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Short Communication

Genomic analysis of a Kpi (pilus system)-positive and CTX-M-15-producing *Klebsiella pneumoniae* belonging to the high-risk clone ST15 isolated from an impacted river in Brazil

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ABSTRACT

Convergence of resistance and virulence in *Klebsiella pneumoniae* is a critical public health issue worldwide. A multidrug-resistant CTX-M-15-producing *K. pneumoniae* (TIES-4900 strain) was isolated from a highly impacted urban river, in Brazil. The genome was sequenced by MiSeq Illumina platform and *de novo* assembled using Unicycler. *In silico* prediction was accomplished by bioinformatics tools. The size of the genome is 5.4 Mb with 5145 protein-coding genes. TIES-4900 strain belonged to the sequence type ST15, yersiniabactin sequence type YbST10, ICEKp4, KL24 (*wzi*-24) and O1v1 locus. Phylogenomics confirmed genomic relatedness with ST15 clones from human and animal hosts. Convergence of broad resistome (antibiotics, heavy-metals and biocides) and virulome, including the Kpi pilus system involved in host-pathogen interaction and persistence of ST15 clone to hospital environments, were predicted. Virulent behavior was confirmed in the *Galleria mellonella* infection model. This study may give genomic insights on the spread of critical-priority WHO pathogens beyond hospital settings.

1. Introduction

Extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* has been categorized by the WHO as a critical priority pathogen [1], since is an important cause of serious hospital- and community-acquired infections worldwide. Worryingly, convergence of multidrug resistance and virulence in international *K. pneumoniae* clones has emerged as One Health threat, as evidenced by genomic studies [2,3].

Among high-risk clones of *K. pneumoniae*, the sequence type ST15 has been documented as an international lineage associated with ESBL and/or carbapenemase production [4,5]. Recently, a novel chaperone-usher pili (CUP) system (named Kpi, *kpiABCDEFG*), involved in the host-pathogen interaction through bacterial adherence along with biofilm formation, was associated with the global dissemination and persistence of the ST15 clone to the hospital setting [6]. Moreover, *K. pneumoniae* virulence has been associated with the production of yersiniabactin siderophore encoded by the *ybt* locus, typically located on a chromosomal mobile integrative conjugative element called ICEKp [7]. Besides *ybt*, *K. pneumoniae* can acquire other siderophore encoding genes, such as *ent* (enterobactin), *iuc* (aerobactin) and *iro* (salmochelin); as well as genotoxin colibactin (*clb*), and *rmpA* and *rmpA2* virulence genes [8,9].

Genomic data analysis is crucial for surveillance and the study of the emergence of multidrug-resistant (MDR) and virulent pathogens within a One Health perspective. In this study, we report the occurrence and genomic features of a virulent Kpi-positive and CTX-M-15-producing

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K. pneumoniae ST15 isolated from an impacted urban river in Brazil, highlighting the spread of critical-priority WHO pathogens beyond hospital settings.

2. Materials and methods

Between February and March 2019, during a Brazilian surveillance study (OneBR project) conducted to characterize the burden of antimicrobial resistance associated with critical WHO priority pathogens in impacted aquatic environments, six surface water samples (500 mL) were collected from different locations (Latitude 23° 31′ 31" S and Longitude 46° 33' 33" W; Latitude 23° 42′ 09" S and Longitude 46° 40' 26" W; Latitude 23° 42′ 09" S and Longitude 46° 40′ 26" W; Latitude 23° 42′ 09" S and Longitude 46° 40′ 26" W; Latitude 23° 31′ 52" S and Longitude 46° 44' 54" W) on the Tietê river, in São Paulo, the biggest city in South America.

The urban river Tietê was screened, in order to investigate the dissemination of WHO critical priority pathogens beyond hospital settings, as a consequence of anthropogenic activities. In this respect, a broad-spectrum cephalosporin-resistant ESBL-producing *K. pneumoniae* strain (TIES-4900) was isolated (Latitude 23° 27′ 16" S and Longitude 46° 54' 36" W) by using MacConkey agar plates containing ceftriaxone (2 μg/mL) (Sigma-Aldrich, St. Louis, MO), a broad-spectrum cephalosporin included in the primary panel for screening potential ESBL producers [10,11]. TIES-4900 was identified by matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS). Antimicrobial susceptibility was determined by Kirby-Bauer method [12].

