Various phenotypic techniques for detection of beta-lactam resistance in Pseudomonas species and Acinetobacter species: a single-center experience

Asmaa Omar Ahmed, Hanan Hareth Abdellatif, Ahmed Elsayed M. Abdallah

Microbiology Unit, Department of Clinical Pathology, Assiut University Hospital, Assiut, Egypt

Correspondence to Ahmed Elsayed M. Abdallah,MSc,
Microbiology Unit, Department of Clinical Pathology, Assiut University Hospital, Assiut, Egypt.
Tel: +201011420362;
Fax: +088-2332278-2080278;
e-mail: ahmedelsayedabdallah1@gmail.com

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Background
The World Health Organization has emphasized that the risk of antibiotic resistance of *Pseudomonas aeruginosa* (PSA) and *Acinetobacter baumannii* (ACB) is due to the extended-spectrum β-lactamase (ESBL) and carbapenemase activity.

Objectives
The study was designed to describe the rates of different β-lactamases, and to assess the best phenotypic method for detection of these resistances.

Methodology
This cross-sectional study included 124 isolates obtained from the patients of Assiut University Hospital. Screening and phenotypic confirmatory tests for resistance were done. The study was approved and monitored by the Medical Ethics Committee, Assiut Faculty of Medicine, IRB 17101464. The antimicrobial-susceptibility tests were done by the Kirby–Bauer disk-diffusion method according to the CLSI 2019 guidelines and by automated Vitek2 Compact 15 system. Also, different phenotypic methods were used.

Results
The highest percentages of β-lactamase enzymes in 52 Pseudomonas isolates (53.8%) were due to both ESBL and carbapenemases (CARBA), whereas isolates with solo ESBL were 19.2% of the total isolates and the least percentages were due to CARBA. The highest percentages of β-lactamase enzymes in 72 Acinetobacter isolates (33.3%) were due to CARBA alone, whereas isolates with both ESBL and CARBA were 16.7% of the total isolates and the least percentages (5.6%) were due to ESBL. The combined-disk test had a high sensitivity and specificity in detection of ESBL and metallo-beta-lactamase (MBL) in PSA, whereas in ACB showed high sensitivity only.

Conclusion
The ESBL and MBL showed the highest percentage among Pseudomonas isolates, whereas among Acinetobacter isolates, the MBL showed the highest percentage. The phenotypic confirmatory tests showed high sensitivity and specificity and proved to be reliable approaches for identification of the β-lactamase resistance.

Keywords:
acinetobacter baumannii, beta-lactam resistance, carbapenemase, phenotypic methods, *Pseudomonas aeruginosa*

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Introduction

The incidence of nosocomial infections induced by resistant species of Pseudomonas and Acinetobacter in the clinical settings is seriously increased. *Pseudomonas aeruginosa* is the leading cause of infections in immunocompromised patients and it is responsible for most of chronic lung infections in cystic fibrosis patients and severe infections in burn victims [1,2]. The World Health Organization has identified it as a serious pathogen due to the evidence of resistance to multiple drugs that necessitate novel management of the problem [3]. This was mainly due to the inappropriate use of β-lactam antibiotics, which led to appearance of the various β-lactamases [4,5]. Also, *Acinetobacter baumannii* has emerged as a very virulent organism with high mortality and morbidity that mainly affects the patients of burn and intensive-care units [6,7].

Since 1980, hospital-acquired infections were identified to be caused by Pseudomonas sp. and Acinetobacter sp. producing extended-spectrum β-lactamases (ESBLs). They are a cluster of enzymes that inactivate β-lactams and responsible for the resistance to penicillin, third-generation cephalosporins, and the monobactam [8]. Yet, clavulanic acid can inhibit them [9]. Another group of enzymes that play a

major role in the antibiotic resistance are the ampC beta-lactamases (AmpC) that mainly inactivate cephamycin (second-generation cephalosporins), whereas carbapenemases (CARBA) are the category of β-lactamases that provide multiple antibiotic resistance to β-lactam, like penicillins, β-lactam-inhibitor combinations, cephalosporins, and carbapenems [10].

