EMERGENT HOSPITAL PATHOGEN IN NATURAL ENVIRONMENT OF CROATIA

Prof Jasna Hrenović

University of Zagreb, Faculty of Science, Department of Biology,
Division of Microbiology, Zagreb, Croatia

HrZZ
Croatian Science Foundation

Bacteria resistant to antibiotics are global problem of the 21st century.

E. faecium
S. aureus
K. pneumoniae
A. baumanii
P. aeruginosa
Enterobacter spp.

Bad Bugs, No Drugs: No ESKAPE! An Update from the Infectious Diseases Society of America FREE

Helen W. Boucher ✉, George H. Talbot, John S. Bradley, John E. Edwards, David Louis B. Rice, Michael Scheld, Brad Spellberg, John Bartlett

Clinical Infectious Diseases, Volume 48, Issue 1, 1 January 2009, Pages 1–12, https://doi.org/10.1086/595011

Published: 01 January 2009 **Article history** ▾

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ESKAPES: Emerging Pathogens of Concern

By: Nick Barsby, Pervinder Singh Johal
Edited by: Andrew Duong, Dr. Uyen Nguyen

Date : January 5, 2016

Carbapenems are used as a last resort antibiotic for treatment of infections caused by antibiotic-resistant isolates.

Development of bacterial resistance to carbapenems is of global concern.

World Health Organization

Media centre

WHO publishes list of bacteria for which new antibiotics are urgently needed

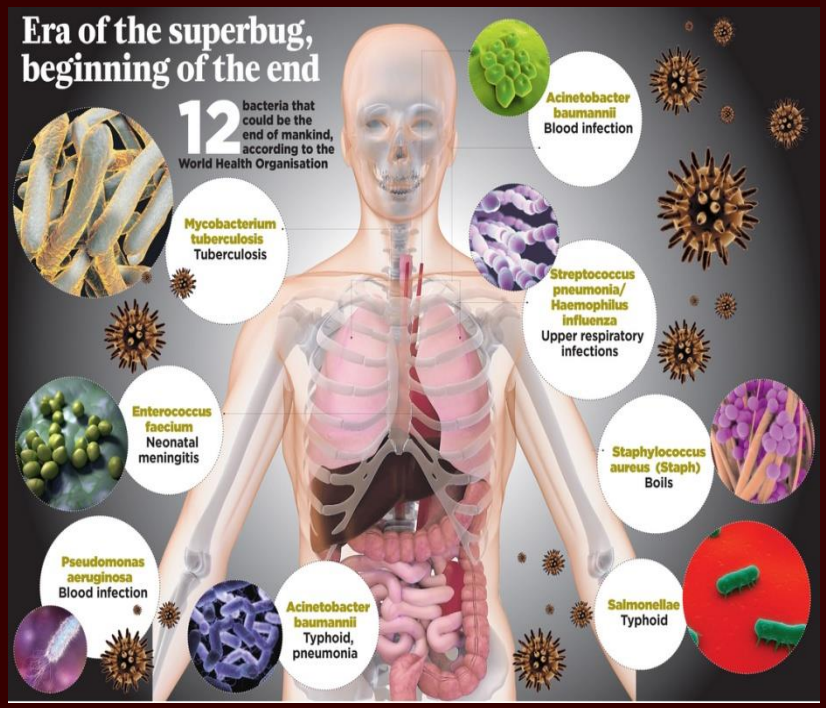
News release

27 FEBRUARY 2017 | GENEVA - WHO today published its first ever list of antibiotic-resistant "priority pathogens" – a catalogue of 12 families of bacteria that pose the greatest threat to human health.

The WHO priority list

PRIORITY: CRITICAL	PRIORITY 2: HIGH	PRIORITY 3: MEDIUM
Acinetobacter baumannii carbapenem-resistant	**Enterococcus faecium** vancomycin-resistant	**Streptococcus pneumoniae** penicillin-non-susceptible
Pseudomonas aeruginosa carbapenem-resistant	**Staphylococcus aureus** methicillin-resistant vancomycin-intermediate and resistant	**Haemophilus influenzae** ampicillin-resistant
Enterobacteriaceae carbapenem-resistant, ESBL-producing	**Helicobacter pylori** clarithromycin-resistant	**Shigella spp.** fluoroquinolone-resistant
	Campylobacter spp. fluoroquinolone-resistant	
	Salmonellae fluoroquinolone-resistant	
	Neisseria gonorrhoeae cephalosporin-resistant fluoroquinolone-resistant	

Source: WHO



Surveillance Atlas of Infectious Diseases

Antimicrobial resistance

Acinetobacter spp. | Carbapenems

Resistant (R) isolates proportion | 2016

Region	Resistant (R) isolates proportion (%)
Austria	12.3
Belgium	2.6
Bulgaria	74.8
Croatia	94.5
Cyprus	71.4
Czech Republic	1.8
Denmark	0.0
Estonia	-

https://ecdc.europa.eu/en/antimicrobial-resistance/surveillance-and-disease-data/data-ecdc

Carbapenem-resistance implies the resistance to other classes of antibiotics.

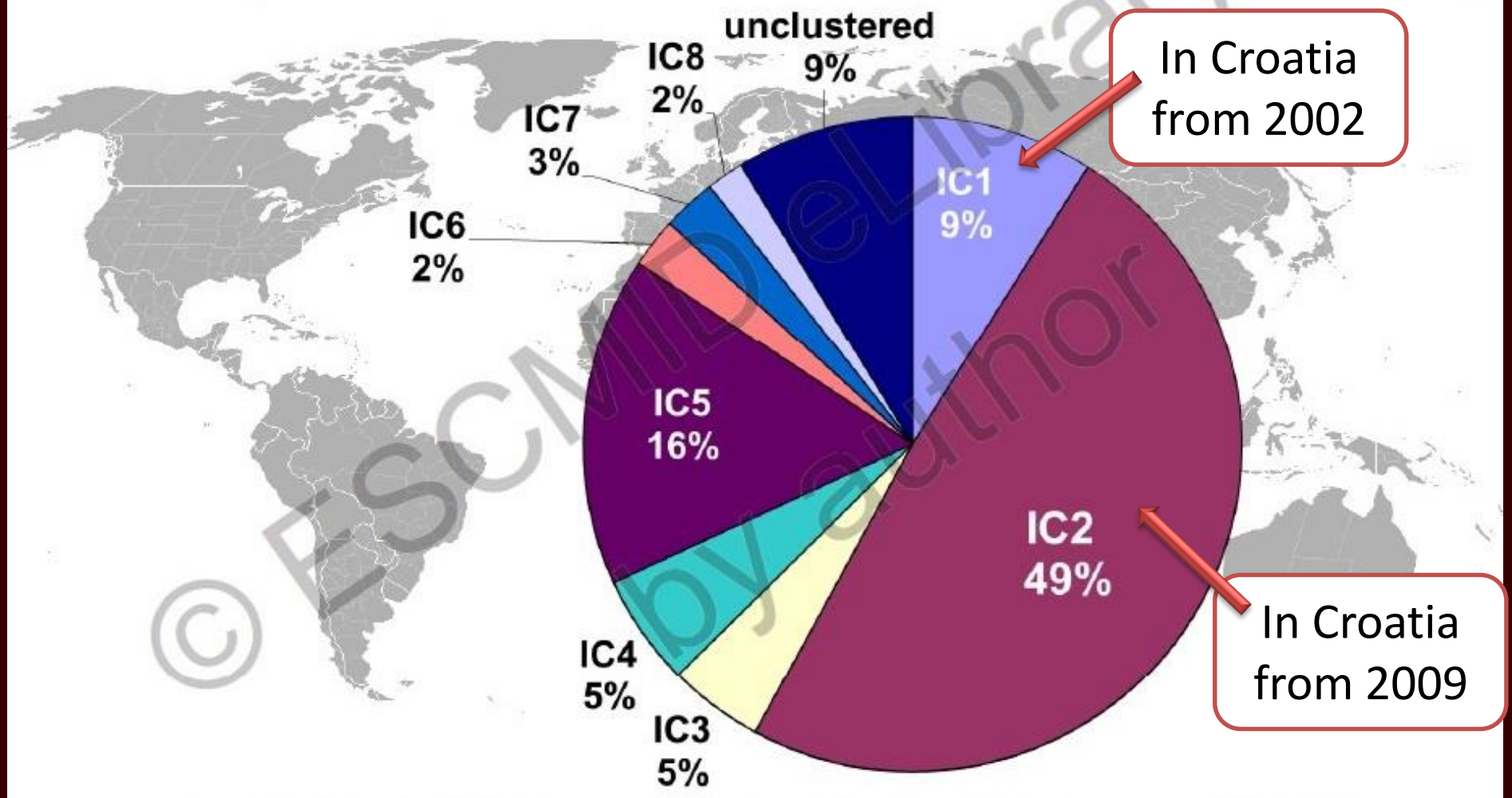
Groups of isolates according to their antibiotic susceptibility profile:
S (susceptible) - susceptible to all antibiotics;
MDR (multidrug-resistant) - non-susceptible to ≥1 agent in ≥3 antimicrobial categories;
XDR (extensively drug-resistant) - non-susceptible to ≥1 agent in all but ≤2 antimicrobial categories;
PDR (pandrug-resistant) - non-susceptible to all agents in all antimicrobial categories.

Clin Microbiol Infect. 2012 Mar;18(3):268-81. doi: 10.1111/j.1469-0691.2011.03570.x. Epub 2011 Jul 27.

Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance.

Magiorakos AP[1], Srinivasan A, Carey RB, Carmeli Y, Falagas ME, Giske CG, Harbarth S, Hindler JF, Kahlmeter G, Olsson-Liljequist B, Paterson DL, Rice LB, Stelling J, Struelens MJ, Vatopoulos A, Weber JT, Monnet DL.

Proportion of carbapenem resistant *A. baumannii* clustering with the International clonal lineages (N = 492)



Higgins PG et al. 2010. Global spread of carbapenem-resistant *Acinetobacter baumannii*. JAC 65:233-8

Genus *Acinetobacter* includes 57 species:

TABLE 1. Updated list of validated named species of *Acinetobacter*

Commonly found human pathogens

- *A. baumannii* (genospecies 2)
- *A. nosocomialis* (genospecies 13TU)
- *A. pittii* (genospecies 3)
- *A. calcoaceticus* (genospecies 1)

Emergent hospital pathogen of 21st century

Uncommon organisms in clinical infections

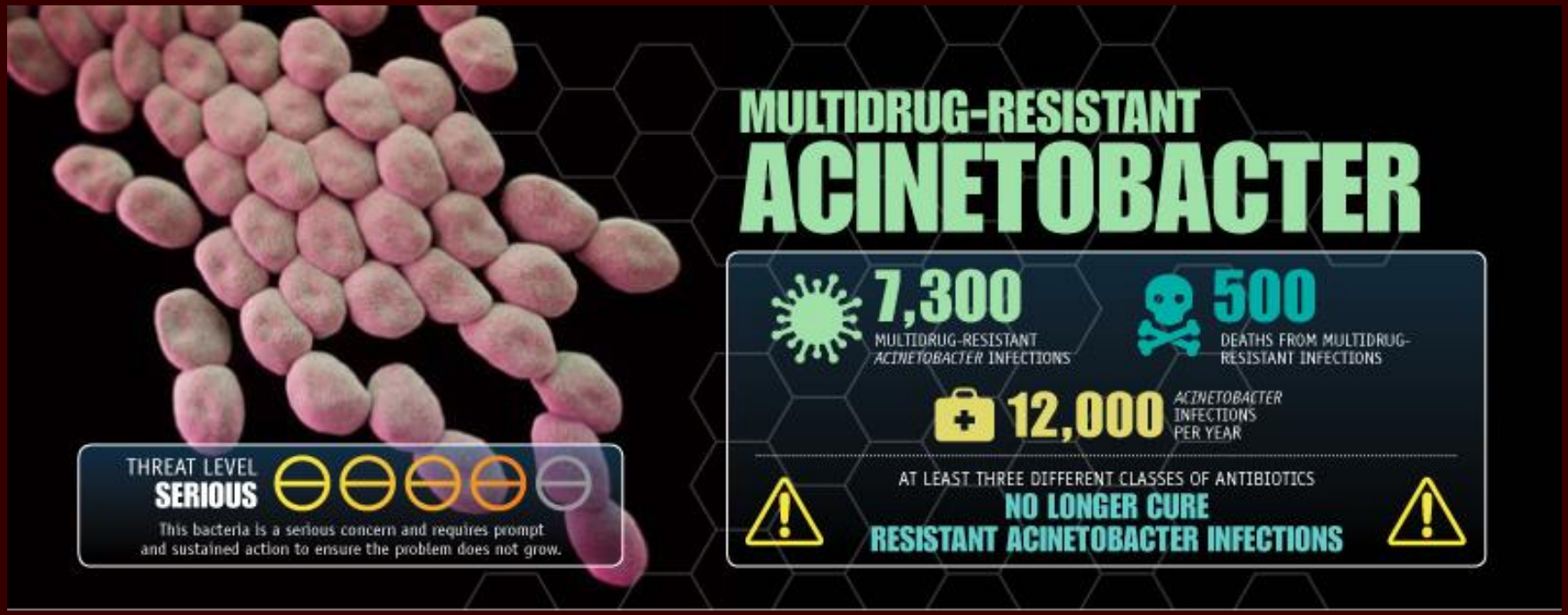
A. baylyi	*A. guillouiae*	*A. lwoffii*	*A. soli*
A. beijerinckii	*A. gyllenbergii*	*A. nectaris*	*A. tandoii*
A. bereziniae	*A. haemolyticus*	*A. parvus*	*A. tjernbergiae*
A. boissieri	*A. harbinensis*	*A. puyangensis*	*A. towneri*
A. bouvetii	*A. indicus*	*A. qingfengensis*	*A. ursingii*
A. brisouii	*A. johnsonii*	*A. radioresistens*	*A. venetianus*
A. gerneri	*A. junii*	*A. rudis*	
A. grimontii[a]	*A. kookii*	*A. schindleri*	

[a]Synonym of *A. junii*.

http://apps.szu.cz/anemec/Classification.pdf

Acinetobacter baumannii is a leading emerging pathogen of the 21st century, which is frequently recovered from patients during hospital outbreaks.

