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MICROBIOLOGICAL AND MOLECULAR CHARACTERIZATION OF CLINICAL CARBAPENEM-RESISTANT *Pseudomonas aeruginosa* ISOLATES

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CONTENTS

ABBREVIATIONS

ABC	ATP-binding cassette
AMI	Amikacin
attI	Cassette integration site
AZT	Aztreonam
bla	Gene encoding β-lactamase
BCE	Before common era
C1	Integron of class 1
CAPITAL	Carbapenem Antimicrobials Pseudomonas Isolate Testing At regional Locations surveillance program
CE	Common era
CFU	Colony-forming unit
CIP	Ciprofloxacin
CI	Chromosomal integron
CIT	Cloxacillin inhibition test
CLSI	Clinical and Laboratory Standards Institute Subcommittee on Antimicrobial Susceptibility Testing
COL	Colistin
CRE	Carbapenem-resistant *Enterobacteriaceae*
DDD	Direct disk diffusion
DNA	Deoxyribonucleic acid
EARS-Net	European Antimicrobial Resistance Surveillance System network
EU	European Union
FEP	Cefepime
Glc*N*Ac and NAG	N-acetylglucosamine acid
HAIs	Hospital acquired infections
I	Intermediate
ICU	Intensive care unit
IMP	Imipenem
IN	Inner membrane
IntI	Integrase gene
LGT	Lateral gene transfer
LPS	Lipopolysaccharide
MATE	Multidrug and toxic compound extrusion family
MBL	Metallo-β-lactamase
Mbp	Megabase-pair
MDR	Multidrug-resistant

MER	Meropenem
MF	Major facilitator family
MFP	Periplasmic membrane fusion protein
MHT	Modified Hodge test
MIC	Minimum inhibitory concentration
MIs	Mobile integrons
MLST	Multi locus sequence typing
Multiplex PCR	Multiplex polymerase chain reaction
Mur*N*Ac or NAM	N-acetylmuramic acid
OM	Outer membrane
PABA	*P*-aminobenzoic acid
PBPs	Penicillin-binding proteins
Pc	Promoter
PFGE	Pulse-field gel electrophoresis
R	Resistant
R/T4	Piperacillin/tazobactam
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division family
S	Susceptible
SMR	Staphylococcal/small multidrug resistance family
ST	Sequence type
TAZ	Ceftazidime
TIC	Ticarcillin
TOB	Tobramycin
TP	Transpeptidation domain
VC	Viable counts
XDR	Extremely drug resistant

INTRODUCTION

Since 1940, antibiotics have significantly transformed medicine and helped treating serious infections caused by bacteria; however, over-consumption and inappropriate use wanes the effectiveness of drugs by slowly development of resistant bacteria strains capable easily spread across continents [1, 2]. No successful antibiotics of new classes have been discovered since 1987, in particularly to treat gram-negative bacteria. A recent database listed the existence of more than 20,000 bacterial resistance genes by warning us about impending health crisis and returning to the pre-antibiotic era [3]. A brand new report commissioned by United Kingdom Prime Minister David Cameron has estimated that antibiotic resistance will kill 300 million people and global costs for treatment will reach more than $100 trillion by 2050 [4]. An alarming increase in resistance has been observed especially in *Clostridium difficile*, carbapenem-resistant *Enterobacteriaceae* (CRE), and *Neisseria gonorrhoeae* [1].

In particular *Pseudomonas aeruginosa* (*P. aeruginosa*) remains one of the major pathogens associated with nosocomial infections, predominantly pneumonia, urinary tract infections, as well as skin and soft-tissue infections [5]. Antibiotic options of *P. aeruginosa* are limited or ineffective due to the predisposition of this bacterium to develop resistance to multiple classes of antibacterial agents [6]. Mortality risk of patients with *P. aeruginosa* infection can exceed over 58.8% including the severity of the underlying condition [7]. Carbapenems are often used as “last-line” agents for treating serious infections caused by multidrug resistant (MDR) *P. aeruginosa* [8]. However, data from European countries, covered by the European Antimicrobial Resistance Surveillance System (EARSS, now EARS-Net), showed an increasing trend in the prevalence of carbapenem-resistant *P. aeruginosa* strains varying between 4.4% and 58.5% among different countries [9]. In Lithuania, the prevalence of carbapenem-resistant strains also increased from 21% to 29% during 2006–2014 [9].

The development of carbapenem resistance in *P. aeruginosa* is multifactorial, but primarily includes several mechanisms such as multidrug efflux system overexpression, lower outer membrane (OM) permeability reduction, or production of chromosomal *ampC* β-lactamase [10]. Besides, the acquisition of carbapenem-hydrolyzing enzymes (also known as metallo-β-lactamases, MBL) is considered as the most significantly mechanism of carbapenem resistance in this bacterium due to an increase in carbapenem use. These enzymes are known to hydrolyze all classes of β-lactams except monobactams [11] .

Verona-integron-encoded metallo-*β*-lactamase (VIM) is one of the MBLs of foremost global importance [12]. Among these types of enzymes, VIM-2 is apparently the most widespread acquired MBL determinant reported worldwide.

The genes responsible for the production of VIM-2 are typically part of an integron and are mobilized by transferable plasmids or integrons. Thus, they can be easily transferred to various types of bacteria [13]. Recently it was reported that VIM-type metallo-*β*-lactamase-producing *P. aeruginosa* is associated with a clone, identified as the internationally spread sequence type 235 (ST235) [14].

To our knowledge, no report on MBL production with VIM-2 enzyme has been published in Lithuania. Until now, no standard method to detect MBL has been established knowing that many counties are likely to have their own reservoirs of resistance genes and strains. Accordingly, we decided to determine the occurrence of MBL-producing *P. aeruginosa* among isolates collected in the Hospital of Lithuanian University of Health Sciences.

Aims and objectives

The aim of this work was to determine molecular mechanisms of resistance to carbapenems in *P. aeruginosa* strains isolated from different clinical specimens.

Objectives of this study:

1. To determine the resistance of carbapenem-resistant *P. aeruginosa* strains to antibiotics of other classes;
2. To evaluate associations between the virulence factors of *P. aeruginosa* strains, such as resistance to serum bactericidal activity as well as biofilm-forming abilities, and resistance to antibiotics;
3. To identify the main resistance mechanisms of *P. aeruginosa* clinical isolates to antibiotics of carbapenem class by different phenotypic methods;
4. To determine the ability of *P. aeruginosa* strains to produce metallo-*β*-lactamases and to identify the prevailing genetic profiles-pulsotypes as well as the structure of integrons and the gene cassette.

Scientific novelty

Despite the increasing rate of carbapenem resistance in Lithuania, there is still lack of a comprehensive understanding of the *P. aeruginosa* mechanisms controlling outbreaks of antimicrobial resistance.

This study was the first to describe the molecular mechanism of carbapenem resistance in *P. aeruginosa* isolates from Lithuania. Our results showed that more than 40% of imipenem- and/or meropenem-resistant isolates possessed MDR and XDR phenotypes. In this work, we report for the first time a detailed genetic analysis demonstrating that MBL VIM-2 was by far the most prevalent acquired β-lactamase in the Hospital of Lithuanian University of Health Sciences. Moreover, a high prevalence of VIM-2 was found to be driven by the dissemination of high-risk clone ST235. Thus, our work adds Lithuania to the growing list of countries documenting epidemic dissemination of this clonal lineage linked to potent β-lactamases. Additionally, our work resulted in the identification of a new GES variant, referred to as GES-27, which has not been described previously.

1. LITERATURE REVIEW

1.1. Drug-resistant gram-negative bacteria – growing health problem

Antibiotics have been always considered one of the greatest medical breakthroughs of the last century, from which the world began the era of antibacterial agents. It is not necessary to reiterate how many lives they have saved and how significantly they have contributed to control infectious diseases associated with increased morbidity and mortality.

Unfortunately, it is inevitable that for more than 60 years of widely used of antibiotic therapy bacteria have adapted and develop resistance to almost all forms of existing antibiotic agents, thus making the drugs less effective and infections more difficult to control.

Nowadays, antimicrobial resistance has become a worldwide problem, leading to increasing healthcare costs and treatment failure and 25,000 deaths of patients in the European Union (EU) [15]. In 2014, the European Antimicrobial Resistance Surveillance System network (EARS-Net) reported increasing antimicrobial resistance percentages in gram-negative bacteria especially in *Escherichia coli* (*E. coli*), *Klebsiella pneumoniae* (*K. pneumoniae*), and *P. aeruginosa* strains [9]. The latest report showed that the prevalence of *E. coli* resistant to third-generation cephalosporins increased significantly from 3% to 36% during the last 4 years. Similar antimicrobial resistance was recorded in *P. aeruginosa* strains, with 14.9% of the isolates being resistant to at least three antimicrobial classes and 5.5% to all five antimicrobial groups under regular EARS-Net surveillance. Such an increased prevalence of combined resistance to multiple antimicrobial groups leaves just few treatment alternatives for patients with infections caused by these pathogens. Being the most potent against gram-positive and gram-negative bacteria, carbapenems have been always used as the "last-line" agent for treatment of infections caused by resistant *Enterobacteriaceae* [6]. Unfortunately, the consumption of this antibiotic that increased up to 45% favored the emergence and spread of carbapenem-resistant bacteria [16], which now became an issue in many southern and eastern European countries [17] as well as other parts of the world [18].

1.2. Antibiotic mechanisms of action

All antibiotics are classified based on their mechanism of action as follows (see Fig. 1.2.1):

1. *Nucleic acid synthesis inhibition.* Deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) replication is the first and most important process in cell division for all living forms. Some antibiotics such as the second generation fluoroquinolones (ciprofloxacin) are known as the most successful antibacterial target to DNA gyrase (type II topoisomerase) enzyme. DNA gyrases are known as enzymes that remove DNA supercoils during the initiation of DNA replication and transcription. Meanwhile fluoroquinolones act by binding the complex of DNA gyrase and DNA. As the results of this binding, quinolones appear to destabilize DNA enzyme complexes, resulting in DNA breaks [19].
2. *Cell membrane function inhibition.* A bacterial cell membrane is a phospholipid bilayer that completely surrounds bacteria cells and carries out many necessary cellular functions including energy generation, protein secretion, chromosome segregation; furthermore it regulates the intra- and extracellular flow of substances [20]. Disruption or damage of the cell membrane significantly affects the fluidity of important solutes, leading to metabolic dysfunction and cell death. The best known example of antimicrobial drugs that can target the microbial cell membrane via direct interaction with the lipid A component of the lipopolysaccharide (LPS) by interrupting functional integrity of outer and inner membranes is polymyxin B [21]. Nevertheless it has been proved that gram-negative bacteria can easily develop resistance to polymyxins through various modifications of the LPS structure that inhibit the binding of polymyxins to LPS. In the meanwhile two scientific groups reported the secondary mode of action of polymixins by inhibiting the activity of inner membrane respiratory enzyme NDH-2 in gram-negative bacteria. This gives new hopes in the development of new antibiotics targeting NDH-2 [22].
3. *Protein synthesis inhibition.* Protein synthesis is one of the most fundamental biological processes by which amino acids are linearly arranged into proteins in the cell through the involvement of three different classes of ribosomes and thus controlling the activities of the cell. It is well known that several types of antibacterial agents such as aminoglycosides, macrolides, lincosamides, streptogramins, chloramphenicol, tetracyclines interact with functional cen-

ters of these ribosomes (30S or 50S subunits) and effectively inhibit formation of synthesized polypeptides [23, 24].

4. *Cell wall synthesis inhibition.* While the cells of humans and animals do not have cell walls, this structure is essential to the survival and prevention the cell from bursting due to osmotic stress in most microorganisms. *β*-Lactams are the best known drugs acting by inhibiting the synthesis of the peptidoglycan layer of bacterial cell walls [25].
5. *Metabolism inhibition.* Bacteria synthesize own folic acid by taking *p*-aminobenzoic acid (PABA) and adding a substance called pteridine to form dihydropteroic acid. Folic acids are necessary for bacteria to make nucleic acids. It is well known that antibiotics such as sulfonamides compete with PABA by inhibiting the synthesis of folic acid that is necessary for growth of bacteria [26].

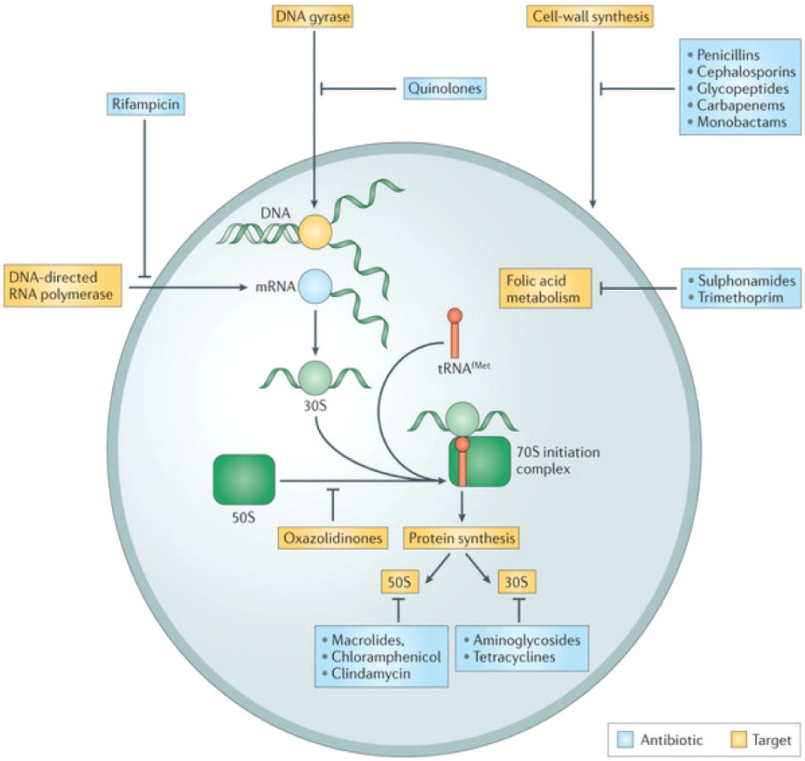

Fig. 1.2.1. *Antibiotic mechanism of action*
(Reproduced from the article by Lewis et al., 2013*) [27]*

Mechanisms of action of *β*-lactam antibiotics – non-enzymatic resistance

The bacteria cell surface (or envelope) is a complex multilayered structure that plays a central role in the protection of cells from its surrounding unpredictable environment. There are three main different layers in the cell envelope: the OM, the peptidoglycan cell wall, and the cytoplasmic or inner membrane (IM) by which the cell envelope is classified into two different groups – gram-positive or gram-negative. Gram-negative bacteria are surrounded by a very thin peptidoglycan (or murein) cell wall, which itself is surrounded by an OM containing LPS and cell membrane. Meanwhile gram-positive bacteria lack an OM, but are surrounded by the cell membrane and more thicker peptidoglycan layer than it is in gram-negative bacteria [28]. Peptidoglycan also known as murein plays an important role of forming the cell wall. Because of its rigidity it gives structural strength to the cell and thus helps to maintain cell integrity due to turgor pressure of the cytoplasm. Peptidoglycan also regulates the passive transport (diffusion) of molecules across cell membrane, the process of cell growth and cell division. For this reason, peptidoglycan is a well-known target for almost all clinically useful antibiotics that inhibit bacterial cell wall synthesis [29, 30]. A characteristic structural feature of peptidoglycan is linear glycan strands made up of alternating *β*-(1,4) N-acetylglucosamine (Glc*N*Ac and NAG) and *β*-(1,4) N-acetylmuramic acid (Mur*N*Ac or NAM) residues that are cross-linked by short amino acids (peptide) [31]. The peptide chain (4 to 5-residues) containing L-alanine ($_{L}$-Ala), D-glutamic ($_{D}$-Glu) acid, 2,6 diaminopimelic acid (m-A_2pm) and D-alanyl-D-alanine ($_{D}$-Ala-$_{D}$-Ala) is attached to the lactyl group during the NAM-NAG unit formation inside the bacteria. In gram-negative bacteria, the cross-linking of two peptide chain (from glycan backbone) is generally formed between carboxyl group of $_{D}$-Ala at position 4 of one peptide and the amino group of m-A_2pm at position 3 of another peptide. These cross-linking reactions are catalyzed by murein synthases also known as a transpeptidase enzyme, which has a catalytic transpeptidation (TP) domain. This domain is well known as penicillin-binding proteins (PBPs) because of their ability to bind penicillins and other *β*-lactams.

The inhibition of murein synthesis by *β*-lactam antibiotics is caused by attack of enzymes needed for the synthesis of the peptidoglycan wall. A *β*-lactam ring (in *β*-lactam antibiotics) is structurally similar to a $_{D}$-Ala-$_{D}$-Ala peptide (serves as a natural substrate for transpeptidase activity during cell wall synthesis) and can be easily recognized by the active site of transpepti-

dase resulting in a weakened cell wall that is sensitive to damaging agents (Fig. 1.2.2) [32].

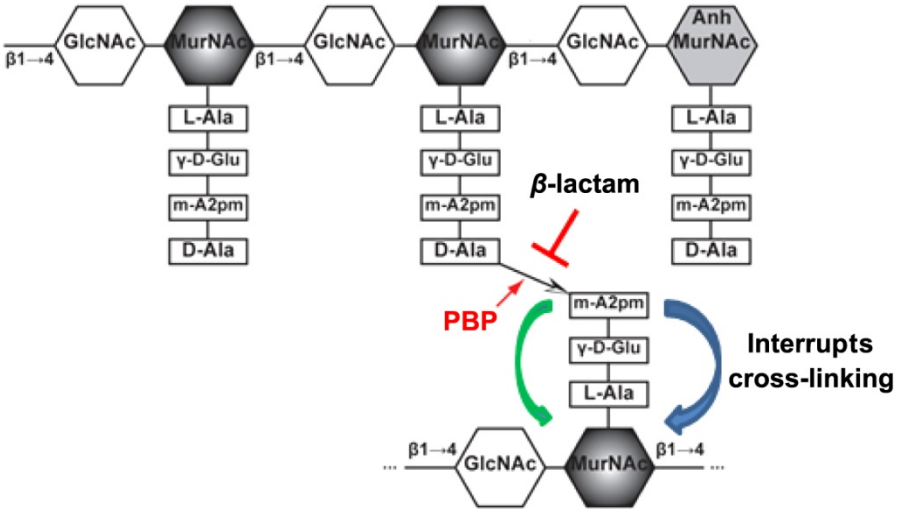

Fig. 1.2.2. *Inhibition of cell wall synthesis in gram-negative bacteria by antibiotics (Reproduced from the article by Zeng et al., 2013)[33]*

1.3. Mechanisms responsible for the emergence of carbapenem resistance in *Pseudomonas aeruginosa*

P. aeruginosa is one of the most important opportunistic pathogen that can cause a wide range of severe acute and chronic infections in hospitalized, immunocompromised hosts. These gram-negative non-fermenting bacteria remain to be the major cause of nosocomial infections, predominantly pneumonia, urinary tract infections, as well as skin and soft-tissue infections. Furthermore, it is the most prevalent pathogen isolated from patients with chronic lung infections, including cystic fibrosis, and is responsible for 10% of all hospital acquired infections (HAIs) [34]. *P. aeruginosa* predisposition to develop resistance to antibiotics contributes to the frequent ineffectiveness of current therapies [35, 36] which is associated with increased morbidity, mortality, and economic impact [37, 38]. Data from EARS-Net continue to show a dramatically increasing prevalence of *P. aeruginosa* strains during the last two decades. It became especially problematic for seriously ill patients in intensive care units (ICUs), where the selective pressure exerted by antibiotics on bacterial populations is very strong [39, 40]. A study conducting in Italy showed that 75% of ICU patients without sepsis received antibiotics with no reason [41]

or just for prophylaxis. Such high frequency of antibiotic use in ICUs favors the development and accumulation of MDR *P. aeruginosa* forms making treatment even more challenging [37, 42].

Carbapenems are regarded as the most potent *β*-lactam antibiotics for treating infections due to MDR *P. aeruginosa*; however, an increased prevalence of resistance to carbapenems among these organisms has been noted worldwide [43, 44]. According to the Carbapenem Antimicrobials Pseudomonas Isolate Testing At regional Locations (CAPITAL) surveillance program in 2010, the prevalence of carbapenem-resistant *P. aeruginosa* ranged from 7.4% to 35.4% in the United States of America [45]. On the other hand, the prevalence of *P. aeruginosa* resistant to all drugs of the carbapenem class (imipenem, meropenem, doripenem, ertapenem) in other countries including Russia, Greece, and Poland was higher than 50% [9].

Compared with other pathogens, *P. aeruginosa* is very difficult to eradicate particularly due to intrinsic or acquired resistance to many antibiotics. Additionally these bacteria have one of the largest genomes among bacterial human pathogens, averaging 6.26 megabase-pair (Mbp) in size (encoding 5567 genes) compared to 4.6 Mbp in *E. coli* or 2.81 Mbp in *Staphylococcus aureus* [46, 47]. Such a large genome size and additional genetic capacity allow this bacterium to easily develop resistance and survive in most hostile environments. Moreover, carbapenem resistance of *P. aeruginosa* is considered to be associated with loss of the porin OprD, overexpression of multidrug efflux pumps, and bacterial production of carbapenemases [48, 43] (Fig. 1.3.1).

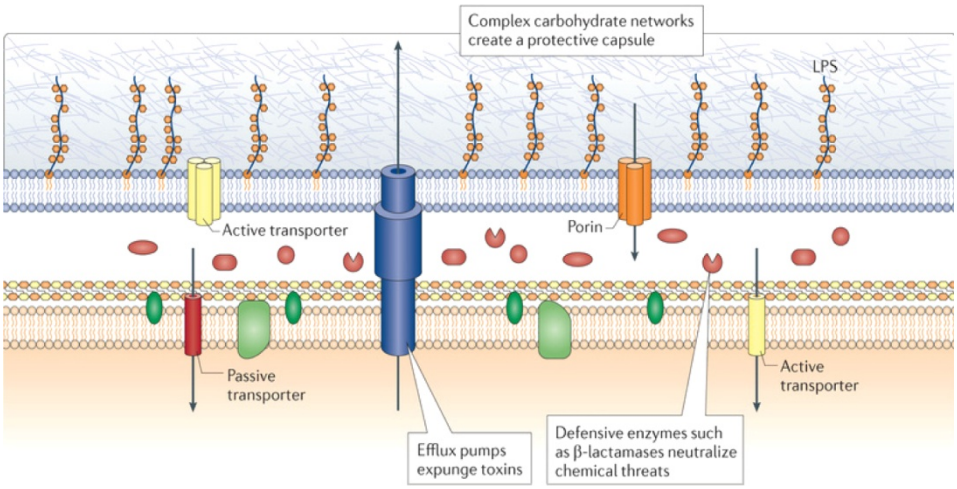

Fig. 1.3.1. *Mechanisms of antibiotic resistance in Pseudomonas aeruginosa (Reproduced from the article by Tommasi et al., 2015) [49]*

1.3.1. OprD-mediated resistance

The bacterial outer membrane (OM) is a very important structure to the cell because it works as a selectively permeability barrier for hydrophobic and hydrophilic compounds [28, 50]. It can be compared to a molecular sieve that favors penetration of very small molecules such as *β*-lactams and quinolone. Therefore, OM plays an important role in the susceptibility of the microorganism to antibiotics [51]. However it was noticed that different bacteria have different membrane permeability. *P. aeruginosa* that is always resistant to a wide range of antibiotic agents has 100 times lower OM permeability compared to *E. coli* [52]. These characteristics of low OM permeability are determined by integral transmembrane proteins better known as porins. Porin proteins are *β*-sheets that are wrapped into aqueous cylinders, which transfer substrates across the cell envelope [53]. Although *P. aeruginosa* produces several different porins, OprD is considered as the most important porin associated with significantly decreased susceptibility to carbapenems, especially to imipenem. The x-ray crystal structure of OprD has shown that this protein is a monomeric 18-stranded *β*-barrel forming a 9-loop structure [54]. Functional studies have revealed that loops 2 and 3 in the OprD contain the binding sites for imipenem [55]. Therefore, mutations such as nucleotide substitutions, deletions, or insertion sequence elements within the *oprD* gene or its promoter region can decrease or cause a loss of OprD production [56–58]. Isolates belonging to OprD-deficient strains have demonstrated a dramatically increased minimum inhibitory concentration (MIC) of carbapenems [59]. Although most reports stress that mutations occurring in porin-encoding genes mostly are found in imipenem-resistant strains, some other studies report that *oprD* mutations can arise in strains susceptible to carbapenems [60]. Interestingly, meropenem resistance is not affected by loss of OprD, indicating that carbapenem resistance seems to be more complex involving other OM carboxylic acid channel or additionally chromosomally encoded mechanism (such as overproduction of AmpC or overexpression of the efflux pumps such as MexAB-OprM, MexXY-OprM, and MexCD-OprJ) [61, 62].

1.3.2. Efflux pump-mediated resistance

While the loss of OprD represents an effective barrier for drug entry into the cell, a reduction in antimicrobial drug accumulation can be also depended on the second intrinsic mechanism such as efflux pumps. The efflux pump system usually is associated with limited OM permeability by making bacteria to MDR [63]. Most efflux proteins are divided into five families: resistance-nodulation-cell division (RND), major facilitator (MF),

staphylococcal/ small multidrug resistance (SMR), ATP-binding cassette (ABC), and multidrug and toxic compound extrusion (MATE) families [64]. Although sequence analysis of *P. aeruginosa* has shown the presence of efflux systems from all 5 superfamilies, the largest number of pumps, i.e., 12, have been described as RND-type efflux systems [65]. In contrast to the ABC superfamily of transporters, which utilize ATP hydrolysis for energy, RND family transporters (as well as remaining superfamilies) are driven by proton motive force. However, a key distinguishing feature between different superfamilies is that RND pumps typically exist as a tripartite system including a periplasmic membrane fusion protein (MFP), OM factor, and cytoplasmic membrane transporter [66]. This homotrimer protein complex forms a channel spanning the OM and the periplasmic space, which exports various dyes, detergents, inhibitors, disinfectants, organic solvents, and lipophilic and amphiphilic drug-like molecules.

There are 10 RND pumps that have been well characterized in *P. aeruginosa*: MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY, MexJK, MexGHI-OpmD, MexVW, MexPQ-OpmE, MexMN, and TriABC [66]. All these types of RND efflux pumps differ only by their substrates (as shown Table 1.3.2.1) and are responsible for a unique phenotype. However, only MexAB-OprM has been considered to be the most important and playing a relevant role in the intrinsic resistance to fluoroquinolones and β-lactam [67, 68]. This efflux system has been also linked to resistance to carbapenems such as meropenem, but not imipenem.

The crystallographic structure analysis of MexAB-OprM has shown that RND systems consist of three RND transported proteins: MexA, MexB, and OprM [69]. These proteins form a tripartite complex that can extrude antibiotic compounds across the cell membrane.

The genes encoding RND MDR efflux pumps are generally organized into operons. The MexAB-OprM efflux operon consists of three genes that are under control of regulatory genes, mostly of the local repressor gene (MexR) belonging to the MarR family of transcription regulatory proteins [70, 71]. Since MexAB-OprM is always expressed in low but detectable amounts, the depression of this operon in the tripartite system leads to over-expression of efflux pumps and increased antibiotic resistance in *P. aeruginosa* [72, 73]. Four mechanisms leading an increased expression of efflux pumps have been characterized. This include mutations that occur in the local expressor gene, global regulatory gene, or promoter region of the transported gene and insertion elements of the transporter gene [74, 75]. Nevertheless, mutations in the local repressor gene are frequently observed in clinical isolates with a MDR phenotypic profile. Detailed analysis of isolates from the University Hospital in France showed that 12 MDR

isolates overexpressed MexAB-OprM due to the mutation in the regulatory genes [76]. Overproduction of MexAB-OprM was also observed in more than 82% of MDR clinical isolates from Germany [77]. Finally, knockout mutants of MexAB-OprM confirmed the role of pumps in an intrinsic resistance mechanism as the mutants become multidrug susceptible to different classes of antibiotics [66].

