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Diagnostic Microbiology and Infectious Disease xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Note

Comparison of phenotypic tests for detecting BKC-1–producing Enterobacteriaceae isolates

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ARTICLE INFO

Article history:
Received 12 September 2015
Received in revised form 18 November 2015
Accepted 25 November 2015
Available online xxxx

Keywords:
Carbapenemase
BKC-1
Klebsiella pneumoniae
Antimicrobial resistance

ABSTRACT

Carbapenemase-producing Enterobacteriaceae may exhibit in vitro susceptibility to carbapenems, especially those producing weak carbapenemases. Routine clinical laboratories have employed phenotypic tests for screening such isolates. BKC-1 is a recently reported carbapenemase that shows weak carbapenemase activity. In this study, we aimed to evaluate the behavior of distinct phenotypic methods against BKC-1–producing Enterobacteriaceae.

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Carbapenem resistance among Gram-negative bacilli has emerged an important health public problem in many geographic regions including Latin America. To date, several mechanisms of carbapenem resistance have been described including production of beta-lactamases, porin loss, penicillin-binding protein alteration, and/or overexpression of efflux systems (Gupta et al., 2011). The production of carbapenemases represents the most important mechanism of carbapenem resistance in Enterobacteriaceae. The last decade was marked by the emergence and spread of carbapenemases like KPC-, NDM-, or OXA-48 among Enterobacteriaceae isolated worldwide (Nordmann and Poirel, 2013).

Unfortunately, carbapenemase-producing Enterobacteriaceae isolates may show low imipenem and/or meropenem MICs, being miscategorized as susceptible to carbapenems, especially by automated susceptibility testing systems. In this manner, routine microbiological laboratories must rely on phenotypic tests for accurate detection of carbapenemase producers. Modified Hodge Test (MHT), combined disk, and double-disk synergy test (DDST) were phenotypic tests widely employed for carbapenemase detection (Hammoudi et al., 2014). Although these tests are easy, simple, and widely available, they are not rapid and reliable. MHT has shown low sensitivity and specificity rates failing to detect NDM-1- (false negatives), ESBL, and/or AmpC-producing Enterobacteriaceae (false positives) (Carvalhaes et al., 2010). In this manner, CarbaNP, Blue-Carba, and Carbapenembac™ (Probac Brazil) have been employed for rapid detection of carbapenemase-producing Gram-negative bacilli (Martino et al., 2015; Nordmann et al., 2012; Pires et al., 2013). Detection of carbapenem hydrolysis by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI TOF MS) has also been successfully used for detection of carbapenemase activity (Carvalhaes et al., 2013).

Recently, a new carbapenemase encoding gene, bla_{BKC-1}, was described in *Klebsiella pneumoniae* clinical isolated from Southeast Brazil. BKC-1 is a weak serine class A carbapenemase that interestingly shows higher hydrolytic activity towards oxacillins (Nicoletti et al., 2015). In this study, we aimed to evaluate the behavior of distinct phenotypic methods against BKC-1–producing Enterobacteriaceae.

Six isolates previously characterized as BKC-1 producers (5 *K. pneumoniae* clinical isolates and 1 *Escherichia coli* DH5α harboring p10Kb with bla_{BKC-1}, named T3) were selected for this study (Table 1). We compared the results of 9 different phenotypic methods: MHT, phenylboronic acid/disk combination (AFB/DC), EDTA/disk combination (EDTA/DC), cloxacillin/disk combination (Cloxa/DC) (ANVISA, 2013; CLSI, 2015b), imipenem hydrolysis testes by spectrophotometry (Picão et al., 2008), ertapenem hydrolysis by MALDI-TOF MS (Bruker Daltonic, Bremen, Germany) (Carvalhaes et al., 2013), CarbaNP Test (Nordmann et al., 2012), Blue-Carba (Pasteran et al., 2015; Pires et al., 2013), and Carbapenembac™ Test (Martino et al., 2015). The tests were carried out as previously described. NDM-1–producing *K. pneumoniae* ATCC BAA-2146, KPC-2–producing *K. pneumoniae* Kp13, and OXA-72–producing *Acinetobacter baumannii* A30235 were tested as positive quality controls (QC), while *K. pneumoniae* ATCC 700603 was tested as negative QC. Additionally, we performed the antimicrobial susceptibility testing to carbapenems by disk diffusion and broth microdilution methodologies according CLSI guideline (CLSI, 2015b). The results were interpreted following CLSI recommendations (CLSI, 2015a).

