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Original paper

Antibiotic resistance determinants of Acinetobacter baumannii strains isolated from nosocomial infections

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Abstract

Over the years, intensive use or the misuse of antibiotics triggered the selection of multidrug resistant bacteria making it harder to treat common infectious diseases. Some of the most dangerous microbial threats are commonly united under the acronym "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species). Among these, *A. baumannii* has acquired resistance to a vast array of antimicrobials in recent decades. This capacity is partly dependent on the ability of this bacterium to acquire resistance genes, often by horizontal gene transfer. In our study, a total of 25 clinical strains of *A. bumannii* were isolated during 2015-2016 from patients hospitalized at the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu. The selected strains were isolated from nasal (n=11) and tracheal secretions (n=14). Strain identification and antibiotic susceptibility testing were performed using Vitek2 system in the Microbiology laboratory of the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu. Molecular analysis of ß-lactam resistance genes revealed the presence of the genes encoding class D β-lactamases such as $bla_{\text{OXA-51}}$ (32% of the investigated strains), $bla_{\text{OXA-23}}$ (24%), $bla_{\text{OXA-24}}$ (8%) and metallo-β-lactamases encoded by $bla_{\text{VIM-2}}$ gene(16% of the strains), the most frequently encountered ones, followed by the less encountered bla_{IMP} (12%) and $bla_{\text{OXA-24}}$ (8%) genes. One strain of *A. baumannii* exhibited resistance to tetracycline encoded by *tetA* (4%) and one to quinolones determined by the presence of the qnrS gene (4%). On the other hand, qnrA, qnrB and tetB were not found among *A. baumannii* isolates. We show here that 50% of analysed strains were isolated from tracheal secretion and nasal exudates and 16% of *A. bumannii* strains exhibited the Multidrug Resistance phenotype (MDR).

Keywords *Acinetobacter baumannii*, multidrug resistance, nosocomial strains.

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Introduction

Over the last decades, the intensive use of antibiotics has boosted the selection of multidrug resistant bacteria making it harder to treat common infectious diseases (OANCEA & STOIA [1]). Consequently this has lead to prolonged illness with increased rates of morbidity and mortality. Some of the most dangerous microbial threats are reunited under the acronym "ESKAPE" (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* species) (ALMAHDAWY & al [2], GHEORGHE & al [3], CHELARIU & al [4], MEREZEANU & al [5]). These clinically important pathogens often contain mobile genetic elements, facilitating the spread of resistant organisms as well as the ability to develop biofilms on host tissues or inert substrata (i.e. medical devices) (MARINESCU & al [6], SALA & al [7]). As their acronym suggest, these pathogens are able to "escape" the biocidal action of antimicrobial agents and they are major culprits of nosocomial infections linked to the highest risk of mortality and elevated health care costs.

