mg), ceftriaxone (30mg), aztreonam (30mg) and cefpodoxime (10mg). Any zone diameter within the "grey zone" must be considered as a probable ESBL producing strain requiring phenotypic confirmatory testing. Each *Klebsiella pneumoniae*, *K. oxytoca*, or *Escherichia coli* isolate should be considered a potential ESBL-producer.

Table 1: MIC and Inhibition Zone Criteria for the Detection of ESBLs in *K. pneumoniae* and *E. Coli*

Antibiotic	Zone diameter		MIC	
	Sensitive (mm)	ESBL (mm)	Sensitive (mg/L)	ESBL (mg/L)
Azt	≥22	≤27	≤8	≥2
Cefota	≥23	≤27	≤8	≥2
Cefpod	≥21	≤22	≤8	≥2
Ceftaz	≥18	≤22	≤8	≥2
Ceftri	≥21	≤25	≤8	≥2

*adapted from NCCLS document M100-S88; Azt=Aztreonam (30mg); Cefota=Cefotaxime (30mg); Cefpod=Cefpodoxime (10mg); Ceftaz=Ceftazidime (30mg); Ceftri=Ceftriaxone (30 mg)

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. Currently, the NCCLS recommends an initial screening by testing for growth in a broth medium containing 1 µg/ml of one of five expanded-spectrum β-lactam antibiotics. This screen is then followed by a phenotypic confirmatory test.

2.2. CLSI Phenotypic Confirmatory Methods

Ceftazidime (30mg) versus ceftazidime/clavulanic (30/10mg) and cefotaxime (30mg) versus (cefotaxime/clavulanic acid (30/10mg) are placed

onto a Muller-Hinton agar plate lawned with the test organism and incubated. Regardless of the zone diameters, a ≥ 5mm increase in a zone diameter for an antimicrobial agent tested in combination with clavulanic acid versus its zone size when tested alone, indicates probable ESBL production. A similar test was designed by Jacoby and Han, in which 20 µg of sulbactam was added to susceptibility disks containing one of the oxyimino-β-lactam antibiotics. An increase of 5 mm in the zone of inhibition in a disk containing sulbactam compared to the drug alone was considered a positive test. Although many ESBL-producing strains were detected with this method, a significant number of strains were not detected. In addition, a number of Amp C-producing strains also showed an enhancement of the zone diameter with the addition of sulbactam.

2.3. Double Disc Synergy/Disk Approximation Method[11]

In this test, the organism is swabbed onto a Mueller-Hinton agar plate with a suspension (adjusted to 0.5 McFarland turbidity standards that have been vortexed) made from an overnight agar plate of the test strain. A susceptibility disk containing amoxicillin-clavulanate is placed in the center of the plate, and disks containing oxyimino-β-lactam the standard ceftazidime (30ug), ceftriaxone (30mg), aztreonam (30mg) or cefpodoxime (10mg) are placed 30 mm (center to center) from the amoxicillin-clavulanate disk. Plates are then incubated overnight at 35^{o}C. Enhancement of the zone of inhibition of the oxyimino-β-lactam caused by the synergy of the clavulanate in the amoxicillin-clavulanate disk is a positive result. This test remains a reliable method for the detection of ESBLs. It is vital to place disc at the precise distance as recommended.

2.4. E-test ESBL Strips[18]

Two E-test combination strips e.g. ceftazidime/ceftazidime-clavulanic acid and cefotaxime/cefotaxime-clavulanate are employed to perform the phenotypic confirmatory testing. Etest ESBL strips are two-sided strips that contain a gradient of ceftazidime on one end and ceftazidime plus clavulanate on the other end.

These strips are inoculated on the surface of the agar plate and incubated overnight. Any reduction of > 3 log 2 (doubling) dilution is considered as positive (Figure-3). This test was shown to be more sensitive than the double-disk approximation test in detecting ESBLs in clinical isolates. This method is convenient and easy to use, but it is sometimes difficult to read the test when the MICs of ceftazidime are low because the clavulanate sometimes diffuses over to the side that contains ceftazidime alone.

