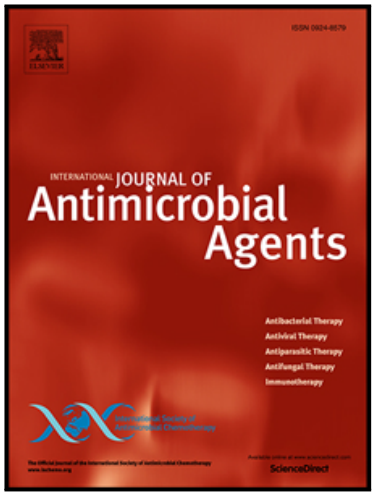
Editor: Stefania Stefani

Journal Pre-proof



Insight into phylogenomic bias of bla$_{VIM-2}$ or bla$_{NDM-1}$ dissemination amongst carbapenem resistant Pseudomonas aeruginosa

Gianuario Fortunato , Ivone Vaz-Moreira , Ina Gajic , Célia M. Manaia

PII: S0924-8579(23)00071-7
DOI: https://doi.org/10.1016/j.ijantimicag.2023.106788
Reference: ANTAGE 106788

To appear in: *International Journal of Antimicrobial Agents*

Received date: 3 August 2022
Accepted date: 8 March 2023

Please cite this article as: Gianuario Fortunato , Ivone Vaz-Moreira , Ina Gajic , Célia M. Manaia , Insight into phylogenomic bias of bla$_{VIM-2}$ or bla$_{NDM-1}$ dissemination amongst carbapenem resistant Pseudomonas aeruginosa, *International Journal of Antimicrobial Agents* (2023), doi: https://doi.org/10.1016/j.ijantimicag.2023.106788

Highlights

bla$_{VIM-2}$ was mostly associated with ST233 and ST111, in Europe

bla$_{NDM-1}$ was mostly reported in Asia, distributed by different sequence types

Significant protein prevalence differences in *bla*$_{VIM-2}$+ or *bla*$_{NDM-1}$+ accessory genomes

bla$_{VIM-2}$ gene frequently inserted in Tn402-like and Tn21 transposons

bla$_{NDM-1}$ gene frequently flanked by *ISAba125* or IS91 insertion sequences

Insight into phylogenomic bias of *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ dissemination amongst carbapenem resistant *Pseudomonas aeruginosa*

Gianuario Fortunato[1], Ivone Vaz-Moreira[1], Ina Gajic[2], Célia M. Manaia[1*]

[1] Universidade Católica Portuguesa, CBQF - Centro de Biotecnologia e Química Fina – Laboratório Associado, Escola Superior de Biotecnologia, Rua de Diogo Botelho 1327, 4169-005 Porto, Portugal

[2] Institute of Microbiology and Immunology, Faculty of Medicine, University of Belgrade, 11000 Belgrade, Serbia

***Corresponding author**

Célia M. Manaia, PhD

Escola Superior de Biotecnologia

Universidade Católica Portuguesa

e-mail: cmanaia@ucp.pt

Abstract

Pseudomonas aeruginosa are ubiquitous opportunistic pathogens that combine intrinsic and acquired multidrug resistance phenotypes. Carbapenem resistance has been expanding in this species, due to different types of acquired genes. In this study, we hypothesised that the spread of carbapenem-resistance among *P. aeruginosa* is influenced by phylogenomic features, being distinct for different genes. To test this hypothesis, we compared the genomes of *P. aeruginosa* harbouring *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ genes. The gene *bla*$_{VIM-2}$ was selected because, although frequent, it is almost restricted to this species and *bla*$_{NDM-1}$ due to its wide interspecies distribution. A group of genomes harbouring the genes *bla*$_{VIM-2}$ (n=116) or *bla*$_{NDM-1}$ (n=27), available in the GenBank, was characterized based on core phylogenomic analysis, functional categories in the accessory

genome and mobile genetic elements flanking the selected genes. Most *bla*$_{VIM-2}$ gene hosts belonged to multilocus sequence types (ST) ST111 (n=32/116) and ST233 (n=27/116) and were reported in Europe (n=75/116). The *bla*$_{NDM-1}$ gene hosts were distributed by different ST (ST38, ST773, ST235, ST357, ST654), frequently from Asia (n=11/27). Significant differences in the prevalence of functional protein/enzyme annotations per number of accessory genomes were observed between *bla*$_{VIM-2}$+ or *bla*$_{NDM-1}$+. The *bla*$_{VIM-2}$ gene was frequently inserted in Tn402-like and Tn21 transposons family and rarely in IS6100, while *bla*$_{NDM-1}$ gene was preferentially flanked by *ISAba125* and *ble*$_{MBL}$ genes or associated with IS91 insertion sequence. The hypothesis that carbapenem resistance gene acquisition is not random among phylogenomic lineages was confirmed, suggesting the importance of phylogeny in the dissemination of antibiotic resistance genes.

Keywords: Beta-lactamases, Mobile genetic elements, Phylogenetic diversity, Antibiotic resistance

1. Introduction

Pseudomonas aeruginosa is one of the 12 priority pathogens listed by the World Health Organization (WHO) [1]. International surveillance programs [2] show the high rates of *P. aeruginosa* resistance against frontline antibiotics, particularly beta-lactams and carbapenems. High carbapenem resistance prevalence has been reported in clinical *P. aeruginosa* worldwide - Europe (8 to 58% of the isolates), South and East Asia (8 to 36% of the isolates) and North America (10 to 31% of the isolates) [3].

With genome sizes ranging 5.5 to 7.0 million base pairs [4], *P. aeruginosa* has the potential to adapt to changing environments and successfully compete in complex microbial communities [5,6]. A recent study that analysed 1 311 genomes of *P. aeruginosa* showed that among the 54 272 genes identified, only nearly 1% represented the core, while about 50% were unique genes [4]. According to Freschi et al. 2019 [7] the core genome was dominated by functions related to RNA processing (COG cluster A), chromatin structure and dynamics (COG cluster B), cell division and partitioning (COG cluster D), metabolism and transport of amino acids (COG cluster E), nucleotides (COG cluster F), coenzymes and lipids (COG cluster H and I), transcription and translation (COG cluster J and K). In contrast, drug resistance, secretion systems (COG cluster L, U and V), and other functions mainly related to survival in changing environments have been frequently observed in the accessory genome [4,8]. Core features and the dynamic gene acquisition revealed by the pangenome analyses support the ubiquitous character of the species [9], thriving across distinct natural environments (soil, water, plants) and animal or human bodies, including clinical settings [10].

The first reports of acquired carbapenems resistance were in *Enterobacteriaceae,* and later in other *Gammaproteobacteria*, including *P. aeruginosa* [11–13]. The gene encoding the Verona integron-encoded metallo-beta-lactamase (VIM) was reported for the first time in *P. aeruginosa* in 1997, isolated from a Hospital inpatient in Verona (Italy), as an integron-borne gene [14]. To date, at least 76 bla_{VIM} variants have been reported, mostly in *P. aeruginosa*, with occasional reports in *P. putida* and *Enterobacteriaceae* [13,15]. The variant $bla_{VIM\text{-}2}$ is the most frequent in *P. aeruginosa*, reported in more than 4% of chromosomes and whole-genome sequences (WGS) available at

Comprehensive Antibiotic Resistance Database (CARD) [15]. Whereas *bla*$_{VIM}$ can be considered a gene originally dispersed by *P. aeruginosa*, the gene encoding the New Delhi metallo-beta-lactamase (NDM-1), another class B beta-lactamase, is widely disseminated through other bacterial groups, mainly within the order *Enterobacterales*. The *bla*$_{NDM}$ gene was reported for the first time in Sweden in a hospital patient who travelled from India, diagnosed with an infection associated with a multidrug resistant *Klebsiella pneumoniae* [16]. The first report of *bla*$_{NDM}$ in *P. aeruginosa* resulted from routine screening of carbapenemase-producing Gram-negative bacteria in the Military Medical Academy in Belgrade, Serbia, in 2010 [17]. Currently, *bla*$_{NDM-1}$ is reported in about 3.7% of the chromosomes and 0.7% of the whole genome sequences of *P. aeruginosa* available at CARD [15]

Given the liability of *P. aeruginosa* for antibiotic resistance acquisition, and the distinct patterns of dissemination of the genes *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$, these were considered interesting models to investigate possible phylogenomic drivers of resistance acquisition hypothetically reflected on the accessory genome and geographic distribution. Given the importance of geographic distribution, we used genomes available in public databases.

