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ARTICLE

Presence of carbapenem-resistant bacteria in soils affected by illegal waste dumps

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ABSTRACT

The carbapenem-resistant bacteria (CRB) are currently at the top of the WHO priority list of bacteria that pose the greatest threat to human health. Considering that soil is one of the important environments for the emergence of antibiotic-resistant bacteria, we isolated and quantified cultivable CRB in soils across Croatia, including ones affected by illegal dumps.

We cultivated CRB at two temperatures, distinguishing between the intrinsically resistant CRB (37°C, mostly *Stenotrophomonas* spp.) and the ones that are presumably human-associated and clinically relevant (42°C, *Acinetobacter* sp., *Enterobacteriaceae, Burkholderia* spp.). Our study demonstrated that distinguishing between the two offers a better insight into the diversity of CRB in the environment. The ones cultivated at 37°C were found in almost all soil samples, while the presumably clinically relevant ones were absent from uncontaminated pasture and grassland, indicating that human-associated CRB are unlikely to be found in soils spared from anthropogenic influence.

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Antibiotics; resistance; soil pollution; environment; dissemination

Introduction

Antibiotic-resistant bacteria (ARB) are considered to be the consequence of selective pressures exerted by antimicrobials used in clinical practice, animal food, and agriculture (Levy 2002). The term *antimicrobial* is not limited to antibiotics but includes many other disinfecting agents such as triclosan, chlorhexidine, or quaternary ammonium compounds. Disinfectant/Antibiotic cross-resistance in bacteria is well documented (Levy 2002; Gnanadhas et al. 2013; Wesgate et al. 2016). Therefore, the release of antimicrobials in the environment may be as important for ARB emergence as is patient treatment with antibiotics (Levy 2002).

Furthermore, there are bacteria that are intrinsically resistant to antibiotics without ever having been exposed to them in their environment. For example, genes carrying resistance to commercial antibiotics have been found in cultivable bacteria from a cave isolated from anthropogenic influence for 4 million years (Bhullar et al. 2012) or in a DNA isolated from a 30,000-year-old permafrost sediment (D'Costa et al. 2011).

Of all types of environment, the soil probably has the largest and most divergent resistome (a collection of all the antibiotic resistance genes), which includes both the bacteria with intrinsic

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and the ones with acquired antibiotic resistance. In fact, many of the clinically resistant bacteria arise from the soil bacteria (D'Costa et al. 2006, 2007; Alkhaleefah 2015).

In 2017, the World Health Organisation issued its first ever list of antibiotic-resistant 'priority pathogens' – a catalogue of 12 families of bacteria that pose the greatest threat to human health (WHO 2017). Among those, carbapenem-resistant bacteria (CRB), namely *Acinetobacter baumannii, Pseudomonas aeruginosa*, and *Enterobacteriaceae* are on the top of the list. In fact, carbapenem resistance is increasing rapidly and becoming a major healthcare problem worldwide (Meletis 2016).

In Croatia, the prevalence of carbapenem-resistant isolates among all clinical isolates has increased from 34% to 86% for *A. baumannii*, 12–21% for *P. aeruginosa*, and from none to detectable (1%) for *Enterobacteriaceae* between 2010 and 2016 (CAMS 2017 – Antibiotic resistance in Croatia, 2010 & 2016).

CRB were initially considered exclusively hospital-acquired pathogens, but recent literature describes community-acquired infections (Dexter et al. 2015), which are not rare (Tang et al. 2016). Tang et al. (2016) reported that almost one-third of human infections with carbapenem-resistant *Enterobacteriaceae* were community-acquired, while 10% were colonisations. The origins of community-acquired CRB infections are unknown (Kelly et al. 2017). Also unknown are the ways by which CRB enter and colonise hospitals worldwide. In any case, the existence of community-acquired infections points to living environments as possible reservoirs of CRB (Eveillard et al. 2013). Recent studies have started to monitor and report CRB occurrence in hospital or municipal wastewaters (Picão et al. 2013; Bengtsson-Palme et al. 2016; Hrenovic et al. 2017a). The fact that both ARB and antibiotic-resistance genes are readily disseminated to the environment through wastewaters and wastewater treatment plants is sufficiently described (Bengtsson-Palme et al. 2016; Yang et al. 2016; Hrenovic et al. 2017a; Dal 2018). In contrast, the data about CRB in soils are still scarce, and to the best of our knowledge, carbapenemase genes and CRB were reported in soil by Gudeta et al. (2016), Alkhaleefah (2015), and Hrenovic et al. (2014, 2017b). The last two found CRB in soils influenced by illegally disposed solid waste of external origin, including hospital waste, which raised concern about an association between Gram-negative CRB in soils and illegal waste dumping in Croatia, especially in view that the central Balkans (including Croatia) is considered one of the world's epicentres of CRB global spread (Meletis 2016).

