sciendo

PROCEEDINGS OF THE LATVIAN ACADEMY OF SCIENCES. Section B,
Vol. 75 (2021), No. 2 (731), pp. 142–148.

DOI: 10.2478/prolas-2021-0022

RETROSPECTIVE STUDY OF GENETIC DIVERSITY OF *ACINETOBACTER BAUMANNII*-RESISTANT STRAINS ISOLATED FROM PATIENTS IN RĪGA EAST UNIVERSITY HOSPITAL IN LATVIA

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Acinetobacter baumannii *is an aerobic gram-negative opportunistic bacterial pathogen, an emerging cause of healthcare-associated infections, associated with increased morbidity, mortality and healthcare costs. It has been widely found in the hospital environment, exhibiting high resistance to antimicrobials, affecting the spread of healthcare-associated infections and preventing effective infection control. The role of virulence factors in the pathogenesis of* A. baumannii *related human infections remains unclear. Therefore, molecular testing of pathogenic bacteria is an important tool for improving infection control measures against* A. baumannii *with combined resistance. The aim of this study was to analyse* A. baumannii *infection cases, antimicrobial resistance profiles and to characterise the genetic heterogeneity of isolates. In general, outbreaks occurring in hospitals are presumed to be clonal, with patient-to-patient transmission of essentially identical strains. Treatment decisions are based on a combination of* in vitro *susceptibility assays and empirical results based on patient outcomes.*

Key words: A. baumannii*, virulence factors, whole genome sequencing.*

INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest public health challenges of our time, already in a stage of crisis attributed to the overuse and misuse of these medications, as well as a lack of new drug development by the pharmaceutical industry due to reduced economic incentives and challenging regulatory requirements. According to the World Health Organisation (WHO), approximately 700 000 deaths were reported worldwide in 2014 due to infections caused by resistant pathogens. The number of such deaths is projected to rise to 10 million in 2050, if the current situation does not change significantly (O'Neill *et al*., 2017).

The biggest concern is imposed by the 'ESKAPE' pathogens comprised of highly multi-, extended- or pan-drug resistant, such as vancomycin-resistant *Enterococcus faecium*; methicillin-resistant *Staphylococcus aureus*; *Klebsiella pneumoniae*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, which are multi-drug resistant, including resistance to carbapenems; and *Enterobacter* spp., which contain extended-spectrum beta-lactamases (ESBLs) and carbapenemases (Rice *et al*., 2008).

In 2017, WHO compiled a list of "priority" pathogens posing the greatest threat to human health to help guide and promote research and the development of new antibiotics. One of the bacteria that has been given the first or critical priority grade is the carbapenem-resistant *A. baumannii* (Anonymous, 2017).

A. baumannii is an opportunistic bacterial pathogen, an emerging cause of healthcare-associated infections (HAIs),

associated with increased morbidity, mortality and healthcare costs (Mazumder, 2021).

The pathogen was first mentioned in 1986 by Bouvet and Grimont. Based on DNA-DNA hybridisation studies, the authors discovered three new *Acinetobacter* species: *Acinetobacter baumannii*, *Acinetobacter johnsonii*, and *Acinetobacter junii* (Bouvet *et al*., 1986).

A. baumannii is one of the pathogens with a high ability to acquire antimicrobial resistance. The pathogen is characterised by a number of resistance mechanisms, including β-lactamases, aminoglycoside-modifying enzymes, the ability to cause pore permeability defects in the cytoplasmic membrane, and an altered "target" in the cell. The coexistence of several of these mechanisms gradually reduces the number of antimicrobials that could be used to treat *A. baumannii* infection (Lee *et al*., 2017).

