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SAKARYA UNIVERSITY
INSTITUTE OF HEALTH SCIENCE

DETECTION OF BIOFILM FORMATION IN CARBAPENAMASE RESISTANCE *KLEBSIELLA* SPP.

M.Sc. THESIS

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Institute Department: Medical Microbiology

Thesis Supervisor: Assoc. Prof. Dr. Tayfur DEMİRAY

JUNE-2021

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"This thesis was accepted unanimously / by all of votes by the jury below on .... / .... / 2021."

JURY MEMBER	OPINION	SIGNATURE

DECLARATION

This study was conducted by T.C. Sakarya University, Institute of Health Sciences. It has been approved by Ethical Committee of Sakarya Medical Faculty on the date of 30.3.2021. This thesis is my own work, from planning to writing, I do not have any unethical behavior at any stage, and all the information in the thesis is academic, and all the information and comments that I have obtained meets the ethical rules that were obtained through the sources, and I cite the source and list these sources in the sources list, and the thesis work and writing. I declare that during my writing, there is no Patent infringement and copyright rights.

…../…/….

Hevi Seerwan GHAFOUR

APPRECIATION

To begin with, I'd like to express my gratitude to my supervisor, Dr. Tayfur DEMİRAY, whose guidance was helpful in developing the research objectives and technique during my study. I want to thank him for his informative advice, which encouraged me to focus on my thinking and elevate my work, as well as for all of the possibilities he provided to advance my study and to finalize my thesis.

I'd like to express my appreciation to Prof. Dr. Mustafa ALTINDIŞ for his patient assistance. Also, I'd want to show my thankfulness to the Clinical Microbiology Laboratory's personal assistants of Sakarya Training and Research Hospital, as well as the clinic's employees, for their valuable contributions to this study.

Finally, without the help of my family and friends, I would not have been able to finish this dissertation. So, I want to express my gratitude to my parents for their great advice and sympathy. You were always been there for me. Thank you.

Best regards

APPROVAL

This study proposal has been approved by Faculty of Medicine Ethics Committee of the Sakarya University on 30.03.2021. The strains recovered as part of standard research, and epidemiologic studies evidence was collected retrospectively from clinical reports. Also anonymized studies were conducted.

CONTENT

ABBREVIATIONS AND ICONS

CDC:	Center for Disease Control and Prevention
ESBL:	Extended-spectrum Beta-lactamase
QS:	Quorum Sensing
MR/K:	Mannose-Resistance *Klebsiella*
cKP:	Classical *Klebsiella pneumonia*
hv-Kp:	Hypervirulent *Klebsiella pneumonia*
MDR:	Multi-Drug Resistance
AMR:	Antimicrobial Resistance
UTIs:	Urinary Tract Infections
CAUTIs:	Catheter Associated Urinary Tract Infections
KPC:	*Klebsiella pneumonia* carbapenamase
VIM:	Verona Integron-encoded Metallo beta-lactamase
NDM-1:	New Delhi Metallo beta-lactamase
IMP:	Imipenemase Metallo beta-lactamase
CAPs:	Community Acquired Pneumonias
HAPs:	Hospital Acquired Pneumonias
WHO:	World Health Organization
CR-Kp:	Carbapenem Resistance *Klebsiella pneumonia*
LPS:	Lipopolysaccharide
CA:	Community Acquired
ICU:	Intenisive Care Unit
MBLs:	Metallo Beta-Lactamases
CRE:	Carbapenem Resistant *Enterobacteriacea*
PBPs:	Penicillin Binding Proteins
AmpC:	Ampicillin-Resistance Gene group C
OD:	Optical Density
TEM:	Transmission Electron Microscope

MIC:	Minimum Inhibitory Concentration
OXA-48:	Oxacillinase-48
SAM:	Ampicillin/ Sulbactam
TZP:	Piperacillin/tazobactam
CXM:	Cefuroxime
FOX:	Cefoxitin
CAZ:	Ceftazidime
CRO:	Ceftriaxone
FEP:	Cefepime
ERP:	Ertapenem
IMP:	Imipenem
MEM:	Meropenem
AMK:	Amikacin
GEN:	Gentamicin
CIP:	Ciprofloxacin
TGC:	Tigecycline
CST:	Colistin
EMB:	Eosin Methylene Blue

FIGURES

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ÖZET

Karbapenama Direnci Klebsıella Spp'de Biyofilm Oluşumunun Tespiti.

GİRİŞ VE AMAÇ: Karbapenem dirençli *Klebsiella pneumoniae* (CR-Kp), direnç genleri ve biyofilmler gibi virülans faktörlerini barındırması nedeniyle önemli halk sağlığı sorunu haline gelmiştir. Bu çalışmanın amacı CR-Kp suşları arasında biyofilm oluşumunu tespit etmek ve *K. pneumoniae suşlarında* karbapenem direnç genleri ile biyofilm oluşumu arasındaki korelasyonu belirlemektir.

GEREÇ VE YÖNTEM: 58 CR-Kp bu çalışmada incelendi. İzolatların identifikasyonu ve antimikrobiyal duyarlılık testleri Maldi-TOF MS ve VITEK 2 ® sistemleri; karbapenem direnç genleri tesbiti ise Gene X-pert sistemi kullanılarak gerçekleştirildi. Biyofilm oluşumunu tespit etmek için mikro plaka yöntemi kullanıldı. İzolatların kaynağı, direnç genleri ile biyofilm oluşum oranı istatistiksel olarak araştırıldı.

BULGULAR: CR-Kp'lerin %98,2'inde biyofilm saptandı. Enfeksiyon bölgesine göre biyofilm oluşumu: %60,30 rektal sürüntü, %27,50 kan, %1,72 balgam, %5,17 yara, %3,44 İdrar, %1,72 kateter şeklinde idi. Biyofilm saptanan CR-Kp'lerde direnç genlerinin oranı %42,2 NDM-1, %28.80 OXA-48, %13.30 KPC, %15.5 NDM-1 +OXA 48 olarak saptandı

TARTIŞMA VE SONUÇ: CR-Kp izolatlarının neredeyse tamamında biyofilm oluşumu saptandı. Rektal sürüntü örnekleri ve kan enfeksiyonundan izole edilen suşların, NDM-1, OXA-48 ve KPC direnç geni içerenlerinde orta ve yüksek düzey biyofilm saptandı. Bu sonuçlar, yoğun bakım hastalarının ve kan akımı enfeksiyonu olan hastaların yüksek ölüm oranı ile daha yüksek risk altında olduğunu göstermektedir.

ANAHTAR KELİMELER: *Klebsiella pneumonia*, Biyofilm oluşumu, Direnç Genleri, Antibiyotik Direnci.

ABSTRACT

INTRODUCTION AND AIM: Carbapenem resistant *Klebsiella pneumoniae* (CR-Kp) become major public health concern due to harboring resistance genes and virulence factors like biofilms. The aim of this study was to detect biofilm formation among (CR-Kp) strains and to determine correlation between carbapenem resistance genes and biofilm formation in *K. pneumoniae.*

MATERIALS AND METHOD: 58 (CR-Kp) involved in this study, identification of the isolates and antibiotic susceptibility testing was performed by using Maldi-TOF MS and VITEK 2 ® system and resistant gene detection held by Gene X-pert system. Microplate method used to detect biofilm formation. The ratio of biofilm formation related to the source of infection and harboring resistance genes were investigated statistically.

RESULTS: 98.2% of (CR-Kp)s were positive biofilm producers. 46.5% isolates were moderately biofilm-producer, 37.9% fully established biofilms, 13.7% weak biofilms, 1.72% were non-biofilm producers. Biofilm formation according to infection site: 60.30% surveillance rectal swabs, 27.50% blood, 1.72% sputum, 5.17% wound, 3.44% Urine, 1.72% catheter. KPCs harboring resistance Gene biofilm formation ratio: 42.2% NDM-1, 28.80% OXA 48, 13.30% KPC, 15.5% NDM-1 +OXA 48.

DISCUSSION AND CONCLUSION: Almost all CR-Kp isolates are biofilm producers. Surveillance rectal swabs and blood infection source strains, resistance genes NDM-1, OXA 48 KPCs had higher ability forming moderate to fully established biofilms These results indicates that ICU patients and patients who have blood stream infections are at higher risk with high mortality rate.

