Phenotypic detection of extended-spectrum β-lactamase production in Enterobacteriaceae: review and bench guide

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ABSTRACT

Strains of Enterobacteriaceae producing an extended spectrum β-lactamase have become a concern in medical bacteriology as regards both antimicrobial treatment and infection control in hospitals. Extended-spectrum β-lactamase (ESBL) detection tests should accurately discriminate between bacteria producing these enzymes and those with other mechanisms of resistance to β-lactams, e.g., broad-spectrum β-lactamases, inhibitor-resistant β-lactamases and cephalosporinase overproduction. Several phenotypic detection tests, based on the synergy between a third-generation cephalosporin and clavulanate, have been designed: the double-disk synergy test (DDST), ESBL Etests, and the combination disk method. These tests often need to be refined in order for them to detect an ESBL in some bacterial strains, such as those that also overproduce a cephalosporinase. The sensitivity of the DDST can be improved by reducing the distance between the disks of cephalosporins and clavulanate. The use of cefepime, a fourth-generation cephalosporin that is less rapidly inactivated by cephalosporinase than by ESBL, improves the detection of synergy with clavulanate when there is simultaneous stable hyperproduction of a cephalosporinase; alternatively, the cephalosporinase can be inactivated by performing phenotypic tests on a cloxacillin-containing agar. Some β-lactamases can hydrolyse both third-generation cephalosporins and carbapenems, such as the metallo-β-lactamases, which are not inhibited by clavulanate, but rather by EDTA. The production of an ESBL masked by a metallo-β-lactamase can be detected by means of double inhibition by EDTA and clavulanate. Since extended-spectrum Ambler class D oxacillinases are weakly inhibited by clavulanate and not inhibited by EDTA, their detection is difficult in the routine laboratory.

Keywords β-Lactamase, Enterobacteriaceae, extended-spectrum β-lactamases, phenotypic detection, review

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INTRODUCTION

Since the first reports of strains of *Klebsiella* spp. resistant to third-generation cephalosporins [1] and the first descriptions of the mechanism of resistance involved [2–5], the epidemiological success of Enterobacteriaceae producing extended-spectrum β-lactamases (ESBLs) has become a concern in the field of medical bacteriology [6–9].

Because of the risk of treatment failure with third-generation cephalosporins or with aztreonam [10–12], it is often recommended to report ESBL-producing Enterobacteriaceae as resistant to these antibiotics, even when the strains appear susceptible according to standard breakpoints (http://www.sfm.asso.fr/). Moreover, the detection of ESBL-producing strains may prompt the implementation of isolation procedures to prevent cross-transmission to other patients. The ESBL phenotypic detection tests, therefore, have to accurately discriminate between ESBL-producing strains and those having other acquired mechanisms of resistance to β-lactam antibiotics. Among Enterobacteriaceae, the three main non-ESBL resistance patterns are those caused by: (a) broad-spectrum β-lactamases (TEM-1, TEM-2, SHV-1, etc.), which confer high-level resistance to amino- and carboxy-penicillins and are characterised by a marked synergy between these

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antibiotics and β-lactamase inhibitors such as clavulanate or sulbactam; (b) inhibitor-resistant β-lactamases (TEM derivatives), which confer resistance to amino- and carboxy-penicillins, and are characterised by an absence of synergy between these antibiotics and inhibitors, resulting in resistance to the combinations amoxicillin–clavulanate, ampicillin–sulbactam and ticarcillin–clavulanate; and (c) cephalosporinase overproduction, which confers resistance to amino- and carboxy-penicillins, as well as to second- and third-generation cephalosporins and aztreonam, and is characterised by a lack of synergy between these antibiotics and inhibitors.

The main characteristics of ESBL-mediated resistance in Enterobacteriaceae are: (a) the resistance to amino- and carboxy-penicillins, as well as to second-generation and one or several third- and fourth-generation cephalosporins or aztreonam; and (b) a synergy between these antibiotics and β-lactamase inhibitors, particularly clavulanate. Resistance, at breakpoint, is not always obvious to all third- or fourth-generation cephalosporins, whether based on disk-diffusion in agar, MIC determination or automated systems [13]. Since the 1980s, specific phenotypic tests have therefore been developed to detect ESBL production. All are based on the use of a third-generation cephalosporin, usually cefotaxime or ceftazidime, and a β-lactamase inhibitor, usually clavulanate, and rely on the reduction of the MIC of the former in the presence of the latter, demonstrating synergy.

This review describes the phenotypic methods for ESBL detection, illustrates the results obtained at the bench and analyses their advantages and limits.

DESCRIPTION OF THE ESBL DETECTION TESTS

Double-disk synergy test

The first test specifically designed to detect ESBL production in Enterobacteriaceae was the double-disk synergy test (DDST) [7]. It was initially designed to differentiate between cefotaxime-resistant strains, i.e., those overproducing cephalosporinase, and those producing ESBLs. The test is performed on agar with a 30-μg disk of cefotaxime (and/or ceftriaxone and/or ceftazidime and/or aztreonam) and a disk of amoxicillin–clavulanate (containing 10 μg of clavulanate) positioned at a distance of 30 mm (centre to centre), i.e., at the distance provided by several types of disk-dispenser (Fig. 1). The test is considered as positive when a decreased susceptibility to cefotaxime is combined with a clear-cut enhancement of the inhibition zone of cefotaxime in front of the clavulanate-containing disk, often resulting in a characteristic shape-zone referred to as 'champagne-cork' or 'keyhole'. Figs 2 and 3 give several examples of positive DDSTs for different enzymes and Enterobacteriaceae species. The DDST was first used in epidemiological studies to assess the spread of ESBL-producing Enterobacteriaceae in French hospitals [8,9]. It has been shown to work well with a wide range of Enterobacteriaceae species and ESBL types, and it is generally regarded as a reliable method for the detection of ESBLs, although it is sometimes necessary to adjust the disk spacing. It is important to note that reducing the distance between the clavulanate-containing disk and the third-generation cephalosporin disk (e.g., to 20 mm) significantly improves the test sensitivity [14,15]. Since the antibiotic disks are routinely spaced 30 mm apart by several types of marketed disk-dispenser, it is necessary, when the result of the test is equivocal (i.e., clear decrease in susceptibility to third-generation cephalosporins without clear synergy), to perform an additional test by arranging the disks by hand with narrower distances (Fig. 4).

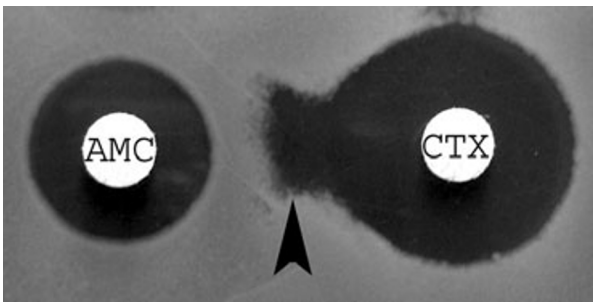

Fig. 1. A positive double-disk synergy test as described in the initial publication [7]. Cefotaxime (CTX) and amoxicillin–clavulanate (AMC) disks are placed at a distance of 30 mm from one another. The inhibition zone is enhanced between those two disks, indicating synergy between cefotaxime and clavulanate.

