






# Detecting KPC-2 and NDM-1 Coexpression in *Klebsiella pneumoniae* Complex from Human and Animal Hosts in South America

 Felipe Vásquez-Ponce,<sup>a</sup> Karine Dantas,<sup>a</sup> Johana Becerra,<sup>a</sup> Gregory Melocco,<sup>b</sup> Fernanda Esposito,<sup>b</sup> Brenda Cardoso,<sup>a</sup> Larissa Rodrigues,<sup>a</sup> Keila Lima,<sup>b</sup> Aline V. de Lima,<sup>b</sup> Fábio P. Sellera,<sup>c,d</sup> Renata Mattos,<sup>e</sup> Lucas Trevisoli,<sup>e</sup> Marco A. Vianello,<sup>f</sup> Thais Sincero,<sup>g</sup> Jose Di Conza,<sup>h</sup> Eliana Vespero,<sup>i</sup>  Gabriel Gutkind,<sup>h</sup> Jorge Sampaio,<sup>b,j</sup>  Nilton Lincopan<sup>a,b</sup>

<sup>a</sup>Department of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil

<sup>b</sup>Department of Clinical Analysis, School of Pharmacy, Universidade de São Paulo, São Paulo, Brazil

<sup>c</sup>Department of Internal Medicine, School of Veterinary Medicine and Animal Science, Universidade de São Paulo, São Paulo, Brazil

<sup>d</sup>School of Veterinary Medicine, Metropolitan University of Santos, Santos, Brazil

<sup>e</sup>Laborclin, Pinhais, Brazil

<sup>f</sup>Natal Garrison Hospital, Brazilian Army, Natal, Brazil

<sup>g</sup>Department of Clinical Analysis, Health Sciences Center, Federal University of Santa Catarina, Florianópolis, Brazil

<sup>h</sup>Facultad de Farmacia y Bioquímica, Instituto de Investigaciones en Bacteriología y Virología Molecular, Universidad de Buenos Aires, Buenos Aires, Argentina

<sup>i</sup>Department of Pathology, Clinical and Toxicological Analysis, Health Sciences Center, University Hospital of Londrina, Paraná, Brazil

<sup>j</sup>Fleury Medicine and Health, Microbiology Section, São Paulo, Brazil

Felipe Vásquez-Ponce and Karine Dantas contributed equally to this article. Author order was determined in order of decreasing seniority.

**ABSTRACT** Reports of Gram-negative bacteria harboring multiple carbapenemase genes have increased in South America, leading to an urgent need for appropriate microbiological diagnosis. We evaluated phenotypic methods for detecting *Klebsiella pneumoniae* carbapenemase 2 (KPC-2) and New Delhi metallo- $\beta$ -lactamase-1 (NDM-1) coexpression in members of the *K. pneumoniae* complex (i.e., *K. pneumoniae*, *K. quasipneumoniae*, and *K. variicola*) isolated from human and animal hosts, based on inhibition of ceftazidime-avibactam (CZA) and aztreonam (ATM) by dipicolinic acid (DPA), EDTA, or avibactam (AVI). While the presence of *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> genes was confirmed by whole-genome sequencing, PCR, and/or GeneXpert, coexpression was successfully detected based on the following: (i) a  $\geq 5$ -mm increase in the zone diameter of ATM (30  $\mu$ g) disks plus AVI (4 or 20  $\mu$ g) and  $\geq 4$ -mm and  $\geq 10$ -mm increases in the zone diameters for “CZA 50” (30  $\mu$ g ceftazidime [CAZ] and 20  $\mu$ g AVI) and “CZA 14” (10  $\mu$ g CAZ and 4  $\mu$ g AVI) disks, respectively, when we added DPA (1 mg/disk) or EDTA (5 mM) in a combined disk test (CDT); (ii) a positive ghost zone (synergism) between ATM (30  $\mu$ g) and CZA 50 disks and between CZA 50 and DPA (1 mg) disks, using the double-disk synergy test (DDST) at a disk-disk distance of 2.5 cm; (iii)  $\geq 3$ -fold MIC reductions of ATM and CZA in the presence of AVI (4  $\mu$ g/mL), DPA (500  $\mu$ g/mL), or EDTA (320  $\mu$ g/mL); and (iv) immunochromatography. Although our results demonstrated that inhibition by AVI, DPA, and EDTA may provide simple and inexpensive methods for the presumptive detection of coexpression of KPC-2 and NDM-1 in members of the *K. pneumoniae* complex, additional studies are necessary to confirm the accuracy of these methodologies by testing other Gram-negative bacterial species and other KPC and NDM variants coexpressed by WHO critical priority pathogens detected worldwide.

**IMPORTANCE** Alerts regarding the emergence and increase of combinations of carbapenemases in *Enterobacteriales* in Latin America and the Caribbean have recently been issued by PAHO and WHO, emphasizing the importance of appropriate microbiological diagnosis and the effective and articulated implementation of infection prevention and control programs. In this study, we evaluated methods based on

**Editor** Maria Antonia De Francesco, Institute of Microbiology, University of Brescia

**Copyright** © 2022 Vásquez-Ponce et al. This is an open-access article distributed under the terms of the [Creative Commons Attribution 4.0 International license](https://creativecommons.org/licenses/by/4.0/).

Address correspondence to Felipe Vásquez-Ponce, felipe.vasquez@icb.usp.br, or Nilton Lincopan, lincopan@usp.br.

The authors declare no conflict of interest.

**Received** 4 April 2022

**Accepted** 12 July 2022

**Published** 18 August 2022

inhibition of ceftazidime (CAZ), ceftazidime-avibactam (CZA), and aztreonam (ATM) by dipicolinic acid (DPA), EDTA, and avibactam (AVI) inhibitors for the identification of KPC-2- and NDM-1-coexpression in members of the *K. pneumoniae* complex recovered from human and animal hosts. Our results demonstrate that inhibition by AVI, DPA, and EDTA may provide simple and inexpensive methods for the presumptive detection of coexpression of KPC-2 and NDM-1 in members of the *K. pneumoniae* complex.

**KEYWORDS** carbapenemases, coproduction, avibactam, aztreonam, *K. quasipneumoniae*, *K. variicola*, combined disk test, disk approximation test, immunochromatography

**D**uring the COVID-19 pandemic, the incidence of carbapenem-resistant *Enterobacteriales* (CRE) has increased in South America and the Caribbean (1, 2). In fact, according to an epidemiological alert of the Pan American Health Organization (PAHO) in October 2021, coexpression of different classes of carbapenemases are expanding in different countries (3). In this regard, coproduction of *Klebsiella pneumoniae* carbapenemase (KPC) and New Delhi metallo- $\beta$ -lactamase-1 (NDM-1) seems to be the major threat to public health (1, 3, 4).

Coproduction of KPC-2 and NDM-1 in South America was first detected in Brazil in members of the *Enterobacter cloacae* complex in 2013 (5). Noteworthy, from 2020 to 2021, coexpression of these enzymes was extended among *K. pneumoniae* isolates from Argentina, Uruguay, Ecuador, and Paraguay (3), and lately this coexpression has been detected in hospital sewage samples in Brazil (6).

KPC belongs to class A carbapenemases, which share a serine residue at their active site that confers hydrolytic properties (7) that can be inhibited by avibactam (AVI), vaborbactam, and relebactam (8), whereas NDM enzymes belong to class B metallo-beta-lactamases (M $\beta$ Ls), which depend on Zn<sup>2+</sup> in their catalytic site (9) and can be inhibited by ethylenediaminetetraacetic (EDTA) and dipicolinic acid (DPA) (10). Strikingly, M $\beta$ Ls are unable to hydrolyze aztreonam (ATM) (11). As a result, bacterial species that produce NDM-type M $\beta$ Ls exhibit *in vitro* susceptibility to this antibiotic. However, despite aztreonam not being hydrolyzed by M $\beta$ Ls, frequently such isolates harbor additional cephalosporinases, like AmpC and extended-spectrum  $\beta$ -lactamases (ESBLs).

Phenotypic methods to detect carbapenemases have been based on the use of inhibitors, where an increase in size of the inhibition zone of carbapenem-containing disks is observed by using combined disk (CDT) methods (12, 13), whereas the presence of a ghost zone (synergism) between carbapenem-containing disks and inhibitor-containing disks can be observed by using the double-disk synergy test (DDST) (14). Additionally, production of carbapenemases can be evaluated quantitatively based on the reduction of carbapenem MICs in the presence of specific inhibitors (15). In brief, a modified carbapenem inactivation test (mCIM), colorimetric methods (with Carba NP or Blue Carba), or inhibition tests using synergy with boronic acid or EDTA have also been routinely used, as recommended by CLSI or EUCAST guidelines (16, 17).

