Optimization and Screening of *Wab*G and *Acr*AB genes in the clinical isolates of *Klebsiella pneumoniae*

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Master of Philosophy
In
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بسم الله الرحمن الرحيم
إبراهيم أبو عوف
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DEDICATION

I would like to dedicate this thesis to my beloved parents and siblings, who have always been incredibly supportive.

DECLARATION

All the data gathered and all the accompanying materials within this thesis reflect my original efforts. No prior presentation or submission of any portion of this work for any other academic qualification.

Vaneeza Arshad Mughal

CERTIFICATE

This thesis, submitted by **Vaneeza Arshad Mughal**, 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 ABBREVIATIONS

UTIs	Urinary Tract Infections
MBEC	Minimum Biofilm Eradication Concentration
MBIC	Minimum Biofilm Inhibition Concentration
MIC-p	Minimum Inhibition Concentration-planktonic
MHA	Muller Hington Agar
MHB	Muller Hington Broth
KP	*Klebsiella pnemoniae*
MDR	Multi-Drug Resistant
CDC	Centers for Disease Control and Prevention
CLSI	Clinical and Laboratory Standard Institute
CV	Crystal Violet
DOX	Doxycycline
ddH2O	Doubled distilled water
K. Pnemoniae	*Klebsiella Pnemoniae*
DNA	Deoxyribonucleic Acid
Kfu	Klebsiella ferric ion uptake
WHO	World Health Organization
ELSIA	Enzyme-Linked Immunosorbent Assay
EDTA	Ethylenediaminetetraacetic Acid
ESBLs	Extended-spectrum beta-lactamases
FDA	Food and Drug Administration
CTAB	cetyltrimethylammonium bromide
HAIs	Healthcare-Associated Infections
ICU	Intensive Care Unit
LPS	Lipopolysaccharides

LFH	Laminar flow hood
hvkp	Hyper virulent Klebsiella pnemoniae
Mbps	Millions of base pairs

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VANEEZA ARSHAD MUGHAL

ABSTRACT

Klebsiella pneumoniae causes a variety of infections including community-acquired pneumonia, hospital-associated infections, and urinary tract infections. It has garnered attention from the World Health Organization (WHO) due to its ranking among the top three pathogens exhibiting multidrug resistance. This resistance is influenced by various factors, including the acquisition of mobile genetic elements carrying specific resistance genes, mutations within the ribosomal binding site, and chromosomal mutations leading to increased resistance. Other mechanisms involve the efflux system, exemplified by the *acr*AB gene, and the formation of biofilms, driven by the *wab*G gene. This study is focused on screening the *acr*AB and *wab*G genes in *K. pneumoniae*. Further, we investigated doxycycline on the planktonic and biofilm of *K. pneumoniae*. We investigated Minimum Biofilm Eradication Concentration (MBEC), Minimum Biofilm Inhibitory Concentration (MBIC), and sub-MIC concentrations of doxycycline on *K. pneumoniae* isolates. Screening of n=156 isolates revealed occurrence of 33% for the *acr*AB gene (Acriflavine resistance channel protein A and B) which encodes for AcrA and AcrB proteins that act as the periplasmic adaptor protein (PAP) in several RND tripartite efflux pumps, and as a proton-drug antiporter respectively to provide characteristic intrinsic resistance to antimicrobials and 25% for the *wab*G gene which is involved in attachment to α-l-glycero-d-manno-heptopyranose II (l,d-HeppII) at the O-3 position of an α-d-galactopyranosyluronic acid (α-d-GalAp) residue. The co-occurrence of these genes was found to be 8%. We tested doxycycline for MIC-p and MIC-b measurements. Sensitivity testing against planktonic cells indicated MIC values ranged from 1-4μg/ml. We assessed biofilm-forming capacity by microtiter plate assay and identified two strong biofilm formers. Biofilms are generally associated with higher levels of virulence and resistance which was reflected in MBEC (512-256μg/ml) and MBIC (128-64μg/ml) in this study. At subminimal concentrations, a reduction in the biofilm-forming ability of the selected isolates was also observed. The most effective doxycycline concentration against our *K. pnemoniae* isolates showed to be 0.5μg/ml. Our results confirm a higher tolerance of *K. pneumoniae* to doxycycline in biofilm form.

INTRODUCTION

INTRODUCTION

Klebsiella pneumonia is a common Gram-negative, rod-shaped bacterium that belongs to the family Enterobacteriaceae (Ashurst *et al.*, 2023). This pathogenic microorganism has a storied history, dating back to its discovery in the late 19th century. First identified by the German microbiologist Carl Friedländer in 1882, after he isolated it from the lungs of a deceased patient from pneumonia (Ashurst *et al.*, 2023). Initially, it was named Friedlander bacillus, later in 1886 it was named *K. pneumoniae*. Bacterium was initially recognized as the causative agent of pneumonia, earning its name. Since then, this bacterium has emerged as a significant opportunistic pathogen, capable of causing various infections in both hospital and community settings (Bryan Corrin *et al.*, 2011).

This bacterium, recognized as a significant cause of hospital-acquired infections worldwide, exhibits a mucoid colony appearance and possesses the ability to ferment lactose. As an opportunistic pathogen, it is found in various locations, including the intestines, skin, and mouth, and can also persist on hospital equipment and surfaces (Wyres *et al.*, 2020). Individuals with compromised immune systems are at risk of developing respiratory infections, soft tissue infections, pyogenic liver abscesses, and urinary tract infections due to this bacterium. Additionally, it is responsible for surgical wound infections, endocarditis, cystitis, and septicemia (Paczosa *et al.*, 2016).

Distinguishing between pneumonia caused by *Klebsiella pneumoniae* and *Streptococcus pneumoniae* is important due to the distinct characteristics of their associated sputum. In Streptococcus-associated pneumonia, the sputum often appears rust-colored and may contain blood due to inflammatory damage to the lung tissue (Effah *et al.*, 2020). Conversely, in *K. pneumoniae*-caused pneumonia, the sputum is described as "currant jelly" because of the presence of necrotic tissue resulting from the bacterium's ability to cause excessive tissue necrosis in the lungs. However, it is essential to rely on proper diagnostic methods, as sputum appearance alone may not definitively differentiate between the two types of pneumonia, and accurate identification is crucial for effective treatment (Ebringer *et al.*, 1978).

Klebsiella pneumoniae has been known to cause outbreaks in healthcare settings, particularly in neonatal units, where it has been a significant cause of infections, especially in Low- and Middle-Income Countries. However, the concerning aspect is its increasing adaptability and ability to develop resistance to multiple antibiotics over time, contributing to the growing global alarm over antimicrobial resistance (Munoz-Price *et al.*, 2010).

The presence of medical implants, such as urinary catheters and respiratory support equipment, heightens the risk of infections caused by *K. pneumoniae* (Murphy *et al.*, 2013). Klebsiella species, including *K. pneumoniae*, exhibit an array of virulent factors that play a crucial role in their ability to cause disease. Among these factors, the polysaccharide capsules enveloping the bacterial cells protect host immune responses and facilitate adherence to surfaces, promoting the formation of biofilms is considered the most important factor. Other essential virulence factors include fimbriae, lipo-polysaccharides, and siderophores, which aid in its survival and protect it from the host's immune system (Highsmith *et al.*, 1985). The collective action of these virulence factors enhances Klebsiella's ability to cause infections and evade the host's immune defenses, presenting significant challenges in effectively managing infections caused by this opportunistic pathogen (Karampatakis *et al.*, 2023).

Carbapenem-resistant Klebsiella species have been associated with varying mortality rates in different regions (Candan *et al.*, 2015). In European and North American populations, the mortality rate has been reported to be around 20-45% (Durdu *et al.*, 2016), whereas, in China, it can be as high as 26% (Tian *et al.*, 2016) and may even reach up to 50%. These bacteria are a leading cause of ventilator-acquired pneumonia, posing a significant threat to vulnerable patients in healthcare settings (Kalanuria *et al.*, 2014). Klebsiella species are also a major cause of bloodstream infections, ranking second after *Escherichia coli* (Lake *et al.*, 2017). The reported death rate associated with Klebsiella bloodstream infections ranges from 20% to 30% (Farhan *et al.*, 2016.). On a population scale, the estimated death rate is approximately 1.3 per 100,000 persons. Furthermore, Klebsiella species are recognized as the third major cause of

hospital-acquired infections in the United States, following Clostridium difficile and Staphylococcus aureus (Magill *et al.*, 2014).

In 2019, out of an estimated 13.7 million infection-related deaths worldwide, approximately 7.7 million were attributed to 33 bacterial pathogens under study. These bacterial infections accounted for 13.6% of all global deaths. Among these pathogens, five were responsible for a significant proportion of deaths, making up 54.2% of the total. These five pathogens are *Staphylococcus aureus, Escherichia coli, Streptococcus pneumoniae, K. pneumoniae, and Pseudomonas aeruginosa*. *Staphylococcus aureus* ranked as the pathogen associated with the most deaths globally, contributing to 1.1 million fatalities. The other four pathogens each caused more than 500,000 deaths: *Escherichia coli* with 950,000 deaths, *Streptococcus pneumoniae* with 829,000 deaths, *K. pneumoniae* with 790,000 deaths, and *Pseudomonas aeruginosa* with 559,000 deaths. Remarkably, the distribution of deaths associated with these leading bacterial pathogens was relatively similar between females and males (Harvard.edu, 2019). This highlights the significant impact these infections have on global health and underscores the need for continued efforts to prevent, control, and treat bacterial infections to reduce their burden on public health. These concerning statistics underscore the urgent need for effective infection control measures and continued research to combat the rise of antimicrobial resistance and enhance patient outcomes in the face of Klebsiella-associated infections (Podschun *et al.*, 1998).

Upon entry into the host, *K. pneumoniae* primarily colonizes mucosal surfaces such as the nasopharynx and the gastrointestinal tract. However, the rate of colonization varies according to the specific body site. For instance, the colonization rate in the nasopharynx is reported to be between 3% to 15%, with higher rates observed in adults compared to children (Cocker *et al.*, 2023). On the other hand, the colonization rate in the gastrointestinal tract differs but can reach up to 20%. These variations in colonization rates highlight the importance of understanding the dynamics of *K. pneumoniae* colonization in different body sites, as it can impact the risk of infection and transmission, particularly in healthcare settings (Bengoechea *et al.*, 2018).

Klebsiella species, including *K. pneumoniae*, exhibit a remarkable ability to form biofilms, which significantly contributes to their ability to cause infections. Biofilms are complex bacterial communities that adhere to surfaces and are surrounded by a protective matrix of polysaccharides, proteins, and DNA (Anderl *et al.*, 2000). In the context of Klebsiella infections, biofilm formation plays a vital role in disease progression and poses challenges for effective treatment. When Klebsiella biofilms develop on medical devices like urinary catheters, ventilators, or intravenous catheters, they create a shielded environment that protects the bacteria from the host's immune response and conventional antimicrobial agents. This protection allows the bacteria to persist and lead to recurrent and chronic infections such as Catheter-Associated Urinary Tract Infections that are difficult to eliminate. Urinary tract infections associated with catheters impose a significant economic burden due to prolonged hospital stays. These infections can escalate, spreading to the kidneys and bloodstream, thereby increasing the complexity of treatment, and making them harder to manage effectively (Guerra *et al.*, 2022).

Additionally, the proximity of bacteria within the biofilm promotes the exchange of genetic material, facilitating the development of antibiotic-resistant strains (Singla *et al.*, 2012). As a result, Klebsiella biofilms pose significant difficulties in clinical settings, contributing to increased morbidity, mortality, and the growing concern about antimicrobial resistance. Understanding the mechanisms of biofilm formation and devising strategies to disrupt or prevent them are crucial in combatting Klebsiella-associated infections effectively (Zahller *et al.*, 2002). Alarmingly, it has been estimated that nearly 60% of bacterial infections and a staggering 80% of chronic infections result from the formation of biofilms. Understanding and addressing biofilm formation is vital for combating microbial infections and managing antimicrobial resistance effectively. The ability of *K. pneumonia* to form biofilms on soft tissues is the reason for respiratory, gastrointestinal, and urinary tract infections (Anderl *et al.*, 2000).

The origins of antibiotic resistance can be traced back to the discovery of penicillin by Sir Alexander Fleming in 1928, which marked a groundbreaking advancement in

medicine by effectively treating bacterial infections. However, shortly after the introduction of antibiotics, the emergence of resistance in bacteria followed due to their remarkable adaptability. Bacteria's rapid reproduction and large population size allowed genetic mutations to occur, conferring resistance to antibiotics. As susceptible bacteria were killed by antibiotics, the surviving resistant bacteria multiplied and passed on their resistance genes, leading to the dominance of antibiotic-resistant strains through natural selection (Grossman *et al.*, 2016). The misuse and overuse of antibiotics, inappropriate prescriptions, incomplete courses, and agricultural practices further accelerated the development of resistance. Moreover, bacteria's ability to horizontally transfer resistance genes between different species facilitated the rapid spread of resistance in the bacterial population (Julian Davies *et al.*, 2010).

The scarcity of new antibiotics discovery exacerbated the situation as existing drugs were increasingly relied upon, driving further resistance development (Martinez *et al.*, 2014). Bacterial biofilms, which offer protective environments, proved more resistant to antibiotics compared to free-floating bacteria, complicating treatment efforts. Consequently, antibiotic resistance has become a major global health concern, demanding responsible antibiotic use, infection prevention and control measures, development of new antibiotics, and alternative therapies research to address this pressing public health issue (Salam *et al.*, 2023).

The widespread and excessive use of antibiotics over the past 70 years has given rise to a global issue in modern medicine: antibiotic resistance. Therefore, multiple drug-resistant and extremely drug-resistant strains of Enterobacteriaceae have emerged, acquiring resistance to a majority of available antibiotics, leaving limited treatment options (Ghosh *et al.*, 2019). The growing prevalence of infections caused by Enterobacteriaceae species is particularly concerning because these organisms are natural inhabitants of our microbiome. Infections attributed to these resistant strains pose a grave threat, as they are associated with high mortality rates and prolonged hospital stays, amplifying the challenges faced by healthcare providers in managing these infections effectively (Bengtsson-Palme *et al.*, 2017).

Klebsiella pneumoniae, a significant member of the Enterobacteriaceae family, has become a concerning pathogen due to its development of antibiotic resistance (Ramsamy *et al.*, 2018). The rise of multi-drug-resistant strains of *K. pneumoniae* is linked to higher mortality rates, and in some cases, these strains progress to extensively drug-resistant forms, severely restricting treatment options. The 2014 World Health Organization (WHO) Global Report on Surveillance of Antimicrobial Resistance identified *K. pneumoniae* as one of the top three pathogens of international concern, highlighting the urgent need for effective strategies to combat its growing threat in healthcare settings (Zhen *et al.*, 2019).

Pakistan is no exception to the global challenge of antibiotic resistance. Studies conducted in different cities of Pakistan provide evidence of the prevalence of multi-drug-resistant *K. pneumoniae* isolates. For instance, a study in Faisalabad, Pakistan, in 2019, revealed a 17% prevalence of multi-drug resistant *K. pneumoniae* isolates (Chaudhry *et al.*, 2020). Similarly, another study in Lahore, Pakistan, during the same year, highlighted a significant resistance of *K. pneumoniae* isolates to cefoxitin, ampicillin, and meropenem (Murray *et al.*, 2020). Furthermore, a study conducted in a medical center in Shanghai, China, from 2014 to 2015, reported the presence of resistance to multiple antibiotics, including ertapenem, ceftazidime, imipenem, piperacillin/tazobactam, ceftriaxone, ampicillin, cefazolin, ampicillin/sulbactam, aztreonam, and cefotetan. These findings underscore the urgent need for vigilant surveillance and appropriate measures to address the growing problem of antibiotic resistance in these regions (Munoz-Price *et al.*, 2013).

The global spread of drug-resistant bacteria, with a particular focus on *K. Pnemoniae*, poses a significant danger. *K. pneumoniae* has emerged as a particularly concerning nosocomial infection linked with drug resistance. It belongs to the critically important multidrug-resistant (MDR) strains and the ESKAPE pathogen group (Sun *et al.*, 2020). Klebsiella species typically carry plasmids containing resistance genes to important antibiotics, such as -lactams like extended-spectrum cephalosporins and carbapenems. This has severely limited available treatment choices, frequently necessitating the use of last-resort medications such as fluoroquinolones. However, resistance to

fluoroquinolones in *K. pneumoniae* has recently risen and been reported. Fluoroquinolone resistance in *K. pneumoniae* is connected to DNA gyrase and topoisomerase IV mutations, as well as the upregulation of multidrug efflux mechanisms (Hasdemir *et al.*, 2004). Data from the European Antimicrobial Resistance Surveillance Network demonstrates that K. pneumoniae is less susceptible to four important medication classes: carbapenems, third-generation cephalosporins, fluoroquinolones, and aminoglycosides. The diverse dissemination of resistance genes has aided in the creation of extensively drug-resistant (XDR) K. pneumoniae strains, complicating the treatment of infections caused by this powerful disease. (Srinivasan *et al.*, 2014).

Efflux pump-mediated antibiotic resistance frequently goes unrecognized and undiscovered in clinical microbiology tests. The importance of the resistance-nodulation-division (RND) efflux mechanisms in giving resistance to aminoglycosides and tetracyclines is well established. In addition to enzymatic changes, overexpression of multidrug efflux systems contributes considerably to drug resistance in bacteria. Because they may eject a wide range of substrates, including therapeutically relevant medicines, efflux pumps, particularly multidrug efflux pumps, are a key problem in drug treatment. These pumps are crucial in modulating resistance to many kinds of medicines and biofilm formations. Notably, efflux pumps do more than only expel drugs from bacterial cells; they also function as virulence factors and adaptive responses that give antimicrobial resistance during infections. (Chetri *et al.*, 2019).

Several efflux systems, including AcrAB, OqxAB, and KexD, as well as additional efflux genes such as EefAB, KetM, and CepA, have been implicated in antibiotic resistance in *K. pneumoniae*. However, the AcrAB efflux pump produced by the AcrAB gene receives special attention (Mazzariol *et al.*, 2002). These efflux systems are critical in the development and persistence of antibiotic resistance in *K. pneumoniae*, posing difficulties in the treatment of infections caused by this bacterium. The AcrAB efflux pump is an important member of the resistance-nodulation-division (RND) family, which is present in Gram-negative bacteria like *K. pneumoniae*. It is made up of three major proteins: AcrA, AcrB, and TolC. AcrA acts as an outer

membrane fusion protein, connecting the inner membrane transporter AcrB to the outer membrane channel TolC (Padilla *et al.*, 2010b). Using the energy obtained from the proton motive force, AcrB actively pumps numerous substrates, including antibiotics, out of the bacterial cell. TolC functions as an escape channel for these substrates, generating a tunnel-like structure that extends from the periplasmic region to the external environment, connecting the AcrAB efflux pump to the bacterial surface. This tripartite complex, when combined, allows for the effective evacuation of substrates from the bacterial cell, contributing to antibiotic resistance and playing an important role in *K. pneumoniae* and other Gram-negative bacteria pathogenicity. It is important to emphasize that these efflux mechanisms are not always easily discovered in traditional antibiotic susceptibility testing, emphasizing the necessity of raising knowledge about the role of efflux pumps in the context of antibiotic resistance. (Jabbar *et al.*, 2019).

Klebsiella pneumoniae has emerged as a concerning superbug in clinical settings. The bacterium's prominent capsule, known as a K type, imparts a mucoid phenotype to the isolate and serves as a crucial virulence factor (Yu *et al.*, 2006). Notably, *K. pneumoniae* has been classified into 79 capsular serotypes, with serotypes K1 and K2 being particularly associated with bacteremia and linked to high mortality rates in regions like Taiwan, Europe, and North America. The presence of capsule-associated genes significantly contributes to the pathogenicity of *K. pneumoniae* isolates. Key virulence factors, such as *wab*G (responsible for core lipopolysaccharide biosynthesis), *uge* (uridine diphosphate galacturonate 4-epimerase), and *ycf*M (the outer membrane lipoprotein), play crucial roles in capsule production, enhancing the bacteria's ability to resist phagocytosis and promoting infection (Lin *et al.*, 2010). The *wca*G gene is another important virulence gene responsible for *K. pneumoniae* capsule biosynthesis. Its presence enhances the bacterium's capacity to evade phagocytosis by macrophages. Notably, bacterial characteristics can undergo variations over time and vary according to geographical regions, leading to changes in their pathogenic properties. As *K. pneumoniae* continues to evolve, understanding the dynamics of these virulence factors

becomes vital in addressing the challenges posed by this versatile and virulent pathogen.

The *wab*G gene is also involved in the synthesis of lipopolysaccharides (LPS) in *K. pneumoniae*. Specifically, it plays a crucial role in linking the first outer core residue (D-GalA) to the O-3 position of the L, D-HeppII residue in the LPS structure. Mutants of *K. pneumoniae* lacking the *wab*G gene were generated in a study, resulting in the complete absence of the outer core LPS. Therefore, these mutants displayed increased sensitivity to antimicrobial agents such as sodium dodecyl sulfate (SDS) and polymyxin B. The outer core LPS contributes to the integrity of the bacterial cell membrane, and its absence made the mutants more susceptible to damage and cell lysis caused by SDS, while also increasing their vulnerability to polymyxin B-mediated cell death hence, proving its role in antibiotic resistance (Izquierdo *et al.*, 2003b).

The *wab*G gene has also been associated with regulating capsule formation and hypermucoviscosity in *K. pneumoniae*, contributing to its hypervirulent phenotype. Additionally, it is involved in the Klebsiella ferric iron uptake (Kfu) system, which is responsible for transporting iron into the bacterial cell and supporting its survival in iron-limited environments. Understanding the multifaceted role of *wab*G in LPS synthesis, capsule formation, hypermucoviscosity, and iron transport is crucial in unraveling the pathogenicity and antimicrobial resistance mechanisms of *Klebsiella pneumoniae* (Prokesch *et al.*, 2016).

A study conducted across hospitals in Asian countries during 2008-2009 revealed *K. pneumoniae* as the most common cause of nosocomial infections, particularly ventilator-associated pneumonia (Mohd Asri *et al.*, 2021). The global problem of antibiotic resistance necessitates the optimization of existing antibiotics for treating infections due to the lack of new antibiotic discoveries. The prevalence of antibiotic resistance in Klebsiella spp. Is notably high in Asia, surpassing 60%, indicating a worrisome increase in resistance to this bacterium. As such, the use of new, broad-spectrum antibiotics should be limited if narrower-spectrum and effective antibiotics are still available (Mohd Asri *et al.*, 2021).

Different countries have reported varying prevalence rates of *K. pneumoniae* infections and antibiotic resistance (Heidary *et al.*, 2018). For instance, the prevalence of *K. pneumoniae* infection was found to be 13% in America, 5% in Pakistan, 64.2% in Nigeria, 33.9% in India, 17.4% in Denmark, and 14.1% in Singapore. The prevalence of *K. pneumoniae* Extended-Spectrum Beta-Lactamase (ESBL) infection was found to be 35.35% in Indonesia and 38.5% of all isolates in a specific hospital in Surabaya. Mortality rates associated with *K. pneumoniae* bacteremia have been reported to be around 20-47.9% in different studies conducted in Turkey and Taiwan (Effah *et al.*, 2020).

The *acr*AB and *wab*G genes of *K. pneumoniae* play pivotal roles in the bacterium's antibiotic resistance and virulence mechanisms. The *acr*AB gene encodes for an efflux pump that expels a wide range of substrates and antibiotics, contributing to multidrug resistance. Its detection is of great significance as it sheds light on the specific mechanisms *K. pneumoniae* employs to evade antimicrobial agents. Understanding the prevalence and distribution of *acr*AB in clinical isolates can provide crucial insights into the local patterns of antibiotic resistance and guide appropriate treatment strategies (Zhang *et al.*, 2021). Similarly, the *wab*G gene is involved in the synthesis of lipopolysaccharides, which are crucial components of the outer membrane that contribute to virulence and host-pathogen interactions. Detection of *wab*G can elucidate the bacterium's capacity to evade the host immune response and adapt to changing environments, making it a key factor in pathogenicity.

The detection of *acr*AB and *wab*G genes In *K. pneumoniae* from clinical samples in Islamabad holds immense importance due to the scarcity of data on these genes in the selected strains. Published data on the prevalence and significance of *acr*AB and *wab*G genes is limited in emerging hypervirulent strains. Uncovering the presence and distribution of these genes can provide valuable insights into the local patterns of antibiotic resistance and virulence traits of *K. pneumoniae*, assisting healthcare professionals in making informed decisions regarding patient treatment and infection control measures. Additionally, the data obtained from this research can contribute to broader studies on the emergence and spread of antibiotic resistance in virulent *K.*

pneumoniae at a global scale, ultimately aiding in the development of effective intervention strategies to combat the threat of multidrug-resistant infections.

In the face of the global threat posed by carbapenemase-producing organisms, including *K. pneumoniae*, there is an urgent need for research on effective treatment options. In this context, antibiotics like ciprofloxacin, cotrimoxazole, and doxycycline, which are classified as WHO-essential drugs due to their efficacy, minimal side effects, and cost-effectiveness, remain crucial in managing various Gram-negative infections, including those caused by *K. pneumoniae*. However, on the verge of increasing antibiotic resistance, it is essential to explore and optimize combination therapy regimens for better treatment outcomes. While colistin, tigecycline, and some aminoglycosides have shown activity against carbapenem-hydrolyzing β-lactamases producing Enterobacteriaceae *in vitro*, further studies are needed to determine their efficacy in real-world clinical scenarios.

Given the limited treatment options for carbapenemase-producing organisms, exploring alternative antibiotics like doxycycline, which has shown potential in inhibiting certain KP isolates *in vitro*, presents an exciting avenue for further investigation (Cunha, Sibley, *et al.*, 1982). Understanding the susceptibility of *K. pneumoniae* to doxycycline, along with optimizing its dosage and exploring combination therapy potential, is crucial in guiding clinicians to make well-informed treatment decisions in hospital settings. Research on doxycycline's efficacy, safety, and effectiveness in clinical practice is vital to solidify its role as a viable treatment option and address the challenges posed by antibiotic-resistant infections (Riond *et al.*, 1988).

Moreover, conducting a comprehensive assessment of doxycycline's activity in real-world clinical scenarios can provide valuable insights into its effectiveness in treating infections caused by *K. pneumoniae*. By rigorously studying doxycycline's role in managing carbapenemase-producing organisms, healthcare professionals can gather essential data to optimize treatment protocols, improve patient outcomes, and combat the growing threat of antibiotic resistance in the region. The fact that doxycycline is still prescribed as an effective treatment option with fewer side effects adds to its importance in the battle against Klebsiella infections, highlighting the need for

continuous research to strengthen evidence-based guidelines and tailored treatment strategies.

Recent research has shown promising results for doxycycline combined with amikacin *against K. pneumoniae* isolates, indicating the potential of combination therapy in managing drug-resistant infections. Therefore, it is crucial to investigate and evaluate the *in vitro* antibacterial activity of doxycycline and other antibiotics against ESBL- and carbapenem-resistant clinical isolates from tertiary care hospitals in Islamabad to develop effective therapeutic strategies and address the challenges posed by antibiotic-resistant *K. pneumoniae* infections.

The prevalence and significance of the AcrAB efflux pump and virulence factors like WabG in the context of antibiotic resistance against various antibiotics, especially in Pakistan, are poorly documented, and no data is available regarding their phenotypic and genotypic screening. This knowledge gap highlights the urgent need for research in this area, as these genes play a crucial role in *K. pneumonia's* antibiotic resistance. Exploring these aspects opens new research avenues, such as investigating gene mutations, inhibiting their activity, or finding ways to make them recessive, all of which can be instrumental in overcoming antibiotic resistance challenges.

A project was designed with these key considerations in mind. The fundamental goal of this research is twofold. To begin, it intends to examine certain genes, specifically *acr*AB and *wab*G, to determine their significance and probable role in antibiotic resistance processes in *K. pneumonia*. Second, this study will look at the effect of Doxycycline on the biofilm development of *K. pneumonia*. These goals add to our understanding of both genetic mechanisms involved with resistance and Doxycycline's potential therapeutic effectiveness against biofilm-related infections to outline appropriate empiric treatment choices. This thorough study provided a better understanding of the complex link between biofilm development and antibiotic resistance in *K. pneumoniae*.

AIMS AND OBJECTIVES

AIMS AND OBJECTIVES

The primary objective of this study was to assess the prevalence of *acr*AB and *wab*G genes among clinical isolates of *Klebsiella pneumoniae* obtained from a tertiary care hospital in Islamabad, Pakistan. Additionally, the study aimed to investigate the impact of doxycycline on robust biofilm-forming isolates that display susceptibility to these antibiotics in their planktonic state.

To address these objectives, the following key tasks were undertaken:

1. Molecular detection of the presence of *acr*AB and *wab*G genes through multiplex PCR.
2. Classification of isolates as sensitive, resistant, or of intermediate susceptibility to doxycycline using the Disk Diffusion Assay.
3. Determination of Minimum Inhibitory Concentrations (MICs) of doxycycline against the isolates utilizing the microdilution plate method.
4. Generation of biofilms from *K. pneumoniae* isolates and differentiation of robust biofilm producers among the susceptible isolates.
5. Assessment of the influence of doxycycline on strong biofilms formed by susceptible *K. pneumoniae* isolates.
6. Investigation of the impact of sub-minimal concentrations of doxycycline on the biofilm-forming capability of *K. pneumoniae* isolates.

LITERATURE REVIEW

LITERATURE REVIEW

Klebsiella pneumoniae, a common rod-shaped, non-motile, lactose-fermenting Gram-negative pathogen belonging to the family Enterobacteriaceae, presents a significant clinical challenge worldwide. When cultured on agar medium, *K. pneumoniae* forms mucoid colonies due to the presence of a polysaccharide capsule outside its bacterial membrane (Bagley *et al.*, 1985). During the pre-antibiotic era, *K. pneumoniae* commonly caused pneumonia, especially among individuals with diabetes and alcoholism. Nevertheless, the introduction of antibiotics significantly altered its epidemiology, leading to a shift in its role as a major agent responsible for infections acquired within healthcare settings (Tzouvelekis *et al.*, 2012).

Some strains of this bacterium possess opportunistic properties, making them capable of causing diseases in hospitalized, critically ill, and immunocompromised individuals. Consequently, *K. pneumoniae* is predominantly associated with nosocomial infections, including ventilator-acquired pneumonia, catheter-associated urinary tract infections, and bloodstream infections. Children admitted to pediatric wards and facing immunosuppression and malnutrition are vulnerable to the harmful effects of *Klebsiella pneumoniae*, which can give rise to severe conditions such as sepsis and meningitis, particularly in preterm infants. Conversely, in community settings, *K. pneumoniae* often manifests as urinary tract infections among children with robust immune systems (Hany Sahly *et al.*, 2002).

Notably, certain strains of *K. pneumoniae* are considered hypervirulent, enabling them to infect even healthy individuals, leading to conditions such as pyogenic liver abscess, endophthalmitis, and meningitis. Furthermore, bloodstream infections by *K. pneumoniae* have been implicated in the development of cancer (Russo *et al.*, 2019).

In recent times, the majority of *K. pneumoniae* infections have been attributed to two distinct types: "classic" *K. pneumoniae* (cKp) and hypervirulent *K. pneumoniae* (hvKp) (Kishibe *et al.*, 2016; Cubero *et al.*, 2016). The "classic" cKp strains demonstrate a notable affinity for hospitals.

settings, leading to infections predominantly affecting vulnerable patient populations. On the other hand, the hvKp variant was initially observed in the Asian Pacific Rim and has gained recognition for causing invasive and metastatic infections within the community (Shon, Bajwa, and Russo, 2013). Notably, hvKp infections often present as liver abscesses, endophthalmitis, meningitis, and septic arthritis, with a particular predilection for individuals with diabetes and young individuals possessing uncompromised immune systems (Struve *et al.*, 2015).

