Short Communication

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A rapid and simple method to detect ESBL in *Enterobacter cloacae* based on MIC of cefepime

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ABSTRACT

Introduction: The aim of this study was to identify a rapid and simple phenotypic method for extended-spectrum β-lactamase (ESBL) detection in *Enterobacter cloacae*. **Methods:** A total of 79 consecutive, non-repeated samples of *E. cloacae* were evaluated. Four phenotypic methods were applied for ESBL detection, results were compared to multiplex polymerase chain reaction (PCR) as the gold standard reference method: 1) ceftazidime and cefotaxime disks with and without clavulanate, both with boronic acid added; 2) disk approximation using cefepime and amoxicillin/clavulanate; 3) ESBL screening by minimum inhibitory concentration (MIC) ≥ 16μg/mL and 4) by MIC ≥ 2μg/mL for cefepime. **Results:** Method 4 showed the best combination of sensitivity (100%) and specificity (94%). **Conclusions:** MIC ≥ 2μg/mL for cefepime would be very useful for the phenotypic detection of ESBL in samples of *E. cloacae.*

Keywords: *Enterobacter cloacae*. ESBL. Phenotypic detection.

Enterobacter spp. are important opportunistic pathogens known to cause hospital acquired infections. A major cause of resistance to third-generation cephalosporins by *Enterobacter* spp. is the expression of AmpC-type β-lactamase. In addition, resistance in many clinical samples is due to the presence of conjugative plasmids encoding the gene extended-spectrum β-lactamase (ESBL)[1].

Until the late 1990s, most ESBLs detected belonged to either the Temoniera (TEM) or sulfhydryl variable (SHV) types. More recently, the cefotaximases (CTX-M) type has rapidly spread and has been detected in samples from various countries[2]. The presence and types of ESBLs in Brazil, however, has not been well studied. We previously reported that the frequency of ESBL in *Enterobacter* spp. isolates was higher than was observed in both *Escherichia coli* and *Klebsiella* spp[3].

The standard tests for ESBL detection were developed by the Clinical and Laboratory Standards Institute (CLSI), and are based on the ability of clavulanic acid (CA) to inhibit the enzyme. However, this test is complicated by the presence of AmpC in *Enterobacter* spp. isolates, because CA also causes induction of AmpC[1]. To overcome β-lactamase interference in ESBL detection, the use of AmpC inhibitors has been proposed. However, the proposed methods have proved difficult to implement in routine laboratory testing. The aim of the present study was to identify the best and simplest phenotypic method for ESBL detection in clinical samples of *Enterobacter cloacae.*

This prospective study was conducted over a period of two years (2009-2011) in a 240-bed tertiary care hospital in Brazil. We used a total of 79 consecutive, non-repeated samples of *E. cloacae* recovered from clinical samples, including urine (44%), blood (21%), secretions (19%), and colonization swabs (16%). *E. cloacae* isolates were obtained primarily from patients in a 24-bed adult intensive care unit. All samples, belonging to 55 clones, were typed by the enterobacterial repetitive intergenic consensus-polymerase chain reaction (ERIC-PCR) molecular technique[4]. Isolates were judged to belong to the same genetic clone if the Dice correlation coefficient was 0.93 or greater. In this study we examined all independent isolates, because samples judged to belong to the same clone by ERIC-PCR may have different sensitivity profiles and show different phenotypic test results. Additionally, there is no standard procedure for selecting which isolates could be considered representative of their clonal groups for further analysis (**Figure 1**).

The identification and tests for antimicrobial sensitivity of isolates were carried out using an automated system (BD Phoenix™, Becton, Dickinson and Company, Sparks, MD, USA). Minimum inhibitory concentrations (MIC) of ceftazidime, ceftriaxone, and cefepime were assessed by the agar dilution method[5] in Mueller Hinton Agar (Becton, Dickinson and Company) plates. *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as controls.