In this study, we evaluated methods based on inhibition of ceftazidime (CAZ), ceftazidime-avibactam (CZA), and ATM by DPA, EDTA, and AVI inhibitors for the identification of KPC and NDM coexpression by *K. pneumoniae* complex members (i.e., *K. pneumoniae*, *K. quasipneumoniae*, and *K. variicola*) recovered from human and animal hosts in South America.

## RESULTS

**Coproduction of NDM-1 and KPC-2 and KPC variants conferring resistance to ceftazidime-avibactam among *K. pneumoniae* complex members.** Fifteen *K. pneumoniae*-related species, including *K. pneumoniae*, *K. quasipneumoniae*, and *K. variicola*, displaying resistance to broad-spectrum cephalosporins and ceftazidime-avibactam were identified in human and animal hosts (Table 1). Regarding carbapenem resistance, all isolates were resistant to ertapenem, imipenem, and meropenem, with the exception of

**TABLE 1**  $\beta$ -Lactam resistance profiles and carbapenemases and cephalosporinases produced by *Enterobacteriales* used in this study

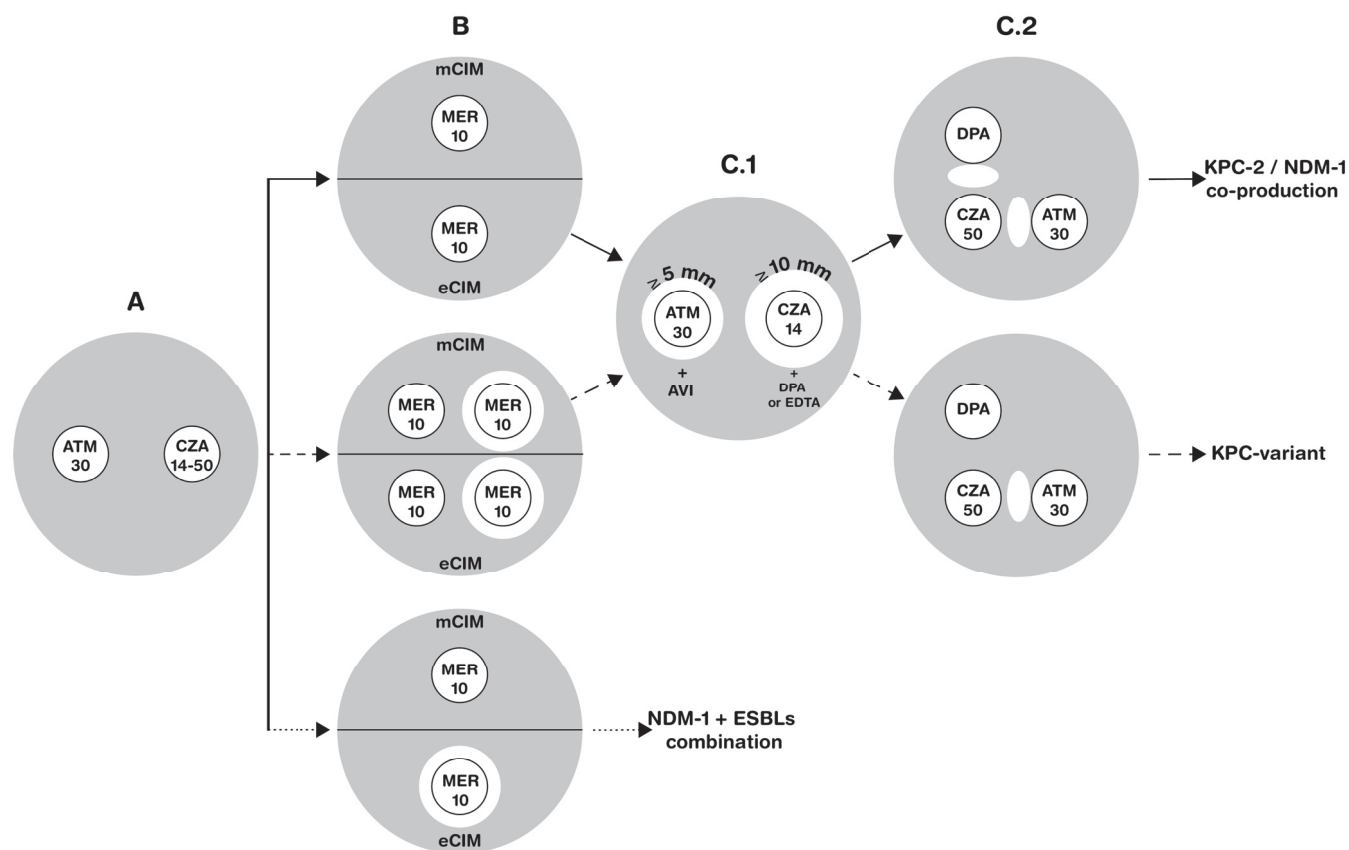
Strain (ST) <sup>a</sup>	Origin (yr)	Country	$\beta$ -Lactam resistance profile <sup>b</sup>	Carbapenemase(s) <sup>c</sup>	Cephalosporinase(s) <sup>d</sup>
<i>K. pneumoniae</i> Kp9417 (ST147)	Human (2021)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	CTX-M-15
<i>K. pneumoniae</i> Kp9270 (ST147)	Human (2021)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. quasipneumoniae</i> 795b (ST1308)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	CTX-M-15
<i>K. quasipneumoniae</i> 868 (ST1308)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, CZA, MER, CZA	KPC-2, NDM-1	CTX-M-15
<i>K. quasipneumoniae</i> 883b (ST1308)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, CZA, MER, CZA	KPC-2, NDM-1	None
<i>K. quasipneumoniae</i> FAI130 (ST1308)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. quasipneumoniae</i> FAI131 (ST1308)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	CTX-M-15
<i>K. variicola</i> L221385 (ND)	Human (2019)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. pneumoniae</i> 14A (ST437)	Human (2018)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. pneumoniae</i> 435AR (ND)	Human (2019)	Argentina	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. pneumoniae</i> 338AR (ND)	Human (2019)	Argentina	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-2, NDM-1	None
<i>K. pneumoniae</i> MV931658 (ST11)	Human (2019)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER	KPC-3	None
<i>K. pneumoniae</i> MV940851 (ST11)	Human (2019)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, MER, CZA	KPC-31	None
<i>K. pneumoniae</i> 330 (ST16)	Human (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	KPC-113	None
<i>K. pneumoniae</i> 331 (ST11)	Human (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, CZA	KPC-114	None
<i>K. pneumoniae</i> IBL2.4 (ST11)	Environment (2013)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER	KPC-2	None
<i>C. freundii</i> PG4 (ST214)	Animal (2020)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ETP, IPM, MER, CZA	NDM-1	CMY-48
<i>K. pneumoniae</i> Kp183 (ST1639)	Human (2017)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	NDM-1	CTX-M-15
<i>E. coli</i> 2ECMBL (ST155)	Human (2017)	Peru	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM, ETP, IPM, MER, CZA	NDM-1	PER-2
<i>K. pneumoniae</i> PRETA (ST307)	Animal (2018)	Brazil	AMC, CEF, CRO, CAZ, CTX, CFO, CPM, ATM	None	CTX-M-15, SHV-28
<i>E. coli</i> Em1cro (ST457)	Animal (2016)	Brazil	AMC, CEF, CFO, ATM	None	CMY-2

<sup>a</sup>ST, sequence type predicted by MLST 2.0 (<https://cge.food.dtu.dk/services/MLST/>); ND, not determined.

<sup>b</sup>Resistance profile determined by disk diffusion, Vitek 2, or broth microdilution methods. AMC, amoxicillin-clavulanic acid; CEF, cephalothin; CRO, ceftriaxone; CAZ, ceftazidime; CTX, cefotaxime; CFO, ceftiofex; CPM, cefepime; ATM, aztreonam; ETP, ertapenem; IPM, imipenem; MER, meropenem; CZA, ceftazidime-avibactam.

<sup>c</sup>Detected by PCR, GeneXpert, immunochromatography, and/or WGS.

<sup>d</sup>Detected by PCR and/or WGS.



