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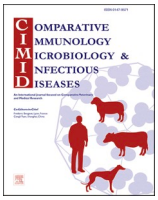

Phenotypic and genotypic characterization of carbapenem encoding genes among carbapenem-resistant Gram-negative bacteria isolated from North Casablanca, Morocco

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ABSTRACT

Carbapenem resistance genes in Gram-negative bacteria (CR-GNB) are a major cause of critical infections and are considered an urgent public health concern. The present study aimed to describe the prevalence of CR-GNB and the dissemination of extended-spectrum beta-lactamase (ESBL) and carbapenemase genes in clinical isolates from Casablanca, Morocco. Firstly, the strains were collected and identified using phenotypic and biochemical methods, then the antibiotic susceptibility was evaluated by the disc diffusion assay to screen isolates resistant to carbapenems. Secondly, three traditional methods, the carbapenem inactivation method, the modified Hodge, and the in-house carba-NP, were performed to predict the carbapenemase production by the included strains. Finally, conventional PCR was utilized to validate and detect the carbapenemase- and ESBL-related genes. Concerning the results, out of the identified 122 strains, 48 were CR isolates, including 30 *Klebsiella pneumoniae*, 13 *Escherichia coli*, and 5 *Pseudomonas aeruginosa*. Furthermore, these strains presented a high level of resistance. Moreover, the prediction of carbapenemase production by the phenotypic methods showed variable results. Also, the PCR analysis revealed a high occurrence of β-lactamase (ESBL and carbapenemase) genes in the included clinical strains, and most strains harbored multiple resistance genes. Our findings suggest that the three existing methods have some limitations, and a validation study is still necessary for the carbapenemase diagnostics.

1. Introduction

Carbapenems, which belong to the β-lactam antibiotics, have an extended broad-spectrum and play an important role in treating infections due to multidrug-resistant Gram-negative bacteria. Additionally, they are often reserved for use as the last line of defense due to their safety profiles. To establish their effect, carbapenems target penicillin-binding proteins (PBPs) to disrupt cell wall biosynthesis, induce autolysis, and bacterial death [1]. The prevalence of carbapenem-resistant Gram-negative bacteria (CR-GNB) represents a worldwide problem. Within that group, carbapenem-resistant *Pseudomonas aeruginosa* (*P. aeruginosa*) and *Enterobacteriaceae* significantly contribute to nosocomial infections [2]. This spread constitutes an urgent threat to health caused by the limited treatment choices, high morbidity and mortality cases, high hospitalization expenses, and elevated challenges in the prevention and control worldwide of nosocomial infections [2]. Furthermore, these organisms are among the most concerning antibiotic-resistant pathogens. They are responsible for a wide range of serious infections including urinary tract infections, meningitis, bloodstream infections, intra-abdominal infections, respiratory tract infections, malignant external otitis, and wound infections [3]. Accordingly, various Moroccan studies reported the terrible situation of carbapenemase-producing bacterial organisms [4]. Between 2010 and 2012, the results of 82 uropathogenic *Klebsiella pneumoniae* (*K. pneumoniae*) isolates collected from laboratories distributed over six Moroccan cities demonstrated a worrisome rate of isolates that were

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carbapenemase producers [5].

The growing CR-GNB can be attributed to the presence of mobile component genes, "plasmid-mediated", encoding for enzymes called "carbapenemases". These enzymes hydrolyze carbapenem antibiotics. The most common gene types are *bla*$_{NDM}$, *bla*$_{VIM}$, *bla*$_{KPC}$, *bla*$_{OXA-48}$, and *bla*$_{IMP}$. Furthermore, these genes confer co-resistance to other antimicrobial classes such as aminoglycosides, sulfonamides, and quinolones, resulting in a multidrug resistance profile [6]. Other resistance mechanisms are implicated, such as PBP mutations, upregulated efflux, and the alteration of outer membrane proteins leading to loss or reduced expression of porins [1]. Accurate investigation of carbapenem resistance is crucial for directing the line of treatment and establishing reliable infection control [3]. Numerous analysis strategies for the sensitive and rapid detection of the carbapenemase production have been developed [3]. These methods contribute to protecting public health, promoting rational antibiotic use, and providing crucial information for the rapid implementation of epidemic-control interventions [7]. The need for laboratories to identify and detect *in vitro* carbapenemase production has led to the development of several methods. Early phenotype-based techniques, such as the Hodge Test, are not extremely specific and sensitive [8]. The Carbapenem Inactivation Method (CIM) is another technique that allows the prediction of enzyme type production. On the other hand, colorimetric tests rely on visual detection to identify positivity for hydrolysis [8]. Molecular detection is expensive and requires substantial expertise, but it contributes to more effective use of antibacterials and better initial management of patients [9].