Table 1.3.2.1. *Substrates and general regulatory genes of major efflux pumps in Pseudomonas aeruginosa*

Operon	Regulator and functions	Substrates	References
mexAB-OprM	MexR (repressor)[a] NalD[a] NalC[b]	Fluoroquinolones, *β*-lactams, *β*-lactamase inhibitors, tetracyclines, chloramphenicol, macrolides, novobiocin, trimethoprim, sulfonamides	[78] [79] [80]
mexCD-OprJ	NfxB (repressor)[a]	Fluoroquinolones, *β*-lactams, tetracycline, chloramphenicol, macrolides, trimethoprim, novobiocin	[81]
mexEF-OprN	MexT (activator)[a] MexS (repressor)[b] MvaT[b]	Fluoroquinolones, chloramphenicol, trimethoprim	[82] [83]
mexXY	MexZ (repressor)[a]	Fluoroquinolones, *β*-lactams, tetracycline, aminoglycosides, macrolides, chloramphenicol	[84]
mexJK	MexL (repressor)[a]	Tetracycline, erythromycin	[85]
mexGHI-OpmD	SoxR[a]	Fluoroquinolones	[86]
mexVW-OprM	NI	Fluoroquinolones, tetracycline, chloramphenicol, erythromycin	[87]
mexPQ-OpmE	NI	Fluoroquinolones, tetracycline, chloramphenicol, macrolides	[88]
mexMN-OprM	NI	Chloramphenicol, thiamphenicol	[88]
triABC-OpmH	NI	Triclosan	[89]

[a]Regulatory proteins that directly control the expression of efflux operons. [b]Proteins that indirectly activate operon expression when mutated. NI, not identified.

1.3.3. Resistance due to *β*-lactamase production. Class B MBLs

β-lactamases can be classified according to functional, molecular, and clinical properties. According to the classification based on the amino acid sequence, *β*-lactamases are divided into class A, C, and D enzymes, which utilize a serine residue for hydrolysis of the *β*-lactam ring, and class B

metallo enzymes, which require one or two divalent zinc ions for substrate (carbapenem) hydrolysis [90, 91]. On the other hand, based on functional classification, which takes into account substrate and inhibitor profiles, all *β*-lactamases are currently divided into three groups: group 1, class C cephalosporinases; group 2, classes A and D broad-spectrum, inhibitor-resistant, and extended-spectrum *β*-lactamases and serine carbapenemases; and group 3, MBLs [90, 92].

Carbapenemases are specific *β*-lactamases with the ability to hydrolyze carbapenems, cephalosporins, and penicillins, except monobactams. During the last decade, several classes A, B, and D carbapenemases have been detected in *P. aeruginosa* [93]; however, class B MBLs are characterized as the most clinically significant carbapenemases found in clinical *P. aeruginosa* isolates (Fig. 1.3.3.1) [94]. The enzymes of this class are subdivided into three subclasses (B1, B2, B3, the most recent B4) based on sequence and structural characteristics of the active site or function (subgroups 3a, 3b) [95]. B1 and B3 MBLs contain two Zn^{2+} ions bound to their active sites. The first Zn^{2+}-binding site is formed by three His residues (H116, H118, and H196) and a water/hydroxide molecule arranged in a tetrahedral geometry. Meanwhile, zinc-binding site 2 in B1-type MBLs is composed of one His-263 and one Cys-221 (ligand residue), and in B3 enzymes, ligand Cys221 is replaced by His121 [96]. In contrast, MBLs of the B2 subgroup require only one Zn^{2+} ion thus explaining a narrow-spectrum activity only on carbapenems [97] (Fig. 1.3.3.2).

Hydrolysis of *β*-lactams by class B MBL occurs by opening the *β*-lactam ring [11, 98].

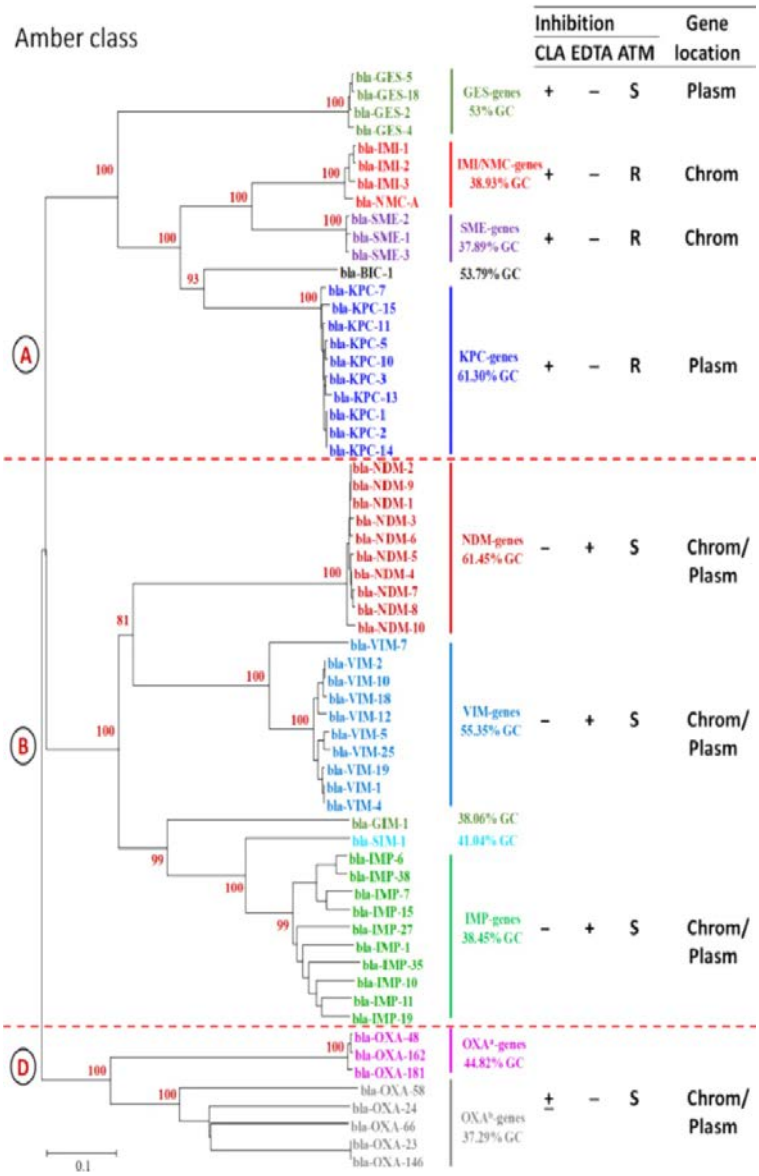

Fig. 1.3.3.1. *Phylogenetic tree of metallo-carbapenemase and serine carbapenemase genes found in gram-negative bacilli included: Enterobacteriaceae, Pseudomonas, and Acinetobacter species GES, KPC, OXAs, IMP, VIM, NDM and SPM enzymes frequently found in Pseudomonas aeruginosa*
(Reproduced from the article by Diene et al., 2014) [94]

[b]OXA genes described only in Acinetobacter species. ATM, aztreonam; CLA, clavulanic acid; R, resistant; S, sensitive.

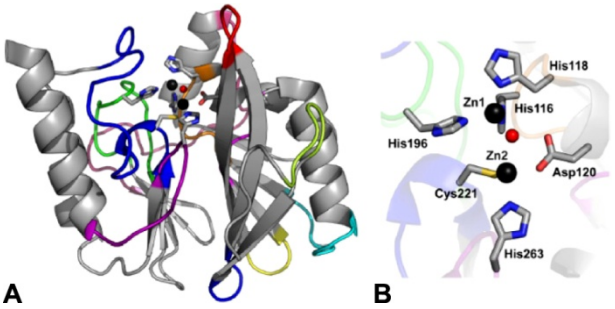

Fig. 1.3.3.2. *Structure of VIM-2*
(Reproduced from the article by Meine et al., 2014) [99]

(A) Three-dimensional structure of VIM-2 enzyme. (B) Active site of the di zinc structure of VIM-2 enzyme. Ions are represented as dark gray spheres bound by the indicated ligand residues (shown as sticks) and a bridging water/hydroxide molecule (red sphere).

Class B MBLs are encoded either by chromosomal genes or acquired genes, which are located in transferable genetic elements such as plasmids, integrons, or transposons through horizontal gene transfer [100, 101]. Chromosomally encoded MBLs are found in several gram-positive and gram-negative bacteria; meanwhile, acquired MBLs are frequently detected in *P. aeruginosa* and other gram-negative non-fermenters. These transferable MBLs are encoded by *bla* genes that are divided into at least 12 types of MBLs, accordingly: IMP, VIM, NDM, SPM, GIM, SIM, DIM, KHM, TMB, FIM, SMB, and AIM [102-112].

Clinically IMP- and VIM-type enzymes are the most important MBL class carbapenemases. The first IMP-type enzyme was described in Japan in 1988 [113], and since then 53 variants have been reported with 33 being detected in *P. aeruginosa* in China, Australia, USA, Canada, and some European countries (Table 1.3.3.3). These enzymes have broad substrate specificity to carbapenems (only to imipenem) and cephalosporins.

Table 1.3.3.3. *Imipenem-type enzymes found in Pseudomonas aeruginosa among European countries*

Enzyme	Gene location	Country of isolation	Ref
IMP-5	Class 1 integron	Portugal	[114]
IMP-7	Class 1 integron	Check Republic	[115]
	Unknown Unknown	Denmark Slovakia	[116] [117]
IMP-8	Class 1 integron	Germany	[118]
IMP-13	Class 1 integron	Italy, France, Belgium	[119–121]
IMP-15	Class 1 integron	Spain	[122]
	Unknown	Germany	[123]
IMP-19	Class 1 integron	Italy	[124]
IMP-22	Class 1 integron	Italy	[125]
IMP-29	Class 1 integron	France	[126]
IMP-31	Unknown	Germany	[127]
IMP-33	Class 1 integron	Italy	[128]
IMP-35	Class 1 integron	Germany	[129]
IMP-37	Unknown	France	JX131372

Contrarily to IMP type enzymes, VIM-type MBLs are mostly predominant in Europe particularly in the countries belonging to the Mediterranean region (Table 1.3.3.4). VIM-1 MBLs were originally detected in Verona, Italy [130]. For VIM-type enzymes, 46 allelic variants are known so far, of which approximately 24 variants are widely described in *P. aeruginosa* [131]. Based on amino acid similarities, all VIM enzymes could be divided into 5 clusters: VIM-1, VIM-2, VIM-7, VIM-12, and VIM-13.

Table 1.3.3.4. *Verona integron-encoded enzymes found in Pseudomonas aeruginosa among European countries*

Enzyme	Gene location	Country of isolation	Ref
VIM-1	Class 1 integron	Italy, France, Greece,	[130–133]
	Unknown	Germany	[123]
VIM-2	Class 1 integron	Spain, Hungary, France, Greece, Italy, Portugal, Croatia, Poland, Sweden and Norway, Denmark **Lithuania**	[134–144]
	Unknown	Belgium, Germany, Turkey	[123]
VIM-4	Class 1 integron	Greece, Sweden, Hungary, Poland	[145–148]
	Unknown	France	[123]
VIM-5	Class 1 integron	Turkey	[149]
VIM-9	Unknown	United Kingdom	AY524988
VIM-10	Unknown	United Kingdom	[150]
VIM-11	Unknown	Italy	AY635904
VIM-13	Class 1 integron	Spain	[151]
VIM-14	Class 1 integron	Italy	[152]
	Unknown	Spain	EF055455
VIM-15	Class 1 integron	Bulgaria	[153]
VIM-16	Class 1 integron	Germany	[153]
VIM-17	Class 1 integron	Greece	[154]
VIM-20	Unknown	Spain	[155]
VIM-30	Class 1 integron	France	JN129451
VIM-36	Unknown	Belgium	JX982635
VIM-37	Unknown	Poland	JX982636
VIM-38	Unknown	Turkey	KC469971
VIM-44	Unknown	Portugal	KP681696
VIM-46	Unknown	Germany	KP749829

1.3.3.1. Epidemiology of VIM-2

Despite the large amount of VIM alleles and their clinical relevance, VIM-2 has emerged as a dominant MBL variant worldwide (Table 1.3.3.4). Although $bla_{\text{VIM-2}}$ gene was discovered for the first time in France in 2000 [136], the earliest record about this gene was done in Portugal in 1995 [139]. It must be noted that VIM-2 shares ~90% amino acid similarity with the VIM-1 cluster [156]. Nevertheless, the $bla_{\text{VIM-2}}$ gene has shown to display better hydrolytic efficiencies toward penicillins, cephalosporins,

cephalothin, cefoxitin, cefotaxime, and moxalactam compared to all other MBLs [157].

Most VIM-2-positive bacteria are mainly resistant to other antibiotic classes and carry other resistance mechanism.

The *bla*$_{\text{VIM-2}}$ gene is found in more than 37 countries. In southern Europe, particularly in Spain and Greece, a large outbreak of VIM-2-producing MDR *P. aeruginosa* has been reported [158, 159]. Besides, these VIM-2 metallo enzymes were also reported in countries with low-level resistance to carbapenems including Sweden, Denmark, and Norway [142, 143]. The *bla*$_{\text{VIM-2}}$ gene has been extensively documented in our neighboring country Poland [123] (Fig. 1.3.3.1.1). Clinical spread of *bla*$_{\text{VIM-2}}$ gene is a matter of great concern for carbapenem resistance in Russia as well as Belarus and Kazakhstan [160].

The spread of VIM-2 producers is increasing at an alarming rate. However, the actual prevalence of carbapenemase producers is still unknown because many countries that are likely to be their main reservoirs have not established any search protocol for their detection yet.

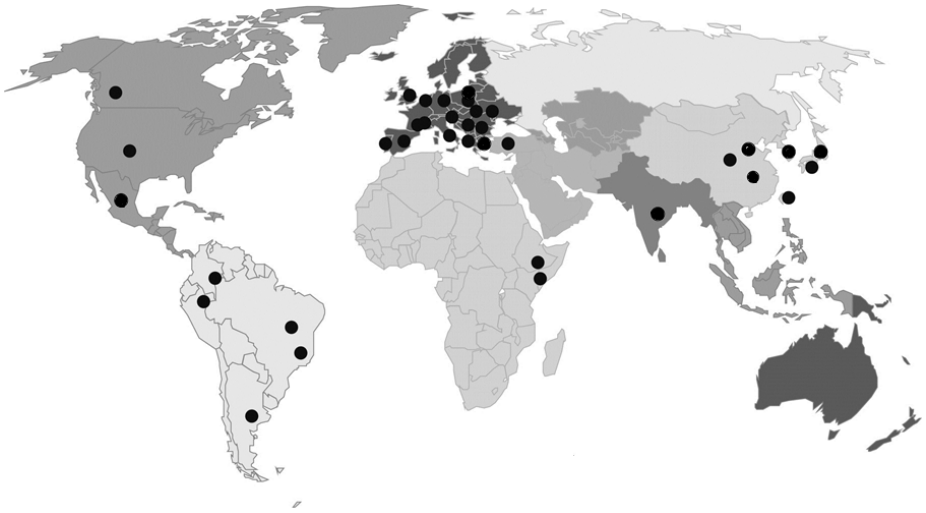

Fig. 1.3.3.1.1. *Spread of the bla*$_{VIM-2}$*-encoding gene (Reproduced from the article by Hawkey et al., 2009) [161]*

Population structure of *P. aeruginosa* has shown that isolates with VIM- type MBLs are geographically scattered and belong to the diverse sequence type (ST). Nevertheless, most of the VIM-2 MBL-producing isolates belonging to ST235 and ST111 clones are responsible for the dissemi-

nation of carbapenem-resistant *P. aeruginosa* isolates in European countries [162, 163].

1.3.3.2. Genetic environment of VIM-2

It is well known that the development of resistance is associated with the ability of bacteria rapidly to evolve into strains that are resistance to antibiotics. Acquisition of foreign resistance genes results from a successful gene mutation and/or genetic exchange via conjugation mechanism of horizontal gene transfer [164]. Most probably, horizontal gene transfer occurs largely due to existence of mobile genetic elements such as plasmids, transposable elements (transposons), integrons, or genomic islands. Extensive studies have shown that these gene transfer system elements play a significant role in the distribution and spread of antimicrobial resistance gene between species or genera of bacteria. However, integron-mediated resistance has been widely detected in gram-negative bacteria especially in *P. aeruginosa.*

Integrons are defined as genetic elements that able to capture individual antibiotic resistance genes including those encoding various β-lactamases. They consist of three elements: the integrase gene (*intI*), the cassette integration site (*attI*), and the promoter (P_c). The integrase gene (*intI*) encodes tyrosine recombinase, which catalyzes recombination reactions between the incoming gene cassette and the integron recombination site (*attI*). Once the gene cassette has been recombined, it is transcribed and expressed by P_c promoter [165].

Integrons are classified into two categories: mobile integrons (MIs) and chromosomal integrons (CIs) [166]. CIs can carry a hundred of gene cassettes, which are usually not involved in antibiotic resistance. These type integrons have capability to spread by lateral gene transfer (LGT) between different lineages and are mostly found in the environmental source. In contrast to CIs, MIs have limited number of gene cassettes, which mobility is associated with transposons or plasmids. They are mostly found in a clinically relevant pathogen with the presence of an MDR phenotype [166, 167].

A gene cassette, existing as a small, without promoter, autonomous, non-replicating double stranded circular DNA molecule, is part of an integron. It is inserted within the variable region of integron, between *attI* and *attC* recombination sites [167]. Most of the cassettes encode resistance genes. More than 130 distinct antibiotic resistance genes, characterized by unique *attC* site, are known [168]. Nevertheless some gene cassettes appear

to encode bacterial functions associated with virulence and host relationships.

Class 1 (C1) integrons are most common and widely distributed among *P. aeruginosa* bacteria. Such elements have a structure consisting of three genetic regions, two of which are highly conserved (CS) and a variable center section. The 5′-CS end accommodates the *intI* gene, *attI* site and P_c. The other end 3′-CS has the *qacEΔ1*gene, which mediates resistance to quaternary ammonium compounds, followed by *sulI* gene encoding resistance to sulphonamides, and two other open reading frames, orf5 and orf6, bearing unknown functions [167] (Fig. 1.3.3.2.1). Genes carried by integrons usually are responsible for multiple resistance mechanisms such as resistance to *β*-lactams, aminoglycosides, and other antimicrobial agents [169].

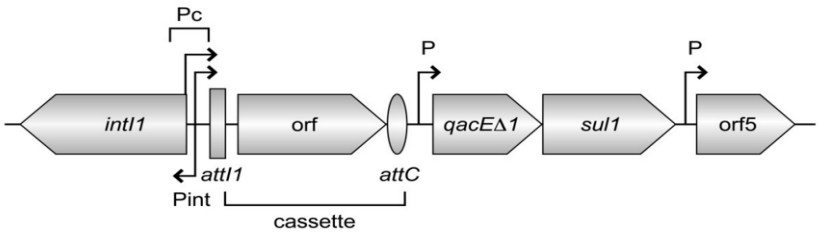

Fig. 1.3.3.2.1. *Schematic presentation of the class 1 integron carrying bla_{VIM-2} gene cassette*
(Reproduced from the article by Larouche et al., 2011) [170]

Different cassette arrays can be integrated in the variable region of the integron. Numerous combinations of gene cassettes are known [171]. A permanently updated list of the structure of integrons from isolates producing bla_{VIM-2} is available from the INTEGRALL database [172]. Several structures of the variable regions of class 1 integrons carrying bla_{VIM-2} gene cassettes of *P. aeruginosa* are represented in Fig. 1.3.3.2.2.

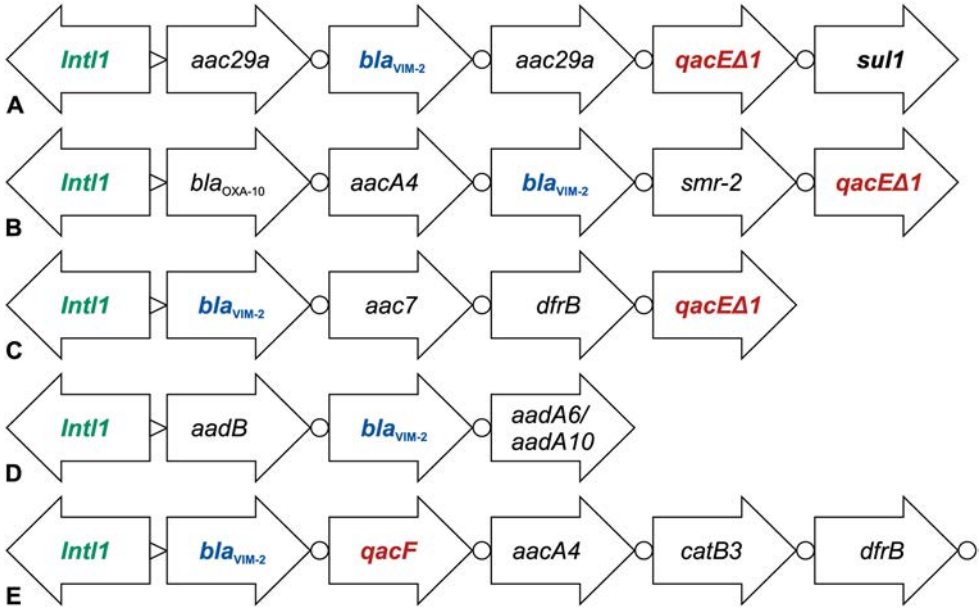

Fig. 1.3.3.2.2. *Structure regions of class 1 integron carrying bla_{VIM-2} metallo-β-lactamase gene cassette*

(A, B, C) Greece, accession no. EU118149 [173], KC527014, KC527015 [174]; (D) Poland, accession no. AM087405 [175], (E) accession no. GU137304

IntI1, class 1 integrase gene; *qac*, gene encoding resistance to quaternary ammonium compounds; ▷ – *attI*, integron gene cassette insertion site; ❍ – *attC*, gene cassette insertion sequence; sul1, gene conferring resistance to sulphonamides; bla_{VIM-2} gene encoding a VIM metallo-*β*-lactamase; *aacA* and *aadA*, genes encoding resistance to aminoglycosides; *dfrB*, conferred resistance to trimethoprim; *cat*, gene associated with chloramphenicol resistance; *smr*, causes resistance to quaternary ammonium compounds.

2. MATERIALS AND METHODS

2.1. Sampling and characterization of study population

A total of 121 *P. aeruginosa* strains isolated and identified by standard microbiological procedures were included in the study. All these strains were isolated from clinical samples of patients treated in the Hospital of Lithuanian University of Health Sciences Kaunas Clinics from January 1, 2011, to June 31, 2012. Specimens were obtained using aseptic techniques to avoid contamination and were promptly transported to the laboratory in a sterile swab under ice-cold conditions. Only one isolate per patient was included in the study from the following sites of infection: bloodstream, upper and lower respiratory tract, urine, skin, or wound. All isolates were collected from the different departments of the Hospital including Internal Medicine, Surgery, and Intensive Care Unit (ICU).

2.2. Study design of experiments

A total of 121 samples recovered from different sources of clinical samples were included in this study. The study consisted of three parts:

- In the first part of the study, all the isolates were examined for susceptibility to 12 antimicrobial drugs with the agar disk diffusion method proposed by Bauer et al. [176] and recommended by the Clinical and Laboratory Standards Institute (CLSI) Subcommittee on Antimicrobial Susceptibility Testing, and the commercial system E-test to determine the MIC, respectively. Isolates detected to be highly resistant to each carbapenem – imipenem or/ and meropenem – were tested further by the broth microdilution assay for 12 anti-pseudomonal agents by using TREK Diagnostic Systems™ Sensititre™ susceptibility gram-negative MIC plates (United Kingdom). Regardless of susceptibility pattern of the bacteria, all isolates were subjected to measurement of the bactericidal activity of patient's serum and biofilm formation ability *in vitro*.
- The second part of this study was design to detect carbapenemase producers by using different phenotypic tests and molecular confirmation methods. For this all *P. aeruginosa* isolates with reduced susceptibility to carbapenems was next assessed with the cloxacillin inhibition test (CIT) and the MHT. Additionally all isolates were submitted to molecular confirmatory test by using multiplex polymerase chain reaction (multiplex PCR). For isolates that were

PCR positive for carbapenemase were selected to differentiate the carbapenemase subtypes by using specific primers based on detected genes (Fig. 2.2.1).

- Our goal in the third part of this study was to analyze the epidemiologic relatedness of selected MBL-producing isolates determined by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST) technique. Carbapenemase-positive PCR amplicons and isolates displaying variation in PFGE pattern were subjected to sequencing by end primers in an ABI3130xl sequencer (Applied Biosystems, Life Technologies Italia, Milan, Italy) and the obtained sequences were compared to the corresponding sequences in the NCBI database.

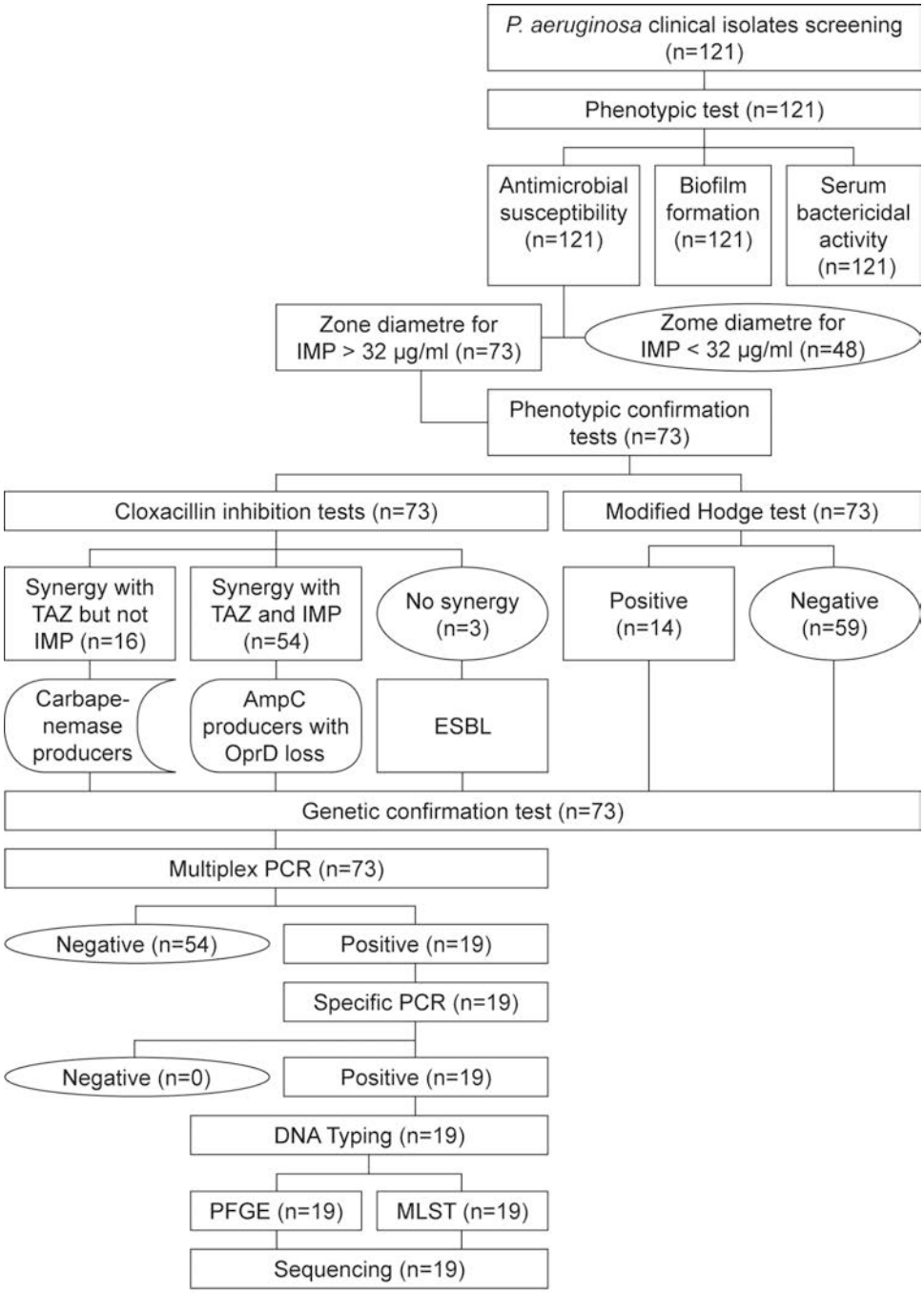

Fig. 2.2.1. *Study design*

IMP, imipenem; ESBL, extended spectrum β-lactamase; PCR, polymerase chain reaction; PFGE, pulse-field gel electrophoresis; TAZ, ceftazidime; MLST, multilocus sequence typing.