Infections caused by the *A. baumannii* include healthcare-associated pneumonia, urinary tract, bloodstream, central nervous system (especially meningitis) and surgical wound infections (O'Neill *et al*., 2017). The main risk factors for such infections are long-term hospital stay, treatment in the intensive care unit, various invasive procedures, including lung ventilation, central venous catheters, urinary catheters, as well as various chronic health conditions, for example, diabetes and obesity (Diancourt *et al*., 2010; Anonymous, 2016b). *A. baumanii* can be detected in various swab specimens, different body fluids (urine, blood), catheter tips, etc. If the bacterium is identified in sputum or urine samples, it is more likely to indicate colonisation, not active infection (Anonymous, 2016a; Liepiņš *et al*., 2016).

According to the European Centre for Disease Prevention and Control Infectious Disease Surveillance Atlas of the situation in Latvia in 2016, it can be concluded that 73.2% of all *A. baumannii* isolates were resistant to carbapenems (CRAB) and 67.2% exhibited combined resistance (XDR – multidrug or extensively drug resistant *A. baumannii*). The worst situation in Europe in 2016 was in Greece, where CRAB was identified in 95.4% and XDR in 84% of *A. baumannii* related cases. In contrast, the best situation in 2017 was observed in Norway, Finland, Netherlands, and Denmark, where CRAB and XDR accounted for 0% of the total number of *A. baumannii* isolates. Compared to 2017, in 2015 in Latvia CRAB isolates were found in 79.4% and XDR — in 75.0% of cases (Fournier *et al*., 2006; Liepiņš *et al*., 2016).

In 2016, results on the prevalence of XDR at Rīga East University Hospital between 2009 and 2015 were published. In 2009, 71 cases of XDR were detected, in 2013 this number reached a maximum of 217 cases, and in 2015 it decreased to 113 cases. In the period from 2013 to 2015, the number of cases of invasive *A. baumannii* infection and colonisation was studied. In 2013, in the case of invasive infections, 33 cases of XDR were confirmed by detecting pathogens in blood and cerebrospinal fluid materials. In 2015, this number decreased to 19 isolates. All patients with sepsis and CNS infections received colistin. In 2013, AMR mostly exceeded 80%, and in 2014 it was already 90%. It should be noted that resistance to colistin was 0% in 2013 and 4% in 2014 (Anonymous, 2016a).

Distinguishing contamination/colonisation from invasive infection is essential in choosing the principal treatment strategy, as colistin is not indicated in cases of contamination or colonisation. Unjustified initiation of colistin may increase the risk of subsequent AMR. Such strains exhibit resistance to all currently available antimicrobial agents and combinations, including colistin/imipenem, colistin/meropenem, colistin/rifampicin, colistin/tigecycline, colistin/sulbactam, colistin/teicoplanin and imipenem/sulbactam (Maragakis *et al*., 2008).

In the treatment of drug-resistant *A. baumannii* infections, colistin is indicated in cases of XDR, while amikacin is preferred in patients with CRAB.

In general, outbreaks occurring in hospitals are presumed to be clonal, with patient-to-patient transmission of essentially identical strains. Treatment decisions are based on a combination of *in vitro* susceptibility assays, and empirical results are based on patient outcomes. Molecular biology methods such as pulsed field gel electrophoresis (PFGE) and polymerase chain reaction (PCR) are the basis for strain typing and evaluation of relatedness, but in some cases identical molecular typing signature is not sufficient to distinguish closely related strains. Until now the highest discriminatory power between two strains can be achieved by whole genome sequencing (WGS). Analysis of WGS can be useful in monitoring *A. baumannii* clonality and genetic relatedness as well as characterisation of antimicrobial gene profile and virulence factors to select appropriate treatment (Adams *et al*., 2010; Fishbain *et al*., 2010).

The aim of the study was to analyse cases of *A. baumannii* infection, antimicrobial resistance profiles and to characterise genetic heterogeneity of *A.baumannii* isolates.

