Occurrence of *Bla*_{ndm-7} And Association With *Bla*_{kpc-2}, *Bla*_{ctx-M15}, *Aac*, *Aph*, *Mph(A)*, *Catb3* And Virulence Genes In A Clinical Isolate of *Klebsiella Pneumoniae* With Different Plasmids In Brazil.

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Short Report

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Abstract

To characterize phenotypically and genotypically an isolate of Multidrug Resistant (MDR) *K. pneumoniae* from a patient with septicemia in a hospital in Recife-PE, Brazil, resistance and virulence genes were investigated by using PCR and sequencing the amplicons and the plasmid DNA was also sequenced. The K74-A3 isolate was resistant to all β -lactams, including carbapenems, as well as to aminoglycosides and quinolones. By conducting a PCR analysis and sequencing, the variant *bla*_{NDM-7} associated with *bla*_{KPC-2} and the *cps*, *wabG*, *fimH*, *mrkD* and *entB* virulence genes were identified. The analysis of plasmid revealed the presence of *bla*_{CTX-M15}, *aac(3)-IVa*, *aph(3')-Ia*, *aph(4)-Ia*, *aac(6')ib-cr*, *mph(A)* and *catB3*, and also the plasmids IncX3, IncFIB, IncQ1, ColRNAI and ColpVC. To our knowledge, this is the first report of the *bla*_{NDM-7} gene in Brazil and we suggest that this variant is located in IncX3. These results alert us to the risk of spreading an isolate with a vast genetic arsenal of resistance, in addition to which several plasmids are present that favor the horizontal transfer of these genes.

Highlights

- First report of the *bla*_{NDM-7} gene in *Klebsiella pneumoniae* in Brazil.
- *Klebsiella pneumoniae* co-produced *bla*_{NDM-7} and *bla*_{KPC-2} associated with other resistance and virulence genes and different plasmids.
- Plasmid sequencing of *Klebsiella pneumoniae* revealed the presence of the *bla*_{NDM-7} variant in a genetic environment similar to that determined in an *E. coli* harboring *bla*_{NDM-7} in IncX3.

Introduction

New Delhi Metallo- β -lactamase (NDM) confers resistance to all β -lactams except aztreonam and since it was first reported to be in isolates of *Klebsiella pneumoniae* and *Escherichia coli*, it has been identified in several countries. Most of the *bla*_{NDM} transport occurs by means of conjugative plasmids with a variety of replicons, which facilitates its rapid dissemination between different species [1].

To date, there are 24 distinct variants of NDM with different levels of enzyme activity [1]. In Germany, *E. coli* transformants with NDM-7 have been reported to have higher MIC values for β -lactams when compared to transformants with NDM-1 [2]. While in Brazil, we have shown the association of different resistance and virulence mechanisms in isolates of *K. pneumoniae* with *bla*_{NDM-1} [3]. However, until now, the *bla*_{NDM-7} variant has not been detected in Brazil, although it is widespread in some regions of Asia. Multidrug Resistant (MDR) *K. pneumoniae* are frequently involved in healthcare-associated infections (HAIs), and the high frequency and diversity of the resistance and virulence factors present in *K. pneumoniae*, which co-produces *bla*_{NDM} and *bla*_{KPC}, make their strains resistant to several antibiotics, thus limiting therapeutic options [4, 5]. The objective of this study was to characterize phenotypically and genetically an isolate of Multidrug Resistant (MDR) *K. pneumoniae* from a patient with bloodstream infection who was in a hospital in Recife-PE, Brazil.

