Optimization and Screening of *entB* and *uge* genes in the clinical isolates of *Klebsiella pneumoniae*

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Dedication

I express heartfelt dedication to my parents who always encouraged me to work harder. I could never do this without your support, affection, and prayers. Thank you so much for believing in me.

Declaration

The material and information contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Aqsa Hashmi

Certificate

This thesis submitted by Aqsa Hashmi is accepted in its present form by the Department of Microbiology, Quaid-i-Azam University, Islamabad, Pakistan; as satisfying the thesis requirements for the degree of Master of Philosophy in Microbiology.

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List of Acronyms

ATTC	American-type test culture
BLI	Beta-lactam Inhibitors
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standard Institute
CV	Crystal Violet
ddH_2O	Double Distilled Water
DNA	Deoxyribonucleic acid
ESBLs	Extended-spectrum-beta-lactamases
HAIs	Hospital Acquired Infections
ICU	Intensive Care Unit
LPS	Lipopolysaccharides
LFH	Laminar Flow Hood
MDR	Multidrug Resistant
MIC	Minimum Inhibitory Concentration
MHB	Muller Hinton Broth
MHA	Muller Hinton Agar
OD	Optical density
ODc	Cutoff Optical density
PBPs	Penicillin-binding proteins
PBS	Phosphate Buffer Solution
QS	Quorum Sensing
RNA	Ribonucleic acid
TSI	Triple Sugar Iron
TSA	Tryptic soya agar
UTIs	Urinary tract infections
VAP	Ventilator-associated pneumonia
XDR	Extended drug-resistant

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All praises to Almighty ALLAH, the Omnipotent, the most compassionate, and all respects andDarood-o-Salam to Holy Prophet Hazrat MUHAMMAD (S.A.W.W), the most exalted among all human beings ever born on the surface of the earth, who is everlastingly a wellspring of direction and information for all mankind as a whole.

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Aqsa Hashmi

Abstract

Klebsiella pneumoniae is listed as an ESKAPE pathogen by the World Health Organization (WHO) and has been recently associated with higher morbidity and mortality. *K. pneumoniae* exhibits multidrug resistance mechanisms which is influenced by various factors that includes the mutations within the ribosomal binding site, acquisition of mobile genetic elements carrying resistance genes. Along with the presence of other types of resistance mechanisms, one is the ribosomal protection and biofilm formation exemplified by the *uge* and *entB* genes. There were 156 isolates of *K. pneumoniae* in all, collected from tertiary care hospitals in Islamabad, Pakistan. So, this study focused on the phenotypic analysis and genotypic screening of *uge* and *entB* genes in *K. pneumoniae*. Gram staining and colony morphology were used to determine morphological characteristics. Following that, biochemical identification was accomplished through a series of assays. Further, the doxycycline on planktonic and biofilm of *K. pneumoniae* was tested. This study involved the use of Minimum Biofilm Eradication Concentration (MBEC), Minimum Biofilm Inhibition Concentrations (MBIC), and sub minimal concentration of doxycycline on isolates of *K. pneumoniae.* Screening of 156 isolates revealed that 38% isolates carried *uge* gene which encodes for enzyme UDP glucose 4 epimerases involved in extracellular polysaccharides synthesis and 13% isolates carried *entB* gene (Enterobactin synthase component B) which is used to scavenge iron to provide characteristic role in biofilm formation. While 14 out of 156 total isolates (9%) carried both genes. Then confirmatory tests including disk diffusion and minimum inhibitory concentration were performed. Our disk diffusion results showed that 11 out of 80 (11%) were doxycycline resistant isolates while 24 out of 80 (30%) were doxycycline sensitive isolates. Further, confirmatory tests to determine the minimal inhibitory doses for doxycycline includes MIC-p and MIC-b measurements. The results were found to be almost same as disk diffusion results i.e., 24 out of 30 (30%) isolates were found sensitive at MIC value ranged from 1-4ug/ml. Further, biofilm forming capacity was determined by microtiter plate assay. We identified two strong biofilm formers, one moderate biofilm former and five weak biofilm former isolates. Biofilms are generally associated with the increase in virulence and resistance of bacteria as our study indicated increase in MBEC (512-128ug/ml) and MBIC (128-32ug/ml) value of

doxycycline after biofilm formation. Further, this study indicated an increase in the biofilm forming ability of isolates with time when exposed to doxycycline at sub minimal concentrations. Hence, our findings indicated a greater susceptibility of *K. pneumoniae* to doxycycline in biofilm form.

Introduction

1.1 Introduction

Klebsiella pneumoniae, a non-motile rod-like microorganism, that is Gram negative has become disproportionately common in hospital settings worldwide (Sandhyarani, 2011). It is an opportunistic pathogen causing infections in humans, particularly in people who are immunocompromised or have underlying medical conditions. In 1882, German microbiologist Carl Friedländer initially discovered and named this bacterium as Friedländer's bacillus. It was later renamed *K. pneumoniae* in the honor of its co-discoverer, Edwin Klebs (Sathiya, 2018).

It is normally found in the soil, air and water. It is also a normal inhabitant of the human digestive tract and can cause infections in the urinary tract, the lungs and the other parts of the body. When *K. pneumoniae* grown on isolation medium i.e., MacConkey agar and Nutrient Agar, produces a large colony of mucoid consistency (Bruce *et al.*, 1981). This species is classified as a facultative anaerobic organism because it can develop in both the presence or absence of free oxygen.

The pneumonia caused due to *K. pneumoniae* should be differentiated from the *streptococcus pneumoniae* associated pneumoniae is of rust color and contains blood while sputum of *K. pneumoniae* caused pneumonia is characterized as currant jelly as *K. pneumoniae* cause excessive necrosis of the surrounding tissues. It is also the causative agent of surgical wound infections, endocarditis, cystitis and septicemia (Ashurst *et al.*, 2018). *K. pneumoniae* can cause outbreaks in health care facilities especially in the neonatal units. It was primarily considered as the cause of infection in neonates especially in the low and middle income countries (Dramowski *et al.*, 2017).

Klebsiella pneumoniae infections are commonly found in the healthcare settings, mainly in intensive care units (ICUs) and the long-term care facilities. The incidence of infections caused by *K. pneumoniae* differs depending on the population studied and the geographic region, but it is estimated that *K. pneumoniae* is accountable for up to 10% of the entire healthcare-related infections (Lin *et al.*, 2013). *K. pneumoniae* has a thick polysaccharide capsule that plays significant part in its virulence. This capsule provides protection to the bacterium from immune defenses of the host like phagocytosis. The

mortality rate reported due to carbapenem resistant *Klebsiella* species is about 20 to 40% in the European countries and North American populations whereas it is about 26% in China and it can be as high as 50% in some parts of the world. Bacteria are the leading cause of ventilator acquired pneumonia. *Klebsiella species* are stated to be the second key reason of the bloodstream infections after *Eschericha coli* and the *Klebsiella* associated bloodstream infections is about 20% to 30% and population mortality rate is predictable to be 1.3% in one million persons. These are also considered as the 3rd main reason of the hospital associated infections after *Clostridium difficile* and *staphylococcus aureus* in the United States (Li *et al.*, 2019).

The colonization rate of *K. pneumoniae* can vary depending on the body site. Mostly these species attach to the mucosal surfaces, like the nasopharynx, the gastrointestinal tract and urinary tract (Martin *et al.*, 2018). The rate of colonization in the nasopharynx is reported to be 3% to 15% and this rate is comparatively higher in adults than children. The rate of colonization in the gastrointestinal tract differs but can be reached up to 20% (Munoz-Price *et al.*, 2010).

In *Klebsiella pneumoniae*, biofilm is produced by several factors which includes the adhesins expression, the extracellular polysaccharide production, and the gene expression regulation through the quorum sensing (Singh *et al.*, 2021). These mechanisms help the *K. pneumoniae* to adhere to surfaces and establish protective community structures that can persist in the environment. The microorganism having ability of biofilm formation directly influences its survival and pathogenic potential. As compared to the individual cells, biofilms forming bacteria have advantages such as increased host immune system evasion, high resistance to antimicrobial agents and effective communication through the quorum sensing (Mooney *et al.*, 2018). As the bacteria are present in close proximity within the biofilms, so there are more chances of genetic material exchange through conjugation and thereby elevating the likelihood of generating antibiotic-resistant strains (Lerminiaux *et al.*, 2019). It has been estimated that almost 60% of bacterial infections and 80% of total chronic infections are due to the formation of biofilms (Bjarnsholt, 2013).

Klebsiella pneumonia exhibit one of the key virulence factors that is linked to the formation of biofilm on the soft tissues including the respirational tract, gastrointestinal tract, and urinary tract which enables it to effectively colonize and persist in the host. In the respiratory tract, *K. pneumoniae* biofilms can form on the lung tissues surfaces, resulting in the development of pneumonia. In the gastrointestinal tract, *K. pneumoniae* biofilms can produce on the intestinal lining surfaces, resulting in enteritis. In the urinary tract, *K. pneumoniae* biofilms can develop on bladder and urethra surfaces, resulting in urinary tract infections. It can also help in biofilm formation on the medical devices like catheters which can lead to the development of Catheter Linked Urinary Tract Infections (Guerra *et al.*, 2022). These catheters associated urinary tract infections because economic burden in the form of prolonged hospital stays. So, *K. pneumoniae* becomes more resistant towards the host immune system along with antibiotics once it forms a biofilm. The extracellular polymeric substances play a role as a physical barrier, preventing antibiotics and immune cells from penetrating the biofilm and reaching the bacteria. Additionally, *K. pneumoniae* within the biofilm can undergo changes in gene expression and metabolic activity, which can alter their susceptibility to various antibiotics like gentamicin, ciprofloxacin and cefiderocol etc (De Oliveira *et al.*, 2020).

Biofilms are intricate communities of microorganisms that are embedded in a self-synthesized EPS matrix, comprising exopolysaccharides and enzymes. The dense nature of enzymes matrix and exopolysaccharides in biofilms provides a physical impediment and sequesters nutrients, making it difficult for antibiotics to penetrate and access to underlying cells. Furthermore, enzymes present in the matrix can also break down or modify antibiotics, further reducing their efficacy. Consequently, biofilms are often more resistant to antibiotics compared to their planktonic counterparts, which can lead to persistent infections and posing treatment more challenging (Gędas *et al.*, 2020).

The discovery of penicillin in 1928, laid down the foundation of the modern medicine and marked the initiation of antibiotics usage in the treatment of many infections (Lobanovska *et al.*, 2017). Antibiotic therapy for infectious diseases differs geographically according to the local resistance patterns. Inappropriate usage of antibiotics fosters the antibiotic resistance development and hence lead to diminishing

the choice of antibiotics for the effective treatment of many infection causing diseases (Barbosa *et al.*, 2000).

The persistent and extensive use of antibiotics for more than 70 years has transformed antibiotic resistance into a worldwide concern within the modern medicine. Resistance to antibiotics depends on multiple factors predominantly due to the inappropriate bacterial exposure with the antibiotics where the selective pressure may result into the development and increase of multiple antibiotic resistant and extremely antibiotic resistant *Enterobacteriaceae* species, that gain resistance to most of the available antibiotics and hence there will be very less treatment options available. The increases incidence of infections caused *Enterobacteriaceae* species is of major and serious concern as these organisms are the natural inhabitants of our microbiome. The infections attributed to these strains also have elevated mortality rates and prolonged hospital admission days (Aslam *et al.*, 2018).

Klebsiella pneumoniae which is a most important member belonging to the *Enterobacteriaceae* family, has appeared as a clinically significant pathogen with increasing drug resistance (Navon-Venezia *et al.*, 2017). The existence of multidrug-resistant *K. pneumoniae* strains is strongly related to the high rates of mortality. Some strains have even developed widespread drug resistance, thus severely limiting the available treatment options. Remarkably, the 2014 WHO Global Statement on Investigation of Antimicrobic Resistance identified *K. pneumoniae* as one of the topmost three pathogens of international concern. In 2019, studies conducted in Pakistan, Faisalabad and Lahore, shown occurrence of approximately 17% of multi-drug resistant *K. pneumoniae* isolates, with a large number exhibiting resistance to cefoxitin, ampicillin, and meropenem. Furthermore, an investigation including samples from the Shanghai Medical Center in China during the period of 2014 and 2015 showed resistance in forty-one samples to various antibiotics, such as ertapenem, ceftazidime, imipenem, piperacillin/tazobactam, ceftriaxone, ampicillin, cefazolin (Organization, 2014).

The antibiotic resistance mechanism in *K. pneumoniae* involves several genetic and physiological factors (Hughes *et al.*, 2017). The primary resistance mechanism is the acquisition of drug inactivating genes that encode enzymes known as beta-lactamases

having the ability to destroy and break-down beta-lactam antibiotics which includes the penicillin's and the cephalosporins. *Klebsiella pneumoniae* has ability to produce various types of beta-lactamases, like carbapenemases and extended-spectrum beta-lactamases (ESBLs). ESBLs exhibit hydrolytic activity against broader range of beta-lactam antibiotics, while carbapenemases confer resistance to even more potent antibiotics called carbapenems. Among β-lactamases, one important concern is the Amp C β-lactamases production. According to structural classification of beta-lactamases by Ambler, Amp C belongs to class C whereas according to system of classification by Bush based on the functionality, these enzymes belong to the group 1. Amp C β-lactamases have capacity to hydrolyze the penicillin, cephalosporins, oxyimino cephalosporins, monobactams and cephamycin's. Different types of beta lactamase inhibitors like ampicillin/clavulanic acid and piperacillin/tazobactam have no activity against these enzymes. The molecular masses of Amp C enzymes generally range from 30-40 kDa and their isoelectric point is greater than 8.0 (Bedenić *et al.*, 2022).

One more significant resistance mechanism in *K. pneumoniae* is alteration or mutation of target sites for antibiotics. For instance, changes in cell wall protein of bacteria known as penicillin-binding protein (PBP) can decrease the affinity of drugs like penicillin's and cephalosporins for their target sites, rendering them less effective. These alterations can occur due to mutations in the genes encoding PBPs (Navon-Venezia *et al.*, 2017). Efflux pumps are another mechanism employed by *K. pneumoniae* to resist antibiotics. Proactive pumps in the bacterial cell membrane function to prevent the accumulation of toxic antibiotics by pumping out their antibiotic-producing proteins. By doing so, efflux pumps reduce the effectiveness of various antibiotics classes which includes the fluoroquinolones, the tetracyclines, and the aminoglycosides (Padilla *et al.*, 2010).

Additionally, *Klebsiella pneumoniae* can develop resistance through the modification of metabolic pathways or the overproduction of certain enzymes. For example, the bacterium can modify the enzymes involved in synthesizing folic acid, a crucial nutrient for bacterial growth. By altering these enzymes, *K. pneumoniae* becomes less prone to drugs which targets the folic acid synthesis, like the sulfonamides and the trimethoprim (Giedraitienė *et al.*, 2011).

Efflux pumps have five major families that are known for their diverse characteristics and dispersal in bacterial organisms. The families that can be identified are major facilitator superfamily (MFS), the small multidrug resistance family (SMR), and the resistance nodulation cell division family. Different families exhibit differences in their morphology, energy source availability, and selection of excreta or substrates, as well as the type of bacterial organisms they are typically found on (Singh *et al.*, 2021).

Tetracyclines have recently gained prominence as the effective antibiotic and have been widely utilized to treat variety of bacterial infections, including those initiated by *K. pneumoniae* (Doi, 2019). One of the classic and well-known examples of efflux-mediated resistance is tetracycline resistance, where the Tet efflux pumps that belongs to the MFS family expel tetracyclines through exchange of proton as energy source. Over 20 diverse *tet* genes have been identified thus far, with most of them being located within mobile genetic elements. Tet (K) and Teţ (L) are the only genes that occur in gram negative organisms, with their high diversity being observed in all types of gram negative bacteria (Kaatz, 2002). Most of these pumps are effective in marking the tetracycline and doxycyclines, but they do not reduce the susceptibility of minocyclins or tigecyclinates as they cannot serve as substrates for these compounds.

Additionally, both artificial plasmids and chromosomal genes encode tetracycline resistance and pBR322 is renowned example of artificial plasmid that was first widely used cloning vectors and contains genes encoding resistance to various antibiotics, including tetracycline. In pBR322, *tetR* is the tetracycline resistance gene that codes for a repressor protein and impedes the antibiotic activity. By doing so, this gene prevents binding of tetracycline to the bacterial ribosomes, which are responsible for protein synthesis. Consequently, the bacteria carrying the pBR322 plasmid with the *tetR* gene are resistant to the effects of tetracycline (Lenski *et al.*, 1994).

While the chromosomal genes that are responsible for tetracycline resistance includes *tetA* gene present on the bacterial chromosome. This *tetA* gene codes for an efflux pump that actively expels tetracycline out of bacterial cell, thereby preventing the antibiotic from reaching its target in the ribosomes. This efflux pump mechanism is commonly observed in bacteria as one of the ways they develop resistance (May *et al.*, 2009).

Two major chromosomal mediated genes of our concern and part of this study are *entB* and *uge*. The aim is to gain a deeper understanding of relationship between these genes and biofilm formation in *K. pneumonia*. In recent study, *entB* gene in *K. pneumoniae* is linked with siderophore production, that plays life-threatening role in biofilm formation. The production of siderophores by *K. pneumoniae,* facilitated by the *entB* gene, permits bacteria to scavenge and acquire iron from environment more efficiently. By accessing this crucial nutrient*, K. pneumoniae* can enhance its growth and persistence in the biofilm structure, leading to increased biofilm formation and maturation. Biofilms provide bacterial communities with defense from immune system of host and antibiotics. The presence of abundant iron, due to siderophore production through the *entB* gene, further enhances the resilience and survival of *K. pneumoniae* within the biofilm matrix, making it more challenging to eradicate (Cao *et al.*, 2023).

While the *uge* gene encodes for the enzyme UDP-glucose 4-epimerase, which act a chief role in formation of biofilm process. *K. pneumoniae* strains with a functional *uge* gene are more proficient in forming biofilms. EPS are a complex matrix of polysaccharides, proteins, and DNA that are secreted by bacteria during biofilm formation. These substances are essential for the structural integrity and stability of the biofilm. When the *uge* gene is active and producing UDP-glucose 4-epimerase, it leads to an increased production of EPS, resulting in enhanced biofilm formation. So, *K. pneumoniae* biofilms formed due to the activity of the *uge* gene can contribute to persistence of infections and capability of the bacteria to cause chronic and recurrent infections. Overall objective of this study was to highlight the occurrence of *uge* and *entB* genes in the clinical isolates of *K. pneumoniae*. Further, this study aimed to investigate the effect of doxycycline on strong biofilm forming isolates.