Table 2: ESBL detection techniques by Clinical Microbiology

Test	Advantages	Disadvantages
Standard interpretive criteria	Easy to performed in lab	ESBLs not always "resistant"
ESBL confirmatory test	Easy to use and interpret	Sensitivity depends on choice of oxyimino-cephalosporin
Double-disk approximation test	Easy to use & interpret	Distance of disk placement for optimal sensitivity not standardized
Three-dimensional test	Sensitive & easy interpret	Non-specific for ESBLS, labor intensive
E-test ESBL strips	Easy to use	Not easy to interpret, not as sensitive as double-disk test
Vitek ESBL test	Easy to use & interpret	Reduced sensitivity
HMRZ-86	detect ESBLs within 2-15 min	Can not differentiate ESBLs, AmpC and MBL

2.5. Three Dimensional Test[16]

Another method suggested for the detection of ESBLs is the three-dimensional test described by Thomson and Sanders. In this test, following inoculation of the test organism onto the surface of a Mueller-Hinton agar plate, a slit is cut into the agar, into which a broth suspension of the test organism is introduced. Subsequently, antibiotic disks are placed on the surface of the plate 3 mm from the slit. Distortion or discontinuity in the expected circular zone of inhibition is considered a positive test. This test was determined to be very sensitive in detecting ESBLs, but it is more technically challenging and labor intensive than other methods.

2.6. VITEK System[13]

The automated microbial susceptibility test system Vitek has also produced an ESBL test that utilizes either ceftazidime or cefotaxime alone and in

combination with clavulanic acid (4µg/ml). A predetermined reduction in growth in wells containing clavulanate compared to those containing drug alone indicates the presence of an ESBL. Furthermore, updated computer algorithms in the new Vitek system have also been shown to categorize the β-lactamases present in many gram-negative clinical isolates based on the phenotype of susceptibility patterns with various β-lactam antibiotics.

Table 3: ESBL detection techniques by Molecular detection

DNA probes	Specific for gene family (e.g.,TEM or SHV)	Labor intensive, can't distinguish between ESBLs and non-ESBLs & variants of TEM or SHV
PCR	Easy to perform, specific for gene family (e.g., TEM or SHV)	Can't distinguish between ESBLs and non-ESBLs & variants of TEM or SHV
Oligotyping	Detects specific TEM variants	Requires specific oligonucleotide probes, labor intensive, can't detect new variants
PCR-RFLP	Easy to perform, can detect specific nucleotide changes	Nucleotide changes must result in altered restriction site for detection
PCR-SSCP	distinguish SHV variants	Requires electrophoresis
LCR	distinguish SHV variants	Requires oligonucleotide primers
Nucleotide sequencing	The gold standard, can detect all variants	Labor intensive, technically challenging, difficult to interpret manual methods
PFGE	Can detect all variants	Labor intensive

2.7. HMRZ-86 (The Cica Beta Test 1)

This kit originally designed for rapid detection of ESBLs and metallo beta lactamases (MBLs) in gram negative rods directly from isolated colonies. The kit consists of plastic strip with a paper pad and solution substrate-Chromogenic Cephalosporin HMRZ-86. HMRZ-86 is a new Chromogenic cephalosporin. A carboxypropyl-oxyimino group bonded to the side chain at position 7 in the compound protects the beta lactam ring (lactamases differentiation ring) from a range of traditional beta lactamases. But this carboxypropyl-oxyimino group cannot protect the cephalosporin from hydrolysis by ESBLs or MBLs. A Chromogenic substance-conjugate located at position 3 bonded by double bond. Hydrolysis of the beta lactam ring by these enzymes changes the wavelength absorbed by the conjugated double bond located at position 3, shifting the color of the compound from yellow to red. ESBL positive strains gives red color within 2-15 minutes. While each of these tests has its merit, none of these methods can accurately detect all strains producing ESBLs[18] showed that the Etest ESBL test with ceftazidime only detected 81% of ESBLs tested in their laboratory, compared to 97 and 91% for the double-disk test and the three-dimensional test, respectively. In a survey of detection of ESBLs in clinical isolates[15] found that only 18% of laboratories correctly identified challenge organisms as potential ESBL producers using susceptibility to one or more expanded-spectrum β-lactam antibiotics as the method of detection. However, none of the detection tests that are based on the phenotype of the β-lactamase produced is 100% sensitive or specific for the accurate detection of ESBLs among clinical isolates of gram-negative bacteria.

3.0. Molecular Detection Methods

The tests described above only presumptively identify the presence of an ESBL. The task of identifying which specific ESBL is present in a clinical isolate is more complicated.

3.1. Isoelectric point

In the early days of studying ESBLs, determination of the isoelectric point was usually sufficient to identify the ESBL that was present. However, with >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[2].

3.2. DNA probes

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[5].