Acute community-acquired human infections suggest a source of this pathogen outside of the hospital settings.



Up to 2010 *A. baumannii* was considered as an exclusively hospital pathogen.

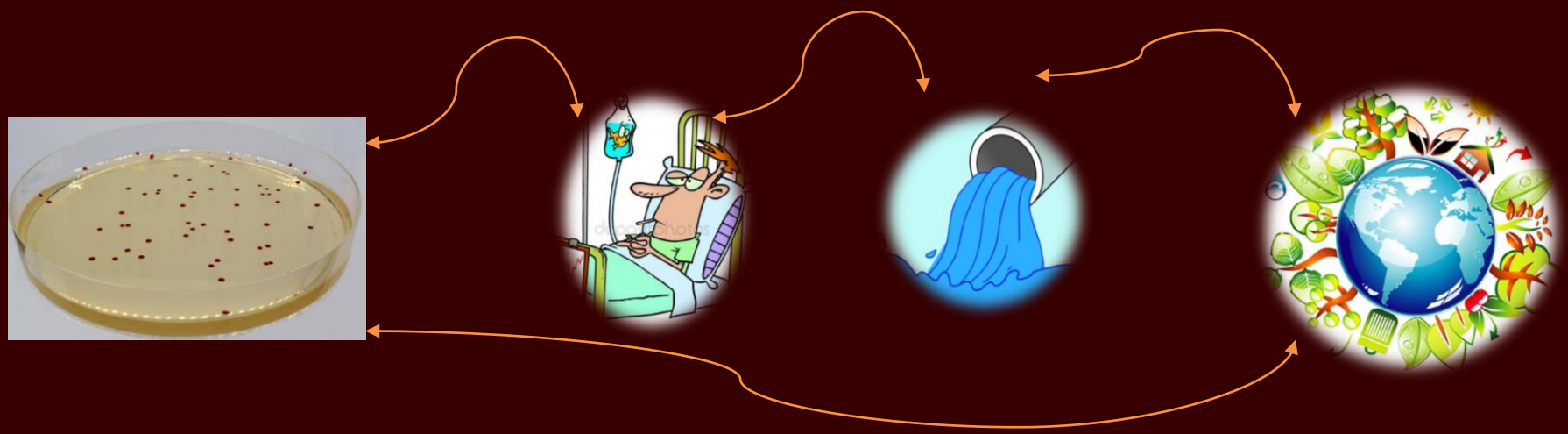
After 2010 onwards, there are reports on its occurrence outside hospital settings:

- Water of Seine River (2010) – 1 isolate
- Untreated hospital wastewater in Brazil (2011) – 3 isolates
- Untreated and chlorinated hospital wastewater in China (2013) – 9 and 1 isolate
- Natural environment in Croatia (2014 onwards)

The significance of environmental isolates in the epidemiology of *A. baumannii* is under a great concern worldwide.

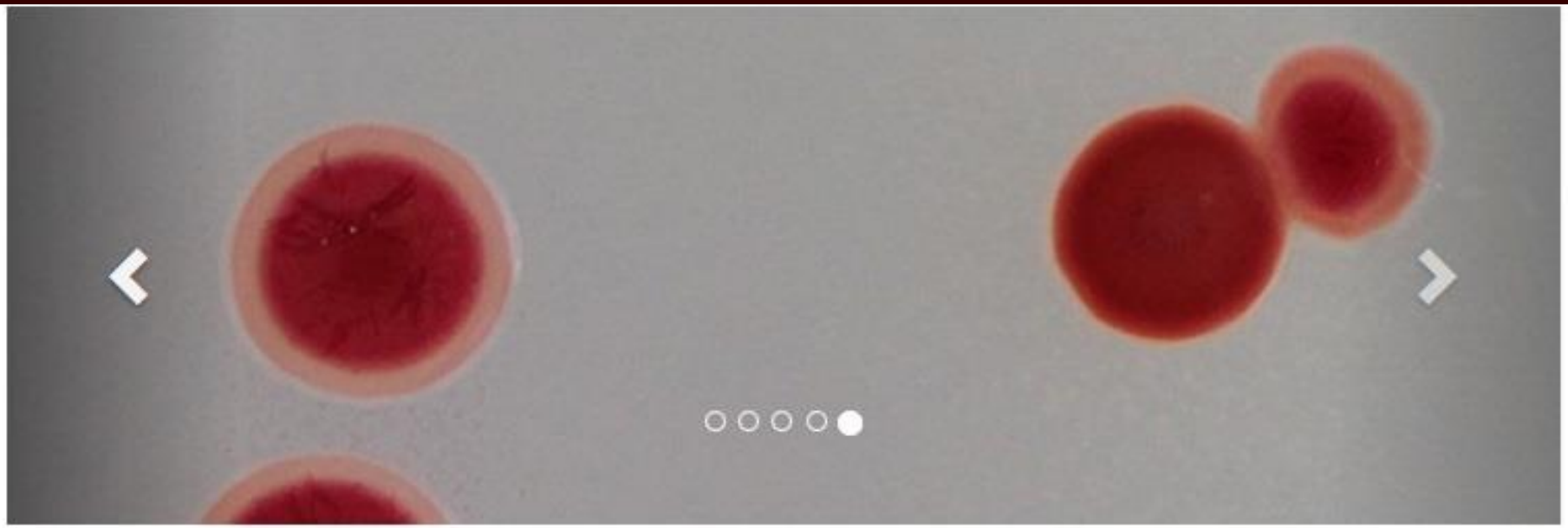
There is no clear evidence about:

- the way of introduction of *A. baumannii* into hospital environment,
- its propagation from hospital settings to the natural environment,
- its natural habitat outside hospitals.



Human waste is generally recognized as a source of different pathogens, which could be spread in natural environment, representing the public health risk.

Aim: the overview of the presence of *A. baumannii* in natural environment of Croatia.



Natural habitat of clinically important Acinetobacter baumannii

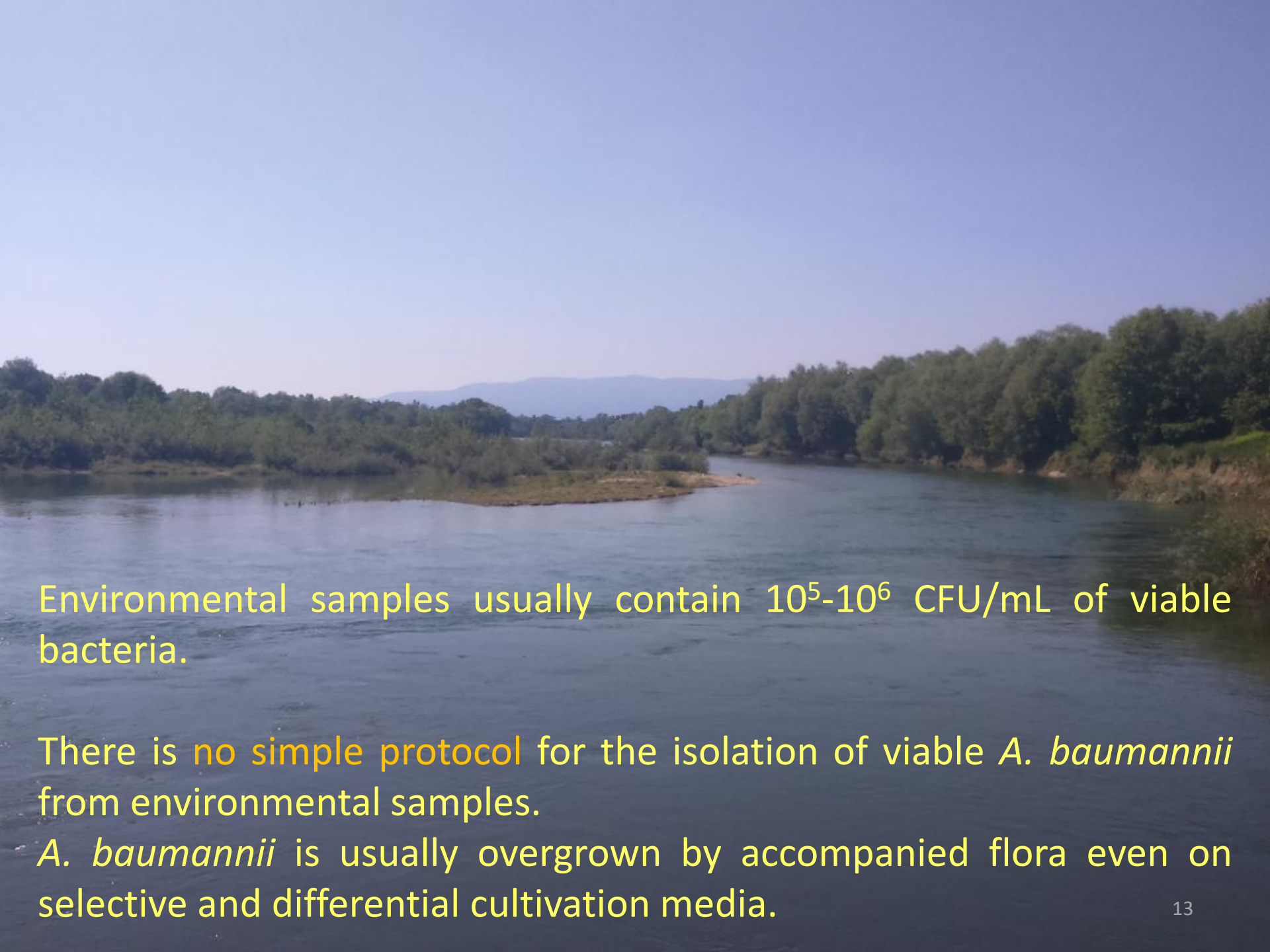
Funding source: Croatian Science Foundation

Duration: 01. 09. 2015 – 31. 08. 2019

Principal investigator: **Prof. Dr. Jasna Hrenović**

Budget: 999,210.00 HRK

Project no.: IP-2014-09-5656



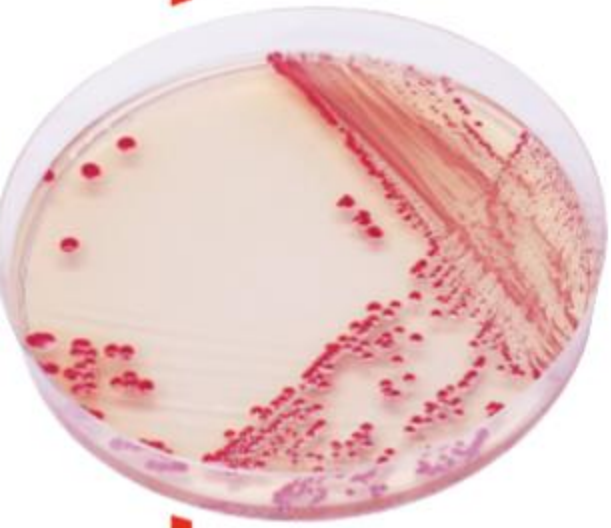
Environmental samples usually contain 10^5-10^6 CFU/mL of viable bacteria.

There is no simple protocol for the isolation of viable *A. baumannii* from environmental samples.

A. baumannii is usually overgrown by accompanied flora even on selective and differential cultivation media.

CHROMagar™ Acinetobacter

www.CHROMagar.com



For detection of *Acinetobacter* and MDR *Acinetobacter* sp.

Background

Common bacteria widely spread in the nature, *Acinetobacter* has the capacity to survive in dry as well as moist environments. It becomes a source of infection in hospital environment when colonizing medical equipments, human skin and sometimes foodstuff. *Acinetobacter* species are generally not pathogenic for healthy people but are life threatening in compromised patients. It is often isolated in nosocomial infections cases, intensive care units, and can for instance cause nosocomial pneumonia, bacteraemia, and meningitis.

Especially, *Acinetobacter baumannii* is becoming a major hospital-acquired infection issue because of its often multi-drug resistance (MDR : resistance to C3G, quinolones, carbapenem etc). This contributes to the increase of morbidity and mortality.
Active surveillance is necessary to control its spread in the facilities, to reduce the risk of cross-contamination, and to identify the carriers. Rapid identification of patients that are colonized with *Acinetobacter* would lead to infection control practices aimed at preventing spread of the organisms.

Plate Reading

For detection of *Acinetobacter* sp.:

- *Acinetobacter* sp.
→ red
- Other gram (-)
→ blue or mostly inhibited
- Gram(+) bacteria and yeasts
→ inhibited

For detection of MDR *Acinetobacter sp.* (if using the optional supplement CR102):
- MDR *Acinetobacter*
→ red

Medium Performance

1. **One unique Red colour:** Detection of *A. baumanii* from traditional culture media might be a difficult and tedious task due to the abundance of background flora found in collected specimens, especially when using media based on differentiation by the lactose/non-lactose fermentation ability. To overcome these difficulties, CHROMagar Acinetobacter was designed as a highly selective medium, allowing the growth of *Acinetobacter* in conspiciously red colonies, after overnight incubation.