Aims and Objectives

2.1 Aims and Objectives

The key objective of this study was to investigate the prevalance of *uge* and *entB* genes among the clinical isolates of *Klebsiella pneumoniae* collected from the tertiary care hospital in Islamabad, Pakistan. This study also intends to identify the sensitive isolates through disk diffusion tests and then to further explore the ability of doxycycline through Minimum Inhibitory Concentration (MICs). Moreover, the research aimed to investigate the effect of doxycycline on the strong biofilm formers that are sensitive towards this antibiotic in the planktonic form. Following key tasks were set to achieve these objectives:

1. To determine the morphological and biochemical characteristics.
2. To optimize and screen *uge* and *entB* genes.
3. To find out the occurrence of these genes through the molecular detection using multiplex PCR.
4. To find out the sensitive, resistant and intermediate isolates against doxycycline through Disk Diffusion Assay.
5. To determine Minimal Inhibitory Concentrations (MICs) of doxycycline against the isolates by microtiter plate method.
6. To make the biofilms of *K. pneumoniae* isolates that are sensitive towards the doxycycline and selection of strong biofilm formers via Optical Density (OD) measurement.
7. To find out the effect of doxycycline on the strong biofilms of *K. pneumoniae* isolates at MBEC and MBIC level.
8. To assess the impact of doxycycline on potent biofilms of *K. pneumoniae* isolates at sub-minimal concentrations.

Literature Review

3.1 *Klebsiella pneumoniae*

Klebsiella pneumoniae is a significant human pathogen recognized for causing a variety of infections, especially in healthcare settings. It is commonly found in the skin, respiratory tract, the human gastrointestinal tract and respiratory tract (Bengoechea *et al.*, 2019). *K. pneumoniae* is a gram-negative, non-motile bacterium. The distinguishing feature of *K. pneumonia* is its encapsulated capsule surrounding the rod-shaped cell which aids in evading the immune system of host. On MacConkey agar and nutrient agar, it shows mucoid consistency which is attributed to thick capsular polysaccharide adhered to outer membrane of bacteria (Priyanka *et al.*, 2020). While other *K. pneumoniae* strains are opportunistic pathogens causing infections in hospitalized, seriously ill and immunocompromised persons. Hence, they are primarily linked to the nosocomial infections like ventilator acquired pneumonia, catheter linked urinary tract infections and blood stream infections. The population-based surveillance study reported by Meatherall *et al* for a period of 8 years reported that about 7.1 persons out of 100,000 people may get bloodstream infections by *K. pneumoniae.* Some of the strains are hypervirulent, means that these can infect healthy people and can cause pyrogenic liver abscess, endophthalmitis, and meningitis. Blood stream infections by *K. pneumoniae* may often lead to the development of cancer (Sawatwong *et al.*, 2019).

3.2 History

Klebsiella pneumoniae was first revealed and named in 1882 by German microbiologist Carl Friedländer. This bacterium gained prominence during 1980s and 1990s as a main leading cause of hospital-acquired infections, mainly in ICUs. These infections were often associated with the use of invasive medical equipment's like ventilators, catheters, and intravenous lines. Additionally, the bacterium is also notorious for causing infections in people with weakened immune systems or chronic illnesses such as diabetes. During the course of time, *K. pneumoniae* has acquired resistance to various frequently used antibiotics, making it difficult for treatment. In recent years, some *K. pneumoniae* strains have established

resistance to even the most powerful drugs including carbapenems, leading to the term "carbapenem-resistant *K. pneumoniae*" or CRKP. The infections caused by CRKP can be extremely challenging to treat leading to high mortality rates (Roshdi Maleki *et al.*, 2021).

3.3 Clinical Epidemiology

Klebsiella pneumoniae is known for causing various infections, like pneumonia, the urinary tract infections, the bloodstream infections, and the wound infections. It is considered as significant healthcare-associated pathogen, as it is frequently developed in healthcare settings and can result in serious infections in patients with weakened immune systems. The clinical epidemiology of *K. pneumoniae* involves analyzing the patterns and traits of the disease in diverse populations (Munoz-Price *et al.*, 2013). This includes different aspects like the incidence and prevalence of infections of *K. pneumoniae*, the specific type of infection it causes, the risk factors for infection, and the outcomes of infection. The infections of *K. pneumoniae* are commonly observed in healthcare settings, mainly in intensive care units (ICUs) and long-term care facilities. The incidence of infections of *K. pneumoniae* differs depending on the population under study and the geographical region, but it is predicted that around 10% of all healthcare-associated infections are caused by *K. pneumoniae*. According to studies, the rate of incidence of hospital acquired pneumonia ranges from 4.4 to 8.8 cases per 10,000 patient-days in intensive care units (ICUs) and 1.7 to 5.6 cases per 10,000 patient-days in non-ICU wards (Kołpa *et al.*, 2018). While the incidence of *K. pneumoniae* in hospitalized patients for bloodstream infections lies in the range of 0.4 to 3.3 cases per 1,000 patient-days. This bacterium is one of the major causes of urinary tract infections in healthcare settings. The colonization (i.e., the bacteria present in the body without causing symptoms) of *K. pneumoniae* prevalence can vary based on the population being studied. For instance, colonization rates in healthy individuals range from 1.3% to 8.8%. The colonization rate for the prevalence of *K. pneumoniae* in hospitalized patients might differs based on the population being examined reaching level as high as 30% in some settings. The prevalence of colonization of *K. pneumoniae* in long-term care facility inhabitants can be as high as 50%. The rates of incidence and prevalence of *K. pneumoniae*

infections are also influenced by elements like antibiotics use, infection control practices, and geographic place (Tängdén *et al.*, 2015). *K. pneumoniae* can cause various infections that includes pneumonia, urinary tract infections, and bloodstream infections. These infections can be severe and leading to high rates of mortality, mostly in patients with underlying medical issues or compromised immune system. The risk of *Klebsiella* infection is higher in patients who have certain risk factors, such as prolonged hospitalization, previous antibiotic use, invasive medical procedures (e.g., intubation or catheterization), and underlying medical conditions such as diabetes or chronic kidney disease.

3.4 Genome characteristics

Klebsiella pneumoniae is known to contain various mobile genetic elements, like plasmids, transposons, and the integrons. These elements can transfer genes between bacteria, including antibiotic resistance genes, and can play a part to the spread of MDR strains. The genome of *K. pneumoniae* is approximately 5-7 million base pairs in length that encode almost 5500 genes. Analysis of large number of *K. pneumoniae* isolates by Holt *et al*., 2015 indicated that more than 95% of isolates possess 2000 genes that are common to them. These 200 genes were then considered as the core genome. The remaining 3000 genes were different in different isolates thus these are considered as the accessory genome. The accessory genome may be possessed by the transfer of horizontal gene in different bacterial species as indicated by the presence of transferable plasmids and other mobile genetic elements in the *K. pneumoniae* genome. Presence of such a huge accessory genome as compared to the core genome indicates that *K. pneumoniae* has access to a vast majority of gene pool and it is considered that the genome of *K. pneumoniae* is open i.e., there could be more of the accessory genes that need to be identified (Snitkin *et al.*, 2012).

Accessory genome is important for bacteria as it helps it to encode different antibiotic resistance enzymes. The accessory genes help bacteria in certain other aspects like nitrogen fixation as well as colonization and infection in the specific host sites. Other functions performed by the accessory genome include carbohydrate metabolism, heavy metal stress

adaptation, and production of polysaccharide capsule. Presence of different types or groups of the accessory genes in different isolates of *K. pneumoniae* is accountable for the differences in their metabolic capabilities which help them to adapt in different types of habitats. A study conducted by Paczosa and Mecsas in 2016 showed the presence of plasmid mediated *lac operon* for the utilization of lactose sugar in the genome of almost 50% *K. pneumoniae* isolates and it was strongly associated with the presence of these isolates in the dairy products (Moynihan). Antibiotic resistance genes like *entB* and *uge* are mostly associated with the chromosomal DNA (Soni *et al.*, 2022).

Although majority of the antibiotic resistance factors arise from the mobile genetic elements, resistance against ampicillin, Fosfomycin and quinolone nalidixic acid is due to the core genome as it possesses bla_{SHV} gene, *fos*A and *oqx*AB genes as part of their core genome. Core genome also contains genes *acr*AB and *oqx*AB for efflux pumps that when overexpressed confer resistance against fluroquinolones and tigecycline. *K. pneumoniae* genome also contains genes *rmpA* and *magA* that code for RmpA and MagA proteins. These proteins are responsible for the hypermucoviscous phenotype of *K. pneumoniae* that is hypervirulent and can cause disease in even healthy patients (Shen *et al.*, 2020).

As with any other genome, *K. pneumoniae* genome is also susceptible to mutations and these mutations confer resistance to *K. pneumoniae* against a wide range of antimicrobial agents. Mutations in the genes *ompK*35 and *ompK*36 that code for outer membrane proteins result in the resistance against fluroquinolones, extended spectrum cephalosporins and even carbapenems. Mutations in the genes encoding for GyrA and ParC DNA gyrase subunits also result in the resistance against fluroquinolone. Mutation due to insertion or deletion of the nucleotides in the genes in *pmrHFIJKLM* operon and in the gene *crrB* give rise to the resistance of *K. pneumoniae* against colistin that is otherwise considered as an effective antibiotic for fluroquinolone and carbapenem resistant isolates (Shields *et al.*, 2017).

3.5 Pathogenesis

The first is the host's immune system, and the second is the organism itself, which are both important elements in pathogenesis. To cause an infection, *K. pneumoniae* must first attach to a host cell and then colonize it. The most common route of entrance is through the respiratory tract, but because of its widespread nature, it is nearly impossible to determine where it came from. The factors can be split into two groups: those that aid in *K. pneumoniae* attachment to host cells, such as fimbriae and flagella, and those that aid in tissue invasion and decrease immunological responses (Highsmith *et al.*, 1985).

3.5.1 Virulence factors

Virulence factors of microorganisms are important for their pathogenesis. These are the microbial surface structures or molecules that help them in the colonization and disease progression in the eukaryotic host. Virulence factors may also be present in the non-pathogenic strains in avirulent form possibly due to the mutation of virulence genes.

Klebsiella pneumoniae possess different types of virulence factors like siderophores, polysaccharide capsule, fimbriae, Pilli, lipopolysaccharide, urease and type IV secretion system. These virulence factors help *K. pneumoniae* to establish disease in its host.

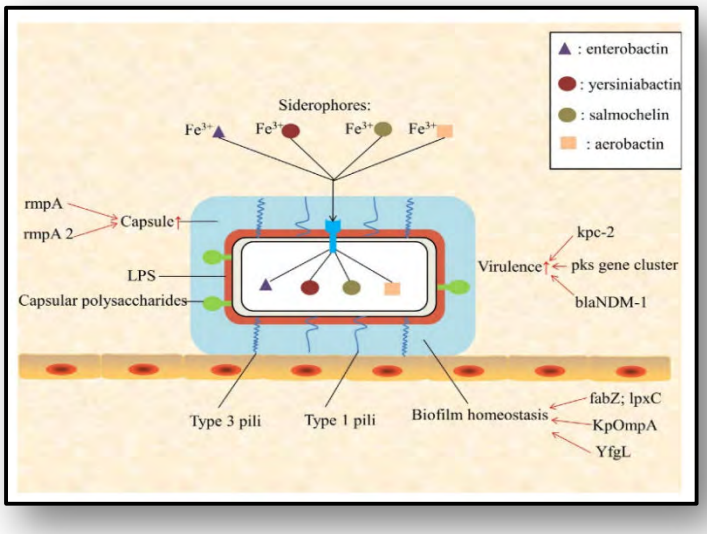


Figure 3.1: Schematic presentation of Klebsiella pneumoniae virulence factors, and biofilm homeostasis.

Table 3. 1: Different virulence factors and their role in the pathogenesis of *Klebsiella pneumoniae*

Factors	Functions
Siderophores	Help to facilitate in the iron acquisition process
Capsular polysaccharide	Opsonization and phagocytosis against macrophages, neutrophils and Dendritic cells
Flagella	Binding to TLRs allows IL-8 to move, attach, and activate.
Exotoxin A	Causes protein synthesis inhibition in host body andorganism dissemination
Urease	Helps bacteria in its survival by causing the hydrolysis of urea and increasing its PH
Fimbriae	In order to attach the host and stimulate the expression of pro-inflammatory genes.
Quorum sensing	Coordinate gene expression and promotes biofilm formation
lipopolysaccharides	An endotoxin that protects the *k. pneumoniae* from the host antimicrobial peptides as well as the polymyxins antibiotics.

3.5.2 Siderophores

Siderophores are small molecules with low molecular weight and high affinity that are secreted by certain bacteria, including *K. pneumoniae*, to facilitate their iron acquisition process (Holden *et al.*, 2015). One common iron chelating molecule is the enterobactin that

has high affinity for iron than any other siderophore. Mammalian innate immune system involves the production of lipocalin 2 that binds with the enterobactin and thus blocking the iron uptake system of bacteria. In order to avoid this, *K. pneumoniae* secretes other types of siderophores like aerobactin, yersiniabactin and salmochelin. Siderophores help the bacteria in iron uptake as well as in the progression of pathogenesis process. Yersiniabactin is prevalent in in most of the human *K. pneumoniae* infections and is associated with the pulmonary infections. Aerobactin is also considered as a main virulence factor of K. *pneumoniae* (Bachman *et al.*, 2009).

3.5.3 Capsular polysaccharide

Capsular polysaccharide is the most significant virulence factor of *K. pneumoniae*. It is present on the bacterial cell surface outside to the outer membrane. It is acidic polysaccharide as it contains uronic acid along with the four to six repeating units of sugar molecules. Although there are 78 different antigenic types of capsules of *K. pneumoniae,* only small number of capsules are related to the diseases in humans. The capsular polysaccharide synthesis (*cps*) gene cluster synthesize the capsule. This gene cluster is about 21-30 kb size and contains 16 to 25 genes that are responsible for the capsule synthesis, its assembly as well as its transport to the outside toward the external surface of the bacterial cell. The 5' terminal of the cps gene cluster contains six conserved genes that are *galF, orf2, wzi, wza, wzb* and *wzc*, whereas 3' terminal contains *gnd* conserved gene. The *wzi* is highly variable and is now being used for the serotyping of different capsular *K. pneumoniae* isolates (Highsmith *et al.*, 1985).

The polysaccharide capsule of *K. pneumoniae* protects it from the processes of opsonization and phagocytosis by the host macrophages, neutrophils and dendritic cells. Neutrophils have the capacity to engulf hypervirulent *K. pneumoniae* isolates through phagocytosis. However, these particular strains of hypervirulent *K. pneumoniae* can evade intracellular destruction and migrate to remote locations, such as the liver, where they can initiate disease progression. This ability of hypervirulent *K. pneumoniae* to escape from neutrophil-

mediated intracellular killing and disseminate to distant organs underscores its potential to cause harm in the body. Capsules possess the anti-inflammatory properties as it can inhibit the interleukin-8 expression through TLR2 and TLR4 signalling (Li *et al.*, 2014).

3.5.4 Urease

It is a nickel containing enzyme that can hydrolyze urea into ammonia and carbamate. Urease is an important virulence factor for those bacteria that colonize the tissue surfaces especially in the urinary tract and indwelling catheters. Urease enzyme causes hydrolysis of urea and increase the PH that leads to the precipitation of inorganic salts and encrustations in the tissue and abiotic surfaces. This situation helps bacteria in its survival and disease progression as urine flow of the host would disturb hence bacteria could avoid clearance due to urine flow and also the formed encrusts help it to form biofilms. *K. pneumoniae* along with other gut pathogens has ability to produce urease that metabolize urea t form nitrogen. Inactivation of urease will affect the growth of *K. pneumoniae* in the gastrointestinal tract (Morris *et al.*, 1999).

3.5.5 Fimbriae

To initiate the process of colonization and infection, *K. pneumoniae* requires adhesion to the host cell. This is achieved by the help of Fimbriae that are thread like structures present on the bacterial cell surface. There are four main types of fimbriae that have been recognized in *K. pneumoniae.* These are Type 1 fimbriae, Type 3 fimbriae, *kpc* fimbriae and KPF-28 fimbriae (Li *et al.*, 2014).

3.5.5.1 Type 1 fimbriae

These are the thin and rigid thread-like structures that are present on outer membrane of *K. pneumoniae*. These are made up of repeating units of FimA molecules and tip contains adhesion molecule FimH. *K. pneumoniae* isolate possessing Type 1 fimbriae adhere to host cells that contain mannose sugars through the FimH adhesion molecule. These are responsible for the urinary tract infections and play part in the gastrointestinal tract and respiratory tract infections (Rosen *et al.*, 2008).

3.5.5.2 Type 3 fimbriae

These are 0.5-2um long structures. Gene *mrkA* synthesize the molecule that polymerize to form the helical shaft. These help *K. pneumoniae* to form biofilms and are involved in catheter associated urinary tract infections through the formation of biofilms. They have no role in the intestines and respiratory tract colonization.

3.5.5.3 *Kpc* fimbriae

These are present in the hypermucoviscous *K. pneumoniae* isolates and are synthesized and assembled by *kpcABCD* operon. These are made up of repeating subunits of *kpcA* along with some molecules of *KpcS* and *KpcI*. These are also involved in the biofilm forming ability of the *K. pneumoniae.*

3.5.5.4 KPF-28 adhesins

These are long and flexible fimbriae. The gene involved in their synthesis is located on the R plasmid that codes for the CAZ-5/SHV-4 β-lactamase. These are considered to be helpful for *K. pneumoniae* in the colonization of human gastrointestinal tract (Raja, 2008).

3.5.6 Quorum sensing

Quorum sensing is the important component of the biofilms. It is the chemical communication system through production of some molecules by the cells of bacteria that help them to regulate their gene expression according to their requirement. It is the transmission mechanisms for bacterial biofilms in the extreme environment. Quorum sensing also helps in the formation of biofilms.

