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Expansion and Evolution of a Virulent, Extensively Drug-Resistant (Polymyxin B-Resistant), QnrS1-, CTX-M-2-, and KPC-2-Producing *Klebsiella pneumoniae* ST11 International High-Risk Clone

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In this study, we report the early expansion, evolution, and characterization of a multiresistant *Klebsiella pneumoniae* clone that was isolated with increasing frequency from inpatients in a tertiary-care university hospital in Brazil. Seven carbapenem- and quinolone-resistant and polymyxin B-susceptible or -resistant *K. pneumoniae* isolates isolated between December 2012 and February 2013 were investigated. Beta-lactamase- and plasmid-mediated quinolone resistance (PMQR)-encoding genes and the genetic environment were investigated using PCR, sequencing, and restriction fragment length polymorphism (RFLP). Clonal relatedness was established using XbaI–pulsed-field gel electrophoresis (PFGE), multilocus sequence typing (MLST), and phylogenetic group characterization. Plasmid analyses included PCR-based replicon typing (PBRT) and hybridization of the *S1*-PFGE product, plasmid MLST, and conjugation experiments. Virulence potential was assessed by PCR by searching for 10 virulence factor-encoding genes (*ureA*, *fimH*, *kfuBC*, *uge*, *wabG*, *magA*, *mrkD*, *allS*, *rmpA*, and *cf29a*) and by phenotypic tests to analyze the hypermucoviscous phenotype. The genetic context of a multidrug-resistant and extensively drug-resistant *K. pneumoniae* ST11-KpI clone harboring IncFIIk-Tn*4401a*-bla_{KPC-2}, *qnrS1*, and $bla_{CTX-M-2}$ was found. Moreover, three isolates displayed high resistance to polymyxin B (MICs = 32, 32, and 128 mg/liter) as well as mucous and hypermucoviscous phenotypes. These bacteria also harbored *ureA*, *fimH*, *uge*, *wabG*, and *mrkD*, which code for virulence factors associated with binding, biofilm formation, and the ability to colonize and escape from phagocytosis. Our study describes the association of important coresistance and virulence factors in the *K. pneumoniae* ST11 international high-risk clone, which makes this pathogen successful at infections and points to the quick expansion and evolution of this multiresistant and virulent clone, leading to a pandrug-resistant phenotype and persistent bacteria in a Brazilian hospital.

Multiresistance in Gram-negative bacilli (GNB) and the spread of resistance determinants have been great problems in the worldwide treatment of bacterial infections and are recognized as a public health problem (1). In South America, carbapenem-resistant *Enterobacteriaceae* (CRE) show relevant occurrence, and *Klebsiella pneumoniae* carbapenemase (KPC) producers seem to be the main problem (2). Recently, New Delhi metallo-beta-lactamase (NDM) producers have been detected in South America (3), including in Brazil (4), which worries the scientific and health communities. In Brazil, São Paulo metallo-beta-lactamase (SPM)-producing *Pseudomonas aeruginosa* strains are endemic carbapenem-resistant bacteria (5). In addition, since 2006, KPC-producing *K. pneumoniae* has caused outbreaks and has become one of the more prevalent multiresistant bacteria in Brazil (6). *K. pneumoniae* is an opportunistic pathogen that is highly adapted to the hospital environment and is associated mainly with pneumonia, bloodstream infections, and urinary tract infections (UTI). Little is known about the virulence potential of KPC producers, and *K. pneumoniae* is a concern (7, 8). This bacterial species shows pathogenic mechanisms involved, for instance, in escape from phagocytosis and biofilm formation. Of special concern, hypervirulent (mainly hypermucoviscous) *K. pneumoniae* strains have emerged and are capable of causing severe infections in healthy and ambulatory individuals (9–11). The *K. pneumoniae* clonal complex 258/11 (CC258/11) and the sequence type 258 (ST258) and ST11 have been detected worldwide as the main, international high-risk clones (HiRC) (12, 13) because they (i) harbor epidemic/transmissible plasmids from incompatibility group FII (IncFII), IncN, IncA/C, and IncL/M (14) and (ii) are associated with carbapenemase production, e.g., KPC and OXA-48 (13, 15). The association of genetic determinants of resistance with virulence is a problem in the treatment of infections. For instance, bacteria with multiresistance and the ability to colonize create an opportunity for the selection of highly resistant and persistent bacterial pathogens (16, 17). This study reports the early expansion, evolution, and characterization of a multiresistant *K. pneumoniae* clone that has been increasingly isolated from inpatients in a hospital from Brazil.

