Diagnostic Microbiology and Infectious Disease 96 (2020) 114912

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

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

Performance of distinct phenotypic methods for carbapenemase detection: The influence of culture media

Jhonatha Rodrigo Cordeiro-Moura [a,1], Lorena Cristina Corrêa Fehlberg [a,*,1], Carolina Silva Nodari [b], Adriana Pereira de Matos [a], Vinicius de Oliveira Alves [a], Rodrigo Cayô [a,b], Ana Cristina Gales [a]

[a] Universidade Federal de São Paulo - UNIFESP, Laboratório Alerta, Division of Infectious Diseases, Departament of Internal Medicine, Escola Paulista de Medicina - EPM, São Paulo, - SP, Brazil
[b] Universidade Federal de São Paulo - UNIFESP, Laboratório de Imunologia e Bacteriologia - LIB, Setor de Biologia Molecular, Microbiologia e Imunologia, Departamento de Ciências Biológicas - DCB, Instituto de Ciências Ambientais, Químicas e Farmacêuticas - ICAQF, Diadema, - SP, Brazil

ARTICLE INFO

Article history:
Received 12 August 2019
Received in revised form 24 September 2019
Accepted 7 October 2019
Available online xxxx

Keywords:
MacConkey agar
Blue-Carba
CarbaNP
Modified Hodge test
CHDL
KPC

ABSTRACT

We evaluated the performance of five phenotypic tests [Modified Hodge Test (MHT); combined-disk test (CDT) using phenylboronic acid, EDTA, and cloxacillin; CarbaNP and CarbAcinetoNP; Blue-Carba, Carbapenembac™ and Carbapenembac Metallo™] for carbapenemase detection in Gram-negative bacilli (GNB). A total of 73 carbapenemase producers and 27 non-carbapenemase producers were tested. All GNB were subcultured onto Müeller-Hinton agar (MHA), MacConkey agar (MAC), and sheep blood agar (SBA). High sensitivity (100%) and specificity (100%) was observed for MHA using CarbaNP, Blue-Carba, and Carbapenembac™. The sensitivity and specificity of CarbaNP (98.6%/100%), Blue-Carba (97.1%/91.0%), and Carbapenembac™ (100%/96.5%) were slightly lower for SBA. In contrast, unacceptable sensitivity rates of CarbaNP (71.1%) and Blue-Carba (66.6%), but not Carbapenembac™ (97.3%), were observed for MAC. The colorimetric methods showed high sensitivity and specificity to detect carbapenemase production from isolates grown on MHA or SBA. However, colonies obtained from MAC must not be tested for carbapenemase detection by colorimetric methods.

1. Introduction

Infections caused by carbapenem-resistant Gram-negative bacilli (CR-GNB) are a major public health problem because they are associated with high mortality rates (Esterly et al. 2012). According to the Centers for Disease Control and Prevention (CDC), carbapenem-resistant *Enterobacteriales* can cause 9300 infections and 600 deaths per year in the USA (CDC 2013). The resistance to carbapenems in GNB is mainly associated with the production of distinct classes of carbapenemases and, in a lower extent, by overproduction of chromosomally encoded AmpC or production of extended-spectrum β-lactamases (ESβLs) coupled with outer membrane impermeability and/or overexpression of RND (Resistance-Nodulation-Division) efflux pumps (Jean et al. 2015).

CR-GNB are usually multidrug resistant (MDR) and, generally, polymyxins and tigecycline are drugs of last resort to treat the infections caused by such pathogens. Since therapeutic failure has been associated when a single *in vitro* active antimicrobial agent is prescribed, combination therapy is frequently recommended for the treatment of infections caused by these pathogens, especially for infections caused by KPC (Jean et al. 2015; Iovleva and Doi 2017). Thus, a reliable and rapid detection of carbapenemase production in GNB by microbiological routine laboratory is of crucial importance for adjustment of antimicrobial therapy and introduction of effective hospital infection control measures.

In this context, several phenotypic and genotypic tests have been developed for carbapenemase detection (Lutgring and Limbago 2016). Some phenotypic tests are based on double-disk synergy test (DDST) using specific β-lactamase inhibitors, chromogenic culture medium, carbapenem hydrolysis activity assays (pH change, spectrometry or electrochemical tests), or immunochromatographic assays (Glupczynski et al. 2016; Aguirre-Quiñonero and Martínez-Martínez 2017). However, the detection of specific group of carbapenemases may be affected depending on the sensitivity of the phenotypic test employed (Lutgring and Limbago 2016). Thus, the detection of carbapenemases by microbiological routine is a challenge due to the variety of enzymes produced by distinct bacterial species. These enzymes show distinct hydrolytic activity against carbapenems, and inhibition profiles by β-lactamase inhibitors (Iovleva and Doi 2017). In addition, reports of some carbapenemases showing low MICs for carbapenems due to mutations on carbapenemase encoding genes or variation in their genetic context can lead to low hydrolytic activity towards carbapenems making difficult their phenotypic detection (Oueslati et al. 2019).

* Corresponding author at: Tel./fax: +55-11-55715180.
E-mail address: lorenafehlberg@gmail.com (L.C.C. Fehlberg).
[1] Both authors have equally contributed to this work.

https://doi.org/10.1016/j.diagmicrobio.2019.114912
0732-8893/

Indeed, the knowledge about local epidemiology of each hospital is of crucial importance, as it will help in the selection of the best methodology for screening carbapenemase-producing GNB. Ideally, this test should be fast, cheap, easily performed, and highly accurate. Herein, we aimed to evaluate the performance of distinct phenotypic methods for carbapenemase detection using different culture media against a well characterized collection of carbapenemase-producing GNB.

2. Methodology

2.1. Bacterial isolates

A total of 73 previously characterized carbapenemase-producing GNB, including *Enterobacteriales* ($n = 51$), *Acinetobacter baumannii* ($n = 14$), and *Pseudomonas aeruginosa* ($n = 8$), showing resistance or reduced susceptibility to imipenem and/or meropenem (Table 1S), according to EUCAST breakpoints (http://www.eucast.org/) were selected for this study. Initially, all strains had their genotypes confirmed by PCR followed by amplicon sequencing. The carbapenemase activity was also checked by ertapenem hydrolysis assay using MALDI-TOF MS (Bruker Daltonic, Bremen, Germany), as previously published (Carvalhaes et al. 2013). In addition, 27 non-carbapenemase-producing GNB, *Enterobacteriales* ($n = 23$), *A. baumannii* ($n = 2$), and *P. aeruginosa* ($n = 2$), were included in this study as a negative controls and tested exactly in same conditions described below. All non-carbapenemase-producing GNB were susceptible to imipenem and meropenem, except three isolates of *K. pneumoniae* that were resistant to at least one carbapenem tested. These three isolates were ESBL producers and showed changes in outer membrane porins (Table 1S).

2.2. Phenotypic tests used for carbapenemase detection

Five distinct phenotypic methods were tested: (i) Modified Hodge Test (MHT); (ii) phenylboronic acid-, EDTA-, and cloxacillin-combined-disc tests (CTD); (iii) CarbaNP Test and its modification; (iv) Blue-Carba Test; and (v) Carbapenembac™ Test followed by Carbapenembac Metalo™ (Probac do Brasil, São Paulo, Brazil). MHT, CarbaNP, and Blue-Carba were performed, as previously described (Nordmann et al. 2012; Pires et al. 2013; CLSI 2015; Pasteran et al. 2015; CLSI 2016). For *A. baumannii* strains, the CarbAcinetoNP was tested, as previously described (Dortet et al. 2014a).

