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Multidrug-resistant ESBL-producing *Klebsiella pneumoniae* complex in Czech hospitals, wastewaters and surface waters

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Abstract

Background Multidrug-resistant (MDR) bacteria pose a significant challenge to the treatment of infectious diseases. Of particular concern are members of the *Klebsiella pneumoniae* species complex (KpSC), which are frequently associated with hospital-acquired infections and have the potential to spread outside hospitals via wastewaters. In this study, we aimed to investigate the occurrence and phylogenetic relatedness of MDR KpSC from patients with urinary tract infections (UTIs), hospital sewage, municipal wastewater treatment plants (mWWTPs) and surface waters and to evaluate the clinical relevance of the KpSC subspecies.

Methods A total of 372 KpSC isolates resistant to third-generation cephalosporins and/or meropenem were collected from patients ($n=130$), hospital sewage ($n=95$), inflow ($n=54$) and outflow from the mWWTPs ($n=63$), river upstream ($n=13$) and downstream mWWTPs ($n=17$) from three cities in the Czech Republic. The isolates were characterized by antimicrobial susceptibility testing and whole-genome sequencing (Illumina). The presence of antibiotic resistance genes, plasmid replicons and virulence-associated factors was determined. A phylogenetic tree and single nucleotide polymorphism matrix were created to reveal the relatedness between isolates.

Results The presence of MDR KpSC isolates (95%) was identified in all water sources and locations. Most isolates (99.7%) produced extended-spectrum beta-lactamases encoded by $bla_{\text{CTX–M–15}}$. Resistance to carbapenems (5%) was observed mostly in wastewaters, but carbapenemase genes, such as $bla_{\text{GES–51}}$ ($n=10$), $bla_{\text{OXA–48}}$ ($n=4$), $bla_{\text{NDM–1}}$ ($n=4$) and $bla_{\text{KPC–3}}$ ($n=1$), were found in isolates from all tested locations and different sources except rivers. Among the 73 different sequence types (STs), phylogenetically related isolates were observed only among the ST307 lineage. Phylogenetic analysis revealed the transmission of this lineage from patients to the mWWTP and from the mWWTP to the adjacent river and the presence of the ST307 clone in the mWWTP over eight months. We confirmed the frequent abundance of *K. pneumoniae* (*K. pneumoniae sensu stricto* and *K. pneumoniae* subsp. *ozaenae*) in patients suffering from UTIs. *K. variicola* isolates formed only a minor proportion of UTIs, and *K. quasipneumoniae* was not found among UTIs isolates; however, these subspecies were frequently observed in hospital sewage communities during the first sampling period.

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Conclusion This study provides evidence of the transmission and persistence of the ST307 lineage from UTIs isolates via mWWTPs to surface waters. Isolates from UTIs consisted mostly of *K. pneumoniae*. Other isolates of KpSC were observed in hospital wastewaters, which implies the impact of sources other than UTIs. This study highlights the influence of urban wastewaters on the spread of MDR KpSC to receiving environments.

Keywords Urinary tract infections, Wastewater treatment plants, *Klebsiella* spp. subspecies, $bla_{CTX-M-15}$

Introduction

Multidrug-resistant (MDR) *Klebsiella pneumoniae*, which produces extended-spectrum beta-lactamases (ESBLs), is recognized among the leading pathogens of nosocomial infections and is responsible for 30% of all gram-negative bacterial infections in Europe [4, 41]. After *Escherichia coli*, *K. pneumoniae* is the second most common cause of healthcare-associated urinary tract infections (UTIs) but is also responsible for other severe infections [46, 65].

Most causes of klebsiella-induced nosocomial UTIs are connected with low-virulence strains called "classic *K. pneumoniae*", while "hypervirulent *K. pneumoniae*" strains cause severe diseases and disseminate within hospitals and communities. One of the main selective advantages of hypervirulent clones is the ability to produce a broad range of virulence-associated factors (VAFs), e.g., siderophores, fimbriae or capsules [53]. Capsule production plays the most important role in the virulence of *Klebsiella* spp. To date, more than 80 types of capsules have been identified serologically and additional capsule types are presumptively identified via genomic analysis tools (not serologically confirmed). Out of capusule producers, the most clinically important are hypervirulent *Klebsiella* spp. strains producing capsule types of K1, K2, K16, K28, K57 and K63 [30, 53].

Klebsiella pneumoniae species complex (KpSC) contains seven phylogenetic groups (*K. pneumoniae* (Kp1), *K. quasipneumoniae* subsp. *quasipneumoniae* (Kp2), *K. variicola* subsp. *variicola* (Kp3), *K. quasipneumoniae* subsp. *similipneumoniae* (Kp4), *K. variicola* subsp. *tropica* (Kp5), *K. quasivariicola* (Kp6) and *K. africana* (Kp7)), some of which are difficult to distinguish by phenotypic identification by routine MALDI-TOF MS diagnostics [15]; therefore, there is only limited information about the clinical relevance of particular subspecies. Moreover, clinically important *K. pneumoniae* subsp. *ozaenae* causing severe disease is difficult to distinguish from other subspecies of *K. pneumoniae* [20]. Molecular biology approaches, such as whole-genome sequencing, are used in clinics for the diagnosis of high-risk clones but not on a routine basis; therefore, there is still great confusion in nomenclature, which results in the underestimation of the clinical relevance of some strains [43].

Recently, an increasing occurrence of carbapenem-resistant KpSC of certain sequence types (STs) has been reported [49, 55]. One of the most discussed globally emerging lineages with the ability to persist and adapt to the human host and health-case settings is ST307. The coexistence of two different carbapenemase-encoding genes (bla_{NDM-1} and bla_{KPC-3}) in one ST307 strain has been reported [13]. Other worrisome studies described the transmission of ST147, which carries bla_{NDM} and bla_{OXA-48} via hospital sewage, to municipal wastewater treatment plants (mWWTPs) and surface water [32]. mWWTPs are recognized as major hotspots where diverse bacterial populations could share genetic material. Effective, yet not sufficient, wastewater treatment leads to the subsequent discharge of potentially clinically relevant clones or antibiotic resistance genes to adjacent surface water [2, 45].

The aims of this study were (i) to evaluate the occurrence and phylogenetic relatedness of clinically relevant MDR KpSC recovered from patients with UTIs, hospital sewage and mWWTPs as well as receiving river water; (ii) to distinguish subspecies within our dataset via digital DNA–DNA hybridization and to evaluate the clinical relevance of different subspecies; and (iii) to enlarge the dataset used in this study by including KpSC isolates collected in the Czech Republic and other European countries to assess the phylogenetic relatedness of predominant STs and their genetic background.

Materials and methods

Sample collection

Sampling was performed in three large cities across the Czech Republic (locations A, B, and C) during two sampling periods (August 2020 and April 2021) to explore differences between locations and seasonality effects. Sampling locations were chosen according following criteria: (a) locations covered both Bohemia and Moravia to cover surveillance data from the Czech Republic, (b) hospitals represented the largest facilities in different regions in the Czech Republic, were the biggest healthcare facility in the city and had to have the Department of Microbiology as a part of healthcare setting, (c) hospitals were located in the basin of the big river where leads the wastewater treatment plants outlet, as described previously [16].

ESBL/carbapenemases producing MALDI-TOF identified isolates of *Klebsiella* spp. from UTIs obtained by during rutine diagnostics as pure cultures, over a two-week period were collected by microbiological departments in cooperating hospitals (A, B, C). Isolates from patients

hospitalized in infection disease departments were not included because sewage water from this department is treated at hospital WWTPs, and after the treatment process, no viable cells remain [34]. At the end of the two weeks of UTIs isolates sampling, water samples were collected from 5 sources: raw hospital wastewater, inflow and outflow from the mWWTPs, and river water collected from the mWWTPs upstream and downstream. Water samples were collected into sterile one-liter glass flasks, transported to the laboratory at 4 °C, and processed immediately.