Therefore, the aim of our study was to look deeper into the issue by isolating and quantifying cultivable CRB in soils affected by illegal dumps across Croatia and to compare the findings with those in unaffected soils and soils under the influence of other human activities that might contribute to the CRB spread. To do that, we used a novel method that would be able to distinguish between native, intrinsically resistant soil bacteria and those that are presumably of anthropogenic origin, therefore clinically relevant. This methodology has been successfully used in our previous studies where CRB were monitored in wastewater treatment plant (Hrenovic et al. 2017a, 2017c) and now it was, to best of our knowledge, implemented for soil samples for the first time. This methodology is based on cultivation of environmental samples on 37 and 42°C in parallel. The latter supresses the growth of *Stenotrophomonas* sp. (Denton and Kerr 1998) which are intrinsically carbapenem-resistant and ubiquitous in soil and therefore dominant in soil samples cultivated at 37°C. Cultivation at 42°C allows the detection of other CRB that are usually present in lower abundance than *Stenotrophomonas* sp.

Materials and methods

Soil sampling

Twenty-two soil samples were taken at 14 locations (8 illegal dump sites, 1 coke factory, 1 thermal power plant, 2 arable lands, 1 pasture, and a grassland) in period from April 2014 to

July 2017 (Table 1). Except for the pasture on the island of Mljet and grassland at the slopes of Medvednica, the remaining 12 locations were selected from the Soil Monitoring Programme (Mesic et al. 2008) for potentially contaminated soil in Croatia. Figure 1 shows some of the locations.

Samples for bacteriological analysis were taken aseptically in a sterile plastic container and prepared in the laboratory within 6 h of collection. Soil water content was measured gravimetrically, and the pH was measured in soil suspension in distilled water (1:2.5 m/v ratio).

Bacteriological analysis

The soil samples were first suspended in sterile peptone water (10 g of soil per 100 mL) and then filtered three times on sterile membrane filters of 0.45 μm pore size (10 mL aliquot). Next, membrane filters were placed on adequate cultivation media as follows:

Intestinal enterococci were determined according to the current Croatian standard (HRN ISO 7899-2, 2000): membrane filters were incubated on Slanetz Bartley agar (Biolife, Milano, Italy) at 37°C for 72 h and confirmed on Bile esculin azide agar (Sigma-Aldrich, Taufkirchen, Germany) after incubation at 44°C for 4 h.

The CRB were cultivated by placing membrane filters on selective CHROMagar™ Acinetobacter medium (CHROMagar, Paris, France): agar: 15 g L^{-1}; peptone and yeast extract: 12 g L^{-1}; salts: 4 g L^{-1}; chromogenic mix: 1.8 g L^{-1}, supplemented with CR102 (one dose per litre) (CHROMagar 2018). For each sample, two incubation temperatures were used, 37 and 42°C (Hrenovic et al. 2017a, 2017c). All the colonies that grew on the plates were labelled CRB and labelled as CRB37 or CRB42. Referencing to our previous work (Hrenovic et al. 2017a, 2017c), the CRB37 are considered to be natively present and intrinsically carbapenem-resistant while CRB42 are considered to be of anthropogenic origin, and potentially clinically relevant, carrying acquired resistance.

The CR102 supplement allows for the growth of carbapenem-resistant *Acinetobacter* sp. and other carbapenem-resistant Gram-negative bacteria, belonging mostly to the *Enterobacteriaceae, Pseudomonas* spp., and *Stenotrophomonas* genera (Figure 2) (Barsoumian et al. 2013; Song et al. 2013). Just to confirm that all bacteria cultivated on CR102 plates can be considered carbapenem-resistant, random isolates were tested using the Vitek® automated system for determination of antibiotic susceptibility (Biomerieux, Marcy-l'Étoile, France). Each tested isolate (N = 20) had minimum inhibitory concentrations of imipenem and meropenem of 8 up to ≥16 mg L^{-1}, which classified them as carbapenem-resistant according to the EUCAST criteria for clinical isolates (EUCAST 2017). This confirmed that all bacteria cultivated on CHROMagar™ Acinetobacter plates supplemented with CR102 can be labelled as CRB.

