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Transmission and survival of carbapenem-resistant *Acinetobacter baumannii* outside hospital setting

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Summary. *Acinetobacter baumannii* origin and its epidemiology is under a great concern worldwide since this microorganism has become a leading nosocomial pathogen of the 21st century among the “ESKAPE” group of microorganisms. The aim of the study was to monitor and explore the epidemiology of this important hospital pathogen in the second largest clinical university hospital in Croatia. The presence of *A. baumannii* in hospital wastewater, as a route for possible transmission outside of the hospital setting, as well as its survival in environmental conditions including seawater, was investigated. During the examination period, ten both carbapenem and multidrug-resistant isolates of *A. baumannii* were recovered from hospital wastewater and compared to the clinical isolates originating from the same monitoring period. Multiplex PCR confirmed that four wastewater isolates harboured *bla*_{OXA-23-like} while five wastewater isolates harboured *bla*_{OXA-40-like} genes sharing 100% sequence identity with *bla*_{OXA-72} sequence described in the same hospital in 2009, confirming the presence of an endemic cluster. Survival of *A. baumannii* in natural seawater was examined during 50 days of monitoring and to the best of our knowledge, was performed for the first time.

Keywords: *Acinetobacter baumannii* · hospital wastewater · transmission · seawater.

Introduction

Among the “ESKAPE” pathogens, *Acinetobacter baumannii* is the most frequently encountered microorganism in the hospital setting causing serious infections, especially in the intensive care units. As a leading nosocomial pathogen of the 21st century, having the ability to acquire resistance to almost all antimicrobial agents, origin and epidemiology of this emerging hospital pathogen is under a great concern worldwide [15].

A. baumannii can cause various infections like nosocomial pneumonia, bacteraemia, meningitis, skin and soft tissue, and urinary tract infections. The incidence of serious infections (blood stream infections and ventilator-associated pneumonia) caused by multidrug-resistant *A. baumannii* ranges between 47% and 93%, with mortality rates between 30% and 70% [2].

University Hospital of Split (UHS) is the leading medical centre with 1400 beds in the Southern Croatia and is situated on two locations (500 m distance) in the middle of Adriatic coast. Hospital wastewaters are discharged without any pre-treatment into the combined urban sewage system. The urban sewage undergoes the mechanical treatment through the coarse screens and is afterwards discharged into the Adriatic Sea.

Since 2009, UHS has a growing problem in the number of infections caused by carbapenem-resistant isolates of *A. baumannii*, which is now almost endemically present in most of the intensive care units inside the hospital [7,8]. The coop-

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eration with Croatian Committee for Antibiotic Resistance Surveillance (CARS) of the Croatian Academy of Medical Sciences (CAMS) for the monitoring of sensitivity and resistance of clinical isolates of *A. baumannii* was established in the last decade. According to recently published data, during the last three months in 2015, a total of 120 clinical isolates of *A. baumannii* were isolated from hospitalized patients in UHS, mainly from respiratory samples in intensive care units [16]. In the last decade, the resistance rates of *A. baumannii* to carbapenems in UHS have increased significantly, from 10 % in the 2006 to almost 97% in 2016 [16]. Besides hospital-acquired infections, community-acquired *A. baumannii* infections, particularly pneumonia in tropical regions of the world, have been recently described [4,1]. Therefore, the aim of the study was to monitor and explore the epidemiology of this important hospital pathogen in Croatia. The presence of *A. baumannii* in hospital wastewater as a route for possible transmission outside of the hospital setting, as well as survival of this pathogen outside the hospital environment was investigated.

