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Phenotypic Carbapenemase Production and *bla*$_{OXA}$ detecting by PCR in *Acinetobacter baumannii* isolates from a Hospital of Infectious Diseases from North-East Romania

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Abstract

***Introduction**: In the last 40 years, Acinetobacter baumannii has been among the bacteria known to acquire multiple mechanisms of antibiotic resistance and, as a result, it is now one of the pathogens involved in healthcare-associated infections with multidrug resistant strains. Our study aimed to assess the production of carbapenemases in carbapenem-resistant A. baumannii by means of phenotypic methods and polymerase chain reaction technique (PCR), as well as to appraise the performances of carbapenemase detection by phenotypic tests compared to the PCR approach. **Materials and Methods**: We used phenotypic methods (E-test MBL, CIM, MHT, Rosco® Kit/OXA/MBL, OXA-23 K-SeT® assay) to investigate the production of carbapenemases in 43 carbapenem-resistant A. baumannii isolates, and PCR to screen for the genes bla*$_{OXA-23}$*, bla*$_{OXA-24}$*, bla*$_{OXA-58}$*, bla*$_{OXA-51}$*, bla*$_{VIM}$*, bla*$_{IMP}$ *and bla*$_{NDM}$*. **Results**: The carbapenem inactivation method (CIM) at 2 hours, CIM at 4h, OXA-23 K-SeT® assay, Rosco® Kit/OXA, and modified Hodge test (MHT) identified 26%, 63%, 65%, 81%, and 42% carbapenemase-producing isolates, respectively. The phenotypic E-test MBL detected metallo-β-lactamase (MBL) production in 79% of strains. PCR revealed bla*$_{OXA-51}$ *in all the isolates, bla*$_{OXA-23}$ *in 35/43 (81%), bla*$_{OXA-24}$ *in 28/43 (65%), bla*$_{VIM}$ *in 7/43 (3%) and bla*$_{OXA-58}$*, bla*$_{IMP}$*, bla*$_{NDM}$ *were not detected. **Conclusion**: Because phenotypic tests do not highlight all the carbapenemase-producing strains, their results must be interpreted with caution relative to their level of performance, and negative results should be confirmed by means of PCR.*

***Keywords**: Acinetobacter baumannii, genotyping, carbapenemase production, OXA-23, OXA-24*

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Introduction

The rising incidence of antibiotic resistance is currently considered a major public health challenge worldwide (1). Infections caused by multidrug-resistant bacteria are associated with increased morbidity, mortality, and costs of care, given the unavailability of new therapeutic alternatives (1,2).

The frequent use of β-lactam antibiotics has prompted *A. baumannii* strains to develop resistance fairly swiftly which, in turn, has led to the administration of carbapenems. The occurrence and spread of carbapenem-resistant strains have undermined the efficacy of these antibiotics in neutralizing β-lactamase-producing bacteria (3). These enzymes are called carbapenemases due to their ability to hydrolyze carbapenems, and this key mechanism is encoded by plasmids (4).

In 2017, the World Health Organization included carbapenemase-producing *A. baumannii* to the list of critical agents, thus flagging it as a priority for scientific research as well as for the development of new antimicrobial therapies (5).

The principal carbapenem-resistance mechanism at *A. baumannii* is the production of class D carbapenemases (6,7). Several oxacillinases (OXA) which hydrolyze carbapenems have been described for *A. baumannii*, the most commonly known being OXA-23, OXA-24/40, OXA-58, OXA-143, OXA-235, and OXA-51 (6-8). Of these, OXA-23 is the most widely occurring (6,7).

Generally, as their expression is often modest, OXA hydrolyze carbapenems only weakly and, as such, they produce low-level resistance. These enzymes can, however, cause an increased resistance when the insertion sequence IS*Aba1* is present upstream of the promoter region of bla_{OXA} genes, enhancing their expression (8-10). The insertion sequence IS*Aba1* has been found in *A. baumannii* strains upstream of the genes bla_{OXA-23}, bla_{OXA-51}, bla_{OXA-58}, and cephalosporinase bla_{ampC} (11).

