A novel hypermucoviscous *Klebsiella pneumoniae* ST3994-K2 clone belonging to Clonal Group 86

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ABSTRACT

Emergent hypervirulent *Klebsiella pneumoniae* has been responsible for severe diseases, representing a serious threat to public health. We report the whole-genome sequencing of a novel ST3994-K2 clone, a single locus variant of ST86 K2, which is considered a worrying hypervirulent clone that emerged in several parts of the world. The strain *K. pneumonia* Kpi144 was isolated in 2013 from a blood culture of a 69-year-old male patient admitted to a tertiary hospital in Teresina, state of Piauí, northeastern Brazil. The strain was susceptible to 41 antibiotics tested, presented hypermucoviscous phenotype, and a virulent behavior was observed in the *Galleria mellonella* infection model. Moreover, the virulome showed several virulence genes. To the best of our knowledge, this is the first worldwide report of a novel ST3994-K2 *K. pneumoniae* clone, an SLV of ST86 K2, which is considered a worrying virulent clone that has emerged in several parts of the world, including South America and Brazil.

Keywords: *Klebsiella pneumoniae*; virulence; Clonal Group 86; ST3994.

INTRODUCTION

Emergent hypervirulent *Klebsiella pneumoniae* (HvKp) has been responsible for severe diseases, posing a severe public health threat. The infections caused by this clinically significant opportunistic pathogen have been increasingly reported globally, including in Brazil (Guerra *et al*. 2020). HvKp strains are possessing the hypermucoviscosity phenotype that has been responsible for severe disseminated infections, including pyogenic liver and other severe illnesses (Lee *et al*. 2017). Additionally, when associated with several virulence factors such as regulators of the mucoid phenotype (*rmpA* and *rmpA2*), siderophores like aerobactin and yersiniabactin (*iuc* and *ybt*), the phospholipase D family protein (PLD1), and outer membrane porin (*KpnO*) (Catalán-Nájera *et al*. 2019), integrative conjugative element (ICEKp) that allows the *ybt* locus mobilisation and could also carry salmochelin or the colibactin locus (Lam *et al*. 2018). Several reports have shown that *K. pneumoniae* strains with K1 and K2 serotypes are strongly associated with hvKp. These strains are more resistant to phagocytosis and intracellular death by macrophages and neutrophils (Lee *et al*. 2017). Usually, hvKp strains with serotype K1 and ST23 have often been the causative agent pyogenic liver abscesses, while hvKp strains with serotype K2 and ST65 have been correlated with various invasive infections (Lee *et al*. 2017). In particular, hvKp belonging to complex clonal 23 (CG23) has been strongly related to the capsular serotypes K1 (Lee *et al*. 2017). The hvKp strains of serotype K2 have been described mainly in Asia, but few reports exist in Latin America (Catalán-Nájera *et al*. 2019). Recently, an hvKp serotype K2 clone belonging to ST86 isolated from marmosets was reported for the first time in Brazil (Guerra *et al*. 2020). This work aimed to characterise a virulent *K. pneumoniae* genomically.

MATERIAL AND METHODS

Bacterial strain data and identification

Here, we evaluated a *K. pneumoniae* isolated in August 2013 from a blood culture of a 69-year-old male patient admitted to a tertiary hospital in Teresina, state of Piauí, northeastern Brazil. The strain named KpPi144 was identified by the Vitek 2 system (biomérieux, France).

Antimicrobial susceptibility testing

The antimicrobial susceptibility tests were determined by disc diffusion method as recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020) using 40 different antibiotic disks of all classes: amoxicillin-clavulanate (AMC), amikacin (AMI), ampicillin-sulbactam (APS), aztreonam (ATM), ceftazidime (CAZ), cefaclor (CFC), cefixime (CFM), cefoxitin (CFO), cefazolin (CFZ), ciprofloxacin (CIP), chloramphenicol (CLO), cefepime (CPM), ceftriaxone (CRO), cefuroxime (CRX), ceftaroline (CTL), cefotetan (CTT), cefotaxime (CTX), doripenem (DOR), doxycycline (DOX), ertapenem (ERT), streptomycin (EST), fosfomicin (FOS), gentamicin (GEN), imipenem (IPM), levofloxacin (LEV), lomefloxacin (LMX), minocycline (MIN), meropenem (MPM), nalidixic acid (NAL), netilmicin (NET), nitrofurantoin (NIT), norfloxacin (NOR), ofloxacin (OFX), piperacillin-tazobactam (PIT), sulphonamide (SUL), trimethoprim-sulfamethoxazole (SUT), ticarcillin-clavulanate (TAC), tetracycline (TET), tobramycin (TOB), trimethoprim (TRI). Colistin (COL) was tested colistin by microdilution (CLSI, 2020).

