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FIRST ISOLATION OF CARBAPENEM-RESISTANT *ACINETOBACTER BEIJERINCKII* FROM AN ENVIRONMENTAL SAMPLE

JÚLIA RADÓ[1], EDIT KASZAB[1]*, TIBOR BENEDEK[2], BALÁZS KRISZT[1] and SÁNDOR SZOBOSZLAY[1]

[1]Department of Environmental Safety and Ecotoxicology, Szent István University, Gödöllő, Hungary
[2]Regional University Center of Excellence, Szent István University, Gödöllő, Hungary

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The emergence of opportunistic *Acinetobacter* spp. in healthcare settings poses a significant threat to public health. The major reasons for nosocomial spread of these species are their abilities to develop and transfer drug resistance against various classes of antibiotics. Considering that *Acinetobacter* spp. are ubiquitous in nature, can utilize several carbon sources, and reach humans via various pathways, our aim was to obtain information about the environmental strains of this genus. Our first step was to develop and test a multistep isolation procedure based on traditional scientific methods. Antibiotic resistance patterns of the isolated strains were determined, as susceptibility to 12 antibiotics of 7 classes was tested by MIC Test Strip method. Altogether 366 samples (groundwater, surface water, and soil) of 24 sites were investigated and a collection of 37 *Acinetobacter* isolates was obtained. Among others, clinically important human pathogen *Acinetobacter* spp., such as *A. baumannii, A. johnsonii*, and *A. gyllenbergii* were identified. Three environmental strains were determined as multidrug-resistant including a carbapenem-resistant, hemolytic *Acinetobacter beijerinckii* strain isolated from a hydrocarbon-contaminated groundwater sample. In summary, it has been found that the applied multistep isolation procedure is applicable to isolate various species of *Acinetobacter* genus. Based on the antibiotic resistance assay, we can conclude that environmental representatives of *Acinetobacter* spp. are able to develop multidrug resistance, but at a lower rate than their clinical counterparts.

Keywords: *Acinetobacter*, environmental samples, antibiotic resistance, isolation protocol

*Corresponding author; E-mail: kaszab.edit@mkk.szie.hu

Introduction

The necessity of simultaneous investigations of environmental and clinical isolates of opportunistic species is a rising issue in our days, since pathways between clinical and non-clinical environments are both globally and locally opening. In the case of some clinically important species such as *Pseudomonas aeruginosa*, comparative studies are available regarding virulence factors, genomic diversity [1], or antibiotic resistance of strains isolated from various environmental sources. However, *Acinetobacter* strains of clinical and environmental habitats are still considered and examined in various ways.

Acinetobacter species are ubiquitous in natural habitats (soil and water). Their presence was verified in the normal skin, throat, and rectal flora as well as in food and body lice [2]. The representatives of this genus can cause serious respiratory, urinary, and biliary tract infections; empyema; bacteremia; meningitis; endocarditis; burn infections; and wound sepsis [3, 4]. *Acinetobacter* strains related to nosocomial infections are mainly found in intensive care units and in burns or high dependency units treating severely ill or debilitated patients [5]. In addition, infections caused by *Acinetobacter baumannii* have been reported in the case of military personnel of combat regions such as Afghanistan or Iraq [6].

Among all *Acinetobacter* species, the *Acinetobacter calcoaceticus–baumannii* complex (ACB) has the greatest influence on clinical healthcare; therefore, ACB is the critical subject of the current scientific investigations. The members of the ACB complex (*A. baumannii, A. calcoaceticus, Acinetobacter pittii*, and *Acinetobacter nosocomialis*) are in close phylogenetic relation; therefore, their differentiation solely on phenotypic characterization is difficult [7, 8].

Acinetobacter-related infections can pose serious clinical concerns with high mortality of patients. Due to the high rate of multidrug-resistant (MDR) *Acinetobacter* strains in clinical settings [9], some *Acinetobacter* species such as *A. baumannii* are mentioned as “emerging nosocomial superbugs” [10].

Regarding antibiotic resistance features, the emergence of MDR *Acinetobacter* strains has been reported from hospital settings all over the world, and the number of infections caused by these strains is continuously increasing [2, 5]. The treatment of MDR *Acinetobacter* strains is complicated because (due to the production of OXA-type carbapenemases) the majority of clinical *Acinetobacter* spp. is resistant to carbapenems, a commonly used group of first-line antimicrobial agents [11]. Moreover, some *A. baumannii* strains were reported to be resistant to all available antibiotics [12], due to their great set of various antibiotic resistance mechanisms, which lead to the emergence of untreatable infections.

Although clinical *Acinetobacter* spp. are well characterized, the available information regarding antibiotic resistance patterns of *Acinetobacter* strains from environmental samples is much limited and controversial, despite the recognition of environment as a potential reservoir for *Acinetobacter* spp.-resistant isolates [13]. The major reason for this is possibly the difficult isolation and identification of ACB from environmental samples. Upon the information of scientific literature, the existing methods for the isolation of *Acinetobacter* spp. are moderately effective and are not standardized.

The available procedures for identification are also limited and occasionally are not verified by molecular genetic methods [13]. Recent publications about *Acinetobacter* species used only classical microbiological methods or mass spectrometry [10, 14] for identification; however, none of these research works used the reliable 16S rRNA-based molecular method for identification.

