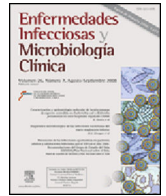




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Original article

Molecular characterization of KPC-producing *Klebsiella pneumoniae* isolated from patients in a Public Hospital in Caracas, Venezuela



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ABSTRACT

Introduction: *Klebsiella pneumoniae* carbapenemase (KPC)-producing bacteria are amongst the most important causative agents of nosocomial infections worldwide. Isolates of this bacterium have been identified in Venezuela but little is known about their local spread. The aim of this study was to perform the molecular characterization of KPC-producing strains isolated from 2012 to 2013 in a public hospital in Caracas, Venezuela.

Methods: Twenty-two *K. pneumoniae* clinical isolates phenotypically classified as KPC producing were subjected to PCR screening for the presence of *bla*_{KPC} genes and their location within transposon *Tn*4401. The *bla*_{KPC} PCR product was sequenced to identify the KPC alleles. Genotypic analysis was performed by means of repetitive extragenic palindromic PCR (rep-PCR) and Multi Locus Sequence Typing (MLST). Finally, conjugation and electroporation assays were used to determine whether the *bla*_{KPC} genes were found in plasmids.

Results: All isolates contained the *bla*_{KPC-2} variant, and 21 of the 22 were associated with the *Tn*4401b isoform. The strains were distributed in 8 sequence types (ST), three of which were new. Conjugation and electroporation assays indicated that 95.5% ($n=21/22$) of the isolates contained the *bla*_{KPC} gene in plasmids.

Conclusions: This study on circulating bacterial strains and the identification of KPC alleles may help to understand the routes of dissemination and control their spread within this hospital.

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Caracterización molecular de aislados de *Klebsiella pneumoniae* productores de carbapenemasas tipo KPC provenientes de pacientes de un hospital público en Caracas, Venezuela

RESUMEN

Introducción: *Klebsiella pneumoniae* productora de carbapenemasas tipo KPC es uno de los principales agentes causantes de infecciones nosocomiales a nivel mundial. En Venezuela se han identificado aislados de esta bacteria, sin embargo, se conoce poco sobre su dispersión. El objetivo de este estudio fue realizar la caracterización molecular de aislados de *K. pneumoniae* productores de KPC provenientes de un hospital público en Caracas, Venezuela, durante los años 2012 y 2013.

Métodos: Se analizaron 22 aislados de *K. pneumoniae* clasificados fenotípicamente como productores de KPC, se les realizó la detección del gen *bla*_{KPC} así como su ubicación en el transposón *Tn*4401 mediante PCR. El producto de PCR del gen *bla*_{KPC} se secuenció para identificar los alelos circulantes. El análisis genotípico se realizó empleando las técnicas de amplificación por PCR de las secuencias repetidas

Palabras clave:

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extragénicas palindrómicas (rep-PCR) y la secuenciación de múltiples *loci* (MLST). Mediante ensayos de conjugación o electroporación, se determinó si los genes *bla_{KPC}* se encontraban en plásmidos.

Resultados: Los 22 aislados contenían una variante del gen *bla_{KPC-2}* pero 21 de ellos estaban asociados a la isoforma *Tn4401b* y se distribuyeron en 8 secuencias tipo (ST), siendo tres de ellas nuevas. Los ensayos de conjugación y electroporación indicaron que el 95,5% ($n = 21/22$) de los aislados portaban al gen *bla_{KPC}* en plásmidos.

Conclusión: El estudio de aislados clínicos circulantes así como la identificación de los alelos del gen *bla_{KPC}* podría ayudar a conocer las rutas de diseminación y a controlar su propagación en este hospital.