MATERIALS AND METHODS

This study was conducted at Rīga East University Hospital between May 2015 and October 2016. Isolates, determined as *A. baumannii,* were obtained from patient samples for microbiological investigations, and cultivated on selective MacConkey agar (*HiMedia,* India) according to laboratory methodologies. Culture identification and phenotypic antimicrobial susceptibility were confirmed by VITEK®2 GN identification card (bioMerieux, France) to the following antimicrobial agents: ciprofloxacin, piperacillin/tazobactam, ampicillin/sulbactam, ceftazidime, imipenem, meropenem, gentamicin, amikacin, and colistin. According to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints (v. 5.0, valid from 2015-01-01), all isolates were categorised as susceptible (S), intermediate (I) or resistant (R). Based on the obtained resistance patterns to the mentioned antimicrobial agents, all *A. baumannii* iso-

lates were divided into three groups: 1) fully susceptible *A. baumannii* (no resistance to ciprofloxacin, piperacillin/tazobactam, ampicillin/sulbactam, ceftazidime, imipenem, meropenem, gentamicin, amikacin and colistin); 2) carbapenem resistant *A. baumannii* (CRAB) — resistant to resistant to all antimicrobials, except aminoglycosides and colistin; and 3) multidrug or extensively drug resistant *A. baumannii* (XDR) — resistant to all antimicrobials, except colistin.

Whole genome sequencing (WGS) was used to analyse the genome of *A.baumannii* isolates. Sequencing and data analysis were performed at the Institute of Food Safety, Animal Health and Environment "BIOR".

DNA extraction and sequencing. Selected isolates were cultivated on nutrient agar (Biolife, Italy) at 37 °C for 24 hours. For genomic DNA extraction one colony from each isolate was selected, resuspended in 180 µl lysis buffer from the QIAamp DNA Mini kit (QIAGEN Manchester Ltd. Manchester, United Kingdom) and treated according to manufacturer protocol for Gram-negative bacteria. Concentration of DNA was measured on Qubit and 1 ng was used for library construction using the Nextera XT Library preparation kit (Illumina, San Diego, California (CA), United States (US)). Sequencing runs were performed on an Illumina Miseq using V3 chemistry for 2×300-bp paired end reads. For genome assembly, Velvet 1.1.04 (Zerbino *et al*., 2008) was used; each sequence was trimmed until the average Phred quality was 30 in a window of 20 bp and genome de novo assembly was done. Expected genome size was 3.9 Mb and targeted coverage was 70×. The genome assembly was assessed by N50 (minimum 10 000) and the mean genome coverage (minimum 30×). Samples with parameters below quality settings were excluded from analysis (Zerbino *et al*., 2008).

Data analysis. Genetic relatedness of *A. baumannii* genomes was analysed by the Ridom SeqSphere+ 5.0.0 software (Ridom, Muenster, Germany) (Jünemann *et al*., 2013). Comparison of genomes was done based on a multilocus sequence typing (MLST) scheme elaborated in the Pasteur Institute [5] and core genome (cg) MLST with 2390 targets and Accessory MLST with 1083 targets schemes (Jünemann *et al*., 2013., Higgins *et al*., 2017) . The newly identified alleles were submitted to the cgMLST nomenclature server (www.cgmlst.org) maintained by Ridom.

The presence of virulence factors was determined by screening assembled contigs against VFDB, version 18 March 2018 with ABRicate (https://github.com/tseemann/abricate), using the default settings. For gene presence determination, the cut-off values were 85% for sequence identity and 50% for sequence length (Chen *et al*., 2016).

For calculation of statistical significance, the chi-squared test and Fischer's exact test were used where applicable to calculate the odds ratios (OR) and 95% confidence intervals (CI) by using two-by-two frequency tables of the respective overlaps. A p value of 0.05 was considered statistically significant.

Permit No. 9-A/14 of the Medical and Biomedical Research Ethics Committee of Rīga East University Hospital Support Fund had been issued for this research (Rīga, 10.07.2014).

