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ARTIGO ORIGINAL

High-risk clones of ESBL-producing *Klebsiella pneumoniae* colonizing ICU patients in Natal, northeastern Brazil

Clones de alto risco de Klebsiella pneumoniae produtores de ESBL colonizando pacientes de UTI em Natal, Nordeste do Brasil

Clones de alto riesgo de Klebsiella pneumoniae productores de BLEE que colonizan pacientes de UCI en Natal, noreste de Brasil

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ABSTRACT

Background and objectives: colonization by extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* in Intensive Care Unit (ICU) patients is considered a risk factor for infections, and poses as a source of spreading these strains in hospital facilities. This study aimed to perform the genetic characterization of ESBL-producing *K. pneumoniae* isolates recovered from surveillance swabs in an ICU in northeastern Brazil. **Methods:** the isolates were recovered between 2018-2019 from the nasal, axillary, and rectal sites of 24 patients admitted to the ICU. Bacterial identification was performed by traditional biochemical tests. Antimicrobial susceptibility was assessed by disk

diffusion, and ESBL phenotype was detected by double-disc synergy test. Polymerase chain reaction (PCR) for *bla*$_{CTX-M}$, *bla*$_{SHV}$, and *bla*$_{TEM}$ genes, PFGE, and MLST were carried out in representative isolates. **Results:** a total of 27 isolates were recovered from 18 patients (75%). The ESBL production was detected in 85% of isolates. Resistance to ciprofloxacin, sulfamethoxazole/trimethoprim and most of the β-lactams tested was recurrent, except for carbapenems. The *bla*$_{SHV}$, *bla*$_{TEM}$, and *bla*$_{CTX-M}$ genes were found in high frequency, and the CTX-M-(1, 2 and 9) groups were identified. Seven sequence types (ST11, ST14, ST17, ST395, ST709, ST855, and ST3827) were described, most of them considered high-risk. **Conclusion:** these findings emphasize the potential threat of well-established high-risk clones in an ICU, and highlight the importance of monitoring these clones to prevent infections.
Keywords: *Klebsiella pneumoniae*. *Intensive Care Unit*. *Infection Control*. *Drug-resistance*. *Beta-lactamases*.

RESUMO

Justificativa e objetivos: a colonização por *Klebsiella pneumoniae* produtora de β-lactamase de espectro estendido (ESBL) em pacientes de Unidade de Terapia Intensiva (UTI) é considerada um fator de risco para infecções, e representa uma fonte de disseminação dessas cepas em instalações hospitalares. Este estudo objetivou realizar a caracterização genética de isolados de *K. pneumoniae* produtores de ESBL recuperados de *swabs* de vigilância em uma UTI no Nordeste do Brasil. **Métodos:** os isolados foram recuperados entre 2018-2019 dos sítios nasal, axilar e retal de 24 pacientes internados na UTI. A identificação bacteriana foi realizada por testes bioquímicos tradicionais. A suscetibilidade antimicrobiana foi avaliada por disco-difusão, e o fenótipo ESBL foi detectado pelo teste de sinergia de duplo-disco. *Polymerase chain reaction* (PCR) para os genes *bla*$_{CTX-M}$, *bla*$_{SHV}$ e *bla*$_{TEM}$, PFGE e MLST foram realizados em isolados representativos. **Resultados:** foram recuperados 27 isolados de 18 pacientes (75%). A produção de ESBL foi detectada em 85% dos isolados. A resistência à ciprofloxacina, sulfametoxazol/trimetoprima e à maioria dos β-lactâmicos testados foi recorrente, exceto para os carbapenêmicos. Os genes *bla*$_{SHV}$, *bla*$_{TEM}$ e *bla*$_{CTX-M}$ foram encontrados em alta frequência, e os grupos CTX-M-(1, 2 e 9) foram identificados. Sete *sequence types* (ST11, ST14, ST17, ST395, ST709, ST855 e ST3827) foram descritos, a maioria deles considerados de alto risco. **Conclusão:** esses achados enfatizam a ameaça potencial de clones de alto risco bem estabelecidos em uma UTI, e destacam a importância do monitoramento desses clones para prevenir infecções.
Palavras-chave: *Klebsiella pneumoniae*. Unidade de Terapia Intensiva. Controle de Infecção. Resistência à Droga. Beta-lactamases.