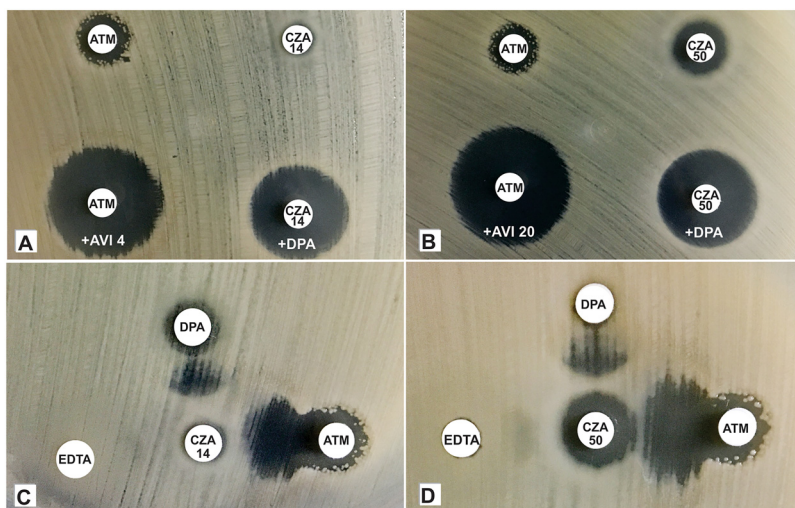
**FIG 1** Workflow proposed for identification of KPC-2 and NDM-1 coexpression in members of the *K. pneumoniae* complex. (A) Isolates displaying resistance to ATM (30  $\mu\text{g}/\text{disk}$ ) and CZA 14 (ceftazidime at 10  $\mu\text{g}/\text{disk}$ , avibactam at 4  $\mu\text{g}/\text{disk}$ ) or 50 (ceftazidime 30  $\mu\text{g}/\text{disk}$ , avibactam 20  $\mu\text{g}/\text{disk}$ ) were submitted for mCIM and eCIM tests. (B) Isolates coexpressing KPC-2 and NDM-1 exhibited positive mCIM and negative eCIM results (solid arrow). Variable mCIM and eCIM results are indicative of the presence of KPC variants conferring resistance to CZA (dashed arrow). Positive mCIM and eCIM results indicated the presence of NDM-1 and ESBL coexpression (dotted arrow). (C.1) In the combined disk test (CDT), a  $\geq 5$  mm increase in the zone diameter of ATM (30  $\mu\text{g}$ ) disks plus AVI (4 or 20  $\mu\text{g}$ ), and a  $\geq 4$  or  $\geq 10$  mm increase in the zone diameter of CZA 50 and CZA 14 disks, respectively, when added DPA (1 mg/disk) or EDTA (5 mM) was added, was indicative of KPC-2 and NDM-1 coproduction or a KPC variant conferring resistance to CZA. (C.2) By using the double-disk synergy test (DDST), a positive ghost zone (synergism) between ATM (30  $\mu\text{g}$ ) and CZA (50  $\mu\text{g}$ ) disks and between CZA (50  $\mu\text{g}$ ) and DPA (1 mg) disks, at a disk-disk distance of 2.5 cm, was indicative of KPC-2 and NDM-1 coproduction (solid arrow), whereas a positive ghost zone between ATM (30  $\mu\text{g}$ ) and CZA (50  $\mu\text{g}$ ) disks alone was indicative of KPC variant conferring resistance to CZA (dashed arrow).

*K. pneumoniae* strains 331 (susceptible to imipenem and meropenem) and MV940851 (susceptible to imipenem).

Initially, metallo- $\beta$ -lactamase and serine carbapenemase production was screened by using mCIM and EDTA-modified carbapenem inactivation (eCIM) (Fig. 1). In this regard, while 13 *Klebsiella* spp. showed mCIM<sup>+</sup> eCIM<sup>-</sup> results, 2 *K. pneumoniae* strains displayed an indeterminate result (i.e., mCIM<sup>-</sup> eCIM<sup>-</sup>). It is important to emphasize that eCIM is not an accurate method to detect suspected coproduction of class A and class B carbapenemases, as it only detects M $\beta$ Ls if both the mCIM and eCIM are positive, whereas mCIM<sup>+</sup> eCIM<sup>+</sup> results may be caused by NDM or M $\beta$ Ls plus AmpC.

Strikingly, immunochromatography revealed coproduction of NDM- and KPC-type carbapenemases in 11 members of the *K. pneumoniae* complex, whereas 2 of 4 CZA-resistant *K. pneumoniae* strains displayed positive bands for KPC production alone (see Table S1 in the supplemental material). Coproduction of NDM and KPC was confirmed by PCR and/or GeneXpert, and further genomic analysis predicted *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub> genes. On the other hand, genomic analysis of four CZA-resistant *K. pneumoniae* strains confirmed the presence of *bla*<sub>KPC-3</sub>, *bla*<sub>KPC-31</sub>, *bla*<sub>KPC-113</sub>, and *bla*<sub>KPC-114</sub> variants. Expression of KPC-31 and KPC-114 was not detected by immunochromatography.

**Detection of KPC-2 and NDM-1 coexpression by the combined disk test.** For the CDT, with different EDTA and DPA concentrations tested, 5 mM EDTA/disk and 1,000  $\mu\text{g}$



**FIG 2** Positive results of a combined disk test (CDT) and double-disk synergy test (DDST) for *K. pneumoniae* strain FAI131 coproducing KPC-2 and NDM-1 carbapenemases. (A) A  $\geq 5$ -mm increase in the zone diameter of an ATM (30  $\mu\text{g}$ ) disk plus AVI (4  $\mu\text{g}$ ) and of a  $\geq 10$ -mm increase in the zone diameter of a CZA 14 disk plus DPA (1 mg/disk) was observed in the CDT. (B) A  $\geq 5$ -mm increase in the zone diameter of ATM (30  $\mu\text{g}$ ) disk plus AVI (20  $\mu\text{g}$ ) and of a  $\geq 4$ -mm increase in the zone diameter of a CZA 50 disk plus DPA (1 mg/disk) was observed in the CDT. (C) A positive ghost zone (synergism) between ATM (30  $\mu\text{g}$ ) and CZA (14  $\mu\text{g}$ ) disks, between CZA (14  $\mu\text{g}$ ) and DPA (1 mg) disks, and a negative ghost zone between CZA (14  $\mu\text{g}$ ) and EDTA (5 mM) disks, at a disk-disk distance of 2.5 cm, was observed in the DDST. (D) A positive ghost zone (synergism) between ATM (30  $\mu\text{g}$ ) and CZA (50  $\mu\text{g}$ ) disks, between CZA (50  $\mu\text{g}$ ) and DPA (1 mg) disks, and a negative ghost zone between CZA (50  $\mu\text{g}$ ) and EDTA (5 mM) disks, at a disk-disk distance of 2.5 cm, was observed in the DDST.

DPA/disk were chosen for inhibition activity of  $M\beta L$ , since these concentrations showed no inhibitory activity across the bacterial growth of all screened isolates when sterile blank disks impregnated with 10  $\mu\text{L}$  of 0.1 M EDTA and 10 mg/mL DPA were tested. On the other hand, in order to detect expression of NDM enzymes, ceftazidime-avibactam at 14  $\mu\text{g}$ /disk (10  $\mu\text{g}$  CAZ and 4  $\mu\text{g}$  AVI; "CZA 14") and 50  $\mu\text{g}$ /disk (30  $\mu\text{g}$  CAZ and 20  $\mu\text{g}$  AVI; "CZA 50") were used, in accordance with guidelines for disk-diffusion antimicrobial susceptibility tests of EUCAST and CLSI, respectively.

For KPC-2-positive NDM-1-positive *K. pneumoniae* complex isolates, an increase of  $\geq 10$  mm in the size of inhibition zones were observed around CZA 14 disks containing 10  $\mu\text{L}$  of 0.1 M EDTA or 10  $\mu\text{L}$  of 10-mg/mL DPA, in comparison to the inhibition zones of CZA disks without EDTA or DPA. Otherwise, for the same KPC-2<sup>+</sup> NDM-1<sup>+</sup> isolates, increases of  $\geq 4$  mm in the size of inhibition zones were observed around CZA 50 disks containing 10  $\mu\text{L}$  of 0.1 M EDTA or 10  $\mu\text{L}$  of 10-mg/mL DPA, in comparison to the inhibition zones of CZA disks without EDTA or DPA. Additionally, for KPC-2 and NDM-1 coproducers, increases of  $\geq 5$  mm in the size of inhibition zones were observed around the ATM 30- $\mu\text{g}$  AVI 4- $\mu\text{g}$  disks, in comparison to the inhibition zones of ATM disks without AVI (Fig. 2A and B). Exceptionally, two CZA-resistant *K. pneumoniae* isolates (strains 330 and 331), which were NDM-1 negative and carried *bla*<sub>KPC-113</sub> or *bla*<sub>KPC-114</sub> gene variants, exhibited an increase of  $\geq 10$  mm in the inhibition zones around CZA-DPA and displayed no increase in the inhibition zones around CZA-EDTA disks. On the other hand, the *bla*<sub>KPC-31</sub>-positive *K. pneumoniae* strain MUV940851 displayed an increase of  $\geq 10$  mm in the inhibition zones around CZA-DPA and CZA-EDTA disks, while no increase around the ATM-AVI disk was detected, supporting the prediction that coproduction of KPC-2 and NDM-1 must be based on a positive synergistic effect shown using both CZA-EDTA and ATM-AVI disk combinations and not a single combination in the CDT test.

