**JÉSSICA NOGUEIRA ROSA**

# CHARACTERIZATION OF *Klebsiella* spp FROM HEALTHY SWINE: VIRULENCE, RESISTANCE TO ANTIMICROBIALS AND GENE TRANSFER MEDIATED BY EXTRACELLULAR VESICLES

Thesis submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

Adviser: Denise Mara Soares Bazzolli

Co-advisers: Hilário Cuquetto Mantovani
Paulo Henrique Furtado Campos

**VIÇOSA – MINAS GERAIS**
**2023**

**Ficha catalográfica elaborada pela Biblioteca Central da Universidade Federal de Viçosa - Campus Viçosa**

T
R788c
2023

Rosa, Jéssica Nogueira, 1994-
Characterization of *Klebsiella spp* from health swine: virulence, resistance to antimicrobials and gene transfer mediated by extracelular vesicles / Jéssica Nogueira Rosa. – Viçosa, MG, 2023.
1 tese eletrônica (118 f.): il. (algumas color.).

Texto em inglês.
Orientador: Denise Mara Soares Bazzolli.
Tese (doutorado) - Universidade Federal de Viçosa, Departamento de Microbiologia, 2023.
Inclui bibliografia.
DOI: https://doi.org/10.47328/ufvbbt.2023.476
Modo de acesso: World Wide Web.

1. Intestinos - Microbiologia. 2. Suínos - Fisiologia. 3. Bactérias patogênicas. 4. Marcadores genéticos. I. Bazzolli, Denise Mara Soares, 1974-. II. Universidade Federal de Viçosa. Departamento de Microbiologia. Programa de Pós-Graduação em Microbiologia Agrícola. III. Título.

CDD 22. ed. 573.37359

Bibliotecário(a) responsável: Euzébio Luiz Pinto CRB-6/3317

JÉSSICA NOGUEIRA ROSA

# CHARACTERIZATION OF *Klebsiella* spp FROM HEALTHY SWINE: VIRULENCE, RESISTANCE TO ANTIMICROBIALS AND GENE TRANSFER MEDIATED BY EXTRACELLULAR VESICLES

Thesis submitted to the Agricultural Microbiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of *Doctor Scientiae*.

APPROVED: July 28, 2023

Assent:

Jéssica Nogueira Rosa
Jéssica Nogueira Rosa
Author

Denise Mara Soares Bazzolli
Adviser

## AGRADECIMENTOS

Agradeço aos meus pais, Margareth e Célio, por acreditar e sonhar junto comigo a realização do mestrado e doutorado. Por acreditar que um dia eu chegaria aqui e poderia ir além.

À professora Denise por todos esses anos de orientação. Sempre serei grata pela oportunidade e ensinamentos. Levarei sempre para minha vida.

À Universidade Federal de Viçosa, ao Departamento de Microbiologia e ao Programa de Pós-graduação em Microbiologia Agrícola por todo o suporte durante esses anos.

Este estudo foi parcialmente financiado pela Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Código Financeiro 001.

As agências de fomento brasileiras: CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) e FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais) pelo suporte financeiro que me permitiu o desenvolvimento deste trabalho.

Aos professores da Pós-graduação em Microbiologia Agrícola que ajudaram em minha formação profissional e trajetória na pós-graduação.

Aos funcionários do Departamento de Microbiologia e do Bioagro, por terem sido tão solícitos sempre que precisei.

Agradeço ao Núcleo de Estudos em Microbiologia Agrícola - NEMA pela coordenação de um grupo de extensão universitária. Todas as decisões tomadas em equipe me ajudaram a crescer como pessoa.

Agradeço ao Newton Moreno Sanches (Newtinho), por todas as lições aprendidas no curto tempo que fiquei ao seu lado. Foram muitos ensinamentos que carregarei para sempre em minha vida. Você sempre terá um espaço no meu coração (pequenininja).

Agradeço a toda equipe do laboratório de Genética Molecular de Bactérias (LGMB). Aos novos membros e aos antigos, tudo foi aprendizado. Em especial, agradeço à Patrícia e ao Giarlã por sempre ajudar quando precisei e pelas conversas.

Agradeço a todos os meus professores, desde o jardim de infância até o mestrado! Vocês são parte do caminho que trilhei. Esse agradecimento, vai com carinho especialà professora Maria Aparecida (por me alfabetizar), Vanderci (por me motivar a ser astronauta, com 10 anos), Alexandre e Flávio (por me inspirarem a ser professora).

Finalmente, agradeço aos meus amigos de caminhada: Matheus, Patricia, Giarlã, Maria Eduarda, Bruna, Mariana e Osiel. Vocês deixarem meu dia melhor.

A todos vocês, muito obrigada!

## RESUMO

ROSA, Jéssica Nogueira, D.Sc., Universidade Federal de Viçosa, julho de 2023. **Caracterização de *Klebsiella* spp em suínos saudáveis: virulência, resistência a antimicrobianos e transferência de genes mediada por vesículas extracelulares**. Orientadora: Denise Mara Soares Bazzolli. Coorientadores: Hilario Cuquetto Mantovani e Paulo Henrique Furtado Campos.

Bactérias do gênero *Klebsiella* pertencem à família Enterobacteriaceae, são Gram-negativas e exibem diferentes habitats e funções ecológicas, especialmente em diferentes hospedeiros. A espécie mais estudada é *Klebsiella pneumoniae* uma bactéria encontrada naturalmente na microbiota intestinal e importante patógeno oportunista. Os fatores de virulência atribuídos a *K. pneumoniae* são produção e secreção de sideróforos, fímbrias do tipo I e III, cápsula polissacarídica, lipopolissacarídeos (LPS), sistema de secreção (T6SS) e vesículas extracelulares (EVs). Fatores envolvidos com virulência e resistência a antimicrobianos são variáveis e codificados por diferentes genes. Esses genes podem ser adquiridos por transferência horizontal (THG) com a participação efetiva de elementos genéticos móveis (MGE), como plasmídeos e elementos integrativos conjugativos (ICE). Suínos apresentam *Klebsiella* spp como membros da microbiota intestinal, e podem ser reservatórios deste gênero com marcadores genéticos de virulência e resistência. Diante disso, este trabalho teve como objetivos: isolar *Klebsiella* spp de suínos saudáveis em fase de crescimento II; caracterizar o fenótipo e o genótipo de virulência e de resistência a antimicrobianos; investigar a contribuição de ICEs para seu nicho ecológico assim como, a transferência horizontal de genes mediada por vesículas extracelulares (EVs). Como resultados do capítulo 1, foram obtidos 144 isolados de *Klebsiella* spp, destes 77 % identificados como *K. pneumoniae*, 14,5 % *Klebsiella aerogenes* e 8,5% *Klebsiella variicola*. Nos isolados obtidos foram observados o seguinte perfil de resistência: β-lactâmicos (100 %), cefalosporinas (75,5 %), fluoroquinolonas (51 %), fenicol (48,8 %), tetraciclina (55,5 %), macrolídeo (37,7%), sulfonamida (35,5 %), aminoglicosideo (8,8%), trimetoprim (4,5 %). No geral, 88,8 % dos isolados foram caracterizados como multirresistentes a antimicrobianos (MDR). Importantes fenótipos e genótipos de virulência foram identificados como produção de sideróforos (*entB, iucB, iutA, kfu e ybtS/irp2*) e biofilme, onde 39 % são classificados como produtores fortes, 45 % moderados e 16 % como produtores fracos. O fenótipo hipermucoviscoso não foi verificado nos isolados investigados, no entanto o gene marcador *iutA* foi identificado em 19 isolados (42,2 %). A partir dos resultados

apresentados, 11 isolados de *K. pneumoniae* MDR foram selecionados para investigação do tipo de sequência (STs) e os mesmos foram classificados como: ST25, ST147, ST616, ST691 e ST6208. Desta forma, dois isolados foram selecionados para o sequenciamento de genomas (capítulo 2), *K. pneumoniae* HS-144 e *K. pneumoniae* HS-13. A anotação funcional revelou classes como metabolismo de carboidratos, aminoácidos e proteínas (37,6%); transporte de membrana (5,7%); regulação e sinalização celular (3,5%); metabolismo de DNA (3,5%); virulência, doença e defesa (3,1%); parede celular e cápsula (5,2%) e outros. *Klebsiella pneumoniae* HS-144 e *K. pneumoniae* HS-13 carregam plasmídeos. Das duas linhagens, somente na linhagem HS-144 foram identificados genes envolvidos com o aparato de conjugação completo (T4SS, T4CP, relaxase e *oriT*), e um fago que transporta o gene $bla_{SHV2.}$ Para esta linhagem (HS-144) ainda foi confirmado o fenótipo ESBL - β-lactamase de espectro estendido. Assim, um ensaio de conjugação biparental utilizando a *K. pneumoniae* HS-144 como doadora e *E. coli* J53 como receptora (seleção com colistina). Foram obtidos três transconjugantes (MIC para colistina de >64mg.mL$^{-1}$), confirmando a natureza mobilizável de determinantes genéticos envolvidos com resistência à colistina, um fármaco utilizado como último recurso em tratamentos de *K. pneumoniae* na clínica humana. No capítulo 3, foram investigados 949 genomas completos de *K. pneumoniae* visando a caracterização de elementos integrativo e conjugativo na espécie. De todos os genomas analisados, 501 genomas apresentaram uma ou mais regiões potenciais com capacidade de auto transferência, sendo que 18,2% destes apresentam coocorrências com outros ICEs. Detectamos 19 novos candidatos a ICEs. Os novos ICEs carregam genes relacionados à adesão e absorção de ferro, biossíntese da cápsula, parede celular e LPS, resposta ao estresse, componentes da membrana e resistência ao arsênico, β-lactâmico, polimixina e novobiocina. Além disso, também mapeamos quatro novos ICEs que carregam a ilha altamente patogênica de *Yersinia* (HPI). No capítulo 4, investigamos a importância das vesículas extracelulares (EVs) na transferência de genes entre *Klebsiella* spp de origem humana e de suino. As EVs-Kp13 (*K. pneumoniae* Kp13) foram obtidas pelo método de filtração hidrostática. As EVs obtidas apresentaram tamanho médio de 182 nm e morfologia arredondada. Neste trabalho foi confirmado que as EVs-Kp13 carreiam genes de resistência a sulfonamida (*sul2*), cefalosporinas ($bla_{CTXM2}$, $bla_{SHV}$, $bla_{TEM}$), cabapenemos ($bla_{KPC}$) e trimetoprim (*dfrA15*). Além disso, foi detectado que as EVs-Kp13 carreiam genes que codificam para o sideróforo yersiniabactina. Nossos resultados de vesidução foram positivos quando utilizada a linhagem *K. aerogenes* 270 como receptora, isolada de suíno saudável. Foram obtidos quatro transvesidantes que apresentaram resistencia para cefalosporinas (terceira

e quarta geração) e trimetoprim, além dos genes marcadores do plasmídeo pKP13f. Podemos concluir que suínos clinicamente saudáveis apresentam *Klebsiella* spp com fenótipo MDR e de virulência relevantes, do ponto de vista clínico, em sua microbiota normal. *Klebsiella pneumoniae* pode ainda carregar elementos genéticos móveis como plasmídeos e ICEs que auxiliam seu fitness em diferentes ambientes. E por fim, a vesidução pode ser um importante meio de disseminação de genes de resistência em *Klebsiella* spp. Esse estudo elucida importantes rotas de fluxo gênico entre espécies e reservatórios diferentes contribuindo expressivamente para a abordagem da saúde unificada.

Palavras-chave: One health. Multirresistência. Fitness microbiano. Vesidução.

## ABSTRACT

ROSA, Jéssica Nogueira, D.Sc., Universidade Federal de Viçosa, July, 2023. **Characterization of *Klebsiella* spp from healthy swine: virulence, resistance to antimicrobials and gene transferer mediated by extracellular vesicles**. Adviser: Denise Mara Soares Bazzolli. Co-advisers: Hilario Cuquetto Mantovani and Paulo Henrique Furtado Campos.

Bacteria of the *Klebsiella* genus belong to the Enterobacteriaceae family, are Gram-negative and exhibit different habitats and ecological functions, especially in other hosts. The most studied species is *Klebsiella pneumoniae*, a bacteria naturally found in the intestinal microbiota and na important opportunistic pathogen. The virulence factors attributed to *K. pneumoniae* are the production and secretion of siderophores, type I and III fimbriae, polysaccharide capsules, lipopolysaccharides (LPS), secretion system (T6SS) and extracellular vesicles (EVs). Factors involved with virulence and antimicrobial resistance are variable and encoded by different genes. Theses genes can be acquired by horizontal transfer (THG) with the effective participation of mobile genetic elements (MGE), such as plasmids and conjugative integrative elements (ICE). Swine have *Klebsiella* spp as members of the intestinal microbiota and may be reservoirs of this genus with genetic markers of virulence and resistance. Therefore, this work had the following objectives: to isolate *Klebsiella* spp from healthy swine in growth phase II; characterize the phenotype and genotype of virulence and antimicrobial resistance; to investigate the contribution of ICEs to their ecological niche as well as horizontal gene transfer mediated by extracellular vesicles (EVs). As a result of chapter 1, 144 *Klebsiella* spp isolates were obtained, of which 77% were identified as *K. pneumoniae*, 14.5% *Klebsiella aerogenes*, and 8.5% *Klebsiella variicola*. In the isolates obtained, the following resistance profile was observed: β-lactams (100 %), cephalosporins (75.5 %), fluoroquinolones (51 %), phenicol (48.8 %), tetracycline (55.5 %), macrolide (37.7%), sulfonamide (35.5%), aminoglycoside (8.8%), trimethoprim (4.5%). Overall, 88.8% of the isolates were characterized as multidrug-resistant (MDR). Important virulence phenotypes and genotypes were identified as siderophore production (*entB, iucB, iutA, kfu* and *ybtS/irp2*) and biofilm, where 39% are classified as strong producers, 45% moderate and 16% as weak producers. The hypermucoviscous phenotype was not verified in the investigated isolates, however the *iutA* marker gene was identified in 19 isolates (42.2%). From the presented results, 11 isolates of *K. pneumoniae* MDR were selected for investigation of the type of sequence (STs) and they were classified as ST25, ST147, ST616, ST691 and ST6208. Thus, two isolates were selected for genome sequencing (chapter 2), *K.*

*pneumoniae* HS-144 and *K. pneumoniae* HS-13. Functional annotation revealed classes such as metabolism of carbohydrates, amino acids, and proteins (37.6%); membrane transport (5.7%); cell regulation and signaling (3.5%); DNA metabolism (3.5%); virulence, disease and defense (3.1%); cell wall and capsule (5.2%) and others. *Klebsiella pneumoniae* HS-144 and *K. pneumoniae* HS-13 carry plasmids. Of the two strains, only genes involved in the complete conjugation apparatus (T4SS, T4CP, relaxase and *oriT*) and a phage carrying the $bla_{SHV2}$ gene were identified in the HS-144 strain. For this strain (HS-144) the ESBL - extended-spectrum β-lactamase phenotype was also confirmed. Thus, a biparental conjugation assay using *K. pneumoniae* HS-144 as a donor and *E. coli* J53 as recipient (selection with colistin). Three transconjugants were obtained (MIC for colistin >64mg.mL$^{-1}$), confirming the mobilizable nature of genetic determinants involved with resistance to colistin, a drug as a last resort in the treatment of *K. pneumoniae* in the human clinic. In chapter 3, 949 complete genomes of *K. pneumoniae* were investigated in order to characterize integrative and conjugative elements in the species. Of all the analyzed genomes, 501 genomes presented one or more potential regions with self-transfer capability, and 18.2% of these present co-occurrences with other ICEs. We detected 19 new ICE candidates. The new ICEs carry genes related to iron adhesion and absorption, capsule biosynthesis, cell wall and LPS, stress response, membrane components and resistance to arsenic, β-lactam, polymyxin and novobiocin. In addition, we also mapped four new ICEs that carry the highly pathogenic Island Yersinia (HPI). In Chapter 4, we investigated the importance of extracellular vesicles (EVs) in gene transfer between human and swine *Klebsiella* spp. EVs-Kp13 (*K. pneumoniae* Kp13) were obtained by the hydrostatic filtration method. The EVs obtained had an average size of 182 nm and rounded morphology. In this work, it was confirmed that EVs-Kp13 carry genes for resistance to sulfonamide (*sul2*), cephalosporins ($bla_{CTXM2}$, $bla_{SHV}$, $bla_{TEM}$), carbapenems ($bla_{KPC}$) and trimethoprim (*dfrA15*). Furthermore, it was detected that EVs-Kp13 carry genes that code for the siderophore yersiniabactin. Our blistering results were positive when using the *K. aerogenes* 270 strain as recipient, isolated from healthy swine. Four transvesiculants were obtained that showed resistance to cephalosporins (third and fourth generation) and trimethoprim, in addition to the marker genes of plasmid pKP13f. We can conclude that clinically healthy swine have *Klesiella* spp with a clinically relevant MDR and virulence phenotype in their normal microbiota. *Klebsiella pneumoniae* can also carry mobile genetic elements such as plasmids and ICEs that help their fitness in different environments. And finally, vesiduction can be an important means of disseminating resistance genes in *Klebsiella* spp. This study elucidates important gene flow

routes between different species and reservoirs, significantly contributing to the unified health approach.

Keywords: One health. Multidrug resistant. Microbial fitness. Vesiduction.

**SUMMARY**

## Introduction

Pork stands out as one of the most consumed animal protein sources globally. In this scenario, Brazil is in fourth place in the ranking of the world's largest producers, giving Brazilian pig farming an important socioeconomic role at a global level. The use of growth promoters (AGPs) in production animals has been debated on a global scale. Even with all the concern and efforts to promote the rational use of antimicrobials in swine feed, the use of these substances categorized as growth promoters (low doses and constant consumption) is widely discussed as being a possible important determinant for the selection of multiresistant bacteria to antimicrobials commonly used in clinical practice and playing a role in the context of one health

The increasing number of resistant microorganisms is considered a threat to public health by the World Health Organization (WHO). In this context, the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* and *Enterobacter* spp) comprises the main bacteria related to the global challenge of difficult-to-treat infections. In production animals, the panorama and sharing of resistance genes and multidrug resistance (MDR) phenotype is not completely established, being little explored especially in clinically healthy animals. The genus *Klebsiella* consists of bacteria naturally found in the normal intestinal microbiota of humans and animals, some of which may be opportunistic pathogens.

The niche established by *K. pneumoniae* in different hosts is defined by genetic and phenotypic aspects, and by the conditions inherent to the host itself. In this context, in humans, two important variants are described for *K. pneumoniae*: i) the so-called classic (cKp), which carries multiple antimicrobial resistance and is commonly associated with nosocomial infections; and ii) hypervirulent (hvKp), associated with systemic community infections. *Klebsiella pneumoniae* cKp and hvKp evolved in isolation and separated into clonal groups (GC) of importance and global distribution. The concomitant emergence of multidrug-resistant and hypervirulent isolates has been documented in the literature, which demonstrates its importance in the context of global public health.

*Klebsiella* spp has several virulence factors: production of siderophores (enterobactin, yersiniabactin, salmochelin and aerobactin), type I and III fimbriae, production of polysaccharide capsule, lipopolysaccharides (LPS), Type IV secretion system (T6SS) and extracellular vesicles (EVs). These factors may vary from isolate to isolate, given the ability of these organisms to acquire genes by horizontal transfer (HTG) with the effective participation

of mobile genetic elements (MGE), such as plasmids and Integrative Conjugative Elements (ICE).

In the current context of information, the central axis of studies involves *K. pneumoniae*, in which the focus is on clinical isolates of human origin, with few studies involving isolates from other animals, especially production ones, such as swine. Swine may be involved in cycles in which MDR pathogen dissemination pathways may occur, and little is known about this. Thus, the hypothesis of this work is: *Klebsiella* spp isolated from clinically healthy swine reservoir and under stress conditions present phenotype and genotypes of antimicrobial resistance and virulence. Furthermore, mobile genetic elements such as plasmids and ICEs contribute to the fitness of *Klebsiella pneumoniae* as well as horizontal gene transfer mediated by extracellular vesicles (EVs). The objectives of this work were: to isolate *Klebsiella* spp from healthy pigs in growth phase II; characterize the phenotype and genotype of virulence and antimicrobial resistance; to investigate the contribution of ICEs to their ecological niche, as well as conjugation and vesiduction.

**Swine production: world panorama**

Pork stands out as the second most-consumed source of animal protein globally (WORLDWATCH INSTITUTE, 2022). World pork production for the beginning of the first half of 2023 is 114.3 million tons (USDA, 2023). Brazil is the fourth country that produces the most pork in the world, and in 2022 production reached the mark of 4.983 million tons (ABPA, 2023; USDA, 2023). The production scenario for Brazil by the end of 2023 is promising and promises to rise by around 2% (USDA, 2023). The state of Minas Gerais is the fourth Brazilian state in the pork production ranking in 2022, contributing about 9.44% for the same year (ABPA, 2023). In terms of pork exports, Brazil is the third country listed in the USDA 2023 report.

The change in the ranking of pork production and exports has been affected in recent years by political and sanitary issues (Carvalho, 2021). China is the largest producer, consumer and importer of pork in the world (USDA, 2023). The increasing reports of African swine fever (ASF) in Chinese territory caused a drop in pork production and consequently increased the import of this protein (Carvalho, 2021). In this way, Brazil has been gaining relevance in the global market, since ASF has not been documented in the country.

The growing global demand for pork requires maintaining an adequate health status and ensuring animal welfare during the production process (Fischer et al., 2019; Sato et al., 2022). In the intensive production model, animals can be exposed to stressors such as stalls with high

animal densities, thermal stress, inadequate water and food supply, and constant interaction with human beings (Sato et al., 2022). These stressors affect the animal's homeostasis and, consequently, the efficiency of the immune response (Sato et al., 2022). Thus, respiratory diseases and intestinal disorders represent significant impacts on animal health and pig farming (Silva et al., 2019; Sato et al., 2022).

The use of antimicrobials in intensive production systems has different purposes: treatment of bacterial diseases; prophylaxis; metaphylaxis and growth promotion (AGP), from the use of antibiotics as a food additive (Stella et al., 2020). The use of AGP was introduced in pig farming more than 50 years ago and provides advantages to the herd, such as a decrease in cases of clinical infections and an increase in the efficiency of absorption of nutrients by the intestinal mucosa (Vieites et al., 2020). However, the use of APC is not enough to control infections, therefore, it is necessary to use antibiotics in therapeutic and prophylactic doses (Stella et al., 2020; Vieites et al., 2020).

In this context, researchers have questioned the use of AGP, there is a view that over the years, the constant use of AGP in low doses can provide an ideal environment for the selection of bacteria resistant to antimicrobials in the gastrointestinal tract and, therefore, it contributes to the spread of resistant microorganisms in the environment (Barton, 2014; Fang et al., 2018; Burow et al., 2019). Thus, given the requirements imposed by the consumer market, especially the exporter, Brazilian regulations have been gradually adapting to the use of AGPs.

**Stages of development of production pigs**

Large-scale system production is the main Brazilian pig production system (Sato et al., 2022). In this system, animals after birth are confined in small physical spaces that become ideal environments for the dissemination of microorganisms (Silva et al., 2019; Sato et al., 2022). This fact results in an increase in cases of clinical infections in the herd (Sato et al., 2022). The environment directly influences the quality of animal life and the succession of microorganisms that make up its intestinal microbiota (Wang et al., 2019). In addition, the growth phase in which the pigs are, changing the diet and using antimicrobials can remodel their intestinal microbiota (Davis & Price 2016; Yan et al., 2019; Wang et al., 2019; Sun et al., 2020).

The average gestation period of the sows is 114 days (Figure 1). The period of lactation (maternity) is essential for newborn animals and demands great energy expenditure from the sows to maintain milk production (Tokach & Dial 1992). After 28 days of birth, the weaning of the animals begins (nursery). Post-weaning is a critical period, during nursery the piglets are

separated from the sows and directed to another confinement environment (Silva et al., 2019). Diet modification, weaning stress, and low immunity can alter the intestinal microbial balance resulting in disturbances in the commensal microbiota (Sato et al., 2022). These changes can lead to clinical manifestations such as diarrhea, reduced feed conversion rate, and production performance (Sato et al., 2022). The use of AGP in nursery phasis is proposed to reduce populations of pathogenic microorganisms and improve animal performance (Gaviolli et al., 2013).

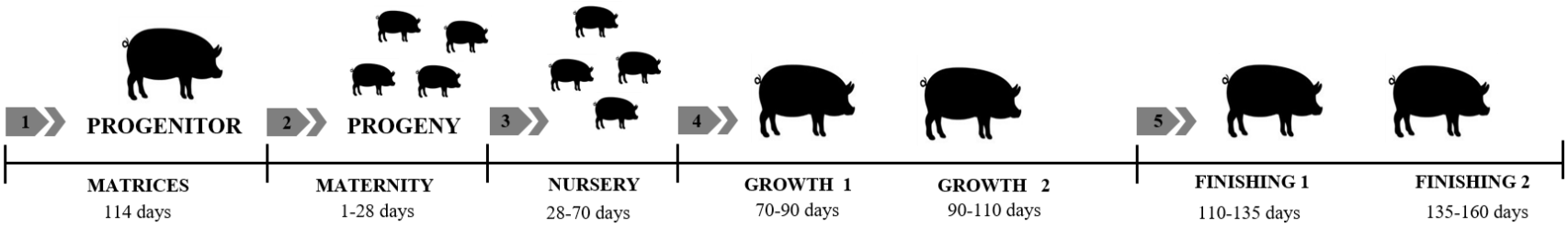



**Figure 1:** Stages of animal development in pig farming. 1) Pregnancy of the matrices. In 2 and 3 we have the phases of greater animal stress. And in 4 and 5 the final stages in the animal's cycle, are growth and finishing.

Subsequently, between the period of 70 to 110 days, the animals enter the growth phase I and II, followed by the finishing phase I and II (110 to 160 days). In these phases, the intestinal microbiota is well-established (Wang et al., 2019). However, diet may influence the succession pattern of intestinal microorganisms (Wang et al., 2019). The age of the animals is a determinant of gut maturity, and functional factors such as metabolism, immunity, hormone secretion, and bone and muscle development are associated (Moeser, Pohl & Rajput 2017; Wang et al., 2019). It is in the finishing phase II that the animals are ready to be slaughtered and marketed.

**Use of antimicrobials such as growth promoter (AGPs)**

Growing concern about the emergence of multidrug-resistant microorganisms in production animals has led producing countries to gradually reduce the use of AGPs (Brito 2022). In Europe, countries such as Sweden, Denmark and the United Kingdom have abolished the use of any antimicrobial as a growth promoter (Cogliani et al., 2011; Ritchie et al., 2017). Since 1992, Brazil has initiated actions aimed at controlling the use of antimicrobials such as tetracyclines, penicillins, chloramphenicol and systemic sulfonamides in order to comply with international requirements (MAPA). As the years went by, new regulations enacted by the Ministério da Agricultura e Pecuária (MAPA) came into force, such as Regulation No. 11, of 11/24/2004 (Olaquindox); regulation nº 35, of 11/14/2005 (carbadox-based products); regulation nº 14, of 05/17/2012 (spiramycin and erythromycin) and regulation nº 45, of 11/22/2016 (colistin sulfate) (MAPA). The last action was in January 2020, with regulation No. 1, of 01/13/2020, which controls the administration of tylosin, lincomycin and tiamulin, classified as important in human medicine as AGPs.

Currently, the use of the following antimicrobials as AGPs is permitted: virginiamycin (aerobic and anaerobic Gram-positives), flavomycin (Gram-positives), salinomycin (Gram-positives), avilamycin (enteric Gram-positives), zinc bacitracin (Gram-positives) and bacitracin methylene disalicylate - BMD (Gram-positive) (Sato et al., 2022). In addition to the regulations established by MAPA, the “Plano de Ação Nacional de Prevenção e Controle da Resistência aos Antimicrobianos no âmbito da Agropecuária – PAN-BR AGRO” was recently created with the purpose of assessing risks and occurrences of dissemination of resistance to antimicrobials in farm animals in Brazil (MAPA, 2021).

Faced with this problem, preventive measures become essential. Recent work suggests that the human gut microbiota and resistance gene profile may be altered in relation to exposure to the animal husbandry environment, such as swine (Davis, Price, 2016; Sun et al., 2020; Young et al., 2020), which reinforces the theory of occupational transfer of resistant

microorganisms, such as members of the Enterobacteriaceae family, between herd and worker (Davis, Price, 2016; Young et al., 2020; Leangapichart et al., 2021). The works mentioned contribute significantly to the Unified Health approach, highlighting the connection between human, animal and environmental health.

## *Klebsiella* spp.: taxonomy, ecology and projection in animals

The genus *Klebsiella* is composed of bacillary, Gram-negative, polysaccharide capsule-forming, facultatively anaerobic bacteria that belong to the Enterobacteriaceae family (Podschun & Ullmann 1998). Currently, the genus includes 27 species and 8 subspecies (Meier-Kolthoff et al., 2022). The species that make up this genus can be found in different environments and hosts, such as some species of plants, insects and mammals (Wyres & Holt 2018). The species commonly associated with animals and humans are *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Klebsiella varicolla* and *Klebsiella grimontii* (Campos-Madueno et al., 2021; Kugelev et al., 2021). However, *K. pneumoniae* is the main microorganism frequently reported in the literature, because of its involvement with important infections and antimicrobial resistance phenotypes used in human and animal clinical practice.

*Klebsiella pneumoniae* can establish an interspecific ecological commensal relationship with humans and animals through colonization of the intestine and respiratory tract, but it can also be associated with invasive or localized infections, which makes it an emerging opportunistic pathogen (WHO, 2017; Wyres & Holt 2018; Ludden et al., 2020; Zhu et al., 2021). In sick animals, *K. pneumoniae* have causing urinary, enteric, mammary, reproductive and respiratory disorders (Ribeiro et al., 2022). In addition, it may be associated with other clinical conditions such as abscesses, otitis, hepatitis, pyoderma, conjunctivitis and sepsis (Ribeiro et al., 2022).

Molecular epidemiology studies use multilocus sequence typing (MLST) and genome-wide multilocus sequence typing (cgMLST) methods to monitor medically important *K. pneumoniae* isolates and global occurrence (Holt et al., 2015). The methods allow to include *K. pneumoniae* strains in clonal groups (GC) based on the sharing of alleles (≥ 594 of 694) of the core genome with at least one other strain (cgMLST), or by groups that share the same variations in 7 alleles specific genes such as *gapA, infB, mdh, pgi, phoE, rpoB* and *tonB* called MLST (Bialek-Davenet et al., 2014). Clonal groups (CG) represent subpopulations of *K. pneumoniae* that share a common ancestor (Wyres, Lam & Holt 2020). Clones can be differentiated from each other based on the content of accessory genes, which may explain their adaptation to different environments (Holt et al., 2015; Wyres, Lam & Holt 2020).

In South America, the most prevalent GCs in clinical isolates of *K. pneumoniae* are CG258 and CG15, with the sequence type ST258 and ST11 being the most widespread and with the potential to carry genes for resistance to carbapenems and 3rd generation cephalosporin (Mathers, Peirano & Pitout 2015; Wyres, Lam & Holt 2020). The data cited above are in humans, in animals, studies with an epidemiological approach of *K. pneumoniae* strains are scarce, but there are reports of the occurrence of ST25, ST11, and ST290 in sick swine (Bowring et al., 2017; Liu et al. al., 2019; Chang et al., 2021; Leangapichart et al., 2021); ST11 in domestic birds (Zhang et al., 2018) and ST15 in dogs and cats (Marques et al., 2019), all types of sequences are common in humans (Wyres, Lam & Holt 2020). In the current literature, little information on molecular typing and sequenced genomes of *Klebsiella* spp in pigs is available. Resistance and virulence mechanisms are also little explored for representatives of the genus in production animals, such as swine. In this context, the characterization of *Klebsiella* spp of animal origin, members of the normal microbiota in healthy swine, is important to understand their dispersion.

## Population genetics of *Klebsiella pneumoniae*

### Pathogenicity and virulence factors

In the human context, the natural colonization of the host by *K. pneumoniae* occurs by encoding a set of chromosomal genes that code for siderophores, type I and III fimbriae, type VI secretion system (T6SS), capsule, extracellular vesicles (EVs) and lipopolysaccharides (LPS) (Holt et al., 2015; Zhang et al., 2021). The severity of infections caused is associated with the presence of accessory genes involved with adherence and resistance to antimicrobials (Wyres, Lam & Holt 2020).

From a clinical point of view, human isolates of *Klebsiella pneumoniae* can be categorized into two important pathotypes, the so-called classic (cKP) and the hypervirulent (hvKp) (Lan et al., 2020; Joseph et al., 2021; Ye et al., 2021). cKPs strains are categorized as opportunistic pathogens that cause infections in hospitalized patients and are resistant to multiple antimicrobials (Navon-venezia et al., 2017). Infections caused by cKps strains are established in the occurrence of intestinal microbiota dysbiosis events (Joseph et al., 2021). It is known that essential factors for successful colonization and the population increase of cKps strains in the intestine and lung are related to genes that encode polysaccharides of type I and III capsule and fimbriae (Lagrafeuille et al., 2018).

The hvKps strains are considered more virulent than cKps and may be associated with systemic infections acquired in the community, in addition to nosocomial infections (Navon-

Venezia et al., 2017). The main virulence factor associated with the phenotype found in hvKps strains is the type VI secretion system (T6SS), which facilitates intestinal colonization and extra-intestinal dissemination (Joseph et al., 2021). The T6SS secretion system is found in all *K. pneumoniae* however, the combination of virulence genes helps in the success of the infection (Joseph et al., 2021). The production of siderophores such as enterobactin (*ent* gene), yersiniabactin (*ybt* gene), aerobactin (*iuc* gene) and salmochelin (*iro* gene) may be transported with hvKps strains (Joseph et al., 2021). Siderophores are essential for the replication and virulence of opportunistic pathogens in the host and, in *K. pneumoniae*, they are strongly associated with systemic dissemination and sepsis in humans and animals (Namikawa et al., 2022). The presence of *rmpA* (a regulator of mucoid phenotype) and *magA* genes regulate capsule production and have been linked to successful colonization in the host (Russo & Marr 2019).

The presence of mobile genetic element (MGE) as integrative conjugative element (ICE) and/or virulence plasmids significantly contributes to the convergence of cKps and hvKps phenotypes (Wyres, Lam & Holt 2020). Fifteen ICEs are described for *K. pneumoniae*, all carrying genes related to the production of siderophores (yersiniabactin and salmochelin). Some ICEs may also carry the gene for the colibactin toxin (ICE*Kp10*) and the *rmpA* gene that regulates the hypermucoviscosity phenotype (ICE*Kp1*) (Lin et al., 2008; Lam et al., 2018; Liu et al., 2019). In animals, the element ICE*KpSL1* has been described for *K. pneumoniae*, isolated from sick swine ICE*KpSL1* also carries genes that code for the yersiniabactin siderophore (Liu et al., 2019).

Recently, studies with extracellular vesicles (EVs) produced by hvKps and cKp strains revealed their involvement in the pathogenesis of *K. pneumoniae* by generating an immune response with high production of pro-inflammatory cytokines and causing pyroptosis in macrophages (Ye et al., 2021; Zhang et al., 2021).

The dynamics of colonization and infection by clinical strains of *K. pneumoniae* in humans and animals represent the main focus of current studies. Epidemiological data on diseases caused by *K. pneumoniae* cKPs and hvKPs in production animals, such as swine, are scarce and mostly descriptive. In sick animals, *K. pneumoniae* is a causal agent in urinary, enteric, mammary, reproductive and respiratory infections (Ribeiro et al., 2022). In addition, it may be associated with other clinical conditions such as abscesses, otitis, hepatitis, conjunctivitis, pyoderma, encephalitis and sepsis (Bidewell et al., 2018; Ribeiro et al., 2022). The main virulence genotypic markers used to identify hvKp strains refer to genes carried by

these plasmids, such as *iutA* (aerobactin siderophore biosynthesis), *peg-344* (metabolic transporter of unknown function), *iroB* (salmocheline siderophore biosynthesis) and *rmpA* and *rmpA2* (regulators that increase capsule production). However, some strains of low virulence also carry these markers (Wyres, Lam & Holt 2020).

Thus, phenotypic and genotypic characterization studies of *K. pneumoniae* isolates are needed to understand more about the genetic determinants of this opportunistic pathogen in production animals, especially in the context of clinically healthy animals.

**Antimicrobial resistance**

*Klebsiella pneumoniae* is one of the bacteria representing the ESKAPE group (*Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* e *Enterobacter* spp). This group of bacteria has representatives with the potential to "escape" the biocidal action of different classes of antibiotics. Therefore, they are considered critical priority pathogens by the World Health Organization (WHO, 2017).

Multidrug-resistant (MDR) *K. pneumoniae* pose a threat to public health, and an aggravating factor is the growing worldwide spread of pan-drug-resistant (PDR) strains (Magiorakos et al., 2012; Xu et al., 2020; Alghoribi et al., 2021). The main mechanisms associated with resistance may be enzymatic inactivation and/or modification (antimicrobial target), increased efflux (mutations in genes encoding efflux pumps) and porin losses (Holt et al., 2015).

