Diagnostic Microbiology and Infectious Disease 94 (2019) 316–319

Contents lists available at ScienceDirect

Diagnostic Microbiology and Infectious Disease

journal homepage: www.elsevier.com/locate/diagmicrobio

Letters to the Editor

Molecular characterization and pathogenicity determination of hypervirulent *Klebsiella pneumoniae* clinical isolates serotype K2 in Mexico

Juan Carlos Catalán-Nájera [a], Humberto Barrios-Camacho [a], Josefina Duran-Bedolla [a], Alan Sagal-Prado [a], Rigoberto Hernández-Castro [b], Jorge García-Méndez [c], Rayo Morfín-Otero [d], María del Rosario Velázquez-Larios [e], Vianney Ortíz-Navarrete [f], Lourdes Gutierrez-Xicotencatl [a], Celia Alpuche-Aranda [a], Jesús Silva-Sánchez [a,*], Ulises Garza-Ramos [a,*]

[a] Centro de Investigación Sobre Enfermedades Infecciosas (CISEI), Instituto Nacional de Salud Pública (INSP), Cuernavaca, Morelos, México
[b] Departamento Ecología de Agentes Patógenos, Hospital General "Dr. Manuel Gea González", Ciudad de México, México
[c] Departamento de Posgrado y Educación Médica Continua, Instituto Nacional de Cancerología, Ciudad de México, México
[d] Instituto de Patología Infecciosa y Experimental, Centro Universitario de Ciencias de la Salud, Hospital Civil de Guadalajara "Fray Antonio Alcalde", Universidad de Guadalajara, Guadalajara, Jalisco, México
[e] Instituto Nacional de Cardiología, Ciudad de México, México
[f] Departamento de Biomedicina Molecular, Centro de Investigación y Estudios Avanzados (CINVESTAV), Ciudad de México, México

ARTICLE INFO

Article history:
Received 6 November 2018
Received in revised form 11 January 2019
Accepted 18 January 2019
Available online 31 January 2019

Keywords:
Klebsiella pneumoniae
K2 serotype
Hypervirulent

ABSTRACT

Hypervirulent *Klebsiella pneumoniae* have been rarely described in Latin America. This work describes the characterization of hypervirulent *K. pneumoniae* isolates capsular serotype K2 belonging to sequence types 86 and 380. The assays showed the hypervirulent *K. pneumoniae* highly virulent, which is determined by the plasmid borne virulence genes. At this time, the hypervirulent *K. pneumoniae* clinical isolates in Mexico are extensively susceptible to antibiotics.

Traditionally, *Klebsiella pneumoniae* is an *Enterobacteriaceae* species considered as an opportunistic pathogen (Podschun and Ullmann, 1998). Today, 2 *K. pneumoniae* variants have been described: the classic *K. pneumoniae* (cKpn) which is responsible of urinary tract infections, pneumonia, bacteremia, and sepsis in immunocompromised patients (Catalan-Najera et al., 2017; Paczosa and Mecsas, 2016) and the hypervirulent *K. pneumoniae* (hvKpn), which is mainly community acquired and causes invasive infection such as liver, lungs, prostatic, kidney, and bones abscesses followed by *K. pneumoniae* dissemination to different organs (Fung et al., 2002). The infections caused by hvKpn have been described around the world (Catalan-Najera et al., 2017; Paczosa and Mecsas, 2016).

Since it was described for the first time, hypermucoviscosity was associated to hypervirulence (Paczosa and Mecsas, 2016). However, the hypermucoviscous *K. pneumoniae* (hmvKpn) does not strictly reflect that the strains are hypervirulent, indicating that both are 2 different phenotypes (Catalan-Najera et al., 2017). The hvKpn strains are associated to several virulence factors such as K1, K2, K5, K20, K54, and K57 capsular serotypes, regulators of mucoid phenotype (*rmpA* and *rmpA2*), siderophores like aerobactin and yersiniabactin (*iucA* and *irp2*), the phospholipase D family protein (PLD1), and outer membrane porin (*KpnO*) (Catalan-Najera et al., 2017; Paczosa and Mecsas, 2016; Podschun and Ullmann, 1998).