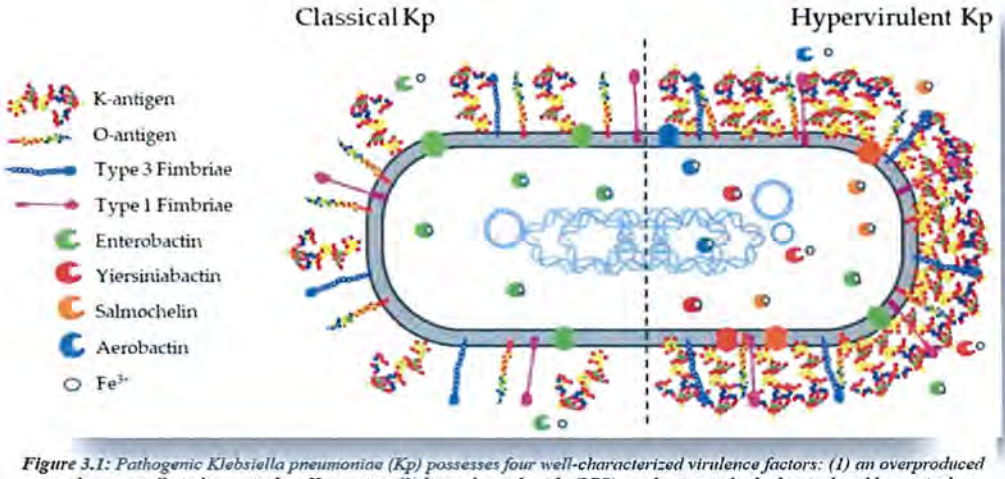

Figure 3.1: *Pathogenic Klebsiella pneumoniae (Kp) possesses four well-characterized virulence factors: (1) an overproduced capsule, especially in hypervirulent Kp strains, (2) lipopolysaccharide (LPS) production in both classical and hypervirulent strains, (3) adhesive structures known as type 1 and type 3 fimbriae, and (4) iron-scavenging siderophores, with enterobactin commonly produced by all Kp strains and other siderophores typically secreted by hypervirulent strains(Arato et al., 2021).*

The increasing prevalence of multidrug-resistant *K. pneumoniae* strains presents significant challenges in managing infections caused by this opportunistic pathogen. Consequently, there is an urgent imperative to further explore its pathogenesis and unravel the mechanisms behind antimicrobial resistance (Gorrie *et al.*, 2022). The surge in interest in studying *K. pneumoniae* is further fueled by the emergence of novel multidrug-resistant clones and the global spread of hypervirulent hvKp strains (Zhu *et al.*, 2021). A population-based surveillance study conducted by Meatherall et al. spanning 8 years found that approximately 7.1 individuals out of 100,000 were affected by bloodstream infections caused by *K. pneumoniae* (Meatherall *et al.*, 2009).

The primary focus of this literature review is to comprehensively analyze the current state of knowledge concerning *K. pneumoniae*. Specifically, we will explore its clinical impact, molecular characteristics, and virulence factors, while also investigating the latest therapeutic strategies employed to combat its infections. By synthesizing and critically evaluating existing research on *K. pneumoniae*, this review aims to shed light on the challenges posed by this bacterium and identify potential avenues for future research and clinical intervention. Gaining insights into the intricacies of *K. pneumonia's* behavior and response to treatments will undoubtedly contribute to the development of effective strategies for managing and controlling its infections in both hospital and community settings.

3.1 Structure

Klebsiella pneumoniae, a member of the Gram-negative family Enterobacteriaceae, possesses a characteristic cellular structure that plays a critical role in its pathogenicity and survival. The bacterium's cell wall is composed of peptidoglycan, providing structural support and protection while conferring resistance to osmotic pressure changes (Holt *et al.,* 2015). Additionally, lipopolysaccharides (LPS) within the cell wall act as potent endotoxins, contributing to their virulence. A defining feature of *K. pneumoniae* is its thick, mucoid capsule composed of polysaccharides, acting as a crucial virulence factor. This capsule aids in evading host immune responses by inhibiting phagocytosis, making it challenging for the immune system to clear the pathogen.

Some *K. pneumoniae* strains possess flagella, whip-like appendages that confer bacterial motility (Swathi *et al.,* 2016). Additionally, hair-like appendages on the bacterial surface called fimbriae and pili not only contribute as virulence factors but contribute to adherence to host tissues and the formation of biofilms. Biofilms, sticky bacterial communities, can develop on various surfaces, including medical devices, fostering the persistence and antibiotic resistance of *K. pneumoniae*. Understanding the intricate cellular structure of *K. pneumoniae* is vital in combating its pathogenicity and

devising effective strategies to combat infections caused by this opportunistic pathogen (Swathi *et al.*, 2016).

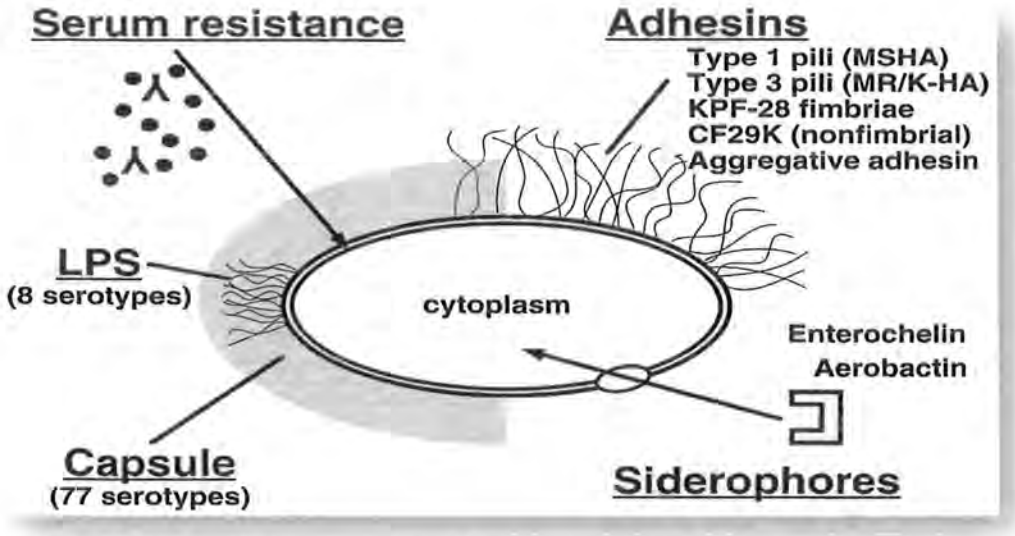

Figure 3.2: Schematic representation of Klebsiella pathogenicity factors (Podschun and Ullmann, 1998b).

3.2 Epidemiology

Klebsiella pneumoniae is mostly seen in humans, where it may colonize the gastrointestinal system and the nasopharynx. *K. pneumoniae* colonization varies in the general population, with 5% to 38% of people having the bacteria in their feces and 1% to 6% carrying it in their nasopharynx (Lee *et al.*, 2016). The primary sources of infection are patients' gastrointestinal tracts and hospital personnel's hands, resulting in epidemics in healthcare settings. Individuals of Chinese origin and those with persistent drinking have been shown to have greater rates of colonization (Jarvis *et al.*, 1985).

The carrier rate for *K. pneumoniae* in hospitalized patients is much higher than in the general population, with some studies indicating rates as high as 77% in hospitalized persons' feces, which is commonly connected with the use of several medications. Pneumonia produced *by K. pneumoniae* is divided into two types: community-acquired pneumonia and hospital-acquired pneumonia. While community-acquired pneumonia is widespread in Western societies, *K. pneumoniae* infections are uncommon, accounting

for just 3% to 5% of cases. However, in underdeveloped countries such as Africa, *K. pneumoniae* might account for up to 15% of all pneumonia infections (Yigit *et al.*, 2001).

K. pneumoniae is responsible for around 11.8% of hospital-acquired pneumonia cases globally, accounting for 8% to 12% of pneumonia infections in ventilated patients and 7% in non-ventilated patients. In patients with underlying alcoholism and septicemia, the death rate from *K. pneumoniae* can range from 50% to 100%. The global prevalence of *K. pneumoniae* and its accompanying illnesses varies, with varying frequencies seen in various locations. According to one research, Chinese people colonized at a rate of 66%, Malay people at 14.3%, and Indians at 7.9%. According to the Centers for Disease Control and Prevention, *Klebsiella* species are responsible for 3% of all epidemic outbreaks. It accounts for around 15% of all pneumonia cases in underdeveloped areas such as Africa (Kaiser *et al.*, 2013).

3.3 Taxonomy of genus Klebsiella

Klebsiella belongs to the Enterobacteriaceae family, which includes well-known species such as *E. coli*, as well as infamous human diseases like Salmonella, Yersinia, Serratia, Enterobacter, Citrobacter, Kluyvera, Leclercia, Raoultella, and Cronobacter. *K. pneumoniae* species complex (KpSC) and other Klebsiella species such as *K. indicia, K. terrigena, K. spallanzanii, K. huaxiensis, K. oxytoca, K. grimontii, K. pasteurii,* and *K. michiganensis* are all members of the Klebsiella genus. The KpSC is a collection of closely related organisms that have a high degree of genetic similarity with *K. pneumoniae* sensu stricto, with nucleotide similarities ranging from 95% to 96%. *K. pneumoniae* (Kp1), *K. quasipneumoniae subsp. quasipneumoniae* (Kp2), *K. variicola subsp. variicola* (Kp3), *K. variicola subsp. variicola* (Kp4), *K. variicola subsp. variicola* (Kp5), *K. variicola subsp. variicola* (Kp6), *K. africana* (Kp7) (Boye *et al.*, 2003).

Klebsiella taxonomy has experienced a complicated development, exhibiting its colorful taxonomic past. Originally, the genus Klebsiella was divided into three species based on the illnesses they produced within the family Enterobacteriaceae: *K.*

pneumoniae, K. ozaena, and K. rhinoscleromatis. As taxonomy advanced, such as numerical approaches, the species classification underwent many modifications, culminating in three primary categories by Cowan, Bascomb, and Orskov (Ristuccia *et al.*, 1984).

Klebsiella isolates from the environment, previously known as "Klebsiella-like organisms" (groups J, K, L, and M), were reclassified into provisional taxa in the early 1980s, leading to the discovery of four new species: *K. terrigenous, K. ornithinolytica, K. planticola, and K. trevisanii*. However, because of substantial DNA sequence similarity, *K. planticola and K. trevisanii* were eventually merged into one species, *K. planticola* (Boye *et al.*, 2003).

K. terrigena and *K. planticola,* formerly thought to be non-clinically relevant and restricted to aquatic, botanical, and soil settings, have now been found in human clinical specimens. Notably, *K. planticola* has been identified with unexpected frequency from human illnesses, accounting for 3.5% to 18.5% of clinical isolates among *Klebsiella* species. Many of these isolates were isolated from respiratory tract secretions, with wound and urine isolates following. However, because many isolates were recovered from polymicrobial tissues, determining their specific involvement as illness-causing organisms remains difficult (Ristuccia *et al.*,1984).

3.4 Risk factors associated with *Klebsiella Pneumoniae* infections.

Several risk factors enhance susceptibility to *Klebsiella pneumoniae* infections, with underlying illnesses being a major influence. Chronic liver illness, gastrointestinal disorders, renal transplants, genital-urinary malignancies, hematologic malignancies, and cancer have all been recognized as major risk factors for *K. pneumoniae* infection. Furthermore, old age has been linked to an increased risk of infection, particularly in senior people over the age of 70 and 80, according to (Weatherall *et al.*, 2009).

The duration of hospitalization and the use of mechanical ventilation devices in healthcare settings have been linked to the incidence of Carbapenem-resistant *K. pneumoniae* isolates, indicating that hospital-acquired infections may be more prevalent in these conditions, according to the study by (Liu *et al.*, 2018).

Children referred to pediatric hospitals, particularly those with immunosuppression and malnutrition, are more prone to the severe consequences of *K. pneumoniae* infections, such as sepsis and meningitis, with preterm newborns being especially vulnerable (Meatherall *et al.*, 2009). In contrast, *K. pneumoniae* causes urinary tract infections more frequently in children with strong immune systems in community settings (Gasink *et al.*, 2009).

Notably, certain *K. pneumoniae* strains are regarded as hypervirulent, allowing them to infect healthy people and cause illnesses such as pyogenic liver abscess, endophthalmitis, and meningitis. Furthermore, *K. pneumoniae* bloodstream infections have been associated with the development of malignancy (Meatherall *et al.*, 2009).

Overall, recognizing the numerous risk factors for *K. pneumoniae* infections is critical for adopting preventative measures and optimizing treatment options, especially in vulnerable populations and hospital settings (Gasink *et al.*, 2009).

3.5 *Klebsiella pneumonia* genome

Klebsiella pneumonia's genome is 5.5 Mbps in size and encodes roughly 5500 genes containing 57.38% GC and two plasmids of size 211,813 bp and 172,619 bp of GC content 52.4% and 52.6%, respectively. Holt et al. (2015) examined a large number of *K. pneumoniae* isolates and discovered that more than 95% of them contain 2000 similar genes, comprising the core genome. The accessory genome is made up of the remaining 3000 genes that differ between isolates. The presence of a large accessory genome in comparison to the core genome shows that *K. pneumoniae* has access to a diverse set of genetic resources, indicating that its genome is accessible and more accessory genes may potentially be discovered (Martin *et al.*, 2018).

The accessory genome is critical to the adaptability and survival of *K. pneumoniae*. It has genes that encode antibiotic resistance enzymes, nitrogen fixation, colonization, and infection in particular host locations. Furthermore, the auxiliary genome is involved in glucose metabolism, heavy metal stress adaptation, and polysaccharide capsule synthesis, all of which contribute to pathogenicity. Variations in accessory genes across *K. pneumoniae* isolates account for variances in metabolic capacity,

allowing them to flourish in a variety of environments. Paczosa and Mesas, for example, discovered in 2016 that around 50% of *K. pneumoniae* isolates have a plasmid-mediated lac operon for utilizing lactose sugar, showing their adaptation to dairy products (Martin *et al.*, 2018).

Certain antibiotic resistance factors, including *bla*kpe, *bla*oxa-as, *bla*Nom-1, and *bla*c-M-15, are in the core genome and relate to mobile genetic elements such as transposons and plasmids, allowing their transmission across bacterial species. Resistance to antibiotics like ampicillin, fosfomycin, and nalidixic acid, on the other hand, is caused by genes found in the core genome, such as *fos*A, and *ogx*AB. Furthermore, the core genome genes *acr*AB and *oq*XAB encode efflux pumps that provide resistance to fluoroquinolones and tigecycline (Snitkin *et al.*, 2012).

The hypermucoviscosity phenotype, which causes certain *K. pneumoniae* strains to be hypervirulent and capable of causing illness even in healthy people, is affected by proteins RmpA and MagA, which are coded for by the *rmp*A and *mag*A genes. The genome of *K. pneumoniae* is prone to alterations, which can lead to resistance to a wide range of antimicrobial drugs. Resistance to fluoroquinolones, extended-spectrum cephalosporins, and even carbapenems is conferred by mutations in genes encoding outer membrane proteins (ompK35 and ompK36) (Snitkin *et al.*, 2012). Fluoroquinolone resistance is also caused by mutations in genes encoding DNA gyrase subunits (*Gyr*A and *Par*C). Resistance to colistin, a previously effective antibiotic against fluoroquinolone and carbapenem-resistant bacteria, is caused by mutations in the *cr*B gene.

Overall, the *Klebsiella pneumoniae* genome exhibits significant genetic diversity, with the accessory genome crucial to the bacterium's adaptability, virulence, and antibiotic resistance, and the core genome contributing to essential resistance factors and the hypermucoviscous phenotype. Understanding *K. pneumonia's* genetic composition is critical for controlling infections and developing successful treatment techniques (Snitkin *et al.*, 2012).

3.6 Virulence factors

Virulence factors are important components of bacteria that have a role in pathogenesis. These microbial surface features or chemicals aid in eukaryotic host colonization and disease development. Interestingly, even non-pathogenic strains may have avirulent versions of these components, most likely due to virulence gene changes. *K. pneumoniae*, for example, has a virulence factor repertoire that includes the polysaccharide capsule, fimbriae, pili, siderophores, urease, and the type IV secretion system (Podschun *et al.*, 1998). These virulent factors work together to help *K. pneumoniae* develop and cause illness in its host. *K. pneumonia's* pathogenic success can be due to the interaction of several virulence factors, which allows it to avoid the host's defenses and cause life-threatening sepsis (Piperaki *et al.*, 2017).

3.6.1 Polysaccharide capsule

The polysaccharide capsule is one of the most important virulence factors *of K. pneumoniae*, a well-known human pathogen that causes a variety of illnesses. This acidic polysaccharide, located outside the outer membrane of bacterial cells, is made up of uronic acid and repeating units of sugar molecules (Piperaki *et al.*, 2017). Although *K. pneumoniae* possesses 78 different antigenic capsule types, only a handful of them are linked to human infections. The capsular polysaccharide synthesis *(ps)* gene cluster governs capsule synthesis, which includes 16-25 genes involved in capsule manufacture, assembly, and transport to the bacterial cell's external surface (Ramos *et al.*, 2012). The *wi* gene is the most variable of the genes in this cluster and is now utilized. The *wi* gene is notably variable among the genes in this cluster and is now employed for serotyping diverse capsular *K. pneumonia* isolates (Brisse *et al.*, 2013).

The polysaccharide capsule acts as a crucial barrier, shielding *K. pneumoniae* from host immunological responses. It aids in the avoidance of opsonization and phagocytosis by host macrophages, neutrophils, and dendritic cells (Cortes *et al.*, 2003; Pan *et al.*, 2011; Evrard *et al.*, 2010). The capsule's capacity to withstand intracellular death by neutrophils is particularly notable in hypervirulent *K. pneumoniae* isolates, allowing them to spread to distant sites of infection, such as the liver, and cause severe illness (Wu *et al.*, 2008).

In addition to its protective function, the capsule has anti-inflammatory qualities. Through TLR2 and TLR4 signaling, it reduces interleukin-8 expression, reducing the host's inflammatory response to the bacteria (Lawlor *et al.*, 2006). *K. pneumonia's* capacity to remain in the host and produce severe infections is enhanced by the combination of immune evasion and anti-inflammatory actions.

Hypervirulent *K. pneumoniae* strains have bigger capsule diameters as compared to classic strains. This trait allows them to survive and be more virulent within the host (Yoshida *et al.*, 2000; Lawlor *et al.*, 2005). The bulky hyper capsule, on the other hand, can act as a physical barrier, limiting the reception of foreign DNA and blocking horizontal gene transfer. As a result, hypervirulent strains are less likely to carry antimicrobial resistance genes than less virulent bacteria (Piperaki *et al.*, 2017).

Understanding the capsule's role and influence on bacterial behavior is critical in unraveling *K. pneumonia's* pathogenicity and developing tailored tactics to treat infections caused by hypervirulent strains. Researchers can create strategies and treatments to combat infections caused by these extremely virulent strains by learning more about the relationships between the capsule and other virulence characteristics.

The increase in antibiotic resistance complicates the fight against *K. pneumoniae* infections. Antibiotic abuse and overuse have resulted in the rise of multidrug-resistant strains of *K. pneumoniae*, posing a substantial public health hazard globally. Finding alternate means to combating *K. pneumoniae* infections is critical since the development of new antibiotics has lagged the evolution of resistance.

One possible approach is to target the capsule as a weakness in hypervirulent strains. Researchers may be able to design innovative therapies that disrupt or neutralize the capsule's function in immune evasion and virulence by leveraging the capsule's participation in these processes. Targeting the synthesis or assembly of the capsule, for example, might make the bacterium more sensitive to host immunological responses or accelerate its removal by host defenses.

Furthermore, knowing the genetic and molecular pathways behind capsule variation may give useful insights for vaccine development. Vaccines targeting certain capsule

types or conserved parts of the *ps* gene cluster might protect against hypervirulent *K. pneumoniae* strains and lower infection load (Piperaki *et al.*, 2017).

3.6.2 Lipopolysaccharide

LPS is a critical virulence component in *Klebsiella pneumoniae*, a Gram-negative bacterium that causes a variety of illnesses in humans. *K. pneumoniae* is an opportunistic pathogen that can cause serious illnesses including pneumonia, urinary tract infections, bloodstream infections, and even life-threatening sepsis, especially in vulnerable people with weakened immune systems or underlying medical disorders. Understanding the function of LPS in *K. pneumoniae* pathogenesis is critical for designing effective anti-infection measures against this opportunistic pathogen (Cortes, 2002; Regué *et al.*, 2005).

LPS, commonly known as endotoxin, is a key component of Gram-negative bacteria's outer membrane. It is essential in the bacterium's interactions with the host, influencing immune responses and promoting colonization and survival within the host environment (Regué *et al.*, 2005).

LPS has three major components: the innermost lipid A region, the core polysaccharide, and the outermost O-antigen. The lipid A region is a highly conserved component across Gram-negative bacteria that is crucial for activating innate immunological responses in the host upon identification by pattern recognition receptors such as Toll-like receptor 4 (TLR4). The core polysaccharide gives LPS structural stability and participates in complement resistance, inhibiting the activation of the host's complement system, which is a vital component of the immune system (Cortes *et al.*, 2002).

The O-antigen is the most variable component of LPS and is responsible for the wide range of serotypes found in *K. pneumoniae*. Serotypes 01, 02, and O3 are the most often connected with human infections of the nine recognized serotypes. Because of the diversity in the O-antigen, *K. pneumoniae* can avoid detection by the host immune system, making it more difficult for the immune system to recognize and develop an effective defense against the bacterium. The bacterium's capacity to produce chronic

infections and stay in the host may also be influenced by serotype diversity (Cortes *et al.*, 2002).

LPS and its components are synthesized by distinct gene clusters inside the bacterial genome. The *waa* gene cluster, for example, is responsible for the assembly of O-antigen repeating units and thereby synthesizes the O-antigen. *Waa* gene clusters synthesize the core polysaccharide, with type 1 and type 2 varying only by two genes. The LPS production gene clusters are critical for the bacterium's pathogenicity and capacity to cause illness (Regué *et al.*, 2005).

LPS's capacity to shield the bacteria from host immune responses is one of the most important functions it plays in *K. pneumoniae* pathogenesis. LPS operates as a physical barrier, preventing antimicrobial peptides and antibiotics, such as polymyxins, from entering the bacterial cell. This defense mechanism permits *K. pneumoniae* to live and multiply in the host's circulation, resulting in severe bloodstream infections and sepsis (Piperaki *et al.*, 2017).

Furthermore, LPS aids *K. pneumonia's* resistance to phagocytosis and other immunological responses by host macrophages, neutrophils, and dendritic cells. The presence of LPS, particularly the O1 serotype, confers serum resistance to *K. pneumoniae*, allowing the bacterium to bypass the host's complement system and other immune defenses. LPS also has anti-inflammatory characteristics, suppressing the production of pro-inflammatory cytokines including interleukin-8 via TLR2 and TLR4 signaling.

LPS-mediated immune evasion is a significant element in the pathogenicity of *K. pneumoniae* and the development of severe infections. The presence of LPS on the bacterial surface permits *K. pneumoniae* to remain and multiply within the host, resulting in chronic infections and recurring illnesses (Regué *et al.*, 2005).

Despite its involvement in immune evasion, LPS has several drawbacks for bacteria. Some hypervirulent *K. pneumoniae* strains can develop a thick hypercapsule that acts as a physical barrier, inhibiting DNA absorption and horizontal gene transfer. As a result, hypervirulent strains are less likely to carry antimicrobial resistance genes than other strains. Understanding the interactions between the capsule and LPS, as well as

their effect on bacterial behavior, gives vital insights into *K. pneumonia'* pathogenicity and the evolution of antibiotic resistance in diverse strains (Regué *et al.*, 2005).

3.6.3 Fimbriae

To initiate colonization and infection, *Klebsiella pneumoniae* relies on adhesion to host cells, a process facilitated by fimbriae, thread-like structures present on the bacterial cell surface. Four main types of fimbriae have been identified in *K. pneumoniae*: Type 1 fimbriae, Type 3 fimbriae, kpc fimbriae, and KPF-28 fimbriae (Cortes *et al.*, 2002).

Type 1 fimbriae are thin and rigid thread-like structures composed of repeating subunits of FimA molecules, with the adhesion molecule FimH at the tip. *K. pneumoniae* isolates possessing Type 1 fimbriae adhere to host cells containing mannose sugars through FimH (Rosen *et al.*, 2008). These fimbriae are responsible for urinary tract infections but play no significant role in gastrointestinal or respiratory tract infections (Struve *et al.*, 2008).

Type 3 fimbriae are longer structures, approximately 0.5-2 mm in length. The gene mrkA synthesizes the molecules that polymerize to form the helical shaft of Type 3 fimbriae (Murphy *et al.*, 2012). These fimbriae are involved in the formation of biofilms, particularly in Catheter-associated urinary tract infections (Murphy *et al.*, 2013). However, they do not contribute significantly to colonization in the intestines or respiratory tract (Struve *et al.*, 2009).

Kpc fimbriae are present in hypermucoviscous *Klebsiella pneumonia* isolates and are synthesized and assembled by the KcABCD operon (Wu *et al.*, 2010). Composed of repeating subunits of kpcA along with some molecules of kpcS and kpcI, Kpc fimbriae are also associated with the formation of biofilms in *K. pneumoniae* (Wu *et al.*, 2010).

KPF-28 adhesins are long and flexible fimbriae, and the gene responsible for their synthesis is located on the R plasmid, which also codes for the CAZ-5/SHV-4 B-lactamase (Di Martino *et al.*, 1996). KPF-28 fimbriae are beneficial for *K. pneumoniae* in the colonization of the human gastrointestinal tract (Di Martino *et al.*,2007).

3.6.4 Siderophores

Both hosts and bacteria rely on iron, a crucial metal required for a variety of metabolic activities, in their complicated interaction. However, the restricted availability of iron in

the human body's extracellular fluid serves as a defense mechanism against bacterial development. Bacteria have evolved strategies to circumvent this difficulty and collect iron for survival and multiplication. The insolubility of free iron under physiological circumstances makes iron absorption by human hosts challenging. Bacteria have developed a siderophore-dependent iron acquisition pathway in response, allowing them to compete with the host for iron resources (Miethke and Marahiel, 2007).

Siderophores are virulence factors that play an important part in the iron acquisition process. Bacteria manufacture these tiny iron-binding molecules, which are released outside the cells and capable of binding to ambient iron. They bind to certain receptors on the bacterial outer membrane and are then transferred to the periplasm and ultimately to the inner membrane. Iron is transferred into the bacterial cytoplasm via an ABC-transporter mechanism and becomes available for utilization by the bacterium (Miethke *et al.*, 2007). Siderophores play an important role in bacterial proliferation and have been recognized as important contributions to bacterial pathogenicity.

Hypervirulent *K. pneumoniae* (hvKP) strains produce considerably more siderophores than classic *K. pneumoniae* (cKP) strains (Russo *et al.*, 2014).

These hvKP strains produce four forms of siderophores: enterobactin, yersiniabactin, salmochelin, and aerobactin, all of which help the bacteria's growth efficiency. Understanding the role of siderophores in pathogenesis gives important insights into the bacterium's capacity to adapt and prosper in the iron-restricted environment of the host. Developing innovative treatment techniques to fight *K. pneumoniae* infections by targeting siderophore-mediated iron acquisition processes may hold promise (Zhu *et al.*, 2021). Siderophores, also known as hemophores, are tiny iron-chelating molecules with a low molecular weight and high affinity that are released by bacteria to help in iron acquisition. *K. pneumoniae* also has a variety of siderophores (Holden *et al.*, 2015).

Enterobactin is a common iron-chelating molecule that has a stronger affinity for iron than any other siderophores. However, the mammalian innate immune system releases lipocalin 2, which binds to enterobactin, preventing bacteria from iron absorption (Bachman *et al.*, 2009). To counteract this defense mechanism, *K. pneumoniae* secretes

additional forms of siderophores such as aerobactin, yersiniabactin, and salmochelin. These siderophores are essential for iron absorption and the advancement of the pathogenic process. Yersiniabactin is found in the majority of human *K. pneumoniae* infections and has been linked to lung infections (Bachman *et al.*, 2011). Aerobactin is also thought to be an important virulence factor for *Klebsiella pneumoniae*.

3.6.5 Urease

Urease is an important virulence component in *K. pneumoniae*, contributing to its pathogenicity and capacity to infect the host. This enzyme oversees catalyzing urea hydrolysis into ammonia and carbon dioxide. Urease is encoded by a group of genes in *K. pneumoniae* that are involved in its synthesis and assembly (Derakhshan *et al.*, 2008; Piperaki *et al.*, 2017).

Urease's participation in nitrogen metabolism is one of its primary roles in *K. pneumoniae*. *K. pneumoniae* may use urea as a nitrogen source for growth and survival by hydrolyzing urea, which is a nitrogenous waste product in the human body. This offers the bacteria an edge in nitrogen-limited habitats like the urinary system and other human tissues (Bradbury *et al.*, 2014).

K. pneumonia's potential to produce urinary tract infections (UTIs) is tightly linked to the presence of urease. UTIs are among the most prevalent infections caused by *K. pneumoniae*, and the bacterium's production of urease aids in colonization and persistence in the urinary tract. *K. pneumoniae* provides an alkaline environment in the urinary tract by hydrolyzing urea to ammonia, which helps neutralize the acidic pH of urine. This alkaline environment helps the bacteria survive and reproduce in the urinary system, allowing it to elude the host's immune defenses and form a chronic infection (Piperaki *et al.*, 2017).

However, ammonia synthesis by urease-expressing *K. pneumoniae* can be harmful to the host. Ammonia is harmful to host cells and can induce epithelial damage in the urinary system, resulting in inflammation and tissue damage. This inflammation can aid in the spread of the bacteria and worsen the severity of the infection (Derakhshan *et al.*, 2008).

Furthermore, *K. pneumoniae* urease production has been associated with the development of urinary stones or calculi. The ammonia produced by urea hydrolysis combines with other components in the urine, causing mineral precipitation and stone formation. These stones can function as a reservoir for the bacteria, creating a haven for *K. pneumoniae*.

These stones can function as a reservoir for the bacteria, creating a haven for *K. pneumoniae* to thrive and defy antibiotic therapy. Furthermore, the presence of urinary stones might restrict urine flow, generating favorable circumstances for bacterial development and recurrent UTIs (Bradbury *et al.*, 2014).

Urease has been linked to *K. pneumonia's* potential to cause infections in various areas, including the respiratory tract and the gastrointestinal tract, in addition to its function in urinary tract infections. Urease is essential for the colonization of tissue surfaces, particularly those in the urinary system and indwelling catheters. Urease aids in the production of inorganic salts and encrustations in tissues and abiotic surfaces by promoting urea hydrolysis and elevating pH. This condition promotes bacterial survival and illness. By escaping clearance owing to interrupted urine flow and encouraging biofilm development, this condition supports the bacterium's survival and illness progression (Bradbury et al, 2014).

Klebsiella pneumoniae, like other gut infections, may create urease, which is required for development in the GI tract. Urease inactivation can have a substantial impact on the bacterium's capacity to grow in this environment.

3.6.6 Pili

Researchers have focused on certain proteins found in *K. pneumoniae* pili to better understand how the bacteria penetrate the human body. These proteins are required by bacteria for them to attach to cell surface receptors and sugar molecules on cell membranes. The 38.6 kDa protein identified in *K. pneumoniae* pili is one such protein of interest. The researchers wanted to see if this protein might act as both a hemagglutinin (producing red blood cell clumping) and an adhesin (increasing attachment to host cells).

To isolate and identify the 38.6 kDa protein, the researchers used a procedure known as SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis). Once identified, they performed hemagglutination and adhesion tests on erythrocytes and enterocytes from patients. Once identified, they performed hemagglutination and adhesion assays in BALB/C mouse erythrocyte cells and enterocytes. These experiments were critical in revealing the protein's dual function as a hemagglutinin and an adhesion protein.

The findings of the study indicated that the pili protein, particularly the 38.6 kDa protein, had both hemagglutination and adhesion capabilities. This discovery emphasizes how important this protein is to the bacterium's pathogenicity. Understanding these pathways gives information on *K. pneumoniae* pathophysiology and identifies prospective targets for treatment approaches.

3.7 Biofilm

In *Klebsiella pneumoniae* infections, biofilm formation is a critical virulence mechanism in which bacteria form organized communities attaching to surfaces with a protective extracellular matrix. This matrix acts as a barrier to host immune responses and medications, making biofilm-associated illnesses extremely difficult to cure (Wang *et al.*, 2020). The ability of *K. pneumoniae* to create biofilms increases its ability to cause persistent and chronic infections by sticking to numerous surfaces within the host, such as lung tissues, urinary catheters, and medical equipment. The biofilm's durability is related to its enhanced antibiotic resistance, immune system evasion, and facilitation of horizontal gene transfer, which promotes the spread of antibiotic resistance and virulence factors. Biofilms formed by *K. pneumoniae* and *Pseudomonas aeruginosa* in catheters are known to cause alkaline urine and possibly urinary tract infections when biofilms detach from the catheter. Furthermore, biofilm development allows for long-term colonization while impairing host cell function, worsening infection severity. Infections caused by biofilms are very common. They are responsible for a high number of bacterial illnesses, accounting for approximately 60%

of overall bacterial infections and 80% of human chronic infections (Hentzer *et al.* 2005).

