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Whole-genome sequencing of MDR *K. pneumoniae* circulating in Moroccan river ecosystems: a comprehensive genomic analysis of environmental isolates

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Abstract

Background *Klebsiella pneumoniae* is a major opportunistic pathogen and a recognized contributor to the global burden of antimicrobial resistance (AMR). Although traditionally associated with healthcare settings, it is increasingly detected in environmental compartments influenced by anthropogenic activities. Aquatic ecosystems may act as reservoirs and dissemination hubs for multidrug-resistant (MDR) lineages and resistance genes. However, genomic data on environmental *K. pneumoniae* in Morocco remain scarce. This pilot study aimed to characterize a targeted subset of MDR *K. pneumoniae* isolates recovered from Moroccan rivers using whole-genome sequencing (WGS), with a focus on resistance determinants, virulence-associated loci, plasmid content, and phylogenetic relationships.

Results The main findings revealed marked genetic heterogeneity among the environmental *K. pneumoniae* isolates. Of the 44 isolates recovered from six Moroccan rivers, 11 MDR isolates were selected for WGS and belonged to nine distinct sequence types, including the high-risk clones ST147 and ST307. Resistance to extended-spectrum β-lactams was mainly associated with ESBL genes, particularly $bla_{CTX-M-15}$, whereas carbapenem resistance was linked to bla_{OXA-48} and bla_{NDM-14} in a subset of isolates. The genomes also carried multiple determinants associated with resistance to other major antimicrobial classes. In addition, the isolates showed diverse plasmid backgrounds, heterogeneous virulence-associated loci, and substantial capsular diversity. Phylogenetic and pangenome analyses further indicated high intraspecific variability and extensive genome plasticity across the collection.

Conclusions This pilot study provides the first whole-genome-based characterization of river-derived MDR *K. pneumoniae* in Morocco. It demonstrates that aquatic ecosystems harbor genetically diverse lineages carrying clinically relevant resistance and virulence determinants. These findings underscore the imperative of integrating environmental surveillance into national AMR monitoring strategies within a One Health framework.

Keywords *Klebsiella pneumoniae*, Multidrug resistance, Whole-genome sequencing; River ecosystems, One Health, Morocco

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Background

K. pneumoniae is a Gram-negative, encapsulated opportunistic pathogen and a major cause of severe infections, including pneumonia, urinary tract infections, and bloodstream infections [1]. Notably, *K. pneumoniae* is a member of the ESKAPE group of priority pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter* spp.), which are frequently associated with difficult-to-treat infections and a substantial antimicrobial resistance (AMR) burden [2]. The global expansion of multidrug-resistant (MDR) *K. pneumoniae* has become a major public health concern, largely driven by the dissemination of extended-spectrum β-lactamases (ESBLs) and carbapenemases that compromise last-line therapies and contribute to increased morbidity and mortality [3]. Importantly, MDR *K. pneumoniae* is often associated with internationally disseminated high-risk clones, such as ST147 and ST307, which have been linked to epidemic spread and the acquisition of clinically relevant resistance determinants [4].

In addition to AMR, *K. pneumoniae* is characterized by a broad virulence repertoire, including capsule-associated factors, adhesins, siderophore systems, and biofilm-related traits that promote colonization, persistence, and immune evasion [5]. The convergence of MDR with enhanced virulence is an alarming global trend. For instance, recent genomic studies have revealed that MDR *K. pneumoniae* clones frequently acquire additional virulence genes, thereby increasing their epidemic potential [6]. This co-occurrence of resistance and virulence not only complicates treatment options but also increases the risk of severe disease and subsequent transmission, reinforcing the need for integrated surveillance approaches that simultaneously track resistance, virulence, and high-risk clonal backgrounds [7].

Although traditionally viewed as a healthcare-associated bacterium, *K. pneumoniae* is increasingly recognized beyond clinical settings, particularly in aquatic ecosystems [8]. Recent studies have documented the presence of environmental *Klebsiella* in surface waters and highlighted aquatic ecosystems as important reservoirs and dissemination pathways for AMR [9]. Rivers affected by anthropogenic activities, including urban discharge, surface runoff, and wastewater treatment plant (WWTP) effluents, may serve as environmental reservoirs where resistant bacteria and mobile resistance determinants persist and disseminate [10]. In Morocco, similar pressures affect river systems and may contribute to microbial contamination and the persistence of resistant bacteria in surface waters [11]. This environmental dimension is central to the One Health framework, as freshwater ecosystems connect human activity with AMR dynamics and facilitate the environmental circulation of clinically relevant lineages [12].

However, despite the importance of environmental AMR surveillance, genomic evidence of MDR *K. pneumoniae* circulating in Moroccan river ecosystems remains limited. To address this gap, the present pilot study applies WGS to MDR *K. pneumoniae* isolates recovered from Moroccan rivers. By combining phenotypic susceptibility testing with genome-based analyses, we aimed to define antimicrobial resistance profiles, characterize resistance and virulence gene contents, investigate plasmid replicon types, and resolve phylogenomic relationships to determine population structure and clonal relatedness. Overall, this integrated approach was designed to provide an initial genomic snapshot of selected MDR environmental *K. pneumoniae* isolates and to explore their relevance for One health surveillance of AMR in Moroccan aquatic ecosystems.

Table 1 Sampling sites, locations, and geographic coordinates

River	City	Latitude (*N*)	Longitude (W)
Bouregreg river	Rabat	33.993436	6.801565
Bouskoura river	Casablanca	33.519931	7.663718
El Maleh river	Mohammedia	33.679071	7.410369
Merzeg river	Casablanca	33.535407	7.789357
Souss river	Agadir	30.341391	9.593026
Tensift river	Marrakech	31.698979	8.063650

Methods

Sampling sites and collection

As part of a pilot study on environmental AMR surveillance, surface water samples were collected between February and June 2024 from six major rivers located in urban and peri-urban areas of Morocco (Table 1), including Bouregreg river (Rabat), Bouskoura river (Casablanca), El Maleh river (Mohammedia), Merzeg river (Casablanca), Souss river (Agadir), and Tensift river (Marrakech). These rivers were selected to represent diverse hydrological environments and varying degrees of anthropogenic pressure, particularly domestic wastewater discharge, industrial effluents, and agricultural runoff. To assess spatial variation in contamination, discrete grab samples were collected at three points along each river (upstream, midstream, and downstream), resulting in a total of 18 water samples. At each site, approximately 500 mL of surface water was collected at a depth of ~ 30 cm using sterile containers. The samples were immediately placed in an insulated container at 4 °C, transported to the laboratory, and processed within 6 h of collection to preserve microbial integrity. Standard environmental water sampling protocols were followed throughout the study [13].