Materials And Methods

An isolate of *K. pneumoniae* (K74-A3) was analyzed that had been taken from a female patient who was admitted to the general emergency department of a public hospital in Recife-PE, Brazil on 08/10/2018. The isolate was

selected for the study because it was involved in bloodstream infection. It was MDR and presented resistance to all β -lactams. Its biochemical composition and its profile of susceptibility to antimicrobials were identified using the automated BD Phoenix^{TM} system. A MALDI-TOF analysis was performed to confirm the species. The culture was preserved in 20% glycerol at -70°C. Susceptibility to several classes of antimicrobials (Amikacin, Ampicillin, Ampicillin-Sulbactam, Cefazolin, Cefepime, Cefoxitin, Ceftazidime, Ceftriaxone, Ciprofloxacin, Colistin, Ertapenem, Gentamycin, Imipenem, Levofloxacin, Meropenem, Piperacillin-tazobactam, Tigecycline, and Trimethoprim-sulfamethoxazole) was interpreted in accordance with the criteria of the Clinical and Laboratory Standards Institute [6]. The MIC for colistin was determined by the Policimbac® test, according to manufacturer's instructions [7].

The genomic DNA of the K74-A3 isolate was extracted using the Wizard Genomic DNA Purification Kit (Promega). The genes encoding resistance to carbapenems (*bla*_{KPC} and *bla*_{NDM}) and virulence genes (*cps*, *wabG*, *magA*, *rmpA*, *fimH*, *mrkD*, *irp2* and *entB*) were investigated by PCR. The description of the primers and amplification conditions used are presented in (Table 1). Negative and positive controls were included in each PCR.

Table 1
Primers used in PCR and sequencing of the PCR product to detect resistance, virulence genes and Incs in the *K. pneumoniae* isolate.

Gene	Primer	Sequence (5-3)	Temp. ^(a)	Amplicon size (bp) ^(b)	Reference
<i>bla</i> _{NDM}	NDM-F	GGTTTGGCGATCTGGTTTTTC	52°C	439	Poirel <i>et al</i> [8]
	NDM-R	CGGAATGGCTCATCACGATC			
<i>bla</i> _{KPC}	KPC-1a	TGTCACTGTATCGCCGTC	63°C	800	Yigit <i>et al</i> [9]
	KPC-1b	CTCAGTGCTCTACAGAAAACC			
<i>cps</i>	cps -F	TCCCAATTGTGACCGAAATC	63°C	437	Hennequin and Forestier [10]
	cps -R	GCTCGCGGCACCAGCTGA			
<i>magA</i>	magA-F	GGTGCTCTTTACATCATTGC	54°C	1283	Turton <i>et al</i> [11]
	magA-R	GCAATGGCCATTTGCGTTAG			
<i>rmpA</i>	rmpA -F	ACTGGGCTACCTCTGCTTCA	54°C	536	Al-jailawi <i>et al</i> [12]
	rmpA -R	CTTGCATGAGCCATCTTTCA			
<i>wabG</i>	wabG-F	CGGACTGGCAGATCCATATC	53°C	683	Brisse <i>et al</i> [13]
	wabG-R	ACCATCGGCCATTTGATAGA			
<i>fimH</i>	fim-H F	TCCACAGTCGCCAACGCTTC	62°C	903	Stahlhut <i>et al</i> [14]
	fim-H R	GCTCAGAATCAACATCGGTAAC			
<i>mrkD</i>	mrkD-F	CCACCAACTATTCCCTCGAA	60°C	226	Sahly <i>et al</i> [15]
	mrkD-R	ATGGAACCCACATCGACATT			
<i>irp2</i>	irp2 F	ATTTCTGGCGCACCATCT	65°C	1000	Guilvout <i>et al</i> [16]
	irp2 R	GCGCCGGGTATTACGGACTTC			
<i>entB</i>	entB- F	ATTCCTCAACTTCTGGGGC	57°C	371	El Fertas Aissani <i>et al</i> [17]
	entB – R	AGCATCGGTGGCGGTGGTCA			
(a) Temp, annealing temperature. (b) base pairs					

