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Improved Blue Carba test and Carba NP test for detection and classification of major Class A and B carbapenemases, including dual producers, among Gram-negative bacilli

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ABSTRACT Prompt and precise identification of carbapenemase-producing organisms is crucial for guiding clinical antibiotic treatments and limiting transmission. Here, we propose modifying the Blue Carba test (BCT) and Carba NP-direct (CNPd) to identify molecular carbapenemase classes, including dual carbapenemase strains, by adding specific Class A and Class B inhibitors. We tested 171 carbapenemase-producing Gram-negative bacilli strains—21 in Class A (KPC, NMC, SME), 58 in Class B (IMP, VIM, NDM, SPM), and 92 with dual carbapenemase production (KPC+NDM, KPC+IMP, KPC+VIM), all previously positive with BCT or CNPd. We also included 13 carbapenemase non-producers. β-lactamases were previously characterized by PCR. The improved BCT/CNPd methods detect imipenem hydrolysis from an imipenem-cilastatin solution, using pH indicators and Class A (avibactam) and/or Class B (EDTA) inhibitors. Results were interpreted visually based on color changes. CNPd achieved 99.4% sensitivity and 100% specificity in categorizing carbapenemases, while BCT had 91.8% sensitivity and 100% specificity. Performance varied by carbapenemase classes: both tests classified all Class A-producing strains. For Class B, the CNP test identified 57/58 strains (98.3%), whereas the BCT test, 45/58 strains (77.6%), with non-fermenters posing the greatest detection challenge. For Classes A plus B dual producers, both tests performed exceptionally well, with only one indeterminate strain for the BCT. The statistical comparison showed both methods had similar times to a positive result, with differences based on the carbapenemase class or bacterial group involved. This improved assay rapidly distinguishes major Class A or Class B carbapenemase producers among Gram-negative bacilli, including dual-class combinations, in less than 2 hours.

IMPORTANCE Rapid and accurate identification of carbapenemase-producing organisms is of vital importance in guiding appropriate clinical antibiotic treatments and curbing their transmission. The emergence of negative bacilli carrying multiple carbapenemase combinations during and after the severe acute respiratory syndrome coronavirus 2 pandemic has posed a challenge to the conventional biochemical tests typically used to determine the specific carbapenemase type in the isolated strains. Several initiatives have aimed to enhance colorimetric methods, enabling them to independently identify the presence of Class A or Class B carbapenemases. Notably, no previous efforts have been made to distinguish both classes simultaneously. Additionally, these modifications have struggled to differentiate between carriers of multiple carbapenemases, a common occurrence in many Latin American countries. In this study, we introduced specific Class A and Class B carbapenemase inhibitors into the Blue Carba test (BCT) and Carba NP-direct (CNP) colorimetric assays to identify the type of carbapenemase, even in cases of multiple carbapenemase producers within these classes. These updated assays demonstrated exceptional sensitivity and specificity (≥ 90%) all within a

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rapid turnaround time of under 2 hours, typically completed in just 45 minutes. These in-house enhancements to the BCT and CNP assays present a rapid, straightforward, and cost-effective approach to determining the primary carbapenemase classes. They could serve as a viable alternative to molecular biology or immuno-chromatography techniques, acting as an initial diagnostic step in the process.

KEYWORDS carbapenemase, Enterobacterales, KPC, MBL

The emergence and global spread of carbapenemase-producing organisms (CPO) have become an epidemiological risk for healthcare systems and a serious threat to antimicrobial treatment (1). During the first wave of coronavirus disease 2019 (COVID-19) (2020) in Argentina, carbapenemase-producing Enterobacterales increased by 50% in prevalence according to the National Surveillance System WHONET-Argentina (2). Additionally, during the period spanning from March 2020 to September 2021, a total of 692 confirmed cases of carbapenemase-producing Enterobacterales were reported in Argentina (3). Among these cases, notably, 20.3% tested positive for a combination of carbapenemases, specifically *bla*KPC plus *bla*NDM. The remaining isolates were single carbapenemases producers, with the majority being *bla*NDM (48.7%) or *bla*KPC (27.4%). Class D carbapenemases were detected at a much lower rate, only 3.6%. Following this concerning trend, the Pan-American Health Organization-World Health Organization issued a regional alert, drawing attention to the emergence of Enterobacterales and *Pseudomonas aeruginosa* strains concurrently producing KPC and NDM carbapenemases. These incidents were notably observed during the COVID-19 pandemic and were documented in multiple countries besides Argentina, including Uruguay, Ecuador, Guatemala, and Brazil (4). A recent report confirms the pronounced trend of increasing CPO incidence among hospital-acquired infections in Latin America, with NDM and KPC plus NDM dual carbapenemase producers accounting for a growing percentage annually, while OXA producers remain rare among Enterobacterales isolates (5).

Phenotypic detection of double carbapenemase producers has become a significant challenge in routine laboratory screenings. A recent assessment of the modified Carbapenem Inactivation Method (mCIM/eCIM) in dual producer Enterobacterales, identified 91% of the strains as expressing only a Class A carbapenemase, while the remaining 9% showed results consistent with the expression of only an MBL (2). A double disc synergy test for carbapenemase classification based on boronic acid and EDTA inhibitors for Class A and Class B carbapenemase, respectively, widely used in low- and middle-income countries (LMIC), failed to accurately identify the simultaneous production of Class A (KPC) and Class B (MBL) carbapenemases in 80.5% of cases, leading to misclassification as single carbapenemase producers (2).