MATERIALS AND METHODS

Bacterial isolates. From December 2012 to February 2013, seven carbapenem-resistant, quinolone-resistant, and polymyxin B-susceptible or -re-

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TABLE 1 Patient data and phenotypic and genotypic characteristics of the QnrS1- and KPC-2-producing *K. pneumoniae* ST11-KpI clone

Isolate/patient code	Patient data					Isolate data							Plasmid analyses[c]	
							Antimicrobial susceptibility[a] MIC (mg/liter)							
	Gender[g]/age (yrs)	Subjacent disease	Hospital ward	Clinical sample	Outcome	Pulsotype	AMI	FOS	TET[b]	TGC	PB		Approx sizes (kb)	Inc group(s)/ pMLST
RP01	F/23	Encephalomyelitis	Neurology	Urine	Alive	A	16	4	**12**	**4**	≤0.5		100, 388, 450	FIIk, FIA
RP04	M/70	Psoriasis, arthritis, Parkinson's disease	Dermatology, intensive care unit	Tracheal secretion	Death	A	3	4	**12**	1	≤0.5		100, 388	FIIk
RP29	F/75	Acute myeloid leukemia	Hematology	Blood	Death	A	3	4	**12**	**4**	**32**		100, 388, 450	FIIk, FIA
RP59[d,e]	M/36	Acute myeloid leukemia	Hematology	Blood	Death	A	3	4	2	1	**32**		100, 388	FIIk
RP60	M/64	Chronic arterial disease, diabetes mellitus, renal insufficiency	General medical, cardiology	Blood	Alive	A	3	4	**12**	1	≤0.5		100, 388	FIIk
RP62[e]	F/66	Chronic arterial disease, diabetes mellitus, renal insufficiency	Geriatrics	Venous catheter	Alive	A	8	4	2	**6**	**128**		100, 388	FIIk
RP66	M/55	Aortic aneurysm (surgery)	Vascular, intensive care unit	Urine	Death	A1[f]	3	4	**12**	1	≤0.5		100, 388	FIIk

[a] Antimicrobial testing and interpretation were performed according to the CLSI criteria of 2012, except for polymyxin B (criteria for colistin were used) and tigecycline, in which the EUCAST criteria of 2012 were applied. MICs in boldface represent resistant or intermediate results. AMI, amikacin (susceptible [S] ≤ 16, intermediate [I] = 32, resistant [R] ≥ 64); FOS, fosfomycin (S ≤ 64, I = 128, R ≥ 256); TET, tetracycline (S ≤ 4, I = 8, R ≥ 16); TGC, tigecycline (S ≤ 1, R > 2); PB, polymyxin B (S ≤ 2, R > 2). For all isolates, MICs of ≥256 (resistant to), piperacillin-tazobactam, cefotaxime, ceftazidime, cefoxitin, cefepime, aztreonam, gentamicin, and chloramphenicol; MIC of ≥32 (resistant to), trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, levofloxacin, moxifloxacin, ertapenem, imipenem, meropenem, and doripenem.

[b] Intermediate MICs were considered as nonsusceptible.

[c] Underlining indicates size and Inc group of the plasmid carrying the bla_{KPC-2} gene.

[d] Hypermucoviscous phenotype.

[e] RP59 and RP62 did not harbor the $bla_{CTX-M-2}$ gene.

[f] Pulsotypes were defined by capital letter A. Subtype (A1) was designated by number 1, indicating the number of bands that differed from the index isolate (RP01).

