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FENOTIPO HETERORRESISTENTE A COLISTÍN Y SU RELACIÓN CON LA EXPRESIÓN CAPSULAR EN CEPAS DE *Klebsiella pneumoniae* PRODUCTORAS DE β-LACTAMASAS DE ESPECTRO EXTENDIDO

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RESUMEN

En los últimos años, se ha observado un aumento en la prevalencia de infecciones provocadas por enterobacterias, como *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia spp*., *Proteus spp*. portadoras de determinantes múltiples de resistencia antibiótica, los cuales son consideradas una amenaza mundial dada la escasa disponibilidad de nuevos antibióticos efectivos. Al respecto, las especies que comprenden el complejo *Klebsiella pneumoniae*, incluyendo *K*. *pneumoniae* sensu stricto y *K*. *variicola* subsp. *variicola,* han emergido como importantes patógenos oportunistas multirresistentes, relacionadas con **I**nfecciones **A**sociadas con la **A**tención en **S**alud (IAAS). A lo anterior, destaca la alta prevalencia de especies portadoras de genes codificantes de β-lactamasas de espectro extendido (BLEE) tipo bla_{CTX-M}, bla_{SHV}, bla_{TEM-2}, *y* carbapenemasas principalmente bla_{KPC}, para las cuales existen pocas opciones de tratamiento. En este contexto, colistín resulta como una última opción de tratamiento efectiva. Pese a lo anterior, resulta preocupante el hecho de la existencia de cepas portadoras de un fenotipo heterorresistentes (HR) a colistín (CST), lo cual podría llevar a la selección de cepas más resistentes con un posible incremento en la tasa de mortalidad.

Actualmente, los mecanismos involucrados con el fenotipo heterorresistente a colistín (CST-HR) no están completamente dilucidados. Sin embargo, la evidencia existente estima que son las mutaciones del gen regulador *mgrB* o mutaciones en alguno de los genes del sistema de dos-componentes como *phoPQ*, *pmrAB*, *pmrC* o c*rrAB* los cuales están relacionados con la expresión de HR. Por su parte, es de sumo interés interesante indagar que otros mecanismos de resistencia no cromosómicos, tales como la hiperproducción capsular, pueden contribuir a la existencia del fenotipo HR. Así, el objetivo de esta tesis fue

caracterizar los mecanismos involucrados en el fenotipo CST-HR en cepas de *K. pneumoniae* productoras de BLEE. Para esto, se identificó la presencia de cepas HR por medio de un análisis de perfil poblacional (PAP) sobre 60 cepas de *K. pneumoniae* productoras de BLEE, todas susceptibles a colistín. Las cepas HR fueron aisladas y se realizaron pasos sucesivos durante 5 generaciones. Posteriormente, se determinaron los parámetros de crecimiento bacteriano y se realizó la secuenciación del genoma completo (Illumina® MiSeq) utilizando Spades V.3.9. para su posterior ensamblaje. Con los datos obtenidos, se realizó el estudio de los mecanismos de resistencia a colistín empleando el software Galaxy web Server utilizando una base de datos local empleando *K. pneumoniae* MGH78578 como genoma de referencia para una cepa susceptible a colistín. El estudio anterior fue complementado con la determinación de mutaciones en reguladores *wzi*, *wzc*, *rcsABC y lon,* los cuales se relacionan con la expresión capsular. Adicionalmente, se determinó el nivel de expresión relativa de los genes *phoPQ, pmrABD y mgrB* y se comparó la cantidad de polisacárido capsular y el potencial Z (Zetasizer Nano ZS90 - Malvern Instrument®) entre las cepas susceptibles y resistentes, empleando el método fenol-agua-ácido sulfúrico y la movilidad electroforética, respectivamente. Finalmente, en el mismo grupo de 60 cepas de *K. pneumoniae* portadoras de BLEE, se estudió la existencia del fenotipo hipermucoviscoso e hipervirulento realizando un screening con el test de filancia y la búsqueda de genes de virulencia *magA, rmpA/A2, ybtS*, entre otros, mediante PCR múltiple. En las 60 cepas de *K. pneumoniae* se encontró ocho cepas con un fenotipo CST-HR, con MIC_{50} de colistin igual a 50 µg/mL. Además, estas cepas poseen múltiples genes de resistencia destacando la presencia de $bla_{CTX\text{-}M\text{-}15}$ y $bla_{CTX\text{-}M\text{-}2}$; $bla_{SHV\text{-}12}$ y $bla_{OXA\text{-}10}$. Se determinó que las cepas pertenecen a tres linajes diferentes (ST11; ST25 y ST1161) siendo el K.-locus 2 el más prevalente. Por otra parte, se destaca la primera descripción de CST-HR en cepas de ST1161, ST de descripción endémica en Chile. Sobre los mecanismos de resistencia a

colistin, se identificó la presencia de múltiples mutaciones en los genes cromosómicos *pmrB*, *phoPQ* y *mgrB*, algunos de los cuales no han sido descritos previamente. Por su parte, se determinaron cambios en los niveles de expresión de los genes reguladores, destacando la disminución en la expresión relativa en *mgrB* y *pmrBD*, las cuales concuerdan con la expresión de resistencia a colistín. Por otro lado, se determinó que las cepas CST-HR poseen una carga superficial menos electronegativa y probablemente, esta no se encuentra relacionada con la cantidad de polisacárido capsular. Por su parte, se determinó la relación causal entre la existencia de mutaciones en *crrB* y la mayor cantidad de polisacárido observada en las cepas HR. Además, se identificó la presencia de dos cepas, una de *K. pneumoniae* y otra de *K. variicola*, portadoras de un fenotipo y genotipo hipermucoviscoso e hipervirulento, siendo esta la primera descripción formal en Chile. Finalmente, como conclusión, fue posible identificar la existencia de cepas de *K. pneumoniae* productoras de BLEE, portadoras de un fenotipo estable de heterorresistencia a colistín, la cuales evidencian niveles elevados de resistencia al antibiótico. De las cepas identificadas, se logró establecer los mecanismos genéticos responsables de la expresión fenotípica de resistencia a colistín las cuales se relacionan con la existencia de alteraciones en el nivel de expresión de genes claves en la incorporación de resistencia a colistín, probablemente, como una consecuencia de las mutaciones identificadas. Se determinó que las cepas heterorresistentes producen una mayor cantidad de polisacárido capsular en comparación a las cepas susceptibles, y que el serotipo capsular presente en las cepas de *Klebsiella pneumoniae* estudiadas no guardan relación con la existencia del fenotipo HR.

ABSTRACT

In recent years, an increase in the prevalence of infections caused by enterobacteria such as *Klebsiella pneumoniae, Escherichia coli, Serratia spp*. and *Proteus spp*. carrying multiple antibiotic resistance determinants has been observed, which are considered a global threat given the scarce availability of new effective antibiotics. In this regard, the species comprising the Klebsiella pneumoniae complex, including *K. pneumoniae* sensu stricto and *K. variicola* subsp. *variicola*, have emerged as important multidrug-resistant opportunistic pathogens associated with healthcare-associated infections (HAIs). In addition, there is a high prevalence of species carrying genes coding for extended-spectrum β-lactamases such as bla_{CTX-M}, bla_{SHV}, bla_{TEM-2}, and carbapenemases, mainly bla_{KPC}, for which there are few treatment options. In this context, colistin is an effective last treatment option. Despite the above, the existence of strains carrying a heteroresistant (HR) phenotype to colistin is of concern, which could lead to the selection of more resistant strains with a possible increase in the mortality rate.

Currently, the mechanisms behind the colistin heteroresistant phenotype (CST-HR) are not fully elucidated. However, existing evidence suggests that mutations in the *mgrB* regulatory gene or mutations in one of the two-component system genes such as *phoPQ, pmrAB, pmrC, crrAB* are related to the expression of heteroresistance. On the other hand, it is of great interest to investigate that other non-chromosomal resistance mechanisms, such as capsular hyperproduction may contribute to the existence of the phenotype. Thus, the aim of this thesis was to characterize the mechanisms involved in colistin heteroresistance in Klebsiella pneumoniae strains producing extended-spectrum β-lactamases. For this, the presence of HR strains was identified by population profiling analysis (PAP) on 60 *K.pneumoniae* - $BLEE^{+}$ strains, all susceptible to colistin. HR strains were isolated and successive passages were performed for 5 generations. Subsequently, bacterial growth parameters were determined and whole genome sequencing (Illumina® MiSeq) and subsequent sequence assembly was performed using Spades V.3.9. With the data obtained, the study of colistin resistance mechanisms was carried out using Galaxy web Server software using a local database with *K. pneumoniae* MGH78578 as reference

genome for a strain susceptible to colistin. The above study was complemented with the determination of mutations in *wzi, wzc, rcsABC* and *lon* regulators, which are related to capsular expression. Additionally, the relative expression level of *phoPQ, pmrABD* and *mgrB* genes was determined and the amount of capsular polysaccharide and Z-potential (Zetasizer Nano ZS90 - Malvern Instrument®) were compared between susceptible and resistant strains, using the phenol-water-sulfuric acid method and electrophoretic mobility, respectively. Finally, the existence of K. pneumoniae - $BLEE^{+}$ strains carrying a hypermucoviscous and hypervirulent phenotype was studied by screening with the filance test and the search for virulence genes *magA, rmpA/A2, ybtS*, among others by multiplex PCR. Of the 60 strains studied, eight of them have an HR phenotype to colistin, with CST-MIC_{50} = 50 µg/mL. The strains possess multiple resistance genes with $bla_{CTX\text{-}M\text{-}15}$ *and* $bla_{CTX\text{-}M\text{-}2}$ *;* $bla_{SHV\text{-}12}$ *and* $bla_{OXA\text{-}10}$. It was determined that the strains belong to three different lineages (ST11;ST25 and ST1161) with K.-locus 2 being the most prevalent. On the other hand, the first description of CST-HR in ST1161 strains of endemic description in Chile is highlighted. Regarding the mechanisms of resistance to colistin, the presence of multiple mutations in the chromosomal genes *pmrB, phoPQ and mgrB* was identified, some of which have not been previously described. On the other hand, changes in the expression level of the regulatory genes were determined, highlighting a decrease in the relative expression of *mgrB* and *pmrBD,* which are consistent with the expression of colistin resistance. On the other hand, it was determined that the CST-HR strains have a less electronegative surface charge and this is probably not related to the amount of capsular polysaccharide. On the other hand, the casual relationship between the existence of mutations in *crrB* and the higher amount of polysaccharide observed in HR strains was determined. Finally, during the development of this work, the presence of two strains, one of *K. pneumoniae* and the other of *K. variicola*, carrying a hypermucoviscous and hypervirulent phenotype and genotype was identified, being this the first formal description in Chile. Finally, it was possible to identify the existence of BLEE-producing strains of *K. pneumoniae*, carriers of a stable phenotype of heteroresistance to colistin, which show high levels of resistance to the antibiotic. Of the strains identified, it was possible to establish the genetic mechanisms responsible for the phenotypic expression of colistin resistance. It was possible to establish the existence of alterations in the expression level of *pmrABD, phoPQ* and *mgrB* genes, probably as a consequence of the mutations identified. It was determined that the heteroresistant strains produce a greater amount of capsular polysaccharide compared to the susceptible strains,

and that the capsular serotype present in the Klebsiella pneumoniae strains studied is not related to the existence of the HR phenotype.

CAPÍTULO I: INTRODUCCIÓN

Durante los últimos años, el aumento en la prevalencia de infecciones provocadas por microorganismos resistentes, principalmente enterobacterias, como *Klebsiella pneumoniae*, *Escherichia coli*, *Serratia spp*., *Proteus spp*., entre otras, así como también bacilos Gram negativos no fermentadores como *Acinetobacter baumannii* y *Pseudomonas aeruginosa*, son consideradas una amenaza mundial, ya que cada vez son menos los antimicrobianos disponibles para tratar infecciones producidas por estos microorganismos.(WHO, 2017) En cuanto a las causas que han llevado a esta crisis actual, es posible mencionar la sobreutilización y el uso irracional de los antibióticos en medicina humana, industria agrícola y producción animal.(Allen et al., 2010) Al respecto, el uso intensivo de los antibióticos ha ejercido a través del tiempo, una enorme presión selectiva, provocando la selección de microorganismos resistentes a múltiples antimicrobianos. Si al aumento en la prevalencia de bacterias resistentes se le suma la escasez de nuevos compuestos, con mecanismos de acción diferentes a los ya conocidos y que, además, sean activos frente a estos microorganismos, nos enfrentamos a un panorama en el cual es posible encontrar infecciones provocadas por bacterias para las cuales no existen tratamientos efectivos disponibles.

Respecto a lo anterior, podemos mencionar, por ejemplo, la amplia distribución de enterobacterias multirresistentes (MDR) y extensivamente resistentes (XDR), como por ejemplo *K. pneumoniae* productoras de β-lactamasas de espectro extendido (BLEE), que

confieren resistencia a cefalosporinas de tercera generación y cepas productoras de carbapenemasas, que confieren resistencia a carbapenémicos entre otras, reduciendo sustancialmente las opciones terapéuticas disponibles.

El complejo *Klebsiella pneumoniae*, incluyendo las especies relacionadas *K*. *pneumoniae* sensu stricto, *K*. *quasipneumoniae* subsp. *quasipneumoniae*, *K*. *variicola* subsp. *variicola y K*. *africana,* entre otras, (Rodrigues et al., 2019) han emergido como importantes patógenos oportunistas comúnmente involucrados en infecciones asociadas a la atención en salud (IAAS). (Barrios-Camacho et al., 2019; Wyres et al., 2020) En la actualidad, tanto *K. pneumoniae* como *K. variicola*, son considerados patógenos oportunistas, relacionados con diferentes cuadros clínicos, que pueden ir desde neumonía e infecciones del tracto urinario (ITU) hasta infecciones graves como bacteriemias, meningitis, abscesos entre otros. (Halaby et al., 2016)

De especial relevancia resulta *K. pneumoniae,* la cual, desde un punto de vista epidemiológico, surge como un importante patógeno intrahospitalario, debido a la facilidad que posee para adquirir nuevos genes que confieren resistencia a prácticamente todos los antimicrobianos en uso clínico. A esto, destaca la resistencia mediada por la expresión de genes codificantes de diferentes β-lactamasas como por ejemplo BLEE (bla_{CTX-M}, bla_{SHV}, bla_{TEM-2}), carbapenemasas como serino–β-lactamasas (principalmente bla_{KPC}), metalo-β -lactamasas (bla_{VIM}, bla_{IMP} y bla_{NDM}) y oxazilinasas de la clase D de Ambler, entre otras. En este contexto, es que colistín emerge como un antibiótico de última línea para el manejo de infecciones provocadas por microorganismos multirresistentes y, especialmente, aquellas producidas por *K. pneumoniae* XDR, ya que colistín es un antibiótico que posee buena

actividad antimicrobiana sobre bacilos Gram negativos (Jones et al., 2013) y destacables propiedades farmacodinámicas. (Petrosillo et al., 2019)

Sin embargo, resulta preocupante el hecho de la existencia de cepas portadoras de un fenotipo HR a diferentes antibióticos, principalmente carbapenémicos y colistín, lo cual resulta complejo, ya que la existencia de este fenómeno en el ámbito clínico daría paso a la selección de cepas más resistentes durante el tratamiento antibiótico, aumento en infecciones recurrentes o crónicas, junto con un posible incremento en la tasa de mortalidad. (Andersson et al., 2019; El-Halfawy & Valvano, 2015) A lo anterior, hay que destacar que las técnicas rutinarias para el estudio de la susceptibilidad bacteriana *in vitro*, no siempre son capaces de identificar la existencia de subpoblaciones HR, lo cual agrava el problema. (Andersson et al., 2019)

Actualmente, los mecanismos que conllevan al fenotipo de CST-HR no están completamente dilucidados. Sin embargo, múltiples trabajos lo relacionan con los mecanismos clásicos de resistencia a colistín, los cuales se vinculan con mutaciones del gen regulador *mgrB* o mutaciones en alguno de los genes del sistema de dos-componentes como *phoPQ*, *pmrAB*, *pmrC*, *crrAB* entre otros. Respecto a lo anterior, de especial importancia resultan las mutaciones en *phoPQ* o *pmrAB*, las cuales pueden generar una sobrexpresión constitutiva de los operones *pmrHFIJKLM, pmrC* o *arnBCADTEF-pmrE,* los cuales están involucrados en la biosíntesis y transferencia de fosfoetanolamina (PetN) o 4-amino-4-deoxi-L-arabinosa (L-Ara4N) al LipA, incrementando la carga positiva del LPS, resultando en una disminución de la afinidad del antibiótico por el LPS. (Formosa et al., 2015) Por su parte, alteraciones en *mgrB* debido a deleciones, mutaciones o interrupciones mediadas por secuencias de inserción del tipo IS*1-like*, IS*3-like* o IS*5-like*, representan el más importante de los mecanismos descritos para la existencia del fenotipo CST-HR en *K. pneumoniae.*

(Baron et al., 2016; Jeannot et al., 2017) Sin embargo, resulta especialmente interesante que el fenotipo CST-HR pueda estar relacionado con otros mecanismos de resistencia no cromosómicos, diferentes a los clásicamente descritos, como por ejemplo, la hiperproducción capsular, la cual disminuye la interacción del antibiótico con la superficie bacteriana; y el incremento en la expresión de bombas de expulsión, especialmente del tipo RND, entre otros mecanismos. (Ernst et al., 2020; Jayol et al., 2017)

Sobre la hiperproducción capsular como posible mecanismo de resistencia, algunos autores postulan que la presencia de una cápsula prominente y de carácter aniónica de polisacárido, actuaría como una barrera protectora entre el LPS y la molécula de colistín, confiriendo así resistencia al antibiótico. Por su parte, algunos autores han demostrado que, *K. pneumoniae* sometida a concentraciones subinhibitorias de colistín, es capaz de regular la producción capsular estableciendo un posible vínculo entre la resistencia a antibióticos policatiónicos, la expresión de la cápsula y la presencia de alteraciones en el grado de virulencia. (Campos et al., 2004; Formosa et al., 2015)

Así, en este trabajo se pretende dilucidar si la existencia del fenotipo CST-HR, detectado en cepas de *K. pneumoniae* productoras de BLEE, se relaciona con los mecanismos clásicos de resistencia a colistín o bien, guardan relación con la existencia de un polisacárido capsular abundante.

CAPÍTULO II: ANTECEDENTES GENERALES

2.1.- Características generales del complejo *Klebsiella pneumoniae*

K. pneumoniae es un bacilo Gram negativo, perteneciente a la familia de las *Enterobacteriaceae.* Generalmente posee un polisacárido capsular que le confiere una apariencia mucosa característica a las colonias cuando crece en medio de cultivo sólido. Es una bacteria inmóvil, fermentadora de lactosa y productora de lisina descarboxilasa, difícil de diferenciar de otros miembros del complejo solo por sus características metabólicas y fenotípicas. (Wyres et al., 2020)

K. pneumoniae conforma el complejo *Klebsiella pneumoniae,* el cual está constituido por miembros estrechamente relacionados que comparten el 90% del índice de nucleótidos promedio (Average Nucleotide Identity – ANI). (Rodrigues et al., 2018) Así, el complejo *Klebsiella pneumoniae* comprende 7 filogrupos diferentes (Figura 1) incluyendo *K. pneumoniae* sensu stricto (Kp1), *K. quasipneumoniae* subsp. *quasipneumoniae (Kp2)*, *K. quasipneumoniae* subsp. *similipneumoniae (Kp4)*, *K. variicola* subsp. v*ariicola (Kp3)*, *K. variicola* subsp. *tropica (Kp5)*, *K. quasivariicola (Kp6)*, y *K. africana (Kp7). (Rodrigues et al., 2018; Wyres et al., 2020)*

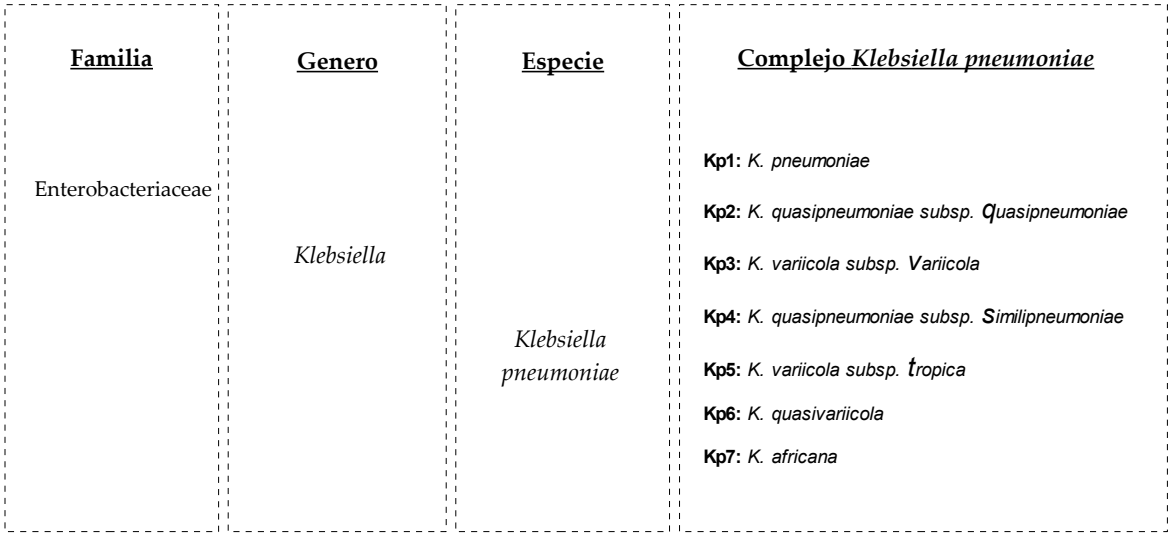

Figura 1.- Clasificación taxonómica del complejo Klebsiella pneumoniae. (Adaptado de (Wyres et al., 2020)

El complejo *Klebsiella pneumoniae* es ubicuo, encontrándose en ambientes como suelos, aguas, plantas, insectos y aves; pero también, colonizando las mucosas de los mamíferos. (Podschun & Ullmann, 1998) En estos últimos y, específicamente, en humanos se encuentra colonizando a nivel del tracto gastrointestinal y orofaringe*. (Paczosa & Mecsas, 2016; Podschun & Ullmann, 1998; Pragasam et al., 2017; Wyres et al., 2020)* Al respecto, se estima que, en humanos el 5 % de la población es portador nasofaríngeo de *K. pneumoniae*, mientras que entre el 5 % y 38 % lo es en la mucosa intestinal. Por su parte, el porcentaje de portación de *K. pneumoniae* aumenta considerablemente en pacientes hospitalizados respecto de la portación comunitaria, constituyéndose el principal reservorio de transmisión intrahospitalario, la portación del tracto gastrointestinal y en manos del personal sanitario. (Ashurst & Dawson, 2018; Podschun & Ullmann, 1998)

Por su parte, *K. pneumoniae*, corresponde al patógeno de mayor relevancia clínica, siendo la principal causa de infecciones en humanos entre los distintos miembros de este complejo (Rodrigues et al., 2018) Se estima que *K. pneumoniae* es parte de al menos 85%

de los aislados clínicos identificados desde múltiples tipos de infecciones, principalmente IAAS, que pueden ir desde neumonía e infecciones del tracto urinario (ITU) hasta infecciones graves como bacteriemias, meningitis, abscesos entre otros. (Paczosa & Mecsas, 2016; Podschun & Ullmann, 1998) Por ejemplo, *K. pneumoniae* es un importante agente etiológico de neumonías intrahospitalaria, como la neumonía asociada a ventilación mecánica (NAVM), estimando su prevalencia en 12 % dependiendo de la región geográfica. (Ashurst & Dawson, 2018) También*, K. pneumoniae* es la principal causa de infecciones invasivas tales como neumonía y bacteriemias y en menor medida, absceso hepático invasivo, todos asociados a pacientes con comorbilidades tales como alcoholismo, diabetes mellitus, enfermedad pulmonar obstructiva crónica (EPOC), malignidad (cáncer de mama, próstata, entre otros) usuarios de fármacos inmunosupresores en el contexto de enfermedades autoinmune o trasplantes de órganos entre otros. A lo anterior, se estima una mortalidad comprendida entre 20 % a 50 % de los casos de pacientes con infecciones provocadas por *K. pneumoniae* MDR. (Vading et al., 2018; Xu et al., 2017)

2.2.- β-lactamasas de espectro extendido en *K. pneumoniae*

En los últimos años, el complejo *K. pneumoniae,* especialmente *K. pneumoniae* y *K. variicola* supb. *variicola (*en adelante *K. variicola)*, han emergido como patógenos de importancia global, debido a la gran plasticidad de su genoma. (Martin & Bachman, 2018) Esta característica fundamental le ha permitido como especie, adquirir nuevos y múltiples genes de resistencia antibiótica. (Kumar et al., 2011) Así, en la actualidad, existe una alta diseminación de especies de *K. pneumoniae* resistentes a múltiples antibióticos, siendo clasificada como MDR cuando el aislado es capaz de resistir al menos tres de los doce grupos o familias de antibiótico consensuados por el comité de experto de la Red Latinoamericana de Vigilancia de la Resistencia a los Antibióticos – ReLAVRA y que se detallan en la Tabla 1. (Jiménez Pearson et al., 2019)

Tabla 1 Antibióticos utilizados para la clasificación de *K. pneumoniae* y otras Enterobactericeae, como multirresistentes (MDR), resistencia extendida (XDR) y panresistentes (PDR)

Código ATC	Grupo farmacológico	Antibiótico
J01CR	Combinación de penicilina más inhibidor de β-lactamasas	Amoxicilina - ácido clavulánico o ampicilina - sulbactam
		Piperacilina - tazobactam
J01DD J01DE	Cefalosporinas de tercera o cuarta generación	Ceftazidima o cefotaxima/ceftriaxona o cefepima
J01DH	Carbapenémicos	Imipenem o meropenem
J01DF	Monobactámico	Aztreonam
J01GB	Aminoglicósido	Gentamicina
		Amikacina
J01MA	Fluoroquinolona	Ciprofloxacino
J01EE	Sulfonamida -Trimetoprim	Trimetoprim - sulfametoxazol
J01XX	Otros antibióticos	Fosfomicina
J01AA	Tetraciclina	Tigeciclina
J01XB	Polimixina	colistín

ATC: Sistema de clasificación anatómica, terapéutica química. MDR: Resistente al menos a tres de los grupos de antibióticos; XDR: Resistente a todos los grupos excepto a dos de ellos; PDR: Resistente a todos los antibióticos

K. pneumoniae es intrínsecamente resistente a ampicilina, debido a la producción de β-lactamasas cromosómicas de la clase A de Ambler, codificada por el gen bla_{SHV} en *K. pneumoniae* y bla_{LEN} en *K. variicola*. A raíz de múltiples eventos móviles, por ejemplo, por la inserción de IS*26*, se produjeron mutaciones puntuales las cuales permitieron ampliar su espectro de hidrólisis conformando enzimas con actividad BLEE.