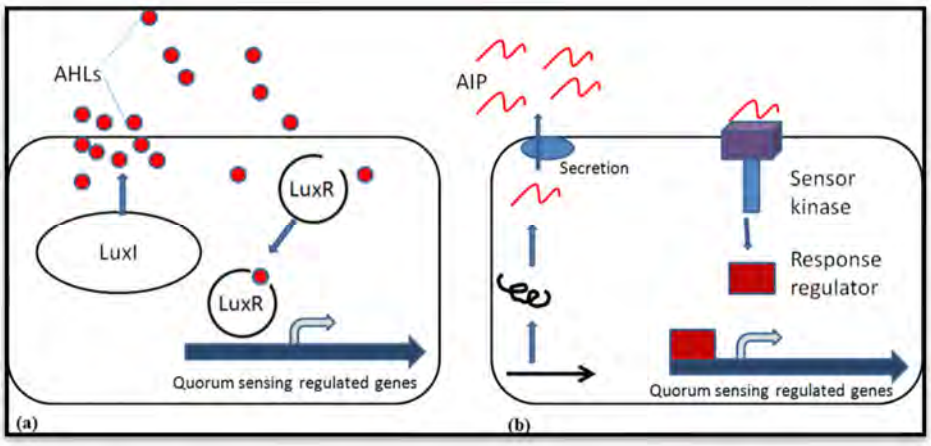


Figure 3. 2: Schematic presentations of quorum sensing systems in Klebsiella pneumoniae.

In *K. pneumoniae*, there are several QS Systems that activate a variety of genes. Two of these are acyl homoserine lactone (AHL)-dependent, meaning that when intracellular AHL levels rise, their transcriptional regulators become active. LasR/LasI and RhIR/RhII systems are two of them. *K. pneumoniae* virulence is being targeted with the QS System. Furanone is a chemical molecule that interferes with QS, causing its pathogenicity to be suppressed. Their more than 300 genes are controlled by QS system that encodes membrane proteins, putative enzymes, transcriptional factors, two component regulatory systems, molecular transporter, virulence factors and other secondary metabolites. Bioluminescence genes are also transcribed by QS system. The Lux gene regulates light emission, and bioluminescence *K. pneumoniae* strains have been employed in a variety of in vivo and in vitro research, including determining pathogenicity in different infections (Chen *et al.*, 2020).

3.5.7 Lipopolysaccharides

Below the capsule, there lies the outer membrane of *K pneumoniae* that is made up of lipopolysaccharides also known as the endotoxin. Lipopolysaccharides is composed of three main parts. First is the innermost lipid A region that is conserved part. Above the lipid A, core polysaccharide is present and then the outermost part is the O-antigen that is highly variable (Farhana *et al.*, 2022). Based on the variability of the O-antigen, there are nine serotypes of *K. pneumoniae.* Out of these nine O-serotypes, three serotypes O1, O2, and O3

are prevalent in the 80% of the infections. O-antigen of the lipopolysaccharide is synthesized by the *wb* gene cluster that contains six genes *wzm, wzt, wbbM, glf, wbbN* and *wbbO.* There are two types of core polysaccharide i.e., type 1 and type 2. These are synthesized from two different *wa* gene clusters. These two gene clusters differ only by the 2 genes. Type 1 has *wabI* and *wabJ* genes while type 2 has *wabK* and *wabM* genes. Other 11 genes of these gene clusters are conserved and same (Clarke *et al.*, 2018).

Lipopolysaccharides protect the *K. pneumoniae* from the host antimicrobial peptides as well as the polymyxins antibiotics. O-antigen prevents the compliment mediated killing by inhibiting the activators. In isolates that lack the endotoxin, capsule protects the organism from the host compliment system. Serum resistance *K. pneumoniae* is also due to the presence of endotoxin especially O1 serotype is highly serum resistant. Lipid A provides protection to the *K. pneumoniae* from the host innate immune responses and antimicrobial peptides. Study conducted on different animal model reported that mutation in the genes of lipid A synthesizing enzymes lead to the absence of virulence from the *K. pneumoniae* isolates (Kidd *et al.*, 2017).

3.6 Biofilm formation

Most of the pathogens have ability to produce biofilms on the soft tissues, respiratory tract, gastrointestinal tract and medical implants. Biofilms are the aggregates or the collection of bacterial cells that are enclosed in the self-produced matrix of exopolysaccharides, proteins and extra cellular DNA. The composition of this extracellular matrix and concentration of matrix constituents vary according to the bacterial species. Biofilms are considered important as they provide protection to the bacterial cells from the host immune responses and antibiotics. These are also involved in the formation of resistant strains as transfer of resistant genes is much easier in the biofilms (Flemming *et al.*, 2010). Almost 60% of the total bacterial infections and 80% of the human chronic infections are due to the formation of biofilms. *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* make the urine alkaline

that facilitate these to form biofilm in the catheters and when these biofilms are detached from the catheter, urinary tract may also get infected.

3.6.1 Stages of biofilm formation

Biofilm formation is the cyclic process and these are formed through the following five stages. First is the attachment through which bacteria become attached to the living or non-living surfaces through the weak interactions like Van der Waals interactions. Second stage is the colonization in which bacteria irreversibly attached to the surfaces with the help of exopolysaccharides, lipopolysaccharides and collagen-binding adhesive proteins. This colonization is achieved through the hydrophobic and hydrophilic interactions. Motility of the bacteria is also reduced in this stage and secretion of exopolysaccharides is maximum as it helps in the trapping of essential nutrients. Third stage is the development of biofilm in which cells arranged in multiple layers through proliferation and exopolysaccharides are produced. Next stage is the maturation in which three dimensional and stable communities are formed having proper distribution of nutrients and signaling molecules through the channels. Fifth stage is the active dispersal in which bacterial cells detach from the biofilms and move to the other locations. This may be due to the factors related to the biofilms itself or maybe due to the host. In this cyclic process, various bacterial enzymes are involved that along with biofilm formation and dispersal also help to degrade the matrix (Tolker-Nielsen, 2015).

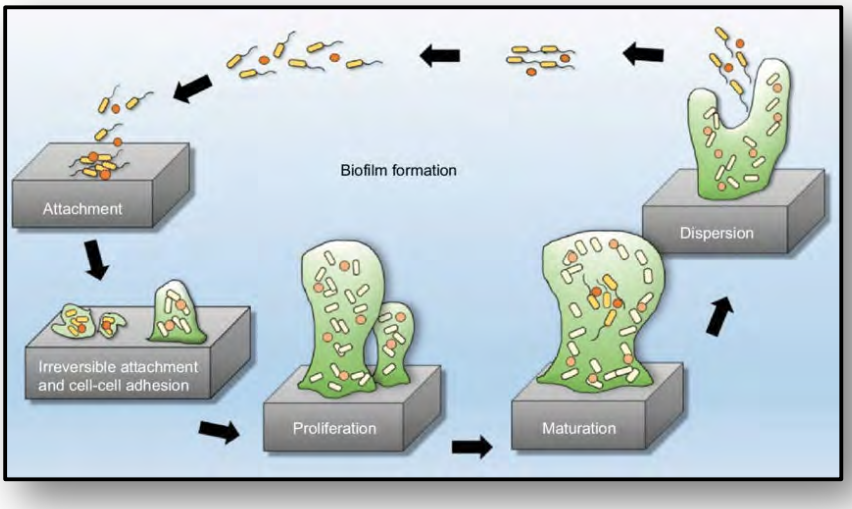


Figure 3. 3: Shows five stages of biofilm development.

3.7 MDR *Klebsiella pneumoniae*

In the process of effective management of antimicrobial resistance, MDR *K. pneumoniae* poses a great risk to the public health as these organisms are recalcitrant to available treatment options. The MDR strains of *Klebsiella* have emerged due to acquisition of diverse beta-lactamases and carbapenemases that has led to the resistance against beta-lactam and carbapenem. First ESBL enzyme was identified in mid 1980s. Up to date diverse beta-lactamases have been identified and categorized into broad-spectrum and extended spectrum Beta-lactamases. Among them CTXM is the most prevalent beta-lactamase worldwide whereas CTXM-14 and CTXM-15 are frequently encountered in clinically significant isolates. These ESBL genes are plasmid borne and part of accessory genome and mobilized by many genetic elements among strains. These MDR strains have potential to hydrolyze third generation oxyimino-cephalosporins including third generation cephalosporin and aztreonam. ESBL production also positively impacts the fitness of the strains. Due to antibiotic selective pressure, carbapenem resistance in *K. pneumoniae* has dramatically increased and *klebsiella* MDR strains are the most common carbapenem resistant Enterobacteriaceae (CRE). According to the reports of CDC (2013), CRE was declared as a

major threat to public health and among 9000 infections 80% of the infections were caused by MDR carbapenem resistant Enterobacteriaceae. It was estimated by The United States Centers for Disease Control and Prevention that above two million people in the United States get infected by multi drug resistant pathogens each year and out of these two million people, almost 23,000 patients die. The genes of carbapenemases also reside on plasmid and an integral part of bacterial accessory genome. According to studies it was also revealed that carbapenem resistance also emerges due to some chromosomal mutations in the core genome and that is driven by the up-regulation of efflux pumps and alteration in the outer-membrane porin channels (Bassetti *et al.*, 2018).

3.8 Emergence of antibiotic resistance in *Klebsiella pneumoniae*

Klebsiella pneumoniae is significant multi-drug resistant pathogen that contributes to the majority of nosocomial infections and associated with high rates of mortality. The widespread and inappropriate use of antibiotics is the major reason for antibiotic resistance development in the microorganisms. It was reported by the World Health Organization time trend analysis that there is a continuous rise in the drug resistance against beta-lactams, carbapenems and colistin. Under immense pressure of antibiotics, the bacterium is evolving through accumulation of multiple antibiotic resistance genes ARGs which has led to the development of extremely drug resistant strains (XDR), which harbors super resistome (Hennequin *et al.*, 2016).

The global emergence of MDR and XDR strains involve multi-factorial process which are dissemination of high-risk multi-resistant genes, acquisition of transposon mediated antibiotic resistance genes, mobilization of multi-resistant plasmids among strains. Many MDR and XDR strains possess self-transmissible genetic elements termed as mobilome. The presence of these diverse mobilome and resistome assists MDR strains to evolve in both epidemic and sporadic sequence type clones (Effah *et al.*, 2020).

According to a surveillance study by the European Antimicrobial Resistance Surveillance Network from 2005 to 2015 that resistance rates in *Escherichia coli* and *Klebsiella*

pneumoniae is continuously increasing and this increase has been more prominent in case of *K. pneumoniae* as compared to *E. coli.*

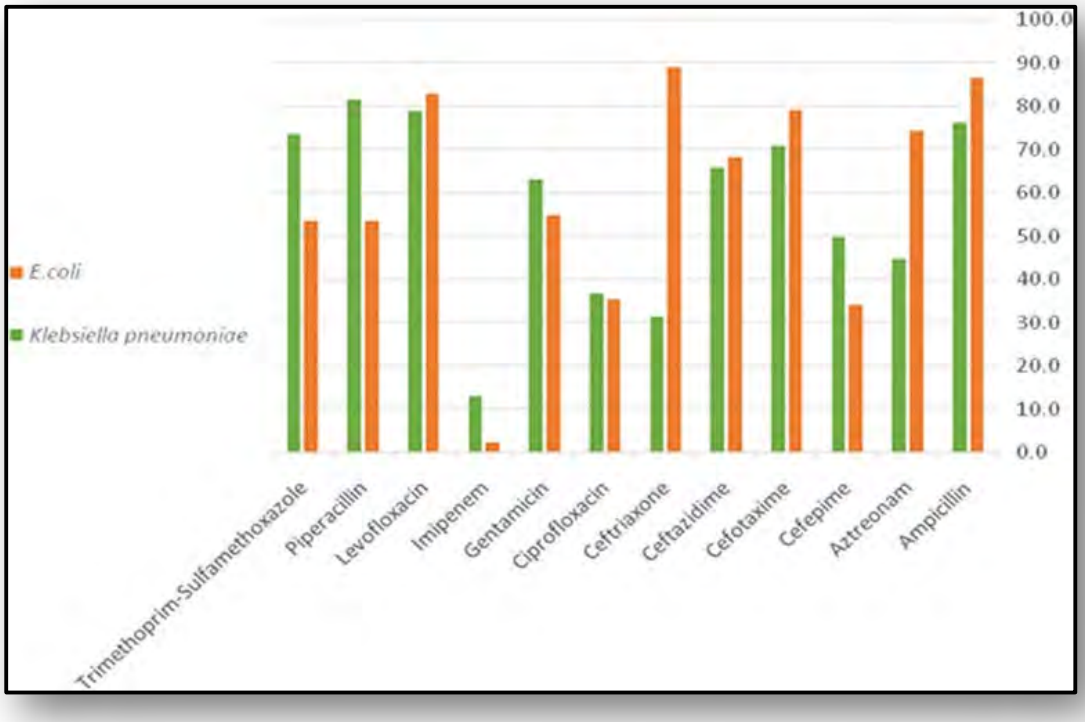


Figure 3. 4: Shows rates of antimicrobial resistance in the collected E. coli and K. pneumoniae in UTIs.

The resistance rate particularly against carbapenems in *K. pneumoniae* was 5% in 2005 and almost 70% in 2015. Similarly, a study conducted in The United States reported that resistance rates among *Enterobacteriaceae* isolates were continuously increase during the period of 1999 to 2011 as 1.39% in 1999 and 3% in 2010-2011. Although *K. pneumoniae* is about 7.7% of total *Enterobacteriaceae* strains in the study but it constitutes almost 33.1% of ESBL associated resistance. Multi drug resistant *K. pneumoniae* caused infections are associated with the increased rates of mortality like 40-50% particularly among the patients that are hospitalized and critically ill. 41 samples from the Shanghai medical center China were collected and all of these were Multi drug resistant. A study conducted in Faisalabad Pakistan, reported that 15% of the total *K. pneumoniae* isolates were MDR (Navon-Venezia *et al.*, 2017).

Emergence of drug resistance is due to the presence of mobile genetic elements carrying various factors for resistance against antibiotics. Resistance mechanism in *K. pneumoniae* may involve the production of ESBL's, efflux pumps, and alteration or absence of target sites (Stokes *et al.*, 2011).

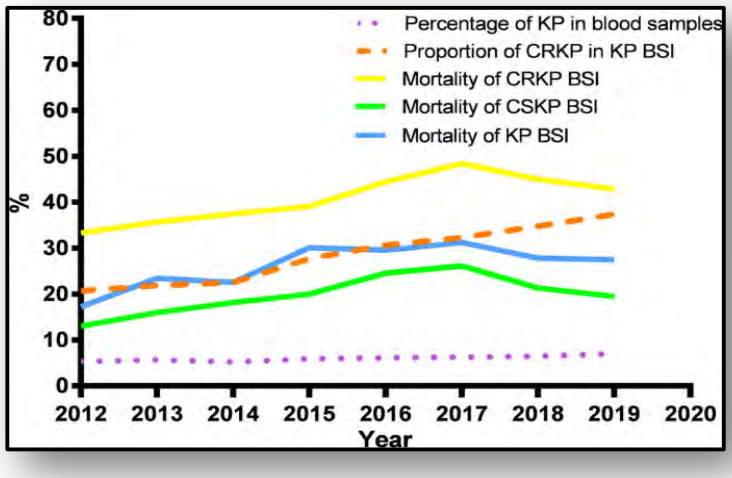


Figure 3. 5: Shows trends in prevalence and mortality of Klebsiella pneumoniae bloodstream infections in Xiangya Hospital, Hunan Province, China, 2012–2019.

3.8.1 Tigecycline Resistance

The tigecycline was initially considered as the potent drug due to its broad-spectrum activity. However, after its extensive use *K. pneumoniae* evolved due to various effective tactics and complicated mechanism of resistance. These include alteration of drug-target site involving modification in both 30S and 16S subunits, alteration in cell membrane permeability, upregulation of efflux pump e.g., AcrAB-TolC and OqxAB. All these strategies adapted by MDR and XDR *K. pneumoniae* has significantly contributed in increase resistance to Tigecycline. The rate of resistance was also higher in case of Ompk35 porin loss (Elgendy *et al.*, 2018).

3.8.2 Resistance against Trimethoprim and Sulfonamides

Sulfonamides is used alone or in combination and can be considered as a treatment option for the *K. pneumoniae* infections. These drugs i.e., sulfonamides inhibit the dihydropteroate synthetase activity whereas trimethoprim inhibits the dihydrofolate synthetase activity. So, by acting synergistically, these drugs affect the synthesis of folic acids. Resistance against these drugs is achieved through the mutations in the chromosomal genes *sul* and *dfr*. *K. pneumoniae* strains also showed the resistance against sulfonamides and trimethoprim (Eliopoulos *et al.*, 2001).

3.8.3 Colistin resistance

Colistin is the drug of choice for most of the MDR pathogens. It disrupts the bacterial plasma membrane by binding with the lipid A. *K. pneumoniae* gained resistance against colistin by modifying its lipopolysaccharides with the addition of 4-amino-4-deoxy-L-arabinose in the Lipid A component. Colistin resistance may be also due to the mutations in the three chromosomal genes that are *mgrB, phoQ, and ccrAB*. Plasmid mediated enzyme phosphoethanolamine transferase MCR-1 has also role in colistin resistance in *K. pneumoniae* (Wright *et al.*, 2015).

3.8.4 β- Lactam resistance

β- Lactam antibiotics are a broad group of antibiotics that are extensively used and include penicillin's, cephalosporins, carbapenems and monobactams. These antibiotics have characteristic β-lactam ring in their structure. The mechanism for resistance against β-lactam antibiotics have the production of β-lactamases that destroy the β-lactam ring. Alexander flemming was the first one to notice that some pathogens including *E. coli* could not get inhibited or destroyed by penicillin and then later on it was discovered that it was due the production of some bacterial enzymes. *K. pneumoniae* is considered as naturally non-susceptible to β-lactams as it can produce SHV-1 type β-lactamase through the chromosomal genes (García-Fernández *et al.*, 2010). Study conducted in Lahore, Pakistan in 2017 reported that almost 82.4% of *K. pneumoniae* samples were β-lactamase producers. Use of β-

lactamase inhibitors like clavulanic acid is effective in treating the infections caused by β-lactamases producer organisms as clavulanic acid can destroy the activity of β-lactamases (Idrees *et al.*, 2022).

β- Lactamase enzymes are the bacterially produced enzymes that can hydrolyze the β-lactam ring in different antibiotics like penicillin's, cephalosporins, carbapenems and monobactams. It is considered that penicillin binding proteins may be evolved due to selective pressure to form the β-lactamase enzymes. There are four major classes of β-lactamases according to their structure i.e. A, B, C and D and these classes include different ESBLs, cephalosporinases and carbapenemases (Alfei *et al.*, 2022).