2. Materials and Methods

2.1. *P. aeruginosa* genomes selection

P. aeruginosa genomes containing the genes *bla*$_{VIM-2}$ (n=272) or *bla*$_{NDM-1}$ (n=40) (Supplementary Table S1), available at NCBI Pathogen Detection isolates browser database (https://www.ncbi.nlm.nih.gov/pathogens/isolates/; accessed on 17.11.2020), were collected for this study. Five additional genomes, which at that time were not available at NCBI, one containing the *bla*$_{VIM-2}$ [18], and four containing the *bla*$_{NDM-1}$ gene [19], were also included (assembly accession numbers: GCA_020404715.1, GCA_022559565.1, GCA_020404785.1, GCA_020404825.1, and GCA_020404705.1) (Supplementary Table S1). Presumable duplicated genome sequences were eliminated whenever were deposited by the same authors/institution, had the same Multi Locus Sequence Type (ST), determined according to the MLSTFinder (https://cge.cbs.dtu.dk/services/MLST/) and the same sample origin. In these cases, was selected

the one with the lowest number of contigs and highest genome coverage. After data trimming, a total of 116 *bla*$_{VIM-2}$+ and 27 *bla*$_{NDM-1}$+ genome sequences, produced with different DNA sequencing technologies, were considered for the study (Supplementary Table S1, highlighted in light blue). In addition, aiming a broader overview of carbapenemase encoding genes, the same database was queried for *bla*$_{IMP-1}$+ (n=258) and *bla*$_{SPM}$+ (n=35) genomes (accessed at 28-01-2023), which were typed based on ST and geographic origin.

2.2. Genome analysis

The genomes were annotated using RASTtk pipeline [20], which output was used as input to the Roary: pan-genome pipeline [21]. The Roary pipeline used default settings, allowing the splitting of paralogs into clusters, and performing a core alignment using MAFFT [22]. The sequence identity threshold was settled by default at 95% and the core-genome was established for genes shared by 99% of the genomes (Supplementary Table S2). The *bla*$_{VIM-2}$+ or *bla*$_{NDM-1}$+ strains genomes were first used to create a wgMLST scheme, including the common genes based on the default BLAST score ratio value of 0.6. From the wgMLST scheme was created the cgMLST, based on genes common to all the analysed genomes with sequence identity values of 99%, using chewBBACA tool [23]. The cgMLST scheme was then represented as a minimum spanning tree using the software Grapetree [24].

2.3. Functional annotation of the accessory genome

The accessory genome, comprised of genes shared by < 95% of the analysed genomes, was annotated in each set of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes, based on functional categories. Functional annotation of deduced aminoacid sequences used the EggNOG database [25] based on the parameters 95% sequence coverage and 100% sequence identity, providing a Cluster of Orthologous Groups (COG) assignment for each protein-coding gene. For each functional category were identified (based on similar protein functions in UniProt (https://www.uniprot.org/) subcategories of the most represented protein families. The respective relative abundance over the total proteins annotated in each category was expressed as proteins percentage values and

graphically represented using the ggplot2 R package [26]. Kolmogorov-Smirnov means comparison was used to analyse the difference in protein distribution in *bla*$_{VIM-2}$+ or *bla*$_{NDM-1}$+ strains functional annotations using SPSS 26.0 (IBM SPSS Inc., Chicago, IL).

2.4. Other antibiotic resistance genes

The core- and accessory- *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes were screened for antibiotic resistance genes, using the CARD Resistance Gene Identifier (https://card.mcmaster.ca/analyze/rgi) [15]. The prevalence of the identified genes among *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes was represented graphically as a barplot and Venn diagram using the R packages ggplot2 and Venn.diagram [27]. Moreover, the antibiotic resistance gene prevalence in the analysed genomes was compared with the data available for all *P. aeruginosa, bla*$_{IMP}$+ and carbapenems resistant *P. aeruginosa* available at CARD databases, based on NCBI deposited genomes (accessed on November 2021).

2.5. *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ genetic environment and transposons comparative analysis

The flanking regions of the *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ genes were analysed for 80 genomes, which permitted this inspection. For the other 63 (55 *bla*$_{VIM-2}$+ and 8 *bla*$_{NDM-1}$+) genomes, the targeted genes were in short contigs (800 to 3000 bp), hindering a proper analysis. Insertion sequences and mobile genetic elements were annotated using the IS finder (https://isfinder.biotoul.fr/blast.php) [28]. Transposons were identified by alignment against reference transposons described in the literature using MAFFT [22] and manually annotated using NCBI blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The structure of the transposons associated with *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ genes was depicted using the R package gggenes (available at https://github.com/wilkox/gggenes).

3. **Results**

Most of the 116 *bla*$_{VIM-2}$+ and 27 *bla*$_{NDM-1}$+ non-repetitive genomes examined were of clinical origin, and only 7 *bla*$_{VIM-2}$+ and 1 *bla*$_{NDM-1}$+ of environmental sources (*e.g.* home and hospital environments, animals associated, hospital effluent, or other unidentified sources) (Supplementary

Table S1). The *bla*$_{VIM-2}$+ genomes were from strains isolated in Europe (n=64), Asia (n=17), South. America (n=22), Africa and the Middle East (n=5), North America (n=7), and one of unknown origin. The *bla*$_{NDM-1}$+ genomes were from strains isolated in Asia (n=11), Europe (n=9), North America (n=3), South America (n=1), Africa (n=1), and two of unknown origin (Supplementary Table S1).

3.1. Phylogenetic diversity and genome features

The analysis of the whole gene set among *bla*$_{VIM-2}$+ genomes revealed 33 527 genes (3 142 present in ≥ 99%, 1 157 in 95-99%, 4 276 in 15-94%, and 24 952 in <15% of the strains). In the *bla*$_{NDM-1}$+ genomes were identified 14 080 genes (4 599 present in ≥ 99%, 468 in 95-99%, 2 990 in 15-94%, and 6 023 in <15% of the strains) (Supplementary Table S2). The phylogenomic relationships among *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}^{+}$ genomes were assessed based on the core genome MLST (cgMLST) and Pasteur MLST, being the identified sequence types (STs) instrumental in the analysis of the results. As expected, the cgMLST-based phylogenetic analysis distributed the genomes according to the MLST types. Phylogenomic analysis suggested a close relationship among specific STs, for instance, between ST111 and ST233, ST175 and ST179, ST235 and ST357, or ST823, ST308 and ST773 (Figure 1). Despite the wide geographic distribution of the predominant phylogenetic lineages, various clades were mostly populated by genomes of some regions. For instance, ST823, ST308 and ST773 in Asiatic countries, or ST244, ST175, ST179, ST17 in European countries (mainly Portugal, Spain and Poland) (Supplementary Figure 1), which may suggest some degree of endemicity. However, the sample size and the fact that most genomes were of clinical isolates, with only 8 (7 *bla*$_{VIM-2}$+ and 1 *bla*$_{NDM-1}$+) from environmental origin scattered by different STs - ST111 (n=2), ST175, ST179, ST233, ST386, and ST823, and ST308 (*bla*$_{NDM-1}$+) (Supplementary Table S1, Figure 1), limits this interpretation. Identical core genomes (100%) were observed only for six pairs of genomes (ST111, ST244, ST233, ST823, ST175, ST235), most of the times of the same country except in the case of ST235.

The diversity of STs was proportional to the number of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1+}$ genomes. The *bla*$_{VIM-2}$+ genomes were distributed by 29 STs, being ST111 (n=32, from Europe, South America, and USA), ST233 (n=28, from Europe, Africa, South America, Asia, and USA), ST235 (n=14, from

Europe, Asia, and USA), and ST823 (n=8, from Asia/Oceania and USA), the predominant (Figure 1A; Supplementary Figure S1 and Table S1). The *bla*$_{NDM\text{-}1}^{+}$ genomes were distributed by 9 STs, mainly in lineages closely related to ST823, most of which were represented by ST308/ST773 (n=5, from Asia, and n=5, from Europe, Asia, USA, and unknown origin, respectively) and ST235/ST357 (n=4, from Europe, and n=4, from Asia, and Africa, respectively). Other *bla*$_{NDM\text{-}1}^{+}$ genomes were observed in lineages closely related to ST111, specifically ST654 (n=4, from Europe, North America, and South America) and ST233 (n=2, from Myanmar and an unspecified location) (Figure 1; Supplementary Figure S1 and Table S1). Unique STs harbouring the *bla*$_{NDM\text{-}1}^{+}$ gene (ST316, ST1023, or ST234) were closely related to the same lineages ST823 and ST111.

3.2. Functional annotation of core and accessory genome

Core and accessory genomes retrieved from comparative genomics analysis were functionally annotated using orthologs databases based on a 99% sequence identity criterion. The core-genome functional analysis revealed negligible differences in the type of gene abundance between *bla*$_{VIM\text{-}2}$+ and *bla*$_{NDM\text{-}1}$+ genomes (data not shown). Based on the strict parameters used (95% coverage, 100% identity), the proteins annotated in accessory genomes were 3040 for *bla*$_{VIM\text{-}2}$+ and 775 for *bla*$_{NDM\text{-}1}$+. For each functional category were identified (based on similar protein functions) subcategories of the most represented protein families (Figure 2). Aiming to assess if the accessory genome was distinct in both groups of genomes, the prevalence of major proteins (proteins per number of genomes) included in different functional subcategories was compared. Proteins involved in transport (*e.g.* carbohydrate transport, extracellular transport), metabolism (*e.g.* permeases, dehydrogenases, or ATPases) and transcriptional regulators were observed to be significantly (*p-value* < 0.05) more prevalent among *bla*$_{VIM\text{-}2}$+ than *bla*$_{NDM\text{-}1}$+ genomes. Protein subcategories like coenzyme catalysis, conjugation, virulence, ABC transporters, membrane-binding protein and pilus, secretion related proteins were observed significantly (*p-value* < 0.05) more abundant in *bla*$_{NDM\text{-}1}$+ than in *bla*$_{VIM\text{-}2}$+ genomes.