An important ESKAPE pathogen is represented by *Acinetobacter* sp. A glucose-non-fermentative, non-motile, non-fastidious, catalase-positive, oxidative-negative, aerobic Gram-negative coccobacillus. Among *Acinetobacter* species, *A. baumannii* is the most important member associated with hospital-acquired infections worldwide (LIN, M.F. & al [8]). This aerobic Gram-negative coccobacillus had been regarded as a low-grade pathogen, but it is a successful pathogen responsible for opportunistic infections of the skin, bloodstream, urinary tract, and other soft tissues (PELEG A.Y. & al [9]). Most *A. baumannii* infections occur in critically ill patients in the intensive care unit (ICU) setting (FOURNIER, P.E. & al [10]). For the past 30 years, strains of *Acinetobacter* sp. have acquired resistance to newly developed antimicrobial agents; and these strains are known as multidrug resistant (MDR). *Acinetobacter* sp. became prevalent in many hospitals all over the world and has been recently recognized as a leading nosocomial pathogen (ABBO, A & al [11]; KANAFANI, A & al [12]). Different terminology like MDR, extensive drug resistant (XDR), and pan-drug resistant (PDR) have been used with various definitions to describe the degree of antimicrobial resistance for *Acinetobacter* sp. MDR *Acinetobacter* sp. can refer to being resistant to a minimum of three classes of antimicrobial drugs like all penicillins and cephalosporins, fluoroquinolones, and aminoglycosides (MAGIORAKOS A.P.& al [13]). *A. baumannii* has acquired resistance to a vast array of antimicrobials in recent decades. This capacity is partly dependent on the ability of this bacterium to acquire resistance genes, often by horizontal gene transfer (HGT) (ADAMS MD & al [14]). *A. baumannii* is well suited for genetic exchange and belongs to a unique class of Gram-negative bacteria labelled as "naturally transformable". This remarkable capacity to aquire foreign genetic material, especially antibiotic resistance genes (ARGs) is possible (GALLAGHER, L.A & al [15]), and enzyme-mediated degradation (β-lactamases), genetic manipulations (mutations, acquiring or leaving a gene, upregulation or down regulation of gene expression), and efflux pumps are all different strategies adopted by *Acinetobacter* sp. to escape from destruction of antibiotics (MARTÍNEZ-GUITIÁN, M & al [16]). *A. baumannii* has become one of the significant and prosperous pathogens in healthcare centres due to its high ability to develop antimicrobial resistance (AR). Many strains of *A. baumannii* are extremely resistant to most clinically available antibiotics (LIN, M.F. & al [8]). Importantly, inhibition of β-lactams by β-lactamases represents an essential AR mechanism in *A. baumannii* strains. Recent researches have revealed that *A. baumannii* has the natural competence to incorporate external DNA and that its genome contains foreign DNA at high frequencies, which triggers repetitive HGT in this pathogen (RAMIREZ, M & al [17]; TOUCHON, M & al [18]; TRAGLIA & al [19]). In addition, albumin, which is the main protein in the blood, promotes the natural efficiency of *A. baumannii* (TRAGLIA & al [20]). Acquired OXA-type carbapenemases are the mainstay against carbapenems, a treatment of choice, followed by metallo-β-lactamases (MBLs) (THOMSON JM & al [21]), *A. baumannii* also possess an intrinsic class D oxacillinases encoding by $bla_{\text{OXA-51-like}}$ which includes over then 40 sequence variants. The ubiquitous nature of $bla_{\text{OXA-51like}}$ genes in *A. baumannii* has led to this gene becoming an important genetic marker for the identification at the species level (TURTON, J F. & al [22]). OXA-51 enzymes are able to hydrolyze penicillins (benzyl penicillin, ampicillin, ticarcillin and piperacillin) and carbapenems (imipenem and meropenem) but do so only very weakly (PELEG AY & al [9]). Overexpression of the $bla_{\text{OXA-51-like}}$ gene in *A. baumannii* was responsible for carbapenem resistance. The overexpression is due to the acquisition of a promoter provided by an insertion sequence (IS) element, ISAba1, inserted upstream of the carbapenemase gene [POIREL & al [23]). Number of class A β-lactamases, including TEM, SHV, GES, CTX-M, SCO, PER, VEB, KPC, CARB, were identified in *A. baumannii* isolates. Part of these enzymes, like CARB-4, TEM-1, and SCO-1, are narrow-spectrum β-lactamases, while other enzymes (TEM-92, PER-1, 2, 7, CARB-10, SHV-5, CTX-M-2, CTX-M-15, GES-14, VEB-1) are extended spectrum β-lactamases (ESBLs). Four examples of MBL are known in *A. baumannii*, including New Delhi Metallo-β-lactamase

(NDM), Imipenemase (IMP), Seoul Imipenemase (SIM) and Verona integron-encoded metallo-β-lactamase (VIM) (BONNIN & al [24]), In general, *A. baumannii* has been gained resistant to a wide range of antimicrobials in recent decades, this ability is partly dependent on the mastery of these bacteria to obtained resistance genes, in predominantly through the HGT (ADAMS MD & al [14]) Major mechanism for resistance to quinolones is mutations in the gyr_A and par_C genes, which results in phenotypic changes in DNA gyrase and topoisomerase IV, leading to reduced drug affinity (UGOLOTTI & al [25]) Drug influx and efflux system encoded by chromosomal DNA mediates reduced expression of OMPs involved in drug influx and increased expression of efflux proteins resulting in active drug expulsion; these are also responsible for quinolone resistance (CHARRIER & al [26]) Plasmid-mediated quinolone resistance markers (PMQRM) *qnrA, qnrB,* and *qnrS* have also been identified in *A. baumannii* that protect DNA by inhibiting binding of quinolones to DNA gyrase and topoisomerase (YANG H & al [27], LING, B-D & al [28]).

