Laboratory Tests in the Detection of Extended Spectrum Beta-lactamase Production: National Committee for Clinical Laboratory Standards (NCCLS) Screening Test, the E-Test, the Double Disk Confirmatory Test, and Cefoxitin Susceptibility Testing

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Extended spectrum beta-lactamase (ESBL) production by *Klebsiella* sp. and *E. coli* is an emerging problem. In this study, 107 clinical isolates (53 *E. coli*, 47 *K. pneumoniae* and 7 *K. oxytoca*) screened as ESBL producers by the NCCLS disk diffusion procedure were submitted to a double disk confirmatory test (DDT) and to the E-test double strip for confirmation of ESBL production by demonstration of clavulanic acid inhibition effect (CAIE). Only 72/107 (67%) of the isolates were confirmed as ESBL producers by DDT, with diverse results among species. By the E-test, 58/107 (54%) isolates were confirmed as ESBL producers, and 18/107 (17%) were not determinable. Susceptibility to cefoxitin was found in 57/68 (83%) of strains that did not show CAIE. ESBL detection remains a controversial issue and clinical laboratories are in need of a simple and effective way to recognize strains with this kind of resistance.

Key Words: ESBL, double disk confirmatory test, cefoxitin susceptibility testing, screening test.

The extended spectrum beta-lactamases (ESBLs) enable certain Gram-negative bacteria to inactivate cephalosporins as well as broad-spectrum penicillins and monobactams (aztreonam). The microorganisms that produce ESBLs most frequently are *Klebsiella* sp. and *E. coli*. ESBL production by Gram-negative organisms is an emergent, worldwide problem [1-6] and the presence of these enzymes has an impact on the efficacy of β-lactam therapy [7,8].

ESBLs can present variations in the *in vitro* pattern of resistance to β-lactam agents conferred to the samples that encode them [9]. Some enzymes (TEM-3 and SHV-2) confer high levels of resistance to cephalosporins, whereas others, such as TEM-7 and TEM-12, confer low levels of resistance, which possibly makes it even more difficult to detect them through the susceptibility tests routinely used in microbiology laboratories. Clinical laboratories must accurately recognize ESBL producers to better support therapy. In 1999, the National Committee for Clinical Laboratory Standards issued recommendations for ESBL detection in *E. coli* and *Klebisella* sp. [10]. Recommendations include a screening test, followed by a confirmatory test, both adapted to diffusion and dilution formats. The confirmatory test is based on the demonstration of inhibition by clavulanic acid. However, other mechanisms of β-lactam resistance, including AmpC type enzymes [11,12], porin changes [13-15], and variants of the original ESBL enzymes [16,17], may be present and even coexist with ESBL, interfering in the results of these tests.

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The aim of this study was to evaluate phenotypic tests, the E-test for detection of ESBL, the Double Disk and cefoxitin susceptibility testing for ESBL production in *E. coli* and *Klebsiella* sp.

Material and Methods

Bacterial strains

A total of 107 clinical isolates (53 *Escherichia coli,* 47 *Klebsiella pneumoniae* and 7 *Klebsiella oxytoca*) were included in the study. Isolates were obtained from patients at Complexo Irmandade Santa Casa de Misericórdia, a 1,100 bed, tertiary care, university hospital, from June 99 to December 99, and identified by the Negative Combo 21 Panel (DadeBehring, West Sacramento, Califórnia, USA). All isolates were screened as ESBL producers by the NCCLS disk diffusion procedure [10]. Isolates showed reduced zones of inhibition to at least one of the following antimicrobials: ceftazidime 30µg (CAZ) ≤ 22mm, ceftriaxone 30 mg (CRO) ≤ 25 mm, aztreonam 30 µg (ATM) ≤ 27 mm and cefotaxime 30 µg (CTX) ≤ 27 mm.

Quality control was performed using *E. coli* ATCC 25922, and *Klebsiella pneumoniae* ATCC 700603.

Susceptibility testing

<u>Confirmation of ESBL phenotype</u>. Double Disk Confirmatory Test (DDCT): The recommendations of Jarlier et al., (1988) were followed with modifications, and of NCCLS (2001) using ceftazidime + clavulanic acid (CAZ+CA); cefotaxime + clavulanic acid (CTX+CA).