RESULTS

A total of 93 *A. baumannii* isolates were included in the study. Colonisation/contamination was observed in 66% (n = 61) of cases, while *A. baumannii* infection was detected in 34% (n = 32) of cases. The colonisation/contamination criteria for *A. baumannii* were as follows: *A. baumannii* had no clinical significance for the patient's disease, as no signs of disease were observed; the empirical antimicrobial treatment, which was not directed against *A. baumannii*, was successful; and *A. baumannii* obtained by screening patients (nasopharyngeal or rectal swab) as a result of an investigation into an outbreak of healthcare associated infections. The most prevalent department of isolation was the intensive care unit (60%). Presence of the *A. baumannii* microorganism mainly was confirmed on the 20^{th} day of hospitalisation

The distribution of infections was as follows: pneumonia — 17%, deep surgical wound infection — 7%, meningitis, sepsis, tracheobronchitis, central venous catheter associated bacteremia — 2% each, and urinary tract infection and osteomyelitis — 1% each. Infection cases were defined according to case definitions of healthcare-associated infections by the European Centre for Disease Prevention and Control, Point prevalence survey of healthcare-associated infections and antimicrobial use in European acute care hospitals, protocol version 5.3 (Anonymous, 2016b).

It was found that in 81% (n = 72) of cases the identified *A. baumannii* strains were XDR, in 15% (n = 13) — CRAB and only in 4.5% (n = 4) of cases *A. baumannii* was sensitive to all tested antimicrobials.

ST and cgMLST were determined using WGS data of all 93 *A. baumannii* isolates and 76 out of 93 were identified as ST 2, although in total five different STs were found (Fig. 1). Analysis of ST 2 isolates revealed at least six previously reported cgMLSTs, the largest group including 43 isolates while several cgMLSTs were represented by only one isolate. Between individual ST2 isolates, difference from 0 to 35 of the analysed 2390 alleles was observed with average distance 9.36 alleles (Table 1, Fig. 1). We found strong association between ST and AMR status, most of the MRAB isolates belonging to ST2 (p 0.0001) while CRAB isolates were more common in ST1 and ST826.

There were eight cases when the second *A. baumannii* isolate was obtained from the same patient after at least three months. Analysis of isolates demonstrates that the majority of the second isolates represented the same ST and even the same cgMLST as the first isolate, but in two cases, two isolates with different ST were observed.

To evaluate pathogenicity of *A. baumannii* isolates, the presence of previously known virulence genes was analysed

