

NDM-producing *enterobacteriaceae* strains among hospital in Brasília, Brazil

Abstract

Carbapenem-resistant *Enterobacteriaceae* (CRE) strains have spread worldwide frequently driven by clonal spread. Additionally, plasmid-borne carbapenemase genes (bla_{KPC} and bla_{NDM}) have broadened the variability of species expressing resistance to carbapenems. This study aimed to characterize the susceptibility profile and bla genes in CRE strains recovered between 2012 and early 2014 in hospitals in Brasília, Brazil. Eighty eight CRE strains recovered from 19 medical settings were analyzed. *Klebsiella pneumoniae* positive for bla_{KPC} accounted for the most of the CRE isolates ($n=47$; 53.4%). Seven bla_{NDM} -positive strains (including *K. pneumoniae*, $n=4$; *Proteus mirabilis*, $n=1$; *Escherichia coli*, $n=1$; and *Providencia rettgeri*, $n=1$) were recovered from patients in six hospitals. The first detected bla_{NDM-1} -positive strain was *P. rettgeri*. Thereafter, bla_{NDM-1} -positive *K. pneumoniae* strains showing indistinguishable Random Amplified Polymorphic DNA (RAPD) profiles were recovered in three hospitals. The susceptibility profile of bla_{NDM-1} -positive *K. pneumoniae* strains was commonly restricted to amikacin, aztreonam and tigecycline. These dates highlighted the emergence of bla_{NDM-1} -positive *K. pneumoniae* strains marked by a single RAPD type among hospitals in Brasília, Brazil.

Keywords: ndm-producing strains, carbapenemase, *klebsiella pneumoniae*, metallo- β -lactamase

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Introduction

The resistance to carbapenems has become a serious world public health issue since the early 2000's.¹ In that time, the world spread of *Klebsiella pneumoniae* carbapenemase (KPC)-producing strains was supported by the predominance of a well-adapted clone of *K. pneumoniae* (ST258) among hospitals around the world. Moreover, the bla_{KPC} gene became easily mobilized by conjugative plasmids among Enterobacteria species.² NDM-1 (New Delhi metallo- β -lactamase-1) is the most recently discovered molecular class B β -lactamase encoded on transferable, plasmid-borne genes (bla_{NDM}).³ The hydrolysis mechanism of NDM relies on the interactions between β -lactam molecules and zinc ions in the enzyme's active site. Therefore, NDM enzymes are inhibited by zinc-chelating agents such as EDTA.⁴ NDM can hydrolyze all β -lactam antibiotics (penicillins, cephalosporins and carbapenems), excepting monobactams.³ Additionally, most NDM-positive strains are broadly resistant to other antibiotic classes, and carry a wide diversity of resistance mechanisms against other antibiotics, such as aminoglycosides and fluoroquinolones, rendering these strains extremely resistant to the available treatments.¹ NDM-1 was first described in *K. pneumoniae* and *Escherichia coli* strains isolated in Sweden in 2008 from an Indian patient who had been transferred from a hospital in New Delhi, India.⁵ Nowadays, NDM has also been detected in a broad variety of other *Enterobacteriaceae* species including *K. oxytoca*, *Proteus mirabilis*, *Enterobacter cloacae*, *Citrobacter freundii* and *Providencia* spp as well as in aerobic bacilli such as *Pseudomonas* spp. and *Stenotrophomonas* spp.³ This wide distribution of the bla_{NDM} gene reflects its association with promiscuous plasmids.⁶ Regardless the purposes, whether medical or otherwise, international travels have played a significant role in the dissemination of NDM producers,

given that, most of the first reports on NDM-positive strains were epidemiologically linked to travels to Indian and Pakistani regions.⁷ In Brazil, the first NDM-producing strain was isolated in 2013 in the South region state, Rio Grande do Sul.⁸ Beside bla_{KPC} and bla_{NDM} , other carbapenemase genes, such as bla_{VIM} , bla_{IMP} and bla_{OXA-48} , have been reported in Enterobacteria world-wide, including in Brazil. However, these carbapenemase genes have not been associated with large spreads or epidemic events.⁹

The aim of this study was to define the profile of carbapenemase genes in CRE strains assessing whether NDM-producing strains have reached hospitals in Brasília, the federal capital of Brazil. Moreover, the study evaluated the role of bacterial clones in spreading of bla_{NDM} among hospital.

