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Multidrug resistance genes, including *bla*$_{KPC}$ and *bla*$_{CTX-M-2}$, among *Klebsiella pneumoniae* isolated in Recife, Brazil

Adriane Borges Cabral[1], Rita de Cássia de Andrade Melo[1], Maria Amélia Vieira Maciel[1] and Ana Catarina Souza Lopes[1]

[1]. Departamento de Medicina Tropical, Universidade Federal de Pernambuco, Recife, PE.

ABSTRACT

Introduction: The prevalence of cephalosporins and carbapenem-resistant *Klebsiella pneumoniae* strains is rising in Brazil, with potential serious consequences in terms of patients' outcomes and general care. **Methods**: This study characterized 24 clinical isolates of *K. pneumoniae* from two hospitals in Recife, Brazil, through the antimicrobial susceptibility profile, analyses of β-lactamase genes (*bla*$_{TEM,}$ *bla*$_{SHV,}$ *bla*$_{CTX-M}$, *bla*$_{KPC}$, *bla*$_{VIM}$, *bla*$_{IMP,}$ and *bla*$_{SPM}$), plasmidial profile and ERIC-PCR (Enterobacterial repetitive intergenic consensus-polymerase chain reaction). **Results**: ERIC-PCR and plasmidial analysis grouped the isolates in 17 and 19 patterns, respectively. Six isolates from one hospital presented the same pattern by ERIC-PCR, indicating clonal dissemination. All isolates presented *bla*$_{SHV}$, 62.5% presented *bla*$_{CTX-M-2}$, 29% *bla*$_{TEM}$, and 41.7% *bla*$_{KPC}$. Metallo-β-lactamase genes *bla*$_{VIM}$, *bla*$_{IMP,}$ and *bla*$_{SPM}$ were not detected. Eleven isolates were identified carrying at least 3 β-lactamase studied genes, and 2 isolates carried *bla*$_{SHV,}$ *bla*$_{TEM}$, *bla*$_{CTX-M-2}$, and *bla*$_{KPC}$ simultaneously. **Conclusions**: The accumulation of resistance genes in some strains, observed in this study, imposes limitations in the therapeutic options available for the treatment of infections caused by *K. pneumoniae* in Recife, Brazil. These results should alert the Brazilian medical authorities to establish rigorous methods for more efficiently control the dissemination of antimicrobial resistance genes in the hospital environment.

Keywords: *Klebsiella pneumoniae*. *bla*$_{KPC}$. *bla*$_{CTX-M-2}$. ERIC-PCR.

INTRODUCTION

Klebsiella pneumoniae is an opportunistic pathogen responsible for many nosocomial infections including septicemia, urinary tract infections, and pneumonia, especially in immunocompromised individuals[1]. Nosocomial infections are caused by multidrug resistant *K. pneumoniae* strains, mainly extended-spectrum β-lactamases (ESBLs) producers. Extended-spectrum β-lactamases are enzymes often clavulanate-susceptible that can hydrolyze oxyimino-cephalosporins (cefotaxime, ceftazidime, ceftriaxone, cefepime) and monobactams, but not cephamycins and carbapenems[2]. Most of ESBLs are derived from the TEM (Temoniera) and SHV (sulphydryl variable) groups, however, since around 2008 CTX-M (cefotaximase) ESBLs have become dominant in many countries, including Brazil, mainly in *K. pneumoniae* nosocomial isolates[3-5].

The carbapenems are often used for the treatment of infections caused by *K. pneumoniae* ESBLs producers. However, this species has presented an efficient mechanism of resistance to carbapenems, known as *Klebsiella pneumoniae* carbapenemase (KPC), which was initially detected in the United States and Israel and later in other countries[6]. In Brazil, KPC enzymes have been described since 2009[7] when originally reported in isolates of *K. pneumoniae* from Recife[8]. Considering that Monteiro et al.[8], reported the one-time identification of KPC enzymes in Recife and analyzed only 4 *K. pneumoniae* isolates and 3 of them were clones, is of utmost importance the study of the occurrence of this resistance genes in a larger number of isolates from Recife, with no clonal relationship. Therefore, the objective of this study was to genetically characterize clinical isolates of *K. pneumoniae* from patients in two hospitals, in the City of Recife, Brazil, through the investigation of *bla*$_{TEM,}$ *bla*$_{,SHV,}$ *bla*$_{CTX-M}$, *bla*$_{KPC,}$ *bla*$_{VIM}$, *bla*$_{IMP,}$ and *bla*$_{SPM}$ and through molecular typing by ERIC-PCR (enterobacterial repetitive intergenic consensus-polymerase chain reaction) and plasmidial profile.