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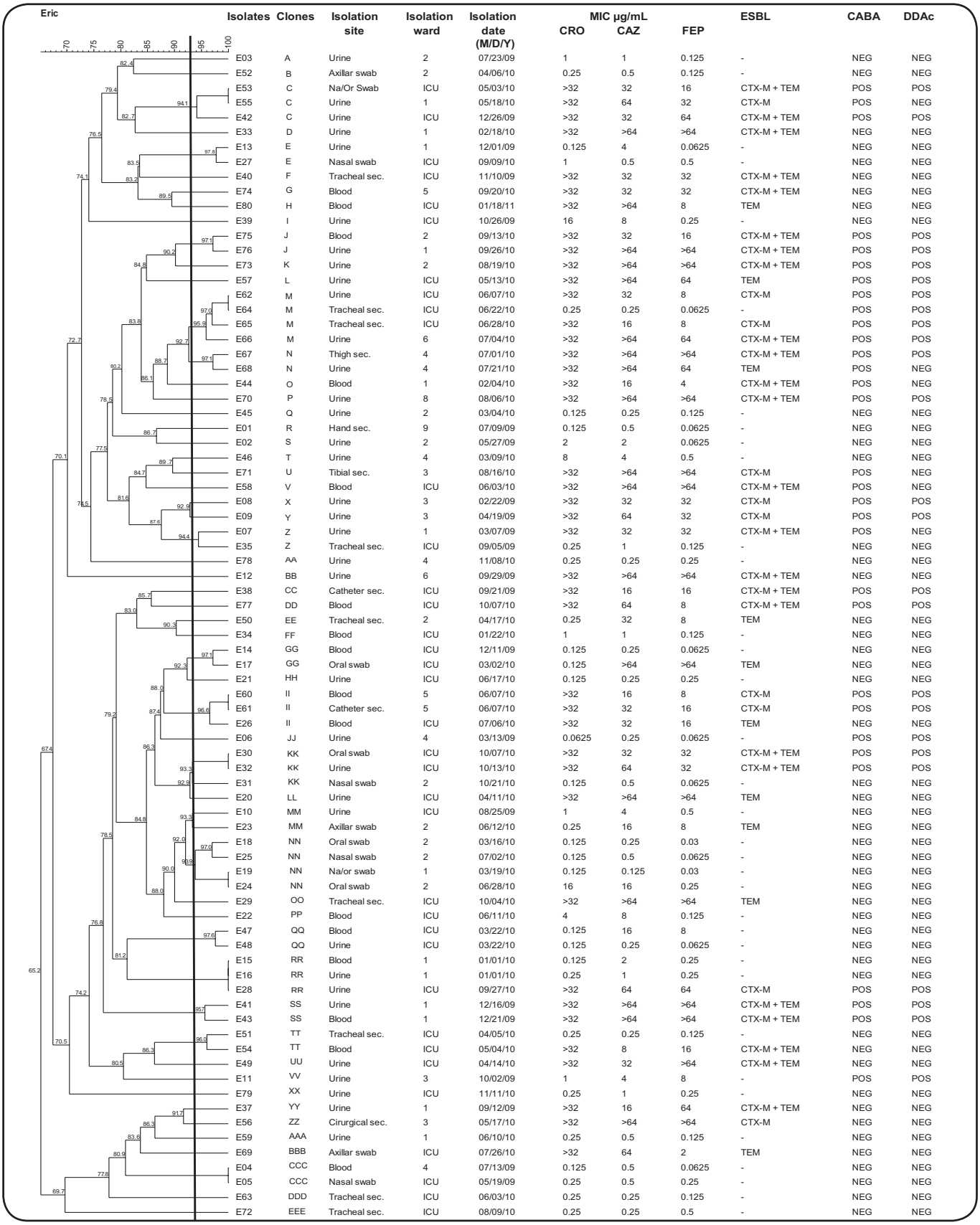