Materials and methods

From 2012 to 2014, a regional surveillance program was conducted by the Public Health Laboratory (LACEN-DF) in order to assess carbapenem resistance in *Enterobacteriaceae* isolates recovered from hospitals in Brasília. We have identified 88 carbapenem-resistant *Enterobacteriaceae* (CRE) strains recovered from patients attended in nineteen medical centers. Identification and antimicrobial susceptibility tests were accomplished using the MicroScan WalkAway™ system (Dade Behring, USA) and Vitek MS system (Matrix-assisted laser desorption ionization-time of flight mass spectrometry - MALDI-TOF MS system - BioMerieux) in accordance to the manufacturer's instructions. In order to assess the clinical susceptibility of bacterial isolates, *in vitro* antibiogram test results were interpreted in accordance to the breakpoints established by the Clinical and Laboratory Standards Institute (CLSI) document published in January 2014. The production of carbapenemase was tested using the modified

Hodge Test (MHT) employing ertapenem disk adsorbed with 10µg of the antibiotic as described by CLSI.¹⁰ Metallo-β-lactamase production was tested with carbapenem-containing disks (10µg meropenem or imipenem) adsorbed with 100mM EDTA.¹¹ Control disks containing only carbapenem were used to evaluate the enlargement of inhibitory zones attributed to the EDTA effect. Specific primers were used in standard polymerase chain reactions (PCR) to detect the following carbapenemase genes: *bla*_{KPC} (F, 5'TGTCAGTGTATCGCCGTC and R, 5'CTCAGTGCTCTACAGAAAACC),¹² *bla*_{NDM} (F, 5'GGTTTGGCGATCTGGTTTTC and R, 5'GGCCTTGCTGCCTTGATC), *bla*_{IMP} (F1, CATTTCATAGCGACAGCAC; F2, 5'AACACGGTTTGGTGGTCTT and R, 5'GGACTTTGGCCAAGCTTCTA), *bla*_{VIM} (F, 5'GATGGTGTGGTGCATATC and R, 5'CTCGATGAGAGTCCTCTAGAG) and *bla*_{OXA-48} (F, 5'GCGTGGTTAAGGATGAACAC and R, 5'ATCATCAAGTTCAACCAACC). The primers used for amplification of *bla*_{NDM}, *bla*_{IMP}, *bla*_{VIM} and *bla*_{OXA-48} were described in this study. PCR products were submitted to DNA sequencing (ABI 3130 Genetic Analyzer- Applied Biosystems®) in order to confirm the identity of the amplified genes. Clonal relatedness among isolates was examined by Random Amplified Polymorphic DNA (RAPD) performed with the primer OPA-2 (5'TGCCGAGCTG) (Operon Technologies, Alameda, CA, USA).¹³ The band patterns were analyzed by visual interpretation, applying the criteria established by Belkum et al.¹⁴ In addition, RAPD patterns were analyzed and dendograms were built employing PyElph software system (version 2.6.5).¹⁵

Results and discussion

Among *Enterobacteriaceae* strains reported to the Public Health Laboratory (LACEN-DF) in Brazil, *K. pneumoniae* was the most frequently CRE detected (n=61/88; 69.3%), followed by species of *Enterobacter* (n=15/88; 17.1%).

The emergence of CRE species has increased the demand on old or outdated antibiotics.¹⁶ In this scenario, the increasing interest on colistin (polymyxin) as an evaluable treatment has driven the emergence of species intrinsically resistant to colistin including *Proteuss* spp., *Serratia* spp., *Morganella* spp. and *Providencia* spp.¹⁷ In our study, intrinsically colistin-resistant strains accounted for 9.0% of the CRE isolates and they included *S. marcescens* (n=4/88; 4.5%), *P. mirabilis* (n=3/88; 3.4%) and *P. rettgeri* (n=1/88; 1.1%).

In relation to carbapenemase genes, *bla*_{KPC} was the most frequently detected gene in the tested CRE strains (n=59/88; 67.0%), followed by *bla*_{NDM} (n=7/88; 8.0%). Additionally, carbapenemase genes with minor epidemiological relevance were also tested (*bla*_{VIM}, *bla*_{IMP} and *bla*_{OXA-48}), but they were not detected among the CRE isolates. Focusing on NDM genes, we firstly isolated a *bla*_{NDM}-positive *P. rettgeri* strain from a necrotic ulcer affecting a 75-year-old male patient in May 2013. The patient had received treatment in two hospitals, both located in Brasília, and had not reported travelling abroad in the six previous years. The *P. rettgeri* strain showed *in vitro* resistance to all tested antimicrobial agents with the exception of gentamicin. The sequence analysis (Basic Local Alignment Search Tool) of the *bla*_{NDM} amplicon showed an identity of 100% (435/435 base-pairs) with

previous reported *bla*_{NDM-1} genes (GeneBank Number: KJ150691.1). Additionally, two distinct bands of plasmid DNA were found in the *P. rettgeri* strain (data not shown). PCR assays carried out with purified plasmid DNA showed that *bla*_{NDM-1} gene was located on the high-molecular-weight plasmid (molecular weight >50 Kb). Interesting, Carvalho-Assef et al.⁸ also recovered a NDM-producing *P. rettgeri* strain from a diabetic foot infection in early 2013, but differently the *bla*_{NDM-1} gene was chromosomally integrated.