Las BLEE, corresponde a un grupo de enzimas pertenecientes a la clase A de Ambler, capaces de hidrolizar penicilinas, aminopenicilinas y cefalosporinas de tercera generación. (Shaikh et al., 2015) Fueron descritas posterior a la introducción de los nuevos compuestos oximinos-β-lactámicos como aztreonam y las cefalosporinas de tercera generación. (De Angelis et al., 2020) La mayoría de ellas derivan de mutaciones puntuales de las enzimas parentales (SHV-1 en *K. pneumoniae* y TEM-1 en *E. coli)*, que originaron sustituciones aminoacídicas como por ejemplo Arg164 en enzimas de la familia TEM, las cuales confirieron cambios estructurales en el sitio activo, ampliando de esta forma su espectro de hidrólisis a las cefalosporinas de tercera y cuarta generación. Más reciente es la descripción de la familia de enzimas denominadas CTX-M, siendo en la actualidad, la familia de mayor diseminación global en *Enterobacteriaceae. (De Angelis et al., 2020; Shaikh et al., 2015)*

CTX-M comprende 5 grupo de enzimas (CTX-M-1; CTX-M-2; CTX-M-8; CTX-M-9 y CTX-M-25) conformando en conjunto más de 230 variantes. (Naas et al., 2017) Su origen se centra en enzimas cromosómicas existentes originalmente en especies de *Kluyvera,* y que por múltiples eventos de recombinación y movilización de $bla_{\text{CTX-M}}$ en plataformas genéticas altamente movilizables como plásmidos (IncF, IncK, IncN, etc), transposones e integrones presentes en clones altamente exitosos (CC258, CC15, CC20 etc) han permitido su persistencia y diseminación global. (Cantón et al., 2012; Wyres et al., 2020) A nivel mundial, CTX-M-14 y CTX-M-15 son las enzimas de mayor presencia en Europa, Asia, África y Estados Unidos (De Angelis et al., 2020), mientras que, en Sudamérica, CTX-M-2 destaca como la BLEE más predominante. (Cantón et al., 2012; Guzmán-Blanco et al., 2014; Villegas et al., 2008)

2.3.- Factores de virulencia en el complejo *K. pneumoniae* y característica del fenotipo hipervirulento (hv)

La gran plasticidad del genoma de *K. pneumoniae* no solo le ha permitido adquirir múltiples genes de resistencia antibiótica, sino que también, le ha permitido adquirir nuevos genes de virulencia. (Wyres et al., 2020) De esta forma, resulta preocupante la reciente descripción de un fenotipo especial conocido como hipervirulento, descrito por primera vez en *K. pneumoniae* (hvKp) y, más recientemente, en *K. variicola* (hvKv). (Lee et al., 2017; Paczosa & Mecsas, 2016; Rodríguez-Medina et al., 2019) El fenotipo hipervirulento fue descrito tradicionalmente como responsables de abscesos piogénicos, meningitis graves, endoftalmitis, fascitis necrotizante y en general, asociado con complicaciones metastásicas. (Lee et al., 2017)

Desde su primera descripción en 1986, (Liu et al., 1986) la existencia de hvKp se relacionaba con aislados que conservaban un buen perfil de susceptibilidad antibiótica. (Paczosa & Mecsas, 2016) Así, tradicionalmente se establece una clara diferencia entre dos fenotipos bien caracterizados; el fenotipo de *K. pneumoniae* “clásico” (cKp) y el fenotipo “hipervirulento” (hvKp).

Pese a lo descrito tradicionalmente, y en especial al hecho sobre el fenotipo hvKp considerado como sensible a la mayoría de los antibióticos; la creciente cantidad de publicaciones de casos donde se reporta la coportación de múltiples genes de resistencia antibiótica, generan un cambio del paradigma tradicional, resultando en una situación epidemiología de alarma global de máxima gravedad. Tradicionalmente se reconoce a la existencia de hvKp con una alta endemicidad en Asia; sin embargo, cada vez son más los reportes publicados en Europa, América del Norte y Sudamérica. (Lee et al., 2017) En Chile, Morales-León F. et al., recientemente realizó la descripción de dos cepas de *Klebsiella pneumoniae* y *Klebsiella variicola* portadoras de un fenotipo hipermucoviscoso e hipervirulentas. (F. Morales-León et al., 2021)

2.3.1.- Factores de virulencia presentes en el complejo *K. pneumoniae*

En general, todos los miembros del complejo *K. pneumoniae* poseen 4 factores de virulencia bien descritos. A saber, estos son: a) lipopolisacárido (LPS), b) sideróforos, c) fimbria (adhesinas o pilli) y d) cápsula. (Paczosa & Mecsas, 2016) A continuación, se describen los aspectos más importantes de cada uno de estos factores.

a) Lipopolisacárido (LPS)

Estructuralmente, el LPS constituye el principal componente de la membrana celular externa de la bacteria, compuesto típicamente por el antígeno-O, core y el Lípido A (LipA). El LPS se puede agrupar en 12 tipos diferentes según el tipo antígeno-O, donde los serotipos O1 y O2 son los de mayor prevalencia clínica. (Paczosa & Mecsas, 2016; Wyres et al., 2020) Como factor de virulencia, su rol principal está vinculado a la protección de la actividad del complemento, específicamente la formación de C3b. (Paczosa & Mecsas, 2016) Sin

embargo, el LipA (endotoxina) actúa como ligando de TLR4, lo cual lo convierte en un potente activador de la respuesta celular.

b) Sideróforos

El hierro es un factor fundamental para el crecimiento bacteriano. Sin embargo, en los mamíferos la biodisponibilidad de este elemento es baja. Así, las bacterias, incluyendo *K. pneumoniae*, requieren de moléculas quelantes del hierro, denominados sideróforos, que les permite asegurar la disponibilidad de este elemento esencial. (Paczosa & Mecsas, 2016; Podschun & Ullmann, 1998)

K. pneumoniae posee cuatro sistemas de sideróforos. a) Enterobactina (*ent*), presente en el cromosoma de todas las cepas de *K. pneumoniae,* (Marr & Russo, 2019) b) yersiniobactina (*ybt*), c) aerobactina (*iuc*) y d) salmochelina (*iro*). Estos últimos tres sistemas de sideróforos, no se encuentran en todas las cepas de *K. pneumoniae,* ya que son adquiridos en plásmidos de virulencia específicos. (Wyres et al., 2020) De esta forma, la presencia de sideróforos, como, por ejemplo enterobactina, resultan indispensable para el crecimiento bacteriano en cualquier medio. Por su parte, la presencia de sideróforos adquiridos como yersiniobactina, aerobactina y salmochelina, se relacionan con diferentes cuadros infecciosos, donde aerobactina (*iucA*) es considerado un buen marcador de hipervirulencia. (Marr & Russo, 2019)

c) Fimbria

La fimbria es un importante mediador de la adhesión bacteriana. En *K. pneumoniae*, la fimbria tipo 1 y tipo 3, son las principales estructuras de adhesión. (Paczosa & Mecsas, 2016)

La fimbria tipo 1, corresponde a una delgada protrusión de la superficie bacteriana, expresada en el 90% de los aislados clínicos y ambientales de todas las Enterobacteriaceae. Su expresión es dependiente del gen *fimA*, que codifica la principal estructura, la subunidad FimA, la cual termina es su extremo con la presencia de una proteína de adhesión (FimH). La fimbria tipo 1 se adhiere a glicoproteinas D-manosiladas, desde donde deriva su denominación común como "manosa-sensible". Respecto a su función, se sabe que contribuye a la invasión del tejido epitelial de la vejiga, contribuyendo a la formación de biopelículas en este mismo tejido como también en superficies abióticas. Por otra parte, la fimbria tipo 1 ejerce un rol contradictorio, ya que contribuye a la respuesta inmune al incrementar la unión a los mastocitos, favoreciendo el reclutamiento de los neutrófilos.

La fimbria tipo 3, es una estructura "manosa-resistente", codificada en el operon *mrkABCD.* (Paczosa & Mecsas, 2016) Estructuralmente, la fimbria tipo 3 está conformada por filamento MrkA, unido a la membrana externa, y una porción terminal MrkD, al igual que MrkA se encargan de la unión a la superficie extracelular de los tejidos y superficies abióticas. Estudios han demostrado que la existencia de la fimbria tipo 3 es fundamental para la formación de biofilms y para la colonización a superficies abióticas como, por ejemplo, un catéter o dispositivos médicos. Por su parte, la fimbria tipo 3 contribuye a la respuesta inmune, estimulando la expresión de especies reactivas del oxígeno (ROS) por parte de los neutrófilos. (Paczosa & Mecsas, 2016)

d) Cápsula

La cápsula es una matriz de polisacárido unida covalentemente a la membrana externa bacteriana. (Nwodo et al., 2012) Posee una fuerte carga negativa, de carácter ácido, conformando una masa gelatinosa donde la mayor parte de ella está constituida por agua (Figura 2).

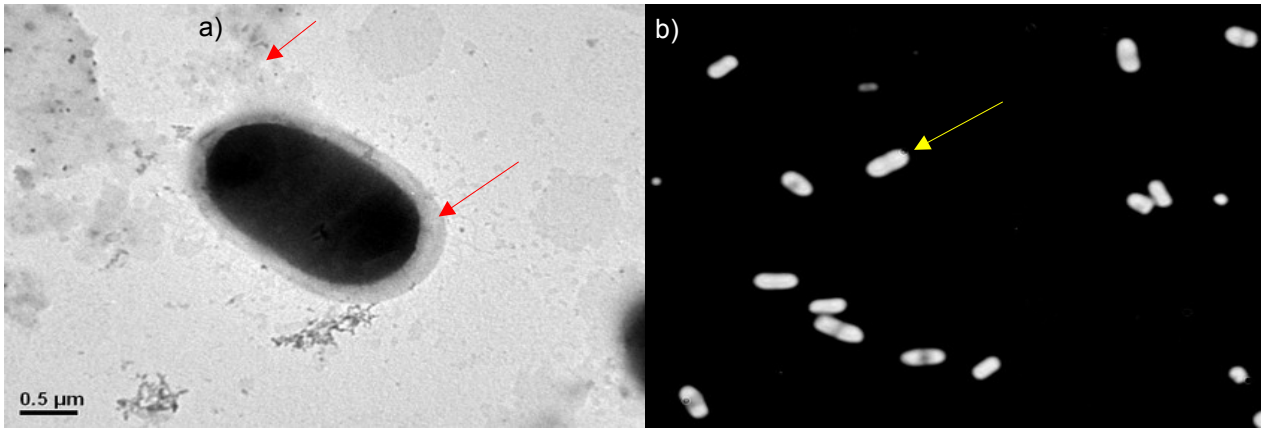

Figura 2. Imágenes representativa de la capsula bacteriana en *Klebsiella pneumoniae (Fuente propia).*
a) Microscopia electrónica de transmisión (TEM) de un cultivo fresco (menos de 24 h) de *K. pneumoniae* UCO505. En la imagen (flecha roja) se observa la estructura capsular gelatinosa y adherida a la membrana externa bacteriana junto a restos de una masa gelatinosa de polisacárido capsular. b) Microscopía óptica 100x de tinción negativa con nigrosina 1%. La estructura blanca (flecha amarilla) corresponde a la frontera externa de la cápsula de UCO505.

La cápsula posee una estructura diversa. (Wen & Zhang, 2014) Así, basado en la existencia de los diferentes genes que codifican los monosacáridos constituyentes, existen, hasta el momento 141 combinaciones posible o K-locus diferentes. (Patro et al., 2020) De estos 141 K-locus, solo algunos pueden ser identificados por los métodos serológicos tradicionales, conformando solo 77 serotipos capsulares (K-types) distintos. (Patro et al., 2020; Wick et al., 2018; Wyres et al., 2020) Pese a esta amplia diversidad, los serotipos más prevalentes se limitan solo a los serotipos K1 y K2, encontrados frecuentemente en aislados de infecciones de origen comunitario, neumonía, infecciones diseminadas y absceso hepático, entre otros (Paczosa & Mecsas, 2016) Adicionalmente, los serotipos K5, K20, K54 y K57 son aislados frecuentemente en diferentes cuadros infecciosos, la mayoría, con una amplia distribución en Asia y Europa. (Follador et al., 2016; Turton et al., 2010) Respecto a la

prevalencia de los serotipos capsulares de *K. pneumoniae* en nuestro país, no existen estudios epidemiológicos ni moleculares al respecto.

Como factor de virulencia, la cápsula posee múltiples efectos protectores. Entre ellos, previene de la fagocitosis y opsonisación por bloqueo de la activación de C3, previene el reconocimiento por parte del sistema inmune el hospedero a través del enmascaramiento de los antígenos y también, incrementa la tolerancia y resistencia a la acción de péptidos antimicrobianos, como β-defensina humana 1 y 3, y lactoferrina; entre otras moléculas bactericidas. (Paczosa & Mecsas, 2016)

d.1) Biosíntesis y regulación de la expresión capsular

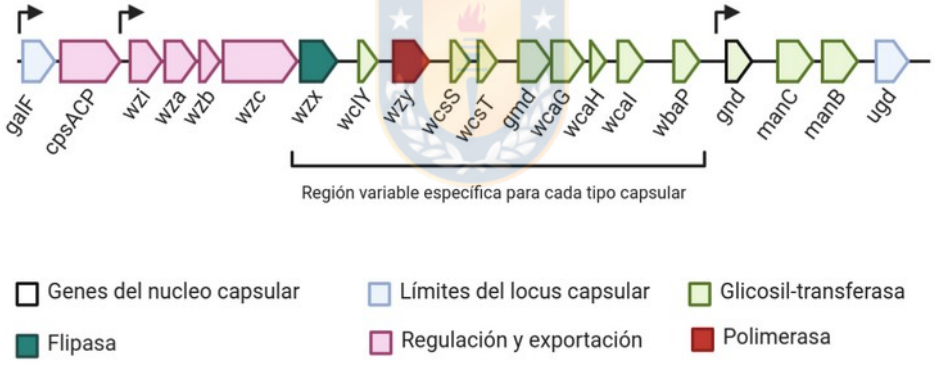

Figura 3.- Esquema de la organización del locus cápsular K1 (K-locus) de *Klebsiella pneumoniae*. Adaptado de: (Rendueles, 2020; Walker & Miller, 2020)

Desde el punto de vista molecular, la biosíntesis del antígeno K se encuentra codificado en el locus *cps* (*capsule polysaccharide synthesis*). (Li et al., 2014) Estos genes están encargados de la síntesis de subunidades de azúcares y posterior formación y exportación de las unidades de polisacáridos. En *K. pneumoniae,* el locus *cps* (figura 3) tiene un tamaño de 21 a 30 Kb, albergando entre 16 a 25 genes. Como se observa en la figura 3, el extremo

5’ terminal, conocido como K-locus, posee 6 genes conservados, *galF, orf2, wzi, wza, wzb*, *wzc*, *gnd* y *ugd*. Por su parte, la región central del locus es altamente variable, codificando la síntesis de los azúcares-específicos de cada uno de los tipos capsulares distintos además de los genes encargados de la síntesis de proteínas para la exportación y el ensamblaje del polisacárido. Estas funciones son realizadas por una flipasa (Wzx) y polimerasa (Wzy*)*, respectivamente. (figura 4) (Wyres et al., 2016) Así, la amplificación por PCR o secuenciación del gen *wzi,* permiten identificar los principales serotipos de mayor importancia clínica, (Pan et al., 2015) mientras que, utilizando los datos de secuenciación de genoma completo, específicamente, comparando el porcentaje de similitud nucleotídica del K-locus con un genoma de referencia, es posible identificar alrededor de 141 tipos capsulares distintos. (Patro et al., 2020; Wick et al., 2018; Wyres et al., 2016)

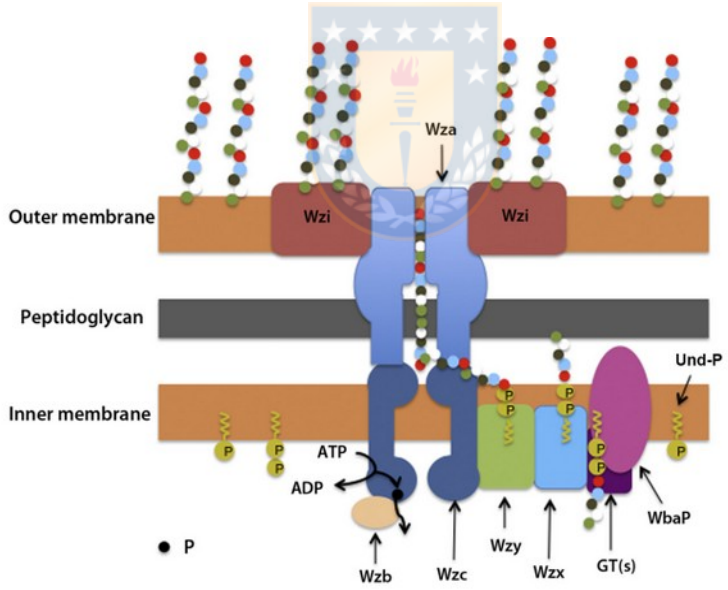

Figura 4.- Modelo de la síntesis cápsular por medio de polimerización dependiente de Wzy en *Klebsiella pneumoniae*. Tomado de:(Wen & Zhang, 2014)

La regulación de la producción capsular es un proceso complejo, el cual es controlado a nivel transcripcional por numerosas proteínas. (Walker & Miller, 2020) Se sabe que ciertas

condiciones ambientales pueden regular la expresión capsular, principalmente, vía activación de un sistema de dos componentes atípico, el sistema RcsAB. (Wall et al., 2018)

Así, por ejemplo, frente a modificaciones que alteren la estructura del lipopolisacárido, peptidoglican o lipoproteínas de superficie por acción de compuestos antibióticos, se genera la activación de RcsF, una proteína de membrana externa que desencadena la vía de fosforilación de RcsAB, activando, por ejemplo, la expresión capsular. (Wall et al., 2018) De esta forma, se podría pensar que la sola exposición de *K. pneumoniae* a concentraciones subinhibitorias de antibióticos disruptores de membrana, de naturaleza policatiónica como los aminoglicósidos y colistín, podrían desencadenar una respuesta bacteriana fisiológica, mediada, entre otros, por RcsAB, que resultaría en una mayor expresión capsular y por tanto, una disminución de la susceptibilidad al antibiótico. (Campos et al., 2004; Duperthuy, 2020)

También se sabe que en *E. coli*, la regulación de la síntesis de ácido colánico, responsable de la característica mucoide de la cápsula, puede estar determinado por acción de RcsAB y RcsF en respuesta a la temperatura. (Wall et al., 2018) Otro ejemplo de activación de la síntesis capsular vía RcsAB, ocurre por medio del aumento de la concentración de Fe^{2+}, donde su acumulación resulta tóxica para la bacteria. Así, el regulador de la absorción de fierro Fur (Ferric uptake regulator), un regulador transcripcional que se une a Fe^{2+} libre, puede regular la expresión de *rcsA* y por tanto, la cantidad de polisacárido capsular. (Yuan et al., 2020)

Según la cantidad de polisacárido capsular presente en una cepa de *K. pneumoniae*, es posible agruparlas arbitrariamente en fenotipos acapsulares, hipocapsulares, capsulares e hipercapsulares. (Ernst et al., 2020) A diferencia de lo tradicionalmente aceptado para las

cepas hipocápsulares o acápsulares, las cuales son consideradas como avirulentas. (Paczosa & Mecsas, 2016) Por su parte, en otro trabajo se determinó el rol de *K. pneumoniae* acapsular en la infección urinaria persistente, debido a la capacidad de estas cepas para ingresar y permanecer al interior de las células del epitelio urinario. (Ernst et al., 2020) Por el contrario, las cepas hipercápsulares se relacionan fuertemente con un mayor grado de virulencia (Dorman et al., 2018) y, posiblemente, con valores de menor susceptibilidad a péptidos antimicrobianos o antibióticos policatiónicos. Tradicionalmente, la existencia de un fenotipo de *K. pneumoniae* hipermucoviscoso (hmKp), se asocia con la existencia del gen *magA* (*wzy_k1* polimerasa específica del serotipo K1*)* (Fang et al., 2010) y del gen cromosómicos *rmpA* (regulator of mucoid phenotype A) y su variante *rmpA2,* que comprenden reguladores transcripcionales del locus *cps*. (Walker & Miller, 2020)

Como fue mencionado, la producción capsular puede ser regulada por acción de múltiples reguladores transcripciones, como por ejemplo, el regulador de la síntesis capsular A y B (*rcsA* y *rcsB*), el regulador kvrAB, RmpC, entre otros. (Paczosa & Mecsas, 2016; Walker & Miller, 2020) Sin embargo, al igual que lo observado en otras especies bacterianas como, por ejemplo, *Acinetobacter baumannii y K. pneumoniae,* la existencia de ciertas mutaciones puntuales en el gen *wzc del operon cps* (figura 4), ha sido relacionado con la hiperproducción capsular. (Ernst et al., 2020)

Así, la cápsula no solo cumple un rol como factor de virulencia, sino que también, ejerce su efecto actuando como un posible mecanismo de resistencia frente a compuestos policatiónicos tales como péptidos antimicrobianos (Band & Weiss, 2014) y antibióticos considerados como la última opción de tratamiento en infecciones por *K. pneumoniae* MDR o PDR. (Mlynarcik & Kolar, 2019)

2.4.- Polimixinas. Mecanismos de acción y resistencia

Las polimixinas (polimixina B y polimixina E o colistín), corresponden a una familia de antibióticos de espectro reducido con actividad exclusiva sobre bacterias Gram negativas. Estructuralmente corresponden a heptapéptidos cíclicos de carácter policatiónico. Polimixina B y polimixina E se diferencian entre sí por un sustituyen en el carbono 6 y 7 del anillo peptídico (Figura 5). Así, la polimixina B posee en el C6 un sustituyente D-fenilalanina mientras que colistín posee un sustituyente D–leucina, con variaciones respecto al aminoácido presente en C7. (Aguayo et al., 2016; Gallardo-Godoy et al., 2019)

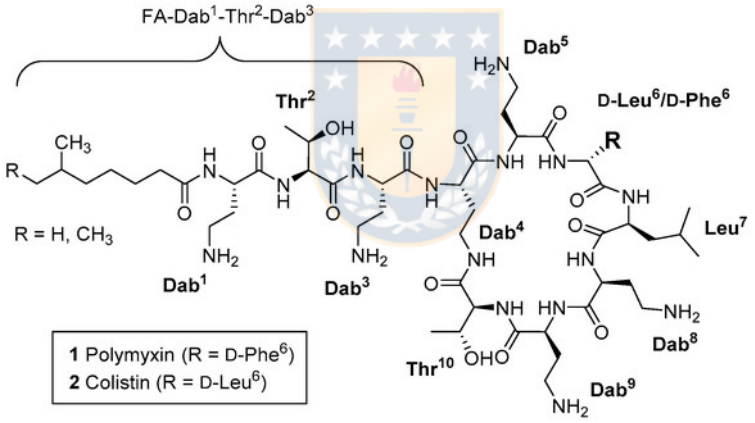

Figura 5.- Modelo estructural de las polimixinas. Dab: ácido α, γ-diaminobutírico; Sustituyente en C6 (R) confiere diferencias entre los dos representantes de la familia. Polimixina B posee un sustituyente en C6 de D-fenilalanina (D-Phe) y polimixina E o colistín D-leucina (D-Leu). Imagen tomada de: (Gallardo-Godoy et al., 2019)

2.4.1.- Mecanismo de acción de las polimixinas

Las polimixinas ejercen su efecto antibacteriano dependiente de la interacción electrostática entre la carga positiva del antibiótico, conferida por los residuos de ácido α, γ-diaminobutírico (Dab), con la carga negativa de la membrana externa de la bacteria otorgada

por los grupos fosfatos ubicados en posición 1' y 4' del disacárido de glucosamina del LipA en el LPS. La interacción entre el antibiótico y la membrana externa de la bacteria provoca el desplazamiento de los cationes Ca^{2+} y Mg^{2+}, los cuales se encuentran estabilizando esta estructura, provocando la desorganización del LPS, incrementando así la permeabilidad de la membrana externa. En la actualidad, no existe claridad respecto al mecanismo de acción exacto de colistín; sin embargo, lo más aceptado es que, posterior a la interacción electrostática entre el antibiótico y el LPS, las regiones apolares de la molécula de colistín, conferidas por un residuo de ácido graso de 6 a 9 átomos de carbono más el sustituyente D-leucina en C6, se insertan en la cara externa del LPS generando la ruptura de la membrana externa y la posterior lisis bacteriana. (Azzopardi et al., 2013; Poirel et al., 2017) De esta manera, colistín ejerce un potente efecto bactericida, concentración dependiente, frente a la mayoría de las bacterias Gram negativas.

Algunos autores afirman que colistín altera la integridad de la membrana citoplasmática, modificando funciones relacionadas con la respiración celular e inhibición de enzimas como NADH oxidasa, NADH citocromo y NADH-quinona oxidoreductasa (NDH-3). (Poirel et al., 2017; Trimble et al., 2016) Otros estudios señalan que las polimixinas inducen la generación de especies reactivas del oxígeno (en inglés: *reactive oxygen species* “ROS”) lo cual induciría la muerte celular dependiendo del estrés oxidativo. Sin embargo, este mecanismo es debatido por otros investigadores ya que se ha evidenciado actividad bactericida de colistín bajo condiciones aeróbicas como anaeróbicas. (Trimble et al., 2016)

2.4.2.- Mecanismos de resistencia a colistín

Las bacterias Gram negativas emplean al menos 3 mecanismos para resistir al efecto de colistín. Entre estos mecanismos se encuentran: a) la expresión de bombas de expulsión,

conformado por transportadores activos de carácter inespecíficos, que confieren resistencia a múltiples antibióticos incluyendo colistín, b) la formación de una cápsula de polisacáridos, la cual impide la interacción entre el antibiótico y la membrana externa y c) la modificación del sitio blanco, específicamente aquellas relacionadas con la incorporación de grupos sustituyentes que modifican las propiedades del LPS. (Poirel et al., 2017; Trimble et al., 2016) A continuación, se expondrán las principales características de cada uno de ellos.

a) Expresión de bombas de expulsión

La resistencia a colistín conferida por la expresión de bombas de expulsión aún no es del todo comprendida. A considerar, los sistemas de expulsión en bacterias Gram negativas proveen niveles significativos de resistencia múltiple a distintas familias de antimicrobianos. Estos sistemas se componen de tres elementos, a) transportador de membrana interna, b) proteína periplásmica y c) canal de membrana externa. En enterobacterias se describen diferentes familias de transportadores que confieren resistencia múltiple a distintos antibióticos, entre los que se incluyen *Resistance-Nodulation-Division* (RND), *ATP-Binding Cassette* (ABC), *Facilitador Mayor Superfamily* (MFS) y *multidrug and toxic compound extrusion* (MATE). Respecto a la resistencia a polimixinas conferidas por bombas de expulsión, tenemos, por ejemplo, la presencia en *K. pneumoniae* de sistemas tipo KpnEF y KpnGH, y la existencia de sistemas de expulsión del tipo MATE que confieren resistencia a colistín en ausencia de otros mecanismos moleculares de mayor importancia como las modificaciones del sitio blanco. (Aghapour et al., 2019; El-Sayed Ahmed et al., 2020) Así, por ejemplo, Ni y et al. estudiaron la contribución de las bombas de expulsión en la resistencia a colistín en aislados de *A. baumannii, K. pneumoniae, Pseudomonas aeruginosa y*

Stenotrophomonas maltophilia. Los autores determinaron que la actividad de 3-chlorofenilhidrazona como agente inhibidor de la actividad de bombas de expulsión, permitían una disminución significativa de los valores de CMI al antibiótico en los aislados tratados. (Ni et al., 2016)

b) Polisacárido capsular

Como ya fue mencionado, la cápsula actúa como una barrera protectora externa de la bacteria. Algunos autores postulan que la hiperproducción de una cápsula aniónica de polisacárido, actuaría como una barrera protectora entre el LPS y la molécula de colistín, contribuyendo a la resistencia al antibiótico. Así, por ejemplo, Formosa et al., analizaron el efecto de colistín sobre la cápsula de cepas de *K. pneumoniae* susceptibles y resistentes al antibiótico, señalando que, en el caso de las cepas susceptibles, estas poseían una única y delgada capa de polisacárido la cual, puede ser irrumpida por el antibiótico. Por su parte, en el caso de cepas resistentes a colistín, no se observaron alteraciones estructurales significativas del LPS, debido a que la cápsula en estos aislados posee una conformación rígida y organizada en múltiples capas. De esta forma, los autores proponen que la formación de una cápsula madura de polisacárido en cepas de *K. pneumoniae* estaría implicada en la resistencia a colistín. (Formosa et al., 2015) En estudios anteriores dirigidos por Campos et al., se demostró que en presencia de colistín, *K. pneumoniae* incrementaba la biosíntesis de polisacáridos vía regulación de la transcripción del operon *cps* (Campos et al., 2004) probablemente vía activación del sistema de dos componentes RcsAB en respuesta a la perturbación estructural de la membrana externa u otro estímulo aún no identificado. (Wall et al., 2018) De esta forma, se establece un probable vínculo entre el aumento de polisacárido capsular, el desarrollo de resistencia a colistín y un aumento en el grado de virulencia. Adicionalmente, la evidencia señala una relación entre el gen *ugd* y la expresión de

resistencia a colistín. El gen *ugd* es parte del operón *cps* (Figura 4), el cual puede ser fosforilado por acción de *wzc* para la síntesis de UDP-glucosa-dehidrogenasa, importante enzima que contribuye a la síntesis del polisacárido capsular y del ácido colánico. Sin embargo, también se ha demostrado su rol en la síntesis de un precursor de 4-amino-4-deoxi-L-arabinosa (UDP-L-ara4N) contribuyendo de esta forma a la expresión de resistencia a polimixinas. (Jayol et al., 2014; Lacour et al., 2008; Mlynarcik & Kolar, 2019) Hasta este momento, el rol de la cápsula de *K. pneumoniae* como mecanismos de resistencia a polimixinas es aún un punto de debate. Existen algunos autores que señalan que la cápsula no contribuye a la resistencia de antibióticos policatiónicos. Mularski et al. determinaron que en cepas de *K. pneumoniae,* la presencia de la cápsula solo confiere una protección marginal frente al efecto de colistín, ya que, al ser expuestas a diferentes concentraciones del antibiótico, se observa un reordenamiento en la estructura del polisacárido, seguido del desplazamiento de los cationes divalentes del LPS y posterior extrusión de la cápsula, dando paso final a la lisis celular. (Mularski et al., 2017)

c) Modificaciones del sitio blanco

Finalmente, el tercer y más importante de los mecanismos de resistencia a polimixinas es aquel relacionado con las modificaciones del sitio blanco, en especial, alteraciones en el LPS que conllevan a una disminución de la carga covalente del LipA y por tanto una disminución de la interacción entre el antibiótico y su sitio de acción. (Borsa et al., 2019; Pragasam et al., 2017)

Entre las modificaciones descritas se encuentran hidroxilaciones, deacilaciones y sustitución de los cationes divalentes Ca^{2+}/Mg^{2+} por adición enzimática de galactosamina,

fosfoetanolamina (PetN) y/o 4-amino-4-deoxi-L-arabinosa (L-Ara4N) al grupo 4'-fosfato del LipA o 1-fosfato-KDO en el LPS. (Jeannot et al., 2017)

Estos complejos mecanismos de resistencia se encuentran controlados por la activación de sistemas de dos componentes, como por ejemplo los sistemas PhoPQ y PmrAB. Al respecto, estos sistemas de dos componentes comprenden de un sensor transmembrana autofosforilante del tipo histidina-quinasa (PhoQ y PmrB) y un regulador de respuesta citoplasmático (PhoP y PmrA) que actúa como promotor de diversos genes para monitorear y responder a estímulos ambientales, incluyendo modificaciones en el pH, cambios en las concentraciones de Fe^{3+}, $Zn2^{+}$, Mg^{2+}, o, a la presencia de péptidos antimicrobianos policatiónicos entre otros. Una vez que ocurre la activación del sistema de dos componentes, se produce un incremento en la expresión de diversos genes, entre ellos, los implicados en la introducción de las modificaciones del LipA. (Beceiro et al., 2011; Jeannot et al., 2017) En el caso de algunas enterobacterias, en especial *E. coli*, las condiciones del medio de crecimiento como, por ejemplo, baja concentración de Mg^{2+}, pH ácido, alta concentración de $Fe^{3+,}$ inducen la activación del sensor de membrana PhoQ generando resistencia a colistín.