Intrinsic carbapenem-resistance among clinically relevant species:

Table 2. Intrinsic resistance in non-fermentative Gram-negative bacteria. Non-fermentative Gram-negative bacteria are also generally intrinsically resistant to benzylpenicillin, first and second generation cephalosporins, glycopeptides, fusidic acid, macrolides, lincosamides, streptogramins, rifampicin, daptomycin and linezolid

Rule no.	Organisms	Ampicillin	Amoxicillin-Clavulanic acid	Ampicillin-sulbactam	Ticarcillin	Ticarcillin-clavulanic acid	Piperacillin	Piperacillin-tazobactam	Cefazolin, Cefalothin Cefalexin, Cefadroxil	Cefotaxime	Ceftriaxone	Ceftazidime	Cefepime	Aztreonam	Ertapenem	Imipenem	Meropenem	Ciprofloxacin	Chloramphenicol	Aminoglycosides	Trimethoprim	Fosfomycin	Tetracyclines	Tigecycline	Polymyxin B/Colistin
2.1	*Acinetobacter baumannii*, *Acinetobacter pittii*, *Acinetobacter nosocomialis* and *Acinetobacter calcoaceticus* complex	R	R	Note[1]					R	R	R			R	R						R	R	R[2]	Note[2]	
2.2	*Achromobacter xylosoxydans*	R							R	R	R				R										
2.3	*Burkholderia cepacia* complex[3]	R	R	R	R	R	R	R	R	R	R			R	R			R	R	R[4]	R	R			R
2.4	*Elizabethkingia meningoseptica*	R	R	R	R	R	R		R	R	R	R	R	R	R	R	R								
2.5	*Ochrobactrum anthropi*	R	R	R	R	R	R	R	R	R	R	R	R	R	R										
2.6	*Pseudomonas aeruginosa*	R	R	R					R	R	R				R										
2.7	*Stenotrophomonas maltophilia*	R	R	R	R		R	R	R	R	R			R	R	R	R								

Growth at 37°C, but not 42°C

R = resistant

[1] *Acinetobacter baumannii* may appear to be susceptible to ampicillin-sulbactam due to activity of sulbactam with this species.

[2] *Acinetobacter* is intrinsically resistant to tetracycline and doxycycline but not to minocycline and tigecycline.

[3] *Burkholderia cepacia* complex includes different species. Some strains may appear susceptible to some β-lactams *in vitro* but they are clinically resistant and are shown as R in the table.

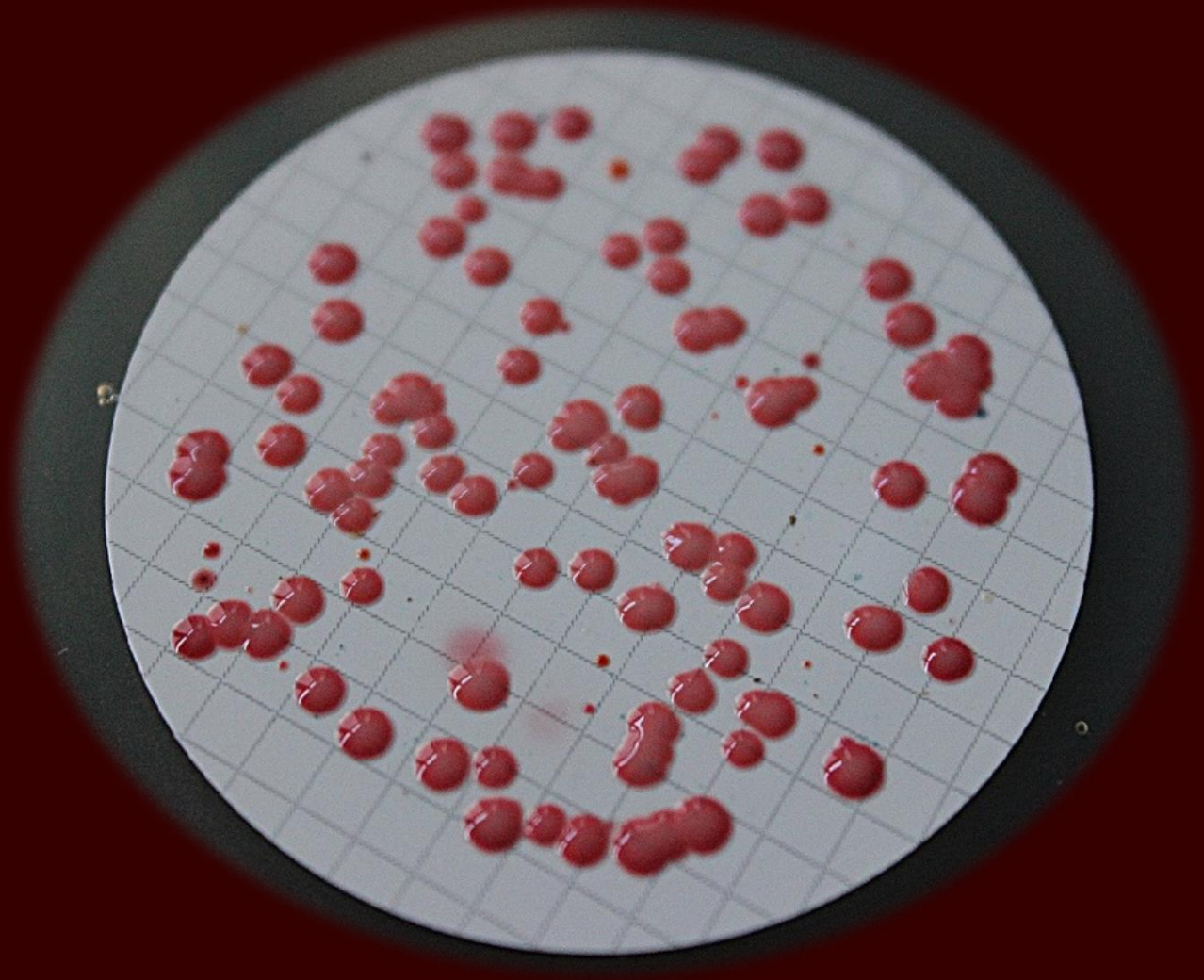
[4] *Burkholderia cepacia* and *Stenotrophomonas maltophilia* are intrinsically resistant to all aminoglycosides. Intrinsic resistance is attributed to poor permeability and putative efflux. In addition, most *Stenotrophomonas maltophilia* produce the AAC(6')Iz enzyme.

[5] *Pseudomonas aeruginosa* is intrinsically resistant to kanamycin and neomycin due to low level APH(3')-IIb activity.

[6] *Stenotrophomonas maltophilia* typically is susceptible to trimethoprim-sulfamethoxazole, but resistant to trimethoprim alone.

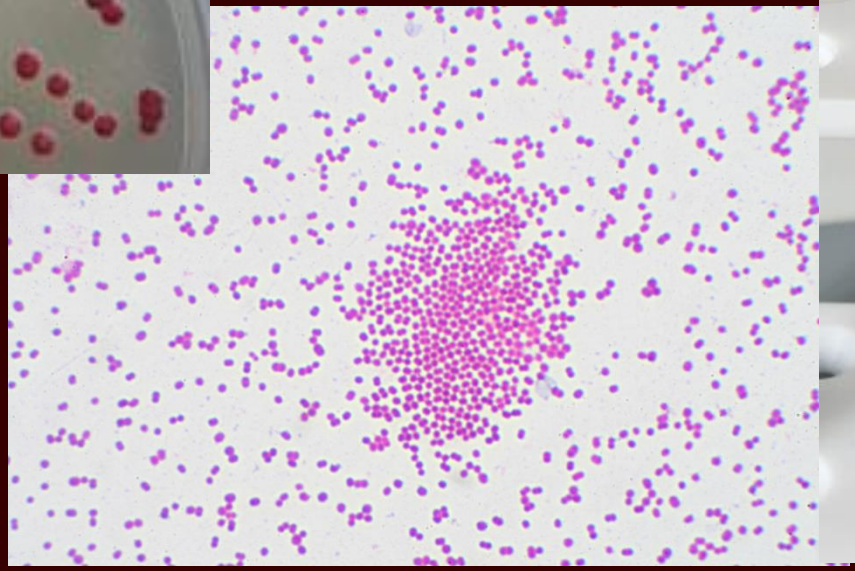
[7] *Stenotrophomonas maltophilia* is intrinsically resistant to tetracycline but not to doxycycline, minocycline and tigecycline.

The recovery of *A. baumannii* was performed on commercial CHROMagar Acinetobacter supplemented with 15 mg/L of cefsulodin sodium salt hydrate after incubation at 42°C/48h.



Identification of environmental isolates I

Phenotypically by routine bacteriological techniques: Gram negative coccobacilli, with typical negative reaction on the Kligler Iron Agar, oxidase negative, catalase positive.



Identification of environmental isolates II

- ✓ Matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) on cell extracts

AnalyteName	AnalyteID	Organism(best match)	ScoreValue	Organism(second best match)	ScoreValue
B1(++) (A)	Š 2/6	Acinetobacter baumannii	2.232	Acinetobacter baumannii	2.195
B2(++) (A)	Š 2/5	Acinetobacter baumannii	2.067	Acinetobacter baumannii	2.046
B3(++) (A)	OB 3929	Acinetobacter baumannii	2	Acinetobacter baumannii	1.978
B4(++) (A)	Š 2/7	Acinetobacter baumannii	2.102	Acinetobacter baumannii	2.048
B5(++) (A)	Š 2/10	Acinetobacter baumannii	2.231	Acinetobacter baumannii	2.191

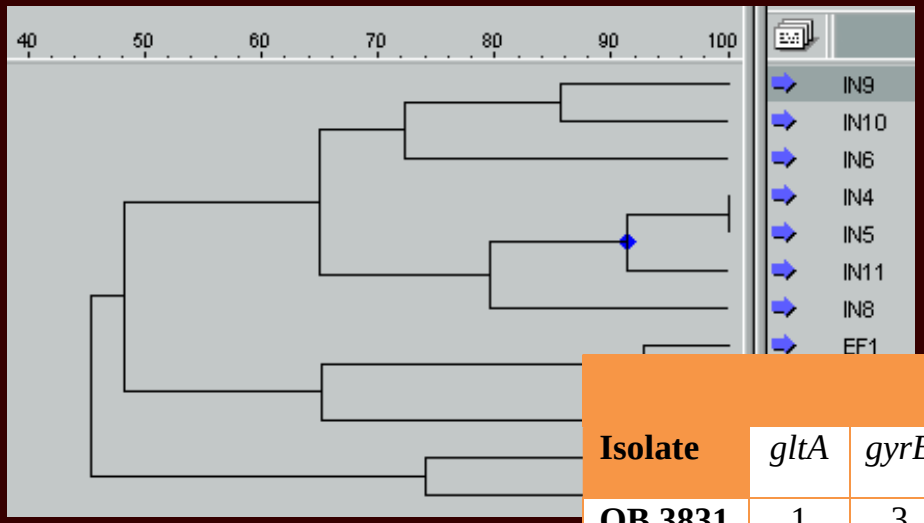
Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	not reliable identification	(-)	red

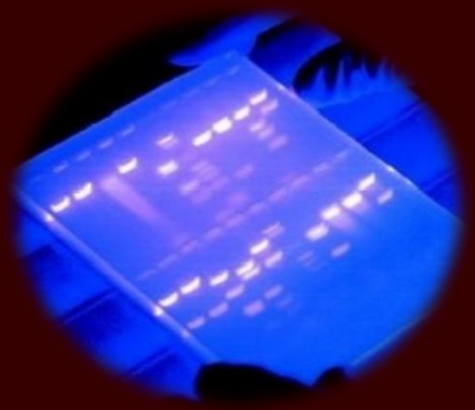
Identification of environmental isolates III

- ✓ amplification and sequencing of *rpoB* gene

Genetic relationship of environmental and clinical isolates

- ✓ PFGE (Pulsed field gel electrophoresis)
- ✓ MLST (Multilocus sequence typing) analysis of seven housekeeping genes (*cpn60, fusA, gltA, pyrG, recA, rplB,* and *rpoB*)



