T.R.
ONDOKUZ MAYIS UNIVERSITY
INSTITUTE OF GRADUATE STUDIES
DEPARTMENT OF MICROBIOLOGY

DETERMINATION OF SOME VIRULENCE GENES AND ANTIBIOTIC RESISTANCE PROFILES OF *KLEBSIELLA PNEUMONIAE* STRAINS OF CATS AND DOGS ORIGIN

Master's Thesis

ANWAR OMER ELFAROUF ELHASSAN AHMED

Supervisor
Prof. Dr. Arzu FINDIK

SAMSUN
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2023

.ACCEPTANCE AND APPROVAL OF THE THESIS

The study entitled **"DETERMINATION OF SOME VIRULENCE GENES AND ANTIBIOTIC RESISTANCE PROFILES OF *KLEBSİELLA PNEUMONIAE* STRAINS OF CATS AND DOGS ORIGIN"** prepared by, **Anwar Omer Elfaroug Elhassan AHMED** and supervised by **Prof. Dr. Arzu FINDIK**, was found successful and unanimously accepted by committee members as Master thesis, following the examination on the date20.02.2024.

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Prof. Dr. Ahmet TABAK
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DECLARATION OF COMPLIANCE WITH SCIENTIFIC ETHIC

I hereby declare and undertake that I complied with scientific ethics and academic rules in all stages of my Master's Thesis, that I have referred to each quotation that I use directly or indirectly in the study and that the works I have used consist of those shown in the sources, that it was written in accordance with the institute writing guide and that the situations stated in the article 3, section 9 of the Regulation for TÜBİTAK Research and Publication Ethics Board were not violated.

Is Ethics Committee Necessary?

Yes ☐ (If it necessary, please add appendices.)

No ☒

28/12 / 2023
Anwar Omer Efaroug
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DECLARATION OF THE THESIS STUDY ORIGINALITY REPORT

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ÖZET

KEDİ VE KÖPEK KÖKENLİ *KLEBSİELLA PNEUMONIAE* SUŞLARININ VİRÜLENS GENLERİNİN VE ANTİBİYOTİK DİRENÇ PROFİLİNİN BELİRLENMESİ

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Yüksek Lisans, Şubat/2023
Danışman: Prof. Dr. Arzu FINDIK

Klebsiella pneumoniae, insanlarda ve hayvanlarda önemli enfeksiyonlara neden olan bir oportünistik patojendir. Klinik olarak sağlıklı ve hastalıklı köpek ve edilerden sıkça izole edilen *K. pneumoniae* suşlarının çoğunun çoklu ilaç direncine sahip olduğu bildirilmiştir. Köpeklerde ve kedilerde, *K. pneumoniae*'nin idrar yolu enfeksiyonları, pyometra, üst solunum yolu enfeksiyonları ve septisemi gibi ekstraintestinal enfeksiyonlara neden olduğu belirtilmiştir. Bu enfeksiyonların tedavisi, antibiyotik direncinin ortaya çıkması nedeniyle genellikle zordur ve yüksek morbidite ve mortalite oranları ile ilişkilidir. Son yıllarda, dünya çapında çoklu ilaca dirençli (MDR), karbapenem dirençli *K. pneumoniae* (CRKP) ve hipervirulent suşlar (hvKP) önemli bir halk sağlığı tehdidi haline gelmiştir. *K. pneumoniae*'nin patojenitesi, enfeksiyonun başlamasına ve immün sistemden kaçışına yardımcı olan çeşitli virülans faktörlerine bağlıdır. Bu çalışma, köpeklerden ve kedilerden izole edilen *K. pneumoniae* suşlarının çeşitli virülens genlerini ve çoklu ilaç direnç durumlarını belirlemeyi ve hipervirulent *K. pneumoniae* suşlarının varlığını saptamayı amaçlamaktadır. Bu kapsamda, 32 *K. pneumoniae* suşu "iplik" testi ile hvKP açısından test edilmiş ve yalnızca bir izolat pozitif bulunmuştur. İzolatların antibiyotik duyarlılıkları Kirby-Bauer disk difüzyon yöntemi kullanılarak belirlenmiştir. İzolatlar, amoksisilin+klavulanik asit'e %53,1, imipeneme %3,1, ampisiline %62,5, penisiline %43,7, sefoperazona %12,5, gentamisine %40,6, amikasine %9,3, siprofloksasine %40,6, enrofloksasine %37,5, nitrofurantoin'e %65,6, trimetoprim+sulfametoksazole %37,5, oksitetrasikline %46,8, sefkinoma %34,3 ve kloramfenikole %28,1 direnç gösterirken, tüm izolatlar meropeneme karşı duyarlıydı. Ayrıca, izolatların %62,5'i çoklu ilaca dirençli bir profil sergiledi. İzolatlar, *magA*, *fimH*, *rpmA*, *mrkD* ve *entB* gibi önemli virülans genlerinin varlığı açısından PCR ile incelenmiştir. *rpmA* geni 32 izolatın hiçbirinde bulunmazken, *magA*, *fimH*, *entB* ve *mrkD* genleri sırasıyla 4, 9, 5, 5 ve 15 izolatta tespit edilmiştir. İplik testi pozitif izolatın yalnızca *mrkD* genine sahip olduğu belirlenmiştir. Sonuç olarak, bu çalışma kedilerden ve köpeklerden izole edilen *K. pneumoniae* suşlarında hipervirulent bir fenotipin düşük olasılığını göstermektedir, ancak bu fenotiple ilişkilendirilen diğer genlerin ve antibiyotik direnç profiliyle ilişkilendirilmesi için daha fazla araştırmaya ihtiyaç vardır.

Anahtar Sözcükler: Antibiyotik direnci, Çoklu ilaç direnci, Kedi, *Klebsiella pneumoniae*, Köpek.

ABSTRACT

DETERMINATION OF SOME VIRULENCE GENES AND ANTIBIOTIC RESISTANCE PROFILES OF *KLEBSIELLA PNEUMONIAE* STRAINS OF CAT AND DOG ORIGIN

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Klebsiella pneumoniae, a multidrug-resistant pathogen, poses a significant threat in both human and animal infections. Its pathogenicity arises from various virulence factors and antibiotic resistance, leading to treatment challenges and failure. The emergence of hypervirulent *K. pneumoniae* strains (hvKP) in recent years, particularly affecting immunocompromised patients, heightens health risks. Virulence of hvKP is attributed to increased capsular or extracapsular polysaccharide production compared to classical *K. pneumoniae* (cKp), along with iron acquisition system. Key genes linked to hypermucoviscosity, including siderophore-encoding genes (e.g., enterobactin) and capsular polysaccharide regulatory genes (*rmpA* and *rmpA2*), contribute to the enhanced virulence of hvKP. Strains of hvKP possess virulence factors such as *rmpA*, *entB*, *fimH*, and *mrkD*, promoting biofilm formation and binding to the extracellular matrix. This study aimed to explore the antibiotic resistance profile and key virulence genes (*magA*, *fimH*, *rpmA*, *mrkD*, and *entB*) in *K. pneumoniae* isolates from dogs and cats in Samsun. Among 32 isolates tested for hvKP using the "string" test, only one isolate showed positive results. Antibiotic susceptibility testing revealed resistance rates, to amoxicillin+clavulanic acid 53.1%, imipenem 3.1%, ampicillin 62.5%, penicillin 43.7%, cefoperazone 12.5%, gentamicin 40.6%, amikacin 9.3%, ciprofloxacin 40.6%, enrofloxacin 37.5%, nitrofurantoin 65.6%, trimethoprim+sulfamethoxazole 37.5%, oxytetracycline 46.8%, cefquinome 34.3% and chloramphenicol 28.1%, cefquinoma, 25% cefepime and 43.75% to cefoxitin. Notably, all isolates remained susceptible to meropenem, while 62.5% exhibited a multidrug resistance profile. PCR analysis of virulence genes showed the absence of *rpmA* in all isolates, while *magA*, *fimH*, *entB* and *mrkD* genes were detected in 4, 9, 5, 5 and 15 isolates, respectively. The string test-positive isolate was identified to possess only the *mrkD* gene. In conclusion, this study indicates a low probability of hypervirulent phenotype among *K. pneumoniae* strains from cats and dogs. However, further investigation into genes associated with this phenotype and their correlation with antibiotic resistance profiles is warranted.
Keywords: Antibiotic resistance, Cat, Dog, *Klebsiella pneumoniae*, Multi-drug

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Anwar Omer Elfaroug Elhassan AHMED

CONTENTS

SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degrees Celsius
Mμ	:	Micrometer
Bp	:	Base pairs

FIGURES LEGENDS

TABLES LEGENDS

1. INTRODUCTION

Klebsiella spp. are recognized as rod-shaped, gram-negative, immobile and capable of fermenting lactose. They are facultatively anaerobic and belong to family Enterobacteriaceae. These bacteria are prevalent in various environments such as soil, water, manure and plants. Additionally, they are part of the microbiota found in the enteric tract, skin, and mouth of both animals and humans (Ribeiro et al., 2022). *Klebsiella pneumoniae* (*K. pneumoniae*) is a well-known opportunistic pathogen that often leads to hospital-acquired infections, including pneumonia, urinary tract infections, meningitis and bloodstream infections. Moreover, *K. pneumoniae* can cause infections in healthy people outside of hospitals, such as endophthalmitis, liver abscess, and meningitis (Zhu et al., 2021).

The interplay between humans, animals, and the environment can affect public health by serving as a reservoir for diseases. One of the pathogens, *Klebsiella pneumoniae*, falls under the WHO definition of zoonoses, which are diseases that can be naturally transmitted from animals to humans through direct contact, food, water, or the environment. Zoonotic diseases account for at least 61% of all human pathogens, and they make up a significant proportion (75%) of newly discovered or emerging infectious diseases over the last decade, identified or emerging infectious diseases as well as existing infectious diseases during the past decade (Carvalho et al.,2020; Hu et al., 2021).

K. pneumoniae can be categorized into two; classical *K. pneumoniae* (cKp) which is responsible for nosocomial pneumonia and hospital-acquired urinary tract infections, and hypervirulent *K. pneumoniae* (hvKp). The latter has gained significance as an important pathogen in recent in decades, capable of causing both community-acquired and increasingly, hospital-acquired infections. In contrast to classical *K. pneumoniae* (cKp), hypervirulent *K. pneumoniae* (hvKp) commonly infects healthy people. Initially, hvkp was identified as the cause of pyogenic liver abscesses in Asia, but its ability to cause other diseases is increasingly recognized. The prevalence of hvkp among *K. pneumoniae* varies, but in areas where hvkp is endemic, the rate can be as high as 12-45%, as reported by Choby et al. (2020). The string test to determine the hypermucoviscous phenotype (string length ≥5 mm) is a common method to identify hypervirulent *K. pneumoniae* (hvkp). This test is believed to have

an approximate 90% accuracy rate for predicting clinical hvkp strains (Zhu et al., 2021).

K. pneumoniae's pathogenicity is typically attributed to important factors such as antimicrobial resistance and virulence (Xu et al., 2018). According to Fasciana et al. (2019), the World Health Organization (WHO), the US Centers for Disease Control and Prevention (CDC) and the UK Department of Health have identified *K. pneumoniae* as an immediate threat to human health due to its status as a multidrug resistant (MDR) microorganism. Carvalho et al. (2020) stated that multidrug resistant (MDR) microorganisms can be transmitted from pets to their owners and veterinary staff, and subsequently spread to the wider community. Zhu et al. (2021) indicate that there has been a recent emergence of multidrug-resistant and hypervirulent (MDR-hv) strains, which is mainly attributed to the horizontal transfer of plasmid-mediated resistance or virulence.

Two main types of antibiotic resistance are frequently observed in *K. pneumoniae*. The first one involves the production of extended β-lactamases (ESBLs), which make bacteria resistant to cephalosporins and monobactams. The second, more concerning mechanism of resistance is the production of carbapenemases by *K. pneumoniae*, which makes bacteria resistant to nearly all available β-lactams, including the carbapenems (Paczosa and Mecsas, 2016).

It has been reported that potential virulence factors, such as mucoviscosity-associated gene A (*magA*) and regulator of mucoid phenotype A (*rmpA*), have been linked to hypermucoviscousphenotype. Additionally, genes such as *fimH* and *mrkD* which encode type 1 and type 3 fimbriae respectively, as well as siderophores like enterobactin (*Ent*), are responsible for attaching to host cells. These genetic factors assist these bacteria in evading the immune system and causing a variety of disease (Fu et al.,2018, Mirzaie and Ranjbar, 2021; Rastegar et al., 2021).

OBJECTIVES OF THE STUDY:

1. Determine the presence of virulence genes in *Klebsiella pneumoniae* strains isolated from cats and dogs.

2. Characterize the specific virulence gene profile to understand the pathogenic potential of the isolates.

3. Evaluate the antimicrobial resistance patterns of *Klebsiella pneumoniae* from companion animals.

4. Identify the spectrum of antibiotics to which these isolates may exhibit resistance.

5. Contribute valuable insights into the virulence and antibiotic resistance dynamics of *Klebsiella pneumoniae* in companion animals.

6. Enhance our understanding of the potential risks associated with infections originating from cats and dogs.

7. Provide information that may guide improved management and treatment strategies for infections related to these companion animals.

2.LITERATURE REVIEW

2.1. What differentiates Hypervirulent Pneumoniae (hvKp) from Classical Pneumoniae (cKp):

Hypervirulent (hvKp) and classical (cKp) are the two distinct strains of *K. pneumoniae*. The acquisition of antibiotic-resistant genes has been observed in the classical type, cKp. The string test, which is suggestive of high mucoviscosity, is usually used to identify hypervirulent *K. pneumoniae* (hvKp) strains. Severe infections contracted in the community, including pyogenic liver abscesses and bloodstream infections, have been associated with these strains. Particularly especially, immunocompetent younger adults (Liu and Guo, 2018). Table 1.1 displays the variations between these two categories.

However, there is disagreement on defining hvKp solely based on the string test. Previous studies have suggested that in both laboratory and real-world model settings, certain strains of hmvKp are not substantially linked to increased virulence. Therefore, it may not be adequate to differentiate between hvKp and cKp alone based on the hypermucoviscosity phenotype (Figure 2.1) (Liu and Guo, 2018). It has been shown that the most accurate markers for differentiating between hvKp and cKp strains are those present on the virulence plasmid. The acquisition of conjugal plasmids or transposons containing resistance genes has increased the resistance of hvKp isolates, which were previously vulnerable to antibiotics. Additionally, the hvKp virulence plasmid can now be acquired by cKp strains. The combination of hypervirulence and extreme drug resistance (XDR) possesses the potential to be fatal and extremely harmful (Russo and Gulick, 2019).

