Susceptibility profiles and resistance genomics of Pseudomonas aeruginosa isolates from European ICUs participating in the ASPIRE-ICU trial

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Abstract

Objective. To determine the antimicrobial susceptibility profiles and the resistome of *P. aeruginosa* isolates from European ICUs during a prospective cohort (ASPIRE-ICU). **Methods**. A total of 723 isolates from respiratory samples or perianal swabs of 402 patients from 29 sites in 11 countries were studied. MICs for a panel of 13 antibiotics were determined by broth microdilution. Horizontally-acquired β-lactamases were analyzed through phenotypic and genetic assays. The first respiratory isolate from the 105 patients providing such samples were analyzed through whole genome sequencing (WGS), including the analysis of the resistome and the assessment of a previously defined genotypic resistance score. Additionally, spontaneous mutant frequencies and of genetic basis of hypermutation were assessed. **Results**: When analyzing the first isolate per patient, all agents except colistin showed resistance rates above 20%, including novel combinations ceftolozane/tazobactam and ceftazidime/avibactam. 24.9% of the isolates met the XDR criteria with a wide intercountry variation (0-62.5%). 13.2% of the isolates met the recently proposed DTR (Difficult to Treat Resistance) classification. 21.4% of the isolates produced ESBLs (mostly PER-1) or carbapenemases (mostly NDM-1, VIM-1/2 and GES-5). WGS showed that these determinants were linked to high-risk clones (particularly ST235 and ST654). WGS revealed a wide repertoire of mutation-driven resistance mechanisms, with multiple lineage-specific mutations. The most frequently mutated genes were *gyrA, parC, oprD, mexZ, nalD and ParS*, but only 2 of the isolates were classified as hypermutable. Finally, a good accuracy of the used genotypic score to predict susceptibility (91-100%) and resistance (94-100%) was documented. **Conclusions**: An overall high prevalence of resistance is documented European ICUs, but with a wide intercountry variability determined by the dissemination of XDR high-risk clones, arguing for the need of reinforcement of infection control measures.

Introduction

The growing prevalence of nosocomial infections produced by multidrug-resistant (MDR) and particularly extensively drug-resistant (XDR) *P. aeruginosa* strains is associated with significantly increased morbidity and mortality, since it compromises the available effective therapeutic options [1,2]. This increasing threat results from the extraordinary capacity of *P. aeruginosa* for developing resistance to nearly all available antibiotics, conferred by mutations in chromosomal genes and by a growing amount of transferable resistance determinants. Of particular concern are those coding for carbapenemases or extended-spectrum β-lactamases (ESBLs), frequently co-transferred with aminoglycoside-modifying enzymes determinants [3]. The dissemination of MDR/XDR global strains, the high-risk clones, in multiple hospitals worldwide adds further concern [4–8]. Moreover, beyond classical molecular epidemiology and phenotypically-targeted resistance mechanisms assessment, recent whole genome sequencing (WGS) studies are providing relevant information for building up the complex and evolving resistome of MDR/XDR *P. aeruginosa* high-risk clones [9–15].On the other hand, the recent introduction of novel β-lactam-β-lactamase inhibitor combinations, such as ceftolozane/tazobactam or ceftazidime/avibactam, which are quite stable against mutation-driven resistance mechanisms, partially alleviate the urgent clinical need of new agents for combating infections by MDR/XDR *P. aeruginosa* [16–18]. However, emergence of resistance to these antibiotics has been found rapidly after their introduction and should therefore be closely monitored [19,20]. Although susceptibility data from European countries is reported in some initiatives, such as the ECDC EARS-Net, information on the involved resistance mechanisms is scarce. Moreover, most genomic surveys so far have been performed at the single hospital or country level [13,21,22]. Thus, here we describe the first large scale survey of antimicrobial susceptibility profiles and resistome analysis from European ICUs. This work is part of the ASPIRE-ICU (Advanced Understanding of *Staphylococcus aureus* and *Pseudomonas aeruginosa* infections in Europe-Intensive Care Units) study [23] and has been presented as a poster in the 31st ECCMID.

Material and Methods

Clinical isolates, susceptibility testing and characterization of resistance mechanisms

A total of 723 isolates obtained from respiratory samples or perianal swabs (PAS) of 402 mechanically-ventilated ICU patients enrolled in the ASPIRE-ICU trial (NCT02413242) from 29 different sites in 11 different countries, from 2016 to 2021, were studied. As part of the ASPIRE-

ICU protocol, PAS and respiratory (endotracheal aspirate or sputum) samples for *P. aeruginosa* culture were obtained at ICU admission and twice weekly thereafter. From patients who were diagnosed with pneumonia, additional respiratory samples were collected at the day of diagnosis and 7 days post-infection. Peri-anal swabs in skimmed milk medium and untreated respiratory samples were stored at −80 °C until shipment to the Central lab at the University of Antwerp until further analysis. Culture of peri-anal swabs was performed by inoculating the swabs directly on CHROMID *P. aeruginosa* Agar (BioMérieux, France) and blood agar (BBL®Columbia II Agar Base (BD Diagnostics, USA) supplemented with 5% defibrinated horse blood (TCS Bioscience, UK)). Patient respiratory samples were blended (30,000 rpm, probe size 8 mm, steps of 10 s, max 60 s in total), diluted 1:1 v/v with Lysomucil (10% Acetylcysteine solution) (Zambon S.A, Belgium) and incubated for 30 min at 37 °C with 10 s vortexing every 15 min. Followed by culture on CHROMID *P. aeruginosa* Agar and blood agar. Plates were incubated at 37 °C for 24 h. Plates without growth were further incubated for 48 h and 72 h. Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS, Bruker Daltonics) was used to identify *P. aeruginosa* isolates, which were stored at −80 °C until further use.