Bacterial isolation and identification

Water samples were processed using a selective enrichment procedure. Briefly, 500 µL of each sample was subcultured into Brain Heart Infusion (BHI) broth (Bio-Rad) to promote bacterial growth and incubated at 37 °C for 24 h. After enrichment, 10 µL of the enriched culture was inoculated onto Brilliance UTI Clarity Agar (Oxoid), a chromogenic medium supplemented with ertapenem (0.5 µg/mL) to screen for carbapenem-resistant isolates, or with ceftazidime (3 µg/mL) to select isolates with an ESBL phenotype. The plates were incubated aerobically at 37 °C for 18–24 h. Presumptive *K. pneumoniae* colonies were identified on the basis of their characteristic appearance on Brilliance UTI Clarity Agar, which presented as blue mucoid colonies in accordance with the manufacturer's guidelines. The selected colonies were purified by repeated streaking until pure cultures were obtained. Species identification was confirmed using the VITEK 2 system (bioMérieux, Marcy-L'Etoile, France), and confirmed isolates were stored in BHI broth with glycerol at − 80 °C for further analyses.

Antimicrobial susceptibility testing

The antimicrobial susceptibility of all the confirmed isolates was performed using the disk diffusion method on Mueller–Hinton agar (Bio-Rad) in accordance with the guidelines of the Antibiogram Committee of the French Society for Microbiology (CA-SFM) [14]. The following antibiotic disks were tested: amoxicillin–clavulanic acid (20/10 µg), cefoxitin (30 µg), ceftazidime (10 µg), cefotaxime (5 µg), cefepime (30 µg), aztreonam (30 µg), ertapenem (10 µg), imipenem (10 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), and trimethoprim–sulfamethoxazole (1.25/23.75 µg) (Oxoid Ltd., Hampshire, United Kingdom).

ESBLs production was confirmed using the double-disk synergy test (DDST) [15]. Quality control was performed using *K. pneumoniae* ATCC 23,357. Isolates resistant to at least three antimicrobial classes were classified as MDR according to standardized international criteria [16]. Intrinsic resistance to ampicillin and ticarcillin in *K. pneumoniae* was excluded from the MDR classification in accordance with EUCAST expected phenotype [17].

Isolates showing reduced susceptibility to at least one carbapenem were further screened using the NG-Test CARBA 5 (NG Biotech, France), an immunochromatographic assay for the rapid detection of KPC, NDM, VIM, IMP, and OXA-48-like carbapenemases, following the manufacturer's instructions [18].

DNA extraction and whole-genome sequencing

From the 44 environmental *K. pneumoniae* isolates recovered in this study, a subset of 11 MDR isolates was selected for WGS. Genomic DNA was extracted from overnight cultures grown in Luria–Bertani (LB) broth at 37 °C, using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's protocol. DNA quantity was determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, USA), and purity was assessed by spectrophotometry using a ScanDrop^2 instrument (Analytik Jena, Germany).

WGS was conducted at the Genomic Sequencing Laboratory, Institut Pasteur du Maroc (Casablanca, Morocco). Sequencing libraries were prepared using the Illumina DNA Prep Kit (Illumina, Eindhoven, Netherlands) and sequenced on the Illumina NextSeq 2000 platform (Illumina, San Diego, CA, USA) to generate paired-end reads of 2 × 150 bp, with an average sequencing depth of approximately 100× per genome.

Quality reads and genome assembly

Raw paired-end sequencing reads were first assessed for quality using FastQC (v0.12) [19] to evaluate sequencing performance and detect potential adapter contamination. Low-quality bases and adapters were trimmed using Trimmomatic (v0.39) [20]. The resulting high-quality reads were *de novo* assembled into draft genomes via SPAdes (v4.2.0) [21]. Assembly quality was evaluated using QUAST (v5.3.0) [22]. Contigs shorter than 200 bp were removed using BBMap (v39.01) [23] to improve assembly reliability. Genome completeness and contamination were subsequently assessed with CheckM (v1.2.2) [24]. Assemblies were retained for downstream analyses when they met the following criteria: contig count < 500, genome size within the expected range for *K. pneumoniae* (4,969,898–6,132,846 bp), G + C content between 56.35% and 57.98%, completeness > 95%, and contamination < 5%. Descriptive assembly statistics, including genome size, N50, GC content, and estimates of genome completeness and contamination for all sequenced isolates, are presented in Supplementary Table S1.

Genomic characterization and annotation

Multilocus sequence typing (MLST), capsular (K-locus) and lipopolysaccharide O-antigen (O-locus) typing, as well as antimicrobial resistance and virulence-associated genes, were determined using Kleborate (v3.2.4) [25], this tool integrates Mash-based genomic distance thresholds for species assignment within the *K. pneumoniae* species complex [26], and Kaptive (v3.1.0) for locus typing [27]. As shown in Supplementary Table S2, all isolates were assigned to *K. pneumoniae* sensu stricto, with a strong species match confidence for all genomes. Using this standardized framework, genome assemblies were screened for major *K. pneumoniae* virulence loci, including the siderophore systems yersiniabactin (*ybt*) and aerobactin (*iuc*), as well as colibactin (*clb*), salmochelin

(*iro*), and hypermucoidy-associated loci (*rmpADC* and *rmpA2*). Plasmid replicon types were identified using PlasmidFinder (v2.1) [28], and plasmid sequences were subsequently reconstructed and clustered with MOB-suite (v3.1.9) [29]. In parallel, draft genomes were functionally annotated using the Bacterial and Viral Bioinformatics Resource Center (BV-BRC) platform [30] to generate consistent gene predictions. Finally, to examine the genetic contexts of $bla_{\text{CTX-M-15}}$ and $bla_{\text{OXA-48}}$, MOB-suite extracted plasmid sequences carrying these genes were compared using Clinker [31], enabling alignment and visualization of conserved gene organization and associated mobile genetic elements (MGEs) across plasmids.

Pangenome and phylogenetic analysis

Pangenome analysis was performed using the anvi'o (v8) platform following its comparative genomics workflow [32]. Genome assemblies were first reformatted and filtered to retain contigs longer than 2,500 bp. For each genome, anvi'o contig databases were created and processed through the built-in annotation workflow, including hidden Markov model (HMM)-based annotation, assignment of functional categories, detection of tRNA genes, and single-copy core gene taxonomy profiling. Annotated genomes were combined into a genome storage database to generate the pangenome, with homologous gene clusters identified and classified into core, accessory, and unique components. Genome relatedness was further assessed by average nucleotide identity (ANI) calculated with pyANI (v0.2.12) (Supplementary Table S3) [33], with the anvi'o pangenome database used as input. Pangenome structure and genomic similarity were visualized using anvi'o interactive plots, enabling comprehensive assessment of genomic conservation and diversity among the analyzed isolates.

