Research article

Carbapenem-resistant isolates of *Acinetobacter baumannii* in a municipal wastewater treatment plant, Croatia, 2014

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Acinetobacter baumannii **is an emerging hospital pathogen. Whereas *A. baumannii* isolated from patients or hospitals has been reported, there are few data regarding propagation of viable *A. baumannii* in the natural environment. This study investigates the occurrence and antimicrobial susceptibility of viable *A. baumannii* in municipal wastewater and its persistence through the wastewater treatment process. A total of 21 *A. baumannii* isolates were recovered at a secondary type of municipal wastewater treatment plant in Zagreb, Croatia: 15 from raw influent wastewater and six from final effluent. All isolates were carbapenem- and multidrug-resistant. Among 14 isolates tested for bla_{OXA} genes, all harboured the constitutive $bla_{OXA\text{-}51\text{-}like}$ gene, while the acquired $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}40\text{-}like}$ genes were found in 10 and three isolates respectively. Six *A. baumannii* isolates recovered from effluent wastewater multiplied and survived in sterilised effluent wastewater up to 50 days. These findings support the idea that multidrug-resistant *A. baumannii* can occur and have the ability to survive in the environment.**

Introduction

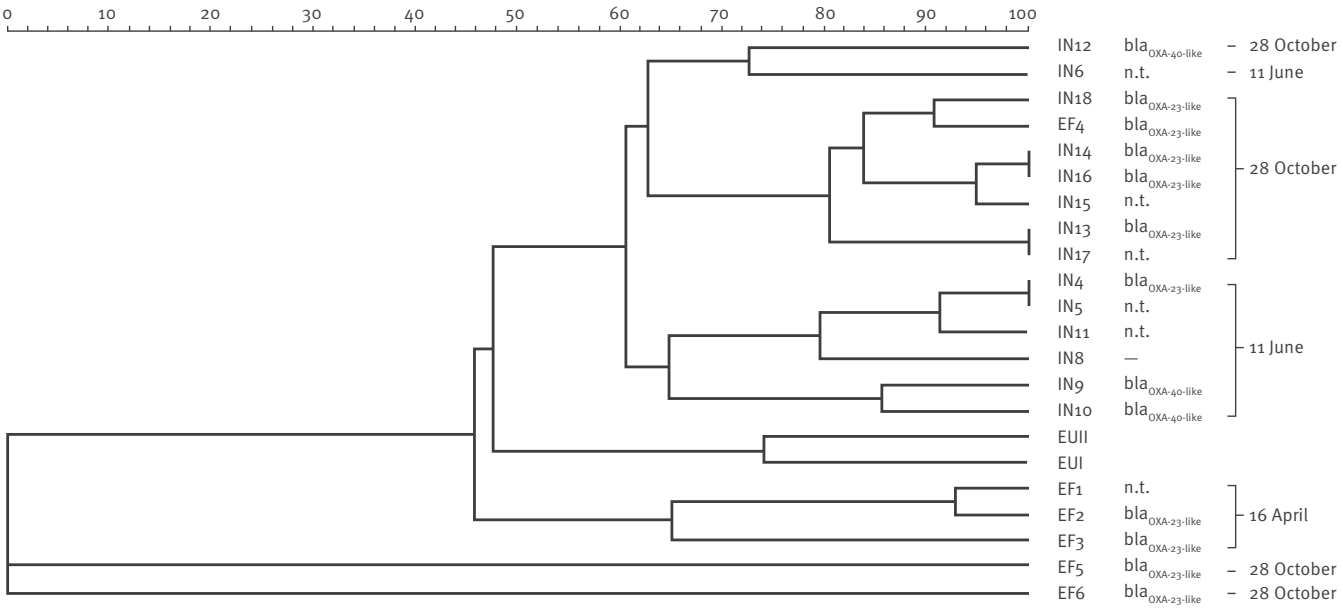
Over the last decade, hospital-acquired infections due to *Acinetobacter baumannii* have increased dramatically worldwide including in Croatia [1,2]. Difficulties caused by this pathogenin the hospital setting are exacerbated by its abilities to form biofilms on abiotic or biotic surfaces and to cope with different environmental conditions, including desiccation and disinfectants [3-5]. Non-susceptibility to commonly used antimicrobials [6] has also been observed, with carbapenem resistance becoming a global problem since 2000[7,8]. In Croatia, proportions of carbapenem-resistant *A. baumannii* strains in clinical samples increased drastically from 2008 to 2012 (10% to 73% of isolates respectively), with some individual hospitals recording a rate of 90% [2,9].

The most important mechanism of carbapenem resistance in *A. baumannii* involves OXA-type carbapenemases, which are encoded by bla_{OXA} lineage genes. Five main phylogenetic subgroups including OXA-23-like, OXA-40-like, OXA-51-like, OXA-58-like, and OXA-143-like have been recognised [10]. Aside from OXA-143-like strains, whose prevalences in certain areas remain to be determined [11], *A. baumannii* strains of all these subgroups are globally distributed. Carbapenemase genes in *A. baumanii* are moreover constitutive (e.g. OXA-51-like) or acquired (e.g. OXA-23-like, OXA-40-like, OXA-58-like). In Croatia, *A. baumannii* isolates with constitutive OXA-51-like carbapenemases have been described [8,12,13]. Moreover, since 2009, isolates with acquired OXA-40-like carbapenemases dominate in most hospitals [13,14], while isolates with acquired OXA-23-like and OXA-58-like enzymes are sporadically reported [14].