MICs for a panel of 12 antipseudomonal agents were determined by broth microdilution using EUCAST 2021 breakpoints (www.eucast.org). Multidrug resistant (MDR), extensively drug resistant (XDR) and pandrug resistant (PDR) profiles were defined as suggested by Magiorakos *et al*. (2012) [24]. However, according to current EUCAST instructions only truly resistant (R) isolates were considered, as opposed to previous recommendations to use I+R. Additionally, difficult to treat resistance (DTR) phenotypes were defined as described previously [25]. The occurrence of horizontally-acquired carbapenemases and Extended Spectrum β-lactamases (ESBLs) was analysed through phenotypic and genetic (PCR and sequencing) assays [12]. Imipenem and ceftazidime resistance cloxacillin inhibition test was initially used for screening chromosomal β-lactam resistance mechanisms (inactivation of OprD and/or the overexpression of AmpC) and was followed by double-disk synergy tests (DDST) for the detection of class B carbapenemases (EDTA) and/or class A carbapenemases or ESBLs (clavulanic acid), following previously established procedures [12].

Whole genome sequencing and resistome analysis

Whole genome sequencing was performed on the first respiratory isolate from each of the patients (n=105).

Library preparation and whole-genome sequencing (WGS). Strains were cultured overnight at 37°C on Mueller Hinton agar plates, transferred to Mueller Hinton broth and incubated overnight at 37 °C. DNA was extracted from 2ml of culture using the MagAttract HMW DNA Kit (Qiagen, Germany) according to manufacturer's instructions. Multiplexed DNA libraries were prepared using the Nextera XT Library and Sample Preparation Kit followed by v2 2×150-bp paired-end sequencing on a MiSeq instrument (Illumina Inc., USA). The primary sequencing analysis was done using BacPipe v.2.6.1 and checkM was used for determining cross contamination.

***De novo* assembly.** Paired-end reads were *de novo* assembled using SPAdes v3.13.1 (http://cab.spbu.ru/files/release3.13.1/)

Variant calling. Previously defined and validated protocols were used with slight modifications [26]. Briefly, paired-ended reads were mapped to the *P. aeruginosa* PAO1 reference genome (NC_002516.2) with Bowtie 2 v2.2.4 and pileup and raw files were obtained by using SAMtools v0.1.16 and PicardTools v1.140, using the Genome Analysis Toolkit (GATK) v3.4-46 for realignment around InDels. From the raw files, SNPs were extracted if they met the following criteria: a quality score (Phred-scaled probability of the samples reads being homozygous reference) of at least 50, a root-mean-square (RMS) mapping quality of at least 25 and a coverage depth of at least 3 reads, excluding all ambiguous variants. MicroInDels were extracted from the totalpileup files when meeting the following criteria: a quality score of at least 500, a RMS mapping quality of at least 25 and support from at least one-fifth of the covering reads. Filtered files were converted to vcf and SNPs and InDels were annotated with SnpEff v4.2. [27]. Gene absence was also investigated using the SeqMonk program (https://www.bioinformatics.babraham.ac.uk/projects/seqmonk/). Finally, as different sequence variants of OprD have been described [28], the *de novo* assemblies were used to first classify the *oprD* gene according to their similarity to PAO1, LESB58, UCBP-PA14, MTB-1, FRD1 or F23197 reference sequences and to further investigate their structural integrity. The presence of horizontally-acquired antimicrobial resistance determinants was also investigated using the web tool ResFinder (https://cge.cbs.dtu.dk/services/ResFinder/).

Assessment of *P. aeruginosa* genotypic resistance scores. The presence of acquired resistance determinants and mutations located within 40 chromosomal genes involved in mutational resistance *(gyrB, mexR, mexA, mexB, oprM, ampDh3, oprD, parS, parR, mexY, mexX, mexZ, galU, mexS, mexT, mexE, mexF, oprN, dacB, gyrA, nalD, nalC, dacC, pbpA, mpl,*

ampR, ampC, fusA1, ftsI, ampD, oprJ, mexD, mexC, nfxB, pmrA, pmrB, parC, parE, armZ, ampDh2) were scored for determining the genotypic resistance scores values [29].

Spontaneous mutant frequencies and characterization of mutator strains. The frequencies of mutation to rifampicin (300 µg/ml) resistance were determined as described previously [30]. For each strain, independent aliquots containing approximately 10^3 cells were inoculated into five flasks containing 10 ml of Mueller-Hinton broth and incubated at 37°C and 180 rpm for 16 to 18 h and serial 1:10 dilutions were plated on Mueller-Hinton agar plates and Mueller-Hinton agar plates supplemented with 300 µg/ml of rifampicin. Mutant frequencies were then calculated by dividing the median numbers of mutants by the median numbers of total cells. The breakpoint used to define hypermutable strains was a frequency of mutation to rifampicin resistance of $>2 \times 10^{-7}$, as established previously [30]. Additionally, as a control, frequencies of mutants to rifampin resistance were determined with reference strain PAO1 and its DNA mismatch repair deficient *mutS* derivative (PAOMS) [30]. To explore the genetic basis for the mutator phenotypes, complementation studies were performed with all hypermutable strains. Plasmid pUCPMS harbouring PAO1 wild-type *mutS*, plasmid pUCPML harbouring PAO1 wild-type *mutL*, and plasmid pUCP24, a control cloning vector, were electroporated into the hypermutable isolates and transformants were selected on Luria-Bertani agar plates containing 50 µg/ml of gentamicin [30]. Complementation was demonstrated by reversion of the increased rate of mutation to rifampin resistance in two independent transformant colonies for each strain. Additionally, the genetic basis of hypermutation was investigated from whole genome sequence data, through the analysis of an exhaustive panel of 15 mutator genes (mutome) as described previously [31].