Phenylboronic acid-, EDTA-, and cloxacillin-CTD is a methodology standardized by the Brazilian Health Surveillance Agency – ANVISA for screening of carbapenemase-producing GNB (ANVISA 2013). Inhibitor solutions of 75 mg/mL cloxacillin, 40 mg/mL phenylboronic acid, and 0.1 M EDTA (Sigma-Aldrich, St. Louis, MO, USA) were previously sterilized using a 0.22 μm filter membrane (Millipore, Darmstadt, Germany) and stored at −20 °C. For the test, 10 μL of each inhibitor solution was added directly to 10 μg imipenem and 10 μg meropenem disks (Thermo Fisher Scientific, Basingstoke, UK) and let dry at room temperature. Imipenem and meropenem disks with and without inhibitor were placed onto Müeller-Hinton agar (Thermo Fisher Scientific, Basingstoke, UK) plates inoculated with a 0.5 McFarland bacterial suspension. The influence of each the beta-lactamase inhibitor itself on strains growth was

Table 1
Comparison of five phenotypic methodologies for carbapenemase detection according to culture medium and group of microorganisms.

Methodologies/Microorganisms (n° of isolates tested)	Sensitivity (%)			Specificity (%)			Positive Predictive Value (%)			Negative Predictive Value (%)		
	SBA	MAC	MHA	SBA	MAC	MHA	SBA	MAC	MHA	SBA	MAC	MHA
MHT												
All isolates (100)	nt	nt	87.3	nt	nt	100	nt	nt	100	nt	nt	74.3
Enterobacteriales (74)	nt	nt	92.1	nt	nt	100	nt	nt	100	nt	nt	85.2
A. baumannii (16)	nt	nt	71.4	nt	nt	100	nt	nt	100	nt	nt	33.4
P. aeruginosa (10)	nt	nt	83.4	nt	nt	100	nt	nt	100	nt	nt	50.0
CDT[a]												
All isolates (100)	95.1	nt	nt	71.0	nt	nt	84.3	nt	nt	90.0	nt	nt
Enterobacteriales (74)	94.1	nt	nt	100	nt	nt	100	nt	nt	88.4	nt	nt
A. baumannii (16)	100	nt	nt	15.4	nt	nt	21.4	nt	nt	100	nt	nt
P. aeruginosa (10)	100	nt	nt	100	nt	nt	100	nt	nt	100	nt	nt
CarbaNP Test[c]												
All isolates (100)	98.6	71.1	100	100	68.4	100	100	86.0	100	96.4	46.4	100
Enterobacteriaceae (74)	98.0	87.2	100	100	64.7	100	100	85.0	100	95.8	69.0	100
A. baumannii (16)	100	0.0	100	100	100	100	100	-[b]	100	100	20.0	100
P. aeruginosa (10)	100	60.0	100	100	-[b]	100	100	100	100	100	0.0	100
Blue-Carba Test												
All isolates (100)	97.2	69.5	100	90.5	66.6	100	97.2	100	100	90.5	17.6	100
Enterobacteriales (74)	98.0	90.6	100	88.9	100	100	96.1	100	100	94.1	25.0	100
A. baumannii (16)	92.8	10.0	100	100	100	100	100	100	100	66.7	18.2	100
P. aeruginosa (10)	100	50.0	100	100	-[b]	100	100	100	100	100	-[b]	100
Carbapenembac™												
All isolates (100)	100	97.6	100	96.3	88.9	100	98.6	96.0	100	100	92.3	100
Enterobacteriales (74)	100	100	100	100	87.0	100	100	94.5	100	100	100	100
A. baumannii (16)	100	85.7	100	50.0	100	100	93.4	100	100	100	50.0	100
P. aeruginosa (10)	100	100	100	100	100	100	100	100	100	100	100	100
Carbapenembac Metalo™												
All isolates (24)[d]	100	100	100	100	100	100	100	100	100	100	100	100
Enterobacteriales (13)	100	100	100	100	100	100	100	100	100	100	100	100
A. baumannii (3)	100	100	100	100	100	100	100	100	100	100	100	100
P. aeruginosa (8)	100	100	100	100	100	100	100	100	100	100	100	100

Abbreviations: SBA, sheep blood agar; MAC, MacConkey agar; MHA, Müeller-Hinton agar; nt, not tested.
[a] Phenylboronic acid/disk combination, EDTA/disk combination, cloxacillin/disk combination.
[b] Denominator was zero; not calculated.
[c] Including CarbAcineto NP results, when applicable.
[d] Values calculated only for metallo-β-lactamase-producing isolates.

evaluated by dropping the same inhibitor amount on Oxoid™ blank disks (Thermo Fisher Scientific, Basindstoke, UK). The inhibition zones were measured after incubation for 16–18 hours at 35 ± 2 °C. The results were interpreted as follows: (i) an increase of ≥5 mm inhibition zone diameter of imipenem and/or meropenem disks containing EDTA in comparison to those disks without EDTA was suggestive of metallo-β-lactamase (MβL) production; (ii) an increase of ≥5 mm inhibition zone diameter of imipenem and/or meropenem disks containing phenylboronic acid in comparison to the same disks without this inhibitor was suggestive of class A carbapenemase production; and (iii) an increase of ≥5 mm inhibition zone diameter of imipenem and/or meropenem disks containing phenylboronic acid and cloxacillin in comparison with the same disks without inhibitors was suggestive of AmpC production (ANVISA 2013).

Carbapenembac™ Test (Probac do Brasil, São Paulo, Brazil) was carried out as previously described with some modifications (Martino et al. 2015). This commercial method is based on carbapenem hydrolysis detection from strips impregnated with 100 μg of imipenem and starch. Initially, the isolates were suspended in a 1 mL of Müeller-Hinton broth pH 7.3 (Probac do Brasil, São Paulo, Brazil). The inoculum was adjusted to 10 McFarland scale, wherein 150 μL was inoculated over the strips and incubated at 35 ± 2 °C for 1 hour. Subsequently, 200 μL of an iodine solution was added over the strip that acquired a dark color. For carbapenamase-producing isolates, the strips became clear and the dark color disappeared. If the dark color persisted, it suggested a non-carbapenemase production. The reading time was approximately 30–60 minutes. In addition, positive isolates by Carbapenembac™ Test (Probac do Brasil, São Paulo, Brazil) were further screened for MβL production by testing another strip, Carbapenembac Metallo™ Test (Probac do Brasil, São Paulo, Brazil). The inoculum was adjusted to 10 McFarland scale in a 1 mL of Müeller-Hinton broth pH 7.3 supplemented with EDTA (Probac do Brasil, São Paulo, Brazil). The next steps were identical to those described for Carbapenembac™ Test. The strips remained dark when the tested isolates produced MβL (inhibition by EDTA), while they became clear when the isolates produced other groups of carbapenemases.

