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Research article

Genomic characterization of a WHO critical priority isolate *Enterobacter kobei* ST2070 harboring OXA-10, KPC-2, and CTX-M-12 recovered from a water irrigation channel in Ecuador

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ABSTRACT

The discharge of untreated or partially treated wastewater can have detrimental impacts on the quality of water bodies, posing a significant threat to public health and the environment. In Ecuador, previous research indicates a high prevalence of antimicrobial resistant (AMR) bacteria in surface waters affected by human activities, including irrigation channels. In this study, we analyzed sediment samples collected from an irrigation channel utilized for agricultural purposes in northern Ecuador, using microbiological techniques and whole-genome sequencing (WGS). Our investigation revealed the first documented occurrence of *E. kobei* in Ecuador and the initial report of environmental *E. kobei* ST2070. Furthermore, we identified the coexistence of OXA-10-type class D β-lactamase and KPC-2-type class A β-lactamase in the *E. kobei* isolate (UTA41), representing the first report of such a phenomenon in this species. Additionally, we detected various antibiotic resistance genes in the *E. kobei* UTA41 isolate, including $bla_{CTX\text{-}M\text{-}12}$, *fosA*, *aac (6')-lb*, *sul2*, *msr(E)*, and *mph(A)*, as well as virulence genes such as bacterial efflux pump and siderophore biosynthesis genes. We also identified two intact prophage regions (Entero_186 and Klebsi_phiKO2) in the isolate. Our study presents the first evidence of *E. kobei* isolate containing two carbapenemase-encoding genes in environmental samples from Latin America. This finding indicates the potential spread of critical-priority bacteria in water samples originating from anthropogenic sources, such as urban wastewater discharges and livestock facilities.

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1. Introduction

Antimicrobial resistance (AMR) is a major global public health threat, as reported by the World Health Organization (WHO) [1]. With an estimated of 1.27 million annual deaths globally attributed to AMR, it is estimated that this figure will rise to 10 million by 2050 [2,3]. Anthropogenic activities, such as the misuse of antimicrobials in human, veterinary, and agricultural medicine, as well as the release of agricultural, household, and pharmaceutical waste into the environment, have significantly impacted the evolution and spread of AMR [4,5]. Aquatic environments, including surface and groundwater, have become a breeding ground for AMR evolution, exacerbating the issue [6]. Given the interconnectedness of humans, animals, and the environment, AMR requires a One Health approach to understand its evolution and dissemination.

World Health Organization has published list of priority AMR pathogens that require urgent need for new antibiotic therapies [7]. The critical priority group include members of the Enterobacteriaceae family that exhibit resistance to carbapenems and contain extended-spectrum β-lactamases. These findings highlight the urgent need for the development of new antimicrobial strategies to address the risks posed by these organisms. *Enterobacter* spp. is the third most frequently occurring pathogenic species in the *Enterobacteriaceae* family [3]. In particular, the *Enterobacter cloacae* complex (ECC) that comprise at least five species (*E. cloacae*, *E. kobei*, *E. asburiae*, *E. hormaechei* and *E. ludwigii*) is commonly associated to nosocomial infections such as bacteraemia, pneumonia, or urinary tract infections [8]. The widespread use of carbapenems in clinical practice and the potential of its resistance genes to be transferred through mobile genetic elements have facilitated the emergence of carbapenem-resistant ECC strains [9]. The emergence of carbapenem resistance is frequently accompanied by concurrent resistance to all conventional β-lactams and other antibiotic classes, thereby exemplifying a prototypical manifestation of multidrug-, pan drug-, or extensively drug-resistant phenotype [10].

One of the most significant problems facing Latin America in terms of water quality is the lack of adequate sewage treatment. Many countries in the region have inadequate or nonexistent sewage treatment facilities, leading to the discharge of untreated or partially treated wastewater into rivers, lakes, and other water bodies [11]. The discharge of untreated or partially treated wastewater can have significant impacts on the quality of water bodies, including increased levels of pollutants, bacteria, and other pathogens that can pose a risk to public health and the environment [12]. In Ecuador, recent studies indicated high prevalence of AMR bacteria in surface waters impacted by anthropogenic activities, including irrigation channels [13]. In this study, we analyzed sediment samples obtained from an irrigation channel used for agricultural purposes in the Province of Pichincha in Ecuador using microbiological techniques and whole genome sequencing (WGS).