The cases of liver abscesses produced by hvKpn variants showed a hypermucoviscous phenotype and belonged to K1 serotype, positive to *rmpA* and aerobactin genes. These characteristics were associated to ST23 that is related worldwide to K1 serotype hypervirulent *K. pneumoniae* (Coutinho et al., 2014; Vila et al., 2011). *K. pneumoniae* serotype K2 strains have mainly been described in Asia but not in Latin America countries, where the few reports of hvKpn describe the K1, K5, and K19 serotypes in Brazil and serotype K1 in Argentina (Moura et al., 2017; Pereira and Vanetti, 2015; Vila et al., 2011). Our work is the first report of hvKpn serotype K2 in America. The aims of this work were to characterize the *K. pneumoniae* isolates with hypermucoviscous phenotype, identify the virulence factors, and determine their pathogenicity through *in vitro* and *in vivo* analysis.

A total of 350 clinical isolates of *K. pneumoniae* were collected from 16 hospitals during the period of 2006 to 2016. From these, 200

* Corresponding author. Tel.: +52-7773-29-30-00x2406; fax: +52-7771-01-29-25.
E-mail addresses: jsilva@insp.mx (J. Silva-Sánchez), ulises.garza@insp.mx (U. Garza-Ramos).

0732-8893/

Table 1
General characteristics of hypermucoviscous *K. pneumoniae* isolates.

Strain	Hospital[a]	Date of isolation	Origin of sample	string Test	Capsular genotype	*rmpA*/*rmpA2*/*terW*	*iucA*/*iroB*/*irp2*/*entB*	Plasmids (kb)[d]	Incompatibility group	MLST[e]
10271	1	2014	Liver abscess	+	K2 **(KL2)**[b]	+/+/+	+/+/−/+	220	IncHI1b	ST86
Δp-10271	NA	NA	NA	−	K2	−/−/−	−/−/−/+	-	NA	ND
6263	2	2015	Urine	+	K2 **(KL2)**[b]	+/−/−	+/+/−/+	200	IncFIB(K)	ST380
9295	3	2015	Surgical wound	+	K2	+/−/−	+/+/+/+	200	IncFIB(K)	ST380
11401	3	2015	Sputum	+	K2 **(KL2)**[b]	+/−/−	+/+/+/+	200	ND	ST380
13131	**3**	**2016**	**Tracheal aspiration**	−	**ND**[c]	−/−/−	−/−/−/**+**	**-**	**NA**	**ND**

ND = not determined; NA = not applied; + = positive; − = negative.
[a] Hospitals: 1, Hospital "Dr. Manuel Gea González", México City; 2, Instituto Nacional de Cardiología, México City; 3, Hospital Civil de Guadalajara "Fray Antonio Alcalde", Guadalajara, Jalisco, Mexico.
[b] The serotype was identified using the Kaptive tool (http://kaptive.holtlab.net/).
[c] The 13131 clinical isolate was negative to K1, K2, K5, K20, K54, and K57 capsular serotypes screened.
[d] The plasmid-borne *rmpA* and *terW* genes were identified by Southern hybridization assay, and the mating experiment showed nonconjugative 220- and 200-kb plasmids.
[e] Sequence type (ST) was determined *in silico* except for strain 9295, which was determined by Sanger sequencing (Podschun et al., 1993).