These biofilms are typically discovered on medical equipment such as catheters and can cause infections throughout the body. Biofilm production occurs in phases, beginning with initial adhesion and progressing through colony formation, maturity, and final release of free-living bacteria. Bacteria cling to surfaces and each other within the biofilm, forming a dense and organized population protected by the EPS. Because it provides resistance to the host's immune system and antimicrobial medicines, this protective matrix makes biofilm-associated infections extremely difficult to cure (Wang *et al.*, 2020).

Biofilm development in *K. pneumoniae* is dependent on two essential components: type 3 pili and capsular polysaccharides (CPs). Type 3 pili allow bacteria to cling to surfaces and begin the creation of biofilms. Capsular polysaccharides impact the structure of the biofilm as well as bacterial communication within the community. The biofilm is adaptable and has dynamic gene regulation that is controlled by quorum sensing. Bacterial communication is enabled through quorum sensing, which results in synchronized changes in behavior and gene expression. Quorum sensing causes unique genetic responses that boost the biofilm's resistance and flexibility as the biofilm expands and the bacterial population rises (Hentzer *et al.*, 2005).

The EPS matrix of biofilms protects against the host's immunological response by preventing antibodies and antimicrobial compounds from being accessed, lowering the immune system's efficiency. Certain gene alterations, such as those that affect the outer membrane proteins *YfgL*, *Wab-G*, *BamB*, and *Kp OmpA*, affect biofilm development, adherence, and interaction with the host's immune response. By altering membrane proteins and bacterial activity, oxidative stress can also harm and destabilize biofilms. Understanding the complexities of biofilm production is critical (Wang *et al.*, 2020).

3.8 Biofilm composition

Biofilms are groups or micro-organisms in which microbes produce an extracellular polymeric substance (EPS) such as proteins DNA polysaccharides and RNA in addition to these components, water which is a major part of biofilm responsible for the flow of nutrients inside the biofilm matrix (Vuotto *et al.*, 2014).

Sr. no.	Components	Percentage of matrix
1.	Microbial cells	2-5%
2.	DNA and RNA	<1-2%
3.	polysaccharides	1-2%
4.	proteins	<1-2%
5.	water	Up to 97%

Table 3.1:Biofilm composition.

3.9 Stages of biofilm formation

Biofilm production is a complex process in which cells change from a floating (planktonic) to a fixed (sessile) growth state. It is suggested that genes play a critical role in biofilm development. Once microbes adhere to the surface, this complicated phenomenon develops in a series of actions involving diverse changes. The creation of a biofilm may be divided into many critical phases.

1. Initial attachment to a surface
2. Micro-colony formation
3. 3D structure formation and maturation
4. Detachment (dispersal)

3.9.1. Attachment

When a bacterial cell approaches a surface, its movement slows progressively, finally resulting in a reversible link between the cell's poles and that surface. Solid-liquid interfaces, such as blood or water, provide a perfect habitat for bacteria to adhere and multiply in the creation of biofilms. Following reversible attachment, cells move to

irreversible attachment, in which surface proteins such as SadB or LapA, combined with extracellular polymeric substances (EPS), enhance cell-surface adhesion. An intracellular second messenger known as bis-(3′-5′)-cyclic dimeric guanosine monophosphate (c-di-GMP) plays a critical role during this transition to irreversible attachment. C-di-GMP is produced by bacteria and affects EPS synthesis and motility in opposing ways. Another second messenger, cAMP, plays a role in the transition from reversible to irreversible attachment. Environmental variables such as carbon and oxygen levels regulate the amounts of c-di-GMP and cAMP, altering surface attachment depending on the environment.

Variations in attachment capacities have been found to contribute to the process of speciation, emphasizing the importance of surface attachment in microbial communities. Surfaces that are rough, hydrophilic, and coated provide a more favorable environment for attachment and biofilm development. Increased attachment may also come from minor changes in parameters like water flow velocity, temperature, or nutrient contents that are just below critical thresholds. Furthermore, the presence of locomotor structures on cell surfaces, such as flagella, pili, fimbriae, proteins, or polysaccharides, is significant and can provide benefits in biofilm development, particularly in mixed populations.

3.9.2 Micro-Colony Formation

Bacterial cells produce microcolonies after irreversible attachment by aggregating previously attached cells and conducting cell division. These microcolonies grow via the proliferation of cells and the production of extracellular polymeric substances (EPS). When a bacterial cell firmly attaches to the surface, the genetic process responsible for exopolysaccharide synthesis is activated when the signal strength exceeds a certain threshold. As a result, this chemical signal orchestrates bacterial cell divisions within the surrounding exopolysaccharide matrix, resulting in the development of microcolonies.

3.9.3 3D Structure Formation and Maturation

Specific genes related to biofilm development become activated after the creation of microcolonies. These genes over the see generation of extracellular polymeric

substances (EPS), which are the major structural components of biofilms. EPS generally accounts for more than 90% of the dry mass in mature biofilms and is composed of a range of components such as polysaccharides, nucleic acids, proteins, lipids, and other biopolymers. EPS plays various important roles within the biofilm structure. It serves as an adhesive that adheres the biofilm to surfaces, as a scaffold that connects cells and is critical in sustaining the biofilm's complicated three-dimensional design. Furthermore, the EPS enclosing the biofilm serves as a protective barrier, sheltering bacterial cells from a variety of stressors such as antimicrobial chemicals, host immunological responses, oxidative factors, and metallic cations. EPS keeps essential molecules inside the biofilm, including quorum sensing (QS) signaling chemicals, extracellular enzymes, and metabolic products. As a result, EPS is critical in promoting cell-to-cell communication and aiding drug breakdown. Surprisingly, research has demonstrated that bacterial adhesion may cause the creation of an extracellular matrix on its own. Following matrix development, water-filled tubes are formed to carry nutrients inside the biofilm. According to Sauer et al. in 2022, these water channels act similarly to a circulatory system, supplying various nutrients to the microcolonies inside the biofilm and eliminating waste products.

3.9.4 Detachment and Dispersal

Researchers have frequently witnessed bacteria disengaging from biofilms after the creation of these biofilms. This self-detachment allows bacteria to reproduce and disperse quickly. Planktonic bacterial cells detach from the biofilm in a predetermined way with a natural rhythm.

Mechanical forces can occasionally dislodge bacteria from biofilms and release them into the surrounding environment. However, usually, some bacteria stop producing extracellular polymeric substances (EPS) and depart from the biofilm. Biofilm cell dispersal can occur via two basic mechanisms: detachment of freshly formed cells from the expanding biofilm or dispersion of biofilm aggregates controlled by variables such as fluid flow effects or quorum sensing. Importantly, dispersion not only marks the end of one biofilm lifecycle but also the beginning of another. Dispersal may be classified into two types: "active dispersal" and "passive dispersal." Active dispersion is

dependent on cell motility or EPS breakdown. Cell motility genes, such as those involved in flagella formation or chemotaxis, and EPS degradation genes, such as those involved in dispersion secretion, are activated during this process. Genes involved in EPS generation (for example, polysaccharide synthesis) and attachment (for example, fimbriae synthesis) are downregulated. The intracellular second messenger c-di-GMP is important in the regulation of these genes. The levels of intracellular c-di-GMP are dynamically controlled by environmental factors. A drop in intracellular c-di-GMP levels, in contrast to the attachment stage, facilitates the shift from a planktonic to a biofilm lifestyle by regulating genes involved in dispersion. A rise in intracellular c-di-GMP levels, on the other hand, promotes the transition to a biofilm lifestyle. As a result, c-di-GMP functions as a key regulator of the transition between planktonic and biofilm forms of bacterial life.

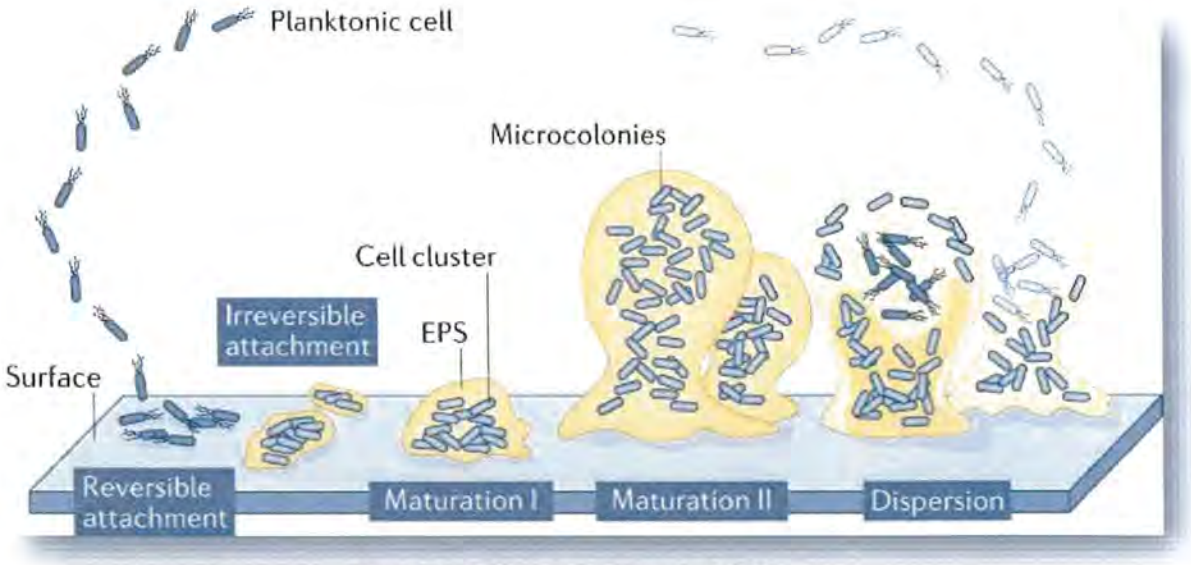

***Figure 3.3**: the biofilm cycle (Sauer et al., 2022)*

3.10 Role of biofilm in antibiotic resistance

Biofilms provide bacteria with various mechanisms to develop resistance against antibiotics, making them highly challenging to treat (Hathroubi *et al.,* 2017). Firstly, biofilms act as physical barriers, preventing the penetration of antibiotics due to the

presence of cationic and anionic molecules like uronic acids, glycoproteins, glycolipids, and extracellular DNA that bind to the antibiotics (Nadell *et al.*, 2015). Secondly, bacterial cells within biofilms exhibit different physiological states, making some cells more susceptible to antibiotics during the growth phase while others remain unaffected in the stationary phase (Bjarnsholt *et al.*, 2013). The presence of persister cells, which are genetically similar but physiologically different from the rest, contributes to higher antibiotic resistance (Lewis 2012). Additionally, efflux pumps in biofilms reduce the concentration of antibiotics within the biofilm environment, protecting bacterial cells (Sun *et al.*, 2014).

Klebsiella pneumoniae is a pathogen known for forming biofilms on endotracheal tubes and urinary catheters (Song *et al.*, 2013; Chatterjee *et al.*, 2014). Different strains of *K. pneumoniae* exhibit variations in biofilm formation dynamics and kinetics, with some strains completing all stages, including the dispersal phase, within 48 hours, while others take longer (Bandeira *et al.*, 2014). Type 1 and type 3 fimbriae, as well as the capsule of *K. pneumoniae*, contribute to the formation of stable biofilms. Environmental factors such as pH, temperature, and nutrient availability influence the dynamics of biofilm formation (Ribeiro *et al.*, 2015; Chien *et al.*, 2013). *K. pneumoniae* biofilms are significantly more resistant to antibiotics compared to planktonic cells (Van Laar *et al.*, 2015).

Resistance to antibiotics in biofilms is attributed to the limited diffusion of drugs through the biofilm matrix, leading to inadequate drug reaching individual bacterial cells (Zahller *et al.*, 2002). Furthermore, cells at the periphery of biofilms may be more susceptible to antibiotics, while cells in the central core region remain protected due to reduced metabolism and limited nutrient and oxygen supply (Anderl *et al.*, 2003).

As bacteria form biofilms, they become even more resistant to antibiotic treatment, with resistant strains exhibiting increased resistance potential (Lin *et al.*, 2015). The resistance level of gentamicin-resistant KPC-producing strains increased significantly after biofilm formation, while gentamicin-susceptible strains retained their susceptibility levels (Naparstek *et al.*, 2014). Additionally, the time elapsed after biofilm formation influences the level of antibiotic resistance, with older biofilms

showing higher resistance, possibly due to increased concentrations of exopolysaccharides and other constituents (Singla *et al.*, 2014).

3.11 Treatment for *Klebsiella pneumonia* infections

In managing *Klebsiella pneumoniae*-associated infections, adherence to standard guidelines for antibiotic therapy is crucial. Before administering antibiotics, it is essential to test the bacterial strains in vitro to determine their susceptibility to specific drugs. Improper antibiotic treatment can lead to severe consequences, as demonstrated by a study where 41.6% of *Klebsiella pneumoniae*-infected patients died within 30 days due to inadequate antibiotic therapy (Tumbarello *et al.*, 2012).

Currently, the recommended regimen for treating *K. pneumoniae* infections involves a 14-day course of third or fourth-generation cephalosporins or respiratory quinolones as monotherapy. Alternatively, combination therapies that include cephalosporins with or without the addition of aminoglycosides are also used (Ashurst *et al.*, 2020). For neonatal bacterial infections, including pneumonia, the World Health Organization guidelines suggest using intramuscular gentamicin along with oral amoxicillin (World Health Organization. 2015. Guidelines). Patients allergic to penicillin can be treated with respiratory quinolones or aztreonam (Liu *et al.*, 2018; Mitharwal *et al.*, 2016). Combination therapy is more effective in improving survival rates compared to monotherapy (Qureshi *et al.*, 2012).

In specific cases, a combination of tigecycline, meropenem, and colistin has been proven effective in eradicating *K. pneumoniae* infections (Kaur *et al.*, 2018). It is essential to tailor the antibiotic therapy to the individual patient and the specific strain of *Klebsiella pneumoniae* to ensure optimal treatment outcomes and minimize the development of antibiotic resistance.

3.12 Advent of antibiotic resistance

The development of antibiotic resistance in microorganisms is primarily attributed to the inappropriate and excessive use of antibiotics. Over the years, there has been a significant increase in antibiotic consumption, with a 36% rise in antibiotic units

consumed during the last decade (Van Bockel *et al.*, 2014). As a result of this overuse, there has been a continuous increase in antibiotic resistance against important classes of antibiotics, including beta-lactams, carbapenems, and colistin, as reported by the World Health Organization (WHO. Global Report on Surveillance. 2014). This rise in antibiotic resistance is a global concern, as it poses significant challenges in treating bacterial infections, impacting human life (Sherry *et al.*, 2019).

Among the resistant pathogens, *Klebsiella pneumoniae*, belonging to the Enterobacteriaceae family, has gained particular attention due to its ability to develop resistance against a wide range of available antibiotics. It is classified as an ESKAPE pathogen, along with other troublesome bacteria such as *Enterococcus faecium, Staphylococcus aureus, Acinetobacter baumannii, Pseudomonas aeruginosa, and Enterobacter spp.*, as these pathogens can resist or evade antibiotic treatments (Boucher *et al.*, 2009).

Surveillance studies have indicated a continuous increase in resistance rates among *Escherichia coli* and *Klebsiella pneumoniae*, with the latter showing a more significant increase, particularly against carbapenems (Navon-Venezia *et al.*, 2017). In the United States, resistance rates among Enterobacteriaceae isolates have also been on the rise, with Klebsiella pneumoniae contributing significantly to Extended Spectrum Beta-Lactamase (ESBL) associated resistance (Logan *et al.*, 2014). Multi-drug-resistant *Klebsiella pneumoniae* infections have been associated with increased mortality rates, particularly among hospitalized and critically ill patients (Xu *et al.*, 2017).

Research from various regions has reported the emergence of multi-drug-resistant *K. pneumoniae* isolates. In a study conducted in Shanghai, China, all 41 samples collected were found to be multi-drug resistant (Zhang *et al.*, 2018). Similarly, a study in Faisalabad, Pakistan, reported that 15% of total *K. pneumoniae* isolates showed multi-drug resistance (Chaudhry *et al.*, 2019).

The emergence of resistance is often attributed to the presence of mobile genetic elements carrying various factors responsible for antibiotic resistance (Li *et al.*, 2014). *K. pneumoniae* may employ several resistance mechanisms, including the production of Extended Spectrum Beta-Lactamases, efflux pumps, and alterations or absence of target

sites, to evade the effects of antibiotics. Understanding these resistance mechanisms is crucial in developing effective strategies to combat antibiotic resistance and ensure successful treatment of infections caused by multi-drug resistant *K. pneumoniae*.

3.12.1 Resistance against Polymyxin E

Polymyxin-E class antibiotics such as Colistin have emerged as crucial antibiotics for the treatment of infections caused by multidrug-resistant (MDR) pathogens. It works by disrupting the bacterial plasma membrane, binding specifically to the lipid A component of the lipopolysaccharides in Gram-negative bacteria (Ah *et al.*, 2014). However, the increasing use of colistin has led to the development of resistance in some bacterial strains, including *K. pneumoniae.*

One of the main mechanisms of resistance in *K. pneumoniae* involves modifying its lipopolysaccharides. Specifically, the bacterium adds 4-amino-4-deoxy-L-arabinose to the lipid A component, which alters the structure and reduces colistin's binding affinity, rendering it less effective in disrupting the bacterial membrane (Ah *et al.*, 2014).

Moreover, colistin resistance can also arise due to mutations in three chromosomal genes: mgrB, phoQ, and crAB (Wright *et al.*, 2015). These mutations can lead to changes in the bacterial membrane, limiting colistin's ability to interact with lipid A and weaken its antimicrobial action.

Additionally, plasmid-mediated resistance plays a significant role in colistin resistance in *K. pneumoniae*. The enzyme phosphoethanolamine transferase MCR-1, encoded by a plasmid, modifies the bacterial lipopolysaccharides, resulting in reduced binding of colistin to the membrane and decreasing its efficacy (Liu *et al.*, 2015).

The spread of polymyxin E (colistin) resistance among bacterial populations is a concerning global health issue. As colistin remains one of the last-resort antibiotics for treating MDR infections, the emergence of resistance poses a serious threat to effective treatment options. Therefore, it is crucial to monitor and understand the mechanisms of colistin resistance in bacteria like *K. pneumoniae* to implement effective infection control measures and develop alternative treatment strategies to combat resistant infections.

3.12.2 Resistance to Fluoroquinolones

Fluoroquinolones are a widely used class of antibiotics due to their broad spectrum of activity, favorable pharmacokinetic properties, and generally good safety profile (Grillon *et al.*, 2016). These antibiotics work by interfering with the DNA replication cycle of bacteria, ultimately inhibiting their growth and causing cell death (Lautenbach *et al.*, 2001).

However, over time, bacteria have developed resistance mechanisms against fluoroquinolones, which pose a challenge to their effectiveness in treating infections. One common mechanism of resistance is through mutations in specific regions of bacterial DNA called quinolone resistance-determining regions (QRDRs) within the gyrA and parC genes of the bacterial chromosome. These mutations lead to changes in the target enzymes, reducing the binding affinity of fluoroquinolones and rendering them less effective.

Another mechanism of resistance involves plasmid-associated resistance, where resistance genes are located on plasmids, small circular DNA molecules that can transfer between bacteria. This enables the rapid spread of resistance among bacterial populations (Lautenbach *et al.*, 2001).

Furthermore, bacteria may develop resistance by altering or reducing the expression of porin proteins, which are channels in the bacterial cell membrane that facilitate the entry of antibiotics. By reducing the permeability of the cell membrane, bacteria can limit the entry of fluoroquinolones into the cell, making them less susceptible to the antibiotic.

Increased expression of efflux pumps is another common mechanism of fluoroquinolone resistance. These pumps act as "efflux" systems that actively pump out the antibiotic from the bacterial cell, reducing its intracellular concentration and thus its effectiveness.

Interestingly, the presence of siderophores, which are iron-chelating molecules produced by some bacteria to scavenge iron, has also been linked to increased resistance against fluoroquinolones. A study demonstrated that among Klebsiella

pneumonia isolates producing siderophores, a significant proportion showed resistance to ciprofloxacin (Zhang *et al.*, 2017).

3.12.3 Resistance to Sulphonamides and Trimethoprim

The emergence of resistance genes that encode enzymes with dual resistance to both sulphonamides and trimethoprim is indeed a significant concern in the field of antimicrobial therapy. These enzymes, known as dihydrofolate reductase-sulphonamide resistance genes (DHFR-Sul), represent a unique mechanism of resistance that severely limits the effectiveness of both drug classes.

DHFR-Sul genes are found in certain bacteria and are responsible for producing altered dihydrofolate reductase enzymes that have reduced binding affinity for both sulphonamides and trimethoprim. As a result, these enzymes are no longer inhibited by either drug, rendering combination therapy with sulphonamides and trimethoprim ineffective in treating infections caused by bacteria carrying these resistance genes.

The presence of DHFR-Sul genes poses a considerable challenge for clinicians, as it reduces the available treatment options for infections caused by these resistant bacteria. Sulphonamides and trimethoprim have historically been used in combination to enhance their effectiveness and prevent the development of resistance. However, with the emergence of DHFR-Sul genes, this combination therapy has become much less effective (https://grepmed.com/GeraldMD, 2018).

The development and spread of DHFR-Sul genes are often linked to the overuse and misuse of sulphonamides and trimethoprim in clinical settings, as well as in agriculture. The selective pressure exerted by these drugs promotes the survival and proliferation of bacteria carrying these resistance genes, leading to the spread of resistance within bacterial populations.

Sulphonamides work by mimicking the structure of para-aminobenzoic acid (PABA), which is a precursor in the synthesis of folic acid. By acting as a competitive inhibitor, sulphonamides prevent the synthesis of folic acid, leading to the inhibition of nucleic acid and protein production in bacteria.

Trimethoprim, on the other hand, inhibits the enzyme dihydrofolate reductase (DHFR), which is responsible for converting dihydrofolic acid to tetrahydrofolic acid.

Tetrahydrofolic acid is crucial for DNA synthesis in bacteria. Resistance to trimethoprim arises mainly due to the acquisition of resistant genes encoding for altered DHFR enzymes with reduced binding affinity for the drug.

3.12.4 Resistance to β-lactams

Beta-lactam antibiotics, including penicillin, cephalosporins, carbapenems, and monobactams, are widely used antibiotics characterized by their beta-lactam ring structure. However, resistance to beta-lactams has emerged due to the production of beta-lactamases, enzymes that destroy the beta-lactam ring. Alexander Fleming first observed that certain pathogens, like *E. coli*, were not affected by penicillin, and later, it was discovered that this was due to the production of bacterial enzymes, such as beta-lactamases.

Klebsiella pneumoniae is naturally less susceptible to beta-lactams because it can produce SHV-1 type beta-lactamase through its chromosomal genes. A study in Lahore, Pakistan, in 2017 found that approximately 82.4% of *K. pneumoniae* samples were beta-lactamase producers. To combat beta-lactamase-producing organisms, beta-lactamase inhibitors like clavulanic acid are used, as they can inactivate the beta-lactamases, thus restoring the effectiveness of beta-lactam antibiotics (Paterson *et al.*, 2005).

3.12.5 Resistance against tetracycline.

Resistance against tetracycline, a commonly used antibiotic, has become a significant concern in the medical community. Over time, bacteria have developed various mechanisms to resist the effects of tetracycline, reducing its effectiveness in treating infections. In Europe, extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella species* (spp.) have shown high levels of tetracycline resistance, with prevalence rates of 66.9% and 44.9%, respectively (Jones *et al.*, 2014). Similarly, methicillin-resistant *Staphylococcus aureus* (MRSA) and *Streptococcus pneumoniae* globally have demonstrated resistance rates of 8.7% and 24.3%, respectively (Mendes *et al.*, 2015). Tetracycline resistance is classically associated with three general mechanisms: efflux, ribosomal protection, and enzymatic inactivation of tetracycline drugs. Efflux pumps actively remove tetracyclines from the bacterial cell, reducing

their intracellular concentration. Ribosomal protection proteins bind to the ribosome and prevent tetracyclines from inhibiting protein synthesis. Lastly, enzymes can chemically modify tetracycline drugs, rendering them inactive and ineffective against the bacteria. Furthermore, chromosomal mutations can lead to increased expression of intrinsic resistance mechanisms within the bacteria, further contributing to tetracycline resistance. Additionally, some bacteria can develop resistance by reducing the permeability of their cell membrane. This reduced permeability limits the entry of tetracycline into the bacterial cell, making it less susceptible to the antibiotic's action. Environmental factors, such as the selective pressure exerted by the overuse or misuse of tetracycline in clinical and agricultural settings, can also contribute to the development of resistance in bacteria (https://grepmed.com/GeraldMD, 2018).

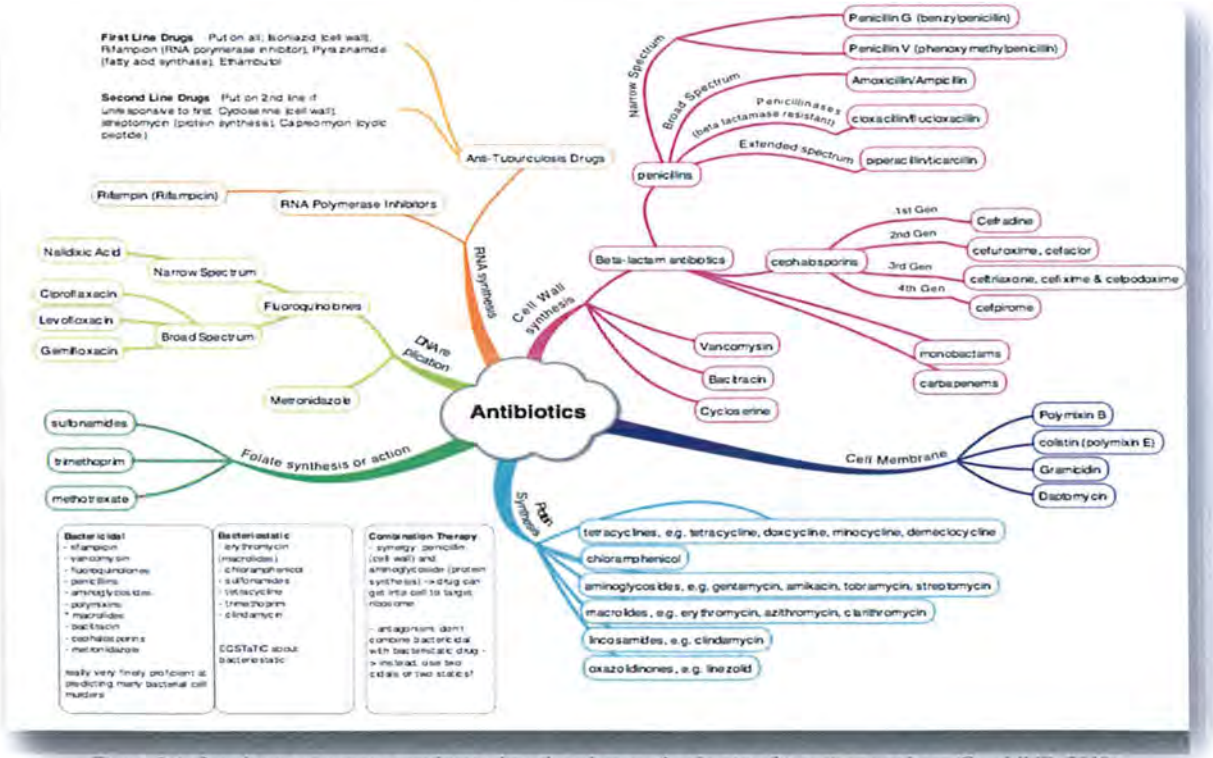

Figure 3.4: flowchart representing antibiotics based on their mode of action (https://grepmed.com/GeraldMD, 2018).

3.13 Antibiotic Destructases

Antibiotic destructases are a type of enzyme that chemically alters the structure of antibiotics, rendering them useless. The antibiotic's antibacterial effects are lost because of this change, enabling resistance to the microbes that manufacture these enzymes. These destructases vary from metabolic enzymes that process foreign chemicals in the host because they have different regulation, catalytic efficiency, pace, and substrate specificity. Antibiotic destructases have developed especially to impart resistance, as opposed to xenobiotic-modifying enzymes, which predominantly detoxify foreign substances in the host. They are highly specialized, with great specificity and effectiveness in deactivating certain antibiotic classes. Beta-lactamases, which degrade beta-lactam antibiotics, and aminoglycoside-inactivating enzymes, which alter aminoglycoside medications, are two well-known examples of antibiotic destructases. There are several kinds of antibiotic destructases, each specialized in functioning on a distinct antibiotic substrate. Peptidase, hydrolases, thioltransferases, epoxidases, cyclopropanes, acyl transferases, methyl transferases, nucleotidylyl transferases, ADP-ribosyl transferases, glycosyltransferases, phosphotransferases, lyases, and oxidoreductases are among the enzyme types. More antibiotic destructases with different substrates are anticipated to be uncovered in the future as antibiotic discovery research progresses.

3.13.1. β-Lactamase Enzymes

Beta-lactamase enzymes are produced by bacteria and can hydrolyze the beta-lactam ring found in various antibiotics, such as penicillin, cephalosporins, monobactams, and carbapenems. It is believed that the evolution of penicillin-binding proteins may have occurred under selective pressure, leading to the development of beta-lactamase enzymes (Meroueh *et al.*, 2003). There are four major classes of beta-lactamases based on their structural characteristics, namely Class A, B, C, and D (Ambler 1980). These classes include different types of extended-spectrum beta-lactamases, cephalosporinases, and carbapenemases.

3.13.1.a Extended Spectrum Beta Lactamases

Extended Spectrum Beta Lactamases (ESBLs) are enzymes capable of hydrolyzing a wide range of antibiotics, such as penicillins, cephalosporins, ceftazidime, cefotaxime, ceftriaxone, cefepime, and aztreonam, but they are unable to degrade carbapenems and cephamycins. ESBL-producing strains often exhibit resistance to other antibiotics, including fluoroquinolones, aminoglycosides, and sulfonamides, as these resistance genes can be found on the same plasmid as the ESBL genes. Clavulanate and tazobactam can inhibit ESBLs. The prevalence of ESBLs in Gram-negative pathogens, particularly in *K. pneumoniae*, is increasing and poses a significant concern for the treatment of infectious diseases. Studies from various countries, including China and India, have reported a rise in ESBL-positive *K. pneumoniae* isolates over the years. In Pakistan, multiple studies have also shown a high prevalence of ESBL-producing *K. pneumoniae* clinical isolates, with percentages reaching up to 96.1%. This growing trend of ESBL production highlights the urgent need for effective strategies to combat antibiotic resistance in clinical settings.

3.13.2 AmpC -Lactamases

The discovery of the AmpC β-lactamase enzyme in 1940 marked the first bacterial enzyme capable of degrading penicillin (Abraham & Chain, 1940). AmpC enzymes belong to Class C in the structural classification of β-lactamases by Ambler and Group 1 in the classification system by Bush based on functionality. Continuous exposure to cefoxitin and cefotetan, along with β-lactamase inhibitors, is believed to contribute to the emergence of AmpC type β-lactamase enzymes (Medeiros, 1997). AmpC enzymes function as cephalosporinases, conferring resistance against various cephalosporins, cephamycins like cefoxitin and cefotetan, aztreonam, and oxyimino cephalosporins such as ceftazidime, cefotaxime, and ceftriaxone. They also show resistance to β-lactam/β-lactamase inhibitor combinations like ampicillin/clavulanic acid and piperacillin/tazobactam (Hemalatha *et al.*, 2007). The increasing prevalence of AmpC β-lactamases is a major concern due to the expanded resistance status of bacterial isolates associated with these enzymes. AmpC β-lactamases are relatively small proteins with a molecular weight of approximately 34-40 kDa. Their isoelectric points

are generally greater than 8.0, except for FOX enzymes, which have an isoelectric point in the range of 6.7-7.2.

In the GenBank database, the AmpC β-lactamase genes are classified under COG 1680, which stands for Cluster of Orthologous genes. COG 1680 also includes other proteins alongside the Amp genes, such as penicillin-binding proteins and proteins from archaea.

