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SHORTOMICS

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Genome sequence analysis of a hypermucoviscous/hypervirulent and MDR CTX-M-15/K19/ST29 *Klebsiella pneumoniae* isolated from human infection

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One sentence summary: This draft genome sequence can provide valuable information to better understand the evolution of hypermucoviscous/hypervirulent and multidrug-resistant lineages of *Klebsiella pneumoniae*.

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ABSTRACT

The emergence of hypervirulent *Klebsiella pneumoniae* (hvKP) with multidrug resistance (MDR) profile is a worrisome public health issue. We report the first draft genome sequence of a hypermucoviscous (positive string test) and MDR *K. pneumoniae* serotype K19, belonging to ST29, isolated from human infection. This strain harboured multiple antimicrobial resistance genes, including $bla_{\text{CTX-M-15}}$, besides yersiniabactin and type 3 fimbriae virulence genes. *In vivo* experiments carried out with the *Galleria mellonella* infection model revealed that *K. pneumoniae* K19/ST29 killed 100% of the larvae at 24 h post-infection, in a similar way to the known hypermucoviscous hvKP K1/ST23 lineage.

Keywords: whole genome sequence; *K. pneumoniae*; hvKP; extended-spectrum beta-lactamase; *Galleria mellonella*; Brazil

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Klebsiella pneumoniae is recognised as an urgent problem of public health, where the emergence and dissemination of multidrug-resistant (MDR) lineages has contributed to the establishment of nosocomial infection outbreaks (Holt *et al.* 2015; Paczosa and Mecsas 2016). On the other hand, hypervirulent *K. pneumoniae* (hvKP) has been worldwide reported as a cause of serious infections in both hospitals and the community (Shon, Bajwa and Russo 2013; Bialek-Davenet *et al.* 2014). Most hvKP strains display a hypermucoviscous phenotype that confers resistance to phagocytosis and intracellular killing (Lin *et al.* 2004; Wang *et al.* 2017). Survival within phagocytic cells contributes to the establishment of distant metastasis, through bloodstream dissemination, which is considered a distinctive ability of hvKP (Lin *et al.* 2010). Hypermucoviscosity is a phenotypic feature characterised by the formation of a viscous filament ≥ 5 mm (positive string test), when a bacterial colony is streched by a bacteriological loop (Catalán-Nájera, Garza-Ramos and Barrios-Camacho 2017).

Despite most of the hvKP strains are susceptible to several classes of antimicrobials, some MDR-hvKP strains have been described, raising important therapeutic challenges and accounting for remarkable morbidity and mortality rates (Shon, Bajwa and Russo 2013; Paczosa and Mecsas 2016; Zhan *et al.* 2017). Indeed, nowadays both carbapenemase (KPC-2)- and extended-spectrum beta-lactamase -producing hvKP strains have been reported in some countries of Europe, Asia and South America (Cejas *et al.* 2014; Surgers *et al.* 2016; Zhang *et al.* 2016; Yu *et al.* 2017). However, whole genome sequence reports, necessary to perform a meticulous investigation of strains of this sort, have been limited (Shankar *et al.* 2016; Kislichkina *et al.* 2017). In this study, we report the draft genome sequence of *K. pneumoniae* KPHU468, a hypermucoviscous/hypervirulent and MDR (CTX-M-15-positive) clinical strain.

The *K. pneumoniae* strain KPHU468 was isolated from an urine sample of a 50-year-old male patient with severe sepsis, and that had previous pyogenic liver abscesses (PLAs), hospitalised in a teaching hospital in the city of São Paulo, Brazil. The strain exhibited a hypermucoviscous phenotype, as confirmed by a positive string test (Fig. 1A), displaying a MDR profile to tobramycin, gentamicin, streptomycin, aztreonam, cephalotin, ceftriaxone, cefotaxime, ceftazidime, cefepime, nalidixic acid, ciprofloxacin, trimethoprim-sulfamethoxazole, tetracycline and doxycycline (CLSI 2017).