Hypermucoviscosity test

The hypermucoviscous phenotype was investigated by the string test according to Wiskur *et al*. (2008). The strain was grown on Muller Hinton Agar, and an isolated

colony was touched with an inoculation loop and raised vertically to verify the formation of viscous filament. The formation of a filament equivalent to 5 mm or more in length indicates the presence of a hypermucoviscous phenotype. The strain *Klebsiella pneumoniae* NTUH-K2044 was used as a positive control for the string test (Wu *et al*. 2009).

Virulence potential

The virulent potential of the KpPi144 strain was assessed by using the *Galleria mellonella* infection model (Moura *et al*. 2017). Virulent behavior was compared with the hypervirulent *K. pneumoniae* (hvKP) K1/ST23 strain A58300 and the non-virulent *K. pneumoniae* ATCC 13883 (Coutinho *et al*. 2014). 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. Briefly, each group was inoculated with 10^6 CFU per larvae, and survival was monitored every hour for 96 hours. All assays were performed in three independent experiments. Survival curves were plotted using the Kaplan-Meier method. Statistical analyses were performed by the log-rank test with $p< 0.05$, indicating statistical significance (GraphPad Software, La Jolla, CA, EUA).

Whole genome sequencing

The genomic DNA was extracted using a PureLinkTM Quick Gel Extraction Kit (Life Technologies, CA). Their quality and quantity were assessed using a Qubit1 2.0 fluorometer (Life Technologies). The bacterial genome was sequenced via Illumina MiSeq (Illumina Inc., San Diego, CA), using 250-bp paired-end (PE) reads. The reads

were *de novo* assembled using Unicycler v0.4.0 (Wick *et al*., 2017) and annotated using the Prokaryotic Genome Annotation Pipeline v.3.2 (PGAP) at NCBI.

Sequence type, resistome and virulome

Multilocus sequence typing (MLST), antimicrobial resistance genes (ARG), virulence factors including yersiniabactin, colibactin, aerobactin and salmochelin, as well as *wzi* type, K-locus, and mutations in quinolone resistance-determining regions were identified using Kleborate v2.1.0 (Lam *et al*. 2021). Presence/absence of the well characterized MGEs harboring the key virulence genes (ICEKp and virulence plasmids) were confirmed by BLASTn search. These elements and their adjacent regions were manually curated using the Bandage v0.8.1 (Wick *et al*. 2015), Artemis v18.1.0 (Carver *et al*. 2012) and CLC Genomics Workbench v12.0.3 (QIAGEN), using the reference mapping tool with default threshold to identified the paired-end short-read coverage to ensure these regions do not belong to an assembly artefact (Cerdeira *et al.* 2011).

Phylogenetic comparison with ST86

Genome assemblies of *Klebsiella pneumoniae* were downloaded from GenBank, based on an MLST search on BacWGSTdb (http://bacdb.cn/BacWGSTdb). Since *K. pneumoniae* ST3449 is a novel sequence type and there are no other assemblies of other CC86 isolates available, only assemblies of ST86 were downloaded.

CSI Phylogeny v1.4 (https://cge.cbs.dtu.dk/services/CSIPhylogeny) was used with default settings to build an approximately maximum-likelihood phylogenetic tree with KpPi144 along with all 56 genomes downloaded from GenBank, using the assembly of *K. pneumoniae* ST86 strain CG43 (RefSeq accession number GCF_000474015.1) as a reference genome. iTOL v6 (https://itol.embl.de) was used to

root the tree at the midpoint, revealing separate clades for ST86 and ST3994. iTOL was also used to annotate the tree with data from ABRicate and Kleborate.

RESULTS AND DISCUSSION

The strain *K. pneumonia* Kpi144 studied here was isolated in 2013 from a blood culture of a 69-year-old male patient admitted to a tertiary hospital in Teresina, state of Piauí, northeastern Brazil. For ethical reasons, these were the only data provided by the hospital. Therefore, the patient's clinical evolution was not informed to us after the isolation of the strain.