METHODS

Bacterial isolates

Twenty-four clinical isolates of *K. pneumoniae* from different patients from the City of Recife, State of Pernambuco (PE), Brazil, were analyzed. Ten isolates were from different units of a public hospital (Hospital A) and 14 were from the intensive care unit of a private hospital (Hospital P), from 2007 and 2008, respectively **(Table 1)**. All isolates were identified by Vitek 2 (bioMérieux, Marcy l'Etoile, France).

Antimicrobial susceptibility and phenotypic detection of ESBL and KPC

The antimicrobial susceptibility was tested on Mueller-Hinton agar by the disk diffusion method[9]. Commercially available disks (Oxoid) loaded with the following antimicrobial were used: ampicillin (AMP), amoxicillin (AMO), amoxicillin/clavulanate (AMC), ampicillin/sulbactam (APS), aztreonam (ATM), cefepime (CPM), cefoxitin (CFO), cefotaxime (CTX), ceftazidime (CAZ), cefalotin (CFL), ciprofloxacin (CIP), gentamicin (GEN), imipenem (IMP), meropenem (MPM), piperacilina/tazobactam (PIT), trimethoprim/sulfamethoxazole (TSU), amikacin (AMI), nalidixic acid (NAL), chloramphenicol (CLO), streptomycin (EST) and tetracycline (TET). The isolates were further examined by the double-disk synergy test (DDST) and Modified Hodge Test (MHT) for ESBLs and KPC detection, respectively, as previously described by Vercauteren et al.[10] and Anderson et al.[11].

Address to: Dra. Ana Catarina S. Lopes. Deptº. Medicina Tropical/UFPE. Av. Prof. Morais Rego s/n, 50732-970 Recife, PE, Brasil.
Phone: 55 81 2126-8526; Fax: 55 81 2126-8528
e-mail: ana.lopes.ufpe@gmail.com
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TABLE 1 - Origin and antimicrobial resistance profile of clinical isolates of *Klebsiella pneumoniae* from two hospitals in the years 2007 and 2008 of the City of Recife, State of Pernambuco, Brazil.

Isolates	Origin	Antimicrobial resistance profile
K1A	catheter	AMO, AMP, AMC, ATM, CMP, CTX, CAZ, CIP, GEN, PIT, TSU, NAL, CLO*
K2A	urine	AMO, AMP, AMC, ATM, CMP, CTX, CAZ, CIP, GEN*, PIT, TSU, NAL, EST*, TET
K3A	wound	AMO, AMP, AMC, CTX*, TSU, NAL*, TET
K4A	tracheal aspirate	AMO, AMP, AMC, CTX*, NAL, TET
K5A	urine	AMO, AMP, AMC, ATM, CMP, CTX, CAZ, GEN, PIT*, TSU, CLO, TET
K6A	urine	AMO, AMP, AMC, ATM*, CIP, GEN*, NAL, CLO, TET
K7A	tracheal aspirate	AMO, AMP, AMC, ATM, CMP, CTX, CAZ, CIP, GEN*, PIT*, TSU, NAL, EST*, TET
K9A	tracheal aspirate	AMO, AMP, AMC, ATM, CMP, CTX, CAZ, PIT*, TSU, CLO
K10A	urine	AMO, AMP, AMC, ATM, CPM, CTX, CFO*, CAZ, CIP, PIT, TSU, AMI*, NAL, CLO, TET
K12A	urine	AMO, AMP, AMC, CMP, CFO, CIP, MPM, PIT, TSU, AMI
K1P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, IMP, MPM, PIT, TSU, NAL, CLO, TET
K3P	bone fragment	AMO, AMP, AMC*, APS, ATM, CMP, CTX, CAZ, CFL, PIT, TSU
K5P	blood	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, IMP, MPM, PIT, TSU, CLO, TET
K8P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, CIP, GEN, IMP, MPM, PIT, TSU, AMI, NAL, CLO, TET*
K9P	abscess secretion	AMO, AMP, AMC, APS, ATM, CMP, CTX, CAZ, CFL, CIP, PIT, TSU, NAL, CLO, EST*, TET
K10P	abscess secretion	AMO, AMP, AMC, APS, ATM, CMP, CTX, CAZ, CFL, CIP, IMP, MPM, PIT, TSU, NAL, CLO*
K12P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, CIP, GEN, IMP, MPM, PIT, TSU, AMI, NAL, CLO, TET*
K13P	tracheal aspirate	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO*, CAZ, CFL, CIP, GEN, IMP, MPM, PIT, TSU, NAL, CLO*, TET*
K14P	blood	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, CIP, GEN, PIT, TSU, NAL, CLO, EST, TET
K15P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CAZ, CFL, CIP, IMP, MPM, PIT, TSU, NAL, CLO*, TET*
K16P	catheter	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, IMP, MPM, PIT, TSU, NAL, CLO, TET*
K19P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO*, CAZ, CFL, CIP, PIT, TSU, NAL, CLO*, EST, TET
K20P	urine	AMO, AMP, AMC, APS, ATM, CMP, CTX, CFO, CAZ, CFL, CIP, GEN, IMP, MPM, PIT, TSU, NAL, CLO, EST*, TET
K22P	tracheal aspirate	AMO, AMP, AMC, APS, ATM, CMP, CTX, CAZ, CFL, CIP, IMP, MPM, PIT, TSU, NAL, CLO*, EST*, TET*