Phylogenetic analysis was performed by comparing whole genomes using the alignment-free split K-mer Analysis (SKA2) (v0.5.1) [34] (Supplementary Table S4), which detects core-genome single-nucleotide polymorphisms (SNPs) from k-mer variation. Split k-mers were generated from genome assemblies using a k-mer size of K = 31. Pairwise distances were computed while allowing ambiguous bases and applying a minimum frequency threshold of 1. An SKA-derived genome-wide SNP alignment was then constructed and used to infer a maximum-likelihood phylogeny with RAxML (v8.2.12) [35], under the GTRCAT approximation, with 100 rapid bootstrap replicates used to assess branch support. To contextualize our environmental *K. pneumoniae* genomes, we compared them against 102 publicly available genomes from the Pathogenwatch database, as previously described for genomic surveillance and lineage contextualization [36]. Metadata for the reference genomes are provided in Supplementary Table S5. The resulting phylogenetic trees were visualized and annotated using iTOL v6 [37].

Statistical analysis

Statistical analyses were performed in R (v4.4.3). The antimicrobial resistance gene load (ARG load) was defined as the total number of acquired resistance genes detected per isolate. Differences in ARG load among rivers were assessed using the Kruskal-Wallis test, and a two-sided p-value < 0.05 was considered statistically significant. Antimicrobial resistance phenotypes were further explored using heatmap visualization based on a binary resistance matrix (resistant or sensitive) derived from phenotypic susceptibility testing. The heatmap was generated using the ComplexHeatmap package in R and used to compare resistance patterns across isolates, with row annotations indicating the sampling source and city of origin.

Results

Selection and antimicrobial susceptibility patterns of environmental *K. pneumoniae*

During the sampling campaign, a total of 44 environmental *K. pneumoniae* isolates were recovered from the investigated aquatic sites. Following phenotypic screening, 11 isolates (25.0%) were selected for WGS based on their broad resistance profiles spanning multiple antimicrobial classes, in line with previous applied MDR criteria. These isolates exhibited resistance to the extended-spectrum β-lactams including aztreonam, cefepime, ceftazidime, cefotaxime, and amoxicillin–clavulanate, in all strains (100%, 11/11). High resistance rates were also observed for fluoroquinolones (81.8%, 9/11 for ciprofloxacin and ofloxacin), aminoglycosides (90.9%, 10/11 for gentamicin and tobramycin), and trimethoprim–sulfamethoxazole (90.9%, 10/11). Regarding carbapenems, resistance to ertapenem was detected in 63.6% (7/11) of the isolates, whereas imipenem resistance occurred in only one isolate (9.1%) (Fig. 1).

MLST and phylogenetic analysis

Multilocus sequence typing revealed a high level of genetic diversity among the 11 MDR *K. pneumoniae* isolates, which were assigned to nine distinct sequence types (STs). Two STs were represented by more than one isolate, including ST2185 (KP8 and KP19) and ST307 (KP26 and KP81), whereas the remaining isolates belonged to ST322, ST4007, ST3430, ST35, ST1418, ST405, and ST147 (one isolate each). Core genome SNP-based phylogenetic reconstruction, performed using SKA2 and RAxML, resolved the isolates into distinct lineages, consistent with their MLST classification (Fig. 2). The closest genomic relationships were observed

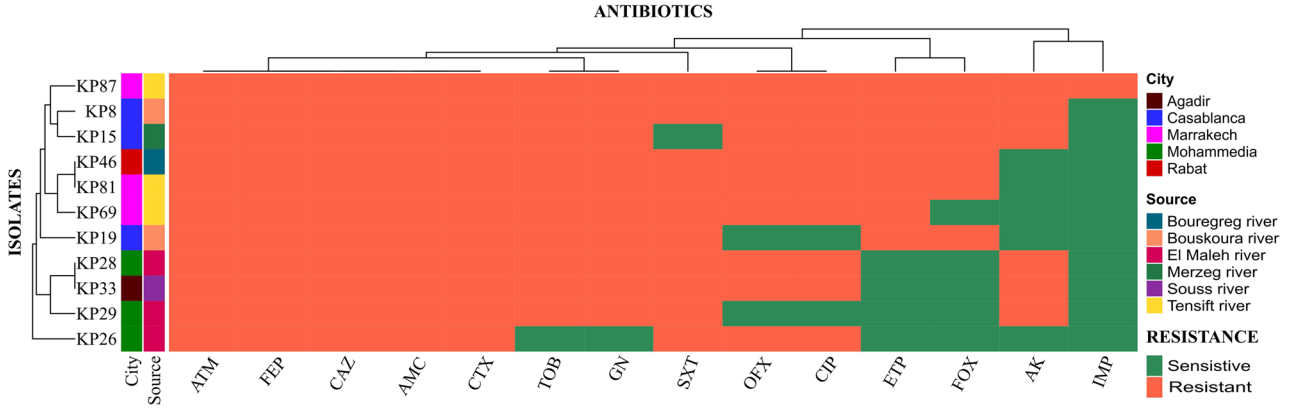

Fig. 1 Heatmap of the phenotypic antimicrobial resistance profiles of environmental *K. pneumoniae* isolates. Hierarchical clustering of antimicrobial susceptibility patterns is presented, with isolates displayed along the vertical axis and antimicrobial agents along the horizontal axis. Green indicates susceptibility, whereas red indicates resistance. The side annotation bars represent the city of origin and the environmental source of each isolate. Dendrograms illustrating the similarity of resistance profiles among isolates and antibiotics. ATM, aztreonam; FEP, cefepime; CAZ, ceftazidime; AMC, amoxicillin–clavulanate; CTX, cefotaxime; TOB, tobramycin; GN, gentamicin; SXT, trimethoprim–sulfamethoxazole; OFX, ofloxacin; CIP, ciprofloxacin; ETP, ertapenem; FOX, cefoxitin; AK, amikacin; IMP, imipenem

