



BRIEF REPORT

Genomic Epidemiology of MBL-Producing *Pseudomonas putida* Group Isolates in Poland

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ABSTRACT

Introduction: *Pseudomonas putida* group are described as low-incidence opportunistic pathogens, but also as a significant reservoir of antimicrobial resistance (AMR) genes, including those of metallo- β -lactamases (MBLs). Our objective was the molecular and genomic characterization of MBL-producing *P. putida* (MPPP) group isolates from Poland, focusing on population structures, successful genotypes and MBL-encoding integrons.

Methods: During a country-wide MBL surveillance in *Pseudomonas* spp., 59 non-duplicate MPPP isolates were collected from 36 hospitals in 23 towns from 2003 to 2016. All of the isolates were subjected to whole-genome sequencing (WGS), followed by species identification, multi-locus sequence typing (MLST),

single-nucleotide polymorphism (SNP)-based phylogenetic/clonality analysis, resistome determination, and susceptibility testing.

Results: The study collection comprised 12 species, of which *P. alloputida* ($n = 19$), *P. monteilii* ($n = 15$), and *P. asiatica* ($n = 11$) prevailed, while the others were *P. kurunegalensis*, *P. putida*, *P. soli*, *P. mosselii*, *P. juntendi*, and four potentially new species. MLST classified the isolates into 23 sequence types (STs) of which 21 were new, with three main clones, namely *P. alloputida* ST69, *P. monteilii* ST95 and *P. asiatica* ST15. The isolates produced VIM-like MBLs only, largely VIM-2 ($n = 40$), encoded by 24 different class 1 integrons (ten new), a number of which occurred also in *P. aeruginosa* and/or Enterobacterales in Poland. The plasmid pool was dominated by IncP-9, IncP-2, and pMOS94-like types. Multiple isolates were extensively drug-resistant.

Conclusions: This study, being one of the most comprehensive analyses of MPPP so far, has shown high diversity of the isolates in general, with three apparently international lineages, each internally diversified by MBL-encoding structures.

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Key Summary Points

Why carry out this study?

Pseudomonas putida group are a low-incidence, though emerging opportunistic pathogens, and have been described as a potentially significant reservoir of antimicrobial resistance (AMR) genes, including those of metallo- β -lactamases (MBLs), for *Pseudomonas aeruginosa* or other Gram-negatives. So far, the data on population structures, and possible hospital-adapted and broadly spreading lineages, has been scarce.

What did the study ask?

The study was carried out on a unique group of MBL-producing *P. putida* (MPPP) isolates, collected by a routine surveillance from all over Poland between 2003 and 2015, and its main objectives were to reveal genome-based clonal structures of individual species populations, to compare these to all isolates available in international sequence databases, and to analyze their AMR, mainly MBL, genetic determinants against Polish MBL-producing *P. aeruginosa* and Enterobacterales isolates.

What were the study outcomes/conclusions?

The MPPP collection turned out to be highly diverse, however, three major species, *P. alloputida*, *P. monteilii* and *P. asiatica*, apparently have segregated international phylogroups or lineages, possibly adapted to nosocomial settings. The isolates were extensively multi-drug-resistant and largely shared their MBL determinants with Polish *P. aeruginosa* and Enterobacterales isolates from the same period; however, the hypothesis of the MPPP being an MBL reservoir for the other organisms seems to be rather unlikely in Poland.

What was learned from the study?

Several species of the *P. putida* group form populations of dynamic structures, producing lineages spreading over large distances and adapting to nosocomial environments. Readily acquiring resistance, they contribute to pools of AMR genes circulating among pathogens in these settings.

INTRODUCTION

Pseudomonas is one of the most abundant and diverse genera in the bacterial kingdom, consisting of nearly 300 valid species (<https://lpsn.dsmz.de/genus/pseudomonas>; accessed on April 8, 2022) [1, 2]. All these have been split into 16 groups, of which those of *P. fluorescens* and *P. putida* are the most numerous [1]. The latter one comprises common rhizosphere and freshwater dwellers, capable of metabolizing a wide range of biogenic and xenobiotic compounds [3], however, also capable to cause infection [4–7]. These may demonstrate multi- or extensive drug resistance (MDR and XDR, respectively), namely non-susceptibility to at least one agent in at least three classes of antipseudomonadal drugs, and non-susceptibility to at least one agent in all but one or two classes, respectively [8]. MDR and XDR are associated with various antimicrobial resistance (AMR) determinants, including class B carbapenemases or metallo- β -lactamases (MBLs), which hydrolyze most of β -lactams and are not inhibited by any of the currently used β -lactamase inhibitors [9, 10]. Of all MBLs, VIM and IMP types, usually encoded by class 1 integrons, are the most frequent in pseudomonads [4–6, 9–15]. In several cross-sectional population studies, it has been suggested that the *P. putida* group may act as a significant AMR reservoir [4, 11, 13, 16], however, not all authors have shared this opinion [17]. Out of 51 species currently included in the *P. putida* group, a small portion have only been repeatedly identified in nosocomial infections, namely *P. alloputida*, *P. monteilii*, *P. asiatica*, *P. kurunegalensis*, and *P. juntendi* [4, 6, 18]. For the

majority of those, the broader population clonal structures and potentially epidemic genotypes remain almost unexplored [19]. The aim of this analysis was to reveal the species composition and molecular epidemiology of the MBL-producing *P. putida* (MPPP) group isolates, collected in Poland over a 13-year period, following our previous studies on MBL-producing *P. aeruginosa* (MPPA) [20–22].

