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Research Article

Austria-wide survey on resistant, potentially pathogenic bacteria at Austrian bathing sites, 2017

Österreichweite Studie zum Vorkommen resistenter potenziell pathogener Bakterien in österreichischen Badestellen, 2017

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Summary

There is growing concern about human-induced antibiotic resistance and on the occurrence of antibiotic-resistant, potentially pathogenic bacteria in the environment. The aim of this study was to investigate the incidence of resistant, clinically relevant bacteria at bathing sites. In total, 27 of 263 bathing sites authorized under the EU Bathing Water Directive (3 per Austrian state) were sampled during the summer of 2017. Samples were tested for antibiotic-resistant bacteria by enrichment in thioglycollate broth and cultivation on chromogenic media. The screening for potentially pathogenic antibiotic-resistant bacteria was negative in 23 of the 27 samples. Antibiotic-resistant bacteria were detected from 4 of the 27 bathing sites: one *Pseudomonas aeruginosa* and three resistant *Enterobacteriaceae* (piperacillin/tazobactam-resistant *Enterobacter cloacae* with high-level expression of AmpC beta-lactamase, carbapenem-resistant *Enterobacter mori*, extended-spectrum beta-lactamase-producing *Escherichia coli*). Despite the occurrence of resistant bacteria, we consider the public health risk at Austrian bathing sites as low.

Keywords: antimicrobial resistance, surface water, pathogens, risk assessment, public health

Zusammenfassung

Die Besorgnis über vom Menschen verursachte Antibiotikaresistenzen und das Auftreten von antibiotikaresistenten, potenziell pathogenen Bakterien in der Umwelt wächst. In Österreich gibt es 263 Badestellen, die als EU-Badegewässer definiert sind. Ziel dieser Studie war es, das Vorkommen von resistenten, klinisch relevanten Bakterien in derartigen Badegewässern zu untersuchen. In der Badesaison 2017 wurden 27 EU-Badestellen (drei Badestellen pro Bundesland) beprobt. Die Wasserproben wurden durch Anreicherung in Thioglykolat-Bouillon und Kultivierung auf chromogenen Nährmedien auf antibiotikaresistente Bakterien getestet. Dreiundzwanzig der 27 Proben waren frei von antibiotikaresistenten, potenziell pathogenen Bakterien. Resistente Bakterien wurden für vier der 27 Badestellen nachgewiesen: einmal *Pseudomonas aeruginosa* und dreimal resistente *Enterobacteriaceae* (piperacillin/tazobactam-resistenter *Enterobacter cloacae* mit AmpC-Überexpression, carbapenem-resistenter *Enterobacter mori*, Extended-Spectrum-Beta-Lactamase-produzierendes *Escherichia coli*). Trotz dem Auftreten resistenter Bakterien in österreichischen Badestellen stufen wir das dadurch bedingte Gesundheitsrisiko als niedrig ein.

Schlagworte: Antibiotikaresistenz, Oberflächengewässer, Pathogene, Risikobewertung, öffentliche Gesundheit

1. Introduction

Nowadays, antimicrobial resistance (AMR) is a major health threat, and according to the World Health Organization (WHO), we are in severe danger of entering a post-antibiotic era, where simple bacterial infections will become untreatable (WHO, 2017). AMR is a phenomenon that has probably existed since microorganisms exist. Microorganisms produce antimicrobials to outcompete other microorganisms in their struggle for limited resources. The discovery of antimicrobials by man, their use, and overuse in our fight against infectious disease and the resulting environmental pollution caused by diverse anthropogenic activities (human medicine, veterinary medicine, agriculture) are factors that have been facilitating the development and spread of AMR. The development of AMR and the emergence of resistant microorganisms are linked to the use of antimicrobials (Goossens et al., 2005). The dramatic health threat caused by AMR is illustrated by the development of a common "One Health" global action plan on AMR by the WHO (WHO, 2015), by the World Organization for Animal Health (OIE, 2016), and the Food and Agriculture Organization (FAO, 2016) with the aim to minimize the impact of AMR. The focus areas of this global action plan include implementing AMR surveillance and antimicrobial residue monitoring in the environment (FAO, 2016). There is an increasing number of reports on the occurrence of clinically relevant multidrug-resistant (MDR) pathogens in the aquatic environment (Zurfluh et al., 2013; Mahon et al., 2017; Zarfel et al., 2017; Khan et al., 2018; Lepuschitz et al., 2017, 2018, 2019). Water is one of the most important habitats for bacteria. It is also a major medium for bacteria to disseminate and potentially to exchange among different environmental compartments, such as waste, surface, and drinking water (Vaz-Moreira et al., 2014). Studies increasingly emphasize the importance of aquatic systems as antibiotic resistance reservoirs, including antibiotic residues, antibiotic-resistant bacteria, and antibiotic-resistant genes, which can be exchanged between pathogenic and non-pathogenic bacteria (Baquero et al., 2008; Zhang et al., 2009; Rizzo et al., 2013; Manaia et al., 2016). However, at present, it is not clear to what extent environmental bacteria are a source for novel resistance mechanisms or which circumstances force them to spread antibiotic resistance. Therefore, the question how antibiotic resistance in the water environment affects human health still needs to be investigated and discussed (Vaz-Moreira et al., 2014).