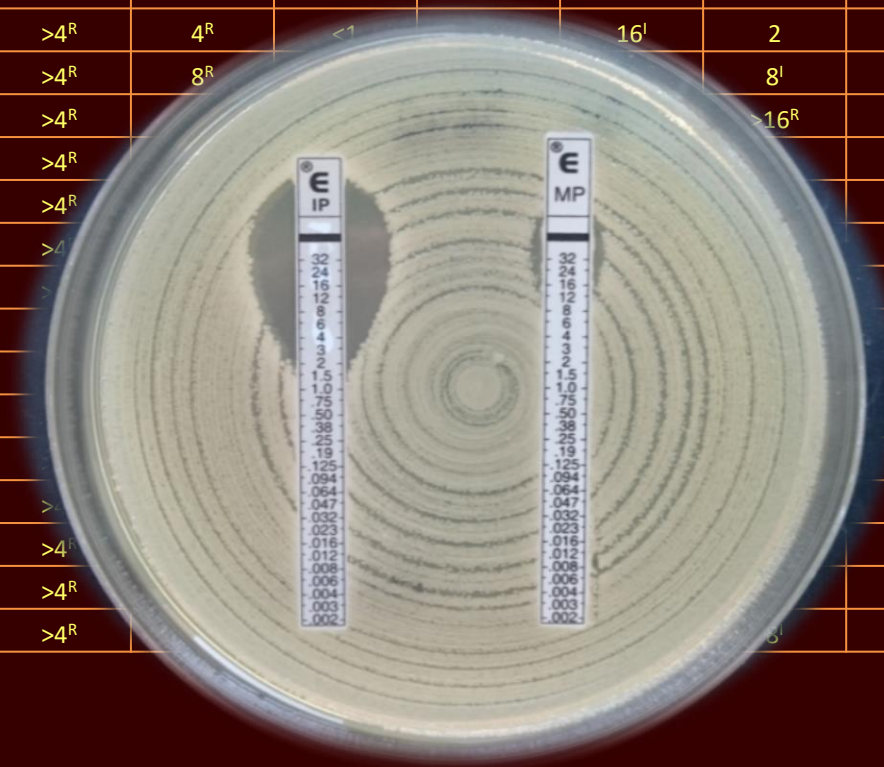


Isolate	Gene locus/allele							Sequence type	Clonal complex	IC type
	gltA	*gyrB*	*gdhB*	*recA*	*cpn60*	*gpi*	*rpoD*			
OB 3831	1	3	3	2	2	96	119 [a]	1421 [a]	92	2
OB 3929	1	3	3	2	2	96	3	195	92	2
OB 3930	1	3	3	2	2	100	3	425	92	2
OB 4027	1	3	3	2	2	96	119 [a]	1421 [a]	92	2
OB 4138	1	3	3	2	2	96	3	195	92	2
S2/1	1	3	3	2	2	96	3	195	92	2
S2/2	1	3	3	2	2	96	3	195	92	2
IN32	1	3	3	2	2	96	3	195	92	2

Antibiotic resistance profile I

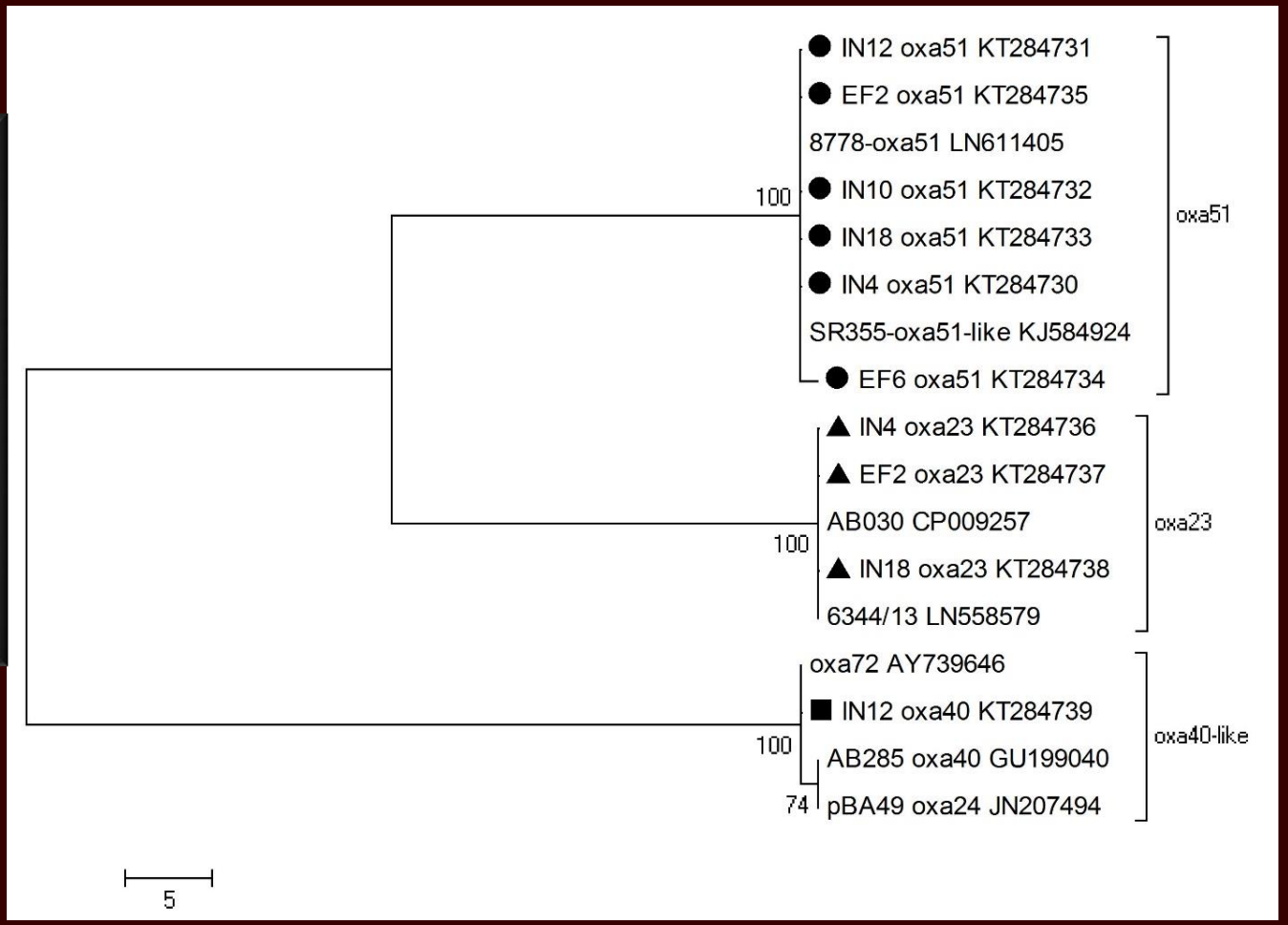
- ✓ Vitek2 system, E-test and broth microdilution
- ✓ interpretation according to EUCAST and CLSI criteria for clinical isolates of *A. baumannii*

Isolate	MIC values of antibiotics (mg/L)											
	MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST
OB 3831	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	8^{I}	16^{I}	128^{R}	>320^{R}	<0.5
OB 3929	>16^{R}	>16^{R}	>4^{R}	4^{R}	>16^{R}	>16^{R}	>64^{R}	2	16^{I}	128^{R}	>320^{R}	<0.5
OB 3930	>16^{R}	>16^{R}	>4^{R}	4^{R}	<1		16^{I}	2	16^{I}	128^{R}	>320^{R}	<0.5
OB 4027	>16^{R}	>16^{R}	>4^{R}	8^{R}				8^{I}	4	>128^{R}	>320^{R}	<0.5
OB 4138	>16^{R}	>16^{R}	>4^{R}					>16^{R}	16^{I}	128^{R}	<20	<0.5
S2/1	>16^{R}	>16^{R}	>4^{R}						8	128^{R}	>320^{R}	<0.5
S2/2	>16^{R}	8^{I}	>4^{R}						<2	128^{R}	>320^{R}	>16^{R}
S2/3	>16^{R}	>16^{R}	>4						8	>128^{R}	>320^{R}	<0.5
S2/4	8^{I}	>16^{R}							4	64^{I}	>320^{R}	>16^{R}
S1/1	>16^{R}	>16^{R}							<2	>128^{R}	>320^{R}	<0.5
S2/5	>16^{R}	>16^{R}							>32^{R}	>128^{R}	<20	<0.5
S2/6	>16^{R}	>16^{R}							>32^{R}	>128^{R}	<20	<0.5
S2/7	>16^{R}	>16^{R}							>32^{R}	>128^{R}	<20	<0.5
S2/8	>16^{R}	>16^{R}	>						>32^{R}	>128^{R}	<20	<0.5
S2/9	>16^{R}	>16^{R}	>4						16^{I}	>128^{R}	160^{R}	<0.5
S2/10	8^{I}	>16^{R}	>4^{R}						4	64^{I}	>320^{R}	>16^{R}
IN32	>16^{R}	>16^{R}	>4^{R}						16^{I}	128^{R}	>320^{R}	<0.5



Antibiotic resistance profile II

- ✓ In carbapenem-resistant isolates the acquired oxacillinases: $bla_{OXA\text{-}23\text{-}like}$, $bla_{OXA\text{-}40\text{-}like}$, $bla_{OXA\text{-}58\text{-}like}$, $bla_{OXA\text{-}143\text{-}like}$ are searched by multiplex PCR



A single isolate of *A. baumannii* was incidentally recovered in the abandoned quarry near City of Pula, from 0.1g of acid paleosol (pH=2.55) influenced by illegally disposed solid waste.

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Occurrence of an Environmental *Acinetobacter baumannii* Strain Similar to a Clinical Isolate in Paleo

Jasna Hrenovic,[a] Goran Durn,[b] Ivana Goic-Barisic,[c] Ana Kovacic[d]

University of Zagreb, Faculty of Science, Division of Biology, Zagreb, Croatia[a]; University
Croatia[b]; Department of Clinical Microbiology, University Hospital Centre Split and Univ
and Dalmatia County, Split, Croatia[d]

**Over the past decade, bacteria of the genus *Acinetobacter* have eme
breaks of *Acinetobacter* infections are considered to be caused excl
ronments. The natural habitats of clinically important multiresist
report an incidental finding of a viable multidrug-resistant strain
paleosol from Croatia. The environmental isolate of *A. baumannii*
hospital in this geographic area and was resistant to gentamicin, t
cin. In paleosol, the isolate was able to survive a low pH (3.37), des
of *A. baumannii* in paleosol is illegally disposed waste of external
The bacteria could have been leached from waste by storm water a**

Bacteria of the genus *Acinetobacter* have been recognized as significant hospital pathogens since the late 1970s, but at that time they were easily treated, because they were susceptible to commonly used antimicrobials. *Acinetobacter* spp. have an increasing ability to develop resistance to commonly used antimicrobial agents, leading to limited options for antibiotic treatment (1). Three major overlapping populations of bacteria of the genus *Acinetobacter* are known: multiresistant isolates from hospitals and hospitalized patients (*Acinetobacter baumannii*, *Acinetobacter*

Minimum inhibitory concentration (MIC) values of tested antibiotics[a] against environmental isolate of *A. baumannii*.

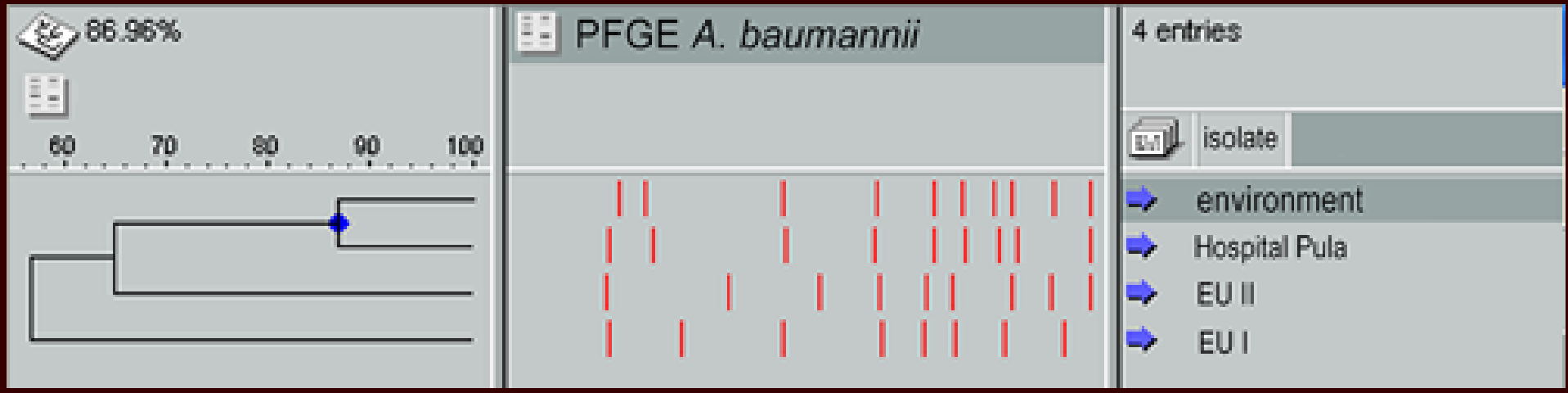
[a] carbapenems (MEM-meropenem, IPM-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), penicillins/β-lactamase inhibitors (SAM-ampicillin/sulbactam), folate pathway inhibitors (SXT- trimethoprim/sulfamethoxazole), polymyxins (CST-colistin). [R] – resistant according to EUCAST and CLSI criteria.

Isolate	MIC values of antibiotics (mg/L)									
	MEM	IPM	CIP	LVX	TOB	GEN	AMK	SAM	SXT	CST
Paleosol	≤0.5	≤0.5	≥4[R]	4[R]	≤1	>16[R]	2	4	160[R]	≤0.5

Multidrug-resistance (MDR) to fluoroquinolones, gentamicin and trimethoprim-sulfamethoxazole

MDR *A. baumannii* from paleosol is related to a clinical isolate from hospital in Pula.

Probable source: illegally disposed solid waste.



Three isolates of *A. baumannii* were recovered from 0.01-1g of technosol at a dump site Sovjak situated above City of Rijeka in a karst pit.

Sci Total Environ. 2017 Dec 31;607-608:1049-1055. doi: 10.1016/j.scitotenv.2017.07.108. Epub 2017 Jul 27.