METHODS

Bacterial Isolates

Pseudomonas putida group isolates were sent along with *P. aeruginosa* to the National Reference Centre for Susceptibility Testing (NRCST) in Warsaw as putative MBL producers within the MBL surveillance in *Pseudomonas* spp. [20, 22]. These were tested with MBL phenotypic and molecular assays, as previously described [22, 23]. Between January 2003 and January 2016, 59 confirmed MPPP isolates were collected from 34 hospitals and two outpatient clinics in 23 cities in 14/16 main administrative regions of Poland (Table 1). Most of the strains were isolated from infections ($n = 51$, 86%), with urinary tract infections (UTIs) being the most frequent ($n = 26$, ~ 50%), followed by bloodstream infections ($n = 9$, ~ 17.5%). One MPPP isolate from 2003 (isolate 2596/03) was partially characterized before [20]. The study was considered to be exempt for approval by a Polish ethical commission since it was an in vitro retrospective study on bacterial isolates cultured during routine medical procedures and collected for epidemiological purposes, not involving patients or their personal data.

Molecular Analyses and Plasmid Profiling

All isolates were typed by pulsed-field gel electrophoresis (PFGE), as previously described [22], and their VIM-encoding integrons were analyzed by PCR and sequencing, as reported [20]. New integron variants were submitted to the INTEGRALL database for numbering and characterization of new gene cassettes [24]. Plasmid

profiling was done with the S1 nuclease (TaKaRa, Otsu, Japan) assay [25].

Antimicrobial Susceptibility Testing

Custom MICRONAUT-S plates with 14 antipseudomonadal antimicrobials (Merlin Diagnostika GmbH, Berlin, Germany) were used to evaluate minimum inhibitory concentrations (MICs). Results were interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations (<https://euca.st.org>), except for gentamicin, for which the Clinical Laboratory Standards Institute (CLSI) criteria (<http://clsi.org>) were used.

Whole-Genome Sequencing and In Silico Analysis

All study isolates were short-read sequenced using Illumina HiSeq platform (Illumina, San Diego, CA, USA). Reads were trimmed by Cutadapt 1.16 (<https://cutadapt.readthedocs.io/en/stable/>) and assembled with SPAdes 3.10.0 [26]. Species identification was done using type strains genome collection (Table S1), and applying a two-step approach [1]: calculation of the average nucleotide identity scores (ANI; cut-off, $\geq 96.5\%$) with FastANI v. 1.32 [27], followed by digital DNA-DNA hybridization (dDDH; cut-off $\geq 70\%$) [28]. The in-sample single-nucleotide polymorphism (SNP)-based clonal analysis was performed for three main species, using BioNumerics version 7.6.3, with an oldest MPPP isolate of each species as a reference. All isolates of the *Pseudomonas* genus ($n = 11,836$) were downloaded from GenBank and PubMLST (<https://pubmlst.org/organisms/pseudomonas-putida>) databases on September 1, 2021. These were identified to the species level, and then all *P. putida* group isolates of the species identified among the Polish isolates ($n = 265$) were subjected to multi-locus sequence typing (MLST) using mlst (<https://github.com/tseemann/mlst>) [29]. The phylogenetic analysis of Polish and international isolates was done within each species group by kSNP v. 3.1.2 [30]. This tool was also used to

Table 1 MPPP isolates assigned to the species level, with basic epidemiological data, genotypes, integron variants/types, and plasmid content

