Institute of Clinical Microbiology
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Characterization of medically important *Pseudomonas aeruginosa* isolates

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LIST OF PUBLICATIONS

This thesis is based on the following publications:

I. Ratkai C, Quinteira S, Grosso F, Monteiro N, Nagy E, Peixe L.: **Controlling for false positives: interpreting MBL Etest and MBL combined disc test for the detection of metallo-beta-lactamases.** *J Antimicrob Chemother*. 2009;**64**:657-8

II. Ratkai C., Nagy E., Peixe L., Bertalan V., Hajdu E**.: Isolation and characterization of an imported extremely-resistant *Pseudomonas aeruginosa* producing three different extended-spectrum β-lactamases and hyperproducing two multidrug-efflux pumps**. *J Inf* 2010, **61**:511-512

III. Ratkai C, Peixe LV, Grosso F, Freitas AR, Antunes P, Fodor E, Hajdú E, Nagy E.: **Successful application of the DiversiLab repetitive-sequence-based PCR typing system for confirmation of the circulation of a multiresistant *Pseudomonas aeruginosa* clone in different hospital wards**. *Diagn Microbiol Infect Dis*. 2010 ;**67**:202-206.

IV. Ratkai C., Nagy E., Peixe L., Szabó Á., Hajdu E.: **Characterisation of *Pseudomonas aeruginosa* strains isolated from patients suffering from cystic fibrosis in South-East Hungary**
under publication

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Abbreviations

bp	base pair
CC	clonal complex
CF	cystic fibrosis
CLSI	Clinical and Laboratory Standard Institute
CFTR	cystic fibrosis transmembrane regulator
EDTA	ethylene-diamin-tetraacetic acid
ESBL	extended spectrum β-lactamase
GIM	german imipenemase
IMP	imipenemase
IS	insertion sequence
ICU	intensive care unit
IM	internal medicine ward
MBL	metallo- β-lactamase
MLST	multilocus-sequence typeing
Mex	multidrug efflux
MDR	multidrug resistant
MICU	medical ICU
NE	neurology ward
NS	neurosurgical ward
OXA	oxacillinase
Opr	outer membrane protein
PFGE	pulsed-field gel-electrophoresis
Rep-PCR	repetitive-element-based PCR
RT-PCR	Real-time PCR
SICU	general surgical ICU
SICU2	trauma and neurosurgical ICU
SICU3	cardiac sugical ICU
SPM	São-Paolo metallo- β-lactamase
ST	sequence type
Tn	transposon
VIM	Verona-imipenemase
VAP	ventilation-associated pneumonia

1. INTRODUCTION

1.1. Features of *Pseudomonas aeruginosa*

Pseudomonas aerugionosa (*P. aruginosa*) is a non-fermentative, aerobic Gram-negative rod, measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Almost all strains are motile by means of a single polar flagellum. It normally lives in moist environments, and uses a wide range of organic compounds for growth, thus giving it an exceptional ability to colonize ecological niches where nutrients are limited, from water and soil to plant and animal tissues. Typical biochemical features of *P. aeruginosa* isolates are: positive oxidase test, growth at 42 °C, hydrolysis of arginine and gelatine, and nitrate reduction. *P. aeruginosa* strains produce two types of soluble pigments, pyoverdin and pyocyanin. The latter blue pigment is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is characteristic for suppurative infections caused by *P. aeruginosa* [1]. The bacterium is ubiquitous in soil, variety of aqueous solutions, including disinfectants, soaps, eye drops, as well as sinks and respiratory equipments. *P. aeruginosa* is a highly adaptable organism. It has a large genome containing 6.26 Mbp (encoding 5567 genes) compared to 4.64 Mbp (4279 genes) in *Escherichia coli* [3]. An approximate calculation of the number of genes needed for cell growth and division in a minimal medium, is around 1500. *P. aeruginosa* therefore has considerable additional genetic capacity. This explains its highly adaptable nature, including the ability to develop resistance against antibiotics.

Occasionally, *P. aeruginosa* can colonise human body sites, with a preference for moist areas, such as the perineum, axilla, ear, nasal mucosa and throat, as well, as stools. The prevalence of colonisation in healthy individuals is usually low, higher colonisation rates can be encountered following hospitalisation, especially among patients treated with broad-spectrum antibiotics. Normally, for an infection to occur, some disruption of the physical barriers (skin or mucous membrane), or by-passing of them (invasive devices), and/or an underlying dysfunction of the immune defence mechanisms is necessary. Therefore, *P. aeruginosa* is mostly a nosocomial pathogen. Infections associated with this bacterium are nosocomial respiratory tract infections including ventilator-associated pneumonia (VAP), dermatitis, soft tissue infections, bacteraemia, bone and joint infections, gastrointestinal infections and a variety of systemic infections, particularly in immunosuppressed patients (AIDS), or patients with severe burns or cancer. Community acquired infections caused by *P. aeruginosa* are uncommon. The most frequent ones are: urinary tract infections, otitis externa,

folliculitis acquired in swimming pools, keratitis due to wearing contact lenses. The mucoid phenotype of *P. aeruginosa* frequently chronically colonises and infects patients with cystic fibrosis causing damage of the lung tissue and decreased pulmonary function. *P. aeruginosa* has an abundance of virulence factors, including flagella, pili, lipopolysaccharides, alginate, alkaline protease, elastase, phospholipase C, exotoxin A, quorum sensing mechanisms, type III secretion system, pyocianin, pyoverdin, and produces a number of toxic proteins which not only cause extensive tissue damage, but also interfere with the human immune system's defence mechanisms [1].

1.2. Antibiotic resistance in *P. aeruginosa*

P. aeruginosa is one of the main organisms responsible for drug-resistant nosocomial infections, and is one of the leading causes of bacteraemia and pneumonia in hospitalised patients. In addition being intrinsically resistant to several antimicrobial agents, *P. aeruginosa* can easily develop resistance to all conventional antipseudomonal antibiotics via different mechanisms [2] (**Table 1.**).

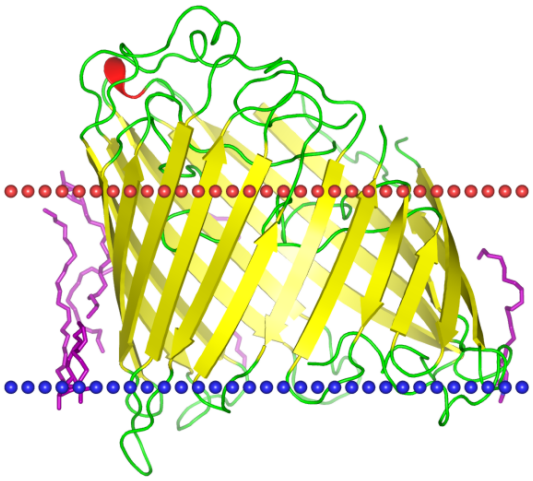
The aminoglycosides inhibit protein synthesis by binding to the 30S subunit of the ribosome. Quinolones bind to the A subunit of DNA gyrase enzyme, which maintains the ordered structure of the chromosome inside the cells. The β-lactams inhibit the peptidoglycan-assembling transpeptidases located on the outer face of the cytoplasmic membrane. Finally the polymyxins bind to phospholipids in the cytoplasmic membrane, destroying its barrier function. There are three basic mechanisms by which *P. aeruginosa* resists the action of the above antimicrobial agents: restricted uptake and efflux; enzymatic drug inactivation and mutations in the targets [3].

1.2.1. Restricted uptake: porins, efflux pumps

All of the antibiotics have to cross the cell wall, to reach their targets. Failure of antibiotics to accumulate in the organism is due to the combination of restricted permeability of the outer membrane and the efficient removal of antibiotic molecules by efflux pumps. The outer membrane of *P. aeruginosa* is an important barrier of the penetration of antibiotics (has low permeability, approximately 8% that of *E. coli*) [4], excluding the larger molecules. Small hydrophilic molecules, such as β-lactams and quinolons can only cross the outer membrane by passing through the channels provided by porin proteins. Investigation of porins has revealed that they contain transmembrane anti-parallel β-strands that wrap into a barrel, and

this β-barrel is embedded into the outer membrane bilayer (**Figure 1.)**. The central area of the barrels of the general and specific porins contains stretches of amino acids from one of the interconnecting regions, that folds back into the channel region and give this region many of its important characteristics.

Figure 1. The structure of the outer membrane porin OprD. (Source of picture is: opm.phar.umich.edu/protein.php?pdbid=2odj)

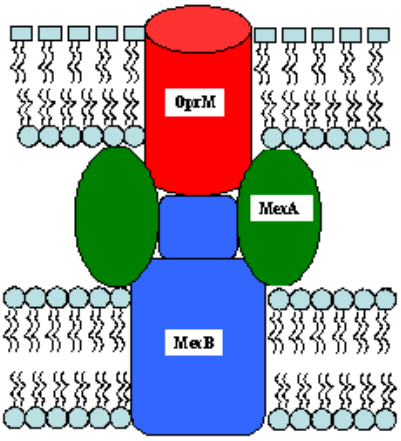


In *P. aeruginosa* there are 163 known or predicted porin proteins based on its genome sequence, 64 are found as part of 3 families of porins: the OprD-specific porin family, the TonB-dependent gated porin family, and the OprM efflux family. OprF is the major channel of *P. aeruginosa* for larger substrates, and is considered to be general, nonspecific porin [4]. Although mutants lacking OprF have been reported, loss of OprF has not been found to be a major cause of antibiotic resistance. Other proteins, that function as substrate-specific porins can serve as general channels for small substrates. OprD is a specialized porin which has a specific role in the uptake of e.g. positively charged molecules such as lysine, or certain carbapenems. Lack of OprD in *P. aeruginosa* is due to a mutation, which can occur at relatively high frequency (10^{-7}), and can be due to deletion, substitution or insertions that cause inactivation of the *oprD* gene. Loss of OprD is frequently associated with resistance to imipenem, which requires this porin to cross the outer membrane. Interestingly, meropenem is not affected by loss of OprD, indicating that the different carbapenems cross the outer membrane by different channels. The aminoglycosides and colistin do not cross the outer membrane via porin channels. Instead they promote their own uptake by binding to the

lipopolysaccharide (LPS) on the outer face of the membrane, which destroys the permeability barrier of the outer membrane and allows the antibiotics to penetrate through the wall to the cytoplasmic membrane. Resistance to aminoglycosides and colistin could be due to the overexpression of OprH protein, which protects the LPS from binding the antibiotics, although this type of resistance mechanism was observed only in laboratory strains [3]. Acquired resistance to polymyxins has been occasionally described in *P. aeruginosa* isolates from cystic fibrosis patients treated for a long time with this drug, and seems to be related to mutations in the outer membrane structure [5].

The multidrug efflux system of *P. aeruginosa* are composed of three protein components, an energy-dependent pump located in the cytoplasmic membrane, an outer membrane porin and a peripheral cytoplasmic membrane linker (sometimes called as membrane fusion protein). Each of these proteins is highly conserved, at around 20% or greater identity level. *P. aeruginosa* has 18 outer membrane proteins with putative function in efflux. Eleven of these, including OprM, OprN, OprJ, fall into one phylogenetic subclass, and are parts of multiple antibiotic efflux systems [4]. The MexAB-OprM system (**Figure 2.**) is constitutively expressed in virtually all isolates, and substrates for this pump include fluoroquinolones, tetracycline, chloramphenicol, and β-lactams (including carbenicillin, piperacillin, ceftazidime, cefepime, and aztreonam). Imipenem does not appear to be a substrate for MexAB-OprM, but because of its hydrophobic side chain, meropenem can be affected by this system. The expression of MexAB-OprM system is negatively controlled by the repressor MexR. Mutational inactivation of a second regulatory gene *nalC* increases the expression of the PA3719 protein, which then increases MexAB-OprM expression. A third regulatory gene of the *mexAB-oprM* operon *nalD*, has also been identified; mutations in this gene have been observed in clinical isolates overexpressing MexAB-OprM [4].

Figure 2. Schematic representation of the assembly of efflux pump MexAB-OprM in the cell wall of *P. aeruginosa*. The pump is composed of three protein components: an energy-dependent pump located in the cytoplasmic membrane (MexB), an outer membrane porin (OprM) and a peripheral cytoplasmic membrane linker (MexA).



Fluoroquinolones and the antipseudomonal β-lactams (piperacillin, cefepime, and meropenem, but not carbenicillin, aztreonam, ceftazidime, or imipenem) are substrates for the MexCD-OprJ system, although this efflux pump is not typically expressed under normal growth conditions. A third efflux system, MexEF-OprN, can export fluoroquinolones, trimethoprim, and chloramphenicol. Finally, MexXY-OprM may contribute to fluoroquinolone, aminoglycoside and selected β-lactam (piperacillin, cefepime, and meropenem but not carbenicillin, ceftazidime, or imipenem) resistance. MexXYOprM expression can be induced with growth in the presence of tetracycline or aminoglycosides [4].

1.2.2. Drug inactivation: enzymatic resistance

All *P. aeruginosa* strains have the *ampC* gene of the inducible β-lactamase. However, induction alone probably does not account for resistance, instead overexpression of the enzyme due to spontaneous mutation in its regulatory gene, which has occurred particularly where ceftazidime therapy was used. Although the enzyme is normally located in the periplasm, it has been detected in sputum during antipseudomonal treatment [3]. The enzyme is probably released from high-level producers in the lungs, and would protect low-level

producers by reducing the local concentration of certain β-lactam antibiotics, like cephalotin or ampicillin.

Many other acquired β-lactamases and aminoglycoside modifying enzymes have been noted in *P. aeruginosa*. The most frequently acquired β-lactamases are PSE-1 and PSE-4. Like classical TEM and OXA enzymes, which also occur in *P. aeruginosa*, these enzymes are not effective against carbapenems, oxyimino-aminothiazolyl cephalosporins (ceftazidime, cefepime, cefpirome) and monobactams. However, β-lactamases that give wider resistance are emerging in *P. aeruginosa*, such as PER-1 β-lactamases, TEM, SHV and OXA ESBLs and carbapanamases and the metallo- β-lactamases [2,10] (**Table 1.**).

PER-1, an Ambler's molecular class A β-lactamase confers high-level resistance to ceftazidime, but it has little in vitro effect on piperacillin. It was recovered in 1991 from France, later was recognized to be widespread in Turkey, and disseminated in Italy, Poland, Japan and Romania [6]. Up to this day only a few sporadic cases of PER-1 producer *P. aeruginosa* has been detected in Hungary, interestingly almost all of them were imported cases [7,8,**article II**].

The presence of OXA-type ESBLs is quite frequent among pseudomonads [11] (**Table 1.**). Like TEM and SHV ESBLs, the OXA ESBLs have minor sequence substitutions that greatly extend their hydrolytic spectra. They confer resistance to carboxypenicillins and ureidopenicillins, but not to ceftazidime. There are five groups of these enzymes, namely OXA group I (including OXA-5; OXA-7; OXA-10 and its derivates: OXA-11, OXA-14, OXA-16, OXA-17, OXA-74; OXA-13 and its derivates: OXA-19, OXA-28), OXA group II (including OXA-2; OXA-3; OXA-15; OXA-20), OXA group III (including OXA-1, OXA-4, OXA-30, ESBLs, OXA-31), OXA group IV (defined by OXA-9) and OXA group V (containing LCR-1). In addition, OXA-18 does not belong to any of these groups [11].

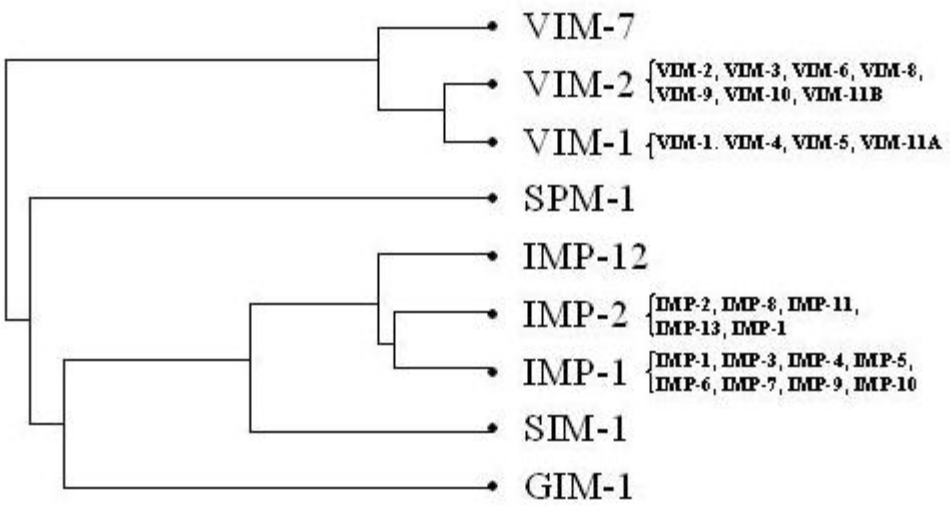
Carbapenems, such as imipenem and meropenem have a very broad spectrum of activity, and the drugs resist hydrolysis by most of the β-lactamases, including ESBLs, and derepressed chromosomal ampC β-lactamases. Resistance to carbapenems could be mediated by different enzymes [12,13], namely most of the metallo- β-lactamases together with some class A and D enzymes. β-lactameses of molecular classes A, C and D use a catalytically active serine residue for inactivation of the β-lactam drug. OXA-type carbapenemases [12] belong to molecular class D, and are usually encoded by chromosomal genes. Some of these carbapenemases are widely distributed in *P. aeruginosa*, and although these enzymes show only week carbapenemase activity, carbapenem resistance may results from a combination of an OXA-type carbapenemase and secondary resistance mechanism, such as porin deficiency

or efflux pumps. Class B metallo-β-lactameses (MBLs), however are enzymes with versatile hydrolytic capabilities, namely the ability to hydrolyze all β-lactam antibiotics, with the exception of monobactams. Four groups of MBLs have so far been reported in *P. aeruginosa*, namely IMP (imipenemase), VIM (Verona imipenemase), SPM (São Paolo metallo-β-lactamese), ans GIM (German imipenemase). These four classes of enzymes together with the SIM isolated from Acinetobacter comprise the MBLs (**Table 1.**).

The common feature of MBLs is the principal zinc binding motif histidine-X-histidine-X-aspartic acid in the active site, which coordinates the arrangement of two H_2O molecules that are important in the hydrolysis. Hence, chelation of zinc by EDTA or mercaptopropionic acid, impairs β-lactam hydrolysis and restores susceptibility to carbapenems. MBLs have a wide and plastic active site, which let all β-lactams to accomodate in there, except aztreonam. β-lactamase inhibitors such as clavulanic acid, tazobactam and sulbactam are also hydrolised by MBLs.

Of the four classes of MBLs only the VIM and IMP classes have so far been found to have three subgroups, and several enzyme variants (**Figure 3.**), which are as follows: VIM-7 group, VIM-2 group, VIM-1 group and IMP-12, IMP-1, IMP-2 group, IMP-1 group [14].

Figure 3. Phylogenetic relationship between the five groups of acquired MBLs, and the subgroups of VIM and IMP.



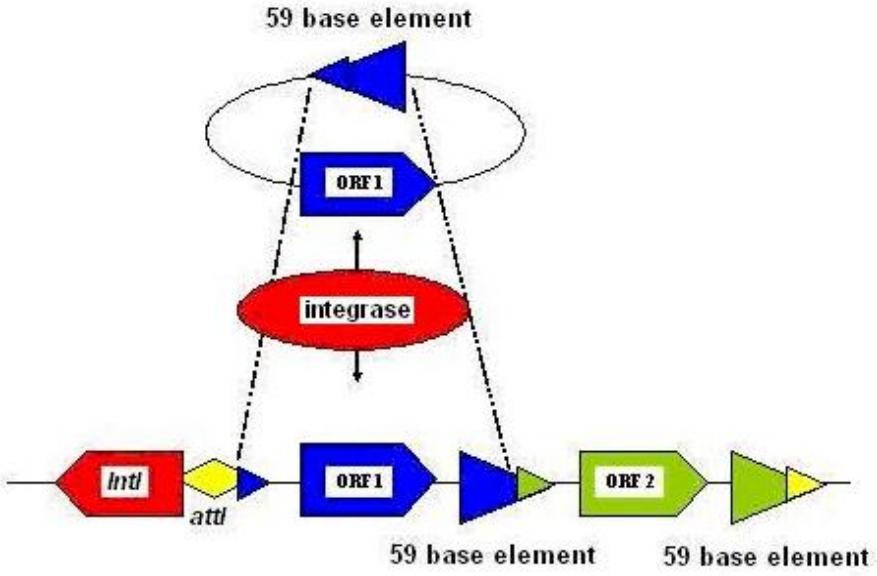
While GIM and SPM have so far been reported from a restricted geographical area, the IMP and VIM enzymes have reached a worldwide dissemination [14]. The IMP enzymes

were originally detected in Asia, but later spread to Europe, to the United States and to Australia, while the VIM gene was first found in Europe, and shortly after emerged to other continents. Despite the worldwide dissemination of these two groups of enzymes, the tendency of the dominancy of the IMP enzyme in Asia, and the VIM enzyme in Europe prevails [14].

Significant differences in the hydrolytic capacity of the different MBL groups are observed. SPM-1 is a very efficient, while GIM-1 is rather a weak carbapenemase. Within the IMP group it has been observed, that IMP-6 and IMP-3 have low capacity of imipenem hydrolysis, while within the VIM group VIM-1 hydrolyse meropenem, ceftazidime and piperacillin more efficiently, than VIM-2 [14]. Still, the majority of MBL producers are highly resistant to carbapenems and cephalosporins.

The genes encoding the MBLs are almost always located on class 1 integrons [15,16]. Integrons are genetic elements that although unable to move themselves, contain gene cassettes that can be mobilized to other integrons or to secondary sites in the bacterial genome. The majority of integrons described belong to class 1. These integrons consist of two conserved regions (5'CS and 3'CS) and a variable region, where different gene cassettes can be inserted. The 5'CS part contains an integrase gene (*intI*), an adjacent recombination site (*attI*) and a common promoter. The 3'CS region usually consist of a partially deleted gene, encoding a quaternary ammonium compound efflux pump (*qacEΔ1*) fused with a sulphonamide resistance gene (*sulI*). In the variable region different β-lactamases, usually MBLs, and/or aminoglycoside-resistance genes, between the genes recombination sites (also known as 59-base elements) are found. The 59-base elements are not highly conserved, and contain imperfect inverted repeats. Integration of new gene cassettes, which is mediated by the integrase can take place either between the gene cassettes and *attI*, or between two gene cassettes 59-base elements. The integrase excises the gene cassettes as covalently closed circular molecules. The recombination takes place close to one end of the 59-base element. Due to the integration of circular gene cassette, part of the 59-base element ends up at the 5'side of the coding sequence of the gene cassette to which it belongs [15,16] (**Figure 4**).