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Introduction

Carbapenemase-producing *Klebsiella pneumoniae* (KPC) strains are increasingly reported worldwide.¹ Bacteria producing these enzymes are generally susceptible only to a few remaining antibiotics and are associated with high mortality, especially in patients with bloodstream infections.² In addition to being resistant to all β -lactams available, KPC carbapenemases have a high capacity to spread, as the KPC genes are contained within transposons such as *Tn4401*, which are carried on transferable plasmids.^{3,4} Although an alarming dispersion of carbapenem-resistant *K. pneumoniae* has been reported in the hospitals of several Venezuelan cities,⁵ the epidemiology and clinical impact of Venezuelan KPC-producing *K. pneumoniae* have not been described. The aim of this study was to characterize the predominant resistant clones circulating in a tertiary-care public hospital located in the Capital District of Caracas. Twenty two KPC-producing *K. pneumoniae* strains isolated from clinical specimens during the years 2012 and 2013 were studied to determine their evolutionary relatedness, identify their KPC alleles and the *Tn4401* flanking regions and document the presence of *bla_{KPC}*-carrying plasmids.

Methods

Hospital settings

“Dr. Domingo Luciani” is a large tertiary-care public hospital belonging to the Venezuelan Institute of Social Security that has 769 beds (727 inpatient and 42 intensive care), with a surgical preponderance. It is the principal public hospital in the eastern part of Caracas, the capital of Venezuela.

Study population

A cross-sectional study was conducted that included carbapenem-resistant *K. pneumoniae* strains isolated from January 2012 to December 2013. Molecular analyses were performed on the first clinical bacterial isolate recovered from a patient during their hospitalization. A total of 22 isolates were obtained, of which 16 were isolated in 2012 and 6 were isolated in 2013. Clinical and epidemiological information was obtained from the medical records for each patient and made irreversibly anonymous. This research had the approval of the Bioethics Committees of the Hospital and the Instituto Venezolano de Investigaciones Científicas.

Bacterial strains and antibiotic susceptibility testing

The study included twenty two *K. pneumoniae* isolates with intermediate or full resistant to carbapenems, according to CLSI 2012 cutoffs concentrations.⁶ The cut-off points for carbapenem sensitivity were Minimal Inhibitory Concentrations of ≤ 1 $\mu\text{g/ml}$

for meropenem and imipenem, and ≤ 0.5 $\mu\text{g/ml}$ for ertapenem. The species identification of isolates and determination of their antibiotic susceptibilities were performed with the automated MicroScan System (Siemens Healthcare Diagnostics Inc., CA, USA) in the hospital's clinical laboratory. All strains were tested for sensitivity to amikacin, gentamicin, imipenem, meropenem, ciprofloxacin, levofloxacin, tetracycline, tigecycline and trimethoprim/sulfamethoxazole. The modified Hodge Test was used to detect phenotypic carbapenemase production.⁷ For this test, the negative control was *Escherichia coli* ATCC 25922 and the positive control was a KPC – producing *Klebsiella pneumoniae* obtained from a previous study.⁸

Detection of genes encoding carbapenemases

Polymerase chain reaction (PCR) was performed with the primers listed in Table S1 to screen for the carbapenemase genes *bla_{KPC}*,⁹ *bla_{IMP}*, *bla_{VIM}*, *bla_{NDM}* and *bla_{OXA-48}*.¹⁰ After PCR amplification the fragments were sequenced with the corresponding primers in the forward and reverse directions by Macrogen, Korea, and the results were compared with sequences available in the Lahey database (www.lahey.org/Studies/) and GenBank®, using the Basic Local Alignment Search Tool (BLAST) (www.ncbi.nlm.nih.gov/blast/).

Genetic environment of *bla_{KPC}* gene

The genetic environment of the *bla_{KPC}* gene was determined by PCR, using previously described primers¹¹ specific for the *Tn4401* transposon (Table S1).

