Co-occurrence of carbapenem and aminoglycoside resistance genes among multidrug-resistant clinical isolates of *Acinetobacter baumannii* from Cracow, Poland

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**Source of support:**
Scientific work was partially supported by the Polish government as a research grant (no. N N405 672140) in the years 2011–12 and by the Jagiellonian University Medical College with funds from maintenance of the research potential of the Department of Pharmaceutical Microbiology JU MC

**Background:** *Acinetobacter baumannii* is a significant hospital pathogen, possessing a considerable degree of antimicrobial resistance. *A. baumannii* resistance to carbapenems and aminoglycosides is mostly conferred by class D OXA carbapenemases and aminoglycoside-modifying enzymes, respectively. The aim of this study was to determine the prevalence of selected genes encoding OXA carbapenemases and aminoglycoside-modifying enzymes in multidrug-resistant strains of *A. baumannii*.

**Material/Methods:** The study included 61 carbapenem-resistant and aminoglycoside-nonsusceptible *A. baumannii* isolates, collected between 2009 and 2011 in Cracow, Poland. Selected resistance genes, including: bla*OXA-51-like*, bla*OXA-23-like*, bla*OXA-40-like*, bla*OXA-58-like*, aac(6')-Ih, aac(3)-Ia, aac(3)-IIa, aac(6')-Ib, aph(3')-Ia and aph(3')-VI, were detected by PCR method.

**Results:** The bla*OXA-51-like* genes were detected in all isolates, while acquired carbapenemase encoding genes were found in 96.7% of tested strains. Presence of bla*OXA-40-like* and bla*OXA-23-like* genes was observed among 65.6% and 27.9% of isolates, respectively. Assayed aminoglycoside resistance genes were found to harbor 98.4% of isolates. Among tested strains, we observed the following percentages of resistance determinants: aac(3)-Ia – 78.7%, aph(3')-VI – 78.7% and aph(3')-la – 27.9%. Analysis of co-occurrence of carbapenem and aminoglycoside resistance genes revealed the highest percentage of strains possessing bla*OXA-40-like*, aac(3)-Ia, and aph(3')-VI genes (44.3%).

**Conclusions:** The bla*OXA-40-like* and aac(3)-Ia/aph(3')-VI were the most prevalent genes encoding acquired OXA carbapenemases and aminoglycoside-modifying enzymes, respectively, among *A. baumannii* strains in Cracow, Poland. Genes conferring resistance to carbapenems and aminoglycosides coexisted in the clinical strains of *A. baumannii*. The phenomenon of *A. baumannii* resistance indicates the necessity of monitoring for the presence of the resistance genes.

**Keywords:** *Acinetobacter baumannii* • carbapenem resistance detection • aminoglycoside resistance detection

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Background

Over the last 30 years the incidence of hospital infections caused by *Acinetobacter* spp. has increased greatly. It is connected with the growing number of highly specialized medical procedures that affect patient immunity. The incidence and seriousness of *Acinetobacter* spp. nosocomial infections is furthermore associated with the simplicity of its nutritional requirements and low susceptibility to adverse environmental conditions, as well as the remarkable ability of these bacteria to acquire resistance determinants to a wide range of antimicrobial agents [1–3]. The most clinically important species belonging to the *Acinetobacter* genus and responsible for over 90% of infections is *Acinetobacter baumannii*. Infections caused by this bacterium are associated with significant mortality [3]. The main problems caused by *A. baumannii* isolates in the hospital environment concern patients hospitalized in intensive care and burn units [1,2,4].

*A. baumannii* strains exhibit a wide spectrum of antimicrobial resistance mechanisms, both intrinsic and acquired. These mechanisms include: outer membrane permeability modifications, efflux pumps, production of drug inactivating/modifying enzymes, and target-site modifications. *A. baumannii* can become resistant due to modification of existing genes, their regulation mechanisms, or acquisition of exogenous DNA by horizontal gene transfer. Diversity and coexistence of mechanisms of resistance in this bacterium result in multiple drug resistance and can cause difficulties in treatment [5–8].

Among antimicrobials frequently used in treatment of *A. baumannii* infections, carbapenems and aminoglycosides are notable. Carbapenems produce synergistic bactericidal activity in combination with aminoglycosides; therefore, carbapenems are often used in combination therapy with aminoglycosides.