The aim of this study was to determine the AR pattern and molecular prevalence of bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24}, bla_{VIM-2}, bla_{IMP} , tetA and PMQRM among *A. bumannii* nosocomial strains isolated from nasal and tracheal secretions in patients hospitalized in the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu.

Material and Methods

1. Bacterial Strains

A total of 25 *Acinetobacter baumannii* were isolated during 2015 and 2016 from patients hospitalized in the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu. The selected strains were isolated from tracheal and nasal swabs.

2. Identification

Strain identification was performed using the automated VITEK 2 system. The phenotypic identification of the 25 strains were based on their characteristics on the culture media and biochemical tests. The samples were cultivated on MacConkey Agar media (for Gram-negative rods), for detection of lactose fermentative species and incubated for 24 hours at 37°C in aerobic conditions. Lactose fermenting strains grow as red or pink and may be surrounded by a zone of acid precipitated bile. The red colour is due to production of acid from lactose, absorption of neutral red and a subsequent colour change of the dye when the pH of medium falls below 6.8. Lactose non-fermenting strains, are colourless and transparent and typically do not alter appearance of the medium, and also used biochemical test including (Oxidase and catalase) tests and further identification was done using API 20E.

3. Antimicrobial Susceptibility Testing

The antimicrobial susceptibility testings for *A. baumannii* were performed with Vitek system in the Microbiology laboratory of the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu and the obtained results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) guidelines 2016-2017.

4. Molecular analysis: molecular detection of the antibiotic resistance genes (ARGs)

4.1. DNA extraction and molecular detection

The genetic support of β-lactam, quinolone and tetracycline resistance *(*bla_{OXA-23}, bla_{OXA-24}, bla_{OXA-51}, bla_{IMP}, bla_{VIM}, qnrA, qnrB, qnrS, tetA and tetB) [Table (2)] was investigated by conventional PCR multiplex/simplex using a total volume of 20 µl (PCR Master Mix 2x, Thermo Scientific) containing 1 µl of bacterial DNA extracted using an adapted alkaline extraction method. For this purpose, 1-5 colonies of bacterial culture were resuspended in 1.5 ml tubes containing 20 µl solution of NaOH 0,05M (sodium hydroxide) and SDS 0,25% (sodium dodecyl sulphate) and heated on a thermo block at 95°C for 15 min. for the permeabilization of bacterial cell wall. In the next step was added 180 µl of TE buffer (TRIS+EDTA) 1X and centrifuge at 13000 rpm for 3 minutes. PCR was performed in a final volume of 20 µL with 10 µL Master Mix (Thermo Scientific), 0.5 µL 0.5 µM primers,1 µl bacterial DNA. PCR was performed according to the conditions described by the author for each set of primers used Table 1.

4.2. Detection of antimicrobial resistance genes by PCR

All PCR reactions were performed using the Thermal Cycler machine Corbet for the detection of carbapenemases encoded by bla_{IMP}, bla_{VIM}, class D β-lactamases encoded by bla_{OXA-51}, bla_{OXA-23}, bla_{OXA-24}, tetracycline resistance genes (tet_A, tet_B), and PMQRM, (qnrA, qnrB, qnrS) genes.

The PCR reactions were initiated with 1 cycle of denaturation at 95°C for 2 min, followed by 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 1 min and a final elongation step at 72°C for 7 min (Table 3). The amplification products were visualized by electrophoresis on a 1% agarose gel, the size of the product compared to a DNA Ladder molecular weight 100bp, stained with the specific weight marker (100bp, Ladder Bench Top, Promega, USA) (Table 3). Primers sequences used in simplex and multiplex PCR assays for carbapenemases in *A. bumannii* strains are presented in Table 2.

