Hypervirulent and hypermucoviscous extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* and *Klebsiella variicola* in Chile

F. Morales-León a,b,c, A. Opazo-Capurro a,b, C. Caro a,c, N. Lincopan d,e, A. Cardenas-Arias d, F. Esposito d,e, V. Illesca f, M. L. Ríoseco g, M. Domínguez-Yévenes a, C. A. Lima a,b, H. Bello-Toledo a,b, and Gerardo González-Rocha a,b

“Laboratorio de Investigación en Agentes Antibacterianos, Facultad de Ciencias Biológicas, Universidad de Concepción, Concepción, Chile; bMillennium Nucleus for Collaborative Research on Bacterial Resistance, Chile; cDepartamento de Farmacia, Universidad de Concepción, Concepción, Chile; dDepartment of Microbiology, Institute of Biomedical Sciences, Universidade de São Paulo, São Paulo, Brazil; eDepartment of Clinical Analysis, School of Pharmacy, Universidade de São Paulo, São Paulo, Brazil; fUnidad de Microbiología, Hospital Dr. Hernán Henríquez Aravena, Temuco, Chile; gLaboratorio de Microbiología, Hospital de Puerto Montt, Puerto Montt, Chile

**ABSTRACT**

Convergence of virulence and antibiotic-resistance has been reported in *Klebsiella pneumoniae*, but not in *Klebsiella variicola*. We, hereby, report the detection and genomic characterization of hypervirulent and hypermucoviscous *K. pneumoniae* and *K. variicola* recovered in Chile from healthcare-associated infections, which displayed resistance to broad-spectrum cephalosporins. One hundred forty-six *K. pneumoniae* complex isolates were screened by hypermucoviscosity by the “string test.” Two hypermucoid isolates, one hypermucoviscous *K. pneumoniae* (hmKp) and one *K. variicola* (hmKv), were further investigated by whole-genome sequencing. In vivo virulence was analyzed by the *Galleria mellonella* killing assay. In *silico* analysis of hmKp UCO-494 and hmKv UCO-495 revealed the presence of multiple antibiotic-resistance genes, such as *bla* CTX-M-1, *bla* SHV-1, and *bla* LSEN-25 among others. This genetic features confer a multidrug-resistant (MDR) phenotype in both strains. Moreover, virulence in *silico* analysis confirmed the presence of the aerobactin gene *iutA*, in addition to yersiniabactin and/or colicin V encoding genes, which are normally associated with high virulence in humans. Furthermore, both isolates were able to kill *G. mellonella* and displayed higher virulence in comparison with the control strain. In summary, the convergence of virulence and the MDR-phenotype in *K. pneumoniae* complex members is reported for the first time in Chile, denoting a clinical problem that deserves special attention and continuous surveillance in South America.

**ARTICLE HISTORY**

Received 21 July 2020 Revised 19 November 2020 Accepted 25 November 2020

**KEYWORDS**

*Klebsiella pneumoniae* complex; virulence; hypermucoviscous; ESBL; multidrug-resistance

**INTRODUCTION**

*Klebsiella pneumoniae* complex includes *K. pneumoniae* sensu stricto, *K. quasipneumoniae* subsp. *quasipneumoniae*, *K. quasipneumoniae* subsp. *similipneumoniae*, *K. variicola* subsp. *variicola*, *K. variicola* subsp. *tropica*, *K. variicoli* *la*, and *K. africana*, respectively [1]. Among members of this complex, *K. pneumoniae* and *K. variicola* have been widely recognized as important opportunistic human pathogens commonly involved in hospital-acquired infections (HAIs) [2,3]. The clinical importance of these species has been associated with multidrug-resistance, mediated by the expression of extended-spectrum β-lactamases (ESBLs) and carbapenemases [4,5], and more recently with colistin resistance [6,7]. Lately, convergence of virulence and antibiotic-resistance has been reported in *K. pneumoniae* [8]. In this regard, hypervirulent *K. pneumoniae* (hvKp) isolates have been defined under the following criteria: i) occurrence of the hypermucoviscous (hmKp) phenotype, as detected by a positive “string test”; ii) presence of the *rmpA* gene, which regulates the capsule biosynthesis; and iii) presence of the aerobactin genes *iucA/iutA* [9,10]. Similarly to *K. pneumoniae*, *K. variicola* can also display the hypermucoviscous (hmKv) and/or hypervirulent (hvKv) phenotypes [1]. Currently, hvKp isolates have been reported mainly in Asia, Europe and North America, and more recently in South America [9], where sporadic reports have been restricted to Argentina and Brazil [10–12]. Hence, the aim of our study was to detect and characterize hypervirulent and hypermucoviscous ESBL-producing *K. pneumoniae* and *K. variicola* isolates recovered from Chilean hospitals.

**CONTACT** Gerardo González-Rocha ggonzal@udec.cl

© 2020 The Author(s). Published by Informa UK Limited, trading as Taylor & Francis Group.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
Materials and methods

*K. pneumoniae complex isolates and antibiotic susceptibility testing*

One hundred forty-six non-repetitive *K. pneumoniae* complex isolates collected between 2011 and 2018 in Chile, were investigated. All isolates were recovered from nosocomial infections and were initially identified by each hospital laboratory as third-generation cephalosporin-resistant *K. pneumoniae*. Species identification was confirmed by conventional PCR according to previously described [13]. Antibiotic susceptibility testing to imipenem, ertapenem, meropenem, ceftriaxone, cefpodoxime, cefotaxime, ceftazidime, amoxicillin/clavulanic acid, amikacin, tobramycin, kanamycin, gentamicin, ciprofloxacin, levofloxacin and tetracycline was performed by the Kirby-Bauer method. ESBL-production and colistin susceptibility were determined by the combined disc test and the broth microdilution method, respectively [14].