While for ATM-AVI disks a negative synergistic activity was expected against the KPC-31



producer, due to its resistance to CZA, positive synergistic activities against KPC-113- and KPC-114-producing *K. pneumoniae* strains suggested that combinations of monobactams and AVI produced inhibitory effects on some KPC variants, in a similar way as for NDM-type carbapenemases. In this respect, since AVI is able to covalently bind to some bacterial penicillin-binding proteins (PBPs), synergistic activity of ATM-AVI against KPC-113 and KPC-114 may be related with different activities on multiple PBP targets (18). It is important also to emphasize that despite there being an “*in vitro* synergy” between ATM-CZA disks in KPC variants resistant to CZA, this doesn't mean that the combination is clinically active. Finally, synergistic activity of CZA-DPA against KPC-113- and KPC-114-producing *K. pneumoniae* strains deserves additional investigation.

#### Detection of KPC-2 and NDM-1 coexpression by the double-disk synergy test.

For all KPC-2<sup>+</sup> NDM-1<sup>+</sup> isolates ( $n = 11$ ), a positive ghost zone (synergism) was observed between CZA 50 and ATM disks and between CZA 50 and DPA disks, with disks positioned at a disk-disk distance of 2.5 cm (Table 2; Fig. 2D). On the other hand, while all KPC-2<sup>+</sup> NDM-1<sup>+</sup> isolates exhibited a ghost zone between CZA 14 and DPA disks (Fig. 2C), only 9 KPC-2<sup>+</sup> NDM-1<sup>+</sup> isolates exhibited a positive ghost zone between CZA 14 and ATM disks, with disks positioned at a disk-disk distance of 2.5 cm (Table 2). For all KPC-2<sup>+</sup> NDM-1<sup>+</sup> isolates, a negative ghost zone between EDTA and CZA 14 disks was observed, whereas 4 KPC-2 and NDM-1 coproducing isolates showed a positive ghost zone between EDTA and CZA 50 disks, with disks positioned at a disk-disk distance of 2.5 cm (Table 2). Otherwise, while 5 KPC-2 and NDM-1 coproducing isolates showed a positive ghost zone between CZA 14 and EDTA disks, 8 KPC-2 and NDM-1 coproducing isolates showed a positive ghost zone between CZA 50 and EDTA disks at a disk-disk distance of 1.5 cm (Table 2).

Although NDM-1<sup>+</sup> CTX-M-15<sup>+</sup> *K. pneumoniae* KP183 and NDM-1<sup>+</sup> PER-2<sup>+</sup> *Escherichia coli* 2ECMBL control strains displayed a positive ghost zone between CZA and ATM and between CZA and DPA disks, it is very important to highlight that this positive DDST result was related to ESBL production, since these enzymes hydrolyze ATM, which is inhibited by AVI. In fact, the positive mCIM and eCIM results displayed by these strains confirmed production of M $\beta$ L alone, as it was not necessary to perform CDT and DDST for NDM-1 KPC-2 coproduction. Therefore, for both CDT and DDST interpretation, we highly recommended the following conditions: (i) ATM and CZA resistance is observed; (ii) KPC-2 and NDM-1 coproducers are mCIM<sup>+</sup> and eCIM<sup>-</sup>, and (iii) KPC variants conferring resistance to CZA could be susceptible to meropenem, displaying an indeterminate mCIM and eCIM result, as interpreted by CLSI guidelines. All CDT and DDST results are summarized in Table 2; see also Table S2.

**Reduction of aztreonam and ceftazidime-avibactam MICs in the presence of AVI, EDTA, or DPA as an indicator of KPC-NDM coproduction.** For MIC reduction assays, the final concentrations of EDTA, DPA, and AVI were fixed at 320, 500, and 4  $\mu$ g/mL, respectively, since these concentrations produced no antibacterial activity against any screened isolates, allowing us to observe a  $\geq 3$ -fold decrease in ATM and CZA MICs among NDM-1 and KPC-2 coproducers in the presence of inhibitors. In Table 3 and Table S3, results of reproducible replicates, performed three times on three distinct occasions, are shown.

## DISCUSSION

The emergence of carbapenem-resistant clinical isolates has become a serious clinical challenge due to the limited treatment options, and the coproduction of multiple carbapenemases by isolates aggravates this issue. There are only limited effective antibiotics against such strains. Combinations of CZA with meropenem and colistin seem to show potential synergism against these isolates. On the other hand, combinations of ATM plus meropenem-vaborbactam or plus CZA have demonstrated synergy against M $\beta$ L and ATM-resistant NDM-producing *Enterobacterales*. Thus, the combination of aztreonam plus avibactam appears to be a promising option against *Enterobacterales* isolates coproducing class A and class B  $\beta$ -lactamases while awaiting development of new antimicrobials (19–24).

Epidemiological alerts have been released by PAHO and WHO in view of the

**TABLE 2** Detection of KPC-2 and NDM-1 coproduction in *K. pneumoniae* complex-related species

Strain	Resistance <sup>a</sup>				CDT <sup>b</sup>				DDST <sup>c</sup>				mCIM <sup>d</sup> eCIM <sup>d</sup>				
	EUCAST		CLSI		ATM + AVI 4		ATM + AVI 20		CZA 14 + DPA		CZA 50 + DPA			CZA 14 + EDTA		CZA 50 + EDTA	
	ATM	CZA 14	ATM	CZA 50	ATM	AVI 4	ATM	AVI 20	CZA 14	DPA	CZA 14	DPA		EDTA	EDTA	EDTA	EDTA
<i>K. pneumoniae</i> Kp9417	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> Kp9270	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. quasipneumoniae</i> 795b	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. quasipneumoniae</i> 868	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. quasipneumoniae</i> 883b	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. quasipneumoniae</i> FA1130	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. quasipneumoniae</i> FA1131	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. varicola</i> L221385	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> 14A	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> 435AR	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> 338AR	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> MV931658	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> MV940851	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> 330	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> 331	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>K. pneumoniae</i> IBL2.4	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>C. freundii</i> PG4	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	
<i>K. pneumoniae</i> Kp183	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>E. coli</i> 2ECMBL	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	
<i>K. pneumoniae</i> PRETA	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	Gray	
<i>E. coli</i> /Em1cro	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	White	

<sup>a</sup>Gray squares indicate resistance. White squares indicate susceptibility. ATM, aztreonam; CZA-14, ceftazidime at 10 µg/disk and avibactam at 4 µg/disk; CZA 50, ceftazidime at 30 µg/disk and avibactam at 20 µg/disk.

<sup>b</sup>Gray squares indicate a positive result for the test; white squares indicate a negative result for the test. A ≥4-mm or ≥10-mm increase in the zone diameter of CZA 50 and CZA 14 disks, respectively, in the presence of DPA (1 mg/disk) or EDTA (5 mM) was interpreted as a positive CDT result. ATM, aztreonam; AVI 4, avibactam 4 µg/disk; AVI 20, avibactam 20 µg/disk; CZA 14, ceftazidime at 10 µg/disk and avibactam at 4 µg/disk; CZA 50, ceftazidime 30 µg/disk and avibactam 20 µg/disk; DPA, dipicolinic acid.

<sup>c</sup>DDST, double-disk synergy test. Gray squares indicate a positive result for the test; white squares indicate a negative result for the test. A positive ghost zone (synergism) between ATM (30 µg) and CZA (14 or 50 µg) disks, CZA and DPA (1 mg) disks, and CZA and EDTA (5 mM) disks was interpreted as a positive DDST result. CZA (14 or 50) disks were placed 2.5 cm center to center from DPA (1,000 µg), EDTA (5 mM), and ATM (30) disks. \*, EDTA (5 mM) and CZA (14 or 50) disks tested at 1.5 cm center to center.

<sup>d</sup>Modified carbapenem inactivation (mCIM) and EDTA-modified carbapenem inactivation (eCIM) tests. Gray squares indicate a positive result for the test. White squares indicate a negative result for the test.

