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Emergence of *Pseudomonas aeruginosa* with class 1 integron carrying bla_{VIM-2} and bla_{VIM-4} in the University Clinical Hospital of Białystok (northeastern Poland)*

Zagrożenia szczepami *Pseudomonas aeruginosa* zawierającymi geny bla_{VIM-2} i bla_{VIM-4} w integronie klasy 1 w Uniwersyteckim Szpitalu Klinicznym w Białymstoku (północno-wschodnia Polska)

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- C Statistical Analysis
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Summary

The effectiveness of carbapenems, considered as last-resort antimicrobials in severe infections, becomes compromised by bacterial resistance. The production of metallo- β -lactamases (MBLs) is the most significant threat to carbapenems activity among *Pseudomonas aeruginosa*. The aim of this study was to assess the presence and type of MBLs genes in carbapenem-resistant *P. aeruginosa* clinical strains, to identify the location of MBLs genes and to determine genetic relatedness between MBL-producers using pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST).

The first identified MBL-positive (with bla_{VIM} genes) *P. aeruginosa* strains were isolated from patients hospitalized in the University Clinical Hospital of Białystok in the period from September 2012 to December 2013. Variants of MBLs genes and variable integron regions were characterized by PCR and sequencing. PFGE was performed after digesting of bacterial genomes by *Xba*I enzyme. By MLST seven housekeeping genes were analyzed for the determination of sequence type (ST). Three strains carried the bla_{VIM-2} gene and one harbored the bla_{VIM-4} gene. The bla_{VIM} genes resided within class 1 integrons. PCR mapping of integrons revealed the presence of four different cassette arrays. Genetic relatedness analysis by PFGE classified VIM-positive strains into four unrelated pulsotypes (A–D). MLST demonstrated the presence of four (ST 111, ST27, and ST17) different sequence type including one previously undescribed new type of ST 2342. Antimicrobial susceptibility testing showed that VIM-positive strains were resistant to carba-

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Keywords	penems, cephalosporins, aminoglycosides, and quinolones, intermediate to aztreonam, and susceptible only to colistin. Integrons mapping, PFGE, and MLST results may point to different origin of these strains and independent introduction into hospitalized patients. <i>Pseudomonas aeruginosa</i> • pulsed-field gel electrophoresis (PFGE) • Verona integron-encoded metallo-β-lactamase (VIM) • integron cassette array • multilocus sequence typing (MLST)
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INTRODUCTION

Pseudomonas aeruginosa, one of the most important microorganism causing nosocomial infections, is well known for its ability to obtain antimicrobial resistance through combination of intrinsic non-susceptibility, selection of chromosomal mutations, and transfer of mobile genetic elements harboring resistance genes [8,12]. Moreover, *P. aeruginosa* is able to produce exopolysaccharides (Pel, Psl and alginate) building biofilm matrix. The tissue colonization is promoted by diverse proteins: aminopeptidase, chitinase, PasP, and protease IV [11].

Carbapenems, antibiotics with excellent stability in the presence of most β -lactamases, are used in clinical setting for the treatment of severe pseudomonal infections [13]. Wide usage of these lifesaving antibiotics contributes to selective pressure on hospital microorganisms. Consequently, an increase in prevalence of *P. aeruginosa* resistant to carbapenems is reported all over the world [1]. The resistance to carbapenems among *P. aeruginosa* strains could be combined with many factors, such as plasmid or integron-encoded carbapenemases, increased expression of efflux systems, increased chromosomal cephalosporinase activity, and reduced porin expression [10]. The most widespread acquired mechanism of carbapenem resistance is the production of MBLs, mainly VIM (Verona Integron-encoded Metallo- β -lactamase) and IMP (Imipenemase) [22]. NDM (New Delhi metallo- β -lactamase), frequently reported among *Enterobacteriaceae*, has been also detected in *P. aeruginosa* strains, which could indicate the transmission of this resistance mechanism [18]. Due to the emergence of carbapenem-resistant *P. aeruginosa* in our hospital, the objective of this study was to determine the prevalence of VIM, IMP, and NDM carbapenemases, to identify the localization of resistance genes within integrons, and

to assess clonal relatedness of MBL-positive isolates by pulsed-field gel electrophoresis (PFGE), and multilocus sequence typing (MLST).

MATERIALS AND METHODS

In total, 261 non-duplicated *P. aeruginosa* strains were isolated from patients hospitalized in University Clinical Hospital of Białystok, Poland during 16 months (September 2012 - December 2013). Among them, 45 strains were resistant to carbapenems, representing 17.2% of all isolated strains. All strains resistant to carbapenems were routinely examined for production of MBL carbapenemases using phenotypic tests. Most of carbapenem-resistant *P. aeruginosa* strains were isolated from upper respiratory tract specimens (bronchoalveolar lavage fluid, bronchial secretion, and bronchial aspirate) from patients hospitalized mainly on Intensive Care Unit (62.2%), Surgery Ward (17.8%), and Hospital Emergency Department (8.9%).