Despite the clinical concerns of acinetobacters, some *Acinetobacter* strains are considered as industrially important [15] due to their ability to degrade several pollutants or industrial products [16–18] such as diesel oil [19]. However, the clinically important features of these strains such as antibiotic resistance or virulence are barely described.

Only a few scientific articles have discussed the antibiotic resistance of environmental *Acinetobacter* strains. Dhakephalkar and Chopade [20] examined different environmental sources (soil, river water, domestic sewage, treated industrial effluent, and laboratory effluent) to isolate *Acinetobacter* strains and to determine their antibiotic and metal resistance. Berlau et al. [21] isolated *Acinetobacter* strains from vegetables and fruits to determine minimum inhibitory concentrations (MICs) of six antimicrobial agents.

Based on the overview of the available scientific information on environmental acinetobacters, our aim was to widen our knowledge on antibiotic/multidrug resistance of naturally occurring *Acinetobacter* isolates, which can be a possible source of non-nosocomial infections [22]. To reach our main objective, we aimed to establish a culture collection of environmental *Acinetobacter* strains and to determine their antibiotic resistance profiles.

Material and Methods

Environmental sample collection

Sampling period was between 2008 and 2013. Environmental samples were taken in accordance with the relevant Hungarian standards MSZ 21464: 1998 [23] and MSZ 12750/2-71: 1971 [24]. Altogether 366 samples of 24 Hungarian sites

were collected for further examinations. Samples with a detectable number of *Acinetobacter* strains (Table I) were originated from hydrocarbon-contaminated and non-contaminated environments as follows:

- Groundwater: 15 samples with total petroleum hydrocarbons (TPH; Dunavarsány, Füzesabony, Polgár, Siklós, Tiszaújváros, Vác, and Zalaegerszeg), 1 sample with methyl tert-butyl ether (MTBE; Marcali) contamination, and 10 non-contaminated samples (Bugyi, Siklós, and Vác);
- Soil: two samples with polycyclic aromatic hydrocarbons (PAHs; Pétfürdő);
- Surface water: six samples from non-contaminated fishpond with no direct use of antibiotics (Hőgyész, Kölesd, and Varsád).

Isolation of Acinetobacter *species*

Successful isolation of *Acinetobacter* strains was carried out based on classical microbiological methods described in scientific literature. The recommended media were examined with two *Acinetobacter* strains (*Acinetobacter beijerinckii and Acinetobacter johnsonii*) formerly isolated in our department. The colony morphology of *Acinetobacter* spp. was examined in cetrimide agar, tryptone glucose yeast (TGY) agar, nutrient agar, McConkey agar [18] and Leeds *Acinetobacter* Medium [25, 26] with different incubation parameters (28, 30, and 42 °C). Furthermore, *Acinetobacter* sp. enrichment liquid medium [27] was tested in combination with TGY and Leeds *Acinetobacter* Medium agar.

Based on these preliminary investigations, the following protocol was implemented in the case of environmental samples: each incoming sample was diluted in tenfold series with physiological saline solution (10^1–10^4) and 1–1 ml of diluted samples were inoculated into tubes containing *Acinetobacter* sp. enrichment liquid medium (9 ml) [27]. After 48 h of incubation (28–30 °C and 160 rpm) agarized Leeds *Acinetobacter* Medium [25] plates were poured containing 1 ml of the shaken enriched cultures. After another incubation period of 72 h, colonies resemble *Acinetobacter* spp. (mauve, round, intact edge, and shiny) on the surface of agar plates were purified using streak plate method and subsequently inoculated onto nutrient agar slants (MERCK 105450) for conservation and further investigations.

Identification of strains

Before 16S rRNA gene base identification, pure cultures of *Acinetobacter* candidates were examined with oxidase and catalase tests [28]. Strains with oxidase-negative and catalase-positive profiles were used for further examinations (16S rRNA gene sequencing and antibiotic resistance investigations).

Table I. The isolated and identified environmental *Acinetobacter* strains with the sampling data