The global spread of hvKP and carbapenem-resistant (CRKP) *K. pneumoniae* strains has become more noticeable and alarming in recent times, raising serious concerns for public health both in China and worldwide (Gundogan, 2014; Zhang et al., 2022). One of the known traits that sets apart hvKp is its ability to produce noticeably higher levels of iron-acquisition molecules, notably siderophores, in contrast with strains of cKp. Although hvKp can produce four distinct siderophores (enterobactin, salmochelin, yersiniabactin, and aerobactin), it is important to note that aerobactin accounts for more than 90% of siderophore production overall (Russo and Gulick, 2019). In the 1980s, the first evidence of the link between aerobactin and pathogenicity emerged. However, recent results have directly demonstrated the critical role that aerobactin—

which is different from the other siderophores—plays in the ex vivo growth and survival of hvKp as well as in the virulence that hvKp exhibits in in vivo settings (Figure 2.1).

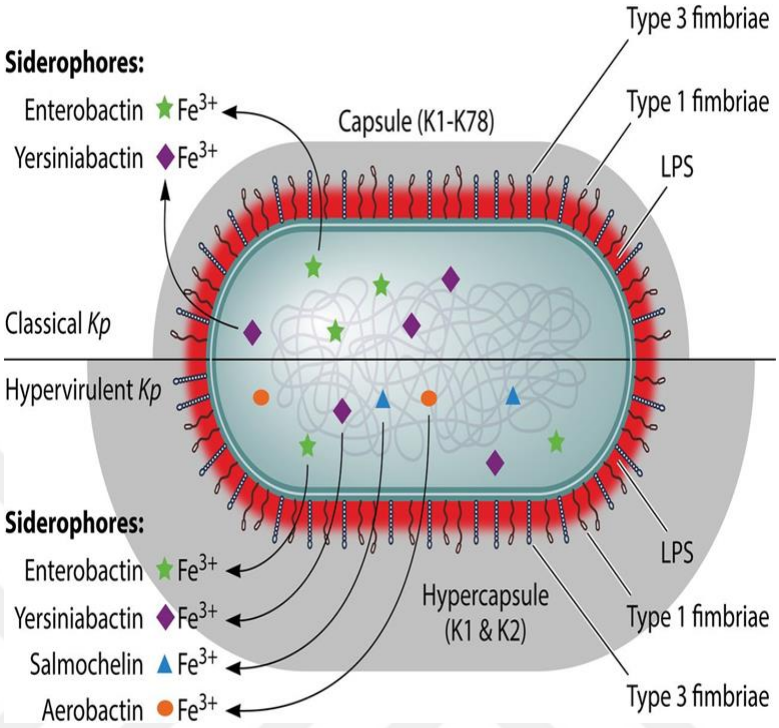

Figure 2. 1. Virulence factors of *K. pneumoniea* (Paczosa and Mecsas, 2016).

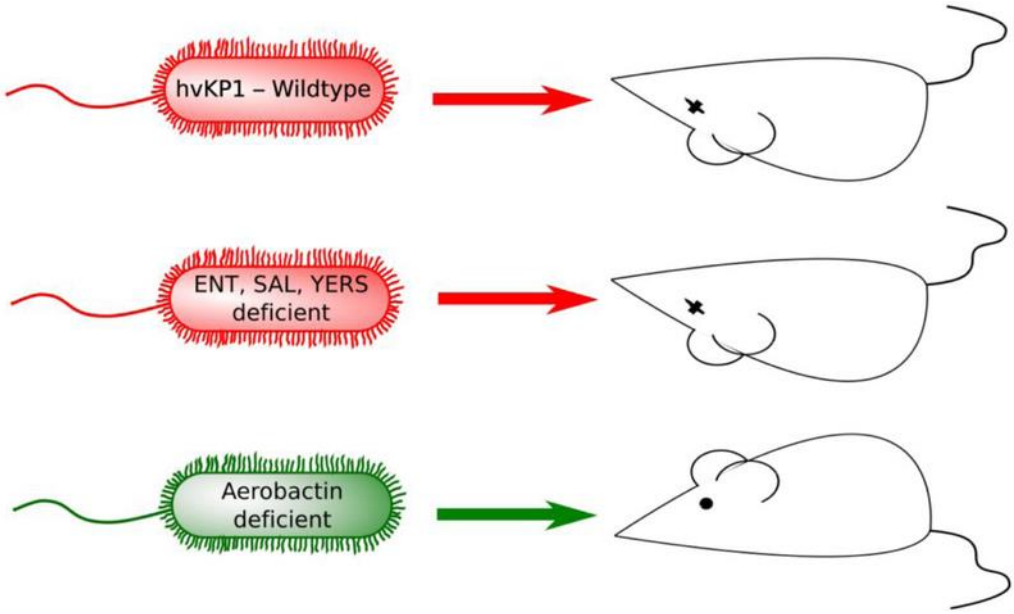

Figure 2. 2. hvKp exhibits siderophores in vivo and in the ex vivo settings (Russo and Gulick, 2019).

Table 1. 1. Main characteristic of cKP, hvKP, and MDR-hvKP (Zhu et al., 2021)

Characteristics	cKP	hvKP	MDR-hvKP	References
Infections	Nosocomial acquisition, predominantly in immunocompromised patients globally. Infectious sites include urinary tract, pneumonia, and bloodstream infections, often polymicrobial. Metastasis is uncommon.	community Host: healthy adults,' Southeast Asia Infectious sites: pyogenic liver abscess, meningitis, endophthalmitis, necrotizing usually monomicrobial at sites of infection Metastasis: Common	nosocomial and community Host: usually immunocompromised patients, in Asia (especially China) Infection sites: pyogenic liver abscess, bloodstream infections, urinary-tract infections	Harada et al., 2019; Russo and Marr, 2019; Liu C. et al., 2020; Tang et al., 2020
Phenotypes	Non-hypermucoviscosity and string < 5mm	Hypermucoviscosity and string ≥ 5mm	Hypermucoviscosity or non-hypermucoviscosity	Russo et al., 2018
Common serotypes	K1–K79	K1, K2, K5, K16, K20, K54, K57, KN1	k1, K2, K16, K20, K54, K62, K64, K47	Pan et al., 2008, 2015; Yang et al., 2021
Siderophores	Enterobactin, yersiniabactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Enterobactin, yersiniabactin, salmochelin, and aerobactin	Russo et al., 2015; Lam et al., 2018b; Choby et al., 2020

cKp: Classical *K. pneumoniae,* hvKP: Hypervirulent *K. pneumoniae*; MDR-hvKP: Multidrug-resistant hvKP

2.2. Virulence factors of *K. pneumoniae*

As shown in Table 1.2, the virulence of *Klebsiella* is linked to a number of substances, including capsules, adhesins, LPS, extracellular toxins such as cytotoxins and enterotoxins, hemolysins, and siderophores. In addition, findings by Samanta and Bandyopadhyay (2020) have shown a strong correlation between serum resistance and the ability of several Gram-negative bacteria to spread in the human bloodstream.

Table 1. 2. Virulence factors and genes produced by *Klebsiella* (Samanta and Bandyopadhyay, 2020).

Virulence factors	**Function**
Capsule	It prevents phagocytosis. Serum resistanceIt inhibits the differentiation and functional capacity of macrophages in vitro.
Mucoviscosity-associated gene A (*magA*)/*wzyKpK1*	It helps in capsular polysaccharide formation and it is associated with *K. pneumoniae* strains causing primary liver abscess.
rmpA	It is associated with hypermucoid strains causing pyogenic liver abscess (PLA).
Pili/fimbriae (a) Type I pili (b) Type III pili (c) KPF-28 fimbriae	The pili help in binding of the bacteria to mucus or the epithelial cells of the urogenital, respiratory and intestinal tracts. Type 1 fimbriae were found to be associated with experimental urinary tract infection in mice. Type 3 fimbriae were detected to promote biofilm formation.
Adhesin: CF 29K (R-plasmid-encoded) of *K. pneumoniae*	It helps to mediate adherence to the human intestinal cell lines.
Lipopolysaccharide (LPS)	In some strains, O-side chain of LPS produces steric hindrance of lytic complement action (MAC formation) and promotes serum resistance.
Enterobactin, aerobactin, yersiniabactin, Kfu/phosphoenolpyruvate sugar phosphotransferase system (PTS), haemin and transferrin transporters	Acts as siderophore to help in iron acquisition. Enterobactin is necessary for penetration to deeper tissues. Yersiniabactin allows the growth of bacteria in the airways
celB	The gene encodes putative cellobiose-specific PTS. It is associated with PLA-caus.
allS	It is detected in K1 strains of *K. pneumoniae*. It helps in anaerobic metabolism of allantoin which acts as the source of carbon, nitrogen and energy under aerobic or anaerobic condition.

2.2.1. Capsule

A crucial component of *K. pneumoniae*'s pathogenicity linked to its viscous phenotype is the capsule that envelops the bacteria's surface (Figure 2.1). The polysaccharide structure of capsule assists in colonization, adhesion, and survival.

Furthermore, resistance to phagocytosis, desiccation, destroying human serum, and phage attacks is conferred. According to Gundogan (2014), *Klebsiella* species, such as *K. pneumoniae,* establish complex acidic polysaccharide capsules that facilitate adhesion and colonization in the urinary and respiratory tracts.

Based on differences in structures and antigens, as well as the diversity of polysaccharide components in the capsule, *K. pneumoniae* can be divided into at least 79 serotypes (Pan et al., 2015; Paczosa and Mecsas, 2016). Eight distinct capsule types have been discovered in hvKP; K1, K2, K5, K16, K20, K54, K57, and KN1 (Zhu et al., 2021). hvKp are most commonly associated with the K1 capsule locus, which is followed by K2, K5, and K57 capsule types. It is crucial to remember that capsule types do not cause hypervirulence on their own because they frequently coexist with other virulence genes.

The K1, K2, K5, and K57 capsule types do not include repeated sugars that cause phagocytosis, in contrast to other capsule types like K1 and K2. Thus, these kinds of capsules aid in avoiding lectinophagocytosis and killing macrophages. In comparison to cKp isolates, studies have demonstrated that K1 isolates are more resistant to both external and intracellular neutrophil killing. According to Choby et al. (2020), this enhanced resistance may inhibit the body's ability to respond to extracellular antibiotics and assist in the infection's spread.

As virulence factors, capsules are essential because they assist in developing the mucoid phenotype as appears in *K. pneumoniae*. The plasmid gene *rmpA* (regulator of mucoid phenotype A; AB289644) causes *K. pneumoniae* to produce more capsular polysaccharides, resulting in to a hypermucoviscous phenotype. A string test, that produces lengthy mucous strings (more than 5 mm) when colonies are touched with a bacteriological loop, is a simple way to identify *K. pneumoniae* strains that are hypermucoviscous (Derakhshan et al., 2016).

Mice infected with capsular strains of *K. pneumoniae* were found to have a significantly longer life time and reduced bacterial density in their bloodstream and lungs when compared to those infected with non-capsular strains in an intranasal infection model. This implies that the capsule might offer defense against immune reactions. Rather of being aggressive, the capsule's resistance to bactericidal effects is mostly defensive. For example, by blocking bacterial binding, the capsule enables *K. pneumoniae* to evade phagocytosis, complement, antimicrobial peptides, and some

antibodies. Nonetheless, it is uncommon to witness active immune cell attack and inhibition caused by the capsule (Zhu et al., 2021).

Whether the *K1* and/or *K2* capsule types confer higher virulence in contrast to non-*K1/K2* types is the focus of several research. The results of these studies have been inconsistent, with some indicating that the *K1/K2* groups have a lower rate of diabetes mellitus and higher metastatic spread. On the contrary, it is clear that non-*K1/K2* hypervirulent strains of *Klebsiella pneumoniae* (hvKp) can cause serious illness, metastatic dissemination, and multisite infection. Furthermore, the hypervirulent phenotype is not observed in *K1/K2* capsule type classical *K. pneumoniae* (cKp) strains unless there is an increase in capsule synthesis and/or the presence of additional hvKp virulence factors. Consequently, hypervirulence is not restricted to specific capsule types, however capsule type may affect the overall virulence potential of hvKp (Russo and Marr, 2019).

A chromosomal operon known as *cps* includes the genes that generate capsules in both conventional and hypervirulent strains of *K. pneumoniae*. As compared to *E. coli*, this cluster of *cps* genes shows conservation in both gene organization and sequencing. As shown in Figure 2.4, a number of genes within the *cps* gene cluster are involved in capsule synthesis. These genes include *wzi*, *wza*, *wzb*, *wzc*, *gnd*, *wca*, *cpsB*, *cpsG*, and *galF* (Paczosa and Mecsas, 2016). *K. pneumoniae K* antigens have historically been identified by serological techniques. However, sequencing the *wzi* locus has become a common method for K-antigen typing in recent years.

All capsular *K. pneumoniae* strains contain the *wzi* locus and specific genes within this locus are strongly linked to different *K* antigens. A surface protein that is involved in the capsule's attachment to the outer membrane is encoded by the *wzi* gene. A bacterium without a capsule is the result of losing this protein. While *wza*, *wzc*, *orf5*, and *orf6* assist in the formation of capsules on the bacterial surface, other genes, such *wzy* (*orf4*), are engaged in the polymerization of capsular polysaccharides (Paczosa and Mecsas, 2016; Zhu et al., 2021).

Subsequently, three *rmpA/A2* gene alleles that effectively stimulate the expression of the *cps* gene were discovered. These alleles appear differently in different geographic areas and serotypes. Three alleles are chromosomal genes, while two are plasmid-borne. A DNA binding domain found in the C-terminal region of *R mpA* suggests that it plays a role in binding to the promoter regions of *cps* genes and activating them. *K. pneumoniae* has a hypermucous phenotype in response to

excessive *RmpA* synthesis, and *RmpA* elevates the expression of *cps* promoter regions. *K. pneumoniae* exhibits decreased synthesis of capsular polysaccharides in an iron-rich environment. In *K. pneumoniae*, the Fur protein restricts the transcription of *rmpA* and other genes linked to iron uptake. Thus, the expression of virulence genes, in particular those that produce capsules in clinical isolates, can be impacted by the amount of iron present in the environment (Paczosa and Mecsas, 2016).