Efflux pumps are membrane proteins that in *K. pneumoniae* may be related to the active excretion of drugs from the intracellular environment, generating resistance to some classes of antimicrobials (Zheng et al. 2018). Overexpression of the AcrAB system, an efflux pump of the RND (resistance-nodulation-division cell) family, may be responsible for resistance to β-lactams, fluoroquinolones, aminoglycosides and tetracyclines in *K. pneumoniae* (Zheng et al. 2018). Mutations in the transcriptional regulatory genes of efflux pumps such as *ramR*, *acrR* and *rpsJ* can also lead to resistance to tigelicin and fluoroquinolones (Chiu et al. 2017). Outer membrane proteins (OMPs) and porins are expressed in high amounts in Gram-negatives and form channels between the lipid bilayers (Holt et al., 2015; Liakopoulos, Mevius, Ceccarelli 2016). In *K. pneumonae* ompK35 and ompK36 control the influx of hydrophilic drugs such as β-lactams and fluoroquinolones, and insertion sequences (IS) can interrupt promoter regions and consequently cause a decrease in the expression of these genes (Wu et al. 2020). Changes in antimicrobial targets, such as lipid A, where changes in its charge lead to a loss of affinity

for polymyxins (Bassetti et al., 2019; Cain et al., 2018; Li et al., 2019). The same occurs with MurA target protein of the antimicrobial fosfomycin (affects cell wall biogenesis) and the topoisomerases *parC* and *gyrA* in this case, targets of fluoroquinolones (Xu et al., 2019; Nicolas-chanoine et al., 2018; Li et al., 2019).

Another mechanism is alteration of the drug by inactivating enzymes produced by the bacteria (Wyres, Lam & Holt 2020). In *K. pneumoniae*, an example is the B-lactamase enzymes that hydrolyze the B-lactam ring of antimicrobials (Li et al., 2022). B-lactamases are enzymes associated with resistance to penicillins, cephalosporins, and carbapenems (Li et al., 2022). Many other genes can encode products that are capable of altering the target molecule, such as *mcr-1* (colistin), *qnrABDS* (fluoroquinolones), *ereAB* (macrolides), *catA* (chloramphenicol), *sul1* (sulfonamides), *tetA* (tetracycline), $bla_{\text{TEM1/OXA1}}$ (carbapenemase) and *dfrA* (trimethoprim) (Li et al., 2022).

In general, in *K. pneumonaie* the genes responsible for antimicrobial resistance can be of chromosomal origin or acquired by horizontal gene transfer (HGT) (Holt et al., 2015). HGT can occur by conjugation, transduction, transformation, or vesiduction (Soler & Forterre 2020; Dell'annunziata et al., 2021). The process of vesiduction consists of packaging genetic material by extracellular vesicles (EVs) and transferring it to a recipient bacterium (Soler & Forterre 2020). It has been shown that EVs produced by *K. pneumoniae* can transfer resistance genes to intraspecies β-lactams and even to other members of the ESKAPE group (Gill et al., 2019; Dell'annunziata et al., 2021). However, further studies are needed to understand molecular aspects of vesiduction in the transfer of resistance and virulence genes in *K. pneumoniae.*

The scenario of antimicrobial resistance non-clinical isolates of *K. pneumoniae* from animals, especially those for production, such as swine, is still not comprehensive, which highlights the importance of further studies.

## Referências

Associação Brasileira de Proteína Animal, ABPA. Relatório Anual, 2023.

Barton, M. D. Impact of antibiotic use in the swine industry. *Current opinion in microbiology*. **2014**, *19*, 9-15. DOI:10.1016/j.mib.2014.05.017

Bassetti, M., Peghin, M., Vena, A., Giacobbe, D. R. Treatment of infections due to MDR Gram-negative bacteria. *Frontiers in medicine*. **2019**, *6*, 74. DOI:10.3389/fmed.2019.00074

Bialek-Davenet, S., Criscuolo, A., Ailloud, F., Passet, V., Jones, L., Delannoy-Vieillard, A. S., Brisse, S. Genomic definition of hypervirulent and multidrug-resistant *Klebsiella pneumoniae* clonal groups. *Emerging infectious diseases*. **2014**, *20*(11), 1812. DOI:10.3201/eid2011.140206

Bidewell, C. A., Williamson, S. M., Rogers, J., Tang, Y., Ellis, R. J., Petrovska, L., AbuOun, M. Emergence of *Klebsiella pneumoniae* subspecies pneumoniae as a cause of septicaemia in pigs in England. *PLoS One*. **2018**, *13*(2), e0191958. DOI:10.1371/journal.pone.0191958

Bowring, B. G., Fahy, V. A., Morris, A., Collins, A. M. An unusual culprit: *Klebsiella pneumoniae* causing septicaemia outbreaks in neonatal pigs?. *Veterinary microbiology*. **2017**, *203*, 267-270. DOI:10.1016/j.vetmic.2017.03.018

Burow, E., Rostalski, A., Harlizius, J., Gangl, A., Simoneit, C., Grobbel, M., Käsbohrer, A. Antibiotic resistance in *Escherichia coli* from pigs from birth to slaughter and its association with antibiotic treatment. *Preventive veterinary medicine*. **2019**, *165*, 52-62. DOI:10.1016/j.prevetmed.2019.02.008

Bruchmann, S., Feltwell, T., Parkhill, J., Short, F. L. Identifying virulence determinants of multidrug-resistant *Klebsiella pneumoniae* in *Galleria mellonella*. *Pathogens and disease*. **2021**, *79*(3), ftab009. DOI:10.1093/femspd/ftab009

Cain, A. K., Boinett, C. J., Barquist, L., Dordel, J., Fookes, M., Mayho, M., Thomson, N. R. Morphological, genomic and transcriptomic responses of *Klebsiella pneumoniae* to the last-line antibiotic colistin. *Scientific reports*. **2018**, *8*(1), 9868. DOI:10.1038/s41598-018-28199-y

Campos-Madueno, E. I., Moser, A. I., Risch, M., Bodmer, T., & Endimiani, A. Exploring the global spread of *Klebsiella grimontii* isolates possessing *bla*$_{\text{VIM-1}}$ and *mcr-9*. *Antimicrobial agents and chemotherapy*. **2021**, *65*(9), 10-1128. DOI:10.1128/AAC.00724-21

Cogliani, C., Goossens, H., Greko, C. Restricting antimicrobial use in food animals: lessons from Europe. *Microbe*. **2011**, *6*(6), 274. DOI:10.1128/MICROBE.6.274.1

Chiu, S. K., Chan, M. C., Huang, L. Y., Lin, Y. T., Lin, J. C., Lu, P. L., Yeh, K. M. Tigecycline resistance among carbapenem-resistant *Klebsiella pneumoniae*: clinical characteristics and expression levels of efflux pump genes. *PloS one*. **2017**, 12(4), e0175140. DOI:10.1371/journal.pone.0175140

Davis, G. S., and Price, L. B. Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Current environmental health reports*. **2016**, *3*, 128-135. DOI:10.1007/s40572-016-0089-9

Dell'Annunziata, F., Dell'Aversana, C., Doti, N., Donadio, G., Dal Piaz, F., Izzo, V., Franci, G. Outer membrane vesicles derived from *Klebsiella pneumoniae* are a driving force for horizontal gene transfer. *International Journal of Molecular Sciences*. **2021**, *22*(16), 8732. DOI:10.1101/2021.07.06.451238

Fang, H., Han, L., Zhang, H., Long, Z., Cai, L., Yu, Y. Dissemination of antibiotic resistance genes and human pathogenic bacteria from a pig feedlot to the surrounding stream and agricultural soils. *Journal of hazardous materials*. **2018**, *357*, 53-62. DOI:10.1016/j.jhazmat.2018.05.066

Fischer, A., Barros, E., Machado, I., Miele, M., Pinheiro, R., de Medeiros, S. A. F., Guth, T. Novos caminhos na suinocultura: uma visão ampla e atual dos desafios e oportunidades da gestão nas granjas. 2019.

Gavioli, D. F., de Oliveira, E. R., da Silva, A. A., Romero, N. C., Lozano, A. P., Silva, R. A. M., da Silva, C. A. Efeito de promotores de crescimento para suínos sobre o desempenho zootécnico, a qualidade intestinal e a eficiência da biodigestão dos dejetos. *Semina: Ciências Agrárias*. **2013**, *34*(2), 3983-3997. DOI: 10.5433/1679-0359.2013v34n6Supl2p3983

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Thomson, N. R. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Sciences*. **2015**, *112*(27), E3574-E3581. DOI:10.1073/pnas.1501049112

Joseph, L., Merciecca, T., Forestier, C., Balestrino, D., Miquel, S. From *Klebsiella pneumoniae* colonization to dissemination: an overview of studies implementing murine models. *Microorganisms*. **2021**, *9*(6), 1282. DOI:10.3390/microorganisms9061282

Kugelev, I. M., Kapay, N. A., Davydenkova, O. V., Russkin, A. S. (2021, March). The effectiveness of various antibacterial drugs in the treatment of swine respiratory diseases. In *IOP Conference Series: Earth and Environmental Science* (Vol. 723, No. 2, p. 022065). IOP Publishing. DOI:10.1088/1755-1315/723/2/022065

Lam, M. M., Wick, R. R., Wyres, K. L., Gorrie, C. L., Judd, L. M., Jenney, A. W., Holt, K. E. Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICEKp in *Klebsiella pneumoniae* populations. *Microbial genomics*. **2018**, *4*(9). DOI:10.1099/mgen.0.000196

Lan, P., Shi, Q., Zhang, P., Chen, Y., Yan, R., Hua, X., Yu, Y. Core genome allelic profiles of clinical *Klebsiella pneumoniae* strains using a random forest algorithm based on multilocus sequence typing scheme for hypervirulence analysis. *The Journal of infectious diseases*. **2020**, *221*(Supplement_2), S263-S271. DOI:10.1093/infdis/jiz562

Lagrafeuille, R., Miquel, S., Balestrino, D., Vareille-Delarbre, M., Chain, F., Langella, P., Forestier, C. Opposing effect of *Lactobacillus* on in vitro *Klebsiella pneumoniae* in biofilm and in an in vivo intestinal colonisation model. *Beneficial Microbes*. **2018**, *9*(1), 87-100. DOI:10.3920/BM2017.0002

Leangapichart, T., Lunha, K., Jiwakanon, J., Angkititrakul, S., Järhult, J. D., Magnusson, U., Sunde, M. Characterization of *Klebsiella pneumoniae* complex isolates from pigs and humans in farms in Thailand: population genomic structure, antibiotic resistance and virulence genes. *Journal of Antimicrobial Chemotherapy*. **2021**, *76*(8), 2012-2016. DOI:10.1093/jac/dkab118

Li, Y., Kumar, S., Zhang, L., Wu, H Klebsiella pneumonia and its antibiotic resistance: a bibliometric analysis. *BioMed Research International*. **2022**. DOI: 10.1155/2022/1668789

Liu, D., Yang, Y., Gu, J., Tuo, H., Li, P., Xie, X., Zhang, A. The Yersinia high-pathogenicity island (HPI) carried by a new integrative and conjugative element (ICE) in a multidrug-resistant and hypervirulent *Klebsiella pneumoniae* strain SCsl1. *Veterinary microbiology*. **2019**, *239*, 108481. DOI:10.1016/j.vetmic.2019.108481

Liakopoulos, A., Mevius, D., Ceccarelli, D. A review of SHV extended-spectrum β-lactamases: neglected yet ubiquitous. *Frontiers in microbiology*. **2016**, *7*, 1374. DOI:10.3389/fmicb.2016.01374

Ludden, C., Moradigaravand, D., Jamrozy, D., Gouliouris, T., Blane, B., Naydenova, P., Peacock, S. J. A One Health study of the genetic relatedness of *Klebsiella pneumoniae* and their mobile elements in the East of England. *Clinical Infectious Diseases*. **2020**, *70*(2), 219-226. doi.org/10.1093/cid/ciz174

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Monnet, D. L. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection*. **2012**, *18*(3), 268-281. DOI:10.1111/j.1469-0691.2011.03570.x

Mathers, A. J., Peirano, G., Pitout, J. D. The role of epidemic resistance plasmids and international high-risk clones in the spread of multidrug-resistant Enterobacteriaceae. *Clinical microbiology reviews*. **2015**, *28*(3), 565-591. DOI:10.1128/CMR.00116-14

Marques, C., Menezes, J., Belas, A., Aboim, C., Cavaco-Silva, P., Trigueiro, G., Pomba, C. *Klebsiella pneumoniae* causing urinary tract infections in companion animals and humans: population structure, antimicrobial resistance and virulence genes. *Journal of Antimicrobial Chemotherapy*. **2019**, *74*(3), 594-602. DOI:10.1093/jac/dky499

Moeser, A. J., Pohl, C. S., & Rajput, M. Weaning stress and gastrointestinal barrier development: Implications for lifelong gut health in pigs. *Animal Nutrition*. **2017**, *3*(4), 313-321. DOI: 10.1016/j.aninu.2017.06.003

Navon-Venezia, S., Kondratyeva, K., Carattoli, A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS microbiology reviews*. **2017**, *41*(3), 252-275. DOI:10.1093/femsre/fux013

Nicolas-Chanoine, M. H., Mayer, N., Guyot, K., Dumont, E., Pagès, J. M. Interplay between membrane permeability and enzymatic barrier leads to antibiotic-dependent resistance in *Klebsiella pneumoniae*. *Frontiers in microbiology*. **2018**, *9*, 1422. DOI:10.3389/fmicb.2018.01422

Plano de Ação Nacional de Prevenção e Controle da Resistência aos Antimicrobianos, no Âmbito da Agropecuária – PAN-BR AGRO. Ministério da Agricultura, Pecuária e Abastecimento (MAPA). Acesso: https://portalarquivos2.saude.gov.br/ Acesso em: 14 de junho 2021.

Podschun, R., and Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical microbiology reviews*. **1998**, *11*(4), 589-603. DOI:10.1128/CMR.11.4.589

Rice, L. B. Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *The Journal of infectious diseases*. **2008**, *197*(8), 1079-1081. DOI:10.1086/533452

Ritchie, H. How do we reduce antibiotic resistance from livestock? Our World in Data. 2017. *Our World in Data*.

Russo, T. A. and Marr, C. M. Hypervirulent *Klebsiella pneumoniae*. *Clinical Microbiology Reviews*. **2019**, v. 32, n. 3, p. e00001-19. DOI:10.1128/CMR.00001-19

Russo, T. A., Olson, R., Fang, C. T., Stoesser, N., Miller, M., MacDonald, U., Johnson, J. R. Identification of biomarkers for differentiation of hypervirulent *Klebsiella pneumoniae*

from classical *K. pneumoniae*. *Journal of clinical microbiology*. **2018**, *56*(9), 10-1128. DOI:10.1128/jcm.00776-18

Silva, G. S., Leotti, V. B., Castro, S. M., Medeiros, A. A., Silva, A. P., Linhares, D. C., Corbellini, L. G. Assessment of biosecurity practices and development of a scoring system in swine farms using item response theory. *Preventive veterinary medicine*. **2019**, *167*, 128-136. DOI:10.1016/j.prevetmed.2019.03.020

Sofiana, E. D., Pratama, J. W. A., Effendi, M. H., Plumeriastuti, H., Wibisono, F. M., Hartadi, E. B., Hidayatullah, A. R. A review of the presence of antibiotic resistance problems on *Klebsiella pneumoniae* acquired from pigs: Public health importance. *Systematic Reviews in Pharmacy*. **2022**, *11*(9), 535-543. DOI:10.31838/srp.2020.9.14

Soler, N., and Forterre, P. Vesiduction: the fourth way of HGT. *Environmental Microbiology*. **2020**, *22*(7), 2457-2460. DOI:10.1111/1462-2920.15056

Sun, J., Liao, X. P., D'Souza, A. W., Boolchandani, M., Li, S. H., Cheng, K., Liu, Y. H. Environmental remodeling of human gut microbiota and antibiotic resistome in livestock farms. *Nature communications*. **2020**, *11*(1), 1427. DOI:10.1038/s41467-020-15222-y

Stella, A. E., de Oliveira, A. F., Moreira, C. N., Viali, K. G. Uso de antimicrobianos na suinocultura: indicações e restrições. *Veterinária e Zootecnia*, *27*. **2020**, 1-14. DOI: 10.35172/rvz.2020.v27.492

Tokach, M. D., & Dial, G. D. Managing the lactating sow for optimal weaning and rebreeding performance. *Veterinary Clinics of North America: Food Animal Practice*. **1992**, *8*(3), 559-573. DOI: 10.1016/S0749-0720(15)30704-0

United States Department of Agriculture (USDA). 2021. Livestock and Poultry: World Markets and Trade. https://www.fas.usda.gov/data/livestock-and-poultry-world-markets-and-trade Acesso em: 18 de julho de 2023.

Vieites, F. M., Souza, C. S., Castro, A. C. S., de Melo Júnior, A. M., Ferreira, M. H., Ferreira, S. E., Oliveira, G. P. Aditivos zootécnicos na alimentação de suínos–Revisão de Literatura. *Brazilian Journal of Development*. **2020**, *6*(7), 45880-45895. DOI:10.34117/bjdv6n7-276

Wang, X., Tsai, T., Deng, F., Wei, X., Chai, J., Knapp, J., Zhao, J. Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria. *Microbiome*. **2019**, *7*(1), 1-18. DOI: 10.1186/s40168-019-0721-7

Worldwatch Institute. 2023. https://www.statista.com/statistics/237632/production-of-meat-worldwide-since-1990/ Acesso em: 18 de julho de 2023.

Wu, L. T., Guo, M. K., Ke, S. C., Lin, Y. P., Pang, Y. C., Nguyen, H. T. V., Chen, C. M. Characterization of the genetic background of KPC-2-Producing Klebsiella pneumoniae with Insertion Elements Disrupting the ompK36 Porin Gene. *Microbial Drug Resistance*. **2020**, 26(9), 1050-1057. DOI:101089/mdr.2019.0410

Wyres, K. L., Wick, R. R., Judd, L. M., Froumine, R., Tokolyi, A., Gorrie, C. L., Holt, K. E. Distinct evolutionary dynamics of horizontal gene transfer in drug resistant and virulent clones of *Klebsiella pneumoniae*. *PLoS genetics*. **2019**, *15*(4), e1008114. DOI:10.1371/journal.pgen.1008114

Wyres, K. L., Lam, M. M., Holt, K. E. Population genomics of *Klebsiella pneumoniae*. *Nature Reviews Microbiology*. **2020**, *18*(6), 344-359. DOI:10.1038/s41579-019-0315-1

Wyres, K. L. and Holt, K. E. *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current opinion in microbiology*. **2018**, *45*, 131-139. DOI:10.1016/j.mib.2018.04.004

Xu, Q., Jiang, J., Zhu, Z., Xu, T., Sheng, Z. K., Ye, M., Wang, M. Efflux pumps AcrAB and OqxAB contribute to nitrofurantoin resistance in an uropathogenic *Klebsiella pneumoniae* isolate. *International journal of antimicrobial agentes*. **2019**, *54*(2), 223-227. DOI:10.1016/j.ijantimicag.2019.06.004

Yan, H., Zhang, L., Guo, Z., Zhang, H., & Liu, J. Production phase affects the bioaerosol microbial composition and functional potential in swine confinement buildings. *Animals*. **2019**, *9*(3), 90. DOI: 10.3390/ani9030090

Yang, F., Deng, B., Liao, W., Wang, P., Chen, P., Wei, J. High rate of multiresistant *Klebsiella pneumoniae* from human and animal origin. *Infection and Drug Resistance*. **2019**, 2729-2737. DOI:10.2147/IDR.S219155

Young, T. M., Bray, A. S., Nagpal, R. K., Caudell, D. L., Yadav, H., Zafar, M. A. Animal model to study *Klebsiella pneumoniae* gastrointestinal colonization and host-to-host transmission. *Infection and immunity*. **2020**, *88*(11), 10-1128. DOI:10.1128/IAI.00071-20

Zhang, J., Zhao, J., Li, J., Xia, Y., Cao, J. Outer membrane vesicles derived from hypervirulent *Klebsiella pneumoniae* stimulate the inflammatory response. *Microbial Pathogenesis*. **2021**, *154*, 104841. DOI:10.1016/j.micpath.2021.104841

Zhang, S., Yang, G., Ye, Q., Wu, Q., Zhang, J., Huang, Y. Phenotypic and genotypic characterization of *Klebsiella pneumoniae* isolated from retail foods in China. *Frontiers in microbiology*. **2018**, *9*, 289. DOI:10.3389/fmicb.2018.00289

Zheng, J. X., Lin, Z. W., Sun, X., Lin, W. H., Chen, Z., Wu, Y., Yu, Z. J. Overexpression of OqxAB and MacAB efflux pumps contributes to eravacycline resistance and heteroresistance in clinical isolates of *Klebsiella pneumoniae*. *Emerging microbes & infections*. **2018**, *7*(1), 1-11. DOI:10.1038/s41426-018-0141-y

Zhu, J., Wang, T., Chen, L., Du, H. Virulence factors in hypervirulent *Klebsiella pneumoniae*. *Frontiers in microbiology*. **2021**, *12*, 642484. DOI:10.3389/fmicb.2021.642484

## Chapter 1

### *Klebsiella* spp in clinically healthy swine: antimicrobial resistance and virulence factors

(Manuscript written according to the guidelines of the Microorganisms - an Open Access Journal from MDPI)

Jéssica Nogueira Rosa[1]; Giarlã Cunha da Silva[1]; Patrícia Pereira Fontes[1]; Matheus Machado Guidini[1]; Rúzivia Pimentel Oliveira[1]; Denise Mara Soares Bazzolli[1*]

[1]Laboratório de Genética Molecular de Bactérias, Departamento de Microbiologia, Instituto de Biotecnologia Aplicada à Agropecuária - Bioagro, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

*Corresponding author: dbazzolli@ufv.br

**Abstract**

Production animals such as swine can be important reservoirs of opportunistic pathogens, since many bacteria with this characteristic are part of their microbiota, such as *Klebsiella* spp. *Klebsiella* spp from animals may have genetic determinants for antimicrobial resistance and different virulence factors. The current scenario presents few studies involving the profile of *Klebsiella* spp in healthy animals. Thus, the objective of this work was to investigate the phenotype and genotype of resistance and virulence of *Klebsiella* spp isolated from the rectum of healthy swine (growth phase II) with and without the addition of antimicrobials and under stress condition. As a result, we obtained 144 isolates of *Klebsiella* spp. About 77 % were *K. pneumoniae*, 14.5 % *K. aerogenes* and 8.5 % *K. variicola*. We mapped isolates of *Klebsiella* spp with the same genetic profile in different animals and stalls. We found resistance to the following classes of antimicrobials: β-lactams (100%), cephalosporins (75.5%), fluoroquinolones (51%), amphenicol (48.8%), tetracycline (55.5%), polymyxin (37.7%), sulfonamide (35.5%), aminoglycoside (8.8%), trimethoprim (4.5%%) and no resistance to carbapenems. Overall, 68.8% of the isolates are multidrug-resistant (MDR). Important virulence genetic markers were detected as to siderophore production (*entB, iucB, iutA, kfu, and ybtS/irp2*). To biofilm formation 39% of the isolates were classified as strong producers, 45% as moderate, and 16% as weak producers. None of the isolates were classified to hypermucoviscous phenotype. We selected 11 *K. pneumoniae* MDR to investigate and the sequence type (STs) identified were ST25, ST147, ST616, ST691, and ST6208. We compared the virulence and persistence of these 11 isolates in *Galleria mellonella* model. The behavior of the 11 bacteria was similar to virulence and persistence parameters. Most isolates showed

the virulence phenotype and increased multiplication potential in the initial hours of infection in *G. mellonella* compared to Kp13 from human. However, after 24 hours of infection some *K. pneumoniae* isolates had their populations reduced inside the larva. We can conclude with this work that healthy swine are reservoirs of *Klesiella* spp MDR and relevant virulence phenotypes, and the sanitary challenge can influence the dispersion of *Klebsiella* spp. between different animals and treatments.

**Keywords:** Enterobacteriaceae; healthy clinically swine; multidrug resistant

## Introduction

The genus *Klebsiella* is composed of bacillary, Gram-negative, encapsulated bacteria and belongs to the Enterobacteriaceae family (Podschun & Ullmann, 1998). It harbors a wide range of species with different ecological niches, and are found in diverse environments and hosts, such as plants, insects, and mammals (Kugelev et al., 2021; Dong et al., 2022). Currently, the genus comprises 27 species and 8 subspecies (Meier-Kolthoff et al., 2022). Species of the genus commonly described in animals and humans are *Klebsiella pneumoniae*, *Klebsiella variicola*, *Klebsiella oxytoca*, *Klebsiella aerogenes* and *Klebsiella grimontii* (Campus-Madueno et al., 2021).

The growing reports of dissemination of resistance and virulence genes among opportunistic pathogens list, *K. pneumoniae* as one of the most studied species of the genus and grouped as ESKAPE (*Enterococcus faecium, Staphylococcus aureus, K. pneumoniae, Acinetobacter baumannii, Pseudomonas aeruginosa* e espécies de *Enterobacter* spp) (Rice, 2008). The ESKAPE group is composed of bacteria with a high potential to escape the biocidal action of different classes of antimicrobials (Rice, 2008; Navon-Venezia, 2017).

*Klebsiella pneumoniae* has a chromosomal gene arsenal associated with its virulence as genes encoding siderophores (*entB*), type I and III fimbriae, lipopolysaccharides (LPS) and capsule (Wyres, Lam & Holt, 2020). The intense gene flow of mobile genetic elements (MGE) between species of the genus and other Enterobacteriaceae impacted the evolutionary context and contributed to the emergence of two pathotypes, *Klebsiella* spp classic (cKP) and hypervirulent (hvKp) (Joseph et al., 2021). Classical *K. pneumoniae* (cKP) are strains described as opportunistic pathogens that cause nosocomial infections and have a high gene load of antimicrobial resistance, either by the acquisition of MGE or accumulation of mutations (Shankar et al., 2019; Wyres et al., 2020; Qi et al., 2023). On the other hand, hvKp are associated with the ability to cause severe infections in healthy and/or immunocompromised

hosts (Russo & Marr 2019). Thus, they are more virulent than cKp, and can cause systemic infections acquired in the community (Mukherjee et al., 2023). In many cases, hvKp has a hypermucoviscous phenotype related to overexpression of genes related to capsule production (*rmpA, rmpA2* and *magA*) (Mukherjee et al., 2023). The hvKp phenotype is a consequence of the acquisition of virulence plasmids (Russo et al., 2018) and integrative and conjugative elements (ICEs) that can carry genes that encode different types of siderophores (yersiniabactin, salmochelin, and aerobactin) and capsule regulators gene (Lin et al., 2008; Mukherjee et al., 2023).

Despite the divergence of these populations in the evolutionary context, currently, the convergence of hvKp and cKp phenotypes is frequently reported in Asia, Europe and growing in South America including Brazil (Gu et al., 2018; Wyres, Lam & Holt, 2020). Molecular epidemiology studies have used multilocus sequence typing (MLST) to monitor the spread of emerging clones of *K. pneumoniae* on a global scale (Diancourt et al., 2005). *Klebsiella pneumoniae* cKp is related to STs such as ST11, ST147, and ST15 (Wyres, Lam & Holt, 2020). cKp clones are globally disseminated in hospital environments and are related to the increase in the frequency of infections in these environments (Chang et al., 2021). For hvKp clones, the STs disseminated on a global scale are ST307 and ST258 (Wyres, Lam & Holt, 2020). The clones hvKp are associated with sepsis, pneumonia, and hepatic abscesses in humans (Chang et al., 2021).

In sick animals, *K. pneumoniae* causes enteric, mammary, reproductive and respiratory infections (Ribeiro et al., 2022). In addition, they can cause other clinical conditions such as abscesses, otitis, conjunctivitis, sepsis and encephalitis (Ribeiro et al., 2022). Little is known about the population characteristics of *K. pneumoniae* in healthy farm animals, although for sick animals it is possible to detect some STs such as ST25 in swine, ST11 in poultry, ST23 in horses, and ST15 in companion animals (Wyres, Lam & Holt, 2020; Ribeiro et al., 2022).

The current scenario presents few studies involving the profile of *Klebsiella* spp in healthy animals, making it difficult for us to understand other representative species of the genus. Thus, the objective of this work was to investigate the phenotype and genotype of resistance and virulence of *klebsiella* spp isolated from the rectum of clinically healthy swine with and without the addition of antimicrobial and under sanitary challenge.

## Materials and methods

### Ethical considerations

All experimental procedures involving animal handling were performed in accordance with the guidelines of the Ethics Committee on the Use of Production Animals of the Universidade Federal de Viçosa (CEUAP-UFV N°27/2021).

### Samples collection and isolation of *Klebsiella* spp

Collections were performed using the swab technique (Choudhury et al., 2019) and samples were obtained from the rectal region of 96 clinically healthy animals in the growth phase II. The genetics DanBred animals were kept under an "all in-all out" management system and fed with a commercial diet (Nutron Cargill), added (treatment 1, n=48) or not (treatment 2, n=48) of 55 ppm zinc bacitracin and 5 ppm enramycin. The animals remained under sanitary challenge until the time of collection. After collection, the swabs were immediately transferred to tubes containing Stuart transport medium (KASVI, SÃO PAULO, SP, BRASIL). The swabs were exhausted into Petri dishes containing Simmons Citrate agar (HIMEDIA, MUMBAI, MAHARASHTRA, INDIA) added with 1% inositol (SIGMA, ST. LOUIS, MO, USA) and subsequently incubated at 37°C for 48 hours (Van et al., 1984). Colonies with the phenotypic appearance of *Klebsiella* spp were isolated and stored in Brain Heart Infusion broth (BD, FRANKLIN LAKES, NEW JERSEY, USA) with 25% glycerol and kept at -80°C. Another strain used in some experiments was *K. pneumoniae* Kp13 (Ramos et al., 2012).

### Identification of *Klebsiella* spp

Species identification was performed by sequencing the 16S ribosomal gene. To obtain the amplicon, primers 27F and 1492R (Lane, 1991) were used. The 25ul reactions were standardized with: Platinum Taq High Fidelity Buffer 1X; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.2 uM of each primer; 1.25 U of Taq DNA polymerase enzyme (PROMEGA, MEDISON, WISCONSIN, USA) and 25ng of DNA. The conditions for amplification of the fragments were: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 95°C for 1 minute, 61°C for 30 seconds and 72°C for 1 minute and 30 seconds, with final elongation for 10 minutes at 72°C. The amplicons had both DNA strands sequenced by the company Macrogen (SEOUL, SOUTH KOREA). The sequencing results were analyzed by BLAST sequence search (Basic Local Alignment Search Tool) using the NCBI (National Center for Biotechnology Information) database.

**Genetic polymorphism**

Evaluation of the genetic profile of the isolates was performed by the fingerprint technique using the primer BOXA1R (5'-CTACGGCAAGGCGACGCTGACG-3') (Versalovic et al., 1994). The 25ul reaction for amplification contained the following reagents: Platinum Taq High Fidelity Buffer 1X; 1.5 mM $MgCl_2$; 0.2 mM dNTPs; 0.5 uM primer; 1.25U of Taq DNA polymerase enzyme (PROMEGA, MEDISON, WISCONSIN, USA) and 25 ng of DNA. Amplification was performed using the following cycles: initial denaturation for 5 minutes at 95°C, followed by 35 cycles of 95°C for 1 minute, 50°C for 1 minute, and 68°C for 7 minutes, with a final elongation of 10 minutes at 68°C. Amplified samples were evaluated by 1.8% agarose gel electrophoresis.

**Dendrogram construction**

With the results of the fragments amplified with the primer BOXA1R, a binary matrix was constructed, with a value of (1) being assigned to the fragments for the presence or (0) for the absence. The binary matrix was submitted to a similarity analysis using the Jaccard coefficient and the unweighted pair group method with arithmetic mean – UPGMA was used for constructing the dendrogram using the PaSt statistical software (Hammer, Harper & Ryan, 2001).

**Hemolysis test**

To evaluate hemolytic activity (Quiblier et al., 2011) Petri dishes containing 5% sheep blood agar (LABORCLIN, MINAS GERAIS, BRASIL) were used. For each isolate, composite streaks were performed to obtain isolated colonies. Cultures were incubated at 37°C for 24 hours. The hemolysis pattern was evaluated as α-hemolysis, ß-hemolysis, or γ-hemolysis.

**String test**

To identify the hypermucoviscous phenotype, a string test was performed (Lee et al., 2006). The isolates were grown on 5% sheep blood agar at 37°C for 24 hours. Subsequently, with an inoculation loop, the colonies were gently touched and lifted. The test was positive when a mucosal cord ≥ 5mm was formed.

**Production of siderophores**

The production of siderophores was determined by the chromium azurol S assay (Louden, Haarmann & Lynne 2011). The glasswares were treated with 6M HCl for 2 hours and then rinsed with ultrapure water to remove any traces of iron. Discs of 8 mm diameter of sterile

filter paper were added under the Petri dishes containing the solidified culture medium. Subsequently, the isolates were grown overnight in Mueller Hilton II broth and adjusted to $DO_{600}$ 0,5. A 10ul aliquot was added above the filter paper discs deposited in the culture medium. The inoculated CAS agar plates were incubated at 37°C for 24 hours. The level of siderophore production was determined by measuring the diameter of the halo formed. The experiments were carried out in biological triplicates.

**Adhesion in abiotic surface**

Biofilm production in a 96-well microplate was performed as previously described (Stepanović et al., 2007) with some modifications. An initial $OD_{600}$ inoculum of 0.01 was prepared in 5 mL TSB overnight at 37°C. From the overnight culture, we added 2ul in 198 uL (1:100) of TBS broth to the microplates and incubated them at 37°C for 24 hours. After the determined time, the culture medium was removed and the wells were washed 2 times with 200 uL of phosphate-buffered saline (PBS). 200 uL of 0.1% crystal violet was added for 30 minutes. The plates were washed with distilled water. Subsequently, 200 uL of 95% ethanol was added for 40 minutes. The biofilm was transferred to another microplate and quantified on a Multiskan FC plate reader (THERMO SCIENTIFIC, WALTHAM, MASSACHUSETTS, EUA) at a wavelength of 570 nm. The isolates were classified as weak, moderate, and strong producers (Stepanović et al., 2007). Thus, the optical density cut-off (ODc) was assigned as a mean OD of negative controls + (3× standard deviation (SD) of negative controls). Isolates with DO ≤ DOc were categorized as non-biofilm producers. The other parameters used were for the weak producer if ODc < OD ≤ 2 × ODc; moderate producer 2 × ODc < OD ≤4 × ODc; and strong producer if OD > 4 × ODc. The experiments were carried out in biological triplicates.

**Detection of virulence genes**

The isolates were inspected for the presence of virulence genes: *rmpA, rmpA2, iutA, iroNB, peg344, maga, kfu, ybtS, irp2, entB, iucB, mrkD, fimH, ycfm, cf29, clbA, clbQ, clbN, k2a, k20, k5a, k54, k57, allS, ugeA, wcaG, wabG, hly, ureA*. PCRs for the genes was performed with the primers listed in supplementary table 2.

**Antimicrobial susceptibility test**

The antimicrobial susceptibility of *Klebsiella* spp (n=45) was determined by the diffusion disc method according to Clinical and Laboratory Standards Institute guidelines (CLSI VET01S ED5:2020). For antimicrobials not used in animals, the CLSI criteria (2016)

were used. Ten classes of antimicrobials were tested, covering the following antimicrobials: cefepime (30ug); cefitriaxone (30 ug); cefotaxime (30 ug); meropenem (10ug), imipemen (10ug); aztreonam (30ug); ampicillin (10ug); amoxicillin (10ug); oxacillin (1ug); enroflorxacin (5ug); ciproflorxacin (5ug); levoflorxacin (5ug); sulfonamide (300Mcg); colistin (10ug); chloramphenicol (30ug); tetracycline (30ug); streptomycin (10ug) and trimethoprim (5ug). Interpretation of results for colistin was based on CLSI (2006) cutoffs established for the closest organism and for reproducibility of our screening assays, colistin susceptibility was also assessed by agar dilution as described by Turlej-Rogacka and collaborators (2018). The control strain used was *E. coli* ATCC25922. The experiments were carried out in biological duplicates. The isolates were classified into multidrug-resistant – MDR (Magiorakos et al. 2012) and evaluated the multiple antimicrobial resistance index – IRMA (Krumperman, 1983).

**Resistance gene detection**

The isolates were inspected for the presence of resistance genes: *bla*$_{\text{OXA}}$, *bla*$_{\text{OXA-2}}$, *bla*$_{\text{OXA-23}}$, *bla*$_{\text{OXA48}}$, *bla*$_{\text{CTX-M}}$, *bla*$_{\text{CTX-M-1}}$, *bla*$_{\text{CTX-M-2}}$, *bla*$_{\text{CTX-M-8}}$, *bla*$_{\text{CTX-M-9}}$, *bla*$_{\text{IMP}}$, *bla*$_{\text{NDM,}}$ *bla*$_{\text{VIM}}$, *ampC*, *mcr-1, sul2, dfrA, qnrS tetB, strA, erm, ant, aac, fosA, ereA* e *cat*. PCRs for the genes was performed with the primers listed in supplementary table 3.

**Multilocus Sequence Typing (MLST)**

The *rpoB, gapA, mdh, pgi, phoE, infB* and *tonB* genes from 11 *K. pneumoniae* isolates were amplified by PCR with primers and protocols available on the Pasteur Institute and University College Cork website (https://bigsdb.pasteur. fr/klebsiella/klebsiella.html). The amplicons had both DNA strands sequenced by the company Macrogen (SEOUL, SOUTH KOREA). Subsequently, we submitted the set of amplicons to the public database Pasteur Institute and University College.

**Virulence assay using *Galleria mellonella* as an alternative infection model**

**Survival analysis**

The larvae of *G. mellonella* (Lepdoptera: Pyralidae) used were kept in the Laboratory of Molecular Genetics of Bacteria, Bioagro, UFV in a continuous production system. The larvae remained with a restricted diet based on brewer's yeast, soy flour, honey, glycerin, and beeswax. The experiment was carried out with larvae in their last instar. Bacterial strains were grown in Luria Bertani broth (LB) to $OD_{600}$ 0,5. Subsequently, $1x10^4$ CFU was applied to the penultimate pseudopod of the larva using the SGE 25ul syringe (TRAJAN SCIENTIFIC AND MEDICAL).

