VILNIUS UNIVERSITY

JUSTAS POVILONIS

TRANSFERABLE ANTIBIOTIC RESISTANCE DETERMINANTS IN GRAM NEGATIVE BACTERIAL PATHOGENS

Summary of doctoral dissertation

Physical sciences, biochemistry (04 P)

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This study was carried out at the Department of Biochemistry and Molecular Biology, Faculty of Natural Sciences, Vilnius University during 2008 – 2012.

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INTRODUCTION

Antibiotics were one of the great discoveries of the 20th century, however the high levels of antibiotics in human medicine and use of antimicrobials in the livestock for prevention and treatment of animal diseases have led to the emergence and spread of resistant bacterial pathogens, which in recent years have become a problem worldwide (Davies, 2007). One of the major factors contributing to the development of resistance to antibiotics is the remarkable ability of bacteria to share genetic resources via Horizontal Gene Transfer (HGT) (Stokes and Gillings, 2011). Bacteria have evolved many different methods of gene exchange – plasmids, transposons, integrons, gene cassettes, integrative and conjugative elements. The transfer of genes encoding antibiotic resistance within and/or between human and animal commensal and pathogenic bacteria could take place and pose the risk for the occurrence of resistant-pathogen-associated infections (Leverstein-Van Hall *et al*., 2002; van Essen-Zandbergen *et al*., 2007). Multidrug-resistant (MDR) strains of zoonotic bacteria, mostly members of the *Enterobacteriaceae* family, represent a common agent of foodborne human illnesses (Swartz, 2002), cause exceptional risk for public health.

Nosocomial infections (or hospital-acquired infections) are notorious for their potential risk to the patients of immunocompromised status. Of particular concern is *Acinetobacter baumannii* as an emerging nosocomial pathogen worldwide (Gordon and Wareham, 2010). The proportion of health care-associated *A. baumannii,* resistant to a broad spectrum antibiotics such as carbapenems, has dramatically risen during the last decade, particularly in the hospital intensive-care-units (ICUs), becoming the most problematic pathogen and leaving almost no options for infection treatment (Peleg *et al*., 2008).

The most common agents of the emergence and dissemination of MDR determinants among bacteria are integrons, which represent the efficient gene capture and expression structures (Stokes and Hall, 1989; Rowe-Magnus and Mazel, 2001). The insertion of resistance genes as gene cassettes into integrons leads to various combinations of MDR pattern (Collis and Hall, 1995). The presence of integrons on the mobile elements such as plasmids and transposons greatly facilitates the spread of antibiotic resistance among bacteria and contributes to the evolution of these resistance vehicles through recombination process (White *et al.,* 2001; Mazel, 2006). Integrons of

classes 1 and 2 are the most frequent and widely distributed among *Enterobacteriaceae* and are thought to be mainly responsible for the spread of MDR phenotypes (Davies, 2007). However, many questions still arise regarding the extent of integrons in the different environments and their transmission routes between distinct ecological niches.

Pathogenic bacteria may acquire and express new mechanisms of resistance due their genomic plasticity. Among them, *A. baumannii* shows particular capabilities. It is able to rapidly obtain various genetic vehicles such as plasmids, transposons and integrons, which harbour the resistance genes (Peleg *et al.,* 2008). The resistome harboured on these vehicles represents a powerful arsenal for the dissemination of resistance. Therefore, it is important to elucidate the diversity of transmissible resistance determinants in *A. baumannii.* Very limited information is available about the plasmids circulating in *Acinetobacter* species, partially, due to the complicated isolation of plasmid DNA and significant differences of replicons of *Acinetobacter* plasmids compare to those of other prokaryotic species. Of particular interest, plasmid-mediated acquisition of carbapenem-hydrolyzing class D β-lactamases (CHDLs) may play an important role in the development of carbapenem resistance in *Acinetobacter* (Poirel *et al*., 2010).

Plasmids play an important role in the spread of antimicrobial resistance. Understanding the molecular epidemiology of resistance plasmids recently became a new issue. The certain plasmid families are detected more frequently among the *Enterobacteriaceae* and play a major role in the dissemination of specific resistance genes (Carattoli, 2011). Tracing the prevalence of resistance plasmids may provide useful insights into the nature of horizontal transfer of antimicrobial drug resistance; replicon identification may provide useful clues to the evolution of these resistance plasmids. The particular focus should be considered on the occurrence of novel mechanisms such as site-specific recombination system XerC/XerD which could play a role in the plasticity of *Acinetobacter* plasmids and in the mobilization of resistance genes (D′Andrea *et al.*, 2009; Merino *et al*., 2010).

The great challenge is to understand the processes and genetic elements which contribute to dissemination of antimicrobial resistance determinants among pathogenic species. However, the prevalence and nature of elements responsible for transmission of antibiotic resistance-associated genes among Gram negative bacteria in

clinical and agricultural environments in Lithuania is largely unknown and the molecular basis of resistance has poorly been investigated yet.

Aim of the dissertation work was to investigate the presence, distribution, and the structure of antibiotic resistance-associated mobile genetic elements prevalent among the pathogenic Gram negative bacterial isolates collected in clinical and agricultural environment in Lithuania.

The following tasks had been formulated:

- to investigate the prevalence of class 1 and class 2 integrons in *E. coli, S. enterica, P. aeruginosa,* and *A. baumannii* isolates from human and animal origin;
- to determine the profiles of resistance gene cassettes, localization, and transferability of class 1 and class 2 integrons;
- to investigate the content of plasmid types circulating in antibiotic-resistant *A. baumannii* and the *Enterobacteriaceae*
- to elucidate the genetic structure of a novel *A. baumannii* plasmid pAB120 conferring resistance to carbapenems.

SCIENTIFIC NOVELTY

We present here the first molecular epidemiology study of 859 Gram negative bacterial isolates from Lithuanian hospitals and farms with the emphasis on the characteristics of antibiotic resistance-associated transferable elements – integrons and plasmids. The genetic data obtained in this work could serve as a starting basis for the further monitoring of the emergence and dissemination of antibiotic resistance elements in pathogenic and commensal bacteria in Lithuania and in the neighboring Eastern European countries.

Three novel gene cassette arrays of class 1 intregron were elucidated in this study, which endorses the plasticity of bacterial genome as a response to the selective pressure and therefore responsible for efficient dissemination of resistance determinants and adaptation to the clinical environment. The analysis of a new gene cassette arrangement of class 1 integron from imipenem resistant *P. aeruginosa* isolate revealed a new OXA-type β-lactamase, assigned as an OXA-205.

The genotyping results revealed that majority of *A. baumannii* isolates from all hospitals belonged to European clonal lineages I and II. Isolates of these clonal lineages dominate in the health care settings of most European countries (Higgins *et al.,* 2010). Clonal spread of ECII strains with a newly observed plasmid pAB120 carrying OXA-72 carbapenemase coding genes was largely responsible for a dramatic increase in the rate of carbapenem-resistant *A. baumannii* in the country tertiary care hospitals. To the best of our knowledge, pAB120 is the first described plasmid of carbapenem-resistant *A. baumannii* isolate harbouring two copies of $bla_{\text{OXA-24/40-like}}$ gene encoding β-lactamase $bla_{\text{OXA-72}}$. The abundance of XerC/XerD recognition sites, their positions in pAB120 strongly support the hypothesis that Xer recombination may be responsible for $bla_{\text{OXA-24/40-like}}$ determinants acquisition and suggest its role in the rearrangement of plasmids via common genetic platforms (D′Andrea *et al.*, 2009; Merino *et al*., 2010). The most prevalent plasmid *rep* gene homology groups detected in this study clearly contributes to the indication of replicon types which seem to be successfully maintained and circulated in clinical isolates of *A. baumanii* (Towner *et al*., 2011).

Defensive statements

- The prevalence of integrons and a variety of their antibiotic resistance gene cassettes show the extent of MDR phenotype among Gram negative bacteria of clinical and animal sources in Lithuania and reflects the response to the treatment by aminoglycosides, sulfonamides/trimethoprim, β-lactams, macrolides;

- Common gene cassettes in integrons and their conjugal transfer represent the potential spread of antibiotic resistance determinants from animals to human via zoonotic microorganisms;

- Two major clonal groups of *A. baumannii* belonging to epidemic European clones I and II with significant differences in the genetic profile of plasmids are prevalent in Lithuanian hospitals. IncF plasmids are prevalent among integron-positive *Enterobacteriaceae*;

- Spread of carbapenem-resistant *A. baumannii* in Lithuanian hospitals mainly caused due to the novel *A. baumannii* 11 kb plasmid pAB120 with two copies of genes coding for OXA-72 β-lactamase;

- The genetic structure of pAB120 indicates the potential role of Xer-based recombination for the acquisition of $bla_{\text{OXA-24/40-like}}$ determinants in *A. baumannii* and represents a potential for the rearrangement of plasmids via common genetic platforms.

Dissertation contents:

The dissertation is written in Lithuanian and contains the following parts: Introduction, Literature review, Materials and Methods, Results, Discussion, Conclusions, List of References, List of publications. Total 158 pages.

Materials and Methods

Bacterial isolates

A total of 217 *E. coli*, 87 *S. enterica*, 111 *P. aeruginosa,* and 444 *A. baumannii* isolates resistant to at least two unrelated antimicrobials were selected for integron studies and plasmid content analysis. *E. coli* (n=85) and *P. aeruginosa* (n=111) human clinical isolates were collected randomly from diverse units of nine hospitals located in different regions of Lithuania and were recovered from various clinical specimens. *A. baumannii* isolates were recovered from various clinical specimens collected from patients hospitalized in intensive care units (ICU) and other wards (non-ICU) of Hospital of Lithuanian University of Health Sciences Kaunas Clinics (n=304), Vilnius University Emergency Hospital (n=103), Šiauliai Hospital (n=37). *S. enterica* human isolates (n=37) were obtained from fecal samples of patients with clinical diagnosis of salmonellosis. The *E. coli* isolates (n=132) of animal origin were obtained from 18 farms from different regions of the country from feces or from internal organs. *S. enterica* isolates (n=50) of animal origin were isolated from swine and chicken meat collected in slaughterhouses.

Antibiotic susceptibility testing was performed by disc diffusion method and by Phoenix Automated Microbiology System (BD Phoenix™) according to Clinical and Laboratory Standards Institute (CLSI) guidelines in hospitals where isolates were collected.

Preparation of DNA templates

All bacterial DNA templates used in PCR were prepared by boiling bacterial colonies suspended in 200 µl of sterile water. The boiled samples were centrifuged briefly and the supernatants used as DNA templates. When needed, genomic DNA was extracted using an Arrow™ magnetic workstation (NorDiag, Norway) according to the manufacturer's instructions.