2.3. Identification of *P. aeruginosa* strains

2.3.1. Automated microbiology systems

The BD BACTEC FX blood culture system (BD, USA) was used to detect microbial growth in blood specimens. Blood cultures were obtained from two peripheral sites by nurses. All blood samples were inoculated into aerobic medium and processed using the BD BACTEC blood culture system. When fully automated systems exhibited bacterial growth in the BD BACTEC Plus Aerobic/F bottles, the experiment continued under part 2.3.2.

2.3.2. Conventional culture methods and fundamental bacteriological laboratory technique used to differentiate bacterial species

Based on infection symptoms and infection localization, all other samples, except blood samples, wound, catheter, ear or eye secretions, urine, purulent matter or bronchial and sputum were cultured BBL Columbia Agar with 5% Sheep Blood (BD, USA), BBL Chocolate II Agar (BD, USA) and MacConkey (Oxoid, UK) agar. Isolates suspected to be *P. aeruginosa* were identified by using Pseudomonas Cetrimide agar (Liofilchem, Italy), according to the manufacturer's instructions for *P. aeruginosa* identification. Cetrimide as a well-known antimicrobial agent with a good inhibitory characteristic against a wide variety of bacterial species including *Pseudomonas* species was chosen primarily. All positive *P. aeruginosa* strains were confirmed with the BD Phoenix™ Automated Microbiology System (BD, USA).

2.4. Antimicrobial susceptibility testing

In the first part of the study, we evaluated the antimicrobial susceptibility profile of 121 *P. aeruginosa* isolates to 12 antimicrobial agents by direct disk diffusion (DDD) testing and Etest method.

2.4.1. Disk diffusion test

Disk diffusion susceptibility testing was performed in accordance with the recommendations of CLSI document M02-A11 [177]. Cartridges containing commercially prepared 6-mm absorbent paper disks of piperacillin/tazobactam, cefepime, tobramycin, and aztreonam were obtained from (BD, BBL USA), stored between 8°C and –20°C, and allowed to come to room temperature before to use. Three or four colonies selected from

overnight culture incubated at MacConkey agar (Oxoid, UK) were transferred to 0.5 mL of phosphate buffer saline (PBS). Suspension was adjusted to achieve a turbidity equivalent to a 0.5 McFarland standard scale (1.5×10^8 CFU/mL). Within 15 min of adjusting turbidity, a sterile cotton swab was dipped into the inoculum suspension and rotated several times against the upper side wall of the tube to express excess liquid. Mueller-Hinton plates (BD, BBL, USA) were streaked three times, turning the plate 60° between streaks to obtain even inoculation. Inoculated plates were allowed to stand for 3 to 15 min before applying the disks. Five antimicrobial agents not closer than 24 mm to each other were applied with multidisc-dispensing device with a pressure ensured complete contact with the agar. All inoculated plates were incubated for a total of 16–20 h at 35°C±1°C in an ambient-air incubator after inoculation with organisms and placement of disks. Zones of inhibition were measured from the back of the plate to the nearest whole millimeter using a ruler. Zone diameters of inhibition were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations as follows: susceptible (S), intermediate (I), or resistant (R) (Table 2.4.2.1) [178].

Table 2.4.1.1. *Breakpoint tables for interpretation of zone diameters according to Eucast Version 2.0*

Antimicrobial agent	**Susceptibility testing**	**Concentration μg/mL**	**Zone Diameter Interpretive Standards (mm)**		
			Resistant	**Intermediate**	**Susceptible**
Aztreonam	Test Discs	30	<16	–	≥50
Cefepime	Test Discs	30	<18	–	≥18
Piperacillin/ tazobactam	Test Discs	100/10	<19	–	≥19
Tobramycin	Test Discs	10	<16	–	≥16

2.4.2. Etest strips

Etest strips were purchased from Liofilchelm (Italy) and were used in accordance with the manufacturer's instructions. They were stored at –20°C and brought to room temperature before use. Mueller-Hinton agar plates (BBL, USA) were inoculated for the disk diffusion method. The strips were applied manually to the surface of each Mueller-Hinton plate with the pressure that ensured complete contact with the agar. The plates were incubated up to 18-h period at 35°C±1°C in an ambient-air incubator. MIC

values were directly read at the point where ellipse of organism growth inhibition intercepts the strip. Detected minimal inhibitory concentrations were evaluated according to EUCAST breakpoints (Table 2.4.2.1) [178].

Results from all of the methods were validated using American Type Culture Collection quality control strains: susceptible, wild-type *P. aeruginosa* ATCC 27853, or *E. coli* ATCC 25922.

Table 2.4.2.1. *Breakpoint tables for interpretation of MICs according to Eucast Version 2.0*

Antimicrobial agent	Susceptibility testing	Concentration μg/mL	Zone Diameter Interpretive Standards (mm)		
			Resistant	Intermediate	Susceptible
Amikacin	MIC Test Strip	0.016–256	>16	–	≤1
Ceftazidime	MIC Test Strip	0.016–256	>8	–	≤8
Ciprofloxacin	MIC Test Strip	0.002–32	>1	–	≤0.5
Gentamicin	MIC Test Strip	0.016–256	>4	–	≤4
Imipenem	MIC Test Strip	0.002–32	>8	–	≤4
Meropenem	MIC Test Strip	0.002–32	>8	–	≤2
Piperacillin	MIC Test Strip	0.016–256	>16	–	≤16

2.4.3. Microdilution assay

A total of 73 carbapenem-resistant *P. aeruginosa* clinical isolates determined by the standard E-test method were additionally tested to evaluate MICs by using TREK Diagnostic Systems™ Sensititre™ susceptibility gram negative MIC plates (UK). The antibiotics used and their potencies are shown in Table 2.4.3.1.

Three or four colonies selected from overnight culture incubated at Muller-Hinton agar (Sigma-Aldrich, USA) were transferred to 0.5 mL of sterile water (TREK, UK). A 0.5 McFarland suspension of each isolate was prepared using Sensititre demineralized water (TREK, UK) and diluted with 11 mL of Muller-Hinton broth (Oxoid, UK) to give final inoculum of 1×10^5 colony-forming unit/mL (CFU/mL), for the Sensititre susceptibility MIC plates. Sensititre susceptibility MIC plates were inoculated using the microchannel pipette of 50 μL of bacterial suspension and incubated at 36°C±1°C for 18–24 h in an ambient-air incubator. Sensititre susceptibility MIC plates were read manually. Breakpoints were applied following EUCAST recommendations, 2015.

Testing included two positive controls manufactured (not published) but used to asses test validity.

Table 2.4.3.1. *Breakpoint tables for interpretation of MICs according to Eucast Version 5.0*

Antimicrobial agent	Susceptibility testing	Concentration μg/mL	Zone Diameter Interpretive Standards (mm)		
			Resis-tant	Interme-diate	Suscep-tible
Amikacin	MIC plates	2–64	>16	8–16	≤8
Aztreonam	MIC plates	1–128	>16	1–16	≤1
Cefepime	MIC plates	0.5–64	>8	–	≤8
Ceftazidime	MIC plates	0.5–64	>8	–	≤8
Ceftolozane/ tazobactam	MIC plates	0.25–32	>4	–	≤4
Ciprofloxacin	MIC plates	0.12–16	>1	0.5–1	≤0.5
Colistin	MIC plates	0.5–16	>4	–	≤4
Imipenem	MIC plates	0.5–64	>8	4–8	≤4
Meropenem	MIC plates	0.25–32	>8	2–8	≤2
Piperacillin/ tazobactam	MIC plates	2–256	>16[2]	–	≤16[2]
Ticarcillin	MIC plates	4–512	>16	–	≤16
Tobramycin	MIC plates	0.25–32	>4	–	≤4

2.5. Phenotypic investigation of *P. aeruginosa* pathogenicity factors

2.5.1. Biofilm formation

The tube method described by Christensen et al. was used to detect biofilm formation [179]. One or two overnight growth colonies of tested microorganisms were inoculated to 10 mL of trypticase soy broth (EMAPOL, Poland). The tubes were incubated at 37°C for 24 h. After incubation tubes were washed with phosphate buffer saline (PBS; pH 7) and air-dried. Subsequently, the tubes were stained with crystal violet (0.1%) for 20 min. Afterwards, the excess of stain was removed and washed with deionized water. Tubes were dried in an inverted position in room temperature. Biofilm formation was considered positive, when a visible film lined in the bottom of the tube. The strains were grouped to non-biofilm producers (no visible film line), moderate-biofilm producers (medium intense film line), and high-biofilm producers (intense film line). Experiments were performed in triplicate and repeated three times.

2.5.2. Serum bactericidal assay

The ability of *P. aeruginosa* strains to resist to serum bactericidal effect was tested as described earlier [180, 181]. Bacteria were grown in nutrient broth, harvested during the early logarithmic phase of growth, and adjusted to a concentration of 2×10^6 bacteria/mL of phosphate buffer saline (PBS; pH 7). Bacterial suspension (25 μL) was mixed with 75 μL of pooled normal human serum in microtiter plates. Bacterial ability to stay viable under human serum effect was evaluated after 1 h, 2 h, and 3 h of incubation at 37°C in an ambient-air incubator. Afterward, 100 μL of bacteria and human blood serum suspension were streaked on a BBL Columbia agar with 5% sheep blood (BD, USA) and incubated at 36°C±1°C for 18–24 h in an ambient-air incubator.

P. aeruginosa strains assigned to 1–4 level were interpreted as serum sensitive and to 5–6 level, as serum resistant. Responses were graded as follows: for grade 1, the viable count (VC) after 1 and 2 h was 10% lower of the inoculum; after 3 h, it was 0.1% lower. For grade 2, the VC after 1 h was up to 100 lower of the inoculum %; after 3 h, it was 10% lower of compared to 1 and 2 hours. For grade 3, the VC after 1 h was 100% higher of the inoculum, but after 2 and 3 h, it was 100% lower. For grade 4, the VC after 1 and 2 h was 100% higher, but after 3 h, the VC was 100% lower of the inoculum. For grade 5 and 6, the VC after 1, 2, and 3 h was 100% higher of the inoculum. Every strain of *P. aeruginosa* strain was tested 3 times. A strain was considered sensitive or resistant if the detected level was the same in all experiments.

2.6. Phenotypic detection of carbapenemase-producing *P. aeruginosa*

The MHT and the CIT were applied to discriminate between carbapenem-producing and carbapenemase non-producing strains among all carbapenem-resistant *P. aeruginosa* isolates.

2.6.1. Cloxacillin inhibition test

AmpC overproduction was confirmed by using 10-μg imipenem (BD, USA) and 30-μg ceftazidime (BD, USA) disks placed on 500 mg/mL of cloxacillin (Vademecum, Spain) containing MH agar plates, as described [182–184]. Briefly, 0.5 McFarland suspension of the test isolate was inoculated evenly on both cloxacillin-containing and non-containing medium. Imipenem (10 μg) and ceftazidime (30 μg) disks were placed on a lawn of the tested organism and incubated at 36°C±1°C for 16–18 h in an

ambient-air incubator. An organism that demonstrated a defined increase in a zone diameter around the ceftazidime (TAZ) and imipenem (IMP) disks with cloxacillin impregnated MH agar compared with non cloxacillin-impregnated agar was considered to be an AmpC producer coupled with OprD deficiency. On the contrary, when the strain was still resistant in the presence of cloxacillin, it was suspected to produce an acquired β-lactamase. If cloxacillin did not work only for ceftazidime indicated extended spectrum beta-lactamase (ESBL)-producing strain.

2.6.2. Modified Hodge test

The MHT was performed on all carbapenem-resistant isolates as described by Lee et al. [185, 186] using *E. coli ATCC 25922* as an organism sensitive to carbapenems, *P. aeruginosa* 18524928.2 (negative control) and a *bla*$_{\text{VIM-1}}$-producing *Enterobacter cloacae 91419421* strain as positive control. Two imipenem and ceftazidime sensitive strains were also chosen as additional negative controls. The surface of a Mueller Hinton agar plate was inoculated using a cotton swab with an overnight culture suspension of the *E. coli* 0.5 McFarland Standard. After several minutes of drying 10-μg meropenem (BD, USA) disk was placed at the center of the Muller Hinton (MH) plate, and tested isolates from the overnight cultures were streaked heavily from the drug disk to the edge of the agar plate. Results were recorded after the overnight incubation at 36°C±1°C in an ambient-air incubator. The true positive results were regarded as appearance of "clover-leaf" inhibition zone due to enhanced growth of indicator strain toward the meropenem disk alongside the test strain.

2.7. Molecular detection of carbapenemase-producing *P. aeruginosa*

2.7.1. DNA extraction

DNA extraction was done by using a DNeasy Tissue kit (Qiagen, DEU) as recommended by a manufacturer. DNA template preparation was performed as follows:

1. The organism was inoculated into 5 mL of trypticase soy broth (Difico, USA) and incubated for 20 h at 36°C±1°C in an ambient-air incubator.
2. Cells from 1.5 mL of an overnight culture were harvested by centrifugation at 3000 rpm for 15 min (Eppendorf, DEU).
3. After the supernatant was discarded, the bacterial pellet was re-suspended in 180 μL of tissue lysis buffer (Qiagen, DEU), and

20 μL of proteinase K solution (Qiagen, DEU) was added, mixed, and incubated at 55°C until cells were completely lysed (approximately for 1 h).

4. After incubation 200 μL of lysis buffers (Qiagen, DEU) was added into the cell suspension and incubated at 70°C for 10 min.
5. After the final incubation, 200 μL of ethanol (96–100%) was added into the cell suspension.
6. The resulting solution was transferred to a DNeasy mini column (Qiagen, DEU) and centrifuged 2 times with 500 μL of washing buffers (Qiagen, DEU) at 12000 rpm for 1 min.
7. DNA was diluted with 100 μL of distilled water and after centrifugation the supernatant was used as a source of template for amplification.
8. DNA-containing flow-through was stored at 4°C.

2.7.2. Multiplex PCR

All carbapenem-resistant *P. aeruginosa* isolates were tested for the presence of carbapenemases by the multiplex polymerase chain reaction (PCR) targeting the different classes of carbapenemase genes (bla_{NDM}, bla_{OXA-48} like, bla_{KPC}, bla_{VIM}, $bla_{IMP,}$ bla_{GES}).

The primers used for multiplex PCR amplification was carried out on a MJ Research PTC-200 thermal cycler instrument (BioRad, USA) with the Taq DNA Polymerase (NEB, UK) were described previously by Doyle et al. [187].

A total of 1 μL of DNA template was added to the reaction mixture solution containing 200 μM concentration of each dNTP, 0.3 μM of each for $bla_{KPC,}$ $bla_{IMP,}$ and $bla_{VIM,}$ 0.4 μM of $bla_{NDM,}$ and 0.5 μM of bla_{OXA-48} like primers, 1.5 mM of $MgCl_2$ and 2.5 U of Taq DNA polymerase. The cycling parameters were as follows: 95°C for 30 s followed by 30 cycles of denaturation at 95°C for 30 s, primer annealing at 60°C for 45 s, extension at 68°C for 1 min, and followed by a final extension at 68°C for 5 min. After the last cycle, the products were analyzed by electrophoresis with 1.0% TopVision Agarose gel (Thermo Fisher Scientific, LT) in 0.5× Tris-borate-EDTA (TBE) buffers. The gel were stained with SYBR Safe DNA gel Stain (Invitrogen, Sc) and visualized with Luminescent Image Analyzer (Fujifilm, JP). The primer sequences and amplicon sizes are shown in Table 2.3.2.5.

2.7.3. Specific PCR

Additional PCR to characterize class B carbapenemases using primers specific for *bla*$_{\text{IMP-1}}$, *bla*$_{\text{IMP-2}}$, *bla*$_{\text{VIM-1}}$, *bla*$_{\text{VIM-2}}$ and *bla*$_{\text{GES-5}}$ gene was performed following the study by Gutierrez et al. [188, 189]. Molecular identification of ESBL genes was done using specific primers for β-lactamases *bla*$_{\text{PER}}$-1 and *bla*$_{\text{VEB-1}}$ genes, as described by Juan et al. and Strateva et al. [190, 191] (Table 2.7.3.1).

Primers were amplified using an AmpliTaq Gold PCR master mix kit (Applied Biosystems at Life Technologies, Hammonton, NJ). DNA template was added to the PCR reaction mixture containing 200 μM concentration of each dNTP, 0.5 μM of each primers, 3% of DMSO, 1.5 mM of $MgCl_2$, and 2.5 U of Taq DNA polymerase. The cycling conditions were as follows: 94°C for 12 min followed by 35 cycles of denaturation at 94°C for 45 s, primer annealing at 60°C for 45 s, extension at 72°C for 1 min, and followed by a final extension at 72°C for 10 min. For *bla*$_{\text{VEB}}$ and *bla*$_{\text{PER}}$, the procedure was as follows: initial denaturation (94°C, 10 min) followed by 35 cycles of denaturation (94°C, 60 s), annealing (60°C for *bla*$_{\text{PER-1}}$ and 62°C for *bla*$_{\text{VEB-1}}$, 60 s), extension (72°C, 45 s) and final extension at 72°C for 10 min.

Table 2.7.3.1. *Primers used for detection of carbapenemase genes and the lengths of PCR products*

Target gene	Primer sequences (5' to 3')	Function	Amplicon size (bp)	References
bla$_{\text{IMP}}$	F GAAGGCGTTTATGTTCATAC R GTACGTTTCAAGAGTGATGC	Am, Seq	587	[187]
bla$_{\text{NDM}}$	F GCAGCTTGTCGCGCATGCGGGC R GGTCGCGAAGCTGAGCACCGCAT	Am, Seq	782	
bla$_{\text{OXA-48}}$	F GCGTGGTTAAGGAATGAACAC R CATCAAGTTCAACCCAACCG	Am, Seq	438	
bla$_{\text{VIM}}$	F GTTTGGTCGCATATCGCAAC R AATGCGCAGCACCAGGATAG	Am, Seq	389	
bla$_{\text{KPC}}$	F TGTCACTGTATCGCCGTC R CTCAGTGCTCTACAGAAAACC	Am, Seq	900	
bla$_{\text{GES}}$	F CACTCTGCATATGCGTCGGA R CTATTTGTCCGTGCTCAGGATG	Am, Seq	825	
bla$_{\text{VIM-1}}$	F GTTAAAAGTTATTAGTAGTTTATTG R CTACTCGGCGACTGAGC	Am, Seq	799	[188]
bla$_{\text{VIM-2}}$	F ATGTTCAAACTTTTGAGTAAG R CTATTTGTCCGTGCTCAGGATG	Am, Seq	801	

Table 2.7.3.1. *Continued*

Target gene	Primer sequences (5' to 3')	Function	Amplicon size (bp)	References
bla$_{IMP-1}$	F ATGAGCAAGTTATCTGTATTC R TTAGTTGCTTGGTTTTGATGG	Am, Seq	741	[188]
bla$_{IMP-2}$	F ATGAAGAAATTATTTGTTTTATG R TTAGTTACTTGGCTGTGATG	Am, Seq	741	
bla$_{GES-5}$	F CACTCTGCATATGCGTCGGA R CTATTTGTCCGTGCTCAGGATG	Am, Seq	799	[189]
bla$_{VEB}$	F CGACTTCCATTTCCCGATGC R GGACTCTGCAACAAATACGC	Am, Seq	782	[190]
bla$_{PER}$	F TGTTAAAAGAGCAAATTGAATCC R GCAACCTTGCGCAATGATAGC	Am, Seq	683	[191]
acsA	F ACCTGGTGTACGCCTCGCTGAC R GACATAGATGCCCTGCCCCTTGAT	Am	842	[192]
	F GCCACACCTACATCGTCTAT R GTGGACAACCTCGGCAACCT	Seq	390	
aroE	F TGGGGCTATGACTGGAAACC R TAACCCGGTTTTGTGATTCCTACA	Am	825	
	F ATGTCACCGTGCCGTTCAAG R TGAAGGCAGTCGGTTCCTTG	Seq	495	
guaA	F CGGCCTCGACGTGTGGATGA R GAACGCCTGGCTGGTCTTGTGGTA	Am	940	
	F AGGTCGGTTCCTCCAAGGTC R TCAAGTCGCACCACAACGTC	Seq	372	
mutL	F CCAGATCGCCGCCGGTGAGGTG R CAGGGTGCCATAGAGGAAGTC	Am	940	
	F AGAAGACCGAGTTCGACCAT R ATGACTTCCTCTATGGCACC	Seq	441	
nuoD	F ACCGCCACCCGTACTG R TCTCGCCCATCTTGACCA	Am	1042	
	F ACGGCGAGAACGAGGACTAC R TTCACCTTCACCGACCGCCA	Seq	336	
ppsA	F GGTCGCTCGGTCAAGGTAGTGG R GGGTTCTCTTCTTCCGGCTCGTAG	Am	989	
	F GGTGACGACGGCAAGCTGTA R TCCTGTGCCGAAGGCGATAC	Seq	369	
trpE	F GCGGCCCAGGGTCGTGAG R CCCGGCGCTTGTTGATGGTT	Am	811	
	F TTCAACTTCGGCGACTTCCA R GGTGTCCATGTTGCCGTTCC	Seq	441	

2.7.4. Pulse-field gel electrophoresis

PFGE was performed on all MBL-producing *P. aeruginosa* isolates by using a mirror modification of the procedures published by Grothues and Tümmler [193] and Barth and Pitt [194].

One to two colonies grown overnight on MacConkey agar (Oxoid, UK) were inoculated in 5 mL of brain heart infusion broth (BHI) (BD, USA) and incubated for 16–20 h at 36°C±1°C in an ambient-air incubator. The cell suspension left overnight was centrifuge at 5000 rpm for 2 min and the supernatant was collected. Afterwards, the cell pellets were suspended in 500 μL of PIV buffer. The cell suspension was mixed with an equal volume of 1.6% certified low-melt agarose (Bio-Rad, USA) and was allowed to solidify in plug mold. The agarose plugs were incubated overnight with 1 mL of lysis buffer (EC) at 36°C±1°C in a shaking water bath and were then incubated with 1 mL of proteolysis buffer (ESP) overnight with gentle shaking at 50°C. The plugs were washed four times for 30 min with TE buffer at 50°C (Sigma-Aldrich, USA). After washing, the plugs were transferred to tubes containing 1 mL of preheated distilled water and incubated at 37°C for 10 min. One-third of a slice of each plug was cut and was placed in 200 μL of RE (restriction buffer) and incubated at 37°C for 16–20 h. Then samples were pre-equilibrate in 200 μL of TE buffer at 37°C for 1 h and loaded and electrophoresed in 1% of certified megabase agarose (Bio-Rad, USA) with 2.2 L of standard 0.5× TBE running buffer. The macrorestriction fragments were separated on a CHEF-DR II apparatus (Bio-Rad, Laboratories, USA). Electrophoresis conditions were as follows: 5 to 40 s for 26 h at 6 V/cm. The gel were stained for 30 min with SYBR Safe DNA gel Stain (Invitrogen, Sc), visualized in a transilluminator and photographed. The banding pattern in each lane was compared by visual examination. Band differences were interpreted according to Tenover et al. [195] (Table 2.7.4.1). Chemical composition used in the PFGE protocol is shown in Table 2.7.4.2.

Table 2.7.4.1. *Solutions composition*

Name of solutions	Reagents and Stocks	Final concentration
PIV	TRIS-HCl (pH 7.6) NaCl Distilled water	10 mM 1 M
EC (Lysis Buffer)	TRIS-HCl (pH 7.6) NaCl EDTA (pH 7.6) Brij58 Sodium deoxycholate Ribonuclease A Lysozyme Distilled water	6 mM 1 mM 100 mM 0.5% 0.2% 20 μg/mL 100 μg/mL
ESP (proteolysis buffer)	EDTA (pH 9.0–9.5) Proteinase K N-Lauroylsarcosine	0.5 M 50 μg/mL 1%
TE	EDTA (pH 7.6) TRIS-HCl (pH 7.6) Distilled water	1 mM 10 mM
RE (restriction buffer)	*SpeI* Buffer Distilled water	10 U/μL
TBE	Boric Acid TRIS EDTA (pH 7.6) Distilled water	0.089 M 0.089 M 0.002 M

Table 2.7.4.2. *Criteria for the interpretation of PFGE profiles*

No. Band differences	No. Genetic differences	Category	Epidemiological interpretation
0	0	Indistinguishable	Isolates are same strain type
2–3	1	Closely related	Isolates are probably related
4–6	2	Possibly related	Isolates are possibly related
≥7	≥3	Different	Isolates are different

2.7.5. Multilocus sequence typing

In order to study the relative distribution we performed MLST in one isolate of each *P. aeruginosa* PFGE clone.

MLST was performed according to the protocol published by Curran et al. [192] by using seven housekeeping genes (*acsA, aroE, guaA, mutL, nuoD, ppsA, and trpE*) and conditions as follows: initial denaturation at 96°C for 1 min; 30 cycles of denaturation at 96°C for 1 min, primer

annealing at 55°C for 1 min, extension at 72°C for 1 min; followed by a final extension step of 72°C for 10 min. Each 50 μL of amplification reaction mixture contained 2.0 μL of chromosomal DNA (5–20 ng/μL), 2.0 μL of forward primer (10 pmol/μL), 2.0 μL of reverse primer (10 pmol/μL), 5.0 μL of 10× PCR buffer (Qiagen, contains 15 mM $MgCl_2$), 1.0 μL of dNTP solution (Qiagen, 10 mM each dNTP), 0.25 μL of Taq polymerase (Qiagen, 5 units/μL) and 37.75 μL of PCR-grade water. All Qiagen solutions were ordered from PCR CORE Kit (Qiagen, DEU). The amplification product was then purified using MinElute UF plates (Qiagen, DEU) following the manufacturers protocol before being used in a sequencing reaction.

The allelic profile for each isolate was determined. Isolates were then assigned a sequence type (ST) according to their allelic profiles. Isolates were considered genetically identical and hence the same ST if they were identical at all analyzed loci. Allele profiles and STs were found at http://pubmlst.org/paeruginosa.