KEY WORDS: Klebsiella pneumonia, Biofilm formation, Resistance Genes, Antibiotic Resistance.

1. INTRODUCTION AND AIM

The genus *Klebsiella* is included in the *Enterobacteriaceae* family which can live in the natural environments like soil and water as well as on the surfaces of mucosal sites of animals. In healthy individuals, the gastrointestinal tract, skin, respiratory system, and genitourinary tract are all typical colonization sites. *Klebsiella pneumoniae* has been a major cause of the infections acquired in hospitals, particularly amongst neonatal intensive-care unit patients, where fatality ratio reaches 70%. These Infections caused by multidrug-resistant (MDR) bacteria which have raised dramatically since the previous two-decade span. (Gupta et al., 2003)

K. pneumoniae is a widespread infective agent in indwelling urinary catheters patients and is a primary cause of enterobacterial nosocomial acquired UTIs (Urinary Tract Infections). CAUTIs (Catheter-associated Urinary-Tract Infections) is capable of introducing organisms invading the bloodstream resulting in severe infection, and significant morbidity and fatality in these at-risk individuals, also pneumonia, urinary tract infection, septicemia, and surgical wound infection are among most prevalent of these infections. Furthermore, UTIs are becoming more common among people in long-term care facilities. As a result, an epidemiology of *Klebsiella* CAUTIs, UTIs suggests that elements which cause a decline in the host immune system's efficacy such as the implantation of a catheter or other implantable tool increases one's vulnerability to infection by these bacteria significantly. Moreover, *K. pneumoni*ae capability of biofilm formation has been linked to the nosocomial *K. pneumoniae* infections pathogenesis, notably on medical equipments. (Clegg & Murphy, 2016; Wilksch et al., 2011)

Biofilms are bacterial aggregation that are securely locked in extracellular matrixes consist of nucleic acids, enzymes, proteins, polysaccharides, allowing the bacteria for irreversible anchoring to any surfaces. Antibiotic resistance is conferred by this matrix by the mechanism which is the production of resistant genes that are chromosomally coded, antibiotic limitation and growth rate decrease that all contribute to counteracting host immunity.

KPC (*Klebsiella pneumoniae* carbapenemase) had expanded amongst gram negative bacteria, particularly since its discovery in the United States in 1996 which had been resulting epidemics in Europe, Asia, South America. KPC enzymes can hydrolyze numerous antimicrobial drugs from the beta-lactam antibiotic family beside carbapenems, also, severely limiting therapy choice is a factor in KPC's high mortality ratio. According to researches conducted in Greece and Italy, mortality rates are 34 percent, 41.6 percent, respectively. Tigecycline, polymyxins, aminoglycosides, and fosfomycin were the antibiotics of choice for the infections caused by these pathogens. However, because of their pharmacokinetic features and toxicity, their usage has been restricted, beside this the major source of concern is the rise of KPC producing bacteria which are resistant to tigecycline, polymyxin (Campos et al., 2016)

The relationship among resistance and virulence in *K. pneumoniae* is a complicated topic since a comprehensive analysis of its population structure is currently missing beside the rise and development of carbapenem resistant burden amongst *K. pneumonia* biofilm former which is a main public health care priority nowadays, and because of the high resistance to almost all available antibiotics, we conducted this research the aim of this study was to detect biofilm formation among carbapenem resistance *K. pneumonia* isolates also to determine the correlation between *K. pneumonia* harboring carbapenem resistance Gene isolates and source of infection impact on biofilm formation ratio which can directly affect the antibiotic treatments.

2. GENERAL INFORMATION

2.1. *KLEBSIELLA PNEUMONIAE*

Klebsiella pneumoniae belongs *Enterobacteriaceae* family which includes 6 species and subspecies and is the most common human pathogen with strains categorized as classical or hypervirulent (HV). (*Bacterial Pathogens and Their Virulence Factors | Douglas I. Johnson (Auth.) | Download*, n.d.) Carl Friedlander isolated K. *pneumoniae* in 1882 for the first time in the lungs of patients who had died after pneumonia. according to him it was the most serious cause of bacterial pneumonia while this theory was quickly refuted in favor of pneumococcus but major hospitals in the United States reported 10 to 50 cases of pneumonia due to *Klebsiella pneumonia* per year from the 1930s to the 1960s. (Ko et al., 2002) The encapsulated bacterium was originally called Friedlander's bacillus after the founder's name, but was renamed *Klebsiella* in 1886. Other than the human gastrointestinal tract it colonized in nasopharynx and skin as well which was later identified as a saprophyte microorganism able to cause biliary, urinary tract infections, osteomyelitis, with bacteremia. (Vuotto et al., 2014)

K. pneumoniae is a significant nosocomial pathogen that lives on the surface of water, soil, also plants and a commensal resident of the mammalian nasopharynx and gastrointestinal tract. The gastrointestinal tract acts like a reservoir for infections which is often the origin of latent infection. This Gram negative, rod in shape, facultative anaerobic, lactose fermenting bacilli which is nonmotile and non-flagellated with a prominent capsule causing urinary tract infections, pneumonia, bacteremia, with wound infection. *Klebsiella* species are responsible for a small percentage of bacterial pneumonias (1%). In debilitated patients, *K. pneumonia* can cause severe lung hemorrhagic necrotizing consolidation, as well as focal lesions, and is found in feces and respiratory tract of around 5 percent of healthy people, which are also amongst top ten bacterial pathogen accountable for hospital acquired infections. Fever, cough (especially cough generating sputum), infected lymph node, Hemoptysis, dyspnea, malaise, and pleuritic pain on X-ray with consolidations are all signs and symptoms of bacterial pneumonia also cause general fatigue, reduced appetite, altering mental state,

incontinence or decompensation caused by underlying diseases particularly in elderly patients with multiple comorbidities. These infectious diseases are undoubtedly of great concern due to their occurrence following the widely usage of antibiotics especially in nosocomial settings. (Alcántar-Curiel et al., 2013; *Clinical Management of Bacterial Pneumonia | Antoni Torres, Catia Cillóniz (Auth.) | Download*, n.d.; *Jawetz, Melnick, & Adelberg's Medical Microbiology, Twenty-Fifth Edition (LANGE Basic Science) | Geo. Brooks, Karen C. Carroll, Janet Butel, Stephen Morse, Timothy Mietzner | Download*, n.d.; El Fertas-Aissani et al., 2013)

K. pneumoniae pneumonias are categorized into two types: CAPs (Community Acquired Pneumonias), HAPs (Hospital Acquired Pneumonias) HAPs of *K. pneumoniae* are much more common than CAPs of *K. pneumoniae.* Hospital Acquired Pneumonias is characterized as pneumonia which appears a minimum of 48 hours after being admitted to the hospital in people who had no signs prior to admission with pneumonia, bacterial HAPs are among most common forms of nosocomial infections also they are main reason of death amongst nosocomial infection. *K. pneumoniae* is the main cause of 11.8 percent of HAP. (Paczosa & Mecsas, 2016) *K. pneumoniae* was thought to be a major cause of community-acquired pneumonia in trials from the 1920s to the 1960s. (Ko et al., 2002) Community-acquired pneumonia (CAP) is characterized as signs, symptoms, and radiographic evidence of pneumonia that occur within 48 hours of hospital admission in patients that come from the community. Despite the widespread use of this dualistic classification for pneumonia, evidence from a number of infectious mechanisms suggest that these categorizations may have significant flaws. (*Nosocomial Pneumonia: Strategies for Management | Jordi Rello (Ed.) | Download*, n.d.)

