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Short report

First evidence of KPC-producing ST258 *Klebsiella pneumoniae* in river water

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SUMMARY

This paper reports the first case of *Klebsiella pneumoniae* carbapenemase (KPC)-2-producing *K. pneumoniae* in river water in Croatia. In total, four KPC-2-producing *K. pneumoniae* isolates were analysed. All isolates shared a similar genetic background, belonging to ST258. Isolates displayed uniform, multi-drug-resistant profiles susceptible to colistin. bla_{SHV-1}, *aac(3')-II*, *aac(6')-Ib* and *aph(3')-Ia* genes were detected in all isolates. In all isolates, the bla_{KPC-2} gene was localized on a single non-conjugative IncFII plasmid that varied in size (~140, ~230, ~225 and ~220 kb). *K. pneumoniae* was viable in river water for up to 50 days, confirming its ability to survive and disseminate in the environment.

Introduction

Extensive use of carbapenems in clinical practice has inevitably led to the emergence of carbapenem-non-susceptible or carbapenem-resistant Enterobacteriaceae. Ongoing global spread of carbapenemase-producing Enterobacteriaceae is particularly concerning because carbapenemases readily hydrolyse carbapenems, and carbapenemase-encoding genes are frequently associated with high-risk bacterial clones [1,2].

Klebsiella pneumoniae carbapenemase (KPC), a class A beta-lactamase, is one of the most prominent carbapenemases capable of hydrolysing virtually all beta-lactams, including carbapenems [2]. Although described in a wide spectrum of Enterobacteriaceae, KPC enzymes are primarily reported in *K. pneumoniae*, which is, mainly due to pandemic spread of the ST258 clone, responsible for dissemination of this carbapenemase on a global level [2]. Association of bla_{KPC} genes with a wide spectrum of plasmids is usually linked to multi-drug resistance and, more importantly, horizontal transfer of bla_{KPC} genes [1,2].

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The first case of KPC-producing *K. pneumoniae* in Croatia was reported in 2011 [3]. Although this isolate was identified as an ST37 clone, further dissemination was exclusively associated with the ST258 clone [3]. Unlike nearby countries (i.e. Greece and Italy), spread of KPC-producing *K. pneumoniae* remained mainly sporadic [2,3].

At present, medical institutions are predominantly recognized as the primary reservoir of KPC-producing Enterobacteriaceae, but their occurrence in other reservoirs, such as animals and the aquatic environment, is increasing [4]. River water is recognized as a putative reservoir of multi-drug-resistant bacteria due to the increasing prevalence of antibiotic-resistant bacteria which most likely originate from anthropogenic sources (i.e. hospital and municipal effluents) [5]. Studies on rivers as potential reservoirs of carbapenemase-producing Enterobacteriaceae are scarce but indicate that there is a growing number of carbapenemase-producing bacteria in the environment; however, insight into this complex issue remains limited [5].

This study reports the first case of KPC-producing *K. pneumoniae* in river water in Croatia. The aim of this study was to provide detailed characterization of KPC-producing *K. pneumoniae* and to shed light on the genetic background of bla_{KPC} genes in isolates retrieved from the Krapina River in continental Croatia.

Methods

Sampling and physicochemical and bacteriological analyses of water

In April 2017, water was sampled from the Krapina River in continental Croatia, 300 m downstream from the Zabok General Hospital sewage outlet (46°00'55.7"N, 15°56'06.8"E). Raw untreated hospital wastewaters are discharged directly into the river at this point. Ten millilitres of river water was collected aseptically in sterile plastic bottles for bacteriological analyses. Samples were processed in the laboratory within 4 h of collection.