The larvae were left in the oven at 37°C and monitored at 24, 48, 72, and 96 hours after infection. Larvae that showed no stimulus to touch were considered dead. All tests were performed with biological and experimental triplicates (n=10 caterpillars per experimental replicate). Survival curves were plotted using the Kaplan-Meier method (Bland and Altman 1998) and differences in survival were calculated using the log-rank test, with a significance level of 1% probability, using the software GNUMERIC© (GNU General Public License). Negative controls were larvae inoculated with PBS.

**Health status of infected larvae**

The health assessment of infected larvae was conducted according to Loh et al. (2013). The criteria were categorized into survival, mobility, melanization and cocoon formation (Supplementary table 7). For each criterion a score was applied, the higher, the healthier the larvae.

**Colonization test**

The persistence test was performed according to the methodology described by Pereira and collaborators (2015) with some modifications. The larvae were inoculated with $1x10^4$ CFU as described in the previous topic. Subsequently, we collected 10ul of hemolymph from each larva (n=10 larvae), aseptically, in previously siliconized tubes using Sigmacote® (Sigma-Aldrich SL21) at 0h, 2h, 4h, 6h, and 24h. After collection, a serial dilution was performed and plated on Simmons citrate agar (HIMEDIA, MUMBAI, MAHARASHTRA, INDIA) added with 1% inositol. Two biological replications were performed.

**Results**

We obtained 144 isolates of *Klebsiella* spp with the isolation method used (Figure 1). We obtained 144 *Klebsiella* spp isolates with the isolation method used. About 77% are *K. pneumoniae*, 14.5% *K. aerogenes* and 8.5% *K. variicola* (Supplementary Table 1). After removing the overlap of isolates from the same animal, using the fingerprint technique, the isolates were reduced to forty-five (Figure 2A). Twenty-three isolates were found in treatment 1 and twenty-two in treatment 2 (Figure2C). According to species identification by 16S rDNA gene sequencing, 40 isolates are *K. pneumoniae*, 3 *K. aerogenes* and 2 *K. variicola* (Figure 2A). We mapped the dispersion of these isolates among the animals in their respective stall (Figure 2B). We observed isolates with the same genetic profile in different animals within each treatment (Figure 2B and C). In addition, isolates with the same genetic profile were detected

in animals from different treatments and confined in stalls far apart (Figure 2B). As reported in the dendrogram (Figure 3) the isolates: 144 and 108; 26D and 267; 26C and 86b; 34A and 27; 1 and 270; 51A and 257C were grouped with 100% similarity (Figure 3). These findings reinforce the spread of these isolates in confined animals. Likewise, other isolates that were grouped in the dendrogram: 34C, 5A and 107; 153, 234, 238 and 288; 254, 45e and 252 (Figure 3).

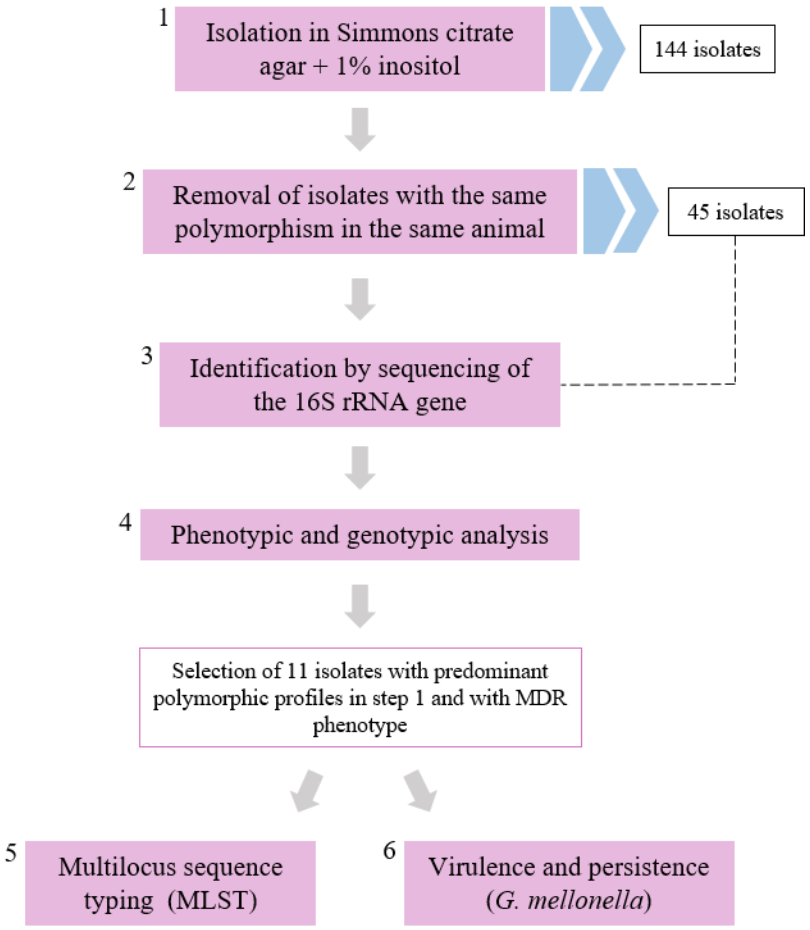



**Figure 1:** Experimental strategy used in this work. In 1, we have the number of isolates obtained in Simmons citrate agar supplemented with inositol (1%). In 2, we have the number of isolates obtained after overlap removal by fingerprinting. Subsequently, species identification by sequencing of the 16S rRNA gene (3) and performance of phenotypic and genotypic analyses (4). Selection of 11 isolates to perform MLST and analysis on *G. mellonella* (5 and 6).

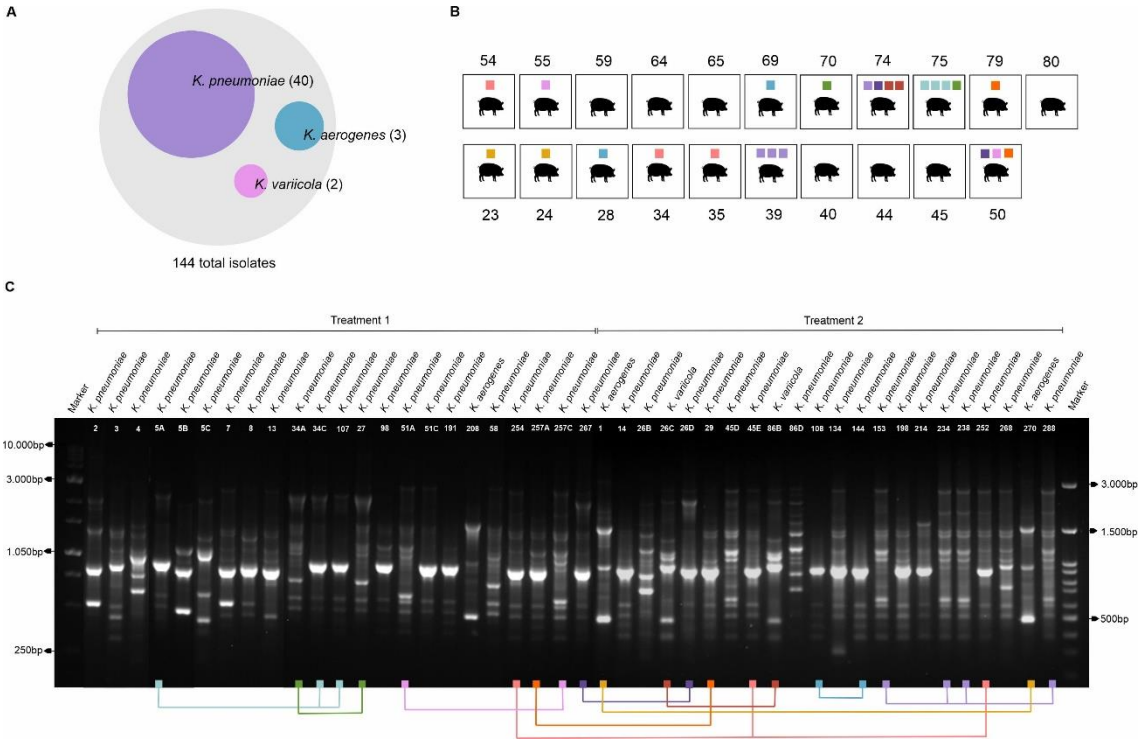



**Figure 2:** Overview of the isolates obtained in this work. A) Number of species representing the genus *Klebsiella*. B) Design of the physical space of the swine farm organized by stall. Each number corresponds to a stall. Squares of the same color represent *Klebsiella* isolates found in different animals or in different pens. C) Fingerprint BOX-PCR of all isolates obtained after removing overlapping isolates present in the same animal. Representation of the isolates obtained in treatments 1 and 2.

Phenotypic analyzes to assess hemolytic potential and capsule overproduction (hypermucoviscosity) were negative, as well as for the virulence marker genes: *hly*, *rmpA, rmpA2*, *iroNB, peg344* and *magA* with the exception of the *iutA* gene (Figure 3; Supplementary table 4).

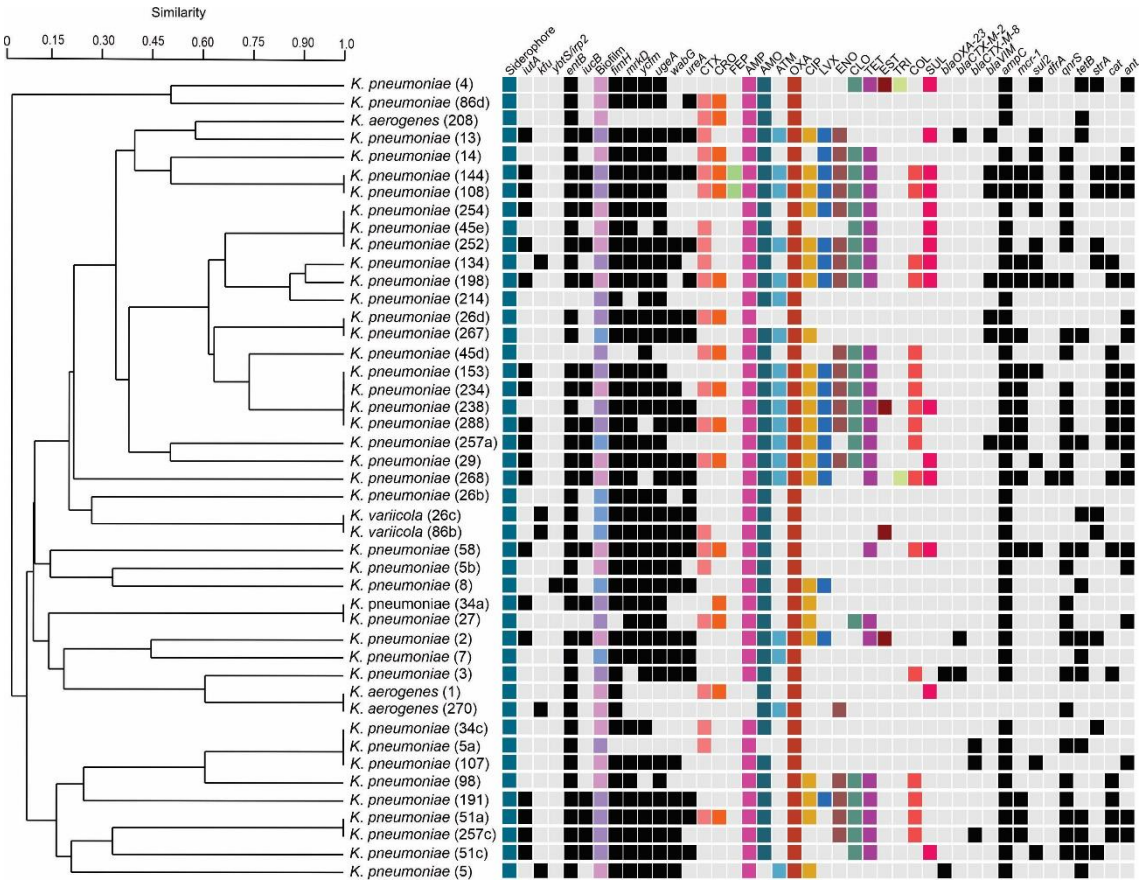



**Figure 3:** Dendrogram of isolates obtained in this work associated with phenotypic and genotypic profiles. Gray-filled squares indicate the absence of phenotypes or genotypes. Colored squares represent characteristics associated with phenotypic tests, and those filled in black are related to PCRs performed to detect genes (genotype). CTX: cefotaxime, CRO: ceftriaxone, FEP: cefepime, AMP: ampicillin, AMO: amoxicillin, ATM: Aztreonam, OXA: oxacillin, CIP: ciprofloxacin, LVX: levofloxacin, ENO: enrofloxacin, CLO: chloramphenicol, TET: tetracycline, EST: streptomycin, TRI: trimethoprim, COL: colistin, SUL: sulfonamide.

The production of siderophores was positive for all isolates (Figure 3; Supplementary Figure 2). We also investigated the presence of genes related to siderophore production and about 93.3% of the isolates carry the *entB* gene, 40% the *iucB* and *iutA* gene, 11.1% the *kfu* gene and 2.2% carry the *ybtS/irp2* genes (Figure 3; Supplementary table 4). We did not find the *iroNB* gene (Salmochelin) in the isolates. The biofilm production according to Stepanović et al. (2007) resulted in the classification in which 39% of the isolates are strong producers, 45% are moderate producers and 16% are weak producers (Supplementary figure 1). Some adhesion-

related genes such as *mrkD* (84.4%), *fimH* (88.8%) and *ycfm* (82.2%) were detected. As well as genes related to capsule biosynthesis (*ugeA* – 86.6% and *wcaG* – 2.2%), lipopolysaccharide – LPS (*wabG* – 53.3%) and invasin (*ureA* – 48.8%) (Figure 3; Supplementary table 4). Other genes related to allantoin metabolism (*allS* – 2.2%) and capsular serotypes (*k54* – 2.2% and *k2a* – 15.5%) were also detected among the isolates (Supplementary table 2). The *cf29, clbA, clbQ, clbN* genes were not detected (Supplementary table 4).

Resistance phenotypes to 10 classes of antimicrobials were evaluated (Figure 4). Overall, 75.5% of the isolates showed resistance to cephalosporins; 100% β-lactams; 51% the fluoroquinolone; 48.8% amphenicol; 55.5% tetracycline; 37.7% polimyxin; 35.5% sulfonamide; 8.8% aminoglycoside; 4.5% trimethoprim and no resistance to carbapenems was detected. Regarding the cephalosporins tested, 48.8% of the isolates were resistant to cefotaxime; 35.5% cefotriaxone and 4.4% resistants to cefepime. Of the cephalosporin resistance-related genes inspected, only one isolate carries the $bla_{\text{CTX-M-8}}$ gene (Figure 3). The isolates were tested for the following β-lactams where, 91.1% were resistant to ampicillin, 93.3% to amoxicillin, 100% to oxacillin and 40% to aztreonam. Of these, 41 isolates were positive for the *ampC* gene and only 3 isolates had the $bla_{\text{OXA-23}}$ gene (Supplementary table 5). Of the fluoroquinolones evaluated, the isolates in general showed 46.6% resistance to ciproflorxacin, 40% to enroflorxacin and 35.5% resistant to levofloxacin. Of these, 28 isolates were positive for the *qnrS* gene (Supplementary table 5). Overall, the isolates were resistant to trimethoprim (4.4%), tetracycline (55.5%), streptomycin (8.8%), sulfonamide (35.5%), chloramphenicol (51.1%) and colistin (37.7%).

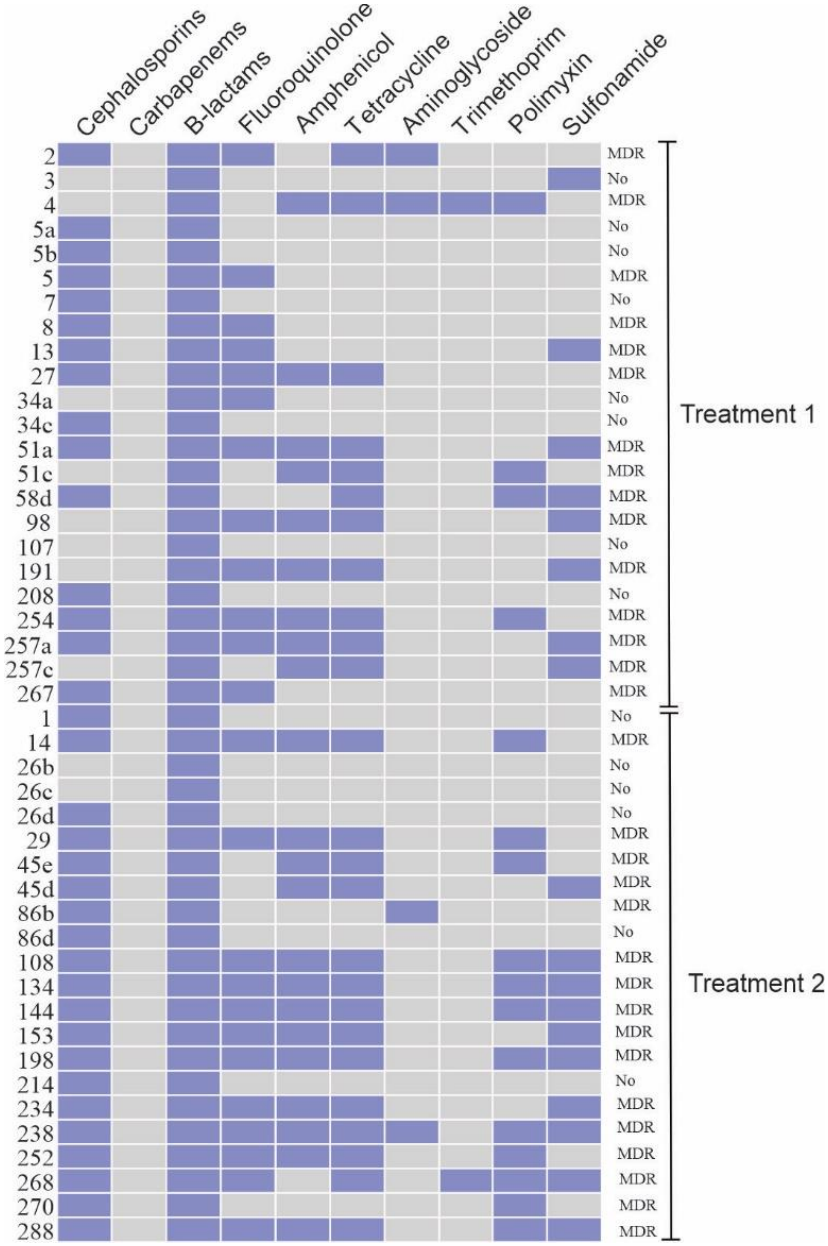



**Figure 4:** Classification of isolates according to resistance phenotype. For each isolate, rectangles filled in gray indicate the absence of resistance to the antimicrobials tested for the represented class. Rectangles filled in blue indicate resistance to one or more antimicrobials tested for the class.

After the phenotypic analysis of resistance, two types of classification were performed. The first is the classification of multidrug resistant (MDR) where 68.8 % of the isolates are grouped. And the second analysis is the multiple antimicrobial resistance index (IRMA) where we have an interval from 0.2 to 0.55, corroborating the results of the MDR classification.

Of the 45 isolates obtained in this work, we selected 11 *K. pneumoniae* MDR with virulence genes of interest to investigate the type of sequence (STs) to which they belong. Of these, 45.6% are ST25; 27.4% for ST147 and 9% for ST616, ST4691 and ST6208.

We also evaluated the virulence of 11 *K. pneumoniae* MDR in *Galleria mellonella*. We used the *K. pneumoniae* strain Kp13 (diseased human reservoir) considered a cKp strain with hypermucoviscous phenotype (Ramos et al., 2012) to compare the behavioral pattern in *G. mellonella*. As a result, larvae inoculated with isolates 288 and 153 had a survival rate of 38% (Figure 5). For isolates 29, 45e, and 13, we have a 35% survival rate (Figure 5). And for isolates 234, 238, 5, and 257 the survival rate was 25% (Figure 5). Finally, isolates 51a and 144 had 55% and 10% larval survival rates, respectively (Figure 5). All isolates were statistically different from the PBS control. And isolate 144 was the only isolate that showed a statistical difference between Kp13 and 51a (Supplementary table 6). The virulence analyzes of the isolates were compatible with the parameters for evaluating the health of the larvae (Supplementary table 7).

The persistence test of *K. pneumoniae* isolates in *G. mellonella* was conducted at times of 0, 2, 4, 6 and 24 hours (Figure 5). All swine isolates selected for this analysis showed similar patterns of persistence where they multiplied more easily within 2-4 hours and stabilized or decreased after 6 hours of infection.

We did not observe any visible characteristics of the phenotypes and genotypes evaluated in this work that can be related to the addition of the growth promoter to the animal diet. However, the sanitary challenge directly influenced the dispersion of *Klebsiella* sp between different animals and different treatments (Figures 2B and C).

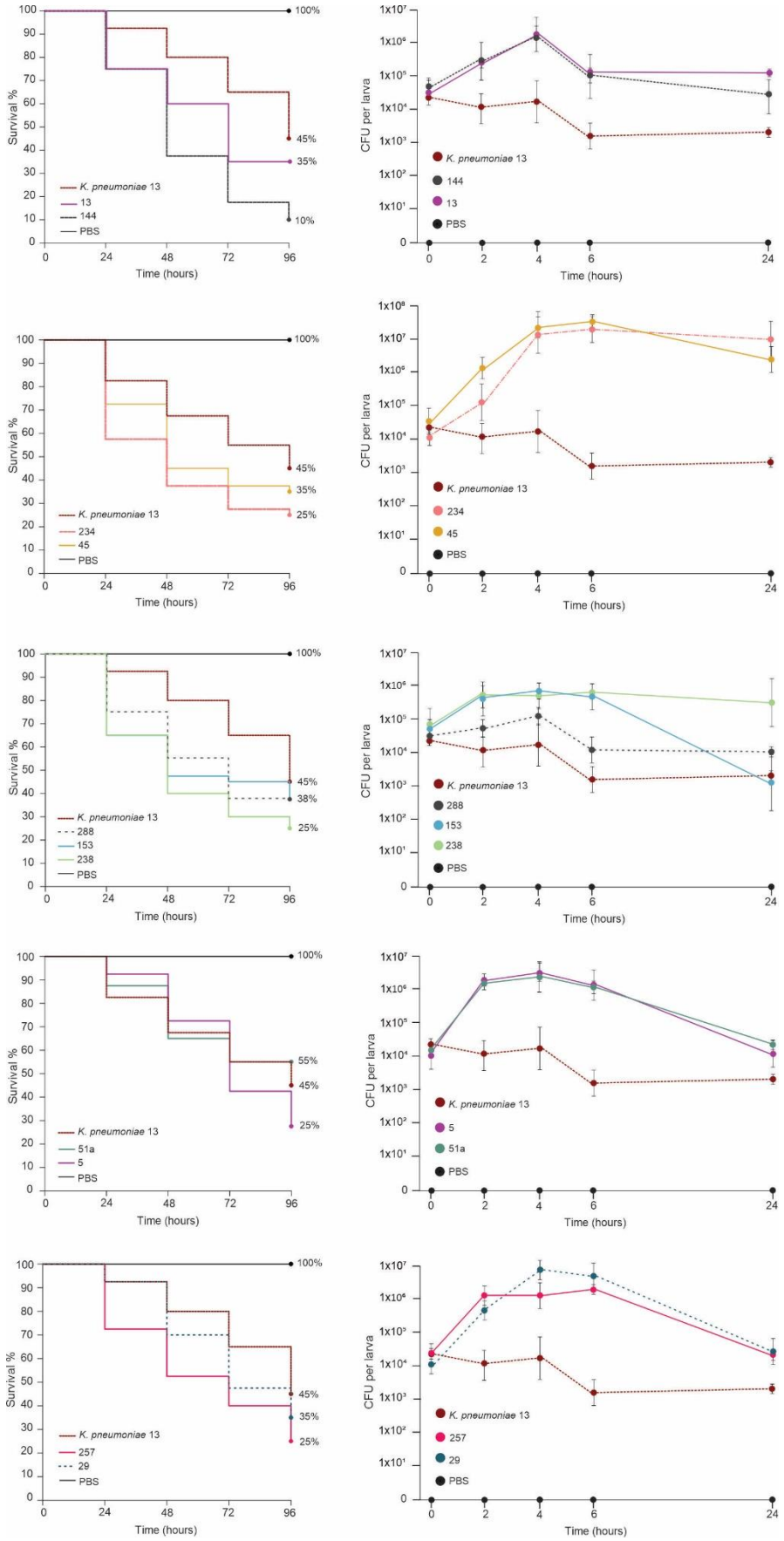



**Figure 5:** Analysis of survival and persistence in *Galleria mellonella*.

**Discussion**

The health of the host and the environment where it is found are determining factors for the balance between commensal and opportunistic microbial communities. The high gene flow of MGE in *Klebsiella* spp contributes to its ecological role in the host (Wyres, Lam and Holt 2020). Thus, this work mapped and investigated the phenotype and genotype of *Klebsiella* spp isolated from the rectum of clinically healthy swine.

Our results suggest possible dissemination of *Klebsiella* spp between different animals within the same stalls and in distant stalls (Figure 2B and C) via fecal and/or oral route. The genetic profiles presented here were used as potential indicators of dispersion (the animals were not related to each other). In some cases, isolates such as 267 and 26d, 1 and 270 that have the same genetic profile but were isolated from different animals, have been shown to have different resistance phenotypes (Figure 3). This finding reinforces how environmental variables, in this case, the health challenge, confinement, and host, can affect the phenotype and genotype of *Klebsiella* spp (Young et al., 2020, Wang et al., 2023). The difference in these phenotypes can be associated with mutations and, mainly, mobile elements that are acquired between intraspecies or interspecies within the host itself (Maclean & San Millan, 2019). Thus, as future prospects, the study of MGE carried by these isolates can be further investigated.

Siderophores are essential for the replication and virulence of opportunistic pathogens in the host and, in *K. pneumoniae*, they are strongly associated with systemic dissemination and sepsis in animals and humans (Zhu et al., 2021; Namikawa et al., 2022). Here, we found four important siderophore component genes in *Klebsiella spp*: yersiniabactin, enterobactin, aerobactin, and the additional system for obtaining iron by the *kfu* gene. Aerobactin can be encoded mainly via virulence plasmids or chromosomal recombination (Di Lorenzo & Stork, 2015; Li et al., 2021; Huang et al., 2022). Interestingly, 40% of the isolates had aerobactin genes, a very common siderophore in hypervirulent *K. pneumoniae* (Huang et al., 2022). Another iron chelator found was yersiniabactin (*ybtS/irp2*) which is very common to be found in virulence plasmids or integrative and conjugative elements (ICEs) (Dai and Hu 2022; Wyres, Lam & Holt 2020; Liu et al., 2019). The presence of these genes is a strong indicator of mobile genetic elements (MGE).

In the process of adapting to the stressful environment found in the host, the biofilm helps colonization and prevents the action of cells of the immune system (Mohamed et al., 2018). Biofilm formation by *Klebsiella* spp is complex and *in vitro* and *in vivo* models have limitations since the nature of biofilm production in the host co-occurs with other

microorganisms (Guerra et al., 2022). In this work, the isolates showed important genes for biofilm production, however, we did not observe a correlation between the presence of these genes for the isolate to be a strong producer (Supplementary figure 1). Thus, we reinforce the need for new models to study the complexity of biofilm formation by *Klebsiella* spp.

We detected important *K. pneumoniae* serotypes such as K54 and K2 (Supplementary table 4). These serotypes are prevalent in hypervirulent *K. pneumoniae* populations (Russo and Marr 2019; Russo et al., 2018; Wyres, Lam & Holt 2020). However, only one hypervirulence marker gene (*iucB*) was detected (Supplementary table 4). Interestingly, all isolates belonging to serotype K2 belong to ST25. ST25 is associated with populations of *K. pneumoniae* that cause sepsis in swine in Australia and England (Bowring et al., 2017; Bidewell et al., 2018).

Another ST found is 147, which has a strong relationship with MDR phenotypes in *K. pneumoniae* from clinical samples and few associated with production animals (Rodrigues et al., 2022). The population dynamics of lineages belonging to the CG174 clonal group, as is the case with ST147 isolates, is complex, and throughout their evolutionary process, they acquired many accessory genes, becoming an emerging group on a worldwide scale (Rodrigues et al., 2022).

The *K. pneumoniae* isolates that presented the MDR phenotype, and whose STs were investigated, showed to be more aggressive in the initial stages of infection, and after 24 hours their population is controlled, but they still cause deaths and consequently phenotypic changes in *G. mellonella* (Figure 5). No correlation with virulence genes could be related to these patterns. Our results once again reinforce the complexity of the *K. pneumoniae* infection process, as the combined action of several genes results in distinct virulent phenotypes (Bruchmann et al., 2021; Pereira et al., 2020; Short et al., 2020).

We found resistance phenotypes to cephalosporins (third and fourth-generation), quinolones, and polymyxin, antimicrobials categorized by the World Health Organization - WHO (2019) as “high priority”. These findings are curious because these antimicrobials are not used in pig farming as growth promoters. Currently, they can be used as prophylactic, metaphylactic, and therapeutic measures (Braun et al., 2018; Rocha et al., 2022). It is important to point out that in this study the animals were not treated with the antimicrobials which the *Klebsiella* spp showed resistance. The additives used in treatment 1 (zinc bacitracin and enramycin) are restricted to Gram-positive bacteria. Thus, we believe that populations of *Klebsiella* spp were not impacted as we did not observe phenotypic changes in both treatments.

This work allowed us to explore a little more about the phenotypic and genotypic aspects of *Klebsiella* spp from clinically healthy swines in a stressful growth environment (sanitary challenge). Here, we detected important resistance and virulence phenotypes in *Klebsiella* spp. Thus, we conclude that clinically healthy swine are reservoirs of *Klesiella* spp MDR with relevant virulence phenotypes. The analyzes carried out in this work contribute significantly to the one health approach.

**Funding information**

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Programa de Excelência Acadêmica-Código Financeiro 001 and Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG.

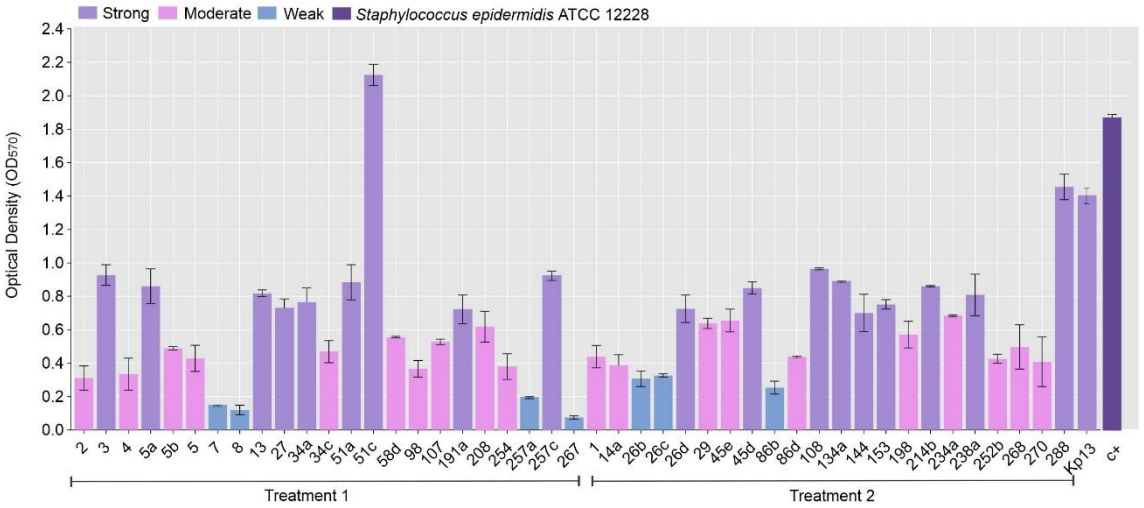
**Author contributions**

R. N. J, S. C. G, F. P. P, O. P. R and G. M. M. sample collection and processing. R. N. J and S. C. G analysis of results. R. N. J performed the edition of figures. S. C. G and B. S. M. D helped in the correction of the manuscript and English. R. N. J wrote the manuscript. All authors discussed the results and their implications. All authors edited and approved the final manuscript.

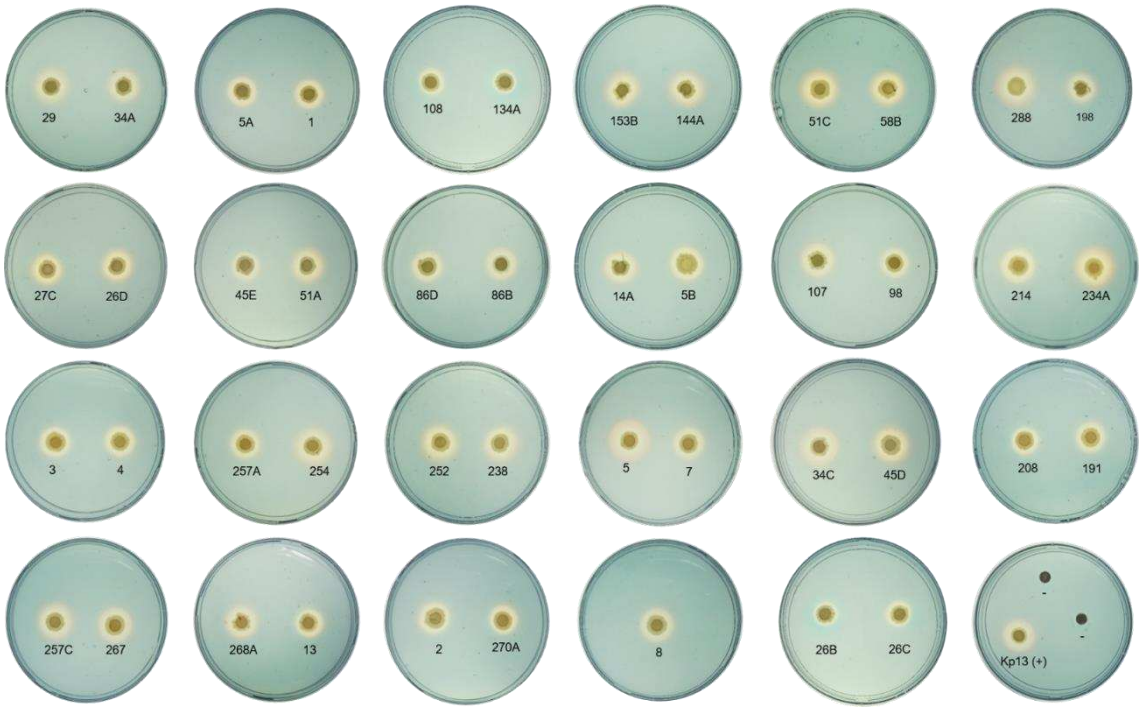
**Conflicts of interest**

The authors declare no competing interests.

## Supplementary material




**Supplementary figure 1:** Phenotypic analysis of biofilm production on abiotic surface. The isolates were separated by treatments 1 and 2 and further classified according to biofilm production. The positive control used was *K. pneumoniae* Kp13 and *S. epidermidis* ATCC 12228




**Supplementary figure 2:** Analysis of siderophore production in CAS agar. As a positive control, the *K. pneumoniae* Kp13 strain was used. For the negative control, we used *S. epidermidis* ATCC 12228.

**Supplementary tables available at the link:**

https://docs.google.com/spreadsheets/d/1ZvafgPRwG54iVCqL9xbBTwCBnTjg3piQ/edit?usp=drive_link&ouid=118022498442200222122&rtpof=true&sd=true

## Reference


Braun, G., Cayo, R., Matos, A. P., de Mello Fonseca, J., Gales, A. C. Temporal evolution of polymyxin B-resistan*t Klebsiella pneumoniae* clones recovered from blood cultures in a teaching hospital during a 7-year period. *International Journal of Antimicrobial Agents.* **2018**, 51(3):522-527. DOI:10.1016/j.ijantimicag.2017.08.031

Bidewell, C. A., Williamson, S. M., Rogers, J., Tang, Y., Ellis, R. J., Petrovska, L., AbuOun, M. Emergence of *Klebsiella pneumoniae* subspecies pneumoniae as a cause of septicaemia in pigs in England. *PloS one*. **2018**,*13*(2), e0191958. DOI:10.1371/journal.pone.0191958

Bowring, B. G., Fahy, V. A., Morris, A., Collins, A. M. An unusual culprit: *Klebsiella pneumoniae* causing septicaemia outbreaks in neonatal pigs?. *Veterinary microbiology*. **2017**, *203*, 267-270.

Bland, J. M., Altman, D. G. Survival probabilities (the Kaplan-Meier method). *Bmj*. **1998**, *317*(7172), 1572-1580. DOI:10.1136/bmj.317.7172.1572

Bruchmann, S., Feltwell, T., Parkhill, J., Short, F. L. Identifying virulence determinants of multidrug-resistant *Klebsiella pneumoniae* in *Galleria mellonella*. *Pathogens and disease*. **2021**, *79*(3), ftab009. DOI:10.1093/femspd/ftab009

Campos-Madueno, E. I., Moser, A. I., Risch, M., Bodmer, T., Endimiani, A. Exploring the global spread of *Klebsiella grimontii* isolates possessing bla VIM-1 and mcr-9. *Antimicrobial agents and chemotherapy*. **2021**, *65*(9), e00724-21. DOI:10.1128/aac.00724-21

Chang, D., Sharma, L., Dela Cruz, C. S., Zhang, D. Clinical epidemiology, risk factors, and control strategies of *Klebsiella pneumoniae* infection. *Frontiers in Microbiology*. **2021**, 3955. DOI:10.3389/fmicb.2021.750662

Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals.* 5th ed. CLSI supplement VET01S (ISBN 978-1-68440-092-8 [Print]; ISBN 978-1-68440-093-5 [Electronic]). Clinical and Laboratory Standards Institute, USA, 2020.

