Emission of extensively-drug-resistant *Acinetobacter baumannii* from hospital settings to the natural environment

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Background: Acinetobacter baumannii is a leading emerging pathogen that is frequently recovered from patients during hospital outbreaks. The role of environmental *A. baumannii* reservoirs is therefore of great concern worldwide.
Aim: To investigate the connection between *A. baumannii* causing hospital outbreaks and environmental isolates from hospital wastewater, urban sewage and river water as the final natural recipient of wastewaters.
Methods: Clinical isolates from patients with hospital-acquired pneumonia and environmental isolates from water were collected during a two-month monitoring period. Recovery of *A. baumannii* was performed using CHROMagar Acinetobacter plates, incubated at 42°C for 48 h. Identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry and analyses of *rpoB* gene. The antibiotic resistance profiles were interpreted according to criteria given for clinical isolates of *A. baumannii*. The sequence types (ST) were retrieved by multi-locus sequence typing.
Results: Fourteen of 19 isolates recovered from patients, hospital wastewaters, urban sewage and river water belonged to ST-195. The remaining five isolates recovered from patients and river water were assigned to ST-1421. All isolates showed very strong relatedness and clustered into CC92, which corresponds to IC2. All isolates were non-susceptible to at least one agent in all but two or fewer antimicrobial categories, and thus were classified as 'extensively-drug-resistant' (XDR). Heteroresistance to colistin was found in two isolates from hospital wastewater.
Conclusion: Close relatedness of clinical and environmental isolates suggests the emission of XDR *A. baumannii* via the untreated hospital wastewater in the natural environment.
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Introduction

Acinetobacter baumannii is a leading emerging pathogen. It is mainly recovered from hospitalized patients, sporadically or

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during hospital outbreaks, but is also an occasional cause of acute community-acquired infections [1]. The successful emergence of *A. baumannii* is ascribed to the development of resistance to antibiotics [2] and to its ability to survive for prolonged periods in adverse environmental conditions [3]. Consequently, both colonized and infected patients, and hospital environments have been implicated in the transmission of *A. baumannii*.

The first environmental multi-drug-resistant (MDR) isolate of *A. baumannii* that was related to a clinical isolate was described from the water of the Seine River [4]. Subsequently, three MDR *A. baumannii* isolates were obtained from untreated hospital wastewater in Brazil [5]. In China, nine MDR *A. baumannii* were recovered from untreated hospital wastewater, and one MDR *A. baumannii* was recovered after disinfection of wastewater by chlorination, but the strains were not related to clinical isolates [6]. One MDR *A. baumannii* related to a clinical isolate was found in acid paleosol from Croatia, probably related to illegally-dumped solid waste [7]. Viable MDR *A. baumannii* related to clinical isolates were also recovered from municipal wastewater in Zagreb, Croatia, both before and after passage through the secondary wastewater treatment process [8,9]. MDR *A. baumannii* has been reported as an emerging nosocomial pathogen in veterinary clinics [10,11]. Moreover, *A. baumannii* has also been reported to be found in the air in intensive care units [12,13].

However, despite the above observations concerning the ubiquity of *A. baumannii*, there is a lack of clear evidence about the relationship between hospital settings and the natural environment in the propagation of this increasingly important pathogen. The aim of this study was to screen the hospital wastewater in Zagreb, Croatia for the presence of viable *A. baumannii* over a period when the number of nosocomial infections with these bacteria was increasing. The propagation of *A. baumannii* was followed from untreated hospital wastewater to urban sewage and the river as the natural recipient of these wastewaters.

Materials and methods

Sampling

Clinical isolates of *A. baumannii* were recovered from sputum and tracheal and bronchial aspirates of patients with hospital-acquired pneumonia in the Special Hospital for Pulmonary Diseases in Zagreb. In this hospital, the number of clinical isolates of *A. baumannii* increased from 33 in 2014 to 53 in 2015, with a consistently high rate of carbapenem resistance in the two years (84% and 83%, respectively). Hospital wastewater was collected at the central manhole at 09.00 am on 27th August 2015 and 6th October 2015. Hospital wastewater is discharged into the urban sewage system without pre-treatment. The urban sewage system in Zagreb is a mixture of hospital, domestic, industrial and storm wastewaters. Some urban sewage undergoes secondary treatment at the wastewater treatment plant, and the remaining urban sewage is discharged directly into the natural recipient, the Sava River. Urban sewage was collected at the influent of the central wastewater treatment plant in Zagreb on 23rd September 2015. Surface water of the Sava River was collected on 11th October 2015 downstream of Zagreb and the discharge site for the urban wastewaters.