Identification and susceptibility testing were conducted using VITEK 2 System (bioMérieux, Marcy l'Etoile, France). Minimum inhibitory concentrations (MICs) of doripenem, imipenem, meropenem, and aztreonam were determined using Etests (bioMérieux) on Mueller-Hinton agar (Oxoid, Basingstoke, United Kingdom) with *P. aeruginosa* ATCC 27853 as a control strain. Results of the susceptibility testing were interpreted according to the EUCAST criteria [15].

Total bacterial DNA was isolated with the Genomic Mini kit (A&A Biotechnology, Gdansk, Poland). To identify genes encoding VIM, IMP, and NDM β -lactamases, polymerase chain reaction (PCR) was performed with primers and conditions described previously [3]. *Pseudomonas aeruginosa* NCTC 13437 (VIM-10 positive), *Escherichia coli* NCTC 13476 (IMP-type positive), and *Klebsiella*

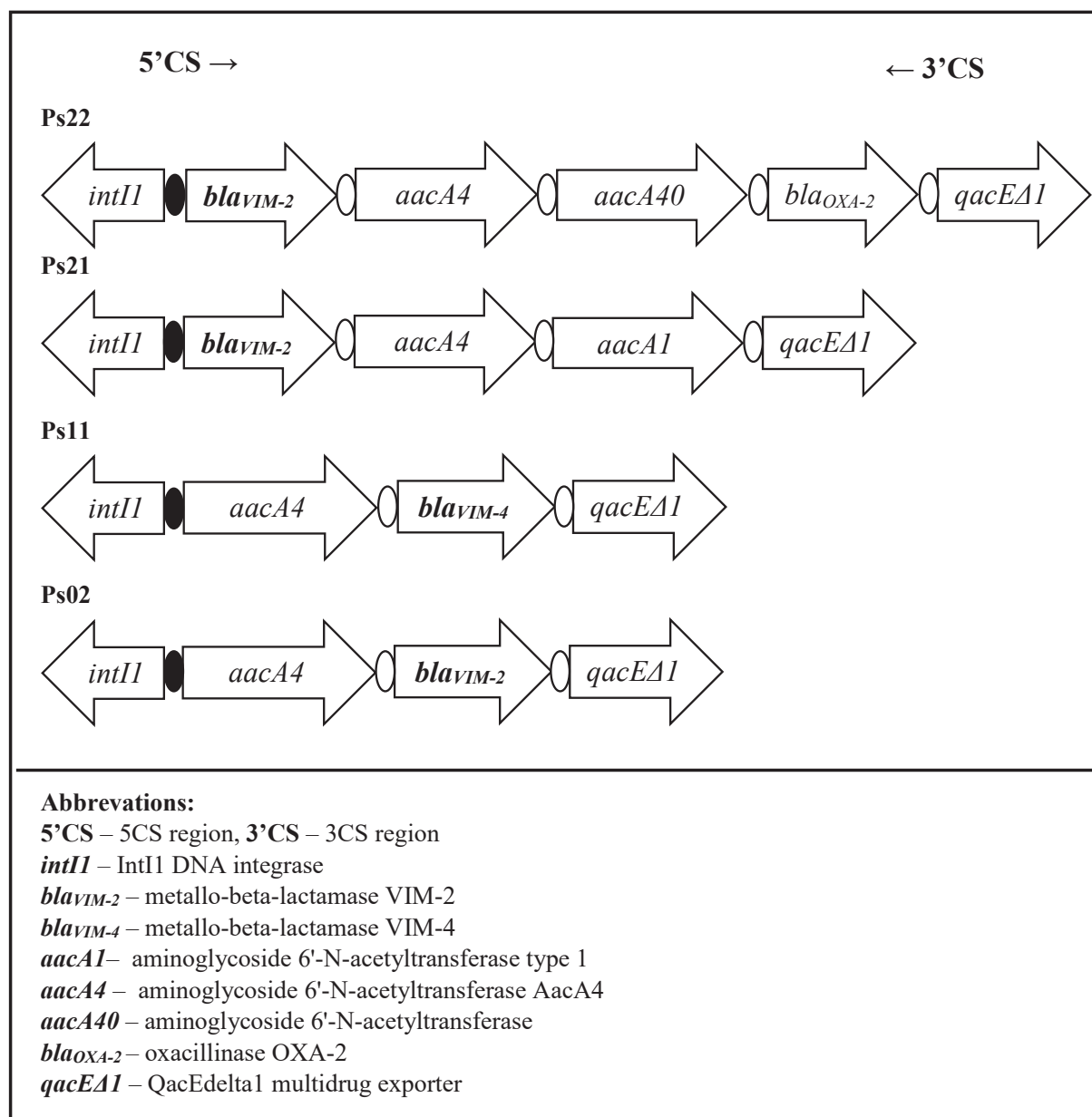


Fig. 1. Integron gene cassette array among VIM - positive *P. aeruginosa* isolates