PCR

The PCR mix (total 25 µl) included 2 µl of template DNA, 1×PCR buffer, 0.5 U of DNA *Taq* Polymerase (Thermo Scientific, Lithuania), 200 µM of each of the deoxynucleotides, 0.4 µM of each of the primers and 2.5 mM $MgCl_2$. DNA amplification

was carried out in a DNA Engine Dyad (BIO-RAD, US) or TPersonal (Biometra, Germany) using the following conditions: a 5 min initial denaturation at 94 °C followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at various temperatures for 30 s and extension at 72 °C for different time intervals.

When needed, long range PCR was performed using Long PCR Enzyme Mix (Thermo Scientific, Lithuania) in a DNA Engine Dyad (BIO-RAD, US) according manufacturer's recommendations.

PCR products were fractionated by gel electrophoresis (0.7-2% agarose in Tris-acetate EDTA buffer with 0.5 mg/ml of ethidium bromide) and visualized by UV transilluminator.

Restriction analysis of PCR products

The restriction mixture (total 20 µl) contained 15 µl of PCR product (amplicon DNA) and 5 U of restriction enzyme in the buffer recommended by the supplier. DNA was incubated for 2 hours at recommended temperature. Restriction products were fractionated by gel electrophoresis (1-2 % agarose in Tris-acetate EDTA buffer with 0.5 mg/ml of ethidium bromide). The length of restriction fragments was determined by comparing them with the migration of GeneRuler 100 bp DNA Ladder (Thermo Scientific, Lithuania).

DNA sequencing and sequence analysis

PCR products and plasmid full length amplicons were gel purified using GeneJet Gel Extraction Kit (Thermo Scientific, Lithuania) or Perfectprep Gel Cleanup Kit (Eppendorf, Germany) and submitted for DNA sequencing at the DNA sequencing centre of Institute of Biotechnology (Vilnius, Lithuania).

Detection of integron-associated elements

Genes coding for class 1 and 2 integron cassettes, IntI1, IntI2, and Sul1 determinants were detected by PCR as described by Mazel *et al*., 2000; Lanz *et al*., 2003; Chen *et al*., 2004; Levesque *et al*., 1995; White *et al*., 2001. Representative amplicons of integron variable region were analyzed by restriction fragment-length polymorphism (RFLP) analysis using restriction endonucleases *Alu*I, *Bme1390*I, and

*Hinf*I (Thermo Scientific, Lithuania). Amplification products of representative integron were gel purified and submitted for DNA sequencing.

Genotyping

Molecular typing of *A. baumannii* isolates was performed by PFGE-*Apa*I restriction analysis according to standard methods with some modifications. Briefly, agarose embedded cells were lysed and total genomic DNA was digested in 200 µl volume with 30 U of restriction endonuclease *Apa*I for 6 h at 37 °C. DNA was electrophoresed on 1% agarose gels by CHEF DR-II Pulsed-Field Electrophoresis System (BIO-RAD) using the initial switch time of 5 s and final switch time of 30 s at an angle of 120 ° at 6 V/cm for 20 h. Gel images were analyzed by using Bionumerics software with the Dice coefficient and band tolerance set at 1.5%. Identification of European clonal lineages I-III was carried out by amplified fragment length polymorphism (AFLP) according Turton *et al.*, 2007. Genotyping by multilocus sequence typing according to the scheme of the Institut Pasteur (MLST-IP) was undertaken with the primers and conditions described on the Pasteur website (http://www.pasteur.fr/mlst).

To confirm the presence of *S. enterica* serotype *Typhimurium* and definitive phage type DT104, amplification of gene specific for *Salmonella typhimurium* (Lin and Tsen, 1999) and a 162-bp 16Sto- 23S spacer region specific for *Salmonella typhimurium* of definitive phage type 104 (DT104) was performed (Pritchett *et al*., 2000). The DNA region of the left and the right junction of SGI1 was confirmed according Boyd *et al.,* 2001.

Plasmid replicon typing

A. baumannii plasmid replicons were typed by PCR-based replicon typing (AB-PBRT) method according Bertini *et al.*, 2010. Replicase gene homology groups, (GRs) were detected by PCR with specific primers targeting 27 different plasmid *rep* genes, originally identified from the published sequences of partially or fully sequenced plasmids isolated from *Acinetobacter* spp. (Bertini *et al*., 2010).

PCR-based replicon typing (PBRT) has been used for detection of plasmid replicon types of *Enterobacteriaceae* (Carattoli *et al.*, 2005). Eighteen pairs of primers

were used to perform 5 multiplex- and 3 simplex-PCRs, targeting the replicons of the major plasmid families occurring in *Enterobacteriaceae* (FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA).

Plasmid analysis

A. baumannii plasmids were analysed by PFGE-*S*1 method. Briefly, agarose-embedded cells were lysed and total genomic DNA was digested in 200 µl volume with 5 U of nuclease S1 for 4 h at 37 °C. DNA was electrophoresed on 1% agarose gels by CHEF DR-II Pulsed-Field Electrophoresis System (BIO-RAD) using the initial switch time of 0.06 s and final switch time of 21.8 s at an angle of 120° at 6 V/cm for 13 h.

A. baumannii plasmid pAB120 DNA was extracted with GeneJet Plasmid Miniprep Kit (Thermo Scientific, Lithuania) and amplified by Long PCR Enzyme Mix (Thermo Scientific, Lithuania) with GR-specific primers according manufacturer's recommendations. Amplicons were digested with *Eco*RI, *Eco*RV, *Eco*47I, *Eco*57I, *Eco*88I, *Bst*XI, *Bsr*DI, *Dra*III, *Hind*III, *Pae*I, *Pst*I, *Xba*I, *Sal*I, *Xho*I restriction endonucleases (Thermo Scientific, Lithuania) according manufacturer's recommendations and analysed on 1-2 % agarose gels. Restriction profiles were compared *in silico* with fully and partially sequenced *Acinetobacter* plasmids at GenBank (http://www.ncbi.nlm.nih.gov/). Plasmid amplicons were purified and sequenced by primer walking strategy.

Plasmid transformation and minimal inhibitory concentration (MIC) assay

pAB120 plasmid DNA was extracted with GeneJet Plasmid Miniprep Kit (Thermo Scientific, Lithuania) and transformed into electrocompetent carbapenem-susceptible *Acinetobacter baumannii* strain K53 by electroporation. Transformants were selected on LB agar plates containing 10 µg/ml imipenem. MICs determination of transformants, donor, and recipient strains was performed by agar dilution method on Mueller-Hinton agar (Oxoid, England) according CLSI (CLSI, 2009).

Conjugation experiments

Conjugation experiments were conducted by mating *E. coli* or *S. enterica* strains harboring integrons with different array types as donor strains and rifampicin-resistant *E. coli* HK225 as the recipient strain at a donor:recipient ratio 1:5 as described by Kadlec and Schwarz (Kadlec and Schwarz, 2008). Transconjugants were selected on LB agar plates containing rifampicin (100 mg/l) and trimethoprim (10 mg/l) or rifampicin (100 mg/l) and spectinomycin (100 mg/l). Transconjugants were tested for the presence of class 1 and class 2 integrons by PCR and RFPL analysis of amplicons.

DNA hybridization

Determination of integron localization and analysis of pAB120 genetic structure were performed by Southern blotting. Electrophoresis gels were transferred onto blotting membrane Hybond-N (Amersham Pharmacia Biotech, United Kingdom) according to standard protocols (Sambrook *et al.*, 1989) and hybridized to the ^{32}P-labeled DNA (integron localization determination) or biotin-labeled probes (pAB120 analysis) for genes *sulI* and *intI2*, specific for class 1 and class 2 integrons, respectively, and to the *aadA1* probe for class 1 integrons harboring *aadA1* cassette but lacking *sulI*, and to the $bla_{\text{OXA-72}}$ probe for pAB120. The DNA probes were labeled using DecaLabel DNA Labeling Kit and Biotin DecaLabel DNA Labeling Kit (Thermo Scientific, Lithuania). After washing, the membranes were exposed to the phosphoimager plate for 6 h and were scanned with a FLA5100 Phosphoimager (FujiFilm, USA) (radioactive labeling) or detection was performed using Biotin Chromogenic Detection Kit (Thermo Scientific, Lithuania) (biotin labeling).

Statistical analysis

Chi-square or Fisher exact test was used for the comparison of categorical variables. A p-value of <0.05 was considered to be statistically significant. Statistical analyses were performed by GraphPad Prism.

Accession number

DNA sequences of newly observed integron gene cassette arrays *aadA7-aadA7*, *dfrA17-aadA5D-IS26-DintI1-aadB-aadA1-cmlA* and a novel plasmid pAB120 were deposited in the GenBank database under accession numbers FJ980455, GQ402463, and JX069966, respectively.

RESULTS

Occurrence of class 1 and class 2 integrons in *E. coli, S. enterica, P. aeruginosa,* and *A. baumannii* isolates of human and animal origin

Thirty-seven percent (113/304) of the selected *E. coli* and *S. enterica* isolates, obtained from human and animal sources and resistant to at least two unrelated antibiotics, harboured integrons of classes 1 and 2. Single class 1 and class 2 integrons were observed at the frequencies of 32% (98/304) and 6% (19/304), respectively. Two *S. enterica* isolates harbored two different class 1 integrons. Four *E. coli* isolates carried integrons of both classes. Fourteen percent (16/114) of *intI*-positive isolates and 37% (11/30) of *intI2*-positive isolates lacked variable regions containing gene cassettes (VRs). Eight percent (9/114) of *intI1*-positive isolates lacked *sulI*. The frequency of class 1 and class 2 integrons in *E. coli* and *S. enterica* isolates within groups regarding the origin (clinical and animal isolates) are shown in Figure 1, A and B.

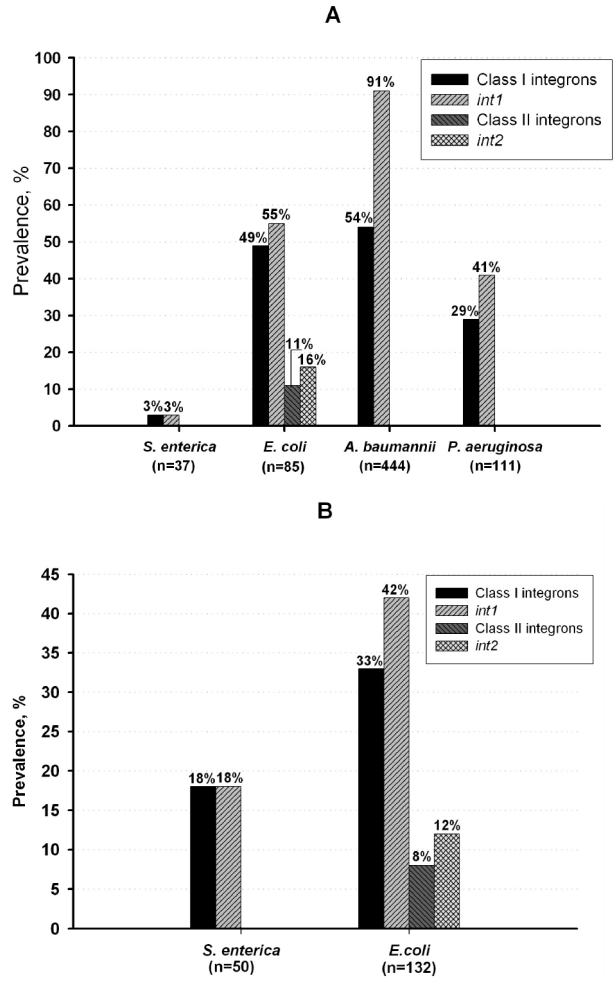

Fig. 1. Prevalence of class 1/class 2 integrons and *intI1/intI2* genes among *E. coli, S. enterica*, *P. aeruginosa*, and *A. baumannii* isolates from clinical (**A**) and animal (**B**) origin.