2.3. Culture medium

To evaluate if the type of culture medium could influence the phenotypic detection, CarbaNP, CarbAcinetoNP, Blue-Carba, Carbapenembac™, and Carbapenembac Metallo™ tests were performed by testing colonies grown on sheep blood agar (SBA; bioMeriéux, Rio de Janeiro, Brazil), Oxoid™ MacConkey agar (MAC; Thermo Fisher Scientific, Basingstoke, UK), and Oxoid™ Müeller-Hinton agar (MHA; Thermo Fisher Scientific, Basingstoke, UK). In order to simulate usual microbiology practices, bacterial lawn of MHA was used to prepare 0.5 McFarland suspension, which was used to further inoculation of other MHA plates in order to perform the MHT. On the other hand, colonies grown on SBA were used for the CDT as recommended by ANVISA. For MHT and CDT, all bacterial strains were grown on Oxoid™ MHA (Thermo Fisher Scientific, Basingstoke, UK) and SBA (bioMeriéux, Rio de Janeiro, Brazil), respectively (ANVISA 2013; CLSI 2016).

2.4. Statistical Analyses

The sensitivity, specificity, positive and negative predictive values (SN, SP, PPV and NPV, respectively) were calculated for each test, as previously described (Lalkhen and McCluskey 2008).

3. Results

The sensitivity and specificity rates, as well as the positive and negative predictive values calculated for all phenotypic tests, according to the bacterial species tested, were shown in Table 1. Between the agar based phenotypic tests CDT showed, in general, higher sensitivity rates (94.1–100.0%) for detecting carbapenemase-producing GNB than those observed for MHT (83.4–92.1%). In contrast, general specificity percentage demonstrated by CDT (71.0%) was lower than those of MHT (100.0%), especially for *A. baumannii* (15.4% *versus* 100.0%, respectively) as shown in Table 1. The positive predictive values (PPV) were 100% for both tests when Enterobacteriales and *P. aeruginosa* were tested. However, these values were highly discrepant for *A. baumannii* (100.0% *versus* 21.4% for MHT and CDT, respectively).

For all strains evaluated, CarbaNP and Blue-Carba tests showed very high and identical sensitivity and specificity rates (100% for both), independent of the bacterial species tested, when colonies were grown on MHA. When colonies were grown on SBA and directly tested by CarbaNP and Blue-Carba, a slight variation in the sensitivity and specificity rates was observed for both tests, Blue-Carba (97.2%/90.5%) and CarbaNP (98.6%/100%). In contrast, these tests were highly affected by the growth of bacterial isolates on MAC, with overall sensitivity of 69.5% for Blue-Carba. Similar results were also observed for CarbaNP Test, in which lower sensitivity (71.1%) and specificity (68.4%) rates were detected, as displayed in Table 1. Very low negative predictive values were observed for Blue-Carba (17.6%) and CarbaNP (46.4%) tests by picking colonies grown on MAC. Sensitivity rates of 0.0% and 10.0% for modified CarbaNP and Blue-Carba, respectively, were observed for *A. baumannii*. For *Enterobacteriales*, the sensitivity rates were higher for Blue-Carba test (90.6%) than that observed for CarbaNP (87.2%) when bacterial isolates were tested on MAC (Table 1).

For *Enterobacteriales* and *P. aeruginosa* subcultured in SBA and MHA, sensitivity and specificity were 100% when Carbapenembac™ and Carbapenembac Metallo™ were evaluated. All 24 MβL-producing strains included in this study were correctly detected, regardless of enzyme or bacterial species, by Carbapenembac Metallo™ (sensitivity and specificity rates, 100%). Low specificity (50.0%) rates were also obtained by Carbapenembac Metallo™ when *A. baumannii* strains subcultured onto SBA were tested (Table 1).

False negative, false positive or inconclusive results were considered discordant results and listed in Table 2. No false positive results were observed for MHT or CDT. In contrast, nine and 11 false negative results were detected by MHT and CDT when testing colonies grown on MHA or SBA, respectively. Among the nine discordant results observed for MHT, the majority occurred with *A. baumannii* and *K. pneumoniae* (four strains each). While 3 of 4 *K. pneumoniae* carried $bla_{\text{BKC-1}}$ and $bla_{\text{CTX-M-2}}$, the other one harbored $bla_{\text{KPC-2}}$ and $bla_{\text{CTX-M-15}}$. In addition, all *K. pneumoniae* showed loss of OmpK35 and/or OmpK36. The 4 *A. baumannii* isolates falsely identified as not producers of carbapenemases by MHT carried MβL ($bla_{\text{IMP-1}}$or $bla_{\text{IMP-10}}$) or CHDL ($bla_{\text{OXA-58}}$ or $bla_{\text{OXA-72}}$) encoding genes. The three BKC-1-producing *K. pneumoniae* (Strains KP60134, KP60135, and KP60136) were not identified as carbapenemase producers by CDT either. A higher number of *A. baumannii* carrying CHDL ($bla_{\text{OXA-23}}$, $bla_{\text{OXA-25}}$, $bla_{\text{OXA-26}}$, $bla_{\text{OXA-58}}$, $bla_{\text{OXA-72}}$, $bla_{\text{OXA-143}}$, or $bla_{\text{OXA-231}}$) encoding genes were not detected as carbapenemase producers by CDT either. Although three inconclusive results were observed when testing *P. aeruginosa* with MHT, two isolates carrying $bla_{\text{SPM-1}}$, and another one carrying $bla_{\text{OXA-18}}$ only; no inconclusive results were seen with CDT.

Fifty-one discordant results (6 false positives, 16 false negatives, and 29 inconclusives) were observed when CarbaNP was employed for detection of carbapanemase production. Discordant results occurred with all species tested mostly when they were recovered from MAC (98.0%) as can be observed in Table 2. In contrast, a single false negative result was observed for a *K. pneumoniae* strain carrying $bla_{\text{KPC-2}}$, $bla_{\text{CTX-M-15}}$, and $bla_{\text{TEM-1}}$ coupled with OmpK35 loss, which was subcultured onto SBA. Inconclusive results for CarbaNP were found in 29 strains (56.8%), being most of them *K. pneumoniae* ($n = 16$; 55.1%), followed by *A. baumannii* ($n = 6$; 20.7%), *P. aeruginosa* ($n = 5$; 17.2%), and *E. coli* ($n = 2$; 6.9%). All carbapanemase-producing *A. baumannii* strains ($n = 16$) subcultured onto MHA or SBA were correctly identified by CarbaNP. However, when this pathogen was subcultured onto MAC

Table 2
Discordant results obtained by the five phenotypic methodologies tested for the detection of carbapenemase production.