The physicochemical parameters of river water were measured according to the standard procedure [6]. Bacteriological analyses were performed in triplicate after suspension and dilution of river water in sterile peptone water. Aerobically grown total heterotrophic bacteria were determined on nutrient agar (Biolife, Chieti, Italy) after incubation at 22°C for 72 h [6]. Intestinal enterococci were detected on Slanetz-Bartley agar (Biolife) at 37°C for 72 h, and confirmed on bile esculin azide agar (Sigma-Aldrich, St Louis, MO, USA) after incubation at 44°C for 4 h (HRN ISO 7899-2, 2000). Carbapenem-resistant bacteria were cultured on CHROMagar Acinetobacter medium supplemented with CR102 (CHROMagar, Paris, France), intended for the cultivation of clinically relevant carbapenem-resistant bacteria, after incubation at 37°C for 72 h and 42°C for 48 h (CRB37 and CRB42, respectively). Cultivation of carbapenem-resistant bacteria was performed at 42°C to suppress the growth of environmental autochthonous species with intrinsic resistance to carbapenems, which grow at 37°C but not at 42°C [6]. The numbers of heterotrophic bacteria, enterococci and carbapenem-resistant bacteria were determined as colony-forming units (cfu), logarithmically calculated, and expressed as log cfu per mL water.

Identification of K. pneumoniae

Blue colonies, presumed to be *K. pneumoniae*, were isolated from the plates on which carbapenem-resistant bacteria grown at 42°C were counted. The colonies were recultivated (42°C for 24 h) on CHROMagar Acinetobacter medium supplemented with CR102 and then on nutrient agar. *K. pneumoniae* were identified on a Vitek2 system (BioMérieux, Marcy l'Etoile, France) following the routine bacteriological procedure, and confirmed with matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) (Microflex LT Version 3.0; Bruker Daltonics, Billerica, MA, USA) on the cell extracts.

Antibiotic susceptibility testing and analysis of antibiotic resistance genes

Antimicrobial susceptibility testing of KPC-producing isolates was performed by the disc diffusion method (Mast Group, Bootle, UK), gradient E-test (BioMerieux) and broth microdilution (Mikrolatest MIC; Erba Lachema, Brno, Czech Republic). Susceptibility results were interpreted according to the EUCAST guidelines [7]. *Escherichia coli* ATCC 25922 and colistin-resistant *E. coli* NCTC 13846 were used as quality control strains. Carba NP test was used for detection of carbapenemase production in all isolates. Phenotypic identification of class A (KPC) and class B carbapenemases was performed by an inhibitor-based method using combination disk testing, and detection of extended-spectrum beta-lactamase production was performed using double-disk synergy test [8].

Polymerase chain reaction (PCR) detection of bla_{KPC} genes was performed using KPC-F (5'-AGTTCTGCTGTCTTGTCT -3') and KPC-R primers (5'-CTTGTCATCCTTGTTAGG -3'). Detection of other genes conferring resistance to different families of antimicrobial agents was also performed by PCR: (i) beta-lactam agents: class A (bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{VEB}, bla_{GES}, bla_{PER}, bla_{SME}), class B (bla_{VIM}, bla_{IMP}, bla_{NDM}), plasmid bla_{AmpC} and class D (bla_{OXA-48}); (ii) quinolones: *qnr* genes; (iii) aminoglycosides: *aadA1/2*, *aadA5*, *aac(3)-II*, *aph(3')-Ia*, *aph(3')-II*, *aac(6')-Ib*, *armA* and *rmt(A)-rmt-(F)*; (iv) tetracycline: *tet(A)-tet(E)*; (v) chloramphenicol: *cmlA* and *floR*; (vi) sulphonamides: *sul1* and *sul2*; and (vii) colistin: *mcr-1 – mcr-4* [3,9]. All beta-lactamase genes detected by PCR were sequenced by ABI310 using BigDye v1.1 (ThermoFisher Scientific, Colorado Springs, CO, USA).

Pulsed-field gel electrophoresis and multi-locus sequence typing

Genetic relatedness of KPC-producing isolates was determined by pulsed-field gel electrophoresis (PFGE) of XbaI digested genomic DNA using the CHEF-DR III System (Bio-Rad Laboratories, Hercules, CA, USA), as described previously [3]. Multi-locus sequence typing (MLST) of all isolates was performed according to the protocol described on the

K. pneumoniae MLST website (https://bigsdb.pasteur.fr/klebsiella/klebsiella.html) [3].

Plasmid characterization and transfer of resistance

The number and size of large plasmids (≥ 50 kb) in all KPC-producing isolates were determined using S1-nuclease digested genomic DNA, separated by PFGE [3]. Subsequently, plasmid DNA was blotted on to positively charged nylon membrane and hybridized with bla_{KPC}-specific probe using DIG-High Prime DNA Labeling and Detection Starter Kit I (Roche, Mannheim, Germany). Plasmids of KPC-producing isolates were classified according to their incompatibility group by PCR-based replicon typing [3].