In light of this need, the present study described the carbapenem susceptibility among Gram-negative bacterial isolates in Casablanca, Morocco. Therefore, we conducted a comparative evaluation on three phenotypic and biochemical methods for the prediction of carbapenemase-producing Gram-negative bacteria: the Hodge test, the modified Carbapenem Inactivation Method (mCIM), and the Carba NP test. Furthermore, the determination of extended-spectrum beta-lactamase (ESBL) and carbapenemase genes was assessed by PCR. To the best of our knowledge, no data have been published up to now in Morocco regarding the use of these three methods: MHT, CIM, and Carba-NP for detecting carbapenemase activity.

2. Materials and methods

2.1. Isolation and identification of carbapenem Gram-negative bacteria

2.1.1. Sample collection, isolation, and identification of bacterial strains

A total of 122 Gram-negative bacterial isolates were collected from various private laboratories in Casablanca between 12th May 2022 and 1st February 2023. The isolates were obtained from cultures of different human clinical specimens, including urine, blood, sputum, and other body fluids. All the clinical samples were subjected to isolation and identification of the strains *Escherichia coli* (*E. coli*), *K. pneumoniae,* and *P. aeruginosa* using standard manual conventional methods and bacteriological investigation, including phenotypic and biochemical assays.

Samples were inoculated on blood, MacConkey, and Cled agar. After an overnight incubation at 37°C, microscopy of Gram-stained preparations was conducted from growth plates, and biochemical test analysis using the Vitek-2 automated system with the AST-GN card (bioMerieux, USA) according to the manufacturer's instructions was used to complete the diagnosis. Bacteria were stored in Luria-Bertani with 50 % glycerol at 20 °C until use. Glycerol stocks were streaked on nutritive agar to obtain fresh cultures.

2.1.2. Screening of carbapenem-resistant isolates: the antibiotic susceptibility profile

Following the identification of the GNB, the isolates were exposed to antimicrobial susceptibility testing by the Kirby-Bauer disk diffusion method. A broth culture of the isolate with turbidity equivalent to the MacFarland standard (0.5) was cultured over the Muller Hinton agar (MHA) plate and left to dry for 15 min. Then, the antibiotic disks were implemented and incubated overnight at 37 °C. The following antibiotics were investigated in this study: ampicillin (10 μg), piperacillin/tazobactam (100 μg/10 μg), cephalothin (30 μg), cefoxitin (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ertapenem (10 μg), imipenem (10 μg), amikacin (30 μg), gentamicin (10 μg), tobramycin (10 μg), nalidixic acid (30 μg), ciprofloxacin (5 μg), ofloxacin (5 μg), nitrofurantoin (300 μg), trimethoprim-sulfamethoxazole (1.25 μg/23.75 μg). The plates were incubated at 37 °C for 24 h, and the diameters of the zones of complete inhibition were measured to the nearest millimeter to determine resistance and sensitivity states. The interpretation followed the European Committee on Antimicrobial Susceptibility Testing guidelines to measure and interpret zone diameter [10]. Strains that demonstrated resistance to at minimum one of the carbapenems were recorded as CR-GNB, while strains that were not susceptible to at least one antibiotic in three or more classes were considered MDR [11,12].

2.1.3. Molecular Identification of carbapenem-resistant bacteria by 16S rDNA sequencing

All isolates were cultured in Luria-Bertani media and incubated for 24 h at 37 °C, and the overnight bacterial cultures were then used for DNA extraction. The extraction of bacterial DNA was performed using the Maxwell® RSC Cell DNA Kit provided by Promega (Ref AS1370, Madison, WI, USA). Briefly, bacterial cells were first lysed, then the lysate was loaded onto prefilled cartridges of the Maxwell® RSC instrument, which performs automated DNA purification through magnetic particle-based separation. The eluted DNA was collected in nuclease-free elution buffer and stored at − 20 °C until further use.

The Polymerase Chain Reaction (PCR) was conducted using the extracted DNA. This PCR process followed the methodology previously described by Sakoui et al. [13]. The PCR mixture, with a total volume of 20 μL, consisted of approximately 100 ng of genomic DNA, 10 mmol/L of deoxynucleotide triphosphates (dNTPs), 5X PCR buffer, 25 mmol/L $MgCl_2$, 10 mmol/L of each primer, and 5 U of Taq polymerase (Promega). The amplification was carried out in a DLAB TC1000-G thermocycler. The PCR thermal profile was set as follows: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation at 95 °C for 40 s, annealing at 55 °C for 40 s, and extension at 72 °C for 1 min; this was concluded with a final extension at 72 °C for 5 min and then held at 4 °C. Quantity and purity of DNA were measured by using nanodrop TM 1000 spectrophotometer. 260/280 nm and 260/230 nm wavelengths were used to measure genomic DNA's purity. The PCR products were purified using the ExoSAP-IT Purification Kit (GE Healthcare, UK), and sequencing was performed using the BigDye Terminator Kit version 1.0 (Applied Biosystems, UK). The chromatograms were read and corrected using the FinchTV software (1.4). The 16S rRNA gene was amplified from each isolate, and the amplicons were sent to CNRST for sequencing. Sequence homologies were determined using nucleotide blast at www.ncbi.nlm.nih.gov/BLAST/and, and isolates were identified at the species level.

