TITLE PAGE

SUCCESSFUL CONTROL OF A LARGE OUTBREAK OF COLONIZATION AND INFECTION BY CARBAPENEM-RESISTANT *Klebsiella pneumoniae* CARBAPENEMASE PRODUCER IN ILL NEONATES

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ABSTRACT

Background: Failures in prevention and control measures in Neonatal Intensive Care Units (NICUs) facilitate the transmission of microorganisms like *Klebsiella pneumoniae*, causing outbreaks of colonization and invasive infections in neonates**. Objective**: We describe a large nosocomial outbreak of carbapenemase-producing *K. pneumoniae* in the neonatal unit of a university hospital in Brazil. **Methods:** Between July 2023 and February 2024, carbapenem-resistant *K. pneumoniae* (CRKP) samples were identified and tested for antimicrobial susceptibility using an automated identification and susceptibility testing system. The bla_{KPC}, bla_{NDM}, and $bla_{\text{CTXM-Gp-1}}$ genes were investigated by polymerase chain reaction (PCR), and clonality was explored by pulsed-field gel electrophoresis (PFGE). **Results**: Thirty-six patients with CRKP were identified. 72.2% (26/36) were colonized, 33.3% (12/36) had infections, and 5.5% (2/36) had both. 37.5% (6/16) of the isolates harbored the bla_{KPC} gene, 6.25% (1/16) bla_{NDM}, and 12.5% (2/16) $bla_{\text{CTXM-Gp-1}}$. Genotyping revealed five distinct PFGE profiles. Among the colonization samples, two clones were predominant: clone A with five isolates and clone C with six isolates. **Conclusion:** This study described a large nosocomial outbreak of carbapenemase-producing *K. pneumoniae* in the neonatal unit of a university hospital in Brazil. Although this outbreak was severe, spreading through the hands of healthcare professionals, it also highlights the potential of the infection control system to manage the outbreak.

Keywords: *Klebsiella pneumoniae;* Infectious Disease Outbreak; Neonates; Pulsed-Field Gel Electrophoresis; bla_{KPC}; Infectious Disease Horizontal Transmission.

HIGHLIGHTS

- The *bla*$_{KPC}$ gene is predominant in hospital outbreaks caused by *Klebsiella pneumoniae*.
- Negligence with biosafety and hygiene measures in ICUs leads to the proliferation of clones of the same species.
- Neglect of biosafety and hygiene measures in neonatal ICUs can cause the spread of the pathogen to other sectors of the hospital.

BACKGROUND

Because of the critical condition and compromised immunity of neonates, the Neonatal Intensive Care Unit (NICU) has consistently been a primary hospital source of multidrug-resistant bacteria. [1] Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) strains have spread rapidly with the increasing overuse of wide-spectrum antibiotics. [2, 3]

According to the literature, one of the possible causes for the spread of CRKP in the hospital environment has been the lack of prevention and control measures directed at strains of this phenotype. [4] Unfortunately, these strains tend to acquire different resistant genes, including genes encoding carbapenems and Extended-spectrum beta-lactamases (ESBLs) in plasmids. [5] In Brazil, the occurrence of outbreaks of this phenotype in NICUs represents a major problem due to its high mortality rate. [6, 7]

From August 2023 physicians at the Clinical Hospital of the Federal University of Uberlandia (HC-UFU), Minas Gerais, Brazil, began to see a spike in neonatal infections and colonization by CRKP. Unfortunately, an outbreak occurred between July 2023 and February 2024, so this study aims to analyze this recent outbreak of CRKP harboring

bla$_{KPC}$, *bla*$_{NDM}$, and *bla*$_{CTXM-GP-1}$ genes occurring between the second half of 2023 and the first quarter of 2024 in the NICU of a Brazilian teaching hospital.

