Extended-Spectrum Beta-Lactamases (ESBL)

Screening and Confirmatory Tests for Extended-Spectrum Beta-Lactamases (ESBL)

Transferable plasmid-mediated beta-lactamases that produce resistance towards third generation cephalosporins and monobactams (e.g. aztreonam) have been described in strains of *Klebsiella pneumoniae*, *K. oxytoca*, *E. coli* and other Enterobacteriaceae. These enzymes are classified as extended-spectrum beta-lactamases (ESBL) and they have been implicated in clinical resistance to monobactams and broad-spectrum cephalosporins such as ceftazidime (CAZ), cefotaxime (CTX), and ceftriaxone (CTR).

Some ESBLs confer high-level resistance to these beta-lactams and are easily detected as resistant (or intermediate) by disk (tablet) diffusion testing. But the ESBL may provide low levels of resistance (MIC 1-2 µg/ml) to monobactams and third generation cephalosporins that can be easily overlooked by routine susceptibility methods and current interpretative criteria (1). These latter isolates may not reach current CLSI breakpoints for resistance, yet can be clinically resistant to beta-lactam therapy (2).

Since some ESBLs are more active on CAZ, while others are more active on CTX, the choice of cephalosporins tested can also affect the ability of laboratories to detect resistant strains (3).
Most ESBLs are inhibited by clavulanic acid, tazobactam or sulbactam and can be readily detected by the double-disk (tablet) synergy test (4).

Double disk (tablet) synergy test

Inoculate a Mueller-Hinton plate as for susceptibility testing and apply Ceftriaxone (CTR) Neo-Sensitabs, Cefotaxime (CTX) Neo-Sensitabs, Ceftazidime (CAZ) Neo-Sensitabs, Cefepime (FEP) Neo-Sensitabs and Aztreonam (AZT) Neo-Sensitabs **at approximately 20 mm** (30 mm from tablet center to tablet center) from a tablet containing Amoxycillin+Clavulanate Neo-Sensitabs (AMC) using a dispenser. Incubate overnight at 35 °C.

Extension of the zone of inhibition (synergism) towards the tablet containing AM+CL, indicates the presence of an extended spectrum beta-lactamase (ESBL).

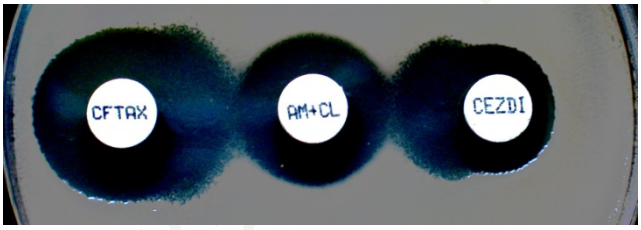


Plate 1.1.0-a. *Klebsiella pneumoniae* (ATCC 700603) producing extended-spectrum beta-lactamases (ESBL). Note the synergy between cefotaxime Neo-Sensitabs (CTX), ceftazidim Neo-Sensitabs (CAZ) and Amoxycillin+Clavulanate Neo-Sensitabs (AMC).

Another possibility of screening for ESBL is the use of lower MIC breakpoints for ceftazidime and aztreonam. Livermore et al (5) showed that most ESBL producers were resistant to CAZ at 2 µg/ml and AZTRM at 1 µg/ml. The corresponding zones with Neo-Sensitabs using McFarland 0.5 inoculum are 24 mm (CAZ and CTR) and 27 mm (AZT). As a consequence, *Klebsiella* spp, *E. coli* and *Salmonella* spp. showing zones < 24 mm with Ceftazidime, Cefepime and/or Ceftriaxone Neo-Sensitabs and/or ≤ 27 mm with Aztreonam and/or Cefotaxime Neo-Sensitabs, should be suspected of ESBL production. The CLSI has adopted practically all the same MIC breakpoints.

Cefpodoxime 10 µg may also be used in the screening of ESBL. Zones < 17 mm should be suspected of strains with ESBL production (18). Recently, the CLSI changed their Cefpodoxime screening breakpoints for ESBL from ≥ 2 to ≥ 8 µg/ml (18).
In a study comparing several ESBL screening methods, Vercauteren et al. (10) found that the double tablet synergy test using Neo-Sensitabs detected 96.9 % of ESBL producers while the E-test ESBL Screen detected 81.2 %.

De Gheldre et al showed that synergism between ceftazidime and cefepime with clavulanate (Neo-Sensitabs) was very useful to detect ESBL in *Enterobacter aerogenes* from Belgian hospitals (13).