*Phenotypic identification of hypermucoviscous isolates*

The hypermucoviscous phenotype was determined by the “string test” [15]. In brief, when a bacteriological loop was able to generate a viscous filament ≥5 mm in length by stretching bacterial colonies growth at 37°C by 18–24 h on a blood agar plate, the isolate was considered as positive, thus defined as hypermucoviscous. Two isolates resulted positive for the “string test,” therefore, subsequent experiments included both strains.

*Whole-genome sequencing (WGS) and in silico analyses of hypermucoviscous isolates*

Total DNA of both hypermucoviscous isolates was extracted for whole-genome sequencing (WGS) using the Wizard® Genomic DNA Purification kit (Promega, USA) following the manufacturer’s protocol. Sequencing was performed by the Illumina MiSeq platform (2 x 250 bp paired end reads) with libraries prepared by the NexteraXT kit (Illumina), with a coverage of 30x.

De novo assembly was carried out by using the SPAdes software, version 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/) with default values. Later, the assembled genomes were used to screen for genes for antibiotic-resistance, plasmids and virulence using the ResFinder v3.2, Plasmid Finder v2.1 and Virulence Finder v2.0 tools available at the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/). Resistome (antibiotics, heavy metals, and disinfectants) was further predicted by the comprehensive antibiotic resistance database (CARD) (https://card.mcmaster.ca/), and ABRicate v0.9.8 (https://github.com/tseemann/abricate) using the BacMet2 database (http://bacmet.biomedicine.gu.se), respectively, considering a ≥90% similarity criteria. Genome annotation was accomplished using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) web-service (http://www.ncbi.nlm.nih.gov/genome/annotation_prok). Sequence types (STs) were determined for *K. pneumoniae* and *K. variicola* through the bioinformatic tools available at https://cge.cbs.dtu.dk/services/MLST and http://mlstv.insp.mx, respectively. Capsular serotypes (K locus) and phylogenetic analysis of the ybt locus were predicted by Kleborate (https://github.com/katholt/Kleborate).

We studied mutations in *wzc, rcsAB*, and *lon* genes in UCO-494 utilizing the *K. pneumoniae* (accession numbers LT174540 and JCMB01, respectively) genome as reference [16]. For all mutation, bioinformatic analysis was performed using the UGENE 1.32.0 Software.

Both UCO-494 and UCO-495 genomes have been deposited at DDBJ/ENA/GenBank under the accession numbers VSY00000000.1 and VSSZ0000000.1, respectively.

*Serum bactericidal assay and virulence behavior in the Galleria mellonella infection model*

Serum bactericidal activity was analyzed according to previously described [17], with minor modifications. Briefly, 250 μL of a bacterial inoculum of 5 x 10^6 CFU/ml were mixed with 750 μL of fresh human serum. Then, viable bacterial cell count was performed in tryptone soy agar (TSA) plates. A *K. pneumoniae* isolate that was previously characterized as hypervirulent in our laboratory was used as positive control, while serum inactivated at 56°C for 30 min was utilized as blank. All experiments were performed in triplicate. A bacterial survival of <1% after 3 h of incubation with serum was considered as susceptible. On the other hand, survival percentages of 1–90% or >90% were considered as intermediate and resistant, respectively [18]. Additionally, in order to compare the levels of virulence of hmKp UCO-494 and hmKv UCO-495, the G. mellonella infection model was utilized [19]. *K. quasipneumoniae* subsp. *similipneumoniae* ATCC700,603 and hvKp k1/ST23 UCO-448 [10] were used as negative and positive hypervirulent controls, respectively. Larvae survival was analyzed during 96 h, and Kaplan-Meier killing curves of *G. mellonella* were
generated using the log rank test with \( p < 0.05 \). Each assay was performed in triplicate.

**Capsular-polysaccharide (CPS) quantification and estimation of capsular size**

Total capsular-polysaccharide (CPS) of hmKp UCO-494 and hmKv UCO-495 was estimated according to the phenol-sulfuric acid method, after extraction using zwittergent 3–14 [20], and incubated in tryptone soy broth (TSB) at 37°C for 18 h with agitation. The estimation of capsular size was carried out by transmission electron microscopy (TEM) of a bacterial inoculum incubated at 37°C for 24 h [21]. Prior to microscopy, the samples were centrifuged at 3,000 rpm for 5 min and washed once with PBS buffer.

**Biofilm assay**

Biofilms quantification was performed as previously described [22]. In brief, a colony from each strain was grown overnight in TSB at 37°C. From this culture, 10 μL of a bacterial suspension was used to inoculate 96-well polystyrene plates containing 90 μL of TSB, and these plates were incubated at 37°C for 24 h. Subsequently, the medium was removed from the plates and each well was washed three times with water. Immediately, the samples were stained with 125 μL of 0.1% crystal violet for 15 min. Excess dye was removed by rinse 4 times in water, and dried during 10 min at 65°C. Afterward, 125 μL of acetic acid solution (30% v/v) were added and then incubated for 15 min at room temperature. Then, 125 μL of the solubilized crystal violet were transferred to a new 96-well polystyrene plate and color intensity was determined at a 550 nm using a spectrophotometer. *K. pneumoniae* ATCC 700603 strain was used as positive control, and acetic acid solution (30% v/v) was used as a negative control. Biofilm formation abilities were defined as follows: i) absorbance values between 0.084 and 0.168 (2x – 4x blank absorbance) were considered as low biofilm-forming strains; ii) values ranging between 0.168 and 0.252 (4x – 6x blank absorbance) were considered as medium biofilm-forming strains, whereas iii) strains displayed absorbance values higher than 0.252 (>6x blank absorbance) were classified as high biofilm formers [23].