Carbapenem resistance in *A. baumannii* principally involves the molecular class D OXA-type serine oxacillinas, but may also be mediated by other mechanisms such as porin modification or loss, and efflux of antimicrobial and modification of penicillin-binding proteins [9]. The most widespread acquired carbapenemas in *A. baumannii* are the class D oxacillinases, represented by 3 main phylogenetic subgroups: OXA-23, OXA-40, and OXA-58, which are often encoded by genes localized in chromosomes or on plasmids. Furthermore, carbapenem resistance can be mediated by intrinsic *A. baumannii*, chromosomely harbored OXA-51-like enzymes, but only when the insertion sequence IS*Aba*1 element is inserted upstream of the gene [9]. The ubiquitous nature of OXA-51-like encoding genes in *A. baumannii* resulted in use of *bla*<sub>OXA-51-like</sub> gene detection in identification of this bacterium to the species level [10]. However, recent studies by Lee et al. [11] revealed the presence of *bla*<sub>OXA-51-like</sub> genes in carbapenem-resistant non-*baumannii* species of *Acinetobacter*: *Acinetobacter nosocomialis* and *Acinetobacter* genomic species “Close to 13TU”. With regard to these findings, the identification of *A. baumannii* strains cannot be based only on the detection of *bla*<sub>OXA-51-like</sub> gene, but must be supported by other identification methods [11].

Mechanisms responsible for *A. baumannii* resistance to aminoglycoside antibiotics include decreased antibiotic uptake and accumulation, modification of the ribosomal target, efflux of antibiotic, and enzymatic modification of aminoglycosides. One of the most important mechanisms attributed to aminoglycoside resistance in *A. baumannii* is the production of aminoglycoside-modifying enzymes, including aminoglycoside acetyltransferases (AAC), phosphoryltransferases (APH), and adenylyltransferases (ANT) [12].

The genes encoding resistance to carbapenems and aminoglycosides may be located in mobile genetic elements such as transposons, integrons, and/or plasmids [8].

The aim of this study was to determine the prevalence of selected genes encoding OXA carbapenemas and aminoglycoside-modifying enzymes in multidrug-resistant strains of *A. baumannii*.

Material and Methods

Bacterial strains

A collection of 61 non-repetitive (1 per patient) carbapenem-resistant (imipenem and meropenem) and aminoglycoside-nonsusceptible (amikacin, gentamicin, and tobramycin) *A. baumannii* strains were isolated in 2009 (n=27), 2010 (n=20), and 2011 (n=14), respectively, from patients hospitalized in the Specialized Hospital in Cracow, Poland. The isolates were identified as *A. baumannii* by the Vitek 2 Compact automatic system (bioMérieux, Poland) and PCR amplification of the *bla*<sub>OXA-51-like</sub> gene. Most tested isolates originated from Intensive Care Unit patients (36; 59.0%) and Burn Therapy Unit patients (18; 29.5%). The isolates were recovered from various clinical specimens including: (in descending frequency) respiratory tract samples (30; 49.2%), wound swabs (13; 21.3%), urine (9; 14.8%), blood (6; 9.8%), and other specimens (3; 4.9%). While phenotypic identification and antibiotic susceptibility testing, as well as molecular detection of *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-23-like</sub>, *bla*<sub>OXA-40-like</sub>, and *bla*<sub>OXA-58-like</sub> genes, among 27 and 20 strains recovered in 2009 and 2010, respectively were described previously [13], 14 additional isolates derived from the Specialized Hospital in Cracow, Poland in 2011 were examined in the current study.

Susceptibility testing

*A. baumannii* susceptibility testing concerning minimal inhibitory concentration (MIC) were performed using the VITEK-2

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*A. baumannii* susceptibility testing concerning minimal inhibitory concentration (MIC) were performed using the VITEK-2
Compact system and relevant antimicrobial susceptibility testing ing cards (bioMérieux, Poland), interpreted according to standard CLSI criteria [14]. A. baumannii ATCC 19606, P. aeruginosa ATCC 27853, and E. coli ATCC 25922 were used as reference strains for quality control. The isolates were stored in a freezer at −80°C for further analysis.