K: *Klebsiella pneumoniae;* **A:** public hospital; **P:** private hospital; **AMO:** amoxicillin; **AMP:** ampicillin; **AMC:** amoxicillin-clavulanate; **ATM:** aztreonam; **CPM:** cefepime; **CTX:** cefotaxime; **CAZ:** ceftazidime; **CIP:** ciprofloxacin; **GEN:** gentamicin; **PIT:** piperacilina/tazobactam; **TSU:** trimethoprim/sulfamethoxazole; **NAL:** nalidixic acid; **CLO:** chloramphenicol; **EST:** streptomycin; **TET:** tetracycline; **CFO:** cefoxitin; **APS:** ampicillin/sulbactam; **IMP:** imipenem; **MPM:** meropenem; **CFL:** cefalotin; **AMI:** amikacin; * intermediate resistance.

DNA extraction and PCR for β-lactamase genes

Genomic deoxyribonucleic acid (DNA) of the *K. pneumoniae* isolates was extracted according to Maniatis et al.[12] and quantified by comparison with known amounts of lambda DNA digested with Hind III in 1% agarose gel. The β-lactamase genes were investigated by PCR using specific primers[13-17] **(Table 2)**. The amplification reactions were prepared in a total volume of 25µl, containing 5ng of genomic DNA, 2.0U of *Taq* DNA polymerase (Promega, USA), 200µM of each dNTP (Promega, USA), 1.5mM of $MgCl_2$, 1µM of each primer and 1X reaction buffer (final concentration). The PCR amplifications were performed in a thermocycler (Biosystems) as follows: 95ºC for 5min and 35 cycles of 1min at 95ºC, 1min at specific temperature for each primer **(Table 2)** and 1min at 72ºC. A final extension step of 10min at 72ºC was performed (genes bla_{SHV}, bla_{TEM}, bla_{CTX-M} e bla_{KPC}) and 5min at 68°C (genes bla_{VIM}, bla_{IMP} e bla_{SPM}). In each PCR (Polymerase chain reaction) were included positive control strains for genes: bla_{SPM-1} (*P. aeruginosa* PSA319) supplyed by *Laboratório Alerta*, *Universidade Federal de São Paulo* (UNIFESP); and bla_{IMP} (*P. aeruginosa* 48-1997A), bla_{VIM} (*P. aeruginosa* VIM-1), bla_{SHV}, bla_{TEM}, bla_{CTX-M} (*K. pneumoniae* K16R) and bla_{KPC} (*K. pneumoniae* K1P), from the culture collection of the *Laboratório de Microbiologia, Departamento de Medicina Tropical*, *Universidade Federal de Pernambuco* (UFPE). The amplifications products were analyzed by electrophoresis in 1% agarose gels in TBE buffer.

Plasmid analysis and ERIC-PCR

Plasmid DNA was extracted by the UltraClean Endotoxin-Free Mini Plasmid Prep Kit (Mo Bio Lab, USA). The primers described by Duan et al.[18] were used for ERIC-PCR. The amplification reactions were prepared in a total volume of 25µl, containing 100ng of genomic DNA, 1.0U of *Taq* DNA polymerase (Promega, USA), 200µM of each dNTP (Promega, USA), 1.52mM of $MgCl_2$, 0,4µM of each primer and 1X reaction buffer (final concentration). The PCR amplifications were performed in a thermocycler (Biosystems) as follows: 95ºC for 3min

TABLE 2 - Primers used for polymerase chain reaction to detect β-lactamase genes.