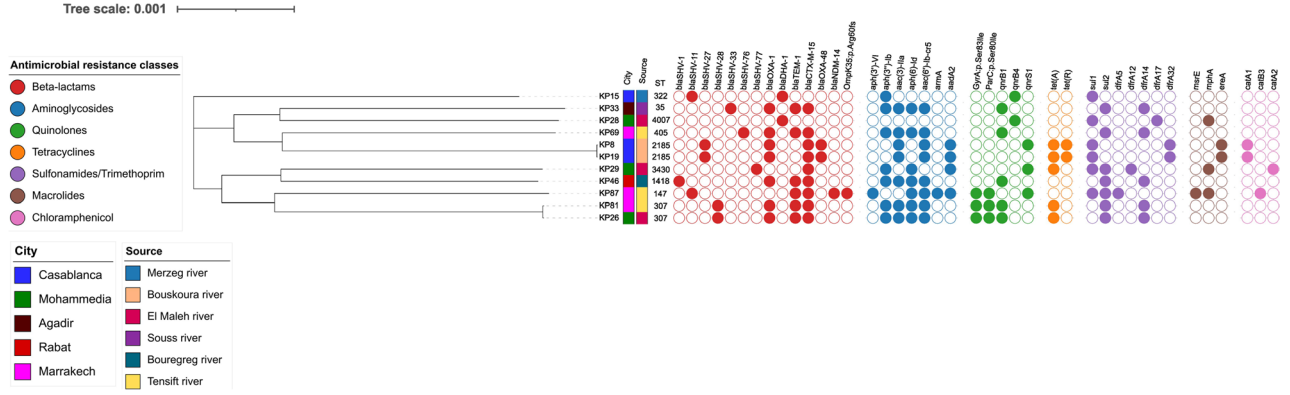

Fig. 2 Phylogenetic relationships and associated characteristics of 11 MDR environmental K. pneumoniae isolates. Sequence types (STs) are indicated alongside isolate identifiers. The adjacent annotation matrix summarizes antimicrobial resistance features. Filled circles indicate the presence of an acquired antimicrobial resistance gene, whereas the open (white) circles indicate its absence. Resistance genes are grouped by antimicrobial class. Isolates are additionally annotated according to the sampling city and environmental source, as indicated in the legend

within the ST-defined pairs, with 12 SNPs separating the ST2185 isolates and 37 SNPs separating the ST307 isolates. In contrast, pairwise genomic distances between isolates belonging to different STs exceeded 10,000 SNPs, indicating substantial genomic divergence across the environmental *K. pneumoniae* population. Additionally, comparison of our environmental MDR *K. pneumoniae* genomes with a global collection of publicly available genomes using Pathogenwatch, revealed phylogenetic dispersion rather than clustering into a single environmental lineage. The Moroccan isolates were positioned among genomes originating from Africa, Europe, Asia, North America, and Oceania (Fig. 3), and they were distributed across multiple lineages which was consistent with their MLST assignments. Isolates belonging to the same sequence type clustered together, whereas genomes from different STs were separated by large core-genome distances.

Resistome distribution across environmental isolates

Whole-genome analysis showed that the 11 environmental MDR *K. pneumoniae* isolates carried a diverse set of acquired antimicrobial resistance genes spanning multiple drug classes (Fig. 2). Among the β-lactamases, $bla_{CTX\text{-}M\text{-}15}$ was the most frequent determinant (9/11, 81.8%), followed by $bla_{OXA\text{-}1}$ (7/11, 63.6%) and $bla_{TEM\text{-}1}$ (6/11, 54.5%); carbapenemase genes were detected less often, with $bla_{OXA\text{-}48}$ in 2/11 isolates (18.2%) and $bla_{NDM\text{-}14}$ in 1/11 (9.1%). Aminoglycoside resistance determinants were common, including *aac(6′)-Ib-cr5* (8/11, 72.7%), *aph(3′′)-Ib* (7/11, 63.6%), *aac(3)-IIa* (7/11, 63.6%), *aph(6)-Id* (7/11, 63.6%), and *aadA2* (4/11, 36.4%), whereas *aph(3′)-VI* and the 16 S rRNA methyltransferase *armA* were each identified in 1/11 isolates (9.1%). Among the other classes, the sulfonamide resistance genes *sul1* (6/11, 54.5%) and *sul2* (7/11, 63.6%) were frequently detected, the plasmid-mediated quinolone resistance

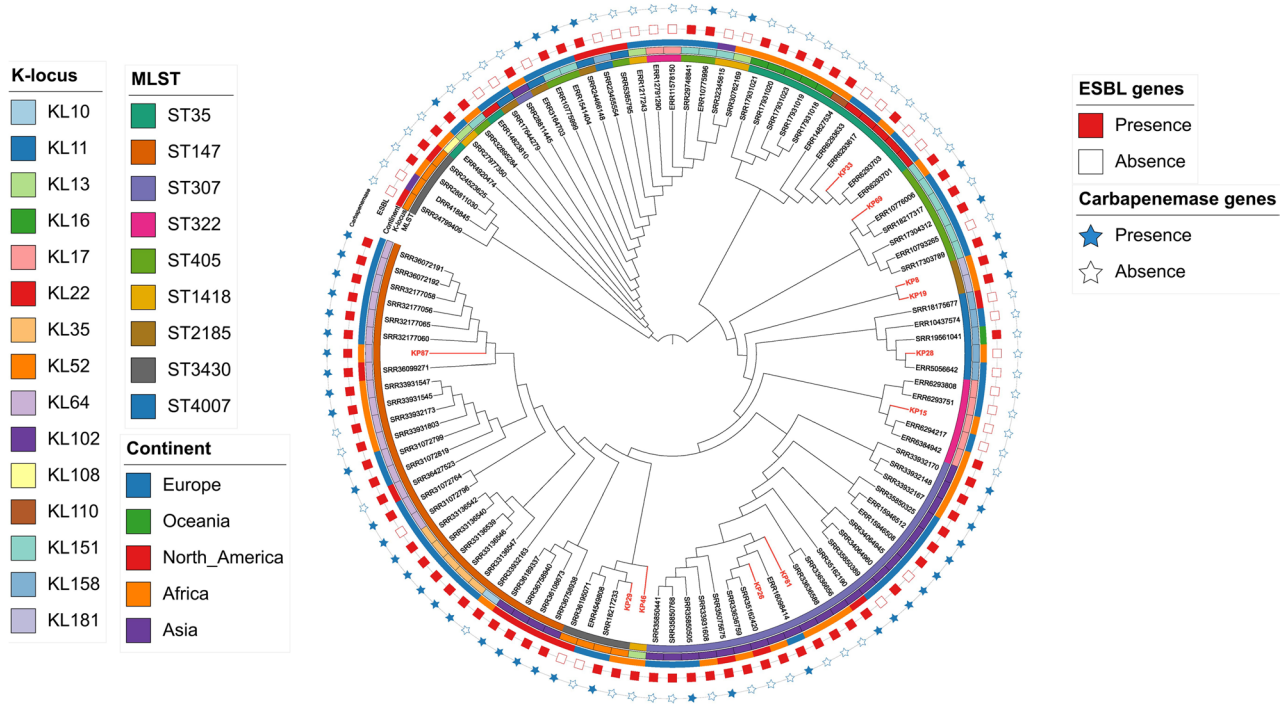

Fig. 3 Phylogenetic tree of *K. pneumoniae* isolates generated using Pathogenwatch. The Moroccan environmental isolates from this study are highlighted in red. Concentric annotation layers indicate (from inside to outside) MLST, capsular (K) locus, continent of origin, and the presence or absence of ESBL and carbapenemase genes. Branch lengths are not to scale