Plasmid DNA was extracted by using the PureYield™ Plasmid Miniprep System (Promega). The plasmid DNA extracted was quantified in Nano Drop and by the Qubit fluorimetric platform (ThermoFisher Scientific). The libraries were assembled using the TruSeq DNA PCR Free Kit (Illumina) and quantified using qPCR. Sequencing was performed using Miseq (Illumina) equipment. Data were processed to remove low-quality reads using Trimmomatic. The filtered reads were then de novo assembled using the Velvet tool, whose parameters were optimised through the Velvet Optimiser programme. The Velvet results were also used as input for another assembly programme (CAP3) to improve the assemblies. Prediction and gene annotation were performed using Prokka. Plasmid DNA sequences were analyzed using Artemis Sanger software. For the observation of resistance genes, the *Resfinder* platform was used (<https://cge.cbs.dtu.dk/services/ResFinder/>) and to determine the plasmid incompatibility group, the Plasmidfinder database was used (<https://cge.cbs.dtu.dk/services/PlasmidFinder/>).

Results

The K74-A3 isolate presented resistance to all drugs tested, except amikacin, colistin and tigecycline (Table 2). PCR analysis and amplicon sequencing showed the presence of the *bla*_{KPC-2} and *bla*_{NDM-7} resistance genes and the *cps*, *wabG*, *fimH*, *mrkD* and *entB* virulence genes. The *magA*, *rmpA* and *irp2* genes were not found. The sequencing revealed point mutations in the *bla*_{NDM-1} (FN396876) gene at positions 388 (G → A) and 460 (A → C), thus configuring the *bla*_{NDM-7} gene. The nucleotide sequence was deposited in the GenBank, under accession number: **MW036197**.

Table 2

Phenotypic and genotypic characterization and origin of the clinical isolate K74-A3 from Recife-PE, Brazil.

Identification/ Harvest date (dd/mm/yyyy)	Isolation source/ Section	Sensibility Profile				Resistance genes (likely relationship described in the literature)
		Pharmacological group	Antimicrobials	MIC (mg/L)	Interpretation	
K74-A3/ (07/11/2018)	Blood/ ER ^a	Aminoglycosides	Gentamycin Amikacin	>8 16	R ^b S ^c	<i>aac(3)-IVa</i> <i>aph(3')-Ia</i> <i>aph(4)-Ia</i> <i>aac(6')ib-cr</i>
		Cephalosporin	Cefazolin Cefepim Cefoxitin Ceftazidime Ceftriaxone	>8 >16 >16 >16 >4	R R R R R	<i>bla</i> _{CTXM-15} <i>bla</i> _{KPC-2} <i>bla</i> _{NDM-7}
		Carbapenems	Imipenem Meropenem Ertapenem	>8 >32 >1	R R R	<i>bla</i> _{KPC-2} <i>bla</i> _{NDM-7}
		Penicillins	Ampicillin Ampicilin- Sulbactam Piperacillin- tazobactam	>16 >16/8 >64/4	R R R	<i>bla</i> _{CTXM-15} <i>bla</i> _{KPC-2} <i>bla</i> _{NDM-7}
		Quinolones and fluoroquinolones	Levofloxacin Ciprofloxacin	>4 >2	R R	<i>aac(6')ib-cr</i>
		Sulfonamide	Sulfamethoxazole- trimethoprim	>2/38	R	ND
		Tetracycline	Tigecycline	2	S	ND
		Polymyxin	Colistin	0,5	S	ND

a: ER-Emergency room; b: R-Resistant; c: S-Sensible; d: ND-Not determined.

Identification/ Harvest date (dd/mm/yyyy)	Isolation source/ Section	Sensibility Profile				Resistance genes (likely relationship described in the literature)
		Pharmacological group	Antimicrobials	MIC (mg/L)	Interpretation	
		Macrolide	ND	ND	ND ^d	<i>mph(A)</i>
		Phenicol	ND	ND	ND	<i>catB3</i>
a: ER -Emergency room; b: R -Resistant; c: S -Sensible; d: ND -Not determined.						

