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Research article

Decoding the resistome, virulome and mobilome of clinical *versus* aquatic *Acinetobacter baumannii* in southern Romania

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ABSTRACT

Acinetobacter baumannii, a notorious opportunistic pathogen, presents a formidable challenge in both clinical and environmental fields due to its resilience and ability to acquire resistance. This study undertook a comprehensive analysis of 183 *A. baumannii* isolates collected between 2019 and 2022 from intra-hospital infections (IHI), hospital sewages (Hs), wastewater treatment plants (WWTP), and adjacent river waters from two Southern cities, focusing on their resistome, virulome, and mobilome through isolation on chromogenic media, identification by MALDI-TOF-MS and antibiotic susceptibility testing by disk diffusion) followed by genotypic characterization [Whole Genome Sequencing (WGS), 3rd generation sequencing through the MinION (ONT) platform, pangenome description, and respectively horizontal gene transfer through conjugation assays]. Our findings reveal significant genomic plasticity and the prevalence of high-risk international clones, underlining the potential of these isolates to act as reservoirs for antibiotic resistance genes (ARGs) that could be dynamically exchanged between clinical and environmental settings through mobile genetic elements (MGEs) such as the pMAL1 plasmids and the critical role of WWTPs in the persistence and spread of *A. baumannii*. Moreover, our study presents the first report of the co-occurrence of $bla_{\text{OXA-23}}$ and $bla_{\text{OXA-72}}$ in *A. baumannii* ST2 clone. Thus, our research underscores the necessity for integrated surveillance and targeted interventions across healthcare and environmental sectors to mitigate the risk posed by this adaptable pathogen.

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1. Introduction

Antimicrobial resistance (AMR) precipitates significant increases in morbidity and mortality rates, escalates healthcare expenses, incurs economic detriments, undermines infectious disease control efforts, and portends a reversion to the pre-antibiotic era [1,2]. The preponderance of challenging bacterial infections among hospitalized or immunocompromised individuals is attributed to ESKAPE pathogens (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa, Enterobacter* spp.), identified by the Centers for Disease Control and Prevention (CDC) as presenting urgent and serious threats, necessitating targeted research and management strategies [3]. AMR in ESKAPE pathogens is propelled by diverse mechanisms,

Abbreviations

AK	Amikacin
AMR	Antimicrobial resistance
ARGs	Antibiotic resistance genes
ARB	Antibiotic resistant bacteria
ATM	Aztreonam
BHI	Brain heart infusion
BRG	Biocide resistance genes
CAZ	Ceftazidime
CDC	Centers for Disease Control and Prevention
Cf	Conjugation frequency
CIP	Ciprofloxacin
CLSI	Clinical and Laboratory Standards Institute
CN	Gentamicin
CP	Carbapenemase
CRAB	Carbapenem-resistant *Acinetobacter baumannii*
CRGs	Carbapenem resistance genes
DOR	Doripenem
EF	Effluent
ERIC-PCR	Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction
ESBL:	Extended-spectrum β-lactamases
FEP	Cefepime
Hs	Hospital sewages
IC	International Clone
IHI	Intra-hospital infections
IMP	Imipenem
IN	Influent
MDR	multidrug-resistant
MEM	Meropenem
MGE	Mobile genetic element
MH	Minocycline
MLST	Multilocus Sequence Typing
PDR	pandrug resistance
Rm. Vâlcea	Râmnicu Vâlcea
SRA	GenBank Sequence Read Archive
SAM	Ampicillin sulbactam
ST	Sequence type
SW	Surface water
SW DO	Surface water downstream region
SW UP	Surface water upstream region
T	Târgoviște
VFs	Virulence factors
WHO	World Health Organization
WGS	Whole Genome Sequencing
WW	Wastewater
WWTP	Wastewater treatment plants
XDR	Extensively drug-resistant

including drug inactivation or alteration, drug target modifications, cell permeability changes, and biofilm formation, often leading to multidrug resistance (MDR) profiles [4]. The World Health Organization (WHO) categorizes carbapenem-resistant *Acinetobacter baumannii* (CRAB) as a pathogen of critical concern [5], with Romania reporting high MDR prevalence in clinical *A. baumannii* isolates, harboring especially fluoroquinolones, carbapenems, and aminoglycosides resistance [6].

Chemical pollutants, including antibiotics and heavy metals, may augment mutation, recombination, and horizontal gene transfer rates, thereby enriching the resistome and mobilome of clinical and environmental reservoirs [7,8]. Thus, a One Health approach is needed to evaluate AMR risks across a spectrum of environments beyond clinical settings, including the vicinities of healthcare facilities, communities, agricultural and aquacultural settings, and natural reservoirs like water and soil, to preempt the recirculation of AMR to humans and animals [9]. Wastewater Treatment Plants (WWTPs), serving as intermediaries between human habitation and aquatic ecosystems, accumulate effluents from diverse sources and emerge as critical nodes for the convergence of antibiotic-resistant bacteria (ARB), antibiotic resistance genes (ARGs), heavy metals, biocides, and biocide-resistant bacteria (BRB), imposing selective pressures on downstream aquatic ecosystems [10–13]. Many studies have demonstrated heightened levels of ARB and ARGs in the downstream effluents of WWTPs, attributable to mobile genetic elements (MGEs) such as integrative and conjugative elements, transposons, and integrons [14–17].

Whole Genome Sequencing (WGS) emerges as a valuable tool within the One Health framework for elucidating antibiotic resistance patterns at the confluence of human, animal, and environmental interfaces [18–20], emphasizing the importance of high-resolution genomic tools in combating AMR. In this regard [20], stated that we should try to understand interactions between human and non-human *A. baumannii* populations instead of seeing them as separate entities, a fact of paramount importance in the public health implications awareness. Furthermore, integrating surveillance, reporting, and dynamic studies of multidrug-resistant (MDR) pathogens, alongside fostering awareness, education, and informed policy-making, represent critical elements of One Health's approach to addressing antibiotic resistance. In this context, our study aimed to conduct a comparative analysis of the resistome, virulome, and mobilome in *A. baumannii* isolated over four consecutive years (2019–2022) from intra-hospital infections and various aquatic compartments (wastewater [WW] and surface water [SW] networks) in two southern Romanian districts, for elucidating the impact of internationally circulating high-risk clones on the natural aquatic microbiota.

2. Materials and methods

2.1. Resistant bacteria isolation and identification

The analyzed isolates were recovered from 35 water samples collected between March 2019–August 2022, encompassing various sources: hospital sewage (Hs, $n = 4$), wastewater treatment plant (WWTP) inputs (influent, $n = 6$; sludge, $n = 4$; effluent, $n = 7$—all

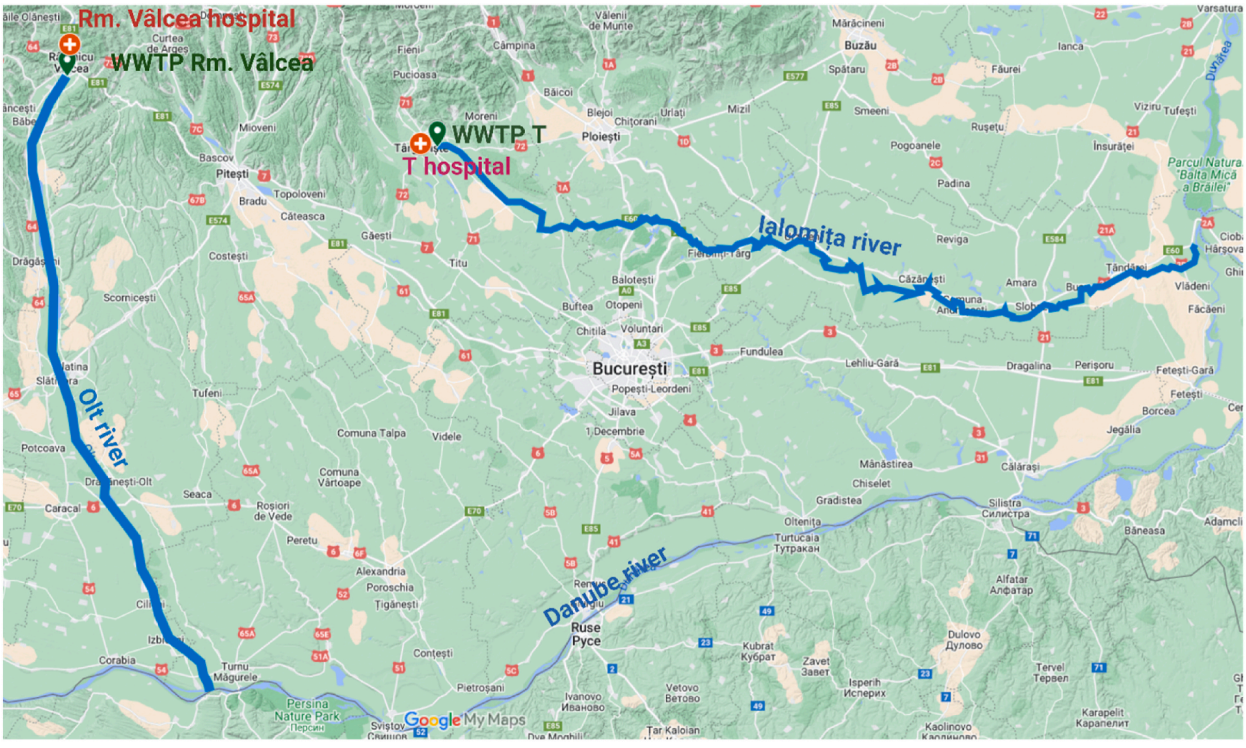


Fig. 1. Sampling points highlighting the rivers collecting the WWTPs effluents, both tributaries to Danube River.

treated as a unified collection group), and surface water (SW)—specifically upstream (n = 4) and downstream (n = 10, located approximately 200 m from the WWTP) sectors, from two southern Romanian localities, namely Târgoviște along the Ialomița River, and Râmnicu Vâlcea (Rm. Vâlcea) on the Olt River (Fig. 1). Serial dilutions of the water samples, up to a factor of 10^-5, were subjected to membrane filtration, followed by inoculation on CHROMagar Acinetobacter, CHROMagar ESBL, and CHROMagar CARBA (CHROMagar, Paris, France), subsequently incubated at 37 °C for 24 h under aerobic conditions. From each sample, 6–10 colonies were selected for taxonomic verification *via* MALDI-TOF-MS (Bruker system). Concurrently, *A. baumannii* isolates were recovered from intra-hospital infections (IHIs) to facilitate the comparative analysis between clinical and environmental isolates. Within a maximum of ten days of the water sampling, the clinical isolates were collected from two emergency hospitals that discharge wastewater into the sampled WWTPs from Târgoviște and Râmnicu Vâlcea. The collected clinical samples, represented by the respiratory tract and wound secretions, were seeded on blood agar and Cystine Lactose Electrolyte Deficient (CLED) agar. The recovered isolates were identified using the MALDI-TOF-MS Bruker system and included in the microbial collection of the Research Institute of the University of Bucharest, ensuring no personal patient data was linked to the samples. The selection criteria included resistance to carbapenems and the two most frequent intra-hospital infections diagnosed in the selected medical settings.

