Genomic insights into the diversity, virulence and resistance of *Klebsiella pneumoniae* extensively drug resistant clinical isolates

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**Abstract**

*Klebsiella pneumoniae* has been implicated in wide-ranging nosocomial outbreaks, causing severe infections without effective treatments due to antibiotic resistance. Here, we performed genome sequencing of 70 extensively drug resistant clinical isolates, collected from Brazil’s hospitals (Brazil) between 2010 and 2014. The majority of strains (60 out of 70) belonged to a single clonal complex (CC), CC258, which has become distributed worldwide in the last two decades. Of these CC258 strains, 44 strains were classified as sequence type 11 (ST11) and fell into two distinct clades, but no ST258 strains were found. These 70 strains had a pan-genome size of 10,366 genes, with a core-genome size of ~4476 genes found in 95% of isolates. Analysis of sequences revealed diverse mechanisms of resistance, including production of multidrug efflux pumps, enzymes with the same target function but with reduced or no affinity to the drug, and proteins that protected the drug target or inactivated the drug. β-Lactamase production provided the most notable mechanism associated with *K. pneumoniae*. Each strain presented two or three different β-lactamase enzymes, including class A (SHV, CTX-M and KPC), class B and class C AmpC enzymes, although no class D β-lactamase was identified. Strains carrying the NDM enzyme involved three different ST types, suggesting that there was no common genetic origin.

**DATA SUMMARY**

Genomic data generated in this study are publicly available and can be accessed at the EMBL-EBI European Nucleotide Archive: accession number ERP010411.

**INTRODUCTION**

*Klebsiella pneumoniae* is a Gram-negative bacterium that has been implicated in extensive outbreaks of nosocomial infections, ranging from pneumonia to urinary tract infections, meningitis, sepsis and abscesses [1–4]. *K. pneumoniae* is considered by the World Health Organization as a ‘priority pathogen’ due to its high levels of antimicrobial resistance [5], including the increasingly common emergence of extensively drug resistant (XDR) strains with very few therapeutic options. These XDR *K. pneumoniae* strains (i.e. those that are susceptible to at most only two antimicrobial classes) have been reported globally, especially in hospital settings [6–9]. Overall, many antibiotic-resistance mechanisms can be identified in *K. pneumoniae*, with resistance to β-lactams having the greatest impact on treatment. Strains demonstrate intrinsic resistance to β-lactams mediated through the SHV β-lactamase encoded on the chromosome. In addition, plasmid-mediated β-lactamases, such TEM-1 and AmpC, as well as extended-spectrum β-lactamases (ESBLs) further contribute to its resistance repertoire [10]. Due to a lack of effective antimicrobial agents and underlying comorbidities of patients, ESBL-producing and carbapenem-resistant...
K. pneumoniae infections often reach mortality rates ranging from 23 to 75%, representing far higher rates of morbidity and mortality than infections with non-resistant bacteria [11, 12].

Over the last two decades, a group of hypervirulent K. pneumoniae isolates with hypermucoviscosity has emerged to cause highly invasive infections. These hypervirulent K. pneumoniae strains are capable of infecting healthy individuals, causing pyogenic liver abscess, bacteraemia, lung, neck and kidney abscesses, pneumonia, cellulitis, necrotizing fasciitis, myositis, meningitis, and endophthalmitis [5]. Recent evolutionary genomic analyses of >2200 K. pneumoniae genomes from around the world indicated that multidrug resistant (MDR) lineages are highly diverse, with high levels of chromosomal recombination, distinct surface polysaccharide loci and a variety of plasmids. In contrast, hypervirulent clones exhibit rare chromosomal recombination and limited plasmid diversity, indicating reduced potential for horizontal gene transfer [13]. This suggests that MDR lineages, and particularly XDR strains, pose a huge threat since they have greater potential to acquire virulence genes than the hypervirulent clones have to acquire antibiotic resistance.

Here, we report the genome sequences of 70 XDR K. pneumoniae isolates collected from Brasilia’s hospitals (Brazil) between 2010 and 2014 to characterize their resistance mechanisms and genomic diversity. We performed pan-genome analysis and compared these Brazilian isolates with isolates from around the world to identify their phylogenetic lineages. We found that our collection is largely predominated by clonal complex 258 (CC258) and sequence types (STs) ST11 and ST340.