(57%) were classified as multidrug resistant and extended-spectrum β-lactamase producers, and 150 (43%) were identified as not multidrug resistant. The hypermucoviscous phenotype was retrospectively analyzed mediated the string test (Kumabe and Kenzaka, 2014), the percentage for isolates identified was of 1.14% (4/350). The hypermucoviscous 10271, 6263, 9295, and 11401 isolates were identified as *K. pneumoniae* by molecular test (multiplex-PCR [M-PCR-1]) (Garza-Ramos et al., 2015a). The origins of isolates were from hepatic abscess, urine, surgical wound, and sputum, respectively, and the main characteristics are summarized in Table 1. The hmvKpn isolates were PCR screened by virulence factors associated to hypervirulence, such as capsular serotypes K1, K2, K5, K20, K54, and K57; regulators of hypermucoviscous phenotype *rmpA* and *rmpA2*; and siderophores *iucA* (aerobactin), *entB* (enterobactin), *iroB* (salmochelin), *irp2* (yersiniabactin), and *terW* (tellurium resistance) genes (Supplementary file). The 4 hmvKpn isolates were positive to K2 serotype and *rmpA,* and siderophores aerobactin, salmochelin, and enterobactin genes. The siderophore yersiniabactin was only found in the 9295 and 11401 isolates (Table 1).

The results of genetic analysis of plasmid profile (Kieser, 1984), southern blot hybridization (labeled with an alkaline phosphatase, Amersham Gene Images AlkPhos Direct Labeling and Detection System), and mating experiments (Miller, 1972) showed that hmvKpn 10271 contained a plasmid of approximately 220 kb (p10271V) and the remaining hmvKpn isolates harbored a plasmid of approximately 200 kb (Table 1). The *rmpA* and *terW* genes were identified in plasmid-borne genes by southern blot hybridization; however, the mating experiment was not successful in the tests carried out (Table 1).

The incompatibility group, plasmid structure, virulome, resistome, and sequence type were explored by whole genome sequencing of hmvKpn *K. pneumoniae* 10271, 6263, and 11401 clinical isolates. The plasmid incompatibility groups identified were determined *in silico* (https://cge.cbs.dtu.dk/services/PlasmidFinder/). The 220-kb plasmid from 10271 isolate corresponded to IncHI1b, and the 200-kb plasmid identified in the 6263 and 9295 isolates corresponded to IncFIB(K) (Table 1). The plasmid structure of 200- and 220-kb plasmids was obtained by BLAST Ring Image Generator. The plasmids showed a high similarity against pLVPK plasmid previously described in the *K. pneumoniae* CG43 (Chen et al., 2004; Tang et al., 2010), as well as in the plasmids described in the hvKpn *K. pneumoniae* Kp52.145 and SB2390 with K2 serotype and hvKpn *K. pneumoniae* NTHU-2044 K1 serotype (Lery et al., 2014) (Supplementary figure).

The virulome analyzed *in silico* (http://www.genomicepidemiology.org) revealed a complete operon of siderophores enterobactin, salmochelin, and aerobactin. The yersiniabactin was present only in 11401 genome. The fimbriae *mrkABCD* was present in 10271, 6263, and 11401 genomes, and the *kfuABC* only in the 6263 genome. The serotype using the Kaptive tool (http://kaptive.holtlab.net/) was confirmed as KL2 for the hmvKpn *K. pneumoniae* 10271, 6263, and 11401 genomes (Table 1) (Supplementary file). In the 3 genomes analyzed, the resistome showed that the SHV-11 (chromosomal β-lactamase), *oqxA*/*oqxB* and *fosA* genes confer resistance to ampicillin, fluoroquinolones, and fosfomycin, respectively. The hmvKpn 10271 isolate was positive to genes of resistance to tellurium, copper, lead, and silver, and the 6263 and 11401 genomes were positive to silver resistance gene. Some of the virulence factors were shared with hvKpn Kp52.145, SB2390, and NTUH-2044 (Supplementary file). The sequences types identified *in silico* were ST86 for 10271 isolate and ST380 for 6263 and 11401 isolates. In the 11401 isolate, the ST380 sequence type was identified by PCR sequencing (Diancourt et al., 2005) (Table 1).

