The Brazilian Journal of
INFECTIOUS DISEASES
www.elsevier.com/locate/bjid

Letters to the Editor

Misidentification of pan drug-resistant *Klebsiella pneumoniae* clinical isolates as a metallo-β-lactamase producers by the EDTA/DDST test

Dear Editor,

Carbapenemase-producing Enterobacteriaceae may exhibit susceptibility to carbapenems. For this reason with the recent spread of NDM-1 among *Enterobacteriaceae*, the phenotypic detection of metallo-beta-lactamase (MβL)-producing has been recommended by Brazilian Agency of Sanitary Surveillance (ANVISA).[1]

In 2013, two pan drug-resistant *Klebsiella pneumoniae* (KPN1 and KPN2) isolates were recovered from urine (cystostomy) of a 75-year-old male patient hospitalized in a tertiary teaching hospital in Florianópolis, Santa Catarina, Brazil. Both isolates were phenotypically identified as MβL producers by ethylenediamine tetraacetic acid (EDTA)/double-disk synergy test (DDST) and forwarded to our laboratory for further characterization. Identification of both isolates as *K. pneumoniae* was confirmed by MALDI-TOF MS (Bruker Daltonics, Germany), according to the manufacturer's recommendations. Both isolates showed an identical pattern by ERIC-PCR. The minimal inhibitory concentrations (MICs) for selected antimicrobial agents were determined by broth microdilution according to Clinical and Laboratory Standards Institute – CLSI. The MICs were interpreted according to the CLSI guidelines,[2] except for tigecycline, which interpretation was performed according to the European Committee on Antimicrobial Susceptibility Testing – EUCAST. Both isolates were fully resistant to all broad-spectrum cephalosporins, aztreonam, gentamicin, fluoroquinolones, meropenem, ertapenem, and polymyxin B as shown in Table 1. The isolates showed intermediate resistance to imipenem and amikacin. While KPN1 was susceptible to tigecycline, KPN2 became resistant to this agent.

The isolates were also screened for ESβL production by disk approximation and the synergism was observed only when amoxicillin/clavulanic acid disk was tested 15 mm a part of ceftriaxone, ceftazidime and cefepime disks. The phenotypic detection of MβL was carried out by the EDTA/DDST and confirmed by ertapenem hydrolysis assay using MALDI-TOF MS (Bruker Daltonics, Germany) as previously reported.[3] Although both isolates showed an increase in the inhibition zone of ceftazidime/EDTA (6 mm) and meropenem/EDTA (7 mm) disks compared to the ceftazidime and meropenem disks, respectively, hydrolysis of ertapenem was not observed, suggesting that both isolates were not MβL producers.

The search for β-lactamase encoding genes was carried out by PCR followed by DNA sequencing of amplicons. Both *K. pneumoniae* isolates carried $bla_{\text{CTX-M-15}}$, and the narrow spectrum-β-lactamases encoding genes, $bla_{\text{SHV-11}}$, $bla_{\text{TEM-1}}$ and $bla_{\text{OXA-1}}$. The presence of the plasmid mediated *qnrS1* gene and a mutation Ser83Ile in *gyrA* were also detected, justifying the quinolone resistance exhibited by both KPN isolates. The analysis of the outer membrane proteins profile by SDS–PAGE showed that both isolates lost the major porins OmpK35 and OmpK36. Sequencing analysis of the *ompk35* and *ompk36* genes revealed the presence of the insertion sequence IS*1* between the promoter region and the start codon of the *ompK35* gene, and the presence of the IS*903* disrupting the *ompK36* gene. These results corroborated with the absence of the major porins on the SDS–PAGE gels, since the presence of the IS resulted in non-functional OmpK35 and OmpK36 porins.

The misidentification of two pan-resistant *K. pneumoniae* isolates as MβL producers by the EDTA/DDST is in agreement with previous study that reported false-positive results when EDTA was employed for identification of the MβL production.[4] This fact may result from the bactericidal effect of EDTA which acts increasing the membrane permeability.[5] Based on that, a disk contained only 100 mM of EDTA was also tested in the present study, confirming their bactericidal effect, since considerable inhibition diameter zones of 18 mm for KPN1 and 16 mm for KPN2 were observed.

This study reported a misidentification of MβL producers by EDTA/DDST in *K. pneumoniae* clinical isolates, as recommended by ANVISA.[1] The pan-resistant phenotype observed between the two *K. pneumoniae* strains isolated in our territory is worrisome, since very few therapeutic options are

Table 1 – Antimicrobial susceptibility profile and resistant determinants among the *K. pneumoniae* isolates misidentified as MβL producers by EDTA/DDST.