The aim of this study has been to identify the antibiotic-resistant phenotypes, carbapenemase production and the presence of the bla_{OXA-23}, $bla_{OXA-24,}$ $bla_{OXA-58,}$ $bla_{VIM,}$ bla_{IMP} and bla_{NDM} genes of *A. baumannii* strains carbapenem-resistant isolated at the "Sf. Parascheva" Hospital of Infectious Diseases Iași, Romania, in order to update our understanding of the spread of resistant phenotypes in the North-Eastern region of the country, as well as to evaluate the performances of phenotypic tests compared to polymerase chain reaction technique (PCR).

Materials and Methods

Bacterial Isolates

The study investigated 43 non-duplicate carbapenem-resistant *A. baumannii* strains, which were isolated from various clinical specimens (3 from sputum, 6 from tracheobronchial aspirate, 6 from blood, 11 from urine, 13 from pus, 3 from cerebrospinal fluid, 1 from catheter tip) collected from patients admitted at the "Sf. Parascheva" Clinical Hospital of Infectious Diseases in Iași, Romania during May 2017 - April 2019. We selected and included in the study only the strains with resistance of imipenem, meropenem, doripenem (43/55). Strains isolated repeatedly from the same patient and those non-resistant to carbapenems were excluded. All the subjects who provided isolates for this research were kept anonymous.

The isolates were identified based on microscopic and biochemical characteristics, and it was subsequently confirmed by Real Time PCR (qPCR) testing with specific primers for bla_{OXA-51} gene, a distinctive feature present in all isolates belonging to the species *A. baumannii* (12).

Antimicrobial Susceptibility

Antibiotic susceptibility was determined by the disk diffusion method using the following antibiotics: ciprofloxacin, 5 μg (CIP), levofloxacin, 5 μg (LEV), gentamicin 10 μg (G), tobramycin,

10 µg (TOB), amikacin, 30 µg (AK), imipenem, 10 µg (IMI), meropenem, 10 µg (MEM), and trimethoprim-sulfamethoxazole, 1.25/23.75 µg (SXT). For colistin (CT), we employed the broth microdilution method using MICRONAUT MIC-Strip (MERLIN Diagnostika Gmbh, Bornheim, Germany). The CT concentrations on the strips ranged from 0.0625 µg/ml to 64 µg/ml. For quality control, we used *Pseudomonas aeruginosa* ATCC 27853 and *Escherichia coli* ATCC 25922 reference strains, and for the minimum inhibitory concentration (MIC) control of CT we used *E. coli* NCTC 13846. The data were interpreted in accordance with the *European Committee on Antimicrobial Susceptibility Testing* (EUCAST) guidelines, breakpoint tables in use at the time of testing (https://www.eucast.org/ast_of_bacteria/previous_versions_of_documents/).

Phenotypic Detection of Carbapenemase Production

E-test MBL (Epsilometer – test metallo-β-lactamase)

The test was performed as described by Khosravi et al. (13). We used Etest® MBL strips (bioMérieux Inc., Marcy L'Etoile, France); at one end, these contain IMI in concentrations decreasing from 256 to 4 µg/ml and, at the other end, they have a variable amount of IMI ranging from 1 to 64 µg/ml in combination with a fixed concentration of ethylenediamine-tetraacetic acid (EDTA). The results were interpreted according to the manufacturer's instructions: a ratio ≥ 8 between the MIC of IMI and that of MIC with EDTA was considered a positive result indicative of metallo-β-lactamase (MBL) production.

The modified Hodge test (MHT)

The MHT was performed as described by Amjad et al. (14). We used the reference strain of *E. coli* ATCC 29522 from which we prepared a 5 ml bacterial suspension in saline solution with a standard density of 0.5 McFarland. A 1:10 dilution of this suspension was inoculated uniformly onto Mueller-Hinton agar plate. A 10 µg MEM disk was placed in the centre of the plate and then each isolate was inoculated in a straight line from the centre towards the exterior of the plate, without touching the MEM disk. The plates were incubated for 18-24 hours at 37°C. After incubation, they were examined for any cloverleaf-like indentations in the MEM disk inhibition area at the intersection between the tested strain and the *E. coli* ATCC 29522 indicator strain; the presence of such indentations was interpreted as positive for carbapenemase production by the respective strain.