How *A. baumannii* is introduced into the hospital environment remains incompletely understood [5,15]. Acute community-acquired human infections have been reported [16] and suggest a source of the pathogen outside of the hospital. Even if such infections are mainly reported from tropical and subtropical areas and represent only a minor proportion of all *A. baumannii* infections worldwide, community-acquired infections underline the importance of searching for possible environmental reservoirs of the pathogen and their potential consequences. In nature, multidrug-resistant (MDR) *A. baumannii* strains have been isolated from hospital wastewaters [17,18]. Aside from reports of *A. baumannii* in wastewaters close to hospitals, only few

Figure 1

Pulsed-field gel electrophoresis dendogram based on *Apa*I-digested DNA from isolates of *Acinetobacter baumannii*, Zagreb, Croatia, 2014 (n=20 isolates)[a]



EF: Isolates derived from effluent wastewater; EUI: international clonal lineage I (RUH 2037); EUII: international clonal lineage II (RUH 134); IN: isolates derived from influent wastewater; n.t.: not tested for *bla*OXA genes; –: no acquired *bla*OXA-like gene.

The isolates' names according to their origin (influent or effluent wastewater), as well as the carbapenemase they harboured and date of collection are shown on the right hand side of the dendogram.

[a] A total of 21 A. *baumannii* isolates were obtained throughout the study but the *Apa*I digestion failed for one, hence 20 isolates were further analysed by pulsed-field gel electrophoresis and presented in the dendogram.

studies report detection elsewhere in the environment [19,20].

The aim of this study was to screen municipal wastewater for the presence of viable *A. baumannii*, characterise the recovered strains as well as investigate their potential for survival in the environment after passage through the wastewater treatment process.

Methods

Sampling and characterisation of wastewater

Wastewater was collected at the largest Croatian wastewater treatment plant of Zagreb. This plant (capacity 1,200,000 population equivalents) is designed for the secondary treatment of municipal wastewater, which originates from a combined sewage system of domestic, hospital, industrial and storm water. The central treatment plant receives wastewater from all nine clinical hospitals of Zagreb. In accordance with the national legislation, sanitary hospital wastewaters are released into the sewage system without pre-treatment, and only radioactive wastewaters and infective solid waste are disposed of separately. The sewage system of Zagreb is an underground network of sewer with different retention time of wastewater depending on the inhabitants' behaviours and stormwater runoff.

Composite 24h samples of influent and effluent wastewater were collected in June and October 2014 and April and October 2014, respectively. The physicochemical parameters of water samples were measured according to the Standard Methods for Examination of Water and Wastewater [21]. Temperature was measured online and average values of composite samples were calculated.