The genomic DNA of KPHU468 was extracted using PureLink Quick Gel Extraction & PCR Purification Combo Kit (Life Technologies, Carlsbad, CA). The Illumina paired-end library of 150 bp was constructed using a Nextera XT DNA Library Preparation Kit (Illumina Inc., Cambridge, UK), and the whole genome sequencing was performed by using an Illumina NextSeq platform (Illumina Inc.). *De novo* genome assembly was carried out using Velvet version 1.2.10, and the contigs were annotated by NCBI Prokaryotic Genome Annotation Pipeline (PGAP) version 3.2 (http://www.ncbi.nlm.nih.gov/genome/annotation_prok/). Resistance genes and plasmid incompatibility groups were predicted using ResFinder 2.1 (https://cge.cbs.dtu.dk/services/ResFinder) and PlasmidFinder 1.3 (https://cge.cbs.dtu.dk/services/PlasmidFinder/), respectively. Capsular type, multilocus sequence type, virulence genes, efflux systems and regulators, and heavy metal genes were determined through the *K. pneumoniae* database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html).

A total of 25 013 798 paired-end reads, assembled into 113 contigs, were produced with ×625 coverage. The genome size was calculated at 5238 980 bp, with 4830 protein-coding

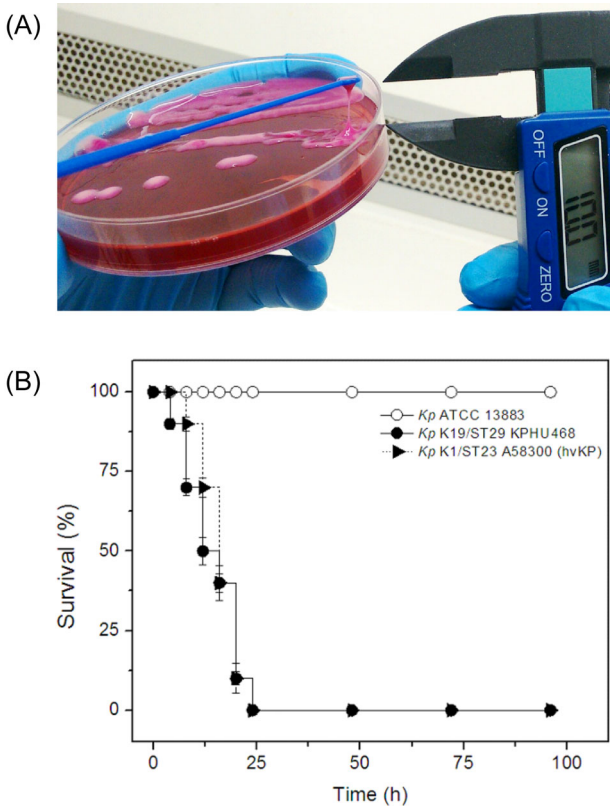

Figure 1. Hypermucoviscosity and hypervirulent behaviour of MDR extended-spectrum β-lactamase (CTX-M-15)-producing *K. pneumoniae* K19/ST29 isolated from a human infection. (**A**) Hypermucoviscosity was confirmed by a positive string test, characterised by the formation of a viscous filament ≥ 5 mm, when a bacterial colony was stretched by a bacteriological loop. (**B**) Kaplan-Meier survival curves of *G. mellonella* infected with 10^5 CFU/larva of the hypermucoviscous *K. pneumoniae* KPHU468 strain (filled circles), the non-virulent *K. pneumoniae* ATCC 13883 strain (open circles) and the clinical hvKP K1/ST23 A58300 strain (filled triangles). The *K. pneumoniae* K19/ST29 killed 100% of the larvae at 24 h post-infection, in a similar way to the hypervirulent *K. pneumoniae* K1/ST23, resulting in significantly higher mortality rate compared to the non-virulent *K. pneumoniae* ATCC 13883 strain ($P < 0.05$). For each strain, groups of *G. mellonella* containing five larvae were evaluated in two separate experiments. In this regard, for all groups, independent experiments were reproducible.

