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Insights on the performance of phenotypic tests versus genotypic tests for the detection of carbapenemase-producing Gram-negative bacilli in resource-limited settings

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Abstract

Background: Carbapenemase-producing Gram-negative (CPGN) bacteria impose life-threatening infections with limited treatment options. Rigor and rapid detection of CPGN-associated infections is usually associated with proper treatment and better disease prognosis. Accordingly, this study aimed at evaluating the phenotypic methods versus genotypic methods used for the detection of such pathogens and determining their sensitivity/specificity values.

Methods: A total of 71 CPGN bacilli (30 *Enterobacterales* and 41 non-glucose-fermenting bacilli) were tested for the carbapenemase production by the major phenotypic approaches including, the modified Hodge test (MHT), modified carbapenem inactivation method (mCIM), combined disk test by EDTA (CDT) and blue-carba test (BCT). The obtained results were statistically analyzed and correlated to the obtained resistant genotypes that were determined by using polymerase chain reactions (PCR) for the detection of the major carbapenemase-encoding genes covering the three classes (Class A, B, and D) of carbapenemases.

Results: In comparison to PCR, the overall sensitivity/specificity values for detection of carbapenemase-producing organism were 65.62%/100% for MHT, 68.65%/100% for mCIM, 55.22%/100% for CDT and 89.55%/75% for BCT. The sensitivity/specificity values for carbapenemase-producing *Enterobacterales* were, 74%100% for MHT, 51.72%/ 100% for mCIM, 62.07%/100% for CDT and 82.75%/100% for BCT. The sensitivity/specificity values for carbapenemase-producing non-glucose fermenting bacilli were, 62.16%/100% for MHT, 81.57%/100% for mCIM, 50/100% for CDT and 94.74%/66.66% for BCT. Considering these findings, BCT possess a relatively high performance for the efficient and rapid detection of carbapenemase producing isolates. Statistical analysis showed significant association ($p < 0.05$) between bla_{NDM} and/or bla_{VIM} genotypes with MHT/CDT; bla_{KPC}/bla_{GIM} genotypes with CDT and bla_{GIM} genotype with BCT.

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Conclusion: The current study provides an update on the performance of the phenotypic tests which are varied depending on the tested bacterial genera and the type of the carbapenemase. The overall sensitivity/specificity values for detection of CPO were 65.62%/100% for MHT, 68.65%/100% for mCIM, 55.22%/100% for CDT and 89.55%/75% for BCT. Based on its respective diagnostic efficiency and rapid turnaround time, BCT is more likely to be recommended in a resource-limited settings particularly, when molecular tests are not available.

Background

Carbapenemases are β-lactamases produced by carbapenem-resistant (CR) bacteria as their major antimicrobial-resistance mechanisms [1, 2]. Owing to their broad spectrum of activity and stability towards the majority of β-lactamases, carbapenems are considered one of the last lines of therapy to treat severe infections caused by multi-drug resistant (MDR) bacteria [2]. However, the emergence of CR among clinically relevant Gram-negative bacilli (GNB) as *Enterobacterales* (such as *E. coli, K. pneumoniae*) and non-glucose fermenting bacilli (such as *A. baumannii, Pseudomonas aeruginosa*) has become globally recognized as one of the most serious challenges to clinicians in dealing with community-acquired and health-care-associated infections [3, 4].

Generally, mechanisms contributing to CR could be through overexpression of efflux pump, decrease in outer membrane permeability coupled with hyper-production of AmpC β-lactamases, and finally through the production of carbapenemase enzymes as the most predominant resistance mechanism [1, 5]. Carbapenemases are β-lactamases that belong to different Ambler classes (A, B, and D). Class A (KPC) and class D (OXA) are serine carbapenemases that depends on serine as an enzyme active center while, class B (NDM, IMP, GIM, VIM) are metallo-β-lactamase enzymes that require Zinc for their activity [6–8]. By far infections caused by carbapenemase-producing organisms (CPOs) either *Enterobacterales* (CPE) or non-glucose fermenting bacilli (CP-NF) are associated with higher mortality rates ranging from 40 to 50% [9] and widespread dissemination as the majority of carbapenemase genes are carried on mobile genetic elements [10], compared to non-CPOs. The CPOs are also resistant to commonly used antibiotics such as aminoglycosides, fluoroquinolones and tetracyclines that will consequently reduce therapeutic options and prolong hospitalization [11, 12]. Accordingly, a variety of laboratory methods is urgently required to allow prompt screening, identification, and implantation of appropriate infection control measures to limit spread of these difficult to treat CPOs.

Despite of being the gold standard in identification of well-known carbapenemase encoding genes, molecular tests are often expensive and require specialized staff, rendering these techniques unavailable for routine clinical practice especially in resource limited settings [13]. To overcome these challenges, several simple and affordable phenotypic tests were recommended by clinical and laboratory standard institute (CLSI) that allow effective detection of CPOs particularly, CPGN pathogens. Currently, growth-based assays and colorimetric hydrolysis methods (blue carba test: BCT, Carba Nordmann/ Poirel: Carb NP) that depends on the growth of the organism in presence of carbapenem antibiotics and degradation of carbapenem products, respectively are commonly used in clinical practice [1, 7, 14]. Of note, CLSI had archived MHT, while other methods as mCIM and Carba NP were endorsed.

Unsuccessful and slow in the diagnosis of CPGN-associated infections is complicated by the treatment failure and patient death. These urges evaluating and regular updates on the sensitivity/specificity values of the existed phenotypic and genotypic diagnostic methods of the most common circulating CPGN pathogens. Although, the phenotypic detection and molecular characterization of CPGN pathogens had been previously reported in literature [13–16], still more studies are urgently required to validate the laboratory performance (sensitivity/specificity/positive predictive value/negative predictive value) of the respective phenotypic methods and to correlate them with the different types of carbapenemase enzymes [1, 14]. Therefore, in this study we aimed to evaluate the performance of four major phenotypic approaches namely MHT, mCIM, BCT, combined disk test by EDTA (CDT) for detecting CPGN pathogens. This was followed by PCR detection of the mostly abundant carbapenemases for exploring the correlation between the associated genotypes and phenotypic tests. The obtained findings of this research will be of a particular importance for determining the most convenient method(s) for rapid and accurate detection of CPGN pathogens particularly, for laboratories with resource-limited settings.