Table 1. Reaction components used in the PCR experiments

Concentration						Final volume
primer	$MgCl_2$	dNTP	Taq-pol	Reaction buffer	DNA	
0,5 µM	1,2 mM	2 µM	0,2U	1x	10x	20 µl

Table 2. Primer sequences and amplicons of the investigated ARGs

Gene	Primers	Primer Sequence s (5′→3′)	Product length (bp)	Ref
$bla_{\text{OXA-23}}$	OXA-23-F OXA-23-R	5'-CCCCGAGTCAGATTGTTCAAGG-3' 5'-TACGTCGCGCAAGTTCCTGA-3'	501	(BOGAERTS, P. & al [29])
$bla_{\text{OXA-24/40}}$	OXA-24/40-F OXA-24/40-R	5' GCAGAAAGAAGTAAARCGGGT3' 5' CCAACCWGTCAACCAACCTA3'	242	(BOGAERTS, P. & al[29])
$bla_{\text{OXA-51}}$	OXA-51-F OXA-51-R	5′-TAATGCTTTGATCGGCCTTG-3′ 5′-TGGATTGCACTTCATCTTGG-3′	390	(WOODFORD, & al [30])
bla_{IMP}	IMP-F IMP-R	5′-GGAATAGAGTGGCTTAAYTCTC-3′ 5′-GGTTTAAYAAAACAACCACC-3′	232	(POIREL, L. & al [31])
bla_{VIM}	VIM -F VIM-R	5′-GATGGTGTTTGGTCGCATA-3′ 5′-CGAATGCGCAGCACCAG-3′	390	(POIREL, L. & al [31])
tetA	tetA-F tet A-R	5′-GCGCGATCTGGTTCACTCG-3′ 5′-AGTCGACAGYRGCGCCGGC-3′	164	(AMINOV, R. & al [32])
tetB	$\text{tet}_{\text{B-F}}$ $\text{tet}_{\text{B-R}}$	5′-TACGTGAATTTATTGCTTCGG-3′ 5′-ATACAGCATCCAAAGCGCAC-3′	206	(AMINOV, R. & al [32])
qnrA	qnrA-F qnrA-R	5′-AGAGGATTTCTCACGCCAGG-3′ 5′-TGCCAGGCACAGATCTTGAC-3′	580	(CATTOIR, V. [33])
qnrB	qnrB-F qnrB-R	5′-GGMATHGAAATTCGCCACTG-3′ 5′-TTTGCYGYYCGCCAGTCGAA-3′	264	(CATTOIR, V. [33])
qnrS	qnrS-F qnrS -R	5′-GCAAGTTCATTGAACAGGGT-3′ 5′-TCTAAACCGTCGAGTTCGGCG-3′	428	(CATTOIR, V. [33)

Table 3. Amplification program for PCR reactions

Genes	Amplification program				
	Initial denaturation	No. of cycle	Denaturation	Annealing	Extension
$bla_{\text{OXA-23}}$, $bla_{\text{OXA-24/40}}$ $bla_{\text{OXA-51}}$, bla_{IMP}, bla_{VIM} *tet*A, tetB, qnrA, qnrB, qnrS	94°C for 10 min	36	95°C 30 sec	52°C 40 sec	72°C 5 min

Results and Discussion

1. Identification of the strains

This study was conducted on a total of 25 clinical strains isolated during 2015-2016 from patients hospitalized in the National Institute for Cardiovascular Diseases Prof. C.C. Iliescu. The selected strains were isolated from tracheal (n=14) and nasal s (n=11).

The respiratory tract is an important site of *A. baumannii* colonization and the most common site of infection. Moreover, the incidence of *A. baumannii* colonization in the respiratory tract increases during stays in the intensive care unit. The crude mortality rate of ventilation-associated and community-acquired pneumonia caused by *A. baumannii* has been estimated to be between 40 and 70% (MCCONNELL MJ, al [34]). As a result of its increasing resistance to many currently available antibiotics (BOUCHER HW, & al [35]).