The cps gene cluster include the mucoviscosity-associated gene (*magA*), which codes for a polymerase required for *K1* biosynthesis. A similar allele was first discovered to be present in additional *K* serotypes, according to current data, and was initially utilized for the PCR-based identification of *K1*-positive strains. The renaming of the *K1* serotype-specific allele as *wyz_K1* raises the possibility that *wyz* alleles are serotype-specific yet functionally equivalent (Clegg and Murphy, 2016).

By transferring mannose to fucose, the *magA* gene stimulates the formation of capsules and may improve the bacteria's resistance to being phagocytosed by macrophages. Plasmid-based, the *rmpA* gene promotes the formation of capsular polysaccharides and is linked to the hypermucoviscous phenotype. The *magA* and *rmpA* genes initially associated with invasive infections (Jazayeri Moghadas et al., 2018).

There are a number of ways in which hypervirulent *K. pneumoniae* strains can produce more capsules than average. One method involves the expression of two transcriptional regulators encoded by the virulence plasmid pKP100, namely *RmpA* and *RmpB* (Choby et al., 2020). Furthermore, the expression of both *rmpA2* and the chromosomal copy of the regulator of mucoid phenotype A (*rmpA*) gene can increase the production of capsules. Furthermore, enhanced capsule production may also be affected by the control of the genes responsible for capsule synthesis, *A* and *B* (*rcsA* and *rcsB*). This is also possible for capsule synthesis to increase in response to external stimuli. For example, high glucose levels cause the *RmpA*-mediated upregulation of capsule formation, whereas increased extracellular iron concentrations cause the downregulation of capsule production. It is noteworthy that a significant number of HV *K. pneumoniae* strains (between 55% and 100%) exhibit one or more copies of *rmpA* or *rmpA2*, whereas non-HV *K. pneumoniae* strains show a lower frequency of this expression (between 7% and 20%) (Lai et al., 2003; Li et al., 2014).

The association between hypermucoviscosity (HMV) and capsule in HV *K. pneumoniae* is important, however it is currently being investigated. Through recent

genomic study, *RmpC*, a novel transcriptional regulator, was discovered in an HV *K. pneumoniae* strain. *RmpC* primarily controls capsule synthesis, whereas *RmpA* seems to coordinate HMV and capsule production. According to Choby et al. (2020), a mutant lacking *RmpC* shows decreased capsule formation but maintains hypermucoviscosity.

In addition, the production of the capsule is dependent on a critical transcription factor termed *RfaH*. *RfaH* inhibits transcription termination, which contributes to the activation of the cps operon. *KvrA* and *KvrB*, two recently discovered regulators, have an impact on the virulence of *K1/K2* hvKP strains as both can trigger the production of capsule genes, a characteristic that is lacking in cKP strains. It's important to note that, opposed to the mechanism linked to *KvrA*, *KvrB*'s influence on capsule expression might depend on how it acts at the *rmpA* promoter, as noted in the research by Zhu and colleagues (2021).

Even in the absence of *rmpA* or *rmpA2*, chromosomal mucoviscosity-associated gene A (*magA*) can cause the development of hypercapsules (Yu et al., 2006). While aiming to identify the genes necessary for the hypercapsule characteristic in *K. pneumoniae* strains acquired from cases of invasive liver disease, the gene *magA* was discovered in 2004. In particular, *magA* was found in just 29% of the strains linked to noninvasive cases, while it was found in 98% of the strains linked to invasive infections (Fang et al., 2004). Further studies showed that *magA* is distinct to *K1* strains and that it performs a similar polymerase function to *wzy*, being critical to the synthesis of the capsule. Later studies published that *magA* was formally renamed as *wzy_K1*, and different serotypes contain different forms of *wzy*. For example, a particular *wzy* variation linked to *K2* strains has been suggested to (Paczosa and Mecsas, 2016).

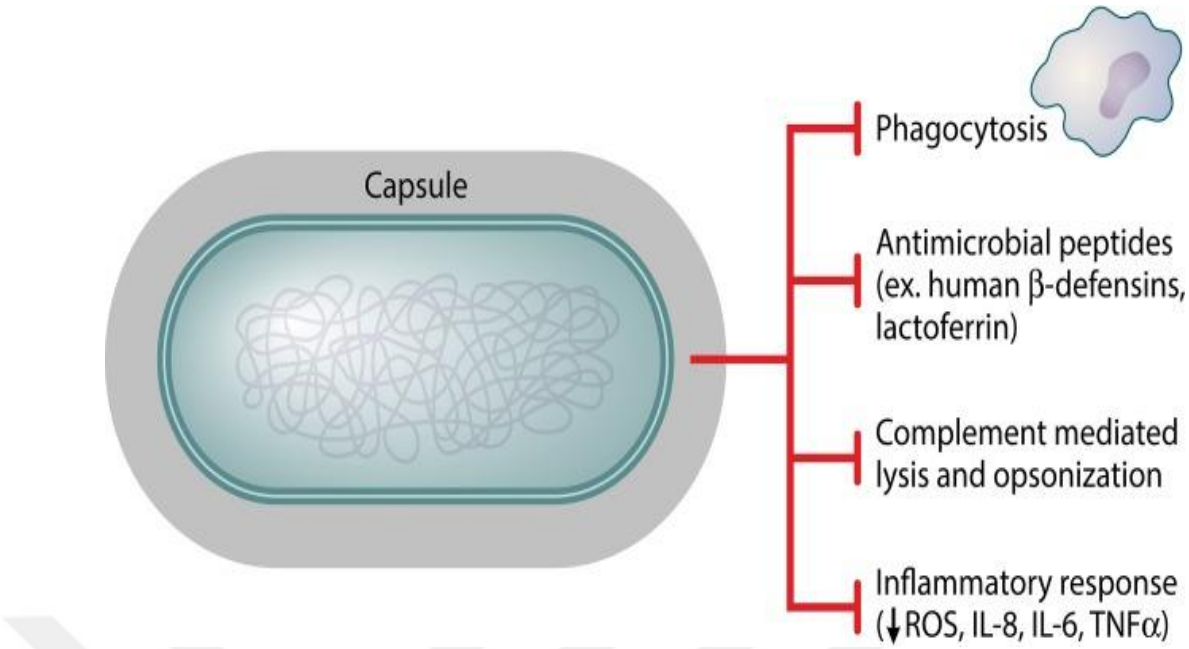

Figure 2. 3. The roles of capsule in *K. pneumoniae* virulence (Paczosa and Mecsas, 2016).

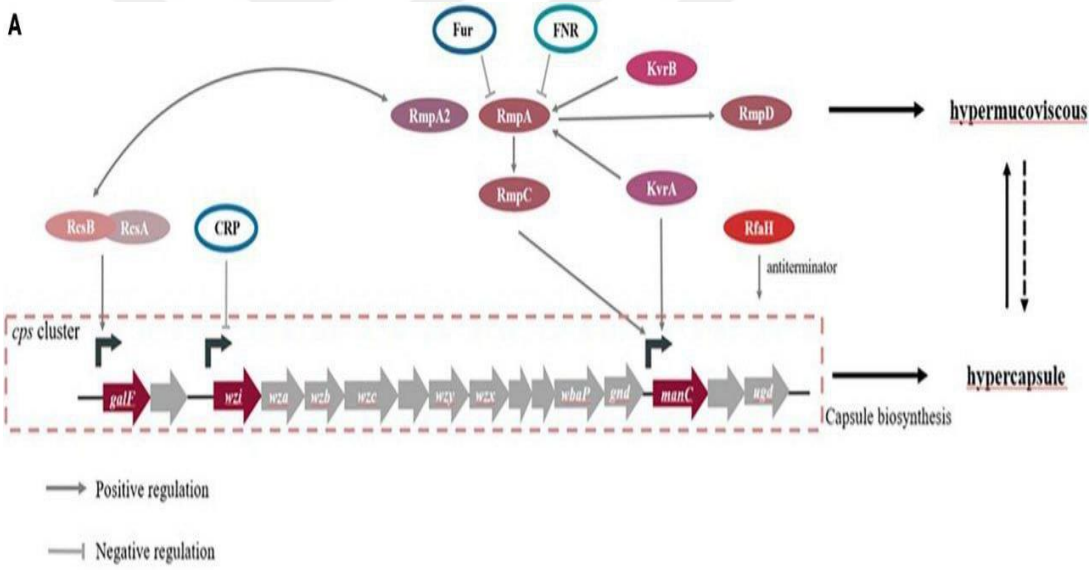

Figure 2. 4. Genes within the *cps* gene cluster which involved in capsule synthesis (Zhu et al., 2021).

2.2.2. Type 1 and 3 Fimbriae

Additionally, several species of *Klebsiella* produce a class of virulence factors named fimbrial adhesins, which are protein structures with the ability to detect different molecular patterns. They make it possible for the bacteria to attach themselves to certain host tissue surfaces. According to Gundogan (2014), this first attachment to mucosal and epithelial surfaces is frequently the first step in the

formation of colonization and infection. Analyzing the genome sequences of several *K. pneumoniae* strains online indicates the presence of multiple gene clusters that may encode fimbrial and non-fimbrial adhesion factors, which is consistent with other *Enterobacteriaceae* family members. It appears that the majority of enteric bacteria typically have these many gene clusters. Nevertheless, thorough investigations on the functions of most of these genes and the products they produce have only examined a small number of variables. As an example, the *K. pneumoniae* genome comprises up to eleven putative fimbrial gene clusters, although as Clegg and Murphy (2016) have shown, only a small number of these have been connected to certain adherence traits.

Two unique kinds of fimbrial adhesins, known as type 1 and type 3 fimbriae, are typically produced by the majority of clinical *K. pneumoniae* isolates (Figure 2.5). Inducing mannose-sensitive hemagglutination, type 1 fimbriae play a major role in urinary tract infections. As described by Gundogan (2014), type 3 fimbriae, on the other hand, are classified as mannose-resistant *Klebsiella*-like fimbriae and are well-known for their ability to agglutinate tannin-treated erythrocytes.

A minimum of two genes—one encoding a periplasmic chaperone and the other encoding a scaffolding protein—are present in fibriae synthesized via the chaperone/usher pathway. There may be more than one periplasmic chaperone-encoding gene in some gene clusters, but the scaffolding or usher protein-producing gene is always present in only one gene cluster. These proteins mediate donor-strand complementation and donor-strand exchange, which facilitate the formation of a functional fimbrial structure (Clegg and Murphy, 2016). On the surface of bacteria, type 1 fimbriae are thin, stiff structures that resemble hairs. Their assembly is facilitated by chaperones and intercellular routes, and their creation is regulated by the *fim* gene cluster. The small-terminal adhesin subunit is encoded by the *fimH* gene within this cluster, whereas the major structural component is encoded by the *fimA* gene.

Notably, *Escherichia coli* lacks the *fimK* gene, which is unique to *K. pneumoniae*. *FimK* is essential for controlling type 1 fimbriae; when it is absent, type 1 fimbriae expression is unsuccessful (Riwu et al., 2022). Type 1 fimbriae are found in almost all Enterobacteriaceae members and are present in approximately 90% of clinical and environmental *K. pneumoniae* isolates (Schembri, 2000; Stahlhut et al., 2009).

Additionally, the spiral assembly of type 3 fimbriae is caused by the *mrkABCD* gene cluster, which might be based on a plasmid or a chromosome. In the meantime, the type 1 and type 3 fimbriae-associated genes *fimH* and *mrkD* each have functions in adhering to host cells (Riwu et al., 2022). These factors are known to play an aggressive role and are responsible for the start of invasion, colonization, and pathogenicity.

Surprisingly some research has shown the rise of hypervirulent, multi-drug resistant *K. pneumoniae* isolates, including those that are resistant to carbapenem and this raises serious concerns for public health (Guo et al., 2017; Zhang et al., 2016). These specific fimbriae are also essential for *K. pneumoniae* to form biofilms, adhere to tissues, and bind with affinity. While *MrkD* can adhere to the extracellular matrix, *MrkA* can attach to non-biological surfaces such as medical equipment both before and after it is placed within the patient's body (Sugawara et al., 2016; Riwu et al., 2022).

Studies using *K. pneumoniae CG43* as a model have shown that iron levels influence type 3 fimbriae activity and biofilm development. *fur* regulates this process favorably, revealing that type 3 fimbriae may not be as important in hvKP infections in vivo when iron levels are low. According to recent research, serotype *K1* hypermucoviscous isolates have reduced initial adhesion because the hypercapsule may cover the type 3 and type 1 fimbriae, even though they have the *mrkD* and *fimH* genes (Zhu et al., 2021).

Through two different procedures, type 3 fimbriae help *K. pneumoniae* colonize endotracheal tubes and cause lung infections. *MrkA* directly binds to plastic surfaces, while *MrkD* makes it easier for adhesion to surfaces coated with collagen or extracellular matrix made from bronchial cells (Paczosa and Mecsas, 2016).

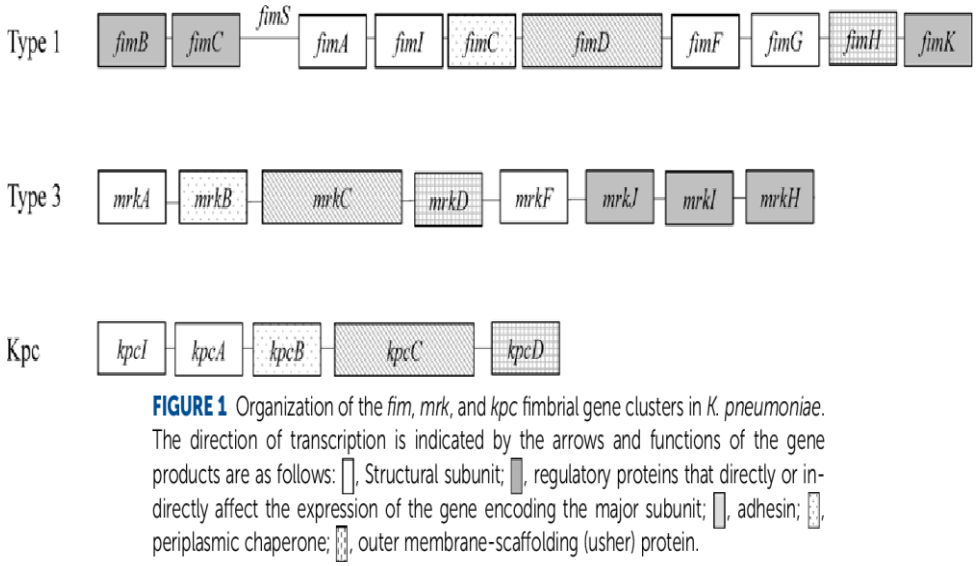

FIGURE 1 Organization of the *fim*, *mrk*, and *kpc* fimbrial gene clusters in *K. pneumoniae*. The direction of transcription is indicated by the arrows and functions of the gene products are as follows: , Structural subunit; , regulatory proteins that directly or indirectly affect the expression of the gene encoding the major subunit; , adhesin; , periplasmic chaperone; , outer membrane-scaffolding (usher) protein.