Isolates	Clones	Isolation site	Isolation ward	Isolation date (M/D/Y)	MIC µg/mL CRO	CAZ	FEP	ESBL	CABA	DDAc
E03	A	Urine	2	07/23/09	1	1	0.125	-	NEG	NEG
E52	B	Axillar swab	2	04/06/10	0.25	0.5	0.125	-	NEG	NEG
E53	C	Na/Or Swab	ICU	05/03/10	>32	32	16	CTX-M + TEM	POS	POS
E55	C	Urine	1	05/18/10	>32	64	32	CTX-M	POS	NEG
E42	C	Urine	ICU	12/26/09	>32	32	64	CTX-M + TEM	POS	POS
E33	D	Urine	1	02/18/10	>32	>64	>64	CTX-M + TEM	NEG	NEG
E13	E	Urine	1	12/01/09	0.125	4	0.0625	-	NEG	NEG
E27	E	Nasal swab	ICU	09/09/10	1	0.5	0.5	-	NEG	NEG
E40	F	Tracheal sec.	ICU	11/10/09	>32	32	32	CTX-M + TEM	NEG	NEG
E74	G	Blood	5	09/20/10	>32	32	32	CTX-M + TEM	NEG	NEG
E80	H	Blood	ICU	01/18/11	>32	>64	8	TEM	NEG	NEG
E39	I	Urine	ICU	10/26/09	16	8	0.25	-	NEG	NEG
E75	J	Blood	2	09/13/10	>32	32	16	CTX-M + TEM	POS	POS
E76	J	Urine	1	09/26/10	>32	>64	>64	CTX-M + TEM	POS	POS
E73	K	Urine	2	08/19/10	>32	>64	>64	CTX-M + TEM	POS	POS
E57	L	Urine	ICU	05/13/10	>32	>64	64	TEM	POS	POS
E62	M	Urine	ICU	06/07/10	>32	32	8	CTX-M	POS	POS
E64	M	Tracheal sec.	ICU	06/22/10	0.25	0.25	0.0625	-	POS	POS
E65	M	Tracheal sec.	ICU	06/28/10	>32	16	8	CTX-M	POS	POS
E66	M	Urine	6	07/04/10	>32	>64	64	CTX-M + TEM	POS	POS
E67	N	Thigh sec.	4	07/01/10	>32	>64	>64	CTX-M + TEM	POS	POS
E68	N	Urine	4	07/21/10	>32	>64	64	TEM	POS	NEG
E44	O	Blood	1	02/04/10	>32	16	4	CTX-M + TEM	POS	NEG
E70	P	Urine	8	08/06/10	>32	>64	>64	CTX-M + TEM	POS	POS
E45	Q	Urine	2	03/04/10	0.125	0.25	0.125	-	NEG	NEG
E01	R	Hand sec.	9	07/09/09	0.125	0.5	0.0625	-	NEG	NEG
E02	S	Urine	2	05/27/09	2	2	0.0625	-	NEG	NEG
E46	T	Urine	4	03/09/10	8	4	0.5	-	NEG	NEG
E71	U	Tibial sec.	3	08/16/10	>32	>64	>64	CTX-M	POS	NEG
E58	V	Blood	ICU	06/03/10	>32	>64	>64	CTX-M + TEM	POS	NEG
E08	X	Urine	3	02/22/09	>32	32	32	CTX-M	POS	POS
E09	Y	Urine	3	04/19/09	>32	64	32	CTX-M	POS	POS
E07	Z	Urine	1	03/07/09	>32	32	32	CTX-M + TEM	POS	NEG
E35	Z	Tracheal sec.	ICU	09/05/09	0.25	1	0.125	-	NEG	NEG
E78	AA	Urine	4	11/08/10	0.25	0.25	0.25	-	NEG	NEG
E12	BB	Urine	6	09/29/09	>32	>64	>64	CTX-M + TEM	NEG	NEG
E38	CC	Catheter sec.	ICU	09/21/09	>32	16	16	CTX-M + TEM	POS	POS
E77	DD	Blood	ICU	10/07/10	>32	64	8	CTX-M + TEM	POS	POS
E50	EE	Tracheal sec.	2	04/17/10	0.25	32	8	TEM	NEG	NEG
E34	FF	Blood	ICU	01/22/10	1	1	0.125	-	NEG	NEG
E14	GG	Blood	ICU	12/11/09	0.125	0.25	0.0625	-	NEG	NEG
E17	GG	Oral swab	ICU	03/02/10	0.125	>64	>64	TEM	NEG	NEG
E21	HH	Urine	ICU	06/17/10	0.125	0.25	0.25	-	NEG	NEG
E60	II	Blood	5	06/07/10	>32	16	8	CTX-M	POS	POS
E61	II	Catheter sec.	5	06/07/10	>32	32	16	CTX-M	POS	POS
E26	II	Blood	ICU	07/06/10	>32	32	16	TEM	NEG	NEG
E06	JJ	Urine	4	03/13/09	0.0625	0.25	0.0625	-	POS	POS
E30	KK	Oral swab	ICU	10/07/10	>32	32	32	CTX-M + TEM	POS	POS
E32	KK	Urine	ICU	10/13/10	>32	64	32	CTX-M + TEM	POS	POS
E31	KK	Nasal swab	2	10/21/10	0.125	0.5	0.0625	-	NEG	NEG
E20	LL	Urine	ICU	04/11/10	>32	>64	>64	TEM	NEG	NEG
E10	MM	Urine	ICU	08/25/09	1	4	0.5	-	NEG	NEG
E23	MM	Axillar swab	2	06/12/10	0.25	16	8	TEM	NEG	NEG
E18	NN	Oral swab	2	03/16/10	0.125	0.25	0.03	-	NEG	NEG
E25	NN	Nasal swab	2	07/02/10	0.125	0.5	0.0625	-	NEG	NEG
E19	NN	Na/or swab	1	03/19/10	0.125	0.125	0.03	-	NEG	NEG
E24	NN	Oral swab	2	06/28/10	16	16	0.25	-	NEG	NEG
E29	OO	Tracheal sec.	ICU	10/04/10	>32	>64	>64	TEM	NEG	NEG
E22	PP	Blood	ICU	06/11/10	4	8	0.125	-	NEG	NEG
E47	QQ	Blood	ICU	03/22/10	0.125	16	8	-	NEG	NEG
E48	QQ	Urine	ICU	03/22/10	0.125	0.25	0.0625	-	NEG	NEG
E15	RR	Blood	1	01/01/10	0.125	2	0.25	-	NEG	NEG
E16	RR	Urine	1	01/01/10	0.25	1	0.25	-	NEG	NEG
E28	RR	Urine	ICU	09/27/10	>32	64	64	CTX-M	POS	POS
E41	SS	Urine	1	12/16/09	>32	>64	>64	CTX-M + TEM	POS	POS
E43	SS	Blood	1	12/21/09	>32	>64	>64	CTX-M + TEM	POS	POS
E51	TT	Tracheal sec.	ICU	04/05/10	0.25	0.25	0.125	-	NEG	NEG
E54	TT	Blood	ICU	05/04/10	>32	8	16	CTX-M + TEM	NEG	NEG
E49	UU	Urine	ICU	04/14/10	>32	32	>64	CTX-M + TEM	NEG	NEG
E11	VV	Urine	3	10/02/09	1	4	8	-	POS	POS
E79	XX	Urine	ICU	11/11/10	0.25	1	0.25	-	NEG	NEG
E37	YY	Urine	1	09/12/09	>32	16	64	CTX-M + TEM	NEG	NEG
E56	ZZ	Cirurgical sec.	3	05/17/10	>32	>64	>64	CTX-M	NEG	NEG
E59	AAA	Urine	1	06/10/10	0.25	0.5	0.125	-	NEG	NEG
E69	BBB	Axillar swab	ICU	07/26/10	>32	64	2	TEM	NEG	NEG
E04	CCC	Blood	4	07/13/09	0.125	0.5	0.0625	-	NEG	NEG
E05	CCC	Nasal swab	ICU	05/19/09	0.25	0.5	0.25	-	NEG	NEG
E63	DDD	Tracheal sec.	ICU	06/03/10	0.25	0.25	0.125	-	NEG	NEG
E72	EEE	Tracheal sec.	ICU	08/09/10	0.25	0.25	0.5	-	NEG	NEG