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http://dx.doi.org/10.1016/j.diagmicrobio.2015.11.021
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Please cite this article as: Martins WMBS, et al, Comparison of phenotypic tests for detecting BKC-1–producing Enterobacteriaceae isolates, Diagn Microbiol Infect Dis (2015), http://dx.doi.org/10.1016/j.diagmicrobio.2015.11.021

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Table 1
Molecular and phenotypic characteristics of BKC-1–producing isolates.

Isolates	Bacteria species	β-Lactam resistance genes	MIC (μg/mL)			Disk diffusion (mm)			AFB/DC (mm)		EDTA/DC (mm)	
			ETP	IPM	MEM	ETP	IPM	MEM	IPM + AFB	MEM + AFB	IMP + EDTA	MEM + EDTA
KP60134	*K. pneumoniae*	$bla_{CTX-M-2}$; $bla_{SHV-110-like}$; bla_{BKC-1}	64 [R]	8 [R]	16 [R]	11 [R]	20 [I]	18 [R]	21 [Neg.]	20 [Neg.]	22 [Neg.]	19 [Neg.]
KP60135	*K. pneumoniae*	$bla_{CTX-M-2}$; $bla_{SHV-110-like}$; bla_{BKC-1}	64 [R]	4 [R]	8 [R]	14 [R]	23 [S]	20 [I]	23 [Neg.]	22 [Neg.]	24 [Neg.]	21 [Neg.]
KP60136	*K. pneumoniae*	$bla_{CTX-M-2}$; $bla_{SHV-110-like}$; bla_{BKC-1}	>256 [R]	128 [R]	32 [R]	13 [R]	7 [R]	6 [R]	14 [Pos.]	19 [Pos.]	12 [Pos.]	16 [Pos.]
KP68324	*K. pneumoniae*	$bla_{CTX-M-2}$; $bla_{SHV-110-like}$; bla_{BKC-1}	32 [R]	32 [R]	8 [R]	12 [R]	20 [I]	21 [I]	23 [Neg.]	20 [Neg.]	22 [Neg.]	19 [Neg.]
KP69725	*K. pneumoniae*	$bla_{SHV-110-like}$; bla_{BKC-1}	64 [R]	32 [R]	32 [R]	7 [R]	15 [R]	13 [R]	18 [Neg.]	16 [Neg.]	19 [Neg.]	15 [Neg.]
T3 isolate	*E. coli*	bla_{BKC-1}	64 [R]	8 [R]	8 [R]	12 [R]	18 [R]	19 [R]	20 [Neg.]	23 [Neg.]	18 [Neg.]	19 [Neg.]
KP13 (KPC + QC)	*K. pneumoniae*	bla_{KPC-2}; $bla_{CTX-M-2}$, bla_{SHV-11}, bla_{OXA-9}	NT	NT	NT	6 [R]	6 [R]	6 [R]	22 [Pos.]	19 [Pos.]	15 [Pos.]	10 [Neg.]
KP ATCC BAA-2146 (NDM + QC)	*K. pneumoniae*	bla_{NDM-1}	NT	NT	NT	6 [R]	10 [R]	6 [R]	9 [Neg.]	8 [Neg.]	31 [Pos.]	30 [Pos.]
ACB 30235 (OXA-72 + QC)	*A. baumannii*	bla_{OXA-72}; bla_{OXA-51}	NT	NT	NT	6 [R]	12 [R]	6 [R]	16 [Neg.]	8 [Neg.]	14 [Neg.]	15 [Pos.]