Figure 2. 5. Type 1 and type 3 fimbriae clusters genes

2.2.3. Siderophores

For both hosts and bacteria, iron is a necessary element since it is involved in many important metabolic processes. As a sort of broad immune defense, however, the limited amount of iron in extracellular fluid poses a problem for bacterial iron uptake. Bacteria need to find ways around this iron acquisition obstacle in order to survive and multiply (Zhu et al., 2021). *K. pneumoniae* depends on iron, a scarce but necessary material, to reproduce during infection. But as part of the non-specific immune response, the host's defense mechanisms, such sequestration, work to restrict iron's availability, making it less available to possible pathogens (Bullen 1972, Carniel 2001).

Host plasma usually has very little free iron because it is bound by iron transport molecules such as transferrin. Mammalian hosts can further reduce the availability of iron in response to bacterial infections by shifting iron binding to lactoferrin, an inherent defense protein found in body fluids. As a result, *K. pneumoniae*, like many other bacterial pathogens, must use techniques to obtain iron from the host in order to maintain its survival and growth while infecting mammals (Bullen 1972, Miethke and Marahiel 2007; Paczosa and Mecsas 2016). *Klebsiella* produce four siderophore which are enterobactin, yersiniabactin, salmochelin, and aerobactin Figure 1.1.

Ferric iron is transported into bacterial cells via a series of proteins called *ATP-binding cassette (ABC)* transporters, *TonB*, and *Exb*. An outer-membrane receptor that is dependent on *TonB* recognizes the iron-siderophore complex, allowing for simpler

diffusion into the periplasm. Following its binding to a periplasmic protein, the siderophore aids in the transport of iron to an *ABC* transporter, which subsequently allows the reduction of ferric iron to ferrous iron and its transit through the inner membrane. Although there is still much to learn about the specific function of iron-chelating systems in uropathogenic *K. pneumoniae*, bacteremic investigations and airway infection models show that mutants with lower iron absorption capacity are less virulent (Clegg and Murphy 2016; Zhu et al. 2021).

Enterobactin is a crucial and primary factor for iron acquisition in both cKP and hvKP. Among the four siderophores, it has the largest affinity for *Fe3+* due to its three catechol rings (Bachman et al. 2012; Palmer and Skaar 2016; Zhu et al. 2021). The *ent* cluster on the chromosome contains the genes that produce enterobactin, and *fepA* encodes the receptor for this molecule (Zhu et al. 2021). While the expression of the other siderophores varies significantly, enterobactin is almost always present in both traditional and HV *K. pneumoniae* strains. As a result, it is thought to be *K. pneumoniae'*s main iron uptake mechanism (Pedschun et al. 1993; El Fertas-Aissani et al. 2013).

Remarkably, hvKP strains were found to produce more siderophores than cKP strains when siderophores were quantitatively measured in them (Russo et al. 2011, 2014; Russo and Marr 2019). Indeed, in a mouse model of systemic infection, a total siderophore concentration greater than 30 μg/ml was associated with increased mortality and served as a powerful signal of an isolate being hvKP. Additionally, even though aerobactin can make four distinct siderophores, it accounts for over 80–90% of total production (Russo and Marr 2019; Zhu et al. 2021).

One important regulatory protein that adjusts gene expression in response to iron levels is the ferric-uptake regulator, or *Fur*. When iron levels are high (iron-replete circumstances), *Fur* can bind to particular DNA sequences called the *Fur* box found in the promoter regions of genes that control iron. The target genes' transcription is suppressed as a result of this binding, and de-repression happens when iron is in short supply. *Fur* thus acts as a comprehensive regulator of enterobacteria's iron-regulated genes. *Fur* has been shown to affect the expression of genes involved in iron acquisition systems and capsule production in *K. pneumoniae*. For example, take into account the *Fur-regulated* genes *rmpA2* and *rcsA*, as well as at least six of the eight genes linked to iron uptake. In *K. pneumoniae*, deletion mutants of *Fur* show changes in adhesin synthesis as well as decreased siderophore production. *Fur* therefore has

the ability to affect the expression of several genes in *K. pneumoniae*, several of them encode proteins essential for colonization and growth in the host (Clegg and Murphy, 2016).

The enterobactin (*Ent*) system and frequently yersiniabactin (*Ybt*) on their chromosomes are present in both the hvKp and cKP strains. However, compared to cKp isolates, hvKp isolates produce more siderophores because they frequently create the aerobactin (*Iuc*) and salmochelin (*Iro*) systems (Choby et al., 2020). *K. pneumoniae* strains frequently contain enterobactin, however lipocalin 2 (the host protein) neutralizes its action. Therefore, enterobactin's role in systemic infection is unlikely (Russo and Marr, 2019). Some *K. pneumoniae* strains have developed modified siderophores in a clever way to evade binding by lipocalin-2. Among these are salmochelin, a highly glycosylated enterobactin, and yersiniabactin, a non-catecholate siderophore. Essentially, strains that are conjugated with yersiniabactin and salmochelin obtain the iron required to survive even when lipocalin-2 is present (Bachman et al., 2012; Zhu et al., 2021).

The enterobactin-producing genes in *K. pneumoniae* are located on the chromosome in the *ent-ABCDEF* gene cluster. The proteins that bind it are encoded by the *fep-ABCDG* gene cluster, whereas the uptake receptor is particularly encoded by *fepA*. Interestingly, there is evidence of elevated *fepA* expression during *K. pneumoniae* infection, suggesting a possible increase in enterobactin activity. However, it's important to note that the host-produced molecule lipocalin-2 inhibits enterobactin insignificant (Paczosa and Mecsas, 2016).

Enterobactin that has been c-glucosylated is basically salmochelin. Genes on a chromosome or a plasmid, namely those in the *iroA* gene cluster, *iroBCD*, control this alteration. Notably, this process involves *IroN* and *IroB*, which are similar to glycosyltransferases and the enterobactin receptor *FepA* (Paczosa and Mecsas, 2016; Zhu et al., 2021). Furthermore, strain *NTUH-K2044*'s DNA segment contains a chromosomal form of *iro*, referred to as *ICEKp1* (Zhu et al., 2021). Salmochelin that is packed with iron is transported more easily by the *IroN* protein. This modification is important because it prevents lipocalin-2 from binding salmochelin, which in inhibits siderophores from being neutralized and inflammation caused by lipocalin-2 from occurring. It follows that salmochelin could promote *K. pneumoniae* colonization in the nasopharynx of a host that has enough lipocalin-2. Presumably, patient populations infected with strains carrying salmochelin genes are generally less

immunocompromised, and these strains may exhibit increased virulence, since salmochelin expression allows *K. pneumoniae* to colonize the nasopharynx in hosts capable of producing lipocalin-2. Salmochelin is found in just 2–4% of hospital-acquired *K. pneumoniae* strains, whereas it is significantly more common in hypervirulent *K. pneumoniae* strains, which supports this assumption. Interestingly, more than 90% of hypervirulent *K. pneumoniae* strains linked to pyogenic liver abscesses had it, according to a study (Hsieh et al., 2008; Bachman et al., 2011; El Fertas-Aissani et al., 2013).

Within a genetic area referred to as the Yersinia high-pathogenicity island, the Gram-negative bacterial pathogen Yersinia was first found to harbor Yersiniabactin. However, other bacteria, such as *K. pneumoniae*, have also been found to have this siderophore (Bach et al., 2000). It seems likely that the *ybt* and *fyu* genes control the transport mechanisms that enable the release of this siderophore, while the *irp* genes regulate the proteins required for the synthesis of yersiniabactin. Roughly 90% of clinical isolates of extremely pathogenic *K. pneumoniae* exhibit this pattern (Hsieh et al., 2008).

Although its precise role in *K. pneumoniae* is yet unknown, *ybtQ* specifies the receptor responsible for yersiniabactin intake (Bach et al., 2000; Lawlor et al., 2007 Hsieh et al., 2008; Paczosa and Mecsas, 2016). Remarkably, only roughly 18% of classical strains have yersiniabactin identified. Yeersiniabactin is present in 93% to 100% of hyper virulent *K. pneumoniae* isolates, whereas it is only present in about 6% of conventional *K. pneumoniae* strains (El Fertas-Aissani et al., 2013; Paczosa and Mecsas, 2016; Zhu et al., 2021).

Similarly, aerobactin rather than hypermucoviscosity is a more accurate measure of hvKP. The virulence plasmid contains the genes *iucABCD* and *iutA*, which are involved in the synthesis and transportation of aerobactin. Interestingly, *K. pneumoniae*'s *iuc* locus showed variation in its genetic structure, generating six distinct iuc lineages. With the exception of iuc4, which was located on the chromosome of *K. pneumoniae* subspecies rhinoscleromatis, the majority of these lineages were carried by different plasmids (Zhu et al., 2021).

Aerobactin is invariably associated with the presence of a hypercapsule, however this siderophore is not present in all hypercapsulated strains. The reason for this connection is that the virulence plasmid that likewise includes the enhancer of capsule production, *rmpA*, also contains the aerobactin transporter, *iutA*, and the

aerobactin gene cluster, *iucABCD*. Interestingly, aerobactin may be the main siderophore expressed in particular HV *K. pneumoniae* strains that cause lung infections, even though it is rarely seen in classical strains that cause lung infections (Paczosa and Mecsas, 2016).

2.2.4. Lipopolysaccharide

Like other members of the *Enterobacteriaceae* family, *K. pneumoniae* has three different segments in its LPS structure: Lipid A, core, and O-Ag polysaccharide (see Figure 2.6). The substance known as lipid A, or endotoxin, acts as an anchor to bind the LPS molecule to the outer membrane. It has long been known that endotoxin plays a major role in the pathogenicity of *K. pneumoniae* and other Gram-negative bacteria. Endotoxin can be released during bacterial lysis and cell development, which can then indirectly release a variety of inflammatory mediators or cytokines (Gundogan, 2014).

Lipopolysaccharide (LPS) is a characteristic shared by hypervirulent *K. pneumoniae* (hvKP) and classical *K. pneumoniae* (cKP). Whether the LPS generated by hvKP strains plays a specific role in hypervirulence is still unknown. The O-antigen is the outermost part of LPS and is the pathogen's first point of interaction with the host's innate immune system. Its function is to prevent complement-mediated pathogen killing. In particular, the O antigen blocks the creation of pores that could rupture the bacterial membrane by interacting with complement component C3b (Shankar-Sinha et al., 2004; Zhu et al., 2021). Genes within the *wb*, *waa*, and *lpx* gene clusters determine these elements (De Majumdar et al., 2015; Raetz et al., 2009; Riwu et al., 2022).

Compared to the wide range of 77 known *K* antigens for *K. pneumoniae* capsule, only nine different forms of O-antigens have been identified in isolates of *K. pneumoniae*, with O1 being the most common (Hansen et al., 1999). The O-antigen present in *O1:K2* cKP strains may increase the virulence and mortality of bacteria by inhibiting macrophage activation and promoting bacteremia (Zhu et al., 2021). Hsiehet al. (2012) have stated that the isolates from *Klebsiella* liver abscess (KLA) are more likely than non-tissue-invasive isolates to have the O1 serotype. In addition, KLA mutants with no O1 antigen showed less pathogenicity than their parent strains. However, in *K1* hypervirulent *K. pneumoniae* (hvKP) strains, in which the K1 capsule obscures the O1 antigen, its function remains unclear (Zhu et al., 2021).

Certain strains of *K. pneumoniae* utilize their capsule to partially conceal their lipoprotein from TLR recognition. Studies reveal that bacteria expressing *K1*, *K10*, and *K16* antigens are able to mask their LPS, while strains expressing *K2* antigens are unable to achieve this (Merino et al., 1992). Similar results are seen in hypervirulent *K. pneumoniae* strains, where the hypercapsule minimizes the *TLR4* signal (Paczosa and Mecsas, 2016).

K. pneumoniae demonstrates its flexibility in particular mammalian tissue settings, such as the lungs, by changing its lipid A structure to a 2-hydroxyacyl modification via a LpxO-dependent, PhoPQ-regulated mechanism. This change increases *K. pneumoniae*'s in vivo pathogenicity by decreasing the activation of the inflammatory response in comparison to the native lipid A, similar to other bacteria with modified LPS, such Yersinia (Montminy et al., 2006; Llobet et al., 2015). Furthermore, the lipid A section in the lipopolysaccharide (LPS) of *K. pneumoniae* enhances its pathogenicity. A mutant strain of *K. pneumoniae* with altered lipid A acylation showed decreased pathogenicity in a mouse model of pneumonia. Additionally, studies in living things as well as laboratory tests have shown that lipid A acts as a barrier against specific cationic antimicrobial peptides (Clements et al., 2007; Llobet et al., 2015).

The primary protection against the complement system is lipopolysaccharide (LPS). Often referred to as "smooth LPS," bacterial strains possessing whole O antigen chains exhibit resistance against complement-mediated lysis. However, strains with shortened or incomplete O chains, also referred to as "rough LPS," are still susceptible to complement-mediated death even when they have a capsule (Merino et al., 1992). Because of the LPS O-antigen's protective function, complement-resistant strains are able to participate in the complement cascade without being killed. By attaching itself to C3b, a dual-function complement component involved in opsonization and pore creation, the O antigen protects against C3 and prevents pore formation by keeping it away from the bacterial membrane (Merino et al., 1992; Merle et al., 2015; Shankar-Sinha et al., 2004b).