En enterobacterias como *E. coli*, *Salmonella enterica* y *Klebsiella* spp., se ha observado resistencia a colistín debido a la presencia de mutaciones en los sistemas PhoPQ y/o PmrAB, que conllevan a una sobrexpresión constitutiva de los operones *pmrCAB* o *arnBCADTEF-pmrE*, los cuales participan en la biosíntesis y posterior transferencia de PetN y L-Ara4N al LipA induciendo resistencia a polimixinas. (Formosa et al., 2015) Recientemente, se ha descrito el rol del sistema de dos componentes CrrAB en la contribución de un alto nivel de resistencia a colistín (> 16 µg/mL) en cepas de *K. pneumoniae*, descrito hasta ahora, exclusivamente en las secuencias tipo (ST) ST11, ST29 y

ST258. A saber, la presencia de mutaciones puntuales en *crrB* induce un aumento en la expresión de *crrC*, el que, a su vez, aumenta la expresión del operón *pmrHFIJKLM* y *pmrC,* de la misma forma que lo hace PhoPQ y PmrAB, introduciendo modificaciones en el LPS. Recientemente, se asoció la mutación de *crrB* con la expresión bombas de expulsión putativas tipo RND que contribuyen a la resistencia en colistín.(Y. H. Cheng et al., 2018; Mmatli et al., 2020)

Sin duda a lo ya señalado, es necesario destacar el rol del gen *mgrB*, gen conservado de 141 nucleótidos que codifica una pequeña proteína kinasa transmembrana de 47 aa, como el principal mecanismo de resistencia a colistín en cepas de *K. pneumoniae*. Según algunos autores, la prevalencia de modificaciones en *mgrB* en cepas de *K. pneumoniae* es de al menos un 25 %. (Baron et al., 2016) MgrB actúa como un regulador negativo de los sistemas de dos componentes PhoPQ y PmrAB, por lo cual, se han observado que, mutaciones puntuales en el gen *mgrB*, pérdida o disrupción por la incorporación de secuencias de inserción tipo IS5-like y IS1-like, entre otras, provocan la activación constitutiva de una cascada de señalización sobre PhoQP y PmrAB desarrollando resistencia a colistín. (Baron et al., 2016; Jeannot et al., 2017) Finalmente, algunos autores han establecido una relación entre la adquisición de resistencia a β-lactámicos, especialmente resistencia a cefalosporinas de tercera generación y carbapenémicos, con la adquisición de resistencia a colistín por disrupción del gen *mgrB* provocado por la transferencia de IS*Ecp1* presente en genes tipo $bla_{\text{OXA-181}}$ y $bla_{\text{CTX-M-15}}$, entre otros. (Baron et al., 2016)

Es necesario recordar que la modificación del LPS con la consiguiente alteración de la carga electronegativa de la membrana externa no sería el único mecanismo de resistencia a

a colistín, por lo cual, algunos autores proponen que la modificación del LPS conlleva a una serie de alteraciones biofísicas en la membrana externa, entre ellas, alteraciones en la carga neta, que resultan en la perdida de la atracción entre el antibiótico y la bacteria. Por ejemplo, Velkov y et al. estudiaron algunas propiedades biofísicas de cepas de *K. pneumoniae,* estableciendo que no existía una diferencia significativa en la carga neta del LPS, determinado según el potencial Z de membrana, entre cepas susceptibles y resistentes a colistín (Velkov et al., 2014) Por su parte, Soon et al., determinó el potencial Z de cepas de *A. baumannii*, estableciendo que la carga neta de la membrana se encuentra entre -60 mV para cepas susceptibles y -50 mV para aquellas que son resistentes al antibiótico.(Soon et al., 2011) Adicionalmente, Esposito et al. estudiaron el efecto de EDTA sobre la inhibición de fosfoetanolaminotransferasa, enzima codificada en genes *mcr* y que confieren resistencia a colistín, demostrando que, en presencia del inhibidor, el potencial Z de los aislados de *E. coli* y *K. pneumoniae* evidenciaban una disminución significativa del potencial Z como de la susceptibilidad al antibiótico. Así, los autores establecieron una relación directa entre el nivel de susceptibilidad a colistín y el potencial Z. (Esposito et al., 2017)

Todos los mecanismos de resistencia antes mencionados corresponden a mecanismos cromosómicos no transferibles. Sin embargo, a mediados del año 2016 se hace la primera descripción de bacilos Gram negativos de origen animal, ambiental y humano, con mecanismos transferibles de resistencia a colistín. Estos mecanismos se encuentran codificados en el gen *mcr*, de los cuales se han descrito 10 variantes (*mcr-1* a *mcr-10)*, que codifican para enzimas de la familia fosfoetanolaminotransferasa, las cuales catalizan una reacción entre fosfatidiletanolamina y el grupo 4'- fosfato del LipA para formar PPE-4-LipidA, un derivado con menor afinidad por el antibiótico. El hallazgo de este mecanismo transferible de resistencia a colistín resulta trascendental, si se considera la elevada posibilidad de

diseminación de microorganismos portadores de genes *mcr* que confieren resistencia a uno de los últimos antibióticos disponibles.

Hasta la fecha, la descripción mundial de *mcr-1* se incrementa exponencialmente en comparación con el resto de las variantes. En sudamericana, existen reportes de genes *mcr-1* en Argentina, Brasil, Colombia, Ecuador, Perú y Bolivia. En Chile, a fines del año 2017 se realiza la primera descripción de *mcr-1* en una cepa de *E. coli* aislada desde infección urinaria. (Legarraga et al., 2018) Pese a lo anterior, la prevalencia de genes *mcr-1* en enterobacterias resistentes a colistín aisladas de humanos alcanza el 1 %, mientras que en muestras de origen animal superan el 20 %.

La resistencia a colistín, mediada por cualquiera de los mecanismos antes descritos, constituye un panorama altamente preocupante, sobre todo en el contexto de la multirresistencia. Sin embargo, a este complejo panorama, es necesario sumar la creciente descripción de casos de cepas heterorresistente a colistín, la cual, debido a su dificultad para identificarlas, constituye un desafío clínico importante que puede relacionarse con posibles fallas terapéuticas.

2.5.- Fenómeno de heterorresistencia

La heterorresistencia a los antibióticos fue observada por primera vez en un cultivo de *Haemophilus influenzae* en 1947. (El-Halfawy & Valvano, 2015) Sin embargo, no fue hasta principios de la década de los 70, cuando se introduce formalmente el término de heterorresistencia (HR), al describir la existencia de cepas de *S. aureus* con diferentes niveles de susceptibilidad a cefalexina. (El-Halfawy & Valvano, 2015; Kayser et al., 1970) Así, desde ese entonces, el fenómeno de HR ha sido ampliamente descrito en patógenos Gram positivos, (Tato et al., 2010) principalmente en cepas de *S. aureus*. Sin embargo, de reciente interés resulta la descripción de fenotipos HR a importantes grupos de antibióticos en patógenos Gram negativos de relevancia clínica, destacando la descripción de HR a colistín en *Klebsiella pneumoniae*. (Ballestero-Téllez et al., 2017; El-Halfawy & Valvano, 2015; Silva et al., 2016; Tato et al., 2010)

2.5.1.- Definiciones de heterorresistencia

Actualmente, se pueden identificar múltiples definiciones de HR; sin embargo, la definición que mejor refleja este fenotipo es la que considera la preexistencia de subpoblaciones isogénicas de células bacterianas con un elevado nivel de resistencia a un antibiótico en particular, en comparación al resto de la población celular del mismo cultivo. (El-Halfawy & Valvano, 2015) En otras palabras, la HR describe la existencia de poblaciones bacterianas que poseen diferencias genotípicas, principalmente asociada a la presencia de uno o varios mecanismos de resistencia al antibiótico, y que poseen valores de

concentración mínima inhibitoria (CMI) en un rango amplio de diluciones, donde una pequeña proporción de ellas, es considerada resistente. (Figura 6).

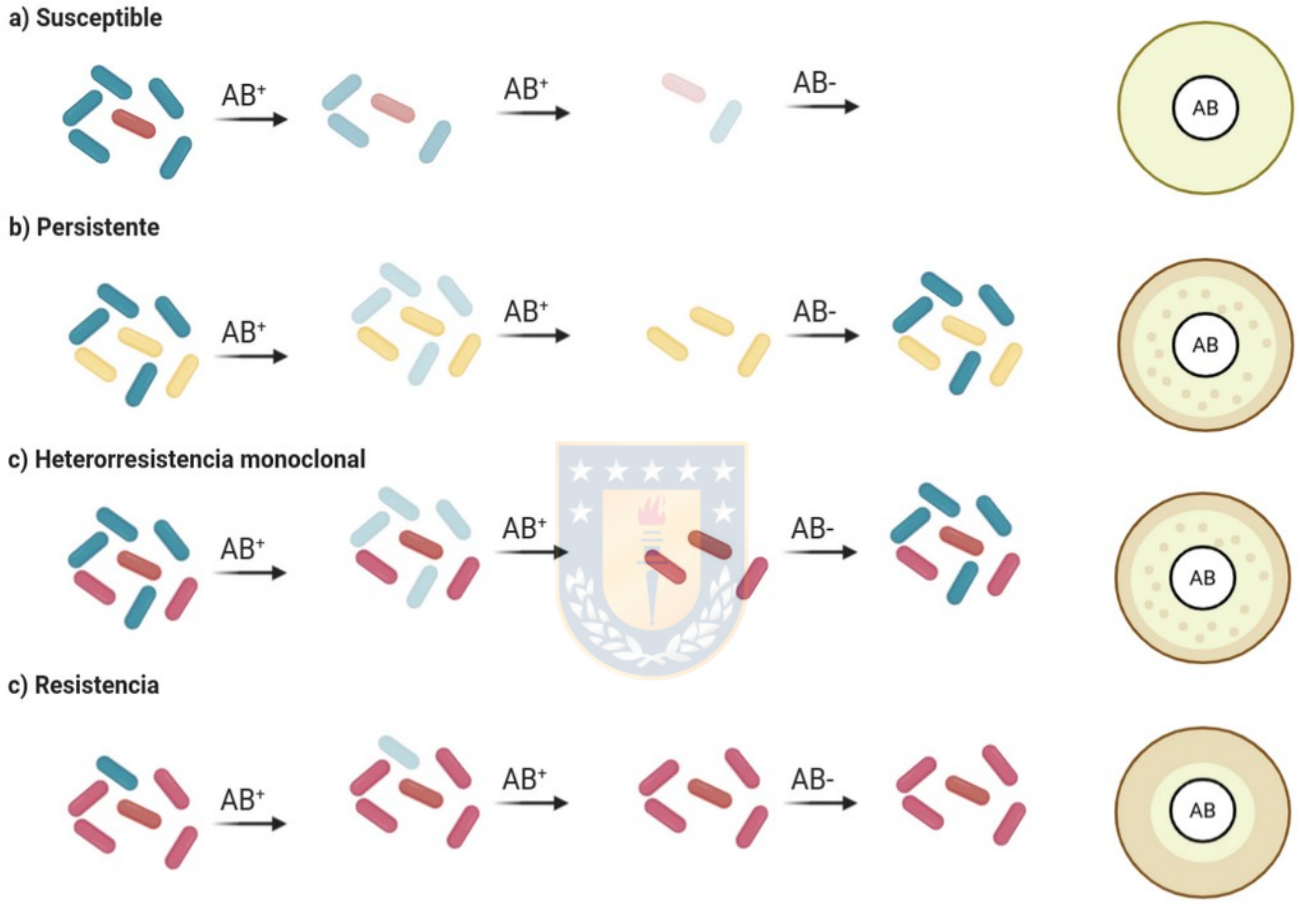

Figura 6.- Esquema general que representa diferentes fenómenos poblacionales relacionados con diferencias en el perfil de susceptibilidad. **a)** Susceptible; **b)** Persistente. En este caso no existen cambios ni mecanismos de resistencia involucrados. **c)** heterorresistencia monoclonal. En este caso, existen una pequeña proporción de células bacterianas que poseen uno o varios mecanismos de resistencia al antibiótico AB y **d)** Resistencia. Existe una proporción mayoritaria de células que poseen uno o varios mecanismos de resistencia al antibiótico AB. Adaptado de: (Dewachter et al., 2019)

Es importante destacar que, según algunos autores, la existencia de isogenicidad en el concepto de HR no es un requisito excluyente, ya que el fenómeno de HR puede resultar de la presencia de infecciones mixtas provocados por diferentes clones que posean distintos

niveles de resistencia, caso conocido como HR policlonal. Sin embargo, este fenómeno aún no ha sido descrito en *K. pneumoniae*.(Andersson et al., 2019)

Un elemento importante es diferenciar el fenómeno de HR con el de persistencia (Figura 8b). Si bien, el resultado final en ambos casos puede ser comparables, en el contexto que en ambas circunstancias, una población bacteriana es capaz de sobrevivir a concentraciones muy superiores a la CIM de un determinado antibiótico, la diferencia principal radica en que una cepa persistente emerge de manera espontánea y se asocia con la activación de mecanismos fisiológicos de regulación metabólica, los cuales pueden estar asociados con un crecimiento lento y un metabolismo reducido. (Andersson et al., 2019; Dewachter et al., 2019; Silva et al., 2016) Por su parte, en las bacterias heterorresistentes, las células pueden evidenciar múltiples cambios genotípicos estables, que tendrán como consecuencia final la expresión de un aumento en el valor de CMI a un antibiótico determinado, respecto de la población original (Figura 6). (Andersson et al., 2019; Band & Weiss, 2019)

2.5.2.- Métodos empleados para la identificación de HR

Uno de los principales problemas relacionados con HR es la dificultad para su detección de forma rutinaria en el laboratorio, por medio de los métodos tradicionales para el estudio de la susceptibilidad *in-vitro*. Esta limitación se debe, en parte, al bajo tamaño del inóculo empleado en la realización de los ensayos *in-vitro* respecto a la proporción de subpoblaciones resistentes presentes en un inóculo. (Walsh et al., 2001) Por tanto, para la identificación de cepas portadoras del fenotipo HR, es necesario emplear métodos específicos que permitan pesquisar con buena sensibilidad, pequeñas subpoblaciones de bacterias con valores de CMI elevadas, pero que se encuentran presentes en una baja proporción. Así, los métodos utilizados para la identificación de HR pueden agruparse en

métodos cualitativos y cuantitativos. (El-Halfawy & Valvano, 2015; Satola et al., 2011; Sherman et al., 2019)

a) Métodos cualitativos

Estos métodos solo permiten identificar la presencia de cepas HR y, por tanto, no aportan información adicional respecto a la proporción de cepas HR presentes en la muestra. Entre los métodos cualitativos, encontramos todos los métodos tradicionales para el estudio de susceptibilidad antibiótica *in-vitro,* destacando la microdilución en caldo o agar, difusión en agar y la epsilometría o E-test.

a.1) Microdilución en caldo

La microdilución en caldo al igual que la dilución en agar, corresponden a los métodos oficiales y recomendado para la determinación de las concentraciones mínima inhibitorias (CIM) de un determinado antibiótico (CLSI, 2019) En el caso de la microdilución, es empleado rutinariamente para el estudio de la susceptibilidad en bacterias de crecimiento rápido. Se emplean diluciones seriadas a log2 de un antibiótico determinado sobre un inóculo bacteriano estandarizado correspondiente a $5x10^4$ UFC/mL. El método tradicional no permite identificar fenotipos HR, ya que la probabilidad de que en el inóculo diluido existan subpoblaciones HR es baja. Algunos autores han propuesto modificaciones al método, utilizando por ejemplo, inóculo bacterianos mayores sobre concentraciones crecientes del antibiótico; sin embargo, los datos publicados son escasos. (Lo-Ten-Foe et al., 2007)

a.2) Difusión en agar

El método de difusión en agar, además de permitir establecer la susceptibilidad bacteriana a un determinado antibiótico, permite identificar la presencia de colonias pequeñas que pueden crecer inmersas en el interior del halo de inhibición del antibiótico, lo que revela la existencia de subpoblaciones resistentes. Habitualmente, la pesquisa de cepas HR por medio de la difusión en agar, se realiza de forma rutinaria durante su aplicación en el estudio de susceptibilidad de una muestra.

a.3) Epsilometría

Al igual que en la difusión en agar, la presencia de un fenotipo HR se revela bajo la formación o crecimiento de pequeñas colonias en torno al halo de inhibición epsilométrico formado bajo acción de una gradiente de concentración del antibiótico en cuestión.

Cabe destacar que los métodos cualitativos, poseen como desventaja la baja sensibilidad en la pesquisa de cepas HR (Satola et al., 2011)debido a la baja proporción de cepas portadoras del fenotipo en un cultivo bacteriano estandarizado en las pruebas, el cual, por lo general, no supera las 10^4 UFC/mL. (Sherman et al., 2019) Para subsanar este problema, algunos autores han implementado modificaciones a los métodos mencionados, desarrollando variantes denominadas comúnmente como macrométodos. En ambos casos, la idea consiste en aumentar la concentración del inóculo empleado, preferentemente a niveles de turbidez equivalentes a 2.0 McFarland o $6x10^8$ UFC/mL aproximadamente. Esto permite aumentar la probabilidad de pesquisar poblaciones de bacterias portadoras del fenotipo HR. Es importante señalar que estas variantes, solo han sido empleadas en la identificación de *S.aureus* hVISA y solo algunos reportes de estudio en enterobacterias, en trabajos comunicados en congresos. (El-Halfawy & Valvano, 2015; Gravey et al., 2017; Satola et al., 2011)

b) Método cuantitativo

b.1) Análisis del perfil poblacional (PAP) (Population Analysis Profiling)

El PAP constituye el método de referencia para la determinación de HR. (El-Halfawy & Valvano, 2015) Este método consiste en cuantificar la proporción de bacterias que son capaces de sobrevivir a concentraciones por sobre la CMI del antibiótico. De los métodos ya descritos, el PAP corresponde a un método cuantitativo, ya que los anteriores solo se limitan a la identificación fenotípica de HR, pero no permiten establecer la proporción de células existentes en un determinado cultivo bacteriano. (Sherman et al., 2019) Operativamente, en el PAP, un inóculo estandarizado de bacterias es sometido a una gradiente de concentraciones del antibiótico, el cual puede realizarse en un medio de cultivo sólido o líquido. (Falagas et al., 2008) Después de 48 h de incubación, se realiza el recuento de bacterias en cada concentración de antibiótico, graficando posteriormente el número de células sobrevivientes versus la concentración del antibiótico. La principal ventaja de este método radica en la capacidad de cuantificar la proporción de aislados HR, la cual puede ser igual o mayor a 10^5 células en un inóculo. Por su parte, las principales desventajas de PAP se encuentran el alto consumo de insumos de laboratorio sumando con el prolongado tiempo operativo para su ejecución. (Sherman et al., 2019)

Hasta el momento, la mayoría de los estudios existentes sobre los mecanismos de resistencia a colistín en cepas de *K. pneumoniae* con fenotipo HR, se reservan a los mismos mecanismos moleculares ya descritos, pudiendo destacar la existencia de mutaciones

puntuales en sistemas de dos componentes PhoPQ, PmrAB, CrrAB y MgrB. (Borsa et al., 2019)

Sin embargo, en algunos casos, estos mecanismos no pueden ser completamente identificados o simplemente, no permiten responder del todo la existencia del fenómeno. En este sentido, se han propuesto como un posible mecanismo que contribuye a la existencia del fenotipo HR a colistín en cepas de *K. pneumoniae*, la producción aumentada del polisacárido capsular.

CAPÍTULO III: HIPÓTESIS, OBJETIVO GENERAL Y OBJETIVOS ESPECÍFICOS

HIPÓTESIS

- El fenotipo heterorresistente a colistín, detectado en cepas de *Klebsiella pneumoniae* productoras de β-lactamasas de espectro extendido, se debe a mecanismos moleculares distintos a aquellos que introducen modificaciones estructurales en el LPS.

- El serotipo y cantidad de polisacárido capsular de cepas de *Klebsiella pneumoniae* susceptibles a colistín es similar al de las cepas heterorresistentes a este antibiótico.

OBJETIVOS

Objetivo general

Caracterizar los mecanismos involucrados en la heterorresistencia a colistín en cepas de *Klebsiella pneumoniae* productoras de β-lactamasas de espectro extendido

Objetivos específicos

- Identificar las mutaciones en los genes *mgrB, phoPQ, pmrAB y crrAB* en cepas de *K. pneumoniae* productoras de BLEE heterorresistente a colistín
- Establecer el nivel de expresión de genes *mgrB, phoPQ, pmrAB y crrAB* en cepas de *K. pneumoniae* productoras de BLEE heterorresistente a colistín
- Comparar el potencial Z en cepas de *K. pneumoniae* productoras de BLEE susceptible y heterorresistente a colistín, con y sin cápsula
- Determinar el serotipo capsular y la cantidad de polisacárido capsular en cepas de *K. pneumoniae* productoras de BLEE susceptibles y heterorresistentes a colistín

CAPÍTULO IV: Comparative analysis of three methods to identify colistin-heteroresistance in *Klebsiella pneumoniae*

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Running Title: Tree comparative methods for colistin-HR identification

Keywords: *Heteroresistance, PAP, colistin, Klebsiella pneumoniae.*

Abstract

K. pneumoniae carbapenems resistance and 3rd generation cephalosporin-resistance were a critical priority where the last-line antimicrobials treatment are limited to few options,including colistin. Colistin-heteroresistance has become as serious problem, as the usual susceptibility tests fail to detected it. There are multiple methods for identified HR, nevertheless, the population analysis profile (PAP) is a gold standard method. A major disadvantage is the time required and the amount of material needed. In this work, we compared methods described in literature to colistin-HR *Klebsiella pneumoniae* detection. We tested MDR-colistin susceptible *K.pneumoniae* and colistin-HR strain. For E-test and disk diffusion, we use a reference method recommended by CLSI and in disk diffusion macromethod and in Modified-PAP, single plate-serial spotting method determined the sensitivity, specificity and concordance among others. We determined that modified-PAP, was the only alternative method to PAP for colistin-HR identification. Finally, we describe a fast and low-cost alternative method to PAP, with good sensitivity, specificity and concordance in colistin-HR *Klebsiella pneumoniae.*

In the last year, the multidrug resistant (MDR) *enterobacteriales* are global epidemic. The who World Health Organization (WHO) defined as critical priority the E*nterobactericeae* carbapenems resistance and 3rd generation cephalosporin-resistance. *(WHO, 2017)* In this sence, *Klebsiella pneumoniae* it is a most important and common human pathogen in healthcare setting, usually related with MDR infections due by extended-spectrum β-lactamase (ESBL)-producing and carbapenem-resistant infection. (Petrosillo et al., 2019) to respect, the last-line antimicrobials for treatment of MDR - *K.pneumoniae* infection are limited to few options including colistin. (Jones et al., 2013)

Heteroresistant (HR) it is an emerging problem describe in Enterobactericeae, where carbapenems-HR and colistin-HR has become as serous problem. (Band et al., 2021) To respect, HR is phenomenon where sub-populations of seemingly isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic. (Andersson et al., 2019; El-Halfawy & Valvano, 2015) The prevalence of Enterobactericeae colistin-HR is unknown, but some authors estimate it to be 10 to 20% dependent of the multidrug-resistance profile and the method used for identification. (Cheong et al., 2019; Meletis et al., 2011; Morales-León et al., 2020; Poudyal et al., 2008) About this, the HR is a challenging phenomenon for the clinical laboratories, since the usual susceptibility tests fail to detect HR and cause misclassification of susceptible isolated may lead to inappropriate therapy. (Band et al., 2021) This could lead to intra-treatment selection of more resistant isolates, recurrent and chronic infections and, finally, increased mortality. (Band & Weiss, 2019)

In the literature, are many methods for identified HR subpopulation like E-test, disk diffusion, microdilution test, among other. (El-Halfawy & Valvano, 2015; Sherman et al., 2019) All of this method have poor sensitivity, thus the population analysis profile (PAP) is a gold

standard for detection of HR. (Silva et al., 2016) However, the PAP allows the detection and quantification of resistant sub-populations, but an important disadvantage is the time required for its execution, as well, the amount of material needed making it an impractical method for routine use. For this, in this work we compared the E-test, disk diffusion, disk diffusion macromethod. (El-Halfawy & Valvano, 2015; Satola, Farley, Anderson, Patel, et al., 2011) and a modified PAP method for colistin-HR *Klebsiella pneumoniae* detection, applied in the routine work of the clinical laboratory. For this, we tested 146 aleatory *Klebsiella pneumoniae* strain collected between 2011 and 2014, previously classified like MDR. (Jiménez Pearson et al., 2019) All of this strains was classified like colistin-susceptible by a microdilution reference method. (Institute Clinical & Laboratory Standards, 2018) Subsequently, we identified colistin-HR by PAP method. Briefly, the isolates were inoculated in Müeller–Hinton (MH) broth and incubated under agitation (350 rpm) for 12–18 h at 35 °C ± 2 °C. Then, the grown cultures was standardized to 0.5 of the McFarland standard and diluted from 10^{-1} to $10^{-6.}$ Later, were inoculated on the surfaces of MH agar plates containing 0, 0.5, 1, 2, 4, 8, and 32 µg/mL of colistin. After 48 h of incubation, the colonies were counted, and the log10 CFU/mL was plotted against the colistin concentration. (Halaby et al., 2016)

For modified-PAP, we use a previously described single plate-serial spotting method (Thomas et al., 2015). Later, 20 µL approximately of same previously diluted inoculum, were used to inoculated in 6 spots on the surfaces of MH agar plates supplemented with colistin. For this, we are using a multi point inoculator made in polylactic acid (PLA) plastic by a 3D printer. The multi point inoculator was validated. (supplement material)

For E-test and disk diffusion, we use a reference method recommended by CLSI. (Institute Clinical & Laboratory Standards, 2018) In case of disk diffusion macromethod, we used a same official procedure but using a 2.0 McFarland Turbidity Standard. We analyzed in triplicate, 20 aleatory strains included 8 colistin-HR *K. pneumoniae* (Morales-León et al., 2020). For details of the procedures used in this work, consult the supplementary material. In all methods, we determined a sensitive, specificity, positive predictive value (PPV) and negative predictive value (Trevethan, 2017). A major error (ME) was considered a colistin-HR strain by tested method, in negative or susceptible strain determined by a references' method. A very major error (VME) was considered as negative HR strain by tested method, in colistin-HR determined by a references' method (PAP). In all case, we considered a significative *p-value* at $p < 0,05$ in Fisher's exact test.