2.2. Antibiotic susceptibility assays

A total number of 183 *A. baumannii* isolates recovered from aquatic and clinical samples were tested for antibiotic susceptibility using the standard disc diffusion method, following the protocols outlined in the current editions of the Clinical and Laboratory Standards Institute (CLSI) guidelines pertinent to the respective year of isolation [21–24]. The antibiotic resistance profiles of these isolates were classified into the three categories: non-MDR (sensitive to all tested antibiotics or resistant to one or two antibiotic classes), MDR (resistant to three or four classes of antibiotics), and extensively drug-resistant (XDR, resistant to five to seven classes of antibiotics), according to Magiorakos et al., 2012 [25].

2.3. Whole genome sequencing (WGS) and bioinformatics

From the 183 isolates, 70 *A. baumannii* clinical, wastewater, and environmental isolateshave been selected for WGS sequencing. The criteria used for the selection of the sequenced isolates included the antimicrobial susceptibility profiles (MDR isolates), isolation sources (from clinical settings to WWTP effluent receiving river), geographic location (isolates from both locations), the presence of β-lactamase encoding genes (positive for OXA-23 and/or OXA-72 CP) and Enterobacterial Repetitive Intergenic Consensus Polymerase Chain Reaction (ERIC-PCR) profiles (representative isolates from all encountered ERIC-PCR profiles) (data not shown).

Total DNA was isolated using DNeasy UltraClean Microbial Kit (Qiagen) and subjected to Illumina (Nextera DNA Flex Library Prep Kit) sequencing on MiSeq and NextSeq platforms (V3, 600 cycles). *De novo* sequencing assembly, adapter trimming, and polishing were performed with Shovill v1.1.0 pipeline (shovill: -keepfiles –trim) [26]. Various Nucleotide/Protein sequence databases, such as CARD, ResFinder, CGE, BacMet, BacAnt, Phaster, VFDB, etc., have been downloaded locally and further used as input for Diamond for predictions [27–32]. Transeq from EMBOSS was used for the translation of nucleotide sequences. Diamond software v2.0.8 was used to compare the contig sequences against the protein databases by BlastX (using the following parameters: diamond blastx –max-target-seqs 0 –more-sensitive –id 70 -p 8 –subject-cover 90) [33]. An in-house Python program has been used to do the cluster analysis based on the predictions on ARGs, biocide resistance genes (BRG), virulence factors (VFs), and MGEs. In-house AWK, Python, and Excel scripts were further used to filter the resulting data. The ST classification has been performed with Multilocus Sequence Typing (MLST) [34], according to the Pasteur scheme [35,36]. The annotation of the selected isolates' sequences has been performed with Prokka v1.14.6, and the resulting output from Prokka (using the following parameters: prokka –cpus 8 –gcode 11 –rnammer –compliant –centre XXX) was further used as input for Roary v3.13.0 (the following options have been used: "*-g 80000 -e –mafft -p 8 -r -qc -r -z -f*") [37,38]. The representations of the resulted Newick pangenome tree from Roary, together with the metadata [sequence type (ST), sampling points, isolation period), the core and accessory genes, have been done using the online tool Phandango [39], FigTree v1.4.4 and Affinity Designer software. Heap's law has been estimated for each data set using Seth Commichaux's Python script [40]. Gene ontology classification of the pangenome data has been performed with DeepNOG [41] and COG Classifier tools [42]. Venn diagrams showing the distribution of ARGs and BRGs (by isolation sources and circulating *A. baumannii* clones) in the analyzed isolates by city have been generated [43]. The graphic representations have been further polished using Affinity Designer software.

2.4. Chromosomal or plasmid location of carbapenem resistance genes (CRGs)

From the total of the 70 *A. baumannii* sequenced isolates, 18 *A. baumannii* carrying CRGs were selected for 3rd generation sequencing experiments, aiming to cover all isolation sources, geographic locations and studied years. DNA extraction was performed according to the manufacturer's instructions using the Wizard® HMW DNA Extraction Kit, Promega. Sequencing was performed using a nanopore sequencing platform [MinION, Oxford Nanopore Technologies (ONT)] and the Rapid Sequencing DNA V14-barcoding kit (SQK-RBK114.24). Base calling was performed with MinKNOW software v23.04.6, followed by reads assembly using the Unicycler pipeline [44]. ARG annotation was performed using Abricate [45] with the NCBI database (v march 2024). Contigs/unitigs harboring ARGs were manually inspected using Blast against the NCBI database for chromosomal/plasmid identities. The insertion sequences were annotated using ISFinder [46].

2.5. Conjugation assays

Transferability of bla_{OXA-23} and bla_{OXA-24} genes by conjugation was tested using the liquid mating method, with rifampicin (RIF) induced resistant *Acinetobacter baylyi* as the recipient. Briefly, equal amounts (500 μL) of overnight cultures of the donor *A. baumannii* isolates (n = 14) and recipient isolate were mixed and incubated in Brain heart infusion (BHI). Cells were resuspended in broth medium and selected on BHI agar plates containing RIF (31.2 μg/mL) and meropenem (MEM) (0.5 μg/mL) [47,48]. Characterization of transconjugants was carried out by evaluating the culture and colony characteristics on a selective medium (BHI + RIF + MEM) for determining the conjugation frequency (Cf) and by molecular methods (DNA extraction from the transconjugants, multiplex PCR check of CRG transferability and 3rd generation sequencing for selected transconjugants, in order to confirm the plasmid dissemination). The Cf was evaluated using the following relationships:

$$Cf = \frac{colonies\ number}{3} \times dilution\ factor$$

2.6. GenBank accession numbers

The whole genome sequencing data (raw data) corresponding to studied samples were submitted to GenBank Sequence Read Archive (SRA) and can be accessed under the PRJNA1083781 code (https://www.ncbi.nlm.nih.gov/sra/PRJNA1083781). The assembled genomes are available upon request.

3. Results and discussion

3.1. Comparative analysis of the antimicrobial resistance phenotypes of A. baumannii isolates with different isolation sources

Acinetobacter spp. represents a significant component of the heterotrophic microbiota in surface water and WWTPs, the microbial loads depending on the treatment stage. The specific genetic characteristics and the functional and pathogenic potential of *A. baumannii* isolates were not linked to their isolation source, its ubiquitous distribution being a reflection of its generalist lifestyles. However, MDR and XDR *A. baumannii* isolates harboring different virulence profiles are reported to be prevalent globally, including in

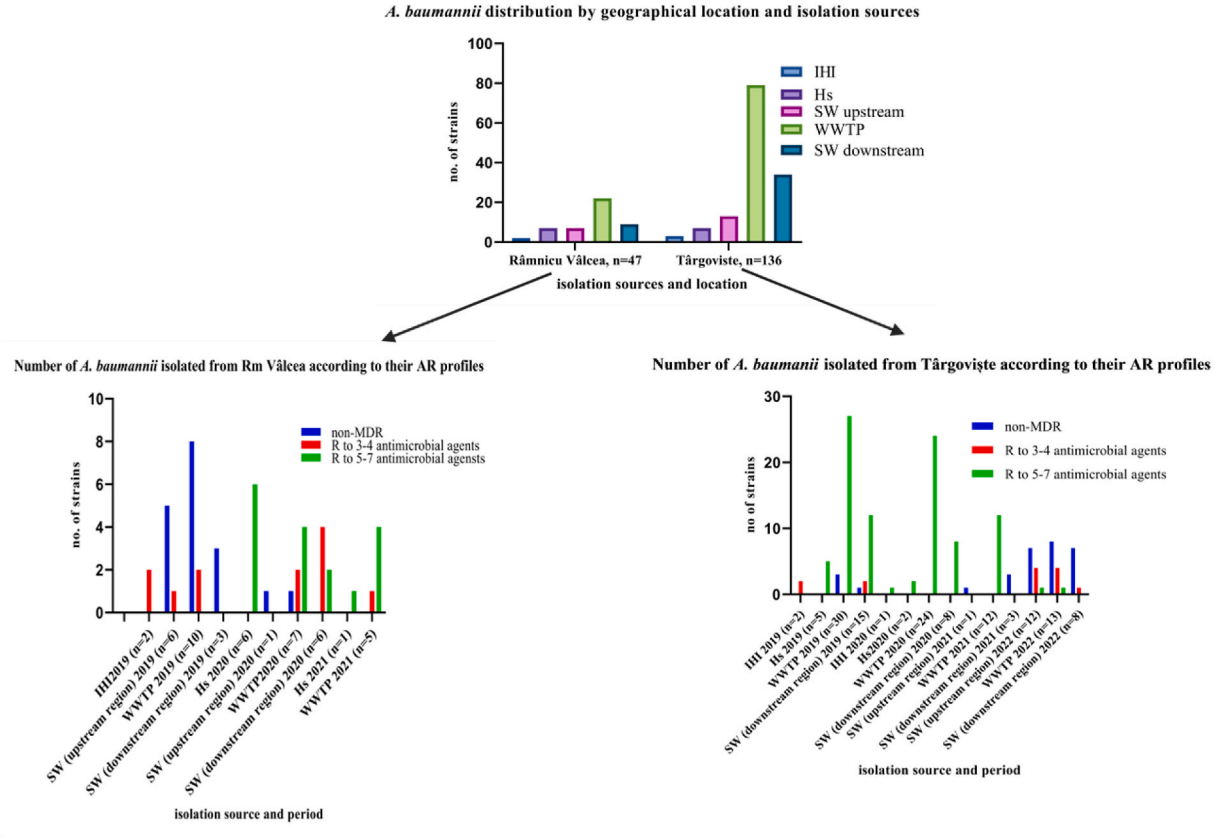


Fig. 2. Prevalence of *A. baumannii* isolates according to their source, time and geographic locations.

Romania [49–62]. Previous studies performed by our team revealed the presence of XDR and CRAB isolates isolated from hospitalized and ambulatory patients, harboring OXA-23 or OXA-24 and belonging to different ICs [48,63]. Other studies performed in Romania revealed an alarming prevalence of MDR isolates from blood samples in Southern Romania between 2017 and 2020 [64].