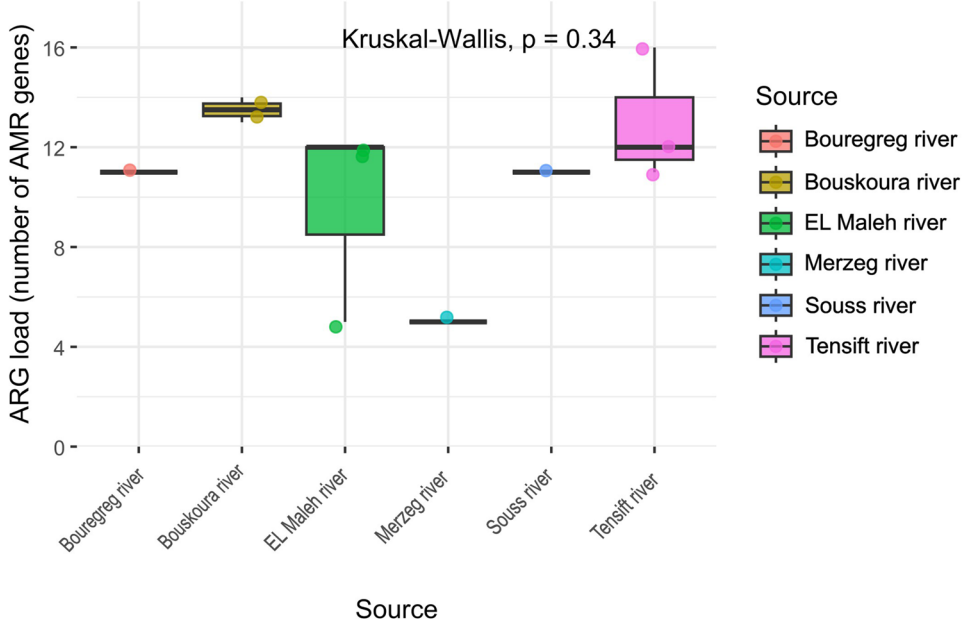

Fig. 4 Antimicrobial resistance gene (ARG) load by sampling source

genes included *qnrB1* (5/11, 45.5%), *qnrB4* (2/11, 18.2%), and *qnrS1* (3/11, 27.3%), the tetracycline resistance gene *tet(A)* occurred in 5/11 isolates (45.5%), and the trimethoprim resistance genes *dfrA12* and *dfrA17* were each detected in 1/11 isolates (9.1%).

Antimicrobial resistance gene burden across environmental sources

The number of acquired antimicrobial resistance genes varied across environmental sources, with ARG loads ranging from 5 to 16 genes per isolate (Fig. 4). The isolates recovered from Bouskoura river showed relatively

high ARG counts (median ≈ 13–14 genes), while those from Tensift river presented broader variability, with values spanning from approximately 11–16 genes. In contrast, isolates from Merzeg river displayed lower ARG counts, with a median of five genes, whereas Bouregreg river, El Maleh river, and Souss river displayed intermediate values, generally around 11–12 genes per isolate. Comparative analysis using the Kruskal–Wallis test revealed no statistically significant differences in ARG load among the environmental sources ($p = 0.34$), indicating that these site-level patterns should be regarded as descriptive observations within the sequenced collection.

Boxplots summarizing the distribution of ARG load, defined as the total number of acquired antimicrobial resistance genes per isolate, among environmental *K. pneumoniae* strains recovered from different sampling sites. Individual isolates are shown as points. Boxes represent the interquartile range (IQR), with horizontal lines indicating median values and whiskers extending to 1.5×IQR.

Virulence-associated traits and capsular (K-locus) diversity

Whole-genome virulence profiling revealed heterogeneous carriage of siderophore systems and marked capsular locus diversity among the 11 environmental MDR *K. pneumoniae* isolates (Table 2). The yersiniabactin locus was detected in 5/11 isolates (45.5%) and occurred across multiple K-loci (KP15, KP29, KP46, KP69, and KP87), encompassing distinct *ybt* lineages and ICE*Kp* variants, including *ybt13 (*ICE*Kp2*), *ybt14 (*ICE*Kp5*), *ybt10 (*ICE*Kp4*), *ybt27* (ICE*Kp22)*, and *ybt9 (*ICE*Kp3)*. In contrast, the aerobactin cluster was identified in a single isolate (1/11, 9.1%), KP87 (KL64), which carried *iuc1* and the hypermucoidy-associated locus *rmpA2*. Capsular typing showed substantial heterogeneity, with nine distinct K-loci observed across the collection. Two capsule types predominated, KL181 (2/11, 18.2%; KP19 and KP8) and KL102 (2/11, 18.2%; KP26 and KP81), while the remaining loci (KL17, KL158, KL52, KL22, KL13, KL151, and KL64) were each detected in a single isolate (9.1% per type).

Table 2 Virulence-associated traits and capsular (K-locus) diversity among environmental *K. pneumoniae* isolates

Isolate	K-locus type	Siderophore system	Hypermucoidy-associated loci
KP15	KL17	*ybt13* (ICE*Kp2*)	–
KP19	KL181	–	–
KP26	KL102	–	–
KP28	KL158	–	–
KP29	KL52	*ybt14* (ICE*Kp5*)	–
KP33	KL22	–	–
KP46	KL13	*ybt10* (ICE*Kp4*)	–
KP69	KL151	*ybt27*(ICE*Kp22*)	–
KP8	KL181	–	–
KP81	KL102	–	–
KP87	KL64	*ybt9* (ICE*Kp3*) ; *iuc1*	*rmpA2*

Plasmid replicon types and conserved genetic environments of key resistance genes

Plasmid replicon profiling revealed 11 distinct plasmid types among the environmental *K. pneumoniae* isolates (Fig. 5), with IncF family replicons predominating. IncFIB(K) was detected in 6/11 isolates, frequently co-occurring with IncFII(K) in 5/11 isolates, whereas additional IncF variants including IncFIB(pNDM-Mar), IncFIB(pKPHS1), and IncFIB(pQil) were identified in 1 isolate each. The IncHI1B(pNDM-MAR) replicon was present in 3/11 isolates, and the IncL/M plasmid was detected in 2/11 isolates. Other replicon types included IncR (2/11 isolates) and multiple Col-type plasmids, including ColRNAI and Col440I (each 4/11 isolates) and Col(pHAD28) (2/11 isolates).

Comparative mapping of resistance-associated regions showed that $bla_{CTX\text{-}M\text{-}15}$ was consistently embedded within IncFII/IncFIB plasmid backbones, flanked upstream by the insertion sequence *ISEcp1* and downstream by genes encoding *tryptophan synthase* subunits, forming a conserved genetic module across all $bla_{CTX\text{-}M\text{-}15}$ carrying isolates. In contrast, $bla_{OXA\text{-}48}$ was uniformly located on IncL/M plasmids, within a conserved genetic environment bordered by a *LysR*-family transcriptional regulator, displaying highly similar gene organization across the $bla_{OXA\text{-}48}$ positive isolates.

