Temporo-spatial Variations in Resistance Determinants and Clonality of *Acinetobacter Baumannii* and *Pseudomonas Aeruginosa* Strains From Romanian Hospitals and Wastewaters

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Abstract

Background: Romania is one of the European countries reporting very high antimicrobial resistance (AMR) rates and consumption of antimicrobials. We aimed to characterize the AMR profiles and clonality of 304 multi-drug resistant (MDR), carbapenemase (CP) and extended spectrum beta-lactamase (ESBL) producing *Acinetobacter baumannii* (*Ab*) and *Pseudomonas aeruginosa* (*Pa*) strains isolated during two consecutive years (2018 and 2019) from hospital settings, hospital collecting sewage tanks and the receiving wastewater treatment plants (WWTPs) located in the main geographical regions of Romania.

Methods: The strains were isolated on chromogenic media and identified by MALDI-TOF-MS. Antibiotic susceptibility testing and confirmation of ESBL- and CP- producing phenotype were performed. The genetic analyses consisted of PCR for ESBL and CP genes, horizontal gene transfer experiments, whole-genome sequencing (WGS), assembling, annotation and characterization.

Results: Both clinical and aquatic isolates exhibited high MDR rates, especially the *Ab* strains isolated from nosocomial infections and hospital effluents. The phenotypic resistance profiles and MDR rates have largely varied by sampling point and by geographic location. The highest MDR rates in aquatic isolates were recorded in Galați WWTP followed by Bucharest. The *Ab* strains harbored mostly *bla*_{OXA-23}, *bla*_{OXA-24}, *bla*_{SHV}, *bla*_{TEM} and *bla*_{GES}, while *Pa* strains *bla*_{IMP}, *bla*_{VIM}, *bla*_{NDM}, *bla*_{VEB}, *bla*_{GES} and *bla*_{TEM}, with high variations depending on the geographical zone and the sampling point. The WGS analysis revealed the presence of antibiotic resistance genes (ARGs) to other antibiotic classes, such as aminoglycosides, tetracyclines, sulphonamides, fosfomycin, phenicols, trimethoprim-sulfamethoxazole as well as class 1 integrons. The molecular *Ab* and *Pa* analyses highlighted: i) the presence of epidemic clones such as ST2 for *Ab* and ST233 and ST357 for *Pa*; ii) the relatedness between clinical and hospital wastewater strains and iii) the possible dissemination of clinical *Ab* strains belonging to ST2 (also proved in the conjugation assays to transfer *bla*_{OXA-23} or *bla*_{OXA-72} genes), ST79 and ST492 and of clinical *Pa* strains belonging to ST357, ST640 and ST621 in the wastewaters.

Conclusion: The prevalent clones were strongly associated with the presence of class 1 integrons, suggesting that those isolates could be a significant reservoir of ARGs and are able to persist into the environment.

Introduction

Antimicrobial resistance (AMR) is an increasing worldwide concern. Romania is one of the European countries reporting elevated AMR rates and the country with the third highest consumption of antibacterials for systemic use in the community sector (according to data from in 2020) [1]. Antibiotics are one of the most popular pharmaceuticals used in human medicine, veterinary care, and farming [2-4]. Unfortunately, they are also frequent contaminants in domestic wastewater, municipal sewage and wastewater treatment plant (WWTP) effluents from where they dissipate into the environment [5-7]. Hospitals generate huge amounts of wastewater per day, high in pathogenic microorganisms, antibiotics and other pharmaceutical or toxic substances, which transform WWTPs in one of the key sources of both antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs). ARGs are disseminated *via* mobile genetic elements (MGEs) to other non-resistant bacterial strains [8-10] during the wastewater treatment steps. The WWTPs standard procedures only partially remove ARGs, ARBs and other resilient pollutants. The remaining contaminants are contributing to the pollution of the natural environments, facilitating the selection and dissemination of ARGs and ARB [7, 11-13] into crops,

animals and humans, from which they could be (re)introduced into the medical environment [14]. Also, the ARGs can be transmitted to the aquatic microbiota *via* horizontal gene transfer (HGT), which is increased in bacterial biofilms formed in natural environments and under stress caused by various pharmaceutical and heavy metal contamination [15].

The lack of surveillance of non-clinical reservoirs is considered one of the main contributors to the spread of AMR, particularly in developing countries. In this context, during the last years, international authorities have made considerable efforts to improve the monitoring of AMR in different environments, underlining the necessity to strengthen the intersectoral human, animal and agricultural cooperation. One of the priority topics of The Joint Programme Initiative on Antimicrobial Resistance (JPIAMR) is represented by the elucidation of the role of the environment as a source for the selection and dissemination of AMR. One important goal is mapping the distribution of multidrug-resistant (MDR) pathogens and plasmids of different genomic lineages in different clinical and aquatic compartments. This important insight could be translated into policy measures to monitor AMR and control the emergence and spread of ARB [16, 17].

Acinetobacter baumannii (*Ab*) and *Pseudomonas aeruginosa* (*Pa*) are members of the initially designed “ESKAPE”, then “ESCAPE” (*Enterococcus faecium*, *Staphylococcus aureus*; *Clostridioides difficile*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacteriaceae*) group [18, 19]. Centers for Disease Control and Prevention (CDC) classified them as hazard urgent level due to their often MDR features [20] and therefore requiring concerted research and management efforts [21]. *Ab* and *Pa* are exhibiting all known AMR mechanisms, such as drug inactivation/alteration, modification of drug binding sites/targets, cell permeability modification and biofilm development [22]. One of the most clinically relevant mechanisms of resistance in *Ab* and *Pa* strains is represented by the production of antibiotic inactivating hydrolytic enzymes, especially carbapenemases (CPs) and extended-spectrum β -lactamases (ESBLs).

