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Carbapenemase Production of Clinical Isolates *Acinetobacter baumannii* and *Pseudomonas aeruginosa* from a Bulgarian University Hospital

Atanaska P. Petrova^{1,2,3}, Irina D. Stanimirova^{1,2}, Ivan N. Ivanov⁴, Michael M. Petrov¹,
Tsonka M. Miteva-Katrandzhieva⁵, Vasil I. Grivnev¹, Velichka S. Kardjeva⁶,
Todor V. Kantardzhiev⁴, Mariana A. Murdjeva^{1,2,3}

¹ Department of Microbiology and Immunology, Faculty of Pharmacy, Medical University of Plovdiv, Plovdiv, Bulgaria

² Laboratory of Microbiology, St George University Hospital, Plovdiv, Bulgaria

³ Technology Center of Emergency Medicine, Plovdiv, Bulgaria

⁴ National Reference Laboratory for Control and Monitoring of Antimicrobial Resistance, National Center of Infectious and Parasitic Diseases, Sofia, Bulgaria

⁵ Department of Social Medicine and Public Health, Faculty of Public Health, Medical University of Plovdiv, Plovdiv, Bulgaria

⁶ Department of Life Science, 'Aquachim' JSCo, Sofia, Bulgaria

Correspondence:

Atanaska P. Petrova, Department of Microbiology and Immunology, Faculty of Pharmacy, Medical University, Plovdiv; Laboratory of Microbiology, St George University Hospital, Plovdiv; Technology Center of Emergency Medicine-Plovdiv, 15A Vassil Aprilov Blvd., 4002 Plovdiv, Bulgaria
E mail: atanasia_petroff@abv.bg
Tel: +359 32 602273

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Background: Production of Bla OXA-23, OXA-24, OXA-58 and hyperexpression of OXA-51 due to ISAb₁ insertion sequence are the leading causes of carbapenem resistance in *Acinetobacter baumannii*. The loss of OprD transmembrane protein and the overexpression of some efflux pumps are considered to be the main factors for carbapenem resistance in *Pseudomonas aeruginosa* whereas metallo-enzymes' production has a secondary role.

Aim: To examine the carbapenem resistance due to carbapenemase production among clinically significant Gram-negative non-fermenters from St George University hospital, Plovdiv: *A. baumannii* and *P. aeruginosa*.

Materials and methods: Forty three *A. baumannii* and 43 *P. aeruginosa* isolates, resistant or with intermediate resistance to imipenem and/or meropenem were included in the study. They were collected from patients admitted in 14 various hospital wards between 2010 and 2014. Both phenotypic and genetic methods were used for identification and antimicrobial susceptibility testing.

Results: All *A. baumannii* demonstrated carbapenemase production determined by a modified Hodge test whereas *P. aeruginosa* isolates did not show this phenomenon. OXA-23 genes were determined in 97.7% (42 out of 43) of *A. baumannii* isolates indistinguishable from the sequence of the classical ARI-1 gene. OXA-24, OXA-58 and overexpression of OXA-51 were not registered in any of the isolates. All *P. aeruginosa* were negative for blaVIM and blaIMP genes.

Conclusion: The leading cause of carbapenem resistance in *A. baumannii* isolates from our hospital is the carbapenemase production due to the expression of OXA-23 gene, whereas in *P. aeruginosa* - the loss of transmembrane OprD protein and the efflux pumps' hyperexpression are suspected to be the main mechanisms.

BACKGROUND

Pseudomonas aeruginosa and *Acinetobacter baumannii* are clinically significant opportunistic human pathogens, which are associated with nosocomial infections worldwide. Infections caused by these

bacteria are typically serious and difficult for treatment due to their intrinsic and acquired resistance to a great variety of antimicrobials.¹ In the last 20 years a huge number of clinical isolates have been reported to be resistant to the most effective bacte-

ricidal antimicrobial agents such as carbapenems. The two most frequently applied representatives of this group - imipenem and meropenem, are the last line drugs for empirical therapy of severe multi-drug resistant infections. Therefore the resistance against them imposes insuperable therapeutic restrictions.