The aim of our study was to assess the burden of AMR caused by *Enterobacteriales*, enterococci, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas* spp. (organisms commonly considered microbial indicators for water contamination (WHO, 2003)) in Austrian bathing sites, an aqueous environment supposedly less prone to anthropogenic influence than regular surface waters.

2. Materials and methods

2.1 Sampling, enrichment, and cultivation of resistant bacteria

In July and August 2017, 27 water samples were collected (according to ÖNORM M 6230, 2015) from 27 bathing sites, all of which fulfilled the criteria set by the EU Bathing Water Directive. Three sites were arbitrarily chosen per state. Water samples were collected in a sterile 500-ml glass flask, 30 cm below the river/lake surface, 2 m from the bank. A 100-ml water sample aliquot was filtered using 0.45-µm pore-sized membranes (Microfil® S device; Merck, Vienna, Austria), and the filtrate was incubated in thioglycollate broth (Becton Dickinson, Franklin Lakes, NJ, USA) at 37°C overnight. To detect vancomycin resistance and screen for carbapenemase-producing and extended-spectrum beta-lactamase (ESBL)-producing isolates, 50 µl of overnight cultures was subcultivated on selective chromogenic media (chromID™ VRE, chromID™ CARBA, and chromID™ ESBL (bioMérieux, Marcy-l'Étoile, France). For the detection of methicillin-resistant staphylococci (MRSA), the overnight cultures were cultivated on BBL™ CHROMagar™ MRSA II (Becton Dickinson, Vienna, Austria). Subcultivated single colonies were identified at species level by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) mass spectrometry using an MALDI Biotyper (Bruker, Billerica, MA, USA).

2.2 Antimicrobial susceptibility testing

In vitro susceptibility testing was performed with VITEK 2 Compact System (bioMérieux, Marcy-l'Étoile, France) using VITEK® 2 AST196 and AST-P586 cards interpreted according to the European Committee on Antimicrobial Susceptibility Testing (EUCAST) criteria for *Enterobacteriaceae*, *Pseudomonas* spp., and *Enterococcus* spp. (European Committee on Antimicrobial Susceptibility Testing,

EUCAST Clinical Breakpoint Tables v. 8.0, valid from January 01, 2018).