Methodologies (total of discordant isolates)	Species (n° of isolates)	Genotypic profile (n° of isolates/strain number)	Culture medium
MHT[a, b, c] **(12)**			
False positives (0)			
False negatives (9)	*A. baumannii* (4)	bla_{OXA-51} + bla_{IMP-1} (1/A18680)	MHA
		bla_{OXA-51} + bla_{IMP-10} (1/A3101)	MHA
		bla_{OXA-51} + bla_{OXA-58} (1/A45063)	MHA
		bla_{OXA-51} + bla_{OXA-72} (1/A38939)	MHA
	K. pneumoniae (4)	ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{TEM-1} (1/K1–1)	MHA
		ΔOmpK35/36 + bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (1/KP60136)	MHA
		ΔOmpK36+ bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (2/KP60134, KP60135)	MHA
	P. aeruginosa (1)	bla_{SPM-1} (1/P4265)	MHA
Inconclusive (3)	*P. aeruginosa* (3)	bla_{SPM-1} + bla_{OXA-56} + $bla_{OXA-50h}$ (2/P1088, P3448)	MHA
		bla_{OXA-18} (1/91–2)	MHA
CDT[a,b] **(14)**			
False positives (0)			
False negatives (14)	*A. baumannii* (11) [c]	bla_{OXA-51} + bla_{OXA-72} (3/83, 383, A38939)	SBA
		bla_{OXA-51} + bla_{OXA-23} (2/A142, 54)	SBA
		bla_{OXA-51} + bla_{OXA-23} + $bla_{OXA-143}$ (1/A41902)	SBA
		bla_{OXA-51} + $bla_{OXA-231}$ (1/31)	SBA
		bla_{OXA-51} + $bla_{OXA-143}$ (1/81)	SBA
		bla_{OXA-51} + bla_{OXA-25} (1/327009)	SBA
		bla_{OXA-51} + bla_{OXA-26} (1/4737)	SBA
		bla_{OXA-51} + bla_{OXA-58} (1/A45063)	SBA
	K. pneumoniae (3)	ΔOmpK36+ bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (2/KP60134, KP60135)	SBA
		ΔOmpK35/36 + bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (1/KP60136)	SBA
Inconclusive (0)			
CarbaNP Test **(51)**			
False positives (6)	*K. pneumoniae* (6)	ΔOmpK36 + bla_{SHV-11} (2/A35870, A51281)	MAC
		ΔOmpK35/36 + $bla_{CTX-M-15}$ + bla_{TEM-1} + bla_{SHV-11} + bla_{OXA-1} + *qnrS1* (1/KPN-1)	MAC
		ΔOmpK36 + bla_{LEN-3} (1/A58243)	MAC
		ΔOmpK36 + bla_{SHV-27} (1/A58238)	MAC
		ΔOmpK36 + bla_{SHV-28} (1/A58236)	MAC
False negatives (16)	*A. baumannii* (8)	bla_{OXA-51} + bla_{OXA-72} (3/83, 383, A38939)	MAC
		bla_{OXA-51} + bla_{OXA-23} (2/A142, 54)	MAC
		bla_{OXA-51} + bla_{OXA-23} + $bla_{OXA-143}$ (1/A41902)	MAC
		bla_{OXA-51} + bla_{IMP-10} (1/A3101)	MAC
		bla_{OXA-51} + bla_{OXA-58} (1/A45063)	MAC
	K. pneumoniae (6)	ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{TEM-1} (1/K1–1)	SBA
		ΔOmpK36 + bla_{IMP-1} (3/A13005, A13006, A13007)	MAC
		bla_{KPC-2} (1/336417)	MAC
		bla_{KPC-2} + bla_{TEM-1} + bla_{SHV-11} + $bla_{CTX-M-2}$ (1/A28008)	MAC
	P. aeruginosa (2)	bla_{SPM-1} (1/P4265)	MAC
		bla_{SPM-1} + bla_{OXA-56} + $bla_{OXA-494}$ (1/P12117)	MAC
Inconclusive (29)	*K. pneumoniae* (16)	ΔOmpK36 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{SHV-11} (2/A43178, A54970)	MAC
		ΔOmpK35/36 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{SHV-11} (1/A54972)	MAC
		ΔOmpK36 + bla_{IMP-1} (3/A13008, A13009, A11775)	MAC
		ΔOmpK36 + bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (2/KP60134, KP60135)	MAC
		ΔOmpK35/36 + bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (1/KP60136)	MAC
		ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{TEM-1} (1/K1–1)	MAC
		ΔOmpK36 + bla_{SHV-11} (1/A58237)	MAC
		ΔOmpK36 + bla_{SHV-36} (1/A35557)	MAC
		ΔOmpK36 + bla_{LEN-25} (1/A58240)	MAC
		ΔOmpK36 + $bla_{LEN-like}$ (1/A35174)	MAC
		bla_{KPC-2} + bla_{TEM-1}+ bla_{SHV-12}+ $bla_{SHV-110}$ + bla_{OXA-9}+ $bla_{CTX-M-2}$ (1/KP-13)	MAC
		bla_{NDM-1} (1/ATCC 2146)	MAC
	A. baumannii (6)	bla_{SIM-1} (1/YNC03/9/T104)	MAC
		bla_{OXA-51} + bla_{IMP-1} (1/A18680)	MAC
		bla_{OXA-51} + bla_{OXA-25} (1/327009)	MAC
		bla_{OXA-51} + bla_{OXA-26} (1/4737)	MAC
		bla_{OXA-51} + $bla_{OXA-143}$ (1/81)	MAC
		bla_{OXA-51} + $bla_{OXA-231}$ (1/31)	MAC
	P. aeruginosa (5)	bla_{SPM-1} (1/346161)	MAC
		bla_{SPM-1} + bla_{OXA-56} + $bla_{OXA-50h}$ (2/P8281, P7790)	MAC
		bla_{OXA-18} (1/91–2)	MAC
		susceptible isolate (1/ATCC 27853™)	MAC
	E. coli (2)	susceptible isolates (2/413703, 418,386–2)	MAC
Blue-Carba Test **(68)**			
False positives (2)	*K. pneumoniae* (2)	ΔOmpK35/36 + $bla_{CTX-M-15}$ + bla_{TEM-1} + bla_{SHV-11}+ bla_{OXA-1} (2/KPN-1, KPN-2)	SBA
False negatives (15)	*A. baumannii* (9)	bla_{OXA-51} + bla_{IMP-10} (1/A3101)	MAC/SBA
		bla_{OXA-51} + bla_{OXA-72} (3/83, 383, A38939)	MAC
		bla_{OXA-51} + bla_{OXA-23} + $bla_{OXA-143}$ (1/A41902)	MAC
		bla_{OXA-51} + bla_{OXA-23} (1/A142)	MAC
		bla_{OXA-51} + bla_{OXA-25} (1/327009)	MAC
		bla_{OXA-51} + bla_{OXA-58} (1/A45063)	MAC
		bla_{OXA-51} + bla_{IMP-1} (1/A18680)	MAC