2. Material and methods

2.1. Bacterial isolation

On October 21, 2018, three water samples (1.0 L) and three sediment samples (100 g, depth 1 cm) was collected from an irrigation channel located in Quebrada Portada, Cayambe, province of Pichincha (0.003667 N 78.172417 W). The samples were placed in sterile containers and transported to the laboratory in a chilled cooler. Upon arrival at the laboratory, the sample was immediately processed for microbiological analysis.

The sediment samples were previously homogenized in PBS at pH 7.4 (1:10 w/v). Water samples and homogenized sediments were subjected to serial dilutions and filtered through 0.45 μm cellulose membrane filters (Merck Millipore; Darmstadt, Germany) and were plated on MacConkey agar supplemented with ceftriaxone (5 μg/mL) (Merck Millipore; Darmstadt, Germany). Both lactose positive and negative colonies in MacConkey agar were purified and cryopreserved with 15% glycerol. To prevent the isolation of clonal bacteria, up to 10 colonies were isolated per sample.

2.2. PCR detection of carbapenemase-encoding genes and bacterial identification

All isolates were screened by PCR for the presence of carbapenemase-producing genes: bla_{KPC}, bla_{VIM}, bla_{IMI}, bla_{OXA}, and bla_{NDM}. PCR amplification was performed in a total volume of 12.5 μL using 6.3 μL of DreamTaq Green PCR Mastermix (Thermo Fisher Scientific; Waltham, MA, USA), 0.3 μL of each primer (10 μM stock), 4.3 μL of nuclease-free water (Thermo Fisher Scientific; Waltham, MA, USA), and 1.3 μL of total DNA extracted by heat shock method. PCR was carried out as follows: initial denaturation at 95 °C for 1 min; 35 cycles of 95 °C for 30 s, annealing at 56 °C for 30 s, extension at 72 °C for 1 min; and a final elongation step at 72 °C for 7 min. PCR products obtained were evaluated by agarose gel electrophoresis and stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, USA) Primer.

2.3. Whole genome sequencing

Whole genome sequencing of isolate UTA41 was done using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Raw sequences were assembled into contigs using QIAGEN CLC Genomics Workbench version 10.0 (Qiagen, Hilden, Germany). The sequence data are available in the GenBank databases under accession number NZ_JAIUDO010000152. Molecular identification, multilocus sequence type (MLST), plasmid typing, resistome and phage analysis were conducted using SpeciesFinder 2.0, MLST 2.0, PlasmidFinder 2.0, ResFinder 4.1 and PHAge search tool–Enhanced Release (PHASTER) databases, respectively [14–17]. The sequence identity threshold was set between 98 and 100%. Ribosomal MLST (rMLST) phylogeny was generated using Galaxy Sciensano platform [18,19], considering our *E. kobei* isolate and an international collection of 226 *E. kobei* whole genome sequences, selected according to

isolate source, country and year of isolation (Fig. 1),. iTOL V.65 was used to edit and visualize the phylogenetic tree [20].

2.4. Antibiotic susceptibility testing

The antimicrobial susceptibility of the carbapenemase-producing strains was determined using the Sensititre System (Thermo Fisher Scientific, Waltham, USA) for piperacillin/tazobactam (TPZ), ceftazidime (CAZ), cefotaxime (CTX), cefepime (FEP), aztreonam (ATM), ertapenem (ETP), imipenem (IPM), meropenem (MEM), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), and Trimethoprim/sulfamethoxazole (SXT). Colistin susceptibility (COL) was performed by the broth microdilution method, following the guidelines of the Clinical & Laboratory Standards Institute [21]. The double-disk synergy test was used to evaluate phenotypic β-lactamase (ESBL) production [22]. The carbapenem inactivation method was used to detect carbapenemases activity (mCIM) [23]. Phenotypic testing for carbapenemase type KPC was performed via the boronic acid combined-disk test (PBA) [24]. An ethyl-enediaminetetraacetic acid (EDTA)-disk synergy test was used to identify metallo-β-lactamase-producing isolates [25].

