Silent Circulation of BKC-1-producing *Klebsiella pneumoniae* ST442: Molecular and Clinical Characterisation of an Early and Unreported Outbreak

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Running Title: Early Outbreak of BKC-1-producing *K. pneumoniae*

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Abstract

Objective: This study aimed to describe the undetected circulation of an epidemic *Klebsiella pneumoniae* ST442 clone, producer of BKC-1, occasioning the first reported outbreak of the infrequent carbapenemase BKC-1.

Methods: Six hundred forty-seven *K. pneumoniae* isolates (2008-2017) with reduced susceptibility to carbapenems were screened for $bla_{\text{BKC-1.}}$ BKC-1 positive isolates were typed using PFGE and MLST. Susceptibility profiles were determined by broth microdilution and additional antimicrobial resistance genes (ARG) investigated by PCR. Some isolates were submitted to the full-genomic characterization by WGS (Illumina MiSeq and MinIon) and virulence assays as biofilm detection and *in vivo* infection by *Galleria mellonella* model evaluated.

Results: Sixteen (2.5%) *K. pneumoniae*, from 15 patients, between 2010 to 2012 were found carrying $bla_{\text{BKC-1}}$. Among these patients, all cause mortality rate was 54.5%. A major clone A1-ST442 (13/16) was isolated during the time period. The BKC-producing isolates had a multidrug resistance phenotype, remaining susceptible only to gentamicin (87.5%) and ceftazidime-avibactam (100%). The presence of two carbapenemases, $bla_{\text{BKC-1}}$ and $bla_{\text{KPC-2}}$, was detected in six isolates increasing significantly the β-lactam MICs. Additionally, other ARG were identified on A1-ST442 and B1-ST11 clones. The B1-ST11 clone was more virulent than A1-ST442 clone.

Conclusion: An undetected outbreak predominantly caused by a BKC-1 positive A1-ST442 clone between 2010-2012 was retrospectively captured ten years later in a Brazilian Hospital. The misidentification of BKC-1 might have triggered worsening events to antimicrobial resistance, which reinforces the need of correct and rapid identification of antimicrobial resistance mechanisms in our hospitals.

Key-words: infection, carbapenem resistance, carbapenemase, outbreak.

1. Introduction

Carbapenemases are the most versatile β-lactamase enzymes, representing the main mechanism responsible by carbapenem resistance, and are generally able to hydrolyse most clinical available β-lactams [1]. In South America, the carbapenemases KPC-like and NDM-like are frequently reported in *K. pneumoniae* isolates, with KPC-2 specifically endemic in Argentina, Brazil, and Colombia [2]. The endemicity of KPC-2, especially in Brazil, is attributed to the clonal expansion of clonal complex 258 (CC258), also responsible for the rapid dissemination and stability of KPC-enzymes in the world [2,3]. In the last few years, new carbapenemases have been characterized such as BKC-1 [4], FRI-1 [5], and VCC-1 [6]; however, few studies have been reported the clinical significance of infections caused by pathogens producing these enzymes [5,7-9].

Brazilian *Klebsiella* Carbapenemase 1 (BKC-1) is a serine carbapenemase with weak hydrolytic activity against carbapenems initially described in carbapenem-resistant *K. pneumoniae* isolates from 2008 in São Paulo, Brazil [4]. To date, two variants of BKC were described, BKC-1 and BKC-2. While BKC-1 has been described in few *K. pneumoniae* isolates and, more recently, in *Citrobacter freundii* , BKC-2 was detected in *Enterobacter hormaechei* subsp. *xiangfangensis* [4,7,10]. In *K. pneumoniae*, BKC-1 seems to be related to CC442 with all reports linked to ST442 or ST1781. $bla_{\text{BKC-1}}$ is plasmid-mediated and associated with the insertion sequence IS*Kpn23* and a phosphotransferase, *aph(3')-VIi* (also called *aph(3')-VIa*), which is able to confer amikacin and kanamycin resistance [4,7]. In addition, other resistance mechanisms have been reported in BKC-1 producers such as ESBL production or OmpK36 loss, increasing β-lactam resistance, or chromosomal mutations occasioning polymyxin resistance [4,7].

The aim of this study was to assess the frequency of BKC-producing *K.*

pneumoniae from clinical isolates cultured from a teaching hospital in São Paulo, Brazil over a 10-year period (2008-2017). In addition, the microbiological, genomic characterization and the *in vivo* virulence were assessed for BKC-positive isolates .