**TABLE 3** MIC reductions induced by AVI, DPA, and EDTA for detection of NDM-1 and KPC-2 coproduction in *K. pneumoniae* complex-related species

Strain	MIC ( $\mu\text{g/mL}$ ) <sup>a</sup>					
	ATM	ATM + AVI	CAZ	CZA	CZA + DPA	CZA + EDTA
<i>K. pneumoniae</i> Kp9417	>256	0.25	>256	>256	0.5	0.25
<i>K. pneumoniae</i> Kp9270	>256	0.25	>256	>256	0.25	0.25
<i>K. quasipneumoniae</i> 795b	>256	0.25	>256	>256	0.25	0.125
<i>K. quasipneumoniae</i> 868	>256	0.25	>256	>256	0.25	0.25
<i>K. quasipneumoniae</i> 883b	>256	0.25	>256	256	0.125	1
<i>K. quasipneumoniae</i> FAI130	>256	0.25	>256	>256	0.25	0.25
<i>K. quasipneumoniae</i> FAI131	>256	0.25	>256	>256	0.25	0.25
<i>K. variicola</i> L221385	>256	0.25	>256	>256	0.25	0.25
<i>K. pneumoniae</i> 14A	8	0.25	>256	>256	0.5	0.25
<i>K. pneumoniae</i> 435AR	>256	2	>256	>256	0.5	0.5
<i>K. pneumoniae</i> 338AR	>256	0.25	>256	>256	0.25	0.125
<i>K. pneumoniae</i> MV931658	>256	0.25	>256	8	0.5	1
<i>K. pneumoniae</i> MV940851	16	0.25	>256	>256	64	16
<i>K. pneumoniae</i> 330	>256	0.25	>256	64	0.5	4
<i>K. pneumoniae</i> 331	>256	0.25	256	64	8	0.125
<i>K. pneumoniae</i> IBL2.4	>256	0.25	128	2	0.25	0.25
<i>C. freundii</i> PG4	4	0.25	>256	>256	<0.5	0.25
<i>K. pneumoniae</i> Kp183	128	0.25	>256	>256	<0.5	0.25
<i>E. coli</i> 2ECMBL	128	1	>256	>256	<0.5	0.25
<i>K. pneumoniae</i> PRETA	64	0.25	32	0.25	0.25	0.25
<i>E. coli</i> Em1cro	8	0.25	32	0.25	0.25	0.25

<sup>a</sup>MICs were determined by broth microdilution method according to CLSL and EUCAST guidelines (18, 19). The MIC reduction of ATM (aztreonam), CAZ (ceftazidime), and CZA (ceftazidime-avibactam) was evaluated in the presence of avibactam (AVI; 4  $\mu\text{g/mL}$ ), dipicolinic acid (DPA; 500  $\mu\text{g/mL}$ ), and EDTA (320  $\mu\text{g/mL}$ ). All assays were performed in triplicate on distinct dates.

emergence and increase of clinically relevant carbapenem-resistant bacteria coproducing KPC and NDM  $\beta$ -lactamases in Latin America and the Caribbean, which has been related to the increased use of broad-spectrum antibiotics in patients with COVID-19. These concerns emphasize the importance of appropriate microbiological diagnosis and the effective and articulated implementation of infection prevention and control programs (3, 25).

In this study, we identified 15 carbapenem- and CZA-resistant isolates belonging to the *K. pneumoniae* complex, of which 11 coproduced NDM-1 and KPC-2 carbapenemases. Since conventional phenotypic methods failed to detect serine carbapenemase and M $\beta$ L coproduction, we tested modifications of the DDST and CDT methods based on use of avibactam, EDTA, and DPA as inhibitors, with aztreonam and ceftazidime-avibactam as enzymatic substrates. These modifications were carried out considering that M $\beta$ Ls (including NDM-1) are susceptible to aztreonam and are inhibited by EDTA or DPA (11, 26), whereas KPC-2 serine carbapenemases are susceptible to CZA and are inhibited by AVI (27). Indeed, we observed that *Klebsiella* isolates coproducing KPC-2 and NDM-1 displayed a positive CDT, with  $\geq 4$ -mm inhibition zones around CZA 50 with DPA or CZA 50 with EDTA disks and  $\geq 5$ -mm inhibition zones around ATM-AVI disks containing 4  $\mu\text{g/mL}$  AVI. For CZA 14 with DPA or CZA 14 with EDTA disks, a  $\geq 10$ -mm inhibition zone was defined as indicative of NDM-1 production. In Fig. 1, a workflow for detection of NDM-1 and KPC-2 coproduction in *Enterobacteriales* is proposed.

Since all isolates coproducing NDM-1 and KPC-2 displayed a positive ghost zone in the DDST, by using CZA 50-ATM and CZA 50-DPA disk combinations, at a 2.5-cm disk-disk distance, it was evident that use of DPA was more efficient than EDTA, even when a 1.5-cm disk-disk distance was used for CZA 50-EDTA disk combinations, as previously suggested (28). On the other hand, for the DDST, use of a CZA 14 disk is not recommended. All these results were confirmed based on  $\geq 3$ -fold reductions of aztreonam and ceftazidime-avibactam MICs in the presence of the inhibitors AVI, EDTA, or DPA.

Although CZA-resistant isolates producing KPC variants displayed a positive CDT with CZA-DPA or CZA-EDTA disks, similar to M $\beta$ L producers, it is important to consider that these isolates presented indeterminate mCIM and eCIM results, which could be associated with low resistance levels for meropenem (29–31). In fact, it has been reported that some



CZA-resistant *Klebsiella* spp. producing KPC variants display susceptibility or low MICs to imipenem and/or meropenem (29–31). On the other hand, these CZA-resistant KPC variants can be presumptively detected by DDST, where a positive ghost zone was observed between ATM and CZA disks and no ghost zone observed between CZA and DPA disks. In brief, it is important to test both CZA and ATM to detect KPC variants or carbapenemase-coproducing organisms, even if in some countries those drugs are not used for clinical treatment. Likewise, strains that are mCIM<sup>+</sup> eCIM<sup>+</sup> and resistant to aztreonam should go through testing to rule out additional enzymes.

Isolates coproducing NDM-1 and ESBLs could show positive CDT and DDST results for ATM-AVI and CZA-ATM combinations. However, it is important to highlight that positive mCIM and eCIM tests must be observed for NDM-1 and ESBL coproducers, whereas a positive mCIM and a negative eCIM must be observed for NDM-1<sup>+</sup> KPC-2<sup>+</sup> strains.

Although immunochromatography methods can rapidly detect coproduction of KPC and NDM carbapenemases, they can fail to identify variants and other combinations of carbapenemases, such as Australian imipenemase, Guiana extended-spectrum  $\beta$ -lactamase, German imipenemase, imipenem-hydrolyzing  $\beta$ -lactamase, Seoul imipenemase, *Serratia marcescens* extended-spectrum  $\beta$ -lactamase, and/or São Paulo metallo- $\beta$ -lactamase (32). In addition, immunochromatography methods are more expensive than other methods (33). Otherwise, methods based on disk combinations, disk elution, and disk prediffusion are valuable and useful in low-resource settings that routinely use disk diffusion for susceptibility testing due to affordability (12, 34, 35). Specifically, CDT and DDST methods have strong potential to identify KPC variants and other combinations of carbapenemases that are undetectable by immunochromatography methods (32, 36, 37). Moreover, the inclusion of a CZA-ATM combination in CDT and DDST methods also has clinical significance because this combination has shown effectiveness against pathogens coproducing carbapenemases (38–41). However, disadvantages of CDT and DDST can include the long turnaround time for results. Since the detection of rare carbapenemases is still problematic with most of the commercially available tests, the combination of methods will enable most laboratories to detect these rare variants and, along with performing accurate antimicrobial susceptibility testing, this could help to optimize patient treatment and limit the further spread of carbapenemase producers (36).

In this study, immunochromatography did not detect KPC-31-positive or KPC-114-positive isolates exhibiting resistance to CZA, which could be a limitation of this method. Therefore, for CZA-resistant isolates, additional testing is recommended. On the other hand, a limitation of this study is the reduced numbers of isolates coproducing NDM and KPC tested and the lack of isolates showing coproduction mediated by other  $M\beta$ L and KPC variants. However, this limitation is due to the recent observation of coproduction phenomena in Latin America. Nevertheless, our results demonstrate that inhibition by AVI, DPA, and EDTA may provide simple and inexpensive methods for the presumptive detection of coexpression of KPC and NDM in members of the *K. pneumoniae* complex in human and veterinary diagnostic laboratories. Therefore, additional studies are necessary to confirm the accuracy of these methodologies by testing other Gram-negative bacterial species or other KPC- and NDM-coexpressing variants. Moreover, further studies should be performed using different brands of disks and with Mueller-Hinton agar. Finally, since class A and class B carbapenemases may travel together as well in mobile genetic elements (42–45), clinical laboratories should test such strains by using those methodologies to demonstrate accuracy, whereas measures should be taken to closely monitor and control the spread of critical priority WHO pathogens coproducing carbapenemases worldwide.

**Conclusion.** In recent years, several studies have reported the emergence of pathogens coproducing multiple carbapenemases. In this regard, while coproduction of OXA-48 and NDM-1 has been previously reported (46, 47), coproduction of KPC-2 and NDM-1 among *K. pneumoniae* isolates increased during the COVID-19 pandemic as a major challenge for clinical laboratories (3, 4, 19, 43–45). CZA has demonstrated both

excellent *in vitro* and *in vivo* activities against class A carbapenemase producers. However, there is increasing evidence of *in vivo* selection of CZA-resistant strains that have developed mutations in KPC, AmpC, CTX-M, OXA-48, VEB, and/or PER  $\beta$ -lactamases (48, 49). Therefore, guidelines regarding methods to screen coproduction of carbapenemases and variants of enzymes conferring resistance to CZA require an urgent update, especially after the SARS-CoV-2 pandemic. In regions and hospitals with high circulation of KPC mutants, genomic investigation is highly recommended. If such tools are not available, resistance profiles to CZA and ATM using traditional antimicrobial susceptibility testing and screening using inhibition by AVI and DPA could be a viable alternative.