In this paper, we investigated the ARGs, BRGs, VFs, MGE, and phylogeny of *A. baumannii* isolates for four consecutive years (2019–2022) from intra-hospital infections, wastewater, and surface water samples in Southern Romania using state-of-the-art WGS alongside third-generation sequencing techniques. The impetus for conducting this longitudinal study stemmed from our earlier findings, which highlighted the occurrence of PDR, XDR, and MDR phenotypes along with CRGs and/or ESBL encoding genes in *A. baumannii* clinical and aquatic isolates recovered during 2019–2021 in Southern Romania [65,66]. Our results highlighted the role of WWTPs in the dissemination of AMR from anthropic sources into natural aquatic environments and pointed out the potential impact of the COVID-19 pandemic (directly correlated with the resistance level of the isolates recovered in 2020) on the *A. baumannii* resistance evolution. We have therefore undertaken a comprehensive phenotypic and genotypic analysis of 183 *A. baumannii* isolates recovered simultaneously from environmental and clinical sources in two cities in Southern Romania. The isolates were cultured on chromogenic media to quantify the total number of *Acinetobacter* and, specifically, the prevalence of *A. baumannii* producing ESBL and CP. The selection of two WWTPs aimed to reflect distinct pollution sources, i.e., industrial in Rm. Vâlcea and animal waste (e.g., from a dog shelter) in Târgoviște. From these locations, 35 water samples were collected (22 from Târgoviște and 13 from Rm. Vâlcea) for *A. baumannii* isolation, with species identification conducted *via* MALDI-TOF mass spectrometry.

Our analysis revealed a notable decrease in the AMR of *A. baumannii* wastewater isolates from 2019 to 2022. The resistance levels observed at the first location (Târgoviște) followed a decreasing pattern from WWTPs to the downstream (DO) surface water (SW) region, upstream (UP) SW region, hospital sewage (Hs), and intra-hospital infections (IHI). A similar trend was observed in Rm. Vâlcea, albeit with equal AR levels noted between the SW UP region and Hs, both surpassing IHI (Fig. 2). A more detailed comparison in Târgoviște during the study period highlighted the presence of PDR in isolates from Hs2019, IHI2020, and WWTP2021, while a strain from SW UP2021 exhibited susceptibility to all antibiotics except ATM. Isolates recovered from SW samples in 2022 displayed susceptibility to almost all tested antibiotics, the highest susceptibility level being identified for carbapenems (IMP, MEM, and DOR), aminoglycosides (CN), and cephalosporins and quinolones (FEP and CIP) (Fig. 2, Additional file 1: Supplementary Table S1).

At the Rm. Vâlcea site, the highest AMR among the *A. baumannii* isolates, was recorded in 2020 and 2021 for Hs and WWTP samples, while most isolates from 2019 were non-MDR. In particular, isolates from Hs2020, SW DO2020, and Hs2021 showed the highest resistance to carbapenems (Fig. 2, Additional file 2: Supplementary Table S2). The highest number of resistance markers was identified in isolates from 2020 (Hs) and 2021 (WWTP) (Fig. 2).

The implications of our previous studies are significant, underscoring the varied resistance profiles of *A. baumannii* across different sources and time frames, highlighting the need for ongoing surveillance and targeted antimicrobial stewardship strategies.

3.2. WGS analysis of A. baumannii with different isolation sources

In this study, 70 *A. baumannii* isolates from Târgoviște and Rm. Vâlcea were sequenced and analyzed utilizing various bioinformatics tools. The molecular analysis results allowed us to characterize the STs and lineage distribution, antibiotic and biocide resistance, virulence genes, and MGEs and perform comparative analyses in different isolation sources and frame sequences of the two locations.

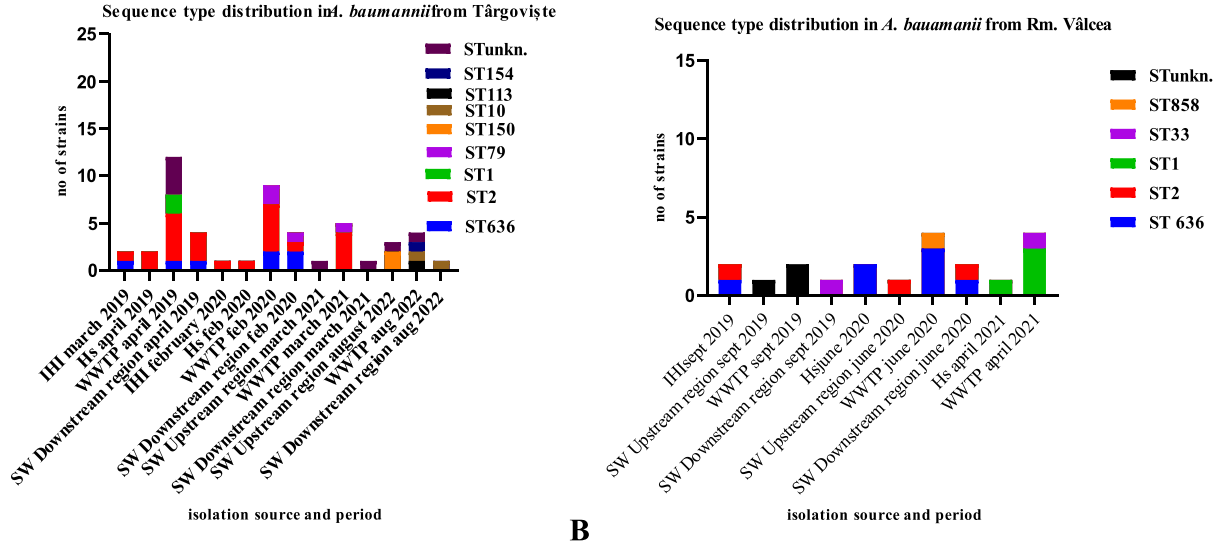


Fig. 3. Sequence types distribution of clinical, WW and SW *A. baumannii* according to isolation source and period in Târgoviște (A) and respectively in Rm. Vâlcea (B).

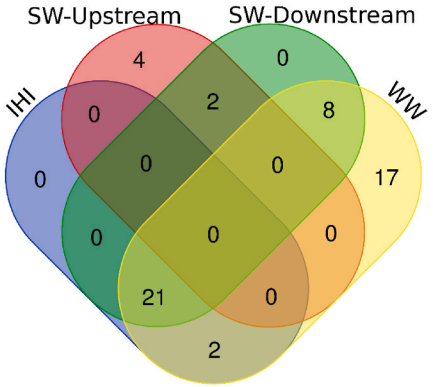
3.2.1. ST and lineage distribution

A considerable proportion (78 %) of *A. baumannii* isolates from Târgoviște, encompassing IHI, WW and SW isolates, were classified within the International Clone 2 (IC2) (ST2 46 %). This was followed by non-IC ST636 (14 %), IC5 (ST79, 8 %), IC1 (ST1, 4 %), IC8 (ST10, 4 %), and IC7 (ST113, 2 %) (Fig. 3A). Additionally, two non-IC clones were detected: one in a SW sample from the UP region of the WWTP in Târgoviște (ST150, 4 %) and another in the effluent of the same WWTP (ST154, 2 %) in 2022. In the second location under study, Rm. Vâlcea, the most predominant ST was non-IC ST636 (35 %), followed by IC1 (ST1) accounting for 20 % and IC2 (ST2, 15 %). Furthermore, two non-IC clones were discovered in SW samples from the downstream region of the WWTP in Rm. Vâlcea (ST33, 10 %) in 2019 and in the WWTP effluent (ST858, 5 %) sampled in 2020 (Fig. 3B). Our findings suggest that WWTPs from Southern Romania may serve as reservoirs for *A. baumannii* resistant and virulent clones, potentially facilitating their spread into the WWTPs' effluent receiving rivers, Ialomita and Olt. These rivers, tributaries of the Danube, have been previously found to harbor antibiotic-resistant bacteria (ARB) and distinctive ARGs, compared to other regions in Romania [65].

3.2.2. ARGs

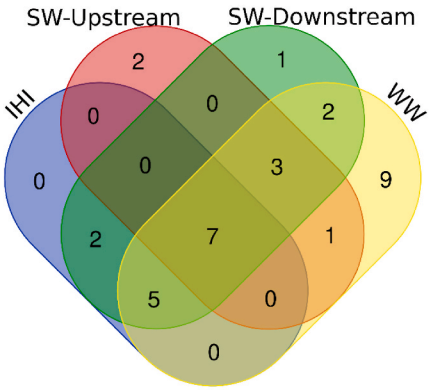
The analysis of ARGs distribution across different sources and time frames revealed the following patterns:
In the first location, Târgoviște, the analysis of 50 *A. baumannii* isolates revealed.

i) clinical *A. baumannii* isolates from 2019 exhibited ARGs profiles embedding carbapenemases (OXA-23 and OXA-24), ESBL (TEM-12). Chromosomal cephalosporinases were represented by ADC-30 and -74. All carried a comprehensive range of ARGs for aminoglycoside-modifying enzymes (AMEs), sulphonamides, phenicols, tetracyclines, macrolides and streptogramin B



A

Sampling points	Total	ARG Genes (Targoviste)										
IHI SW–Downstream WW	21	aph(3')-VIa	ant(2")-Ia	mph(E)	sul1	blaOXA-72	ant(3")-IIa	blaOXA-23	aadA1	blaOXA-66	aac(3)-I	blaTEM-12
		sul2	blaADC–74	aph(3")-Ib	armA	catA1	aph(3')-IIb	tet(B)	msrE	aph(6)-Id	blaADC–30	
IHI WW	2	catB8	aph(3')-Ia									
SW–Downstream SW–Upstream	2	blaADC–165	blaOXA–730									
SW–Downstream WW	8	blaOXA–65	blaADC–76	blaOXA–58	catB7	cat1	blaADC–73	aac(3)-IIa	blaTEM–1			
SW–Upstream	4	blaADC–117	blaOXA–378	blaOXA–121	blaADC–163							
WW	17	blaADC–57	aac(6')-Ian	blaOXA–64	blaADC–1	cmlB1	blaADC–160	blaOXA–558	blaOXA–92	blaTEM–101	aac6'-Ib	blaOXA–69
		blaADC–32	blaOXA–120	fosA	blaADC–5	tet(A)	blaADC–81					