Because of *K. pneumoniae* potential to induce nosocomial infections, it become one of the most clinically active and commonly isolated bacterial strains. Virulent *K. pneumoniae* serotypes, alternatively can induce neonatal sepsis in hospital intensive care unit patients, immunocompromised patients, or in general within healthy individuals resulting in life-threatening infections in all cases. Various *K. pneumoniae* clinical isolates are highly resistant to widely utilized antibiotics causing increased

mortality rate. The tendency of microorganism to generate biofilm on medical instruments, such as catheters, and biotic surfaces such as wounds, is one of the main factors leading to antibiotic treatment resistance. (Caneiras, Lito, Melo-Cristino, et al., 2019; Chen et al., 2014) It is now well established that most bacteria grow as organized biofilm communities in order to colonize surfaces in natural, industrial, and medical environments.(Di Martino et al., 2003) Biofilm formation, in particular, is a common reason of implant failure and shortens the lifespan of many implanting medical instruments. Bacteria are protected from opsonization and phagocytosis by extracellular polymeric compounds, after they have established themselves in the biofilm. bacteria with biofilm susceptibility to antibiotics are lower than planktonic bacteria according to in vitro studies. (Anderl et al., 2000)

2.2. VIRULENCE FACTORS OF *K. PNEUMONIAE*

The “permeability” of *K. pneumoniae* bacterium of mobile genetic molecules is a critical aspect in their spread not only in terms of antibiotic resistance, but also in developing more resistance phenotype due to Genes which may give a survival advantage to the microorganism. However, the relationship among resistance and virulence in *K. pneumoniae* is a complicated topic since a comprehensive analysis of its population structure is currently missing. This makes detecting the development of new clones challenging which might be beneficial way for developing epidemiologic surveillance programs and avoiding epidemics, Specifically bacteria that have developed resistance to carbapenem (carbapenem resistant *K. pneumoniae*, CR-Kp). (Fasciana et al., 2019)

Some virulence factors including capsule polysaccharide, lipopolysaccharide, type 1 and type 3 fimbriae, outer membrane proteins, and iron absorption determinants, nitrogen source utilization are all found in *K. pneumoniae*, which exploited these virulence factors to survive also avoid detection by the immune system during infections as well as for the formation of biofilm. (Nirwati et al., 2019) which all play a vital role in dissemination of the infections This knowledge can help to support attempts to manage the bacterium's risk to human health by recognizing or

understanding the development of clinically significant clones within this extremely genetically varied species. Although none has been known about the virulence Genes carried by *K. pneumoniae* expressing beta-lactamases and less is understood about the virulence capability of KPC producers. (Caneiras, Lito, Mayoralas-Alises, et al., 2019)

The thick polysaccharide capsule and mucus that surrounds the cell envelope in *K. pneumoniae* protects the cell from phagocytosis and as a result are substantially essential for the cell's persistence and ability to cause infection. The existence of a capsule is considered to be important for *K. pneumoniae* virulence; There have been 77 distinct capsular forms examined so far, and *Klebsiella* species without a capsule are often less virulent. however, the size of the capsule and the rate of capsular polysaccharide (CPS) synthesis have been exhibiting to always have a significant impact on virulence, demonstrating the presence of 2 pathogenic mechanisms: Protection against phagocytosis of the pathogens and immediate alteration of immune system response. (Highsmith & Jarvis, 1985) LPS (Lipopolysaccharide) is made up of lipids and a polysaccharide, inner with outer core, and O-polysaccharide antigens, which all are required for the bacterium to survive complement-mediated lysis. (Ashurst & Dawson, 2018; Cortés et al., 2002)

The capacity of bacteria to attach to host tissue surface is an important stage in the establishment of infections. Most clinical isolates of *K. pneumoniae* express 2 kinds of fimbrial adhesins, type one and type three fimbriae. The majority of enterobacterial species have well-defined Type 1 fimbriae which facilitate adhesion to mannose-containing structures on host cells or in the extracellular matrix which are an important virulence factor in urinary tract infection caused by *K. pneumoniae*. Type 3 fimbriae are non-channeled, narrow (4 to 5 nm in diameter). The MR/K (mannose resistant *Klebsiella* like) hemagglutinins or ‘‘Mrk proteins" are responsible for the production of *K. pneumoniae* biofilms which differentiated by the capability to agglutinate tannic acid treated erythrocytes in a form of mannose resistant which is also known as mannose resistant *Klebsiella* like hemagglutination, Mrk proteins are encoded by the Genes mrkABCDF which are the part of the mrkABCDF operon. Mrk proteins adhere to surfaces in the form of type 3 fimbriae. Lung surfactant exposure causes type 3

fimbria mediated biofilm development in *K. pneumoniae* which have been proven to have a key role in the development of K. pneumoniae biofilms. (Struve et al., 2009; Wilksch et al., 2011) Type 3 fimbriae (Mrk fimbriae) of *K. pneumoniae* have been widely investigated and contribute in cell adherence to a variety of biotic, abiotic substrates, involving type IV and type V collagen, silicone, hard plastics. Though type three fimbriae aren't involved directly in *K. pneumoniae* virulence many studies have shown that they are necessary for colonization along with persistence in catheter associated urinary tract infection (CAUTI). (Willsey et al., 2018)

Porins, which are proteins locating the outer membrane of gram negative bacteria, are linked in resistance to beta-lactam antibiotics. (Gutmann et al., 1985) Iron is required for both in-vitro, in-vivo bacterial development. Iron chelation is thought to promote the development of these extracellular infections within tissues and during subsequent systemic distribution. various gut pathogens, involving *K. pneumoniae* may manufacture cytoplasmic urease which hydrolyzes urea to ammonia and carbon dioxide as a nitrogen source for development. (Li et al., 2014; Nassif & Sansonetti, 1986)

2.3. BIOFILM AND BIOFILM FORMATION IN *KLEBSIELLA* SPP.

Microorganisms typically reside in the shelter of increasingly moist biofilms in nature which is most widespread and successful forms of life on Earth and produces a favorable habitat for cells to stick together and onto many types of biotic and abiotic substrates because microorganisms inside this shelter make a cement like structure which is functioning as "biological superglue" to stick or trap onto various substrates. With biofilms being associated in 65 percent of bacterial infections allowing cells to survive and increasing antibiotic resistance which are a serious challenge in healthcare, due to their superior resistance to macrophages and antibiotics. biofilm infections on implants or indwelling devices for example, are hard to destroy that results in serious clinical consequences many of which are fatal. Bacterial biofilm formation on the inner, exterior substrates of the catheter has been recognized as the most prominent cause of CAUTIs in addition, according to *K. pneumoniae* (CAUTIs) are most

prevalent (HAIs) resulting in higher patient morbidity. It is a crucial concern in the health establishment because it forms on medical indwelling, in human tissue, and is implicated in a variety of dangerous chronic infections. In general, Biofilm is a population of bacteria attach to a substrate and growing in a self-producing matrix of extracellular polymeric molecules. (Abebe, 2020; Desai et al., 2019) *K. pneumoniae* and the biofilm-forming phenomena were initially described by LeChevallier et al. in 1988. (Nirwati et al., 2019)

Biofilms are complex of extracellular polymeric substance that consist of assemblage of cells attached to one another or to a surface, instill in a self-synthesized matrix. DNA, proteins and polysaccharide are essential components of this complex extracellular polymeric matter. *K. pneumoniae* biofilms that formed inside indwelling instruments and catheters are more considerable clinically, as well as it engages with respiratory, urinary and gastrointestinal colonization and leading to serious infections in immunocompromised patients. *K. pneumoniae* biofilm outgrowth on solid surfaces starts with attachment of cells to develop microcolonies then maturation and lastly spreading of vegetating cells. (Piperaki et al., 2017) Bacteria gets benefit from biofilm in several ways: including improved interspecific metabolic cooperation, quorum sensing, better tolerance to host immunological responses, demanding greater antibiotic doses, and enhanced capability for bacterial conjugation. By functioning as a barrier, biofilm protects bacteria from hard environmental conditions such as excessive temperature, pH, high salinity and pressure, nutrition deficiency, antibiotics, and so on when compared to planktonic bacteria. Antibiotic resistance is mostly determined by structural barriers and persistent cells inside biofilms. Microbial cells in a biofilm are near enough so they interact with one another via chemicals, allowing cells to communicate and respond to any ecological, environmental, or host related signals. Biofilm development is usually considered as a cooperative business where strains with species coordinate together for a similar goal, according to Oliveira et al. Cell to cell contact is required for this cooperative action. Quorum sensing is a cell to cell communication mechanism within a microbial population. (Abebe, 2020; Surgers et al., 2019)