No.	Designation of strains	Sampling place	Isolation source	Contamination of isolation source	Time of sampling	Identified species	Nearest neighbor upon 16S rRNA	Sequence homology (%)
ACB complex								
1.	Sk-V/3	Siklós	Groundwater	N/A	2011.06.21	*A. baumannii*	ATCC 19606 (T)	99.62
2.	HT-4/1	Hőgyész	Fishpond	N/A	2011.08.01	*A. baumannii*	ATCC 19606 (T)	100
3.	HT-4/3	Hőgyész	Fishpond	N/A	2011.08.01	*A. baumannii*	ATCC 19606 (T)	99.66
4.	II HT-3/1	Varsád	Fishpond	N/A	2011.08.05	*A. baumannii*	ATCC 19606 (T)	99.68
5.	TN6	Tiszaújváros	Groundwater	TPH	2008.11.19	*A. calcoaceticus*	DSM 30006 (T)	98.63
6.	PT2/2	Pétfürdő	Soil	PAH	2010.04.30.	*A. calcoaceticus*	DSM 30006 (T)	100
7.	PT3/6	Pétfürdő	Soil	PAH	2010.04.30.	*A. calcoaceticus*	DSM 30006 (T)	99.38
8.	PT3/9	Pétfürdő	Soil	PAH	2010.05.03.	*A. calcoaceticus*	DSM 30006 (T)	99.57
9.	1/2	Vác	Groundwater	TPH	2011.06.23	*A. calcoaceticus*	DSM 30006 (T)	99.70
10.	5/06/4	Vác	Groundwater	N/A	2011.06.23	*A. calcoaceticus*	DSM 30006 (T)	99.62
11.	PM5	Polgár	Groundwater	TPH	2011.07.05	*A. calcoaceticus*	DSM 30006 (T)	100
12.	PM7	Polgár	Groundwater	TPH	2011.07.05	*A. calcoaceticus*	DSM 30006 (T)	99.85
13.	PM13	Polgár	Groundwater	TPH	2011.07.05	*A. calcoaceticus*	DSM 30006 (T)	99.70
14.	PM7/3	Polgár	Groundwater	TPH	2011.08.09	*A. calcoaceticus*	DSM 30006 (T)	100
15.	DM-5/1	Dunavarsány	Groundwater	TPH	2011.09.20	*A. calcoaceticus*	DSM 30006 (T)	100
16.	Sk-V	Siklós	Groundwater	N/A	2012.05.29	*A. calcoaceticus*	DSM 30006 (T)	99.84
17.	5/06	Vác	Groundwater	N/A	2012.06.12	*A. calcoaceticus*	DSM 30006 (T)	99.85
18.	22	Vác	Groundwater	TPH	2012.06.12	*A. calcoaceticus*	DSM 30006 (T)	100
19.	24	Vác	Groundwater	TPH	2012.06.12	*A. calcoaceticus*	DSM 30006 (T)	100
20.	42	Vác	Groundwater	TPH	2012.06.12	*A. calcoaceticus*	DSM 30006 (T)	99.69
21.	J5	Siklós	Groundwater	N/A	2012.11.06	*A. calcoaceticus*	DSM 30006 (T)	100
22.	J7	Siklós	Groundwater	N/A	2012.11.06	*A. calcoaceticus*	DSM 30006 (T)	100
23.	TV-11/J4	Füzesabony	Groundwater	TPH	2012.11.13	*A. calcoaceticus*	DSM 30006 (T)	99.18
24.	SV/1	Kölesd	Fishpond	N/A	2013.06.27	*A. calcoaceticus*	DSM 30006 (T)	100
25.	PM15/3	Polgár	Groundwater	N/A	2011.08.09	*A. pittii*	CIP 70.29 (T)	99.85

(Continued)

Table I. The isolated and identified environmental *Acinetobacter* strains with the sampling data *(Continued)*

No.	Designation of strains	Sampling place	Isolation source	Contamination of isolation source	Time of sampling	Identified species	Nearest neighbor upon 16S rRNA	Sequence homology (%)
26.	II HT-1/1	Kölesd (Sárvíz)	Fishpond	N/A	2011.08.05	*A. pittii*	CIP 70.29 (T)	99.83
27.	BUT-8/1	Bugyi	Groundwater	N/A	2011.10.04	*A. pittii*	CIP 70.29 (T)	99.85
28.	FK-3/J2	Marcali	Groundwater	MTBE	2013.05.15	*A. pittii*	CIP 70.29 (T)	99.73
29.	T3N6	Tiszaújváros	Groundwater	TPH	2009.03.04	*A. beijerinckii*	58a (T)	99.69
30.	Z4N3	Zalaegerszeg	Groundwater	TPH	2009.05.28	*A. beijerinckii*	58a (T)	99.24
31.	6/2	Varsád	Fishpond	N/A	2013.06.27	*A. gyllenbergii**	1271 (T)	97.47
32.	Z4SZ2	Zalaegerszeg	Groundwater	TPH	2009.05.28	*A. johnsonii*	CIP 64.6 (T)	98.64
33.	ST-2	Siklós	Groundwater	TPH	2012.02.20	*A. johnsonii*	CIP 64.6 (T)	98.36
34.	J6	Siklós	Groundwater	N/A	2012.09.18	*A. johnsonii*	CIP 64.6 (T)	98.82
35.	6/1	Varsád	Fishpond	N/A	2013.06.27	*A. johnsonii**	CIP 64.6 (T)	97.91
36.	J3	Siklós	Groundwater	N/A	2012.09.18	*A. radioresistens*	DSM 6976 (T)	99.74
37.	II HT-4/2	Hőgyész	Fishpond	N/A	2011.08.05	*A. soli*	B1 (T)	100

Note: ACB: *Acinetobacter calcoaceticus–baumannii* complex; TPH: total petroleum hydrocarbon contamination >100 μg/L; PAH: policyclic aromatic hydrocarbon contamination >2 μg/L; MTBE: methyl tert-butyl ether contamination 31.4 μg/L.

*Pairwise similarity (%) is the highest with *A. oryzae* [23], a still invalid species with 99.30% (strain "6/2") and 98.86% (strain "6/1") homology. Names indicated in this table are the first valid species by EzTaxon [32].