Cultivation of resistant KpSC, antibiotic susceptibility and beta-lactamase production testing

Clinical isolates were subcultured on MacConkey agar (MCA; Oxoid, UK) supplemented with cefotaxime (2 mg/L) or meropenem (0.125 mg/L) to select cephalosporin- or carbapenem-resistant isolates, respectively. Species identification was again conducted via a MALDI-TOF mass spectrometer (Microflex LT, Bruker Daltonics, Germany). The purified isolates were stored at -80 °C.

Water samples were ten-fold serially diluted or concentrated by filtering (0.22 µm; Sigma–Aldrich, US) before plating (depending on the expected amount of viable bacterial colonies in the various types of samples collected during optimalization process). Next, they were cultivated on chromogenic media Klebsiella ChromoSelect Selective Agar Base (Millipore, USA) at 37 °C overnight. The suspected *Klebsiella* spp. isolates were subcultured on MCA supplemented with cefotaxime (2 mg/L) or meropenem (0.125 mg/L) in combination with zinc and bicarbonate to obtain cephalosporin-resistant isolates or isolates with decreased susceptibility to carbapenems. Colonies were identified via MALDI–TOF mass spectrometry, and up to 30 *Klebsiella* spp. colonies per water sample were collected and stored at -80 °C. Additionally, KpSC isolates obtained on meropenem-enhanced media were subjected to PCR to confirm the presence of carbapenemase-encoding genes (bla_{GES}, bla_{IMI}, bla_{NMC-A}, bla_{IMP}, bla_{KPC}, bla_{NDM}, $bla_{OXA48-like}$, bla_{VIM}), described by Davidova-Gerzova et al. [16].

For all the isolates, the production of beta-lactamases was tested by MastDiscs AmpC, ESBL & Carbapenemase Set D72C (Mast Diagnostics, UK), and the isolates suspected for carbapenemase production were additionally tested by MastDiscs Combi Carba Plus D73C (Mast Diagnostics, UK). For the final KpSC collection, we included only isolates harboring AmpC, ESBL, and / or carbapenemase-encoding genes. The minimal inhibitory concentrations (MICs) against 24 antimicrobials were determined via MIC G-I and MIC G-II assays (Erba Lachema, Czech Republic), followed by categorization of the isolates into resistant, intermediate, or susceptible phenotypes according to the clinical breakpoints from EUCAST (2023) or the CLSI (2020) for chloramphenicol, tetracycline, cefoperazone, and netilmicin (Additional file 1). Isolates showing resistance to at least one antimicrobial within three or more classes of antibiotics were considered MDR [44].

DNA extraction, whole-genome sequencing and data processing

DNA from all obtained isolates was extracted via a NucleoSpin Tissue Kit (Macherey-Nagel, Germany) and subjected to library preparation (Nextera XT DNA Library Preparation Kit, Illumina, Inc., USA) and sequencing (HiSeq 4000, Illumina, Inc., USA) following the manufacturer's protocol. The data were processed as described by Kutilova et al. [34]. Raw Illumina paired-end reads were adapted, and quality ($Q \geq 20$) reads were trimmed via Trimmomatic v0.39 [9], and high-quality trimmed reads were assembled via the *de novo* assembler SPAdes v3.15.5 [5].

Genomic data analysis and phenotype/genotype correlation

The assembled contigs were analyzed via ABRicate v0.9.8 ([59] Abricate; available at https://github.com/tseemann/abricate) in ResFinder (version no. 4.4.2, [10]) and PointFinder (2022-08-08, [70]) to identify the most common antibiotic resistance genes (ARGs) and mutations associated with antibiotic resistance; the CARD database (version 4.0.2) was used to detect genes for resistance to other antimicrobials and disinfectants; the PlasmidFinder (2022-03-30, [14]) and pMLST tools were used for analyses of plasmid replicons and plasmid sequence types; and the virulence factor database (core dataset, versions 2022-12-23) [42] was used to assign virulence-associated genes (VAGs). The STs of the KpSC isolates were determined via MLST 2.0 [37]. The serotype was determined via Kaptive [35]; the defined serotypes were labeled K1–68, and the predicted K loci identified from the genomes were labeled KL102–183.

Phenotypic manifestations were compared to the genetic background of each isolate according to the current ResFinder database (version no. 4.4.2). Disconcordance was evaluated as (i) gene presence where a genetic marker was present but no phenotypic resistance to relevant antibiotics was detected or (ii) gene absence where resistance to a specific antimicrobial was manifested but no genetic marker was detected.

Precise identification of a particular strain was performed by assessing the closest type strain (https://www.ezbiocloud.net), performing genome-based classification of the strain (Type (Strain) Genome Server; [48]), computing the estimated average nucleotide identity (Average Nucleotide Identity; [54]), and finally performing digital

DNA–DNA hybridization (Genome-to-Genome Distance Calculator 3.0; [47]).

Phylogenetic and SNP analysis

Prokka-annotated [58] sequences were generated, core and pangenomes were created with PIRATE 1.0.4 [7], and a maximum-likelihood tree was built via RAxML [60]. The matrices of single nucleotide polymorphisms (SNPs) distances were determined via snp-dists 0.6.3 (available at Seemann, T., snp-dists; https://github.com/tseemann/snp-dists) on the basis of the core gene alignment. The threshold of 21 SNPs distances was set for clonal transmission between two KpSC isolates [6], and up to 35 SNPs were considered the same transmission cluster [22]. Phylogenetic trees and individually computed SNPs matrices were constructed for ST307, ST337 and ST1083, which presented up to 21 SNPs differences within our dataset.

To select lineages with the potential to circulate in health-care settings, microbiological departments in hospitals in locations A (bioproject no. PRJNA1130712) and B (PRJNA1138523) provided sequencing data of KpSC collected from 2018 to 2022. For STs obtained from both our study and retrospective data from at least one hospital (ST14, ST17, ST23, ST25, ST45, ST54, ST219, ST307, ST321, ST405, ST433, ST551, and ST1271), phylogenetic trees and SNP matrices were individually constructed. Finally, the ST307 dataset was enhanced with genomic data available in the Pasteur database to determine whether strains in the Czech Republic share specific characteristics and differ from those spreading in other European countries.

Visualization and statistics

Basic data analysis and visualizations were performed in Excel (Microsoft), and further data computing and visualizations were conducted in the R Core Team (2023, version 4.3.2). The phylogenetic tree was visualized in iTOL [40], version 6.9. Comparisons of categorical variables were performed via Fisher's exact test, and a p value < 0.05 was considered to indicate statistical significance.

Results

Antibiotic resistance profiling

A total of 372 KpSC isolates were obtained from patients with UTIs ($n=130$), hospital wastewater ($n=95$), inflow ($n=54$) and outflow from the mWWTP ($n=63$), river upstream ($n=13$) and downstream of the mWWTP ($n=17$). Resistant isolates were present in all tested sources, and the differences between sampling, location or source were statistically insignificant ($p>0.05$) (Additional file 1). No statistical computing was performed on river water isolates, as no isolates were recovered from river water during the second sampling period or on isolates from location C because only two sources were examined there (Fig. 1A). Most (95%) isolates presented a MDR resistance profile, with resistance to cephalosporins (100%), trimethoprim (91%), ciprofloxacin (87%), aminoglycosides (80%) and tetracyclines (66%) as the most common resistance phenotypes. Resistance to last-line drugs such as ertapenem (16%) and meropenem (2%) or colistin (8%) was also observed. The isolates were producers of ESBL (371/372), AmpC beta-lactamases (19/372) and carbapenemases (17/372). Overall information about metadata, antibiotic resistance and genetic background is available in the Additional file 1.