The QS system is a process by which bacteria adjust Gene expression profiles in response to the size of their microbial community resulting in creation of various biofilms. Quorum sensing is a broad term for the mechanism which bacteria generate and detect signal elements by it, allowing them to coordinate their activity in a density dependent mode. In addition to communication these strong relationships between microbial populations allow them to share Genetic materials, also the frequency of gene transfer is even higher than in their natural state. as a consequence, horizontal microbial gene transfer and the production of biofilms are interlinked. Exopolysaccharides play a vital role in biofilm development and have been linked to QS in several studies. Substrate situations, chemical with physical growth stimuli, cellular architectures, and any other difficulties can all have an impact on biofilm formation. Its development is determined by the interplay of these and other elements which may be classified into number of stages ranging from the initial adherence of bacteria to the substrate up to the production of grown biofilm with a distinctive 3-dimensional design. At each phase, many bacterial activities are necessary including adhesion, stress response, transport, motility, metabolic pathway activation and extracellular matrix formation. (Abebe, 2020; De Araujo et al., 2010)

The *K. pneumoniae* capsular polysaccharide is required for late biofilm maturation and is involved in substrate adherence, spacing, and ordering of bacteria in the early phase of biofilm formation. Though the initial bacterial attachment to a substrate is an important stage in biofilm development, another significant aspect in the establishment of a biofilm is the dissemination of microorganisms on the surface. Biofilm development is frequently seen as a serious issue due to sessile bacteria's capacity to endure external stress better than planktonic bacteria and hence to survive. (Dos et al., n.d.) Antibiotic resistance limiting the number of viable therapies available because of pathogen's capacity to colonize medical equipment and tools such as catheters, only immunocompromised individuals and individuals in medium to long-term recovery settings were first thought of great concern to be at risk. (D'Apolito et al., 2020)

2.4. RESISTANCE GENES AND ANTIBIOTIC RESISTANCE

According to WHO (World Health Organization) Antibiotic-Resistant Bacteria Priority List to guide Research, Discovery, and Development of New Antibiotics which has been published in 2017 *K. pneumoniae* listed as one of the top three pathogens of worldwide concern along with *Acinetobacter baumani* and *Pseudomonas aeruginosa* and it is the second most common etiological agent in (CAUTIs). There are "classic" and hypervirulent strains of *K. pneumoniae*, "Classic" non-virulent *K. pneumoniae* (c-KP) strains are usually associated with pneumonia, urinary tract infection, nosocomial infections, and neonatal sepsis in immunocompromised patients. Hypervirulent strains of *K. pneumoniae* (hv-KP) were first recognized in Taiwan in the last twentieth century and caused liver abscesses, meningitis, and endophthalmitis in previously healthy adult patients. Currently, hvKP strains are being spread in different parts of the world, High virulence of hv-KP is associated mainly with enhanced capsule production. In addition to c-KP and hv-KP, in recent years the third type of *K. pneumoniae* was detected which characterized by a combination of antibiotic resistance and hypervirulence. Antibiotic resistance of bacteria is the main cause of non-effective therapy of nosocomial infections and sepsis. The antibiotic resistance of *K. pneumoniae* strains is associated mainly with the production of ESBL (extended spectrum beta lactamase) which was discovered in 1983 in Europe and 1989 in the United States. KPCs was originally found in gram-negative bacteria by Alexander Fleming in 1929 which has been studied extensively since then, and it has been discovered that it produces a beta-lactamase which is the enzyme that causes antibiotic beta-lactam ring to hydrolyze and have been recovered worldwide especially in intensive care units (ICU). The prevalence of ESBL-producing strains of *K. pneumoniae* is 23% in the USA, and up to 85–100% in some European countries. because of ESBLs ability to hydrolyze oxyimino cephalosporins, third generation cephalosporins, they considered incompetent in the treatment against these bacterial diseases, for this reason Carbapenems become a therapy option for ESBL as a result of these resistance. However, later on it shown up that *K. pneumoniae* was responsible for almost 80% of the carbapenem-resistant *Enterobacteriaceae* infection according to documents reported to CDC (Center for Disease Control and Prevention) in 2013. Penicillin's, cephalosporins, monobactams, carbapenems, and even beta-lactamase

inhibitors are all effectively hydrolyzed by KPCs which their distribution epidemiology differs by geographic area. Bacteria that produce these enzymes are often sensitive of only a few antibiotics and patient with bloodstream infection due to these microorganisms have a high fatality rate. Several bacteria that have these enzymes are sensitive to colistin, tigecycline, and one or more aminoglycosides although few are resistant to all of them. Furthermore, only a few antibiotics targeting KPC-positive bacteria are currently developing. In *K. pneumoniae* two major kinds of antibiotic resistance have been described. The production of (ESBLs), cephalosporins and monobactams is one mechanism and AmpC (Ampicillin-Resistance Gene group C) which make bacteria resistant to ampicillin and carbenicillin is the second one. Bacterial enzyme (ESBLs) provide resistance to a variety of beta-lactam antibiotic classes. The presence of beta-lactam-insensitive cell wall transpeptidases causes the synthesis of beta-lactamase enzymes, or the spontaneous release of the beta-lactam elements from Gram negative bacteria is one of the most common signs of beta-lactam antibiotic resistance. (Ashurst & Dawson, 2018; Caneiras, Lito, Melo-Cristino, et al., 2019; Igrejas et al., 2019; Khaertynov et al., 2018; Munoz-Price et al., 2013; Paczosa & Mecsas, 2016)

Until beta-lactamases (ESBL, MBL) expressing isolates were discovered beta-lactams were wonders treatments. ESBLs are plasmid mediated enzymes which can hydrolyze oxyimino-cephalosporins (ceftriaxone, cefotaxime, and ceftazidime) and monobactams (aztreonam), but not cephamycin or carbapenems. In vitro, they are blocked by clavulanate." Metal chelating compounds such as EDTA inhibit metallo-beta-lactamases (MBLs) which hydrolyze carbapenems. (Dumaru et al., 2019) Carbapenems amongst beta-lactams are antibiotics that are used to treat bacterial infections caused by *Enterobacteriaceae* also the most efficient agent against both Gram positive and negative bacteria with a wide range of antibacterial activity. CRE (carbapenem resistance Enterobacteriaceae) increased in recent years, *Klebsiella* sp. was shown to be one of the most prevalent recent MDR rises between 2001 and 2011, notably the Patients that are infected with Enterobacteriaceae that produce carbapenemase such as *K. pneumoniae* have a significant mortality rate. Carbapenems all have the same beta-lactam ring and work by adhering and inactivating the penicillin binding proteins (PBPs) which are involved in bacterial cell wall construction. for

severe infections caused by (ESBLs) Enterobacteriaceae antibiotics like imipenem and meropenem are suggested as first-line therapy. The rise of carbapenem-resistant enterobacteria is particularly concerning as antimicrobial therapy choices are severely limited as a consequence. Carbapenem resistance may be caused by a combination of mechanisms: alterations in the permeability of the outer membrane and up-regulation of efflux systems, Hyperproduction of AmpC lactamases (cephalosporinases: AmpC-lactamases are therapeutically relevant cephalosporinases found on the chromosomes of many *Enterobacteriaceae* and a few other bacteria, where they cause resistance to cephalothin, cefazolin, cefoxitin, most penicillin's, and beta-lactamase inhibitor beta-lactam combination.) which ESBLs is linked to this condition, synthesize (carbapenemases) which is a specific carbapenem hydrolyzing β lactamases. The inclusion of a carbapenem ring in addition to the beta-lactam ring gives them a distinctive molecular structure this combination are resistance to majority of beta-lactamases involving ampicillin and carbenicillin (AmpC) as well as (ESBLs). (Jacoby, 2009; Meletis, n.d.; Nordmann et al., 2009; Wasfi et al., 2016)