**Results**

Two hypermucoviscous isolates exhibiting a positive string test were identified as *K. pneumoniae* (UCO-494) and *K. variicola* (UCO-495) (Table 1). UCO-494 and UCO-495, belonging to the ST1161 and ST173 lineages, respectively, were isolated from blood and catheter cultures of ICU patients, admitted at two different hospitals located in southern Chile (Table 1). Both isolates were resistant to aminoglycosides and broad-spectrum cephalosporins. UCO-494 was additionally resistant to ertapenem, levofloxacin and ciprofloxacin, remaining susceptible to imipenem, meropenem, and tetracycline. Additionally, colistin-resistance in UCO-494 and UCO-495 was associated with MIC values of 8 and 16 μg/mL, respectively (Table 1). Resistome analysis revealed the presence of the ESBLs and cephalosporinases encoding genes bla_{CTX-M-1}, bla_{SHV-12} and bla_{TEM-1} in *K. pneumoniae* UCO-494 and bla_{TEM-12} and the bla_{LEN-25} genes in *K. variicola* UCO-495 (Table 1). Moreover, ertapenem resistance in *K. pneumoniae* UCO-494 was associated with a deletion in the *ompK35* gene, leading to porin deficiency, and also linked to the presence of the *ompK37* gene, which has been associated with reduced permeability to carbapenems [24,25]. Additionally, *K. pneumoniae* UCO-494 harbored the aac(6’)-Ib-bac, aac(6’)-Ib-cr, aadA1 and aadA2 and *K. variicola* UCO-495, the aph(3’)-Ia, aph(6’)-Id and aph(3’)-Ib aminoglycosides resistance genes (Table 1).

Importantly, both isolates were resistant to colistin (Table 1). From WGS data, we predicted in *K. pneumoniae* UCO-494 (colistin_{MIC} 8 μg/mL) a Gly256Arg (G766C) amino acid substitution in PmrB, while in *K. variicola* UCO-495 (colistin_{MIC} 16 μg/mL) we predicted a Ser170Ala (G508T) amino acid substitution in PmrB, Thr146Ala (A436G) in PmrA and Asp152Glu (T456G) in PhoQ. All amino acid substitutions were neutral by PROVEAN.

Fluoroquinolone resistance in *K. pneumoniae* UCO-494 strain was mediated by aac(6’)-Ib-cr, aqxA, aqxB, qnrB19 and qnrB4 genes and gyrA (83 L, 87Y) and parC (80I) mutations. Moreover, *K. pneumoniae* UCO-494 strain harbored the ColRNI, Inca/C2, IncFIB and Inc FII plasmids. On the other hand, *K. variicola* UCO-495 was susceptible to fluoroquinolones and additionally carried IncF-like plasmids (Table 1).

Furthermore, in *K. variicola* UCO-495, we found diverse metal-resistance systems, such as the arsenic (*arsBCRD*), cobalt/manganese (*corC*), cobalt/magnesium (*mgtA*), magnesium/cobalt/nickel/manganese (*corA*) and tellurium resistance genes *terW* and *terZCD*. Moreover, *K. pneumoniae* UCO-494 contained the arsenic (*arsCDBAH*) and magnesium/cobalt/nickel/manganese (*corA*) systems. Likewise, we identified the presence of resistance genes to glyphosate (*phnMLKII*) and quaternary ammonium compounds (*emrD – qacEd1*) in *K. pneumoniae* UCO-494.

In *K. pneumoniae* UCO-494, phylogenetic analysis of the ybt locus revealed 14 lineages (*ybt locus sequence type Ybst 327–1LV*) with ICEKp5 element, were K-locus KL19 and O-locus O1v2, were also identified. On the other hand, we designated a new ST to MLST *K. variicola*, which corresponded to ST173 (allelic profile *leu*S10; *pgi* 9; *pgk* 6;
| Phenotype                  | UCO-494          | UCO-495          |
|---------------------------|------------------|------------------|
| Origin                    | Blood            | Catheter         |
| year                      | 2012             | 2012             |
| String test               | +                | +                |
| K-locus*                  | KL19             | KL25             |
| ybt                       | ybt14 ICEKp5     | -                |
| O-locus                   | O1v2             | -                |
| ESBL combined disc test   | +                | +                |
| **WGSS data**             |                  |                  |
| Contig number             | 462              | 226              |
| Genome size               | 6,400,426        | 5,982,509        |
| GC%                       | 56,4%            | 56,1%            |
| CDS; pseudogenes; tRNA    |                  |                  |
| **Resistance profile**    |                  |                  |
| ERT, CIP, LEV, AMK, KAN, GEN, TOB, AMP, CTX, CAZ, AMC | TET, STX, W, CPD, CRO, AMK, KAN, GEN, TOB, AMP, CTX, CAZ, FEP, AMC |
| MC colistin               | 8 μg/mL          | 16 μg/mL         |
| **Resistome**             |                  |                  |
| Antibiotic resistance genes | sul1; sul2;    |                  |
| colistin                  |                  |                  |
| mutation gen              |                  |                  |
| arsenic (arsCDBAH);        |                  |                  |
| magnesium/cobalt/nickel/manganese (corA) |                  |
| glyphosate (phnMLKIJ);      |                  |                  |
| quaternary ammonium (emrD - gacEα) |                  |
| PmrB: Gly256Arg (G766C)   |                  |                  |
| PmrA: Thr146Ala (A436G); PmrB: Ser170Ala (G508T); PhoO: Asp152Glu (T456G); |                  |
| arsenic (arsBCRD), cobalt/manganese (corC), cobalt/magnesium (mgtA), magnesium/cobalt/nickel/manganese (corA); tellurium resistance gen (terW and terZCD) |                  |
| **Virulome**              |                  |                  |
| Virulence genes           |                  |                  |
| Enterobactin (entB; entF; ycbH; entD), urea (ureA), alolantoin (als), aerobactin (iatA), fimbria type 1 (fimABCDFEGH), fimbria type 3 (mraABCDF), yersiniabactin (iyp1; iyp2; fyuA; ybtAES), colicin V (cypV; cvaA), biofilm (treC; sugE), ECP (ecpABCDE) | Urea (ureA), alolantoin (als), aerobactin (iatA), fimbria type 1 (fimABCDFEGH), fimbria type 3 (mraABCDF), colicin V (cypV; cvaA), biofilm (treC; sugE), ECP (ecpABCDE), KFU &kuABC) |
| Plasmids                  | CoIRNA; IncAV/C; IncFIB (B); IncFII | IncFIB; IncFII; IncHI2; IncHI2A |