2.2. Characterization and identification of carbapenem-resistant genes

2.2.1. Phenotypic identification of carbapenemase-producing isolates: the Hodge-modified test

Modified Hodge test (MHT), a phenotypic test used to identify carbapenemase production among GNB [14]. This test was conducted in the same manner as previously described by Dwomoh [15]. A 0.5 McFarland suspension of the overnight culture of *E. coli* ATCC 25922 (used as both an indicator strain and quality control) was prepared and subsequently diluted 1:10 in sterile saline. The resulting suspension was then uniformly inoculated as a lawn on Mueller–Hinton agar (MHA) plates. A 10 μg meropenem, ertapenem, or imipenem susceptibility disk was positioned in the center of the test plate. The test organisms were streaked straight from the edge of the central disk to the edge of the plate using sterile swabs, and thereafter the plate was incubated for 16–24 h at

37 °C in ambient air. MHT positive test (the production of carbapenemases) was indicated by the appearance of cloverleaf-like indentation and enhanced growth of indicator *E. coli* strains. MHT negative test showed no growth of the *E. coli* ATCC 25922 along the test organism growth streak within the diffusion zone [16].

2.2.2. Biochemical identification of carbapenemase-producing isolates: the modified carbapenem inactivation method

The modified carbapenem inactivation method was performed as reported by CLSI recommendations [17]. For this purpose, 1 μL loopful of bacteria from fresh culture and suspended in a tube containing 1 mL tryptic soy broth, then the bacterial suspension was mixed for 10–15 s. Subsequently, the 10 μg meropenem, ertapenem, or imipenem susceptibility disk was carefully immersed in the inoculum, and disks without bacteria were used for control purposes. Then, incubated at 35°C ± 2 for four hours. After the incubation step, the disk was detached and placed on an MHA plate inoculated with the quality control strain *E. coli* ATCC 25922 with turbidity equivalent to 0.5 McFarland, which was then incubated in an upturned position at 35 °C ± 2 for 18–24 h. The diameters of the inhibition zones around each disk were measured in millimeters.

According to Pierce VM et al. (2017) criteria, strains with no inhibition zone or an inhibition zone diameter of 6–10 mm were considered carbapenemase positive. A zone diameter of 11–19 mm was interpreted as an intermediate result. In contrast, disks incubated in suspensions that did not contain carbapenemases yielded a clear inhibition zone diameter of ≥ 20 mm and were interpreted as negative (not producing carbapenemases). A narrow ring of growth bordering the disk, representing the carryover of the test bacteria from the medium, was neglected [18].

2.2.3. Biochemical identification of carbapenemase-producing isolates: in-house Carba NP test

The iCarba NP test was carried out according to the method of Nordmann et al. (2012), with some slight modifications. Two solutions were made: solution A (control solution) containing 6 mg of imipenem-cilastatin + 0.1 mmol/L $ZnSO_4$ + 0.5 % phenol red solution at pH 7.8, and solution B (test solution) containing 0.1 mmol/L $ZnSO_4$ + 0.5 % phenol red solution at pH 7.8. Then, 100 μL of solution A and 100 μL of solution B were added in the first and adjacent columns, respectively. Two calibrated loops (10 μL) of the tested isolates from the Mueller-Hinton agar (MHA) plate were put in a 96-well U-bottom microtiter plate in two adjacent wells as duplicates. The plate was incubated at 37°C with shaking at 150 rpm for 2 h. *K. pneumoniae* ATCC1383 was used as a negative control, respectively. The change of color from red to orange or red to yellow of the test solution was recorded as a positive test result, whereas red-orange was considered indeterminate. No color change was considered negative. However, the color change in solution A was considered an invalid result.

2.2.4. Molecular identification of ESBL- and carbapenemase-encoding genes

The carbapenem-resistant isolates were screened for the presence of genes encoding carbapenemases and ESBLs at the Department of Veterinary Medicine and Animal Production, University of Naples "Federico II". The presence of ESBLs bla_{TEM}, bla_{PER}, $bla_{CTX\text{-}M}$, bla_{SHV} genes, and metallo-β-lactamase (carbapenemase) bla_{IMP}, $bla_{OXA\text{-}48}$, bla_{NDM}, bla_{VIM}, bla_{GES} genes was studied using the conventional PCR as previously described.

DNA extraction and PCR DNA preparation were performed by suspending a colony of each bacterial strain in 100 μL of distilled water, boiling at 98°C for 10 min. Then, 1 μL of DNA was amplified in a reaction mixture containing 12.5 μL of 2X Hot Start PCR Master Mix (biotechrabbit GmbH, Berlin, Germany), 1 μL of each specific primer, and 10.5 μL of distilled water. The amplification reactions were prepared in a total volume of 25 μL per tube. Sequences of the primers synthetized by Eurofins Genomics GmbH (Ebersberg, Germany), melting temperatures, and the expected product sizes [19]. are presented in Table 1.