3.3. Other antibiotic resistance genes in *P. aeruginosa* bla_{VIM-2}+ and bla_{NDM-1}+

Other antibiotic resistance mechanisms were part of the core-genome of both bla_{VIM-2}+ and bla_{NDM-1}+ *P. aeruginosa*, with efflux pumps representing 85% and 76% of the resistance genes identified, respectively. The other respective 15% and 24% resistance mechanisms, included beta-lactamase encoding and other genes (Supplementary Table S3). The identified efflux pump encoding genes were mainly part of *Opr*, *Opm*, *Mex* and *Mux* operons, representing 56% in the bla_{VIM-2}+ and 54% in the bla_{NDM-1}+, of the total resistance genes. The prevalence of the genes encoding the efflux pumps observed in bla_{VIM-2}+ and bla_{NDM-1}+ genomes was not different from that reported in *P. aeruginosa* genomes available in the public database NCBI (n=7813), >90% (Supplementary Table S3). The beta-lactamase encoding genes observed in most/all the bla_{VIM-2}+ and bla_{NDM-1}+ genomes were represented by bla_{PDC-3} and $bla_{OXA-850}$, while these genes have been reported in 26% and 32% of the *P. aeruginosa* isolates available at NCBI, respectively.

Regarding the accessory genome, the bla_{VIM-2}+ and bla_{NDM-1}+ genomes shared ~ 53% of the antibiotic resistance genes (Figure 3). These were mostly related to aminoglycoside resistance (47%), with beta-lactams resistance representing 6% of the genes (Figure 3). The prevalence of antibiotic resistance genes observed in the bla_{VIM-2}+ and bla_{NDM-1}+ genomes was compared with that of 7813 *P. aeruginosa* genomes available in the CARD database. As expected, the bla_{VIM-2}+ and bla_{NDM-1}+ genomes presented a higher prevalence of antibiotic resistance genes than the average for the species (Supplementary Table S3). The clearest examples of these were the gene *aac*(3)-I associated with aminoglycoside resistance, present in 77% of the bla_{VIM-2}+ genomes and*aph*(3')-III/IV/VI/VII (aminoglycoside resistance), *cat* and floR, associated with phenicol resistance, and *qnrB*, quinolone, respectively, present in 66, 70, 74 and 52%of the bla_{NDM-1}+ genomes, in comparison with < 1% of the average of all *P. aeruginosa* genomes (Supplementary Table S3). These values are also higher than those observed in the average of the carbapenemase encoding genomes (Supplementary Table S3). Indeed, aminoglycoside resistance seems to be part of the background resistance profile of carbapenem resistance strains. In genomes harbouring other carbapenemase encoding

genes, aminoglycoside resistance was the most prevalent resistance genotype, mainly represented by the families *aac*(6'), aph(3') and *aadA*, observed in 53%, 55% and 66% of the genomes (Supplementary Table S3). The co-occurrence of other carbapenem-resistance genes with bla_{VIM-2}+ or bla_{NDM-1}+, suggested that redundancy for this type of resistance may occur, as bla_{VEB} and bla_{CTX-M} was observed in 1% of the bla_{VIM-2}+, and bla_{VEB} and bla_{GES} in 15% and 7% of the bla_{NDM-1}+ genomes, respectively. However, such redundancy is not comparable with the observed for bla_{IMP-1} genomes where the co-occurrence of bla_{VEB} was observed in all the 31 genomes analysed (Supplementary Table S3). Among the bla_{VIM-2}+ genomes analysed in this study, bla_{IMP} or bla_{KPC} were observed in two ST111 genomes, in a distinct genomic environment of bla_{VIM-2}. Also, two ST654 bla_{NDM-1}+ genomes contained the bla_{GES} gene integrated into the same mobile genetic element of bla_{NDM-1}.

3.4. Transposons and mobile genetic elements associated with bla_{VIM-2} and bla_{NDM-1} genes

The genetic environment of the genes bla_{VIM-2} and bla_{NDM-1} inserted in contigs with >3000 bp was analysed. This analysis unveiled five main genomic environments, three including bla_{VIM-2} or bla_{NDM-1}, and two identified only in bla_{NDM-1}^{+} genomes. In the group of 61 bla_{VIM-2}^{+} genomes that gene was included in Tn21 transposon structures (n=17), Tn402-like transposons (n=23), or in insertion sequences IS6100 (n=19), while seven could not be identified (*e.g.* related to Tn3 family). In the group of 27 bla_{NDM-1}^{+} genomes that gene was flanked by ISAba125 and the bleomycin resistance gene ble_{MBL} (n=8) and IS91 (n=19), 8 of which included also an IS6100 sequence (Figure 4). The most commonly observed structure including bla_{NDM-1} was represented by IS91 which flanked the gene on both sides (Figure 4B). The Tn21 depicted in Figure 4, besides the *mer* operon, transposase (tniA) and resolvase, includes an integron containing bla_{VIM-2} or bla_{NDM-1} and other antibiotic resistance genes, mostly aminoglycoside resistance genes. A particular Tn21 structure (Figure 4C) was observed in a bla_{NDM-1}+ genome (GCA_020404785.1, whose genome was sequenced for this study) where that gene was flanked by ISAba125 and ble_{MBL} inserted upstream to an integron containing another carbapenemase resistance gene, bla_{GES}. The Tn402-like element presented a structure similar

to the Tn21 in transposase and resolvase, including an integron containing *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ and other antibiotic resistance genes (Figure 4). As observed for Tn21, also the Tn402-like was described including *bla*$_{NDM-1}$ flanked by ISAba125 and *ble*$_{MBL}$ as the unique ARG. The complete transposons Tn21 and Tn402-like were associated with *bla*$_{NDM-1}$ only in 9 of the 27 genomes. Finally, the insertion sequence IS6100 was observed upstream or downstream of integrons including *bla*$_{VIM-2}$ (Figure 4) or the *bla*$_{NDM-1}$ genetic environment. The same genetic environment was observed in *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes, sometimes without other genes or including other antibiotic resistance genes. The only exception was ISAba125 and IS91 associated only with *bla*$_{NDM-1}$ (Figure 4).

4. Discussion

P. aeruginosa is a ubiquitous multidrug resistant pathogenic species distributed worldwide [29]. Despite the large number (>3000) of sequence types deposited in the public database PubMLST (available at https://pubmlst.org/organisms/pseudomonas-aeruginosa), beta-lactamase-producing *P. aeruginosa* belongs mostly to ST235, ST111 and ST233 [30,31]. The frequent and broad beta-lactam resistance observed in this species has motivated the therapeutical use of carbapenems, against which acquired resistance has increased in prevalence and expanded geographically [30]. The genes *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ analysed in this study exemplify distinct modes of carbapenem resistance evolution in *P. aeruginosa*. The gene *bla*$_{VIM-2}$ is a genetic variant of a gene originally detected in this species, the *bla*$_{VIM-1}$, that has considerably higher activity against carbapenems, which may explain the fact that VIM-2 is a dominant carbapenemase produced by *P. aeruginosa* [30]. Although spread among *P. aeruginosa*, the variant VIM-2 is not frequently detected in other species [32], although have been reported sporadically in *Enterobacteriaceae* and *Burkholderiales* [33,34]. In contrast, *P. aeruginosa bla*$_{NDM-1}$+ was firstly reported in *Enterobacteriaceae* having now a broad phylogenetic diversity of hosts. While *bla*$_{VIM-2}$ has been associated with ST111 and European countries [30], *bla*$_{NDM-1}$ has been frequently identified in ST235, and also in ST233, ST111, or ST654 strains [35,36]. Also globally disseminated is the *bla*$_{IMP-1}$ gene, associated with *P. aeruginosa* ST357 and many other distinct lineages of this species (e.g. ST235, ST234, ST621,

ST664) and of distantly related taxa, such as *Enterobacterales* and *Acinetobacter baumannii*, and presenting a global distribution [30]. In contrast, the bla_{SPM-1} gene associated with the *groEL* gene and the mobile genetic element, IScr4, was firstly reported in *P. aeruginosa* in 2002, with evidence of a restricted local (mainly Brazil) and phylogenetic (mainly ST277) occurrence [37]. Curiously, such a limited distribution has been noted to other class B metalo-beta-lactamases described in *P. aeruginosa* isolates [30].