16S rRNA gene-based identification of strains

Acinetobacter candidates were identified based on 16S rRNA gene sequence analysis. The 16S rRNA gene was amplified from chromosomal DNA. DNA of strains from liquid cultures (24 h) was extracted and purified using the MOBIO Ultra Clean Microbial DNA Isolation Kit (MOBIO Laboratories, USA) following the manufacturer's instructions, detected by 1% agarose gel electrophoresis and visualized under ultraviolet light.

For amplification of 16S rRNA genes, universal 27f (5′ GAG TTG ATC CTG GTC AG 3′) forward and 1492r (5′ TAC GGG TAC CTT GTT ACG ACT T 3′) reverse primers were used [29]. The reaction parameters were as follows:

98 °C for 5 s, 32 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 45 s, and then a final extension at 72 °C for 10 min. PCR reactions were performed in 50 µl reaction volumes containing 5 µl Taq-Buffer (Fermentas, Lithuania), 10 µl (1 mM) dNTP, 0.25 µl Taq DNA polymerase, 0.5 µl (1 µM) forward primer (27f), 0.5 µl (1 µM) reverse primer (1492r), 1 µl DNA template, and 32.75 µl nuclease-free water. Amplicons were detected by agarose gel electrophoresis, purified by NucleoSpin Extract II DNA Clean-up set (Macherey-Nagel, Germany) and used as a template for sequencing reactions using 27f as sequencing primer. The nucleotide sequence determination was performed using the Big Dye Terminator version 3.1 (Applied Biosystems, USA). Cycle Sequencing Kit (Applied Biosystems) and sequences were analyzed using ABI 3130 Genetic Analyzer (Applied Biosystems). Prior capillary gel electrophoresis products of the sequencing reactions had been purified using ethanol precipitation method. The obtained sequences were edited and assembled using MEGA5 software [30], and then homology BLAST searches [31] were performed in the GenBank database. The determination of closest type strain was carried out by EzTaxon-e server [32, 33]. Species-level identification was accepted above 98% sequence homology. Sequencing data obtained in this study were submitted to the European Nucleotide Archive database and can be found under accession numbers HG810368–HG810404.

Antibiotic resistance examination of environmental strains

The antibiotic sensitivity tests of environmental *Acinetobacter* strains were performed according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [34].

For the determination of MIC values, MIC Test Strip (Liofilchem, Italy) was applied with 12 antibacterial agents belonging to 7 antibiotic groups (Table II). In the first step, bacterial suspensions of 0.5 McFarland value [35] were spread onto the surface of Mueller–Hinton agar (MERCK 1.05435.0500) in accordance with

Table II. The examined antibacterial agents

Antibiotic group	Antibacterial agent	MIC gradient (μg/ml)
Carbapenems	Imipenem	0.002–32
	Meropenem	0.002–32
Aminoglycosides	Gentamicin	0.016–256
Fluoroquinolones	Ciprofloxacin	0.002–32
	Ofloxacin	0.002–32
Cephalosporins	Cefotaxime	0.016–256
	Ceftazidime	0.016–256
	Ceftriaxone	0.016–256
	Cefepime	0.016–256
Glycylcyclines	Tigecycline	0.016–256
Penicillins	Piperacillin	0.016–256
Rifamycins	Rifampicin	0.002–32

Note: MIC: minimum inhibitory concentration.

the recommendation of the manufacturer. MIC Test Strips were positioned on the surface of the inoculated medium. *Escherichia coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used for quality control of test strips. After an incubation period of 24 h at 35 °C, MIC values were determined and evaluated according to CLSI's recommendation. Strains were classified into resistant (R), intermediate resistant (I), and sensitive (S) categories.

Results and Discussion

As it was described in "Methods" section, a multistep isolation protocol was implemented upon the experienced effectivity of the examined media. The main steps of this selection procedure for acinetobacters are summarized in Figure 1.

The multiple, parallel checks of examined media showed that our protocol is suitable for selective isolation of environmental *Acinetobacter* strains with a rate similar to other available procedures [13], but without the addition of antibiotics, as selective agents. The main advantage of avoidance of antibiotics during isolation is their possible effect on the antibiotic resistance profile of a given strain; therefore, our procedure appears to be more reliable regarding antibiotic resistance profiling than other methods. On the whole, this protocol can be recommended for other researchers for further testing and adaptation to reach a good detection rate of environmental acinetobacters with a decreasing rate of false identifications and/or with a negative effect on antibiotic resistance profiling.

During our examination period, altogether 37 environmental strains belonging to the genus *Acinetobacter* were isolated with the aforementioned multistep protocol from groundwater (26 isolates), soil (3 isolates), and surface water – fishpond