Extensively and multi drug-resistant Acinetobacter baumannii recovered from technosol at a dump site in Croatia.

Hrenovic J[1], Durn G[2], Music MS[1], Dekic S[1], Troskot-Corbic T[3],

⊕ Author information

Abstract

In a karst pit above City of Rijeka in Croatia the hazard
was periodically used as an illegal dump site. The surfa
geochemically and bacteriologically. From the technos
Acinetobacter baumannii were recovered. Isolates from
isolates: the affiliation to IC1 and 2, multi-drug resistan
carbapenem resistance mediated by bla_{OXA72} and bla
isolates were able to survive in contact with technosol
technosol was the illegally disposed hospital waste. Pr
the spread of clinically important A. baumannii in natur

KEYWORDS: Bacteria; Environment; Hydrocarbons; Tar; Waste

PMID: 28724243 DOI: 10.1016/j.scitotenv.2017.07.108

Minimum inhibitory concentration (MIC) values of tested antibiotics[a] against environmental isolates of *A. baumannii*. [R] - resistant, [I] - intermediate according to EUCAST and CLSI criteria.

[a] carbapenems (MEM-meropenem, IMI-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), tetracyclines (MIN-minocycline), penicillins/β-lactamase inhibitors (SAM-ampicillin/sulbactam, TIM-ticarcillin/clavulanic acid), folate pathway inhibitors (SXT- trimethoprim/sulfamethoxazole), polymyxins (CST-colistin).

Isolate	MALDI TOF score value	MIC values of antibiotics (mg/L)											
		MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST
Sovjak1	2.036	≥16[R]	≥16[R]	≥4[R]	4[R]	≤1	≤1	32[R]	≤1	16[I]	≥128[R]	≤20	≤0.5
Sovjak2	2.086	≥16[R]	≥16[R]	≥4[R]	4[R]	≤1	≤1	16[I]	≤1	16[I]	≥128[R]	≤20	≤0.5
Sovjak3	2.000	≥16[R]	≥16[R]	≥4[R]	4[R]	≤1	≤1	>64[R]	8[I]	16[I]	≥128[R]	≥320[R]	≤0.5

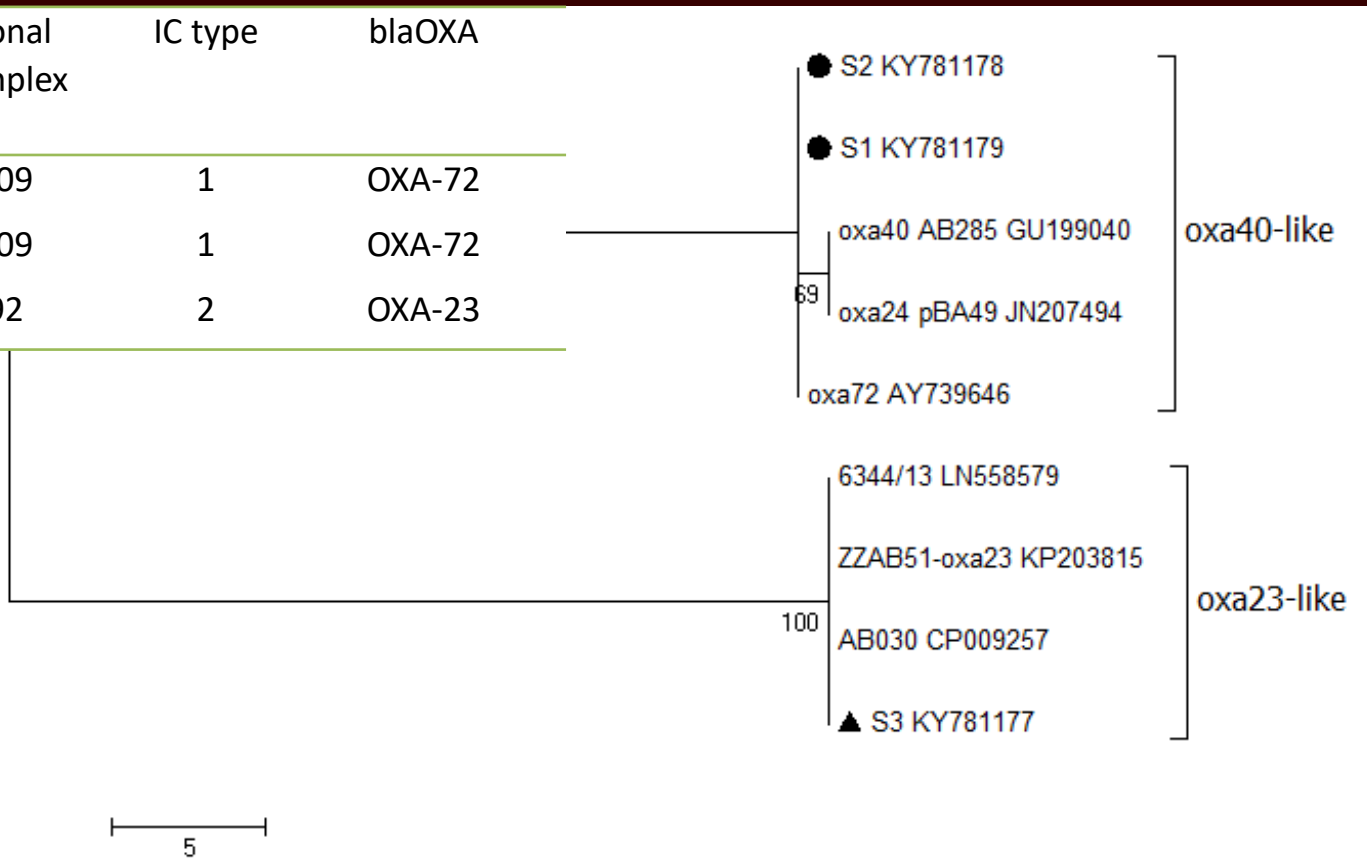
Two isolates (Sovjak 1, 2) multidrug-resistant (MDR)
One isolate (Sovjak 3) extensively drug-resistant (XDR)

A. baumannii from technosol share features characteristic for clinical isolates:

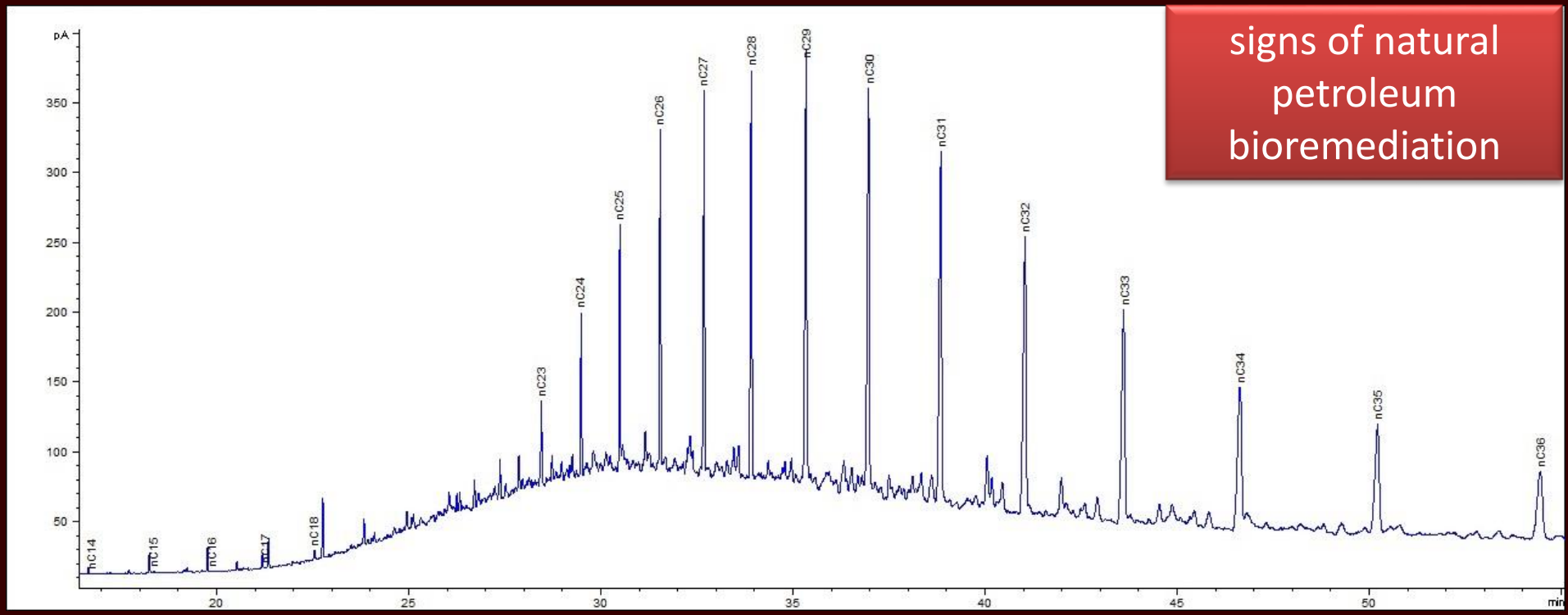
- MDR/XDR antibiotic resistance profile
- Affiliation to IC1 and 2
- Resistance to carbapenems mediated by acquired bla_{OXA72} and bla_{OXA23} genes

Izolat	Sequence type	Clonal complex	IC type	blaOXA
Sovjak 1	231	109	1	OXA-72
Sovjak 2	231	109	1	OXA-72
Sovjak 3	195	92	2	OXA-23

Probable source: illegally disposed hospital solid waste.



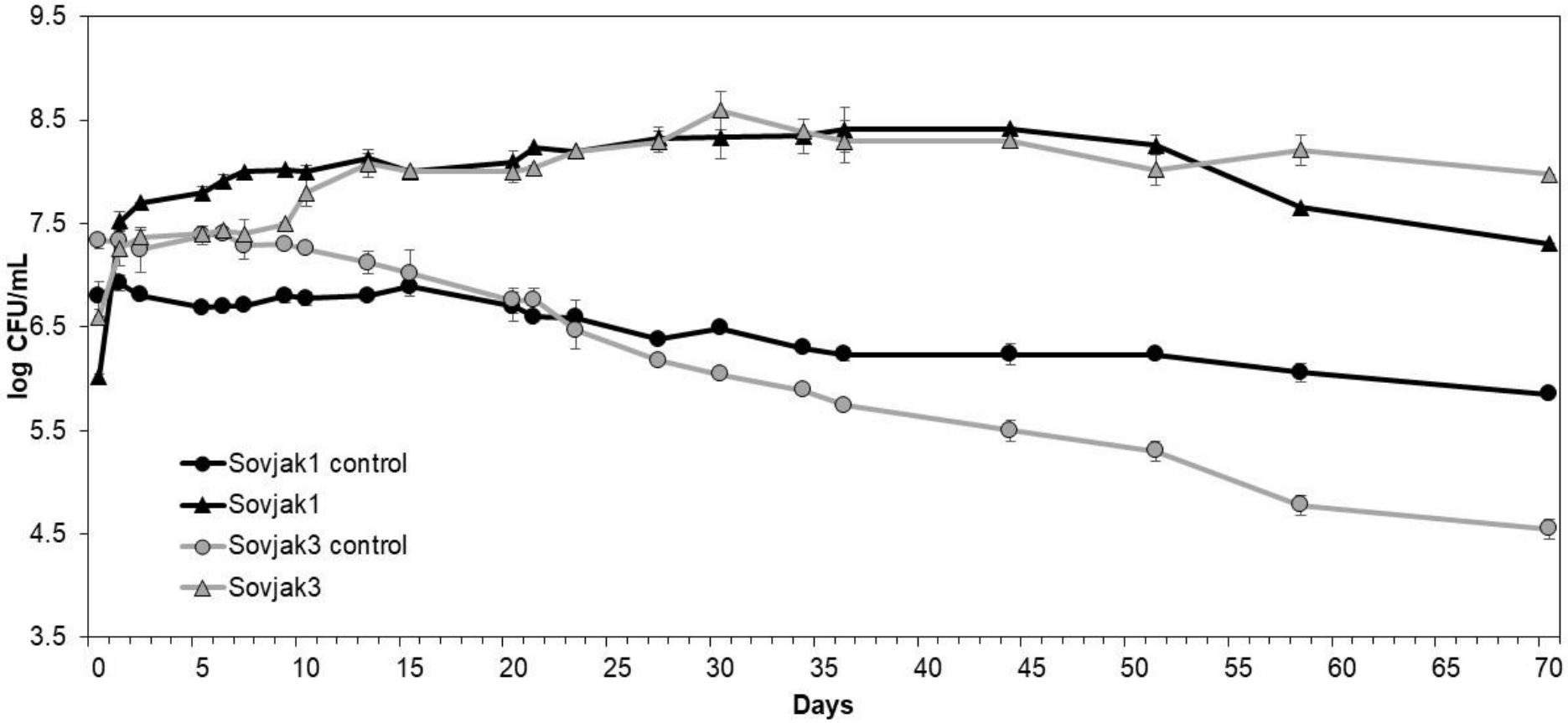
Technosol at a dump site was rich in heavy metals and contained 76% of petroleum hydrocarbons.



signs of natural petroleum bioremediation

Gas chromatogram of alkane fraction in fresh technosol.

Two *A. baumannii* isolates multiplied and survived in autoclaved technosoil during 70 days.



Survival of *A. baumannii* isolates in natural spring water (control) and natural spring water with 10% of technosol.

Hospital wastewater was collected at the central manhole of one hospital in Zagreb.