3.8.5 Aminoglycoside resistance

From 1940-1980 aminoglycoside resistance were actively used against MDR *K. pneumoniae* and replaced third generation cephalosporin, carbapenem and quinolones. During this period, another unique and versatile mechanism of antibiotic resistance has emerged in *K. pneumoniae* strains which involve alteration in the drug structure. The modification of the drug is achieved through the diverse mechanism including acylation, adenylation, and phosphorylation (Yan *et al.*, 2004). Another significant mechanism of aminoglycoside resistance involves the production of 16Sr RNA methylase, belonging *armA* gene family. The mechanism of this enzyme involves the inhibition of drug to bind to its target site i.e., 16Sr RNA. Elevated resistance against aminoglycosides is also chromosomal mediated. Major chromosomal resistance mechanism involves alteration in cell permeability, upregulation of efflux pump such as AcrAB-TolC and down-regulation of major porins such as Ompk35 and Ompk36 (Galani *et al.*, 2019).

3.8.6 Fluoroquinolones Resistance

Due to their spectrum of activity, their phenotypic profiles and their generally good therapeutic index, fluoroquinolones are most widely used antibiotics. Fluoroquinolones inhibit the bacterial growth by interfering with the DNA replication cycle (Drlica, 1999). Resistance against fluoroquinolones is mediated through the mutations in quinolone

resistance determining regions in the *gyrA* and *parC* genes in the chromosomes, plasmids associated resistance, alterations or minimized expression of the porin proteins, and increased expression of the efflux pumps (Martinez-Martinez *et al.*, 2008). The prevalence of plasmid mediated *qnr* genes in *K. pneumoniae* isolates were reported to be 3.9% in Taiwan. It is also proposed that fluoroquinolones resistance is largely associated with efflux pumps rather any other mechanism. Presence of siderophores is also responsible for the increased resistance against ciprofloxacin. The study conducted by Wenli Zhang *et al*., 2017 demonstrated that among *K. pneumoniae* isolates having siderophores, 69.23% were resistant against ciprofloxacin (Yang *et al.*, 2008).

3.9 A targeted approach for the investigation of gene mutation in Type II Topoisomerase

The presence of resistance in quinolone resistant determining region (QRDR) significantly enhance the level of resistance against fluoroquinolones, as these DNA segments encode for the primary and secondary target of the drug. A substitution of single amino acid in this region alters the overall architecture of the target resulting in the reduced affinity of the drug towards the target site. Mutation in the *gyrA* and *parC* are the most commonly observed as compared to the *gyrB* and *parE* subunits. The amount of resistance caused by single amino acid substitution in DNA gyrase or topoisomerase varies depending upon the specific quinolone drug and bacterial species. Target site mutation results in 16 folds increase in resistance whereas concomitant mutation in DNA gyrase and Topoisomerase IV increases MIC value up to 100 folds (Lascols *et al.*, 2007).

3.10 An overview of tetracycline's

Tetracyclines have become increasingly popular, as widely used antimicrobials, and are currently being used to treat bacterial infections, including those caused by *K. pneumoniae*. The initial tetracyclines were derived from the fermentations of actinomycetes. They are preferred due to their efficient tolerance, the mode of action and body excretion rate. However, it is important to note that strains of *K. pneumoniae* can vary in their susceptibility

patterns and may develop resistance to tetracycline antibiotics through mechanisms such, as efflux pumps, ribosomal protection proteins and enzymatic inactivation (Santajit *et al.*, 2016). Recent surveillance studies have shown that 66.9% of selected countries have reported tetracycline resistance while ESBL producing Escherichia coli and *Klebsiella* species (spp.) exhibit resistance rates of 69.1% and 24.3% respectively. Additionally, MRSA (Methicillin Staphylococcus aureus) and Streptococcus pneumoniae show occurrence rates of 8.7% and 22.3% respectively (Fritsche *et al.*, 2007).

Resistance to tetracycline is often achieved through the resistance genes acquisition. The efflux pumps, which are encoded by genes on plasmids and other mobile genetic elements, actively expel tetracycline from the bacterial cell, thereby decreasing its intracellular concentration and hindering its effectiveness. A resistance gene called *tet(M)* encodes a ribosomal protection protein that binds to the underlying RNA and prevents direct oxidation of proteins (Tetracycline) from inhibiting protein synthesis (Roberts, 2005). Excluding the recently described mosaic tetracycline resistance genes, 23 genes have been identified that encode efflux pumps and 11 genes are encoded as ribosomal protection proteins. Earlier clinical studies have demonstrated that the *tetB* gene, which is located on highly mobile genetic elements that can migrate between bacterial species, is the most prevalent determinant of inherited nephrogenic clefties. Various incompatibility groups on conjugative plasmids contain the gene *tetA*. Acquired resistance genes and mutations in the bacterial genome are also responsible for tetracycline resistance in *K. pneumoniae*. The impact of these mutations can vary depending on the component(s) involved in tetracycline binding or transport, resulting in altered permeability of the bacterial cell membrane or reduced affinity of an antibiotic for its target sites (Chopra *et al.*, 2001).

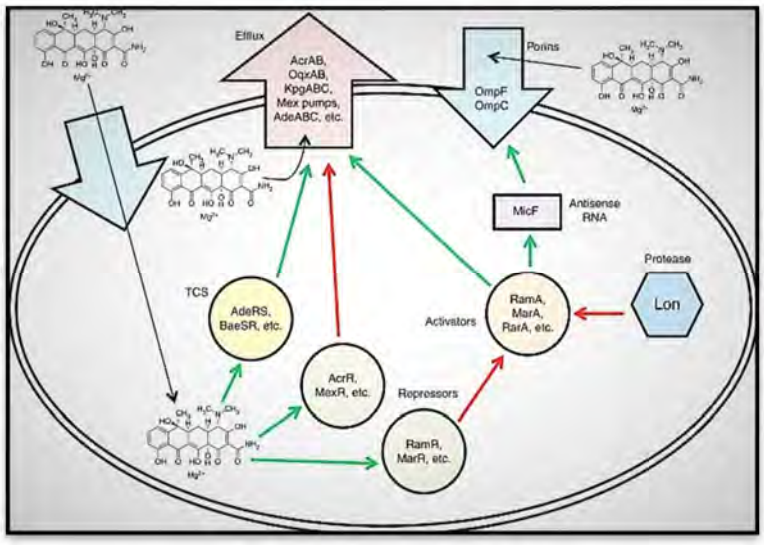


Figure 3. 6: Demonstrates regulation of the expression of intrinsic multidrug resistance mechanisms in Gram-negative bacteria that affect tetracyclines. The known regulatory mechanisms that affect tetracycline susceptibility are listed. Tetracycline resistance is "increased" in the interactions with green arrows, and "reduced" in the interactions with red arrows.

3.10.1 Mechanism of action of tetracycline

Tetracyclines work by preventing bacteria from making proteins, which is one of their mechanisms of action. The A-site, which is where incoming aminoacyl-tRNA molecules typically bind during protein synthesis, is where tetracycline binds reversibly to the 30S ribosomal subunit of the bacterial ribosome. Tetracycline inhibits the attachment of aminoacyl-tRNA to the mRNA-ribosome complex by interacting with the ribosome. This interference messes up protein synthesis's elongation phase, which ultimately stops bacterial growth. Tetracycline works by preventing aminoacyl-tRNA from accessing the ribosome's acceptor site (A-site), which stops new amino acids from being added to the peptide chain as it grows. Protein synthesis is consequently stopped, which prevents bacterial replication and ultimately causes the infection to be eradicated (Chukwudi, 2016).

Tetracycline can also impact the structure and permeability of the cell membrane apart, from its effects on factors. Depending on the concentration and specific circumstances this

disruption can contribute to either inhibiting growth or killing bacteria. It's important to note that tetracycline operates differently in cells compared to cells, which explains why it only affects bacterial cells. One of the ways in which tetracycline effectively treats infections while minimizing harm to the host is, by targeting bacterial protein synthesis (Chukwudi, 2016).

3.10.2 Structure of Tetracycline

Tetracycline is a broad-spectrum antibiotic. Tetracycline has a structural structure that is composed of tetracycle nuclei, which is made up of four rings. The chemical name of tetracycline is 4 dimethylamino(s): 1,4,4a,5,5a; 11,12 and 12 amines: 3,6,10,12,11 pentahydroxy-6 methyl-12 naphthacenecarboxamide. Tetracycline's core consists of three congruent six-membered rings (A, B and C) and one five-mitgliededic ring (C). The letters A, B, C, and D represent the rings that start on the left side of the molecule. The moiety of naphthalene is found in ring A, which is fused to rhizosphere B via a carbon carbon bond. Fused ring B is connected to a pair of rings called rnds c and d, where reliant radii (ring D) are connected with rut C. The letters A, B, C, and D represent the rings that start on the left side of the molecule. The moiety of naphthalene is found in ring A, which is fused to rhizosphere B via a carbon carbon bond. Ring B is fused to ring C, and re-ring D is linked to the same cylinder. These two rings combine to form a unique tetracyclic compound known as 'tiracin'. Multiple functional groups are present in tetracycline at different locations within the cytoskeleton. Four hydroxyl groups (OH) and an amino group (NH2) are present at positions 4, 5, 6, and 7 in the three compounds. The antibiotic activity of tetracycline is dependent on its functional groups. The dimethylamino group (N (CH3)2) is present in ring D of tetracycline at position 4a. The molecule's stability and solubility are further enhanced by this substituent (Zakeri *et al.*, 2008).

Doxycycline:

Tetracycline is the primary antibiotic used, and doxycyclines are also widely used.' This synthetic derivative is a modification of natural antibiotics that contain tetracyclines. Various

bacterial pathogens, such as *K. pneumoniae*, can be targeted with the broad-spectrum activity of doxycycline. Its primary function is to inhibit the bacterial protein production by targeting their ribosomes.

The bacterial subunit's 30S ribosomal subunit is targeted by the antibiotic drug doxycycline, which blocks the aminoacyl terminus' binding site to an end of their 30-lig proteins. The bacteria's protein synthesis is prevented from continuing to synthesize more amino acids due to this. Bacterial growth and reproduction are hindered by the disruption of protein synthesis, which contributes to the bacteriostatic effect of antibiotics. Tetracycline is the primary antibiotic used, and doxycyclines are also widely used.' This synthetic derivative is a modification of natural antibiotics that contain tetracyclines. Various bacterial pathogens, such as *K. pneumoniae*, can be targeted with the broad-spectrum activity of doxycycline. Its primary function is to inhibit the bacterial protein production by targeting their ribosomes (Saifi *et al.*, 2013).

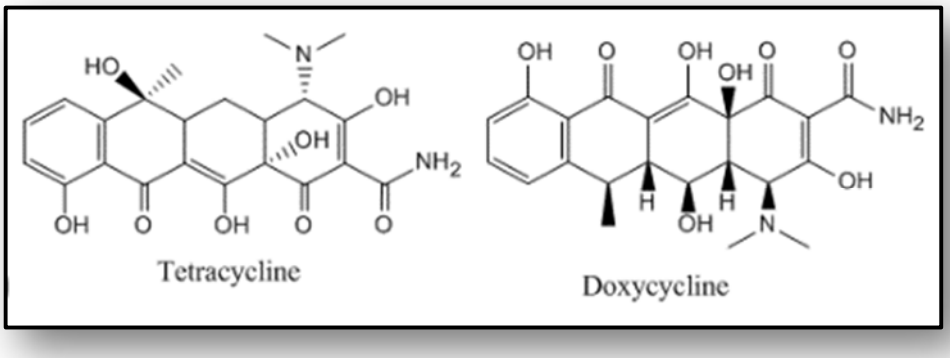


Figure 3. 7: Shows chemical structure of tetracycline and doxycycline.

3.10.3 Regulation of tetracycline resistance genes

Tet (A), Tetra (B), and Tet (C) are the typical resistance genes of tetracycline in *K. pneumoniae,* while other proteins like ribosomal protection are present as well. The presence of tetracycline in nature and other factors play a role in the regulation of these recurrent opioids, particularly resistance genes. The resistance genes are typically suppressed or low-pitched up when tetracycline is not present. Conversely, exposure to tetracycline triggers the

activation of regulatory mechanisms that activate these genes, leading to antibiotic resistance (Wang *et al.*, 2014).

This is largely dependent on the TetR transcriptional regulators, which are among the primary contributory systems. Regulators often serve as suppressors, binding to particular DNA sequences known as operator sites upstream of resistance genes. The binding of TetR proteins to the operator sites occurs in the absence of tetracycline, leading to resistance gene Tetracycline resistance in *K. pneumoniae* transcription. When tetracycline is present, the antibiotic binds to the TetR protein, which causes a conformational change that disrupts its binding to operator sites. RNA polymerase is activated to initiate transcription and generate tetracycline resistance proteins (Deng *et al.*, 2013).

The use of two component signal transduction systems is an additional regulatory mechanism. The system comprises a sensor protein found in the bacterial membrane and cellular receptor protein that regulates gene expression to specific signals. The regulation of tetracycline resistance in *K. pneumoniae* is associated with two component systems, PhoP PhiQ and BaeS BaE. It is still uncertain how tetracycline resistance genes are controlled by these systems.

In addition, tetracycline resistance in *K. pneumoniae* can be affected by global regulatory systems like the LysR family of regulators and the CpxAR two component system. Their regulatory networks can indirectly impact the expression of tetracycline resistance genes, and these systems are responsible for controlling many cellular processes. Importantly, the regulation of tetracycline resistance genes in *K. pneumoniae* can be multi-dimensional and involve interactions with multiple regulatory systems. Specific and interspecific interactions between regulatory mechanisms and *K. pneumoniae* strains may differ (Srinivasan *et al.*, 2013).

The role of tetracycline resistance genes in *K. pneumoniae* is being investigated to determine the most effective treatment options for antibiotic resistance. Targeting these regulatory mechanisms may increase the susceptibility of tetracycline to antibiotic treatment and

improve the effectiveness of treating *K. pneumoniae* infections. Also, understanding these regulatory mechanisms can assist in developing new that can inhibit the expression of tetracycline resistance genes and minimize the spread of antibiotic resistance in clinical settings.

3.11 Role of *uge and entB* genes in biofilm formation

This research aims to shed light on the roles of *uge* and *EntB* in biofilm development in *K. pneumonia* as there is limited data reported on these genes and also how these genes specifically influence the biofilm formation process in *K. pneumonia*. So, the major concern of this study is to gain a deeper understanding of the relationship between the *uge* and *entB* genes and biofilm formation in *K. pneumonia*. By investigating their contribution to biofilm development could potentially lead to better strategies for managing biofilm-related infections and combating antibiotic resistance in this pathogen.

***uge* (UDP-glucose 4-epimerase):** UDP glucose 4 epimerases, which plays a role in the biosynthesis of extracellular polysaccharides, is encoded by this gene. The biofilm matrix in *K. pneumoniae* is composed of these polysaccharides. Structured communities of bacteria attached to surfaces, known as biofilms, are crucial for the virulence and antibiotic resistance of these microorganisms. *uge* plays a crucial role in the conversion of UDP glucose into UDE galactose, which is utilized in producing polysaccharides essential for their stability and growth (Das, 2022).

***entB* (Enterobactin synthase component B):** *entB* gene is a key component of the enterobactin synthase enzyme, which plays clerical roles in producing enterotic bacteria. The enzyme enterobactin is a type of iron chelating molecule that is produced by *K. pneumoniae* and used to scavenge for iron from the surrounding environment. Iron is an essential nutrient for bacterial growth, and siderophores provide this energy to bacteria in iron-scarce environments such as the human body, where they can survive. By promoting the growth of bacteria and providing a competitive edge against nearby microorganisms, enterobactin can indirectly influence the formation of biofilms (Hamzaoui *et al.*, 2018).

3.12 Some novel approaches to combat the effect of tetracycline resistance

Targeting the regulatory mechanisms involved in tetracycline resistance can be a potential strategy to restore susceptibility to tetracycline and improve the effectiveness of antibiotic treatment against *Klebsiella pneumoniae* infections (Nelson *et al.*, 1999). Here are some approaches that can be explored:

1. Repressor-based strategies: One approach is to target the repressor proteins, such as TetR, that bind to the operator sites and prevent the expression of tetracycline resistance genes. Small molecules or compounds can be designed to disrupt the binding of the repressor proteins to the operator sites, thereby allowing the expression of the resistance genes to be suppressed even in the presence of tetracycline. By inhibiting the function of these repressors, tetracycline resistance can be diminished, rendering the bacteria susceptible to the antibiotic (Ramos *et al.*, 2005).

2. Two-component system inhibitors: Another potential strategy is to develop inhibitors that target the sensor or response regulator proteins of two-component systems involved in tetracycline resistance regulation. By blocking these proteins' activity, it may be possible to disrupt the signaling pathways that lead to the activation of tetracycline resistance genes. This approach requires a thorough understanding of the specific two-component systems involved in tetracycline resistance regulation in *K. pneumoniae* and the development of targeted inhibitors against them (Worthington *et al.*, 2013).

3. Global regulatory system modulation: Global regulatory systems, such as the LysR family regulators or the CpxAR two-component system, can indirectly influence the expression of tetracycline resistance genes. Modulating these global regulators can potentially affect the overall regulatory network, leading to reduced tetracycline resistance. Identifying specific targets within these systems and designing

modulators or inhibitors to alter their activity may offer a promising avenue for intervention (Davin-Regli *et al.*, 2021).

4. Combination therapies: Combining tetracycline with other antibiotics or adjuvants that target different aspects of bacterial resistance mechanisms can enhance the effectiveness of treatment against *K. pneumoniae* infections. For example, combining tetracycline with an efflux pump inhibitor can prevent the active efflux of tetracycline, thereby increasing its intracellular concentration and overcoming resistance. Additionally, combining tetracycline with drugs that target other resistance mechanisms, such as those affecting cell wall synthesis or protein translation, can synergistically improve the antibacterial effect (Dhanda *et al.*, 2023).

5. Alternative therapies: Exploring alternative therapeutic options, such as phage therapy or the use of bacteriocins, can provide alternative treatment strategies against tetracycline-resistant *K. pneumoniae* infections. Bacteriophages can specifically target and kill bacteria, including antibiotic-resistant strains, while bacteriocins are antimicrobial peptides produced by bacteria that can selectively inhibit the growth of certain pathogens. These approaches can bypass the need to restore susceptibility to tetracycline and directly target the resistant bacteria (Corrêa *et al.*, 2020).

Chapter 04

Material & Methods

4.1 Materials and Methods

All the experiments were performed in the laboratory of Genomics and Molecular epidemiology, Department of Microbiology, Quaid-e-Azam University Islamabad.