3.3. 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-lactamase 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. Several molecular methods that will aid in the detection and differentiation of ESBLs without sequencing have been suggested[2].

3.4. Oligotyping method[12]

The first molecular method for the identification of β-lactamase was the oligotyping method developed by Ouellette *et al.*, which was used to discriminate between TEM-1 and TEM-2. This method used oligonucleotide probes that are designed to detect point mutations under stringent hybridization conditions. Subsequently, Mabilat and Courvalin developed additional oligonucleotide probes to detect mutations at six positions within the bla_{TEM} gene. 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.

3.5. PCR-RFLP[1]

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 enzyme endonucleases (*Nhe*I), and the subsequent fragments were separated by electrophoresis. The sizes of the fragments generated by each restriction enzyme indicate point mutations within the bla_{TEM} structural gene, 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.

3.6. PCR-SSCP

Another method used to characterize SHV-type ESBLs is PCR single-strand conformational polymorphism (PCR-SSCP) analysis. This method has been used to detect a single base mutation at specific locations within the bla_{SHV} gene. In this test, a 475-bp amplimer is generated using oligonucleotide primers that are internal to the coding sequence of the bla_{SHV} gene, digested with restriction enzyme *Pst*I. The fragments are then denatured and separated on a 20% polyacrylamide gel. Genes for SHV-1, -2, -3, -4, -5, and -7 β-lactamases can be identified by the electrophoretic pattern of the digested amplimer[9].

3.7. LCR[7]

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 complimentary to the target sequence and hybridize adjacent to each other. 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[17].

3.8. Nucleotide sequencing

Nucleotide sequencing remains the standard for determination of the specific β-lactamase gene present in a strain. However, this too can give variable results depending on the method used. It is possible that some of the variability seen in the sequences for some of the SHV β-lactamases was due to compressions and difficulty in reading traditional sequencing autoradiographs, rather than actual differences in the sequence[2].

3.9. Pulsed field gel electrophoresis (PFGE)

Many investigators are using molecular methods pulsed-field gel electrophoresis (PFGE) for detection of ESBLs[10]. Whole chromosomal DNA digests in agarose with *Xba*l, and restricted fragments separate in a CHEF MAPPER XA apparatus. Pulsed-field gel electrophoresis (PFGE) is performed under the following conditions: 0.5X Tris-Borate-EDTA buffer, 1% agarose, at 13^0 C and 200 V for 23 h, with the switch interval ramped. Gels stain with ethidium bromide and photograph. All bands have to mach exactly to classify isolates as indistinguishable. Patterns differ by 1-3 bands were designed as highly related. Isolates that differs by 4-6 bands is considered as possible related. Isolates with more than 6 bands different is considered as different types[18].

4.0. Medical Significance of Detection of ESBLs

It is generally thought that patients having infections caused by an ESBL-producing organism are at an increased risk of treatment failure with an expanded-spectrum β-lactam antibiotic. Therefore, it is recommended that any organism that is confirmed for ESBL production is reported as resistant to all expanded-spectrum β-lactam antibiotics, regardless of the susceptibility test result. While some ESBL-producing strains have overt resistance to expanded-spectrum β -lactam antibiotics, many isolates will not be phenotypically "resistant" according to guidelines such as those previously used by the NCCLS. Therefore, it is important for the clinical microbiology lab to be aware of isolates that may show increased MICs of oxyimino-cephalosporin even though they may not be reported as resistant[2].

5.0. Problems in detection

ESBLs producer organisms are a major challenge for the clinical microbiology laboratory. ESBLs can be difficult to detect because they have different levels of activity against various cephalosporins, some ESBL isolates may appear susceptible to a third generation cephalosporin in vitro[10]. Extended-spectrum b-lactamases (ESBLs) and AmpC b-lactamases are of increasing clinical concern[14]. The diffusion of metallo-beta-lactamases (MBLs) among clinically important human pathogens represents a therapeutic issue of increasing importance[15]. ESBLs, AmpC beta-lactamases and MBLs are resistant to the most of the extended spectrum cephalosporin.

6.0. Conclusion

No one single method can identify all types of ESBLs. Phenotypic confirmatory method, double disc synergy test and Etest ESBL strip test are easy to practice in laboratory. HMRZ-86 is very easy to perform in laboratory and take only 15 minutes time. HMRZ-86/Chromogenic cephalosporin positive strains are ESBLs, AmpC β-lactamases and Metallo-β-lactamases which are resistant to cephalosporins and most of other antibiotics.

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