Table 1.- Sensitivity and specificity for the different methods used in determining colistin-HR

Method	SEN (%)	ESP (%)	CA (%)	EM (%)	EMM (%)	PPV (%)	NPV (%)	p-value*
Disk Diffusion (0.5 McFarland)	4	100	62	0	38	100	61	0,401
Disk Diffusion macromethod (2.0 McFarland)	4	83	52	10	23	14	57	0,987
E-test (0.5 McFarland)	13	100	65	0	35	100	63	0,059
Modified-PAP	88	100	95	0	5	100	92	< 0,01

SEN: Sensitivity; ESP: Specificity, CA:Concordance; PPV: Positive predictive value; NPV: Negative predictive value; *contingence test. p-value significative at p-value < 0,05; ** Reference method PAP.

In comparative analyses (table 1), we determined that modified-PAP, was the only alternative method to gold standard PAP analyzes for colistin-HR identification. This method showed acceptable sensitivity (88%) with high specificity and concordance (95%). Furthermore, the modified-PAP method had a high PPV a NPV. The main advantage of modified-PAP method was a low material consumption and fast laboratory procedure, because this method is based on the single plate-serial dilution spotting method. (Thomas et al., 2015) Previously, we confirmed that our modified-PAP method, had a good precision and accuracy in bacterial CFU estimation (supplementary material).

Respect to other compared methods, disk diffusion and E-test showed a low sensitivity with high EMM to colistin-HR identification. In general, all diffusion method are not recommended by CLSI to colistin *in-vitro* antimicrobial susceptibility testing. (Institute Clinical & Laboratory Standards, 2018) This recommendation is made because all polymyxins (polymyxin B and colistin) are cationic polypeptides compound, how could interact with acid or sulfate group of agar or plastic in the Petri dish, which can resulting in poor diffusion of antibiotic (Figure S3) and smaller inhibition zones. (Uwizeyimana et al., 2020) Nevertheless, some works demonstrated that agar dilution method are not problem *in-vitro* susceptible determination of colistin (Poirel et al., 2017) while other authors, propose a modified agar with the incorporation of surfactant (polysorbate 80), (Uwizeyimana et al., 2020) to minimize the antibiotic absorption to plastic or improve diffusion problem. (Turlej-Rogacka et al., 2018; Villanueva et al., 2017)

Other problem in the HR identification is the very low frequency of antibiotic-resistance subpopulation. All in-vitro susceptible test used routinely in the laboratory, employ a standard inoculum, about 10^4 CFU/mL, from a dilution of a standard 0.5 McFarland. This is so low

inoculum size respect to the low frequency of resistance cell or antibiotic resistance-subpopulation for that, usually are misclassified as colistin-susceptible. In our case, the colistin-HR frequency was 10^{-6} colistin-resistance cell which is consistent with other works (Andersson et al., 2019; Morales-León et al., 2020). In this sense, some authors recommended the used a high inoculum size, like 2.0 McFarland, equivalent to $6x10^{8}$ cell/mL (Satola, Farley, Anderson, & Patel, 2011), however, there is no information about this.

Finally, the heteroresistance is an unappreciated phenomenon with a major impact on the outcome of antimicrobial therapy. For that, early identification of strains with colistin-HR is fundamental to avoid therapeutic failures. In this work, we describe a fast and low-cost alternative method to PAP, with good sensitivity, specificity and concordance in colistin-HR *Klebsiella pneumoniae*. However, further work needed to develop a simple, fast and inexpensive method for screening in daily laboratory practice.

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CAPÍTULO V: Colistin heteroresistance among extended spectrum β-lactamases-producing *Klebsiella pneumoniae*

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Abstract: Colistin-heteroresistant (CST-HR) Enterobacterales isolates have been identified recently, challenging the clinical laboratories, since routine susceptibility tests fail to detect this phenotype. In this work, we describe the first CST-HR phenotype in extended-spectrum β-lactamase (ESBL)-producing *Klebsiella pneumoniae* isolates in South America. Additionally, we determine the genomic mechanisms of colistin heteroresistance in these strains. The CST-HR phenotype was analyzed by the population analysis profile (PAP) method, and mutations associated with this phenotype were determined by whole-genome sequencing (WGS) and the local BLAST+ DB tool. As a result, 8/60 isolates were classified as CST-HR according to the PAP method. From WGS, we determined that the CST-HR isolates belong to three different Sequence Types (STs) and four K-*loci*: ST11 (KL15 and KL81), ST25 (KL2), and ST1161 (KL19). We identified diverse mutations in the two-component regulatory systems PmrAB and PhoPQ, as well as a disruption of the *mgrB* global regulator mediated by IS1-*like* and IS-5-*like* elements, which could confer resistance to CST in CST-HR and ESBL-producing isolates. These are the first descriptions in Chile of CST-HR in ESBL-producing *K. pneumoniae* isolates. The emergence of these isolates could have a major impact on the effectiveness of colistin as a 'last resort' against these isolates, thus jeopardizing current antibiotic alternatives; therefore, it is important to consider the epidemiology of the CST-HR phenotype.

Keywords: colistín heteroresistance; *Klebsiella pneumoniae*; extended-spectrum β-lactamases

1. Introduction

In recent years, the prevalence of multidrug-resistant (MDR) Enterobacterales isolates in clinical settings has been increasing alarmingly. Among this group, extended-spectrum β-lactamase (ESBL)-producers and carbapenem-resistant *Klebsiella pneumoniae* represent a serious threat to public health [1,2]. As such, polymyxin antibiotics, such as colistin (CST), are considered to be a 'last-line therapy' to treat infections caused by highly-resistant *K. pneumoniae* due to their microbiological activity [3] and pharmacodynamic properties [1,4]. Worryingly, colistin-heteroresistant (CST-HR) Enterobacterales isolates have been recently identified. Heteroresistance (HR) is a phenomenon in which sub-populations of isogenic bacteria exhibit a range of susceptibilities to a particular antibiotic [5,6]. Currently, the mechanisms of CST-HR are not fully understood; however, diverse publications relate this phenotype to traditional CST-resistance mechanisms, which correspond to mutations in the regulatory gene *mgrB* and/or in two-component systems, such as PhoPQ, PmrAB, or PmrC, among others [6]. Specifically, *phoPQ* or *pmrAB* mutations can lead to the constitutive over-expression of the *pmrHFIJKLM, pmrC,* or *arnBCADTEF-pmrE* operons involved in phosphoethanolamine (PetN) and 4-amino-4-deoxy-L-arabinose (L-Ara4N) biosynthesis and their transfer to LipA, thereby increasing the positive charges of the LPS and resulting in a decreased affinity to polymyxins [7]. On the other hand, alterations in *mgrB* due to deletion or disruption mediated by IS1-like, IS3-like, and IS5-like elements are the most important CST-HR mechanisms in *K. pneumoniae*. In this sense, *mgrB* is a conserved gene 141 nucleotides in length, which encodes a small transmembrane protein of 47 amino acids that exerts a negative feedback on the PhoPQ and PmrAB systems [8,9]. Specifically, when *mgrB* is disrupted, this inactivated gene is reported to up-regulate the PhoPQ or PmrAB systems, thus

conferring resistance to CST [9]. Moreover, a novel CST-resistance mechanism has been described in *K. pneumoniae* ST11, ST29, and ST258 lineages, which is related to CrrAB mutations, similar to PhoPQ or PmrAB alterations [10,11]. Interestingly, the CST-HR phenotype may be associated with other no-chromosomal mechanisms, such as capsule-hyperproduction, reducing the interactions of CST with bacterial surfaces, as well as increased expression of RND-type efflux pumps [12,13]. Notably, efflux-pump mediated CST-resistance has not yet been related to CST-HR phenotype expression [6]. CST-HR in *K. pneumoniae* was described previously [14–18]in clinical highly-resistant strains, and this phenotype was related to an increase in morbidity and mortality [14], mainly associated with a loss of colistin activity.

Therefore, the identification of CST-HR is a challenging problem for microbiology laboratories, since the usual susceptibility tests fail to detect HR strains. This could lead to the intra-treatment selection of more resistant isolates, recurrent and chronic infections, and, ultimately, increased mortality [14]. There are some published methods to detect HR sub-populations. Among these methods, population analysis profile (PAP) is the *'gold standard'* technique, as it allows the detection and quantification of resistant sub-populations [19]. Due to the above, the aim of this study was to characterize the CST-HR phenotype among a collection of ESBL-producing *K. pneumoniae* clinical strains collected in Chilean hospitals.

2. Materials and Methods

2.1. Bacterial isolates and antibiotics-susceptibility tests

Sixty CST-susceptible and third-generation cephalosporin (3GC)-resistant *K. pneumoniae* isolates collected between 2011 and 2014 from seven Chilean hospitals were included. Bacterial data, such as the type of infection, hospital section, and pathological product, were recorded. Individual patient data were omitted. All isolates were originally identified as *K. pneumoniae* by each hospital laboratory. Antibiotic-susceptibility tests were performed on carbapenems (imipenem [IPM], meropenem [MEM] and ertapenem [ETP]), cephalosporins (ceftriaxone [CRO], cefotaxime [CTX], ceftazidime [CAZ] and cefepime [FEP]), amoxicillin/clavulanic acid [AMC], aminoglycosides (amikacin [AMK] and gentamicin [GEN]), fluoroquinolones (ciprofloxacin [CIP] and levofloxacin [LEV]), tetracycline [TET], and sulfamethoxazole/trimethoprim (SXT). Susceptibility tests were performed by the Kirby–Bauer method, whereas ESBL production was determined by a combined disc test [20]. In all strains positive for ESBL production, β-lactamases genes were screened by conventional-PCR [21]. CST-susceptibility was determined via the broth microdilution method according to the EUCAST guidelines, which considers a minimum-inhibitory concentration (MIC) value of ≤ 2 μg/mL as CST-susceptible [22].

2.2. Detection of colistin-heteroresistant (CST-HR) sub-population

CST-HR was determined by the PAP technique for all CST-susceptible and ESBL-producing *K. pneumoniae* strains, according to Halaby et al. [15], with the modifications published by Thomas et al. [23]. Briefly, the isolates were inoculated in Müeller–Hinton (MH) broth and incubated under agitation (350 rpm) for 12–18 h at 35 °C ± 2 °C. Then, the grown cultures were standardized to 0.5 of the McFarland standard and diluted from 10^{-1} to 10^{-6} in sterile distilled water. Later, between 10 and 15 micro-drops (2 μL approximately) were inoculated in 6 spots on the surfaces of MH agar plates containing 0, 0.5, 1, 2, 4, 8, and 32 μg/mL of CST. After 48 h of incubation at 35 °C ± 2 °C, the colonies were counted, and the

$\log_{10}$ CFU/ml was plotted against the CST concentration. The limit of quantification (LOQ) was 2.6 $\log_{10}$ CFU/mL. Afterward, we determined the stability of the CST-resistant sub-populations through consecutive sub-cultures of single colonies that appeared on the agar plates supplemented with the highest CST concentration in the PAP experiment. Sub-cultures were performed for up to five generations in MH agar without CST. Then, we determined the CST-MIC of the resulting sub-cultured colonies as described above.

2.3. Molecular strain typing

To ascertain isogenicity, we determined the clonal relationship between the resulting CST-susceptible and CST-HR sub-populations via ERIC-PCR using an ERIC2 primer (5′-AAGTAAGTGACTGGGGTGAGCG-3′) [24]. The amplified products were separated by 1.5% agarose gel electrophoresis at 60 V for 3 h. A band profile analysis was performed utilizing the GelJ software [25], and the resulting dendrogram was built by the unweighted pair group mean method (UPGMA) using the Dice similarity coefficient and a 1% band position tolerance. The Dice coefficient considered a >90% similarity as indistinguishable strains [26].

2.4. Growth curves

Bacterial growth curves were determined in an MH broth (MHB) that was Mg^{2+}/Ca^{2+} cation adjusted. Briefly, overnight cultures of *K. pneumoniae* isolates were diluted in MHB to reach a turbidity equal to 0.5 McFarland. Then, 100 µL of each diluted culture was pipetted into a 96-well plate with 100 µL MHB supplemented with Mg^{2+} and Ca^{2+} at 10 and 25 mg/L, respectively. Following this, OD_{650nm} was measured for 18 h at 37 °C. Each isolate was cultured in triplicate. The growth parameters of K, $N_{0,}$ and g were determined by the GrowthCurve R software, and the Wilcoxon test was used to compare the means between the susceptibly and resistance parameters, with significance set at $p < 0.05$ [27].

2.5. Whole-genome sequencing (WGS) and in silico genome analyses

Total DNA for whole-genome sequencing (WGS) was extracted using the Wizard™ Genomic DNA Purification kit (Promega, USA) following the manufacturer's protocol. DNA concentration and integrity were verified by a spectrophotometer (Take3 BioTek Instruments™). Sequencing was performed by the Illumina MiSeq platform (2 × 250 bp paired end reads) with libraries prepared by NexteraXT kit (Illumina), with coverage of 30x. *De novo* assembly was carried out using SPAdes assembler version 3.9 (https://cge.cbs.dtu.dk/services/SPAdes/). The assembled genomes were used to screen for antibiotic-resistance determinants with the ResFinder v3.2 tools available at the Center for Genomic Epidemiology server (https://cge.cbs.dtu.dk/services/). In addition, 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* using bioinformatic web tools (https://cge.cbs.dtu.dk/services/MLST/), and capsular serotypes were identified by the Kaptive web tool [28].

CST-resistance mechanisms were the focus of the analysis of the *mgrB, phoPQ, pmrAB* genes. Their sequences were analyzed with the local BLAST+ DB using the UGENE software version 1.32.0, including the *K. pneumoniae* MGH78578 (Genbank accession number NC_009648.1) genome as a CST-susceptible reference. The PROVEAN (Protein Variation Effect Analyzer, available at http://provean.jcvi.org/index.php) software was later used to predict whether the amino acid substitutions resulting from missense mutations in *mgrB, pmrAB,* and *phoPQ* might affect the functions of these proteins. These genomes have been deposited at the DDBJ/ENA/GenBank under the accession numbers JAAIWP01, JABJWF01, JAAIWO01, JAAIWQ01, JAAIWR01, JABJUO01, JAAFZD01, and JABJUP01.

2.6. Transcriptional analysis by qRT-PCR

Quantitative real-time PCR (qRT-PCR) was used to determine the relative expression of *phoP* (phoP-F 5'-CAG GGA AGC GGA CTA CTA TCT-3'; phoP-R 5'-GCG GCG GAT CAG TGA TAA AAA-3'), *phoQ* (phoQ-F 5'-CCG ACG GTG ACC CTT ATC TAA-3'; phoQ-R 5'-CCA TTG CGT TTC AGC CAT TCC-3'), *pmrD* (pmrD-F 5'-GCA ATC TGG TAT CGC CTT CTA-3'; pmrD-R 5'-CCG GGC AAC AGG ATT ACA-3'), *pmrA* (pmrA-F 5'-AAT CAG CGT CGG CAA TCT-3'; pmrA-R 5'-GAC AGC AGG GCA TAC TCT TTT-3'), *pmrB* (pmrB-F 5'-TGG CGA TGC GAC GTT AAT-3'; pmrB-R 5'-CAT CAG GCC CGC TTT CAA-3') and *mgrB* (mgrB-F 5'-CTG CCT GTT GCT GTG GAA-3'; mgrB-R 5'-GTG CAA ATG CCG CTG AAA-3'). The primers were designed using the Primer3 design tool.

qRT-PCR reactions were carried out as follows: A culture volume of 5 mL was taken after 18 h incubation at 37 °C (150 rpm) in cation-adjusted MHB with (heteroresistant strains) or without CST (susceptible strain). Next, 2 mL of each culture was centrifuged at 4000 rpm for 15 min. Later, the resulting pellets were used for total RNA extraction with a Trizol® according to the manufacturer's instructions (Invitrogen, ThermoFisher™). qRT-PCR experiments were carried out using a StepOne™ cycler (Applied Biosystems™), and a Kapa SYBR Fast qPCR master mix (KapaBiosystems™) was used as a signal reporter. For each determination, sterile distilled water was used as a blank. The relative gene expression was measured based on the real-time PCR efficiency (E) and ΔCT from the target and control genes, using *rpoB* (rpoB-rtF 5'-CGCGCAGACCAACGAATATG-3'; rpoB-rtR 5'-CGCCTGAGCGATAACGTAG-3') as an endogenous control.

3. Results

3.1. Strain characteristics and antimicrobial susceptibility

Sixty ESBL-producing *K. pneumoniae* determined by a combined disc test and CST-susceptible isolates (CST-MIC_{50} = 1 µg/mL) recovered from seven different Chilean hospitals were included. Of these isolates, 38% (24/60) were collected from medical units and 33% (20/60) from intensive-care units (ICUs). The remaining samples were recovered from ambulatory, surgical, and pediatric units (13% (8/60), 8% (5/60), and 5% (3/60), respectively). Moreover, 52% (31/60) were isolated from urine, 17% (10/60) from blood, 13% (8/60) from sputum, 11% (7/60) from skin and soft tissue, and 7% (4/60) from pleural effusion samples. Antibiotic-susceptibility tests revealed that about 90% were resistant to 3GC (54/60 CRO, 55/60 CTX, 54/60 CAZ), 85% (51/60) were resistant to FEP, 27% to carbapenems (9/60 MEM, 40/60 ETP and all isolates were susceptible to IPM), 98% to fluoroquinolones (59/60 CIP and 58/60 LEV), and 67% to aminoglycosides (28/60 AMK and 52/60 GEN). Moreover, 48% (29/60) of the isolates harbored genes encoding for enzymes of the $bla_{CTX\text{-}M\text{-}2}$ ESBL-group, whereas 33% (20/60) contained genes of the $bla_{CTX\text{-}M\text{-}1}$ ESBL-group.

3.2. Colistin-heteroresistant sub-populations and drug resistance

As mentioned above, the population analysis profile (PAP) is the *'gold standard'* method for the detection of heteroresistant sub-populations [5]. Accordingly, the PAP assay showed the presence of the CST-HR phenotype in 8/60 strains (UCO505, UCO509, UCO511, UCO513, UCO515, UCO517, UCO519, and UCO521) (Table 1). Figure 1A shows the bactericidal curve for the detected sub-populations, evidencing their ability to grow under the highest CST concentration (16 µg/mL). From these results, we determined the frequency of CST-resistant colonies, which oscillated between 10^{-5} and 10^{-7}. Interestingly, after five CST-free sub-cultures in MH agar, the resulting CST-$MICs_{50}$ totaled 64 µg/mL in a wide range from 8 to > 64 µg/mL, which suggests that the heteroresistant phenotype is stable (Table 2).

Furthermore, the growth curves revealed that all strains had the same growth rate constant ($K_{CST\text{-}susceptible}$ = 0.02 min^{-1} ± 0,004 versus $K_{CST\text{-}HR}$ = 0.02 min^{-1} ± 0.003; p = 0.672) and generation time ($g_{CST\text{-}susceptible}$ = 32.8 min ± 5.9 versus $g_{CST\text{-}HR}$ = 33.6 min ± 4.2; p = 0.675), indicating that the heteroresistant phenotype does not affect bacterial fitness (Figure 1B).

Table 1. Data of the CST-HR ESBL-producing *K. pneumoniae* isolates collected in Chilean hospitals.

Strain	Year of isolation	Location	Unit	Sample	GenBank Accession number
UCO511	2012	Concepcion	ICU	Urine	JAAIWP01
UCO521	2013	Santiago	ICU	Catheter	JABJWF01
UCO509	2012	Los Angeles	MED	Blood	JAAIWO01
UCO513	2012	Valparaiso	ICU	Blood	JAAIWQ01
UCO515	2013	Santiago	ICU	Urine	JAAIWR01
UCO519	2013	Santiago	SUR	Blood	JABJUO01
UCO505	2012	Pto. Montt	MED	Bronquial	JAAFZD01
UCO517	2012	Santiago	MED	Urine	JABJUP01

ICU: Intensive-care unit; MED: medicine; SUR: surgery.

Table 2. Main characteristics CST-HR *K. pneumoniae* strains (N=8). The table includes antibiotic-susceptibility patterns, clonal lineages, and antibiotic-resistance genes.

Strain	ST	K-locus	CST-MIC (g/mL)		CST-HR Frequency (10^{-6})	Antibiotic resistance genes and resistance patterns		
			S	HR		**β-lactamases (*bla*)**	Aminoglycosides resistance genes	Resistance pattern
UCO511	11	81	2	64	5,30	CTX-M-2; OXA-2; SHV-182; TEM1	aac(3)-Iia; aac(6´)-Ib3; aadA2; aph(3'')-Ib; aph(6)-Id	ERT; CPD; CRO; CTX; CAZ; FEP; AMC; GEN; AMK; LEV; CIP
UCO521	11	15	2	64	6,70	CTX-M-15; OXA-1; SHV-182	aac(3)-Iia; aac(6')-Ib-cr; aadA2; aph(3')-Ia	CPD; CRO; CTX; CAZ; FEP; GEN; LEV; CIP
UCO509	25	2	1	64	3,30	SHV-110; SHV-81; TEM-1B	aph(3´´)-Ib; aph(6)-Id	CAZ; FEP; AMC; LEV; CIP
UCO513	25	2	0,5	32	5,60	CTX-M-2; OXA-10; SHV-12; TEM1B	aadA1; aph(3'')-Ib; aph(6)-Id	ERT; CPD; CRO; CTX; CAZ; FEP; AMC; GEN;LEV; CIP
UCO515	25	2	2	> 64	2,80	CTX-M-15; OXA-1; OXA-10; SHV-110; TEM-1B	aac(3)-Iia; aac(6´)-Ib-cr; aadA1; aph(3´´)-Ib; aph(6)-Id	ERT; CPD; CRO; CTX; CAZ; FEP; AMC; GEN; AMK; LEV; CIP
UCO519	25	2	2	32	3,30	CTX-M-15; OXA-1; OXA-10; SHV-110; SHV-81, TEM-1B	aac(3)-IIa; aac(6´)-Ib-cr; aadA1; aph(3'')-Ib; aph(6)-Id	ERT; CPD; CRO; CTX; CAZ; FEP; AMC; GEN; AMK; LEV; CIP
UCO505	1161	19	2	64	0,32	CTX-M-2; OXA-2; TEM-1B	aac(3)-Iia; aac(6')Ib3; aac(6')-Ib-cr	ERT; CPD; CRO, CTX; CAZ; FEP; AMC; GEN; AMK; LEV; CIP
UCO517	1161	19	0,5	8	0,14	OXA-10; OXA-9; SHV-187; TEM-1A	aac(6')-Ib; aadA1; rmtG	ERT; CPD; CRO; CTX; CAZ; FEP; AMC; GEN; AMK; LEV; CIP

ST: Sequence-type, HR: heteroresistant, S: susceptible, HR: heteroresitance strain, imipenem [IPM], meropenem [MEM], ertapenem [ETP], ceftriaxone [CRO], cefotaxime [CTX], ceftazidime [CAZ], cefepime [FEP], amoxicillin/clavulanic [AMC], amikacin [AMK], gentamicin [GEN], ciprofloxacin [CIP], levofloxacin [LEV], tetracycline [TET], sulfamethoxazole/trimethoprim (SXT)

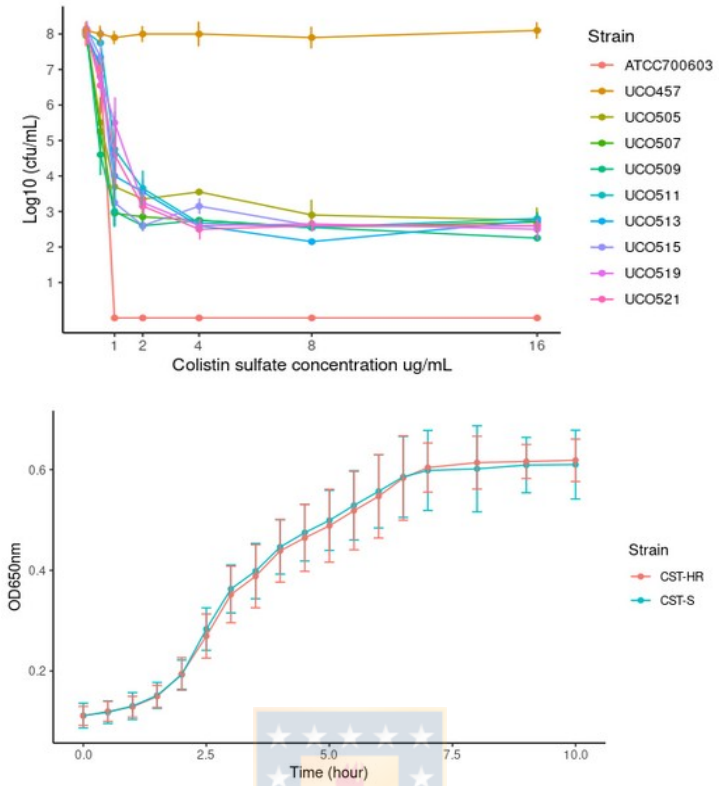

Figure 1. A. Population analysis profiles (PAPs) for CST-HR strains. ATCC 700603 (*Klebsiella quasipneumoniae subsp. similipneumoniae*) was used as a negative control (CST-susceptible) and *E. coli* UCO457 as a positive control (CST-resistant *mcr-1* positive); **B.** bacterial growth curves for CST-susceptible (CST-S) and heteroresistant (CST-HR) strains.

The MLST analysis showed that the eight CST-HR *K. pneumoniae* strains belonged to three different lineages: ST11 (UCO511; UCO521), ST25 (UCO509; UCO513; UCO515; UCO519), and ST1161 (UC505; UCO517) (Table 2). Importantly, ST11 is the most disseminated MDR lineage worldwide, whereas ST1161 is apparently endemic to Chile and has not been previously related to CST resistance. On the other hand, ST11 isolates differ in their K-loci (KL-15 and KL-81 to UCO521 and UCO511, respectively), whereas ST25 is related to the same and most prevalent capsular serotype (KL-2) (Table 2). For the clonal relationship between the susceptible and CST-HR-derived strains, ERIC-PCR showed that

both are closely genetically related, with a Dice's similarity coefficient up to 90%, confirming that both are isogenic strains (Appendix 1).