Choudhury, R., Middelkoop, A., Bolhuis, J. E., Kleerebezem, M. Legitimate and reliable determination of the age-related intestinal microbiome in young piglets; rectal swabs and fecal samples provide comparable insights. *Frontiers in microbiology*, **2019**, 10, 1886. DOI: 10.3389/fmicb.2019.01886

Dai, P., Hu, D. The making of hypervirulent *Klebsiella pneumoniae*. *Journal of Clinical Laboratory Analysis*. **2022**, e24743. DOI:10.1002/jcla.24743

Di Lorenzo, M., Stork, M. Plasmid-Encoded Iron Uptake Systems. *Plasmids: Biology and Impact in Biotechnology and Discovery*. **2015**, 577-597. DOI:10.1128/9781555818982.ch29

Diancourt, L., Passet, V., Verhoef, J., Grimont, P. A., Brisse, S. Multilocus sequence typing of *Klebsiella pneumoniae* nosocomial isolates. *Journal of clinical microbiology*. **2005**, *43*(8), 4178-4182. DOI:10.1128/JCM.43.8.4178-4182.2005

Dong, N., Yang, X., Chan, E. W. C., Zhang, R., Chen, S. *Klebsiella* species: taxonomy, hypervirulence and multidrug resistance. *EBioMedicine.* **2022**, 79: 103998. DOI: 10.1016/j.ebiom.2022.103998

Francisco, A. P., Bugalho, M., Ramirez, M., Carriço, J. A. Global optimal eBURST analysis of multilocus typing data using a graphic matroid approach. *BMC bioinformatics*. **2009**, *10*(1), 1-15. DOI:10.1186/1471-2105-10-152

Gu, D., Dong, N., Zheng, Z., Lin, D., Huang, M., Wang, L., Chen, S. A fatal outbreak of ST11 carbapenem-resistant hypervirulent *Klebsiella pneumoniae* in a Chinese hospital: a molecular epidemiological study. *The Lancet infectious diseases*. **2018**, *18*(1), 37-46. DOI: 10.1016/S1473-3099(17)30489-9

Guerra, M. E. S., Destro, G., Vieira, B., Lima, A. S., Ferraz, L. F. C., Hakansson, A. P., Converso, T. R. *Klebsiella pneumoniae* Biofilms and Their Role in Disease Pathogenesis. *Frontiers in cellular and infection microbiology*. **2022**, 555. DOI: 10.3389/fcimb.2022.877995

Hammer, Ø., Harper, D. A. T., Ryan, P. D. PAST: Paleontological statistics software package for education and data analysis. *Palaeontologia Electronica*. **2001**, *4*(1),1-9.

Huang, C., Tao, S., Yuan, J., Li, X. Effect of sodium hypochloride on biofilm of Klebsiella pneumoniae with diferente drug resistance. *American Journal of Infection Control*. **2022**, *50(8)*, 922-928. DOI:10.1016/j.ajic.2021.12.003

Jorjão, A. L., de Oliveira, F. E., Leão, M. V. P., Jorge, A. O. C., de Oliveira, L. D. Effect of *Lactobacillus rhamnosus* on the response of *Galleria mellonella* against *Staphylococcus aureus* and *Escherichia coli* infections. *Archives of microbiology*. **2018**, *200*(3), 383-389. DOI: 10.1007/s00203-017-1441-7

Joseph, L., Merciecca, T., Forestier, C., Balestrino, D., Miquel, S. From *Klebsiella pneumoniae* colonization to dissemination: an overview of studies implementing murine models. *Microorganisms*. **2021**, *9*(6), 1282. DOI:10.3390/microorganisms9061282

Kugelev, I. M., Kapay, N. A., Davydenkova, O. V., Russkin, A. S. The effectiveness of various antibacterial drugs in the treatment of swine respiratory diseases. In *IOP Conference Series: Earth and Environmental Science.* **2021**, (Vol. 723, No. 2, p. 022065). IOP Publishing. DOI: 10.1088/1755-1315/723/2/022065

Krumperman, P. H. Multiple antibiotic resistance indexing of Escherichia coli to identify high-risk sources of fecal contamination of foods. *Applied and environmental microbiology*. **1983**, 46(1), 165-170. DOI:10.1128/aem.46.1.165-170.1983

Lane, D. J. 16S/23S rRNA sequencing.(eds. Stackebrandt E. Goodfellow M). *Nucleic Acid Tech. Bact. Syst.* **1991**, 115–175.

Lee, H.C., Chuang, Y.C., Yu, W.L., Lee, N.Y., Chang, C.M., Ko, N.Y., Wang, L.R., Ko, W.C. Clinical implications of hypermucoviscosity phenotype in *Klebsiella pneumoniae* isolates: association with invasive syndrome in patients with community-acquired bacteraemia. *J. Intern Med.* **2006**, *259*(6):606–614. DOI:10.1111/j.1365-2796.2006.01641.x

Leangapichart, T., Lunha, K., Jiwakanon, J., Angkititrakul, S., Järhult, J. D., Magnusson, U., Sunde, M. Characterization of *Klebsiella pneumoniae* complex isolates from pigs and humans in farms in Thailand: population genomic structure, antibiotic resistance and virulence genes. *Journal of Antimicrobial Chemotherapy***. 2021**, *76*(8), 2012-2016. DOI: 10.1093/jac/dkab118

Li, C., Pan, D., Li, M., Wang, Y., Song, L., Yu, D., Shen, X. Aerobactin-mediated iron acquisition enhances biofilm formation, oxidative stress resistance, and virulence of *Yersinia pseudotuberculosis*. *Frontiers in microbiology*. **2021**, *12*, 699913. DOI:10.3389/fmicb.2021.699913

Liu, D., Yang, Y., Gu, J., Tuo, H., Li, P., Xie, X., Zhang, A. The Yersinia high-pathogenicity island (HPI) carried by a new integrative and conjugative element (ICE) in a multidrug-resistant and hypervirulent *Klebsiella pneumoniae* strain SCsl1. *Veterinary microbiology*. **2019**, *239*, 108481. DOI:10.1016/j.vetmic.2019.108481

Lin, T. L., Lee, C. Z., Hsieh, P. F., Tsai, S. F., & Wang, J. T. Characterization of integrative and conjugative element ICE Kp1-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess. *Journal of bacteriology*. **2008**, *190*(2), 515-526. DOI: 10.1128/jb.01219-07

Louden, B. C., Haarmann, D., Lynne, A. M. Use of blue agar CAS assay for siderophore detection. *Journal of microbiology & biology education*. **2011**, *12*(1), 51-53. DOI: 10.1128/jmbe.v12i1.24

Loh, J. M., Adenwalla, N., Wiles, S., Proft, T. *Galleria mellonella* larvae as an infection model for group A streptococcus. *Virulence*. **2013**, *4*(5), 419-428. DOI:10.4161/viru.24930

Lu, Y., Feng, Y., McNally, A., Zong, Z. Occurrence of colistin-resistant hypervirulent *Klebsiella variicola*. *Journal of Antimicrobial Chemotherapy*. **2018**, *73*(11), 3001-3004. DOI: 10.1093/jac/dky301

MacLean, R. C., San Millan, A. The evolution of antibiotic resistance. *Science*, **2019**. *365*(6458), 1082-1083. DOI:10.1126/science.aax3879

Magiorakos, A. P., Srinivasan, A., Carey, R. B., Carmeli, Y., Falagas, M. E., Giske, C. G., Harbarth, S., Hindler, J. F., Kahlmeter, G. Olsson-Liljequist, B., Paterson, D. L., Rice, L. B., Stelling, J., Struelens, M. J., Vatopoulos, A., Weber, J. T., Monntet, D. L. Multidrug-resistant, extensively drug-resistant and pandrug-resistant bacteria: an international expert proposal for interim standard definitions for acquired resistance. *Clinical microbiology and infection*. **2012**, 18(3), 268-281. DOI:10.1111/j.1469-0691.2011.03570.x

Meier-Kolthoff, J.P., Sardà C. J., Peinado-Olarte, R.L., Göker, M. TYGS and LPSN: a database tandem for fast and reliable genome-based classification and nomenclature of prokaryotes. *Nucleic Acids Research*. **2022**, 50, D801-D807. DOI:10.1093/nar/gkab902

Mohamed, S. H., Mohamed, M. S. M., Khalil, M. S., Azmy, M., & Mabrouk, M. I. Combination of essential oil and ciprofloxacin to inhibit/eradicate biofilms in multidrug-resistant Klebsiella pneumoniae. *Journal of applied microbiology*. **2018**, *125*(1), 84-95. DOI:10.1111/jam.13755

Mukherjee, S., Bhadury, P., Mitra, S., Naha, S., Saha, B., Dutta, S., Basu, S. Hypervirulent *Klebsiella pneumoniae* Causing Neonatal Bloodstream Infections: Emergence of NDM-1-Producing Hypervirulent ST11-K2 and ST15-K54 Strains Possessing pLVPK-Associated Markers. *Microbiology Spectrum*. **2023**,*11*(2), e04121-22. DOI:10.1128/spectrum.04121-22

Namikawa, H., Niki, M., Niki, M., Oinuma, K. I., Yamada, K., Nakaie, K., ... & Shuto, T. Siderophore production as a biomarker for *Klebsiella pneumoniae* strains that cause sepsis: A pilot study. *Journal of the Formosan Medical Association*, **2022**.*121*(4), 848-855. DOI: 10.1016/j.jfma.2021.06.027

Navon-Venezia, S., Kondratyeva, K., Carattoli, A. *Klebsiella pneumoniae*: a major worldwide source and shuttle for antibiotic resistance. *FEMS microbiology reviews*. **2017** May 1;41(3):252-75. DOI:10.1093/femsre/fux013

OMS. Critically important antimicrobials for human medicine: 6th revision, 2019. <https://www.who.int/publications/i/item/9789241515528>

Pereira, M. F., Rossi, C. C., de Queiroz, M. V., Martins, G. F., Isaac, C., Bosse, J. T., Li, Y., Wren, B. W., Terra, V. S., Cuccui, J., Langford, P. R., Bazzolli, D. M. S. *Galleria mellonella* is an effective model to study *Actinobacillus pleuropneumoniae* infection. *Microbiology*. **2015**, *161*(2), 387-400. DOI:10.1099/mic.0.083923-0

Pereira, M. F., Rossi, C. C., da Silva, G. C., Rosa, J. N., & Bazzolli, D. M. S. *Galleria mellonella* as an infection model: an in-depth look at why it works and practical considerations for successful application. *Pathogens and Disease*. **2020**, *78*(8). DOI:10.1093/femspd/ftaa056

Podschun, Rainer; Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews*. **1998**, 11(4), 589-603. DOI:10.1128/CMR.11.4.589

Qi, Y., Xue, J. Z., Li, S. S., Elken, E. M., Haqmal, M. A., Li, X. S., Ma, H. X. Analysis of an IncR plasmid carried by carbapenem-resistant *Klebsiella pneumoniae*: a survey of swine *Klebsiella pneumoniae* in Jilin Province. *Journal of Global Antimicrobial Resistance*. **2023**. DOI: 10.1016/j.jgar.2023.04.018

Quiblier, C., Zinkernagel, A. S., Schuepbach, R. A., Berger-Bächi, B., Senn, M. M. Contribution of SecDF to *Staphylococcus aureus* resistance and expression of virulence factors. *BMC microbiology*. **2011**, *11*(1), 1-12. DOI:10.1186/1471-2180-11-72

Ramos, P. I. P., Picão, R. C., Vespero, E. C., Pelisson, M., Zuleta, L. F. G., Almeida, L. G. P. Gerber, A. L., Vasconcelos, A. T., Gales, A. C., Nicolas, M. F. Pyrosequencing-based analysis reveals a novel capsular gene cluster in a KPC-producing *Klebsiella pneumoniae* clinical isolate identified in Brazil. *BMC microbiology.* **2012**, 1-12. DOI:10.1186/1471-2180-12-173

Ribeiro, M. G., de Morais, A. B. C., Alves, A. C., Bolaños, C. A. D., de Paula, C. L., Portilho, F. V. R., Paes, A. C. *Klebsiella*-induced infections in domestic species: a case-series study in 697 animals (1997–2019). *Brazilian Journal of Microbiology*. **2022**, *53*(1), 455-464. DOI: 10.1007/s42770-021-00667-0

Rocha, J., Ferreira, C., Mil-Homens, D., Busquets, A., Fialho, A. M., Henriques, I., Manaia, C. M. Third generation cephalosporin-resistant *Klebsiella pneumoniae* thriving in patients and in wastewater: what do they have in common?. *BMC genomics*. **2022**, *23*(1), 1-14. DOI: 10.1186/s12864-021-08279-6

Rodrigues, C., Desai, S., Passet, V., Gajjar, D., Brisse, S. Genomic evolution of the globally disseminated multidrug-resistant *Klebsiella pneumoniae* clonal group 147. *Microbial genomics*. **2022**, *8*(1). DOI:10.1099/mgen.0.000737

Rodríguez-Medina, N., Barrios-Camacho, H., Duran-Bedolla, J., Garza-Ramos, U. *Klebsiella variicola*: an emerging pathogen in humans. *Emerging microbes and infections*. **2019**, *8*(1), 973-988. DOI:10.1080/22221751.2019.1634981

Russo, T. A., Olson, R., Fang, C. T., Stoesser, N., Miller, M., MacDonald, U., Johnson, J. R. Identification of biomarkers for differentiation of hypervirulent *Klebsiella pneumoniae* from classical *K. pneumoniae*. *Journal of clinical microbiology*. **2018**, *56*(9), e00776-18. DOI: 10.1128/JCM.00776-18

Russo, T. A., & Marr, C. M. Hypervirulent *Klebsiella pneumoniae*. *Clinical microbiology reviews*. **2019**, *32*(3), e00001-19. DOI:10.1128/CMR.00001-19

Shankar C, Kumar S, Venkatesan M, Veeraraghavan B. Emergence of ST147 *Klebsiella pneumoniae* carrying blaNDM-7 on IncA/C2 with ompK35 and ompK36 mutations in India. *Journal of Infection and Public Health*. **2019**, 12:741–3. DOI:10.1016/j.jiph.2019.03.023

Shankar, C., Nabarro, L. E., Muthuirulandi Sethuvel, D. P., Raj, A., Devanga Ragupathi, N. K., Doss, G. P., Veeraraghavan, B. Draft genome of a hypervirulent *Klebsiella quasipneumoniae subsp. similipneumoniae* with novel sequence type ST2320 isolated from a chronic liver disease patient. *J Glob Antimicrob Resist*. **2017**, *9*, 30-31. DOI:10.1016/j.jgar.2017.01.004

Stepanović, S., Vuković, D., Hola, V., Bonaventura, G. D., Djukić, S., Ćirković, I., Ruzicka, F. Quantification of biofilm in microtiter plates: overview of testing conditions and practical recommendations for assessment of biofilm production by *staphylococci*. *Apmis*. **2007**, *115*(8), 891-899. DOI:10.1111/j.1600-0463.2007.apm_630.x

Short, F. L., Di Sario, G., Reichmann, N. T., Kleanthous, C., Parkhill, J., Taylor, P. Genomic profiling reveals distinct routes to complement resistance in *Klebsiella pneumoniae*. *Infection and immunity*. **2020**, *88*(8), e00043-20. DOI:10.1128/IAI.00043-20

Turlej-Rogacka, A., Xavier, B. B., Janssens, L., Lammens, C., Zarkotou, O., Pournaras, S., Malhotra-Kumar, S. Evaluation of colistin stability in agar and comparison of four methods for MIC testing of colistin. *European Journal of Clinical Microbiology & Infectious Diseases.* **2018**, *37*(2), 345-353. DOI:10.1007/s10096-017-3140-3

Van Kregten, E. R. I. C., Westerdaal, N. A., Willers, J. M. New, simple medium for selective recovery of *Klebsiella pneumoniae* and *Klebsiella oxytoca* from human feces. *Journal of Clinical Microbiology.* **1984**, *20*(5), 936-941. DOI:10.1128/jcm.20.5.936-941.1984

Versalovic, J., Schneider, M., De Bruijn, F. J., Lupski, J. R. Genomic fingerprinting of bacteria using repetitive sequence-based polymerase chain reaction. *Methods in molecular and cellular biology*. **1994**, *5*(1), 25-40.

Wang, H., Qi, J. F., Qin, R., Ding, K., Graham, D. W., Zhu, Y. G. Intensified livestock farming increases antibiotic resistance genotypes and phenotypes in animal feces. *Communications Earth & Environment*, **2023**. *4*(1), 123. DOI:10.1038/s43247-023-00790-w

Wu, J., Chen, J., Wang, Y., Meng, Q., Zhao, J. Siderophore iucA of hypermucoviscous *Klebsiella pneumoniae* promotes liver damage in mice by inducing oxidative stress. *Biochemistry and Biophysics Reports*. **2022**, *32*, 101376. DOI:10.1016/j.bbrep.2022.101376

Wyres, K. L., Lam, M., Holt, K. E. Population genomics *of Klebsiella pneumoniae*. *Nature Reviews Microbiology*. **2020**,*18*(6), 344-359. DOI:10.1038/s41579-019-0315-1

Wyres, K. L., Nguyen, T. N., Lam, M., Judd, L. M., van Vinh Chau, N., Dance, D. A., Holt, K. E. Genomic surveillance for hypervirulence and multi-drug resistance in invasive *Klebsiella pneumoniae* from South and Southeast Asia. *Genome medicine*. **2020**, *12*(1), 1-16. DOI: 10.1186/s13073-019-0706-y

Young, T. M., Bray, A. S., Nagpal, R. K., Caudell, D. L., Yadav, H., Zafar, M. A. Animal model to study *Klebsiella pneumoniae* gastrointestinal colonization and host-to-host transmission. *Infection and immunity*. **2020**, 88(11), e00071-20. DOI:10.1128/IAI.00071-20

Zhu, J., Wang, T., Chen, L., Du, H. Virulence factors in hypervirulent *Klebsiella pneumoniae*. *Frontiers in Microbiology*. **2021**, 12, 642484. DOI:10.3389/fmicb.2021.642484

# Chapter 2

## Genomic characterization of *Klebsiella pneumoniae* MDR from healthy swine

(Manuscript written according to mBio Journal)

Jéssica Nogueira Rosa[1]; Giarlã Cunha da Silva[1]; Patrícia Pereira Fontes[1]; Matheus Machado Guidini[1]; Denise Mara Soares Bazzolli[1*]

[1]Laboratório de Genética Molecular de Bactérias, Departamento de Microbiologia, Instituto de Biotecnologia Aplicada à Agropecuária - Bioagro, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

*Corresponding author: dbazzolli@ufv.br

### Abstract

Antimicrobial resistance is a growing concern, and the indiscriminate use of antimicrobials in healthcare and production systems can escalate this process. The use of antimicrobials such as colistin sulfate, tetracycline, tylosin, and others to promote animal growth is prohibited by current Brazilian legislation. Gram-negative bacteria such as *Klebsiella pneumoniae* are naturally found in the gut microbiota of human and animals. In general, members of Enterobacteriaceae family, as *K. pneumoniae,* can contributes significantly to the exchange of genetic material among species of this family. Thus, our objective was characterizing *K. pneumonaie* MDR genomes from clinically healthy swine under sanitary challenge and to investigate the presence of plasmids with resistance genes. The genomes were identified as *K. pneumoniae* and the strains were named HS-144 and HS-13, respectively. The average genome size was 5.45 Mpb, 57 % GC content and the coding sequences average number was 5148. The assembly allowed us to obtain 35 contigs for isolate HS-144 and 41 for HS-13. The functional annotation highlights classes such as metabolism of carbohydrates, amino acids, and proteins (37.6%); membrane transport (5.7%); cellular regulation and signaling (3.5%); DNA metabolism (3.5%); virulence, disease, and defense (3.1%); cell wall and capsule (5.2%). In general, the functions of genes related to virulence were hyper adherence, bacteriocin, quorum sensing response; biofilm formation, iron acquisition, and others. HS-144 and HS-13 genomes carry plasmids, but only HS-144 has regions with complete conjugation apparatus, positive phenotype for extended-spectrum β-lactamase – ESBL, and a putative prophage that carries the $bla_{SHV2}$ gene. Thus, we performed the conjugation assay using the HS-144 strain as a donor and the antimicrobial colistin to select transconjugants. We obtained three transconjugants with plasmid and *mcr-1* and $bla_{TEM1}$ genes. In addition, the transconjugants had a colistin MIC of

>64mg.mL$^{-1}$ close to the HS-144 donor. Thus, we conclude that *K. pneumoniae* MDR from the healthy swine carry an important genetic arsenal for its adaptation to different hosts and antimicrobial resistance. In addition, they are important sources of mobile elements such as plasmids that can carry and disseminate resistance genes to other bacteria.

**Keywords:** one health; colistin resistance; ESBL

## Introduction

Swine in an intensive production system, during the development stages, go through stressful conditions and may have their health compromised by bacterial agents (Effendi et al., 2022; Vieites et al., 2020). Thus, the use of antimicrobials as growth promoter is an alternative to reduce cases of clinical infections and increase the efficiency of nutrient absorption by the intestinal mucosa (Sun et al., 2020). However, there is ongoing concern about the role of antimicrobials as growth promoters and the selection of multiresistant bacteria (Davis, Price 2016; Leangapichart et al., 2021). Thus, the use of many antimicrobials as growth promoters in animal production systems is declining worldwide and this trend is even observed in Brazil (Vieites et al., 2020). *Klebsiella pneumoniae* (Gram-negative bacterium) is an opportunistic pathogen belonging to the Enterobacteriaceae family (Podschun & Ullmann 1998), acting as a reservoir of resistance genes and contributing significantly to their dissemination (Wyres, 2018). In general, antimicrobial resistance in *K. pneumoniae* can be determined by the presence of mobile genetic elements (MGE) such as plasmids, phages, and insertion sequence (IS) (Wyres, Lam & Holt 2020). These MGEs can be acquired and/or transferred to other Enterobacteriaceae species by conjugation, transformation, transduction, or vesiduction (Soler & Forterre 2020; Zhu et al., 2021). Furthermore, mutations in essential genes such as *gyrA* and *parC*, as well as efflux pumps are commonly associated with resistance to fluoroquinolones, cephalosporins, and carbapenems in *K. pneumoniae* (Wyres, Lam & Holt 2020). All these factors contribute to the emergence of strains with multidrug-resistant (MDR) phenotypes (Dell'annunziata et al., 2021). The inappropriate use of broad-spectrum cephalosporins for the treatment of human and animal infections contributes to the emergence of *K. pneumoniae*-producing extended-spectrum β-lactamase (ESBL) enzymes (Girijan & Pillai 2023). The ESBL phenotype in *K. pneumoniae* is associated with the expression of genes located on the chromosome (*bla*$_{SHV}$) or on plasmids (*bla*$_{CTX}$ and *bla*$_{TEM}$) (Girijan & Pillai 2023; Sudatip et al., 2023).

The virulence factors in *K. pneumoniae* are related to the production of capsules, lipopolysarides (LPS), siderophores, adhesins and extracellular vesicles (EVs). These are essential determinants for the development of infections in hospital environments and in animal production (Wyres, Lam & Holt 2020). Mobile genetic elements (MGE) such as virulence plasmids and integrative and conjugative elements (ICEs) are part of the accessory genome of *K. pneumoniae* and are responsible for the hypervirulent phenotype (Kot et al., 2023). ICEs can carry genes that encode for siderophores such as yersinabactin and salmochelin, toxins (colibactin), and hypermucoviscosity regulators (Wyres, Lam & Holt 2020). Virulence plasmids can also carry the genes responsible for the mentioned siderophores with the inclusion of aerobactin besides resistance genes (Wyres, Lam & Holt 2020). The diversity of genes associated with antimicrobial resistance and virulence reinforces the importance of genomic analyzes of *K. pneumoniae* from different sources.

Studies related to the characterization of *K. pneumoniae* genomes are very well elucidated in clinical isolates from human. Currently, little is known about the genetic potential of *K. pneumoniae* coming from different niches, such as clinically healthy swine. Thus, the objective of this work is to characterize genomes of *K. pneumonaie* MDR from clinically healthy swine under sanitary challenge and to investigate the presence of resistance plasmids.

## Materials and methods

### Bacterial strains, growth and maintenance conditions

The *Klebsiella pneumoniae* strains HS-144 and HS-13 used in this work were isolated from the rectum of healthy swine in december 2019. The strains belong to the Laboratory of Molecular Genetics of Bacteria at the Universidade Federal de Viçosa (UFV). Another strain used was *Escherichia coli* J53 (Matsumura, et al 2018) and *Escherichia coli* ATCC25922.

### Whole genome sequencing (WGS) and in silico analysis

DNA from the strains was obtained with the Wizard Genomic DNA Purification kit (Promega, Madison, Wisconsin, USA) and inspected for quality by the NanoDrop® 2000 spectrophotometer (Thermo Scientific, Walthan, Massachusetts, USA) and by gel electrophoresis agarose. Samples were sent to Macrogen (Seoul, South Korea) for sequencing (WGS) and genome assembly. The Illumina TruSeq platform was used. The library was built with the TrueSeq Nano DNA kit and genome assembly was performed using the De Novo method. Quality control of genome assembly was performed using BUSCO (version 3.0.2) and identification at the species level was performed using ANI (Average Nucleotide Index)

analysis. Finally, genome alignment was performed using the BLASTn tool. Genome annotation was performed using the RAST server (http://rast.nmpdr.org) and the genes were grouped into functional categories. Genomes were inspected for resistance genes by the CARD (Alcock et al., 2020) and ResFinder (Bortolaia et al., 2020) antibiotic resistance database. Virulence genes were searched using the VirulenceFinder database (Kleinheinz, Joensen, Larsen 2014) and VFDB (Liu et al., 2022). Finally, the genomes were submitted to a database such as Mobile Element Finder v1.0.2 (Johansson et al., 2020), PlasmidFinder (Carattoli et al., 2014) and oriTFinder v1.0 (Li et al., 2018) to search for genes related to plasmids. To search for phages, we used the Phaster predictor (Arndt et al., 2016).

**Extended-spectrum β-lactamase (ESBL) phenotype test**

*Klebsiella pneumoniae* HS-144 and HS-13 strains were subjected to the phenotypic test for ESBL production according to Tzelepi et al. (2000). Bacterial suspensions were prepared with turbidity of 0.5 (MacFarland scale) in 0.8% saline solution. Then, the suspensions were homogenized and plated on Mueller-Hinton agar plates (BD, Franklin Lakes, New Jersey, USA). To carry out this test, we used amoxicillin/clavulanic acid discs (30ug), aztreonam (30ug), cefotaxime (30ug), ceftazidime (30 µg) and cefepime (30 µg). Bacteria were considered ESBL producers when the formation of an irregular zone of inhibition (ghost-zone) is visible between the disc composed of amoxicillin/clavulanic acid and the others. The negative control strain used was *E. coli* ATCC 25922. The experiments were performed in biological replicates.

**Minimal inhibitory concentration (MIC)**

MIC for colistin was performed as per the Clinical and Laboratory Standards Institute (CLSI 2020 - Vet) guidelines. The dilution range used was from 128 ug.ml$^{-1}$ to 0.25 ug.ml-1. Bacteria were grown in Mueller Hinton broth to an $OD_{600}$ of 0.5. Subsequently, they were transferred to 96-well microplates containing the tested antimicrobial. The bacteria were placed in an oven at 37°C for 24 hours. For the final evaluation, 50ul of 0.1% resazurin was added and the plate was incubated for 2 to 3 hours at 37°C. The MIC value was considered as the lowest dilution of the antimicrobial that did not change color. The experiment was conducted in biological triplicates.

**Conjugation assay**

*Klebsiella pneumoniae* HS-144 (colistin resistance) strain was used as donor and *E. coli* J53 (Azide resistant) as the recipient strain. The procedure was performed according to Franco

et al. (2015) with some modifications. Bacteria were grown on Luria Bertani (LB) agar overnight at 37°C. Subsequently, a 2% inoculum we prepared and left it in a shaker at 180 rpm at 37°C until reaching an $OD_{600}$ of 0.5. A 1x PBS wash was performed and the pellet was resuspended with LB medium. Donor and recipient bacteria were mixed in a 3:1 ratio, respectively. The mixture was conditioned in an oven at 37°C for 4 hours. Subsequently, they were seeded in LB agar containing colistin 8 $ug.ml^{-1}$ and sodium azide 100 $ug.ml^{-1}$ and left overnight in an oven at 37 °C. Grown colonies were picked on new plates containing colistin 8$ug.ml^{-1}$ and sodium azide 100$ug.ml^{-1}$.

**Confirmation of transconjugants**

We performed the polymerase chain reaction (PCR) technique to confirm the presence of the *mcr-1* and *bla*$_{TEM}$ genes. The primers used to detect the *mcr-1* gene were: *mcr1*-F 5' GGGTGTGCTACCAAGTTTGC 3' and *mcr1*-R 5' CATTGGCGTGATGCCAGTTT 3', which generate an amplicon of 1.126 bp (Paveenkittiporn et al., 2017). For the detection of a 461 bp amplicon representing the *bla*$_{TEM}$ gene, we used the primers *bla*$_{TEM}$-F 5' ACAGCGGTAAGATCCTTGAGAG 3' and *bla*$_{TEM}$-R 5' GAAGCTAGAGTAAGTAGTTCG 3' (Yu et al., 2018). The positive control for these genes was DNA from the *K. pneumoniae* HS-144 strain.

**Pulsed field gel eletroforesis (PFGE)**

The preparation of the plugs for the PFGE was carried out according to Barton et al. (1995) with some modifications. Bacteria were grown on LB agar with or without colistin (8 $\mu g.mL^{-1}$) overnight at 37°C. Subsequently, an initial inoculum of 2% was made in 10 ml of LB with or without colistin (8 $\mu g.mL^{-1}$), and placed in a shaker at 180 rpm at 37°C until reaching an $OD_{600}$ of 0.5. After growth, the 10 ml were centrifuged for 10 minutes at 5,000 rpm and washed with 2 ml of solution 1 (1M NaCl and 10mM Tris-HCl pH8). We centrifuged again (10 min at 5,000 rpm) and resuspended in 500ul of solution 2 (1M NaCl; 100mM EDTA; 6 mM Tris-HCl pH8; 0.5% Sarcosyl; 0.2% Deoxycholate and 0.5% Brij). After the previous step, we added 500 ul of the suspension with 500ul of 2% agarose containing lysozyme ($1mg.mL^{-1}$) and RNase ($20mg.mL^{-1}$) and poured it into the molds. The plugs were soaked with solution 2 added with lysozyme ($1mg.mL^{-1}$) and RNase ($20mg.mL^{-1}$) and left in a water bath at 37°C for 40 minutes. We removed the previous solution and added the NDS buffer (EDTA 0.45M; Tris-HCl pH9; sarkosyl 1% and proteinase K 2 $mg.mL^{-1}$) and left for 24 hours at 50°C. The next day, the plugs were washed with 5 ml of 50mM EDTA added with 1% sarkosyl for 30 minutes

at 50°C. The run was performed on a 1.2% gel in a 0.5% TBE running buffer for 20 hours. A voltage of 210 and an amperage of 130 were used. The parameters for 14 hours with a pulse every 45 seconds and 6 hours with a pulse every 25 seconds were used. We used the Pulse MarkerTM (Sigma, St. Louis, Missouri, USA).

## Results

### Characterization of sequenced genomes

The genomes of the *K. pneumoniae* HS-144 and *K. pneumoniae* HS-13 isolates, were sequenced and characterized (Table 1). All alignments performed by ANI (Average Nucleotide Index) gave values above 99.3% and coverage above 90% (Table 1). The genome size is 5.3 e 5.6 Mpb, as well as the GC content, is 57% (Table 2). The average number of coding sequences is 5148 and about 87 RNAs (Table 2). For the genome of the *K. pneumoniae* HS-144 strain, 35 contigs were obtained and for the *K. pneumoniae* HS-13 strain, 41 contigs were obtained (Table 2). Regarding the taxonomy of these strains, they belong to the domain: Bacteria, phylum: Pseudomonadota, class: Gammaproteobacteria, order: Enterobacterales, family: Enterobacteriaceae, genus: *Klebsiella*, species: *Klebsiella pneumoniae*, strains *K. pneumoniae* HS-144 and *K. pneumoniae* HS-13.

**Table 1:** ANI (Average Nucleotide Index) analysis

| Strains | ANI analysis | | | |
|---|---|---|---|---|
| | Ranking | Similar genome | ANI (%) | Cobertura do alinhamento (%) |
| *K. pneumoniae* HS-144 | 1 | GCA_947662965.1_*K.pneumoniae*_6044 | 99.95 | 92.78 |
| | 2 | GCA_027915375.1_*K.pneumoniae*_ | 99.93 | 93.39 |
| | 3 | GCA_027913955.1_*K.pneumoniae*_ | 99.93 | 93.80 |
| | 4 | GCA_027913835.1_*K.pneumoniae*_ | 99.92 | 93.80 |
| | 5 | GCA_027915315.1_*K.pneumoniae*_ | 99.92 | 93.78 |
| *K. pneumoniae* HS-13 | 1 | GCA_027913925.1_*K.pneumoniae*_ | 99.35 | 91.58 |
| | 2 | GCA_027915395.1_*K.pneumoniae*_ | 99.35 | 91.64 |
| | 3 | GCA_027920145.1_*K.pneumoniae*_KP0213 | 99.32 | 90.07 |
| | 4 | GCA_027919515.1_*K.pneumoniae*_CHRF_KPN_03 | 99.32 | 91.39 |
| | 5 | GCA_027918915.1_*K.pneumoniae*_CHRF_KPN_02 | 99.31 | 90.09 |

**Table 2:** *K. pneumoniae* HS-144 and HS-13 genomes features

| Parameters | Strains | |
|---|---|---|
| | *K. pneumoniae* HS-144 | *K. pneumoniae* HS-13 |
| Size genome (Mbp) | 5.6 | 5.3 |
| Number of contigs | 35 | 41 |
| GC% content | 57.04 | 57.16 |
| Number of Coding Sequences | 5.148 | 5.148 |
| Number of RNAs | 87 | 86 |
| BioSample ID | SAMN35723120 | SAMN35723125 |

The functional annotation of the genes showed the most prevalent categories such as carbohydrate metabolism (19.1%); amino acids (11.8%); cofactors and vitamins (8.7%); protein metabolism (6.7%); membrane transport (5.7%); cell wall and capsule (5.2%); RNA metabolism (5.5%); breathing (4.1%); stress response (3.9%); cellular regulation and signaling (3.5%); DNA metabolism (3.5%); virulence, disease, and defense (3.1%); and iron metabolism and acquisition (2.2%) (Figure 1; Supplementary Table 1).

Functions related to virulence are identified as: hyper adherence (*yidE*), bacteriocin (colicin), choline tolerance system, lysozyme inhibitors, iron acquisition (aerobactin and enterobactin), capsule, lipopolysaccharides (LPS), type VI secretion system, and response quorum sensing and biofilm formation (*lsrACDBFGE* operon). Other important genes for adaptation are related to the response to stress, namely osmotic, oxidative, thermal shock, cold shock, detoxification (tellurite and selenite), and periplasmic (*degS*) (Supplementary Table 1).

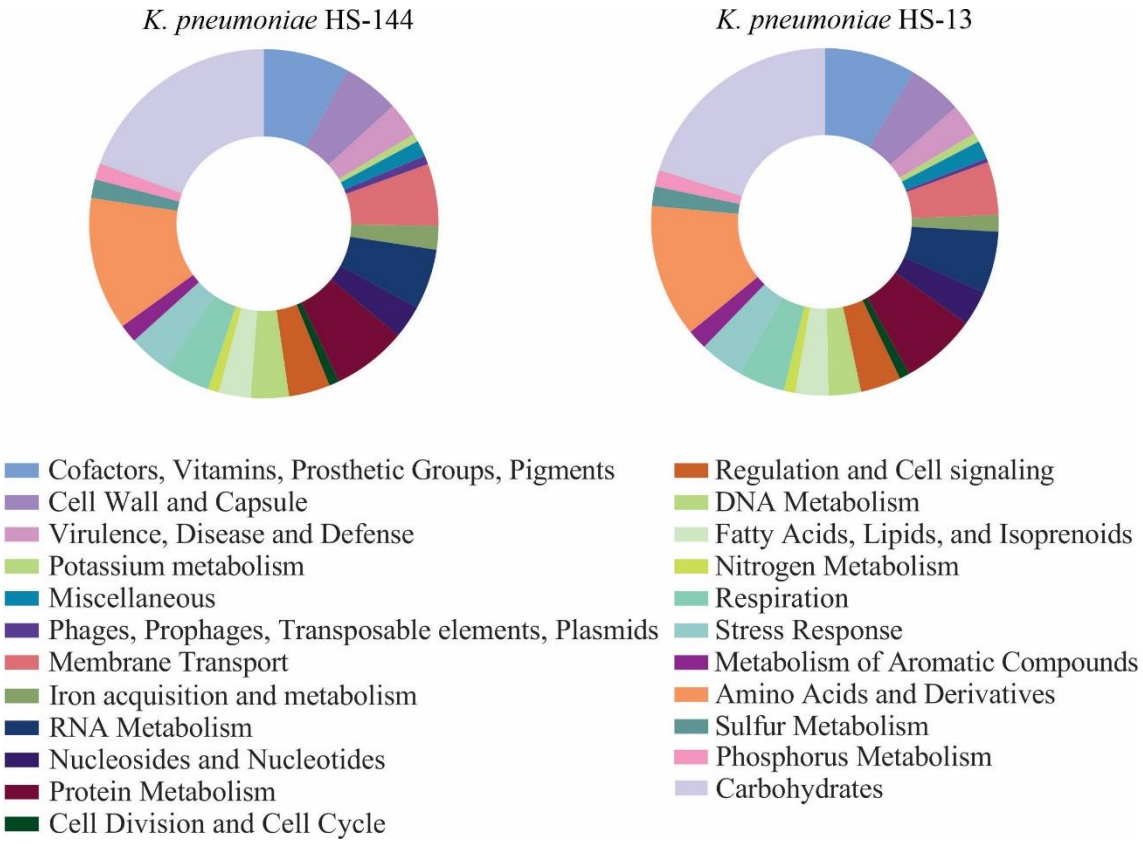



**Figure1:** Functional classes of genes identified in the *K. pneumoniae* HS-144 and HS-13 strains genomes performed by the RAST server.

