Screening and confirmatory assays for detection of ESBLs (extended-spectrum ß-lactamases) production by *Klebsiella pneumoniae* isolates

Métodos de triagem e de confirmação para detecção de BLEAs (beta lactamases de espectro ampliados) em isolados de *Klebsiella pneumoniae*

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Abstract

ESBLs (extended-spectrum ß-lactamases) are enzymes produced by *Enterobacteriaceae*, especially *Klebsiella pneumoniae*, which mediate resistance to extended-spectrum ß-lactams and aztreonam. Considering the importance of detecting ESBLs producing strains, in this study, usual screening and confirmatory assays were compared. One hundred and seven strains of *K. pneumoniae*, isolated from patients with urinary tract infection, were screened for ESBLs production according to NCCLS 2005 guidelines, using broth microdilution in the Microscan Walkaway (Dade Behering) and Kirby-Bauer disk diffusion (KBD) test. Fifty-five strains selected as possible ESBL producers (51,4%), were submitted to confirmatory assays: E-test, double disk (DD) approximation and clavulanic acid (CA) association. Production of ESBLs was detected by the five methods used in 38 (69%) strains and 10 (18,2%) were negative only in DD. Seven strains (12,7%) did not present suggestive breakpoints in the Microscan Walkaway and were negative in the DD test, whereas the KBD values for the same strains obtained were indicative of ESBLs production. These results suggest that the combination of KBD breakpoints determination and CA association can be used as an efficient, alternative and less expensive system than the E-test.
Key words: *K .pneumoniae*. Extended-spectrum ß-lactamases (ESBLs). Screening.

Resumo

BLEAs (ß-lactamases de espectro estendido) são enzimas produzidas por várias espécies de *Enterobacteriaceae*, principalmente *Klebsiella pneumoniae*, as quais conferem resistência aos ß-lactâmicos de espectro ampliado e aztreonam. Considerando a importância da detecção de BLEAs, neste estudo foram comparados métodos de triagem e de confirmação. As 107 amostras de *K. pneumoniae*, isoladas de pacientes com infecção urinária foram triadas quanto à produção de BLEAs, segundo normas do NCCLS 2005, utilizando microdiluição em caldo no Microscan Walkaway e teste de disco difusão de Kirby-Bauer (KBD). As 55 cepas selecionadas foram submetidas a ensaios de confirmação como possíveis produtoras de BLEAs (51,4%): E-test, aproximação com discos duplos (DD) e associação com ácido clavulânico (CA). A produção de BLEAs foi detectada pelos cinco métodos em 38 (69%) amostras e 10 amostras (18,2%) foram negativas para o teste DD. Sete amostras (12,7%) não apresentaram valores sugestivos no método automatizado e também foram negativos no teste de DD, mas os valores dos halos no teste de KBD foram indicativos da produção de BLEAs. Estes resultados sugerem que o uso combinado do método KBD e associação com CA podem ser utilizados como um sistema alternativo eficiente e menos dispendioso que o E-test.
Palavras-chave: *K.pneumoniae.* Beta-lactamases de espectro ampliado (BLEAs). Triagem.

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Introduction

Production of extended-spectrum ß-lactamases (ESBLs) is one of the most important mechanisms of resistance to third-generation cephalosporins among *Enterobacteriaceae*, especially *Klebsiella pneumoniae*. Bacteria producing ESBLs, frequently present resistance to others classes of antibiotics, resulting in difficult-to-treat infections (MACKENZIE et al., 2002; STÜRENBURG; MACK, 2003). As outbreaks of nosocomial infections due to strains of *Klebsiella* ESBL producing occur worldwide in intensive care units, the identification of such strains is desirable so that infection control measures can be investigated and applied (YAGI et al., 2000; SILVA et al., 2001; FARREL et al., 2003; TZELEPI et al., 2003; COLODNER et al., 2004; VILLEGAS et al., 2004; HERNÁNDEZ et al., 2005). Risk factors associated with ESBL production include: prolonged hospital or intensive care unit stay, prior hospitalization (BISSON et al., 2002), multiple courses of antimicrobial therapy particularly extended-spectrum cephalosporin (LUCET et al., 1996; LAUTENBACH et al., 2001). Additional risks involve: indwelling devices as central venous and urinary catheters (LUCET et al., 1996; KIM et al., 2002); intubation and assisted mechanical ventilation (LIN et al., 2003) and severe underlying disease as malignancy and heart failure (HO et al., 2002). The degree of resistance to third-generation cephalosporins can be highly variable. While some ESBLs confer frank resistance to expanded-spectrum cephalosporins, many isolates when tested in laboratory may present intermediate resistance or they are susceptible to one or more of these antimicrobials, despite the carriage of ESBL genes. Therefore, considering the increase of ESBL producing strains, the routine clinical microbiology laboratories should choose sensitive and precise detection methods for this purpose (STÜRENBURG; MACK, 2003).

