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Multiclonal Expansion of *Klebsiella pneumoniae* Isolates Producing NDM-1 in Rio de Janeiro, Brazil

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ABSTRACT We characterized NDM-1-producing *Klebsiella* isolates from Rio de Janeiro, Brazil. PCR was applied for resistance and virulence determinants. The genetic context of bla_{NDM} was determined by S1 nuclease pulsed-field gel electrophoresis (PFGE) and hybridization. Genotyping was performed by PFGE and multilocus sequence typing (MLST). Most isolates carried multiple resistance genes and remained susceptible to amikacin, fosfomycin-trometamol, polymyxin B, and tigecycline. The spread of NDM-1-producing *Klebsiella pneumoniae* was not associated with clonal expansion and appears to be associated with Tn*3000*.

KEYWORDS *Klebsiella pneumoniae*, New Delhi metallo-β-lactamase, molecular typing, MLST, NDM carbapenemase

New Delhi metallo-β-lactamase (NDM) is a clinically significant carbapenemase that is capable of inactivating almost all β-lactams (except aztreonam) (1, 2). Since the first description of NDM in 2009, in *Klebsiella pneumoniae* and *Escherichia coli* isolates from Sweden (1), NDM-producing bacteria have been reported worldwide (3).

The first description of NDM in Brazil was in *Providencia rettgeri* isolated from Rio Grande do Sul State (southern Brazil), in 2013 (4). Despite the chromosomal gene location, the hospital where that isolate was recovered began a retrospective and prospective search for NDM-producing bacteria, identifying *Enterobacter hormaechei* subsp. *oharae* isolates carrying the bla_{NDM-1} gene in plasmids (5). Active surveillance was subsequently initiated in the country, and there have been other reports involving NDM-producing *Enterobacteriaceae*, such as *Enterobacter cloacae*, *Morganella morganii* (6), *E. coli*, and *K. pneumoniae* (7). A draft genome of a NDM-1-producing *K. pneumoniae* (NPKP) isolate from Rio de Janeiro (GenBank accession no. JSER00000000) revealed several resistance genes for different antimicrobial classes (8). That isolate (CCBH13327) was included in this study for epidemiological analysis.

Here, we characterized 16 NDM-1-producing *Klebsiella* isolates from Rio de Janeiro State (southeast region of Brazil). The isolates were received by the Laboratório de Pesquisa em Infecção Hospitalar (Oswaldo Cruz Institute, Rio de Janeiro, Brazil). They were collected between July 2013 and November 2014 from eight health care institutions (H1 to H8) in three cities in the region (Rio de Janeiro, Niterói, and Campos dos Goytacazes) and were recovered from rectal swab ($n = 7$), urine ($n = 3$), blood ($n = 4$), catheter tip ($n = 1$), and cerebrospinal fluid (CSF) ($n = 1$) samples from nonconsecutive patients.

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The isolates were identified as *K. pneumoniae* by conventional biochemical techniques, and the isolate CCBH16302 was identified as *Klebsiella quasipneumoniae* (9) by whole-genome sequencing. The MICs for meropenem, imipenem, tigecycline, and polymyxin B were determined by Etest (AB Biodisk, Sweden), and susceptibility to other antimicrobial agents was determined by agar diffusion. Phenotypic detection of carbapenemases was based on enzymatic blocking using EDTA and phenyl boronic acid (10) and *in vitro* analysis of imipenem hydrolysis by the Carba NP test (11).

Pulsed-field gel electrophoresis (PFGE) typing using the restriction enzyme XbaI and multilocus sequence typing (MLST) were performed for all isolates as indicated in the Pasteur Institute MLST databases (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html). PCR assays were used to detect the β-lactamase-encoding genes bla_{NDM} (12), bla_{KPC}, $bla_{OXA-48-like}$, bla_{IMP}, bla_{VIM} (13), bla_{CTX-M} (14), bla_{SHV}, bla_{TEM}, and bla_{CMY} (15) and the plasmid-mediated quinolone and aminoglycoside resistance determinants *qnrA*, *qnrB*, *qnrS* (16), *aadA* (17), *aadB* (18), *aac(3′)-IIa* (19), *aac(6′)-Ib* (20), *rmtA*, *rmtB*, *rmtC*, *armA*, *npmA* (21), and *rmtD* (22). The PCR products of bla_{NDM}, bla_{KPC}, and *aac(6′)-Ib* were subjected to DNA sequencing. The virulence factor-encoding genes *cf29a*, *mrkD*, *fimH* (23), *entB*, *ycfM* (24), *iroN* (25), *kfu* (26), *magA* (27), *allS* (28), and *ybtS* (29) were also searched by PCR. The hypermucoviscosity phenotype was detected by the string test (30).