B

Sampling points	Total	ARG Genes (Rm. Valcea)						
IHI SW–Downstream SW–Upstream WW	7	aph(3')-VIa	sul1	blaOXA–23	blaOXA–66	aph(3")-Ib	tet(B)	aph(6)-Id
IHI SW–Downstream WW	5	blaOXA–72	aadA1	blaADC–74	cat1	blaTEM–1		
SW–Downstream SW–Upstream WW	3	blaOXA–120	blaADC–156	catA1				
IHI SW–Downstream	2	sul2	blaADC–30					
SW–Upstream WW	1	blaADC–73						
SW–Downstream WW	2	ant(3")-IIa	aac(3)–I					
SW–Upstream	2	mph(E)	msrE					
SW–Downstream	1	blaADC–25						
WW	9	ant(2")-Ia	blaOXA–92	aac6'-Ib	blaADC–95	blaADC–79	blaOXA–51	aph(3")-Ia	tet(A)	blaADC–81

Fig. 4. Venn diagrams showing the distribution of ARGs by isolation sources in analyzed bacteria and by city (A - Târgoviște and B - Rm. Vâlcea).

resistance. In 2020, similar profiles were noted with the presence of OXA-23 carbapenemase and ADC-30 cephalosporinase alongside a similar spectrum of ARGs;

ii) Hs isolates from 2019 to 2020 were 100 % positive for OXA-23, with varied presence of ADC cephalosporinases (ADC-73 and ADC-30) and AMEs over the two years. Resistance genes for phenicols, sulphonamides, tetracyclines, and macrolides and streptogramin B were detected, showing a shift in resistance patterns between 2019 and 2020;

iii) SW bacteria isolated in 2021 and 2022 revealed the presence of carbapenemases (OXA-121, OXA-378, and OXA-710) and chromosomal cephalosporinases (ADC-165 in 2021, respectively ADC-117 which possess an ESBL spectrum and ADC-163 in 2022), indicating a diverse carbapenem resistance profile across the two years;

iv) WWTP isolates from 2019 to 2022 were consistently positive for OXA-23, OXA-72 carbapenemases and TEM-1 β-lactamase over multiple years, while the chromosomal cephalosporinases profiles shifted from ADC-73, -30, −74, −5, −81/-1 (in decreasing frequency order) in 2019/2020 to ADC-73 and -5 in 2021 to ADC-76, -160, −32, −57 in 2022. The AMEs, phenicols, sulphonamides, tetracyclines, and macrolides and streptogramin B resistance genes also showed variability in presence across the years, indicating dynamic changes in resistance patterns;

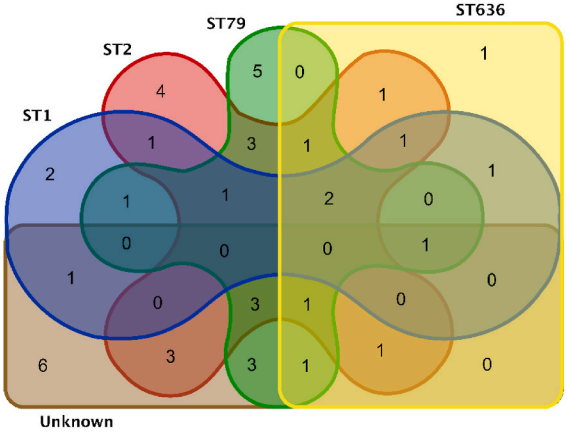
v) Downstream SW isolates showed a constant presence of the acquired $bla_{\text{OXA-23}}$, $bla_{\text{OXA-72}}$ over the four years along with chromosomal $bla_{\text{ADC-73}}$, $bla_{\text{ADC-30}}$, $bla_{\text{ADC-74}}$ in 2019–2020, $bla_{\text{ADC-165}}$ in 2021 and $bla_{\text{ADC-76}}$ in 2022, as well as with genes encoding for AMEs, phenicols, sulphonamides, tetracyclines, and macrolides and streptogramin B resistance (Additional files 3 Supplementary Table S3).

In the second location, Rm. Vâlcea, the analysis of 20 *A. baumannii* isolates revealed that.

i) clinical *A. baumannii* isolates from 2019 harbored OXA-23 and OXA-72 carbapenemases, TEM-1 β-lactamase and chromosomal cephalosporinases, ADC-30 and ADC-74, and ARGs for aminoglycosides, phenicols, sulphonamides, and tetracyclines;

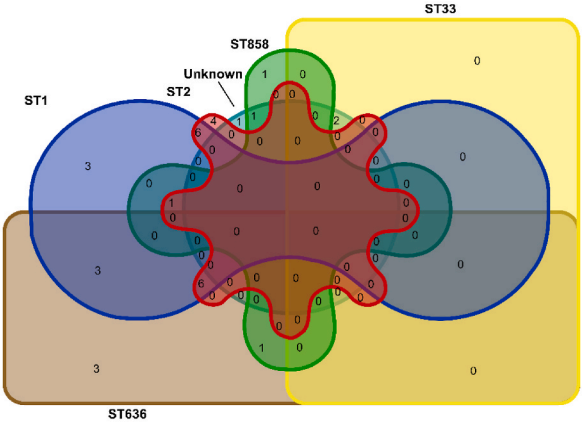
ii) Hs isolates from 2020 to 2021 showed a broad spectrum of β-lactam, aminoglycosides, phenicols, sulphonamides, and tetracyclines resistance genes, indicating a comprehensive array of resistance mechanisms;

iii) SW isolates from 2019 to 2020 demonstrated the acquisition of OXA-23, OXA-120 carbapenemases, presence of ADC-156 and ADC-73 chromosomal cephalosporinase, and a suite of genes for AMEs, phenicols, sulphonamides, and macrolides and streptogramin B resistance;



A

Subtypes (ST)	Total	ARG Genes (Targoviste)					
ST1 ST2 ST636 ST79	2	ant(2")–Ia	ant(3")–IIa				
ST1 ST636 ST79 Unknown	1	blaOXA–72					
ST2 ST636 ST79 Unknown	1	aph(3')–VIa					
ST1 ST2 ST79	1	aac(3)–I					
ST1 ST2 ST636	1	sul1					
ST2 ST636 ST79	1	aadA1					
ST2 ST79 Unknown	3	blaOXA–23	armA	aph(6)–Id			
ST2 ST636 Unknown	1	blaOXA–66					
ST636 ST79 Unknown	1	aph(3')–Ia					
ST1 ST2	1	aac6'–Ib					
ST1 ST79	1	aph(3')–IIb					
ST1 ST636	1	catA1					
ST1 Unknown	1	blaADC–81					
ST2 ST79	3	blaADC–1	aph(3")–Ib	aac(3)–IIa			
ST2 ST636	1	catI					
ST2 Unknown	3	mph(E)	tet(B)	msrE			
ST79 Unknown	3	blaOXA–65	blaADC–5	blaTEM–1			
ST1	2	blaOXA–92	tet(A)				
ST2	4	catB8	blaTEM–101	blaTEM–12	blaADC–30		
ST79	5	aac(6')–Ian	cmlB1	catB7	fosA	sul2	
ST636	1	blaADC–74					
Unknown	6	blaADC–117	blaOXA–378	blaOXA–558	blaADC–32	blaOXA–120	blaADC–73



B

Subtypes (ST)	Total	ARG Genes (Rm. Valcea)					
ST1 ST2 ST858	1	blaOXA–23					
ST1 ST2 ST636	6	aph(3')–VIa	sul1	aadA1	blaOXA–66	aac(3)–I	catI
ST1 ST2	6	sul2	aph(3")–Ib	tet(B)	aph(6)–Id	blaTEM–1	blaADC–30
ST1 ST636	3	blaOXA–72	aph(3')–Ia	blaADC–74			
ST636 ST858	1	ant(3")–IIa					
ST858 Uknown	1	blaOXA–51					
ST33 Uknown	2	blaOXA–120	blaADC–156				
ST1	3	blaOXA–92	aac6'–Ib	blaADC–81			
ST2	4	mph(E)	tet(A)	blaADC–73	msrE		
ST858	1	blaADC–79					
ST636	3	ant(2")–Ia	blaADC–25	catA1			
Uknown	1	blaADC–95					

Fig. 5. Venn diagrams showing the distribution of ARGs by circulating clones in the analyzed isolates and by city (A - Târgoviște and B - Rm. Vâlcea).

Table 1
VFs associated with isolated A. *baumannii* from different isolation sources and geographic locations.

Geographical location	STs	β-lactamases	Isolation source	Virulence encoding genes involved in several processes							
				Adherence	Biofilm formation	Effector delivery systems	Exotoxins	Exoenzymes	Immune modulation	Metabolic	Regulation
Târgoviște and Rm. Vâlcea	**2**	**OXA-66; OXA-23; ADC-30; ADC-73; TEM-1**	IHI T, Hs T, WWTP T, SW DO region T, IHI Rm. Vâlcea, SW UP region Rm. Vâlcea, WWTP Rm Vâlcea	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M, tagX*	*plcD, plc1, plc2*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hem O, entE*	*bfmR, bfmS*
	636	**OXA-66; OXA-72; ADC-74**	IHI T, WWTP T, SW DO region T, IHI Rm Vâlcea, Hs Rm. Vâlcea, WWTP Rm. Vâlcea, SW DO region Rm Vâlcea	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, A, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, H, K, L, M, tagX*	*plcD, plc1, plc2,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, entE, hemO*	*bfmR, bfmS*
	1	**OXA-66; OXA-23; OXA-72; OXA-92; ADC-30,74, 81; TEM-1**	WWTP T, WWTP Rm. Vâlcea, Hs Rm Vâlcea	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M, tagX*	*plcD, plc1, plc2,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hemO, enE*	*bfmR, bfmS,*
	79	**OXA-65; OXA-23; OXA-72; ADC-5; TEM-1**	WWTP T, SW DO region T,	*pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, tssA, B, C, D, E, F, G, K, L, M,*	*plcD, plc1,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, pgi, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, enE*	*bfmR, bfmS,*
	150	**OXA-121; ADC-163**	SW UP T	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M,*	*plcD, plc2,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, ompA*	*bauA-bauE, barA, barB, basA-bas D, bas F, bas H, entE*	*bfmR, bfmS,*
	10	**OXA-68; ADC-76**	SW DO region T	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M,*	*plcD, plc2,*	*cpaA,*	*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, galU, tviB, ompA*	*bauA-bauE, barA, barB, basA-bas D, bas F, bas H, entE*	*bfmR, bfmS,*
	154	**OXA-69; ADC-160**	WWTP T	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M, tagX*	*plcD, plc1,*	*cpaA*	*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, galU, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hemO*	*bfmR, bfmS,*
	113	**OXA-64; ADC-57**	WWTP T	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, K, L, M,*	*plcD, plc1,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, galU, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hem O, entE*	*bfmR, bfmS,*
	33	**OXA-120; ADC-156**	SW DO region Rm. Vâlcea	*pilB, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaB, C, D,*	*gspC, D, E, F, G, H, K, L, M, N,*	*plcD, plc1,*	*cpaA*	*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB,*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hem O, entE*	*bfmR, bfmS,*
	858	**OXA-23; OXA-51; ADC-79**	WWTP Rm. Vâlcea	*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P, Q, R, S, W, X, Y, fimT, tsaP*	*bap, csuA, csuC, csuD, csuE, pgaA, B, C, D,*	*gspD, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, H, K, L, M, tagX*	*plcD, plc1,*		*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, tviB, ompA*	*bauA-bauE, barA, barB, bas A-bas D, bas F, bas H, hem O, entE*	*bfmR, bfmS,*

iv) WWTP isolates from 2019 to 2021 harbored OXA-72 and OXA-23 carbapenemases, a diverse profile of chromosomal cephalosporinases (ADC-95/2019, ADC-74, -79/2020 and ADC-156, -73, −81/2021), AMEs and sulphonamides genes, underscoring the persistence of these ARGs over time;
v) downstream SW isolates from 2019 to 2020 showed a high diversity of ARGs for β-lactams, aminoglycosides, phenicols, sulphonamides, and tetracyclines, highlighting the complexity of resistance profiles in this environment (Additional files 4 Supplementary Table S4).