**DNA extraction**

Genomic DNA was extracted using the Sherlock AX (A&A Biotechnology, Poland) isolation kit according to the manufacturer’s recommendations. DNA quantification was performed by spectrophotometry at 260 nm. The purity of DNA was evaluated by the ratio of the absorbance at 260 nm and 280 nm (A260/A280) (Biometra, Germany). DNA extraction was performed in 100 µl of genomic DNA, 20 pM of each primer (Sigma Aldrich, Poland), 10 µl of GoTaq® buffer, 4 µl of MgCl₂ solution, 1 µl of PCR nucleotide mix, 2 µl of each primers, 0.25 µl GoTaq® DNA Polymerase (Promega, USA) and 5 µl of DNA (Promega, USA). A single reaction mixture contained: 10 µl of GoTaq® buffer, 4 µl of MgCl₂, 1 µl dNTPs and 0.25 µl GoTaq® DNA Polymerase (Promega, USA) in a final volume of 50 µl. Initial denaturation at 94°C for 3 min was followed by 30 cycles of amplification consisting of 94°C for 25 s, 52°C for 40 s, 72°C for 50 s, and a final extension step at 72°C for 5 min.

**Molecular determination of aminoglycoside resistance**

Detection of blaOXA-51-like, blaOXA-23-like, blaOXA-40-like, and blaOXA-58-like genes was performed by multiplex PCR, as described previously [15]. The PCR was carried out in a thermocycler T personal (Biometra, Germany). A single reaction mixture contained: 10 µl of GoTaq® buffer, 4 µl of MgCl₂ solution, 1 µl of PCR nucleotide mix, 2 µl of each primers, 0.25 µl GoTaq® DNA Polymerase, and 5 µl of DNA (Promega, USA). Amplification reactions were performed in a T Personal thermal cycler (Biometra, Germany) with the following parameters: 1) triplex PCR at -95°C for 2 min, following 35 cycles: of 30 s at 95°C, 40 s at 58°C, 90 s at 72°C, and 5 min at 72°C; 2) duplex PCR at -95°C for 2 min, following 35 cycles: of 30 s at 95°C, 40 s at 55°C, 90 s at 72°C, and 5 min at 72°C; and 3) single PCR at -95°C for 2 min, following 35 cycles: of 30 s at 95°C, 40 s at 49°C, 90 s at 72°C, and 5 min at 72°C.

**PCR products detection**

All obtained amplicons were subjected to electrophoresis with 2% agarose gel stained with ethidium bromide, visualized, and digitalized. The size of PCR products was compared with the molecular weight standard O’Gene Ruler 100 bp DNA Ladder Plus (Thermo Scientific, USA).

**Results**

**OXA carbapenemases genes**

Phenotypic identification of 14 A. baumannii strains (originated from 2011) was confirmed in all isolates by presence of blaOXA-51-like genes. Furthermore, analysis of occurrence of acquired OXA carbapenemase genes among strains isolated from 2009, 2010, and 2011 revealed the presence of blaOXA-23-like gene in 10 (71.4%) strains, and blaOXA-58-like gene was present in 3 (21.4%) strains. Moreover, only 1 strain did not carry any assayed acquired OXA carbapenemase genes. Figure 1 shows results of a previously performed analysis [13] and the present analysis of occurrence of OXA encoding genes over the selected period of time (2009, 2010, 2011). The most prevalent genes encoding OXA carbapenemases among strains isolated in 2009, 2010, and 2011 were blaOXA-40-like genes.

**Aminoglycoside resistance genes**

All 61 A. baumannii strains were screened for occurrence of selected genes encoding aminoglycoside-modifying enzymes (aac(6′)-Ib, aac(3)-Ia, aac(3)-IIa, aac(6′)-Ib, aph(3′)-Ia, aph(3′)-VI). Our studies revealed the presence of at least 1 gene in 60 strains (98.4%). We observed the following percentages of genes aac(3)-Ia – 78.7%, aph(3′)-VI – 78.7% and aph(3′)-Ia – 27.9% among analyzed strains. Furthermore, 47 (77%) isolates...
simultaneously possessed more than 1 gene encoding aminoglycoside-modifying enzymes. We noted genotypes with different combinations of genes encoding aminoglycoside-modifying enzymes, such as: 1) \textit{aac(3)-Ia}, \textit{aph(3')-Ia}, \textit{aph(3')-VI}; 2) \textit{aac(3)-Ia}, \textit{aph(3')-VI}; 3) \textit{aph(3')-Ia}, \textit{aph(3')-VI}, respectively, in 6 (9.8%), 30 (49.2%), and 11 (18.1%) strains (Table 1). The frequency of occurrence of analyzed genes encoding aminoglycoside-modifying enzymes over the selected period of time (2009, 2010, and 2011) is presented in Figure 2. Taking into account the year of strain isolation, we observed in 2009 and 2010 the prevalence of \textit{aac(3)-Ia}, \textit{aph(3')-VI} genotype in 63% (17) and 50%, respectively (10). Among strains isolated in 2011, the dominant aminoglycoside resistance genotype consisted of \textit{aac(3)-Ia} gene, which was present in 50% (7). Analysis of aminoglycoside resistance genotypes and susceptibility patterns to amikacin, gentamicin, and tobramycin is presented in Table 1. The most prevalent aminoglycoside resistance genotype (\textit{aac(3)-Ia}, \textit{aph(3')-VI}) was present in 30 (49.2%) strains, which were characterized by resistance to amikacin – 26 isolates, gentamicin – 30 isolates, and tobramycin – 16 isolates.