Rapid and accurate classification of CPOs is critical for guiding clinical antibiotic therapy to prevent treatment failures. Rapid biochemical techniques have been employed to identify carbapenem hydrolysis as an indirect indicator of the presence of a carbapenemase (6). These methodologies rely on detecting a change in the color of the culture medium due to the presence of a pH indicator. When carbapenem antibiotics, typically imipenem-cilastatin, undergo hydrolysis by a carbapenemase, the medium becomes more acidic, leading to this color change phenomenon. The Blue Carba test (BCT) employs bromothymol blue as the pH indicator, which changes from blue to green/yellow when a carbapenemase is present (7). Conversely, the Carba NP-direct test (CNPd) relies on a phenol red indicator, which changes color from red to orange/yellow in the presence of carbapenemase activity (8). Both tests are cost-effective and highly reliable methods for definitively detecting contemporary major carbapenemases, particularly those from Class A (such as KPC) and Class B (including NDM, VIM, IMP, etc.), with Class D (such as OXA-48-like) posing the greatest detection challenge, as previously reported (7–9).

Currently, available manual colorimetric tests on the market fall short of effectively inferring the molecular Class of carbapenemases. This is particularly crucial when it

comes to the prudent use of expensive antibiotics such as the new β-lactamase inhibitor combinations, primarily designed to target Class A enzymes. MBLs are enzymes that depend on zinc ions for their catalytic activity and, therefore, can be inhibited by metal-chelating agents like EDTA (10). Recently introduced combinations of β-lactam antibiotics with β-lactamase inhibitors (e.g., ceftazidime-avibactam, imipenem-cilastatin-relebactam, meropenem-vaborbactam) are effective against most serine carbapenemases (Class A). However, these β-lactams/β-lactamase inhibitors are not effective against MBLs, making them a valuable tool for carbapenemase classe identification (11).

The incorporation of specific carbapenemase inhibitors has the potential to enhance the identification of distinct molecular carbapenemase classes. In line with this, Oviaño et al. introduced an innovative modification to the CNP test by utilizing imipenem/relebactam to confirm the presence of Class A carbapenemase-producing Enterobacterales (12). Concurrently, De Oliveira Santos et al. suggested the inclusion of EDTA inhibitors in the CNP and BCT, enabling differentiation between Class A and Class B carbapenemase production in *P. aeruginosa* (13). Nevertheless, these test modifications were not challenged with dual carbapenemase producers, and some of them were associated with some performance limitations.

The objective of this study is to propose a modification of the BCT and CNPd through the addition of specific inhibitors for Class A and Class B carbapenemases classification. This modification aims to facilitate the identification process of the major molecular Classes of carbapenemases A and B, including the detection of dual carbapenemase strains within clinical isolates of Gram-negative bacilli.

MATERIALS AND METHODS

Bacterial isolates

Our study includes a panel of 184 clinical isolates, comprising 171 carbapenemase-producing strains and 13 non-producers. All 171 isolates exhibited non-susceptibility to at least one carbapenem (imipenem or meropenem, or, in the case of Enterobacterales, ertapenem). All carbapenemase-producing isolates included in the study had previously tested positive using the BCT and/or CNPd. Class D enzymes did not meet the prerequisite of producing a positive colorimetric test and were therefore excluded from the inhibitor validation process. These isolates were sourced from the collections of the Servicio Antimicrobianos at the Instituto Nacional de Enfermedades Infecciosas and the CDC Antimicrobial Resistance Isolate Bank. Strains were chosen with a focus on ensuring diversity in species/enzyme combinations and considering the varied geographical origins to minimize the likelihood of clonality. Specifically, for dual producers, predominantly associated with the *Klebsiella pneumoniae* species, representatives of distinct pulse types were deliberately selected, employing Pulse Field Gel Electrophoresis with *Xbal*. For the *Pseudomonas* spp. population, since Classes A plus B double-producing strains were not available in our collection, a combination of individual producers (1:1) was tested. Isolates had been previously identified using matrix-assisted laser desorption ionization-time of flight mass spectrometry (Bruker) (14). Species included were (n) *Aeromonas* spp. (1), *Acinetobacter* spp. (8), *Citrobacter* spp. (3), *Enterobacter cloacae* (6), *Escherichia coli* (14), *Klebsiella aerogenes* (2), *Klebsiella oxytoca* (2), *K. pneumoniae* (97), *Morganella morganii* (1), *Pseudomonas* spp. (45), *Proteus mirabilis* (1), and *Serratia marcescens* (4). For β-lactamase characterization, we employed PCR analysis followed by DNA sequencing of the amplicons whenever possible, which was considered the gold standard in our investigation (15). The panel comprised 21 producers of Class A carbapenemases (KPC, NMC, SME), 58 of Class B (IMP, VIM, NDM, SPM), 92 with dual carbapenemase production (KPC+NDM, KPC+IMP, KPC+VIM), and 13 carbapenemase non-producers despite being carbapenem-resistant (detailed information on the resistance mechanisms of the panel isolates is depicted in Table S1).

Strains were subculture overnight from frozen stocks on Trypticase soy or Columbia Blood agar plates at 35°C in an aerobic atmosphere with previous testing by CNPd or BCT.