[g] F, female; M, male.

sistant *K. pneumoniae* strains were isolated from a tertiary-care university hospital in Ribeirão Preto, São Paulo, Brazil. The isolates were selected during a routine surveillance project conducted in the clinical microbiology laboratory at the hospital to screen for multiresistant *Enterobacteriaceae*, based on the antimicrobial susceptibility profile (18). Initially, bacteria were identified, and antimicrobial susceptibility was tested using the Vitek2 system (bioMérieux). All *K. pneumoniae* isolates were from inpatient clinical samples (Table 1) and were designated RP01, RP04, RP29, RP59, RP60, RP62, and RP66. This study was authorized by the research ethics committee, CEP/FCFRP 100/2011. *K. pneumoniae* ST258-KpA harboring IncFII-Tn*4401a*-bla_{KPC-2}, *K. pneumoniae* ST437-KpD harboring IncN-Tn*4401b*-bla_{KPC-2} (19), *Escherichia coli* ATCC 25922, and azide-resistant *E. coli* J53 (20) were used as control strains for the experiments. Control strains for incompatibility groups from plasmids were also used (21).

Determination of the MICs. The MICs of antimicrobials tested against the studied bacteria were evaluated using Etest strips (bioMérieux) according to the manufacturer's instructions. The beta-lactam antimicrobials piperacillin-tazobactam, cefotaxime, ceftazidime, cefoxitin, cefepime, aztreonam, ertapenem, imipenem, meropenem, and doripenem and the non-beta-lactam antimicrobials gentamicin, amikacin, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, ciprofloxacin, levofloxacin, moxifloxacin, tetracycline, and tigecycline were evaluated. In addition, the MIC values were determined using the agar dilution method and criteria applied for *E. coli* isolated from the urinary tract. For these antimicrobials, the interpretation of susceptibility profiles was performed using breakpoints recommended by the Clinical and Laboratory Standards Institute (CLSI) in 2012. Moreover, polymyxin B MIC values were determined using the broth dilution method and then realized and interpreted according to the recommendation of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in 2012.

Investigation of resistance genes and genetic environment. The beta-lactamase-encoding genes were investigated using PCR and sequencing, with searching for bla_{KPC}, bla_{GES}, bla_{SPM}, bla_{IMP}, bla_{VIM}, bla_{NDM}, bla_{OXA-48}, bla_{OXA-58}, and bla_{OXA-23} (coding for carbapenemases) (22, 23), bla_{CTX-M}, bla_{TEM}, bla_{SHV}, and bla_{BES} (coding for extended-spectrum beta-lactamases [ESBLs]) (20), and bla_{CMY} (coding for AmpC) (24). Moreover, plasmid-mediated quinolone resistance (PMQR) was verified by PCR and sequencing based on the presence of the *qnrA*, *qnrB*, *qnrS*, *qepA*, and *aac(6′)-Ib-cr* genes (25). The investigation of the genetic environment of the bla_{KPC} genes was performed as previously described (26, 27) by searching for the classical transposon Tn*4401* using the long-PCR approach. In addition, the *tnp*R-*tnp*A, IS*Kpn7*-bla_{KPC}, and bla_{KPC}-IS*Kpn6* amplicons, which were obtained using long-PCR of Tn*4401*, were analyzed by restriction fragment length polymorphism (RFLP) after enzymatic restriction using the PstI, BamHI, and HindIII enzymes according to the manufacturer's instructions. The generated Tn*4401*-RFLPs were compared among the studied isolates and compared to control strains.

Plasmid analyses and conjugation experiments. Plasmids were searched following the PCR-based replicon typing (PBRT) scheme (21) targeting replicons of the major Inc plasmids occurring in *Enterobacteriaceae*. Besides S1-nuclease digestion followed by pulsed-field gel electrophoresis (*S1*-PFGE), Southern blotting and hybridization with specific probes were performed to search for the bla_{KPC-2} gene in plasmid as well as to identify the incompatibility group of the plasmid (detected in the PBRT scheme) carrying bla_{KPC-2}. In addition, the *S1*-PFGE approach was able to determine number and size of the plasmid content from studied *K. pneumoniae* isolates. Conjugation experiments to transfer genes coding for beta-lactamases and PMQR harbored in conjugative or cotransferred plasmids were performed by liquid-medium mating (20) using azide-resistant *E. coli* J53 (which is lactose fermentation positive and plasmid-free). Transconjugants were selected using sodium azide (100 mg/liter) and ceftazidime (2 mg/liter) for beta-lactamase-encoding genes or nalidixic acid (6 mg/liter) for PMQR-encoding genes. Transconjugants were analyzed by PCR with searching for bla_{KPC} and the PMQR genetic determinants. Moreover, the IncFII-like plasmids from the transconjugants