The last group, metallo-beta-lactamase (MBL), differs from the three others by the fact that it possesses in the active site metallic ions, whereas the others are serine-active enzymes. This group exhibits a broad-spectrum hydrolysis, including all beta-lactams, except aztreonam [11]. Although polymerase chain reaction (PCR) is the most accurate method for detection of the enzymes, the phenotypic tests in clinical laboratories are very sensitive and special and are better appropriate for routine testing [12]. There are unmet needs to estimate the prevalence of these virulent enzymes in those two organisms in our region. So, this study was designed to detect the distribution of different beta-lactamases between isolates of Pseudomonas and Acinetobacter species and to compare between different phenotypic methods for detection of B-lactamases.

Methodology

This cross-sectional study was done in the Microbiology Unit of Clinical Pathology Department at Assiut University Hospital. We included 124 isolates in the period between June 2018 and April 2020 from several clinical specimens (blood, urine, sputum, and pus). The ethical committee of the Faculty of Medicine in Assiut University approved our study under IRB number 17101464. The informed consents were provided from the patients before recruitment of the isolates. All isolates were identified by the standard microbiological tests.

The antimicrobial-susceptibility tests were done by the Kirby–Bauer disk-diffusion method according to the CLSI 2019 guidelines and by Vitek2 Compact 15 system [13].

Detection of ESBLs

Isolates that showed resistance to third-generation cephalosporin were suspected to be ESBL producers and confirmed by phenotypic tests (ChromID™ ESBL agar, ESBL combined-disk test, and E-Test). We used the ESBL E-Test as a gold-standard test [14].

ChromID™ ESBL agar (BioMérieux)

This is a selective chromogenic medium used according to the manufacturer's instructions for identification of enterobacteria with ESBL. Pseudomonas sp. appear as straw-colored, reddish-brown, or colorless colonies, Acinetobacter sp. appear as pale-white colonies or translucent colonies.

Combined-disk test (Oxoid)

The test evaluates the synergy between an oxyimino cephalosporin and clavulanic acid. Disks of ceftazidime (30 μg) alone and ceftazidime + clavulanic acid (30 μg/10 μg) were used [13].

ESBL test of Vitek2 compact 15 (BioMérieux)

This is a novel method to rapidly detect ESBL formation based on simultaneous evaluations of cefepime, cefotaxime, and ceftazidime-inhibitory effects alone and in the presence of clavulanic acid [15].

E-Test (BioMérieux)

Cefotaxime/cefotaxime + clavulanic acid (CT/CTL) and ceftazidime/ceftazidime + clavulanic acid (TZ/TZL) were used according to the manufacturer's instructions to detect the clavulanic acid-inhibitable ESBL.

Detection of carbapenemases

Isolates that showed resistance to carbapenems were suspected to be carbapenemase producers and confirmed by phenotypic tests (ChromID® CARBA SMART agar, modified Hodge test (MHT), and Rapidec Carba NP Test). Sensitivity and specificity could not be calculated for these tests due to the inability to perform PCR that is the gold-standard test [16].

Those isolates were also tested for MBL production by combined-disk test and E-test. The E-test was taken as a gold-standard test [17].

ChromID® CARBA SMART agar (BioMérieux)

This is a selective chromogenic medium used according to the manufacturer's instructions for the identification of carbapenemase produced by bacteria.

The presence of typical colonies in the CARB compartment: suspicion of CP bacteria. The presence of typical colonies in the OXA compartment: suspicion of OXA-48-type CP bacteria. In Pseudomonas sp., isolates appear as greenish or reddish-brown colonies, and in Acinetobacter sp., isolates appear as reddish-yellow colonies.

Modified Hodge test (MHT)

The tested microorganism inactivates the carbapenem by carbapenemase that diffuses from the disk, after it has been placed on the Mueller Hinton Agar [18] (Fig. 1).