In the analysis of *A. baumannii* isolates from Rm. Vâlcea, Venn diagrams indicated a relatively homogenous distribution of ARGs. Specifically, seven ARGs were ubiquitous across all IHI, WW, and SW isolates, whereas nine ARGs were exclusive to the WW isolates. In contrast, the Târgoviște isolates demonstrated no universal ARGs across all collection points. Instead, a pattern emerged where most ARGs were common among clinical isolates, downstream water, and WW samples, with WW isolates exhibiting 17 unique ARGs (Fig. 4A and B). The prevalent clonal types identified included ST1, ST2, ST636, and ST79, with a conspicuous absence of ST79 among the Rm. Vâlcea and its notable prevalence in Târgoviște isolates (Fig. 5A and B). Furthermore, Târgoviște isolates displayed an additional diversity with four distinct subtypes (ST10, ST150, ST154, ST113), each characterized by two beta-lactam resistance genes exclusive to their respective subtype (Table 1). Conversely, the analysis for Rm. Vâlcea revealed a significant overlap in ARGs among ST1, ST2, and ST636, with these subtypes containing the most of unique genes, suggesting a reduction in strain diversity within this geographic location.

The comparative analysis of *A. baumannii* isolated from the two locations revealed both common and unique features. Our findings indicate that the *A. baumannii* isolates harbored ARGs encoding for different antibiotic classes such as β-lactams, aminoglycosides, phenicol, sulphonamides, tetracyclines, fosfomycin, macrolides and streptogramin B (see Supplementary Tables 3 and 4). The isolates with the highest resistance were identified in 2019, 2020, and 2021, particularly from three sources of isolation (WW/IHI/WW) in Târgoviște and in 2019 for Hs isolates in Rm. Vâlcea. This trend could be partly attributed to the behavioral changes influenced by the COVID-19 pandemic. In both Târgoviște and Rm. Vâlcea, *A. baumannii* isolated in 2020 from SW samples collected downstream of WWTPs in both Ialomița and Olt rivers, showed lower resistance levels as compared to WWTP. This finding is consistent with prior research that pointed to a surge in ARGs following the release of effluents from WWTPs [67–69].

Both similarities and particularities between the two locations and different isolation sources have also highlighted a variable diversity of ARGs or the presence of unique genes detected across different sources and time frames, with a notable presence of pandrug - resistant isolates in specific years. More specifically, while some ARGs are homogeneously distributed across all IHI, WW, and SW isolates, at least in one of the two sampled locations, other ARGs were exclusive to the WW isolates. Also, most ARGs were common among clinical and environmental isolates.

Both locations showed a significant presence of carbapenemases (e.g., OXA-23, OXA-72), ESBL (e.g., TEM-1, TEM-12), cephalosporinases (e.g., ADC-30, ADC-74) and aminoglycoside-modifying enzymes (AMEs) genes [e.g. *aac3(I)*; *aadA1*; *ant (3″)-IIa*; *aph(6)-Id*]. A meta-analysis of 29 studies published between 2015 and 2020 regarding the presence of CP in *A. baumannii* isolates and the geographic dissemination of circulating clones revealed also that the most common CP identified were OXA-23 and OXA-72 [70]. Other studies conducted in different geographical regions such as Pakistan, Philippines or Tunisia revealed the predominance of OXA-23 production among CRAB isolates [71,72].

In our study, the prevalent clonal types included ST1, ST2, and ST636. Furthermore, *A. baumannii* belonged to ten phylogenetic groups in different isolations sources in Târgoviște and Rm. Vâlcea: ST2-*bla*$_{\text{OXA-66}}$; *bla*$_{\text{OXA-23}}$; *bla*$_{\text{ADC-30}}$; *bla*$_{\text{ADC-73}}$; *bla*$_{\text{TEM-1}}$ (IHI Târgoviște; Rm. Vâlcea, Hs Târgoviște, WWTP Târgoviște; Rm. Vâlcea, SW Târgoviște; Rm. Vâlcea); ST636-*bla*$_{\text{OXA-66}}$; *bla*$_{\text{OXA-72}}$; *bla*$_{\text{ADC-74}}$ (IHI Târgoviște; Rm. Vâlcea, Hs Rm. Vâlcea, WWTP Târgoviște; Rm. Vâlcea, SW Târgoviște; Rm. Vâlcea); ST1-*bla*$_{\text{OXA-66}}$; *bla*$_{\text{OXA-23}}$; *bla*$_{\text{OXA-72}}$; *bla*$_{\text{OXA-92}}$; *bla*$_{\text{ADC-30,74,81}}$; *bla*$_{\text{TEM-1}}$ (Hs Rm. Vâlcea, WWTP Târgoviște; Rm. Vâlcea); ST79-*bla*$_{\text{OXA-65}}$; *bla*$_{\text{OXA-23}}$; *bla*$_{\text{OXA-72}}$; *bla*$_{\text{ADC-5}}$; *bla*$_{\text{TEM-1}}$ (WWTP Târgoviște, SW Târgoviște); ST150-*bla*$_{\text{OXA-121}}$; *bla*$_{\text{ADC-163}}$ (SW Târgoviște); ST10-*bla*$_{\text{OXA-68}}$; *bla*$_{\text{ADC-76}}$ (SW Târgoviște); ST154-*bla*$_{\text{OXA-69}}$; *bla*$_{\text{ADC-160}}$ (WWTP Târgoviște); ST113-*bla*$_{\text{OXA-64}}$; *bla*$_{\text{ADC-57}}$ (WWTP Târgoviște); ST33-*bla*$_{\text{OXA-64}}$; *bla*$_{\text{ADC-57}}$ (SW Rm. Vâlcea); ST858-*bla*$_{\text{OXA-23}}$; *bla*$_{\text{OXA-51}}$; *bla*$_{\text{ADC-79}}$ (WWTP Rm. Vâlcea). In both investigated Romanian locations, the most frequent CRGs, associated with widespread clones encountered in clinical and aquatic *A. baumannii*, were *bla*$_{\text{OXA-72}}$-ST1, *bla*$_{\text{OXA-23}}$-ST2 and *bla*$_{\text{OXA-72}}$-ST636, also reported in different neighboring countries. For e.g. *bla*$_{\text{OXA-23}}$-ST2 was reported in a clinical *A. baumannii* isolates recovered from a burn wound infection in Albania [73]. In Serbia *bla*$_{\text{OXA-23}}$-ST2 and *bla*$_{\text{OXA-72}}$-ST636 were recovered from inpatients sampled in 2018 [74]; *bla*$_{\text{OXA-23}}$-ST2 in a mucoid XDR *A. baumannii* isolated from a cystic fibrosis patient in Rome [75]. The ST2 epidemic clone has been also identified in Poland, Russia, Philippines, Latin America and China, highlighting the need to give special importance to this clone [70,71,72,76,77,78]. In Tunisia, the PDR *A. baumannii* ST2 recovered from patients admitted to ICU revealed high resistance rates and the presence of 14 β-lactamases/CP and *mcr* (*mcr-1* to *mcr-5*) genes [79]. In China, a study including 70 *Acinetobacter* spp. isolates recovered from untreated Hs samples revealed that almost 60 % of the isolates were MDR, ST2 being the predominant type among these CRAB isolates, with Tn*2006* and Tn*2009* being the key MGE encoding carbapenem-resistance) [80], opposite to our results that revealed the chromosomal or plasmid location of *bla*$_{\text{OXA-23}}$ gene (flanked by IS*Aba1* upstream and downstream, in 1–3 copies and respectively in a pA105-like plasmid flanked by IS*Aba1* upstream and downstream.

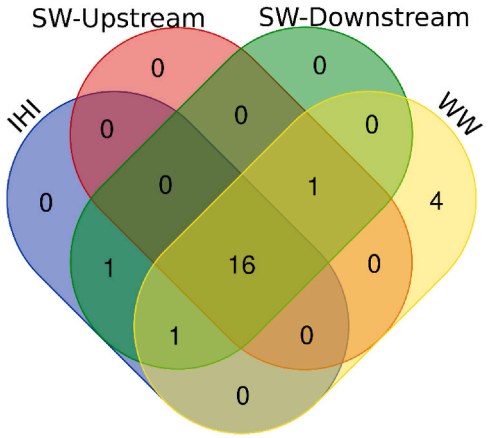
For *A. baumannii* isolated from Târgoviște in 2021, in ST2 subtype, there were encountered three isolates harboring simultaneously *bla*$_{\text{OXA-23}}$ and *bla*$_{\text{OXA-72}}$, two from WWTP influent and one from the WWTP effluent. Additionally, in 2019, an isolate belonging to an unknown subtype (but close to ST2, according to the molecular phylogeny assay) was recovered from WWTP active sludge, harboring the same gene association, indicating that OXA-23 producing isolates acquired a *bla*$_{\text{OXA-72}}$ containing pMAL-like plasmid. To the best of our knowledge, there were no reports of co-occurrence of both carbapenemases elsewhere, thus highlighting the ability of *A. baumannii* to acquire multiple resistance determinants, as well as the necessity of continuous surveillance of ARB isolates in different

environments.

The discovery of the less frequent *bla*OXA-120-ST33 clone in two environmental carbapenem susceptible *A. baumannii* isolates, collected from a SW sample downstream of WWTP (Olt river) in 2019 and from Rm. Vâlcea WWTP influent in 2021, is a significant finding. This clone, previously reported in clinical isolates in France [81], underscores the potential of environmental isolates as reservoirs and vectors of important clinical genes. The ST154 and ST113 identified in the case of one isolate from WWTP Târgoviște effluent and respectively from the influent in 2022, were previously reported also in clinical samples [82,83].