3.13.3 TetX – the flagship tetracycline-inactivating enzyme

In 1984, enzymatic inactivation of tetracyclines was proposed as a resistance mechanism. A plasmid from Bacteroides fragilis was found to confer tetracycline resistance to *E. coli* under aerobic conditions and contained a putative tetracycline efflux pump and a gene, *tet*X, encoding a potentially novel tetracycline resistance enzyme. The heterologous expression of *tet*X, *tet*X1, and *tet*X2 in *E. coli* revealed that *tet*X and *tet*X2 are active flavin-dependent monooxygenases (FMOs) that degrade tetracyclines, while TetX1 is a non-functional variant. TetX was shown to inactivate first, second, and third-generation tetracyclines, including tigecycline, leading to the formation of various degradation products, which may attenuate the biological activity of tetracyclines. X-ray crystal structures of TetX bound to different tetracyclines confirmed its class A FMO mechanism, utilizing NADPH to reduce the flavin cofactor and transfer a hydroxyl group to the nucleophilic C11a of the tetracycline enol. These findings shed light on the enzymatic basis of tetracycline resistance mediated by TetX enzymes (Yang *et al.*, 2004; Volkers *et al.*, 2011, 2013).

3.14 Tetracyclines

Tetracyclines, a class of potent broad-spectrum antibiotics, have been integral to modern medicine ever since their discovery in the mid-20th century. These remarkable drugs are derived from a natural product produced by the bacterium Streptomyces and demonstrate exceptional effectiveness against a wide range of bacterial infections. By targeting the process of protein synthesis within bacteria, tetracyclines impede their ability to proliferate and cause harm. As a result, they have proven to be highly effective in treating various bacterial ailments, encompassing respiratory, urinary tract,

skin, and sexually transmitted infections. Nevertheless, the growing concern of antibiotic resistance looms large, urging healthcare practitioners to exercise caution in their use and prompting continuous research efforts to fully harness the potential of tetracyclines in combatting bacterial diseases effectively (Askari Rizvi, 2018).

Recent surveillance investigations in particular European nations have indicated worrying levels of tetracycline resistance among select bacterial species. Tetracycline resistance was identified in Escherichia coli and Klebsiella species (spp.) that manufacture extended-spectrum-lactamases (ESBLs) at rates of 66.9% and 44.9%, respectively. Global resistance rates for methicillin-resistant Staphylococcus aureus (MRSA) and Streptococcus pneumoniae were 8.7% and 24.3%, respectively. Tetracycline resistance is commonly attributed to a variety of mechanisms, such as the acquisition of mobile genetic elements containing tetracycline-specific resistance genes, mutations within the ribosomal binding site, or chromosomal mutations that result in increased expression of intrinsic resistance mechanisms. Importantly, three class-specific mechanisms have been identified: efflux, ribosome protection, and enzymatic inactivation of tetracycline medicines. Several recent analyses have fully investigated tetracycline-specific resistance factors and their frequency in clinical and environmental settings, providing important insights into the issues faced by antibiotic resistance. (Roberts 2005, 2011; Jones et al. 2008; Thaker et al. 2010).

3.14.1 Structure

Tetracycline is characterized by its rigid structure consisting of four rings with various chemical groups attached to its upper and lower sides. By modifying these regions of the tetracycline molecule, chemists have created first and second-generation compounds with varying levels of activity. The antibiotic properties of tetracyclines are particularly dependent on the presence of a dimethylamine group at position C4 in ring A, which is essential for their antibacterial activity. Chemical modifications in the upper and lower peripheral zones of the tetracycline structure can either reduce or enhance their antibiotic and non-antibiotic characteristics. Semi-synthetic tetracyclines, such as minocycline and doxycycline, have been developed through these modifications, leading to increased selectivity and effectiveness against specific

biological targets, especially at positions C7 through C9 of the D ring (Askari Rizvi, 2018).

Tetracyclines' efficacy, potency, and antibacterial activities are closely connected to changes in both the upper and lower sections of their molecular structure. A linear configuration of the DCBA naphthacene ring system, with an A-ring matching the C1-C3 substructure and an exocyclic C2 carbonyl and amide group, is required to ensure that a tetracycline molecule is physiologically active and antibacterial. The presence of an amino group at position C4 of the A ring, as well as the presence of ketoenolic tautomers at locations C1 and C3, is critical for suppressing protein synthesis and providing antibiotic action to tetracyclines. Certain changes, such as changing the native 4S isomer of the C4-dimethylamino group to its 4R isomer, might, however, reduce its antibacterial activity against Gram-negative bacterial strains. Maintaining antibacterial efficacy in the bottom portion of the molecule necessitates the existence of a C10-phenol and a C11-C12 keto-enol substructure, as well as a 12a-OH group.

Changes in the C5 through C9 locations allow for the creation of a variety of tetracycline derivatives, each with specific antibacterial properties. It is possible to improve selectivity towards certain biological targets for antifungal purposes by modifying the R1, R2, and R3 groups, albeit these alterations may not have a substantial impact on antibacterial activity. Tetracycline D-rings are highly susceptible to changes that affect antibacterial activities. Changes to the R4, R5, and R6 groups become critical for establishing high specificity against bacteria and producing significant pharmacokinetic changes. In conclusion, painstaking chemical changes in both the upper and lower portions of the tetracycline structure have played a critical role in the development of a broad variety of tetracycline derivatives, each with distinct pharmacological features. (Askari Rizvi, 2018).

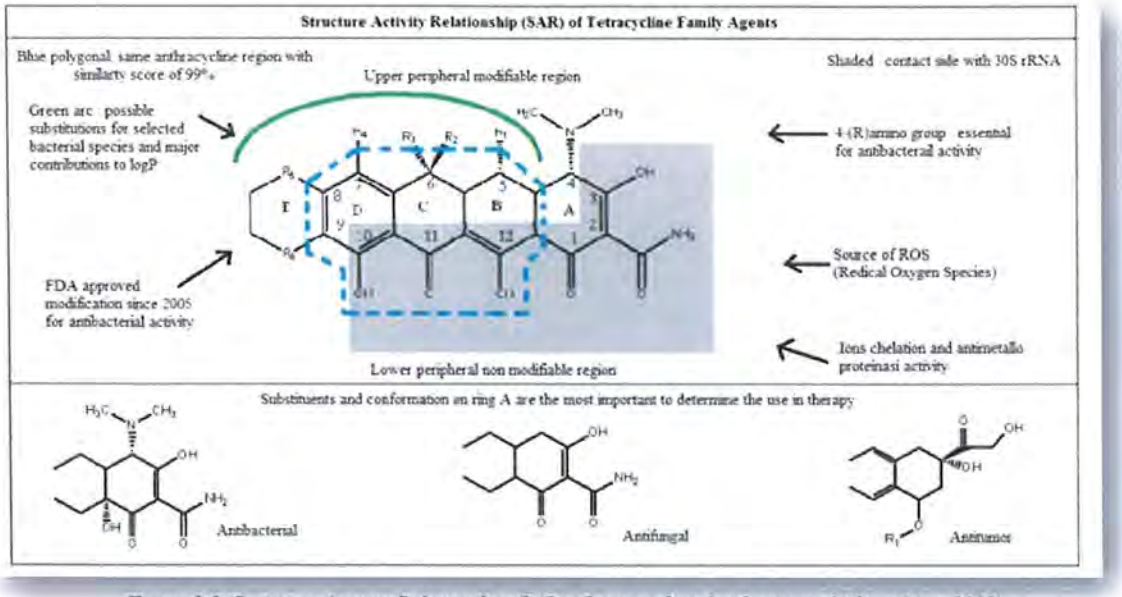

Figure 3.5: Structure-Activity-Relationship (SAR) of tetracycline family agents (Askari Rizvi, 2018)

3.14.2 Pharmacokinetics

Nikaido and Thanassi (1993) investigated and described the method by which tetracyclines are taken up by Gram-negative organisms such as E. coli. Tetracycline molecules enter Gram-negative bacteria by passive diffusion through the outer membrane porins OmpF and OmpC, most likely when complexed with Mg2+. This is corroborated by the discovery that mutants missing these outer membrane porins are more resistant to tetracyclines (Pugsley and Schnaitman 1978). Tetracycline accumulates in the periplasm because of the Donnan potential formed across the outer membrane. Tetracycline dissociates from Mg2+ at this point and assumes a weakly lipophilic, uncharged state, allowing it to pass through the inner membrane and enter the bacterial cytoplasm. The process of absorption into the cytoplasm is partially energy dependent, with factors such as passive diffusion, use of the proton motive force, and phosphate bond hydrolysis. (McMurry and Levy 1978; Smith and Chopra 1984; Yamaguchi et al. 1991).

Tetracyclines, such as doxycycline, minocycline, and tigecycline, have diverse pharmacokinetic properties that may be classified into three groups. Tetracyclines, oxytetracyclines, chlortetracyclines, demeclocyclines, lymecyclines, methacyclines, and rolitetracyclines are all found in Group 1. These medications are normally taken orally and are absorbed in the stomach, duodenum, and small intestine. However, the development of insoluble compounds with calcium, magnesium, iron, and aluminum might impede their absorption. Furthermore, protein, fat, and carbohydrate-containing meals might limit their absorption. Doxycycline and minocycline, both of which are taken orally, belong to Group 2. Doxycycline is well-absorbed and has little dietary interference, leading to a high bioavailability. It also has significant tissue penetration and is mostly eliminated intact by the kidneys and bile. Minocycline, on the other hand, is well-absorbed but might be hampered by iron and antacids. It differs from other tetracyclines in that it produces a variety of metabolites. A tiny percentage is expelled in the urine, but the majority is eliminated in the feces.

Group 3 includes glycylcycline and amino methyl cycline, with limited pharmacokinetic data available on tigecycline. Tigecycline shows protein binding of around 73% to 79%, with a small portion excreted in urine and renal clearance accounting for more than 20%. In the realm of pharmacodynamics, tetracyclines have been less extensively studied compared to other classes of drugs like β-lactams, fluoroquinolones, and aminoglycosides.

The minimum inhibitory concentration (MIC) is the most important metric for determining tetracycline efficacy. This measure depicts the link between the antibacterial activity of the medicine and its potential toxicity within the body. The time-kill curve approach was used to explore the pharmacodynamic characteristics of doxycycline, minocycline, and tigecycline, offering useful insights into their bactericidal capabilities. However, further study in this area is needed to get a better knowledge of tetracycline pharmacodynamics and improve its therapeutic use.

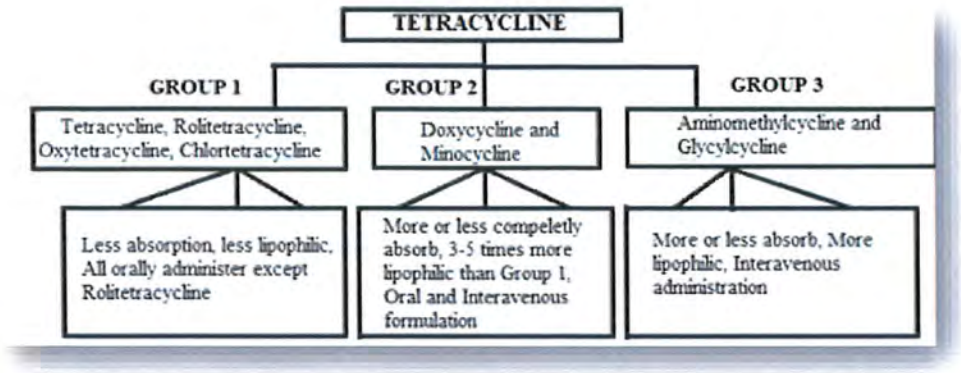

Figure 3.6: Groups of tetracyclines based on pharmacokinetics. (Askari Rizvi, 2018)

Name of Drug	Clinical Data	Pharmacokinetics					Chemical Data	
	Administration Routes	Bioavailability	Protein Binding	Metabolism	Biological Half-life	Excretion	Formula	Molecular mass
Tetracycline	Oral, Topical	75%	-	Not metabolized	8-11hrs			
57-108hrs	Urine (≥60%), Feces	C22H24N2O8	444.4					
Chlorotetracycline	Oral, Topical	30%	50-55%	Hepatic	5.6-9hrs	Renal, biliary	$C_{22}H_{23}ClN_2O_8$	478.8
Oxytetracycline	Oral, Ophthalmic	-	-	-	6-8hrs	Renal	$C_{22}H_{24}N_2O_9$	460.3
Demclocycline	Oral	60-80%	41-50%	Hepatic	10-17hrs	Renal	$C_{21}H_{21}ClN_2O_8$	464.5
Doxycycline	Oral, Intravenous	100%	90%	Hepatic	15-25hrs	Urine (40%)	$C_{22}H24N_2O_8$	444
Lymecycline	Oral	100%	-	-	10hrs	Renal	$C_{25}H_{33}N_4O_{10}$	597
Methacycline	Oral	-	-	-	-	Renal	$C_{22}H_{22}N_2O_8$	442
Rolitetracycline	Oral	-	-	-	-	Renal	$C_{27}H_{39}N_3O_8$	527
Minocycline	Oral	100%	-	Liver	11-12hrs	Renal, Feces	$C_{23}H_{27}N_2O_7$	431
Tigecycline	-	-	71-89%	Not metabolized	42.4hrs	Biliary, Renal	$C_{29}H_{39}N_5O_8$	585

Figure 3.7: Clinical data, chemical data, and pharmacokinetics study of tetracyclines (Askari Rizvi, 2018)

3.14.3 Mechanism of action

Tetracyclines are generally considered bacteriostatic, meaning they inhibit bacterial growth without directly killing the bacteria. However, some studies have shown that in specific organisms and isolates, tetracyclines can exhibit bactericidal activity in vitro (Norcia et al. 1999; Petersen et al. 2007; Bantar et al. 2008; Noviello et al. 2008). It is important to note that in vivo outcomes may not necessarily align with in vitro bactericidal assessments, as seen in a mouse model where tigecycline, a tetracycline derivative, demonstrated bactericidal activity *against E. coli* and *K. pneumoniae* (Tessier *et al.*, 2013).

Tetracyclines are an antibiotic class that predominantly targets bacterial ribosomes. They bind to a well-preserved portion of the 30S ribosomal subunit's 16S ribosomal RNA (rRNA). This contact impairs translation by preventing aminoacyl-transfer RNA (tRNA) from properly binding when it docks during elongation. (Maxwell 1967; Brodersen et al. 2000; Pioletti et al. 2001).

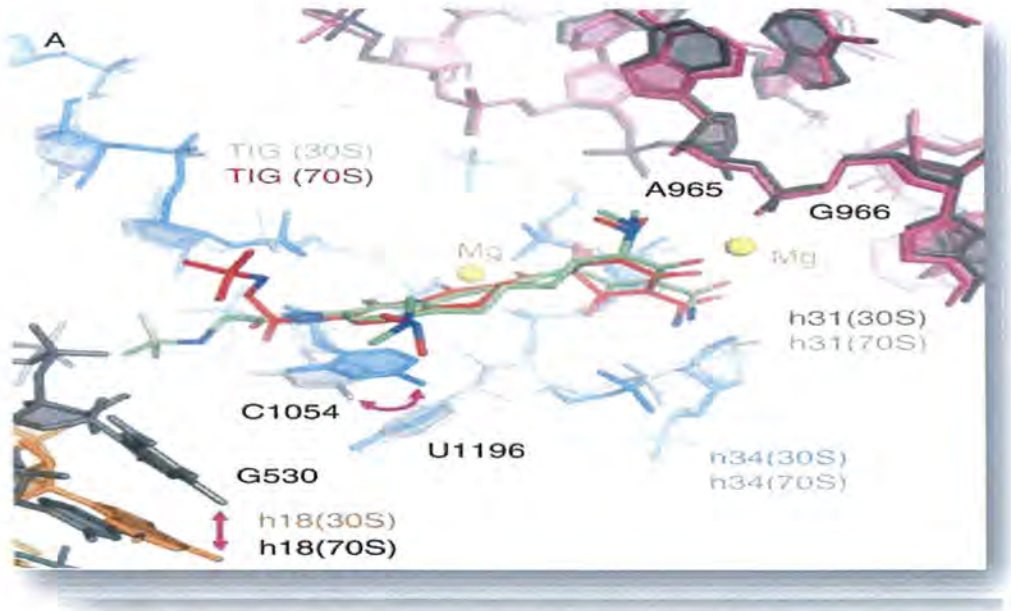

Figure 3.8: *Alternative binding modes of tigecycline at the primary ribosomal-binding site. Alternative tigecycline-binding modes in the 30S (green) and 70S (red) structures are shown, superimposed within the primary tetracycline-binding site. Key nucleotides (G530, A965, G966, C1054, U1196) and helices (h18, h31, h34) are shown in both structures. (From Schedlbauer et al. 2015)*

3.14.3.a ribosomal binding

Researchers used crystallography to identify a high-occupancy binding site for tetracycline (known as Tet-1) as well as five less prominent binding sites inside the Thermus thermophilus 30S ribosomal subunit. Tetracycline is expected to bind to the Tet-1 site, where it forms a complex with two Mg2+ ions in a cavity nestled between helices h34 and h31. This location is close to the A-site, where aminoacyl-tRNA generally hooks onto the 30S subunit. The relevance of the other tetracycline-binding sites discovered inside the 30S subunit structure is unknown. Tigecycline, a tetracycline derivative, was found to bind solely to the Tet-1 site. In comparison to tetracycline, it displayed higher binding affinity and stronger anti-translational efficacy. In comparison to tetracycline, it displayed higher binding affinity and stronger anti-translational efficacy. The orientation of the tigecycline's side chain changed when examined in the 30S and 70S ribosome structures. This shows that throughout the decoding process, tigecycline must respond to conformational changes. Competition assays with several tetracycline derivatives revealed that they all preferentially bind to a single major location. Eravacycline and tigecycline had the greatest binding affinity among these compounds, followed by minocycline, omadacycline, and tetracycline.

3.14.3.b Intrinsic multidrug resistance mechanisms

In bacteria, intricate intrinsic regulatory networks control the uptake and intracellular accumulation of various antibiotics, including tetracyclines. Mutations that influence the expression or function of key repressors, activators, pumps, or porins can have a simultaneous impact on the susceptibility to a wide range of antibiotic classes.

1. AraC Transcriptional Activators

The transcriptional activators MarA, RamA, SoxS, RobA, and the recently identified RarA are all members of the "AraC-family." These proteins are critical in allowing Gram-negative bacteria to adapt to diverse environmental stressors, including antibiotic exposure. Each activator oversees regulating a particular collection of genes in response to various stimuli. For example, MarA regulates the "mar regulon," a group of approximately 60 genes that contribute to multiple antibiotic resistance.

These activators work by binding to a 20-base pair consensus DNA sequence via two helix-turn-helix motifs present within the DNA-binding domain. This binding occurs in the promoter region of stress-responsive genes, such as the "marbox" for *Mar*A. Furthermore, AraC-family activators can bind to their promoters, resulting in a feedback loop that causes their expression to be activated. It's worth mentioning that mutations that result in constitutive expression of AraC-family regulons are prevalent mechanisms in bacteria that contribute to multidrug resistance. (Grossman, 2016c).

Recent research on *Klebsiella pneumoniae* has highlighted AraC-family activators, notably *ram*A, as important contributors to clinically relevant resistance to tetracycline drugs. Bratu et al. (2009) discovered a link between lower sensitivity to tigecycline in *K. pneumoniae* clinical isolates from New York City and the expression of *ram*A and *sox*S in one investigation. Surprisingly, neither *mar*A nor *acr*AB showed this association. Surprisingly, in vitro-selected mutants with lower tigecycline resistance showed higher expression of *mar*A and *acr*B but not *sox*S and *ram*A. This shows that these regulators have a complicated interaction in tigecycline resistance. Another study utilizing tigecycline phase 3 trial isolates discovered that isolates with higher MIC values for tigecycline were substantially related to greater expression of *ram*A and, to a lesser extent, *acr*A (Ruzin et al. 2008). Various studies from Germany, Turkey, Singapore, Chile, Pakistan, Italy, and China have also linked tigecycline resistance in K. pneumoniae to mutations in multidrug resistance genes (such as *ram*R and *acr*R), increased expression of AraC-family activators (rarA, marA, ramA), and efflux pump subunits (acrB, oqxB). Interestingly, tigecycline-resistant K. pneumoniae isolates without *ram*A overexpression have been found, indicating the presence of other resistance mechanisms. The identification of *rar*A, a previously unknown AraC-family regulator, has given information on its function in multidrug resistance and tigecycline resistance in *K. pneumoniae* and other bacteria. The *oqx*AB operon, which encodes a multidrug resistance efflux pump, is hypothesized to be activated by *rar*A. Its overexpression has also been connected to the control of the expression of *acr*AB and *omp*F. These findings highlight the complexities of tigecycline resistance pathways in

K. pneumoniae and highlight the need to understand the function of AraC-family activators in antibiotic resistance. (Grossman *et al.*, 2016c).

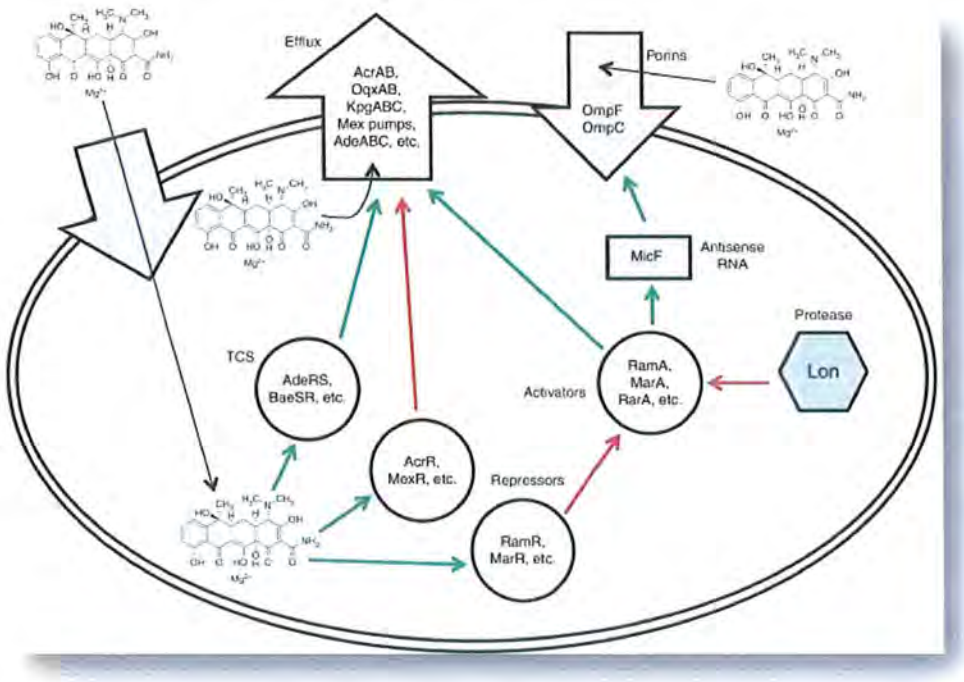

Figure 3.9: *Regulation of expression of Gram-negative intrinsic multidrug-resistance mechanisms affecting tetracyclines. A summary of known regulatory mechanisms affecting tetracycline susceptibility is shown. Green arrows indicate interactions in which tetracycline resistance is "increased," and red arrows indicate interactions in which tetracycline resistance is "reduced." See the text for details. TCS, Two-component signal transduction system. (Grossman, 2016c)*

2. Two-component system

TCSs (two-component signal transduction systems) are the most common signal transduction mechanism in bacteria. A TCS is typically composed of a histidine kinase, which is normally incorporated in the bacterial membrane, and a response regulator. These systems allow for the transfer of a phosphate group, which activates the response regulator. This activated response regulator frequently acts as a transcription factor, influencing gene expression in response to external inputs (Bem *et al.*, 2015). Several TCSs in bacteria have been linked to the control of susceptibility to tetracycline-class

antibiotics in both Gram-negative and Gram-positive bacteria. These TCSs are thought to impact antibiotic sensitivity by modifying bacterial cell membrane permeability or influencing the expression of intrinsic multidrug efflux mechanisms.

(Ruzin et al. 2007) revealed constitutive overexpression of the AdeABC efflux pump in two clinical isolates with high tigecycline minimum inhibitory concentrations (MICs) of 4 g/ml. In each of these isolates, this overexpression was linked to the presence of an insertion element in the *ade*S gene. (Sun *et al.*, 2014) provided additional support for these findings by studying genetically heterogeneous clinical isolates of A. baumannii from Taiwan. The Taiwanese investigation discovered that tigecycline resistance, defined as MIC values of 8 g/mL, was connected to *Ade*ABC overexpression in the majority of the isolates. This overexpression appears to be caused by mutations in the *ade*R and *ade*S genes. (Grossman *et al.*, 2016c).

3. Lon protease

The development of multidrug resistance by AraC-family regulators in Gram-negative bacteria is post-translationally regulated by Lon, a cytoplasmic ATP-dependent serine protease (Tsilibaris *et al.*, 2006). Lon's major function within the cell is to destroy unstable or misfolded proteins. Lon binds to the amino-terminal residues of activators such as MarA, RamA, and SoxS under normal circumstances (Griffith *et al.*, 2004; Nicoloff *et al.*, 2006; Ricci *et al.*, 2014). This contact causes the activators to be degraded by protease. As a result, this procedure allows for the fast reversal of stress-related phenotypes. However, mutations in the lon gene can extend the stability of these stress-responsive activators. As a result, increased expression of resistance genes such as acrAB may develop, potentially leading to antibiotic resistance. (Grossman*et al.*, 2016c).

The Lon protease enzyme has been linked to the development of antibiotic resistance in bacteria. Studies on E. coli cultures demonstrated that mutants with lon mutations or deletions may flourish in the presence of modest levels of tetracycline and chloramphenicol. These mutants frequently include other mutations in genes like *mar*R or *acr*R, or they have tandem amplifications of the acrAB region, which all contribute to their antibiotic-resistant phenotype. Lon protease also contributes to the stability of transposase enzymes associated with IS elements and transposons, which can result in

increased transposition and genomic instability in lon mutant strains. For example, one study found an *E. coli* mutant with *acr*R (A191V) and lon: IS186 mutations in a urinary tract infection (UTI). While this mutant showed decreased sensitivity to tigecycline (with a MIC of 0.25 g/mL), the amount of resistance was not considered clinically important. Another investigation found a frameshift mutation in the lon coding area in a tigecycline-resistant clinical isolate of *K. pneumoniae*. This mutant, however, also exhibited a *ram*R deletion, which likely contributed to its tigecycline-resistant phenotype, with a MIC of 8 g/ml. In the same investigation, *K. pneumoniae* lon mutants produced by transposon mutagenesis had tigecycline MICs that were 8- to 32-fold higher than the original strain (0.5 g/mL). These data imply that lon mutations can lead to increased tigecycline resistance, but that other genetic variables may also play a role in establishing the overall resistance phenotype. While lon mutations have been linked to resistance in experimental conditions, it is unclear how they contribute to clinically meaningful resistance to tetracyclines or other antibiotics during infections. Although clinical isolates of *E. coli and K. pneumoniae* with lon mutations have been identified, further study is needed to completely understand the function of lon in clinically relevant resistance levels. (Grossman *et al.*, 2016c).

4. Intrinsic efflux

Multiple efflux pumps belonging to the resistance-nodulation-division (RND) superfamily have been associated with reduced susceptibility or resistance to tigecycline and tetracyclines in various Gram-negative bacteria. AcrAB is the major pump found in Enterobacteriaceae and has been implicated in tigecycline resistance in *E. coli*, *Enterobacter spp.*, *K. pneumoniae*, *Morganella morganii*, and *Proteus mirabilis*. Additional pumps like OqxAB and KpgABC in *K. pneumoniae* and SdeXY-HasF and SmdAB in Serratia marcescens have also been linked to tigecycline resistance. In non-fermenter and anaerobic Gram-negative bacteria like *A. baumannii* and *Pseudomonas aeruginosa*, RND-type pumps such as AdeABC and MexAB-OprM are known to confer reduced susceptibility to tigecycline and other tetracyclines. In Gram-positive bacteria, the MATE-family pump MepA in *S. aureus* and NorB have demonstrated specificity for tetracyclines and have been associated with reduced susceptibility to tigecycline. While

these efflux pumps play a significant role in intrinsic resistance to tetracyclines, their clinical significance in certain bacteria and the precise regulatory mechanisms governing their expression require further investigation.

3.14.4 Doxycycline

Doxycycline hyclate, a water-soluble tetracycline antibiotic, has a broad spectrum of antibacterial activity against both gram-positive and gram-negative bacteria. Because of its flexibility, it is a good therapy choice for a variety of bacterial illnesses. Doxycycline hyclate is often used to manage and treat a variety of illnesses, including acne, malaria (both prevention and therapy), skin infections, and sexually transmitted infections such as chlamydia, syphilis, gonorrhea, and pelvic inflammatory disease. It is also used to treat Lyme disease. Furthermore, doxycycline hyclate is useful in suppressing disease outbreaks such as cholera, mycoplasma infections, tularemia, typhus, and Rickettsia infections. Doxycycline, like other tetracyclines, has immunomodulating capabilities in addition to antibacterial qualities, making it a powerful tool for treating inflammation associated with disorders such as rheumatoid arthritis. Doxycycline has also been shown to be effective in treating a variety of skin problems, including acne vulgaris, rosacea, bullous dermatoses, granulomatous disease, and livedo vasculitis. Doxycycline hyclate is especially advised in the setting of adult periodontal disease due to its capacity to exhibit anti-collagenase and anti-matrix metalloproteinase action in gingival crevicular fluid. Notably, no indication using doxycycline hyclate for this reason alters the antibiotic sensitivity of normal periodontal flora or opportunistic bacteria. This emphasizes its focused therapeutic impact while protecting the oral microbiota balance. (Graber et al, 2021).

3.14.3.a Mode of action

Doxycycline hyclate can cross numerous membranes and successfully reach diverse tissues due to its high lipophilicity. It functions as a cationic coordination complex in gram-negative bacteria, allowing passage via porin channels such as OmpF and OmpC. In gram-positive bacteria, on the other hand, it occurs in an electroneutral, lipophilic form that permits it to pass the cytoplasmic membrane. This energy-dependent uptake mechanism is propelled by the proton motive force (Graber, 2021). Doxycycline hyclate

acts as a bacteriostatic agent once within bacterial cells. It accomplishes this by allosterically attaching to the 30S bacterial ribosomal unit during protein synthesis. This interaction inhibits charged aminoacyl-tRNA from binding to the ribosomal A site, causing protein elongation to stop and, eventually, bacterial death. (Graber *et al.,* 2021). Doxycycline hyclate has immunomodulatory activities in addition to antibacterial capabilities. It, for example, limits leukocyte mobility during inflammation by interfering with calcium-dependent microtubular formation and decreasing lymphocytic proliferation. It commences anti-inflammatory activities in situations such as osteoarthritis by blocking nitric oxide synthase. Tetracycline resistance, including doxycycline resistance, can arise through a variety of methods. These methods might include efflux pumps, enzymatic degradation, or alterations in bacterial ribosomal RNA (rRNA) mediated by Tet(O) and Tet(M) ribosomal protection proteins. These proteins inhibit tetracyclines from binding to the ribosome's main site, allowing protein synthesis to proceed while giving antibiotic resistance.

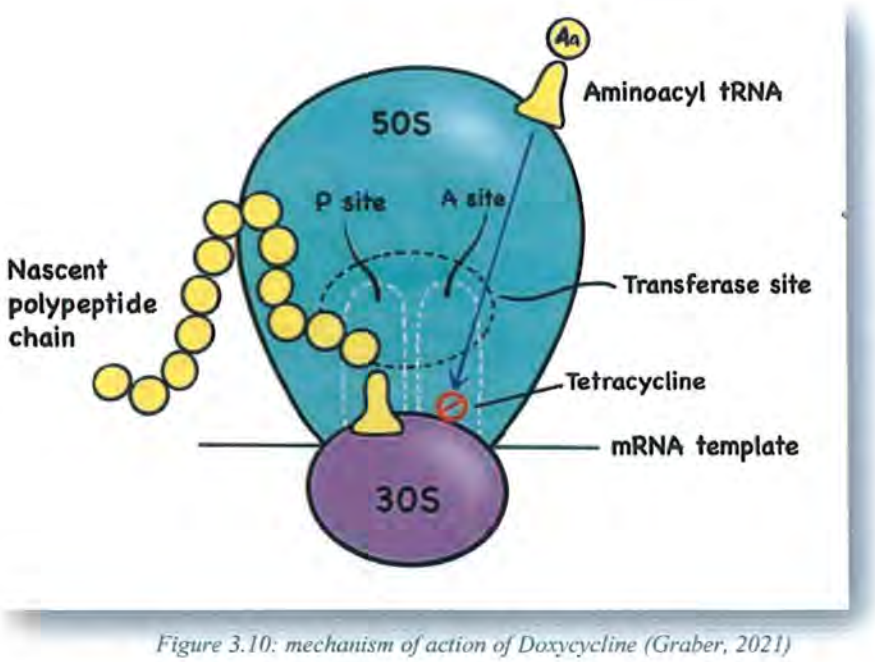

Figure 3.10: mechanism of action of Doxycycline (Graber, 2021)

3.15 Genetic evaluation of *acr*AB and *wab*G in antibiotic resistance

The rise of numerous drug-resistant characteristics in pathogenic bacteria such as *Klebsiella pneumoniae* is a major and widespread clinical problem across the world. These characteristics are frequently associated with increased activity of membrane transport systems, with a focus on AcrAB-TolC, a member of the resistance-nodulation-division (RND) family of transporters (Mazzariol *et al.*, 2002). AcrA, a periplasmic membrane-fusion protein; AcrB, an inner membrane protein; and TolC, an outer membrane channel compose the AcrAB-TolC efflux pump (Mazzariol *et al.*, 2002). These three components work together to allow diverse chemicals to escape. The genes encoding these components, particularly acrA, acrB, and tolC, are critical for the pump's correct operation. (Chetri *et al.*, 2019).