The aerobically grown total heterotrophic bacteria were determined by counting all grown colonies on tryptone glucose yeast agar (Biolife, Milano, Italy) after appropriate serial dilution and incubation at 22°C for 72 h (APHA, 2005).

The counts of total heterotrophic bacteria (He), intestinal enterococci (Ie), and CRB (CRB37 and CRB42) were expressed in colony forming units per 1 g of wet soil (CFU g^{-1}). Enumeration of bacteria was done in triplicate.

Identification of CRB isolates

For insight in bacterial species carrying carbapenem-resistance in tested soil samples, certain isolates were identified to genus and/or species level. The identification was not performed systematically, meaning not every CRB colony was identified, but random morphologically different colonies were subjected to further analysis. This approach does not give complete picture of population density, as

Table 1. List and description of collected soil samples.

No	Sample name	Description	Location[1] (x/y)	Depth	Sampling date (dd/mm/yy)
1	Mljet	Pasture	700,322.05/4738,419.01	0–20 cm	06/10/14
2	Susak 1	Illegal dump site	445,503.27/4928,432.91	0–20 cm	11/04/14
3	Susak 2			20–40 cm	
4	Samobor	Illegal dump site	555,430.88/5065,621.95	0–20 cm	07/07/14
5	Bakar 1	Coke factory sanitary landfill	464,358.38/5016,331.27	0–10 cm	05/10/16
6	Bakar 2**				
7	Sovjak 1	Illegal dump site	452,276.54/5024,073.81	0–20 cm	
8	Sovjak 2**				
9	Plomin	Thermal power plant	434,397.75/4998,174.70		
10	Sv. Lovrec 1***	Quarries and illegal dump site	403,571.26/5002,171.95	0–20 cm	29/10/16
11	Sv. Lovrec 2***				
12	Sv. Lovrec 4***			0–10 cm	
13	Loborika 1	Illegal dump site	412,839.14/4975,388.41	0–10 cm	
14	Loborika 2	Illegal dump site	413,154.55/4975,424.01		
15	Morinje 1	Illegal dump site with visible medical waste	576,132.35/4838,169.02	0–10 cm	13/12/16
16	Morinje 2**				
17	Zaton	Illegal dump site next to a freeway	565,047.34/4849,530.86	0–20 cm	
18	Medjimurje 1	Agricultural soil fertilised with swine derived manure	598,883.32/5141,976.56	0–30 cm	05/07/17
19	Medjimurje 2			30–60 cm	
20	Medjimurje 3	Agricultural soil fertilised with poultry derived manure	603,917.42/5142,341.49	0–30 cm	
21	Medjimurje 4			30–60 cm	
22	Medvednica	Grassland	583,565.55/5085,759.93	0–20 cm	17/10/16

**Second soil sample was taken at the same location within 10 m distance. ***Three soil samples were taken at the same location within 20 m distance. [1]WGS 84/UTM zone 33N.

Figure 1. Photographs of several sites from which soil samples were collected. The samples are described in Table 1.

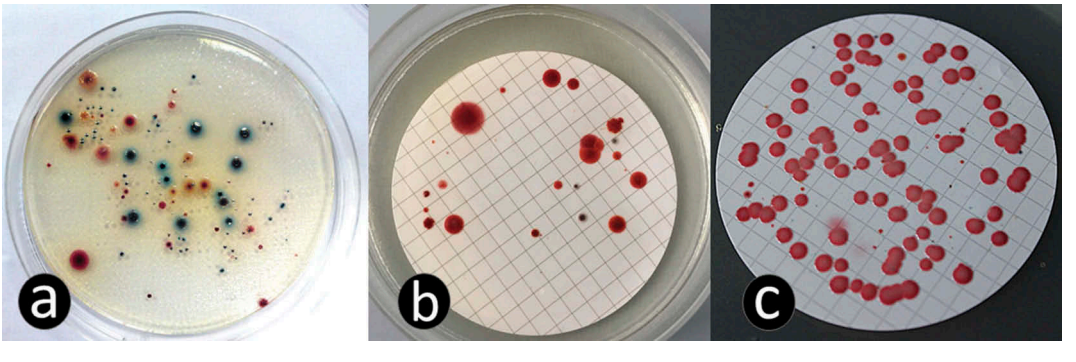

Figure 2. (a) An example of CHROMagar AcinetobacterTM plate (without CR102 supplement for cultivation of carbapenem-resistant bacteria) inoculated with wastewater sample after incubation at 37°C for 48 h. The blue colonies are usually *Enterobacteriaceae* and red colonies are *Stenotrophomonas* sp., *Acinetobacter* sp., *Pseudomonas* sp., or other Gram-negative bacteria. (b and c) – example of CHROMagar AcinetobacterTM (with CR102 supplement) from here presented study, inoculated with soil sample and incubated at 37°C (b) and 42°C (c).

could be determined by molecular techniques, but it gives information about presence of cultivable CRB in soil that can be of clinical relevance and potential risk to public health.