The genomic DNA was extracted from overnight cultures using the PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. DNA concentration was evaluated by Qubit® 2.0 fluorometer (Life Technologies, Carlsbad, CA). Genomic library was constructed using a Nextera XT DNA Library preparation Kit (Illumina Inc., Cambridge, UK) and, subsequently, sequenced using 2 × 150 paired-end library on a MiSeq platform (Illumina). Read with a PHRED quality score below 20 were discarded and adapters were trimmed using TrimGalore v0.6.5 (https://github.com/FelixKrueger/TrimGalore). *de novo* genome assembly was performed with Unicycler v0.4.8 (https://github.com/rrwick/Unicycler). Sequences were annotated using NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v. 3.2 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/) and Rapid Annotation System Technology (RAST) pipeline (https://github.com/MG-RAST). Additionally, Circos plot was performed using Circular Genome Visualization Tool from Kbase (https://www.kbase.us/) and subsystems obtained from RAST.

To assess phylogenetic relationship, we performed a search for *K. pneumoniae* ST15 genomes on bacWGSTdb (http://bacdb.cn/BacWGSTdb). Using search results, 363 genome assemblies of isolates containing data for country, source of isolation and collection date were downloaded from NCBI GenBank. FastANI v1.32 (https://github.com/ParBLiSS/FastANI) was used to select the 30 genomes with highest average nucleotide identity (ANI) to TIES-4900, for further phylogenetic comparison. Additionally, 3 genomes of *K. pneumoniae* ST15 (GenBank assembly accession numbers: GCA_005222595.1, GCA_012241595.1 and GCA_014500865.1) isolated from human and animal hosts, in Brazil, were included in the phylogenetic analysis. Next, a maximum-likelihood tree based on SNP alignment was generated using default settings of CSI Phylogeny (https://cge.cbs.dtu.dk/services/CSIPhylogeny), where the chromosome sequence of *K. pneumoniae* ST15 strain BP327 (RefSeq assembly accession number: NZ_CP036335.1) was used as reference.

Antibiotic resistome and plasmidome were predicted by ResFinder 4.1 and PlasmidFinder 2.0, respectively (http://genomicepidemiology.org/), whereas the detection of genes encoding for heavy metal and biocides tolerance were performed by BacMet database (http://bacmet.biomedicine.gu.se/). Additionally, biocides tolerance genes were identified by *in silico* comparative analysis against an *in-house* database. For all predicted genes, a ≥ 98% for sequence identity threshold was used as filter for identification.

Table 1
Genomic and epidemiological data of *Klebsiella pneumoniae* strain TIES-4900.

Strain	TIES-4900
Source	Aquatic environment
Genome size (Mbp)	5.4
No. of CDS[a]	5145
G + C content (%)	57.25
tRNA (*n*)	77
rRNA (*n*)	3
Non-coding RNA (*n*)	10
Pseudogenes	87
CRISPR	2
MLST (ST)[b]	15
K-locus/O-locus	KL24/O1v1
wzi	24
Resistome	
β-lactams	$bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}1}$, $bla_{OXA\text{-}1}$
Aminoglycosides	*aac(6′)-Ib-cr*, *ant (3″)-Ia*
Fluoroquinolones	*oqxA*, *oqxB*, *qnrB1*, *gyrA* (S83F, D87A), *parC* (S80I)
Tetracyclines	*tet(A)*
Trimethoprim	*dfrA14*
Heavy metal	*silA*, *silB*, *silC*, *silE*, *silF*, *silG*, *silP*, *silR*, *silS*
Biocides	*oqxA*, *oqxB*, *smvR*
Virulome	*fyuA*, *kfuA*, *kfuB*, *kfuC*, *mrkA*, *mrkB*, *mrkC*, *mrkD*, *mrkF*, *mrkI*, *mrkJ*, *irp1*, *irp2*, *ybtA*, *ybtE*, *ybtP*, *ybtQ*, *ybtS*, *ybtT*, *ybtU*, *ybtX*
Plasmidome	IncFII, Col440I, Col440II, IncFIB(K), IncR
OneBR ID	ONE210
GenBank accession number	JABUOR000000000

[a] CDSs, coding sequences.
[b] MLST, Multilocus sequence type. ST, sequence type.