2.3 DNA isolation and whole genome sequencing

From subcultivated isolates, the DNA was extracted using the MagAttract High-Molecular-Weight DNA Kit (Qiagen, Hilden, Germany) and quantified fluorometrically on a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) using a target-specific Qubit assay for double stranded deoxyribonucleic acid (dsDNA BR Assay Kit, Thermo Fisher Scientific). The Nextera XT DNA library preparation kit (Illumina, San Diego, CA, USA) was used to prepare libraries for whole genome sequencing (WGS) according to manufacturer's protocol. Paired-end sequencing (2 × 300 bp) of genomic libraries was performed using the Illumina Miseq instrument. Sequencing coverage calculator (www.illumina.com/CoverageCalculator) was used to calculate for a desired mean coverage of at least 100-fold. *De novo* assembly of raw reads was performed using SPAdes (version 3.9.0) (Bankevich et al., 2012) and next generation sequencing (NGS) data interpretation was carried out using SeqSphere⁺ software (Ridom, Münster, Germany). Primary species confirmation was performed using rMLST (ribosomal multilocus sequence typing) (Jolley et al., 2012). For further phylogenetic analysis, the classical MLST (multi-locus sequence type) (Jolley et al., 2004) was extracted from the WGS data. PlasmidFinder 1.3 (Carattoli et al., 2014), SerotypeFinder 2.0 (Joensen et al., 2015), and VirulenceFinder 2.0 (Joensen et al., 2014) available from the Center for Genomic Epidemiology web server (http://www.genomicepidemiology.org) and the Comprehensive Antibiotic Resistance Database (CARD) (Jia et al., 2017) were used to identify *Escherichia coli* serotypes and to search for the presence of plasmids, virulence genes, or genes conferring antibiotic resistance.

3. Results

3.1 Strain isolation and primary species identification

The screening for antimicrobial-resistant bacteria yielded negative results in 23 of the 27 samples (Figure 1). Four water sample subcultures yielded growth on one chromogenic medium each: chromID™ CARBA (water sample K3) and chromID™ ESBL (water samples B1, NOE2, V2). No growth was observed on chromID™ VRE and on BBL™ CHROMagar™ MRSA II plates. Primary species identification using MALDI-TOF-MS identified one bacterial species in each of the four water samples (Table 1).

3.2 Whole genome sequencing analysis

The *Pseudomonas aeruginosa* isolate was assigned to classical ST2698; no plasmids were detected. The *Enterobacter mori* isolate was assigned to classical ST1009; no plasmids were identified. WGS analysis of the *Enterobacter cloacae* isolate was assigned to classical ST102 and the presence of three plasmids (IncHI2A (accession no. BX664015), pSL483 (CP001137), IncFII (CP001919)) was identified. Subtyping of the *E. coli* isolate was assigned to classical ST10 (Warwick scheme), serotype H40, and the presence of the plasmid IncI1 (AP005147) was identified. The analysis of the *E. coli* isolate with VirulenceFinder revealed the presence of one virulence gene *iss* (increased serum survival, CP001509) and the absence of Shiga-toxin genes.

3.3 *In vitro* and *in silico* antimicrobial resistance analysis

In Table 2, the *in vitro* susceptibility testing results of the four resistant water isolates (B1, K3, NOE2, and V2) using VITEK 2 Compact System method are summarized. The *P. aeruginosa* isolate harbored 51 AMR gene loci

Table 1. Antibiotic-resistant bacteria detected at Austrian bathing sites
Tabelle 1. Vorkommen von antibiotikaresistenten Bakterien in österreichischen Badegewässern

MALDI-TOF-MS	Water sample ID	Agar plate yielding isolate	Collection date	Federal state	Bathing site
Pseudomonas aeruginosa	B1	chromID™ ESBL	11.07.2017	Burgenland	Stausee Forchtenstein
Enterobacter mori	K3	chromID™ CARBA	28.08.2017	Carinthia	Ossiachersee Bodensdorf
Enterobacter cloacae	NOE2	chromID™ ESBL	10.07.2017	Lower Austria	Donaualtarm Greifenstein
Escherichia coli	V2	chromID™ ESBL	05.09.2017	Vorarlberg	Bregenz Wocherhafen