Modified BCT and CNPd tests

Selection of the inhibitor concentrations for carbapenemase classification

We determined the optimal inhibition conditions by conducting experiments with a panel of four *K. pneumoniae* strains, which included, ZAQ1 (KPC-2 producer), ZCD1 (NDM-1 producer), ZAQ6 (KPC-2 plus NDM-5, dual producer), and ATCC 700603, as a non-carbapenemase, extended-spectrum β-lactamase (ESBL) producer for reference. The genomes of these clinical strains were sequenced to confirm that the indicated carbapenemase gene was the sole contributor to the test result. Each strain was subjected to testing in duplicate using the protocols for BCT and CNPd described below. We evaluated various concentrations of EDTA (ICN) by introducing 5 µL, 7.5 µL, 10 µL, 12.5 µL, 15 µL, or 20 µL of corresponding 12.5 mM, 25 mM, and 50 mM solutions into the reaction mixture. As for sodium avibactam (Molekula, Cat. 90023818), we incorporated 5 µL, 10 µL, 15 µL, or 20 µL of the respective 0.5 mg/mL, 2 mg/mL, and 10 mg/mL solutions. The minimum inhibitor concentration, capable of effectively inhibiting the specific carbapenemase class throughout the entire assay, was chosen. This selection ensured that the inhibitor did not interfere with carbapenemase classes beyond its inhibition profile, and it maintained the pH of the reaction unaltered. In conclusion, the chosen inhibitor concentrations were as follows: (i) avibactam: a minimum final concentration of 45 µg/mL in each well was necessary to inhibit KPC production in the selected strains. To achieve this concentration, each well had to receive 10 µL of an extemporaneous avibactam solution at a concentration of 0.5 mg/mL. The addition of other reaction reagents and bacterial inoculum will lead to the dilution of this well, reaching the specified concentration (refer to details below); (ii) EDTA: inhibition of the MBL-producing strain required a minimum final concentration of 1.14 mM in the well. To achieve this concentration, each well had to receive 10 µL of an extemporaneous 12.5 mM EDTA solution. The subsequent addition of reaction reagents and bacterial inoculum to this well, following the specified volumes outlined below, will lead to EDTA dilution to the designated concentration.

Technique description

The modified procedure for conducting the BCT and CNPd test involved the following steps:

Preparation of test solutions

For BCT: the test followed previously reported conditions (7). Briefly, an extemporaneous "revealing solution" was prepared using an aqueous solution of bromothymol blue at a concentration of 0.04% (Biopack), along with 0.1 mmol/L ZnSO4 (Sigma). The pH was adjusted to 7.0. The "working solution" was prepared daily, by adding 6 mg/mL of imipenem-cilastatin in an injectable form (Celtyc) to the revealing solution.

For CNPd: the test followed previously reported conditions (8). An extemporaneous aqueous "revealing solution" was prepared comprising 0.05% phenol red (Biopack), 0.1 mmol/L ZnSO4, and 0.1% (vol/vol) Triton X-100 (Mallinckrodt, St. Louis, MO), with a pH adjusted to 7.8. The "working solution" was prepared by adding 12 mg/mL of imipenem-cilastatin in an injectable form to the revealing solution.

Test setup

The test was carried out using a multi-well plate (Star). Well #1 was filled with 50 µL of the BCT or CNPd revealing solution, which contained only the pH indicator solution (serving as the internal negative control). Wells #2 to #5: each received 50 µL of freshly

prepared working solution (the revealing solution supplemented with imipenem). Wells #2 serves as internal positive control. Well #3 received 10 µL of an extemporaneous solution of avibactam at a concentration of 0.5 mg/mL (Class A inhibition well). The final concentration of avibactam in each well was at least 45 µg/mL. The avibactam stock solution was aliquoted and kept in the freezer for the duration of the study. Well #4 received 10 µL of an extemporaneous 12.5 mM EDTA solution (Class B inhibition well). The final concentration of EDTA in each well was at least 1.14 mM. Well #5 received 10 µL each of the avibactam 0.5 mg/mL and EDTA 12.5 mM solutions (Class A plus Class B inhibition well). Schematic representation of test setup is depicted in Fig. 1A.

Inoculation

A bacterial suspension was prepared using half of a 10 µL loop, which was sampled from 3 to 5 colonies of pure culture and dissolved in 2 mL of sterile distilled water for all Gram-negative bacilli, excluding *Pseudomonas* spp. For the latter genus, the bacterial suspension was prepared using a 10 µL whole loop. Following this, wells #1 to #5 were inoculated with 50 µL of the bacterial suspension. It is important that all wells receive the same inoculum size.

Incubation

The plates were incubated at 35°C with agitation for up to 150 minutes. Visual observation was performed at 15-minute intervals, monitoring color changes in the wells.

Plates were independently interpreted by two operators.

Test interpretation

BCT: a shift from blue to green or yellow within the wells containing antibiotics indicated a positive result. Conversely, if the well's color remained unchanged after 150 minutes, the tests were deemed negative.

CNPd: a color change from red to orange or yellow within the antibiotic-containing wells was considered a positive result. Conversely, if the well's color remained unchanged after 150 minutes, the tests were deemed negative.

To classify the type of carbapenemase present, the following criteria were applied (a schematic representation is depicted in Fig. 1B):

Class A carbapenemase: it was deduced if a positive BCT or CNPd result was observed for the internal positive control (well #2) and the Class B inhibition well (well #4), with negative results for the internal negative control (well #1), the Class A inhibition well (well #3), and the Classes A plus B inhibition well (well #5).

Class B carbapenemase: it was deduced if a positive result was observed for the internal positive control (well #2) and the Class A inhibition well (well #3), with negative results for the internal negative control (well #1), the Class B inhibition well (well #4), and the Classes A plus B inhibition well (well #5).

Classes A plus B dual carbapenemase producer: it was deduced if a positive BCT or CNPd result was observed for the internal positive control (well #2), the Class A inhibition well (well #3), and the Class B inhibition well (well #4), along with negative results for the internal negative control (well #1) and the Classes A plus B inhibition well (well #5).

Indeterminate result: indicating an unclassified carbapenemase, was inferred when a positive BCT or CNPd result was observed for the internal positive control (well #2), the Class A inhibition well (well #3), the Class B inhibition well (well #4), and the Classes A plus B inhibition well (well #5), with a negative result for the internal negative control (well #1). Likewise, an indeterminate result was defined as a positive result in the internal positive control (well #2) and negative results in the internal negative control (well #1), and all wells containing inhibitors (wells #3 to #5).