The AcrAB operon, which encodes for the AcrAB-TolC efflux pump, is regulated by two repressor proteins, AcrR and MarR. AcrR, encoded by the *acr*R gene, acts as a repressor, and binds to the operator site of the *acrAB* operon, leading to operon repression (Chetri *et al.*, 2019). On the other hand, the operon is positively regulated by MarA, a transcriptional activator, whose expression is controlled by the dissociation of MarR repressor from the operator site of the marRAB operon (Izquierdo *et al.*, 2003a).

Mutations in the genes encoding AcrR and MarR have been found to result in the overactivity of the AcrAB-TolC pump (Mazzariol *et al.*, 2002), leading to increased antibiotic resistance in bacteria. This research aimed to investigate the presence of mutations in the acrR gene and measure the expression levels of acrA and acrB in specific clones (C14 and C16) to understand the underlying mechanisms of antibiotic resistance in these strains (Chetri *et al.*, 2019; Izquierdo *et al.*, 2003a).

The *wab*G gene is also involved in the synthesis of lipopolysaccharides (LPS) in *Klebsiella pneumoniae (Jabbar et al., 2019).* Specifically, it plays a crucial role in linking the first outer core residue (D-GalA) to the O-3 position of the L, D-HeppII residue in the LPS structure. Mutants of *Klebsiella pneumoniae* lacking the wabG gene were generated in a study, resulting in the complete absence of the outer core LPS (Aljanaby Jabbar Jaloob, 2019). Therefore, these mutants displayed increased

sensitivity to antimicrobial agents such as sodium dodecyl sulfate (SDS) and polymyxin B (Izquierdo *et al.*, 2003b). The outer core LPS contributes to the integrity of the bacterial cell membrane, and its absence made the mutants more susceptible to damage and cell lysis caused by SDS, while also increasing their vulnerability to polymyxin B-mediated cell death hence, proving its role in antibiotic resistance (Izquierdo *et al.*, 2003a).

The *wab*G gene has also been associated with regulating capsule formation and hypermucoviscosity in *Klebsiella pneumoniae*, contributing to its hypervirulent phenotype (Izquierdo *et al.*, 2003b). Additionally, it is involved in the Klebsiella ferric iron uptake (Kfu) system, which is responsible for transporting iron into the bacterial cell and supporting its survival in iron-limited environments. Understanding the multifaceted role of WabG in LPS synthesis, capsule formation, hypermucoviscosity, and iron transport is crucial in unraveling the pathogenicity and antimicrobial resistance mechanisms of *K. pneumoniae* (Jabbar et al., 2019).

The mutation of the *wab*G gene in *K. pneumoniae* leads to significant changes in the composition of lipopolysaccharide (LPS), a crucial component of the bacterial outer membrane and an essential immune molecule (Izquierdo *et al.*, 2003a). Comparative analysis of LPS from mutant and wild-type strains revealed a complete loss of Galacturonic acid (GalA) and approximately 30% reduction in glucosamine (GlcN) in the wabG mutant strains. Further analysis of the LPS core region of the wabG mutant showed the presence of a molecular structure consistent with one hexose, two heptoses, and one Kdo unit (Jabbar*et al.*, 2019).

The *wab*G gene plays a key role in the biosynthesis of LPS in *K. pneumoniae*, where LPS is an essential structural element of the outer membrane and contributes to bacterial pathogenicity (Izquierdo *et al.*, 2003b). *K. pneumoniae* expresses O antigen and capsule polysaccharide (K antigen) on its surface, which is critical for its virulence. The O antigen, located on the outermost part of LPS, is composed of repeating units of oligosaccharide polymers, and genetic variations in O antigen biosynthesis genes are found in the WabG cluster.

The importance of monotherapy cannot be overlooked in the battle against antibiotic-resistant organisms like *Klebsiella pneumoniae*. While combination therapies are explored, the efficacy of individual antibiotics like doxycycline remains crucial, especially in resource-limited settings like Islamabad. This WHO-essential drug has proven effective, with minimal side effects and cost-effectiveness, making it a valuable treatment option. However, the rising antibiotic resistance necessitates continuous research to optimize dosages and genetic roles ensuring their continued efficacy in clinical practice.

In response to the knowledge gap concerning the *acr*AB and *wab*G genes' significance in antibiotic resistance among *Klebsiella pneumoniae*, a comprehensive research project was undertaken. The project aimed to screen clinical isolates for these genes using phenotypic and genotypic methods, shedding light on their association with biofilm formation, a key factor in antibiotic resistance. This critical investigation holds the potential to identify therapeutic strategies, ultimately advancing efforts to combat antibiotic-resistant infections.

In summary, with limited treatment options for such organisms, the exploration of alternative antibiotics like doxycycline is essential. By understanding its susceptibility, optimizing dosages, and exploring combination therapy potential, clinicians can make well-informed decisions to effectively manage *Klebsiella pneumoniae* infections. Additionally, investigating the presence of a*cr*AB and w*ab*G genes and their impact on biofilm formation can provide valuable insights into combatting antibiotic resistance. Overall, this research project contributes to the development of tailored treatment strategies and identifies potential solutions to address the challenges posed by antibiotic-resistant *Klebsiella pneumoniae* infections in Islamabad and beyond.

STUDY DURATION

STUDY DURATION

All the experiments performed, and results collected were during the 01-year duration of the last year of the M.Phil. program i.e., September 2021- September 2023, in the laboratory of Genomics and Molecular Epidemiology Department of Microbiology, Quaid-e-Azam University Islamabad under the supervision of Dr. Javed Iqbal Dasti.

MATERIALS AND METHODS

Materials and methods

5.1 Material

5.1.1 Glassware: This included Petri plates, test tubes, beakers, conical flasks, glass slides, graded cylinders, and pipettes.

5.1.2 Equipment: Autoclave, laminar flow hood, digital balance, incubator, refrigerator, microscope, PCR, water bath, centrifuge, vortex, and gel electrophoresis equipment were used to perform the experiments properly.

5.1.3 Chemicals: ethanol, distilled water, tryptic soy Agar, MacConkey Agar, Mueller Hinton Agar, Simon's citrate media, safranin, TSI media, urease broth, peptone, oxidase reagent, hydrogen peroxide, crystal Violet, phosphate buffered saline, methanol, gram. iodine and oil for microscopy were used. All these chemicals were laboratory-grade.

5.1.4 Miscellaneous: inoculating loop, tweezers, sterilized swabs, aluminum foil, Scotch tape, permanent marker, test tube racks, match sticks, burner, filter paper, toothpicks, scissors, and measuring rulers.

5.2 Method

5.2.1 Sample collection and preservation

A total of 175 isolates of *Klebsiella pneumonia* were collected and carefully preserved in 40% glycerol stock from a tertiary care hospital in Rawalpindi and Islamabad Pakistan.

5.2.2 Sub-culturing from the collected samples

The glycerol-preserved cultures were initially revived in nutrient broth for enrichment and then plated on freshly prepared McConkey agar plates.

5.2.2.1 Culture refreshment in nutrient broth for identification

The following protocol was followed to refresh *K. pneumonia.*

- For each of the 175 *K. pneumonia* isolates, 1 ml of nutrient broth was taken in a separate autoclaved Eppendorf tube.
- 20-15uL of sample from the glycerol stock was taken and then inoculated in the autoclaved Nutrient broth.

- Eppendorf tubes were then placed in a shaking incubator at 37°C and 175 pm for 24 hours.
- Eppendorf tube containing only nutrient broth was kept as a negative control.
- Next day, turbidity in the Eppendorf tubes was considered as bacterial growth when compared to the negative control Eppendorf as it was transparent showing the sterility of our media and proving the growth of our isolates.

5.2.2.2 Culturing on MacConkey agar plates

To confirm the growth of *K. pneumonia* we streaked them on a selective media i.e., MacConkey agar, and the following protocol was followed:

- Freshly prepared MacConkey agar (Oxoid TM MacConkey Agar) plates were prepared and then labeled with the sample numbers to be streaked.
- With the help of sterile toothpicks, cultures from the broth were inoculated on the MacConkey agar plates very carefully.
- The plates were then incubated at 37°C for the next 18-24 hours.
- Growth on the plates was observed the next day showing *Klebsiella pneumonia*.

5.2.3 Identification of *Klebsiella pneumonia*

For efficient preliminary identification of the strains, it was divided into two categories i.e., morphological, and chemical identification.

5.2.3.1 Morphological Identification

Colony morphology: After the incubation period the colonies were carefully examined in a Laminar flow hood to ensure aseptic conditions and to determine the morphological characteristics. Morphological characteristics of *Klebsiella pneumonia* colonies like shape, color, and presence of mucus were observed as the identification mark.

Table 5.1: characteristics of klebsiella on MacConkey Agar plate

CHARACTERISTICS	MACKONKEY AGAR
color	Pink, yellow
texture	Smooth mucoid
shape	circle
elevation	convex
size	2-3mm
surface	mucoid

Gram Staining (microscopy):

Gram staining was performed to identify the shape of bacteria i.e., gram-negative rods of *Klebsiella pneumonia*. After 24 hours of incubation, an isolated colony was picked from each sample plate with the help of a sterilized toothpick. A drop of saline was placed on the glass strip using a dropper and the already picked-up colony was mixed with the saline forming a spread smear. After this the smear was air dried and a few drops of crystal Violet were added to the slide after one minute. This was done by holding the slide in an inclined position. After a minute it was rinsed with water and a drop of gram iodine modern was loaded onto the slide for one minute. This was also rinsed with water. 95% ethanol was applied for a few seconds and rinsed off to decolorize and lastly, safranin was added for one minute and rinsed. After all this, the slide was air dried properly followed by heat fixation for a couple of seconds and was observed under a 100X lens of the microscope by using emulsion oil.

5.2.3.2 Biochemical Identification

Oxidase test

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

Procedure: Initially, we inoculated our bacteria on a tryptic soy agar plate via streaking. Then we incubated for 24-48 hours at 37°C. After incubation, a few drops of

reagent i.e., p-amino dimethylaniline oxalate were added to the surface of the culture of our microorganism. Within 10-30 seconds we observed the color change.

Catalase test

Principle: This test detects the presence of a catalyst enzyme. The bacteria produce catalase enzymes and use this to break down H202 to release free oxygen.

Procedure: We took nutrient agar in a Petri plate and used the sterile method of streak inoculation to inoculate Bacillus cereus. Then we incubated it for 24 to 48 hours at 37C. After the growth of bacteria on the plates, we put three to four drops of 3% hydrogen peroxide on the Petri plates and let it flow over the entire surface. Then we saw the formation of bubbles which indicated that oxygen is produced, and the degradation of hydrogen peroxide has taken place.

Triple sugar iron (TSI) Test

Principle: This test is used to check the fermentation capabilities of different bacteria along with hydrogen sulfide production. It helps to distinguish Enterobacteriaceae from other gram-negative bacteria.

Procedure: First, we Inoculated the experimental organism into its appropriately labeled tube using the stab or streak method using an aseptic technique. Then we incubated it for 18-24 hours at 37°C. After that, we examined the color of the broth, both the butt and the slant. Based on observations, we will easily be able to determine the type of reaction that has taken place (acid, alkali, or non), and whether the carbohydrate has been fermented or not. We will also observe the presence of any black precipitate within the medium and whether the organism was capable of producing H2S or not. The acid-base indicator "phenol-red" is also incorporated to detect carbohydrate fermentation through a change of color of the medium from orange-red to yellow, in the presence of acids.

Indole test

Principle: This test is used to detect the enzyme tryptophanase which breakdown stripped a fan into ammonia, indoor, and pyruvic is it. The indoor production is confirmed by adding Kovacs reagent.

Procedure: Using aseptic techniques, first we inoculated the experimental organism into the appropriate labeled deep tube using stab inoculation. Then we incubated the tubes for 24-36 hours at 37°C. After incubation growth was visible so we added 10 drops of Kovac's reagent and agitated the culture gently. On observations, we determine the record of whether the organism can hydrolyze the tryptophan.

Citrate Utilization Test

Principle: The fundamental concept behind this test is to assess an organism's capability to metabolize citrate as its exclusive carbon source, leading to the production of alkalinity. The enzyme citrate breaks down citrate into oxaloacetic acid and acetic acid.

Procedure: Initially we freshly prepared plates of Simmon citrate agar medium. After the plates were solidified, we incubated the plates at 37°C for 24 -48 hours. The next day an obvious color change was observed indicating the presence of *Klebsiella pneumonia.*

5.2.4. Preservation in glycerol stock

Once all the isolates of *Klebsiella pneumonia* were isolated, identified, and confirmed glycerol stock solution was made for preservation. For preservation initially, the colonies were incubated in 500µl broth in sterilized Eppendorf's and incubated at 37°C for 24 hours. On the next day, 40% glycerol was carefully poured into the tubes. These tubes were then vortexed, labeled, and stored at -20°C.

5.2.5. Molecular detection of *AcrAB* and *WabG* genes

As discussed previously, the project was designed to evaluate the role of *AcrAB* and *WabG* genes in antibiotic resistance, and for this purpose, we initially did molecular detection of these genes. For this, the following protocol was followed.

5.2.5.1 CTAB (Cetyl trimethylammonium bromide) method of DNA extraction

We employed the CTAB DNA extraction method which is a widely employed technique for isolating DNA from bacterial cells. The principle of this method involves the use of the ionic detergent CTAB to disrupt the bacterial cell membranes, leading to the release of intracellular components. CTAB functions by solubilizing the lipids in the bacterial cell membrane, effectively breaking it apart. Subsequently, a mixture of

chloroform and isoamyl alcohol is added to the lysate, creating two phases: an organic phase and an aqueous phase. The contaminants and cellular debris, along with lipids and proteins, are partitioned into the organic phase, while the nucleic acids, including bacterial genomic DNA, remain in the aqueous phase. This method enables the efficient extraction of high-quality bacterial DNA suitable for various molecular biology studies. The step-by-step protocol followed is as follows:

Solutions required.

T.E Buffer, 5M NaCl, CTAB, chloroform, phenol-chloroform, Na-acetate, ice-cold absolute ethanol, 70% ethanol

Protocol

1. we added 1ml of sterile nutrient broth in autoclaved and labeled Eppendorfs, after this we added freshly streaked colonies in the media of the respectively labeled Eppendorfs with the help of the sterile toothpicks and placed them in the shaker incubator for 24hrs at 37°C and 175 pm.
2. The next day we centrifuged Eppendorfs at 13,000 rpm for 10 minutes. This results in the formation of 2 layers i.e., the supernatant and the pallet. We retained the pallet and discarded the supernatant.
3. After this we added 80µl of TE buffer and 20µl of 5M NaCl into the pallet.
4. To again resuspend the pallet, we vortexed it thoroughly and then added 10µl of CTAB.
5. We incubated the Eppendorfs in the water bath at 60°C for 20 minutes.
6. After incubation we added 100µl of chloroform, vortexed it, and then again incubated it on ice for 30 minutes.
7. After incubation, we microfuged it at 10,000 rpm for 10 minutes to produce two layers.
8. We transferred the supernatant into the newly labeled and autoclaved Eppendorfs and discarded the rest.
9. Then we added 100µl of phenol-chloroform very carefully into the supernatant, vortexed it to get a milky solution, and then again microfuge at top speed (14,800) for 5 minutes to get 02 layers.

10. We then transferred the supernatant to the newly labeled and autoclaved Eppendorf, added 100µl of chloroform, and vortexed.

11. 02 layers were produced, we again separated the supernatant into newly labeled epis, discarded the rest, and then added 10µl of Na-acetate.

12. 200µl of ice-cold ethanol was added for the precipitates to form and then incubated at -20°C for 1 hour.

13. We again microfuged at 13,000 rpm for 10 minutes to produce two layers and discard the supernatant in this step.

14. We then resuspend the pallet by adding 70% ethanol (100µl) and microfuged it again at top speed for 5 minutes to obtain the DNA pallet.

15. We air-dried the ethanol by keeping the lids open and after that, as the last step, we resuspended the pallet in 20µl of TE buffer and stored it at -20°C.

5.2.5.2 Confirming DNA extraction via gel tank electrophoresis and illuminator.

To confirm that the extraction was done successfully, we performed gel tank electrophoresis and visualized the results, The following protocol was followed.

Gel electrophoresis

1. The agar conc. depends upon the gene size, but as in this step, we were only confirming the presence of the DNA, so we used an average amount i.e., 1.5%. For this purpose, we took 36ml of distilled water, 4ml of TBE buffer, and 0.6g of agar powder and mixed this concoction in a beaker.
2. We covered the beaker with aluminum foil to prevent evaporation and boiled the gel until all the particles dissolved and a clear solution was visible.
3. Then we removed the foil and allowed it to cool down until we were able to place the beaker on our wrists without burning, added Ethidium Bromide very carefully as it is carcinogenic, and mixed it thoroughly.
4. During the cooling process of the gel, we prepared the casting tray by cleaning it with distilled water and placing combs and casting dams firmly to prevent any leakage and well formation.

5. After the gel was cooled, we poured it into the tray and allowed it to solidify before loading. During the solidification, we added TBE buffer into the gel tank just enough for the gel to be submerged.
6. When the gel solidified, we removed the comb and the gel dams, carefully placed the casting tray into the tank, and started our loading process.
7. For this purpose, we added 1-1.5µl of the loading dye and 3-3.5µl of the extracted DNA, mixed them thoroughly via reverse pipetting, and then carefully loaded the mixture into the wells.
8. After successfully loading all the isolates we tuned on the gel tank with the setting of 400Ampere currents, 75volts for 35 minutes.

- **Visualization**

After the gel tank stopped, we carefully removed our gel from the casting tray, placed it on the UV lamp for visualization, and confirmed the extraction of our DNA via the CTAB method.

5.2.5.3 Polymerization Chain Reaction (PCR)

Primer Dilution

As the first step of PCR, we dilute the forward and the reverse primers as they are concentrated. For this purpose, we add 500µl of PCR water in the forward and reverse primer stock. After this, we add 10µl of the main stock into newly labeled epis and add 40µl of the PCR water. We use this for our PCR.

Optimization of the genes

Now we optimized our genes via PCR and for this purpose we initially make the reaction mixture, the recipe of which is shown in the table. After preparing the reaction mixture, we take our PCR tubes and label them with the respective isolate numbers. After this, we place our rack with the PCR tubes onto the ice trays and add 1.5µl in the respective PCR tube. Then we added 8.5µl of the reaction mixture in every PCR tube containing DNA very carefully to prevent cross-contamination. After this, we placed the PCR tubes in the respective zones that had been set on the PCR machine according to the temperature ranges on which we optimized the gene and started the cycle. Lastly, we removed the PCR tubes from the ice racks after the cycle was completed.

Table 5.2: PCR recipe

ingredients	For 10 samples
PCR water	63µl
Taq Buffer	10µl
MgCl2	6µl
dNTPs	2µl
F. primer	4µl
R. primer	4µl
Taq polymerase	1µl

Gel electrophoresis and visualization.

Overall, we followed the same protocol of the gel electrophoresis as mentioned above for DNA visualization but in this case, we made the gel concentration according to the size of the gene. For a smaller-sized gene i.e., *wab*G in this case we made 2% gel i.e., 0.6-0.7g of gel powder while for a larger-sized gene i.e., *acr*AB in this case we made 1% gel i.e., 0.4g of agar powder. After the gel electrophoresis, we visualized our gel under the UV lamp for visualization.

5.2.6 Phenotypic detection of Klebsiella pneumonia strains against doxycycline

5.2.6.1 Disk Diffusion Assay

Initially to attain some qualitative data as to which isolates were resistant and sensitive to the

drug ceftriaxone, disk diffusion of 150 isolates was done through the Kirby-Bauer method in

Laminar Flow Hood (LFH).

Protocol:

1. **Preparation of Muller Hinton Agar (MHA) Plate:** First, we measure the amount of distilled water needed in a measuring cylinder and pour it into a flask. After that we weigh the media i.e., Muller Hinton Agar (MHA) based on the given calculation of the

media bottle and add it to the distilled water then mix properly. After mixing we cover the opening with aluminum foil and seal it with the help of scotch tape. After proper labeling, we place it in the autoclave for sterilization. After being autoclaved, it is carefully poured into the sterilized petri plates and left for solidification.

2. **MacFarland preparation:** 2-3 colonies of pseudomonas aeruginosa were picked from the cultured plates and were suspended in 500µl of sterile saline in autoclaved Eppendorf. This mixture is then vortexed to achieve a smooth suspension. The turbidity is adjusted to a 0.5 MacFarland standard either by adding more colonies if the suspension is too dilute or saline is added if the suspension created is too concentrated. Then we leave it for 10-15 minutes before lawning.
3. **Inoculation of the MHA palate:** For inoculation on the agar plate that was left to solidify, we placed a sterile swab in the inoculum tube. Then to inoculate the dried surface of the MHA plate, we streak the swab three times over the entire Agar surface. By rotating the swab against the tube site, we ensure removal access and avoid highly concentrated inoculation on the plate. To achieve a uniform distribution of the inoculum, the plate is rotated 60 degrees each time. Finally, we dispose of the swab in a suitable container.
4. **Antibiotic disc placement:** To place the antibiotic disc, we used sterilized forceps. With the help of these forceps, we carefully remove one disc at a 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 at 35 degrees centigrade in an air incubator for 24 hours.
5. **Zone determination:** To identify the resistant and sensitive isolates CLSI guidelines were followed according to which if.

   Zone of inhibition $\geq$ 14mm = sensitive

   Zone of inhibition $\leq$ 10mm or no zone = resistant

   Zone of inhibition = 11-13mm = intermediate

5.2.6.2 Microdilution method

Through the microdilution method, our main goal was to determine the minimum inhibitory concentration (MIC) of the doxycycline and to build a susceptibility profile of *K. pneumonia* against doxycycline. MIC is the lowest concentration of the drug which can inhibit bacterial growth and it was done to explore the ability of doxycycline against *K. pneumonia.*

Protocol

1. Stock preparation of ceftriaxone: To prepare the stock solution of ceftriaxone, CLSI guidelines were followed according to which the stock solution was made in distilled water for doxycycline. The formula used for stock solution preparation is.

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

   Where W is the weight of the powdered drug, C is the concentration of the drug, V is the total volume of the stock, and P is the potency of the drug. To prepare 10ml of stock solution i.e., 5120µg/ml initially, we took 5ml of water in a falcon tube, then we added 0.058g of the powdered doxycycline. After mixing it we slowly added the remaining 5ml of distilled water to obtain a clear stock solution which is then properly labeled and stored at 4°C after wrapping it in a foil.
2. **Preparing primary suspensions:** To make primary suspensions, we took autoclaved Eppendorfs, labeled them with the isolate numbers, and poured 1ml of Muller Hinton Broth (MHB). Then with sterilized toothpicks, we picked up 2-3 colonies of the colonies from the freshly streaked MacConkey plates submerged them in the respectively labeled Eppendorfs and placed them in the incubator for 4-5 minutes.
3. **Preparation of working solution:** To make a working solution, the autoclaved test tube was used and placed on the test tube stand within the LFH, in that test tube initially 4.5ml of Muller Hinton Broth (MHB) was added with the help of a pipette. After that 0.5ml of the stock solution was added to make a total of 5ml working solution which is to be used afterward.

4. **Preparation of secondary suspensions:** For this initial test tubes were autoclaved, labeled with the isolated numbers, and placed inside the LFH on the test tube stand. In each test tube total of 2µl of solution is needed. For this, we added 1.98ml of MHB and 20µl of the previously made primary suspension resulting in the formation of the secondary suspensions.
5. **MIC via microtiter plates:** To minimize any sort of contamination, we placed all the necessary equipment such as the microtiter plate, marker, pipettes, etc. in the LFH under UV for about one minute. We labeled the wells (rows) from 512µl/ml to 0.25µlml based on dilutions and out of the 08 columns we labeled only 07 with the isolate number whose dilutions are to be performed. The last row is left for adding the controls at the end. Just to be sure we label three wells in pairs for growth control, media control, and working solutions control to compare our results in the end. After that, we initially added 100µl of media (MHB) in all the rows leaving the first columns (labeled as 512µl/ml) and the last row empty. After adding the media, we added 200µl of the working solution in the first column (labeled as 512µl/ml) leaving the last row empty again. When the working solution has been added, we work on the individual rows one by one. From each row,100µl from the first well (512µl/ml) is taken with the help of the pipette and added into the neighboring well (256µl/ml) and mixed properly with repeated pipetting, then from the well labeled as 256µl/ml again 100µl is taken and added into the next neighboring well and this process is kept on until the last well i.e., 0.25 µl/ml. and from the last well of the row 100µl is discarded. This process is repeated for all seven isolates. Lastly, 100µl of the secondary suspensions are added in all the wells except the last row. Lastly, we add our controls in the designated wells as labeled, and by putting the lid on we cover it with cling film to be extra sure to label it and leave it in the incubator for 24 hours at 37°C.
6. **Determination of results:** The results were observed and recorded following the CLSI guidelines. Dilutions with no growth (in terms of turbidity) were considered MIC. Based on CLSI guidelines the breakpoints for doxycycline are.

MIC ≤ 4μg/ml = sensitive

MIC ≥ 16μg/ml = resistant

5.2.6.3 Identifying strong biofilm formers.

For the sensitive isolates against doxycycline that were confirmed via Disk diffusion assay and MIC, the biofilm-forming potential was determined by using the microtiter plate method (de Rossi *et al.*,2009). Standardized 0.5 McFarland suspension of sensitive isolates were produced by taking 2-3 colonies from a 24-hour culture plate with the help of sterile toothpicks. These were then mixed in 1ml of Mueller Hinton Broth. 200ul of each isolate's suspension was added in the separate wells of the microtiter plate and the plate was incubated at 37°C for 18-24 hours. The next day, broth and suspended bacterial cells were removed from the wells very carefully by not touching the bottom of the wells to prevent disruption of any biofilm produced. After discarding the media, the wells were washed with Phosphate buffer saline three times followed by the addition of 150μl of 70% methanol for the fixation of the biofilms. It was made sure that the PBS added was at room temperature and not very hot after autoclaving to prevent damage to the biofilms. After leaving the methanol for 15 minutes, the wells were allowed to air dry inside the LFH which was followed by the addition of 2% crystal violet for about 10-15 minutes to stain the biofilms, which was then removed. The excess stain was then washed with the help of PBS (3 times) and finally after letting it air dry for about 5 minutes 150 μl of 95% ethanol was added into each well. Biofilms were then quantified by taking their optical density values at 540nm or 492 nm in an ELIZA plate reader. For each isolate, this process was performed in triplicates. After taking their optical density readings, Standard deviations, and cut-off Optical density i.e., ODc values are determined by using the formula.

ODc= 3SD + Avg (CV controls)

The biofilms were characterized as follows.

Weak biofilm Former: OD= 2XODC

Moderate biofilm former: OD= Optical density- less than 4XODc

Strong biofilm former: OD= Optica density= 4xODc or greater.

5.2.6.4 Minimum Biofilm Eradication Concentration (MBEC)

Procedure

The lowest concentration of antimicrobial agent in this case the doxycycline that eliminates bacterial growth and its biofilms is known as MBEC. To begin, on day 01 we streaked our strong biofilm formers on freshly prepared MacConkey agar plates to be used the next day. On the next day, we prepared the standardized bacterial suspension i.e., MacFarland by dispersing 2-3 colonies of the freshly prepared strong biofilm isolates into the Eppendorf containing MHB. After this, we added 75 µl of the standardized bacterial suspension (MacFarland) into the 96-well flat-bottomed microtiter plate and incubated it for 24 hrs. at 37°C. The next day we initially observed the growth and looked for turbidity in the wells. The wells were all turbid showing bacterial growth and biofilm formation. After this, we very carefully removed all the media including the planktonic cells from the wells and washed the wells 3 times with PBS. Now two-fold serial dilutions were prepared starting from 2048µg/ml to 0.25µg/ml and were applied to each isolated number independently. We again left the plate in the incubator for incubation for the next 24 hours at 37°C. The next day i.e., the fourth day we observed our plate and looked for clear wells as the clear wells indicated the eradication of the biofilm formation at that concentration but to double check our results biofilm from the bottom of the well was scraped with a toothpick and thoroughly mixed in 1mI PBS in a sterile Eppendorf tube. For each well, separate Eppendorf tubes with PBS were used. Afterward, the biofilm was disrupted by vortexing the Eppendorf tubes. The sample from Eppendorf was streaked on a new MacConkey plate using sterilized toothpicks and incubated for 24 hours at 37°C.

MBEC interpretation

The sections of MacConkey where no growth occurred after incubation were designated as MBEC. It signifies that the antibacterial agent (doxycycline) has completely stopped bacteria from growing.

5.2.6.e Minimum Biofilm Inhibition Concentration (MBIC)

Protocol

The protocol of MBIC till Day 03 is the same as MBEC which has been previously discussed. The variation in the protocol starts from day 04. On day 04 after removing the microtiter plate from the incubator, we carefully removed the media from the wells and washed those wells 03 times with PBS. After washing we add 100µl of freshly prepared MHB into the wells to provide fresh nutrients and to check if there will be any sort of growth in these wells. We then place the plate in an incubator for 24hrs at 37°C.

MBIC Interpretation

The next day the first well that is completely clear and has shown no growth will be designated as the MBIC of the doxycycline showing that the doxycycline inhibited the growth of bacteria at this concentration.

5.2.6.5 Effect of doxycycline on the biofilm formation of the Klebsiella pneumoniae at sub-minimal concentration

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 to doxycycline were taken. After this 0.5 McFarland suspension was prepared and 100µl was added to each good microtiter plate. For one isolate 1 row was used. 100µl of antibiotic dilutions ranging from 0.5 g/ml to 0.0625ug/ml were added in triplicates into wells. Then the doxycycline was added in wells respective to their dilutions. For different time intervals, separate plates were prepared. Drug and media-containing wells were taken as negative controls and bacterial suspension without antibiotic dilutions was considered as positive control. At different time intervals, like 2 hours, 4 hours, 6 hours, and 24 hours, plates 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 to 15 minutes. The excess stain was washed with the help of PBS. Then 150µl of 95% ethanol was added into each well. Biofilm was quantified by taking optical density readings from the ELIZA plate reader at 540m. An increase or decrease in the OD values of biofilms with time was observed to determine the inhibitory or stimulatory effect of doxycycline on the biofilm-forming ability of *K. pneumoniae*.

RESULTS

RESULTS

6.1 Culturing from the collected samples

To form a stock of the collected *Klebsiella Pnemoniae* isolates, differential media i.e., Mackonkey agar was used to streak the isolates. Five different isolates were streaked on a plate plate for subculturing.

Figure 6.1: MacConkey Agar plate showing streaked isolates from collected samples.

6.2 Glycerol stock preparation

To form a stock of our collected isolates for use, we took previously streaked isolates and preserved them in 50% glycerol for further use.

Figure 6.2: Glycerol stock of *Klebsiella Pnemoniae*.

6.3 Colony Morphology

Different morphological characteristics of the colonies such as shape, color, size, surface, etc. were observed on the MacConkey agar plates for the *Klebsiella pnemoniae* isolates.