Data availability

Sequence files will be deposited in the ASPIRE-ICU NCBI BioProject PRJNA768775

Results

Figure 1 shows the antimicrobial susceptibility data considering a single isolate from each of the 402 enrolled patients. When available, the first respiratory isolate was considered (n=105) whereas the first PAS isolate was used for those patients showing no positive respiratory samples (n=297). Lowest resistance rates were documented for colistin (1.2%),

distantly followed by amikacin (12.9%). Up to 28.4% of the isolates were resistant to tobramycin and 37.4% to ciprofloxacin. Among β-lactams, resistance rates were highest for imipenem (48%), followed by piperacillin/tazobactam (30.6%), meropenem (27.6%), cefepime (27.1%) and ceftazidime (26.4%). It is noteworthy that resistance rates for the novel antipseudomonal combinations ceftolozane/tazobactam (23.4%) and ceftazidime/avibactam (21.4%) were not much lower. Moreover, aztreonam showed similar resistance rates (21.4%). Up to 32.9% of the isolates were MDR, 24.9% XDR and 0.7% PDR. On the other hand, 13.2% were classified as DTR. Figure 2 shows the distribution of the resistance profiles in the different countries. The prevalence of XDR phenotype was highest in hospitals from Serbia (62.5%), followed by those in Hungary (35.3%), Bulgaria (27.5%) and Czech Republic (15.8%). XDR isolates were not detected among isolates from Germany, UK, Turkey and Estonia.

Figure S1 shows the susceptibility data for the 402 isolates according to sample types. As shown, resistance rates were higher for respiratory isolates than for PAS isolates. For instance, up to 41.9% of the respiratory isolates showed an XDR profile, whereas only 18.9% of PAS isolates did. However, it appears not to be an intrinsic feature of the sample type itself, since an analysis of paired isolates from the 45 patients contributing both respiratory and PAS isolates showed no major differences in resistance rates (Figure 3a).

Another aspect analyzed was the potential emergence of resistance during the course of the colonization/infection. For this purpose, susceptibility data were analyzed for 118 early (first) and late (last) pairs of isolates from 100 patients showing multiple PAS and/or respiratory samples and results are shown in Figure 3b. An overall tendency for increased resistance was observed with the highest (>10%) increase for imipenem and piperacillin/tazobactam.

The β -lactam resistance mechanisms were analyzed through phenotypic and molecular (PCR + sequencing) assays. As shown in Figure 4, β-lactam resistance mechanisms were found in close to one third (30.6%) of the isolates, 21.4% showing acquired β-lactamases and 9.2% only mutation-driven resistance mechanism (positive inhibition of ceftazidime resistance with cloxacillin suggesting AmpC hyperproduction, and/or positive inhibition of imipenem resistance with cloxacillin suggesting OprD inactivation). Regarding horizontally acquired β-lactamases, 48.6% of the isolates producing such enzymes showed a PER-1 ESBL, alone or together with an OXA-10. Besides PER-1, another single isolate from Bulgaria produced a VEB-1 ESBL. Regarding carbapenemases, 21.4% of the isolates producing horizontally-acquired β-lactamases produced VIM (either VIM-1 or VIM-2), 17.8% GES-5, 15.5% NDM-1, and 2.4%

IMP-7. It is noteworthy that 9.5% of the isolates producing acquired β-lactamases coproduced NDM-1 and GES-5.

WGS was performed on the 105 respiratory isolates. MLST analysis revealed up to 47different STs, with ST235, considered to be a high-risk clone, being by far the most frequent one, detected in 33 isolates. Besides ST235, 15 clones/clonal complexes were detected in at least 2 patients (2 to 7). Table 1 shows the distribution of the main horizontally-acquired and mutation driven resistance mechanisms for the complete collection of isolates and for those clones detected in at least 2 patients. Conversely, Figure 5 summarizes the resistome of the 54 (51.4%) MDR/XDR/PDR isolates and Dataset S1 provides susceptibility data and resistome analysis for all tested isolates. Globally, ST235 was the clone more frequently associated with horizontally-acquired and mutation-driven resistance mechanisms and therefore strongly associated with MDR/XDR/PDR profiles, and it was frequently detected in patients from Serbian hospitals. The detection of acquired β-lactamases was concordant with the above PCR analysis. All PER-1 and VIM-2 producing isolates belonged to ST235, whereas GES-5 was detected among ST235 and ST654 isolates. Moreover, all NDM-1 producing isolates belonged to ST654 and coproduced GES-5. The most frequent horizontally-acquired aminoglycoside modifying enzyme was AadB being detected in 25.7% of the isolates, mainly from ST235 and ST175 clones. Regarding the mutational resistome, the most frequently mutated target included the QRDR regions of *gyrA* (52.4% of the isolates) or *parC* (41.9%), the carbapenem porin OprD (49.5%) the negative regulator of MexXY efflux pump MexZ (44.8%), the negative regulator of MexAB-OprM NalD (27.6%) and ParS from the ParRS two component system (21.9%) (Table 1). As shown in Figure 5, specific resistome patterns were observed for the different MDR/XDR/PDR clones. It is noteworthy that ST235 isolates from Serbian hospitals showed multiple different resistome signatures including both horizontally-acquired and mutation-driven resistance. On the other hand, a single resistome pattern was observed for ST654, the second most frequent genotype. Also noteworthy, ST175 isolates from Spain and Hungary showed the same previously described OprD and *ampR* mutations responsible for β-lactam resistance in this clone.

In order to determine whether frequent mutation-driven resistance was linked to mutator phenotypes, spontaneous mutant frequencies were determined for the 105 respiratory isolates and results are shown in Figure 6. Only 2 (1.9%) of the isolates showed a mutator phenotype and only one of them showed an XDR profile associated with a large number of resistance mutations, while the other showed only imipenem resistance due to an *oprD* mutation.

Complementation assays with wild-type mutator genes revealed that one of the isolates was deficient in *mutS* and the other in *mutL*. Genomic analysis of a previously described panel of genes involved in mutator phenotypes (mutome) revealed the presence of specific mutations in *mutS* (T493P) and *mutL* (A272D), respectively. Curiously, the *mutL* mutation originated a novel MLST allele within CC298 clonal complex. On the other hand, diverse polymorphisms apparently not involved in increased mutation rates were documented in mutator genes of wild-type isolates (Table 1S).