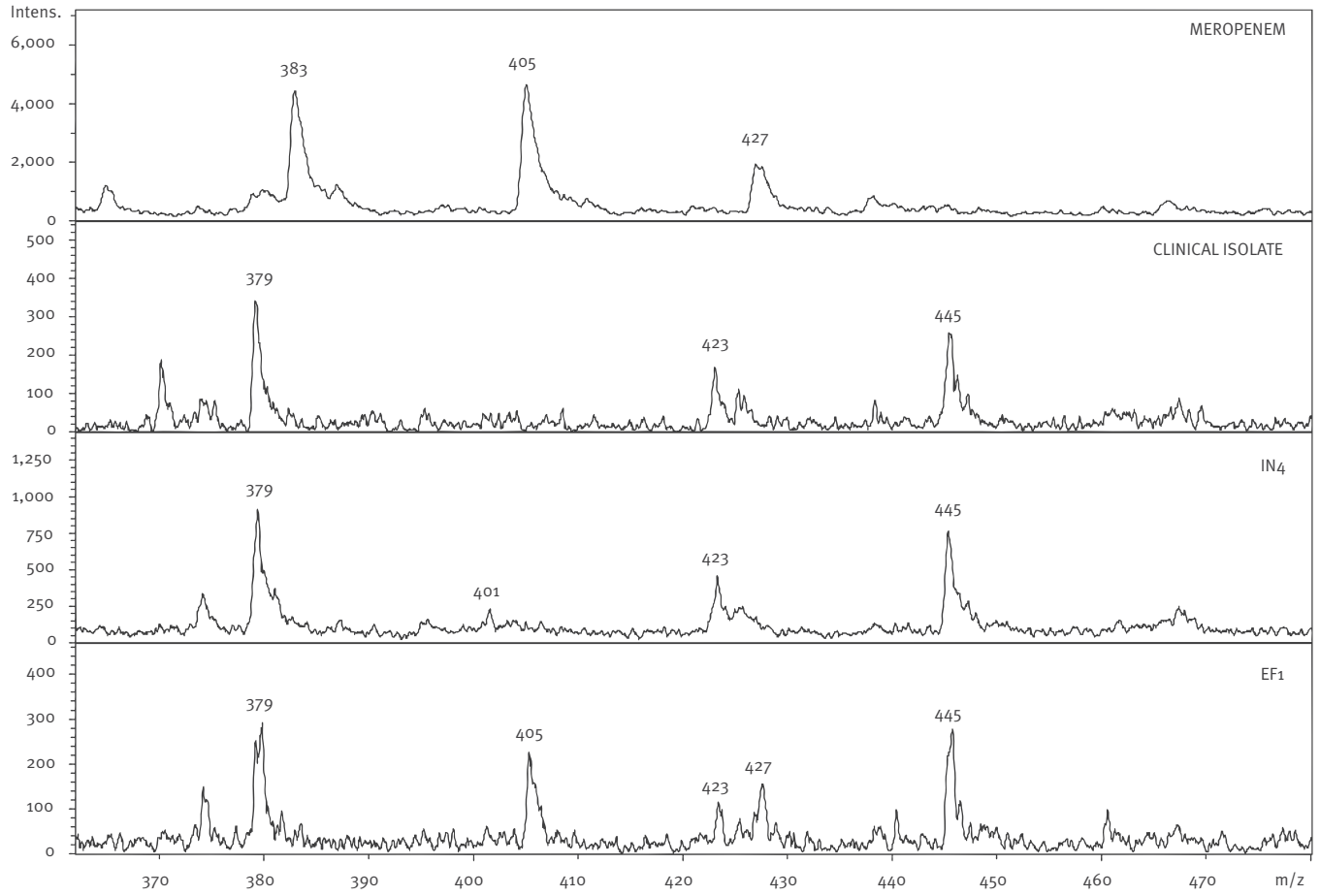
Bacteriological analyses

For bacteriological analyses, wastewater was aseptically sampled in sterile 1L glass bottles and transferred to the laboratory within 1h. The wastewater samples were concentrated on sterile membrane filters of pore size 0.45µm in triplicate both before and after dilution in sterile peptone water.

Intestinal enterococci were determined according to HRN ISO 7899–2 [22]. Membrane filters were incubated on Slanetz Bartley agar (Biolife) at 37 °C for 72h and subsequent confirmation of intestinal enterococci (i.e. colonies with dark brown halo) was done on bile esculin azide agar (Sigma-Aldrich) after incubation at 44 °C for 4h.

Figure 2

MALDI-TOF MS spectra of meropenem and its degradation products after 2.5h of contact with isolates of *Acinetobacter baumannii*



Intens.: intensity; MALDI-TOF MS: matrix-assisted laser desorption ionization-time of flight mass spectrometry.

The top panel entitled MEROPENEM represents the negative control solution with only meropenem and characteristic peaks indicating no degradation of this antibiotic (m/z 383 meropenem molecule; m/z 405 meropenem sodium salt; m/z 427 meropenem disodium salt). The panel below entitled CLINICAL ISOLATE represents the positive control, whereby a clinical carbapenem-resistant *A. baumannii* strain fully degrades meropenem leading to characteristic peaks in the spectra (m/z 379 decarboxylated sodium salt of meropenem; m/z 401 meropenem with a disrupted amide bond; m/z 423 sodium salt of meropenem with a disrupted amide bond; m/z 445 disodium salt of meropenem with a disrupted amide bond). The two lower panels (IN4 and EF1) show the degradation of meropenem in presence two respective wastewater isolates, recovered in this study. While the IN4 strain induces full meropenem degradation, in the same way than the clinical strain, the degradation by EF1 is incomplete.

Aerobically grown total heterotrophic bacteria were determined on Nutrient agar (Biolife) after incubation at 22 °C for 72h [21].

Carbapenem-resistant bacteria were determined on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar), which allows the growth of carbapenem-resistant isolates after incubation at 42 °C for 48h.

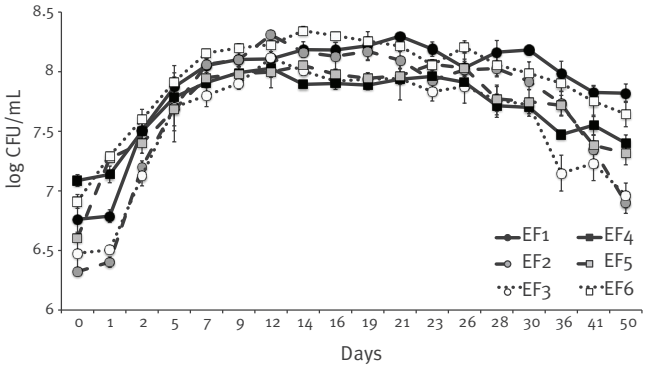
The numbers of intestinal enterococci, aerobically grown total heterotrophic bacteria, and carbapenem-resistant bacteria were determined as colony forming units (CFU), logarithmically transformed, and expressed as log CFU per 1 mL of water. The prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria was calculated.