The pathogenicity of hmvKpn isolates was evaluated through *in vivo* and *in vitro* assays. The identification of lethal dose median (LD_{50}) was carried out in groups of 6 female BALB/c mice; these were inoculated PBS bacteria doses in the range of 100 CFU/mL at 100,000 CFU/mL intraperitoneally (Reed and Muench, 1938). The hmvKpn isolates 10271, 6263, 9295, and 11401 showed an LD_{50} of 100 CFU/mL, and the cKpn 13131 isolate had an LD_{50} of >100,000 CFU/mL (control strain) (Table 2). Only mice inoculated with hmvKpn isolates showed signs of illness at 24 h, and they died 48 h after bacterial inoculation. Resistance to human serum from healthy volunteers was performed using the established method (Podschun et al., 1993), and the results were interpreted according to Hughes et al. (1982). The isolates showed a resistance phenotype in different degrees to the components of human serum. The hmvKpn isolates were classified as grade 6 and 5, meanwhile the cKpn like grade 1 (Table 2). The neutrophil-mediated phagocytosis resistance was performed according methods established by Hampton et al. (1994). In terms of the ability to evade phagocytosis mediated by neutrophils, all hmvKpn isolates were classified as resistant to phagocytosis (Table 2), compared to the control strains *S. aureus* USA 300 and cKpn 13131 were phagocytized by neutrophils. According to previous assays, the hmvKpn 10271, 6263, 9295, and 11401 isolates are considered as hvKpn variant with serotype K2 (Table 2).

In order to evaluate the contribution of plasmid in the hypermucoviscous phenotype and virulence, the hvKpn 10271 was selected**.** The 220-kb (p10271V) plasmid curing was carried out according to the methodology proposed by El-Mansi et al. (2000). The assay consisted of growing bacteria in LB broth–SDS 5% (w/v) as curing agent and selection on an LB-agar plate supplemented with 32 μg/mL of tellurium. Colonies of hmvKpn 10271 isolate susceptible to tellurium were obtained (Δp-10271 strain). Several colonies were analyzed by plasmid profile and PCR analysis against virulence genes. The absence of the plasmidic-borne *rmpA*, *rmpA2, terW*, *iucA*, and *iroB* genes was confirmed; however, the *entB* gene was present in the Δp-10271 strain (Table 1). Then, pathogenicity assays showed that the Δp-10271 strain became nonvirulent because it was unable to kill mice and its resistance

Table 2
Virulence properties of hypervirulent-*K. pneumoniae* isolates and strains.

Strain	LD_{50} (CFU/mL)[a]	Serum resistance[b] (grade)	Neutrophil-mediated phagocytosis[c]	Biofilm production[d]	Adherence to HeLa cells[e] (%)	Phenotype
10271	100	R (6)	R	No producer	1.7	hvKpn
Δp-10271	>**100,000**	R (5)	IS	No producer	1.9	**NA**
Cp-10271	100	R (5)	R	No producer	2.9	hvKpn
6263	100	R (5)	R	No producer	2.3	hvKpn
9295	100	R (5)	R	No producer	1.9	hvKpn
11401	100	R (5)	R	No producer	2.2	hvKpn
13131	>**100,000**	**S (1)**	**S**	**No producer**	**2.6**	**cKpn**

Abbreviations: hvKpn = hypervirulent *K. pneumoniae*; cKpn = classical *K. pneumoniae*; NA = not applied; − = negative.
[a] Determined by intraperitoneal injection of bacteria to groups of 6 female BALB/c mice.
[b] Interpreted as R, resistant; IS, intermediately sensitive; and S, sensitive.
[c] Interpreted as R, resistant and S, phagocytosis and intracellular death sensitive.
[d] Identified as nonproducers since they showed a biofilm index <1.
[e] Percentage of adhered bacteria from an initial inoculum of 6×10^5 CFU/mL.

to serum decreased to grade 5 and became intermediately sensitive to phagocytosis (Table 2). The complementation of p10271V plasmid to Δp-10271 strain was carried by transformation. The Cp-10271 strain recovered both the hypermucoviscous phenotype and the virulence determined in the *in vivo* and *in vitro* assays (Table 2).