Figure 4. General structure of integron-gene cassette system. The *intI* gene codes for a tyrosine recombinase (integrase) that catalyzes site-specific recombination between 59-base elements and the *attI* site, resulting in integration or excision of gene cassettes. Cassettes in the free circular form are transcriptionally silent.



Integrons are usually located on transposons, although chromosomal fixation can also occur, and they can also be found on conjugative plasmids, conferring horizontal dissemination. Integrons encoding MBLs often harbour other resistance genes, most frequently aminoglycoside resistance-genes, thus increasing the likelihood of dissemination of multiple resistances.

Aminoglycosides are a vital component of antipseudomonal chemotherapy implicated in the treatment of a variety of infections, particularly pulmonary infections in cystic fibrosis patients with aerosolized tobramycin. The first reports of aminoglycoside-resistant isolates are from the 1960s, and today are all too common and present in virtually all areas in the world, but particularly in Europe and Latin America [17]. Resistance typically results from drug inactivation by plasmid- or chromosome encoded enzymes, although enzyme-independent resistance from defect in uptake and accumulation also occur, particularly in CF patients and in intensive care units. Traditionally aminoglycoside inactivation in resistant strains involves their modification by enzymes that phosphorylate (aminoglycoside-phosphoryltransferase), acetylate (aminoglycoside-acetyltransferase), or adenylate (aminoglycoside-adenyltransferase) these antibicrobials. *P. aeruginosa* resistance to aminoglycosides owing to

enzymatic N-acetylation has been known for a long time. Acetylation of aminoglycosides can occur at the 1, 3, 6`, and 2`amino groups and involves all medically useful compounds. Inactivation of aminoglycosides such as kanamycin, neomycin, and streptomycin by a phosphotransferase, that modify the 3`-OH of these antimicrobials, is well known since the 1980s. The adenylation of streptomycin and gentamycin has been described first 20 years ago. While the specificity of aminoglycoside-modifying enzymes has in the past tended to compromise the use of only selected aminoglycosides, leaving the others still effective, increased the prevalence of strains harboring multiple aminoglycoside-modifying enzymes [17].

1.2.2.1. Problems with MBL-detection

The international epidemiology of MBL-producing *P. aeruginosa* is not quite clear. The prevalence of these enzymes is still unknown in most countries, due to the lack of proper screening recommendations. Several methodologies have been proposed for the routine detection of MBL-producing *P. aeruginosa*, including microdilution test, iodometric polyacrylamide gel method, particularly the MBL E-test and the MBL combined disc test [18-25], although there are concerns regarding the latter two method's reliability. All of these methods have to be available for routine diagnosis; most of these are phenotypic methods, based on the fact that the activity of MBLs can be experimentally inhibited by metal chelators, such as EDTA, due to the Zn^{2+} in the active site. Recently, in order to increase the reliability of these tests, some authors suggested the inclusion of ceftazidime-resistance beside meropenem- and/or imipenem-resistance to the MBL-screening criteria [19]. Nevertheless, in very specific circumstances such as the case of mucoid *P. aeruginosa* isolates, these methods are stated as inefficient. Relying on EDTA for MBL inactivation, these methods are prone to false-positive results since the chelating agent itself can increase membrane permeability, thus increasing the chance of a bactericidal effect. Given the significance of MBL-producing *P. aeruginosa* isolates, reliable and rapid detection of MBLs would be crucial for patient management and appropriate infection control procedures.

1.2.3 Changes in targets: mutational resistance

This mechanism of resistance results from mutational changes in target enzymes which result in maintenance of their vital role in cell metabolism, but resistance to the action of selective inhibition by antibiotics. In *P. aeruginosa* it is most commonly seen in the quinolones through mutation in the *gyrA* gene, encoding the A subunit of the target enzyme,

DNA gyrase [26]. Changes in the structure of the ribosome 30S subunit, which is the target of the aminoglycosides, influence streptomycin sensitivity, while alteration in the penicillin-binding proteins of *P. aeruginosa* could result resistance to β-lactams. Mutations leading to the increased production of the AmpC β-lactamase can occur at frequencies of 10^{-7}-10^{-9}, and may result decreased susceptibility to penicillins, cephems and monobactams [27] (**Table 1.**).

Table 1. A summary of the resistance mechanisms of *P. aeruginosa* against anti-pseudomonal agents.

Resistance mechanism		**Affected anti-pseudomonal agents**
Restricted uptake: porins, efflux pumps	OprD loss	Imipenem
	MexAB-OprM upregulation	Fluoroquinolones, tetracycline, chloramphenicol, carbenicillin, piperacillin, ceftazidime, cefepime, and aztreonam, meropenem
	MexCD-OprJ upregulation	Fluoroquinolones, piperacillin, cefepime, and meropenem
	MexEF-OprN upregulation	Fluoroquinolones, trimethoprim, and chloramphenicol
	MexXY-OprM upregulation	Fluoroquinolone, aminoglycoside, piperacillin, cefepime, and meropenem
Drug inactivation: enzymatic resistance	AmpC derepression	Penicillins, cephems, monobactams
	Narrow spectrum class A β-lactamases (PSE-1, PSE-4, TEM-1) and class D β-lactamases (OXA-3)	Penicillins, cefoperazone
	Extended spectrum class A β-lactamases (PER-1, VEB-1, GES-1, GES-2, TEM, SHV) and class D β-lactamases (OXA-11, OXA-14, OXA-18, OXA-28)	Penicillins, cephems, monobactams
	Metallo-β-lactamases class B (IMP, VIM, GIM, SPM)	All β-lactams except monobactams
	Aminoglycoside-acetyltransferase AAC(3)-I	Gentamicin
	Aminoglycoside-acetyltransferase AAC(3)-III, aminoglycoside-adenyltransferase ANT(2'')-I	Gentamicin, tobramycin
	Aminoglycoside-acetyltransferase AAC(3)-III, AAC(3)-VI, AAC(6')-II	Gentamicin, tobramycin, netilmicin
	Aminoglycoside-acetyltransferase AAC(6')-I	Tobramycin, netilmicin, amikacin
	Aminoglycoside-adenyltransferase ANT(4')-II	Tobramycin, amikacin
	Aminoglycoside-phosphoryltransferase APH(3')-VI	Amikacin
Changes in targets: mutational resistance	Membrane changes	Polymyxins, aminoglycosides
	Mutations in *gyrA* and/or *parC* genes	Fluoroquinolones

1.2.4. Multi-, and pandrug resistance of *P. aeruginosa* - clinical impact

As a consequence of all the above mentioned resistance mechanisms the repertoire of antimicrobial agents that can be used against *P. aeruginosa* infections is relatively limited. The most important antipseudomonal agents include some β-lactams, (ticarcillin, ureidopenicillins, piperacillin, cefoperazone, ceftazidime, cefepime, aztreonam, imipenem and meropenem), aminoglycosides (gentamicin, tobramycin, netilmicin and amikacin) and fluoroquinolones (of which ciprofloxacin remains the most active compound). Polymyxins (polymyxin B and polymyxin E - colistin) are also active, but due to their higher toxicity, are usually considered only for multi-, or pandrug resistant strains, although newer studies shows a better safety profile of polymyxins, than indicated in older reports [28]. The term multidrug resistant (MDR) *P. aeruginosa* is defined by most authors as being resistant to at least 3 antipseudomonal antibiotic-groups. The term pandrug-resistant *P. aeruginosa* has been introduced for isolates being resistant to all antipseudomonal agents, except the polymyxins. Concerning the β-lactam–β-lactamase inhibitor combinations, piperacillin–tazobactam is preferable to ticarcillin-clavulanate for the treatment of *P. aeruginosa* infections, because of the more favourable pharmacokinetics of tazobactam, the superior antipseudomonal activity of piperacillin, and the fact that unlike tazobactam, clavulanate usually induces the production of AmpC enzyme and could antagonize the antimicrobial effect of ticarcillin [27].

In vitro susceptibility data are essential for the selection of antimicrobial chemotherapy for *P. aeruginosa* infections, because of the frequency and variability of acquired resistance mechanisms of the clinical isolates. As *P. aeruginosa* infection could be lethal, empirical regimens adequate for *P. aeruginosa* coverage should always be initiated prior to the results of cultures and susceptibility testing when infections by this pathogen are suspected. For the empirical therapy several aspects should be considered, such as the nature and source of infection, pharmacokinetic parameters, underlying risk factors and diseases. Antibiotic monotherapy is usually recommended for non-complicated urinary tract infections, whereas combination therapy with at least two different anti-pseudomonal agents is normally recommended for the treatment of severe infections, such as nosocomial pneumonia, bacteraemia [27]. The preferred combination remains the combination of aminoglycosides with β-lactams. In cystic fibrosis patients, early aggressive combination therapy is currently recommended for initial colonization episodes to delay as long as possible the chronic *P. aeruginosa* infection. Once chronic infection is developed, maintenance chemotherapy based on the administration of antipseudomonal agents at regular intervals can significantly improve the survival of these patients [27].

Several factors indicate that the emergence and spread of drug-resistant *P. aeruginosa* can be related to the overuse of antimicrobial agents, although the risk differs for the different agents. A strong association between use and resistance has been documented for carbapenems. In a cohort study comparing the relative risks for emergence of resistant *P. aeruginosa* in patients treated with different anti-pseudomonal agents, imipenem was found to be associated with a significantly higher risk of emergence of resistance. On the other hand ceftazidime, piperacillin and ciprofloxacin had the lowest risk to develop resistance [27].

1.3. Determination of different genotypes of *P. aeruginosa* – bacterial typing methods

Bacterial typing has its own vocabulary, although the use of this terminology is not always consistent, and can be confusing sometimes. The terms "isolate" and "strain" is often used interchangeably, but not always appropriately. An **isolate** is a population of bacterial cells in pure culture derived from a single colony. In clinical microbiology, isolates are usually derived from the primary culture of a clinical specimen obtained from an individual patient. A **strain** is the descendant of a single isolate in pure culture, usually derived from a single initial colony on a solid growth medium. A strain may be considered an isolate or group of isolates that can be distinguished from other isolates of the same genus and species by phenotypic and genotypic characteristics. Cultures of a particular microorganism, isolated at the same time from multiple body sites of a patient, and indistinguishable by typing, also considered to be a single strain [29].

The ability to quickly and reliably differentiate among related bacterial isolates is essential for epidemiological surveillance systems. There are several typing methods used in laboratories today. These range from methods based on simple phenotypic features to DNA sequencing [29]. Previously, the comparison of phenotypic characters, such as colony morphology, color, odor, antibiogram-based typing, biotyping, serotyping and the ability of growing in the presence of specific substances, were used for differentiation. Today these methods are becoming obsolete, because they require strict standardization of experimental conditions, since phenotypes are quite susceptible to the environmental conditions. Instead, more reliable genotyping methods have been developed [29]. Genotypic assays are based on the comparison of genomic DNA differences in the composition of bacterial DNA (e.g., the presence or absence of plasmids), in the overall structure (e.g., restriction endonuclease profiles), or in the precise nucleotide sequences of one or more genes or intergenic regions.

1.3.1. Pulsed-field-gel-electrophoresis (PFGE)

A variety of molecular genetic methods have been used to type *P. aeruginosa* strains, each varying in their discriminatory potentials. Pulsed-field gel electrophoresis (PFGE) allows separation of large DNA fragments generated with restriction endonucleases in agarose gels, by periodic alteration of the angle of the electric field's direction. PFGE has a remarkable discriminatory power and reproducibility; therefore it is commonly used, and is the gold-standard method for the comparative typing of most bacterial species including *P. aeruginosa*. However, 2-4 days are required to obtain results, and relatively expensive PFGE equipment is needed. Gels need to be analyzed closely and carefully, even after digitalization and computerized processing [30,31]. When compared by PFGE, two isolates differing by one mutational event (single nucleotide substitution, insertions, and deletions) may differ by zero. When there are no observed band differences, the isolate should be termed as "indistinguishable" rather than "identical", and assigned to the same type. Subtypes will be assigned to isolates that differ by one to eight bands. Isolates differ by at least nine bands should be assigned as different types [29]. Tenover *et al*.[32] suggested that in case of health-care associated outbreaks, isolates differing by one to four bands should be assigned as "closely related", and therefore "probably part of the outbreak", while isolates differing five to eight bands are "possibly related", and therefore "possibly part of the outbreak".

1.3.2. Multilocus sequence typing (MLST)

In recombining populations it is essential to obtain information regarding isolate characterization from multiple chromosomal locations that are unlikely to be co-inherited in a single genetic event. Additionally, it is recommended to avoid parts of the genome that are evolving rapidly due to strong selection pressures, such as antibiotic use or immunological selection. The approach to use housekeeping genes was established for multi-locus enzyme electrophoresis (MLEE). Although MLEE played a central role in bacterial epidemiology, it is technically cumbersome and has not been adopted for routine surveillance. The multilocus sequence typing (MLST) was built on the success of MLEE targeting the variation present at multiple housekeeping loci, however in the case of MLST typing is achieved by nucleotide sequence determination of the gene fragments. In 2004, Curran and colleagues adopted the MLST scheme for *P. aeruginosa* which is based on the allelic differences in the following housekeeping genes: *acsA, nuoD, trpE, mutL, guaA, aroE, ppsA* [33]. The advantage of the method is mainly the interpretative, no cost software (e.g., eBURST), and the freely available database on the internet. This method is recently becoming more popular as a bacterial typing

method to compare clones spreading in different geographical areas [30,34,35]. The implications for population genetics and dynamics are more significant than those for bacterial epidemiology, since polymorphism in the slowly evolving genes, which are its targets, may not be high enough for useful epidemiological comparisons. Additionally, the genes in question are unlikely to have any direct relevance to virulence or drug resistance.

1.3.3. Repetitive-element-based PCR (rep-PCR) assays

Rep-PCR assays use primers targeting highly conserved, non-coding repetitive sequence elements in the bacterial genome, and is an established approach for subspecies classification and strain delineation [36,31,37]. Recently, rep-PCR has been adapted to an automated format by Healy and colleagues [38], known as DiversiLab system (BioMerieux) to provide a reliable PCR-based typing method for clinical laboratories. The standardized rep-PCR and quality-controlled reagents in a kit format, automated detection and analysis using micro-fluids for rapid detection, and digitized the corresponding information in a software package allows simplistic data archiving, retrieval, and reporting, producing the automated microbial DNA typing system [38].

The surveillance systems of nosocomial infections have to be rapid, reliable, and able to differentiate among related and unrelated bacterial isolates, especially in highly critic areas, such as Intensive Care Units (ICUs). ICUs are often the scene of sporadic appearance of multiresistant pathogens, such as *P. aeruginosa.* An outbreak can be defined as a temporal increase in the incidence of infection or colonization by a certain bacterial strain, caused by enhanced, patient-to-patient transmission [29]. Often the sporadic isolation of multiresistant strains does not draw the attention in time to determine the nosocomial spread of one clone. The collection of multiresistant, potential nosocomial pathogens for further typing may help to discover hidden reservoirs, and the circulation of outbreak clones in different wards. Only a few typing methods evaluate outbreaks in real time, provide widespread epidemiological data, and have data-archiving capability, all of which are required to build libraries and share data among laboratories.

1.4. Cystic fibrosis and the role of *P. aeruginosa* in this clinical entity

Cystic fibrosis (CF) is an autosomal recessive disorder that occurs approximately in 1:2000 Caucasian children [39], in Hungary the rate is 1:4000. The disease is also present in Hispanic and black population, but at a much lower rate. The highest incidence of CF patients

in the world, 1:1353, is found in Ireland [40]. Cystic fibrosis is caused by abnormal functioning of the CFTR protein, the Cystic Fibrosis Transmembrane Regulator, which is a cAMP induced chloride channel, and is expressed on the apic membrane of the cells. The gene encoding the CFTR protein is expressed mainly in epithelial cells in the airway, kidneys, pancreas, bile ducts, reproductive organs, and the bowel. The main function of CFTR protein is to transport chloride ions across the cell membrane. CFTR has two nucleotide-binding domains, both of them is capable to bind and hydrolyze ATP, which helps to open and close the channel. An alteration of the *cftr* gene causes the disease. The alteration in *cftr* gene, therefore the amino acid residues, can lead to a change in diameter size of the pore, causing loss in function of the channel. The mutations can occur in different areas of the *cftr* gene, with different consequences in the synthesis of the CFTR protein. Each mutation is associated with a different phenotype, and results in a different prognosis for the patient. In Europe, the most common mutation is the ΔF508, which refers to a deletion of a phenylalanin at position 508 in the protein.

The initial diagnosis of the disease is made via sweat test, since CF patients produce higher levels of sodium chloride in their sweat (>60 mmol/L). Many newborns are screened for CF if they present with meconium ileus. Due to the reduction in pancreatic enzymes the patient may have a reduced ability to absorb fats, which has an effect on the growth of the CF patients. If the screening sweat test is positive, additional confirmatory tests are carried out via molecular methods to detect the exact alteration of the *cftr* gene. The different mutations cause different symptoms. The pathophysiology of this disease is poorly understood, and symptoms range from gastrointestinal or nutritional abnormalities, reproductive problems as an adult, chronic sinusitis, endobronchial disease. In the lungs the loss of the CFTR protein function affects the volume and viscosity of the airway surface liquid, which cause poor mucociliary clearance. The high salt concentration of the CF lungs causes inactivation of natural antimicrobials, such as defensins. There is an ongoing debate whether the CF lung becomes first infected by bacteria followed by inflammation, or vice versa, but it is a fact that bacteria infect the respiratory tract usually early in the course of cystic fibrosis disease, often fail to be eradicated. The bacterial infections together with an aggressive host inflammatory response are the key players in the irreversible airway damage from which most patients die. These infections usually lead to chronic colonisation of the lung with exacerbations over time. There is a pattern of the colonisation of the CF lung, which usually involves *Staphylococcus aureus* in infancy, followed by *Haemophilus influenzae,* as a child, *P. aeruginosa, Stenotrophomonas maltophilia, Achromobacter xylosoxidans* in teenage years, *Burkholderia*

cepacia complex, non-tuberculous mycobacteria in early twenties, and several newer species, including moulds, are becoming more common [41].

Staphylococcus aureus. *S. aureus* is relatively common in early childhood, and is often the first bacterium infects the CF lung. There is ongoing debate about preventing the infection with antibiotic is useful. Small colony variants have been described and are commonly associated with persisting infections and co-infections with other pathogens, such as *P. aeruginosa*. Resistant strains, particularly MRSA (methicillin resistant *S. aureus*) cause more problems for CF patients and centres.

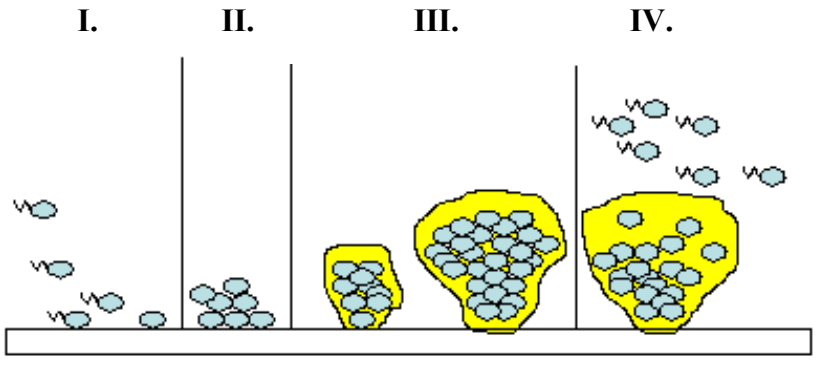
Pseudomonas aeruginosa. With the advent of effective anti-staphylococcal antimicrobial therapy, *P. aeruginosa* emerged as the most important bacterial pathogen in lung disease of CF patients, with a high prevalence in adults, up to 80%. *P. aeruginosa* possesses and develops a wide range of strategies by which it evades host defences and ensures the survival within the CF respiratory tract.

Bacteria use specific adhesins to bind to receptors on the cell surface. *P. aeruginosa* has several classes of adhesins, including pili and flagellae, which are also used for motility. The majority of *Pseudomonas* strains isolated from recently infected patients with CF are pilated [41], which supports the role of adherence at this stage. Once chronic infection has been developed, the pilin genes are down-regulated. The receptor for both pili and flagellae has been identified as a disaccharide, GalNAcβ1-4Gal. These receptors are more abundant on the surface of CF respiratory epithelial cells, which may be related to the abnormal function of sialyltransferase enzymes within CF cells, and that causes greater adherence of *P. aeruginosa* to CF cells, than to others. It has long been described, that adherence of the organism to this receptor led to an NFκB-mediated increase in expression of pro-inflammatory cytokines, such as interleukin-8. This adherence hypothesis explains the high prevalence of *P. aeruginosa* (and *S. aureus*, which also binds to this receptor) as opposed to other common respiratory organisms, such as *S. pneumoniae*, and explains the inflammatory response also [41].

After first infecting the host, *P. aeruginosa* has to survive host defences, and repeated courses of antibiotic treatment. The bacterium has a repertoire of immunoevasive strategies includes the secretion of exoproducts, antibiotic-resistance proteins, and phenotypic changes, which make them virtually unrecognizable from the bacteria, that was first isolated. Elastase and alkaline-protease protect against immune destruction by cleaving immunoglobulins,

complement components, and cytokins. Exotoxin A inhibits phagocytosis and supresses the cell-mediated immune response. The siderophores, such as pyocianin, break down intercellular tight junctions, slow the ciliary beat frequency, therefore affect mucociliary clearance. *P. aeruginosa* strains found in the CF lung are unusually hypermutable, they can react promptly to their environment, not only by switching genes on and off, but by an increased frequency of mutation events within the genome. One such mutation triggers conversion to a mucoid phenotype. Over-producing mucoid exopolysaccharide (alginate) surrounds and protect them from external affects, such as mucociliary clearance, immune response or antibiotics. Another highly successful survival strategy involves the formation of biofilms (**Figure 5**.). Many bacteria protect themselves in this way, on biological surfaces in diseases such as endocarditis and osteomyelitis, or on synthetic materials such as catheters, prosthetic valves, and on environmental structures such as rocks in rivers. In the CF lung *P. aeruginosa* and *Burkholderia cepacia* isolates exist in biofilms, which protects them from phagocytosis, and prevents penetration of antobiotics. This phenotypic change plays a major role in the persistence of pseudomonas infection in the majority of CF patients, despite best medical attempts at eradication [41].

