T.C.

KIRSEHIR AHI EVRAN UNIVERSITY

HEALTH SCIENCES INSTITUTE

DEPARTMENT OF MOLECULAR MEDICINE

MOLECULAR DETECTION OF FIM H, MRKD, AND WCAG GENES RESPONSIBLE FOR BIOFILM FORMATION IN *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT SITES OF INFECTION

NOOR ABDUL JABAR

MASTER THESIS

KIRŞEHİR / 2022

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SUPERVISOR

Prof. Dr. ERGIN KARİPTAŞ

II. SUPERVISOR

Dr. ESTABRAQ ALI MAKLEF

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THESIS STATEMENT

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NOOR ABDUL JABAR

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December, 2022 **NOOR ABDUL JABAR**

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LIST OF SYMBOLS AND ABBREVIATIONS

Abbreviations	Explanations
AMR	**:** Antimicrobial resistance
AST	**:** Antibiotic sensitivity test
BHI	**:** Brain heart infusion
BHIB	**:** Brain heart infusion broth
BHIG	**:** Brain heart infusion broth with glucose
CPS	**:** Capsular poly saccharide
CV stain	**:** Crystal violet stain
DCs	**:** Dendritic cells
ESBL	**:** Extended spectrum beta lactamase
LPS	**:** Lipopolysaccharide
MIC	**:** Minimum inhibitory concentration
MTP	**:** Microtiter plate
OMPs	**:** Outer membrane Proteins
PBS	**:** Phosphate buffer saline
TBE	**:** Tri borate EDTA buffer solution
TSB	**:** Tryptic soy broth
UTI	**:** Urinary tract infection
VAP	**:** Ventilator associated pneumonia

ABSTRACT

MASTER THESIS

MOLECULAR DETECTION OF FIM H, MRKD, AND WCAG GENES RESPONSIBLE FOR BIOFILM FORMATION IN *KLEBSIELLA PNEUMONIAE* ISOLATED FROM DIFFERENT SITES OF INFECTION

NOOR ABDUL JABAR

Kırşehir Ahi Evran University
Health Sciences Institute
Department of Molecular Medicine

Advisor: Prof. Dr. Ergin KARİPTAŞ
Co-Advisor: Asst. Dr. Estabraq Ali MAKLEF

Background: *Klebsiella pneumoniae* leads to hospital and community-acquired illnesses with substantial morbidity and death. *K. pneumonia's* biofilm production is connected to virulence genes. Resistance is enhanced by biofilm development. This research investigated sodium hypochlorite as well as carnation extract against *K. pneumoniae* biofilm. Materials and methods: About 100 *Klebsiella pneumoniae* isolates were obtained from various clinical specimens taken from hospitals in the Medical City of Baghdad, Iraq, between June 2021 and January 2022. This bacterium was isolated by used traditionally laboratory methods and identification by used VITEK-2 automated system, biofilm-related genes (*fimH, wcaG* and *mrkD*) were detection by conventional-PCR as well as biofilm evaluation by three different methods (Tryptic Soy Broth TSB, Congo Red Agar CRA and Brain Heart Infusion Broth BHIB). Additionally, the impact of anti-biofilm materials (sodium hypochlorite and carnation) on biofilm was measured. Results: In this investigation, *K. pneumoniae* isolates obtained from sputum, burns, urine, and blood formed biofilms. Pus, ear fluid, and fluid isolates were less likely to form biofilms. When compared to the use of Congo red agar and the brain-heart infusion broth techniques, tryptic soy broth is readily quantified and may be a reliable way to identify biofilm growth.

The high frequency of the *mrkD and fimH* genes in isolates was also a strong correlation among the capacity to produce biofilm along with the presence of *mrkD and fimH* genes. In addition, sodium hypochlorite and carnation, in particular Syzygium aromatics, revealed a strong inhibitory influence on the biofilm establishment in *K. pneumoniae* isolates. Conclusion: *mrkD and fimH* genes in *K. pneumoniae* are associated with biofilm formation, making them interesting appropriate treatment options. The tryptic soy broth technique is a more efficient and dependable way to identify biofilm-forming bacteria in laboratories. Sodium hypochlorite and carnation, in particular Syzygium aromatics, inhibited and removed *K. pneumoniae* biofilm, and the impact increased with a level in the both bactericidal and bacteriostatic spectrum.