So far, five main groups of acquired chromosomal or plasmid located class D β -lactamases (CHDLs) with different geographic distribution have been identified in *Ab* strains, i.e. OXA-23, OXA-24/-40, OXA-58, OXA-143 and OXA-235 [23]. In *Pa* strains, there were identified eleven families of class B or of metallo- β -lactamases (MBL) [Verona integron-encoded MBL (VIM); imipenemases (IMPs); New Delhi Metallo- β -lactamase (NDM); Australian imipenemase (AIM); Central Alberta MBL (CAM); Dutch imipenemase (DIM); Florence imipenemase (FIM); German imipenemase (GIM); Hamburg MBL (HMB); São Paulo MBL (SPM) and Seoul imipenemase (SIM)], chromosomal/plasmid encoded or integron-borne, the VIM, IMP and NDM types being distributed worldwide [24].

Presently, there are insufficient data on the *Ab* and *Pa* release from hospital settings and survival into the wastewater and eventually into the natural recipient. Despite the huge burden of AMR presence in Romania and its significant overall impact on European AMR rates, the genetic relationships between the bacterial strains isolated from different aquatic environmental and clinical compartments were not investigated at national level.

Our aim was to perform a phenotypic and molecular characterization of the acquired resistome of a significant number of MDR, CP and ESBL producing *Ab* and *Pa* strains isolated in the same temporal sequence from the hospital environments and the receiving wastewater network from different counties in Romania.

Methods

Phenotypic characterization of the *Ab* and *Pa* strains

Sampling location

The water samples collection took place from September 2018 to August 2019. The collection points were represented by sewage tanks from eight hospital units and their wastewater collecting WWTPs. The eight sampling locations covered several Romanian regions such as the Southern region, with sampling locations in Bucharest (C/H - Glina municipal WWTP 2018/2019), WWTP Târgoviște (E) and WWTP Râmnicu-Vâlcea (J); Central and Western regions: WWTP Cluj (I) and WWTP Timișoara (F); Northern and Eastern regions: WWTP Iași (G) and WWTP Galați (D) (Fig. 1).

The different sampling points from the selected locations were: hospital/WWTP effluent (EF), WWTPs influent (IN), activated sludge (AS) and returned sludge (RS), where all isolated strains were considered within a single group.

Isolation and characterization of *Ab* and *Pa* strains

The water samples were collected in sterile glass sample containers, transported to the laboratory at 5 ± 3 °C and processed within the first 24h. The water samples were diluted and filtered through 0.45 µm pore size membrane filters (Millipore, France), as described in SR EN ISO 9308-2/2014 (for coliform bacteria) and then cultivated on ChromID ESBL agar and ChromID CARBA agar (BioMérieux, France). The resistant colonies obtained after cultivation at 37° C for 24 hours in aerobic conditions were subsequently inoculated on the corresponding antibiotic-enriched media for the confirmation of ESBL- or CP- producing phenotype. All resistant strains were identified by MALDI-TOF-MS (Bruker system). In the same time frame with the collection of the water samples (i.e., during a ten-day period prior to the water sampling), clinical strains were isolated belonging to *Ab* and *Pa* from intra-hospital infections that occurred in the units discharging the wastewater in the sampled WWTPs.

The antibiotic susceptibility profiles of the identified strains (220 *Ab* and 84 *Pa*) were determined using the standard disc diffusion method according to Clinical and Laboratory Standards Institute (CLSI guidelines) for 2018 and 2019 [25, 26].

PCR for ESBL and CP genes

The *Ab* and *Pa* strains were screened for CP genes (*bla*_{VIM}, *bla*_{IMP}, *bla*_{NDM}, *bla*_{KPC} for *Pa* and *bla*_{OXA-51/69-like}, *bla*_{OXA-23-like}, *bla*_{OXA-24-like}, *bla*_{OXA-58-like}, *bla*_{OXA-143}, *bla*_{OXA-235} genes for *Ab* strains) and ESBL genes (*bla*_{CTX-M}, *bla*_{TEM}, *bla*_{SHV}, *bla*_{PER}, *bla*_{VEB}, *bla*_{GES}) using previously described primers and PCR protocols [27, 28].

Mating experiments

Transferability of *bla*_{OXA-23} and *bla*_{OXA-24} genes by conjugation was tested in solid mating using a rifampicin (RIF) resistant *Acinetobacter baylyi* ADP1 as recipient. Briefly, equal amounts (100 µL) of overnight cultures of the donors (n=40 *Ab* strains from all isolation sampling points) and recipient strains were mixed and incubated in Brain heart infusion agar plates, cells were resuspended in saline solution and selected in plates containing RIF (300 mg/L) and meropenem (MEM) (0.5 mg/L) [29]. Characterization of the transconjugants were conducted by PCR.

Whole-genome sequencing (WGS), assembling, annotation and characterization

To determine the genetic relationships between the clinical and wastewater isolates, 54 strains (*Ab*, n=34 and *Pa*, n=20) were selected for WGS, with both clinical and water isolates available for the same geographical location. Total DNA was isolated using DNeasy UltraClean Microbial Kit Qiagen and subjected to Illumina (Nextera DNA Flex Library Prep Kit) sequencing on MiSeq platform. Raw reads were checked for quality using FastQC [30], assembled using Shovill pipeline [31] and primarily annotated using Prokka [32], while the prediction of resistance profiles was performed by using ABRicate [33], ResFinder, PlasmidFinder [34-37], PathogenFinder [38], CARD [39]. Strain relatedness was investigated using MultiLocus Sequence Typing (MLST) [35, 40] and Single Nucleotide Polymorphism (SNP) [41]. Comparative gene analyses were performed using Roary [42] and the output was used to infer phylogenies using RAxML (Maximum Likelihood inference using bootstrap value N = 1000) [43] and visualized using iTOL [44].

Results

Identification and antibiotic susceptibility profiles of *Ab* and *Pa* strains

A total number of 220 *Ab* and 84 *Pa* were isolated from clinical and water samples during the two consecutive years.

Two pilot sampling campaigns were performed, in Bucharest in 2018 from which 66 *Ab* and 50 *Pa* strains were isolated (Table 1).

In 2019, the sampling campaign was extended including, in addition to Bucharest, six other cities that are representative for the main country regions, i.e., North-East (Iași, Galați), Central-West (Cluj, Timișoara) and South (Târgoviște, Râmnicu Vâlcea). A total of 154 *Ab* and 34 *Pa* resistant isolates were recovered (see Table 1).