4.2 Chemicals and Glassware

4.2.1 Glassware

Petri plates, test tubes, beakers, conical flasks, glass slides, graded cylinders, and pipettes were among the glassware.

4.2.2 Equipment

Autoclave, Laminar Flow Hood (LFH), digital balance, incubator, refrigerator, microscope, water bath, centrifuge, vortex, PCR and gel electrophoresis equipment were used to perform the experiments properly.

4.2.3 Miscellaneous

Inoculating loop, sterilized swabs, aluminum foil, scotch tape, permanent marker, test tube racks, matchsticks, burner, syringe, micropipette tips, filter paper, toothpicks, scissors, and measuring rulers were used.

4.2.4 Chemicals

All the chemicals used were laboratory grade and included ethanol, distilled water, tryptic soya agar, MacConkey agar, Muller Hinton agar, Simon's citrate media, TSI media, Urease broth, peptone, oxidase reagent, hydrogen peroxide, crystal violet, phosphate buffered saline, methanol, Gram Iodine, oil for microscopy and safranin.

4.3 Collection of Samples

156 samples of *Klebsiella pneumoniae* were collected from tertiary care hospitals in Rawalpindi and Islamabad, Pakistan. These samples were then carefully preserved in 40% glycerol solution.

4.4 Culturing of *Klebsiella pneumoniae*

Glycerol preserved cultures were revived in the nutrient broth for enrichment and then plated on the MacConkey agar plates. The plates were incubated for 24 hours at temperatures ranging from 37 to 42 degrees Celsius and then these plates were checked for bacterial growth after the incubation durations.

4.5 Morphological Identification

This included colony morphology and Gram staining.

4.5.1 Colony morphology

Klebsiella pneumoniae isolates were streaked on MacConkey agar plates. The plates were streaked and then incubated for 24 hours at 37 degrees Celsius. Colonies were carefully examined in Laminar Flow Hood (LFH) after incubation to ensure aseptic conditions and to evaluate morphological traits.

4.5.2 Gram Staining-microscopy

Gram staining was used to determine the morphology of bacteria so that gram-positive and gram-negative bacteria could be distinguished. A sterilized toothpick was used to retrieve an isolated colony from each sample plate after 24 hours of incubation. The procedure was carried out on a glass slide. Using a dropper, a drop of saline was applied to the glass slide. The colony was picked up and mixed with saline to make a large smear. For a few minutes, the smear was allowed to air dry. After drying, a few drops of Crystal Violet were applied to the slide, which was then rinsed with water after one minute by holding the slide in an inclined position. The mordent, Gram's Iodine was put onto the slide for one minute before being washed with water. To decolorize, 95% ethanol was applied for a few seconds and then rinsed off. Finally, Safranin was located for one minute before being rinsed. After all of these applications, the slide was fully air dried before being heat fixed for a few seconds. Using emulsion oil, the slide was examined under a microscope with a 100X lens.

4.6 Biochemical identification

4.6.1 Protocol for oxidase test

Principle: This test detects the presence of cytochrome C oxidase, an enzyme in the bacteria's electron transport chain.

Procedure: Tetramethlyl-p-phenylendiaminedihydrochloride, an oxidase reagent, was freshly produced by dissolving 0.1g of the reagent in 10 ml of distilled water. The reagent was applied on the filter paper, and a few colonies from fresh cultures were rubbed on it. Change in color of the colonies to blue or purple indicates positive result.

4.6.2 Protocol for catalase test

Principle: This test is used to determine whether or not catalase enzyme is present. Bacteria generate the catalase enzyme, which breaks down H2O2 and releases free oxygen.

Procedure: This experiment was carried out on a glass slide. A sterile toothpick was used to place a drop of 3% H2O2 on the slide, then a single and pure colony from fresh culture was picked up and combined with H2O2 on the slide, where bubbling was observed

4.6.3 Protocol for citrate test

Principle: This test is used to detect the microorganisms that have the ability to utilize as a carbon source.

Procedure: By carefully measuring the media, Simmons citrate agar slants were created. The slants were mixed with *K. pneumoniae* test strains and cultured for 24 hours at 37 degrees Celsius. Next day, change in color of media was observed. No change in color or no growth on slant was considered as negative result.

4.6.4 Protocol for Indole test

Principle: This test is used to detect the enzyme tryptophanase which breakdown tryptophan into ammonia, indole and pyruvic acid. The indole production is confirmed by adding KOVAC's reagent

Procedure: Peptone water was prepared in sterilized test tubes. The test tubes were inoculated with test strains and incubated overnight. After incubation, KOVAC's reagents was added to the tubes to detect indole production. Control was left un-inoculated and cherry red colored ring formation on the top of broth culture was noted.

4.6.5 Protocol for urease test

Principle: This test is used for determination of ability of microbes to produce urease enzyme and hydrolyze urea into ammonia and carbon dioxide.

Procedure: Test strains were streaked on agar slants and incubated for 24 hours at 37 degrees centigrade on urea agar slants made in sterilized test tubes by sterilizing an inoculating loop. The controls were not inoculated. There was observed a color change.

4.7 Molecular Identification of *Klebsiella pneumonia*

After identification through morphological features and biochemical testing, molecular techniques were used to confirm the bacterial isolate's identity. These techniques involve polymerase chain reaction (PCR), but before PCR DNA extraction was done through CTAB method. After PCR, gel electrophoresis was done to visualize and decipher PCR results.

4.7.1 Isolation of bacteria

To isolate pure culture, inoculum was taken from the sample that was identified morphologically and biochemically and streaked on MacConkey agar plates, incubated at 37 degrees Celsius for 18 to 34 hours.

Then next day, pure isolated colonies were picked using sterile inoculating loop and then suspended into 1ml Eppendorf containing saline or nutrient broth. The bacterial suspension

was centrifuged 2 to 3 times to obtain heavy pallet. After achieving the desired pallet, normal saline or nutrient broth was drained out.

4.7.2 Genomic DNA extraction

CTAB method:

The extraction was done using CTAB method which include following steps;

1. First make culture of the respective bacteria overnight (1ml).
2. Next day centrifuge it at 13,000 revolution per minute (rpm) for 5 minutes. At a result of this centrifugation, supernatant and pallet will be produced. And after that discard the supernatant.
3. Now, add 80ul TE buffer and 20ul of NaCl (5M) into the pallet. Again, re-suspend by vortexing. Now, add 10ul CTAB to each Eppendorf tube and incubate these at 60 degrees Celsius for 20 minutes.
4. Now, add 100ul of chloroform in Eppendorf tubes and vortex it. Again, incubate these tubes on ice for 30 minutes.
5. Then, centrifuge in a microfuge at 10,000rpm for 10 minutes. Again, supernatant and pallet will be produced.
6. Now transfer the supernatant to the newly labelled Eppendorf tubes. Add 100ul of phenol-chloroform to the tubes and vortex to get a milky solution at top speed for 5 minutes.
7. Again, add supernatant to the newly labelled Eppendorf.
8. Now, add 10ul of Na-acetate and 200ul of 100% ice-cold ethanol to the precipitates and incubate ate -20 degree Celsius for 1 hour.
9. After completion of time, take out samples from the fridge and centrifuge at top speed for 10 minutes.
10. Now, discard the supernatant and re-suspend the pallet by adding 70% of ethanol (100ul). Then centrifuge again to obtain the DNA pallets. After centrifugation, drain out the ethanol from Eppendorf and keep their lids open for drying.

11. And lastly, again add 20ul of TE buffer in the suspended pallet and then store DNA at -20 degree Celsius.

4.7.3 Ethidium bromide fluorescence quantification

In this method of DNA quantification, small amount (3.5ul) of DNA is run on the agarose gel which is subsequently stained with Ethidium bromide. This method is simple and instrument free i.e., does not require any fancy equipment of quantifying DNA such as Spectrophotometer and Nanodrop.

The gel-based Ethidium bromide fluorescence is based on the principle that Ethidium bromide emits red/orange light in response to excitation by UV light and the emission is highly increased when DNA subunits are intercalated by Ehidium bromide.

Procedure:

First of all, 4ul Ethidium bromide was mixed with 1% agarose that was prepared in 1X TBE buffer. Upon polymerizing the gel, the 2-3ul extracted DNA was mixed with 1.5ul of Thermo scientific's 6X loading dye and loaded into wells. A thermo scientific 1Kb ladder was utilized as a DNA marker and loaded into wells. A 1X TBE buffer was used to run the gel at a current of 400mA, 100volts, and time of 35-40 minutes. The process was repeated with water. Then, after the modified time interval, gel was examined under UV trans illuminator to see DNA bands.' The BIO RAD documentation system captured images of the bands.

4.7.4 Spectrophotometric quantification

It is the most commonly used method in laboratories to determine average concentration of DNA or RNA as well as the purity of the sample.

Principle:

The principle of spectrophototometric quantification is that it is based on Beer Lambert equation and the fact that the nucleic acid has the maximum absorption of 262 nm.

Procedure:

Different dilution of DNA was made in 1X TE buffer of distilled water and can also be used to quantify DNA spectrophotometrically. DNA concentration was calculated by following formula:

dsDNA concentration ug/ml= abs 260 x 50ug/ml

Following formula was used for concentration of diluted samples.

dsDNA concentration ug/ml= abs260 x 50ug/ml x Dilution factor

4.8 Detection of chromosomal mediated *uge* and *entB* genes

4.8.1 PCR optimization

PCR (Polymerase Chain Reaction) optimization is a crucial process to ensure the efficiency of amplifying DNA fragments. The goal is to find the right conditions for temperature, primer concentration, and other reaction components to achieve optimal amplification. Here's a detailed protocol for PCR optimization:

4.8.1.1 Primer dilution

There is a main stock of forward and reverse primers and they are quite concentrated, so for dilution, add 500ul of PCR water in both of them. For this purpose, take Eppendorf and label them with forward and reverse primer and add 40ul of PCR water in both of them. Then add 10ul main primer stock containing 500ul of PCR water.

4.8.1.2 Setting up PCR reaction mixture

Master Mix of SOLID BIODYNE 5X FIREPOL was used for PCR reaction mixture. It contains all the reagents required for PCR i.e., buffer, $MgCl_2$, dntps, Taq polymerase except DNA template, forward and reverse primers, and PCR water. Total reaction volume was kept 10ul. Following schemes of volumes was used in making of PCR reaction mixture.

Reaction mixture	For 10 samples	For 5 samples
PCR water	63ul	32.5ul
Taq buffer	10ul	5ul
MgCl2	6ul	3ul
dNTPs	2ul	1ul
Forward primer	4ul	2ul
Reverse primer	4ul	2ul
Taq polymerase	1ul	0.5ul

4.8.1.3 Basic protocol

First of all, autoclave all the necessary apparatus. Now place the respective trays on ice racks and label all PCR tubes. Now add 0.7ul PCR water in each PCR tube and then add 0.8ul of DNA in each tube. Now, take another PCR tube to make the reaction mixture (10ul) as mentioned above and also label it with respective gene symbol. Then add 8.5ul reaction mixture in each PCR tube. In each PCR tube, total volume should be 10ul. Now turn on the PCR machine (Thermo cycler) and place the PCR samples in the respective zones. Now set the temperature of each zone accordingly and start the process by turning on the machine. After completion of time, remove all the PCR tubes and place them on ice rack.

4.8.1.4 PCR Conditions

Initially for molecular examination of *K. pneumoniae*, the PCR samples were kept at 95°Cfor 5 minutes followed by 35 cycles of denaturation at 95°C for 45 seconds, annealing at 50-65°C (varies depending upon the genes sequence) for 45 seconds, extension at 72°C for 1 minute and final extension at 72°C for 10 minutes in applied Bio System Thermo cycler 2700 and end at 4 ∞.

4.8.1.5 Gel formation

The agarose gel concentration depends upon the gene size. (For a small size gene, 2(0.8g) gel formation and for a large size gene, 1% (0.6g) gel formation). Now, to make a gel, take 36ml distilled water and 4ml TBE buffer. Weigh powdered gel on weighing balance and mix in a beaker containing distilled water and TBE buffer and then place over the heating plate. Don't allow to boil much, remove at intervals. And boil the gel until all particles have been dissolved. Now cool down the gel until you are able to place it over your wrist and then add 4ul Ethidium bromide in the beaker. Then pour into casting tray and fix combs in row 1 and row 3. Remove any bubble produced via tip. Allow it to solidify completely. Add diluted TBE buffer into the tank and remove combs from the casting tray and put gel intact.

4.8.1.6 Loading in wells

For this purpose, 2-3ul amplified DNA was mixed with 2ul of 6X loading dye (Thermo Scientific) and loaded into wells. DNA marker (Thermo Scientific 1Kb ladder) was loaded into wells. The gel was then run at current 400mA, voltage 100 volts, and time 35-40 minute in 1X TBE buffer. After the adjusted time period, gel was visualized under UV trans-illuminator to examine DNA bands. Bands were photographed by BIO RAD documentation system.

4.9 Phenotypic identification of *Klebsiella pneumonia*

4.9.1 Disk diffusion Assay (Kirby-Bauer)

Disk diffusion of 80 different isolates of *Klebsiella pneumoniae* through Kirby-Bauer was done under Laminar Flow Hood (LFH).

Protocol

Preparation of Muller Hinton Agar (MHA) plate: First weigh the media (MHA) properly and dissolve it carefully in the measured amount of distilled water in the beaker and seal the lid with foil. MHA must be autoclaved before pouring. Once autoclaved, MHA is poured into petri plate and then let it solidify.

Inoculum preparation: In Eppendorf, a 500uL suspension of sterile water was used to pick up 2/3 of the bacteria, which were carrying two colonies of small inoculating agents. The suspension is smoothed by vortexing the saline tube.' Next, bring this suspension's turbidity up to an acceptable level (a 0.5 McFarland standard) by adding more organism if it is too light, or filling the suspension with sterile saline unless it's too heavy.). Use this suspension within 15 minutes of preparation.

Inoculation of the MHA plate: Place a sterile swab into the inoculum tube to initiate the process. Then, inoculate the dried surface of an MH agar plate, by streaking the swab against the side of the tube three times over the entire surface. After that, rotate the plate 60 degrees each time for achieving uniform distribution of the inoculum. Finally, dispose of the swab in a suitable container.

Antibiotic disc placement: To place the antibiotic disc, we used sterilized forceps. With the help of these forceps, we carefully remove one disc at time from the cartridge with the help of forceps, we gently pressed the disc on the Agar surface to make total contact. To avoid the agar surface from encountering the room air as much as possible we make sure to work in the LFH and keep the lid on when not placing the disc. Once the disc is in place, we place it in 35 degrees centigrade in an air incubator for 24 hours.

Determination of zone size: To identify the resistant and sensitive isolates CLSI guidelines were followed according to which if

Zone of inhibition ≥ 14mm = sensitive

Zone of inhibition < 10 or no zone = resistant

Zone of inhibition 11-13mm = intermediate

4.9.2 Minimum Inhibitory concentration (MIC) of Doxycycline

Minimum inhibitory concentration is the lowest concentration of antibiotic that can inhibit the growth of bacteria. Doxycycline MIC was used to look for the susceptibility profile of *klebsiella pneumoniae*. To determine the MIC, broth and micro dilution methods were used.

Stock/ Drug suspension preparation of Doxycycline

The CLSI-recommended technique was utilized to prepare Doxycycline stock. Normally saline is utilized, however for this stock, distilled water (dH2O) was used. To make a stock solution, use the following formula:

$$W = C \times V \times 100/P$$

Where, W is the weight of powdered drug, C is total concentration, V is the volume of stock solution, P is the potency of drug.

To prepare 10ml of Doxycycline stock solution i.e., 5120 ug/ml, 0. 058g of Doxycycline powdered drug was measured. First it was dissolved in 5mL of distilled water, vortex gently and then add remaining 5mL of dH2O to make it 10mL, so clear solution is made. The stock was then stored at 4°C after wrapping it in the foil.

Preparation of working solution and antibiotic dilutions

First 12 test tubes were autoclaved and stacked on the rack to prepare antibiotic dilutions. The concentrations in these tubes ranged from 512ug/mL to 0.25ug/ml. Starting at 0.25ug/mL, 1 ml of MHB was added to each tube (in reverse manner). To prepare the working solution, 4.5ml of MHB was placed into a tube labelled 512ug/mL, and then 500uL (0.5mL) of drug solution was added into W.S to make by it 5ml. the drug solution and media was fixed properly repeated pipetting and then 3ml was discarded out of this tube. For serial dilution, 1mL out of 2ml in the working solution was transferred into 256 labeled tubes by using pipette. It was mixed and step was repeated until 0.25, after mixing 1mL was discarded. Additional 2 tubes were used, media control and drug + media control. The whole procedure was done in aseptic conditions under laminar flow hood, LFH.

4.9.2.1 MIC by test tube method

Preparation of bacterial culture suspension for tube method

With the help of pippete, 500ul (5ml) of MHB was added into sterilized Eppendorf tube and 2-3 colonies of freshly cultured *K. pneumoniae* were mixed in it and then incubated for 15 minutes. In a large size test tube, 19.8 ml of MHB was added after which, 200 ml of bacterial

culture solution was added to make it 20 ml in total [19 mL + (800uL+200uL)]. Then 12 test tubes were arranged and labeled from 512ug/mL-0.25ug/mL. The procedure mentioned above was performed. After serial dilution, one 1ml of culture suspension was added into all tubes to make the 2 ml volume of the tubes and were sealed properly. The procedure was performed in LFH and results were noted after 24 hours of incubation.

4.9.2.2 MIC by microtiter plate method

Preparation of bacterial culture suspension for plate method

The working solution was made by pouring 1mL + 440ul (total=1440ml) of MHB into the 512 tube, followed by 160ul of the drug solution. The rest of the dilution method follows the same steps as before. 500ul of MHB was added to an Eppendorf labelled with the isolate number, and 2-3 colonies of bacteria were suspended in it to make the primary suspension. 0.5 McFarland standards were used to match the turbidity. For 15-20 minutes, the Eppendorf's were incubated. In a test tube, 20ul of primary suspension was mixed to 1mL+980 ml of MHB for secondary suspension, which was used for plating. The plate was labeled from 512 -0.5 ug/ml horizontally. Vertically it was labeled with isolate number. 100ul of serially diluted drug was added into each column and 100ul of bacterial suspension was added into each respective row. The column was left intentionally for media control to check the sterility. The plate was incubated after loading at 37°C for 24 hours. It was performed in LFH.

Determination of results

The drug dilution with no growth in terms of turbidity was considered MIC.