MATERIAL AND METHODS

Bacterial samples, species identification, and antimicrobial susceptibility tests

Clinical isolates of infection and colonization of *Klebsiella pneumoniae* were collected between 2023 and 2024 in the NICU of the HC-UFU located in the eastern region of Uberlândia City, Minas Gerais, Brazil. Identification and antimicrobial sensitivity tests were performed with an automated identification and susceptibility testing system (Vitek® 2 - bioMérieux and MALDI-TOF – Bruker). The identified bacterial samples were stored at -20 °C in Brain Heart Infusion (BHI) broth with 15% glycerol. Colonization was defined as the presence of bacteria in the rectal mucosa. Infection was defined as the detection of the organism in clinical cultures, associated with clinical signs of infection. The endemic level of colonization and infection by KPC/1000 patient-days was calculated using a method described by Arantes and colleagues (2023) [8]. Epidemiological and demographic data were obtained from hospital infection control service reports.

Phenotypic evaluation of hypermucoviscosity using the string test

The phenotypic evaluation of hypermucoviscosity was performed by the string test according to the criteria described by Catalán-Nájera and colleagues (2017). [9] The samples were grown on Tryptic Soy Agar (TSA) plates and incubated overnight at 37°C.

After the bacterial growth, the formation of a viscous filament was evaluated when touching a sterile loop to the surface of a colony. Filament formation ≥ 5mm characterized the strain as hypermucoviscous.

Molecular techniques

Genomic DNA extraction

To execute molecular analyses, DNA extraction was performed by thermal lysis. The samples were cultured in BHI agar for 24 hours, pure colonies were resuspended in 150µL of Tris-Edta buffer (TE; Tris [1M] + EDTA [0.5 M]) [1X] and placed in a boiling water bath (100ºC) for 10 minutes. The tubes were then placed in an ice bath for 10 minutes, and centrifuged at 20,000xg for 1 minute, and the supernatant containing the DNA was recovered. The extracted DNA was quantified by spectrophotometry (Nanodrop®) and stored at -20 ºC until use.

Detection of resistance and virulence genes by polymerase chain reaction (PCR)

To evaluate the presence of resistance and virulence genes, the technique of conventional polymerase chain reaction (PCR) was performed with consensus primers for each gene. The primers used in the reactions are described in **Table I**, and the reagents used, and the reaction volumes are described in **Table II**. Amplification was performed on an Eppendorf Mastercycler® thermal cycler programmed according to the following conditions: initial denaturation at 95ºC for 2 minutes; followed by 30 cycles with denaturation at 95ºC for 30 seconds; annealing at the specific temperature of each primer

(**Table I**) for 1 minute, extension at 72ºC for 1 minute, and final extension at 72ºC for 5 minutes. Electrophoresis was run at 90V for 40 minutes on a 2% agarose gel in TBE running buffer (Tris, Boric acid, and EDTA) [0.5x]. The gel was stained with 2µL of SYBER® Safe (Applied Biosystems®) and photographed using the L-Pix EX photo documentation system (Loccus Biotecnologia®).

Evaluation of genetic similarity by Pulsed-Field Gel Electrophoresis (PFGE)

All strains were typed according to the protocols described previously. [10] Following digestion of intact genomic DNA with XbaI restriction enzyme (Ludwig Biotec) for *K. pneumoniae* DNA fragments were separated on 1% (w/v) agarose gels in 0.5% TBE [Tris–borate–ethylene diamine tetra-acetic acid (EDTA)] buffer using a CHEF DRIII apparatus (Bio-Rad, USA) with 6 V/cm, pulses from 5s to 40s, for 21h at 12ºC. Gels were stained with ethidium bromide and photographed under ultraviolet light. Computer-assisted analysis was performed using a transilluminator and photographed using the L-Pix EX photo documentation system (Loccus Biotecnologia®). Comparison of the banding patterns was accomplished by the unweighted pair-group method with arithmetic averages (UPGMA) using the Dice similarity coefficient.

Ethical statement

The samples were obtained from the microbiological collection of the Laboratory of Molecular Microbiology at the Federal University of Uberlandia. Throughout the study, patient identities and other unique characteristics remained protected, ensuring no

risk of exposure. At all stages, the principles of confidentiality and non-maleficence were strictly followed.

RESULTS

Thirty-six patients involved in the outbreak of CRKP were identified during the surveillance period, 72.2% (26/36) were colonized, 33.3% (12/36) had infections, and 5.5% (2/36) exhibited both. It was observed that patients were hospitalized for an average of 44.52 days. (**Table III**).