Materials and methods

Bacterial isolates and identification

Over a period of 12 months (January 2021-December 2021), a total of 71 GNB isolates with reduced susceptibility to carbapenem (ertapenem, imipenem and

meropenem) by disk diffusion method were collected from the microbiology laboratories of El-Demerdash a tertiary care hospital with 3200 beds, Cairo, Egypt [17]. This study was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Faculty of Pharmacy, Ain Shams University Research ethics committee, (ACUC-FP-ASU RHDIRB2020110301 REC #72).

The respective isolates were recovered from unidentified clinical specimens including urine (a midstream urine specimen was collected from patients suffered from urinary tract infection), blood (In case of blood stream infections) and sputum (In case of pneumonia) as a routine care of patients admitted to the hospital. Based on the hospital guidelines, an informed consent was obtained from all patients and/or their legal guardian(s) after clarifying to them the objective of this study. For preliminary isolation of GNB, a set of nutrient agar, blood agar, chocolate agar and MacConkey agar were used. For subsequent studies, isolates were preserved at -80 °C. Identification of collected clinical isolates was checked based on microscopic, macroscopic and conventional biochemical tests as mentioned in Bergey's manual of determinative bacteriology [18]. Briefly, macroscopic evaluation involves description of size, shape, elevation, texture, margin and optical character of colonies. A set of biochemical tests including oxidase test, sugar fermentation, triple sugar iron, indole production/ methyl red / Voges Proskauer and citrate utilization (IMVC test) were used to identify Gram negative bacteria. The identification of the recovered isolates was confirmed using the automated microbial identification system, Vitek-2 system (bioMérieux, Marcy L'Etoile, France).

Antimicrobial susceptibility tests

The antibiotic susceptibility testing was determined by Kirby-Bauer disk diffusion method and a panel of 19 antibiotics disks including amoxicillin/clavulanic acid (AMC: 20 µg/10µg), ampicillin (AM:10 µg), amikacin (AK: 30 µg), aztreonam (AZM: 30 µg), cefotaxime (CTX: 30 µg), ciprofloxacin (CIP: 5 µg), ceftriaxone (CRO: 30 µg), ceftazidime (CAZ: 30 µg), cefepime (FEP: 30 µg), cefoxitin (FOX: 10 µg), colistin (CT: 10 µg), ertapenem (ETP: 10 µg), gentamicin (CN: 10 µg), imipenem (IPM: 10 µg), levofloxacin (LEV: 10 µg), meropenem (MEM: 10 µg), ampicillin/ sulbactam (SAM: 10/10 µg), trimethoprim/sulfamethoxazole (SXT: 1.25/23.75ug) and tigecycline (TGC: 15 µg) obtained from Oxoid, Basingstoke, United Kingdom were tested. According to the CLSI guidelines [17], isolates that showed resistance to at least one of the above-mentioned carbapenems were considered CR- GNB while, isolates that were not susceptible to at least one agent in three or more antimicrobial categories were recorded as MDR- GNB [19]. To confirm CR, minimum inhibitory concentration (MIC) of meropenem was determined by broth microdilution method. Isolates that tested intermediate or resistant to current CLSI breakpoints of meropenem (MIC≥2–4 µg/ml for CPE and ≥4–8 µg/ml for CP-NF) were considered as potential carbapenemase producers [16]. A flow chart for detection of CPO was shown in supplementary file Fig S1.

Phenotypic tests for detection of carbapenemase producing organisms (CPO)

-Modified Hodge test (MHT)

An overnight culture of *E. coli* ATCC 25,922 (quality control/indicator strain) equivalent to 0.5 McFarland was diluted 1:10 using sterile saline solution and thereafter was streaked as a lawn on a Mueller Hinton agar plate. After placing meropenem or ertapenem disk at center of plate, 3±5 colonies of overnight tested isolates were streaked from edge to the central disk using sterile swabs and thereafter plates were incubated at 37 °C for 24 h in ambient of air. The production of carbapenemase enzymes were indicated by the appearance of clover leaf like indentation and enhanced growth of indicator *E. coli* strains [20]. Phenotypic tests namely MHT, mCIM, BCT and CDT were done in duplicate to ensure reproducibility.

-Modified carbapenem inactivation method (mCIM)

An overnight culture of tested bacteria (1 µl loopful for *Enterobacterales* and 10 µl for non-glucose fermenting GNB including, *P. aeruginosa*, *A. baumannii* and *Stenotrophomonas maltophilia*) was suspended in 2ml trypticase soy broth (TSB). Meropenem disk was added to the vortexed suspension and broth was incubated for 4 h at 37 °C. Just prior to completion of 4 h incubation, an overnight culture of *E. coli* ATCC 25,922 (adjusted to 0.5 McFarland), was streaked over the surface of Mueller-Hinton agar plate and meropenem disk was introduced to center of plate. Plates were incubated in ambient air at 37 °C for 18 to 24 h. Tested isolates that showed an inhibition zone between 6 and 15 mm or presence of pinpoint colonies within 16–18 mm zone were considered CPO. Tested isolates that showed an inhibition zone≥19 mm were considered carbapenemase negative [20].

-Blue-carba test (BCT)

The BCT is a rapid biochemical test that depends on in vitro hydrolysis of imipenem and release of an acid that can be detected by bromothymol blue indicator. In a microtiter plate, a loopful of tested bacterial culture was added to both tested solution (0.04% bromothymol blue, 0.1mmol/liter ZnSO4 and 3 mg/ml of imipenem with a final pH adjusted to 7) and negative control solution (0.04% bromothymol blue adjusted to pH=7). After about 2 h, the presence of CPO caused a shift in the pH

and turned the tested solution into either yellow or green color, while non-CPO remained blue in color [21–23].

-Combined disk test (CDT)

A Mueller-Hinton agar was over-streaked by an overnight culture of the tested bacterium adjusted to 0.5 McFarland. Imipenem and imipenem/ EDTA disks (Oxoid, Basingstoke, United Kingdom) were placed on the surface of the agar and thereafter plates were incubated at 37 °C for 24 h. Production of class B carbapenemase was indicated by enhancement of inhibition zone ≥ 5 mm for latter disk when compared to former disk [24].