Isolation and characterisation of *Acinetobacter baumannii* isolates

There is no simple protocol for the isolation of viable *A. baumannii* from environmental samples. *A. baumannii* is usually overgrown with accompanying flora even on selective and differential media. For the isolation of carbapenem-resistant *A. baumannii* from wastewater selective CHROMagar Acinetobacter supplemented with commercial supplement CR102 was used. Cefsulodin sodium salt hydrate (Sigma-Aldrich) was added at 15 mg/L to suppress the growth of *Pseudomonas* and *Aeromonas* spp. The isolation of *A. baumannii* was performed at 42 °C for 48h to suppress the growth of most other species of *Acinetobacter* and *Stenotrophomonas* with intrinsic resistance to carbapenems, as well as species of *Pseudomonas*. Single

Figure 3

Survival of six *Acinetobacter baumannii* isolates (EF1–6) recovered from effluent wastewater in the autoclaved effluent wastewater during 50 days, Croatia, 2014



CFU: colony forming units.

colonies of *A. baumannii* were isolated from plates inoculated with 0.01–0.1mL of influent water and 0.1–1.0mL of effluent water. Presumptive colonies of *A. baumannii* were recultivated (42 °C for 24h) on the same selective plates and then on Nutrient agar.

Pure cultures of presumptive *A. baumannii* grown at 42 °C on Nutrient agar were firstly characterised by routine bacteriological techniques to assess the following characteristics: Gram negative coccobacilli, negative oxidase, positive catalase reaction, no reaction on the Kligler Iron Agar (Biolife). Further identification of presumptive *A. baumannii* was carried out by ATB 32GN and Vitek 2 systems (BioMerieux) [23,24].

Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) which was shown to be a reliable method for rapid identification of *A. baumannii* [25,26] was used for confirmation on cell extracts according to Sousa et al. [27]. In brief, overnight cultures pre-grown on Nutrient agar were suspended in 70% ethanol. After centrifugation and removal of the supernatant, cultures were extracted with 70% formic acid and an equal volume of acetonitrile. The suspension was centrifuged and cell extracts were spotted onto a MALDI target plate and air dried at room temperature. Subsequently, the sample was overlaid with a saturated solution of α-cyano-4-hydroxycinnamic acid in 50% acetonitrile and 2.5% trifluoroacetic acid as a matrix, dried, and subjected to analysis. Spectra were obtained by using Microflex LT (Bruker Daltonics) in the linear positive mode in the range 2–20 kDa. Each recorded spectrum is the result of six series of 40 single laser shots in different locations. Identification of isolates was performed in technical triplicate and biological duplicate. Recorded mass spectra were processed with the MALDI Biotyper 3.0 software package (Bruker Daltonics) using standard settings.

The genetic relatedness of *A. baumannii* isolates from wastewater to clinical isolate was assessed by pulsed-field gel electrophoresis (PFGE). Representatives of *A. baumannii* belonging to the international clonal lineage I (RUH 2037) and II (RUH 134), which have been confirmed as two dominant clones causing outbreaks in Croatia since 2002 and which are still present in Croatian hospitals [8,9,12-14], were used as PFGE reference strains. PFGE analysis was performed using CHEF-DRII/III system (Bio-Rad) with *Apa*I (New England BioLabs) as restriction enzyme. The images of gel-electrophoresed restriction products were processed using Gel-Doc 1000 system (Bio-Rad) and Compar software. PFGE profiles were analysed and compared using Molecular Analyst Software for Fingerprinting (Bio-Rad). Matching and dendogram were performed with the unweighted pair group method with arithmetic averages (UPGMA) analysis [28,29] using Dice similarity coefficient with optimisation and a position tolerance of 1.0%. The isolates were classified into clusters based on their genetic similarity (cut-off of ≥ 90%).

Antibiotic resistance of *Acinetobacter baumannii* isolates

Susceptibility to β-lactams (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (amikacin, gentamicin, tobramycin), and trimethoprim/sulfamethoxazole were determined by disc-diffusion tests. The minimum inhibitory concentration (MIC) values were confirmed using AST-XN05 and AST-N233 testing card for Vitek2 system or using E-tests (AB Biodisk) for colistin (which was also included for susceptibility testing), and interpreted according to the European Committee on Antimicrobial Susceptibility Testing criteria [30].

The carbapenemase-induced carbapenem degradation was confirmed by MALDI-TOF MS as described by Burckhardt and Zimmermann [31] and Hrabak et al. [32], with slight modifications. In brief, a loopful of overnight bacterial culture was added to 0.5mL of 0.2mM meropenem trihydrate (Tokyo Chemical Industry Co., Ltd.) in 0.05M NaCl. The solutions were incubated at 37 °C for 2.5h with stirring at 150rpm. After centrifugation, the supernatants were spotted onto a MALDI-TOF MS targets by a sandwich method using 2,5-dihydroxybenzoic acid (DHB) as a matrix, and subjected to analysis. A carbapenem-resistant clinical isolate of *A. baumannii* carrying the OXA-72 carbapenemase [13] was used as a positive control, while a meropenem solution without addition of bacteria served as the negative control.

The presence of the genes of bla_{OXA} lineage which encode OXA-type carbapenemases was checked in 14 isolates of *A. baumannii*, selected on the basis of PFGE pattern and antibiotic susceptibility profiles. Multiplex polymerase chain reaction (PCR) with specific respective primers was used to amplify $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes, according to Woodford et al. [33].