The main mechanisms of carbapenem resistance in *P. aeruginosa* are the loss of the outer-membrane OprD protein and the hyperexpression of the efflux pumps belonging to the Resisto-Nodular Division (RND) such as MexAB-OprM and MexXY-OprM sometimes combined with overexpression of intrinsic AmpC.^{2,3} The production of carbapenem-hydrolyzing enzymes as metallo-enzymes especially blaVIM and blaIMP plays a secondary role.⁴ On the other hand carbapenemase production in *A. baumannii* facing class oxacillinases (group 2df beta-lactamases) including OXA-23, OXA-24, OXA-58 and the hyperexpression of OXA-51 is the leading cause of this type of resistance in many cases combined with other mechanisms as impaired influx and/or increased efflux.⁵

AIM

The aim of this study was to examine the structure of carbapenem resistance among *P. aeruginosa* and *A. baumannii* clinical isolates from our hospital. This would help us to elucidate the therapeutic options and antibiotic stewardship strategies hence such kind of investigations had never been performed till this moment in the hospital.

MATERIALS AND METHODS

MATERIALS

Forty three *P. aeruginosa* and 43 *A. baumannii* clinical isolates collected between 2010 and 2014 from different clinical units of St George University Hospital in Plovdiv were examined in the study (**Table 1**). The greatest number of isolates was obtained from the Intensive Care Unit (ICU) following by the Department of Burn Care, Cardiac surgery and Pediatrics. The including criteria were: confirmed identification, resistance or intermediate susceptibility to imipenem and/or meropenem, different resistance type for isolates from the same unit, precise clinical and epidemiological data. Isolates which didn't match the above criteria were excluded from the study.

METHODS

Both phenotypic and molecular methods were used for identification, screening the presence of

carbapenemases and typing.

The identification of all bacterial isolates was confirmed using VITEK 2 automatic system and API20-NE strip tests (BioMerieux, France) with automatic interpretation performed by BIOMIC V3 (Giles scientific, USA). It was followed by PCR detection of the intrinsic OXA-51 (biochemical methods are not reliable in distinguishing *A. baumannii* from some genomic species). The antibiotic susceptibility to imipenem (10 µg) and meropenem (10 µg), (Liofilchem) was determined by Bauer-Kurby disk-diffusion test, E-tests with imipenem and meropenem and automatically obtained gradient MIC by VITEK 2 system. The results were assessed on the basis of CLSI criteria which were still in use in our country until 2014.

PHENOTYPIC TESTS FOR CARBAPENEMASE DETECTION

A modified Hodge test (MHT) was used for screening of common carbapenemase production as previously described^{6,7} followed by double-disk combined test

Table 1. *A. baumannii* and *P. aeruginosa* isolates collected from different units in St George University Hospital, Plovdiv

Department	Number of <i>A. baumannii</i> isolates	Number of <i>P. aeruginosa</i> isolates
ICU	15 (35%)	10 (23%)
Cardiac Surgery	6	4
Burn-care	6	6
Neurosurgery	3	1
Vascular Surgery	3	-
First Surgical clinic	3	-
Traumatology	2	-
Invasive Cardiology	1	-
Urology	1	5
Obstetrics and Gynaecology	1	3
Pediatrics	1	7
Thoracic and Abdominal Surgery	1	4
Ear-Nose-Throat	-	2
Infectious Diseases	-	1
TOTAL	43	43

with disks imipenem (10 µg) and imipenem/EDTA (10/760 µg), (Liofilchem),⁸ and combined E-test with imipenem (4-256 µg/mL) and imipenem (1-64 µg/mL) + constant level of EDTA (Liofilchem) for detection of metallo-β-lactamases.^{9,10}

MOLECULAR TESTS

Omega bio-tek E.Z.N.A Bacterial DNA kit (VWR) was used for DNA extraction of isolates after 18 hours of cultivation on 5% Columbia agar (Biolife). The average concentration of DNA was 22 µg/ml. The amplification process was performed on Applied Biosystems 7300 Real-Time PCR machine. Primers for PCR screening of carbapenemases are listed in **Table 2**.