Several screenings for the presence of ESBLs have been suggested: modified breakpoints for standard methods of susceptibility testing by the National Committee for Clinical Laboratory Standards (NCCLS, 2005); double disk approximation test (JARLIER et al., 1988); combined disk method (JACOBY; HAN, 1996; BABIC et al., 2006), and E-test ESBL strips (CORMICAN et al., 1996). Each method presents disadvantages as already described by different authors (CORMICAN et al., 1996; BROWN et al, 2000; M'ZALI et al., 2000).

Therefore, this study attempts to compare the use of breakpoints in broth microdilution (Microscan Walkaway) and the Kirby-Bauer disk diffusion test (KBD), as well as Double disk approximation test (DD), clavulanic acid association test (CA), and E-test strips for detecting ESBL producing among 55 strains of *K. pneumoniae*, selected from 107 strains isolated from patients with urinary tract infection.

Materials and Methods

Bacterial strains

A collection of 107 *K. pneumoniae* strains isolated from urinary tract infection, at a University Hospital in the city of Londrina, were studied. Strains were initially identified with Microscan Walkaway (Dade Behring, Sacramento, USA), followed by confirmation with API 20E (Bio-Merieux, Marcy I'Etoile, France), and stored in brain heart infusion (BHI) broth +20% glicerol at –20^{0}C. Among these isolates, 55 strains were selected by the resistance phenotypes presented, suggesting resistance to ß-lactams and other classes of antimicrobial agents. *K. pneumoniae* ATCC 700603 was used as a positive control, and *Escherichia coli* HB 101 as a negative control.

Detection of ESBL producing strains

1. ESBL screening was performed by

a) Broth microdilution: according to the manufacturer's recommendations commercial dehydrated panels neg/urine combo type 3 (NUC 3) provided by Microscan Walkaway (Dade Behring, Sacramento, USA) was used. Results obtained in

screening breakpoints were considered according to NCCLS, 2005, ESBL as: for cefpodoxime MIC ≥ 8 μg/mL and for ceftazidime, aztreonam and cefotaxime MIC ≥ 2 μg/mL.

b) Kirby-Bauer disk diffusion (KBD) test: by using Mueller-Hinton agar (BBL Microbiology Systems, Cockeysville, USA); diminished zones of inhibition around third-generation ß-lactam disks were considered suggestive of ESBL production. According to NCCLS (2005) the following antibiotics ß-lactams and zone diameters indicative of resistance were used to indicate ESBL production: cefpodoxime ≤ 17 mm (30 μg) (Oxoid, Basingstoke, UK); cefotaxime (30 μg) (Oxoid, Basingstoke, UK) and aztreonam ≤ 27 mm (30 μg) (Bristol-Myers Squibb, São Paulo, Brasil); ceftazidime ≤ 22mm (30 μg) (Oxoid, Basingstoke, UK).