Molecular genotyping

The genetic relationships between the carbapenem-resistant isolates were determined by repetitive element palindromic-PCR (rep-PCR) and Multi-Locus Sequence Typing (MLST). rep-PCR was performed using previously described primers REP1 and REP2.¹² PCR fingerprinting was analyzed with the BioNumerics software package version 7.5 (Applied Maths NV/Inc.) using the Dice binary coefficient and Unweighted Pair Group Method (UPGM) arithmetic average with settings of 1% tolerance and 1% optimization to create dendrograms. The positions of bands on each gel were normalized using the 100-bp DNA ladder (New England Biolabs® Inc.) as an external reference standard. MLST was performed as described by Diancourt et al.¹³ (Table S1). After PCR amplification, the fragments were sequenced with the corresponding primers in the forward and reverse directions by Macrogen, Korea. Allele numbers and sequence types (STs) were assigned by the *Klebsiella pneumoniae* MLST web site (<http://www.pasteur.fr/recherche/genopole/PF8/mlst/Kpneumoniae.html>).

Table 1

Antibiotic susceptibility in KPC-producing *K. pneumoniae* isolates and clinical characteristics of patients from whom carbapenem-resistant *K. pneumoniae* isolates were obtained.

Isolate	Year	IMP	MEM	ETP	AK	GN	CIP	LEX	SXT	TE	TYG	Gender	Patient type	Infection site	Hospitalization area
HDL1	2012	4	8	>4	≤16	>8	>2	>4	>2/38	≤4	≤2	M	P	Skin and soft tissue	Pediatrics
HDL2	2012	8	>8	>4	32	>8	≤1	≤2	>2/38	≤4	≤2	F	P	Pneumonia	Pediatrics
HDL3	2012	8	>8	>4	≤16	>8	>2	>4	>2/38	≤4	≤2	F	P	Cerebrospinal fluid	Pediatrics
HDL4	2012	>8	>8	>4	≤16	≤4	≤1	≤2	≤2/38	≤4	≤2	F	A	Skin and soft tissue	Surgery
HDL5	2012	>8	>8	>4	16	>8	>2	>4	>2/38	≤4	≤2	F	A	Skin and soft tissue	Surgery
HDL6	2012	>8	>8	>4	≤16	>8	>2	>4	>2/38	-	-	M	A	UTI	Internal medicine
HDL7	2012	8	>8	>4	≤16	≤4	>2	>4	>2/38	≤4	≤2	F	P	Skin and soft tissue	Surgery
HDL9	2012	>8	>8	>4	≤16	≤4	>2	>4	>2/38	≤4	≤2	M	A	Intra-abdominal	Surgery
HDL10	2012	>8	>8	>4	32	>8	-	-	>2/38	-	-	F	P	Cerebrospinal fluid	Pediatrics
HDL11	2012	>8	>8	>4	≤16	>8	>2	4	>2/38	≤4	≤2	M	P	Catheter-associated UTI	Pediatrics
HDL12	2012	>8	>8	>4	>32	>8	>2	>4	>2/38	>8	≤2	F	A	Surgical site	Surgery
HDL13	2012	>8	>8	>4	≤16	≤4	>2	>4	≤2/38	≤4	≤2	M	A	Pneumonia	Intensive care
HDL14	2012	>8	>8	>4	≤16	≤4	>2	>4	≤2/38	≤4	≤2	M	A	Pneumonia	Intensive care
HDL15	2012	>8	>8	>4	>32	>8	>2	>4	>2/38	>8	≤2	M	P	Skin and soft tissue	Surgery
HDL17	2012	>8	>8	>4	>32	>8	>2	>4	>2/38	>8	≤2	M	A	Catheter-related bloodstream	Internal medicine
HDL20	2012	>8	>8	>4	≤8	≤4	>2	>4	>2/38	≤4	≤2	M	P	Pneumonia	Pediatrics
HDL23	2013	>8	>8	>4	≤16	8	>2	≤2	>2/38	≤4	≤2	M	P	Cerebrospinal fluid	Pediatrics
HDL24	2013	>8	>8	>8	≤16	>8	>2	4	≤2/38	>8	≤2	M	A	Intra-abdominal	Surgery
HDL25	2013	8	8	>4	>32	>8	>2	>4	≤2/38	>8	≤2	M	A	Intra-abdominal	Internal medicine
HDL26	2013	2	8	>4	≤16	≤4	2	≤2	>2/38	≤4	≤2	M	P	Pneumonia	Intensive care
HDL27	2013	>8	>8	>8	>32	≤4	>2	>4	>2/38	≤4	≤2	M	A	Pneumonia	Intensive care
HDL31	2013	>8	>8	>4	≤16	≤4	≤1	≤2	>2/38	≤4	≤2	F	A	Intra-abdominal	Surgery