Finally, to assess the correlation between phenotypes and genotypes of antibiotic resistance, a recently described genotypic resistance score [29] was applied for ceftazidime, ceftolozane/tazobactam, meropenem, ciprofloxacin and tobramycin, and results are presented in Table 2. Three of the 105 isolates were not assessable since they were genomic outliers [29]. For the remaining 102 isolates the phenotypic-genotypic correlation was high. A genotypic score below 0.5 predicted susceptibility in 100% of the isolates for ceftazidime, ceftolozane/tazobactam, ciprofloxacin and tobramycin and in 90.9% for meropenem. Conversely, a genotypic score equal or higher than 1 predicted resistance in 93.9% (meropenem) to 100% (tobramycin and ceftolozane/tazobactam) of the cases. Additionally, from 3 to 12 isolates, depending on the antibiotic, showed scores ≥0.5 and <1 and were classified as undetermined resistance genotype.

Discussion

The susceptibility profiles to a panel of 13 antipseudomonal agents was evaluated in over four hundred *P. aeruginosa* isolates from the ICUs of hospitals from 11 European countries. Nearly one fourth of the isolates met the ECDC/CDC XDR criteria [24] but a wide geographic variation was found, with some countries reporting over 60% of XDR isolates while others had none. In addition to the classical MDR/XDR/PDR ECDC/CDC definitions, recently proposed DTR criteria (resistance to all first line agents including classical antipseudomonal β-lactams and fluoroquinolones) were also applied [25], since direct comparisons between both definitions are currently lacking. DTR prevalence was established at 13.2% in European ICUs, well above the 2.1% reported for US hospitals in a recent study [25]. Still, prevalence of DTR phenotypes was nearly half of that of XDR phenotypes. Indeed, up to 49.5% of XDR isolates were classified as non-DTR, but 100% of DFT isolates were classified as XDR (Dataset S1). A high prevalence (over 20%) of resistance to the novel β-lactam β-lactamase inhibitor combinations was observed as well. Moreover, up to 83% of the XDR isolates were resistant to ceftolozane/tazobactam and/or ceftazidime/avibactam. These findings contrast with those

documented in some recent national European surveys [13,22,32] and is correlated with the high prevalence of horizontally-acquired broad spectrum β-lactamases (ESBLs and/or carbapenemases) in our isolate collection. Similar to XDR profiles, we observed a wide inter-country variation in the distribution of such concerning β-lactamase enzymes, with specific countries showing an extremely high number and diversity of isolates producing horizontally-acquired β-lactamases, while their presence was not detected in several other countries. One of the limitations of our survey is, however, the wide variation of patients and strains contributed by each of the participating countries ranging from 4 to 104 (Figure 2).

One intriguing finding of the study was that respiratory isolates showed more frequent resistant phenotypes than PAS isolates. Although the investigation of the underlying factors falls out of the scope of this work and will be addressed separately in the clinical part of the ASPIRE-ICU trial [23], it appears not to be an intrinsic feature of the sample origin itself, since an analysis of paired isolates from the patients contributing both respiratory and PAS isolates showed no major differences in resistance rates. Thus, differences should reside in the characteristics of patients contributing only PAS isolates but not respiratory samples and may potentially include nosocomial acquisition, antibiotic exposure, length of admission, or ICU characteristics among others.

In addition to the high primary resistance rates documented, a clear tendency towards increased resistance during the course of the colonization/infection was evidenced. These findings are in agreement with previous experiences and likely reflect within host evolution of resistance during antibiotic exposure [33–35]. Interestingly, this increased resistance appeared not to occur for the novel β-lactam β-lactamase inhibitor combinations, consistently with their reported higher stability against *P. aeruginosa* mutation-driven resistance mechanisms [18].

WGS resistome analysis of respiratory isolates showed a high proportion and diversity of horizontally-acquired resistance genes and mutations, frequently linked to major international widespread high-risk clones [5,8]. Indeed, ST235 was particularly dominant and was found to be associated with multiple horizontally-acquired β-lactamases as well as with diverse patterns of mutation-driven resistance, even among isolates from a single center, denoting the dissemination of multiple independent ST235 lineages. One remarkable finding was the coproduction of NDM-1 class B and GES-5 class A carbapenemases among ST654 isolates, apparently not previously described so far [8].

A vast repertoire of resistance mutations was also evidenced, with multiple genes including *oprD*, *gyrA*, *parC*, *mexZ*, *nalD* and *parS* being mutated among over 20 to 50 percent of the isolates, indicating that they are under strong selective pressure. However, with the exception of classical QRDR mutations, all others were clone specific. Interestingly, previously described *oprD* and *ampR* specific mutations responsible for β-lactam resistance in ST175 isolates widely disseminated in Spanish hospitals [9,12,13] were also identified in the single isolate from this clone recovered from a patient admitted to a hospital in Hungary. Conversely, widespread clone ST235 was associated with multiple different resistance mutations in *oprD, mexZ*, or MexAB-OprM regulators (*mexR*, *nalC* or *nalD*) even among isolates from a single hospital.

In order to determine whether frequent mutation-driven resistance was linked to mutator phenotypes, spontaneous mutant frequencies were determined, but only 2% of the isolates were *mutS* or *mutL* deficient mutators. This low prevalence of mutators among *P. aeruginosa* ICU respiratory isolates is in agreement with a previous study in a single Spanish ICU [36] and contrasts with the very high prevalence (30-60%) of mutators documented in chronic respiratory infections [30,37–39]. Strong association with mutation-driven resistance leading to an XDR phenotype was only evidenced in one of the two patients. In the other, only mutation-driven carbapenem resistance was evidenced. Curiously, in this patient the *mutL* mutation originated a novel MLST allele as described previously for other *mutL* deficient mutators [40].

Finally, we attempted to assess whether the resistance genotype correlated well with the susceptibility phenotypes, and for this purpose we used a recently described genotypic resistance score that had been developed using a Spanish multicenter cohort [29]. Overall, we observed a good capability of the genotypic score to predict susceptibility (90.9 to 100%) and resistance (93.9-100%) to 5 antipseudomonal agents in this international cohort. These scores were quite similar to those previously documented for the Spanish collection, confirming a broad applicability of the described scoring system. The lowest performance was however documented, as in the previous study, for the prediction of meropenem resistance. Indeed, unlike the other agents, the assessment of meropenem phenotype-genotype correlation is complicated by the existence of an I category (MICs 4-8), that microbiologically correlates with a non-wildtype population (low level resistance).