Regarding predictors of resistance genes, CARD and ResFinder detected genes related to resistance to classes of antimicrobials such as cephalosporins, aminoglycosides, macrolides, sulfonamides, fluoroquinolones, tetracyclines, phenicols, carbapenems, fosfomycin and β-lactams (Supplementary Table 2 and 3). Point mutations were also found among the genes *ramR*, *ompK36*, *ompK37*, *parC*, *gyrA*, and *acrR* (Supplementary Table 4). These mutations may contribute to resistance to cephalosporins, ciprofloxacin, fluoroquinolones, and carbapenems (Supplementary Table 4). The PlasmidFinder and Mobile Genetic Element Finder predictors found varied plasmid-associated replicons in the HS-144 and HS-13 genomes (Supplementary Table 5 and 6). For the HS-144 genome, we have the IncX4 replicons in contg18 (*mcr-1* resistance gene); IncFII at contig8 (*iucC* virulence gene); Col440I in contig26 and IncFIA in contig21 (Supplementary Table 5 and 6). For the HS-13 strain we have Col440I replicons in contig31; IncR on contig27 and IncFIB on contig30 (Supplementary Table 5 and 6). The next step was to investigate regions with conjugation-related genes, for which we used the oriTFinder predictor (Supplementary Table 7). We detected complete conjugation apparatus (*oriT*, relaxase, T4CP, and T4SS) only in the HS-144 strain genome was detected in the contig8 (Supplementary Table 7).

To investigate the presence of prophages in these genomes we used Phaster (Supplementary Table 8). We found five intact phages in the HS-144 strain, one of which is responsible for transporting the *blaSHV2* gene present in contig8 (Supplementary Table 8). For the HS-13 strain, two intact phages were detected.

**Plasmid detection, ESBL phenotype and conjugation assay**

To validate the presence of plasmid replicons detected by the predictors used in this work, we performed the PFGE technique (Figure 2A). It was possible to verify that both strains carry plasmids, however, only the HS-144 strain carries a plasmid with a complete conjugation system, as showed by genomic analyses. We performed the ESBL phenotype detection test on the HS-144 and HS-13 strains (Figure 2B). The choice of this phenotypic analysis was based on the fact that the prediction of resistance genes presents the related genetic markers. The combined disc analysis showed that only HS-144 strain is positive for the ESBL phenotype (Figure 2B).

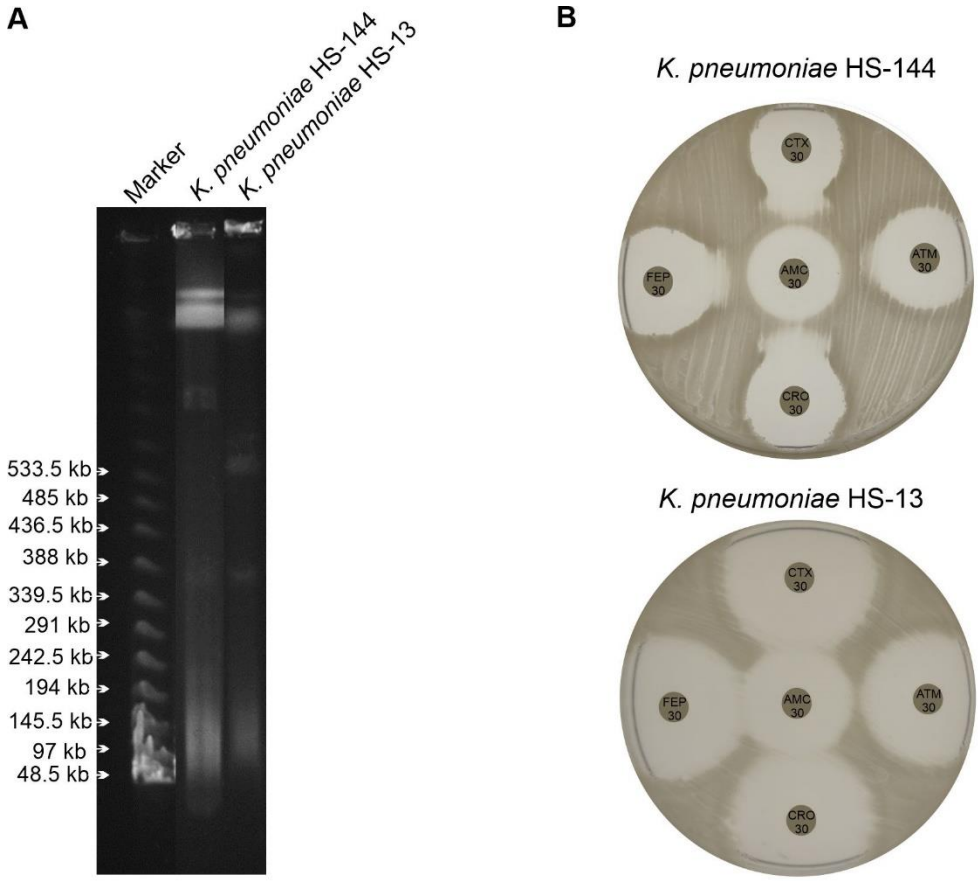



**Figure 2:** Genotypic and phenotypic characterization of HS-144 and HS13 strains. A) Pulsed-field electrophoresis (PFGE) for plasmid detection. B) Combined disc test for ESBL phenotype detection.

We know that the IncX4 replicon that is in contig18 and that this contig also has the *mcr-1* gene (Supplementary Table 6) and that at least one of the plasmids carried by the HS-144 strain is conjugative. Thus, we performed the conjugation assay with *E. coli* J53 as receptor and selection in colistin ($8ug.ml^{-1}$) to transconjugants counter-selection. As a result, we obtained three transconjugants (Figure 3A). These transconjugants were transferred for 3 generations to new LB agar plates with colistin and sodium azide. The presence of the *mcr-1* and $bla_{TEM1}$ genes in the transconjugants (Figure 3B) was confirmed by PCR indicating that the genetic marker was transfered (Figure 3B). The PFGE gel shows the presence of plasmid in the transconjugants (Figure 3C).

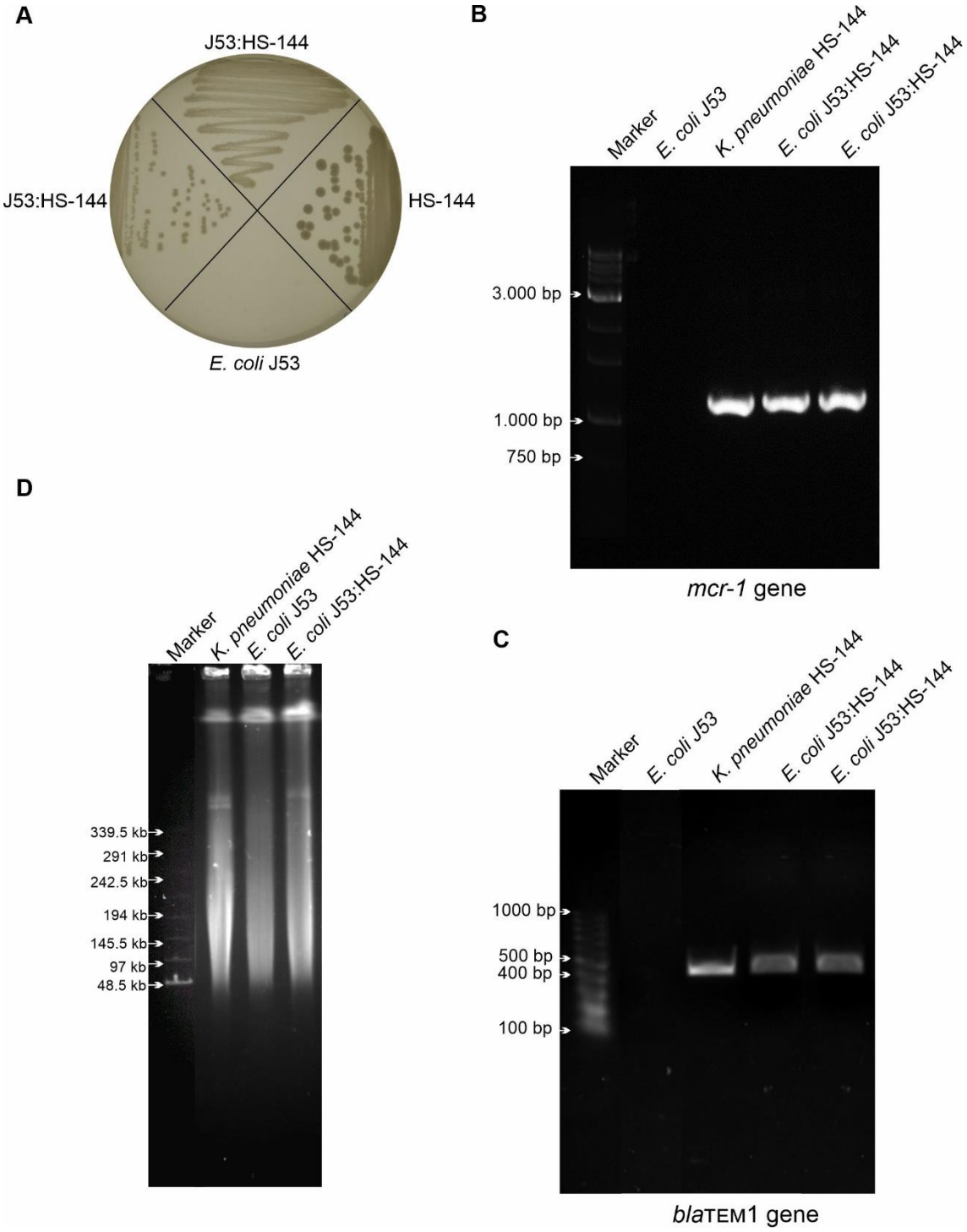



**Figure 3:** Phenotypic and genotypic characterization before and after conjugation assay. A) Transconjugants in LB agar with colistin 8 ug.mL$^{-1}$. B) *mcr-1* gene detection by PCR. C) *bla*$_{TEM1}$ gene detection by PCR. D) Pulsed-field electrophoresis (PFGE) for plasmid detection.

After detecting the resistance gene and the plasmid, we performed the minimum inhibitory concentration (MIC) test (Table 3). After conjugation, the transconjugants presented

MIC >64mg.L$^{-1}$. Prior to conjugation, the J53 receptor had a MIC of 2 mg.L$^{-1}$ (Table 3). The transconjugants did not show the ESBL phenotype.

**Table 3:** Minimum inhibitory concentration (MIC) of the strains used in this work before and after the conjugation assay

| Isolates | Minimal inhibitory concentration (MIC) |
|---|---|
| | Colistin (mg.L$^{-1}$) |
| *K. pneumoniae* 144 | 128 |
| *E. coli J53* transconjugant | >64 |
| *E. coli* J53 transconjugant | >64 |
| *E. coli* J53 transconjugant | >64 |
| *E. coli* J53 | 2 |

**Discussion**

In this work, we sequenced two genomes of *K. pneumoniae* MDR from clinically healthy swine under sanitary challenge, the strain HS-144 and HS-13. We found classes of genes responsible for antimicrobial resistance (ESBL) and virulence (aerobactin) phenotypes possibly present in plasmids.

The sequencing of the genomes allowed us to reach the level of contigs, 35 for the HS-144 strain and 41 for the HS-13 (Table 2). The fragmentation of genomes into contigs makes it difficult to predict mobile genetic elements such as plasmids and integrative and conjugative elements (Van der Graaf-Van Boolois, Wagenaar & Zomer 2021). Here, we detected 3 to 4 plasmid replicons for the investigated strains (Supplementary Tables 5 and 6). However, it was not possible to define it. We investigated regions with genes from conjugative systems and the origin of transfer (*oriT*) and detected one in the HS-144 strain (Supplementary Table 7). The plasmids carried by the HS-13 strain should be investigated more precisely in the future, however, we know that the IncR replicon is not conjugative as well as probably the others detected in the prediction.

In a previous study, the HS-13 and HS144 strains showed resistance phenotypes to some antimicrobials of the cephalosporin, sulfonamide, fluoroquinolone, polymyxin, amphenicol, tetracycline, and B-lactam classes (chapter 1). Here we confirmed the ESBL phenotype for the HS-144 strain (Figure 2). In *K. pneumoniae* this phenotype is increasingly reported in production animals in clinical conditions (Abrar et al., 2019; Effendi et al., 2022). The $bla_{\text{CTXM}}$, $bla_{\text{SHV}}$, $bla_{\text{OXA}}$, and $bla_{\text{TEM}}$ genes are the main ESBL encoders, with $bla_{\text{TEM}}$ being the most

associated with livestock (Abrar et al., 2019). Many plasmids are reported to disseminate genes related to the ESBL phenotype (Sudatip et al., 2023). We did not detect the ESBL phenotype in the transconjugants, which allows us to state that possibly the *bla*$_{SHV2}$ gene may play an important role in the phenotype in the HS-144 strain.

The *bla*$_{SHV2}$ gene was detected in contig8, the same site at which the Phaster predictor detected the presence of a phage (PHAGE_Escher_RCS47_NC_042128) (Supplementary Table 8). By inspecting the predicted regions for the phage, we concluded that the *bla*$_{SHV2}$ gene is within the element. This phage can be integrated into the genome or even present in plasmids, as already reported in the literature (Tran et al., 2023).

The conjugation assay showed us that the HS-144 strain carries a conjugative plasmid with the *mcr-1* gene that is transferred to *E. coli* J53 (Figure 3). We chose to use colistin as selective marker due to the fact that the IncX4 replicon is located in the same contig as the *mcr-1* resistance gene (Supplementary Table 5). In the literature, it is known that IncX4 plasmids are conjugative (Sadek et al., 2021). As a result, we obtained transconjugants with *mcr-1* and *bla*$_{TEM}$ genes, and colistin resistance phenotype as the donor strain (Figure 3; Table 3).

Brazilian swine farming has been decreasing the use of antimicrobial growth promoters over the years (Gharaibeh & Shatnawi 2019). Colistin sulfate was withdrawn from circulation in livestock in Brazil since 2016 (Longo et al., 2019). However, many reports of colistin resistance had already been described in European Asian countries and South America (Longo et al., 2019). Livestock is the main reservoir environment for the *mcr-1* gene (Gharaibeh & Shatnawi 2019). Colistin resistance mechanisms occur by the spread of plasmids with *mcr* genes or by mutations in the lipid A portion of lipopolysaccharide (LPS) (Gharaibeh & Shatnawi 2019).

We detected mutations in efflux pumps, porins, and DNA replication genes (Supplementary Table 4). These mutations change the reading frame and replace an amino acid the main mutated genes were *ompK36*, *ompK37*, *ramR*, *parC*, *gyrA*, and *acrR*. Mutations can affect resistance phenotypes to fluoroquinolones, cephalosporins, and carbapenems. Phenotypically, resistance to carbapenems has not been confirmed to both strains (chapter 1). The AcrAB efflux system contributes not only to resistance to fluoroquinolones but also to chloramphenicol, tetracycline, and erythromycin (Zhang et al., 2023). In addition, it may be associated with virulence since it confers resistance to antimicrobial peptides that are components of the innate immune system (Padilla et al., 2010; Zhang et al., 2023).

The swine from which the HS-144 and HS-13 strains were isolated were in an environment under sanitary challenge and did not show clinical signs or disease history. However, the environment without inappropriate management can be ideal for the dissemination of *K. pneumoniae* MDR (chapter 1). Thus, we suggest that *K. pneumoniae* is an important health marker microorganism in livestock.

We conclude that clinically healthy production pigs are sources of *K. pneumoniae* MDR, as conjugative plasmids, which are capable of carrying and disseminating resistance genes such as *mcr-1* and $bla_{\text{TEM1}}$. *Klebsiella pneumoniae* MDR may still have an important chromosomal genetic arsenal for its pathogenicity.

**Funding information**

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Programa de Excelência Acadêmica-Código Financeiro 001 and Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG.

**Author contributions**

R.N.J, S.C.G, F.P.P, and G.M.M. helped in the development of experiments and analysis of results. R.N.J wrote this article and edited the images. S. C. G and B. S. M. D helped correct the manuscript and the English. All authors discussed the results and their implications. All authors edited and approved the final manuscript.

**Conflicts of interest**

The authors declare no competing interests.

**Supplementary tables:**

https://docs.google.com/spreadsheets/d/1TTZvXacoGLMFSsy3e529MAGmOrkivvQE/edit?usp=sharing&ouid=118022498442200222122&rtpof=true&sd=true

## References


Arndt, D., Grant, J. R., Marcu, A., Sajed, T., Pon, A., Liang, Y., Wishart, D. S. PHASTER: a better, faster version of the PHAST phage search tool. *Nucleic acids research*. **2016**, 44(W1), W16-W21.

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleics acids research*. **2018**, 48, D517-D525. DOI:10.1093/nar/gkz935

Barton, Bret M.; Harding, Gordon P.; Zuccarelli, Anthony J. A general method for detecting and sizing large plasmids. *Analytical biochemistry*, v. 226, n. 2, p. 235-240, 1995.

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Aarestrup, F. M. ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother*. **2020**, 75, 3491–3500. DOI:10.1093/jac/dkaa345

Carattoli, A., Zankari, E., García-Fernández, A., Voldby Larsen, M., Lund, O., Villa, L., Hasman, H. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrobial agents and chemotherapy*. **2014**, 58(7), 3895-3903. DOI:10.1128/aac.02412-14

Davis, Gregg S.; Price, Lance B. Recent research examining links among *Klebsiella pneumoniae* from food, food animals, and human extraintestinal infections. *Current Environmental Health Reports*, v. 3, n. 2, p. 128-135, 2016. DOI:10.1007/s40572-016-0089-9

Dell'Annunziata, F., Dell'Aversana, C., Doti, N., Donadio, G., Dal Piaz, F., Izzo, V., Franci, G. Outer membrane vesicles derived from Klebsiella pneumoniae are a driving force for horizontal gene transfer. *International Journal of Molecular Sciences*. **2021**, *22*(16), 8732. DOI:10.3390/ijms22168732

Effendi, M. H., Hartadi, E. B., Witaningrum, A. M., Permatasari, D. A., Ugbo, E. N. Molecular identification of blaTEM gene of extended-spectrum beta-lactamase-producing *Escherichia coli* from healthy pigs in Malang district, East Java, Indonesia. *Journal of Advanced Veterinary and Animal Research*. **2022**, 9(3), 447. DOI:10.5455/javar.2022.i613

Gharaibeh, M. H., and Shatnawi, S. Q. An overview of colistin resistance, mobilized colistin resistance genes dissemination, global responses, and the alternatives to colistin: A review. *Veterinary world*. **2019**, 12(11), 1735. DOI:10.14202/vetworld.2019.1735-1746

Girijan, S. K., and Pillai, D. Genetic diversity and prevalence of extended-spectrum beta-lactamase-producing *Escherichia coli* and *Klebsiella pneumoniae* in aquatic environments receiving untreated hospital effluents. *Journal of Water and Health*. **2023**, 21(1), 66-80. DOI:10.2166/wh.2022.194

Johansson, M. H., Bortolaia, V., Tansirichaiya, S., Aarestrup, F. M., Roberts, A. P., Petersen, T. N. Detection of mobile genetic elements associated with antibiotic resistance in

Salmonella enterica using a newly developed web tool: MobileElementFinder. *Journal of Antimicrobial Chemotherapy*. **2021**, 76(1), 101-109. DOI:10.1093/jac/dkaa390

Kot, B., Piechota, M., Szweda, P., Mitrus, J., Wicha, J., Grużewska, A., Witeska, M. Virulence analysis and antibiotic resistance of *Klebsiella pneumoniae* isolates from hospitalised patients in Poland. *Scientific Reports*. **2023**, 13(1), 4448. DOI:10.1038/s41598-023-31086-w

Kleinheinz, K. A., Joensen, K.G., Larsen, M. V. Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and *E. coli* virulence genes in bacteriophage and prophage nucleotide sequences. *Bacteriophage*. **2014**, DOI:10.4161/bact.27943

Leangapichart, T., Lunha, K., Jiwakanon, J., Angkititrakul, S., Järhult, J. D., Magnusson, U., Sunde, M. Characterization of *Klebsiella pneumoniae* complex isolates from pigs and humans in farms in Thailand: population genomic structure, antibiotic resistance and virulence genes. *Journal of Antimicrobial Chemotherapy*. **2021**, *76*(8), 2012-2016. DOI:10.1093/jac/dkab118

Li, X., Xie, Y., Liu, M., Tai, C., Sun, J., Deng, Z., Ou, H. Y. oriTfinder: a web-based tool for the identification of origin of transfers in DNA sequences of bacterial mobile genetic elements. *Nucleic acids research*. **2018**, 46(W1), W229-W234. DOI:10.1093/nar/gky352

Liu, B., Zheng, D., Jin, Q., Chen, L., Yang, J. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*. **2019**, 47, D687-D692. DOI:10.1093/nar/gky1080

Longo, L. G., de Sousa, V. S., Kraychete, G. B., Justo-da-Silva, L. H., Rocha, J. A., Superti, S. V., Moreira, B. M. Colistin resistance emerges in pandrug-resistant *Klebsiella pneumoniae* epidemic clones in Rio de Janeiro, Brazil. *International journal of antimicrobial agentes*. **2019**, 54(5), 579-586. DOI:10.1016/j.ijantimicag.2019.08.017

Matsumura, Yasufumi, Gisele Peirano, Johann DD Pitout. Complete genome sequence of *Escherichia coli* J53, an azide-resistant laboratory strain used for conjugation experiments. *Genome Announcements.* **2018**, 6.21e00433-18. DOI: 10.1128/genomeA.00433-18

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J. A., Albertí, S. *Klebsiella pneumoniae* AcrAB efflux pump contributes to antimicrobial resistance and virulence. *Antimicrobial agents and chemotherapy*. **2010**, 54(1), 177-183. DOI:10.1128/aac.00715-09

Paveenkittiporn, W., Kerdsin, A., Chokngam, S., Bunthi, C., Sangkitporn, S., Gregory, C. J. Emergence of plasmid-mediated colistin resistance and New Delhi metallo-β-lactamase genes in extensively drug-resistant Escherichia coli isolated from a patient in Thailand. *Diagnostic microbiology and infectious disease*. **2017**, *87*(2), 157-159. DOI: 10.1016/j.diagmicrobio.2016.11.005

Podschun, R., and Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical microbiology reviews*. **1998**, 11(4), 589-603. DOI:10.1128/CMR.11.4.589

Franco, A., Leekitcharoenphon, P., Feltrin, F., Alba, P., Cordaro, G., Iurescia, M., Battisti, A. Emergence of a clonal lineage of multidrug-resistant ESBL-producing *Salmonella infantis* transmitted from broilers and broiler meat to humans in Italy between 2011 and 2014. *PloS one*. **2015**, 10(12), e0144802. DOI:10.1371/journal.pone.0144802

Sadek, M., Ortiz de la Rosa, J. M., Abdelfattah Maky, M., Korashe Dandrawy, M., Nordmann, P., Poirel, L. Genomic features of MCR-1 and extended-spectrum β-lactamase-producing Enterobacterales from retail raw chicken in Egypt. *Microorganisms*. **2021**, 9(1), 195. DOI:10.3390/microorganisms9010195

Soler, N., and Forterre, P. Vesiduction: the fourth way of HGT. *Environmental Microbiology*. **2020**, 22(7), 2457-2460. DOI:10.1111/1462-2920.15056

Sun, J., Liao, X. P., D'Souza, A. W., Boolchandani, M., Li, S. H., Cheng, K., Liu, Y. H. Environmental remodeling of human gut microbiota and antibiotic resistome in livestock farms. *Nature communications*. **2020**, 11(1), 1427. DOI:10.1038/s41467-020-15222-y

Sudatip, D., Mostacci, N., Tiengrim, S., Thamlikitkul, V., Chasiri, K., Kritiyakan, A., Hilty, M. The risk of pig and chicken farming for carriage and transmission of *Escherichia coli* containing extended-spectrum beta-lactamase (ESBL) and mobile colistin resistance (*mcr*) genes in Thailand. *Microbial genomics*. **2023**, 9(3). DOI: 10.1099/mgen.0.000951

Tran, T., Thorn, J., Scott, A., Robertson, J., Nash, J., Carrillo, C., Topp, E. Genetic characterization of extended-beta-lactamase (ESBL) plasmids captured from dairy manures. *bioRxiv*, 2023-03. DOI:10.1101/2023.03.20.533445

Tzelepi, E., Giakkoupi, P., Sofianou, D., Loukova, V., Kemeroglou, A., Tsakris, A. Detection of extended-spectrum β-lactamases in clinical isolates of Enterobacter cloacae and *Enterobacter aerogenes*. *Journal of clinical microbiology*. **2000**, *38*(2), 542-546. DOI:10.1128/jcm.38.2.542-546.2000

Van der Graaf-Van Bloois, L., Wagenaar, J. A., Zomer, A. L. RFPlasmid: predicting plasmid sequences from short-read assembly data using machine learning. *Microbial genomics*. **2021**, 7(11). DOI:10.1099/mgen.0.000683

Vieites, F. M., Souza, C. S., Castro, A. C. S., de Melo Júnior, A. M., Ferreira, M. H., Ferreira, S. E., Oliveira, G. P. Aditivos zootécnicos na alimentação de suínos–Revisão de Literatura. *Brazilian Journal of Development*. **2020**, 6(7), 45880-45895. DOI:10.34117/bjdv6n7-276

Wyres, K. L., and Holt, K. E. *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current opinion in microbiology*. **2018**, 45, 131-139. DOI:10.1016/j.mib.2018.04.004

Wyres, K. L., Lam, M. M., Holt, K. E. Population genomics of *Klebsiella pneumoniae*. *Nature Reviews Microbiology*. **2020**, 18(6), 344-359. DOI:10.1038/s41579-019-0315-1

Yu, F., Lv, J., Niu, S., Du, H., Tang, Y. W., Pitout, J. D., Chen, L. Multiplex PCR analysis for rapid detection of *Klebsiella pneumoniae* carbapenem-resistant (sequence type 258 [ST258] and ST11) and hypervirulent (ST23, ST65, ST86, and ST375) strains. *Journal of clinical microbiology*. **2018**, *56*(9), e00731-18. DOI:10.1128/JCM.00731-18

Zhang, Z., Morgan, C. E., Bonomo, R. A., Yu, E. W. Cryo-EM Structures of the *Klebsiella pneumoniae* AcrB Multidrug Efflux Pump. *MBio*. **2023**, e00659-23. DOI:10.1128/mbio.00659-23

Zhu, J., Wang, T., Chen, L., Du, H. Virulence factors in hypervirulent *Klebsiella pneumoniae*. *Frontiers in microbiology*, **2021**, 12, 642484. DOI:10.3389/fmicb.2021.642484

# Chapter 3

## Integrative and Conjugative Elements (ICEs) in *Klebsiella pneumoniae* contribute to adaptive diversity in different reservoir

(Manuscript written according to the guidelines of the Microbial Genomics)

Jéssica Nogueira Rosa[1], Giarlã Cunha da Silva[1], Osiel Silva Gonçalves[2], Matheus Machado Guidini[1], Patrícia Pereira Fontes[1], Rúzivia Pimentel Oliveira[1], Mateus Ferreira Santana[2], Denise Mara Soares Bazzolli[1*]

[1]Laboratório de Genética Molecular de Bactérias, Departamento de Microbiologia, Instituto de Biotecnologia Aplicada à Agropecuária- Bioagro, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brasil.

[2]Grupo de Genômica Evolutiva Microbiana, Laboratório de Genética Molecular de Microrganismos, Instituto de Biotecnologia Aplicada à Agropecuária- Bioagro, Universidade Federal de Viçosa, Minas Gerais, Brasil.

[*] Corresponding author: dbazzolli@ufv.br

### Abstract

*Klebsiella pneumoniae* is an opportunistic pathogen in humans and animals. Its genomics versatility allows it to explore a wide range of environments and reservoirs. Currently, efforts are focused on the study of human reservoir strains of *K. pneumoniae* due to the increasing spread of multiple antimicrobial resistance. However, the understanding of how integrative and conjugative elements (ICEs) contribute to their ecological role in other reservoirs is poorly studied. Thus, the objective of this work was to investigate the presence of ICEs in *K. pneumoniae* genomes to understand how these elements can modulate their ecological niche in different reservoirs. We analyzed 949 complete genomes of *K. pneumoniae*, the most from human (74.3%), animal (5.7%), environmental (3%), and food (0.1%) reservoirs. Overall, 501 analyzed genomes had one or more potential regions with self-transfer capability, and 18.2% of these present co-occurrences between ICEs. We detected 19 novel ICE candidates. The novel ICEs carry genes related to adhesion and iron uptake, capsule biosynthesis, cell wall and LPS, stress response, membrane components, and resistance to arsenic, β-lactam, polymyxin, and novobiocin. In addition, we also mapped four novel ICEs that carry the highly pathogenic island of Yersinia (HPI). Despite the limited number of *K. pneumoniae* genomes from non-human reservoirs, it was possible to find that ICEs may contribute to adaptation in stressful environments. Taken together, we conclude that the diversity of genes carried by ICEs in *K.*

*pneumoniae* may help their adaptive process to the different reservoirs/environments where they can inhabit.

**Keywords:** Ecological niche, Adaptation, Microbial fitness, One health.

**Abbreviations**

ABC, ATP-binding cassette; CDS, coding DNA sequence; CG, clonal group; cKP, classic *Klebsiella pneumoniae*; DMT, drug metabolite transporter; EVs, extracellular vesicles; EPs, efflux pumps; HGT, horizontal gene transfer; hvKP, hypervirulent *Klebsiella pneumoniae*; HPI, high pathogenicity island of Yersinia; ICE, integrative and conjugative element; LPS, lipopolysaccharide; MGE, mobile genetic element; MFP, mating pair formation; MFS, major facilitator superfamily; NCBI, National Center for Biotechnology Information; ORF, open reading frame; RND, resistance-nodulation-cell division; ST, sequence type; T4CP, type IV coupling proteins;T4SS, type IV secretion system.

**Impact Statement**

*Klebsiella pneumoniae* is a commensal bacterium that inhabits different hosts such as humans, animals, and plants. In addition, it is an opportunistic human and animal pathogen. The gene arsenal of *K. pneumoniae* plays an important role in modulating its ecological niche. These gene determinants can be related to their core genome or acquired by horizontal gene transfer (HGT). HGT-mediated antimicrobial resistance contributes to the spread of MDR strains, which makes *K. pneumoniae* a declared emerging pathogen by the World Health Organization. Integrative and conjugative elements (ICEs) contribute to the aptitude of *K. pneumoniae* as an opportunistic pathogen. It is now known that ICEs carry genes encoding colibactin and the highly pathogenic island of Yersinia (HPI). Studies of ICEs in *K. pneumoniae* isolates from non-human reservoirs are limited and therefore essential to understand how these elements contribute to the ecological role of this microorganism. The diversity of environments inhabited by *K. pneumoniae* reflects the plasticity of its genome. Thus, this work investigated the presence of ICEs in *K. pneumoniae* genomes available in a public database. This work contributes to the understanding of the relationship between this complex microorganism and its respective hosts with a focus on the one health approach.

**Data summary**

All genomes used in this work belong to the National Center for Biotechnology Information (NCBI) public database. All generated information is supported by supplementary data files.

## Introduction

*Klebsiella pneumoniae* is a Gram-negative bacterium member of the Enterobacteriaceae family and an important opportunistic pathogen of the human and animal respiratory system (Podschun & Ullmann 1998; Wyres & Holt 2018). It is found in water, soil, food, and plants, which makes it ubiquitous in nature (Campos-madueno et al., 2021). *Klebsiella pneumoniae* may present as an endophytic microorganism or a disease-causing agent in plants (Fouts et al., 2008). Some isolates found in soil and water contaminated with heavy metals have the potential for bioremediation (Kumar et al., 2021). Dissemination of *K. pneumoniae* by hospital routes, and contamination of soils with sewage and in livestock are mentioned increasingly, and recently, an isolate causing disease in humans and animals was reported as the causative agent of disease in maize, sorghum, and bananas (Tot et al., 2021; Huang et al., 2016). The niche established by *K. pneumoniae* in different hosts can be defined by its genetic-phenotypic aspects and by the conditions inherent to the host/environment itself (Pope et al., 2019).

In this scenario, two important variants are described for *K. pneumoniae*: i) the so-called "classic" *K. pneumoniae* (cKp), which carries genes for resistance to multiple antimicrobials and is commonly associated with nosocomial infections; and ii) "hypervirulent" *K. pneumoniae* (hvKp), mainly associated with systemic community infections. However, the spread of strains with both phenotypes is increasing (Bidewell et al., 2018; Liu et al., 2019). These *K. pneumoniae* strains can be monitored using techniques that allow them to be included in clonal groups (CG). Strains belonging to CG258 and CG15 are the most widespread on a global scale and have the potential to carry genes of resistance to critically important antimicrobials (Wyres, Lam & Holt 2020). As well as CG23 and CG65 strains that represent mostly hypervirulent clones (Liu et al., 2019; Leangapichart et al., 2021). *Klebsiella pneumoniae* clones can also be differentiated based on the content of accessory genes, which may explain their adaptation to different environments and hosts (Wyres, Lam & Holt 2020).

The virulence factors of *K. pneumoniae* are associated with the production of different types of siderophores (enterobactin, yersiniabactin, salmochelin, and aerobactin), type I and III fimbriae, capsules, lipopolysaccharides (LPS), extracellular vesicles (EVs) and mobile genetic elements: ICEs (integrative and conjugative elements) and plasmids (Holt et al., 2015; Wyres, Lam & Holt 2020). ICEs and plasmids significantly contribute to the convergence of the hvKPs and cKPs phenotypes (Wyres, Lam & Holt 2020). Currently, the ICEs described for *K. pneumoniae* actively contribute to its virulence and are closely related to clinical isolates from the human reservoir (Lin et al., 2008; Liu et al., 2019; Lam et al., 2018). These elements carry

high pathogenicity islands of Yersinia (HPI), colibactin toxin, and the *rmpA* gene that regulates the hypermucoviscosity phenotype (Lin et al., 2008; Liu et al., 2019; Lam et al., 2018). The only report of an ICE in *K. pneumoniae* isolates from an animal reservoir describes the ICE*KpSL1* found in sick pigs that also carry HPI (Liu et al., 2019).

The low frequency of complete *K. pneumoniae* genomes from other reservoirs makes it difficult to understand how ICEs contribute to their ecological role in these reservoirs. The spread of MGEs among species of the Enterobacteriaceae family is recurrent and, therefore, it is necessary to monitor elements such as ICEs. Thus, this work aimed to investigate the presence of ICEs in *K. pneumoniae* genomes available in a public database and to understand how these elements contribute to the ecological niche of *K. pneumoniae* from different reservoirs.

## Materials and methods

### Dataset, detection, and delimitation of ICEs

Complete genomes of *K. pneumoniae* deposited at the NCBI until July 2021 were used. Information on the deposited genomes such as country, year, sequence type (ST), reservoir, and collection source were recorded (Supplementary Table 1). The BIGSdb-Pasteur platform (Jolley & Maiden 2010) was used to search genomes that did not have sequence types (STs) in their depository in NCBI. The search for conjugative modules was performed using MACSyFinder CONJscan (Cury et al., 2020). Subsequently, we inspected the genomes for relaxases, type IV coupling proteins (T4CP) and clusters of genes encoding the type IV secretion system (T4SS) using the oriTfinder tool (Li et al., 2018). ICEs sequences already validated for *K. pneumoniae* were retrieved from the ICEberg database 2.0 (Liu et al., 2018). Comparative analyses with the ICEberg data allowed the identification of new putative elements of ICE. We considered conjugative the elements that presented the T4SS gene group, a relaxase, T4CP and type-specific genes related to the formation of mating pairs (MFP). For the delimitation of direct repeat regions (*attR* and *attL*), the Repeat Finder plugins feature was used in Geneious Prime® version 2020. The integrase family was sorted by searching conserved domains using CD-Search (Marchler-bauer & Bryant 2004). The tRNA genes were identified by tRNAscan-SE (Chan, Lowe, 2019). The search for the relaxase family was carried out by MOBscan (Garcillán-barcia et al., 2020).

**Characterization, comparative analysis, and dispersion of ICEs**

We used GeneMarkS (Besemer et al., 2001) to annotate protein sequences. The functional annotation of new ICEs was performed using the eggNOG-mapper database (Cantalapiedra et al., 2021). We then performed a global analysis of the ICEs mapped to the genomes (size, GC%, and number of open reading frames (ORFs). A sequence identity matrix was constructed using nucleotide sequences from the novel ICEs and the ICEs described for the species. Alignment of these sequences was performed in Clustal Omega (Sievers & Higgins 2014) and a heatmap was generated using the ggplot2 R package. To trace the evolutionary history of these elements, we used the gene for integrase (*int*) and relaxase (*mob*). The sequence of int and mob genes were aligned in ClustalW and later, phylogenetic trees of maximum likelihood (GTR model and 1000 bootstrap) were built. Alignments were performed in MEGA X and the phylogenetic tree edited in iTOL (Letunic & Bork 2019). The search for resistance genes was performed using the comprehensive antibiotic resistance database CARD (Alcock et al., 2020) and ResFinder (Bortolaia et al., 2020). And the search for virulence genes we used the VirulenceFinder (Kleinheinz, Joensen & Larsen 2014) and VFDB (Liu et al., 2022) databases. Finally, we performed a MegaBLAST alignment with the ICEs described for the species, establishing a >90% cutoff for identity and coverage to investigate its spread.