Thereafter the first detection of *bla*_{NDM}, six other *bla*_{NDM}-positive strains (*K. pneumoniae*, n=4; *P. mirabilis*, n=1; *E. coli*, n=1) were isolated in three hospitals. All isolates were resistant to β-lactams (with exception of aztreonam and cefotetan), quinolones, nitrofurantoin and trimethoprim-sulfamethoxazole; and showed variable susceptibility profiles against aminoglycosides, tetracycline and tigecycline (Table 1). Interesting, all NDM-producing strains showed negative results for the carbapenemase expression assay MHT. However, NDM-producing strains are positive in the EDTA test confirming the production of metallo-β-lactamases (Table 1). Negative or weakly positive results in MHT have been already reported for NDM-producing strains.¹⁸ However, these findings are worrisome once phenotypic detection of carbapenemase in MHT is recommended for the clinical microbiology laboratories as epidemiological screening assay for detection of CRE isolates.¹⁰

Four strains of NDM-producing *K. pneumoniae* were isolated from patients treated in three hospitals; therefore, it was tested if these strains were clonally unrelated as commonly reported for *bla*_{NDM}-positive strains.¹⁹⁻²¹ However, all NDM-producing *K. pneumoniae* strains tested in this study were considered as genetically indistinguishable on RAPD analyses, showing the same amplified polymorphic DNA pattern (Figure 1B & 1C). Two of these strains were isolated from two patients (patients 2 and 3) assisted in the same hospital (hospital B), warning for the possibility of cross infections (Table 1 & Figure 1). The other two clonal strains of *K. pneumoniae* were isolated from two patients (patients 5 and 6) treated in two different hospitals (hospital C and D) (Table 1 & Figure 1). Moreover, because of a prolonged colonization period (2 months and 10 days) with *bla*_{NDM}-positive strains (*K. pneumoniae* and *E. coli*), the patient 6 had the opportunity of translocating two NDM-producing enterobacterial species into two different hospitals (Table 1). Additionally, the isolation of different bacterial species positive for *bla*_{NDM} from the patient 6 (Table 1) endorses the idea on the promiscuous nature of mobile genetic elements carrying *bla*_{NDM} genes.³ These findings reinforce the role of patient transfer in spreading NDM-producing bacteria among hospitals,²² and endorses the need of rapid communication that alerts about the presence of infected or colonized patients with NDM-producing strains in Brazilian hospitals.

As occurs in Brasília, Brazilian hospitals have frequently reported outbreaks involving CRE strains mainly associated with *bla*_{KPC}-positive *K. pneumoniae* strains belonging to the clonal complex 258 (ST 11).^{23,24} The clone ST11 of *K. pneumoniae* has been characterized for causing large outbreaks,²⁵ and has been also responsible for spreading *bla*_{NDM} gene in Greece.²⁶ Taken together, these data warn about the possibility of a worst-case scenario, in which, the epidemic clone ST11 would acquire the *bla*_{NDM} gene and spread among Brazilian hospital.

Table 1 NDM-producing strains isolated in Brasília hospitals

Patient	Species	Susceptible phenotype ^a	Hospitals	Isolation Date	Assays for carbapenemase detection		
					Phenotypic assay for carbapenemase (MHT)	Phenotypic assay for metallo-β-lactamase (EDTA test)	PCR for bla _{NDM}
1	<i>Providencia rettgeri</i>	GEN, TET	A	03/06/2013	Negative	Positive	Positive
2	<i>Klebsiella pneumoniae</i>	AMI, AZT, TET, TGN	B	20/08/2013	Negative	Positive	Positive
3	<i>Klebsiella pneumoniae</i>	AMI, TGN	B	07/09/2013	Negative	Positive	Positive
4	<i>Proteus mirabilis</i>	AMI, CTE, GEN, TOB,	B	30/10/2013	Negative	Positive	Positive
5	<i>Klebsiella pneumoniae</i>	AMI, TGN	C	3/11/2013	Negative	Positive	Positive
6	<i>Klebsiella pneumoniae</i>	AMI, AZT, TET, TGN	D	11/11/2013	Negative	Positive	Positive
6	<i>Escherichia coli</i>	AMI, AZT, GEN, NIT, TGN	E	21/01/2014	Negative	Positive	Positive