E-test

The determination of the Minimum Inhibitory Concentration (MIC) was performed through the technique of the E-test (AB Biodisk, Solna, Sweden), on double strips containing ceftazidime (0.5-32 µg/mL) and ceftazidime/ clavulanic acid (0.064-4 µg/mL) in Mueller-Hinton agar. Isolates were considered ESBL producers when clavulanate caused a ≥3 twofold-concentration decrease (ratio ≥8) in the MIC. Additionally, a strain was considered an ESBL producer if a phantom zone or a deformation of the ceftazidime zone could be observed, independent of the ratios or MICs. The outcome of the test was indeterminate when both MICs were outside the test range of the test device. This phenomenon suggests the presence of an inhibitor-resistant TEM or AMPC enzymes. Manufacturer's instructions were followed for performing and interpreting the test.

Susceptibility to cefoxitin

Sixty-eight samples of *E. coli* and *Klebsiella* sp. were used, which were positive in the ESBL screening test yet were negative in the confirmatory test. The samples were submitted to a cefoxitin susceptibility test (30µg), according to Steward et al. [21].

Results and Discussion

Table 1 shows the results of the DDCT using ceftazidime, cefotaxime, and both drugs. Overall, the clavulanic acid inhibition effect (CAIE) was observed in 72/107 (67%) of the isolates originally screened as ESBL producers by disk diffusion. CAIE was more frequently observed among isolates of *K. pneumoniae* (75%), however when *E coli* and *K. oxytoca* were tested 62% and 57% of the isolates, respectively, were confirmed as ESBL producers. Demonstration of CAIE was more evident with CTX-CA. A strategy based exclusively on CAIE by CAZ-CA would have confirmed only 17/53 (32%) of *E. coli*, 24/47 (51%) of *K. pneumoniae*, and 1/7 (14%) of *K. oxytoca* isolates. Results show that confirmation of ESBL production by DDCT varies according to the substrate used (CTX or CAZ), and that the screening procedure presents a variable performance when compared to the DDCT, according to the organism tested. Better results were obtained when *K. pneumoniae* was tested.

Table 1. Results of the Double Disk Confirmatory Test with ceftazidime (CAZ) + clavulanic acid (CA) and cefotaxime (CTX) + clavulanic acid (CA) in isolates of *E. coli, Klebsiella pneumoniae*, and *Klebsiella oxytoca* screened as extended spectrum beta-lactamase producers by disk diffusion

Organism	CAZ + CA			CTX + CA			CAZ + CA or CTZ + CA		
	P (%)	N (%)	T (%)	P (%)	N (%)	T (%)	P (%)	N (%)	T (%)
E. coli	17 (32.1)	36 (67.9)	53 (100)	25 (47.2)	28 (52.8)	53 (100)	33 (62.3)	20 (37.7)	53 (100)
K. pneumoniae	24 (51.1)	23 (48.9)	47 (100)	29 (61.7)	18 (38.3)	47 (100)	35 (74.5)	12 (25.5)	47 (100)
K oxytoca	1 (14.3)	6 (85.7)	7 (100)	4 (57.1)	3 (42.9)	7 (100)	4 (57.1)	3 (42.9)	7 (100)
Total	**42 (39.3)**	**65 (60.7)**	**107 (100)**	**58 (54.2)**	**49 (45.8)**	**107 (100)**	**72 (67.3)**	**35 (32.7)**	**107 (100)**

P = positive; N = negative; T = total.

Table 2. Results of E test strips containg ceftazidime (CAZ) and ceftazidime + clavulanic acid (CAZ+CA) in isolates of *E. coli, Klebsiella pneumoniae*, and *Klebsiella oxytoca* screened as extended spectrum beta-lactamase (ESBL) producers by disk diffusion

	E-test strips containing CAZ and CAZ+CA				
	Pos (%)	Neg (%)	ND[a]	Induction	Total
E. coli	25 (46.9)[b]	15 (28.3)	12 (22.6)	1 (1.89)	53 (100)
K. pneumoniae	31 (65.9)	12 (25.5)	3 (6.38)	1 (2.13)	47 (100)
K. oxytoxa	2 (28.6)[b]	2 (28.6)	3 (42.9)	0	7 (100)
Total	**58 (54.2)**	**29 (27.1)**	**18 (16.8)**	**2 (1.87)**	**107 (100)**

[a] Not determinable, due to off scale results (below the test ranges).
[b] One isolate showing "phantom" inhibition zone, compatible with ESBL production.