The carbapenem inactivation method (CIM)

The CIM test was performed as described by van der Zwaluw et al. (15). The strain to be tested, cultured on a Mueller-Hinton agar plate, was inoculated with a 10 µl loop in 400 µl of distilled water to obtain a bacterial suspension in which a 10 µl MEM disk was immersed and incubated at 35°C for 2 hours and 4 hours, respectively. The CIM test was performed in duplicate. After incubation, the MEM disk was placed on the surface of a Mueller-Hinton agar plate already inoculated with the *E. coli* ATCC 29522 indicator strain. The absence of a bacterial growth inhibition area for the indicator strain around the MEM disk after 18-24 hours of incubation at 37°C indicates that the test strain is producing carbapenemase.

The combined disk method (Rosco® Kit/OXA/MBL)

This method has been used to identify the production of MBL and OXA using the Confirm Kit 98025 (Versions 2) (ROSCO® Diagnostica, Taastrup, Denmark). This kit contains tablets of IMI (10µg) and IMI in association with various β-lactamase inhibitors. To determine MBL and OXA production for *A. baumannii* strains, we

used tablets of IMI with dipicolinic acid (DPA) and with EDTA, respectively. The surface of a Mueller-Hinton agar plate was inoculated with the bacterial suspension of the strain to be tested at a density of 0.5 McFarland, and then the tablets were added according to the instructions. After incubation, 18-20 hours at 37°C, the results were interpreted according to the instructions of the test manufacturer (https://www.rosco.dk/gfx/pdf/98025%20-%20Print%20Insert%202017.pdf - accessed in February 2019).

The immunochromatographic detection of OXA-23 production

For this test, we used the OXA-23 *K*-SeT® (Coris BioConcept, Gembloux, Belgium) (https://www.bioconnections.net/uploads/4/8/8/4/48842659/resist__oxa-23.pdf - accessed March 2019). The test uses monoclonal antibodies directed against an epitope of OXA-23 carbapenemase, adsorbed on a nitrocellulose membrane, and monoclonal antibodies marked with colloidal gold, directed against another epitope of OXA-23 carbapenemase. In principle, if a sample contains OXA-23 carbapenemase, its epitopes will interact with the anti-OXA-23 antibodies and a colored test line will appear.

PCR Detection of carbapenem resistance genes

The bla_{OXA} and MBL genes which encode carbapenem resistance were targeted.

We extracted bacterial DNA using the Promega kit from gram negative bacteria (Promega, Wizard® Genomic DNA Purification Kit) according to the manufacturer's instructions (https://www.promega.ro/resources/protocols/technical-manuals/0/wizard-genomic-dna-purification-kit-protocol/ - accessed June 2021).

The *A. baumannii* isolates were screened for the genes $bla_{OXA-23,}$ $bla_{OXA-24,}$ bla_{OXA-58} and bla_{OXA-51} by means of qPCR using specific probes and primers (see Table I) (12). The total volume per PCR reaction was 23µl: 10 µl of Master Mix (Promega), 0.6 µl per primer, 0.4 µl per probe, 8.4 µl of nuclease-free water and 3 µl of DNA matrix. Amplification was done using Mx3005P Stratagene and the following protocol: one cycle at 95°C for 10 min, followed by 40 cycles of 15

Table I. Primers and probes used to PCR for *Acinetobacter baumannii*

Primers/Probe	5'-3' sequence	Amplicon size	Ref.
OXA-23 probe	FAM-CCAGTCTATCAGGAACTTGCGCGA-BHQ_1	116 bp	(12)
OXA-23-FOR	GACACTAGGAGAAGCCATGAAG		
OXA-23-REV	CAGCATTACCGAAACCAATACG		
OXA-51 probe	HEX-ACTTGGGTACCGATATCTGCATTGCC-BHQ-2	112 bp	
OXA-51-FOR	TGTCTAAGGAAGTGAAGCGTG		
OXA-51-REV	AACTGTGCCTCTTGCTGAG		
OXA-24 probe	FAM-AGTAACACCCATTCCCCATCCACTTTT-IABkFQ	151bp	
OXA-24-FOR	GATGACCTTGCACATAACCG		
OXA-24-REV	CAGTCAACCAACCTACCTGTG		
OXA-58 probe	HEX-TGGACCAATACGACGTGCCAATTCT-IAbRQSp	141bp	
OXA-58-FOR	AAGATTTTACTTTGGGCGAAGC		
OXA-58- REV	CAACTTCCGTGCCTATTTGC		
IMP-FOR	TTGACACTCCATTTACTGCTA	172bp	(16)
IMP-REV	TCATTTGTTAATTCAGATGCATA		
VIM-FOR	GAGTTGCTTTTGATTGATACAG	247bp	
VIM-REV	TCGATGAGAGTCCTTCTAGA		
NDM-FOR	AACACAGCCTGACTTTCG	111bp	
NDM-REV	TGATATTGTCACTGGTGTGG		