IMP: imipenem, MEM: meropenem, ETP: ertapenem, AK: amikacin, GN: gentamicin, CIP: Ciprofloxacin, LEX: levofloxacin, SXT: trimethoprim-sulfamethoxazole, TE: tetracycline, TYG: tigecycline, (-): no data, (-): not detected, M: male, F: female, A: adult, P: pediatric, UTI, urinary tract infection.

Plasmid analysis

Conjugation experiments were performed to determine whether the carbapenemase resistance of the *K. pneumoniae* isolates was attributable to the carriage of *bla*_{KPC} genes on plasmids. The recipient cells were *Escherichia coli* strain J-53 (F⁻, *met*⁻ *pro*⁻, Azi^R). For the isolates from which transconjugants were not obtained, plasmid DNA was extracted by the Kier extraction method¹⁴ and electroporated with a Bio-Rad Gene Pulser[®] into *Escherichia coli* XL1-Blue (F⁺, *proAB*, *lacIq*ΔM15, Tc^R) recipient cells. Transconjugants were selected on LB agar plates supplemented with ampicillin (100 μg/ml) and sodium azide (200 μg/ml), while transformants were selected on LB agar plates supplemented with ampicillin (100 μg/ml). The presence of the *bla*_{KPC} gene was confirmed by PCR.

Results

Clinical and epidemiological characteristics

We analyzed 22 carbapenem-resistant *K. pneumoniae* strains isolated from the clinical specimens of an equal number of inpatients in a public hospital in Caracas, Venezuela. The patients' clinical characteristics are summarized in Table 1. Of the 22 total patients, 12 (54.5%) were adults and 14 (63.6%) were male. All infections were health care associated, with 8 (36.4%) from the surgical unit. The most common sites of infections were intra-abdominal, pneumonia, skin and soft tissue, with 5 patients each (22.7%), followed by catheter-related bloodstream and cerebrospinal fluid, with 2 patients each (9.2%) (Table 1).

Antibiotic susceptibility testing of *K. pneumoniae* isolates

All carbapenemase-producing *K. pneumoniae* isolates were resistant to ertapenem and meropenem while 21 (95.5%) were resistant to imipenem (Table 1). All isolates were positive with the Hodge Test, indicating the presence of a carbapenemase. The percentages of isolates resistant to the non-β-lactam

drugs were as follows: gentamicin, 54.5% (*n* = 12/22); amikacin, 22.7% (*n* = 5/22); ciprofloxacin, 81% (*n* = 17/21); levofloxacin, 66.6% (*n* = 14/21); trimethoprim-sulfamethoxazole, 72.3% (*n* = 17/22) and tetracycline, 20% (*n* = 5/20), while 100% were susceptible to tigecycline (Table 1).

Detection of genes encoding carbapenemases

The *bla*_{KPC-2} gene was detected in all isolates. None of the isolates harbored *bla*_{VIM}, *bla*_{IMP}, *bla*_{OXA-48} or *bla*_{NDM} genes encoding others carbapenemases.

Genetic environment of *bla*_{KPC} gene

In 21 of the 22 isolates (95.5%) from which *bla*_{KPC-2} gene was amplified, the *Tn*4401 primers produced the 703-bp PCR product associated with *Tn*4401 variant 'b'.¹¹ The *ISKpn6* and *tnpA* genes were amplified and were also found in 21 of the 22 isolates. The inverted repeat sequences of the region flanking *Tn*4401 were not amplified with the specific primers described by Naas et al.^{4,15} There was only one isolate, HDL4, from which neither *ISKpn6* nor the *tnpA* gene nor the nonconserved region of *Tn*4401 could be amplified.