Species	Isolate	City (center symbol) ^a	Specimen	PFGE	ST ^b	Integron variant (type) ^c	Plasmid content	
							SI-PFGE	Inc group ^d
<i>P. allopautida</i>	2596/03	Warsaw (HW3)	Urine	D	ST69	In461 (In461)	~ 460 kb, < 50 kb	IncP-2; IncP-9ε
	4481/15	Pruszków (HW6)	Urine			In461 (In461)	~ 320 kb	IncP-2
	2153/05	Poznań (HP1)	Urine			In1446	~ 100 kb	IncP-1β1; IncP-9ε
	579/11	Gdynia (HG7)	Urine			In1446	~ 80 kb	IncP-6-like; IncP-9ε
	2441/06	Kościerzyna (HG3)	Sputum			In2136 (In2136)	~ 80 kb	IncP-9ε
	891/07	Kościerzyna (HG3)	Urine			In2136 (In2136)	~ 80 kb	IncP-9ε
	5033/13	Warsaw (HW2)	Urine			In2136 (In2136)	~ 80 kb	IncP-9ε
	2291/10	Starogard Gdański (HG6)	BAL			In2136 (In2136)	< 50 kb	IncP-9ε
	9276/10	Cracow (HK2)	Stomach content			In2133 (In2136)	~ 80 kb	IncP-9ε
	1498/14	Chorzów (HS2)	Stool			In2133 (In2136)	~ 90 kb	IncP-9ε
	3760/14	Rzeszów (HR3)	Urine			In1654	–	–
	5594/09	Rzeszów (HR9)	Urine			In1663	< 50 kb	IncP-9ε
	24/14	Warsaw (HW25)	Blood			In2138	–	–
	3088/05	Poznań (HP1)	Urine			In56	~ 80 kb	IncP-1β1; IncP-9ε
5768/13	Warsaw (HW22)	Urine			In1654	< 50 kb	–	
5075/10	Suwałki (HB1)	Ulcer swab		N	ST76	In461 (In461)	~ 420 kb, < 50 kb	IncP-2
1537/10	Warsaw (HW3)	Urine		AF	ST77	In238 (In238)	~ 80 kb	IncP-9ε
6396/12	Opole (HO5)	Stool		W	ST78	In1008 (In1008)	~ 160 kb, < 50 kb	IncQ-1α-like; pMOS94-like
733/14	Chorzów (HS2)	Stool		S	ST79	In1008 (In1008)	~ 375 kb, ~ 160 kb	IncP-2; IncQ-1α-like

Table 1 continued

Species	Isolate	City (center symbol) ^a	Specimen	PFGE	ST ^b	Integron variant (type) ^c	Plasmid content		
							SI-PFGE	Inc group ^d	
<i>P. monteilii</i>	8712/11	Suwalki (HB1)	Pus	O	ST95	In2135	-	-	
	1259/13	Warsaw (HW10)	Blood			In238 (In238)	-	-	
	10873/ 11	Warsaw (HW17)	Stool			In2138	-	-	
	4421/11	Legnica (HD5)	Stool			In1008 (In1008)	< 50 kb	pMOS94-like	
	5219/11	Opole (HO5)	Blood			In1008 (In1008)	< 50 kb	pMOS94-like	
	985/06	Warsaw (HW1)	Urine		L	ST95	In1662	-	
	135/16	Sanok (HR18)	Urine			In238 (In238)	-	-	
	6266/12	Poznań (HP9)	Urine			In1646	-	-	
	2219/13	Poznań (HP9)	Urine			In1646	-	-	
	811/11	Poznań (HP6)	Urine		K	ST94	In237 (In238)	~ 330 kb, ~ 80 kb	IncP-9γ
	5622/11	Nowa Sól (HF1)	Blood			In1660 (In461)	~ 380 kb, ~ 250 kb, ~ 65 kb	IncP-9α	
	2188/10	Koszalin (HZ6)	Urine			In461 (In461)	~ 360 kb, ~ 85 kb	IncP-9ε	
	4182/12	Łódź (HE10)	Sputum		AD	ST95	In461 (In461)	~ 420 kb, ~ 250 kb	IncP-2; pMOS94-like
	160/14	Biała Podlaska (HL2)	Sputum		A	ST95	In238 (In238)	< 50 kb	pMOS94-like
	5530/11	Poznań (HP13)	Peritoneal fluid		M	ST110	In1661	-	-