The hmvKpn isolates nonhypervirulent were not identified in the present study. Likewise, the percentage of hypermucoviscosity identified in this work in *K. pneumoniae* could not correspond to the real percentage because the isolates included in the study are not the total of isolates obtained in each hospital. However, the hypermucoviscous *Klebsiella variicola* and *Klebsiella quasipneumoniae* nonhypervirulent isolates have been described previously in Mexico (Garza-Ramos et al., 2015b, 2016).

None of the hvKpn was considered as a biofilm producer (Bandeira et al., 2014) since they showed a biofilm index less than 1 and obtained a minimum percentage of adhered bacteria (1.7–2.3% range) compared to the control strain (20%). The degree of bacterial adhesion to eukaryotic cells was evaluated as described by Letourneau et al. (2011). All isolates had a minimum percentage of adhered bacteria of 1.7%, 2.3%, 1.9%, and 2.2%, for hvKpn 10271, 6263, 9295, and 11401, respectively, (Table 2) compared to the control strain of diffusely adherent *Escherichia coli* with a 20% of adhered bacteria.

In conclusion, this work describes for the first time the hypervirulent *K. pneumoniae* isolates with hypermucoviscous phenotype with serotype K2 in Latin America. These hypermucoviscous/hypervirulent *K. pneumoniae* isolates are highly virulent isolates and contain the pLVPK-like plasmid, which is responsible for conferring the hypermucoviscous and virulence phenotype. However, the hvKpn isolates do not have the ability to produce biofilm or adhesion to eukaryotic cells. At this time, the hvKpn *K. pneumoniae* K2 clinical isolates in Mexico are extensively susceptible to antibiotics but contains the virulence factors related to hypervirulent *K. pneumoniae* K1 and K2 described in other regions in the world.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

Catalán-Nájera is a doctoral student from Programa de Doctorado con Área de Concentración en Enfermedades Infecciosas at Instituto Nacional de Salud Pública (INSP). The present study was supported by grants 256988 and 256927 from SEP-CONACyT (Secretaría de Educción Pública-Consejo Nacional de Ciencia y Tecnología). The authors thank Dra. Gabriela Echaniz for kindly providing *S. aureus* USA 300 strain and Dra. María de Jesús González Orozco and Alejandro Sánchez Pérez for their technical assistance.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diagmicrobio.2019.01.013.