were typed according to protocols for multilocus sequence typing of plasmids (pMLST) (http://pubmlst.org/plasmid). In addition, to investigate the transferability of resistance to tigecycline and polymyxins, the transconjugants were also searched using tigecycline (0.1 mg/liter) and polymyxin B (0.4 mg/liter). Plasmid analyses were also used in the transconjugant strain to confirm the results.

Bacterial clonal relatedness. Bacterial clonal relatedness was established by XbaI genome enzymatic macrorestriction followed by PFGE using the CHEF DRIII PFGE system (Bio-Rad), and the results were analyzed using the Tenover criteria (28). MLST was also performed according to the *K. pneumoniae* MLST website (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html) to determine phylogenetic relationships. In addition, phylogenetic group characterization was performed (29).

Investigation of virulence context. For virulence investigation, PCR assays were performed to search for 10 virulence factor-encoding genes (*ureA*, *fimH*, *kfuBC*, *uge*, *wabG*, *magA*, *mrkD*, *allS*, *rmpA*, and *cf29a*) that have been associated with the virulence phenotype in *K. pneumoniae* (9). In addition, the hypermucoviscous phenotype was tested by evaluating the formation of a viscous string (positive test, >0.5 cm in length) that was stretched using an inoculation loop (30).

RESULTS

Patient data and clinical outcome. The patients were primarily elderly (median age of 55 years, range of 23 to 75 years) and severely ill, with multiple underlying diseases (Table 1). All had prolonged length of stay (median of 68 days, range of 30 to 217 days) and had been submitted to multiple invasive procedures, such as central venous catheterization, mechanical ventilation, and urinary catheterization. Four of the seven patients also had nosocomial infections due to bacterial species other than carbapenem and quinolone-resistant *K. pneumoniae*. Moreover, multiple antimicrobials were used in the course of their hospital stay.

Antimicrobial resistance profile and resistance genetic context. Three isolates displayed resistance to polymyxin B (*K. pneumoniae* RP29, -59, and -62, with MICs of 32, 32, and 128 μg/ml, respectively), whereas other bacteria were polymyxin B susceptible (RP01, -04, -60, and -66). Moreover, a heteroresistance phenomena, characterized here as pinpoint bacterial colonies within an antimicrobial inhibition zone, was observed for at least one cephalosporin, cephamycin, or carbapenem (for all isolates), and antimicrobial susceptibility was observed only to amikacin and fosfomycin (for all isolates) and tetracycline (RP59 and -62) and tigecycline (RP04, -59, -60, and -66) (Table 1).

The bla_{KPC-2}, bla_{TEM-1}, bla_{SHV-1}, bla_{OXA-9}, and *qnrS1* genes were detected in all isolates. In addition, $bla_{CTX-M-2}$ (except for RP59 and -62) was found. The investigation of the genetic environment of KPC-2-encoding genes demonstrated that bla_{KPC-2} was located on transposon Tn*4401* variant "a."

Transferability characteristics. Tn*4401a*-bla_{KPC-2} was harbored on an ~100-kb IncFII plasmid. bla_{KPC-2} was transferred to a recipient strain, yielding transconjugant bacteria that were resistant to all beta-lactam antimicrobials and maintained susceptibility to all non-beta-lactam antimicrobials. Nevertheless, $bla_{CTX-M-2}$ and *qnrS1* were not transferred to a recipient strain, and no transconjugant resistance to tigecycline or polymyxin was recovered. Moreover, the IncFII-like plasmids were determined by pMLST as FIIk, which is a virulence plasmid from *K. pneumoniae* (Table 1).

Bacterial clone and virulence potential. The XbaI-PFGE pulsotypes A and A1, which are the same sequence type, ST11, and the phylogenetic group KpI were found (Table 1).