RESUMEN

Justificación y objetivos: la colonización por *Klebsiella pneumoniae* productora de β-lactamasas de espectro extendido (BLEE) en pacientes de Unidades de Cuidados Intensivos (UCI) se considera un factor de riesgo para infecciones, y se presenta como una fuente de propagación de estas cepas en instalaciones hospitalarias. Este estudio tuvo como objetivo realizar la caracterización genética de aislamientos de *K. pneumoniae* productores de BLEE recuperados de hisopos de vigilancia en una UCI en el noreste de Brasil. **Métodos:** los aislamientos se recuperaron entre 2018-2019 de sitios nasales, axilares y rectales de 24 pacientes ingresados en la UCI. La identificación bacteriana se realizó mediante pruebas bioquímicas tradicionales. La susceptibilidad antimicrobiana se evaluó mediante difusión en disco, y el fenotipo BLEE se detectó mediante la prueba de

sinergia de doble-disco. La *polymerase chain reaction* (PCR) para los genes *bla*$_{CTX-M}$, *bla*$_{SHV}$ y *bla*$_{TEM}$, PFGE y MLST se llevaron a cabo en aislamientos representativos. **Resultados:** se recuperaron 27 aislamientos de 18 pacientes (75%). La producción de ESBL se detectó en 85% de los aislamientos. La resistencia a ciprofloxacino, sulfametoxazol/trimetoprima y a la mayoría de los β-lactámicos evaluados fue recurrente, excepto a los carbapenémicos. Los genes *bla*$_{SHV}$, *bla*$_{TEM}$ y *bla*$_{CTX-M}$ se encontraron en alta frecuencia, y se identificaron los grupos CTX-M-(1, 2 y 9). Se describieron siete *sequence types* (ST11, ST14, ST17, ST395, ST709, ST855 y ST3827), la mayoría consideradas de alto riesgo. **Conclusión:** estos hallazgos enfatizan la amenaza potencial de los clones de alto riesgo bien establecidos en una UCI, y resaltan la importancia de monitorear estos clones para prevenir infecciones.
Palabras clave: *Klebsiella pneumoniae*. Unidad de Cuidados Intensivos. Control de Infección. Resistencia a Medicamentos. Beta-lactamasas.

INTRODUCTION

The *Klebsiella pneumoniae* complex consists of closely related species standing as opportunistic pathogens and related to a wide range of infections, including urinary tract infections, bloodstream infections, and pneumonia, mainly among hospitalized and immunocompromised patients.[1] In the last 30 years, *K. pneumoniae* has become a major cause of healthcare-related infections globally, especially due to its ability to acquire and transfer antimicrobial resistance mechanisms.[2] Brazilian epidemiological and surveillance data highlight *K. pneumoniae* as the most reported Gram-negative microorganism associated with laboratory-confirmed bloodstream infections in adult Intensive Care Units (ICUs) between 2018 and 2019, along with the extended-spectrum β-lactamase (ESBLs) production as the most prevalent phenotype.[3]

The spread of plasmid-mediated ESBLs conferring resistance to oxyimino-cephalosporins and monobactams are considered a major threat to global public health, and have been increasingly described among members of Enterobacterales, including *K. pneumoniae.*[4,5] Detection rates of ESBL-producing *K. pneumoniae* strains reach up to 80% in some countries, and have been associated with enhanced risk of treatment failure, patient mortality, and hospital costs.[4] In Brazil, over 70% of *K. pneumoniae* isolates recovered from infected patients were ESBL producers.[3]

Regardless of the infection site, the first stage in some hospital-acquired infections caused by *K. pneumoniae* consists of colonization in patients' gastrointestinal tract, which is important, as it may precede and possibly serve as a source of subsequent clinical infection for patients as well as pose as a reservoir within healthcare institutions.[1,6]

Multidrug-resistant (MDR) *K. pneumoniae* colonizing patients admitted to ICUs is considered a significant risk factor for subsequent infection. Patients who carry this pathogen are more likely to progress to infection when compared to non-carriers.[1,6] Furthermore, colonization and subsequent infection may be concerning due to the dissemination and establishment of MDR high-risk clones in healthcare facilities, especially in ICUs.[7]

Most surveillance research using genotyping prioritizes strains originating from sites of infection rather than colonization. However, screening of patients at risk for colonization along with the genetic characterization of ESBL-producing *K. pneumoniae* isolates may assist infection control programs.[8] In this regard, the present study describes the phenotypic and genotypic features of ESBL-producing *K. pneumoniae* isolates recovered from surveillance cultures of ICU inpatients in a tertiary hospital in the city of Natal, northeastern Brazil.