Molecular characterization of carbapenemase producing GNB

Overnight cultures of tested isolates were grown in Luria Bertani (LB) broth containing 25 µg/mL meropenem. PCR detection and conditions of carbapenemase-encoding genes was carried out using PCR as previously described [25]. The set of 5 primers with the corresponding annealing temperatures and expected amplicon sizes was shown in supplementary file (Table S1). The molecular characterization tests were performed from the colonies of the same plate where phenotypic tests were performed whenever possible to ensure uniformity, reproducibility and effective comparison of the results.

Phenotypic analysis using heatmap signature

Morpheus online software (https://software.broadinstitute.org/morpheus/ accessed on 25 March 2022) was used to generate a dendrogram showing heatmap signatures of the isolates, to determine their phenotypic relatedness based on the antimicrobial resistance pattern and the production of carbapenemase enzymes as determined by MHT, mCIM, BCT, CDT by EDTA and PCR. An excel file of required data including isolate code, antibiotic sensitivity, results of 4 phenotypic tests and carbapenemase genes was uploaded and then the interactive tools of Morpheus was used to create hierarchical clustering, annotations and display chart.

Statistical analysis

The sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of each phenotypic test along with their 95% confidence interval (CI) were calculated using the free software VassarStats (http://vassarstats.net/; accessed on 1 February 2022). The clinical research calculator namely calculator 1 was used to determine the previously mentioned values along with 95% CI. Pearson's chi-square test was used to analyze the association of different types of carbapenemase enzymes with each phenotypic test. Spearman's correlation was performed to determine the strength between all possible phenotypes and genotypes combinations. The strength of Spearman's correlation coefficient varies between +1 and −1 value. Values between 0 ± 0.3, 0.4 ± 0.6 and 0.7 ± 1 indicates a weak, moderate strength and strong relationship, respectively. Minitab version 19 was used for statistical analysis, P-value < 0.05 were considered statistically significant results.

Results

Overview on the carbapenem-resistance Gram-negative bacilli (CR-GNB) isolates and antimicrobial susceptibility testing

Of 71 CR-GNB included in the study, 32 (45%), 22 (30.9%), and 17 (23.9%) were derived from sputum, blood, and urine specimens, respectively.

The antibiogram analysis of CR-GNB against various classes of antimicrobial agents including β-lactam group (AMC, AM, AZM, CTX, CRO, CAZ, FEP, FOX, IPM, MEM, SAM, ETP), aminoglycosides (AK, CN), quinolones (CIP, LEV), polymyxins (CT), glycylcyclines (TGC) and sulfonamides/diaminopyrimidines (SXT) was depicted in Fig. 1. The results revealed that the antimicrobial resistance profile of the tested CRE and CR-NF had exceeded 80% for all β-lactam group, aminoglycosides, quinolones and sulfonamides/diaminopyrimidines. On the other hand, the highest susceptibility profile was recorded towards CT ranging from 90.3 to 93.4% and followed by TGC ranging from 39.1 to 46.7%. About 98.6% (70/71) of the tested isolates had shown acquired resistance to at least one agent in three or more antimicrobial classes and were considered MDR. Overall, 95.77% (68/71) of the tested CR-GNB isolates were not susceptible to meropenem MIC and were defined as potential CPO.

Out of 71 CR-GNB isolates, 67 (94.36%) were CPO. Of these carbapenemases, bla_{KPC} was the most frequent (49, 73.13%), followed by bla_{OXA-48} (35, 52.23%), bla_{VIM} (23, 34.32%), bla_{NDM} (10, 14.92%) and bla_{GIM} (4, 5.97%) as depicted in Fig. 2. Out of 67 CPO isolates, 44 (65.67%) produced more than one carbapenemase genes. Co-existence of bla_{KPC} and bla_{OXA-48} was the most dominant (34%), followed by co-detection of class A and class B (27.2%), then class B and class D (20.4%) and finally 3 classes (18.1%). The 4 non-CPO isolates comprised 2 isolates of *A. baumannii* and 1 isolate of *K. pneumoniae* and *P. aeruginosa*.

Performance of phenotypic tests for detection of CPO

The performance of various phenotypic assays (MHT, mCIM, BCT and CDT by EDTA) for detection of CPO were shown in Table 1. Growth based method as MHT and mCIM had shown a sensitivity of 65.62% and 68.65%, respectively for detection of CPO while, both tests had shown 100% specificity. The BCT had a sensitivity of 89.55% and specificity of 75% while; CDT by EDTA had

Table 1 Evaluation of sensitivity, specificity, positive predictive value and negative predictive value of phenotypic tests