Table 1

Physical, chemical and bacteriological characteristics of influent and effluent wastewater from a secondary water treatment plant, by sampling date, Zagreb, Croatia, 2014

Parameter	Influent		Effluent	
	11 June	28 October	16 April	28 October
Water flow (m^3/day)	250,540	509,994	248,038	495,356
Temperature (°C)	18.6	15.7	16.7	16.9
Dissolved O_2 (mg/L)	0.09	6.80	8.59	9.54
pH	7.76	7.92	7.73	7.83
COD (mg O_2/L)	353	177	26	24
BOD_5 (mg O_2/L)	184	88	3	5
Total nitrogen (mg/L)	31.3	30.9	23.9	20.7
Total phosphorus (mg/L)	5.46	2.68	1.94	1.29
Intestinal enterococci (log CFU/mL)	4.5±0.2	4.7±0.2	2.0±0.2	1.8±0.2
Total heterotrophic bacteria (log CFU/mL)	7.5±0.1	7.3±0.1	4.9±0.1	4.8±0.1
Carbapenem-resistant bacteria (log CFU/mL)	4.3±0.2	2.5±0.2	1.1±0.2	1.4±0.2
Prevalence of carbapenem-resistant bacteria (%)[a] ± SD	57±2	34±2	22±4	29±4

CFU: colony forming unit; COD: chemical oxygen demand; BOD_5: biochemical oxygen demand in five days; SD: standard deviation.

[a] Prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria was calculated as (log CFU/mL $_{\text{carbapenem resistant}}$/log CFU/mL $_{\text{heterotrophic}}$) x 100.

Survival of *Acinetobacter baumannii* in effluent wastewater

Survival of six isolates of *A. baumannii* (isolates EF1–6) recovered from effluent wastewater was monitored in the sterilised effluent wastewater collected on 28 October 2014. Effluent wastewater was filtered through filter paper (blue band) and membrane filter of pore size 0.45µm. Wastewater was partitioned per 200mL into Schott bottles and autoclaved (121°C for 20min). The isolates of *A. baumannii* were pre-grown separately on CHROMagar Acinetobacter at 42 °C for 24h. The biomass from each plate was then vortexed in sterile 0.05M NaCl, and 1mL of each suspension was inoculated into bottles containing the effluent wastewater. The bottles were sealed with a sterile gum cap and aeration was insured by a central hole through which filtered air (1L/min) was provided. The bottles were incubated at 16.7±0.2 °C in a thermostat (Memmert IPP400) with stirring (120 rpm) during 50 days. Any loss of the solution volume caused by evaporation was compensated by refilling with sterile 0.05M NaCl. For each measurement of bacterial numbers 1mL was taken from the bottle.

Numbers of *A. baumannii* were counted in triplicate after dilution in 0.05M NaCl on Nutrient agar after incubation at 42 °C for 24h. The numbers of CFU were logarithmically transformed and reported as log CFU per 1 mL of water.

Results

Physical, chemical and bacteriological characteristics of wastewater

Temperatures of pH-neutral wastewater were relatively constant (Table 1). The physico-chemical characteristics of wastewater slightly varied on two sampling occasions of influent or effluent. Most evident however, were variations in inflow and outflow and concentration of dissolved oxygen where the influent in June was anoxic (0.09 mg dissolved O_2/L). Both in June and in October, the ratio of five-day biochemical oxygen demand (BOD_5) to chemical oxygen demand (COD) in the influent was 0.5, which is considered high (0.3–0.6) and suggests nutrient rich biodegradable influent wastewater [34]. Concentrations of nutrients in effluent wastewater were however in compliance with the national emission standards [35].

National emission standards do not require the monitoring of microorganisms at a described type of wastewater treatment plant. High levels (› 1.8 log CFU/mL) of intestinal enterococci (Table 1) that are reliable indicators of faecal pollution [36] were found in the influent and effluent water at each sampling occasion. The prevalence of carbapenem-resistant bacteria among total heterotrophic bacteria ranged from 34 to 57% in influent and 22 to 29% in effluent.

Isolation and characterisation of *Acinetobacter baumannii* isolates

For the whole study, a total of 21 *A. baumannii* were isolated; 15 from influent and six from effluent wastewater. Colonies of *A. baumannii* were circular, convex, smooth, red with a paler central area. All isolates of *A.*

Table 2

Minimum inhibition concentration values of tested antibiotics and presence of acquired genes of bla_{OXA} lineage[a] in isolates of *Acinetobacter baumannii*, Zagreb, Croatia, 2014