2. Material and Methods

2.1 BKC-1-producing Isolates Screening and Identification

A 10-year retrospective surveillance study (January 2008 to December 2017) was conducted by testing 647 *K. pneumoniae* clinical isolates with reduced susceptibility to ertapenem, imipenem or meropenem (MICs ≥ 0.5 mg/mL), from a biological collection of a teaching hospital located in São Paulo, Brazil. The isolates were initially screened for $bla_{\text{BKC-1}}$ by PCR as previously described [4], and subsequently submitted to DNA sequencing by Sanger. All $bla_{\text{BKC-1}}$ positive *K. pneumoniae* isolates were subject to identification confirmation by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF MS) and selected for further microbiological and genetic characterization.

2.2. Clinical Data Collection

Clinical data of patients with BKC-1 positive cultures was extracted from the medical records in a standardized case form. The variables collected included patient demographic data, underlying diseases, Charlson comorbidity index, Pitt bacteremia score (when applicable), antibiotic use, ICU admission, type of ward, presence of septic shock, and outcome (mortality) data at 3 days, 14 days and 30 days.

2.3. *Genetic Relatedness and Plasmid Analysis*

The clonal relationship of BKC-1 positive isolates was initially determined by Pulsed Field Gel Electrophoresis (PFGE) using SpeI as restriction enzyme and interpreted according to Tenover and colleagues (1995) [11]. Multilocus sequence typing (MLST) was performed as previously described [12]. To verify the plasmid profile, S1 nuclease-PFGE was performed [13], followed by direct in-gel P32-radiolabelled hybridisation for location of the carbapenemase-encoding genes.

2.4. *Antimicrobial Susceptibility Testing*

In vitro antimicrobial susceptibility testing was performed to sixteen antimicrobial agents (Table 1) by broth microdilution and interpreted following EUCAST guidelines.

2.5. *Investigation of Additional Antimicrobial Resistance Determinants*

Antimicrobial resistance genes (ARG) were investigated to identify the presence of other resistance mechanisms to β-lactams, aminoglycosides, polymyxins, and quinolones as previously described [4,7,14]. All antimicrobial resistance genes were investigated by PCR (Table S1) followed by DNA Sanger sequencing.

2.6. *Conjugation experiments*

Conjugation experiments were carried out in six representative isolates (A45517, A47758, A48120, A48295, A48524, and A48907B). Briefly, 3h-broth cultures of BKC-1-producing *K. pneumoniae* (donor strains) were mixed with the receptor strain *E. coli* J53 AziR (ratio 1:3) and incubated overnight at 37ºC without agitation [15]. Transconjugants were selected on MacConkey agar plates supplemented with azide (100 µg/mL) plus ampicillin (100 µg/mL) and confirmed by $bla_{\text{BKC-1}}$ PCR.

Conjugation frequency was determined by the ratio between the numbers of transconjugants and donors.

2.5. *In vivo Virulence Study using Galleria mellonella model*

Representatives of ST11 harbouring *bla*$_{\text{BKC-1}}$ (A48295 and A48906), ST442 isolates harbouring only *bla*$_{\text{BKC-1}}$ (A45517, A47758, and A52034), or *bla*$_{\text{BKC-1}}$ and *bla*$_{\text{KPC-2}}$ (A46209 and A48120) were selected for *in vivo* virulence studies using the *Galleria mellonella* wax moth larvae model. An initial standardised suspension containing $1x10^9$ bacteria/mL was prepared and 10 µL of selected dilutions (reaching 10^6, 10^5 and 10^4 bacteria) was injected into the hemocoel of the larvae, through the rear left pro-leg using a Hamilton syringe. Each suspension was injected in 10 larvae, and incubated at 37ºC for 72 hours. Experiments were performed in triplicate. Furthermore, the isolates A58300 (K1 strain), KP13 (ST442, KPC-producer, capsular type KL107), P12 (ST11, KPC-producer, capsular type KL64) and, P41 (ST11, KPC-producer, capsular type KL15) were used for comparative purposes [16].

2.6. *Biofilm Assay*

Biofilm measurements were performed using the 96-well plate method of Stepanovic and colleagues [17]. Briefly, 2 µL of fresh bacteria culture was inoculated on 198 µL of TSB supplemented with 1% glucose, poured in a well and incubated for 24 h at 37ºC. After incubation, each well content was washed three times with saline 0.85% and posteriorly fixated with 150 µL of methanol. Biofilm was stained with 2% crystal violet for 15 minutes at room temperature and the Optical Density (OD_{550nm}) was measured. Each assay was performed in biological and technical triplicates.