ESBL Etests

ESBL Etests have been developed in order to quantify the synergy between extended-spectrum

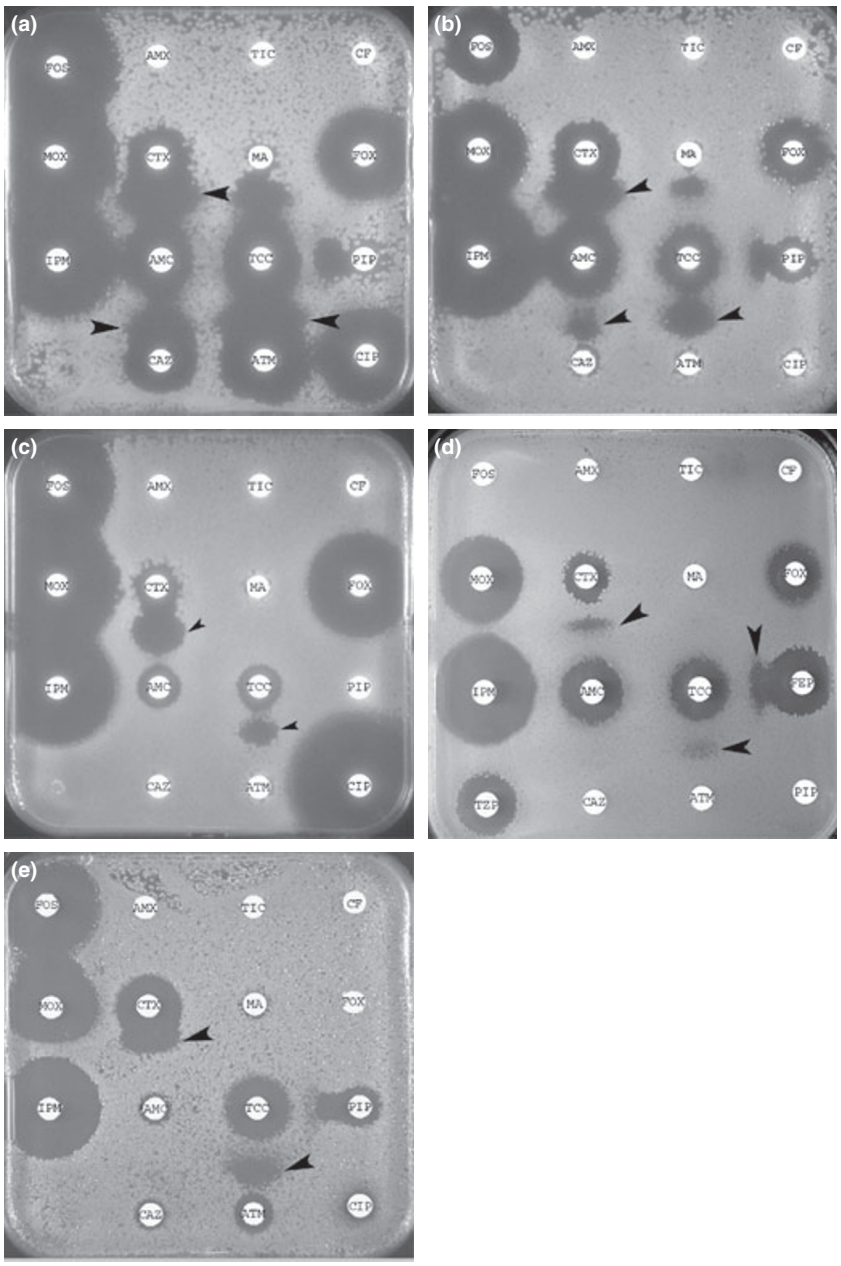

Fig. 2. Double-disk synergy tests for several SHV-derivative extended-spectrum β-lactamases in several Enterobacteriaceae. Synergy between cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM) or cefepime (FEP), and clavulanate (amoxicillin–clavulanate (AMC) or ticarcillin–clavulanate (TCC)), is indicated by arrows. (a) *Escherichia coli* SHV-2. (b) *Klebsiella pneumoniae* SHV-4. (c) *Salmonella* Enteritidis SHV-12. (d) *K. pneumoniae* SHV-12. (e) *Enterobacter cloacae* producing inducible cephalosporinase and SHV-12.

cephalosporins and clavulanate. The Etests called CT/CTL, TZ/TZL and PM/PML are two-sided strips containing gradients of cefotaxime (CT), or ceftazidime (TZ) or cefepime (PM), either alone (at one end of the strip), or combined with clavulanate 4 mg/L (on the other end). The ESBL test is considered as positive when the MIC value of the tested drug is reduced by more than three doubling dilution steps (MIC ratio ≥8) in the presence of clavulanate [16]. The test is also considered as positive when there is either: (a) a rounded zone (phantom zone) just below the lowest concentration of CTL, TZL or PML gradients, or (b) a deformation of the CT, TZ or PM inhibition ellipse at the tapering end. The presence of a phantom zone or an ellipse deformation indicates ESBL production. Interpreting results of the ESBL Etest strips is delicate and requires training. In a recent study, it has been reported that laboratories may fail to interpret correctly the inhibition ellipse in *c.* 30% of cases [17]. In addition, ESBL detection by Etest may fail when