Isolate	Minimum inhibition concentration values of antibiotics (mg/L)									*bla* OXA-like
	MEM	IPM	LVX	CIP	TOB	GEN	AMK	SXT	CST	
IN4	>16^R	>16^R	>8^R	4^R	>16^R	8^R	4	20	0.50	*bla* OXA-23-like
IN5	>16^R	>16^R	>8^R	4^R	>16^R	>16^R	4	20	0.25	Not tested
IN6	>16^R	>16^R	>8^R	16^R	2	>16^R	8	10	0.19	Not tested
IN8	>16^R	>16^R	>8^R	8^R	2	>16^R	8	10	0.25	–[b]
IN9	>16^R	>16^R	>8^R	16^R	8^R	2	2	20	0.25	*bla* OXA-40-like
IN10	>16^R	>16^R	>8^R	8^R	>16^R	>16^R	2	16	0.25	*bla* OXA-40-like
IN11	>16^R	>16^R	>8^R	8^R	>16^R	>16^R	2	16	0.25	Not tested
IN12	>16^R	>16^R	>8^R	4^R	8^R	>16^R	4	16	0.50	*bla* OXA-40-like
IN13	>32^R	>32^R	>8^R	8^R	4^R	>16^R	4	20	0.50	*bla* OXA-23-like
IN14	>16^R	>16^R	>8^R	4^R	16^R	>16^R	8	16	0.75	*bla* OXA-23-like
IN15	>16^R	>16^R	>8^R	4^R	2	>16^R	4	20	0.50	Not tested
IN16	>16^R	>16^R	4^R	16^R	4^R	4^R	4	20	0.50	*bla* OXA-23-like
IN17	32^R	>32^R	>8^R	16^R	16^R	>16^R	8	16	0.50	Not tested
IN18	>16^R	>16^R	>8^R	8^R	8^R	>16^R	4	20	0.50	*bla* OXA-23-like
IN19	>16^R	>16^R	4^R	4^R	8^R	8^R	16^R	16	0.50	Not tested
EF1	>16^R	>16^R	4^R	4^R	>16^R	>16^R	4	20	0.50	Not tested
EF2	>16^R	>16^R	4^R	8^R	8^R	8^R	4	20	0.25	*bla* OXA-23-like
EF3	>16^R	>16^R	>8^R	4^R	4^R	>16^R	4	20	0.50	*bla* OXA-23-like
EF4	>32^R	>32^R	>8^R	4^R	>16^R	>16^R	16^R	32	0.75	*bla* OXA-23-like
EF5	>16^R	>16^R	>8^R	4^R	>16^R	>16^R	32^R	32	0.50	*bla* OXA-23-like
EF6	>16^R	>16^R	>8^R	4^R	>16^R	>16^R	64^R	32	0.50	*bla* OXA-23-like

AMK: amikacin; CIP: ciprofloxacin; CST: colistin; GEN: gentamicin; IPM: imipenem; LVX: levofloxacin; MEM: meropenem; SXT: trimethoprim-sulfamethoxazole; TOB: tobramycin; R: resistant according to European Committee on Antimicrobial Susceptibility Testing criteria.

[a] The constitutive gene $bla_{OXA\text{-}51\text{-}like}$ was confirmed in all isolates.

[b] No acquired $bla_{OXA\text{-}like}$ gene.

baumannii from wastewater gave reliable score values (range: 2.048–2.409) when compared with *A. baumannii* strains in MALDI Biotyper database. All isolates were also determined by Vitek 2 system as *A. calcoaceticus-baumannii* complex.

An influent isolate named IN19 appeared resistant to *Apa*I digestion and was therefore not subjected to subsequent PFGE cluster analysis. For the remaining *A. baumannii* isolates recovered from wastewater, none were similar to isolates belonging to the international clonal lineage I or II based on the PFGE dendrogram (Figure 1). Three effluent wastewater samples collected on 16 April clustered together as well as all but one of the influent samples from 11 June, and all but one of the influent samples from 28 October. The effluent samples from 28 October, however, showed the absence of clusterisation.

Isolates IN4 and IN5, IN13 and IN17, IN14 and IN16 showed pairwise 100% identical PFGE profile. These PFGE identical pairs were respectively collected on the same date, all from influent water.

Antibiotic resistance of *Acinetobacter baumannii* isolates

According to MIC values of tested antibiotics (Table 2) all 21 *A. baumannii* isolates from wastewater were resistant to carbapenems. Although always susceptible to trimethoprim-sulfamethoxazole and colistin, all isolates were resistant to at least five of the nine antibiotics tested, and therefore could be classified as MDR. The isolates identical in PFGE profile (pairs IN4 and IN5, IN13 and IN17, IN14 and IN16, Figure 1) displayed differences in phenotypic antimicrobial sensitivity profiles. Isolate IN4 showed lower MIC value for gentamicin and higher MIC value for colistin as compared with isolate IN5. Differences in MIC values of amikacin, ciprofloxacin, meropenem, trimethoprim-sulfamethoxazole and tobramycin were observed for isolates IN13 and IN17. Isolates IN14 and IN16 showed differences in MIC values of all tested antibiotics, except for imipenem and meropenem (Table 2).

The carbapenemase-induced carbapenem degradation by *A. baumannii* isolates from wastewater was confirmed by MALDI-TOF MS. No spontaneous degradation of meropenem was observed and characteristic peaks (m/z 383, 405, 427) were evident after 2.5h

of incubation (Figure 2). All isolates from wastewater degraded the meropenem within 2.5h.