Test	GNB	TP[a]	FP[b]	FN[c]	TN[d]	Sensitivity	Specificity	PPV	NPV
95% confidence interval [95% CI]									
MHT	*Enterobacterales* (n = 28)	20	0	7	1	74% [53.40-88.12]	100% [5.46–100]	100% [79.95–100]	12.50% [0-53.32]
	Non glucose fermenting bacilli (n = 40) *A. baumannii* *P. aeruginosa*	23 16 6	0 0 0	14 8 0	3 2 1	62.16% [44.78–77.06] 66.66% [44.69–83.57] 50% [22.28–77.71]	100% [31–100] 100% [19.78–100] 100% [5.46–100]	100% [82.19–100] 100% [75.92–100] 100% [51.68–100]	17.64% [4.67–44.19] 20% [3.54–55.78] 14.28% [0.78-58]
	total tested = 68	43	0	21	4	65.62% [52.61–76.75]	100% [39.57–100]	100% [89.56–100]	15.38% [5.04–35.72]
m-CIM	*Enterobacterales* (n = 30)	15	0	14	1	51.72% [32.89–70.10]	100% [5.46–100]	100% [74.65–100]	6.66% [3.49–33.96]
	Non glucose fermenting bacilli (n = 41) *A. baumannii* *P. aeruginosa*	31 19 11	0 0 0	7 6 1	3 2 1	81.57% [65.10-91.67] 76% [49.65–85.50] 91.66% [62.08–99.60]	100% [31–100] 100% [19.78–100] 100% [5.46–100]	100% [86.27–100] 100% [79.07–100] 100% [67.85–100]	30% [8.09–64.63] 25% [4.45–64.42] 50% [2.66–97.33]
	total tested = 71	46	0	21	4	68.65%, [56.02–79.13]	100%, [39.57–100]	100%, [90.39–100]	16%, [5.25–36.91]
BCT	*Enterobacterales* (n = 30)	24	0	5	1	82.75% [63.51–93.47]	100% [5.46–100]	100% [82.82–100]	16.66% [1.05–70.12]
	Non glucose fermenting bacilli (n = 41) *A. baumannii* *P. aeruginosa*	36 23 12	1 0 1	2 2 0	2 2 0	94.74% [80.93–99.08] 92.56% [72.49–98.60] 100% [69.87–100]	66.66% [12.53–98.23] 100% [19.78–100] 0% [0-94.53]	97.29% [84.19–99.85] 100% [82.19–100] 92.30% [62.08–99.59]	50% [9.18–90.81] 50% [9.18–90.81] NA
	total tested = 71	60	1	7	3	89.55% [79.06–95.34]	75% [21.94–98.68]	98.36% [90.01–99.91]	30% [8.09–64.63]
CDT	*Enterobacterales* (n = 30)	18	0	11	1	62.07 [42.36–78.69]	100% [5.46–100]	100% [78.12–100]	8.33% [0-40.24]
	Non glucose fermenting bacilli (n = 41) *A. baumannii* *P. aeruginosa*	19 15 4	0 0 0	19 10 8	3 2 1	50% [33.65–66.34] 60% [38.89–78.18] 33.33% [11.27–64.56]	100% [31–100] 100% [19.78–100] 100% [5.46–100]	100% [79.07–100] 100% [74.65–100] 100% [39.57–100]	13.63% [3.58–35.96] 16.66% [2.94–49.11] 11.11% [0.58–49.32]
	total tested = 71	37	0	30	4	55.22%, [42.63–67.21]	100%, [39.57–100]	100%, [88.28–100]	11.76% [3.83–28.39]

TP[a], true positive, FP[b], False positive, FN[c], False negative, TN[d], true negative; Sensitivity = a/(a + c), Specificity = d/(b + d), PPV (Positive predictive value) = a/(a + b), NPV (Negative predictive value) = d/ (c + d); MHT, modified Hodge test, mCIM, modified carbapenem inactivation method, CDT, combined disk test by EDTA ; BCT, blue-carba test

* Non glucose fermenting included *A. baumannii*, *P. aeruginosa* and *Stenotrophomonas maltophilia* isolates

a sensitivity of 55.22% and specificity of 100%. For CPE, the sensitivity, specificity, PPV and NPV of four phenotypic tests was 51-82.75%, 100%, 100% and 6.66%-16.66%, respectively. For CP-NF, the sensitivity, specificity, PPV and NPV of four phenotypic tests had ranged from 50 to 94.74%, 66-100%, 97.29-100% and 13.63-50%, respectively.

Heat-map showing different antimicrobial resistance patterns and carbapenemase enzymes (BCT, CDT, mCIM, MHT, PCR) among 71 CR-GNB (Fig. 3). The heat map analysis of the 71 CR-GNB isolates showed that they were not clonal (Fig. 3).

The statistical association between carbapenemase enzyme and phenotypic tests with their respective *p* values was shown in Table 2. The results had indicated that CPO having bla_{KPC} or bla_{GIM} genotypes had shown a statistical significance with CDT by EDTA at *p* value 0.02 and 0.04, respectively. The CPO having bla_{NDM} and bla_{VIM} had also shown statistically significant relation with CDT by EDTA and MHT. Isolates producing bla_{OXA-48} did not show statistical correlation with any of the tested phenotypic tests. Strength of Spearman's correlation coefficient among all possible genotypes and phenotypes combinations was shown in Table 3. The results revealed a significant Spearman's correlation coefficient (0.515–0.774) between bla_{NDM} and/or bla_{VIM} genotypes with isolates giving positive results with MHT and/or CDT by EDTA (supplementary file, Table S2-S8).