Gene coding for IntI1 was detected in 41% (46/111) of *P. aeruginosa* and in 91% (406/444) of *A. baumannii* isolates from human origin whereas VRs were determined in 29% (32/111) and 54% (238/444) of isolates, respectively (Figure 1, A). No *intI2* genes were detected in *S. enterica, P. aeruginosa,* and *A. baumannii.*

Characterization of class 1 and class 2 integron gene cassettes

A total of 31 different gene cassettes were identified in 26 different types of class 1 and class 2 integrons (Figure 2, Table 1). Among *Enterobacteriaceae* isolates, the most frequent were those coding for variants of aminoglycoside adenylyltransferases (*aadA*) conferring streptomycin – spectinomycin resistance and/or dihydrofolate reductases (*dfr*) conferring trimethoprim resistance (Figure 2, A and B). The most common *aadA* variants were *aadA1* and *aadA5*, consisted 42% (23/55) and 49% (27/55) of total *aad* gene cassettes detected in integron–positive *Enterobacteriaceae* isolates from clinical samples, respectively. *aadA1* was also prevalent among *E. coli* and *S. enterica* from animal origin (50/63, 79%). Five *dfr* gene variants were distributed at the following order (number of total gene cassettes; human and animal origin, respectively): *dfrA1* (19/49, 39% and 31/40, 78%), *dfrA17* (27/49, 55% and 5%, 2/40), *dfrA12* (2/49, 4% and 4/40, 10%), *dfrA14* (0%, 0/49 and 3/40, 3%), and *dfrA5* (1/49, <1% and 0%, 0/40). Other genes found on the cassettes in *E. coli* and *S. enterica* integrons conferred resistance to the aminoglycosides (*aacA4* and *aadB*), β-lactams ($bla_{\text{PSE-1}}$, $bla_{\text{OXA-1/30}}$), chloramphenicol (*catB3* and *cmlA*), erythromycin (*ereA2*), and coded for an esterase of unknown function (*estX*) (Figure 2, A and B).

Genes conferring resistance to the aminoglycosides were also prevalent among *P. aeruginosa* and *A. baumannii* isolates. Two variants of *aacA* genes were detected among *P. aeruginosa,* the dominant variant being *aacA4* (16/17, 94%) (Figure 2, C). Aminoglycoside resistance genes *aadA6* and *aadB* as well as gene of unknown function *gcuD* were also among the most frequently detected in integrons of *P. aeruginosa* (Figure 2, C). The vast majority of *A. baumannii* class 1 integrons harboured genes *aacC1* (237/238, 99%) and *aadA1* (213/238, 89%). *orfX′* and *orfX″* – open reading frames of unknown function – were also frequent in class 1 integrons of *A. baumannii* (Figure 2, D).

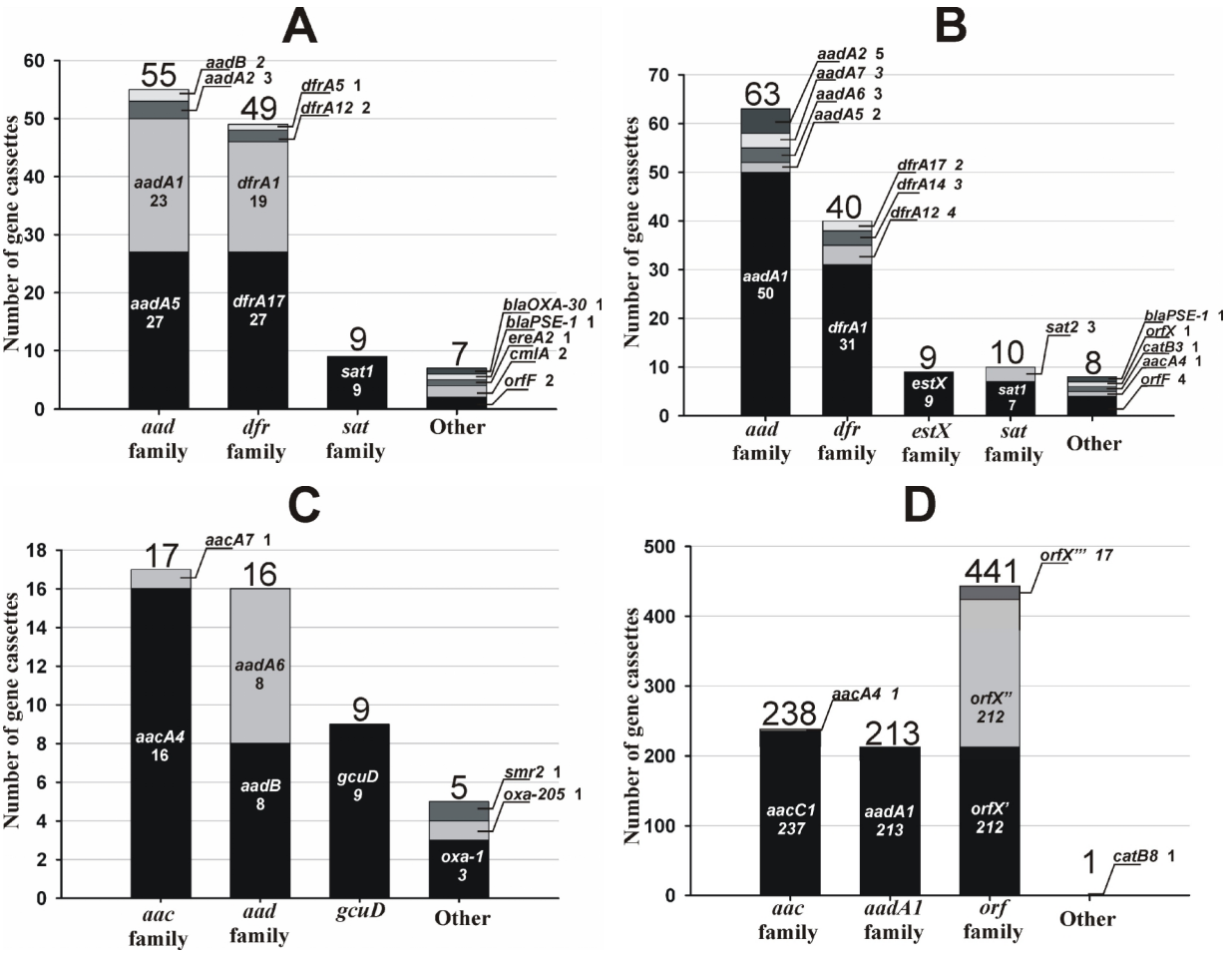

Fig. 2. Prevalence of antibiotic resistance genes and their variants in class 1/class 2 integrons among *E. coli, S. enterica* (**A** – from clinical sources, **B** – from animal sources), *P. aeruginosa* (**C**), and *A. baumannii* (**D**) isolates.

Table 1. Prevalence, diversity, and genome location of class 1 and class 2 integrons from *E. coli, S. enterica, P. aeruginosa,* and *A. baumannii* isolates of human and animal origin in Lithuania.

Bacteria (no of isolates)		***E. coli* (217)**						***S. enterica* (87)**			***P. aeruginosa* (111)**	***A. baumannii* (444)**
Source	**Integron location**	**Human (85)**	**Poultry (54)**		**Swine (35)**	**Cattle (43)**		**Human (37)**	**Poultry (31)**	**Swine (19)**	**Human (111)**	**Human (444)**
			Hl. (27)	**Ds. (27)**	**Ds. (35)**	**Hl. (17)**	**Ds. (26)**					
Class 1 integrons												
aadA1	P	1	3	1	2	1	2			1		
dfrA1-aadA1	P	9	2	3	1	6	10		1			
dfrA17-aadA5	P	24				1	1					
dfrA12-orfF-aadA2	P	2	2							2		
dfrA17-aadA5Δ-IS26-ΔintI1-aadB-aadA1-cmlA	P	2										
dfrA5-ereA2	P	1										
$bla_{OXA-1/30}$ - *aadA1*	C	1										
aadA2 + bla_{PSE-1}	C							1	1			
dfrA14-aadA6	C					1	2					
aadA7-aadA7	P									3		
estX-aadA1	P				4	1				1		

Table 1 continuation

Bacteria (no of isolates)	Integron location	*E. coli* (217)						*S. enterica* (87)			*P. aeruginos* (111)	*A. baumannii* (444)
Source		Human (85)	Poultry (54)		Swine (35)	Cattle (43)		Human (37)	Poultry (31)	Swine (19)	Human (111)	Human (444)
			Hl. (27)	Ds. (27)	Ds. (35)	Hl. (17)	Ds. (26)					
aacA4-catB3-dfrA1-orfX	ND			1								
aacA4	ND										13	
aadB	ND										6	
aadA6-gcuD	ND										7	
aacA4-bla$_{OXA-1/30}$	ND										3	
aadB-bla$_{OXA-205}$	ND										1	
aacA7-smr2-gcuD	ND										1	
aadB + aadA6-gcuD	ND										1	
aacC1-orfX'-orfX''-aadA1	ND											195
aacC1-?	ND											25
aacC1-orfX'-orfX''-orfX'''-aadA1	ND											17
aacA4-catB8-aadA1	ND											1
Total		**40**	**7**	**5**	**7**	**10**	**15**	**1**	**2**	**7**	**32**	**238**
Class 2 integrons												
dfrA1-sat1-aadA1	P	7	1		2		3					
dfrA1-sat1	P			1								
estX-sat2-aadA1	C				1							
Total		**7**	**1**	**1**	**3**		**3**					
Class 1 + Class 2 integrons												
dfrA1-aadA1 + dfrA1-sat1-aadA1	P/P	1										
dfrA17-aadA5 + dfrA1-sat1-aadA1	P/P	1										
aadA1 + estX-sat2-aadA1	P/C				2							
Total		**2**			**2**							

Hl. – healthy animals; **Ds**. – diseased animals; **P** – plasmid; **C** – chromosome; **ND** – not determined.