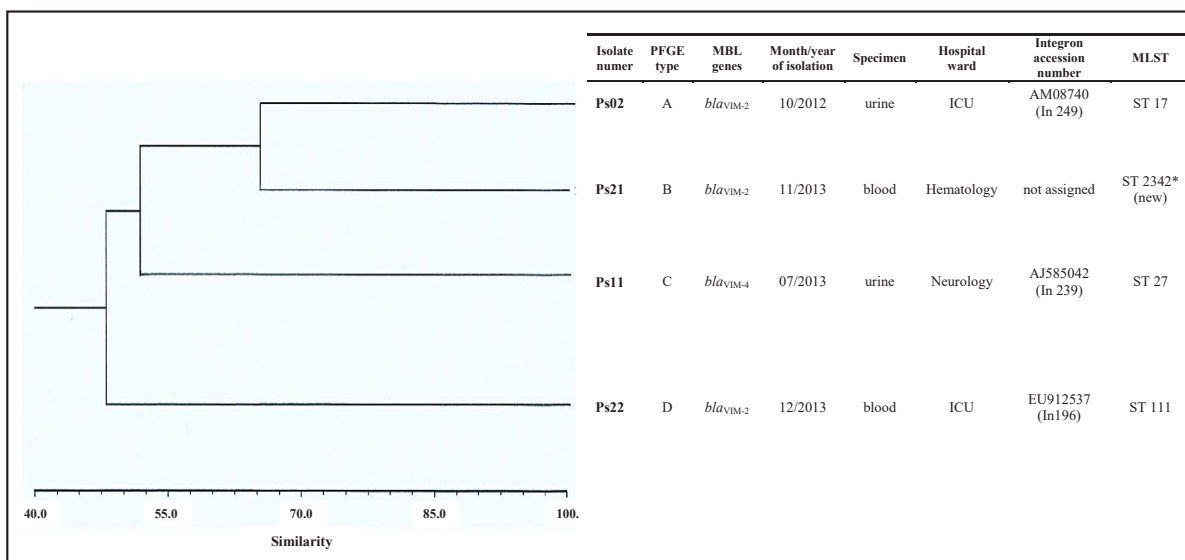
pneumoniae ATCC BAA-2146 (NDM-1 positive) were used as control strains.

PCR and sequencing revealed the presence of *bla_{VIM}* gene among four strains. PCR mapping of integrons in these VIM-positive strains was performed according to the protocol by Lévesque et al. [6] and genetic relatedness was examined by PFGE with the CHEF Mapper XA Chiller System (Bio-Rad, Hercules, CA, USA).

Bacterial DNA was embedded in agarose plugs and digested with 20U of *Xba*I endonuclease (EurX, Gdansk, Poland) per each plug for 6h at 37°C. PFGE was performed under following conditions: switch time, 4s-35s; gradient, 6.0V/cm; temperature, 14°C; run time, 20h.

Banding patterns were interpreted according to the criteria published by Tenover et al. [17]. A dendrogram was prepared using NTSYS-pc 2.02g software (Exeter Software, East Setauket, NY, USA) with Dice's similarity coefficient and the unweighted pair group method with arithmetic mean (UPGMA) set at 1.0% position tolerance and 1.0% optimization.

MLST based on sequences of seven *P. aeruginosa* housekeeping genes (*acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *ppsA*, and *trpE*) was performed with primers and conditions described by Curran et al. [2] and published at the PubMLST website [15]. Nucleotide BLAST was used to determine the matching of corresponding data with the appropriate housekeeping gene of *P. aeruginosa*. Both for-



Abbreviations: ICU, Intensive Care Unit; ST, sequence type, * sender *Sacha P.*, Department of Microbiological Diagnostics and Infectious Immunology, Medical University of Bialystok, Poland.