Amplicon products were visualized after running for 90 min at 100 V on a 1.5 % agarose gel containing GreenSafe DNA gel stain (Canvax Reagents SL, 47151 Valladolid, Spain) (Fig. 1). A 100 bp DNA ladder (biotechrabbit GmbH, 12489 Berlin, Germany) was utilized as a size marker.

2.2.5. Statistical analysis

The specificity, sensitivity, negative predictive value (NPV), positive predictive value (PPV), and 95 % confidence intervals (CI) for each of these measures using VassarStats (http://vassarstats.net/prop1.html). This analysis was performed to determine the performance of the phenotypic methods in the detection of carbapenemase using PCR results as the gold standard method. Prism 8.1 (GraphPad, San Diego, CA, USA) and Excel (Microsoft, Redmond, WA, USA) were used for statistical analyses.

3. Results

3.1. Phenotypic, biochemical, and molecular identification

In the present study, one hundred twenty-two (122) clinical isolates were identified by using phenotypic and biochemical methods, the VITEK automated system (bioMerieux, USA), and molecular identification was done by 16S rRNA gene sequencing. All strains' sequencing data were visualized using FinchTV software and subsequently analyzed for identification with the Genius Prime software.

The most predominant isolated pathogens were 45.9 % (n = 56) *K. pneumoniae,* 35.2 % *E. coli* (n = 43), and 18.8 % *P. aeruginosa* (n = 23). Considering the gender distribution of the participants from whom the strains were considered, 56.8 % were males and 43.2 % were females. The included participants were between five months and 92 years old. The overall mean age was 41.4 ± 24.5 for females, and that for males was 49.1 ± 27 years. Most of the isolates were from patients above the age of 60 years (52 %), followed by the age range of 41–60 (27.4 %), and the range of 21–40 (10.9 %) with the least number of specimens collected from those within the age range of 0–20 years (9.5 %). The majority of the bacteria were isolated from the urine specimens (88.8 %), followed by pus (4.09 %), sputum (2.45 %), and blood (1.64 %).

3.2. Antimicrobial resistance profiles of the retrieved strains

Antimicrobial susceptibility testing was performed to screen carbapenem-resistant isolates. The results were analyzed according to the most recent CLSI guidelines [17] for antimicrobial susceptibility interpretation. Susceptibility testing revealed a high profile of resistance to all antibiotics tested, demonstrating that out of 122 strains screened for carbapenem resistance, 62 strains were identified as carbapenem resistant, reporting an occurrence of 50 %. Also, out of the 56 *K. pneumoniae* screened, 37 were CR. A total of 7 *P. aeruginosa* of the 23 strains were CR, and out of 43 *E. coli* strains, 18 were CR-*E. coli* (Table 2). The highest antibiotic resistance levels among the 122 isolates were recorded for ampicillin (84.42 %) and ceftazidime (65.57 %). However, 63.93 % of strains were resistant to cefoxitin and cefotaxime, while 63.11 % to cephalothin. Amikacin was the most susceptible antibiotic (40.98 %) in the present study (Fig. 1 and Table 2). The majority of resistant carbapenem strains presented a high level of resistance towards the tested antibiotics, with more than 3 antibiotics from three different classes indicating the multidrug-resistant (MDR) phenotype (Fig. 1). Results are summarized in Table 2.

3.3. Phenotypic and biochemical identification of carbapenemase-producing isolates by the three tests

All 48 isolates characterized as carbapenem-resistant were subjected

Table 1
Primer sequences, amplicon sizes, and amplification programs of carbapenemases and ESBL genes.

Gene	Primer Sequence (5′-3′)	Size of Product (bp)	Melting temperature (°C)
bla_{SHV}	F:ATGCGTTATATTCGCCTGTG R:TGCTTTGTTATTCGGGCCAA	747	52 °C
bla_{IMP}	F:ACAYGGYTTRGTDGTKCTTG R:GGTTTAAYAAARCAACCACC	387	58 °C
bla_{NDM}	F:ACTTGGCCTTGCTGTCCTT R:CATTAGCCGCTGCATTGAT	603	52 °C
bla_{OXA-48}	F:ATGCGTGTATTAGCCTTATCG R:CATCCTTAACCACGCCCAAATC	265	58 °C
bla_{GES}	F:CTGGCAGGGATCGCTCACTC R:TTCCGATCAGCCACCTCTCA	600	55 °C
bla_{VIM}	F:TGTCCGTGATGGTGATGAGT R:ATTCAGCCAGATCGGCATC	437	60 °C
bla_{CTX-M}	F:ATGTGCAGYACCAGTAARGTKATGGC R:TGGGTRAARTARGTSACCAGAAYCAGCGG	593	58 °C
bla_{TEM}	F:TCGCCGCATACACTATTCTCAGAATGA R:ACGCTCACCGGCTCCAGATTTAT	445	60 °C
bla_{PER}	F:AGTGTGGGGGCCTGACGAT R:GCAACCTGCGCAATRATAGCTT	725	56 °C