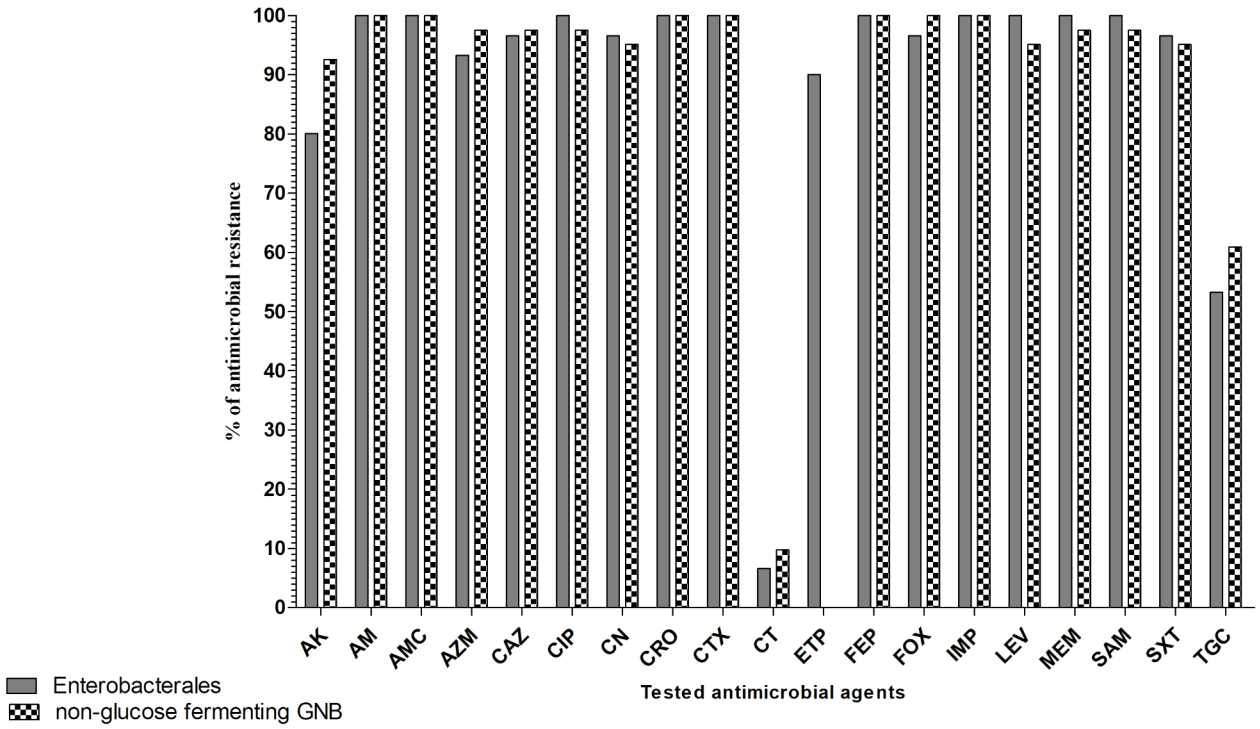

Fig. 1 The antibiogram analysis of Carbapenemase-producing Gram-negative (CPGN) pathogens (n = 71) including, *Enterobacterales* and non-glucose-fermenting Gram-negative bacilli (GNB) against various classes of antimicrobial agents

Discussion

Given their alarming prevalence across the globe including Egypt, infections caused by CR-GNB especially CPO poses an ongoing public health threat that is associated with higher mortality rates, longer hospitalization and increased healthcare costs [26]. Of note, World Health Organization (WHO) had listed CR-NF namely *A. baumannii* complex and *P. aeruginosa* along with CRE as critical pathogens with the most tenacious resistant problem for which innovative new treatments are urgently required [27]. Therefore, this study was conducted to determine the significant association of the molecular and phenotypic tests to determine the most reliable phenotypic test to guide the physician in selecting the appropriate empirical antibiotic therapy, particularly in life-threatening infections such as meningitis and pneumonia or bloodstream infections caused by this nightmare pathogens.

In this study, we aimed to detect the prevalence of CPO across 71 CR-GNB, after checking their resistance profiles by disk diffusion method and determining potential CPO by broth microdilution assay against meropenem antibiotic. Our results revealed that the previously mentioned CR-NF along with *K. pneumoniae* were the most frequently isolated, reflecting their bioburden and ensuring their importance as life threating human pathogens. The antibiogram analysis of CR-GNB clinical isolates revealed high resistance pattern to β-lactam group, aminoglycosides, quinolones and sulfonamides/diaminopyrimidines while, colistin and tigecycline had shown improved susceptibility pattern. Additionally, 98.6% of tested CR-GNB expressed MDR phenotypes, which was in accordance with other recently published national studies that underscore the considerable resistant of CPO [28, 29]. Our analysis of the meropenem MIC profile among distinct bacterial genera revealed that majority of CRE (29/30, 96.66%) and CR-NF (39/41, 95.12%) isolates displayed MIC values of ≥2–4 μg/ml and ≥4–8 μg/ml, respectively and were considered as potential CPO as recommended by CLSI. Despite of meropenem enhanced performance as an initial screening test, still carbapenemase enzymes as $bla_{\text{OXA–48}}$ and variants of bla_{KPC} with weak activity against carbapenems can be underestimated [30–32].

Of concern, the dissemination of these perturbing enzymes including OXA–48, KPC and class B metallo β-lactamases within our hospital settings had called for prompt detection of CPO to allow implementing effective infection control measures and prescribing of appropriate antimicrobial regimen [33, 34]. In context of this, a series of phenotypic tests including MHT, mCIM, BCT and CDT was proposed to evaluate their performance, in comparison to gold standard PCR. Our study revealed that MHT had shown relatively reduced sensitivity and

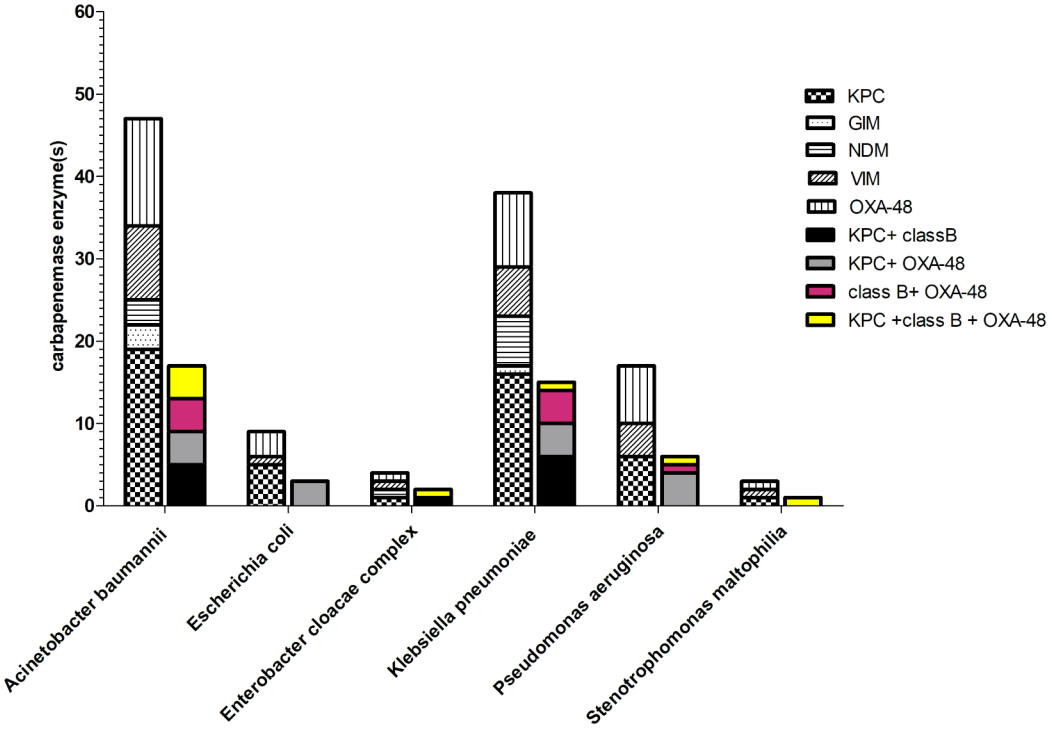

Fig. 2 Prevalence of different carbapenemase genotypes among the recovered carbapenem-resistance Gram-negative bacilli (CR-GNB; n=71).). *Bla*$_{KPC}$, *gene coded for Klebsiella pneumoniae* carbapenemases (KPC); *bla*$_{NDM}$, a gene coded for New Delhi metallo-β-lactamase (NDM); imipenem-resistant *Pseudomonas*-type carbapenemases (IMP); *bla*$_{VIM}$, a gene coded for Verona integron-encoded metallo-β-lactamase (VIM); *bla*$_{GIM}$, a gene coded for German imipenemase and *bla*$_{OXA-48,}$ oxacillinase (OXA-48-like) types