Quality control

(1) *K. pneumoniae* ATCC BAA 1705, positive control.
(2) *K. pneumoniae* ATCC BAA 1706, negative control.

Rapidec Carba NP test (BioMérieux)

It is a ready-to-use strip for the rapid detection of carbapenemase production. The test is used according to the manufacturer's instructions and based on the detection of carbapenem hydrolysis by carbapenemase as hydrolysis acidifies the medium that changes the color of the pH indicator (Fig. 2).

Detection of metallo-β-lactamases was done by

Combined-disk test (Oxoid)

The test evaluates the synergy between carbapenem and EDTA. Two disks – 10 μg of meropenem and meropenem/EDTA (10 μg + 750 μg) were used [19].

E-test (IP/IPI) imipenem and imipenem-EDTA (BioMérieux)

Strips were used according to the manufacturer's instructions to confirm the presence of EDTA-inhibitable MBL enzymes.

Results

From the 124 isolates that were included in the study, there were 52 Pseudomonas sp. and 72 Acinetobacter sp. isolates. Screening and confirmation tests of the phenotypes of various beta-lactamases were done.

Results of phenotypic screening tests

The antibiotic-resistance pattern by Vitek2 and disk-diffusion method was almost the same; there was mild variation in resistance to ampicillin, imipenem, cefepime, and ceftriaxone. In Acinetobacter isolates, there was a difference in antibiotic resistance to imipenem between Vitek2 and disk-diffusion method as in Table 1.

Results of phenotypic confirmatory tests in Pseudomonas isolates

ESBL phenotypic confirmatory tests

Among the phenotypic confirmatory tests, the combined-disk test showed the highest sensitivity and specificity, and the chromogenic media showed the lowest specificity (Table 2).

Carbapenemases' phenotypic confirmatory tests

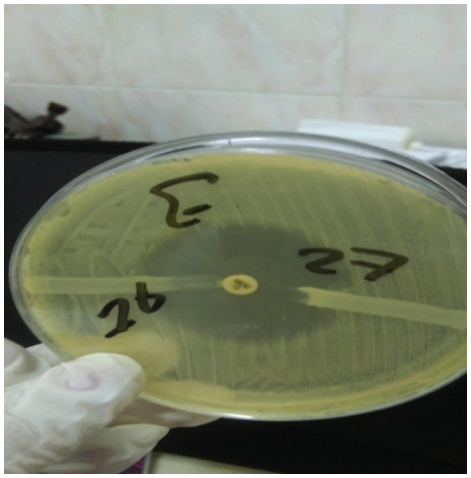
Among the phenotypic confirmatory tests, the ChromID® CARBA SMART agar detected the highest percentage of carbapenemase producer among the phenotypic confirmatory tests, then Carba NB, and last MHT. Their percentages were 96.3%, 77.8%, and 70.4%, respectively.

The combined-disk test showed sensitivity (76.5%) and specificity (80%) as a phenotypic confirmatory test for the detection of the MBL.

Distribution of different beta-lactamases among Pseudomonas isolates

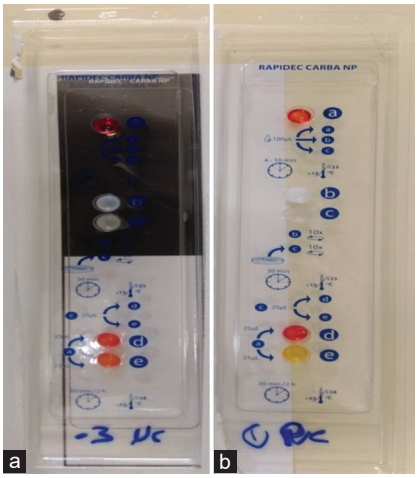
The highest percentage of B-lactamase enzymes (53.8%) was due to both ESBL and CARBA, whereas isolates with solo ESBL were 19.2% of the total isolates of

Figure 1



Modified Hodge test: (26) negative result, (27) positive result.

Figure 2



Rapidec Carba NP: (a) negative result, (b) positive result.