hmKP, hypermucoviscous *Klebsiella pneumoniae*; hvKP, hypervirulent *Klebsiella pneumoniae*; *Capsular polysaccharide concentration in OD650nm 2.0. Significative difference with p-value equal to 0.0001 in t test. ERT, ertapenem; CIP, ciprofloxacin; LEV, levofloxacin; AMK, amikacin; KAN, kanamycin; GEN, gentamicin; TOB, tobramycin; AMP, ampicillin; CTX, cefotaxime; CAZ, ceftazidime; FEP, cephalixin; ; CPD, cefpodoxime; CRO, ceftriaxone; AMC, amoxicillin-clavulanic acid; W, trimethoprim; TET, tetracycline; SXT, sulphamethoxazole-trimethoprim.
**phoE** 1; **pyrG** 11; **rpoB** 1; **fusA** 2), whereas *K. pneumoniae* UCO-494 belonged to ST1161 (Table 1).

Virulome analysis of hvKv UCO-495 revealed the presence of the ferric uptake system kfuABC, which has been associated to hypervirulent *Klebsiella* strains [15]. Both isolates contained the aerobactin gene *iutA*, mannose-sensitive type 1 fimbriae (*fimABCD* operon), the mannose-resistant *Klebsiella*-like (type III) fimbriae cluster (*mrkABCDFHII*), and the *E. coli* common pilus operon (*ecpABCDE*) and biofilm related (*treC, sugE*) genes, which are associated with mucoviscosity and CPS production [26]. Only hmKp UCO-494 carried additionally the enterobactin (*entB, entF*, and *ycfH*), yersiniabactin siderophore cluster *ybtAEPQSTUX* and the siderophore genes *irp1* and *irp2*, which are considered as genetic markers for high-pathogenicity island [27] (Table 1). It is important to highlight that in both strains the presence of *rmpA/A2* was not identified.

Interestingly, hmKv UCO-495 was resistant to the bactericidal activity of human serum, while hmKp UCO-494 was susceptible, with 1% survival after 1 h interaction (Figure 1). Curiously, *K. pneumoniae* UCO-494 produced more CPS (155.44 ± 3.68 µg/mL) than *K. variicola* UCO-495 (30.26 ± 0.11 µg/mL). Likewise, UCO-494 displayed a capsular thickness of 0.124 ± 0.017 µm, whereas capsule thickness of UCO-495 was 0.097 ± 0.019 µm (Figure 2). Interestingly, in UCO-494 we predicted a F573S (T1718C) and R608T (G1823C) amino acid substitutions in *wcz* (deleterious by PROVEAN). Moreover, S35N (G104N) amino acid substitution in *crsA* in addition to E142Q (G424C) and R517C (T843C) in *lon* gen was identified. All of these genes were related with hypercapsule production [16].

On the other hand, *K. variicola* UCO-495 killed >75% *G. mellonella* larvae at 24 h post-infection, while *K. pneumoniae* UCO-494 killed 50% of the larvae at 24 h post-infection. Moreover, 100% mortality was observed at 36

![Figure 1](image-url)

**Figure 1.** A) Serum bactericidal activity. *K. quasipneumoniae* subsp. *similipneumoniae* ATCC 700603 as negative control; *K. pneumoniae* hypervirulent UC-448 as positive control. b) *K. pneumoniae* UCO-494 and *K. variicola* UCO-495; Kaplan-Meier killing curves of *G. mellonella* larvae; ATCC 700603 as negative control; *K. pneumoniae* hypervirulent UC-448 as positive control; The assay was made with blank, inoculated the larvae with NaCl 0.9%. Data no showed.
Discussion

Traditionally, *K. variicola* has been considered as susceptible to most antibiotic classes, but this description has change over time, due to an increase in the MDR-*K. variicola* reports [1]. In South America, there is a single report in Colombia describing a KPC-2-producing *K. variicola* strain, which was resistant to all β-lactams [5].

Worryingly, it is the emergence of hypervirulent-MDR phenotype, especially in *K. variicola* isolated. In this regard, Farzana R et al. describe a fatal MDR-hvKp outbreak in neonates in Bangladesh. The isolates contained a *bla*<sub>CTX-M-15</sub> and *bla*<sub>NDM-1</sub> genes, among others, in addition to several virulence genes like siderophore (*kfuABC*) and Enterobactin (*entABCDEFHIJ*) associated with hypervirulent phenotype [6]. On the other hand, Lu et al. described the first hvKp isolated from blood from a patient with cholangitis in China, which was resistant to colistin (MIC = 8 ug/mL) [28]. These are concordant with our study since we identified an MDR *K. variicola* that was resistant to colistin. In the case of *K. pneumoniae*, colistin-resistant hvKp isolates has been reported previously. Specifically, Lu et al. reported five colistin-resistant hvKp strains recovered from blood samples in China [29]. Similar to our findings, these isolates were colistin-resistant and carbapenem-susceptible. Moreover, Huang et al. characterized diverse colistin-resistant hvKp isolates that were also resistant to carbapenems, since they produced the KPC-2 carbapenemase [30].