2.7.6. Sequencing

Selected interested samples of amplicons were submitted to DNA sequencing with a BigDye Terminator kit (Applied Biosystems Inc., Foster City, CA) in an MJ Research PTC-200 DNA Engine thermal cycler (Bio-Rad Laboratories, Waltham, USA), and sequences were analyzed on an ABI Prism 3100 genetic analyzer (ABI, Foster City, USA). Resulting sequences were compared using software available at the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov).

2.7.7. Sensitivity and specificity

The performance of the various disks for detection of different β-lactamases was determined with genotypically defined carbapenem resistance mechanisms as the reference standard. For each test and category of β-lactamase, sensitivity was calculated from the number of true-positive bacteria, whereas specificity was calculated from the number of true-negative bacteria according to the method of Ilstrup et al. [196].

2.8. Statistical analysis

Statistical analysis was performed by using the IBM SPSS Statistics Version 20 for Windows statistical package. Proportions were compared using nonparametric chi-square criterion or Fisher's exact test. Differences between groups were considered significant if P was <0.05. The IBM SPSS Statistics Version 20 statistical package was used for the data analysis.

3. RESULTS

3.1. Sampling and characterization of study population

During the 16-month period, 121 of *P. aeruginosa* strains were isolated from the patients hospitalized in different clinical departments. The highest rate of infection due to *P. aeruginosa* was documented in ICUs (40.6%) and internal medicine wards (33.8%) (Table 3.1.1). *P. aeruginosa* strains were isolated from various clinical samples, but wound/pus (43.8%) and lower respiratory tract (33.1%) were the predominant sources of *P. aeruginosa* strains, followed by urine (16.5%), upper respiratory tract (4.1 %) and blood (2.5%) (Table 3.1.1).

Table 3.1.1. *Distribution of Pseudomonas aeruginosa strains by source and medical department (n=121)*

Source	Department			
	Surgery Medicine	**Intensive care Units**	**Internal Medicine**	**Total**
Blood stream	–	2 (1.7)	1 (0.8)	**3 (2.5)**
Lower respiratory tract	4 (3.3)	29 (24.0)	7 (5.8)	**40 (33.1)**
Upper respiratory tract	–	–	5 (4.1)	**5 (4.1)**
Urine	3 (2.5)	4 (3.3)	13 (10.7)	**20 (16.5)**
Wound or skin	24 (19.8)	14 (11.6)	15 (12.4)	**53 (43.8)**
Total	**31 (25.6)**	**49 (40.6)**	**41 (33.8)**	**121 (100.0)**

Values are number

3.2. Antimicrobial susceptibility patterns

All *P. aeruginosa* strains (n=121) tested by the Kirby-Bauer disk diffusion susceptibility test and the E-test were divided into two major groups. The first group consisted of 65 (53.7%) carbapenem-resistant strains (resistant to imipenem and meropenem) and the second group comprised 56 (46.2%) carbapenem-sensitive *P. aeruginosa* strains (sensitive to meropenem and imipenem). Antibiotic resistance patterns (against different groups of antibiotics) between carbapenem-resistant and carbapenem-sensitive strains are represented in Table 3.2.1.

Drug susceptibility testing in our study revealed statistically significant differences between carbapenem-sensitive and carbapenem-resistant *P. aeruginosa* strains. Carbapenem-resistant *P. aeruginosa* strains were

resistant to the majority of tested antibiotics more frequently than carbapenem-sensitive strains (Table 3.2.1).

Table 3.2.1. *Resistance of carbapenem-resistant and carbapenem-sensitive P. aeruginosa strains to various antibiotic groups/antimicrobial groups*

Antibiotics by class	Antimicrobial Agent	CRS, n (%)	CSS, n (%)	χ^2	*P* value
Penicillins	Piperacillin	46/65 (70.8)	16/56 (28.6)	21.44	**<0.001**
	Piperacillin/ Tazobactam	16/38 (42.1)	7/43 (16.3)	6,62	**0.01**
Cephalosporins	Ceftazidime	35/65 (53.8)	14/56 (25.0)	10.39	**0.001**
	Cefepime	11/38 (28.9)	7/42 (16.7)	1.73	0.189
	Cefoperazone/ sulbactam	19/38 (50.0)	11/43 (25.6)	5.16	**0.023**
Fluoroquinolones	Ciprofloxacin	57/65 (87.7)	12/56 (21.4)	53.90	**<0.001**
Aminoglycosides	Gentamicin	51/65 (78.5)	10/56 (17.9)	44.20	**<0.001**
	Amikacin	25/65 (38.5)	3/56 (5.4)	18.54	**<0.001**
	Tobramycin	21/37 (56.8)	4/41 (9.8)	19.73	**<0.001**
Monobactams	Aztreonam	9/59 (15.3)	4/51 (7.8)	1.44	0.23

CRS, carbapenem-resistant strains; CSS, carbapenem-sensitive strains.

The carbapenem-resistant *P. aeruginosa* strains were recovered from the urinary tract significantly more often than carbapenem-sensitive *P. aeruginosa* strains, 75.0% and 25.0%, respectively (*P*=0.037). Carbapenem sensitive *P. aeruginosa* strains were recovered from the respiratory tract significantly more frequently than carbapenem-resistant strains, 60.0% and 40.0%, respectively (Table 3.2.2).

Table 3.2.2. *Proportion of P. aeruginosa strains recovered from various sources in relation to carbapenem resistance*

Source	Carbapenem-resistant strains, n (%)	Carbapenem-sensitive strains, n (%)	χ^2	*P* value
Urinary tract (N=20)	15 (75.0)	5 (25.0)	4.37	**0.037**
Wounds (N=53)	31 (58.5)	22 (41.5)	0.86	0.353
Blood (N=3)	1 (33.3)	2 (66.7)	–[#]	0.596
Respiratory tract (N=45)	18 (40.0)	27 (60.0)	5.42	**0.020**

–[#] Fisher exact test was employed for small sample size.

A total of 73 carbapenem-resistant clinical *P. aeruginosa* isolates determined by standard susceptibility testing methods were selected for evaluation of their minimum inhibitory concentrations (MICs). Additionally, 8 strains were included to the group of carbapenem-resistant isolates on the inconclusive results of the E-test method. Antimicrobial susceptibility profiles of the 73 *P. aeruginosa* isolates are shown in Table 3.2.3. The MIC values are summarized in Fig. 3.2.1–3.2.8.

According to EUCAST interpretative categories, all strains showed a high level of resistance against at least one tested antimicrobial agent. Imipenem was found to be the least active antimicrobial agent as 97.3% (n=71) of the *P. aeruginosa* isolates were resistant to it, followed by aztreonam (91.8%, n=67), ticarcillin (83.6%, n=61), meropenem (83.6%, n=61), and ciprofloxacin (72.6%, n=53). Moreover, 10 (13.7%) isolates were resistant against all the tested antibiotics except colistin. Colistin was the most effective antibiotic as 99% (n=72) of the isolates were susceptible to it (Table 3.2.3). Of the 73 isolates, 54 (74%) were found to be MDR, while 36 (49.3%) were extensively drug-resistant (XDR). Only one isolate was found to be resistant to colistin and showed a pandrug-resistant (PDR) profile (Table 3.2.3).

Table 3.2.3. *Antimicrobial susceptibility profiles of carbapenem-resistant P. aeruginosa strains*

Antibiotics by class	**Antimicrobial agent**	**Carbapenem-resistant strains (N=73)**		**MDR (N=54)**	**XDR (N=36)**	**PDR (N=1)**
		Susceptible, n (%)	**Resistant, n (%)**			
Penicillins	Ticarcillin	12 (16.4)	61 (83.6)	x	x	x
Penicillins + *β*-lactamase inhibitors	Piperacillin/ tazobactam	28 (38.4)	45 (61.6)		x	x
Monobactams	Aztreonam	6 (8.2)	67 (91.8)	x	x	x
Cephalosporins	Ceftazidime	32 (43.8)	41 (56.2)		x	x
	Cefepime	50 (68.5)	23 (31.5)			x
Cephalosporins + β-lactamase inhibitors	Ceftolozane/ tazobactam	53 (72.6)	20 (27.4)			x
Carbapenems	Imipenem	2 (2.7)	71 (97.3)	x	x	x
	Meropenem	12 (16.4)	61 (83.6)		x	x

Table 3.2.3. *Continued*

Antibiotics by class	Antimicrobial agent	Carbapenem-resistant strains (N=73)		MDR (N=54)	XDR (N=36)	PDR (N=1)
		Susceptible, n (%)	Resistant, n (%)			
Fluoroquinolones	Ciprofloxacin	20 (27.4)	53 (72.6)		x	x
Aminoglycosides	Tobramycin	41 (56.2)	32 (43.8)			x
	Amikacin	50 (68.5)	23 (31.5)			x
Polymyxins	Colistin	72 (98.6)	1 (1.4)			x

Criteria for defining MDR, XDR, and PDR in *Pseudomonas aeruginosa*. MDR, nonsusceptible to ≥1 agent in ≥3 antimicrobial categories; XDR, nonsusceptible to ≥1 agent in all but ≤2 categories; PDR, nonsusceptible to all antimicrobial agent listed; x, nonsusceptible to an antimicrobial agent.

The MIC values in carbapenem-resistant strains were compared among seven classes of antibiotics. In the penicillin class of antibiotics (Fig. 3.2.1, 3.2.2), the MIC distribution diagrams showed the increased MIC of penicillin against carbapenem-resistant *P. aeruginosa* strains. Among 61 ticarcillin-resistant *P. aeruginosa* strains (Fig. 3.2.1), 46.6% (n=34) of the isolates had MICs greater than 64 μg/mL using EUCAST breakpoints. However, the MIC values of penicillins combined with a β-lactamase inhibitor (Fig. 3.2.2) did not inhibit the bacterial growth of carbapenem-resistant *P. aeruginosa* (n=45; 63%) even the concentration of piperacillin/tazobactam was >16 μg/mL.

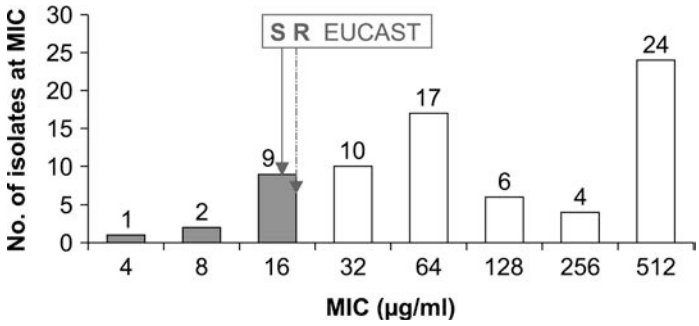

Fig. 3.2.1. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of ticarcillin*

S, sensitive; R, resistant.

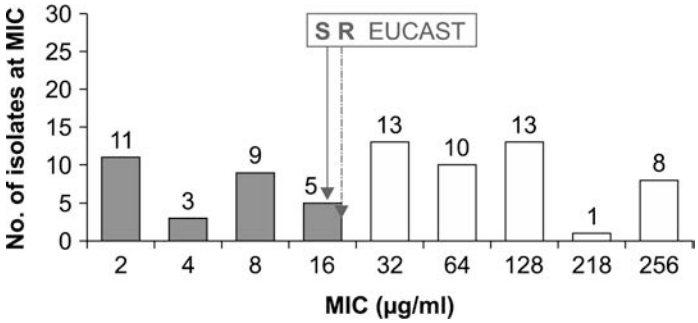

Fig. 3.2.2. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of piperacillin/tazobactam*

S, sensitive; R, resistant.

Analysis of the distribution of *P. aeruginosa* isolates by the MICs of monobactam (Fig. 3.2.3) revealed that aztreonam was the second least potent agent after imipenem: 10 (13.4%) strains were resistant at the MIC of ≥32 µg/mL and 57 (86.6%) were intermediately resistant at the MICs ranging between 2 and 16 µg/mL.

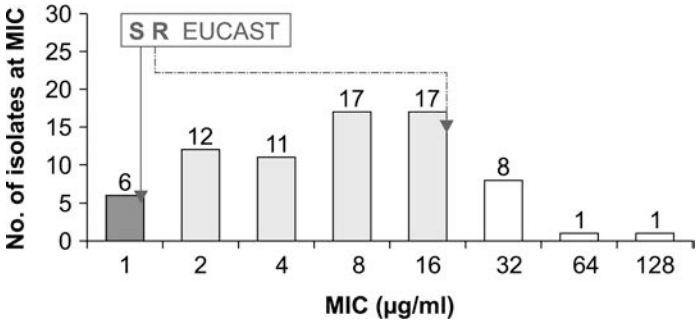

Fig. 3.2.3. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of aztreonam*

S, sensitive; R, resistant.

In the cephalosporin class of antibiotics, MIC distribution values of ceftazidime shown in Fig. 3.2.4 demonstrated that 41 (56.0%) carbapenem-resistant strains were resistant to ceftazidime at the MIC values of 16 µg/mL and more; of them, 6 (14.6%) isolates showed high-level resistance to ceftazidime at the MIC of 64 µg/mL.

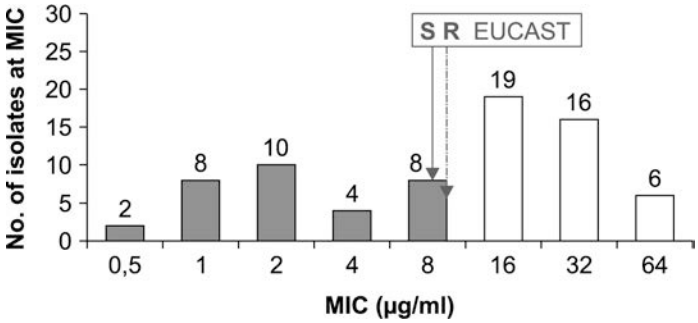

Fig. 3.2.4. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of ceftazidime*

S, sensitive; R, resistant.

Despite the fact that cefepime had the highest activity among antimicrobial agents of this class and inhibited 68% (n=50) of carbapenem-resistant *P. aeruginosa* strains, 32.9% (n=24) of these isolates were sensitive to cefepime at the MIC value very close to the resistance zone (Fig. 3.2.5).

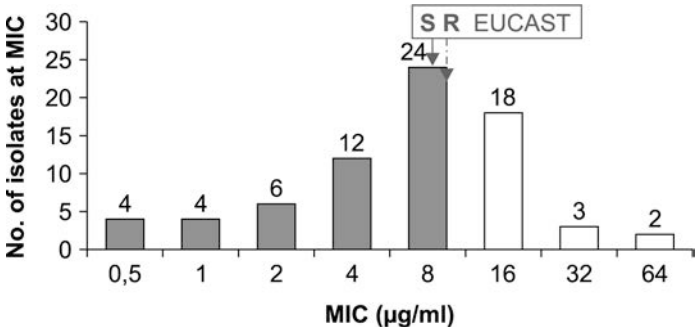

Fig. 3.2.5. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of cefepime*

S, sensitive; R, resistant.

Contrary, the combination of cephalosporin and β-lactamase inhibitor inhibited 53 of the 73 carbapenem-resistant *P. aeruginosa* strains at the concentration of less than 8 μg/mL (Fig. 3.2.6).

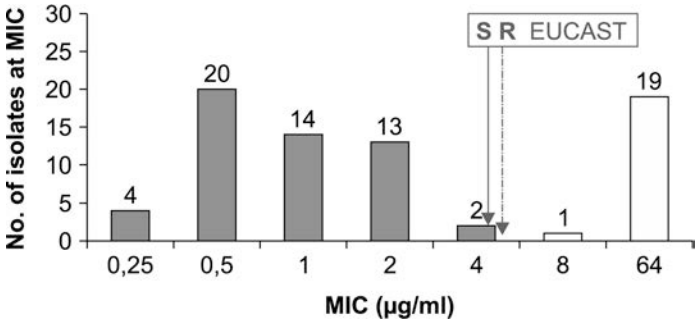

Fig. 3.2.6. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of ceftolozane/tazobactam*

S, sensitive; R, resistant.

MIC diagrams showed that imipenem was the least potent agent against carbapenem-resistant *P. aeruginosa* isolates. Of the 71 (97.3%) strains showing resistance, 59 (83.0%) were resistant at the MIC of >8 μg/mL and 12 (16.9%) were intermediately resistant at the MICs ranging between 32 and 64 μg/mL (Fig. 3.2.7). A total of 17 strains were resistant at the MIC above the break point recommended by EUCAST.

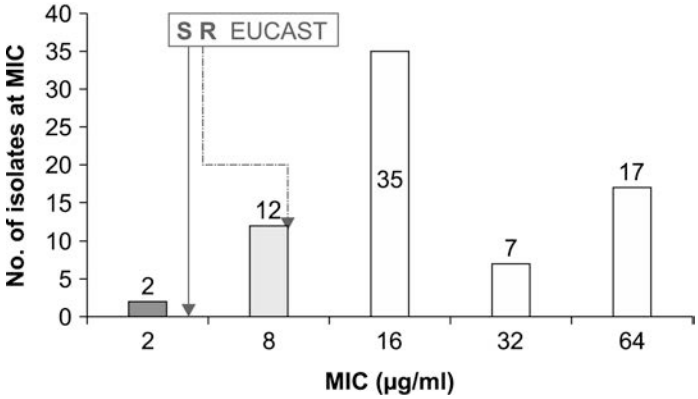

Fig. 3.2.7. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of imipenem*

S, sensitive; R, resistant.

As illustrated in Fig. 3.2.8, meropenem was slightly more potent than imipenem. Although the MICs values of meropenem were lower compared with those of imipenem, resistance to meropenem still reached more than 80%. According to the EUCAST breakpoints, 59% (n=36) of the strains showed intermediate resistance at the MICs of 4 and 8 μg/mL.

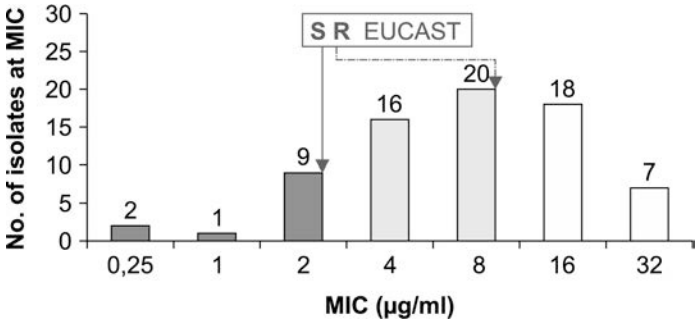

Fig. 3.2.8. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of meropenem*

S, sensitive; R, resistant.

Of the 73 *P. aeruginosa* isolates, 53 (72.6%) showed *in vitro* resistance to fluoroquinolones (Fig. 3.2.9). Our study showed that more than 50% (n=28) of the isolates were resistant to ciprofloxacin at the MIC of 16 μg/mL.

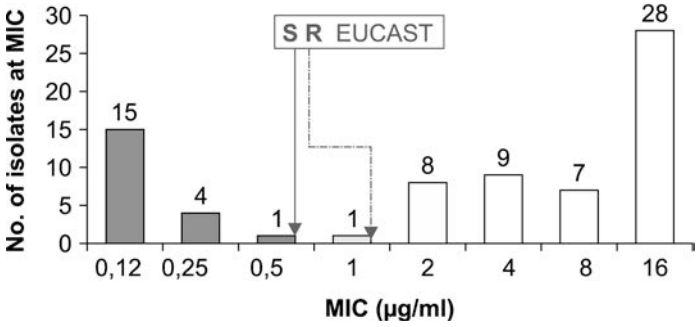

Fig. 3.2.9. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of ciprofloxacin*

S, sensitive; R, resistant.

Fig. 3.2.10 and 2.3.11 display the distribution of *P. aeruginosa* strains by the MICs to aminoglycosides. Tobramycin had activity against 56% (n=41) of the tested isolates; of them, 24 were inhibited at the MIC below 1 μg/mL. However, more than 65% (n=21) of the strains were resistant to tobramycin at the MICs of more than 16 μg/mL (Fig. 3.2.10).

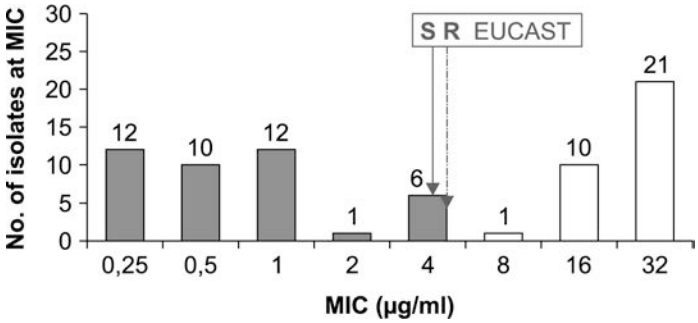

Fig. 3.2.10. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of tobramycin*

S, sensitive; R, resistant.

Amikacin was active *in vitro* against 68.5% of the isolates with the greatest percentage of the strains being inhibited at the MIC of 2 μg/mL. The greatest percentage (57.9%) of the strains was resistant to amikacin at the MIC of 32 μg/mL (Fig. 3.2.11).

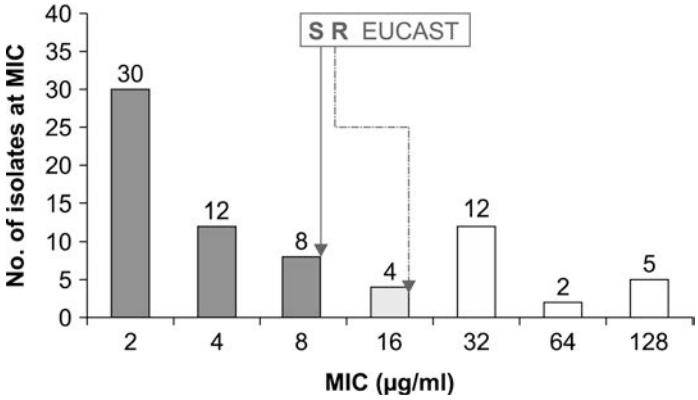

Fig. 3.2.11. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of amikacin*

S, sensitive; R, resistant.

Colistin was found to be the only antimicrobial agent having good activity in vitro: the growth of even 99% of the *P. aeruginosa* strains was inhibited at the MICs of 4 μg/mL and lower (Fig. 3.2.12).

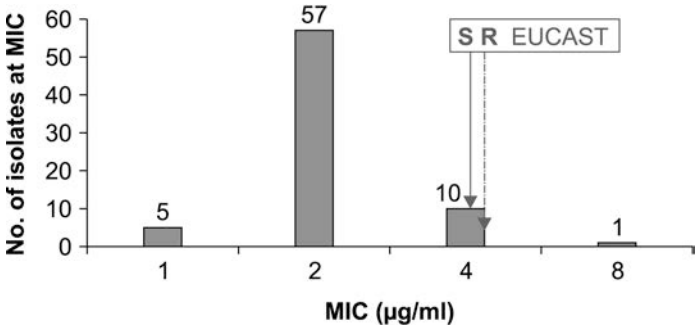

Fig. 3.2.12. *Distribution of clinical P. aeruginosa isolates by minimum inhibitory concentrations (MICs) of colistin*

S, sensitive; R, resistant.

3.3. Human serum and biofilm-formation effect on the survival of *P. aeruginosa* isolates

All 121 *P. aeruginosa* strains in this study were tested for human serum bactericidal effect. A total of 85 (70.2%) and 36 (29.8%) of *P. aeruginosa* strains were found to be sensitive and resistant to serum, respectively. Table 3.3.1 depicts the resistance of serum-sensitive and serum-resistant *P. aeruginosa* strains to various antimicrobial agents. There were no statistically significant differences in the resistance to all antimicrobial agents, listed in the table, between serum-sensitive and serum-resistant *P. aeruginosa* strains.

Table 3.3.1. *Resistance of serum-sensitive and serum-resistant P. aeruginosa strains to various antibiotics*

Antibiotics by class	***P. aeruginosa* strains**			χ^2	***P* value**
	Antimicrobial agent	**Serum sensitive (N=85), n (%)***	**Serum resistant (N=36), n (%)****		
Carbapenems	Imipenem	48 (56.5)	17 (47.2)	0.87	0.351
Penicillins	Piperacillin	41 (48.2)	21 (58.3)	1.03	0.310
Cephalosporins	Ceftazidime	32 (37.6)	17 (47.2)	0.96	0.327
Fluoroquinolones	Ciprofloxacin	50 (58.8)	19 (52.8)	0.38	0.539
Aminoglycosides	Gentamicin	45 (52.9)	17 (47.2)	0.33	0.565

*Grades 1–4;** grades 5–6.

Application of the tube method (TM) showed that among 121 clinical *P. aeruginosa* isolates, 52 (43.0%), 30 (24.8%), and 39 (32.2%) were found to be nonbiofilm producers, moderate and high biofilm producers, respecti-

vely. The resistance of nonbiofilm producers, moderate and high biofilm producers to various tested antimicrobial agents is shown in Table 3.3.2. In this study we did not found any statistically significant differences between resistance to carbapenems and serum resistance and ability to produce biofilms in *P. aeruginosa* strains. Biofilm formation of *P. aeruginosa* strains had no statistically significant associations with resistance to any tested antibiotic as well.

Table 3.3.2. *Resistance of P. aeruginosa strains with different biofilm production level to various antibiotics (n=121)*

Antibiotics by class	**Antimicrobial agent**	***P. aeruginosa* strains**			***P* value**
		Nonbiofilm producer (N=52), n (%)	**Moderate biofilm producer (N=30), n (%)**	**High biofilm producer (N=39), n (%)**	
Carbapenems	Imipenem	33 (63.5)	15 (50.0)	17 (43.6)	0.152
Penicillins	Piperacillin	31 (59.6)	15 (50.0)	16 (41.0)	0.212
Cephalosporins	Ceftazidime	28 (53.8)	18 (60.0)	16 (41.0)	0.260
Fluoroquinolones	Ciprofloxacin	32 (61.5)	18 (60.0)	19 (48.7)	0.441
Aminoglycosides	Gentamicin	19 (36.5)	12 (40.0)	18 (46.2)	0.651

We did not find any differences in biofilm production among the *P. aeruginosa* strains recovered from different sources (data not shown).

3.4. Phenotypic detection of carbapenemase production in clinical isolates of *P. aeruginosa*

All imipenem- and/or ceftazidime-resistant *P. aeruginosa* isolates were screened for phenotypic detection of the presence of chromosomal resistance (AmpC +/– OprD) or acquired β-lactamases by the CIT and the MHT.

According to results of the CIT, β-lactam resistance was caused by chromosomal mechanisms (AmpC +/– OprD) in 54 (74%) isolates out of 73 (Fig. 3.4.1 A). On the other hand, in 16 (22%) CIT was negative for both ceftazidime and imipenem, suggesting the presence of a carbapenemase (Fig. 3.4.1 B). The remaining 3 isolates were negative only for ceftazidime, suggesting the presence of an ESBL (Fig. 3.4.1 C).