Five virulence-encoding genes were detected (*ureA*, *fimH*, *uge*, *wabG*, and *mrkD*) in all isolates. Furthermore, superior mucosity was observed in RP29, and a hypermucoviscous phenotype was detected in the RP59 bacteria; however, these facts were observed after initial isolation of the bacterium, and, thereafter, these characteristics decreased.

DISCUSSION

The fact that different susceptibility profiles were displayed by bacteria belonging to the same clone (ST11; KpA and KpA1 pulsotypes) is intriguing; however, mutations, altered permeability, and regulation of gene expression could be factors responsible for this phenomena. It is worrying that *K. pneumoniae* RP29 displayed an extensively drug resistance (XDR) phenotype (18) because it is a dangerous and potentially pandrug-resistant (PDR) pathogen. Moreover, the other studied bacteria also presented a multidrug resistance (MDR) phenotype (18).

The heteroresistance phenomena (31–33) that was observed in the studied isolates and mainly in transconjugants may be explained by genetic factors associated with carbapenem resistance (34) and/or the role that alterations in porin expression play in the resistance level and in MIC values (35), which is becoming a problem for microbiological diagnostic laboratories and physician initiatives. The IncFIIk-Tn*4401a*-bla_{KPC-2} genetic context was able to be transferred to recipient bacteria, showing that the bla_{KPC-2} genetic determinant of resistance to carbapenems may be easily disseminated and representing a danger due to its presence in virulence plasmids that can be present alone or coreside and be compatible with other FII-positive resistance plasmids within the same bacterial cell. Furthermore, Tn*4401* is the most common genetic environment that supports bla_{KPC} genes, which may also be easily recombined with diverse incompatibility groups of plasmids in *Enterobacteriaceae* and nonfermenting Gram-negative bacilli (36, 37). However, different platforms also support bla_{KPC} worldwide (34, 38, 39), including in Brazil (19, 27). Tn*4401* variants "a" and "c" were related (19, 40), and the "b" variant has been described as predominant in *K. pneumoniae* (41) and in other genera and species (19, 42) from Brazil. The Tn*4401* variant "a" has been detected since 2007 (19) in hospitals and seems to be the single genetic environment/variant that supports bla_{KPC-2} in this health care center.

The absence of transconjugant bacteria resistant to tigecycline or polymyxin B indicates chromosome-encoded resistance, which is also suggested by the different MIC values for tigecycline and polymyxin B and by the plasmid content of the isolates. High MIC values were found to polymyxin B (Table 1), and the resistance mechanism has been described in *K. pneumoniae*, mainly as an increased production of capsule polysaccharide of the bacterial outer membrane (blocking the target site of antimicrobial action). However, bacteria may develop polymyxin resistance due to a mechanism that involves modification of the bacterial outer membrane, mainly through alteration of the lipopolysaccharide moiety, in the course of antimicrobial therapy (43, 44). Furthermore, the hypermucoviscous phenotype, which is related to the virulence phenotype (30) and resistance to polymyxins (44), was detected in RP59. However, this characteristic is generally present in $magA^+$ and enables the overproduction of the exopolysaccharide web, and $rmpA^+$ acts as a regulator of the mucous phenotype (9, 30), but these genes were not found in this study. Thereby, this hypermucoviscous phenotype could be regulated by other genetic

mechanisms, and the control of gene expression could play an important role in this characteristic. Polymyxin resistance is a nonfrequent phenotype in *Enterobacteriaceae*. Nonetheless, this resistance has been increasingly reported in *K. pneumoniae* and seems to be a great problem in Italy (45) and the United States (46), because it is generally associated with KPC producers and the ST258 clone.

Concerning the detected genes that code for virulence factors, *ureA* is related to the urease operon, which is involved in urea metabolism and required for efficient bacterial gastrointestinal colonization. *fimH* encodes adhesin and influences the expression of type 1 fimbriae, which mediates binding, invasion, biofilm formation, and the ability to colonize during a UTI. *uge* contributes to the expression of smooth lipopolysaccharide (LPS) with O antigen molecules and capsule polysaccharide (K antigen) on the cell surface, yielding the ability to produce a UTI and virulence during sepsis and pneumonia. *wabG* is involved in the cell attachment of capsular polysaccharide, which contributes to the biosynthesis of the core LPS and encapsulated cell, resulting in virulence. *mrkD* encodes the type 3 fimbriae adhesin, which facilitates adhesion to the basement membranes of several human tissues (9). This virulence context was also detected in Canada in KPC-2-producing *K. pneumoniae* strains that were imported from Greece (47). These virulence factors could promote and partly explain the epidemiological success of ST11. Unfortunately, these advantageous traits have not been researched extensively, and few reports regarding the pathogenic potential and virulence factors of KPC-producing *K. pneumoniae* have been published (8, 47–49), reflecting a lack of knowledge of the epidemiologic scenario and prospects for medical prognosis.