Selected types of spectra are shown in Figure 2. The positive control carbapenem-resistant clinical isolate of *A. baumannii* yielded a typical spectrum of complete meropenem degradation with characteristic peaks at m/z 379, 401, 423, and 445. Three isolates (IN4, IN5, IN8) of *A. baumannii* from wastewater degraded the meropenem as efficiently as the clinical isolate, as represented by the spectra of isolate IN4 in Figure 2. In the spectra of the remaining *A. baumannii* isolates recovered from wastewater in this study, residues of meropenem sodium and disodium salts (m/z 405 and 427) were visible in addition to the meropenem degradation products. This type of meropenem degradation is represented by the spectra of isolate EF1 in Figure 2 and suggests that such wastewater isolates degraded meropenem slower than the clinical isolate. A contact time of more than 2.5h was needed for these environmental isolates to fully degrade 0.2mM of meropenem. These isolates degraded the meropenem molecule first, followed by the degradation of meropenem sodium and disodium salts.

Monitoring of the meropenem degradation by MALDI-TOF MS does not provide detection of the type of carbapenemase carried nor detection of other carbapenem-resistance mechanisms [31]. The presence of genes of bla_{OXA} lineage which encode OXA-type carbapenemases was further confirmed by PCR. All of 14 tested *A. baumannii* isolates harboured the constitutive $bla_{OXA\text{-}51\text{-}like}$ gene. Among these, 10 isolates had the acquired $bla_{OXA\text{-}23\text{-}like}$, three the acquired $bla_{OXA\text{-}40\text{-}like}$ genes and one isolate did not have any acquired bla_{OXA} gene (Table 2).

For the three pairs of identical isolates by PFGE, the bla_{OXA} genes in two pairs were only determined for one isolate respectively. For the remaining pair comprising IN14 and IN16, the acquired $bla_{OXA\text{-}23\text{-}like}$ gene was confirmed for both isolates in the pair (Table 2 and Figure 1). The $bla_{OXA\text{-}23\text{-}like}$ gene was moreover found in two tested isolates from the three effluent samples of 16 April, which clustered together by PFGE. It was also present in the four of six tested influent water sample isolates from 28 October, which together formed a separate in PFGE. The four remaining isolates with $bla_{OXA\text{-}23\text{-}like}$ gene did not cluster together by PFGE and included three effluent isolates from 28 October and one influent isolate from 11 June. Although influent samples from 11 June formed a larger PFGE group, this group contained a sub-cluster with two isolates harbouring $bla_{OXA\text{-}40\text{-}like}$ genes. The third isolate with $bla_{OXA\text{-}40\text{-}like}$ gene was from an influent water sample collected on 28 October.

Survival of *Acinetobacter baumannii* in effluent wastewater

Six isolates of *A. baumannii* recovered from effluent wastewater showed excellent survival in the autoclaved effluent wastewater from 28 October (Figure 3). The low concentration of nutrients in this effluent wastewater was sufficient to support the multiplication of bacteria. Multiplication of isolates occurred after one day of incubation, and log phase of growth was evident up to nine days of incubation. The stationary phase of growth was continued up to 30 days, after which a slight decrease of log CFU was observed up to 50 days of incubation. After 50 days of monitoring the numbers (log CFU) of *A. baumannii* isolates were 4.4 to 15.7% higher than initial numbers.

Discussion

In this investigation, wastewater collected at a water treatment plant in Zagreb in April, June and October 2014 tested positive for *A. baumannii* at each occasion. A previous study in 2002 [37] reported the presence of eight phosphate-accumulating *A. baumannii* strains in the activated sludge of a municipal wastewater treatment plant in Izmir, Turkey. The strains in the latter study were however only characterised biochemically (API 20NE identification kit) so they could have belonged to other species of the genus *Acinetobacter* considered important for enhanced biological phosphate removal from wastewaters [24,27,38]. In contrast, none of the *A. baumannii* isolates in the current study were able to accumulate intracellular polyphosphate granule as confirmed by Neisser staining (data not shown).

The prevalences of carbapenem-resistant bacteria among total heterotrophic bacteria, which were estimated each time the water was sampled, ranged from 34 to 57% in influent water and 22 to 29% in effluent water. As carbapenems are heavily used in clinical environments, such prevalences suggest hospital wastewater in the influent water and bacteria of clinical origin in the effluent. The finding of OXA-40-like carbapenemase in three of 14 *A. baumannii* isolates recovered from wastewater strengthens this hypothesis. Indeed, OXA-40-like carbapenemases constitute the dominant mechanism of carbapenem resistance in clinical isolates of *A. baumannii* from most Croatian hospitals [9,13,14]. In addition to this, a number of strains isolated from wastewater degraded meropenem in the same way as a control clinical isolate. The dominance (10 of 14) of wastewater isolates harbouring the $bla_{OXA\text{-}23\text{-}like}$ gene was nevertheless unexpected, since the clinical isolates of *A. baumannii* which produce OXA-23-like carbapenemases have only been observed sporadically in the country [14]. It is possible that isolates carrying the $bla_{OXA\text{-}23\text{-}like}$ gene are more resistant to adverse environmental conditions in sewage as well as in the wastewater treatment process, thus surviving longer, but this needs to be further examined.