Rodriguez-Villalobos et al. (20) and Fluit et al. (21) showed that the double disk (Neo-Sensitabs) synergy test has a higher sensitivity for the detection of ESBL than all combination disks (Oxoid, E-test).

Florijn et al (16) conclude that the use of ceftazidime, ceftriaxone and amoxycillin+clavulanate as Neo-Sensitabs is a cheap and reliable method for detection of *E. coli*, *Klebsiella* spp. and *P. mirabilis* isolates suspected of carrying ESBL. It performs better in a routine setting than the E-test, which often yields a result that cannot be interpreted.

Pitout et al. (22) recommend the use of cefepime and piperacillin+tazobactam when testing against strains with high level expression of AmpC beta-lactamases (*E. cloacae*, *E. aerogenes*, *C. freundii*, *S. marcescens)*. Synergism between cefepime and tazobactam indicates presence of an ESBL.

Synergism between Ticarcillin + Clavulanate and aztreonam/ceftazidime/cefepime permit the detection of ESBL producing strains of *Ps. aeruginosa* (SFM 2001). These strains show currently no zone around Ceftazidime Neo-Sensitabs (14).

Confirmatory Tests for ESBL (combination disks)

The CLSI (9) recommends the use of Ceftazidime in combination with Clavulanic acid: Ceftazidime+Clavulanate Neo-Sensitabs, as a phenotypic confirmatory test for the presence of ESBL. Perform the antibiogram using Mueller Hinton Agar and McFarland 0.5 inoculum. Test both Ceftazidime+Clavulanate, Cefepime+Clavulanate and Ceftazidime/ Cefepime Neo-Sensitabs.
An increase in zone diameter of ≥ 5 mm for the combination Ceftazidime+Clavulanate or Cefepime+Clavulanate compared to Ceftazidime/ Cefepime alone is confirmatory of the presence of an ESBL.

Steward et al (12) showed that synergism between cefepime and clavulanate (Cefepime + Clavulanate Neo-Sensitabs) is very useful to detect ESBL in *Klebsiella pneumoniae*, differentiating strains producing ESBL (synergy between cefepime and clavulanate) from strains producing Amp C or hyperproducers of beta-lactamase.

Enterobacter, Serratia, *Morganella morganii*, Providencia, *Citrobacter freundii* and *Pseudomonas aeruginosa* produce chromosomally encoded inducible **Amp C** beta-lactamase. High level expression of Amp C may prevent the recognition of ESBL. Cefepime is practically not affected by Amp C and consequently Cefepime Neo-Sensitabs should be included as an ESBL screening agent when testing Enterobacter, Serratia ect. Synergism between Amox-Clav and Cefepime will indicate ESBL production (11,12,13,17,19). Strains with Cefepime zones < 24 mm should be suspected of ESBL production.

Recently Schwaber et al (32) found that the Vitek 2, Advanced Expert System identified the ESBL phenotype in only 62.5 % isolates of *Enterobacter* spp. and erroneously reported cephalosporin susceptibility in 28 %.

Cefepime+Clavulanate (and cefepime) Neo-Sensitabs should be used in the confirmatory tests for ESBL, because they are effective in detecting ESBL in strains of Klebsiella, *E. coli* etc. that may produce Amp C or are hyperproducers of beta-lactamase (31).

ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa* (30). Cloxacillin 500 µg or Boronic acid Diatabs can be used to inhibit AmpC, for example by prediffusing (1 hour) one of these compounds on the agar before inoculation and before adding the antibiotic tablets (Neo-Sensitabs), placed on the same spots.

With *Klebsiella oxytoca*, synergism between Amoxycillin+Clavulanate (AMC) and Aztreonam or Ceftriaxone but not with ceftazidime indicates the presence of hyperproduction of K-1 chromosomal beta-lactamase (but **negative** for ESBL). Strains producing ESBL show synergism between AMC and ceftazidime (Use Ceftazidime+Clavulanate).
The use of cefotaxime, ceftriaxone, cefepime, aztreonam with AMC may result in **false positive** results for ESBL in *Klebsiella oxytoca* (Vitek,41).

The emergence of ESBL in Salmonellae merits concern. They cause frequently neonatal meningitis in many developing countries and are often already resistant to ampicillin and chloramphenicol (7).
Karas et al (8) reports clinical failure due to ESBL, in spite of the organism being susceptible with disk diffusion and MIC test (CTX MIC 0.75 µg/ml). The double disk diffusion test indicated the presence of an ESBL, but the test was first performed when therapy with cefotaxime was stopped, due to treatment failure.
The laboratory report should indicate that ESBL-producing strains may be resistant clinically to all penicillin, cephalosporins and aztreonam (9).