Figure 5. Steps thought to be involved in biofilm formation. I.: Single planktonic bacterial cells are inhaled, and settle onto a surface, such as respiratory mucosa. II.: They multiply into microcolonies. III.: These microcolonies evolve into a mushroom-shape structure, and biofilm matrix is produced (in yellow). IV.: On occasion the biofilm matrix breaks, a shower of organism leave the biofilm, which might cause respiratory exacerbations.



Molecular epidemiology of *P. aeruginosa* revealed the species to have an epidemic population structure, where highly successful clones arise occasionally. One of the most

successful clones of *P. aeruginosa* is clone C, which had been found to spread throughout Europe in the aquatic environment and in clinical samples [42,43,44]. Clone C strains were found to be especially successful in infecting patients with cystic fibrosis. In a survey of CF population of the Medical school of Hannover, 30 % of the CF patients found to harbour clone C isolates [42], and it was also common in France [44], United Kingdom [46], Sweden [45], Canada [42], Belgium [45] and in Germany [44] as well. However, Vosahlikova et al. observed a high diversity among isolates from Czech CF patients [45], the prevalence of the cluster genotypes was significantly lower than that of the epidemic strains found in other centres, there were no indications of wide spread of a predominant epidemic strain. Similar results have been found in Vancouver, British Columbia and Porto Alegre (Brazil) [45].

The factors which determine the successful transmission and colonization of pathogens in environmental niches are mainly unknown as it is a complex interplay between virulence potential and environmental spread. Romling *et al*. [42] investigated several features of clone C *P. aeruginosa* isolates, which can determine the capability for spreading and causing infections, especially in CF patients. Neither enhanced biofilm formation, nor enhanced antibiotic resistance was observed within clone C isolates, and previous studies revealed, that they do not produce pyocianin, or bacteriocins either, so there is still no answer to that very complex and still remains a challenging question.

***Burkholderia cepacia* complex**. *Burkholderia cepacia* complex led to severe outbreaks in CF subjects worldwide during the 1980s, with substantial morbidity and mortality. Currently 3-4% of CF patients are infected with this organism, although the prevalence in certain centres are much higher. There are 17 species that is now referred to as the *Burkholderia cepacia* complex. Within cystic fibrosis *B. cenocepacia, B. multivorans, B. vietnamiensis* are responsible for the majority of the infections. Identifying the *Burkholderia cepacia* complex species can be difficult, since they are easily confused with other non-fermenting Gram-negative rods. Infections with *Burkholderia cepacia* complex is an independent negative prognostic indicator in CF. Patients could experience rapid respiratory decline, necrotising pneumonia, or develop the so called “cepacia syndrome”, in which organisms invade systemically, and cause endotoxic shock, multi-organ failure, and in many cases, death. What makes *Burkholderia cepacia* complex unique and dangerous for a CF patient are as follows: the high potential of transmissibility, antibiotic multi-resistance, host-defence survival strategies, producing biofilms and the associations with severe clinical outcome.

2. AIMS OF THE STUDY

The aims of this study were to investigate clinically relevant *P. aeruginosa* isolates, namely:

- to determine the different resistance mechanisms, especially those causing resistance to carbapenems, in multi- and pan-resistant isolates,
- to study the molecular relatedness between isolates, comparing different molecular typing methods,
- to investigate *P. aeruginosa* isolates infecting/colonising patients suffering from cystic fibrosis.

3. METHODS AND MATERIALS

3.1. Bacterial isolates

3.1.1. Multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

From January of 2004 until November 2008 altogether 4761 *P. aeruginosa* isolates were recovered from non-cystic-fibrosis patients, hospitalized in different hospital wards in South-Hungary (Szeged). 1200 (25.69 %) of them were isolated at an Intensive Care Unit. 2199 (47.07 %) were isolated from respiratory samples, 824 (17.64 %) from urogenital tract specimens, 328 (7.02 %) from blood cultures, 1107 (23.69 %) from wound cultures, 25 (0.5 %) from cerebrospinal fluids, 158 from other clinical samples. Of the 4671 clinical isolates 1502 (32 %) were imipenem and/or meropenem resistant, 364 (7 %) were multiresistant. After excluding isolates obtained from the same patients, 51 isolates were selected for this study for further analyses. The selection criteria were as follows: all 51 isolates were multidrug resistant, being non-susceptible to at least three different antipseudomonal antibiotic groups multidrug resistant, including carbapenems (imipenem and meropenem MIC $\geq$ 16 mg/L), and with one exception all exhibited ceftazidime resistance, which was used as a marker for potential MBL production, as suggested by Samuelsen et al [19]. The investigated isolates are listed in **Table 2**. Within this timeframe, three different outbreaks/case accumulations were detected; 5, 7 and 29 patients were included respectively. The isolates from the outbreak including the 29 patients were examined in the epidemiological analysis, listed in chapter 3.1.1.1.

Identification of the isolates by VITEK 2 system (bioMérieux) and susceptibility test by disk-diffusion method and E-test if required were performed, according to CLSI guidelines. After isolation, the strains were stored at -70 °C in CryoBank medium (Copan diagnostic Inc., California, USA) for further investigation. *P. aeruginosa* ATCC 27853 was used for quality control.

3.1.1.1. Isolates of the epidemiological analysis; environmental screening

During the period of 17 months from June 2007 to November 2008 multiresistant *P. aeruginosa* strains were isolated from 29 different patients during their stay in different wards including the medical ICU (MICU), general surgical ICU (SICU1), surgical ICU for trauma patients and neurosurgery (SICU2) and cardiac surgery ICU (SICU3) in the University

Hospital of Szeged, Hungary (isolates are listed in **Table 2**. marked with *). All the wards are situated in different buildings. Twenty-eight isolates had the same antibiotic-resistance profile (only susceptible to amikacin, netilmicin and polymixin B), while the remaining one isolate (54688) had slightly different profile (only susceptible to amikacin and polymixin B). These isolates have been tested during the epidemiological analysis.

Environmental sampling was carried out in the MICU in August 2008, in connection with the regular hygienic control of the ward. A total of 12 sites and the tap water were sampled from the patients' environment, including tubes of the mechanical ventilation system, blood gas machines, monitors, equipment trolley, sinks, computers of the clinicians, hands of nurses. The sites were sampled by swabs moistened by sterile saline solution. Sampling of the tap water was performed according to the Hungarian Government Decree 201/2001. (X. 25.).

Table 2. List of the examined multidrug resistant *P. aeruginosa* isolates, the date and site of isolation, and the results of antibiotic susceptibility testing.

Strain	Date of isolation	Hospital ward	Site of isolation	TZP	CFP	CAZ	FEP	IPM	MEM	CN	TOB	AK	NET	CIP	LEV	MXF	PB
82114	26.10.2004	UH	urine	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S
7025	24.01.2005	UH	urine	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S
72982	07.10.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S
78065	24.10.2005	MICU	tracheal asp.	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S
79577	02.11.2005	SICU2	nasoph.swab	**I**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**I**	**R**	**R**	**R**	S
15939	17.11.2006	SB	bile	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S
95490	14.12.2004	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	S
95149	12.12.2004	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	S	S	S	S
2740	11.01.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**I**	S	S	S	S
12064	11.02.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	S
9541	03.02.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S
23611	27.03.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	S	**R**	S	S	S	S
30831	25.04.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**I**	**R**	**R**	S	S	**R**	S
83712	16.11.2006	SICU2	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	**R**	**R**	**R**	S
49010	28.06.2005	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S
57006	02.08.2005	MICU	blood culture	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S
64007	27.11.2007	MICU	tracheal asp.	**R**	**R**	**I**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
7238	26.01.2005	PS	urine	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S	S	S	S	S
8768	01.02.2005	PN	urine	S	**I**	**I**	**R**	**R**	**R**	**R**	S	**R**	**R**	S	S	S	S
22495	22.03.2005	PICU	tracheal asp.	S	**I**	**I**	**I**	**R**	**R**	**R**	**I**	**R**	**R**	S	S	**R**	S
8107	30.01.2006	PICU	nasoph. swab	S	**I**	S	**I**	**R**	**R**	**R**	S	**R**	S	S	S	**R**	S
44744	20.06.2006	PICU	tracheal asp.	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	S	S

Antibiotics: TZP: piperacillin-tazobactam, CFP: cefoperazon, CAZ: ceftazidime, FEP: cefepime, IPM: imipenem, MEM: meropenem, CN: gentamycin, TOB: tobramycin, AK: amikacin, CIP: ciprofloxacin, LEV: levofloxacin, MXF: moxifloxcain, PB: polimyxin B.
Hospital wards: MICU: medical ICU; SICU1: general surgical ICU; SICU2: trauma and neurosurgical ICU; SICU3: cardiac sugical ICU; NS: neurosurgical ward; NE: neurology ward; IM: internal medicine ward, PS: pediatric surgery; PN: pediatric nephrology; PICU: pediatric ICU, SB: surgery; UH: urology. Isolates marked with * are selected for the epidemiological analysis (chapter 3.1.1.1)

Table 2. cont'd

Strain	Date of isolation	Hospital ward	Site of isolation	TZP	CFP	CAZ	FEP	IPM	MEM	CN	TOB	AK	NET	CIP	LEV	MXF	PB
32174*	13.06.2007	MICU	tracheal asp.	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
38979*	20.07.2007	SICU3	tracheal asp.	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
64152*	27.11.2007	MICU	tracheal asp.	**R**	**I**	**I**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
56*	02.01.2008	MICU	blood culture	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
1321*	08.01.2008	MICU	blood culture	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
45611*	09.07.2008	SICU2	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
47671*	17.07.2008	SICU1	wound	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
48968*	24.07.2008	SICU2	tracheal asp.	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
48974*	23.07.2008	SICU2	tracheal asp.	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
48964*	24.07.2008	SICU2	nasoph.swab	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
50626*	28.07.2008	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
52432*	04.08.2008	MICU	tracheal asp.	**R**	**R**	**R**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
51356*	05.08.2008	SICU2	MiniBal	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
53818*	12.08.2008	SICU2	nasoph. swab	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
54688*	15.08.2008	SICU2	nasoph. swab	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	**R**	**R**	**R**	**R**	S
57258*	27.08.2008	SICU3	nasoph. swab	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
57866*	29.08.2008	MICU	tracheal asp.	**R**	**R**	**I**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
59371*	04.09.2008	NE	tracheal asp.	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
59985*	05.09.2008	SICU1	blood culture	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
63272*	18.09.2008	SICU1	tracheal asp.	**R**	**I**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
66401*	28.09.2008	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
67609*	07.10.2008	MICU	tracheal asp.	**R**	**I**	**I**	**I**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
68241*	07.10.2008	SICU1	tracheal asp.	**R**	**R**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
70436*	11.10.2008	MICU	tracheal asp.	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
71228*	15.10.2008	SICU2	nasoph. swab	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
71032*	17.10.2008	SICU1	drain amylase	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
73127*	19.10.2008	MICU	tracheal asp.	**R**	**I**	**I**	**R**	**R**	**R**	**R**	**R**	S	S	**I**	**R**	**R**	S
73558*	22.10.2008	IM	urine	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S
73608*	22.10.2008	SICU2	nasoph. swab	**R**	**R**	**R**	**R**	**R**	**R**	**R**	**R**	S	S	**R**	**R**	**R**	S

3.1.2. *P. aeruginosa* isolates recovered from cystic fibrosis patients

From 2003 to 2007 149 *P. aeruginosa* were isolated from 16 patients suffering from cystic fibrosis, attending the CF centre in the Children's Hospital of Szeged, Hungary. In this period of time all isolates from the 16 patients were stored for further analysis, regardless their antibiotic susceptibility. 78 isolates (52%) had mucoid colony morphology. Identification of the isolates by VITEK 2 system (bioMérieux) and susceptibility test by disk-diffusion method and E-test if required were performed, according to CLSI guidelines. After isolation, the strains were stored at -70 °C in CryoBank medium (Copan diagnostic Inc., California, USA) for further investigation.

To verify whether strains found in different patients may have been transmitted from patient-to patient, we tracked possible contacts among the different patients with the MedSol patients' data system used in the hospital, and by contacting the patient's clinicians.

3.2. Determination of different antibiotic-resistance mechanisms

3.2.1. Resistance mechanisms in multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

3.2.1.1. Detection of ESBLs

3.2.1.1.1. Phenotypical methods

Conventional double-disk synergy test (DDST) was performed to detect ESBLs in a representative *P. aeruginosa* strain from each genotype using amoxicillin-clavulanate, expanded spectrum cephalosporins (cefotaxie, ceftazidime), aztreonam and cefepime. Modifications of DDST were applied according to Jiang *et. al* [50], namely shorter distance (20 mm instead of 30 mm) between the discs, and 250 μg/ml cloxacillin and Phe-Arg β-naphthylamide dihydrochloride (MC-207,110) was applied into the Mueller-Hinton agar. Using cloxacillin, that inhibits the activities of the AmpC enzyme, and MC-207,110 that inhibits the efflux pumps produced by *P. aeruginosa* increases the specificity of this test.

3.2.1.1.2. Genotypical methods

The genes of the extended spectrum β-lactamases (PER-1 [51], PER-2, TEM, SHV, GES, VEB-1, OXA groups [11] were sought by PCR methods in a representative *P.*

aeruginosa strain from each genotype (the primers and PCR conditions used are listed in **Table 4.)**, confirmed by sequencing and restriction-fragment-length-polymorphism (RFLP) assay was performed on PCR products, as described previously [11]. To investigate the location of the PER-1 β-lactamase gene plasmid purification (QIAGEN Plasmid Midi Prep, QIAGEN Inc), transformation assay using Stratagene XL-10 Gold Ultracomponent cells (Agilent Technologies, USA) were used according to the manufacturer's instruction. PCR detection of the Tn1213 specific IS element were performed with the primers and conditions used by Poirel *et al* [51].

3.2.1.2. Detection of metallo-lactamases

3.2.1.2.1. Phenotypical methods

In contrast to serine β-lactamases, MBLs can be experimentally inhibited with metal chelators, such as EDTA or 2-mercapto-propionic acid. Based on this phenomenon several methodologies have been proposed for the routine detection of MBL-producing *Pseudomonas aeruginosa*, particularly the MBL E-test [18,20] and the MBL combined disc test [19,47]. The MBL E-test (AB BIODISK, Solna, Sweden) consists of a plastic strip containing an imipenem gradient in one end, and imipenem+EDTA in the other end. The MBL E-test is considered positive in case of a reduction of the imipenem MIC by ≥3 twofold dilution in the presence of EDTA. MBL-combined disc-test (using 750 μg EDTA) on Mueller–Hinton agar media were performed, as described by Yong et. al [47]. MDL combined disc test was considered positive if the zone diameter difference between imipenem+EDTA and imipenem discs was larger than 7 mm.

Bioassay and carbapenem-hydrolysis assays with cell-free extracts were performed in a representative *P. aeruginosa* strain from each genotype, in order to detect carbapenemase enzyme activity as described previously [25,48].

3.2.1.2.2. Genotypical methods

MBL presence or absence was confirmed by PCR in a representative *P. aeruginosa* strain from each genotype, aimed the $bla_{\text{SPM-1}}$, $bla_{\text{GIM-1}}$, and the most common bla_{VIM} and bla_{IMP} genes [49]. PCR master mix was prepared according to the standard protocol: 1 x PCR buffer, $MgCl_2$ 2,5 mM, dNTP 200 μM, primers 1-1 μM, Taq polymerase 1 U/reaction, sterile water. The primers and PCR conditions used are listed in **Table 3.** Strain HMV-2, a $bla_{\text{VIM-2}}$

producer, kindly provided by Prof. Luis Martinez-Martinez, was used as positive control for VIM detection.

3.2.1.3. Detection of integrons

Acquired metallo-β-lactamases (MBLs) and some other antiobiotic resistance markers (especially aminoglycoside-resistance genes) are mostly encoded by integron-borne genes. Therefore class 1 integrons were sought, using PCR (primers that were used are listed in **Table 3.**), characterized by sequencing, and restriction fragment-length polymorphism (RFLP) using 10 µl integron-PCR product, 2 µl *Alu*I restriction enzyme, 1,5 µl NEB2 buffer, 1,5 µl water, incubated overnight at 37°C, and examined with 2% gel-electrophoresis run at 70V for 2hrs.

3.2.1.4. Efflux pump, porin channel examinations

The expression of the chromosomal genes encoding the *opr*D, *mex*B, *mex*X, *mex*C was studied in a representative isolate from each genotype with real-time reverse transcriptase PCR assays according to Quale et al. [52]. RNA was isolated using the method by Palagyi-Meszaros *et al* [53]. Briefly, the isolate was grown in 60 mL of liquid medium in a hypovial to A600 nm = 1–1.5; 15 mL of culture was centrifuged at 15 000 g for 2 min, the pellet was suspended in 300 µL of SET buffer [20% sucrose, 50 mm EDTA (pH 8.0) and 50 mm Tris ⁄ HCl (pH 8.0)] and 300 µL of SDS buffer was added [20% SDS, 1% (NH4)2SO4, pH 4.8]; 500 µL of saturated NaCl was added next, the sample was centrifuged at 20 000 g for 10 min and the clear supernatant was transferred into a new tube. 2-Propanol (70% of the total volume of the supernatant) was added to the solution and the mixture was centrifuged at 20 000 g for 20 min. The pellet was washed twice with 1 mL of 70% ethanol. The dried pellet was suspended in 20 µL of diethylpyrocarbonate-treated water.

The expression of mRNA for the genes of interest was optimised to that of the housekeeping gene *rps*L. This gene is known to be expressed consistently in *P. aeruginosa* [54]. Normalized expression of each gene was calibrated to the mRNA expression of the reference strain *P. aeruginosa* PAO1, results are given as the relative expression of the mRNA compared to that of *P. aeruginosa* PAO1. The following values were considered to represent overexpression compared to the control strain: for *mex*B ≥3-fold; for *mex*X≥10-fold; *mex*C≥2-fold; and reduced expression of *opr*D≤0,7-fold [52].

3.2.2. Detection of different antibiotic-resistance mechanisms in *P. aeruginosa* isolates recovered from cystic fibrosis patients

From the 149 isolates 26 were resistant to at least one carbapenem. These imipenem and/or meropenem resistant isolates were selected for the following tests: MBL detection using the PCR method as described in section 3.3.1.2**.** PCR detection of class 1 integrons as described in section 3.3.1.3. (primers and PCR conditions are listed in **Table 3**).

From the 149 isolates 5 were resistant to ceftazidime and to cefepime. ESBL genes were searched from these isolates as described in section 3.3.1.1. (primers and PCR conditions are listed in **Table 4**).

Seven isolates were selected for RT-PCR analysis as described in section 3.3.1.4., examining efflux pumps and porins based on the following criteria:

- isolates resistant only to imipenem were tested for OprD deficiency;
- isolates resistant to imipenem and/or meropenem and cefepime, but not to ceftazidime were tested for OprD deficiency and for overexpression of MexXY-OprM;
- isolates resistant imipenem and/or meropenem, cefepime and to ceftazidime were tested for OprD deficiency and for overexpression of MexXY-OprM, MexAB-OprM, MexCD-OprJ.

Table 3. Primers used for the detection of the MBL genes, and searching for Class I integrons.

Primer	Sequence of primer	Size of product	Reaction conditions	Homology with genes:
VIM B VIM F	5' ATG GTG TTT GGT CGC ATA TC 3' 5' TGG GCC ATT CAG CCA GAT C 3'	261 bp	94°C 2´, (94°C 1´, 51°C 1´, 72°C 3´) 35x, 72°C 7´	VIM 2,18,1,3,6,13,4,11,14,10,9,12 MBL
VIP 1 VIP 2	5' ACT CAC CCC CAT GGA GTT TT 3' 5' ACG ACT GAG CGA TTT GTG TG 3'	800 bp	Multiplex PCR: 94°C 2´	VIM 2,18,3,6,11,14,8,10,9,12 MBL
IMP-F IMP-R	5' CTA CCG CAG AGT CTT TG 3' 5' AAC CAG TTT TGC CTT ACC AT 3'	600 bp	(94°C 2´, 55°C 1´, 72°C 3´) 35x 72°C 7´	IMP 6,10,4,1,7,5,25 MBL
VIM-DIA F VIM-DIA R	5' CAG ATT GCC GAT GGT GTT TGG 3' 5' AGG TGG GCC ATT CAG CCA GA 3'	523 bp	Multiplex PCR: 96°C 2´	VIM 2,18,1,3,6,11,14,8,10,9 MBL
IMP-DIA F IMP-DIA R	5' GGA ATA GAG TGG CTT AAT TCT C 3' 5' GTG ATG CGT CYC CAA YTT CAC T 3'	361 bp	(94°C 1´, 52°C 1´, 72°C 1´) 30x 72°C 10´	IMP 6,10,1,4,8,25,13,9,22,24,8,9,18,5,7,21,19,20,15,14,11
GIM R GIM F	5' ACT CAT GAC TCC TCA CGA GG 3' 5' AGA ACC TTG ACC GAA CGC AG 3'	753 bp	94°C 5´, (94°C 20´´, 57°C 45´´, 72°C 30´´) 35x 72°C 6´	German imipenemase
SPM F SPM R	5'-CCTACAATCTAACGGCGACC-3' 5'-TCGCCGTGTCCAGGTATAAC-3'	629 bp	94°C 10´, (94°C 1´, 55°C 1´, 72°C 1´) 38x 72°C 10´	São-Paolo metallo beta lactamase
Integron 5'CS 3'CS	5' GGC ATC CAA GCA GCA AG 3' 5' AAT GCG GAT GTT GCG ATT AC 3'	variable	94°C 6´, (94°C 1´, 53°C 1´, 72°C 1´) 35x 72°C 16´	class 1 integron
Int 1 F Int 1 R	5' GCC ACT GCG CGG TTA CCA CC 3' 5' GGC CGA GCA GAT CCT GCA CG 3'	898 bp	Multiplex PCR:	Integrase 1
sul 1 F sul 1 R	5' CGG CGT GGG CTA CCT GAA CG 3' 5' GCC GAT CGC GTG AAG TTC CG 3'	433 bp	94°C 5´, (94°C 30´´, 69°C 30´´, 72°C 1´) 30x	sulfonamide resistance gene 1
sul 2 R sul 2 F	5' GCG TTT GAT ACC GGC ACC CGT 3' 5' GCG CTC AAG GCA GAT GGC ATT 3'	293 bp	72°C 8´	sulfonamide resistance gene 2

Table 4. Primers used for the detection of the different ESBL genes in *P. aeruginosa*.