ETP = ertapenem; IPM = imipenem; MEM = meropenem; NT = no tested; [R] = resistant; [S] = susceptible; [Neg.] = negative; [Pos.] = positive.

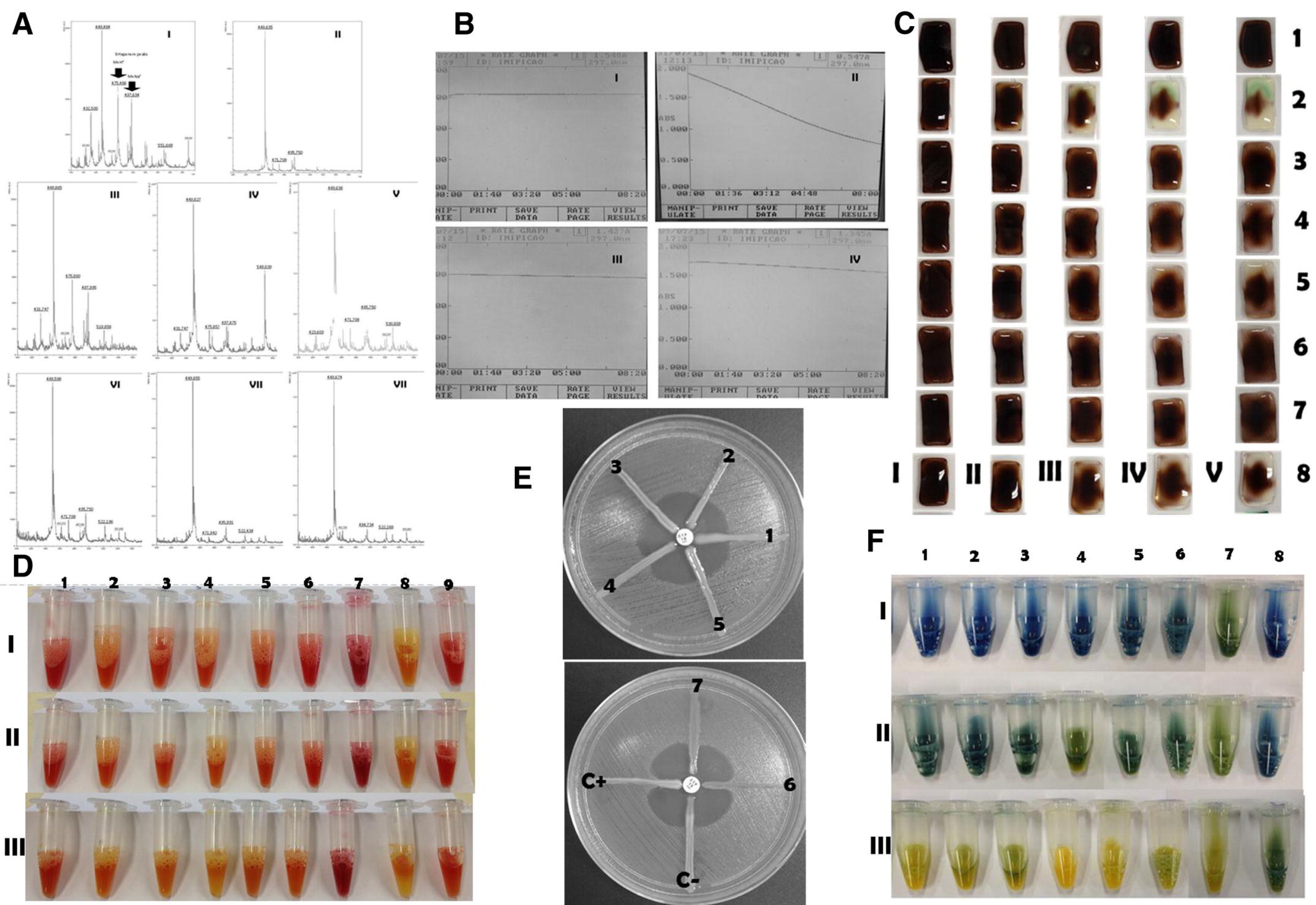

Fig. 1. Distinct methodologies applied for screening BKC-1–producing Enterobacteriaceae. **(A)** Pattern of ertapenem hydrolysis (0.25 mg/mL) by MALDI-TOF. I, negative control; II, positive control; III, IV, and V, common profile of hydrolysis pattern showed by BKC-1–producing *K. pneumoniae* isolates after 15, 60, and 120 minutes of incubation; VI, VII, and VIII, profile of T3 after 15, 60, and 120 minutes of incubation. **(B)** Pattern of imipenem hydrolysis (3 mg/mL) by spectrophotometry after 8 minutes of incubation. I, *K. pneumoniae* ATCC 700603 (negative QC); II, *K. pneumoniae* KP13 (positive QC)—incubation of imipenem with KPC-2–producing strain; III, common profile of hydrolysis pattern showed by BKC-1–producing *K. pneumoniae* isolates; IV, profile of T3 isolate. **(C)** Carbapenembac™ tests. Columns I–IV, 15, 30, 60, and 120 minutes of incubation, respectively. Line 1, *K. pneumoniae* ATCC 700603 (negative QC); line 2, *K. pneumoniae* KP13 (positive QC); line 3, KP60136; line 4, KP60135; line 5, KP60134; line 6, KP68324; line 7, KP69725; and, line 8, T3. **(D)** CarbaNP Test. Lines I–III, 30, 60, and 120 minutes of incubation, respectively. Line 1, KP60136; line 2, KP60135; line 3, KP60134; line 4, KP68324; line 5, KP69725; line 6, T3, line 7, only Carba-NP solution; line 8, *K. pneumoniae* ATCC 700603 (negative QC); and line 9, *K. pneumoniae* KP13 (positive QC). **(E)** MHT. 1, KP60136; 2, KP60135; 3, KP60134; 4, KP68324; 5, KP69725; 6, T3; C+, *K. pneumoniae* KP13 (positive QC); and, C−, *K. pneumoniae* ATCC 