Since the early 2000s, multidrug-resistant *K. pneumoniae* strains have been increasingly disseminating carbapenemases of Classes A, B, and D among *Enterobacteriaceae*. The most concerning carbapenemases are the Class A KPCs which were first detected in the United States in 1996 and are the ultimate alarming carbapenemases for their mild to high level carbapenem resistant because of their global appeal they present a challenge to antibiotic-based drugs that are currently available, beta-lactamases encoded by the KPC are mainly plasmid-encoded enzymes which are now expanding across the globe even though their expansion is depending on the geographical area. Furthermore, the Verona integron-encoded metallo beta-lactamase (VIM), imipenemase metallo beta-lactamase (IMP), and New Delhi metallo beta-lactamase (NDM-1) are zinc-dependent of Class B metallo beta-lactamases (MBLs) all described in 2008 as a novel carbapenemases which are encoded on highly transmissible plasmids that rapidly spread between bacteria instead of depending on clonal replication. In particular, NDM-1 which originated in Asia and spread throughout nearly every continent within a year of its discovery in India and plasmid-expressed Class D carbapenemases of the oxacillinase-48 (OXA-48) type which was first isolated from Turkey in 2004 and is now endemic in Turkey, as well as Lebanon

and Belgium. (Endimiani et al., 2016; Nordmann et al., 2009; Vuotto et al., 2014; Yin Chung, 2016; Zhu et al., 2016) (Desai et al., 2019; Nordmann et al., 2009)

CRKP isolates Carbapenem MICs (Minimum Inhibitory Concentration) can range from 0.12 to >256 mg/L depending on the geographical origin of the bacteria and the form of carbapenemase produced. about the fact that VIM enzymes have high carbapenem hydrolytic function their carbapenem MICs are low in a proportion of VIM-producing *K. pneumoniae* isolates. Similarly, carbapenem sensitivity in IMP-producing *K. pneumoniae* isolates can be reduced only slightly. Carbapenem MICs are higher in isolates containing the NDM-1 metallo-beta-lactamase. (*Carbapenemase-Producing Klebsiella Pneumoniae: (When) Might We Still Consider Treating with Carbapenems? | Elsevier Enhanced Reader*, n.d.)

Treatment of infections caused by multidrug-resistant bacteria is thus a significant issue for physicians and the capacity of bacterial pathogens which build biofilms might complicate this issue even more, the mortality rate from KPC-producing *K. pneumoniae* is substantial (50%) in individuals with bloodstream infection because KPC strains are resistant to all beta-lactam antibiotics including carbapenems which are normally employed as a last option. Failure to treat as a result of multidrug resistance relates to higher rates of morbidity and death. (Ashurst & Dawson, 2018; *Predictors of Outcome in ICU Patients with Septic Shock Caused by Klebsiella Pneumoniae Carbapenemase–Producing K. Pneumoniae | Elsevier Enhanced Reader*, n.d.; Ribeiro et al., 2015) Despite these challenges a new molecule, ceftazidime-avibactam has just been available. This medicine is a mixture of ceftazidime and a novel beta-lactamase inhibitor capable of inhibiting ESBLs, AmpC, and class A carbapenemases including KPCs but not MBLs. (Meletis, n.d.)

2.5. IMPACT OF BIOFILM ON ANTIMICROBIAL TREATMENT

In the ongoing antibiotic resistance crisis, (KPC) has become one of the most significant contemporary pathogens, particularly in endemic areas and management of infections caused by multidrug-resistant bacteria has become a major modifier of health costs. (*Management of KPC-Producing Klebsiella Pneumoniae Infections | Elsevier Enhanced Reader*, n.d.) Biofilms reduce the efficacy of antibiotics, defend

against host defense mechanisms and promote bacterial interaction allowing virulence determinants to be expressed. (Lavender et al., 2004)

Because of the connection between biofilm development and the capacity to withstand antibiotic therapy, the mode of growth of biofilms in antibiotic treatment decisions is extremely important in clinical procedure. This high antibiotic resistance in biofilms was initially attributed to a physical barrier developed by the extracellular polymeric substance which prevented drug penetration into the biofilm core. Several studies have linked antibiotic resistance to biofilm formation in *K. pneumonia* clinical isolates, in 2000 Anderl and his colleagues used an in vitro model system to assess the impact of ampicillin and ciprofloxacin on *K. pneumoniae* biofilms which was formed on microporous membranes with agar nutrient medium using wild-type and beta-lactamase-deficient mutant *K. pneumoniae*, wild-type biofilms avoided killing after being exposed to all antibiotics for an extended period of time. The investigators tested the antibiotics' diffusion and found that ampicillin did not reach wild-type *K. pneumoniae* biofilms while ciprofloxacin did. Ampicillin could only penetrate biofilms produced by beta-lactamase-deficient mutant, showing that increased resistance to ampicillin and ciprofloxacin in both wild-type and mutant *K. pneumonia* biofilms could not be due to poor diffusion, using transmission electron microscopy (TEM) the penetration of ampicillin and ciprofloxacin by *K. pneumoniae* biofilms was verified. The researchers used an agar plate to image cells in biofilm after antibiotic treatment defining certain regions of the biofilm that could exceed a 10-fold minimum inhibitory concentration (MIC) with ciprofloxacin or ampicillin, If the MIC assay shows that an antibiotic is insufficient, it is considered clinically insignificant. (Desai et al., 2019; Naparstek et al., n.d.; Singla et al., 2013; Vuotto et al., 2014) Infections caused by *K. pneumoniae* strains that can form biofilms are more complex to handle since mature bacterial biofilms have antibiotic resistance 10–1,000 times that of planktonic bacteria and bacteria in biofilms can withstand phagocytosis rendering them difficult to eradicate. (Potempa et al., 2018)

Antimicrobial penetration through a biofilm is supposed to be influenced by biofilm thickness the agent's successful diffusivity in the biofilm, the agent's reactivity in the biofilm, the biofilm's sportive potential for the agent, the dosage concentration and

length, as well as external mass transfer properties. Biofilm cell density and biofilm age are normally highly associated. (*Microbial Biofilms | Mahmoud A. Ghannoum, Matthew Parsek, Marvin Whiteley, Pranab K. Mukherjee (Eds.) | Download*, n.d.)

3. MATERIAL AND METHOD

3.1. BACTERIAL ISOLATES

Obtaining 120 various clinical samples within -80 °C freezing degree that come from different clinical origins from Sakarya teaching hospital, located in Sakarya-Turkey. The included isolated were carbapenem resistance strains generally which were previously stored there for further studies. All isolated were subjected to MaldiTOF-MS (Matrix-assisted laser esorption ionization-time of flight mass spectrometry, bioMérieux, France) for isolate identification also VITEK 2 ® system (bioMérieux, Marcy l'Etoile, France) used for antimicrobial susceptibility testing. *K. pneumoniae* strains were screened for resistance to these antibiotics: (SAM), (TZP), (CXM), (FOX), (CAZ), (CRO), (FEP), (ERP) (IMP), (MEM), (AMK), (GEN), (CIP), (TGC), (CST) results were interpreted depending on EUCAST 2010, EUCAST 2013 guidelines (*European Committee on Antimicrobial Susceptibility Testing Breakpoint Tables for Interpretation of MICs and Zone Diameters*, n.d.) Carbapenemase-Related gene determination Was performed using Gene X-pert for their harbored resistance Genes detection.

3.2. RECULTERING THE ISOLATES

Collecting all the stock isolates and set to the room temperature 25 c, then reculturing isolates by inoculating storing balls into 2 ml TSB and incubated for overnight growth at 37°C. after observing next day results clear broth appearance results inoculated and incubated again for another overnight growth to yield more positive turbid results, turbid appearance broth transferred onto Blood, EMB agar and incubated overnight at 37 C° in order to obtain the single pure colonies. According to colonies physical characteristics on agar media, isolate identification performed once more and the suspicious ones subjected to Maldi-TOF MS to exclude any possible contamination also to confirm presence of *klebsiella* genus.