2.7. *Whole-genome Sequencing*

Based on previous analysis, the whole-genome sequencing (WGS) was performed on two representative isolates (A52034 and A48295) using the Illumina MiSeq platform (Illumina Inc., San Diego, CA). DNA libraries were prepared for paired-end sequencing (2x300 cycles) using Nextera XT V2. Quality control of raw sequence reads included fastqc (0.11.2), and quality and adaptor trimming were performed using Trim galore (0.4.3). Reads were assembled in contigs using the *de novo* assembler SPAdes (3.9.0) and were aligned to the original fastq reads using BWA aligner (0.7.15). The investigation of antimicrobial resistance genes and plasmid replicons were assessed by ResFinder v.3.2 and PlasmidFinder v.2.1 (https://cge.cbs.dtu.dk/services/) and virulence determinants were investigated using a previous *Klebsiella* virulome dataset [16] and Kapitive (https://kaptive-web.erc.monash.edu/).

In addition, a transconjugant TcA48295.9 was sequenced using a hybrid sequencing approach. Total genomic DNA was extracted and libraries were prepared using a PCR free rapid barcoding kit (SQK RBK004) and sequenced with a R9.4 flow cell on a MinION (Oxford Nanopore technologies, UK).

3. Results

3.1. General Microbiological and Epidemiological data

Among 647 *K. pneumoniae* isolates screened by PCR, 16 (frequency of 2.5%) harboured $bla_{\text{BKC-1}}$, and were all confirmed as *K. pneumoniae* by MALDI-TOF MS. The 16 BKC-1-producing *K. pneumoniae* were isolated from 15 patients hospitalized between October 2010 and March 2012. It was not possible to retrieve clinical data of four patients. The clinical description and temporal distribution of infected patients by

BKC-1 producers are shown in Figure 1. The most frequent clinical specimens culturing *K. pneumoniae* were blood (10 isolates; 62.5%) and tracheal aspirate (3 isolates; 18.8%). The three remaining isolates were recovered from wound skin, urine, and central venous catheter tip (6.3% each) (Figure 1). The average age of patients was 56.8 years-old (±21.6), with 10 males (66.6%) and 5 females (33.4%). Six of eleven patients with clinical data died within 30 days of infection. The first case (index case) was described in October 2010, in a female patient admitted in the neurology ICU, who presented the BKC-1-producing *K. pneumoniae* blood infection after four months of hospitalization.

3.2. Isolates Genetic Relatedness

PFGE analysis revealed the presence of a major clone, named A1, recovered from 13 isolates of 12 patients, distributed along the three years (2010-2012). Besides that, an isolate (A47758) belonged to clone A2, while the two remain isolates recovered from the emergency room (A48295) and nephrology (A48906) belonged to clone B1 (Table 1). All clone A isolates were identified as ST442, while the clone B isolates belonged to the ST11.

3.3. Antimicrobial Susceptibility Profile

The BKC-1-producing *K. pneumoniae* isolates showed a multidrug resistance (MDR) profile with high MICs for monobactam, cephalosporins, and carbapenems (Table 1). Polymyxin B resistance was detected in 11 of 16 isolates (68.8%) isolates ($MIC_{50/90}$, 32 and 64 mg/L). The most potent *in vitro* antimicrobial agents against BKC-1 clone were ceftazidime-avibactam ($MIC_{50/90}$, 4/4 and 8/4 mg/L) and gentamicin ($MIC_{50/90}$, 0.5/64 mg/L). Interestingly, five isolates showed elevated MICs for

ceftazidime-avibactam (8/4 mg/L), however, no isolates were categorized as resistant.

3.4. Antimicrobial Resistance Genes and Plasmid Mobilization Analysis

The β-lactamase *bla*$_{\text{SHV-like}}$ and the phosphotransferase *aph(3')-VIi* were detected in all BKC-1-producing *K. pneumoniae* isolates, while *bla*$_{\text{CTX-M}}$ and *bla*$_{\text{TEM}}$ were only amplified from clone B1-ST11 isolates. The simultaneous presence of *bla*$_{\text{BKC-1}}$ and *bla*$_{\text{KPC-2}}$ was detected in six isolates (Table 1). The mechanism responsible for polymyxin resistance was not identified; however, our data suggested that *mgrB* alterations were not the mechanism involved in polymyxin resistance in those isolates. S1-PFGE analysis revealed the presence of a single 10-Kb plasmid carrying *bla*$_{\text{BKC-1}}$ in all BKC-1 producers. In contrast, *bla*$_{\text{KPC-2}}$ was located in a different plasmid on the isolates harbouring *bla*$_{\text{BKC-1}}$ and *bla*$_{\text{KPC-2}}$ (data not shown).