J Hosp Infect. 2017 Aug;96(4):323-327. doi: 10.1016/j.jhin.2017.04.005. Epub 2017 Apr 11.

Emission of extensively-drug-resistant Acinetobacter baumannii from hospital settings to the natural environment.

Seruga Music M[1], Hrenovic J[2], Goic-Barisic I[3], Hunjak B[4], Skoric D[1], Ivankovic T[1].

⊕ Author information

Abstract

BACKGROUND: Acinetobacter baumannii is a leading emerging pathogen that is frequently recovered from patients during hospital outbreaks. The role of environmental A. baumannii reservoirs is therefore of great concern worldwide.

AIM: To investigate the connection between A. baumannii causing hospital outbreaks and environmental isolates from hospital wastewater, urban sewage and river water as the final natural recipient of wastewaters.

METHODS: Clinical isolates from patients with hospital-acquired pneumonia and environmental isolates from water were collected during a two-month monitoring period. Recovery of A. baumannii was performed using CHROMagar Acinetobacter plates, incubated at 42°C for 48 h. Identification was performed by matrix-assisted laser desorption ionization-time of flight mass spectrometry and analyses of rpoB gene. The antibiotic resistance profiles were interpreted according to criteria given for clinical isolates of A. baumannii. The sequence types (ST) were retrieved by multi-locus sequence typing.

RESULTS: Fourteen of 19 isolates recovered from patients, hospital wastewaters, urban sewage and river water belonged to ST-195. The remaining five isolates recovered from patients and river water were assigned to ST-1421. All isolates showed very strong relatedness and clustered into CC92, which corresponds to IC2. All isolates were non-susceptible to at least one agent in all but two or fewer antimicrobial categories, and thus were classified as 'extensively-drug-resistant' (XDR). Heteroresistance to colistin was found in two isolates from hospital wastewater.

CONCLUSION: Close relatedness of clinical and environmental isolates suggests the emission of XDR A. baumannii via the untreated hospital wastewater in the natural environment.

10 isolates were recovered from 0.001 - 0.01 mL hospital wastewater.

Isolate	Origin	Date	Sequence type	International clonal lineage
OB 3929	Tracheal aspirate	18. 9. 2015	195	2
OB 4138	Bronchial aspirate	2. 10. 2015	195	2
S2/1	Hospital wastewater	27. 8. 2015	195	2
S2/2			195	2
S2/3			195	2
S2/4			195	2
S1/1		6. 10. 2015	195	2
S2/5			195	2
S2/6			195	2
S2/7			195	2
S2/8			195	2
S2/9			195	2

A. baumannii from wastewater and clinical isolates belong to the same ST.

Isolate	MIC values of antibiotics (mg/L)											
	MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST
OB 3929	>16^{R}	>16^{R}	>4^{R}	4^{R}	>16^{R}	>16^{R}	>64^{R}	2	16^{I}	128^{R}	>320^{R}	<0.5
OB 4138	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	>16^{R}	16^{I}	128^{R}	<20	<0.5
S2/1	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	4	8	128^{R}	>320^{R}	<0.5
S2/2	>16^{R}	8^{I}	>4^{R}	>8^{R}	>16^{R}	8^{R}	>64^{R}	2	<2	128^{R}	>320^{R}	80^{R}
S2/3	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	4	8	>128^{R}	>320^{R}	<0.5
S2/4	8^{I}	>16^{R}	>4^{R}	>8^{R}	8^{R}	>16^{R}	>64^{R}	4	4	64^{I}	>320^{R}	20^{R}
S1/1	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	8^{I}	<2	>128^{R}	>320^{R}	<0.5
S2/5	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	8	8^{I}	>32^{R}	>128^{R}	<20	<0.5
S2/6	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	8	>16^{R}	>32^{R}	>128^{R}	<20	<0.5
S2/7	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	8	8^{I}	>32^{R}	>128^{R}	<20	<0.5
S2/8	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	8	8^{I}	>32^{R}	>128^{R}	<20	<0.5
S2/9	>16^{R}	>16^{R}	>4^{R}	>8^{R}	>16^{R}	>16^{R}	8	8^{I}	16^{I}	>128^{R}	160^{R}	<0.5

All isolates extensively drug-resistant (XDR)

Urban wastewaters in Zagreb are consisted of: domestic, hospital, industrial and storm waters.

Monitoring was performed at the central wastewater treatment plant.

A. baumannii in urban wastewaters

Water Research 140 (2018) 261–267

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

Characterization of *Acinetobacter baumannii* from water and sludge line of secondary wastewater treatment plant

Paul G. Higgins [a, b, 1], Jasna Hrenovic [c, *, 1], Harald Seifert [a, b], Svjetlana Dekic [c]

[a] Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Cologne, Germany
[b] German Centre for Infection Research (DZIF), Partner Site Bonn-Cologne, Cologne, Germany
[c] Faculty of Science, University of Zagreb, Zagreb, Croatia

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ABSTRACT

The elimination of potentially pat
much attention in public health. R
tobacter baumannii in wastewater
objective of this study was to chara
secondary WWTP in Zagreb, Croati
using CHROMagar Acinetobacter p
microdilution and results interpre
Molecular characterization was pe
urban wastewater is constantly re
resistance, and emitting them via ef
are incorporated into activated sluc
process of anaerobic mesophilic sl
bilized sludge. The majority (102
antibiotic-susceptible isolates (17/
resistant isolates belonged to inte
72, while the susceptible isolates
otics, together with the appearanc
compared to influent wastewater.
discharge into the natural recipien

RESEARCH ARTICLE

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

J Hrenovic [1], I Goic-Barisic [2], S Kazazic [3], A Kovacic [4], M Ganjto [5], M Tonkic [2]
1. University of Zagreb, Faculty of Science, Department of Biology, Zagreb, Croatia
2. University Hospital Centre Split, Department of Clinical Microbiology and University of Split School of Medicine, Split, Croatia
3. Ruđer Boskovic Institute, Division of Physical Chemistry, Zagreb, Croatia
4. Institute of Public Health of Split and Dalmatia County, Split, Croatia
5. Zagreb Wastewater - Management and Operation Ltd., Zagreb, Croatia

Correspondence: Jasna Hrenovic (jasna.hrenovic@biol.pmf.hr)

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Hrenovic J, Goic-Barisic I, Kazazic S, Kovacic A, Ganjto M, Tonkic M. Carbapenem-resistant isolates of Acinetobacter baumannii in a municipal wastewater treatment plant, Croatia, 2014. Euro Surveill. 2016;21(15):pii=30195. DOI: http://dx.doi.org/10.2807/1560-7917.ES.2016.21.15.30195

Article submitted on 05 February 2015 / accepted on 14 April 2016 / published on 14 April 2016

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 per-**

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,

Isolate	Sequence type	International clonal lineage
Influent	ST-195	IC2
	ST-195	IC2
	ST-1604	IC1
	ST-1523	unclustered
Activated sludge	ST-195	IC2
	ST-195	IC2
	ST-1524	IC5
Digested sludge	ST-195	IC2
	ST-195	IC2
	ST-231	IC1
	ST-1525	unclustered
Effluent	ST-195	IC2
	ST-195	IC2
	ST-195	IC2
	ST-195	IC2
	ST-1523	unclustered

A. baumannii recovered from each stage of wastewater treatment, except alkaline lime-treated stabilised sludge (pH 12).

Clonal lineage	Acquired bla_{OXA}	Intrinsic bla_{OXA}
IC2	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
	bla_{OXA-23}	bla_{OXA-66}
IC1	bla_{OXA-72}	bla_{OXA-69}
	bla_{OXA-72}	bla_{OXA-69}
IC5	-	bla_{OXA-65}
unclustered	-	bla_{OXA-51}
	-	$bla_{OXA-208-like}$
	-	$bla_{OXA-117-like}$

Carbapenem-resistant isolates belonged to IC2 carrying the acquired OXA-23 (dominant) or IC1 carrying OXA-72.

Susceptible isolates belonged to IC5 or were unclustered.

Oxacillinases from carbapenem-resistant environmental isolates are highly related to those from clinical isolates.

MICROBIAL DRUG RESISTANCE
Volume 22, Number 7, 2016

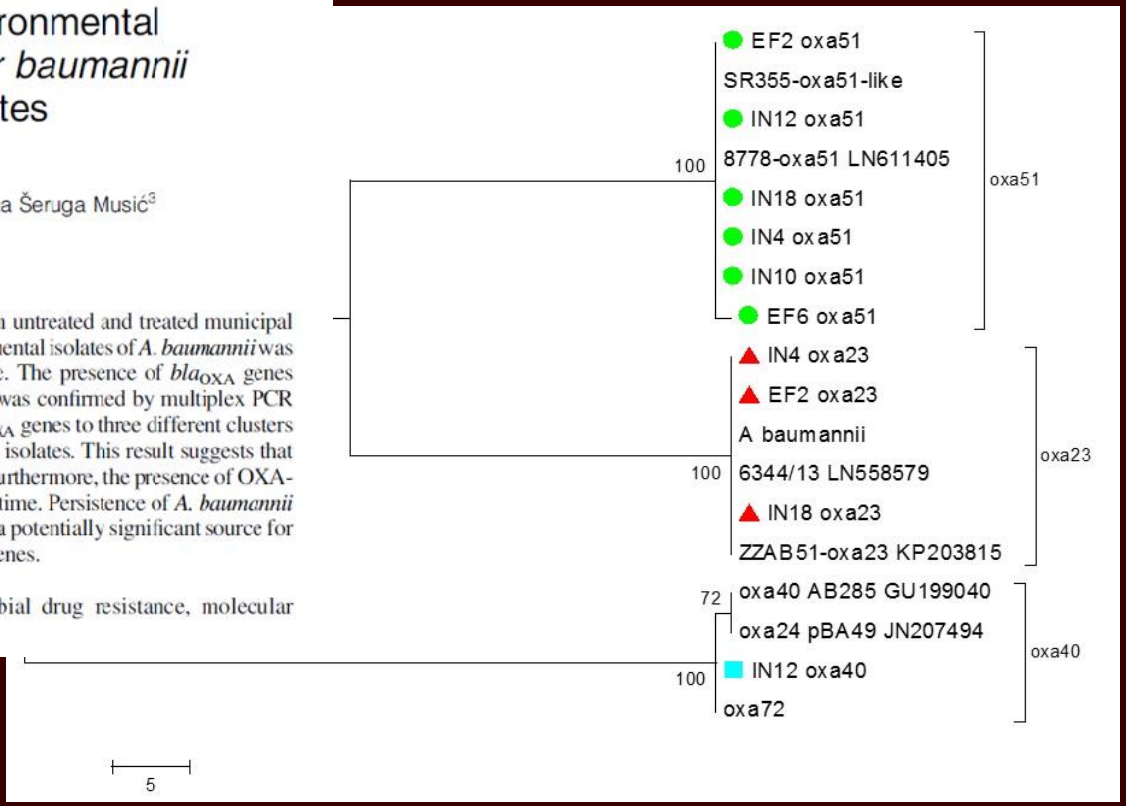
DOI: 10.1089/mdr.2015.0275

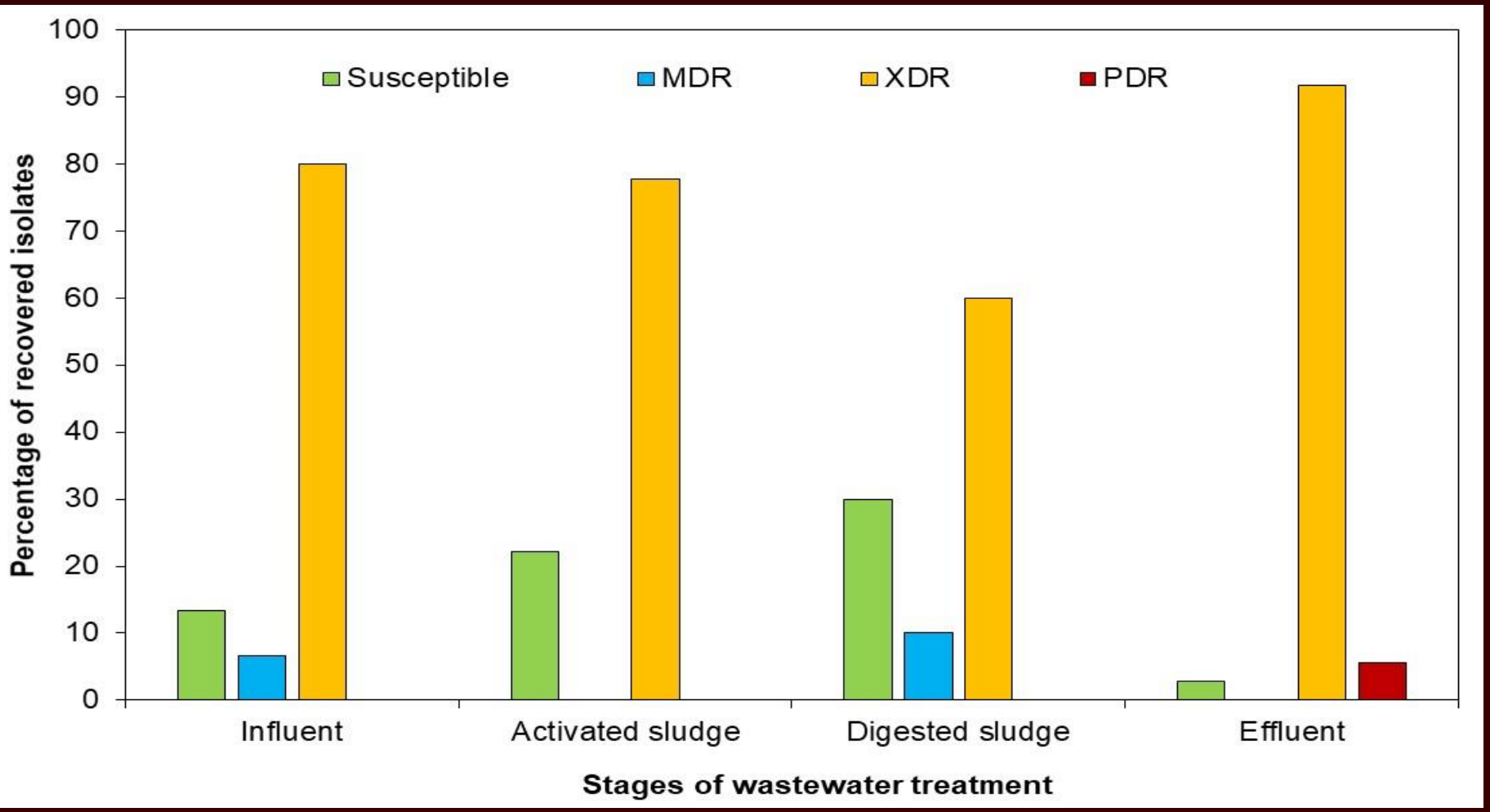
Emergence of Oxacillinases in Environmental Carbapenem-Resistant *Acinetobacter baumannii* Associated with Clinical Isolates