Fig. 2. PFGE dendrogram and MLST results of VIM-producing *P. aeruginosa* isolates

Table 1. MIC and breakpoint values of VIM-positive *P. aeruginosa* isolates

Strain number	MIC (mg/l) – Etest method					Breakpoint (mg/l) – VITEK 2 System						
	DOR	IMM	MEM	ATZ	CTZ	CEP	GEN	NET	TOB	CIP	LEV	COL
Ps02	>32 R	>32 R	>32 R	2I	>64 R	>64 R	>16 R	>32 R	8 R	4 R	8 R	≤0.5 S
Ps21	>32 R	>32 R	>32 R	3I	>64 R	16 R	16 R	16 R	16 R	4 R	8 R	2 S
Ps11	4 R	>32 R	>32 R	2I	>64 R	>64 R	>16 R	>32 R	>16 R	4 R	8 R	2 S
Ps22	8 R	32 R	16 R	6I	64 R	16 R	16 R	16 R	16 R	2 R	4 R	2 S

DOR - doripenem; IMM - imipenem; MEM - meropenem; ATZ - aztreonam; CTZ - ceftazidime; CEP - cefepime; GEN - gentamicin; NET - netilmicin; TOB - tobramycin; CIP - ciprofloxacin; LEV - levofloxacin; COL - colistin; S - susceptible; - intermediate; R - resistant.

ward and reverse sequences were queried against the PubMLST sequence definition database.

RESULTS

Among genes encoding MBLs, only *bla_{VIM}* gene was detected in four isolates, which represents 8.9% of 45 isolates resistant to carbapenems. None of the stra-

ins resistant to carbapenems carried *bla_{IMP}* and *bla_{NDM}* genes. Sequencing revealed the presence of *bla_{VIM-2}* in three isolates and *bla_{VIM-4}* in one isolate. PCR mapping of integrons combined with sequencing demonstrated that all of *bla_{VIM}* genes were located within the class 1 integrons. Integron’s DNA sequences were analyzed against the Integrall database [7]. By the PCR mapping of integrons we found the presence of four different cas-

sette arrays (Figure 1). Entire sequenced fragment from isolate Ps11 (ST 27) was identical to the integron In239 harboring *bla*_{VIM-4} gene, previously described in Poland [14]. Also, gene cassette array from isolate Ps02 (ST 17) showed structure identical to the In249 reported earlier in Poland [4]. Interestingly, entire sequenced fragment from isolate Ps22 was identical to the integron In196 described in Mexico [5]. Isolate Ps21 (new ST 2342) harboring *bla*_{VIM-2} gene showed unique cassette composition which have not been described previously in *P. aeruginosa*. The PFGE genetic relatedness investigation revealed the presence of four separate pulsotypes (A–D) among VIM-positive isolates (Figure 2). The MLST results correlated with data obtained in PFGE analysis. All *P. aeruginosa* strains subjected to MLST displayed different sequence types (Figure 2). Antimicrobial-susceptibility testing (Table 1) showed that all carbapenem-resistant isolates were resistant also to cephalosporins, aminoglycosides, and quinolones. All VIM-positive isolates showed intermediate resistance to aztreonam, and were susceptible to colistin.

DISCUSSION

In Poland, the first *P. aeruginosa* strain with *bla*_{VIM-2} gene was described in 2003 [21]. In larger study, involving seventeen hospitals in Poland, the predominant MBL was VIM-2 [4]. The first description of IMP-producing *P. aeruginosa* in Poland, and in our hospital, was in 2007 [16]. Here we report the emergence of *bla*_{VIM-2} and *bla*_{VIM-4} genes among *P. aeruginosa* isolates resistant to carbapenems in northeastern Poland.

PFGE revealed high clonal diversity among VIM-positive strains, which suggested no clonal dissemination at the single-center level. MLST analysis also showed high genetic diversity, as there were no strains with the same

allelic profile. Moreover, the combinations of alleles in Ps21 VIM-positive strain represented new sequence type ST2342 (Poland; date entered to MLST Database 2016-10-05).

In the present study, none of tested isolates harbored *bla*_{IMP} gene, which suggest eradication of this clone from hospital environment. Amplification and sequencing with specific primers demonstrated that all of *bla*_{VIM} genes were located within the class 1 integrons. PFGE types correlated well with the polymorphism of integrons, which imply that each VIM-positive strain came from different origin. Similar results were obtained in Japan, where MBL-producing *P. aeruginosa* showed no genetic relatedness [20]. This situation is quite rare, because in most studies researchers report intra- or inter-hospital dissemination of MBL-producing isolates [21]. MLST analysis revealed four sequence types, which may point to local presence of unrelated VIM-positive strains. Antimicrobial-susceptibility testing results obtained in our research are consistent with previously published studies. *P. aeruginosa* isolates producing MBLs are reported to be resistant to β -lactams, quinolones, and aminoglycosides, which indicates the copresence of multiple resistance mechanisms [22]. In this case, colistin remains therapeutic option for the treatment of pseudomonal infections caused by carbapenem-resistant isolates [9]. Due to the serious threat posed by MBL-positive *P. aeruginosa*, there is an urgent need for effective methods to monitor and prevent spreading of multi-drug-resistant microorganisms. Furthermore, to avoid the development of resistance among bacterial strains, hospital formulary should be frequently revised to select optimal antibiotic regimens.

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