In summary, an overall high prevalence of antimicrobial resistance is documented among *P. aeruginosa* isolates from European ICUs. However, a high inter-country variability was documented, with wide dissemination of ESBL- and/or carbapenemase- producing high-risk

XDR clones in some of them, arguing for the need of reinforcement of infection control measures.

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Transparency declaration

Omar Ali and Alexey Ruzin are employees of AstraZeneca. All other authors none to declare

Legends to Figures

Figure 1. Susceptibility rates to 13 antipseudomonal agents (A), and prevalence of MDR/XDR/PDR (B) and DTR (C) profiles among 402 *P. aeruginosa* isolates (one per patient) from the ASPIRE-ICU study.

Figure 2. Prevalence of MDR/XDR/PDR profiles for the 11 countries participating in the ASPIRE-ICU study.

Figure 3. (A) Comparative analysis of susceptibility profiles between paired PAS and respiratory samples from 45 patients. (B) Comparative analysis of susceptibility profiles between paired early and late isolates from 118 patients/sample types.

Figure 4. Prevalence (%) of β-lactam resistance mechanisms among 402 *P. aeruginosa* isolates (one per patient) from the ASPIRE-ICU study

Figure 5. WGS resistome analysis of 54 MDR/XDR/PDR respiratory isolates from the ASPIRE-ICU study. Countries codes: (BG) Bulgary, (CZ) Czech Republic, (ES) Spain, (NL) Netherlands, (RS) Serbia. Colour codes: Inactivating loss-of-function mutations and well-characterized gain-of-function mutations are indicated in black whereas any other aminoacid substitution are indicated in grey.

Figure 6. Rifampicin resistance mutant frequencies and genetic basis for hypermutation for the 105 respiratory isolates from the ASPIRE-ICU study. PAO1 and its *mutS* defficient derivative were used as controls.

Figure S1. Susceptibility rates and MDR/XDR/PDR profiles for (A) respiratory (n=105) and (B) PAS (n=297) isolates from the ASPIRE-ICU study.

Dataset S1. Susceptibility profiles and genomic information for the collection of *P. aeruginosa* isolates studied.

Table 1. Distribution of acquired resistance determinants and mutated genes among the 105 respiratory isolates and the clones detected in two or more patients.

Gene/mutation	N isolates (%)	ST235 n=33	ST253 n=7	ST654 n=6	ST274 n=3	ST308 n=3	CC17 n=2	ST108 n=2	ST111 n=2	ST175 n=2	CC244 n=2	ST252 n=2	CC298 n=2	ST348 n=2	ST633 n=2	ST2211 n=2	ST2601 n=2
PSE-1	3 (2.9)								2								
OXA-2	25 (23.8)	24															
OXA-544	1 (0.9)	1															
OXA-10	2(1.9)						1		1								
PER-1	24 (22.9)	24															
GES-5	10 (9.5)	4		6													
IMP-7	1(0.9)																
NDM-1	6 (5.7)			6													
VIM-2	4 (3.8)	4															
aacA4	13 (12.4)	8	1				1		1								
aacA7	3 (2.9)	3															
aacCA3	4 (3.8)	4															
aacCA5	4 (3.8)	4															
aadA2	6 (5.7)	4							2								
aadA6	16 (15.2)	14	1											1			
aadB	27 (25.7)	24								2							
aph3')-VIa	6 (5.7)			6													
aph3')-XV	23 (21.9)	23															
aph3´)-VIb	24 (22.9)	24															
mexR	8 (7.6)					1										2	
nalD	29 (27.6)	21		6			1							1			
nalD-394Δ4	19 (18.1)	21															
nalD-L153Q	6 (5.7)			6													
nalC	9 (8.6)	5															2
nalC-nt199Δ1	5 (4.8)	5															
mexA	6 (5.7)											1				2	
mexB	7 (6.7)	2															

oprM	3 (2.9)																
mexZ	47 (44.8)	29	2	6			1			2							
mexZ-569Δ10	4 (3.8)	4															
mexZ-291Δ11	4 (3.8)	4															
mexZ-E8X	19 (18.1)	19															
mexZ-A2T	6 (5.7)			6													
mexZ-V48A	3 (2.9)	1							2								
mexZ-G195D	2 (1.9)									2							
armZ	19 (18.1)						1	2		2					2		
mexY	16 (15.2)						1		2							2	2
mexX	5 (4.8)	1															2
mexS	12 (11.4)						1							2		2	2
mexT	9 (8.6)															2	2
mexE	3 (2.9)															2	
mexF	4 (3.8)															2	
oprN	7 (6.7)															2	
nfxB	7 (6.7)		1			1										2	
mexC	8 (7.6)																
mexD	10 (9.5)	8															
mexD-554InsG	5 (4.8)	5															
oprJ	8 (7.6)															2	
PBP2	4 (3.8)															2	
PBP3	4 (3.8)						1	2									
PBP4	6 (5.7)															2	
PBP5	2 (1,9)															2	
ampC	4 (3.8)						1										
ampR	8 (7.6)					1				2				2			2
ampR-G154R	2 (1.8)									2							
ampD	12 (11.4)	4					1				1						
ampDh2	4 (3.8)						1	2									
ampDh3	6 (5.7)																
Mpl	6 (5.7)										1						
oprD	52	27	2	6			1			2						2	

	(49.5)																
oprD-630Δ1	15 (14.3)	15															
oprD-1301Δ1	3 (2.9)	3															
oprD-W65X	5 (4.8)	5															
oprD-W138X	2 (1.9)		2														
oprD-Q142X	2 (1.9)									2							
oprD-W277X	3 (2.8)						1									2	
oprD-G316D	6 (5.7)			6													
oprD-G916D	4 (3.8)	4															
gyrA	55 (52.4)	33	1	6	1	1	2		2	2				2			
gyrA-T83I	54 (51.4)	33	1	6	1	1	2		2	2				2			
gyrB-S466F	1 (0.9)																
parC	44 (41.9)	29	1	6		1	1		2	2				1			
parC-S87L	43 (41.0)	29	1	6		1	1		2	1				1			
parE	7 (6.7)	4							2					1			
parE-S457G	4 (3.8)	4															
parE-E459G	2 (1.9)								2								
parS	23 (21.9)	8					1	2	2								2
parS-L137P	3 (2.9)								2								
ParR	6 (5.7)						1						2				
parR-M59I	1 (0.9)																
pmrA	1 (0.9)																
pmrB	2 (1.9)																
galU	1 (0.9)						1										
fusA1	4 (3.8)															2	

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Table 2. Distribution of the resistance genotypic scores values among 102 *P. aeruginosa* respiratory isolates.