Virulome was predicted by using the Institute Pasteur BIGSdb database for *K. pneumoniae* (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). In addition, Kpi operon was detected by using BLASTn against the clinical reference strain Kp3380 (GenBank accession number: PITM00000000.1), using Geneious Prime version 2020.04 (Biomatters, New Zealand).

Kleborate was used to screen assemblies to confirm the species designation, multilocus sequence type (ST), ICEKp associated virulence loci [yersiniabactin (*ybt*), colibactin (*clb*)], capsule synthesis (*wzi*), K-locus, and O antigen (LPS) serotypes (https://github.com/katholt/Kleborate). The ICEKp4 was detected and its structure was inferred using BLASTn, and manual curation using Geneious Prime version 2020.04 (Biomatters, New Zealand), against the ICEKp4 of the *K. pneumoniae* 16703568 strain, recovered from a human patient (GenBank accession number. KY454629.1).

The virulent potential of the TIES-4900 strain was evaluated by using the *Galleria mellonella* infection model [13]. Virulent behavior was compared with the non-virulent *K. pneumoniae* ATCC 13883 and the hypervirulent *K. pneumoniae* (hvKP) K1/ST23 strain A58300 [14]. Groups of *G. mellonella* containing ten larvae (0.25–0.35 g; supplied by the Institute of Biomedical Sciences, University of São Paulo, Brazil) per strain were used for the survival assay. In brief, each group was inoculated with 10^6 CFU per larvae, and survival was monitored every hour, for 96 h. Survival assays were performed in two independent experiments.

3. Results and discussion

The *K. pneumoniae* strain TIES-4900 displayed a MDR profile to aztreonam, piperacillin/tazobactam, cefoxitin, cefepime, ciprofloxacin, levofloxacin, gentamicin, tobramycin, tetracycline, trimethoprim/sulfamethoxazole; remaining susceptible to ertapenem, imipenem, meropenem, and amikacin. Genomic data comprised a total of 978,934