A conjugal-transfer experiment was performed by the filter-mating method using rifampicin-resistant *E. coli* A15R strain as a recipient. Transconjugants were selected on LB-agar plates supplemented with 256 mg/L rifampicin and 0.5 mg/L meropenem.

K. pneumoniae survival assay

The survival of one *K. pneumoniae* isolate (9/1) in water from the Krapina River was monitored for 50 days at room temperature (22±2°C). Overnight bacterial culture was suspended in duplicate in test tubes containing 40 mL of autoclaved river water. Tubes were rotated at 3 rpm using a Stuart Tube Rotator SB3 (Stone, Staffordshire, UK). The number of bacteria was determined on nutrient agar incubated at 42°C for 24 h, and bacterial concentration was expressed as log cfu/mL.

Results

Physicochemical and bacteriological analyses of water

The river water had a neutral pH of 7.72. Chemical parameters indicated the presence of nutrients in the river water: chemical oxygen demand 22 mg/L; total organic carbon 13 mg/L; total nitrogen 2.7 mg/L and total phosphorus 0.3 mg/L. The bacterial count of total heterotrophs was highest, followed by intestinal enterococci, CRB37 and CRB42 (Table I). The number of total heterotrophs was in the expected range for the pH-neutral natural river water. The relatively low number of intestinal enterococci indicates moderate faecal pollution of water. The prevalence and absolute number of CRB42 were lower compared with CRB37, suggesting selection through elevated incubation temperature. Four isolates (named 9/1, 9/2, 9/3 and 9/4) from the CRB42 culture plates had MALDI-TOF scores of 2.141–2.299, identifying them as *K. pneumoniae*.

Table I
Bacteriological characteristics of sampled river water

Group of bacteria	Log cfu/mL (prevalence %)
Total heterotrophs	6.4±0.5
Intestinal enterococci	0.8±0.2
CRB37	0.8±0.0 (0.0002%)
CRB42	0.2±0.1 (0.00004%)

cfu, colony-forming unit; CRB37, CRB42, carbapenem-resistant bacteria grown at 37 and 42°C, respectively.
Mean values of triplicate measurements and standard deviations are presented.
Prevalence of CRB among total heterotrophic bacteria was calculated as percentage ratio of absolute numbers (cfu_{CRB} /$cfu_{heterotrophic}$) x 100.
Detection limit was 1 cfu/mL.

Antibiotic susceptibility and presence of antibiotic resistance genes

All KPC-producing isolates were resistant to ampicillin, amoxicillin/clavulanic acid, piperacillin/tazobactam, all cephalosporins, all carbapenems, trimethoprim-sulfamethoxazole, ciprofloxacin and both aminoglycosides tested (gentamicin and amikacin), but remained susceptible to colistin. The bla_{KPC-2} gene was detected in all isolates, and the presence of other carbapenemase-encoding genes was not observed. In addition to the bla_{KPC-2} gene, all isolates carried bla_{SHV-1}, *aac(3')-II*, *aac(6')-Ib* and *aph(3')-Ia* genes.

Molecular epidemiology

The PFGE results showed that XbaI PFGE banding patterns exhibited a high level of genetic relatedness (>85% similarity), and all were assigned to a single cluster. MLST analysis revealed that all isolates belonged to ST258.

Plasmid analysis and transfer of resistance

S1-nuclease-PFGE analysis revealed variable plasmid profiles among the analysed isolates. Southern blot hybridization with bla_{KPC} and the IncFII-specific probe showed that bla_{KPC} genes were located on a single IncFII plasmid of ~140, ~230, ~225 and ~220 kb in isolates 9/1, 9/2, 9/3 and 9/4, respectively. All isolates failed to transfer carbapenem resistance to *E. coli* A15R by filter mating.