Table 1 continued

Species	Isolate	City (center symbol) ^a	Specimen	PFGE	ST ^b	Integron variant (type) ^c	Plasmid content	
							SI-PFGE	Inc group ^d
<i>P. asiatica</i>	6466/12	Poznań (HP9)	Urine	Q	ST15	In1008 (In1008)	< 50 kb	pMOS94-like
	3658/15	Warsaw (HW17)	Urine			In2137 (In238)	-	-
	6180/09	Zielona Góra (AF2)	Urine			In1649	-	-
	2869/09	Warsaw (HW1)	Hospital environment			In461 (In461)	~ 250 kb	-
	4695/09	Warsaw (HW61)	Urine			In461 (In461)	-	-
	4837/09	Warsaw (HW13)	Urine			In461 (In461)	-	-
	4798/12	Warsaw (HW1)	Urine			In461 (In461)	~ 250 kb	-
	537/14	Warsaw (HW22)	Urine			In461 (In461)	~ 440 kb, ~ 290 kb, < 50 kb	IncP-2; pMOS94
	8332/10	Warsaw (HW10)	Blood			In461 (In461)	~ 330 kb, < 50 kb	IncP-2; pMOS94-like
	5519/10	Poznań (HP1)	-	Z	ST15	In1008 (In1008)	~ 450 kb, < 50 kb	IncP-2; pMOS94-like
<i>P. kurumegalensis</i>	4147/15	Warsaw (HW21)	Blood	AB	ST97	In2134 (In1008)	~ 200 kb, < 50 kb	pMOS94-like
	3456/12	Warsaw (HW21)	Catheter blood	AA	ST114	In528	-	-
	3044/13	Warsaw (HW21)	Bile			In528	-	-
	4034/13	Gdańsk (HG1)	Blood	T	ST46	In238 (In238)	~ 65 kb	IncP-9η
<i>P. putida</i>	4591/12	Gryfice (HZ3)	Wound swab	E	ST111	In1649	~ 450 kb	IncP-2
	5241/13	Bielsko-Biała (HS3)	Pleura	C	ST112	In70	~ 170 kb, < 50 kb	pMOS94
	4533/14	Kurno (HE13)	Skin lesion	I	ST116	In461 (In461)	-	-
<i>P. juntedi</i>	4025/15	Ruda Śląska (AS10)	Urine	G	ST98	In249	-	-
	4849/14	Gryfice (HZ3)	Blood	F	ST115	In2015 (In238)	-	-
<i>P. soli</i>	4264/15	Warsaw (HW17)	Ulcer swab	Y	ST117	In2138	-	-
	1173/11	Warsaw (HW21)	Pus	X	ST70	In461 (In461)	~ 250 kb	-

Table 1 continued

Species	Isolate	City (center symbol) ^a	Specimen	PFGE	ST ^b	Integron variant (type) ^c	Plasmid content	
							SI-PFGE	Inc group ^d
<i>Pseudomonas</i> sp. #12 ^e	4491/12	Opole (HO5)	Urine	J	ST96	In1008 (In1008)	< 50 kb	IncQ-1 α -like; pMOS94-like
	542/15	Biala Podlaska (HL2)	Rectal swab	B	ST99	In2136 (In2136)	~ 170 kb	-
	795/08	Suwalki (HB1)	Pleural fluid	nd ^f	ST109	In336	-	-
<i>Pseudomonas</i> sp. #15 ^f	760/13	Warsaw (HW17)	BAL	R	ST113	In461 (In461)	~ 440 kb, ~ 70 kb	IncP-2; IncP-9 ϵ

^aThe first letter in a center symbol indicates hospital (H) or outpatient clinic (A)

^bNew STs are bolded

^cNew integrons are bolded. Integron types were arbitrarily distinguished, as previously described [22]

^dPlasmid replicons with both the nucleotide coverage and identity values between 75 and 95% are defined as “-like”

^eNumber of this putative novel species is derived from earlier work [1]

^fNumber of this putative novel species is according to the numbering proposed in the earlier work [1]

^gnd, not determined

calculate SNP numbers between the isolates and their species-specific references (defined as above). Phylogenetic trees were plotted by iTOL v5 (<https://itol.embl.de/>). Resistomes of the study and international isolates were determined with AMRFinderPlus [31]. Plasmid incompatibility groups (Inc) were assigned by ABRicate (<https://github.com/tseemann/abricate>), using PlasmidFinder [32] and custom databases (including IncP-2, -7, -9, pMOS94 lineage *rep* genes and/or *oriV* regions) [12, 33, 34], with detailed classification to individual Inc subgroups [33, 35].

Nucleotide Sequence Accession Numbers

Sequences of new integrons have been published under following accession numbers: In1659-In1663, OL880459-OL880463; and In2133-In2138, OL880464-OL880469. A new VIM variant, VIM-77, has been assigned the number MZ947163.1. Genomes and SRA data was deposited under the GenBank BioProject PRJNA788750 (JAJSP0000000000-JAJSRU000000000; SRR17284949-SRR17285007).

RESULTS

Species Distribution and General Clonal Analysis

When compared to *P. aeruginosa*, the *P. putida* group constitute a small fraction of all MBL-producing pseudomonads in Poland (~ 5%) [22]; however, the 59 MPPP isolates turned out to be highly diverse, representing 12 different species (Tables 1 and S2). The most abundant were *P. alloputida* ($n = 19$), recently derived from *P. putida* [36], *P. monteilii* ($n = 15$), and *P. asiatica* ($n = 11$), being a heterotypic synonym of *P. shirazica* [36, 37]. The remaining isolates were of the recently reported *P. kurunegalensis* [1], and *P. putida* ($n = 3$ each), and *P. soli*, *P. juntendi* and *P. mosselii* ($n = 1$ each). Five isolates were of four new species, separated based on ANI and dDDH approaches: *Pseudomonas* sp. #10 and #12 described previously [1], and #14

and #15 distinguished here. *Pseudomonas* sp. #14 is related to *P. sichuanensis* (ANI score, 93.9%), whereas #15 is similar to #4 (95.7%) [1] and *P. peradeniyensis* (95.6%). MLST classified the organisms into 23 species-specific STs, of which 21 were new [29].