3. Results

3.1. PCR screening and antimicrobial susceptibility profile of the strain

A total of 60 isolates with *Enterobacteriaceae*-like morphology were recovered from the sediments and water samples. A single isolate (UTA41) recovered from sediment samples was positive for bla_{OXA} and bla_{KPC} genes. Positive results for mCIM and PBA were exhibited by UTA41, demonstrating the expression of carbapenemases and the KPC gene. However, the EDTA test showed negative for the presence of metallo-β-lactamases. Phenotypic ESBL production was confirmed using antimicrobial susceptibility testing. Table 1 shows the susceptibility to the 16 antibiotics evaluated. The isolate showed the expected phenotypic profile according to the encoded carbapenemases.

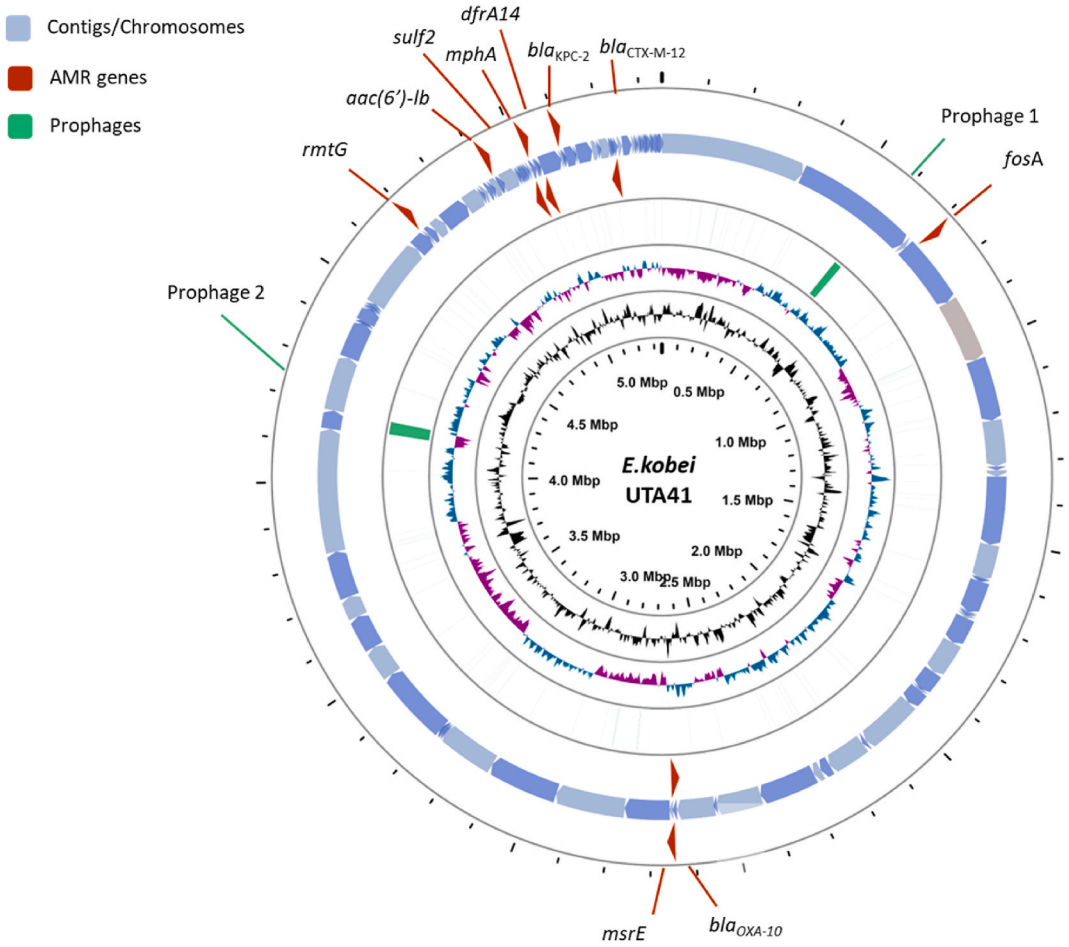

Fig. 1. Genomics features of the *E. kobei* isolate. Circular representation of the *E. kobei* isolate. Rings represent the following features labeled from inside to outside: ring 1 and 2, average GC content; ring 3, green blocks correspond to prophages; ring 4, contigs and AMR genes.