Analysis of the genetic environment of the bla_{NDM} gene was performed by PCR mapping, as described previously (12), and S1 nuclease PFGE, followed by Southern blot hybridization with a bla_{NDM} probe. For further analysis, three representative isolates (CCBH13327, CCBH15949, and CCBH16302), belonging to different sequence types (STs), were subjected to whole-genome sequencing. The genomic DNA of the three strains was extracted with the QIAamp DNA Blood minikit (Qiagen, Germany) and was sequenced using an Illumina MiSeq platform (Illumina, USA). A genomic library was constructed by transposon tagmentation with the Nextera XT DNA sample preparation kit (Illumina). Sequence reads were then trimmed and filtered using a Phred score of >20. Geneious v.6.1.7 software (Biomatters, New Zealand) was used to perform *de novo* assembling. Genome annotation was performed with Rapid Annotation using Subsystem Technology (RAST) v.2.0 (http://rast.nmpdr.org).

The isolates were nonsusceptible to all β-lactams (100%) except for aztreonam (69%) and gentamicin, ciprofloxacin, and sulfamethoxazole-trimethoprim (75%) and remained susceptible to amikacin (81%) and fosfomycin-trometamol (94%), as determined according to CLSI guidelines (11). In accordance with EUCAST guidelines (31), the strains were considered susceptible to polymyxin B (87.5%) and tigecycline (69%) (Table 1). As reported previously, fosfomycin, polymyxin B, and tigecycline are the antimicrobials with the highest activities against NDM-1 producers (32).

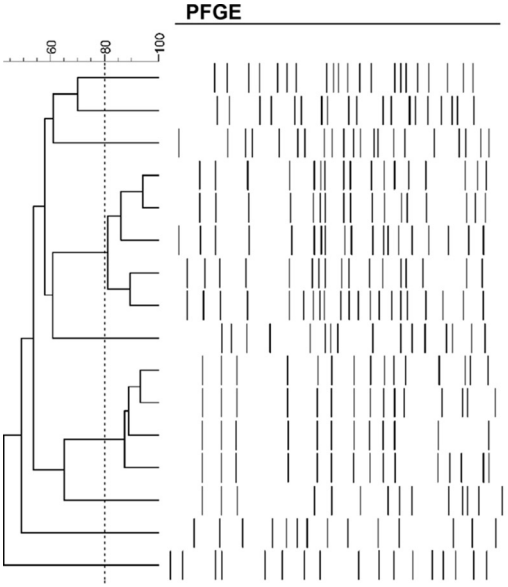
All strains were phenotypically positive for metallo-β-lactamase production and harbored bla_{NDM-1}. Other resistance genes (Table 1), including bla_{KPC-2}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrA*, *qnrB*, *qnrS*, *aadA*, *aadB*, *aac(3)-IIa*, *aac(6′)-Ib*, and its variants, were also found. The coproduction of bla_{NDM-1} with other β-lactamase genes and genetic determinants related to quinolone and aminoglycoside resistance was often detected (12). We also detected two strains (CCBH16059 and CCBH16505) carrying the bla_{NDM-1} and bla_{KPC-2} genes in separate plasmids; this finding has been described in *E. hormaechei* in Brazil (33), but this is the first description of *K. pneumoniae* coharboring bla_{NDM-1} and bla_{KPC-2} in this country. This finding is worrisome, since KPC-2-producing *K. pneumoniae* is already endemic in Brazil (34) and the spread of bla_{NDM-1} could be facilitated when associated with this successful pathogen.

The virulence genes observed included *entB*, *fimH*, *yfcM*, *mrkD* (100%), *kfU* (75%), and *ybtS* (25%). Four isolates were positive for the hypermucoviscosity phenotype (Table 1). However, no specific virulence factor has been associated with NDM-type producers. Apparently, the difficulty of treating infections caused by carbapenemase-producing *Enterobacteriaceae* is the most significant issue (32, 35).