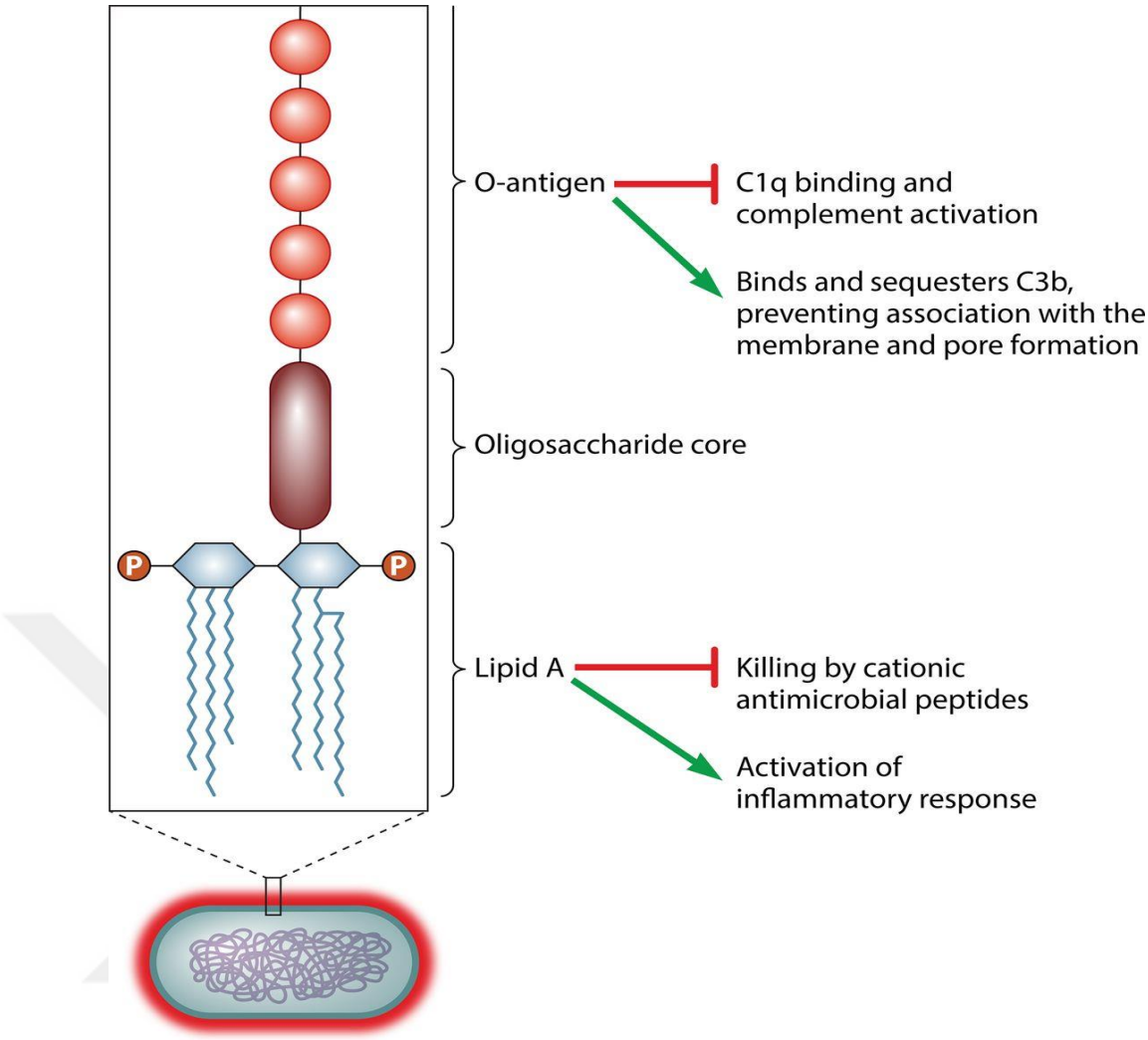

Figure 2. 6. LPS structure: Lipid A, core, and O-Ag polysaccharide (Paczosa and Mecsas, 2016).

2.3. Antimicrobial Resistance

Antibiotic resistance in bacteria is partly caused by the extensive use of antibiotics in animal husbandry. Given the similarities across these antibiotics and those used in human medicine, this is quite concerning. Because *Klebsiella* consistently expresses a chromosomal class A β-lactamase, it is considered inherently resistant to ampicillin, amoxicillin, and carbenicillin but not extended-spectrum β-lactam antibiotics. Extensive use of antibiotics, long stay in an intensive care unit or nursing home, medical equipment, and catheter use are important risk factors (Gundogan, 2014).

In *Klebsiella*, transposons and plasmids are examples of mobile genetic elements that spread antibiotic resistance genes Figure 2.7. Specific recombination genes are carried by integrons in plasmids or transposons, which mobilize genes related to

antibiotic resistance. Superintegrons and antibiotic resistance integrons (*ARIs*) are examples of integrons. Based on the integrase (*intI*) gene, *ARIs* are classified into four classes: Clinical *K. pneumoniae* that produces *ESBLs* often belongs to Class 1, whereas Classes 2 and 3 are uncommon. Class 1 integrons promote diverse resistance phenotypes by linking to resistance to aminoglycosides and β-lactams. The presence of integrons is inversely correlated with resistance to nitrofurantoin and streptomycin (Samanta and Bandyopadhyay, 2020).

According to Choby et al. (2020), there have been two observed convergent events: cKp strains gaining virulence genes or entire virulence plasmids, and hvKp strains acquiring antibiotic resistance genes on their chromosomes or plasmids. Strains of cKp that have virulence genes have the potential to develop into extremely dangerous pathogens. A cKp strain's capacity for virulence is determined by its type and quantity of virulence genes it acquires; distinct gene acquisitions produce differing degrees of pathogenicity. The deadly outbreak in five individuals that resulted from the acquisition of a ~170 kb *pLVPK*-like plasmid by a *ST11* cKp strain serves as an illustration of the serious consequences of such acquisitions. This epidemic underscore the destructive effect of acquiring full virulence plasmids (Gu et al., 2018).

Extended-spectrum beta-lactamases (*ESBLs*) are a major factor in the resistance of *Klebsiella* isolates to beta-lactam antibiotics, such as cephalosporins, monobactams, and penicillins, but not cephamycins and carbapenems. Resistance to other antibiotic families, including trimethoprim-sulfamethoxazole, aminoglycosides, and fluoroquinolones, is frequently linked to *ESBLs*. The *TEM* and *SHV* beta-lactamase families account for the majority of *ESBLs*, yet more recently, beta-lactamases from the *CTX-M*, *PER*, and *KPC* families have also been discovered (Gundogan, 2014).

K. pneumoniae was found to have resistance patterns during a 14-month period in Beijing, China between 2010 and 2011. Among them, 61.4% were multidrug-resistant (MDR), 22% were extensively drug-resistant (XDR), and 1.8% were pandrug-resistant (PDR). Most MDR *K. pneumoniae* isolates were resistant to quinolones and aminoglycosides, produced carbapenemase (KPC) and/or extended-spectrum β-lactamase (ESBL), and belonged to certain clones such as *CC258* (which includes *ST258, ST11, ST512, ST340,* etc.), *CC15*, and *CC14*. Drug-resistant and hypervirulent *K. pneumoniae* populations have historically remained apart, however some drug-resistant hypervirulent *K. pneumoniae* cases have recently been reported (Hennequin and Robin, 2016).

The expression of genes linked to antibiotic resistance has been found to be regulated by a possible set of regulatory variables that was identified using bioinformatic research. Using this method, scientists have identified *RarA*, an *AraC*-type regulator that regulates the expression of the *oqxAB* operon, which codes for an efflux pump in *Klebsiella pneumoniae*. A multidrug-resistant (MDR) phenotype is produced by overexpressing *RarA*, suggesting that a functioning efflux pump is required for this resistance. Notably, *RarA*'s regulatory effects extend beyond *K. pneumoniae* as it is also projected to be present in the genomes of *Enterobacter* and *Serratia* species. Thus, *RarA* becomes one of the transcriptional regulators that have been shown to regulate the activity of the *AcrAB* efflux pump, along with *SoxS*, *RamA*, *MarA*, and *Rob* (Veleba et al., 2012).

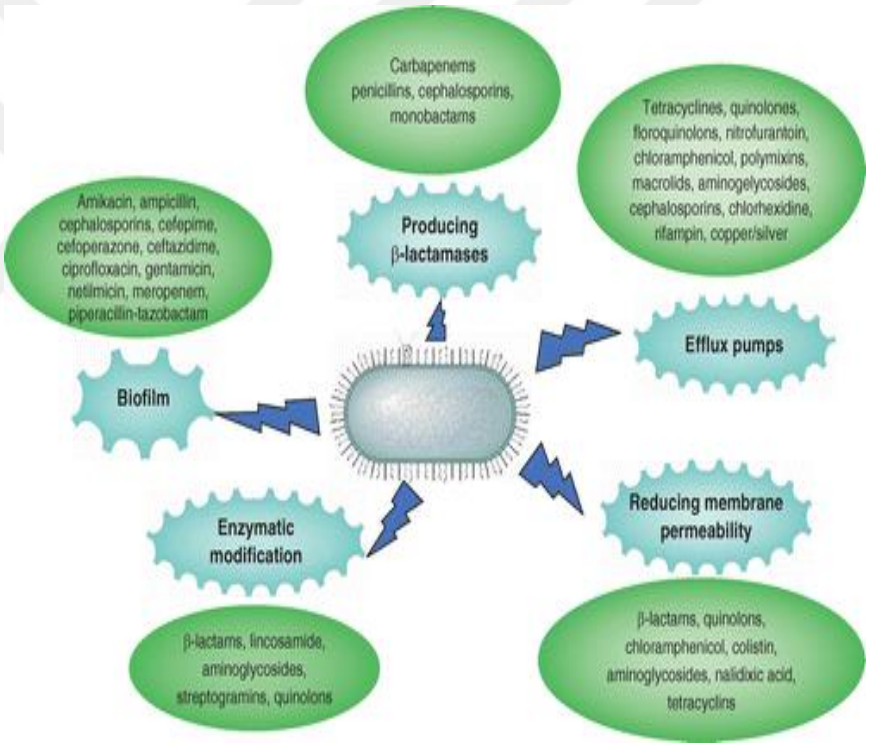

Figure 2. 7. β-lactamase synthesis, efflux pumps, enzymatic modification, biofilm formation, and decreased membrane permeability are the main mechanisms by which *K.pneumoniae* develop resistance to antibiotics (Karami-Zarandi et al., 2023).

2.3.1. Resistance to Beta lastimase/ beta lactamoes inhibitors

Penicillins, cephalosporins, monobactams, carbapenems, and β-lactamase inhibitors are among a number of commonly used antibiotics that fall under the class of β-lactamase antibiotics. The chemical structures of these antibiotics have a similar β-lactam ring. The synthesis of β-lactamase enzymes is the first step *in K. pneumoniae*'s major resistance mechanism against β-lactams. This resistance mechanism can then involve changes in membrane permeability and active ejection via efflux pumps (Hennequin and Robin, 2016).

Suicidal effect is the mode of action for β-lactamase inhibitors such as clavulanic acid, sulbactam, and tazobactam. Within their structures, these inhibitors have a similar β-lactam ring. To be more precise, ESBLs are among the many class A β-lactamases that clavulanic acid targets. Sulbactam and tazobactam, on the other hand, have a wider range of action and block both class C cephalosporinases and class A β-lactamases. The efficiency of these inhibitors, which include clavulanic acid, sulbactam, and tazobactam, against serine carbapenemases and metallo-β-lactamases is insufficient. Other β-lactamase inhibitors that do not have a β-lactam ring but are nevertheless efficient at blocking β-lactamases include avibactam, vabrobactam, and relebactam. Among them, avibactam exhibits activity against all of the β-lactamases that *K. pneumoniae* produces, with the exception of metallo-β-lactamases such as *IMP*, *NDM*, and *VIM* enzymes (Karami-Zarandi et al., 2023).

A broad spectrum of β-lactam antibiotics, including cephalosporins, penicillins, cephamycins, monobactams, and even β-lactamase inhibitors like clavulanic acid, can cause resistance in organisms that produce AmpC β-lactamase (source: "Scientific Opinion on the Public Health Risks of Bacterial Strains Producing Extended-Spectrum β-Lactamases and/or AmpC β-Lactamases in Food and Food-Producing Animals," 2011).

2.3.2. Extended-spectrum β-lactamases (ESBLs)

Since *Klebsiella pneumoniae*'s chromosome encodes *SHV-1* β-lactamase, the bacteria naturally exhibit resistance to ampicillin and carbenicillin. The first extended-spectrum β-lactamase (ESBL) that could hydrolyze oxyimino-cephalosporins was discovered in the early 1980s (Hennequin and Robin, 2016). Since then, *ESBLs* have been found in most genera of the *Enterobacteriaceae* family, including *Klebsiella*, and

around the world. Three groups include the most common *ESBLs*: *TEM*, *SHV*, and *CTX-M* (Samanta and Bandyopadhyay, 2020).

Since then, the development of *ESBLs* has been the main factor contributing to resistance to third-generation cephalosporins. By hydrolyzing antibiotics, these enzymes—which are usually transferred by plasmids—provide resistance against penicillins, first-, second-, and third-generation cephalosporins, as well as aztreonam. β-lactamase inhibitors can suppress them, although they are unaffected by cefoxitin and carbapenems. Furthermore, they are frequently carried on plasmids that contain extra resistance mechanisms, including as resistance to aminoglycosides, cotrimoxazole, and fluoroquinolones (Hennequin and Robin, 2016).

Since *K. pneumoniae* was first linked to penicillin resistance in the 1960s, the pathogen has experienced significant evolution. As a result of acquiring many ESBL elements, it is now known to be a major source of nosocomial infections. The first ESBL in *K. pneumoniae* was identified as the *SHV-2* enzyme in 1983. *TEM-3* was later discovered in 1987. Plasmid-borne *TEM* and *SHV* enzymes were the main ESBL variations found in clinical *K. pneumoniae* isolates for the next ten years as the bacteria continued to accumulate ESBL elements (Karami-Zarandi et al., 2023).

2.3.3. Carbapenemases

While the impact of *ESBL* generation on clinical outcome is still debatable, KP carbapenemase (KPC) is the most prevalent carbapenemase in KP strains and is a well-known independent risk factor in the early mortality of KP infections (Kim et al., 2019). Research on carbapenem resistance mechanisms to date indicates that the most common mechanism of carbapenem resistance in *K. pneumoniae* is the production of carbapenemase (KPC), which is encoded by the plasmidic gene *blaKPC*. The rapid dissemination of this KPC has usually been caused by the clonal expansion of clonal complex (*CC*) 258 strains, including *ST258* and *ST512* (Bowers et al., 2015; Fasciana et al., 2019; Pitout et al., 2015).