Ivana Goic-Barisic,[1,2] Jasna Hrenovic,[3] Ana Kovacic,[4] and Martina Šeruga Musić[3]

Six carbapenem-resistant isolates of *Acinetobacter baumannii* were recovered from untreated and treated municipal wastewater of the capital city of Zagreb, Croatia. Molecular identification of environmental isolates of *A. baumannii* was performed by amplification, sequencing, and phylogenetic analyses of *rpo*B gene. The presence of bla_{OXA} genes encoding OXA-type carbapenemases (OXA-51-like, OXA-23, and OXA-40-like) was confirmed by multiplex PCR and sequencing. Phylogenetic analyses corroborated the affiliation of detected bla_{OXA} genes to three different clusters and showed association of environmental OXAs with those described from clinical isolates. This result suggests that isolates recovered from municipal wastewater are most probably of clinical origin. Furthermore, the presence of OXA-40-like (OXA-72) in an environmental *A. baumannii* isolate is reported for the first time. Persistence of *A. baumannii* harboring the clinically important OXAs in the wastewater treatment process poses a potentially significant source for horizontal gene transfer and implications for wider spread of antibiotic resistance genes.

Keywords: *Acinetobacter baumannii*, carbapenemase, oxacillinanase, microbial drug resistance, molecular characterization, public health





Antibiotic susceptibility profile of *A. baumannii* isolates recovered from different stages of the wastewater treatment process. Number of isolates: influent 45; activated sludge 18; digested sludge 20; effluent 36; all stages (total) 119.

Microb Drug Resist. 2016 Oct 28. [Epub ahead of print]

Pan Drug-Resistant Environmental Isolate of Acinetobacter baumannii from Croatia.

Goic-Barisic I[1,2], Seruga Music M[3], Kovacic A[4], Tonkic M[1,2], Hrenovic J[3].

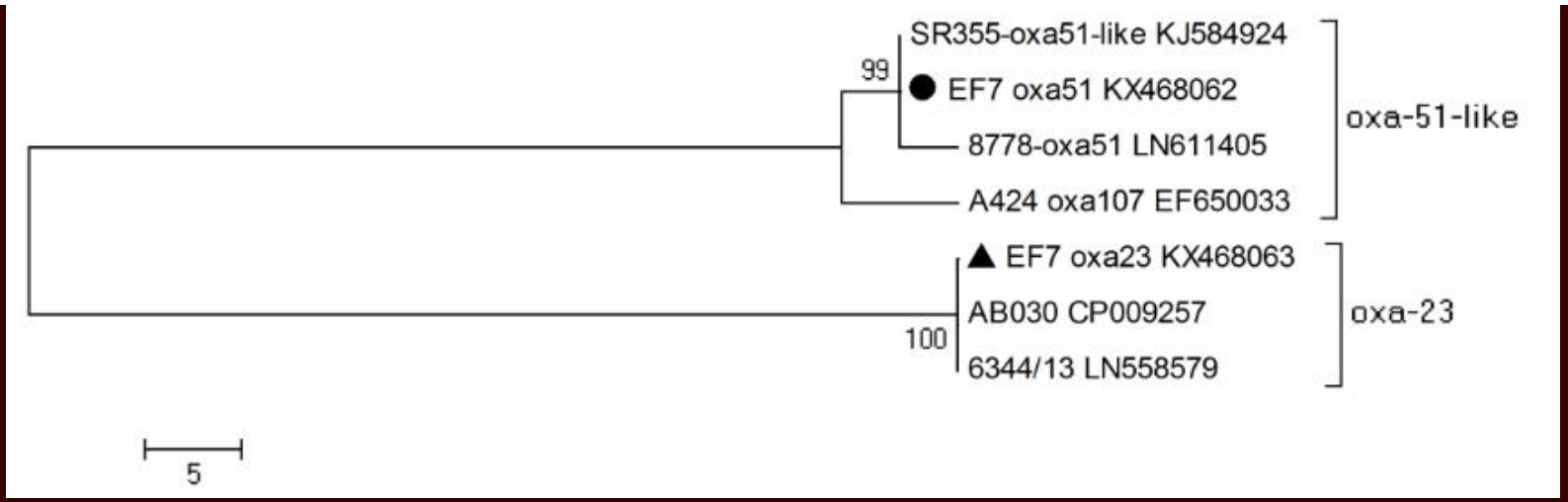
⊕ Author information

Abstract

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/β-lactamase inhibitors, carbapenems, fluoroquinolones, aminoglycosides, folate pathway inhibitors, and polymyxins, except intermediately susceptible to minocycline and tigecycline. Intrinsic chromosomally located $bla_{OXA-51-like}$ gene and acquired plasmid-located $bla_{OXA-23-like}$ 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.

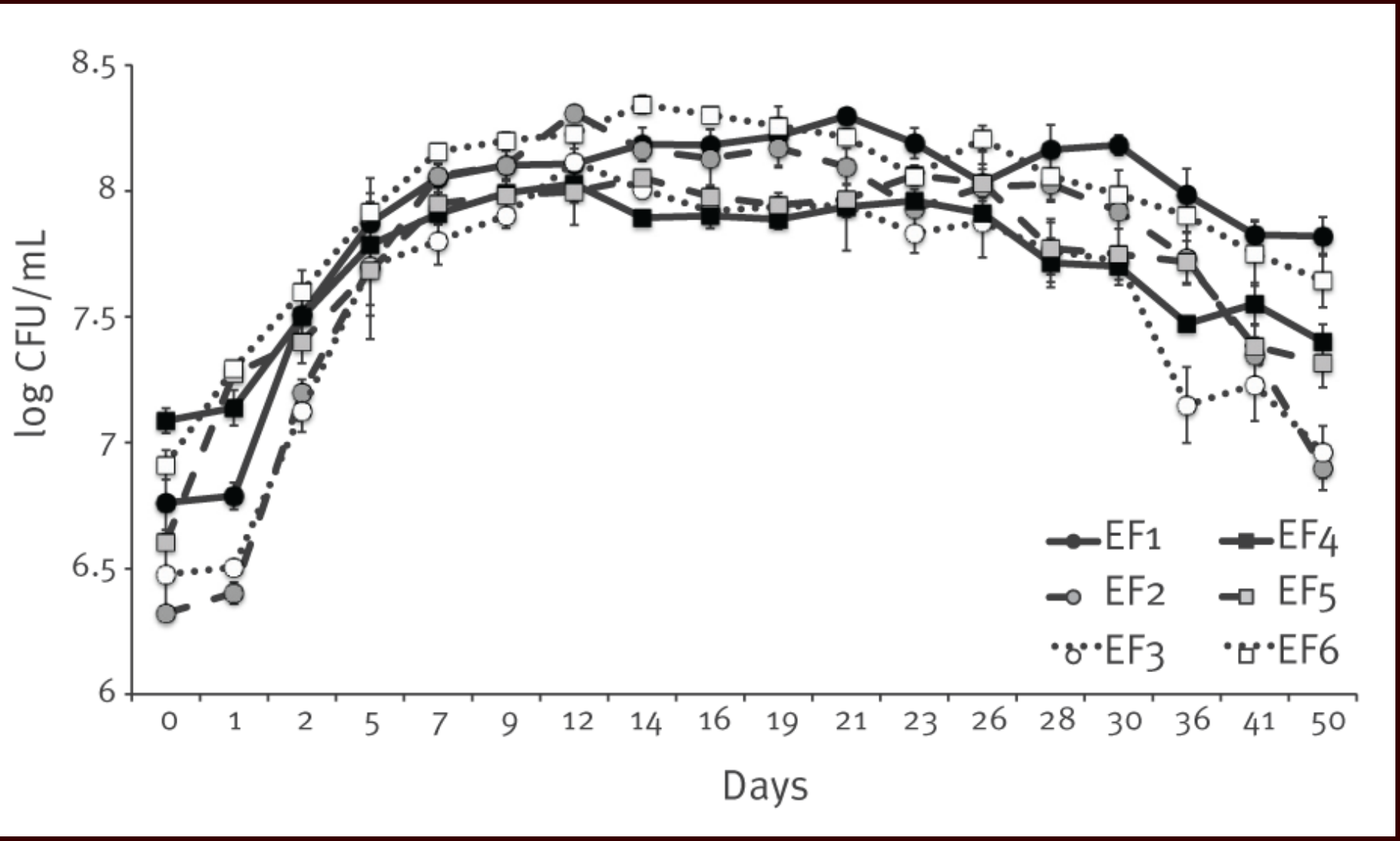
KEYWORDS: Acinetobacter baumannii; antibiotics; microbial drug resistance; public health; wastewater

PMID: 27792476 DOI: 10.1089/mdr.2016.0229



87% of isolates carbapenem-resistant, IC 1 i 2 = clinically important

13% of antibiotics-sensitive isolates, unclustered = native strains in natural habitat?



Six *A. baumannii* isolates multiplied and survived in autoclaved effluent water during 50 days.

Four isolates of *A. baumannii* were recovered from 10mL of water from Sava River downstream the City of Zagreb, after discharge of the urban wastewaters into the natural recipient.

Journal of Hospital Infection

Available online 11 April 2017

In Press, Corrected Proof— Note to users

Emission of extensively-drug-resistant *Acinetobacter baumannii* from hospital settings to the natural environment

M. Seruga Music[a], J. Hrenovic[a], I. Goic-Barisic[b], B. Hunjak[c], D. Skoric[a], T. Ivankovic[a]

Show more

https://doi.org/10.1016/j.jhin.2017.04.005

Summary

Background

Acinetobacter baumannii is a leading emerging pathogen that is frequently recovered from patients during hospital outbreaks. The role of environmental *A. baumannii* reservoirs is therefore of great concern worldwide.

Aim

To investigate the connection between *A. baumannii* causing hospital outbreaks and environmental isolates from hospital wastewater, urban sewage and river water as the final natural recipient of wastewaters.

Minimum inhibitory concentration (MIC) values of tested antibiotics[a] against environmental isolates of *A. baumannii*. [R] - resistant, [I] - intermediate according to EUCAST and CLSI criteria.

[a] carbapenems (MEM-meropenem, IMI-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), tetracyclines (MIN-minocycline), penicillins/β-lactamase inhibitors (SAM-ampicillin/sulbactam, TIM-ticarcillin/clavulanic acid), folate pathway inhibitors (SXT- trimethoprim/sulfamethoxazole), polymyxins (CST-colistin).

Isolte	**MIC values of antibiotics (mg/L)**											
	MEM	IPM	CIP	LVX	TOB	GEN	AMK	MIN	SAM	TIM	SXT	CST
Sava3	>16[R]	>16[R]	>4[R]	>8[R]	>16[R]	>16[R]	>64[R]	4	16[I]	>128[R]	>320[R]	<0.5
Sava4	>16[R]	>16[R]	>4[R]	>8[R]	<1	8[R]	16[I]	8[I]	8	>128[R]	>320[R]	<0.5
Sava5	>16[R]	>16[R]	>4[R]	>8[R]	>16[R]	>16[R]	>64[R]	8[I]	8	>128[R]	<20	<0.5
Sava6	>16[R]	>16[R]	>4[R]	>8[R]	>16[R]	>16[R]	>64[R]	4	16[I]	>128[R]	>320[R]	<0.5

All isolates extensively drug-resistant (XDR)

A. baumannii in river

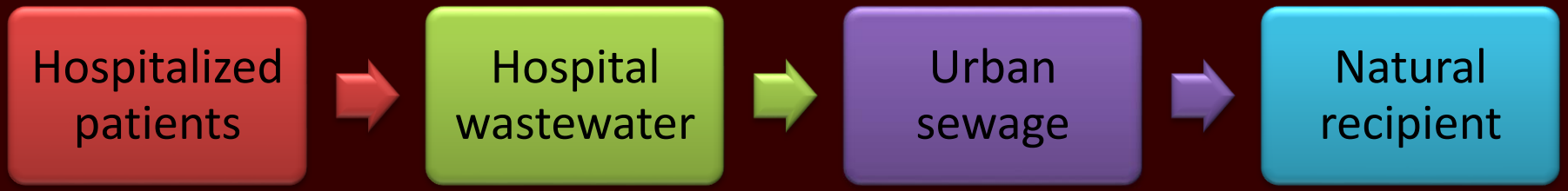
Isolate	Origin	Date	Sequence type	International clonal lineage
OB 3831	Sputum	11. 9. 2015	1421 [a]	2
OB 3929	Tracheal aspirate	18. 9. 2015	195	2
OB 4027	Sputum	24. 9. 2015	1421 [a]	2
OB 4138	Bronchial aspirate	2. 10. 2015	195	2
S2/1	Hospital wastewater	27. 8. 2015	195	2
S2/2			195	2
S2/3			195	2
S2/4			195	2
S1/1		6. 10. 2015	195	2
S2/5			195	2
S2/6			195	2
S2/7			195	2
S2/8			195	2
S2/9			195	2
IN32	Urban sewage	23. 9. 2015	195	2
Sava3	Sava River	11. 10. 2015	1421 [a]	2
Sava4			195	2
Sava5			1421 [a]	2
Sava6			1421 [a]	2

A. baumannii from Sava, urban sewage, hospital wastewaters, and clinical isolates belong to the same ST.

[a] new ST

Hospital wastewaters are discharged into the urban sewage system without pre-treatment.
Urban wastewater, treated or not, is discharged into the Sava River.