Primer name	Primer sequence (5'-3')	Temp*	Reference	Gene
SHV.For	GGGTTATTCTTATTTGTCGC	62°C	Rasheed et al.[13]	bla_{SHV}
SHV.Rev	AGCGCGAGAAGCATCCTG			
T1F	ATAAAATTCTTGAAGACGAAA	57°C	Rasheed et al.[13]	bla_{TEM}
T2R	GACAGTTACCAATGCTTAATC			
CTX-MA1	SCSATGTGCAGYACCAGTAA	61°C	Saladin et al.[14]	bla_{CTX-M}
CTX-MA2	CCGCRATATGRTTGGTGGTG			
CTX-M13U	GGTTAAAAAATCACTGCGTC	60°C	Saladin et al.[14]	$bla_{CTX-M-1}$
CTX-M13L	TTGGTGACGATTTTAGCCGC			
CTX-M25U	ATGATGACTCAGAGCATTCG	62°C	Saladin et al.[14]	$bla_{CTX-M-2}$
CTX-M25L	TGGGTTACGATTTTCGCCGC			
KPC-1a	TGTCACTGTATCGCCGTC	63°C	Yigit et al.[15]	bla_{KPC}
KPC-1b	CTCAGTGCTCTACAGAAAACC			
VIM-DIA F	CAGATTGCCGATGGTGTTTGG	62°C	Dong et al.[16]	bla_{VIM}
VIM-DIA R	AGGTGGGCCATTCAGCCAGA			
IMP-DIA F	GGAATAGAGTGGCTTAATTCTC	60°C	Dong et al.[16]	bla_{IMP}
IMP-DIA R	GTGATGCGTCYCCAAYTTCACT			
SPM-DIA F	CCTACAATCTAACGGCGACC	60°C	Gales et al.[17]	bla_{SPM}
SPM-DIA R	TCGCCGTGTCCAGGTATAAC			
ERIC-1	ATGTAAGCTCCTGGGGATTAAC	36°C	Duan et al.[18]	
ERIC-2	AAGTAAGTGACTGGGGTGAGCG			

***Temp**: annealing temperature. All primer names were described according the respective references **F (and For, 1, U, a)**: sequence forward; **R (and Rev, 2, L, b)**: sequence reverse.

and 40 cycles of 1min at 92ºC, 1min at 36°C and 8min at 72ºC. A final extension step of 16min at 72ºC was performed. The products of plasmid extraction and ERIC-PCR were analyzed by electrophoresis on agarose gel 0.7% and 1.5%, respectively. The band patterns generated by ERIC-PCR were analyzed according to Tenover et al.[19] and the software DARwin 5.0 was used to generate dendrogram.

RESULTS

Antimicrobial susceptibility profile

The antimicrobial susceptibility profiles of the 10 *K. pneumoniae* isolates from the public hospital (Hospital A) showed that the carbapenems presented the highest levels of activity; imipenem inhibited all isolates and meropenem inhibited 9 (90%) isolates. The aminoglycosides, amikacin and streptomycin inhibited 80% of the isolates, and gentamicin inhibited 50% of the isolates. The other antimicrobials inhibited less than 50% of the isolates. The aminoglycosides were the most effective antimicrobials against the isolates from the private hospital (Hospital P), followed by the carbapenems. Interestingly, the isolates K2A and K7A, and K8P and K12P showed pair wise identical antimicrobial resistance profiles and were not clones according to the ERIC-PCR results.

ERIC-PCR

Six *K. pneumoniae* isolates from Hospital P (K10P, K12P, K13P, K15P, K16P, and K22P) presented the same band pattern by ERIC-PCR (profile E11) indicating clonality **(Figure 1)**. All other isolates from both hospitals showed distinct genetic profiles **(Table 3)**, with a maximum of 60% similarity **(Figure 1)**. These other isolates were considered genetically unrelated by differing in at least seven bands, which indicates changes that are consistent with the occurrence of three or more independent genetic events.

β-lactamase phenotypic and genotypic detection

Out of the 24 *K. pneumoniae* clinical isolates studied, 9 (37.5%) were ESBL producers by the DDST (double-disc synergy test). The non-ESBL producers by DDST presented at least one of the three studied genes, bla_{SHV}, $bla_{TEM,}$ or bla_{CTX-M}. All isolates that were positive by PCR for bla_{CTX-MA} were positive for $bla_{CTX-M-2}$. The gene $bla_{CTX-M-1}$ was not detected. All 24 *K. pneumoniae* isolates presented the bla_{SHV}, 15 (62.5%) presented the $bla_{CTX-M-2}$, and 7 (29%) had the bla_{TEM} **(Table 3)**. Based on two characteristics, the presence of the $bla_{CTX-M-2}$ gene (15 isolates) and a positive DDST (K3P), 67% (16/24) of the isolates were identified as ESBL producers. The most frequently observed combination of genes was bla_{SHV} and $bla_{CTX-M-2}$ (42%), followed by bla_{SHV} and bla_{TEM} (8%); the $bla_{CTX-M-2}$ and bla_{TEM} combination was not observed. The three β-lactamase genes studied were identified simultaneously in five isolates (21%): K1A, K5A, K10A, K1P, and K14P **(Table 3)**. The ERIC-PCR results showed that these isolates were not clones.