Our findings described the convergent hypervirulent phenotype and colistin-resistance in *K. pneumoniae* and *K. variicola* MDR strains. In this sense, the mutations in genes involved in colistin-resistance might be mediating this phenotype. As described previously, point mutations or deletions in *pmrA* or *pmrB* genes result in the addition of phosphoethanolamine to the lipid A [31]. Moreover, it has been demonstrated in vivo the role of *PmrAB* system, in which it has been associated to intra-macrophage survival and virulence in *K. pneumoniae* [32]. In case of hvKp UCO-494, we identified a point mutation in *pmrB*, similarly to the description of Lagerbäck et al., where a NDM-1-producing *K. pneumoniae* isolate presented an amino acid substitution in G256R in the *pmrB* gene [33], which was related with colistin-resistance *K. pneumoniae* [34]. Furthermore, it is important to highlight that the mechanism of colistin-resistance in hvKp UCO-495 was mediated by chromosomal mutations in the two-component system PhoPQ, especially in the D150G substitution in PhoP. Even though mutations in these systems are associated to colistin-resistance [30], general data of molecular mechanisms of colistin-resistance in *K. variicola* are scarce; therefore, our results describe a non-classical *pmrAB* and *phoQ* mutations in this species [7]. In this regard, we determined that these
mutations are neutral according to in silico models, in consequence, in vivo studies should be performed in order to determine if they have an impact on colistin resistance [16,35].

WGS analyses reflect a widely diverse resistome. In this sense, the bla$_{LEN-25}$ gene was detected in the K. variicola UCO-495 genome, which corresponds to an intrinsic-chromosomal β-lactamase. Furthermore, we found that hvKv UCO-495 strain was resistant to cephalosporins, which might be mediated by bla$_{SIV-12}$, while hvKp UCO-494 resistance was mediated by bla$_{CTX-M-1}$. In this case, there are some reports of convergence of hypervirulent phenotype and ESBL genes in K. pneumoniae. For instance, hypervirulent and ESBL-producing have been linked to several ESBLs genes, such as bla$_{CTX-M-14}$, bla$_{CTX-M-18}$, bla$_{CTX-M-3}$ and bla$_{SIV-12}$ [3637,38].

In case of heavy-metal resistance genes, we found in hvKv UCO-495, the tellurium resistance genes terW and terZCD, which are related to the plasmid pKV8917 [39] in hvKp and hvKv strain [1,40]. These genes were not detected in hvKp UCO-494. Relevantly, we identified the presence of the quaternary-ammonium resistance gene emrD in K. pneumoniae UCO-494. As note, these compounds have been heavily used during the SARS-CoV-2 pandemic as disinfectants, which could have an important ecological impact on selecting MDR-bacterial isolates due to selective pressure [41].

Furthermore, Moura et al. identified a K. pneumoniae serotype K19 isolate in Brazil [10]. In this study, the authors determined that this serotype has a similar killing ability compared to hypervirulent K1-isolates [10]. Moreover, the Brazilian isolate produced the ESBL CTX-M-15, which belongs to the same group of the ESBL detected in hvKp UCO-494 isolate (CTX-M-1) [42]. These findings suggest that this serotype could be endemic to South America, where it could be disseminated through the region. In addition, molecular epidemiology determined by MLST revealed that hvKp UCO-494 belonged to the ST1161, which is apparently endemic to Chile since it has been detected previously in the country [43]. In the case of hvKv UCO-495, it was designated as ST173, which corresponds to a new ST that could be endemic to this geographical area. In consequence, further epidemiological studies are needed, in order to understand their prevalence and epidemiology in South America.

In the case of siderophore production, it has been demonstrated that yersiniabactin, salmochelin, and aerobactin are the most predominant in K. pneumoniae and K. variicola [44]. Specifically, the aerobactin system has four biosynthetic enzymes, iucABCD, and an outer membrane transporter, iutA [44]. Interestingly, epidemiological studies have shown a significant relationship between iucABCD-iutA with the hvKp phenotype; therefore, aerobactin is considered a substantive virulence factor in hvKp isolates [45]. However, the occurrence of multiple siderophore systems in hvKp strains suggests that siderophore systems in addition to Iuc-system play important roles in the pathogenesis of these microorganisms during either colonization or invasive processes [46].

Although all Klebsiella pneumoniae complex species could form mucoid colonies, it is well recognized the existence of two well-defined phenotypes. The classical (cKp/cKv) and hypermucoviscous (hmKp/kKv) phenotypes, both differentiated by their ability of forming a viscous and adhesive mucous string in solid media. Because of this, it is important to elucidate the mechanisms of CPS-production in hypermucoviscous K. pneumoniae strains that lack the rmpA/rmpA2 genes and do not belong to the predominant K1 or K2 serotypes [47]. In this sense, Ernst et al. studied the impact of single-nucleotide polymorphisms of the wzc gene in the capsule biosynthesis, which could confer a hypercapsule production phenotype, enhancing virulence [16]; and additionally, contribute to the resistance to polycationic peptides, such as colistin [48]. On the other hand, diverse mechanisms are related with hypercapsule production, such as mutation in wzc, rcsAB and lon protease genes [49]. Our results showed a mutation in all of this gen in hvKp UCO-494. In this sense, some authors suggest that a single amino acid substitution in wzc, rcsA or lon protease genes could increase capsule production [16], and this mechanism could be related to the hypermucoviscous phenotype in K. pneumoniae UCO-494; however, this phenomenon has not been studied in K. variicola.