Primer	Sequence of primer	Size of product	Reaction conditions	Homology with genes:
PER-1-F PER-1-R	5'ATG AAT GTC ATT ATA AAA GC 3' 5'AAT TTG GGC TTA GGG CAG AA 3'	933 bp	94 °C 10´, (94 °C 1´, 50 °C 1´, 72 °C 1´) 32x 72 °C 7´	PER-1 ESBL
PER-2 F PER-2 R	5'TGT GTT TTC ACC GCT TCT GCT CTG 3' 5'CAG CTC AAA CTG ATA AGC CGC TTG 3'	~900 bp	94 °C 10´, (94 °C 1´, 56 °C 1´, 72 °C 1´) 32x 72 °C 7´	PER-2 ESBL
VEB-1 F VEB-1 R	5'CGA CTT CCA TTT CCC GAT GC 3' 5'GGA CTC TGC AAC AAA TAC GC 3'	642 bp	94 °C 10´, (94 °C 1´, 56 °C 1´, 72 °C 1´) 32x 72 °C 7´	VEB -1 ESBL
GES-1 F GES-1 R	5'ATG CGC TTC ATT CAC GCA C 3' 5'CTA TTT GTC CGT GCT CAG G 3'	~800 bp	94 °C 10´, (94 °C 1´, 56 °C 1´, 72 °C 1´) 32x 72 °C 7´	GES ESBL
TEM-1 F TEM-1 R	5'ATG AGT ATT CAA CAT TTC CG 3' 5'CTG ACA GTT ACC AAT GCT TA 3'	867 bp	94 °C 12´, (94 °C 1´, 58 °C 1´, 72 °C 1´) 35x 72 °C 10´	TEM ESBL
OXA III F OXA III R	5'TTT TCT GTT GTT TGG GTT TT 3' 5'TTT CTT GGC TTT TAT GCT TG 3'	427 bp	96 °C 5´, (96 °C 30´´, 55 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA III group (OXA 1, 4, 30, 31) ESBL
OXA I F OXA I R	5' TCA ACA AAT CGC CAG AGA AG 3' 5' TCC CAC ACC AGA AAA ACC AG 3'	276 bp	96 °C 5´, (96 °C 30´´, 53 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA I group (OXA 5,7,10,11,14,16,17,13,19,28,47) ESBL
OXA II F OXA II R	5' AAG AAA CGC TAC TCG CCT GC 3' 5' CCA CTC AAC CCA TCC TAC CC 3'	427 bp	96 °C 5´, (96 °C 30´´, 58 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA II group (OXA 1,4,30,31) ESBL
OXA-5 F OXA-5 R	5' AGC CGC ATA TTT AGT TCT AG 3' 5' ACC TCA GTT CCT TTC TCT AC 3'	664 bp	96 °C 5´, (96 °C 30´´, 53 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA-5 ESBL
OXA-18 F OXA-18 R	5' CGA TTA CGG CAA CAA GGA 3' 5' TTA GGC GGG CGA AGA CGA 3'	322 bp	96 °C 5´, (96 °C 30´´, 53 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA-18 ESBL
OXA-20 F OXA-20 R	5' AGA GCG GTG ACT ACT GGA TA 3' 5' AAA GCA TTG ACG GAT TGA AG 3'	308 bp	96 °C 5´, (96 °C 30´´, 53 °C 30´´, 72 °C 1´) 30x 72 °C 5´	OXA-20 ESBL
SHV 1 SHV 2	5'GGG TTA TTC TTA TTT GTC GC 3' 5'TTA GCG TTG CCA GTG CTC 3'	~900 bp	96 °C 10´, (94 °C 1´, 56 °C 1´, 72 °C 1´) 30x 72 °C 10´	SHV ESBL

3.3. Molecular typing methods

3.3.1. Molecular typing of multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

3.3.1.1. Pulsed-field gel-electrophoresis

PFGE analysis was performed as described previously by Deplano *et al*. with a slight modification [55]. All 51 multidrug resistant clinical isolates were digested with *Spe*I, and the fragments were separated by electrophoresis in 1% agarose gels with a CHEF- DR III System (Bio-Rad Laboratories) for 12-12 h, with the switch times ranging from 4 s to 12 s, and from 15 s to 36 s in a Tris-borate-EDTA buffer containing 50 µM thiourea. Gels for PFGE were stained in an aqueous solution containing 0.5 µg ethidium-bromide and photographed under UV translumination. Patterns of PFGE were visually analysed according to the criteria described previously [29,32].

3.3.1.2. Repetitive-element-based PCR, DiversiLab

The 51 multi-resistant *P. aeruginosa* isolates were fingerprinted using the automated, rep-PCR based DiversiLab system (bioMériux), version 3.3. DNA was extracted from the bacteria using the Mo Bio Ultra Clean^{TM} Microbial DNA Isolation Kit (MoBio Laboratories Inc) kit according to the manufacturer's instructions. Fingerprinting of the isolates was performed with the Pseudomonas kit (DiversiLab) following the instructions of the rep-PCR worksheet. After the amplification, the amplicons were loaded on a microfluids chip and detected, using the Agilent 2100 Bioanalyzer. The results were sent via internet to the hospital's secured DiversiLab website. The analysis of the data was performed using the DiversiLab software using the Pearson correlation coefficient. Reports were automatically generated, included dendograms, virtual gel images, similarity matrix, scatter plots and selectable demographic fields.

3.3.1.3. Repetitive-element-based (ERIC) PCR assay

ERIC (enterobacterial repetitive intergenic consensus) PCR was performed on a selection of multi-drug resistant isolates to confirm the PFGE and/or DiversiLab results. The reactions were performed as described by Syrmis *et al.* [58]. The results were analyzed visually.

3.3.1.4. Multi-locus sequence typing

Four multidrug resistant isolates (82114, 49010, 56, 95149) were selected for MLST analysis based on the antibiotic-resistance profile and the typing results obtained with PFGE and DiversiLab. The clonal relatedness at global level of the above multidrug-resistant clinical isolates was investigated as described previously by Curran *et al* [33]. Briefly, the genomic DNA was purified using the Mo Bio Ultra Clean™ Microbial DNA Isolation Kit according to the manufacturer's instructions. DNA amplification and sequencing of the seven housekeeping genes (*acsA, nuoD, trpE, mutL, guaA, aroE, ppsA*) were performed. The nucleotide sequences were determined by using previously published primers [30,33] and were compared to existing sequences in the MLST database (www.pubmlst.org/paeruginosa). The eBURST algorithm (http://eburst.mlst.net/) was used for phylogenetic analysis [56,57].

3.3.2. Molecular typing of *P. aeruginosa* isolates recovered from cystic fibrosis patients

For bacterial typing with PFGE and DiversiLab methods 44 isolates were selected from the 149 isolates, based on different antibiotic susceptibility profile. Six isolates were randomly selected from different patients for MLST analysis. The methods were used for bacterial typing are detailed in section 3.2.1.

4. RESULTS

4.1. Antibiotic resistance

4.1.1. Anibiotic resistance in multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

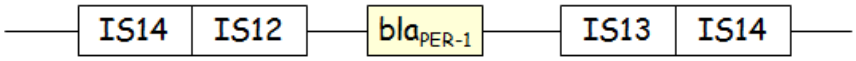
From 2004 to 2008 altogether 51 multidrug resistant *P. aeruginosa* have been selected for further analyses from different, non-cystic fibrosis patients. All isolates were resistant to imipenem and meropenem, and additionally at least for two more antipseudomonal antibiotic groups. The rate of susceptibility to the different antipseudomonal antibiotics were as follows: piperacillin/tazobactam: 25 %, cefoperazone: 0 %, ceftazidime: 8,3 %, cefepime: 0 %, imipenem: 0 %, meropenem: 0%, gentamycin: 16 %, tobramycin: 25 %, amikacin: 41,6 %, netilmicin: 41,6 %, ciprofloxacin: 50 %, levofloxacin: 50 %, moxifloxacin: 33,3 %, polymyxin B: 100 %. Isolates 82114, 15939 and 49010 were pandrug-resistant, being susceptible only for polymyxin B. Molecular typing methods revealed 12 different genotypes; a representative strain from each type has been selected for further testing, such as ESBL detection, MBL detection, efflux-pump analyses.

4.1.1.1. ESBL detection

With phenotypical ESBL-detecting methods positive results was observed in case of isolate 49010. This strain was obtained from a 20 year old poly-traumatised patient transported from Temesvár, Romania, where she had been treated for two months after a car accident, into our Surgical ICU. The microbiological results of the endotracheal aspirate, wound sample, blood culture and urine sample were positive for this extremely-drug resistant *P. aeruginosa* isolate [**article II**]. PCR experiments revealed the presence of $bla_{\text{PER-1}}$, $bla_{\text{OXA-I, II group}}$ –type genes in this isolate. Sequencing of the coding region identified the genes of PER-1 and OXA-74 ESBL enzymes, the latter corresponds to the $bla_{\text{OXA-I group}}$. The product of $bla_{\text{OXA-II group}}$ PCR was investigated with RFLP analysis [11], which revealed $bla_{\text{OXA-2}}$ gene. To investigate the location of the PER-1 β-lactamase gene plasmid purification, transformation assay were used. The pan-resitant strain did not appear to harbour plasmids, and failed to transfer ceftazidime resistance marker by conjugation to other bacterial hosts, suggesting that the $bla_{\text{PER-1}}$ gene was chromosomally located. In order to learn more about the genetic environment of the $bla_{\text{PER-1}}$ gene, IS elements characteristic to the composite transposon Tn1213, were searched for and found with PCR. The Tn1213 was first described

from *P. aeruginosa* by Poirel *et al.* in 2005 [51], containing the IS*Pa14* and IS*Pa12* IS elements upstream from the bla_{PER-1} gene, and the IS*Pa13*, IS*Pa14* and a glutathione-S-transferase gene (*gst*) downstream from the bla_{PER-1} gene (**Figure 6**).

Figure 6. The location of bla_{PER-1} gene on Tn1213 transposon.



4.1.1.2. MBL detection

The selected isolates were all resistant to both imipenem and meropenem (MIC ≥ 16 mg/L), and with one exception, additionally exhibited ceftazidime resistance/reduced susceptibility, which was used as a marker for potential MBL production [19]. MBL detection results, both by MBL-E-test and MBL combined disc test, were negative for two isolates (49010, 8107), the remaining isolates fulfilled the necessary criteria for identification as a MBL producing strain (a reduction of imipenem MIC by ≥3 twofold dilution in the presence of EDTA for the E-test, and a zone diameter difference between imipenem+EDTA and imipenem discs larger than 7 mm for the imipenem-EDTA disc method) (**Table 5**.) In contrast, the bioassay with cell-free extracts as well as the carbapenem-hydrolysis assay with spectrophotometric analysis produced negative results. This pattern was later confirmed by PCR, since no MBL genes were detected.

Table 5. Comparison of MBL detection methods in different carbapenem-resistant *P. aeruginosa* strains.

Strains	E-test (mg/L)		Imipenem-EDTA disk-method (mm)			
	IPM	IPM+EDTA	IPM	IPM+EDTA	[(IPM+EDTA)-IPM]	EDTA
82114*	32	1.5	6	17	11	11
56*	32	1.5	10	20	10	17
95149*	32	1.5	10	25	15	12
49010	16	12	10	11	1	10
57006	32	1.5	9	21	12	10
64007	NT	NT	9	20	7	13
7238	24	3	10	17	7	12
8768	16	1.5	12	19	7	10
22495	32	1.5	10	26	16	14
8107	16	3	12	18	6	12
44744	32	1.5	6	18	12	10

IPM: imipenem; EDTA: ethylenediaminetetraacetic; NT: not tested, *: representative strains from three different outbreaks/case accumulations

4.1.1.3. Detection of integrons

PCR detection of class 1 integron-specific elements (*Int*1, *sul*1, *sul*2 genes) were performed on a representative isolates from each genotype. The PCR reactions revealed four presumably different integron-types within these strains (**Table 6**). The integrons that had the same size but were isolated from different genotypes, were examined with RFLP, and proved to be the same integron-type.

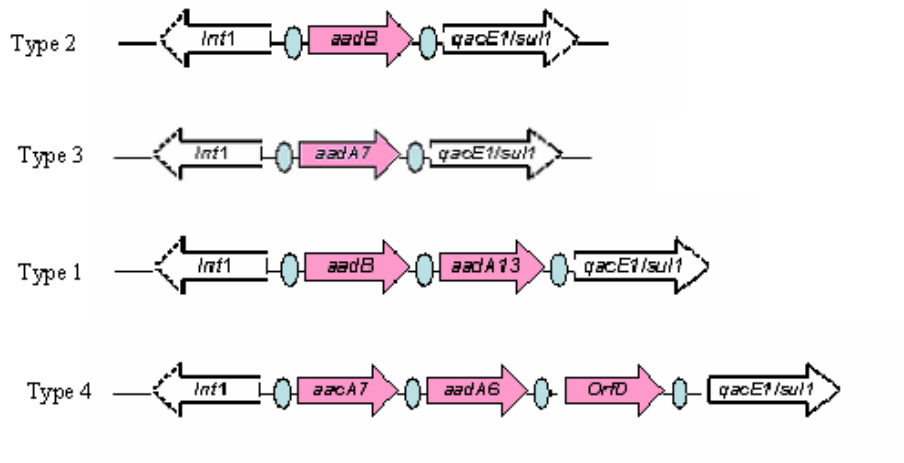
Table 6. The results of integron-isolation, the size of the integrons, and the results of the RFLP tests

Strains	Strain genotype	Integron	Integron RFLP type
82114*	I	1535 bp	Type 1
56*	II	689 bp	Type 2
95149*	III	-	-
49010	IV	-	-
57006	V	1858 bp	Type 4
64007	VI	689 bp	Type 2
7238	VII	-	-
8768	VIII	-	-
22495	IX	916 bp	Type 3
8107	X	-	-
44744	XI	-	-

Isolates marked with *: representative isolates from the outbreaks/case accumualtion

In order to detect the different antibiotic-resistance genes located on the integrons, nucleotide sequencing was performed, and resulted different aminoglycoside-resistance genes (**Figure 7**).

Figure 7. The structure of the four different class 1 integrons isolated from multidrug resistant *P. aeruginosa*. The following aminoglycoside-resistance genes were encoded: ***aad*B**: aminoglycoside-2''-O-adenyltransferase, confers resistance to gentamycin, tobramycin, kanamycin; ***aad*A7**: aminoglycoside-3'-adenyltransferase, confers resistance to streptomycin, spectinomycin; ***aad*A13**: aminoglycoside (3'')-O-adenyltransferase, confer resistance to streptomycin, spectinomycin; ***aac*A7**: aminoglycoside 6'-acetyltransferase, confers resistance to amikacin, isepamicin; ***aad*A6**: aminoglycoside-adenyltransferase, confers high level resistance to streptomycin, spectinomycin.



4.1.1.4. Efflux pump, porin channel examinations

A representative isolate from each genotype was examined for the expression-level of the following efflux-pumps and porins: MexAB-OprM, MexXY-OprM, MexCD-OprJ and OprD. The expression of mRNA of the genes of interest was detected by RT-PCR assays, the results are shown in **Table 7**. In case of two strains (64007, 44744) despite of repeating the RT-PCR tests, inconclusive results were seen. Significant increase in the expression of MexAB-OprM genes was only seen in the case of a pandrug-resistant isolate (49010). Upregulation of the MexXY-OprM genes occurred in 4 strains (82114, 56, 49010, 22495), there was a particularly high, 128 fold increase in case of the above mentioned pandrug-resistant isolate (49010). In case of the isolate 57006, the increase was 9,17 fold, close to the 10 fold level, which is considered to be positive. Overexpression of the MexCD-OprJ genes was detected in case of two isolates (95149, 22495). With the exception of two (49010, 8107), all the multidrug-resistant strains down-regulated the expression of the OprD gene, in five cases the decrease-level were less than 0,1 fold compared to the PAO1 control strain.

Table 7. Relative gene-expression values of the multidrug-resistant *P. aeruginosa* isolates, compared to *P. aeruginosa* PAO1 and the expression level of the *rps*L housekeeping gene.

Strains	Strain genotype	MexAB-OprM	MexXY-OprM	MexCD-OprJ	OprD
82114*	I	0,36 x	**16x**	0,07 x	**0,03 x**
56*	II	0,44 x	**10,68 x**	0,31 x	**0,01 x**
95149*	III	0,25 x	0,19 x	**3,23 x**	**0,34 x**
49010	IV	**6,68 x**	**128 x**	0,4 x	0,75 x
57006	V	0,09 x	9,17 x	0 x	**0,03 x**
64007	VI	IR	IR	IR	IR
7238	VII	0,51 x	0,4 x	0,16 x	**0,02 x**
8768	VIII	0,33 x	0,49 x	0,14 x	**0,15 x**
22495	IX	0,74 x	**29,24 x**	**2,51 x**	**0,09 x**
8107	X	0,87 x	1,13 x	0,85 x	3,29 x
44744	XI	IR	IR	IR	IR

Isolates marked with * represents the isolates from the outbreaks/case accumulations, IR: inconclusive results. The positive results are written in red.

4.1.2. Antibiotic resistance in *P. aeruginosa* isolates recovered from cystic fibrosis patients

From 2003 to 2007 149 *P. aeruginosa* were collected from 16 different patients suffering from cystic fibrosis, attending the CF centre in Szeged. The percentage of the isolates susceptible to the tested agents were as follows: piperacillin/tazobactam: 100 %, cefoperazone: 87,5 %, ceftazidime: 93,75 %, cefepime: 68,8 %, imipenem: 81,3 %, meropenem: 87,5 %, gentamycin: 37,5 %, tobramycin: 68,8 %, amikacin: 38,8 %, netilmicin: 62,5 %, ciprofloxacin: 93,75 %, levofloxacin: 68,8 %, moxifloxacin: 43,8 %, polymixin B: 100%. The highest resistance was seen in case of gentamycin, amikacin and moxifloxacin.

Of the 149 isolates 26 were resistant to at least one carbapenem. These imipenem and/or meropenem resistant isolates were selected for MBL detection and PCR detection of class 1 integrons. No MBL genes were found with PCR. From isolate RN62639 a 689 bp class 1 integron was amplified, encoding an *aac*A4 aminoglycoside-acethyltransferase gene (AAC(6')II), that confers resistance to tobramycin and amikacin. Of the 149 isolates 5 were resistant to ceftazidime and to cefepime. ESBL genes were searched, and in isolate RN64944

an OXA-10 ESBL were found. No other β-lactamase or aminoglycoside-resistance genes have been detected.

Seven isolates were selected for RT-PCR analysis, examining efflux pumps and porins, based on the criteria detailed in section 3.3.2. One isolate showed inconclusive results. OprD deficiency was detected in case of 5 of the 6 cases, hyperproduction of the MexXY-OprM efflux pump was detected in 3 isolates, overexpression of MexAB-OprM was detected in one isolate at a relatively high level, 148 fold. No isolates hyper-produced the MexCD-OprJ efflux pump.

4.2. Bacterial typing

4.2.1. Molecular typing of multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

From 2004 to 2008 51 multidrug-resistant, non-mucoid *P. aeruginosa* isolates were selected for further analyses from different non-cystic fibrosis patients. Within this timeframe at least three different outbreaks/case accumulations were detected; in one of the outbreaks 29 different patients were included, which isolates were examined in the epidemiological analysis (in section 4.2.1.1). The remaining 22 multidrug resistant isolates were typed by macrorestriction analyses (PFGE with *SpeI* digestion), which method is considered to be the gold standard method of bacterial typing. With this method the isolates proved to belong to 11 different pulso-types. The isolates were also typed by the automated typing system, DiversiLab, with this method 14 different genotypes were determined. The only difference between the PFGE and the DiversiLab typing results was among 3 isolates that were occurred in a smaller outbreak in the MICU department (PFGE pulso-type is designated as C, DiversiLab Clone 3, **Table 8**). To confirm the PFGE or DiversiLab results, another molecular typing method, namely the ERIC PCR was used for these isolates, which method confirmed the PFGE results, namely that these isolates belong to the same genotype. Isolate 83712 was determined as pulso-type C' (C variant) with PFGE; with DiversiLab it showed 87% homology with Clone 3 isolates; and with ERIC PCR it belonged to type 3, although the ERIC results were analyzed visually, which does not allow to differentiate between slight differences.

Based on the above different molecular typing methods, the 22 multidrug resistant *P. aeruginosa* isolates belong to 11 different genotypes. Three representatives of the 11

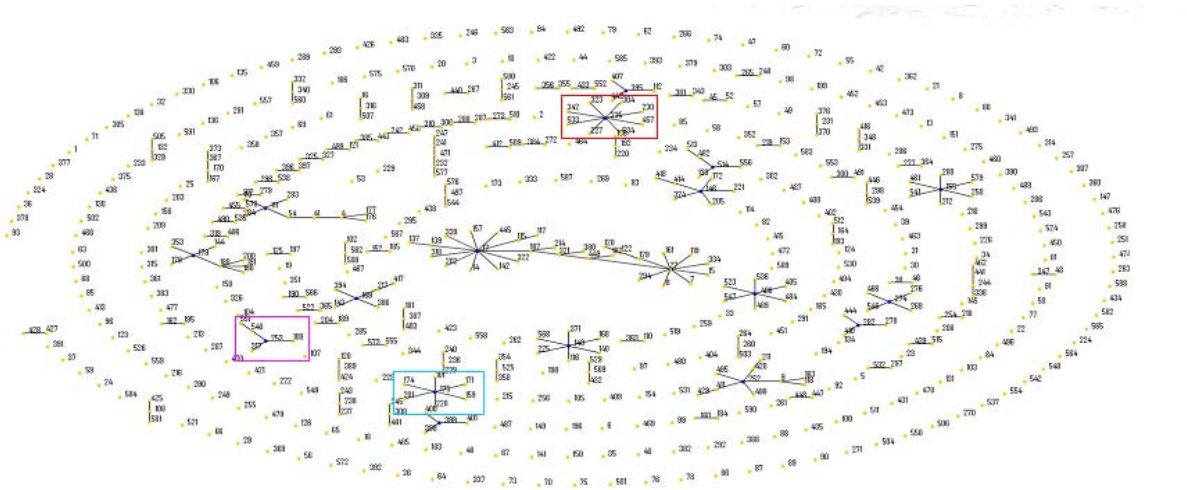
genotypes were selected for MLST analysis, one isolate from each outbreaks/case accumulations (pulso-type A 82114 and pulso-type C 95149) and a pandrug-resistant clinical isolate: 49010 (isolates marked with * in **Table 8**). As the result of the MLST analysis they proved to be different ST types (ST235, ST253, ST619), all of them are a member of different clonal complexes (**Figure 8**.). The data of the investigated strains are available at: http://pubmlst.org/paeruginosa. According to the MLST typing analyses, the pandrug resistant isolate 49010 (ST235) belongs to a clonal complex, previously identified in VIM metallo-β-lactamase-, and also PER-1 producers in Hungary, namely CC11 [7,56].