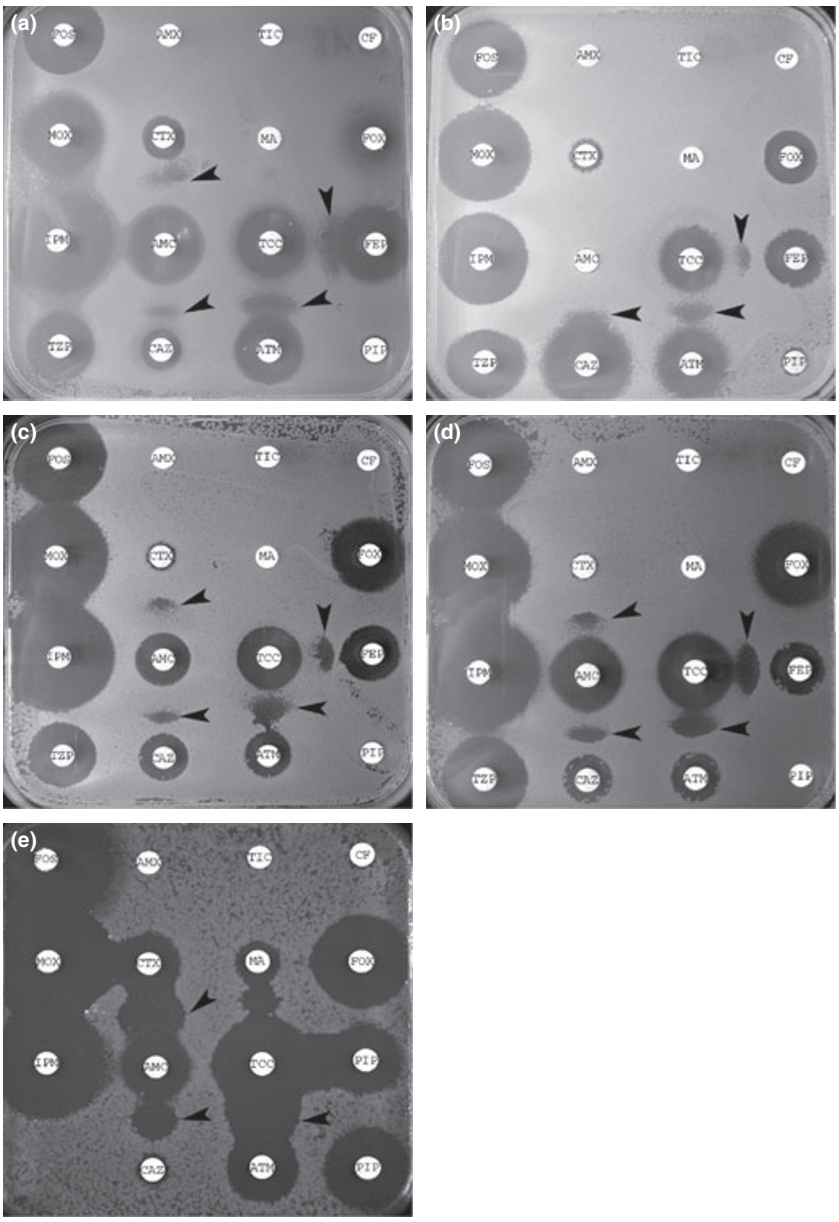

Fig. 3. Double-disk synergy tests for TEM, CTX-M and PER extended-spectrum β-lactamases. Synergy between cefotaxime (CTX), ceftazidime (CAZ), aztreonam (ATM) or cefepime (FEP), and clavulanate (amoxicillin–clavulanate (AMC) or ticarcillin–clavulanate (TCC)), is indicated by arrows. (a) *Citrobacter diversus* TEM-3. (b) *Serratia marcescens* producing inducible cephalosporinase and CTX-M3. (c) *Escherichia coli* CTX-M-15. (d) *E. coli* CTX-M-27. (e) *Proteus mirabilis* PER-1.

the MIC values for cephalosporins fall outside the range of MICs available on the test strip [18], as will be shown below.

Combination disk method

Several manufacturers have developed ESBL detection tests based on the combination disk method. The principle of this method is to measure the inhibition zone around a disk of cephalosporin and around a disk of the same cephalosporin plus clavulanate. Depending on the disk type, a difference of ≥5 mm between the two diameters (i.e., corresponding to a two-fold dilution), or a zone expansion of 50% are considered as indicating ESBL production [19,20]. The test is easy to perform and its interpretation is straightforward. Sensitivity and specificity for this method were first reported to be 96% and 100%, respectively [18]. Carter *et al.* [19] evaluated the performance of the Oxoid cefpodoxime 10 ng ± 1 μg clavulanate combination disks to distinguish ESBL producers from AmpC overproducers and *Klebsiella oxytoca* isolates

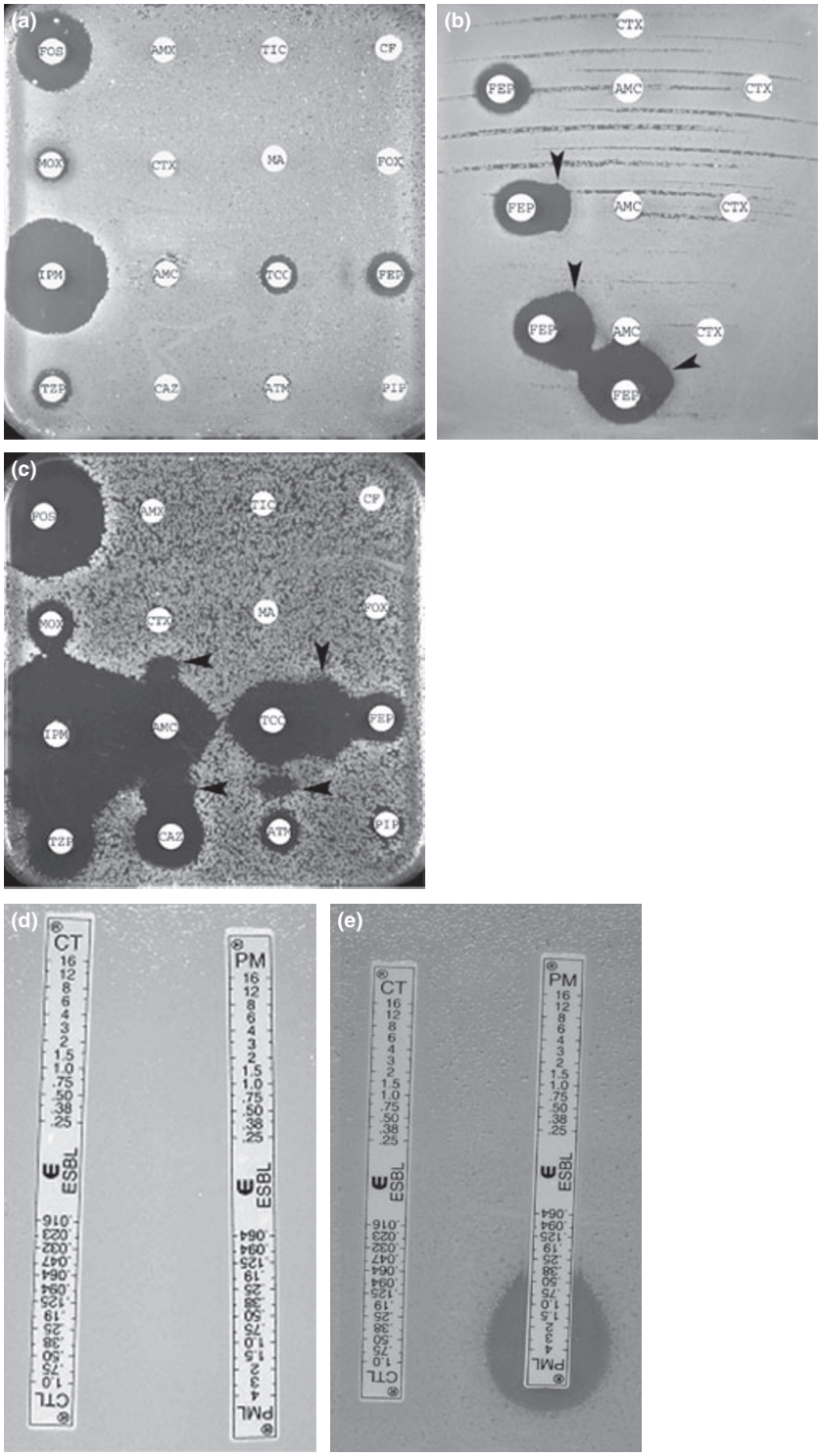

Fig. 4. Detection of extended-spectrum β-lactamase (ESBL) production in an *Enterobacter cloacae* strain that stably overproduces a cephalosporinase, by combining several tests. CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; AMC, amoxicillin–clavulanate; TCC, ticarcillin–clavulanate. (a) Disk diffusion test on conventional Mueller–Hinton (MH) agar. No synergy is detected between cefotaxime (CTX), ceftazidime (CAZ) or cefepime (FEP) and a clavulanate-containing disk (amoxicillin–clavulanate (AMC) or ticarcillin–clavulanate (TCC)). (b) Positive disk synergy test (arrows) using narrowed distances (<30 mm) between the clavulanate-containing disk (amoxicillin–clavulanate) and the disks of third- or fourth-generation cephalosporins (cefotaxime or cefepime). (c) Positive disk synergy test (arrows) on cloxacillin-containing agar (200 mg/L). (d) ESBL Etests on conventional MH agar. CT, cefotaxime; CTL, cefotaxime with clavulanate 4 mg/L; PM, cefepime; PML, cefepime with clavulanate 4 mg/L. All MICs are over range. (e) Positive ESBL Etest on agar containing cloxacillin (200 mg/L). MICs are: CT >16 mg/L, CTL >1 mg/L, PM >16 mg/L, PML 0.5 mg/L.

overexpressing K1 enzyme. The presence of clavulanate enlarged the zone of inhibition by ≥5 mm for all 180 ESBL-producing organisms, and by ≤1 mm for AmpC overproducers and *K. oxytoca* isolates overexpressing K1 enzyme.