TABLE 1 Resistance and virulence features of NDM-1-producing *Klebsiella* spp. from Rio de Janeiro[a]

Isolate	Pulsotype	ST	Resistance genes	Size of plasmid harboring bla_{NDM-1} (kb)	Virulence features	MIC (μg/ml)				Disk diffusion results	
						IPM	MEM	PMB	TGC	Nonsusceptible	Susceptible
CCBH13327	E	323	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrA*, *aadA*, *aac(3)-IIa*, *aac(6′)-Iq*	~190	*entB*, *fimH*, *ycfM*, *mrkD*	>32	>32	0.5	2.0	ATM, CAZ, CTX, ETP, FEP, TZP, AMK, CIP, GEN, SXT	FOT
CCBH15668	D	37	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~306	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	2.0	8.0	0.5	0.5	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH15669	D	37	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*		*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	2.0	2.0	0.5	0.5	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH15779	I	365	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~346	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	2.0	2.0	0.5	0.5	CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, ATM, FOT
CCBH15948	D	37	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~346	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*; positive string test	4.0	4.0	0.5	1.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH15949	D	37	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~346	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*; positive string test	4.0	4.0	2.0	2.0	ATM, CAZ, CTX, ETP, FEP, TZP, AMK, CIP, GEN, SXT	FOT
CCBH16046	F	1588	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~296	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*, *ybtS*	>32	>32	0.5	1.0	CAZ, CTX, ETP, FEP, TZP, FOT	AMK, ATM, CIP, GEN, SXT
CCBH16047	G	340	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrA*, *aac(3)-IIa*, *aac(6′)-Ib*	~290	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*; positive string test	>32	>32	0.5	1.0	CAZ, CTX, ETP, FEP, TZP, AMK, CIP, GEN, SXT	ATM, FOT
CCBH16059	C	906	bla_{NDM-1}, bla_{KPC-2}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~172	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*, *ybtS*; positive string test	>32	>32	1.0	1.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH16302	A	138	bla_{NDM-1}, bla_{TEM}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~346	*entB*, *fimH*, *ycfM*, *mrkD*	4.0	>32	0.5	8.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH16328	F	1588	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~430	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	>32	>32	1.0	1.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH16385	B	1803	bla_{NDM-1}, bla_{TEM}, *aadA*	~178	*entB*, *fimH*, *ycfM*, *mrkD*	32	>32	0.5	1.0	CAZ, CTX, ETP, FEP, TZP, GEN	AMK, ATM, CIP, FOT, SXT
CCBH16505	F	1588	bla_{NDM-1}, bla_{KPC-2}, bla_{TEM}, bla_{SHV}, *qnrB*, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~296	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*, *ybtS*	>32	>32	1.0	1.0	ATM, CAZ, CTX, ETP, FEP, TZP	AMK, CIP, GEN, FOT, SXT
CCBH16528	D	37	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, *aadA*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~306	*entB*, *fimH*, *ycfM*, *mrkD*, *ybtS*	2.0	2.0	4.0	2.0	CAZ, CTX, ETP, FEP, TZP	AMK, ATM, CIP, GEN, FOT, SXT
CCBH16784	H	2279	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *qnrB*, *aadA*, *aadB*, *aac(3)-IIa*, *aac(6′)-31*	~143	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	>32	>32	32	1.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT
CCBH16886	F	1588	bla_{NDM-1}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, *armA*, *qnrB*, *aac(3)-IIa*, *aac(6′)-Ib-cr*	~87	*entB*, *fimH*, *ycfM*, *mrkD*, *kfu*	>32	>32	0.5	8.0	ATM, CAZ, CTX, ETP, FEP, TZP, CIP, GEN, SXT	AMK, FOT

[a]ST, sequence type; AMK, amikacin; ATM, aztreonam; CAZ, ceftazidime; CIP, ciprofloxacin; CTX, cefotaxime; ETP, ertapenem; FEP, cefepime; FOT, fosfomycin-trometamol; GEN, gentamicin; IPM, imipenem; MEM, meropenem; PMB, polymyxin B; SXT, trimethoprim-sulfamethoxazole; TGC, tigecycline; TZP, piperacillin-tazobactam.