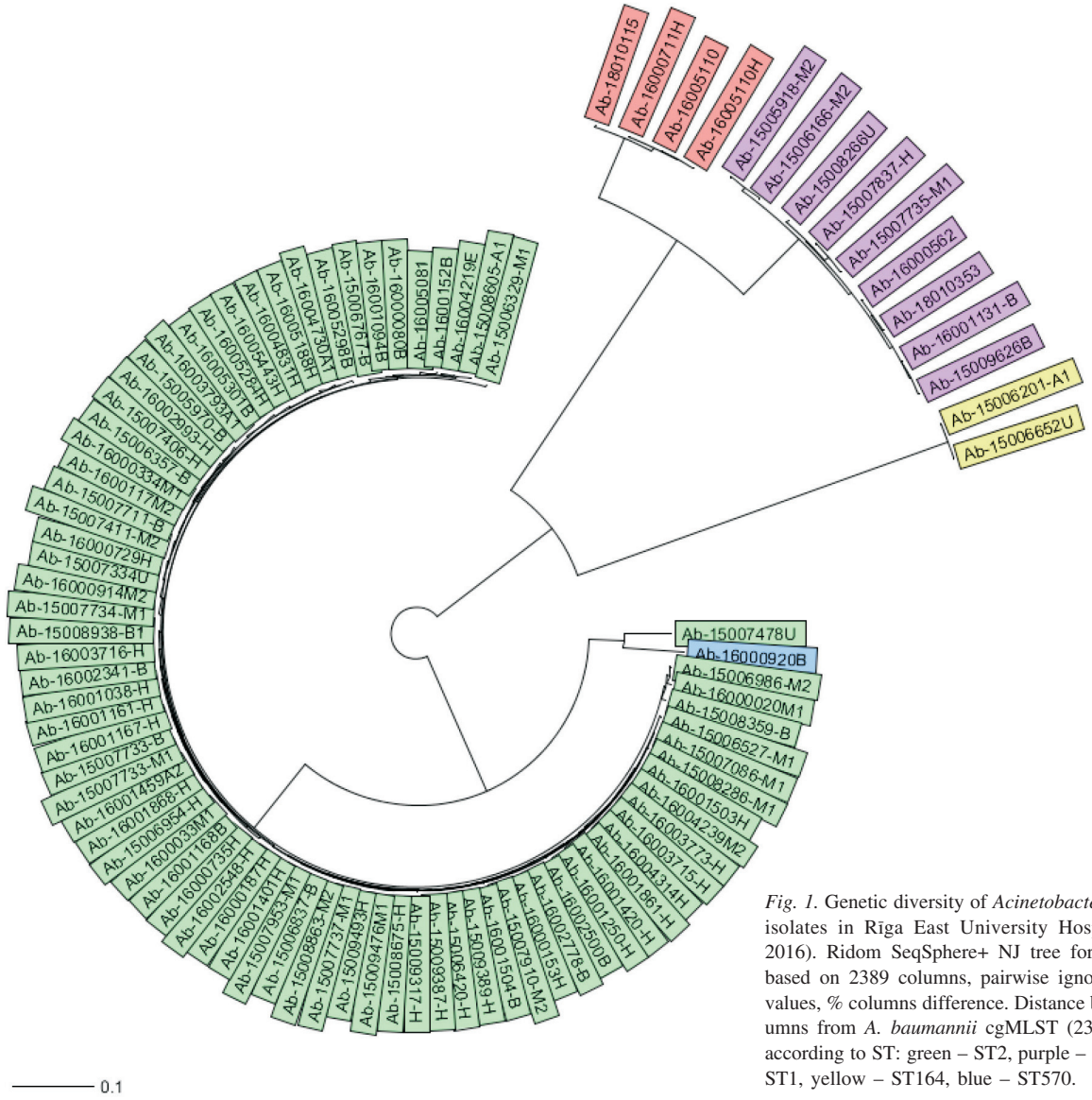

Fig. 1. Genetic diversity of *Acinetobacter baumannii* isolates in Rīga East University Hospital (2015–2016). Ridom SeqSphere+ NJ tree for 93 samples based on 2389 columns, pairwise ignoring missing values, % columns difference. Distance based on columns from *A. baumannii* cgMLST (2389). Colours according to ST: green – ST2, purple – ST286, red – ST1, yellow – ST164, blue – ST570.

Table 1. Heterogeneity of *A. baumannii* isolates based on the Pasteur MLST 7 gene scheme and cgMLST scheme based on 2389 genes

ST	cgMLST	Isolates, n	Distance between isolates (alleles)	XDR	CRAB	Sensitive
ST2	cgMLST 1534	4	0–3	3	0	0
	cgMLST 1948	3	3–4	2	0	0
	cgMLST 2161	43	0–17	38	2	0
	cgMLST 2838	3	7–8	3	0	0
	cgMLST 2839	17	0–10	13	1	1
	cgMLST 2841	2	22	2	0	0
	cgMLST 2842	1	-	0	1	0
	cgMLST 2843	1	-	1	0	0
	cgMLST 2844	1	-	1	0	0
	cgMLST 2845	1	-	1	0	0
ST826	cgMLST 2840	2	0	0	2	0
	new	7	7–53	2	4	0
ST1	new	4	0–22	2	0	1
ST164	cgMLST 1428	2	4	0	2	0
ST570	new	1	-	1	0	0

in WGS data. In total, 71 genes representing various functions in *A. baumannii* pathogenesis like adherence, invasion, induction of apoptosis, serum resistance, biofilm formation and persistence were analysed (listed in Table 2). Each isolate contained from 53 to 71, median 59 of selected virulence genes. We identified several genes like *bap*, *bauA*, *abaR* that were more often found in the XDR group than in the CRAB group (Table 3). Two of them, *bap* and *bauA*, were exclusively found in ST2 isolates and were missing in isolates from other common STs–ST1 and ST826.