CONVENTIONAL PCR SCREENING FOR CARBAPENEMASE-ENCODING GENES

PCR reactions were carried out in 25 µl volumes containing 1x PCR buffer with 1.5 mM MgCl₂; 0.2 mM dNTP; 1.5 U Taq polymerase (VWR); 3 µl DNA; 50 pmol forward and reverse primer per reaction.

PCR conditions included 30 cycles of amplification under the following conditions: denaturing at 95°C (30s); annealing for 60s at primer specific

temperatures (**Table 2**), extension at 72°C (1 min/kb product) and final extension at 72°C (10 min). PCR products were resolved on 1% agarose gel stained with Gel-Red (Biotium) and photographed under UV light illumination. 100-bp DNA ladder (Biolabs, New England) was used to assess the product size.

RAPD-PCR ANALYSIS

We applied our own experimental RAPD protocol using ERIC 1R-(5'-AAGCCTCCTGGGGATTCA-3') and ERIC 2-(5'-AAGTAAGTGACTGGGGT-GAGCG-3') primers^{11,12} which turned out to be applicable to both microorganisms: *A. baumannii* and *P. aeruginosa*. Fragments were separated by QiAxcel capillary electrophoresis (Qiagen, Germany) and analyzed by CLIQS 1D PRO software (TotalLab, England).

PCR reactions were carried out in 25 µl volumes containing 1x PCR extra buffer with 1.5 mM MgCl₂; 0.2 mM dNTP; 1.5U µl Taq; 4 µl DNA; 100 pmol of ERIC 1R primer and ERIC 2 primer per reaction.

PCR conditions included initial denaturing at 95°C (5 min) followed by 45 cycles of amplifica-

Table 2. Primers used in this study with their nucleotide sequence, annealing temperature and product size

Primer	Nucleotide sequence (5'-3')	Annealing t°C	Product size (bp)	Source of reference
Bla VIM-F	TTTGGTCGCATATCGCAACG	66	500	Hujer KM et al. ¹³
BlaVIM-R	CCATTCAGCCAGATCGGCAT			
Bla IMP-F	GTTTATGTTTCATACWTCG	45	432	Hujer KM et al. ¹³
Bla IMP-R	GGTTTAAAYAAAACAACCAC			
OXA-51-like R	TGGATTGCACTTCATCTTGG	52 (this study)	1000	Turton JF et al. ¹⁴
ISAbal-R	GCTCACCGATAAACTCTCT			Merkier et al. ¹⁵
OXA-23-like F	GATCGGATTGGAGAACCAGA	51	501	Turton JF et al. ¹⁴
OXA-23-like R	ATTCTGACCGCATTTCAT			
OXA-24-like F	GTACTAATCAAAGTTGTGAA	53	246	Merkier et al. ¹⁵
OXA-24-like R	GGAAGTCTGACAATGC			
OXA-51-like F	TAATGCTTTGATCGGCCTTG	47	353	Turton JF et al. ¹⁴
OXA-51-like R	TGGATTGCACTTCATCTTGG			
OXA-58 A	CGATCAGAATGTTCAAGCGC	53	599	Poirel et al. ¹⁶
OXA-58 B	ACGATTCTCCCCTCTGCGC			

tion under the following conditions: denaturing at 95°C (35s); annealing at 42°C (35s), extension at 72°C (35s) and final extension at 72°C (10 min).

SEQUENCING ANALYSIS.

The bidirectional sequencing of OXA-23 gene products (501bp) was performed with the same amplification primers (**Table 2**) on Genome Lab GeXP system (Beckman Coulter, USA) and Sequencher 5.4.5 (Gene Codes, USA) software was used to analyze the results.

PCR reactions were carried out in 12.5 µl volumes containing 2 µl DTCS mix; 1.5 µl 5xSeq buffer (0.4 M Tris-HCl, 10 mM MgCl₂, pH=9); 0.035 mM Bovine Thrombin (Sigma-Aldrich, USA); 1M Betaine; 2 µl DNA; 3.5 µl distilled water and 10 pmol of primers.