sequences, and a GC content of 57.4%. Overall, 45 tRNAs, 1 rRNA, 8 ncRNAs and 354 pseudogenes were identified. Resistance genes to aminoglycosides (*aac(3)-IIa*, *strA* and *strB*), β-lactams (bla_{SHV-83}, bla_{TEM-1B}, $bla_{CTX-M-15}$, and bla_{OXA-1}), quinolones (*oqxA*, *oqxB*, *qnrB66* and *aac(6')Ib-cr*), fosfomycin (*fosA*), phenicol (*catB3*), sulphonamide (*sul2*), tetracycline (*tetA*) and trimethoprim (*dfrA14*) have been identified. The results from PlasmidFinder detected the incompatibility groups IncFII and IncFIB. The strain was assigned to capsular type *wzi19*-K19 and sequence type ST29. Additionally, genes encoding virulence determinants for type 3 fimbriae (*mrkA*, *mrkB*, *mrkE*, *mrkF*, *mrkI* and *mrkJ*) and the siderophore yersiniabactin (*ybtA*, *ybtP*, *ybtQ*, *ybtS*, *ybtT* and *ybtU*) have also been identified. Moreover, the presence of efflux system and regulator genes (*acrR*, *envR*, *fis*, *marA*, *marR*, *ramA*, *ramR*, *sdiA*, *soxR*, *soxS*), as well as copper resistance genes (*pcoA*, *pcoB*, *pcoC*, *pcoE*, *pcoR*, *pcoS*), was confirmed.

In order to evaluate the virulent potential of the hypermucoviscous KPHU468 strain, *in vivo* experiments were carried out with the *Galleria mellonella* infection model (Junqueira 2012; Insua *et al.* 2013), using the non-virulent *K. pneumoniae*