In spite of this, all wastewater *A. baumannii* isolates recovered in this study were MDR and showed comparable levels of antibiotic resistance to clinical *A. baumannii* isolates in Croatia [12]. As environmental bacterial isolates are generally more susceptible to antibiotics than clinical isolates [39], these results also point to

hospitals as the likely source of MDR *A. baumannii* in the wastewaters considered. In a preliminary screening, we confirmed the presence of MDR *A. baumannii* in the wastewater of one hospital in Zagreb (data not shown). The observation of MDR *A. baumannii* in hospital wastewaters has also been previously reported in Brazil and China [17,18].

High numbers of intestinal enterococci that are reliable indicators of faecal pollution [36] were also found in the wastewater together with *A. baumannii*, suggesting the presence of sanitary water and bacteria of faecal origin. MDR *A. baumannii* has been found in digestive tracts of hospitalised patients [40] as well as in animal faeces and urine [41]. The digestive tracts of colonised patients and animals could be important epidemiological reservoirs of MDR *A. baumannii*, from which bacteria could migrate through wastewater into the natural environment. The wastewater from veterinary clinics [41] and stormwater, which could leach the bacteria from solid waste [20] may also represent a source of *A. baumannii*. Multilocus sequence typing (MLST) of housekeeping genes should be further performed in order to assess the genetic relatedness of *A. baumannii* isolates from municipal wastewater and clinical isolates, as well as isolates from hospital wastewaters in Zagreb.

When allochthonous bacterial species come in the environment, some abiotic and biotic ecological factors have been shown to determine their survival and multiplication. Little is known however about the influence of environmental factors on *A. baumannii* strains which are significant for public health in wastewater. Six isolates of *A. baumannii* recovered from effluent wastewater multiplied and survived in the effluent wastewater for up to 50 days of monitoring. The average annual water flow velocity of the Sava River, which receives the effluent wastewater, is 0.75m/s, and 12 days are needed for the water mass to travel from the location of effluent discharge to the firth of the Sava into the Danube River. Therefore, the viable MDR *A. baumannii* could possibly spread through the natural water bodies or accumulate in river sediments. A separate study finding a MDR *A. baumannii* isolate related to a clinical isolate in the Seine River in France further supports this possibility [19].

Zhang et al. [39] reported that the tertiary wastewater treatment process contributed to the selective increase of MDR *Acinetobacter* spp. in final effluent and natural recipient. Lower numbers of carbapenem-resistant bacteria together with lower prevalence of such bacteria among total heterotrophic bacteria in effluent than in influent wastewater does not support this in the case of our secondary wastewater treatment process. Higher numbers of viable MDR *A. baumannii* isolated per sampling occasion from influent than from effluent wastewater rather suggest a beneficial role of the secondary wastewater treatment process on the distribution of clinically important bacteria in the environment. However, certain numbers of carbapenem-resistant bacteria and *A. baumannii* are released after the secondary treatment through the effluent wastewater into the natural recipient, which is the Sava River. The presence of viable MDR *A. baumannii* in both raw and treated municipal wastewater poses a serious concern about the spread of this emerging pathogen in nature.

Disinfection of effluent water has been suggested as a promising tool for prevention of the accumulation of MDR bacteria in waterbodies [42]. However, currently the standards for discharge of treated municipal wastewater do not prescribe the elimination of MDR bacteria. Furthermore, carbapenem-resistant MDR *A. baumannii* were isolated from hospital wastewaters both prior and after disinfection by chlorination [18]. Genes encoding New Delhi metallo-beta-lactamase-1 (NDM-1) were present in domestic and industrial wastewater and each stage of the wastewater treatment process, including the chlorinated effluent [43]. Although the viable bacteria were not determined, these NDM-1 genes were 99.8% identical to the NDM-1 genes carried by *A. baumannii*. The findings of MDR *A. baumannii* and NDM-1 genes after the process of chlorination suggest that conventional disinfection of effluent may not be the best strategy for mitigating the propagation of *A. baumannii* in the environment. Other disinfection techniques or barrier approaches at the source of contamination may be a more promising approach.

Tracking the source and further dispersion of *A. baumannii* contamination is needed to initiate any response plan for its control in the environment. The isolates of *A. baumannii* recovered from the environment should be compared with hospital, veterinary/livestock and community-acquired strains by using additional types of molecular assays such as whole genome sequencing [16], whereby there may be identifiable differences between these strains. Larger screening and epidemiological studies should also be performed to investigate the impact of human waste on the spread of MDR *A. baumannii* in the natural environment. In the current era when new antimicrobials for MDR Gram-negative microorganisms are scarce, it is most important to understand the epidemiology of this human pathogen. The results of this study represent a step forward in this direction.

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Conflict of interest

None declared

Authors' contributions

J Hrenovic designed the study. J Hrenovic, I Goic-Barisic, S Kazazic, A Kovacic, and M Ganjto, performed the experiments and analysed data. M Tonkic interpreted part of the results. J Hrenovic wrote the first draft. All authors reviewed, provided comments and approved the final version of manuscript.

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