2. Phenotypic confirmatory testing

a) Double-disk (DD) approximation test (JARLIER et al., 1988): briefly, the culture was swabbed onto a Mueller-Hinton Agar (BBL Microbiology Systems, Cockeysville, USA) plate. A susceptibility disk containing amoxacillin (20 μg)/ clavulanate (10 μg) (Oxoid, Basingstoke, UK) was placed in the center of the plate, and disks containing aztreonam (30 μg-Bristol-Myers Squibb, São Paulo, Brasil), ceftazidime (30 μg-Oxoid, Basingstoke, UK), cefotaxime (30 μg-Oxoid, Basingstoke, UK) were placed 25 mm apart from the amoxicillin-clavulanate disc. Enhancement of the inhibition zone of the oxyimino- ß-lactam, resulting from the synergy of the clavulanate in the amoxicillin-clavulanate disk, indicates an ESBL producer strain.

b) Clavulanic acid association test (JACOBY; HAN, 1996): for the combined disk method, disks containing cefpodoxime (30 μg), ceftazidime (30 μg) and cefotaxime (30 μg) all supplied by Oxoid, Basingstoke, UK with and without clavulanic acid (10 μg), were used. The resulting inhibition zones were compared. A test was considered positive when the difference of zone diameters between the ß-lactam disk and disk containing antibiotic associated with clavulanic acid was ≥ 5mm.

c) ESBL E-test strips (AB Biodisk, Solna, Sweden) (CORMICAN et al., 1996): the strips carry two antibiotic gradients: ceftazidime (0,5 a 32 μg/ml), and ceftazidime with clavulanic acid (0,064 to 4 μg/mL). The E-test method was carried out according to manufacturer's instructions. Colonies from 18-24h culture, in Mac Conkey Agar, were emulsified in sterile, normal saline to an inoculum turbidity equivalent to a 0,5 McFarland standard. This suspension was swabbed onto a Mueller-Hinton Agar (BBL Microbiology Systems, Cockeysville, USA) plate and it was allowed to dry for 10-15min at room temperature. An ESBL E-test strip was then applied to each plate with sterile forceps and the plate was incubated at 37^0C for 18h. After incubation, the MICs were read according to the manufacturer's guidelines and the ratio determined. A MIC ratio ≥ 8 is indicative of ESBL production. It was considered gold standard.

Results and Discussion

A key problem in the detection of ESBL producing bacteria is the variable MIC and zone diameters values by disk diffusion testing possibility due to the low-level expression of the enzyme or the inoculum effect. Consequently, isolates may be reported by the laboratory as susceptible to third-generation cephalosporins whereas treatment failure may occur (D'AGATA et al., 1998).

The 107 strains of *K. pneumoniae* here studied were initially tested for antimicrobial susceptibility, according to NCCLS 2005 guidelines, for breakpoints in a broth microdilution method using Microscan Walkaway and KBD test. In broth microdilution, 48 strains yielded positive results suggestive of ESBL producers. Using KBD, performed with the 107 strains, 55 presented inhibition zones for β-lactams suggestive of ESBL producers, including seven (12,7%) strains that did not present breakpoints suggestive of ESBL by broth microdilution test. These

strains showed resistance also to first-generation cephalosporins (cephalotin and cefuroxime), aminoglycosides and quinolones in the same test. Moland et al. (1998) suggest that *Klebsiella* isolates with hidden resistance to extended spectrum cephalosporins and aztreonam, might not be detected if the specific pannel is not used in Microscan Walkaway or the strains produce low amounts of ESBL.

When KBD assay is highly controlled, the diameters of the inhibition zones can be converted to MIC values (MACKENZIE et al., 2002). However, Freitas et al. (2003) suggest that the KB method was useful only as a screening since it provided several false positive results. In this study, results obtained in KBD were better indicators of ESBL production than the breakpoints in automated system of microdilution method.

Table 1. Results obtained with 17 *K. pneumoniae* strains not identified as ESBLs in broth microdilution and DD test.