700603 (negative QC). **(F)** Blue-Carba. Lines I–III, 15, 30, and 60 minutes of incubation, respectively. Column 1, KP60136; column 2, KP60135; column 3, KP60134; column 4, KP68324; column 5, T3 isolate; column 6, KP69725; column 7, *K. pneumoniae* KP13 (positive QC); and column 8, *K. pneumoniae* ATCC 700603 (negative QC).

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MHT using ertapenem disk failed to detect the production of BKC-1, except for T3 isolate (positive) and KP68324 (weak positive) (Fig. 1). However, when we replaced the ertapenem by the meropenem disk, some BKC-producing isolates showed a weak positive result. These results could be attributed to the BKC-1 poor hydrolytic activity against ertapenem when compared to that of meropenem (Nicoletti et al., 2015). Weak positive results are difficult to interpret because they depend on the reader's expertise. Previous studies also have shown that MHT is a reliable test to detect KPC and OXA-48 but fail in identify MβL and cabapenemases with poor hydrolytic activity (Doyle et al., 2012; Hrábak et al., 2014). Three BKC-1–producing isolates showed carbapenem inhibition zones within the susceptibility or intermediate breakpoint ranges and could not have been recognized by routine microbiology clinical laboratories using disk diffusion. A single BKC-1–producing isolate, KP60136, was detected as carbapenemase producer by AFB/DC. Nonetheless, we also verified EDTA/DC false-positive result for this isolate. *K. pneumoniae* isolates exhibiting porin loss (OmpK35 and/or OmpK36) maybe falsely recognized as carbapenemase producers (Cassu-Corsi et al., 2015). We did not verify false-positive tests with Cloxa/DC test against BKC-1 producers.