## MATERIALS AND METHODS

**Bacterial isolates, identification, and susceptibility profiles.** From 2018 to 2021, 15 carbapenem-resistant and/or ceftazidime-avibactam-resistant isolates belonging to the *K. pneumoniae* complex were recovered from human and animal hosts (Table 1). Initially, identification and susceptibility profiles were obtained by use of matrix-assisted laser desorption/ionization–time of flight (Bruker), and Vitek-2 (bioMérieux) instruments and disk diffusion methods, respectively. Specifically, ceftazidime-avibactam (CAZ-AVI) disks (Liofilchem) containing CZA 14 and CZA 50 were tested and interpreted according to EUCAST and CLSI breakpoints, respectively (50, 51). *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as control strains.

**Carbapenemase detection.** The presence of carbapenemase-encoding genes was evaluated by whole-genome sequencing (WGS) with an Illumina NextSeq platform and/or by GeneXpert (52), or by PCR methods using specific primers (53). Expression of KPC and/or NDM carbapenemases was evaluated by using the modified carbapenem inactivation (mCIM) and EDTA-modified carbapenem inactivation (eCIM) assays (54) and by the NG-Test Carba 5 (NG Biotech, Guipry, France) immunochromatographic method.

**CDT and DDST for detection of KPC and NDM coproduction.** Both the CDT and DDST were adapted from methods previously described for the detection of carbapenemases (55). ATM 30, CZA 14, or CZA 50 disks were used as substrates for carbapenemase activity, whereas EDTA and DPA were used as inhibitors of  $M\beta L$  activity and AVI was used as an inhibitor of KPC activity. In brief, while 10  $\mu$ L of 100 mM EDTA or 10  $\mu$ L of 10 mg/mL DPA was added to CZA (14 and 50) disks (56, 57), 10  $\mu$ L of 400 or 2,000  $\mu$ g/mL AVI was added to ATM disks. In this way, for each screened isolate, ATM disks without and with AVI (4 or 20  $\mu$ g/disk) and CZA disks without and with EDTA or DPA were placed onto Mueller-Hinton agar plates (Becton, Dickinson, Le Pont de Claix, France) previously inoculated with a 0.5 McFarland standard bacterial suspension (Fig. 1). Inhibition zone diameters around the antibiotic disks (with and without EDTA, DPA, or AVI) were measured and compared after 18 to 24 h of incubation at 37°C. Blank disks containing 5 mM EDTA, 1,000  $\mu$ g DPA, or 4 or 20  $\mu$ g AVI were used as controls. For the DDST, CZA (14 or 50) disks were placed 2.5 cm apart (center to center) from DPA (1,000  $\mu$ g), EDTA (5 mM), and ATM (30  $\mu$ g) disks onto Mueller-Hinton agar plates previously inoculated (Fig. 1). Additionally, EDTA and CZA disks were placed 1.5 cm apart (center to center), as previously suggested (28). Results were analyzed 18 to 24 h after incubation at 37°C. Isolates previously characterized by WGS as KPC-2 (*K. pneumoniae* IBL2.4), NDM-1 and CMY-48 (*Citrobacter freundii* PG4), NDM-1 and CTX-M-15 (*K. pneumoniae* Kp183), NDM-1 and PER-2 (*Escherichia coli* 2ECMBL), CTX-M-15 and SHV-28 (*K. pneumoniae* PRETA), and CMY-2 (*E. coli* Em1cro) producers were used as controls (Table 1). All assays were performed in triplicate on distinct dates.

**MIC reductions in the presence of EDTA, DPA, or AVI.** For MIC determinations, ATM, CAZ, EDTA, and DPA were purchased from Sigma-Aldrich, and avibactam was purchased from Selleckchem. All MICs were determined by the broth microdilution methodology outlined in ISO 20776 (50, 51). In brief, bacterial inoculum was adjusted to a 0.5 McFarland turbidity standard and diluted to a ratio of 1:10 in Mueller-Hinton broth (Becton, Dickinson, France). All isolates were tested in serial dilutions of ATM and CAZ, ranging from 0.06 to 256  $\mu$ g/mL. For MIC reduction assays, the final concentrations of EDTA and DPA were fixed at 320 and 900  $\mu$ g/mL, respectively, since these concentrations showed no antibacterial activity against any of the screened isolates. Avibactam was tested at a final concentration of 4  $\mu$ g/mL. *E. coli* ATCC 25922 and *K. pneumoniae* ATCC 700603 were used as susceptible controls (Table 3). All assays were performed in triplicate on distinct dates. MIC interpretation was performed according to CLSI and EUCAST breakpoints (50, 51).

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**SUPPLEMENTAL FILE 1**, XLSX file, 0.01 MB.

**SUPPLEMENTAL FILE 2**, XLSX file, 0.02 MB.

**SUPPLEMENTAL FILE 3**, XLSX file, 0.01 MB.

## ACKNOWLEDGMENTS

We thank Cefar Diagnóstica Ltda (São Paulo, Brazil) and CEFAP-GENIAL facility for kindly supplying antibiotic disks for susceptibility testing and Illumina sequencing, respectively.

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo (grants 2020/08224-9 and 2019/15578-4) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (grants 422984/2021-3 and 314336/2021-4). J.D.C. and G.G. are members of Carrera del Investigador Científico, CONICET. F.V.-P. is a research fellow of the Agency for Research and Development (ANID) Scholarship Program Doctorado Becas Chile (2020-72210089). N.L. is a research fellow of CNPq (grant 314336/2021-4).

We declare that we have no competing interests.