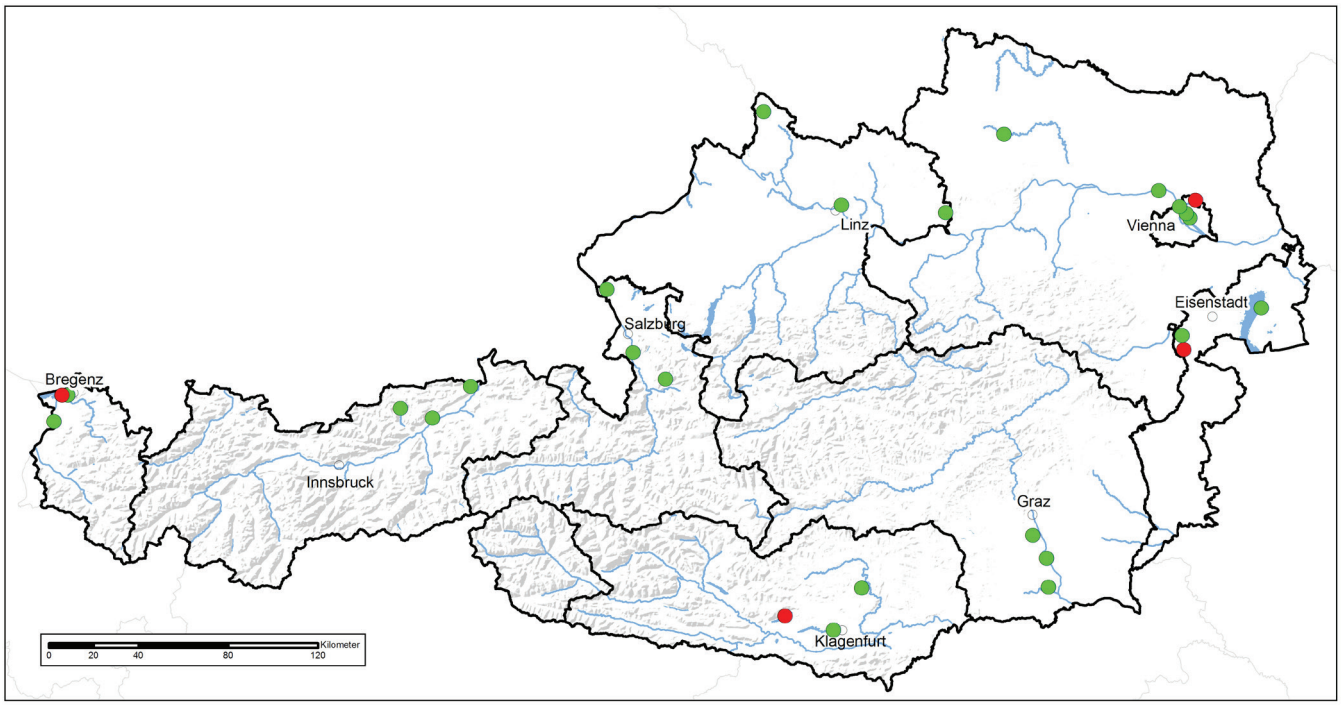

Figure 1. Sampling points of 27 EU-bathing sites (three per state) collected during the summer of 2017. Green dots represent bathing sites negative for antibiotic-resistant bacteria; red dots represent bathing sites positive for antibiotic-resistant bacteria.
Abbildung 1. Probenahmestellen der 27 EU-Badestellen (drei Badestellen pro Bundesland), die in der Badesaison 2017 beprobt wurden. Grüne Punkte: Badestellen die frei von antibiotikaresistenten Bakterien waren, rote Punkte: Badestellen in denen antibiotikaresistenten Bakterien nachgewiesen wurden.

in total, of which 42 were associated with antibiotic efflux, 4 with target alteration, and 5 with antibiotic inactivation (FosA, OXA-50, PDC-3, APH(3')-IIb, and catB7). The isolate was susceptible to piperacillin/tazobactam, ceftazidime, cefepime, imipenem, meropenem, amikacin, gentamicin, and ciprofloxacin and intermediately resistant to aztreonam (MIC 16 mg/L). The *E. mori* isolate was resistant to imipenem and meropenem. Additionally, it was resistant to ampicillin, amoxicillin/clavulanic acid, and moxifloxacin. It harbored 23 AMR gene loci in total, of which 15 were associated with antibiotic efflux, 4 with target alteration, and 4 with antibiotic inactivation (NmcR, FosA2, ACT-29, and IMI-2). The *E. cloacae* isolate was an AmpC producer. It was resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, cefotaxime, ceftazidime, aztreonam, and fosfomycin. It harbored 25 AMR gene loci in total, of which 14 were associated with antibiotic efflux, 7 with target alteration, 1 with target replacement (sul1), and 3 with antibiotic inactivation (FosA2, aadA2, and ACT-24). The *E. coli* isolate produced ESBL and showed resistance to ampicillin, cefuroxime axetil, and cefotaxime and intermediate resistance to cefepime (minimum inhibitory concentration (MIC) = 2 mg/L) and aztreonam (MIC 4 mg/L). This isolate V2 harbored 53 AMR gene loci in total, of which 40 were associated with antibiotic efflux, 10 with