Within the same time frame, clinical *Ab* and *Pa* clinical strains were isolated in hospital units from which wastewater samples were collected. The hospital wastewater was collected and treated by the corresponding WWTPs from the same town.

Table 1. The distribution of *Ab* and *Pa* strains selected for this study.

Year	Romanian region	Isolation source	Number (n) of isolated species	
			<i>Ab</i>	<i>Pa</i>
2018	South	Bucharest hospital 1 (Clinical Institute Fundeni-CIF + Institute for Cardiovascular Emergencies Prof. C.C. Iliescu- IBC CCI)	n=10	n=15
		Bucharest hospital (H) 1 EF	-	n=11
		Bucharest hospital (H) 2 (NIIDMB)	n=4	n=3
		Bucharest hospital 2 EF	n=49	n=21
		Bucharest WWTP C EF	n=3	-
		Total 2018	n=66	n=50
2019	South	Bucharest hospital 2	n=5	-
		Bucharest hospital 2 EF	n=21	-
		Bucharest WWTP - H	n=8	-
		Târgoviște hospital	n=2	n=1
		Târgoviște hospital EF	n=5	-
		Targoviște WWTP - E	n=30	-
		Vâlcea hospital	n=2	n=1
		Vâlcea hospital EF	-	n=5
		Valcea WWTP - J	n=10	-
	Central-West	Cluj hospital	n=2	n=2
		Cluj hospital EF	-	n=3
		Cluj WWTP - I	n=5	n=2
		Timișoara hospital	-	n=2
		Timișoara WWTP - F	n=6	n=2
	North-East	Iași hospital	n=1	-
		Iași hospital EF	n=1	n=8
		Iași WWTP - G	n=15	n=2
		Galați hospital	-	n=4
		Galați WWTP - D	n=41	n=2
		Total 2019	n=154	n=34

The analysis of MDR rates from the hospital to the collecting WWTP in the first sampling campaign from 2018 has revealed the following aspects: 1) *clinical isolates* - all *Ab* and the majority of *Pa* strains (93.3-100%) were MDR (Supplemental Tables 2 and 3); 2) *hospital EF* - all *Ab* isolates were MDR (Supplemental Table 2), while the *Pa* strains exhibited various MDR rates (from 25% to 100%) (Supplemental Table 3); 3) *WWTP C, collecting the two hospital EFs* – all *Ab* isolates were MDR (Supplemental Table 2).

In 2019, the MDR rates from hospital to the collecting WWTP were as follows: 1) *clinical isolates* – with one exception, all *Ab* isolates were MDR (Supplemental Table 2), while the *Pa* strains have shown a high variation of MDR rate (from 0 to 100%) with the geographical location (Supplemental Table 3); 2) *hospital EF* – all *Ab* isolates were MDR (Supplemental Table 2), while the *Pa* strains exhibited various MDR rates (from 0% to 100%) (Supplemental Table 3); 3) *WWTPs, collecting the sewages of the sampled hospital units effluents* – the MDR resistance rates varied from 0 to 100% for both *Ab* and *Pa* strains.

Table 2 and 3. AR profiles in clinical and wastewater *Ab* and *Pa* strains.

CP and ESBL encoding genes in clinical and environmental *Ab* and *Pa* strains

a. Profiles of CP and ESBL genes from the clinical to the aquatic environment in 2018 *versus* 2019

The *Ab* strains from the clinical settings to the WWTP effluent and receiving river exhibited different profiles of CP and ESBLs in the two consecutive years, i.e.: 1) *clinical strains* – *Ab* strains recovered in 2018 were OXA-23 and OXA-24 producers (64.28/42.85%) and only 7.14% were positive for *bla*_{TEM}, while in 2019, 41.66% of all intra-hospital *Ab* strains were OXA-23 and OXA-24 producers, 25% were positive for *bla*_{SHV}, 16.66% for *bla*_{VIM} and *bla*_{VEB} and 8.33% for *bla*_{TEM} and *bla*_{GES} (Supplemental Table 2); 2) *hospital sewage* – the identified carbapenem-hydrolyzing class D β -lactamases (CHLDs) were represented in the two consecutive years by OXA-23 (67.34/96.29%) and OXA-24 (2.04/0%), MBLs by VIM (4.08/14.81%) and ESBLs by TEM (20.41/0%), SHV and PER (each one 0/3.70%), GES (0/18.51%) (Supplemental Table 2); 3) *WWTPs* – some of the enzymes were common for the strains isolated in 2018 and 2019 [i.e. OXA-24 (57.14/10.25%); OXA-23 (42.85/53.84%); TEM (28.57/10.89%); SHV (14.28/0.64%)], while some other were different [i.e. NDM in *Ab* strains from 2018 (14.28%) and GES (19.87%); VEB (8.97%); CTX-M (2.56%) and PER (0.64%) in 2019].

The CP and ESBL genes identified in the *Pa* isolates during the transmission chain from the clinical sector to the WWTP effluent and receiving river in the two consecutive years were the following: 1) *clinical strains* – the *Pa* strains were positive for *bla*_{IMP} (66.66/0%), *bla*_{VIM} (33.33/20%) and *bla*_{VEB} (38.88/30%); 2) *hospital sewage* – in the two consecutive years the following MBLs were identified in the *Pa* strains: VIM (9.37/18.75%); IMP (9.37/0%); NDM (0/12.5%); while the ESBLs were represented by GES (6.25/6.25%); VEB (43.75/12.5%) and TEM (0/31.25%); 3) *WWTPs* – only the *Pa* strains isolated in 2019 were positive for ESBL encoding genes [*bla*_{TEM} (16.66%); *bla*_{GES} and *bla*_{VEB} (8.33% each)] (Supplemental Table 3).

b. Geographic distribution of CP and ESBL genes in clinical and water *Ab* and *Pa* isolates

The 2018 pilot study was limited to the Bucharest region, and then it was extended during 2019 to other regions of the country, allowing us to perform a comparative analysis regarding the geographic distribution of the CP and ESBL encoding genes in the *Ab* and *Pa* strains.