KPC phenotypic and genotypic detection

The gene bla_{KPC} was identified in 10 (71%) isolates from the Hospital P, on the other hand, this gene was not detected in the isolates from the hospital A. The isolate K14P did not show resistance to carbapenems, was negative in the MHT, however, it carried the bla_{KPC} gene **(Table 3)**. The K8P, K12P, K16P, and K20P isolates showed negative MHT results but presented resistance to carbapenems and contained the bla_{KPC} gene. The isolate K5P was resistant to carbapenems, however, it was MHT negative and did not carry the bla_{KPC} gene. The bla_{VIM}, bla_{IMP}, and bla_{SPM} genes were not detected in any of the isolates analyzed in this study.

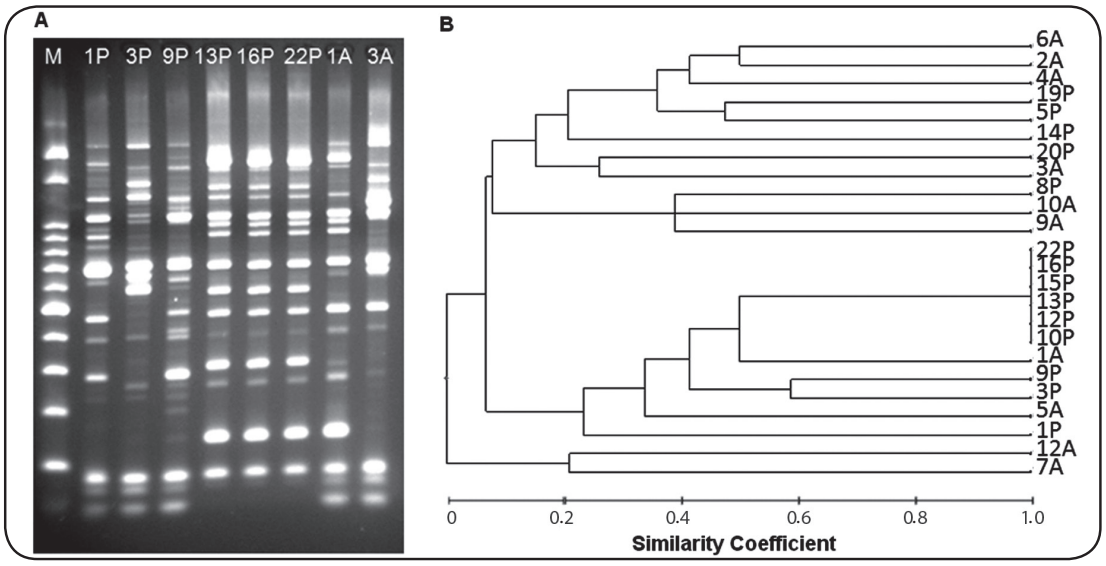

FIGURE 1 - Efficacy of Enterobacterial Repetitive Intergenic Consensus-polymerase chain reaction (ERIC-PCR) method in discriminating epidemiologically related and non-related isolates of *Klebsiella pneumoniae*. A: Agarose gel (1.5%) electrophoresis of amplification products. **B:** Dendrogram generated through software DARwin 5.0, showing the genetic similarity. **Lane 1:** M: molecular weight marker (100-bp DNA ladder); **Lanes 2 to 9:** clinical isolates of *Klebsiella pneumoniae*.

TABLE 3 - Phenotypic and molecular typing of *Klebsiella pneumoniae* isolates from Recife, Brazil.