Table 1 Antibiotic-resistance pattern of Pseudomonas spp. and Acinetobacter sp. isolates by disk-diffusion method and Vitek2

Antibiotic	Acinetobacter sp.		Pseudomonas sp.	
	Resistance (%) by disk-diffusion method	Resistance (%) by Vitek2	Resistance (%) by disk-diffusion method	Resistance (%) by Vitek2
Ampicillin	39 (93%)	37 (88.1%)	26 (96.3%)	22 (81.5%)
Ampicillin/sulbactam	–	20 (47.6%)	–	12 (46.2%)
Piperacillin/tazobactam	37 (88.1%)	37 (88.1%)	19 (70.5%)	22 (81.5%)
Cefazolin	39 (93%)	42 (100%)	–	25 (93%)
Cefoxitin	39 (93%)	24 (57.1%)	–	25 (93%)
Ceftazidime	39 (93%)	38 (90.5%)	23 (85.2%)	23 (85.2%)
Ceftriaxone	42 (100%)	36 (85.7%)	27 (100%)	15 (55.5%)
Cefepime	38 (90.5%)	39 (93%)	26 (96.3%)	19 (70.5%)
Imipenem	31 (73.8%)	27 (64.3%)	20 (74%)	15 (55.5%)
Meropenem	40 (95.2%)	40 (95.2%)	23 (85.2%)	23 (85.2%)
Amikacin	–	29 (69%)	–	6 (22.2%)
Gentamicin	–	29 (69%)	–	16 (59.3)
Tobramycin	–	34 (81%)	–	20 (77%)
Ciprofloxacin	35 (83.3%)	35 (83.3%)	18 (66.7%)	18 (66.7%)
Levofloxacin	39 (93%)	39 (93%)	20 (77%)	20 (77%)
Trimethoprim/sulfamethoxazole	26 (62%)	27 (64.3%)	12 (46.2%)	12 (46.2%)
Azetronam	39 (93%)	–	23 (85.2%)	–

Table 2 Sensitivity, specificity, positive predictive value, and negative predictive value of phenotypic confirmatory tests for ESBL detection in Pseudomonas isolates

Confirmatory test	Sensitivity	Specificity	PPV	NPV
Combined-disk test	82.4%	57.1%	82.4%	57.1%
Chromogenic media	82.4%	42.9%	77.8%	50%

ESBL, extended-spectrum β-lactamase; NPV, negative predictive value; PPV, positive predictive value

Pseudomonas and the least percentages were due to CARBA (Table 3 and Fig. 3).

Results of phenotypic confirmatory tests in Acinetobacter isolates

ESBL phenotypic confirmatory tests

Among the phenotypic confirmatory tests, the combined-disk test showed the same sensitivity and specificity as the chromogenic media in Acinetobacter isolates (Table 4).

Carbapenemases' phenotypic confirmatory tests

Among the phenotypic confirmatory tests, the ChromID® CARBA SMART agar detected the highest percentage of carbapenemase producer among the phenotypic confirmatory tests, then Carba NB, and last MHT (Table 5).

The combined-disk test showed high sensitivity (87.5%) but low specificity (36.4%) as a phenotypic confirmatory test for the detection of the MBL.

Distribution of different beta-lactamases among Acinetobacter isolates

The highest percentage of B-lactamase enzymes (33.3%) was due to CARBA alone, whereas isolates with both ESBL and CARBA were 16.7% of the total isolates of Acinetobacter and the least percentages (5.6%) were due to ESBL and AmpC (Table 6 and Fig. 4).