PCR conditions included initial DNA denaturing at 96°C (30s) followed by 35 cycles of amplification (denaturing at 96°C for 20s; annealing at 50°C for 20s and extension at 60°C for 4 min).

STATISTICAL ANALYSIS

To identify the dynamics of carbapenem resistance in *A. baumannii* and *P. aeruginosa* isolates Time series analysis and forecasting were applied. Data manipulation and graphical representation as well as

descriptive and statistical analyses were undertaken using statistical software package SPSS v.19 (IBM Corp. Chicago, IL, USA).

RESULTS

All *A. baumannii* and *P. aeruginosa* isolates exhibited resistance to more than 3 groups of antimicrobials which determined them as multi-drug resistant. Their antibiotic susceptibility pattern is shown in **Fig. 1**.

The susceptibility testing to carbapenems revealed 8 different profiles. Thirty six (84%) of *A. baumannii* isolates and 21 (49%) of *P. aeruginosa* were resistant to both imipenem and meropenem (**Table 3**). Forty two out of 43 *A. baumannii* (97.7%) isolates demonstrated positive result for carbapenemase production using MHT (**Fig. 3**), whereas all 43 *P. aeruginosa* isolates were negative for such phenomenon. Neither *A. baumannii* nor *P. aeruginosa* showed positive metallo-β-lactamase profile using double-disk combined method and combined E-test.

There is no trend of the dynamics and it is not possible to identify a model for forecasting a future change of the resistance to both antibiotics imipenem and meropenem for *Pseudomonas* and *Acinetobacter* in our ICU during the period 2010-

Figure 1. Antimicrobial resistance profile of all *A. baumannii* and *P. aeruginosa* isolates included in the study.

* Trimethoprim-sulfamethoxazole in *Pseudomonas* was tested for diagnostic purposes

Table 3. Antibiotic susceptibility profile to imipenem and meropenem of all *A. baumannii* and *P. aeruginosa* isolates included in this study

№	Carbapenem susceptibility	Number of <i>A. baumannii</i> isolates	Number of <i>P. aeruginosa</i> isolates
1.	Im-R ; Mer-R	36 (84%)	21 (49%)
2.	Im-R ; Mer-I	1	9
3.	Im-R; Mer-S	-	6
4.	Im-I; Mer-R	2	2
5.	Im-S ; Mer-R	1	-
6.	Im-I; Mer-I	2	4
7.	Im-I; Mer-S	1	1
8.	Im-S; Mer-I	-	-
Total number of isolates		43	43

Im: imipenem; Mer: meropenem; S: sensitive; R: resistant; I: with intermediate susceptibility

Figure 2. Imipenem and meropenem dynamic resistance profile of all isolated *A. baumannii* and *P. aeruginosa* from ICU at St George University Hospital, Plovdiv between 2010 and 2014.

2014. Nevertheless the levels of resistance still remain high – more than 76% for *Acinetobacter* and 43% for *Pseudomonas* for both carbapenems. The dynamics for the pointed period is shown in **Fig. 2**.

All *A. baumannii* isolates were subjected to PCR screening for the presence of OXA-23, OXA- 24,

OXA-58 and hyperexpression of OXA-51 due to upstream situated ISAbal. Forty two out of 43 (97.7%) were positive for OXA-23 (**Fig. 4**). 100% correlation between the MHT results and the genetic screening was established. All isolates were negative for the presence of OXA-24, OXA-58 and hyper-expression of the intrinsic OXA-51.

Figure 3. Presence of clover leaf type of inhibition (MHT) near the tested organism (marked with an arrow) was interpreted as positive for carbapenemase production. *E.coli* ATCC R 25922 was used as an indicator strain.

All *P. aeruginosa* isolates were screened for the presence of VIM and IMP metallo-enzymes' encoding genes and all of them were negative.