Samanta and Bandyopadhyay (2020) reported that KPC genes are present on transposons (based on *Tn3*), plasmids with different sizes, and incompatibility groups in clinically relevant *K. pneumoniae* clonal types (*ST 258*). The first KPC-positive *K. pneumoniae* in Italy, which belonged to *ST258* strain, was discovered in Florence in 2008. It has been clear ever since that these strains are spreading. Italy is actually an endemic country for CR-Kp due to the fact that the most recent European

Antimicrobial Resistance Surveillance Network study indicated an average incidence of up to 33.9% (Fasciana et al., 2019).

Clonal complexes of hvKP and MDR strains are thought to be non-overlapping at the moment. In the past ten years, however, there have been reports of epidemic hvKP strains acquiring multiple antibacterial resistance. These strains include a hvKP *ST23K1* clone that harbors the *blaKPC-2* gene in Argentina, the *blaCTX-M-3* gene in France, and the *blaCTX-M-15* gene in South Korea; a hvKP *ST1797K1* clone that acquired the *blaKPC-2* carbapenemase gene in China; and a hvKP *ST86* clone that acquired the cephalosporinase gene *blaCTX-M-14/15* from Australia (Lev et al., 2018).

The percentage of invasive *K. pneumoniae* isolates that demonstrated resistance to carbapenems and third-generation cephalosporins was between 5 and 10% and 25 to 50%, respectively, based on EARS-Net data for Portugal in 2017 (Carvalho et al., 2020). The most prevalent class A enzyme variation in *Enterobacteriaceae* include *K. pneumoniae* carbapenemases (KPC), class *D OXA-48*, and the metallo-β-lactamases *NDM*, *IMP*, and *VIM*. In 1991, the carbapenemase of *K. pneumoniae* was initially identified as *IMP-1* metallo-enzyme. Furthermore, during the course of the following years, various carbapenemases, including *KPC, OXA-48, NDM*, and *VIM*, were identified in isolates of *K. pneumoniae*. A *K. pneumoniae* strain containing *OXA-48* was identified in Turkey in 2001 and proved to be resistant to all β-lactam antibiotics, marking the first instance of this resistance. Though their carbapenemase activity is less, *OXA-48*-like β-lactamases exhibit a clinically-relevant resistance to carbapenems when they coexist with impermeability, *ESBLs*, and/or *AmpC*. Furthermore, 2009 saw the discovery of *NDM-1* with Indian ancestry (Karami-Zarandi et al., 2023b).

In addition to the appearance of distinct carbapenemase enzymes, *CRKP* has been linked to a few different pathways. For example, ertapenem susceptibility is correlated with expression of the *AcrAB* efflux pump or with the combination of *ESBL* generation and loss of *OmpK36* porin. Moreover, *CRKP* has been connected to the expression of plasmid-mediated *AmpC, blaACT-1*, or overexpression of efflux pumps in addition to the loss of *OmpF* porin protein (Bradford et al., 1997; Karami-Zarandi et al., 2023b).

3. MATERIALS AND METHODS

3.1. Revival of *K. pneumoniae* isolates

Isolates previously isolated and stored in culture collection at -20°C were inoculated into Tryptone Soy Agar (TSA) medium and incubated at 37°C for 24 hours.

3.2. String Test

In order to determine the phenotype of hypermucoviscous, the isolates were sub-cultured into Tryptone Soy Agar (TSA) 37ºC /24h. with an inoculation loop was pressed into the surface of the colonies and lifted slowly. The presence of >5 mm thread-shaped elongation was considered positive (Wiskur et al., 2008).

3.3. Antibiotic Sensitivity Tests

Antibiotic susceptibility profiles of the isolates were determined by Kirby-Bauer Disk Diffusion Test. For this purpose, a total of 17 antibiotic disks from 7 different groups were used. Briefly, suspensions of all bacterial isolates grown on TSA agar were prepared in tubes containing physiological saline and their turbidity was adjusted to McFarland 0.5. 100µ of suspensions were inoculated onto Mueller Hinton Agar plates by spreading method. Then, the antibiotic disks specified in Table 3.1 were placed on the agar surface and incubated at 37°C for 18-24 hours. At the end of the incubation period, the diameter of the inhibition zones formed around the antibiotic disks was measured and evaluated according to CLSI (2018) criteria.

Table 3. 1. Antibiotic Discs Used in the Antibiotic Sensitivity Tests.

Antibiotic Agent	**Smbol**	**Antibiotic Agent**	**Symbol**
Oxytetracycline	OT	Amoxicillin+clavulanic acid	AMC
Cefquinome	SEQ	Imipenem	IPM
Chloramphenicol	C	Ampicillin	AMP
Cefoxitin	FOX	Penicillin	PEN
Cefepime	SEP	Cefoperazone	SEF
Ciprofloxacin	CIP	Meropenem	MR
Enrofloxacin	ENR	Gentamicin	CN
Nitrofurantoin	N	Amikacin	AMK
		Trimethoprim+sulfamethoxazole	STX

3.4. Determination of Virulence Genes

3.4.1. DNA Extraction

DNA extraction of *Klebsiella pneumoniae* isolates was performed by the boiling method. For this purpose, several colonies were taken from the cultures on TSA agar and suspended in sterile distilled water. The suspension was then boiled in a water bath at 100 ºC for 10 minutes and then centrifuged at 10000 rpm for 10 minutes. After centrifugation, the supernatants were removed and transferred to DNase/RNase-free tubes.

3.4.2. Multiplex PCR for *magA, rmpA, mrkD, entB* genes

In the Polymerase Chain Reaction (PCR) for our study, the reaction mixture for 32 samples comprised the following materials: 427.2 µL of water (H_2O), 80 µL of 10X buffer, 96 µL of 25 mM MgCl2, 16 µL of 10 mM dNTP, 2.4 µL each of *magA-F* and *magA-R* primers, 1.6 µL each of *rmpA-F* and *rmpA-R* primers, and 0.8 µL each of *mrkD-F*, *mrkD-R*, *entB-F*, and *entB-R* primers shown in Table 3.2. Additionally, 9.6 µL of Taq Polymerase (5 U) and 160 µL of DNA template were included, resulting in a total reaction volume of 800 µL.

For the amplification of specific target gene regions, the following primer sets were utilized: *magA-F* and *magA-R*, *rmpA-F* and *rmpA-R*, *mrkD-F* and *mrkD-R*, *entB-F* and *entB-R*. The PCR cycling conditions consisted of an initial denaturation step at 95°C for 15 minutes, followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 90 seconds, and extension at 72°C for 60 seconds. A final extension step was performed at 72°C for 10 minutes. This cycle was repeated 30 times shown in Table 3.3.

The amplicons were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Table 3. 2. Primers used for multiplex PCR assays.

Target gene	Nucleotide sequence (5′-3′)	Size of amplicon(bp)	Reference
rmpA_ for *rmpA_rev*	CATAAGAGTATTGGTTGACAG CTTGCATGAGCCATCTTTCA	461	(Compain et al., 2014)
magA_for *magA_rev*	GGTGCTCTTTACATCATTGC GCAATGGCCATTTGCGTTAG	1283	
mrkD_for *mrkD_rev*	AAGCTATCGCTGTACTTCCGGCA GGCGTTGGCGCTCAGATAGG	340	
entB_for *entB_rev*	GTCAACTGGGCCTTTGAGCCGTC TATGGGCGTAAACGCCGGTGAT	400	

Table 3. 3. Cycling condition for multiplex PCR.

Primers (forward and reverse)	Initial denaturation	Denaturation	Cycling conditions		
			Annealing	Extension	Final extension
magA-F *magA-R* *rmpA- F* *rmpA- R* *mrkD-F* *mrkD-R* *entB-F* *entB-R*	95°C 15 min	94°C 30 s Repeated for 30 cycles	60°C 90 s	72°C 60 s	72°C 10 min

3.4.3. PCR for *fimH* gene

For the PCR procedure in our investigation, the following materials and corresponding volumes were employed: 252 µL of water (H2O) serving as the solvent, 340 µL of 2X Master Mix, 10.2 µL each of *FimH-F* and *FimH-R* primers

shown in Table 3.4, and 68 μL of DNA template. The total reaction volume was precisely measured at 680 μL.

For *fimH*_for and *fimH*_rev, the cycling conditions included an initial denaturation at 95°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, extension at 72°C for 45 seconds, and a final extension at 72°C for 10 minutes shown in Table 3.5.

The amplicons were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Table 3. 4. Primers used for *fimH* gene PCR.

Target gene	**Nucleotide sequence (5′-3′)**	**Size of amplicon(bp)**	**Reference**
*fimH*_for *fimH*_rev	ATGAACGCCTGGTCCTTTGC GCTGAACGCCTATCCCCTGC	688	(Massinga et al., 2021)

Table 3. 5. Cycling condition for *fimH* PCR.

Cycles	**Initial denaturation**	**Denaturation**	**Annealing**	**Extension**	**Final extension**
25	95℃/5 min	94℃/1 min	60℃/1 min	72℃/45 s	72℃/10 min

4. RESULTS

4.1. String test

Out of 32 strains, only 1 isolate was string test positive, hypermucoviscous.

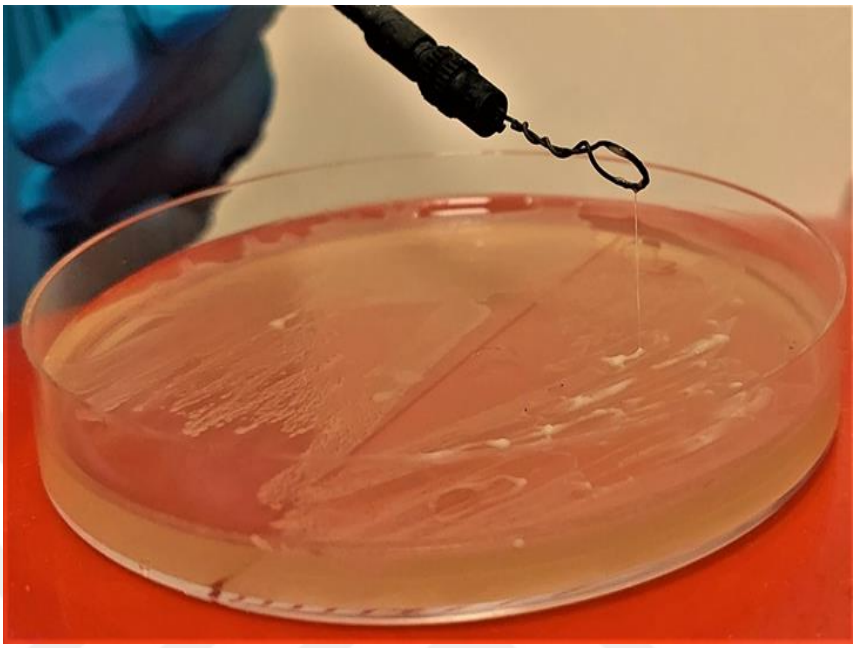

Figure 4. 1. String test positive isolate showing >5 mm thread-shaped elongation.

4.2. Antibiotic Susceptibility Tests

As a result of antimicrobial susceptibility tests, the resistance rates of the isolates to the beta-lactam antibiotics used were as follows; 53.1% of the isolates were resistant to amoxicillin-clavulanic acid, 3.1% to imipenem, 62.5% to ampicillin, 43.75% to penicillin, 12.5% to cefoperazone, 34.3% to cefquinoma, 25% to cefepime and 43.75% to cefoxitin. In addition, all isolates were susceptible to meropenem.

Resistance rates of the isolates to aminoglycoside antibiotics were 40.6% to gentamicin and 9.3% to amikacin. Resistance rates for fluoroquinolone antibiotics were 40.6% to ciprofloxacin and 37.5% to enrofloxacin. In addition, 65.6% were resistant to nitrofurantoin, 37.5% to trimethoprim+sulfamethoxazole, 46.8% to oxytetracycline and 28.1% to chloramphenicol which is shown in Figure 4.2.

Antibiotic susceptibility tests revealed that 20 (62.5%) of all isolates showed resistance to more than one antibiotic. In particular, 6 isolates (18.75%) were resistant to three antibiotic classes, 4 isolates (12.5%) to four antibiotic classes, 6 isolates (18.75%) to five antibiotic classes and 4 isolates (12.5%) to six antibiotic classes. Among all isolates, the number of isolates exhibiting multiple antibiotic resistance was

(62.5%) which is shown in Table 4.1.

Table 4. 1. Number of antibiotic classes to which the isolates showed resistance.

