

# Pan Drug-Resistant Environmental Isolate of *Acinetobacter baumannii* from Croatia

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*Acinetobacter baumannii* is an emerging nosocomial pathogen with also emerging resistance to different antibiotics. Multidrug and pan drug-resistant clinical isolates were reported worldwide. Here we report the first evidence of pan drug-resistant environmental isolate of *A. baumannii*. The isolate was recovered from the effluent of secondary treated municipal wastewater of the City of Zagreb, Croatia. The isolate was resistant to penicillins/ $\beta$ -lactamase inhibitors, carbapenems, fluoroquinolones, aminoglycosides, folate pathway inhibitors, and polymyxins, except intermediately susceptible to minocycline and tigecycline. Intrinsic chromosomally located *bla*<sub>OXA-51-like</sub> gene and acquired plasmid-located *bla*<sub>OXA-23-like</sub> gene were related to clinical isolates. Pan drug-resistant *A. baumannii* can occur in natural environments outside of the hospital. Secondary treated municipal wastewater represents a potential epidemiological reservoir of pan drug-resistant *A. baumannii* and carbapenem resistance gene.

**Keywords:** *Acinetobacter baumannii*, antibiotics, microbial drug resistance, public health, wastewater

## Introduction

MULTIDRUG-RESISTANT (MDR) *Acinetobacter baumannii* has emerged as one of the most common nosocomial pathogens worldwide, with the ability to survive under a wide range of environmental conditions, and to persist for extended periods of time on biotic and abiotic surfaces.<sup>1,2</sup> *A. baumannii* is considered an equivalent to methicillin-resistant *Staphylococcus aureus* and, therefore, called “Gram-negative MRSA.”<sup>3</sup> Increasing antimicrobial resistance leaves very few therapeutic options, especially in carbapenem-resistant *Acinetobacter* isolates that are reported worldwide. These isolates are susceptible only to polymyxins—peptide antibiotics that are not routinely used because of earlier reports about toxicities. Polymyxin-resistant *A. baumannii* occurred almost exclusively in patients who had received colistin for treatment of carbapenem-resistant, colistin-susceptible *A. baumannii* infections.<sup>4</sup> Pan drug-resistant clinical isolates that demonstrate resistance to all antimicrobial agents, including polymyxins, have also been reported in the literature, making treatment of these infections extremely difficult and in some cases impossible with fatal outcome.<sup>4,5</sup>

In Croatia, the first clinical carbapenem-resistant isolate of *A. baumannii* was recovered in 2002 in southern part of

Croatia.<sup>6</sup> Over the period of 7 years, carbapenem resistance in clinical isolates of *A. baumannii* did not exceed 30%, but since 2009, incidence of carbapenem resistance drastically increased, especially in two biggest clinical centers in northern and southern parts of Croatia.<sup>7</sup> Carbapenem resistance in *A. baumannii* has rapidly spread throughout Croatia, and in 2014 nonsusceptibility to carbapenems reached 82%.<sup>8</sup> This resulted in a change of treatment options for infections caused by MDR *A. baumannii*, foregrounding ampicillin/sulbactam and colistin as the only therapeutic choice. Recently published data confirmed the occurrence of environmental carbapenem-resistant isolates of *A. baumannii* related to clinical isolates in untreated and treated wastewater in Croatia.<sup>9</sup> However, to date there is no report on the occurrence of either clinical or environmental pan drug-resistant isolates of *A. baumannii* in Croatia. Here we report the first evidence of pan drug-resistant *A. baumannii* in treated wastewater in Zagreb, Croatia.