## REFERENCES

- García-Betancur JC, Appel TM, Esparza G, Gales AC, Levy-Hara G, Cornistein W, Vega S, Nuñez D, Cuellar L, Bavestrelo L, Castañeda-Méndez PF, Villalobos-Vindas JM, Villegas MV. 2021. Update on the epidemiology of carbapenemases in Latin America and the Caribbean. *Expert Rev Anti Infect Ther* 19:197–213. <https://doi.org/10.1080/14787210.2020.1813023>.
- Farfour E, Lecuru M, Dortet L, Le Guen M, Cerf C, Karnycheff F, Bonnin RA, Vasse M, Lesprit P, SARS-CoV-2 Hospital Foch study group. 2020. Carbapenemase-producing Enterobacterales outbreak: another dark side of COVID-19. *Am J Infect Control* 48:1533–1536. <https://doi.org/10.1016/j.ajic.2020.09.015>.
- Pan American Health Organization. 2021. Epidemiological alert: emergence and increase of new combinations of carbapenemases in Enterobacterales in Latin America and the Caribbean, 22 October 2021. PAHO/WHO, Washington, D.C. <https://www.paho.org/en/documents/epidemiological-alert-emergence-and-increase-new-combinations-carbapenemases>.
- Josa M D, Leal R, Rojas J, Torres M I, Cortés-Muñoz F, Esparza G, Reyes LF. 2022. Comparative evaluation of phenotypic synergy tests versus RESIST-4 O.K.N.V. and NG test carba 5 lateral flow immunoassays for the detection and differentiation of carbapenemases in Enterobacterales and *Pseudomonas aeruginosa*. *Microbiol Spectr* 10:e0108021. <https://doi.org/10.1128/spectrum.01080-21>.
- Pereira PS, Borghi M, Albano RM, Lopes JCO, Silveira MC, Marques EA, Oliveira JCR, Asensi MD, Carvalho-Assef APD. 2015. Coproduction of NDM-1 and KPC-2 in *Enterobacter hormaechei* from Brazil. *Microb Drug Resist* 21:234–236. <https://doi.org/10.1089/mdr.2014.0171>.
- Pereira AL, de Oliveira PM, Faria-Junior C, Alves EG, de Castro e Caldo Lima GR, da Costa Lamounier TA, Haddad R, de Araújo WN. 2022. Environmental spreading of clinically relevant carbapenem-resistant gram-negative bacilli: the occurrence of blaKPC- or -NDM strains relates to local hospital activities. *BMC Microbiol* 22:6–12. <https://doi.org/10.1186/s12866-021-02400-1>.
- Stoesser N, Sheppard AE, Peirano G, Anson LW, Pankhurst L, Sebra R, Phan HTT, Kasarskis A, Mathers AJ, Peto TEA, Bradford P, Motyl MR, Walker AS, Crook DW, Pitout JD. 2017. Genomic epidemiology of global *Klebsiella pneumoniae* carbapenemase (KPC)-producing *Escherichia coli*. *Sci Rep* 7: 1–11. <https://doi.org/10.1038/s41598-017-06256-2>.
- Vázquez-Ucha JC, Arca-Suárez J, Bou G, Beceiro A. 2020. New carbapenemase inhibitors: clearing the way for the  $\beta$ -lactams. *Int J Mol Sci* 21: 9308–9332. <https://doi.org/10.3390/ijms21239308>.
- Feng H, Liu X, Wang S, Fleming J, Wang DC, Liu W. 2017. The mechanism of NDM-1-catalyzed carbapenem hydrolysis is distinct from that of penicillin or cephalosporin hydrolysis. *Nat Commun* 8:2242. <https://doi.org/10.1038/s41467-017-02339-w>.
- Chen AY, Thomas PW, Stewart AC, Bergstrom A, Cheng Z, Miller C, Bethel CR, Marshall SH, Credille CV, Riley CL, Page RC, Bonomo RA, Crowder MW, Tierney DL, Fast W, Cohen SM. 2017. Dipicolinic acid derivatives as inhibitors of New Delhi metallo- $\beta$ -lactamase-1. *J Med Chem* 60:7267–7283. <https://doi.org/10.1021/acs.jmedchem.7b00407>.
- Shakil S, Azhar EI, Tabrez S, Kamal MA, Jabir NR, Abuzenadah AM, Damanhour GA, Alam Q. 2011. New Delhi metallo- $\beta$ -lactamase (NDM-1): an update. *J Chemother* 23:263–265. <https://doi.org/10.1179/joc.2011.23.5.263>.
- Khan A, Erickson SG, Pettaway C, Arias CA, Miller WR, Bhatti MM. 2021. Evaluation of susceptibility testing methods for aztreonam and ceftazidime-avibactam combination therapy on extensively drug-resistant gram-negative organisms. *Antimicrob Agents Chemother* 65:e00846-21. <https://doi.org/10.1128/AAC.00846-21>.
- Pournaras S, Zarkotou O, Poulou A, Kristo I, Vrioni G, Themeli-Digalaki K, Tsakris A. 2013. A combined disk test for direct differentiation of carbapenemase-producing Enterobacteriaceae in surveillance rectal swabs. *J Clin Microbiol* 51:2986–2990. <https://doi.org/10.1128/JCM.00901-13>.
- Şekowska A, Bogiel T, Kaczmarek A. 2020. Evaluation of the usefulness of selected methods for the detection of carbapenemases in *Klebsiella* strains. *J Med Microbiol* 69:792–796. <https://doi.org/10.1099/jmm.0.001202>.
- Hrabák J, Chudáčková E, Papagiannitsis CC. 2014. Detection of carbapenemases in Enterobacteriaceae: a challenge for diagnostic microbiological laboratories. *Clin Microbiol Infect* 20:839–853. <https://doi.org/10.1111/1469-0691.12678>.
- Sood S. 2014. Identification and differentiation of carbapenemases in *Klebsiella pneumoniae*: a phenotypic test evaluation study from Jaipur, India. *J Clin Diagn Res* 8:6–8. <https://doi.org/10.7860/JCDR/2014/7027.4614>.
- Bialvaei AZ, Kafil HS, Asgharzadeh M, Memar MY, Yousefi M. 2016. Current methods for the identification of carbapenemases. *J Chemother* 28:1–19. <https://doi.org/10.1179/1973947815Y.0000000063>.
- Asli A, Brouillette E, Krause KM, Nichols WW, Malouin F. 2016. Distinctive binding of avibactam to penicillin-binding proteins of Gram-negative and Gram-positive bacteria. *Antimicrob Agents Chemother* 60:752–756. <https://doi.org/10.1128/AAC.02102-15>.
- Bes T, Nagano D, Martins R, Marchi AP, Perdigão-Neto L, Higashino H, Prado G, Guimaraes T, Levin AS, Costa S. 2021. Bloodstream infections caused by *Klebsiella pneumoniae* and *Serratia marcescens* isolates co-harboring NDM-1 and KPC-2. *Ann Clin Microbiol Antimicrob* 20:57. <https://doi.org/10.1186/s12941-021-00464-5>.
- Köle M, Sesli Çetin E, Şirin MC, Cicioğlu Arıdoğan B. 2022. Evaluation of in vitro efficacy of ceftazidime-avibactam, meropenem, and colistin single and binary combinations against carbapenem resistant *Klebsiella pneumoniae* strains isolated from various clinical specimens. *Mikrobiyol Bul* 56: 230–250. <https://doi.org/10.5578/mb.20229804>.
- Belati A, Bavaro DF, Diella L, De Gennaro N, Di Gennaro F, Saracino A. 2022. Meropenem/vaborbactam plus aztreonam as a possible treatment strategy for bloodstream infections caused by ceftazidime/avibactam-resistant *Klebsiella pneumoniae*: a retrospective case series and literature review. *Antibiotics (Basel)* 11:373. <https://doi.org/10.3390/antibiotics11030373>.
- Rawson TM, Brzeska-Trafny I, Maxfield R, Almeida M, Gilchrist M, Gonzalo X, Moore LS, Donaldson H, Davies F. 2022. A practical laboratory method to determine ceftazidime-avibactam-aztreonam synergy in patients with New Delhi metallo-beta-lactamase (NDM)-producing Enterobacterales infection. *J Glob Antimicrob Resist* 29:558–562. <https://doi.org/10.1016/j.jgar.2022.01.025>.
- Morroni G, Bressan R, Fioriti S, D'Achille G, Mingoa M, Cironi O, Di Bella S, Piazza A, Comandatore F, Mauri C, Migliavacca R, Luzzaro F, Principe L, Lagatolla C. 2021. Antimicrobial activity of aztreonam in combination with old and new  $\beta$ -lactamase inhibitors against MBL and ESBL co-producing Gram-negative clinical isolates: possible options for the treatment of complicated infections. *Antibiotics* 10:1341. <https://doi.org/10.3390/antibiotics10111341>.
- Mauri C, Maraolo AE, Di Bella S, Luzzaro F, Principe L. 2021. The revival of aztreonam in combination with avibactam against metallo- $\beta$ -lactamase-producing Gram-negatives: a systematic review of in vitro studies and clinical cases. *Antibiotics* 10:1012. <https://doi.org/10.3390/antibiotics10081012>.