Table 8. The 22 multidrug resistant isolates, and the results of the different molecular typing metods, namely the PFGE, DiversiLab and the ERIC PCR.

Strain	Date of isolation	Hospital ward	Site of isolation	PFGE pulso-type	DiversiLab Clone	ERIC PCR type	Typing results-based on PFGE, DiversiLab, and ERIC PCR
82114*	26.10.2004	UH	urine	A	1	NT	I
7025	24.01.2005	UH	urine	A	1	NT	I
72982	07.10.2005	MICU	tracheal asp.	A	1	NT	I
78065	24.10.2005	MICU	tracheal asp.	A	1	NT	I
79577	02.11.2005	SICU2	nasoph.swab	A	1	NT	I
15939	17.11.2006	SB	bile	B’	2	NT	II
95490	14.12.2004	MICU	tracheal asp.	C	3	c	III
95149*	12.12.2004	MICU	tracheal asp.	C	12	c	III
2740	11.01.2005	MICU	tracheal asp.	C	3	c	III
12064	11.02.2005	MICU	tracheal asp.	C	3	c	III
9541	03.02.2005	MICU	tracheal asp.	C	13	c	III
23611	27.03.2005	MICU	tracheal asp.	C	14	c	III
30831	25.04.2005	MICU	tracheal asp.	C	3	c	III
83712	16.11.2006	SICU2	tracheal asp.	C′	3-87%	c	III
49010*	28.06.2005	MICU	tracheal asp.	D	4	NT	IV
57006	02.08.2005	MICU	blood cult.	E	5	NT	V
64007	27.11.2007	MICU	tracheal asp.	F	6	NT	VI
7238	26.01.2005	PS	urine	G	7	NT	VII
8768	01.02.2005	PN	urine	H	8	NT	VIII
22495	22.03.2005	PICU	tracheal asp.	I	9	NT	IX
8107	30.01.2006	PICU	nasoph.swab	J	10	NT	X
44744	20.06.2006	PICU	tracheal asp.	K	11	NT	XI

The isolates marked with * were selected for an MLST analysis.

Figure 8 . The eBURST diagram of *P. aeruginosa* isolates generated by software available at http://eburst.mlst.net with the three examined isolates. Isolate 82114 is ST619, belonging to an unknown clonal complex circled in blue; isolate 95149 is ST253 belonging to an unknown clonal complex circled in pink; isolate 49010 is ST235 belonging to clonal complex 11 cirled in red.



4.2.1.1. Investigation of multidrug resistant isolates of the epidemiological analysis; environmental screening

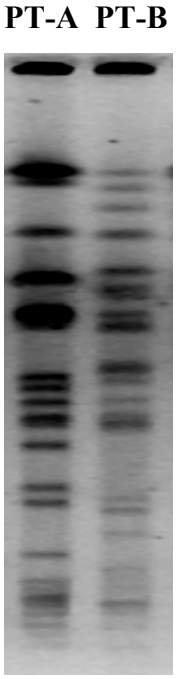
From June 2007 to November 2008 29 multiresistant clinical *P. aeruginosa* were obtained from an outbreak, that showed resistance to all antibiotics except polymixin B, amikacin, and ± netilmicin. In addition to similar resistance patterns, they had similar colony morphology as well. The 29 different patients, 14 females and 15 males, were admitted to different clinical wards including four ICUs (**Table 9**). Twenty patients developed infection based on clinical evidence, such as isolation of the strain from blood culture or from lower respiratory tract specimens (endotracheal aspirates or protected bronchoalveolar lavage) together with fever, increased procalcitonin and/or CRP levels, increased white-blood cell sedimentation and granulocytes in the lower respiratory tract specimens. Nine patients displayed simple colonization. The first patient, from whom the multiresistant *P. aeruginosa* was isolated, was admitted to the MICU from another hospital ward (cardiac surgical ICU).

Table 9. Data of the 29 multiresistant *P. aeruginosa* strains and patients involved in the epidemiological analysis

Patients	First date of isolation	Wards where the patient was before isolation of the strain	after	Infection/ Colonization	PFGE	Type with DiversiLab
32174	13/06/2007	SICU3	-	Infection	PT-A	Clone 1
38979	20/07/2007	-	-	Infection	PT-A	Clone 1
64152	27/11/2007	SICU3	-	Colonization	PT-A	Clone 1
56	02/01/2008	SICU3	-	Infection	PT-A	Clone 1
1321	08/01/2008	-	-	Infection	PT-A	Clone 1
45611	22/07/2008	-	-	Colonization	PT-A	Clone 1
47671	23/07/2008	-	-	Infection	PT-A	Clone 1
48968	24/07/2008	NS	-	Colonization	PT-A	Clone 1
48974	23/07/2008	-	-	Infection	PT-A	Clone 1
48964	24/07/2008	MICU	MICU	Infection	PT-A	Clone 1
50626	28/07/2008	SICU1	-	Infection	PT-A	Clone 1
51356	05/08/2008	NE	NE	Infection	PT-A	Clone 1
52432	08/08/2008	-	-	Infection	PT-A	Clone 1
53818	12/08/2008	NS	-	Colonization	PT-A	Clone 1
57258	27/08/2008	-	-	Infection	PT-A	Clone 1
57866	01/09/2008	-	-	Colonization	PT-A	Clone 1
59371	04/09/2008	SICU2	-	Infection	PT-A	Clone 1
59985	10/09/2008	-	-	Infection	PT-A	Clone 1
63272	18/09/2008	MICU	MICU	Infection	PT-A	Clone 1
66401	20/09/2008	-	-	Infection	PT-A	Clone 1
67609	21/09/2008	-	-	Colonization	PT-A	Clone 1
68241	23/09/2008	-	-	Infection	PT-A	Clone 1
70436	24/09/2008	-	-	Infection	PT-A	Clone 1
71032	24/09/2008	-	-	Colonization	PT-A	Clone 1
71228	26/09/2008	-	-	Colonization	PT-A	Clone 1
73127	29/09/2008	-	-	Infection	PT-A	Clone 1
73558	01/10/2008	SICU2	MICU	Infection	PT-A	Clone 1
73608	22/10/2008	-	-	Infection	PT-A	Clone 1
54688	15/08/2008	-	-	Colonization	PT-B	Clone 2

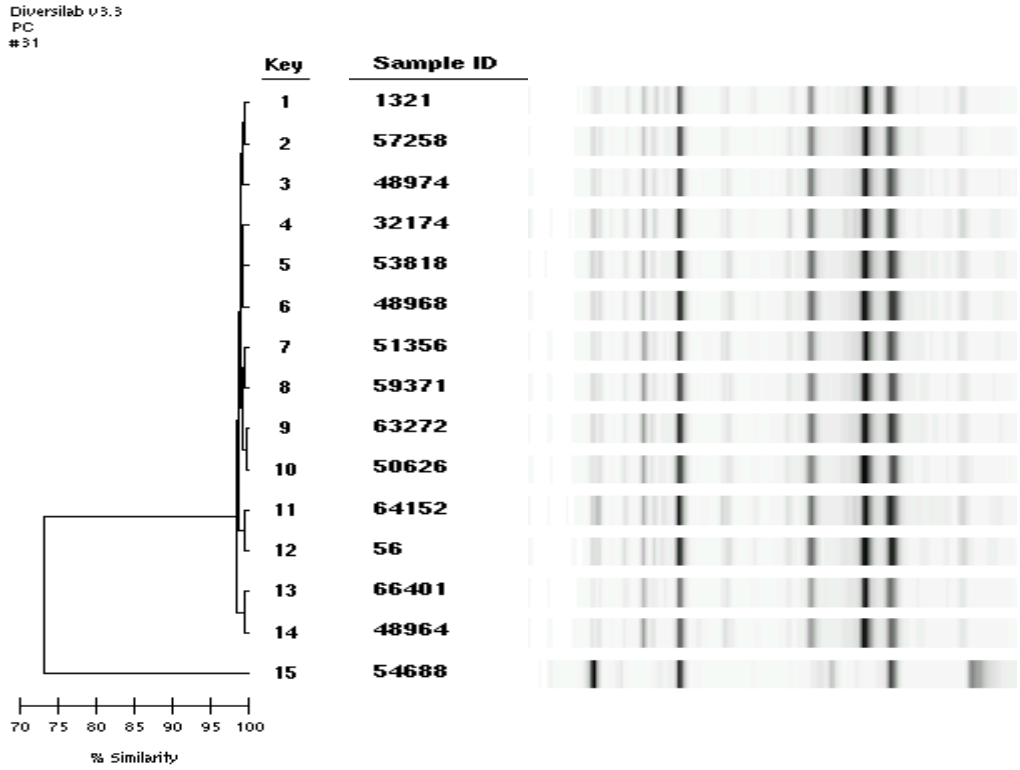
PFGE was used as a gold standard for determining similarities between the multiresistant strains obtained in different time points from patients treated in different wards. SpeI digestions of the 29 clinical *P. aeruginosa* isolates were performed, resulting two unique patterns (designated as pulso-types, PT-A and PT-B), PT-A was observed in 28 of the 29 strains (**Table 9.**). PT-B corresponds to the strain with the different antibiotic-resistance profile. An example of the two banding patterns is shown in **Figure 9**.

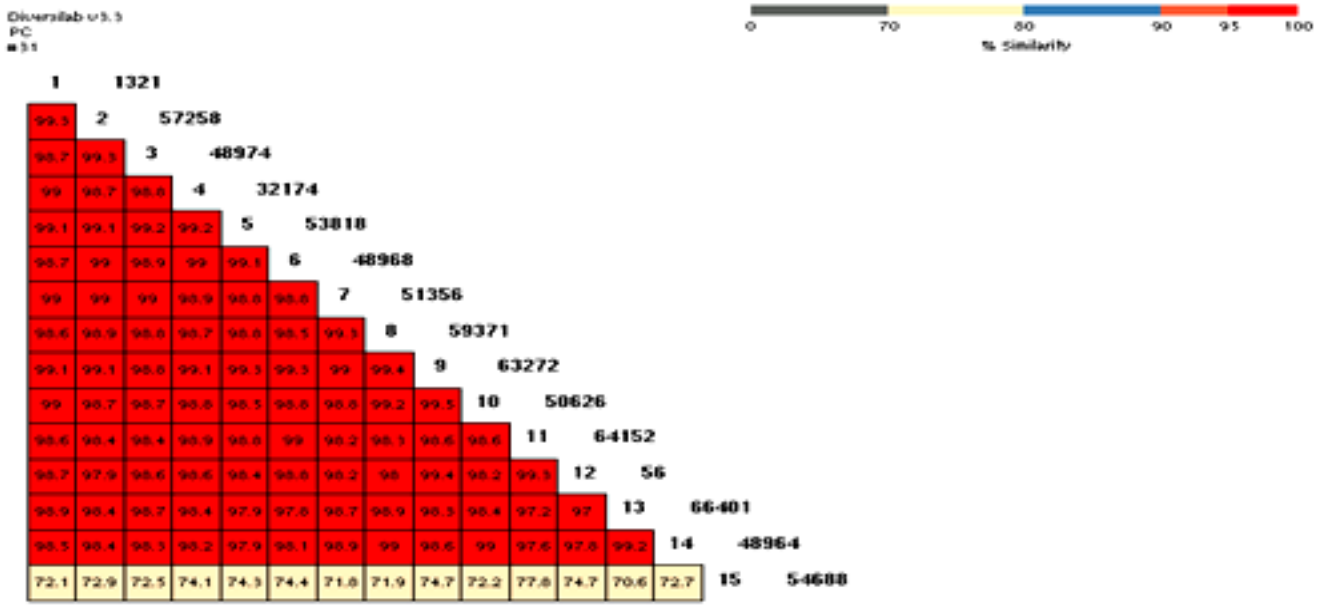
Figure 9. An example of the DNA banding profile of the two pulsotypes, PT-A and PT-B following the PFGE analysis. PT-A: pulsotype A, PT-B: pulsotype B (corresponds to the strain with the different antibiotic-resistance profile).



All clinical isolates and five *P. aeruginsa* strains obtained from the environment were analyzed with the DiversiLab Pseudomonas kit. Twenty-eight of the 29 clinical isolates were clustered together (**Table 9.**). The remaining one isolate had the unique antibiotic-susceptibility. **Figure 10.** shows the similarity matrix and the virtual gel images of 14 representatives of the clustered strains, and the one with the different pattern.

Figure 10. Dendogram, virtual gel image and the similarity matrix of some representatives of the 29 investigated *P. aeruginosa* isolates typed by the DiversiLab. 1-14: related *P. aeruginosa* isolates (Clone 1), 15: unrelated *P. aeruginosa* isolate with the different antibiotic-resistance profile (Clone 2).





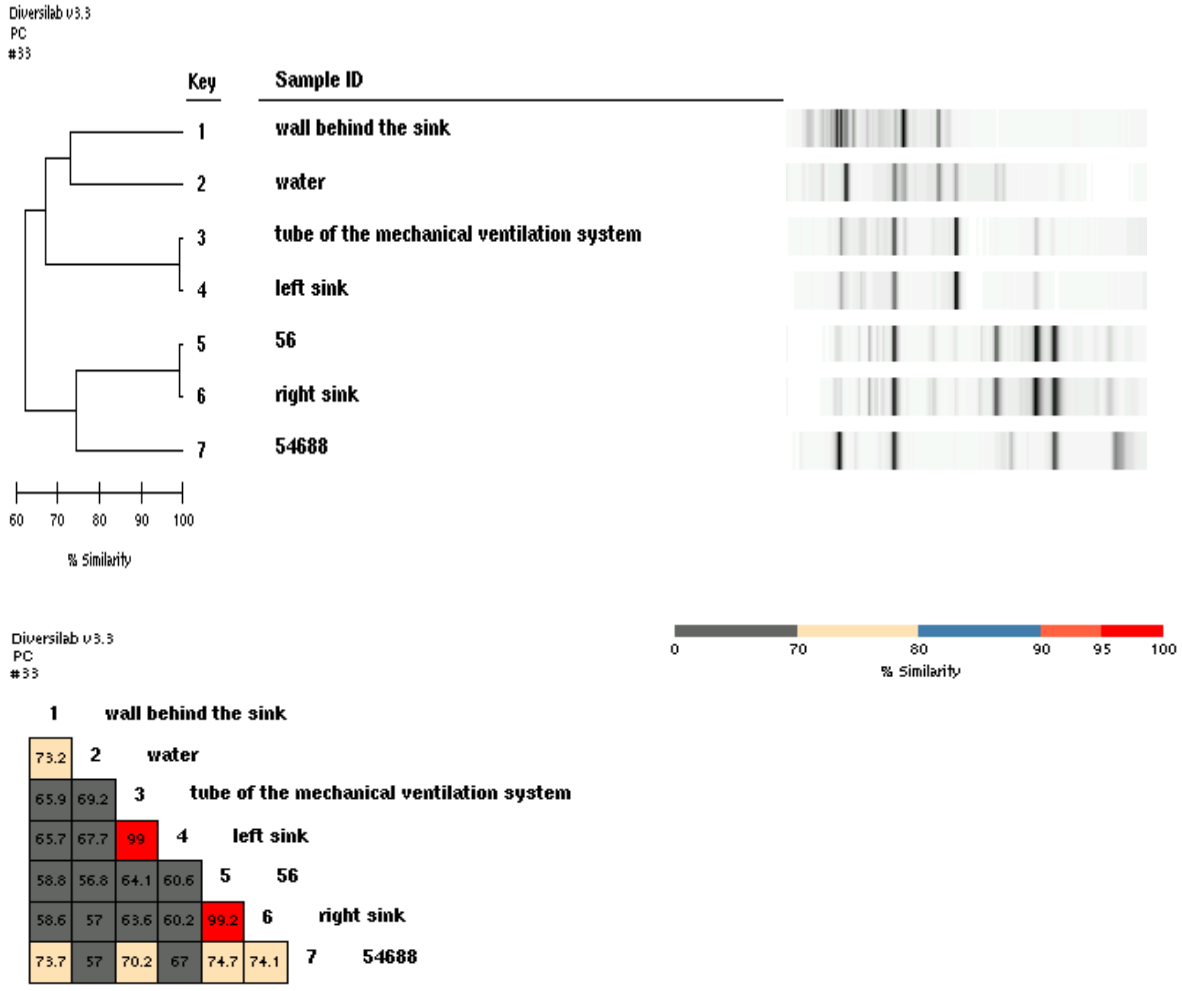
Environmental sampling was carried out at the ICU department in order to detect the source of the *P. aeruginosa* strain responsible for the outbreak. Of the 13 environmental samples (**Table 10**) five were found to be contaminated by *P. aeruginosa*. All the environmental isolates were typed by DiversiLab system, **Figure 11.** shows the similarity matrix and the rep-PCR patterns created for them. Altogether four different clones were found among the environmental strains. One of these isolates had the same rep-PCR profile as a representative strain of the circulating multiresistant clinical *P. aeruginosa* (Clone 1). The strain that is presumably responsible for the outbreak was found in the right sink drain. Two environmental *P. aeruginosa* were also clonally related (Clone 3), but they were unrelated from the strain colonising or infecting 29 patients during the 17 month period.

Allelic profiles were determined for a representative isolate circulating in the different wards including ICUs, corresponding to the PT-A, and Clone 1. The MLST revealed a new ST, namely ST618, belonging to a clonal complex previously hasn't been described, containing six STs. The data of the investigated strain are available at: http://pubmlst.org/paeruginosa.

Table 10. Environmental samples, the isolated microbes, and the results of typing of the *P. aeruginosa* isolates by DiversiLab system. NT: not tested

Enviromental samples	**Isolated microbe(s)**	**Type with DiversiLab**
Right sink drain	*P. aeruginosa*	Clone 1
Left sink drain	*P. aeruginosa*	Clone 3
Tube of the mechanical ventilation system	*P. aeruginosa*	Clone 3
Equipment trolley	Coag.neg. *Staphylococcus*	NT
Computer of clinicians	*E. faecalis*	NT
Wall behind the sink	*P. aeruginosa*	Clone 4
	E. coli	NT
	Enterobacter spp	NT
Blood gas machine 1	Coag.neg. *Staphylococcus*	NT
Blood gas machine 2	Coag.neg. *Staphylococcu s*	NT
Monitor 1	*Candida* spp	NT
	E. faecalis	NT
Monitor 2	sterile	
Monitor 3	Coag.neg. *Staphylococcus*	NT
Hand of the nurse	Coag.neg. *Staphylococcus*	NT
Tap water	*P. aeruginosa*	Clone 5

Figure 11. Dendogram, virtual gel image and the similarity matrix of the environmental *P. aeruginosa* isolates, and the representatives of PT-A and PT-B typed by the Diversilab system. 1-4, 6: environmental isolates, 5: representative isolate from the outbreak (corresponds to PT-A, Clone 1), 7: the isolate with the different antibiotic-resistance profile (corresponds to PT-B, Clone 2)



4.2.2. Molecular typing of *P. aeruginosa* isolates recovered from cystic fibrosis patients

From 2003 to 2007 149 *P. aeruginosa* were isolated from 16 patients suffering from cystic fibrosis, 78 isolates (52%) had mucoid colony morphology. Fourty-four isolates were selected for bacterial typing by PFGE and rep-PCR-based DiversiLab methods, 27 (61 %) of them had mucoid colonies. In average ~3 (2,75) isolates per patient were typed. With PFGE 16 different genotypes were determined. In case of 13 patients one genotype was recovered per patient from their sputum samples, in case of three patients, two different strains caused the infection/colonisation. With PFGE the same genotypes, designated as pulso-type K determined typed from two different patients, such as pulso-type L, and pulso-type F (**Table 11**).

With DiversiLab 20 different genotypes were determined. The results with the exception of two patients were contradictory with the PFGE results. With DiversiLab the same genotype (designated as CloneA, CloneC, CloneD) were determined from more than one patient in three cases (data not shown).

Six CF isolates were randomly selected from different patients for MLST analysis. Sequencing the seven alleles resulted six different STs, namely ST343, ST614, ST277, ST625, ST626 and ST627. The results of the MLST analyses are available at http://pubmlst.org/paeruginosa. With the exception of ST625, which is a member of an uncharacterized clonal complex, all STs are singletons.

Table 11. Different CF patients colonized/infected with the same pulso-type of *P. aeruginosa*, and the result of MLST analysis, if it was performed.

Patient	Strain	Year of isolation	Mucoid	PFGE pulso-type	MLST
DM	63282	2005	+	F	NT
LA	61644	2005	+	F	NT
BoT	27013	2003	-	K	NT
	83650	2005	-	K	ST626
TZ	23513	2003	-	K	NT
	23918	2005	-	L	ST625*
KT	63285	2005	-	L	NT

NT: not tested, * marks the ST that is a member of a clonal complex.

5. DISCUSSION

5.1. Antibiotic resistance

5.1.1. Antibiotic resistance of multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

Within a period of four years 51 multidrug resistant *P. aeruginosa* have been collected from different, non-cystic fibrosis patients. The selection criteria were being resistant to carbapenems and at least for two more antipseudomonal antibiotic groups. Molecular typing methods revealed 12 different genotypes; a representative strain from each type has been selected for further testing. Interestingly, there were differences in the antibiotic resistance profile within a genotype. Isolate 15939 was designated as PFGE pulso-type B', and according to the DiversiLab, it is 86% related to the pulso-type B isolates from the epidemiological analysis. However their antibiotic-resistance profile is different, since isolate 15939 is a pandrug resistant isolate, while all 28 pulso-type B isolates are susceptible to amikacin and netilmicin. Isolates designated as pulso-type C were proved to belong to the same genotype and caused a smaller outbreak at the MICU, but showed differences in the aminoglycoside-resistance profile. No class 1 integrons could be found in these isolates, which are known to harbour different antibiotic-resistance markers, especially aminoglycoside-resistance genes and MBL genes. No further investigation was carried out to characterize these differences. Isolates designated as pulso-type A on the other hand had no differences in the antibiotic-resistance pattern, although they were isolated from three different hospital wards, geographically far from each other.