In the period of 2007 to 2011, there was a prevalence of KPC-2-producing *K. pneumoniae* ST258 causing outbreaks in the studied hospital, and only one *K. pneumoniae* ST11 isolate was detected from a colonized inpatient. Within this period, the genetic context corresponding to IncFII-Tn*4401a*-bla_{KPC2} was also detected; however, the clones were fully susceptible to polymyxin B (19), and PMQR clones were not detected. The use of polymyxins has increased in the last 10 years due to carbapenem-resistant bacteria, leading to the use of this antimicrobial category as a last-therapy option to treat patients with infections by these bacteria. Not surprisingly, in late 2011, polymyxin B-resistant *K. pneumoniae* emerged as pathogens implicated in serious infections in the studied hospital. Furthermore, when comparing the *K. pneumoniae* ST11 strain isolated in 2009 with the seven *K. pneumoniae* ST11 studied here, significant differences were observed between the detected XbaI-PFGE pulsotypes and those that acquired the *qnrS1* gene; however, the same virulence factors were found (data not shown). Other polymyxin B-resistant KPC-2-producing *K. pneumoniae* ST11 isolates have been increasingly isolated in this hospital as well as in other hospitals from the same region (data not shown). *K. pneumoniae* ST11 is broadly associated with the dissemination of bla_{KPC}, is dominant in China (50), has become endemic in Taiwan (51), and seems to be the prevalent KPC-2-producing *K. pneumoniae* clone in Brazil (41, 52, 53). Moreover, *K. pneumoniae* ST11 has been associated with outbreaks and dissemination of other carbapenemases, such as OXA-48 (54, 55) and NDM (56, 57). In Brazil, the bla_{NDM} genes were initially related to non-*K. pneumoniae* species (4, 58), and we believe that the meeting of these genes with ST11 could cause the national dissemination of NDM producers toward the endemicity of this clone in Brazil.

The management of clinical infections due to multiresistant KPC-producing *K. pneumoniae* remains a challenge. Although the isolates of this study display susceptibility to amikacin and fosfomycin (Table 1), the pharmacokinetics (PK) and pharmacodynamics (PD) of these drugs make individual usage unlikely for the successful treatment of severe infections other than those restricted to the inferior urinary tract. Because of this, the combination of two to three drugs, including amikacin, tigecycline, as well as polymyxins and meropenem, could be utilized after optimization of the use of these drugs based on the newest PK/PD perspectives (59–62). The virulence context found in these bacteria also represents a problem for medical treatment. Multiresistant KPC-2-producing *K. pneumoniae* strains were isolated from clinical samples related to UTIs, bloodstream infections, or pneumonia (Table 1), which are diseases that are certainly benefited by virulence mechanisms that contribute to the clinical outcomes of morbidity and mortality of patients infected with these bacteria. Moreover, these virulence factors improve the conditions for bacterial intestinal colonization and persistence in these patients and in the hospital.

The association of multiple resistance determinants in bacteria has been broadly reported; however, to our knowledge, no description of coresistance of bla_{KPC-2}, $bla_{CTX-M-2}$, and *qnrS1* in *K. pneumoniae* has been reported.

A virulent and multiresistant *K. pneumoniae* ST11 clone has emerged and quickly expanded in the studied hospital as well as neighboring regional health care centers. ST11 corresponds to a successful HiRC, and the coresistance (KPC-2, CTX-M-2, and QnrS1) plus high polymyxin resistance and virulence factors that were found could be a problem. This fact seems to lead to a PDR phenotype and persistent bacteria in Brazilian hospitals.

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