2. Antimicrobial Susceptibility Testing

The strains identification was performed using an automated VITEK 2 system. The phenotypic identification was based on their characteristics on the culture media and biochemical features (MacConkey Agar for differentiate lactose fermentative species by lactose non-fermentative ones, mini galleries API 20NE). The results of the identification are summarized in Table 4. 50% of analysed strains were from tracheal secretion and the rest from nasal exudate; 16% of *A. baumannii* exhibited the Multidrug Resistance phenotype (MDR).

Table 4. The bacterial strains and their isolation sources

Hospital code	Isolation source	Phenotype
1	Tracheal secretion	
2	Tracheal secretion	
3	Tracheal secretion	
4	Tracheal secretion	MDR
5	Tracheal secretion	
6	Tracheal secretion	
7	Tracheal secretion	
8	Tracheal secretion	
9	Tracheal secretion	
10	Tracheal secretion	
11	Tracheal secretion	
759	Tracheal secretion	
2055	nasal	MDR
1840	nasal	
768	nasal	
1213	nasal	
1715	nasal	MDR
617	nasal	
244	nasal	
340	nasal	
1629	nasal	MDR
617	nasal	
236	nasal	
758	Tracheal secretion	
393	Tracheal secretion	

3. Molecular detection

The *A. baumannii* strains harbored genes encoding for different β-lactamases : 24% of analysed isolates contained the bla_{OXA-23} gene (n=6); 8% of the investigated strains presented bla_{OXA-24} (n=2) (Figure 1 and 2). Concerning the intrinsic bla_{OXA-51} gene it was observed that 32% of the nosocomial isolates were positives (n=8) (Fig. 3) and carbapenemases were revealed by bla_{VIM-2} obtained in 16% of the strains (n=4); and by bla_{IMP} in 12% of investigated isolates (n=3) (Fig. 4). Regarding the quinolone and tetracycline resistance in *A. baumannii* isolates only 4% showed the qnrS gene (Fig. 5) and encoding quinolone resistance and 4% tetA tetracycline resistance (Fig. 6).

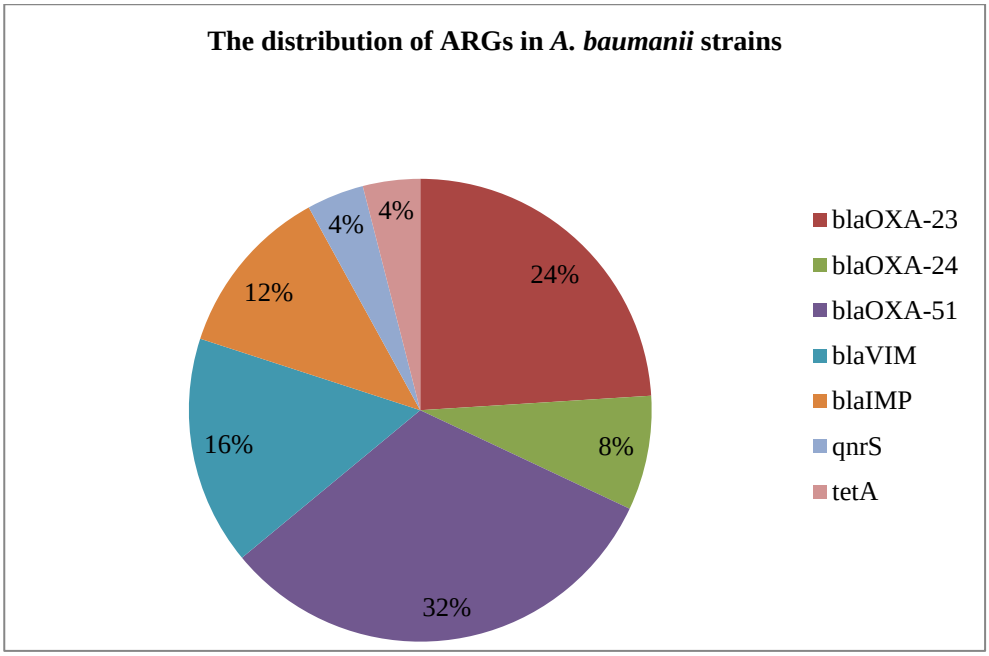

Figure 1. Distribution of the ARGs in *A. baumannii* analysed strains.