BKC-1–producing isolates were detected as carbapenemase producers by both spectrophotometry and MALDI-TOF MS. However, an extended period of incubation was necessary for detection of BKC-1 carbapenemase activity by both methodologies, spectrophotometry (8 versus 3 minutes for NDM and KPC QC controls) and MALDI-TOF MS (2–4 hours versus 15 minutes NDM and KPC QC controls, except for the T3 isolate, that was detected after 15-minute incubation; Fig. 1). Different results observed for T3 strain could be attributed to a higher expression of $bla_{\text{BKC-1}}$ in a distinct host. Interestingly, a characteristic hydrolysis curve was observed for BKC-1–producing isolates on the spectrophotometer (Fig. 1).

CarbaNP Test, Blue-Carba, and Carbapenembac™ are relatively new tests that have been employed for rapid identification of carbapenemase producers. These tests are based on the reduction of pH due to beta-lactam ring hydrolysis by carbapenemase. Due to its high sensitivity and specificity, CarbaNP has been recommended by CLSI for phenotypic detection of carbapenemase-producing Enterobacteriaceae (CLSI, 2015a). In our study, CarbaNP showed positive results only after 2-hour incubation period (Fig. 1). In contrast, Blue-Carba was faster than Carba-NP to identify BKC-1–producing isolates, resulting in positive results after 15 minutes of incubation. Carbapenembac™ is a test developed by Probac, Brazil, for detection of carbapenemases, mainly KPC (Martino et al., 2015). Carbapenembac™ consists of a strip that is impregnated with a concentration of 100 μg imipenem and starch, and which shows distinct end staining after bacteria incubation (change of dark becoming clear) due to carbapenemase activity (Martino et al., 2015). The presence of BKC-1 was detected by this methodology; however, different to what was previously observed by Martino et al. (2015), who tested only KPC-producing Enterobacteriaceae, our results showed that the test reading was easier after 3 hours of incubation to identify BKC-1–producing isolates.

BKC-1 encoding gene was reported only in Enterobacteriaceae isolated from the city of São Paulo, but we believe that this enzyme might be encountered in other Enterobacteriaceae species or geographic regions since this gene has been associated with *IS*Kpn23, an insertion sequence reported for the first time in *K. pneumoniae*, but commonly found among soil bacteria (Nicoletti et al., 2015). Poor hydrolytic activity of BKC-1 against carbapenems seems to be the main cause of MHT and AFB/DC negative results. This fact consists the principal challenge to clinical routine laboratories in screening BKC-1 producers. The difference on carbapenems MICs observed could be attributed to the presence of additional mechanisms of beta-lactam resistance detected in these isolates (Nicoletti et al., 2015). The identification of bla_{BKC} by molecular tests is the gold standard methodology for confirming its presence. However, adequate phenotypic tests are of fundamental importance for accurately screening BKC-1–producing isolates. Although a few number of isolates were tested in our study, due to the paucity of BKC-1–producing isolates described so far, our results showed that rapid tests, Carba-NP, Blue-Carba, and Carbapenembac™ were reliable for phenotypic detection of BKC production.

Conflict of interests

A.C.G. has recently received research funding and/or consultation fees from AstraZeneca, MSD, and Novartis. The others authors have nothing to declare.

Acknowledgments

We would like to thank the National Council for Science and Technological Development (CNPq) for providing financial support to A.C.G. (process number: 305535/2014-5).

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Please cite this article as: Martins WMBS, et al, Comparison of phenotypic tests for detecting BKC-1–producing Enterobacteriaceae isolates, Diagn Microbiol Infect Dis (2015), http://dx.doi.org/10.1016/j.diagmicrobio.2015.11.021

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