sec at 95°C and 1 min at 60°C. The data were analysed with the ΔtCt method by the software of the qPCR machine.

For the bla_{VIM}, bla_{IMP} and bla_{NDM} genes, we performed the conventional PCR, using specific probes and primers (see Table I) (16). The total volume per PCR reaction was 20µl: 10 µl of Master Mix 2X (Promega), 0.6 µl per primer, 6.8 µl of nuclease-free water and 2 µl of DNA matrix. Amplification was done using GeneAmp PCR System 9700 and the following protocol: one cycle at 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C, 30 sec at 60°C, 30 sec at 72°C and one cycle 10 min at 72°C. The amplified DNA was analyzed using 2.5% agarose gel electrophoresis and we visualized the amplicon by UV transluminator.

For qPCR and conventional PCR the positive control was a fragment of synthetic DNA (g Blocks® Gene Fragments), while negative control was a *P. aeruginosa* ATCC 27853 strain. We used PCR water as no template control.

Research ethics

The study observes the international ethical principles and recommendations of the Helsinki Declaration by the World Health Organization with regard to medical research on human subjects. The study was approved by the Research Ethics Committee of "Grigore T. Popa" University of Medicine and Pharmacy Iași Romania (No. 12825) and by the Ethics Committee of "Sf. Parascheva" Hospital of Infectious Diseases Iași, Romania (No. 23). All the data were collected from the records of the Medical Laboratory of the hospital and patient information was anonymized.

Statistical analysis

The data were processed using version 18.0 of the software SPSS by SPSS Inc. 2009, Chicago. The ANOVA and t-Student tests with a 95% confidence interval were performed to compare sample means. The Kruskal Wallis test was used to compare the categories of variables from the same group. To compare two or more frequency distributions from the same population, we chose the test c^2. The asymmetry and Kurtosis tests ($-2 < p < 2$) helped to assess normal distribution of variables. The threshold for statistical significance was set at $p \leq 0.05$ (17).

Results

Characteristics of Isolates

The studied *A. baumannii* strains originated from a range of clinical specimens. Most of the isolates (33/43) came from non-sterile sites: 3 from sputum, 6 from tracheobronchial aspirate, 13 from pus from wounds, and 11 from urine. Another 10 strains were isolated from sterile sites: 6 from blood, 3 from cerebrospinal fluid, and 1 from a catheter tip culture. In the case of the 11 strains isolated from urine, 5 were collected from urethral probes.

The age of the patients who provided the studied strains varied widely from 33 to 88 years. The results of the Skewness and Kurtosis tests within the interval [-2; 2] and the groups mean (66.21 *vs* 63.91 years at p=0.434) were close to the median value (66-67 years), suggesting that the series of values regarding the patients' age were homogenous, so tests of significance for continuous variables could be used. With regard to the patients' gender, most were men (55.8%).

Antibiotic Resistance Profiles

The antibiotic susceptibility test revealed that 42 (98%) of the isolates were resistant to at least 1 antibiotic from each tested class and, as such, they were defined as *extensively drug resistant* (XDR); of these, 34 (81%) proved sensitive only to CT and 8 to CT and aminoglycosides. A single strain was *pandrug resistant* (PDR), presenting resistance to all antibiotics used in the test, including CT (18). All 43 strains were resistant to fluoroquinolones and to SXT (see Table II).

Phenotypic and PCR Detection of carbapenemase

The highest percentage of positivity was obtained for the Rosco® Kit/OXA/MBL that identified OXA production in 35 of the tested strains (81%). The same test showed that 8 isolates (19%) were MBL producing. Other tests: MHT, CIM test at 2h and CIM test at 4h identified carbapenemase-producing in 18 (42%), 11 (26%), and 27 (63%) strains, respectively. With the E-test MBL, we identified the MBL production in 34 isolates (79%) (see Table II).