References

Bandeira M, Carvalho PA, Duarte A, Jordao L. Exploring dangerous connections between klebsiella pneumoniae biofilms and healthcare-associated infections. Pathogens 2014;3(3):720–31.
Catalan-Najera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermucoviscosity: two different but complementary Klebsiella spp. phenotypes? Virulence 2017;8(7):1111–23.
Chen YT, Chang HY, Lai YC, Pan CC, Tsai SF, Peng HL. Sequencing and analysis of the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43. Gene 2004;337:189–98.
Coutinho RL, Visconde MF, Descio FJ, Nicoletti AG, Pinto FC, Silva AC, et al. Community-acquired invasive liver abscess syndrome caused by a K1 serotype Klebsiella pneumoniae isolate in Brazil: a case report of hypervirulent ST23. Mem Inst Oswaldo Cruz 2014;109(7):970–1.
Diancourt L, Passet V, Verhoef J, Grimont PA, Brisse S. Multilocus sequence typing of Klebsiella pneumoniae nosocomial isolates. J Clin Microbiol 2005;43(8):4178–82.
El-Mansi M, Anderson KJ, Inche CA, Knowles LK, Platt DJ. Isolation and curing of the Klebsiella pneumoniae large indigenous plasmid using sodium dodecyl sulphate. Res Microbiol 2000;151(3):201–8.
Fung CP, Chang FY, Lee SC, Hu BS, Kuo BI, Liu CY, et al. A global emerging disease of Klebsiella pneumoniae liver abscess: is serotype K1 an important factor for complicated endophthalmitis? Gut 2002;50(3):420–4.
Garza-Ramos U, Silva-Sanchez J, Martinez-Romero E, Tinoco P, Pina-Gonzales M, Barrios H, et al. Development of a multiplex-PCR probe system for the proper identification of Klebsiella variicola. BMC Microbiol 2015a;15:64.
Garza-Ramos U, Silva-Sanchez J, Barrios H, Rodriguez-Medina N, Martinez-Barnetche J, Andrade V. Draft genome sequence of the first hypermucoviscous Klebsiella variicola clinical isolate. Genome Announc 2015b;3(2).
Garza-Ramos U, Silva-Sanchez J, Catalan-Najera J, Barrios H, Rodriguez-Medina N, Garza-Gonzalez E, et al. Draft genome sequence of a hypermucoviscous extended-spectrum-beta-lactamase-producing Klebsiella quasipneumoniae subsp. similipneumoniae clinical isolate. Genome Announc 2016;4(4).
Hampton MB, Vissers MC, Winterbourn CC. A single assay for measuring the rates of phagocytosis and bacterial killing by neutrophils. J Leukoc Biol 1994;55(2):147–52.
Hughes C, Phillips R, Roberts AP. Serum resistance among Escherichia coli strains causing urinary tract infection in relation to O type and the carriage of hemolysin, colicin, and antibiotic resistance determinants. Infect Immun 1982;35(1):270–5.
Kieser T. Factors affecting the isolation of CCC DNA from Streptomyces lividans and Escherichia coli. Plasmid 1984;12(1):19–36.
Kumabe A, Kenzaka T. String test of hypervirulent Klebsiella pneumonia. QJM 2014;107(12):1053.
Lery LM, Frangeul L, Tomas A, Passet V, Almeida AS, Bialek-Davenet S, et al. Comparative analysis of Klebsiella pneumoniae genomes identifies a phospholipase D family protein as a novel virulence factor. BMC Biol 2014;12:41.
Letourneau J, Levesque C, Berthiaume F, Jacques M, Mourez M. In vitro assay of bacterial adhesion onto mammalian epithelial cells. J Vis Exp 2011;51, e2783.
Miller JM. Experiments in molecular genetics. NY: Cold Spring Harbor Laboratory; 1972.
Moura Q, Esposito F, Fernandes MR, Espinoza-Munoz M, Souza TA, Santos SR, et al. Genome sequence analysis of a hypermucoviscous/hypervirulent and MDR CTX-M-15/K19/ST29 Klebsiella pneumoniae isolated from human infection. Pathog Dis 2017;75(9).
Paczosa MK, Mecsas J. Klebsiella pneumoniae: going on the offense with a strong defense. Microbiol Mol Biol Rev 2016;80(3):629–61.

Pereira SC, Vanetti MC. Potential virulence of Klebsiella sp. isolates from enteral diets. Braz J Med Biol Res 2015;48(9):782–9.
Podschun R, Ullmann U. Klebsiella spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. Clin Microbiol Rev 1998;11(4):589–603.
Podschun R, Sievers D, Fischer A, Ullmann U. Serotypes, hemagglutinins, siderophore synthesis, and serum resistance of Klebsiella isolates causing human urinary tract infections. J Infect Dis 1993;168(6):1415–21.
Reed LJ, Muench H. A simple method of estimating fifty per cent endpoint. Am J Epidemiol 1938;27(3):493–7.
Tang HL, Chiang MK, Liou WJ, Chen YT, Peng HL, Chiou CS, et al. Correlation between Klebsiella pneumoniae carrying pLVPK-derived loci and abscess formation. Eur J Clin Microbiol Infect Dis 2010;29(6):689–98.
Vila A, Cassata A, Pagella H, Amadio C, Yeh KM, Chang FY, et al. Appearance of Klebsiella pneumoniae liver abscess syndrome in Argentina: case report and review of molecular mechanisms of pathogenesis. Open Microbiol J 2011;5:107–13.