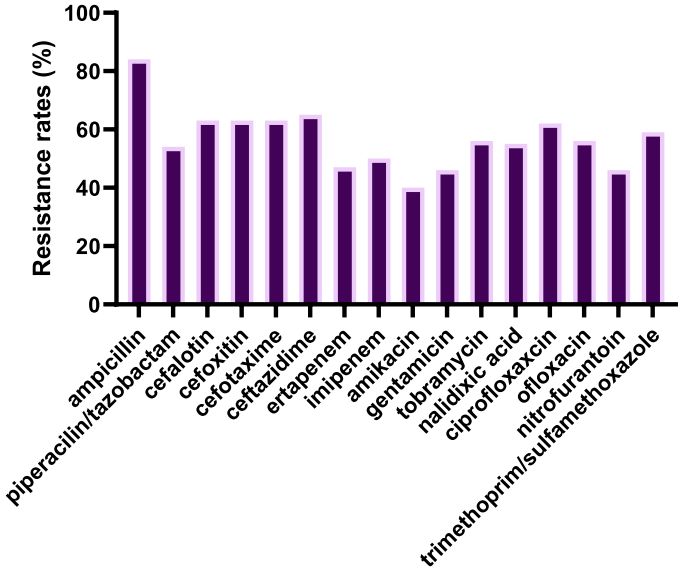

Fig. 1. Antimicrobial resistance profiles of all the study isolates.

to the three phenotypic assays: the Modified Hodge Test (MHT), the Carbapenem Inactivation Method (CIM), and the Carba NP test. Across the different carbapenems tested (imipenem, ertapenem, and meropenem). For Imipenem, using the MHT, 13 of 48 isolates were positive, 17 were negative, and 18 were indeterminate. However, the ICM showed that among the 48 strains, 31 were positive, 9 were negative, and 8 were indeterminate. Using ERT, the MHT demonstrated that 7 strains were positive, 33 were negative, and 8 were indeterminate. Furthermore, the CIM revealed that 22 were positive, 15 were negative, and 11 were indeterminate. Employing MEM, the MHT illustrated that 6 isolates were positive, 29 were negative, and 13 were indeterminate. The CIM indicated that 26 strains were positive, 12 were negative, and 10 were indeterminate. Concerning the In Carba-Np test, twenty-seven isolates were screened for carbapenemase production, and 21 were negative among the 48 tested isolates. These results show that CIM consistently detected a higher number of carbapenemase-producing strains compared to both the MHT and the Carba NP test, suggesting a higher sensitivity across all carbapenems tested. The results are presented in Fig. 2 below.

Tables (Supplementary material) show the specificity, sensitivity, PPV, NPV, and the 95 % confidence intervals (CI) calculated for each phenotypic method for predicting the presence of carbapenemase-associated genes in the included strains. The different methods demonstrated that the best sensitivity was obtained in detecting OXA-48 and NDM, 83 %, 66 %, and 64 %, with ICM. MHT and Carba-NP, respectively. For the prediction of GES, we noted that MHT using ERT obtained the greatest specificity (70 %) and the best sensitivity (66 %) with the ICM, while the Carba-NP test presented 33 % specificity and sensitivity. Furthermore, to assess the reliability and accuracy of these methods, 95 % confidence intervals (CI95 %) were calculated, and Tables (Supplementary material) summarize the results obtained.

3.4. Genotypic characterization of ESBL- and carbapenemase-producing isolates by PCR

Molecular analysis revealed that all 48 carbapenem-resistant strains (100 %) were ESBL producers, primarily of class A Serine β-lactamase, including bla_{SHV}, bla_{CTX-M}, and bla_{TEM}, except for bla_{PER}, with a detection rate of 14 % ($n = 7$). Meanwhile, for carbapenemases, all strains were positive, harboring bla_{NDM}. However, only 16 % ($n = 8$) were bla_{VIM}-positive; none of the screened strains harbored the bla_{IMP} gene. In addition, all strains were bla_{OXA-48}-positive producers. And only 3 strains were producers of bla_{GES}. Several strains harbored multiple carbapenemase genes; for example, 45 strains carried two types among the four carbapenemase genes screened, while three strains contained 3 carbapenemase genes. Regarding ESBL genes, all the included strains were found to carry three different ESBL genes, 12 strains harbored four of them, and 4 isolates with five different ESBL genes. The distribution of ESBL and carbapenemase genes across the three species is presented in Tables 3 and 4.

4. Discussion

CR-GNB presents a critical global healthcare crisis in recent years. Furthermore, CR-GNB infections have become progressively complex in terms of treatment, given increased mortality risks, and the limited therapeutic choices [20]. In this light, carbapenems have earned clinical value as a last-resort treatment for critical Gram-negative bacterial infections. However, over the past few years, the emergence of CR has dramatically augmented [21]. Herein, we assessed the prevalence of CR-GNB from clinical strains collected from different clinical samples of patients in Casablanca. The results revealed that among the 122 strains, forty-eight were carbapenem resistant, which is higher than the rate signaled in Morocco by [22]. And those obtained in Marrakech [23].