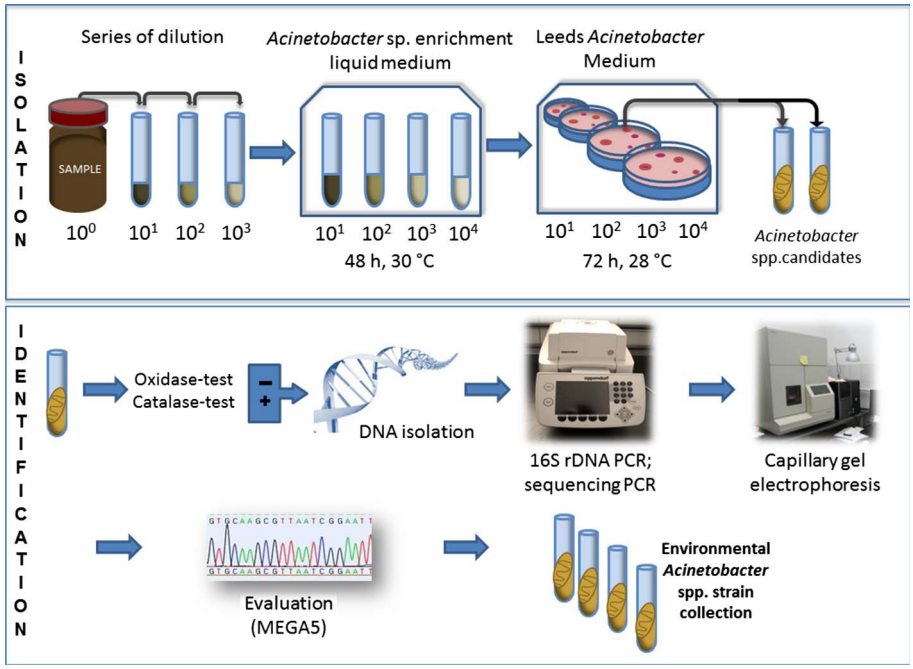

Figure 1. The multistep method for selective isolation and identification of *Acinetobacter* strains

(8 isolates) samples (Table I). Among environmental isolates, the overwhelming occurrence of *A. calcoaceticus*-related strains ($n = 20$) was observed followed by representatives of species *A. baumannii* ($n = 4$), *A. johnsonii* ($n = 4$), *A. pittii* ($n = 4$), *A. beijerinckii* ($n = 2$), *Acinetobacter gyllenbergii* ($n = 1$), *Acinetobacter radioresistens* ($n = 1$), and *Acinetobacter soli* ($n = 1$). Fifteen strains were isolated from TPH-contaminated sites, 3 strains from PAH-contaminated soil, 1 strain from MTBE-contaminated groundwater, and 18 strains from non-contaminated samples (10 strains from groundwater and 8 strains from fishponds). As we know, *Acinetobacter* species can degrade different hydrocarbons [15–17, 36, 37], but based on our results, their adaptation and degradation abilities are not determining factors regarding their environmental frequency. About 75.6% of the identified *Acinetobacter* spp. were the members of the ACB complex with verified human health concerns and three of the remaining species (*A. beijerinckii, A. johnsonii,* and *A. gyllenbergii*) are considered as emerging human pathogens that may become increasingly important in the future [4].

Surprisingly, the numbers of *A. baumannii* strains in the examined environmental samples were quite low, i.e., only 4 of 37 strains (Table I), while previous studies reported environmental *A. baumannii* in greater numbers [14, 20, 21].

However, the 16S rRNA-based identification was missed from those papers and identification was based on phenotypic methods or matrix-assisted laser desorption ionization–time of flight mass spectrometry accepted with relatively low (2.005–2.418) score values. Considering the different identification procedures, it is possible that the real environmental frequency of *A. baumannii* is lower than it was previously expected, and our results reflect the proportion closer to the reality. *A. baumannii* was detected mainly in fishponds (three of four strains), but the low detection rate does not let us draw general conclusions. Definite correlation between the occurence of *A. baumannii* and the level or type of the contamination of environmental samples could not be found. Further investigations may help us to inform about the effects of the available nutrients, oxygen, or other biogeochemical factors of the environmental samples on the frequency of *A. baumannii*.

The results of antibiotic susceptibility tests and the minimal inhibitory concentrations of antibiotics in the case of the examined environmental strains are summarized in Table III.

The results of the MIC test indicated that the majority of the investigated environmental acinetobacters were sensitive to the most widely used antibiotics. Imipenem, meropenem, gentamicin, ciprofloxacin, ofloxacin, and ceftazidime proved to be highly effective antibiotics, inhibiting the growth of nearly all tested acinetobacters (from 89% to 97%).

According to reports of National Center for Epidemiology (NCE) in Hungary, since 2010 there has been a steady increase in the prevalence of clinical acinetobacters resistant to imipenem, meropenem, gentamicin, ciprofloxacin, and ceftazidime [38]. According to NCE, 49.4% of clinical ACB complex strains exhibited resistance to imipenem and 53% against meropenem in 2012. Approximately 20.1% of other clinical acinetobacters (other than the members of ACB complex) showed resistance against both tested carbapenems [39]. On the contrary, 96.5% of the environmental strains with a closer phylogenetic relationship to the ACB complex were susceptible to the tested carbapenems. Solely *A. pittii* "II HT-1/1" strain (isolated from a fishpond sample) exhibited intermediate resistance against meropenem. Based on the NBS Annual Reports [39], ciprofloxacin resistance among clinical ACB complex strains and other clinical acinetobacters was 80.4% and 41.3% in Hungary, respectively. Conversely, 100% of our environmental ACB complex strains showed susceptibility to ciprofloxacin. Although there are no available data from 2012, in 2011, 84.5% of clinical ACB complex strains and 32.3% of other clinical acinetobacters exhibited resistance to ceftazidime [40].