3.3. BIOFILM FORMATION ASSAY

In vitro assessment of bacterial attachment and biofilm formation is done using microtiter plate assay which is considered as the gold standard. (Cusumano et al., 2019) Culture stocks were used to obtain isolates, after streaking on Blood agar fresh isolates obtained from blood sheep agar and inoculated into TSB with 0.05% glucose. Using 1\20 rate to dilute bacterial suspension, 200 microliters of the dilution was pipetted into 96 flat-bottom microtiters well. *E.coli* 25922 standard was used as positive control because it is strong biofilm producer according to (Crémet et al., 2013; Naves et al., 2008), while broth only was used as a negative-control and incubated at 37°C for overnight growth. The contents of the wells were emptied after the incubation duration and the wells were washed three times with 250 µL of distilled water, shaken, and inverted to clear the non-binding bacteria from the plate. The bacteria that had adhered to the wells were fixed for 15 minutes in 200 µL of 99 percent methanol. The wells were then stained for 5 minutes with 200 µL of a 1 percent crystal violet solution. The wells were washed and air-dried to clear any remaining stain. 200 µL of glacial acetic acid, 33 percent (v/v), was used to resolubilize dye attached to the wells. Using an ELISA auto reader, the optical density (OD) of each well was estimated at 570 nm. The isolates were examined in three separate 96 flat-bottom microtiter wells, and all measurements were performed three times. By measuring the absorbance of the crystal violet stain obtained for each biofilm, the biofilm-forming ability of each test isolate was compared to the positive and negative controls. Non-binding strains had an OD less than the negative -control, weakly binding strains had an OD up to twofold, moderate binding strains had an OD between twofold and fourfold, and strongly binding strains had an OD greater than fourfold. (Vatan et al., 2018)

2.4. STATISTICAL ANALYSIS

The average and median values were calculated after three runs of each test. *SPSS* software version 27 (IBM corp.) was used for statistical analysis, the strains were categorized into four groups as: strong, median, weak and none biofilm formers. And

the ratio of biofilm formation according to the source of infection and the harboring resistance genes were investigated statistically, to compare between isolate groups t tests were performed, *P-value* of ≤0.05 was considered as significant statistically. (Ali Rahdar et al., 2019; Vatan et al., 2018)

4. RESULTS

4.1. BACTERIAL ISOLATES

Isolates identified and confirmed previously according to Maldi-TOF MS identification methods as *K. pneumonia* strains, also VITEK 2 ® system assured antimicrobial susceptibility and defined *K. pneumonia* isolates as MDR (Multidrug resistance) against following antibiotics: (SAM), (TZP), (CXM), (FOX), (CAZ), (CRO), (FEP), (ERP) (IMP), (MEM), (AMK), (GEN), (CIP), (TGC), (CST) especially carbapenems antibiotics of widespread penicillin groups. Table 1

While reculturing all 120 isolates from -80 c freezing degree at 37c for 24 hr. 50.8% (n=61) isolates yielded as positive results after overnight cultures incubation. A turbidity equivalent to 0.5 McFarland standard (Mcfarland, 1907) used for inoculation of the isolates onto EMB and blood agar plates to obtain the pure single colony, during visualizing the physical characteristics (shape and appearance) of isolates colony on EMB and blood agar (Fig.1) which by extended incubation, *Klebsiella* colonies become large and mucoid, and they begin to coalesce. suspicious isolates subjected once again to Maldi-TOF MS to confirm gaining pure *K. pneumonia* isolates, after excluding doubt a total of 48.3% (n=58) isolates obtained for more studying. Among all 58 isolates, most of *K. pneumonia* isolates were from rectal surveillance swab sources 60.3% (n=35), followed by blood origins 27.5% (n=16), wound 5.17% (n=3), urine 3.44% (n=2), sputum 1.72% (n=1), catheter 1.72% (n=1).

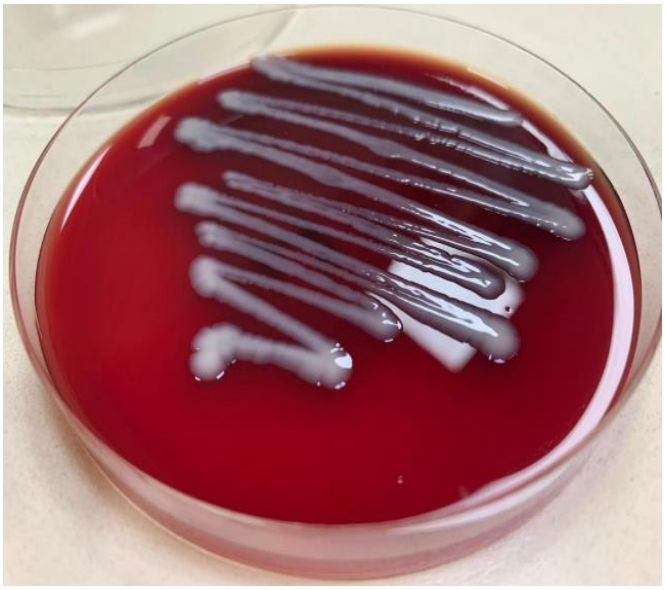

Figure 1: Klebsiella pneumonia on Blood agar

Carbapenemase producing isolates: Among 58 *K.pneumoniae* isolates all were carbapenem resistant confirmed by VITEK 2 ® system, carbapenemase genes were detected in 77.5% (n: 45) of isolates by Gene X-pert molecular method. Distribution of carbapenemase genes among carbapenem resistant cases were as follow accordingly: KPC 10.3% (n=6), NDM-1 32.7% (n=19), OXA-48 22.4% (n=13), NDM-1-OXA-48 12% (n=7), Negative resistance Genes 10.3% (n=13) were determined as resistance (Carbapenemase-Related Genes). Table 1

Table 1: Identification of Klebsiella isolates by Vitek, Maldi-TOF MS system

Kp dentification	Vitek, Maldi-TOF MS	Clinical Source	GENE X-PERT	Kp Identification	Vitek, Maldi-TOF MS	Clinical Source	GENE X-PERT
K1	KPC-KP	Blood	NDM-1	K30	KPC-KP	Blood	NEGATIVE
K2	KPC-KP	Blood	NDM-1	K31	KPC-KP	Blood	X PERT All NEGATIVE
K3	KPC-KP	Blood	NDM-1	K32	KPC-KP	Rectal Sur. Swab	NDM-1
K4	KPC-KP	Blood	OXA-48	K33	KPC-KP	Rectal Sur. Swab	NDM-1
K5	KPC-KP	Rectal Sur. Swab	NDM-1	K34	KPC-KP	Rectal Sur. Swab	NEGATIVE
K6	KPC-KP	Rectal Sur. Swab	NDM-1	K35	KPC-KP	Rectal Sur. Swab	NEGATIVE
K7	KPC-KP	Blood	NDM-1	K36	KPC-KP	Rectal Sur. Swab	NEGATIVE
K8	KPC-KP	Rectal Sur. Swab	NDM-1	K37	KPC-KP	Rectal Sur. Swab	KPC
K9	KPC-KP	Rectal Sur. Swab	NDM-1	K38	KPC-KP	Rectal Sur. Swab	OXA-48
K10	KPC-KP	Blood	NDM-1	K39	KPC-KP	Rectal Sur. Swab	NEGATIVE
K11	KPC-KP	Rectal Sur. Swab	X PERT All NEGATIVE	K40	KPC-KP	Wound	OXA-48
K12	KPC-KP	Rectal Sur. Swab	NDM-1 + OXA-48	K41	KPC-KP	Rectal Sur. Swab	NEGATIVE
K13	KPC-KP	Blood	NDM-1	K42	KPC-KP	Blood	OXA-48
K14	KPC-KP	Rectal Sur. Swab	NDM-1 + OXA-48	K43	KPC-KP	Blood	OXA-48
K15	KPC-KP	Rectal Sur. Swab	NDM-1 + OXA-48	K44	KPC-KP	Rectal Sur. Swab	NDM-1
K16	KPC-KP	Blood	NDM-1 + OXA-48	K45	KPC-KP	Catheter	NEGATIVE
K17	KPC-KP	Rectal Sur. Swab	NDM-1	K46	KPC-KP	Blood	NDM-1-OXA 48
K18	KPC-KP	Rectal Sur. Swab	NDM-1	K47	KPC-KP	Rectal Sur. Swab	OXA-48
K19	KPC-KP	Blood	NDM-1	K48	KPC-KP	Rectal Sur. Swab	OXA 48
K20	KPC-KP	Rectal Sur. Swab	NDM-1	K49	KPC-KP	Sputum	NDM-1
K21	KPC-KP	Urine	NDM-1	K50	KPC-KP	Rectal Sur. Swab	NDM-1-OXA 48
K22	KPC-KP	Rectal Sur. Swab	OXA-48	K51	KPC-KP	Rectal Sur. Swab	NDM-1-OXA 48
K23	KPC-KP	Urine	X PERT All NEGATIVE	K52	KPC-KP	Rectal Sur. Swab	OXA-48
K24	KPC-KP	Blood	OXA-48	K53	KPC-KP	Rectal Sur. Swab	OXA-48
K25	KPC-KP	Blood	X PERT All NEGATIVE	K54	KPC-KP	Rectal Sur. Swab	KPC
K26	KPC-KP	Wound	OXA-48	K55	KPC-KP	Rectal Sur. Swab	KPC
K27	KPC-KP	Rectal Sur. Swab	OXA 48	K56	KPC-KP	Rectal Sur. Swab	KPC
K28	KPC-KP	Rectal Sur. Swab	NEGATIVE	K57	KPC-KP	Rectal Sur. Swab	KPC
K29	KPC-KP	Wound	X PERT All NEGATIVE	K58	KPC-KP	Rectal Sur. Swab	KPC