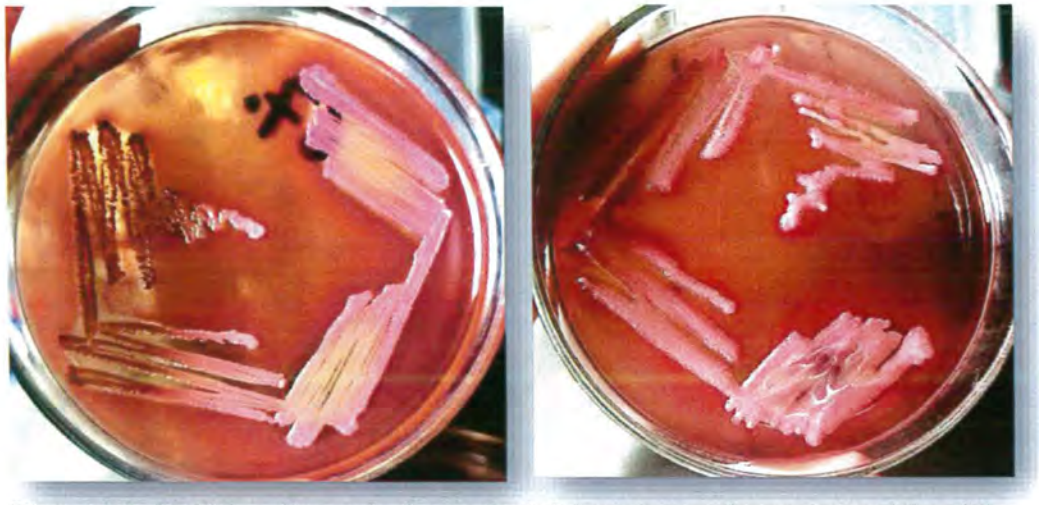

Figure 6.3: Pinkish-yellow colonies on the left (non-lactose fermenter) and the pink (lactose fermenter) smooth mucoid colonies on the right.

6.4 Gram Staining

Gram staining was performed to identify the shape of bacteria i.e., gram-negative rods of *Klebsiella pneumonia* isolates.

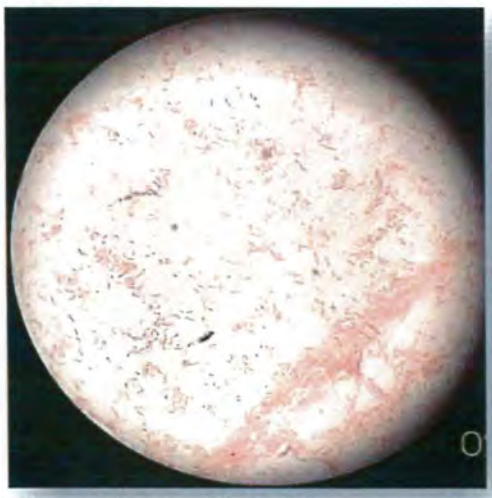

Figure 6.4: Gram staining of *Klebsiella pneumonia* showing rods.

6.5 Biochemical Tests

Various biochemical tests were performed for the identification and characterization of the *Klebsiella pnemoniae* isolates. The tests performed are as follows.

a. Oxidase Tests

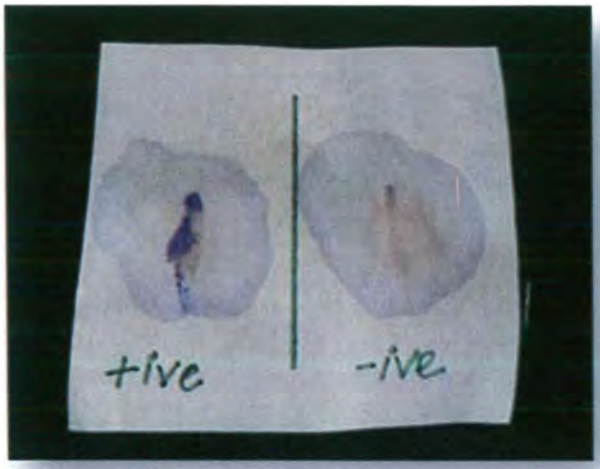

Figure 6.5(a): Klebsiella isolates showing the negative result on the right with no color change in comparison to the positive control.

b. Catalase Test

Figure 6.5(b): *Klebsiella pneumonia* isolates showing positive results on the right in comparison with the negative control on the left.

c. Tripple Sugar Iron Test

Figure 6.5(c): On the left, positive result from Klebsiella isolates and negative control on the right.

d. Indole Test

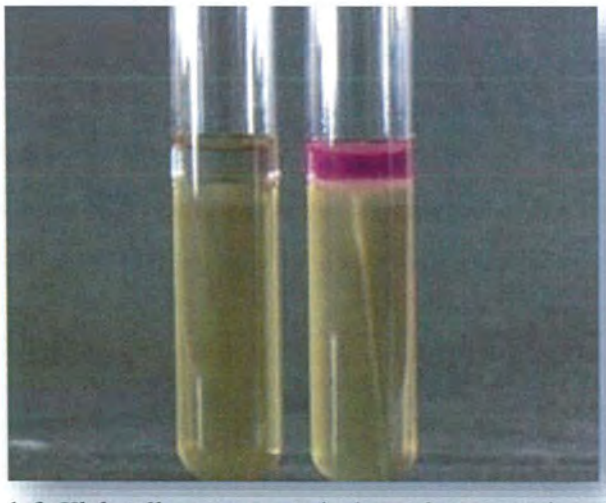

Figure 6.5(d): On the left *Klebsiella pneumonia* isolates show negative results while on the right is the positive control.

e. Citrate Utilization Test

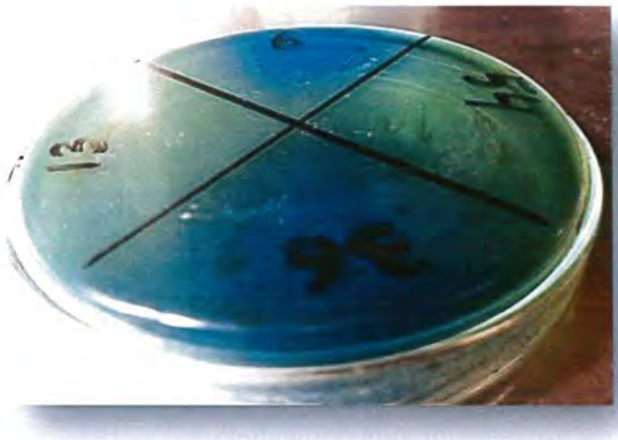

Figure 6.5(e): test on isolates 6 and 36 of Klebsiella showing positive results as compared to the two negative controls of 13 and 54 (*E. coli*).

6.6 DNA extraction and visualization

DNA extraction of the *Klebsiella pnemoniae* isolates was done via the CTAB extraction method to ensure accuracy. After extraction of n=156 isolates, we confirmed the DNA extraction via gel electrophoresis and UV lamp. The results of which are shown below in figure 6.6.

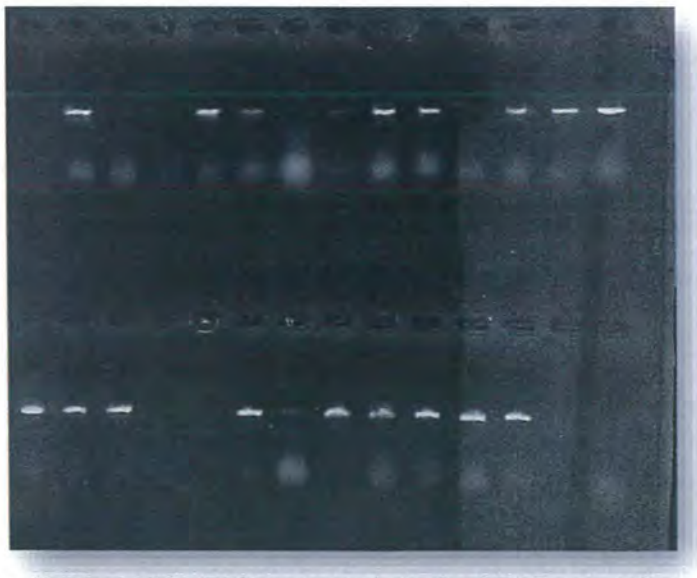

Figure 6.6: Visualization of the DNA extracted via the CTAB extraction method.

6.7. Molecular detection of the *acr*AB gene in *Klebsiella Pnemoniae*.

We screened a total of 156 *Klebsiella pneumonia* isolates for the *acr*AB and *wab*G genes. The gene of size 397bp (*acr*AB) was optimized at 67.5°C on 1-1.5% agarose gel while the gene *wab*G of size 375bp was optimized at 55.5°C on 2% agarose gel. In doing so we found out that **n=52** isolates were positive for the *acr*AB gene while **n=39** was positive for *wab*G.

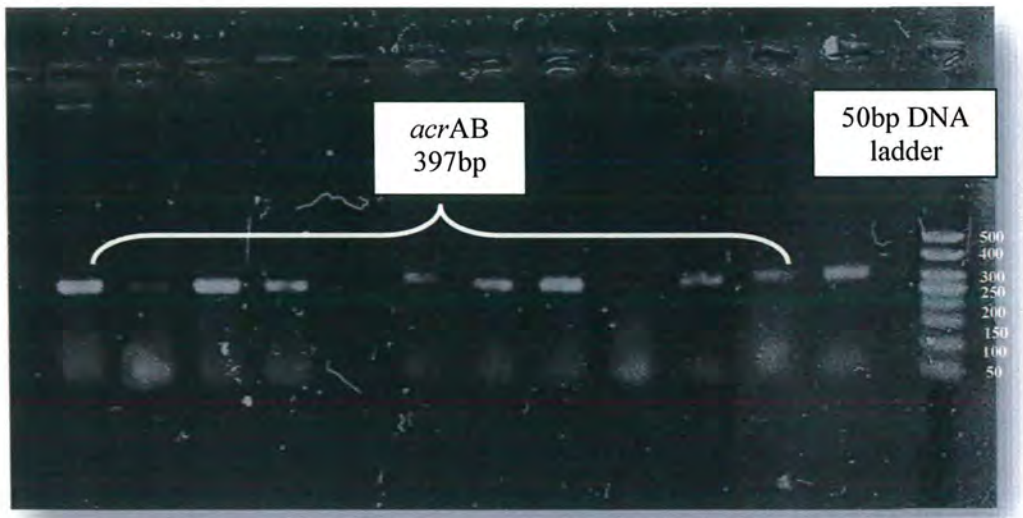

Figure 6.7(a): Figure shows the gene *acr*AB of size 397bp polymerized and optimized at 67.5°C against the ladder.

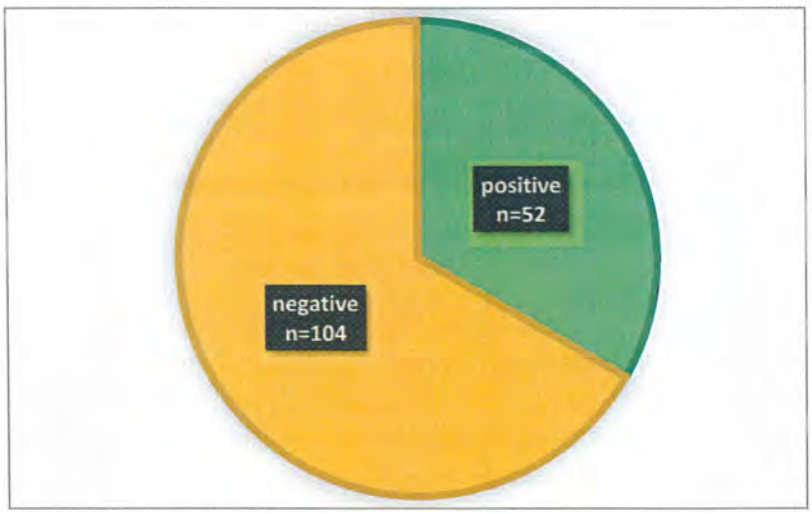

Figure 6.7(b): *acr*AB gene prevalence in 156 *Klebsiella pneumoniae* isolates

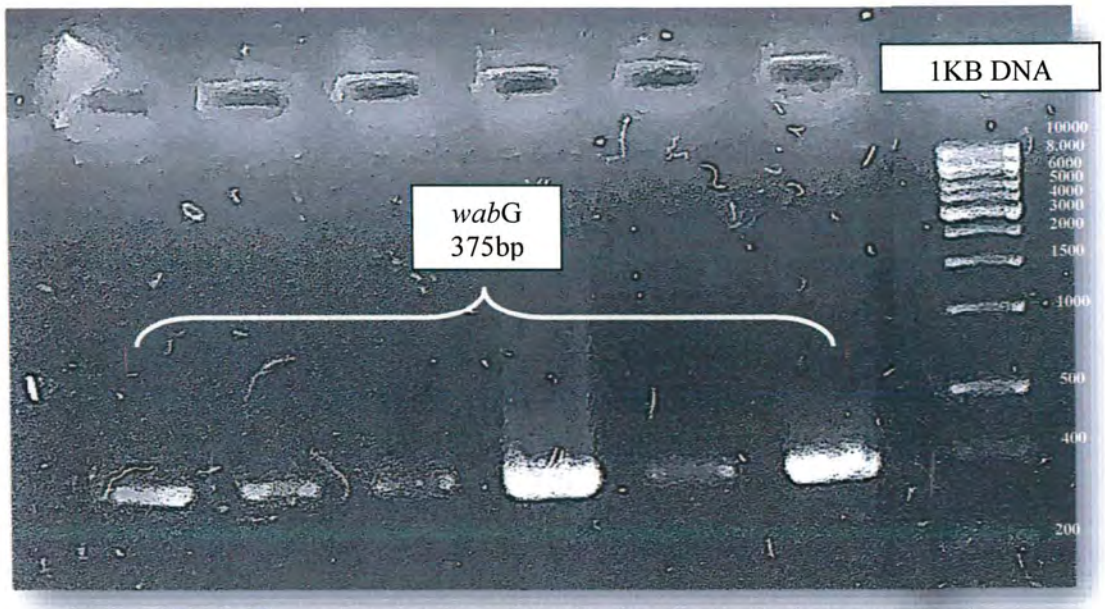

Figure 6.7(c): Figure showing the polymerized and optimized gene *wab*G of size 375bp (55.5°C) against the ladder.

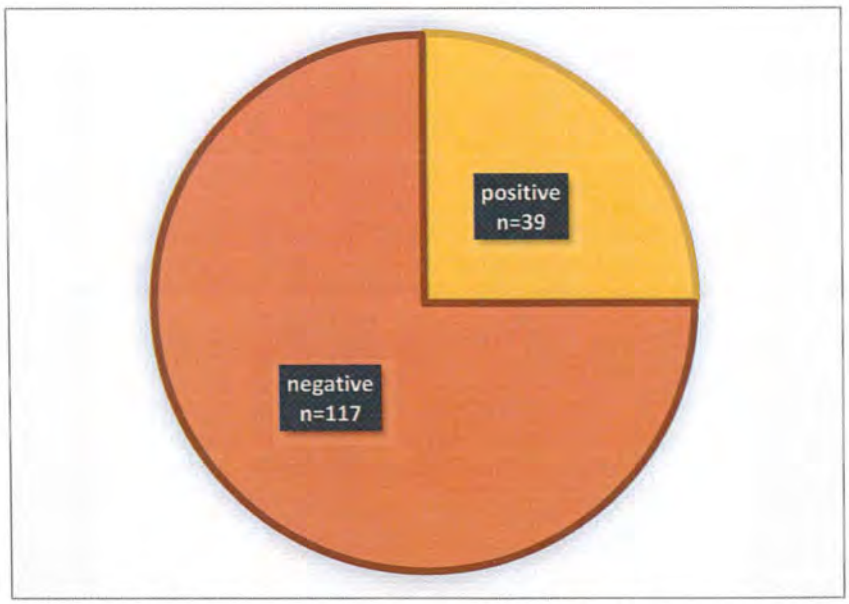

Figure 6.7(d): *wab*G gene prevalence in 156 *Klebsiella pneumonia* isolates

6.8. Occurrence and co-occurrence of the *acr*AB and *wab*G genes in 156 *Klebsiella pneumonia* isolates

The graph below shows initially the percentage of the prevalence of the two genes *acr*AB (33%) and *wab*G (25%) in the 156 isolates of *Klebsiella pneumonia* and is followed by the percentage of the co-occurrence of the two genes (8.3%) in our collected 156 *Klebsiella Pnemoniae isolates.*

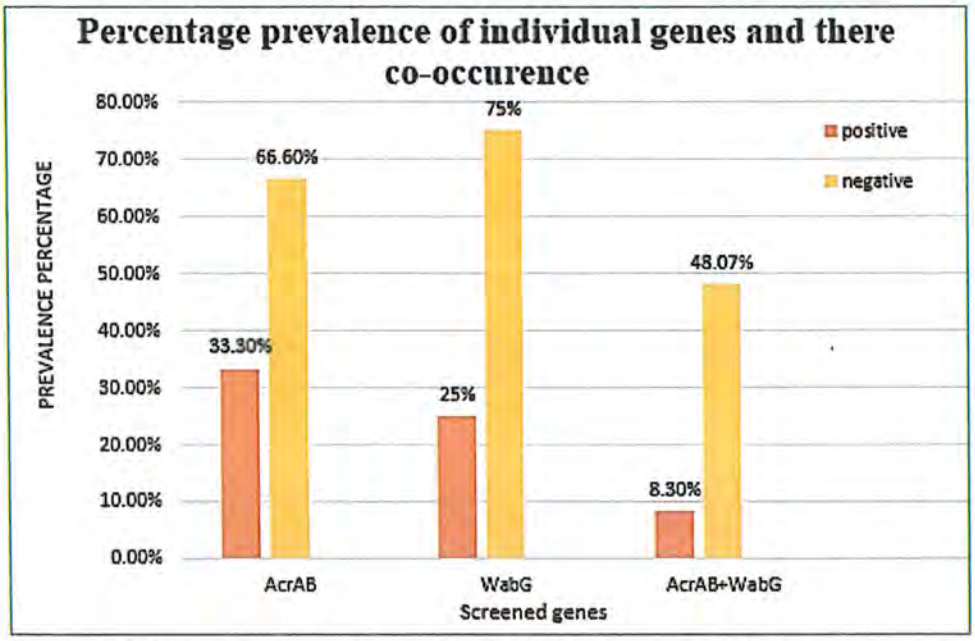

Figure 6.8: Percentage prevalence of the individual genes and their co-occurrence. *acr*AB (33.3%) gene was more prevalent as compared to the *wab*G (25%) gene in our isolates and the co-occurrence came out to be only 8.3%.

Table 6.1: Occurrence and Co-occurrence of the screened genes.

GENES	PRESENCE	NO. OF ISOLATES
***acr*AB + *wab*G**	Both positive	13
	Both negative	75
***acr*AB**	Positive	52
	Negative	104
***wab*G**	Positive	39
	Negative	117

6.9. Scrutinization of the isolates via Disk Diffusion Assay

Out of n=80, we scrutinized our isolates with the help of the disc diffusion method. In doing so we found out that n=24 was sensitive, n=11 was resistant and n=44 was intermediate.

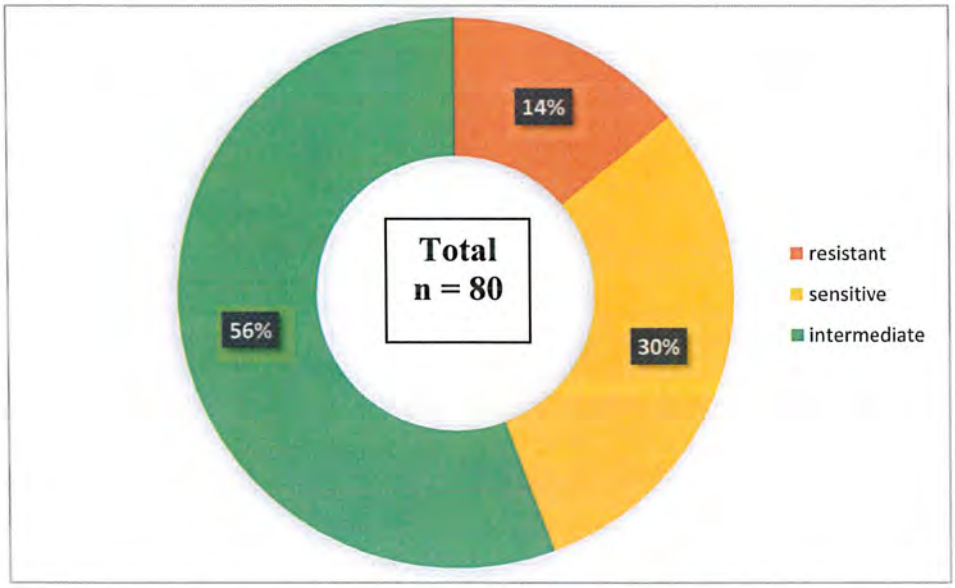

Figure 6.9(a): Graphical representation of the results obtained via Disk Diffusion

Figure 6.9(b): resistant isolates of klebsiella against the standard disc of the drug Doxycycline.

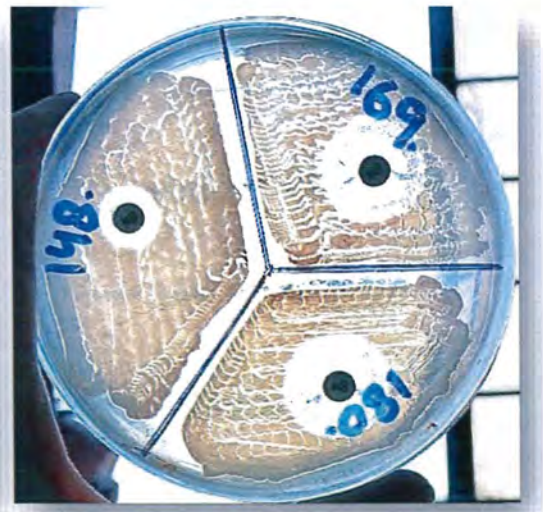

Figure 6.9(c): Sensitive klebsiella against the standard disc of doxycycline.

6.10. Determination of Minimum Inhibition Concentration (MIC) of sensitive isolates

After initial scrutinization as sensitive, resistant, and intermediate, we further performed a Microtiter plate Assay on the sensitive isolates i.e., n=24 against doxycycline to check the concentrations at which it was effective against the *Klebsiella Pnemoniae* isolates. In doing so we found out that 67% were sensitive at 4µg/ml, 21% at 2µg/ml, and 12% at 1µg/ml.

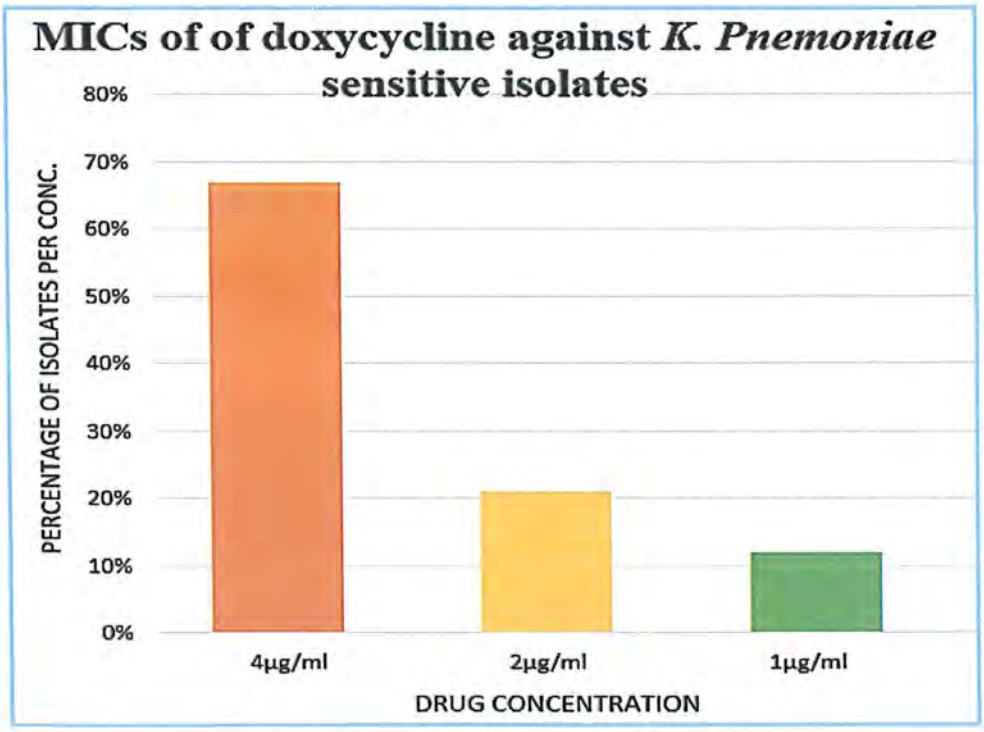

Figure 6.10(a): Percentage of isolates per MIC.

Table 6.2: No. of sensitive isolate obtained per drug concentration.

Total Number of sensitive isolates	Minimum Inhibitory Concentrations (MICs)	Number of isolates per concentration.
24	**4µg/ml**	**16**
	2µg/ml	**5**
	1µg/ml	**3**

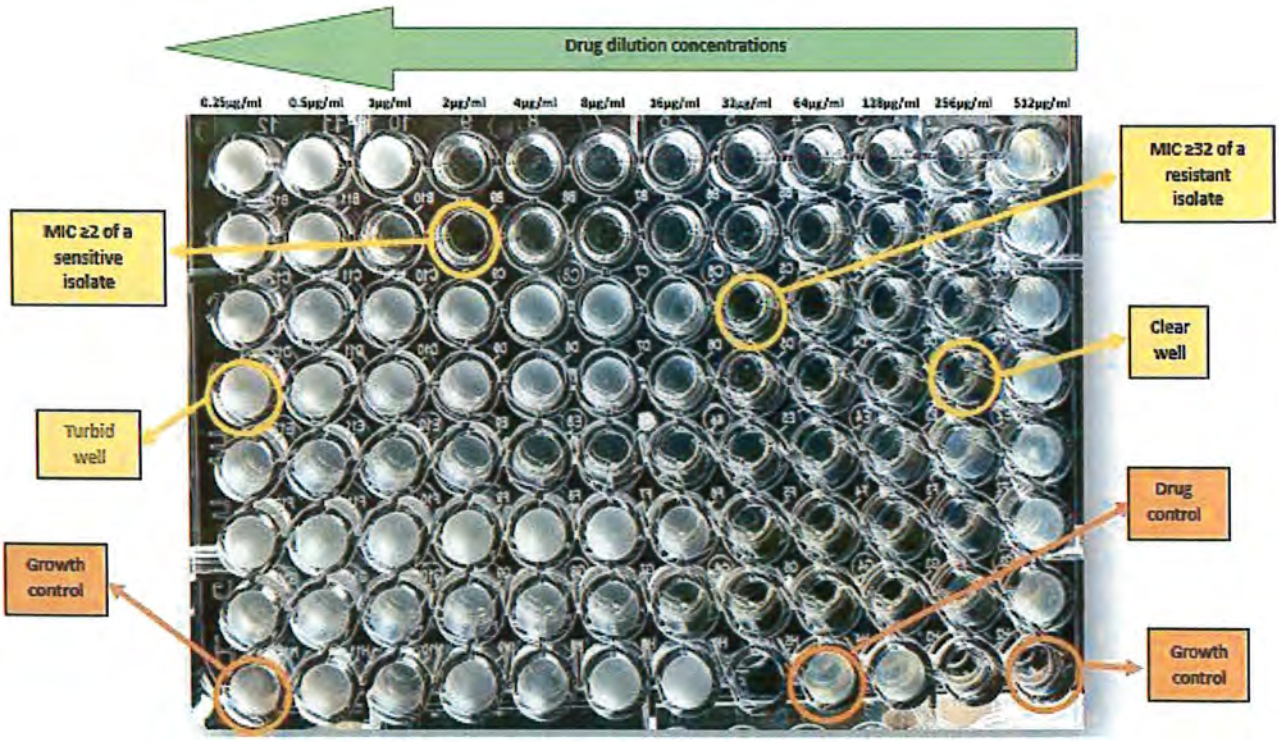

Figure 6.10(b): Microtiter plate showing the MIC results of the resistant and sensitive isolates of klebsiella against doxycycline at various drug concentrations.

6.11. Optical density of the sensitive isolates

After obtaining the MIC values of the sensitive isolates, we checked the biofilm-forming ability of n=8, out of which n=2 were strong biofilm formers, n=1 was moderate biofilm former and n=5 were weak biofilm formers. We checked the biofilm-forming ability of these isolates by checking their optical density at two wavelengths i.e., 492nm and 630nm. KP180 and kP169 showed to be the strong biofilm formers while KP94 was a moderate biofilm former leaving the remaining five to be the weak biofilm formers.

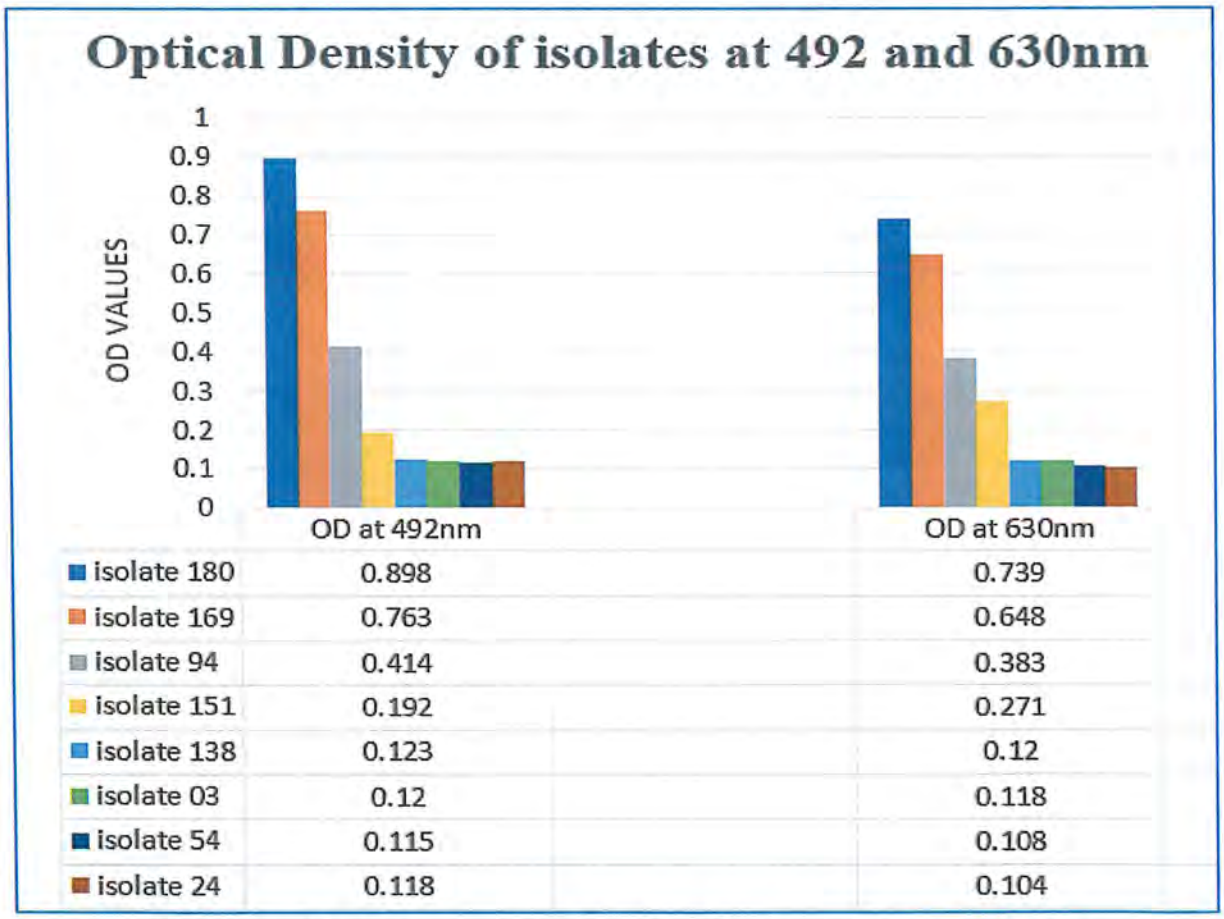

Figure 6.11(a): Graphical representation of the selected isolates and their optical density at two wavelengths i.e., 492nm and 630nm. As shown above, KP180 and KP169 showed to be strong biofilm formers and had the highest OD values, KP94 showed to be a moderate biofilm former while the remaining isolates were weak biofilm formers.