Material and methods

Isolation and characterization of *A. baumannii* isolates. Samples of wastewater were collected from the University Hospital of Split (UHS), situated at two locations (A and B) in the town of Split. Wastewater was sampled for five times, in the period from October 2014 until April 2015, on both locations. The samples were taken in 500 ml sterile bottles and processed within two hours. They were diluted in sterile peptone water and filtered through membrane filters of pore size 0.45 μm . The filters were placed on CHROMagar Acinetobacter supplemented with CR102 (CHROMagar) and 15 mg/L of cefsulodin sodium salt hydrate (Sigma-Aldrich) [13]. The plates were incubated at 42°C/48 h. Presumptive colonies of *A. baumannii* were recultivated (42°C/24 h) on the same selective plates and afterwards on nutrient agar. Identification of *A. baumannii* was performed by routine bacteriological techniques and confirmed by matrix-assisted laser desorption ionization-time of flight mass spectrometry - MALDI-TOF MS (software version 3.0, Microflex LT, Bruker Daltonics) on cell extracts [18]. Antibiotic susceptibility was assessed by disk diffusion method. The MICs values were confirmed by Vitek2 system or gradient E-tests (AB Biodisk), and interpreted according to the EUCAST criteria, except for ampicillin/sulbactam and tigecycline [3,5].

Molecular characterization of *A. baumannii* isolates. The relatedness of isolated *A. baumannii* to clinical isolates was assessed by using pulsed-field gel electrophoresis (PFGE). The PFGE results of *A. baumannii* isolates recovered from the wastewater were compared to four clinical isolates collected from routine surveillance cultures and respiratory samples (tracheal aspirates) of patients in Intensive Care Units (ICUs) of

UHS during October 2015 and April 2016. PFGE analysis was performed using CHEF-DRII/III system (Bio-Rad) with *Apal* (New England BioLabs) and *Salmonella* serotype Braenderup strain H9812 as marker. 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). Dendrogram was created with the unweighted pair group method with arithmetic averages analysis (UPGMA) using Dice similarity coefficient with optimisation and a position tolerance of 1.5%. The isolates were classified into clusters based on their genetic similarity (cut-off of $\geq 90\%$). The presence of genes of *bla*_{OXA} lineage, which encode OXA-type carbapenemases, was further confirmed in selected *A. baumannii* strains, based on PFGE patterns and antibiotic susceptibility profiles. Multiplex PCR was used to amplify *bla*_{OXA-51-like}, *bla*_{OXA-40-like}, *bla*_{OXA-23-like} and *bla*_{OXA-58-like} genes, according to Woodford et al [22]. In the same PCR reaction, primers for *bla*_{OXA-143-like} were added, according to Higgins [12]. All obtained amplicons of *bla*_{OXA} genes were sequenced on both strands (commercial service Macrogen Europe, the Netherlands). Raw nucleotide sequences were assembly and manual editing using Sequencher™ 4.7 software (<http://www.genecodes.com/>). Together with available sequences retrieved from the Genbank, *bla*_{OXA} sequences obtained in the scope of this study were aligned with ClustalX 2.0 [19]. Subsequent phylogenetic analyses were performed by using MEGA 7 software [14], with neighbour-joining method and number of differences model. In order to estimate the stability of nodes and to support the inferred clades, bootstrap analyses of 500 replicates were performed.

Survival of *Acinetobacter baumannii* in seawater. Survival of *A. baumannii* in natural seawater was followed for three isolates (2, 8, and 16). Overnight bacterial cultures were suspended in 100 mL of autoclaved natural seawater. Bacterial suspensions were incubated at 20°C with 170 rpm during 50 days of monitoring. After specified period of time, bottles were shaken, sub-samples were diluted in sterile saline solution, inoculated onto Nutrient agar plates, and bacterial colonies were counted after incubation at 42°C/24h. Number of viable bacteria was determined as colony forming units (CFU), logarithmically transformed, and expressed as log CFU per 1 mL of seawater. Experiments were performed in technical triplicate with mean values presented. Survival of bacteria was calculated as $((\log \text{CFU}/\text{mL}_{\text{time}} : \log \text{CFU}/\text{mL}_{\text{start}}) * 100)$, where $\log \text{CFU}/\text{mL}_{\text{time}}$ represents the number of bacteria on a day of measurement and $\log \text{CFU}/\text{mL}_{\text{start}}$ the initial number of bacteria.