Antibiotic[a]	N (%) Isolates S/I/R[b]		
	Score <0.5 (susceptible genotype)	**Score 0.5 - <1 (undetermined genotype**	**Score ≥1 (Resistant genotype)**
CAZ	44 (100)/ 0 (0)	7 (58.3)/ 5 (41.7)	2 (4.3)/ 44 (95.7)
TOL/Tz	58 (100)/ 0 (0)	1 (33.3)/2 (66.6)	0(0)/ 41 (100)
MER	30 (90.9)/ 2(6.1) / 1(3)	12 (60)/ 7 (35)/ 1 (5)	0 (0)/ 3 (6.1)/ 46 (93.9)
CIP	33 (100)/ 0 (0)	9 (81.8)/ 2 (18.2)	1 (1.7)/ 57 (98.3)
TOB	51 (100)/ 0 (0)	9 (100)/ 0 (0)	0 (0) / 42 (100)

[a] CAZ, ceftazidime; TOL/Tz ceftolozane/tazobactam; MER, meropenem; CIP, ciprofloxacin; TOB, tobramycin

[b] I/R for CAZ and CIP, S/R for TOL/Tz and TOB, S/I/R for MER according to EUCAST breakpoints

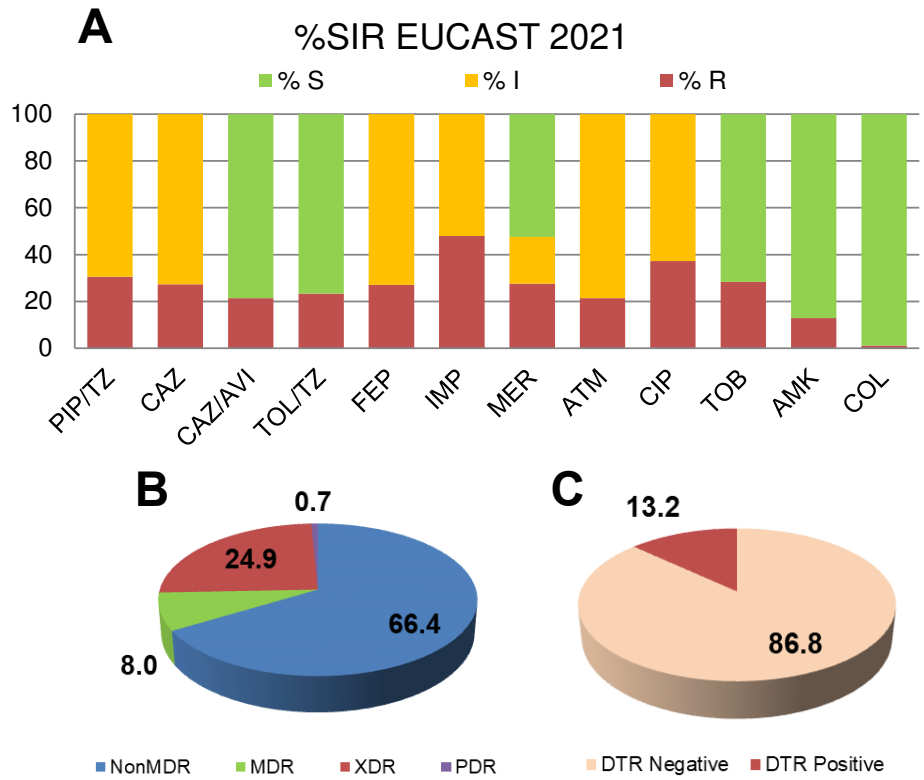


Figure 1. Susceptibility rates to 12 antipseudomonal agents (A), and prevalence of MDR/XDR/PDR (B) and DTR (C) profiles among 402 *P. aeruginosa* isolates (one per patient) from the ASPIRE-ICU study.