A total of 103 variants of ARGs encoding resistance to 10 different antibiotic classes were found. Resistance to cephalosporins was encoded mainly by $bla_{\text{CTX-M-15}}$ (362/372). Genes encoding the AmpC-type beta-lactamases $bla_{\text{DHA-1}}$ and $bla_{\text{CMY-2}}$ were found in five and one isolate, respectively. Carbapenemase-encoding genes, including $bla_{\text{GES-51}}$ ($n=10$), $bla_{\text{OXA-48}}$ ($n=4$), $bla_{\text{NDM-1}}$ ($n=4$) and $bla_{\text{KPC-3}}$ ($n=1$), were found in isolates from all tested locations, sampling periods and all sources except rivers. Other widely abundant ARGs were *oqxA/oqxB* (phenicols/quinolones), *sul2* (sulfonamides), *aph(6)-Id* (aminoglycosides), *dfr14* (trimethoprim), *qnrB1* (quinolones) and *aac(6')-Ib-cr* (aminoglycosides/quinolones) were found (Fig. 1B). Within our dataset, no *mcr* gene encoding colistin resistance was detected (Additional file 1).

At least one point mutation with known phenotype function was observed in all but one isolate. The average number of point mutations was 18 per sample, and in total, we observed point mutations at 45 different positions (twenty-six in *ompK36*, nine in *acrR*, five in *ompK37* and five in *ramR*). Interestingly, no known point mutations in *par* or *gyr* were detected. More than 90% of the isolates presented mutations in the *acrR*, *ompK36* and *ompK37* genes. We observed no concordance in the presence of point mutations or resistance to any tested antibiotics.

A discrepancy between phenotypic and genotypic resistance (one point mutation excluded) was detected in 95% of the isolates. Presence of ARGs *oqxAB*, $bla_{\text{OXA-1}}$, and various variants of *qnrS* and *qnrB* genes was not phenotypically manifested in 76%, 39% and 13% isolates, respectively. Isolates that were resistant to an antibiotic without the presence of ARGs were observed mostly for tigecycline (30%), ertapenem (12%), tetracycline (9%) and colistin (8%). The production of specific carbapenemases was not always supported by the presence of related genes, especially OXA-48 and metallo-beta-lactamase production (Fig. 1B; Additional file 1).

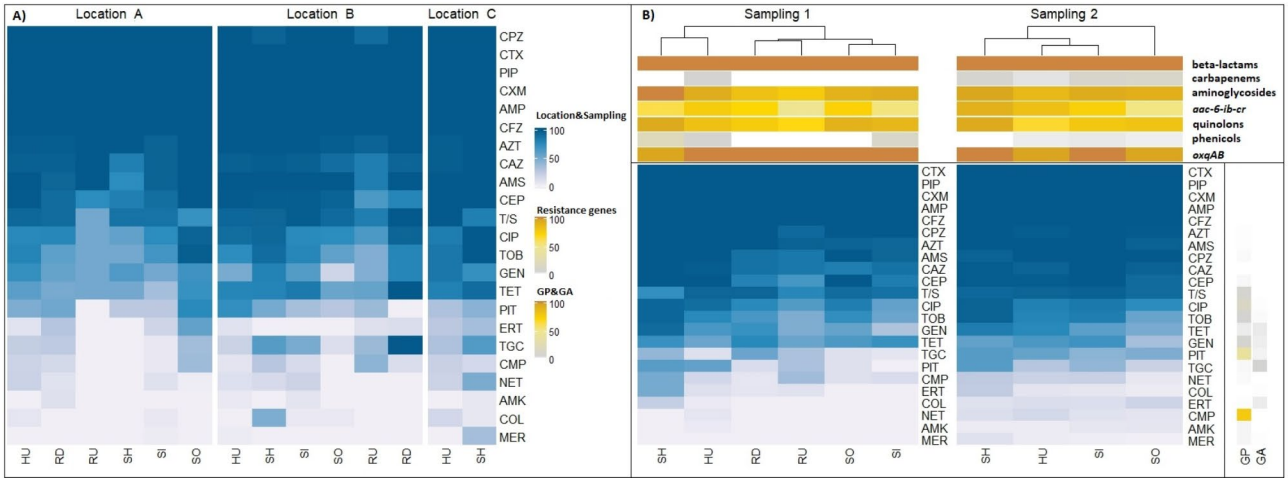

Fig. 1 Antibiotic resistance and ARGs across sources, samplings and locations. 1 **A** Heatmap displays differences in antibiotic resistance profiles between locations A, B, and C. Shades of blue indicates resistance to particular antibiotics, the darker the colour is the more isolates were resistant. At location C, only UTIs isolates and isolates from hospital wastewater were collected. 1**B** Central heatmap with side charts showing differences in antibiotic resistance between the first and second sampling periods. Shades of blue indicates resistance to particular antibiotics, the darker the colour is the more isolates were resistant. During the second sampling period, no isolates from river water were obtained. The upper chart shows the presence of ARGs related to resistance to particular antibiotics. The right side of the chart represents the discrepancy between the presence of ARGs and the phenotypic profile. GP (gene presence) - presence of an antibiotic resistance gene without phenotypic manifestations. The observed resistance of GA (gene absence) to related antibiotics was not supported by its genetic background. Color ramps for the upper chart and ride side chart were created as follows: 0% "white", 5% "light gray", 50% "khaki", 70% "gold", 99% "goldenred", and 100% "red".Sources HU – UTIs isolates, RD – downstream river water, RU – upstream river water, SH – hospital wastewater, SI - mWWTPs inflow, SO – mWWTPs outflow. Antibiotics: AMK, amikacin; AMP, ampicillin; AMS, ampicillin + sulbactam; AZT, aztreonam; CAZ, ceftazidime; CEP, cefepime; CFZ, cefazolin; CIP, ciprofloxacin; CMP, chloramphenicol; COL, colistin; CPS, cefoperazone + sulbactam; CPZ, cefoperazone; CTX, cefotaxime; CXM, cefuroxime; ERT, ertapenem; GEN, gentamicin; MER, meropenem; NET, netilmicin; PIP, piperacillin; PIT, piperacillin + tazobactam; T/S, cotrimoxazole; TET, tetracycline; TGC, tigecycline; TOB, tobramycin

Genetic background assessment

KpSC subspecies relevancy

MLST analysis and digital DNA-DNA hybridization revealed that within the set of KpSC isolates, most of them belonged to *K. pneumoniae*, namely, *K. pneumoniae sensu stricto* ($n=220$; 42 different STs) and *K. pneumoniae* subsp. *ozaenae* ($n=101$; 20 different STs), followed by *K. quasipneumoniae* subsp. *similipneumoniae* ($n=26$; 4 different STs), *K. variicola* ($n=22$; 5 different STs), *K. quasipneumoniae sensu stricto* ($n=2$; 2 different STs) and one isolate distantly related to *K. quasivariicola* (Fig. 2).

Overall, 73 different STs were assigned: 68 STs with already defined MLST types and five novel combinations (Additional file 2). UTIs-associated isolates formed *K. pneumoniae* (*K. pneumoniae sensu stricto* and *K. pneumoniae* subsp. *ozaenae)*. In hospital sewage, predominated *K. pneumoniae sensu stricto*, followed by *K. quasipneumoniae* subsp. *similipneumoniae* and *K. variicola*. In mWWTPs we detected especially *K. pneumoniae* and only minor proportion of other strains. Apart from *K. pneumoniae*, none of the strains were recovered from a river water (Additional file 1).