Water samples were collected aseptically in sterile 1-L glass bottles and transferred to the laboratory within 1 h.

Isolation and characterization of *A. baumannii* isolates

The isolation of *A. baumannii* was performed on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar, Paris, France) and 15 mg/L of cefsulodin sodium salt hydrate (Sigma-Aldrich, St Louis, MO, USA) after incubation at 42°C for 48 h [8]. Presumptive colonies of *A. baumannii* were subcultured at 42°C for 24 h on the same selective plates and then on Nutrient agar (Biolife, Milano, Italy). Identification of *A. baumannii* was performed using routine bacteriological techniques and Vitek2 system (BioMerieux, Marcy l'Etoile, France). Identification was confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS Version 3.0; Microflex LT, Bruker, Billerica, MA, USA) on cell extracts [14]. Molecular identification was performed by amplifying a fragment of *rpo*B gene encoding RNA polymerase β-subunit by using *rpo*B+1627/*rpo*B-2231 primer pair [15]. Obtained amplicons were sequenced directly on both strands using a commercial service (Macrogen, Seoul, South Korea). Raw nucleotide sequences were assembled and edited using Sequencher 4.7 software (http://www.genecodes.com/) and representative sequences were deposited in GenBank. Sequences were aligned with ClustalX 2.0 [16] and phylogenetic analyses were performed with MEGA 7 software [17] using the neighbour-joining method with the number of differences. Bootstrap analyses were performed (500 replicates) to estimate the stability of nodes and to support the inferred clades.

The genetic relatedness of *A. baumannii* isolates from waters to clinical isolates, as well as their population structure, was determined by multi-locus sequence typing (MLST). Fragments of seven housekeeping genes (*gltA, gyrB, gdhB, recA, cpn60, gpi* and *rpoD*) were amplified by polymerase chain reaction (ProFlex 96-Well PCR System, Applied Biosystems, Foster City, CA, USA) using specific primers according to the procedures listed in the MLST database (http://pubmlst.org/abaumannii/). Amplified fragments were sequenced, edited and analysed as described above for *rpo*B gene. The sequence type (ST) together with allele sequences and profiles were retrieved from the *A. baumannii* MLST website (http://pubmlst.org/perl/bigsdb/bigsdb.pl?db=pubmlst_abaumannii_oxford_seqdef).

Susceptibilities to carbapenems (meropenem, imipenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (tobramycin, gentamicin, amikacin), tetracyclines (minocycline), penicillins/β-lactamase inhibitors (ampicillin/sulbactam, ticarcillin/clavulanic acid), folate pathway inhibitors (trimethoprim/sulfamethoxazole) and polymyxins (colistin) were determined by minimum inhibitory concentration (MIC) values obtained by the AST-XN05 and AST-N233 testing card for Vitek2 system. Colistin resistance was confirmed by gradient dilution E-test (AB Biodisk, BioMerieux) and the broth microdilution method recommended by the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [14]. MICs were interpreted according to EUCAST criteria [18] for all antibiotics with defined breakpoints for *Acinetobacter* spp., while the breakpoints of the Clinical and Laboratory Standards Institute [19] were used for penicillins/β-lactamase inhibitors and minocycline.

Results

Over the two-month monitoring period, a total of 19 isolates of *A. baumannii* were collected, comprising four clinical isolates, 10 isolates from hospital wastewater, one isolate from urban sewage, and four isolates from the Sava River (Table I). Single colonies were recovered from plates inoculated with 0.001–0.01 mL of hospital wastewater, 0.01 mL of urban sewage and 10 mL of water from the Sava River. Phylogenetic analysis of the c. 600-bp-long fragments of *rpo*B gene were used to confirm the identity of all isolates with 100% sequence identity to the reference sequences of *A. baumannii* strain AC29 (CP007535) from GenBank as *A. baumannii* (data not shown).

According to the MLST analysis using the Oxford scheme, 14 isolates (two clinical, 10 from hospital wastewaters, one from urban sewage and one from the Sava River) were identified as ST-195 (Table I). Another five isolates (two clinical and three isolates from Sava River) showed the same MLST profile, which differed from ST-195 by a single nucleotide change (G to T) in the *rpoD* allele, and were assigned to ST-1421. Isolates belonging to ST-195 and ST-1421, a DLV (double locus variant), showed very strong relatedness and clustered into clonal complex 92 (CC92), which corresponds to international clone 2 (IC2) in the MLST scheme (Table I).