Pangenome analysis of environmental *K. pneumoniae* isolates

Pangenome analysis of the 11 environmental *K. pneumoniae* genomes identified a total of 8,154 gene clusters, reflecting substantial genomic diversity across the isolate collection (Fig. 6). Core genome-based clustering separated the strains into multiple phylogenomic groupings. Pairwise average nucleotide identity (ANI) values ranged from 98.70% to 99.99%, indicating high overall genomic similarity among the isolates. The highest ANI values were observed for the two ST-defined isolate pairs, namely ST2185 (KP8 and KP19) at 99.99% and ST307 (KP26 and KP81) at 99.93%, whereas the lowest value was recorded between KP8 and KP87 (98.70%). Most pairwise comparisons fell within a narrow range of approximately 98.80%–99.20%. Overall, the combination of high ANI values and a large pangenome supports the presence of a conserved genomic backbone together with considerable diversity in accessory gene content.

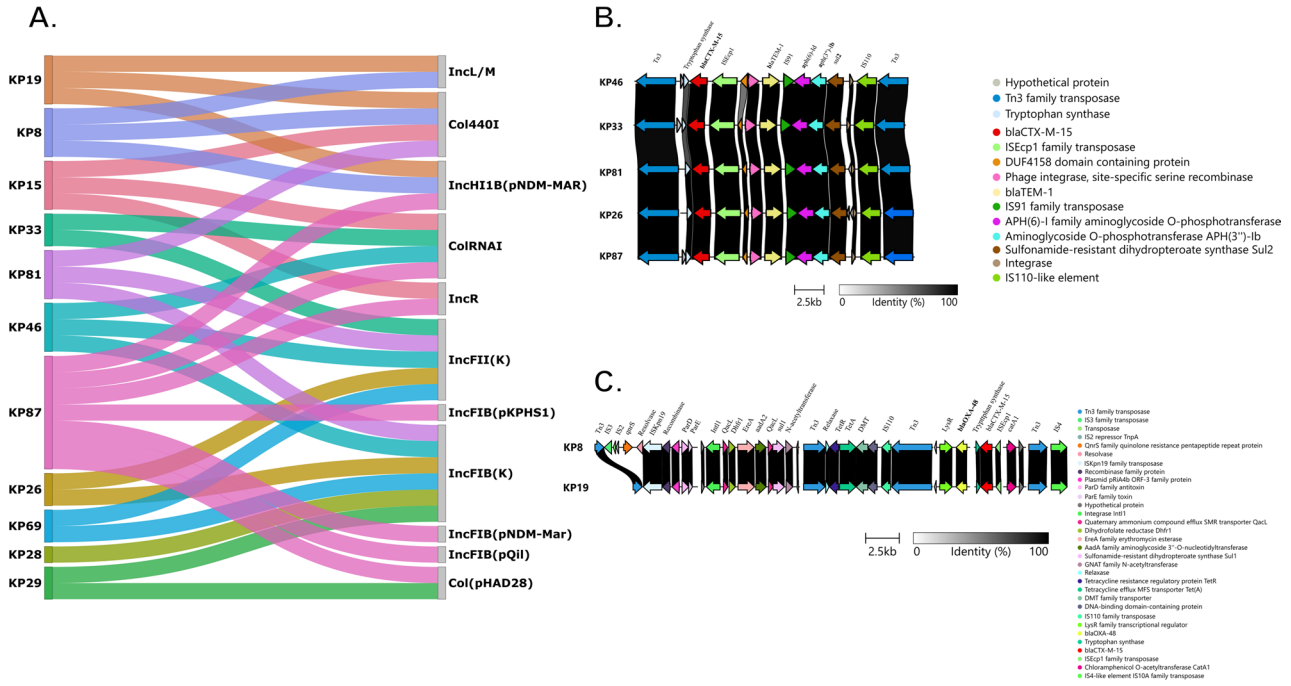

Fig. 5 Plasmid replicon diversity and comparative gene-cluster organization of $bla_{CTX\text{-}M\text{-}15}$ and $bla_{OXA\text{-}48}$ among environmental *K. pneumoniae* isolates. **A** Sankey diagram summarizing the distribution of plasmid replicon types across the isolates. **B** Comparative gene-cluster maps of IncFII/IncFIB plasmid regions carrying $bla_{CTX\text{-}M\text{-}15}$. **C** Comparative gene-cluster maps of IncL/M plasmid regions carrying $bla_{OXA\text{-}48}$. In panels B and C, arrows represent predicted coding sequences. Arrow colors indicate functional categories, including ARGs, insertion sequences and transposases, conjugative transfer-associated genes, and other genes. Shaded regions indicate nucleotide sequence identity between homologous plasmid regions

Discussion

This pilot study provides whole-genome-based insights into a targeted subset of MDR *K. pneumoniae* circulating in Moroccan river ecosystems. Rather than representing the broader environmental *K. pneumoniae* population, the sequenced collection was intentionally enriched for isolates with MDR phenotypes. Within this selected dataset, we identified lineages and resistance backgrounds closely related to those reported in clinical settings. Freshwater systems influenced by anthropogenic pressures are increasingly recognized as reservoirs and mixing points for resistant Enterobacterales and MGEs, supporting their critical relevance within One Health surveillance frameworks [38]. In Morocco, environmental genomic data on *K. pneumoniae* remain limited; the distribution and genetic diversity of MDR lineages in surface waters are still insufficiently characterized. The recovery of MDR isolates from various river sites expands the available genomic evidence and supports the inclusion of river ecosystems in national AMR monitoring strategies.

Phenotypic analyses showed broad resistance across multiple antimicrobial classes, consistent with the observed MDR profiles. Genomic characterization revealed a resistome dominated by $bla_{CTX\text{-}M\text{-}15}$, together with $bla_{OXA\text{-}1}$ *and* $bla_{TEM\text{-}1}$, reflecting ESBL-associated resistance patterns widely described in MDR *K. pneumoniae* strains from both clinical and environmental sources [39]. Similar findings have been documented in Tunisia, where ESBL-producing *K. pneumoniae* isolated from surface water samples frequently carried $bla_{CTX\text{-}M\text{-}15}$ as the predominant ESBL determinant [40]. This convergence indicates that clinically relevant ESBL profiles can persist in freshwater ecosystems under anthropogenic influence, supporting the growing body of evidence that surface waters act as environmental reservoirs contributing to the potential dissemination of ESBL-producing Enterobacterales beyond healthcare settings [41]. Furthermore, the detection of carbapenemase genes notably $bla_{OXA\text{-}48}$ and $bla_{NDM\text{-}14}$, is particularly significant given their central role in limiting therapeutic options for severe infections. In Morocco, carbapenemase-producing *K. pneumoniae* have been repeatedly reported in hospital settings, with $bla_{OXA\text{-}48}$ and bla_{NDM} variants representing the predominant mechanisms of carbapenem resistance [42, 43]. However, in our collection, ertapenem resistance was observed in more isolates than those carrying recognized carbapenemase genes, suggesting that additional non-carbapenemase mechanisms, such as permeability defects or porin alterations acting together with β-lactamases, may also contribute to reduced carbapenem susceptibility [44]. The identification of these determinants in river isolates extends the established clinical epidemiology into the environmental