All CST-HR and ESBL-producing *K. pneumoniae* strains (N=8) exhibited an MDR phenotype and were resistant to several antibiotic-groups, including third-generation cephalosporins (7/8 CRO, 7/8 CTX, 8/8 CAZ), FEP (8/8), fluoroquinolones (8/8 CIP and 8/8 LEV), aminoglycosides (5/8 AMK and 6/8 GEN), and SXT (7/8) (Table 2). Interestingly, all were susceptible to IMP, 4/8 to MEM, and 6/8 to ETP. These data are concordant with the *in silico* analysis, in which we identified the ESBL-encoding genes $bla_{\text{CTX-M-15}}$ (3/8), $bla_{\text{CTX-M-2}}$ (3/8), $bla_{\text{SHV-12}}$ (1/8), $bla_{\text{SHV-110}}$ (3/8), and $bla_{\text{OXA-10}}$ (1/8), which mediate resistance to third-generation cephalosporins and FEP (Table 2). Additionally, we detected non-ESBL enzyme genes, such as $bla_{\text{TEM-1}}$, $bla_{\text{OXA-2}}$, and others (Table 2). Moreover, none of the carbapenemase-encoding genes were identified, which suggests that ETP and MEM non-susceptibility may be related to a combination of more than a single mechanism, such as porin loss and ESBL production [29]. In the case of aminoglycoside resistance in CST-HR strains, the *in silico* analysis revealed the presence of multiple aminoglycosides-modifying enzyme genes, such as N-acetyltransferases (*aac(3)-Ia, aac(6')-Ib3,* and *aac(6')-Ib-cr*), O-nucleotydyltransferases (*ant(3")-Ia, aadA1* and *aadA2*), and O-phosphotransferases (*aph(3')-Ia, aph(3'')-Ib, aph(6)-Ib,* and *aph(6')-Ib*) (Table 2). These results are congruent with the resistant patterns, where a single strain (UCO509) was susceptible to AMK and GEN. Interestingly, the CST-HR UCO517 strain harbored the 16S rRNA methyltransferase *rmtG* gene (Table 2), which is reported to be highly prevalent in South America [30] and was previously reported in a *K. pneumoniae* strain in Chile [30]. The presence of the N-acetyltransferases variant *aac(6')-Ib-cr* is importance since it mediates additional resistance to fluoroquinolones [31]. This gene was present in 6/8 of the CST-HR isolates, which was previously reported in Chile in ESBL-producing of *E. coli* and *K. pneumoniae* strains [32]. Accordingly, all CST-HR strains were

resistant to CIP and LEV (Table 2); this resistance could be mediated by the multiple resistance determinants detected, including the *aac(6')-Ib-cr* gene, *oqxAB* genes, and *qnrB1* and *qnrB2* genes. On the other hand, no gyrase-mutations associated with quinolone-resistance were detected in these strains (Table 2).

3.3. Colistin-resistance in colistin-heteroresistant *K. pneumoniae* strains.

To characterize the mechanisms of CST-resistance in the CST-HR-derived isogenic strains, the strains were subjected to WGS. The *in silico* analysis revealed the presence of a mutation in *phoP* in all ST25 CST-HR isolates, corresponding to a T104A amino acid substitution (Table 3). Moreover, this mutation was classified as neutral by the PROVEAN software. For the PhoPQ two-components system, a single isolate (UCO517) had the A351N mutation in PhoQ (Table 3), in which the CST-HR derived strain showed a 16-fold increase of the CST-MIC. From the PROVEAN analysis, this substitution was predicted to have a deleterious effect on the protein function. Additionally, UCO519 presented a P95L amino acid substitution in the PmrB protein (a 16-fold CST-MIC increase), whereas A256G was detected in both UCO511 and UCO521, in which a 32-fold CST-MIC increase was detected (Table 3). This mutation was also considered to be deleterious by PROVEAN. Furthermore, the UCO511 and UCO521 strains belong to the ST11 lineage; however, they differ in their K-loci (KL-81 and KL-15m respectively) (Table 2). Moreover, the ERIC-PCR analysis showed a <90% similarity; consequently, these isolates were considered genetically unrelated (Appendix 1). Four CST-HR-derived isolates showed alterations on the *mgrB* gene, which is known to mediate resistance to CST (Table 3). Specifically, a single nucleotide modification was identified in UCO521, which corresponds to the deletion of 27-bp in the gene, whereas the other three CST-HR-derived strains (UCO505, UCO511, and UCO513) displayed a

disruption of the *mgrB* gene mediated by the insertion sequences IS5-like and IS1-like (Table 3). Additionally, no amino acid substitutions were identified in the PmrA and PmrD proteins in these isolates, and *mcr*-like genes were also absent.

Table 3. Modification of CST-resistance genes in CST-HR-derived *K. pneumoniae* isolates.

		MIC (µg/mL)		PmrB		PhoP	PhoQ	MgrB	
Strains	ST	S	HR	95	256	104	351	39	IS
UCO505	1161	2	64						IS5-like
UCO517	1161	0.5	8				Ala → Asp		
UCO509	25	1	64			Thr → Ala			
UCO513	25	0.5	32			Thr → Ala			IS1-Like
UCO515	25	2	> 64			Thr → Ala			
UCO519	25	2	32	Pro → Leu		Thr → Ala			
UCO511	11	2	64		Arg → Gly				IS1-Like
UCO521	11	2	64		Arg → Gly			Cys X	

ST: Sequence-type; HR: heteroresistant; MIC: minimum-inhibitory concentration; IS: insertion sequence, Cysx. stop codon.

3.4. qRT-PCR analysis

The expression levels of CST-related resistance genes were analyzed for CST-susceptible and CST-HR-derived strains to evaluate the effect of *pmrB, phoP,* and *mgrB* mutations (Figure 2). Accordingly, we observed a decrease of the relative *mgrB* expression in heteroresistant UCO505, UCO511, and UCO519, which are related to IS element insertion but not significant modifications of expression in UCO521, which presented a premature stop codon (Figure 2).

On the other hand, all isolates, except for UCO509, displayed a significantly increased relative expression of the *phoP* gene in their CST-HR-derived isogenic strains (Figure 2), which is related to LPS modification mediated by the *pmrFHIJKLM* operon [9]. The *phoP* gene has been associated with increased expression of *prmD* [9], which was observed in CST-HR-

derived UCO505 and UCO517 isolates. Furthermore, both UCO505 and UCO519 showed a significantly increased relative expression of *phoQ* (Figure 2), a sensor kinase, which activates *phoP* via phosphorylation [13]. Moreover, we observed an increase in the expression of *pmrB*, especially in CST-HR-derived UC511, UCO519, and UCO521 strains (Figure 2), which may be due to amino acid substitution detected by WGS (Table 2). In addition, only the CST-HR UCO521 strain evidenced a significant increase in *pmrA* expression (Figure 2), which was reported to induce LipA modifications in CST-resistant isolates [31].

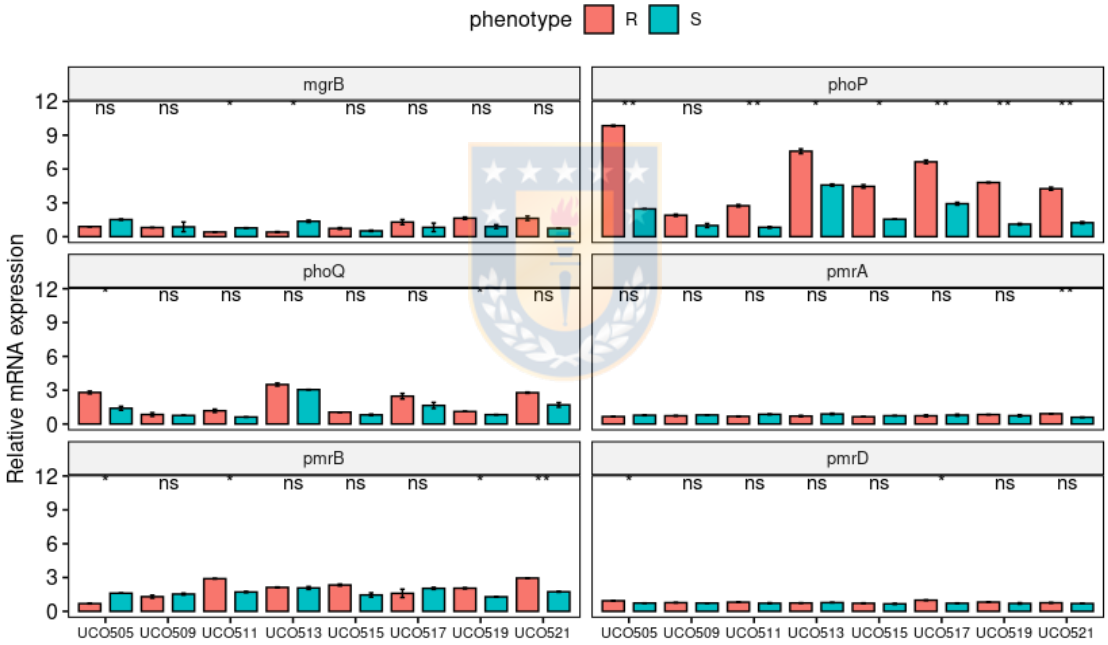

Figure 2. Relative expression (RT-qPCR) of the CST-resistance genes *mgrB*, *phoPQ,* and *pmrABD* in CST-susceptible (S) and CST-HR-derived (R) *K. pneumoniae* strains. (* $p < 0.05$; ** $p < 0.01$, ns: not significant).

4. Discussion

K. pneumoniae is an important human pathogen, which is classified as a common opportunistic hospital-associated bacteria involved in multiple infections, including urinary tract infections, cystitis, pneumonia, and surgical wound infections [33]. During the last decade, there has been a notable increase in the reports of highly-resistant *K. pneumoniae* isolates worldwide [34]. In our study, we included 60 *K. pneumoniae* MDR-strain that displayed resistance to multiple antibiotics. In highly-resistant *K. pneumoniae* isolates, one of the most important resistance mechanisms corresponds to the presence of ESBLs enzymes, which have a global prevalence of 20% to 70% [35]. Specifically, the CTX-M ESBL family is the most prevalent family globally, in which the variant CTX-M-2 group is the predominant ESBL in *K. pneumoniae* isolates from South America [32,36], which is concordant with our findings.

Even though the HR phenotype was reported 50 years ago, it has been extensively studied only during the last 5 years in Enterobacterales members [6]. In general terms, antibiotic-HR is a process in which subpopulations of isogenic bacteria display different ranges of susceptibilities to a certain drug [18]. Even though the mechanisms involved in HR are unclear, it has been proposed that this phenomenon could be promoted by the intra-treatment usage of antibiotics [14]. Even though antibiotic-HR affects clinical outcomes [14], its study is limited mainly because it cannot be easily explored in standard clinical laboratories [18]. Because the PAP test, considered to be the '*gold standard*', is time-consuming and costly, other methods have been proposed, such as combined microscopy and microfluidic methods, simplified PAP and differential antibiotic-susceptibility tests. However, Andersson *et al.* proposed a new technique combining susceptibility tests and different inoculum sizes,

which has been shown to improve the detection of HR bacteria [6]. Due to the above, few epidemiological data exist on HR prevalence [14]. Thus, our study provides more information on this phenomenon in clinically relevant pathogens. Specifically, our study is the first CST-HR *K. pneumoniae* description in South America. Although the global frequency of this phenotype is still unknown, our study identified 13% CST-HR in a collection of 60 non-repetitive *K. pneumoniae* samples. Previously, Cheong et al. investigated the presence of the CST-HR phenotype in South Korea in a collection of 231 CST-susceptible isolates recovered from bloodstream infections and found a frequency of 1.3% [18], which is significantly lower compared to the 72% frequency reported by Meletis G et al. [17]. Nicoloff et al. identified 27% of clinical isolates that show a CST-HR phenotype in four different bacterial species, including *K. pneumoniae* [37]. These inconsistencies may be due to the difficulty in detecting CST-HR, partly because of the different detection methodologies employed [6]. In our study, we utilized the PAP technique, which is considered the *'gold standard'* method for identifying heteroresistant sub-populations, as other methods, such as E-tests, fail to detect CST-HR sub-populations because the proportion of HR cells is normally low [17,38]. Poudyal et al. reported a CST-HR proportion in *K. pneumoniae* between $6{,}03 \times 10^{-9}$ and $1{,}29 \times 10^{-5}$ [16], whereas an HR proportion between 10^{-3} and 10^{-6} was reported in carbapenem-resistant and CST-HR *K. pneumoniae* [17]. Our results are concordant with those previously reported since we determined a low CST-HR proportion varying between 10^{-5} and 10^{-7} cells. On the other hand, the observed HR phenotype is different to the persistence phenomenon, which mediates antibiotic tolerance at the associated cost of bacterial growth [14,39]. Remarkably, our results showed no differences in the growth rates between parental (CST-susceptible) and CST-resistant sub-populations, which suggests that the observed CST-HR phenotype does not correspond to CST-tolerance, as reported for CST-HR *Acinetobacter baumannii* [40]. Furthermore, our results confirm that the CST-HR phenotype is stable since we

observed an increase in CST-MIC values by at least 2-fold after more than five generations without antibiotic selective pressure [6,39]. As mentioned in the results section, we were able to generate CST-resistance isogenic strains on eight ESBL-producing *K. pneumoniae* isolates. To determine the genetic mechanisms involved in this process, we investigated the two-components systems of PmrAB and PhoPQ, in addition to the global regulator MgrB, which corresponds to the main chromosomal mechanisms involved in CST-resistance in this species [41]. Mutations in the *pmrB* gene have been widely described in *K. pneumoniae* isolates that are resistant or have reduced susceptibility to CST [42]. Pitt et al. determined that a punctual mutation in *pmrB* of an extensively drug-resistant (XDR) *K. pneumoniae* ST258 isolate can yield amino acid substitution in P95L, which is consequently related to a significant increase of the MICs to CST, demonstrating that a single mutation can confer resistance to this drug [43]. Moreover, Jayol et al. described a single W157P amino acid substitution in the *pmrB* of six CST-resistant isolates. The authors suggested that this substitution could have an impact on the dimerization process of PmrB, which might induce the constitutive activation of PmrA, thereby producing the overexpression of *pmrC*, and ultimately leading to CST-resistance [44]. The role of *pmrAB* mutations in the CST-HR phenotype has been demonstrated in *A. baumannii* and *Pseudomonas aeruginosa*, in which a single amino acid substitution in PmrB was associated with HR [45,46]. Remarkably, mutations in *pmrB* may arise after low-dosage CST exposure, which could produce a stable CST-resistant phenotype without a fitness cost [47]. Our results showed that the UCO519 isolate (ST25) possessed a P95L amino acid substitution in PmrB, whereas an A256G amino acid substitution in UCO511 and UCO521 (both ST11) was identified without an evident fitness cost. Cheng et al. demonstrated that the same substitution was not sufficient to alter the MICs to CST; secondary factors contributing to CST-resistance are likely necessary [47].

PhoP is a member of the PhoPQ two-component system involved in CST-resistance since it activates the *pmrHFIJKLM* operon, thereby conferring resistance to this drug [31]. Our findings revealed an amino acid substitution in position 104 in PhoP, which was detected in the CST-HR isolates belonging to ST25. To the authors' best knowledge, this mutation has not been previously described in CST-resistant *K. pneumoniae* isolates. Thus, further studies are required to determine its role in CST resistance. For the HR isolate that belongs to ST1161 (UCO517), we identified an A351D amino acid substitution in PhoQ. Jayol et al. reported a CST-HR mechanism in *K. pneumoniae* that involves a single substitution in PhoQ (A191Y), which generates a structural modification in this protein, thereby producing the CST-HR phenotype [48]. Furthermore, Halaby et al. identified an A21S substitution in PhoQ in a CST-HR and ESBL-producing *K. pneumoniae* isolate, which produced an 8-fold increase of CST-MIC from 2 to 16 µg/mL, demonstrating that this amino acid substitution plays an important role in the CST-HR phenotype [15]. Moreover, Kim et al. described multiple amino acid substitutions in PhoPQ, PmrB, and MgrB that emerged after exposure to CST, such as during the development of CST-HR [49]. In addition to the previously described mechanisms, alterations in the MgrB global regulator have been identified as the main chromosomal mechanism of CST-resistance in *K. pneumoniae* [49,50]. Specifically, the *mgrB* gene encodes a negative-feedback regulator of the PhoPQ two-component system, whose inactivation is associated with the up-regulation of the PhoPQ system, resulting in modifications of the LPS and, consequently, resistance to CST [41]. In this respect, it has been observed that *mgrB* inactivation by IS-like elements reduces *mgrB* mRNA levels, which is associated with CST-resistance [49]. Our results showed that three CST-HR isolates possessed IS5-like (UCO505) and IS1-like (UCO511 and UCO513) transposons—insertion sequences that were previously described in CST-resistant isolates [49]. These results are congruent with the *mgrB* expressions levels determined by RT-qPCR.

5. Conclusions

CST-HR represents a threat to treating serious infections caused by relevant nosocomial pathogens, since CST is a *'last-resort'* alternative to control highly resistant infections. This is the first report on eight CST-HR ESBL-producing *K. pneumoniae* isolates in South America. Although the proportion of CST-HR detected in our study was low, it remains of special concern since CST-HR was not previously identified in a clinical setting in Chile. Here, the resulting CST-resistant isolates were stable, displaying high levels of resistance to CST. The genetic mechanisms involved in this resistance included mutations in PhoPQ and PmrAB, some of which had not been previously identified. The disruption of MgrB was identified, while plasmid-contained *mcr*-like genes were not found. Since this phenotype has been poorly researched, further studies are necessary to understand its mechanisms and epidemiology, since the rise of CST resistance will leave limited therapeutic options to treat this important pathogen.

Author Contributions: Methodology, F.M-L, A.O-C, and CAL.; software, F.M-L and A.O-C; formal analysis, F.M-L, A.O-C, CAL, H.B-T, and G.G-R.; investigation, F.M-L and A.O-C.; resources, F.M-L and A.O-C.; data curation, F.M-L, A.O-C, and H.B-T; writing—original draft preparation, F.M-L and A.O-C.; writing—review and editing, A.O-C, CAL, H.B-T, and G.G-R.; visualization, F.M-L.; supervision, A.O-C, H.B-T, and G.G-R.; project administration, F.M-L and G.G-R.; funding acquisition, F.M-L, G.G-R, and A.O-C. All authors read and agreed to the published version of the manuscript.

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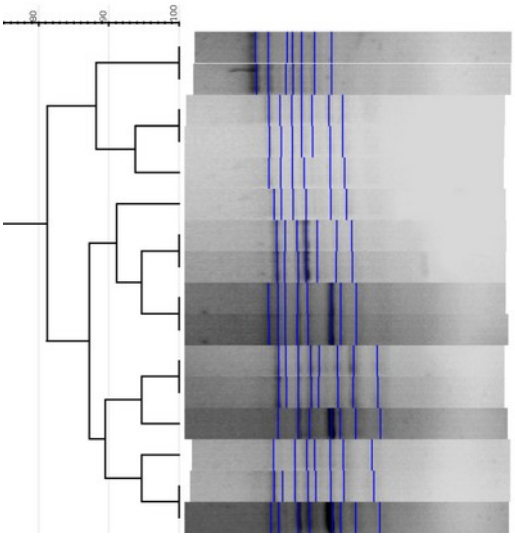
Conflicts of Interest: The authors declare no conflict of interest.

Appendix A



Strain Number	MLST	K-locus	Location	Sample	Phenotype
UCO521_R	11	15	Santiago	catheter	CST-HR
UCO521		15	Santiago	catheter	CST-S
UCO515_R	25	2	Santiago	orine	CST-HR
UCO515		2	Santiago	orine	CST-S
UCO513_R	25	2	Valparaiso	blood	CST-HR
UCO513		2	Valparaiso	blood	CST-S
UCO511_R	11	81	Concepcion	Orine	CST-HR
UCO511		81	Concepcion	orine	CST-S
UCO517_R	1161	19	Pto. Montt	Orine	CST-HR
UCO517		19	Pto Montt	orine	CST-S
UCO509_R	25	2	Los Angeles	blood	CST-HR
UCO509		2	Los Angeles	blood	CST-S
UCO505_R	1161	19	Pto. Montt	Bronquial	CST-HR
UCO519_R	25	2	Santiago	blood	CST-HR
UCO519		2	Santiago	blood	CST-S
UCO505		19	Pto. Montt	bronquial	CST-S

Figure 1- Appendix A. Dendrogram for ERIC-PCR between susceptible and heteroresistant subpopulations. CST-S: colistín susceptible; CST-HR: colistín heteroresistant. A Dice's index of > 90% similarity was considered indistinguishable (dotted line).

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CAPÍTULO VI: Hypervirulent and Hypermucoviscous Extended-spectrum β-lactamase-Producing *Klebsiella pneumoniae* and *Klebsiella variicola* in Chile

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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 *variicola* recovered in Chile from health-care 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 analysed 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 blaCTX-M-1, blaDHA-1 and blaLEN-25 among others clinically relevant resistance determinants, including mutations in a two-component regulatory system related to colistin resistance. These genetic features confer a

multidrug-resistant (MDR) phenotype in both strains. Moreover, virulome 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 to 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 deserve special attention and continuous surveillance in South America.

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. quasivariicola*, 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 determined 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 detected and characterise hypervirulent and hypermucoviscous ESBL-producing *K. pneumoniae* and *K. variicola* isolates recovered from Chilean hospitals.

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, 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 37ºC 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 × 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, PlasmidFinder 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://mlstkv.insp.mx, respectively. Capsular serotypes (K-locus) and phylogenetic analysis of the *ybt* locus were predicted by Kleborate (https://github.com/katholt/Kleborate). Mutations in chromosomal genes *mgrB*, *phoPQ* and *pmrAB* were analyzed with local BLAST+ DB using *K. pneumoniae* MGH78578 or *Klebsiella variicola* DSM 15968 (accession number NC_009648.1 and NZ_CP010523.2) genomes as colistin-susceptible references. In order to predict the functional effect of amino acid substitutions, we used the PROVEAN web server (http://provean.jcvi.org/index.php).

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 were 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 VSSY00000000.1 and VSSZ00000000.1, respectively.

Serum bactericidal assay and virulence behaviour 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 $5x10^6$ 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 56º 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 *Galleria mellonella* infection model was utilised [19]. *K. quasipneumoniae* subsp. *similipneumoniae* ATCC700603 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 37ºC for 18h with agitation. The estimation of capsular size was carried out by transmission electron microscopy (TEM) of a bacterial inoculum incubated at 37ºC for 24h [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 37º 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 37º C for 24 h. Subsequently, the medium was removed from the plates and each wells 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 65º C. Afterwards, 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 plates and color intensity was determined at a 550 nm using a spectrophotometer. 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 - 0.168 (2x - 4x blank absorbance) were considered as low biofilm-forming strains; ii) values ranging between 0.168 - 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*$_{SHV-187}$ and *bla*$_{DHA-1}$ in *K. pneumoniae* UCO-494 and blaSHV-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 harboured the *aac(6')-Ib*; *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 (colistinMIC 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*, *oqxA*, *oqxB*, *qnrB19* and *qnrB4* genes and *gyrA* (83L, 87Y) and *parC* (80I) mutations. Moreover, *K. pneumoniae* UCO-494 strain harbored the ColRNAI, IncA/C2, IncFIB and IncFII 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, were identified the presence

of resistance genes to glyphosate (phnMLKJI) and quaternary ammonium compounds (emrD – qacEΔ1) 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 leuS10; pgi 9; pgk 6; 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 (mrkABCDFHIJ), 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 and 48 h, respectively (Figure 1). Finally, hmKp UCO-494 displayed a low biofilm-forming ability, since it showed an OD550 nm 0.130 ± 0.003, whereas hmKv UCO-495 was classified as medium biofilm-producer, since it displayed an OD550 nm value of 0.246 ± 0.021 [23].

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 et al describe a fatal MDR-hvKv outbreak in neonates in Bangladesh. The isolates contained the a $bla_{\text{CTM-M-15}}$ and $bla_{\text{NDM-1}}$ 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 hvKv isolated from blood from a patient with cholangitis in China, which was resistant to colistin (MIC = 8 µg/mL) [28]. These are concordant with our study, since we

identified a 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 hmKp strains recovered from blood samples in China [28]. Similar to our findings, these isolates were colistin-resistant and carbapenems-susceptible. Moreover, Huang et al characterized diverse colistin-resistant hmKp 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 gen [33], which was related with colistin-resistance *K. pneumoniae* [34]. Furthermore, it is important to highlight that the mechanism of colistin-resistance in hvKv UCO-495 was mediated by chromosomal mutations in the two-components 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 blaLEN-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*$_{SHV-12}$, 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*$_{SHV-12}$ [36,37].

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 [40] in hvKp and hvKv strain [1,41]. 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 [42].

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) [39]. These findings suggest that this serotype could be endemic to South America, where could being 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 hmKv UCO-495, it was designated as ST*173*, 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 hmKp 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/Kv) 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 Enterobacterales [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 mrkABCDFHIJ [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 hmKp 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 fimbriae, 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 reaffirms 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/hypervirulent phenotype of *K. pneumoniae*-complex isolates is the results 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.