**METHODS**

**K. pneumoniae strains**

Ninety-five K. pneumoniae clinical isolates resistant to carbapenem were retrieved from 12 hospitals from Brasilia, Brazil, during the period of 2010 to 2014, and stored at Brasilia’s Laboratório Central (LACEN-DF) at −80 °C (Table S1, available with the online version of this article). Species identification and antibiotic-resistance profiling were determined using the vitek 2 system (bioMérieux); sequencing subsequently identified one isolate as Klebsiella quasipneumoniae.

**Growth conditions and DNA isolation**

*K. pneumoniae* isolates were streaked onto MacConkey agar plates (Sigma-Aldrich), and sub-cultured into liquid Luria-Bertani (LB) medium (Sigma-Aldrich) for overnight growth at 37°C. The DNA was isolated using the cationic detergent cetyltrimethylammonium bromide (CTAB) protocol, as described by Petersen and Scheie [14], with the minor modifications specified below. Bacterial cells from 2 ml of overnight culture were collected by centrifugation, and resuspended in 300 µl 2× CTAB lysis buffer (100 mM Tris-HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA, 2% CTAB and 0.2% β-mercaptoethanol). The tube containing the suspension was then placed in a 65°C water bath for 30 min to lyse the cells. Following incubation, one volume of chloroform:isoamylalcohol (24:1) was added to each sample. The samples were centrifuged and the aqueous phase was transferred to a new centrifuge tube. The genomic DNA in the aqueous phase was precipitated with ~400 µl cold isopropanol overnight, washed with 1 ml 70% ethanol, and then resuspended in 100 µl Milli-Q water (Millipore). DNA concentration was assessed by fluorimetry using a Qubit system (Thermo-Fisher Scientific). DNA purity was assessed using a Thermo-Fisher Scientific NanoDrop 2000c spectrophotometer.

**Genome sequencing and assembly**

Genome sequencing was performed according to Holt et al. [15]. Purified genomic DNA was sequenced via Illumina HiSeq 2000 at the Wellcome Trust Sanger Institute. Index-tagged paired-end Illumina sequencing libraries were prepared, combined into pools of uniquely tagged libraries and sequenced on the Illumina HiSeq 2000 to generate tagged 125 bp paired-end reads. Only isolates (n=79) with sequencing depth greater than 40× coverage were de novo assembled (Table S2). Sequencing quality of raw Illumina sequencing reads was assessed using FastQC v0.11.8 (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/), followed by trimming with trimmomatic v0.39 [16] using the default parameters to remove Illumina adapters, low-quality bases and any reads less than 36 bp. Trimmed paired-end reads that overlapped (due to the original DNA fragments being shorter than twice the length of reads) were merged to generate longer single-end reads using FLASH v1.2.11 [17]. The remaining paired-end trimmed reads and the merged reads were subsequently used in de novo assembly with SPAdes v3.13.2 [18], followed by removal of contigs less than 200bp with SeqKit v0.11.0 [19]. Genome assemblies were further assessed using QUAST v5.0.2 [20]. However, a subset of isolates with assembled genomes (n=5) did not include any associated sample.
metadata information (such as LACEN ID, sampling time and sample isolation sites) and were removed for all subsequent analyses. Isolates excluded from this analysis and the rationale for exclusion have been listed in Table S3. Raw sequencing reads have been deposited in the European Nucleotide Archive under accession number ERP010411.

**Genome annotation**

All assembled genomes were annotated with the prokaryotic genome annotation pipeline, Prokka, v1.12 [21].

**Identification and classification of multilocus sequence type (MLST), virulence genes and resistance genes**

To identify MLST, virulence genes and resistance genes from each isolate, we took two different approaches: (a) using raw sequencing reads with snippy v4.6.0 (https://github.com/tseemann/snippy), a rapid molecular typing tool; and (b) analysing assembled genomes with Kleborate v0.4.0-beta (https://github.com/katholt/Kleborate), a tool for screening Klebsiella genome assemblies for important features [23–25]. The MLST marker genes for analysis were retrieved from the Klebsiella Pasteur MLST sequence definition database (https://bigdb.pasteur.fr/klebsiella/). The rationale behind using multiple methods was to generate a robust set of MLSTs, virulence genes and resistance genes, especially given that the quality of genome assemblies would impact on allele identification, given the varied sequencing depth of a number of the isolates. The K and O locus types were subsequently detected from assemblies using Kleborate v0.4.0-beta [26].