For serious systemic infections, even if the isolate appears susceptible to Amoxycillin+Clavulanate, Ticarcillin+ Clavulanate or Piperacillin+Tazobactam, do not report it as susceptible, because resistant mutants may be selected during therapy.

For Q.C. use *Klebsiella pneumoniae* ATCC 700603: zone of Ceftazidime+Clavulanate and Cefepime+Clavulanate is ≥ 5 mm larger than Ceftazidime/Cefepime Neo-Sensitabs (see document **1.1.1**)

Detection of ESBLs using Neo-Sensitabs™

ENTEROBACTERIACEAE

At present there are no CLSI guidelines available for detection of ESBL in other species than E.coli, K.pneumoniae, P.mirabilis and Salmonella spp.

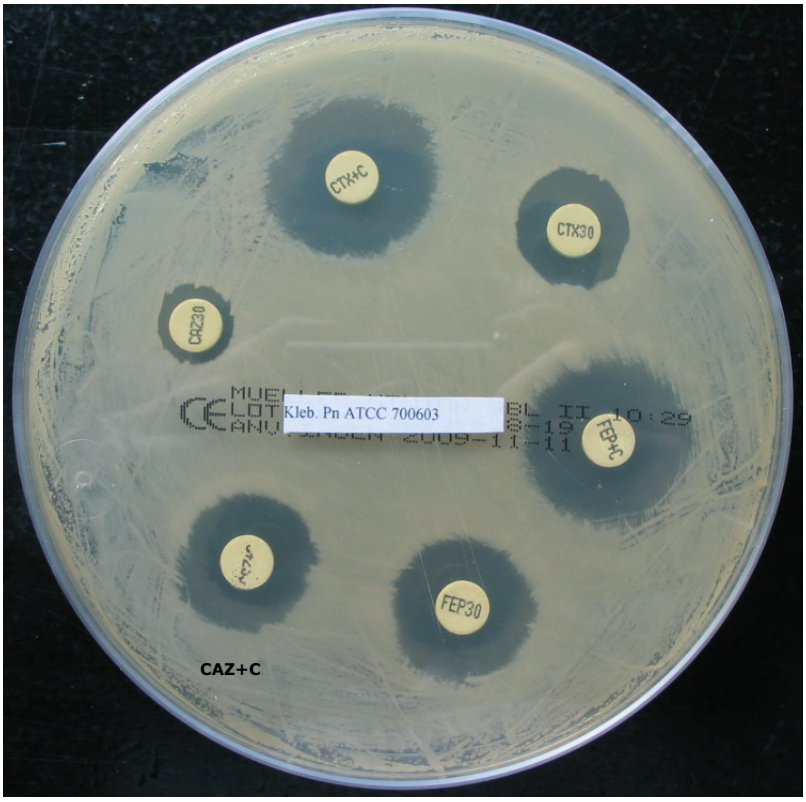
ESBLs are also found in other Enterobacteriaceae such as Enterobacter spp. etc (22)

Strains showing cefotaxime and/or ceftazidime MICs ≥ 1 µg/ml, showing reduced susceptibility to amoxicillin + clavulanate should be tested further for the presence of ESBLs.

Procedure 1 (Double disk synergy test)

Mueller Hinton agar plates are inoculated with the strain to be tested and Neo-Sensitabs applied onto the agar. Cefotaxime, Ceftazidime and Cefepime Neo-Sensitabs at a distance of 15-20 mm (edge to edge) from Amoxycillin + Clavulanate Neo-Sensitabs.

Procedure 2 (Combination disks synergy test). ESBL Confirm ID kit



K. pneumoniae ATCC 700603, ESBL positive

Use Cefotaxime, Ceftazidime and Cefepime and their combinations with Clavulanate (ESBL Confirm ID kit).

Interpretation

A key hole or ghost zone between Amoxycillin + Clavulanate and any of Cefotaxime, Ceftazidime or Cefepime Neo-Sensitabs indicates the presence of an ESBL.
When using the combination disks, a ≥ 5 mm larger zone for any of the combinations compared to the corresponding single antimicrobial indicates the presence of an ESBL.

Cefpodoxime and Cefpodoxime + Clavulanate may be used for screening purposes.
Klebsiella oxytoca hyperproducing K-1 beta-lactamase may show a false positive result (potentiation of cofotaxime and /or cefepime). Only when the strain is resistant to ceftazidime and shows synergism between ceftazidime and clavulanate should it be reported as ESBL positive.