Transconjugants were only obtained for A48295 (ST11), and A48907B (ST442) isolates. The conjugants cells TcA48295.9 and TcA48907B.15 carrying *bla*$_{\text{BKC-1}}$ showed conjugation frequency of 1.7×10^{-7} and 4.2×10^{-8}, respectively (Table S2). B-lactams MIC for the transconjugants demonstrated higher values for TcA48295.9 (Table S2). To verify the mechanism of *bla*$_{\text{BKC-1}}$ plasmid mobilization and the reason for the higher MICs, we chose the transconjugant TcA48295.9 for MinIon sequencing. The plasmid carrying *bla*$_{\text{BKC-1}}$ was the same 10-Kb IncQ1 plasmid detected in previous studies (p60136) [15]. The IncQ1-*bla*$_{\text{BKC-1}}$ plasmid was co-mobilized by a 68.6 Kb IncM1-plasmid named pA48295A which also carried other ARG as *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{TEM-1A}}$, and *qnrE1*.

3.5. Galleria mellonella Virulence Testing and Biofilm production

In vivo virulence assay analysis revealed that larvae infected by BKC-1 producer

ST11 isolates had lower survival rates than those infected by BKC-1 ST442 isolates ($p<0.0001$) (Figure 2A). Besides, BKC-1 ST11 with capsule serotype KL64 seem to show higher virulence profiles than ST11 isolates harbouring different capsule serotypes (Figure 2A).

No difference was noticed on biofilm production of ST442 and ST11. In general, the isolates were classified as weakly adherent (n= 7 isolates) and moderately adherent (n= 9 isolates), however A47758 (ST442, A2 pulsotype) had a higher capacity for biofilm production (Figure 2B).

3.6. Genetic Analysis of the two BKC-1 clones

WGS of two representative isolates A48295 (B1-ST11) and A52034 (A1-ST442) provided the general genomic features of each clone evaluated during this study. Molecular capsule analysis indicated the capsule type KL64 for A48295 and KL107 for A52034. The isolates carried similar virulence-encoding genes, except for the presence of *traT* (humam serum resistance) and *clpK* (thermotolerance) only identified on B1-ST11 clone (Table S3). Resfinder analysis resulted in the presence of multiple ARG collaborating to MDR phenotype in both isolates. Acquired antimicrobial resistance genes identified in each isolate as so as the antimicrobial resistance classes are present on Table S3. Mutational analysis on two-component systems (TCS) of A52034 (polymyxin B resistant isolate) revealed mutations in the follow TCS: CrrA (E192V), CrrB (C68S and Q296L), and PmrB (T246A). According to the analysis using PROVEAN, the mutation E192V in CrrA was predicted to be deleterious for the protein function. As expected, no mutations on TCS were found on polymyxin-susceptible A48295 isolate.

4. Discussion

The description of BKC-1 has not gained attention, because this enzyme has been infrequently reported and restricted to clinical isolates recovered from São Paulo [4,7]. However, the identification of multiple *K. pneumoniae* carrying *bla*$_{BKC-1}$ is a cause of concern because reveals a silent and undetected circulation of a resistant clone in the studied hospital.

In our study, most BKC-1 producers belonged to ST442 (CC442) as the first reported isolates [4,7]. Moreover, two BKC-1-producing *K. pneumoniae* ST11 were detected, representing the first description of *bla*$_{BKC-1}$ out of the CC442 in *K. pneumoniae*. The acquisition of the IncQ1 plasmid carrying *bla*$_{BKC-1}$ by the epidemic ST11 clone, is worrisome since its high virulence capacity is well established [18,19]. The frequency of BKC-1 producers in this work was almost ten times higher when compared to the results of our previous study which determined the frequency of BKC-1 from Brazilian isolates in 2016 [7].