To understand the genomic context of the carbapenemase (bla\_(KPC)\_4401) gene and its associated replicative transposon Tn4401, we used TETyper [27] to track sequence variation within Tn4401 to infer transposition events and potential \( \text{bla}_{\text{KPC}} \) transmission. We used the Tn4401b sequence from pKPC\_UVA01 (CP017937) as our reference sequence, and provided both assembled genomes as well as raw FASTQ reads to identify transposon structural variation, as well as the genomic context of Tn4401b.

**Pan-genome and phylogenetic analyses**

To perform pan-genome and gene presence–absence analyses, we further excluded two assemblies with >800 contigs (2714082 and 3919904) as contig breaks can impact gene presence–absence. An additional assembly (4213265) was removed due to poor species match in Kleborate and a larger than expected genome size (10.7 Mb). Thus, we performed pan-genome analyses for a final 70 assembled and annotated isolates with associated metadata (i.e. sample isolation sites, year or antibiotic-resistance profiles) using the rapid large-scale prokaryote pan-genome analysis pipeline Roary [28]. We also included 20 randomly selected reference genomes from diverse clonal groups of MDR, hypervirulent and unassembled lineages (Table S4) [13] to understand the distribution of these strains within the global context. Using GFF3 files from our Prokka-annotated de novo assemblies, and the National Center for Biotechnology Information (NCBI) reference genomes, a core-genome alignment was generated using Roary. Core-genome SNPs were extracted from this core-genome alignment using the SNP Sites program (https://github.com/sanger-pathogens/snp_sites) [29], and used as input to generate a maximum-likelihood tree with RAxML [30]. To create the best-scoring tree with bootstrapping support, we used the GTRCAT model with the –f a and the –N autoMRE options in RAxML. A gene presence–absence matrix was mapped onto the phylogenetic tree using the roary_plot function. The phylogenetic tree with associated \( K \), \( O \) and antimicrobial-resistance genes was visualized using ggtree (https://bioconductor.org/packages/devel/bioc/vignettes/ggtree/inst/doc/ggtree.html).

**Pan-genome and phylogenetic analyses of ST11 isolates**

Given that the majority of our isolates belonged to ST11, we performed additional analyses on ST11 isolates by comparing Brasilia ST11 isolates to all publicly available ST11 reference-quality genomes. We downloaded 677 reference-quality \( K.\_pneumoniae \) genomes from the National Center for Biotechnology Information (NCBI) (Table S5). To identify reference-quality isolates that belonged to the ST11 group rapidly, we used mlst v2.19.0 (https://github.com/tseemann/mlst) and data from PubMedST [31] to identify 128 ST11 reference genomes (Table S5). The associated country of isolation (‘geo_loc_name’) was pulled from each associated BioProject using Entrez-direct v13.9 [32] as this geographical data was more complete than latitude and longitude (‘lat_lon’) data. Pan-genome analyses of Prokka-annotated Brasilia ST11 and reference ST11 genomes were performed using Roary as described above. Core-genome SNPs were extracted as above, followed by creating a best-scoring maximum-likelihood tree with RAxML and visualization using ggtree.

**SNP differences between the ST15 isolate and the PMK1 reference**

Multi-FASTA alignment of the core-genome SNPs between the ST15 isolate (6175670) and PMK1 was extracted from the above and visualized in MView [33]. Direct SNP calling via snippy v4.6.0 (https://github.com/tseemann/snippy), using PMK1 as the reference genome, was also performed.

**RESULTS**

**Genome sequencing and assembly of \( K.\_pneumoniae \) clinical isolates**

Genome sequencing of 95 clinical isolates was performed. One isolate (5121467) was classified as \( K.\_quasipneumoniae \) subsp. \( quasipneumoniae \) (Table S6), and thirteen isolates had insufficient sequencing depth (less than 40×) for de novo assemblies. Additional isolates with no associated metadata or with poor assemblies were removed from subsequent comparative genomics analyses (see Methods). The final collection of 70 assembled genomes (Table S2) had a median of 71 contigs (minimum of 43 contigs and maximum of 238 contigs) and a median N50 (i.e. the shortest contig length that
covers 50% of the genome) for contig lengths of ~270 746 bp (Table S7). The median genome size was ~5.68 Mbp, with a range of 5.28 – 6.41 Mbp. These strains were isolated from twelve hospitals, and predominately came from urine (15) or urinary catheters (12), blood (14), pulmonary fluids (14) and a variety of other sites (15) (Table S1, Fig. S1a).