The array type of 1500 bp *dfrA1-aadA1* was most frequent among *Enterobacteriaceae* and was detected in 34% (33/98) of class 1 integron–positive *E. coli* isolates from human clinical samples, poultry, cattle, swine, and from *S. enterica* (poultry and swine) (Table 1). The second most common array type was 1600 bp *dfrA17- aadA5*, which prevailed in clinical *E. coli* isolates (25/42, 60%) and was carried by two *E. coli* isolates from cattle. The others class 1 integron types shared among different groups of isolates were 1000 bp *aadA1*, 2000 bp *dfrA12-orfF-aadA2*, 2000 bp *estX-aadA*, 1000 bp *aadA2*+1200 bp *bla*$_{PSE-1}$. The remaining integron types were present within single groups of isolates (Table 1).

In *A. baumannii*, the most prevalent was class 1 integron of 2500 bp *aacC1-orfX' -orfX'' -aadA1* (82%, 195/238) while other three gene cassette arrays also have

been detected, although in less extent (Table 1). None of them were found in *Enterobacteriaceae.* Six gene cassette arrays were identified in *P. aeruginosa* isolates with the most frequent representatives of 800 bp *aacA4* (41 %, 13/32), 750 bp *aadB* (22 %, 7/32), and 1300 bp *aadA6-gcuD* (25 %, 8/32) (Table 1).

Of the three different array types of class 2 integrons, the 2200 bp *dfrA1-sat1-aadA1* array was most frequent, being found in clinical *E. coli* isolates (9/9, 100%), diseased cattle (3/3, 100%), and diseased swine (2/5, 40%), as well as in a single isolate from healthy poultry (Table 1).

Novel variants of class 1 integrons

Class 1 integrons with the newly identified gene cassette arrays were observed. Three *S. enterica* isolates from swine carried class 1 integron with gene cassette array *aadA7-aadA7* (1800 bp) (GenBank accession number FJ980455) (Table 1).

Two *E. coli* isolates from urinary tract infection harboured class 1 integron 4800-bp *dfrA17-aadA5*Δ-IS*26*-Δ*intI1-aadB-aadA1-cmlA* (GenBank accession number GQ402463) (Figure 3). Within the gene array, the IS*26* element was inserted 157 bp downstream the 5′-end of the *aadA5* gene as the second gene cassette. At the right insertion site of IS*26*, the 3′- end-truncated integrase gene (*intI1*) was observed, which was followed by the resistance gene cassettes *aadB*, *aadA1,* and *cmlA*. DNA sequence analysis downstream the *qacE*Δ*1* and *sul1* genes revealed the presence of the complete *orf5* and *orf6*, which were followed by macrolide inactivation gene cluster *mphR-mrx-mphA*, flanked by the IS*6100* element and additional copy of IS*26*. The transposition genes *tnpR* and *tnpM*, characteristic for Tn*21*, were found upstream the 5′-CS, the 3′-end of *tnpR* being truncated by one more copy of IS*26* (Figure 3).

Analysis of a novel 1503 bp gene cassette array of integron (named as a In671) from imipenem resistant *P. aeruginosa* isolate revealed two cassettes, including *aadB* and a cassette encoding a new OXA-type β-lactamase, assigned as an OXA-205. Within the deduced 266 amino acid sequence, all the conserved motifs, typical for class D enzymes were found. Comparison of amino acid sequence of OXA-205 with other class D β-lactamases revealed that it belongs to the OXA-2 sublineage (Figure 4, A), showing the highest sequence similarity with OXA-type enzymes OXA-118 and OXA-119, found in uncultured bacteria from a wastewater plant and *Burkholderia cepacia*

clinical isolate (97 % and 96 % sequence identity, respectively) (Figure 4, B). OXA-205 exhibits 92 % sequence identity with the well-described narrow spectrum class D β-lactamase OXA-46. Although OXA-205 producing *P. aeruginosa* isolate is resistant to imipenem, OXA-205 shares weak relationship with the carbapenem-hydrolyzing class D β-lactamases (<40 % sequence identity).

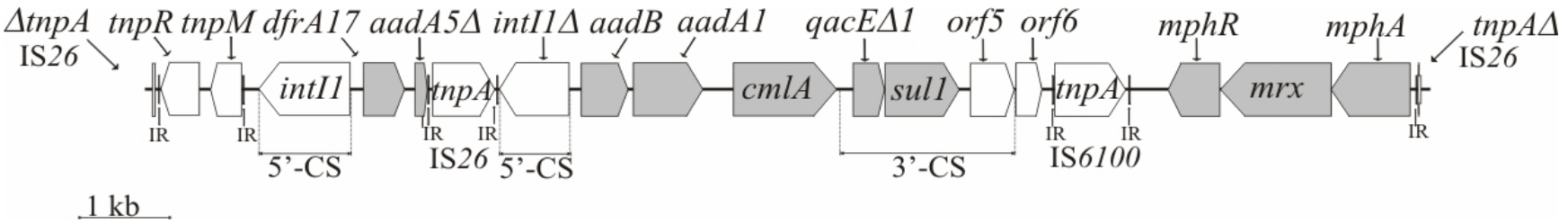

Fig. 3. Schematic representation of the arrangement of variable region (accession number GQ402463) of class 1 integron from human *Escherichia coli* isolates. 5′-CS and 3′-CS of class 1 integron are shown by arrows. Inverted terminal repeats (IR) of IS*26* and IS*6100* are indicated by vertical bars. Grey colour represents antibiotic resistance-associated genes.

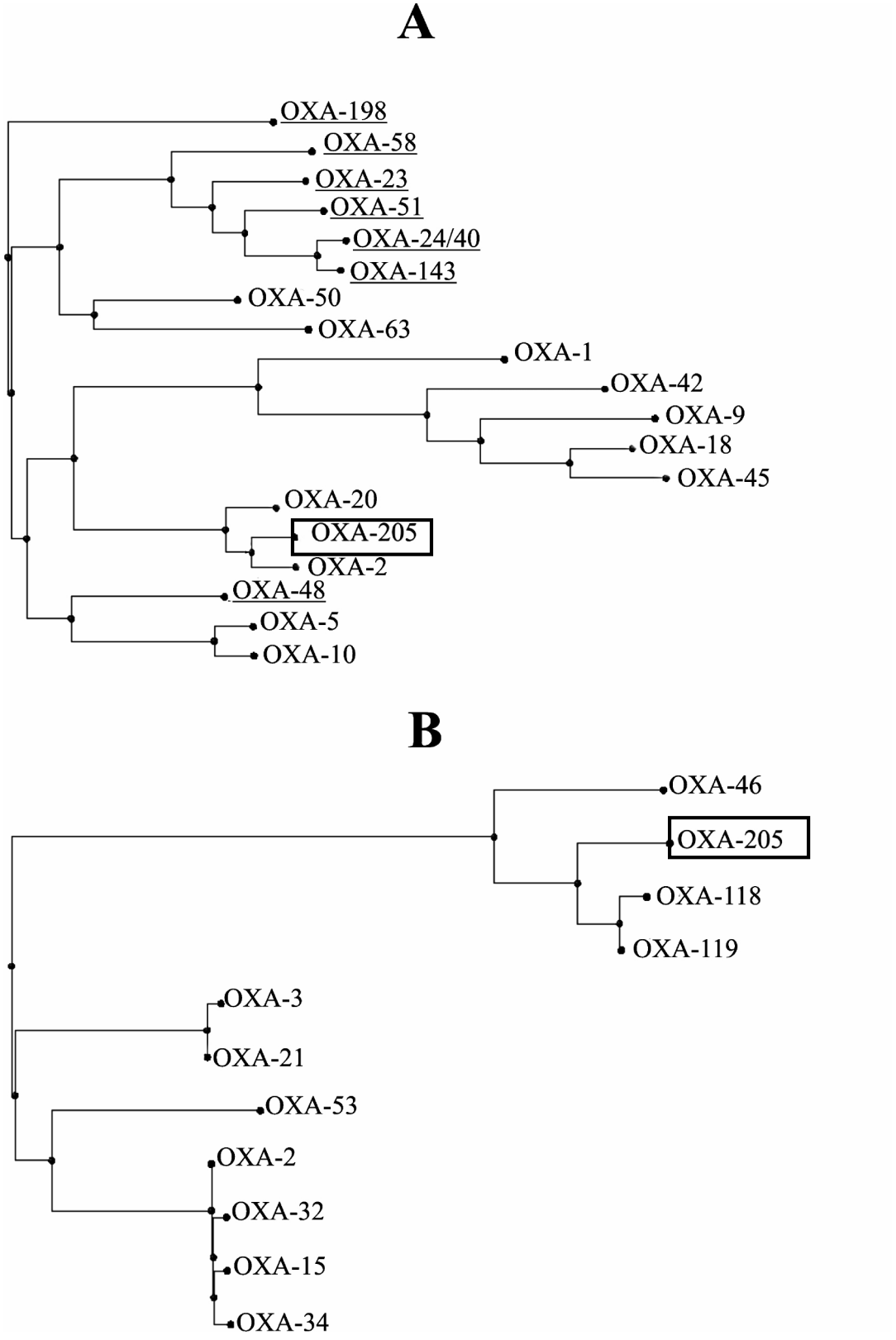

Fig. 4. Dendrogram obtained for class D β-lactamases including CHDLs (underlined) (**A**); obtained for class D β-lactamases belonging to OXA-2 lineage (**B**). The alignment used for the tree calculation was undertaken with the COBALT multiple protein sequence alignment tool.

Conjugal transfer and genomic location of integrons

Conjugal transfer was confirmed for 8 of 13 different types of class 1 integrons and for two of three different types of class 2 integrons detected in *Enterobacteriaceae*. Transconjugants were *intI1* or *intI2* positive, and displayed an identical size of amplicons and RFLP pattern of variable region as the parental strains. Of transferable class 1 integron types, all eight were found located on plasmids of size >50 kb as determined by DNA hybridization (data not shown) (Table 1).

Chromosomal location of class 1 integrons with gene cassettes 1000 bp *aadA2* and 1200 bp $bla_{\text{PSE-1}}$ in the *S. enterica* serotype *Typhimurium* DT104 isolates was shown by DNA hybridization and PCR, targeting the SGI1 flanking regions (data not shown). Nontransferable class 1 integrons with gene cassette arrays 1600 bp *dfrA14-aadA6* and $bla_{\text{OXA-1/30}}$ − *aadA1* and class 2 integron with gene cassette array 2500 bp *estX-sat2-aadA1* were also found located in chromosomal DNA (Table 1).