Isolates	DDST*	*bla*$_{SHV}$	*bla*$_{TEM}$	*bla*$_{CTX-M}$	MHT**	*bla*$_{KPC}$	*bla*$_{VIM}$, *bla*$_{IMP}$, and *bla*$_{SPM}$	Plasmidial profile	ERIC-PCR
K1A	+	+	+	+	NT	-	NT	P6	E3
K2A	+	+	-	+	NT	-	NT	P7	E1
K3A	-	+	-	-	NT	-	NT	P2	E7
K4A	-	+	-	-	NT	-	NT	P1	E4
K5A	+	+	+	+	NT	-	NT	P3	E5
K6A	-	+	-	-	NT	-	NT	P4	E6
K7A	+	+	-	+	NT	-	NT	P1a	E10
K9A	+	+	-	+	NT	-	NT	P8	E2
K10A	+	+	+	+	NT	-	NT	P1	E9
K12A	-	+	-	-	NT	-	NT	P5	E8
K1P	-	+	+	+	+	+	-	P15	E14
K3P	+	+	-	-	NT	-	-	P12	E15
K5P	-	+	-	+	-	-	-	P13	E12
K8P	-	+	+	-	-	+	-	P14	E13
K9P	+	+	-	+	NT	-	-	P16	E17
K10P	-	+	-	+	+	+	-	P10a	E11
K12P	-	+	+	-	-	+	-	P9	E11
K13P	-	+	-	+	+	+	-	P9	E11
K14P	-	+	+	+	-	+	-	P11	E19
K15P	-	+	-	+	+	+	-	P10	E11
K16P	-	+	-	+	-	+	-	P10	E11
K19P	+	+	-	+	NT	-	-	P11	E16
K20P	-	+	-	-	-	+	-	P17	E18
K22P	-	+	-	-	+	+	-	P9a	E11

K: *Klebsiella pneumoniae;* **A:** public hospital; **P:** private hospital; **DDST**: double-disc synergy test; MHT: modified Hodge test; **ERIC-PCR**: Enterobacterial Repetitive Intergenic Consensus-polymerase chain reaction; **ESBLs**: extended-spectrum β-lactamases; **KPC**: *Klebsiella pneumoniae* carbapenemase; **NT:** not tested; *Detection of ESBLs through the double-disc synergy test; **Detection of KPC through the modified Hodge test.

Plasmidial profile analysis

All isolates harboured 1 to 10 plasmids of different sizes **(Table 4)**. Plasmids of >150 kb, 150kb, and ≤3.4kb were identified in 50%, 54%, and 83% of the isolates, respectively. Plasmids of 100kb, 80kb, and 12kb were identified in 57%, 78%, and 50% of the isolates from the Hospital P, suggesting a dissemination of these plasmids. It should be noted that the isolates K4A and K10A; K12P and K13P; K14P and K19P; and K15P and K16P, presented the same plasmidial profile, defined by the number and molecular weight of the bands **(Table 4)**.

TABLE 4 - Plamidial profile of 24 *Klebsiella pneumoniae* clinical isolates from Recife, Brazil.

Isolates	Plasmid number	Plasmid size	Plasmidial profile
K1A	2	4kb; <3.4kb	P6
K2A	6	>150kb; 150kb; 90kb; 4kb; two <3.4kb	P7
K3A	5	>150kb; 150kb; 3.4kb; two <3.4kb	P2
K4A	3	>150kb; 150kb; <3.4kb	P1
K5A	3	>150kb; 150kb; 3.4kb	P3
K6A	3	>150kb; 10kb; <3.4kb	P4
K7A	4	>150kb; 150kb; 3.4kb; <3.4kb	P1a
K9A	8	>150kb; 150kb; 100kb; 80kb; 10kb; three <3.4kb	P8
K10A	3	>150kb; 150kb; <3.4kb	P1
K12A	1	<3.4kb	P5
K1P	2	100kb; 8kb	P15
K3P	2	80kb; 12kb	P12
K5P	1	<3.4kb	P13
K8P	5	100kb; 80kb; 3.4kb; two <3.4kb	P14
K9P	2	8kb; <3.4kb	P16
K10P	9	Two >150kb; 150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb	P10a
K12P	8	150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb; <3.4kb	P9
K13P	8	150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb; <3.4kb	P9
K14P	4	80kb; 8kb; two <3.4kb	P11
K15P	10	Two >150kb; 150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb; <3.4kb	P10
K16P	10	Two >150kb; 150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb; <3.4kb	P10
K19P	4	80kb; 8kb; two <3.4kb	P11
K20P	5	80kb; 75kb; 3,4;kb; two <3.4kb	P17
K22P	9	>150kb; 150kb; 100kb; 80kb; 65kb; 15kb; 12kb; 9kb; <3.4kb	P9a

K: *Klebsiella pneumoniae;* **A:** public hospital; **P:** private hospital.