Against the background endemicity of this bacterium (**Table III**) the rates of colonization and/or infection with carbapenem-resistant strains per 1000 patient-days during the period from November 2023 to February 2024 reached the upper alert limit established, 2σ above the average incidence of colonization/infection (**Fig. 1**).

The outbreak's first peak in November presented 88.9% (8/9) of the samples as colonization cases, with three samples tested for carbapenem resistance. Among these, one sample was positive for *bla*$_{\text{KPC}}$, another for *bla*$_{\text{NDM}}$, and the third tested negative for all genes (**Fig. 1 and Fig. 2**). The infection/colonization rate was 6.25 per 1000 patient-days in early November 2023, dropping to 0.67 per 1000 patient days in December 2023 for the nine cases of colonization and/or infection that subsequently occurred. In early January 2024, the rate of CRKP increased to 8.73 per 1000 patient-days (the second peak). Still, it declined to 2.87 per 1000 patient-days by the end of February. By this point, the outbreak was deemed to be over (**Fig. 1**).

Of the total samples analyzed by PFGE (n=16), 37.5% (6/16) of the isolates involved in the outbreak harbored the *bla*$_{\text{KPC}}$ gene, 6.25% (1/16) *bla*$_{\text{NDM}}$, 12.5% (2/16) *bla*$_{CTXM\text{-Gp-1}}$ demonstrated by PCR (**Fig. 2**).

Genotyping of *K. pneumoniae* isolates revealed six distinct PFGE profiles. Among the colonization samples, two clones were predominant: clone A, with five isolates, and clone C, with six isolates. Interestingly, the highest frequency of KPC-producing strains was in clone A. Notably, clones A and C spread beyond the NICU to adult patients admitted to other units (**Fig. 2**)

DISCUSSION

To the best of our knowledge, this is one of the biggest outbreaks of CRKP in a NICU reported in Brazil. [5, 6, 11] Previous studies found that neonates, especially those with low birth weight, are often associated with nosocomial infections caused by carbapenem-resistant strains. [12] Unfortunately, this bacterium is endemic in Brazilian tertiary hospitals, and consequently, there has been an increase in the frequency of associated outbreaks. [13, 14, 15]

As usual, *K. pneumoniae* harbors different resistance genes, including bla_{KPC}, bla_{NDM}, and $bla_{\text{CTXM-Gp1}}$, for which the samples of this study had a significant positivity rate. Such genes produce resistance enzymes belonging to the groups of β-lactamases and metalo-β-lactamases and are the important cause of the ineffectiveness of a wide range of antibiotics, and their detection is important to find alternatives for better treatment. [16, 17, 18]

In the hospital environment, colonized and infected patients are considered the main reservoirs of multi-resistant microorganisms harboring different resistance genes. [19] In our hospital, previous studies have reported high endemic prevalence rates of CRKP in colonization and infection, favoring its dissemination in the environment. [20]

In this study, we observed a polyclonal outbreak with two predominant clones involving CRKP and a rapid expansion of these clones to multiple patients. Notably, there was a rapid initial spread of the pathogen, however, the drop in cases in December indicates that transmission was being contained, which brought a false sense of security, leading to a loosening of measures and consequently an increase in cases in January/2024.

However, this part efforts to improve the situation it was necessary to close the NICU to new admissions and contain the outbreak. Unfortunately, we must assume that the spread of this strain was due to poor compliance with prevention and control measures, especially failures in hand hygiene and contact precautions. This was probably also the cause that other patients outside the NICU have been affected by *K. pneumoniae* presenting the same clone observed in neonates.

Upon identifying the lack of control of the outbreak, the hospital intensified the implementation of some containment measures to control the situation, such as providing the immediate closure of the unit for new admissions, carrying out urgent repairs to the air conditioning system, relocating affected patients, and reinforcing a deep cleaning with ATP testing (detection of adenosine triphosphate), as well as contact precautions and hand hygiene. All neonates were tested and bathed with chlorhexidine, and the staff was trained in safety measures. After the joint implementation of these measures, the outbreak was contained.