The presence of two carbapenemases (BKC-1 and KPC-2) had impact on β-lactam MICs, with those isolates being highly resistant to β-lactams compared with isolates producing BKC-1 alone. Interestingly, MICs of 8/4 mg/L to ceftazidime-avibactam were also observed in those isolates carrying *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$. The co-production of serine carbapenemases has been shown to increase MICs to ceftazidime-avibactam [20]. It is highly probable that the presence of *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$ be the cause of the low inhibitory efficacy of avibactam, although other mechanisms (as permeability defects) could not be discarded [20]. As the genetic background of *bla*$_{BKC-1}$ is highly conserved, with *bla*$_{BKC-1}$ being flanked by the phosphotransferase *aph(3')-VIi* (downstream) and IS*Kpn23* (upstream), the pattern of resistance to amikacin and kanamycin and the remaining susceptibility to gentamicin was something expected in

ST442 clone [7]. In fact, gentamicin was an important therapeutic option for the patients evaluated in this retrospective study. Four of the five discharged patients, who had a favourable outcome, had received gentamicin. However, the clone B1-ST11 was highly resistant to gentamicin (MICs, 64 and >64 mg/L) likely due to the codification of different aminoglycoside modifying enzymes such as *aac(3)-IIa*, *aac(6')-Ib-cr*, *aadA1*, *addA2*, *aph(3')-VIi* .

Although multiple mechanisms have been shown to cause polymyxin B resistance in our hospital, the inactivation of MgrB is the most common mechanism detected thus far [16,21]. In contrast, our polymyxin-resistant isolates did not show alterations in *mgrB*. Analysis of A52034 (single A1-ST442 polymyxin B resistant) revealed deleterious mutations on the TCS CrrA. Although we have generated WGS data on a single representative isolate for both clones analysed, this finding could evidence that polymyxin resistance in this clone could be mediated by mutations on TCS.

Virulence analysis confirmed a high virulence profile of ST11-KL64 isolates with high larvae mortality. Analysis conducted using *K. pneumoniae* ST11 with two types of capsules (KL64 and KL15) showed that the survival rates of ST11-KL64 was lower when compared to that of ST11-KL15. In a recent study conducted in China, a novel sub-clone of carbapenem-resistant *K. pneumoniae* ST11-KL64 carrying *rmpA*/*rmpA2*, was responsible for increased 30-day mortality rate in Chinese patients [22]. Different of the Chinese isolates, the studied ST11-KL64 clone did not present important virulence-encoding genes such as *rmpA/rmpA2* or aerobactin [22]. We believe that the difference of virulence between the two clones could be explained by different capsule types (KL64 vs KL107).

In conclusion, we reported for the first time a silent outbreak caused by BKC-1-

313 producing *K. pneumoniae*, capturing clinical, microbiological and genomic data. The
314 detection of this outbreak almost 10 years after its start reinforces the challenge to
315 monitor and control AMR in real time. In addition, the misidentification of new
316 carbapenemase variants may have worsened the spread of resistant clones and led to
317 dissemination in other hospitals. The impact of this information on the patient outcome
318 remains unclear; however, the role of BKC-1 infections can hardly be determined if
319 these isolates are misidentified as possessing other mechanisms of resistance.

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Conflict of interests

A.C.G. has recently received research funding and/or consultation fees from Cristália, Enthasis Therapeutics, InfectoPharm, Eurofarma, Pfizer, MSD, and Zambon. Other authors have nothing to declare.

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Ethics approval

Ethical approval for this study was obtained from Research Ethics Committee from Universidade Federal de São Paulo - UNIFESP/São Paulo Hospital (Process number: 8567211118). Waiver of Informed Consent was granted by REC because this study involved a retrospective chart review with patients' confidentiality and anonymity were preserved.

References

1. Bush K. Past and Present Perspectives on β-Lactamases. Antimicrob Agents Chemother 2018;62:e01076-18.

2. Escandón-Vargas K, Reyes S, Gutiérrez S, Villegas MV. The epidemiology of carbapenemases in Latin America and the Caribbean. Expert Rev Anti Infect Ther 2017;15:277-297.

3. Sampaio JL, Gales AC. Antimicrobial resistance in Enterobacteriaceae in Brazil: focus on β-lactams and polymyxins. Braz J Microbiol 2016;47 Suppl 1:31-37.

4. Nicoletti AG, Marcondes MF, Martins WM, et al. Characterization of BKC-1 class A carbapenemase from *Klebsiella pneumoniae* clinical isolates in Brazil. Antimicrob Agents Chemother 2015;59:5159-5164.

5. Dortet L, Poirel L, Abbas S, Oueslati S, Nordmann P. Genetic and Biochemical Characterization of FRI-1, a Carbapenem-Hydrolyzing Class A β-Lactamase from Enterobacter cloacae. Antimicrob Agents Chemother 2015;59:7420-7425.

6. Mangat CS, Boyd D, Janecko N, et al. Characterization of VCC-1, a Novel Ambler Class A Carbapenemase from *Vibrio cholerae* Isolated from Imported Retail Shrimp Sold in Canada [published correction appears in Antimicrob Agents Chemother. 2016 May;60(5):3263]. Antimicrob Agents Chemother 2016;60:1819-1825.