In order to investigate the β-lactam resistance within these isolates, different pheno- and genotypical methods were applied. All 12 strains were resistant not only to carbapenems, but with one exception, also for ceftazidime, which was used as a marker for MBL production. MBL screening was performed first with widely-used phenotypical methods, namely the MBL E-test and the MBL combined disc test. The results were negative for two isolates; the remaining 10 fulfilled the necessary criteria for identification of a MBL producing strain (**Table 5.**). However, neither MBL enzyme function with bioassay, nor MBL genes with molecular methods has been detected; and the isolated integrons only contained aminoglycoside-resistance genes. Therefore a high-rate of false-positive results was obtained with MBL E-test and MBL-combined disk-test, even when the inclusion of the most recent MBL-screening criteria was adopted. Our results stress the importance of conducting a control

test using a disc containing solely EDTA, since the increased inhibition zone around the disk containing IPM-EDTA was most likely due to the membrane permeabilizing effect of EDTA, rather then a chelating action upon any hypothetical MBLs. The same effect could be observed in case of the E-test method. Among our clinical *P. aeruginosa* isolates the susceptibility to EDTA was common, the inhibition zones around the disks containing only EDTA were varying from 10 mm to 17 mm. These false-positive results unambiguously preclude the recommendation for using the E-test or the combined disk-test as a carbapenemase-screening method [**article I**]. An easy-to-perform bioassay using cell-free extracts, where the interference of EDTA is avoided, is preferable for routine carbapenemase-activity screening for clinical microbiology laboratory.

In fact, the resistant phenotype to carbapenems observed in the examined strains could be linked to the overexpression of efflux pumps involved in the extrusion of carbapenems and/or OprD deficiency, rather than the production of MBLs, as it was suspected by the interpretation of the MBL E-test and MBL combined disc test. One of the examined genotypes hyper-produced MexAB-OprM efflux pump, four strains over-expressed the genes of MexXY-OprM and two of them the MexCD-OprJ. Eight strains had significantly down-regulated the genes of the OprD porin channel (**Table 7.**). In a pandrug resistant strain (49010), which was resistant to all anti-pseudomonal antibiotics with the exception of polymyxin-B, beside the overexpression of two different efflux-pump genes, the production of three ESBL enzymes did extensively contribute to β-lactam resistance [13].

5.1.2. Antibiotic resistance of *P. aeruginosa* isolates recovered from CF patients

Within a period of four years 149 *P. aeruginosa* were isolated from 16 different patients suffering from cystic fibrosis, attending the CF centre in Szeged. In this period of time all isolates from the 16 patients were stored for further analysis, regardless their antibiotic susceptibility.

Among these isolates the highest resistance was seen in case of gentamycin, amikacin and moxifloxacin (62,5 %, 61,2 %, 56,2 % respectively). Otherwise the strains had a good in vitro susceptibility, which results do not follow the international tendency [9,59,60]. Among our CF isolates were no pan- or multidrug resistant *P. aeruginosa*, and no developing resistance was observed due to the antimicrobial treatments [**article IV**].

No MBLs were found, only one class 1 integron, and an OXA-10 ESBL was found among the isolates. Isolate RN64944 producing the OXA-10 ESBL was resistant to cefepime,

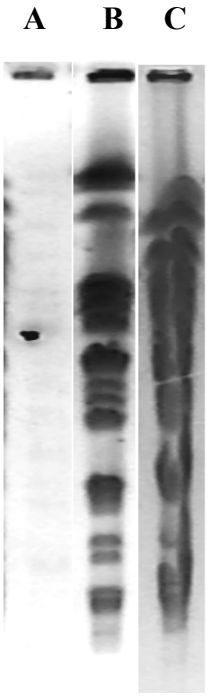
but susceptible to all other β-lactams. Isolate RN62639 harbouring the class 1 integron was resistant to all aminoglycosides. Isolates being only resistant to imipenem were relatively prevalent, the porin channel analyses proved, that this could be the effect of OprD deficiency. Isolate BoT83704, which proved to overexpress the MexAB-OprM efflux pump, was resistant to both carbapenems and all fluoroquinolones. These antibiotic resistance patterns correspond with the determined resistance mechanisms.

5.2. Comparison of three different molecular typing methods

5.2.1. Molecular typing of multidrug-resistant *P. aeruginosa* isolates recovered from non-cystic-fibrosis patients

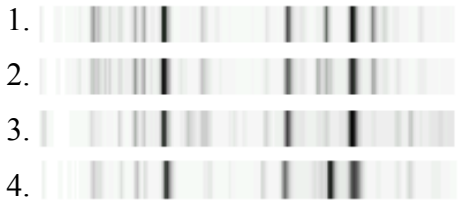
In this study we used different molecular methods for bacterial typing in order to determine their applicability in typing of *P. aeruginosa*. A total of 51 multiresistant *P. aeruginosa* were selected for molecular typing. All 51 isolates have been typed by PFGE. Our results confirmed that PFGE, which is known to be the gold-standard method of bacterial typing, has a high discriminatory power; and reliable to determine the different bacterial genotypes in case of local outbreaks. However, it is limited by technical complexity, relatively expensive PFGE equipment is required, and the prolonged turnaround times (2-4 days) for results [29,31], which may not cause problem if late confirmation of the circulation of a clone could be carried out. However gels need to be analyzed carefully and quality control is essential. There are several factors that can affect the results of a PFGE, such as the amount of DNA embedded in the agarose, the effectiveness of the enzymatic restriction, the quality of the electrophoresis, or mucoidity of the isolate (**Figure 12**).

Figure 12. Examples for technical errors of PFGE. **A**: not enough DNA in the gel, **B:** too much DNA in the gel, **C**: mucoid *P. aeruginosa* isolate.



Beside PFGE, all 51 *P. aeruginosa* were typed with the repetitive-element-PCR-based DiversiLab instrument as well, and contradictory results only occurred in case of three isolates (**Table 8.**). ERIC PCR was used in case of these isolates and confirmed the PFGE results, namely that the isolates belonged to the same genotype (PFGE pulso-type C). In case of these isolates the DiversiLab virtual gel image showed too many bands with similar sizes, during the PCR reaction too many products have been amplified due to an unknown reason (**Figure 13**).(The same technical problem occurred in case of the CF isolates, see section 5.2.2.).

Figure 13. DiversiLab virtual gel image showing the pattern of those 3 isolates that had contradictory results with PFGE (1.: 6541, 2.: 23611, 3.: 95149) and an isolate from PFGE pulso-type C (4.: 95490). ERIC PCR confirmed, that the above four isolates belong to the same genotype. According to the DiversiLab the similarity of the above three isolates compared to the fourth one are respectively: 66%, 51%, 72,8%.



In case of the epidemiological 29 *P. aeruginosa* isolated from an outbreak, the results of the PFGE and the DiversiLab system were the exact same (**Table 9.**), namely with the exception of one, all isolates belonged to the same genotype. Collecting multiresistant isolates potentially involved in nosocomial infections could be important for later analysis of the epidemiological situation by using typing methods. The DiversiLab typing system helped us in the detection of the circulation of *P. aeruginosa* in our hospital wards. It confirmed that the 29 different patients, with one exception, were infected/colonised by the same genotype. The strain successfully spread among patients and wards and survived in different environments. DiversiLab also helped us to detect the source of the strain, which was found in the right sink drain (**Figure 11.**). None of the other environmental samples were contaminated with this strain at the time the environmental sampling was carried out. It is also interesting to note, that two other environmental *P. aeruginosa* isolates, sampled from the left sink drain, and from the tube of the ventilation system were found, with a different rep-PCR pattern. That means that another *P. aeruginosa* strain was present in the patients' immediate environment, but have not colonised/infected any patients yet.

In conclusion of DiversiLab, although the equipment is quite expensive, it is simple, fast, reproducible, having high discriminatory power, and providing information about similarity of the isolates in real time. Using the data-base for a longer period of time it is

possible to follow and compare outbreak isolates locally. Therefore it can be an effective tool for a microbiological laboratory during epidemiological analyses [**article III**].

A representative isolate from each outbreak/case accumulation (82114, 95149, 56), and the 49010 pandrug resistant isolate, which is proved to produce three different ESBLs and hyperproduces two efflux-pumps, were selected for an MLST analysis. *P. aeruginosa* 82114 isolated from a case accumulation (of 5 cases) proved to be ST619, a member of a clonal complex that has not been described previously. *P. aeruginosa* 95149 isolated from an outbreak of 8 cases proved to be ST253, which is also a member of a clonal complex. *P. aeruginosa* 56 isolated from an outbreak of 28 cases, is an ST618, also a member of an unknown clonal complex (**Figure 8.**). It is known that *P. aeruginosa* has a non-clonal population structure punctuated by successful epidemic clones, or clonal complexes [33]. Therefore, our MLST results draw the attention of the epidemiologists on account of the gathering evidence that some clonal lineages are more likely spread among patients and cause infections/outbreaks.

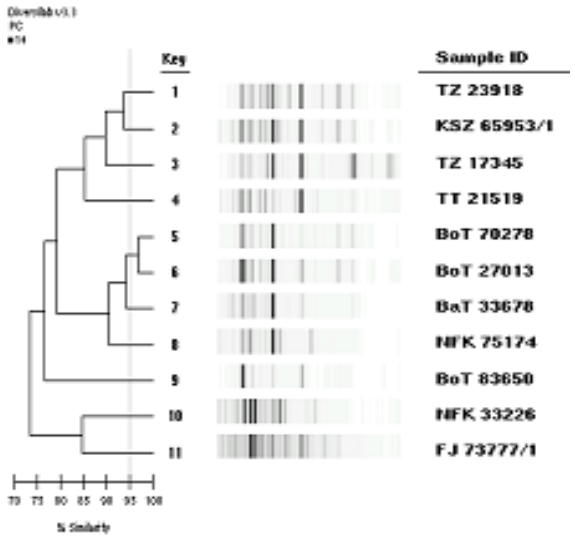
With MLST analysis the pandrug resistant isolate (49010) is proved to be ST235, a member of the CC11 clonal complex. Interestingly, a PER-1 producer strain was isolated from an outbreak in Poland [6], with the same ST, and also from Hungary and Serbia [7]. Although, our strain was isolated from a Romanian patient, regarding the virulence of the CC11 members, and the geographical closeness of Hungary, Romania, Serbia and Poland, we can not exclude the possibility that the same strain is circulating in these countries.

In this study we compared three different typing methods for the molecular typing of clinical *P. aeruginosa* isolates. By using the MLST method we can gain information about the clonal relationship of the bacterial isolates, as it has been proved previously [30,33]. PFGE and rep-PCR-based methods nevertheless have higher discriminatory power, than MLST [30], making it possible to also differentiate between isolates with similar antibiotic patterns, and to find the environmental presence of the same epidemic strain. Therefore the implication of MLST for population genetics and dynamics may be more significant than those for bacterial epidemiology, since polymorphism in the slowly evolving genes, which are its targets, may not be high enough for useful epidemiological comparisons.

5.2.2 Molecular typing of *P. aeruginosa* isolates recovered from CF patients

Based on the different antibiotic susceptibility profile 44 isolates were selected for bacterial typing. With the PFGE method 16 different genotypes were determined. In case of 13 patients one genotype were recovered per patient from their sputum samples, so these patients were colonised/infected with the same strain from 2003 to 2007. In case of three patients, two different genotypes could be isolated from their sputum, so they were colonised/infected with different strains. Overall a high diversity was observed, which corresponds to the international literature [45]. The isolates were also typed by the DiversiLab system. The virtual gel image showed too many bands with similar sizes in almost all of the strains due to an unknown reason (**Figure 14**). Results did not correspond neither with the antibiotic susceptibility pattern, nor with the PFGE results, which is considered to be the gold standard method of bacterial typing.

Figure 14. The virtual gel image of some of the examined CF isolates shows the presence of too many bands in case of almost all of the isolates.



The same genotype (designated as CloneA, CloneC, CloneD, data not shown) were determined from more than one patient in three cases. PFGE analysis could not confirm these results, and also the clinical background studies showed that these patients were not hospitalised at the same time or did not participate the same CF camp, so the cross-infection could be excluded. Considering all the above results, we concluded that during the molecular

typing of CF isolates the PFGE method is found to be more accurate and reliable than the DiversiLab system.

Until recently, CF patients were thought to be colonised with their own unique *P. aeruginosa* strain acquired from the environment. Cross-infection was considered a relatively infrequent source of acquisition. With PFGE the same genotype (designated as pulso-type F, data not shown) was isolated from patients DM and LA from 2005 (**Table 11.**), when they participated in the same CF-camp. Pulso-type L was isolated from patients TZ and KT from 2005. At that time, they were hospitalised at the same time in the same hospital ward. Pulso-type K recovered from patients BoT and TZ. The strains were isolated in 2003, and according to their clinical background the two patients attended the same CF-camp in Budapest, Hungary. Interestingly, the MLST results suggest that the latter strain, being a member of a clonal complex, and not a singleton, could be a member of an epidemic clone (**Table 11**). However the remaining five strains examined with MLST proved to be singletons with this method, which suggests that the prevalence of the cluster genotypes is significantly lower among these CF isolates. With our results we proved, that the transmission of a certain strain from patient to patient in a hospital ward or in a CF camp is expected, but not inevitable. Nevertheless the incidence of the cross-infections in the CF centre in Szeged were relatively low due to the infection control measures applied to prevent patient-to-patient contacts, such as scheduling appointments with the clinicians at different time points, trying to hospitalize the patients in different hospital rooms [**article IV**].

6. CONCLUSIONS

Based on the present study we can conclude, that in case of *P. aruginosa* isolates obtained from non-CF patients:

- The presence of multi- or pandrug resistant isolates among patients hospitalised in the University Hospital of Szeged between 2004-and 2008 was relatively low (7 %).
- Routine detection of the different antibiotic resistance mechanisms, especially the ones with epidemiological consequences, such as MBL production, is vital for the hospital laboratories, therefore a cheap, fast and reliable method is required. MBL-E-test is used worldwide, but in this study is proved to be inaccurate due to the effects of EDTA.
- Determination of the different resistance mechanisms against antipseudomonal β-lactams revealed that in contrast with the international tendency, MBL production in clinical *P. aeruginosa* isolates is not widespread in our University Hospital.
- In case of hospital outbreaks a fast, reliable molecular typing method is required in order to localize the outbreak, and possibly to detect the source of the isolate responsible. In the present study DiversiLab typing system is proved to be as accurate and reliable as the gold-standard PFGE. Additional advantage of the system is the long-term, internet-based database.
- Following the international tendency our epidemic *P. aeruginosa* strains proved to be members of clonal complexes based on the MLST analysis.

Based on the present study we can conclude that the *P. aeruginosa* isolates obtained from CF patients:

- have an overall good in vitro susceptibility, which results are in contrast with the international tendency. Among our isolates there were no MBL producer, no multi-or pandrug resistant isolate, and no therapy-induced resistance was observed.
- During the molecular typing of CF isolates the DiversiLab system is found to be too discriminative and not as reliable as the PFGE method.
- These isolates have a high genetic diversity, no clonality was observed, which corresponds to the international literature.
- With the exception of a few sporadic cases of transmission, there were no highly epidemic strain isolated, which is expected to be a result of the good infection control measures taken by the clinicians, nurses of the CF centre, and of the good patient compliance.

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Controlling for false positives: interpreting MBL Etest and MBL combined disc test for the detection of metallo-β-lactamases

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Sir,

Metallo-β-lactamases (MBLs) are enzymes with versatile hydrolytic capabilities, namely the ability to hydrolyse all β-lactam antibiotics, with the exception of monobactams. Consequently, bacteria producing MBLs often cause infections that are difficult to treat due to the ineffectiveness of β-lactams. Often, the genes encoding MBLs are located on integrons that frequently harbour other resistance determinants and that may be associated with mobile genetic elements, thus increasing the likelihood of their dissemination. MBLs are particularly associated with Gram-negative pathogens, and their occurrence requires continuous monitoring due to their potential impact on patient management and the associated implication for infection control.

Several methodologies have been proposed for the routine detection of MBL-producing *Pseudomonas aeruginosa*, particularly the MBL Etest and the MBL combined disc test, although there are concerns regarding their reliability.[1–3] Recently, in order to increase the reliability of the test, some authors suggested the inclusion of ceftazidime, meropenem or imipenem resistance in the MBL-screening criteria.[2] Nevertheless, in very specific circumstances, such as the case of mucoid *P. aeruginosa* isolates, these methods are stated as inefficient.[1] Relying on EDTA for MBL inactivation, these methods are prone to false-positive results since the chelating agent itself can increase membrane permeability, thus increasing the chance of a bactericidal effect.[4] Given the significance of MBL screening for routine laboratories, it seems important to present data that highlight problems with established methodologies.

In the present work we assessed the reliability of the MBL Etest and combined disc test methods for the detection of MBL-producing *P. aeruginosa* isolates.

From 2004 to 2008, 51 multidrug-resistant, non-mucoid *P. aeruginosa* isolates were recovered from different non-cystic fibrosis patients, hospitalized in nine distinct wards in South Hungary (Szeged). Within this time frame, three different outbreaks were detected at the intensive care unit, involving 5, 7 and 29 isolates each, and presenting identical inhibition zone profiles. Strain HMV-2, a $bla_{\text{VIM-2}}$ producer (kindly provided by Professor Luis Martinez-Martinez), was used as a positive control for MBL detection, and *P. aeruginosa* ATCC 27853 was used both as a recommended control for susceptibility tests and as a negative control for MBL assays. Identification was performed using the VITEK 2 system and susceptibility was determined by the disc diffusion method. MBL detection included the MBL Etest (AB Biodisk, Solna, Sweden) and the MBL combined disc test, performed on Mueller–Hinton agar medium using 10 μg imipenem discs (Oxoid Ltd, Basingstoke, UK) and imipenem discs supplemented with EDTA (750 μg) in parallel with blank paper discs supplemented with EDTA alone. A bioassay and a carbapenem hydrolysis assay with cell-free extracts were performed, in order to detect carbapenemase activity as previously described.[5] MBL presence was confirmed by PCR aimed at detecting $bla_{\text{SPM-1}}$, $bla_{\text{GIM-1}}$ and the most common bla_{VIM} and bla_{IMP} genes.[6] Class 1 integrons were characterized by PCR and sequencing. Strain clonal relatedness was assessed by PFGE.[6]

All isolates were resistant to both imipenem and meropenem (MIC $\geq$ 16 mg/L), and, with one exception, additionally exhibited ceftazidime resistance, which was used as a marker for potential MBL production.[2] PFGE typing revealed no clonal relatedness among the isolates. MBL detection results, both by MBL Etest and MBL combined disc test, were negative for two isolates. The remaining isolates fulfilled the necessary criteria for identification as an MBL-producing strain (a reduction of imipenem MIC by three or more 2-fold dilutions in the presence of EDTA for the Etest, and a zone diameter difference between imipenem + EDTA and imipenem discs of >7 mm for the MBL combined disc test).

In contrast, the bioassay with cell-free extracts as well as the carbapenem hydrolysis assay with spectrophotometric analysis

Table 1. Comparison of MBL detection methods in different carbapenem-resistant *P. aeruginosa* strains

	Etest (mg/L)		IPM/EDTA disc method (mm)				Assay with cell-free extract	
Strain (no. of isolates)[a]	IPM	IPM+EDTA	IPM	IPM+EDTA	(IPM+EDTA)−IPM	EDTA	bioassay	hydrolysis assay
A (5)	32	1.5	6	17	11	11	−	−
8768	16	1.5	12	19	7	10	−	−
22495	32	1.5	10	26	16	14	−	−
7238	24	3	10	17	7	12	−	−
B (29)	32	1.5	10	20	10	17	−	−
57006	32	1.5	9	21	12	10	−	−
44744	32	1.5	6	18	12	10	−	−
C (7)	32	1.5	10	25	15	12	−	−
49010	16	12	10	11	1	10	−	−
8107	16	3	12	18	6	12	−	−
HMV-2	>256	6	6	25	19	NT	+	+
ATCC 27853	<4	1	22	22	0	NT	−	−

IPM, imipenem; NT, not tested.
[a]A, B and C are representative strains from three different outbreaks.

produced negative results. This pattern was later confirmed by PCR, since no MBL genes were detected. Nevertheless, it was found that four strains harboured different sized class 1 integrons carrying only aminoglycoside resistance genes. Summarized results are presented in Table 1.

A high rate of false-positive results was obtained with both the MBL Etest and MBL combined disc test, even when the most recent MBL-screening criteria were adopted.[1,2] Our results stress the importance of conducting a control test using a disc containing solely EDTA. In fact, the obtained EDTA inhibition zone diameters proved to be significantly bigger than expected [*t*-test against constant (0): $N=10$, $t=16.58$, $P<0.001$]. Additionally, the sole usage of EDTA embedded discs produced growth inhibition effects similar to the difference between the inhibition zones obtained from imipenem alone and imipenem+EDTA [*t*-test for dependent samples: EDTA average=11.80, (imipenem+EDTA)−imipenem average=9.70, $N=10$, $t=1.50$, $P=0.17$]. The combination of the above results clearly shows that the increased inhibition zones obtained for imipenem+EDTA seem to be due to a bactericidal effect of EDTA, probably through a membrane permeabilizing effect, rather then a chelating action upon any hypothetical MBL. In fact, the resistance phenotype to carbapenems observed in the studied strains could be linked to the overexpression of efflux pumps involved in the extrusion of carbapenems and/or OprD deficiency (data not shown) rather than the production of MBLs as could be suspected by the interpretation of the MBL Etest and MBL combined disc test.

These results highlight the need for considerable caution, at least in some specific geographical regions, in the routine application of the Etest or the combined disc test as a carbapenemase-screening method. Alternatively, an easy-to-perform assay using cell-free extracts, where EDTA interference is avoided, seems preferable (see Table 1).

Acknowledgements

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We wish to thank Professor Luis Martinez-Martinez for providing a control strain (VIM-2-producing *P. aeruginosa*) used in this study.

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Transparency declarations

None to declare.

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ARTICLE II.

Isolation and characterization of an imported extremely-resistant *Pseudomonas aeruginosa* producing three different extended-spectrum β-lactamases and hyperproducing two multidrug-efflux pumps

Dear Editor,

The Journal of Infection has recently published reports on the spread of multi/extremely/pan-resistant *Pseudomonas aeruginosa* strains in clinical settings,[1] as well as we face the problem in different hospitals in Hungary.[2,3] We describe here the isolation and characterization of an extremely-resistant *P. aeruginosa*, from a poly-traumatized 20-year-old woman transported to our ICU on 28 June 2007 from Timisoara, Romania, where she had been treated for 2 months after a car accident. Shortly after admission, for reconstruction surgery of her tracheo-esophageal fistula, she needed mechanical ventilation because of double pneumonia, confirmed by chest X-ray. On the day of admission, the endotracheal aspirate and wound sample were positive for extremely-drug-resistant *P. aeruginosa* and MRSA, and the blood culture was positive for *Candida glabrata*. On 6 July the blood culture, on 18 July the urine sample were positive for *P. aeruginosa*. She was treated with amphotericin-B (0.5 mg/kg/day intravenously for 10 days), colistin (2 million UI intravenously 8-hourly for 7 days) and vancomycin (1 g intravenously 12-hourly for 14 days). Some laboratory data on days 1, 8, 15 and 20, procalcitonin: 5.44, 4.46, 0.19 and 0.17 ng/l, WBC: 8.72, 7.3, 5.59 and 7.01 G/l, and transaminases (aspartate aminotransferase/alanine aminotransferase): 152/117, 111/139, 46/82 and 23/59 U/l. By day 17 of therapy her fever decreased and mechanical ventilation was not needed, but the large tracheo-esophageal fistula necessitated a tracheal tube. On day 21 of therapy, the trachea tube could not be replaced and, despite 45-min attempted cardio-respiratory resuscitation, she died.