In addition to the Hungarian reports of NCE, other European [41] and American [42] study groups observed a high rate of resistance among clinical *Acinetobacter* spp. to carbapenems (12% and 47%), to quinolones (51% and 67%), and to the third-generation cephalosporins (47% and 62%, respectively).

Table III. Antibiotic susceptibility patterns of the isolated *Acinetobacter* strains investigated with MIC Test Strips method

	Antibiotic resistance (R/I/S), MIC values																							
Groups of antimicrobial agents	Carbapenems				Aminoglycosides		Fluoroquinolones				Cephalosporins								Glycylcyclines		Penicillins		Rifamycins	
	IMI		MRP		GM		CIP		OFX		CTX		CAZ		CRO		FEP		TGC		PIP		RD	
Antimicrobial agents concentration (μg)	0.002–32		0.002–32		0.016–256		0.002–32		0.002–32		0.016–256		0.016–256		0.016–256		0.016–256		0.016–256		0.016–256		0.002–32	
ACB complex																								
A. baumannii Sk-V/3	S	0.19	S	0.25	S	1.5	S	0.19	S	0.75	S	8	S	3	I	12	S	2	S	0.094	S	16	I	3
A. baumannii HT-4/1	S	0.125	S	0.125	S	0.5	S	0.012	S	0.125	S	4	S	3	S	8	S	1.5	S	<0.016	S	0.094	S	0.5
A. baumannii HT-4/3	S	<0.002	S	0.19	S	<0.016	S	0.094	S	0.25	S	0.75	S	2	S	0.75	S	2	S	0.047	S	16	S	<0.016
A. baumannii II HT-3/1	S	0.19	S	0.125	S	0.5	S	0.064	S	0.19	S	8	S	6	S	3	S	8	S	0.047	I	24	I	3
A. calcoaceticus TN6	S	0.19	S	0.25	S	1	S	0.38	S	0.38	I	24	S	6	I	48	I	16	S	0.023	I	24	I	2
A. calcoaceticus PT 2/2	S	0.125	S	0.38	S	1	S	0.25	S	0.38	I	16	S	4	I	32	S	3	S	0.032	I	24	I	1.5
A. calcoaceticus PT 3/6	S	0.125	S	0.25	S	1.5	S	0.19	S	0.25	I	12	S	4	I	24	S	4	S	0.047	I	32	I	1.5
A. calcoaceticus PT 3/9	S	0.125	S	0.25	S	0.38	S	0.19	S	0.25	S	8	S	3	I	16	S	2	S	0.047	I	32	I	2
A. calcoaceticus 1/2	S	0.19	S	0.25	S	0.75	S	0.094	S	0.38	I	16	S	8	I	48	S	4	S	0.032	I	32	I	1.5
A. calcoaceticus 5/06/4	S	0.125	S	0.25	S	3	S	0.19	S	0.25	I	24	S	6	I	24	S	6	S	0.032	I	48	I	1.5
A. calcoaceticus PM 5	S	0.19	S	0.19	S	1	S	0.19	S	0.38	I	24	S	6	I	48	S	6	S	0.047	R	>256	I	1.5
A. calcoaceticus PM 7	S	0.25	S	0.25	S	2	S	0.19	S	0.5	I	16	S	8	I	16	S	4	S	0.047	I	64	I	3
A. calcoaceticus PM 13	S	0.19	S	0.38	S	0.75	S	0.19	S	0.25	I	24	S	6	I	32	S	4	S	0.032	R	>256	I	2
A. calcoaceticus PM 7/3	S	0.125	S	0.25	S	1	S	0.125	S	0.38	I	12	S	4	I	24	S	2	S	0.047	S	16	I	1.5
A. calcoaceticus DM-5/1	S	0.5	S	0.25	S	2	S	0.5	S	0.25	I	24	S	4	I	16	I	12	S	0.094	I	24	I	1.5
A. calcoaceticus Sk-V	S	0.5	S	0.38	S	1	S	0.38	S	0.19	I	16	S	6	S	12	S	8	S	0.094	I	32	I	3
A. calcoaceticus 5/06	S	0.75	S	0.38	S	1	S	0.38	S	0.25	I	48	S	8	I	32	I	16	S	0.094	I	32	I	3
A. calcoaceticus 22	S	0.5	S	0.19	S	0.5	S	0.38	S	0.19	I	12	S	4	I	16	S	8	S	0.125	I	32	I	2
A. calcoaceticus 24	S	0.5	S	0.25	S	1.5	S	0.38	S	0.19	I	24	S	4	I	24	S	8	S	0.094	I	32	I	3
A. calcoaceticus 42	S	0.75	S	0.38	S	0.75	S	0.5	S	0.25	I	32	S	8	I	16	I	12	S	0.125	I	32	R	4
A. calcoaceticus J5	S	0.75	S	0.5	S	0.75	S	0.5	S	0.25	I	24	S	6	I	16	I	16	S	0.064	I	32	I	3
A. calcoaceticus J7	S	1	S	0.25	S	0.5	S	0.75	S	1	I	32	S	4	I	24	I	12	S	0.125	I	32	I	3
A. calcoaceticus TV-11/J4	S	0.75	S	0.38	S	1	S	0.38	S	0.125	I	32	S	8	I	16	I	16	S	0.094	I	24	R	4
A. calcoaceticus SV/1	S	1	S	0.19	I	8	S	0.5	S	0.25	I	32	S	4	I	16	S	8	S	0.064	I	24	I	3
A. pittii PM 15/3	S	0.19	S	0.25	S	1	S	0.19	S	0.25	I	12	S	2	I	12	S	2	S	0.064	I	32	I	2
A. pittii II HT-1/1	S	0.5	I	48	S	0.125	S	0.125	S	8	S	4	S	6	S	4	S	0.047	S	0.38	S	0.125	I	3
A. pittii BUT-8/1	S	1	S	0.38	S	0.75	S	0.25	S	0.25	I	12	S	3	S	8	S	4	S	0.125	I	24	I	2
A. pittii FK-3/J2	S	1	S	0.25	S	1.5	S	0.25	S	0.25	I	16	S	4	I	12	S	6	S	0.064	I	24	R	4
A. beijerinckii T3N6	R	>32	R	>32	S	0.38	S	0.75	S	0.38	R	>256	R	>256	R	>256	R	48	S	0.023	I	24	S	0.75
A. beijerinckii Z4N3	S	0.094	S	0.75	S	0.19	S	0.125	S	0.25	S	1.5	S	3	S	4	S	1.5	S	0.094	S	12	S	0.75
A. gyllenbergii 6/2	S	0.75	S	0.064	S	0.125	S	0.38	I	4	R	128	R	128	I	24	R	32	S	0.023	R	>256	S	1
A. johnsonii Z4SZ2	S	0.125	S	0.19	S	0.5	S	0.047	S	0.19	S	6	I	12	S	3	S	8	S	<0.016	S	8	S	0.5
A. johnsonii ST-2	S	0.75	S	0.38	S	0.75	S	0.125	S	0.094	S	8	S	8	S	1.5	I	16	S	0.047	I	24	I	1.5
A. johnsonii J6	S	0.5	S	0.19	S	0.19	I	2	S	2	R	>256	R	>256	R	128	R	>256	S	0.094	S	6	I	1.5
A. johnsonii 6/1	S	0.5	S	0.38	S	0.75	R	6	S	0.25	R	>256	R	>256	R	>256	I	24	S	0.023	R	>256	S	0.5
A. radioresistens J3	S	3	S	0.094	S	1	S	0.094	S	0.19	S	1	S	0,5	S	1.5	S	0.5	S	0.032	R	>256	I	3
A. soli II HT-4/2	S	0.064	S	0.125	S	0.25	S	0.032	S	0.38	S	3	S	1.5	S	2	S	4	S	<0.016	R	>256	I	1.5