Automated method

The VITEK 2 ESBL test (bioMérieux, Marcy l'Etoile, France) is based on the simultaneous assessment of the antibacterial activity of

cefepime, cefotaxime and ceftazidime, measured either alone or in the presence of clavulanate. This test relies on card wells containing 1.0 mg/L of cefepime, or 0.5 mg/L of cefotaxime or ceftazidime, either alone or associated with 10 or 4 mg/L of clavulanate, respectively. After inoculation, cards are introduced into the VITEK 2 machine, and for each antibiotic tested, turbidity is measured at regular intervals. The proportional reduction of growth in wells containing a cephalosporin combined with clavulanate is then compared with that achieved by the cephalosporin alone and is interpreted as ESBL-positive or -negative through a computerised expert system (Advanced Expert System).

The automated Phoenix ESBL test (Becton Dickinson, Sparks, MD, USA) also relies on the growth response to selected expanded-spectrum cephalosporins. This test is composed of five wells, each containing a cephalosporin alone or in combination with clavulanic acid (cefpodoxime, ceftazidime, ceftazidime with clavulanic acid, cefotaxime with clavulanic acid and ceftriaxone with clavulanic acid). In this system, the results are also interpreted through a computerised system.

Many studies have evaluated the ability of automated systems to detect ESBL-producing Enterobacteriaceae ([13]) and some of these studies are summarised below. Sanders *et al.* [21] evaluated the VITEK 2 automated system and the DDST using well-characterised ESBL-producing strains of *Escherichia coli* (n = 176) and *Klebsiella pneumoniae* (n = 157). The performance of both methods was found to be similarly good, with sensitivity and specificity values of 99.5% and 100% for the VITEK vs. 98% and 99.5% for the DDST. More recently, Schwaber *et al.* [22] assessed the performance of the VITEK 2, using 40 ESBL-producing clinical isolates of *Enterobacter* spp. This system identified only 25 of these 40 isolates (62.5%) as ESBL producers, indicating that the method is less efficient for AmpC-inducible species than for *E. coli* and *K. pneumoniae*. Sanguinetti *et al.* [23] evaluated the Phoenix automated test for ESBL detection using 510 clinical isolates of Enterobacteriaceae, including 319 ESBL producers belonging to a large range of species. Among these ESBL producers, 59 belonged to AmpC-inducible species such as *Enterobacter aerogenes*, *Enterobacter cloacae*, *Citrobacter freundii* and *Providencia stuartii*. They reported 100% sensitivity and 98.9% specificity.

An important and recent study by Wiegand *et al.* [24] compared these two automated systems with: (a) a third automated system (Microscan WalkAway-96 System, Dade Behring); (b) the DDST using four cephalosporin disks (cefotaxime, ceftazidime, cefpodoxime and cefpirome); (c) the combination disk method using cefotaxime, ceftazidime and cefpodoxime disks containing or not containing clavulanic acid; and (d) the E-test method. A collection of 144 Enterobacteriaceae isolates (85 ESBL producers) was included in the study. The main results are presented in Table 1. It should be noted that for the Microscan system, the ESBL detection was limited to *E. coli* and *Klebsiella* spp. and that VITEK 2 was used with the conventional cards and not with the ESBL-specific cards containing cephalosporin–clavulanate combinations. The results of this study show that the overall sensitivity was above 90% with the Phoenix system and all agar diffusion methods, and reached 94% or more for the subgroup *E. coli* and *Klebsiella* spp. For the subgroup producing inducible AmpC enzyme (*Enterobacter* spp., *C. freundii* and *Serratia marcescens*), the sensitivity reached 90% or more with Phoenix, VITEK 2 and the DDST. The overall specificity

Table 1. Sensitivity and specificity of phenotypic methods for detection of extended-spectrum β-lactamase (ESBL) production in 144 Enterobacteriaceae (85 ESBL-producing strains) [24]

Species studied	Method	Sensitivity (%)	Specificity (%)
All species (n = 144)	Phoenix	98.8	52.2
	VITEK 2	85.9	78.0
	MicroScan	83.5	72.9
	DDST	94.1	81.4
	CDM	92.9	96.6
	ESBL Etests	94.1	84.7
Escherichia coli, *Klebsiella* spp. (n = 104)	Phoenix	100.0	51.5
	VITEK 2	84.5	93.9
	MicroScan	98.6	51.5
	DDST	94.4	72.7
	CDM	94.4	97.0
	ESBL Etests	98.6	72.7
Enterobacter, *Citrobacter* and *Serratia* spp. (n = 28)	Phoenix	90.0	33.3
	VITEK 2	100.0	38.9
	MicroScan	0.0	ND
	DDST	90.0	100.0
	CDM	80.0	100.0
	ESBL Etests	60.0	100.0

DDST, double-disk synergy test; CDM, combination disk method; ND, not determined.

reached 90% only with the combination disk method, whereas the specificity reached this level with VITEK 2 for the subgroup *E. coli* and *Klebsiella* spp., and with DDST and Etest for the subgroup producing inducible AmpC. It should be noted that for the AmpC-inducible subgroup, the specificity of VITEK 2 and Phoenix was below 40%. Very recently, Thomson *et al.* [25] evaluated VITEK 2 and Phoenix, both with ESBL-specific cards, on 102 well-characterised strains of *E. coli*, *K. pneumoniae* and *K. oxytoca*, including 76 ESBL producers. Sensitivity and specificity for ESBL detection were 96% and 81%, respectively for Phoenix, and 89% and 85%, respectively for VITEK 2.

Screening method

The CLSI recommends a two-step method, the first step being a screening test for reduced susceptibility to more than one of the indicator cephalosporins (cefotaxime, ceftriaxone, ceftazidime, cefpodoxime and aztreonam). Reduced susceptibility indicates a positive result. A subsequent confirmation of ESBL production is then given by the demonstration of synergy between ceftazidime or cefotaxime and clavulanate. The presence of an ESBL is confirmed in *E. coli*, *Proteus mirabilis*, *K. pneumoniae* or *K. oxytoca* if: (a) the MIC values in the presence of clavulanate are reduced by at least three two-fold dilutions; or (b) the diameter of the inhibition zone is increased by at least 5 mm when the tested cephalosporin is combined with clavulanate [26]. The major drawback of the CLSI guideline is the absence of recommendations for the interpretation of ESBL testing results for those species of Enterobacteriaceae that are also good AmpC producers, such as *C. freundii*, *Enterobacter* spp., *Morganella morganii*, *P. stuartii*, and *Serratia* spp.