**K. pneumoniae** diversity and pan-genome analysis

Genomic studies on global *K. pneumoniae* populations have illustrated their diversity, with hundreds of phylogenetic lineages or ‘clones’ [13, 15]. To gain further insights into these regional Brazilian isolates, we performed pan-genome analyses of these isolates together with 20 representative reference *K. pneumoniae* strains from diverse clonal groups to identify all genes found within this collection of strains (i.e. the pan-genome) [13] (Table S4). Specifically, we investigated the core-genome that contains genes shared by all strains, and the accessory genome that encodes genes found only in subsets of strains. Each genome assembly was annotated using Prokka, followed by Roary, to cluster orthologous coding sequences to create a matrix of gene presence or absence across the entire collection together with the 20 representative references genomes (Fig. 1). When we only considered the 70 Brasília isolates, we identified a core-genome of 4476 genes that were present in ≥95% of isolates, with a large pan-genome of 10 366 genes (total number) (Table S8). When placed within the context of the larger *K. pneumoniae* species complex, by including the 20 reference genomes, we identified 4173 core genes present (~84% of the total number of genes in each strain) in >95% of strains. Furthermore, this identified an additional 14 316 genes present in the accessory genome (Table S8, Fig. S2). Core-genome SNPs were subsequently extracted from the Roary core-genome alignment and used to reconstruct a maximum-likelihood tree (Fig. 1) to reveal evolutionary relationships between strains. Overall, isolates from different hospitals and different time periods often grouped together in the phylogeny, and showed similar gene presence–absence ‘blocks’ (Figs 1b and S3). Thus, while...
Closely related STs with single locus variation are further strains cannot cause disease in murine infection models, ST437 was identified (Table S6). The core-genome analyses K synthesis loci (K loci) and molecular distinct K-types. In addition, nucleotide sequences of capsule (K-loci) are defined by serology, with 77 immunologically and phagocytosis. Traditionally, the inflammatory response, and reducing complement-mediated killing plays key defensive roles in suppressing the host inflammatory response when compared to capsulated strains [35]. The capsule also is a key virulence factor, since isogenic non-capsulated strains from China (Fig. S4). This clade is distinct from the Brasilia groupings, with the largest clade including most of the isolates distribution of all ST11 strains from around the world. We included in Fig. 1, we included an additional 128 ST11 from around the world. In addition to the reference sequences standing their phylogenetic relationships with ST11 isolates belonged to the ST11 group, we were interested in understanding their phylogenetic relationships with ST11 isolates from around the world. In addition to the reference sequences included in Fig. 1, we included an additional 128 ST11 reference-quality genomes in order to reconstruct a detailed core-genome phylogeny to visualize the geographical distribution of all ST11 strains from around the world. We observed that global ST11 isolates fell largely into many groupings, with the largest clade including most of the isolates from China (Fig. S4). This clade is distinct from the Brasilia ST11 isolates, which were further separated into two distinct groups: ST11.A and ST11.B (Figs 1a and S4).

**Brasilia ST11 isolates in relation to the global distribution of ST11**

Given that the majority of strains within our collection belonged to the ST11 group, we were interested in understanding their phylogenetic relationships with ST11 isolates from around the world. In addition to the reference sequences included in Fig. 1, we included an additional 128 ST11 reference-quality genomes in order to reconstruct a detailed core-genome phylogeny to visualize the geographical distribution of all ST11 strains from around the world. We observed that global ST11 isolates fell largely into many groupings, with the largest clade including most of the isolates from China (Fig. S4). This clade is distinct from the Brasilia ST11 isolates, which were further separated into two distinct groups: ST11.A and ST11.B (Figs 1a and S4).

**K. pneumoniae virulence**

There are four well-characterized *K. pneumoniae* virulence factors: capsule, lipopolysaccharide (LPS), fimbriae (type 1 and type 3) and siderophores [5]. The polysaccharide capsule is a key virulence factor, since isogenic non-capsulated strains cannot cause disease in murine infection models, when compared to capsulated strains [35]. The capsule also plays key defensive roles in suppressing the host inflammatory response, and reducing complement-mediated killing and phagocytosis. Traditionally, *Klebsiella* capsule types (K-types) are defined by serology, with 77 immunologically distinct K-types. In addition, nucleotide sequences of capsule synthesis loci (K loci) and molecular K-typing schemes have been used to provide finer resolution and discrimination than serological classification. Recent genomics studies [23] have further improved on these methods by establishing a curated K locus reference database with clear nomenclature and an efficient bioinformatics tool, Kaptive, to enable analysis [23, 26].