Note: Shaded cells represent antibiotic resistance. MIC: minimal inhibitory concentration; IMI: imipenem; MRP: meropenem; CN: gentamicin; CIP: ciprofloxacin; OFX: ofloxacin; CTX: cefotaxime; CAZ: ceftazidime; CRO: ceftriaxone; FEP: cefepime; TGC: tigecycline; PIP: piperacillin; RD: rifampicin; S: sensitive; I: intermediate resistant; R: resistant.

Moreover, a recent study on *A. baumannii* originated from a sewage treatment plant verified that 82% of the isolates were resistant to carbapenems, fluoroquinolones, and colistin [14].

Until the early 1970s, gentamicin (aminoglycoside) was successfully applied against nosocomial *Acinetobacter* infections [42]. According to Towner [5], acinetobacters usually show a higher rate of aminoglycoside resistance than other pathogens, which can correlate with the results of NCE. In 2012, 69% of clinical ACB complex strains and 34.1% of other clinical *Acinetobacter* strains were resistant to gentamicin in Hungary [39], but our environmental strains showed nearly complete susceptibility to gentamicin (97.3%).

When the aforementioned antibiotics are not effective in nosocomial *Acinetobacter*-related infections, tigecycline is considered as the drug of choice [43]. In this study, tigecycline was the sole antibiotic that inhibited the proliferation of all environmental *Acinetobacter* strains (100% efficacy).

Cefotaxime, ceftriaxone, cefepime, piperacillin, and rifampicin were the least effective antibiotics against the proliferation of the investigated environmental *Acinetobacter* strains. Acinetobacters obtained in this study widely exhibited intermediate resistance/resistance against the tested the third-generation cephalosporins (70% resistance to cefotaxime, 65% to ceftriaxone, and 32.4% to cefepime). Furthermore, 78% of strains were resistant to piperacillin and 81% to rifampicin. The rifampicin resistance of strains presented in this study is in contrast with the findings of Dhakephalkar and Chopade [20] who obtained rifampicin susceptibility higher than 90% among environmental acinetobacters.

Regarding multidrug resistance, *A. baumannii* that usually exhibits an elevated ability to rapidly develop antibiotic resistance [44] showed the lowest antibiotic resistance among all tested environmental strains (susceptibility against 10–12 antibiotics) followed by *A. pittii* and *A. calcoaceticus* (susceptibility from 7 to 10 antibiotics).