Results

During the examination period, 10 isolates of *A. baumannii* were recovered from hospital wastewater and 4 from hospital-

ized patients. MALDI-TOF MS analysis of all isolates gave the reliable score values above 2.0, identifying them as *A. baumannii*. All 14 isolates displayed a high level of resistance (Table 1) to both carbapenems (imipenem and meropenem) with MICs >64 mg/L, and susceptibility to colistin (MIC<0.5 mg/L) and some of them to ampicillin/sulbactam (MIC 8-16 mg/L). The resistance phenotype was very similar for all isolates (resistant to carbapenems and the majority of tested antibiotics). According to the PFGE analysis (Fig. 1) 9 isolates were grouped in 4 clusters with a similarity of at least 95% and they were selected for further molecular characterization. We also chose strain 2A for further analysis since it turned out that it was not in the same cluster with isolate 2. Four clinical isolates (c.i. 13-16) from respiratory samples (BAL and tracheal aspirates) from patients in intensive care units of UHS were molecular analysed by PFGE

and multiplex PCR together with wastewater isolates. Phenotypic and genotyping characteristics of carbapenem-resistant isolates of *A. baumannii* recovered from hospital wastewater and clinical isolates from patients hospitalised in the University Hospital of Split are presented in Table 1.

Multiplex PCR confirmed that besides from the fact that all isolates were positive for OXA-51-like genes, wastewater isolates 2, 4, 7 and 9 harboured *bla*_{OXA-23-like}, while wastewater isolates 3, 6, 8, 11 and 12 harboured *bla*_{OXA-40-like} genes (Fig. 2). Clinical isolates 13-15 harboured *bla*_{OXA-40-like} and clinical isolate 16 *bla*_{OXA-23-like} gene. Phylogenetic analyses of all amplified and sequenced *bla*_{OXA} fragments clearly supported the affiliation of detected *bla*_{OXA} genes to two different clusters identical as those from clinical isolates (c.i. 13-16) and available in GenBank (Fig. 3). Clinical (13-15) and wastewater isolates 3, 6, 8, 11 and 12 shared 100%

Table 1. Phenotypic and genotyping characteristics of carbapenem-resistant isolates of *A. baumannii* recovered from hospital wastewater and clinical isolates from patients hospitalised in the University Hospital of Split

Number of Isolate (all)	Resistance phenotype	Genotype	Sampling
2,4,7,9 c.i. 16	AB, IP, MP, AK, TB, GM, CP, LV, SXT	<i>bla</i> _{OXA-51} , <i>bla</i> _{OXA-23}	OCT-15 JAN-16
3,6,8,11,12 c.i. 13,14,15	IP, MP, AK, GN, CP, LV, SXT	<i>bla</i> _{OXA-51} , <i>bla</i> _{OXA-40}	OCT-15, FEB-16 DEC-15, MAR-16

AB, ampicillin-sulbactam; AK, amikacin; IP, imipenem; MP, meropenem; GM, gentamicin; TB, tobramycin; CP, ciprofloxacin; LV, levofloxacin; SXT, trimethoprim/sulfamethoxazole; JAN, January; FEB, February; MAR, March; OCT, October; DEC, December; 15, 2015; 16, 2016; c.i. clinical isolate

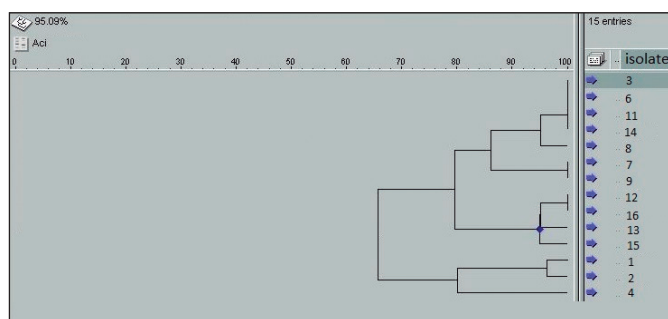


Fig. 1. Results of genotyping by pulsed-field gel electrophoresis (PFGE) from wastewater isolates (1-12) and clinical isolates (13-16) of *Acinetobacter baumannii*. The majority of isolates (9/10) were grouped in 4 clusters with a similarity of at least 95%.