**Results**

**Characteristics of the dataset**

We obtained a dataset of 949 complete genomes distributed on a global scale (Fig.1A). Of these, 9% presented information regarding the STs of *K. pneumoniae* strains (Supplementary Table 1). Most of the genomes obtained are from human (74.3%), animal (5.7%), environmental (3%), and food (0.1%) reservoirs (Fig.1B). Different collection sources such as feces, blood, and urine stand out in the human and animal reservoirs (Supplementary Fig.1A and C). Soils and water are the most environmentally relevant collecting sources (Supplementary Fig.1B). The majority of *K. pneumoniae* genomes obtained belong to diseased reservoirs, whether human or animal. Furthermore, these strains showed multiple resistance to antimicrobials or hypermucoviscous phenotypes. By performing the STs analysis of these genomes, it was possible to determine that in human reservoirs the ST258 and ST11 groups are the most prevalent (Supplementary Table 1). For the other reservoirs, no prevalent STs were observed (Supplementary Table 1).

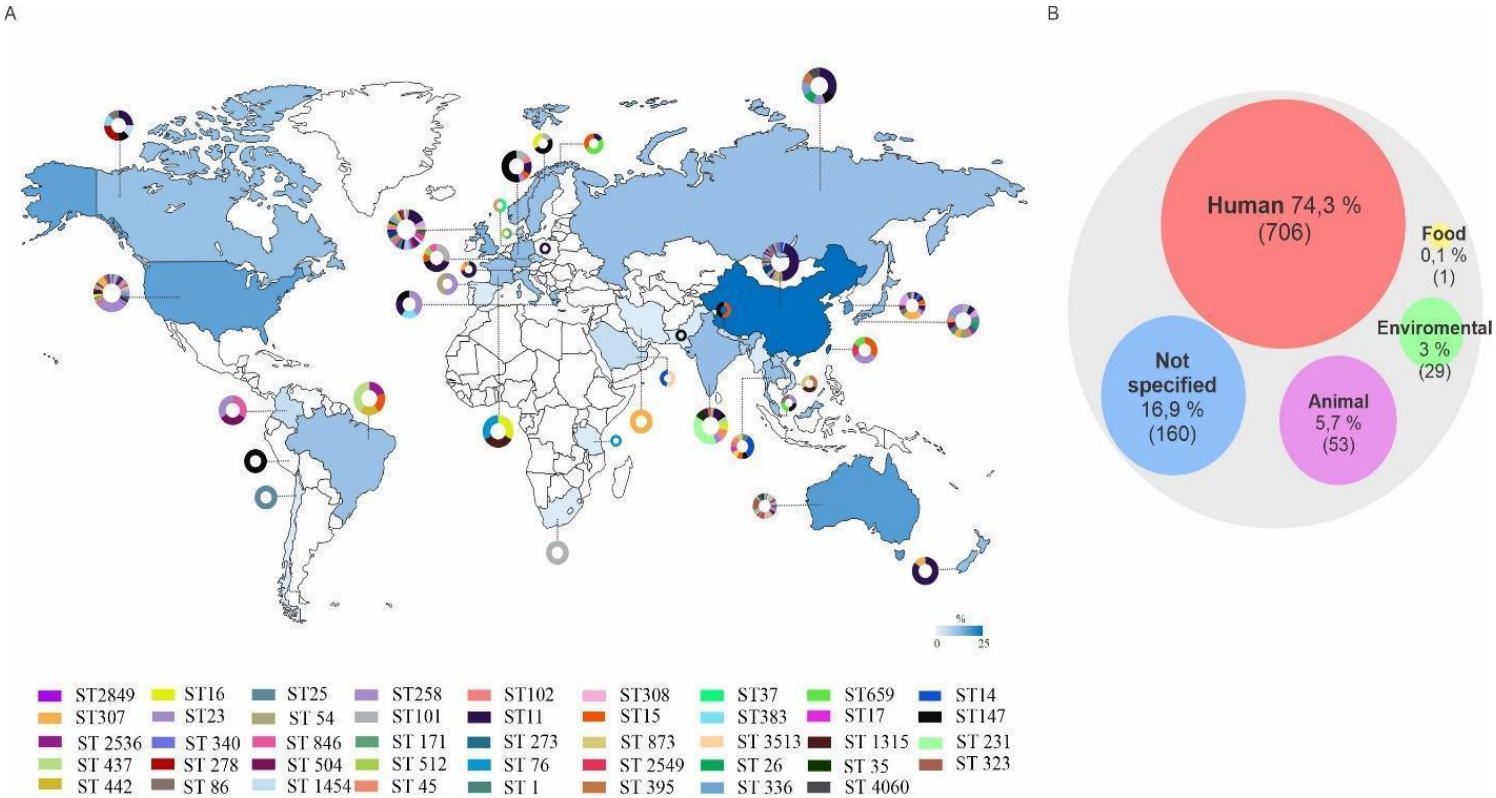



**Figure. 1.** Set of data used in this work. A) Geographical distribution of the complete *K. pneumoniae* genomes found. The heat map was drawn based on the number of genomes across countries. The distribution of the predominant STs in each country is represented by the color coding of the graphs, as indicated in the legend. B) Percentage of *K. pneumoniae* genomes from human, animal, environmental and food reservoirs.

### *Klebsiella pneumoniae* genomes carry regions with potential auto-transfer capability

The results obtained by oriTfinder identified that 501 analyzed genomes had 1 or more regions with potential capacity for self-transfer (Fig. 2A; Supplementary Table 2). From these results, 483 relaxases, 452 T4CP, 188 transfer origins, 255 G-type and 396 T-type conjugation systems were predicted (Fig. 2B). We found no evidence of a specific relationship between the number of elements in *K. pneumoniae* genomes and reservoirs. From the regions predicted by oriTfinder, two analyzed genomes showed plasmid integration (Supplementary Table 2).

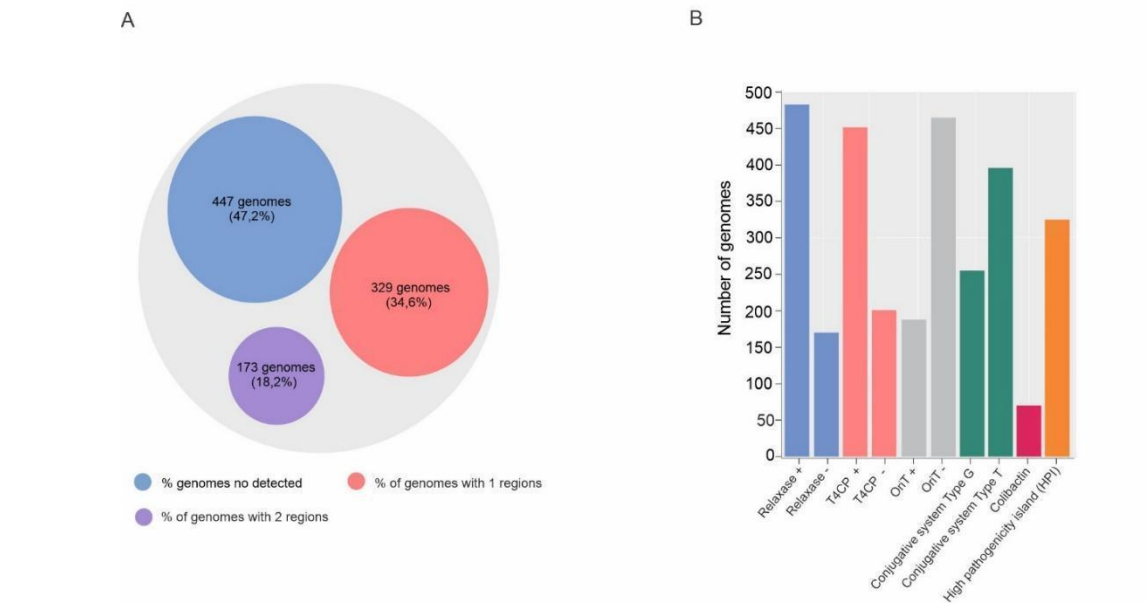



**Figure. 2.** *Klebsiella pneumoniae* genomes carry regions with potential autotransfer capability. A) Percentage of genomes that presented candidate regions for ICEs predicted. B) Typical regions of ICEs detected in the analyzed genomes.

### An arsenal of novel ICEs is present in *Klebsiella pneumoniae* genomes

The regions predicted by oriTfinder that did not represent ICEs described in the literature were inspected and demonstrated the novelty of 19 putative ICEs (Fig. 3A; Table1). These novel elements were identified in *K. pneumoniae* isolates from human, animal, and environmental reservoirs (Fig. 3B).

The novel ICEs have an average size of 60 kb and encode 24 to 162 ORFs. The GC content of these element ranges from 43% to 54%. The identity matrix showed greater differences among putative novel ICEs (Fig. 3D). The *attL* and *attR* sequences showed common regions conserved for the ICEs described (Supplementary Fig. 3A). tRNA sites are preferred regions chosen for the integration of ICEs. And encode integrases belonging to the XerC, XerD or Tyr family (Supplementary Fig. 3B and E). They also have relaxase from the $MOB_H$, $MOB_C$,

or $MOB_P$ family (Supplementary Fig. 3C; Supplementary Table 5), and the conjugative system type G or type T (Supplementary Fig. 3D).

The novel ICEs have protein-coding regions categorized into ICE core-genes, cellular processes, mobile elements, virulence, resistance, and unknown function. Most encoded proteins have functions related to replication, membrane components, stress response, resistance to antimicrobials and/or heavy metals (Fig. 3C).

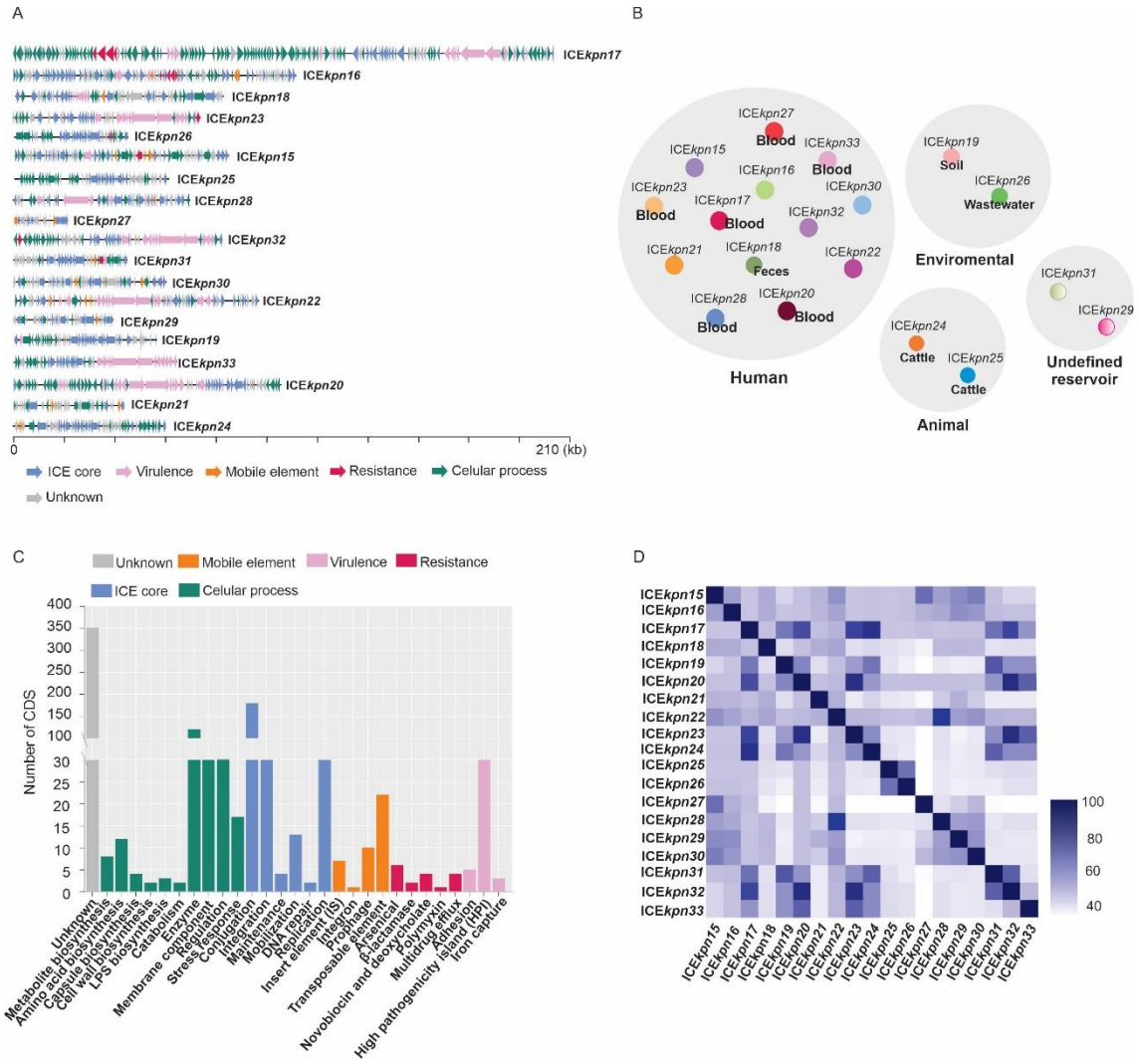



**Figure. 3.** Characterization of the novel ICEs described in this work. A) Representation of putative ICEs. The size of the ICE is indicated on the scale bar (Kb) and the ORFs functions are represented by colored arrows below the figure. B) Reservoirs of the new identified ICEs and the respective sources of the isolates. C) Number of CDS regions found for each subcategory of processes found for novel ICEs. D) Heatmap based on the identity matrix (40% to 100% of nucleotide identity) of the novel ICEs.

**Table 1:** ICEs putative identified in this work.

| Name | Reservoir | Access number | Range | Size (kb) |
|---|---|---|---|---|
| ICE*kpn15* | - | NZ_CP029738.1 | 3997336..4078188 | 80.85 |
| ICE*kpn16* | Human | NZ_CP024482.1 | 724820..832256 | 107.44 |
| ICE*kpn17* | Human | NZ_CP047633.1 | 1670024..1877802 | 207.78 |
| ICE*kpn18* | Human | NZ_CP047677.1 | 565942..645099 | 79.16 |
| ICE*kpn19* | Environmental | NZ_CP025541.3 | 3519217..3572657 | 53.44 |
| ICE*kpn20* | Human | NZ_CP036320.1 | 5105553..5208479 | 102.93 |
| ICE*kpn21* | Human | NZ_CP040861.1 | 2239236..2280150 | 40.91 |
| ICE*kpn22* | Human | NZ_CP012743 | 787509..880170 | 92.66 |
| ICE*kpn23* | Human | NZ_CP031734.1 | 1752487-1823992 | 71.51 |
| ICE*kpn24* | Animal | NZ_CP057069.1 | 1769738..1827461 | 57.72 |
| ICE*kpn25* | Animal | NZ_CP057069.1 | 3353357..3412935 | 59.58 |
| ICE*kpn26* | Environmental | NZ_CP056275.1 | 1735471..1777474 | 42.00 |
| ICE*kpn27* | Human | NZ_CP052372.1 | 755983..775627 | 19.64 |
| ICE*kpn28* | Human | NZ_CP052375.1 | 4939503..5007790 | 68.29 |
| ICE*kpn29* | - | NZ_CP011980.1 | 740488..777550 | 37.06 |
| ICE*kpn30* | Human | NZ_CP032833.1 | 440905..498547 | 57.64 |
| ICE*kpn31* | - | NZ_CP032167.1 | 1396218..1438248 | 42.03 |
| ICE*kpn32* | Human | NZ_CP063003.1 | 1786876 - 1866091 | 79.22 |
| ICE*kpn33* | Human | NZ_CP003999.1 | 1831302 - 1893066 | 61.77 |

**Comparative analyzes reveals a heterogeneity and genic potential of Kp ICEs**

Global analysis of previously reported ICEs, and the putative ones described here, indicate size ranges of 19 to 207 kb and encode between 24 to 162 ORFs with GC content of 43% to 54% (Supplementary Fig. 4A). Altogether, these elements present 15.4% of CDS regions associated with virulence such as adhesion, iron uptake, and HPI (Fig. 4A). We also identified that 1.5% of the CDS regions can be related to resistance to arsenic, β-lactams, polymyxin and novobiocin (Fig. 4B). Furthermore, 24.6% of the CDS are related to cellular processes that code for proteins of capsule biosynthesis, cell wall, and LPS; stress response and membrane components such as major facilitator superfamily (MFS) protein family efflux

pumps, drug metabolite transporter (DMT), ATP-binding cassette (ABC) and resistance-nodulation-cell division (RND) (Fig. 4B; Supplementary Table 3). ICE*kpn19* was the only element that carried resistance genes to fluoroquinolone and tetracycline.

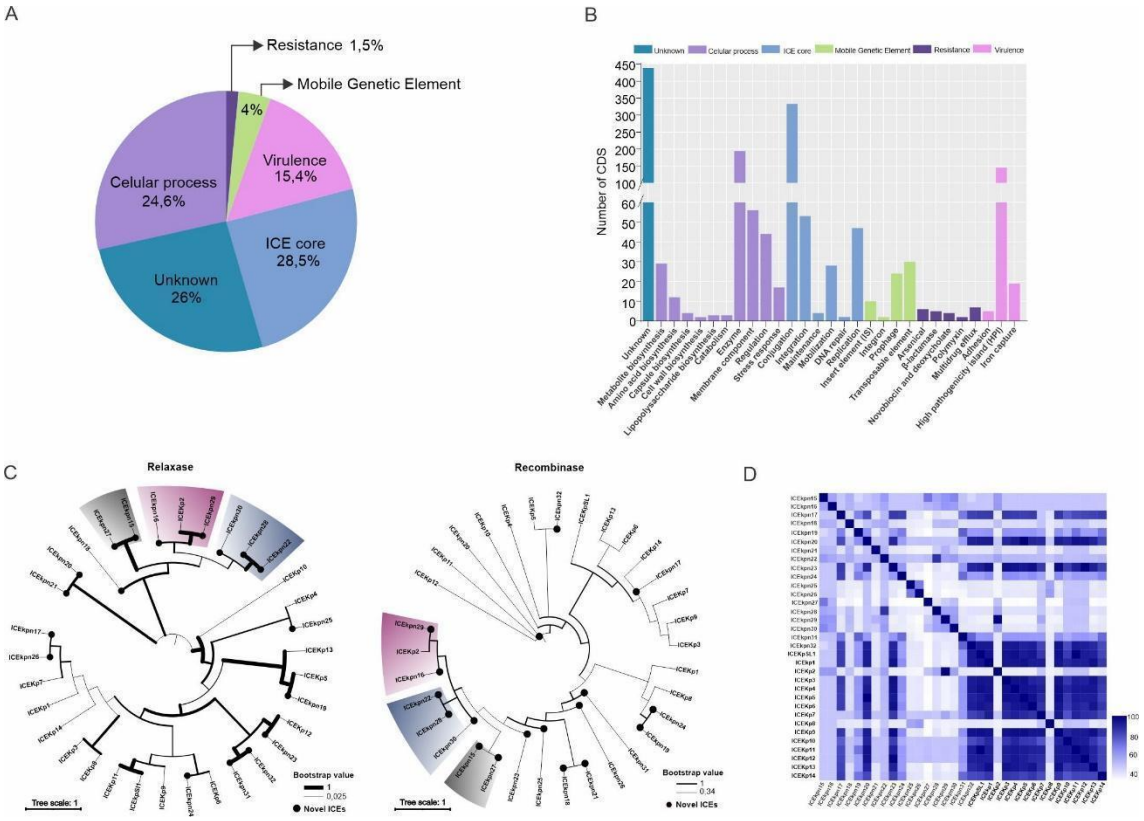



**Figure. 4.** Characterization of all ICEs described for *K. pneumoniae*. A) Percentage of each functional category referring to all ICEs described in *K. pneumoniae*, including those in this work. B) Number of CDS regions found for each subcategory of processes found for all ICEs found for *K. pneumoniae.* C) A maximum likelihood phylogenetic tree was constructed with the relaxase and integrase genes for all ICEs described for the species. D) Heatmap based on the identity matrix (40% to 100% of nucleotide identity) of the novel ICEs and the ICEs described in the literature for the species.

The phylogenies of the relaxase and integrase genes were shown to be conserved in ICE*kpn15* and ICE*kpn27*; ICE*Kpn22*, ICE*kpn28,* and ICE*kpn30*. Furthermore, ICE*kpn16* and ICE*kpn19* were shown to be in a conserved cluster in both phylogenies with ICE*kp2* (Fig. 4C). The ICE*kpn17*, ICE*kpn20*, ICE*kpn32,* and ICE*kpn23* elements carry HPI, as well as the other ICEs described in the literature for the species. Likewise, the identity matrix generated with all ICEs showed that the ICE*kp1,* ICE*kp3,* ICE*kp4,* ICE*kp5,* ICE*kp6,* ICE*kp7,* ICE*kp8,* ICE*kp9,* ICE*kp10,* ICE*kp11,* ICE*kp12,* ICE*kp13,* and ICE*kp14* elements have sequences closer to each other (Fig. 4D).

Overall, 18.2% of the analyzed genomes showed co-occurrence between ICEs described for the species (Supplementary Table 2). In *K. pneumoniae* genomes from animal reservoirs, ICE*kp2* co-occurred with ICE*kp4* in 1.3%. The ICE*kp2* and ICE*kp4* elements are well-characterized ICEs for *K. pneumoniae*.

**The high pathogenicity island of Yersinia (HPI) is widely found in Kp ICEs**

In addition, 325 genomes carry the HPI and 70 carry the colibactin genotoxin in the predicted regions for ICE (Fig. 2B). Of the genomes inspected for ICEs, 38.8% carry ICE*kp2*. In addition, we found ICE*kp1* (1.1%), ICE*kp3* (25%), ICE*kp4* (14.1%), ICE*kp5* (5.1%), ICE*kp6* (1.3%), ICE*kp10* (14.3%), ICE*kp11* (3.5%), ICE*kp12* (5.5%) and ICE*kpSL1* (0.3%) (Supplementary Fig.2). Furthermore, some ICE*kp* variations were detected (Supplementary Table2). We consider as variants the elements that do not compromise the typical modules of the characterized ICE but that may contain gaps or small additional regions that do not code for known genes (Supplementary Table 2).

***Klebsiella pneumoniae* ICEs are disseminated in diverse species worldwide**

The alignments developed using Megablast, with cutoff of 90% for identity and coverage showed that these elements are globally disseminated and found in different reservoirs (Fig. 5). Furthermore, some ICEs and/or variants were found in species such as *Escherichia coli*, *Salmonella enterica*, *Enterobacter cloacae*, *Klebsiella quasipneumoniae*, *Klebsiella aerogenes*, *Citrobacter koseri*, *Citrobacter freundii, Proteus mirabilis*, *Providencia stuartii* and *Morganella morganii* (Supplementary Table 4).

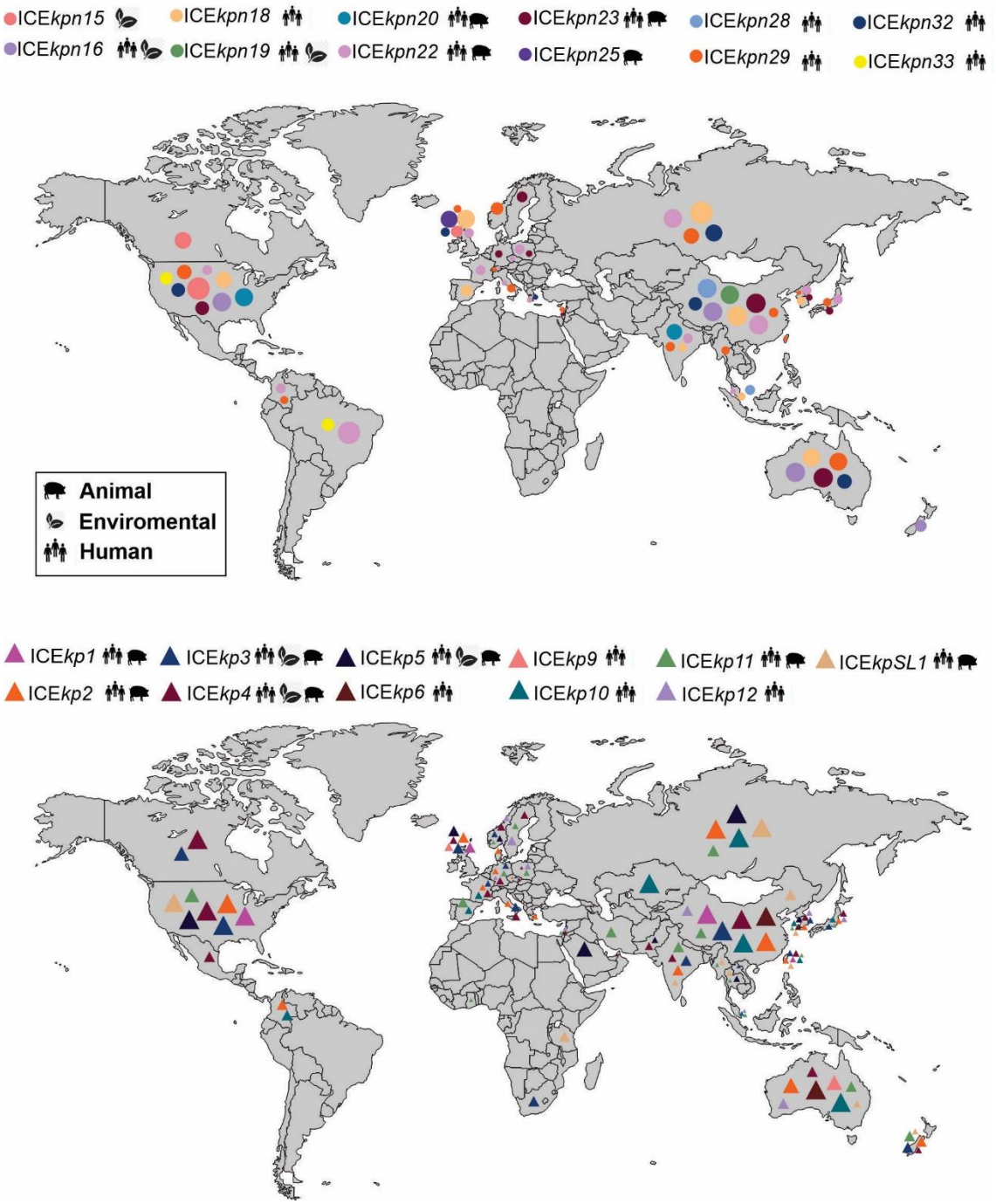



**Figure. 5.** Dispersion of the ICEs described for *K. pneumoniae*. Colored circles represent the novel ICEs identified in this work. Colored triangles represent the ICEs described in the literature. The figures in the table represent reservoirs of *K. pneumoniae*.

## Discussion

*Klebsiella pneumoniae* has enormous population complexity, and it is currently known that the accessory genome significantly contributes to the ecological niche in the human reservoir (Wyres, Lam, Holt 2020). Despite many efforts, few studies are carried out with *K. pneumoniae* from animal reservoirs, environmental, and food samples, with a proposal to identify mobile genetic elements, such as ICEs.

Here, we investigated the presence of ICEs in complete genomes of *K. pneumoniae* from different reservoirs available in a public database (NCBI). Surprisingly, our results showed that about 501 analyzed genomes had 1 or more regions with potential capacity for self-transfer (Supplementary Fig.2A). The data point to the co-occurrence of ICEs in approximately 172 genomes, being more common in human reservoirs, perhaps due to their abundance in the dataset. However, in *K. pneumoniae* genomes from an animal reservoir, ICE*kp2* can co-occur with ICE*kp4* (Fig. 2A). Thus, we suggest that the presence of these elements in this reservoir is common, despite limited data.

The co-occurrence of ICEs is reported in *Shewanella sp.* and, it has a fundamental role in the ecological niche of this microorganism in aquatic and/or hospital environments (Noto et al., 2019). This phenomenon was also identified in *K. pneumoniae* that carries ICE*kp1* and ICE*kp2*, where the ICE*kp2 mob2* gene increases the efficiency of ICE*kp1* mobilization (Ferzand et al., 2019). Thus, is possible that this event may occur in other cases of co-occurrence with ICE*kp2* in *K. pneumoniae* from different reservoirs.

The novel ICE*kpn17*, ICE*kpn20*, ICE*kpn32,* ICE*kpn33* and, ICE*kpn23* carry HPI as well as the other ICEs described in the literature for the species (Supplementary Fig. 4C) (Lin et al., 2008; Lam et al., 2018; Liu et al., 2019). These elements are widely dispersed in *K. pneumoniae* except for ICE*kpn17* which was found in a single human reservoir genome. However, ICE*kpn17* carries genes important for host colonization that are related to resistance to novobiocin, bile salts, capsule production, and type I fimbriae.

Previous work has shown that HPI is found in more than a third of *K. pneumoniae* isolates from the human reservoir and is highly associated with strains that cause invasive infections such as liver abscesses and bacteremia (Lam et al., 2018). Our results show that HPI-carrying ICEs in *K. pneumoniae* are widely dispersed in the human reservoir from blood samples (Supplementary Table 3, Supplementary Fig. 1A). Recently, ICE*kpSL1*, which carries HPI, was identified in *K. pneumoniae* from a diseased animal reservoir (Liu et al., 2019). When evaluating the dispersion of ICE*kpSL1*, we observed the predominance of only human reservoir

isolates (Supplementary Table 3). This reinforces the importance of monitoring these elements in *K. pneumoniae* isolates from animal reservoirs since sick animals can carry them.

The absence of important data such as clonal group (CG) or type of sequence (ST) referring to the analyzed genomes did not allow us to reach an epidemiological pattern of dispersion of these ICEs. However, we observed that most genomes belong to ST11 and ST258, both from clonal group 258. A study that evaluated the frequency of HPI in *K. pneumoniae* belonging to CG258 concluded that HPI is present in 40% of the isolates evaluated (Lam et al., 2018). Here, HPI is present in 34% of the inspected genomes (Fig. 2B). CG258 has global clinical significance as it is associated with carbapenemases (Wyres, Lam, Holt 2020).

The functional annotation of all ICEs showed that most of the genes transported by these elements are related to membrane components (Fig. 4B). Among them are efflux pumps (EPs) of the major facilitator superfamily (MFS), drug metabolite transporter (DMT), ATP-binding cassette (ABC) and resistance-nodulation-cell division (RND) proteins. EPs strongly contribute to the emergence of multidrug resistance due to their potential to expel a wide range of antimicrobials (Li, Plésiat, Nikaido 2015). EPs of the MFS family are known to contribute to resistance and virulence in *E. coli*, *Acinetobacter baumanni* and *K. pneumoniae* (Liu et al., 1999; Sharma et al., 2016; Pasqua et al., 2019).

The genes present in the HPI encode proteins from the MFS family (*ybtX*) and the ABC transport family (*ybtPQ*) (Farzand, 2017). Interestingly, the deletion of the *ybtPQ* genes changes the status of resistant *K. pneumoniae* to being sensitive to antimicrobials, such as colistin, novobiocin, and tetracycline (Farzand, 2021). These results suggest that ABC membrane transporters found in new ICEs can affect *K. pneumoniae* resistance in different reservoirs.

The putative ICE*kpn16* carries arsenic resistance and stress response genes. This element was identified in the genome of *K. pneumoniae* from a human reservoir, and to be present in other species such as *Citrobacter freundii* and *Enterobacter cloacae* from the same reservoir. In *Enterobacter cloacae* the coding regions for arsenic were interrupted by insertion sequences. Interestingly, we found ICE*kpn16* in a genome of *Klebsiella sp.* endophytic (Supplementary Table 3), isolated from *Alhagi sparsifoliano*, which exhibits the ability to promote the growth of plants under water stress (Zhang et al., 2017). Isolates of *K. pneumoniae* obtained in sewage, soil, and water contaminated with arsenic, showed potential for bioremediation in these environments (Kumar et al., 2021; Abbas et al., 2014). However, the absence of complete genomes hinders our understanding of the potential role of ICEs in *K.*

*pneumoniae* in these environments and hosts. This result suggests that the ICE*kpn16* may contribute to the adaptation of *Klebsiella sp.* to these environments.

Interestingly, the ICE*kpn25* element was found in a genome of *K. pneumoniae* and a genome of *C. freundii*, both from the same sample of cattle feces (Supplementary Table 3). ICE*kpn25* transports membrane components as efflux pumps of the major facilitator superfamily (MFS) protein family, however, its functionality is not known. This finding reinforces the great potential for the transference of ICEs in *K. pneumoniae* in livestock.

Other promising elements were ICE*kpn18* and ICE*kpn28*. We found both in a genome of *K. aerogenes*, in the human reservoir. The ICE*kpn18* element is widely dispersed in *K. pneumoniae* isolated from the Asian continent. This element carries genes involved in biofilm adhesion/formation such as type I fimbriae. ICE*kpn28* found in *K. pneumoniae* and *Proteus mirabilis* in human reservoirs carries receptor genes for ferric coprogen and ferric-rodotorulic acid and adhesion autotransporters.

Taken together, some questions remain unclear. Why is an ICE that carries arsenic resistance genes found in human reservoir isolates? What environmental conditions inherent to the host lead to the excision and mobilization of these elements? With the rise of the one health approach studies like this one are increasingly needed.

The search and monitoring of ICEs in *K. pneumoniae*, allows us to understand the role of these elements in the context of health and in understanding the propagation of genes involved in virulence and resistance. The diversity of genes carried by ICEs in *K. pneumoniae* may help them adapt to adverse environments. The limitation of sequenced genomes of *K. pneumoniae* from the non-human reservoir makes it difficult to monitor ICEs related to virulence and resistance. In summary, the gene arsenal of the ICEs described for *K. pneumoniae* may help the adaptation and significantly contributes to the fitness in different reservoirs and environments.

**Funding information**

This research was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico-CNPq, Coordenação de Aperfeiçoamento de Pessoal de Nível Superior/Programa de Excelência Acadêmica-Código Financeiro 001 and Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG.

**Author contributions**

R. N. J, G. N. M, O. R and F. P. P helped in data collection. R. N. J, S. C. G and G. S. O helped in the analysis of results. R. N. J performed the edition of figures. S. F. M and B. S. M. D helped

in the correction of the manuscript and English. R. N. J wrote the manuscript. All authors discussed the results and their implications. All authors edited and approved the final manuscript.

**Conflicts of interest**

The authors declare no competing interests.