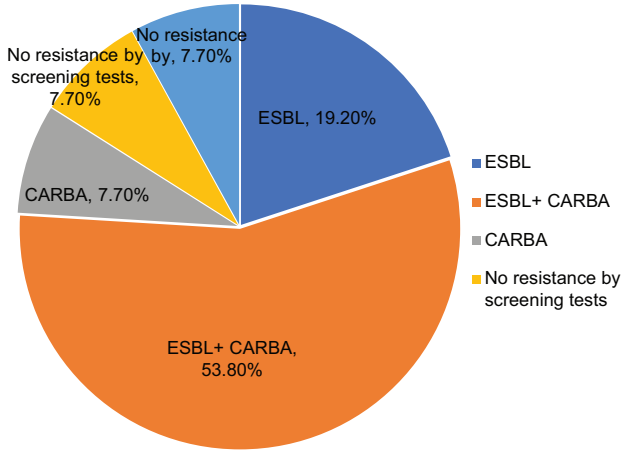
Discussion

Resistance to antibiotics has grown as a major worldwide concern and is affecting nearly all bacterial species. Beta-lactamase enzyme could destroy the antimicrobial activity of penicillin and cephalosporin groups through the disruption of their 4-membered β-lactam rings. The production of β-lactamase may be chromosomal or plasmid-associated [20].

In addition to optimum patient management and the immediate implementation of appropriate infection-control measures to prevent the spread of those pathogens and also to avoid infections and community outbreaks acquired in the clinical environment, the early and correct determination of beta-lactamases producing Gram-negative bacilli is mandatory [21].

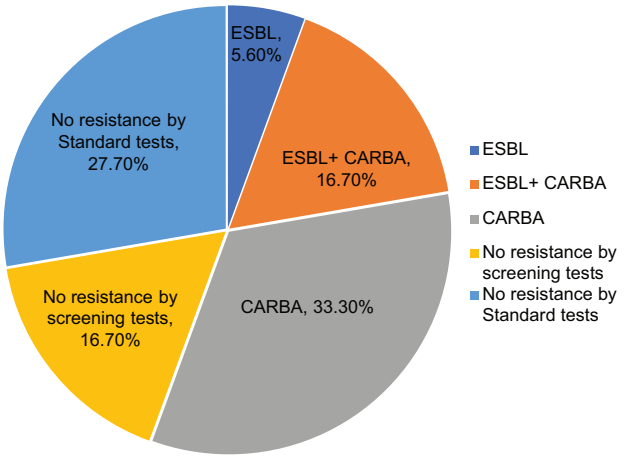
In the current study, we found that the results of antibiotic-susceptibility tests by Vitek2 compact and disk-diffusion method were almost the same (there was mild variation in resistance to different antibiotics), but the Vitek2 compact required less technical time per test, and provided earlier results than disk-diffusion method. This agrees with Mazzoriol *et al.* [22] study, which proved that Vitek2 and disk method produced very similar overall susceptibility-category agreements in Pseudomonas. On the other hand, Rechenchoski *et al.* [23] compared the Vitek2® automated system and disk-diffusion method, with using the broth microdilution as gold standard, and

Figure 3



Pie chart of the distribution of different beta-lactamases among the Pseudomonas isolates.

Figure 4



Pie chart of the distribution of different beta-lactamases among the Acinetobacter isolates.

Table 3 Distribution of different beta-lactamases among the Pseudomonas isolates

Type of enzyme	Positive (*n*=52) (positive (%=100%))
ESBL	10 (19.2%)
ESBL+CARBA	28 (53.8%)
CARBA	6 (11.5%)
No resistance by screening tests	4 (7.7%)
No resistance by standard tests	4 (7.7%)

ESBL, extended-spectrum β-lactamase.

Table 4 Sensitivity, specificity, positive predictive value, and negative predictive value of phenotypic confirmatory tests for ESBL detection in Acinetobacter isolates

Confirmatory test	Sensitivity	Specificity	PPV	NPV
Combined-disk test	100%	13.3%	38.1%	100%
Chromogenic media	100%	13.3%	38.1%	100%

ESBL, extended-spectrum β-lactamase; NPV, negative predictive value; PPV, positive predictive value.

Table 5 Percentage of carbapenemase detection by phenotypic confirmatory tests in Acinetobacter isolates

Confirmatory test	ChromID® CARBA SMART	Rapidec Carba NP Test	MHT
Percentage of carbapenemase detection	97.1	82.9	71.4

MHT, Modified Hodge test.