We also obtained a high percentage of positive results to the OXA-23 immunochromatographic *K*-SeT® assay, which found OXA-23 carbapenemase in 28/43 isolates (65%).

Table II. The phenotypic and genotypic profile of *Acinetobacter baumannii* carbapenem-resistant strains

No. samples	Antibiotic resistance profile	Phenotypic tests						Genotypic tests		
		MBL E-test	CIM 4h	MHT	Rosco® test		OXA-23 *K*-SeT®	*bla*$_{OXA-23}$	*bla*$_{OXA-24}$	*bla*$_{VIM}$
					MBL	OXA				
2	CIP, LEV, G, TOB, AK, SXT	+	-	-	-	+	+	+	+	+
2	CIP, LEV, G, TOB, AK, SXT	+	-	-	-	+	+	+	+	-
3	CIP, LEV, G, TOB, AK, SXT	+	-	-	-	+	+	+	-	-
5	CIP, LEV, G, TOB, AK, SXT	+	+	+	-	+	-	+	+	-
1	CIP, LEV, G, TOB, AK, SXT	+	+	+	-	+	-	+	+	+
3	CIP, LEV, G, TOB, AK, SXT	+	+	-	-	+	+	+	-	-
2	CIP, LEV, G, TOB, AK, SXT	+	+	-	-	+	+	+	+	-
2	CIP, LEV, G, TOB, AK, SXT	+	+	+	-	+	+	+	-	-
1	CIP, LEV, G, TOB, AK, SXT	+	+	+	-	+	+	+	+	-
2	CIP, LEV, G, TOB, AK, SXT	-	-	-	-	+	+	+	+	-
1	CIP, LEV, G, TOB, AK, SXT	-	-	-	-	+	+	+	-	-
2	CIP, LEV, G, TOB, AK, SXT	+	+	+	-	+	-	-	+	-
1	CIP, LEV, G, TOB, AK, SXT	+	+	+	+	-	-	-	-	-
1	CIP, LEV, G, TOB, AK, SXT	-	+	-	-	+	+	+	-	-
1	CIP, LEV, G, TOB, AK, SXT	-	+	-	-	+	+	+	+	-
1	CIP, LEV, G, TOB, AK, SXT	+	+	-	+	-	+	+	+	-
1	CIP, LEV, G, TOB, AK, SXT	+	+	-	+	-	+	+	-	-
1	CIP, LEV, G, TOB, AK, SXT	+	-	+	-	+	-	-	+	+
1	CIP, LEV, G, TOB, AK, SXT	+	-	+	-	+	-	-	+	-
1	CIP, LEV, SXT	+	+	-	-	+	+	+	-	-
1	CIP, LEV, SXT	+	+	-	+	-	+	+	-	-
1	CIP, LEV, G, TOB, AK, SXT	-	-	-	+	-	-	+	+	-
1	CIP, LEV, G, TOB, AK, SXT, CT	-	-	-	-	+	+	+	+	-
1	CIP, LEV, G,TOB, SXT	-	-	-	-	+	+	+	+	-
1	CIP, LEV, G, SXT	+	-	-	+	-	+	+	-	-
1	CIP, LEV, G, AK, SXT	+	+	+	-	+	-	-	+	+
1	CIP, LEV, G, AK, SXT	+	+	+	+	-	-	-	+	+
1	CIP, LEV, G, TOB, AK, SXT	-	+	+	-	+	+	+	+	-
1	CIP, LEV, TOB, SXT	+	+	+	+	-	-	-	+	+

CIP: ciprofloxacin; LEV: levofloxacin; G: gentamicin; TOB: tobramycin; AK: amikacin; SXT: trimethoprim-sulfamethoxazole; CT: colistin; CIM: carbapenem inactivation method; MHT: modified Hodge test; OXA-23 *K*-SeT®: oxacillinase-23 immunochromatographic test; OXA: oxacilinase; MBL: metallo-β-lactamase

The detection of class D carbapenemases by qPCR revealed the presence of the bla_{OXA-51} gene in all isolated tested. Also, we detected the bla_{OXA-23} gene at 35/43 (81%) of strains and the bla_{OXA-24} gene at 28/43 strains (65%); 21/43 (49%) of the strains harbored both the bla_{OXA-23} and bla_{OXA-24} genes. The bla_{OXA-58} was not detected. The conventional PCR detected 7/43 (3%) *A. baumannii* strains bla_{VIM} positive, but bla_{IMP} and bla_{NDM} were not detected.