METHODS

Study design and bacterial identification

This is a descriptive study of resistance surveillance conducted with ICU inpatients at a referral hospital for infectious diseases, located in the city of Natal, Rio Grande do Norte State, Brazil, from August 2018 to March 2019. A total of fifty-four samples were obtained from nasal (n=24), axillary (n=24), and rectal (n=6) swabs that were collected from 24 ICU inpatients that were admitted to hospital for at least seven days, regardless of age and clinical conditions. The present study was approved by the Research Ethics Committee of the *Universidade Federal do Rio Grande do Norte*, under CAAE (*Certificado de Apresentação para Apreciação Ética* - Certificate of Presentation for Ethical Consideration) 45184115.5.0000.5537.

For collection, the swabs were soaked in sterile saline solution (0.85%) and were introduced 1 cm into the nostril or rectum with circular movements. For the axillary site, swabs were rubbed in the armpit with rotational movements, comprising 2 cm^2 of area, approximately. After collection, swabs were transported in a Cary-Blair transport medium (HiMedia, India) for further processing at the *Universidade Federal do Rio Grande do Norte*'s Mycobacterial Laboratory. Swabs were transferred to Brain Heart Infusion (BHI) broth (Sigma-Aldrich, United States) and incubated at 35ºC for eight hours, followed by culture in 5% Sheep Blood Agar and MacConkey Agar (KASVI, Brazil) media, and incubation at 35ºC for 24 hours.

K. pneumoniae isolates were identified through the macroscopic characteristics of the colonies, Gram stain, and classical biochemical tests, such as growth on triple sugar-iron (TSI) agar, sulfide and indole production, motility, citrate use, lysine, ornithine, and arginine decarboxylation, urease production, phenylalanine deaminase test.[9] Afterwards, isolates were stored at -20ºC in BHI broth with 10% glycerol and 10μg/ml of ampicillin.

Phenotypical and molecular antimicrobial susceptibility-related assays

Antimicrobial susceptibility test was determined by disk diffusion method for amikacin (30 μg), amoxicillin/clavulanate (30 μg), aztreonam (30 μg), cefepime (30 μg), cefotaxime (30 μg), ceftazidime (30 μg), ceftriaxone (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), imipenem (10 μg), meropenem (10 μg), sulfamethoxazole/trimethoprim (25 μg), and tetracycline (30 μg). Methodology and interpretation were performed according to the Clinical and Laboratory Standards Institute breakpoints.[10] ESBL phenotypical detection was performed using the double-disc synergy test (DDST) described elsewhere.[11] *Escherichia coli* ATCC 25922 was used as a control for both assays.

Eleven non-repetitive (one per selected patient) isolates exhibiting phenotypic resistance to 3rd generation cephalosporins were randomly selected for further genetic characterization, following a criterion of equal representation within the collection period. (KP02-JOVN, KP03-LFN, KP09-FMSN, KP12-MFON, KP14-RHRSN, KP17JMN from nasal site; KP05-ABAJA, KP10-JFSA, KP11-FHSA, KP15-JAA from axillary site; and KP20-FNCR from rectal site). Molecular detection of ESBL-related genes - $bla_{\text{CTX-M}}$, and $bla_{\text{CTX-M-1}}$, $bla_{\text{CTX-M-2}}$, $bla_{\text{CTX-M-8}}$, $bla_{\text{CTX-M-9}}$ groups,[12,13] bla_{SHV}, and bla_{TEM} [14] – was performed by polymerase chain reaction (PCR) according to the references, following quality patterns and using internal control strains.

Genotyping by pulsed-field gel electrophoresis (PFGE) and multilocus sequence typing (MLST)