Fig. 2. Multiplex PCR results from wastewater isolates (2-12) and clinical isolates (13-16) with positive controls (K1-3). In wastewater isolate 1 only *bla*_{OXA-51}-like gen was detected.

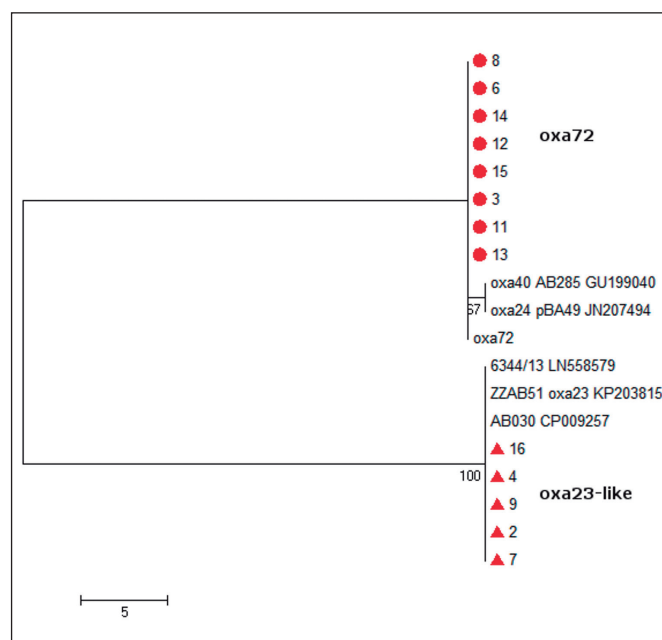


Fig. 3. Neighbour-joining phylogenetic tree inferred on *bla*_{OXA} genes fragments amplified from wastewater isolates (2-12) and clinical isolates (13-16) of *Acinetobacter baumannii*. GenBank accession numbers are given next to the name of each strain.

sequence identity with *bla*_{OXA-72} sequence described in the same hospital in 2009, confirming the presence of an endemic cluster.

Three isolates of *A. baumannii* survived in seawater during 50 days of monitoring (Fig. 4). No multiplication of bacteria was observed as compared to initial numbers. After 7 days of contact, a decrease in bacterial numbers was observed up to 28 days, but there was no further sharp decrease up to 50 days of contact. After 50 days in seawater, survival of *A. baumannii* isolates ranged from 55-67%, corresponding to 3.8-4.5 log CFU/mL.

Discussion

The epidemiology of emerging hospital pathogen *A. baumannii* still remains unclear even though numerous studies have investigated nosocomial outbreaks during the last two decades [1, 20]. Unfortunately together with unclear epidemiology, the overuse of carbapenems has rapidly resulted in the worldwide dissemination of carbapenem resistant *A. baumannii* strain, including Croatia. In the last decade, drastic increase in resistance rate of carbapenem-resistant *A. baumannii* isolates is not isolated problem in only one hospital, but is already a leading national health problem requiring wider attention. Once endemic in the hospital setting, *A. baumannii* has become extremely difficult to eradicate [20]. Carbapenem resistant *A. baumannii* carrying OXA-72 oxacillinase spread in the UHS after transfer of a patient colonized by international clonal lineage II (IC II) strain from General Hospital Mostar (Bosnia and Herzegovina) to the UHS at the beginning of 2009 [8, 9]. Clinical isolates 13-15 in this study shared 100% sequence identity with the *bla*_{OXA-72} sequence described in the same hospital in 2009, confirming the presence of an endemic cluster. Since OXA-72 within OXA-40-like group was described as dominant

mechanism of resistance in clinical isolates of *A. baumannii* in 2009 inside UHS, this investigation also revealed oxacillinase belonging to OXA-23-like group (c.i.16) which contributed to the resistance rate to carbapenems of 90% in the last two years in UHS. Carbapenem resistance in *A. baumannii* has rapidly spread throughout Croatia since 2008 and high rates of non-susceptibility to imipenem (87%) and meropenem (88%) are unfortunately not unexpected [16]. The mechanism of carbapenem resistance in other hospitals in Croatia was similar as previously described in UHS from 2002 to 2008, and during 2009 [21].