Regarding the *Ab* strains, the Southern region shows the largest spectrum and positivity of CP and ESBL encoding genes, both in *clinical* [i.e., *bla*_{OXA-23} (44.44%); *bla*_{OXA-24} (33.33); *bla*_{VIM} (22.22%); *bla*_{TEM} and *bla*_{SHV} (11.11%)] and the *aquatic isolates* [*hospital sewage*: *bla*_{OXA-23} (100%); *bla*_{GES} (19.23%); *bla*_{VIM} (15.38%); *bla*_{VEB} and *bla*_{SHV} (3.84%) and *WWTPs*: i.e. *bla*_{OXA-23} (44.44%); *bla*_{GES} (32.09%); *bla*_{TEM} (18.51%); *bla*_{OXA-24} (14.81%); *bla*_{VEB} (13.58%); *bla*_{CTX-M} (4.93%) and *bla*_{SHV} (1.23%)].

Central-Western region revealed the presence of the following CP and ESBL encoding genes: 1) *in clinical settings* all *Ab* strains were *bla*_{VEB} positive; 50% were *bla*_{OXA-23} and *bla*_{OXA-24} positive; 2) *the aquatic isolates* recovered from the two sampled WWTPs were *bla*_{OXA-23} and *bla*_{VEB} positive (23.07% each of them).

In the North-Eastern region the CP and ESBL identified in *Ab* strains from the hospitals to the WWTP were the following: 1) all *clinical Ab* strains were OXA-24 producers; while in the sampled WWTPs the *Ab* strains harbored OXA-23 (72.58%); OXA-24 (6.45%); TEM (3.22%) and PER (1.61%) (Fig. 2).

The geographical distribution of the CP/ESBLs found in *Pa* strains isolated from intra-hospital infections, the hospital sewage tank and the sampled WWTP from the corresponding city: in the Southern region 50% of nosocomial *Pa* strains were VEB producers, while the wastewater *Pa* strains harbored *bla*_{VEB} (40%), *bla*_{NDM} (40%) and *bla*_{GES} (20%); in the North-Eastern region, 50% of clinical *Pa* strains were VEB producers; 62.5% of the

hospital sewage strains were positive for *bla*_{TEM}; 50% respectively 25% of the WWTPs were *bla*_{VEB} and *bla*_{TEM} positive. The *Pa* strains from the Central-Western regions revealed different CP/ESBLs in clinical/hospital sewage (VIM) and in the sampled WWTPs (14.28% were GES positive) (Fig.3).

c. WGS analysis of clinical and wastewater *Ab* and *Pa* isolates

Acquired resistome

The WGS analysis allowed detailed insights in the acquired resistome of the selected strains.

In case of nine *Ab* strains recovered in 2018 in Bucharest from intra-hospital infections (n=2), hospital sewage EF (n=5) and the corresponding WWTP EF (n=2) the WGS demonstrated the presence of OXA-72 and OXA-23 in the IN and the EF of the collecting sewage tank and of genes encoding aminoglycoside modifying enzymes (AMEs) i.e. aph(3'')-VIa, ant(3'')-IIa, sulphonamides (sul1) and class 1 integrons (qacEΔ1), in all investigated samples (Table 4).

Table 4. ARGs in clinical and wastewater *Ab* strains isolated in Bucharest in 2018.

ARGs classes	<i>Ab</i> (2018)	Bucharest H2 clinical (%)		Bucharest H2 sewage EF (%)		Bucharest WWTP C EF (%)		STs			
			no of strains		no of strains		no of strains	1	2	492	636
β-lactams	<i>bla</i> _{OXA-46}	100	1	100	1	50	1				
	<i>bla</i> _{OXA-72}	100	1	40	1	100	1				
	<i>bla</i> _{OXA-23}	0		80	1	0					
	<i>bla</i> _{ADC-30}	100	1	20	1	0					
	<i>bla</i> _{OXA-92}	0		0		50	1				
	<i>bla</i> _{ADC-73}	0		80	1	0					
	<i>bla</i> _{ADC-74}	0		0		50	1				
	<i>bla</i> _{ADC-81}	0		0		50	1				
	<i>bla</i> _{TEM-42}	50	1	0		50	1				
Aminoglycosides	aph(6)-Id	100	1	100	1	0					
	aph(3'')-VIa	50	1	100	1	50	1				
	aph(3'')-Ia	0		0		50	1				
	aph(3'')-Ib	100	1	100	1	0					
	aac(3)-I	50	1	0		100	1				
	aac(6'')-Ib	50	1	0		0					
	aadA1	50	1	0		100	1				
	aadA2	0		20	1	0					
	ant(3'')-IIa	100	1	100	1	100	1				
	armA	0		100	1	0					
	ant(2'')-Ia	50	1	20	1	100	1				
Phenicol	catA1	0		80	1	100	1				
Sulphonamides	sul1	50	1	100	1	100	1				
	sul2	100	1	20	1	0					
Trimethoprim	dfpA12	0		20	1	0					
Tetracyclines	tet(B)	100	1	100	1	0					
	tet(A)	0		0		50	1				
Class 1 integrons	qacEΔ1	50	1	100	1	100	1				
Macrolides and Streptogramin B	mph(E)	0		100	1	0					
	msrE	0		100	1	0					

The WGS analysis of 12 *Ab* clinical strains isolated in 2019 from two hospital units (Bucharest H2 and Târgoviște), the collecting sewage tank of Bucharest hospital 2 and the sampled corresponding WWTPs from

Bucharest (H) and Târgoviște (E) revealed the presence of *bla*_{OXA-23} in all sampled points from Bucharest. The presence of both *bla*_{OXA-23} and *bla*_{OXA-72} was shown in clinical *Ab* strains from Târgoviște hospital unit and in the corresponding WWTP (i.e., of *bla*_{OXA-23} in EF and of *bla*_{OXA-72} in the RS) (Supplemental Table 5). Similarly, the AMEs (i.e. aph(6)-Id, ant(3'')-IIa), sulphonamides resistance (sul 1) and class 1 integrons (qacEΔ1) were present in all sampled sites from Bucharest. The WGS of nine *Ab* clinical and wastewater strains from Eastern and Northern Romania revealed a high diversity of ARGs, with differences between different cities, i.e.: *bla*_{OXA-23}, aph(6)-Id, aph(3'')-Ib, armA, sul 1, tet(B), mph(E), msrE and qacEΔ1 in Galați WWTP (D) and *bla*_{OXA-72}, aph(3')-Ia, aac(3)-I, aadA1, ant(2'')-Ia, sul1 and qacEΔ1 in Iași WWTP (G) (Supplemental Table 5).