### Table 1. Analysis of occurrence of aminoglycoside resistance genotypes and susceptibility patterns to amikacin, gentamicin and tobramycin among 61 A. baumannii clinical strains.

| Resistance genotype | Isolates | No. of isolates resistant (R)/intermediate (I) |
|---------------------|----------|------------------------------------------|
|                     | No.      | %                     | Amikacin | Gentamicin | Tobramycin |
| \textit{aac(3)-Ia}  | 30       | 49.2                   | 26/4     | 30/0       | 16/14      |
| – \textit{aph(3')-VI} | 11     | 18.1                   | 5/6      | 11/0       | 3/8        |
| \textit{aac(3)-Ia}  | 12       | 19.7                   | 11/1     | 12/0       | 10/2       |
| \textit{aph(3')-VI} | 6        | 9.8                    | 4/2      | 6/0        | 3/3        |
| – – –                | 1        | 1.6                    | 1/0      | 1/0        | 1/0        |

#### Co-occurrence of OXA carbapenemases and aminoglycoside resistance genes

Analysis of coexistence of genes encoding OXA carbapenemases and aminoglycoside-modifying enzymes is presented in Table 2. Our studies revealed the highest percentage of A. baumannii strains carrying simultaneously \textit{bla\textsubscript{OXA-40-like}}, \textit{aac(3)-Ia}, and \textit{aph(3')-VI} genes – 44.3% (27). We also noticed the prevalence of isolates possessing 1) \textit{bla\textsubscript{OXA-23-like}}, \textit{aph(3')-Ia}, \textit{aph(3')-VI} and 2) \textit{bla\textsubscript{OXA-40-like}}, \textit{aac(3)-Ia}, which accounted for 18.1% (11) of both genotypes.

### Discussion

A. baumannii is an opportunistic pathogen often associated with nosocomial infections worldwide. Strains of this species have high ability to develop resistance to different groups of antimicrobial drugs both by upregulation of intrinsic resistance mechanisms and acquisition of determinants of resistance. Multidrug-resistant A. baumannii strains are responsible for severe infections with high rates of morbidity and mortality, mainly affecting immunocompromised patients [6,10,17,18]. Carbapenems and aminoglycosides are antimicrobial drugs frequently used in treatment A. baumannii infections, often in combination therapy. Although the prevalence of resistance to these agents has become an increasing therapeutic problem, there is limited data concerning Poland [4,19].

The \textit{bla\textsubscript{OXA-51-like}} genes naturally occur in the genome of A. baumannii but have been recently detected in carbapenem-resistant non-baumannii species of Acinetobacter. The detection of \textit{bla\textsubscript{OXA-51-like}} gene can be used as a supplementary tool to identify the organism to the species level, confirmed by additional methods [10,11].
Table 2. Co-occurrence of genes encoding OXA carbapenemases and aminoglycoside-modifying enzymes among A. baumannii strains (n=61).

| Co-occurrence of genes mediating resistance to carbapenems | Resistance to aminoglycosides | Isolates |
|------------------------------------------------------------|-------------------------------|---------|
| bla<sub>bla</sub>OXA-23-like | aac(3)-Ia | – | aph(3')-VI | 1 | 1.6 |
| – | – | aac(3)-Ia | aph(3')-VI | 11 | 18.1 |
| – | – | aph(3')-Ia | aph(3')-VI | 5 | 8.2 |
| – | – | aac(3)-Ia | – | 2 | 3.3 |
| – | – | aac(3)-Ia | aph(3')-VI | 27 | 44.3 |
| – | – | – | – | 11 | 18.1 |
| – | – | aac(3)-Ia | – | 1 | 1.6 |
| – | – | aac(3)-Ia | aph(3')-VI | 1 | 1.6 |
| – | – | – | aph(3')-VI | 1 | 1.6 |