Of the main species, *P. alloputida* and *P. monteilii* have been observed in multiple Polish regions in contrast to *P. asiatica*, limited almost exclusively to Warsaw (Table 1, Figure S1). In each of these, a spread clone/subclone could be found. In *P. alloputida* it was ST69 of a single pulsotype (pulsotype D; $n = 15/19$), in *P. monteilii* it was ST95 of two pulsotypes (O and L; $n = 9/15$), and in *P. asiatica* it was ST15 of a single pulsotype (Q; $n = 9/11$). The oldest isolate in the collection, 2596/03 [20], represented the *P. alloputida* ST69 major clone.

MBL Types and VIM-Encoding Integrons

The isolates produced six VIM-like MBLs, of which VIM-2 prevailed ($n = 41$), followed by VIM-4 ($n = 13$) (Table 1). A single *P. asiatica* ST97 isolate had a new variant, VIM-77, differing by a single mutation (N165D) from VIM-2.

Twenty-four, including ten new VIM-encoding class 1 integrons were distinguished; similar elements, differing from each other by one cassette/mutation only, were clustered into types (Tables 1 and S3). The variety of the integron content was observed even within the more prevalent *P. alloputida* ST69, *P. monteilii* ST95 and *P. asiatica* ST15 clones. The most prevalent types were In461 ($n = 15$), In238 ($n = 8$), In1008 ($n = 8$), and In2136 ($n = 7$), all spread across the species and clones. In461 occurred in six species/eight STs, with a cluster of *P. asiatica* ST15 isolates from Warsaw ($n = 6$). It has been a Poland-specific structure observed since 2003, including the early *P. alloputida* ST69 2596/03 isolate [20]. In461 has disseminated broadly in Polish MPPA with highly conjugative IncP-2-like plasmids [21], however, in MPPP this correlation was weaker (Table 1). In238 types (In237, In238, In2015, In2137), all with a 169-bp duplication in their *bla*_{VIM-1}-like cassettes [22, 38, 39], were found in five species/six STs. In238 had been identified first in the

Polish index MPPA from 1998 [39], and together with related elements, over years it has diffused in *P. aeruginosa* and Enterobacterales in Poland, Hungary and Greece [22, 38, 40–42]. In1008 types (In1008, In2134) were observed in isolates of four species/six STs. These have spread in Polish *P. aeruginosa* and Enterobacterales since 2001 [20, 22, 38]; interestingly, In1008 was reported also in *P. monteilii* in Spain [6]. The new In2136 types (In2133, In2136) with duplicated *bla*_{VIM-2} cassettes, occurred mainly in a cluster of *P. allopitida* ST69 ($n = 6$). Similar duplications, though of other *bla*_{VIMS}, have been found in *P. aeruginosa* from Japan [43] and Spain [44], and in *Citrobacter freundii* from the US (GenBank acc. No. KP975074). Apart from In461, In238 and In1008 types, several other integrons (In56, In70, In249, In1446, In1654, In1646, In1649) have been observed in Polish MPPA and VIM-positive Enterobacterales too [20, 22, 38] (M. Biedrzycka and R. Izdebski, unpublished data); however, only two elements (In2015 and In1446) were identified first in MPPP and then other organisms. In summary, VIM integrons have been one of the key factors of the MPPP genetic diversity. The high number of common elements with *P. aeruginosa* and Enterobacterales indicates that the *P. putida* group has participated readily in the circulation of AMR determinants among Gram-negatives in Poland [4, 11, 13, 16, 45].

Plasmid Content

The S1 analysis visualized at least one plasmid (< 50 to ~ 460 kb) in ~ 65% of the MPPP isolates ($n = 38$); the highest number of such isolates were in *P. allopitida* (17/19) (Table 1). This was a notably higher score than in MPPA (~ 42%) (results not shown) [22]. Typing revealed a variety of replicons, including IncP-9 ($n = 17$) and IncP-2 ($n = 10$). The self-transmissible IncP-9 plasmids, associated with metabolic functions and/or resistance to drugs and heavy metals, have been divided into eight subgroups (α – θ), with three (α , ε , η) linked with AMR [33]. Four subgroups (α , γ , ε , η) were identified in the study collection, of which ε dominated ($n = 14$). IncP-2 replicons occurred in isolates of five

species with large plasmids in S1 profiles (~ 320 to 460 kb) and often In461, which, like in MPPA [21], might indicate the In461 location on IncP-2 megaplasmids. Such highly transmissible plasmids of multiple functions have been broadly identified in pseudomonads as important AMR platforms [46, 47], and seem to be common in Polish MPPP and MPPA populations (~ 15%) [21]. Twelve isolates harbored plasmids of the recently identified pMOS94 lineage, having 94–100% nucleotide identity to the original *repA-oriV* sequence [12]. These plasmids have been shown to frequently carry MBL genes in pseudomonads from over last 20 years [12]. VIM-encoding plasmids and the direct context of VIM integrons in the MPPP isolates have been subjected to a separate detailed study (P. Urbanowicz and M. Gniadkowski, manuscript in preparation).