## Materials and Methods

The composite 24 hr sample of effluent wastewater was collected on September 9, 2015 at the secondary type municipal wastewater treatment plant of the City of Zagreb, Croatia.<sup>10</sup> The isolation of *A. baumannii* was performed at

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TABLE 1. MIC VALUES OF TESTED ANTIBIOTICS AGAINST ENVIRONMENTAL ISOLATE OF *ACINETOBACTER BAUMANNII*

Antimicrobial category	Antibiotic	MIC (mg/L)
Carbapenems	MEM	>16 <sup>a</sup>
	IMI	>16 <sup>a</sup>
Fluoroquinolones	CIP	>4 <sup>a</sup>
	LVX	>8 <sup>a</sup>
Aminoglycosides	TOB	>16 <sup>a</sup>
	GEN	>16 <sup>a</sup>
	AMK	>64 <sup>a</sup>
Tetracyclines	MIN	8 <sup>b</sup>
	TGC	2 <sup>b</sup>
Penicillins/ $\beta$ -lactamase inhibitors	SAM	>32 <sup>a</sup>
	TIM	>128 <sup>a</sup>
Folate pathway inhibitors	SXT	>320 <sup>a</sup>
Polymyxins	CST	>16 <sup>a</sup>

<sup>a</sup>Resistant.<sup>b</sup>Intermediate according to EUCAST and CLSI criteria.

AMK, amikacin; CIP, ciprofloxacin; CST, colistin; GEN, gentamicin; IMI, imipenem; LVX, levofloxacin; MEM, meropenem; MIC, minimum inhibitory concentration; MIN, minocycline; SAM, ampicillin/sulbactam; SXT, trimethoprim/sulfamethoxazole; TGC, tigecycline; TIM, ticarcillin/clavulanic acid; TOB, tobramycin.

42°C/48 hr on CHROMagar *Acinetobacter* (CHROMagar) supplemented with CR102 and 15 mg/L cefsulodin sodium salt hydrate (Sigma-Aldrich). Identification of isolate was performed by routine bacteriological techniques, Vitek 2 system (BioMerieux), and MALDI-TOF MS (software version 3.0, Microflex LT; Bruker Daltonics) on cell extracts.<sup>11</sup> Molecular identification was performed by amplification of a fragment of *rpoB* gene encoding RNA polymerase  $\beta$ -subunit by using *rpoB*+1627/*rpoB*-2231 primer pair.<sup>12</sup> Amplified fragments were sequenced, edited, and analyzed as described previously.<sup>9</sup>

Susceptibility testing was done by using Vitek 2 system and E-test, and minimum inhibitory concentrations (MICs) were interpreted according to EUCAST and CLSI criteria<sup>13,14</sup> for all antibiotics with defined breakpoints for *Acinetobacter* spp., except tigecycline where breakpoints for Enterobacteriaceae were used. The presence of genes of *bla*<sub>OXA</sub> lineage that encode OXA-type carbapenemases was searched by multiplex polymerase chain reaction (PCR) with specific primers for *bla*<sub>OXA-51-like</sub>, *bla*<sub>OXA-40-like</sub>, *bla*<sub>OXA-23-like</sub>, and *bla*<sub>OXA-58-like</sub> genes.<sup>15</sup>

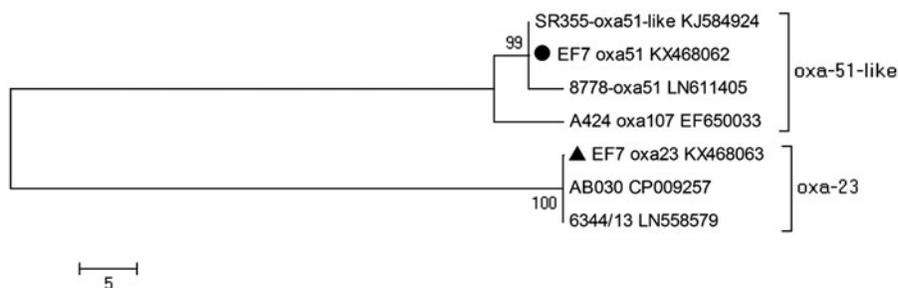
## Results and Discussion

The isolate of *A. baumannii* named EF7 was recovered from 0.5 ml of the secondary treated municipal wastewater

of the City of Zagreb. By phenotypical analyses and Vitek 2 system, the isolate was determined as *A. calcoaceticus*-*A. baumannii* complex. MALDI-TOF MS analysis gave the score value of 2.150, which indicates the secure genus and probable species identification. Phylogenetic analysis of the ~600 bp fragment of *rpoB* gene confirmed the identification of isolate as *A. baumannii* with 100% sequence identity (KX468061) to the reference sequence from GenBank (CP007535).