The most abundant STs included ST307 (52/372), ST405 (19/372), ST29 (17/372) and ST616 (17/372). *K. pneumoniae sensu stricto* ST307 was the most common subspecies detected in all sources but was detected mostly at location B (Fig. 3). *K. pneumoniae* subsp. *ozaenae* ST405 was recovered from all sources and locations but mostly from location B. *K. pneumoniae sensu stricto* ST29 was detected in patients, hospital wastewater and one isolate from a mWWTPs outflow, mostly from location A. *K. variicola* ST616 was also commonly found but only from hospital wastewater effluent at location B during the first sampling period.

Plasmid replicons associated with KpSC

Altogether, 41 different plasmid replicons were found, and at least one plasmid replicon was detected in 98% of isolates. The plasmid replicon FIB(K) predominated in isolates from all sources. Among isolates originating from hospital wastewater, FIB(K) was often accompanied by Col(pHAD28) (71/95). The predominant F-type plasmid was K7:A-:B- (235/372), which was connected with different STs (Fig. 4; Additional file 1). More plasmid replicons (median of 4 per isolate) were observed in isolates from hospital wastewater than in those from other sources (median of 2 per isolate), and the difference was statistically significant ($p<0.05$).

Principal coordinate analysis of the main characteristics of the KpSC

To better understand the mechanisms inside the KpSC dataset, we performed a principal component (PC) analysis to visualize the clustering of isolates on the basis

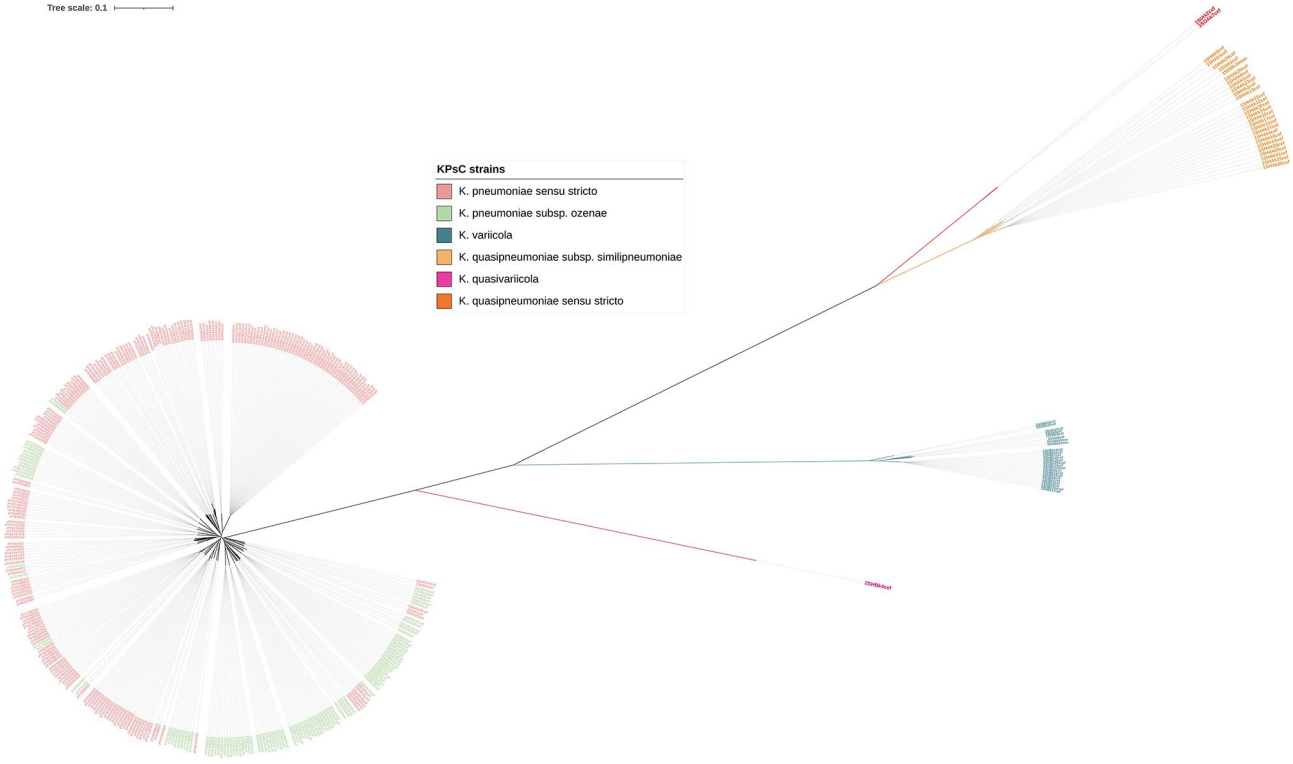

Fig. 2 Phylogenetic distances between different subspecies of KpSC. See legend to distinguish particular subspecies

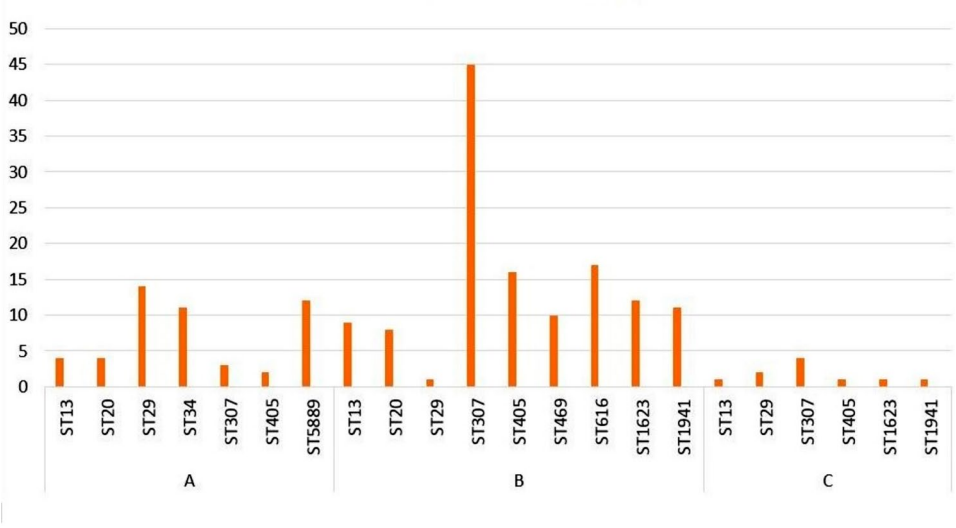

Fig. 3 Distribution of the most abundant STs among different location (**A**, **B**, **C**)

of multiple characteristics. The two main components accounted for only 21% of the influence of the PC1 and 13% of the influence of the PC2 variables, which suggests low inner diversity. The main coordinate PC1 was associated with $bla_{SHV-187}$, KP1_RS17305 (GDP-fucose synthetase associated with antiphagocytosis) and the Col440I plasmid replicon, whereas PCA2 was associated with *qnrB1* (resistance to quinolones), *aac(3)-IIa* (resistance to aminoglycosides) and the plasmid replicon FIB(K) (pCAV1099–144), which means that these characteristics influenced the shape and grouping of the variables. We observed three groups of characteristics that were positively correlated: (a) the *mrk*, *fimB* and *fimK* genes; (b) the fosfomycin resistance gene *fosA6* and the glycosyltransferase KP1_RS17220, KP1_RS17230 and KP1_RS17240 genes; and (c) the bla_{OXA-2}, bla_{OXA-10}, and *fosA7* resistance genes and the FIB (pKPHS1) plasmid replicon (Fig. 5A). When we marked components by metadata, we