Probable source of *A. baumannii* in Sava River: hospital and consequently urban wastewater.



In the City of Split at the costal region of Croatia, hospital wastewaters are discharged into the urban sewage system without pre-treatment. Untreated urban wastewater is discharged into the Adriatic Sea.

Hospitalized patients → Hospital wastewater → Urban sewage → Natural recipient

RESEARCH ARTICLE

International Microbiology 20(4):166-169 (2017)
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www.im.microbios.org

INTERNATIONAL MICROBIOLOGY
OPEN ACCESS

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

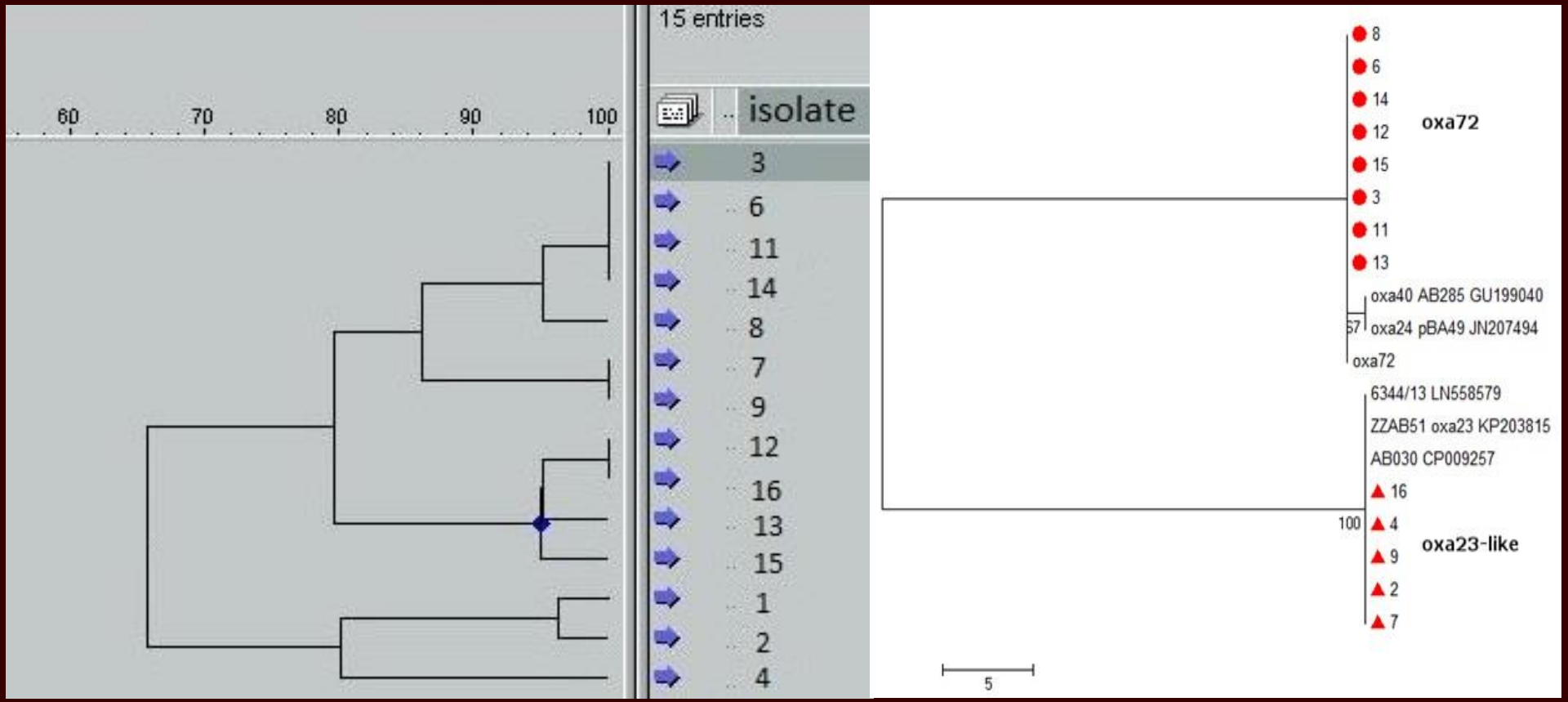
Ana Kovacic[1], Martina Seruga Music[2], Svjetlana Dekic[2], Marija Tonkic[3], Anita Novak[3], Zana Rubic[3], Jasna Hrenovic[2], Ivana Goic-Barisic[1]*

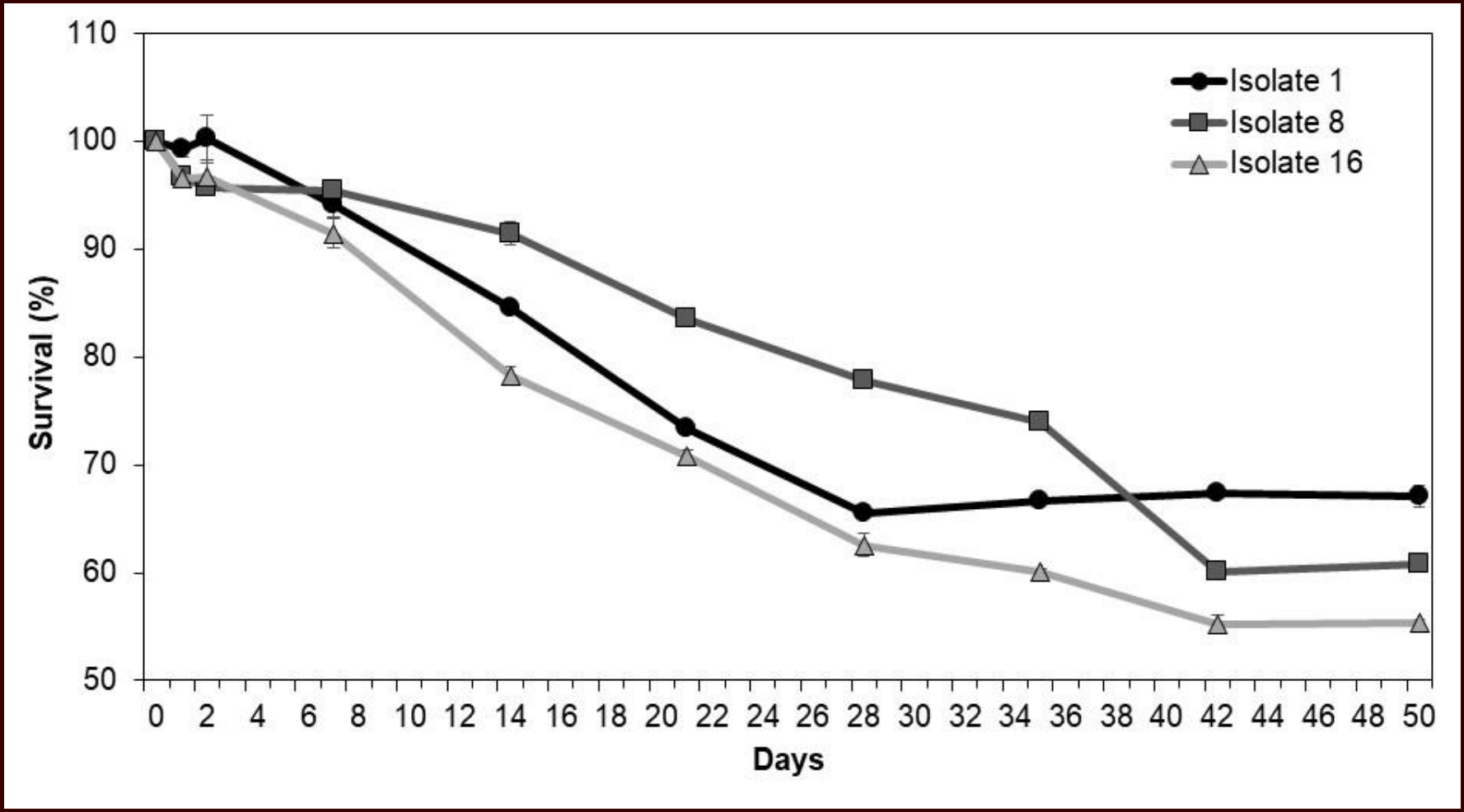
[1]Institute of Public Health of Split and Dalmatia Country, Split, Croatia
[2]Department of Biology, Faculty of Science, University of Zagreb, Zagreb, Croatia
[3]University Hospital of Split and School of Medicine University of Split, Split, Croatia

Received 11 July 2017 · Accepted 20 November 2017

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 $bla_{\text{OXA-23-like}}$, while five wastewater isolates harboured $bla_{\text{OXA-40-like}}$ genes sharing 100% sequence identity with $bla_{\text{OXA-72}}$ 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.

In wastewater from Split hospital 10 carbapenem-resistant *A. baumannii* isolates were recovered (1-10).
Isolates are related to clinical isolates from the same hospital (13-16), and possess the same acquired OXA-23 and OXA-72.





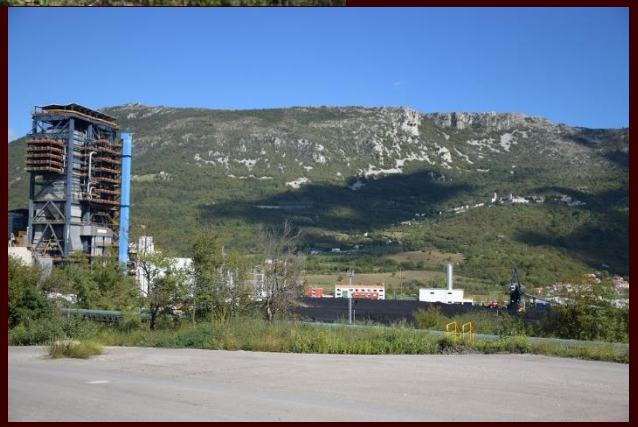
Three *A. baumannii* isolates survived in autoclaved seawater during 50 days.

A. baumannii were searched, but not found:

- In natural waters not influenced by hospital wastewaters: springs, wells, creeks, lakes.
- In soils not influenced by solid waste: meadows, forest, construction and other inert waste in Croatia.







Conclusion:

- Human solid and liquid waste is a source of clinically relevant *A. baumannii* in environment

- Natural environment could represent a secondary habitat of *A. baumannii*

- Measures for prevention of spread of *A. baumannii* in environment:

- proper management and disposal of human solid waste
- novel technologies of disinfection of hospital wastewater.

Thank you for attention!

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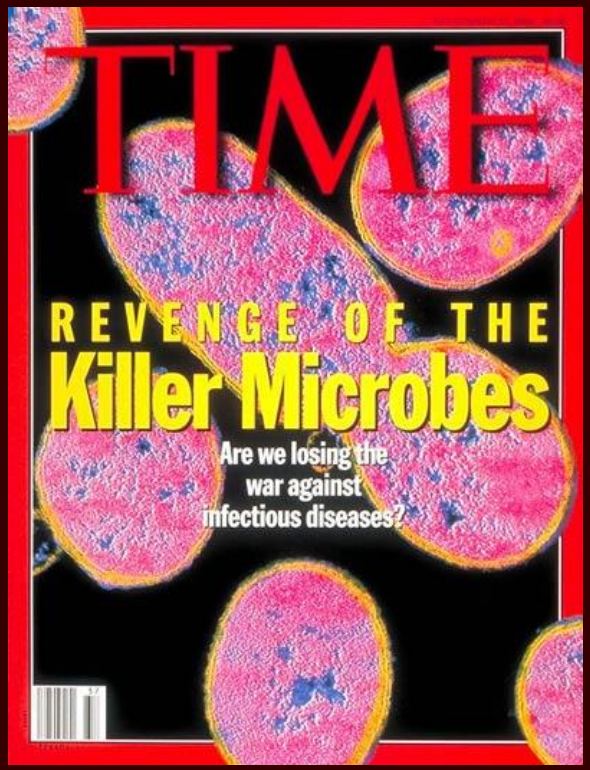
https://www.pmf.unizg.hr/naturaci



HRZZ
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