Table I
Origin, date of isolation and multi-locus sequence typing of *Acinetobacter baumannii* clinical isolates, and isolates from hospital wastewater, urban sewage and river water

Isolate	Origin	Date of isolation	Sequence type	Clonal complex	IC type
OB 3831	Sputum	11.09.2015	1421[a]	92	2
OB 3929	Tracheal aspirate	18.09.2015	195	92	2
OB 4027	Sputum	24.09.2015	1421[a]	92	2
OB 4138	Bronchial aspirate	02.10.2015	195	92	2
S2/1	Hospital wastewater	27.08.2015	195	92	2
S2/2			195	92	2
S2/3			195	92	2
S2/4			195	92	2
S1/1		06.10.2015	195	92	2
S2/5			195	92	2
S2/6			195	92	2
S2/7			195	92	2
S2/8			195	92	2
S2/9			195	92	2
IN32	Urban sewage	23.09.2015	195	92	2
Sava3	River water	11.10.2015	1421[a]	92	2
Sava4			195	92	2
Sava5			1421[a]	92	2
Sava6			1421[a]	92	2

IC, international clone.
All isolates were determined by Vitek 2 system as *A. calcoaceticus-baumannii* complex; matrix-assisted laser desorption ionization-time of flight mass spectrometry score values ranged from 2.021 to 2.282 for *A. baumanni*.
[a] Novel sequence type.

The antibiotic resistance profiles of clinical isolates were comparable to those of environmental isolates from hospital wastewater, urban sewage and the Sava River (Table II). The 19 isolates examined shared resistances to carbapenems, fluoroquinolones, aminoglycosides and penicillins/β-lactamase inhibitors. All were classified as extensively-drug-resistant (XDR) [20]. Six isolates (two clinical, two hospital wastewater, one urban sewage and one river) were susceptible to colistin alone, and another six (one clinical, four hospital wastewater and one river) were only susceptible to colistin and trimethoprim/sulfamethoxazole.

Discussion

In this study, the occurrence of *A. baumannii* over a two-month period was followed from clinical isolates from patients in the Special Hospital for Pulmonary Diseases in Zagreb, through hospital wastewater and urban sewage to river water.

Clinical isolates of *A. baumannii* belonging to IC2 are a frequently reported cause of hospital-acquired infections worldwide, and have been present in Croatia since at least 2009 [21,22]. Carbapenem resistance in clinical isolates of *A. baumannii* is now very frequent in Croatia, having increased drastically from 10% in 2008 to 87% in 2015 [23]. Carbapenem-resistant *A. baumannii* belonging to ST-195 have been reported from hospitalized patients in Asian countries and Denmark [24]. Recently, carbapenem-resistant *A. baumannii* strains susceptible and resistant to polymyxin belonging to ST-195 were described from wounds of hospitalized patients in Malaysia [25]. Therefore, the finding of carbapenem-resistant clinical and wastewater isolates of *A. baumannii* belonging to globally spread CC92 inside IC2 is not unusual. Recovering identical isolates belonging to ST-195 from patients and wastewaters suggests that clinical isolates of ST-195 are disseminated via the hospital wastewater into the natural environment, but this observation could also be due to a chance event. However, the finding of isolates belonging to ST-1421 in both clinical isolates and river water during the same time period lends weight to the hypothesis that there is release of *A. baumannii* from hospital settings into the natural environment. It should be noted that *A. baumannii* was not isolated from water of the Sava River upstream of Zagreb during the same period (data not shown).

All clinical and environmental isolates showed an XRD profile. Heteroresistance to colistin was only detected in two isolates from hospital wastewater. There are two possible explanations for the detection of additional antibiotic resistance in clinically important bacteria recovered from environmental samples. One explanation is that antibiotic resistance could have developed in the hospital and these bacteria discharged directly into the sewage system, where they could survive and propagate. An alternative explanation is that more susceptible bacteria are discharged into the sewage system and acquire additional antibiotic resistance under the influence of emerging water pollutants. Exposure of bacteria to low levels of antibiotics can increase its MIC values by phenotypic adaptation or genetic change [26]. Colistin-resistant clinical isolates were not detected in the hospital during the relatively short monitoring period, but colistin would have been used as therapy for some patients. This suggests that there is a real possibility that heteroresistance to colistin in two isolates from hospital wastewater was developed under the influence of

Table II

Minimum inhibitory concentration (MIC) values of antibiotics[a] tested against isolates of *Acinetobacter baumannii*