low NPV in detecting CPE (74%, 12.50%) and CP-NF (62.16%, 17.64%), respectively. Such finding could be attributed to failure of MHT to detect 21 CPO isolates (7 CPE and 14 CP-NFB) that carry *bla*$_{KPC}$ either alone or in combination with *bla*$_{OXA-48}$ [1, 14]. However, Yan et al. had improved the efficiency of the MHT in detecting KPC-producing *K. pneumoniae* mucoid colonies by using EDTA that primarily lyses bacterial cells to release β-lactamases [35].

To overcome pitfalls of MHT in terms of its subjective interpretation and validity, CLSI had endorsed a more reliable growth based carbapenemase detection test, mCIM. In comparison to MHT, mCIM had shown improved sensitivity in the detection of total CPO (68.65%) and CP-NF (81.75%). Our findings were in tune with Kuchibiro et al., who reported on improved sensitivity of mCIM in detecting CP-*A. baumannii* and CP-*Pseudomonas* spp. at 76.5% and 90%, respectively [36]. In this study, the decreased sensitivity and NPV of mCIM could be related to high number of false negative results (14 CPE and 7 CP-NF), whereas 16 of these strains (10 CPE and 6 CP-NF) produced *bla*$_{KPC}$, accounting for 76.19%. Of note, such isolates produced either *bla*$_{OXA-48}$ or class B carbapenemase along with *bla*$_{KPC}$. This data suggest that further studies could be required to estimate effect of longer incubation period between tested isolate and meropenem disk (>4 h) to ensure detection of carbapenemases with either low level of expression, weak hydrolytic activities, or class B carbapenemase that requires divalent cations for their action.

Metallo-β-lactamases of subclass B1 as *bla*$_{VIM}$ and *bla*$_{NDM}$ are resistance determinant of increased clinical relevance within our hospital settings. The CDT an inhibitor-based approach that depends on suppressing the enzymatic activity by a chelating agent namely EDTA was tested, prior to PCR. In comparison to MHT and mCIM, CDT by EDTA had shown a reduced sensitivity rate for detection of CPE, CP-NF and CPO at 62.07%, 50% and 55.22%, respectively. Out of 30 false negative results, 27 (90%) carry *bla*$_{KPC}$ alone or in combination with *bla*$_{OXA-48}$, that were not inhibited by EDTA. Of particular concern that the other 3 false negative results belong to CP-NF (*A. baumannii*, *P. aeruginosa*, *Stenotrophomonas maltophilia*) that co-produce *bla*$_{KPC}$ + *bla*$_{VIM}$ + *bla*$_{OXA-48}$. Failure to detect *bla*$_{VIM}$ in such isolates could be attributed to inability of EDTA to reach metallo β-lactamase active site that is characterized by being a shallow groove with few contact points [37] or it could be related to inadequate expression of *bla*$_{VIM}$.

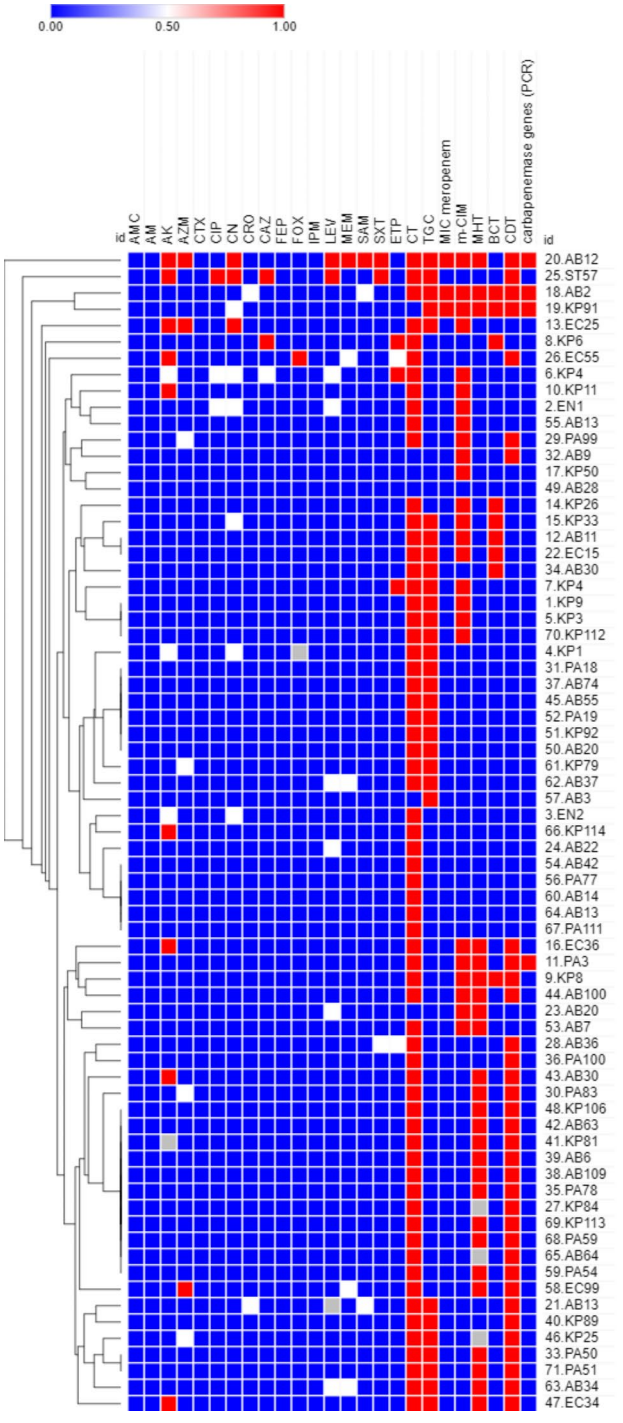

Fig. 3 The heat-map clonal analysis of the among the recovered carbapenem-resistance Gram-negative bacilli (CR-GNB) phenotypes. AMC (amoxicillin/clavulanic acid), AM (ampicillin), AK (amikacin), AZM (aztreonam), CTX (cefotaxime), CIP (ciprofloxacin), CRO (ceftriaxone), CAZ (ceftazidime), FEP (cefepime), FOX (cefoxitin), (CT) colistin, ETP (ertapenem), CN (gentamicin), IPM (imipenem), LEV (levofloxacin), (MEM) meropenem), (SAM) ampicillin/ sulbactam, SXT (trimethoprim / sulfamethoxazole, TGC (tigecycline). MHT (modified Hodge test), mCIM (modified carbapenem inactivation method), CDT, (combined disk test by EDTA), BCT (blue-carba test)