Table 2 *(continued)*

Methodologies (total of discordant isolates)	Species (n° of isolates)	Genotypic profile (n° of isolates/strain number)	Culture medium
	K. pneumoniae (4)	ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{TEM-1} (1/K1–1)	SBA
		bla_{KPC-2} + bla_{TEM-1} + bla_{SHV-11} + $bla_{CTX-M-2}$ (1/A28008)	MAC
		ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{SHV-11} (2/530, 535)	MAC
	P. aeruginosa (2)	bla_{SPM-1} (2/P4265, P1088)	MAC
Inconclusive (51)	*K. pneumoniae* (37)	ΔOmpK36 + bla_{SHV-62} (1/A58242)	MAC/SBA
		ΔOmpK36 + bla_{SHV-36} (1/A35557)	MAC/SBA
		ΔOmpK36 + bla_{LEN-25} (1/A58240)	MAC/SBA
		ΔOmpK36 + bla_{SHV-11} (1/A58237)	MAC/SBA
		ΔOmpK36 + bla_{IMP-1} (6/A13005, A13006, A13007, A13008, A13009, A11775)	MAC
		ΔOmpK36 + bla_{SHV-11} (2/A35870, A51281)	MAC
		bla_{KPC-2} (1/K2–61)	MAC
		ΔOmpK36 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{SHV-11} (2/A43178, A54970)	MAC
		ΔOmpK35/36 + bla_{KPC-2} + $bla_{CTX-M-15}$ + bla_{SHV-11} (1/A54972)	MAC
		ΔOmpK35/36 + $bla_{CTX-M-15}$ + bla_{TEM-1} + bla_{SHV-11} + bla_{OXA-1} (2/KPN-1, KPN-2)	MAC
		ΔOmpK36 + $bla_{CTX-M-2}$ + bla_{TEM-1} + bla_{SHV-11} (1/609)	MAC
		ΔOmpK35/36 + bla_{TEM-1} + bla_{SHV-11} (1/7461)	MAC
		ΔOmpK35/36 + bla_{KPC-2} + $bla_{CTX-M-44}$ + bla_{TEM-1} + bla_{SHV-11} (1/A54973)	MAC
		ΔOmpK36 + bla_{KPC-2} + $bla_{CTX-M-44}$ + bla_{TEM-1} + bla_{SHV-11} (1/A62271)	MAC
		ΔOmpK35 + bla_{KPC-2} + bla_{TEM-1} + $bla_{CTX-M-15}$ (1/K1–1)	MAC
		ΔOmpK36 + $bla_{CTX-M-2}$ + bla_{SHV-11} (1/7008)	MAC
		ΔOmpK36 + bla_{KPC-2} + bla_{SHV-32} (1/A54974)	MAC
		ΔOmpK35 + bla_{KPC-2} + $bla_{CTX-M-2}$ + bla_{SHV-11} (1/532)	MAC
		ΔOmpK36 + bla_{LEN-3} (1/A58243)	MAC
		ΔOmpK36 + bla_{SHV-27} (1/A58238)	MAC
		ΔOmpK36 + bla_{SHV-28} (1/A58236)	MAC
		ΔOmpK36 + $bla_{LEN-like}$ (1/A35174)	MAC
		ΔOmpK36 + bla_{BKC-1} + $bla_{CTX-M-2}$ + $bla_{SHV-110-like}$ (1/KP60135)	MAC
		bla_{SHV-11} (1/A51282)	MAC
		bla_{KPC-2} + bla_{TEM-1} + bla_{SHV-12} + $bla_{SHV-110}$ + bla_{OXA-9} + $bla_{CTX-M-2}$ (1/Kp13)	MAC
		bla_{KPC-2} + bla_{TEM-1}+ $bla_{CTX-M-2}$ + bla_{SHV-12} + bla_{SHV-11} (1/28009)	MAC
		bla_{SHV-11} (1/A58300)	MAC
		OmpK35/36 (1/194)	MAC
		bla_{NDM-1} (1/ATCC 2146™)	MAC
	P. aeruginosa (6)	bla_{SPM-1} + bla_{OXA-56} + $bla_{OXA-494}$ (1/P3448)	MAC/SBA
		bla_{SPM-1} (1/346161)	MAC/SBA
		bla_{OXA-18} (1/91–2)	MAC/SBA
		bla_{SPM-1} + bla_{OXA-56} + $bla_{OXA-494}$ (1/P8281, P7790)	MAC
		susceptible isolate (1/27853)	MAC
	A. baumannii (4)	bla_{OXA-51} + bla_{OXA-23} (1/54)	MAC
		bla_{OXA-51} + bla_{OXA-26} (1/4737)	MAC
		bla_{OXA-51} + $bla_{OXA-143}$ (1/81)	MAC
		bla_{OXA-51} + $bla_{OXA-231}$ (1/31)	MAC
	E. coli (3)	susceptible isolates (3/413703, 418,386–2, 25,922)	MAC
	E. cloacae (1)	susceptible isolate (1/100)	MAC/SBA
***CarbapenemBac™* (6)**			
False positives (4)	*K. pneumoniae* (3)	ΔOmpK36 + $bla_{CTX-M-2}$ + bla_{SHV-11} (1/7008)	MAC
		ΔOmpK36 + bla_{SHV-11} (1/A51281)	MAC
		OmpK35/36 (1/194)	MAC
	A. baumannii (1)	bla_{OXA-51} (1/A70043)	SBA
False negatives (2)	*A. baumannii* (2)	bla_{OXA-51} + bla_{OXA-58} (1/A45063)	MAC
		bla_{OXA-51} + $bla_{OXA-143}$ (1/81)	MAC

Abbreviations: SBA, sheep blood agar; MAC, MacConkey agar; MHA, Müeller-Hinton agar; nt, not tested.

[a] Isolates were grown on SBA for MHT and disk combination test (CDT) according with the guidelines.

[b] Phenylboronic acid/disk combination, EDTA/disk combination, cloxacillin/disk combination.

[c] These isolates were erroneously identified as MβL-producers.

agar, 14 strains showed discordant results, with 8 strains identified as false-negative carbapenemase producers and 6 strains showing inconclusive results (Table 2). Among 68 discordant results observed with Blue-Carba, 51 (75.0%) strains presented inconclusive results. Most of them belonged to *Enterobacteriales* ($n = 41$; 80.4%), mainly *K. pneumoniae* ($n = 37$; 90.2%). All 51 inconclusive results were observed from colonies subcultured onto MAC, being eight (15.7%) also subcultured onto SBA simultaneously (four *K. pneumoniae*, three *P. aeruginosa*, and one *E. cloacae*). All false-positive results ($n = 2$) were obtained by *K. pneumoniae* subcultured onto SBA. All 15 false negative results were observed with strains subcultured onto MAC, except for a *K. pneumoniae* strain subcultured onto SBA. This strain carried bla_{KPC-2}, $bla_{CTX-M-15}$, and bla_{TEM-1} coupled with OmpK35 loss. The *A. baumannii* strain A3101 carrying bla_{IMP-10} was not identified as carbapenemase producer when subcultured on both MAC and SBA (Table 2). The number of discordant results observed for Carbapenembac™ ($n = 6$) was lower in comparison to those obtained with other methodologies evaluated in the present study. Only four false positive results were observed with this test, namely the *A. baumannii* strain A70043, when subcultured onto SBA, and 3 *K. pneumoniae* (7008, A51281, and 194), subcultured onto MAC. In addition, two false negative results were seen for *A. baumannii* strains carrying bla_{OXA-58} and $bla_{OXA-143}$, both subcultured onto MAC. Carbapenembac Metallo™ test correctly detected all MβL-producing strains ($n = 24$; Table 2).

Comparing all groups of carbapenemases tested (Table 3), the detection rates were high for KPC-2, ranging from 97.1% to 100% independent of the tested methodology when the strains were subcultured on SBA or

Table 3
Comparison of five phenotypic methodologies according to each culture medium according to group of carbapenemases tested.