Our results based on cgMLST confirm the phylogenetic distribution of the bla_{VIM-2}+ and bla_{NDM-1}+ genomes evidencing the wider presence of bla_{NDM-1} across a broader phylogenetic amplitude than bla_{VIM-2}. An interesting result was the observation that some lineages may prevail in distinct geographic origins, like the lineage ST823/ST308/ST73 in Asiatic countries or the lineage ST244/ST175/ST179/ST17 in Portugal, Spain and Poland. ST179, although not widely distributed [12,38] has been considered as part of the international high-risk clones [30,36]. The geographic and phylogenetic distribution of acquired carbapenemase encoding genes can contribute to better understand the interplay between the external factors and intrinsic bacterial properties for the dissemination of resistance determinants.

The rich accessory genome of *P. aeruginosa* has been associated with ubiquitous and worldwide distribution and adaptation to different environments [6]. Genes involved in metal or antibiotic resistance, biofilm formation and virulence present in the accessory genome of *P. aeruginosa* can probably explain the ability to survive under challenging conditions and environments [29]. The functional analysis of bla_{VIM-2}+ and bla_{NDM-1}+ accessory genomes showed a similar profile of protein involved in metals tolerance, antibiotic resistance and mobile genetic elements. In particular, it was observed the presence of proteins involved in the resistance to metals like copper (*cop* operon) or mercury (*mer* operon), being these operons described in genomes of bacteria adapted to metal contaminated environments [5,40]. Other proteins with similar prevalence among bla_{VIM-2}+ and bla_{NDM-1}+ accessory genome were represented by mobile genetic elements like transposases, integrases and phage integrases. The consistent presence of mobile genetic elements in the accessory genomes of *P. aeruginosa* highlights the important role they may have in gene sharing and consequent

genome improvement and adaptation processes [5,41]. Virulence factors were instead observed to be more prevalent among *bla*$_{NDM-1}$+ than *bla*$_{VIM-2}$+ accessory genomes, as well as conjugation and secretion systems related proteins. Secretion systems play an important role both in virulence and conjugation [5,42]. The protein abundance in accessory genomes reflects substantial differences between both groups of genomes analysed. In particular, the higher presence of secretion systems, virulence factors and conjugation elements in *bla*$_{NDM-1}$+ may suggest the strong influence of horizontal gene transfer processes and also its clinical relevance.

The mobilization of antibiotic resistance genes enriches the bacterial resistome, sometimes enhancing functions and mechanisms available in the intrinsic resistome [43]. The *bla*$_{VIM-2}$ and *bla*$_{NDM-1}$ are examples of such accessory genes that can increase the bacterial ability to survive to clinical treatments [44]. In *P. aeruginosa*, the intrinsic resistance is often due to efflux pump systems, often with multidrug resistance potential [43]. Most of the antibiotic resistance genes detected in the accessory genome of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes, as well as in other carbapenemase encoding strains, were associated with aminoglycoside resistance, which genes are frequently part of integron structures where other genes may be also transported, explaining the multidrug resistance phenotype [12]. The co-existence of different carbapenemase resistance genes in ubiquitous bacteria like *P. aeruginosa* is emerging as a serious threat [45]. The cases of multiple carbapenemase resistance genes may deserve a deep analysis, mostly defining their genomic environment, to avoid the spread of these lineages.

The mobilization of carbapenemase resistance genes among *P. aeruginosa* can use different mechanisms [30]. Transposons are mobile genetic elements commonly involved in antibiotic resistance gene mobilization, being Tn21 and Tn402-like often associated with carbapenemase resistance genes. These findings were confirmed for the *bla*$_{VIM-2}$+ and also for the *bla*$_{IMP-1}$+ genomes (n=31) belonging to ST235, ST233 and ST111 that we examined in this study. Instead, *bla*$_{NDM-1}$ has been described mostly flanked by insertion sequences IS91 or ISAba125 [36,46]. Interestingly, in genomes of isolates from Serbia, the *bla*$_{NDM-1}$ flanked by

ISAba125 and ble_{MBL} was included in Tn21 or Tn402-like transposons (Figure 4). The Tn21, in particular, was observed to include bla_{NDM-1} flanked by *ISAba125* and ble_{MBL} and another integron containing bla_{GES}. The study of adaptative characteristics of bacteria and the monitoring of the high-risk clones, as well as the mobilization of carbapenem resistance genes, are valuable approaches to controlling the propagation of multidrug-resistant *P. aeruginosa*.

The mobile genetic elements observed to be associated with the genes bla_{VIM-2} and bla_{NDM-1} in this study are present in about 3-4% of the *P. aeruginosa* genomes and have a wide distribution over distinct species (Supplementary Table S4). Besides *P. aeruginosa,* the same genetic mobile elements were observed in *Klebsiella pneumoniae*, *Escherichia coli*, *Citrobacter freundii* and *Acinetobacter baumannii. K. pneumoniae* and *E. coli* were the species with the highest share of the elements Tn21 (19% and 23%), Tn402-like (22% and 20%, respectively) and IS6100 (28% and 26%, respectively). Although these two species shared also the highest proportion of IS91 (22% and 29%, respectively) or ISAba125 (19% and 27%, respectively), *P. aeruginosa* shared 19% and 15% of each and *A. baumannii* 15% of ISAba125 (Supplementary Table S4). Considering that *A. baumannii* is a likely reservoir of ISAba125 [47] a higher percentage of share would be expected. Bonnin et al. [47] suggested that *A. baumannii* that originally had the ISAba125 element, acquired the bla_{NDM} gene through the acquisition of the Tn125 transposon that was after transferred to *Enterobacteriaceae* or *P. aeruginosa*. These observations show that the mobile genetic elements observed to carry the genes bla_{VIM-2} or bla_{NDM-1} are spread over distinct phylogenetic groups, confirming *Enterobacteriaceae* as major reservoirs of transposons and insertion sequences responsible for antibiotic resistance acquisition [11]. These results also confirm the widest phylogenetic distribution of IS91 and ISAba125, observed in this study to be associated with the bla_{NDM-1} gene [36,46].

5. Conclusions

While carbapenem resistance is widespread among *P. aeruginosa,* it is suggested that distinct genes tend to be associated with different mobile genetic elements and phylogenetic lineages. The same mobile genetic elements that carry the genes bla_{VIM-2} and bla_{NDM-1}, are observed in other *Gammaproteobacteria,* suggesting the frequent inter-genus genetic exchange. However, these may have preference for some specific lineages within the *P. aeruginosa* species, seeming to have a non-random geographic distribution. The study of the interplay between phylogenomic lineages, mobile genetic elements and accessory genome is essential to better understand the ecology of antibiotic resistance and, therefore, how it can be controlled.

Declarations

Funding: This work was financially supported by the European Union's Horizon 2020, under the Innovative Training Networks (ITN-ETN) programme Marie Skłodowska-Curie grant (ANtibioticS and mobile resistance elements in WastEwater Reuse applications: risks and innovative solutions) agreement No 675530 and by FEDER through project RISK.AR "Assessing the risks associated with environmental antibiotic resistant bacteria: propagation and transmission to humans" (PTDC/CTA-AMB/28196/2017) – Programa Operacional Competitividade e Internacionalização. The authors thank the scientific collaboration under the FCT project UID/Multi/50016/2019.

Competing Interests: None

Ethical Approval: Not required

Sequence Information: NCBI accession numbers: GCA_020404715.1, GCA_022559565.1, GCA_020404785.1, GCA_020404825.1, GCA_020404705.1

https://www.ncbi.nlm.nih.gov/bioproject/753000

References

[1] World Health Organization. 2019 Antibacterial agents in clinical development: an analysis of the antibacterial clinical development pipeline. Geneva: World Health Organization; 2019. Licence: CC BY-NC-SA 3.0 IGO. 2019.

[2] European Centre for Disease Prevention and Control. Surveillance of antimicrobial resistance in Europe Annual report of the European Antimicrobial Resistance Surveillance Network (EARS-Net) 2017. 2018.

[3] The Center for Disease Dynamics Economics & Policy. ResistanceMap: Antibiotic resistance. 2018. 2021. https://resistancemap.cddep.org/ (accessed December 2, 2021).

[4] Freschi L, Vincent AT, Jeukens J, Emond-Rheault JG, Kukavica-Ibrulj I, Dupont MJ, et al. The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. Genome Biol Evol 2019;11:109–20. https://doi.org/10.1093/gbe/evy259.

[5] Kung VL, Ozer EA, Hauser AR. The Accessory Genome of Pseudomonas aeruginosa. Microbiol Mol Biol Rev 2010;74:621–41. https://doi.org/10.1128/mmbr.00027-10.

[6] Sawa T, Momiyama K, Mihara T, Kainuma A, Kinoshita M, Moriyama K. Molecular epidemiology of clinically high-risk Pseudomonas aeruginosa strains: Practical overview. Microbiol Immunol 2020;64:331–44. https://doi.org/10.1111/1348-0421.12776.

[7] Freschi L, Vincent AT, Jeukens J, Emond-Rheault JG, Kukavica-Ibrulj I, Dupont MJ, et al. The Pseudomonas aeruginosa Pan-Genome Provides New Insights on Its Population Structure, Horizontal Gene Transfer, and Pathogenicity. Genome Biol Evol 2019;11:109–20. https://doi.org/10.1093/gbe/evy259.