DISCUSSION

Multidrug resistance among ESBL-producer isolates is predictable because the genes that encode for ESBLs are usually located in self-transferable plasmids, which can also carry other antimicrobial resistance genes[20]. All isolates were tested against 9 classes of antimicrobials, however, for the majority, the therapeutic options were restricted to a maximum of 4 classes, evidencing the problematic aspects of treatment of multidrug resistant *K. pneumoniae* isolates. It was possible to establish an association between antimicrobial resistance profiles and β-lactamases genes because the isolates K2A and K7A, and K8P and K12P showed the same β-lactamase genes and the same resistance profile. All isolates from the Hospital A that had two or three genes (bla_{SHV}, $bla_{TEM,}$ and $bla_{CTX-M-2)}$ were resistant to the oxyimino-cephalosporins tested (ceftazidime, cefotaxime and cefepime) and to aztreonam, whereas isolates that carried only bla_{SHV} were resistant to only one of these antimicrobials. On the other hand, this association was not observed between the isolates from the Hospital P because they were resistant to all tested cephalosporins and monobactam, regardless of the number of genes carried.

The DDST was effective in detecting the ESBL producers among all isolates from the Hospital A (PCR positive for $bla_{CTX-M-2}$) that were KPC negative. On the other hand, this test failed to detect 7 ESBL producers from the Hospital P. The failure of DDST to detect ESBLs in the K1P, K10P, K13P, K14P, K15P K16P Hospital P isolates can be explained by the concomitant presence of the KPC carbapenemase. Tsakris et al.[21] showed that the phenotypic detection of ESBLs is hampered when the isolates also produce KPC; therefore, these authors suggest the addition of boronic acid to the test to inhibit the KPC enzymes, thus facilitating the detection of the ESBL enzymes.

The negative result in the ESBL detection through the DDST for the isolate K5P, which was positive for the bla_{CTX-M} and negative for the bla_{KPC}, can be explained by the fact that: I) this is not a confirmatory test for the detection of ESBLs[9] because it relies on the bacterial phenotype that can be altered by the conditions of *in vitro* cultivation; II) additionally, the test only detects ESBLs that are inhibited by the clavulanic acid (36 of 135 TEM enzymes, and 5 of 72 SHV enzymes have currently demonstrated resistance to the clavulanic acid and related inhibitors, however, no CTX-M has demonstrated this feature so far)[2]; and III) the molecular methods present higher sensitivity for the detection of ESBLs compared to the phenotypic methods.

The MHT did not detect carbapenemases in five isolates carrying the bla_{KPC} gene and resistant to carbapenems. In Brazil, it is more common to encounter false positive results for carbapenemase production, for example, in isolates of *K. pneumoniae* cefotaximases producers that simultaneously present loss of porins[22]. The problematic detection of KPC in *Enterobacteriaceae* has been reported by other authors, supporting the suggestion of phenotypic detection of KPC using boronic acid[23].

In spite of an early dissemination of KPC-2-producing *K. pneumoniae* strains in Brazil, bla_{KPC} gene was not detected in the isolates from Hospital A. This fact may indicate a good clinical practice in Hospital A. The bla_{KPC} gene occurrence was 71% in the isolates from the Hospital P, which is equal to the occurrence of the ESBL producers in this hospital. Probably, this fact can limit the therapeutic options and increase the mortality rates. Another relevant finding was the lack of expression of resistance to carbapenems by K14P isolate that was positive for bla_{KPC}. Similar results were reported by Peleg et al.[24], showing that only 5 out of 19 isolates carrying carbapenemases genes expressed resistance to carbapenems. The acquisition of genes that encode for carbapenemases is not always associated with high levels of resistance to carbapenems[25]. This variable susceptibility can be explained by a number of factors such as: I) co-presence of other mechanisms of resistance[26]; II) genetic suppression leading to a silenced gene; or III) dosage of the gene that is dependent on the plasmid copy number[24].

The presence of identical plasmid profile in different isolates from Hospital P suggests that plasmids with the same molecular weight may have disseminated among these isolates. Aktas et al.[27], considered that *K. pneumoniae* is a nosocomial reservoir and the source for the transmission of resistance plasmids. According to Sharma et al.[28], there is a correlation between the number of plasmids harbored by an isolate and the multidrug resistance. This association was not observed in this study; the isolates showing resistance to the greatest number of drugs (K8P and K12P) did not carry the greatest number of plasmids, and the isolates resistant to fewer drugs (K3A and K4A) were not the ones carrying the smallest number of plasmids. Additionally, the isolates that presented the same antimicrobial resistance profile did not present the same plasmidial profile.