CIM, TMH, and Carba-NP tests were conducted to predict the isolates' carbapenemase producers. Our findings revealed that ICM detected 83 % (n = 40), while the MHT detected 66 % (n = 32), and the Carba-NP test detected 62 % (n = 30) of the CR isolates. In light of

Table 2
Antibiotic resistance among all included Gram-negative bacterial isolates, with a detailed profile of the 48 carbapenem-resistant strains.

Antimicrobial Agents	Overall Resistance Profile (All Gram-negative Isolates, n = 122)			Resistance Profile of Carbapenem-resistant Isolates by Species (n = 48)			
	Resistant	Susceptible	Intermediate	(*n* = 48)	*K. pneumoniae* Total (*n* = 30)	*E. coli* Total (*n* = 13)	*P. aeruginosa* Total (*n* = 5)
Ampicillin	84.42 % (*n* = 103)	15.57 % (*n* = 19)	0 % (n = 0)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Piperacillin/tazobactam	54.91 % (*n* = 67)	22.95 % (*n* = 28)	22.13 % (*n* = 27)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Cephalothin	63.11 % (*n* = 77)	36.88 % (*n* = 45)	0 % (*n* = 0)	100 % (n = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Cefoxitin	63.93 % (*n* = 78)	36.06 % (*n* = 44)	0 % (*n* = 0)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Cefotaxime	63.93 % (*n* = 78)	36.06 % (*n* = 44)	0 % (*n* = 0)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Ceftazidime	65.57 % (*n* = 80)	34.42 % (*n* = 42)	0 % (*n* = 0)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Ertapenem	47.54 % (*n* = 58)	52.45 % (*n* = 64)	0 % (*n* = 0)	95.83 % (*n* = 46)	93.33 % (*n* = 28)	100 % (*n* = 13)	100 % (*n* = 5)
Imipenem	50.81 % (*n* = 62)	49.18 % (*n* = 60)	0 % (n = 0)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Amikacin	40.98 % (*n* = 50)	40.98 % (*n* = 50)	18.03 % (*n* = 22)	95.83 % (*n* = 46)	96.66 % (*n* = 29)	40 % (*n* = 12)	100 % (*n* = 5)
Gentamicin	46.72 % (*n* = 57)	50 % (n = 61)	3.27 % (*n* = 4)	89.58 % (*n* = 43)	83.33 % (*n* = 25)	100 % (*n* = 13)	100 % (n = 5)
Tobramycin	56.55 % (*n* = 69)	27.04 % (*n* = 33)	16.39 % (*n* = 20)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Nalidixic acid	55.73 % (*n* = 68)	20.49 % (*n* = 25)	23.77 % (*n* = 29)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (n = 13)	100 % (*n* = 5)
Ciprofloxacin	62.29 % (*n* = 76)	29.5 % (*n* = 36)	8.19 % (*n* = 10)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Ofloxacin	56.55 % (*n* = 69)	23.77 % (*n* = 29)	19.67 % (*n* = 24)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Nitrofurantoin	46.72 % (*n* = 67)	20.49 % (*n* = 25)	24.59 % (*n* = 30)	100 % (*n* = 48)	100 % (*n* = 30)	100 % (*n* = 13)	100 % (*n* = 5)
Trimethoprim/ Sulfamethoxazole	59.83 % (n = 73)	40.16 % (*n* = 49)	0 % (*n* = 0)	87.55 % (*n* = 42)	83.33 % (*n* = 25)	92.3 % (*n* = 12)	100 % (*n* = 5)