Three-dimensional tests

Two types of three-dimensional tests, direct or indirect, are proposed by Thomson and Sanders [14]. The direct three-dimensional test is a modification of the disk-diffusion test that generates data on both antimicrobial susceptibility of the tested strain and substrate profile of the β-lactamase produced by this strain. In this test, the studied organism is inoculated onto the surface of an agar plate (inoculum with optical density of 0.5 McFarland) and then dispensed with a higher inoculum (between 10^9 and 10^{10} cells/mL) in a circular slit cut in the agar so that the slit is filled. Afterwards, antibiotic disks are placed on the agar plate, 3 mm outside the strain-containing slit. Enzymic inactivation of each antibiotic is detected by inspection of the margin of the inhibition zone in the vicinity of its intersection with the strain-containing slit. Inactivation of the antibiotic, as it diffuses through the slit, results in a distortion or discontinuity in the expected circular inhibition zone, or the production of discrete colonies in the vicinity of the inoculated slit.

The indirect three-dimensional test is a modification of the direct three-dimensional test, in which the surface of the agar plate is inoculated with a fully susceptible indicator strain (ATCC 25922). With the exception of this modification, the method is the same as that described for the direct three-dimensional test. This test is used for the β-lactams that do not give an inhibition zone by the direct test and for which information on inactivation is therefore not provided. This test provides data on the substrate profile of the β-lactamase produced by the tested strain. Of course, the indirect test does not provide information on susceptibility of the tested strain. Thomson and Sanders compared the combination of the direct and the indirect three-dimensional test and the DDST with 32 strains of *E. coli* and *K. pneumoniae*, 28 of which produced ESBL [14]. They reported detection rates of 93% for the three-dimensional test and 82% for DDST. It has to be noted that no strain overproducing a cephalosporinase was included in this study.

COMBINING SEVERAL ESBL TESTS AT THE BENCH

Adaptation of the DDST to detect an ESBL in strains overproducing cephalosporinase

Several bacterial species (*Enterobacter* spp., *C. freundii*, *M. morganii*, *P. stuartii*, and *S. marcescens*) have inducible chromosomally encoded AmpC cephalosporinase. Either inducibility or stable overproduction of this enzyme, resulting from mutation, can coexist with the production of an acquired ESBL. When the AmpC β-lactamase is inducible, a synergistic effect is detected as easily as in *E. coli* and *Klebsiella* spp., as illustrated

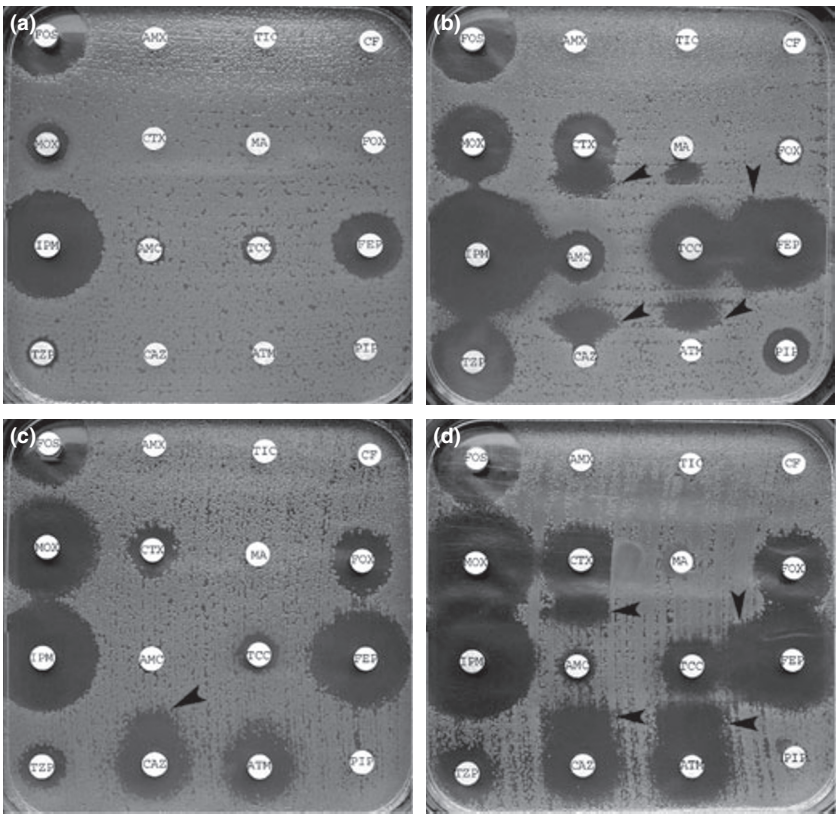

Fig. 5. Extended-spectrum β-lactamase detection by a double-disk diffusion test on agar containing cloxacillin (200 mg/L) for clinical isolates that stably overproduce a cephalosporinase. Synergy between cefotaxime (CTX), ceftazidime (CAZ) or cefepime (FEP) and clavulanate (amoxicillin–clavulanate (AMC) or ticarcillin–clavulanate (TCC)) is indicated by arrows. (a) and (b) *Enterobacter cloacae* with SHV-12 enzyme. (c) and (d) *Serratia marcescens* with TEM-19. (a) and (c) are on conventional Mueller–Hinton agar. (b) and (d) are on cloxacillin-containing agar.

in Fig. 2e (*E. cloacae* SHV-12). In contrast, synergy is often hidden when the AmpC β-lactamase is stably overproduced. As shown in Fig. 4a, no synergy is detected for this clinical strain of *E. cloacae* producing an ESBL and stably overproducing AmpC β-lactamase.

Several modifications of the DDST may facilitate detection when the ESBL phenotype is hidden by a stably overproduced cephalosporinase in this manner:

- Some cephalosporins, e.g., cefepime, are less rapidly inactivated by AmpC cephalosporinases than by ESBLs. In one study, cefepime was used in a DDST approach for the detection of ESBLs in *Enterobacter* spp. strains that also stably overproduce a cephalosporinase. Tzelepi *et al.* [15] reported a sensitivity of 16% only when using cefotaxime, ceftriaxone, ceftazidime and aztreonam disks (positive test with at least one molecule). The use of cefepime increased the sensitivity of the test to 61% when the disk was placed at a standard distance (30 mm) from the clavulanate-containing disk. Sensitivity increased even more, to 90%, when this distance was reduced to 20 mm.
- Performing the DDST on agar containing cloxacillin (200 mg/L) was shown long ago to inhibit cephalosporinase activity [27] and has been shown to enhance the ability of the test to detect ESBL-producing *Acinetobacter baumannii* [28] and *Pseudomonas aeruginosa* [29]. Two examples of ESBL detection on cloxacillin-containing agar are illustrated in Fig. 5; for *E. cloacae* with SHV-12 enzyme in Fig. 5a, b and for *S. marcescens* with TEM-19 in Fig. 5c, d.
- Narrowing the distance between the clavulanate-containing disk and the disks of third-generation cephalosporins to <30 mm can also enhance the detection of ESBLs in AmpC-expressing species [30], as previously mentioned.

Combining several tests for ESBL detection in strains resistant to third-generation cephalosporins through cephalosporinase stable overproduction.