**Supplementary figures:**

https://docs.google.com/document/d/1Fab1uLkd7zNHhN7Oq0haa4bAw4Fdy8Q3/edit?usp=sharing&ouid=118022498442200222122&rtpof=true&sd=true

**Supplementary tables:**

https://docs.google.com/spreadsheets/d/166PsGhi4VF2eITwAGZFyKiCxI30y9ZBJ/edit?usp=sharing&ouid=118022498442200222122&rtpof=true&sd=true

## Reference


Abbas, S. Z., Riaz, M., Ramzan, N., Zahid, M. T., Shakoori, F. R., Rafatullah, M. Isolation and characterization of arsenic resistant bacteria from wastewater. *Brazilian Journal of Microbiology*, 45, 1309-1315 (2014)

Alcock, B. P., Raphenya, A. R., Lau, T. T. Y., Tsang, K. K., Bouchard, M., Edalatmand, A. CARD 2020: antibiotic resistome surveillance with the comprehensive antibiotic resistance database. *Nucleics acids research*. 48, D517-D525 (2020) doi: 10.1093/nar/gkz935

Besemer, J., Lomsadze, A., Borodovsky, M. GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Research*. 29, 2607–2618 (2001)

Bidewell, C. A., Bidewell, C. A., Williamson, S. M., Rogers, J., Tang, Y., Ellis, R. J., Petrovska, L., AbuOun, M. Emergence of *Klebsiella pneumoniae* subspecies pneumoniae as a cause of septicaemia in pigs in England. *PloS One*, 13, 487 e0191958, (2018). doi.org/10.1371/journal.pone.0191958

Bortolaia, V., Kaas, R. S., Ruppe, E., Roberts, M. C., Schwarz, S., Cattoir, V., Aarestrup, F. M. ResFinder 4.0 for predictions of phenotypes from genotypes. *J. Antimicrob. Chemother*. 75, 3491–3500 (2020)

Campos-Madueno, E. I., Moser, A. I., Risch, M., Bodmer, T., Endimiani, A. Exploring the global spread of *Klebsiella grimontii* isolates possessing *bla*$_{\text{VIM-1}}$ and *mcr-9*. *Antimicrobial Agents and Chemotherapy*, 00724-21, (2021) doi.org/10.1128/AAC.00724-21

Cantalapiedra, C. P., Hernández-Plaza, A., Letunic, I., Bork, P., & Huerta-Cepas, J. eggNOG-mapper v2: Functional Annotation, Orthology Assignments, and Domain Prediction at the Metagenomic Scale. *bioRxiv*, 2021. doi: 10.1093/molbev/msab293

Cury, J., Abby, S. S., Doppelt-Azeroual, O., Néron, B. and Rocha, E. P. C. Identifying conjugative plasmids and integrative conjugative elements with CONJscan BT - Horizontal Gene Transfer: Methods and Protocols. in (ed. de la Cruz, F.). Springer US 265–283 (2020)

Chan, P. P. and Lowe, T. M. tRNAscan-SE: Searching for tRNA Genes in Genomic Sequences. *Methods Mol. Biol*. 1962, 1–14 (2019)

Clausen, P., Aarestrup, F. M. and Lund, O. Rapid and precise alignment of raw reads against redundant databases with KMA. *BMC bioinformatics*, 19, 1-8 (2018) doi: 10.1186/s12859-018-2336-6

Farzand, R., Rajakumar, K., Zamudio, R., Oggioni, M. R., Barer, M. R., O'Hare, H. M. ICE*Kp2*: description of an integrative and conjugative element in *Klebsiella pneumoniae*, co-occurring and interacting with ICE*Kp1*. *Scientific reports*, 9, 1-11, (2019) doi: 10.1038/s41598-019-50456-x

Farzand, R., Rajakumar, K., Barer, M. R., Freestone, P. P., Mukamolova, G. V., Oggioni, M. R., O'Hare, H. M. A virulence associated siderophore importer reduces antimicrobial susceptibility of *Klebsiella pneumoniae*. *Frontiers in microbiology*, 12, 52 (2021) doi.org/10.3389/fmicb.2021.607512

Fouts, D. E., Tyler, H. L., DeBoy, R. T., Daugherty, S., Ren, Q., Badger, J. H., Methé, B. A. Complete genome sequence of the N2-fixing broad host range endophyte *Klebsiella pneumoniae* 342 and virulence predictions verified in mice. *PLoS genetics*, 4(7), e1000141 (2008) doi: 10.1371/journal.pgen.1000141

Garcillán-Barcia, M. P., Redondo-Salvo, S., Vielva, L., de la Cruz, F. MOBscan: Automated annotation of MOB relaxases BT - Horizontal gene transfer: Methods and Protocols. in (ed. de la Cruz, F.). Springer US 295–308 (2020)

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Thomson, N. R. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Sciences*, 112, E3574-564 E3581, (2015) doi.org/10.1073/pnas.1501049112

Huang, M., Li, L. I. N., WU, Y. X., Honhing, H., HE, P. F., LI, G. Z., HE, Y. Q. Pathogenicity of *Klebsiella pneumonia* (KpC4) infecting maize and mice. *Journal of integrative agriculture*, 15(7), 1510-1520 (2016) doi:10.1016/S2095-3119(16)61334-5

Jia, B., Raphenya, A. R., Alcock, B., Waglechner, N., Guo, P., Tsang, K. K., McArthur, A. G. CARD 2017: expansion and model-centric curation of the comprehensive antibiotic resistance database. *Nucleic Acids Research*. 45, D566–D573 (2017) doi:10.1093/nar/gkw1004

Jolly, K. A. and Maiden, M. C. J. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*. 2010 doi:10.1186/1471-2105-11-595

Kumar, P., Dash, B., Suyal, D. C., Gupta, S. B., Singh, A. K., Chowdhury, T., Soni, R. Characterization of Arsenic-Resistant *Klebsiella pneumoniae* RnASA11 from Contaminated Soil and Water Samples and Its Bioremediation Potential. *Current Microbiology*, 78, 3258-3267 (2021) doi:10.1007/s00284-021-02602-w

Kumar, S., Stecher, G., Li, M., Knyaz, C. Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547–1549 (2018) doi:10.1093/molbev/msy096

Kleinheinz, K. A., Joensen, K.G., Larsen, M. V. Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and *E. coli* virulence genes in bacteriophage and prophage nucleotide sequences. *Bacteriophage*. (2014). doi 10.4161/bact.27943

Lam, M. M., Wick, R. R., Wyres, K. L., Gorrie, C. L., Judd, L. M., Jenney, A. W., Holt, K. E. Genetic diversity, mobilisation and spread of the yersiniabactin-encoding mobile element ICE*Kp* in *Klebsiella pneumoniae* populations. *Microbial Genomics*, (2018) doi.org/10.1099/mgen.0.000196

Leangapichart, T., Lunha, K., Jiwakanon, J., Angkititrakul, S., Järhult, J. D., Magnusson, U., Sunde, M. Characterization of *Klebsiella pneumoniae* complex isolates from pigs and humans in farms in Thailand: population genomic structure, antibiotic resistance and virulence genes. *Journal of Antimicrobial Chemotherapy*, (2021). doi.org/10.1093/jac/dkab118

Li, X., Plésiat, P., Nikaido, H. The challenge of efflux-mediated antibiotic resistance in Gram-negative bacteria. *Clinical microbiology reviews*, 28, 337-418 (2015) doi:10.1128/cmr.00117-14

Li, X., Xie, Y., Liu, M., Tai, C., Sun, J., Deng, Z., Ou, H. Y. oriTfinder: a web-based tool for the identification of origin of transfers in DNA sequences of bacterial mobile genetic elements. *Nucleic Acids Research*. 46, W229–W234 (2018) doi:10.1093/nar/gky352

Lin, T. L., Lee, C. Z., Hsieh, P. F., Tsai, S. F., Wang, J. T. Characterization of integrative and conjugative element ICE Kp1-associated genomic heterogeneity in a *Klebsiella pneumoniae* strain isolated from a primary liver abscess. *Journal of Bacteriology*, 190, 515-526, (2008). doi.org/10.1128/JB.01219-07

Liu, D., Yang, Y., Gu, J., Tuo, H., Li, P., Xie, X., Zhang, A. The Yersinia high-pathogenicity island (HPI) carried by a new integrative and conjugative element (ICE) in a multidrug-resistant and hypervirulent *Klebsiella pneumoniae* strain SCsl1. *Veterinary Microbiology*, 239, (2019). doi.org/10.1016/j.vetmic.2019.108481

Liu, B., Zheng, D., Jin, Q., Chen, L., Yang, J. VFDB 2019: a comparative pathogenomic platform with an interactive web interface. *Nucleic Acids Research*. 47, D687-D692 (2019) doi:10.1093/nar/gky1080

Letunic, I. and Bork, P. Interactive Tree of Life (iTOL) v4: recent updates and new developments. *Nucleic Acids Research*. 47, W256–W259 (2019) doi:10.1093/nar/gkz239

Marchler-Bauer, A. and Bryant, S. H. CD-Search: protein domain annotations on the fly. *Nucleic Acids Research*. 32, W327-31 (2004) doi:10.1093/nar/gkh454

Parmeciano DI Noto, G., Iriarte, A., Ramírez, M. S., Centrón, D., Quiroga, C. ICE SXT vs. ICE Sh95: Co-existence of Integrative and Conjugative Elements and Competition for a New Host. *Scientific reports*, *9*(1), 1-7 (2019) doi:10.1038/s41598-019-44312-1

Pasqua, M., Grossi, M., Zennaro, A., Fanelli, G., Micheli, G., Barras, F., Prosseda, G. The varied role of efflux pumps of the MFS family in the interplay of bacteria with animal and plant cells. *Microorganisms*, 285, (2019) doi:10.3390/microorganisms7090285

Pope, J. L., Yang, Y., Newsome, R. C., Sun, W., Sun, X., Ukhanova, M., Jobin, C. Microbial colonization coordinates the pathogenesis of a *Klebsiella pneumoniae* infant isolate. *Scientific reports*, 9(1), 1-13, (2019) doi:10.1038/s41598-019-39887-8

Podschun, R. and Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical Microbiology Reviews*, 11, 589-603 (1998) doi:10.1128/CMR.11.4.589

Sievers, F. and Higgins, D. G. Clustal Omega, accurate alignment of very large numbers of sequences BT - Multiple sequence alignment methods. in (ed. Russell, D. J.) Humana Press 105–116 (2014)

Tot, T., Kibel, S., Sardelić, S., Nemer, K., Benčić, A., Vraneš, J., Bedenić, B. Polyclonal spread of colistin resistant *Klebsiella pneumoniae* in Croatian hospitals and outpatient setting. *Germs*, 11(2), 163 (2021) doi:10.18683/germs.2021.1254

Wyres, K. L. and Holt, K. E. *Klebsiella pneumoniae* as a key trafficker of drug resistance genes from environmental to clinically important bacteria. *Current Opinion in Microbiology*, 45, 131-139 (2018) doi.org/10.1016/j.mib.2018.04.004

Wyres, K. L., Lam, M.C. Holt, K. E. Population genomics of *Klebsiella pneumoniae*. *Nature Reviews Microbiology*, 18, 344-359, (2020) doi.org/10.1038/s41579-019-0315-1

Zhang, L., Zhong, J., Liu, H., Xin, K., Chen, C., Li, Q., Shen, X. Complete genome sequence of the drought resistance-promoting endophyte *Klebsiella sp.* LTGPAF-6F. *Journal of biotechnology*, 246, 36-39 (2017) doi:10.1016/j.jbiotec.2017.02.008

## Chapter 4

# Vesiduction: the hotspot of resistance gene transfer among *Klebsiella* spp from human and animal

(Manuscript written according to the guidelines of the Journal Emerging Microbes and Infections)

Jéssica Nogueira Rosa[1]; Giarlã Cunha da Silva[1]; Patrícia Pereira Fontes[1]; Matheus Machado Guidini[1]; Denise Mara Soares Bazzolli[1*]

[1]Laboratório de Genética Molecular de Bactérias, Departamento de Microbiologia, Instituto de Biotecnologia Aplicada à Agropecuária- Bioagro, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brazil.

*Corresponding author: dbazzolli@ufv.br

## Abstract

Extracellular vesicles (EVs) are spherical, nanomeric membrane structures produced in all domains of life. In *Klebsiella pneumoniae*, a Gram-negative and opportunistic bacterium, EVs are considered a potential vehicle for transporting virulence and resistance plasmids in a process known as vesiduction. Thus, this work investigated the transfer of resistance genes by vesiduction among *K. pneumoniae* from human source and other *Klebsiella* spp from healthy pigs. The EVs from *K. pneumoniae* Kp13 (EVs-Kp13) presented an average size of 182 nm and rounded morphology. We confirmed that EVs-Kp13 carry sulfonamide resistance genes (*sul2*), cephalosporins ($bla_{CTXM2}$, $bla_{SHV}$, $bla_{TEM}$), carbapenems ($bla_{KPC}$), and trimethoprim (*dfrA15*), compatible with the plasmids present in this strain. In addition, genes coding for the yersiniabactin siderophore (*ybtS*) were detected within EVs-Kp13. Our vesiduction results were positive when using the *K. aerogenes* 270 isolate (from swine) as recipient. We obtained four transvesiductant that presented phenotypic alterations for cephalosporins (third and fourth generation) and trimethoprim, in addition to detecting of the markers genes of plasmid pKP13f. The results obtained in this work highlight for the first time the transfer of resistance genes interspecies, mediated by vesiduction, between *K. pneumoniae* from a human and *K. aerogenes* from a swine. This study reports an important gene flow route between different species thus significantly contributing to the one health approach.

**Keywords:** extracellular vesicles, cephalosporin resistance, HTG.

## Introduction

*Klebsiella pneumoniae* can present itself as a commensal bacterium or as an opportunistic pathogen, it is a Gram-negative bacterium and an important representative of the Enterobacteriaceae family (Podschun & Ullmann 1998). The phenotypic and genetic diversity of *K. pneumoniae* makes it possible to differentiate them into two important pathotype: classic (cKp) and hypervirulent (hvKp) (Joseph et al., 2021). Basically, cKp strains are related to nosocomial infections acting as opportunistic pathogens (Wyres, Lam & Holt 2020). The hvKp strains, on the other hand, act as true pathogens and are responsible for causing primary diseases in healthy individuals (Wyres, Lam & Holt 2020). However, the phenotypes cKp and hvKp can be found in the same isolate (Joseph et al., 2021). The genetic arsenal of *K. pneumoniae* contributes to its successful colonization and dissemination in the host (Holt et al., 2015). Its virulence factors are associated with capsule production, exopolysaccharides associated with mucoviscosity, lipopolysaccharides (LPS), adhesins, iron uptake systems, and production of extracellular vesicles - EVs (Holt et al., 2015; Ye et al., 2021).

The spread of antimicrobial resistance and virulence genes in *K. pneumoniae* is determined by horizontal gene transfer (HGT) (Wyres et al., 2019). Conjugation, transformation, and transduction are the three most established canonical mechanisms of HGT in the literature (Soler & Forterre 2020). However, a fourth mechanism of HGT has gained focus in recent years, the process is called vesiduction (Soler & Forterre 2020). Vesiduction is a process where EVs can incorporate, transport, and deliver mobile genetic elements (MGE) to recipient bacteria (Soler & Forterre 2020).

In the literature, it is known that vesiduction contributes to the transfer of genes $bla_{\text{OXA24}}$, $bla_{\text{NDM1}}$, $bla_{\text{CTXM15}}$, $bla_{\text{KPC2}}$ and *aac(6')-Ib-cr.* These reports are described interspecies and intraspecies with representatives of the family Enterobacteriaceae (Yeron et al., 2000; Rumbo et al., 2011; Ho et al., 2015; Bitto et al., 2017; Chantterjee et al., 2017; Bielaszewska et al., 2020). In *K. pneumoniae*, vesiduction seems to be a determining event for the convergence of cKp and hvKp phenotypes (Tang et al., 2023). There are reports of vesiduction related to the transfer of virulence genes such as *rmpA2* and *iroBCD/iucaABCD*, important hvKp markers (Li et al., 2022; Hua et al., 2022; Wang et al., 2022), and mainly, resistance genes such as $bla_{\text{KPC2}}$, $bla_{\text{SHV}}$, $bla_{\text{NDM1}}$, $bla_{\text{CTXM15}}$ and *mcr-1* (Dell'Annunziata et al., 2021; Song et al., 2022; Hua et al., 2022; Wang et al., 2022; Tang et al., 2023).

Vesiduction has been massively reported among human reservoir *K. pneumoniae*. Currently, no work reports gene transfer mediated by extracellular vesicles (vesiduction) in *K.*

*pneumoniae* from farm swine. Given the importance of vesiduction in *K. pneumoniae*, this work investigated the transfer of resistance genes, mediated by vesiduction, between *K. pneumoniae* from human sources and other *Klebsiella* spp from animal sources (swine).

## Materials and methods

### Bacterial strain

The *Klebsiella pneumoniae* Kp13 strain was donated by the Universidade Federal do Rio de Janeiro (UFRJ). The genomic characterization of the Kp13 strain is described by Ramos et al. (2012). Other isolates used were *Klebsiella pneumoniae* 107 and *Klebsiella aerogenes* 270 from the rectum of healthy swine and belong to the Laboratório de Genética Molecular de Bactérias na Universidade Federal de Viçosa (UFV). The strain *Escherichia coli* J53 (Matsumura, et al 2018) was also used.

### Growth curve

*Klebsiella pneumoniae* Kp13 was inoculated into 10 mL of Luria-Bertani (LB) and incubated overnight at 37°C under agitation at 180 rpm. Subsequently, an initial inoculum of 2% was performed in 60 mL of LB with or without meropenem (8 $\mu g.mL^{-1}$) under agitation at 180 rpm at 37°C. The $OD_{600}$ was read every 40 minutes for 24 hours. The experiment was performed in biological duplicate.

### Obtaining and purifying extracellular vesicles (EVs)

EVs were obtained using the adapted hydrostatic filtration method (Antenucci et al., 2017). The bacteria were inoculated in 600 mL of LB added with meropenem (8 $\mu g.mL^{-1}$) and cultivated for 16 hours under agitation at 180 rpm at 37°C. The culture was centrifuged at 5,000 xg at 4°C for 30 minutes. An aliquot was taken to count colony-forming units ($CFU.mL^{-1}$). Subsequently, the supernatant was filtered through a 0.45 μm membrane (Millipore, Billerica, MA, USA). The filtrate was added to a 1000 kDa dialysis membrane (Biotech CE Tubing - Spectrumlabs) wrapped in a glass column sealed with a transparent film and incubated overnight at 4 °C. We added 600 mL of PBS to wash the filtrate. For purification, the filtrate was dialyzed into PBS under low agitation for 24 h. Finally, the samples were filtered through a 0.22 μm membrane (Cole-Parmer, Vernon Hills, IL, USA) and concentrated on a 10 kDa Amicon column (Millipore, Billerica, MA, USA). We used 5 uL of the sample to evaluate possible contaminants. In all subsequent analyzes, we added 0.1% tween to the EV samples. Samples were stored at -20°C. The experiment was performed in biological duplicate.

**Morphology**

The purified EVs were diluted 2 and 10 times and visualized by a transmission electron microscope (Zeiss Libra 120) located at the Microscopy and Microanalysis Center of the Federal University of Viçosa. For this analysis, 10 uL of the dilutions were added to copper grids (Sigma, St. Louis, Missouri, USA) and subsequently, the samples were counterstained with 3% uranyl acetate for 1 minute.

**Dynamic light scattering (DLS)**

The size of the EVs was estimated using the dynamic light scattering apparatus - DLS (Zetasinzer Nano ZS, Malvern Instruments, United Kingdom). The data were analyzed using the Malvern Zetasizer software version 7.11 to obtain the average hydrodynamic diameter of the particles in the solution. For this analysis, we used 150 ug of EVs diluted in PBS 1X. The parameters used were: a refractive index of 1.332 and a viscosity of 0.9043.

**Protein quantification and profile**

The samples of EVs and total proteins (pellet) were quantified using the Bradford reagent (Sigma, St. Louis, Missouri, USA), from the standard curve of BSA (0.1 to 1.4 $\mu g.mL^{-1}$) and the Qubit Kit (ThermoFisher, Waltham, Massachusetts, USA). To verify the protein profile of the samples, we used a polyacrylamide gel (12%) stained with Coomassie blue (SAMBROOK et al., 1990). After obtaining the pellets by centrifugation at 6500 xg for 15 min, the cells were added to lysis tubes (Matrix B, MP Biomedicals) and taken to the homogenizer (Precellys® Evolution - Bertin Instruments) twice for 30 seconds. Subsequently, they were centrifuged at 4500 xg for 10 min at 4 °C and the supernatant was collected. Samples were prepared by adding sample buffer (50 $mmol.L^{-1}$ Tris-HCl, pH 6.8; 100 mmol.L-1 dithiothreitol; 2% SDS; 0.1% bromophenol blue; 10% glycerol) and heated at 100 °C for 10 minutes.

**Detection of resistance and virulence genes**

The evaluated resistance and virulence genes were present in two plasmids and in an integrative and conjugative element (ICE*kp33*). The polymerase chain reaction (PCR) was performed for the genes listed in Table 1.

**Vesiduction**

The procedures were performed according to Wang et al. (2022) with some modifications. To conduct the vesiduction process, EVs-Kp13 were treated with RQ1 RNase-Free DNase (Promega, Madison, Wisconsin, USA) to remove foreign DNA. Subsequently, 110

mg.mL$^{-1}$ of treated EVs were added to 100 uL of recipient bacteria at an $OD_{600}$ of 0.5 in 1.5 mL microtubes. The tubes were placed in an oven at 37°C for 5 hours. Then the culture was transferred to LB agar spiked with ceftriaxone (8 μg.mL$^{-1}$) and/or meropenem (8 μg.mL$^{-1}$) for selection. The recipient bacteria were *K. aerogenes* 270, *K. pneumoniae* 107, *E. coli* J53. For isolates grown in selective media, a stability test was carried out for five generations.

**Fingerprint**

To evaluate the genetic profile of the isolates after vesiduction, we used the fingerprint technique with the primer (GTG) 5'-GAGGGTGGCGGTTCT-3' (Lieckfeldt, Meyer and Borner 1993). For the reaction with the GTG5 primer, we performed a PCR mixture with a final volume of 25 uL containing the components: 1x Green buffer (Platinum Taq High Fidelity Buffer), 0.2 mM of dNTP, 1 μM of primer, 1.5 mM of $MgCl_2$ and 2 U of Platinum Taq High Fidelity DNA polymerase enzyme (Promega, Madison, Wisconsin, USA). The conditions in the thermal cycler (BioRad-C1000TM Thermal Cycler) were: 95°C for 2 minutes, 30 cycles with an initial denaturation at 95°C (1 minute); annealing at 40°C (1 minute) and extension at 72°C (6 minutes). The last cycle was followed by a final single extension step at 72°C for 5 minutes. We also performed with the primers ERIC-F:5'-ATGTAAGCTCCTGGGGATTCA C-3' and ERIC-R: 5'-AAGTAAGTGACTGGGGTGAGCG-3' (Versalovic, Koeuth and Lupski, 1991). In a final reaction of 25 uL we added 1x Green buffer (Platinum Taq High Fidelity Buffer), 0.2 mM of dNTP, 0.25 μM of each primer, 1.5 mM of $MgCl_2$ and 1.25 U of Platinum Taq High Fidelity DNA polymerase enzyme (Promega, Madison, Wisconsin, USA). The conditions in the thermal cycler (BioRad-C1000TM Thermal Cycler) were 95°C for 5 minutes, 30 cycles with an initial denaturation at 95°C (1 minute); annealing at 52°C (1 minute) and extension at 65°C (5 minutes). The last cycle was followed by a final single extension step at 65°C for 10 minutes. The DNA used was standardized to 25 ng. PCR products were evaluated using 1.8% agarose gel electrophoresis.

**Antibiogram**

Antimicrobial susceptibility was determined by the diffusion disc method according to the Clinical and Laboratory Standards Institute – Vet guidelines (CLSI 2023). Bacterial suspensions were prepared with turbidity of 0.5 (MacFarland scale) in 0.8% saline solution. Then, the suspensions were homogenized and seeded on Mueller-Hinton agar plates (BD, Franklin Lakes, New Jersey, USA). We used the disks with the following antibiotics: ceftriaxone (CRO 30ug), cefepime (FEP 30ug), cefotaxime (CTX 30ug), and ceftiofur (CTF

30ug). The negative control strain used was *E. coli* ATCC25922. The experiments were performed in biological duplicates.

**Minimal inhibitory concentration (MIC)**

The MIC for the antimicrobial ceftriaxone was performed according to the Clinical and Laboratory Standards Institute guidelines (CLSI 2020). Bacteria were grown in Mueller-Hinton broth (BD, Franklin Lakes, New Jersey, USA) until reaching an $OD_{600}$ of 0.5. The dilution range used was from 256 $ug.ml^{-1}$ to 0.25 $ug.ml^{-1}$. Subsequently, they were transferred to 96-well microplates containing the tested antimicrobial. The bacteria were placed in an oven at 37°C for 24 hours. For the final evaluation, 50 uL of 0.1% resazurin was added and the plate was incubated for 2 to 3 hours at 37°C. The MIC value was considered as the lowest dilution of the antimicrobial that did not change color. The experiment was conducted in biological triplicates.

**Pulsed field gel eletrophoresis (PFGE)**

Bacteria were grown on LB agar with or without ceftriaxone (8 $\mu g.mL^{-1}$) overnight at 37°C. Subsequently, an initial inoculum of 2% was made in 10 ml of LB with or without ceftriaxone (8 $\mu g.mL^{-1}$), and placed in a shaker at 180 rpm at 37°C until reaching an $OD_{600}$ of 0.5. The preparation of the plugs was performed according to Barton et al (1995) with some modifications (Rosa et al., 2023 chapter 2). The run was performed on a 1.2% gel in a 0.5% TBE running buffer for 20 hours. A voltage of 210 and an amperage of 130 were used. The parameters for 14 hours with a pulse every 45 seconds and 6 hours with a pulse every 25 seconds were used. The marker used was the Pulse MarkerTM (Sigma, St. Louis, Missouri, USA).

**Digestion with Not1 and Southern blotting**

We performed DNA digestion with Not1 restriction enzyme (New England Biolabs, Ipswich, Massachusetts, USA). We used 30 units of Not1 to cleave 3ug of total DNA. The reaction was left at 37°C for 18 hours and the inactivation was carried out at 65°C for 20 minutes. Subsequently, the DNAs were added to a 0.9% agarose gel, and the run was conducted overnight in 1X TBE buffer at 30 volts. We used the 1KB molecular weight marker of Kasvi (São José dos Pinhais, Paraná, Brazil). The conduction of southern blotting technique was performed according to Sambrook and Russel (1990). For this, the gel with the cleaved DNAs was washed with the denaturing solution (1M NaCl; 0.4M NaOH) for 1 hour under low agitation. Subsequently, we washed with the neutralization solution (1M NaCl; 0.5 M Tris-HCl

– pH 7.2) for 1 hour under low agitation. The DNAs were transferred to a positively charged nylon membrane (Amersham HybondTM – N+) for 18 hours. Subsequently, the DNAs were fixed on the membrane for 5 minutes and exposed to ultraviolet light (254 nm) at an energy of 770 j/cm2. The probe labeled with digoxigenin was constructed for the *bla*$_{CTM-2}$ gene (Table 1) using the DIG PROB Synthesis kit (Sigma, St. Louis, Missouri, USA) and detection was performed with the NBT/BCIP kit (Roche Applied Sciences, Penzberg, Germany).

## Results

### The *K. pneumoniae* Kp13 strain and its EVs-Kp13 have peculiar characteristics

When analyzing the growth curve of the Kp13 strain after 24 hours, we noticed no difference in growth with and without antibiotics (Figure 1A). The number of colony-forming units at 16 hours for meropem treatment was 1.4 x $10^9$. As for growth without antibiotics, it was 1.1 x $10^9$ CFU.mL$^1$. Although there are no differences in these data, we chose to perform the extraction of the EVs with the addition of meropenem at the point of 16 hours of growth. EVs-Kp13 have a rounded morphology (Figure 1B), a mean size of 182 nm and excess of exopolysaccharides. This last feature contributed to the aggregation of EVs-Kp13 visualized in transmission electron microscopy (Figure 1B). The final yield of EVs-Kp13 was 4.6 mg.mL$^{-1}$ and its protein profile is enriched in some bands (Figure 1C).

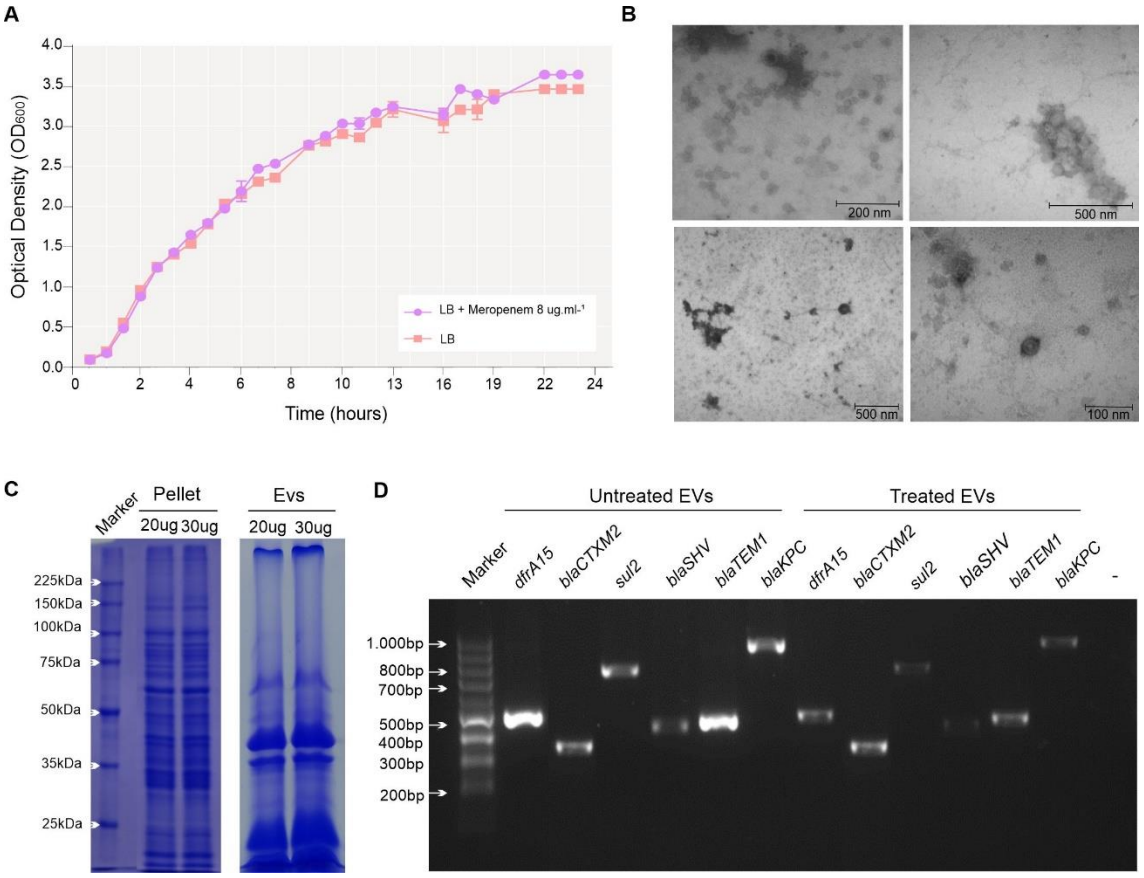



**Figure 1:** Characterization of EVs produceb by *K. pneumoiae* Kp13 (EVs-Kp13). A) Growth curve of *K. pneumoniae* Kp13 with and without the addition of meropenem (8 ug.mL$^{-1}$). B) Transmission electron microscopy (TEM) of EVs-Kp13. The EVs in the 200 nm magnification micrograph were 2-fold diluted. The others were diluted 10 times. C) SDS-PAGE of EVs-Kp13 and *K. pneumoniae* Kp13 pellet. D) 1.5% agarose gel showing gene content of DNase-treated and untreated Kp13-EVs. Cellco 100 bp marker.

## EVs-Kp13 carries resistance and virulence genes

It is known that the *K. pneumoniae* Kp13 strain carries six plasmids (Ramos et al., 2014). One of them has the *bla*$_{KPC}$ resistance gene (pKP13d) and is 45.5 Kb in size. The other carries the resistance genes *bla*$_{CTXM-2}$, *bla*$_{SHV}$, *bla*$_{TEM-1}$, *dfrA15*, and *sul2* (pKP13f) and is 295.4 kb in size. Another mobile genetic element identified in this strain is a possible ICE*Kp33* (Chapter 3) that contains the genes that code for the yersiniabactin siderophore (Supplementary Figure 1). Therefore, after obtaining the EVs-Kp13, the presence of these genes in their internal content was investigated. As a result, we detected all resistance genes (*bla*$_{CTXM-2}$, *bla*$_{SHV}$, b*la*$_{TEM-1}$, *bla*$_{KPC}$, *dfrA15*, and *sul2*) and virulence within EVs-Kp13 (Figure 1D; Supplementary Figure 1).

**Vesiduction contributes to the transfer of resistance genes between *Klebsiella* spp**

The next step was to investigate whether resistance and virulence genes could be transferred by vesiduction to other isolates. For this, we made sure that the donor lines did not present resistance phenotypes and genotypes for these markers. As a result, we obtained four transvesiductants for the *K. aerogenes* 270 isolate on LB agar with 8 ug.mL$^{-1}$ of ceftriaxone (Figure 2A). When LB agar added with meropenem 8 ug.mL$^{-1}$ was used for the selection of transvesiculants, we did not observe growth. Thus, to certify that the four transvesiductants were really a *K. aerogenes* 270 isolate, we performed the fingerprint using two different primers (ERIC and $GTG_5$) (Figure 2B). With this analysis, we found that it is the same recipient isolate (*K. aerogenes* 270). Later, we inspected these isolates and detected by PCR the genes $bla_{CTXM-2}$, $bla_{SHV}$, $bla_{TEM-1}$, *dfrA15* and *sul2* (Figure 2C). The same genes are present in plasmid pKP13f. The *blaKPC* gene and the markers for the yersiniabactin siderophore were not found in the transvesiductants (Figure 2C). The other isolates used as recipients did not grow on the selective media used in this work.

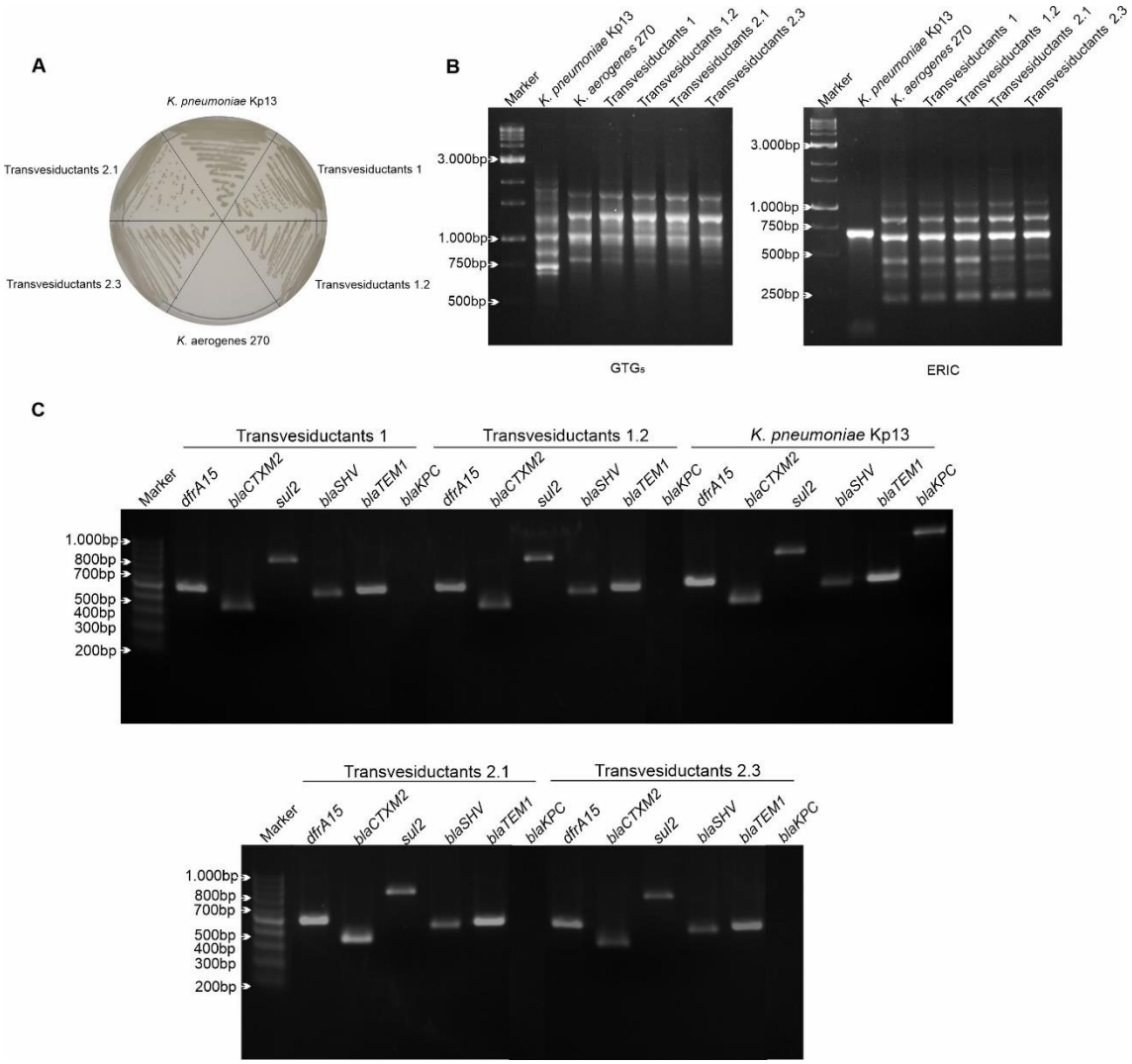



**Figure 2:** Differentiation of transvesiductants. A) Growth phenotype on LB agar with addition of ceftriaxone (8 ug.mL$^{-1}$). B) Fingerprint GTG$_5$ and ERIC. C) 1.5% agarose gel with resistance genes found after vesiduction. Kasvi 1 Kb marker.

***Klebsiella aerogenes* transvesiductants show cephalosporin resistance phenotypes**

The transvesiductants maintained growth on ceftriaxone 8 ug.mL$^{-1}$ for more than 5 generations showing a stable phenotype. When investigating the resistance phenotype of transvesiculants to cephalosporins (third and fourth generation), we observed a change from sensitive to resistant phenotype (Figure 3). The same was observed in the antibiogram performed for trimethoprim (*dfrA15* gene). Subsequently, the minimum inhibitory concentration was performed with the transvesiculants for the antibiotic ceftriaxone (Table 2). Before the vesiduction process, the minimum inhibitory concentration of *K. aerogenes* 270 was ≥2 mg.L$^{-1}$, after the vesidulation event, the minimum inhibitory concentration changed to 64 mg.L$^{-1}$. The MIC of the transvesidctants approximates the *K. pneumoniae* Kp13 donor which is ≥64 mg.L$^{-1}$. This result reinforces vesiduction as an important mechanism of interspecies horizontal transfer of resistance genes.

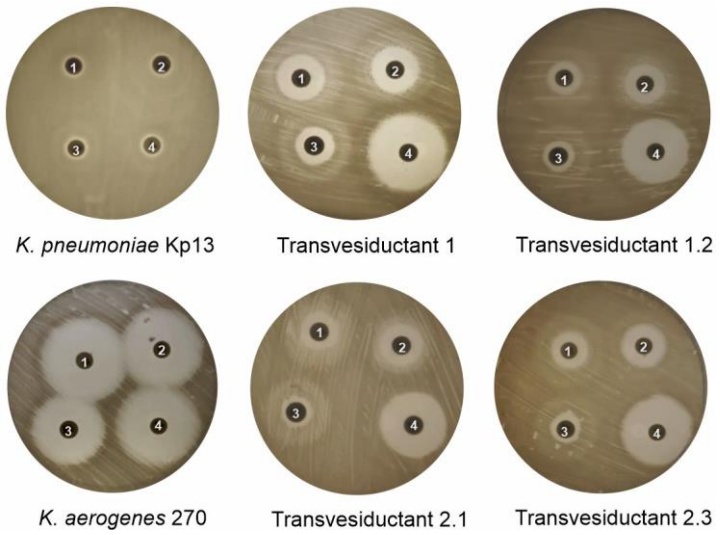



**Figure 3:** Resistance phenotype: donor, recipient, and transvesiculats. Each number indicated on the disc corresponds to a tested cephalosporin. (1) Cefotaxime - CTX 30ug, (2) ceftriaxone - CRO 30ug, (3) Ceftiofur - CTF 30ug and (4) cefepime - FEP 30ug.