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Table 1.- Strain characteristic, MLST, capsular locus type, resistome and virulome of UCO-494 and UCO-495

Phenotype	UCO-494 hmKp	UCO-495 hmKv
Origin	Blood	Catheter
year	2012	2012
String test	+	+
ST	1161	173
*K-locus**	KL19	KL25
ybt	ybt14 ICEKp5	-
ybST	327-1LV	-
O-locus	O1v2	-
ESBL combined disc test	+	+
WGS data		
Contig number	462	226
Genome size (bp)	6400426	5982509
GC%	56,4%	56,1%
CDS; pseudogenes; tRNA	6668;203;95	5968;169;79
Resistance profile	ERT, CIP, LEV AMK, KAN, GEN, TOB, AMP, CTX, CAZ, AMC	TE, STX, W, CPD, CRO, AMK, KAN, GEM, TOB, AMP, CTX, CAZ, FEP, AMC
MIC colistin	8 µg/mL	16 µg/mL
Resistome		
Antibiotic resistance genes	*sul1; sul2; arr-2; dfrA12; aadA1; aadA2; aac(6`)-Ib; aac(6`)-Ib-cr; oqxA/B; qnrB19; qnrB4; $bla_{CTX-M-1}$; bla_{DHA-1}; bla_{OXA-10}; bla_{OXA-9}; $bla_{SHV-187}$; gyrA83L; gyrA87Y; parC80I*	*bla_{LEN-25}; bla_{SHV-12}; bla_{TEM-1B}; oqxA; oqxB; aph(3")-Ia; aph(6)-Id; aph(3")-Ib ; tet(D)*
colistin mutation gen	*PmrB: Gly256Arg (G766C)*	*PmrA: Thr146Ala (A436G); PmrB: Ser170Ala (G508T); PhoQ: Asp152Glu (T456G)*
Heavy-metal resistance genes	arsenic *(arsCDBAH);* magnesium / cobalt / nickel / manganese *(corA);* glyphosate (*phnMLKJI*); quaternary ammonium (*emrD – qacEΔ1*)	arsenic (*arsBCRD*), cobalt/manganese (*corC*), cobalt/magnesium (*mgtA*), magnesium/cobalt/nickel/manganese (*corA*): tellurium resistance gen (t*erW* and *terZCD)*
Virulome		
Virulence genes	Enterobactin (*entB; entF; ycfH; entD), urea(ureA), alantoin (allS), aerobactin (iutA), fimbria type 1 (fimABCDFEGH), fimbria type 3 (mrkABCDF), yersiniabactin (irp1; irp2; fyuA; ybtAES), colicin V (cvpA; cvaA), biofilm (treC; sugE), ECP (ecpABCDE)*	*Urea (ureA), alantoin (allS), aerobactin (iutA), fimbria type 1 (fimABCDFEGH), fimbria type 3 (mrkABCDF), colicin V (cvpA; cvaA), biofilm (treC; sugE), ECP (ecpABCDE), KFU (kfuABC)*
Plasmids	ColRNAI; IncA/C2; IncFIB (3); IncFII	IncFIB; IncFII; IncHI2; IncHI2A

*hmKP: hypermucoviscous Klebsiella pneumoniae, hvKP: hypervirulent Klebsiella pneumoniae; *Capsular polisacharide concentration in OD_{650nm} 2.0. Significative difference with p-value equal to 0.0001 in ttest. ERT: ertapenem, CIP: ciprofloxacin, LEV: levofloxacin; AMK: amikacin; KAN: kanamycin; GEN:gentamicin TOB: tobramycin; AMP: ampicillin: CTX: cefotaxime: CAZ:ceftazidime: AMC: amoxicillin-clavulanic acid; W: trimethoprim; CPD: cefpodoxime; CRO:ceftriaxone*

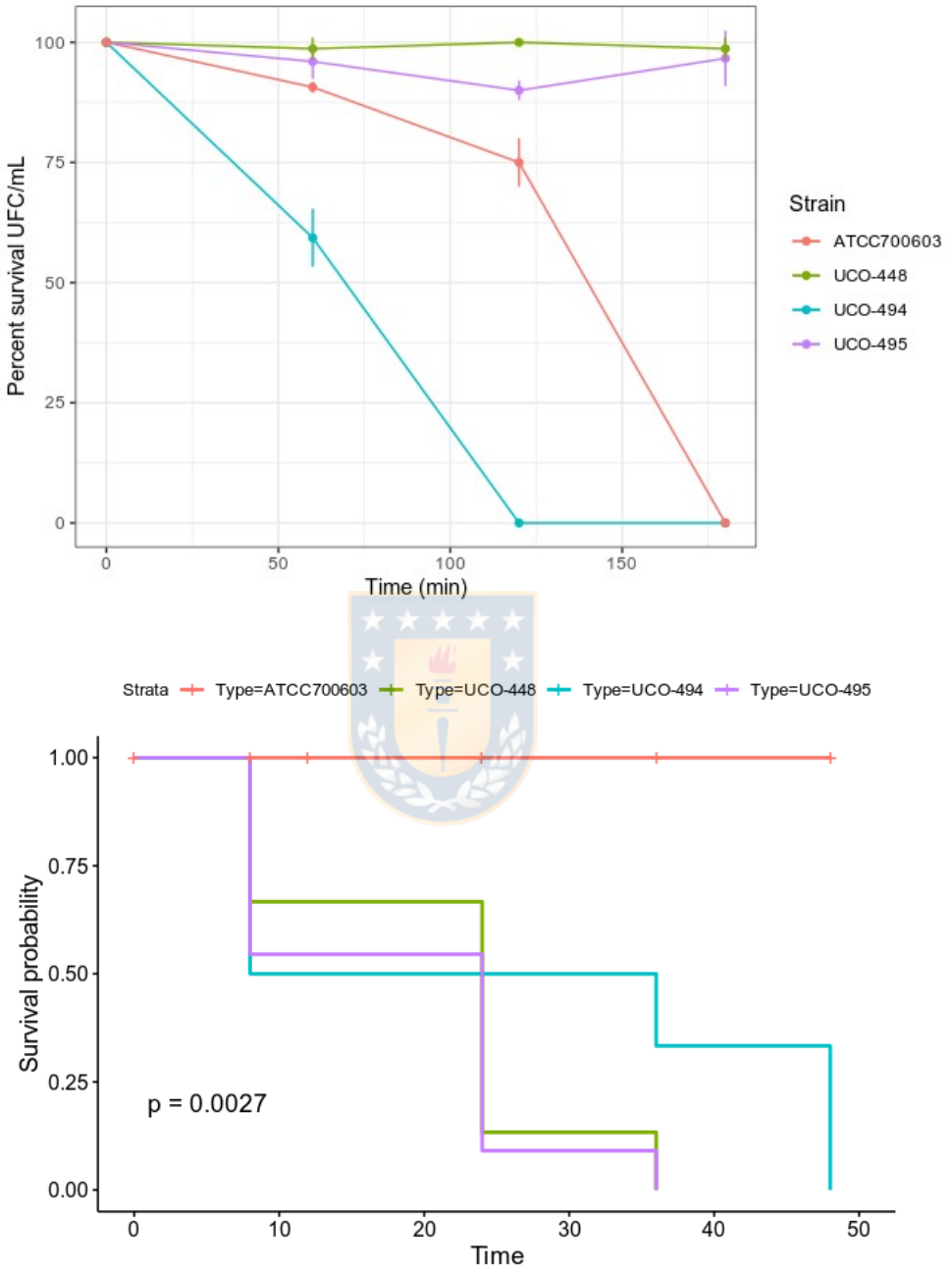

Figure 1.- a) Serum bactericidal activity. *K. quasipneumoniae* Subsp. *similipneumoniae* ATCC 700603 as negative control; *K. pneumoniae* hypervirulent UC-448 as positive control. b) *K. pneumoniae* UCO-494 and *K. variicola* UCO-495; Kaplan-Meier killing curves of *G. mellonella* larvae; ATCC 700603 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.

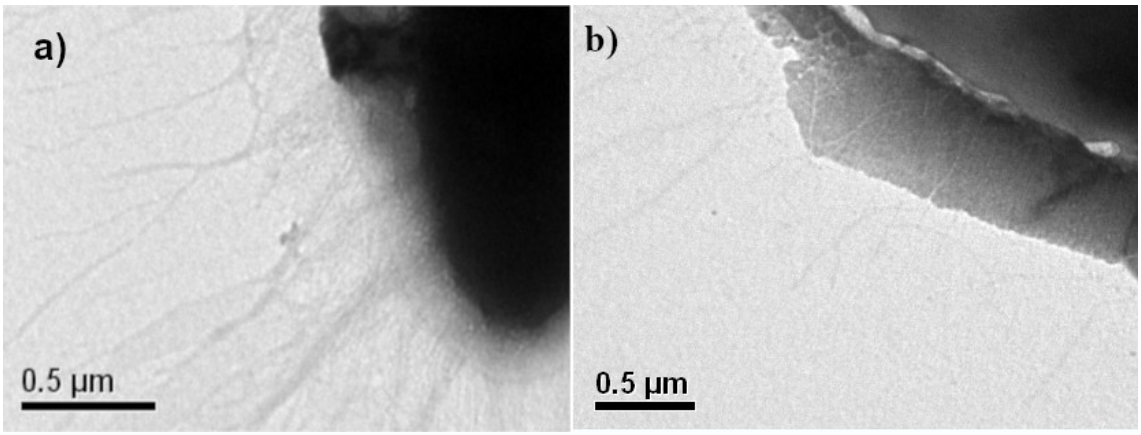

Figure 2.- Representative transmission electronic microscopy images of exopolysaccharide capsular *UCO-494_a (K. pneumoniae UCO-494); UCO-495_a (K. variicola UCO-495) and ATCC700603_a (K. quasipneumoniae negative control) without washes; UCO494_b (K. pneumoniae UCO-494); UCO-495_b (K. variicola UCO-495) and ATCC700603_b (negative control) after washes. We estimated of capsular size in 0.124 ± 0.017; 0.097 ± 0.019 µm and 0.091 ± 0.012 µm for UCO-494_b; UCO-495_b and ATCC700603_b respectively.*

CAPÍTULO VII: Capsular polysaccharide as alternative colistin resistance mechanisms in colistin-heteroresistance ESBL-*K.pneumoniae*

Manuscrito para enviar a Frontier in Microbiology

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Abstract

Multi-drug resistance *Klebsiella pneumoniae,* especially ESBL-producing and carbapenem resistance has emerged as an important opportunistic nosocomial pathogen, considered as a critical in worldwide. In this sense, colistin has emerged like the last-resort antibiotic for treating infections caused by this pathogen but, unfortunately, colistin-heteroresistant ESBL-*Klebsiella pneumoniae* have been recently identified. About mechanisms of CST-HR are not fully understood, but interestingly, the CST-HR phenotype may be associated with other mechanisms such as capsule polysaccharide. For that, in this work, we study alternative mechanism of colistin-resistance related with capsule amount in a recently described ESBL-producing CST-HR *K. pneumoniae*. For this, we study 8 CST-HR previously described. We determined and capsular-polysaccharide (CPS) quantification by phenol-sulfuric acid method and estimation of capsular size by optical microscopy with negative stain. The Zeta potential measurements were determined by the electrophoretic mobility. Whole-genome sequencing (WGS) was performed by the Illumina MiSeq platform and libraries prepared by the NexteraXT kit (Illumina). We studied mutations in *wzi*, *wzc*, *rcsABC* and *lon* genes and finally, we study the amino acid substitution in *CrrAB* gen like colistin-resistance mechanism. Its sequence was analyzed with the local BLAST. From this, we determined that Colistin-heteroresistance *K. pneumoniae* have a less electronegative surface charge and probably its unrelated to the amount of polysaccharides in the capsule, and RcsA and RcsC could be related with capsular polysaccharide amount and colistin-

heteroresistance but CrrB but not MgrB alteration could be related with colistin-heteroresistance and high amount of capsule polysaccharide. In conclusion, in this work we were able to determine that colistin heteroresistant strains produce a greater amount of capsular polysaccharide compared to susceptible ones. However, it was not possible to show that this phenomenon is related to the existence of the heteroresistant phenotype.

Keywords: colistin, heteroresistance, *klebsiella pneumoniae*, capsule

Introduction

Klebsiella pneumoniae has emerged as an important opportunistic and the most common nosocomial pathogen, (Wyres et al., 2020) related with a broad spectra of diseases, as a urinary tract infections, bloodstream, lung, abdominal infections among other. (Navon-Venezia et al., 2017; Paczosa & Mecsas, 2016) In the last year, multi-drug resistance *Klebsiella pneumoniae*, especially ESBL-producing and carbapenem resistance, was considered as a critical pathogen in worldwide due by a worrying increase in prevalence of infections caused and the limited therapeutic options available. (Shiri Navon-Venezia, Kira Kondratyeva, 2017) In this sense, colistin has emerged like the last-resort antibiotic for treating infections caused by multi-drug resistance *Klebsiella pneumoniae (Moubareck, 2020)*.

Colistin and polymyxin B, are cationic polypeptide antibiotic, similar to that of cationic antimicrobial peptides (CAMPs) (Poirel et al., 2017). Polymyxin bind to LPS due by electrostatic interaction between diaminobutyric acid (Dab) residue in policationic antibiotic and negative charge of LPS (Moubareck, 2020; Poirel et al., 2017). This interaction produces competitively displace divalent cations (Ca^{2+}/Mg^{2+}) from the phosphate groups of membrane lipids, which leads to the destabilization of the outer cell membrane causing a cell lysis and fast-bactericidal act. (Moubareck AC., 2020) Unfortunately, colistin-heteroresistant (CST-HR) ESBL-*Klebsiella pneumoniae* have been recently identified (Morales-León et al., 2020). About mechanisms of CST-HR are not fully understood, but there are related with traditional colistin chromosomal mechanism like mutation in the *mgrB* or in two-component systems, such as

p*hoPQ*, *pmrABC* and, recently *crrAB. (Jayol et al., 2015; Pragasam et al., 2017)* Interestingly, the CST-HR phenotype may be associated with other mechanisms such as increased expression of RND-type efflux pumps or capsule polysaccharide (El-Sayed Ahmed et al., 2020; Morales-León et al., 2020).

The bacterial capsule is a protective, negative charged layer external to outer membrane, comprised primarily of long chained repeating polysaccharides. (Band & Weiss, 2014; Wen & Zhang, 2014) *K. pneumoniae* it is a Gram-negative and encapsulated bacteria (Paczosa & Mecsas, 2016) were the capsule provides increased resistance against CAMPs and polymyxin. (Band & Weiss, 2014) Some author, determined the direct correlation between higher amounts of capsular polysaccharides and increased resistance to polymyxin (Campos et al., 2004). Furthermore, studies demonstrated that the exposure of *K. pneumoniae* to peptide antibiotic like polymyxin B, causes the release of polysaccharides from bacterial surface, probably due by a direct damage or a bacterial response to stress. This phenomenon protects bacterial by sequestering antibiotic and could be related with resistance to antibiotic. (Llobet et al., 2008)

For the other hand, the capsule regulator mechanism of capsule expression is a complex regulated genetic network. The K-antigen biosynthesis enzymes are encoded on the capsule polysaccharides synthesis locus (cps) and his biosynthesis pathway is a Wzx/Wzy-dependent polymerization pathway (Patro et al., 2020)and capsule polysaccharide amount is regulated by multiple way. Recently, Ernst et al, studied the mutation in cps locus genes and demonstrated thaw *wcz* mutation its related with increase in cps amount while the mutation in *wbaP* its related with acapsular *K.pneumoniae* phenotype (Ernst et al., 2020). For the other hand, some author related the traditional colistin-resistance mechanisms with capsule modification, like possible colistin resistance contribution (Formosa et al., 2015).For that, in

this work, we study alternative mechanism of colistin-resistance related with capsule amount in a recently described ESBL-producing CST-HR *K. pneumoniae*.

Materials and methods

Strain and capsular-polysaccharide (CPS) quantification and estimation of capsular size

We study eight colistin-heteroresistance ESBL-*Klebsiella pneumoniae*, that were previously characterized. (Morales-León et al., 2020) Total capsular-polysaccharide (CPS) was estimated according to the phenol-sulfuric acid method, after extraction using zwittergent 3-14, (H. Y. Cheng et al., 2010) and incubated in tryptone soy broth (TSB) at 37ºC for 18 h with agitation. The estimation of capsular size was carried out by optical microscopy with negative stain (nigrosin 1%) of a bacterial inoculum incubated at 37ºC for 24 h. (Breakwell et al., 2009)

Z potential measurements

Zeta potential measurements were performed as previously described with minor modifications. (Velkov et al., 2014) Briefly, the bacterial surface was cleansed by washing twice with Milli-Q water by centrifugation (5000rpm / 5°C), and finally resuspended in Milli-Q water at 10^8cfu/ml (0,5 McFarland) and then, diluted 1:4 in Mili-Q water. A capillary zeta cells

was filled with 1000 µL of sample. (ATAScientific, Taren Point, NSW, Australia). The electrophoretic mobility (EPM) of bacterial cells was measured at 25°C with a zeta potential analyzer at 150 V (Zetasizer Nano ZS; Malvern Instruments, Malvern,UK) before being converted to zeta potentials using the Helmholtz–Smoluchowski theory. EPM measurements were performed in six-fold on separately prepared samples. Statistical analyses were conducted using the Student' t-test using Rstudio Software.

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

Total DNA of 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 × 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. All genome have been deposited at DDBJ/ENA/GenBank under the accession numbers (table 1)

Later, genome annotation was accomplished using the rapid prokaryotic genome annotation (Prokka) (Seemann, 2014) and them, the assembled genomes were used to analyzed the *cps* locus to finder mutations in his sequences. We studied mutations in *wzi*, *wzc*, *rcsABC* and *lon* genes. Finally, we study the amino acid substitution in *CrrAB* gen like colistin-resistance mechanism. Its sequence was analyzed with the local BLAST+ DB using the UGENE software version 1.32.0.

In order to predict the functional effect of amino acid substitutions, we used the PROVEAN web server (http://provean.jcvi.org/index.php). Sequence types (STs) were determined for *K. pneumoniae* through the bioinformatic tools available at https://cge.cbs.dtu.dk/services/MLST/ and capsular serotypes (K-locus) was predicted by Kleborate (https://github.com/katholt/Kleborate).

Results

Previously, we identified a stable colistin-heteroresistant phenotype ESBL-*K.pneumoniae* strain, isolated from different source (Morales-León et al., 2020) (Table1). All colistin-heteroresistance phenotype, has a CST-MIC up to 8 µg/mL.

Table 1. Main Characteristic of colistin-heteroresistant ESBL-*K.pneumoniae*

Strain	ST	K-locus	CST-MIC (µg/mL)	Polysaccharide amount (µg/mL)	β-lactamase (*bla*)	Genbank
UCO511	11	81	64	59,1 ± 36,0	CTX-M-2; OXA-2; SHV-182; TEM1	JAAIWP01
UCO521	11	15	64	109,9 ± 64,1	CTX-M-15; OXA-1; SHV-182	JABJWF01
UCO509	25	2	64	77,0 ± 39,4	SHV-110; SHV-81; TEM-1B	JAAIWO01
UCO513	25	2	32	85,2 ± 52,7	CTX-M-2; OXA-10; SHV-12; TEM1B	JAAIWQ01
UCO515	25	2	>64	71,4 ± 26,3	TX-M-15; OXA-1; OXA-10; SHV-110; TEM-1B	JAAIWR01
UCO519	25	2	32	73,7 ± 19,6	CTX-M-15; OXA-1; OXA-10; SHV-110; SHV-81, TEM-1B	JABJUO01
UCO505	1161	19	64	181,1 ± 50,7	CTX-M-2; OXA-2; TEM-1B	JAAFZD01
UCO517	1161	19	8	99,2 ± 29,2	OXA-10; OXA-9; SHV-187; TEM-1A	JABJUP01

The MLST analysis showed that the eight strains belonged to three different lineages (ST11, ST25 and ST1161), and four different K-locus (2, 15, 19 and 81). Four strains were related with the most prevalent capsular serotype K-locus 2 (KL-2) from the same ST25, and the ST1161, wish is endemic from Chile, has the same K-locus 19. In the ST11, which was the most widespread MDR lineage worldwide, has a two different K-locus (KL15 and KL81).

In the antibiotic-resistance context, all strains has a multi-drug resistance pattern, were, β-lactamase are the most important mechanism. To respect, the $bla_{CTX\text{-}M\ 2}$ and $bla_{CTX\text{-}M\text{-}15}$ was the most frequent *bla* gen, beside bla_{SHV} and $bla_{OXA.}$ All strain was colistin-resistance, with CST-MIC between 8 and > 64 µg/mL. The resistance mechanism related was described previously. (Morales-León et al., 2020).

Colistin-heteroresistance *K. pneumoniae* have a less electronegative surface charge and probably it is unrelated to the amount of polysaccharides in the capsule.

We quantified the total capsule in susceptible and colistin-heteroresistance strain, in order to established his relation. From this, we identified a poor relation between total polysaccharide amount and colistin susceptibility patterns. Nevertheless, the colistin-heteroresistance strains had a higher quantity of total polysaccharide compared with colistin-susceptible (Figure 1a and 1b), but this relation was not significant ($p = 0,549$).

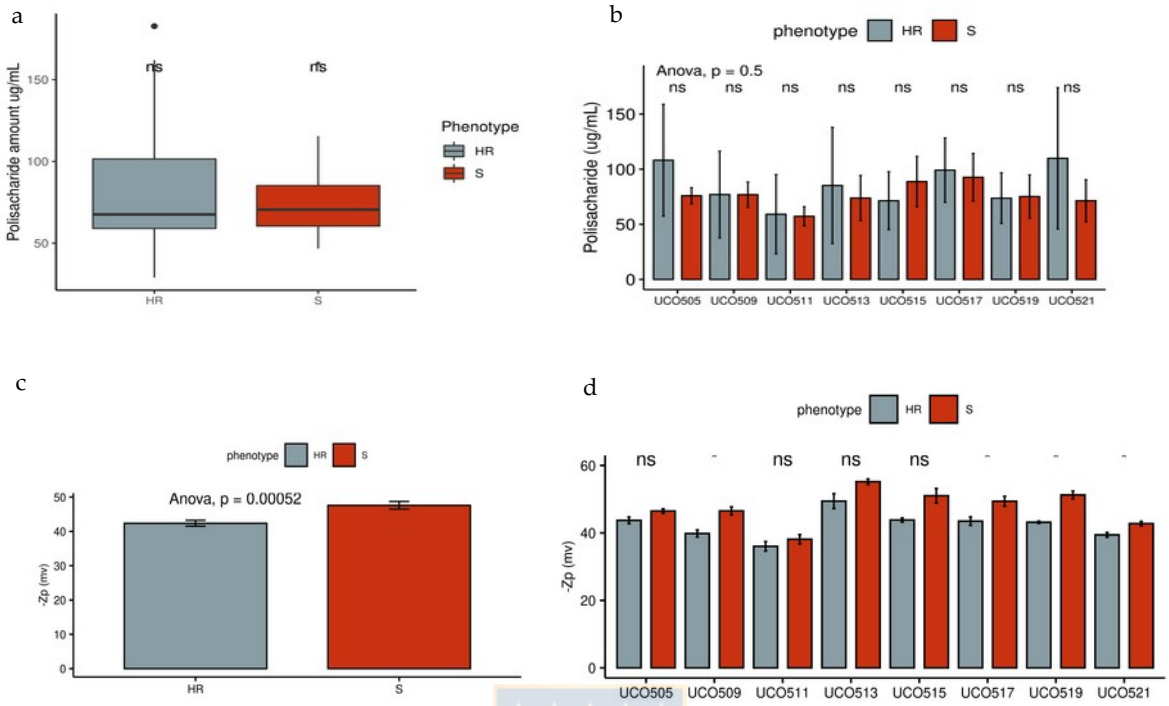


Figure 1. a) Total amount of capsular polysaccharide between susceptible (S) and heteroresistance (HR) strains. b) Total amount of capsular polysaccharide in susceptible (S) and heteroresistance (HR) strains compared with control ATCC700603.

KL: K-locus, *t-student was significative at p-value < 0.05. c) Z potential in -mV between colistin susceptible and heteroresistance strain d) Comparative Zp in all strain. ANOVA was significative at p-value < 0,05

We determined the membrane potentials (Zp) in susceptible and heterorresistente strain. In figure 1c and 1d, we can observe that colistin-heteroresistance had a - 42,37 ± 3,98 mV compare with - 47,60 ± 5,37 mV in susceptible strain ($p < 0,05$). This effect was statistically significance only in UCO509 (- 46.5 ± 2.1 mV versus -39.8 ± 1.8), UCO517 (- 49.4 ± 2.5 versus - 43.5 ± 2.2), UCO519 (-51.3 ± 2.0 versus -43.2 ± 2.0) and UCO521 (- 42.8 ± 1.2 versus - 39.4 ± 1.2 mV), all strain had different KL, and probably, there are no relation between K-locus type and Zp membrane. In the figure 2, we observed the relation between Zp and polysaccharide amount. From this, we could be established a relationship between polysaccharide capsule amount and membrane potential, only in susceptible strains. This could be meaning that a higher polysaccharide capsule amount, higher electronegative

membrane potential. This observation was not clear in colistin-heteroresistance strain, probably due to influence exerted by the LipA modifications and cationic charge in colitis-resistance strain. For this, we could not determine the molecular modifications in colistin-heteroresistance LipA and we were unable to determine the membrane potential in the acapsular strain due to the impossibility of obtaining strains with this phenotype.

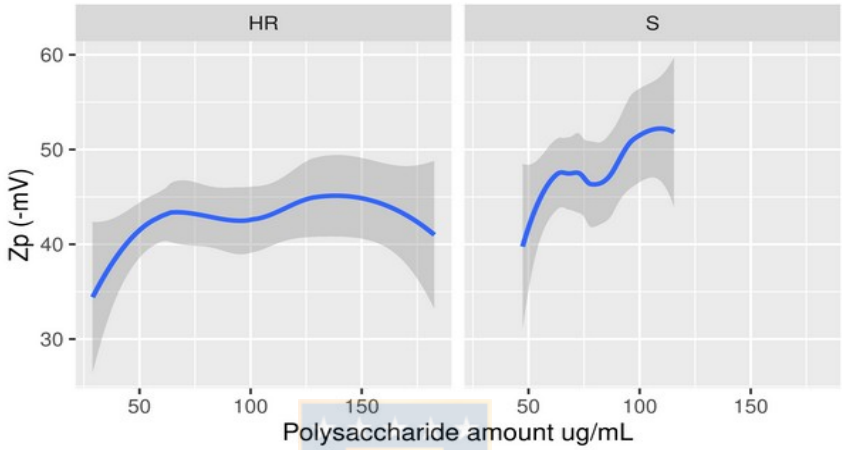


Figure 2.- Relation between capsular polysaccharide amount (µg/mL) and membrane potentials (Zp in -mV) in colistin-susceptible and colistin-heteroresistance *K.pneumoniae* strains.

RcsA and RcsC could be related with capsular polysaccharide amount and colistin-heteroresistance

We identified three different amino acid substitution in Lon protease (Q142E;E259A;C517R) in five different strain (UCO509; UCO513; UCO515; UCO519 and UCO521). Of this, there were two common Lon amino acid substitution, E259A and C517R in KL2 strain. All these substitutions were classified as neutral with provean (-1.464 and 1,325 provean score respectively). In case of UCO521, they have another silent Q142E (-0,924

provean score) Lon amino acid substitution. For the other hand, in RcsA, a sensor kinase of two-component system, only UCO521 had a two amino acid substitution (T174I; P186S) which deleterious effect (-6 and -8 provean score respectively). Finally, we identified one deleterious (-3,589 provean score) amino acid substitution (N79T) in RcsC, another sensor kinase, in four KL2 strains (UCO509, UCO513; UCO515 and UCO519). No other amino acid substitutions were found in RcsB, *wzi,* and *wzc*. For the other hand, not significant differences were found between amino acid substitution and polysaccharide capsule amount in Lon (76,0 ± 25,9 µg/mL in HTR strain versus 83,3 ± 39,8 µg/mL in ΔLon-HTR strain; *p=0,316*), RcsA (76,0 ± 25,9 µg/mL in HTR strain versus 109,6 ± 64,0 µg/mL in ΔRcsA-HTR strain; *p=0,101*) and RcsC (76,0 ± 25,9 µg/mL in HTR strain versus 76,7 ± 32,2 µg/mL in ΔRcsC-HTR strain; *p=0,480*). All of this data are shown in Table 2.

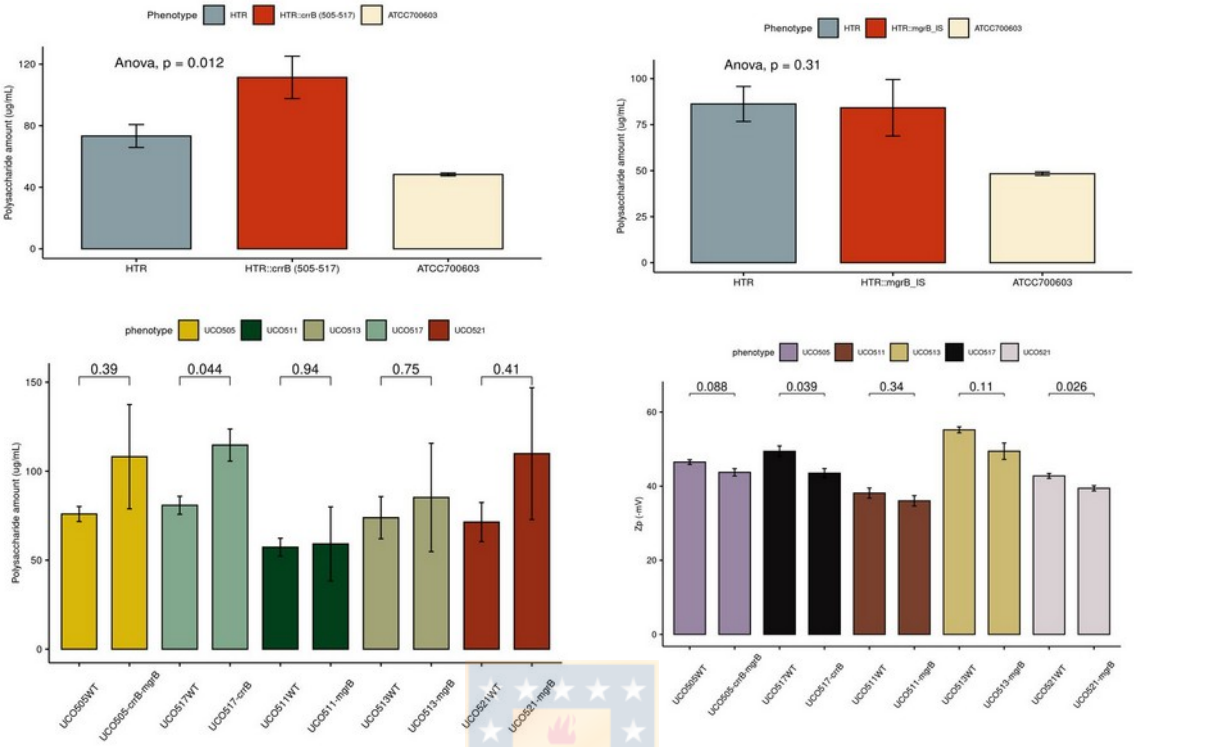
Table 2 .- amino acid substitution in Lon, RcsAC, CrrB and MgrB, serotype and colistin MIC in colistin-heteroresistance in ESBL-*K. pneumoniae*

Heteroresistance strain main characteristic			amino acid substitution				
Strain	**KL**	**CST MIC µg/mL**	**Lon**	**RcsA**	**RcsC**	**MgrB**	**CrrB**
UCO505	19	64				IS*5*-like	I66V Q239H T276A Q287K
UCO509	2	64	E259A C517R		N79T		
UCO511	81	64				IS*1*-like	
UCO513	2	32	E259A C517R		N79T	IS*1*-like	
UCO515	2	>64	E259A C517R		N79T		
UCO517	19	8					I66V Q239H T276A Q287K
UCO519	2	32	E259A C517R		N79T		
UCO521	15	64	Q142E C517R	T174I P186S		CysX	

KL: K-locus identified by Kaptive Web tools; ST: sequence type; CST MIC: colistin minimum inhibitory concentration; Not amino acid substitution were identified in *wzi, wzc* and RcsB. *rmpA/A2* gen was not identified. Lon reference *cp052569;* RcsA reference *cp054780* and RcsC references *cp525691*

CrrB but not MgrB alteration could be related with colistin-heteroresistance and high amount of capsule polysaccharide

We study a possible relation between CrrB and MgrB amino acid substitution and capsule polysaccharide amount (Table 2). To respect, in figure 3a and 3b, we observed a relation with high amount of capsule in CrrB amino acid substitution strains compared with WT: *crrB* strains (103,7 ± 37,3 µg/mL versus 73,3 ± 30,7 µg/mL; *p=0.012*). We did not observe this effect in strain with modification in MgrB. For the other hand, in the individual analysis (figure 3c and 3d), we observed that in UCO517-CrrB, colistin-heteroresistance strain, had more capsule amount (114,7 ± 15,6 µg/mL versus 80,8 ± 8,8 µg/mL capsule; *p= 0,044*) and less Zp (-43,5 ± 2,2 mV versus -49,4 ± 2,5 mV; *p= 0,04*) compare with UCO517WT. These were not significant differences in other analyzed strains.