[8] Mosquera-Rendón J, Rada-Bravo AM, Cárdenas-Brito S, Corredor M, Restrepo-Pineda E, Benítez-Páez A. Pangenome-wide and molecular evolution analyses of the Pseudomonas aeruginosa species. BMC Genomics 2016;17. https://doi.org/10.1186/S12864-016-2364-4.

[9] Nolan LM, Turnbull L, Katrib M, Osvath SR, Losa D, Lazenby JJ, et al. Pseudomonas aeruginosa is capable of natural transformation in biofilms. Microbiology 2020;166:995. https://doi.org/10.1099/MIC.0.000956.

[10] Frimmersdorf E, Horatzek S, Pelnikevich A, Wiehlmann L, Schomburg D. How Pseudomonas aeruginosa adapts to various environments: A metabolomic approach. Environ Microbiol 2010;12:1734–47. https://doi.org/10.1111/j.1462-2920.2010.02253.x.

[11] Potter RF, D'Souza AW, Dantas G. The rapid spread of carbapenem-resistant Enterobacteriaceae. Drug Resist Updat 2016;29:30–46. https://doi.org/10.1016/J.DRUP.2016.09.002.

[12] Botelho J, Grosso F, Quinteira S, Brilhante M, Ramos H, Peixe L. Two decades of bla VIM-2 -producing Pseudomonas aeruginosa dissemination: An interplay between mobile genetic elements and successful clones. J Antimicrob Chemother 2018. https://doi.org/10.1093/jac/dkx517.

[13] Matsumura Y, Peirano G, Devinney R, Bradford PA, Motyl MR, Adams MD, et al. Genomic epidemiology of global VIM-producing Enterobacteriaceae. J Antimicrob Chemother 2017;72:2249. https://doi.org/10.1093/JAC/DKX148.

[14] Lauretti L, Riccio ML, Mazzariol A, Cornaglia G, Amicosante G, Fontana R, et al. Cloning and Characterization of blaVIM, a New Integron-Borne Metallo-β-Lactamase Gene from a Pseudomonas aeruginosa Clinical Isolate. Antimicrob Agents Chemother 1999;43:1584.

[15] Alcock BP, Raphenya AR, Lau TTY, Tsang KK, Bouchard M, Edalatmand A, et al. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. Nucleic Acids Res 2020;48:D517–25. https://doi.org/10.1093/NAR/GKZ935.

[16] Yong D, Toleman M, Giske C, Cho H, Sundman K, Lee K, et al. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother 2009;53:5046–54. https://doi.org/10.1128/AAC.00774-09.

[17] Jovcic B, Lepsanovic Z, Suljagic V, Rackov G, Begovic J, Topisirovic L, et al. Emergence of NDM-1 Metallo-β-Lactamase in Pseudomonas aeruginosa Clinical Isolates from Serbia. Antimicrob Agents Chemother 2011;55:3929. https://doi.org/10.1128/AAC.00226-11.

[18] Vaz-Moreira I, Varela AR, Pereira TV, Fochat RC, Manaia CM. Multidrug Resistance in Quinolone-Resistant Gram-Negative Bacteria Isolated from Hospital Effluent and the

Municipal Wastewater Treatment Plant. Microb Drug Resist 2016;22. https://doi.org/10.1089/mdr.2015.0118.

[19] Kabic J, Fortunato G, Vaz-Moreira I, Kekic D, Jovicevic M, Pesovic J, et al. Dissemination of Metallo-β-Lactamase-Producing Pseudomonas aeruginosa in Serbian Hospital Settings: Expansion of ST235 and ST654 Clones. Int J Mol Sci 2023;24. https://doi.org/10.3390/IJMS24021519.

[20] Brettin T, Davis JJ, Disz T, Edwards RA, Gerdes S, Olsen GJ, et al. RASTtk: A modular and extensible implementation of the RAST algorithm for building custom annotation pipelines and annotating batches of genomes. Sci Rep 2015;5. https://doi.org/10.1038/SREP08365.

[21] Page AJ, Cummins CA, Hunt M, Wong VK, Reuter S, Holden MTG, et al. Roary: rapid large-scale prokaryote pan genome analysis. Bioinformatics 2015;31:3691–3. https://doi.org/10.1093/BIOINFORMATICS/BTV421.

[22] Katoh K, Standley DM. MAFFT Multiple Sequence Alignment Software Version 7: Improvements in Performance and Usability. Mol Biol Evol 2013;30:772. https://doi.org/10.1093/MOLBEV/MST010.

[23] Silva M, Machado MP, Silva DN, Rossi M, Moran-Gilad J, Santos S, et al. chewBBACA: A complete suite for gene-by-gene schema creation and strain identification. Microb Genomics 2018;4:e000166. https://doi.org/10.1099/MGEN.0.000166/CITE/REFWORKS.

[24] Zhou Z, Alikhan NF, Sergeant MJ, Luhmann N, Vaz C, Francisco AP, et al. GrapeTree: visualization of core genomic relationships among 100,000 bacterial pathogens. Genome Res 2018;28:1395–404. https://doi.org/10.1101/GR.232397.117.

[25] Huerta-Cepas J, Szklarczyk D, Heller D, Hernández-Plaza A, Forslund SK, Cook H, et al. EggNOG 5.0: A hierarchical, functionally and phylogenetically annotated orthology resource based on 5090 organisms and 2502 viruses. Nucleic Acids Res 2019;47:D309–14. https://doi.org/10.1093/NAR/GKY1085.

[26] Wickham H. ggplot2 Elegant Graphics for Data Analysis (Use R!). 2016. https://doi.org/10.1007/978-0-387-98141-3.

[27] Chen H, Boutros PC. VennDiagram: a package for the generation of highly-customizable

Venn and Euler diagrams in R. BMC Bioinforma 2011 121 2011;12:1–7. https://doi.org/10.1186/1471-2105-12-35.

[28] Siguier P, Perochon J, Lestrade L, Mahillon J, Chandler M. ISfinder: the reference centre for bacterial insertion sequences. Nucleic Acids Res 2006;34. https://doi.org/10.1093/nar/gkj014.

[29] Moradali MF, Ghods S, Rehm BHA. Pseudomonas aeruginosa Lifestyle: A Paradigm for Adaptation, Survival, and Persistence. Front Cell Infect Microbiol 2017;7:39. https://doi.org/10.3389/FCIMB.2017.00039.

[30] Yoon EJ, Jeong SH. Mobile Carbapenemase Genes in Pseudomonas aeruginosa. Front Microbiol 2021;12:30. https://doi.org/10.3389/fmicb.2021.614058.

[31] del Barrio-Tofiño E, López-Causapé C, Oliver A. Pseudomonas aeruginosa epidemic high-risk clones and their association with horizontally-acquired β-lactamases: 2020 update. Int J Antimicrob Agents 2020;56. https://doi.org/10.1016/j.ijantimicag.2020.106196.

[32] Edelstein M V., Skleenova EN, Shevchenko O V., D'souza JW, Tapalski D V., Azizov IS, et al. Spread of extensively resistant VIM-2-positive ST235 Pseudomonas aeruginosa in Belarus, Kazakhstan, and Russia: A longitudinal epidemiological and clinical study. Lancet Infect Dis 2013;13. https://doi.org/10.1016/S1473-3099(13)70168-3.

[33] Khajuria A, Praharaj AK, Grover N, Kumar M. Emergence of VIM-2 metallo-beta-lactamase producing Ralstonia pickettii clinical isolate in India. Indian J Med Microbiol 2014;32:191–3. https://doi.org/10.4103/0255-0857.129831.

[34] Morfin-Otero R, Rodriguez-Noriega E, Deshpande LM, Sader HS, Castanheira M. Dissemination of a blaVIM-2-Carrying Integron Among Enterobacteriaceae Species in Mexico: Report from the SENTRY Antimicrobial Surveillance Program. Https://HomeLiebertpubCom/Mdr 2009;15:33–5. https://doi.org/10.1089/MDR.2009.0878.

[35] Kocsis B, Gulyás D, Szabó D. Diversity and distribution of resistance markers in pseudomonas aeruginosa international high-risk clones. Microorganisms 2021;9:1–14. https://doi.org/10.3390/microorganisms9020359.

[36] Janvier F, Jeannot K, Tessé S, Robert-Nicoud M, Delacour H, Rapp C, et al. Molecular

Characterization of blaNDM-1 in a Sequence Type 235 Pseudomonas aeruginosa Isolate from France. Antimicrob Agents Chemother 2013;57:3408. https://doi.org/10.1128/AAC.02334-12.

[37] Toleman MA, Simm AM, Murphy TA, Gales AC, Biedenbach DJ, Jones RN, et al. Molecular characterization of SPM-1, a novel metallo-beta-lactamase isolated in Latin America: report from the SENTRY antimicrobial surveillance programme. J Antimicrob Chemother 2002;50:673–9. https://doi.org/10.1093/JAC/DKF210.