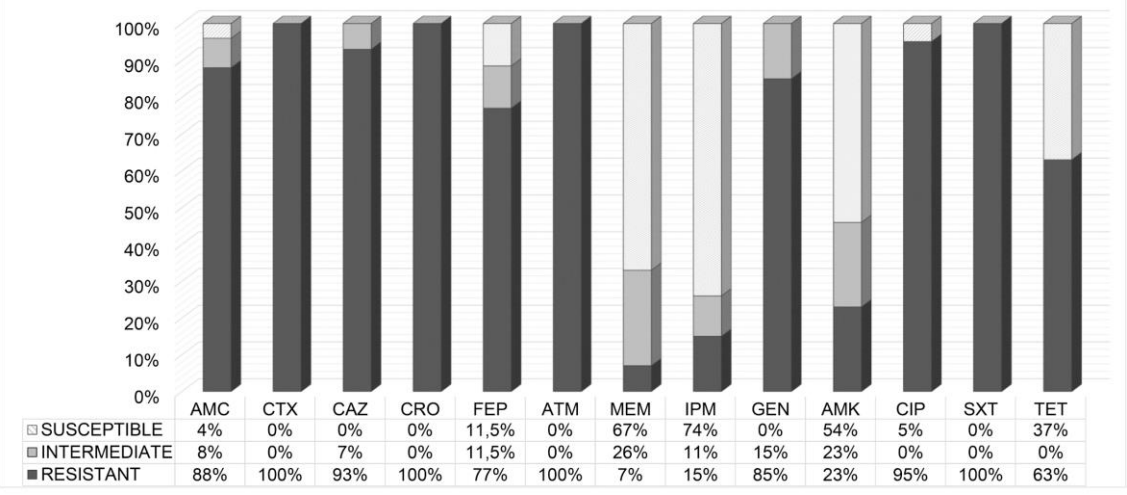
Genetic relatedness of isolates was investigated by PFGE following an adapted protocol.[15] Briefly, genomic DNA was digested with *XbaI*, and fragments separated in 1% agarose gel were submitted to electrophoresis for 23 hours using a CHEF-DR III apparatus (Bio-Rad), with pulses varying from 0.5 to 35 seconds at a voltage of 6 V/cm. After staining with ethidium bromide (0.5 mg/mL), the gels were examined using the GelJ v.2.0. software. Similarity among isolates was estimated using the Dice coefficient with a 1.0% tolerance setting and 85% similarity cut-off.[16]

MLST was performed as described by the Institute Pasteur protocol (https://bigsdb.pasteur.fr/klebsiella/). The housekeeping genes (*gapA*, *infB*, *mdh*, *pgi*, *phoE*, *rpoB*, and *tonB*) were amplified by PCR. Sequencing of PCR products was performed using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Life Technologies) on an ABI Prism 3130 Genetic Analyzer (Applied Biosystems). Determination of allele profiles and sequence types (STs) was achieved by comparing the obtained sequences with the documented data available at the Klebsiella PasteurMLST database (https://bigsdb.web.pasteur.fr/Klebsiella/Klebsiella.html - accessed on June 08, 2022).

RESULTS

A total of 27 isolates identified as *K. pneumoniae* complex were recovered from 18 patients (75%; 18/24), including five from rectal (19%), 10 from nasal (37%), and 12 from axillary (44%) sites. For eight patients, it was possible to recover more than one isolate. All isolates were Gram-negative, Yellow/Yellow on TSI agar, with gas production and no hydrogen-sulfide production. The Citrate, lysine, and urease were positive, and negative for indole and motility. The isolates also were non-susceptible to at least one tested 3rd generation cephalosporin; however, phenotypical detection revealed 23 ESBL-producing isolates (85%; 23/27). A total of 14 of 24 (58%) patients were colonized by ESBL-producing *K. pneumoniae*. Moreover, most isolates were resistant to ciprofloxacin, gentamicin, sulfamethoxazole/trimethoprim, and all tested β-lactams, except carbapenems and amikacin, as shown in Figure 1.

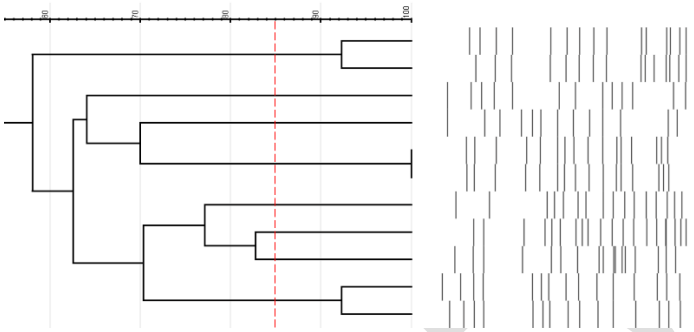
Figure 1. Antimicrobial susceptibility pattern of *Klebsiella pneumoniae* isolated from surveillance swabs in Intensive Care Unit



	AMC	CTX	CAZ	CRO	FEP	ATM	MEM	IPM	GEN	AMK	CIP	SXT	TET
SUSCEPTIBLE	4%	0%	0%	0%	11,5%	0%	67%	74%	0%	54%	5%	0%	37%
INTERMEDIATE	8%	0%	7%	0%	11,5%	0%	26%	11%	15%	23%	0%	0%	0%
RESISTANT	88%	100%	93%	100%	77%	100%	7%	15%	85%	23%	95%	100%	63%

Caption: AMC - amoxicillin/clavulanate; CTX - cefotaxime; CAZ - ceftazidime; CRO - ceftriaxone; FEP - cefepime; ATM - aztreonam; MEM - meropenem; IPM - imipenem; GEN - gentamicin; AMK - amikacin; CIP - ciprofloxacin; SXT - sulfamethoxazole/trimethoprim; TET - tetracycline.