The isolate was resistant to all tested antibiotics (penicillins/ $\beta$ -lactamase inhibitors, carbapenems, fluoroquinolones, aminoglycosides, folate pathway inhibitors, and polymyxins) except intermediately susceptible to tetracyclines (Table 1), thus categorized as pan drug resistant.<sup>16</sup> The isolate remained intermediately susceptible to minocycline (MIC: 8 mg/L), which is not at market in Croatia, and tigecycline (MIC: 2 mg/L), which is recently present at the market in Croatia and is accepted as part of the combined treatment of MDR *Acinetobacter* infections. Environmental isolate EF7 showed very similar antibiotic resistance profile to pan drug-resistant clinical isolate of *A. baumannii* recovered in 2013 in Germany<sup>5</sup> with the exception of tigecycline resistance in the German isolate. The levels of antibiotic resistance of isolate EF7 were comparable with those of the clinical isolates of *A. baumannii* in Croatia,<sup>7,17</sup> with exception that no clinical isolates resistant both to ampicillin/sulbactam and colistin were registered. Ampicillin/sulbactam and colistin were used in recommended therapy in Zagreb's hospitals in recent years, including the period of wastewater sampling. It is possible that the pan drug resistance was developed in hospital and isolate EF7 was discharged into sewage system.

Multiplex PCR and sequencing confirmed the presence of both intrinsic chromosomally located *bla*<sub>OXA-51-like</sub> gene and acquired plasmid-located *bla*<sub>OXA-23-like</sub> gene. Sequences of *bla*<sub>OXA-51-like</sub> (KX468062) and *bla*<sub>OXA-23-like</sub> (KX468063) genes showed 100% identity with sequences from clinical isolates available in GenBank (Fig. 1), which suggest the potential origin of isolate EF7 in hospitalized patients or hospital wastewaters.<sup>18</sup> Untreated hospital wastewaters in Zagreb are directly discharged into sewage system, which is collected and treated at investigated central wastewater treatment plant. Removal of carbapenem-resistant bacteria and *A. baumannii* in the secondary wastewater treatment plant is moderate.<sup>10</sup> Thus, certain number of *A. baumannii* is released through the effluent wastewater into the natural recipient Sava River. In aquatic environmental conditions MDR *A. baumannii* has the potential to multiply and survive up to 50 days.<sup>10</sup> Therefore, the viable pan drug-resistant *A. baumannii* recovered from effluent wastewater could be easily spread through the natural water body, which could represent an environmental reservoir of this emerging



**FIG. 1.** Unrooted phylogenetic tree (NJ method, number of differences) constructed on the basis of *bla*<sub>OXA</sub> genes encoding OXA-type carbapenemases. GenBank accession numbers are given next to the name of each strain. Gene sequences of *bla*<sub>OXA-51</sub> type are marked with a black dot and of *bla*<sub>OXA-23</sub> type with a black triangle.

pathogen. Moreover, since the plasmid-located *bla*<sub>OXA-23-like</sub> gene in isolate EF7 may be self-transferable,<sup>19</sup> the effluent wastewater represents a potential epidemiological reservoir of the carbapenem resistance gene. The impact of secondary treated municipal wastewaters on the occurrence of community-acquired *A. baumannii* infections outside the hospital environment should be further investigated.

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### Compliance with Ethical Standards

This research fully complies with Ethical Standards applicable for this journal and the relevant national and international ethics-related rules and professional codes of conduct.

### Disclosure Statement

No competing financial interests exist.

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