[38] Hishinuma T, Uchida H, Tohya M, Shimojima M, Tada T, Kirikae T. Emergence and spread of VIM-type metallo-β-lactamase-producing Pseudomonas aeruginosa clinical isolates in Japan. J Glob Antimicrob Resist 2020;23:265–8. https://doi.org/10.1016/j.jgar.2020.09.010.

[39] Wang M-G, Liu Z-Y, Liao X-P, Sun R-Y, Li R-B, Liu Y, et al. Retrospective Data Insight into the Global Distribution of Carbapenemase-Producing Pseudomonas aeruginosa. Antibiotics 2021;10. https://doi.org/10.3390/ANTIBIOTICS10050548.

[40] Mathema VB, Thakuri BC, Sillanpää M. Bacterial mer operon-mediated detoxification of mercurial compounds: A short review. Arch Microbiol 2011;193:837–44. https://doi.org/10.1007/S00203-011-0751-4/TABLES/1.

[41] Virolle C, Goldlust K, Djermoun S, Bigot S, Lesterlin C. Plasmid Transfer by Conjugation in Gram-Negative Bacteria: From the Cellular to the Community Level. Genes (Basel) 2020;11:1–33. https://doi.org/10.3390/GENES11111239.

[42] Trokter M, Waksman G. Translocation through the conjugative type IV secretion system requires unfolding of its protein substrate. J Bacteriol 2018;200. https://doi.org/10.1128/JB.00615-17/SUPPL_FILE/ZJB999094662S1.PDF.

[43] López-Causapé C, Cabot G, del Barrio-Tofiño E, Oliver A. The Versatile Mutational Resistome of Pseudomonas aeruginosa. Front Microbiol 2018;0:685. https://doi.org/10.3389/FMICB.2018.00685.

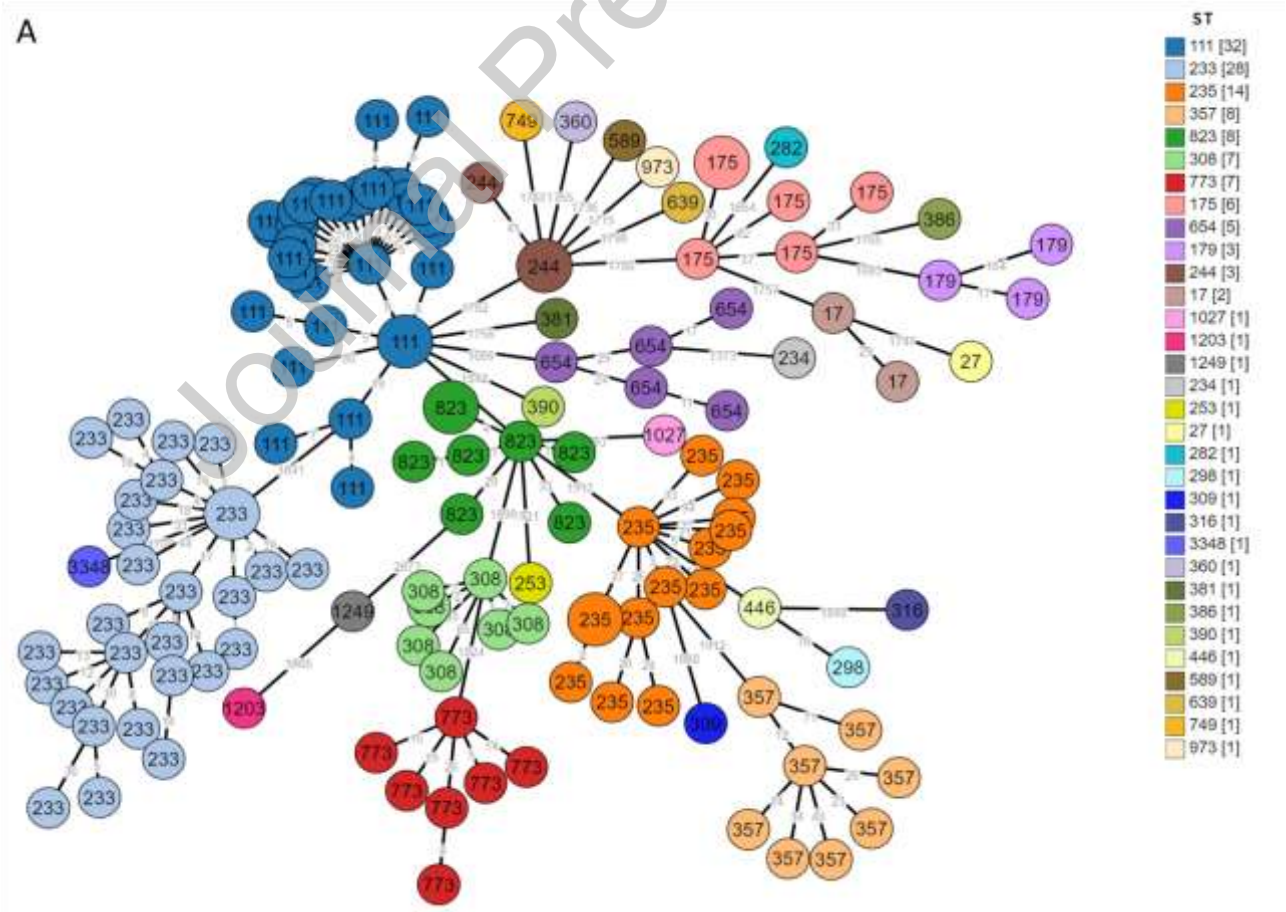
[44] Pacheco T, Bustos-Cruz RH, Abril D, Arias S, Uribe L, Rincón J, et al. Pseudomonas aeruginosa coharboring blaKPC-2 and blaVIM-2 carbapenemase genes. Antibiotics 2019;8. https://doi.org/10.3390/antibiotics8030098.

[45] Ellappan K, Belgode Narasimha H, Kumar S. Coexistence of multidrug resistance mechanisms and virulence genes in carbapenem-resistant Pseudomonas aeruginosa strains from a tertiary care hospital in South India. J Glob Antimicrob Resist 2018;12:37–43. https://doi.org/10.1016/J.JGAR.2017.08.018.

[46] Poirel L, Dortet L, Bernabeu S, Nordmann P. Genetic features of blaNDM-1-positive Enterobacteriaceae. Antimicrob Agents Chemother 2011;55:5403–7. https://doi.org/10.1128/AAC.00585-11.

[47] Bonnin RA, Poirel L, Nordmann P. New Delhi metallo-β-lactamase-producing Acinetobacter baumannii: A novel paradigm for spreading antibiotic resistance genes. Future Microbiol 2014;9. https://doi.org/10.2217/fmb.13.69.

Figures



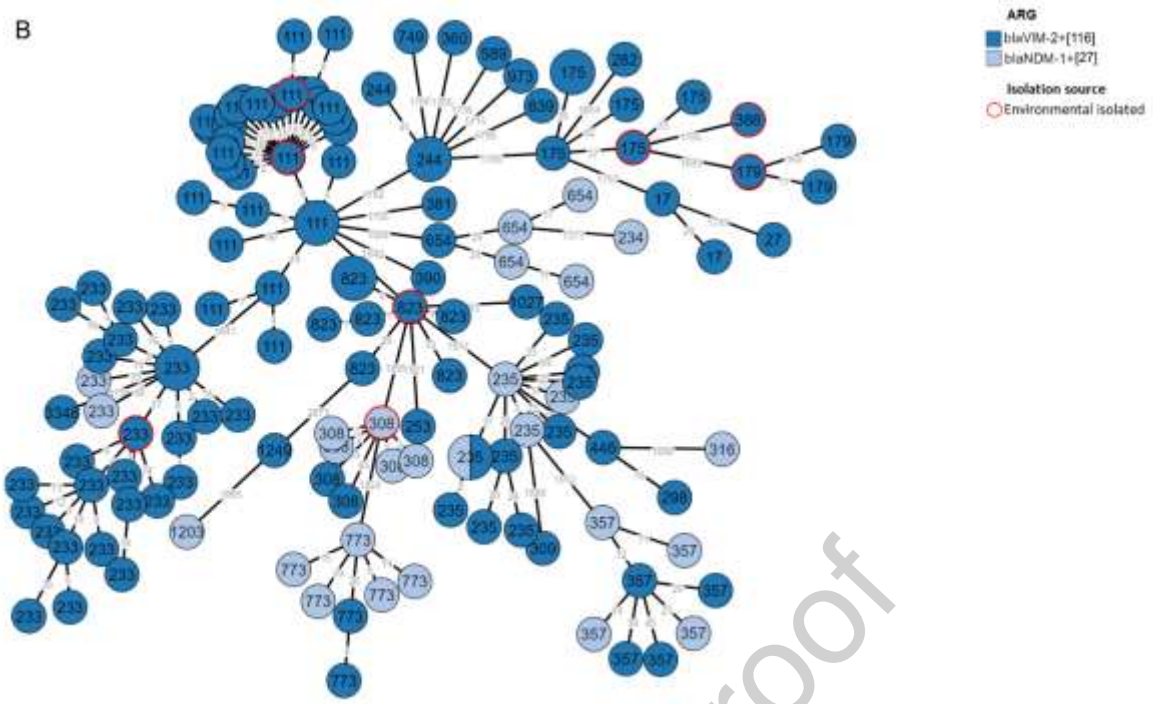


Figure 1. Phylogenetic distribution of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ strains. A) Minimum spanning tree based on cgMLST, reporting the phylogenetic distribution of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ strains identified with the ST (different colors). B) Minimum spanning tree based on cgMLST, reporting the phylogenetic distribution of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ strains identified by ST numbers in the nodes and colored by ARGs harbored. The isolation source is indicated by a red circle around the nodes referring to environmental isolated strains.