The size of the K74-A3 plasmid DNA sequencing was 123.132 bp, with 102 contigs, 133 CDS (Coding Sequence) and an average of 50.97% GC (guanine and cytosine). Sequencing coverage was 333x. In addition, five different plasmids were identified: IncX3, IncQ1, IncFIB, ColRNAI, ColpVC, and so too were identified the *bla*_{CTX-M15}, *aac(3)-IIVa*, *aph(3')-Ia*, *aph(4)-Ia aac(6')ib-cr*, *mph(A)* and *catB3* resistance genes, the confirmed *bla*_{NDM-7} gene. The genetic environment of *bla*_{NDM-7} is shown in Figure 1(a).

Through sequencing, the presence of the *bla*_{KPC-2} gene was also confirmed, and it was possible to determine its genetic environment, The *bla*_{KPC-2} gene was found inserted between a partial *ISKpn6* insertion sequence (Δ *ISKpn6*) with an associated left inverted repeat (IRL) and *tnpR* resolvase. A 21-bp fragment corresponding to the *bla*_{TEM} gene was found upstream of the *bla*_{KPC-2} gene Figure 2. Comparing the genetic environment of the *bla*_{KPC-2} gene with sequences deposited in GenBank, the variant NTE_{KPC}-IId (GenBank accession no. **MG786907**) [18] with ~100% identity for the isolates was observed.

In the same consensus sequence where the variant NTE_{KPC}-IId was located, plasmid mobility proteins (*mobA*, *mobB*, and *mobC*) and replication proteins (*repA*, *repB*, and *repC*) were found with a 100% similarity to the reference pool of the IncQ1 Ea18A (GenBank accession no. **MH000708**) [18].

The *oriV*, *oriT* and *repB* genes were also found inserted in the same replication and mobilization genes contig as the *bla*_{KPC-2} gene. Thus, this result suggests that isolate K74-A3 has the replicon corresponding to plasmid IncQ1 in the same contig of the *bla*_{KPC-2} gene (Figure 2).

Discussion

The first report of NDM-7 was described in France in 2012, in an *E. coli* isolate recovered from a woman with repeated urinary tract infections with a history of travel to Myanmar, Asia [20]. Since then, *bla*_{NDM-7} has emerged in countries such as Australia, Philippines, Japan, South Korea, China, Myanmar, Nepal, India, United Arab Emirates, Gabon, Germany, Spain and Canada [1, 21–27].

In Brazil, Silva et al. (2019) [28] identified the presence of *bla*_{NDM-1} in all isolates tested (n = 81) from different bacterial species from different states. This dissemination when considered together with the absence of previous detection of other variants led this study to suggest the predominance of *bla*_{NDM-1} in Brazil. It was also observed that *K. pneumoniae* was the most frequent bacterial species producing *bla*_{NDM-1} and that the dissemination of this gene in Brazil did not seem to be related to the clonal expansion of this bacterium [29]. In the present study, by sequencing the plasmid of the *K. pneumoniae* isolate, the presence of the *bla*_{NDM-7} variant was seen in a genetic

environment similar to that determined, in Germany, in the *E. coli* isolate harboring *bla*_{NDM-7} in the auto-transferable plasmid IncX3 [2]. IncX3 appears to be the most common type of plasmid carrying *bla*_{NDM} [1], which has led to the belief that it plays a role in this emerging variant becoming acquired and spreading in Brazil.

The MDR profile found in this study in K74-A3 can be justified due to the association of different β -lactamases (*bla*_{NDM-7}, *bla*_{KPC-2} and *bla*_{CTX-M15}), aminoglycoside resistance genes (*aac(3)-IVa*, *aph(3')-Ia* and *aph(4')-Ia*) and quinolones (*aac(6')ib-cr*), in addition to which there is the accumulation of different plasmids (IncX3, Q1, IncFIB, ColRNAI and ColpVC). Bacterial isolates that caused an outbreak in Madrid in Spain also showed a vast genetic arsenal of resistance [21]. It is noteworthy that all of them had the *bla*_{NDM-7} variant in plasmid IncX3, and that most of them were *K. pneumoniae*. In fact, only one of these isolates was *E. coli*, which suggests that IncX3 is shared between species. A study carried out in Canada also indicated the horizontal transfer of IncX3 with *bla*_{NDM-7} between different *Enterobacteriaceae*. Interestingly, one of the patients had recently traveled to India (an endemic location) [25].