Resistome and Susceptibility Testing

The resistome analysis revealed a variety of acquired AMR gene patterns, provided mainly by 14 β -lactamase- and 30 aminoglycoside-modifying enzyme-encoding genes (Table S4). Individual resistomes contained 3–15 genes, and an average isolate had ~ 7.1 genes. Among the main species, *P. asiatica* had the highest number of AMR genes per isolate (~ 9.0) when compared to *P. monteilii* and *P. allopitida* (~ 6.8 and ~ 6.5, respectively). Most of the strains were XDR [8], with almost uniform resistance to β -lactams and fluoroquinolones, and varying resistance to aminoglycosides (Table S5). All isolates were susceptible to colistin.

Clonality and Phylogeny of the *P. allopitida* Isolates

The results of the in-sample SNP comparison were congruent with typing, clustering the ST69 isolates, and separating sporadic clones (Figure S2). The total number of polymorphic positions was 65,097 and SNP numbers between any and the reference isolates ranged from 51 to 51,451 SNPs (Table S6). SNP numbers in pairs of closest-related isolates were 11–51,328 in the whole sample, and 11–109 within the ST69

group, indicating relatedness within that. Inferred phylogeny of the 19 Polish and 73 international isolates defined as *P. alloputida* (Table S7), corresponded to the recent analysis with several clonal complexes (CCs) distinguished (Fig. 1) [19]; however, the CC1 from that report did not meet the ANI/dDDH species inclusion criteria used here, thus was excluded from our study. All Polish ST69 isolates grouped within CC7, together with 14 strains from all over the world, mostly of non-clinical background. This CC was previously described as the most clonal, with the highest number of acquired AMR and virulence factors genes [19]. Two MDR strains from Japan, NBRC111121 and GTC 16473 [19], were more related to the study ST69 isolates, distanced by 581 and 916 SNPs to the early 2596/03 isolate. This may indicate a par excellence international lineage adapted to hospital environments, locally acquiring various AMR determinants. The remaining Polish isolates of sporadic STs grouped together with several others within CC4, making it the second numerous and one of the most diverse *P. alloputida* CCs.

Clonality and Phylogeny of the *P. monteilii* Isolates

The SNP-based phylogeny using the isolate 985/06 as a reference segregated the STs, clustering the main ST95 clone (Figure S3). This population was more homogeneous than *P. alloputida*, with 13,683 polymorphic sites overall, and 56–8,934 SNPs between the reference and any other isolate. Isolates in the ST95 group were related with each other, differing by 38–78 SNPs (Table S6). The international *P. monteilii* isolates sequenced ($n = 32$; Table S8) fell into two main phylogroups; all Polish isolates belonged to one of these, and the ST95 clone formed a specific cluster within this group (Figure S4). The international isolates of the phylogroup were from all over the world, and were mainly clinical isolates, including those from Brazil with VIM or IMP MBLs. The second phylogroup consisted of the isolates of environmental or unknown origins.

Clonality and Phylogeny of the *P. asiatica* Isolates

In the *P. asiatica* sample, the number of polymorphic positions was 37,169, and SNP numbers between any of the isolates and the reference, isolate 2869/09, were 10–36,461 (Table S6). Pairs of closest relatives differed by 12–36,461 in the whole sample, and 12–83 within the major ST15 subclone, evidencing relatedness of its isolates (Figure S5). When compared with the international strains available ($n = 40$; Table S9; Figure S6), remarkably frequent carbapenemase producers, the Polish ST15 isolates formed a clade with five ST15 isolates from other countries, almost all having *bla*_{VIM} genes (in other integrons, though). In general, the study isolates were related mostly to French ones, and in case of the ST15 major subclone it was the strain PC9/HB3267 (distant by 163 SNPs from the reference), showed to be highly resistant and pathogenic in various models [48, 49]. Like in *P. alloputida* ST69, this might indicate an international lineage of *P. asiatica*, particularly adapted to nosocomial settings.

Phylogeny of Minor Species

The results of phylogenetic analyses of the study isolates representing minor species against representatives of these from other countries are shown in Tables S10–S17 and Figures S7–S13. Even though the Polish isolates segregated with some organisms into individual lineages or branches, in general no specifically close relationships were revealed. However, an interesting case was *P. kurunegalensis*, of which nine international sequenced isolates were identified and compared with the three Polish isolates (Table S10). The two ST114 isolates were related to two nosocomial Chinese strains (Figure S7), separated by 143–146 SNPs from the reference isolate 3456/12, and sharing the same VIM integron, In528. The third isolate of ST46 turned out to be related to an MBL-negative isolate from the US (292 SNPs), forming together an outlier branch within the *P. kurunegalensis* phylogenetic tree.