For identification, selected colonies were re-cultivated in pure culture on selective plates (CHROMagar™ Acinetobacter supplemented with CR102) and then on non-selective nutrient plates (tryptone glucose yeast agar). Pure culture grown on non-selective plate was subjected to routine bacteriological techniques (Gram stain, oxidase and catalase test, growth on Kligler-iron agar) and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) (software version 3.0, Microflex LT, Bruker Daltonics) (Saffert et al. 2011; Schauman et al. 2013; Sousa et al. 2014; Singhal et al. 2015). Only the isolates with a valid MALDI-TOF MS score of ≥2.0 (Bizzini et al. 2010), meaning undoubtedly identified to genus/species level, were presented in the results (N_{total} = 100; 63 valid and 37 invalid scores).

Statistical analysis

Before statistical analysis, we logarithmically transformed the CFU (log CFU per 1 g of wet soil) to normalise the distribution. Statistically significant correlations between variables ($p < 0.05$) were determined in Statistica v. 13.1. (TIBCO Software Inc., Palo Alto, CA, USA) using Spearman's rank correlation.

Results

CRB in soil

Table 2 shows the bacterial counts by soil sample. CRB37 were detected in all but two soil samples. Opposite to what one might expect, these two samples were *not* from uncontaminated locations of pasture and grassland but from quarry dump site and coke factory sanitary landfill. The reasons for the absence of CRB37 in sample No. 10 are most likely the extremely low pH of 2.6 (pH of other soils was ranging from 4.9 to 9.7; mean ± sd = 7.4 ± 1.1) and in sample No. 5, the low humidity of 9% (humidity of other soils was ranging from 12.1 to 57.1; mean ± sd = 22.7 ± 11.1). These two samples also had significantly ($p < 0.05$) lower total heterotrophic bacterial counts than other soils.

The presence of CRB37 in non-contaminated pasture and grassland soil (samples No. 1 and 22) confirms that soil in general contains intrinsically resistant CRB, except where conditions are unfavourable for the growth of mesophilic Gram-negative bacteria (extreme pH, low humidity).

Table 2. Numbers of bacteria detected in soil samples (log CFU g^{-1}).

Sample	1	2	3	4	5	6	7	8	9	10	11
He	4.8	7.3	6.9	8.4	5.1	6.8	6.7	6.5	6.8	2.3	6.4
Ie	*	1.9	1.4	6.2	2.8	1.7	3.4	1.6	1.5	*	2.0
CRB37	2.4	nm.	nm.	nm.	*	4.3	3.1	4.1	3.0	*	2.6
CRB42	*	4.7	3.6	4.6	*	3.3	2.0	1.4	2.7	*	1.4
pH	7.3	8.3	8.3	7.3	8.5	7.9	9.7	6.8	7.9	2.6	7.8
W (%)	22.7	18.2	17.7	24.5	9.3	16.3	31.4	21.7	17.8	22.8	25.5
Sample	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**
He	7.9	7.4	7.1	7.5	7.1	7.6	7.7	6.5	7.8	6.9	8.1
Ie	1.3	1.8	2.5	2.4	2.4	1.9	0.0	0.0	0.0	0.0	2.2
CRB37	5.3	3.4	3.7	3.1	3.5	4.5	4.8	4.9	4.3	3.4	3.1
CRB42	1.2	*	1.4	2.3	2.1	3.9	2.6	3.1	3.5	2.4	*
pH	7.7	7.2	6.8	7.2	8.2	7.9	6.1	5.9	4.9	4.9	6.9
W (%)	15.5	57.1	31.6	28.4	28.5	14.6	15.1	16.5	14.8	12.1	42.4

Shaded are the highest numbers of specific type of bacteria across all the samples, as well as lowest pH and W (%) values.
*Below detection limit (<1 CFU g^{-1}). For clarity, only mean values are presented; standard deviations for all samples were in the range ±0.1–0.3. He: Total heterotrophs; Ie: intestinal enterococci; CRB37 and CRB42: carbapenem-resistant bacteria cultivated at 37 or 42°C, respectively; W: water content; nm: not measured.