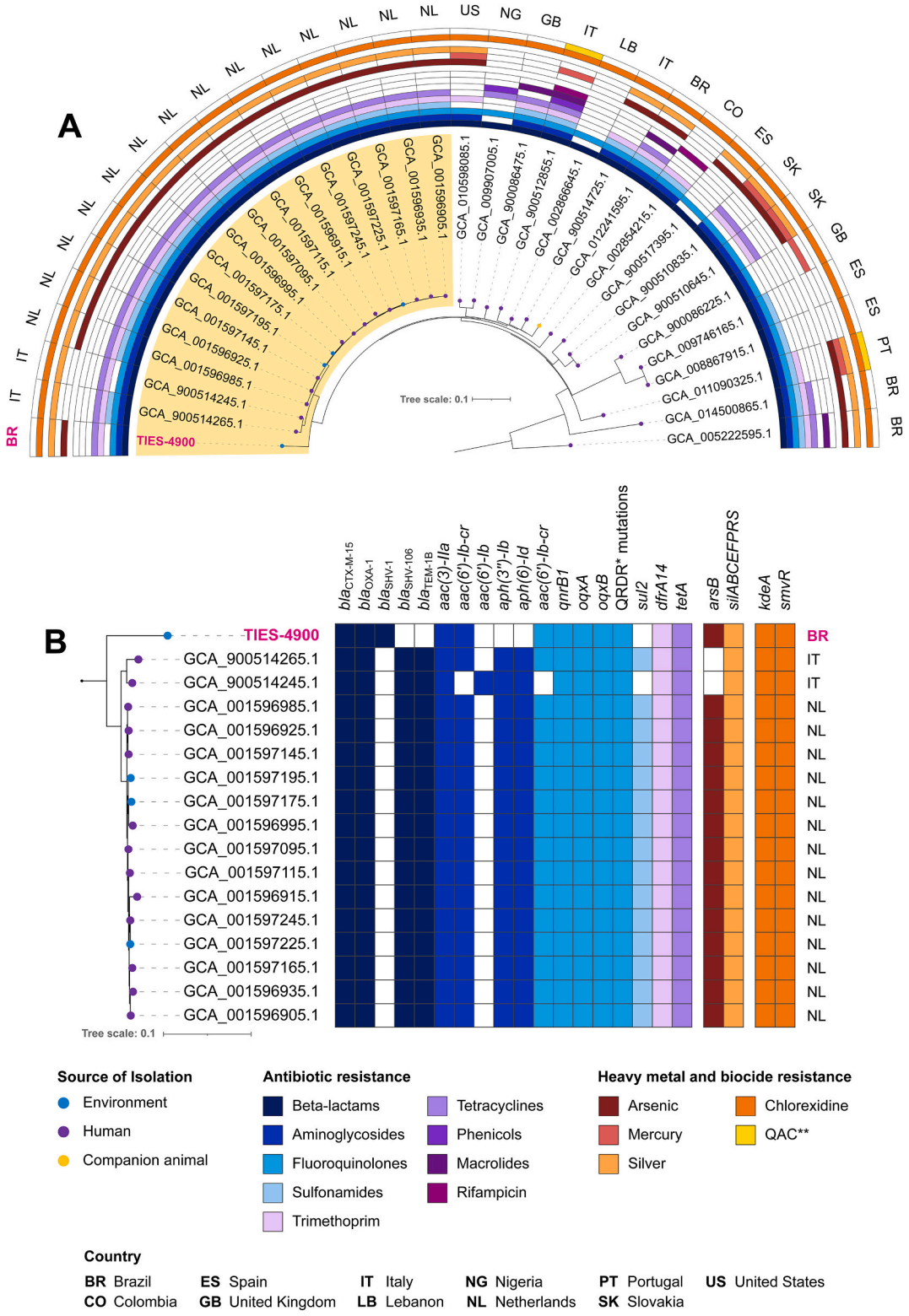

Fig. 1. In A, SNP-based phylogenetic tree of 34 *Klebsiella pneumoniae* ST15 strains isolated globally from human, environmental and companion animal sources, and their predicted resistance phenotypes. The cluster (ANI ≥ 99.9090% and SNP difference ranging from 112 to 125) grouping TIES-4900 strain with 16 *K. pneumoniae* ST15 isolated from human and environmental sources, in Netherlands and Italy, is highlighted. In B, resistome of the *K. pneumoniae* ST15 cluster highlighted in Fig. A. The TIES-4900 environmental strain (GenBank accession number: JABUOR000000000] is represented by magenta colour. *QRDR: quinolone resistance-determining regions. **QAC: quaternary ammonium compounds. ISO 3166-1 Alpha-2 country codes were used. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

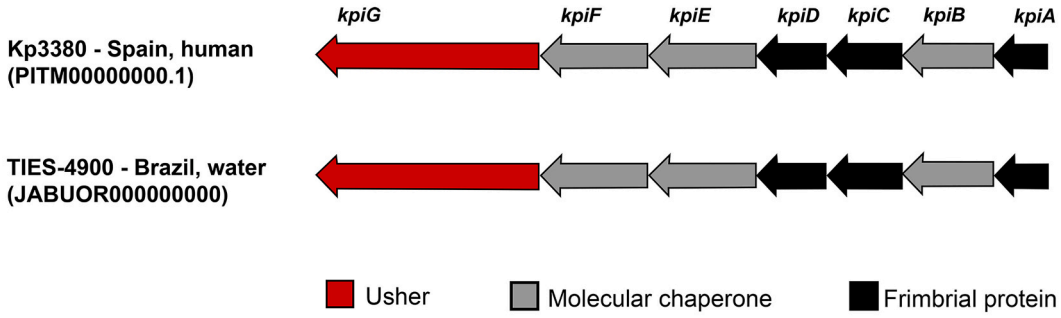

Fig. 2. Comparation of the *Klebsiella pneumoniae* TIES-4900 Kpi operon (accession number: JABUOR000000000) against the reference strain Kp3380 (GenBank accession number: PITM00000000.1).

paired-end reads assembled into 99 contigs, with 179× coverage, and a G + C content of 57.25%. In brief, strain TIES-4900 presented a genome size calculated as 5,479,561 bp, with 5145 protein-coding sequences, 77 tRNAs, 3 rRNAs, 10 non-coding RNAs, and 87 pseudogenes and two CRISPR arrays (Table 1). Circular genome map and subsystem obtained from RAST server were accessed (Supplementary Fig. S1).