Table 1
Minimum inhibitory concentrations (MIC) of the studied strain.

Class	Antimicrobial	Abbreviation	MIC (μg/mL)	Result
Aminoglycosides	Gentamicin	GEN	>8	R
	Amikacin	AMK	>32	R
Fluoroquinolones	Ciprofloxacin	CIP	1	S
	Levofloxacin	LVX	≤1	S
Polymyxin	Colistin	COL	1	S
Sulfonamides	Trimethoprim-sulfamethoxazole	SXT	>4	R
Tetracyclines	Minocycline	MNO	8	I
	Tigecycline	TGC	1	S
β-Lactams	Cefotaxime	CTX	>32	R
	Ceftazidime	CAZ	16	R
	Meropenem	MEM	>8	R
	Imipenem	IPM	8	R
	Piperacillin/tazobactam	TZP	>64	R
	Cefepime	FEP	>16	R
	Ertapenem	ETP	>4	R
	Aztreonam	ATM	>16	R

*R: resistant; S: susceptible; I: intermediate.

3.2. Genomic characteristics

Sequencing analysis showed *E. kobei* isolate had genome size of 5.3 million base pairs (bp), with a CG content of 54,49%, 5169 protein-coding sequence, 75 tRNAs and 86 rRNAs. The genomic analysis revealed that the isolate harbored multiple antibiotic resistance genes (ARGs) including $bla_{\text{CTX-M-12}}$, $bla_{\text{OXA-10}}$, $bla_{\text{KPC-2}}$, *aac(6′)-lb*, *sul2*, *dfrA14*, *fos*A, *msr(E)*and *mph(A)* in its resistome (Fig. 1).The plasmid and virulence content of the isolate are shown in in Table 2. In addition, the isolate harbors two intact prophages sized 24.8 and 38.8 kb, with GC contents of 54,87% and 52,17%, respectively. The two complete prophage regions are similar to PHAGE_Entero_186_NC_001317(15) and PHAGE_Klebsi_phiKO2_NC_005857(15) (Fig. 1).

Multilocus sequence typing (MLST) identified the isolate as ST2070. To complement the information about the related isolates, Fig. 3 includes a Detailed Phylogenetic Clade Representation of *E. kobei* ST54 and ST2070 Isolates. The tree illustrates each genome by its NCBI accession number. The focal isolate of this study, UTA-41, is prominently marked in red. For each genome, the figure displays the corresponding ribosomal Sequence Type (rST), the country of isolation, the year of isolation when available, and the source of the sample. ST54 is a common sequence type found across various countries and years, with isolates derived from diverse sources such as hospital drains, human samples, and environmental locations.

4. Discussion

To our knowledge, this is the first documented occurrence of *E. kobei* in Ecuador and the first report of the ST2070 worldwide. Currently, there are no reports of *E. kobei* ST2070 in scientific literature or in specialized repositories such as Pathogenwatch or PubMLST. Phylogenetic analysis based on ribosomal MLST indicated that isolate UTA41 exhibits significant similarities with *E. kobei*

Table 2
Genomic and epidemiological data of *E. kobei*.

Characteristics	*E. kobei*
Source	Sediments from irrigation channel
Genome size (bp)	5.351.005
No. of CDS	5.169
GC content (%)	54,49
tRNA	75
rRNA	86
MLST	2070
Resistome	
Aminoglycosides	*aac(6′)-lb*
Beta-lactams	$bla_{\text{CTX-M-12}}$ $bla_{\text{OXA-10}}$ $bla_{\text{KPC-2}}$
Folate pathway antagonist	*sul2, dfrA14*
Fosfomycin	*fosA*
Streptogramin b	*msr(E)*
Macrolide	*mph(A)*
Plasmid content	Col440l, IncM1, IncP6, pKPC-CAV1321
Prophages	PHAGE_Entero_186_NC_001317 PHAGE_Klebsi_phiKO2_NC_005857
Virulome	*acrB, fepA, fepD, entA, entB, entE, entF, entS*
GenBank accession number	NZ_JAIUDO010000152

isolates belonging to ST54. It shares identical allele profiles, with the notable exception of the *rplB* locus, which is 84 in ST2070 and 15 in ST54 (Fig. 2). In addition, according to the rMLST profiles, UTA41 belongs to the rST 147493. No evidence of isolates belonging to this ribosomal MLST profile was detected in the specialized database PubMLST [26].