By comparing the results of phenotypic tests identifying carbapenemase production with those of PCR testing, we found that the Rosco® Kit/OXA test and the OXA-23 *K*-SeT® immunochromatographic assay had highest levels of sensitivity in the detection of OXA positive strains: 83% (35/42) and 80% (28/35), respectively. Also, both tests had the highest specificity (100%) (see Figure 1).

The lowest levels of sensitivity were registered for the MHT (40%; 17/42), and CIM at 2h tests (24%; 10/42). By extending the incubation time for the CIM test from 2 hours to 4 hours, we obtained an increase in sensitivity to 62% (26/42). The CIM and MHT test turned out to be non-specific (0%) (see Table II).

For E-test MBL the sensitivity and specificity in the detection of bla_{VIM} positive strains were 100% (7/7) and 25% (9/36), respectively.

According to the data, there were no statistically significant differences between the antibiotic resistance of strains with the gene bla_{OXA-51} and those carrying both bla_{OXA-51} and bla_{OXA-23} (see Table III).

Discussion

By means of qPCR we detected the gene bla_{OXA-51} in all 43 carbapenem-resistant *A. baumannii* strains featured in this study. Moreover, we noticed that the carbapenem resistance for the most of studied strains was mediated by the OXA-23 and OXA-24 carbapenemases, as the qPCR results revealed the presence of the genes bla_{OXA-23} and bla_{OXA-24} in 35 (81%) and 28 (65%) of the tested strains, respectively.

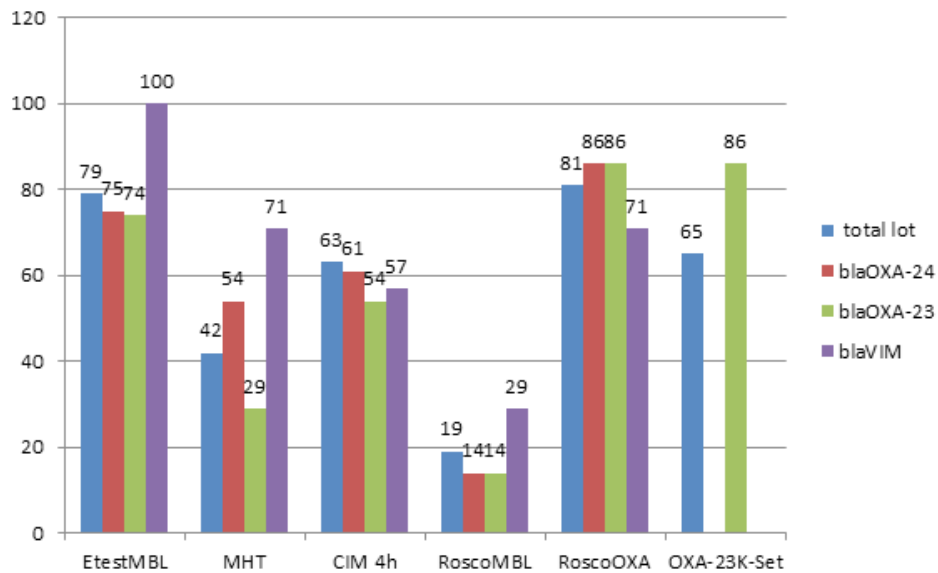

Fig. 1. The positivity rates of phenotypic tests for the detection of carbapenemase production by *Acinetobacter baumannii* carbapenem-resistant strains.