**Is the resistance phenotype found on transvesiductants determined by marker gene or pKP13f plasmid acquisition?**

Our results so far indicate that the transvesiductants were contemplated with the pKP13f plasmid. However, our PFGE results were not conclusive as the content of the plugs showed degradation and it was necessary to improve the protocol (Supplementary Figure 2A). As a second alternative, we chose to use the *Not*1 restriction enzyme. This enzyme cleaved the pKP13f plasmid into 11 fragments, one of which was 6.3 Kb in size, in which the $bla_{\text{CTXM-2}}$ gene is located. In this way, we produced a digoxigenin-labeled probe for the $bla_{\text{CTXM-2}}$ gene. However, upon completion of the hybridization and detection, we noticed that there was probably some problem with the cleavage (Supplementary Figure 2B and C). Despite the technical problems, DNA detection with a labeled probe still shows that the gene is present in the transvesiductants (Supplementary Figure 2C).

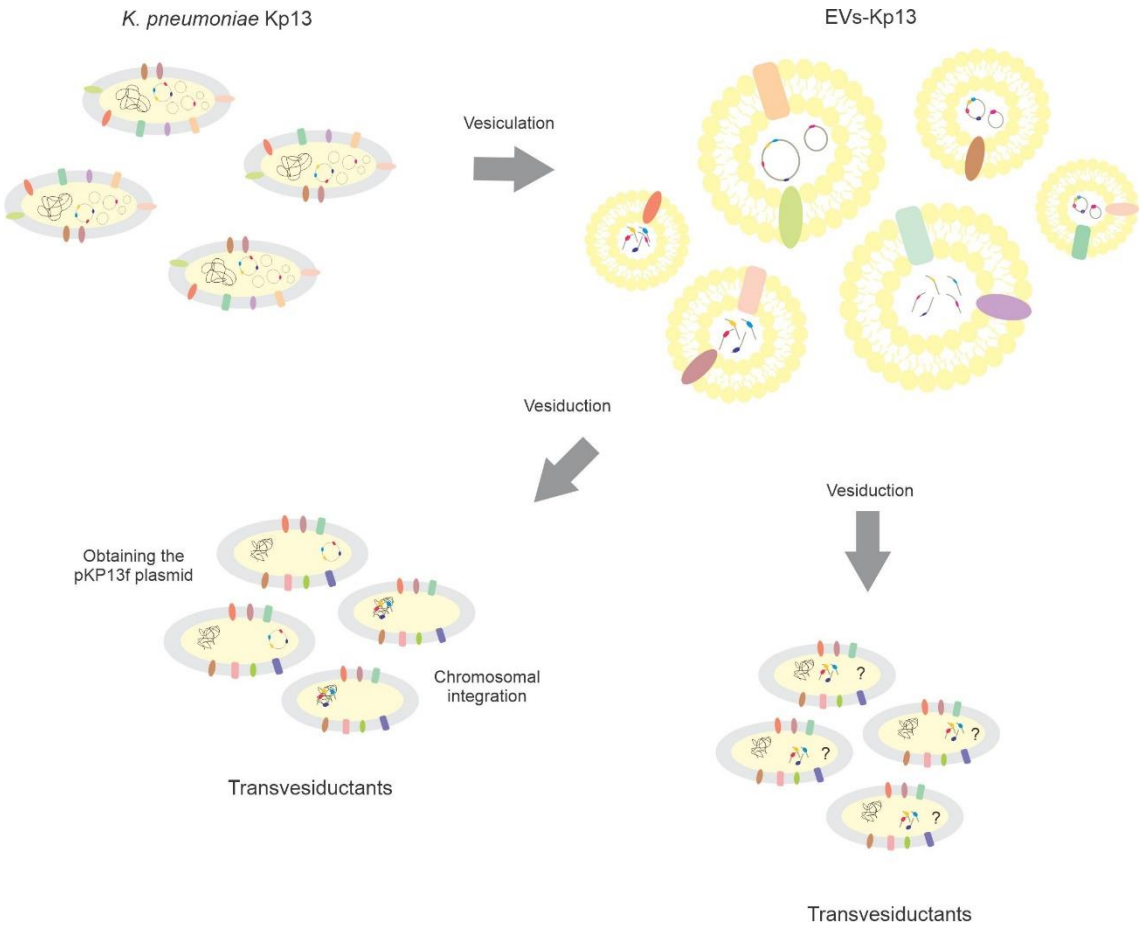



**Figure 4:** Delivery mechanisms related to the vesiduction process between EVs-Kp13 and *K. aerogenes* 270. EVs-Kp13 are produced by the progenitor cell (*K. pneumoniae* Kp13) by vesiculation and carry genes for antimicrobial resistance and virulence. EVs-Kp13 transport, protect and deliver resistance genes to other bacteria by a phenomenon known as vesiduction. The recipient strain is termed transvesiculants

**Table 1:** Resistance and virulence genes used in this work

| Localization | Genes | Name | Sequence 5' ----->3' | Size (bp) | Tm°C | Reference |
|---|---|---|---|---|---|---|
| plasmid pKP13f | *dfrA15* | *dfrA-F* | GTGAAACTATCACTAATGG | 457 | 50 | Navia et al., 2003 |
| | | *dfrA-R* | CCCTTTTGCCAGATTTGG | | | |
| | *sul2* | *sul2-F* | CGGCATCGTCAACATAACCT | 721 | 58 | Boerlin et al., 2005 |
| | | *sul2-R* | TGTGCGGATGAAGTCAGCTC | | | |
| | *bla*$_{CTXM2}$ | *blaCTX-M-2*-F | TGATACCACCACGCCGCTC | 341 | 59 | Xu et al., 2005 |
| | | *blaCTX-M-2*-R | TATTGCATCAGAAACCGTGGG | | | |
| | *bla*$_{SHV}$ | blaSHV-F | ACCTTTAAAGTAGTGCTCTGC | 306 | 57 | Yu et al., 2018 |
| | | blaSHV-R | CACCATCCACTGCAGCAGCTG | | | |
| | *bla*$_{TEM1}$ | *blaTEM1-F* | ACAGCGGTAAGATCCTTGAGAG | 461 | 55 | Yu et al., 2018 |
| | | *blaTEM1-R* | GAAGCTAGAGTAAGTAGTTCG | | | |
| plasmid pKP13d | *bla*$_{KPC}$ | *blaKPC-F* | ATGTCACTGTATCGCCGTC | 879 | 57 | Endimiani et al., 2008 |
| | | *blaKPC-R* | TTACTGCCCGTTGACGCC | | | |
| ICE*kp33* | *irp1* | *irp1-F* | CAGCCTCACGGCCCTTAT | 360 | 55 | Shan et al., 2021 |
| | | *irp1-R* | CGGCGTATGCTCAGTCAGTA | | | |
| | *irp2* | *irp2-F* | TTCCTTCAGCATCGCCTGTTA | 484 | 55 | Shan et al., 2021 |
| | | *irp2-R* | CAAGCCCGACATACTCAATCT | | | |
| | *irp3* | *irp3-F* | TGCTGCTATTGGGTAAACACG | 407 | 55 | Shan et al., 2021 |
| | | *irp3-R* | GCGACAAACAGGCTGGATGA | | | |
| | *irp5* | *irp5-F* | CCCTGCTGTTCGCCTTGT | 789 | 57 | Shan et al., 2021 |
| | | *irp5-R* | CCTGGCTGTGGAGAATAGTGG | | | |
| | ybt-PQ | *ybtP-ybtQ F* | GCCGGGAACGTCAAAGAA | 1.816 | 55 | Bach et al., 2000 |
| | | *ybtP-ybtQ R* | AGGTGAGCTTTCATGTGCCT | | | |

**Table 2:** Antibiogram and minimum inhibitory concentration

| Isolates | Antibiogram | | | | | Minimal inhibitory concentration (MIC) |
|---|---|---|---|---|---|---|
| | FEP (30ug) | CRO (30ug) | CTX (30ug) | CTF (30ug) | CAZ (30ug) | Cefttiaxone ($mg.L^{-1}$) |
| | Cefepime | Ceftriaxone | Cefotaxima | Ceftiofur | Ceftazidima | |
| *Klebsiella pneumoniae* Kp13 | - | - | - | - | - | >64 |
| *Klebsiella aerogenes* 270 | 29mm | 27mm | 29mm | 24mm | 27mm | ≥2 |
| Transvesiculant 1 | 19mm | 11mm | 12mm | 10mm | 9mm | 64 |
| Transvesiculant 1.2 | 20mm | 11mm | 12mm | 10mm | 9mm | 32 |
| Transvesiculant 2.1 | 19mm | 13mm | 9mm | 8mm | 8mm | 64 |
| Transvesiculant 2.3 | 19mm | 10.4mm | 8mm | 9mm | 7mm | 64 |

**Discussion**

Vesiculation is a complex process, and each microorganism responds differently to environmental stimuli (Toyofuko et al., 2023). It is known that stressful conditions such as iron depletion and the use of antimicrobial agents can stimulate vesiculation in *K. pneumoniae* (Ye et al., 2021). Here, we used 8 ug.mL$^{-1}$ meropenem to obtain EVs-Kp13, as we did not observe differences in terms of CFU.mL$^{-1}$ at the 16-hour point of the growth curve (Figure 1A). It is known that the use of carbapenems increases vesiculation in *K. pneumoniae* (Ye et al., 2021) thus, greater chances of DNA packaging.

EVs-Kp13 were obtained by the hydrostatic filtration method, a method used for the first time for *K. pneumoniae*. EVs-Kp13 have peculiar characteristics such formation of aggregates (Figure 1B). These characteristics are probably explained by the excess production of the capsule. However, studies show that the nature of the membrane lipopolysaccharides can be determinant for the aggregation of EVs (Gnopo et al., 2020). A work carried out with EVs of *Porphyromonas gingivalis* used tween 0.1% to reduce autoaggregation (Cecil et al., 2016). Thus, we used tween 0.1% and we had an improvement in the aggregation profile without interfering with the integrity of EVs-Kp13 (Figure 1B).

EVs from Gram-negative bacteria are classified according to their internal and external content (Toyofuko et al., 2023). EVs are classified into outer-membrane vesicles (OMVs), outer-inner membrane vesicles (OIMVs), outer membrane explosive vesicles (EOMVs), and explosive outer-inner membrane vesicles (EOIMVs) (Toyofuko et al., 2023). Briefly, OMVs can transport plasmids and outer membrane proteins, but not other cytoplasmic contents. OIMVs may contain cytoplasmic contents and outer and inner membrane proteins. EOMVs and EOIMVs come from cell lysis caused by phages (Toyofuko et al., 2023). Thus, based on our results, we believe that the EVs produced by *K. pneumoniae* grown in the presence of meropenem may be of the OIMVs and OMVs type, although it is not well elucidated how DNA packaging occurs.

It is estimated that high copy number plasmids are packaged by EVs with greater efficiency compared to low copy number plasmids (Tran & Boedicker 2017). As a result, the transfer of plasmids with high copy numbers via vesiduction is greater (Dell'Annunziata et al., 2021; Tran & Boedicker 2017). Plasmid size can also affect the average size of EVs produced (Dell'Annunziata et al., 2021). We know that EVs-Kp13 have an average size of 183 nm and have the resistance genes present in plasmids pKP13d (45.5 Kb) and pKP13f (295.4 Kb), and virulence genes from a putative ICE (Figure 1D; Supplementary Figure 1). However, we

speculate that only the pKP13f plasmid transfer was performed to the transvesiculants at low efficiency (Figure 2C; Figure4). Plasmid pKP13f (conjugative) is considerably large and its maintenance for the cell is energetically high. We believe that the packaging of this plasmid is smaller and, therefore, the number of transvesiductants was also smaller. However, further studies are needed to confirm this hypothesis.

So far, they indicate that the transvesiductants were contemplated with the plasmid pKP13f. However, our PFGE results were not conclusive as the content of the plugs showed degradation and it was necessary to improve the protocol (Supplementary Figure 2A). In addition to the degradation of the plugs, probably due to the excess production of capsule by the donor bacteria, the plasmid may not be visually detected in the gel or the plasmid may have integrated into the chromosome of the donor bacteria. Thus, we used the Not1 restriction enzyme. The enzyme would cleave the pKP13f plasmid into 11 fragments, one of which is 6.3 kb in size, in which the *bla*$_{\text{CTXM-2}}$ gene is located. In this way, we produced a digoxigenin-labeled probe for the *bla*$_{\text{CTXM-2}}$ gene. At the end of the hybridization and detection, we noticed that there was probably some problem with the cleavage (Supplementary Figure 2B and C). DNA detection with a labeled probe further shows that the gene is present in the transvesiculants (Supplementary Figure 2C). Our approach must be revised to be able to state that the pKP13f plasmid was actually transferred to the transvesiductants. Despite this, the transvesiductants present a stable cephalosporin resistance phenotype as well as the presence of resistance genes (Table 2; Figure 2C). Showing that EVs protect packaged DNAs from degradation by DNases.

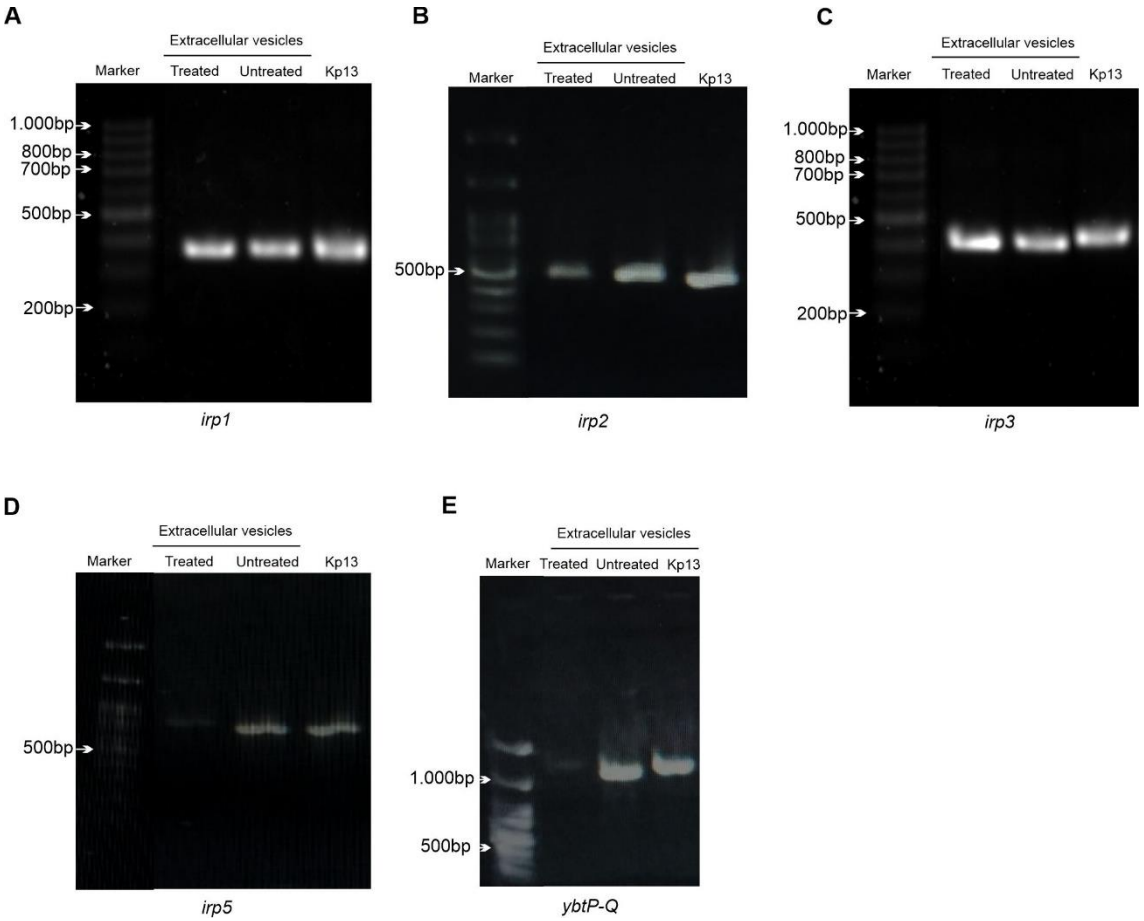
In vitro vesiduction is controlled by ideal conditions for donor cell growth, however, it can be a very common event within the host, where stressful conditions such as competition for nutrients, oxygenation, available iron, and the presence of antimicrobials occur with bigger frequency. Furthermore, because they are stable, EVs can transport DNA over long distances and deliver it to different bacteria (Toyofuko et al., 2023). In this work, we observed that vesiduction occurred only with one isolate of *K. aerogenes* 270, which allows us to state that cargo delivery by EVs-Kp13 has no phylogenetic dependence and is perhaps more related to the compatibility of the genetic element (ICE or plasmid) with the recipient strain.

In summary, Kp13-EVs are produced by the progenitor *K. pneumoniae* Kp13 by vesiculation and carry resistance and virulence genes important for pathogenesis and adaptation. Kp13-EVs transport, protect and deliver resistance genes (*bla*$_{\text{CTXM-2}}$, *bla*$_{\text{SHV}}$, *bla*$_{\text{TEM-1}}$, *dfrA15*, and *sul2*) to *K. aerogenes* 270 (transvesiculants) by a phenomenon known as

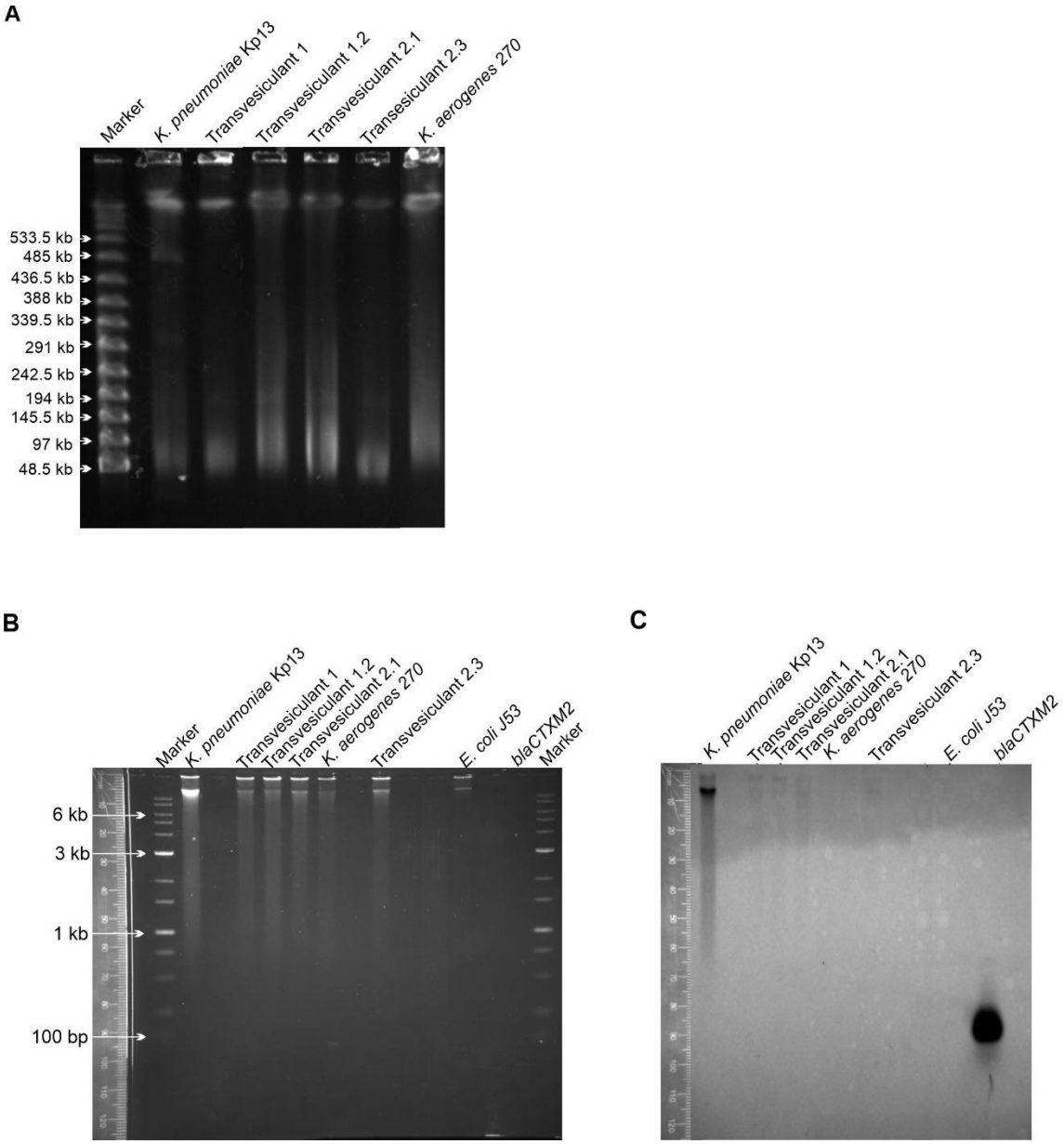
vesiduction (Figure 4). However, we hypothesize that: that: 1) the transvesiculants may have received the plasmid pKP13f and kept it in the cell, or 2) the plasmid pKP13f is integrated into the genome of the transvesiculants, or lastly and least likely, 3) only fragments of genes are in the EVs-Kp13 and these are translated by the recipient cell's translation machinery (Figure 4).

In conclusion, the results obtained in this work highlight for the first time the transfer of interspecies resistance genes, mediated by vesiduction, between *K. pneumoniae* from human reservoir and *K. aerogenes* from swine reservoir. Here we also report for the first time the transfer of $bla_{\text{CTXM-2}}$, *dfrA15*, and *sul2* genes mediated by vesiduction. This study elucidates an important gene flow route between different species and reservoirs, contributing significantly to the one health approach.

## Supplementary material




**Supplementary figure 1:** Virulence genes detected within EVs-Kp13.EVs-Kp13. A) *irp1* gene. B) *irp2* gene. C) *irp3* gene. D) *irp5* gene. E) *ybtP-Q* gene.




**Supplementary figure 2:** Detection of plasmid pKP13f and possible chromosomal integration. A) 1.2% agarose gel used in pulsed-field gel electrophoresis (PFGE) containing plugs from the donor, recipient, and transvesiculating strains. B-C) Product of digestion with Not1 and southern blot with the probe labeled with digoxigenin for the $bla_{CTXM2}$ gene, respectively.

## Reference


Antenucci, F., Fougeroux, C., Bossé, J. T., Magnowska, Z., Roesch, C., Langford, P., Bojesen, A. M. Identification and characterization of serovar-independent immunogens in *Actinobacillus pleuropneumoniae*. *Veterinary research*. **2017**, 48(1), 1-12. DOI:10.1186/s13567-017-0479-5

Barton, Bret M., Harding, Gordon P., Zuccarelli, Anthony J. A general method for detecting and sizing large plasmids. *Analytical biochemistry*, v. 226, n. 2, p. 235-240, 1995.

Bach, S., de Almeida, A., & Carniel, E. The *Yersinia* high-pathogenicity island is present in different members of the family Enterobacteriaceae. *FEMS microbiology letters*. **2000**, 183(2), 289-294. DOI:10.1111/j.1574-6968.2000.tb08973.x

Bitto, NJ., Chapman, R., Pidot, S., Costin, A., Lo, C., Choi, J., D'Cruze, T., Reynolds, EC., Dashper, SG., Turnbull, L. Vesículas de membrana bacteriana transportam sua carga de DNA para as células hospedeiras. *Science Rep.* **2017**, 7, 7072. DOI: 10.1038/s41598-017-07288-4

Bielaszewska, M., Daniel, O., Karch, H., Mellmann, A. Dissemination of the *bla*$_{\text{CTX-M15}}$ gene among Enterobacteriaceae via outer membrane vesicles. J. Antimicrob. Chemother. 2020, 75, 2442–2451. DOI: 10.1093/jac/dkaa214

Boerlin, P., Travis, R., Gyles, C. L., Reid-Smith, R., Heather Lim, N. J., Nicholson, V., Archambault, M. Antimicrobial resistance and virulence genes of *Escherichia coli* isolates from swine in Ontario. *Applied and environmental microbiology*. **2005**, 71(11), 6753-6761. DOI: 10.1128/AEM.71.11.6753-6761.2005

Cecil, J. D., O'Brien-Simpson, N. M., Lenzo, J. C., Holden, J. A., Chen, Y. Y., Singleton, W., Reynolds, E. C. Differential responses of pattern recognition receptors to outer membrane vesicles of three periodontal pathogens. *PLoS One*. **2016**, 11(4), e0151967. DOI:10.1371/journal.pone.0151967

Chatterjee, S., Mondal, A., Mitra, S., Basu, S. *Acinetobacter baumannii* transfere o gene BlaNDM-1 via vesículas de membrana externa. *J. Antimicrob. Chemother.* **2017**, 72, 2201–2207. DOI:10.1093/jac/dkx131

Clinical and Laboratory Standards Institute (CLSI). *Performance Standards for Antimicrobial Disk and Dilution Susceptibility Tests for Bacteria Isolated From Animals.* 5th ed. CLSI supplement VET01S (ISBN 978-1-68440-092-8 [Print]; ISBN 978-1-68440-093-5 [Electronic]). Clinical and Laboratory Standards Institute, USA, 2020.

Dell'Annunziata, F., Dell'Aversana, C., Doti, N., Donadio, G., Dal Piaz, F., Izzo, V., Franci, G. Outer membrane vesicles derived from *Klebsiella pneumoniae* are a driving force for horizontal gene transfer. *International Journal of Molecular Sciences*. **2021**, 22(16), 8732. DOI:10.3390/ijms22168732

Endimiani, A., Carias, L. L., Hujer, A. M., Bethel, C. R., Hujer, K. M., Perez, F., Bonomo, R. A. Presence of plasmid-mediated quinolone resistance in *Klebsiella pneumoniae* isolates possessing *bla*$_{\text{KPC}}$ in the United States. *Antimicrobial agents and chemotherapy*. **2008**, *52*(7), 2680-2682. DOI:10.1128/AAC.00158-08

Gnopo, Y. M., Misra, A., Hsu, H. L., DeLisa, M. P., Daniel, S., Putnam, D. Induced fusion and aggregation of bacterial outer membrane vesicles: Experimental and theoretical analysis. *Journal of colloid and interface Science*. **2020**. 578, 522-532. DOI:10.1016/j.jcis.2020.04.068

Ho, M.-H., Chen, C.-H., Goodwin, JS., Wang, B.-Y., Xie, H. Functional Advantages of *Porphyromonas Gingivalis* Vesicles. *PLoS ONE*. **2015**, 10, e0123448. DOI: 10.1371/journal.pone.0123448

Holt, K. E., Wertheim, H., Zadoks, R. N., Baker, S., Whitehouse, C. A., Dance, D., Thomson, N. R. Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in *Klebsiella pneumoniae*, an urgent threat to public health. *Proceedings of the National Academy of Sciences*. **2015**, 112(27), E3574-E3581. DOI:10.1073/pnas.1501049112

Hua, Y., Wang, J., Huang, M., Huang, Y., Zhang, R., Bu, F., Wang, Q. Outer membrane vesicles-transmitted virulence genes mediate the emergence of new antimicrobial-resistant hypervirulent *Klebsiella pneumoniae*. *Emerging microbes & infections*. **2022**, 11(1), 1281-1292. DOI:10.1080/22221751.2022.2065935

Hussein, M., Jasim, R., Gocol, H., Baker, M., Thombare, V. J., Ziogas, J., Velkov, T. Comparative Proteomics of Outer Membrane Vesicles from Polymyxin-Susceptible and Extremely Drug-Resistant *Klebsiella pneumoniae*. *Msphere*. **2023**, 8(1), e00537-22. DOI:10.1128/msphere.00537-22

Joseph, L., Merciecca, T., Forestier, C., Balestrino, D., Miquel, S. From *Klebsiella pneumoniae* colonization to dissemination: an overview of studies implementing murine models. *Microorganisms*. **2021**, 9(6), 1282. DOI:10.3390/microorganisms9061282

Jolly, K. A. and Maiden, M. C. J. BIGSdb: Scalable analysis of bacterial genome variation at the population level. *BMC Bioinformatics*. 2010 doi:10.1186/1471-2105-11-595

Kumar, P., Dash, B., Suyal, D. C., Gupta, S. B., Singh, A. K., Chowdhury, T., Soni, R. Characterization of Arsenic-Resistant *Klebsiella pneumoniae* RnASA11 from Contaminated Soil and Water Samples and Its Bioremediation Potential. *Current Microbiology*, 78, 3258-3267 (2021) doi:10.1007/s00284-021-02602-w

Kumar, S., Stecher, G., Li, M., Knyaz, C. Tamura, K. MEGA X: Molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol*. 35, 1547–1549 (2018) doi:10.1093/molbev/msy096

Kleinheinz, K. A., Joensen, K.G., Larsen, M. V. Applying the ResFinder and VirulenceFinder web-services for easy identification of acquired antibiotic resistance and *E. coli* virulence genes in bacteriophage and prophage nucleotide sequences. *Bacteriophage*. (2014). doi 10.4161/bact.27943

Li, P., Luo, W., Xiang, T. X., Jiang, Y., Liu, P., Wei, D. D., Zhang, W. Horizontal gene transfer via OMVs co-carrying virulence and antimicrobial-resistant genes is a novel way for the dissemination of carbapenem-resistant hypervirulent *Klebsiella pneumoniae*. *Frontiers in Microbiology*. **2022**, 13, 945972. DOI:10.3389/fmicb.2022.945972

Lieckfeldt, E., Meyer, W., Börner, T. Rapid identification and differentiation of yeasts by DNA and PCR fingerprinting. *Journal of Basic Microbiology*. **1993**, 33(6), 413-425. DOI:10.1002/jobm.3620330609

Lucena, A. C. R., Ferrarini, M. G., de Oliveira, W. K., Marcon, B. H., Morello, L. G., Alves, L. R., Faoro, H. Modulation of *Klebsiella pneumoniae* Outer Membrane Vesicle Protein Cargo under Antibiotic Treatment. *Biomedicines*. **2023**, 11(6), 1515. DOI: 10.3390/biomedicines11061515

Matsumura, Y., asufumi, Peirano, G., Pitout, J. D. D. Complete genome sequence of *Escherichia coli* J53, an azide-resistant laboratory strain used for conjugation experiments. *Genome Announcements*. **2018**, 6-21, e00433-18. DOI:10.1128/genomeA.00433-18

Navia, M. M., Ruiz, J., Sanchez-Cespedes, J., Vila, J. Detection of dihydrofolate reductase genes by PCR and RFLP. *Diagnostic microbiology and infectious disease*. **2003**, 46(4), 295-298. DOI:10.1016/s0732-8893(03)00062-2

Podschun, R., Ullmann, U. *Klebsiella* spp. as nosocomial pathogens: epidemiology, taxonomy, typing methods, and pathogenicity factors. *Clinical microbiology reviews*. **1998**, 11(4), 589-603. DOI: 10.1128/CMR.11.4.589

Ramos, P. I. P., Picão, R. C., Vespero, E. C., Pelisson, M., Zuleta, L. F. G., Almeida, L. G. P., Nicolás, M. F. Pyrosequencing-based analysis reveals a novel capsular gene cluster in a KPC-producing *Klebsiella pneumoniae* clinical isolate identified in Brazil. *BMC microbiology*. **2012** 12,1-12. DOI:10.1186/1471-2180-12-173

Ramos, P. I. P., Picão, R. C., de Almeida, L. G. P., Lima, N. C. B., Girardello, R., Vivan, A. C. P., Nicolás, M. F. Comparative analysis of the complete genome of KPC-2-producing *Klebsiella pneumoniae* Kp13 reveals remarkable genome plasticity and a wide repertoire of virulence and resistance mechanisms. *BMC genomics*. **2014**, 15(1), 1-16. DOI:10.1186/1471-2164-15-54

Rumbo, C., Fernández-Moreira, E., Merino, M., Poza, M., Mendez, J. A., Soares, N. C., Mosquera, A., Chaves, F., Bou, G. Transferência Horizontal do gene *OXA-24* Carbapenemase via Vesículas de Membrana Externa: Um Novo Mecanismo de Disseminação de Genes de Resistência a Carbapenemes em *Acinetobacter baumannii*. *Antimicrobiano. Agentes Chemother.* **2011**, 55, 3084–3090. DOI:10.1128/AAC.00929-10

Sambrook, et al. Molecular cloning. 2ª Edition. Cold Spring Harbor Lab. Press.3 Volumes. 1990.

Song, Z., Qin, Y., Peng, Y., Huang, M., Hua, Y., Jiang, H., Rui, Y. Carbapenem-resistant *Klebsiella pneumoniae* (CRKP) transfers conjugative plasmids containing $bla_{\text{NDM-5}}$ and *mcr-1* genes via outer membrane vesicles (OMVs). **2022**. DOI: 10.21203/rs.3.rs-2234437/v1

Soler, N., & Forterre, P. Vesiduction: the fourth way of HGT. *Environmental Microbiology*. **2020**, 22(7), 2457-2460. DOI:10.1111/1462-2920.15056

Shan, C., Liu, C., Lu, Q., Fu, G., Shah, S. A. H., Akhtar, R. W., Gao, H. High Pathogenicity Island (HPI) Main Structural Genes and their Bioinformatics Analysis in Clinically Isolated *E. coli* from Saba Pigs. **2021**. DOI:10.17582/journal.pjz/20200625160645

Tang, B., Yang, A., Liu, P., Wang, Z., Jian, Z., Chen, X., Liu, W. Outer Membrane Vesicles Transmitting $bla_{\text{NDM-1}}$ Mediate the Emergence of Carbapenem-Resistant Hypervirulent *Klebsiella pneumoniae*. *Antimicrobial Agents and Chemotherapy*. **2023**, 67(5), e01444-22. DOI:10.1128/aac.01444-22

Toyofuku, M., Schild, S., Kaparakis-Liaskos, M., Eberl, L. Composition and functions of bacterial membrane vesicles. *Nature Reviews Microbiology*. **2023**, 1-16. DOI: 10.1038/s41579-023-00875-5

Tot, T., Kibel, S., Sardelić, S., Nemer, K., Benčić, A., Vraneš, J., Bedenić, B. Polyclonal spread of colistin resistant *Klebsiella pneumoniae* in Croatian hospitals and outpatient setting. *Germs*, 11(2), 163 (2021) doi:10.18683/germs.2021.1254

Tran, F., & Boedicker, J. Q. Genetic cargo and bacterial species set the rate of vesicle-mediated horizontal gene transfer. Scientific reports. 2017, 7(1), 8813. DOI: 10.1038/s41598-017-07447-7

Versalovic J, Koeuth T, Lupski R. Distribution of repetitive DNA sequences in eubacteria and application to finerpriting of bacterial enomes. *Nucleic Acids Res.* **1991**, 19(24):6823–31. DOI:10.1093/nar/19.24.6823

Wang, Z., Wen, Z., Jiang, M., Xia, F., Wang, M., Zhuge, X., Dai, J. Dissemination of virulence and resistance genes among *Klebsiella pneumoniae* via outer membrane vesicle: An important plasmid transfer mechanism to promote the emergence of carbapenem-resistant hypervirulent Klebsiella pneumoniae. *Transboundary and Emerging Diseases*. **2022**, 69(5), e2661-e2676. DOI: doi.org/10.1111/tbed.14615

Wyres, K. L., Lam, M. M., Holt, K. E. Population genomics of *Klebsiella pneumoniae*. *Nature Reviews Microbiology*. **2020**, 18(6), 344-359. DOI: 10.1038/s41579-019-0315-1

Xu, L., Ensor, V., Gossain, S., Nye, K., Hawkey, P. Rapid and simple detection of $bla_{\text{CTXM}}$ genes by multiplex PCR assay. *Journal of medical microbiology*. **2005**, 54(12), 1183-1187. DOI:10.1099/jmm.0.46160-0

Yaron, S., Kolling, G. L., Simão, L., Matthews, K. R. Vesicle-Mediated Transfer of Virulence Genes from *Escherichia coli* O157:H7 to Other Enteric Bacteria. *Appl. Ambiente. Microbiol.* **2000**, 66,4414–4420.DOI: 10.1128/AEM.66.10.4414-4420.2000

Ye, C., Li, W., Yang, Y., Liu, Q., Li, S., Zheng, P., Ma, Y. Inappropriate use of antibiotics exacerbates inflammation through OMV-induced pyroptosis in MDR *Klebsiella pneumoniae* infection. *Cell Reports*. **2021**, 36(12). DOI:10.1016/j.celrep.2021.109750

## Conclusions and perspectives

In view of our results, we can conclude that clinically healthy swine under stress conditions are colonized by *Klebsiella* spp MDR with virulence phenotypes and belong to globally important STs such as ST147 and ST25 (chapter 1). Furthermore, these animals are sources of *K. pneumoniae* MDR, as conjugative plasmids, which are capable of carrying and disseminating resistance genes such as *mcr-1* and *bla*$_{\text{TEM1}}$. *Klebsiella pneumoniae* MDR may still have an important chromosomal genetic arsenal for its pathogenicity (chapter 2).

The search and monitoring of integrative and conjugative elements (ICEs) in *K. pneumoniae* allowed us to understand the role of these elements in the context of health and in understanding the propagation of genes involved in virulence and resistance. In summary, the gene arsenal of ICEs described for *K. pneumoniae* can aid in its adaptation and significantly contribute to its fitness in different reservoirs and environments (Chapter 3). Finally, we conclude that *Klebsiella pneumoniae* produces extracellular vesicles (EVs) that transport and protect resistance and virulence genes from enzymatic degradation. Furthermore, Kp13-EVs are involved in the transfer of resistance genes between *K. pneumoniae* (human) and *K. aerogenes* (animal) by a process known as vesiduction. Here, we report for the first time the transfer of the *dfrA15* and *sul2* genes via vesiduction (Chapter 4).

As future prospects we intend to (1) carry out studies aimed at investigating conjugative plasmids present in other representatives of *Klebsiella* spp; (2) conduct further studies with *K. pneumoniae* MDR to further investigate the mobile genetic elements (MGE) found in *K. pneumoniae* HS-144 and HS-13; (3) to investigate factors related to the excision and integration of ICEs in *K. pneumoniae* and (4) to understand which molecular mechanisms are involved in the vesiduction process.