Table 5. ARGs in clinical and wastewater *Ab* strains isolated in 2019.

The WGS analysis of the acquired resistome of the 10 *Pa* strains recovered in 2018 from two Bucharest hospital units and their collecting sewage tanks demonstrated the dissemination of CP *bla*_{IMP-13} and of genes encoding AMEs (aph(3')-IIb, ant(2'')-Ia), fosfomycin (fosA), phenicols (catB7, bcr1) and trimethoprim-sulfamethoxazole (sul1) resistance in the case of one Bucharest hospital and its effluent (Table 6). In the case of the second investigated hospital and the corresponding sewage tank, there has been noticed the presence of genes encoding AMEs (aph(3')-IIb) and phenicols (catB) (Table 6).

Table 6. ARGs in clinical and wastewater *Pa* strains isolated in Bucharest in 2018.

ARGs classes	<i>Pa</i> (2018)	Bucharest H1 clinical (%)	no of strains	Bucharest H1 sewage EF (%)	no of strains	Bucharest H2 clinical (%)	no of strains	Bucharest H2 sewage EF (%)	no of strains	STs
										233 357 395 621 unk
β-lactams	<i>bla</i> _{IMP-13}	100	1	100	1	0		14.28	1	
	<i>bla</i> _{VIM-2}	0		0		0		14.28	1	
	<i>bla</i> _{GES-1}	0		0		0		28.57	1	1
	<i>bla</i> _{VEB-9}	100	1	0		0		85.71	5	1
	<i>bla</i> _{OXA-494}	0		100	1	0		0		
	<i>bla</i> _{OXA-486}	0		0		0		14.28	1	
	<i>bla</i> _{OXA-488}	0		0		100	1	0		
	<i>bla</i> _{OXA-10}	100	1	0		0		85.71	5	1
	<i>bla</i> _{OXA-4}	0		0		0		28.57	1	
	<i>bla</i> _{OXA-50}	100	1	0		0		85.71	5	1
	<i>bla</i> _{PDC-55}	0		100	1	0		0		
	<i>bla</i> _{PDC-11}	100	1	0		0		85.71	5	1
	<i>bla</i> _{PDC-113}	0		0		100	1	0		
	<i>bla</i> _{PDC-119}	0		0		0		14.28	1	
Aminoglycosides	<i>aac</i> (6')-Ib	0		100	1	0		14.28	1	
	<i>aac</i> (6')-II	100	1	0		0		100		
	<i>aadA1</i>	100	1	0		0		85.71	5	1
	<i>aadA2</i>	0		0		0		28.57	1	
	<i>aph</i> (3')-IIb	100	1	100	1	100	1	100	5	1
	<i>ant</i> (2'')-Ia	100	1	100	1	0		100	5	1
	<i>aac</i> (3)-I	0		0		0		14.28	1	
	<i>aph</i> (6)Ic	0		0		0		14.28	1	
Fosfomycin	<i>fosA</i>	100	1	100	1	100	1	100	5	1
Phenicol	<i>catB7</i>	100	1	100	1	100	1	100	5	1
	<i>bcr1</i>	100	1	100	1	0		85.71	5	1
	<i>cmIA6</i>	0		0		0		28.57	1	1
	<i>floR</i>	0		0		0		28.57	1	1
Sulphonamides	<i>sul1</i>	100	1	100	1	0		100	5	1
Trimethoprim	<i>drfA6</i>	0		0		0		28.57	1	1
	<i>drfB5</i>	0		0		0		28.57	1	1
	<i>drfB2</i>	100	1	0		0		85.71	5	1
Tetracyclines	<i>tet</i> (G)	0		0		0		28.57	1	
	<i>tet</i> (A)	100	1	0		0		85.71	5	1
Class 1 integrons	<i>qacEΔ1</i>	0		100	1	0		100	5	1
Quinolones	<i>oqxB10</i>	0		0		0		14.28	1	1
	<i>qnrVC1</i>	0		0		0		14.28	1	1

The ARGs distribution in four *Pa* strains recovered in 2019 from Northern and Eastern Romania revealed the presence of ESBL encoding genes (*bla*_{TEM-40}, *bla*_{VEB-9}), AMEs encoding genes (*aac*(6')-II, *aadA1*, *aph*(3')-IIb) as well as determinants of resistance to fosfomycin (*fosA*), phenicol (*catB7*, *bcr1*), tetracycline [*tet*(A)] and class 1 integrons (*qacEΔ1*) in clinical and wastewater samples. Regarding the six *Pa* clinical and wastewater strains from Central and Western regions of Romania, the presence of resistance genes encoding for fosfomycin (*fosA*) and phenicol (*catB7*, *bcr1*) has been observed in *Pa* strains from almost all sources (Table 7).

Table 7. ARGs in clinical and wastewater *Pa* strains isolated from North - Eastern and Central - Western Romania in 2019.