The observation of multiresistant *A. baumannii* in hospital wastewater has also been previously reported in Brazil, China and Zagreb in Croatia [6, 22, 23]. In Croatia, carbapenem resistant isolates of *A. baumannii* were detected in municipal wastewater treatment plant in capital city of Zagreb during 2014 [13]. This finding has prompted several more researches including this one, focused on transmission of carbapenem resistant clinical isolates through the hospital wastewater into natural environment [10, 17]. In addition, the first pan drug-resistant environmental isolate of *A. baumannii* was recovered from the effluent of secondary treated municipal wastewater of the capital city of Zagreb in Croatia in 2015, harbouring plasmid-located *bla*_{OXA-23-like} gene [11]. This knowledge reveals municipal wastewater as a potential epidemiological reservoir of carbapenem resistant genes. The situation with wastewaters of UHS is even more complex since they are being released to the Adriatic Sea, without any pre-treatment.

Although three isolates of *A. baumannii* selected in this study did not multiply in seawater, they successfully survived *in vitro* investigation which lasted 50 days. The absence of bacterial multiplication could be explained by low nutrient concentration in natural seawater. In case that MDR *A. baumannii* reaches the seawater, it could be disseminated by sea current through the large area of the seawater ecosystem, which poses a serious public health concern. In Croatia, but also in great number of European countries, it is not legally required to treat hospital wastewater before discharging into the sewage system. Survival in the environmental conditions, including seawater especially in the warm period of the year up to 50 days, may also pose a potential epidemiological reservoir of carbapenem resistant genes. To the best of our knowledge, this is the first description of transmission of carbapenem-resistant *A. baumannii* through the hospital wastewater in this geographic area and monitoring of its survival in natural seawater. Our results suggest that the nosocomial pathogen *A. baumannii* is well adapted to different environments, not only to the hospital setting.

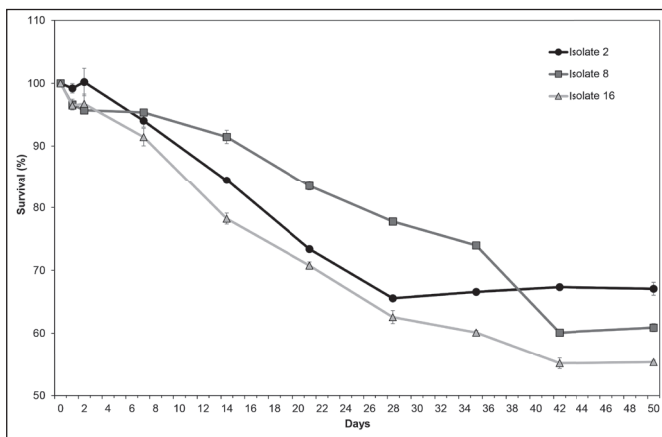


Fig. 4. Survival of *A. baumannii* wastewater isolates 2 and 8, and clinical isolate 16 in seawater during 50 days of monitoring. Initial log CFU/mL: 6.7 ± 0.1 isolate 2; 7.3 ± 0.1 isolate 8; 6.8 ± 0.1 isolate 16. Survival of bacteria was calculated as $((\log \text{CFU/mL}_{\text{time}} : \log \text{CFU/mL}_{\text{start}}) * 100)$, where $\log \text{CFU/mL}_{\text{time}}$ is number of bacteria on day of measurement and $\log \text{CFU/mL}_{\text{start}}$ is initial number of bacteria. Mean values and standard deviations are presented.

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Competing interests. None declared.

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