RAPD analysis of 21 random OXA-23 positive *A. baumannii* isolates from ICU, Cardiac Surgery and Burn-care Clinic revealed 6 RAPD types. The first 11 types exhibited more than 90% similarity which suggests their common clonal lineage and the rest 10 profiles showed approximately 70% similarity which made us assumed they belong to a second clone (lines 1-11), (**Fig. 5**). RAPD analysis of 20 random isolates *P. aeruginosa* from same departments revealed 3 RAPD types. All isolates *Pseudomonas* obtained from the ICU belonged to 2 similar RAPD profiles.

Sequencing analysis of 23 randomly chosen OXA-23 positive *A. baumannii* revealed 100% homology with the classical OXA-23 -ARI-1 gene described by Donald HM et al.¹⁷

DISCUSSION

This is the first detailed molecular and phylogenetic study on the carbapenemases production in clinical isolates *Pseudomonas* and *Acinetobacter* from the

largest Bulgarian hospital - St George University hospital in Plovdiv. The data obtained gave us grounds to assume that the leading cause for carbapenem resistance in our *A. baumannii* isolates was the presence of OXA-23 gene and respectively the production of carbapenemases. This was an expected result in view of that other Bulgarian authors published similar data concerning different University hospitals in the country.¹⁸⁻²⁰ Stoeva et al. organized the first detailed study in our country on multi-drug resistant *A. baumannii* proving the wide spread of OXA-23 among them. OXA-58 and OXA-24 are not that significant for Bulgaria as well as the hyperexpression of the intrinsic OXA-51 due to the upstream situated ISAbal. The latter is still not detected in Bulgarian *A. baumannii* isolates including ours.¹⁸

The phylogenetic analysis of 21 OXA-23 positive *A. baumannii* in our study revealed the persistence of two main clones in the hospital. This correlates with the results from other Bulgarian hospitals.^{18,20-22} The RAPD similarity profiles and same clone affiliation of ICU isolates suggests the co-existence of two strains. The slight genetic variability observed among clone isolates could be explained by the high selective antibiotic pressure in the ICU setting and the long period during which this clone was present. The persistence of one or two clones is not unusual for Bulgarian ICUs.^{18,21} It may predispose to nosocomial outbreaks.

No carbapenemase producing *P. aeruginosa* were detected in this study. Metallo-enzyme producing *Pseudomonas* are not typical for our country. The first case of VIM-positive *P. aeruginosa* was published in 2008 by Keuleyan et al.²³ Since then till 2015 no other cases have been reported even though VIM and/or IMP encoding strains have been determined in all our neighbouring countries – Greece, Serbia, Romania and Turkey except Macedonia.²⁴⁻²⁹

In 2013 Vacheva et al. studied 29 Bulgarian clinical isolates of *Pseudomonas aeruginosa* in

Figure 4. Detection of OXA 23 genes in *A. baumannii* clinical isolates: line 6 - positive control, line 7- negative control, line 8 - DNA ladder, lines 1,2,3,4,5,9-14 - isolates carrying OXA 23 gene.

Legend: Capillary electrophoresis RAPD-bands: Lines 1-5: 1st profile; lines 6-11: 2nd profile; lines 12-17: 3rd profile; line 18: 4th profile; line 19: 5th profile; lines 20-21: 6th profile.

Figure 5. RAPD analysis of 21 isolates OXA-23 positive *A. baumannii* with dendrogram showing the spreading of 2 main clones in St George University Hospital, Plovdiv: lanes 1-11 determining the first one with more than 90% similarity; lanes 12-21 determine the second one with approximately 70% similarity.

collaboration with the Medical University of Palma de Maiorca in Spain.³⁰ In this research they reported about the over-expression of MexXY-OprM and the loss of OprD receptor as a leading cause for carbapenem resistance. Furthermore our own investigations performed for first time in our coun-

try proved that the leading factor for carbapenem resistance in our clinical *P. aeruginosa* isolates is the increased expression or hyperexpression of the MexXY-OprM efflux pump as well. In some cases it was combined with increased expression or hyperexpression of MexAB-OprM with or

without decreased or lost production of the OprD transmembrane receptor (unpublished data).