K. pneumoniae survival assay

The results of the *K. pneumoniae* survival assay are presented in Figure 1. The tested isolate (9/1) survived in autoclaved river water for 50 days of monitoring. An increase in the initial number of bacteria was observed within one day of incubation, and this can be explained by the nutrients available for multiplication of *K. pneumoniae*. A slight decrease in the number of bacteria was detected after 14 days and further

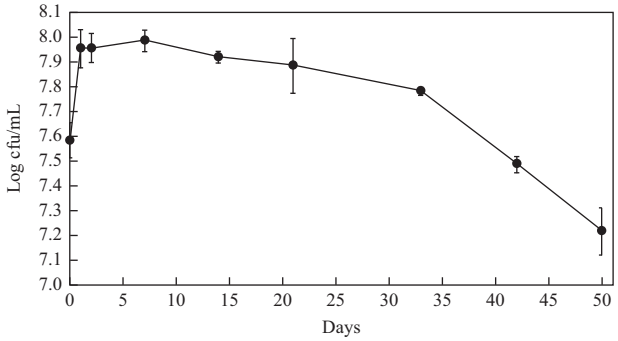

Figure 1. Survival of *Klebsiella pneumoniae* isolate (9/1) in autoclaved river water for 50 days. Mean values and standard deviations are presented.

incubation. After 50 days of contact, bacterial numbers remained high and reduced by just 0.4 log cfu/mL compared with the initial number.

Discussion

This study describes the first case of KPC-producing *K. pneumoniae* isolated from natural river samples in Croatia. Since the first nosocomial outbreak of KPC-producing *K. pneumoniae* ST258 in Croatia, reported at Zabok General Hospital in 2012, this is the first confirmed environmental case of KPC-producing *K. pneumoniae*, highly related to clinical isolates reported in medical institutions from the same geographic area in Croatia [10].

All four analysed bla_{KPC-2}-producing *K. pneumoniae* isolates had identical multi-drug-resistance profiles, remaining susceptible to colistin. The presence of genes conferring resistance to multiple classes of antibiotics was in accordance with observed antibiotic resistance patterns. Association between the bla_{KPC-2} gene and the ST258 clone is well documented at global level, and previous reports have recognized ST258 as an epidemic clone responsible for dissemination of KPC-2-producing *K. pneumoniae* in Croatia, including Zabok General Hospital [2,3].

Although reports of KPC-producing *K. pneumoniae* in water ecosystems are increasing, to the authors' knowledge, only one description of environmental KPC-3-producing ST258 *K. pneumoniae* has been reported in Europe, from a groundwater sample from Italy [5]. Ability to survive in river water for at least 50 days indicates that ST258, as a clinically relevant strain, may have the potential to persist in surface waters, perpetuating its spread via environmental water flows.

The bla_{KPC-2} gene was identified on non-conjugative IncFII plasmids of various sizes. Predominant association of bla_{KPC}-harbouring IncFII plasmid with ST258 *K. pneumoniae* is well documented and is frequently reported in isolates from geographically diverse areas [2]. bla_{KPC-2}-harbouring IncFII plasmid was also associated with an epidemic KPC-2-producing ST258 clone described in Croatia between 2011 and 2013, including Zabok General Hospital [3].

In conclusion, four *K. pneumoniae* isolates from river water shared similar features with clinical isolates registered in the nearby hospital: affiliation to ST258, carbapenem and multidrug resistance, and association of the bla_{KPC-2} gene with an IncFII plasmid. This indicates that *K. pneumoniae* isolates from river water are likely to be of clinical origin, and were likely discharged via the untreated hospital wastewaters into the natural water bodies. The presence of KPC-producing *K. pneumoniae* ST258 in an aquatic environment is of concern in many ways. Although hospital settings are considered to be the primary reservoir of carbapenemase-producing Enterobacteriaceae, the ability of KPC-producing *K. pneumoniae* ST258 to remain viable in river water for an extended period highlights its potential to transfer antibiotic resistance genes among different natural bacterial ecosystems. This report emphasizes the need for further, more systematic, environmental studies to evaluate the presence of carbapenemase-producing Enterobacteriaceae in Croatia.

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Conflict of interest statement
None declared.

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