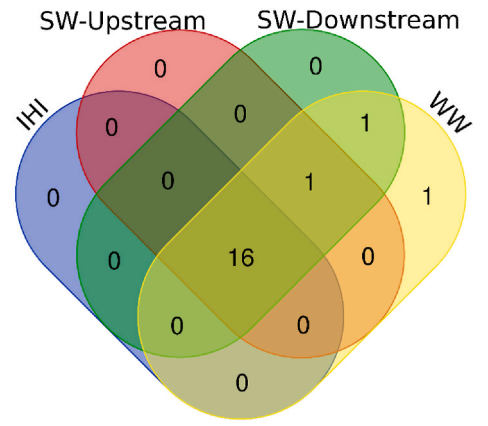
Our results revealed that the *A. baumannii* isolates harbored resistance to other classes of antimicrobials besides β-lactams, including aminoglycosides, macrolides, sulfonamides, tetracyclines, and as well as antibiotic efflux pumps. Similar to our results, several other studies performed in different geographical regions from Europe, Asia and Africa have indicated high resistance levels to aminoglycosides [84–86], but also to macrolides, sulfonamides and tetracyclines [87], encoded by ARGs similar to those detected in our isolates.

Furthermore, it was shown that animal and plant isolates could serve as a significant reservoir of ARGs [88]. They revealed a high repertoire of ARGs in *A. baumannii* isolates resistant to cephalosporins, aminoglycosides, or fluoroquinolones from five countries across three continents, different STs, including epidemic and novel. This underscores the role of these sources in harboring and spreading AMR. Also environmental *A. baumannii* isolates can acquire clinically relevant MGEs and VFs similar to nosocomial isolates and act as reservoirs and vectors of important clinical genes, warranting further research into their ecology and evolution [89].



Sampling points	Total	BRG Genes (Targoviste)									
IHI SW–Downstream SW–Upstream WW	16	adeC	abeM	adeJ	adeH	adeA	nreB	adeI	adeB	amvA	adeF	adeL
		adeR	adeK	abeS	adeG	adeN						
IHI SW–Downstream WW	1	qacEΔ1										
SW–Downstream SW–Upstream WW	1	adeS										
IHI SW–Downstream	1	qacE										
WW	4	triC	triß	triA	soxR							

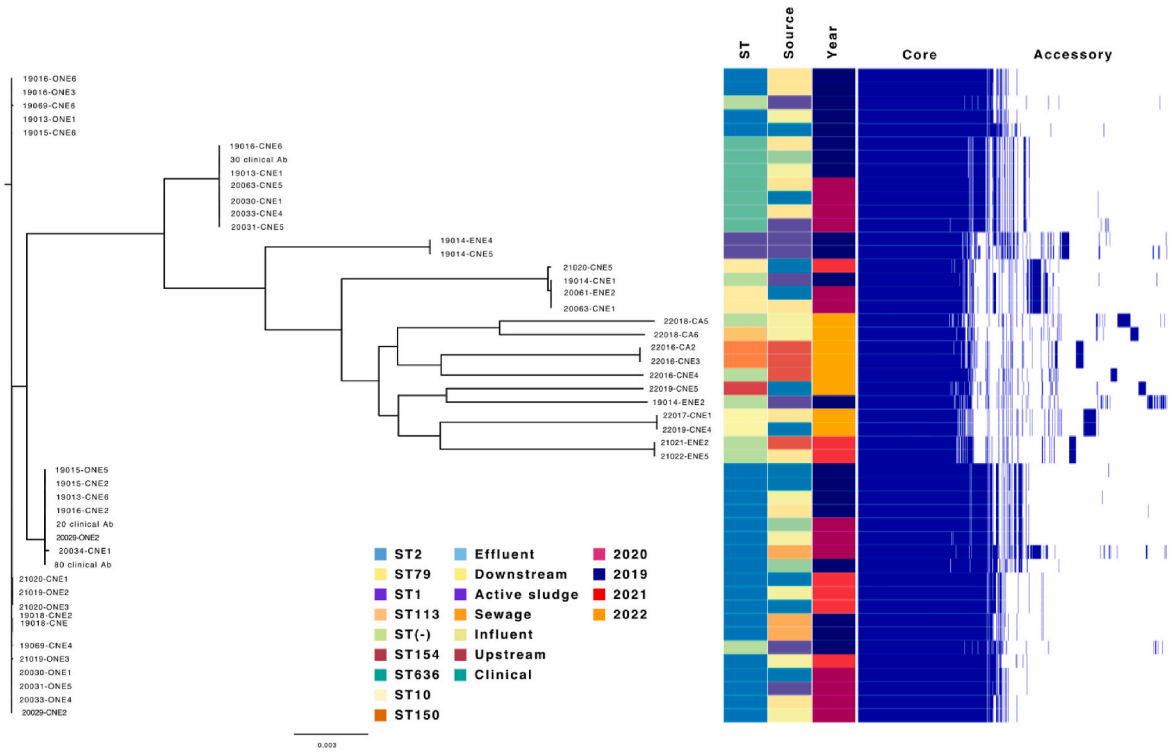
A



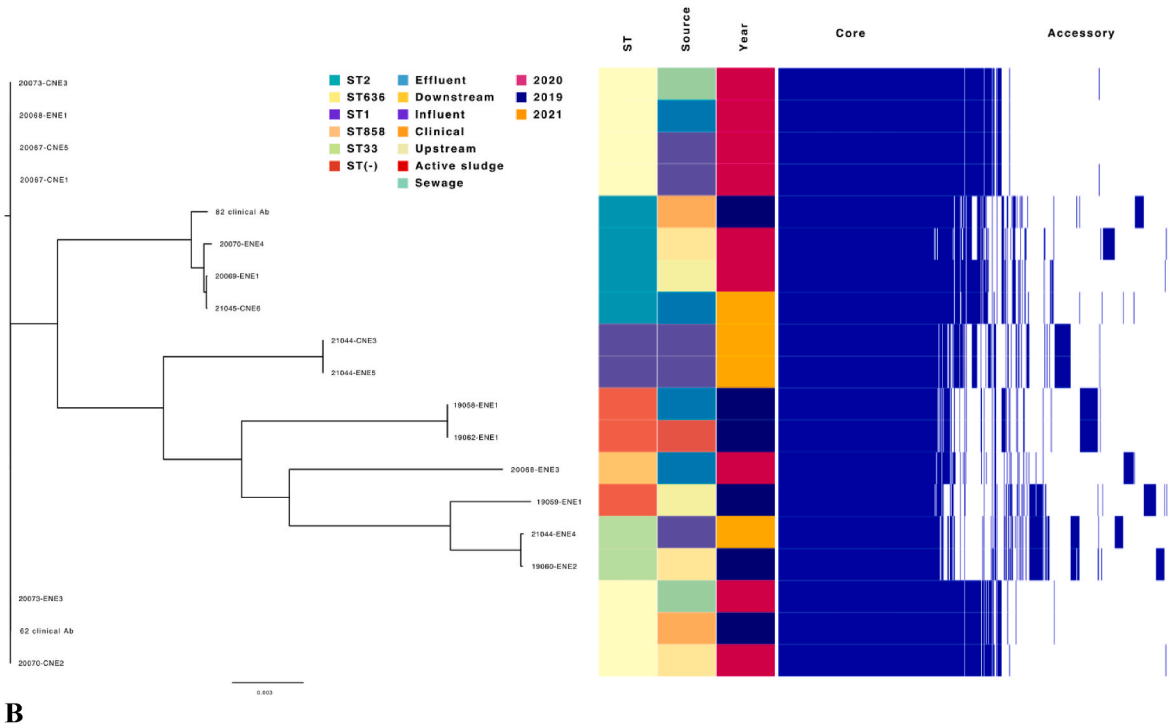
Sampling points	Total	BRG Genes (Rm. Valcea)										
IHI SW–Downstream SW–Upstream WW	16	adeC	abeM	adeJ	adeH	adeA	nreB	adeS	adeI	adeB	adeF	adeL
		adeR	adeK	abeS	adeG	adeN						
SW–Downstream SW–Upstream WW	1	amvA										
SW–Downstream WW	1	qacE										
WW	1	qacEΔ1										

B

Fig. 6. Venn diagrams showing the distribution of BRGs by isolation sources in analyzed *A. baumannii* and by city (A - Târgoviște and B - Rm. Vâlcea).



A



B

Fig. 7. Pangenome tree for clinical, WW and SW *A. baumannii* isolates (A - Târgoviște and B - Rm. Vâlcea).

3.2.3. BRGs

WGS, bioinformatics and BacMet database analysis revealed the presence of a resistance-nodulation-cell division (RND) efflux pump (*adeI J K; acrB, D, E; triC, B, A*), multidrug and toxic compound extrusion (MATE) family efflux pumps (*abeM*), small multidrug resistance (SMR) family efflux pumps (*qacE, qacEΔ1*, and *abeS*), adeFGH efflux pumps (*adeL, G, H, F, C, B, R*) and a major facilitator superfamily (MFS) efflux pump (*amvA, nreB*) in *A. baumannii* genomes. The *adeM* is strongly related to imipenem resistance of *A. baumannii* [90], and *adeN, adeI, J, K, L, G,* and *H* genes were found in all isolation sources from both geographical locations (Fig. 6 A and B and Additional files 5 and 6: Supplementary Table S5 and S6). Notably, an increased diversity of β-lactam and aminoglycoside resistance genes, alongside BRGs, especially efflux systems, which may contribute to both antibiotic and biocide resistance [91], has been registered. A similar BRG profile was observed in the two locations, with comparable gene sharing among all sampling points. In three studies conducted in Poland, Egypt, and Iran, the *qacEΔ1* gene was detected in more than 90 % of the cases, opposite to our results where 39 % of the isolates were positive for the *qacEΔ1* gene [92]. It is well known that overproduction of *adeFGH* and *adeIJK* efflux pumps (two RND efflux family members identified in analyzed isolates from both locations) play an important role in carbapenem resistance in *A. baumannii*, but further studies are required to demonstrate the relative expression.