In the case of virulence, the irp1 (polyketide synthetase) and irp2 (iron acquisition yersiniabactin synthesis enzyme) encode for iron-repressible high molecular weight proteins that are involved in yersiniabactin production [4]. This siderophore system was first described for Yersinia species; however, they could be also present in other Enterobacteriaceles [50]. It is believed that its dissemination occurred via horizontal gene transfer events since the responsible genes have been identified within pathogenicity islands, such as ICEKp, which is frequently identified in K. pneumoniae [2]. The mannose-sensitive type 1 fimbriae are common in K. pneumoniae. These fimbriae are encoded by fim-like genes, in which the major components are fimA and fimH that confer its ability to adhere to human mucosal or epithelial surfaces [51]. Furthermore, other important adhesin in K. pneumoniae is the mannose-resistant Klebsiella-like (type III) codified in the fimbriae cluster mkrABCDFHIJ [52]. This is considered as a virulence factor and contributor to mucous adherence, tissue colonization, and biofilm [53]. In our case, only UCO-494 irp1 and irp2 genes.

Importantly, biofilm-formation ability of hvKp contributes to hypervirulence, since hypervirulent strains
generate more biofilms in comparison with less virulent isolates [54]. Specifically, biofilms provide protection against environmental conditions, such as desiccation, and also protect bacteria from the immune system action [46]. Accordingly, diverse studies associate biofilm phenotype to capsule, and/or fimбриas; however, it has been also demonstrated that the lack of capsule enhances biofilm-formation in K. pneumoniae [46]. Our results revealed that K. pneumoniae UCO-494 presented a low biofilm-formation ability, and at the same time displayed a lower G. mellonella killing ability in comparison to K. variicola UCO-495. Moreover, hvKp UCO-494 was susceptible to the serum activity, in contrary to hvKv UCO-495 that was resistant. However, hvKp UCO-494 produced more CPS in comparison with hvKv UCO-495, which is concordant with the bacterial-size capsule, in which hvKp UCO-494 has a thicker capsule than hvKv UCO-495. These discordant results suggest that more research is needed in order to establish the specific role of biofilm-formation and virulence in Klebsiella species. In this regard, some studies have demonstrated no significant differences in biofilm-formation ability between invasive (more virulent) and non-invasive (less virulent) K. pneumoniae isolates [55]. In another study, K. pneumoniae mutant strains with decreased biofilm production ability did not show any difference in their ability to survive serum activity, which reafirms the need for further studies in this regard.

In conclusion, we identified the convergence of hypermucoviscous phenotype and MDR K. pneumoniae and K. variicola isolates in Chile. It is important to consider the relevance of these phenotypes since they are not normally screened by a routine laboratory. Moreover, our results demonstrate the relevance of K. variicola as pathogen, due to its antibiotic-resistance and virulence features. Moreover, our results suggest that the hypermucoviscous/hypermuc viscosity phenotype of K. pneumoniae-complex isolates is the result of multiple mechanisms, including siderophores and biofilm-production, which have not been well elucidated yet. Our results remark the need for more detailed research of the mechanisms and epidemiology of hypervirulent strains, in order to elucidate the role of high-risk K. pneumoniae-complex lineages.

Disclosure statement
No potential conflict of interest was reported by the authors.

Funding
This study was supported by Universidad de Concepción, Grant [VRID Nº 218.074.061-1.0], the National Agency for Research and Development (ANID)/Scholarship Program/DOCTORADO NACIONAL/2016 21160336 (for FML), the Merck Investigator Studies Program (MISP) USA, NLD-128156, the National Fund for Scientific and Technological Development (FONDECYT) of Chile (FONDECYT-Iniciación, grant number 11190602 to AOC) and by the ANID Millennium Science Initiative/Millennium Initiative for Collaborative Research on Bacterial Resistance, MICROB-R, NCN17_081.

ORCID
F. Morales-León http://orcid.org/0000-0001-7889-2844
A. Opazo-Capurro http://orcid.org/0000-0002-6382-7625
N. Lincopan http://orcid.org/0000-0003-0161-5800
A. Cardenas-Arias http://orcid.org/0000-0003-0553-8881
F. Esposito http://orcid.org/0000-0002-7707-0942
C. A. Lima http://orcid.org/0000-0002-4697-034X
H. Bello-Toledo http://orcid.org/0000-0002-9277-4681
Gerardo González-Rocha http://orcid.org/0000-0003-2351-1236

References
[1] Rodríguez-Medina N, Barrios-Camacho H, Duran-Bedolla J, et al. Klebsiella variicola: an emerging pathogen in humans. Emerg Microbes Infect. 2019;8(1):973–988.
[2] Lam MMC, Wick RR, Wyres KL, et al. Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in Klebsiella pneumoniae populations. Microb Genomics. 2018;4. DOI:10.1099/mgen.0.00196.
[3] Pienpenbrock E, Higgins PG, Wille J, et al. Klebsiella variicola causing nosocomial transmission among neonates: an emerging pathogen? J Med Microbiol. 2020. DOI:10.1099/jmm.0.001143.
[4] Wyres KL, Lam MMC, Holt KE. Population genomics of Klebsiella pneumoniae. Nat Rev Microbiol. 2020;18:344.
[5] Barrios-Camacho H, Aguilar-Vera A, Beltran-Rojel M, et al. Molecular epidemiology of Klebsiella variicola obtained from different sources. Sci Rep. 2019;9:10610.
[6] Farzana R, Jones LS, Rahman MA, et al. Outbreak of hypervirulent multidrug-resistant Klebsiella variicola causing high mortality in neonates in Bangladesh. Clin Infect Dis. 2019;68:1225–1227.
[7] Lu Y, Feng Y, McNally A, et al. Occurrence of colistin-resistant hypervirulent Klebsiella variicola. J Antimicrob Chemother. 2018;73:3001–3004.
[8] Araújo BF, Ferreira ML, de Campos PA, et al. Hypervirulence and biofilm production in KPC-2-producing Klebsiella pneumoniae CG258 isolated in Brazil. J Med Microbiol. 2018;67:523–528.