Methodologies/resistance genes (n° of isolates)	Culture medium	Discordant results (n° of isolates)	False positives (n° of isolates)	False negatives (n° of isolates)	Inconclusive (n° of isolates)	% of isolates correctly identified as carbapenemase producers
Modified Hodge Test						
KPC (35)	MHA	1	0	1	0	97.1
MβL (23)	MHA	5	0	3	2	78.2
CHDL (12)	MHA	2	0	2	0	83.3
BKC-1 (3)	MHA	3	0	3	0	0.0
CDT[a]						
KPC (35)	SBA	0	0	0	0	100
MβL (23)	SBA	0	0	0	0	100
CHDL (12)	SBA	11	11	0	0	8.4
BKC-1 (3)	SBA	3	0	3	0	0.0
CarbaNP Test[b]						
KPC (35)	MHA	0	0	0	0	100
	SBA	1	0	1	0	97.1
	MAC	7	0	2	5	80.0
MβL (23)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	15	0	6	9	34.8
CHDL (12)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	11	0	7	4	8.4
BKC-1 (3)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	3	0	0	3	0.0
Blue-Carba Test						
KPC (35)	MHA	0	0	0	0	100
	SBA	1	0	1	0	97.1
	MAC	14	0	3	11	60.0
MβL (23)	MHA	0	0	0	0	100
	SBA	3	0	1	2	86.7
	MAC	15	0	4	11	34.8
CHDL (12)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	11	0	7	4	8.4
BKC-1 (3)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	1	0	0	1	66.7
Carbapenembac™						
KPC (35)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	0	0	0	0	100
MβL (23)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	0	0	0	0	100
CHDL (12)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	2	0	2	0	83.4
BKC-1 (3)	MHA	0	0	0	0	100
	SBA	0	0	0	0	100
	MAC	0	0	0	0	100

Abbreviations: SBA, sheep blood agar; BKC, Brazilian *Klebsiella* carbapenemase; KPC, *Klebsiella pneumoniae* carbapenemase; MβL, metallo-β-lactamase; CHDL, carbapenem-hydrolyzing class D β-lactamase; MAC, MacConkey agar; MHA, Müeller-Hinton agar.
[a] Phenylboronic acid/disk combination, EDTA/disk combination, cloxacillin/disk combination.
[b] Including CarbAcineto NP results.

MHA. Similar results were observed for MβL-producing strains, except for MHT (78.2%) and Blue-Carba (86.7%), when tested directly from MHA and SBA, respectively. One hundred percent of Class D carbapenemases were detected by Carbapenembac™ and colorimetric methods when colonies were grown on SBA or MHA. In contrast, only 8.4% of Class D carbapenemases were correctly identified by CarbaNP or Blue-Carba Test when bacterial colonies were cultivated onto MAC. In general, the performance of colorimetric methods was also lower than expected for screening other classes of β-lactamases when colonies were obtained from MAC. All *K. pneumoniae* harboring $bla_{\text{BKC-1}}$ were correctly identified as carbapenemase producers by CarbaNP, Blue-Carba Test and Carbapenembac™ by testing colonies from MHA or SBA; however, only Carbapenembac™ was able to identify the BKC-1-producing isolates as carbapenemase producers when colonies were obtained from MAC. It is interesting to notice that neither MHT nor CDT were able to identify BKC-1-producing *K. pneumoniae* as carbapenemase producers. In contrast, although 83.3% of Class D carbapenemase producers were correctly detected by MHT, only 8.4% were by CDT.

4. Discussion

Carbapenems used to be the first line for the empirical treatment against non-fermenting GNB and *Enterobacteriales* in countries where

the frequency of ESβL was high, like in Brazil (Sampaio and Gales 2016). However, the emergence and spread of epidemic carbapenemase-producing lineages in the nosocomial setting, such as the KPC-2-producing *K. pneumoniae* CC258 clone, has jeopardized the clinical use of carbapenems (Iovleva and Doi 2017). Therefore, the detection of carbapenemases in clinical isolates has been of fundamental importance. It can be accomplished by performing several assays, which are mainly based on the hydrolytic activity of carbapenemase towards carbapenems. Rapid diagnostic tests have the potential to reduce time for correctly introduction of antimicrobial treatment for infections caused by such pathogens and to improve local surveillance and rapid implementation of infection control measures (Lutgring and Limbago 2016).

The MHT and the CDT are two phenotypic methods based on disk diffusion agar. The MHT was the first developed phenotypic method to detect carbapenemases, initially described in 2009 (CLSI 2009). It was recommended only for *Enterobacteriales* isolates showing elevated MICs or reduced inhibition zones by disk diffusion to carbapenems (CLSI 2016). However, MHT is not able to distinguish the type of carbapenemase. Previous studies shown that MHT was a reliable test only to detect KPC or OXA-48 enzymes, but demonstrated high false negative results for NDM-1-producing isolates or isolates producing weak carbapenemases (Carvalhaes et al. 2010; Hrábak et al. 2014; Sampaio and Gales 2016). In contrast, the class of carbapenemase can be distinguished by using distinct inhibitors such as EDTA, phenylboronic acid and/or cloxacillin on CDT. In fact, the Brazilian Surveillance Health Agency has recommended this test for detection of carbapenemase producing isolates by routine clinical labs (ANVISA 2013). However, both MHT and CTD have long turnaround times, at least 18 hours and, in this study, were not able to detect BKC-1-producing *K. pneumoniae* isolates. This is maybe one of the reasons why BKC-1-producing isolates have been unfrequently detected in Brazil.

In the present study, 3 *P. aeruginosa* strains, including two coproducers of SPM-1, OXA-56, and OXA-494 (formerly OXA-50 h), as well as one strain producing OXA-18, showed inconclusive results by MHT due to irregular growth. It is important to notice that MHT is not recommended for *P. aeruginosa* (CLSI 2016). Indeed, 97.1% of KPC-2-producing *Enterobacteriales* strains evaluated in the present study were correctly identified by MHT. In contrast, only 83.3% and 78.2% of Class D- or MβL-producing isolates (including *Enterobacteriales, P. aeruginosa* and *A. baumannii*) were phenotypically identified as carbapenemase producers with such methodology. All three NDM-1-producing *K. pneumoniae* tested in this study were correctly detected by MHT (Martins et al. 2016). Previous studies also reported false positive results by MHT with isolates that coproduced ESβL coupled with outer membrane impermeability (Carvalhaes et al. 2010). Although we did not observe such result in the present study, four *K. pneumoniae* strains that coproduced ESβL, a carbapenemase, and also had porin alteration showed false negative results by MHT. Similar results were observed for three *K. pneumoniae* strains with the same genetic background by CDT. False negative results were obtained by MHT for four *A. baumannii* strains, producers of IMP-1, IMP-10, OXA-58, and OXA-72. As expected, 11 *A. baumannii* strains producing distinct class D carbapenemases were erroneously identified as MβL producers by CTD. These isolates showed an increase in the inhibition zones of imipenem and meropenem disks in the presence of EDTA. Previous studies demonstrated that some class D enzymes existed as a highly active dimer at high concentration and as a less active monomer at low concentration (Danel et al. 2001). As divalent cations make the dimeric structure of some OXA enzymes more stable, a chelator agent would affect the activity of such enzymes. Therefore, chelators, such as EDTA, could have influenced the activity of these enzymes, causing the misidentification of such isolates as MβL producers by EDTA-based phenotypic methods (Danel et al. 2001). This fact could justify the lowest specificity (15.4%) exhibited by CDT for *A. baumannii*. This is also one of the reasons why ANVISA has recommended the use of the CTD test only for *Enterobacteriales*.