4.2. BIOFILM FORMATION ASSAY

4.2.1. Biofilm producing isolates

In three different growth mediums containing glucose as a carbon source, biofilm production of *K. pneumoniae* strains was studied. Fig 2. Increases in biomass were shown to be significantly positive. (De Araujo et al., 2010) By measuring the absorbance of the crystal violet stain obtained for each biofilm, the biofilm-forming ability of each test isolate was compared to the positive and negative controls. The strains were divided into four categories: non-adherent (< 29% absorbance compression with negative control), weakly adherent (29% - 37%), moderately adherent (37% - 70%), or highly adherent (> 70%), depending on the OD of the bacterial biofilm. Table 2

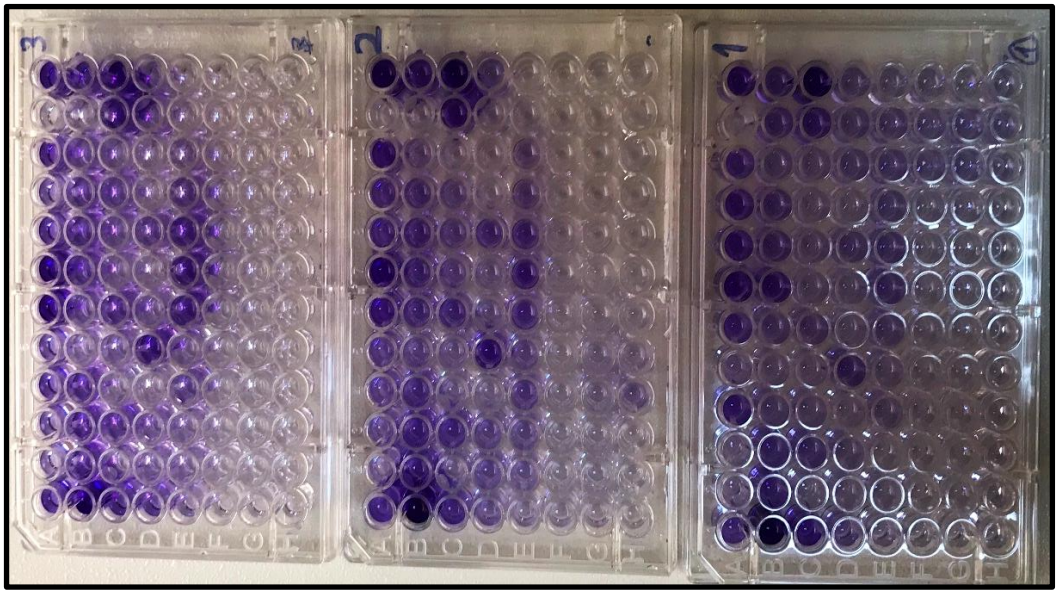

Figure 2: Microtiter plate assay biofilm formation

Table 2: Biofilm formation Classification

Biofilm production %	Classification
< 29	None
29-37	Weak
37- 70	Moderate
> 70	strong

The result shows that 98.2% (n=57) of *K. pneumonia* strains were biofilm producers and were divided into four categories according to the classification described previously. According to the biofilm analysis, 46.5% (n = 27) of the 58 examined *K. pneumoniae* isolates were categorized as moderately biofilm-producing strains, 37.9% (n = 22) formed fully established biofilms, 13.7% (n= 8) produced weak biofilms, and 1.72% (n = 1) of the strains were non-biofilm producers. The strains that were tested for biofilm formation, as well as the results obtained, are outlined in Fig 3, where the capacity to generate biofilm with various *Klebsiella* strains from clinical sources is summarized. The findings suggest that each strain has a different ability to form biofilm under the same experimental conditions.

4.2.2. Biofilm formation and source of infections

The *K. pneumoniae* strains that produced biofilm regarding to the source of infection categorized as follow: the greater ratio 60.30% originated from surveillance rectal swabs, 27.5% blood, 1.72% sputum, 5.17% wound, 3.44% Urine, 1.72% catheter. *K. pneumoniae* isolate when comparing to isolates collected from urine, catheter, wound, and sputum samples rectal swab and blood samples had a significantly greater potential to influence completely and moderately developed biofilms. (Table 3). The biofilm with the highest concentration strain was K13 which was isolated from blood and obvious strong (+++) biofilm-formation ability among the isolates obtained from surveillance rectal swab was more than other isolates significantly ($p \leq 0.05$) Fig 3.

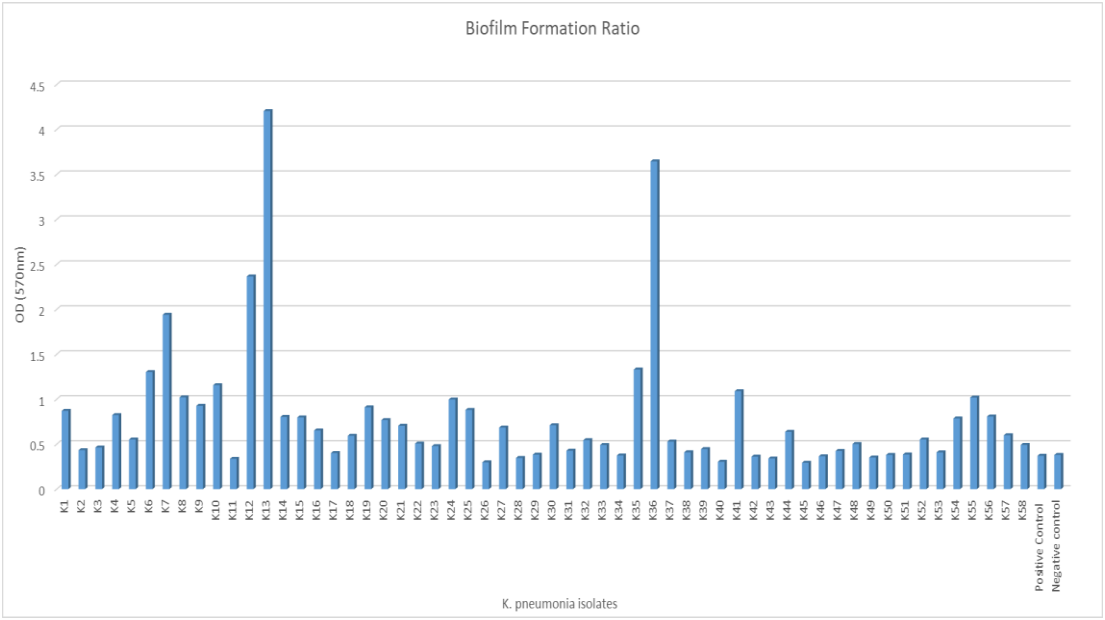

Figure 3: Biofilm formation among K. pneumoniae isolates determined at 570nm OD

Table 3: Biofilm formation and source of infection Correlation

Biofilm production %	Type of specimen					
	Blood	Sputum	Surveillance rectal swab	Wound	Urine	Catheter
None (0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (100%)
Weak (+)	3 (18.7%)	1 (100%)	2 (5.71%)	2 (66.6%)	0 (0%)	0 (0%)
Moderate (++)	4 (25%)	0 (0%)	20 (57.1%)	1 (33.3%)	2 (100%)	0 (0%)
Strong (+++)	9 (56.2%)	0 (0%)	13 (37.1%)	0 (0%)	0 (0%)	0 (0%)
Total	16 (100 %)	1 (100%)	35 (100%)	3 (100%)	2 (100%)	1 (100%)

4.2.3. Biofilm formation and Resistance Genes

The *K. pneumoniae* resistance Genes strains biofilm former divided as follow: 42.2% NDM-1, 28.80% OXA 48, 13.30% KPC, 15.5% NDM-1 +OXA 48. *K. pneumoniae* isolates with OXA 48, NDM-1 resistance Genes obviously had greater ability to form moderate and fully established biofilm in accordance to other resistance Genes that were isolated. Table 4.