Acknowledgments
The authors want to thank the microbiologists of the Hospital Dr. Hernán Henríquez Aravena, Temuco; Hospital Dr. Eduardo Schultz Schroeder, Puerto Montt; Hospital Clínico UC, Santiago; Hospital Clínico San Borja Arriarán, Santiago, Hospital Padre Hurtado, Santiago and Hospital Dr. Leonardo Guzmán Cortes, Antofagasta.
[9] Guerra JM, de Fernandes NCCA, Dos Santos ALM, et al. Detection of hypermucoviscous Klebsiella pneumoniae sequence type 86 capsular type K2 in South America as an unexpected cause of a fatal outbreak in captivity marmosets. bioRxiv. 2020. DOI:10.1101/2020.02.02.0685.

[10] Moura Q, Esposito F, Fernandes MR, 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. DOI:10.1093/femspd/ftx121.

[11] Cejas D, Caniglia LF, Cruz GR, et al. First isolate of KPC-2-producing Klebsiella pneumoniae sequence type 23 from the Americas. J Clin Microbiol. 2014;52:3483–3485.

[12] Coutinho RL, Visconde MF, Descio FJ, 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;109:973–974.

[13] Garza-Ramos U, Silva-Sánchez J, Martínez-Romero E, et al. Development of a Multiplex-PCR probe system for the proper identification of Klebsiella variicola. BMC Microbiol. 2015;15:64.

[14] Institute Clinical & Laboratory Standards. Performance stands for antimicrobial susceptibility testing: twenty-fourth informational supplement CLSI document M100-S24. Wayne: Clinical and Laboratory Standards Institute; 2018.

[15] Catalán-Najera JC, Garza-Ramos U, Barrios-Camacho H. Hypervirulence and hypermuoviscosity: two different but complementary Klebsiella spp. phenotypes? Virulence. 2017;8:1–13.

[16] Ernst CM, Braxton JR, Rodriguez-Osorio CA, et al. Adaptive evolution of virulence and persistence in carbapenem-resistant Klebsiella pneumoniae. Nat Med. 2020;26:705–711.

[17] Ullman U, Fischer A, Podschan R. Expression of putative virulence factors by clinical isolates of Klebsiella planticcola. J Med Microbiol. 2000;49:115–119.

[18] Benge GR. Bactericidal activity of human serum against strains of Klebsiella from different sources. J Med Microbiol. 1988;27:11–15.

[19] Insua JL, Lloret E, Moranta D, et al. Modeling Klebsiella pneumoniae pathogenesis by infection of the Wax Moth Galleria mellonella. Infect Immun. 2013;81:3552–3565.

[20] Cheng HY, Chen YS, Wu CY, et al. RmpA Regulation of Capsular Polysaccharide Biosynthesis in Klebsiella pneumoniae CG43. J Bacteriol. 2010;192:3144–3158.

[21] Schembri MA, Blom J, Krogfelt KA, et al. Capsule and fimbra interaction in Klebsiella pneumoniae. Infect Immun. 2005;73:4626–4633.

[22] O’Toole GA. Microtiter dish Biofilm formation assay. J Vis Exp. 2010. DOI:10.3791/2437.

[23] Cusumano JA, Caffrey AR, Daffinee KE, et al. Weak biofilm formation among carbapenem-resistant Klebsiella pneumoniae. Diagn Microbiol Infect Dis. 2019;95:14877.

[24] Doménech-Sánchez A, Martínez-Martínez L, Hernández-Alles S, et al. Role of Klebsiella pneumoniae OmpK35 porin in antimicrobial resistance. Antimicrob Agents Chemother. 2003;47:3332–3335.

[25] Poulou A, Voulgari E, Vrioni G, et al. Outbreak caused by an ertapenem-resistant, CTX-M-15-producing Klebsiella pneumoniae sequence type 101 clone carrying an OmpK36 porin variant. J Clin Microbiol. 2013;51:3176–3182.

[26] Wu M-C, Lin T-L, Hsieh P-F, et al. Isolation of genes involved in biofilm formation of a Klebsiella pneumoniae strain causing pyogenic liver abscess. PLoS One. 2011;6: e23500.

[27] Amaretti A, Righini L, Candeliere F, et al. Antibiotic resistance, virulence factors, phenotyping, and genotyping of non-escherichia coli enterobacterales from the gut microbiota of healthy subjects. Int J Mol Sci. 2020;21:1847.

[28] Lu Y, Feng Y, McNally A, et al. The occurrence of colistin-resistant hypervirulent Klebsiella pneumoniae in China. Front Microbiol. 2018;9. DOI:10.3389/FMICB.2018.02568.

[29] Huang Y-HY-W, Chou S-H, S-W L, et al. Emergence of an XDR and carbapenem-producing hypervirulent Klebsiella pneumoniae strain in Taiwan. J Anti microb Chemother. 2018;73:2039–2046.