Isolate No	**Number of Antibiotic Classes to which it is Resistant**	**Isolate No**	**Number of Antibiotic Classes to which it is Resistant**
1	6	16	3
2	1	17	4
3	3	18	5
4	2	19	3
5	1	20	4
6	1	21	2
7	1	22	2
8	1	23	5
9	3	24	4
10	6	25	2
11	6	26	5
12	3	27	5
13	3	28	6
14	4	29	5
15	1	31	5
		32	2

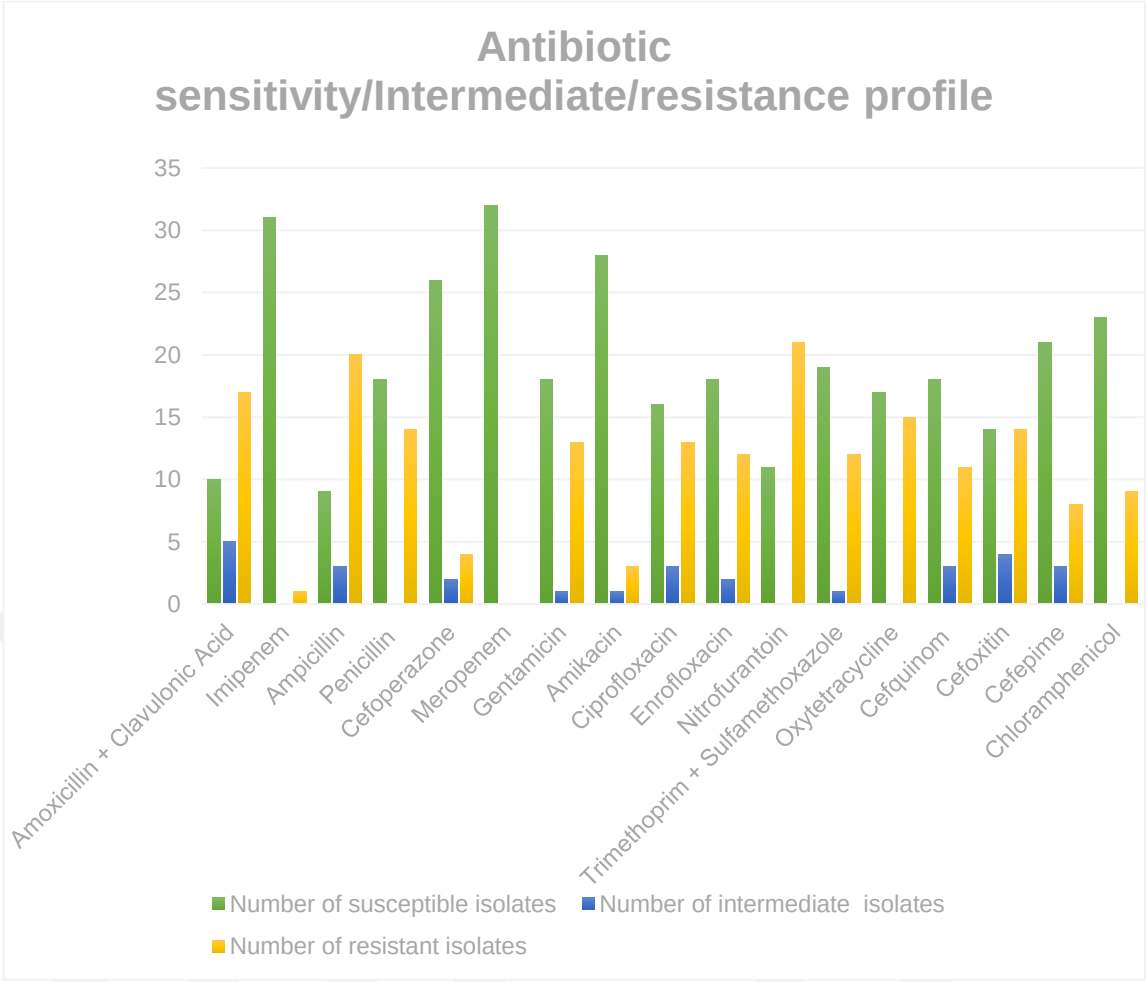

Figure 4. 2. Antibiotic sensitivity/Intermediate/resistance profile.

4.3. Determination of Virulence Genes

Analysis revealed that among the isolates, 4 were positive for the *magA* gene, 15 for the *mrkD* gene, 9 for the *fimH* gene and 5 for the *entB* gene. In contrast, none of the isolates had the *rpmA* gene. The results of multiplex PCR and *fimH*, PCR performed on all isolates can be found in Table 4.2, while Figure 4.3 and Figure 4.4 provide visual representations of these results. Notably, four isolates were observed to have both entB and *mrkD* genes at the same time, while one isolate contained both *magA* and *mrkD* genes. Some isolates harbored more than one gene, as shown in Table 4.3. None of the isolates harbored all of the genes examined. The presence or absence of each virulence gene varied between samples, indicating a different pattern of distribution. The presence of these virulence genes suggests that the respective samples may have specific pathogenic properties associated with these genes.

Table 4. 2. Virulence genes harbored by the isolates.

No	*magA*	*rmpA*	*mrkD*	*entB*	*fimH*	No	*magA*	*rmpA*	*mrkD*	*entB*	*fimH*
1	-	-	+	-	-	**18**	-	-	-	-	+
2	-	-	+	-	-	**19**	-	-	+	-	-
3	-	-	+	-	-	**20**	-	-	-	-	-
4	-	-	+	-	-	**21**	-	-	+	+	+
5	-	-	-	+	+	**22**	-	-	-	-	-
6	-	-	-	-	-	**23**	-	-	+	-	-
7	-	-	+	-	-	**24**	-	-	-	-	-
8	-	-	-	-	-	**25**	-	-	-	-	-
9	-	-	+	+	-	**26**	+	-	-	-	+
10	+	-	-	-	-	**27**	-	-	-	-	+
11	-	-	+	-	-	**28**	-	-	-	-	-
12	+	-	-	-	-	**29**	-	-	+	+	-
13	-	-	-	-	-	**31**	-	-	-	-	+
14	-	-	-	-	-	**32**	-	-	+	-	-
15	-	-	+	-	-						
16	+	-	-	-	-						
17	-	-	+	+	-						

Table 4. 3. Isolates that harbor more than one virulence gene.

Isolates Number	Virulence genes
Isolate 9	*magA, mrkD, entB*
Isolate 12	*magA, mrkD*
Isolate 17	*mrkD, entB*
Isolate 19	*magA, mrkD, entB, fimH*
Isolate 21	*mrkD, entB, fimH*
Isolate 23	*magA, mrkD, fimH*
Isolate 26	*magA, fimH*
Isolate 29	*mrkD, entB*
Isolate 32	*mrkD, fimH*

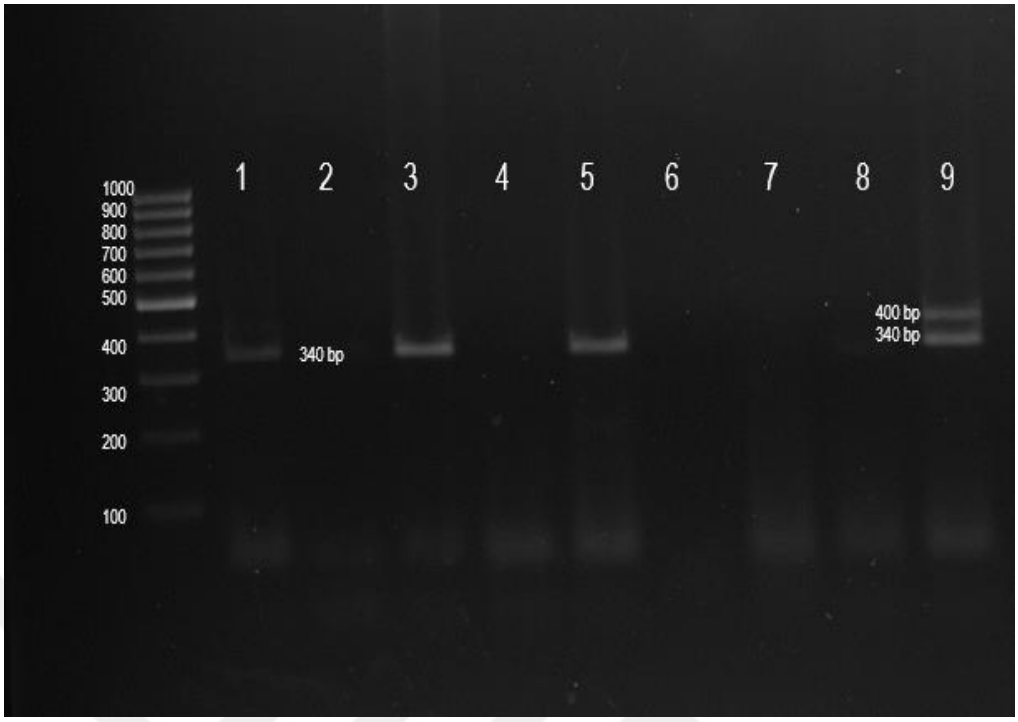

Figure 4. 3. Gel electrophoresis image of bands specific to *entB* and *mrkD* genes. *entB* gene exhibited a 400bp band and *mrkD* gene exhibited a 340bp band.

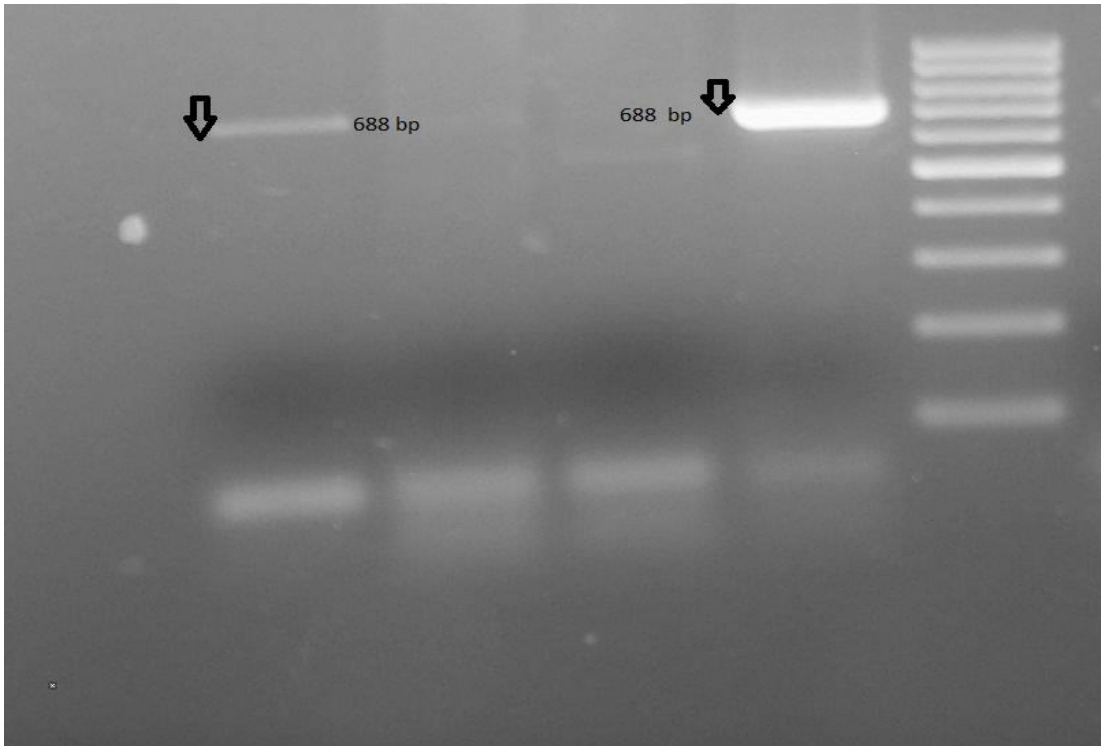

Figure 4. 4 Gel electrophoresis image of the 688bp band specific to the *fimH* gene.

5. DISCUSSION

Liu et al. (1986) reported seven cases of invasive *K. pneumoniae* infection in community members, presenting with hepatic abscess without biliary tract illness or septic endophthalmitis. This seminal clinical report marked the initial recognition of hvKp. The assessment of hvKp often involves the hypermucoviscosity phenomenon, identified through a positive string test. This test, involving the development of viscous strings around 5 mm in length when a loop is used to stretch the colony on an agar plate, serves to differentiate classical cKp from hvKp (Russo and Marr, 2019).

Our study found that only one isolate tested positive for the string test. However, literature suggests that the term "hypermucoviscous" can be problematic as it lacks sensitivity and specificity for hvKp strains; some cKp strains also exhibit this phenotype. Additionally, some studies defined hvKp solely based on a positive string test, leading to misclassifications and contributing to confusion in the literature (Russo and Marr, 2019).

The pathogenicity of *K. pneumoniae* arises from various virulence factors, including capsule production, hypermucoviscosity, the presence of lipopolysaccharides, and iron acquisition systems. These factors collectively enable the bacterium to evade and overcome the innate immunity of mammalian hosts, facilitating the establishment and maintenance of infections (Yang et al., 2019).

Among the genes associated with capsular synthesis, *rmpA* and *rmpA2*, carried on plasmids, play a crucial role. In our study, *rmpA* was not detected in any isolates, aligning with findings reported by Caneiras et al. (2019), Kus et al. (2017), and Rastegar et al. (2021b). This absence suggests potential variations in the hypermucoviscous phenotype among *K. pneumoniae* strains and emphasizes the need for a comprehensive understanding of virulence factors in these bacteria. Additionally, our observation of a single isolate testing positive in the string test did not coincide with the presence of the *rmpA* gene in that isolate. The absence of the *rmpA* gene in phenotypically hypermucoviscous strains suggests the involvement of alternative genes, highlighting discrepancies between observable traits and genetic markers for hvKP, a phenomenon observed by other researchers, including Remya et al. (2019).

The presence of the *magA* gene in *K. pneumoniae* strains is significant. The role of *magA* extends beyond resistance, potentially helping to trace the origin of infectious

diseases caused by *K. pneumoniae*. In addition, it serves as a molecular marker for rapid diagnosis, revealing its potential as a novel therapeutic target (Fang et al., 2004). In our study, the prevalence of *magA* was higher compared to rates reported by Hao et al. (2022), Khalil et al. (2019), Mirzaie and Ranjbar (2021), and Yang et al. (2019). This finding is consistent with the rates reported by Fu et al. (2018) and Kim et al. (2019) at 12.8% and 11.6%, respectively. However, the association between these factors and hypermucoviscosity was not represented in our study, with only one isolate testing positive for the string. Some studies propose the existence of additional mucoid factors beyond *magA* and *rmpA* genes, possibly related to variations in lipopolysaccharide (LPS) composition (Rastegar et al., 2021a; Yang et al., 2019).

Among other virulence-contributing genes, *fimH* and *mrkD* play crucial roles. These genes are associated with bacterial cell adhesive agents, specifically Type 1 fimbriae (*fmH-1*) and Type 3 fimbrial adhesion (*mrkA* and *mrkD*), leading to *K. pneumoniae* adherence to urinary tract epithelial and endothelial cells, resulting in urinary tract infections (Panjaitan et al., 2019). The precise mechanism of Type 3 fibrillar adhesion remains unknown, but studies suggest its importance in *K. pneumoniae* biofilm formation (Khalil et al., 2019). Nevertheless, the impact of type 1 fimbriae on biofilm formation was identified as negligible, while the expression of type 3 fimbriae was determined to significantly enhance biofilm formation and facilitate the occurrence of catheter-associated *K. pneumoniae* infection (Ali and Al-Kakei, 2019). In our study, *fimH* and *mrkD* genes were found at rates of 28% and 46.9%, respectively. These rates were lower than those reported in most other studies (El Fertas-Aissani et al., 2013; Kus et al., 2017; Fu et al., 2018; Kim et al., 2019; Yang et al., 2019; Massinga et al., 2021; Mirzaie and Ranjbar, 2021; Ranjbar et al., 2019; Rastegar et al., 2021a; Hao et al., 2022).