3.2.4. VFs

The sequenced isolates revealed a high repertoire of VFs for adherence (*ata, pilA, B, C, D, E, F, I, J, K, L, M, N, O, P,Q, R, S, W, X, Y, fimT, tsaP*), biofilm formation (*bap, csuA, csuC, csuD, csuE, pgaB, C, D*), effector delivery systems (*gspD, E, F, G, K, L, M, N, tssA, B, C, D, E, F, G, H, K, L, M, tagX*), exotoxins (*plcD, plc1, plc2*), exoenzymes (*cpaA*), immune modulation (*lpxA, lpxB, lpxC, lpxD, lpxL, lpxM, lpsB, galU, tviB, ompA, pgi*), nutrition/metabolic factors (*bauA-bauE, barA, barB, basA-basD, basF, basH, hemO, entE*) and regulation (*bfmR, bfmS*) [32]. The isolates recovered from IHI, WWTP and SW samples in both locations, belonging to IC2 and IC1 revealed the highest number of VFs, all isolates revealed an equal number of VFs involved in adherence, biofilm formation, effector delivery systems, exotoxins, immune modulation, regulation and metabolic factors. The high repertoire of VFs associated with ST2-$bla_{\text{OXA-72}}$, ST2-$bla_{\text{OXA-23}}$, ST636-$bla_{\text{OXA-72}}$ and ST1-$bla_{\text{OXA-72}}$ MDR clones were disseminated by MGEs (pMAL-1linked to $bla_{\text{OXA-72}}$ and pA105-like responsible for the dissemination of $bla_{\text{OXA-23}}$ gene) suggest the potential of clinical and environmental *A. baumannii* to survive using resistance and persistence strategy. In contrast, the *cpaA*, encoding for a coagulation targeting metallo-endopeptidase was associated with *A. baumannii* isolates belonging to ST10, ST154 and ST33 clones (Table 1). Another study involving WGS analysis revealed 41 potential ARGs in clinical, and respectively 32 in the case of the community isolates. In contrast, 68 VFs were commonly seen in isolates from both isolation sources, highlighting the possible transmission threat to public health posed by virulent *A. baumannii* present in the gut of asymptomatic individuals in the community [93].

3.2.5. Pangenome analysis of clinical, WW and SW A. baumannii isolates

Pangenome analysis conducted on 40 *A. baumannii* isolates, with 20 from each of the two locations, identified a comprehensive gene pool comprising 11,499 and 16,757 genes, respectively. Similarly, another comparative analysis of 23 available genomes of *A. baumannii* revealed a pan-genome of 15,883 genes [94]. In another study, 206 *A. baumannii* genomes revealed 12,336 genes, of which 1999 genes were shared by all isolates and 3920 were strain-specific genes. According to Heaps' law, the pan-genome of *A. baumannii* remains open ($\alpha = 0.71$), which means by each newly added genome, the number of new genes will increase the genetic repertoire of the species [95]. As a rule, when $0 < \alpha < 1$, the pan-genome is considered open [96]. In our study, the extensive genetic repertoire suggested the presence of an open pangenome, as inferred from a Heap's law γ value of 0.41. This fact also corroborates the high genomic plasticity already reported for this species, especially considering that this bacterium can achieve new gene content through transposable elements. Ali and collaborators used several bioinformatics tools (e.g., ResFinder and Galaxy Community hub) to identify the genes that were resistant to mutations against antibiotics. They found 2227 core genes in each species of the *A. baumannii* genome. The pan-genome analysis showed a 5-fold increase in the genome of *A. baumannii* in 5 years, and the genome is still open [97].

In Târgoviște isolates, the pangenome analysis, based on accessory gene profiles, revealed three principal clusters: a dominant cluster primarily consisting of ST2 isolates (along with several unidentified sequence types potentially related to ST2), a distinct cluster of ST636 isolates, and a heterogeneous central cluster with isolates from various subtypes, characterized by a significant array of accessory genes (Fig. 7A). Within this pangenome, Roary classified 56.45 % (n = 9460) of the 16,757 genes as hypothetical proteins (HPs), indicative of functions ranging from transcription and mobilome elements (like prophages and transposons) to general cellular processes [38]. Following HPs, the most abundant genes were HTH-type transcriptional regulators (2.38 %). Notably, two isolates, encoded as 20064 CNE4 and 20064 CNE5, were distinguished by their unique accessory genomes, each possessing around 1900 unique genes, with a collective total of 4712 genes, 40.96 % (n = 1930) of which being HPs not found in other isolates. These isolates, sourced from activated sludge and associated with ST2 and ST79 respectively (Additional file 7: Supplementary Table 7), displayed a high proportion of HPs involved in mobility and signal transduction, alongside a variety of virulence factors from *A. baumannii* (ACICU) and *P. aeruginosa* (PAO1 and PA14), suggesting enhanced virulence potential [98].

The Rm. Vâlcea pangenome analysis identified a cohesive ST636 cluster and another central cluster predominantly featuring ST1 and ST2 isolates, with some unidentified sequence types possibly related to ST1 (Fig. 7B). A high percentage of HPs (44.69 %, n = 5139) was also observed here, with the 21049 ENE3 isolate particularly notable for its vast array of unique accessory genes (41.72 %, n = 4798), hinting at possible hyper-virulence feature. This isolates HPs were largely linked to mobility functions, and ARG/BRG/MGE predictions pointed towards a virulence profile derived from *Escherichia coli* phages (Additional file 7: Supplementary Table 7).

Our results underscore the need for in-depth investigations, a task that researchers and professionals in the field of microbiology and infectious diseases are uniquely positioned to undertake to fully comprehend and address the genetic diversity and potential virulence of *A. baumannii* isolates.

3.2.6. Genetic context analysis of clinical, WW and SW A. baumannii isolates

The initial analysis of the genomic context revealed that the predicted genetic elements, including ARGs, BRGs, MGEs and VFs, were identified either as isolated instances within contigs (lacking adjacent elements within a 1000 base pairs (bp) length) or assembled into clusters (with at least two elements situated less than 1000 bp apart). This observation led to the hypothesis that denser clusters of these elements might play roles in mediating resistance, enhancing virulence, or facilitating genetic mobility.

Subsequent evaluations demonstrated that a significant proportion, approximately 82 %, of all predicted elements across the examined isolates, formed clusters comprising up to three elements. The bulk of these, around 55 %, were individual elements positioned a considerable distance apart, ranging from several thousand to tens of thousands of base pairs from other isolated elements or clusters. The remainder, 18 %, constituted larger clusters containing between 4 and 38 elements. The analysis of the lengths of these elements and their clusters revealed that most, about 71 %, spanned from 3000 bp to 10,000 bp within the contig sequences, with a substantial extension covering up to 93 % of this range. A smaller fraction, 7 %, extended over distances between 10,000 and 44,000 bp, with many of these larger clusters potentially contributing to pathogenicity island (PAI) formation, especially those ranging between 10,000 and 13,000 bp, which predominantly consisted of phage-related elements (Fig. 8).

Clusters primarily composed of VFs, yet devoid of additional PAI components, were categorized into two groups based on their lengths: one peaking around 10,000 bp and the other around 26,000 bp. These clusters generally included only VFs and PAIs, with few containing ARGs, particularly when a PAI-associated element was succeeded by an MGE (typically Tn*6166*) within 1000 bp, which then led to a combination of ARGs (notably for streptomycin and tetracycline resistance) and further MGEs (e.g., Tn*6302*, IS*91*), resulting in clusters around 11,000 bp in size. Additionally, resistance clusters, either lacking PAI components or with PAIs situated terminally, featured BRGs (e.g., *mer* and *qacEΔ1* genes) alongside MGEs such as Tn*6022*, Tn*6302*, and IS*91*). It was observed that these ARG clusters, potentially circulating within the population, could exhibit either sense or antisense orientations, necessitating verification in future studies. Predominantly, these ARG/MGE clusters were linked to the ST2 clone, especially within WW, SW (downstream regions), and IHI samples (Additional file 8 and 9: Supplementary Tables 8 and 9).

Târgoviște isolates, identified as 20064 CNE4 and 20064 CNE5, distinguished by their rich accessory genome content, presented extensive clusters interspersed with VFs and PAI components, reaching lengths up to 33,000 bp. Similarly, *A. baumannii* encoded 21049 ENE3 from Rm. Vâlcea, noted for its abundant accessory genes, contained significant clusters, including one spanning 43,000 bp. The longest clusters were primarily identified in isolates from sewage and activated sludge, distributed across ST1, ST2, and ST636, without marked distinctions between the two locations.

Regarding the chromosomal or plasmid locations of CRGs, long-read sequencing data revealed the $bla_{\text{OXA-23}}$ gene flanked by IS*Aba1* elements in varying configurations, including single, double (in 19015 CNE2, 19018 CNE6 *A. baumannii*), and triple copies (in isolates encoded 20030 ONE1, 82 Ab), depending on the genetic context. Additionally, in the ST2 isolate (*A. baumannii* encodes 21045 CNE6), $bla_{\text{OXA-23}}$ was also found within a pA105-like plasmid, bordered by IS*Aba1* elements both upstream and downstream. The $bla_{\text{OXA-72}}$ gene was consistently located within a pMAL1-like plasmid, measuring between 9000 and 10,000 bp across all analyzed isolates (Tables 2 and 3). Different mobile genetic platforms were encountered in *A. baumannii* responsible for the dissemination other non-β-lactam ARGs, for e.g. for aminoglycoside resistance genes: pA105-like plasmid and IS*Aba125* for *aph(3′)-VIa* gene; Tn*5393* for *aph*

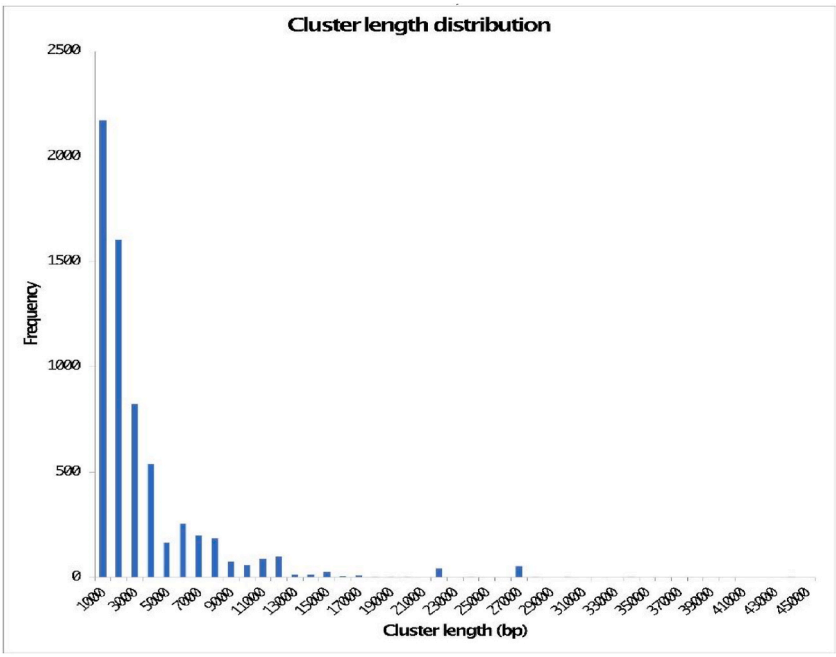


Fig. 8. Cluster length distribution in analyzed isolates.