Molecular genotyping of KPC-producing *K. pneumoniae* isolates

The rep-PCR studies revealed that KPC-2-producing *K. pneumoniae* isolates belonged to several different genetic backgrounds (Fig. 1). MLST genotyping yielded 8 different sequence types: ST11 (45.5%, *n* = 10), ST15 (4.5%; *n* = 1), ST17 (4.5%; *n* = 1), ST833 (4.5%; *n* = 1), ST1271 (18.9%; *n* = 4), and three novel sequence types that were deposited in the *K. pneumoniae* MLST database and assigned the designations ST1857 (4.5%; *n* = 1), ST1859 (9.1%; *n* = 2), and ST1860 (9.1%, *n* = 2) (Fig. 1). The allelic profile of ST1857 is 17-19-39-20-125-4-305 and included a new *tonB* allele, while STs 1859 and 1860 have new alleles combinations (18-22-26-108-98-37-227 and 18-22-18-61-32-13-50, respectively), indicating that they are not SLV of a previously described Sequence Type.

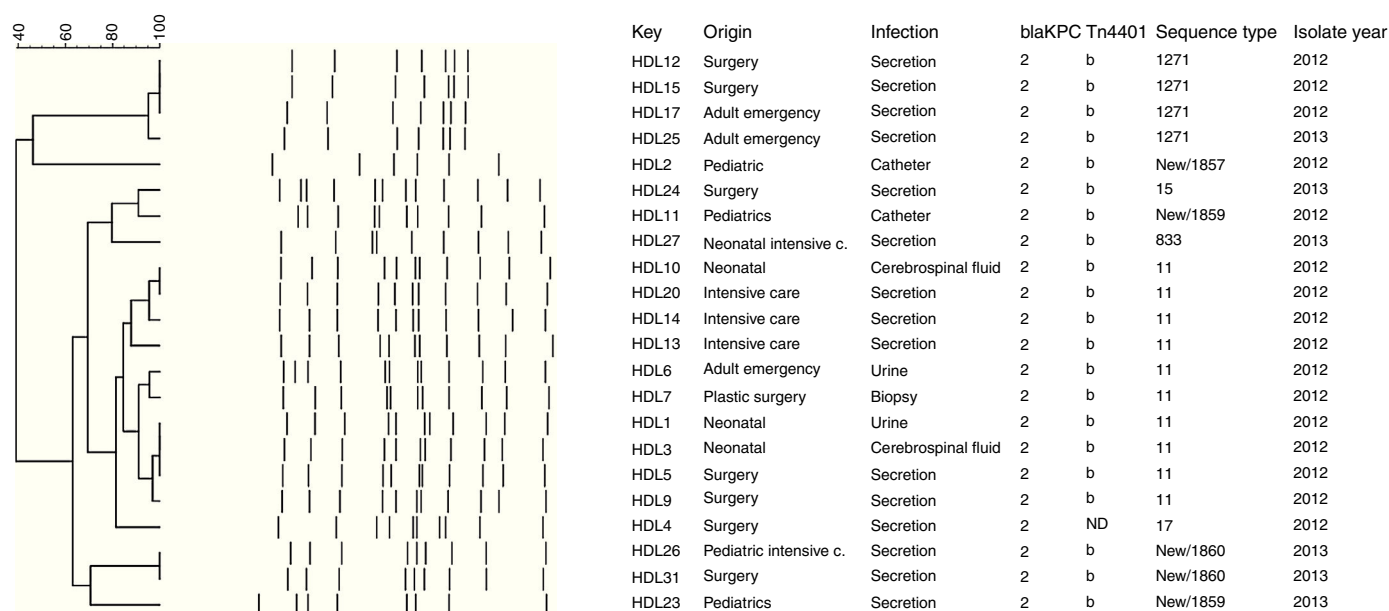


Fig. 1. Genetic relationship of 22 isolates of *bla*_{KPC-2}-harboring *K. pneumoniae* (Bionumerics software package version 7.5, Applied Maths NV/Inc.). The list on the right contains the results from analysis of *bla*_{KPC}, MLST and the nonconserved region of the *Tn4401* element. ND: no determine.