The breakpoint for Doxycycline

According to CLSI guideline 2021, the breakpoint for doxycycline is sensitive $\leq$ 4ug/ml and resistant $\geq$ 16ug/ml.

4.9.3 Biofilm formation protocol

4.9.3.1 Determination of strong biofilm formers

Biofilm forming potential was determined for the sensitive isolates against doxycycline by using the microtiter plate method. First day, streaking of sensitive isolates was done on MacConkey agar plates and then incubated for 24 hours at 37 degrees Celsius. Then next day, standardized 0.5 McFarland suspension of sensitive isolates was made by taking 2-3 colonies from 24-hour culture plate and mixing them in 1ml of Muller Hinton Broth. For 15-20 minutes, the Eppendorf's were incubated. Then 200ul of each isolate's suspension in triplicates was added in the separate wells of microtiter plate and plate was incubated at 37 degrees Celsius for 18-24 hours. Next day, broth media and suspended bacterial cells were carefully removed from the wells and then washing of these wells was done 2-3 times with Phosphate buffer saline (PBS). After washing, 70% methanol (150ul) was added in the wells for the fixation of biofilm. After 15 minutes, methanol was removed from the wells and allowed it to air dry for 5 minutes in the hood. Then for staining, 2% Crystal Violet (150-200ul) was added in the wells. After allowing Crystal Violet to stain for about 15-20 minutes, excessive stain was washed three times with the help of Phosphate buffer saline. After washing, plate was allowed to air dry again for 5 minutes. Then 95% ethanol (150ul) was added in each isolate's well.

4.9.3.2 Optical Density (OD) measurement

Biofilms were then quantified by taking their optical density values twice at 492nm and 630nm in ELIZA plate reader. After taking their optical density readings, Standard Deviation and cut off Optical density (ODc) values are determined and biofilms were categorized as following;

Weak biofilm former: optical density= 2xODc

Moderate biofilm former: optical density= less than 4xODc

Strong biofilm former: optical density= 4xODc or greater. This was done in triplicates for each isolate.

4.10 Determination of doxycycline effect on strong biofilm formers

4.10.1 Minimum Biofilm Eradication Concentration protocol (MBEC):

Two isolates that were sensitive against doxycycline in planktonic form and have ability to produce strong biofilms were taken to determine the effect of doxycycline on their strong biofilms. This was done by first streaking isolates on MacConkey agar and incubating the plates at 37°C for 24 hours.

Then second day, standardized 0.5 McFarland suspension of isolates were made in Muller Hinton broth from 24-hour culture plate and then incubate for 15-20 minutes. Now, add 75ul of the McFarland in each well starting from concentration 2048ug/ml and ending at 0.5ug/ml. Now, incubate the micro titer plate at 37°C for 24 hours for the biofilms to be produced.

Third day, take out the micro titer plate from incubator and check for the turbidity i.e., all wells should be turbid showing strong biofilm formation. Discard the media from the wells and washed twice with the help of phosphate buffer saline. Then, make 2-fold serial dilution of antibiotic (doxycycline in this study) from concentration 2048ug/ml until reaching 0.5ug/ml. For this purpose, test tubes are used which are labelled from 2048ug/ml to 0.5ug/ml. 1ml MHB is poured in all test tubes except first one. Then working solution is made in separate test tube by pouring 4.5ml MHB followed by 500ul drug solution. After that, 2ml is taken from the working solution and added in test tube labelled as 2048ug/ml and from here 1ml of W.S is taken and added in test tube labelled as 1024ug/ml in which 1ml MHB is already present. Then 1ml is again taken from 1024ug/ml test tube and mix in the next test tube and these dilutions continue till 0.5ug/ml. Now, when the dilutions have been made separately in test tubes, add 100ul of these dilutions in the respective well of the

microtiter plate. Media and growth controls are also added in last wells. Now again incubate the plate at 37°C for 24 hours.

Then fourth day, inhibition or eradication of formed biofilms on the wells was observed by visualizing the wells. The clear wells indicated the absence of bacterial growth i.e., the biofilm has been completely eradicated at particular concentration of drug. Antibiotic dilutions from the wells were removed and wells were washed twice with the help of phosphate buffer saline.

While attached biofilms (if any) were scrapped from the wells with the help of tooth picks and suspended in 1ml of PBS in Eppendorf tubes. These tubes were vortexed and the contents were plated on the properly labelled McConkey agar plates. Plates were incubated at 24 hours at 37°C.

Next day absence of growth indicated eradication of biofilms.

4.10.2 Minimum Biofilm Inhibition Concentration protocol (MBIC):

The MBIC protocol is same till third day. At fourth day, all the media is carefully removed from the wells. And washing of these wells is done with phosphate buffer saline (PBS) three times. Then freshly prepared MHB (100ul) is added in each well. The purpose is to provide fresh nutrients to bacteria to check for any growth as if the bacteria has died or not. Then plate is incubated at 37 degrees Celsius for 24 hours.

Next day, check the plate for results. If the wells are turbid, it means that bacteria have shown growth. And the first clear well will show the MBIC concentration.

4.11 Effect of doxycycline on the biofilm forming ability of *klebsiella pneumonia* isolates at sub-Minimal concentrations (sub-MICs)

To study the effect of doxycycline on the biofilm forming ability of *Klebsiella pneumoniae* isolates at sub minimal concentrations, two strong biofilm forming isolates that were

sensitive towards doxycycline were taken. This was done by first streaking isolates on MacConkey agar and incubating the plates at 37°C for 24 hours.

Then second day, standardized 0.5 McFarland suspension of isolates were made in Muller Hinton broth from 24-hour culture plate and then incubate for 15-20 minutes. First microtiter plate was labelled 0.5ug/ml to 0.625ug/ml horizontally and isolates number vertically. Then 100ul of McFarland was added into each well of microtiter plate for respective isolate number. Keep plate aside and cover with lid. Now, antibiotic dilutions were made in six Eppendorf tubes (ranging from 50ug/ml to 0.625ug/ml) in such a way that 450ul MHB was poured in first three Eppendorf tubes and 200ul in next three test tubes. Then 50ul drug solution was added in first Eppendorf labeled as 50ug/ml. For dilutions, pick 50ul from first Eppendorf and mix in next tube labelled as 5ug/ml. Then after mixing, again pick 50ul and pour it to third Eppendorf labelled as 0.5ug/ml. This time 200ul is picked from the 0.5ug/ml labelled Eppendorf and pour into next tube labelled as 0.25ug/ml and continue till 0.625ug/ml with same concentration. Then 100ul of these dilutions ranging from 0.5ug/ml to 0.625ug/ml were added into each well for respective isolate number.

Drug and media containing wells were taken as negative controls and bacterial suspension without antibiotic dilutions were considered as positive control. At different time intervals like 2 hours, 4 hours, 6 hours and 24 hours plate were taken out from the incubator. Media and antibiotic dilutions were removed from the wells and wells were washed with phosphate buffer saline 3 times. After washing, biofilms were fixed by using methanol. These fixed biofilms were then stained with crystal violet for 10-15 minutes. Excess stain was washed with the help of PBS. Then 150ul of glacial acetic acid was added into each well.

Biofilm was quantified by taking optical density reading from ELIZA plate reader at 540nm. Increase or decrease in the OD values of biofilm with time was observed to determine the inhibitory or stimulatory effect of doxycycline on the biofilm forming ability of *K. pneumoniae*

Results

5.1 Samples collection

For the analysis, sample were collected from tertiary care hospital, Rawalpindi. Total number of collected isolates was 156 and sources include; urine, sputum, pus, catheter, blood, fluids and different equipment's.

5.2 Morphological Identification

To identify and confirm the isolates of *Klebsiella pneumoniae*, it was based on colony morphology and gram staining.

5.2.1 Colony morphology

All the isolates of *Klebsiella pneumonia* were freshly streaked on Nutrient agar (NA) and MacConkey Agar plates to visualize the colonies morphology (shape, texture, color).

Figure 5. 1: K. pneumoniae growth on MacConkey aga.

Figure 5. 2: K. pneumoniae growth on Nutrient agar

Table 5. 1: Showing the growth characteristics of K. pneumoniae on NA and McConkey agar.

Characteristics	**Nutrient agar**	**MacConkey agar**
Color	Greyish white	Pink-Red
Texture	Mucoid	Mucoid
Shape	Circular	Circular
Elevation	Dome shaped	Convex
Size	2-3nm	2-3nm

5.2.2 Gram staining

For confirmation of gram-negative short rods of *Klebsiella pneumoniae*, gram staining was performed. Under microscope, bacteria showing purple color were considered as gram positive bacteria while bacteria showing pink color were considered as gram negative. *K. pneumoniae* were short pink rods under microscope.

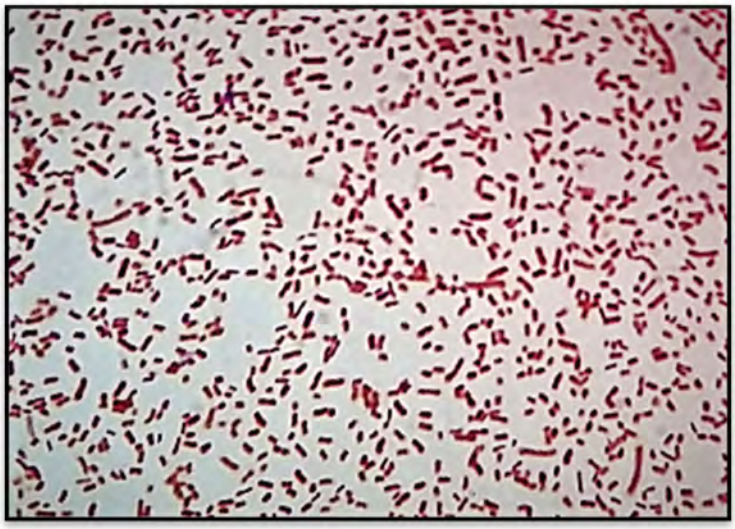


Figure 5. 3: Microscopic image of Klebsiella. Pneumoniae.

5.3 Biochemical identification

5.3.1 Oxidase test

Klebsiella pneumoniae is generally negative for the oxidase test. It lacks the cytochrome c oxidase enzyme required for the color change reaction to occur.

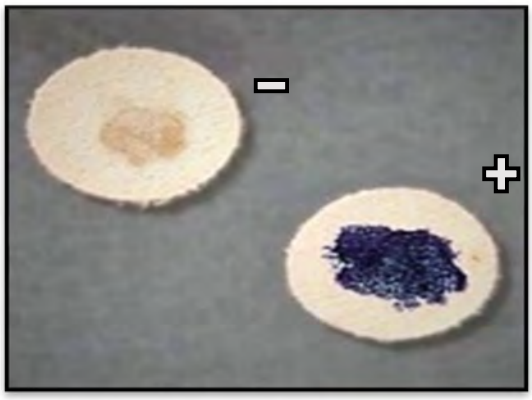


Figure 5. 4: Negative oxidase test in case of test isolate and positive in case of control.

5.3.2 Catalase test

All isolates of *K. pneumoniae* were catalase positive. Bubbles emergence was considered as positive result, while negative results in case of control showed no bubble formation.

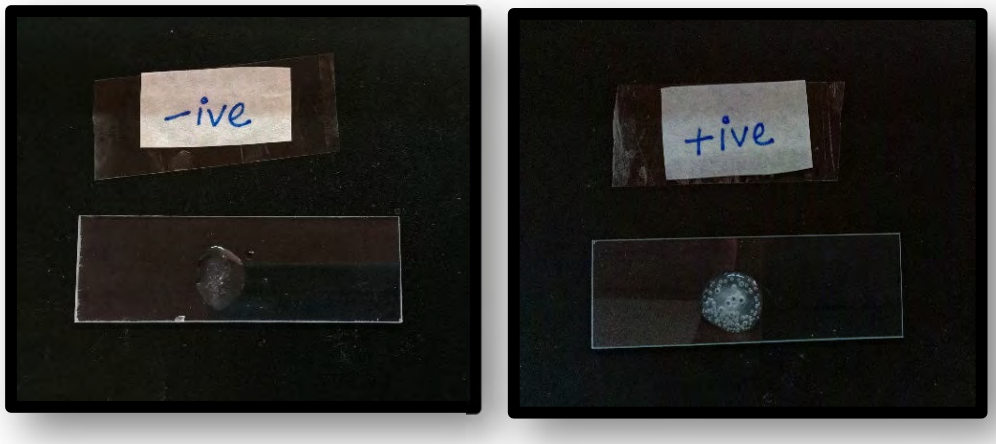


Figure 5.5: Negative catalase test in case of control and positive catalase test in case of test isolate.

5.3.3 Citrate test

Citrate test was used to confirm the isolates able to utilize citrate as Carbon source. Color change was observed in the slants. A change from green to Prussian blue was considered as a positive result for *K. pneumoniae.*

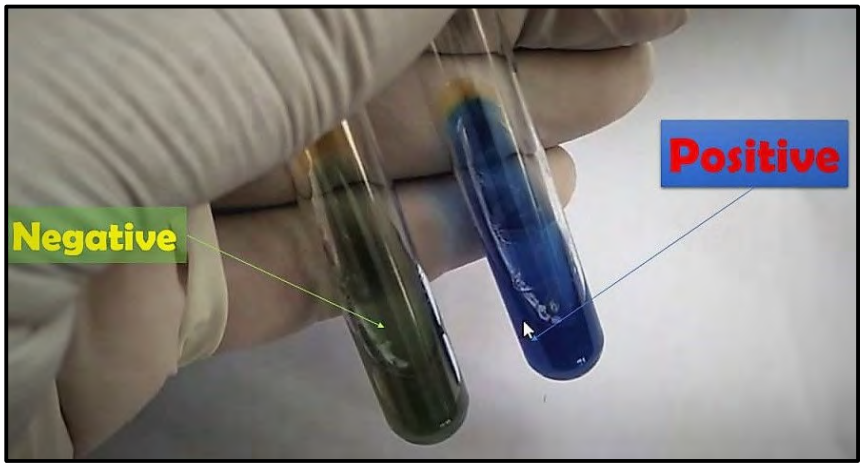


Figure 5. 6: Shows green test tube negative for control, second Prussian blue test tube positive in case of test isolate.

5.3.4 Indole test

Indole test basically check for the presence of tryptophanase enzyme which hydrolyze the tryptophan and produces indole product and KOVAC's reagent confirm the formation of indole test.

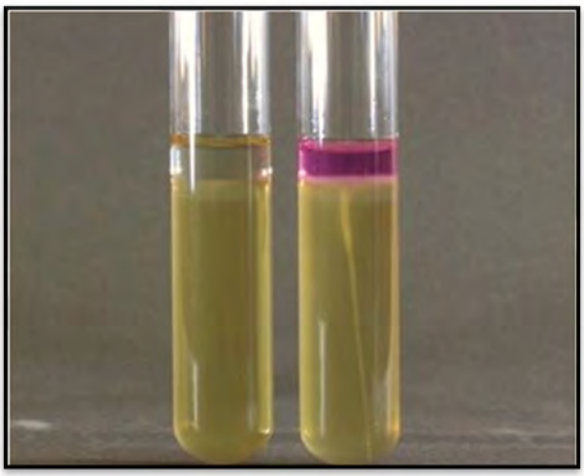


Figure 5. 7: Shows first test tube negative for test isolate, second test tube positive in case of control.

5.3.5 Urease test

This test checks the ability of bacteria to produce urease enzyme. K. pneumoniae is typically positive for the urease test as change in color from yellow to pink was observed.

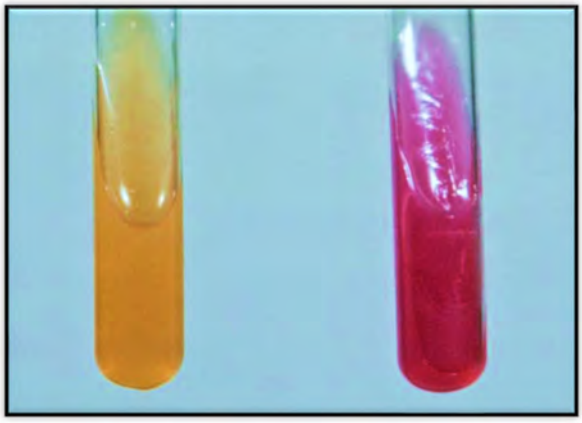


Figure 5. 8: Shows yellow test tube, negative for control, second pink test tube, positive in case of test isolate.

Table 5. 2: Summarizing biochemical tests and its results for the test

Biochemical tests	Results	Identification
Catalase test	Positive	Bubble formation
Oxidase test	Negative	No color change
Citrate test	Positive	Color change
Indole test	Negative	No ring production
Urease test	Positive	Color change

5.4 Results for Genotypic detection

Table 5. 3: summarizing sequence of primers and gene size of *entB*

Primers	Sequence 5'-3'	Annealing ° C	Amplicon (bp)
entB-F	ATTTCCTCAACTTCTGGGGC	58.2	371
entB-R	AGCATCGGTGGCGGTGGTCA		

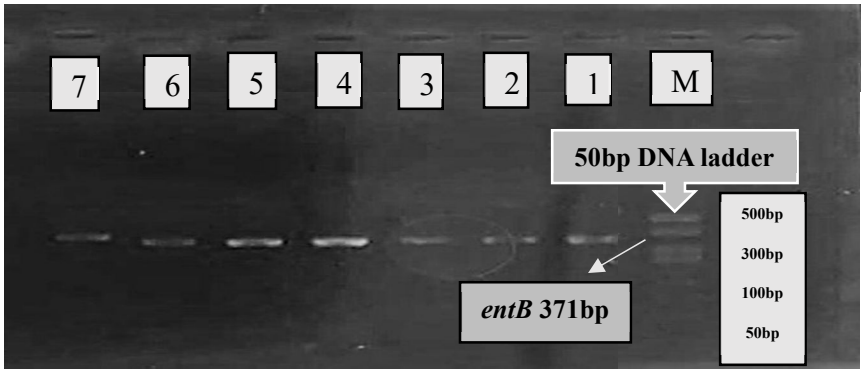


Figure 5. 9: Molecular detection of entB gene (371bp) on 1.5% agarose gel with the 50bp ladder as a marker. 25 out of 156 isolates of Klebsiella pneumoniae carried entB gene.