MHT: Modified Hodge test; CIM: Carbapenem Inactivation Method; OXA-23 K-SeT®: OXA-23 immunochromatographic test; OXA: oxacilinase; MBL: metallo-β-lactamase

Table III. A comparative analysis of the antibiotic resistance profile of *Acinetobacter baumannii* carbapenem-resistant strains

Antibiotic	Resistant strains			
	Total 43 (%)	bla_{OXA-51}&bla_{OXA-23} 35 (%)	bla_{OXA-51} 8 (%)	*p*
CIP	43 (100)	35 (100)	8 (100)	1.000
LEV	43 (100)	35 (100)	8 (100)	1.000
G	40 (93.02)	33 (94.29)	7 (87.5)	0.527
TOB	38 (88.37)	32 (91.43)	6 (75)	0.230
AK	38 (88.37)	31 (88.57)	7 (87.5)	0.933
SXT	43 (100)	35 (100)	8 (100)	1.000

CIP: ciprofloxacin; LEV: levofloxacin; G: gentamicin; TOB: tobramycin; AK: amikacin; SXT: trimethoprim-sulfamethoxazole

Similar results were obtained in studies from several other geographical areas. According to them, the gene bla_{OXA-23} was the first gene associated with carbapenem resistance and it has also been most frequently identified in *A. baumannii* strains (19-21).

A study performed in Northeast Romania by Timofte et al., during the years 2014-2015, reported the presence of OXA 23 and OXA 24 genes in *A. baumannii*, but the bla_{OXA-58}, bla_{VIM}, bla_{IMP}, bla_{NDM} genes were not identified (22). In the same geographical region, the study performed by Mereuta et al. showed the presence of the bla_{VIM-2} gene in 2/16 strains of *A. baumannii* carbapenem-resistant (23).

Bonnin et al. conducted a study that included 13 *A. baumannii* strains isolated in the Western part of our country, between 2009 and 2010. They identified the presence of bla_{OXA-23} and bla_{OXA-58} genes in 11 and 2 strains, respectively (24).

In the Southern Romania, Radu-Popescu et al. reported the presence of the bla_{IMP-1}, bla_{VIM-2}, bla_{OXA-24}, bla_{OXA-51} and bla_{OXA-58} genes, in *A. baumannii* strains, in a study conducted between 2001-2003 (25). Other studies for the same region were performed by Gheorghe et al., in 2017 and 2021. They identified bla_{OXA-23} gene in 50% and 33% of strains, respectively. The bla_{OXA-24} gene was detected in 27% and 67% of strains, respectively. Only in the 2017 study was the presence of bla_{VIM} (1%) and bla_{IMP} (27%) genes reported (26, 27).

The strains tested by us presented high levels of resistance to antibiotics - most were XDR, and one was PDR. For a substantial percentage of XDR strains (81%), the only remaining therapeutic option was CT, while aminoglycosides were a viable alternative to CT in only 19% of XDR isolates. The data were similar with the results from other studies (28).

The sensitivity of MHT in our study was 40%, inferior to that reported by Sun et al. in China (68.4%), or by Abouelfetouh et al. and El-Kazzaz et al. in Egypt (78.4% and 56.9%, respectively) (29-31). On the other hand, Bonnin et al. have reported poor sensitivity for MHT in detection of carbapenemase-producing strains (32).

At the same time, the CIM test was demonstrated to achieve high levels of sensitivity (100%, 68.9%, and 70%) by several teams of researchers (30,33). These are all superior to the level reached in our study (24% for CIM at 2 h, and 62% for CIM at 4 h).

The reduced capacity of phenotypic tests (CIM, MHT) to detect the production of carbapenemase could be due to the low level of activity of OXA-type carbapenemases (34). This may be supported by the fact that in the CIM test we did not obtain complete MEM inactivation, which facilitated the emergence of colonies in the growth inhibition area, suggesting that OXA

exert a limited influence on carbapenems. Our study showed that the incubation period of the MEM disk influenced the number of positive tests (11 strains at 2 hours and 27 strains at 4 hours of incubation, respectively). Our results are supported by the study conducted by Aktaş et al. (35) which found 4 and 7 positive strains after incubation 2 and 4 hours, respectively.

The test Rosco® Kit/OXA/MBL even if not reaching 100% sensitivity, it still found 30 of the 35 $bla_{\text{OXA-23}}$-positive strains and 24 of the 28 $bla_{\text{OXA-24}}$-positive strains to be producers of oxacillinase. However, our results were inferior to those obtained by Uddin et al. (36), who reported 100% sensitivity, but did not differentiate between the classes of carbapenemases present in their *A. baumannii* strains.