ARGs classes	<i>Pa</i> (2018)	Bucharest H1 clinical (%)	no of strains	Bucharest H1 sewage EF (%)	no of strains	Bucharest H2 clinical (%)	no of strains	Bucharest H2 sewage EF (%)	no of strains	STs				
										233	357	395	621	unkw
β-lactams	<i>bla</i> _{IMP-13}	100	1	100	1	0		14.28	1					
	<i>bla</i> _{VIM-2}	0		0		0		14.28		1				
	<i>bla</i> _{GES-1}	0		0		0		28.57		1				
	<i>bla</i> _{VEB-9}	100	1	0		0		85.71	5					
	<i>bla</i> _{OXA-494}	0		100	1	0		0						
	<i>bla</i> _{OXA-486}	0		0		0		14.28		1				
	<i>bla</i> _{OXA-488}	0		0		100	1	0						
	<i>bla</i> _{OXA-10}	100	1	0		0		85.71	5					
	<i>bla</i> _{OXA-4}	0		0		0		28.57		1				
	<i>bla</i> _{OXA-50}	100	1	0		0		85.71	5					
	<i>bla</i> _{PDC-55}	0		100	1	0		0						
	<i>bla</i> _{PDC-11}	100	1	0		0		85.71	5					
	<i>bla</i> _{PDC-113}	0		0		100	1	0						
	<i>bla</i> _{PDC-119}	0		0		0		14.28		1				
Aminoglycosides	<i>aac</i> (6')-Ib	0		100	1	0		14.28		1				
	<i>aac</i> (6')-II	100	1	0		0		100						
	<i>aadA1</i>	100	1	0		0		85.71	5					
	<i>aadA2</i>	0		0		0		28.57		1				
	<i>aph</i> (3')-IIb	100	1	100	1	100	1	100	5					
	<i>ant</i> (2'')-Ia	100	1	100	1	0		100	5					
	<i>aac</i> (3)-I	0		0		0		14.28		1				
	<i>aph</i> (6)Ic	0		0		0		14.28						
Fosfomycin	<i>fosA</i>	100	1	100	1	100	1	100	5					
Phenicol	<i>catB7</i>	100	1	100	1	100	1	100	5					
	<i>bcrl</i>	100	1	100	1	0		85.71	5					
	<i>cmlA6</i>	0		0		0		28.57		1				
	<i>floR</i>	0		0		0		28.57		1				
Sulphonamides	<i>sul1</i>	100	1	100	1	0		100	5					
Trimethoprim	<i>drfA6</i>	0		0		0		28.57		1				
	<i>drfB5</i>	0		0		0		28.57		1				
	<i>drfB2</i>	100	1	0		0		85.71	5					
Tetracyclines	<i>tet</i> (G)	0		0		0		28.57		1				
	<i>tet</i> (A)	100	1	0		0		85.71	5					
Class 1 integrons	<i>qacEΔ1</i>	0		100	1	0		100	5					
Quinolones	<i>oqxB10</i>	0		0		0		14.28		1				
	<i>qnrVC1</i>	0		0		0		14.28		1				

Ab and *Pa* strains molecular phylogeny

The *Ab* strains phylogeny, MLST profiles and SNP analyses highlight the relatedness between the strains which were divided in six groups.

In group I there were included clinical isolates from Iași, Târgoviște and Cluj and in the effluent and downstream of Bucharest WWTP C which belonged to ST636. The SNP analysis suggests the similarities between a clinical isolate sampled in 2019 and the two aquatic strains from this group, sampled in 2018 (harboring 51 and 60 SNPs of the clinical strain); the group II included most of the strains (clinical and wastewater isolates from all investigated regions) belonging to ST2, a successful widespread *Ab* clone. SNP analyses suggest the relatedness between clinical and hospital wastewater strains (189-201+202+203+204, thus less than 20 SNPs

between clinical and hospital sewage), and more intriguing, the relatedness between strains sampled in clinics and urban WWTPs (169-184 =15 SNPs, 187-1803303 =22 SNPs), suggesting the dissemination of clinical *Ab* ST2 strains in the wastewaters (Table 8); the group III was represented by strains isolated from Bucharest hospital and its collecting sewage tank isolates belonging to ST492, all of them being closely related (26 SNPs); in group IV clinical strains and aquatic strains isolated from Bucharest belonging to ST79 were included; these strains were less related (577 SNPs); the group V included wastewater strains isolated from South Romania belonging to ST1, the isolates being also less related (>700 SNPs); group VI included only environmental strains from the Northern region of the country belonging to ST155 and two related novel STs (Fig. 4).

Table. 8. Matrix representation of calculated SNPs distances between the closely related ST2 strains.

Strain code/Isolation source	187 BL3 Ab11	169 DF0462 Ab	18033O3 WWTP C DO	182 19001 CNE1 Ab	183 19003 CNE4 Ab	184 19002 CNE1 Ab	185 19011 CNE6 Ab	186 19012 ENE2 Ab	189 BL3 Ab5	201 18003 CN10 Ab	202 18002 CN6 Ab	203 18001 CN7 Ab
187 BL3Ab11 - clinical Bals												
169 DF0462 Ab - clinical Cluj	110											
18033O3 WWTP C DO	75	140										
182 19001 CNE1 Ab - WWTP D IN	580	140	521									
183 19003 CNE4 Ab - WWTP D EF	589	91	542	64								
184 19002 CNE1 Ab - WWTP D RS	616	109	555	73	129							
185 19011 CNE6 Ab -Bals sewage IN	843	417	798	465	257	463						
186 19012 ENE2 Ab -Bals sewage EF	844	418	800	466	257	463	2					
189 BL3 Ab5 - clinical Bals	732	273	704	423	348	424	353	337				
201 18003 CN10 Ab - Bals sewage IN	921	456	895	593	521	588	345	338	35			
202 18002 CN6 Ab - Bals sewage IN	915	449	888	523	523	589	344	341	48	6		
203 18001 CN7 Ab - Bals sewage EF	922	458	897	525	525	592	348	335	37	4	4	
204 18004 CN10 Ab - Bals sewage EF	934	461	907	525	525	593	334	329	33	4	6	6

Conjugation assays revealed a clear dissemination of the same circulating clones from the hospital units into different aquatic compartments [i.e. ST2 encountered in Bucharest hospital unit and the corresponding sampled

WWTP in 2019 carrying the same CP encoding gene (*bla*_{OXA-23} or *bla*_{OXA-72}) in *Ab* strains; ST2 carrying *bla*_{OXA-72} gene in *Ab* strains from Târgoviște hospital unit and *bla*_{OXA-23} in the EF of the corresponding WWTP E].