Table 2. Antimicrobial susceptibility testing results of four resistant water isolates
Tabelle 2. Ergebnisse der Antibiotika-Resistenztestung der vier resistenten Wasserisolate

Sample ID	AM	AMC	PIP-TAZ	CXM-AX	FOX	CTX	CAZ	FEP	ATM	IPM	MEM	AN	GM	CIP	MXF	TGC	FOS	SXT
N0E2 (*E. cloacae*)	≥32	≥32	≥128	NA	NA	≥64	≥64	≤1	16	≤0.25	≤0.25	≤2	≤1	≤0.25	≤0.25	1	64	≤20
B1 (*P. aeruginosa*)	-	-	8	-	-	-	4	2	16	1	≤0.25	≤2	≤1	≤0.25	-	-	-	-
K3 (*E. mori*)	≥32	≥32	≤4	NA	NA	≤1	≤1	≤1	≤1	≥16	≥16	≤2	≤1	≤0.25	0.5	≤0.5	≤16	≤20
V2 (*E. coli*)	≥32	8	≤4	≥64	≤4	≥64	≤1	2	4	≤0.25	≤0.25	≤2	≤1	≤0.25	≤0.25	≤0.5	≤16	≤20

target alteration, 2 with antibiotic inactivation (AmpC and CTX-M-1), and 1 with target protection (*mfpA*).

Interpretation of MIC breakpoints (mg/L) according to the EUCAST criteria (red = resistant, orange = intermediate, green = sensitive); AM = ampicillin, AMC = amoxicillin/clavulanic acid, PIP-TAZ = piperacillin/tazobactam, CXM-AX = cefuroxime axetil, FOX = cefoxitin, CTX = cefotaxime, CAZ = ceftazidime, FEP = cefepime, ATM = aztreonam, IPM= imipenem, MEM = meropenem, AN = amikacin, GM = gentamicin, CIP = ciprofloxacin, MXF = moxifloxacin, TGC = tigecycline, FOS = fosfomycin, SXT= trimethoprim/sulfamethoxazole; NA = no defined breakpoints available

4. Discussion

In our study, the screening for antimicrobial-resistant bacteria was negative in 23 of the 27 samples. Resistant bacteria were detected from 4 of the 27 bathing sites. Two of the four isolates carried plasmids: *E. cloacae* yielded three plasmids and the *E. coli* isolate one plasmid. These findings indicate that antibiotic resistance genes were acquired by multiple separate acquisition events mediated by plasmids (Villa et al., 2012; Voulgari et al., 2014). The occurrence of plasmids raises concerns about the possibility of the detected strains contributing to the dissemination of resistance genes among bacterial species in the water environment (Cloutier and McLellan, 2017; Rothenheber and Jones, 2018; Schang et al., 2016). Vancomycin-resistant enterococci were not detected in any of the water samples. In Austrian hospitals, these gram-positive bacteria never reached the high clinical importance observed in the United States (BMG, 2017). In 2017, the ratio of vancomycin resistance among blood culture isolates from Austria was 0% for *Enterococcus faecalis* and only 3.2% for *Enterococcus faecium* (BMG, 2017).