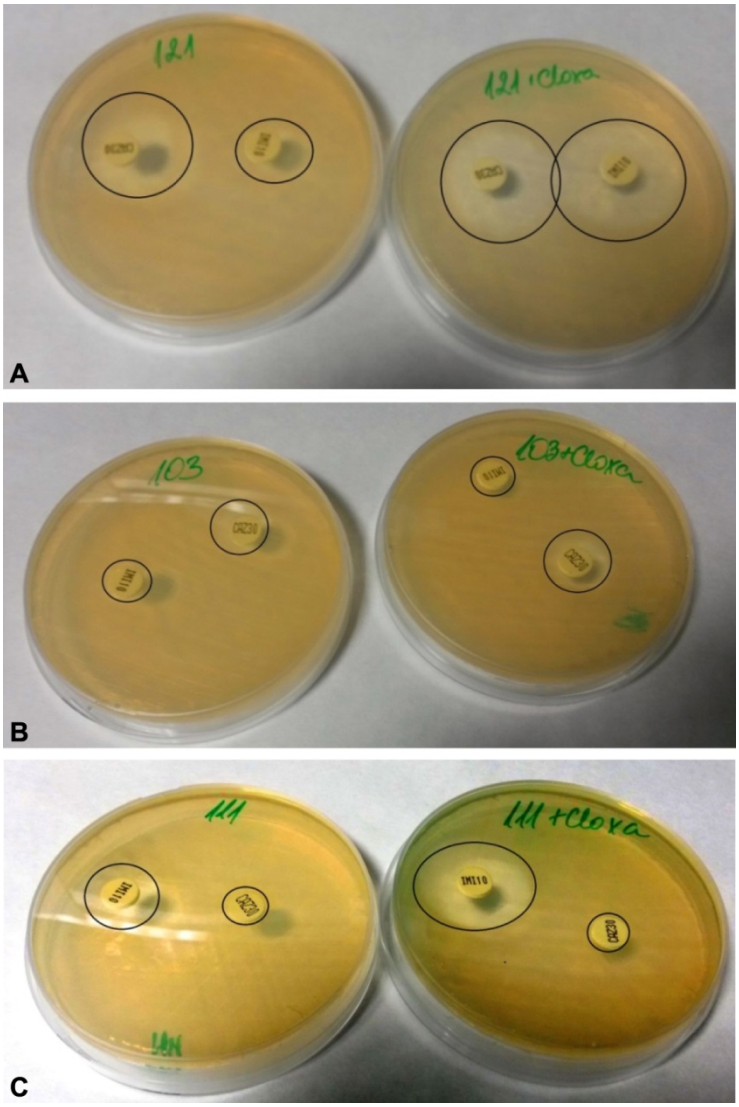

Fig. 3.4.1. *The phenotypic identification of AmpC enzyme conferring carbapenem resistance performed by cloxacillin-containing medium*

A, representative CIT results for carbapenemase negative but AmpC positive in the OprD deficient *P. aeruginosa* isolate; B, carbapenemase-producing *P. aeruginosa* strain; C, ESBL-producing *P. aeruginosa* strains.

A total of 73 resistant *P. aeruginosa* isolates were additionally screened for carbapenemase production by the MHT. The results of this test confirmed CIT results that resistance to carbapenem in 54 isolates was due to a combination of AmpC-production and porin loss (Table 3.4.1). Additionally, 14 strains were positive for carbapenemase in the MHT and negative for ceftazidime and

imipenem with cloxacillin (Fig. 3.4.2). Unexpectedly, 5 strains were negative for both tests and were taken in consideration. None of the isolates in this strain collection were positive with MHT and CIT. The results of CIT and MHT tests are shown in Table 3.4.1.

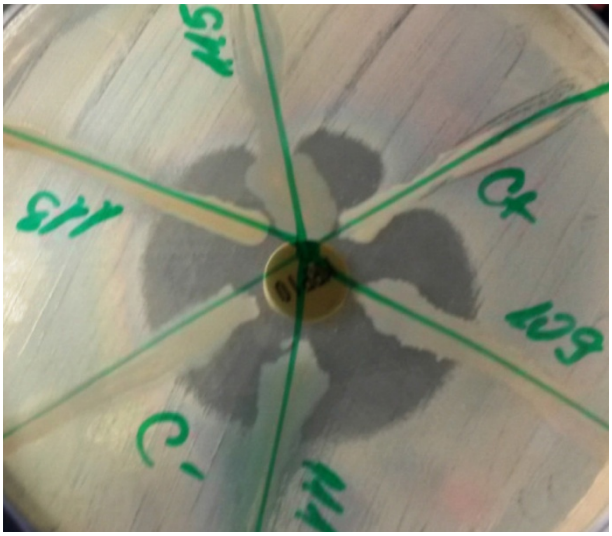

Fig. 3.4.2. *Modified Hodge test (MHT) with meropenem disk*

Interpretation of the MHT results proposed in this study: 115) true-positive MHT, 109;111;113) negative control – *E. coli* ATCC 25922 positive control – *bla*$_{VIM}$-producing *P. aeruginosa.*

Table 3.4.1. *Comparison of combined disk method and modified Hodge test for detection of carbapenemase-producing P. aeruginosa*

No. of strains	Antimicrobial pattern		CIT		MHT	
	Ceftazidime	Imipenem	AmpC		*β*-lactamases	
			Positive N=54 (#)	Negative N=19 (#)	Positive N=19 (#)	Negative N=54 (#)
41	R	R/I	22 (0)	19 (0)*	14 (5)▲	22
30	S	R/I	30 (0)	–	–	30 (0)
2	S	S	2 (0)	–	–	2 (0)

CIT, cloxacillin inhibition test; MHT, modified Hodge test; #, false positive or false negative result. *Including 16 strains with CIT negative for both antibiotics; ▲including two GES-, one PER-, and two VIM-producing strains.

The sensitivity of the CIT for detection of AmpC was 100%, and the specificity was 100% as well if the additional criterion of a negative MHT result was included. In our study, the specificity of MHT was excellent, but the sensitivity was only 73.6%, the reason being that five isolates with no combination of AmpC hyper-production and porin loss showed negative results in MHT. The CIT had a 100% negative predictive value.

The antimicrobial susceptibility of the CIT-negative isolates was significantly higher than that of CIT-positive to all tested antibiotics except for aztreonam, imipenem, and colistin (Table 3.4.2). All CIT-negative isolates were resistant to ceftolozane/tazobactam, while only 1 (1.9%) CIT-positive isolate was resistant to this antibiotic, known to be stable against chromosomal mechanisms of *P. aeruginosa*. Moreover, CIT-negative isolates showed MDR and XDR phenotypes more frequently than CIT-positive isolates.

Table 3.4.2. *Comparative analysis of antibiotic susceptibility profiles of Pseudomonas aeruginosa isolates*

Antimicrobial agent	**No. (%) of nonsusceptible isolates**			
	Total (N=73)	**CIT-positive [Chromosomal (AmpC±OprD)] (N=54)**	**CIT-negative (Carbapenemase/ ESBL producers) (N=19)**	***P value***
TIC	61 (84.0)	42 (77.8)	19 (100)	**0.02**
P/T	46 (63.0)	28 (51.9)	18 (95.0)	**<0.001**
AZT	67 (91.8)	48 (88.9)	19 (100)	0.13
TAZ	41 (56.0)	22 (40.7)	19 (100)	**<0.001**
FEP	23 (32.0)	11 (20.4)	12 (63.2)	**<0.001**
IMP	71 (97.3)	51 (94.4)	19 (100)	0.3
MER	61 (83.6)	42 (77.8)	19 (100)	**0.02**
CIP	53 (72.6)	35 (64.8)	18 (95.0)	**0.01**
TOB	32 (44.0)	15 (27.8)	17 (89.5)	**<0.001**
C/T	20 (27.4)	1 (1.9)	19 (100)	**<0.001**
AMI	23 (31.5)	6 (11.1)	17 (89.5)	**<0.001**
COL	1 (1.0)	0	1 (5.3)	0.28
MDR	56 (76.7)	37 (70.0)	19 (100)	**0.005**
XDR	46 (63.0)	27 (50.9)	19 (100)	**<0.001**

TIC, ticarcillin; P/T, piperacillin/tazobactam; AZT, aztreonam; TAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem, CIP, ciprofloxacin; TOB, tobramycin; C/T, ceftolozane/tazobactam; AMI, amikacin; COL, colistin, MDR, multidrug-resistant; XDR, extensively drug resistant.

3.5. PCR detection of *bla* genes and resistance traits (patterns)

All isolates (n=73) were subjected to the detection of acquired *β*-lactamases through multiplex and specific PCR, followed by sequencing in representative isolates. All 16 CIT-negative isolates were found to be positive for bla_{VIM} gene and also were confirmed to produce MBL enzymes by EDTA double disk synergy test (DDST) (Table 3.5.1).

On the basis of these results, the proportion of isolates producing carbapenemase was 13.22% with regard to all *P. aeruginosa* isolates investigated (n=121), 84.21% with regard to phenotypic test results (n=19), and 21.91% with regard to the isolates with reduced susceptibility to imipenem (n=73).

The results of MICs for MBL, class A carbapenemases and ESBL-producing *P. aeruginosa* isolates are shown in Table 3.5.2. As expected, all investigated strains showed higher level resistance to antibiotics of all classes except polymyxins as their MIC was above the break points recommended by EUCAST (Table 3.5.2). The MIC of imipenem for bla_{VIM}-positive isolates was >64 μg/mL (range 0.5–64), while isolates with a MIC of <64 μg/mL were negative for bla_{VIM}. All VIM producers showed the highest value for ticarcillin (MIC >512 μg/mL) and ceftolozane/tazobactam MICs (>64 μg/mL) compared with bla_{VIM}-negative isolates – the value of inhibition was always below 256 μg/mL and 2 μg/mL, respectively. The VIM producers (except one) were susceptible only to colistin (MIC <2 μg/mL).

All bla_{VIM} positive isolates (n=16) exhibited a XDR phenotype, including resistance to all the tested drugs except colistin (Table 3.5.1). On the other hand, this pan-resistant phenotype was observed in 30 isolates (41%) of bla_{VIM} negative isolates. As shown in Table 3.5.1, VIM producers were recovered from various wards of the hospital, however mainly from intensive care unit (50%) and surgery unit (37.5%). Most of the VIM producers were isolated from wound or skin (37.5%) but they were also recovered from urine and lower respiratory tract (25% each), blood and upper respiratory tract (6.25% each). Isolates positive for CIT test (n=3) were negative only for ceftazidime. One isolate produced PER ESBL enzyme and the other two were positive for GES enzymes. The PER ESBL isolate showed the same resistance profile (XDR) as MBL producers. Two GES-positive *P. aeruginosa* isolates showed reduced resistant profile to imipenem and meropenem (only one) with MIC of 16 μg/mL and 8 μg/mL compared with MBL producing strains.

No bla_{VIM-1}, bla_{IMP-2}, bla_{IMP-1}, bla_{NDM}, bla_{OXA-48}, bla_{KPC}, or bla_{VEB} was amplified from any of the 73 isolates.

Table 3.5.1. *Characteristics of MBL (B class)-, class A carbapenemase-, and extended spectrum β-lactamase (ESBL)-producing P. aeruginosa isolates*

No.	Site of isolation	Ward	Resistant profile	Biofilm production level	Carbapenemase type	Molecular class	PFGE number	PFGE group	ST
5	WS	S	XDR	None	GES-27	A	PT-1	B	ST660
13	WS	ICU	XDR	High	PER-1*	A**	ND	ND	ND
32	WS	S	XDR	Moderate	VIM-2	B (MBL)	PT-2	D	ST235
33	WS	S	XDR	Moderate	VIM-2	B (MBL)	PT-3	D	ST235
46	URT	IM	XDR	None	VIM-2	B (MBL)	PT-4	C	ST1047
47	WS	S	XDR	Moderate	VIM-2	B (MBL)	PT-5	F	ST235
51	WS	S	XDR	None	VIM-2	B (MBL)	PT-6	F	ST235
70	LRT	S	XDR	High	VIM-2	B (MBL)	PT-7	F	ST235
96	U	ICU	XDR	High	VIM-2	B (MBL)	PT-8	F	ST235
98	BS	ICU	XDR	None	VIM-2	B(MBL)	PT-9	F	ST235
99	LRT	ICU	XDR	High	VIM-2	B (MBL)	PT-10	E	ST235
100	LRT	ICU	XDR	High	VIM-2	B(MBL)	PT-11	F	ST235
101	U	ICU	XDR	High	VIM-2	B (MBL)	PT-12	F	ST235
103	LRT	ICU	XDR	High	VIM-2	B (MBL)	PT-13	F	ST235
107	U	S	XDR	Moderate	VIM-2	B (MBL)	PT-14	F	ST235
111	LRT	S	XDR	None	GES-5	A	PT-15	A	ST1628
115	WS	IM	XDR	None	VIM-2	B (MBL)	PT-16	F	ST235
134	U	ICU	XDR	None	VIM-2	B (MBL)	PT-17	D	ST235
135	WS	ICU	XDR	None	VIM-2	B (MBL)	PT-18	D	ST235

ICU, intensive care unit; XDR, extensively drug-resistant; PFGE, pulse-field gel electrophoresis; PT, pulsotypes; ST, sequence type; S, surgery; IM, internal medicine; WS, wound or skin; URT, upper respiratory tract; LRT, lower respiratory tract; U, urine; BS, blood stream. *Non-carbapenemase; **extended-spectrum.

Table 3.5.2. *MICs of antibiotics for MBL-, class A carbapenemase-, and ESBL-producing P. aeruginosa isolates*

No. of isolates	MIC (μg/mL)											
	Penicillins	Penicillins + β-lactamase inhibitors	Monobactams	Cephalosporins		Carbapenems		Fluoroquinolones	Aminoglycosides		Cephalosporins inhibitors	Polymyxins
	TIC >16#	P/T >16#	AZT >16#	TAZ >8#	FEP >8#	IMP >8#	MER >8#	CIP >1#	TOB >4#	AMI >16#	C/T >4#	COL >4#
5▲	128	32	16	64	64	16	8	16	32	64	64	4
13*	512	128	128	64	64	32	32	16	32	32	64	4
32	512	64	16	32	16	64	16	16	32	64	64	2
33	512	32	2	16	16	64	8	4	32	32	64	2
46	512	32	4	16	8	64	8	8	32	128	64	2
47	512	16	2	16	8	64	8	8	32	128	64	2
51	512	32	1	16	8	64	16	4	32	32	64	2
70	512	32	16	32	16	64	32	16	32	32	64	2
96	512	32	2	16	8	64	16	8	32	32	64	2
98	512	32	2	16	16	64	16	8	32	32	64	2
99	512	128	32	32	8	64	16	16	32	128	64	2
100	512	32	8	16	16	64	32	16	32	32	64	2
101	512	32	2	16	8	64	16	8	32	32	64	2
103	512	128	16	32	16	64	32	16	32	32	64	2
107	512	32	16	32	16	64	16	16	32	32	64	2
111▲	256	32	8	64	16	16	4	0.12	32	128	64	2
115	512	256	32	64	32	64	32	16	32	32	64	8
134	512	32	2	16	16	64	16	4	4	4	64	2
135	512	16	4	16	8	64	16	2	4	4	64	2

MIC, minimum inhibitory concentration; TIC, ticarcillin; P/T, piperacillin/tazobactam; AZT, aztreonam; TAZ, ceftazidime; FEP, cefepime; IMP, imipenem; MER, meropenem, CIP, ciprofloxacin; TOB, tobramycin; C/T, ceftolozane/tazobactam; AMI, amikacin; COL, colistin. ▲class A carbapenemase producers; *ESBL producing strains; #strain considered to be resistant above these MIC.

3.6. Molecular characterization of the gene encoding a new *β*-lactamase in clinical *P. aeruginosa* strains

PCR sequence analysis revealed that 16 *P. aeruginosa* isolates carried $bla_{\text{VIM-2}}$. One isolate was found to carry a variant $bla_{\text{PER-1}}$; one, a carbapenem-hydrolyzing enzyme GES-5, and one, a novel variant of class A enzymes.

At a nucleotide level, the highest similarities of new entire sequence were detected with 99% of the MBL bla_{GES} like genes from *P. aeruginosa*. A new allele, with 803 bp (base pair) fragment long and contained 6 open reading frames designated GES-27 enzyme. GES-27 differed from GES-5 by a single amino acid substitution, proline (Pro) 167 was replaced by glutamine (Gln) (Fig. 3.6.1). The importance of these changes in the regulation of the expression of the GES enzyme remains unknown. However, $bla_{\text{GES-27}}$ shows 99% identities with novel GES variants: $bla_{\text{GES-28}}$ described for a *P. aeruginosa* isolate in Germany (GenBank: ALC74967.1), as well as with $bla_{\text{GES-29}}$ (GenBank: ALM96712.1), recovered in Australia. *β*-Lactamase GES-27 differed from GES-28 by the same amino acid substitution plus aspartate (Asp) was replaced to asparagine (Asn) at Amber position 179 (both have similar characteristic) and from GES-29 by only a Gln-to-Ala (glutamine to alanine) change at Amber position 243 (Fig. 3.6.1).

As expected, expression of bla_{GES} genes in *P. aeruginosa* conferred resistance to all *β*-lactams except GES-5 producing strain which showed reduced susceptibility only to aztreonam and ciprofloxacin (Table 3.5.2). However, the MICs of ticarcillin, imipenem, and meropenem were lower for VIM-producing strains than those for GES enzyme-producing strains. In contrast, the MICs of cefepime for *P. aeruginosa* carrying GES-27 were higher than those obtained for *P. aeruginosa* (VIM-2) (Table 3.5.2).

It is noteworthy that both isolates producing GES enzymes (GES-5 and GES-27) showed a negative MHT and a positive CIT result for imipenem, indicating that carbapenem resistance in these two isolates depended mainly on chromosomal mechanisms (OprD + AmpC). However, they both yielded a weakly positive result of ESBL DDST.

Fig. 3.6.1. *Comparison of the amino acid sequences of β-lactamases GES-5, GES-15, GES-27, GES-28 and GES-29*

The differences found between GES-27 and other GES variants are boxed.

3.7. Genetic relationships among *Pseudomonas aeruginosa* strains based on molecular typing methods

Spe*I* restriction digestion applied in carbapenemase-producing *P. aeruginosa* isolates yielded 18 banding patterns, labeled from PT-1 to PT-18 (Table 3.5.1). Studies on genetic relatedness showed that isolates shared ≥90 identity bands. PFGE revealed 2 main clusters with 6 dominance groups, denoted as A-F (Fig. 3.7.1). The largest PT group was F that consisted of 10 $bla_{\text{VIM-2}}$-producing isolates. The other two groups, E and D of the cluster 1, also producing $bla_{\text{VIM-2}}$, were smaller than the group F VIM-producing isolates. All isolates of these groups had almost identical resistance patterns. The last isolate carrying $bla_{\text{VIM-2}}$ belonging to group C did not show any similarity to groups E, F, or D.

The non-MBL isolates were divided into groups A and B. Group A comprised isolates carrying GES-5 *β*-lactamase and cluster B contained one isolate carrying a novel GES variant.

As shown in Table 3.5.1, 15 $bla_{\text{VIM-2}}$ gene encoded isolates belonged to a single genotype, identified as the international high-risk clone ST235 by MLST analyses. The only one VIM-2 isolate was identified as the clone ST1047. Remarkably, most of the isolates belonging to the clone ST235 were isolated from patients admitted to the ICU or reanimation unit. The isolates producing GES-5 and GES-27 enzymes were found to belong to two different PFGE clonal types and MLST clones, i.e., ST1628 and ST660, respectively (Table 3.5.1).

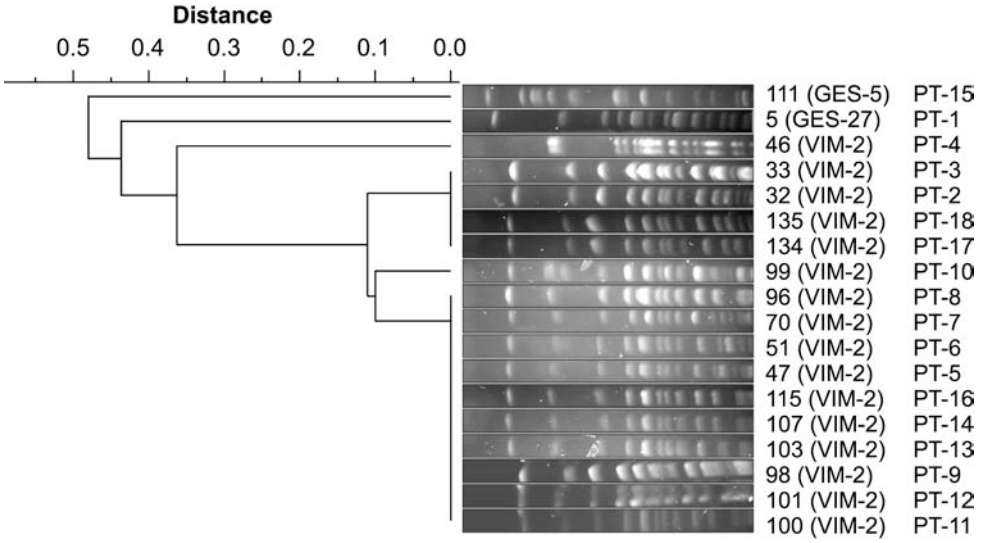

Fig. 3.7.1. *UPGMA-phylogenetic tree showing the relationship among 18 carbapenemase-producing P. aeruginosa isolates*

The tree was constructed based on the DNA macro-restriction fragment patterns obtained by PFGE using *SpeI* restriction enzyme.

3.8. Characteristics of class 1 integron-associated MBL genes

In order to characterize the genetic elements harboring $bla_{\text{VIM-2}}$ in *P. aeruginosa* isolates belonging to ST235 and ST1047 clones, PCR and sequencing of the involved integrons was performed. $bla_{\text{VIM-2}}$ gene identified in isolates from ST235 was located in the previously described class 1 integron In559 (GenBank DQ522233) (Fig. 3.8.1 A).

The predominant integron In559 consists of three regions: a 5′ conserved segment (5′-CS), a variable region, and a 3′ conserved segment (3′-CS). The first position of variable region is occupied by an aminoglycoside-6′-acetyltransferase gene (*aacA7*) responsible for resistance to amikacin and other aminoglycosides. At the next position is the MBL gene $bla_{\text{VIM-2}}$ that confers resistance to carbapenems. In the third position, the dihydrofolate reductase gene (*dfrB*) for trimethoprim resistance, followed by and an aminoglycoside acetyltransferase gene were found. In the 3′-CS end *tni* gene, code for a recombination protein was also identified in all ST235 clones (Fig. 3.8.1 A).

$bla_{\text{VIM-2}}$ from ST1047 showed a different integron structure (Fig. 3.8.1 B). Between its 5′-CS and 3′-CS ends, In599 integron contained only two gene cassettes. Just downstream of the 5′-CS, an *aacA4* gene cassette encoding an

AAC(6′)-I aminoglycoside acetyltransferase was identified, meanwhile the *bla*$_{VIM-2}$ gene cassette was inserted as the second position. The cassette array was followed not by a 3′- CS typical *sul1* gene associated integron but by *tniC* gene typical of transposition model.

bla$_{GES-27}$ was also found to be located in a class 1 integron, immediately downstream of *IntI1*, but multiple attempts to link *IntI1* to *bla*$_{GES-5}$ through PCR consistently failed (data not shown).

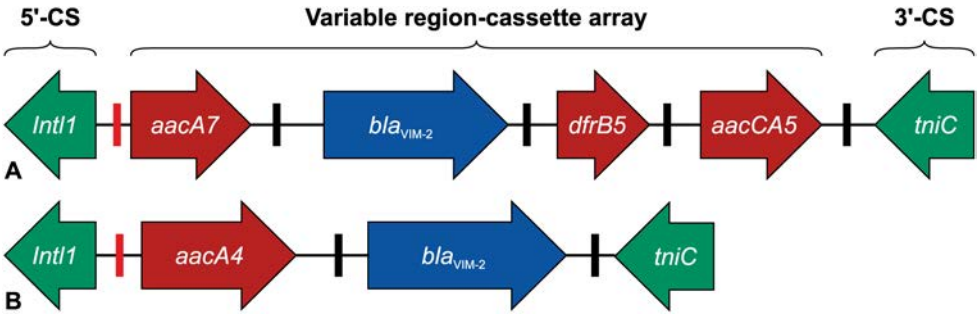

Fig. 3.8.1. *Schematic representation of Pseudomonas aeruginosa reported in this study.*

The gene cassettes are represented by boxed arrows indicating the transcriptional orientation. A red line represents *attII* site, and the black line represents the gene cassette recombination site, *attC* or 59-bp element. Variable region is between the integrase gene (*IntI1*) and the 3′-CS (conserved sequences) – 5′ and 3′ conserved sequences of class 1 integrons are green and MBL (metallo-β-lactamase) gene cassette are blue.

4. DISCUSSION

An increased risk of nosocomial infections in ICUs over the last decade has raised a concern among healthcare professionals and the wider community [197]. In particular, *P. aeruginosa* is one of the most frequent gram-negative pathogens, accounting for 13% of all pathogens that colonize after admission to ICUs [198]. The National Nosocomial Infections Surveillance system (NNIS) in the United States reports *P. aeruginosa* as the leading pathogen in ICUs that causes ventilator-associated pneumonia (VAP), hospital-acquired urinary tract, bloodstream infections and surgical site infection for the last two decades. Similar results were achieved in the Extended Prevalence of Infection in Intensive Care (EPIC II) study conducted in Europe [199]. The data of this large international study indicated *P. aeruginosa* as a predominant pathogen responsible for 30% of pneumonias, 19% of urinary tract infections, and 10% of bloodstream infections. In agreement with these findings, our results demonstrated a high prevalence of *P. aeruginosa* infections in the ICUs (40.5%) at the Hospital of Lithuanian University of Health Sciences. Although we have not analyzed transmission pathways of *P. aeruginosa*, several reports demonstrated the role of exogenous or endogenous reservoirs of nosocomial *Pseudomonas* spp. infections in the ICU [200,201]. Besides all known risk factors, antibiotic selective pressure and patient colonization pressure play a more important role for this pathogen acquisition in ICUs with and endemic level [202].

4.1. Resistance pattern of *P. aeruginosa*

The results of antimicrobial susceptibility testing were reviewed for a collection of 121 *P. aeruginosa* strains isolated from various clinical specimens in the Hospital of Lithuanian University of Health Sciences during the 2-year period. More than half (58.6%, 71/121) of the *P. aeruginosa* isolates were resistant to carbapenem. The percentage of the *P. aeruginosa* isolates exhibiting resistance to carbapenems documented in this study was higher as compare with previous studies conducted in 2003 (21.1%) and 2008 (41.6%) [203]. All carbapenem-resistant isolates, except 12 isolates, were resistant to both imipenem (97.3%) and meropenem (83.6%). Carbapenem-resistant isolates were also found to be significantly more frequently resistant to all β-lactams and non-β-lactams than carbapenem-susceptible strains. Furthermore, 54 (76.0%) and 36 (50.7%) carbapenem-resistant strains were found to be resistant to at least 3 different classes of antimicrobial agents and therefore were considered as MDR and XDR, respectively.

In comparison to the previous study carried out in Lithuania, the present study showed a significantly increased MDR rate from 7.1% in 2003 to 44.6% (54/121) in 2012 (+36.9%) [204]. In this study, the prevalence of MDR *P. aeruginosa* isolates is similar to that reported in southern European countries such as Spain (46.0%) [188], Greece (63.3%) [205], and Croatia (82%) [206], but relatively higher than those reported in neighboring countries such as Poland (14.2%) [207]. However, it is worth noting that MDR rates between present and other studies are difficult to compare due to varying definitions of multidrug resistance.

4.2. Biofilm formation and serum susceptibility in *P. aeruginosa*

Numerous studies indicate that bacteria resistance to various antimicrobial agents is associated with the ability to develop surface attached polymicrobial communities known as biofilms [208, 209]. Hostacka et al. showed that the percentage of biofilm production in the strains sensitive or resistant to ciprofloxacin and aminoglycosides was the same [210]. However, the results of our research showed no association between biofilm formation and resistance of carbapenem-resistant and carbapenem-sensitive *P. aeruginosa* strains. Studies done by Carlos et al. [211] and other authors [212] reported that strains capable of forming biofilms were more frequently observed among isolates with a MDR phenotype. Contrary to the results of these studies, our study did not support this hypothesis as well (Fig. 4.2.1).