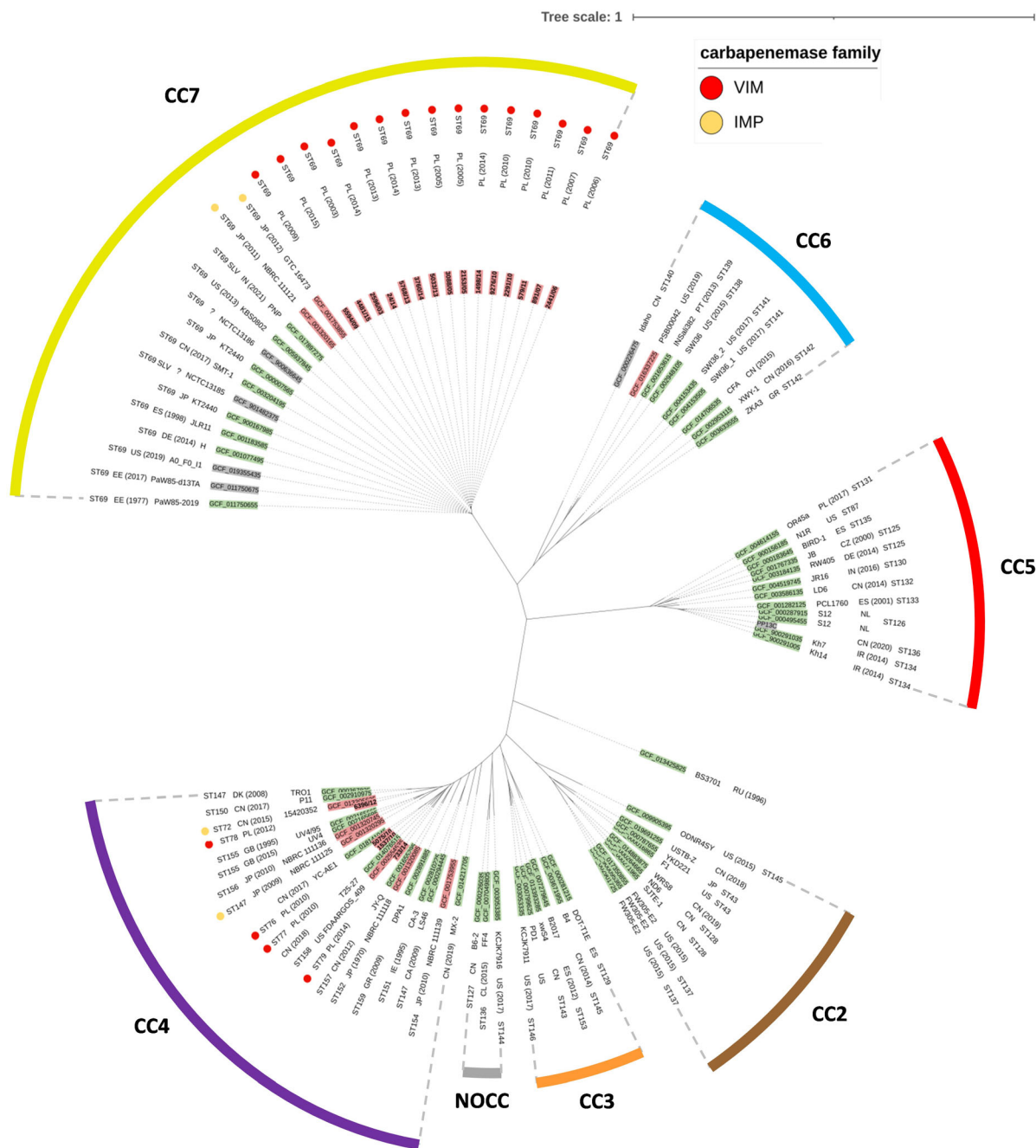


Fig. 1 SNP-based phylogenetic tree of 19 sequenced *P. alloputida* Polish isolates compared with the international genomes available in GenBank and PubMLST. Numbers correspond to original numbers of the study isolates or GenBank assembly numbers. Their red, green, or grey backgrounds indicate clinical, non-clinical or unknown origin, respectively. The strain names, countries and years of isolation, and attributed STs are specified at the corresponding GenBank assembly numbers. The presence of carbapenemase gene is marked with colored circles. The study Polish isolates are *bolded*. Strains analyzed previously [19] are assigned to original clonal complexes (CC2–CC7) and indicated in colors according to that report. The tree was constructed using kSNP v3.1.2 [30] and visualized with iTOL. The names of countries were coded with ISO3166-1 alpha-2 standard