In our studies concerning the group of 61 A. baumannii strains (47 described previously and 14 currently), all carried bla<sub>bla</sub>OXA-51-like genes [13]. A study from Poland, conducted by Wroblewska et al., revealed the presence of intrinsic OXA carbapenemases among all tested strains [19]. The most frequent enzymatic mechanism of carbapenem resistance in A. baumannii is the production of oxacillinases encoded by genes of the bla<sub>bla</sub>OXA-23-like, bla<sub>bla</sub>OXA-40-like, and bla<sub>bla</sub>OXA-58-like lineages [10]. We have reported that genes encoding acquired carbapenemases belonging to OXA-40 group were the most frequent (40; 65.5%) among carbapenem-resistant, aminoglycoside-nonsusceptible A. baumannii isolates (n=61) obtained from the Specialized Hospital in Cracow. While bla<sub>bla</sub>OXA-40-like oxacillinases were dominant in Spain and Taiwan [17,20], bla<sub>bla</sub>OXA-23-like were the most prevalent in Greece and Germany [21,22].

Aminoglycoside resistance in A. baumannii is predominantly associated with modifications of these antimicrobials by enzymes, such as acetyltransferases (AAC), phosphotransferases (APH), and adenylyltransferases (ANT), often coexisting in a single isolate. Our analysis of the distribution of genes encoding aminoglycoside-modifying enzymes among assayed carbapenem-resistant, aminoglycoside-nonsusceptible A. baumannii isolates (n=61) revealed the dominance of aac(3)-Ia (48; 78.7%) and aph(3')-VI (48; 78.7%). Studies by Moniri et al. also highlighted high prevalence of aac(3)-Ia and aph(3')-VI, in 63.3% and 65%, respectively, of isolates [23]. Nemec et al. investigated the occurrence of aminoglycoside resistance genes, and reported high percentages of strains carrying aac(3)-Ia (64%) and aph(3')-VI (52%) [24]. In our study, 77% (47) of isolates possessed 3 combinations of more than 1 gene encoding aminoglycoside-modifying enzymes, with the predominance of aac(3)-Ia, aph(3')-VI genotype. Among 11 combinations of aminoglycoside-modifying enzymes encoding genes, Bakour et al. found 11.3% of strains possessing both aac(3)-Ia and aph(3')-VI genes [25]. Analysis of correlation between the content of resistance genes and resistance phenotypes revealed that strains with aac(3)-Ia, aph(3')-VI genotype (30) were resistant to gentamicin, and had resistant/intermediate susceptibility to amikacin (26/4) and tobramycin (16/14). Strains carrying both aph(3')-Ia and aph(3')-VI genes (11) were resistant to gentamicin and resistant/intermediate susceptible to amikacin (5/6) and tobramycin (3/8). We also noted that 1 isolate did not carry any of the mechanisms assayed, but presented an aminoglycoside-resistant phenotype. To conclude, in some strains there was no correlation between the presence of assayed resistance genes and susceptibility phenotype, which can be explained by the occurrence of other aminoglycoside resistance genes, overexpression of an efflux pump such as AdeABC, or additional resistance mechanisms. These findings are also supported by Salazar De Vegas et al. [18] and Nemec et al. [24].

This study analyzed the occurrence of various genes that confer resistance to carbapenems and aminoglycosides in the group of A. baumannii strains resistant to carbapenems and nonsusceptible to aminoglycosides in Cracow, Poland. Our research revealed the co-occurrence of selected carbapenem and aminoglycoside resistance genes in 8 combinations, with the dominance of isolates carrying bla<sub>bla</sub>OXA-40-like, aac(3)-Ia and aph(3')-VI genes (44.3%). The coexistence of genes accountable for resistance to carbapenems and aminoglycosides in isolates of A. baumannii was also found by Salazar de Vegas et al., Endimian et al., Nigro et al., and Sung et al. [18,26–28].
A. baumannii multidrug resistance appears to be a consequence of accumulation of mutations and the acquisition of resistance determinants by the transfer of plasmids, transposons, and integrons, which may lead to the formation of clusters of resistance genes, termed “resistance islands” [6]. The high genetic plasticity of the A. baumannii genome contributes to high adaptation, virulence capacities, and multidrug resistance results in its ability to persist in the hospital environment and survive antibiotic treatment [29].

Conclusions

1. blaOXA-40-like were the most prevalent genes encoding acquired OXA carbapenemases among A. baumannii strains isolated in Cracow, Poland.

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Acknowledgements

The Microbiological Laboratory of the Department of Laboratory Diagnostics of the Specialized Rydygier's Hospital in Cracow, Poland is gratefully acknowledged for providing the isolates used in this study.