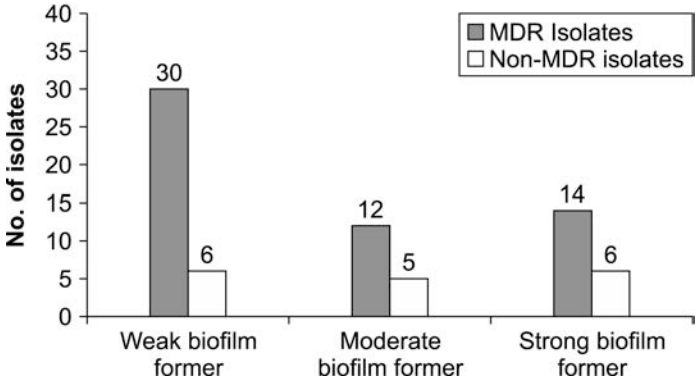

Fig. 4.2.1. *The relation between biofilm formation and multidrug-resistant Pseudomonas aeruginosa*

It is well known that serum sensitivity or resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains [213]. In this study, approximately one-

third of our tested single clinical isolates was resistant to serum bactericidal activity and was high-biofilm producers. However, there was no association between resistance to serum, biofilm formation, and resistance to carbapenems and other classes of the tested antibiotics. Together these findings suggest that single isolates being sensitive *in vitro* to antibiotics probably can become resistant when acting as a member of biofilm in biofilm-producing strains. This could explain why no association was found between in vitro sensitivity to antibiotics and biofilm formation.

4.3. Phenotypic detection methods of carbapenemase production in *P. aeruginosa*

Phenotypic detection of carbapenemase-producing organisms is originally based on tests showing reduced carbapenem susceptibility. It was noticed that MICs of carbapenems could be highly variable (low levels of carbapenem resistance) thus leading to problematic detection of potential carbapenemase producers [214]. Moreover, carbapenem resistance in *Enterobacteriaceae* and *P. aeruginosa* is not only associated with production of carbapenemase enzymes, but is also mediated by other mechanisms such as AmpC β-lactamase production and porin loss [215, 216]. As 71 of the 73 *P. aeruginosa* strains were identified as resistant to carbapenems determined by Sensititre susceptibility MIC plates, we selected all *P. aeruginosa* clinical isolates for putative AmpC production based on reduced susceptibility to cephalosporins and/or carbapenems. Several methods, such as the double disk synergy test (DDTS), combine disk test (CDT), Etest strip, or β-lactamase inhibitor-based methods, have been developed to detect AmpC production [217, 218]. The inhibitor-based synergy assay (such as cloxacillin) is usually performed due to high sensitivity and specificity values of this method [219–221]. Despite the fact that the most accurate in vitro identification of bacteria producing carbapenemases is obtained with the commercial agar tablet/disc diffusion test (RoscoDiagnostica KPC and MBL confirm kit) [182, 222], this method remains to be highly expensive for many diagnostic laboratories. We demonstrated that cloxacillin, a specific inhibitor of class C β-lactamases, in combination with CAZ and imipenem accurately detected 100% (54/54) of AmpC-positive isolates. Increased resistance to MEM due to the overexpression of efflux pumps was the main criteria in our study to change this antibiotic to IMP disk in order to avoid false results. On the other hand, the CIT used proved to be a highly sensitive and specific screening method for the detection of acquired β-lactamases in *P. aeruginosa*. Our data are thus in agreement with the results of the recent study by Fournier et al. [184], which used imipenem and cloxacillin disks to

discriminate between carbapenemase-producing and nonproducing *P. aeruginosa* strains. Moreover, our approach, employing ceftazidime in addition to imipenem, allowed us the detection of not only prevalent MBLs, but also ESBLs and GES-type carbapenemases. Additionally, as described previously [190], resistance to ceftolozane, even if combined with tazobactam, was a good predictor of the presence of acquired *β*-lactamases (ESBLs or carbapenemases) in *P. aeruginosa*; all 19 isolates shown to produce these acquired enzymes were found to be resistant to ceftolozane/tazobactam, whereas only 1 of the 54 isolates showing chromosomal resistance had an MIC greater than 4/4 mg/L.

In our study, we decided to confirm carbapenemase presence by the MHT, which was recommended by the CLSI in 2009 [223]. The test successfully identified 73.68% (14/19) of the carbapenemase producers. False-negative results were obtained for 2 of the 16 VIM-2 producing *P. aeruginosa* strains. The results of our study are largely in agreement with the findings of previous reports by Girlich et al., who demonstrated false-negative results for 7 of the 14 NDM-1-producing *Enterobacteriaceae* strains [224], and Galani et al., who reported a rate of 4.2% of false negative results among 95 MBL-positive isolates [225]. False-negative results of the MHT for 6 of the 14 VIM-2-producing *Pseudomonas* spp. were also reported by Wonkeun et al. [226], although Lee et al. suggested that $ZnSO_4$-containing MacConkey agar may improve the sensitivity of the MHT for detecting IMP or VIM-producing *P. aeruginosa* from 77.4% to 94% [227]. We did not try to modify of the MHT based on observation that the test performance could be highly varied depending on the MBL and species tested [224, 228]. However, class A (GES enzyme) carbapenemase-producing strains (n=2) and one ESBL producer were not detected with MHT test. These findings coincide with other recent studies [160]. Unfortunately, this test could not differentiate the carbapenemase producer and is not highly sensitive and specific [229]. The sensitivity and specificity for detecting MBL-producing *P. aeruginosa* in our study were only 73.6% and 91.5%, respectively.

4.4. Molecular characterization of *P. aeruginosa* resistant to carbapenem antimicrobials

The PCR assay was also used for the detection of the presence of MBL genes in *P. aeruginosa*. Of the 12 MBLs currently defined, the MBL VIM-2 was, by far, the most prevalent acquired *β*-lactamase among *P. aeruginosa* strains isolated in the Hospital of Lithuanian University of Health Sciences.

Based on these results, the proportion of isolates producing this gene was 14.8% with regard to all *P. aeruginos*a isolates investigated and 24.65% with regard to the isolates with reduced susceptibility to carbapenems. Similarly, the prevalence of MBL-producing *P. aeruginosa* was detected in three Greek studies where the prevalence of VIM-2 producers ranged between 16–28% of all tested carbapenem-resistant *P. aeruginosa* [158, 230, 231]. A higher prevalence (49.4%) of VIM-2 production among carbapenem-resistant *P. aeruginosa* isolates was observed in another study conducted in Spain in 2013 [232]. In 2012, Viedma et al. [134] reported a large outbreak of infections caused by a VIM-2-producing MDR *P. aeruginosa* strain that was responsible for 76% of the infections in the Spanish hospital. Nevertheless, the increasing number of MBL-producing *P. aeruginosa* has been recently identified in countries closer to the Lithuanian border. Poland was the first country in Central Europe reporting an growing occurrence of MBL producers [141, 233]. Since then, VIM-4 has become endemic in *P. aeruginosa* isolates obtained from patients hospitalized at the Children's Memorial Hospital, Warsaw, during 1998–2006 [234]. MBL *P. aeruginosa* is increasingly identified in countries considered to have a low prevalence of antimicrobial resistance. Belgium reported a large number of MBL-producing *P. aeruginosa* strains. Of the 127 carbapenem-resistant strains, more than 67% were confirmed to be VIM-2 producers [235]. Infections with VIM-2-producing *P. aeruginosa* have been also reported in Germany [236, 237]. Recently, Schneider et al. have described a novel VIM-2 type variant, VIM-15, and VIM-16 [238] which has repeatedly been detected in other part of Germany [239]. The findings of this study clearly demonstrate the ongoing spread and evolution of this group *β*-lactamases. A nationwide surveillance study performed in 2010–2011 identified 40% of VIM-2 MBL-producers isolated in 11 different hospitals of the Netherlands [240]. MBL has also been detected in another study reported from the Netherlands, where 35 (33%) of the 106 *P. aeruginosa* strains isolated in the ICU were identified as VIM-2 producers [241]. In the Balkans, VIM-2 was found in *P. aeruginosa* isolates from Serbia [242] and Croatia [243], and a VIM-2 variant, VIM-15, was isolated from *P. aeruginosa* strains in Bulgaria [238]. Despite the fact that Scandinavian countries such as Sweden and Norway have so far succeeded in keeping the incidence of major resistant nosocomial pathogens at a low level, several studies have demonstrated VIM-2 producing strains to be associated with international travelling [244].

In our study, the VIM-2 producers were mainly recovered from internal medicine (50%) and surgery units (37.5%). All bla_{VIM}-positive isolates (n=16) exhibited a XDR phenotype. However, in our study, we did not find any association between carbapenem resistance rates and presence of

VIM-2, as 77% of the strains were resistant to imipenem without MBL production. This finding is supported by previous studies from France demonstrating that more than 80% of the imipenem-resistant strains were not associated with MBL production [245]. Similar results were observed among carbapenem-resistant strains isolated in Norwegian and Swedish hospitals [246, 247]. This demonstrates that carbapenem resistance could be also driven by other resistance mechanism including mutations in the *oprD* gene accompanied with AmpC or up-regulation of efflux pumps.

To our knowledge, this study is the first reporting $bla_{\text{VIM-2}}$ incidence in Lithuania. Since there is no similar study conducted and published in Lithuania, we suppose that such isolates carrying MBL genes could be detected in other region of Lithuania, but remained to be undetected until now. Based on other authors' observations, described above, we also believe that these MBL genes could be transmitted from other *P. aeruginosa*, and human travelling contributes to the dissemination of these genes.

Additionally, our work has led to the identification of a previously undescribed GES variant, designated as GES-27. GES-27 differs from carbapenem-hydrolyzing GES-5 by a single amino acid substitution, proline 167, that is replaced by glutamine. Interestingly, the replacement of proline 167 by a different amino acid (serine) has previously been noted in another GES-5 variant (GES-15) [248]. Since GES-5 was also detected in our collection, we wondered whether isolates producing GES-5 and GES-27 would be clonally related, suggesting a recent evolution of GES-27 from GES-5 in this clonal lineage. However, both isolates showed different PFGE profiles ruling out this possibility. Although several variants of GES enzymes have been identified worldwide with reports from Europe [249], Asia [250], Africa [251], and America [252], these enzymes have been most frequently associated with single occurrences contrarily to class B MBLs.

In our study, all VIM-2 producers except one belonged to international clone lineage ST235. Although, VIM-2-producing ST235 isolates have been documented in 17 different countries worldwide [253], this work adds Lithuania to the growing list of countries documenting epidemic dissemination of this clonal lineage linked to potent β-lactamases.

PCR mapping showed that VIM-2 was inserted in a class 1 integron with three gene cassettes, downstream of the *aacA7* gene cassette encoding AAC(6′)-I1 aminoglycoside acetyltransferase explaining aminoglycoside resistance. A similar integron (GenBank accession number EF577406) was detected in a *P. aeruginosa* isolate in Palma de Mallorca, Spain (Juan et al., unpublished data) and Romania [254]. In particular, the previous integron had three gene cassettes in the variable region (*aacA7*, $bla_{\text{VIM-2,}}$ and *dhfrB*) similarly to integrons found in the United States (21), Taiwan [255], Russia

and India [256]. A class 1 integron carrying *aacA7* and *bla*$_{\text{VIM-2}}$ has been described in Norway [257]. This integron was reported to be embedded in a TniC-like transposon from a Ghana *P. aeruginosa* isolate. Instead of the conserved 3′ end with *qacEΔ1* and *sulI* genes, VIM-2 was followed by a dihydrofolate reductase gene for trimethoprim resistance and an aminoglycoside acetyltransferase gene. The 3′ end having the *tniC* gene, which encodes a recombination protein, has recently been found to be associated with a VIM-2 integron from a French *P. aeruginosa* isolate [258]. Based on multiple reports, the extremely high incidence recently reported in Russia and Belarus is of particular concern [259]. Indeed, as found in Russian isolates, *aacA7* preceded *bla*$_{\text{VIM-2}}$ in a class 1 integron in our ST235 isolates, potentially indicating a common origin.

CONCLUSIONS

1. *P. aeruginosa* strains exhibited high-level resistance not only to carbapenems, but also to antibiotics of other classes. A relatively great proportion (74%, n=54) of *P. aeruginosa* strains were multidrug and broad-spectrum resistant. One-third of the tested *P. aeruginosa* strains were resistant to at least 7 of the 12 antimicrobial agents, including ceftolozane/tazobactam, a new antimicrobial drug. Colistin was found to be the most effective antibiotic against *P. aeruginosa*.
2. One-third of the tested *P. aeruginosa* clinical isolates was resistant to serum bactericidal effect and was high-biofilm producers. No associations between resistance to serum bactericidal effect, biofilm formation as well as resistance to carbapenems and other classes of the tested antibiotics were found.
3. Both phenotypic methods – the modified Hodge test and the cloxacillin inhibition test – provided useful information on the mechanisms of drug resistance in *P. aeruginosa*. *P. aeruginosa* isolates with porin loss and hyperproduction of AmpC showed positive synergism of ceftazidime and imipenem with cloxacillin and negative result of the modified Hodge test. Negative results of the modified Hodge test ruled out almost all of the tested VIM isolates.
4. In the Hospital of Lithuanian University of Health Sciences, $bla_{\text{VIM-2}}$ was found to be the most common metallo-β-lactamase gene linked to the international high-risk clone ST235. A new bla_{GES} β-lactamase gene, which has not been described previously, was identified, and a new identification number, GES-27, was assigned in the database.

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LIST OF THE AUTHOR'S PUBLICATIONS

Publications related to the results of dissertation:

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Other publications:

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Biofilm formation and serum susceptibility in Pseudomonas aeruginosa

Research Article

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Abstract: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the most important opportunistic pathogens. The pathogenicity of *P. aeruginosa* has been associated with multiple bacterial virulence factors. The aim of this study was to evaluate the association between *P. aeruginosa* strains obtained from various clinical samples and resistance to antibiotics and pathogenicity factors, such as resistance to serum bactericidal activity and biofilm formation. This study included 121 *P. aeruginosa* strains isolated from clinical samples; 65 of the isolated *P. aeruginosa* strains were carbapenem-resistant, and 56 were carbapenem-sensitive. Carbapenem-resistant *P. aeruginosa* strains were more often resistant to the majority of tested antibiotics, compared to carbapenem-sensitive strains. We did not find any statistically significant difference between resistance to carbapenems and serum resistance and ability of tested P. aeruginosa strains to produce biofilms. Carbapenem-resistant *P. aeruginosa* strains were recovered from the urinary tract significantly more often (75.0%) than carbapenem-sensitive *P. aeruginosa* strains (25.0%). Carbapenem-sensitive *P. aeruginosa* strains were recovered significantly more often from the respiratory tract than carbapenem-resistant strains, 60.0% and 40.0%, respectively. All the *P. aeruginosa* strains recovered from blood were serum-resistant. *P. aeruginosa* strains recovered from the respiratory tract and wounds were significantly frequently serum sensitive, 95.6% and 56.6%, respectively. We did not find any differences in biofilm production among the *P. aeruginosa* strains recovered from different sources.

Keywords: *P. aeruginosa* • *Antibiotic susceptibility* • *Biofilm* • *Carbapenem-resistance* • *Serum-bactericidal activity*

1. Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the most important opportunistic pathogens causing a variety of severe acute and chronic infections in hospitalized, immunocompromised hosts [1]. These gram-negative, non-fermenting bacteria continue to be a major cause of nosocomial infections, predominantly pneumonia and infections of the urinary tract, skin and soft tissue. Furthermore, they are the most prevalent pathogens isolated from patients with chronic lung infections, including cystic fibrosis, with high rates of associated morbidity and mortality [2-4].

The predisposition of *P. aeruginosa* to development of resistance to antibiotics and expression of multiple virulence factors contributes to the frequent ineffectiveness of current therapies. The pathogenicity of *P. aeruginosa* has been associated with multiple bacterial virulence factors, including biofilm formation and the expression of adhesions, endotoxin and hydrolytic exotoxins, which cause tissue destruction. The resistance to serum bactericidal effect is one of the major virulence factors of *P. aeruginosa* [5,6]. The host innate immune system includes serum components, such as antibodies and proteins of the complement system that mediate the bactericidal effect of serum. This phenomenon is seen with a higher frequency of serum resistance among *P. aeruginosa* strains isolated from blood, wounds, urine [7,8] than among strains isolated from the sputum of asymptomatic patients with cystic fibrosis [9-11]. Serum resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains and isolates [12].

Therapy is complicated by the organism's potent ability for adaptation, mutation, and gene acquisition [13]. This diversity of *P. aeruginosa* infections is due

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to the development of various adaptive mechanisms such as the nutritional and metabolic pathways, besides the regulation of gene expression.

P. aeruginosa can form bacterial biofilm that protects the organism from defenses and antimicrobial therapy [14]. *P. aeruginosa* biofilm is difficult to eradicate, and it causes bacterial persistence, leading to infection chronicity and morbidity [15].

In addition, its ability to form biofilm provides greater protection against host immune defense systems and susceptibility to various antimicrobial agents [16,17]. P. aeruginosa is a multidrug resistant (MDR) organism and is considered a phenomenon of bacterial resistance. This is demonstrated by different types of antibiotic resistance. It is also commonly believed that in MDR *P. aeruginosa* isolates, reduced virulence may result due to decreased biofilm. However, recent data suggest otherwise, and MDR *P. aeruginosa* may remain fully pathogenic [18].

The aim of this study was to evaluate the association between the resistance of *P. aeruginosa* strains obtained from various clinical samples and antibiotics and pathogenicity factors such as resistance to serum bactericidal activity and biofilm formation.

2. Materials and methods

2.1 Bacterial strains and susceptibility testing

A total 121 strains of *P. aeruginosa* were included in this study. All these strains were isolated from clinical samples of patients treated in the Hospital of Lithuanian University of Health Sciences during the period 1 January 2011 to 31 June 2012. Sixty-five (53.7%) strains showing resistance to meropenem and/or imipenem by routine disk diffusion method and 56 (46.3%) strains sensitive to meropenem and imipenem were included in this study. Only one strain per patient was included.
The susceptibility testing to meropenem, imipenem, piperacillin, ceftazidime, ciprofloxacxin, gentamicin, and amikacin was performed by the E-test method according to the recommendations of the manufacturer (Liofilchelm, Italy). Detected minimal inhibitory concentrations were evaluated according to The European Committee on Antimicrobial Susceptibility Testing (EUCAST) breakpoints [19]. Minimum inhibitory concentration (MIC) values, which were detected between the sensitive and resistant breakpoint, were interpreted as sensitive. The susceptibility testing to piperacillin/tazobactam, cefepime, cefoperazone/sulbactam, tobramycin, aztreonam was performed by disk diffusion method (BBL, USA), and zones were interpreted according to EUCAST recommendations.

All *P. aeruginosa* strains according to interpreted MIC results were divided into carbapenem-sensitive and carbapenem-resistant groups. The strains resistant to meropenem and imipenem were attributed to the carbapenem-resistant group.

2.2 Serum bactericidal assay

The ability of *P. aeruginosa* strains to resist serum bactericidal effect was tested as described earlier in the literature [20,21].

Bacterial ability to stay viable under human serum effect was evaluated after 1 hour, 2 hours and 3 hours and graduated to 6 levels. *P. aeruginosa* strains assigned to 1-4 levels were interpreted as serum sensitive and at 5-6 levels as serum resistant. Every strain of P. aeruginosa strain was tested 3 times. A strain was considered sensitive or resistant if the detected level was the same in all experiments.

2.3 Biofilm formation

The tube method, described by Christensen, was used to detect biofilm formation [22]. One to two overnight growth colonies of tested microorganisms were inoculated with 10 ml Trypticase Soy Broth (EMAPOL, Poland). The tubes were incubated at 37°C for 24 h. After incubation, tubes were washed with phosphate buffer saline and dried, then stained with crystal violet (0.1%) for 20 min. The excess stain was washed with deionized water. Tubes were dried in an inverted position at room temperature. Biofilm formation was considered positive when a visible film lined the bottom of the tube. The strains were grouped as non-biofilm producers (no visible film line), moderate-biofilm producers (medium intense film line), and high-biofilm producers (intense film line).

2.4 Statistical analysis

Proportions were compared with nonparametric statistical criterion chi-square or Fisher's exact test. Differences between groups were considered significant if P was <0.05. Statistical package IBM SPSS Statistics Version 20 was used for the data analysis.

3. Results

In our study P.aeruginosa strains (n=121) were divided into two groups. The first group consisted of 65 carbapenem-resistant strains (resistant to imipenem and meropenem), and the second group consisted of 56 *P. aeruginosa* carbapenem-sensitive strains.

Carbapenem-resistant *P. aeruginosa* strains were more often resistant to the majority of tested antibiot-

ics, except cefepime and aztreonam, compared to carbapenem-sensitive strains, and the difference between groups was statistically significant (Table 1).

Table 1. Resistance of Carbapenem-Resistant and Carbapenem-Sensitive *Pseudomonas aeruginosa* strains to Various Antibiotics.

Antimicrobial Agent	Carbapenem-Resistant Strains, n (%)	Carbapenem-Sensitive Strains, n (%)	χ^2	*P*
Piperacillin	46/65 (70.8)	16/56 (28.6)	21,44	<0.001
Piperacillin/ tazobactam	16/38 (42.1)	7/43 (16.3)	6,62	0.01
Ceftazidime	35/65 (53.8)	14/56 (25.0)	10,39	0.001
Cefepime	11/38 (28.9)	7/42 (16.7)	1,73	0.189
Cefoperazone/ sulbactam	19/38 (50.0)	11/43 (25.6)	5,16	0.023
Ciprofloxacin	57/65 (87.7)	12/56 (21.4)	53,90	<0.001
Gentamicin	51/65 (78.5)	10/56 (17.9)	44,20	<0.001
Amikacin	25/65 (38.5)	3/56 (5.4)	18,54	<0.001
Tobromycin	21/37 (56.8)	4/41 (9.8)	19,73	<0.001
Aztreonam	9/59 (15.3)	4/51 (7.8)	1,44	0.23

All 121 *P. aeruginosa* strains were tested for human serum bactericidal effect; 85 (70.2%) of *P. aeruginosa* strains were found to be sensitive to serum and 36 (29.8%) were resistant.

The resistance of *P. aeruginosa* serum-sensitive and serum-resistant strains to various tested antimicrobial agents is shown in Table 2. We did not find any statistically significant difference between resistance to carbapenems and serum resistance of tested *P. aeruginosa* strains.

Table 2. Resistance of Serum Sensitive and Serum-Resistant *Pseudomonas aeruginosa* strains to Various Antibiotics.

Antimicrobial agent	*Pseudomonas aeruginosa* strains		χ^2	*P*
	Serum sensitive (grades 1-4) N=85 n (%)	Serum resistant (grades 5-6) N=36 n (%)		
Carbapenems	48 (56.5)	17 (47.2)	0.87	0.351
Piperacillin	41 (48.2)	21 (58.3)	1.03	0.310
Ceftazidime	32 (37.6)	17 (47.2)	0.96	0.327
Ciprofloxacin	50 (58.8)	19 (52.8)	0.38	0.539
Aminoglycosides	45 (52.9)	17 (47.2)	0.33	0.565

Resistance to serum of *P. aeruginosa* strains had no statistically significant correlation with resistance to tested antibiotics.

All 121 *P. aeruginosa* strains were tested for formation of biofilm; 52 (43.0%), 30 (24.8%), and 39 (32.2%) of the strains were found to be no, moderate and high biofilm producers, respectively. The resistance of non-biofilm producers and moderate and high biofilm producers to various antimicrobial agents is shown in Table 3. We did not find any statistically significant differences between carbapenem-resistant and carbapenem-sensitive *P. aeruginosa* groups and their variant ability to produce biofilm.

Table 3. Resistance of *Pseudomonas aeruginosa* Strains with Different Biofilms Production Level to Various Antibiotics.

Antimicrobial agent	*Pseudomonas aeruginosa* strains			*P*
	non-biofilm producer N=52 n (%)	moderate biofilm producer N=30 n (%)	high biofilm producer N=39 n (%)	
Carbapenems	33 (63.5)	15 (50.0)	17 (43.6)	0.152
Piperacillin	31 (59.6)	15 (50.0)	16 (41.0)	0.212
Ceftazidime	28 (53.8)	18 (60.0)	16 (41.0)	0.260
Ciprofloxacin	32 (61.5)	18 (60.0)	19 (48.7)	0.441
Aminoglycosides	19 (36.5)	12 (40.0)	18 (46.2)	0.651

Biofilm formation of *P. aeruginosa* strains also had no statistically significant correlation with resistance to any tested antibiotics.

In our study, 53 out of 121 (43.8%) *P. aeruginosa* strains were recovered from infections of wounds, 45/121 (37.2%) from the respiratory tract, 20/121 (16.5%) from the urinary tract, and 3/121 (2.5%) from blood.

The carbapenem-resistant *P. aeruginosa* strains were recovered from the urinary tract significantly more often than carbapenem-sensitive *P. aeruginosa* strains, 75.0% and 25.0%, respectively, p=0.037. Carbapenem-sensitive *P. aeruginosa* strains were recovered from the respiratory tract significantly more frequently than carbapenem-resistant strains, 60.0% and 40.0%, respectively (Table 4).

Table 4. Proportion of Pseudomonas aeruginosa Strains Recovered From Various Sources in Relation to Carbapenem Resistance.

Source	Carbapenem-Resistant Strains, n (%)	Carbapenem-Sensitive Strains, n (%)	χ^2	*P*
Urinary tract (N=20)	15 (75.0)	5 (25.0)	4.37	0.037
Wounds (N=53)	31 (58.5)	22 (41.5)	0.86	0.353
Blood (N=3)	1 (33.3)	2 (66.7)	–*	0.596
Respiratory tract (N=45)	18 (40.0)	27 (60.0)	5.42	0.020

– Fisher exact test was employed for small sample size.*

The *P. aeruginosa* strains recovered from different sources had different serum resistance (Table 5). All the strains recovered from blood were serum resistant. *P. aeruginosa* strains recovered from respiratory tract and wounds were significantly frequently serum sensitive, 95.6% (n=45) and 56.6%, (n=53), respectively.

Table 5. Serum-Resistance of *Pseudomonas aeruginosa* Strains Recovered From Various Sources.

Source	% of tested *Pseudomonas aeruginosa* strains (n)		χ^2	*P*
	Serum sensitive (grades 1-4)	Serum resistant (grades 5-6)		
Urinary tract (N=20)	60.0 (12)	40.0 (8)	1.20	0.273
Wounds (N=53)	56.6 (30)	43.4 (23)	8.40	0.004
Blood (N=3)	0	100.0 (3)	–*	0.025
Respiratory tract (N=45)	95.6 (43)	4.4 (2)	21.96	<0.001

– Fisher exact test was employed for small sample size.*

We did not find any differences in biofilm production among the *P. aeruginosa* strains recovered from different sources (Table 6).

Table 6. Biofilm Production of *Pseudomonas aeruginosa* Strains Recovered From Various Sources.