Even testing a modification of CarbaNP, CarbAcinetoNP, against *A. baumannii* strains, this test was not good enough for phenotypic detection of carbapenemases when *A. baumannii* isolates were subcultured onto MAC. The same finding was observed, when Blue-Carba was tested. This finding is very important because MAC is commonly employed by routine labs for identification of GNB. In fact, previous studies reported that growth on MAC influenced negatively the detection of carbapenemases (Dortet et al. 2014b). Ramos and colleagues also evaluated by ertapenem hydrolysis assay using MALDI-TOF MS 61 carbapenemase-producing GNB subcultured onto MHA, SBA, MAC, and CPS agar. The authors reported that MAC influenced the results obtained with *A. baumannii* isolates producing distinct class D carbapenemases (OXA-25, OXA-26, and OXA-72) after 4 hours of incubation (Ramos et al. 2016).

Low rates of carbapenemase detection were observed with CarbAcinetoNP (SN, 8.4%) and Blue-Carba (SN, 8.4%) when MAC was used as growth medium, and by CDT performed with colonies recovered from SBA (SN, 8.4%). Strains subcultured onto MAC and MHA tested against Carbapenembac™ and MHT showed similar performance (83.3% and 83.4%, respectively). These results could be explained, in part, by previous studies that described the influence of MAC and the class D carbapenemase structures (Danel et al. 2001; Anderson et al. 2012; Martins et al. 2013; Dortet et al. 2014a). In addition, detection of MβL- and KPC-2-producing strains by CarbaNP and Blue-Carba was also affected when they were subcultured on MAC.

The Carbapenembac™ and Carbapenembac Metallo™ were the best phenotypic tests employed for carbapenemase detection independently of bacterial species or culture medium tested. Our results corroborated with those previously described by Martino and colleagues that showed 100% of sensitivity and specificity among 100 *Enterobacteriales* clinical isolates, mostly KPC-2 producers (Martino et al. 2015). Recently, the Brazilian Committee on Antimicrobial Susceptibility Testing (BrCAST) conducted a multicenter study with a collection of carbapenemase-producing GNB (Gales et al. 2017). Each participate center tested blindly all strains subcultured on MHA, SBA, and CPS agar for Blue-Carba, CarbaNP, Rapidec CarbaNP, and Carbapenembac™. The sensitivity rates for KPC and MβL detection was >90% for Carbapenembac™. However, the sensitivity for other enzymes belonging to classes A and D was very low in comparison with our results (~47.0%). In addition, SBA showed a better performance (54.3%) compared to the results obtained with the other two culture medium tested (Gales et al. 2017).

A high number of inconclusive results, based on slight change of color, was observed for Blue-Carba (75.0%) and CarbaNP (56.8%) tests. However, this phenomenon occurred mainly among those strains that were not carbapenemase producers and when subcultured on MAC. Previous studies demonstrated that OXA-48 did not hydrolyze carbapenems as efficiently as KPC or MβL enzymes do. For this reason, screening of OXA-48 based on carbapenem susceptibility profile is challenging (Iovleva and Doi 2017). A large number of OXA-48- and OXA-48-like producing *Enterobacteriales* ($n = 45$) was included in a previous study that aimed to evaluate the performance of Rapidec CarbaNP® and β-Carba™ Test (Mancini et al. 2017). Among them, 15 OXA-48 producers were correctly identified by Rapidec CarbaNP® and β-Carba™ Test, while both tests were able to detect OXA-48-like production in 24/31 and 6/31 strains, respectively. The authors described that extending the period of incubation in 2 hours significantly increased the correct detection of OXA-48-like enzymes by β-Carba™ Test (Mancini et al. 2017). Similar results were observed in the present study for class D carbapenemases and BKC-1, which were tested using a 2 h incubation period. As observed for OXA-48, BKC-1 is a class A enzyme that exhibits a low carbapenemase activity (Nicoletti et al. 2015). Although OXA-370 was correctly identified by all tests evaluated in the present study, the carbapenem hydrolysis activity of BKC-1 was only detected by Carbapenembac™ test when bacterial colonies were grown on MAC. However, in both cases, the number of strains evaluated was very low. For classes A (except BKC-1) and B carbapenemases, the

detection occurred within 30 minutes of incubation. Recently, an easy and accurate immunochromatographic assay, RESIST-4 O.K.N.V. K-seT (Coris BioConcept, Gembloux, Belgium), has become commercially available for carbapenemase detection. This test is able to identify in a few minutes the presence of bla_{VIM}-like, bla_{KPC}-like, bla_{NDM}-like, and bla_{OXA-48}-like (Greissl et al. 2019). However, this test is expensive to be employed as a screening methodology in some developing countries. In addition, it was recently reported that some KPC variants like KPC-28, which confer resistance to ceftazidime/avibactam but not to carbapenems, continues to be identified as carbapenemase producers by this assay. While genotypic methods still identify bla_{KPC}, phenotypic methods are more sensitive noticing that carbapenemase activity of this variant was lost. Clinically this observation is very important because carbapenems would be probably dismissed as therapeutic options if only genotypic tests were employed (Oueslati et al. 2019).

In conclusion, Carbapenembac™ was the only phenotypic test able to detect 100% of carbapenemase-producing isolates regardless of the media where colonies were grown. Overall CarbaNP and Blue-Carba showed high performance in detecting carbapenemase-producing isolates subcultured on SBA or MHA. Our results corroborate the findings of previous studies indicating that colonies grown on MAC cannot be directly used for testing by colorimetric tests. It was previously suggested that the accumulation of lactic acid in the bacterial isolates that were able to ferment lactose would interfere in the performance of CarbaNP (Lee et al. 2014; Dortet et al. 2014b). As many routine labs perform surveillance cultures for screening carbapenem-resistant Gram-negative bacilli by testing carbapenem disks on MAC plates (Richter and Marchaim 2017), it would be very practical if these colonies could be directly used for preparation of inoculum solution. However, the high number of discordant results obtained when colonies were grown on MAC indicates that this practice must be avoided. Although there are innumerous methodologies available for carbapenemase detection, some of them have limitations to be included in the microbiological routine, such as high cost and the need of technical expertise. Currently, new β-lactamases inhibitors, as avibactam, are available for treatment of infections caused by Class A or OXA-48 carbapenemase-producing isolates. Since such compounds present distinct activities according to type of carbapenemase produced, the correct identification of such enzymes is mandatory. Therefore, we must be careful and reasonable to choose the best methodology according to the local epidemiology and the reality of each microbiology routine.

Acknowledgements

We are grateful to the Probac® do Brazil for providing the Carbapenembac™ and Carbapenembac Metallo™ kits. We also would like to thank the technical support of all students from Laboratório ALERTA and Laboratório Especial de Microbiologia Clínica (LEMC), Universidade Federal de São Paulo (UNIFESP).

Transparency declarations

A.C.G. recently received research funding and/or consultation fees from Bayer, Eurofarma, MSD, Pfizer, and Zambon. Other authors have nothing to declare. This study has not been financially supported by any Diagnostic/Pharmaceutical company.