Plasmids in integron-positive *Enterobacteriaceae*

Among *E. coli* and *S. enterica* isolates carrying class 1 and/or class 2 integrons, plasmids of 15 different Inc groups were detected by PBRT (Figure 5). Different variants of plasmid heterogeneous group IncF were the most prevalent with the prevalence for IncF_repB 76 % (71/94), IncFIB 67 % (63/94), and IncFIA 34 % (32/94). Groups of IncHI1, IncHI2, IncW, IncN, IncFIC were significantly linked ($p<0.01$) to animal isolates (Figure 5). However, none of the Inc group was significantly associated with certain type of integron.

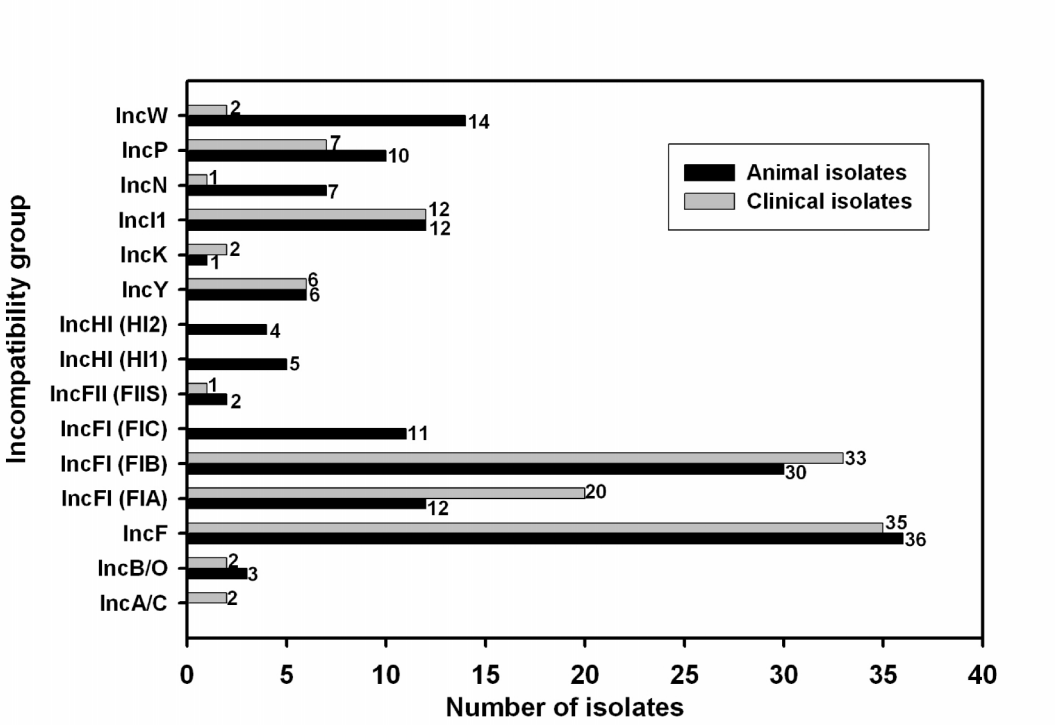

Fig. 5. Distribution of plasmid Inc groups among 94 integron-positive isolates of *E. coli* and *S. enterica* from clinical and animal sources.

Genotyping of *A. baumannii* isolates

PFGE-*Apa*I analysis showed that 444 *A. baumannii* isolates belonged to 74 pulsotypes according to their macrorestriction patterns (Figure 6). Pulsotype groups I-IV accounted for 89% (393/444) of isolates. Other isolates (51/444) were unique (similarity <80 %) or nontypable. The most prevalent in all hospitals were group I and II pulsotypes, comprising *Clusters 1-2* (at 80% Dice similarity cut-off) (Figure 6). Representative isolates from pulsotype groups I and IV were of ST-1 type, isolates from pulsotype groups II and III were of ST-2 type according to MLST-IP.

Of all isolates studied, 230 (52%) and 202 (45%) belonged to ECI and ECII clones, respectively. *Cluster 1* and *2* isolates belonged to ECI and ECII clones, respectively. *Cluster 2* isolates were significantly more resistant to carbapenems whereas their resistance to cefoperazone/sulbactam, ampicillin/sulbactam and aminoglycosides was significantly lower ($p<0.01$) if compare to *Cluster 1* isolates.

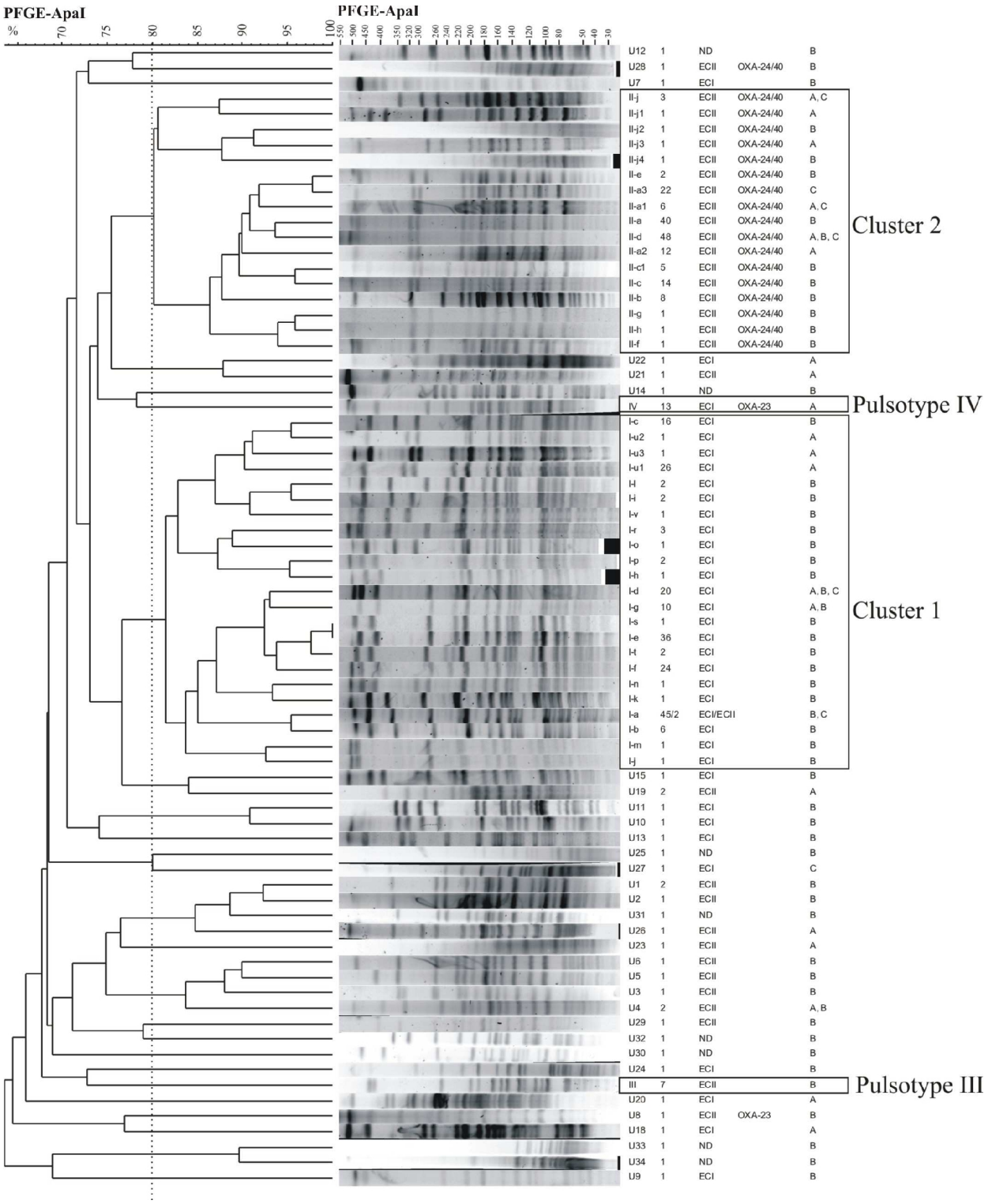

Fig. 6. Pulsed-field gel electrophoresis (PFGE) profiles of *Apa*I-digested genomic DNA for representative isolates of *Acinetobacter baumannii*, including number of isolates belonging to respective pulsotype, European clone I-II (ECI-II), CHDLs determination data and hospitals where isolates were collected. Boxes indicate four major groups of isolates which clustered at a level of 80 % (according Dice coefficient with band tolerance parameter set at 1 %). Hospital **A** – Vilnius University Emergency Hospital; Hospital **B** – Hospital of Lithuanian University of Health Sciences Kaunas Clinics; Hospital **C** – Šiauliai Hospital; ND – not determined.

A. baumannii plasmids

The PCR-based *A. baumannii* plasmid replicon typing (AB-PBRT) showed that 93% (413/444) of all isolates carried from one to two of the 19 known *A. baumannii* replicase gene (*rep*) homology groups, which were represented by GR2 (including *rep*Aci1 and *rep*Aci2) and GR6 (*rep*Aci6) groups, found in 87% (385/444) and 66% (293/444) of isolates, respectively (Table 2). ECI isolates were significantly associated with the carriage of a combination of GR2+GR6 replicons (90%, 206/230), whereas ECII isolates most often had GR2 replicon type (55%, 112/202) ($p<0.01$).

PFGE-*S*1 assay identified in *A. baumannii* isolates five plasmids of 6 kb, 9kb, 11kb, 70kb and 100 kb in size being present from one to four in various combinations (Table 2, Figure 7). Among ECI isolates the combination of 9+70 kb plasmids was dominant (60%, 137/230). ECII isolates showed a greater variety in plasmid size and combinations, however, none of the presently known *A. baumannii* plasmid replicon groups, except for GR2 and GR6, have been detected in these isolates by AB-PBRT (Table 2). In carbapenem-resistant $bla_{\text{OXA 24/40-like}}$-positive *Cluster 2* (ECII) isolates, the 11 kb plasmid was most common, found alone or in different combinations in 92% (154/167) of isolates.