Source	% of tested *Pseudomonas aeruginosa* strains (n)			
	non-biofilm producer	moderate biofilm producer	high biofilm producer	*P*
Urinary tract (N=20)	45.0 (9)	25.0 (5)	30.0 (6)	0.970
Wounds (N=53)	41.5 (22)	22.6 (12)	35.8 (19)	0.739
Blood (N=3)	66.7 (2)	33.3 (1)	0	0.476
Respiratory tract (N=45)	42.2 (19)	26.7 (12)	31.1 (14)	0.933

4. Discussion

Many studies are focused on clinical significance of antibiotic - resistance bacteria. Virulent organisms are able to produce clinical symptoms of infection in human and animal hosts and should therefore be exposed more frequently to antimicrobial drugs, and the risk of resistance is expected to be higher [23]. In the study by Drahovska et al., enterococci isolated from human infections and from the traditional Slovak sheep cheese, bryndza, were compared, and a higher level of resistance was found in clinical than in food strains; differences were found in the distribution of virulence-associated cylA gene, as well [24]. The relationship between antibiotic resistance and virulence factors in urinary enterococcus isolates were found in the study by Baylan et al.: hyaluronidase asa1 gene positive *Enterococcus faecalis* (*E. faecalis*) isolates were more resistant to ciprofloxacin, norfloxacin and levofloxacin; esp gene positive *E. faecalis* isolates were more resistant to doxycycline; and hyl gene positive *E. faecalis* isolates were more resistant to nitrofurantoin than these gene negative isolates [25].

Bacterial strains that have acquired resistance to one antibiotic can develop resistance to other classes of antibiotics. Our results showed that *P. aeruginosa* clinical isolates resistant to carbapenems were more resistant to piperacillin, piperacillin/tazobactam, ceftazidime, cefoperazone/sulbactam, ciprofloxacin and aminoglycosides. The data of Lagatolla et al. [26], similar to those in other studies [27,28], demonstrate that most of the blaVIM positive isolates of *P. aeruginosa* exhibited a multidrug-resistant phenotype, including imipenem, meropenem, ceftazidime, piperacillin, aztreonam, amikacin, gentamicin, tobramycin and ciprofloxacin, except polymixin B. Antibiotic resistance alone cannot explain the virulence of bacteria. A study by Doina et al., concluded that strategies could be developed to target virulence factors of pathogens instead of whole bacteria, such as the development of drugs that target the plasmids containing resistance genes or drugs that target the adhesion of virulent bacteria to tissue [29]. It is very important to detect associations between bacterial pathogenic factors and antibiotic resistance. Many researchers have reported that bacterial biofilm is associated with resistance to a wide range of antimicrobial agents [30]. However, we found one study by Hostacka et al. that did not confirm these findings; it showed the same percentage of production of biofilm in the strains sensitive to ciplofloxacin and aminyglycosides compared with the resistant one [31]. Serum sensitivity/resistance might be an important microbial phenotype, which could conceivably differentiate between invasive and non-invasive strains [12]. Approximately 1/3 of our tested single clinical isolates were resistant to serum bactericidal effect and were high biofilm producers. In our study relationship was not found with resistance to serum, biofilm formations and resistance to carbapenems and other classes of tested antibiotics. A single isolates being sensitive in vitro to antibiotics may run into resistance in case of the bacteria manage to become a productive member of a biofilm producing community. This could explain why no association was found between in vitro sensitivity to antibiotics and biofilm formation. Unfortunately, we did not investigate genes that are responsible for resistance to carbapenems. Further studies are needed to assess the importance of other pathogenicity factors of *P. aeruginosa*. However, Hostacka et al., showed that the resistance to antibiotics has not always been associated with changes in the production of the pathogenicity factors such as motility, biofilm N-acylhomoserine lactone signal molecules production and response to oxidative stress.

5. Conclusion

In our study, the source of *P. aeruginosa* infection was related to carbapenem-resistance. Carbapenem-sensitive strains were isolated most frequently from the respiratory tract, and carbapenem-resistant strains were isolated from the urinary tract. We observed an association between the source of recovery of strains and their resistance to serum bactericidal effect. All *P. aeruginosa* strains isolated from blood were serum-resistant. No correlation was observed between biofilm formation

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ORIGINAL ARTICLE

Nosocomial dissemination of VIM-2-producing ST235 *Pseudomonas aeruginosa* in Lithuania

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Abstract *Pseudomonas aeruginosa* multidrug resistance, and particularly the production of carbapenemases linked to international high-risk clones, is of growing concern. While high levels of carbapenem resistance (>60 %) have been reported in Lithuania, so far, there is no information on the underlying mechanisms. Thus, the aim of this work was to determine the molecular epidemiology and prevalence of acquired carbapenemases among 73 carbapenem-resistant *P. aeruginosa* isolates recovered in a hospital from Kaunas, Lithuania in 2011–2012. The presence of acquired carbapenemases was evaluated through phenotypic (modified Hodge test, cloxacillin inhibition test, double-disc synergy test) and genetic methods [polymerase chain reaction (PCR) and sequencing]. Clonal relatedness was assessed by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST). Acquired β-lactamases were detected in 19 (26 %) of the isolates, whereas resistance was exclusively chromosomal (OprD inactivation±AmpC hyperproduction) in the remaining 54 (74 %) isolates. The acquired β-lactamases detected included 16 VIM-2, one PER-1 and two GES enzymes. PFGE revealed that 15 of the 16 VIM-2 isolates belonged to a single clone, identified as the international high-risk clone ST235 by MLST. bla_{VIM-2} was preceded by *aacA7* in a class I integron, similar to epidemic ST235 isolates described in nearby countries. Additionally, sequencing of bla_{GES} revealed the presence of the carbapenem-hydrolysing enzyme GES-5 in one of the isolates and a novel GES variant, designated GES-27, in the other. GES-27 differed from GES-5 by a single amino acid substitution, proline 167, that was replaced by glutamine. Increasing emergence and dissemination of concerning resistance mechanisms and international clones warrants global surveillance and control strategies.

Introduction

The increasing prevalence of nosocomial infections produced by multidrug-resistant (MDR) or extensively drug-resistant (XDR) *Pseudomonas aeruginosa* strains severely compromises the selection of appropriate treatments and is, therefore, associated with significant morbidity and mortality [1–3]. This growing threat results from the extraordinary capacity of this pathogen for developing resistance to nearly all available antibiotics by the selection of mutations in chromosomal genes and from the increasing prevalence of transferable resistance determinants, particularly those encoding class B carbapenemases [metallo-β-lactamases (MBLs)] or extended-spectrum β-lactamases (ESBLs), frequently cotransferred with genes encoding aminoglycoside-modifying enzymes [4, 5]. For decades, multiple reports have warned of the occurrence of epidemic outbreaks caused by MDR/XDR strains within the hospital environment. More recently, concerning reports have provided evidence of the existence of MDR/XDR global clones disseminated in several hospitals worldwide that have been referred as high-risk clones [6, 7].

While high levels of carbapenem resistance (>60 %) have been reported in Lithuania [8], so far, there is still no information on the underlying mechanisms, the potential association with MDR/XDR profiles or whether high-risk clones might be

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involved in resistance dissemination. Thus, the aim of this work was to determine the molecular epidemiology and mechanisms of carbapenem resistance, with a particular focus on acquired carbapenemases, among *P. aeruginosa* isolates from Lithuania.

Materials and methods

Bacterial strains

A total of 121 non-duplicate *P. aeruginosa* clinical isolates were included in the study. They were isolated from clinical samples of patients, treated in the hospital of Lithuanian University of Health Sciences during the period from 1 January 2011 to 30 June 2012. Preliminary antimicrobial susceptibility data were generated previously [8], and the present work focused on characterisation of the 73 (60.3 %) isolates that were determined to be intermediate or resistant (non-susceptible) to imipenem and/or meropenem.

Susceptibility testing

Preliminary antimicrobial susceptibility data for the whole (121 isolates) collection were available from the previous study [8]. Seventy-three carbapenem-resistant *P. aeruginosa* clinical isolates were additionally tested to evaluate the minimum inhibitory concentrations (MICs) of imipenem, meropenem, ticarcillin, piperacillin–tazobactam, ceftazidime, cefepime, aztreonam, ciprofloxacin, tobramycin, amikacin and colistin by broth microdilution using the customised Sensititre plates (ref. FRCNRP, Thermo Fisher Scientific). Breakpoints were applied following European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (http://www.eucast.org). Consensus recommendations were used for the definition of MDR and XDR profiles [9]. However, fosfomycin susceptibility was not evaluated since neither the EUCAST nor the Clinical and Laboratory Standards Institute (CLSI) have defined breakpoints for this antibiotic.

Phenotypic detection of carbapenemase production

The modified Hodge test (MHT) and cloxacillin inhibition test (CIT) were used to detect the carbapenemase-producing isolates. The MHT was performed on all isolates as described previously [10, 11]. Plates were read after overnight incubation at 37 °C. An isolate was considered carbapenemase-positive when alongside the tested isolate there appeared a clover leaf-shaped inhibition zone, due to the enhanced growth of the *Escherichia coli* strain towards the meropenem disc. The CIT was performed by using 10-μg imipenem and 30-μg ceftazidime discs placed on Mueller–Hinton (MH) agar plates containing or not containing 500 μg/mL cloxacillin. When susceptibility to imipenem and ceftazidime was restored in plates containing cloxacillin, it was interpreted that resistance was caused by AmpC hyperproduction coupled with OprD deficiency [12]. On the contrary, when the strain was still resistant in the presence of cloxacillin, it was suspected to produce an acquired β-lactamase, a carbapenemase if resistance to imipenem (±ceftazidime) was not inhibited by cloxacillin and an ESBL if cloxacillin did not work only for ceftazidime. For the phenotypic detection of MBLs, a double-disc synergy test (DDST) with imipenem and meropenem and EDTA was used. Likewise, for phenotypic assessment of ESBLs, a DDST with ceftazidime, cefepime, aztreonam and amoxicillin–clavulanate was performed in MH agar plates supplemented with 500 μg/mL of cloxacillin.

Detection of carbapenemase genes by PCR

A previously described multiplex polymerase chain reaction (PCR) was performed as first screening for the characterisation of different classes of carbapenemase genes ($bla_{\text{NDM-1}}$, $bla_{\text{OXA-48}}$, bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{GES}) in the isolates showing positive carbapenemase phenotypic tests [13, 14]. The primers used for multiplex PCR amplification and the reaction conditions were slightly modified from those previously described by Doyle et al. [13]. When required, additional PCRs to characterise the class B carbapenemases were performed, using previously described specific primers for $bla_{\text{IMP-1}}$, $bla_{\text{IMP-2}}$, $bla_{\text{VIM-1}}$ and $bla_{\text{VIM-2}}$ genes [14]. Selected amplicons were subjected to DNA sequencing. Sequencing was performed using the BigDye Terminator Cycle Sequencing Kit (PE Applied Biosystems, Foster City, CA) and an ABI PRISM 3100 DNA Sequencer (PE Applied Biosystems). The resulting sequences were then compared with those available in GenBank (http://www.ncbi.nlm.nih.gov/BLAST).

Molecular strain typing

Pulsed-field gel electrophoresis (PFGE) was performed on all MBL-producing *P. aeruginosa* isolates as previously described [15]. Briefly, bacterial DNA was digested with *Spe*I (New England BioLabs, UK) and PFGE separation was performed under the following conditions: 6 V/cm, 14 °C and pulses ranging from 5 to 40 s for 26 h, using a CHEF-DR II System (Bio-Rad Laboratories, USA). PFGE patterns were interpreted according to the classification criteria previously described by Tenover et al. [16]. Clonal relatedness was investigated by MLST in one isolate of each carbapenemase-producing *P. aeruginosa* PFGE clone, following the protocol described by Curran et al [17]. The sequences obtained were assigned allele numbers following comparison of the DNA sequence with the

Table 1 Comparative analysis of antibiotic susceptibility profiles of the studied *Pseudomonas aeruginosa* isolates

Antimicrobial agent[a]	No. of non-susceptible isolates (%)			
	Total (n=73)	CIT-positive[b] [chromosomal (AmpC±OprD)] (n=54)	CIT-negative[b] (carbapenemase/ESBL producers) (n=19)	p-Value
TIC	61 (84.0)	42 (77.8)	19 (100)	0.02
P/T	46 (63.0)	28 (51.9)	18 (95.0)	<0.001
AZT	67 (91.8)	48 (88.9)	19 (100)	0.13
TAZ	41 (56.0)	22 (40.7)	19 (100)	<0.001
FEP	23 (32.0)	11 (20.4)	12 (63.2)	<0.001
IMP	71 (97.3)	51 (94.4)	19 (100)	0.3
MER	61 (83.6)	42 (77.8)	19 (100)	0.02
CIP	53 (72.6)	35 (64.8)	18 (95.0)	0.01
TOB	32 (44.0)	15 (27.8)	17 (89.5)	<0.001
C/T	20 (27.4)	1 (1.9)	19 (100)	<0,001
AMI	23 (31.5)	6 (11.1)	17 (89.5)	<0.001
COL	1 (1.0)	0	1 (5.3)	0.28
MDR	56 (76.7)	37 (70.0)	19 (100)	0.005
XDR	46 (63.0)	27 (50.9)	19 (100)	<0.001

[a] *TIC* ticarcillin; *P/T* piperacillin/tazobactam; *AZT* aztreonam; *TAZ* ceftazidime; *FEP* cefepime; *IMP* imipenem; *MER* meropenem; *CIP* ciprofloxacin; *TOB* tobramycin; *C/T* ceftolozane/tazobactam; *AMI* amikacin; *COL* colistin; *MDR* multidrug-resistant; *XDR* extensively drug-resistant

[b] *CIT* cloxacillin inhibition test

sequences of previously typed strains by using the MLST website (http://www.pubmlst.org). For each isolate, the allele numbers at each of the seven loci defined the allelic profile or sequence type (ST). Dendrogram analysis was performed using CLIQS 1D Pro software version 1.0. (TotalLab Ltd., Newcastle, UK). The unweighted pair group method using arithmetic averages (UPGMA) was used to construct the phylogenetic tree. Dice coefficients were used to calculate the similarity percentage of the band patterns.

Characterisation of the genetic environment of bla_{VIM-2} genes

Integrons harbouring MBL-encoding genes were characterised by PCR and DNA sequencing by using specific primers to amplify the DNA region located between intI and QacEΔ1, and the corresponding MBL-encoding gene [14].

Statistical analysis

Proportions were compared using non-parametric statistical criterion Chi-square or Fisher's exact test. Differences between groups were considered significant for a p-value <0.05. The statistical package IBM SPSS Statistics 20 was used for the data analysis.

Results

Comparative antimicrobial susceptibility profiles of the 73 *P. aeruginosa* isolates are summarised in Table 1. According to the EUCAST interpretative categories, 97.3 % and 83.6 % of the isolates were non-susceptible to imipenem and meropenem, respectively. Regarding the other antibiotics tested, non-susceptibility was highest for aztreonam (91.8 %), followed by ticarcillin (84.0 %) and ciprofloxacin (72.6 %). Up to 56 (76.7 %) and 46 (63.0 %) of the isolates showed MDR and XDR phenotypes, respectively. Moreover, 10 (13.7 %) isolates were resistant against all the tested antibiotics, except colistin. Only one isolate was found to be resistant to colistin and it showed a pandrug-resistant (PDR) profile.

All imipenem- and/or ceftazidime-resistant *P. aeruginosa* isolates were screened for phenotypic detection of the presence of chromosomal resistance (AmpC ± OprD) or acquired β-lactamases by the CIT and MHT. According to the CIT, β-lactam resistance was only caused by chromosomal mechanisms (AmpC ± OprD) in 54 of the 73 isolates (74 %). On the other hand, in 16 (22 %) isolates, the CIT was negative for both ceftazidime and imipenem, suggesting the presence of a carbapenemase. In the remaining three isolates, the CIT was negative only for ceftazidime, suggesting the presence of an ESBL. The MHT was positive only in 14 (all CIT-negative for both ceftazidime and imipenem). Except for aztreonam,

imipenem and colistin, the resistance rates were significantly higher among CIT-negative isolates (Table 1). Particularly noteworthy, all CIT-negative isolates were resistant to ceftolozane/tazobactam, while only 1 (1.9 %) CIT-positive isolate was resistant to this antibiotic, known to be stable against *P. aeruginosa* chromosomal mechanisms [18–20]. Moreover, CIT-negative isolates showed much more frequently an MDR and XDR phenotype.

All CIT-negative isolates (and ten randomly chosen positive isolates as negative controls) were subjected to the detection of acquired β-lactamases through PCR, followed by sequencing in representative isolates. All 16 CIT-negative isolates were positive for $bla_{\text{VIM-2}}$ and were confirmed to produce and MBL through EDTA DDST. Of the three isolates showing CIT results only negative for ceftazidime, one produced a PER-1 ESBL and the other two were positive for GES enzymes. Sequencing revealed the presence of the carbapenem-hydrolysing enzyme GES-5 in one of the isolates and a novel GES variant, designated GES-27, in the other. GES-27 differed from GES-5 by a single amino acid substitution, proline 167, that was replaced by glutamine. It is noteworthy that both isolates producing the GES enzymes showed a negative MHT and a positive CIT result for imipenem, indicating that carbapenem resistance in these two isolates depended mainly on chromosomal mechanisms (OprD + AmpC). However, they both yielded a weakly positive ESBL DDST.

PFGE analysis was performed in the 16 isolates positive for VIM-2 and in the two isolates positive for GES enzymes. As shown in Fig. 1, 15 of the VIM-2 isolates belonged to a single genotype, identified as the international high-risk clone ST235 through MLST. The remaining VIM-2 isolate was identified as the unrelated ST1047. Remarkably, most of the isolates belonging to the ST235 were isolated from patients admitted to the intensive care unit (ICU) or reanimation wards. On the other hand, the isolates producing the GES-5 and GES-27 enzymes were found to belong to two different PFGE clonal types. Moreover, they belonged to a different MLST clone, ST1628 for the GES-5-producing isolate and ST660 for the one producing GES-27.

In order to characterise the genetic elements harbouring $bla_{\text{VIM-2}}$ in ST235 and ST1047 clones, PCR and sequencing of the involved integrons was performed. $bla_{\text{VIM-2}}$ from ST235 was located in the previously described class 1 integron In559 (GenBank DQ522233, *Int*I1-*aaCA7*-$bla_{\text{VIM-2}}$-*dfrB5*-*aacA5*-*tniC*), whereas $bla_{\text{VIM-2}}$ from ST1047 showed a different integron structure (*Int*I1-*aaCA4*-$bla_{\text{VIM-2}}$-*tniC*). $bla_{\text{GES-27}}$ was also found to be located in a class 1 integron, immediately downstream of *Int*I1, but multiple attempts to link *Int*I1 to $bla_{\text{GES-5}}$ through PCR consistently failed.

Discussion

In this work, we have evaluated, through several phenotypic and molecular tests, the prevalence of acquired β-lactamases among carbapenem-resistant *P. aeruginosa* isolates recovered from a Lithuanian hospital. Consistently with previous works [21], the MHT only detected 14 of 18 carbapenemase producers. On the other hand, the CIT used proved to be a highly sensitive and specific screening method for the detection of acquired β-lactamases in *P. aeruginosa*. Our data are, thus, in agreement with recent studies by Fournier et al. [12], who used imipenem±cloxacillin discs. Moreover, our approach, testing ceftazidime in addition to imipenem, allowed us the detection of not only prevalent MBLs but also ESBLs and GES-type carbapenemases. Additionally, as previously described [18–20], ceftolozane resistance, even if combined with tazobactam, was a good predictor of the presence of acquired β-lactamases (ESBLs or carbapenemases) in *P. aeruginosa*; all 19 isolates shown to produce these acquired enzymes were found to be resistant to ceftolozane/tazobactam,

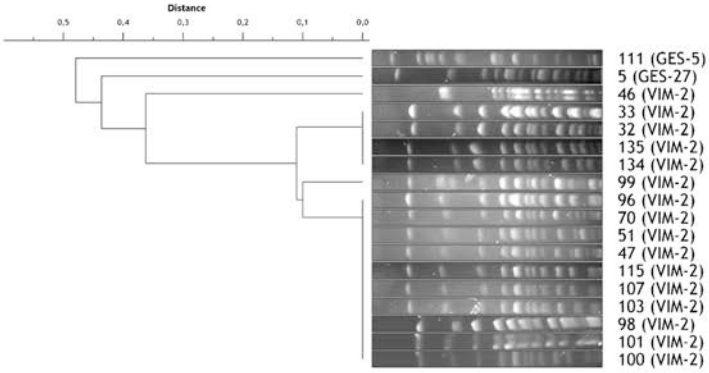

Fig. 1 Unweighted pair group method using arithmetic averages (UPGMA) phylogenetic tree showing the relationship among the 18 carbapenemase-producing *Pseudomonas aeruginosa* isolates. The tree was constructed based on the DNA macrorestriction fragment patterns obtained by pulsed-field gel electrophoresis (PFGE) using *Spe*I restriction enzyme

whereas only one of the 54 isolates showing chromosomal resistance had an MIC greater than 4/4 mg/L.

Genetic analysis revealed that, consistently with findings in other European countries [22], the MBL VIM-2 was, by far, the most prevalent acquired β-lactamase among Lithuanian hospitals. Moreover, high prevalence of VIM-2 was found to be driven by the dissemination of the concerning high-risk clone ST235. Thus, our work adds Lithuania to the growing list of countries documenting epidemic dissemination of this clonal lineage linked to potent β-lactamases. Indeed, VIM-2-producing ST235 isolates have been documented in 17 different countries worldwide [7]. Among multiple reports, the extremely high incidence recently reported in Russia and Belarus is of particular concern [23]. Indeed, as described in Russian isolates, *aacA7* preceded $bla_{\text{VIM-2}}$ in a class 1 integron in our ST235 isolates, potentially indicating a common origin.

Additionally, our work has led to the identification of a previously undescribed GES variant, designated GES-27. GES-27 differed from the carbapenem-hydrolysing GES-5 by a single amino acid substitution, proline 167, that was replaced by glutamine. Interestingly, the replacement of proline 167 by a different amino acid (serine) has been previously noted in another GES-5 variant (GES-15) [24]. Since GES-5 was also detected in our collection, we wondered whether isolates producing GES-5 and GES-27 would be clonally related, suggesting recent evolution of GES-27 from GES-5 in this clonal lineage. However, both isolates showed different PFGE profiles, ruling out this possibility.

In summary, in this work, we have analysed, for the first time, the epidemiology and mechanisms of carbapenem resistance in *P. aeruginosa* isolates from Lithuania. Major findings indicate the dissemination of the MBL VIM-2 linked to the international high-risk clone ST235 and the detection of a novel GES carbapenemase variant designated GES-27.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest.

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SANTRAUKA

Įvadas

Pseudomonas aeruginosa yra vienas iš kliniškai ir epidemiologiškai svarbiausių patogenų, dažniausiai sukeliantis hospitalines kraujo bei šlapimo takų infekcijas imunosupresinės būklės pacientams, sergantiems sunkiomis gretutinėmis ligomis ir gydomiems intensyviose terapijos skyriuose. Nepaisant plataus veikimo antibiotikų ir pasiekimų šiuolaikinės medicinos praktikoje *P. aeruginosa* sukelta bakteremija ir ventiliacinė pneumonija vis dar išlieka viena opiausių šių dienų problemų, susijusių su 18–61 proc. mirtingumu pasaulyje dėl bakterijos padidėjusio atsparumo vienos ar kelių klasių antimikrobinėms medžiagoms. Lietuvos sveikatos mokslų universiteto Laboratorinės medicinos klinikoje atliktos analizės duomenimis pacientų mirtingumas *P. aeruginosa* sukelta bakteremija intensyvios terapijos skyriuose siekia 58,8 proc. Nors sergančiųjų mirtingumas hospitalinės infekcijos atveju priklauso nuo daugelio priežasčių bei rizikos veiksnių tyrimais nustatyta, jog ligos baigtį lemia ne tik sunki ligonio būklė, gretutinės ligos, bet ir uždelstas arba netinkamas pradinio adekvataus empirinio gydymo parinkimas. Šis veikimo faktorius yra glaudžiai susijęs su *P. aeruginosa* įgyto dauginio atsparumo kelių skirtingų klasių antibiotikams vystymosi bei plitimo kintančioje aplinkoje didėjimu.

Nors *P. aeruginosa* atsparumas antibiotikams yra perduodamas chromosomoje užkoduotų atsparumą lemiančių veiksnių bendros veiklos, vis dažniau pasirodo pranešimų apie bakterijų įgytą ir horizontalios genų pernašos būdu perduodamą atsparumą beta laktaminių antibiotikų grupei, o ypatingai karbapenemų klasės pagrindiniams atstovams – imipenemui ir meropenemui. Tyrimais nustatyta, jog atsparumas karbapenemams gali pasireikšti skirtingais molekuliniais mechanizmais, tačiau labiausiai paplitusios ir kliniškai svarbiausios išlieka bakterijų gaminamos ir karbapenemus hidrolizuojančios *β*-laktamazės – karbapenemazės, arba bla_{VIM} genų koduojamos metalo-*β*-laktamazės.

Mikroorganizmų atsparumo antibiotikams ir jų netinkamo vartojimo problemos skatina imtis kontrolinių veiksmų. Siekiant ne tik fenotipiškai, bet ir genotipiškai įvertinti epidemiologijoje svarbių ir virulentinėmis savybėmis pasižyminčių bakterijų atsparumo antibiotikams pobūdį, mastą, jo atsiradimo priežastis, plitimo kelius, bei sukurti atsparumo prevencijos sistemą, pirmiausia reiktų išsiaiškinti atsparių antibiotikams bakterijų atsiradimo genetines priežastis. Išanalizuoti atsparumą antibiotikams lemiančių bakterijų genų ir jų koduojamų baltymų pagrindines atsparumo antibiotikams strategijas.

Darbo tikslas

Įvertinti *P. aeruginosa* padermių, išskirtų iš įvairių klinikinių medžiagų, atsparumo karbapenemų klasės antibiotikams molekulinius mechanizmus.

Tikslui pasiekti buvo iškelti tokie uždaviniai:

1. Nustatyti karbapenemams atsparių *P. aeruginosa* padermių atsparumą kitų klasių antibiotikams.
2. Įvertinti *P. aeruginosa* padermių patogeniškumo veiksnių-atsparumo baktericidiniam serumo poveikiui bei gebėjimo formuoti bioplėveles, sąsajas su atsparumu antibiotikams.
3. Fenotipiniais lyginamosios analizės metodais nustatyti atsparumo karbapenemų klases antibiotikams pagrindinius mechanizmus.
4. Nustatyti *P. aeruginosa* padermių gebėjimą gaminti MBL *β*-laktamazes bei nustatyti vyraujančius genetinius profilius-pulsotipus ir integronų struktūrą.

Darbo mokslinis naujumas

Pateikiamas darbas yra pirmasis, Lietuvoje išskirtų atsparių karbapenemų klasės antibiotikams *P. aeruginosa* bakterijų, išsamus molekulinis tyrimas. Atliktas tyrimas leidžia detaliau pažvelgti į *P. aeruginosa* padermių atsparumo karbapenemų klasės antibiotikams molekulinius mechanizmus.

Šiame darbe panaudoti nauji fenotipiniai metodai, leidžiantys greitai nustatyti *P. aeruginosa* atsparumo antibiotikams pagrindinius mechanizmus.

Tyrimo metu pirmą kartą nustatytas $bla_{\text{VIM-2}}$ genas, lemiantis atsparumą karbapenemų klasės antibiotikams. Iki šiol nebuvo rasta duomenų analizuojančių šio genų paplitimą Lietuvoje. Šiuo tyrimu taip pat pirmą kartą parodėme, jog Lietuvos sveikatos mokslų universiteto klinikinėje ligoninėje vyrauja ST235 tipas, kuris yra paplitęs pasauliniu mastu.