In our study, we did not find any association between the *A. baumannii* virulence gene profile and other parameters like infection type, patient diagnosis and source of isolates.

DISCUSSION

Antimicrobial resistance is a significant problem in hospitals of many countries, including in Latvia (Anonymous, 2016c; Fitzpatrick *et al*., 2016., Kanafani *et al*., 2021).

Table 2. Virulence gene/number of isolates in relation to virulence

Virulence mechanism		Virulence gene/ Number of isolates gene is present
Adherence	Omp	*OmpA(93)*
Biofilm formation	AdeFGH efflux pump	*adeF(93); adeG(93); adeH(93)*
	Bap	***bap(77)***
	Csu fimbriae	*csuA(87); csuA/B(87); csuB(90); csuC(91); csuD(91); csuE(91)*
	PNAG	*pgaA(93); pgaB(93); pgaC(93); pgaD(93)*
Enzyme	Phospholipase C	*plc(93)*
	Phospholipase D	*plcD(93)*
Immune evasion	Capsule	*ACICU_00071(88); ACICU_00072(93); ACICU_00073(91); ACICU_00074(93);* ***ACICU_0087(52); ACICU_00075-00086 (1)***
	LPS	*lpsB(93); lpxA(93); lpxB(93); lpxC(93); lpxD(93); lpxL(93); lpxM(93)*
Iron uptake	Acinetobactin	*barA(93); barB(93); basA(92); basC(83); basD(83); basG (92); basI(83); basJ(91);*
		bauA(77)*; bauB(93); bauC(93); bauD(93); bauE(93); bauF(93); entE(93)*
Regulation	BfmRS	*bfmR(93); bfmS(93)*
	Quorom sensing	*abaI(93); abaR(83)*
Serum resistance	Pbp	*pbpG(93)*

Table 3. Virulence gene presence among XDR *A. baumannii* and carbapenem resistant *A. baumannii* (CRAB)

Virulence gene	XDR (n; %)	CRAB (n; %)	*p* value*
ACICU_00087	47 (63)	3 (23)	0.013
abaR	69 (92)	9 (69)	0.037
Bap	68 (91)	4 (31)	< 0.00001
bauA	68 (91)	4 (31)	< 0.00001
basC	72 (96)	6 (46)	0
basD	71 (95)	6 (46)	0.0001
bas1	67 (89)	11 (85)	0.638

* *p* value calculated for number of XDR and CRAB

A. baumannii resistance also affects the spread of healthcare-associated infections affecting the spread of HAIs and preventing effective infection control. In order to improve the current situation, implementation of an antimicrobial drug management programme (Stewardship programme) and introduction of an AMR monitoring system is necessary, thus promoting more appropriate use of antimicrobials and reducing the problem of pathogen resistance. The introduction of such preventive measures in a hospital would also reduce the incidence of *A. baumannii* strains with combined resistance, with a particular focus on the right choice of drugs to reduce selection pressure and further spread of resistant strains (Owens *et al.*, 2006; Perez *et al.*, 2007).

In our study, culture identification and antimicrobial susceptibility confirmation by the VITEK®2 GN identification card (bioMerieux, France) did not affect the interpretation of the data, because during the study antimicrobial susceptibility of *A. baumannii* was determined according to the EUCAST breakpoints, which was integrated into the VITEK®2 GN identification card (bioMerieux, France).

In our study, 34% of patients had a confirmed *A. baumannii* infection. It is crucial to distinguish colonisation from infection in order to avoid unjustified initiation of antimicrobial therapy.

Clinicians and researchers have often considered that infections caused by *A. baumannii* are not associated with high lethality. Healthcare professionals report that the microorganism has not caused a significant increase in mortality in hospitalised patients, so the mortality caused by *A. baumannii* infection remains a contentious issue with several studies showing controversial results (Park *et al.*, 2013; Xiao *et al.*, 2017).