Table 2
ARGs in IC for which complete genomes were obtained and their chromosomal/plasmid location. Colors indicates the genetic platforms highlighted in Table 3.

Isolation source, geographical location, clone	*blaADC*	*qacEdelta1sul1*	*tet(A)*	*ant(3'')-IIa*	*catA1*	*aac(3)-Ia*	*aadA1*	*aph(3')-Ia*	*armA mph(E) msr(E)*	*blaOXA-72*	*aph(3')-VIa*	*blaTEM-12*	*sul2*	*catB8*	*aph(3'')-Ib*	*aph(6)-Id*	*tet(B)*	*blaOXA-23*
19014 ENE4, rezidual sludge WWTP Târgoviște, **ST1**	C	C	C	C	C	C	C	C		P (pMAL1)	P (pA105-like)							
19015 CNE2, effluent WWTP Târgoviște, **ST2**	C	C		C		C	C		C			C	C	C	C	C	C	C
19018 CNE6, Hs Târgoviște hospital, **ST2**	C			C	C				C						C	C	C	C
19058 ENE1, effluent WWTP Rm. Vâlcea ST unknown	C			C														
19060 ENE2, SW-DO WWTP Rm. Vâlcea **ST33**	C			C														
20030 ONE1 effluent WWTP Târgoviste, **ST2**	C	C		C	C				C						C	C	C	C
20067 CNE1, influent WWTP Rm. Vâlcea, **ST636**	C	C		C	C	C	C	C		P (pMAL1)	P (pA105-like)							
20068 ENE1, effluent WWTP Rm. Vâlcea, **ST636**	C	C		C	C	C	C	C		P (pMAL1)	P (pA105-like)							
20070 ENE4, SW-DO Rm. Vâlcea, **ST2**	C			C									C		C	C	C	
20073 ENE3, Hs Rm. Vâlcea hospital, **ST636**		C		C	C					P (pMAL1)								
21020 CNE5, effluent WWTP Târgoviste, **ST79**										P (pMAL1)								
21020 ONE3, influent WWTP Târgoviste, **ST2**	C	C		C	C				C	P (pMAL1)					C	C	C	C
21044 CNE3, influent WWTP Rm. Vâlcea, **ST1**	C	C	C	C	C	C	C	C		P (pMAL1)	P (pA105-like)	C						
21045 CNE6, effluent WWTP Rm. Vâlcea, **ST2**	C	C		C	C				C		P (pA105-like)				C	C	C	C + P (pA105-like)
22019 CNE5, effluent WWTP Târgoviste, **ST154**	C			C														
62 Ab, IHI - Rm. Vâlcea hospital, **ST636**	C	C		C	C	C	C	C		P (pMAL1)	P (pA105-like)							
82 Ab, IHI - Rm. Vâlcea hospital, **ST2**	C			C		C						C	C		C	C	C	C
64 Ab, IHI - Târgoviște hospital, **ST2**	C	C		C		C	C		C			C		C	C	C	C	C

(6)-Id and *aph(3'')-Ib* genes; IS*1396* for *ant(3'')-IIa*, *aph(3')-Ia*, *ant(3'')-Ih*, and *aac(6')-IId*, *aac(3)-Ia*, *aadA1* (Table 3).

3.3. Conjugation assays

Conjugation experiments demonstrated that CRGs could be transferred from *A. baumannii* donors with different isolation sources (IHI, WW and SW samples) to the recipient *A. baylyi* RIF-R isolate. The Cf of IHI, WW and SW samples isolated from Târgoviște (Table 2) ranged between 5.4×10^{-7} (bacteria isolated from WWTP) and 1.9×10^{-11} (IHI isolate). In the second investigated location, Cf ranged between 2×10^{-9} (isolate from WWTP) and 3.3×10^{-13} (bacteria isolated from WW and Hs), respectively. PCR amplification of bla_{OXA-23} and bla_{OXA-72} genes revealed their presence in transconjugants. The long-read sequencing results showed also that both pMAL1-like and bla_{OXA-72} were detected in selected transconjugants obtained from IHI, Hs and WW in the case of Rm. Vâlcea and WW for Târgoviște isolates and suggested that *A. baylyi* RIF-R obtained carbapenem resistance due to the acquisition, dissemination or regulation of bla_{OXA-72} along with pMAL1-like within the host (Table 4).

3.4. Conclusion

This study analyzed a significant number of clinical and aquatic *A. baumannii* isolated during 2019–2022 from two locations in

Table 3
Mobile genetic platforms carrying ARGs encountered in the *A. baumannii* isolates selected for long-read sequencing. ARGs in brackets indicate variable presence of in the respective mobile genetic platform. Colors are corresponding with the ARGs in Table 2, indicating the carrying platform for each ARG.

Mobile genetic platforms
Tn*As3* (contain ***catA1/catB8, [tet(A)], sul 1, qacEdelta1, [bla$_{TEM-12}$]***)
IS*1396*-***ant(3'')-IIa-aph(3')-Ia-[ant(3'')-Ih/aac(6')-IId]-aac(3)-Ia-aadA1-qacEdelta1-sul1***-IS*1396*
IS*Aba125*-***aph(3')-VIa***-IS*Aba125*
IS*Vsa3*-**tet(B)**-[ISAba1-***bla$_{OXA-23}$***-IS*Aba1*]-**sul2**-IS*Aba1*-[***bla$_{OXA-23}$***-IS*Aba1*]
IS*26*-***bla$_{TEM-12}$***–IS*26*-***aac(3)-Ia***-IS*26*
IS*Aba24*-***mph(E)-msr(E)***-ISEc*28*-***arm(A)***-ISEc*28*
IS*Aba1*-***blaOXA-23***-ISAba1-blaOXA-23-ISAba1-***tet(B)-aph(6)-Id-aph(3'')-Ib***-IS*Aba1*
IS*Aba1*-***blaOXA-23***-ISAba1-***tet(B)-aph(6)-Id-aph(3'')-Ib***-IS*Aba1*
IS*Aba1* - ***bla*OXA-23** - IS*Aba1*
Tn5393 (contains ***aph(6)-Id, aph(3'')-Ib)***

Table 4
Conjugative transfer CRGs in clinical, WW and SW *A. baumannii* isolates.

R	*A. baumannii* D - isolation source, year	D - Geographical region	CP	Conjugation frequency	Transferred CP	Transferered plasmid
A. baylyi RIF R	WW sample - Hs, 2019	Târgoviște	OXA-23, OXA-66	8×10^{-9}	OXA-23	
	WWTP IN, 2019		OXA-72, OXA-66	6.2×10^{-8}	OXA-72	pMAL1
	WWTP - active sludge, 2019		OXA-23, OXA-72, OXA-66	5.4×10^{-7}	OXA-23, OXA-72	pMAL1
	SW sample - DO, 2019		OXA-23, OXA-66	5.2×10^{-8}	OXA-23	
	IHI, 2020		OXA-23, OXA-66	1.9×10^{-11}	OXA-23	
	WW sample - Hs, 2020		OXA-23, OXA-66	4.2×10^{-9}	OXA-23	
	WWTP EF, 2020		OXA-23, OXA-66	5.9×10^{-7}	OXA-23	
	SW sample - DO, 2020		OXA-23, OXA-66	3.3×10^{-8}	OXA-23	
	WWTP EF, 2021		OXA-72, OXA-66	4.1×10^{-9}	OXA-72	pMAL1
	IHI, 2019	Rm. Vâlcea	OXA-72, OXA-66	2.5×10^{-11}	OXA-72	pMAL1
	WW sample – Hs, 2020		OXA-72, OXA-66	3.3×10^{-13}	OXA-72	pMAL1
	WWTP - IN, 2020		OXA-72, OXA-66	2×10^{-9}	OXA-72	pMAL1
	WWTP - EF, 2020		OXA-23, OXA-72, OXA-66	2×10^{-9}	OXA-23, OXA-72	pMAL1
	WW sample –Hs, 2021		OXA-23, OXA-66	2×10^{-9}	OXA-23	

*PCR was performed to detect CP encoding genes in transconjugants and 3rd sequencing of selected transconjugants.

Southern Romania, offering insights into their sequence types, genetic diversity of antibiotics and biocides resistance, virulence factors, and their associated MGEs. The study highlighted the role of these isolates as potential reservoirs of ARGs and the relatedness between environmental and clinical *A. baumannii*. The discovery of genes of clinical significance in environmental bacteria and the demonstration of their transferability highlight the risk of gene exchange across different environments *via Acinetobacter* isolates. Additionally, it was shown that *A. baumannii* can persist after various stages of wastewater treatment processes, and the presence of pMAL1 plasmids seems to play a significant role in the spread of ARGs and VFs. The limitations of this study are: i) the reliance on culture-dependent methods for isolating bacteria limits its ability to fully capture the prevalence of MDR CRAB *A. baumannii* isolates in water bodies, offering only a snapshot of the spread of clinically relevant epidemic clones; ii) some sampling campaigns could not be performed according to the sampling plan due to unforeseen weather conditions; iii) moreover, the number of clinical isolates was lower than that of environmental isolates. However, the selected isolates are representative of the most frequent resistant clones circulating in the respective hospital units, evaluated in our previous studies. Our findings are crucial for understanding the fate of AR from clinical settings to the aquatic environment, laying the groundwork for future efforts to curb AR spread.

Institutional review board statement

All procedures were performed in compliance with relevant laws and institutional guidelines and have been approved by the Bioethics Commission of the University of Bucharest, No. 9/8121; October 2, 2018.

Data availability statement

Raw genome sequences are publicly available in the GenBank Sequence Read Archive (SRA) and can be accessed under the PRJNA1083781 code (https://www.ncbi.nlm.nih.gov/sra/PRJNA1083781). The assembled genomes that support the findings of this study are available upon request from the corresponding author.

CRediT authorship contribution statement

Irina Gheorghe-Barbu: Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Marius Surleac:** Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Ilda Czobor Barbu:** Writing – review & editing, Investigation, Formal analysis, Data curation. **Simona Paraschiv:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Leontina Mirela Bănică:** Writing – review & editing, Methodology, Investigation, Data curation. **Liviu-Iulian Rotaru:** Software, Investigation, Formal analysis, Data curation. **Corneliu Ovidiu Vrâncianu:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Mihai Niță Lazăr:** Writing – review & editing, Validation, Methodology, Investigation. **Dan Oțelea:** Writing – review & editing, Visualization, Supervision. **Mariana Carmen Chifiriuc:** Writing – review & editing, Visualization, Validation, Supervision, Resources, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e33372.

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