The branches label refers to the number of locus differing between the strains reported.

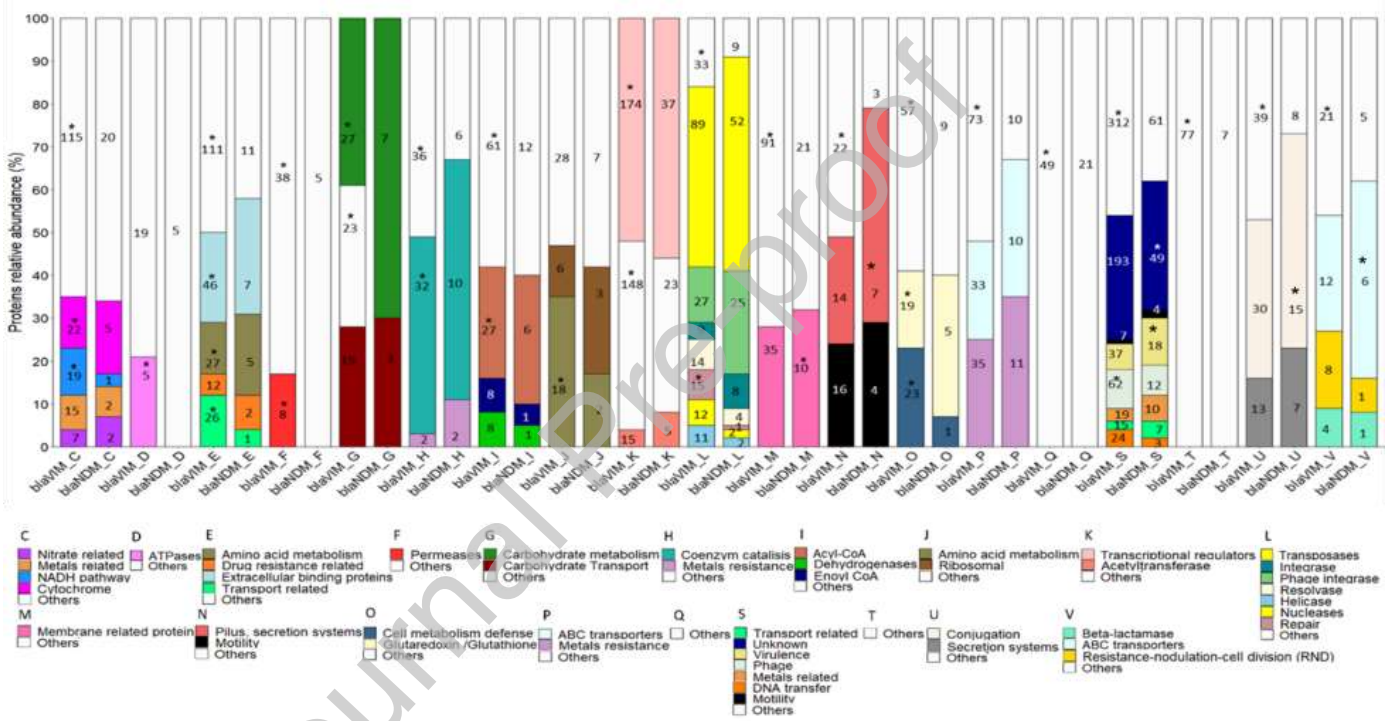


Figure 2. Functional analysis of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ genomes. Barplot with the functional annotation of proteins relative abundance retrieved from the accessory genomes of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ strains. The bars refer to the functional categories: C. Energy production and conversion, D. Cell cycle control and mitosis, E. Amino Acid metabolism and transport, F. Nucleotide metabolism and transport, G. Carbohydrate metabolism and transport, H. Coenzyme

metabolism, I. Lipid metabolism, J. Translation, K. Transcription, L. Replication and repair, M. Cell wall/membrane/envelop biogenesis, N. Cell motility, O. Post-translational modification, protein turnover, chaperone functions, P. Inorganic ion transport and metabolism, Q. Secondary Structure, S. Function Unknown, T. Signal Transduction, U. Intracellular trafficking and secretion, and V. Defense mechanisms.

In the figure are reported the number of proteins in each sub-category and indicated with an * the statistical differences (p-value < 0.05) between *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ functional sub-categories analysed.

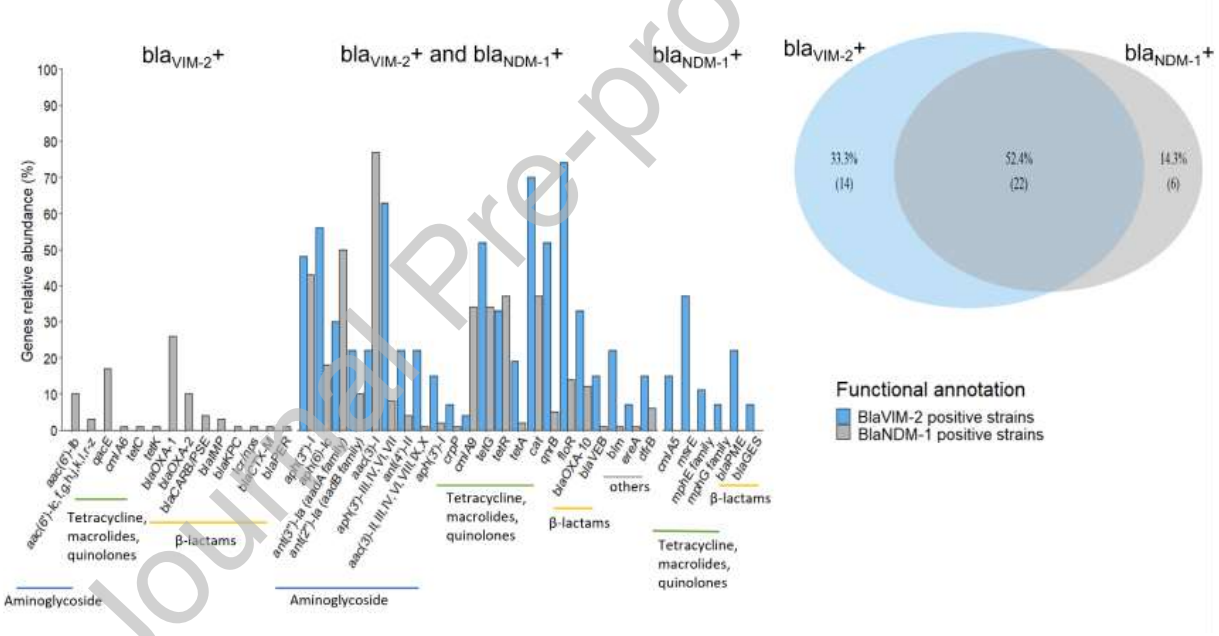
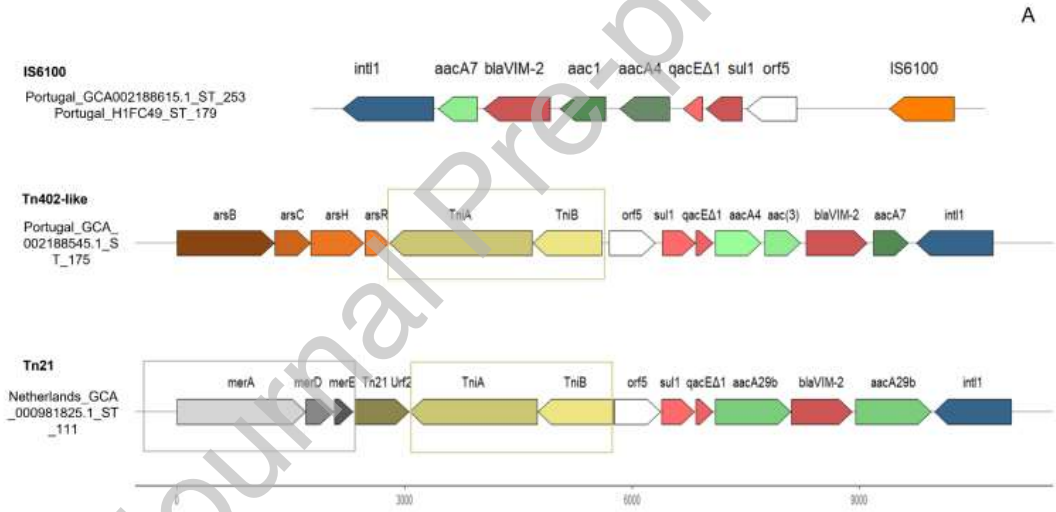
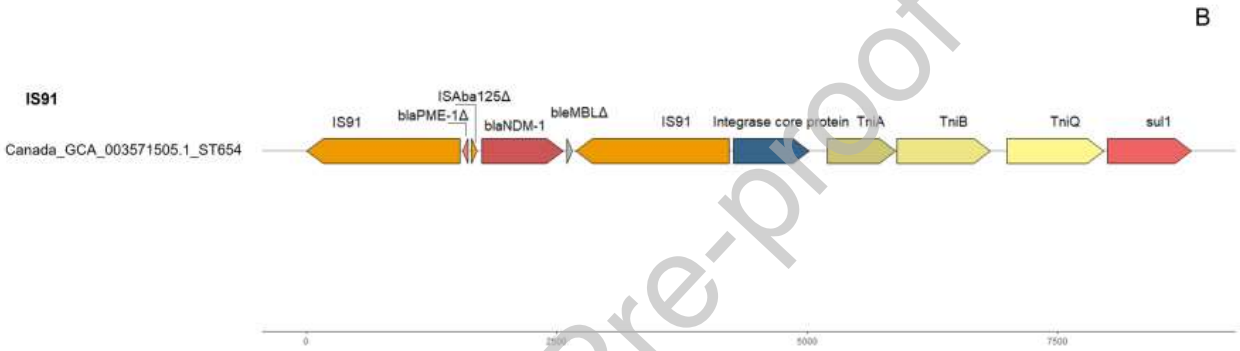