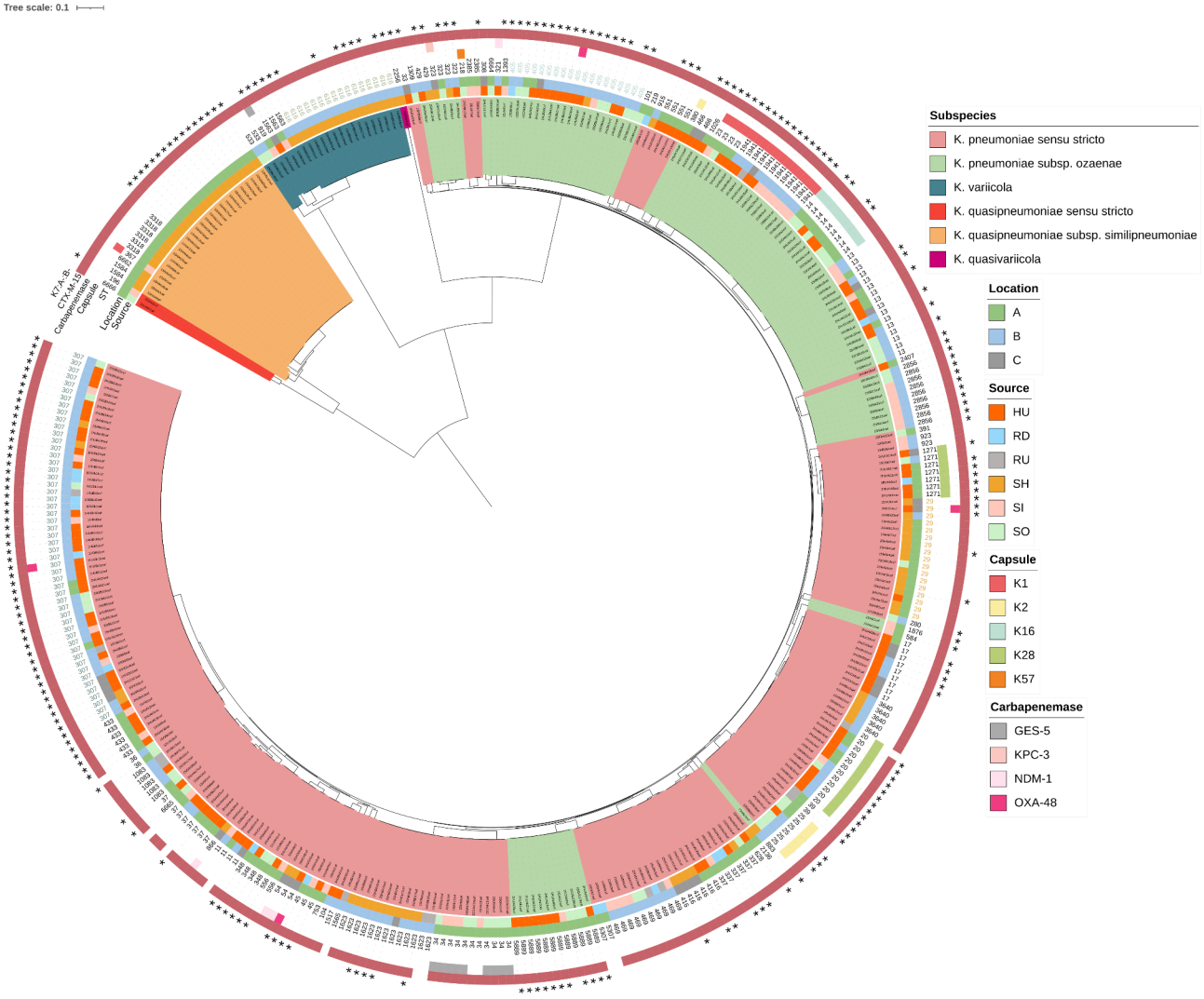

Fig. 4 Phylogenetic tree of the KpSC isolates based on SNP analysis. See the legend color code for information about location, source, ST,s presence of clinically relevant capsule types, F-type plasmid K7:A-:B-, presence of a single variant of CTX-M-15 beta-lactamase and presence of genes encoding resistance to carbapenems. The colored STs were repeatedly mentioned in the manuscript. Read isolates were coded as follows: sampling period (1, 2), HU- UTIs isolates; RD- downstream river water; RU- upstream river water; SH- hospital wastewater; SI- mWWTPs inflow; SO - mWWTPs outflow, location (A-C), number of isolates cultivated on plates with antibiotics (c – cefotaxime/m – meropenem), variant of isolate (optionally, if two morphologically different colonies were taken from one plate)

did not observe grouping based on characteristics such as source, location or STs, but we were able to distinguish groups according to the subspecies within the KpSC (Fig. 5B).

Virulence-associated factors in the KpSC

Virulence-associated factors (VAFs) are diverse without links between sources, locations, sampling periods or specific subspecies. However, we observed an STs-dependent number of virulence factors among the most abundant STs (ST29, ST307, ST409, and ST616), which were usually shared without any connection to the sample origin (Additional file 1). Most of the isolates produced fimbria of both types I (*fimA*) and III (*mrk* gene cluster).

At least six genes from the enterobactin-encoding gene cluster *entABCDEFS* were present in all but two isolates. The yersincin-associated gene cluster (*ybt*; 103/372) was present in isolates of ST405, ST1623 and ST1941, and was associated with the presence of *irp* genes. Interestingly, *the ybtS* and *irp1* genes were present among STs that generally harbored more VAFs. The *fyuA* gene encoding a highly specific yersiniabactin receptor from *Y. enterocolitica* harbored only ST25 and ST1271, but *fyuA/psn* (a similar receptor from *Y. pestis*) harbored 25% of the isolates, including the most prevalent STs.

Out of the 68 different loci for capsular polysaccharides identified in our dataset, 28 belonged to defined serotypes, and 40 were used to predict K loci identified from