Šiame darbe taip pat pirmą kartą aptiktas Europoje mažai paplitęs bla_{GES} genas su unikalia nukleotidų seka, todėl naujam genų dariniui suteiktas naujas identifikacijos numeris $bla_{\text{GES-27}}$.

Akivaizdu viena – dėl *P. aeruginosa* genomo plastiškumo nuolat atsiranda itin agresyvių žmogui padermių, o gausus antibiotikų vartojimas bei globalizacija (žmonių migracija) neabejotinai skatina tokių padermių plitimą tarpkontinentinėse ribose.

Tyrimo eiga

Visos padermės buvo išskirtos iš klinikinės tiriamosios medžiagos pacientams, gydytiems Lietuvos sveikatos mokslų universiteto ligoninėje

nuo 2011 metų sausio mėn. 1 d. iki 2012 metų birželio mėn. 31 d. Tik viena vieno paciento padermė buvo įtraukta į tyrimą.

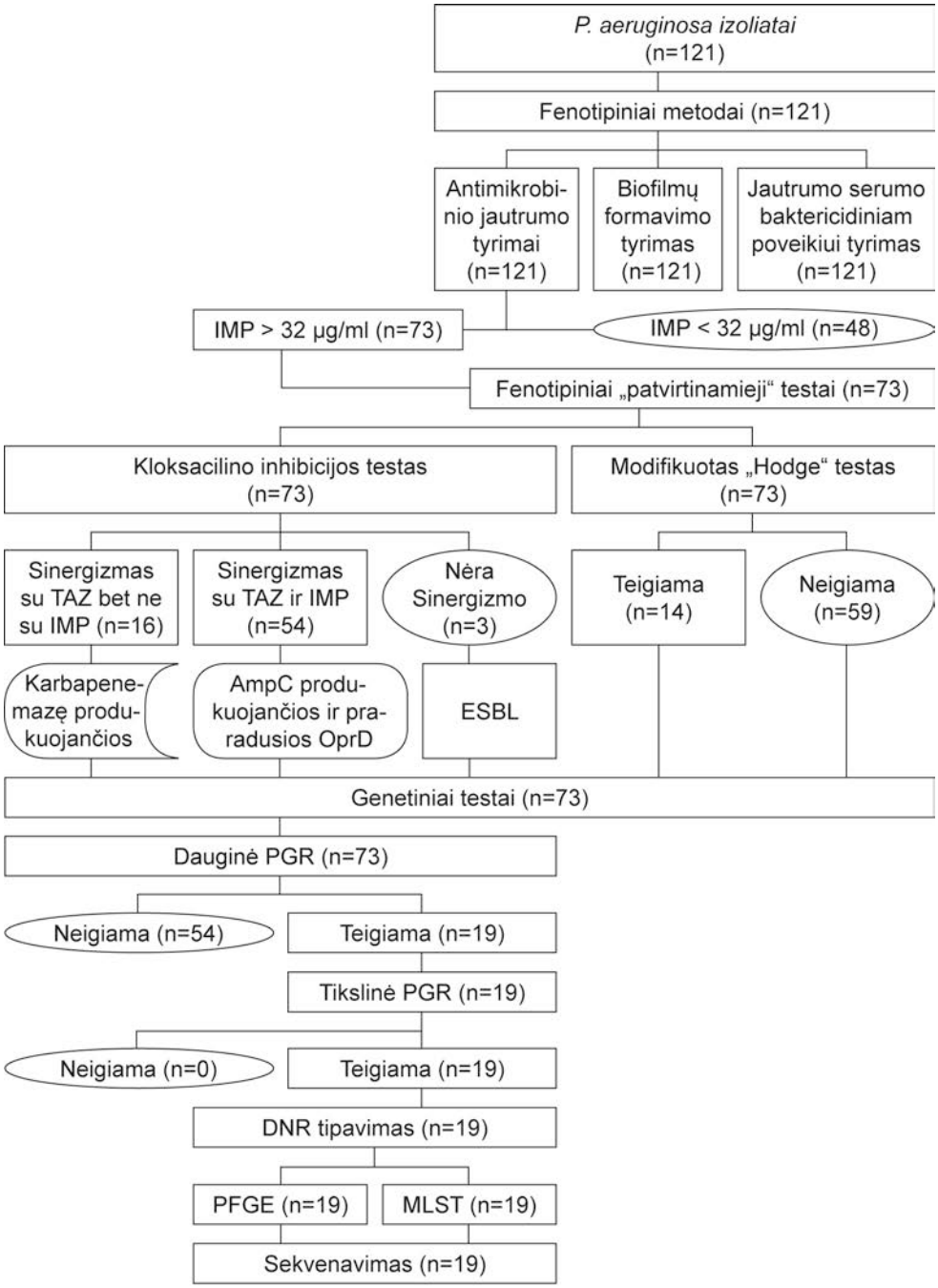
Pirmoje tyrimų dalyje visoms išskirtoms *P. aeruginosa* padermėms nustatytas jautrumas antibakterinėms medžiagoms remiantis E-testų (Liofilhelm, Italija) ir Kirby Bauer diskų metodu. Nustatytos minimalios slopinančios koncentracijos buvo vertintos pagal Europos priešmikrobinių vaistų jautrumo komiteto (EUCAST) rekomenduotus lūžio taškus. Septyniasdešimt trims (n=73) atsparioms karbapenemų klasės antibiotikams padermėms papildomai įvertintas jautrumas penkių klasių antibiotikams (MSK), remiantis sultinio mikropraskiedimo principu, panaudojant komercines Sensititre plokšteles (*TREK Diagnostic Systems*).

Pirmoje tyrimų dalyje taip pat įvertintas visų tirtų *P. aeruginosa* padermių gebėjimas išlikti atspariomis žmogaus baktericidiniam serumo poveikiui ir gebėjimo formuoti bioplėveles.

Antroje tyrimų dalyje atsirinktos tik atsparios karbapenemų klasės antibiotikams padermės. Plataus spektro *β*-laktamazių (ESBL), *ampC β*-laktamazių ir karbapenemazių gamybos fenotipinis nustatymas buvo atliktas remiantis dviejų etapų PVBL (plataus veikimo *β*-laktamazių) nustatymo metodu, sudarytu iš kloksacilino inhibicijos testo (CIT) atlikimo ir rezultatų interpretacijos bei modifikuoto „Hodge“ testo (MHT) atlikimo ir visumos gautų rezultatų vertinimo.

Tolimesniuose tyrimuose atrinktoms padermėms (N=73), atsparioms vienam arba dviem karbapenemų klasės antibiotikams (imipenemui ir /arba meropenemui), nustatytas atsparumą karbapenemų klasės antibiotikams koduojantis genas. Visoms *P. aeruginosa* padermėms, koduojančioms karbapenemazės geną nustatytas vyraujantis pulsotipas bei integronų struktūra. Visi metodai plačiau aprašyti metodikos skyriuje.

Tyrimo eiga pavaizduota 1 pav.



1 pav. *Tyrimo eiga*

IMP – imipenemas; ESBL – plataus spektro *β*-laktamazė; PGR – polimerazės grandininė reakcija; PFGE – pulsuojančio gelio elektroforezė; TAZ – ceftazidimas; MLST – bakterijų tipavimas pagal kelis genomo lokusus.

Rezultatai ir jų aptarimas

P. aeruginosa padermių atsparumo antibiotikams bendroji charakteristika

Į tyrimą įtrauktos 121 *P. aeruginosa* padermės išskirtos iš įvairios klinikinės tiriamosios medžiagos: žaizdų – 53 (43,8 proc.), kvėpavimo takų- 45 (37,2 proc.), šlapimo takų – 20 (16,5 proc.) ir kraujo – 3 (2,5 proc.). Didžiausias išskiriamų *P. aeruginosa* padermių skaičius buvo fiksuotas Intensyvios terapijos – 49 (40,6 proc.) ir vidaus ligų skyriuose – 41 (33,8 proc.)

Ištyrus *P. aeruginosa* padermių jautrumą antibiotikams nustatyta, kad tirtosios padermės pasidalino į dvi atskiras grupes iš kurių 53,71 proc. (n=65) sudarė izoliatai atsparūs vienam arba dviem karbapenemų klasės antibiotikams (imipenemui ir/arba meropenemui) ir 46,28 proc. (n=56) padermės jautrios karbapenemų klasės antibiotikams (imipenemui ir/arba meropenemui).

Tyrimu nustatyta, jog karbapenemams atsparios padermės buvo kur kas dažniau ($p<0,05$) atsparios ir kitų klasių antibiotikams, lyginant su karbapenemams jautriomis padermėmis, atitinkamai: ciprofloksacinui (87,7 proc., n=57 ir 21,4 proc., n=12; $p<0,05$), gentamicinui (78,5 proc., n=51 ir 17,9 proc., n=10; $p<0,05$), tobramicinui (56,8 proc., n=21 ir 9,8 proc., n=4; $p<0,05$), piperacilinui (70,8 proc., n=46 ir 28,6 proc., n=16; $p<0,05$), amikacinui (38,5 proc., n=25 ir 5,4 proc., n=3; $p<0,05$), ceftazidimui (53,8 proc., n=35 ir 25,0 proc., n=14; $p<0,05$), piperacilinui/tazobaktamui (42,1 proc., n=16 ir 16,3 proc., n=7; $p<0,05$), cefoperazonui/sulbaktamui (50,0 proc., n=19 ir 25,6 proc., n=11; $p<0,05$) (1 lentelė).

1 lentelė. *Karbapenemų klasės antibiotikams jautrių ir atsparių Pseudomonas aeruginosa padermių atsparumas kitų klasių antibiotikams*

Antibiotikų klasė	**Antimikrobinė medžiaga**	**KA*, n (%)**	**KJ**, n (%)**	χ^2	***P***
Penicilinai	Piperacilinas	46/65 (70,8)	16/56 (28,6)	21,44	**<0,001**
	Piperacilinas/ Tazobaktamas	16/38 (42,1)	7/43 (16,3)	6,62	**0,01**
Cefalosporinai	Ceftazidimas	35/65 (53,8)	14/56 (25,0)	10,39	**0,001**
	Cefepimas	11/38 (28,9)	7/42 (16,7)	1,73	0,189
Cefalosporinai	Cefoperazonas/ sulbaktamas	19/38 (50,0)	11/43 (25,6)	5,16	**0,023**
Fluorchinolonai	Ciprofloksacinas	57/65 (87,7)	12/56 (21,4)	53,90	**<0,001**
Aminoglikozidai	Gentamicinas	51/65 (78,5)	10/56 (17,9)	44,20	**<0,001**
	Amikacinas	25/65 (38,5)	3/56 (5,4)	18,54	**<0,001**
	Tobramicinas	21/37 (56,8)	4/41 (9,8)	19,73	**<0,001**
Monobaktamai	Aztreonamas	9/59 (15,3)	4/51 (7,8)	1,44	0,23

* – Karbapenemų klasės antibiotikams atsparios *P. aeruginosa* padermės; ** – Karbapenemų klasės antibiotikams jautrios *P. aeruginosa* padermės.

Tyrimu nustatyta, kad statistiškai patikimai didesnė karbapenemams jautrių izoliatų dalis buvo išskirta iš apatinių kvėpavimo takų sekreto, lyginant su karbapenemams atspariomis padermėmis, tai atitinkamai sudarė 60,0 proc., n=27 ir 40 proc. n=18; p<0,05. Tuo tarpu *P. aeruginosa,* išskirtos iš šlapimo takų, buvo dažniau atsparios negu jautrios karbapenemų klasės antibiotikams (75 proc., n=15 ir 25 proc., n=5; p<0,05).

Septyniasdešimt trims (n=73) atsparioms karbapenemų klasės antibiotikams padermėms papildomai įvertintas jautrumas septynių klasių antibiotikams (MSK), remiantis sultinio mikropraskiedimo principu, panaudojant komercines Sensititre plokšteles (*TREK Diagnostic Systems*). Tyrimo metu nustatyta, kad Lietuvos sveikatos mokslų universitete išskirtos karbapenemų klasės antibiotikams atsparios *P. aeruginosa* padermės pasižymėjo dažnu atsparumu ir kitų klasių antibiotikams. Didžiausias atsparumas buvo stebimas imipenemui (97,3 proc., n=71), aztreonamui (91,8 proc., n=67), tikarcilinui (83,6 proc., n=61) ir meropenemui (83,6 proc., n=61). Dauguma *P. aeruginosa* padermių buvo atsparios ciprofloksacinui, piperacilinui/tazobaktamui ir ceftazidimui. Dešimčiai (13,7 proc., n=10) bakterijų nustatytas atsparumas visų tirtų klasių antibiotikams, išskyrus polimiksinų grupę. 99 proc. tirtų *P. aeruginosa* padermių išliko atsparios tik kolistinui (2 lentelė).

Tarp tirtų izoliatų 74 proc. (n=54/73) sudarė padermės, turinčios dauginį atsparumą antibiotikams (2 lentelė). Dauginiu atsparumu pasižyminčios *P. aeruginosa* padermės dažniausiai buvo atsparios 3 klasių antibiotikams 100 proc. (n=54/54). 67 proc. padermių (n=36/54) nustatytas atsparumas penkių klasių antibiotikams, viena padermė pasižymėjo visišku atsparumu septynių tirtų klasių antibiotikams.

Dažniausiai *P. aeruginosa* padermėse nustatytas atsparumo antibiotikams derinys – atsparumas tikarcilinui, aztreonamui ir imipenemui.

2 lentelė. *Karbapenemų klasės antibiotikams atsparių Pseudomonas aeruginosa padermių antimikrobinio atsparumo profiliai*

Antibiotikų klasė	**Antimikrobinė medžiaga**	**Karbapenemams atsparios padermės (N=73)**		**DAA (N=54)**	**DAA>5* (N=36)**	**DAA>7 ** (N=1)**
		Jautru, n (%)	**Atsparu, n (%)**			
Penicilinai	Tikarcilinas	12 (16,4)	61 (83,6)	x	x	x
Penicilinai + *β*-laktamazės inhibitoriai	Piperacilinas/ tazobaktamas	28 (38,4)	45 (61,6)		x	x
Monobaktamai	Aztreonamas	6 (8,2)	67 (91,8)	x	x	x
Cefalosporinai	Ceftazidimas	32 (43,8)	41 (56,2)		x	x
	Cefepimas	50 (68,5)	23 (31,5)			x
Cefalosporinai + *β*-laktamazės inhibitoriai	Ceftolozanas/ tazobaktamas	53 (72,6)	20 (27,4)			x
Karbapenemai	Imipenemas	2 (2,7)	71 (97,3)	x	x	x
	Meropenemas	12 (16,4)	61 (83,6)		x	x
Fluorchinolonai	Ciprofloksacinas	20 (27,4)	53 (72,6)		x	x
Aminogliko-zidai	Tobramicinas	41 (56,2)	32 (43,8)			x
	Amikacinas	50 (68,5)	23 (31,5)			x
Polimiksinai	Kolistinas	72 (98,6)	1 (1,4)			x

DAA – dauginis atsparumas antibiotikams; *– atsparumas penkioms ir daugiau antibiotikų klasėms; **- atsparumas septynioms antibiotikų klasėms.

P. aeruginosa padermių patogeniškumo veiksnių tyrimo duomenys

Bakterijų gebėjimas išlikti gyvybingomis žmogaus serumo poveikyje buvo vertintas po 1 val., 2 val. ir 3 val. ir buvo suskirstytas į 6 lygius. *P. aeruginosa* padermės priskirtos 1–4 lygiui buvo priskirtos serumui jautrioms, 5–6 lygio – serumui atsparioms. Tyrimo metu 70,2 proc. (n=85)

izoliatų išliko jautrūs serumo baktericidiniam poveikiui, atitinkamai 29,8 proc. (n=36) parodė atsparumą kraujo serumui. Tyrimo metu jokio statistiškai reikšmingo skirtumo tarp atsparumo karbapenemams ir atsparumo baktericidiniam serumo poveikiui nebuvo rasta.

Įvertinus *P. aeruginosa* gebėjimą gaminti bioplėveles pagal skirtingus intensyvumo lygius jokio statistiškai reikšmingo skirtumo tarp atsparumo karbapenemams ir gebėjimo gaminti bioplėveles taip pat nebuvo rasta.

P. aeruginosa padermių fenotipinė charakteristika

Remiantis antibiotikų sinergizmo su kloksacilinu tyrimo rezultatais nustatyta, kad 74 proc. (n=54) *P. aeruginosa* padermių atsparumas beta laktaminiams antibiotikams buvo susijęs su C klasės *β*-laktamazių produkcija (AmpC) ir specifinio kanalo OprD praradimu. Šešiolikai tirtų padermių (22 proc.) nenustačius kloksacilino sinergizmo su imipenemo bei ceftazadimo antibiotikais, rezultatas buvo vertinamas kaip neigiamas nurodant, kad atsparumo priežastis tikėtina *β*-laktamazių gamyba. Likusioms trims *P. aeruginosa* padermėms nebuvo pastebėtas kloksacilino sinergizmas tik su ceftazadimu, todėl šis rezultatas buvo vertinamas kaip galima ESBL produkcija.

Tiriant modifikuotu „Hodge testu“ (arba dvigubos difuzijos metodu) karbapenemazių gamybos atveju (n=14) buvo stebima susiliejanti (dobilo lapą primenanti) augimo slopinimo zona. Penkioms padermėms buvo stebimas neigiamas dviejų testų rezultatas.

3 lentelė. *Karbapenemazių gamybos fenotipinis nustatymas remiantis kloksacilino inhibicijos testo ir modifikuoto Hodge testo rezultatais*

Padermių skaičius	**Antimikrobinis profilis**		**KIT**		**MHT**	
	Ceftazidimas	**Imipenemas**	**AmpC**		***β*-laktamazės**	
			Teigiama (N=54)#	**Neigiama (N=19)#**	**Teigiama (N=19)#**	**N (N=54)#**
41	A	A/VA	22 (0)	19 (0)*	14 (5)▲	22
30	J	A/VA	30 (0)	–	–	30 (0)
2	J	J	2 (0)	–	–	2 (0)

A – atsparu; VA – vidutiniškai atsparu; J – jautru; KIT, kloksacilino inhibicijos testas; MHT, modifikuotas Hodge testas; # – gautas klaidingai teigiamas ar klaidingai neigiamas rezultatas.

P. aeruginosa padermių molekulinė charakteristika

Dauginės polimerazės grandininės reakcijos metodu nustatyta, kad LSMU Ligoninėje „Kauno klinikos“ vyravo karbapenemams atsparios *P. aeruginosa* padermės, sintetinančios $bla_{\text{VIM-2}}$ metalo- *β*-laktamazę (21,9 proc., n=16) (4 lentelė). Iš tirtų padermių dvi padermės turėjo bla_{GES}, tuo tarpu tik viena bla_{PER} *β*-laktamazės genus. Nei viena iš tirtų 73 imipenemui ir/ar meropenemui atsparių padermių neprodukavo bla_{IMP}, bla_{KPC}, bla_{NDM} ir $bla_{\text{OXA-48}}$ *β*-laktamazės genų.

Vienai *P. aeruginosa* padermei sintetinančiai bla_{GES} *β*-laktamazės geną nustatyta unikali nukleotidų seka, todėl naujam genui suteiktas naujas GES numeris – GES-27.

Visos $bla_{\text{VIM-2}}$ geną koduojančios padermės pasižymėjo dauginiu atsparumu net 5 klasių antibiotikams (4 lentelė). Reikia atkreipti dėmesį, kad visos $bla_{\text{VIM-2}}$ geną koduojančios padermės, pasižymėjo dideliu atsparumu imipenemui, tikarcilinui ir ceftalozanui/tazobaktamui, kai šių antibiotikų minimali slopinamoji koncentracija buvo aukčiau nustatytų klinikinių lūžio taškų atitinkamai (>64 µg/ml; > 512 µg/ml ir 64 µg/ml).

VIM-2 metalo-*β*-laktamazę gaminančios *P. aeruginosa* padermės buvo aptiktos skirtingose infekcijos vietose/ėminuose, tačiau daugiausiai žaizdose (pūliai, žaizdos sekretas), ir tai sudarė net 37,5 proc. Didžiausias $bla_{\text{VIM-2}}$ geną koduojančių *P. aeruginosa* padermių paplitimas nustatytas intensyvios terapijos skyriuose (50 proc.) (4 lentelė).

Genetiniam tipavimui panaudojus PFGE-*SpeI* metodą nustatyta 18 skirtingų genetinių profilių-pulsotipų, tarpusavyje besiskiriančiu bent vienu mikro-restrikcijos fragmentu. Taikant genetinių profilių 90% panašumo slenkstį visi pulsotipai susigrupavo į šešias grupes ir du klasterius. Didžiausią F grupę sudarė dešimt $bla_{\text{VIM-2}}$ geną koduojančių *P. aeruginosa* padermių. Šešios metalo- *β*-laktamazę koduojančios *P. aeruginosa* padermės buvo priskirtos, atitinkamai E, C ir D pulsotipų grupei. A ir B grupes sudarė *P. aeruginosa* padermės, koduojančius A klasės karbapenemazės genus.

P. aeruginosa sekos tipų (ST) nustatymo tyrimas parodė, kad C pulsotipui priklausanti padermė priklauso ST1047 tipui, o reprezentatyvios D, F ir E pulsotipo padermės (n=15) priklauso tarptautiniam STR235 tipui, kuris priskiriamas didelės rizikos grupei.

Visose metalo-*β*-laktamazę produkuojančiose padermėse (išskyrus vieną) nustatytas I klasės integronas su dažniausiai nustatytu *aacA7*-$bla_{\text{VIM-2}}$-*dfrB5*-*aacA5* genų kasečių deriniu.

4 lentelė. *Metalo-β-laktamazę, plataus spektro β-laktamazę ir A klasės karbapenemazės genus koduojančių P. aeruginosa padermių bendroji charakteristika*

Nr.	Ėminys	Skyrius	DAA*	Gebėjimo gaminti bioplėveles lygis	Karbapenemazės tipas	Molekulinė klasė	PLGE numeris	PLGE grupė	ST
5	Ž	S	>5	Negamina	GES-27	A	PT-1	B	ST660
13	Ž	ITS	>5	Aukštas	PER-1*	A **	ND	ND	ND
32	Ž	S	>5	Vidutinis	VIM-2	B (MBL)	PT-2	D	ST235
33	Ž	S	>5	Vidutinis	VIM-2	B (MBL)	PT-3	D	ST235
46	VKT	VS	>5	Negamina	VIM-2	B (MBL)	PT-4	C	ST1047
47	Ž	S	>5	Vidutinis	VIM-2	B (MBL)	PT-5	F	ST235
51	Ž	S	>5	Negamina	VIM-2	B (MBL)	PT-6	F	ST235
70	AKT	S	>5	Aukštas	VIM-2	B (MBL)	PT-7	F	ST235
96	ŠT	ITS	>5	Aukštas	VIM-2	B (MBL)	PT-8	F	ST235
98	KR	ITS	>5	Negamina	VIM-2	B(MBL)	PT-9	F	ST235
99	AKT	ITS	>5	Aukštas	VIM-2	B (MBL)	PT-10	E	ST235
100	AKT	ITS	>5	Aukštas	VIM-2	B(MBL)	PT-11	F	ST235
101	ŠT	ITS	>5	Aukštas	VIM-2	B (MBL)	PT-12	F	ST235
103	AKT	ITS	>5	Aukštas	VIM-2	B (MBL)	PT-13	F	ST235
107	ŠT	S	>5	Vidutinis	VIM-2	B (MBL)	PT-14	F	ST235
111	APT	S	>5	Negamina	GES-5	A	PT-15	A	ST1628
115	Ž	VS	>5	Negamina	VIM-2	B (MBL)	PT-16	F	ST235
134	ŠT	ITS	>5	Negamina	VIM-2	B (MBL)	PT-17	D	ST235
135	Ž	ITS	>5	Negamina	VIM-2	B (MBL)	PT-18	D	ST235

Ž – žaizdos; VKT – viršutiniai kvėpavimo takai; AKT – apatiniai kvėpavimo takai; KR – kraujas; ŠT – šlapimo takai; S – skubios pagalbos skyrius; ITS – intensyvios pagalbos skyrius; VS – vidaus skyriai. PGLE – pulsuojančio lauko gelio elektroforezė; ST – sekos tipas.

Išvados

1. *P. aeruginosa* padermės buvo atsparios ne tik karbapenemų klasės antibiotikams, bet ir kitų klasių antibiotikams. Palyginti didelė dalis tirtų bakterijų pasižymėjo dauginiu ir plataus spektro atsparumu antimikrobinėms medžiagoms. Vienas trečdalis tirtų *P. aeruginosa* padermių buvo atsparios bent septynioms antimikrobinėms medžiagoms iš 12 tirtų, įskaitant naują antibiotiką – ceftolozaną/tazobaktamą. *P. aeruginosa* didžiausias jautrumas buvo nustatytas tik kolistinui.
2. Vienas trečdalis mūsų tirtų *P. aeruginosa* padermių buvo atsparios serumo baktericidiniam poveikiui ir gebėjo formuoti bioplėveles. Sąsajų tarp atsparumo serumo baktericidiniam poveikiui, gebėjimo formuoti bioplėveles ir atsparumo karbapenemams bei kitų klasių antibiotikams, nenustatėme.
3. Fenotipiniai lyginamosios analizės metodai leido įvertinti numanomus bakterijų atsparumo antibiotikams molekulinius mechanizmus. *P. aeruginosa* padermės, produkuojančios C klasės *β*-laktamazę (AmpC) ir praradusios specifinį OprD kanalą davė teigiamą imipenemo ir ceftazidimo sinergizmą su kloksacilinu bei neigiamą „Hodge“ testo rezultatą. Neigiami „Hodge“ testo rezultatai atmetė beveik visas metalo-*β*-laktamazę neprodukuojančias padermes.
4. Kauno klinikose vyravo padermės, sintetinančios $bla_{\text{VIM-2}}$ metalo-*β*-laktamazę, priklausančią STR235 didelės rizikos grupei. Buvo aptiktas pasaulyje naujas bla_{GES} *β*-laktamazės genas, kuriam duomenų bazėje suteiktas naujas identifikacijos $bla_{\text{GES-27}}$ numeris.

CURRICULUM VITAE

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Education

2011–2015	**Ph. D. studies, Biology.** Lithuanian University of Health Sciences, Kaunas, Lithuania. "Microbiological and molecular characterization of clinical carbapenem-resistant *Pseudomonas aeruginosa* isolates".
2009–2011	**Master of Molecular Biology and Biotechnology.** Vytautas Magnus University, Faculty of Natural Sciences, Program of Molecular Biology and Biotechnology, Kaunas, Lithuania. "Antimicrobial activity of probiotics and effect of prebiotics against enteric pathogens"
2005–2009	**Bachelor of Biology.** Vytautas Magnus University, Faculty of Natural Sciences, program of Biology, Kaunas, Lithuania. Gastrointestinal infections: epidemiology of campylobacter-riosis and salmonellosis in Kaunas and Klaipėda cities.

Job Experience

27 06 2016–now	Quality management specialist at quality control department. National Public Health Surveillance Laboratory. Žolyno 36, LT-10210 Vilnius, Lithuania.
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PADĖKA

Iš visos širdies dėkoju žmonėms, padėjusiems man ruošti šią daktaro disertaciją.

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Norėčiau padėkoti visiems LSMU MA Laboratorinės medicinos klinikos, klinikinės chemijos ir genetikos laboratorijos kolektyvo buvusiems ir esamiems žmonėms už visokeriopą pagalbą per visus čia praleistus metus. Ačiū Irenai už pagalbą ir geranoriškumą atliekant bakterijų tipavimą.

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