DISCUSSION

The *P. putida* group of environmental origin are emerging opportunistic pathogens, usually affecting immunocompromised patients. In recent years these have been increasingly responsible for an array of infections, from keratitis and UTIs in catheterized patients, to catheter-related bacteremia [7]. In a recent report from China, 44 cases of community- and hospital-acquired *P. putida* infections were described, of which 75% were MDR. One fourth of the patients had the urinary catheter inserted [50]. Similarly, a recent paper from Germany underlined the catheter insertion as a risk factor for infection, and out of a total of 89 *P. putida* group strains recovered, 41 isolates (46.1%) harbored the *bla*_{VIM} MBL genes. The frequent and broad AMR of *P. putida* may facilitate successful spread in hospital settings with high antibiotic pressure, exemplifying ability of these organisms to adopt to critical environmental conditions [17]. At the same time, there are no societies' treatment recommendations for *P. putida* infections since these are newly emerging human pathogens with still not many cases. The treatment is usually conducted with antibiotics active against *P. aeruginosa*. However, a high percentage of MDR strains observed worldwide and also in our study makes the choice of effective antibiotic difficult. Susceptibility profiles presented here limited the treatment options mostly to colistin, acting as a last-resort drug.

To the best of our knowledge, this work has been one of the broadest and most comprehensive analyses of MDR nosocomial isolates of the *P. putida* group so far, and one of a few such WGS studies. Its strong element was the use of the most updated taxonomy of the organisms [1], which allowed for the precise classification of the isolates and their targeted comparisons with international strains, and for distinction of two potentially new species. Apart from *P. alloputida* [19], specific data on the other species is still rather scarce and usually concerns single isolates or limited outbreaks [4–7, 11–14, 16, 18, 51]. Moreover, to our knowledge, this is the 1st report on MBL-

producing clinical isolates of *P. soli*, *P. juntendi*, *Pseudomonas* #14 and #15.

The study revealed predominance of *P. alloputida*, *P. monteilii* and *P. asiatica* in the entire group (~ 76% in total), which in general corresponds to other reports [5, 6, 18, 51] and numbers of genomic sequences available at the time of the study. The clonality and phylogenetic analyses revealed the presence of three lineages, one per each of the major species, of the apparently broad geographic distribution, which may be considered to be epidemiologically 'successful' clones. The internal variety of these clones, illustrated not only by the lack of MBLs in many of their international members, but also by the diversity of MBLs and their genetic determinants when present, suggests that the clones have been spreading basically as MBL-negatives, and then locally acquiring different MBL-encoding structures.

The Polish MPPP isolates shared a remarkable number of VIM integrons with MPPA and Enterobacterales circulating in the country [20, 22, 38] (M. Biedrzycka and R. Izdebski, unpublished data), proving extensive on-site exchange of these elements between different populations of Gram-negative bacteria. However, it has been not possible to assign the *P. putida* group the role of the AMR/MBL reservoir, as postulated in several earlier reports [4, 11, 13, 16], because most of the integrons have been found first in the other species. Previously we have analyzed > 1300 Polish MPPA isolates from the same period, identified during the same surveillance program [20–22, 52]. Keeping in mind huge population size differences between the two groups of organisms, it should be noted that the *P. putida* group was relatively more diversified, and seemingly more frequently hosted plasmid DNA. As it was mentioned above, the MPPP and MPPA shared multiple VIM integrons with each other, and in both groups *In461* often correlating with conjugative *IncP-2* megaplasmids was the most frequent element. However, its contribution in MPPP was higher than in MPPA (~ 25% versus ~ 18%) but the correlation with the *IncP-2s* was looser (~ 47% versus ~ 85%) [21]. The MPPA population was remarkably dominated by four STs (ST235, ST111, ST273, ST654; ~

73%), whereas the contribution of the three *P. alloputida*, *P. monteilii* and *P. asiatica* major STs (ST69, ST95 and ST15, respectively) to all MPPP was apparently lower (~ 61%). In both groups most of the main clones internally varied, e.g. by VIM integrons; however, in MPPA these have segregated several bona fide epidemic genotypes (ST-pulsotype-integron) that were responsible for ~ 60% of all MPPA infections. In MPPP only two small clusters of *P. alloputida* ST69 (with In2136 types) and *P. asiatica* ST15 (with In461) might be presumably identified as counterparts of the MPPA epidemic organisms.

This work has a limitation, being the age of the MPPP isolates; however, still this has been a relatively big group representative for specific and rare opportunistic pathogens, collected by a targeted surveillance over > 10 years and all over a country. Therefore, the study has made an important contribution to understanding the taxonomy, phylogeny, and dynamics of clinical populations of emerging pathogens, contributing to a pool of AMR genes.

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Compliance with ethics guidelines. This article does not contain any study on human or animal subjects, material or data. The study was considered to be exempt for approval by a Polish ethical commission since it was an in vitro retrospective study on bacterial isolates cultured during routine medical procedures and collected for epidemiological purposes, not involving patients or their personal data.

Data availability. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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