Sequencing yielded 8 700 000 PE reads (~110X coverage), 41 contigs; genome length was 5 210 444 bp. A total of 5010 protein-coding sequences, 79 tRNAs, 4 rRNAs were annotated in strain KpPi144, 57.60 % of GC content. The genome sequence revealed a novel ST3994 identified by the *K. pneumoniae* database (http://bigsdb.pasteur.fr), a single locus variant (SLV) of the hvKp ST86 K2 clone shows a mash distance confidence of 0.00118 *(K. pneumoniae* CG43, K2-ST86, CP006648.1 accession number). Previous reports have shown ST86 K2 as a hypervirulent clone with susceptibility to antibiotics, causing severe infections in hospitals and acquired in the community (Guerra *et al*. 2020).

The strain was susceptible to all 41 antibiotics tested and Kleborate indicated that it harboured only the core chromosomal AMR gene, blaSHV-1, which confers intrinsic resistance to ampicillin. Furthermore, Kleborate indicated the presence of the KL2 capsule locus and O1v1 O locus associated with the K2 capsule, O1 lipopolysaccharide, respectively, in addition to the chromosomal ICE*Kp1* MGE containing the yersiniabactin locus (*ybt* lineage 2) as well as a truncated *iro* locus (*iro* lineage 3 with premature STOP codon in *iroC*) and the *rmpADC* locus (*rmp* lineage 3).

The presence of ICE*Kp1* was confirmed by BLASTn search of the Kpi144 assembly (100% coverage, 99.96% identity and 75X of paired short-read coverage). Kleborate did not detect any loci associated with *K. pneumoniae* virulence plasmids (*iuc*, *iro* lineages 1, 2, 2a or *rmpA2*) and BLASTn search of the Kpi144 assembly using the pNTUH-K2044 reference plasmid (represents the most common virulence plasmid, KpVP-1) confirmed the absence of any similar replicons in Kpi144 (maximum BLAST hit coverage <3%).

The KpPi144 K2/ST3994 strain demonstrated a hypermucoviscous phenotype, and a virulent behavior was observed in the *Galleria mellonella* infection model. In this regard, both KpPi144 K2/ST3994 and A58300 K1/ST23 strains killed 100 % larvae at 48 h and 24 h post-infection, respectively. In contrast, no mortality was observed in larvae groups infected with the non-virulent *K. pneumoniae* strain ATCC 13883 (Figure 1).

Therefore, the new clone presented here, although the strain shows virulence killing the larvae 48 h post-infection and is SLV of the hvKP ST86 K2 clone, is worrying but cannot be considered hvKP. The absence of aerobactin may be one of the determining factors for this.

The phylogenetic comparison of KpPi144 K2/ST3994 with ST86 showed that the percentage of reference genome covered by all 57 isolates was 86.56 %, corresponding to 4478910 positions. SNP count of KpPi144 and the other 56 isolates variated between 1555 and 1881, with an average and median of 1646 and 1639, respectively. The SNP count among the 56 *K. pneumoniae* ST86 variated between 1 and 643, with average and median of 255.03 and 311, respectively. Figure 2 shows the phylogenetic tree with KpPi144 (ST3994, CG86) and *K. pneumoniae* ST86 isolates.

CONCLUSIONS

To the best of our knowledge, this is the first worldwide report of a novel hypermucoviscous ST3994-K2 *K. pneumoniae*, an SLV of ST86 K2, which is considered a worrying virulent clone that has emerged in several parts of the world, including South America and Brazil.

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ACCESS TO DATA

This Whole Genome Shotgun project has been deposited at GenBank under accession number JAEDYN000000000. Additionally, genomic information of *K. pneumoniae* KpPi144 strain is available on the OneBR platform under the number ID ONE205 (http://onehealthbr.com/).

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ETHICAL APPROVAL AND CONSENT

Ethical approval was received from the School of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo (Ribeirão Preto, SP, Brazil) [approval no. CEP/FCFRP 362; CAEE 36031914.9.0000.5403].

CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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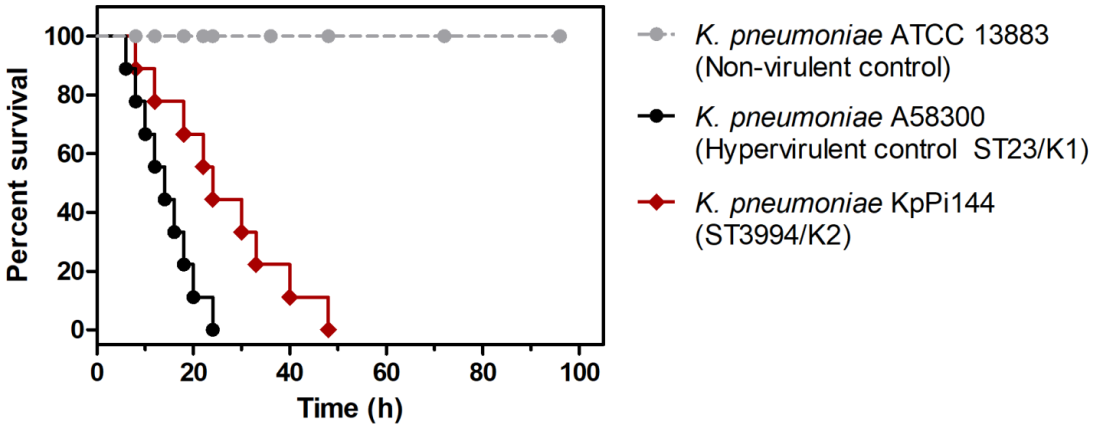