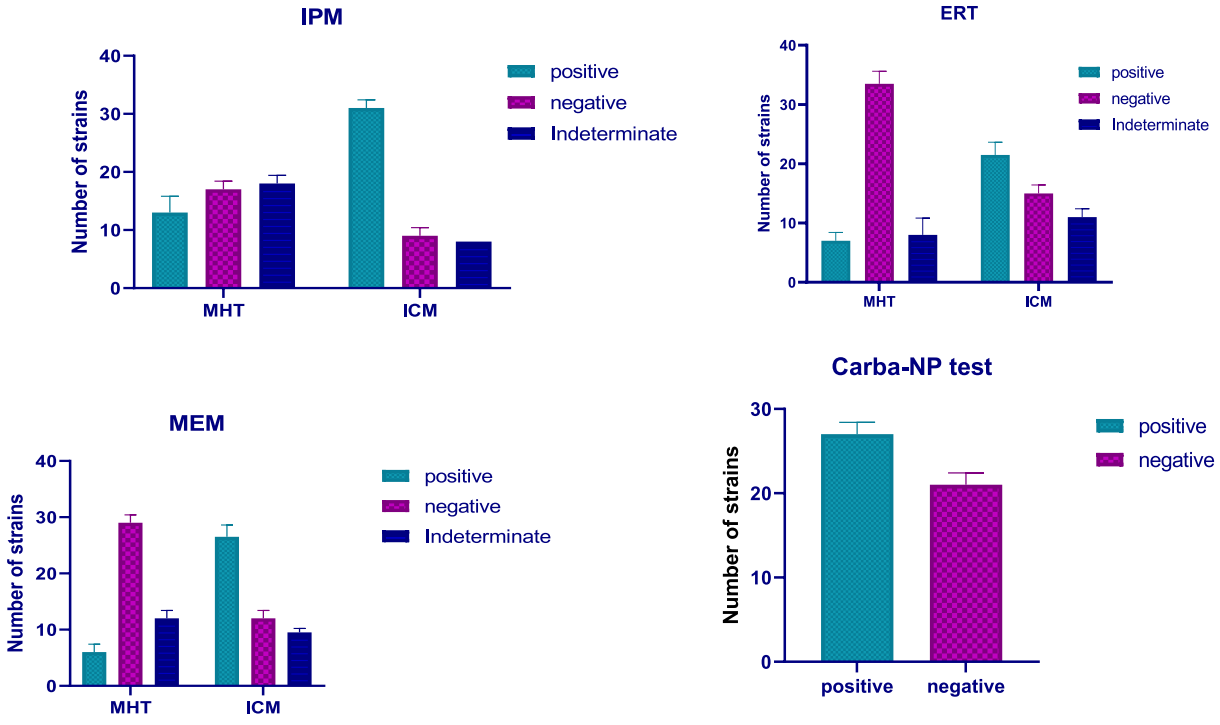

Fig. 2. Number of carbapenemase-producing strains predicted by MHT, CIM, and Carba-NP.

Table 3
Prevalence of ESBLs and carbapenemase enzymes among the carbapenem-resistant isolates.

Species	bla_{SHV}	bla_{CTX-M}	bla_{VIM}	bla_{TEM}	bla_{PER}	bla_{GES}	bla_{OXA-48}	bla_{NDM}	bla_{IMP}
***K. pneumoniae* Total (n = 30)**	30	30	1	30	3	3	30	30	0
***E. coli* Total (n = 13)**	13	13	3	13	0	0	13	13	0
***P. aeruginosa* Total (n = 5)**	5	5	4	5	4	0	5	5	0

Table 4
Distribution of the total number of Gram-negative isolates harboring ≥ 2 resistance genes across *K. pneumoniae*, *E. coli*, and *P. aeruginosa*.

Gene Class	Gene number	Total Strains	*K. pneumoniae* Total (n = 30)	*E. coli* Total (n = 13)	*P. aeruginosa* Total (n = 5)
Carbapenemases	**Strains with 2 genes**	45	27	13	5
	Strains with 3 genes	3	3	0	0
ESBLs	**Strains with 3 genes**	48	30	13	5
	Strains with 4 genes	12	3	4	5
	Strains with 5 genes	4	0	0	4

these results, we can note that the ICM showed a high level of sensitivity for predicting ESBLs and carbapenemase production and may be used as a favorable tool for rapid identification.

PCR was carried out to determine the prevalence of the ESBL- and carbapenemase genes among the clinical isolates. Among the seven genes screened, we found that bla_{NDM}, bla_{OXA-48}, bla_{SHV}, bla_{CTX-M}, and bla_{TEM} were co-harbored in all the isolates. Additionally, bla_{VIM} and bla_{PER} were found in 8 isolates (16.66 %), whereas bla_{GES} was present in 3 isolates (6.25 %). Our findings indicate a diverse distribution of carbapenemase and ESBL genes across the tested strains. This rate is significantly similar to the one signaled in Iran by Abbasi et al. (2023), which demonstrated that the most abundant genes were bla_{TEM} (100 %), bla_{CTX-M1} (98.7 %), bla_{SHV} (98.7 %), $bla_{CTX-M15}$ (94.8 %) for ESBLs; and bla_{OXA-48} (90.1 %) and bla_{NDM} (70.5 %) for carbapenemases [24]. On the other hand, Hamdy Mohammed et al. (2016) indicated that among bla_{VIM}, bla_{TEM}, bla_{SHV}, $bla_{CTX-M-1}$, and bla_{CTX-M}, the bla_{TEM} was the predominant gene (72.7 %), followed by bla_{VIM} , which had 66.7 %, and 15 % of strains showed bla_{SHV}, while 36 % harbored bla_{CTX-M} [25]. Notable carbapenemase genes found in pets include bla_{KPC}, bla_{NDM}, bla_{OXA-48}, and bla_{VIM} , which are the same as those implicated in human infections. Recently, it has been reported that bla_{PER}, bla_{SHV}, bla_{VIM}, and bla_{GES} were the most commonly detected genes in clinical *P. aeruginosa* strains isolated from dogs [26]. The detection of carbapenem-resistant bacteria in companion animals underscores the importance of the One Health approach, which recognizes the interconnectedness of human and animal health, particularly when addressing zoonotic bacteria. Herein, to ensure acquiescence with the latest available guidelines for carbapenemase production, the performance of each test was evaluated. Results highlighted that the performance of these methods showed variability according to the ESBLs and carbapenemases detected and the detection method applied. The highest reliability and accuracy among the tested methods were observed for detecting genes bla_{SHV}, bla_{CTX-M}, bla_{OXA-48}, and bla_{NDM-1} with IC95 % of 0.83, 0.66, and 0.64, respectively, using IMP in the methods ICM, TMH, and Carba-NP for predicting imipenem hydrolytic activity. Similar to our findings, another study by Lee et al. (2022) found that the MHT utilizing the imipenem disc had the best sensitivity (86 %), while that using the meropenem disc had a sensitivity of 64 % for estimating the presence of carbapenemase-related genes [27]. In contrast, it has been recorded that the disc diffusion method by ertapenem is a susceptible index of carbapenemase formation [15]. Despite this, careful consideration should still be given to choosing the ideal carbapenem antibiotic for carbapenemase prediction. Imipenem is usually recorded as a better choice for the because it has only 2 molecular peaks of interest; in contrast, meropenem has 4–6, and ertapenem has 8–10, and avoiding reading errors of mass spectra and fewer peaks makes the analysis of results easy. Additionally, imipenem has a superior hydrolysis rate and higher sensitivity for bla_{OXA-48}, which confers for a more accurate and rapid identification than ertapenem [28].