CONCLUSIONS

In conclusion, this study described an outbreak with two predominant clones of CRKP that mainly produced *bla*$_{\text{KPC,}}$ spreading rapidly among infected and colonized

neonates and persisted for a relatively long time in the NICU of a comprehensive hospital in Brazil.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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Table I. Primers used for amplification of resistance genes from *Klebsiella pneumoniae* samples by Polymerase Chain Reaction

Genes groups targeted	Primer	Sequence	Gene	Pb	Annealing temperature	Reference
Carbapenemase gene	KPC-F*	CGTCTAGTTCTGCTGTCTTG	bla_{KPC}	798	55°C	[21]
	KPC-R†	CTTGTCATCCTTGTTAGGCG				
β-lactamase genes	NDM-F	GGTTTGGCGATCTGGTTTTC	bla_{NDM}	621	55°C	[21]
	NDM-R	CGGAATGGCTCATCACGATC				
	CTXM-Gp1-F	TTAGGAAATGTGCCGCTGTA	$bla_{CTXM-Gp1}$	688	60°C	[22]
	CTXM-Gp1-R	CGATATCGTTGGTGGTACCAT				

*F: forward; †R: reverse

Table II. Reagents for the Polymerase Chain Reaction for gene detection and amplification for gene sequencing

Reagents	Reaction volume for gene detection	Reaction volume for gene sequencing
DNA	1.0 µL	1.0 µL
primer forward (10µM)	0.75 µL	0.9 µL
primer reverse (10µM)	0.75 µL	0.9 µL
GoTaq Green Master Mix, 2x	12.5 µL	15.0 µL
Sterile ultrapure water	10.0 µL	12.2 µL
Final volume	25.0 µL	30 µL

Table III. Relationship between the hospitalization period and the quantity of infections/colonization and genes presented in the NICU.

Month/Year	Mean LOH*	N of patients	Infection/Colonization	Isolates tested/*bla*KPC+/*bla*NDM+/ *bla*CTXMGP-1+/String Test+
Jul/2023	118.0	1	0/1	0/0/0/0/0
Aug/2023	0.0	0	0/0	0/0/0/0/0
Sep/2023	69.5	4	1/3	3/1/0/1/0
Oct/2023	97.4	4	1/3	2/0/0/0/0
Nov/2023	45.3	9	2/8†	3/1/1/0/0
Dec/2023	6.0	1	1/0	1/1/0/0/0
Jan/2024	15.5	11	6/6†	2/1/0/1/0
Feb/2024	4.5	4	1/3	3/2/0/0/1

*LOH: Length of hospitalization; †Patients with infections and colonization.