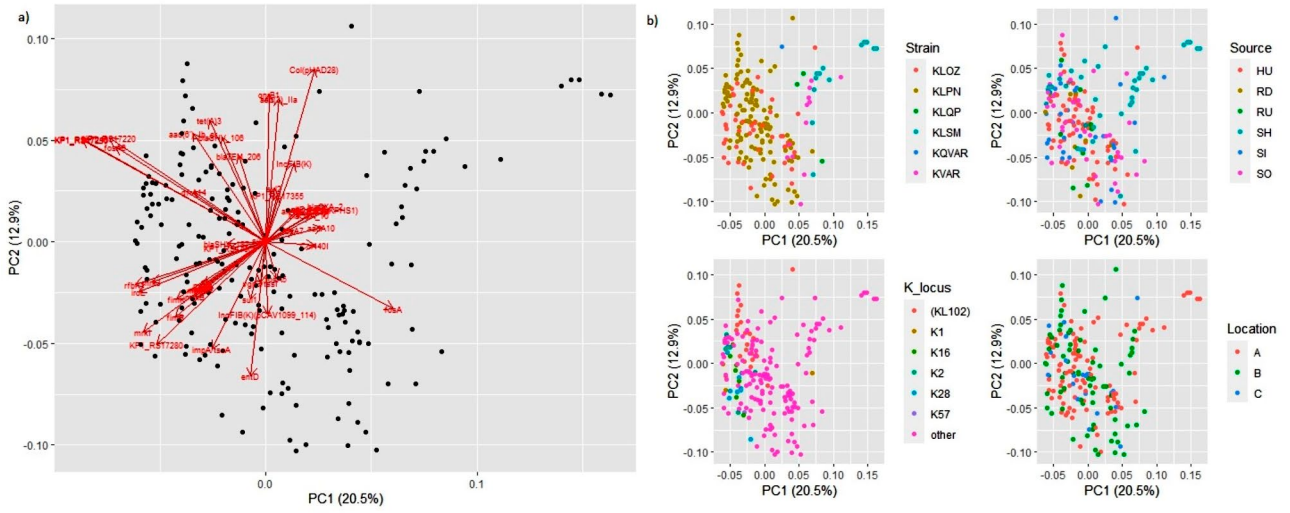

Fig. 5 Principal component analysis. Plots provide essential information from a large dataset and transform it into a few principal components, which convey the most variation in the dataset. 4a Loading plot. PC1 is shown horizontally, and PC2 is shown vertically. The values of the vectors on each PC show how much weight they have on that PC. Angles between the vectors express how characteristics correlate with one another, and grouping together suggests a strong positive correlation between variables. 4b PC clustering with marked strain, source, capsule or location. The dots do not represent each isolate; rather, they represent variation and account for the varied influences of the original characteristics

genomes. The frequency of capsule types in our dataset usually mirrored the frequency of the most abundant STs; therefore, the most frequent capsule types were (KL102) present in ST307 and ST433 and (KL151) present in ST405 and ST2856. The most virulent capsule types, K1 and K2, were detected mostly in the UTIs and mWWTPs isolates, but one isolate was also recovered from river water. K1 capsule type-positive isolates included ST23 and ST1941, which all (except for *iuc* in ST1941) harbored siderophore-encoding genes (*iro*, *irp* and *iuc*) and colibactin-producing *pks* island genes (*clb* gene cluster). The K1 capsule was also detected in isolate ST367; however, it did not confer any VAFs usually associated with the K1 serotype. On the other hand, one isolate, ST380, with a K2 capsule, carried *pks* island genes, which are usually associated with the K1 serotype and siderophore-encoding genes. No other isolates in our dataset carried genes associated with *pks* islands. Isolates with the K2 (ST25) and K20 (ST2385) serotypes often harbored genes encoding the siderophores salmochelin, yersiniabactin, and aerobactin. One isolate, ST258 with K57, harbored genes encoding salmochelin and aerobactin. *The rmpA* gene was present in all the isolates that carried salmochelin-encoding genes. In addition to the K1 and K2 capsule types, other globally reported highly virulent capsule types, such as K16 in ST14, K28 in ST20 and ST1271 and K57 in ST218, were detected in all sources except hospital wastewater. The most frequently detected lipopolysaccharide antigens (O) were O1/O2v2 and O1/O2v1, which were associated with different STs (Additional file 1).

KpSC population structure and phylogenetic analysis

Phylogenetic relatedness in the current study of the KpSC population

Phylogenetic analysis revealed low similarity among isolates within our dataset (SNPs in the range of 1-246985), except for two STs (ST348 and ST1083), where isolates originated from the same transmission cluster (up to 35 SNPs), and one ST, where clonal transmission was observed. The closest relatives of ST348 ($n=4$; SNPs in the range of 18–80) originated from UTIs and mWWTPs outflows and were found at location (A). For ST1083 ($n=5$; SNPs in the range of 25-3023), we observed relatedness between UTI isolates and isolates from the upstream river (SNPs in the range of 25–39) at location (B). These isolates presented similar genomic characteristics and similar antibiotic resistance patterns.

ST307 ($n=52$; SNPs in the range of 17-2027, median of 1029 SNPs) was the predominant lineage recovered from all sources within this study but mostly at location B. We observed several isolates that differed in up to 21 SNPs between clinical isolates, mWWTPs inflow and downstream river water in the first sampling period, which were considered clonal transmissions between sources. Moreover, we observed one persistent clinical clone (location B), which was detected in both the first and second sampling (15 SNPs) periods and was closely related to isolates recovered from river water in the first sampling period (18–20 SNPs). All the isolates produced CTX-M-15, and one clinical isolate from location B was also positive for the OXA-48 carbapenemase. Most isolates harbored *aph(3")-Ib*, *aac(6')-Ib-cr*, *oqxA/B*, *dfrA14*, *fosA6*, *qnrB1*, *sul2* and *tet(A)* resistance genes. All

the isolates presented a similar set of VAGs except two, which did not carry fimbriae type I- or III-producing genes (Additional file 2).

Phylogeny of KpSC strains in the Czech Republic and Europe

For a better assessment of the transmission of resistant KpSC strains in the Czech Republic, we selected STs that were shared by both the current study and at least one cooperating hospital microbiological department. Among the 13 (for ST14, ST17, ST23, ST25, ST45, ST54, ST219, ST307, ST321, ST405, ST433, ST551, and ST1271) individually constructed SNPs matrices (Additional file 3), only ST307 showed relatedness between isolates.

Firstly, we enhanced our ST307 dataset with unpublished data from the hospital microbiological department from location B (n=7; SNPs range from 43 to 1150; median 182 SNPs). Only 43% of the strains harbored $bla_{\text{CTX-M-15}}$, but all harbored $bla_{\text{SHV-106,}}$ encoding ESBL. On the other hand, they all carried $bla_{\text{OXA-48}}$ or $bla_{\text{NDM-1}}$. Most of them harbored several plasmid replicons, which included FII (pKP91), IncHI1B (pNDM-MAR), IncFIB (K), IncFIB (pNDM-Mar), IncL and IncR. Most of these isolates harbored siderophore-encoding genes (*iro*, *irp*, *ybt* and *iuc*). One UTI isolate from the current study differed in 43 SNPs from the UTI isolate from the hospital at location B (Additional file 2).

The low SNPs difference between Czech ST307 isolates led us to investigate whether ST307 in the Czech Republic shares unique characteristics. Therefore, we downloaded publicly available European ST307 data from the Pasteur database and compared them with the above-mentioned Czech ST307 dataset. We observed an overall low SNPs difference among European ST307 strains (n=710; SNPs ranging from 1 to 3090, median 160 SNPs). Human clinical isolates clustered with strains of non-human origin, including those from animals, the environment or even food from various countries, and were collected over several years (Additional file 4). Most European isolates presented very similar characteristics, as described in this study, as most of them harbored the ESBL-encoding genes $bla_{\text{CTX-M-15}}$ and $bla_{\text{SHV-106}}$ and other beta-lactamase genes $bla_{\text{OXA-1}}$ and $bla_{\text{TEM-1B}}$. Carbapenemase-encoding genes were represented especially by $bla_{\text{OXA-48}}$, $bla_{\text{NDM-1}}$ and $bla_{\text{KPC-3}}$ (Additional file 5). The predominant ARGs in the whole dataset consisted of *aph(3")-Ib*, *aac(6')-Ib-cr*, *oqxA/B*, *dfrA14*, *fosA6*, *qnrB1* and *sul2*. The tetracycline resistance gene *tet(A)* was present in 43% of the isolates; other ARGs were detected in less than 20% of the isolates. Most of the strains harbored the FIB(K) plasmid replicon, followed by FII(pKP91), Col(pHAD28) and IncFIB(pQil). Among the F plasmids, the most abundant replicon sequence types were K7:A-:B- and K2:A-:B-. The isolates shared similar VAFs as the isolates detected in the Czech Republic but also harbored mostly siderophore-encoding genes (*iro*, *irp*, *ybt* and *iuc*), and the predominant capsule type was KL102 (Additional file 1).