FIGURE 1 - Dendrogram plot obtained by the *enterobacterial repetitive intergenic consensus*-polymerase chain reaction (ERIC-PCR) analysis of the ESBL isolates. Hospital wards: ICU: intensive care unit; 1, 3, 5, and 8: clinical and surgical patients, differing only in the number of patients/room; 2: SUS (Brazilian National Unified Health System) patients; 4: emergency care; 6: maternity; 9: surgical center. Na/Or swab: nasal or oral swab; MIC: minimum inhibitory concentration; CRO: ceftriaxone; CAZ: ceftazidime; FEP: cefepime; ESBL: extended spectrum β-lactamase; CABA: ceftazidime and cefotaxime disks with and without clavulanic acid + boronic acid; DDAc: disk approximation using cefepime and amoxicillin/CA; CA: clavulanic acid; CTX-M: cefotaximases; TEM: Temoniera; NEG: negative; POS: positive.

For detection and typing of ESBL, we performed multiplex PCR with primers for identification of the predominant ESBL types (TEM, SHV, and CTX-M), as described[6]. We performed four assays of phenotypic detection for ESBL activity. The first was ceftazidime and cefotaxime disks, with and without CA (10μg/disk) added of 20μL of boronic acid (BA) solution at a concentration of 20g/L (Sigma, St. Louis, MO, USA), in which an increase ≥ 5mm in the diameter of the halo surrounding the disk containing the drug + CA + BA compared to the disk containing only the drug + BA was considered a positive result for ESBL[7] (CABA method). The second assay was disk approximation using cefepime and amoxicillin/CA positioned at a distance of 20 mm center to center, in which the presence of a ghost zone (synergism) between the disks was considered a positive result for ESBL activity[8] (DDAc method). The final two tests were a cefepime 16 (CPM16 method) and cefepime 2 (CPM2 method) assay, in which MICs for cefepime ≥ 16μg/mL and ≥ 2μg/mL, respectively, were considered positive tests for ESBL activity[5][9].