We used Kaptive to identify K loci from our whole-genome sequences (Fig. 2). The predominant K loci included KL64 (37), KL15 (10) and KL27 (8). In addition, we also identified KL55 (5), KL151 (5), and one each of KL2, KL7, KL24, KL36 and KL154. The other surface antigen of interest in *Klebsiella* is LPS, which includes lipid A, the core oligosaccharide and the O-antigenic polysaccharide. There are currently 12 distinct O loci identified, with both the O1 and O2 antigens the most common. In our collection, we identified a total of four different O loci including O2v1 (37 isolates), O2v2 (9), O4 (17), OL103 (5), and 1 each of O1v1 and O1v2 (Fig. 2). Surprisingly, while O2 was predominant, the O1 locus was only found in 2/70 strains.

Known *Klebsiella* virulence factors include the polyketide synthesis loci *ybt* and *clb*, which encode the iron-scavenging siderophore yersiniabactin and genotoxic colibactin, respectively. These two loci are located on the mobile genetic element ICE*Kp* [24]. Yersiniabactin plays a critical role in *Klebsiella* virulence since its iron-scavenging ability is not inhibited by human lipocalin-2, enabling it to promote bacterial growth and dissemination, while avoiding activating the host inflammatory response. Yersiniabactin is found in approximately a third of clinical isolates and is associated with strains isolated from bacteraemia and tissue-invasive infections. ICE*Kp* can carry additional genes, including those involved in the synthesis of the siderophore salmonchelin (*iro*) and colibactin (*clb*). Given the emergent threat of MDR and XDR isolates becoming more virulent through the acquisition of virulence genes via acquisition of mobile genetic elements, we investigated the presence of virulence genes and the associated ICE*Kp* lineage in our collection. To look for the above virulence genes known to contribute to hypervirulence in *K. pneumoniae*, we used Kleborate, which assigns virulence STs (YbSTs for yersiniabactins and CbSTs for colibactins) and associated lineages using the locus-specific schemes of BIGSdb (https://bigd.db.pasteur.fr/klebsiella/klebsiella.html) [24]. By these criteria, only five of our clinical isolates carried ICE*Kp*, all of which were variants of ICE*Kp*3 with the yersiniabactin lineage *ybtB*, indicating potential increases in virulence in these isolates (Fig. 3). None of the isolates from this collection carried other virulence factors, such as colibactin, aerobactin (*iuc*), salmonchelin (*iro*) or the *rmpA1/rmpA2* genes associated with hypermucoidy. Interestingly, the five isolates that carried ICE*Kp*3 were sampled from five different hospitals between 2013 and 2014, and isolated from either blood or pulmonary sites.

**Antibiotic-resistance profile**

Given our interest in understanding the XDR profiles of these isolates, we used the Vitek platform to characterize
their antibiotic resistance (Fig. 4, Table S9). Almost all strains were resistant to β-lactam antibiotics (including penicillins, cephalosporins and carbapenems), quinolones, co-trimoxazole (a combination of trimethoprim and sulfamethoxazole), at least one aminoglycoside (although 57/70 showed susceptibility to at least one aminoglycoside) and nitrofurantoin. In contrast, 40 of the 70 clinical isolates were susceptible to the glycyclcline, tigecycline...
Overall, the Brazilian clinical isolates demonstrated resistance to between 17 and 23 of the 24 tested antibiotics, and 8 to 11 of the 11 different tested antibiotic classes.

To understand the genetic basis for the high antimicrobial resistance observed in these isolates, we used the Kleborate program to identify both acquired resistance genes and chromosomal mutations known to be associated with drug resistance. Kleborate uses ARG-ANNOT [36] to classify antimicrobial-resistance genes, with the β-lactamases further delineated into Lahey classes. Kleborate assigns resistance scores from 0 to 3, with 0 as the least resistant (no ESBLs and no carbapenemases) and 3 being the most resistant (presence of carbapenemase with colistin resistance). Our collection contained highly resistant isolates where all except for one isolate expressed a carbapenemase.

Fig. 3. Presence of the yersiniabactin- and colibactin-encoding mobile element ICEKp in a subset of Brasília K. pneumoniae isolates. The yersiniabactin STs (YbSTs) and colibactin STs (CbSTs) were identified by Kleborate. Core-genome SNP phylogeny of these isolates is shown on the left, with the tips of the tree labelled with the year of isolation (indicated by shapes) and hospital (indicated by colours). The majority of Brasilia K. pneumoniae isolates do not have virulence genes.