Table 5. 4: Summarizing sequence of primers and gene size of *uge*

Primers	Sequence 5'-3'	Annealing ° C	Amplicon (bp)
uge-F	TCTTCACGCCTTCCTTCACT	61.5	535
uge-R	GATCATCCGGTCTCCCTGTA		

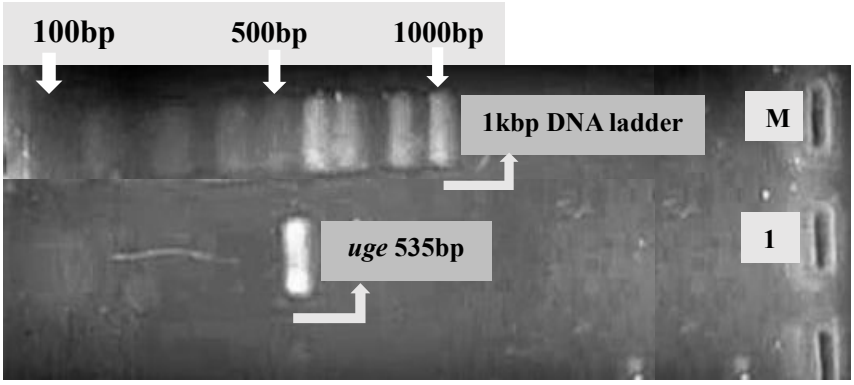


Figure 5. 5: Molecular detection of uge gene (535bp) on 1.5% agarose gel with the 1kbp ladder as a marker.73 out of 156 isolates of Klebsiella pneumoniae carried uge gene.

5.5 Results of disk diffusion assay

According to CLSI guideline 2018, zone of inhibition for doxycycline is greater than or equal to 14 (>14). So, following above guideline, among 80 clinical isolates of *K. pneumonia* on which disk diffusion was performed;

1. 24 isolates (30%) were sensitive to drug doxycycline (zone size was greater than or equal to 14mm in these isolates).
2. 44 isolates (55%) were intermediate (zone size between 11-13mm).
3. Remaining 11(13%) isolates were found resistant to doxycycline (zone size was less than or equal to 10mm in these isolates and in some isolates no zone was observed).

Figure 5.11: Shows zones produced by sensitive isolates.

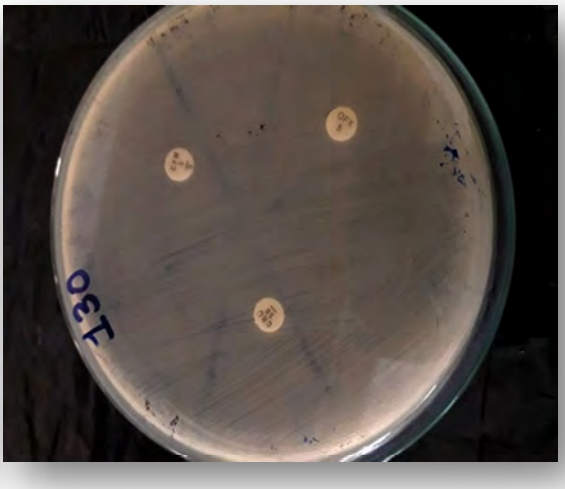


Figure 5.12: Shows no zone formation of resistant isolates.

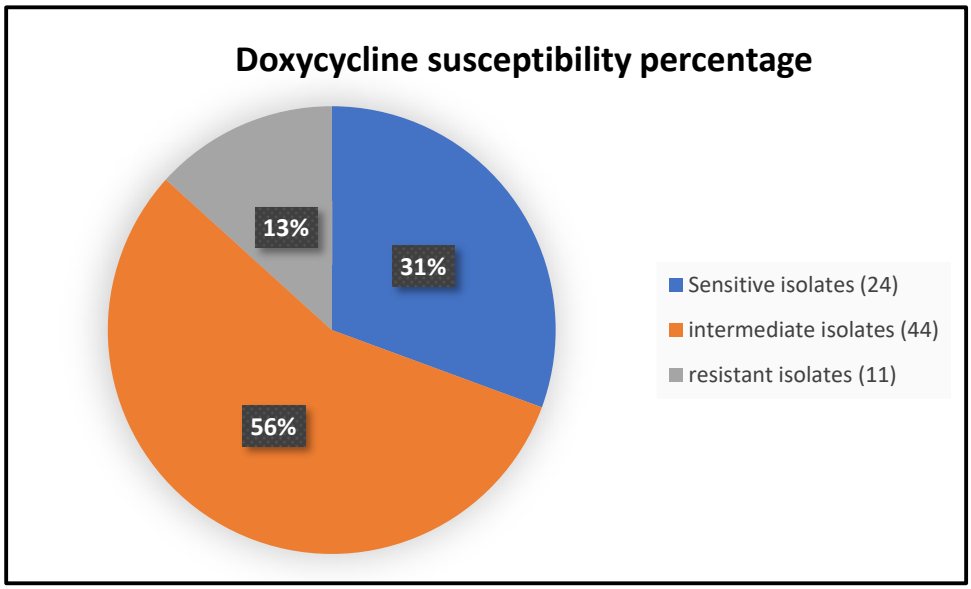


Figure 5. 13: Showing the no. of resistant, sensitive, and intermediate isolates against doxycycline.

5.6 Results of Micro dilution method

Minimum Inhibitory concentration of doxycycline on isolates of *K. pneumoniae* was determined.

5.6.1 MIC by tube method

The results were observed and recorded following the CLSI guidelines 2018. Dilutions with no growth (in terms or turbidity) was considered MIC.

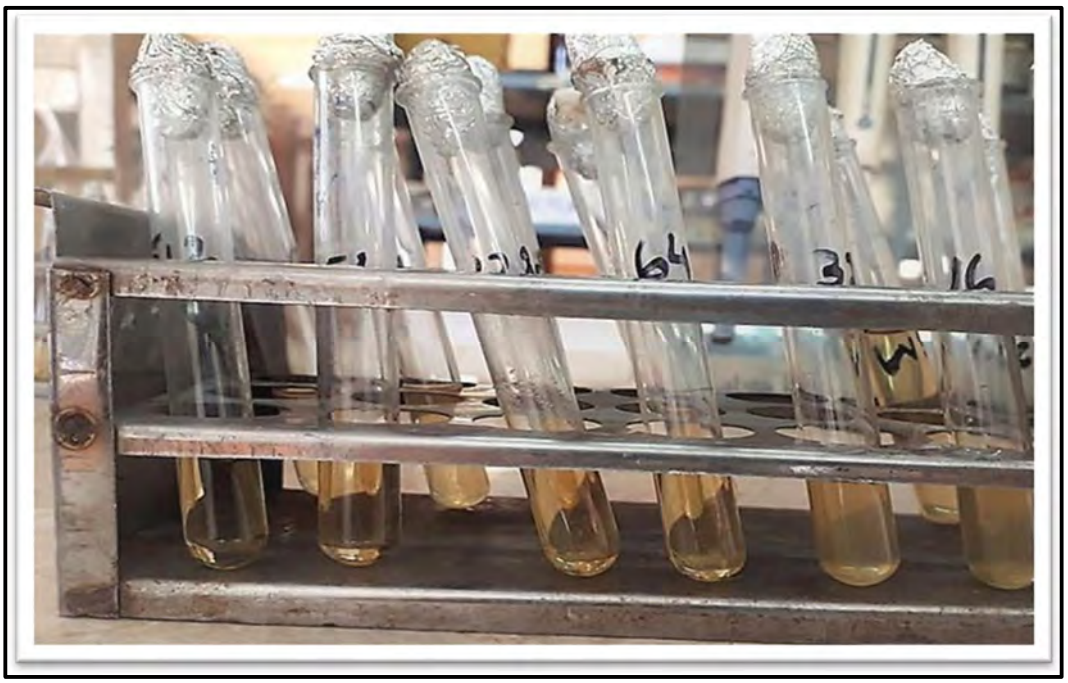


Figure 5.14: MICs showed by test tube method.

5.6.2 MIC by microtiter plate method

MIC for 24 sensitive isolates (30%) of *K. pneumoniae* was performed, and all the results were recorded following the CLSI guidelines. Among them, 16 isolates had MIC ≥4µg/ml, 7 isolates had MIC ≥2µg/ml, while 01 isolate had MIC ≥1µg/ml.

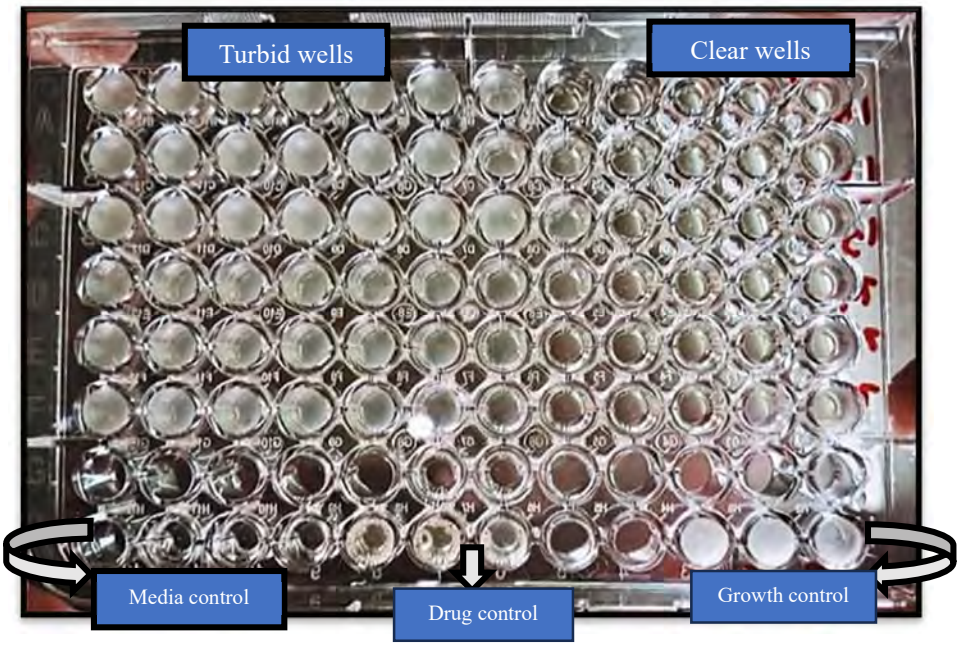


Figure 5.15: Shows MIC of different isolates of K. pneumoniae by microtiter plate method.

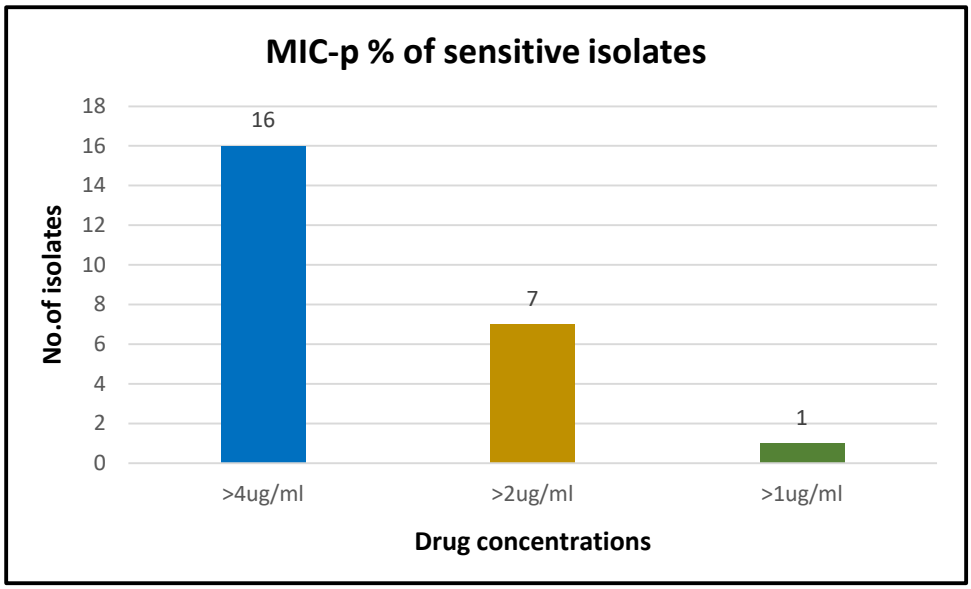


Figure 5.16: Showing the MICs of sensitive isolates against doxycycline.

5.7 Results for detection of biofilm formation

The aim of this study was to investigate the role of *uge* and *entb* genes in biofilm formation among the clinical isolates of *K. pneumoniae.* So, the biofilm forming potential was determined for 8 out of 24 sensitive isolates against doxycycline. Out of 8 isolates, 2 isolates i.e., 120 and 169 were strong biofilm formers because of the presence of uge gene in these isolates. While one isolate i.e., 43 was moderate biofilm former as uge gene (main role in biofilm formation) was absent and entB gene was present for this isolate number and rest of 5 isolates i.e., 3, 54, 154, 139, 143 showed weak biofilm formation because of the absence of both genes in these isolates.

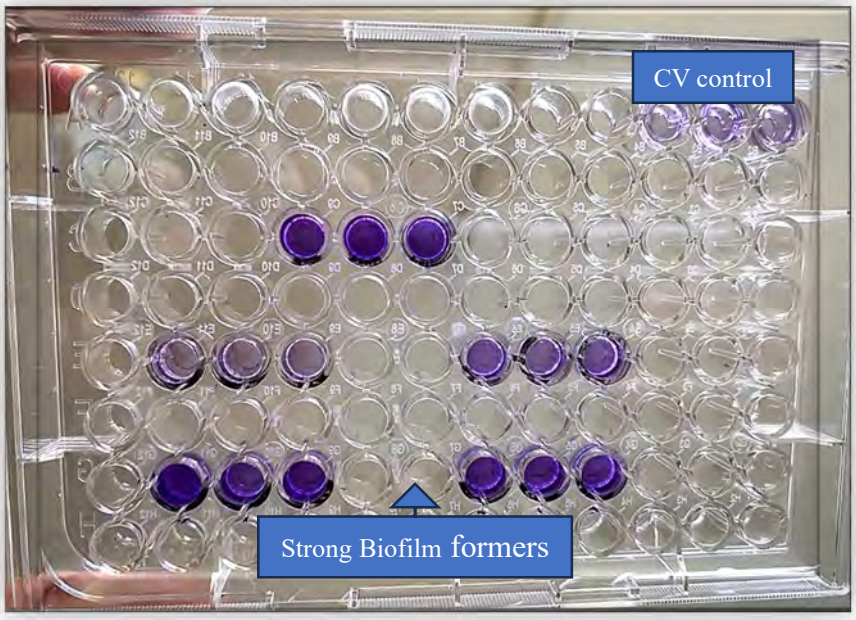


Figure 5.17: Showing biofilm forming potential of sensitive isolates. Few were found to be moderate and weak while some were strong biofilm formers. Specifically, well numbers 2,3,4 and 7,8,9 in row G represents strong biofilm formers. While well no 2,3,4 and 7,8,9 in row E represents moderate biofilm formers. And finally, one isolate in well no 4,5,6 in row C shows weak biofilm former.

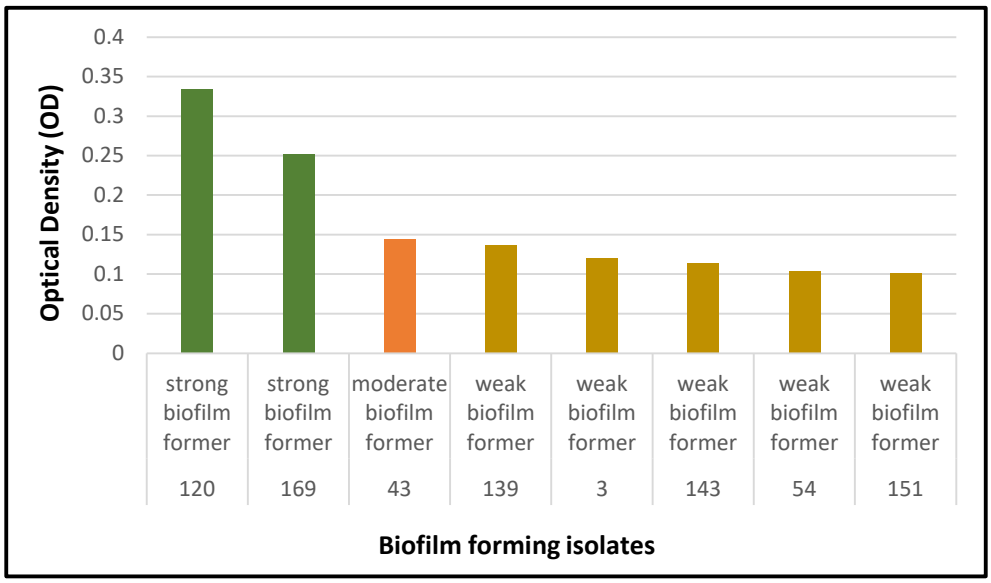


Figure 5.18: Showing biofilm forming potential isolates of Klebsiella pneumoniae at different OD values.

5.8 Determination of doxycycline effect on strong biofilm formers

Minimum Biofilm Eradication Concentration protocol (MBEC):

Out of 5 sensitive isolates, two strong biofilm forming isolates i.e., 169 & 120 were taken to evaluate the effect of doxycycline. Both isolates showed eradications of biofilm till drug concentration ≥512ug/ml. while moderate biofilm former showed eradication of biofilm till drug concentration ≥128ug/ml.

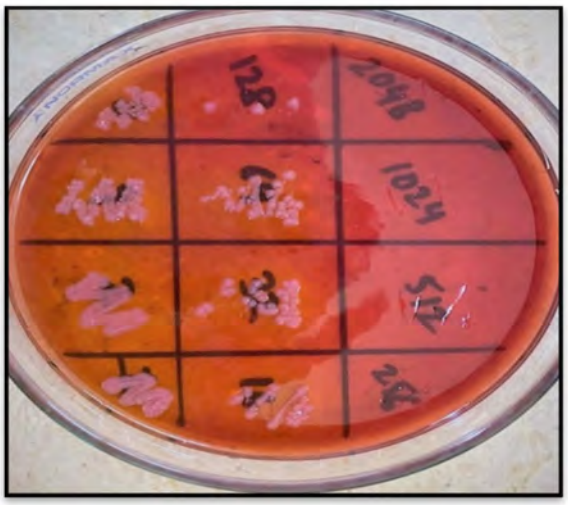


Figure 5.19: Shows the evaluation of doxycycline effect in eradicating biofilm after streaking the isolates on MacConkey agar plate.