Table 4: Biofilm formation and Resistance Genes Correlation

Biofilm production %	Resistance Genes			
	NDM-1	OXA 48	NDM-1- OXA 48	KPC
None (0)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Weak (+)	1 (5.26%)	4 (30.7%)	1 (14.2%)	0 (0%)
Moderate (++)	9 (47.3%)	7 (53.8)	3 (42.8%)	3 (50%)
Strong (+++)	9 (47.3%)	2 (15.3%)	3 (42.8%)	3 (50%)
Total	19 (100 %)	13 (100%)	7 (100%)	6 (100%)

5. DISCUSSION AND CONCLUSION

Foreign polymer objects implanted into the body have become a widespread technique in modern medicine. because the insertion or implantation of medical devices is frequently connected with microbial infections, the use of foreign material has resulted in complications. Device-associated infections contribute considerably to the growing problem of nosocomial infections in terms of morbidity and death. In both community and hospital settings *K. pneumoniae* is a frequent infection of the urinary tract. these infections are more common in hospitalized patients who have been catheterized for a long time. Because of the possibility for urosepsis which can be severe, with a high rate of fatality. The capacity of *Klebsiella* sp. to stick to surfaces and create biofilms is an essential aspect in the pathogenesis of the bacteria. (Maldonado et al., n.d.) *K. pneumoniae* has a wide spectrum of phenotypic and genetic variation. However, little is understood about how genetic diversity is organized within bacterial populations (i.e., species population structure) and how this relates to the organism's ecology or ability to produce various forms of illness. Although a virulence plasmid has been identified which is missing from the vast majority of clinical infection isolates. High-throughput genomic investigations have yielded crucial insights into the population structure of *K. pneumoniae*, allowing us to examine and compare the whole genetic complement of hundreds or thousands of *K. pneumonia* individual that can aid our understanding of how it develops, spreads, and causes disease. (Wyres et al., 2020)

In this present study the stocked isolates recultured but not all isolates were obtained due to contamination, or many freezing and melting actions which increases bacterial mortality according to Hilliard et. al study. (Hilliard & Davis, 1918) the remained isolates were investigated and found out that almost all carbapenamase *K. pneumoniae* 98.2% of the total isolates were biofilm producers, and there is study supporting this outcome with nearly the same results which declares that a substantial association is present between carbapenem resistance and the capacity of the isolates to produce biofilms. (Ali Rahdar et al., 2019) According to Fang et al, carbapenem-sensitive *K. pneumoniae* biofilm development differs from carbapenem-resistant *K. pneumoniae* biofilm development, which carbapenem-sensitive *K. pneumoniae* is less

likely to have high biofilm-forming potential than carbapenem-resistant *K. pneumoniae* due to a lack of certain resistance genes. (Fang et al., 2021) and this shows KPCs high biofilm formation virulence factor and harboring extended-spectrum beta-lactamase (ESBL)-positive genes give them a positive impact on their biofilm forming capacity.

For biofilm formation with their site of infection, a study result showed that an enormous proportion of *K. pneumoniae* isolates from sputum and surgical-wound swabs produce fully established biofilms while *K. pneumoniae* strains isolated from blood exhibited weak tendencies to form massive biofilms. (Seifi et al., 2016) In contrast, our findings exhibited that strong biofilm-formation ability was determined among surveillance rectal swabs and blood clinical samples which they had a remarkably greater ability to form fully and moderately established biofilms compared to other isolates significantly those results might come from difference in sample group numbers included in these studies, or might be due to using stocked isolates in present study that can cause their virulence to decrease gradually. But in contrast and depending on this finding the patients in the ICU might be the most at-risk population because majority of surveillance rectal swabs are obtaining from the ICUs and urgent crucial care are needed there.

A recent study investigating the ability of biofilm formation with resistance Genes had demonstrated that a single plasmid or clone carrying a carbapenemase gene may boost the *K. pneumoniae* bacterial host defense or virulence remarkably. However, the same study reveals that. K. pneumonia carrying NDM-1+ OXA 48 transconjugant Genes improved more virulence and pathogenicity, which shows that presence of two separate carbapenemase genes boost the fitness and pathogenicity of a bacterial host more than the presence of a single gene. (Lee et al., 2020) while our findings determined that NDM-1 especially, then OXA 48 resistance carbapenemase Genes alone had greater ability to form moderate and fully established biofilm, nor their conjugation together. And this declares that isolates with one of these two resistance Genes are alarming concern generally.

The production of biofilms and the development of beta-lactamases both contribute to the widespread spread of multi-drug resistant strains of gram-negative bacilli. They are accountable for the chronicity, persistence, and return of infections, which result in high morbidity and death, creating a significant health concern. In this context, understanding biofilm development and the antibiogram of bacterial isolates is critical for providing patients with reliable empirical antibiotic treatment. (Dumaru et al., 2019)

Many investigations in *K. pneumoniae* carbapenamase carried out in attempt to better understand the processes behind this resistance also to achieve better antibiotic resolution procedures, Generally, this study indicates that carbapenamase K. pneumonia have a great ability to form biofilms, also there is a positive correlation between the biofilm formation with Carbapenamase Resistance Genes harboring isolates where NDM-1, OXA 48 have a high capacity forming biofilms, and the source of infection directly have an effect on the biofilm formation rate where the priority goes to surveillance rectal swabs site, then blood which means that harboring resistance genes has a direct effect on the K. pneumonia virulence. And the patients with blood stream infections and ICU patients are at higher risk according to our outcomes. These results indicate that the eradication of these infections is hard due to their high biofilm formation capacity and their high resistance against antibiotic is because they are really high biofilm producers. physicians and hospital personals should take carbapenem resistance *K. Pneumonia* in great concern due to their high virulence which globe are facing recently.

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APPENDIX

Ethics Committee approval

Evrak Tarih ve Sayısı: 30.03.2021-E.21439

T.C.
SAKARYA ÜNİVERSİTESİ REKTÖRLÜĞÜ
Tıp Fakültesi Dekanlığı

Sayı : E-71522473-050.01.04-21439-196
Konu : Girişimsel Olmayan Etik Kurul Başvuru Dosyası Hk.

Sayın Dr. Öğr. Üyesi Tayfur DEMİRAY

Sağlık Bakanlığı Sakarya Üniversitesi Eğitim ve Araştırma Hastanesi
Tıbbi Mikrobiyoloji Anabilim Dalı

İlgi : 07.02.2021 tarihli 196 sayılı başvurunuz.

Destekleyicisi olduğunuz **"Karbapenem Dirençli Klebsiella İzolatlarında Biyofilm Oluşumunun Saptanması"** isimli çalışmanın ilgili belgeler araştırmanın gerekçe, amaç, yaklaşım ve yöntemleri dikkate alınarak incelenmiş olup; çalışmanın başvuru dosyasında belirtilen şekilde etik ve bilimsel açıdan sakınca bulunmadığına etik kurul üyelerince karar verilmiştir.

Bilgilerinize rica ederim.

Prof. Dr. Hasan Çetin EKERBİÇER
Etik Kurulu Başkanı

Yücel DEMİR
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Güvenli Elektronik
İmzalı Aslı İle Aynıdır.
30../03./20.21

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III- Addresses

IV- Professional Experience

V- Scientific Institutions membership

VI- Scientific Interests

Publications:

A. <u>Sakarya Medical Journal, published original research, article, review within Sakarya Med J scope</u>

A1. Altındiş M , Ghafour H . "Is Blood Type Linked to COVID19 Risk?". Sakarya Tıp Dergisi. vol. 11, no. 1, pp. 207-217 (2021) doi:10.31832/smj.860739.

VII- Scientific Activities

Awards

Projects

Conferences or seminars given

Panels attended (as a panelist)

VIII- Other information's