(see footnote in Table S9).
Fig. 4. Heat map of the antibiotic-sensitivity profile of Brasilia K. pneumoniae clinical isolates as assessed by Vitek 2. Resistance (red) or sensitivity (blue) against a particular antibiotic is defined using the European Committee on Antimicrobial Susceptibility Testing (EUCAST) clinical breakpoint at the time of isolation. Missing antibiotic information is shown in white. Antibiotics tested included antibiotics from the following classes (indicated by Roman numerals). (I) Penicillins, which included ampicillin/sulbactam, ampicillin, aztreonam, piperacillin/tazobactam and piperacillin. (II) Carbapenems, which included ertapenem, imipenem and meropenem. (III) Second-generation cephalosporins, which included cefalotin, cefuroxime and cefotetan. (IV) Third-generation cephalosporins, which included cefotaxime, ceftazidime and ceftriaxone. (V) Fourth-generation cephalosporins, which included cefepime. (VI) Quinolones, which included levofloxacin and ciprofloxacin. (VII) Aminoglycosides, which included amikacin, gentamicin, tobramycin. The complete profile with MIC values is available in Table S7.
and tobramycin (MIC ≥8 µg ml\(^{-1}\); 55/70) was frequently
Kp
Interestingly, the isolate (4022578) with the complete loss of
K. pneumoniae [42]. Point mutations in the binding domains termed
the quinolone-resistance-determining regions (QRDRs) influence resistance. Here, we observed mutations in two
topoisomerases, GyrA-831 and ParC801, in most (69/70)
isolates. In addition, we observed the plasmid-encoded qnr
genes (primarily qnrS1) in 47/70 strains.

**β-Lactam resistance**

The overuse and misuse of antibiotics has led to the rapid
evolution of antibiotic-resistant K. pneumoniae strains. Overall, our XDR clinical isolates were nearly universally
resistant to all tested classes of β-lactam antibiotics,
including penicillins, carbapenems, and second-, third-
and fourth-generation cephalosporins. There was substantial
diversity in resistance mechanisms observed, but each
strain presented two to nine different β-lactamase enzymes
(Table S6, Fig. S5). The most widespread enzyme expressed
was the broad spectrum β-lactamase SHV-11 (present in
67/70 isolates). Similarly, the majority of isolates expressed
a class A β-lactamase KPC-2 (61/70) or a class B metallo-
-ß-lactamase NDM-1 (7/70), while the class A ESBL GES-5
was found in one isolate. To understand the genomic context of
bla\(_{KPC2}\) and its associated replicative transposon
Tn4401, we used TETyper and identified seven instances of
truncations including ST11 strains 1625271, 4577657
and 6292852 (representing both ST11.A and ST11.B),
and strains from four other ST groups including ST15
(4879999), ST25 (1720144), ST37 (4455550) and ST340
(3919904) [27].

NDM was first described in New Delhi, India, in 2009
[43], while the first reports of a NDM in Brazil were in
2013, in Rio Grande do Sul [44, 45], Rio de Janeiro and
Brasilia [46]. The NDM-producing strains identified in this
study were in fact collected from 2013 to 2014, consistent
with the previous reports. Of the seven NDM-producing
strains (Table S6, Fig. S5), five, from four different hospitals,
were all of the ST37 isolates identified and these formed a
distinct phylogenetic group (Fig. 1b), suggesting they were
highly related. The other two came from ST340, part of
CC258, and a quite distinct and unique (in our collection)
strain of ST15 (6175670). This isolate is ~600 core-genome
SNPs and a total of 1359 SNPs different from the NDM-1-
producing reference strain PMK1, the 2011 ‘source’ isolate
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SNPs and a total of 1359 SNPs different from the NDM-1-
producing reference strain PMK1, the 2011 ‘source’ isolate
from a Nepal hospital [47]. Consistent with our findings,
NDM-producing strains from CC258 are rare, with a few
case reports from Oman [48], New Zealand [49], England,
Sweden and India [50].

Class A β-lactamas were also common, being found in
61/70 strains, with up to five enzymes found in two isolates.
These include, in order of observation, LAP-2 (40 isolates)
Frequently observed, in our collection (in conjunction with
Oxa-2 (33), TEM1-D (19), and lower incidences of Oxa-9 (6), Oxa-1 (4), SHV-187 [3]
and SHV-28 (1). ESBLs were identified in more than half
(40/70 isolates), and included CTX-M–2 (18),–15 (15), –8
(5) and –27 (3).
DISCUSSION