Fig. 4 illustrates the different approaches that can be applied at the bench to detect ESBL production in a strain of *E. cloacae* that stably overproduces

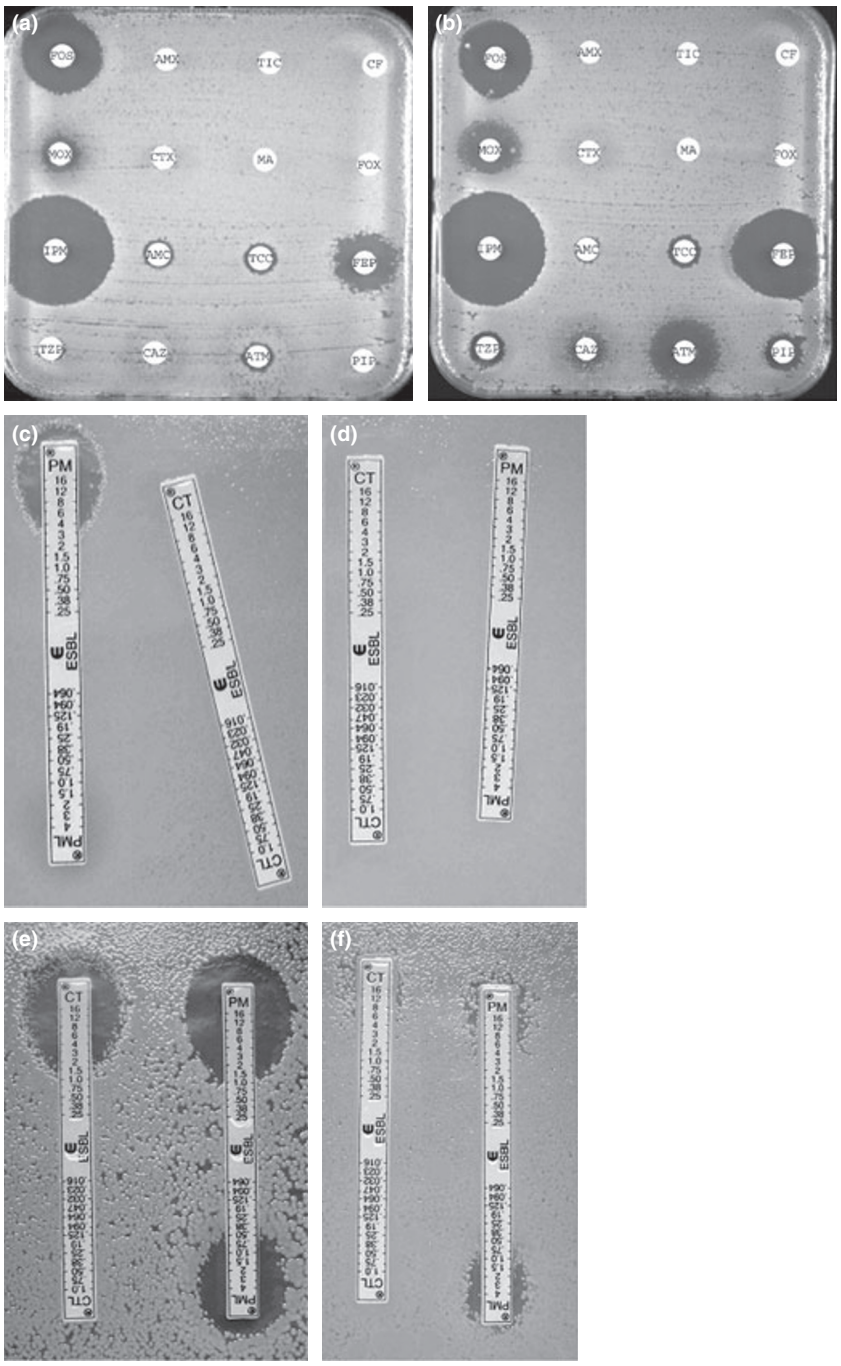

Fig. 6. Combination of several tests for extended-spectrum β-lactamase (ESBL) detection in two *Enterobacter cloacae* strains: one (EC1) stably overproducing a cephalosporinase, the other (EC2) stably overproducing a cephalosporinase and producing an ESBL. (a) Conventional disk test with EC1; no synergy is detected. (b) Conventional disk test with EC2; no synergy is detected. (c) ESBL Etests on Mueller–Hinton (MH) agar with EC1; CT, cefotaxime; CTL, cefotaxime with clavulanate 4 mg/L; PM, cefepime; PML, cefepime with clavulanate 4 mg/L. MICs are: CT >16 mg/L, CTL >1 mg/L, PM 4 mg/L, PML >4 mg/L. (d) ESBL Etests on MH agar with EC2. All MICs are over range. (e) ESBL Etests on agar containing cloxacillin (200 mg/L) with EC1. MICs are: CT 1 mg/L, CTL >1 mg/L, PM 1 mg/L, PML 0.75 mg/L. (f) ESBL Etests on agar containing cloxacillin (200 mg/L) with EC2. MICs are: CT >16 mg/L, CTL >1 mg/L, PM 12 mg/L, PML 1 mg/L.

AmpC β-lactamase. The standard diffusion test shown in Fig. 4a does not provide evidence of ESBL production (no synergy between clavulanate and third-generation cephalosporins or even cefepime). In contrast, synergy is unambiguously detected when the distance between the cefepime and clavulanate disks is reduced (<30 mm) (Fig. 4b) or when the test is performed on cloxacillin-containing plates (Fig. 4c). These examples clearly illustrate the need to modify the standard DDST method when dealing with cephalosporinase overproducers. Combinations of these methods can further improve the detection of ESBLs. For ESBL Etests, the use of cloxacillin-containing media is recommended when the MIC values are higher than those measurable on the strips. This is

