ACINETOBACTER BAUMANNII RECOVERED FROM TREATED MUNICIPAL WASTEWATER IS RELATED TO CLINICAL ISOLATES

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Background:

Over the last decade hospital-acquired infections due to *Acinetobacter baumannii* are increasing worldwide. Clinical isolates of *A. baumannii* are usually multi-drug resistant (MDR), with resistance to carbapenems increasing drastically in Croatia, from 10% in 2008 to 82% in 2014 [1]. The most important mechanism of carbapenem resistance in *A. baumannii* is the enzymatic hydrolysis mediated by oxacillinases encoded by bla_{OXA} genes [2].

Although *A. baumannii* has been isolated from patients and hospitals during outbreaks, how this pathogen is introduced into the hospital environment remains incompletely understood. Crucial questions regarding the epidemiology of *A. baumannii* are not known: are the infected patients and hospital environment the only sources of *A. baumannii*, at which extent *A. baumannii* are released from hospitals in nature, do they survive or even multiply in nature, do they have natural habitat outside hospitals.

Here we report the finding of MDR carbapenem resistant *A. baumannii* in treated municipal wastewater which is related to clinical isolates.

Material/methods:

The isolate was recovered from the effluent of the secondary type of wastewater treatment plant of the City of Zagreb (Fig. 1) where the municipal wastewater treated consists of domestic, industrial, hospital and storm wastewaters. The composite 24h sample of the effluent wastewater was collected on April 2014 in sterile glass bottle and analysed within 2h. The wastewater sample was concentrated on sterile membrane filters of pore size 0.45µm after dilution in sterile peptone water.

Figure 1: Municipal wastewater treatment plant of the City of Zagreb.

The isolation of *A. baumannii* from wastewater was performed at 42°C/48h on CHROMagar Acinetobacter with the addition of commercial supplement CR102 (CHROMagar) which allows the growth of carbapenem-resistant isolates. Cefsulodin sodium salt hydrate (Sigma-Aldrich) was added at 15 mg/L to suppress the growth of *Pseudomonas* and *Aeromonas* spp. Presumptive colony was recultivated (42°C/24h) on the same selective plate (Fig. 2) and then on Nutrient agar.

Figure 2: Pure culture of presumptive *A. baumannii* grown on CHROMagar Acinetobacter. Colonies were large, circular, convex, smooth, red with a paler central area.

Pure culture of presumptive *A. baumannii* was 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 was carried out by Vitek 2 system (BioMerieux). Final identification was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on cell extracts [3]. Recorded mass spectra were obtained by using Microflex LT (Bruker Daltonics) and processed with the MALDI Biotyper 3.0 software package. Molecular identification of isolate was performed by amplification of a fragment of *rpo*B gene encoding RNA polymerase β-subunit by using *rpo*B+1627/*rpo*B-2231 primer pair [4].

Antibiotic resistance was determined according to MIC values obtained by Vitek 2 system and E-test and interpreted according to EUCAST criteria [5]. The presence of bla_{OXA} genes was confirmed by multiplex PCR with specific primers for $bla_{OXA\text{-}51\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}23\text{-}like}$ and $bla_{OXA\text{-}58\text{-}like}$ genes [6]. Survival of isolate was monitored in the batch system containing the autoclaved effluent wastewater from which it was recovered.

Results:

By phenotypical analyses and Vitek 2 system the isolate named EF2 was determined as *A. calcoaceticus-baumannii* complex. MALDI-TOF MS analysis gave the reliable score value of 2.352 identifying it as *A. baumannii* (Table 1). Phylogenetic analysis of the *rpo*B gene fragment confirmed the identity as *A. baumannii* and showed 100% sequence ID to the clinical isolates (Fig. 3).

Isolate was susceptible to amikacin, trimethoprim-sulfamethoxazole and colistin, but resistant to carbapenems (imipenem, meropenem), fluoroquinolones (ciprofloxacin, levofloxacin), aminoglycosides (gentamicin, tobramycin) and therefore could be classified as MDR (Table 2).

Amplification of bla_{OXA} genes by multiplex PCR and sequencing confirmed the presence of intrinsic $bla_{OXA\text{-}51}$ and acquired $bla_{OXA\text{-}23}$ genes. Phylogenetic analyses of bla_{OXA} genes from environmental isolate showed association with those previously described from clinical isolates (Fig. 4).

Isolate multiplied in water up to 50 days of monitoring at 16.7°C when its number was 9% higher than initial number (Fig. 5).

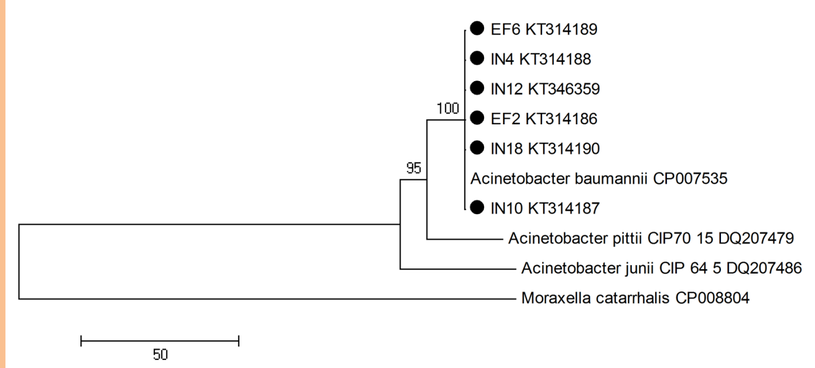

Figure 3: Phylogenetic tree (NJ method, number of differences) constructed on the basis of *rpo*B gene representing the molecular identification of *A. baumannii* isolate. GenBank accession numbers are given next to the name of each strain. *Moraxella catarrhalis rpo*B gene sequence was used as an outgroup to root the tree. Black dots represent the sequences of strains analysed in this study.

