P 0257

OCCURRENCE OF *ACINETOBACTER CALCOACETICUS-BAUMANNII* COMPLEX IN MUNICIPAL WASTEWATER

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Background:

Acinetobacter calcoaceticus-baumannii complex (ABC) includes six genetically closely related but phenotypically undistinguishable species: *A. calcoaceticus, A. baumannii, A. pittii, A. nosocomialis,* genomic species "close to 13TU", and genomic species "between 1 and 3". Human diseases have been attributed mainly to *A. baumannii,* and to a lesser extent to other species of ABC. *A. baumannii* is most often studied as an emerging hospital pathogen causing outbreaks in Croatia since 2002 and is still present in Croatian hospitals. Clinical isolates of *A. baumannii* are usually multi-drug resistant. The carbapenem resistance of all clinical *A. baumannii* isolates monitored in Croatian hospitals dramatically increased in the last few years, from 10% in 2008 to 78% in 2013 [1].

Whereas pathogenic strains of ABC can be readily isolated from patients and hospital environmental sources during outbreaks, there are few data regarding its propagation in the natural environment. Reports on the occurrence of ABC in wastewater treatment plants are scarce. Multi-drug resistant isolates of *Acinetobacter* spp. were reported at each stage of the wastewater treatment processes in China [3,6].

The **objective** of this study was to screen the municipal wastewater of Zagreb, the capital of Croatia, for the presence of viable ABC and for its possible discharge into a natural recipient after the wastewater passage through the treatment process.

Methods:

The sampling was done at the secondary type of wastewater treatment plant of the City of Zagreb (capacity 1,200.000 population equivalents, Fig. 1) where the municipal wastewater treated consists of domestic, industrial, hospital and storm wastewaters.

The composite 24h samples of the influent and effluent wastewater were collected during 6 months in 2014. Wastewater was aseptically sampled in sterile 1L glass bottles and analysed within 2h. The wastewater samples were concentrated on sterile membrane filters of pore size 0.45µm after dilution in sterile peptone water.

Figure 1. Wastewater treatment plant of the City of Zagreb.

The isolation of ABC from wastewater was performed at 42°C/48h on CHROMagar Acinetobacter either without or 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 ABC colonies (Fig. 2) were recultivated (42°C/24h) on the same selective plates and then on Nutrient agar. Single ABC colonies were isolated from plates inoculated with 0.01–0.1mL of influent water and 0.1–1.0mL of effluent water.

Pure cultures of presumptive ABC 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 was carried out Vitek 2 systems (BioMerieux). Final identification of ABC was carried out by matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on cell extracts [4]. Recorded mass spectra were obtained by using Microflex LT (Bruker Daltonics) and processed with the MALDI Biotyper 3.0 software package. Molecular identification of ABC 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 [5].

Antibiotic resistance profiles were determined for carbapenems meropenem and imipenem by Vitek 2 system and interpreted according to EUCAST criteria [2].

Results:

On 6 sampling occasions 34 isolates belonging to ABC, as determined by Vitek 2 system, were recovered: 28 from influent and 6 from effluent wastewater (Table 1). The comparison of ribosomal proteins by MALDI-TOF MS with strains of bacteria in MALDI Biotyper database gave the score values form 2.013 - 2.409 identifying the ABC isolates as *A. baumannii* or *A. pittii*. Phylogenetic analysis of the *rpo*B gene fragment confirmed the identity of isolates as *A. baumannii* or *A. pittii* and showed their close relatedness to the clinical isolates with 100% sequence ID (Fig. 3). Molecular identification of ABC confirmed that 22 and 6 isolates of *A. baumannii* were recovered from influent and effluent wastewater, respectively, while 6 *A. pittii* were recovered only from influent wastewater. The 28/34 isolates (27 *A. baumannii* and 1 *A. pittii*) were resistant to carbapenems (Table 1).

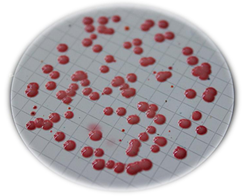

Figure 2. Presumptive ABC colonies grown on CHROMagar Acinetobacter. Colonies of ABC were large, circular, convex, smooth, red with a paler central area.

Table 1. Date of sampling of influent or effluent wastewater, MALDI-TOF MS score values of ABC isolates, and MIC values of carbapenems. Isolates named as IN were isolated from influent wastewater and isolates named as EF were isolated from effluent wastewater. All isolates were determined by Vitek 2 system as *A. calcoaceticus-baumannii* complex. [R] resistant; [I] intermediate according to EUCAST criteria.