[30] Minh-Duy P, Nhu NTK, Achard MES, et al. Modifications in the pmrB gene are the primary mechanism for the development of chromosomally encoded resistance to polymyxins in uropathogenic Escherichia coli. J Antimicrob Chemother. 2017;72:2729–2736.

[31] Cheng HY, Chen YF, Peng HL. Molecular characterization of the PhoPQ-PmrD-PmrAB mediated pathway regulating polymyxin B resistance in Klebsiella pneumoniae CG43. J Biomed Sci. 2010;17. DOI:10.1186/1423-0127-17-60.

[32] Lagerbäck P, Khine WWT, Giske CG. Evaluation of antibacterial activities of colistin, rifampicin and meropenem combinations against NDM-1-producing Klebsiella pneumoniae in 24 h in vitro time–kill experiments. J Antimicrob Chemother. 2016;71:2321–2325.

[33] Huang J, Li C, Song J, et al. Regulating polymyxin resistance in Gram-negative bacteria: roles of two-component systems PhoPQ and PmrAB. Future Microbiol. 20 20;15:445–459.

[34] Poirel L, Jayol A, Polymyxins: NP. Antibacterial activity, susceptibility testing, and resistance mechanisms encoded by plasmids or chromosomes. Clin Microbiol Rev. 2017;30:557–596.

[35] Surgers L, Boyd A, Girard PM, et al. ESBL-producing strain of hypervirulent Klebsiella pneumoniae K2, France. Emerg Infect Dis. 2016;22:1687–1688.

[36] Lin Z, Zheng J, Bai B, et al. Characteristics of hypervirulent Klebsiella pneumoniae: does low expression of rmpA contribute to the absence of hypervirulence? Front Microbiol. 2020;11:436.

[37] Passet V, Brisse S. Association of tellurite resistance with hypervirulent clonal groups of Klebsiella pneumoniae. J Clin Microbiol. 2015;53:1380–1382.

[38] Rodrigues C, Passet V, Rakotondrasoa A, et al. Description of Klebsiella africanaensis sp. nov., Klebsiella variicola subsp. tropicalensis subsp. nov. and Klebsiella variicola subsp. variicola subsp. nov. Res Microbiol. 2019;170:165–170.

[39] Rodriguez-Medina N, Barrios-Camacho H, Duran-Bedolla J, Garza-Ramos U. Klebsiella variicola: an unexpected cause of a fatal outbreak in captivity marmosets. bioRxiv. 2020. DOI:10.1101/2020.02.02.0685.
emerging pathogen in humans. Emerg Microbes Infect. 2019;8:973–988.

[40] Rezazadeh M, Baghchesaraei H, Peymani A. Plasmid-mediated quinolone-resistance (qnr) genes in clinical isolates of Escherichia coli collected from several hospitals of Qazvin and Zanjan Provinces, Iran. Osong Public Heal Res Perspect. 2016;7:307–312.

[41] Barría-Loaiza C, Pincheira A, Quezada M, et al. Molecular typing and genetic environment of the blaKPC gene in Chilean isolates of Klebsiella pneumoniae. J Glob Antimicrob Resist. 2016;4:28–34.

[42] Harada S, Doi Y. Hypervirulent Klebsiella pneumoniae: a call for consensus definition and international collaboration. J Clin Microbiol. 2018;56:e00959–18.

[43] Paczosa MK, Mecsas J. Klebsiella pneumoniae: going on the offense with a strong defense. Microbiol Mol Biol Rev. 2016;80:629–661.

[44] Choby JE, Howard-Anderson J, Weiss DS. Hypervirulent Klebsiella pneumoniae – clinical and molecular perspectives. J Intern Med. 2020;287:283–300.

[45] Cubero M, Grau I, Tubau F, et al. Hypervirulent Klebsiella pneumoniae clones causing bacteremia in adults in a teaching hospital in Barcelona, Spain (2007-2013). Clin Microbiol Infect. 2016;22:154–160.

[46] Aghapour Z, Gholizadeh P, Ganbarov K, et al. Molecular mechanisms related to colistin resistance in Entero bacteriaceae. Infect Drug Resist. 2019;12:965–975.

[47] Gottesman S, Stout V. Regulation of capsular polysaccharide synthesis in Escherichia coli K12. Mol Microbiol. 1991;5:1599–1606.

[48] Carniel E. The Yersinia high-pathogenicity island: an iron-uptake island. Microbes Infect. 2001;3:561–569.

[49] Murphy CN, Mortensen MS, Krogfelt KA, et al. Role of Klebsiella pneumoniae type 1 and type 3 fimbriae in colonizing silicone tubes implanted into the bladders of mice as a model of catheter-associated urinary tract infections. Infect Immun. 2013;81:3009–3017.

[50] Roe CC, Vazquez AJ, Esposito EP, et al. Diversity, virulence, and antimicrobial resistance in isolates from the newly emerging Klebsiella pneumoniae ST101 lineage. Front Microbiol. 2019;10:542.

[51] El Fertas-Aissani R, Messai Y, Alouache S, et al. Virulence profiles and antibiotic susceptibility patterns of Klebsiella pneumoniae strains isolated from different clinical specimens. Pathol Biol. 2013;61:209–216.

[52] Kong Q, Beanan JM, Olson R, et al. Biofilm formed by a hypervirulent (hypermucoviscous) variant of Klebsiella pneumoniae does not enhance serum resistance or survival in an in vivo abscess model. Virulence. 2012;3:309–318.

[53] Soto E, Dennis MM, Beierschmitt A, et al. Biofilm formation of hypermucoviscous and non-hypermucoviscous Klebsiella pneumoniae recovered from clinically affected African green monkey (Chlorocebus aethiops sabaeus). Microb Pathog. 2017;107:198–201.