Invalid result: the test must be discarded if a positive result is obtained for the internal negative control (well #1).

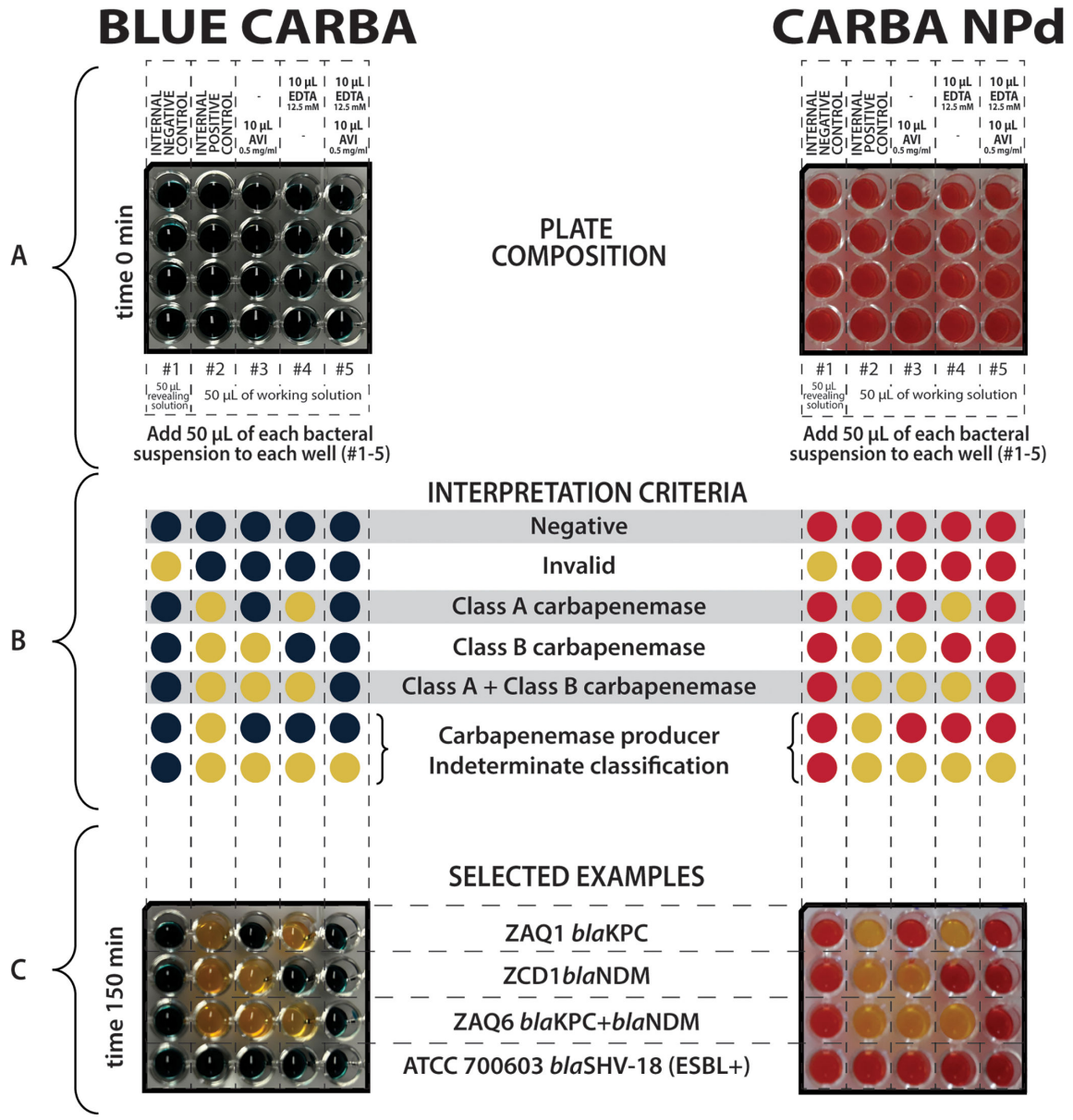


FIG 1 Representative BCT and CNP test with the addition of inhibitors for the classification of Class A, Class B carbapenemases, and their combinations. AVI, avibactam; EDTA, ethylenediaminetetraacetate. (A) Schematic representation of the distribution of reagents, inhibitors, and inoculum for each test. Wells #1 and #2 represent the negative and positive controls for each unknown strain. Well #3 is for Class A inhibition, well #4 is for Class B inhibition, and well #5 is for both Class A and Class B inhibition. The inclusion of well #5 is crucial to prevent false negatives stemming from dual carbapenemase producers in regions where their prevalence is endemic or unknown. (B) Interpretation of the tests. In the BCT, a color change from blue to green or yellow within the wells containing antibiotics indicates a positive result. In the CNP test, a color change from red to orange or yellow within the antibiotic-containing wells is considered a positive result. The presence of Class A, Class B, or both Class A and Class B carbapenemases is determined based on the inhibitory effects of avibactam and/or EDTA as depicted in the graphic representation. (C) Examples of tests performed with different strains, including KPC-2-producing *K. pneumoniae* (ZAQ1), NDM-1-producing *K. pneumoniae* (ZCD1), KPC-2 plus NDM-5 producing *K. pneumoniae* (ZAQ6), and non-carbapenemase-producing ATCC 700603 *K. pneumoniae* producing the extended-spectrum β-lactamase SHV-18. Photographs were taken after a 150-minute incubation period. The colors depicted accurately represent the true tonalities captured, as no adjustments or manipulations were applied to the displayed image.