Regarding the lineage closely related to ST2070, the ST54 lineage has been primarily detected in human and sewage samples [27,28]. This suggests that its presence in environmental settings is likely a result of sewage pollution. This highlights the importance of our findings, particularly in the context of irrigation water safety. Water pollution might include microorganisms considered potentially pathogenic or with antibiotic resistance profiles of critical relevance, which could disseminate through the food chain [29,30]. The discovery of these kinds of microorganisms in environmental samples, particularly those influenced by sewage pollution, serves as a warning sign of the broader ecological impact of human waste management practices [31]. It emphasizes the urgent need to implement and improve sewage treatment processes, along with stricter environmental monitoring, to prevent the spread of potentially pathogenic bacteria and to curb the escalation of AMR [32]. This is particularly crucial in countries like Ecuador, which have a low rate of wastewater treatment coverage [13]. By addressing these environmental factors, we can take a significant step towards mitigating the risks associated with AMR dissemination, ultimately protecting public health and preserving the effectiveness of current antibiotics [33].

The isolate UTA41 co-harbor the OXA-10-type class D β-lactamase and the KPC-2-type class A β-lactamase. This co-carriage was observed on different studies of *Klebsiella pneumoniae* from clinical origin on Sweden, Taiwan, and Russia [34–36]. KPC-2 has become the most clinically important carbapenemases enzyme due to its prevalence in enteric bacteria from human, animal and environmental origin [37–39]. The emergence of KPC-2 dates back to 1996, and since then, it has exhibited extensive dissemination globally, including America, Europe, Southeast Asia, and Africa [40,41]. OXA enzymes, belonging to the class D β-lactamases, demonstrate a distinctive proficiency towards almost all β-lactam antibiotics in *Enterobacteriaceae* [42,43]. These enzymes have been found to be extensively prevalent among the ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter*