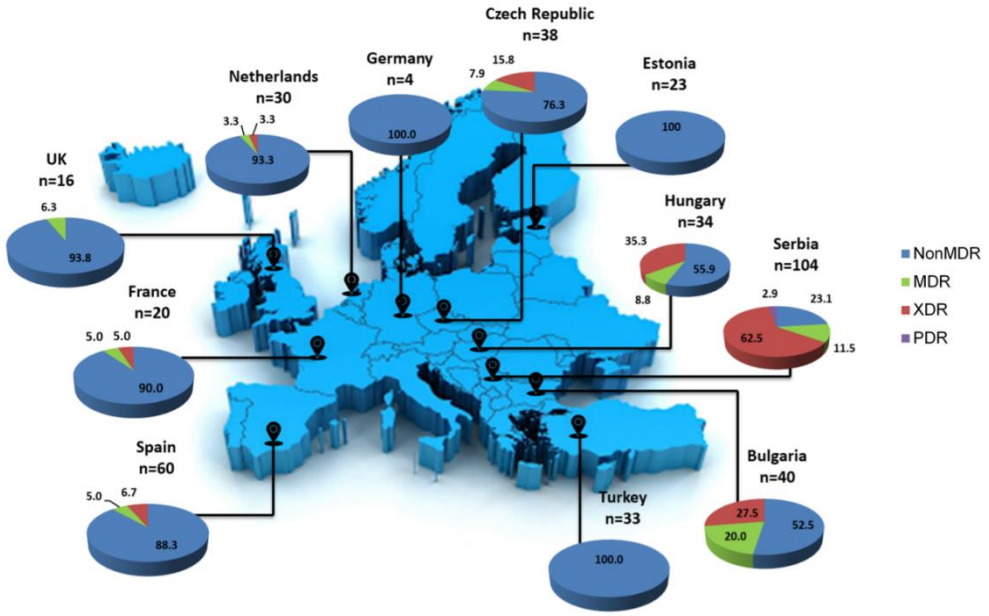
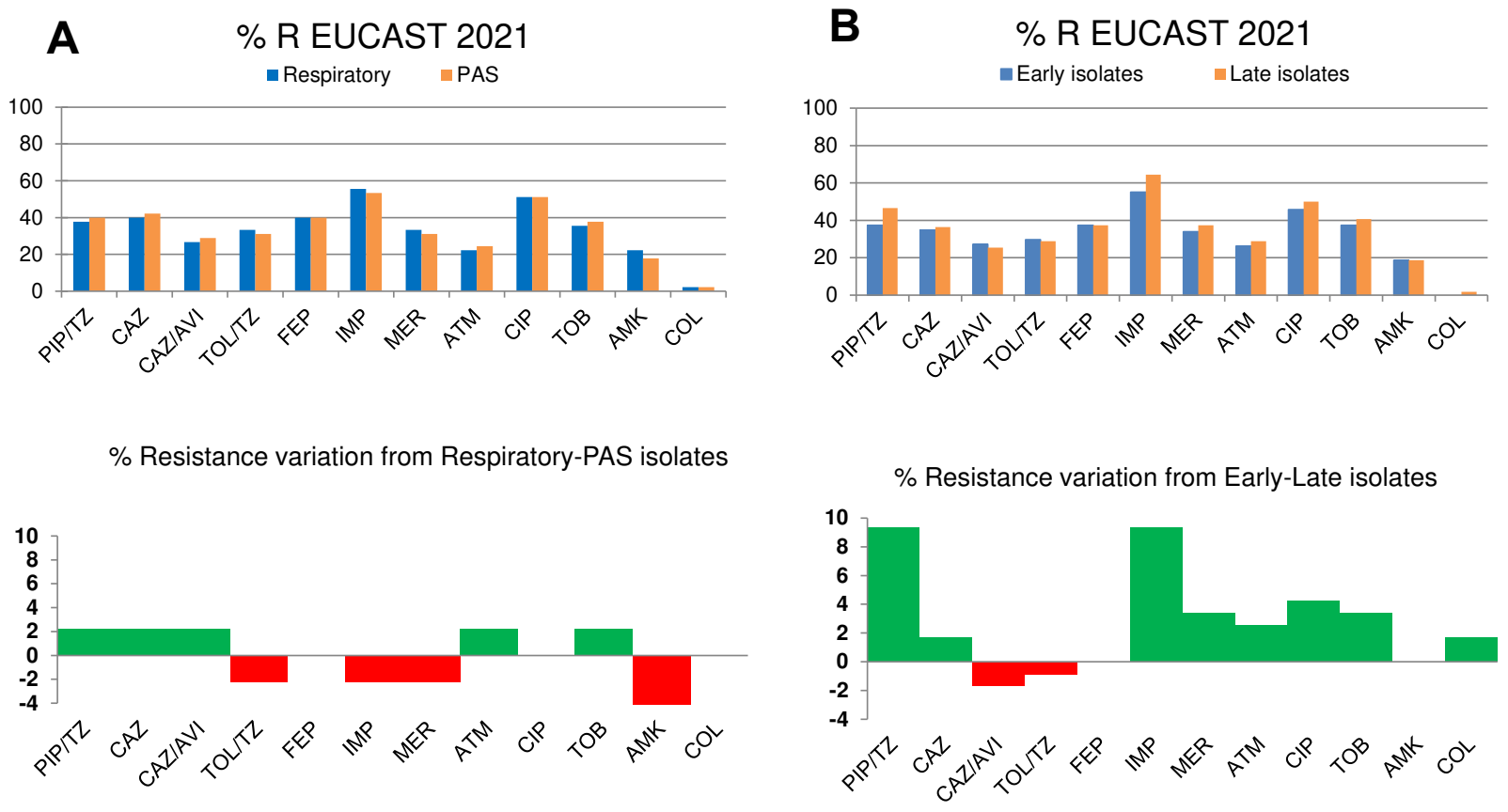


Figure 2. Prevalence of MDR/XDR/PDR profiles for the 11 countries participating in the ASPIRE-ICU study.