Inhibitor stability assays

To assess the stability of the inhibitors under various conditions, we supplemented the wells only with avibactam and EDTA inhibitor solutions, following the concentrations and positions in the plate previously specified (Fig. 1A). Subsequently, we subjected

these plates to different storage conditions, which included: refrigerator at −4°C, room temperature during the spring season (with an average high of 22°C to 24°C, while the minimum registered was from 13°C to 16°C during the study period), and incubator set at 35°C. For the plates placed in the incubator, we conducted evaluations every 2 weeks for 2 months. Meanwhile, those stored at room temperature and in the freezer were assessed monthly for 1 and 3 months, respectively.

Following the storage period, the plates were retrieved from their respective storage locations and allowed to reach room temperature. Once they had equilibrated to room temperature, we continued by adding the suitable revealing and working solutions for either the BCT or CNPd. Subsequently, as outlined above, the plates were inoculated with the aforementioned four control strains of *K. pneumoniae*, each representing distinct molecular classes of resistance mechanisms. The interpretation of the results was conducted in accordance with the procedure described in this study.

Reproducibility studies

Reproducibility assessments were carried out as part of the methodology validation. To achieve this, four isolates of *K. pneumoniae*, including ZAQ1 (KPC-2 producer), ZCD1 (NDM-1 producer), ZAQ6 (KPC-2 plus NDM-5 dual producer), and ATCC 700603, were evaluated across 28 batches over the 20-week study duration. The recorded data included the performance of each carbapenemase class obtained through BCT and CNPd.

Statistical tests

For categorical variables, absolute and relative frequencies were calculated, while for quantitative variables, the median, first, and third quartiles were calculated. To compare the median time to positivity between two groups of paired observations, the non-parametric Wilcoxon rank and sign test was performed, and between more than two groups of independent observations, the Kruskal-Wallis test with the Benjamini–Hochberg correction in post hoc tests. For the comparison of the proportion of positivity over time, the Cochran Q test was used with the Benjamini–Hochberg correction in post hoc tests. For the statistical analysis, the R software (version 4.2.2) was used. A significance level of 0.05 was established for the statistical inference tests.

RESULTS

Analytical performance

Initially, we conducted a confirmation step to ensure that all isolates in the panel tested positive for at least one colorimetric assay (a positive result in well #2). This criterion was met for all strains with the CNPd test but for all except 12 isolates with the BCT, mostly involving *Pseudomonas* spp. (Table S1). Among confirmed positive isolates, a robust color change (from blue to yellow for BCT and from red to yellow for CNPd) was observed in 98.3% to 99.2% of carbapenemase-producing Enterobacterales, but only in 71.8% to 64% of non-fermenting bacilli with BCT and CNPd, respectively (see Table S1 for details). The remaining isolates exhibited a color change from red to orange or from blue to green for CNPd and BCT, respectively. However, both operators achieved complete agreement, with 100% concordance in interpreting the results despite the color variations.

Regarding the classification of carbapenemases classes, CNPd demonstrated remarkable performance, achieving 99.4% sensitivity in correctly categorizing the examined carbapenemases, which included Class A (21/21), Class B (57/58), and Classes A plus B carbapenemases (92/92). The exception was one case involving *Acinetobacter baumannii* carrying NDM-1. This isolate yielded an indeterminate result, as all wells containing the inhibitor showed positive reactions. Carbapenemase non-producing isolates did not hydrolyze imipenem, as demonstrated by the absence of color change in the imipenem solution, thus in the avibactam and/or EDTA wells. This exceptional performance resulted in a specificity of 100% for CNPd (Table S1).

On the other hand, BCT showed a sensitivity of 91.8%, with variations depending on the specific class being evaluated. BCT accurately categorized all 21 Class A carbapenemases (100% sensitivity). Conversely, for Class B carbapenemases, the sensitivity was 77.6% (45/58 strains), with notable differences in performance between Enterobacteriaceae (94.4% sensitivity) and non-fermenting bacilli (70% sensitivity).

Two aspects contributed to this performance: firstly, only one incorrect carbapenemase classification was observed, involving an *A. baumannii* NDM-1+OXA-64 strain with indeterminate results (all wells containing the inhibitor showed positive reactions). Secondly, the remaining false negatives consisted of strains undetected by BCT, including six *Pseudomonas* spp., five *Acinetobacter* spp., and one *Aeromonas hydrophila* CphA, all MBL producers. Almost all isolates co-producing Classes A plus B carbapenemases were correctly classified by BCT (98.9% sensitivity), except one *K. oxytoca* carrying KPC-2+IMP-8 with indeterminate results. Isolates that did not produce carbapenemase did not exhibit imipenem hydrolysis, as evidenced by the lack of a color change in the imipenem solution, both alone and in the presence of inhibitors. The specificity of BCT remained consistently high at 100% (Table S1).

Examples of tests performed with different strains, including KPC-2 producing *K. pneumoniae* (ZAQ1), NDM-1 producing *K. pneumoniae* (ZCD1), KPC-2 plus NDM-5 producing *K. pneumoniae* (ZAQ6), and non-carbapenemase-producing ATCC 700603 *K. pneumoniae* producing the extended-spectrum β-lactamase SHV-18 are depicted in Fig. 1C.