Among the 11 isolates selected for molecular characterization, six (54%) harbored *bla*$_{\text{CTX-M}}$ genes, including *bla*$_{\text{CTX-M-1}}$ (27%), *bla*$_{\text{CTX-M-2}}$ (27%), and *bla*$_{\text{CTX-M-9}}$ (9%) groups. Furthermore, *bla*$_{\text{SHV-like}}$ and *bla*$_{\text{TEM-like}}$ were detected in eleven (100%) and six (54%) isolates, respectively. Most isolates (91%) were detected co-harboring at least two of the searched genes, including one isolate co-harboring *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-9}}$, and *bla*$_{\text{SHV-like}}$ (Figure 2).



Strain ID	Pulsotype	ST	Collection Date	bla_{CTX-M}	bla_{TEM}	bla_{SHV}
KP14-RHRSN	A	3827	10.10.18	CTX-M-Group 2 ; CTX-M-Group 9	NEG	POS
KP12-MFON	A	3827	22.8.18	CTX-M-Group 2	NEG	POS
KP15-JAA	B	855	10.9.18	CTX-M-Group 2	NEG	POS
KP03-LFN	C	17	22.8.18	NEG	NEG	POS
KP02-JVON	D	395	24.9.18	NEG	POS	POS
KP09-FMSN	D	395	24.9.18	NEG	POS	POS
KP20-FNCR	E	11	22.8.18	CTX-M-Group 1	NEG	POS
KP17-JMN	F	709	26.2.19	CTX-M-Group 1	POS	POS
KP10-JFSA	G	11	04.2.19	CTX-M-Group 1	POS	POS
KP11-FHSA	H	14	22.8.18	NEG	POS	POS
KP05-ABAJA	H	14	31.10.18	NEG	POS	POS

Figure 2. Dendrogram and genetic features of representative *K. pneumoniae* of the study
Caption: ID – identification; ST - sequence type; NEG – negative; POS – positive.

Genetic relatedness analysis by PFGE demonstrated the presence of eight pulsotypes (A to H), including indistinguishable strains on clusters A, D, and H. MLST genotyping revealed a genetic background composed of seven distinct STs (ST11, ST14, ST17, ST395, ST709, ST855, and ST3827), of which most were associated with high-risk clonal groups (CG258, CG15, and CG20) (Figure 2). Of these, ST395/D, ST3827/A, and ST14/H clones were found in different patients, the latter two with a difference between the collections of 49 and 70 days, respectively. Such data demonstrate the spread and persistence ability of these clones in ICU settings. Finally, despite the small number of samples, no predominance of any specific clone was observed, demonstrating a considerable level of genetic diversity.

DISCUSSION

Overall, studies that survey *K. pneumoniae* genotype prioritize strains from clinical samples of infected sites over those isolated from colonization samples.[17] Therefore, this study is intended to investigate the prevalence of ESBL-producing *K.*

pneumoniae and circulating clones in patients colonized in an ICU in Natal-RN to better understand the dynamics of dissemination of these clones in ICU settings.

In this study, we examined only one adult ICU, which had nine beds at the time of study collection. A previous study has already shown that adult patients admitted to hospital in critical wards such as the ICU tend to have higher rates of colonization by ESBL producers, with *K. pneumoniae* being the main microorganism involved.[18] Furthermore, colonization rates can be highly variable.[6] In this study, 58% of patients were colonized by ESBL-producing *K. pneumoniae*, by phenotypic identification. It is worth mentioning that colonization by *K. pneumoniae* was found to be a significant risk factor for later infection by the same pathogen. In other studies, with patients admitted to the ICU, most asymptomatic carriers developed infection by this microorganism when compared to non-carriers.[6]

Isolation rates from the rectal (5/6 – 83%) site were higher when compared with the axillary (12/24 - 50%) and nasal (10/24 - 42%) sites. This finding was expected since the surveillance screening of stool specimens, rectal swabs, or perirectal swabs may produce a greater yield of bacterial growth than other body sites such as nostrils or skin.[19] In addition, from colonization of the patient's gastrointestinal tract, contamination of different parts of the body will likely occur through exogenous or endogenous processes.[20] Even so, the colonization of the axillary and nasal sites was considered high, this leads us to the hypothesis that there is high fecal contamination in this ICU, with intense cross-transmission, and precarious hygiene care for bedridden patients.