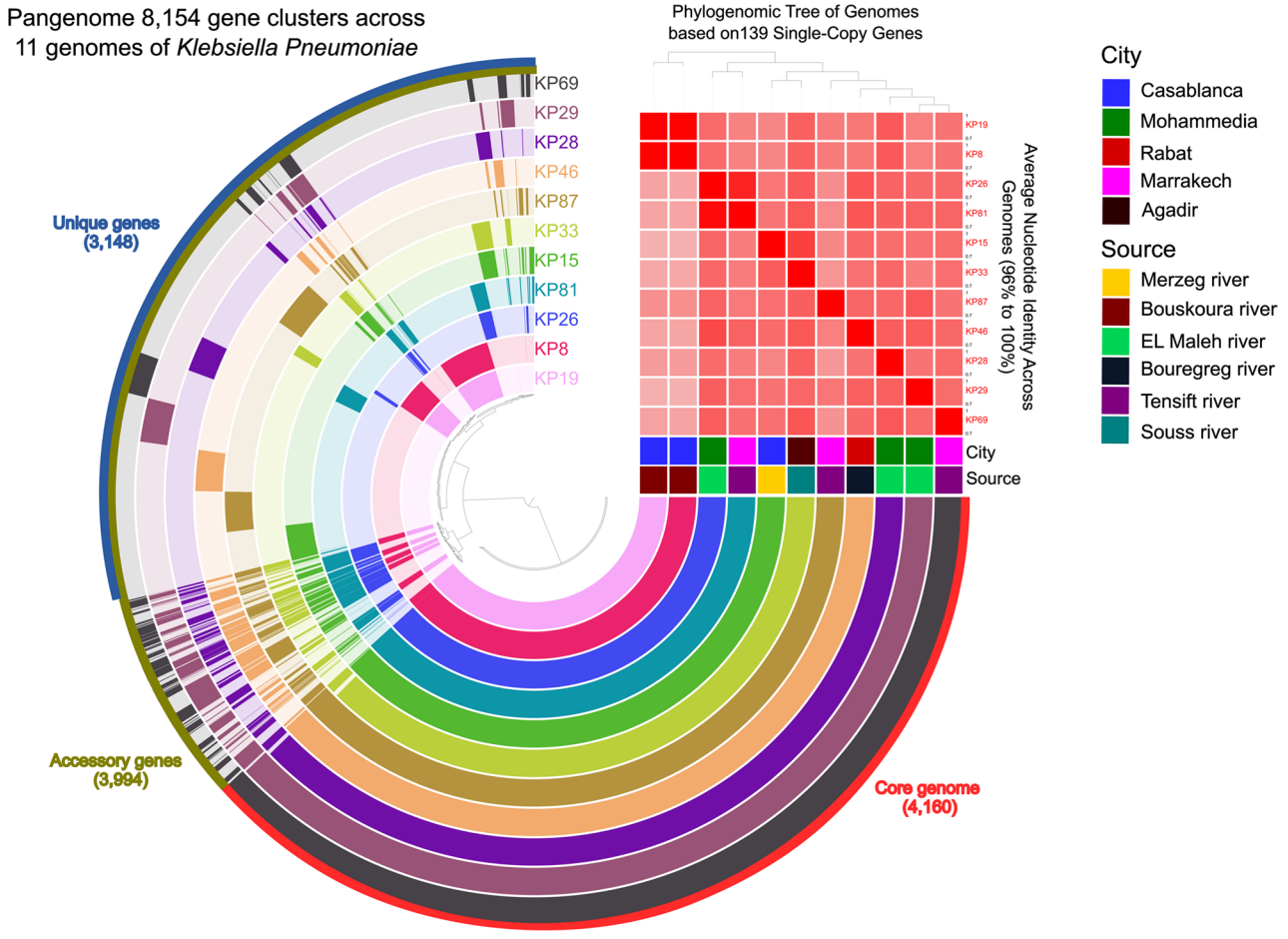

Fig. 6 Pangenome structure and genomic relatedness of environmental *K. pneumoniae* isolates. Pangenome analysis was performed using anvi'o on 11 environmental *K. pneumoniae* genomes. Concentric layers represent individual genomes, organized according to their phylogenomic relationships inferred from 139 single-copy core genes. Within each layer, dark-colored segments indicate the presence of a gene cluster, whereas lighter shading denotes its absence. The pangenome is partitioned into core genome (4,160 gene clusters), accessory genome (3,994 gene clusters), and unique genes (3,148 gene clusters), as indicated in the outermost layers. Pairwise average nucleotide identity (ANI) values among genomes are shown as a heatmap, with higher similarity represented in red and lower similarity in lighter colors, illustrating genomic relatedness across the isolate collection

compartment, suggesting that resistance mechanisms traditionally associated with clinical infections are also persisting within aquatic ecosystems.

Core-genome phylogenetic analysis combined with MLST revealed substantial genetic heterogeneity among the environmental *K. pneumoniae* isolates recovered in this study. The identification of nine distinct sequence types (STs) across the sequenced collection supports a broad population diversity in Moroccan river ecosystems rather than the dominance of a single environmental lineage. This observation is consistent with genomic evidence indicating that *K. pneumoniae* is a highly structured species with extensive clonal diversity across various ecological niches [45]. Notably, the detection of ST147 and ST307 is epidemiologically significant, as both lineages are globally recognized high-risk clones frequently associated with ESBL production and a high capacity for acquiring carbapenemase genes [4], thereby facilitating their widespread international dissemination. In Morocco, ST147 isolates carrying NDM-type carbapenemases, including $bla_{NDM\ 14}$ have been previously documented in community settings [46], reinforcing concerns regarding the dissemination of these lineages beyond healthcare facilities.

Moreover, ST307 has been identified in Moroccan hospital settings and was among the most prevalent STs reported in a neonatal intensive care unit bloodstream infection study [47], further supporting the local establishment of this globally disseminated MDR lineage. The recovery of these lineages from river samples raises the hypothesis that surface waters may contribute to the persistence and dissemination of clinically relevant *K. pneumoniae* lineages outside hospital settings. Consistently, phylogenetic contextualization against publicly available genomes placed our environmental isolates within globally circulating lineages rather than clustering into

discrete, environment-specific clades. Taken together, these results support the observation that the recovered isolates belong to genomic lineages that are widely distributed internationally and are not confined to a distinct, locally restricted background.