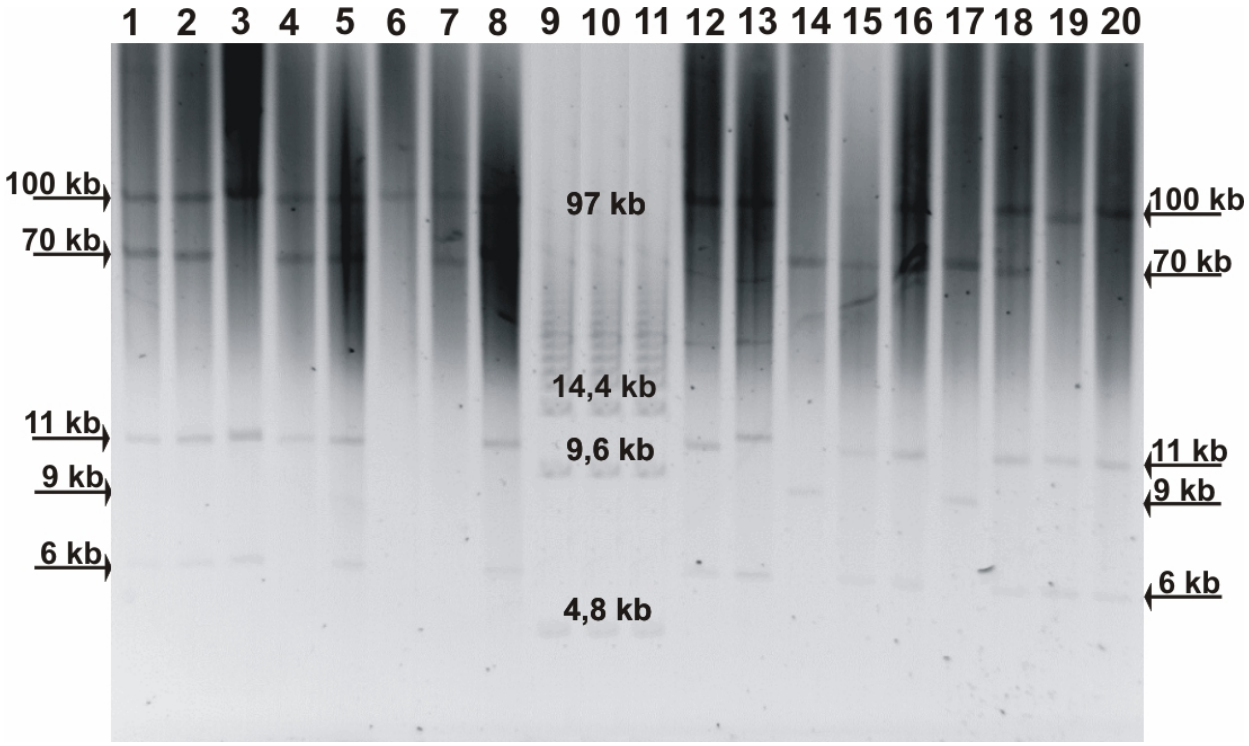

Fig. 7. PFGE-*S*1 analysis of plasmids of *A. baumannii*. **1, 2, 3, 4, 5, 6, 7, 8, 12, 13, 15, 16, 18, 19, 20** – ECII isolates; **14, 17** – ECI isolates; **9, 10, 11** – DNA marker (BIORAD).

Table 2. Plasmids in *A. baumannii* isolates from Lithuanian hospitals

Group by PFGE-*Apa*I (no of isolates)	European clone lineages (no of isolates)	Plasmid replicase gene group (no of isolates)	Plasmids by PFGE-*S*1, kb (no of isolates)
Vilnius University Emergency Hospital (n=103)			
Cluster 1 (39)	ECI (39)	GR2+GR6 (31) GR2 (1) GR6 (4) ND (3)	9+70 (29) 9+11+70 (2) 70 (1) ND (7)
Cluster 2 (38)	ECII (38)	GR2+GR6 (14) GR2 (19) GR6 (1) ND (4)	11+70+100 (4) 11+100 (8) 100 (2) 11+70 (6) 11 (14) 9+11 (2) ND (2)
Pulsotype IV (13)	ECI (13)	GR2+GR6 (12) GR6 (1)	9+70 (12) 9 (1)
Unique (10)	ECI/ECII (4/6)	GR2+GR6 (3) GR2 (1) GR6 (2) ND (4)	6+9+70+100 (2) 9+70+100 (1) 9+70 (3) 11+100 (1) 11 (1) ND (2)
Nontypable (3)	ECII/ND (1/2)	GR2+GR6 (2) ND (1)	9 (1) 11 (1) ND (1)
Hospital of Lithuanian University of Health Sciences Kaunas Clinics (n=304)			
Cluster 1 (159)	ECI/ECII (157/2)	GR2+GR6 (149) GR2 (3) GR6 (5) ND (2)	9+70 (88) 6+9+70 (35) 6+70 (14) 9 (4) 70 (5) 6 (3) ND (10)
Cluster 2 (103)	ECII (103)	GR2+GR6 (20) GR2 (73) GR6 (6) ND (4)	11 (80) 11+100 (10) 11+70+100 (5) 70+100 (2) ND (6)
Pulsotype III (7)	ECII (7)	GR2+GR6 (5) GR6 (2)	9+70 (3) ND (4)
Unique (23)	ECI/ECII/ND (6/10/7)	GR2+GR6 (5) GR2 (6) GR6 (6) ND (6)	9+70 (1) 9 (1) 11 (1) 70 (1) 100 (1) ND (18)
Nontypable (12)	ECI/ECII/ND (3/7/2)	GR2+GR6 (4) GR2 (3) ND (5)	11 (7) ND (5)
Šiauliai Hospital (n=37)			
Cluster 1 (8)	ECI (8)	GR2+GR6 (8)	9+70 (7) 9 (1)
Cluster 2 (26)	ECII (26)	GR2+GR6 (11) GR2 (14) ND (1)	6+11+70+100 (13) 6+11+100 (2) 11+70+100 (1) 6+11 (7) 11 (2) 100 (1)
Unique (1)	ND (1)	ND (1)	ND (1)
Nontypable (2)	ECII (2)	GR2+GR6 (1) GR6 (1)	9+70 (1) 70+100 (1)

ND – not determined.

Novel GR2 type plasmid in carbapenem-resistant *A. baumannii* clinical isolates

Further investigation was performed on the identity of an ubiquitous 11 kb plasmid from the representative *A. baumannii* ECII isolates by PCR-based amplification of full length amplicons using primers specific to replicase gene group GR2, most frequent on 11 kb plasmid carrying isolates. The comparison of the restriction profiles of amplicons *in silico* yielded no matches with those of the known *A. baumannii* plasmid DNA sequences in the data bases. The newly found plasmid designated pAB120 has been purified from the host *A. baumannii* isolate and transferred into the carbapenem-susceptible recipient *A. baumannii* clinical isolate by electroporation. The carriage of $bla_{\text{OXA-24/40-like}}$ and *rep* genes was confirmed by PCR. According to the MIC assay, recipient *A. baumannii* strain carrying pAB120 was resistant to imipenem and meropenem (MIC 32 mg/l), while recipient strain without a plasmid was susceptible to both carbapenems (MIC <4 mg/l). A full length amplicon of pAB120 from K53 was obtained by a long range PCR and sequenced by a primer walking strategy. To confirm the genetic organization of pAB120, a native plasmid was isolated from K53 strain and was subjected to restriction analysis, PCR mapping, and Southern analysis.

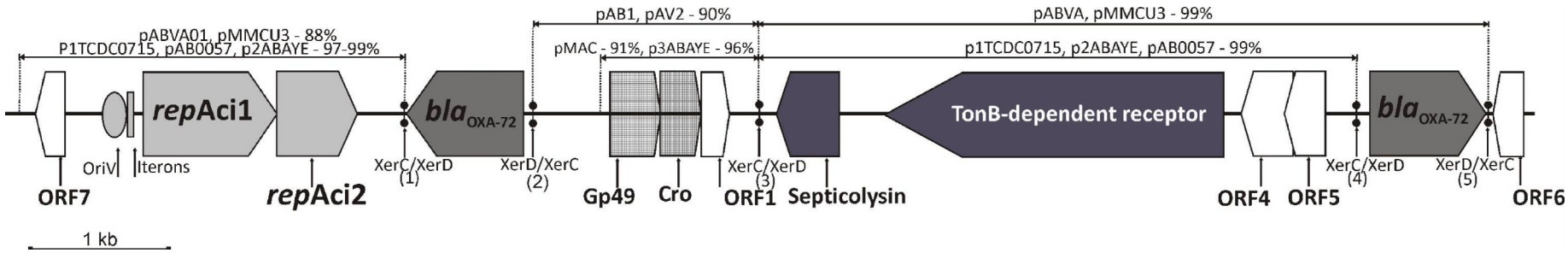

Fig. 8. Linear representation of 10879 bp plasmid pAB120 (GenBank no JX069966) from *A. baumannii* clinical isolates. Nucleotide sequences of plasmids p1TCDC0715, pAB0057, p2ABAYE, pABVA01, pMMCU3, pAB1, pAV2, p3ABAYE, pMAC used for nucleotide identity determination of homologous regions were retrieved from GenBank (CP002523.1, CP001183.1, CU459138.1, FM210331.1, GQ904227.1, CP000522.1, DQ278486.1, CU459140.1, AY541809.1, respectively).

pAB120 was 10879 bp in length and possessed 13 open reading frames (ORFs) including two copies of a gene for OXA-24/40-like β-lactamase OXA-72, two putative toxin-antitoxin systems, consisting of Gp49-like/Cro-like proteins and ORF4/ORF5, respectively (Figure 8). At least five 28-bp lengh XerC/XerD-like binding sites were detected, two pairs of them flanking $bla_{\text{OXA-72}}$ genes. Overall, pAB120 showed the highest sequence similarity to known GR2 plasmids p1ABTCDC0715, p2ABAYE, pAB0057, pABVA01 and pMMCU3 (CP002523.1, CU459138.1, CP001183.1, FM210331.1, GQ904227.1). The major differences between pAB120 and related plasmids were found within regions flanked by XerC/XerD-like sites (1)-(2) and (2)-(3), respectively (Figure 8). Downstream the replicase coding genes *rep*Aci1 and *rep*Aci2, a module surrounded by XerC/XerD-like sites and containing an additional copy of $bla_{\text{OXA-72}}$ gene was present in pAB120, which in plasmids p1TCDC0715, pAB0057 and p2ABAYE is replaced by the region of different composition. Moreover, Gp49 and Cro-like protein coding genes comprising a putative toxin-antitoxin system are located in a region flanked by XerC/XerD-like sites (2)-(3). The DNA module of similar fragment (90-96% sequence identity) was observed in unrelated *Acinetobacter* plasmids pAB1, pMAC, p3ABAYE and pAV2 (CP000522.1, AY541809.1, and CU459140.1). Screening of *A. baumannii* clinical isolates for the presence of pAB120 plasmid by two multiplex PCR using primer pairs targeting the unique sequences in plasmid DNA including both plasmid-borne $bla_{\text{OXA-72}}$ genes, showed that pAB120 was present in 95% (165/174) of carbapenem-resistant $bla_{\text{OXA-24/40-like}}$-positive ECII isolates from all hospitals.