The *bla*_{KPC-2} gene is often found inserted in the Tn4401 transposon, which has different isoforms, but it has also been found in a non-Tn4401 mobile element (NTE_{KPC}). The non-Tn4401 variant of the present study resembles the NTE_{KPC}-Ild variant (Figure 2). These findings corroborate the results obtained by our research group that demonstrated the presence of the *bla*_{KPC-2} gene in the non-Tn4401 element (NTE_{KPC}-Ild) [18, 30], which is transported by small, mobilized and promiscuous plasmids of the IncQ1 type. These data indicate that this type of plasmid may have been responsible for the spread of the *bla*_{KPC-2} gene among *K. pneumoniae* in hospital patients in Recife, Brazil.

In addition to the co-production of the *bla*_{NDM-7} and *bla*_{KPC-2} genes, the K74-A3 isolate also has different virulence factors, which can make the bacterium more pathogenic due to the following capabilities: it prevents phagocytosis due to capsule production (*cps*); it protects against humoral defenses because of the action of lipopolysaccharide (*wabG*); it adheres on abiotic surfaces and forms biofilm due to the presence of fimbriae type I and III (*fimH* and *mrkD*); and it acquires iron from the medium by secreting enterobactin (*entB*), thereby enhancing the permanence of the pathogen in vivo [31]. Similar findings were reported by Pérez-Vázquez et al. (2019) [21].

In conclusion, the emergence of the *bla*_{NDM-7} variant in the *K. pneumoniae* isolate, together with the *bla*_{KPC-2}, *bla*_{CTX-M15}, *aac(3)-IVa*, *aph(3')-Ia*, *aph(4')-Ia*, *aac(6')ib-cr*, *mph(A)*, *catB3* genes and the plasmids IncX3, IncFIB, IncQ1, ColRNAI and ColpVC, shows the potential for horizontal gene transference in this bacterial species. Additionally, the appearance of *bla*_{NDM-7} in Brazil poses a threat to health systems, further limiting therapeutic options. Moreover, this limitation favors the spread of this gene in Brazil, and also to other countries, given the frequency and volume of international and intercontinental travel. Thus, effective infection control measures related to health care by bacteria carrying *bla*_{NDM} must be taken to avoid the spread of this gene and these MDR bacteria.

Declarations

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Conflict of interest The authors declare that they have no conflicts of interest.

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Figures

Figure 1

Schematic representation of the *bla*_{NDM-7} genetic environment. (a) Sequence of the genetic environment (4.7kb) of isolate K74-A3 from the present study. In this, *bla*_{NDM-7} is represented in black, Upstream are the insertion sequence

Δ ISAb125 interrupted by the insertion of IS5, which results in a truncated transposase, followed by IS5 and Δ ISAb125. IS5, insertion sequence 5. Downstream are the *bla*_{MBL} gene that encodes a bleomycin resistance protein; *trpF*, a gene that encodes a supposed phosphoribosylanthranilate isomerase and *dsbC*, a gene that encodes a protein from the DcbC superfamily oxiredutase. (b) Sequence of the genetic environment (5.9 kb) of *E. coli* that hosts *bla*_{NDM-7} in IncX3 (KC567147) (Gottig et al [2]). The vertical lines represent gaps that schematize the end of one contig and the beginning of another contig.

Figure 2

Comparison among non-Tn4401 (NTE_{KPC}-Ild) element carrying the *bla*_{KPC-2} gene in *Klebsiella pneumoniae* K74-A3 isolate and reference sequences deposited in GenBank (NTEKPC-Ild accession No. MG786907 [18] and Tn4401: accession N°. KJ933392 [19]). Protein coding sequences are represented as arrows and labeled with the name of the gene. Vertical lines represent *gaps* schematizing the termination of one contig and the beginning of another contig. Traced in gray represents homologous shared regions (> 95%). The direct repeat sequence of NTE_{KPC}-Ild is represented by a circle.