Figure 3. Antibiotic resistance profile analysis of *bla*$_{VIM-2}$+ and *bla*$_{NDM-1}$+ accessory genomes. The barplot represents the antibiotic resistance genes carried by *bla*$_{VIM-2}$+ (grey bars), *bla*$_{NDM-1}$+ (blue bars) genomes, and shared between both. The bars correspond to the genes relative abundance considering the total number of strains analyzed (116 *bla*$_{VIM-2}$+ and 27 *bla*$_{NDM-1}$+). The antibiotic resistance genes are reported and grouped in the barplot sections from left to right based on the antibiotic class (aminoglycosides, tetracycline, macrolides and quinolones, beta-lactams, and others). The overview of the antibiotic resistance genes in *bla*$_{VIM-2}$+ (grey circle) and *bla*$_{NDM-1}$+ (blue circle) are reported in the Venn diagram.





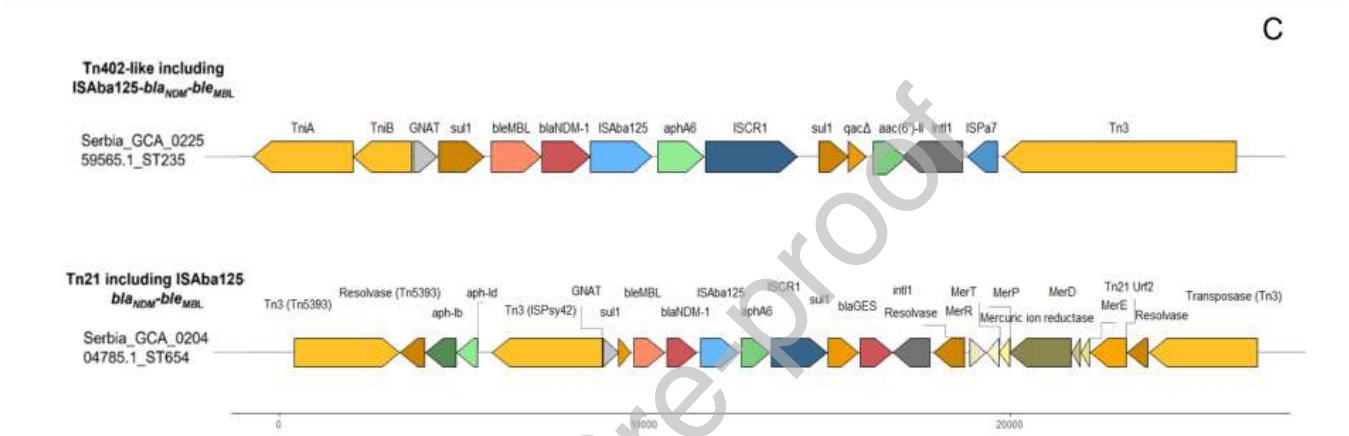
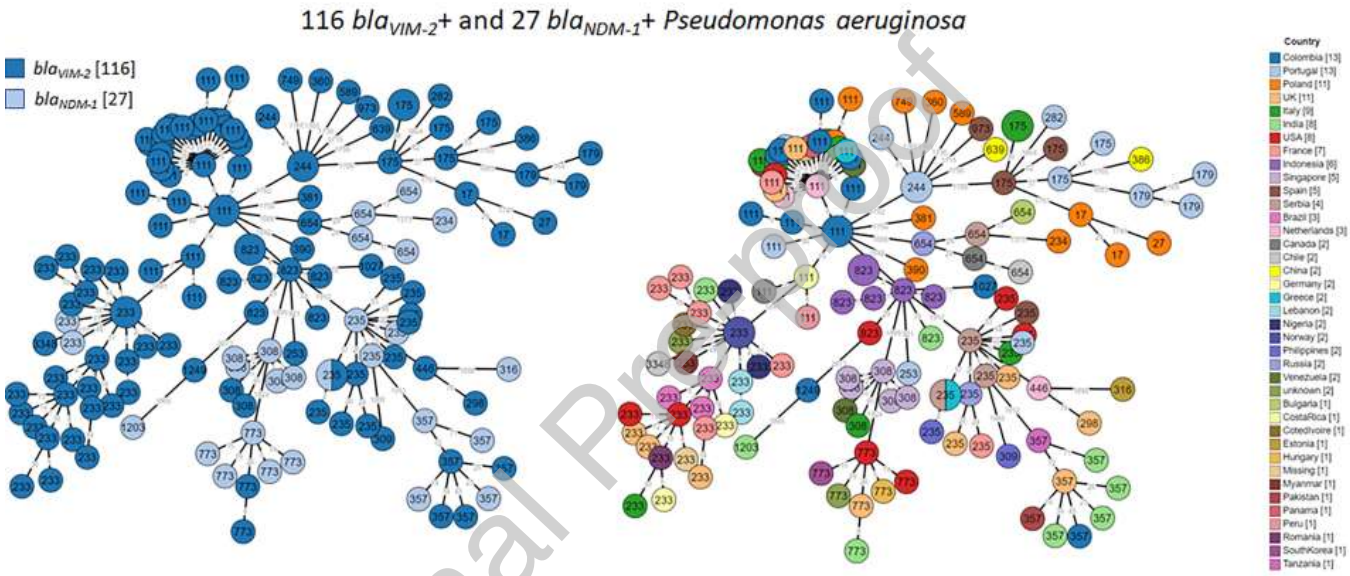


Figure 4. Transposons organization and genetic environment of *bla*$_{VIM-2}$ or *bla*$_{NDM-1}$ in the analyzed *P. aeruginosa* genomes. In the figures are reported the most representative transposons organization and genomic environments of *bla*$_{VIM-2}$or *bla*$_{NDM-1}$ harbored by the analyzed *P. aeruginosa* strains. The gene *bla*$_{VIM-2}$ was described as associated principally with insertion sequences IS6100, Tn402-like and Tn21 transposons (A). The gene *bla*$_{NDM-1}$ was described as associated with the insertion sequence IS91 (B) and Tn21 and Tn402-like transposons including the ISAba125-*bla*$_{NDM}$-*ble*$_{MBL}$ (C). In all the transposons and genetic environments represented, the aminoglycosides resistance genes are colored in green shape, the insertion sequences in orange, antibiotic resistance genes in red, Tni modules in beige, mercury resistance genes in brown, integrase (*intI1*) in blue and other genes in white. The transposons sequences annotation was curated manually using BLAST databases and similar transposons were aligned using MAFFT. The distribution of MGEs analysed in *bla*$_{VIM-2}$ + and *bla*$_{NDM-1}$ + strains are described in Supplementary Table S5.

The class 1 integron including the *bla*$_{VIM,}$ associated with IS6100 (A), was described in a plasmid by Botelho et al. (https://doi.org/10.1093/jac/dkx143) and chromosome (our study) with 100% of sequence identity.

The Tn402 and Tn21 transposons family harbouring *bla*$_{VIM}$ reported in (A) were previously published by Botelho et al. (https://doi.org/10.1093/jac/dkx143) and Zee et al. (https://doi.org/10.3389/FMICB.2018.02057) and selected as examples.

Graphical abstract



Most common	bla_{VIM-2}+	bla_{NDM-1}+
Sequence type	ST233, ST111	ST38, ST773, ST235, ST357, ST654
Geographic distribution	Europe (75/116)	Asia (n=13/27)
Mobile genetic elements	Tn402-like and Tn21	*ISAba125* or *IS91*