The TIES-4900 strain was assigned to the ST15, which has been recognized as a high-risk international clone causing human infections in hospital settings, European, Asian, African, Oceania, North American and South American countries [3,15–17]. More recently, ESBL-producing *K pneumoniae* ST15 has been reported in companion animals in Italy [18], France [19], Portugal [20], Germany [21], and Brazil [4], supporting clonal adaptation and persistence of this lineage at the human-animal-environment interface, constituting a One Health issue.

The 30 genomes selected for phylogenetic analysis revealed an ANI value ranging from 99.9073% to 99.9687%, whereas three Brazilian isolates presented ANI values of 99.9070% (human source), 99.8543% (human source) and 99.8810% (companion animal), in comparison to TIES-4900 (Supplementary Table S1).

SNP-based phylogenetic analysis revealed differences of 0–509 SNPs between all 34 *K. pneumoniae* ST15 genomes, and the phylogenetic tree clustered (ANI ≥ 99.9090% and SNP difference ranging from 112 to 125) TIES-4900 strain with 16 *K. pneumoniae* ST15 isolated from human and environmental sources, in Netherlands and Italy (Fig. 1, Supplementary Tables S1 and S2).

Resistome analysis revealed the presence of genes conferring resistance to β-lactams ($bla_{CTX\text{-}M\text{-}15}$, $bla_{SHV\text{-}1}$, $bla_{OXA\text{-}1}$), aminoglycoside [*aac(6′)-Ib-cr*, *aac(3)-lla*], tetracycline (*tetA*), trimethoprim (*dfrA14*), and phenicols (*catB3*). Moreover, chromosomal mutations in the quinolone resistance-determining region (QRDR) of *gyrA* (S83F and D87A) and *parC* (S80I), and detection of PMQR genes (*oqxA, oqxB* and *qnrB1*) were associated with quinolone resistance (Fig. 1). Genes conferring resistance to heavy metal [silver (*silABCEFPRS*) and arsenic (*arsB*)], and biocides [quaternary ammonium compounds (*oqxA*, *oqxB*) and chlorhexidine (*kdA, smvR*)] were also predicted, which could favor the co-selection to antibiotic resistance. Plasmid replicons IncFII, Col440I, Col440II, IncFIB(K) and IncR were also detected (Table 1).

Regarding the virulent content of the CTX-M-15-producing *K. pneumoniae* ST15, it was detected the novel CUP system, named Kpi (*kpiABCDEFG*), which is involved in the host-pathogen interaction through bacterial adherence along with biofilm formation, being associated with the global dissemination and persistence of the ST15 clone to the hospital setting [6], was identified in TIES-4900. Comparative analysis of Kpi cluster revealed 100% identity with the Kpi element (GenBank accession number: PITM00000000.1), previously identified in clinical European *K. pneumoniae* clones ST15 [6] (Fig. 2).

Moreover, the virulome of the CTX-M-15-producing *K. pneumoniae* ST15 included the *mrkABCDFIJ* cluster (type 3 fimbrial genes mediating adherence to surfaces and host tissues and biofilm formation). In addition, through genomic comparative analysis, the integrative conjugative element ICEKp4-*ybt*10 was identified in the TIES-4900 genome (Fig. 3), as well as in 94.11% (32/34) *K. pneumoniae* genomes analyzed in this study. Moreover, TIES-4900 presented KL24 (*wzi*-24), O1v1 locus, *kfuABC* (ferric uptake system), *fyuA* (yersiniabactin receptor), *irp-1-2*, the *ybt* cluster (*ybtAEPQSTUX*, yersiniabactin siderophore synthesis), and the allelic profile YbST sequence type 10 (YbST10). In this regard, siderophores play an essential role in bacterial virulence as they are involved in mechanisms for scavenging iron from the host, thus increasing the ability of bacteria to persist and develop their cell cycle, resulting in invasive and life-threatening infections [7]. Particularly, the yersiniabactin siderophore has been linked to pathogen survival in the respiratory tract, and human infections with poor outcome [22,23]. The *ybt* operon was identified for the first time in *Yersinia pestis* and *Yersinia enterocolitica*, on the high-pathogenicity island (HPI) [24]; since then, *ybt* variants have been identified among Enterobacterales members, including *Enterobacter cloacae*, *Klebsiella aerogenes* and *Escherichia coli*