Figure 1. The virulent potential of the KpPi144 (*K. pneumoniae* ST3994, CC86). Kaplan-Meier survival curves of *G. mellonella* infected with 10^6 CFU/larva of the hypermucoviscous ST3994-K2 *K. pneumoniae* KpPi144 strain (red rhombus), the clinical hvKP K1/ST23 A58300 strain (black circles) and the non-virulent *K. pneumoniae* ATCC 13883 strain (light-grey circles). KpPi144 and A58300 clinical strains killed 100% of larvae at 48- and 24-hours post-infection, resulting in a significantly higher mortality rate than the non-virulent *K. pneumoniae* ATCC 13883 strain ($p < 0.05$).

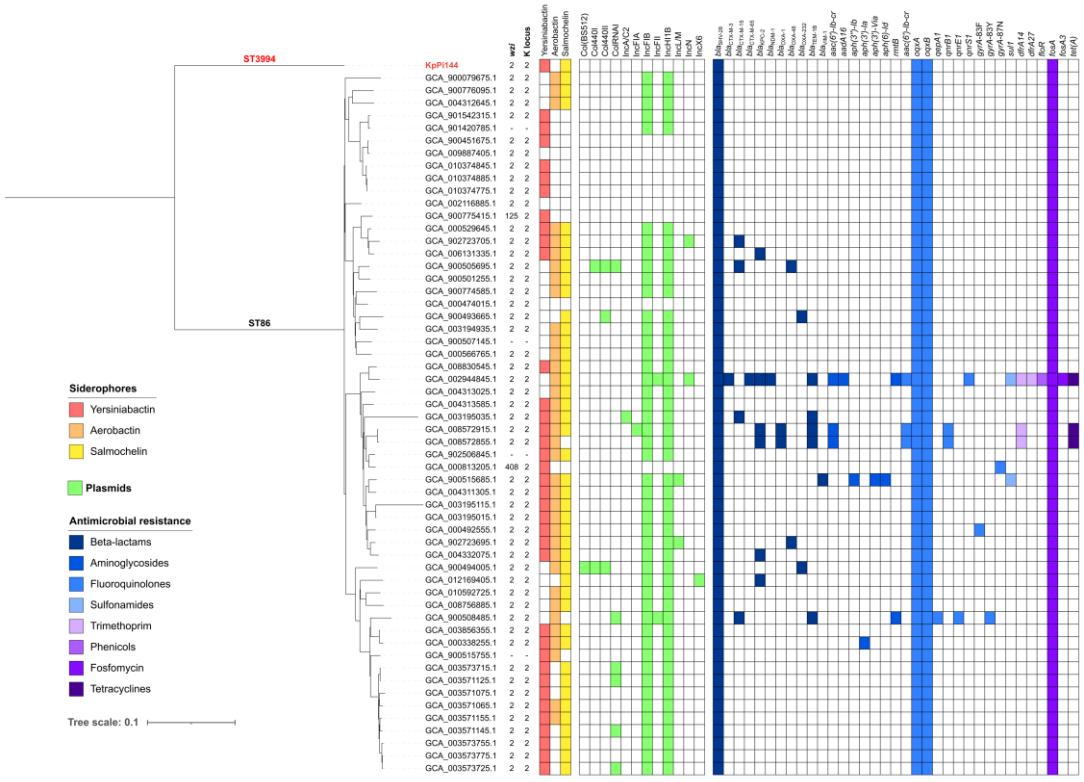

Figure 2. Phylogenetic tree of KpPi144 (*K. pneumoniae* ST3994, CG86) and *K. pneumoniae* ST86 isolates. The presence of the *wzi* types, K-locus, yersiniabactin, aerobactin, salmochelin, siderophores genes, plasmids replicons and ARG.