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crrB-ref-WP_004184324.1   MNKETILSRQILTYMLLLTFVIIAIAILGSYLFYSFLIYYLPGGMNEGSEDAMTFLDWMWILMASITSLVVALFFTVKLSARIFNPLNDVAYSLKQISQGNLSARAFNSSSKLGEMNRLV
UCO-505-crrB              MNKETILSRQILTYMLLLTFVIIAIAILGSYLFYSFLIYYLPGGMNEGSEDAMTFLDWMWILMASVTSLVVALFFTVKLSARIFNPLNDVAYSLKQISQGNLSARAFNSSSKLGEMNRLV
UCO517-crrB               MNKETILSRQILTYMLLLTFVIIAIAILGSYLFYSFLIYYLPGGMNEGSEDAMTFLDWMWILMASVTSLVVALFFTVKLSARIFNPLNDVAYSLKQISQGNLSARAFNSSSKLGEMNRLV
                          ************************************************************:*************************************************

 1     66

crrB-ref-WP_004184324.1   NDFNEMAEKLQTLDAQRNLWNAAIAHELRTPVTIILRGRLQGLVDGVFEPEPSLFRNLLKQTEGLTNLIEDLRVVSSSGGAGYSLMLSEVDLKATISNALDTFWPDFDKKHFKIVTEINQQ
UCO-505-crrB              NDFNEMAEKLQTLDAQRNLWNAAIAHELRTPVTIILRGRLQGLVDGVFEPEPSLFRNLLKQTEGLTNLIEDLRVVSSSGGAGYSLMLSEVDLKATISNALDTFWPDFDKKHFKIVTEINHQ
UCO517-crrB              NDFNEMAEKLQTLDAQRNLWNAAIAHELRTPVTIILRGRLQGLVDGVFEPEPSLFRNLLKQTEGLTNLIEDLRVVSSSGGAGYSLMLSEVDLKATISNALDTFWPDFDKKHFKIVTEINHQ
                          ************************************************************************************************************:*

       239
crrB-ref-WP_004184324.1   YCVCDPLRIIQCLTVLFDNALKYSTSQTLLIKNGITGNDNFIIVQDQGPGIPEELQKSLFQPFQRGEYAKNINPEGCGLGLSVVKAIMCAHGGDVSYSLTPENGSLFKLSWPV
UCO-505-crrB              YCVCDPLRIIQCLTVLFDNALKYSTSQTLLIKNGIAGNDNFIIVQDKGPGIPEELQKSLFQPFQRGEYAKNINPEGCGLGLSVVKAIMCAHGGDVSYSLTPENGSLFKLSWPV
UCO517-crrB               YCVCDPLRIIQCLTVLFDNALKYSTSQTLLIKNGIAGNDNFIIVQDKGPGIPEELQKSLFQPFQRGEYAKNINPEGCGLGLSVVKAIMCAHGGDVSYSLTPENGSLFKLSWPV
                          ***********************************:*********:************************************************************

                         276       287  353
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Figure 3.- Polysaccharide amount (μg/mL) and Zp (-mV) in colistin-heteroresistance *K. pneumoniae*-ESBL. Comparative in CrrB or MgrB mutation strain.

a) Global polysaccharide amount in CrrB mutation strain. **b)** Global polysaccharide amount in MgrB mutation strain. **c)** Polysaccharide amount in colistin-susceptible (WT) and colistin-heteroresistance strain with CrrB and MgrB mutation. **d)** Membrane potential (-mV) in colistin-susceptible (WT) and colistin-heteroresistance strain with CrrB and MgrB mutation. **e)** Multiple amino acid alignment of CrrB in UCO505 and UCO517. WP004184324 as reference sequence. The multiple sequence alignment were made by Clustal-Omega. UCO505 had a CrrB mutation and MgrB mutation due by IS1-like.

Discussion

Colistin is considered as the last-resort antibiotic for multi-drug resistance *Klebsiella pneumoniae* and other MDR-*Enterobactericeae* infections. (El-Sayed Ahmed et al., 2020) Colistin is bactericidal antibiotic with narrow spectrum, but the have a good pharmacokinetics characteristic and good global susceptibility pattern. (Hua et al., 2017; Karaiskos et al., 2015, Jones et al., 2013) However, the recent description of colistin-heteroresistance it will be a new challenge for medical therapy. (Andersson et al., 2019)

In the last year, multiple works reveal the different mechanisms related with colistin-resistance, (Trimble et al., 2016) where the most important of them is the LPS modifications due mutation or interruption of Two-component regulatory systems (Satola et al., 2011) PmrAB, CrrAB, PhoPQ or MgrB, the latter, a small transmembrane lipoprotein that could negative regulation of the PhoPQ. *(Mmatli et al., 2020)* However, in case of colistin-heteroresistance mechanisms, are not entirely clear. Some author related the same traditional mechanisms of colistin-resistances with heteroresistance phenotype, (El-Sayed Ahmed et al., 2020) but in some case, these mechanisms can only give partial response to the phenomenon.

In our case, previously we identified some of this traditional mechanisms in colistin-heteroresistance *K.pneumoniae*-ESBL, could be explained this phenotype. (Morales-León et al., 2020) For example, four colistin-heteroresistance strain presented mutation in *mgrB, d*ue by IS*1*-like and IS*5*-like element or partial sequence deletion. Both mutations, can lead to the

upregulation of the *phoPQ* operon, resulting in enhanced LPS modification. (Mmatli et al., 2020; Morales-León et al., 2020) To respect, the IS*5*-family is the most common mechanism of *mgrB* interruption, further, IS*1*-like, IS*3*-like, IS*Kpn14*, IS*903* among other, have been frequently described. (Berglund, 2019; Halaby et al., 2016) Interesting is Formosa et al. work, how study the nanoarchitecture of the capsule in colistin-resistance *K. pneumoniae*. In this work, the author established that the mutation of the *mgrB* gene in colistin-resistance strain, could affect directly to the organization of capsule, presenting a well-organized multilayered capsule that may interact with colistin and increase a MIC_{CST}. (Formosa et al., 2015; Mmatli et al., 2020) For this, we compared our colistin-heteroresistance strain that they had a mutation in *mgrB* (UCO505, UCO513, UCO511 and UCO521) with capsule amount. In global analysis we could identify a relative relation between high capsule amount and mutated *mgrB.* However, this may mean that mutation in *mgrB* are related to structural capsule organization and not in its expression or synthesis. To respect, we do not study structural aspects of the capsule. The mechanism related in this context, its not clear, but the evidence indicates that the premature polymixin B or colistin exposure of *K. pneumoniae,* induced an upregulation of *cps* operon, *pmrF*, *ugd* or *pagP* and therefore, a increase of capsule amount and colistin-resistance. This mechanism is probably due by a complex regulated mechanism of cps production.(Llobet et al., 2011) In our work, we did not study this aspect.

CrrAB is another recently Two-Component system related with colistin resistance in *K. pneumo*niae. (Y.-H. H. Cheng et al., 2018) Any mutation in *crrB* gen are responsible for the increase in *crrC* expression and this, could be regulated a *pmrC* gen, *pmrHFIJKLM* or *arnBCADTEF* operon inducing colistin-resistance. (Jayol et al., 2017; McConville et al., 2020)

To respect, McConville HT et al., study the effect of amino acid substitution in CrrB in *K. pneumoniae,* (McConville et al., 2020) demonstrated that a CrrB are a positive regulator lead

to the addition of 4-amino-4-deoxy-L-arabinose (L-Ara4N) and phosphoethanolamine (pEtN) to LipA inducing a higher polymixin MIC. Other author suggested amino acid substitutions of the CrrB protein result in increased autophosphorylation of this protein, consequently leading to colistin resistance. (Aghapour et al., 2019) For the other hand, the CrrB amino acid substitution cause a high virulence and fitness cost, probably due by activation of the pentose phosphate pathway. (McConville et al., 2020) For the other hand, Sun Lang et al., study a proteomic impact of *crrB* mutation in *K.pneumoniae*. The author, first showed that colistin impact in multiple biochemical pathways like gluconeogenesis, arginine biosynthesis, enterobactin biosynthesis, and, interesting, in the class A β-lactamase (like TEM and SHV) expression, among other. The same work, showed that *crrB* mutation impact in colistin resistance by activation of *arnBCADTEF* operon by PmrAB, PhoPQ and PagP expression. This activation is related with LipA addition to LPS and regulation of bacterial capsule expression probably due by *barA* and *rcsA* gen expression. (McConville et al., 2020) Finally, the CrrB amino acid substitution may induce a KexD (H239_3064) transcription, a multidrug efflux-pump that contributing tho colistin resistance. (Y. H. Cheng et al., 2018; Sun et al., 2020) In our case, we identified two strain with I66V, Q239H, T276A and Q287K amino acid substitution in CrrB, which are related to greater amount of capsule. For the other hand, previously, we show that all strain have the same growth curve which means that there is no effect on the bacterial fitness cost. (Morales-León et al., 2020) Respect to virulence profile, we don't study this aspect but recently, Ferreira Melina et al., described a first ESBL-producing colistin-resistant hypermucoviscous *K. quasipneumoniae*, with similar amino acid substitution in CrrB (Q239H and Q287K). Like us, the author did not do experiments to confirm the amino acid substitution effect. (Ferreira et al., 2019)

Rcs is a two-component signal transduction system, compound with 5 members (RcsA, RcsB, RcsC and RcsD) related with regulation of the transcription of biofilm, virulence genes and capsule polysaccharide in *Enterobactericeae. (Su et al., 2018; Yuan et al., 2020)*

About the Rcs and capsule polysaccharide expression, RcsC are membrane-bound protein who as server as sensor kinase catalyzing the phosphorylation of RcsD and RcsB. The RcsB phosphorilated act as transcription regulator for exopolysaccharide biosynthesis, flagellar mobility and autoregulation of Rcs. (Peng et al., 2018)

The Rcs system regulates the expression of cps genes in bacteria. For example, the high glucose concentration in the media growth increase the cps amount, via *galF* promoter and increase expression of RcsA. For other hand, the iron homeostasis could be influence on the *cps* gene expression and can also influence on capsule amount. (Walker & Miller, 2020) In this sense, the ∆*fur* strain produces more capsule probably by RcsA way. (Walker & Miller, 2020) Mutation in *rcsA, rcsC* or *lon,* affect the regulation of the capsule production by RcsB, induced increase in capsule amount (Ernst et al., 2020; Gottesman & Stout, 1991; Rozanov et al., 1998). Some work showed that exposure to polymyxin in *K. pneumoniae* could be related with up-regulation system and increase in capsule polysaccharide synthesis mediated by RcsB and PhoPQ system by cps operon activate. (Llobet et al., 2011; Mlynarcik & Kolar, 2019) We identified mutation in Lon protease and RcsA and RcsC but not in RcsB. In this sense, any strain was related with polysaccharide capsule amount.

Conclusion

In this work we were able to determine that colistin heteroresistant strains produce a greater amount of capsular polysaccharide compared to susceptible ones. However, it was not possible to show that this phenomenon is related to the existence of the heteroresistant phenotype. For its part, the expression of the capsular polysaccharide is a complex biochemical network highly regulated by multiple molecular pathways, which are difficult to relate to a single event. Despite the above, it is extremely important to continue with this work, to understand the final role that the polysaccharide capsule plays in the existence of the colistin heteroresistant phenotype.

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CAPÍTULO VIII: DISCUSIÓN GENERAL

Klebsiella pneumoniae complex ha emergido como un patógeno humano altamente relevante, debido a su capacidad para resistir a la acción de las principales familias de antibióticos, incluyendo cefalosporinas de tercera generación, carbapenémicos, aminoglicósidos, fluoroquinolonas, entre otros. (Janssen et al., 2021) Lamentablemente, la disponibilidad de nuevos antibióticos activos para el manejo de infecciones por especies de *K. pneumoniae* complex MDR o PDR, siguen siendo escasas, por lo cual colistín resulta en una opción terapéutica útil y efectiva.

Colistín es un antibiótico considerado de última línea para el manejo de infecciones provocadas por Enterobacterias MDR, ya que, entre otras características, es un antibiótico que mantiene un buen perfil de susceptibilidad. Así, por ejemplo, en el estudio SMART realizado en Taiwan el año 2016, se demostró que en general, *K. pneumoniae* aisladas desde unidades de pacientes críticos posee una buena susceptibilidad, con valores de MIC_{90} inferiores a 0,5 µg/mL. (Lai et al., 2019) Sin embargo, el mismo estudio SMART realizado dos años más tarde, demostró una disminución de la susceptibilidad a colistín con un importante aumento de cepas resistentes a carbapenémicos. Estos datos resultan similares a los publicados en un reporte realizado con cepas aisladas en India entre los años 2014 y 2016. En este reporte se destaca la alta prevalencia de infecciones causadas por cepas de *K. pneumonie* BLEE y portadoras de bla_{KPC}, que mantienen una buena susceptibilidad a colistin. En este artículo, el autor destaca a colistín como un antibiótico promisorio en el manejo de infecciones provocadas por *K. pneumoniae* MDR. (Sabnis et al., 2018) Por su parte, para el

caso de América Latina, un pequeño trabajo realizado en una unidad materno-infantil de un hospital en Perú determinó que la resistencia a colistín en aislados de *K. pneumoniae* había aumentado dramáticamente hacia fines del año 2018, alcanzando un 33% de las cepas estudiadas. (Naomi-Matsuoka et al., 2020) Por su parte, el estudio SENTRY, el cual analizó la susceptibilidad de las enterobacterias más relevantes aisladas de procesos infecciosos durante los años 2008 y 2010, señaló que *Klebsiella* spp posee una buena susceptibilidad a colistín, con valores de $CMI_{90} \leq 0{,}5$ µg/mL y que especialmente, en las cepas provenientes de Chile, se encontró un 97b% de susceptibilidad en las cepas estudiadas. (Gales et al., 2012) Por su parte, es importante destacar la alta prevalencia en Latinoamérica de *K. pneumoniae* productoras de BLEE, cercano al 37 % de los aislados, del cual, Chile presenta la más alta prevalencia de infecciones por *K. pneumoniae* – $BLEE^+$, alcanzando 63 % de positividad en las muestras estudiadas.(Vega & Dowzicky, 2017) En nuestro trabajo, el 41 % (60/145) de las cepas de *K. pneumoniae* - $BLEE^+$ estudiadas poseen una susceptibilidad a colistín mayor a lo reportado en la literatura, con una CMI_{50} igual a 1 µg/mL. (Felipe Morales-León et al., 2020)

A pesar de la buena susceptibilidad a colistín reportada en la literatura, la creciente descripción de multirresistencia y, principalmente, el aumento de casos de resistencia a cefalosporinas de tercera generación y carbapenémicos, (Vega & Dowzicky, 2017) hace que colistín sea un antibiótico que ha experimentado un importante incremento en su consumo (Klein et al., 2018) y, por tanto, una disminución de la susceptibilidad global en prácticamente todas las Enterobacterias. A lo anterior, se suma la preocupación respecto a la creciente descripción de casos de *K. pneumoniae* portadoras de un fenotipo HR a múltiples antibióticos, incluyendo colistín. (Nicoloff et al., 2019; Silva et al., 2016) En la literatura disponible, la frecuencia descrita sobre cepas de *K. pneumoniae* portadoras de un fenotipo

CST-HR es discrepante, ya que las cifras fluctúan entre 1 % y hasta más de 60 %. Así, por ejemplo, Cheong Ha et al., identificaron la existencia de *K. pneumoniae* CST-HR en 1,3 % de las cepas estudiadas. Sin embargo, cabe destacar que la identificación del fenotipo HR fue realizada por medio de un análisis rutinario de susceptibilidad *in-vitro* realizado con E-test. (Cheong et al., 2019) Por el contrario, Meletis et al., estudiaron 16 cepas de *K. pneumoniae* resistentes a carbapenémicos, las cuales fueron obtenidas desde IAAS. Los autores realizaron la identificación de CST-HR por medio del Análisis del Perfil Poblacional (*Population analysis profile* o PAP), identificando el 75 % (12/16) de las cepas como HR. (Meletis et al., 2011) Por otro lado, Barragán-Prada et al., estudiaron cepas de *K. pneumoniae* resistentes a carbapenémicos, obtenidas entre los años 2014 y 2015, desde muestras de hisopado rectal de 14 pacientes hospitalizados. De estas cepas, 9 de ellas fueron clasificadas como susceptibles a colistín. Los autores, empleando PAP, identificaron en 3/9 (33 %) de las cepas de *K. pneumoniae* un fenotipo HR. (Barragán-Prada et al., 2019) Recientemente, Band Victor et al., estudiaron una colección de 408 cepas de *K. pneumoniae* obtenidas durante los años 2012 y 2015, desde ocho hospitales pertenecientes a la red de vigilancia norteamericana de Enterobacterales resistentes a carbapenémicos (CRE). Los autores, utilizando PAP, identificaron la presencia de HR en 10,1 % de las muestras analizadas (41/408),(Band et al., 2021) resultados que fueron similares a los identificados en nuestro trabajo. (Felipe Morales-León et al., 2020) Así, la discrepancia observada en la frecuencia de *K. pneumoniae* CST-HR se puede relacionar en parte, con los métodos empleados en la identificación de este fenotipo. Por ejemplo, se sabe que los métodos basados en técnicas de estudio de susceptibilidad *in-vitro* por difusión en agar, son incapaces de identificar subpoblaciones CST-HR. Así, Nicoloff Hervé et al., estudiaron la existencia de HR a diferentes antibióticos incluyendo colistín, en distintas especies de

enterobacterias, incluyendo *E. coli, K. pneumoniae* y *S*. typhimurium. Los autores estudiaron 766 cepas, detectando mediante E-test que el 36,5 % eran HR. Sin embargo, de estas cepas, solo el 11,4 % pudo ser confirmado como HR con el método *gold standars*, que es PAP. (Nicoloff et al., 2019; Wozniak et al., 2019) Estos hallazgos implican que el E-test, así como otros métodos basados en técnicas de difusión en agar, se asocian con un alto porcentaje de error en la identificación de cepas portadoras de un fenotipo HR, siendo métodos que solo debieran ser recomendados para el estudio *in-vitro* de la susceptibilidad antibiótica. (Sabnis et al., 2018) De igual forma, en esta tesis (capítulo IV), se pudo identificar que los métodos recomendados en la literatura son incapaces de identificar la existencia de *K. pneumoniae* CST-HR y, que probablemente, también lo sean para otros antibióticos. (Felipe Morales-León et al., 2020)

Entre las posibles causas para la incapacidad de los métodos de difusión en pesquisar cepas con fenotipo HR, tenemos la baja proporción de células o subpoblaciones bacterianas con un fenotipo resistentes. Así, los métodos para el estudio de la susceptibilidad *in-vivo* emplean inóculos bacterianos estandarizados, los cuales se encuentran en el rango de magnitud de 10^4 UFC/mL, mientras que la frecuencia de subpoblaciones HR está en el orden de las 10^{-6} células resistentes (rango de 10^{-7} y 10^{-5}). (Nicoloff et al., 2019) Así, la descripción de CST-HR en *K. pneumoniae* resulta en un problema de salud pública de relevancia, ya que la dificultad en su identificación está asociada a una disminución de la susceptibilidad global a colistín (CLSI, 2019) como también, a incrementos en la morbi-mortalidad asociada a infecciones bacterianas y, por tanto, a un costo en salud. (Andersson et al., 2019; Sabnis et al., 2018; Sherman et al., 2019) Por lo tanto, resulta de interés conocer los mecanismos por los cuales se produce el fenómeno de HR, con lo cual es posible contribuir a la comprensión

de este fenómeno y a la posibilidad de desarrollar métodos más sensibles y precisos que permitan su identificación temprana.

Tradicionalmente, la existencia de *K. pneumoniae* CST-HR se asocia a los mecanismos de resistencia comúnmente descritos para el antibiótico, (Felipe Morales-León et al., 2020; Nicoloff et al., 2019) como por ejemplo, la presencia de alteraciones electrostáticas del LipA, lo que ocurre por incorporación y sustitución de los grupos fosfatos por fosfoetanolamina o aminoarabinosa, (Barragán-Prada et al., 2019) lo cual impide la interacción electrostática entre la molécula del antibiótico y el LPS. Así, Poudyal et al., describieron por primera vez la existencia de CST-HR en cepas de *K. pneumoniae*, al identificar la existencia de una pequeña subpoblación de bacterias que eran resistentes al antibiótico (CMI_{CST} entre 32 y 128 µg/mL), en cepas originalmente susceptibles a colistín. (Poudyal et al., 2008) Posteriormente, en el año 2011, Meletis et al., identificaron en cepas de *K. pneumoniae* portadoras de bla_{VIM} y bla_{KPC}, la existencia del fenotipo CST-HR (CMI_{CST} entre 16 a 64 µg/mL). En este caso, el autor realizó la búsqueda dirigida de especies portadoras del fenotipo HR, por medio de un ensayo PAP, estudiando una colección de 20 aislados clínicos de *K. pneumoniae* previamente identificados como susceptibles a colistín. (Meletis et al., 2011) Cabe señalar que estos dos trabajos, corresponden solo a la descripción de los primeros casos de *K. pneumoniae* con fenotipo CST-HR y por tanto, los autores no aportan información sobre los mecanismos moleculares relacionados. Posteriormente, Jayol et al., identificaron por E-test, la existencia de una cepa de *K. pneumoniae* resistente a carbapenémicos bla_{OXA-48} con fenotipo CST-HR. En este caso, los autores identificaron la presencia de una mutación puntual del gen *phoP,* la cual implica una sustitución aminoacídica Asp191Tyr, responsable de la alta resistencia al antibiótico (CMI=128 µg/mL). (Jayol et al., 2015) Por su parte, Halaby et al., identificaron

cepas HR provenientes desde aislados clínicos de *K. pneumoniae* productoras de BLEE, previamente clasificados como susceptibles a colistín. En este caso, los autores determinaron la existencia de una interrupción del gen *mgrB* por la presencia de IS*3-like* e IS*Kpn14*, mutaciones puntuales en gen *phoP* y dos nuevos mecanismos, una mutación en el gen *yciM*, el cual está relacionado con la regulación de la integridad de la membrana externa, y del gen *lpxM*, responsable de la incorporación de una cadena acilo secundaria al LipA. (Halaby et al., 2016) Así, en estos trabajos se señala que mutaciones en genes *yciM* y *lpxM* podrían explicar el aumento observado en la CMI a colistín de las cepas estudiadas. Al respecto, existen reportes recientes de cepas de *K. pneumoniae* resistentes a colistín con presencia de mutaciones en *yciM* y *lpxM. (Boszczowski et al., 2019)* Sin embargo, es necesario señalar que en esta tesis, ambos genes no fueron estudiados. (Felipe Morales-León et al., 2020)

Un trabajo que resulta interesante destacar fue el realizado por Band et al., quienes describieron la existencia de un fenotipo inestable de CST-HR. AL respecto, el fenotipo inestable de HR es aquel que, en ausencia de presión selectiva ejercida por el antibiótico, las cepas recuperan su perfil de susceptibilidad original. Los autores concluyen que la adquisición de un fenotipo inestable de CST-HR implica un deterioro del fitness bacteriano y que, posiblemente, la resistencia al antibiótico se deba a las diferencias en los niveles de expresión de *phoPQ* y *mgrB,* causado por un mecanismo no descrito en este trabajo. (Band et al., 2018) No obstante, Barragán-Prada et al., estudiaron la existencia de CST-HR en aislados clínicos de *K. pneumoniae* – BLEE^{+} y resistentes a carbapenémicos debido a la presencia de $bla_{\text{CTXM-15}}$ y $bla_{\text{OXA-48}}$, respectivamente. Los autores identificaron un fenotipo estable de HR, es decir, al contrario de lo ya señalado, la resistencia a colistín permanece evidente luego de múltiples pasos sucesivos de cultivos bacterianos en ausencia de presión

selectiva. Además, los autores establecieron la existencia de una interrupción de *mgrB* por IS*Kpn18*, siendo este, el mecanismo molecular que contribuye al fenotipo HR. (Barragán-Prada et al., 2019) Estos resultados resultan muy interesante, ya que los autores identificaron resultados discordantes en cuanto al fenotipo CST-HR y el fitness bacteriano, entendido este último como la existencia de diferencias en la curva de crecimiento bacteriano entre una cepa susceptible y otra resistente al antibiótico. (Andersson et al., 2019) En este sentido, es importante destacar con mayor detalle la existencia de estos dos tipos de HR: a) Fenotipo HR estable, es decir, aquel que mantiene su característica de resistencia al antibiótico en ausencia de presión selectiva; y b) la HR inestable, el cual implica que la cepa HR retoma su perfil de susceptibilidad original cuando no está presente el antibiótico. Un aspecto relevante de esto es que, en el caso de la HR estable a colistín, ocurre por mutaciones puntuales en los sistemas de dos componentes tipo PmrAB, PhoPQ y MgrB, entre otros, y que inducen cambios electrostáticos en el LPS, sin modificar el fitness bacteriano. Lo anterior, puede ser explicado por la presencia de mecanismos moleculares compensatorios, los cuales permiten sustituir las funciones fisiológicas bacterianas para mantener su sobrevida. (Band et al., 2018; Barragán-Prada et al., 2019) Así, los datos obtenidos en nuestro trabajo, (Felipe Morales-León et al., 2020) describen la existencia de un fenotipo HR estable, donde se identificaron los mecanismos moleculares de la resistencia a colistín. Sin embargo, es importante precisar que ninguno de estos mecanismos fue demostrado *in vivo* durante de desarrollo de la tesis.