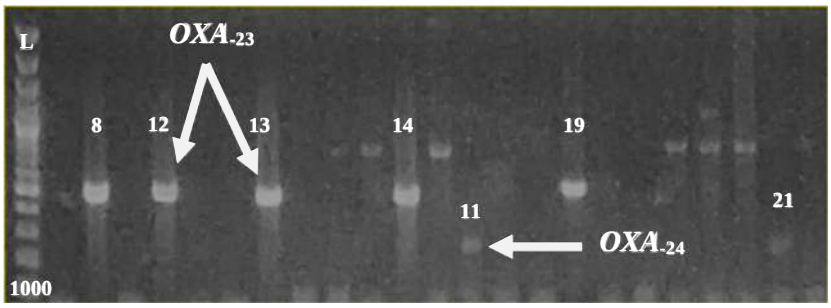

Figure 2. Detection of gene encoding for CHLD producing *A.baumannii* strains. L molecular size ladder, positives isolates for bla_{OXA-23} (501 bp) gene: nr. (14, 12, 13, 24, 19, 8), and bla_{OXA-24} (242 bp) positive strains were (21, 11).

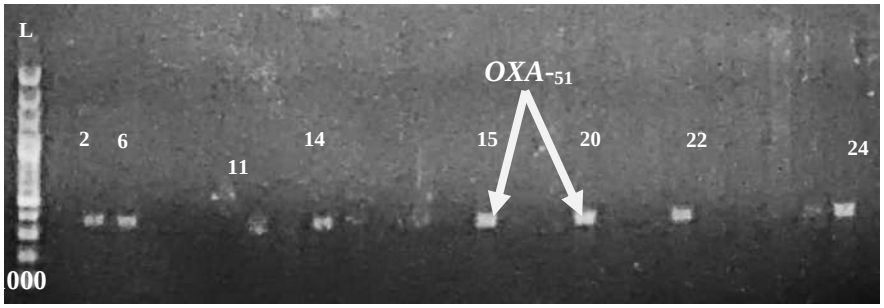

Figure 3. Electrophoresis gel for the gene encoding the intrinsic carbapenemase OXA-51: L molecular size ladder, positives isolates for bla_{OXA-51} (390 bp) gene nr. (24, 15, 2, 22, 11, 14, 20, 6).

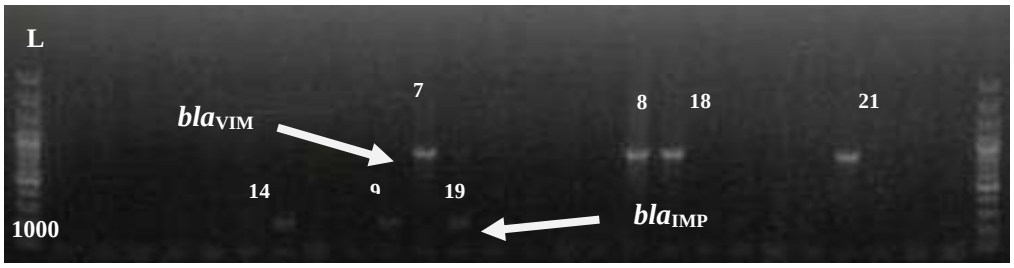

Figure 4. Electrophoresis gel for detection of the genes encoding MBL VIM-2 and IMP. L molecular size ladder, positives isolates for *bla*$_{VIM-2}$ (390 bp) gene no. (7, 8, 18, 21), and the positive isolates for *bla*$_{IMP}$ (232 bp) no. (14, 9, 19).

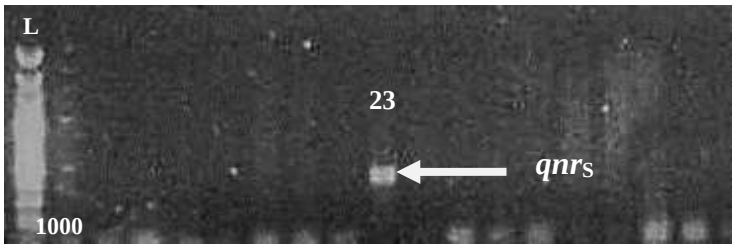

Figure 5. Agarose gel showing the gene encoding for -*qnr*S. L molecular size ladder, the positive isolate for *qnr*S (428 bp) gene was no. (23).