All the phenotypic tests were performed using fresh cultures. The inoculum was adjusted to the 0.5 McFarland standard, which contains approximately 1 to 2 × 10^8 colony forming units (CFU)/mL. This adjustment was necessary because an inoculum-dependent effect is observed in β-lactamase-producing samples[10].

A high (56%) frequency of ESBL gene prevalence was detected from the *E. cloacae* isolates, and all ESBL-positive samples also tested positive in the CPM2 assay. No correlation was identified between clonal genotypes and the absence or presence of ESBL.

Bell et al. and Park et al. proposed screening methods for ESBL in *Enterobacter* spp. based on MIC > 0.25μg/mL and MIC ≥ 1μg/mL for cefepime, respectively[11][12].When applying these criteria to our samples, we did not find similar sensitivity and specificity to these reports. However, when we increased the stringency by raising the cutoff MIC value for cefepime to ≥ 2μg/mL, we obtained 100% sensitivity and 94% specificity (**Table 1**). We therefore conclude that this is the most accurate and easily applicable method for ESBL detection activity in *Enterobacter* spp.

CPM16 revealed greater (100%) specificity with reduced (80%) sensitivity (**Table 1**), but use of this method is limited in clinical routines, where the MIC is determined using automated methods. With these methods, 16μg/mL of cefepime is a concentration not routinely included in dilution panels, whereas 2μg/mL of cefepime is frequently found.

Jeong et al. achieved 98.4% sensitivity for ESBL detection in chromosomal AmpC producers using the CABA method[7]. This is in contrast with the 62% sensitivity and 91% specificity we attained in this study (**Table 1**).

Although Tzelepi et al.[8] demonstrated that the DDAc method using cefepime worked well in species that possessed a chromosomal *ampC* gene[8], in this study we achieved only 49% sensitivity with this method (**Table 1**). This may have been due to differences in interpretation of the results, which can be subjective. In our case, it was difficult to determine whether the β-lactam inhibitory zones were qualitatively altered.

TABLE 1 - Sensitivity and specificity of the four different phenotypic methods used for ESBL detection in *Enterobacter cloacae* isolates.

	Methods			
	CABA	DDAc	CPM16	CPM2
Sensitivity (%)	64.0	50.0	80.0	100.0
Specificity (%)	91.0	91.0	100.0	94.0

ESBL: Extended-spectrum β lactamase; CABA: ceftazidime and cefotaxime disks with and without clavulanic acid + boronic acid; DDAc: disk approximation using cefepime and amoxicillin-clavulanic acid; CPM16 and CPM2: MICs for cefepime ≥ 16 μg/mL and ≥ 2 μg/mL, respectively. MICs: minimum inhibitory concentrations.

Interpretation of results from this assay likely requires expertise of the analyst. Furthermore, the ideal distance between disks depends on the sensitivity profile of the bacteria, as has been previously reported[13]. A modification to this test was recently suggested; adding phenylboronic acid to the cefepime disk would improve detection of ESBL activity in AmpC producing samples[14].

To consider the accuracy of the methods, the CABA and DDAc methods detected three ESBL-like samples that were negative by PCR (**Figure 1**), while the CPM2 method detected two. The discrepancy may be due to the primer sequences, which were designed to recognize the most common ESBL families (TEM, CTX-M, and SHV). However, there are other, less prevalent families that were not assessed in this study[13]. Therefore it is possible that these were not false-positive results from the phenotypic assays, but false-negative results from the PCR-based assay.

In the present study, nine (20%) samples showed sensitivity to cefepime (MIC between 2μg/mL and 8μg/mL) and were positive to ESBL with both CPM2 and the genotypic method (**Figure 1**). These cases demonstrate the necessity for a routine clinical use of a rapid and simple method to detect ESBL activity in *Enterobacter* spp. isolates since cefepime may be the drug of choice for treatment of *Enterobacter* spp. infections[15].

However further clinical studies are needed to evaluate patients treated with cefepime to assess the efficacy of this cephalosporin drug in the treatment of ESBL-producing *Enterobacter* spp. infections. In addition, the rapid detection of ESBL isolates would enable infection control practitioners to implement precautions, avoiding further spread of this pathogen in the hospital setting.