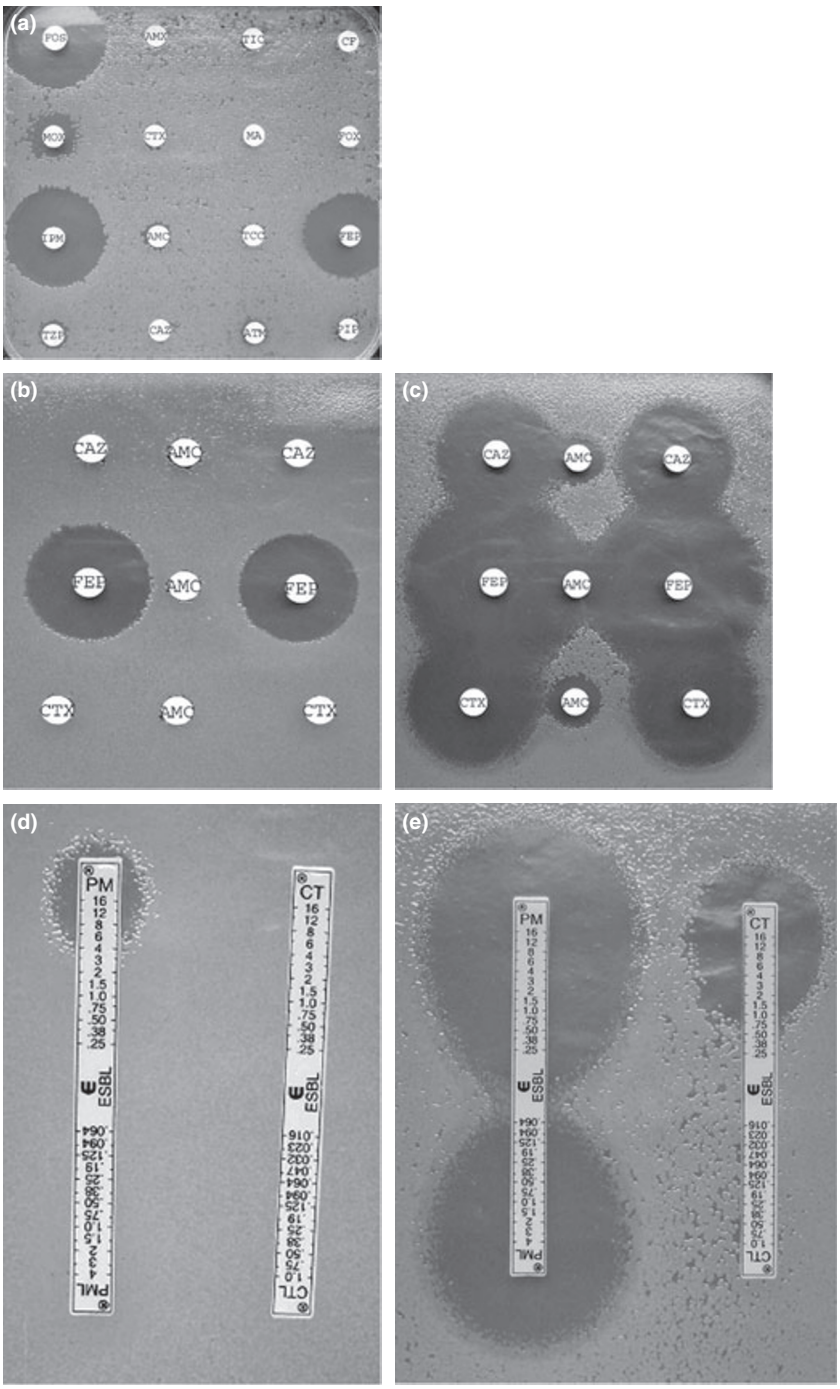

Fig. 7. Combination of several tests for extended-spectrum β-lactamase (ESBL) detection with a non-ESBL-producing *Citrobacter freundii* strain resistant to third-generation cephalosporins through stable AmpC overproduction. (a) Negative double-disk synergy test (DDST) on Mueller–Hinton (MH) agar. (b) Negative DDST despite using narrowed distances (<30 mm) on MH agar. (c) Negative DDST despite using narrowed distances on cloxacillin-containing agar (200 mg/L). (d) Negative ESBL Etests with cefotaxime (CT/CTL, right strip) or cefepime (PM/PML, left strip) on conventional MH agar. (e) Negative ESBL Etests with cefotaxime (CT/CTL, right strip) or cefepime (PM/PML, left strip) despite using cloxacillin-containing agar.

illustrated in Fig. 4d, where the ESBL Etests on Mueller–Hinton agar indicated out-of-range MIC values, whereas those on cloxacillin-containing agar (Fig. 4e) were in range, with a further significant reduction in the presence of clavulanate (ratio MIC cefepime/MIC cefepime–clavulanate ≥8).

Another example of using cloxacillin-containing agar is given in Fig. 6, showing two *E. cloacae* strains isolated from the same sample, both of which stably overproduce a cephalosporinase, but only one of which also produces an ESBL. No clear synergy was detected for either strain in the conventional DDST (Fig. 6a, b). For the

ESBL-producing strain, MICs for cephalosporins fell outside the range of the ESBL Etests for both CT/CTL and PM/PML (Fig. 6d). In contrast, the PM/PML test is positive (MIC ratios ≥8) on cloxacillin-containing agar (Fig. 6f). For the ESBL-non-producing strain, ESBL Etests were not interpretable for either PM/PML or CT/CTL (Fig. 6c). Both tests were negative on cloxacillin-containing agar (Fig. 6e).

The DDST remains clearly negative, despite any modification, for non-ESBL strains overproducing cephalosporinase, as illustrated in Fig. 7 for a non-ESBL-producing *C. freundii* isolate resistant to third-generation cephalosporins through stable overproduction of its cephalosporinase. As shown in Fig. 7a, the disk test does not show any clavulanate synergy even when performed with narrowed disk spacing (Fig. 7b), or on cloxacillin-containing agar (Fig. 7c). Similar results (i.e., no synergy) were obtained with ESBL Etests performed on standard and cloxacillin-containing media (Fig. 7d, e). Another example of a negative DDST for a *K. pneumoniae* clinical isolate producing the plasmid-encoded class C cephalosporinase DHA-1 is given in Fig. 8. No synergy is detected, either with the DDST on conventional Mueller–Hinton agar (Fig. 8a), or with narrowed distances between the disks (Fig. 8b). Rather, the inhibition zones around the cefotaxime or ceftazidime disks are flattened on the side facing the clavulanate-containing disk, when the distances between the disks are shortened, indicating an antagonism related to the induction of the DHA class C cephalosporinase by clavulanate, as previously reported [31].

Adaptation of ESBL tests when the enzyme is not inhibited, or poorly inhibited, by clavulanate

In some cases, strains are resistant to third-generation cephalosporins through the production of metallo-β-lactamases (MBLs). These Ambler class B enzymes (IMP, VIM, GIM and SPM-1) have been isolated from *Pseudomonas* spp., *Acinetobacter* spp. and Enterobacteriaceae strains. They generally hydrolyse third-generation cephalosporins as well as carbapenems, but not aztreonam. The level of carbapenem resistance caused by MBLs is generally higher in *Pseudomonas* spp. than in Enterobacteriaceae, which often appear sensitive to imipenem. Walsh *et al.* [32] proposed an identification procedure for MBLs based on the analysis of MICs. *P. aeruginosa*, *Acinetobacter* spp. and Enterobacteriaceae may be considered as potential MBL producers when the imipenem MIC reaches at least 16, 8 and 2 mg/L, respectively. For a routine approach in clinical laboratories, Walsh *et al*. then recommended use of the Etest MBL, a two-sided strip containing gradients of imipenem alone on one side, and combined with EDTA on the other side. This test is considered as positive when the MIC of imipenem is reduced by at least three doubling dilutions in the presence of EDTA [33]. However, because false-negative results can arise for the

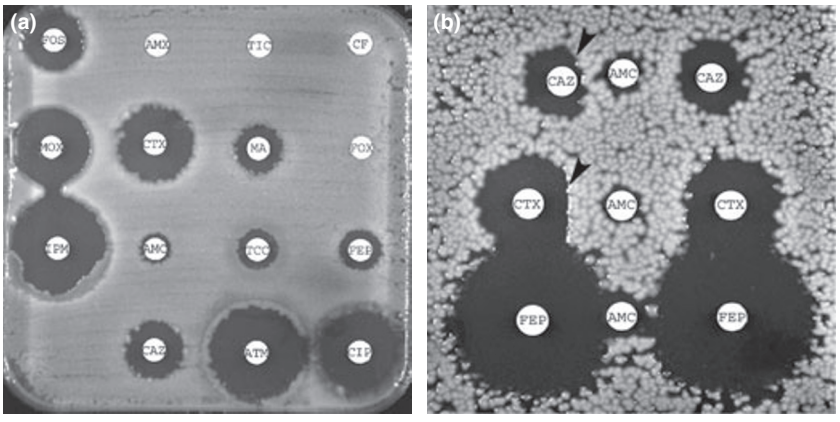

Fig. 8. Combination of several tests for extended-spectrum β-lactamase (ESBL) detection in a *Klebsiella pneumoniae* strain resistant to third-generation cephalosporins owing to production of an inducible DHA-1 class C β-lactamase. (a) Negative double-disk synergy test (DDST) on Mueller–Hinton (MH) agar. (b) Negative DDST despite using narrowed distances (<30 mm) on conventional MH agar. Arrows indicate flattening of inhibition zones, indicating antagonism attributable to the induction of the DHA-1 β-lactamase by clavulanate.