The highest counts of CRB42 were found at the illegal dump site on the island of Susak (Table 2) and in agricultural soil samples fertilised with swine or poultry manure. The pasture on Mljet and grassland at Medvednica, which are considered as uncontaminated, free of any anthropogenic influence, turned out to be free from CRB42 (Table 2).

Statistical analysis showed that the CRB37 and CRB42 counts correlated positively ($p < 0.05$) with each other and the He counts (Table 3) but showed no correlation ($p > 0.05$) with the Ie counts, pH, and water content in soil. The pH correlated positively with Ie ($p < 0.05$), as this type of bacteria better adapts to high pH (Meckes and Rhodes 2004; Ivankovic et al. 2014). This finding also suggests that Ie, even though it is the usual indicator of anthropogenic influence, should not be considered as indicator of CRB presence in soil.

CRB identified in soil

Bacteria identified after cultivation at 37°C were intrinsically resistant to carbapenems with 10 isolates of *Stenotrophomonas* sp. and 1 isolate of *Elizabethkingia meningoseptica* (Zhang et al. 2000; Çıkman et al. 2016; EUCAST 2016). Table 4 shows CRB42 isolates randomly identified across the soil samples. Incubation at 42°C allowed for a greater variety of isolates, but dominant were the bacteria from the *Burkholderia, Acinetobacter*, and *Enterobacteriaceae* genera.

Table 3. The correlations of different groups of bacteria cultivated from soil samples, with each other and with pH and water content (W).

	He	Ie	CRB37	CRB42	pH	W (%)
He	1.000	0.222	0.781	0.599	0.409	0.088
Ie		1.000	−0.184	−0.087	0.745	0.368
CRB37			1.000	0.608	0.173	−0.083
CRB42				1.000	0.049	−0.385
pH					1.000	0.139
W						1.000

He: Total heterotrophs; Ie: intestinal enterococci; CRB37 and CRB42: carbapenem-resistant bacteria cultivated at 37 or 42°C, respectively.
Correlations; *R* values are shown; significant correlations ($p < 0.05$) are shaded.

Table 4. List of bacteria with acquired resistance to carbapenems randomly isolated after cultivation at 42°C.

Species	Number of isolates	Location*
Acinetobacter baumannii	3	8
Acinetobacter sp.	6	21
Burkholderia ambifaria	12	18/19/20
Burkholderia multivorans	2	14
Burkholderia sp.	2	21
Cupriavidus gilardii	3	2/3
Cupriavidus respiraculi	5	2/3
Enterobacter asburiae	1	18
Enterobacter cloacae	3	18/20
Enterobacter ludwigii	1	7
Enterobacter sp.	2	21
Escherichia coli	3	20
Escherichia sp.	1	21
Ochrobactrum intermedium	3	18
Pediococcus sp.	1	20
Providencia sp.	1	19
Providencia stuartii	1	18
Pseudomonas putida	1	2
Sphingobacterium thalpophilum	1	14

Isolates that were undoubtedly identified to genus or species level by valid MALDI-TOF MS score of ≥2.0 are shown. *Location where the bacteria were isolated (referenced to no in Table 1).

Discussion

Our findings confirm that ARB, including carbapenem-resistant ones, are normally present in soil and are a part of natural microflora (Forsberg et al. 2012; Nesme and Simonet 2015; Williams-Nguyen et al. 2016). The resistance genes found in soil are probably intrinsic to many soil-inhabiting bacteria (Nesme and Simonet 2015). Mechanisms of gene transfer from environmental to clinical pathogens, such as the horizontal or lateral, are known, but little is still known about to what extent intrinsic ARB from soil contribute to the global spread of antibiotic resistance. Recent review articles by Nesme and Simonet (2015) and Williams-Nguyem et al. (2016), therefore, clearly stress the need to focus more on environments affected by human activities.

Alkhaleefah (2015) found significantly higher CRB counts in soils from a farm using cattle manure than a farm using inorganic fertilisers, which points to animal manure as a source of ARB associated with anthropogenic activities, in this particular case, feeding the livestock with antibiotic-enriched feed. Our findings from agricultural soils fertilised with swine and poultry manure confirm Alkhaleefah's findings.