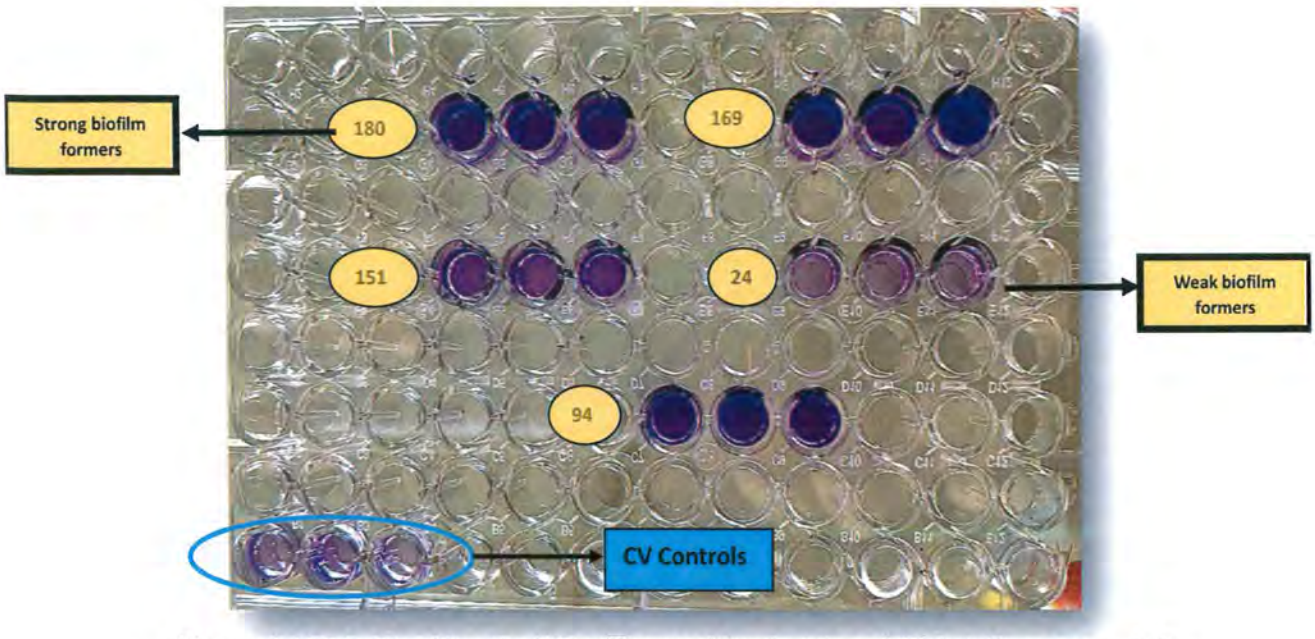

Figure 6.11(b): Checking the OD of the sensitive isolates of *Klebsiella pneumonia* to classify them as strong, weak, and moderate biofilm formers.

6.12. Inhibition and Eradication effect of doxycycline on the strong and moderate biofilm formers

Strong moderate biofilm former was further tested for Minimum Biofilm Inhibition Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC. On comparing the MBICs and MBECs to MIC-p's values of the same isolates it was observed that the MBEC and MBIC values of these isolates were significantly high as compared to the MIC-p values. This comparison showed that the concentration of doxycycline needed to remove the planktonic cells is not enough to completely inhibit the biofilm formation, drug concentration must be. Figure 6.13 shows the comparison of all values of the selected isolates.

Figure 6.3: Comparison of the MBIC, MBEC, and MIC-p values

ISOLATE NO.	BIOFILM FORMING ABILITY	MBIC	MBEC	MIC-p
180	**strong**	**≥64**	**≥256**	**≥2**
169	**strong**	**≥128**	**≥512**	**≥2**
94	**moderate**	**≥128**	**≥128**	**≥1**

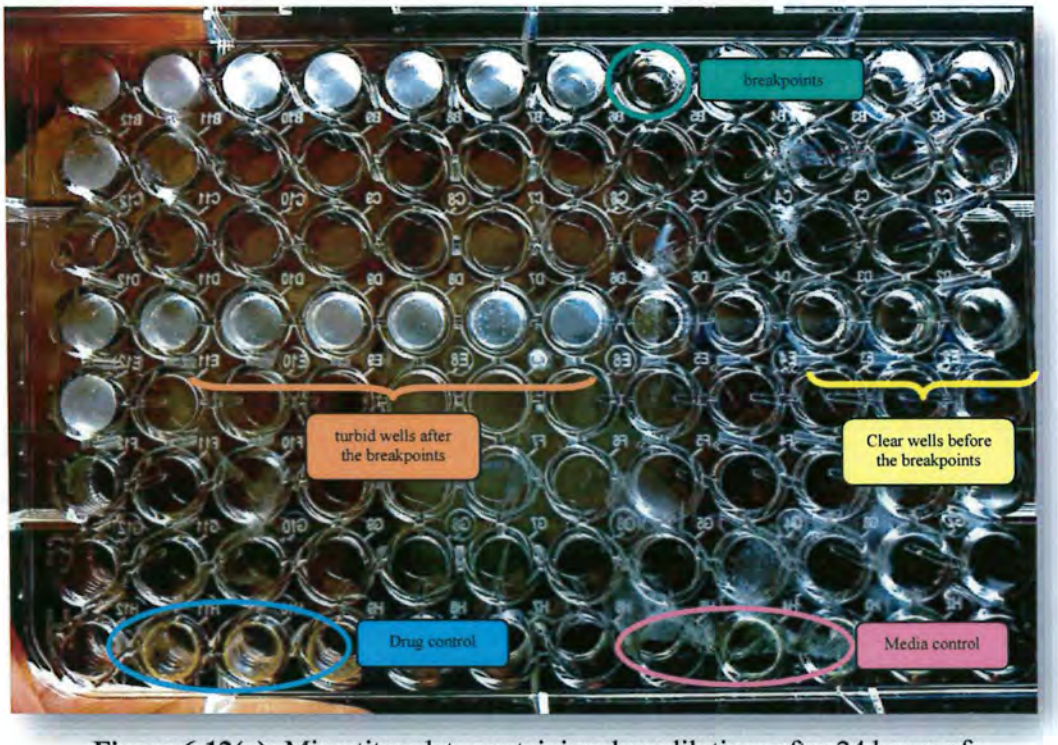

Figure 6.12(a): Microtiter plate containing drug dilutions after 24 hours of incubation showing clear wells indicating no apparent growth because of doxycycline i.e., MBEC and MBIC values.

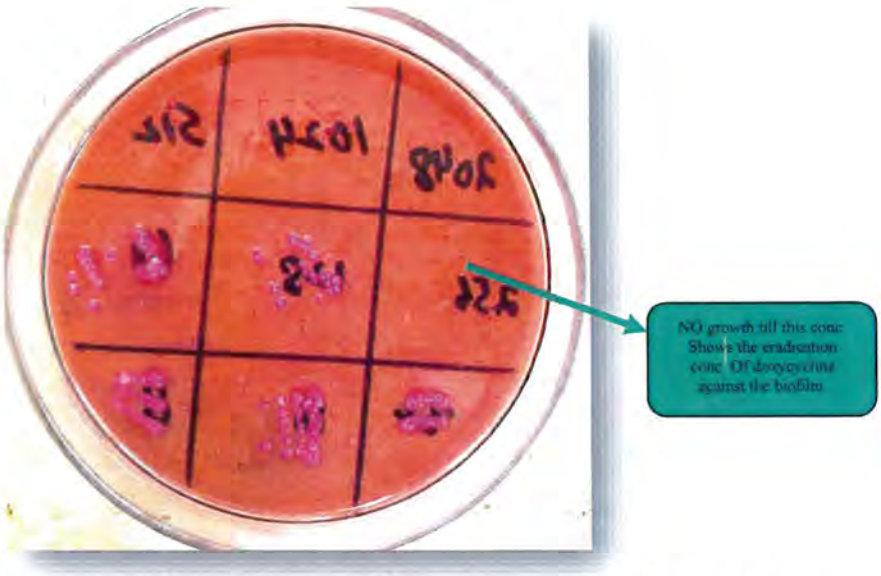

Figure 6.12(b): The streaking result of the MBEC microtiter plate shows that the apparent clear wells still had biofilms formed in them and provided accurate MBEC values.

6.13. Effect of doxycycline on the biofilm formation of the *Klebsiella pneumoniae* at sub-minimal concentration

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 to doxycycline were taken and the effect of doxycycline on their biofilm-forming ability was checked at 2,4,6 and 24hours. The most significant inhibitory effect was observed at 2 and 4 hours but no such effects were observed after 24 hours. The highest level of inhibition was observed at 0.5µg/ml.

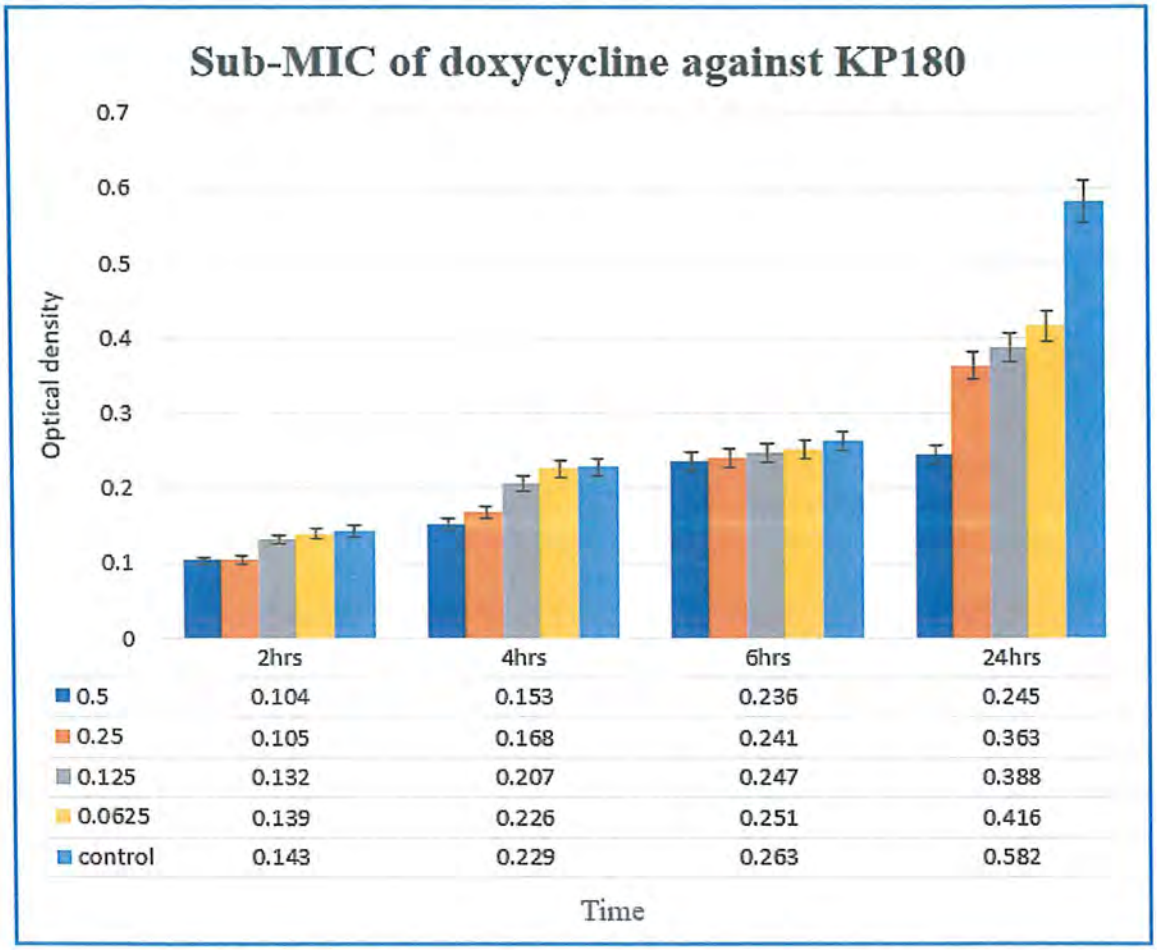

Figure 6.13(a): The biofilm of *Klebsiella pneumoniae* isolate KP180 displayed significant inhibition when exposed to sub-minimal concentrations of doxycycline for 2 and 4 hours, but there was no substantial inhibitory effect observed after 24 hours. The highest level of inhibition was achieved at a concentration of 0.5µg/ml.

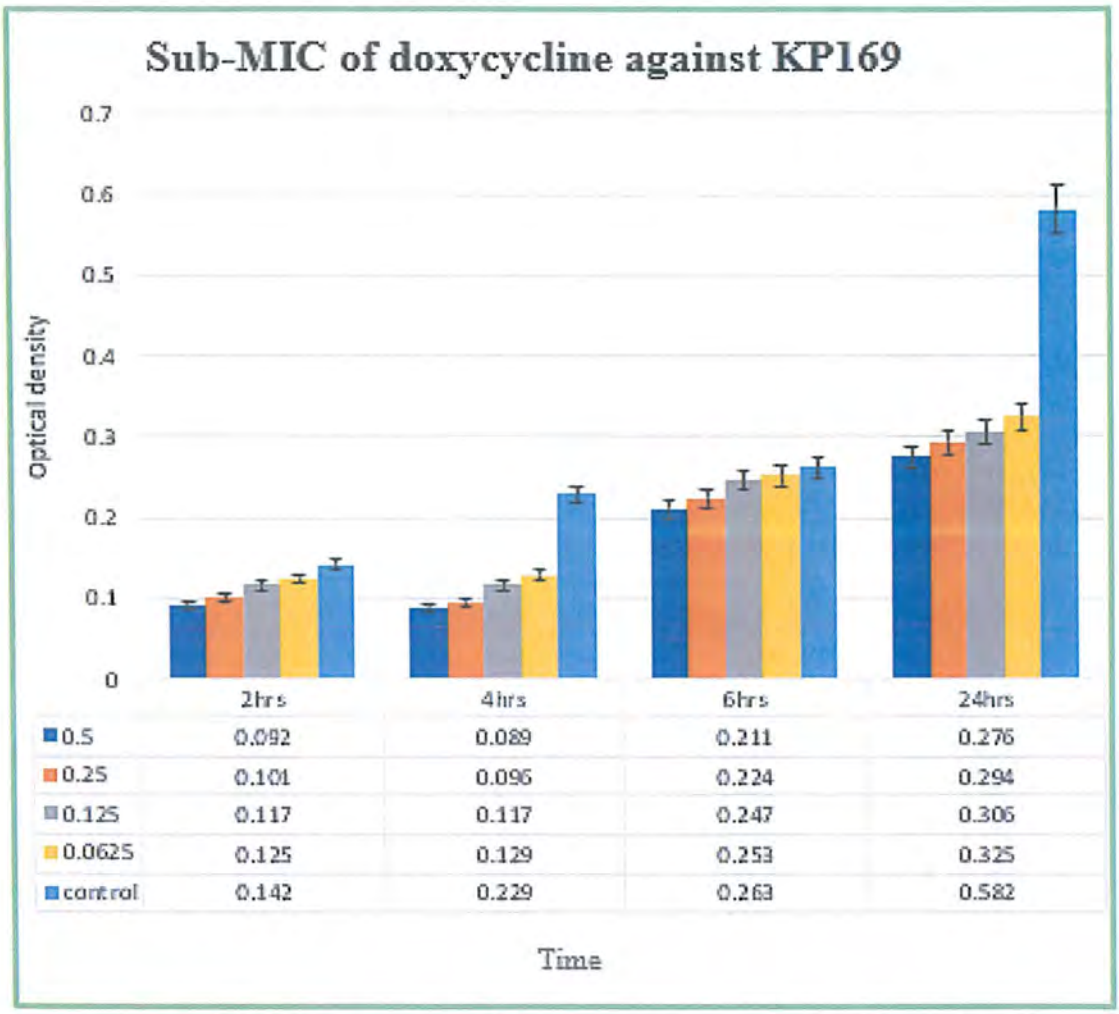

Figure 6.13(b): The biofilm of *Klebsiella pneumoniae* isolate KP*169* displayed significant inhibition when exposed to sub-minimal concentrations of doxycycline for 2 and 4 hours, but there was no substantial inhibitory effect observed after 24 hours. The highest level of inhibition was achieved at a concentration of 0.5µg/ml.

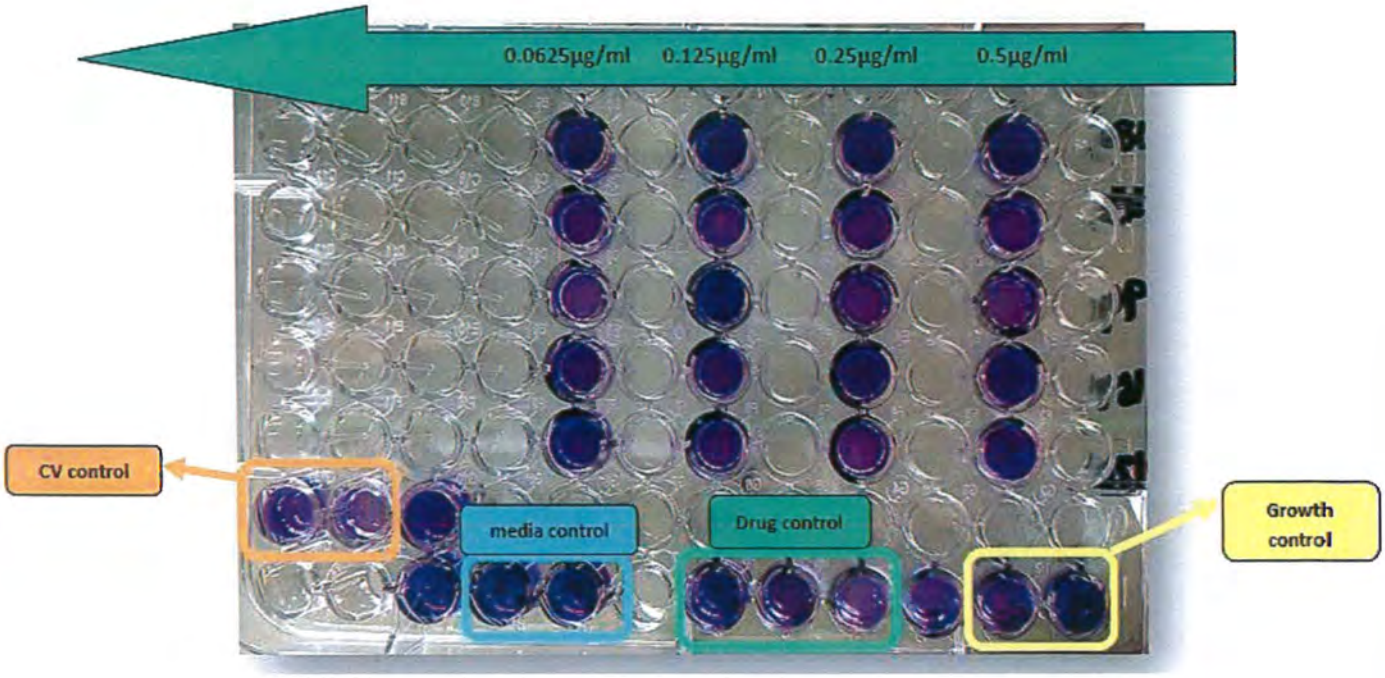

Figure 6.13(c): Microtiter plate showing Sub-mic of doxycycline against the biofilm of *Klebsiella pnemoniae* isolates at 2,4,6 and 24hrs.

DISCUSSION

DISCUSSION

Klebsiella pneumoniae is a bacterium responsible for various infections, including pneumonia, urinary tract infections, and more. While it initially affected primarily immunocompromised individuals, the emergence of stronger strains has made even healthy individuals vulnerable (Paczosa *et al.*, 2016). These strains are also becoming increasingly resistant to antibiotics, posing a significant challenge for treatment. This rise in antibiotic resistance is a global issue and threatens the effectiveness of these life-saving drugs. The overuse and inappropriate use of antibiotics, coupled with limited new drug development by pharmaceutical companies due to economic factors and regulatory hurdles, have contributed to this crisis. Several bacteria, including *Klebsiella pneumoniae*, have been categorized as urgent threats by the Centers for Disease Control and Prevention (CDC), adding to the burden on healthcare systems and patients (Ventola *et al.*, 2015). To address this challenge, coordinated efforts are crucial, including policy changes, increased research, and crisis management strategies. Choosing the right antibiotic therapy should be based on susceptibility results, potentially extending beyond beta-lactam and carbapenem classes to include drugs like colistin, tigecycline, doxycycline, and fosfomycin. Recent research has emphasized the importance of combination therapy for KPC-producing *K. pneumoniae* bloodstream infections, with doxycycline showing promise as a potential component due to its in vitro activity against certain strains and favorable pharmacokinetics (Tang *et al.*, 2019). Even though in 2009, the United Kingdom expressed concerns about variations in the use of water-soluble doxycycline hyclate products for poultry across the European Union, potentially impacting public and animal health, the matter was referred to the European Medicines Agency (EMA) and following a review of available data, the Committee for Veterinary Medicinal Products (CVMP) did not find immediate health risks. However, due to worries about antibiotic resistance, the CVMP recommended changes to doxycycline product information, including prudent use guidelines and proper administration instructions. In June 2010, the European Commission adopted the CVMP's recommendations (Article 35, European Medicine Agency *et al.*, 20101) These changes applied to different strengths of doxycycline hyclate products used in

poultry drinking water throughout the European Union. This again opened up the opportunity to use this drug against such infections. Recent research highlights the significance of using combination therapy to treat bloodstream infections caused by KPC-producing *K. pneumoniae*. Doxycycline has shown promising in vitro activity against specific KPC isolates, making it a potential candidate for combination therapy. Doxycycline offers several advantages, including robust absorption with high bioavailability (average 95%), satisfactory pharmacokinetics with intravenous dosing, oral administration, a broad antimicrobial spectrum, and minimal side effects (Hirsch *et al.*,2010). It has also proven effective in managing multi-drug resistant *K. pneumoniae*-induced urinary tract infections (UTIs). However, there's a notable lack of comprehensive and up-to-date information regarding doxycycline's pharmacokinetics and pharmacodynamics (Musicha *et al.*, 2017). The prevalence of Extended Spectrum Beta-Lactamases (ESBLs) production in bacterial species has limited the use of many beta-lactam antibiotics, including carbapenems, which were once considered last-resort options for treating multi-drug resistant bacteria (Papp-Wallace *et al.*, 2011). In this landscape, doxycycline hyclate emerges as a viable treatment alternative for patients with susceptible Multi-Drug Resistant Urinary Tract Infections (MDR UTIs) (White *et al.*, 2017).

Following the given information, we designed this research to assess the antimicrobial susceptibility of doxycycline concerning clinical *Klebsiella pneumoniae* isolates obtained from tertiary care hospitals in Islamabad, Pakistan. Our findings revealed that out of 80 isolates, 17.5% (14) displayed resistance to doxycycline, while 30% (24) exhibited sensitivity. Our observed susceptibility rate closely aligned with a study conducted in Karachi by Zehra NM *et al.*, 2016, although it notably surpassed the 11.5% resistance rate reported in another Karachi study (Abdullah *et al.*, 2013). A separate investigation in Mumbai, India reported an antibiotic susceptibility rate of 68.4% (S *et al.*, 2020). Doxycycline also demonstrated favorable susceptibility against isolates resistant to amoxicillin (75.9%) and cefuroxime (64.8%) (Swaminathan *et al.*, 2020).

Tetracycline resistance often originates from the acquisition of novel genes that facilitate energy-driven expulsion of tetracyclines or the synthesis of protective proteins, shielding bacterial ribosomes from tetracycline's effects. Many of these genes are associated with mobile plasmids or transposons and can be discerned using molecular methods like DNA-DNA hybridization utilizing oligonucleotide probes and DNA sequencing. In a smaller subset of scenarios, resistance emerges through mutations that modify the permeability of outer membrane porins and/or lipopolysaccharides, adjust the regulation of innate efflux systems, or alter the 16S rRNA (Chopra *et al.,* 2001). Recent surveillance studies have unveiled the prevalence of tetracycline resistance in selected European countries, indicating rates of 66.9% for extended-spectrum β-lactamase (ESBL)-producing *Escherichia coli* and 44.9% for *Klebsiella* species. Moreover, global percentages of tetracycline resistance were observed at 8.7% for methicillin-resistant *Staphylococcus aureus* (MRSA) and 24.3% for *Streptococcus pneumoniae* (Grossman *et al.,* 2016).

Some pivotal genes that emerged as key players in *Klebsiella pneumonia's* resistance dynamics include the *acr*AB genes and the *wab*G *gene*. A further in-depth investigation is warranted in this area. In our study, we diligently examined these specific genes to gain insights. Our findings revealed that out of the 156 isolates studied, 52 demonstrated the presence of the *acr*AB gene, while the remaining 104 were devoid of it. This translates to a prevalence rate of only 33.3%, which is notably lower compared to a study conducted in Iran that reported a prevalence rate of 96.52% for the *acr*AB gene (Ranjbar *et al.,* 2019). Another study showcased a 100% prevalence of the *acr*AB gene within their *Klebsiella pneumoniae* isolates (Ferreira *et al.,* 2019). This recent data unmistakably indicates ongoing dissemination of this gene responsible for resistance in *Klebsiella*. Over time, mutants carrying this gene in their genetic makeup (chromosome) have emerged and actively contributed to the perpetuation of antibiotic resistance. In a similar vein, the *wab*G gene, a key player in biofilm formation—one of the pivotal virulence factors—has emerged as a formidable force in *K. pneumoniae* resistance. A distinct study has unveiled *wab*G*'s* role through meticulous crafting and characterization of nonpolar mutants using *K. pneumoniae* wild-type strains. Profound

insights emerged as analysis of lipopolysaccharide (LPS) samples from these mutants disclosed an absence of O antigen coupled with truncated-core LPS. This implies a pivotal role for *WabG* in tethering the inaugural outer core residue (d-GalA) to the O-3 position of the l,d-HeppII residue (Izquierdo *et al.*, 2003). In our research, we conducted a comprehensive examination of the *wab*G gene, a plasmid-associated gene. Among the 156 isolates we investigated, 39 were found to harbor this gene, resulting in a prevalence rate of 25%. This prevalence stands notably lower when compared to a study conducted in China, which reported a gene prevalence of 77.4% (Zhang *et al.*, 2018). Similarly, a separate study in Japan exhibited a prevalence of 91.5% for this gene (Ikeda *et al.*, 2018). These recent studies collectively underscore the gene's evolutionary transfer over time, leading to the emergence of mutants carrying the *wab*G gene and their consequential contribution to the escalating challenge of antibiotic resistance. Based on our findings, the *acr*AB gene exhibited the highest prevalence when compared to the *wab*G gene. While the simultaneous presence of both genes within a single isolate is a potential scenario, limited research has been conducted on this aspect. Nevertheless, our data highlights that out of the total 156 isolates, 13 demonstrated the co-occurrence of both the *AcrAB* and *WabG* genes.

Klebsiella pneumoniae possesses the ability to amplify both its virulence and resistance by creating biofilms on vulnerable tissues and indwelling catheters. A study carried out by Nirwati et al. in 2019 uncovered that 85.63% of *K. pneumoniae* isolates were proficient in forming biofilms. The formation of biofilms leads to heightened bacterial resilience against antibiotics compared to their free-floating counterparts. Singla et al. in 2013 illustrated that *K. pneumoniae* isolates that developed biofilms resulted in a significant rise, ranging from two to fourfold, in the minimum inhibitory concentration (MIC) values of ciprofloxacin and amikacin when contrasted with planktonic cells. Moreover, biofilm development led to an eightfold increase in the MIC value of piperacillin (Singla *et al.*, 2013). Within our collection of *K. pneumoniae* isolates, a substantial majority exhibit robust biofilm-forming capability. We aimed to investigate the impact of doxycycline, the drug under consideration, on the biofilm-forming potential of sensitive isolates. To achieve this, we initially subjected 156 isolates to the

Disk Diffusion Assay and Microdilution Method to discern sensitivity and resistance. Following these assays, we identified 24 out of 80 isolates (30%) as sensitive. Among these, we selected 8 sensitive isolates for further analysis of their biofilm-forming abilities. Among the selected isolates, KP180 and KP169 demonstrated strong biofilm-forming abilities, while KP94 displayed a moderate capability, and the remaining five isolates (KP3, KP54, KP151, KP138, and KP24) exhibited weak biofilm formation. In their planktonic states, all these isolates exhibited MICs of 1-4μg/ml concerning doxycycline. Notably, even though KP180 and KP169 were sensitive to doxycycline, they managed to produce robust biofilms, requiring significantly higher concentrations to inhibit their biofilm growth. For instance, in the case of KP180, the Minimum Biofilm Eradication Concentration (MBEC) was determined to be ≥256μg/ml, while the Minimum Biofilm Inhibitory Concentration (MBIC) was ≥64μg/ml. Similarly, for KP169, the MBEC and MBIC values were found to be ≥512μg/ml and ≥128μg/ml, respectively. The noteworthy observation is that the minimum inhibitory concentration required to combat biofilm growth increased substantially for strong biofilm-forming isolates. Interestingly, both KP180 and KP169, characterized as potent biofilm formers, were found to possess the *wab*G gene upon our screening. Additionally, KP94, categorized as a moderate biofilm former, also harbored the *wab*G gene. Conversely, the remaining five isolates, classified as weak biofilm formers, lacked the *wab*G gene.

We further investigated the effects of our drug on biofilm formation in *Klebsiella pneumoniae*. Doxycycline concentrations in the range of 2-4μg/ml were found effective against the planktonic cells of our sensitive isolates; however, these concentrations proved ineffective against biofilms. Notably, higher doxycycline concentrations were necessary to inhibit biofilm growth, with MBIC concentrations of ≥64μg/ml and ≥128μg/ml observed for KP180 and KP169, respectively. Similarly, MBEC concentrations of ≥256μg/ml and ≥152μg/ml were reported for KP180 and KP169, respectively. This phenomenon indicated that *K. pneumoniae* isolates sensitive to doxycycline in their planktonic state did not maintain susceptibility when existing within a biofilm structure. Lastly, we studied the sub-minimal concentrations of doxycycline against the strong biofilm formers i.e., KP180 and KP160. On doing so the

biofilm of *Klebsiella pneumoniae* isolate KP160 and KP180 displayed significant inhibition when exposed to sub-minimal concentrations of doxycycline for 2 and 4 hours, but there was no substantial inhibitory effect observed after 24 hours. The highest level of inhibition was achieved at a concentration of 0.5µg/ml. Over time, as the concentration of antibiotics in the bloodstream drops below the minimum inhibitory concentrations required for bacterial inhibition or eradication, the efficacy of the drug diminishes. Consequently, bacteria detect this sub-MIC concentration of antibiotics, triggering fluctuations in their virulence and resistance factors. One of these mechanisms involves an alteration in the bacteria's biofilm-forming ability, which can either increase or decrease in response to sub-MIC levels of antibiotics. Shi and colleagues 2019 observed an escalation in the biofilm-forming capacity and other virulence factors of Pseudomonas aeruginosa under sub-MIC concentrations of ciprofloxacin (Shi et al., 2019). Another mechanism involves the amplification of toxin-antitoxin systems in K. pneumoniae when exposed to sub-MIC levels of gentamicin, leading to augmented stress management abilities and resistance (Andersson & Hughes, 2014).

The heightened resistance to doxycycline following biofilm formation can be attributed to the limited diffusion of the drug into the biofilm matrix and subsequently to the bacterial cells. Moreover, nutrient scarcity within the inner layers of biofilms may force bacteria into a stationary phase, rendering the antibiotic ineffective in eradicating biofilm populations (Naparstek *et al.*, 2014). The utilization of high doses of doxycycline raises concerns about potential cytotoxicity, thereby discouraging its application at elevated concentrations. Prior reports have underscored the enhanced efficacy of combining different antibiotics to treat bacterial infections, compared to their individual use. For future investigations, exploring the synergistic effects of doxycycline in conjunction with other drugs on strong biofilm-forming isolates could be valuable. Lemaître and colleagues 2012 demonstrated this approach against Yersinia pestis, revealing synergistic action (Lemaître *et al.*, 2012).

CONCLUSION

In conclusion, the escalating global challenge of antibiotic resistance underscores the critical necessity for innovative drug development, particularly in addressing formidable pathogens like *Klebsiella pneumoniae*, a prominent member of the ESKAPE group. As our arsenal of effective antibiotics dwindles, the urgency for novel therapeutic options intensifies. This dissertation has shed light on the potential of doxycycline as a promising candidate against Klebsiella infections, emphasizing its notable impact on disrupting Klebsiella biofilm formation, a key virulence factor contributing to its pathogenicity followed by the detection of key virulence genes *acr*AB and *wab*G in this pathogen. The significance of this research extends far beyond the laboratory, highlighting the imperative for continued exploration into alternative antimicrobial strategies and the relentless pursuit of solutions to combat the growing menace of antibiotic-resistant infections.

BIBLIOGRAPHY

Abraham, E. P., & Chain, E. (1988). An enzyme from bacteria can destroy penicillin. 1940. Reviews of infectious diseases, 10(4), 677-678.

Abrar, S., Vajeeha, A., UI-Ain, N., & Riaz, S. (2017). Distribution of CTX-M group I and group III B-lactamases produced by Escherichia coli and Klebsiella pneumoniae pathogenesis, 103, 8-12.
Lahore, Pakistan. Microbial

Ah Y-M, Kim A-J, Lee J-Y (2014) Colistin resistance in Klebsiella pneumonia. Int J Antimicrob Agents 44:8-15

Alvarez, M., Tran, J. H., Chow, N., & Jacoby, G. A. (2004). Epidemiology of conjugative plasmid-mediated AmpC -lactamases in the United States. Antimicrobial agents and chemotherapy, 48(2), 533-537.