The detection of MRSA was negative in all investigated isolates, and there are only a few studies describing the cultivation of MRSA from water samples (Tolba et al., 2008; Boopathy 2017; Lepuschitz et al., 2017, 2018). Up to date there are no official guidelines for the detection of MRSA from water samples, which might lead to the underestimation of MRSA in the environment. According to the definition by Magiorakos et al. (2012), three of the four bacteria isolated in our study on Austrian bathing sites were non-susceptible to at least one agent in three or more antimicrobial categories and therefore categorized as MDR bacteria (Magiorakos et al., 2012). Only the *P. aeruginosa* isolate must not be considered multidrug resistant. Non-fermenting bacteria, such as *P. aeruginosa*, are not only found in the clinical setting but also occur naturally in the aquatic environment (Kittinger et al., 2017). Our findings are in accordance with those of Suzuki et al., who in 2013 postulated that in advanced nations where the majority of the population is urban and where medical services are widespread, antibiotic-resistant bacteria such as *P. aeruginosa* are likely to be widely distributed, even in apparently pristine rivers (Suzuki et al., 2013). In 2017, the ratio of antibiotic resistance among *P. aeruginosa* blood culture isolates from Austria was 5% for aminoglycosides, 8.7% for ceftazidime, 13.5% for piperacillin/tazobactam, 12.3% for fluoroquinolones, and 13.9% for carbapenems (BMG, 2017). Pseudomonads can carry multiple intrinsic and acquired resistance genes and mobile genetic elements, exchange them with other *Enterobacteriaceae*, and are known to be the origin of several carbapenemase families (Pfeifer et al., 2010; Farinas and Martinez, 2013). The risk of intrinsic resistances found in environmental microorganisms being transferred to pathogens is an international concern (Forsberg et al., 2012; Cox and Wright, 2013; Singer et al., 2016). Two of the four antibiotic-resistant bacteria isolated in our study belonged to the genus *Enterobacter*. Although not surveyed under the European Antimicrobial Resistance Surveillance Network (EARS-Net), *Enterobacter* spp. have great relevance as invasive clinical pathogens. In 2017, the WHO published it's first-ever list of antibiotic-resistant "priority pathogens"—a catalog of 12 families of bacteria that pose the greatest threat to human health. The most critical group of all includes *Acinetobacter*, *Pseudomonas*, and *Enterobacteriaceae*. *Enterobacter* spp. have become resistant to a large number of antibiotics, including carbapenems and third-generation cephalosporins—the main antibiotics for treating infections caused by MDR bacteria. The detection of a carbapenem-resistant *E. mori* isolate at a Carinthian bathing site clearly underlines this resistance threat. Recently, the first clinical carbapenemase-carrying *E. mori* isolate was described in Austria (Hartl et al., 2019). This isolate was obtained from a 59-year-old patient suffering from acute otitis externa after visiting a thermal bath, which indicates water as the source of infection with antibiotic-resistant bacteria (Hartl et al., 2019). Also the finding of an ESBL-producing *E. coli* mirrors the situation in clinical microbiology. According to the "Der Österreichische Resistenzbericht" (AURES) report 2017, 49.5% of invasive *E. coli* isolates were resistant to aminopenicillins, 20.5% resistant to fluoroquinolones, 9.6% to third-generation cepha-

losporins, and 7.7% to aminoglycosides (BMG, 2017). Rivers and lakes are considered relevant reservoirs for MDR bacteria, because they pool materials from different origins, such as wastewater plants, water of urban or industrial effluents, agricultural activities, or rain (Lupo et al., 2012; Zurfluh et al., 2013; Kittinger et al., 2017). Our findings reveal that even ecologically pristine waters used for recreational activities can harbor resistant isolates. Exner et al. (2018) who evaluated potential health risks of water bodies contaminated with antibiotic-resistant pathogens reported that bathers without increased vulnerability and bathers with increased vulnerability should be differentiated. Ingestion of bathing water can theoretically lead to colonization of the gastrointestinal tract, but this is considered unlikely, given the low levels of swallowed bathing water or concentrations of antibiotic-resistant pathogens in open water. With regard to bathing in water authorized under the EU Bathing Water Directive, possible exposure to antibiotic-resistant bacteria according to the current knowledge poses no increased health risk for bathers without increased vulnerability; this assumes that the criteria of the EU Bathing Water Directive are met. They include intact skin of the bather and no antibiotics taken, observing the general rules of hygiene. For bathers with increased vulnerability or predisposition, risk of infection cannot be ruled out under the following conditions: open, extensive wounds; extensive skin disease; or prolonged intake of antibiotics. For these reasons, these persons should generally not bathe in open and untreated water irrespective of the presence of antibiotic-resistant pathogens (Exner et al., 2018).

5. Conclusion

We consider the public health risk at Austrian bathing sites authorized under the EU Bathing Water Directive to be low despite the occurrence of MDR bacteria. However, our results confirm the existing risk for dissemination of MDR bacteria via the aquatic environment.

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