The novelty of our approach, however, is a methodology to distinguish between the soil bacteria with the intrinsic and acquired (clinically relevant) carbapenem resistance by incubating the samples at 37 and 42°C, respectively. As our results with the identified isolates show (Table 4), incubating at 37°C blurs the picture of how many CRB species are present in the soil, as the intrinsically resistant *Stenotrophomonas* spp. is dominant and wins the competition with other species. Only at 42°C, when growth of *Stenotrophomonas* spp. is supressed (Denton and Kerr 1998) did we detect isolates associated with human activities. This is probably why Gudeta et al. (2016), unlike us, found no *Enterobacteriaceae, Acinetobacter*, or *Pseudomonas* species in their samples or why Alkhaleefah (2015) found no *Enterobacteriaceae* or *Acinetobacter* spp. Incubation and enrichment temperature of 37 and/or 22°C could also explain why mentioned studies (Alkhaleefah 2015; Gudeta et al. 2016) reported finding carbapenemase genes that were not related to carbapenemases observed in clinical bacteria.

Even though we did not determine carbapenemase genes in our current study, in our earlier study (Hrenovic et al. 2014, 2017b), the *A. baumannii* isolates (from the same soil samples Nos. 8 and 12 in this study) carried acquired class D oxacilinase genes, which were not detected in the studies by Alkhaleefah (2015) and Gudeta et al. (2016). This strongly suggests that incubation of soil samples at 37°C is probably shading clinically relevant carbapenemase genes and CRB in soil. The main limitation of our study is that carbapenemase genes were not defined by molecular techniques and compared between samples incubated at two temperatures that would solidify the assumption that cultivation at higher temperature allows isolation of clinically relevant CRB from soil. We propose further research in this direction.

Thanks to our method for distinguishing intrinsically resistant CRB37 and presumably anthropogenic originated CRB42, we have gained a better insight into the distribution of clinically relevant CRB in soil; none were found in the uncontaminated pasture and grassland soil (samples 1 and 22) and soils with poor growth conditions (samples 5 and 10). The remaining illegal dump sites confirm that they are the most probable source of clinically relevant CRB in soil, which were probably leached from the waste by storm water and infiltrated the soil, where they can survive for long (Hrenovic et al. 2017b). These findings recognise illegal dump sites as potential sites of dissemination of ARB. More stringent monitoring and prevention of such sites as well as (bio) remediation of already contaminated sites is therefore desired.

Conclusions

Our study has demonstrated that distinguishing between the intrinsically resistant and presumably clinically relevant CRB makes a significant difference in analysing environmental samples for CRB, and we propose that future studies of the kind should try this method.

Intrinsically resistant CRB were found in almost all soil samples, while presumably clinically relevant ones were absent from uncontaminated pasture and grassland, indicating that human-associated CRB are unlikely to be found in soils spared from anthropogenic influence.

Since we did not genotype the isolated CRB or search for specific carbapenemase genes, future research could take this direction. Metagenomic analysis of soils conditioned and/or enriched at 42°C could give a more precise insight into the diversity of ARB in the environment.

Conflicts of interest

None to declare.

References

[WHO] World Health Organization website. [accessed 2017 June 27]. http://www.who.int/medicines/publications/WHO-PPL-Short_Summary_25Feb-ET_NM_WHO.pdf?ua=1.

Alkhaleefah FI. 2015. Solation and characterisation of imipenem-resistant bacteria from natural environments and clinical settings. [PhD Thesis]. United Kingdom: University of Hull.

Apha AWWA. 2005. WEF. standard methods for examination of water and 369 wastewater. 21th ed. New York: American Public Health Association.

Barsoumian A, Calvano T, Markelz AE, Cassidy R, Murray CK, Beckius ML, Mende K, Akers KS. 2013. Variations of CHROMagar Acinetobacter to detect imipenem-resistant *Acinetobacter baumannii – calcoaceticus* complex. Scandin J Infect Dis. 45:446–452.

Bengtsson-Palme J, Hammaren R, Pal C, Ostman M, Bjorlenius B, Flach CF, Fick J, Kristiansson E, Tysklind M, Joakim, et al. 2016. Elucidating selection processes for antibiotic resistance in sewage treatment plants using metagenomics. Sci Total Environ. 572:697–712.

Bhullar K, Waglechner N, Pawlowski A, Koreva K, Banks ED, Johnston MD, Barton HA, Wright GD. 2012. Antibiotic resistance is prevalent in an isolated cave microbiome. PLoS ONE. 7:e34953.