Virulence profiling revealed heterogeneous carriage of siderophore systems and marked capsular diversity. The detection of yersiniabactin across multiple ICE*Kp* variants aligns with its known prevalence among MDR *K. pneumoniae* and its frequent association with successful lineages [48]. Although hypervirulence-associated loci were rare overall, the co-occurrence of yersiniabactin, aerobactin (*iuc1*), and *rmpA2* in our ST147 isolate is consistent with the increasingly reported convergence of resistance and virulence traits in *K. pneumoniae* at a global scale [49]. The extensive K-locus diversity observed in our isolates further reflects the genomic plasticity of *K. pneumoniae*; capsule diversification and switching are recognized contributors to ecological adaptability and clonal success, including among high-risk lineages [50]. In our dataset, capsular typing identified nine distinct K-loci. KL181 and KL102 were the most frequent, each detected in two isolates (18.2%), while the remaining loci (KL17, KL158, KL52, KL22, KL13, KL151, and KL64) were each identified in a single isolate (9.1%). Similarly, a high level of capsule heterogeneity has been reported in large-scale genomic analyses, where numerous K-loci were observed even within defined sequence types [51], This illustrates that extensive capsule variation is a hallmark of *K. pneumoniae* populations, with significant implications for environmental persistence and public health. Pangenome-level patterns further support this high genomic plasticity, consistent with population genomics studies describing a large accessory genome [52], that facilitates adaptation to diverse ecological niches, including aquatic environments.

Plasmid replicon profiling in our environmental *K. pneumoniae* isolates revealed a diverse plasmid background (11 replicon types) dominated by the IncF family. Specifically, IncFIB(K) (6/11) frequently co-occurred with IncFII(K) (5/11), alongside the presence of IncL/M plasmids (2/11) and several Col-type plasmids, including ColRNAI (4/11), Col440I (4/11), and Col(pHAD28) (2/11). This profile is consistent with the recognized role of IncF and IncL/M plasmids as major vehicles for clinically important resistance genes, notably the established association of $bla_{CTX-M-15}$ with IncF backbones and bla_{OXA-48} with IncL/M plasmids [53, 54]. These observations indicate that environmental isolates can maintain plasmid replicon types identical to those found in clinical lineages. Furthermore, it suggests that aquatic systems may facilitate the persistence and exchange of MGEs, promoting the horizontal gene transfer of adaptive traits across diverse ecological niches and human-associated settings [55].

This study has limitations that should be acknowledged. The relatively small number of sequenced isolates and their phenotypically targeted selection restrict broad ecological and population-level conclusions regarding the distribution of clones, plasmids, and resistance determinants across Moroccan river ecosystems. In addition, short-read Illumina sequencing imposes important constraints on plasmid reconstruction. Repetitive elements, insertion sequences, and mosaic plasmid regions are frequently fragmented in draft assemblies, preventing full resolution of complex plasmid architectures. Consequently, the assignment of resistance genes to specific plasmid backbones and the reconstruction of their surrounding genetic contexts remain limited. Future long-read sequencing will therefore be valuable to resolve complete plasmid structures and better define the genomic context of key resistance determinants. Conjugation assays should likewise be prioritized to confirm the transferability of the plasmids identified in this study. Finally, while Pathogenwatch provided a strong global contextualization, the current underrepresentation of Moroccan genomic data in public databases may not yet fully capture the extent of local diversity. Despite these constraints, this study represents the first national genomic characterization of river-derived *K. pneumoniae* isolates in Morocco across multiple sites, establishing a vital baseline for aquatic AMR surveillance.

Conclusions

Our study provides an initial whole-genome-based characterization of a targeted subset of MDR *K. pneumoniae* strains recovered from Moroccan river ecosystems and reveals substantial genomic diversity, as reflected by the marked intraspecies heterogeneity observed through MLST and capsular (K-locus) typing. Pangenome analysis further highlighted pronounced genetic variability and genomic plasticity across isolates, while virulence profiling revealed heterogeneous carriage of key virulence-associated loci, including siderophore systems and capsule-related diversity, that can support persistence and adaptation in environmental niches. The detection of clinically relevant resistance determinants including ESBLs and carbapenemases, together with high-risk lineages such as ST147 and ST307, indicates that river environments can harbor resistance and virulence-associated backgrounds of direct clinical concern. Collectively, these findings support integrating aquatic ecosystems into One Health genomic surveillance. Future research should expand environmental sampling across seasons and regions, and strengthen the linkage between environmental and clinical surveillance to better define transmission interfaces and drivers of MDR *K. pneumoniae* dissemination in our country.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-026-12903-8.

Additional file 1. Genome assembly statistics and quality metrics of environmental K. *pneumoniae isolates*. Excel file summarizing genome size, GC content, number of contigs, N50 values, completeness, and contamination estimates for the 11 MDR K. *pneumoniae genomes* generated in this study (corresponding to Supplementary Table S1).

Additional file 2. Comprehensive genomic characterization including MLST, K-locus, resistance and virulence determinants. Excel file containing multilocus sequence types (STs), capsular (K) locus assignments, antimicrobial resistance genes, virulence-associated loci (*ybt, iuc, clb, iro, rmpA/rmpA2*) identified using Kleborate (corresponding to Supplementary Table S2).

Additional file 3. ANI percentage identity matrix among environmental K. pneumoniae isolates. Excel file containing the pairwise ANIb percentage identity matrix for the 11 environmental K. pneumoniae isolates sequenced in this study, used to support species-level assignment and genomic similarity assessment across the isolate collection (corresponding to Supplementary Table S3).

Additional file 4. Core-genome SNP alignment matrix generated using SKA2. Excel file containing the pairwise SNP distance matrix used for phylogenetic reconstruction with RAxML, including SNP distances among environmental isolates (corresponding to Supplementary Table S4).

Additional file 5. Metadata of publicly available reference genomes included in phylogenetic contextualization. Excel file listing accession numbers, country of origin, year of isolation, sequence type of the 102 publicly available K. pneumoniae genomes retrieved from Pathogenwatch for comparative phylogenetic analysis (corresponding to Supplementary Table S5).

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Authors' contributions

AA conceived and designed the study, performed the investigation and sampling, curated the data, conducted the formal analyses, produced the visualizations, and wrote the original draft. IZ and KN supervised the work, contributed to the study design and methodology, provided resources, coordinated project administration, and reviewed and edited the manuscript. AK, HM, and AEH contributed to the methodology. All authors read and approved the final manuscript.

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Data availability

The *Klebsiella pneumoniae* genome sequences generated in this study have been deposited in the DDBJ/ENA/GenBank databases under BioProject accession number PRJNA1420733.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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