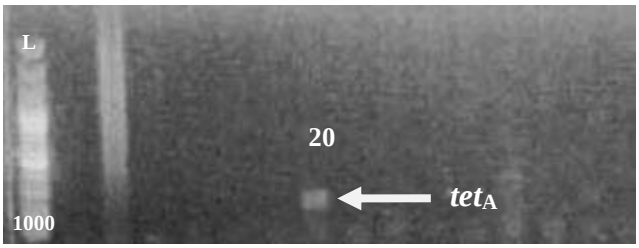

Figure 6. Electrophoresis gel for the detection of gene encoding tetracycline resistance (tetA): L molecular size ladder, the positive isolate for tetA (164 bp) gene was no. (20).

The analyzed strains were positive for genes encoding for different β-lactamases belonging to CHLD class: 24% of analysed isolates revealed *bla*$_{OXA-23}$ gene, 8% of the investigated strains presented *bla*$_{OXA-24}$ gene. Concerning the presence of the intrinsic *bla*$_{OXA-51}$ gene 32% of the investigated strains were positives, carbapenemases were revealed by *bla*$_{VIM-2}$ gene obtained in 16% of the strains, and by the *bla*$_{IMP}$ gene (12%). Regarding the quinolone and tetracycline resistance only 4% revealed the only one PMQRM (*qnr*$_S$ gene) and another 4% of the strains were positives for gene. On the other hand, the *qnr*$_A$, *qnr*$_B$ and *tet*B were not found among *A. baumannii* isolates; consequently, the increase in MDR among *A. baumannii* strains is a global health threat. Long-term, low-dose, or unnecessary consumption of antibiotics leads to the emergence of multidrug-resistant strains (PELEG AY & al [9], DOI Y & al [36]). Moreover, treatment options are limited due the ability of *A. baumannii* strains to develop resistance to newly introduced classes of antibiotics and to spread the genetic determinants of resistance *via* mobile genetic elements (HOWARD A & al [37], EVANS BA & al [38]). In the last two decades, carbapenem resistance in *A. baumannii* isolates has reached high levels worldwide. In this study, the rate of carbapenem resistance among the clinical isolates was 95%. Similarly, data reported by the Central Asia and Eastern European Surveillance of Antimicrobial Resistance revealed that the carbapenem resistance rate was 93% in Turkey (CAESAR, [39]). According to the 2017 report of The European Antimicrobial Resistance Surveillance Network (EARS-Net), the rate of carbapenem resistance was 52.5% in Hungary, 78.7% in Italy, 68.2% in Spain, and 96.2% in Croatia (ECDC [40]).

All these worrying date show the fact that either new therapeutic approaches are necessary in order to combat the problem of antimicrobial resistance. Such alternative approaches to be used in the future may include synergistic drug combinations (UNLU & al [41]), natural compounds (SAVIUC & al [42] and nanoparticles (IBRAHEEM & al [43], NEACSU & al [44]).

Conclusion

A. baumanniii strains isolated from the nasal and respiratory infections showed resistance to carbapenems antibiotics which limited options available to treat the patients. Molecular analysis of ß-lactam resistance genes, have revealed that *bla*$_{OXA-51}$, *bla*$_{OXA-23}$ and *bla*$_{VIM-2}$ genes were the most frequently encountered ones, followed by the

less encountered bla_{OXA-24} and bla_{IMP} genes. One strain of *A.baumannii* exhibited resistance to tetracycline encoded by tetA gene and one to quinolones determined by the presence of the qnr_S gene. Our results highlight the need to monitor the mechanisms of resistance caused by the presence of gene encoding for carbapenems, tetracyclines and quinolones antibiotics among *A. baumannii* strains isolated from clinical patients.

Acknowledgments

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