The *Pa* strains (Fig. 5) were also grouped in six phylogenetic groups: group I included wastewater isolates from Timișoara, and one clinical strain from the Bucharest hospital that belonged to three singleton STs (ST252, ST254 and ST395); group II comprises clinical strains from Central Romania (Cluj hospital) and one collecting sewage tank from a hospital unit in Bucharest that belonged to the epidemic clone ST233; group III included strains isolated from Iași hospital sewage and its collecting WWTP G belonging to ST640; group IV (Bucharest hospital unit collecting sewage tank isolate belonging to ST621); group V (wastewater strains from central Romania that belonged to ST620); group VI contained the majority of the strains (clinical and wastewater isolates from South and East Romania) that belonged to the widespread ST357 and one unknown ST (Fig. 5).

The spread of the same epidemic clone has been noticed, from the hospital unit into the natural aquatic recipient from the East part of the country – Galați hospital unit and the receiving WWTP D (ST357 carrying the *bla*_{VEB-9} ESBL encoding gene). As revealed by the SNPs analyses, the two ST640 strains (Iași hospital and sludge from the Iași urban WWTP) represent the same clone (32 SNPs, the proposed threshold being 37 SNPs). The other *Pa* strains are more diverse, even within the same clone the strains being more distantly related (>100 SNPs); this fact was also suggested by the difference between the core genome (4716 genes) and the pan genome (12395 genes) calculated for all the *Pa* strains included in this study.

Discussion

Hospitals are a concentrated source of MDR bacteria, which besides having clinical consequences (treatment options are limited and expensive), can be released in wastewater and finally into the environment [45]. Previous studies have revealed the presence of beta-lactam, tetracyclines, quinolones and sulfonamides resistance genes in both natural and polluted aquatic environments, indicating that these determinants are released from clinical into aquatic environments, and then further disseminated to opportunistic pathogens [46]. Therefore, rapid identification of high-risk clones is essential for segregation of infected patients, preventing the spreading of resistance and improving the antimicrobial treatment. This requires the knowledge of the genetic environment and the carrying platforms of ARGs, as well as the development of new methods for assessing the spreading potential of ARGs mediated by mobile genetic elements in both aquatic and hospital environments.

In Romania, in contrast to clinical studies [47-50, 27, 51-58, 28, 59-61] there is little information available on the epidemiology of AMR reservoirs in the environment, especially in polluted water and rivers. We have previously shown that MDR, CP and ESBL-producing *K. pneumoniae* isolated from clinics, hospital wastewater and urban WWTPs from different regions of the country exhibit multiple antibiotic and antiseptic resistance, as well as virulence genes, with the ST101 clone being the most frequently encountered in all sampling sites [62]. Also, we have demonstrated the spread of *K. pneumoniae* ST101 from hospital to wastewater influent and its persistence in the wastewater effluent after the chlorine treatment, suggesting its dissemination in the community and in different aquatic compartments [63]. In our previous research, we have shown that enterococci and *Enterobacteriales* strains in four Romanian natural aquatic fishery lowland salted lakes from Natura 2000 Network are carrying a high diversity of resistance markers correlated with class 1 integrons [64]. Other authors have found tetracycline and sulfonamides ARGs in the WWTP and the receiver river from the northwestern

Romania and demonstrated that some ARGs, such as *bla*_{VIM} and *bla*_{SHV} could persist in the chlorinated hospital wastewater being detected both in the influent and chlorinated effluent [65, 66].

The purpose of this study was to characterize the AMR profiles and clonality of two of the most dangerous ESKAPE pathogens, *Ab* and *Pa*, isolated for two consecutive years from hospital settings, hospital collecting sewage tanks and the receiving WWTPs from three different geographical regions of Romania. The clinical and environmental *Ab* isolates recovered from different geographical regions of Romania revealed high AMR and MDR levels. In another study, from a significant number of groundwater, surface water, and soil samples from Hungary, there were isolated different *Acinetobacter* species (i.e., *A. baumannii*, *A. johnsonii*, *A. gyllenbergii* and *A. beijerinckii*, with 8.10% of *A. beijerinckii*) exhibiting a MDR phenotype [67].

In our study, both imipenem-resistant *Ab* and *Ps* strains were also resistant to other classes of clinically important antibiotics, including quinolones (ciprofloxacin) and aminoglycosides (gentamicin). The phenotypic resistance profiles and MDR rates have largely varied by sampling point and by geographic location. The highest MDR rates in aquatic isolates were recorded in Galați WWTP (D), that could be explained by the location of this county on the lower course of Danube River. The Danube River is considered the most important non-oceanic body of water in Europe and the “future central axis for the European Union”, with its Danube Delta being included in the Biosphere Reserve and Ramsar Sites lists. The Danube River crosses ten countries, so this basin represents an optimal pool for resistant pathogens and anthropogenic pollutants dissemination and accumulation throughout large and distant areas, being assigned as a reservoir of AMR. The following two locations with high MDR rates in the aquatic isolates were Bucharest (H) (the capital and largest city) and Târgoviște (E), both located in the Southern part of the country.

The most frequently CPs encountered in clinical and environmental *Ab* strains were OXA-23 and OXA-24, while the ESBLs were represented by SHV, TEM and GES. Hrenoic et al., in 2016 investigated the AMR of *Ab* recovered from the IN and the final EF of a municipal WWTP in Zagreb, Croatia and demonstrated that 66.66 % of the *Ab* isolates were positive for the acquired CP *bla*_{OXA-23} and *bla*_{OXA-24} [68]. In the case of *Pa* clinical strains, the following CPs and ESBLs have been detected: IMP, VIM, NDM, VEB, GES and TEM.