Minimum Biofilm Inhibition Concentration protocol (MBIC): Similar to MBEC results, out of 5 sensitive isolates, same two strong biofilm forming isolates i.e., 169 & 120 were taken to evaluate the effect of doxycycline. Isolate number 120 and 169 showed inhibitions of biofilm till drug concentration ≥32ug/ml and ≥128ug/ml, respectively.

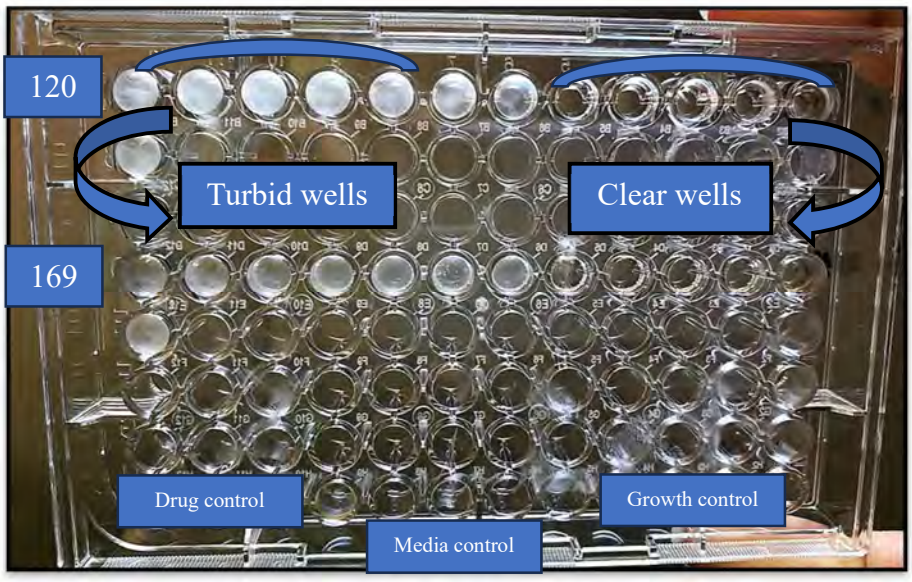
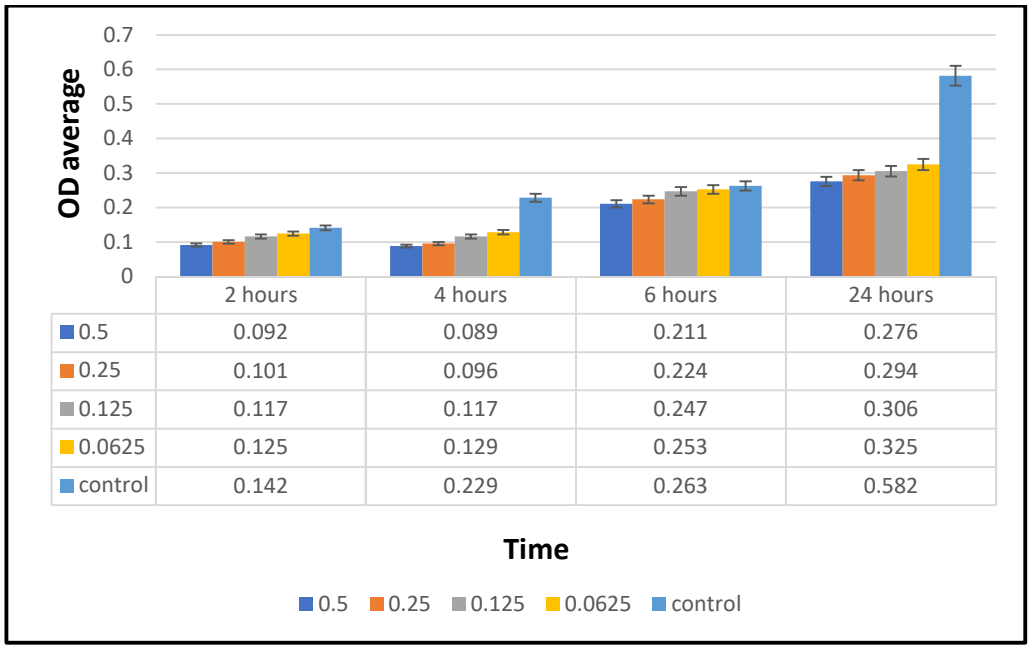


Figure 5.20: Shows the evaluation of doxycycline effect in inhibiting biofilm at different concentrations.

Table 5. 5: Summarizing drug concentration values for MIC-p, biofilm eradication and biofilm inhibition in 02 strong biofilm forming isolates of *Klebsiella pneumoniae.*

Isolate no.	Biofilm forming potential	MIC-p	MBEC	MBIC
120	Strong Biofilm former	≥4ug/ml	≥512ug/ml	≥32ug/ml
169	Strong Biofilm former	≥2ug/ml	≥512ug/ml	≥128ug/ml

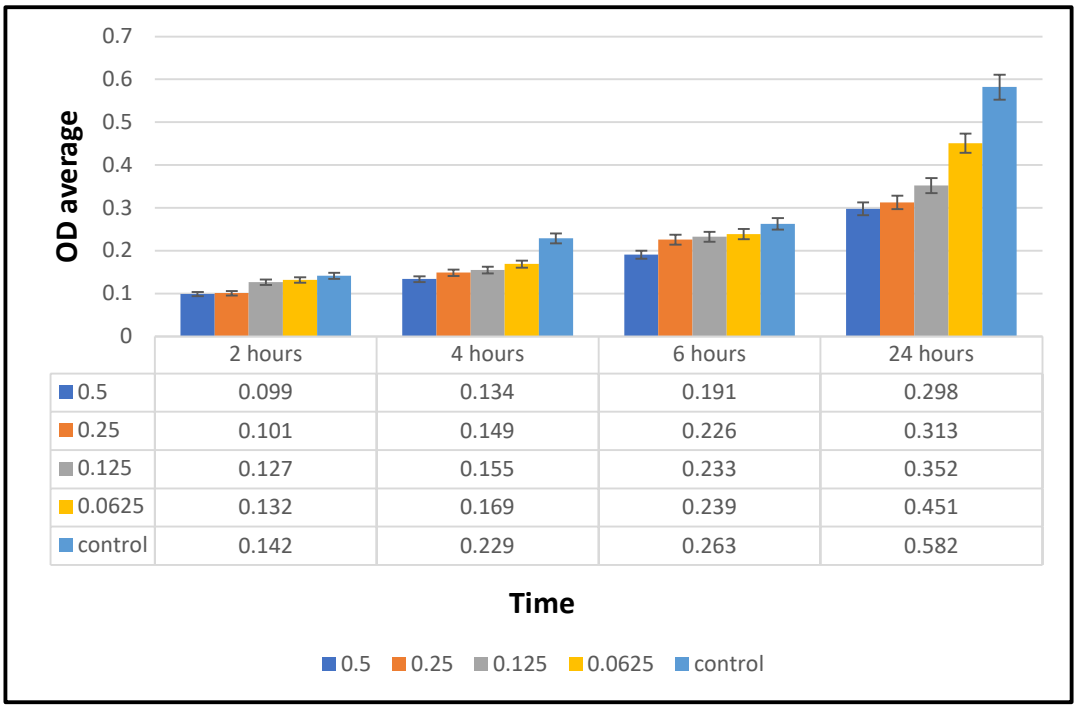
5.9 Effect of doxycycline on the biofilm forming ability of *Klebsiella pneumoniae* isolates at sub minimal concentrations



	2 hours	4 hours	6 hours	24 hours
0.5	0.092	0.089	0.211	0.276
0.25	0.101	0.096	0.224	0.294
0.125	0.117	0.117	0.247	0.306
0.0625	0.125	0.129	0.253	0.325
control	0.142	0.229	0.263	0.582

Figure 5.21 (a): Graphical representation of increase in biofilm forming ability of isolate 169 of Klebsiella pneumoniae with time when exposed to sub-minimal concentrations of doxycycline ranging from 0.5ug/ml to 0.0625ug/ml. Control represents the growth control that only contained the bacterial suspension and no

antibiotic. As the bacteria is continuously growing with time, the Optical density of control wells is increasing.



	2 hours	4 hours	6 hours	24 hours
0.5	0.099	0.134	0.191	0.298
0.25	0.101	0.149	0.226	0.313
0.125	0.127	0.155	0.233	0.352
0.0625	0.132	0.169	0.239	0.451
control	0.142	0.229	0.263	0.582

Figure 5.22 (b): Graphical representation of continuous increase in biofilm forming ability of isolate 120 of Klebsiella pneumoniae with time when exposed to sub-minimal concentration of doxycycline ranging from 0.5ug/ml to 0.0625ug/ml. Control represents the growth control that only contained the bacterial suspension and no antibiotic. As the bacteria is continuously growing with time, the Optical density of control wells is increasing.

Discussion

Discussion

Klebsiella pneumoniae has appeared as a significant pathogen which causes approximately 20% of the infections in hospitals globally. The capacity of *K. pneumoniae* to form biofilm is a common trait which is associated with the increase in virulence of this bacterium. This study mainly focused on the prevalence of different phenotypic and genotypic factors. The majority of isolates used in this study were able to form biofilm. In terms of isolates of *K. pneumoniae* from urinary tract infections, this finding was almost identical to the previous report (Zhao *et al.*, 2020). The emergence of antibiotic resistance via various mechanisms is another factor that is linked to *K. pneumoniae* infection. The present study mainly focuses on the doxycycline resistance among the clinical isolates of *K. pneumoniae* from tertiary care hospital in Islamabad, Pakistan. Though, it is significant to remember that strains of *K. pneumoniae* can vary in their susceptibility patterns and may develop resistance to tetracycline antibiotics through mechanisms such, as efflux pumps, ribosomal protection proteins and enzymatic inactivation. Recent surveillance studies have shown that 66.9% of selected countries have reported tetracycline resistance while ESBL producing *Escherichia coli* and *Klebsiella* species (spp.) show resistance rates of 69.1% and 24.3% respectively, (Jones *et al*. 2014). However, methicillin resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* exhibit percentages for tetracycline resistance were 8.7% and 24.3%, respectively. In another study, (Okafor *et al.*, 2023) showed resistance against tetracycline (69%) and doxycycline (56.6%). These findings are comparatively higher to our results, which indicated that among 80 clinical isolates of *K. pneumonia* on which disk diffusion was performed, the 13% isolates (11 out of 80) showed resistance against doxycycline while 24 isolates (30%) were sensitive to drug doxycycline.

There has been continuous increase in the antibiotic resistance due to the irrational use of antibiotics among *K. pneumoniae* isolates along with other members of *Enterobacteriaceae* and hence the treatment options for combating bacterial infections are gradually becoming very less. Further MIC was performed to evaluate the effect of doxycycline among *K.*

pneumoniae isolates. Out of the 80 isolates, 14 i.e., 17% isolates showed resistance against doxycycline. Among them, 2 isolates showed MIC ≥512µg/ml, 4 isolates showed MIC ≥256µg/ml, 2 isolates showed MIC ≥128µg/ml, 3 isolates showed MIC >64µg/ml, and 3 isolates showed MIC >32µg/ml. All the results were recorded following the CLSI guidelines. While 42 i.e., 52% isolates showed intermediate results against doxycycline. And it was observed that all isolates had MIC ≥8ug/ml. And 24 i.e., 30% isolates showed sensitivity against doxycycline. Among them, 16 isolates had MIC ≥4µg/ml, 7 isolates had MIC ≥2µg/ml, while 01 isolate had MIC ≥1µg/ml. The disk diffusion results and MIC results were almost similar.

The present finding unveiled that the most predominant virulence gene among *K. pneumoniae* species was *uge* (49%) and low occurrence was noticed for *entB* gene (16%). Though, the *uge* gene occurrence among the isolates of *K. pneumoniae* has varied widely in different studies. Remya *et al*. found occurrence of 48.6% harboring the *uge* gene which is almost similar to our findings. While the prevalence of *entB* gene varies from our results as in a study conducted by (Albasha *et al.*, 2020). Isolates of *K. pneumoniae* that were collected from various hospitals in Khartoum State. It is revealed that the important gene for siderophores system i.e., *entB* gene is positive in 93.3% of all isolates of *K. pneumoniae*. One more study conducted in Egypt by (Osama *et al.*, 2023) revealed that the siderophores encoding genes *entB* is prevalent in hmvKp isolates (90.5%). As it is seen that rate of prevalence of *entB* gene is much higher comparatively to our results. The present study demonstrated an association between these genes and biofilm production by *K. pneumoniae* to identify the possible molecular mechanisms during formation of biofilm. The *uge* gene encodes for the enzyme UDP-glucose 4-epimerase, express both capsular polysaccharide with K antigen on the surface and smooth lipopolysaccharide (LPS) with O antigen that act as an important role in process of biofilm formation. While *entB* plays an important role in iron uptake from host and allow it to convert into mineral that is essential for bacterial growth and their stability in the environment. All studies showed strong association between production of siderophore and bacterial virulence (Russo *et al.*, 2014).

Klebsiella pneumoniae can also increase its virulence and resistance by making biofilms on soft tissues and also on the indwelling catheters. Nirwati *et al*., 2019 showed 85.63% prevalence of biofilm forming *K. pneumoniae* isolates. Similarly, Cepas, V. *et al* 2019 showed presence of 37.6% biofilm forming *K. pneumoniae* isolates. Due to biofilm formation, bacteria become more resistant towards antibiotics compared to planktonic forms. The biofilm formation provides protection to species of *K. pneumoniae* against the immune response of the host, the antibiotics action and enhance its persistence. In this study, majority of *K. pneumoniae* isolates have capability to produce biofilm. So, the biofilm forming potential was determined for the sensitive isolates against doxycycline. Out of 24 sensitive isolates, 8 isolates showed results for biofilm formation. Among them, 2 isolates were found strong biofilm formers (120 & 169) at 492nm because of the presence of *uge* gene having major role in biofilm formation among these isolates. And 01 isolate (43) was moderate biofilm former. And 05 isolates produced weak biofilm formation as both *uge* and *entB* genes were absent in these isolates. So, by investigating their contribution to biofilm development could potentially lead to better strategies for managing biofilm-related infections and combating antibiotic resistance in this pathogen.

Two strong biofilm formers KP120 and KP169 that were otherwise sensitive towards doxycycline were selected and checked for the effect of drug on their strong biofilms. In the planktonic form, these two isolates appeared to be sensitive towards doxycycline as the minimum inhibitory concentration required in vitro was 2ug/ml. But as these isolates were capable of producing strong biofilms, the concentration required to eradicate and inhibit their biofilms significantly increased. As for KP120, minimum biofilm eradication concentration (MBEC) value has increased to a level of 512ug/ml against doxycycline. Similarly, MBEC value for KP169 and KP43 has increased up to 512ug/ml and 128ug/ml, respectively. Also, minimum inhibitory concentration values for biofilm (MBIC) were different as compared to MIC values against doxycycline. As for KP120, MBIC value has increased to a level of 32ug/ml against doxycycline. Similarly, MBIC value for KP169 and KP43 has increased up

to 128ug/ml. So, based on these results, we can say that *K. pneumoniae* has more tolerance against doxycycline when these isolates are present in the form of biofilm.

Moreover, these results were confirmed by exposing the isolates of *K. pneumoniae* to doxycycline at sub-minimal concentrations. With time, as the concentration of antibiotics in blood drops than the minimum inhibitory concentrations required for the inhibition or eradication of bacteria, the drug will no longer be effective. Hence, it would only be detected by bacteria and consequently bacteria increase or decrease their virulence and resistance factors in response to the sub minimal concentrations of the antibiotics. One mechanism is the increase in the biofilm forming ability of the bacteria that is sometimes increasing or sometimes decreasing in response to sub-MIC level of the antibiotics. Our study indicated that the effect of doxycycline results in the increased biofilm forming ability of *K. pneumoniae* isolates with time at sub-minimal concentrations ranging from 0.5 to 0.0625ug/ml. The drug showed optimal inhibition of biofilm formation after a 2-hour incubation period at a concentration of 0.5ug/ml for both isolates known to form robust biofilms, namely 169 and 120. However, over time, the impact of the remaining drug concentration on biofilm formation diminished.

Based on our findings, it can be observed that the doxycycline resistance among the isolates of *K. pneumoniae* in planktonic form and biofilm form have a noticeable difference thus far, although more time is needed to know the extent and trajectory of antimicrobial resistance associated with intermittent doxycycline use.

Conclusion

Conclusion

In summary, emergence of antibiotic resistance as a global issue highlights the urgent need for development of innovative drug especially when addressing multi-drug resistant pathogens like *Klebsiella pneumoniae*, a prominent member of the ESKAPE group. As the availability of effective antibiotics diminishes, the imperative for exploring new therapeutic approaches becomes more pronounced and evident. This research has illuminated the potential of doxycycline as a promising option in combating *Klebsiella* infections as it highlights the notable effectiveness of doxycycline to impede the formation of biofilms in *K. pneumoniae*, a pivotal factor contributing to the pathogenicity of this bacterium. Moreover, the identification of crucial virulence genes, specifically *entB* and *uge*, in this pathogen has been effectively achieved. The implications of this study go well beyond the boundaries of the laboratory, emphasizing the essential nature of ongoing investigations into alternative strategies for managing infections and the determined efforts to find solutions to address the growing threat posed by antibiotic resistance. This could provide valuable insights into their more clinical relevance in future.

Future Perspectives

Future perspectives

The following are suggested recommendations for prospective research endeavors, derived from the outcomes of the conducted study.

1. In the realm of vaccine development, it is prudent to prioritize the utilization of reverse vaccinology techniques, along with the power of bioinformatics, to anticipate virulence factors and their variants. This proactive strategy holds the potential in finding conserved molecular targets which could significantly enhance the design of vaccines intended to combat emerging threats. In doing so, it ensures a more thorough and comprehensive approach to protecting against these potential hazards.
2. Future research in the combination therapies for combating antibiotic resistance entails the development and implementation of effective mechanisms and strategies which could be helpful in contributing to more successful treatment outcomes and reduced resistance rates.
3. Another promising avenue of research involves the utilization of synthetic biology to advance safety measures. Researchers can delve into the engineering of attenuated strains, a strategy that holds substantial potential for augmenting the safety profiles of vaccines. This approach facilitates the formulation of vaccines characterized by diminished virulence while concurrently upholding their immunogenic properties.
4. Future studies may also explore innovative techniques to disrupt biofilms, such as the development of biofilm-specific enzymes, nanoparticles, or targeted therapies that can penetrate and eradicate biofilm communities. This approach has the potential to yield valuable insights into the dynamics of biofilm behavior and their intricate interactions, consequently improving empirical and intervention strategies.

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