Bizzini A, Durussel C, Bille J, Greub G, Prod'hom G. 2010. Performance of matrix-assisted laser desorption ionization–time of flight mass spectrometry for identification of bacterial strains routinely isolated in a clinical microbiology laboratory. J Clin Microbiol. 48:1549–1554.

CAMS. 2017. Antibiotic resistance in Croatia, 2010 & 2016. Published by The Croatian Academy of Medical Sciences (CAMS). http://iskra.bfm.hr/hrv/Resistance.aspx?id=66

CHROMagar website. [accessed 12 September 2018]. http://www.chromagar.com/clinical-microbiology-chromagar-acinetobacter-focus-on-acinetobacter-50.html#.W5jFpuQnbcs

Çıkman A, Parlak M, Bayram Y, Güdücüoğlu H, Berktaş M. 2016. Antibiotics resistance of *Stenotrophomonas maltophilia* strains isolated from various clinical specimens. African Health Sci. 16:149–152.

HRN ISO 7899-2; EN ISO 7899-2. 2000. Water quality – detection and enumeration of intestinal enterococci – part 2: membrane filtration method. Croatian Standards Institute, Zagreb, Croatia.

D'Costa VM, Griffiths E, Wright GD. 2007. Expanding the soil antibiotic resistome: exploring environmental diversity. Curr Opin Microbiol. 10:481–489.

D'Costa VM, King CE, Kalan L, Morar M, Sung WL, Schwarz C, Froese D, Zazula G, Calmels F, Debruyne R, et al. 2011. Antibiotic resistance is ancient. Nature. 477:457–461.

D'Costa VM, McGrann KM, Hughes DW, Wright GD. 2006. Sampling the antibiotic resistome. Science. 311:374–377.

Dal KA. 2018. Antibiotic resistance and wastewater: correlation, impact and critical human health challenges. J Environ Chem Eng. 6:52–58.

Denton M, Kerr KG. 1998. Microbiological and clinical aspects of infection associated with *Stenotrophomonas maltophilia*. Clin Microbiol Rev. 11:57–80.

Dexter C, Murray GL, Paulsen IT, Peleg AY. 2015. Community-acquired Acinetobacter baumannii: clinical characteristics, epidemiology and pathogenesis. Expert Rev Anti-Infect Ther. 13:567–573.

EUCAST (European Committee on Antimicrobial Susceptibility Testing) intrinsic resistance and exceptional phenotypes. 2016. Expert rules version 3.1. [accessed 27 August 2018]. http://www.eucast.org/expert_rules_and_intrinsic_resistance/.

EUCAST (European Committee on Antimicrobial Susceptibility Testing) Reading guide. 2017. Version 7.1. [accessed 11 April 2018]. http://www.eucast.org/clinical_breakpoints/

Eveillard M, Kempf M, Belmonte O, Pailhories H, Joly-Guillou ML. 2013. Reservoirs of Acinetobacter baumannii outside the hospital and potential involvement in emerging human community-acquired infections. Int J Infect Dis. 17:802–805.

Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MOA, Dantas G. 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science. 337:1107–1111.

Gnanadhas DP, Marathe SA, Chakravortty D. 2013. Biocides – resistance, cross-resistance mechanisms and assessment. Expert Opin Investig Drugs. 22:191–206.

Gudeta DD, Bortolaia V, Amos GCA, Wellington EMH, Brandt KK, Poirel L, Nielsen JB, Westh H, Guardabassi L. 2016. The soil microbiota harbors a diversity of carbapenem-hydrolyzing β-lactamases of potential clinical relevance. Antimicrob Agents Chemother. 60:151–160.

Hrenovic J, Durn G, Goic-Barisic I, Kovacic A. 2014. Occurrence of an environmental *Acinetobacter baumannii* strain similar to a clinical isolate in paleosol from Croatia. Appl Environ Microbiol. 80:2860–2866.

Hrenovic J, Durn G, Seruga-Music M., Dekic S, Troskot-Corbic T, Skoric D. 2017b. Extensively and multi drug-resistant *Acinetobacter baumannii* recovered from technosol at a dump site in Croatia. Sci Total Environ. 607–608:1049–1055.

Hrenovic J, Ganjto M, Goic-Barisic I. 2017c. Carbapenem-resistant bacteria in a secondary wastewater treatment plant. Water SA. 43:186–191.

Hrenovic J, Ivankovic T, Ivekovic D, Repes S, Stipanicev D, Ganjto M. 2017a. The fate of carbapenem-resistant bacteria in a wastewater treatment plant. Wat Res. 126:232–239.