The *Pseudomonas* isolates were identified by the VITEK 2 system (bioMérieux, Marcy l'Etoile, France) and subjected to susceptibility testing by the disk-diffusion method, using CLSI breakpoints. After isolation, all *P. aeruginosa* isolates were stored at −70 °C in CryoBank medium (Copan Diagnostics Inc., California, USA) as a member of a multi/extremely-resistant and pan-resistant *P. aeruginosa* strain collection for further investigation. All *P. aeruginosa* isolates of this patient proved to be resistant to all anti-pseudomonal antibiotics (piperacillin and tazobactam, cefoperazone, ceftazidime, cefepime, imipenem, meropenem, tobramycin, gentamicin, amikacin, netilmicin, ciprofloxacin, levofloxacin, moxifloxacin and aztreonam) except polymyxin-B, which remained effective against these isolates. The blood culture isolate of this patient was selected for further molecular investigations of the antibiotic resistance.

The presence of metallo-beta-lactamase genes (bla_{SPM-1}, bla_{GIM-1}, and the most common bla_{VIM} and bla_{IMP}) was searched for by PCR.[4] The lack of carbapenemase genes suggested the presence of other mechanisms behind the high-level β-lactam resistance. The genes of the extended-spectrum β-lactamases [PER-1,[5] PER-2, TEM, SHV, GES, VEB-1, and OXA groups[6]] were also sought by PCR method, using specific primers, and the results were confirmed by sequencing and restriction-fragment-length-polymorphism (RFLP) assays on the PCR products, as described previously.[6] PCR revealed the presence of three different extended-spectrum beta-lactamase genes: bla_{PER-1}, bla_{OXA-1} and $bla_{OXA-II\ group}$. Sequencing of the coding region identified the genes of PER-1 and OXA-74 ESBL, the latter corresponding to the $bla_{OXA-1\ group}$. RFLP analysis of the product of $bla_{OXA-II\ group}$ PCR revealed the bla_{OXA-2} gene. No plasmids were isolated from this strain, and it failed to transfer a ceftazidime resistance marker by conjugation to other bacterial hosts, suggesting that the bla_{PER-1} and OXA-type genes were chromosomally located.

To investigate the genetic environment of the bla_{PER-1} gene, IS elements characteristic of the composite transposon Tn1213 were searched for and found by PCR. Tn1213 was first described from *P. aeruginosa* by Poirel et al. in 2005,[5] with the IS*Pa14* and IS*Pa12* IS elements upstream from the bla_{PER-1} gene, and the IS*Pa13*, IS*Pa14* and a glutathione-S-transferase gene (*gst*) downstream from the bla_{PER-1} gene, which we also found.

To seek further resistance mechanisms behind the multiple resistance of the present strain, the expression of the chromosomal genes encoding *opr*D, *mex*B, *mex*X and *mex*C was studied in real-time reverse transcriptase PCR assays according to Quale et al.[7] RNA was isolated by the method of Palágyi-Mészáros et al.[8] The expression of mRNA for the genes of interest was optimized to that of the housekeeping gene *rps*L, known to be expressed consistently in *P. aeruginosa*.[9] The normalized expression of each gene was calibrated to the mRNA expression of the reference strain *P. aeruginosa* PAO1 and the results were given as the expression of the mRNA relative to that of *P. aeruginosa* PAO1. The real-time PCR assays revealed that this strain hyperproduces two multidrug-efflux pumps, MexAB-OprM and MexXY-OprM (6.68-fold and 128-fold increases of expression, respectively, relative to the PAO1 control strain). No change was detected in the rate of production of the MexCD-OprJ efflux pump, or in the OprD outer membrane protein.

The clonal relatedness of our extremely-resistant isolate was investigated by MLST analysis as described by Curran et al.[10] DNA amplification and sequencing of the seven housekeeping genes (*acsA*, *nuoD*, *trpE*, *mutL*, *guaA*, *aroE* and *ppsA*) were performed. The nucleotide sequences, determined by using published primers,[10,11] and were compared with existing sequences in the MLST database (www.pubmlst.org/paeruginosa). The eBURST algorithm (http://eburst.mlst.net/) was used for phylogenetic analysis.[12,13] The MLST analyses indicated that this strain (ST235) belongs to an internationally spreading clonal complex (CC11), previously identified in VIM metallo-β-lactamase and PER-1 ESBL-producing *P. aeruginosa* strains from patients in Hungary in 2008.[2,13] A PER-1, OXA-2, OXA-74 and OXA-50-producer strain with the same ST was isolated from an outbreak in Poland in 2007.[14] Although our strain was probably imported from Romania, the known virulence of the CC11 members of MLST types of *P. aeruginosa*, and the geographical closeness of Hungary, Romania and Poland, suggest that the same clone, capable of acquiring several resistance mechanisms, may be circulating in these countries.

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Diagnostic performance of procalcitonin for bacteremia in patients with bacterial infection at the emergency department

Dear Editor,

Recently, Limper et al published a review article and concluded that procalcitonin (PCT) was helpful in discriminating infectious from non-infectious fever, such as autoimmune, inflammatory, and malignant disease.[1] Fever is a common presentation of patients admitted to the emergency department (ED), and bacterial infection is one of the most important etiologies. Early and accurate identification of bacterial infection is of primary importance, because early institution of an appropriate antimicrobial regimen in infected patients is associated with better outcome. Bloodstream infection or bacteremia is one of the most serious infectious diseases encountered in the ED, but timely identification of these patients is a great challenge

ARTICLE III.

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Successful application of the DiversiLab repetitive-sequence-based PCR typing system for confirmation of the circulation of a multiresistant *Pseudomonas aeruginosa* clone in different hospital wards☆

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Abstract

The applicability of the repetitive-sequence-based PCR (rep-PCR)–based DiversiLab system was tested compared with the pulsed field gel electrophoresis (PFGE) to type a phenotypically similar subset of a large collection of multiresistant *Pseudomonas aeruginosa* strains isolated during a 17-month period from patients treated in different wards including 4 intensive care units (ICUs). Five environmental *P. aeruginosa* isolates obtained from one of the ICUs were also included. The DiversiLab system and the PFGE demonstrated the genetic relationship among the isolates with the same efficacy. One of the environmental isolates had the same rep-PCR type as the circulating clone. Multilocus sequence typing of one of the clinical isolates of the circulating clone proved that it is a member of a clonal complex of *P. aeruginosa* that has not been previously described in clinical samples.

Keywords: Molecular typing methods; Epidemiologic analysis; Multiresistant *P. aeruginosa*

The surveillance systems of nosocomial infections must be rapid, reliable, and capable of differentiating between related and unrelated bacterial isolates, especially in highly critical areas such as intensive care units (ICUs). However, ICUs are often the scene of the sporadic appearance of multiresistant pathogens such as *Pseudomonas aeruginosa*, which can be the origin of nosocomial outbreaks. An outbreak may be defined as a relatively rapid increase in the incidence of infection or colonization by a certain bacterial strain, caused by enhanced patient-to-patient transmission (Belkum et al., 2007). The sporadic isolation of multiresistant strains does not often draw attention on the possible nosocomial spread of 1 clone. The collection in routine laboratories of multiresistant, potential nosocomial pathogens for further typing may help to discover hidden reservoirs and the circulation of possible outbreak clones in different wards. The aim of the present study was to use the pulsed field gel electrophoresis (PFGE) and the DiversiLab system to look for genotypic similarities among phenotypically similar multiresistant clinical *P. aeruginosa* isolates. Multilocus sequence typing (MLST) was also used to compare clonal relationship of our circulating clone with other known *P. aeruginosa* clones frequently isolated in other geographic areas.

From June 2007 to November 2008, multiresistant *P. aeruginosa* was isolated from 29 different patients during their stay in different wards, including 4 ICUs at the University Hospital in Szeged, Hungary. Patient's isolates treated in some other non-ICU wards were also included. Clinical evidences indicated infection in 20 patients, and 9 patients displayed simple colonization (Table 1). The isolates were originally collected for investigation of

☆ Part of this study was presented as a poster, "The applicability of three different methods for the molecular typing of *Pseudomonas aeruginosa*," during the 19th ECCMID in Helsinki 2009 (P519).

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Table 1
Data on the 29 multiresistant *P. aeruginosa* isolates obtained from different patients

Patients	Patient's ward at the time of isolation	First date of isolation	Wards where the patient was		I/C	Typing	
			Before	after		PFGE	DiversiLab
			isolation of the strain				
1	MICU	13/06/2007	SICU3		I	PT-A	Clone 1
2	SICU3	20/07/2007			I	PT-A	Clone 1
3	MICU	27/11/2007	SICU3		C	PT-A	Clone 1
4	MICU	02/01/2008			I	PT-A	Clone 1
5	MICU	09/01/2008			I	PT-A	Clone 1
6	SICU2	07/07/2008			C	PT-A	Clone 1
7	SICU1	15/07/2008			I	PT-A	Clone 1
8	SICU2	21/07/2008	NS		C	PT-A	Clone 1
9	SICU2	23/07/2008			I	PT-A	Clone 1
10	SICU2	24/07/2008	MICU	MICU	I	PT-A	Clone 1
11	MICU	26/07/2008	SICU1		I	PT-A	Clone 1
12	SICU2	30/07/2008	NE	NE	I	PT-A	Clone 1
13	MICU	02/08/2008			I	PT-A	Clone 1
14	SICU2	08/08/2008	NS		C	PT-A	Clone 1
15	SICU3	25/08/2008			I	PT-A	Clone 1
16	MICU	01/09/2008			C	PT-A	Clone 1
17	NE	02/09/2008	SICU2		I	PT-A	Clone 1
18	SICU1	03/09/2008			I	PT-A	Clone 1
19	SICU1	15/09/2008	MICU	MICU	I	PT-A	Clone 1
20	MICU	26/09/2008			I	PT-A	Clone 1
21	MICU	30/09/2008			C	PT-A	Clone 1
22	SICU1	02/10/2008			I	PT-A	Clone 1
23	MICU	09/10/2008			I	PT-A	Clone 1
24	SICU1	10/10/2008			C	PT-A	Clone 1
25	SICU2	13/10/2008			C	PT-A	Clone 1
26	MICU	17/10/2008			I	PT-A	Clone 1
27	IM	20/10/2008	SICU2	MICU	I	PT-A	Clone 1
28	SICU2	20/10/2008			I	PT-A	Clone 1
29[a]	SICU2	13/08/2008			C	PT-B	Clone 2

I/C = infected/colonized; MICU = medical ICU; SICU1 = general surgical ICU; SICU2 = trauma and neurosurgical ICU; SICU3 = cardiac surgical ICU; NS = neurosurgical ward; NE = neurology ward; IM = internal medical ward.

[a] *P. aeruginosa* strain with a different antibiotic resistance profile isolated from the same hospital ward.

the resistance mechanisms behind the multiresistance of the *P. aeruginosa* and comprised a subset of a large collection of carbapenem-resistant isolates obtained in 2007 and 2008. Twenty-eight of the 29 clinical isolates had the same antibiotic resistance profile (susceptible only to amikacin, netilmicin, and polymyxin B), and 1 exhibited a slightly different resistance profile (susceptible only to amikacin and polymyxin B). Environmental sampling was carried out in one of the ICUs during the same period in connection with the regular hygienic control of the ward. Five *P. aeruginosa* strains were isolated from different surfaces and included in this study (Table 2).

PFGE analysis was performed by a slight modification of a procedure described previously (Deplano et al., 2005). All 29 clinical isolates were digested with *Spe*I, and the fragments were separated by electrophoresis for 12 h in 1% agarose gels with a CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA). PFGE patterns were analyzed visually according to criteria described previously (Belkum et al., 2007; Tenover et al., 1995), and 2 unique patterns (designated as pulsotypes, PT-A and PT-B) were found. PT-A was observed in 28 of the 29 isolates, and PT-B corresponded to the isolate with the different antibiotic resistance profile (Table 1). Banding patterns are not shown.

The 29 clinical and the 5 environmental *P. aeruginosa* isolates were fingerprinted by using the automated, repetitive-sequence-based PCR (rep-PCR)–based DiversiLab system, version 3.3. After extraction of the DNA, the isolates were fingerprinted with the Pseudomonas kit (DiversiLab), following the instructions of the rep-PCR worksheet. After the amplification, the amplicons were loaded onto a

Table 2
Environmental *P. aeruginosa* isolates obtained in medical ICU and typed by the DiversiLab system

Environmental sample	Isolates	Type with DiversiLab
Right sink drain	*P. aeruginosa*	Clone 1
Left sink drain	*P. aeruginosa*	Clone 3
Mechanical ventilation system	*P. aeruginosa*	Clone 3
Wall behind sink	*P. aeruginosa*	Clone 4
Tap water	*P. aeruginosa*	Clone 5

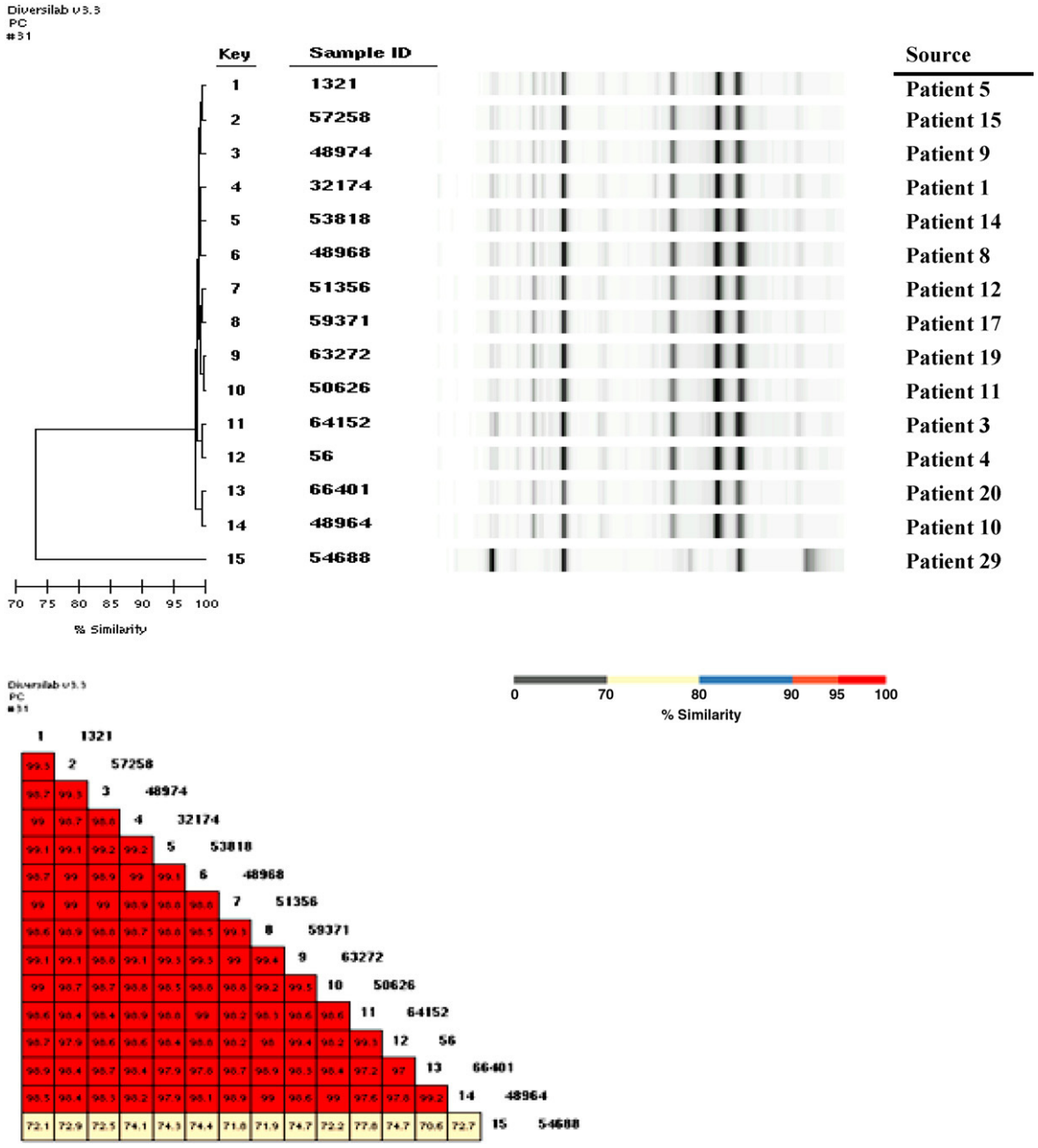

Fig. 1. Dendrogram, virtual gel image, and the similarity matrix of some of the 29 investigated *P. aeruginosa* isolates typed with the DiversiLab. 1–14: related *P. aeruginosa* isolates (clone 1). 15: unrelated *P. aeruginosa* isolate with a different antibiotic resistance profile (clone 2).

microfluids chip and the products were detected with the Agilent 2100 Bioanalyzer (Caretto et al., 2008; Healy et al., 2005). Automatically generated reports included dendrograms, virtual gel images, similarity matrices, scatter plots, and selectable demographic fields. Twenty-eight of the 29 clinical isolates were clustered together. The remaining isolate had a different rep-PCR profile together with the slightly different antibiotic susceptibility (Table 1). Fig. 1 depicts the similarity matrix and the virtual gel images of 14 selected patients isolates clustered together, and the one with the different rep-PCR pattern. Fig. 2 shows the similarity matrix and the rep-PCR patterns created by the DiversiLab for the 5 environmental *P. aeruginosa* isolates with 4 different clones. One of these isolates had the same rep-PCR profile as that of a representative strain of the circulating multiresistant clinical *P. aeruginosa* (clone 1) (Tables 1 and 2). Two environmental *P. aeruginosa* isolates were also clonally related (clone 3), but not related to the strains colonizing or infecting the 28 patients.

MLST typing was performed on 1 selected clinical isolate corresponding to PT-A/clone 1. Amplification and sequencing of the 7 housekeeping genes suggested by Curran et al. (2004)

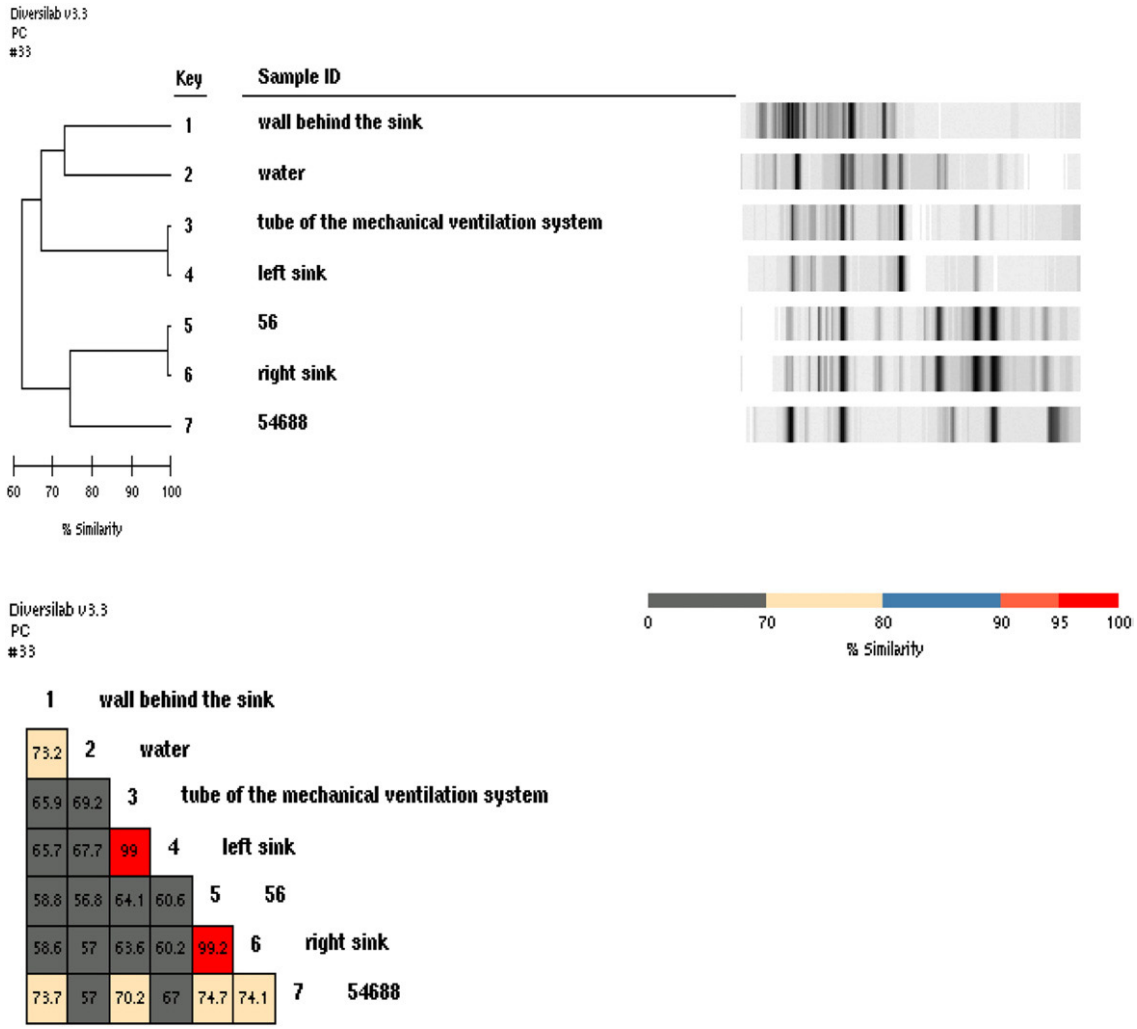

Fig. 2. Dendrogram, virtual gel image and the similarity matrix of the environmental *P. aeruginosa* isolates, and the representative PT-A and PT-B typed with the DiversiLab system. 1–4, 6: environmental isolates. 5: representative patient isolate (patient 12) (corresponds to PT-A, clone 1). 7: isolate with a different antibiotic resistance profile (corresponds to PT-B, clone 2) (patient 29).

were performed. The nucleotide sequences were determined by using previously published primers (Curran et al., 2004; Johnson et al., 2007) and were compared with existing sequences in the MLST database (http://www.pubmlst.org/paeruginosa). The eBURST algorithm (http://www.eburst.mlst.net/) was used for phylogenetic analysis (Feil et al., 2004; Libisch et al., 2008). The MLST revealed a new sequence type (ST), namely, ST618, belonging to a clonal complex previously not described, containing 6 STs. The data on the investigated strain are available at http://www.pubmlst.org/paeruginosa.