CONCLUSIONS

Carbapenem resistance is threatening tendency for severe therapeutic restrictions especially in the ICUs concerning the specificity of services in these clinical units. The main cause determining such type of resistance for our hospital is the persistence of clonal-related OXA-23 producing *A. baumannii* strains, whereas in *Pseudomonas* the increased expression or hyper-expression of key efflux pumps sometimes combined with decreased or lost production of OprD remains as a leading factor.

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Производство карбапенемазы в клинических изолятах *Acinetobacter baumannii* и *Pseudomonas aeruginosa* в болгарской университетской больнице

Атанаска П. Петрова^{1,2,3}, Ирина Д. Станимирова^{1,2}, Иван Н. Иванов⁴,
Михаил М. Петров¹, Цонка М. Митева- Катранджиева⁵, Васил И. Гривнев¹,
Величка С. Карджева⁶, Тодор В. Кантарджиев⁴, Мариана А. Мурджева^{1,2,3}

¹ Кафедра микробиологии и иммунологии, Факультет фармации, Медицинский университет- Пловдив, Пловдив, Болгария

² Лаборатория микробиологии, Университетская больница „Св. Георги“, Пловдив, Болгария

³ Технологический центр неотложной медицины, Пловдив, Болгария

⁴ Национальная референтная лаборатория контроля и мониторинга устойчивости к антибиотикам, Национальный центр инфекционных и паразитарных заболеваний, София, Болгария

⁵ Кафедра социальной медицины и общественного здравоохранения, Факультет общественного здравоохранения, Медицинский университет- Пловдив, Пловдив, Болгария

⁶ Отделение молекулярной биологии, „Аквахим“ АО, София, Болгария

Адрес для корреспонденции:

Атанаска П. Петрова, Кафедра микробиологии и иммунологии, Факультет фармации, Медицинский университет- Пловдив; Лаборатория микробиологии, Университетская больница „Св. Георги“, Пловдив, Технологический центр неотложной медицины, Пловдив, бул. „Васил Априлов“ № 15А, 4002, Пловдив, Болгария
E mail: atanasia_petroff@abv.bg
Тел: +359 32 602273

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Введение: Производство bla OXA-23, OXA-24, OXA-58 и гиперэкспрессия OXA-51 по причине ISAb₁ инсерционной последовательности является основной причиной развития устойчивости к карбапенемам при *Acinetobacter baumannii*. Потеря OprD трансмембранного белка и избыточная экспрессия некоторых эффлюкс-насосов считаются основными факторами развития устойчивости к карбапенемам при *Pseudomonas aeruginosa*, в отличие от производства металлоферментов, которому отводится вторичная роль.

Цель: Исследовать устойчивость к карбапенемам вследствие производства карбапенемазы среди клинически значимых грамотрицательных неферментативных бактерий в Университетской больнице „Св. Георги“ - Пловдив: *A. baumannii* и *P. aeruginosa*.

Материалы и методы: 43 *A. baumannii* и 43 *P. aeruginosa* изолята, устойчивых или со средней устойчивостью к имипенему и меропенему были включены в исследование. Они были взяты у пациентов из 14 различных по профилю больничных отделений, поступивших на лечение в период 2010 – 2014. Для исследования идентификации и антимикробной восприимчивости были использованы методы как фенотипического, так и генетического исследования.

Результаты: Все *A. baumannii* показали производство карбапенемазы, установленной при помощи модифицированного теста Ходжа, в отличие от изолятов *P. aeruginosa*, которые не показали данного феномена. OXA-23 гены были идентифицированы в 97.7% (42 из 43) изолятов *A. baumannii*, неразличимых от последовательности классического ARI-1 гена. OXA-24, OXA-58 и избыточная экспрессия OXA-51 не были установлены ни в одном из изолятов. Все *P. aeruginosa* оказались отрицательными на наличие blaVIM и blaIMP генов.

Заключение: Основной причиной устойчивости карбапенемов в составе изолятов *A. baumannii* в нашей больнице является производство карбапенемазы в результате экспрессии OXA-23 генов, в отличие от *P. aeruginosa*, где предполагается, что основными механизмами являются потеря трансмембранного OprD белка и гиперэкспрессия эффлюкс-насосов.