Sampling date	Isolate name	MALDI TOF identification (score value)	MIC values of antibiotics (mg/L)	
			meropenem	imipenem
16.4.2014	**EF1**	*A. baumannii* (2.262)	>16[R]	>16[R]
	EF2	*A. baumannii* (2.352)	>16[R]	>16[R]
	EF3	*A. baumannii* (2.329)	>16[R]	>16[R]
7.5.2014	**IN1**	*A. pittii* (2.177)	0.25	<0.25
	IN2	*A. pittii* (2.156)	0.25	0.25
	IN3	*A. pittii* (2.222)	0.25	<0.25
11.6.2014	**IN4**	*A. baumannii* (2.231)	>16[R]	>16[R]
	IN5	*A. baumannii* (2.085)	>16[R]	>16[R]
	IN6	*A. baumannii* (2.157)	>16[R]	>16[R]
	IN8	*A. baumannii* (2.168)	>16[R]	>16[R]
	IN9	*A. baumannii* (2.167)	>16[R]	>16[R]
	IN10	*A. baumannii* (2.193)	>16[R]	>16[R]
	IN11	*A. baumannii* (2.409)	>16[R]	>16[R]
29.10.2014	**EF4**	*A. baumannii* (2.191)	>32[R]	>32[R]
	EF5	*A. baumannii* (2.161)	>16[R]	>16[R]
	EF6	*A. baumannii* (2.219)	>16[R]	>16[R]
	IN12	*A. baumannii* (2.190)	>16[R]	>16[R]
	IN13	*A. baumannii* (2.118)	>32[R]	>32[R]
	IN14	*A. baumannii* (2.213)	>16[R]	>16[R]
	IN15	*A. baumannii* (2.121)	>16[R]	>16[R]
	IN16	*A. baumannii* (2.244)	>16[R]	>16[R]
	IN17	*A. baumannii* (2.163)	32[R]	>32[R]
	IN18	*A. baumannii* (2.048)	>16[R]	>16[R]
	IN19	*A. baumannii* (2.090)	>16[R]	>16[R]
5.11.2014	**IN20**	*A. pittii* (2.291)	1.5	0.25
	IN21	*A. baumannii* (2.328)	0.25	0.25
3.12.2014	**IN22**	*A. baumannii* (2.118)	>16[R]	>16[R]
	IN23	*A. pittii* (2.013)	4[I]	0.50
	IN24	*A. baumannii* (2.168)	>16[R]	>16[R]
	IN25	*A. baumannii* (2.041)	> 16[R]	>16[R]
	IN26	*A. baumannii* (2.223)	32[R]	8[R]
	IN27	*A. baumannii* (2.199)	8[R]	2
	IN28	*A. baumannii* (2.085)	16[R]	8[R]
	IN29	*A. pittii* (2.094)	12[R]	8[R]

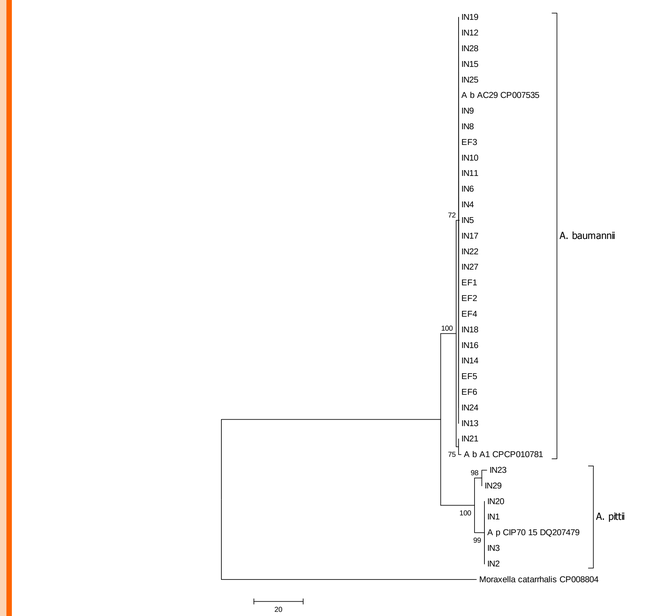

Figure 3. Phylogenetic tree (NJ method, number of differences) constructed on the basis of *rpoB* gene fragment sequence analysis representing the molecular identification of ABC isolates. *Moraxella catarrhalis rpoB* gene fragment was used as an outgroup to root the tree. GenBank accession numbers are given next to the names of reference strains.

Conclusion:

- Municipal wastewaters of Zagreb are continuously polluted with ABC probably due to the input of untreated hospital wastewaters.
- Among the ABC, only *A. baumannii* and *A. pittii* species are present with *A. baumannii* as a predominant species.
- More frequent isolation of *A. baumannii* from influent than from effluent suggests its moderate elimination, but also its persistence in the secondary type wastewater treatment system.
- The absence of *A. pittii* isolation from effluent suggests its complete elimination in the secondary type of wastewater treatment plant.

References:

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Acknowledgements:

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