Discussion

Prevalence of class 1 and class 2 integrons among *E. coli* from human and animal origin, observed in this study, is consistent with the data from other European countries, which ranges between 30% and 80% for the class 1 integrons and between 3 % and 15 % for the class 2 integrons (Povilonis *et al.,* 2010; Goldstein *et al*., 2001; Guerra *et al*., 2003; Heir *et al*., 2004; Sunde and Norström, 2006; Cocchi *et al*., 2007; van Essen-Zandbergen *et al*., 2007; Kadlec and Schwarz, 2008; Machado *et a*l., 2008). Isolates from healthy and diseased animals (poultry and cattle) shared identical integron types, transferable by conjugation. These observations suggest a possible horizontal transfer of integron-associated resistance determinants between commensal and pathogenic *E. coli* (Povilonis *et al.,* 2010).

The occurrence of class 1 integrons in human *S. enterica* isolates was significantly lower compared to clinical *E. coli* isolates (Povilonis *et al.,* 2010). The low prevalence of class 1 integrons was also found in *S. enterica* isolates from poultry, which is thought one of the main sources of drug-resistant *Salmonella* human infections (Miko *et al*., 2005). However, the incidence of class 1 integrons and diversity of their gene cassette arrays among *Salmonella* isolates from swine, produced for slaughter, was significantly higher compared to poultry isolates. Therefore, swine represent a considerable source of *Salmonella*-associated MDR determinants, which could be potentially transferred to humans through the food chain (Povilonis *et al.,* 2010).

The class 1 integron with *dfrA1-aadA1* array was most common and was found in all representative groups of human and animal *E. coli* isolates and in *S. enterica* poultry isolates. This clearly indicates the transferability of this integron type between *Enterobacteriaceae* present in different environments. The global dissemination of *dfrA1-aadA1* integron among *S. enterica* isolates has been recently reported by Krauland *et al*. (2009). The other most commonly observed class 1 integron types were *dfrA17-aadA5* and *aadA1* (Povilonis *et al.,* 2010). These integrons, along with *dfrA1-aadA* integron, appear to be mostly disseminated in *E. coli* isolates recovered from human and animal sources in Europe and United States (Dolejská *et al*., 2008; Kadlec and Schwarz, 2008; Machado *et al*., 2008; Ajiboye *et al*., 2009). *dfrA12-orfF-aadA2* array was less prevalent ($p<0.01$), nevertheless, it was found in *E. coli* isolates from urinary tract

infections, healthy poultry, and in *S. enterica* isolates from swine, indicating its transferability among bacteria, consistent with the recent observations showing that *dfrA12-orfF-aadA2* is the most world widespread class 1 integron type among nontyphoidal *S. enterica* isolates (Krauland *et al*., 2009).

Only two types of class 1 integron were prevalent among *A. baumannii,* both of them present very similar gene cassette arrangement - *aacC1-orfX'-orfX''-aadA1* and *aacC1-orfX'-orfX''-orfX'''-aadA1* (Povilonis *et al.,* 2013). Spread and persistence of these integrons among *A. baumannii,* observed in different European countries during the last decade suggests that formed integron gene cassette arrays have a unique genetic stability. Gene cassettes and their possible transfer may determine the same resistance phenotype among geographically and epidemiologically unrelated *A. baumannii* strains (Nemec *et a*l., 2004; Zarrilli *et al*., 2004; Turton *et al*., 2005; Kraniotaki *et al*., 2006; D'Arezzo *et al*., 2009).

Compared with *A. baumannii,* a larger diversity of class 1 integrons was observed in clinical *P. aeruginosa* isolates. Notably, almost all integrons harboured genes which confered resistance to aminoglycosides. Knowing that *P. aeruginosa* is often naturally resistant to many antibiotics mostly due to the active multidrug-resistance pumps and a permeability barrier, integron-borne aminoglycoside resistant determinants further narrow the spectrum of efficient antibiotics against *P. aeruginosa* infections. This study also reports a novel gene cassette encoding a new OXA-type β-lactamase, OXA-205, harboured by unique integron in a imipenem-resistant *P. aeruginosa* isolate from clinical specimen, which expands diversity of group of OXA type β-lactamases, recently reported in many countries (El Garch *et al*., 2011; Hocquet *et al.,* 2011; Juan *et al*., 2009; Liu *et al*., 2010).

Class 2 integrons were found exclusively in *E. coli*, the *dfrA1-sat1-aadA1* array being the most prevalent among isolates from all sources. The dominance of this integron type is in accordance with the observations reported by other similar studies conducted in Europe (Cocchi *et al.,* 2007; Vinué *et al.,* 2008).

Two human *E. coli* isolates carried a class 1 integron residing on the conjugative plasmid of >50 kb with a novel genetic organization of variable region. The observed gene cassette arrangement of this integron strongly endorses the plasticity of bacterial

genome, where IS elements play an important role for acquisition/mobilization of antibiotic resistance genes as well as formation of composite transposons and therefore responsible for efficient dissemination of resistance determinants and adaptation to the clinical environment (Povilonis *et al.,* 2010).

The origin of the frequent incidence of *aadA* gene cassettes within integrons of classes 1 and 2, found in different environments, where specific selective pressure of relevant antimicrobials varies considerably, is still disputed (Antunes *et al*., 2004; Srinivasan *et al.,* 2008; Barlow *et al.,* 2009). However, an extensive streptomycin usage in the livestock for infection treatment and/or prevention purposes could support such selection. Newly observed class 1 integron-type *aadA7* - *aadA7* carrying streptomycin resistance gene duplication in *S. enterica* isolates of swine origin could be a response to the extensive use of streptomycin in the livestock production (Povilonis *et al*., 2010).

The high frequency of *dfr* gene cassettes within integrons determined in this study, confirms that these genes represent a potential source for horizontal spread of trimethoprim resistance among bacteria present in different environments, including livestock, where trimethoprim is used for antimicrobial therapy and prophylaxis of food-producing animals (Mathew *et al*., 2007; Prescott, 2008). This study showed that integron-associated *dfr* genes are in most part responsible for the trimethoprim resistance of *E. coli* isolates of human and animal origin in Lithuania (Šeputienė *et al*., 2010).

Class 1 and class 2 integrons in *Enterobacteriaceae* which have been transferred by conjugation and found to be located on the plasmids of different size (>50 kb), represent a considerable source for horizontal transfer of a variety of antibiotic resistance genes among *Enterobacteriaceae*. Our observation that certain integron types, however, are common between isolates from different sources suggests the possibility of such transmission route (Povilonis *et al.,* 2010).

Integrons serve not only for the platform of antibiotic resistance genes, but also may be considered as a molecular markers characterizing multidrug-resistance phenotype. Integrons carrying isolates were more frequently resistant to almost all antibiotics tested in this study, compared with those without integrons. This suggests, that integron carrying bacterial strains may have better expressed genome plasticity, they become more susceptible to acquirement of an alien DNA by conjugation or transformation, present a more favourable environment for formation of the new

molecular genetic mechanisms which support the development of resistance to several different classes of antibiotics.

Plasmids are important elements associated with a spread and maintainence of antibiotic resistance in bacteria. Obtained data about the most common plasmid replicon groups (IncF and IncI1) detected in *Enterobacteriaceae* isolates, which carry integrons, correlates with the epidemic plasmids of *Enterobacteriaceae* associated with resistance to antibiotics (Carattoli, 2011). While there are certainly known some plasmid Inc groups related to a transfer of certain resistance gene, we did not find any significant correlation between the Inc group and particular class 1 and class 2 integron types.

The proportion of carbapenem-resistant *A. baumannii* in Lithuanian tertiary care medical centers has increased dramatically (Povilonis *et al.,* 2013). This study revealed that the most spread *A. baumannii* genotypes found in Lithuanian hospitals comprised PFGE genotype *Clusters 1* and *2*, whose isolates belonged to the international clonal lineages ECI and ECII, respectively. Isolates of ECI and ECII clonal lineages displayed characteristic resistance profiles and plasmid content, showing that different resistance mechanisms are developed by *A. baumannii* belonging to these clones (Povilonis *et al.,* 2013). The most prevalent carbapenemase producers, found in all hospitals were ECII isolates belonging to *Cluster 2* and carrying two copies of gene for acquired OXA-72 carbapenemase on the newly observed plasmid pAB120. Our study shows that clonal spread of OXA-72 producing ECII strains was largely responsible for a dramatic increase in the rate of carbapenem resistant *A. baumannii* in Lithuanian tertiary care hospitals (Povilonis *et al*., 2013). OXA-72-producing isolates have been reported only in the limited number of countries including Brazil, Colombia, Taiwan, USA, France and Croatia (Werneck *et al*., 2010; Montealegre *et al*., 2012; Lu *et al*., 2009; Tian *et al*., 2011; Barnaud *et al*., 2010; Franolic´-Kukina *et al*., 2011). In addition to two copies of gene for OXA-72 β-lactamase, flanked by XerC/XerD-like sites, pAB120 also carried genes for two putative toxin-antitoxin systems, which could be involved in the plasmid maintenance. Recent studies have demonstrated the presence of different DNA modules of various intervening regions flanked by XerC/XerD binding sites in *A. baumannii* plasmids (Grosso *et al*., 2012; Tian *et al*., 2011; D′Andrea *et al*., 2009; Merino *et al*., 2010). According to the recent observations, Xer proteins might act as a site-specific recombination system responsible for mobilization of $bla_{\text{OXA-24/40-like}}$ genes among

Acinetobacter species (D′Andrea *et al*., 2009; Merino *et al*., 2010). Indeed, plasmids with $bla_{\text{OXA-24/40-like}}$ gene integrated in different locations flanked by XerC/XerD-like binding sites were recently reported in *A. baumannii* strains from Spain, Italy, Portugal, USA and Colombia (Grosso *et al*., 2012; Tian *et al*., 2011; Montealegre *et al*., 2012; D′Andrea *et al*., 2009; Merino *et al*., 2010). To the best of our knowledge, the pAB120 is the first described plasmid harbouring two copies of gene, coding for OXA-72 β-lactamase observed in carbapenem-resistant *A. baumannii* isolates (Povilonis *et al*., 2013). However, the expression of two $bla_{\text{OXA-72}}$ gene copies in *A. baumannii* carrying pAB120 was not higher if compare to the same *A. baumannii* strain transformed with plasmid pAC92 harbouring single $bla_{\text{OXA-24/40}}$ gene copy as determined by real –time PCR (data not shown).