Strains no	*Broth microdiluition (µg/mL)*				*KBD test (mm)*			*CA association test (mm)*			*E-test ((µg/mL*		*DD test*
	CTX	*CPD*	*ATM*	*CAZ*	*CPD*	*CAZ*	*CTX*	*CPD+CA*	*CAZ+CA*	*CTX+CA*	*CAZ*	*CAZ+CA*	
16*	≤2	≤ 1	≤2	≤2	16	18	20	21	23	25	0,75	0,094	neg
17	>32	2	16	2	13	19	16	18	26	25	1,0	0,094	neg
22*	≤2	≤ 1	≤2	≤2	16	14	20	23	21	25	0,75	0,064	neg
25	4	>2	8	2	16	19	24	25	25	30	0,75	0,064	neg
34	>32	>2	>16	>16	0	0	0	10	19	19	32	0,5	neg
40	4	>2	8	2	15	20	22	23	25	26	1,5	0,094	neg
50*	≤2	≤ 1	≤2	≤2	14	20	25	25	25	30	1,5	0,094	neg
55	>32	>2	>16	>16	17	20	22	29	29	32	0,75	0,064	neg
57*	≤2	≤ 1	≤2	≤2	16	25	20	22	28	28	0,75	0,064	neg
61	4	2	8	8	14	16	21	25	20	31	4,0	0,19	neg
71*	≤2	≤ 1	≤2	≤2	14	20	22	24	25	27	0,75	0,064	neg
76	8	>2	>16	2	16	20	23	28	29	32	2,0	0,19	neg
79	8	>2	>16	2	12	0	0	17	20	21	1,5	0,094	neg
88	16	>2	>1	4	11	20	22	27	28	30	2,0	0,19	neg
89	4	>2	8	2	16	20	22	22	25	28	2,0	0,19	neg
93*	≤2	≤ 1	≤2	≤2	17	20	19	24	25	24	1,0	0,125	neg
105*	≤2	≤ 1	≤2	≤2	15	0	0	23	24	25	0,75	0,094	neg

* strain negative for ESBLs screening in broth microdiluition and DD test; the other ones, negative only in DD test
CTX-cefotaxime, CPD- cefpodoxime, ATM-aztreonam, CAZ-ceftazidime, CA-clavulanic acid

The other strains (38) presented positive results as ESBLs producers in all tests.

Using DD confirmatory test for the 55 strains positive in KBD; negative results were obtained in 17 strains including those seven already negative in broth microdilution (table 1). This means that it was not observed any enhancement of the inhibition zone of the oxyimino- ß-lactam as a result of the synergy of the clavulanate in the amoxicillin-clavulanate disk. However, DD test as proposed by Jarlier et al. (1988), remains a reliable method for ESBL detection and the disadvantage of this method is that the sinergy between the amoxicillin-clavulanate disk and the indicator cephalosporin may be overlooked if the inoculum is too large or the disks are too far from each other. Some authors have proposed a disc distance edge-to-edge of 15mm, as showing greater sensitivity than the 25-30mm apart center to center, frequently used (STÜRENBURG; MACK, 2003; GROVER et al.2006). This could explain the 31% of negative strains, found since it was used 30mm distances. These results are coincident with those obtained by Cormican et al. (1996).

Tests performed with disk association with CA showed 55 (100%) isolates as ESBLs producers. This method has been successfuly used all over the world (M'ZALI et al., 2000; GROVER et al., 2006), although it also presents limitations, like

strains that produce enzymes which are not inhibited by clavulanic acid, will not be distinguished. This method will not detect, as well, ESBLs that are not active against ceftazidime, unless disks containing other β-lactms are included (BROWN et al., 2000).

E-test ESBL has been described as a sensitive and convenient method by some authors (CORMICAN et al., 1996), and it is considered as gold-standard for ESBL detection. In this study, all 55 strains initially screened as positive in KBD were confirmed by this test. However, it requires an extra agar plate to be inoculated, what makes it difficult to incorporate into a routine disk testing system, and it is also comparatively expensive (M'ZALI et al., 2000; MACKENZIE el al., 2002).Recently molecular methods have been described as sensitive and specific, however they are expensive, time consuming and require specialized equipment and expertise (GROVER et al., 2006).

Summarizing our results showed that 100% strains were detected as ESBLs producers by agreement of E-test strips (gold standard), with CA and KBD results; 87 % were detected by broth microdilution (Microscan Walkaway) and 69% by DD. Considering practical reasons and the better results obtained by the use of more than one method, the disk diffusion agar and clavulanic acid association may be suggested for ESBL screening and confirmation in clinical laboratories, as sensitive and less expensive system than E-test.

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