Table 6 Distribution of different beta-lactamases among the Acinetobacter isolates

Type of enzyme	Positive (*n*=72) (positive (%=100%))
ESBL	4 (5.6%)
ESBL + CARBA	12 (16.7%)
CARBA	24 (33.3%)
No resistance by screening tests	12 (16.7%)
No resistance by standard tests	20 (27.7%)

ESBL, extended-spectrum β-lactamase.

reported that the Vitek2® automated system was more sensitive than disk-diffusion method.

In the comparison between different ESBL phenotypic confirmatory methods, among the phenotypic methods, we noted that the combined-disk test showed the same sensitivity (82.4%) for detection of ESBL as the chromogenic media that showed a lower specificity (42.9%) than combined-disk test (specificity 57.1%) in Pseudomonas. These results disagreed with Lin *et al.* [24], who show sensitivity and specificity 7.7% and 100%, respectively, for combined-disk test. Phenotypic detection of ESBLs can be obscured by the chromosomal AmpC cephalosporinase in *P. aeruginosa*.

Although the sensitivity of the combined-disk test was 100%, specificity was 13.3% in Acinetobacter isolates. We noted that the chromogenic media showed the same sensitivity (100%) and specificity (13.3%) for detection of ESBL in Acinetobacter.

Huang *et al.* [25] show the same sensitivity and specificity of chromogenic ESBL media (94.9%), but these results were including Enterobacteriaceae besides Acinetobacter and Pseudomonas isolates. Often, Acinetobacter species show false-positive results due to coexpression of AmpC, so the isolate should be retested by a method that is unaffected by AmpC b-lactamases [26].

Among all methods tested, the ChromID ESBL agar detected ESBL-producing isolates with high sensitivity but showed the lowest specificities. The main advantage of the ChromID ESBL agar sensitivity was enabling the recovery and identification of most ESBL-producing organisms within 24 h, that is, only for screening.

There were 70.8% of Pseudomonas isolates that were positive by E-test (reduction of the MIC by three doubling dilutions (i.e. MIC ratio of ≥8) in the

presence of clavulanic acid). This was concordant with previous study that investigated ESBL-producing Gram-negative bacteria that show 66.7% of Pseudomonas isolates to be ESBL-producing [27]. There were 34.8% of Acinetobacter isolates that were positive. This low percentage needs to be confirmed by cloxacillin-containing plates or at reduced growth temperature [28].

Clavulanate may operate as a high AmpC inducer, leading to false negatives in the identification of ESBL by enhancing screening drug resistance. Tazobactam and a sulbactam, which are far less likely to increase AmpC β-lactamases, are recommended as ESBL-detection inhibitors and cephalosporin of the fourth generation is the better choice as an indicator medicine.

In the presence of AmpC β-lactamases, cefepime is a more reliable ESBL detector because of its stable AmpC β-lactamase.

Thus, in the presence of the AmpC enzyme, it demonstrates the synergy that emerges from the ESBL's suppression. In the current study, we found that the ChromID® CARBA SMART agar detected the highest percentage of carbapenemase producer (96.3% and 97.1%) for Pseudomonas and Acinetobacter, respectively, among the phenotypic confirmatory tests, then Carba NP (77.8% and 82.9%), and last MHT (70.4% and 71.4%).

We found that the chromogenic media was a reliable method for detection of carbapenemase and this agrees with Vrioni *et al.*'s [29] study, which reported that ChromID CARBA was found to be an easily performed and very accurate method for CPE detection in rectal swabs, and agrees with Papadimitriou-Olivgeris *et al.* and Day *et al.* [30,31] studies, which approved that ChromID® CARBA SMART agar is a reliable and accurate method for detection of carbapenemase. The major drawbacks we met at usage of chromogenic media were the short half-life of the media and its high cost, which may be the cause of limitation of the usage of the chromogenic media as a routine method for screening of resistance.