Etest MBL with Enterobacteriaceae, a disk-diffusion test based on the detection of a synergy between a substrate-containing disk (imipenem, ceftazidime or meropenem) and a disk containing an MBL inhibitor (EDTA or mercaptopropionic acid) is recommended in addition [34,35]. MBLs may be co-produced with ESBLs, thus leading to difficulties in identifying the production of each enzyme. In a recent study, Kassis-Chikhani *et al.* [36] reported the detection of a multidrug-resistant *K. pneumoniae* strain producing both the VIM-1 MBL and the SHV-5 ESBL in a French hospital (Fig. 9). The MBL was detected with the EDTA disk-diffusion test when EDTA (4 μL of a 0.5 M solution at pH 8) was added to the imipenem disk (Fig. 9b) as well as with the MBL Etest (Fig. 9c). However, production of MBL enzyme alone could not explain the high level of resistance to aztreonam observed for this strain (Fig. 9a). The addition of EDTA to a clavulanate-containing disk made it possible to demonstrate the production of ESBL by showing synergy between EDTA-clavulanate and a third-generation cephalosporin or cefepime (Fig. 9d). PCR-based screening finally allowed the detection of the class A ESBL.

In addition to the production of class A ESBLs, clinical isolates may be resistant to third-generation cephalosporins through the production of extended-spectrum Ambler class D oxacillinases. Most of these enzymes are point mutants derived

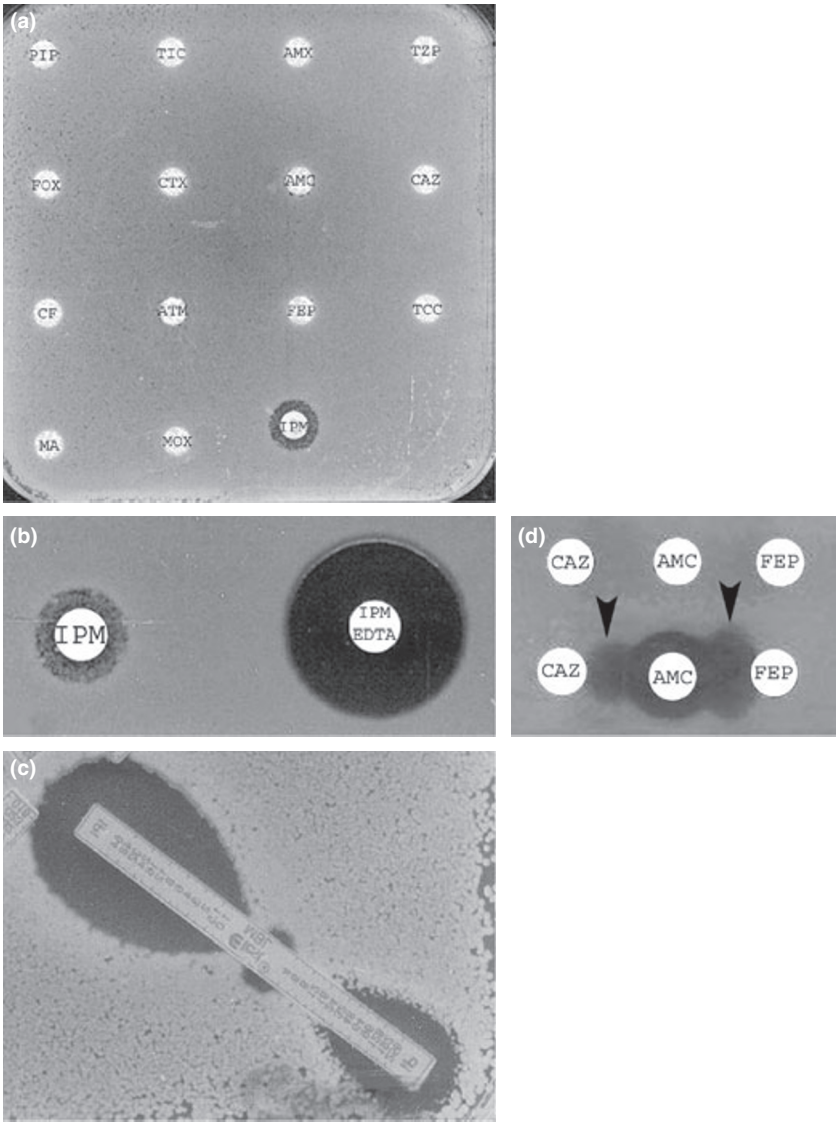

Fig. 9. Detection of an extended-spectrum β-lactamase (ESBL) (SHV-5) in a strain of *Klebsiella pneumoniae* producing the metallo-β-lactamase VIM-1. (a) Negative double-disk synergy test (DDST) on Mueller–Hinton agar. (b) Positive EDTA synergy test showing the production of a metallo-β-lactamase. (c) Positive MBL Etest. (d) Negative conventional DDST (top) and positive modified DDST (bottom), showing synergy between the disks containing ceftazidime or cefepime and a disk containing clavulanate and EDTA (4 μL of a 0.5 M solution buffered at pH 8).

from OXA-2 and OXA-10 β-lactamases. They confer resistance to ceftriaxone, cefepime, cefpirome and, especially, ceftazidime, whereas the cefotaxime MIC is marginally increased [37]. In contrast, OXA-17, which is an OXA-10 derivative, is characterised by an activity against cefotaxime that is significantly higher than that against ceftazidime [38]. Unlike class A ESBLs, OXA-type ESBLs are only weakly inhibited by clavulanic acid, except for OXA-18, and are not inhibited by EDTA. Consequently, most remain almost unidentifiable according to simple inhibition tests [37].

ESBL DETECTION IN THE FUTURE

Despite all the methods developed during the last two decades, the identification of ESBLs by conventional phenotypic methods remains sometimes difficult in practice, and becomes more so as ESBLs are more and more frequently associated with overproduced cephalosporinases, broad-spectrum β-lactamases (TEM-1, TEM-2, SHV-1, SHV-11, etc.) or MBLs. Enzymic mechanisms of resistance, including ESBLs, can also be associated with an outer-membrane permeability defect or an efflux mechanism, complicating further the interpretation of susceptibility data. Consequently, molecular detection and identification of β-lactamases would be essential for a reliable epidemiological investigation of antimicrobial resistance. Molecular detection is usually based on amplification by PCR, followed by sequencing, which relies on the use of specific primers, allowing the amplification of family or group members. Because β-lactamases are characterised by a wide genetic diversity, an exhaustive enzyme characterisation using a PCR approach can be time-consuming, and a microarray approach would be of great help in detecting simultaneously several enzyme-encoding genes from a single strain. Two approaches have already been described: the first for detection at the family level [39], and the second for the identification of the ESBL mutants of TEM-1[40].

CONCLUSION

Because resistance to broad-spectrum β-lactams is linked to various molecular mechanisms involving a wide variety of enzymes, ESBL detection has become a diagnostic challenge in clinical laboratories. In most cases, the standard disk diffusion tests are effective, and they are still recommended for ESBL detection in routine laboratories. Nevertheless, it is often worth combining the standard disk diffusion test with other approaches, such as modified disk tests or Etests, especially for those species of Enterobacteriaceae where AmpC cephalosporinase hyperproduction is likely.

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