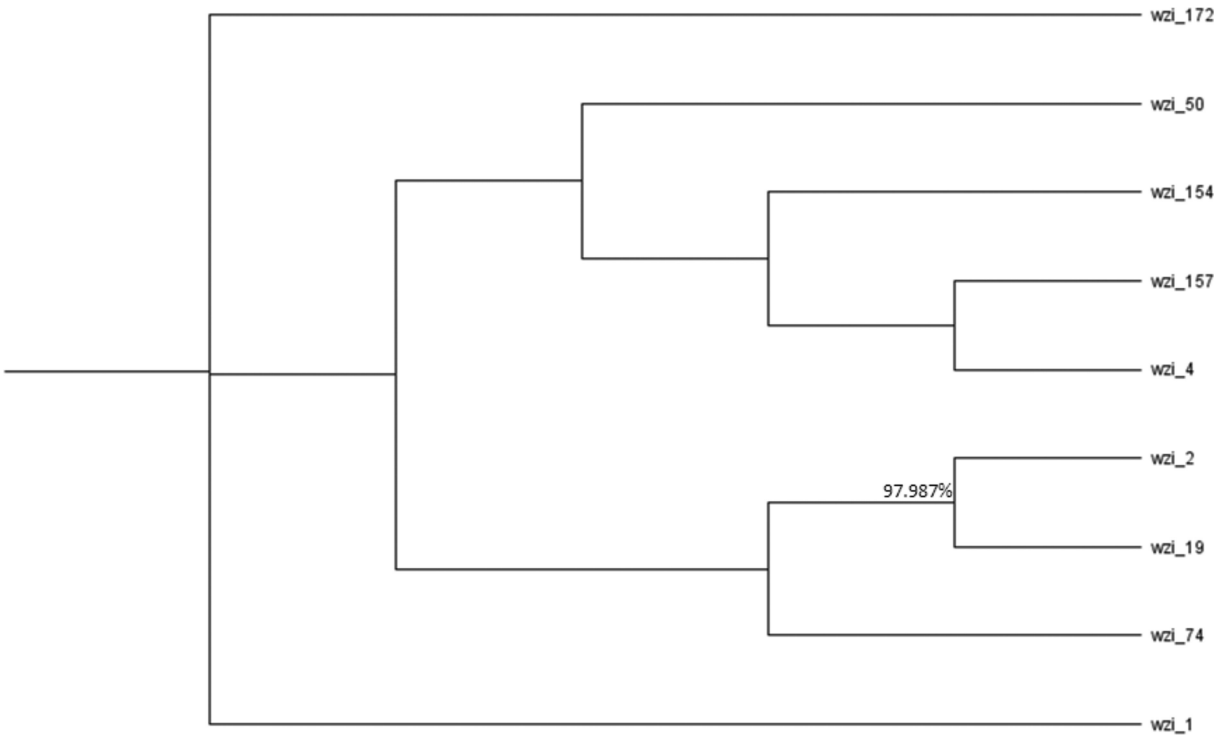

Figure 2. Phylogenetic tree of *wzi* alleles constructed using RAxML with the bootstrap algorithm.

Table 1. SNPs comparative analyses of KPHU468 strain against hypervirulent (NTUH-K2044, Kp52.145, Kp U25) and non-hypervirulent (MGH78578, Kp13, 1084, HS11286) *K. pneumoniae* strains.

Reference strain (accession number)	Genome size (bp)	Total SNPs	SNP ratio (%)	Insertion (%)	Deletion (%)	Transition (%)	Transvertion (%)
NTUH-K2044 (AP006725)	5248520	33149	0.6	0.4	0.6	65.5	27.0
MGH78578 (CP000647)	5315120	33022	0.6	0.5	0.5	65.5	27.3
1084 (CP003785)	5386705	35173	0.7	0.5	1.0	63.9	27.2
Kp13 (CP003999)	5307003	36798	0.7	0.5	0.5	64.7	27.3
Kp U25 (CP012043)	5491870	33733	0.6	0.4	0.4	65.1	27.6
Kp52.145 (FO834906)	5438894	34625	0.6	0.5	0.6	64.5	27.3
HS11286 (NC_016845)	5333942	33197	0.6	0.5	0.5	64.8	27.1

strain ATCC 13883 and the clinical hvKP K1/ST23 strain A58300 (Coutinho *et al.* 2014) as comparative strains. *Galleria mellonella* larvae, of nearly 250 to 350 mg, were inoculated with 10^5 CFU of each strain, and survival analysis was evaluated during 96 h. For each strain, groups of *G. mellonella* containing five larvae were evaluated in two separate experiments, as previuously described (Moura *et al.* 2017). Survival curves were plotted using the Kaplan-Meier method, and data were analysed by the log rank test, with $P < 0.05$ indicating statistical significance (Graph Pad Software, San Diego, CA, USA). In this regard, *K. pneumoniae* K19/ST29 killed 100% of the larvae at 24 h post-infection, in a similar way to the hypervirulent *K. pneumoniae* K1/ST23 (Fig. 1B).

Main virulence determinants of *K. pneumoniae* are pili, lipopolysaccharide, siderophores and capsular polysaccharides (K antigens). In this regard, although K serotypes have been associated to different types of infection, K1 and K2 serotypes have emerged as hvKP variants, being isolated from patients with bacteraemia (usually suffering from PLAs) and without previous invasive procedures (Patel, Russo and Karchmer 2014; Wu *et al.* 2017). Since hypermucoviscosity has become associated with an unusual and aggressive type of infection, strains exhibiting this phenotype are considered hypervirulent. These hypervirulent variants of *K. pneumoniae* have been identified in human or animal infections in North America, Europe, South America and Asia (Cejas *et al.* 2014; Coutinho *et al.* 2014; Cubero *et al.* 2016; Wei *et al.* 2016; Parrott, Shi and Wu 2017; Seguel *et al.* 2017). Interestingly, K1 and K2 *K. pneumoniae* strains isolated from bacteraemia associated to PLAs exhibit a hypermucoviscous phenotype, with a *magA*- and/or *rmpA*-positive genotype (Cubero *et al.* 2016). Most K1 serotypes have been clustered into clonal complex 23 comprising ST23 and ST57, whereas K2 K. *penumoniae* strains have belonged to several STs, such as ST65, ST86, ST375 and ST380 (Coutinho *et al.* 2014; Struve *et al.* 2015; Cubero *et al.* 2016; Hennequin and Robin 2016); more recently, hypermucoviscous KPC-2-producing *K. pneumoniae* of capsular serotype K1 or K20, belonging to ST11, have been identified in nosocomial infections in Asia (Wei *et al.* 2016; Zhan *et al.* 2017).