Regarding the ESBL testing, sometimes, accuracies could be impaired in organisms with multiple β-lactamase enzymes, mainly the coexistence of enzymes from different Ambler classes.[21] Resistance to other antimicrobial classes was common in this study, mainly to ciprofloxacin and tetracycline. ESBL producers are often co-resistant to multiple antibiotic classes and heavy metals, due to the concomitant presence of these genes in the same plasmid, which could improve bacterial fitness.[18]

Although carbapenems were the drugs with the best activity against the isolates in this study, as seen in Figure 1, resistance to these drugs has grown in bacteria that cause hospital-acquired infections, increasing the need to implement strategies to reduce the excessive consumption of carbapenems.[22] Therefore, surveillance of these patients at risk for ICU-acquired infections caused by ESBL-producers can be an important auxiliary measure.

Among the ESBL enzymes, the most frequent in *K. pneumoniae* belong to the plasmid-mediated TEM, SHV, and CTX-M enzyme families.[7] The TEM and SHV families have many variants with a different spectrum of activity, but not all have the ESBL phenotype.[23] In this work, the presence of these genes was identified in many isolates, although the variants were not identified. Some isolates with the ESBL phenotype carried bla_{TEM} and bla_{SHV} and lacked $bla_{\text{CTX-M}}$, suggesting that the TEM and SHV enzymes present could have ESBL activity, or even other uninvestigated enzymes could be responsible for this phenotype. The CTX-M enzymes have a significant hydrolytic capacity for 3rd generation cephalosporins, and can be clustered into five groups, CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25, with representatives of groups 1 and 9 being the most prevalent in the world, while group 2 is frequently reported in Latin America,[23] as confirmed in this study.

The emergence, persistence, and dispersion of certain resistance mechanisms, such as ESBL, are enhanced by the presence of so-called MDR high-risk clones.[24] Of the 11 representative isolates, nine belong to high-risk clones spread worldwide: ST11, ST395, ST855 (CG258), ST14, ST709 (CG15), and ST17 (CG20). All these CGs are known to be associated with multidrug resistance, including carrying genes encoding ESBL and carbapenemases.[7] In Brazil, these clones (mainly CG258) are endemic and associated with the presence of ESBL genes and carbapenemases such as *Klebsiella pneumoniae* carbapenemase (KPC) and New-Delhi Metallo-β-lactamase (NDM).[17,25,26] Besides that, isolates belonging to ST11 were the only ones grouped into two pulsotypes (E and G); this intraclonal variability is a phenomenon commonly described in ST11.[17]

ST3827 was the only non-high-risk clone. Isolates belonging to this clone have already been reported carrying $bla_{\text{SHV-11}}$ in food samples from Europe [27] and $bla_{\text{CTX-M-15}}$ isolated from a native Amazonian fish in Brazil.[28] As far as we know, this is the first time this clone has been associated with human colonization.

Two points deserve to be emphasized - the hospital in which the study was conducted did not carry out surveillance of colonization by MDR bacteria in patients admitted to hospital at the time of the study. Furthermore, this study was performed one year before the spread of the COVID-19 pandemic in Brazil. Therefore, this study could serve as a basis for future adaptations to local measures to prevent and control infections and install protocols of hygiene and as a comparison of the clonal population of *K. pneumoniae* before and after the pandemic period.

It is worth indicating the limitations of this study. Firstly, the low number of rectal swabs occurred because collections were often performed after patient diaper changes; therefore, due to hospital constraints, there were no possibilities for a second diaper change on the same day. Secondly, a random selection of isolates for molecular characterization was necessary due to budgetary constraints of the involved laboratories.

Finally, this study identified a high rate of ESBL-producing *K. pneumoniae* isolates colonizing ICU patients, which suggests reduced hygiene care, allowing intense cross-transmission. Moreover, most of the isolates belonged to high-risk clones and carried highly disseminated resistance determinants, such as $bla_{\text{CTX-M}}$, which increases the risk of possible infections for patients admitted to hospital. These findings warn of the intensification of hygiene, infection control, and surveillance measures as well as monitoring the dynamics of these endemic clones.

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