Lately, a variety of colorimetric methods that explore hydrolytic activity of carbapenemase enzymes within a short turnaround time as Carba-NP and its variant BCT had been proposed. In comparison to PCR, the colorimetric assays always-broader detection of carbapenemase activity and it is a reliable method for rapid detection of CPO directly from solid media or blood cultures [38].

Among the other three evaluated phenotypic tests in this study, BCT had presented the highest sensitivity for detection of CPE, CP-NF, and CPO at 82.75%, 94.74% and 89.55%, respectively. Our results were nearly in agreement with other studies that reported on higher sensitivity of BCT for detection of CPE ranging from 95.3 to 98% [39, 40]. However, a relatively lower specificity (66.66%) and PPV (97.29%) were recorded for BCT among CR- NG in our study. This could be attributed to false positive result exhibited by an *A. baumannii* isolate that carry either $bla_{\text{OXA–48}}$ variants or rare carbapenemase not detected by PCR. On the contrary, no false positive results were recorded with the other three phenotypic tests, rendering perfect specificity and PPV.

Another important aim of our study was to evaluate the correlation between CR genes and different phenotypic tests. Our results recorded a statistically significant difference between CPO isolates having class B carbapenemases and/or bla_{KPC} with CDT by EDTA. Although previous studies had reported on effectiveness of EDTA to inhibit metallo β-lactamase enzymes [41, 42], still there is paucity of data on its capability to interact with bla_{KPC}. However, we should put in consideration that EDTA itself effect membrane permeabilization particularly in non-fermenting bacilli and restore activity of carbapenem leading to false interpretation of CDT [43, 44]. Additionally, CPO having bla_{VIM} or bla_{NDM} had shown statistically significant difference with MHT. This results were comparable to Sultan et al., who reported on effectiveness of MHT to determine bla_{NDM} in clinical laboratories of Pakistan [45]. Finally, *Enterobacterales* isolates producing bla_{GIM} had shown significant statistical association with BCT. In summary, each phenotypic test has its own pros and cons. However, limitation of study includes the inability to evaluate performance of Carba NP and presence of isolates harboring more than one type of carbapenemase enzymes that may impede performance of CDT with EDTA. Another limitation is evaluating performance of MHT that is not currently recommended by CLSI.

Conclusion

The performance of phenotypic tests (sensitivity/specificity/PPV/NPV) varied depending on bacterial genera, genotype, and coexistence of more than one type of carbapenemase. Statistical analysis showed significant association ($p<0.05$) between $bla_{\text{NDM}}/bla_{\text{VIM}}$ genotypes

Table 2 Statistical association between carbapenemase enzyme and phenotypic tests with their respective *p* values

Pearson Chi-Square (two-tailed *P* value)						
Carbapenamase enzyme	Phenotypic Tests	*Enterobacterales*	*A. baumannii*	*P. aeruginosa*	Non-glucose fermenting GNB	Total tested isolates
bla_{KPC}	MHT	P = 0.334	P = 0.126	P = 0.428	P = 0.116	P = 0.096
	mCIM	P = 0.525	P = 0.573	P = 0.715	P = 0.475	P = 0.500
	BCT	P = 0.084	P = 0.693	P = 0.188	P = 0.261	P = 0.088
	CDT	P = 0.192	P = 0.136	P = 0.071	P = 0.021*	P = 0.020*
bla_{NDM}	MHT	P = 0.053	P = 0.145	NA	P = 0.121	P = 0.009*
	mCIM	P = 0.159	P = 0.881	NA	P = 0.707	P = 0.684
	BCT	P = 0.631	P = 0.193	NA	P = 0.072	P = 0.464
	CDT	P = 0.017*	P = 0.100	NA	P = 0.052	P = 0.001*
bla_{VIM}	MHT	P = 0.039*	P = 0.005*	P = 0.014*	P = 0.006*	P = 0.000*
	mCIM	P = 0.056	P = 0.738	P = 0.273	P = 0.526	P = 0.350
	BCT	P = 0.732	P = 0.136	NA	P = 0.159	P = 0.943
	CDT	P = 0.033*	P = 0.001*	P = 0.030*	P = 0.004*	P = 0.000*
bla_{GIM}	MHT	P = 0.519	P = 0.727	P = 0.260	P = 0.738	P = 0.615
	mCIM	P = 0.309	P = 0.512	P = 0.657	P = 0.707	P = 0.523
	BCT	P = 0.041*	P = 0.603	NA	P = 0.613	P = 0.446
	CDT	P = 0.406	P = 0.188	P = 0.118	P = 0.052	P = 0.048*
bla_{OXA-48}	MHT	P = 0.717	P = 0.003*	P = 0.428	P = 0.061	P = 0.115
	mCIM	P = 0.269	P = 0.472	P = 0.715	P = 0.319	P = 0.099
	BCT	P = 0.140	P = 0.495	NA	P = 0.63	P = 0.305
	CDT	P = 0.313	P = 0.816	P = 0.071	P = 0.277	P = 0.287

* Statistically significant, MHT, modified Hodge test, mCIM, modified carbapenem inactivation method, CDT, combined disk test by EDTA ; BCT, blue-carba test. Non glucose fermenting bacilli included *A. baumannii, P. aeruginosa* and *Stenotrophomonas maltophilia* isolates. NA: not applicable

with MHT/CDT; bla_{KPC}/bla_{GIM} genotypes with CDT and bla_{GIM} genotype with BCT. BCT possess a relatively high performance for the efficient and rapid detection of CPO. The overall sensitivity/specificity values for detection of CPO were 65.62%/100% for MHT, 68.65%/100% for mCIM, 55.22%/100% for CDT and 89.55%/75% for BCT. Based on its respective diagnostic efficiency and rapid turnaround time, BCT is more likely to be recommended in our resource limited settings when PCR is not available.