Table 1. Bruker Daltonik MALDI Biotyper classification results for analyte EF2.

Rank(Quality)	Matched Pattern	ScoreValue	NCBIIdentifier
1(+++)	Acinetobacter baumannii LMG 994 HAM	2.352	470
2(+++)	Acinetobacter baumannii ECII RUH_134 HCB	2.339	470
3(++)	Acinetobacter baumannii ECI RUH_875 HCB	2.224	470
4(++)	Acinetobacter baumannii ECIII RUH_5875 HCB	2.219	470
5(++)	Acinetobacter baumannii GEIH_2000 HCB	2.207	470
6(++)	Acinetobacter baumannii DSM 30011 DSM	2.201	470
7(++)	Acinetobacter baumannii 13101_1 CHB	2.031	470
8(+)	Acinetobacter baumannii DSM 30007T HAM	1.956	470
9(+)	Acinetobacter baumannii B389 UFL	1.745	470
10(-)	Acinetobacter baumannii DSM 30007T_QC DSM	1.674	470

Table 2. MIC values of tested antibiotics[a] for the *A. baumannii* isolate EF2. [a]MEM, meropenem; IPM, imipenem; LVX, levofloxacin; CIP, ciprofloxacin; TOB, tobramycin; GEN, gentamicin; AMK, amikacin; SXT, trimethoprim-sulfamethoxazole; CST, colistin.[R] resistant according to EUCAST criteria.

MIC values of antibiotics (mg/L)								
MEM	IPM	LVX	CIP	TOB	GEN	AMK	SXT	CST
>16[R]	>16[R]	4[R]	8[R]	8[R]	8[R]	4	20	0.25

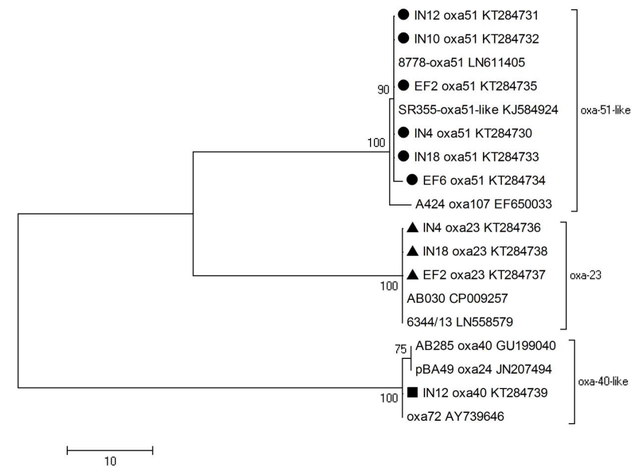

Figure 4: Unrooted phylogentic tree (NJ method, number of differences) constructed on the basis of bla_{OXA} genes encoding OXA-type carbapenemases. GenBank accession numbers are given next to the name of each strain. Gene sequences of $bla_{OXA\text{-}51}$ type are marked with black dots while the black triangles denote $bla_{OXA\text{-}23}$ type gene sequence.

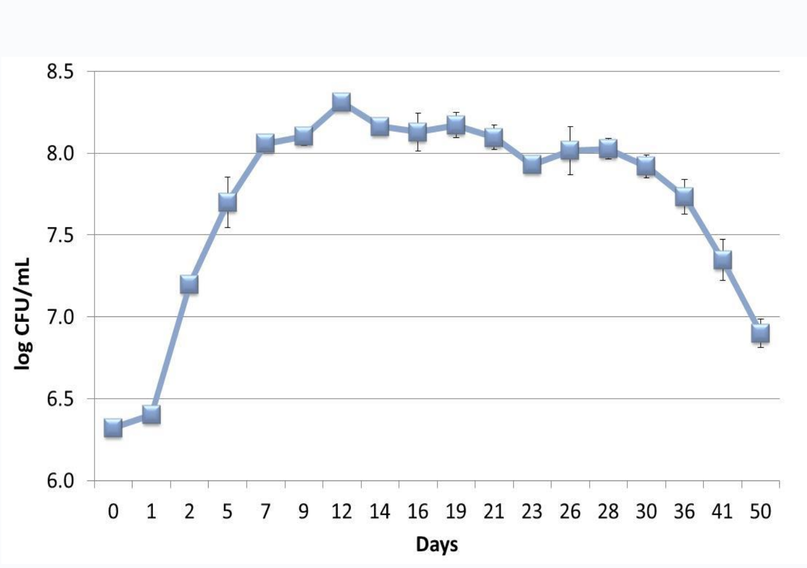

Figure 5: Survival of *A. baumannii* isolate EF2 recovered from effluent wastewater in the autoclaved effluent wastewater during 50 days. Average values and standard deviations of triplicate measurements are presented.

Conclusion:

MDR *A. baumannii* recovered from treated municipal wastewater is most probably of clinical origin and is able to survive in environment outside hospital.

References:

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