Discussion

The objective of this study was to determine whether MDR ESBL-/carbapenemase-producing KpSC clones of clinical origin spread via hospital wastewaters and mWWTPs into receiving river water and to assess their impact on public health and the environment. We revealed high variability in the number of KpSC STs from different niches, confirmed the dissemination of clinically relevant clones into the environment, and investigated similar genomic patterns among clinical isolates from the current study and isolates collected from cooperating hospitals.

In contrast to our previous study focusing on *E. coli* [16], we did not observe a difference in the rate of antibiotic resistance among clinical, wastewater and river water isolates. Notably, these findings are biased by the use of antibiotic-supplemented media for selective cultivation; therefore, the results do not reflect the complete picture, as susceptible isolates were excluded. In both of our studies, ESBL producers were observed in all niches, whereas carbapenemase-producing isolates were solely recovered from hospitals and municipal wastewaters. ESBL was encoded mostly by $bla_{\text{CTX-M-15}}$. The predominance of CTX-M-15 in KpSC clinical isolates has been repeatedly reported in recent studies [8, 31].

Most isolates in this study presented an MDR profile and carried multiple ARGs, but when the resistance profile and genetic background were compared, we observed a discrepancy between the phenotype and genotype. Although previous studies [1, 28] and the ResFinder database link *oqxAB* genes with resistance to multiple antimicrobials, including chloramphenicol, we observed 70% concordance between presence of these genes and chloramphenicol resistance. One of the factors that influences the ability of OqxAB to induce chloramphenicol resistance could be the genomic location of the operon [10]. OqxAB efflux pumps are also recognized for their resistance to ciprofloxacin [10], but a discrepancy between the presence of OqxAB and the resistance phenotype was also observed in our study. We also observed ertapenem-resistant isolates (12%) with controversial genetic backgrounds. Traditionally, carbapenem resistance is associated with carbapenemase genes or point mutations in selected sites of *ompK36* or *ompK7* [17], but unlike in previous studies, point mutations in *ompK36/37* [57] did not lead to ertapenem resistance in our isolates. However, carbapenem resistance is a complex mechanism that depends not only on the presence or absence of ARGs but also on the presence of other genotypic factors,

such as transposons, promoters, copy numbers of resistance-associated genes or the expression of ESBLs [33]. We did not consider the presence of the *ompK37* point mutation, as it was present in all the isolates regardless of susceptibility or resistance to ertapenem. Some studies suggest the impact of point mutations in *ompK35*, but we did not detect any of them [12]. All the isolates carrying the carbapenemase-encoding gene (except bla_{OXA-48}) presented phenotypic resistance to carbapenems; the isolates harboring bla_{GES-51} and bla_{OXA-48} conferred resistance only to ertapenem, whereas bla_{KPC-3} and bla_{NDM-5} resulted in phenotypic resistance to both ertapenem and meropenem. Additionally, all carbapenemase producers (according to the MAST disc test) were resistant to ertapenem or both ertapenem and meropenem. Isolates harboring the recently described bla_{GES-51} [62] presented an ESBL phenotype according to the MAST disc test. We did not identify any colistin-related resistance genes, although we detected several colistin-resistant KpSC isolates. The production of capsule and the number of capsule layers positively influence antibiotic resistance [25]. We checked whether any of the clinically relevant capsule types were involved in colistin resistance in our isolate collection, but we were not able to find any pattern. We hypothesize that the presence of colistin-resistant isolates with no apparent genetic marker could be influenced by some point mutations or not discovered resistance mechanisms that have yet to be investigated.

KpSC strains are difficult to discriminate in routine MALDI-TOF diagnostics, where only *K. pneumoniae* (as a complex) and *K. variicola* can be discriminated by the commonly used commercial database MALDI Biotyper (Vers. 8.0, 7854 MSP, RUO, Bruker Daltonics, Bremen, Germany) and VitekMS DB (v.3.2, bioMérieux, Marcy-l'Étoile, France); therefore, the clinical role of a particular species is consistently underestimated [15, 26]. For rapid discrimination of phylogroups among KpSC, genetic markers were identified on the basis of the presence of *bla* genes; *K. pneumoniae* was related to the presence of bla_{SHV}, *K. quasipneumoniae* with $bla_{OKP,}$ and *K. variicola* was associated with bla_{LEN} [11, 27]. This association was confirmed in our dataset. However, distinguishing clinically relevant subspecies within *K. pneumoniae*, *K. pneumoniae* subsp. *ozaenae* and *K. pneumoniae sensu stricto*, remains possible only by DNA–DNA hybridization or other similar methods.

Recently, *K. variicola* and *K. quasipneumoniae* infections (apart from UTIs), including those caused by isolates carrying carbapenemase genes, have been reported [21, 38, 43]. The absence of *K. quasipneumoniae* in the clinical isolates in the current study could be caused by a lower proportion of this subspecies in the urine samples in general. On the other hand, a high proportion of these genera in hospital wastewaters (at location A) could be caused by their relatively high prevalence in the gut; however, this was not the case in this study. Within this dataset, we also detected one *K. quasivariicola*-like isolate that might represent a novel species, as it is distantly related to *K. quasivariicola*, but further characterization requires more than one isolate of this potential species.

PC counting revealed a positive correlation between different genes and plasmid replicons within our KpSC collection. This method allows us to compute the associations of different components within the dataset. The first group with such associations was formed by isolates with the ability to adhere to host cells via fimbriae type I (*fim*) and III (*mrk*). In particular, clinical isolates produce both fimbriae types that mediate adhesion to several cell types in vitro [61], but in our study, most isolates harbored both fimbriae types regardless of their source or location. The next association group included genes encoding glycosyltransferase and fosfomycin resistance. Glycosyltransferases are coresponsible for various virulence mechanisms, including the adherence and biofilm formation of pathogenic bacteria [51, 68]. Although it has not been reported directly for Enterobacterales, in gram-positive bacteria, *fosB* mediates the production of the specific antioxidant bacillithiol as a nucleophile to destroy fosfomycin [56]. Cluster 3 included a specific plasmid replicon, a fosfomycin resistance gene and genes encoding class D oxacillinases [3]. In our study, we observed IncFIB(K) replicon sequence type K7 to be the most prevalent type among all the KpSC isolates. The IncFIB(K) plasmid replicon has been described as one of the most common KpSC strains from both human [19, 23] and nonhuman sources [17, 66]. Additionally, the specific replicon sequence type formula K7:A-:B-, which was commonly detected in this study (and among European strains generally) was previously described in association with various KpSC STs [69].

The production of VAFs such as enterobactin, fimbriae, lipopolysaccharides or capsules is common in most *K. pneumoniae* isolates but not in other strains within the KpSC. The sole production of the above-mentioned VAFs is not the pledge of successful host colonization. The ability to produce additional siderophores is one of the key factors for hypervirulence [36] and is strongly linked to capsule type. Among other important hypervirulent capsule types, the most pathogenic serotypes are K1 and K2. Capsule production in hypervirulent strains is potentiated by a regulator of mucoid phenotype A, *rmpA* [52], which is consistent with our data in which *rmpA* was observed mostly in the K1 and K2 isolates. These clones can secrete toxins with cytostatic or cytotoxic effects, such as colibactin, which is encoded by polypeptide synthase (*pks*) genes. *pks* islands are composed of 19 genes (*clbA-clbR*), and the presence of all the genes is necessary for colibactin production. In our dataset, most K1

producers and one K2-producing isolate harbored a *pks* island. While the K1 serotype is usually limited to ST23, we also observed this serotype in ST1941 and ST367. The secretion of the siderophores aerobactin (encoded by *iuc*), salmochelin (encoded by *iro*), and yersinicin (encoded by *irp*) also influences the production of colibactin [24] and was recognized among the isolates in our data. Although isolates within the same STs are not specifically related to the same capsule type [50] in our dataset, most isolates of certain STs produced the same capsule type.