Funding

We would like to thank the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) for providing a grant to LCCF (Process number: 2014/12224–3), the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) for providing grants to A.P.M. (PROEX-CAPES), and C.S.N. (DS-CAPES), and to the National Council for Science and Technological Development (CNPq) for providing grants to J.R.C-M. and to A.C.G. (Process number: 305535/2014–5).

Ethical approval

Ethical approval for this study was obtained from Research Ethics Committee from Federal University of São Paulo – UNIFESP/São Paulo Hospital (Process number: 7033200616).

References

Aguirre-Quiñonero A, Martínez-Martínez L. Non-molecular detection of carbapenemases in *Enterobacteriaceae* clinical isolates. J Infect Chemother 2017;23:1–11.

Anderson NW, Buchan BW, Riebe KM, et al. Effects of solid-medium type on routine identification of bacterial isolates by use of matrix-assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2012;50:1008–13.

Brazilian Health Surveillance Agency (ANVISA). Medidas de prevenção e controle de infecções por enterobactérias multirresistentes. Brasília, Brazil: ANVISA; 2013.

Carvalhaes CG, Picão RC, Nicoletti AG, et al. Cloverleaf test (modified Hodge test) for detecting carbapenemase production in *Klebsiella pneumoniae*: be aware of false positive results. J Antimicrob Chemother 2010;65:249–51.

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.

Centers for Disease Control and Prevention (CDC). U.S. Department of health and human services. Antibiotic resistance threats in the United States, 2013.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing nineteen informational supplement. Document M100-S19. Wayne, PA, 2009.

Clinical and Laboratory Standards Institute (CLSI). Performance standards for antimicrobial susceptibility testing; twenty-six informational supplement. Document M100-S26. Wayne, PA, 2016.

Clinical Laboratory Standard Institute (CLSI). Methods for dilution antimicrobial susceptibility test for bacteria that grow aerobically - tenth edition: Approved standard M7-A10. CLSI. Wayne, PA: USA; 2015.

Danel F, Frére JM, Livermore DM. Evidence of dimerization among class D beta lactamases: kinetics of OXA-14 beta lactamase. Biochim Biophys Acta 2001;1546:132–42.

Dortet L, Poirel L, Errera C, et al. CarbAcineto NP test for rapid detection of carbapenemase-producing *Acinetobacter* spp. J Clin Microbiol 2014a;52:2359–64.

Dortet L, Bréchard L, Poirel L, Nordmann P. Impact of the isolation medium for detection of carbapenemase-producing Enterobacteriaceae using an updated version of the Carba NP test. J Med Microbiol 2014b;63:772–6.

Esterly JS, Wagner J, McLaughlin MM, et al. Evaluation of clinical outcomes in patients with bloodstream infections due to gram-negative bacteria according to carbapenem MIC stratification. Antimicrob Agents Chemother 2012;56:4885–90.

Gales AC, Barth A, Carvalho-Assef APD et al. Inter-laboratorial Reproducibility of Phenotypic Methods for Detection of Carbapenemase-producing *Enterobacteriaceae*: A Real-Life Scenario. In: 27th European Congress of Clinical Microbiology and Infectious Diseases- ECCMID 2017, Vienna.

Glupczynski Y, Evrard S, Ote I, et al. Evaluation of two new commercial immunochromatographic assays for the rapid detection of OXA-48 and KPC carbapenemases from cultured bacteria. J Antimicrob Chemother 2016;71:1217–22.

Greissl C, Saleh A, Hamprecht A. Rapid detection of OXA-48-like, KPC, NDM, and VIM carbapenemases in *Enterobacteriales* by a new multiplex immunochromatographic test. Eur J Clin Microbiol Infect Dis 2019;38:331–5.

Hrábak J, Chudáckóva E, Papagiannitsis CC. Detection of carbapenemases in *Enterobacteriaceae*: a challenge for diagnostic microbiological laboratories. Clin Microbiol Infect 2014;20:839–53.

Iovleva A, Doi Y. Carbapenem-resistant *Enterobacteriaceae*. Clin Lab Med 2017;37:303–15.

Jean SS, Lee WS, Lam C, et al. Carbapenemase-producing gram-negative bacteria: current epidemics. antimicrobial susceptibility and treatment options Future Microbiol 2015;10:407–25.

Lalkhen AG, McCluskey A. Clinical tests: sensitivity and specificity. Contin Educ Anaesth Crit Care Pain 2008;8:221–3.

Lee LY, Korman TM, Graham M. Rapid time to results and high sensitivity of the CarbaNP test on early cultures. J Clin Microbiol 2014;52:4023–6.

Lutgring JD, Limbago BM. The problem of Carbapenemase-producing-Carbapenem-resistant-*Enterobacteriaceae* detection. J Clin Microbiol 2016;54:529–34.

Mancini S, Kieffer N, Poirel L, et al. Evaluation of the Rapidec CarbaNP® and β-Carba® tests for rapid detection of Carbapenemase-producing *Enterobacteriaceae*. Diagn Microbiol Infect Dis 2017;88:293–7.

Martino MD, Koga PC, Pasternak J, et al. Evaluation of a new rapid test for carbapenemase detection in carbapenemase resistant *Enterobacteriaceae*. J Microbiol Methods 2015;115:20–1.

Martins AF, Borges A, Pagano M, et al. False positive results in screening for metallo-beta-lactamase are observed in isolates of *Acinetobacter baumannii* due to production of oxacillinases. Braz J Infect Dis 2013;17:500–1.

Martins WMBS, Nicoletti AG, Santos S, et al. Frequency of BKC-1-producing *Klebsiella* species isolates. Antimicrob Agents Chemother 2016;60:5044–6.

Nicoletti AG, Marcondes MF, Martins WM, et al. Characterization of BKC-1 class a carbapenemase from *Klebsiella pneumoniae* clinical isolates in Brazil. Antimicrob Agents Chemother 2015;59:5159–64.

Nordmann P, Poirel L, Dortet L. Rapid detection of carbapenemase-producing *Enterobacteriaceae*. Emerg Infect Dis 2012; 18: 1503–157.

Oueslati S, Iorga BI, Tlili L, et al. Unravelling ceftazidime/avibactam resistance of KPC-28, a KPC-2 variant lacking carbapenemase activity. J Antimicrob Chemother 2019;74:2239–46.
Pasteran F, Veliz O, Ceriana P, et al. Evaluation of the blue-Carba test for rapid detection of carbapenemases in gram-negative bacilli. J Clin Microbiol 2015;53:1996–8.
Pires J, Novais A, Peixe L. Blue-Carba, an easy biochemical test for detection of diverse carbapenemase producers directly from bacterial cultures. J Clin Microbiol 2013;51:4281–3.
Ramos AC, Carvalhaes CG, Cordeiro-Moura JR, et al. Influence of culture media on detection of Carbapenem hydrolysis by matrix- assisted laser desorption ionization-time of flight mass spectrometry. J Clin Microbiol 2016;54:1896–8.
Richter SS, Marchaim D. Screening for carbapenem-resistant Enterobacteriaceae: who, when, and how? Virulence 2017;8:417–26.
Sampaio JL, Gales AC. Antimicrobial resistance in Enterobacteriaceae in Brazil: focus on β-lactams and polymyxins. Braz J Microbiol 2016;47:31–7.