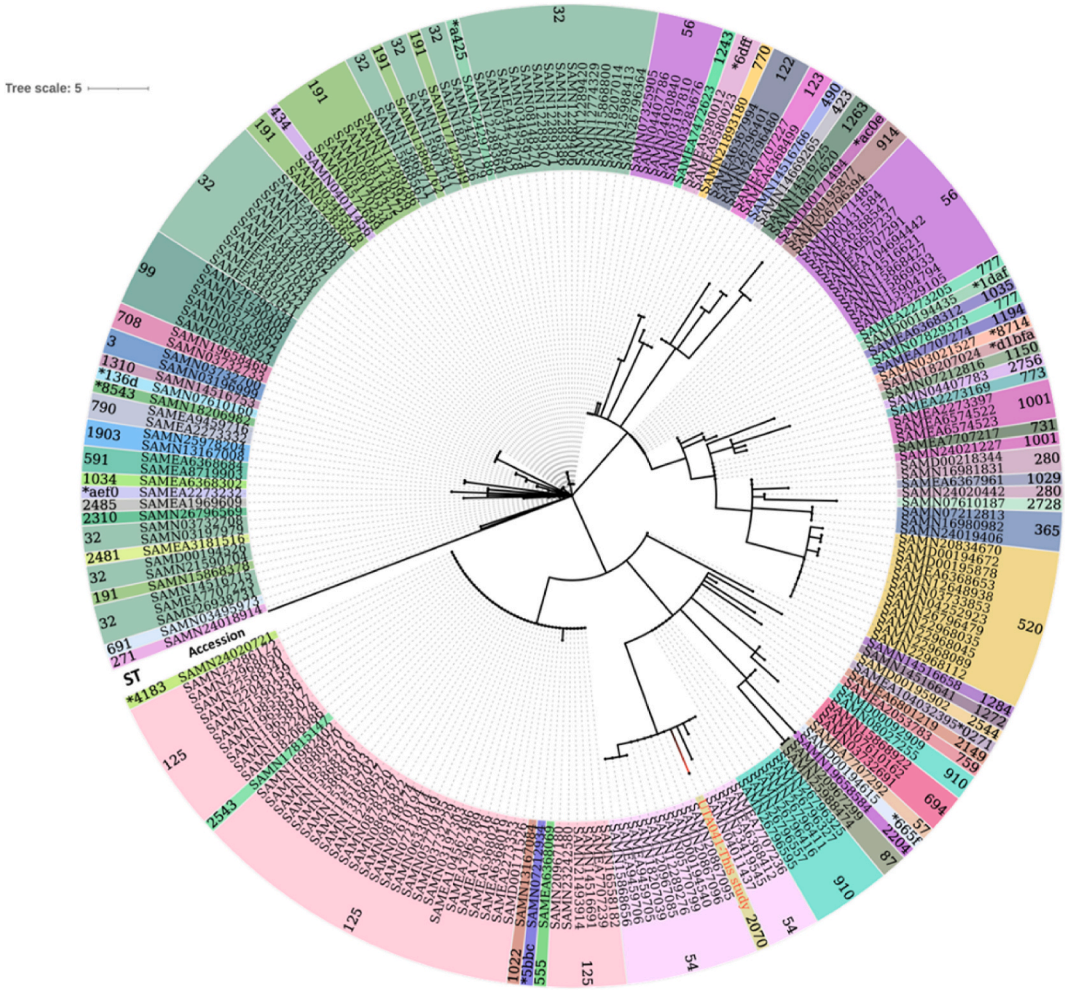

Fig. 2. rMLST-Based Phylogenetic Tree of *E. kobei* Isolates from Various Global Locations. Each genome is represented by its NCBI accession number. The focal isolate, UTA-41, is highlighted in red. Sequence types (STs) within the *E. cloacae* complex are denoted by color coding.

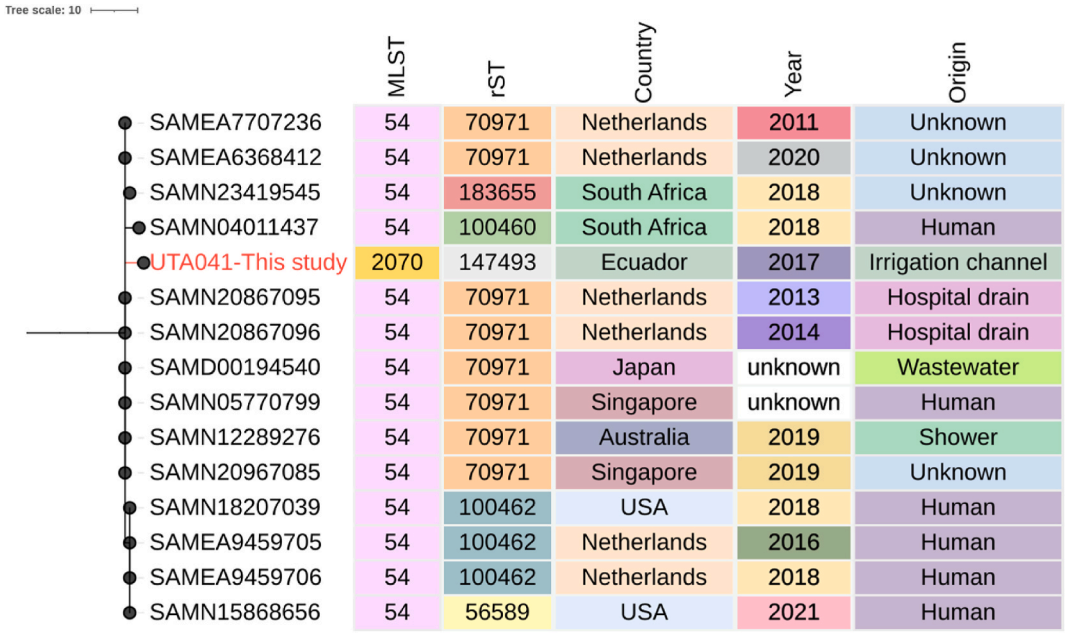

Fig. 3. Phylogenetic Analysis of ST54 and ST2070 *E. kobei* Isolates Indicating Global Distribution, Isolation Years, and Sample Sources. The focal isolate, UTA-41, is highlighted in red.

baumannii, *Pseudomonas aeruginosa* and *Enterobacter* spp.), which are notorious for their association with nosocomial infections and multidrug resistance [44]. The OXA-10 variant has been identified to exhibit a suboptimal level of carbapenemase activity. Nevertheless, the extent to which this variant may contribute to the emergence of antibacterial drug resistance remains unclear and requires further investigation [45,46]. Although primarily associated with clinical settings, its presence in the environment, especially in water used for irrigation, poses a risk for the spread of resistance genes and pathogens in agricultural settings.