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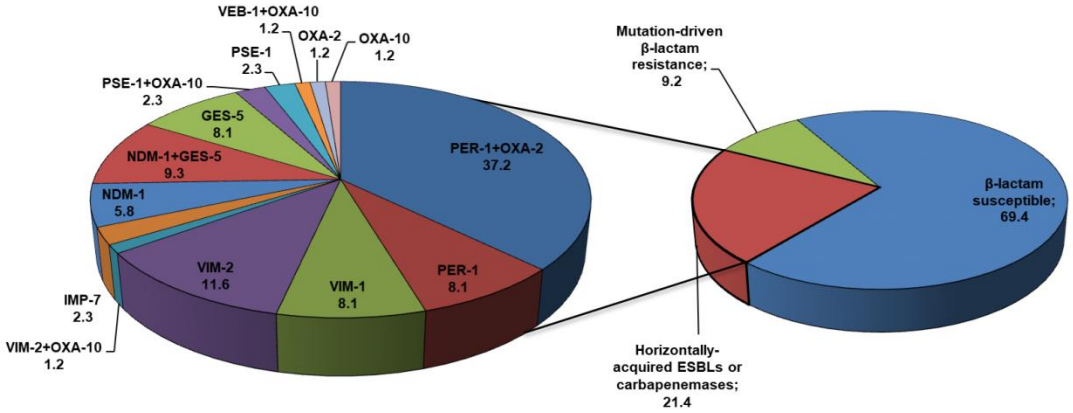
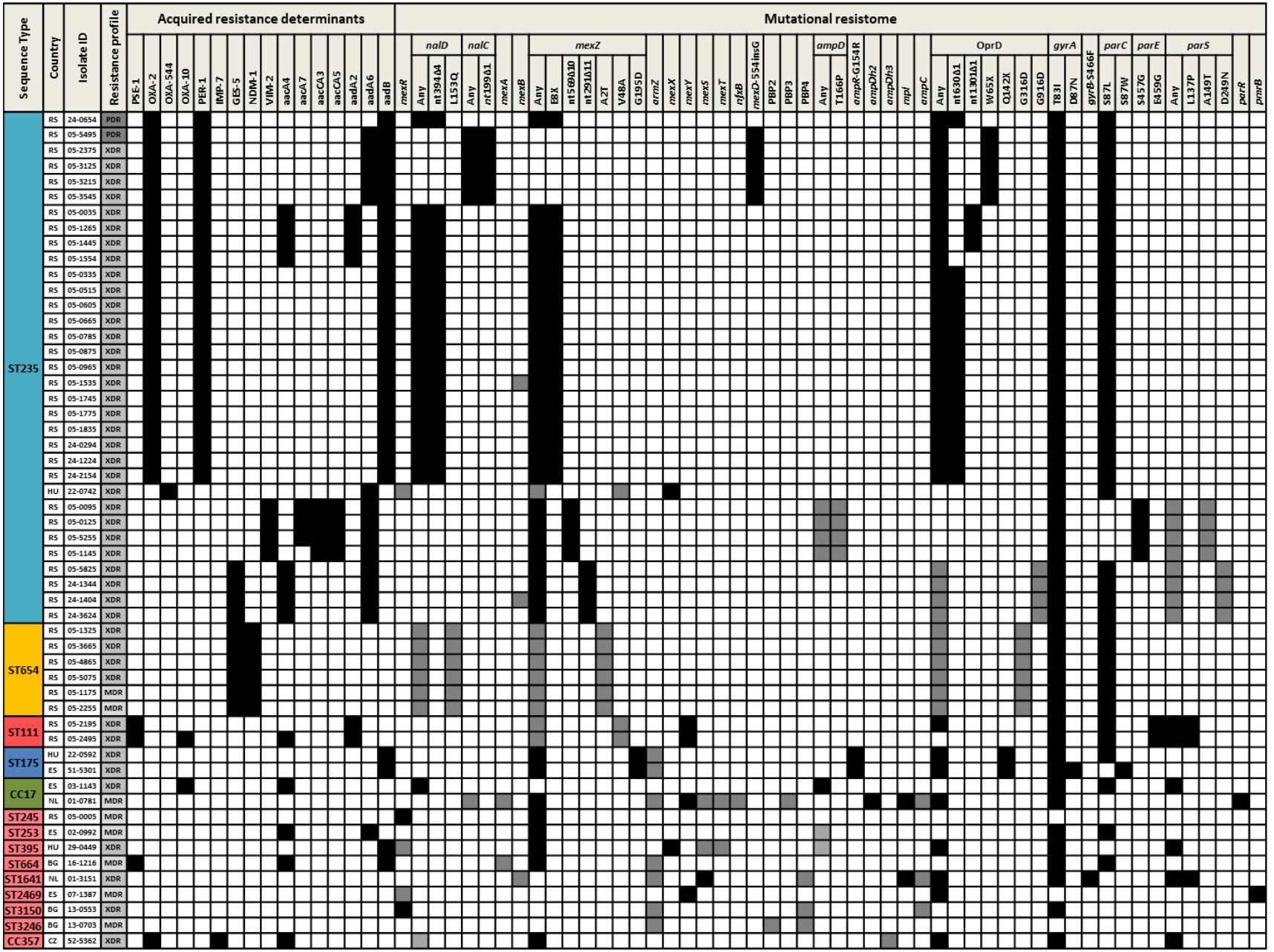


Figure 4. Prevalence (%) of β-lactam resistance mechanisms among 402 *P. aeruginosa* isolates (one per patient) from the ASPIRE-ICU study



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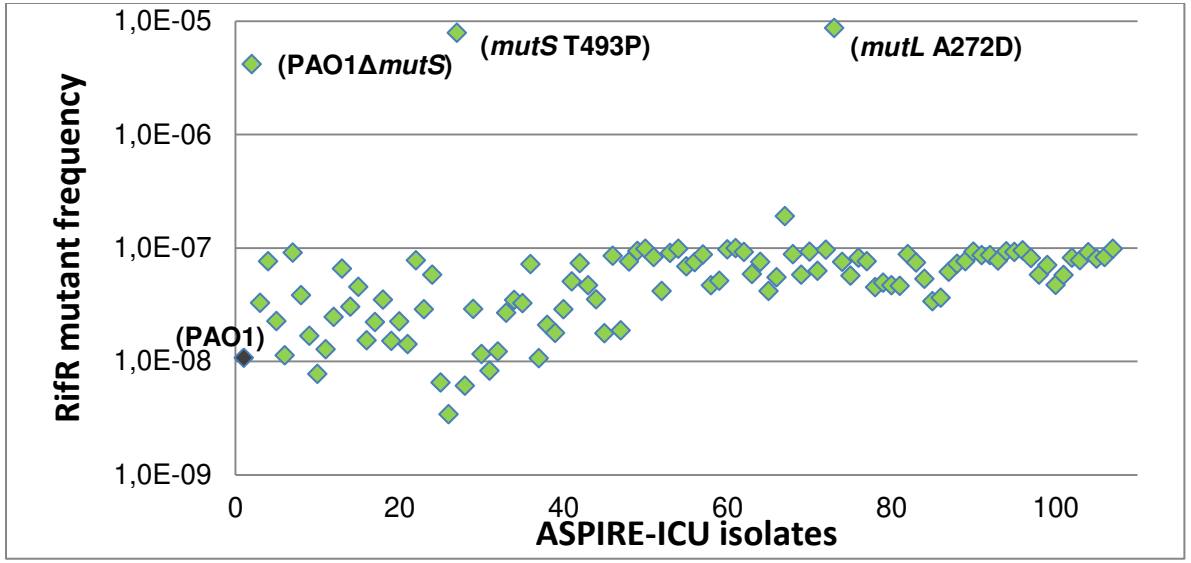

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