Time for a positive result

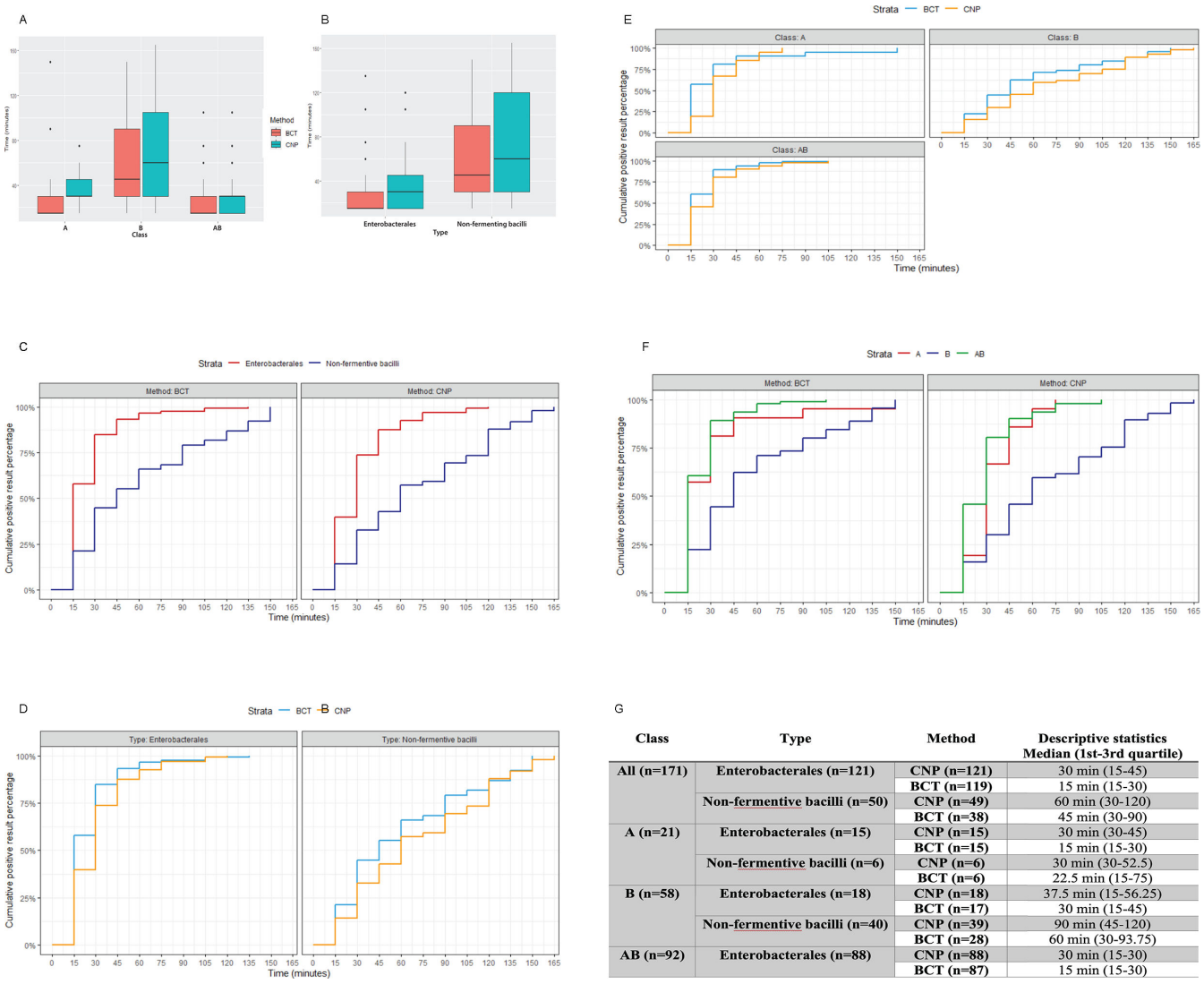
Subsequently, we examined the time required to achieve a positive classification. The median time for the upper quartile to obtain a positive result for dual producers of Classes A and B was just 30 minutes, regardless of the method used (Fig. 2A). For individual carbapenemases, the time for the upper quartile ranged from 30 to 45 minutes (median 15–30 minutes) for Class A, irrespective of the method employed. For Class B, it ranged from 90 to 105 minutes (median 45–60 minutes) (Fig. 2A).

Significant differences in detection times were observed when segregating by bacterial group, with non-fermenting bacilli requiring the longest time for a positive result, showing a mean time increase of 30 minutes for both methods (Fig. 2B and C). BCT was significantly faster than CNPd for Enterobacterales (BCT: 15 minutes vs CNPd: 30 minutes; $P < 0.01$), but both methods had similar response times for non-fermenting bacilli (BCT: 45 minutes vs CNPd: 60 minutes; $P = 0.552$) (Fig. 2D). A significantly faster response was observed for dual producers with BCT (median 15 minutes) compared to CNPd (median 30 minutes) ($P < 0.001$). No significant differences were detected for Class A (BCT: 15 minutes vs CNPd: 30 minutes; $P = 0.205$) or Class B (BCT: 45 minutes vs CNPd: 60 minutes; $P = 0.484$) (Fig. 2E). Remarkably, both methods demonstrated quicker classification of Class A ($P < 0.01$) and Class A plus Class B ($P < 0.01$) enzymes compared to Class B (Fig. 2F). This is due to the higher prevalence of Class B among non-fermenters, which have slower associated times to results. A summary of the descriptive statistics for the total sample and discriminated by carbapenemase type and bacterial group is shown in Fig. 2G.

Outliers, defined as a time to positive exceeding the third quartile, were detected in 8.2% of the panel isolates (6.4% strains using BCT and 8.2% with CNP). In most instances, the outliers were linked to MBL producers in non-fermenting bacilli. However, there was no characteristic pattern observed, whether in terms of species or enzyme type, to explain this delay in positivity. This suggests that inter-isolate variability is likely tied to the levels of enzymes produced (Table S1).