Isolates	Pulsotype	ST	City	Hospital	Date	Specimen
CCBH16302	A	138	Campos dos Goytacazes	H1	5/2014	Rectal swab
CCBH16385	B	1803	Rio de Janeiro	H8	5/2014	Blood
CCBH16059	C	906	Rio de Janeiro	H7	5/2014	Rectal swab
CCBH15668	D	37	Campos dos Goytacazes	H1	3/2014	Urine
CCBH15669	D	37	Campos dos Goytacazes	H1	3/2014	Urine
CCBH16528	D	37	Campos dos Goytacazes	H1	5/2014	Blood
CCBH15948	D	37	Campos dos Goytacazes	H1	4/2014	Blood
CCBH15949	D	37	Campos dos Goytacazes	H1	4/2014	Blood
CCBH13327	E	323	Campos dos Goytacazes	H1	7/2013	Rectal swab
CCBH16046	F	1588	Rio de Janeiro	H5	5/2014	Catheter tip
CCBH16505	F	1588	Rio de Janeiro	H4	7/2014	Urine
CCBH16328	F	1588	Rio de Janeiro	H3	6/2014	Rectal swab
CCBH16886	F	1588	Rio de Janeiro	H3	9/2014	Rectal swab
CCBH16047	G	340	Niterói	H2	5/2014	CSF
CCBH16784	H	2279	Rio de Janeiro	H6	9/2014	Rectal swab
CCBH15779	I	365	Campos dos Goytacazes	H1	3/2014	Rectal swab

FIG 1 Dendrogram and epidemiological characteristics of NDM-1-producing *Klebsiella* spp. from Rio de Janeiro.

PFGE and MLST analysis allowed us to identify nine pulsotypes (pulsotypes A to I) and nine STs (STs 37, 138, 323, 340, 365, 906, 1588, 1803, and 2279) (Fig. 1). The STs 37, 138, 323, and 365 were restricted to the same city and hospital (H1), and an outbreak of ST37 was identified among patients in the adult intensive care unit (ICU). ST1588 was identified in three hospitals in Rio de Janeiro city, and the common procedures for patient and staff transferences between hospitals could explain this finding. Although there are some reports of clonal spread (36, 37), our findings suggest that the spread of NPKP in Rio de Janeiro is not related to specific clones, as described commonly (32, 38). STs 138, 323, and 340 have been described in Brazil in association with KPC production (34); ST340 belongs to clonal complex 258 and was identified from a CSF sample. Furthermore, two new STs, i.e., ST1803 and ST2279, isolated from blood and rectal swab samples, respectively, were observed.

Plasmid analyses were successfully performed for 15/16 isolates. The plasmids carrying the $bla_{\text{NDM-1}}$ gene had different sizes, ranging from ~87 to 430 kb (Table 1). The $bla_{\text{NDM-1}}$ gene was associated with a truncated IS*Aba125* upstream and a ble_{MBL} (bleomycin resistance) gene downstream in all isolates. For the three selected strains, namely, CCBH13327 (GenBank accession no. JSER00000000), CCBH15949 (GenBank accession no. MCGW00000000), and CCBH16302 (GenBank accession no. MDCA00000000), whole-genome sequencing showed that the $bla_{\text{NDM-1}}$ gene was associated with the Tn*3000* transposon (39). This structure was delimited by two copies of IS*3000*; the first copy truncated the 5′ portion of IS*Aba125* upstream of the $bla_{\text{NDM-1}}$ gene, and ble_{MBL} was present downstream, followed by *trpF*, *tat*, *cutA1*, *groES*, and *groEL*, truncated by the second copy of IS*3000*. The same genetic structure was reported previously for NDM-1-producing *E. coli* and *E. hormaechei* from Rio de Janeiro (39). Our findings support the suggestion that Tn*3000* has a key role in the spread of the $bla_{\text{NDM-1}}$ gene, since the gene was found in plasmids of different sizes even in genetically related strains.

The spread of NPKP in Rio de Janeiro involves nonclonal isolates harboring the $bla_{\text{NDM-1}}$ gene in association with Tn*3000* and a variety of determinants of resistance to different antimicrobial classes. To our knowledge, this is the first report of *K. quasipneumoniae* carrying $bla_{\text{NDM-1}}$. We also highlight the possibility that some patients are serving as reservoirs for NDM-producing bacteria, because 44% of isolates were recovered from surveillance rectal swabs, which means that the detection of these isolates may be underestimated.

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