7. Martins WM, Nicoletti AG, Santos SR, Sampaio JL, Gales AC. Frequency of BKC-1-Producing *Klebsiella* Species Isolates. Antimicrob Agents Chemother 2016;60:5044-5046.

8. Meunier D, Findlay J, Doumith M, et al. FRI-2 carbapenemase-producing Enterobacter cloacae complex in the UK. J Antimicrob Chemother 2017;72:2478-2482.

9. Kubota H, Uwamino Y, Matsui M, et al. FRI-4 carbapenemase-producing *Enterobacter cloacae* complex isolated in Tokyo, Japan. J Antimicrob Chemother 2018;73:2969-2972.

10. Martins WMBS, Seco BMS, Sampaio JLM, Sands K, Toleman MA, Gales AC. Detection of BKC-1 in *Citrobacter freundii*: A clue to mobilisation in an IncQ1 plasmid carrying *bla*BKC-1. Int J Antimicrob Agents 2020;56:106042.

11. Tenover FC, Arbeit RD, Goering RV, et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J Clin Microbiol 1995;33:2233-2239.

12. Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. J Clin Microbiol 2005;43:4178-4182.

13. Toleman MA. Direct in Gel Genomic Detection of Antibiotic Resistance Genes in S1 Pulsed Field Electrophoresis Gels. Methods Mol Biol 2018;1736:129-136.

14. Cannatelli A, Giani T, D'Andrea MM, et al. MgrB inactivation is a common mechanism of colistin resistance in KPC-producing *Klebsiella pneumoniae* of clinical origin. Antimicrob

Agents Chemother 2014;58:5696-5703.
15. Martins WMBS, Nicolas MF, Yu Y, et al. Clinical and Molecular Description of a High-Copy IncQ1 KPC-2 Plasmid Harbored by the International ST15 *Klebsiella pneumoniae* Clone. mSphere 2020;5:e00756-20.
16. Andrey DO, Pereira Dantas P, Martins WBS, et al. An Emerging Clone, *Klebsiella pneumoniae* Carbapenemase 2-Producing *K. pneumoniae* Sequence Type 16, Associated With High Mortality Rates in a CC258-Endemic Setting. Clin Infect Dis 2020;7:e141-e150.
17. Stepanović S, Vuković D, Hola V, et al. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. APMIS 2007;115:891-899.
18. Liao W, Liu Y, Zhang W. Virulence evolution, molecular mechanisms of resistance and prevalence of ST11 carbapenem-resistant *Klebsiella pneumoniae* in China: A review over the last 10 years. J Glob Antimicrob Resist 2020;23:174-180.
19. Yao H, Qin S, Chen S, Shen J, Du XD. Emergence of carbapenem-resistant hypervirulent Klebsiella pneumoniae. Lancet Infect Dis 2018;18:25.
20. Aires-de-Sousa M, Ortiz de la Rosa JM, Gonçalves ML, Pereira AL, Nordmann P, Poirel L. Epidemiology of Carbapenemase-Producing *Klebsiella pneumoniae* in a Hospital, Portugal. Emerg Infect Dis 2019;25:1632-1638.
21. Braun G, Cayô R, Matos AP, de Mello Fonseca J, Gales AC. Temporal evolution of polymyxin B-resistant *Klebsiella pneumoniae* clones recovered from blood cultures in a teaching hospital during a 7-year period. Int J Antimicrob Agents 2018;51:522-527.
22. Zhou K, Xiao T, David S, et al. Novel Subclone of Carbapenem-Resistant *Klebsiella pneumoniae* Sequence Type 11 with Enhanced Virulence and Transmissibility, China. Emerg Infect Dis 2020;26:289-297.

413 **Table 1.** Clinical and microbiological features of BKC-1-producing *K. pneumoniae* isolates recovered in this study.