The MHT, which is recommended by CLSI 2014 for carbapenemase confirmation, is cheap and, in principle, simple to perform. Pasteran *et al.*, Willems *et al.*, and Seah *et al.* [32–34] reported that, however, MHT displays significant investigator dependence, it cannot distinguish among the different carbapenemase classes, and it reportedly has low specificity because of AmpC beta-lactamase overproduction and decreased permeability. El-Ghazzawy *et al.* [35] reported high sensitivity of MHT (94.6%), but Doyle *et al.* [36] reported that MHT sensitivity was only 12%.

As regards using Rapidec Carba NP test and MHT for detection of carbapenemase, we found that Rapidec Carba NP test was better than MHT as it detects a higher percentage of carbapenemase and was time-saving, this agrees with Lifshitz *et al.*'s [37] study, which reported that the Rapidec Carba NP was easily performed and accurate and had a faster turnaround time than MHT.

In the present study by using E-test as a gold standard, we found that the combined-disk test is a reliable test for detection of MBL as the sensitivity of the test was 76.5% and specificity was 80% in Pseudomonas isolates, whereas in Acinetobacter isolates, the sensitivity of the test was 87.5% and specificity was 36.4%.

Chu *et al.* [38] stated that the findings of the combined-disk test may be deceptive, because EDTA may have its own bactericidal effect, resulting in increased zones of inhibition, not linked with actual MBL generation. On the contrary, spurious negative findings described by Picão *et al.* [39] may originate from EDTA-caused carbapenem hydrolysis or inactivation. Bartolini *et al.* [40] reported that the presence of MBL in isolates containing more than one carbapenemase gene could not be identified in phenotypical experiments, which might justify false-positive and -negative findings of all phenotypic testing observed in this investigation.

As regards the distribution of different beta-lactamases among Gram-negative bacilli, the rate of ESBL-producing Pseudomonas isolates in the present study was 63% and 19% in Acinetobacter. The percentage among Pseudomonas isolates was close to El-Shouny *et al.* [41], who stated that ESBL-producing isolates represented 56% of the total. The rate in Acinetobacter was lower than that reported in Yong *et al.* [42], who report the presence of 54.6% of isolates as ESBL producers.

In the current study, the rates of MBL-producing Pseudomonas and Acinetobacter isolates were 29.6% and 57.1%, respectively. This result agreed with Kuper *et al.* [43], who stated that although the clinical prevalence of these enzymes is low, they have been reported in Enterobacter and Pseudomonas species, whereas Moosavian *et al.* [44] reported that 53% of Acinetobacter strains were carbapenemase producers.

This discrepancy in the beta-lactamase rate can be ascribed to various antibiotic policies that can help in the selection and/or the stringent

execution of infection-control measures of some antibiotic-resistant organisms rather than others. The limitation of this study was the small sample size, and that PCR could not be used as the gold standard for some tests due to its unavailability and its high cost.

Future studies may be carried out in larger sample size with application of genotypic methods that considered the gold-standard test to increase the sensitivity of the tests. Implementation of proper antibiotic policies, effective stewardship programs, and strict implementation of infection-control measures to decrease antibiotic resistance.

Conclusion

The ESBL and MBL showed the highest percentage among Pseudomonas isolates, whereas among Acinetobacter isolates, the MBL showed the highest percentage. The phenotypic confirmatory tests showed high sensitivity and specificity and proved to be reliable approaches for identification of the beta-lactamase resistance.

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Author contributions:

Hanan Hareth Abdellatif and Asmaa Omar Ahmed designed the study, supervised the implementation, and drafted the paper. Asmaa Omar Ahmed and Ahmed Elsayed M. Abdallah recruited patients and collected the data. Hanan Hareth Abdellatif and Asmaa Omar Ahmed supervised data analysis and interpretation of the results. All authors contributed to writing and editing the final paper.

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Conflicts of interest

The authors have no conflict of interests. Each author listed in the paper had seen and approved the submission of this version of the paper and takes full responsibility for it.

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