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 blaBKC-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 blaBKC-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, blaBKC-1 and blaKPC-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*BKC-1 is plasmid-mediated and associated with the insertion sequence IS*Kpn*23 and a phosphotransferase, *aph(3’)-VIIa* (also called *aph(3’)-VIIa*), 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*BKC-1 by PCR as previously described [4], and subsequently submitted to DNA sequencing by Sanger. All *bla*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 37°C 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_{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 \( \text{bla}^{\text{BKC-1}} \) (A48295 and A48906), ST442 isolates harbouring only \( \text{bla}^{\text{BKC-1}} \) (A45517, A47758, and A52034), or \( \text{bla}^{\text{BKC-1}} \) and \( \text{bla}^{\text{KPC-2}} \) (A46209 and A48120) were selected for in vivo virulence studies using the *Galleria mellonella* wax moth larvae model. An initial standardised suspension containing \( 1 \times 10^9 \) 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 37°C 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 37°C. 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\(_{550\text{nm}}\)) 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 blaBKC-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
3.4. Antimicrobial Resistance Genes and Plasmid Mobilization Analysis

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

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

3.5. Galleria mellonella Virulence Testing and Biofilm production

\textit{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 (human 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*<sub>BKC-1</sub> 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*<sub>BKC-1</sub> out of the CC442 in *K. pneumoniae*. The acquisition of the IncQ1 plasmid carrying *bla*<sub>BKC-1</sub> 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*<sub>BKC-1</sub> and *bla*<sub>KPC-2</sub>. 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*<sub>BKC-1</sub> and *bla*<sub>KPC-2</sub> 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*<sub>BKC-1</sub> is highly conserved, with *bla*<sub>BKC-1</sub> 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')-VIIi.

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

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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


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

| Isolate | Patient | Ward     | MIC (mg/L) | Antimicrobial Resistance genes
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<td></td>
<td></td>
<td>ATM</td>
<td>CFL</td>
</tr>
<tr>
<td>A45517</td>
<td>Pt1</td>
<td>Neurology ICU</td>
<td>A1</td>
<td>442</td>
</tr>
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<td>General ICU</td>
<td>A1</td>
<td>442</td>
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<td>442</td>
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<td>A49436</td>
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<td>Emergence</td>
<td>A1</td>
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1. *bla*\(\text{TEM-1B}\), *bla*\(\text{SHV-110}\), *bla*\(\text{KPC-2}\), *aph(3')-VIi*; 2. *aph(3')-VIi*; *blashv-110*; *blabkc-1; 3. *blasktm-1B*; 4. *bllactx-m-8*; *bllactx-m-15*; 5. *blaskc-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.

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.

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.