The DiversiLab system was recently evaluated by several authors for confirming different outbreaks caused by *Staphylococcus aureus*, *Acinetobacter baumannii*, *Klebsiella* spp., or *P. aeruginosa* (Caretto et al., 2008; Doléans-Jordheim et al., 2009; Fontana et al., 2008; Grisold et al., 2009) or to test the discriminative power of it in the case of known outbreak strains (Grisold et al., 2010; Tenover et al., 2009) in comparison with the PFGE. In most cases, DiversiLab system proved to be a rapid and reliable method for molecular analysis of suspected nosocomial outbreaks cased by methicillin-resistant *S. aureus*, *A. baumannii*, and extended-spectrum β-lactamase–producing *Klebsiella* spp. It was possible to complete typing and cluster analysis automatically in a short period (Grisold et al., 2009). In the case of *P. aeruginosa*, Doléans-Jordheim et al. (2009) emphasize that rep-PCR provided results similar to those obtained by the PFGE-*Spe*I typing method, and it was most reliable on nosocomial outbreak isolates.

At the time of the isolation of our strains, epidemiologic relatedness between the infected or colonized patients treated in different wards was not suspected. The phenotypical similarities draw the attention of possible clonal relatedness of this subset of isolates, which was easily confirmed first by the DiversiLab system and after by PFGE. When we later followed the routes of the patients before and after isolation of the multiresistant *P. aeruginosa* strain retrospectively, it turned out that some of the patients visited different wards where patients, infected or

colonized with the same multiresistant clone, were also present previously (Table 1).

MLST proved that the circulating *P. aeruginosa* strain is a member of a clonal complex that has previously not been described in clinical samples. *P. aeruginosa* is known to have nonclonal population structure punctuated by successful epidemic clones or clonal complexes (Curran et al., 2004). Accordingly, our MLST result may draw the attention of epidemiologists to the possibility that some clonal lineages are more likely to be spread among patients and/or cause infections. It has been shown that only the MLST typing method provides information concerning the clonal relationship of the bacterial isolates (Curran et al., 2004; Johnson et al., 2007; Turner and Feil, 2007; Urwin and Maiden, 2003). PFGE- and rep-PCR–based methods have higher discriminatory power than MLST (Johnson et al., 2007); accordingly, the implication of MLST for population genetics and dynamics may be more significant than for everyday molecular typing because the polymorphism in the slowly evolving target genes may not be high enough for useful epidemiologic comparisons.

In conclusion, in our case DiversiLab system and PFGE-*Spe*I typing of *P. aeruginosa* strains proved with the same efficacy the circulation of a successful multiresistant clone on different wards. According to our experience, the collection by clinical microbiology laboratories of multiresistant isolates potentially involved in nosocomial infections even in nonepidemic situation could be important for the later analysis by molecular typing methods.

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ARTICLE IV.

Characterisation of *Pseudomonas aeruginosa* strains isolated from patients suffering from cystic fibrosis in South-East Hungary

manuscript

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Introduction

Cystic fibrosis (CF) is an autosomal recessive disorder that occurs approximately in 1:2000 Caucasian children, in Hungary the rate is 1:4000 [1]. The highest incidence of CF patients in the world, 1:1353, is found in Ireland [2]. Cystic fibrosis is caused by abnormal functioning of the CFTR protein, the Cystic Fibrosis Transmembrane Regulator, which is a cAMP induced chloride channel, and is expressed on the apic membrane of the cells. The gene encoding the CFTR protein is expressed mainly in epithelial cells in the airway, kidneys, pancreas, bile ducts, reproductive organs, and the bowel. The main function of CFTR protein is to transport chloride ions across the cell membrane. CFTR has two nucleotide-binding domains, both of them is capable to bind and hydrolyze ATP, which helps to open and close the channel. An alteration of the *cftr* gene causes the disease. The alteration in *cftr* gene, therefore the amino acid residues, can lead to a change in diameter size of the pore, causing loss in function of the channel. The mutations can occur in different areas of the *cftr* gene, each mutation is associated with a different phenotype, and results in a different prognosis for the patient. In Europe, the most common mutation is the ΔF508, which refers to a deletion of a phenylalanin at position 508 in the protein. The initial diagnosis of the disease is made via sweat test, since CF patients produce higher levels of sodium chloride in their sweat (>60 mmol/L). Many newborns are screened for CF if they present with meconium ileus. If the screening sweat test is positive, additional confirmatory tests are carried out via molecular methods to detect the exact alteration of the *cftr* gene.

The pathophysiology of this disease is poorly understood, and symptoms range from gastrointestinal or nutritional abnormalities, reproductive problems as an adult, chronic sinusitis, endobronchial disease. In the lungs the loss of the CFTR protein function affects the volume and viscosity of the airway surface liquid, which cause poor mucociliary clearance. There is an ongoing debate whether the CF lung becomes first infected by bacteria followed by inflammation, or vice versa, but it is a fact that bacteria infect the respiratory tract usually early in the course of cystic fibrosis disease, often fail to be eradicated. The bacterial infections together with an aggressive host inflammatory response are the key players in the irreversible airway damage from which most patients die. The contact with these bacteria usually lead to chronic colonisation of the lung with exacerbations over time. There is a pattern of the colonisation of the CF lung, which usually involves *Staphylococcus aureus* in infancy, followed by *Haemophilus influenzae,* as a child, *P. aeruginosa, Stenotrophomonas maltophilia, Achromobacter xylosoxidans* in teenage years, *Burkholderia cepacia* complex,

non-tuberculous mycobacteria in early twenties, and several newer species, including moulds, are becoming more common [3].

P. aeruginosa is one of the main organisms responsible for drug-resistant nosocomial infections, and emerged as the most important bacterial pathogen in lung disease of CF patients, with a high prevalence in adults, up to 80%. In addition being intrinsically resistant to several antimicrobial agents, *P. aeruginosa* can easily develop resistance to all conventional antipseudomonal antibiotics via different mechanisms. After first infecting the host, *P. aeruginosa* has to survive host defences, and repeated courses of antibiotic treatment. The bacterium has a repertoire of immunoevasive strategies includes the secretion of exoproducts, antibiotic-resistance proteins, and phenotypic changes, which make them virtually unrecognizable from the bacteria, that was first isolated, e.g. the siderophores, such as pyocianin that breaks down intercellular tight junctions, slow the ciliary beat frequency, therefore affect mucociliary clearance. *P. aeruginosa* strains found in the CF lung are unusually hypermutable, they can react promptly to their environment, not only by switching genes on and off, but by an increased frequency of mutation events within the genome. One such mutation triggers conversion to a mucoid phenotype. Over-producing mucoid exopolysaccharide (alginate) surrounds and protects them from external affects, such as mucociliary clearance, immune response or antibiotics. Another highly successful survival strategy involves the formation of biofilms. In the CF lung *P. aeruginosa* and *Burkholderia cepacia* isolates exist in biofilms, which protects them from phagocytosis, and prevents penetration of antibiotics. This phenotypic change plays a major role in the persistence of pseudomonas infection in the majority of CF patients, despite best medical attempts at eradication [4].

The aims of this study were to investigate *P. aeruginosa* isolates infecting/colonising patients suffering from cystic fibrosis, namely:

- to determine the different resistance mechanisms, especially those causing resistance to carbapenems
- to study the molecular relatedness between isolates, to find the most reliable typing method in case of these isolates

Methods and materials

Bacterial isolates

From 2003 to 2007 149 *P. aeruginosa* were isolated from 16 patients suffering from cystic fibrosis, attending the CF centre in the Children's Hospital of Szeged, Hungary. In this period of time all isolates from the 16 patients were stored for further analysis, regardless their antibiotic susceptibility. 78 isolates (52%) had mucoid colony morphology at the time of the isolation. Identification of the isolates by VITEK 2 system (bioMérieux) and susceptibility test by disk-diffusion method and E-test if required were performed, according to CLSI guidelines. After isolation, the strains were stored at -70 °C in CryoBank medium (Copan diagnostic Inc., California, USA) for further investigation.

To verify whether strains found in different patients may have been transmitted from patient-to patient, we tracked possible contacts among the different patients with the MedSol patients' data system used in the hospital, and by contacting the patient's clinicians.

Detection of different antibiotic-resistance mechanisms in *P. aeruginosa* isolates

Of the 149 isolates 5 were resistant to ceftazidime and to cefepime. ESBL genes were searched from these isolates. The genes of the extended spectrum β-lactamases (PER-1, PER-2, TEM, SHV, GES, VEB-1, OXA groups [5] were sought by PCR methods in a representative *P. aeruginosa* strain from each genotype, confirmed by sequencing.

Of the 149 isolates 26 were resistant to at least one carbapenem. These imipenem and/or meropenem resistant isolates were selected for further analyses. MBL presence or absence was confirmed by PCR, aimed the $bla_{\text{SPM-1}}$, $bla_{\text{GIM-1}}$, and the most common bla_{VIM} and bla_{IMP} genes [6]. PCR master mix was prepared according to the standard protocol: 1 x PCR buffer, $MgCl_2$ 2,5 mM, dNTP 200 µM, primers 1-1 µM, Taq polymerase 1 U/reaction, sterile water. Acquired metallo-β-lactamases (MBLs) and some other antiobiotic resistance markers (especially aminoglycoside-resistance genes) are mostly encoded by integron-borne genes. Therefore class1 integrons were sought, using PCR.

In order to study the antibiotic resistance due to the efflux-pump overexpression, real-time PCR was performed, based on the following criteria:

- isolates resistant only to imipenem were tested for OprD deficiency;

- isolates resistant to imipenem and/or meropenem and cefepime, but not to ceftazidime were tested for OprD deficiency and for overexpression of MexXY-OprM;
- isolates resistant imipenem and/or meropenem, cefepime and to ceftazidime were tested for OprD deficiency and for overexpression of MexXY-OprM, MexAB-OprM, MexCD-OprJ.

The expression of the chromosomal genes encoding the *opr*D, *mex*B, *mex*X, *mex*C was studied with real-time reverse transcriptase PCR assays according to Quale et al. [7]. RNA was isolated using the method by Palagyi-Meszaros et al [8]. Briefly, the isolate was grown in 60 mL of liquid medium in a hypovial to A600 nm = 1–1.5; 15 mL of culture was centrifuged at 15 000 g for 2 min, the pellet was suspended in 300 μL of SET buffer [20% sucrose, 50 mm EDTA (pH 8.0) and 50 mm Tris ⁄ HCl (pH 8.0)] and 300 μL of SDS buffer was added [20% SDS, 1% (NH4)2SO4, pH 4.8]; 500 μL of saturated NaCl was added next, the sample was centrifuged at 20 000 g for 10 min and the clear supernatant was transferred into a new tube. 2-Propanol (70% of the total volume of the supernatant) was added to the solution and the mixture was centrifuged at 20 000 g for 20 min. The pellet was washed twice with 1 mL of 70% ethanol. The dried pellet was suspended in 20 μL of diethylpyrocarbonate-treated water.

The expression of mRNA for the genes of interest was optimised to that of the housekeeping gene *rps*L. This gene is known to be expressed consistently in *P. aeruginosa* [9]. Normalized expression of each gene was calibrated to the mRNA expression of the reference strain *P. aeruginosa* PAO1, results are given as the relative expression of the mRNA compared to that of *P. aeruginosa* PAO1. The following values were considered to represent overexpression compared to the control strain: for *mex*B ≥3-fold; for *mex*X≥10-fold; *mex*C≥2-fold; and reduced expression of *opr*D≤0,7-fold [7].

Molecular typing of *P. aeruginosa* isolates

For bacterial typing with PFGE and DiversiLab methods 44 isolates were selected from the 149 isolates, based on different antibiotic susceptibility profile.

PFGE analysis was performed as described previously by Deplano *et al*. with a slight modification [10]. The DNA of the isolates were digested with *Spe*I, and the fragments were separated by electrophoresis in 1% agarose gels with a CHEF- DR III System (Bio-Rad Laboratories) for 12-12 h, with the switch times ranging from 4 s to 12 s, and from 15 s to 36 s in a Tris-borate-EDTA buffer containing 50 μM thiourea. Gels for PFGE were stained in an

aqueous solution containing 0.5 µg ethidium-bromide and photographed under UV translumination. Patterns of PFGE were visually analysed according to the criteria described previously [11,12].

The selected *P. aeruginosa* isolates were fingerprinted using the automated, rep-PCR based DiversiLab system (bioMériux), version 3.3. DNA was extracted from the bacteria using the Mo Bio Ultra Clean™ Microbial DNA Isolation Kit (MoBio Laboratories Inc) kit according to the manufacturer's instructions. Fingerprinting of the isolates was performed with the Pseudomonas kit (DiversiLab) following the instructions of the rep-PCR worksheet. After the amplification, the amplicons were loaded on a microfluids chip and detected, using the Agilent 2100 Bioanalyzer. The results were sent via internet to the hospital's secured DiversiLab website. The analysis of the data was performed using the DiversiLab software using the Pearson correlation coefficient. Reports were automatically generated, included dendograms, virtual gel images, similarity matrix, scatter plots and selectable demographic fields.

Six isolates were randomly selected from different patients for MLST analysis, as described previously by Curran et al [13]. Briefly, the genomic DNA was purified using the Mo Bio Ultra Clean™ Microbial DNA Isolation Kit according to the manufacturer's instructions. DNA amplification and sequencing of the seven housekeeping genes (*acsA, nuoD, trpE, mutL, guaA, aroE, ppsA*) were performed. The nucleotide sequences were determined by using previously published primers [14,13] and were compared to existing sequences in the MLST database (www.pubmlst.org/paeruginosa). The eBURST algorithm (http://eburst.mlst.net/) was used for phylogenetic analysis [15,16].

Results

Antibiotic resistance in *P. aeruginosa* isolates

From 2003 to 2007 149 *P. aeruginosa* were collected from 16 different patients suffering from cystic fibrosis, attending the CF centre in Szeged. The percentage of the isolates susceptible to the tested agents were as follows: piperacillin/tazobactam: 100 %, cefoperazone: 87,5 %, ceftazidime: 93,75 %, cefepime: 68,8 %, imipenem: 81,3 %, meropenem: 87,5 %, gentamycin: 37,5 %, tobramycin: 68,8 %, amikacin: 38,8 %, netilmicin:

62,5 %, ciprofloxacin: 93,75 %, levofloxacin: 68,8 %, moxifloxacin: 43,8 %, polymixin B: 100%. The highest resistance was seen in case of gentamycin, amikacin and moxifloxacin.

Of the 149 isolates 26 were resistant to at least one carbapenem. These imipenem and/or meropenem resistant isolates were selected for MBL detection and PCR detection of class 1 integrons. No MBL genes were found with PCR. From isolate RN62639 a 689 bp class 1 integron was amplified, encoding an *aac*A4 aminoglycoside-acethyltransferase gene (AAC(6')II), that confers resistance to tobramycin and amikacin. Of the 149 isolates 5 were resistant to ceftazidime and to cefepime. ESBL genes were searched, and in isolate RN64944 an OXA-10 ESBL were found. No other β-lactamase or aminoglycoside-resistance genes have been detected.

Seven isolates were selected for RT-PCR analysis, examining efflux pumps and porins, based on the criteria detailed in Materials and Methods. One isolate showed inconclusive results. OprD deficiency was detected in 5 of the 6 cases, hyperproduction of the MexXY-OprM efflux pump was detected in 3 isolates, overexpression of MexAB-OprM was detected in one isolate at a relatively high level, (148 fold). No isolates hyper-produced the MexCD-OprJ efflux pump.

Molecular typing of *P. aeruginosa* isolates recovered from cystic fibrosis patients

Of the 149 *P. aeruginosa* isolates 44 were selected for bacterial typing by PFGE and rep-PCR-based DiversiLab methods, 27 (61 %) of them had mucoid colonies. In average ~3 (2,75) isolates per patient were typed. With PFGE 16 different genotypes were determined. In case of 13 patients one genotype was recovered per patient from their sputum samples, in case of three patients, two different strains caused the infection/colonisation. With PFGE the same genotypes, designated as pulso-type K determined typed from two different patients, such as pulso-type L, and pulso-type F (**Table 1**).

With DiversiLab 20 different genotypes were determined. The results with the exception of two patients were contradictory with the PFGE results. With DiversiLab the same genotype (designated as CloneA, CloneC, CloneD) were determined from more than one patient in three cases (data not shown).

Six CF isolates were randomly selected from different patients for MLST analysis. Sequencing the seven alleles resulted six different STs, namely ST343, ST614, ST277, ST625, ST626 and ST627. The results of the MLST analyses are available at

http://pubmlst.org/paeruginosa. With the exception of ST625, which is a member of an uncharacterized clonal complex, all STs are singletons.

Discussion

Among the 149 *P. aeruginosa* isolates the highest resistance was seen in case of gentamycin, amikacin and moxifloxacin (62,5 %, 61,2 %, 56,2 % respectively). Otherwise the strains had a good in vitro susceptibility, which results do not follow the international tendency [17,18,19]. Among our CF isolates were no pan- or multidrug resistant *P. aeruginosa*, and no developing resistance was observed due to the antimicrobial treatments.

No MBLs were found, only one class 1 integron, and an OXA-10 ESBL was found among the isolates. Isolate RN64944 producing the OXA-10 ESBL was resistant to cefepime, but susceptible to all other β-lactams. Isolate RN62639 harbouring the class 1 integron was resistant to all aminoglycosides. Isolates being only resistant to imipenem were relatively prevalent, the porin channel analyses proved, that this could be the effect of OprD deficiency. Isolate BoT83704, which proved to overexpress the MexAB-OprM efflux pump, was resistant to both carbapenems and all fluoroquinolones. These antibiotic resistance patterns correspond with the determined resistance mechanisms.

Based on the different antibiotic susceptibility profile 44 isolates were selected for bacterial typing. With the PFGE method 16 different genotypes were determined. In case of 13 patients one genotype were recovered per patient from their sputum samples, so these patients were colonised/infected with the same strain from 2003 to 2007. In case of three patients, two different genotypes could be isolated from their sputum, so they were colonised/infected with different strains. Overall a high diversity was observed, which corresponds to the international literature [20]. The isolates were also typed by the DiversiLab system. The virtual gel image showed too many bands with similar sizes in almost all of the strains due to an unknown reason (**Figure 1**). Results did not correspond neither with the antibiotic susceptibility pattern, nor with the PFGE results, which is considered to be the gold standard method of bacterial typing.

The same genotype (designated as CloneA, CloneC, CloneD, data not shown) were determined from more than one patient in three cases. PFGE analysis could not confirm these results, and also the clinical background studies showed that these patients were not hospitalised at the same time or did not participate the same CF camp, so the cross-infection could be excluded. Considering all the above results, we concluded that during the molecular

typing of CF isolates the PFGE method is found to be more accurate and reliable than the DiversiLab system.

Until recently, CF patients were thought to be colonised with their own unique *P. aeruginosa* strain acquired from the environment. Cross-infection was considered a relatively infrequent source of acquisition. With PFGE the same genotype (designated as pulso-type F) was isolated from patients DM and LA from 2005 (**Table 1.**), when they participated in the same CF-camp. Pulso-type L was isolated from patients TZ and KT from 2005. At that time, they were hospitalised at the same time in the same hospital ward. Pulso-type K recovered from patients BoT and TZ. The strains were isolated in 2003, and according to their clinical background the two patients attended the same CF-camp in Budapest, Hungary. Interestingly, the MLST results suggest that the latter strain, being a member of a clonal complex, and not a singleton, could be a member of an epidemic clone (**Table 1**). However the remaining five strains examined with MLST proved to be singletons with this method, which suggests that the prevalence of the cluster genotypes is significantly lower among these CF isolates. With our results we proved, that the transmission of a certain strain from patient to patient in a hospital ward or in a CF camp is expected, but not inevitable. Nevertheless the incidence of the cross-infections in the CF centre in Szeged were relatively low due to the infection control measures applied to prevent patient-to-patient contacts, such as scheduling appointments with the clinicians at different time points, trying to hospitalize the patients in different hospital rooms.

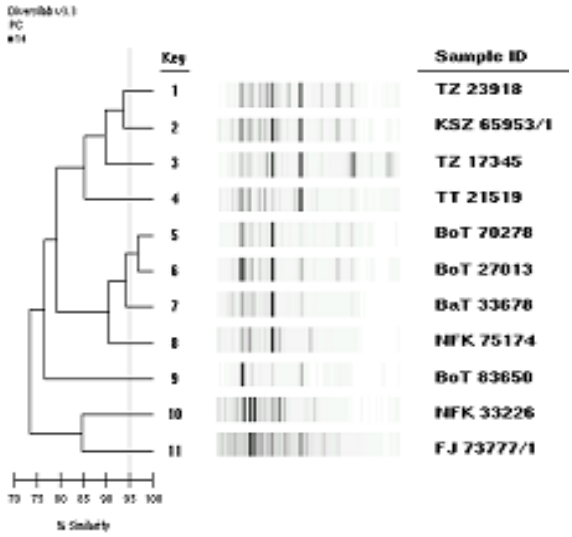
Based on the present study we can conclude that the *P. aeruginosa* isolates obtained from CF patients have an overall good in vitro susceptibility, which results are in contrast with the international tendency. Among our isolates there were no MBL producer, no multi-or pandrug resistant isolate, and no therapy-induced resistance was observed. During the molecular typing of CF isolates the DiversiLab system is found to be too discriminative and not as reliable as the PFGE method. These isolates have a high genetic diversity, no clonality was observed, which corresponds to the international literature. With the exception of a few sporadic cases of transmission, there were no highly epidemic strain isolated, which is expected to be a result of the good infection control measures taken by the clinicians, nurses of the CF centre, and of the good patient compliance.

Table 1. Different CF patients colonized/infected with the same pulso-type of *P. aeruginosa*, and the result of MLST analysis, if it was performed.

Patient	Strain	Year of isolation	Mucoid	PFGE pulso-type	MLST
DM	63282	2005	+	F	NT
LA	61644	2005	+	F	NT
BoT	27013	2003	-	K	NT
	83650	2005	-	K	ST626
TZ	23513	2003	-	K	NT
	23918	2005	-	L	ST625*
KT	63285	2005	-	L	NT

NT: not tested, * marks the ST that is a member of a clonal complex.

Figure 1. The virtual gel image of some of the examined CF isolates shows the presence of too many bands in case of almost all of the isolates.



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