Inhibitor stability studies

The control strains consistently yielded reliable results across the various proposed conditions. Stability checks were conducted for up to 1 month for those stored at room temperature, 2 months for plates incubated at 35°C, and 3 months for those stored in the



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Class	Type	Method	Descriptive statistics Median (1st-3rd quartile)
All (n=171)	Enterobacterales (n=121)	CNP (n=121)	30 min (15-45)
		BCT (n=119)	15 min (15-30)
	Non-fermentive bacilli (n=50)	CNP (n=49)	60 min (30-120)
		BCT (n=38)	45 min (30-90)
A (n=21)	Enterobacterales (n=15)	CNP (n=15)	30 min (30-45)
		BCT (n=15)	15 min (15-30)
	Non-fermentive bacilli (n=6)	CNP (n=6)	30 min (30-52.5)
		BCT (n=6)	22.5 min (15-75)
B (n=58)	Enterobacterales (n=18)	CNP (n=18)	37.5 min (15-56.25)
		BCT (n=17)	30 min (15-45)
	Non-fermentive bacilli (n=40)	CNP (n=39)	90 min (45-120)
		BCT (n=28)	60 min (30-93.75)
AB (n=92)	Enterobacterales (n=88)	CNP (n=88)	30 min (15-30)
		BCT (n=87)	15 min (15-30)

FIG 2 The statistical analysis of the time required to achieve a positive result using two different colorimetric methods, BCT and CNPd, stratified by carbapenemase class [Class A producers (n: 21), Class B producers (n: 58), and Class A plus B co-producers (n: 92)]. (A and B) The box plots display the distribution of times (in minutes) needed for a positive result, with red boxes representing outcomes from the BCT and blue boxes indicating results from the CNPd method. The center line of the box indicates the median positivity times. The top and bottom ends of the box indicate the first and third quartiles, respectively. The length of the box is the interquartile range and corresponds to the difference between these two percentiles. The extending lines represent the expected variance of the data. Outliers are times that differ significantly from the rest of the data set and are plotted as individual points beyond the extremes of the line. (A) Stratified by carbapenemase Classes. (B) Stratified by type of bacteria (Enterobacterales or non-fermenting bacilli). (C) The cumulative percentage of positive results is shown over time (in minutes) for both, the BCT and CNPd, stratified by type of bacteria. The red line represents Enterobacterales and the blue line represents non-fermenting bacilli. (D) The cumulative percentage of positive results is shown over time (in minutes) for each type of bacteria (Enterobacterales and non-fermenting bacilli), stratified by method. The light blue line represents the BCT, and the orange line represents CNPd. (E) The cumulative percentage of positive results is shown over time (in minutes) for each carbapenemase Class (A, B, and A plus B), stratified by method. The light blue line represents the BCT, and the orange line represents CNPd. (F) The cumulative percentage of positive results is shown over time (in minutes) for the BCT and CNPd, stratified by carbapenemase Classes (A, B, and A plus B). The red line represents Class A carbapenemase, the blue line represents Class B carbapenemase, and the green line represents cases of dual carbapenemase production involving Class A and Class B enzymes. (G) Descriptive statistics of time (minutes) until the event (positive result) for the median, first quartile, and third quartile of time until the event (positive result), stratified by type of bacteria (Enterobacterales or non-fermenting bacilli) and by the carbapenemase Classes (A, B, and A plus B).

freezer. It's noteworthy that all these strains were accurately classified within 45 minutes by both colorimetric methods, regardless of the tested conditions.

Reproducibility studies

The study consistently demonstrated 100% repeatability in accurately classifying the four *K. pneumoniae* isolates evaluated, encompassing one KPC-2 producer, one NDM-1 producer, one dual KPC-2 plus NDM-5 producer, and one ESBL producer.

DISCUSSION

As the coronavirus disease pandemic surged through Latin America, causing peak hospitalizations across most countries, the region faced an unprecedented emergence of dual carbapenemase producers, mainly within Enterobacterales (2, 4, 5). The accumulation of carbapenem-resistance mechanisms in clinical isolates poses a significant challenge for phenotypic tests. Previous observations indicated that up to 80% of KPC plus NDM dual producer strains had undetected carbapenemases using the classic disk synergy test based on EDTA and boronic acid disks (2). Similarly, mCIM/eCIM mostly identified (91% of cases) the presence of a single serine enzyme (2). Many LMICs laboratories rely on these initial tests to guide empirical therapeutics as susceptibility to late-line drugs is usually assessed on demand or after this phenotypic screening. This situation has the potential to lead to treatments that may not be suitable for the infecting strain's genotype.

Rapid and accurate classification of CPOs is crucial for guiding clinical antibiotic therapy and preventing therapeutic failures. A recent study evaluated a modified CNP test using imipenem/relebactam as a Class A inhibitor (12). This innovative method, leveraging relebactam's ability to inhibit imipenem hydrolysis, achieved exceptional results with 100% sensitivity and specificity, all within a turnaround time of less than an hour. However, it is important to note that this technique cannot distinguish between MBL producers and Classes A plus B dual producers. While the therapeutic approach may remain similar for these two groups, identifying a reservoir containing multiple carbapenemases is crucial for infection control teams. Moreover, certain minority Class A carbapenemases, like Sme in *S. marcescens* and FRI, IMI, and NMC-A predominantly found in *Enterobacter* spp., exhibit resistance to relebactam (16, 17). The presence of these carbapenemases, if not accounted for, could lead to incorrect classification as non-Class A enzymes when relying on relebactam inhibition. Although their prevalence is relatively low, their circulation has been documented in several countries of the Americas region (18–23).

Researchers previously explored adding EDTA to differentiate *P. aeruginosa*-producing Class A from those with Class B carbapenemases (13). The authors concluded that only the CNP test, when incorporating EDTA, effectively differentiated between Class A and Class B carbapenemases, as the BCT struggled to detect most MBLs (13). Using EDTA alone in our panel would have made all dual producers indistinguishable from Class A carbapenemase isolates, with potentially negative implications for initial therapy selection in scenarios where dual producers are prevalent or emerging.