WGS bioinformatic analysis of *Ab* strains confirmed that the international clone ST2 is broadly spread in our country. Worldwide CP producers are mostly associated with international clone II and OXA-23 [69, 70, 61]. Other clinical and wastewater isolates belonged to ST636, ST1, ST79, ST492 and ST2 and were associated with OXA-72. Other research group demonstrated recently that carbapenem resistant *Ab* isolates recovered from different sampling points of a WWTP in Zagreb, positive for *bla*_{OXA-23} belonged to the international clonal lineage IC2, OXA-72 producers to IC1, while the susceptible ones were unclustered [71]. There have also been noticed the presence of two *A. baumannii* OXA-23 producers belonging to IC2, strains isolated from the sewage of a nursing home in Zagreb [72]. Other authors have demonstrated the association of OXA-23, OXA58, OXA-72 and ST2 in hospital environments from neighboring countries of Romania [73-75]. There has also been pointed out the presence of different *A. baumannii* complex species with MDR phenotypes isolated from environmental samples in one of the Romanian neighbors - Hungary [67].

The WGS analysis of *Pa* strains has shown that ST357 was associated with IMP-13 in one clinical strain; in the sewage effluent, ST357 was associated with VEB-9 and with both IMP-13 and VEB-9; the epidemic clone ST233 was associated with VIM-2 in clinical and wastewater *Pa* strains; ST640 with TEM-40 in hospital sewage and

WWTP; ST621 with IMP-13 in hospital sewage; ST620 with GES-1 in WWTP, while an unknown ST was associated with VIM-2 in a sewage strain. The singletons ST252, 254, 244, 395 were not CP or ESBL producers.

A class 1 integron (*qacEΔ1*) was present in the majority of the identified *Ab* clones. This association could be the result of co-selection processes due to spread of successful clones (such as ST2, ST636 and ST1 *Ab*) that were selected by antibiotic treatment in the hospital settings and that were able to accumulate various CPs and ESBLs (i.e. OXA-23, OXA-72, TEM-12, ADC-30, ADC-74, ADC-73, ADC-81). Class 1 integrons were revealed in 80% of *Pa* strains and 50% of *Pa* belonging to the epidemic clones ST233, 357 and respectively the ST244.

Since the prevalent clones have a great potential of transmission among patients, the observation that those prevalent clones are significantly associated with the presence of class 1 integrons suggests that those isolates could be a significant reservoir of ARGs and are able to persist in the environment.

Conclusion

Our study has shown a high prevalence of MDR *Ab* and *Pa* strains in all investigated points (hospital units, their collecting sewage tanks and the sampled WWTPs), highlighted the most frequent CP and ESBL genes, as well as their association with specific sequence types. The reported data highlight the importance of the *screening* of acquired AMR in the environment and could provide important knowledge for monitoring the ARB and ARGs transmission from hospital into water bodies.

Abbreviations

AMR: Antimicrobial Resistance

WWTPs: Wastewater Treatment Plants

ARB: Antibiotic Resistant Bacteria

ARGs: Antibiotic Resistance Genes

MDR: Multi-Drug Resistant

CP: Carbapenemase

ESBL: Extended Spectrum Beta-Lactamase

Ab: *Acinetobacter baumannii*

Pa: *Pseudomonas aeruginosa*

WGS: Whole-Genome Sequencing

MGEs: Mobile Genetic Elements

HGT: Horizontal Gene Transfer

JPIAMR: Joint Programme Initiative on Antimicrobial Resistance

CDC: Centers for Disease Control and Prevention

CHDLs: carbapenem-hydrolyzing class D β -lactamases

MBL: Metallo- β -Lactamases

VIM: Verona Integron-encoded MBL

IMPs: Imipenemases

NDM: New Delhi Metallo- β -lactamase

AIM: Australian Imipenemase

CAM: Central Alberta MBL

DIM: Dutch Imipenemase

FIM: Florence Imipenemase

GIM: German Imipenemase

HMB: Hamburg MBL

SPM: São Paulo MBL

SIM: Seoul Imipenemase

VEB: Vietnamese extended-spectrum beta-lactamase

PER: *Pseudomonas* extended-resistant

TEM: Temoneira patient's name

SHV: Sulfhydryl Variable enzyme

CTX-M: Cefotaxime first isolated in Munich Carbapenemase

GES: Guyana Extended Spectrum β -lactamase

WWTP C and H: Bucharest WWTP

WWTP E: WWTP Târgoviște

WWTP J: WWTP Râmnicu-Vâlcea

WWTP I: WWTP Cluj

WWTP F: WWTP Timișoara

WWTP G: WWTP Iași

WWTP D: WWTP Galați

EF: Effluent

IN: Influent

AS: Activated Sludge

RS: Returned Sludge

CLSI: Clinical and Laboratory Standards Institute

RIF: rifampicin

MLST: MultiLocus Sequence Typing

MEM: meropenem

IMP: imipenem

CAZ: ceftazidime

ATM: aztreonam

FEP: cefepime

TZP: piperacillin-tazobactam

CIP: ciprofloxacin

GEN: gentamicin

AK: amikacin

MH: minocycline

SAM: ampicillin sulbactam

H: Hospital

SNP: Single Nucleotide Polymorphism

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

All data analyzed or generated during this study are included in this published article and its Supplementary Information files. Any additional information is available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

IG, ICB and MCC designed the study and corrected the manuscript; LM, MP, LIP, ICB, GGP, AB, CS; SG, IL, OS performed the isolation and identification of the samples; IG, AM, CSD, DT, MP, MMM, MIP *screened* the phenotypic and molecular markers of the isolates; MS, SP performed the WGS experiments; ICB and LIP contributed to the assembling, annotation and characterization of the obtained data; IG and ICB analyzed the WGS and performed the molecular epidemiological studies; MCC, MNL, DO, SP, MS, MP read and corrected the manuscript; IG, ICB and MS have equally contributed to this paper as main authors.

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Figures

Figure 1

Geographic distribution of the sampling points.

Figure 2

CP and ESBL encoding genes in clinical and wastewater *Ab* from different geographical regions.

Figure 3

CP and ESBL encoding genes in clinical and wastewater *Pa* from different geographical regions.

Figure 4

Phylogenetic tree of clinical and wastewater *Ab* strains.

Figure 5

Phylogenetic tree of clinical and wastewater *Pa* strains.

Supplementary Files

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