Isolate	Patient	Ward	PFGE	ST	MIC (mg/L)																	Antimicrobial Resistance genes[a]
					ATM	CFL	CFX	CPM	CAZ	CRO	ETP	IMI	MER	PTZ	CAZ-AVI	CIP	LEV	AMK	GEN	TOB	PMB	TGC	
A45517	Pt1	Neurology ICU	A1	442	128	>512	>64	256	8	>512	1	4	2	16/4	2/4	>64	64	1024	0.5	8	32	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A45843	Pt2	General ICU	A1	442	8	>512	>64	64	2	256	≤1	2	0.5	16/4	4/4	>64	64	512	0.5	8	64	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A46209	Pt3	Pediatrics	A1	442	>512	>512	>64	>256	512	>512	512	256	128	512/4	8/4	>64	>64	128	1	16	≤0.125	2	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$
A46313	Pt4	Neurology ICU	A1	442	256	>512	>64	64	128	256	16	8	8	512/4	4/4	>64	64	512	1	4	64	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A47626	Pt5	-	A1	442	256	>512	64	64	64	>512	32	8	8	512/4	2/4	>64	64	512	0.5	4	32	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A47758	Pt6	-	A2	442	256	>512	>64	128	128	512	8	8	8	512/4	4/4	>64	64	256	0.5	4	32	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A48120	Pt7	-	A1	442	>512	>512	>64	>256	256	>512	512	128	512	>512/4	8/4	>64	64	256	0.5	4	128	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$
A48240	Pt8	Emergence room	A1	442	256	>512	>64	128	128	>512	16	8	8	512/4	2/4	>64	64	512	0.5	4	64	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A48295[b]	Pt9	Nephrology	B1	11	512	>512	64	>256	128	>512	64	128	32	512/4	4/4	>64	>64	256	>64	32	≤0.125	0.5	*aph(3')-VIi; bla*$_{SHV-182}$; *bla*$_{BKC-1}$; *bla*$_{CTX-M-8}$; *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1B}$
A48524	Pt10	Nephrology	A1	442	>512	>512	>64	>256	128	>512	512	128	512	>512/4	8/4	>64	64	512	0.5	4	64	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$
A48827	Pt11	-	A1	442	>512	>512	>64	>256	256	>512	256	64	128	>512/4	4/4	>64	32	256	0.5	16	≤0.125	0.25	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$
A48834	Pt12	Pulmonology ICU	A1	442	8	>512	64	64	2	512	≤1	2	1	16/4	4/4	>64	64	512	0.5	4	32	1	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$
A48906	Pt13	Emergence room	B1	11	>512	>512	64	>256	256	>512	64	128	32	>512/4	2/4	>64	>64	256	64	32	≤0.125	0.5	*aph(3')-VIi; bla*$_{SHV-182}$; *bla*$_{BKC-1}$; *bla*$_{CTX-M-8}$; *bla*$_{CTX-M-15}$ and *bla*$_{TEM-1B}$
A48907B	Pt12	Pulmonology ICU	A1	442	>512	>512	>64	>256	128	>512	128	64	256	>512/4	8/4	>64	64	256	0.5	4	≤0.125	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$ and *bla*$_{KPC-2}$
A49436	Pt14	Emergence	A1	442	>512	>512	>64	>256	256	>512	1024	128	512	>512/4	8/4	>64	64	256	0.5	16	32	0.5	*aph(3')-VIi; bla*$_{SHV-110}$; *bla*$_{BKC-1}$