Isolate	MALDI-TOF MS ID	MIC (μg/mL)/susceptibility category[a]													
		CEP	CAZ	CTX	FEP	AZT	IPM	MEM	ETP	AK	GEN	LEV	CIP	TGC	PO
KPN 1	*K. pneumoniae*	≥256[R]	≥256[R]	≥256[R]	≥256[R]	≥32[R]	2[I]	8[R]	64[R]	32[I]	≥64[R]	≥128[R]	≥128[R]	1[S]	32[R]
KPN 2	*K. pneumoniae*	≥256[R]	≥256[R]	≥256[R]	≥256[R]	≥32[R]	2[I]	8[R]	64[R]	32[I]	≥64[R]	≥128[R]	≥128[R]	4[R]	32[R]

Isolate	MALDI-TOF MS ID	Diameter variation with EDTA (mm)		Porin lesion type[b]		Resistant determinants
		CAZ	MEM	OmpK 35	OmpK36	
KPN 1	*K. pneumoniae*	7	6	$IS1_{(-5)}$	$IS908_{(+726)}$	CTX-M-15, TEM-1, SHV-11, QnrS1, OXA-1
KPN 2	*K. pneumoniae*	7	6	$IS1_{(-5)}$	$IS908_{(+726)}$	CTX-M-15, TEM-1, SHV-11, QnrS1, OXA-1

[a] *Abbreviations*: CEP, cephalotin; CAZ, ceftazidime; CTX, cefotaxime; FEP, cefepime; IPM, imipenem; MEM, meropenem; ETP, ertapenem; AK, amikacin; GEN, gentamicin; LEV, levofloxacin; CIP, ciprofloxacin; TGC, tigecyclin; PO, polymyxin B.

[b] Porin lesion type: + and − indicate the position upstream and downstream of start codon; numbers between parentheses correspond the nucleotide insertion position; IS_n indicate the mutation in the promoter region and presence of insertion sequences, respectively.

clinically available for treating such infections. Probably, the isolates also possess other resistance mechanisms that had contributed to their multi-drug resistant profile. The chelating agents such as EDTA can increase the outer membrane permeability, facilitating the entry of antibiotics.[4,5] Based on that, disk contained only 100 mM of EDTA should be used additionally, to confirm their bactericidal effect; therefore attention should be taken by the routine laboratories to avoid the report of false positive results.

Conflicts of interest

A.C.G. recently received research funding and/or consultation fees from AstraZeneca and Merck Sharp & Dohme. Other authors have nothing to declare.

Acknowledgments

We thank Ana Carolina Ramos da Silva for her assistance in the SDS–PAGE gels and to Lorena Cristina Corrêa Fehlberg for reviewing this manuscript.

REFERENCES

1. Agência Nacional de Vigilância Sanitária (ANVISA). Medidas de Prevenção e Controle de Infecções por Enterobactérias Multiresistentes. Brasília, Brasil: ANVISA; 2013.
2. Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; approved standard – twenty fourth edition M100-S24. Wayne: CLSI; 2014.
3. Carvalhaes CG, Cayô R, Assis DM, et al. Detection of SPM-1-producing *Pseudomonas aeruginosa* and class D β-lactamase-producing *Acinetobacter baumannii* isolates by use of liquid chromatography–mass spectrometry and matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol. 2013;51:287–90.
4. Chu YW, Cheung TK, Ngan JYW, Kam KM. EDTA susceptibility leading to false detection of metallo-β-lactamase in *Pseudomonas aeruginosa* by E-test and imipenem-EDTA disk method. Int J Antimicrob Agents. 2005;26:340–1.
5. Ratkai C, Quinteira S, Grosso F, Monteiro N, Nagy E, Peixe L. Controlling for false positives: interpreting MBL Etest and MBL combined disc test for the detection of metallo-beta-lactamases. J Antimicrob Chemother. 2009;64:657–8.

Dandara Cassu-Corsi[*,1], Willames M.B.S. Martins[1]

Laboratório ALERTA, Disciplina de Infectologia, Departamento de Medicina, Universidade Federal de São Paulo - UNIFESP, São Paulo, SP, Brazil

Mara Cristina Scheffer

Laboratório de Microbiologia, Divisão de Análises Clínicas, Hospital Universitário, Universidade Federal de Santa Catarina - UFSC, Florianópolis, SC, Brazil

Rodrigo Cayô, Ana Cristina Gales

Laboratório ALERTA, Disciplina de Infectologia, Departamento de Medicina, Universidade Federal de São Paulo - UNIFESP, São Paulo, SP, Brazil

[*] *Corresponding author.*
E-mail address: dandara.corsi@gmail.com (D. Cassu-Corsi).
[1] These authors contributed equally to this work.

Received 7 August 2014
Accepted 20 August 2014
Available online 13 October 2014

http://dx.doi.org/10.1016/j.bjid.2014.08.008