MDR strains of the Enterobacteriaceae are isolates that are non-susceptible to at least one agent in ≥3 antimicrobial categories as defined by Margiorakos et al. [51] (see table 3 in their paper), while XDR isolates are non-susceptible to at least one agent in all but ≤2 categories. In particular, MDR K. pneumoniae, due to the spread of high-risk clones and widespread resistance plasmids, has become a major bacterial pathogen that causes hospital outbreaks worldwide with high morbidity and mortality [52, 53]. Some of these MDR isolates have evolved to become XDR isolates that have few therapeutic options [54]. By the above criteria, all
<table>
<thead>
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<th>Strain*</th>
<th>ST</th>
<th>Year</th>
<th>Source</th>
<th>Hospital</th>
<th>K locus</th>
<th>O locus</th>
<th>No. of resistance genes/classes</th>
<th>AGM†</th>
<th>FQ‡</th>
<th>Sulf§</th>
<th>Te‡†</th>
<th>Trim-Dfr‡¶</th>
<th>Omp mutants*</th>
<th>β-Lactamase**</th>
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<td>O2v1</td>
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<td>L, I, II</td>
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<td>Al</td>
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<td>1, 2, 3</td>
<td>L, I, II</td>
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<td>L2, O2</td>
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<td>1, 2, 3</td>
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*Bold text indicates that these strains encode yersiniabactin on the ybt9 of the integrative and conjugative element ICEKp3.
†AGMs code 1, AphA6; 2, any Aac; 3, any Aad; 4, other Aph ignored StrAB SatA Rmt.
‡FQ, fluoroquinolone-resistance mutations code 1, GyrA-83I; 2, ParC-80I; 3, Qnr-51; 4, other GyrA; 5, other Qnr.
§Sulfonamide-resistance I, SulI; II, SulII.
||Tetracycline-resistance efflux: A, TetA; D, TetD.
¶Trimethoprim.
#Porin mutations that might affect antimicrobial uptake and susceptibility.
**L2, LAP-2; O2, Oxa-2; T1D, TEM-1D^; O+, OXA-2 or −9; S, SHV-28 or −187.
††For each potential resistance gene identified by Kleborate, this indicates inexact nucleotide and inexact amino acid match; with ^ indicating inexact nucleotide but exact amino acid match; ? for incomplete match.
of the isolates sequenced here are XDR, and arose from a single city during a 4 year period, enabling a detailed study of relationships between \textit{K. pneumoniae} isolates that are highly recalcitrant to therapy. The isolates investigated here were resistant to 17–23/24 tested antibiotics, and 8–11/11 different tested antibiotic classes. Generally speaking, the most effective antibiotics were amikacin, gentamicin and/or tigecycline, but resistance to these antibiotics [based on European Committee on Antimicrobial Susceptibility Testing (EUCAST) definitions] were observed in 28.6, 41.4 and 42.8% of isolates, respectively. This provides a very threatening picture given the continuous increase in resistance trends observed worldwide and evidence that some of these isolates form very closely related clusters suggesting clonal outbreaks.

Interestingly, there has been a worldwide spread of MDR ST258 \textit{K. pneumoniae} that are limiting therapeutic options [52]. However, amongst our XDR isolates from Brasília’s hospitals, ST258 was absent and the major representative ST from CC258 was ST11. Nevertheless, with the continuous antibiotic-resistance development by bacteria, and the fact that there are strains already resistant to one or two last-choice antibiotics, it seems likely that no single therapeutic options will remain possible for all \textit{K. pneumoniae} infections [52].

In these Brazilian hospital isolates, there were three STs, all from CC258, that made up the majority (87%; 61/70) of strains. This included a single ST437 isolate, a cluster of 16 ST340 strains and a large number of ST11 isolates that appeared to form two sequence subgroups (termed ST11.A, comprising 36 strains, and ST11.B, comprising 8 isolates; Fig. 1a). The next largest class was the outgroup ST37, with five isolates, in addition to four other single isolates from ST15, ST25, ST199 and ST2655. These groups were more distantly related to the CC2258 strains, but showed similarity with some reference strains. Thus, the 2014 ST15 isolate 6175670 was closely related to the 2011 Nepalese reference strain PMK1 (with a total of 1359 SNPs different between the two strains), but despite these strains bearing the carbapenemase NDM-1, they were quite distinct from the NDM-1-expressing ST37 cluster of five isolates. Similarly, the 2011 ST25 isolate 1720144 aligned closely with the most antibiotic-resistant hypervirulent reference strain INFO14 [13], presenting the worrying picture that hypervirulent isolates might be evolving to become untreatable.