In the context of *Klebsiella*, siderophores are essential molecules produced by bacterial cells to efficiently bind and acquire ferric iron (Fe3+) from host iron-binding proteins. These high-affinity extracellular chelators play a crucial role in bacterial virulence, especially for extracellular pathogens like *Klebsiella*, which lack direct access to iron in the host's body. In order to overcome the inherent iron limitation encountered in vivo, *Klebsiella* employs siderophores as a strategic mechanism for iron acquisition (El Fertas-Aissani et al., 2013). Our study revealed that the prevalence of isolates carrying the *entB* gene was 15.6%, a rate significantly lower than what has

been reported in the literature. Specifically, studies conducted by Massinga et al. (2021), Kus et al. (2017), Kim et al. (2019), and Ahmed and Alaa (2016) reported rates of 64.9%, 80.86%, 96.2%, and 100%, respectively. Moreover, our findings align with the 4.05% reported by Remya et al. (2019). This observed variation emphasizes the diversity in the prevalence of the *entB* gene among *Klebsiella* isolates in different studies.

Additionally, the activation of the iron-enterobactin outer membrane receptor gene (*fepA*) during infection further underscores the potential significance of enterobactin in the pathogenicity of *Klebsiella* (El Fertas-Aissani et al., 2013). Further research is warranted to elucidate the intricate relationship between enterobactin and *Klebsiella* virulence, considering the variations observed in *entB* prevalence across different studies.

The emergence of MDR *K. pneumoniae* strains poses a significant global threat, leading to serious and potentially life-threatening infections. The widespread use of antimicrobial agents has contributed to the proliferation of MDR *K. pneumoniae* strains. The increasing prevalence of *K. pneumoniae* strains exhibiting resistance to multiple antimicrobials represents an urgent problem with far-reaching implications for public health worldwide (Mirzaie and Ranjbar, 2021). There is an increasing number of reports detailing the emergence of MDR-hvKP strains. MDR-hvKP can develop through two distinct mechanisms. Firstly, hvKP strains may obtain antimicrobial resistance genes or plasmids through horizontal transfer, transforming into MDR-hvKP, categorized as type I MDR-hvKP. Additionally, MDR-hvKP strains can originate from the introduction of a pLVPK-like virulence plasmid into a classic MDR *K. pneumoniae* strain, termed as type II MDR-hvKP. Type I MDR-hvKP strains exhibit the same virulence determinants as the hvKP strains mentioned earlier. In contrast, the heightened virulence in type II MDR-hvKP is primarily linked to increased capsule and siderophore production, resulting from the acquisition of plasmid-mediated virulence determinants, including *rmpA/rmpA2*, *iut*, and *iro* (Zhu et al., 2021). In our study, the prevalence of MDR *K. pneumoniae* isolates was 62.5%, which, although substantial, was lower than the rates reported in some studies. For instance, Mirzaie and Ranjbar (2021) and El Fertas-Aissani et al. (2013) reported higher prevalence rates of 92% and 74.1%, respectively. *K. pneumoniae* has exhibited a notable propensity to develop resistance to a broad spectrum of antibiotics,

particularly third-generation cephalosporins. The acquisition of multidrug resistance by *K. pneumoniae* enhances its virulence capacity. The efficacy of beta-lactam antibiotics, commonly used to combat bacterial infections, is frequently compromised by the production of beta-lactamase enzymes—a common mechanism of bacterial resistance (Shaikh et al., 2015; Vasaikar et al., 2017). Plasmid-mediated carbapenemases, enzymes that hydrolyze all β-lactams, including the last-line carbapenems, have emerged due to the widespread use and abuse of carbapenems (Queenan and Bush, 2007).

The observed resistance of *K. pneumoniae* to cephalosporins may be attributed to the presence of the *kpc* gene, as the KPC enzyme specifically degrades broad-spectrum cephalosporins (Effah et al., 2020). The resistance rate to amoxicillin-clavulanic acid in our study was 53.1%, lower than the rates of 62.3% (Banerjee et al., 2020), 72% (Kus et al., 2017), and 82.9% (Z. Zhang et al., 2022) reported in other studies, but higher than the rate reported by Jazayeri Moghadas et al. (2018). The resistance rate to imipenem (3.1%) closely aligned with that reported by Zhang et al., whereas Mirzaie and Ranjbar (2021) reported the highest resistance rate, in contrast to Banerjee et al. (2020), who found that all isolates were antibiotic-sensitive. Interestingly, all isolates in our study demonstrated susceptibility to meropenem, contrary to the findings of Mirzaie and Ranjbar (2021).

Most *Klebsiella* isolates are inherently resistant to ampicillin due to constitutively expressed chromosomal A beta-lactamase (Gündoğan, 2014). In this study, only 62.5% of the isolates were resistant to ampicillin. This rate contradicts the results of Kus et al. (2017) and Nirwati et al. (2019) who reported that 100% *of K. pneumoniae* isolates were resistant to ampicillin. Although it has been reported that *K. pneumoniae* isolates have a chromosomal gene encoding the production of the beta-lactamase enzyme, that this enzyme is constitutively expressed and always produced by the bacterium, it is possible that the synthesis of the enzyme causing the resistance in question may not have occurred due to various gene mutations or other factors affecting gene expression. Additionally, 43.7%, 12.5%, and 34.3% of the isolates in our study were resistant to penicillin, cefoperazone, and cefquinome, respectively. These findings underscore the complexity and variability in antibiotic resistance patterns, emphasizing the need for vigilant surveillance and tailored treatment strategies. Remya et al. (2019) investigated the distribution of virulence genes in beta-

lactamase-producing isolates, discovering an association between *fimH* and KPC-positive *K. pneumoniae*. Their findings revealed that the solitary KPC producer carried multiple virulence genes, including *ybtS, entB, k fu, uge*, and *fimH*. Unlike our study, which observed resistance to two or three single antibiotics within the Beta-lactam group, clinical samples in studies using KPC-producing *K. pneumoniae* suggested the absence of *magA, k2A, rmpA*, and aerobactin. It is noteworthy that our results differ from these findings. Additionally, contrary to our study where most isolates showed resistance to two or three single antibiotics in the Beta-lactam group, studies have indicated that ESBL-producing *K. pneumoniae* have the ability to produce more fimbrial adhesins (Remya et al., 2019).

When assessing the resistance of *K. pneumoniae* to aminoglycosides in our study, it was identified that 40.6% of the isolates exhibited resistance to gentamicin. This percentage is notably higher than the rates of 31.25% (Ahmed and Alaa, 2016) and 39.6% (Kus et al., 2017) but lower than the reported rates of 54.8% (Banerjee et al., 2020), 62.9% (Eghbalpoor et al., 2019), and 75.7% (Z. Zhang et al., 2022). The resistance rate to amikacin was higher than the 4.8% reported by Pereira and Vanetti (2015) but lower than that reported by Ahmed and Alaa (2016), Derakhshan et al. (2016a), Z. Zhang et al. (2022), and Derakhshan et al. (2016b) Liu et al. (2014), Peerayeh et al. (2014), at 10%, 27%, 28.12%, 31.5%, 31.4%, and 50%, respectively.

In our investigation, 40.6% of the isolates demonstrated resistance to ciprofloxacin, a quinolone antibiotic. This rate surpasses the 32.1% reported by Kus et al. (2017) and is significantly lower than the rates of 75.3% (Eghbalpoor et al., 2019) and 83.9% (Banerjee et al., 2020) reported in two separate studies.

The resistance rate to nitrofurantoin was found to be 65.6% in our study. This percentage is higher than the 35.8% reported by Jazayeri Moghadas et al. (2018) and slightly lower than the 68% reported by Mirzaie and Ranjbar (2021). These variations highlight the diversity in resistance patterns and emphasize the need for a nuanced understanding of antibiotic resistance dynamics influenced by various factors such as geographical location and local antibiotic usage practices.

In the context of antibiotic resistance, the co-occurrence of tetracycline, fluoroquinolones, trimethoprim-sulfamethoxazole, aminoglycosides, and associated resistance factors is commonly identified on plasmids that can be transferred between different bacteria. Plasmids carrying genes encoding carbapenemase and extended-

spectrum beta-lactamases (ESBLs), providing resistance against a broad spectrum of antibiotics, contribute to the dissemination of antibiotic resistance (Russo and Marr, 2019).

In our study, we found that 37.5% of the isolates demonstrated resistance to trimethoprim+sulfamethoxazole, a rate notably lower than the 56.6%, 73%, and 77% reported by Kus et al. (2017), Mirzaie and Ranjbar (2021), and Zhang et al. (2022), respectively. Furthermore, our findings indicated that 46.8% of the isolates exhibited resistance to oxytetracycline. The resistance rate to chloramphenicol in our study was 28.1%, presenting a significant deviation from the 100% resistance reported by Banerjee et al. (2020). These variations underscore the dynamic and context-dependent nature of antibiotic resistance patterns, influenced by factors such as geographical location, population characteristics, and local antibiotic usage practices.

6. CONCLUSION AND RECOMMENDATIONS

The study revealed 31 of isolates are susceptible for Imipenem, and only 1 isolate is resistant to imipenem, nevertheless all 32 isolates are susceptible for Meropenem resistance, indicating high effectiveness against the tested isolates. These findings suggest that Imipenem and Meropenem show notable efficacy with low resistance, making them potentially important antimicrobial agents for the tested isolates.

In contrast, the resistance patterns among *Klebsiella pneumoniae* isolates to different antibiotics, the resistance rates to trimethoprim+sulfamethoxazole and oxytetracycline were 37.5% and 46.8%, respectively. These rates differed from those reported in previous studies, emphasizing the dynamic nature of antibiotic resistance in *K. pneumoniae*.

A significant proportion (40.6%) of isolates exhibited resistance to gentamicin, indicating a concerning trend. The resistance rates to amikacin and ciprofloxacin were also noteworthy, emphasizing the need for vigilant monitoring of aminoglycoside resistance in clinical settings. The prevalence of MDR *K. pneumoniae* isolates was 62.5%, emphasizing the urgent global threat posed by these strains.

The study explored various virulence factors contributing to *K. pneumoniae* pathogenicity. While only one isolate demonstrated a positive string test for hypermucoviscosity, and some studies defined hvKp solely based on a positive string test, leading to misclassifications and contributing to confusion. The absence of *rmpA* in all isolates raised questions about its prevalence, aligning with findings in other studies. The *MagA* gene, associated with high resistance to phagocytosis, showed higher prevalence rates compared to some previous reports. The study identified the presence of the *entB* gene in 15.6% of isolates, indicating *Klebsiella*'s use of siderophores as a strategy for iron acquisition, a crucial aspect of bacterial virulence.

The antibiotic resistance profiles of *K. pneumoniae*, a pathogen with a significant place in the WHO list, are of concern due to their potential spread. Particularly, companion animals like cats and dogs may harbor antibiotic-resistant *K. pneumoniae* strains, serving as potential reservoirs for agents exhibiting these concerning characteristics. This highlights the importance of monitoring and addressing the transmission dynamics of antibiotic-resistant *K. pneumoniae* in both human and animal medicine.

Recommendations

i. Further investigations about the link between fimbira 1 and fimbira 3 genes and biofilm formation in *K. pneumoniae* as a crucial way to understand its pathogenicity must be made.

ii. The association between antibiotic resistance and biofilm formation should be elaborated on by future studies.

iii. Continuous surveillance of MDR *K. pneumoniae* strains is crucial for understanding the evolving landscape of antibiotic resistance. International collaboration and data sharing can provide a comprehensive picture of MDR prevalence globally.

iv. Understanding the dynamics of siderophore production and iron acquisition systems in *K. pneumoniae* can unveil potential targets for therapeutic interventions. Research on siderophores may contribute to novel strategies against bacterial virulence.

v. Implementing stringent antibiotic stewardship programs is crucial to curb the emergence of antibiotic-resistant *K. pneumoniae* strains. Surveillance of local resistance patterns should guide empirical treatment strategies.

vi. Educate pet owners about the risks of *K. pneumoniae* infections and the importance of regular veterinary check-ups and good hygiene practices.

vii. Encourage collaboration and data sharing among veterinary professionals, researchers, and public health authorities to enhance knowledge and response to *K. pneumoniae* infections in animals.

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CURRICULUM VITEA

Experience :

Research assistant, central veterinary research laboratory

• Laboratory management (quality control checks and safety) • training of undergraduates,

graduates and junior lab technical staff • Prepare technical proposals and/or presentations • Assist with creating graphs for presentations and publications

Intern as research assistant, food hygiene and environmental health, virology team, veterinary medicine, university of Helsinki, Finland

• Cell Culture and virus propagation (murine norovirus in RAW cells).

• Food virological experiments.

• Molecular biology (Real-Time PCR. PCR).

• Routine laboratory works.

Training as assistant surgeon and clinical practice, department of surgery and anesthesiology, veterinary medicine, University of Khartoum

• Assist In Surgical Operation

• Diagnosis And Treat Animal

Intern in biochemistry department, veterinary medicine university of Khartoum, Sudan.

• Administering, and collecting lab quizzes and reports

• Supervising Students Regarding, Procedure, Technique and Safety Element of

• Laboratory Session Provide directions and clarify instructions and notify the instructor of injuries or hazard in the lab

Intern in laboratory of physiology department, veterinary medicine university of Khartoum, Sudan.

• Blood Sampling from Different Kind of Animal

• Total Blood Count

• Serum Tests by Spectrophotometer

Team leader, microbiology department veterinary week, faculty of veterinary medicine

- Developed good team working and presentation skills.

• Team leader of microbiology fair

Supervising team member topics and discuss it with them.

Certificates :

• Climate change challenge, The way forward to Reinforcement of livestock systems in Developing countries.

• Sustainable Food Production through livestock.

• Diagnosis of Transboundary Animal Diseases, Food and Agriculture Organization of United Nations.

• The useful of using geographical information system (GIS) in disease surveillance and control.

• Laboratory Biorisk Management Standard Based on CWA15793:2011, Center for Training and Human Resources Development.

• Research methodology and ethics

• Training Course in Camel Health and Husbandry, Camel Research Centre Faculty Of Veterinary, Medicine University of Khartoum

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Publications :

1. **DETERMINATION OF SOME VIRULENCE GENES AND ANTIBIOTIC RESISTANCE PROFILES OF *K. pneumoniae* STRAINS OF CAT AND DOG ORIGIN.**

Won Awards, Incentives and Scholarships

1. **Erasmus+ 2023**

2. **Turkish Scholarships for Foreign Students (YTB) 2021**