Analysis of WGS of *A. baumannii* isolates showed that in 2015–2016 there were several clones of ST2 clones circulating in Rīga East University Hospital. The ST2 genotype is associated with multidrug resistance and was previously reported as an endemic strain in European and USA hospitals, often involved in outbreaks (Fitzpatrick *et al.*, 2016). In our study, comparison of isolates from reinfection in individual patients demonstrated that in some cases the strain was the same as in the first infection episode, but in most cases the genetic distance was large enough to assume unrelated cases of infection. Using WGS, it is possible to follow development of infection within one patient as well as for surveillance of larger intra-hospital outbreaks (Kim *et al.*, 2018; Gramatniece *et al.*, 2019).

In our study we found that the virulence gene profile was more associated with a certain ST and genotype than other parameters of infection. Detailed studies of virulence fac-

tors and their role in pathogenesis allow us to understand the dominant role of certain ST and target new therapies against these factors. In hospitals, widely spread ST2 genotypes carry specific genes like *bap* and *bauA*, which provide an advantage over other STs. *bap* is known to be involved in biofilm formation, and *bauA* is related to iron uptake (Brossard *et al*., 2012; Sefid *et al*., 2015). Further research is necessary to continue the study.

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Received 22 July 2020
Accepted in the final form 13 April 2021

ACINETOBACTER BAUMANNII REZISTENTO CELMU ĢENĒTISKĀS DAŽĀDĪBAS RETROSPEKTĪVS PĒTĪJUMS RĪGAS AUSTRUMU KLĪNISKAJĀ UNIVERSITĀTES SLIMNĪCĀ, LATVIJĀ

Acinetobacter baumannii ir aerobs gramnegatīvs oportūnistisks mikroorganisms, kas ir ar veselības aprūpi saistītu infekciju cēlonis, saistīts ar paaugstinātu saslimstību, mirstību un veselības aprūpes izmaksām. *A. baumannii* piemīt augsta izturība pret antimikrobiāliem līdzekļiem, kas ietekmē ar veselības aprūpi saistīto infekciju izplatīšanos un prasa efektīvu infekciju kontroli. Virulences faktoru loma ar *A. baumannii* saistīto infekciju patoģenēzē joprojām nav skaidra, tāpēc mikroorganisma molekulāri ģenētiskā analīze ir svarīgs līdzeklis, lai uzlabotu infekcijas kontroles pasākumus rezistentu *A. baumannii* identifikācijas gadījumā. Šī pētījuma mērķis bija analizēt *A. baumannii* infekcijas gadījumus, antimikrobiālās rezistences profilu un raksturot *A. baumannii* ģenētisko heterogenitāti un molekulāri bioloģiskos parametrus. Ārstēšanas lēmuma pamatā ir antimikrobiālās jutības testu un empīrisko rezultātu kombinācija. Kopumā pētījumā tika iekļauti 93 pacienti ar *A. baumannii*. *A. baumannii* infekcija tika atklāta 34% (n = 32) gadījumu, savukārt kolonizācija/kontaminācija tika novērota 66% (n = 61) gadījumu. Infekciju sadalījums bija šāds: pneimonija — 17%; dziļā ķirurģiskās brūces infekcija — 7%; meningīts, sepse, traheobronhīts, ar centrālo venozo katetru saistītā bakteriēmija — katra pa 2% un urīnceļu infekcija un osteomielīts — katrs pa 1%. Tika konstatēts, ka 81% (n = 72) gadījumā identificētie *A. baumannii* celmi bija multirezistenti, 15% (n = 13) — karbapenēmu rezistenti un 4,5% (n = 4) gadījumu *A. baumannii* bija jutīgs pret visiem pārbaudītajiem antimikrobiāliem līdzekļiem. Analizējot *A. baumannii* izolātu genomu, noskaidrots, ka pētījuma laikā cirkulēja vairāki ST2 kloni, kas ir saistīti ar daudzu antimikrobiālo līdzekļu rezistenci. Pētījumā mēs neatradām saistību starp *A. baumannii* virulences gēnu profilu un citiem parametriem, piemēram, infekcijas veidu, pacienta diagnozi un mikrorganisma avotu.