ESBL + AmpC beta-lactamases

Current susceptibility tests are not accurate enough for surveillance of ESBL and AmpC producing pathogens. Laboratories testing for ESBL and AmpC need to be aware that some of these organisms may test false positive for ESBL, using CLSI methodology (40).

Combined disk test (ESBL+AmpC). ESBL+AmpC Screen kit

Apply one of each: Cefotaxime (CTX30), Cefotaxime+Clavulanate (CTX+C), Cefotaxime+Cloxacillin (CTXCX) and Cefotaxime+Clavulanate+Cloxacillin (CTXCC) Neo-Sensitabs on a MH plate inoculated (McFarland 0.5) with the strain to be tested.

Interpretation

		Cefotaxime CTX30	Cefotaxime+Clav. CTX+C	Cefotaxime+Cloxa. CTXCX
ESBL	CTX+C or CTXCC	≥ 5 mm -	- <4 mm	- ≥ 5 mm
AmpC	CTXCX or CTXCC	≥ 5 mm -	- ≥ 5 mm	- <4 mm
ESBL+AmpC	CTX+C and CTXCC	<4 mm -	- ≥ 5 mm	- ≥ 5 mm

In strains possessing both chromosomal (Enterobacter, Cit. Freundii etc. (44) or plasmidic AmpC beta lactamases and ESBLs, Boronic acid or Cloxacillin are used as inhibitors of the AmpC beta lactamase.

Combined disk test (KPC+ESBL)

For detection of ESBLs in clinical isolates of KPC carbapenemase-possessing Enterobacteriaceae, the following method is used (43):

Inoculate (McF 0.5) the MH agar plate with the strain to be tested and add:

1 Cefotaxime+Boronic acid and 1 Cefotaxime+Clavulanate+Boronic acid, 1 Ceftazidime+Boronic acid and 1 Ceftazidime+Clavulanate+Boronic acid.

Interpretation

Cefotaxime+Boronic+Clav. zone ≥ 5 mm than Cefotaxime+Boronic and/or Ceftazidime+Boronic+Clav. zone ≥ 5 mm than Ceftazidime+Boronic indicates the presence of an ESBL.

Detection of ESBLs in different strains

The presence of ESBLs may be masked by the overexpression of AmpC beta-lactamases or by the induction of AmpC beta-lactamase by clavulanate used in synergy tests. ESBLs may be confused with enzymes such as *K. oxytoca* chromosomal ß-lactamase (K1). Laboratory staff must be aware of the increasing array of different resistance mechanisms and phenotypes.

NON-FERMENTERS

Here are particularly *P. aeruginosa* and *A. baumannii* that may possess several types of beta-lactamases. Non-fermenters showing reduced susceptibility to ceftazidime and/or cefepime and/or aztreonam should be tested for the presence of ESBLs.

Procedure

Apply Ceftazidime, Cefepime and Aztreonam Neo-Sensitabs. At a distance of approx. 15 mm (edge to edge) apply Ticarcillin + Clavulanate Neo-Sensitabs. Separately apply Ceftazidime + Clavulanate and Cefepime + Clavulanate Neo-Sensitabs.

Interpretation

A key-hole zone or ghost zone between Ticarcillin + Clavulanate and any of Ceftazidime, Cefepime or Aztreonam Neo-Sensitabs indicates the presence of an ESBL.
With the combination disks a ≥ 5 mm larger zone for Ceftazidime + Clavulanate and/or Cefepime + Clavulanate compared to the single antimicrobials indicates the presence of an ESBL.
The prediffusion procedure with Boronic acid may also be used (30) when ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa*.

1) E. coli, Klebsiella spp., Salmonella spp., Proteus mirabilis, Shigella sonnei:

ESBL	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Cefoxitin S.
VEB-1 (ESBL) (25)	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Synergism Imipenem and Ceftazidime and/or Cefoxitin and Ceftazidime.
AmpC plasmid (no ESBL)	No synergism Ceftazidime+Clavulanate and Cefepime+Clavulanate. Cefoxitin R, Ceftazidime R. Synergism Cefotaxime/Ceftazidime and Cloxacillin and Cefotaxime or Cefotaxime and Boronic acid.
DHA (Induc. plasmid AmpC)	Antagonism Clavulanate (AMC) and 3rd generation. Synergism Cefotaxime/Ceftazidime and Cloxacillin or Boronic acid.
DHA+ ESBL (33)	Antagonism Clavulanate (AMC) and 3rd gen. cephalosporins (DHA). Synergism Tazobactam (Piperacillin+Tazobactam) and Ceftazidime/Cefepime.
Amp C + ESBL	Synergism Cefepime+Clavulanate: ESBL (31).Cefoxitin R, Ceftazidime R. Synergism Cefotaxime or Ceftazidime and Cloxacillin or Boronic acid: AmpC.
DHA + ESBL	Synergism Ceftazidime + Clavulanate and/or Cefepime + Clavulanate: ESBL. Antagonism Clavulanate (AMC) and 3rd gen. cephalosporins: DHA/ACT-1.
ESBL + Metallo-beta-lactamases (24,28)	Synergism Aztreonam + Clavulanate (Amoxicillin+Clavulanate): ESBL. Synergism Imipenem+EDTA: metallo-beta-lactamases. Synergism Meropenem and DPA: metallo-beta-lactamases.