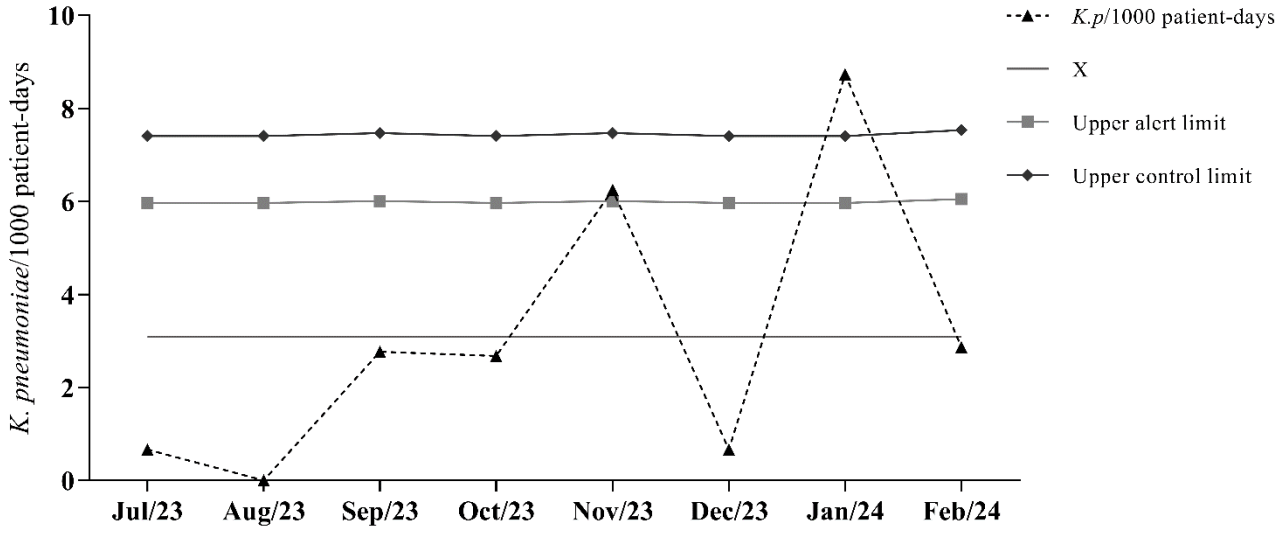

Figure 1. Endemic level of carbapenem-resistant *Klebsiella pneumoniae* infections per 1000 patient-days in neonates from July 2023 to February 2024. Upper Alert Limit (X + 2(σ)); Upper Control Limit (X + 3(σ)); X, central line (mean of infections: 3.23); σ: standard deviation.

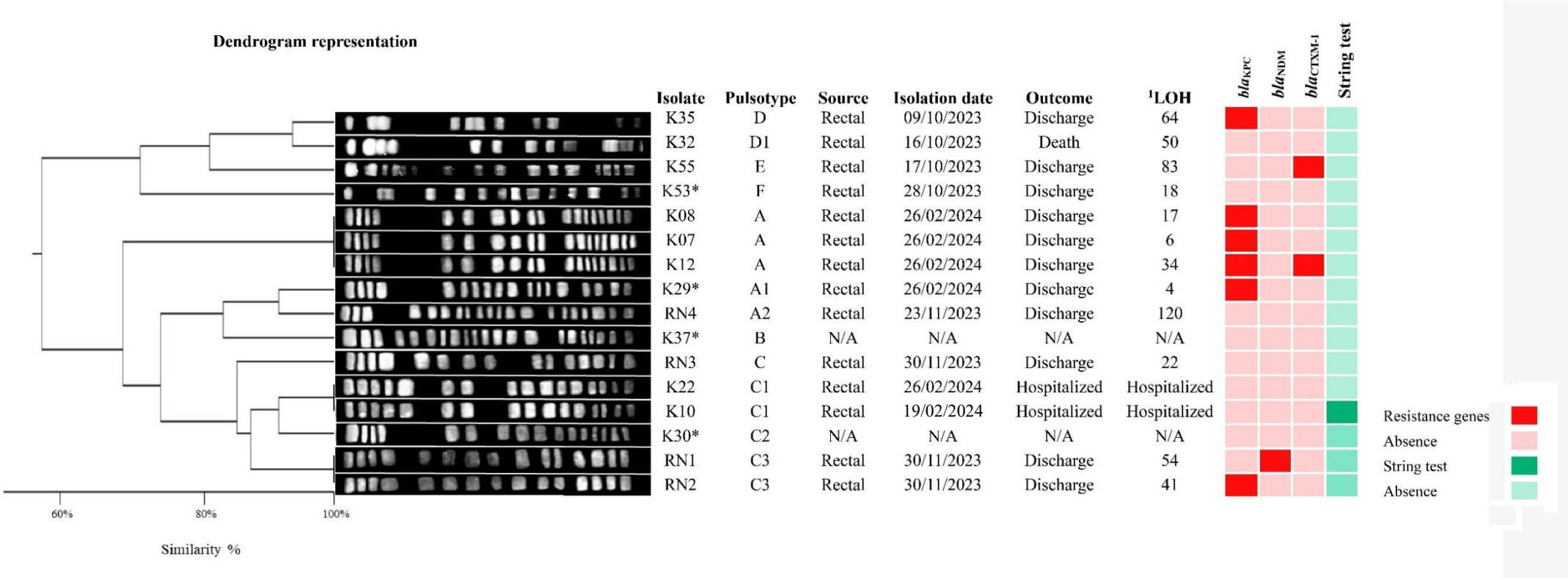

Figure 2. Dendrogram generated by computer analysis of clonal DNA profiles from 16 clinical samples of *Klebsiella pneumoniae* based on pulsed-field gel electrophoresis (PFGE) and heatmap showing resistance genes. The analysis was carried out using the method of Dice/UPGMA (similarity ≥ 80%). [1]LHO: Length of hospitalization, days; *adult patients; N/A: not answered.