Plasmid analysis

Nine of the 22 *K. pneumoniae* isolates (40.9%) transferred β -lactam resistance to *E. coli* recipients in the conjugation assay. Plasmid DNA was isolated from the remaining 13 *K. pneumoniae* isolates and used to electroporate the *E. coli* recipient strain, XL1-Blue. Transformants were obtained with plasmid DNA from 12 of the 13 (92.3%), confirming that the *bla*_{KPC} genes were located on plasmids. Both electroporation and conjugation experiments were unsuccessful for one isolate (HDL11), despite repeated attempts. The presence of the *bla*_{KPC} gene was confirmed by PCR in all transformants and transconjugants.

Discussion

Carbapenems have been the treatment of choice for infections caused by ESBL-producing *Enterobacteriaceae*, but an undesirable consequence has been the selection of carbapenem-resistant strains.¹⁶ Although carbapenem resistance in the *Enterobacteriaceae* is still unusual in Venezuela, KPCs are carried on mobile genetic elements and are easily disseminated, so the rapid detection of KPC-carrying bacteria is essential for effective therapeutic options and control measures.¹⁷ The present study integrated clinical and molecular data on *K. pneumoniae* strains isolated in one large public hospital in Venezuela, and determined the presence of different circulating genotypes and KPC alleles in order to help identify and control routes of dissemination. The majority of the 22 carbapenem-resistant *K. pneumoniae* strains were isolated from the pediatric and surgical services areas and they varied in their antibiotic sensitive or resistance profiles.

The *bla*_{KPC} gene could be amplified from all isolates and although there have been twenty-three variants of the KPC enzyme reported (KPC-2 through KPC-24) (<http://www.lahey.org/studies/other.asp#table1>), sequencing of the *bla*_{KPC} genes amplified from our isolates showed that all possessed the KPC-2 allele variant, one of the most extensively distributed worldwide,^{2,18} including in the South American countries of Colombia,^{19,20} Brazil^{21,22} and Argentina.^{23,24} There have been three previous reports of KPC-producing enterobacteria isolated in Venezuela. A KPC-2-producing *Enterobacter cloacae* isolated from tracheal

secretions of a cancer patient in a private hospital in Caracas²⁵ and a KPC-2-producing *Klebsiella oxytoca* found in a pediatric patient with pneumonia in the intensive care unit at Los Andes University Hospital, Mérida, Venezuela.¹⁷ Finally, in a cross sectional study, Marciano et al.⁵ analyzed enterobacteria from eight hospitals in Caracas isolated between October 15, 2009 and January 15, 2010, and found that 1.9% was *K. pneumoniae* with a KPC producing phenotype,⁵ but the alleles of KPC genes were not identified.

The *bla*_{KPC} gene is generally associated with a *Tn3*-based transposon, *Tn4401*, composed of a transposase gene, a resolvase gene, the *bla*_{KPC} gene and two insertion sequences (*ISKpn6* and *ISKpn7*). Six isoforms (a–e) of *Tn4401* have been described, which differ by polymorphisms located upstream of the *bla*_{KPC} gene.²⁶ We found the *bla*_{KPC-2} gene associated with *Tn4401* variant “b” in all isolates tested except HDL4. The “b” isoform has been observed in the USA,¹⁸ Colombia,¹¹ and Brazil.³ Similar to the report by Pereira et al.,³ the inverted repeat sequences of the flanking regions were not amplified in our isolates, suggesting that their insertion sites may be different from that of *K. pneumoniae* YC described by Naas et al.⁴ In the HDL4 isolate PCR did not amplify either *ISKpn6*, the *tnpA* gene, or the nonconserved region of *Tn4401*, suggesting that the genetic environment of the *bla*_{KPC-2} gene in this isolate is different from that of the other strains isolated from this hospital. A distinct genetic background of the *bla*_{KPC-2} gene has been identified in China, where it was found to be carried on plasmid pKP048. This plasmid contains the integration structure of a *Tn3*-based transposon and partial *Tn4401* segment, with the gene order *Tn3*-transposase, *Tn3*-resolvase, *ISKpn8*, the *bla*_{KPC-2} gene and the *ISKpn6*-like element.²⁷ Genotyping with rep-PCR showed that there were several *K. pneumoniae* genotypes circulating in the hospital, which was confirmed by MLST. The 22 *bla*_{KPC-2}-harboring *K. pneumoniae* isolates belonged to 8 different sequence types, with 45% ($n = 10/22$) belonging to ST11, part of the Clonal Complex CC258 that has been reported in many countries worldwide,¹¹ including China,²⁸ Korea,²⁹ Hungary,³⁰ Poland,³¹ Brazil,^{3,32} Spain,³³ and United Kingdom.³⁴ The second most common ST in our study, ST1271, found in 4 (18.9%) of our isolates, was previously reported in Cuba.³⁵ This is particularly interesting because there are many Cuban medical personnel working in Venezuela. ST15 (4.5%; $n = 1$), belonging to GC15, has been found