25. Beovic B, Dousak M, Ferreira-Coimbra J, Nadrah K, Rubulotta F, Belliato M, Berger-Estilita J, Ayoad F, Rello J, Erdem H. 2020. Antibiotic use in patients with COVID-19: a 'snapshot' infectious diseases international research initiative (ID-IRI) survey. *J Antimicrob Chemother* 75:3386–3390. <https://doi.org/10.1093/jac/dkaa326>.
26. Jackson AC, Zaengle-Barone JM, Puccio EA, Franz KJ. 2020. A cephalosporin prochelator inhibits New Delhi metallo- $\beta$ -lactamase 1 without removing zinc. *ACS Infect Dis* 6:1264–1272. <https://doi.org/10.1021/acscinfed.0c00083>.
27. Zhanel GG, Lawson CD, Adam H, Schweizer F, Zelenitsky S, Lagacé-Wiens PRS, Denisuk A, Rubinstein E, Gin AS, Hoban DJ, Lynch JP, Karlowsky JA. 2013. Ceftazidime-avibactam: a novel cephalosporin/ $\beta$ -lactamase inhibitor combination. *Drugs* 73:159–177. <https://doi.org/10.1007/s40265-013-0013-7>.
28. Programa Nacional de Control de Calidad en Bacteriología Inei Anlis Dr. Carlos G. Malbran. 2021. Alerta epidemiológica: emergencia de Enterobacterales doble productores de carbapenemas. <http://antimicrobianos.com.ar/ATB/wp-content/uploads/2021/05/Alerta-epidemiológica-dobles-productores-de-carbapenemasa-COVID-19-v4.pdf>.
29. Findlay J, Poirel L, Juhas M, Nordmann P. 2021. KPC-mediated resistance to ceftazidime-avibactam and collateral effects in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 65:e00890-21. <https://doi.org/10.1128/AAC.00890-21>.
30. Oueslati S, Iorga BI, Tlili L, Exilie C, Zavala A, Dortet L, Jousset AB, Bernabeu S, Bonnin RA, Naas T. 2019. Unravelling ceftazidime/avibactam resistance of KPC-28, a KPC-2 variant lacking carbapenemase activity. *J Antimicrob Chemother* 74:2239–2246. <https://doi.org/10.1093/jac/dkz209>.
31. Mueller L, Masseron A, Prod'Hom G, Galperine T, Greub G, Poirel L, Nordmann P. 2019. Phenotypic, biochemical, and genetic analysis of KPC-41, a KPC-3 variant conferring resistance to ceftazidime-avibactam and exhibiting reduced carbapenemase activity. *Antimicrob Agents Chemother* 63:e01111-19. <https://doi.org/10.1128/AAC.01111-19>.
32. Kieffer N, Poirel L, Nordmann P. 2019. Rapid immunochromatography-based detection of carbapenemase producers. *Infection* 47:673–675. <https://doi.org/10.1007/s15010-019-01326-1>.
33. Wei M, Wang P, Wang S, Yang C, Gu LI. 2021. Rapid detection and differentiation of KPC and MBL carbapenemases among Enterobacterales isolates by a modified combined-disk test. *Pol J Microbiol* 70:387–394. <https://doi.org/10.33073/pjm-2021-036>.
34. World Health Organization. 2021. WHO integrated global surveillance on ESBL-producing *E. coli* using a "One Health" approach: implementation and opportunities. <https://apps.who.int/iris/rest/bitstreams/1335472/retrieve>.
35. Lima KO, de Lima AV, Rocha DADC, Sampaio SCF, Cappellano P, Sampaio JLM. 2022. A simple disk pre-diffusion test to predict in vitro aztreonam/avibactam activity against NDM-producing *Klebsiella pneumoniae* complex. *J Glob Antimicrob Resist* 28:49–52. <https://doi.org/10.1016/j.jgar.2021.12.009>.
36. Baeza LL, Pfennigwerth N, Greissl C, Göttig S, Saleh A, Stelzer Y, Gatermann SG, Hamprecht A. 2019. Comparison of five methods for detection of carbapenemases in Enterobacterales with proposal of a new algorithm. *Clin Microbiol Infect* 25:1286.e9–1286.e15. <https://doi.org/10.1016/j.cmi.2019.03.003>.
37. Zhu Y, Jia P, Li X, Wang T, Zhang J, Zhang G, Duan S, Kang W, Xu Y, Yang Q. 2021. Carbapenemase detection by NG-Test CARBA 5-a rapid immunochromatographic assay in carbapenem-resistant Enterobacterales diagnosis. *Ann Transl Med* 9:769. <https://doi.org/10.21037/atm-20-8216>.
38. Sun S, Chen K, Kong X, Tian W, Niu S. 2022. Genetic diversity and in vitro activity of aztreonam/avibactam and ceftazidime/avibactam against carbapenem-resistant Enterobacterales: a multi-center study in Southwest China. *Infect Drug Resist* 15:2243–2251. <https://doi.org/10.2147/IDR.S357396>.
39. Wei J, Zou C, Wang D, Huang A, Niu S. 2020. Genetic diversity and in vitro activity of ceftazidime/avibactam and aztreonam/avibactam against imipenem-resistant Enterobacteriaceae isolates in Southwest China: a single-centre study. *J Glob Antimicrob Resist* 22:448–451. <https://doi.org/10.1016/j.jgar.2020.04.023>.
40. Yasmin M, Fouts DE, Jacobs MR, Haydar H, Marshall SH, White R, D'Souza R, Lodise TP, Rhoads DD, Hujer AM, Rojas LJ, Huyen C, Perez F, Edwards A, Bonomo RA. 2020. Monitoring ceftazidime-avibactam and aztreonam concentrations in the treatment of a bloodstream infection caused by a multidrug-resistant *Enterobacter* sp. carrying both *Klebsiella pneumoniae* carbapenemase-4 and New Delhi metallo- $\beta$ -lactamase-1. *Clin Infect Dis* 71:1095–1098. <https://doi.org/10.1093/cid/ciz1155>.
41. Davido B, Fellous L, Lawrence C, Maxime V, Rottman M, Dinh A. 2017. Ceftazidime-avibactam and aztreonam, an interesting strategy to overcome  $\beta$ -lactam resistance conferred by metallo- $\beta$ -lactamases in Enterobacteriaceae and *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother* 61:e01008-17. <https://doi.org/10.1128/AAC.01008-17>.
42. Sadek M, Juhas M, Poirel L, Nordmann P. 2020. Genetic features leading to reduced susceptibility to aztreonam-avibactam among metallo- $\beta$ -lactamase-producing *Escherichia coli* isolates. *Antimicrob Agents Chemother* 64:e01659-20. <https://doi.org/10.1128/AAC.01659-20>.
43. Hao J, Zhang B, Deng J, Wei Y, Xiao X, Liu J. 2022. Emergence of a hypervirulent tigecycline-resistant *Klebsiella pneumoniae* strain co-producing *bla*<sub>NDM-1</sub> and *bla*<sub>KPC-2</sub> with an uncommon sequence type ST464 in Southwestern China. *Front Microbiol* 13:868705. <https://doi.org/10.3389/fmicb.2022.868705>.
44. Li Y, Fang C, Qiu Y, Dai X, Zhang L. 2022. Genomic characterization of a carbapenem-resistant *Citrobacter freundii* cocarrying *bla*<sub>KPC-2</sub> and *bla*<sub>NDM-1</sub>. *J Glob Antimicrob Resist* 29:289–292. <https://doi.org/10.1016/j.jgar.2022.04.014>.
45. Sun Q, Dai Y, Chen J, Yu K, Wang Y, Zhang Y, Kong Y, Cheng J. 2022. Coexistence of two *bla*<sub>KPC-2</sub> genes in a *bla*<sub>NDM-1</sub>-carrying multidrug-resistant ST15 *Klebsiella pneumoniae* isolate recovered from cerebrospinal fluid in China. *J Glob Antimicrob Resist* 29:232–235. <https://doi.org/10.1016/j.jgar.2022.04.006>.
46. Solgi H, Badmasti F, Aminzadeh Z, Giske CG, Pourahmad M, Vaziri F, Havaei SA, Shahcheraghi F. 2017. Molecular characterization of intestinal carriage of carbapenem-resistant Enterobacteriaceae among inpatients at two Iranian university hospitals: first report of co-production of *bla*<sub>NDM-7</sub> and *bla*<sub>OXA-48</sub>. *Eur J Clin Microbiol Infect Dis* 36:2127–2135. <https://doi.org/10.1007/s10096-017-3035-3>.
47. Genç S, Kolaylı F, Özçelik EY. 2022. Molecular characterization of carbapenemase producing *Klebsiella pneumoniae* strains by multiplex PCR and PFGE methods: the first *K. pneumoniae* isolates co-producing OXA-48/KPC and KPC/NDM in Turkey. *J Infect Chemother* 28:192–198. <https://doi.org/10.1016/j.jiac.2021.10.009>.
48. Ortiz de la Rosa JM, Nordmann P, Poirel L. 2019. ESBLs and resistance to ceftazidime/avibactam and ceftolozane/tazobactam combinations in *Escherichia coli* and *Pseudomonas aeruginosa*. *J Antimicrob Chemother* 74:1934–1939. <https://doi.org/10.1093/jac/dkz149>.
49. Xu T, Guo Y, Ji Y, Wang B, Zhou K. 2022. Epidemiology and mechanisms of ceftazidime-avibactam resistance in Gram-negative bacteria. *Engineering* 11:138–145. <https://doi.org/10.1016/j.eng.2020.11.004>.
50. European Committee on Antimicrobial Susceptibility Testing. 2022. Breakpoint tables for interpretation of MICs and zone diameters, version 12.0. Accessed 1 January 2022. [http://www.eucast.org/clinical\\_breakpoints/](http://www.eucast.org/clinical_breakpoints/).
51. Clinical and Laboratory Standards Institute. 2022. Performance standards for antimicrobial susceptibility testing. CLSI supplement M100, 32nd ed. Clinical and Laboratory Standards Institute, Wayne, PA.
52. Jin S, Lee JY, Park JY, Jeon MJ. 2020. Xpert Carba-R assay for detection of carbapenemase-producing organisms in patients admitted to emergency rooms. *Medicine (Baltimore)* 99:e23410. <https://doi.org/10.1097/MD.00000000000023410>.
53. Poirel L, Walsh TR, Cuvillier V, Nordmann P. 2011. Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis* 70:119–123. <https://doi.org/10.1016/j.diagmicrobio.2010.12.002>.
54. Tsai YM, Wang S, Chiu HC, Kao CY, Wen LL. 2020. Combination of modified carbapenem inactivation method (mCIM) and EDTA-CIM (eCIM) for phenotypic detection of carbapenemase-producing Enterobacteriaceae. *BMC Microbiol* 20:315. <https://doi.org/10.1186/s12866-020-02010-3>.
55. Cohen Stuart J, Leverstein-Van Hall MA. 2010. Guideline for phenotypic screening and confirmation of carbapenemases in Enterobacteriaceae. *Int J Antimicrob Agents* 36:205–210. <https://doi.org/10.1016/j.ijantimicag.2010.05.014>.
56. Marchiaro P, Mussi MA, Ballerini V, Pasteran F, Viale AM, Vila AJ, Limansky AS. 2005. Sensitive EDTA-based microbiological assays for detection of metallo- $\beta$ -lactamases in nonfermentative Gram-negative bacteria. *J Clin Microbiol* 43:5648–5652. <https://doi.org/10.1128/JCM.43.11.5648-5652.2005>.
57. Coppi M, Cannatelli A, Antonelli A, Baccani I, Di Pilato V, Sennati S, Giani T, Rossolini GM. 2018. A simple phenotypic method for screening of MCR-1-mediated colistin resistance. *Clin Microbiol Infect* 24:201.e1–201.e3. <https://doi.org/10.1016/j.cmi.2017.08.011>.