Our findings are consistent with previous reports, which revealed that the sensitivity of CIM to identify carbapenemase is higher and that CIM is more accurate compared to MHT [29]. Additionally, the sensitivity of CIM in the present study is also consistent with previous studies in which the CIM test has a significantly superior sensitivity than the Carba NP test and the one shown with MHT in detecting carbapenemase production [30]. Whereas another study by Rizvi et al. [31] revealed that the sensitivity of MHT was higher than that presented by CIM [31]. Compared to another study, in Ghana by Quansah et al., demonstrated that MHT had a less susceptible test for the identification of carbapenemase activity [32]. However, Wilson et al. [27] showed that the MHT is not as susceptible as other conventional and molecular techniques [27]. Meanwhile, comparing the results of the phenotypic tests to the results of the molecular detection of carbapenemases showed that the sensitivity of MHT, CIM, Carba NP was 78.4, 68.9, 56.8, and 70.3 %, respectively [33]. Moreover, the Carba NP test had a superior specificity (90 %), which is different from our results [34]. Our results are in agreement with previous published reports in which MHT has been noted to have less sensitivity (41–89 %) for the identification of MBLs [35]. Following CLSI recommendations, MHT is no longer used as a phenotypic test for carbapenemase detection, due to the lower specificity of the method to detect some ESBL production occurring with porin loss [36]. Previously published reports revealed that the defeat of CIM to identify carbapenemase production might be due to the brief incubation period of the carbapenem disc relative to other studies that recommended six hours of incubation, especially with weak-level carbapenemase effect [33]. Moreover, Carba NP methods exerted less sensitivity for carbapenemase producers. The limited sensitivity of Carba NP was often caused by the low carbapenemase effect of some strains [35]. The comparison of multiple traditional phenotypic methods highlights that phenotypic methods have lower specificity and sensitivity than genotypic characterization for detecting carbapenem-resistant strains, commonly leading to false positives or negatives. In contrast, molecular methods are still recommended because of their sensitivity and rapidity [37].

5. Conclusion

Our investigation provided high rates of carbapenem resistance with a high level of ESBL and carbapenemase genes spread in the carbapenem-resistant isolates, indicating an alarming dissemination of these resistant strains harboring this resistance mechanism. The current situation highlights the need for earlier diagnostic strategies. Moreover,

the findings of previous studies and our study revealed that there is no ˝perfect˝ predictive method and that the phenotypic tests for carbapenemase production detection, while useful, are insufficient for the reliable identification of strains that produce carbapenemases.

CRediT authorship contribution statement

Luisa De Martino: Writing – review & editing, Visualization, Validation, Methodology. **Abdelaziz Soukri:** Writing – review & editing. **Sinem Arslan:** Writing – review & editing. **Yasmina Mouzoun:** Writing – review & editing. **Yassine Zouheir:** Supervision. **Rossana Schena:** Writing – review & editing. **Bouchra El Khalfi:** Writing – review & editing, Visualization, Validation, Supervision, Methodology. **Fatima-Zahra Jouga:** Writing – review & editing. **Fatima Mourabiti:** Writing – review & editing, Writing – original draft, Software, Methodology, Investigation, Formal analysis, Data curation. **Francesca Paola Nocera:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis.

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Declaration of Competing Interest

The authors declare no conflict of interest.

Declaration of Competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.cimid.2025.102399.

Data Availability

The data represents the findings of this study and are available within the article/supplementary materials.

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