E. kobei UTA41 has been found to harbor the ESBL gene $bla_{\text{CTX-M-12}}$, which confers resistance to β-lactam antibiotics like cephalosporins, including cefotaxime. Although CTX-M-12 has been documented mainly in clinical *Enterobacteriaceae* isolates, in many regions of the world, including Asia, Africa and America, its presence in Ecuadorian isolates is not frequent [47–50]. The wider distribution of CTX-M-12 and its variants represent a major public health concern since infections caused by bacteria producing these enzymes are frequently resistant to multiple antibiotics and can pose challenges for clinical management. However, there is only one report of *E. kobei* carrying CTX-M-12 enzyme in *E. kobei* recovered from clinical samples in northeastern China [51]. Our study is the first report showing the presence of the $bla_{\text{CTX-M-12}}$ gene in *E. kobei* in environmental samples from Latin America.

We identified *fosA* gene in our isolate. The *fosA* gene was frequently detected in ECC and has been reported as the cause of enzymatic resistance to fosfomycin, an antibiotic commonly used to treat urinary tract infections [52]. The *aac(6′)-lb* gene encodes for aminoglycoside-modifying enzyme that confers resistance to a broad range of antibiotics, including amikacin and gentamicin [53]. Our isolate had *sul2* gene, which confers resistance to sulfonamides, a class of antibiotics commonly used to treat bacterial infections in humans and animals worldwide. Sulfonamides resistance is considered a tracer for anthropogenic pollution due to its high dissemination [54]. The *dfrA14* gene that encodes resistance to trimethoprim, which is commonly used in combination with sulfonamides [55] was identified in our isolate. In addition, we found *msr*(E) gene that confers resistance to macrolides, such as erythromycin and azithromycin that are commonly used to treat respiratory tract infections [56], and *mph*(A) gene that encodes resistance to macrolides and lincosamides, which are used to treat infections caused by Gram-positive bacteria [57]. Therefore, the presence of these ARGs in bacterial populations from water bodies suggests that the water may be contaminated with human or animal waste, creating a potential route for the dissemination of antibiotic-resistant pathogens to the food chain.

Plasmids are essential genetic elements in bacterial virulence, resistance, and adaptability. In our study on *E. kobei* UTA41, we identified several plasmid incompatibility groups, including Col440l, IncM1, IncP6, and pKPC-CAV1321. Unfortunately, traditional de novo assembly of short reads from total genomic DNA does not enable the separation of assemblies based on their original location (plasmids or chromosome), making the location of antibiotic resistance genes within plasmids unclear. To address this challenge, future studies could analyze the plasmids of this isolate separately or use a hybrid sequencing approach that integrates both short and long read sequences. These approaches would enable accurate bacterial genome assembly and comprehensive plasmid characterization, providing valuable insights into the genetic diversity and antibiotic resistance mechanisms of *E. kobei* UTA41.

Virulence factors (VF) have been of increasing interest because of their relationship with the pathogenesis of invasive infections. *Enterobacteriaceae* can use VF to increase invasiveness, overcome host defenses and cause infections [58]. The UTA41 isolate had the following virulence genes: *acrB* (bacterial efflux pump); *fepA, fepD, entA, entB, entE, entF, entS* (siderophore biosynthesis genes). Similar findings are shown in studies of resistome and virulome in ECC [59,60]. The presence of the AcrB efflux pump confers inherent and

evolved drug resistance to *Enterobacterales*, giving rise to multi-drug resistant (MDR) bacteria by blocking the activity of drugs that cannot accumulate and reach their intracellular targets [61]. On the other hand, siderophores are small molecules that play a critical role in iron acquisition and homeostasis in bacterial physiology. Moreover, they exhibit a range of functions beyond iron binding, such as binding non-iron metals, which can influence the host-pathogen interaction and bacterial fitness [62]. In summary, our findings demonstrate that the UTA41 isolate carries several virulence genes which may contribute to its pathogenicity and multidrug resistance.