Ivankovic T, Hrenovic J, Itskos G, Koukouzas N, Kovacevic D, Milenkovic J. 2014. Alkaline disinfection of urban wastewater and landfill leachate by wood fly ash. Arh Hig Rada Toksikol. 65:365–375.

Kelly AM, Mathema B, Larson EL. 2017. Carbapenem-resistant Enterobacteriaceae in the community: a scoping review. Int J Antimicrob Agent. 50:127–134.

Levy SB. 2002. Factors impacting on the problem of antibiotic resistance. J Antimicrob Chemother. 49:25–30.

Meckes MC, Rhodes ER. 2004. Evaluation of bacteriological indicators of disinfection for alkaline treated biosolids. J Environ Eng Sci. 3:231–236.

Meletis G. 2016. Carbapenem resistance: overview of the problem and future perspectives. Ther Adv Infect Dis. 3:15–21.

Mesic H, Baksic D, Basic F, Cidic A, Durn G, Husnjak S, Kisic I, Klaic D, Komesarovic B, Mesic M, et al. 2008. Croatian soil monitoring programme: project development of the croatian soil monitoring programme with a pilot project LIFE05 TCY/CRO/000105.Zagreb, Croatia: Croatian environment agency; p. 136.

Nesme J, Simonet P. 2015. The soil resistome: a critical review on antibiotic resistance origins, ecology and dissemination potential in telluric bacteria. Environ Microbiol. 17:913–930.

Picão RC, Cardoso JP, Campana EH, Nicoletti AG, Petrolini FVB, Assis DM, Juliano L, Gales AC. 2013. The route of antimicrobial resistance from the hospital effluent to the environment: focus on the occurrence of KPC-producing *Aeromonas* spp. and *Enterobacteriaceae* in sewage. Diagn Microbiol Infect Dis. 76:80–85.

Saffert RT, Cunningham SA, Ihde SM, Monson Jobe KE, Mandrekar J, Patel R. 2011. Comparison of Bruker biotyper matrix-assisted laser desorption ionization–time of flight mass spectrometer to BD Phoenix automated microbiology system for identification of gram-negative bacilli. J Clin Microbiol. 49:887–892.

Schaumann R, Knoop N, Genzel GH, Losensky K, Rosenkranz C, Stingu CS, Schellenberger W, Rodloff AC, Eschrich K. 2013. Discrimination of Enterobacteriaceae and non-fermenting gram negative bacilli by MALDI-TOF mass spectrometry. Open Microbiol. 7:118–122.

Singhal N, Kumar M, Kanaujia PK, Virdi JS. 2015. MALDI-TOF mass spectrometry: an emerging technology for microbial identification and diagnosis. Front Microbiol. 6:791.

Song W, Lee J, Kim TK, Park MJ, Kim HS, Kim JS. 2013. Modified CHROMagar Acinetobacter medium for direct detection of multidrug-resistant *Acinetobacter* strains in nasal and rectal swab samples. Ann Lab Med. 33:193–195.

Sousa C, Botelho J, Silva L, Grosso F, Nemec A, Lopes J, Peixe L. 2014. MALDI-TOF MS and chemometric based identification of the *Acinetobacter calcoaceticus-Acinetobacter baumannii* complex species. Int J Med Microbiol. 304:669–767.

Tang HJ, Hsieh CF, Chang PC, Chen JJ, Lin YH, Lai CC, Chao CM, Chuang YC. 2016. Clinical significance of community- and healthcare-acquired carbapenem-resistant Enterobacteriaceae isolates. PLoS ONE. 11: e0151897.

Wesgate R, Grasha P, Maillard J. 2016. Use of a predictive protocol to measure the antimicrobial resistance risks associated with biocidal product usage. Am J Infect Control. 44:458–464.

Williams-Nguyen J, Sallach JB, Bartelt-Hunt S, Boxall AB, Durso LM, McLain JE, Singer RS, Snow DD, Zilles JL. 2016. Antibiotics and antibiotic resistance in agroecosystems: state of the science. J Environ Qual. 45:394–406.

Yang F, Mao D, Zhou H, Luo Y. 2016. Prevalence and fate of carbapenemase genes in a wastewater treatment plant in Northern China. PLoS One. 11:e0156383.

Zhang L, Li XZ, Poole K. 2000. Multiple antibiotic resistance in *Stenotrophomonas maltophilia*: involvement of a multidrug efflux system. Antimicrob Agent Chemother. 44:287–293.