The main goal of this study was to track KpSC clones from the hospital environment to the adjacent aquatic environment via wastewater. Although the number of similar studies is limited, the clonal relatedness of ST11 and ST985 between patients, wastewater and river water has been reported in Austria [39] and China [71]. However, we observed only low similarity between KpSC isolates, even those from the same STs. We detected only *Klebsiella pneumoniae sensu stricto* ST307, which presented a median range of 84 SNPs between isolates. However, several isolates shared distances of up to 21 SNPs, confirming the transmission of this lineage from UTIs to mWWTPs and from mWWTPs to rivers. Moreover, we detected one clone that was present in the mWWTPs in both the first and second sampling periods, which suggests that the clone has the ability to survive in wastewater, persist in the community or persist in hospital facilities and continuously spread via wastewater to the WWTPs. The higher proportion of ST307 at location B than at location A remains unclear. Possible explanations include some unrevealed source of ST307 at location B or too small sampling time-frame as we used a grab samples instead of composite samples. This lineage was the most abundant in this study and is increasingly reported worldwide [29, 64]. It was previously suggested that with the ability to rapidly acquire ARGs, including carbapenemase genes, ST307 has the potential to become a predominant lineage in the future [67], but in our current study, we observed only one isolate harboring $bla_{\text{OXA-48}}$.

ST307 isolates obtained within this study were put in context with other Czech and European isolates of this particular sequence type. We were able to observe relatedness between clinical isolates collected in the same hospital (location B) even during different collection periods (2018–2021). Such relatedness has also recently been observed in other countries [29] and could suggest interpatient transmission or persistence in hospitals. Compared with the European ST307 strains, we observed very similar genomic characteristics between isolates during the last 12 years across the continent. This finding is in agreement with other studies on ST307 genomics that suggested that the ST307 core genome is highly conserved worldwide and that the main differences remain in the plasmids [64]. In particular, the common presence of carbapenemase-encoding genes is alarming, as has been repeatedly reported [13, 63, 67]. Although it was suggested that *Klebsiella* spp. is not a One Health pathogen [18], closely related ST307 strains found among different sources, including animals [63], wastewaters, the environment and even food, show otherwise.

In this study, we had to address several biases that have been broadly described in Davidova-Gerzova et al. [16]. First, the first sampling period was set up immediately after the local flood (location A), which could influence the total amount of antibiotic-resistant bacteria detected in the river. Also, the river was not accessible, so we had to perform sampling at the first possible access point to the river water which was up to 1,9 km downstream. Second, we used grab samples from all types of water samples, as we wanted to prevent the possibility of bacterial overgrowth in our experiments, although this approach provides only a snapshot of the population and could lead to the under/overestimation of some subspecies in our study. Third, before the second sampling period, we contacted hospital and mWWTPs in location C to cooperate in isolate collection and wastewater sampling to cover a broader area. After difficulties in legal agreements between the mWWTP facility and our institution, we finally performed a second sampling at location C, but only clinical isolates and raw hospital wastewater were obtained.

Conclusion

This study highlights the influence of mWWTPs on the receiving environment in the context of the spread of antibiotic-resistant bacteria and ARGs. We detected ESBL disseminated among isolates from all water sources and locations, whereas carbapenemase genes were present mostly in hospital-related environments and mWWTPs. SNPs analysis provided evidence of the transmission of the ST307 lineage from UTIs isolates via mWWTPs to surface water and even the persistence of this lineage in wastewater for several months. Most isolates from UTIs consisted of *K. pneumoniae* (*K. pneumoniae sensu stricto* and *K. pneumoniae* subsp. *ozaenae*), including hypervirulent isolates, which generally harbored a broader range of VAFs than other isolates within the KpSC. These isolates were, however, observed more often in hospital wastewaters, which implies the impact of sources other than UTIs. This study emphasizes the importance of the surveillance of pathogen-resistant bacteria in hospital and community wastewaters and their spread through mWWTPs to surface water and the environment.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13756-024-01496-0.

Supplementary Material 1: Additional file 1: Summary table of KpSC isolates and its characteristics obtained within this study. S1: Read isolate name as: 1/2: first and second sampling period; HU - clinical isolates (UTIs - urinary tract infections), SH - hospital wastewater, SI - municipal wastewater treatment plants (mWWTPs) inflow, SO - mWWTPs outlet, RU - river upstream, RD - river downstream, A/B/C - location, no. of isolate, cef/mer - selective cultivation on medium enhanced with cefotaxime or meropenem; Serotyping: ND - not defined locus, K-locus: K1-81 defined capsular types, (KL102-184) predicted K loci identified from genomes; Phenotype characteristics: dark blue marking - resistance, light blue - dubious, white - sensitive isolate; Genetic characteristics: + stays for presence of target gene/plasmid replicon, blank for absence of target gene/plasmid replicon, dark grey marking for 100% coverage and identity; beta-lactams: shades of brick red - AmpC, ESBL, carbapenemases, other beta-lactamases. S1_EU: European strains of ST307 used for the purposes of this study obtained from cooperating hospitals in Czech Republic and downloaded from Pasteur database; HU - clinical isolates, NP - not provided; + stays for presence of target gene/plasmid replicon, blank for absence of target gene/plasmid replicon, dark grey marking for 100% coverage and identity; beta-lactams: shades of brick red - AmpC, ESBL, carbapenemases, other beta-lactamases. Statistics: Statistical comparison of antimicrobial resistance according origin of Klebsiella spp. isolates.

Supplementary Material 2: Additional file 2: The main characteristics of ST307 isolates within Czech Republic. See information about Location, Source, Year of sampling, Capsule type and selected genetic characteristics.

Supplementary Material 3: Additional file 3: Matrices of SNP distances. Matrix_all was created for all isolates which were obtained in current study. Other tables were created as an individual SNP tables for STs that a) showed low number of SNPs; b) were found in both current study and at least one cooperating hospital. For ST307 matrix containg only current study isolates and a matrix which was enhanced of both Czech and other European strains were created.

Supplementary Material 4: Additional file 4: Characteristics and phylogeny of ST307 among European ST307. Green marking in the center stays for Czech islates obtained in this study, blue marking represents Czech isolates from other studies. "Other" for carbapenemases covers blaGES-5, blaNDM-7, blaOXA-162, blaOXA-181, blaOXA-232, blaOXA-244, blaVIM-1, blaVIM-4. "Other" for replicon sequence type covers: [F-:A13:B-], [F2:A-:B-], [F29:A-:B-], [K1:A13:B-], [K12:A-:B-], [K13:A-:B-], [K13:A13:B-], [K19:A-:B-], [K2/D181:D334:A-:B-], [K28:A-:B-], [K5:A-:B-], [K7:A-:B70], [K7:A10:B-], [K8:A-:B-], [Y4:A-:B36]. See legend for more information about Source and replicon sequence types.

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Author contributions
LD-G, IS, and MD conducted the research and designed the experiments. LD-G, JL, IS, LN, PK and MK performed the experiment. LD-G, JL, IS and MB provided the data analysis. LD-G wrote the manuscript. MD and others provided revisions. All authors contributed to the article and approved the submitted version.

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Data availability
The datasets supporting the conclusions of this article are included within the article and its Additional files, GenBank repository, Bioproject no. PRJNA1130981 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1130981/) and Pasteur database.

Declarations

Ethical approval
Not required.

Patient consent
Not required.

Permission to reproduce material from other sources
Not required.

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Not required.

Competing interests
The authors declare no competing interests.

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