Ambler, R. P. (1980). The structure of 6-lactamases. Philosophical Transactions of the Roval Society of London. B, Biological Sciences, 289(1036), 321-331.

Anderl, J. N., Zahller, J., Roe, F., & Stewart, P. S. (2003). Role of nutrient limitation and stationery-phase existence in Klebsiella pneumonia biofilm resistance to ampicillin and ciprofloxacin. Antimicrobial agents and chemotherapy, 47(4), 1251-1256.

Ashurst, J. V., & Dawson, A. (2018). Klebsiella pneumoniae.

Bachman M. A., Oyler J. E., Burns S. H., Caza M., Lepine F., Weiser J. N., et al. (2011).

Klebsiella pneumoniae yersiniabactin promotes respiratory tract infection through the evasion of lipocalin 2. Infect. Immun. 79, 3309-3316. 10.1128/1A1.05114-11.

Bachman, M. A., Miller, V. L., & Weiser, J. N. (2009). Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. PLOS Pathog, . 5(10), e1000622.

Abdullah, F.E., Mushtaq, A., Irshad, M., Rauf, H., Afzal, N. and Rasheed, A. (2013). Current efficacy of antibiotics against Klebsiella isolates from urine samples - a multi-.

FUTURE PROSPECTIVES

Following are some of the recommendations for future studies based on the conducted research:

1. Future studies in vaccine development can prioritize harnessing reverse vaccinology, leveraging bioinformatics to predict virulence factors and their variants. This proactive approach can aid in designing vaccines against emerging threats by identifying conserved targets, ensuring more comprehensive protection.
2. The future studies can also delve into dynamic biofilm models. These models can be designed to replicate the intricate processes of biofilm formation and dispersion. Such models, integrated with microfluidic systems and real-time imaging, can provide invaluable insights into biofilm behavior, thereby improving empirical and intervention strategies.
3. Another research avenue involves exploring synthetic biology for enhanced safety. Researchers can investigate the engineering of attenuated strains, which can significantly enhance the safety profiles of vaccines. This approach allows for the design of vaccines with reduced virulence while maintaining their immunogenicity.

centric experience in Karachi. *Pakistan Journal of Pharmaceutical Sciences*, 26(1), pp.11–15.

Aljana by, A., 2017. Role of rmpA, wabG, uge, Ycfm, fimh1, EntB, Ybt-irp2, and kfu genes in pathogenicity of Klebsiella pneumoniae: An overview. International Journal of ChemTech 10, 391–398.

Amin, A., Ghumro, P. B., Hussain, S. and Hameed, A. (2009). Prevalence of antibiotic resistance among clinical isolates of Klebsiella pneumoniae isolated from a Tertiary Care Hospital in Pakistan. *Malaysian Journal of Microbiology*.

Anderl, J.N., Franklin, M.J. and Stewart, P.S. (2000a). Role of Antibiotic Penetration Limitation in Klebsiella pneumoniae Biofilm Resistance to Ampicillin and Ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, 44(7), pp.1818–1824

Anderl, J.N., Franklin, M.J. and Stewart, P.S. (2000b). Role of Antibiotic Penetration Limitation in Klebsiella pneumoniae Biofilm Resistance to Ampicillin and Ciprofloxacin. *Antimicrobial Agents and Chemotherapy*, 44(7), pp.1818–1824.

David L. Paterson, M.D., Ph.D., Feng Yee Chang, M.D. *Klebsiella species (K. pneumoniae, K. oxytoca, K. ozaenae, and K. rhinoscleromatis) - Infectious Disease and Antimicrobial Agents.*

Arato, V., Raso, M.M., Gasperini, G., Berlanda Scorza, F. and Micoli, F. (2021). Prophylaxis and Treatment against Klebsiella pneumoniae: Current Insights on This Emerging Anti-Microbial Resistant Global Threat. *International Journal of Molecular Sciences*, 22(8), p.4042.

Askari Rizvi, S.F. (2018). Tetracycline: Classification, Structure-Activity Relationship and Mechanism of Action as a Theranostic Agent for Infectious Lesions-A Mini Review. *Biomedical Journal of Scientific & Technical Research*, 7(20).

Bagley, S.T. (1985). Habitat association of Klebsiella species. *Infection control: IC*, 6(2), pp.52–8.
Bengoechea, J.A. (2018).

Bengtsson-Palme, J., Kristiansson, E. and Larsson, D.G.J. (2017). Environmental factors influencing the development and spread of antibiotic resistance. *FEMS Microbiology*

Reviews, 42(1).

Boye, K. and Hansen, D.S. (2003). Sequencing of 16S rDNA of Klebsiella: taxonomic relations within the genus and to other Enterobacteriaceae. *International Journal of Medical Microbiology*, 292(7-8), pp.495–503.

Bradbury, R.S., Reid, D.W. and Champion, A.C. (2014). Urease production as a marker of virulence in Pseudomonas aeruginosa. *British Journal of Biomedical Science*, 71(4), pp.175–176

Badulla, W. F., Alshakka, M. & Mohamed Ibrahim, M. I. (2020). Antimicrobial Resistance Profiles for Different Isolates in Aden, Yemen: A Cross-Sectional Study in a Resource-Poor Setting. BioMed Research International, 2020.

Bandeira, M., Carvalho, P. A., Duarte, A., & Jordao, L. (2014). Exploring dangerous connections between Klebsiella pneumonia biofilms and healthcare-associated infections. Pathogens, 3(3), 720-731.

Candan, E.D. and Aksöz, N. (2015). Klebsiella pneumoniae: characteristics of carbapenem resistance and virulence factors. *Acta Biochimica Polonica*, 62(4).

Chaudhry, T.H., Aslam, B., Arshad, M.I., Alvi, R.F., Muzammil, S., Yasmeen, N., Aslam, M.A., Khurshid, M., Rasool, M.H. and Baloch, Z. (2020). Emergence of bla_{NDM-1} Harboring Klebsiella pneumoniae ST29 and ST11 in Veterinary Settings and Waste of Pakistan. *Infection and Drug Resistance*, Volume 13, pp.3033–3043.

Chetri, S., Bhowmik, D., Paul, D., Pandey, P., Chanda, D.D., Chakravarty, A., Bora, D. and Bhattacharjee, A. (2019). AcrAB-TolC efflux pump system plays a role in carbapenem non-susceptibility in Escherichia coli. *BMC Microbiology*, 19(1).

Chopra, I. and Roberts, M. (2001). Tetracycline Antibiotics: Mode of Action, Applications, Molecular Biology, and Epidemiology of Bacterial Resistance. *Microbiology and Molecular Biology Reviews*, 65(2), pp.232–260.

Cocker, D., Chidziwisano, K., Mphasa, M., Mwapasa, T., Lewis, J.M., Rowlingson, B., Sammarro, M., Bakali, W., Salifu, C., Zuza, A., Charles, M., Mandula, T., Maiden, V., Amos, S., Jacob, S.T., Kajumbula, H., Mugisha, L., Musoke, D., Byrne, R. and Edwards, T. (2023). Investigating One Health risks for human colonization with extended-spectrum β-lactamase-producing Escherichia coli and Klebsiella pneumoniae in Malawian households: a longitudinal cohort study. *The Lancet Microbe*, [online] 4(7), pp. e534–e543. Cortes, G. (2002). Molecular Analysis of the Contribution of the Capsular

Polysaccharide and the Lipopolysaccharide O Side Chain to the Virulence of Klebsiella pneumoniae in a Murine Model of Pneumonia. *Infection and Immunity*, 70(5), pp.2583–2590.

Cunha, B.A., Sibley, C.M. and Ristuccia, A.M. (1982). Doxycycline. *Therapeutic Drug Monitoring*, 4(2), p.115.

Derakhshan, S., Sattari, M. and Bigdeli, M. (2008). Effect of subinhibitory concentrations of cumin (Cuminum cyminum L.) seed essential oil and alcoholic extract on the morphology, capsule expression, and urease activity of Klebsiella pneumoniae. *International Journal of Antimicrobial Agents*, 32(5), pp.432–436.

Durdu, B., Hakyemez, I.N., Bolukcu, S., Okay, G., Gultepe, B. and Aslan, T. (2016). Mortality markers in nosocomial Klebsiella pneumoniae bloodstream infection. *SpringerPlus*, 5(1).

Ebringer, R.W., Cawdell, D.R., Cowling, P. and Ebringer, A. (1978). Sequential studies in ankylosing spondylitis. Association of Klebsiella pneumoniae with active disease. *Annals of the Rheumatic Diseases*, 37(2), pp.146–151.

Effah, C.Y., Sun, T., Liu, S. and Wu, Y. (2020a). Klebsiella pneumoniae: an increasing threat to public health. *Annals of Clinical Microbiology and Antimicrobials*, 19(1).

Effah, C.Y., Sun, T., Liu, S. and Wu, Y. (2020b). Klebsiella pneumoniae: an increasing threat to public health. *Annals of Clinical Microbiology and Antimicrobials*, 19(1).

Farhan, E., Abdullah, Sadaf Akber, S., Anis, W. and Syedain, F. (n.d.). *Identification of Klebsiella pneumoniae and Klebsiella oxytoca in urine specimens from a laboratory in Karachi*.

Ferreira, R.L., da Silva, B.C.M., Rezende, G.S., Nakamura-Silva, R., Pitondo-Silva, A., Campanini, E.B., Brito, M.C.A., da Silva, E.M.L., Freire, C.C. de M., Cunha, A.F. da and Pranchevicius, M.-C. da S. (2019). High Prevalence of Multidrug-Resistant Klebsiella pneumoniae Harboring Several Virulence and β-Lactamase Encoding Genes in a Brazilian Intensive Care Unit. *Frontiers in Microbiology*, 9.

Gasink, L.B., Edelstein, P.H., Lautenbach, E., Synnestvedt, M. and Fishman, N.O. (2009). Risk Factors and Clinical Impact of Klebsiella pneumoniae Carbapenemase-Producing K. pneumoniae. *Infection Control & Hospital Epidemiology*, 30(12), pp.1180–1185.

Ghosh, D., Veeraraghavan, B., Elangovan, R. and Vivekanandan, P. (2019). Antibiotic Resistance and Epigenetics: More to It than Meets the Eye. *Antimicrobial Agents and Chemotherapy*, 64(2).

Gorrie, C.L., Mirčeta, M., Wick, R.R., Judd, L.M., Lam, M.M.C., Gomi, R., Abbott, I.J., Thomson, N.R., Strugnell, R.A., Pratt, N.F., Garlick, J.S., Watson, K.M., Hunter, P.C., Pilcher, D.V., McGloughlin, S.A., Spelman, D.W., Wyres, K.L., Jenney, A.W.J. and Holt, K.E. (2022). Genomic dissection of Klebsiella pneumoniae infections in hospital patients reveals insights into an opportunistic pathogen. *Nature Communications*, 13(1).

Graber, E.M. (2021). Treating acne with the tetracycline class of antibiotics: A review. *Dermatological Reviews*.

Grossman, T.H. (2016b). Tetracycline Antibiotics and Resistance. *Cold Spring Harbor Perspectives in Medicine*, 6(4), p.a025387.

Grossman, T.H. (2016c). Tetracycline Antibiotics and Resistance. *Cold Spring Harbor Perspectives in Medicine*, 6(4), p.a025387.

Guerra, M.E.S., Destro, G., Vieira, B., Lima, A.S., Ferraz, L.F.C., Hakansson, A.P., Darrieux, M. and Converso, T.R. (2022). Klebsiella pneumoniae Biofilms and Their Role in Disease Pathogenesis. *Frontiers in Cellular and Infection Microbiology*, [online] 12, p.877995.

Hany Sahly, Rainer Podschun and Ullmann, U. (2002). Klebsiella Infections in the Immunocompromised Host. *Advances in Experimental Medicine and Biology*, pp.237–249.

Harvard.edu. (2019). *Global Burden of 369 Diseases and Injuries in 204 Countries and Territories, 1990-2019: A Systematic Analysis for the Global Burden of Disease Study 2019*.

Hasdemir, U.O., Chevalier, J., Nordmann, P. and Pages, J.-M.. (2004). Detection and Prevalence of Active Drug Efflux Mechanism in Various Multidrug-Resistant Klebsiella pneumoniae Strains from Turkey. *Journal of Clinical Microbiology*,

Hedayatianfard, K., Akhlaghi, M. and Sharifiyazdi, H. (2014). Detection of tetracycline resistance genes in bacteria isolated from fish farms using polymerase chain reaction. *Veterinary Research Forum*, [online] 5(4), pp.269–275.

HEIDARY, M., NASIRI, M.J., DABIRI, H. and TARASHI, S. (2018). Prevalence of Drug-resistant Klebsiella pneumoniae in Iran: A Review Article. *Iranian Journal of Public Health*, 47(3), pp.317–326.

Hentzer, M., Eberl, L. and Givskov, M. (2005). Transcriptome analysis of Pseudomonas aeruginosa biofilm development: anaerobic respiration and iron limitation. *Biofilms*, 2(1), pp.37–61.

Highsmith, A.K. and Jarvis, W.R. (1985). Klebsiella pneumoniae: selected virulence factors that contribute to pathogenicity. *Infection control: IC*, [online] 6(2), pp.75–7.

Hirsch, E.B. and Tam, V.H. (2010). Detection and treatment options for Klebsiella pneumoniae carbapenemases (KPCs): an emerging cause of multidrug-resistant infection. *Journal of Antimicrobial Chemotherapy*, 65(6), pp.1119–1125.

Holt, K.E., Wertheim, H., Zadoks, R.N., Baker, S., Whitehouse, C.A., Dance, D., Jenney, A., Connor, T.R., Hsu, L.Y., Severin, J., Brisse, S., Cao, H., Wilksch, J., Gorrie, C., Schultz, M.B., Edwards, D.J., Nguyen, K.V., Nguyen, T.V., Dao, T.T. and Mensink, M. (2015). Genomic analysis of diversity, population structure, virulence, and antimicrobial resistance in Klebsiella pneumoniae, an urgent threat to public health. *Proceedings of the National Academy of Sciences*, 112(27), pp. E3574–E3581.

https://grepmed.com/GeraldMD (2018). *Antibiotics Mindmap - Major antibiotic families, mechanisms ...* [online] GrepMed.

Hutchings, M.I., Truman, A.W. and Wilkinson, B. (2019). Antibiotics: Past, Present, and Future. *Current Opinion in Microbiology*, 51(1), pp.72–80.

Ikeda, M., Mizoguchi, M., Oshida, Y., Tatsuno, K., Saito, R., Okazaki, M., Okugawa, S. and Moriya, K. (2018). Clinical and microbiological characteristics and occurrence of Klebsiella pneumoniae infection in Japan. *International Journal of General Medicine*, Volume 11, pp.293–299.

Izquierdo, L., Núria Coderch, Núria Piqué, Bedini, E., Maria Michela Corsaro, Merino, S., Fresno, S., Tomás, J.M. and Regué, M. (2003a). The *Klebsiella pneumoniae wabG* Gene: Role in Biosynthesis of the Core Lipopolysaccharide and Virulence. *Journal of Bacteriology*, 185(24), pp.7213–7221.

Izquierdo, L., Núria Coderch, Núria Piqué, Bedini, E., Maria Michela Corsaro, Merino, S., Fresno, S., Tomás, J.M. and Regué, M. (2003b). The *Klebsiella pneumoniae wabG* Gene:

Role in Biosynthesis of the Core Lipopolysaccharide and Virulence. *Journal of Bacteriology*, 185(24), pp.7213–7221.

J, D. and D, D. (2010). *Origins and Evolution of Antibiotic Resistance*. Microbiology and molecular biology reviews: MMBR.

Jarvis, W.R., Munn, V.P., Highsmith, A.K., Culver, D.H. and Hughes, J.M. (1985). The Epidemiology of Nosocomial Infections Caused by Klebsiella pneumoniae. *Infection Control*, 6(2), pp.68–74.

Kaiser, R.M., Castanheira, M., Jones, R.N., Tenover, F. and Lynfield, R. (2013). Trends in Klebsiella pneumoniae carbapenemase-positive K. pneumoniae in US hospitals: report from the 2007–2009 SENTRY Antimicrobial Surveillance Program. *Diagnostic Microbiology and Infectious Disease*, 76(3), pp.356–360.

Kalanuria, A., Zai, W. and Mirski, M. (2014). Ventilator-associated pneumonia in the ICU. *Critical Care*, [online] 18(2), p.208.

Karampatakis, T., Tsergouli, K. and Behzadi, P. (2023). Carbapenem-Resistant Klebsiella pneumoniae: Virulence Factors, Molecular Epidemiology and Latest Updates in Treatment Options. *Antibiotics*, 12(2), p.234.

Lake, J.G., Weiner, L.M., Milestone, A.M., Saiman, L., Magill, S.S. and See, I. (2017). Pathogen Distribution and Antimicrobial Resistance Among Pediatric Healthcare-Associated Infections Reported to the National Healthcare Safety Network, 2011–2014. *Infection Control & Hospital Epidemiology*, 39(1), pp.1–11.

Lautenbach, E., Strom, Brian L., Bilker, Warren B., Patel, J., Edelstein, Paul H. and Fishman, Neil O. (2001). Epidemiological Investigation of Fluoroquinolone Resistance in Infections Due to Extended-Spectrum β-Lactamase–Producing *Escherichia coli* and *Klebsiella pneumoniae*. *Clinical Infectious Diseases*, 33(8), pp.1288–1294.

Lee, C.-R., Lee, J.H., Park, K.S., Kim, Y.B., Jeong, B.C., and Lee, S.H. (2016). Global Dissemination of Carbapenemase-Producing Klebsiella pneumoniae: Epidemiology, Genetic Context, Treatment Options, and Detection Methods. *Frontiers in Microbiology*, 7.

Lin, W.-H.., Wang, M.-C.., Tseng, C.-C.., Ko, W.-C.., Wu, A.-B. ., Zheng, P.-X. . and Wu, J.-J. (2010). Clinical and microbiological characteristics of Klebsiella pneumoniae isolates causing community-acquired urinary tract infections. *Infection*, 38(6), pp.459–

464.

Magill, S.S., Edwards, J.R., Bamberg, W., Beldavs, Z.G., Dumyati, G., Kainer, M.A., Lynfield, R., Maloney, M., McAllister-Hollod, L., Nadle, J., Ray, S.M., Thompson, D.L.,

Wilson, L.E. and Fridkin, S.K. (2014). Multistate Point-Prevalence Survey of Health Care–Associated Infections. *New England Journal of Medicine*, [online] 370(13), pp.1198–1208.

Martin, R.M. and Bachman, M.A. (2018). Colonization, Infection, and the Accessory Genome of Klebsiella pneumoniae. *Frontiers in Cellular and Infection Microbiology*, 8.

Martinez, J.L. (2014). General principles of antibiotic resistance in bacteria. *Drug Discovery Today: Technologies*, [online] 11, pp.33–39.

Mazzariol, A., Zuliani, J., Cornaglia, G., Rossolini, G.M. and Fontana, R. (2002). AcrAB Efflux System: Expression and Contribution to Fluoroquinolone Resistance in *Klebsiella* spp. *Antimicrobial Agents and Chemotherapy*, 46(12), pp.3984–3986.

Meatherall, B.L., Gregson, D., Ross, T., Pitout, J.D.D., and Laupland, K.B. (2009). Incidence, risk factors, and outcomes of Klebsiella pneumoniae bacteremia. *The American Journal of Medicine*, 122(9), pp.866–73

Munoz-Price, L.S., Hayden, M.K., Lolans, K., Won, S., Calvert, K., Lin, M., Sterner, A. and Weinstein, R.A. (2010). Successful Control of an Outbreak of Klebsiella pneumoniae Carbapenemase—Producing K. pneumoniae at a Long-Term Acute Care Hospital. *Infection Control & Hospital Epidemiology*, 31(4), pp.341–347.

Munoz-Price, L.S., Poirel, L., Bonomo, R.A., Schwaber, M.J., Daikos, G.L., Cormican, M., Cornaglia, G., Garau, J., Gniadkowski, M., Hayden, M.K., Kumarasamy, K., Livermore, D.M., Maya, J.J., Nordmann, P., Patel, J.B., Paterson, D.L., Pitout, J., Villegas, M.V., Wang, H. and Woodford, N. (2013). Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. *The Lancet. Infectious diseases*, [online] 13(9), pp.785–796.

Murphy, C.N., Mortensen, M.S., Krogfelt, K.A. and Clegg, S. (2013). Role of Klebsiella pneumoniae Type 1 and Type 3 Fimbriae in Colonizing Silicone Tubes Implanted into the Bladders of Mice as a Model of Catheter-Associated Urinary Tract Infections. *Infection and Immunity*, 81(8), pp.3009–3017.

Murray, C.J.L., Abbafati, C., Abbas, K.M., Abbasi, M., Abbasi-Kangevari, M., Abd-Allah, F., Abdollahi, M., Abedi, P., Abedi, A., Abolhassani, H., Aboyans, V., Abreu, L.G., Abrigo, M.R.M., Abu-Gharbieh, E., Haimed, A.K.A., Abushouk, A.I., Acebedo, A., Ackerman, I.N., Adabi, M. and Adamu, A.A. (2020). Five insights from the Global Burden of Disease Study 2019. *The Lancet*, [online] 396(10258), pp.1135–1159.

Nm, Z., Sr, J., Pns, N., Hospital, S., Karachi, P. and Zehra, N. (2016). *Emergence of Multidrug Resistant Gram-negative Pathogens in an Intensive Care Unit of a Tertiary Care Hospital*.

Nobrega, D.B., Calarga, A.P., Nascimento, L.C., Chande Vasconcelos, C.G., de Lima, E.M., Langoni, H. and Brocchi, M. (2021). Molecular characterization of antimicrobial resistance in Klebsiella pneumoniae isolated from Brazilian dairy herds. *Journal of Dairy Science*, 104(6), pp.7210–7224.

Paczosa, M.K. and Mecsas, J. (2016a). Klebsiella pneumoniae: Going on the Offense with a Strong Defense. *Microbiology and Molecular Biology Reviews*, [online] 80(3), pp.629–661.

Paczosa, M.K. and Mecsas, J. (2016b). Klebsiella pneumoniae: Going on the Offense with a Strong Defense. *Microbiology and Molecular Biology Reviews*, [online] 80(3), pp.629–661.

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J.A. and Albertí, S. (2010a). Klebsiella pneumoniae AcrAB Efflux Pump Contributes to Antimicrobial Resistance and Virulence. *Antimicrobial Agents and Chemotherapy*, 54(1), pp.177–183.

Padilla, E., Llobet, E., Doménech-Sánchez, A., Martínez-Martínez, L., Bengoechea, J.A. and Albertí, S. (2010b). Klebsiella pneumoniae AcrAB Efflux Pump Contributes to Antimicrobial Resistance and Virulence. *Antimicrobial Agents and Chemotherapy*, [online] 54(1), pp.177–183.

Papp-Wallace, K.M., Endimiani, A., Taracila, M.A. and Bonomo, R.A. (2011). Carbapenems: Past, Present, and Future. *Antimicrobial Agents and Chemotherapy*, 55(11), pp.4943–4960.

Piperaki, E.-T., Syrogiannopoulos, G.A., Tzouvelekis, L.S. and Daikos, G.L. (2017). Klebsiella pneumoniae. *The Pediatric Infectious Disease Journal*, 36(10), pp.1002–1005.

Podschun, R. and Ullmann, U. (1998). Klebsiella spp. as Nosocomial Pathogens: Epidemiology, Taxonomy, Typing Methods, and Pathogenicity Factors. *Clinical Microbiology Reviews*, 11(4), pp.589–603.

Prokesch, B.C., TeKippe, M., Kim, J., Raj, P., TeKippe, E.M. and Greenberg, D.E. (2016). Primary osteomyelitis is caused by hypervirulent Klebsiella pneumoniae. *The Lancet Infectious Diseases*, 16(9), pp. e190–e195.

Ramsamy, Y., Essack, S.Y., Sartorius, B., Patel, M. and Mlisana, K.P. (2018). Antibiotic resistance trends of ESKAPE pathogens in Kwazulu-Natal, South Africa: A five-year retrospective analysis. *African Journal of Laboratory Medicine*, 7(2).

Ranjbar, R., Kelishadrokhi, A.F. and Chehelgerdi, M. (2019). Molecular characterization, serotypes, and phenotypic and genotypic evaluation of antibiotic resistance of the Klebsiella pneumoniae strains isolated from different types of hospital-acquired infections. *Infection and Drug Resistance*, Volume 12, pp.603–611.

Regué, M., Izquierdo, L., Fresno, S., Núria Piqué, Maria Michela Corsaro, Naldi, T., Cristina De Castro, Waidelich, D., Merino, S. and Tomás, J.M. (2005). A Second Outer-Core Region in *Klebsiella pneumoniae* Lipopolysaccharide. *Journal of Bacteriology*, 187(12), pp.4198–4206.

Riond, J.L. and Riviere, J.E. (1988). Pharmacology and toxicology of doxycycline. *Veterinary and human toxicology*, [online] 30(5), pp.431–443.

Ristuccia, P.A. and Cunha, B.A. (1984). Klebsiella. *Infection Control & Hospital Epidemiology*, [online] 5(7), pp.343–347.

Russo, T.A. and Marr, C.M. (2019). Hypervirulent Klebsiella pneumoniae. *Clinical Microbiology Reviews*, [online] 32(3), pp. e00001-19.

S, S., G, I., S, A., S, M., R, M., A, M. and Sn, C. (2020). *Susceptibility Pattern of Doxycycline in Comparison to Azithromycin, Cefuroxime, and Amoxicillin Against Common Isolates: A Retrospective Study Based on Diagnostic Laboratory Data*. [online] The Journal of the Association of Physicians of India.

Salam, M.A., Al-Amin, M.Y., Salam, M.T., Pawar, J.S., Akhter, N., Rabaan, A.A. and Alqumber, M.A.A. (2023). Antimicrobial Resistance: A Growing Serious Threat for Global Public Health. *Healthcare*, [online] 11(13), p.1946.

Sauer, K., Stoodley, P., Goeres, D.M., Hall-Stoodley, L., Burmølle, M., Stewart, P.S. and Bjarnsholt, T. (2022). The biofilm life cycle: expanding the conceptual model of biofilm formation. *Nature Reviews Microbiology*, [online] 20(10), pp.608–620.

Shon, A.S., Bajwa, R.P.S. and Russo, T.A. (2013). Hypervirulent (hypermucoviscous)Klebsiella pneumoniae. *Virulence*, 4(2), pp.107–118.

Singla, S., Harjai, K. and Chhibber, S. (2012). Susceptibility of different phases of biofilm of Klebsiella pneumoniae to three different antibiotics. *The Journal of Antibiotics*, 66(2), pp.61–66.

Snitkin, E.S., Zelazny, A.M., Thomas, P.J., Stock, F., Henderson, D.K., Palmore, T.N. and Segre, J.A. (2012). Tracking a Hospital Outbreak of Carbapenem-Resistant Klebsiella pneumoniae with Whole-Genome Sequencing. *Science Translational Medicine*, [online] 4(148), pp.148ra116–148ra116.

Srinivasan, V.B., Singh, B.B., Priyadarshi, N., Chauhan, N.K. and Rajamohan, G. (2014). Role of Novel Multidrug Efflux Pump Involved in Drug Resistance in Klebsiella pneumoniae. *PLoS ONE*, 9(5), p.e96288.

Sun, S., Gao, H., Liu, Y., Jin, L., Wang, R., Wang, X., Wang, Q., Yin, Y., Zhang, Y. and Wang, H. (2020). Co-existence of a novel plasmid-mediated efflux pump with colistin resistance gene mcr in one plasmid confers transferable multidrug resistance in Klebsiella pneumoniae. *Emerging Microbes & Infections*, pp.1–41.

Swaminathan, S., Immanuel, G., and Arora, S. (2020). *Journal of the Association of Physicians of India - JAPI.* [online] japi.org.

Swathi, C.H., Chikala, R., Ratnakar, K.S. and Sritharan, V. (2016). A structural, epidemiological & genetic overview of Klebsiella pneumoniae carbapenemases (KPCs). *The Indian Journal of Medical Research*, [online] 144(1), pp.21–31.

Tang, H.-J., Lai, C.-C., Chen, C.-C. and Weng, T.-C. (2019). Colistin-sparing regimens against Klebsiella pneumoniae carbapenemase-producing K. pneumoniae isolates Combination of tigecycline or doxycycline and gentamicin or amikacin. *Journal of Microbiology, Immunology and Infection*, [online] 52(2), pp.273–281.

Tian, L., Tan, R., Chen, Y., Sun, J., Liu, J., Qu, H. and Wang, X. (2016). Epidemiology of Klebsiella pneumoniae bloodstream infections in a teaching hospital: factors related to the carbapenem resistance and patient mortality. *Antimicrobial Resistance & Infection*

Control, 5(1).

Tzouvelekis, L.S., Markogiannakis, A., Psichogiou, M., Tassios, P.T. and Daikos, G.L. (2012). Carbapenemases in Klebsiella pneumoniae and Other Enterobacteriaceae: An Evolving Crisis of Global Dimensions. *Clinical Microbiology Reviews*, 25(4), pp.682–707.

Ventola, C.L. (2015). The Antibiotic Resistance Crisis: Part 1: Causes and Threats. *P & T: A peer-reviewed Journal for Formulary Management*, [online] 40(4), pp.277–83.

Vuotto, C., Longo, F., Balice, M.P., Donelli, G. and Varaldo, P.E. (2014). Antibiotic Resistance Related to Biofilm Formation in Klebsiella pneumoniae. *Pathogens*, 3(3), pp.743–758.

Wang, G., Zhao, G., Chao, X., Xie, L. and Wang, H. (2020). The Characteristic of Virulence, Biofilm, and Antibiotic Resistance of Klebsiella pneumoniae. *International Journal of Environmental Research and Public Health*, 17(17), p.6278.

White, C.R., Jodlowski, T.Z., Atkins, D.T. and Holland, N.G. (2017). Successful Doxycycline Therapy in a Patient with Escherichia coli and Multidrug-Resistant Klebsiella pneumoniae Urinary Tract Infection. *Journal of Pharmacy Practice*, [online] 30(4), pp.464–467.

Wyres, K.L., Lam, M.M.C. and Holt, K.E. (2020). Population genomics of Klebsiella pneumoniae. *Nature Reviews Microbiology*, 18(6), pp.344–359.

Yigit, H., Queenan, A.M., Anderson, G.J., Domenech-Sanchez, A., Biddle, J.W., Steward, C.D., Alberti, S., Bush, K. and Tenover, F.C. (2001). Novel Carbapenem-Hydrolyzing -Lactamase, KPC-1, from a Carbapenem-Resistant Strain of Klebsiella pneumoniae. *Antimicrobial Agents and Chemotherapy*, 45(4), pp.1151–1161.

Yu, W.-L., Ko, W.-C., Cheng, K.-C., Lee, H.-C., Ke, D.-S., Lee, C.-C., Fung, C.-P. and Chuang, Y.-C. (2006). Association between rmpA and magA genes and clinical syndromes caused by Klebsiella pneumoniae in Taiwan. *Clinical Infectious Diseases: An Official Publication of the Infectious Diseases Society of America*, [online] 42(10), pp.1351–1358.

Zahller, J. and Stewart, P.S. (2002). Transmission Electron Microscopic Study of Antibiotic Action on Klebsiella pneumoniae Biofilm. *Antimicrobial Agents and Chemotherapy*, 46(8), pp.2679–2683.

Zhang, S., Yang, G., Ye, Q., Wu, Q., Zhang, J., and Huang, Y. (2018). Phenotypic and Genotypic Characterization of Klebsiella pneumoniae Isolated from Retail Foods in China. *Frontiers in Microbiology*, 9.

Zhang, Y., Wang, X., Wang, S., Sun, S., Li, H., Chen, H., Wang, Q. and Wang, H. (2021). Emergence of Colistin Resistance in Carbapenem-Resistant Hypervirulent Klebsiella pneumoniae Under the Pressure of Tigecycline. *Frontiers in Microbiology*, 12.

Zhen, X., Lundborg, C.S., Sun, X., Hu, X. and Dong, H. (2019). Economic burden of antibiotic resistance in ESKAPE organisms: a systematic review. *Antimicrobial Resistance & Infection Control*, 8(1).

Zhu, J., Wang, T., Chen, L. and Du, H. (2021). Virulence Factors in Hypervirulent Klebsiella pneumoniae. *Frontiers in Microbiology*, 12.

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