Isolate	Sequence type	MIC values of antibiotics (mg/L)											
		MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST
OB 3831	1421[a]	>16R	>16R	>4R	8R	>16R	>16R	>64R	8I	16I	128R	>320R	<0.5
OB 4027		>16R	>16R	>4R	8R	>16R	>16R	>64R	8I	4	>128R	>320R	<0.5
Sava3		>16R	>16R	>4R	>8R	>16R	>16R	>64R	4	16I	>128R	>320R	<0.5
Sava5		>16R	>16R	>4R	>8R	>16R	>16R	>64R	8I	8	>128R	<20	<0.5
Sava6		>16R	>16R	>4R	>8R	>16R	>16R	>64R	4	16I	>128R	>320R	<0.5
OB 3929	195	>16R	>16R	>4R	4R	>16R	>16R	>64R	2	16I	128R	>320R	<0.5
OB 4138		>16R	>16R	>4R	8R	>16R	>16R	>64R	>16R	16I	128R	<20	<0.5
S2/1		>16R	>16R	>4R	8R	>16R	>16R	>64R	4	8	128R	>320R	<0.5
S2/2		>16R	8I	>4R	>8R	>16R	8R	>64R	2	<2	128R	>320R	80R
S2/3		>16R	>16R	>4R	8R	>16R	>16R	>64R	4	8	>128R	>320R	<0.5
S2/4		8I	>16R	>4R	>8R	8R	>16R	>64R	4	4	64I	>320R	20R
S1/1		>16R	>16R	>4R	8R	>16R	>16R	>64R	8I	<2	>128R	>320R	<0.5
S2/5		>16R	>16R	>4R	8R	>16R	>16R	8	8I	>32R	>128R	<20	<0.5
S2/6		>16R	>16R	>4R	8R	>16R	>16R	8	>16R	>32R	>128R	<20	<0.5
S2/7		>16R	>16R	>4R	8R	>16R	>16R	8	8I	>32R	>128R	<20	<0.5
S2/8		>16R	>16R	>4R	8R	>16R	>16R	8	8I	>32R	>128R	<20	<0.5
S2/9		>16R	>16R	>4R	>8R	>16R	>16R	8	8I	16I	>128R	160R	<0.5
IN32		>16R	>16R	>4R	>8R	>16R	>16R	>64R	8I	16I	128R	320R	<0.5
Sava4		>16R	>16R	>4R	>8R	<1	8R	16I	8I	8	>128R	>320R	<0.5

R, resistant according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical and Laboratory Standards Institute (CLSI) criteria; I, intermediate according to EUCAST and CLSI criteria.

[a] Carbapenems (MEM, meropenem; IMI, imipenem), fluoroquinolones (CIP, ciprofloxacin; LVX, levofloxacin), aminoglycosides (TOB, tobramycin; GEN, gentamicin; AMK, amikacin), tetracyclines (MIN, minocycline), penicillins/β-lactamase inhibitors (SAM, ampicillin/sulbactam; TIM, ticarcillin/clavulanic acid), folate pathway inhibitors (SXT, trimethoprim/sulfamethoxazole), polymyxins (CST, colistin).

colistin residues or other emerging water pollutants in the hospital wastewater. The presence of isolates adapted to colistin concentrations of up to 80 mg/L in hospital wastewater opens up the possibility of their dissemination through the sewage system into the natural environment.

In conclusion, these data suggest that XDR *A. baumannii* that is disseminated within the hospital environment can escape into hospital wastewater which is then discharged into the urban sewage system, part of which is discharged directly into the Sava River. It is concluded that removal of MDR *A. baumannii* in the Zagreb wastewater treatment plant is only moderate, and that considerable numbers of these bacteria are released through the effluent water into the Sava River [8]. However, the hospital wastewater and urban sewage system of Zagreb is a closed underground network of sewer. Thus, the probability of *A. baumannii*-contaminated hospital and urban wastewater being a direct source of hospital outbreaks is very low. Nevertheless, in natural water, *A. baumannii* can survive for up to 50 days [8]. Therefore, the presence of XRD *A. baumannii* in river water could be associated with the occurrence of community-acquired infections [1]. Proper management and disposal of hospital wastewaters is mandatory to prevent the spread of XRD *A. baumannii* in nature. Disinfection of hospital wastewater by chlorination results in only moderate elimination of *A. baumannii* [6]. Novel technologies to disinfect hospital wastewater before its discharge into the urban sewage system may be a promising strategy for mitigating the propagation of pathogens in the environment. The efficacies of different methods, such as H_2O_2, NaOH or heating, on the removal of XDR bacteria should be examined further in the context of global antibiotic resistance.

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