In conclusion, we demonstrate here that for those laboratories that use dilution methods to determine antimicrobial sensitivity, adopting the CPM2 method would be very useful for the phenotypic detection of ESBL in samples of *Enterobacter* spp., while for laboratories that use only the disk-diffusion method, the CABA method is highly effective. Our findings also suggest the need for greater surveillance of ESBL in *Enterobacter* spp. infections for both improved treatment options and to reduce the risk of wider outbreaks.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

1. Crowley B, Ratcliffe G. Extended-spectrum β-lactamases in *Enterobacter cloacae*: underestimated but clinically significant! J Antimicrob Chemother 2003; 51:1316-1317.
2. Bonnet R. Growing Group of Extended-Spectrum β-Lactamases: the CTX-M Enzymes. Antimicrob Agents Chemother 2004; 48:1-14.
3. Menegucci TC, Barros JPRP, Costa BB, Tsuneto PY, Aoki EE, Zarpellon MN, et al. Perfil de sensibilidade e prevalência de bacilos Gram-negativos em um hospital ensino. XIII Congresso Brasileiro de Controle de Infecção e Epidemiologia Hospitalar. Anais J Infect Control 2012; 1:199.
4. Silbert S, Pfaller MA, Hollis RJ, Barth AL, Sader HS. Evaluation of three molecular typing techniques for nonfermentative Gram-negative bacilli. Infect Control Hosp Epidemiol 2004; 25:847-851.
5. Clinical and Laboratory Standards Institute (CLSI). Performance Standards for Antimicrobial Susceptibility Testing. Twentieth Informational Supplement. Approved Standart M100-S23. Wayne, PA: CLSI; 2013.
6. Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae. Apmis 2007; 115:1400-1408.
7. Jeong SH, Song W, Park MJ, Kim JS, Kim HS, Bae IK, et al. Boronic acid disk tests for identification of extended-spectrum β-lactamase production in clinical isolates of Enterobacteriaceae producing chromosomal AmpC β-lactamases. Int J Antimicrob Agents 2008; 31:467-471.
8. Tzelepi E, Giakkoupi P, Sofianou D, Loukova V, Kemeroglou A, Tsakris A. Detection of Extended-Spectrum β-Lactamases in Clinical Isolates of *Enterobacter cloacae* and *Enterobacter* aerogenes. J Clin Microbiol 2000; 38:542-546.
9. European Committee on Antimicrobial Susceptibility Testing (EUCAST). Breakpoint tables for interpretation of MICs and zone diameters. Version 2.0. [Cited 2014 October 2]. EUCAST; 2013. Available at (http://www.eucast.org/clinical_breakpoints).
10. Maglio D, Ong C, Banevicius MA, Geng Q, Nightingale CH, Nicolau DP. Determination of the *in vivo* pharmacodynamic profile of cefepime against extended-spectrum-β-lactamase-producing *Escherichia coli* at various inocula. Antimicrob Agents Chemother 2004; 48:1941-1947.
11. Bell JM, Turnidge JD, Jones RN, SENTRY Asia-Pacific Participants. Prevalence of Extended-Spectrum β-Lactamase-Producing *Enterobacter cloacae* in the Asia-Pacific Region: Results from the SENTRY Antimicrobial Surveillance Program, 1998 to 2001. Antimicrob Agents Chemother 2003; 47:3989-3993.
12. Park YJ, Park SY, Oh EJ, Park JJ, Lee KY, Woo GJ, Lee K. Occurrence of extended-spectrum β-lactamases among chromosomal AmpC-producing *Enterobacter cloacae*, *Citrobacter freundii*, and *Serratia marcescens* in Korea and investigation of screening criteria. Diagn Microbiol Infect Dis 2005; 51:265-269.
13. Oliveira KRP, Barth AL. β-lactamases na família Enterobacteriaceae: métodos de detecção e prevalência. Rio Grande do Sul: UFRGS; 2008. p. 89.
14. Willems E, Verhaegen J, Magerman K, Nys S, Cartuyvels R. Towards a phenotypic screening strategy for emerging β-lactamases in Gram-negative bacilli. Int J Antimicrob Agents 2013; 41:99-109.
15. Endimiani A, Perez F, Bonomo RA. Cefepime: a reappraisal in an era of increasing antimicrobial resistance. Expert Rev Anti Infect Ther 2008; 6:805-824.