^aAbbreviations: AMI, amikacin; GEN, gentamicin; TOB, tobramycin; TET, tetracycline; TGN, tigecycline; AZT, aztreonam; CTE, cefotetan

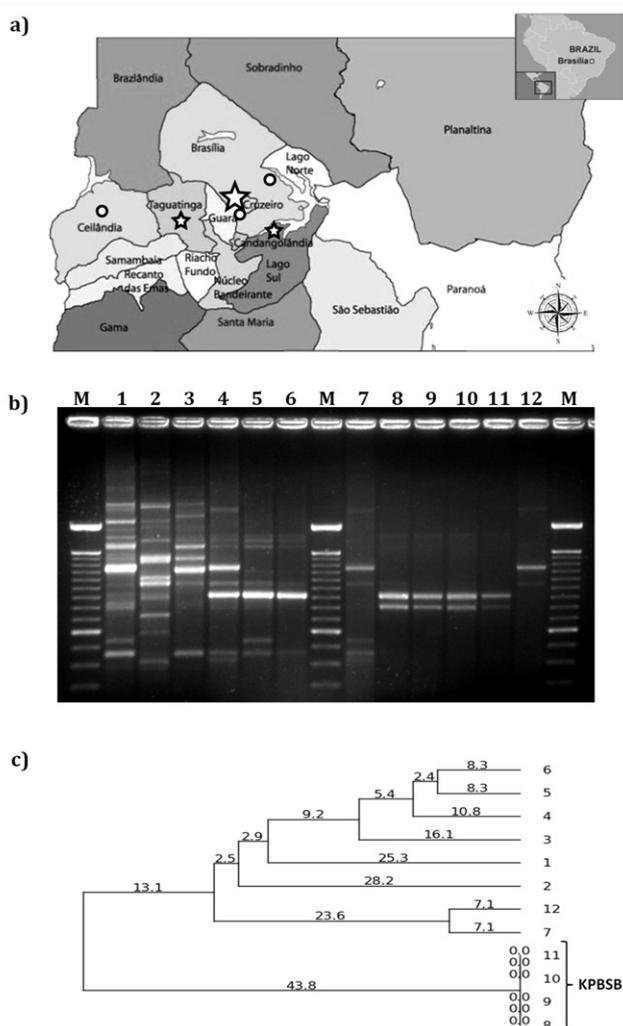


Figure 1 Distribution of cases of NDM-producing *Enterobacteriaceae* strains in Brasília and genetic relatedness of *bla*_{NDM}-producing *K. pneumoniae* strains. **A)** Geographic distribution of the occurrence of NDM-producing strains. Symbols: Stars correspond to the cases associated with *bla*_{NDM}-positive *K. pneumoniae* (small star - 1 case; large star - 2 cases). Circles indicate cases associated with other *bla*_{NDM}-positive strains (*Providencia rettgeri*, *P. mirabilis* and *Escherichia coli*). **B)** RAPD profiles of the carbapenem-resistant *K. pneumoniae* strains. *bla*_{NDM}-positive *K. pneumoniae* showed the same RAPD profile (samples 8-11) and were named KPBSB clone. Sample 1, *K. pneumoniae* IOC4955; sample 2, *K. pneumoniae* ATCC700603; samples 3 to 7 and 12 *bla*_{KPC}-positive strains isolated in different hospitals in Brasília (enrolled to examine the discriminatory power of the RAPD assay); samples 8 and 9, *bla*_{NDM}-positive strains isolated in hospital B; sample 10, *bla*_{NDM}-positive strain isolated in hospital D; and, sample 11, *bla*_{NDM}-positive strain isolated in hospital C. **C)** Dendrogram of the RAPD profiles as analyzed with PyElph software system (version 2.6.5). (see description of figure 1B for sample consultation).

Conclusion

Hospitals in Brazil have reported the isolation of several species of CRE positive for *bla*_{KPC} and, more recently, for *bla*_{NDM} as well. Additionally, the initial spread of *bla*_{NDM}-positive *K. pneumoniae*

strains has been driven by a single clone. Our findings suggest that *bla*_{NDM}-positive strains are being transported among hospitals by inpatient transfers and that they are spreading throughout patient cross infections. Finally, the present results call for an improved surveillance on inpatient transfers, for the molecular detection of CRE strains, and for enforcements in infection control measures.

Conflict of interest

All authors declare to have no conflict of interest.

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