Por otra parte, Band et al., estudiaron las características del LipA en una colección de cepas con fenotipo CST-HR. El autor confirmó la existencia de una modificación de aminoarabinosa en el fosfato terminal y la adicción de fosfoetanolamina al LipA, confirmando

la relación entre múltiples mutaciones moleculares de los sistemas de dos componentes y la resistencia a colistín. (Band et al., 2018)

Pese a lo ya descrito, algunos autores proponen la existencia de mecanismos alternativos a las modificaciones del LipA que también, contribuyen a la existencia de CST-HR. (Andersson et al., 2019) Así, Bardet y et al., estudiaron la existencia de cepas de *K. pneumoniae* hipermucoviscosas, identificando la existencia de subpoblaciones CST-HR (CMI=128 µg/mL). Es importante señalar que la identificación del fenotipo HR fue realizada por E-test y no fue confirmado con el método *gold estándar*, que corresponde a PAP. Pese a lo anterior, resulta interesante destacar que en ese trabajo se describe la observación de la presencia de colonias bacterianas de *K. pneumoniae* resistente a colistín que eran morfológicamente más mucosas y diferentes a las cepas susceptibles. Estos investigadores establecieron que la mutación por inserción de un nucleótido, en este caso G en posición 38 en *mgrB*, provoca un codón de término anticipado, llevando a la expresión de una proteína truncada y a la desrregulación del sistema de dos componentes PhoPQ, confiriendo la resistencia al antibiótico. Además, se menciona la existencia de cepas HR con hiperproducción capsular, siendo este un elemento contribuyente a la resistencia al antibiótico; sin embargo, este hecho no fue estudiado por el autor. (Bardet et al., 2017) En relación con lo anterior, Silva et al., establecieron una relación causal entre la existencia de cepas de *K. pneumoniae* HR y la formación de biopelículas, este último como un posible mecanismo de resistencia alternativo que permita explicar la resistencia al antibiótico. (Silva et al., 2016) Por su parte, algunos autores han establecido una relación entre la existencia de mutaciones o interrupciones del gen *mgrB* y la existencia de cambios en la estructura o conformación capsular, y no en su expresión o biosíntesis. (Aghapour et al., 2019; Mlynarcik

& Kolar, 2019; Trimble et al., 2016)El mecanismo relacionado con la presencia de cambios en la conformación capsular y la modificación de *mgrB* no es del todo clara; sin embargo, la evidencia existente indica que tras la exposición a concentraciones subMIC de colistín, *K. pneumoniae* induce la autoregulación del operón *cps o de* los genes *pmrF, ugd* y *pagP*, estimulando la síntesis del polisacárido capsular. Así, este mecanismo propuesto, corresponde a solo una parte de la compleja regulación molecular de la síntesis capsular. (Bardet et al., 2017) Por su parte, resulta interesante el trabajo realizado por Silva et al., quienes llevaron a cabo un estudio sobre la conformación estructural de la cápsula en cepas de *K. pneumoniae* resistentes a colistín. Estableciendo los autores que la mutación en *mgrB*, afecta directamente a la organización estructural de la cápsula, la cual se presenta como una estructura bien organizada en múltiples capas, interactuando con el antibiótico, lo cual favorece el aumento de los valores de CMI a colistín. (Silva et al., 2016) En relación con lo anterior, en esta tesis (Capítulo VII) se estudió la posible relación entre la presencia de interrupciones en el gen *mgrB* de cepas HR y la cantidad de exopolisacárido capsular. Al respecto, se pudo establecer que las cepas HR expresan cuantitativamente, una cantidad mayor de exopolisacárido capsular respecto a las cepas susceptibles. Sin embargo, no se pudo relacionar esta observación con la existencia de múltiples interrupciones del gen *mgrB*. Por su parte, en un trabajo realizado previamente por nuestro laboratorio (ANEXO 2), se analizaron 146 cepas de *K. pneumonae* - BLEE^{+}, determinando que 40,7 % de ellas correspondían a cepas portadoras del serotipo K2, mientras que 59,3 % fue clasificado como no tipificable. Cabe señalar que este trabajo, solo realizó la búsqueda dirigida de los de tipos capsulares más prevalentes descritos en la literatura, a saber, estos serotipos son K1, K2, K5, K20, K54 y K57. Por su parte, también en este trabajo, se estableció que no existe una relación entre la presencia de resistencia a colistín con la presencia de un serotipo capsular

determinado. (Caro-Zúñiga, 2019) Por otra parte, en un trabajo reciente, se determinó que la existencia de mutaciones en *mgrB* se relaciona con un aumento de la virulencia en *K. pneumoniae*, probablemente debido a un mecanismo similar al que ejerce el polisacárido capsular como factor de virulencia, pero que *mgrB* no se relaciona con la expresión capsular en *K. pneumoniae.* (Mmatli et al., 2020)

Sin duda, resulta interesante la observación planteada en esta tesis sobre la relación existente entre la presencia de mutaciones en el gen *crrB* y la expresión capsular, las cuales si puede guardar una relación causal. Al respecto, CrrAB corresponde a un sistema de dos componentes recientemente descrito en *K. pneumoniae*, y relacionado con la expresión de resistencia a colistín. (Llobet et al., 2011) Algunos autores han establecido que cualquier mutación en el gen *crrB*, sería el responsable del incremento en la expresión del gen *crrC*, el cual, a su vez, puede regular la expresión de *pmrC* y al operon *pmrHFIJKLM* o *arnBCADTEF*, induciendo así la expresión de resistencia a colistín. (Formosa et al., 2015; Mmatli et al., 2020)Por otra parte, McConville et al., demostraron que CrrB es un regulador positivo que lleva a la incorporación de 4-amino-4-deoxi-L-arabinosa (L-Ara4N) y fosfoetanolamina al LipA, con lo cual se induce un aumento en los valores de CMI a colistín. (McConville et al., 2020) Otros autores sugieren que sustituciones aminoacídicas en CrrB, resultan en el incremento de la autofosforilación de esta proteína induciendo resistencia a colistín. (Y. H. Cheng et al., 2018) Por otra parte, la sustitución aminoacídica de CrrB se relacionaba con un aumento en la virulencia y un efecto sobre el fitness bacteriano, probablemente a causa de la activación de la vía de las pentosas, vía alternativa para asegurar la biosíntesis de la glucosa.(Jayol et al., 2017; McConville et al., 2020)

Además, algunos autores han demostraron que la expresión de resistencia a colistín impacta sobre múltiples vías metabólicas tales como la gluconeogénesis, la biosíntesis de arginina, enterobactina y también sobre la expresión de β-lactamasas de la clase A de Ambler (como TEM y SHV), entre otros efectos. Así mismo, McConville et al demostró que mutaciones en *crrB* impactan sobre la resistencia a colistín mediante la activación del operón *arnBCADTEF,* por medio de la expresión de PmrAB, PhoPQ y PagP. También, *crrB* guarda relación con la regulación capsular, probablemente por aumento en la expresión de genes *barA* y *rcsA.* (McConville et al., 2020) Adicionalmente, la sustitución aminoacídica de CrrB puede inducir la transcripción de KexD, una bomba de expulsión múltiple recientemente descrita y que contribuye a la resistencia a colistín. (Aghapour et al., 2019) En esta tesis se identificaron dos cepas portadoras de sustituciones aminoacídicas de CrrB en las posiciones I66V, Q239H, T276A y Q287K, las cuales se relacionaron con una mayor cantidad capsular. Se encontró también que todas las cepas resistentes a colistín, tienen una mayor cantidad de polisacárido capsular con curvas de crecimiento equivalentes, lo cual implica que la presencia de estas sustituciones en CrrB, no tendría un impacto significativo sobre el fitness bacteriano. Así, nuestros resultados concuerdan con lo descrito en la literatura. (McConville et al., 2020) En cuanto al perfil de virulencia, aspecto que no fue incluido en esta tesis, recientemente Ferreira et al., describen el primer caso de *K. quasipneumoniae* - BLEE^{+} hipermucoviscosa, portadora además de un fenotipo CST-HR la cual era portadora de dos sustituciones aminoacídicas en CrrB (Q239H y Q287K), similares a las descritas en nuestra tesis. En este caso, el autor, no estableció la contribución real de estas sustituciones sobre la resistencia a colistín. (Ferreira et al., 2019) Por su parte, recientemente, Wozniak et al., describieron la existencia de una cepa de *K. pneumoniae* HR portadora de un fenotipo hipermucoviscoso e hipervirulento, siendo este el primer reporte de estas características.

(Wozniak et al., 2019) Al respecto, resulta interesante los resultados de este trabajo, ya que los autores, no identificaron la existencia de mecanismos tradicionales de resistencia a colistín relacionados con mutaciones en los sistemas de dos componentes ya descritos. Por su parte, tampoco identificaron la existencia del viruloma clásico, emplea en la definición propuesta por Moura Q. et al., de una cepa hipervirulenta e hipermucoviscosa (Moura et al., 2017)

La creciente descripción de casos de *K. pneumoniae* complex, especialmente *K. pneumoniae* y *K. variicola* dotadas de un fenotipo hipervirulento resulta preocupante, sobre todo, si se considera la convergencia de estos fenotipos hipermucoviscosos o hipervirulentos con la portación de múltiples genes de resistencia a antibióticos. En este sentido, Farzana et al., describieron un brote hospitalario ocurrido en una unidad pediátrica en Bangladesh, causado por un clon de *K. variicola* hipervirulenta con fenotipo MDR susceptible a colistín. En este caso, todas las cepas estudiadas resultaron ser portadoras de $bla_{\text{CTX-M-15}}$ y $bla_{\text{NDM-1}}$, entre otros genes de resistencia. A ellos, se le suma la existencia de múltiples genes de virulencia tales como sideróforos (*kfuABC*) y enterobactina (*entABCDEFHIJ*), que se encuentran ampliamente asociados al fenotipo hipervirulento. (Farzana et al., 2019) Al caso anterior, es necesario sumar la descripción realizada por Lu et al., sobre el primer caso de *K. variicola* hipervirulenta, aislada desde una muestra de sangre, y que además eran resistente a colistín (CMI: 8 μg/mL). Los autores reportaron, además, cinco cepas de *K. pneumoniae* hipermucoviscosa, susceptibles a carbapenémicos, pero con resistencia a colistín. (Lu et al., 2018) Todos estos casos descritos son similares a los descritos recientemente en nuestro trabajo en el cual se describe la convergencia de cepas de *K. pneumoniae* y *K. variicola* MDR con fenotipo hipervirulento y resistentes a colistín. (F. Morales-León et al., 2021) Al respecto, pudimos determinar la existencia de mutaciones en los sistemas de dos componentes *phoPQ*

y *pmrAB* como responsable de la resistencia a colistin. Por su parte, algunos autores han demostrado el rol *in-vivo* del sistema de dos componentes *pmrAB* sobre la contribución de resistencia a colistín, pero también, sobre la virulencia, al verse incrementada la sobrevida intramacrofago de *K. pneumoniae.* (Farzana et al., 2019)

Por otra parte, en esta tesis, al igual que lo descrito por otros autores, se rompe con el paradigma tradicional sobre la existencia de un viruloma único y tradicional relacionado con el fenotipo hipervirulento. (Lu et al., 2018) Al respecto, es importante destacar el rol de la producción de sideróforos en la existencia de estos fenotipos. Así, se ha demostrado que yersiniabactina, salmochelina y aerobactina, son los sideróforos más abundantes en *K. pneumoniae* y *K. Variicola* hipervirulentas. Estudios epidemiológicos han demostrado una relación significativa entre la existencia del sistema de sideróforos i*ucABCD-iutA,* con la existencia del fenotipo hipermucoviscoso e hipervirulento en *K.pneumoniae*, siendo además, considerado por algunos autores como un marcador de hipervirulencia. (F. Morales-León et al., 2021) Por lo tanto, la existencia de múltiples sistemas de sideróforos en los fenotipos hipervirulentos de *K. pneumoniae* sugiere un rol fundamental en la patogénesis de estos microorganismos, principalmente durante la fase de colonización e invasión. (H. Y. Cheng et al., 2010)

Tradicionalmente, todos los miembros del *K. pneumoniae* complex, son reconocidos por su capacidad de formar colonias mucosas. Sin embargo, en este punto es necesario recordar, la existencia de un fenotipo clásico (cKp) y un fenotipo hipermucoviscoso (hmKv/Kp) de *K. pneumoniae* complex, (Imtiaz et al., 2021; F. Morales-León et al., 2021; Sherif et al., 2021) por lo cual, resulta importante comprender el mecanismo de producción capsular en las cepas de *K. pneumoniae* hipermucoviscosas, que no poseen los genes

tradicionalmente descritos como responsables de este fenotipo, como son los reguladores *rmpA/A2*, altamente predominantes en los serotipos K1/K2. (Paczosa & Mecsas, 2016) En este sentido, Ernst et al., estudiaron el impacto de mutaciones puntuales en el gen *wzc*, que confieren un fenotipo hipercapsular e hipervirulento de *K. pneumoniae;* (Ernst et al., 2020) y que, además, contribuyen a la existencia de resistencia a péptidos policatiónico como colistín. (Marr & Russo, 2019; Rodríguez-Medina et al., 2019)Como ya fue señalado, la producción capsular es un sistema complejo altamente regulado, en la cual, la hiperproducción capsular se ha relacionado con la existencia de mutaciones en los genes *wzc, bss, rcsAB* y proteasa Lon, entre otros genes y reguladores transcripcionales. (Cubero et al., 2016) Así, en esta tesis, se identificaron mutaciones en todos estos genes, con excepción de *bss*. Probablemente la existencia de estas mutaciones, sean las responsables de la existencia de cepas de *K. pneumoniae* y *K. variicola* MDR, portadoras de un fenotipo hipermucoviscosos e hipervirulento. (Ernst et al., 2020) Por lo tanto, resulta interesante continuar con los estudios sobre la prevalencia y epidemiología local de *K.pneumoniae* complex MDR, portadora de un fenotipo hipermucoviscoso e hipervirulento, ya que así, se podría establecer la magnitud real del problema, información que permitiría diseñar estrategias en las políticas actuales sobre control de IAAS, como también en los Programas de racionalización del uso de antimicrobianos.

CAPÍTULO IX: CONCLUSIONES

1. Se identificó la existencia de cepas de *K. pneumoniae* productoras de BLEE, portadoras de un fenotipo estable de CST-HR, que presentaron:
    - Mutaciones en los genes *phoPQ*, *pmrAB* y *crrAB,* algunas de las cuales no han sido descrita.
    - Mutaciones e interrupciones en el gen regulador *mgrB* causadas por la existencia de secuencias de inserción.

2. El nivel de expresión de genes *pmrABD*, *phoPQ* y *mgrB*, está alterado, probablemente como consecuencia de las mutaciones identificadas.

3. Las mutaciones y los cambios en los niveles de expresión relativa de los genes *phoPQ*, *pmrACD* y *mgrB*, no afectaron el fitness bacteriano, entendiendo este último como cambios en los parámetros de crecimiento bacteriano.

4. Las cepas de *Klebsiella pneumoniae* productoras de BLEE con fenotipo heterorresistentes a colistín, presentaron una mayor cantidad de polisacárido capsular en comparación a las cepas susceptibles, lo que podría contribuir al aumento en los vales de MIC observado en las cepas heterorresistentes.

5. La mayoría de las cepas de *Klebsiella pneumoniae* productoras de BLEE, con y sin el fenotipo CST-HR presentaron el serotipo capsular K2. Detectándose, además, algunos

serotipos poco frecuentes, como los serotipos KL19 y KL81, no descritos previamente en la literatura como portadores de un fenotipo CST-HR.

6. No fue posible establecer la existencia de una relación significativa entre la cantidad de polisacárido capsular presente y el tipo capsular. La cantidad de muestras analizadas y la diversidad de tipos capsulares que se identificaron en este trabajo no permiten establecer esta relación.

De esta forma y, considerando las conclusiones realizadas, tenemos:

Hipótesis 1:

El fenotipo heterorresistente a colistín, detectado en cepas de Klebsiella pneumoniae productoras de β-lactamasas de espectro extendido, se debe a mecanismos moleculares distintos a aquellos que introducen modificaciones estructurales en el LPS.

Se rechaza la primera hipótesis planteada en base a que los mecanismos de resistencia a colistín identificados en las cepas de *K. pneumoniae* - BLEE^{+} HR, comparten la mayoría de los mecanismos descritos en la literatura.

Hipótesis 2:

El serotipo y cantidad de polisacárido capsular de cepas de Klebsiella pneumoniae susceptibles a colistín es similar al de las cepas heterorresistentes a este antibiótico

Se rechaza parcialmente, considerando que los serotipos capsulares son independientes de la existencia del fenotipo heterorresistente y responde a la frecuencia y diseminación global y no a la existencia de un mecanismo de resistencia determinado. Por su parte, la cantidad de polisacárido capsular expresada en las cepas de *K. pneumoniae* - BLEE^{+} HR a colistín no pudo ser descartada como un posible mecanismo que contribuya a la existencia del fenotipo.

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PROYECCIONES

En los resultados de este trabajo se identificaron mutaciones en el gen *phoP*, las cuales no han sido descritas anteriormente. De esta forma, la presencia de estas mutaciones no permiten ser relacionadas con la expresión fenotípica de resistencia a colistín, por lo cual, se espera evaluar el impacto de estas mutaciones sobre la expresión de modificaciones en la estructura del LPS, lo que conlleven a la existencia de resistencia al antibiótico.

La evidencia determinada en este trabajo, junto con la existente en la literatura, sugieren que la exposición de *K. pneumoniae* a concentraciones sub-inhibitorias de colistín, inducen la activación de reguladores globales tipo CrrAB, RcsABC, entre otros. Estos reguladores producen la activación de vías moleculares relacionadas con la activación de factores de virulencia, regulación en la expresión del polisacárido capsular y por tanto, la consiguiente expresión de resistencias a colistín. Esta hipótesis merece el planteamiento de estudios futuros que podrían cambiar la forma en la cual se emplea colistín.

Finalmente, otro punto que resulta interesante conocer, es aquel que guarda relación con la existencia de cepas portadoras de fenotipos hipervirulentos y su relación con la existencia de fenotipos multirresistentes. De ser así, resultaría interesante conocer el entorno genético de la convergencia entre genes de resistencia a múltiples antibióticos como la de genes de hipervirulencia.

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ANEXO 1. — Material suplementario Capítulo IV

Supplementary materials

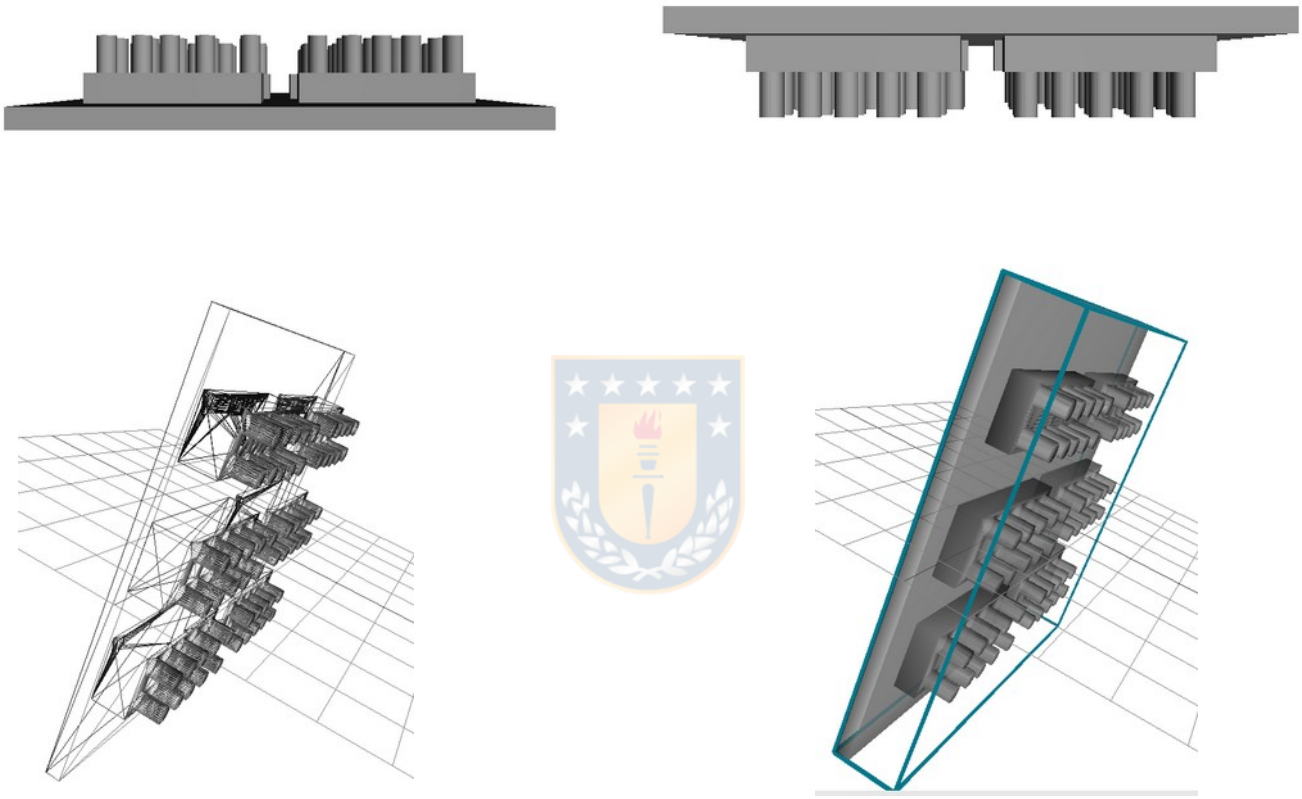

Figure S1.- Multiples views of multipoint inoculator used in the M-PAP. The technical specifications was reserved by patent rights.

Table S1.- Validation of total volume by section

	Mean Vol (µL) (n=5)	SD	p-value
Section 1	20,3	0,2	0,743
Section 2	20,3	1,5	
Section 3	20,1	0,4	
Section 4	20,2	0,5	
Section 5	19,7	0,2	
Section 6	19,8	0,7	

P-value: ANOVA analyses with difference at p-value < 0,05. Reference volume: 20 µL/section.

Table S2.- Comparative analysis of CFU/mL determined by PAP, M-PAP and traditional spread plate method.

Strain	Mean Log CFU/mL for tree method					
	PAP		M-PAP		Control	
	mean	SD	Mean	SD	mean	SD
M1	8,4	0,48	8,30	0,51	8,4	0,59
M2	8,1	0,05	8,32	0,20	8,4	0,45
M3	8,3	0,04	8,05	0,48	8,7	0,16

Control by Spread plate method. Inoculum of 0.5 McFarland equivalent to $1,5x10^8$ UFC/mL. About 8,2 of the logarithmic scale. M1, M2, M3 are ATCC 700603, *K.pneumoniae* K970 and *K.pneumoniae* K1003 reference strains from Laboratory collection.

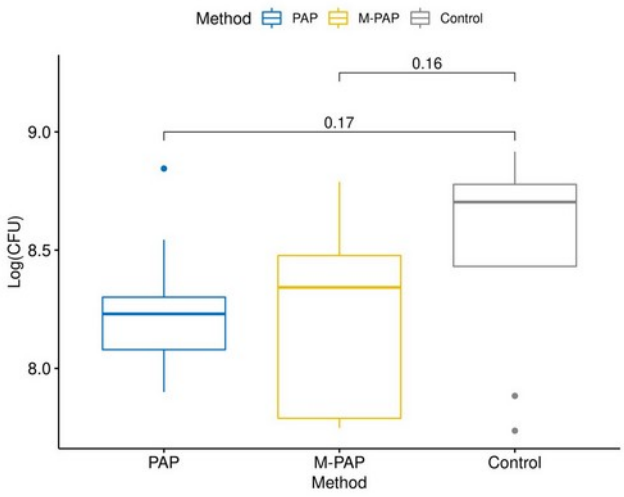

Figure S2.- Comparative analysis of PAP and M-PAP for CFU/mL estimate. PAP. Population analysis profile, M-PAP modified PAP, Control Spread plate method. All assay was made using a standard inoculum of 0.5McFarland or $1{,}5x10^8$ CFU/mL (8,2 in logarithmic scale). Not significative difference in Log (CFU/mL) between Control and tested method. p-value significative at $p < 0{,}05$ in t-student test.

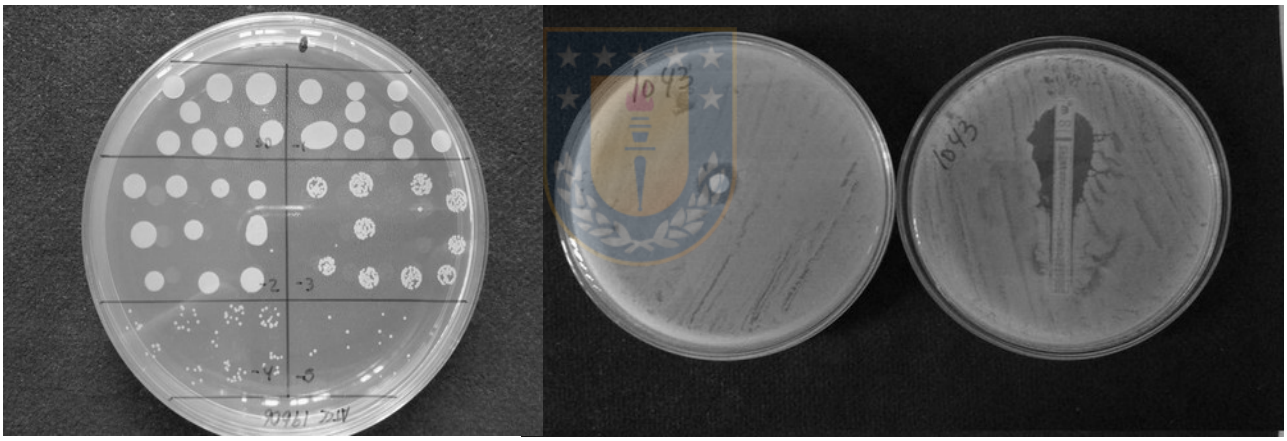

Figure S3.- A) Modified-PAP method. Reference image from ATCC19606 in Agar MH without antibiotic. B) Comparative image to Disk diffusion method and E-test to colistin. We observed irregular antibiotic diffusion in agar.

ANEXO 2. — Pesquisa de Factores de virulencia en Cepas de *Klebsiella pneumoniae* MDR aisladas en Hospitales Chilenos.

El anexo 2 corresponde al Trabajo de Fin de Carrera para optar al título profesional de Químico Farmacéutico de la Srta. Camila Beatriz Caro Zúñiga, realizado durante el año 2018 – 2019 en el marco de esta tesis Doctoral. El trabajo se encuentra como archivo anexo a esta Tesis y está disponible en la Colección de la Biblioteca Central de la Universidad de Concepción.

Prof. Patrocinante: Dra. Helia Bello Toledo.

Profesor Guía: Felipe Morales León.

Resumen

Klebsiella pneumoniae es capaz de evadir la respuesta inmune del huésped a través de la expresión de factores de virulencia. El fenotipo hipervirulento (hvKP) se caracteriza por causar infecciones diseminadas y graves en adultos aparentemente sanos y se encuentra frecuentemente asociado a serotipos capsulares K1, K2, K5, K20, K54 y K57, a sideróforos como aerobactina y yersiniabactina, siendo los genes asociados a su biosíntesis considerados marcadores moleculares de virulencia, además de la expresión de un fenotipo hipermucoviscoso (hmKP). Tradicionalmente las cepas hmKP y hvKP se han asociado a mayores tasas de susceptibilidad a los antibacterianos respecto de los fenotipos clásicos. En esta tesis se trabajó con 146 cepas de *K. pneumoniae* MDR, a las que se realizó estudios del

perfil de susceptibilidad y estudios moleculares mediante PCR convencional, para caracterización del serotipo capsular, en donde se encontró el serotipo capsular K2 en el 47,9 % de las cepas, y determinación de distintos factores de virulencia altamente relacionados con los fenotipos hvKP. Así se identificó la presencia de los marcadores de virulencia ybtS, iucA y iutA en cepas productoras de carbapenemasas en 32 %, 24 % y 52 % y no productoras de carbapenemasas en 44,6 %, 4,1 % y 70,2 %, respectivamente. Así como también se identificó la presencia del fenotipo hmKP en 2 cepas (1,4 %) en que una de ellas presentó un comportamiento hipervirulento *in vitro*. Se pudo concluir que en las cepas mencionadas el serotipo capsular K2 es el prevalente y existe la presencia de distintos factores de virulencia asociados a hvKP