ESBL + 16S rRNA methylases (26)	Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Cefoxitin S. No zone with Amikacin, Gentamicin, Tobramycin Neo-Sensitabs.
ESBL+carbapenemases (not MBL)	Synergism Aztreonam +Clav. (Amox+Clav) Ertapenem I/R, metallo-β-lactamases neg. Positive Hodge test.

2) High level K-1 (*Klebsiella oxytoca*):

(no ESBL)	No synergism Ceftazidime+Clavulanate. Ceftazidime S. Synergy is currently observed with third gen. cephalosporins and Clavulanate as well as with Cefepime+Clavulanate.
K-1 + ESBL (23)	Synergism Ceftazidime+Clavulanate. Ceftazidime I/R.

3a) Enterobacter spp., Serratia spp., Providencia rettgeri, Citrobacter freundii:

ESBL	Synergism Cefepime and Clavulanate (Amoxycyllin+Clavulanate) and/or Ceftazidime and Clavulanate.
ESBL + 16S rRNA methylases	Synergism Cefepime and Clavulanate and/or Ceftazidime +Clavulanate. No zone with Amicacin, Gentamicin, Tobramycin Neo-Sensitabs.
ESBL + Metallo-beta-lactamases	Synergism Aztreonam (or Cefepime)+Clavulanate: ESBL Synergism Imipenem + EDTA and/or Meropenem + DPA: Metallo-beta-lactamases.

3b) Morganella morganii

ESBL	Synergism Cefepime and Tazobactam (Piperazillin+Tazobactam). Synergism Sulbactam (Ampicillin+Sulbactam) and Ceftazidime or Cefotaxime.

4) Pseudomonas aeruginosa:

ESBL	Ceftazidime S or R. Ticarcillin resistant. Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate. Synergism Aztreonam/Ceftazidime or Cefepime with Ticarcillin+Clavulanate
ESBL (PER-1)	Piperacillin S, Ceftazidime R. Synergy as above.
VEB-1 (ESBL)	Synergism between Imipenem and Ceftazidime (or Cefepime) in the presence of Cloxacillin. Synergism Ceftazidime+Clavulanate and/or Cefepime+Clavulanate.
OXA-18 (ESBL)	Ceftazidime R, Ticarcillin R, Aztreonam R, Meropenem I. Synergism Ceftazidime/Cefepime with Ticarcillin+Clavulanate.
ESBL + Metallo-beta-	Synergism Aztreonam (or Cefepime)+Clavulanate: ESBL lactamases Synergism Imipenem + EDTA and/or Meropenem + DPA: Metallo-beta-lactamases.

5) Acinetobacter spp. Use Double Disk Synergy Test

ESBL	Synergism Ceftazidime/Cefepime and Amoxycillin/Clavulanate. Synergism Ceftazidime/Cefepime and Ticarcillin+Clavulanate
PER-1 (ESBL)	Cephalosporins R, Aminoglycosides R. Synergy TIC+Clav, Synergy PIP+TAZO.
VEB-1 (ESBL)	Synergism Cefepime and Ticarcillin+Clavulanate (distance 15 mm). Best at 30 °C in the presence of Cloxacillin or Boronic acid.

Beceiro et al (37) have shown that the double disk synergy test gives the best results with Acinetobacter spp. due to Acinetobacter's intrinsic susceptibility to clavulanic acid.

6) Achromobacter xylosoxidans

VEB-1 (ESBL)	Synergy between Ceftazidime and Clavulanate.

7) Haemophilus influenzae:

ESBL (27)	Compare Cefpodoxime and Cefpodoxime+Clavulanate. Zones: ≥ 5 mm larger with the combination.

Note:

Although the lower cephalosporins MIC breakpoints of EUCAST and those forcoming from CLSI are better able to separate ESBL pos/neg populations of E.coli and Klebsiella spp, significant numbers of ESBLpositive isolates remain below these breakpoints. Confirmation testing of ESBL is necessary (42).

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