This study introduces an innovative method for detecting and classifying carbapenemase-producing Gram-negative bacilli, providing a distinct advantage by identifying dual Class A plus Class B carbapenemase producers—an aspect not addressed in existing literature. Our findings demonstrate that these colorimetric assays serve as exceptional tools for diagnosing dual carbapenemase producers and distinguishing between single Class A or Class B carbapenemase producers. The updated CNPd assay exhibited 99.4% sensitivity and 100% specificity across all studied classes, enabling accurate detection and classification of all carbapenemases. The modified BCT method successfully detected and classified the majority of carbapenemases in the panel, achieving 91.8% sensitivity and 100% specificity, with significant differences in its ability to detect Enterobacteriaceae and non-fermenting bacilli, particularly NDM-producing *Acinetobacter*, which showed poor detection. This misdetection could be due to the lipoprotein nature of the NDM enzyme (24), which requires more powerful methods for its release into the reaction medium. Such methods are provided by Triton-X

supplementation in CNPd (8). Additionally, these assays yield results within a rapid turnaround time of under 2 hours.

Unlike previous reports (13), the incorporation of EDTA did not result in a significant reduction of the BCT's ability to classify MBL. This difference in performance with this previous report (13), likely stems from variations in reaction conditions introduced in this work, by minimizing the potential impact of alkaline solutions like EDTA on the optimal reaction pH. Regarding serine enzyme inhibitors, our preference favors avibactam over relebactam, despite positive experiences with the latter (12). The primary reason for this preference is avibactam's broader availability compared to the recently approved imipenem-cilastatin-relebactam, accessible in fewer countries. Additionally, avibactam, unlike relebactam, allows the detection of less frequent Class A enzymes as mentioned before (16, 17). The utilization of the pharmaceutical formulation vial containing ceftazidime plus avibactam (Pfizer/Allergan) has yielded promising results comparable to pure avibactam powder, thereby expanding the accessibility of this methodology, even more after the expiration of its patent (not shown) (25).

Generally, both methods achieved earlier detection for Enterobacterales than for non-fermenting bacilli, with significant differences, particularly for Class B carbapenemases. Comparing median times in detecting various classes of carbapenemases, revealed that both methods detected Classes A plus B producers earlier. The observation that both systems detect dual producers as quickly as or faster than their components is of vital significance. This implies that when a hydrolytic profile consistent with dual carbapenemase production is detected, the test can be concluded, as there will be no further alteration in interpretation. Likewise, when avibactam or EDTA alone inhibition is evident, there is no need to prolong incubation for fear of missing a co-producer of Classes A plus B, as the dual producer is expected to become positive before or at the same time.

The inhibitors' remarkable stability, observed under varied storage conditions, suggests potential adoption in the industry. Additionally, it facilitates laboratory workflows by allowing for the pre-preparation of plates containing only ready-to-use inhibitors, offering substantial flexibility in time management. In our laboratory, the cost of a complete plate, accommodating analysis for up to 16 isolates, amounted to $4 (equivalent to $0.25 per strain). Demonstrated stability at 35°C for up to 2 months allows re-incubation of pre-prepared plates with inhibitors, ensuring efficient utilization until all reaction opportunities are exhausted. For re-used panels, deactivating inoculated wells with one drop of a 2% hypochlorite solution (20×) post-test completion is recommended.

This study has several limitations. (i) OXA-48 carbapenemases were excluded from this study due to historical limitations in reliably detecting them with the colorimetric assays (6–9, 12). Therefore, understanding the local epidemiology of circulating carbapenemases is crucial before determining the suitability of these methods in regions outside Latin America, where Class D carbapenemases remain uncommon (5). In scenarios where Class D carbapenemases are emerging, we recommend integrating these rapid tests into the interpretative reading of the antibiogram and evaluating them when available. Isolates exhibiting a susceptibility profile suggesting the potential production of a carbapenemase but yielding negative or inconclusive results in colorimetric assays should undergo confirmation using a more sensitive methodology. Additionally, a colorimetric assay suggesting a Class B alone or in combination with Class A carbapenemase with phenotypic resistance to temocillin (26), or a colorimetric assay suggesting a Class A enzyme with phenotypic resistance to imipenem-relebactam (11), should prompt further investigation for Class D co-production. It is important to clarify that the misidentification of a Class D carbapenemase as part of a combination would have epidemiological implications but would not affect patient treatment. Targeted therapies for Class A with ceftazidime-avibactam, Class B, or their combination with aztreonam-avibactam or cefiderocol, would still effectively cover the undetected Class D enzyme (11, 27, 28). (ii) This study was carried out in a single center, which is also the reference center

for antimicrobial resistance. (iii) It is important to highlight that this study serves only as a proof of concept, aimed at validating the feasibility of inferredunequivocally the major molecular classes of carbapenemase, produced alone or even in combinations. The analytical performance showed in this work should not be considered definitive, as it did not consider the pretest probability because the inclusion criteria were based on a prior positive BCT and CNPd. (iv) The panel of dual carbapenemase-producing strains primarily included Enterobacterales, predominantly *K. pneumoniae* isolates. (v) It is essential to recognize that the validity of these findings should be assessed under different conditions, using reagents for various manufacturers, and within diverse epidemiological scenarios.

In conclusion, colorimetric methods have gained widespread acceptance in Latin American laboratories, given their notable performance and cost-effectiveness, particularly when developed in-house. The enhancements made to BCT and CNPd procedures provide a fast, simple, and cost-effective way to deduce the primary carbapenemases belonging to Classes A and/or B. These improved techniques could serve as an alternative to molecular biology or immunochromatography methods, serving as an initial step in clinical diagnosis and guiding the early application of targeted antibiotic therapy in patient care.

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ADDITIONAL FILES

The following material is available online.

Supplemental Material

Table S1 (JCM01255-23-S0001.xlsx). Bacterial species, resistance mechanisms, and results of the colorimetric assays of the analyzed panel.

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