No correlation was observed in a quantitative analysis between the plasmids and the bla_{SVH}, bla_{TEM}, and bla_{CTX-M} genes, because some isolates were positive for only one gene, but carried one to nine plasmids. Other isolates presented two genes with varying numbers of plasmids. K1P was positive for all β-lactamases genes studied ($bla_{SHV,}$ bla_{TEM}, bla_{CTX-M}, and bla_{KPC}) and carried only two plasmids (100kb and 8kb). According to Wei et al.[29], the number of antibiotic resistance genes in plasmids of multidrug-resistant *K. pneumoniae* can reach up to five. The presence of high molecular weight plasmids (60 to 180Kb) in clinical isolates of *K. pneumoniae* is quite common and has often been associated with the production of ESBLs[29,30].

The ERIC-PCR revealed that 6 isolates from the Hospital P showed clonal relationship even when presenting different antimicrobial resistance phenotypes, contents of resistance genes, and plasmidial profiles. Molecular typing data reported in the literature corroborate these results. Ben-Hamouda et al.[31], reported isolates with identical PFGE or ERIC-PCR profiles and different drugs susceptibility profiles. Bennett et al.[32] did not observe any correlation between PFGE profiles and resistance genes. Souza Lopes et al.[30] identified RAPD subtypes correlated with different plamidial profiles. These discrepancies in the typing techniques result from the fact that, the evidences for clonality are best considered as relative instead of absolute, because of the potential of genetic changes detectable only by DNA sequencing or other specific analyses.

The finding that 11 (46%) isolates were carriers of at least 3 of the β-lactamase studied genes is worrying. It should be noted that the isolates K1P and K14P presented the $bla_{SHV,}$ bla_{TEM}, bla_{CTX-M}, and bla_{KPC} genes and were not clones. The simultaneous production of three β-lactamases by *K. pneumoniae* deserves to be highlighted[33].

The accumulation of resistance genes in some strains, observed in this study, imposes limitations in the therapeutic options available for the treatment of infections caused by *K. pneumoniae* in Recife, Brazil. The presence of intra-hospital clones was another remarkable observation. These results should alert the Brazilian medical authorities to establish rigorous methods for detection and thus, more efficiently control the dissemination of antibiotic resistance genes in the hospital environment. Moreover, measures to control the excessive use of antimicrobials in hospitals must also be taken.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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ABSTRACT IN PORTUGUESE

Genes de multirresistência, incluindo bla_{KPC} e $bla_{CTX-M-2}$, em *Klebsiella pneumoniae* isoladas em Recife, Brasil

Introdução: A prevalência de cepas de *Klebsiella pneumoniae* resistentes a cefalosporinas e carbapenêmicos está aumentando no Brasil, com sérias consequências em termos de desfechos dos pacientes e cuidados gerais. **Métodos**: Este estudo caracterizou 24 isolados clínicos de *K. pneumoniae* provenientes de dois hospitais de Recife, Brasil, através do perfil de susceptibilidade a antimicrobianos, análise de genes de β-lactamase ($bla_{TEM,}$ $bla_{SHV,}$ bla_{CTX-M}, bla_{KPC}, bla_{VIM}, $bla_{IMP,}$ and bla_{SPM}), perfil plasmidial e ERIC-PCR (*Enterobacterial repetitive intergenic consensus-polymerase chain reaction*). **Resultados**: A análise da ERIC-PCR e do perfil plasmidial agrupou os isolados em 17 e 19 perfis, respectivamente. Seis isolados de um hospital apresentaram o mesmo padrão de ERIC-PCR, indicando disseminação clonal. Todos os isolados apresentaram bla_{SHV}, 62,5% apresentaram $bla_{CTX-M-2}$, 29% bla_{TEM} e 41,7% bla_{KPC}. Genes de metalo-β-lactamase bla_{VIM}, bla_{IMP} e bla_{SPM} não foram detectados. Onze isolados foram identificados carreando, pelo menos, três dos genes de β-lactamase estudados, dentre estes, dois isolados continham $bla_{SHV,}$ bla_{TEM}, $bla_{CTX-M-2}$ e bla_{KPC} simultaneamente. **Conclusões**: O acúmulo de genes de resistência em algumas cepas, observado nesse estudo, impõem limitações nas opções terapêuticas disponíveis para o tratamento de infecções causadas por *K. pneumoniae* em Recife, Brasil. Estes resultados devem alertar as autoridades médicas brasileiras para estabelecer rigorosos métodos para controlar eficientemente a disseminação de genes de resistência a antimicrobianos no ambiente hospitalar.

Palavras-chaves: *Klebsiella pneumoniae*. bla_{KPC}. $bla_{CTX-M-2}$. ERIC-PCR.

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