Table 3 Spearman correlation coefficient among all possible genotypes and phenotypes combinations

Sample 1	Sample 2	Correlation	95% CI for ρ	*P*-Value
bla_{VIM} + bla_{GIM} + bla_{NDM}	MHT + BCT + EDTA	0.774	[64.0- 86.2]	0
bla_{VIM} + bla_{NDM}	MHT + EDTA	0.763	[62.5–85.5]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	EDTA + mCIM + MHT	0.736	[58.7–83.6]	0
bla_{VIM} + bla_{NDM}	EDTA	0.724	[0.57.1–82.8]	0
bla_{VIM} + bla_{NDM}	MHT + BCT + EDTA	0.718	[0.56.3–82.4]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	MHT	0.711	[54.8–0.82.1]	0
bla_{VIM} + bla_{NDM}	EDTA + mCIM + MHT	0.683	[51.6–0.80.0]	0
bla_{VIM} + bla_{NDM}	MHT	0.679	[50.7–80.0]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	BCT + EDTA	0.678	[50.9–79.7]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	MHT + BCT + EDTA + mCIM	0.653	[47.7–77.9]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	MHT	0.653	[47.3–78.1]	0
bla_{VIM} + bla_{NDM}	MHT + BCT + EDTA + mCIM	0.623	[43.8–75.7]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	MHT + EDTA	0.617	[43.1–75.3]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	EDTA + mCIM + MHT	0.61	[42.1–74.7]	0
bla_{VIM} + bla_{NDM}	Blue Carba + EDTA	0.607	[41.8–74.6]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	MHT	0.6	[40.4–74.3]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT + EDTA	0.593	[40.1–73.5]	0
bla_{VIM} + bla_{GIM}	MHT + EDTA	0.581	[38.6–72.6]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	mCIM + EDTA	0.581	[38.6–72.6]	0
bla_{VIM} + bla_{GIM}	EDTA	0.577	[38.1–72.3]	0
bla_{VIM} + bla_{NDM}	MHT + BCT	0.559	[35.9–71.0]	0
bla_{VIM} + bla_{GIM} + bla_{NDM}	MHT + BCT	0.558	[35.8–70.9]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT + EDTA + mCIM	0.558	[35.8–70.9]	0
bla_{VIM} + bla_{GIM}	MHT + BCT + EDTA	0.548	34.6–70.2]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT	0.544	[34.1–69.8]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	EDTA + mCIM + MHT	0.541	[33.8–69.7]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	MHT + EDTA	0.533	[32.8–69.0]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT + EDTA	0.524	[31.7–68.3]	0
bla_{VIM} + bla_{NDM}	mCIM + EDTA	0.523	[31.6–68.2]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT	0.518	[31.0- 67.9)	0
bla_{VIM}	MHT + EDTA	0.517	[30.7–67.9]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	mCIM + MHT	0.515	[30.6–67.6]	0
bla_{VIM} + bla_{GIM} + bla_{NDM} + bla_{OXA-48}	EDTA	0.515	[30.7–67.7]	0
bla_{VIM} + bla_{NDM} + bla_{OXA-48}	MHT + BCT + EDTA + mCIM	0.509	[29.9–67.2]	0
bla_{VIM}	MHT + BCT + EDTA	0.502	[29.0- 66.8]	0

Bla_{KPC}, *gene coded for Klebsiella pneumoniae* carbapenemases (KPC); bla_{NDM}, a gene coded for New Delhi metallo-β-lactamase (NDM); imipenem-resistant *Pseudomonas*-type carbapenemases (IMP); bla_{VIM}, a gene coded for Verona integron-encoded metallo-β-lactamase (VIM); bla_{GIM}, a gene coded for German imipenemase and bla_{OXA-48}, oxacillinase (OXA-48-like) types. MHT, modified Hodge test, mCIM, modified carbapenem inactivation method, CDT, combined disk test by EDTA; BCT, blue-carba test

List of abbreviations

Bla_{KPC}	the gene coded for *Klebsiella pneumoniae* carbapenemases (KPC).
bla_{NDM}	the gene coded for New Delhi metallo-β-lactamase (NDM).
Bla_{IMP}	the gene coded for the imipenem-resistant *Pseudomonas*-type carbapenemases (IMP).
bla_{VIM}	a gene coded for Verona integron-encoded metallo-β-lactamase (VIM).
bla_{GIM}	the gene coded for German imipenemase
bla_{OXA-48}	the gene coded oxacillinase (OXA-48-like) types
BCT	blue-carba test
CLSI	clinical and laboratory standard institute
CDT	combined disk test by EDTA
CPGN	Carbapenemase-producing Gram-negative (CPGN)
CPO	Carbapenemase-producing organisms
EDTA	ethylene diamine tetra acetic acid
GNB	Gram-negative bacilli
MDR	mlti-drug resistant
MHT	modified Hodge test
mCIM	modified carbapenem inactivation method
FP	False positive
FN	False negative
TP	true positive
TN	true negative
PPV	Positive predictive value
NPV	Negative predictive value
PCR	polymerase chain reaction

Supplementary Information

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Supplementary Material 1

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Authors' contributions
NAK and STT have collected the isolates and performed all experiments incorporated in the manuscript under the supervision and guidance of KMA. KMA has designed the protocol of this study. NAK has written the first draft of manuscript. KMA follow the practical part of regarding the multifactorial design experiments. KMA, and ISY have helped in writing, and revising this manuscript. All authors read and approved the final manuscript.

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Data availability
All data generated or analyzed during this study are included in this published article in the main manuscript.

Declarations

Ethics approval and consent to participate
This study was conducted in accordance with the Declaration of Helsinki, reviewed and approved by the Faculty of Pharmacy, Ain Shams University Research ethics committee, (ACUC-FP-ASU RHDIRB2020110301 REC #72). The informed consent was obtained from all subjects and/or their legal guardian(s).

Consent to publish
not applicable.

Competing interests
The authors declare that they have no competing interests.

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