In our study, the OXA-23 K-SeT® immunochromatographic assay had a high level of sensitivity (80%), and only for 7/35 strains identified by qPCR positive for $bla_{\text{OXA-23,}}$ we acquired false negative results. These data are compatible with the outcomes of a study published recently by Riccobono et al. (37).

The E-test MBL identified MBL production in 34/43 strains (79%). We found that 33 of the 34 strains that tested positive for MBL showed bla_{OXA} genes, and 7/33 bla_{OXA} strains were bla_{VIM} positive also. Usually, a positive E-test MBL result is indicative of MBL presence, but in our study, only 7 strains (3%) were bla_{VIM} positive. The positive E-test MBL results for bla_{OXA} positive strains may be explained by the EDTA ability to facilitate the transformation of OXA in a less active monomeric configuration, the modification leading to the increase in diameter of the growth inhibition area around the disk of IMI and EDTA (38,39). This reasoning is supported by other studies reporting the finding of MBL-positive *A. baumannii* strains by means of the E-test MBL, but the genes encoding these enzymes were not identified when the PCR tests were performed (19,40).

The isolates with a similar antibiotic-resistance profile and the same positive phenotypic test results for the detection of carbapenemase production were grouped together, and we identified clusters of strains that displayed the same phenotypic characteristics. The existence of strains with similar phenotypic features may point to their belonging to the same bacterial clone. Confirming this hypothesis by genotypic analysis could lead to the implementation of measures preventing the proliferation of these microorganisms in hospital environments.

The limitations of our study were represented by the fact that the study was carried out in a single hospital and that we did not have the possibility to determine the clonality of the strains, this aspect remaining to be studied in future research.

Conclusions

The carbapenem-resistance of studied isolates was associated with the presence of the $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-24}}$ genes. The phenotypic tests performed to identify the carbapenemase-producing strains had variable performances. The E-test MBL is easy to carry out, but its results must be interpreted with caution in the case of *A. baumannii* strains. Rosco® Kit/OXA and the OXA-23 *K*-SeT® assay showed high sensitivity and specificity, which makes them useful phenotypic tests for the identification of OXA-producing *A. baumannii* strains.

Characterization of phenotypic profile of *A. baumannii* strains circulating in the North-East of Romania is useful for establishing the appropriate first choice antibiotic therapy. At the same time, knowing the genetic profile of these microorganisms can help limiting the spread of antibiotic-resistant strains. Also, the identification of phenotypic and genotypic characteristics of *A. baumannii* strains can provide valuable information to improve the management of infections caused by these strains.

Abbreviations

AK - amikacin
CIM - carbapenem inactivation method
CIP - ciprofloxacin
CT - colistin
DOR- doripenem
DPA - dipicolinic acid
EDTA - ethylenediamine-tetraacetic acid
EUCAST - European Committee on Antimicrobial Susceptibility Testing
G - gentamicin
IMI - imipenem
IS - insertion sequence
LEV - levofloxacin
MBL - metallo-β-lactamase
MEM – meropenem
MHT - modified Hodge test
MIC - minimum inhibitory concentration
OXA - oxacillinases
OXA-23 *K*-SeT® - OXA-23 immunochromatographic assay
PCR - polymerase chain reaction
PDR - pandrug-resistant
qPCR - Real Time PCR
SXT - trimethoprim-sulfamethoxazole
TOB - tobramycin
XDR - extensively drug-resistant

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Author Contributions

Conceptualization: ERB, LSI and OSD; Methodology: ERB; Software: ERB and IJ; Validation: ERB, IJ and CL; Formal analysis: ERB and CL; Investigation: ERB; resources: ERB and OSD; Data curation: ERB and IJ; Writing-original draft preparation: ERB; Writing-review and editing: ERB, LSI, OSD and CL; Visualization: ERB, LSI and OSD; Supervision: LSI and OSD; Project administration: ERB and LSI; Funding acquisition: ERB and LSI. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement

The study was conducted in accordance with the Declaration of Helsinki. The study was approved by the Research Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy Iași Romania and by the Ethics Committee of the "Sf. Parascheva" Hospital of Infectious Diseases Iași, Romania.

Informed Consent Statement

Bacterial strains were isolated in Microbiology Laboratories from clinical samples harvested for routine diagnosis, therefore the informed consent was not applicable.

Conflicts of Interest

The authors declare no conflicts of interests.

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