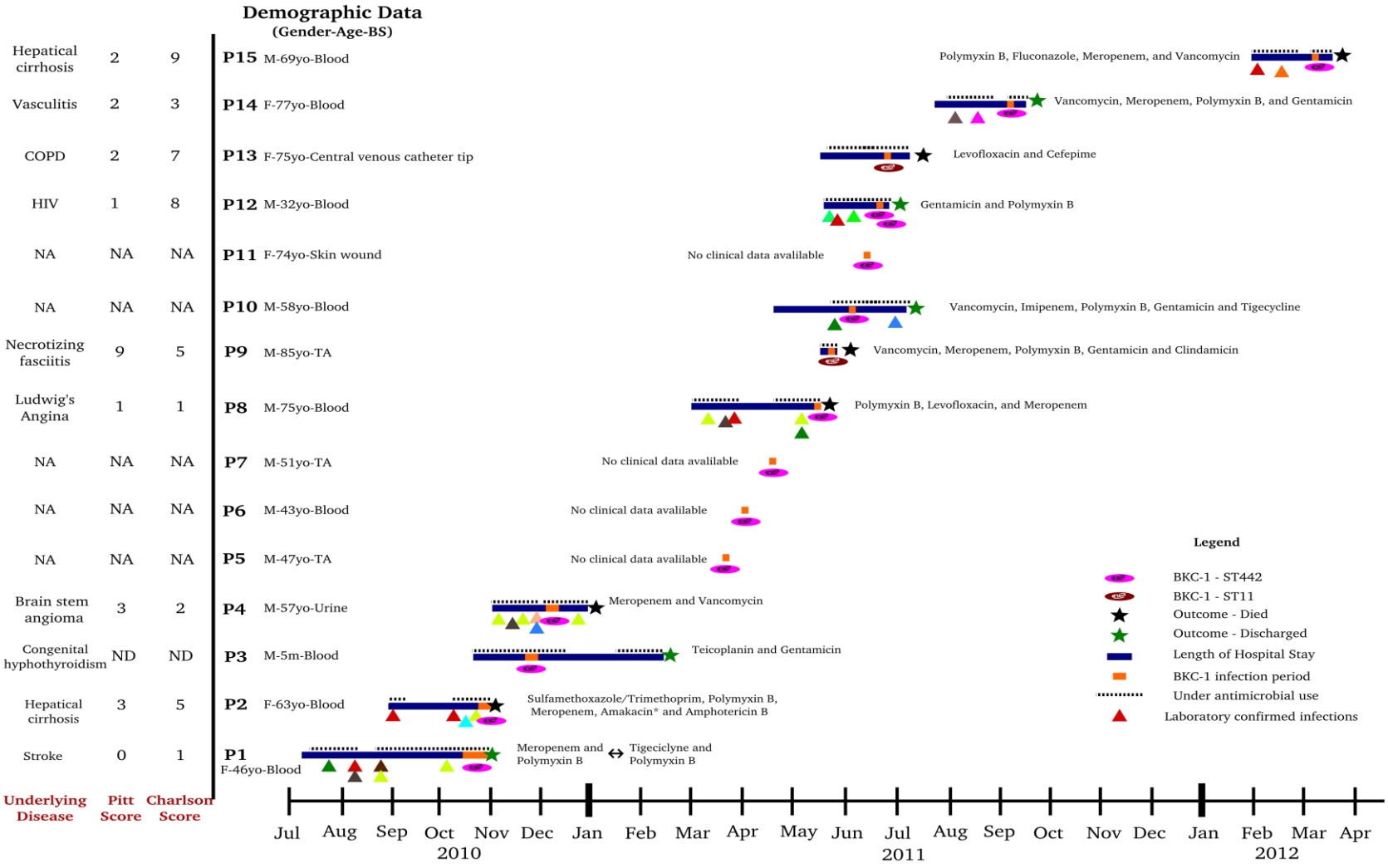
		room																				and bla_{KPC-2}
A52034[b]	Pt15	Gastrology	A1	442	64	>512	>64	128	16	>512	2	4	4	512/4	2/4	>64	64	512	0.5	4	32	0.5	*aph(3')-VIi;* $bla_{SHV-110}$; bla_{BKC-1}

Legend: TA, Tracheal aspirate; ATM, aztreomam; CFL, cephalotin; CFX, cefoxitin; CPM, cefepime; CAZ, ceftazidime; CRO, ceftriaxone; ERT, ertapenem; IMI, imipenem; MER, meropenem; TZP, piperacillin-tazobactam; CAZ-AVI, ceftazidime-avibactam; CIP, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; GEN, gentamicin; TOB, tobramycin; PMB, polymyxin B; TGC, tigecycline.

[a]Antimicrobial resistance genes (Table S1) were initially investigated by PCR followed by Sanger DNA sequencing. The full content of ARG was assessed for two isolates (A48295 and A52034) submitted to Whole-genome sequencing and can be found in Table S2.

[b]Isolates submitted to Whole-genome sequencing.

Figure 1. General demographic data and the length of hospital stay of the patients infected by BKC-1-producing *K. pneumoniae* isolates. Underlying diseases and their respective Pitt and Charlson scores are also provided. Pitt score was just provided in bacteremia cases.



Legend: ND, not determined; NA, not available; P, patient; yo, years-old; COPD, Chronic obstructive pulmonary disease, M, male; F, female; TA, tracheal aspirate.

Figure 2. Virulence assays performed in BKC-1-producing *K. pneumoniae* isolates. (A) Kaplan-Meier plots showing the survival rates of *G. mellonella* over 72 hours post infection with BKC-1 producers detected in the outbreak described in this study. A58300 strain (K1 – *K. pneumoniae* ST23) was used as hypervirulent positive control. In addition, some isolates previously tested [5] (Kp13, P12, and P41) were also used to comparative purposes. Saline solution (NaCl 0.85%) was used as negative control. (B) Biofilm production plot of BKC-1-producing isolates. The isolates were categorized according biofilm production (non-adherent, weakly adherent, moderately adherent, or strongly adherent) using the OD of negative control, following recommendation established by Stepanovic and colleagues (2009) [24]. Different colours were used to represent clones and control isolates. Dark blue, A1-ST442 clone; Light blue, A2-ST442; Red, B1-ST11; salmon, control isolates.