Environmental strains exhibiting simultaneous resistance against several classes of antibiotics were species *A. beijerinckii*, *A. gyllenbergii,* and *A. johnsonii* that are considered as emerging human pathogens [4]. These isolates met the currently accepted definition of multidrug resistance [45]. *A. beijerinckii* "T3N6" isolated from TPH-contaminated groundwater was resistant to cephalosporins and to the tested carbapenem, too. Since carbapenems are often considered as antibiotics of "last resort" in treatment of MDR *Acinetobacter* [46], the occurrence of a multidrug- and carbapenem-resistant environmental *A. beijerinckii* isolate, whose clinical counterparts may cause endocarditis [4], can be worrying. To the best of our knowledge, the antibiotic resistance of *A. beijerinckii* against carbapenems and cephalosporins has never been reported earlier (neither among nosocomial nor among environmental strains).

A. johnsonii strain "6/1" (Table I) isolated from an overwintering fishpond showed the second highest rate of resistance to five antibiotics: ciprofloxacin, cefotaxime, ceftazidime, ceftriaxone, and piperacillin. Therefore, strain "6/1" is considered as MDR. *A. gyllenbergii* "6/2" (originated from the same fishpond as strain "6/1") showed resistance against cephalosporins, piperacillin, and exhibited intermediate resistance to ofloxacin; therefore, it is also considered as an MDR strain. Based on the overview of scientific sources, the resistance of *A. johnsonii* strains against ciprofloxacin, cephalosporins, and piperacillin has never been reported earlier.

In comparison with clinical isolates, it can be summarized that the occurrence of antibiotic resistance among terrestrial and aquatic representatives of the genus *Acinetobacter* does not reach the clinical level, but it needs more attention and continuous monitoring. The detected rates of antibiotic resistance among environmental strains can be explained with several reasons such as natural resistance or horizontal gene transfer with other naturally occurring bacteria. The antibiotic residues in the environment and the effect of the misuse or overuse of antibiotics may also cause problems among environmental strains. However, we did not have the chance to examine these scenarios. Further investigations of fishponds with antibiotic treatment or communal, industrial effluents with antibiotic residues may help us to clarify this possibility. Wastewaters can be important sources of MDR bacteria and antibiotic residues in the environment because antibiotics are not completely metabolized by the human body [47]. Since modern wastewater treatment plants are still not designed to remove antibiotic residues found in domestic wastewaters [48], and urban sewage system can be considered as the secondary habitat of *A. baumannii* [14], the active compounds of antibiotics in the form of micropollutants may trigger the evolution of drug-resistant pathogens.

Regarding environmental contamination, we found that hydrocarbon-contaminated environments (soil and groundwater) may function as reservoirs or incubators for antibiotic-resistant acinetobacters. Considering that catabolic and resistance genes are often located on the same mobile genetic elements (plasmids) and a hydrocarbon pollution may act like an impulsive force for horizontal gene transfer [49], the prevalence of MDR acinetobacters within contaminated environments is still a possibility. However, the low number of isolated ACB strains cannot let us draw general conclusions.

Conclusions

This study provides a new, multistep method for the effective and selective isolation of environmental *Acinetobacter* species, which was checked by

molecular genetic identification based on 16S rRNA. Based on this isolation protocol, a collection of well-characterized environmental acinetobacters was available for further investigations.

We can conclude that environmental habitats may function as reservoirs or incubators for acinetobacters regardless their contamination (TPH, PAH, and MTBE) or matrix (groundwater, fishpond, and soil). As their presence is verified, the chance that environmental *Acinetobacter* strains can reach the infectious dose and may pose a public health risk cannot be excluded.

The antibiotic assay served pieces of evidence for the presence of antibiotic resistance and in some cases, multidrug resistance among the representatives of the environmental *Acinetobacter* strain collection. However, the antibiotic susceptibility patterns of environmental acinetobacters remarkably differ from clinical counterparts. Tigecycline (glycylcycline) proved to be the most effective antibiotic agent against environmental *Acinetobacter* proliferation (100% efficiency).

Based on our investigations, the detection rates and the antibiotic resistance profiles of the examined *Acinetobacter* spp. are not worrying but cannot be ignored and need further investigations. It is a warning sign that one MDR organism *A. beijerinckii* "T3N6" was found in a TPH-contaminated groundwater, which exhibited resistance to antibiotics of the "last resort" and its clinical counterparts can cause life-threatening infection (endocarditis). This result suggests the necessity of the detailed examination of acinetobacters that are used for industrial purposes. As a precaution, it can be suggested that opportunistic or unidentified microorganisms with the chance to cause infections or spread antibiotic resistance should be replaced with safe (Risk Group 1) ones as the classification of 2000/54/EC Directive suggests [50].

Based on our results, environmental acinetobacters other than the members of the clinically important ACB complex, namely *A. beijerinckii*, *A. johnsonii,* and *A. gyllenbergii* require detailed examinations regarding their public health and antibiotic resistance issues.

Our results may give a good basis for further examinations to reveal the significance of the human risk of environmental *Acinetobacter* strains. The improved multistep isolation and identification method may help to reach further results regarding the environmental role of *Acinetobacter* strains of various habitats.

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Conflict of Interest

The authors declare no conflict of interest.

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