Very similar positions of XerC/XerD recognition sites downstream the replicase coding genes in pAB120 and in plasmids pAB0057/ p1ABTCDC0715 allowed us to presume that an ancestral *rep*Aci1/*rep*Aci2 - type plasmid, harbouring recombination targets of XerC/XerD could be employed as a platform for XerC/XerD-mediated recombination system to form plasmids with distinct genetic arrangement. The presence of a large region with another copy of $bla_{\text{OXA-72}}$ gene surrounded by XerC/XerD recognition sites and homologous to its counterparts in plasmids pABVA01 and pMMCU3, suggests that large common genetic platforms flanked by XerC/XerD recognition sites might participate in the dissemination of $bla_{\text{OXA-24/40-like}}$ genes and in the rearrangement of plasmids in *Acinetobacter* spp. (Povilonis *et al*., 2013). Moreover, the presence of another region in pAB120, harbouring the putative toxin-antitoxin system, bracketed with XerC/XerD-like sites and showing the highest identity to the regions in unrelated plasmids pAB1 and pAV2, suggests that different plasmids may exchange not only CHDL coding genes but also another components comprised within a limited region between the two closest XerC/XerD binding sites (Povilonis *et al*., 2013). Altogether, the structure of pAB120 consisting of the two DNA modules (with $bla_{\text{OXA-72}}$ gene each) may be a result of several recombination events, mediated by XerC/XerD recombinases.

XerC/XerD system seems not to be a unique site-specific recombination mechanism involved in the acquisition of CHDLs in plasmids of *A. baumannii.* Several studies reported *A. baumannii* plasmids associated with *rep*Aci1 and *rep*Aci2 genes,

carrying locus with bla_{OXA-58} gene bracketed by two copies of Re27 sequence (Poirel and Nordmann, 2006; Bertini *et al*., 2007; Zarrilli *et al*., 2008). Re27 and XerC/XerD-like binding sites reveal very high degree of sequence identity that further supports their involvement in acquisition of CHDLs by site-specific recombination.

Majority of *Acinetobacter* plasmids seem to be non-self-transmisible with a exception of plasmids belonging to GR6 (*rep*Aci6) carrying putative *tra* gene which may reflect the ability of self-conjugation and possess a potential to mobilize other plasmids carrying resistance genes included those coding for CHDLs (Bertini *et al*., 2010; Towner *et al*., 2011). pAB120 did not possess any elements responsible for mobilization. GR6 (*rep*Aci6) replicon type was significantly less prevalent in carbapenem-resistant *Cluster 2* isolates comparing with carbapenem-susceptible *Cluster 1* ($p<0.01$). Hence we think that wide dissemination of $bla_{OXA-24/40-like}$ genes in clinical isolates of *Cluster 2* may occur via clonal expansion rather than by horizontal transfer supported by helper plasmids belonging to group GR6.

Successful maintenance and long-term persistence of CHDLs harbouring plasmids may be due to the addiction systems based on two putative toxin-antitoxin systems detected in nucleotide sequence of plasmid pAB120. ORFs coding for the Gp49-like and Cro-like proteins show the homology to putative toxin and antitoxin coding genes of RelE and Xre/Cro families, respectively (Makarova *et al.,* 2009). Another pair, ORF5 and ORF4, coding for hypothetical inner membrane and cytoplasmic proteins, belonged to protein families DUF497 and COG3514, respectively, and bioinformatics analysis showed that these proteins might share structural similarity to toxin-antitoxin system of RelBE family (Makarova *et al*., 2009). Jurėnaitė *et al.* confirmed these elements to be functional toxin-antitoxin system (Jurėnaitė *et al.,* 2013).

Conclusions

1. The prevalence of integrons and a variety of their gene cassettes conferring resistance to antibiotics, shows the extent of MDR phenotype among Gram negative bacteria of clinical and animal sources in Lithuania and reflects the response to the treatment by aminoglycosides, sulfonamides/trimethoprim, β-lactams, macrolides;

2. Common gene cassettes in integrons from *Enterobacteriaceae* of clinical and animal origin and their conjugal transfer show that antibiotic resistance determinants of zoonotic microorganisms can be transferred from animals to human;

3. Molecular epidemiology of *A. baumannii* from Lithuanian hospitals shows the predominance of two major clonal groups belonging to epidemic European clones I and II with significant differences in the genetic profile of plasmids. IncF plasmids associated with the spread of antibiotic resistance determinants are prevalent among integron-positive *Enterobacteriaceae*;

4. A rapid spread of carbapenem-resistant *A. baumannii* in Lithuanian hospitals mainly caused due to the novel *A. baumannii* 11 kb plasmid pAB120 with two copies of genes coding for OXA-72 β-lactamase;

5. The genetic structure of pAB120 supports the presumption that Xer-based recombination may be responsible for the acquisition of $bla_{\text{OXA-24/40-like}}$ determinants in *A. baumannii* and represents a potential for the rearrangement of plasmids via common genetic platforms.

List of publications

- **Povilonis J**, Šeputienė V, Ružauskas M, Šiugždinienė R, Virgailis M, Pavilonis A, Sužiedėlienė E (2010). Transferable class 1 and 2 integrons in *Escherichia coli* and *Salmonella enterica* isolates of human and animal origin in Lithuania. *Foodborne Pathog Dis*. 7(10):1185-92.
- Šeputienė V, **Povilonis J**, Ružauskas M, Pavilonis A, Sužiedėlienė E (2010). Prevalence of trimethoprim resistance genes in *Escherichia coli* isolates of human and animal origin in Lithuania. *J Med Microbiol*. 59(Pt 3):315-22.
- **Povilonis J**, Šeputienė V, Krasauskas R, Juškaitė R, Miškinytė M, Sužiedėlis K, Sužiedėlienė E (2013). Spread of carbapenem-resistant *Acinetobacter baumannii* carrying a plasmid with two genes encoding OXA-72 carbapenemase in Lithuanian hospitals. *J Antimicrob Chemother*. 68(5):1000-6.

Conference presentations

- **J. Povilonis**, A. Bogdaitė, V. Šeputienė, R. Plančiūnienė, A. Pavilonis, E. Sužiedėlienė. Identification of a new integron-encoded OXA-type beta-lactamase, OXA-205, in a clinical isolate of *Pseudomonas aeruginosa*. *22 th European Congress of Clinical Microbiology and Infectious Diseases*. London, Great Britain, 2012 03 31 – 04 03.
- V. Šeputienė, **J. Povilonis**, A. Bogdaitė, R. Juškaitė, M. Miškinytė, E. Sužiedėlienė. Carbapenem-resistant *Acinetobacter baumannii* in Lithuanian hospitals. *22 th European Congress of Clinical Microbiology and Infectious Diseases*. London, Great Britain, 2012 03 31 – 04 03.
- **J. Povilonis**, V. Šeputienė, A. Vitkauskienė, E. Sužiedėlienė. Molecular typing of multidrug-resistant *Acinetobacter baumannii* from a tertiary care university hospital in Lithuania. *21th European Congress of Clinical Microbiology and Infectious Diseases/27th International Congress of Chemotherapy*. Milan, Italy, 2011 05 07 – 10.
- V. Šeputienė, A. Bogdaitė, **J. Povilonis**, R. Juškaitė, A. Vitkauskienė, E. Sužiedėlienė. OXA-carbapenemase producing *Acinetobacter baumannii* in tertiary care university hospital in Lithuania. *21th European Congress of Clinical Microbiology and Infectious Diseases/27th International Congress of Chemotherapy*. Milan, Italy, 2011 05 07 – 10.
- **J. Povilonis**, V. Šeputienė, M. Ružauskas, R. Šiugždinienė, A. Pavilonis, E. Sužiedėlienė. Prevalence of class 1 and class 2 integrons among *Escherichia coli* isolates of human and animal origin in Lithuania. *19th European Congress of Clinical Microbiology and Infectious Diseases*. Helsinki, Finland, 2009 05 16 - 19.
- **Oral presentation** „Atsparių antibiotikams *Acinetobacter baumannii* padermių, išskirtų 2010 m. Kauno klinikose, molekulinė epidemiologija“. IV-oji nacionalinė doktorantų mokslinė konferencija„Mokslas – sveikatai**“.** Kaunas, Lithuania, 2011 04 07.

- **Oral presentation** „Dauginis atsparumas antibiotikams: I ir II klasės integronai Lietuvoje paplitusiose patogeniškose *Escherichia coli*, *Salmonella enterica* ir *Shigella* spp. padermėse“ konferencijoje „Mikroorganizmų atsparumas antimikrobinėms medžiagoms: klinikiniai aspektai“. Panevėžys, Lithuania, 2008 11 26.

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Santrauka

Šis darbas yra pirmasis Lietuvoje išskirtų atsparių antibiotikams Gram neigiamų patogeninių bakterijų išsamus molekulinės epidemiologijos tyrimas. Darbe apibūdinti atsparumo antibiotikams pernašos genetiniai elementai iš 859 Gram neigiamų bakterijų padermių, išskirtų iš sergančių žmonių, sveikų ir sergančių gyvūnų.

Tyrime nustatytas integronų paplitimo dažnis (~20 – 50 %) ir jų genų, lemiančių atsparumą antibiotikams, įvairovė parodo dauginio atsparumo antibiotikams fenotipo išplitimo mastą klinikinės ir gyvūninės kilmės Gram neigiamose bakterijose bei liudija apie atsaką gydymui vartojamiems aminoglikozidų, sulfonamidų/trimetoprimo, β-laktamų, makrolidų klasių antibiotikams. Darbe nustatyti trys nauji integronai ir ištirta jų struktūra. *P. aeruginosa* integrone nustatyta nauja D klasės β-laktamazė OXA-205, kuri papildo OXA tipo β-laktamazių, paplitusių antibiotikams atspariose bakterijose, įvairovę. Bendros genų kasetės klinikinės ir gyvūninės kilmės *Enterobacteriaceae* šeimos bakterijų integronuose ir parodyta integronų pernaša liudija, kad atsparumą antibiotikams lemiančios genetinės determinantės per zoonotinius mikroorganizmus gali būti pernešamos iš gyvūnų į žmogaus organizmą

Apibūdinta hospitalinių infekcijų sukėlėjo *Acinetobacter baumannii* populiacija, cirkuliuojanti Lietuvos ligoninėse. Ypatingas dėmesys skirtas *A. baumannii* plazmidžių tyrimams, nes vis dar stokojama duomenų apie šiai rūšiai būdingas plazmides. Parodyta, kad Lietuvos ligoninėse cirkuliuoja *A. baumannii* pandeminių klonų linijos ECI ir ECII, kurioms būdingi skirtingi plazmidžių replikonų tipai ir jų deriniai. Nustatyta nauja *A. baumannii* plazmidė pAB120, turinti atsparumo karbapenemams genus ir pasižyminti rekombinacijos potencialu; parodyta, kad plazmidę turinčios *A. baumannii* padermės išplito mūsų šalies ligoninėse per pastaruosius kelerius metus ir taip lėmė atsparumo karbapenemams šuolį. Šiame darbe nustatyti ir apibūdinti genetiniai elementai gali būti naudojami kaip taikiniai greitam *A. baumannii* klonų nustatymui molekuliniais metodais ir kaip molekuliniai žymenys infekcijų kontrolei.

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