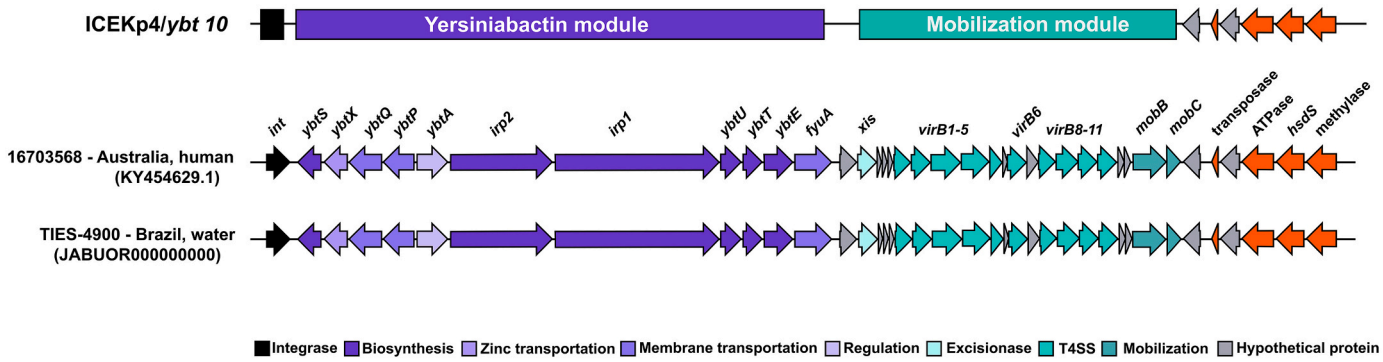

Fig. 3. Comparation of the ICEKp4 structure of *Klebsiella pneumoniae* TIES-4900 (accession number: JABUOR000000000) against the reference clinical strain 16703568 (GenBank accession number: KY454629.1). Squares represent modules of core genes encoding yersiniabactin siderophore (purple), and mobilization proteins (green). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

[25–27].

Interestingly, a virulent behavior of TIES-4900 strain was confirmed in the *Galleria mellonella* infection model. In fact, this strain killed 100% larvae at 30 h post-infection, in a similar way that the hypervirulent K1/ST23 control strain A58300 [15], which achieved 100% mortality at 24 h post-infection. On the other hand, no mortality was observed in larvae groups infected with the non-virulent *K. pneumoniae* strain ATCC 13883.

In summary, the convergence of broad resistome and virulome in the high-risk clone ST15 is a critical issue, which could be contributing with severe infections in human and non-human host [5,15,28], and persistence and adaptation to aquatic environments impacted by anthropogenic activities, including hospital and urban discharges [5,28]. Therefore, this study gives genomic insights on the spread of critical-priority WHO pathogens beyond hospital setting and highlights the urgent need for active genomic surveillance strategies under a One Health approach.

3.1. Nucleotide sequence accession number

This Whole Genome Shotgun project has been deposited at DDBJ/ENA/GenBank under accession number JABUOR000000000. The version described in this paper is the first version. In addition, genomic information of *K. pneumoniae* TIES-4900 strain is available on the OneBR platform under the number ID ONE210 (http://onehealthbr.com/).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygeno.2021.12.007.

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Author statement

We declare that the manuscript "*Genomic investigation of a virulent Kpi-positive (pilus system) and CTX-M-15-producing K. pneumoniae high-risk ST15 isolated from an impacted river in Brazil*" by Brenda Cardoso, Fernanda Esposito, Herrison Fontana, Bruna Fuga, Quézia Moura, Elder Sano, Maria I. Z. Sato, Carlos J. Brandão, Flavio A. Oliveira, Carlos E. Levy and Nilton Lincopan, has not been published before and is not under consideration for publication elsewhere.

All authors made relevant contributions to the development of the research, the manuscript has been read and approved by all named authors and confirm that the order of authors listed in the manuscript has been approved by all of us. We also affirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Declaration of Competing Interest

The authors declare no conflict of interest.

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