Prophages are often involved in host survival strategies and contribute toward increasing the genetic diversity of the host genome. A total of seven prophages were detected into UTA41 using the tool PHASTER. However, only two intact prophage regions were identified (PHAGE_Entero_186_NC_001317 and PHAGE_Klebsi_phiKO2_NC_005857) (Table 2 and Fig. 1). The graphical representation of the prophage regions is shown in Supplementary Fig. S1, where it is mainly noted the presence of regions that codifies phage-related proteins. No ARGs or VF were detected inside the prophage regions, according to the Prokka Annotation method. Prophage Entero_186 has been reported in specific strains of *Klebsiella pneumoniae*, which sometimes harbor antibiotic resistance genes (ARGs) [63]. While the prophage Klebsi_phiKO2 is commonly associated with *K. pneumoniae* isolates, it has also been reported in *Citrobacter portucalensis* [64,65]. For prophage regions that exhibit incomplete matches, re-sequencing procedures or a combination of long and short reads could be useful in elucidating their structure. The application of whole-genome sequencing (WGS) is providing insights into the impact of prophages on bacterial evolution and shaping [66]. Therefore, the integration of these techniques can enhance our understanding of the contribution of prophages to bacterial diversity and adaptation.

The detection of *E. kobei* UTA41 in water bodies, particularly in a country like Ecuador where water pollution control in irrigation channels and other water bodies may be inadequate, is of significant importance. The presence of such pathogens in water used for agricultural purposes poses significant risks not only to crop health but also to public health through the potential for contaminating food supplies [67]. In regions where monitoring and control of water quality are less stringent, the risk of widespread dissemination of antibiotic-resistant bacteria and their resistance genes increases [68]. This scenario is particularly concerning in agricultural communities where irrigation water is a critical resource. This situation highlights the urgent need for enhanced surveillance and control measures in water management systems, particularly in regions with known deficiencies in water pollution control [69]. By prioritizing the detection and monitoring of such pathogens, we can better understand their ecological impact and develop effective strategies to mitigate their spread, ensuring safer agricultural practices and protecting public health.

5. Conclusions

This study documented the first report of *E. kobei* isolate recovered from environmental samples in Latin America harboring two carbapenemases encoding genes. Our findings indicate the potential circulation of critical-priority bacteria in water samples that could have an anthropogenic origin, such as urban wastewater discharges and livestock facilities. The presence of isolate carrying these resistance genes in water used for agricultural purposes indicates the potential public health risk as their dissemination across food and water samples could facilitate their integration into the food chain. Therefore, it is necessary to establish an effective monitoring system that comprehensively evaluates their distribution in the environment and food supply. In conclusion, the emergence of carbapenem-resistant *E. kobei* in environmental samples represents a significant public health concern. Our study highlights the need for increased surveillance and proactive measures to limit the spread of these bacteria in the environment and the food chain. Implementing effective monitoring programs to identify and track these organisms is essential to prevent their spread and protect public health.

Ethics approval

Not applicable.

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Consent to participate

Not applicable.

Consent to publish

Not applicable.

CRediT authorship contribution statement

Joselyn Corrales-Martínez: Writing – original draft, Visualization, Investigation, Data curation. **Katherine Jaramillo:**

Investigation. **Daniel A. Tadesse:** Resources, Investigation. **Carolina Satán:** Investigation. **Fernando X. Villavicencio:** Project administration, Investigation. **Lissette Sánchez-Gavilanes:** Investigation, Formal analysis. **Brenda Rivadeneira-Cueva:** Investigation. **José Luis Balcázar:** Visualization, Formal analysis, Data curation. **William Calero-Cáceres:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

William Calero-Caceres reports financial support and equipment, drugs, or supplies were provided by Technical University of Ambato. Daniel A. Tadesse reports financial support and equipment, reagents, or supplies were provided by US Food and Drug Administration. Katherine Jaramillo, Carolina Satan, Fernando X. Villavicencio reports financial support and equipment, drugs, or supplies were provided by National Institute for Research in Public Health (INSPI). Jose Luis Balcazar reports financial support was provided by Catalan Institute of Investigation of Water.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26379.

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