Table 3. Results of cefoxitin susceptibility tests applied on isolates that did not show clavulanic acid inhibitory effect by double disk confirmatory test

	Cefoxitin			
	Susceptible	Intermediate	Resistant	Total
Escherichia coli	18	1	1	20
Klebsiella pneumoniae	10	0	2	12
Klebsiella oxytoca	3	0	0	3
Total	**31**	**1**	**3**	**35**

Table 4. Results of cefoxitin susceptibility tests applied on isolates that did not show clavulanic acid inhibitory effect by E test strip containing ceftazidime (CAZ) and ceftazidime + clavulanic acid (CAZ+CA)

	E-test results	Susceptible	Cefoxitin Intermediate	Resistant	Total
E. coli	Negative	13	1	1	15
	ND[a]	11	0	1	12
	Induction	1	0	0	1
Klebsiella pneumoniae	Negative	10	1	1	12
	ND[a]	3	0	0	3
	Induction	0	0	1	1
Klebsiella oxytoca	Negative	2	0	0	2
	ND[a]	3	0	0	3
	Induction	0	0	0	0

[a] ND = not determinable, due to off scale results (below the test ranges).

The quantitative format, using E-test strips containing CAZ and CAZ+CA, had problems in confirming ESBL production (Table 2). The CAIE could be demonstrated in only 58/107 (54%) of the isolates and 18/107 (17%) were not determinable due to off-scale results (all results below the test ranges). This was a limitation of the quantitative format that was present in all species studied, since 23% (12/53), 6% (3/47), and 43% (3/7) of the isolates of *E. coli*, *K. pneumoniae*, and *K. oxytoca*, respectively, were included in this category. Among isolates presenting indeterminable results CAIE was evident in 5/12 *E. coli*, 2/3 *K. pneumoniae*, and 2/3 *K oxytoca*. At the time this study was being performed, E-test strips containing a combination of CTX and CTX+CA were not available to us. Based on the results obtained by DDCT, it is expected that E-test strips with CTX and CTX-CA would contribute to demonstrate CAIE in a higher proportion of isolates. Using strips with CTX and CTX+CA and CAZ and CAZ+CA, the occurrence of indeterminate results using the E-test was observed in 4% of isolates in a recent study [20].

What would be the impact of strains not confirmed by DDCT or E-test strip for clinical laboratories? Overall, due to the presence of a high number of false-positives in the screening procedure, the two steps strategy (screening/confirmatory tests) may result in increases in turnaround time and cost for the laboratory. Tests based on molecular detection of ESBL genes by PCR and isoelectric focusing [21,22] are more conclusive in defining ESBL production. These tests, however, are not available for most clinical laboratories. In an algorithm recently proposed [21], a disk diffusion test using cefoxitin is applied to those isolates of *K. pneumoniae* in which the CAIE is not verified. According to this algorithm, cefoxitin resistant isolates would likely have an alternative resistance mechanism, such as Amp-C-type enzyme and/or porin changes. On the other hand, susceptible isolates would suggest hyperproduction of ESBL. Isolates in which CAIE was not demonstrated by both DDCF and the E-test in our study were submitted to a cefoxitin susceptibility test and most organisms presented results with cefoxitin susceptibility, i.e., compatible with ESBL production (Tables 3 and 4). Our results show that the role of the cefoxitin susceptibility tests applied on strains that do not show the CAIE must be better defined.

Strains of *E. coli* and *Klebsiella* sp., may show resistance to β-lactam antibiotics by a diverse and continuously growing group of mechanisms. Clinical

laboratories are constantly challenged by these strains and remain in need of a simple, straightforward method to detect ESBL production in *E. coli* and *Klebsiella* sp.

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