One cluster of interest included ST340 and ST37 strains. ST340 strains were typified by KL15 (10 isolates), KL151 (5) or KL64 (1) capsule types, O4 LPS type, 1–4 AGMs (aminoglycoside modifying genes), common appearance of GyrA-83I and ParC-80I topoisomerase (fluoroquinolone-resistance) mutations, SulI (14/16 isolates), DfrA12 (14/16), few porin mutations (2/16), TEM-1D (10/16), and CTX-M ESBLs of various classes (15/16, most commonly CTX-M-15), as well as KPC-2 carbapenemase. In contrast, ST37 strains were capsule type KL55, O-type OL103, carried the three AGMs Aac3-IIIΔ, AadA2Δ, AadA5, two fluoroquinolone-resistance loci, GyrA-83I, ParC-80I, all had DfrA12 plus other Dfr genes, lacked porin mutations and \(\beta\)-lactamases except carbapenemase NDM-1 and ESBL CTX-M-27 (3/5 strains). These results again are suggestive of highly related strains circulating in multiple hospitals in Brasília (each found in four hospitals), although the first isolation of ST37 strain was in 2013, suggesting this might be a more recently introduced lineage.

A key finding here was the existence of two very distinct clades of Brasília ST11 isolates as mentioned above. Further analysis showed these had quite distinct surface and antibiotic-resistance properties (Table 1). Thus, the 36 ST11.A isolates were all capsule type KL64 (39% with missing genes) and LPS O-antigen type O2v1 (64% with missing genes), while ST11.B isolates were KL27 and O2v2 (with only one missing O-antigen gene). Resistance also varied in a subtype-specific fashion. Thus, ST11.A generally had only the AGM AphA6 (25/36 isolates), with two having two additional AGMs. ST11.B lacked this enzyme and instead had between one and three of the enzymes Aac3-IIIa, AadA2 and AphA3-IIIa. Similarly with regards to fluoroquinolone resistance, while ST11.A strains had the mutations GyrA-83I, ParC-80I and Qnr-S1, ST11.B strains carried the former two mutations as well as GyrA-87G. Similar patterns were observed for other resistance genes: (i) ST11.A strains had 2–3 sulphonamide-resistance genes (sulI, II) with ST11.B strains only having sulI; (ii) 34/36 ST11.A strains carried the tetracycline efflux genes tetAD (one had only tetD and another none), while none of the ST11.B strains had either gene; (iii) 34/36 ST11.A strains had the trimethoprim dihydrofolate reductase gene dfrA1, while 6/8 ST11.B strains carried dfrA12; (iv) all ST11.A strains carried mutations in porins OmpK35 and 35/36 carried OmpK36 mutations, while only 2/8 ST11.B strains carried either; (v) with regards \(\beta\)-lactamases, ST11.A strains carried Lap-2 and 25/36 had Oxa-2; 15/36 had the ESBL CTX-M-2, while 6/8 ST11.B strains carried Oxa-2 and/or TEM-1D, 2/8 carried CTX-M-2, while both ST11.A and ST11.B carried KPC-2 and SHV-11. Thus, it is clear that these are two related but separate lineages. Intriguingly, both were first identified in 2010, and were identified annually until the last sampling period in 2014 in eight (ST11.A) and five hospitals (ST11.B) from multiple types of clinical samples, with both isolates isolated from four common hospitals. In particular, the predominance of KL64 ST11.A strains is of particular interest having been found as early as 2010, since in China this isolate arose much later (2016) [2]. It was curious that clone ST258, found in many Brazilian states, had never been identified in the large city of Brasília (from which these isolates came), despite extensive surveillance since 2010 [55, 56]. Nevertheless, the presence and persistence of a clone of ST11 in the capital, for at least a decade, provides considerable concern since this clone frequently presents as polymyxin resistant, removing one of the last resources against \textit{Enterobacteriaceae} resistant to carbapenems [57].

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Author contributions
W. F. P., D. J. P., R. E. W. H. and O. L. F. conceived the study. W. F. P., C. D. F., S. C. D. and S. A. A. collected the strains and associated metadata, extracted the genomic DNA, as well as performing Vitek 2 antibiotic-susceptibility testing. D. J. P. coordinated the genomic sequencing. A. H. Y. L. performed all analyses and generated the final figures. A. H. Y. L., W. F. P., D. J. P., R. E. W. H. and O. L. F. contributed to the conception and writing of the manuscript. All authors contributed to the article and approved the submitted version.

Conflicts of interest
The authors declare that there are no conflicts of interest.

References


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