Hypermucoviscosity has also been described in serotypes other than K1 and K2, lacking *magA* and *rmpA* genes, such as the K122 serotype (Cubero *et al.* 2016). On the other hand,

hypermucoviscosity-negative K1 serotype has been also identified (Lin *et al.* 2012). In this concern, it has been shown that histone-like nucleoid-structuring protein (H-NS) is a crucial regulator of both type 3 pili and capsular polysaccharide of *K. pneumoniae*, where capsule expression was dereppressed in the absence of H-NS, leading a hypermucoviscous phenotype (Ares *et al.* 2016).

Thus, in order to investigate possible genomic features conferring hypervirulent phenotype to KPHU468, comparative analyses of this strain with other hypervirulent (NTUH-K2044, Kp52.145, hvKP1, Kp U25 and Kp04C62; accession numbers AP006725, FO834906, AOIZ01000000, CP012043 and MIFX01000000, respectively) and non-hypervirulent (MGH78578, Kp13, 1084 and HS11286; accession numbers CP000647, CP003999, CP003785 and NC˙016845, respectively) *K. pneumoniae* strains have been further carried out. At a first step, virulence genes were compared, using the *K. pneumoniae* database (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). It was observed that *mrkA17*, *mrkB22*, *mrkD28*, *mrkH10*, *mrkI7* and *ybtQ5* alleles were unique to KPHU468, whereas *mrkJ6*, *ybtE4*, *ybtP4*, *ybtS4*, *ybtT4* and *ybtT42* alleles present in KPHU468 were also found in the hypervirulent Kp U25 strain, but in none of the non-hypervirulent strains. In this regard, it is possible to infer the association of these allele variants with the development of the hypermucoviscous/hypervirulent phenotype.

Additionally, phylogenetic analysis of *wzi* alleles was conducted in Geneious R10 (Biomatters Ltd, Auckland, New Zealand). Allele sequences were submitted to multiple alignment using ClustalW, and a phylogenetic tree was built using RAxML with bootstrap algorithm. Interestingly, *wzi2*-K2 and *wzi19*-K19 were grouped in the same branch, with 98.987% of identity (Fig. 2), evidencing a high similarity between these two capsular types.

At last, the strains were submitted to single nucleotide polymorphisms (SNPs) comparison. Short read sequences from KPHU468 were trimmed, using FASTX (http://hannonlab.cshl.edu/fastx_toolkit/) with Phred 20, and aligned against the reference sequences, using Bowtie 2 (Langmead and Salzberg 2012). Finally, SNPs variants were identified with Geneious R10 (Biomatters Ltd). KPHU468 presented an SNP ratio of 0.6% when compared with NTUH-K2044, Kp52.145, Kp U25, MGH78578 and HS11286, and 0.7% when compared with 1084 and Kp13 (Table 1). So, it was not detected significant difference between SNP ratio when compared with hypervirulent and non-virulent strains.

In summary, interplay between resistance and virulence seems to be an emergent threat, representing a serious challenge to global public health (Hennequin and Robin 2016). So, the release of this draft genome sequence can provide valuable information to better understand the evolution of hypermucoviscous/hypervirulent and MDR lineages of *K. pneumoniae*.

This whole genome shotgun project has been deposited in DDBJ/ENA/GenBank under the accession no. NBYZ00000000. The version described in this paper is the first version, NBYZ01000000.

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Conflict of Interest. None declared.

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