in France, Italy, Spain and Israel,³⁶ South Korea, Malaysia, Singapore and Thailand,³⁷ Portugal,³⁸ Hungary³⁹ and China.⁴⁰ ST833 (4.5%; $n=1$) has been reported in Israel³⁶ and in Trieste, Italy⁴¹ and also is part of the Clonal Complex CC258. Interestingly, the KPC-2-producing *Klebsiella pneumoniae* ST833 strain was isolated in the Trieste Pediatric Hospital from the blood culture of a three year old patient transferred from a Venezuelan hospital to undergo marrow transplantation. In our on-going epidemiological study of KPC-producing *K. pneumoniae* in Venezuela, we have also found isolates belonging to ST833 in two other Venezuelan states: Zulia (in press) and Anzoátegui.⁸ ST17 has been found in China⁴² and Canada.⁴³ It will be interesting to determine whether the novel sequence type we found – ST1857 (4.5%; $n=1$), ST1859 (9.1%; $n=2$), ST1860 (9.1%, $n=2$) – are widely distributed within Venezuela or merely locally restricted genotypes.

Plasmid analysis of the carbapenemase resistant *K. pneumoniae* isolates indicated that nine (40.9%) contained the *bla*_{KPC-2} gene on plasmids that were readily transferable by conjugation, while in twelve others a resistance conferring plasmid could only be transferred by electroporation. We were unable to transfer carbapenem resistance from strain HD11, with either conjugation or electroporation, although PCR and sequencing confirmed that it contained a *bla*_{KPC-2} gene. Perhaps the *bla*_{KPC} gene in this strain is located on chromosome of this isolate, as described for *Acinetobacter baumannii*⁴⁴ and *Pseudomonas aeruginosa*⁴⁵ strains, but further studies are needed to confirm this possibility.

Antimicrobial resistance in *Klebsiella pneumoniae* is now a serious problem in many Venezuelan hospitals and requires urgent control measures, including strict adherence to hand hygiene and healthcare workers wearing gowns and gloves by when they enter rooms with patients having *K. pneumoniae*-related illnesses. Healthcare facilities also must follow strict cleaning procedures to prevent the spread of *K. pneumoniae*. We hope that the molecular epidemiology provided by this study can aid to control the dissemination of the strains involved, but constant vigilance and on-going monitoring of resistant strains is essential for reducing their presence.

Conclusions

In one large public hospital in Caracas, Venezuela, 22 carbapenem resistant *K. pneumoniae* containing the *bla*_{KPC-2} gene were isolated over a period of two years. These isolates were distributed into several different genotypes but 50% of them belong to CC258, most commonly as the widely distributed ST11, but three novel STs were also identified. This study underscores the need for collaborative institutional, local, national, and regional surveillance of KPC-producing *K. pneumoniae* to assist diagnosis and guide antimicrobial stewardship and infection control efforts.

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Conflict of interest

The authors declare that they have no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.eimc.2017.01.010](https://doi.org/10.1016/j.eimc.2017.01.010).

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