November 2022, 84 Pages

Keywords: *Klebsiella. Pneumoniae* , Biofilm formation, Antibiofilm, Virulence genes

ÖZET

YÜKSEK LİSANS TEZİ

FARKLI ENFEKSİYON YERLERİNDEN İZOLE EDİLEN *KLEBSIELLA PNEUOMONAE*'DA BİYOFİLM OLUŞUMUNDAN SORUMLU FİM H , MRKD , VE WCAG GENLERİNİN MOLEKÜLER SAPTANMASI

NOOR ABDUL JABAR

Kırşehir Ahi Evran Üniversitesi
Sağlık Bilimleri Enstitüsü
Moleküler Tıp Anabilim Dalı

Danışman: Prof. Dr. Ergin KARİPTAŞ
II. Danışman: Dr. Öğr. Üyesi Estabraq Ali MAKLEF

Arka plan: *Klebsiella pneumoniae*, önemli morbidite ve ölümlü hastane ve toplum kaynaklı hastalıklara yol açmaktadır. K. pneumonia'nın biyofilm üretimi, virülans genlerine bağlıdır. Direnç, biyofilm gelişimi ile arttırılmaktadır. Bu araştırma, sodyum hipokloritin yanı sıra karanfil ekstraktını *K. pneumoniae* biyofilmine karşı araştırmıştır. Materyal ve yöntem: Irak, Bağdat Tıp Merkezi'ndeki hastanelerden alınan çeşitli klinik örneklerden Haziran 2021 ile Ocak 2022 arasında yaklaşık 100 *Klebsiella pneumoniae* izolatı elde edilmiştir. Bu bakteri, geleneksel laboratuvar yöntemleri kullanılarak izole edilmiş olup, VITEK-2 otomatik sistemi kullanılarak tanımlama yapılmıştır. Biyofilm ile ilgili genler (fimH, wcaG ve mrkD), konvansiyonel-PCR ile ve ayrıca biyofilm değerlendirmesi, üç farklı yöntem (Triptik Soya Sıvı besiyeri TSB, Kongo Kırmızı Agarı CRA ve Beyin Kalp İnfüzyon Sıvı Besiyeri BHIB) ile tespit edilmiştir. Ayrıca anti-biyofilm maddelerinin (sodyum hipoklorit ve karanfil) biyofilm üzerindeki etkisi ölçülmüştür. Bulgular: Bu araştırmada K. pneumoniae izolatları, balgam, yanık, idrar ve kanda oluşan biyofilmlerden elde edilmiş olup, irin, kulak sıvısı ve sıvı izolatlarının biyofilm oluşturma olasılığı daha düşük çıkmıştır. Kongo kırmızı agar ve beyin-kalp infüzyon besiyeri tekniklerinin kullanımıyla

karşılaştırıldığında, triptik soya sıvı besiyeri kolayca ölçülebilir ve biyofilm büyümesini tanımlamanın güvenilir bir yolu olabilir. İzolatlarda mrkD ve fimH genlerinin yüksek frekansı, mrkD ve fimH genlerinin varlığı ile birlikte biyofilm üretme kapasitesi arasında da güçlü bir korelasyonu ortaya koymuştur. Ek olarak, sodyum hipoklorit ve karanfil, özellikle Syzygium aromatikleri, K. pneumoniae izolatlarında biyofilm oluşumu üzerinde güçlü bir inhibitör etki ortaya çıkarmıştır. Sonuç: K. pneumoniae'deki mrkD ve fimH genlerinin biyofilm oluşumu ile ilişkili olması onları ilginç ve uygun tedavi seçenekleri haline getirmektedir. Triptik soya sıvı besiyeri tekniği, laboratuvarlarda biyofilm oluşturan bakterileri tanımlamanın daha verimli ve güvenilir bir yoludur. Sodyum hipoklorit ve karanfil, özellikle Syzygium aromatikleri, K. pneumoniae biyofilmini inhibe etmiş ve uzaklaştırmış olup, etkisi hem bakterisidal hem de bakteriyostatik spektrumda belirli bir düzyede artış göstermiştir.

Kasım 2022, 84 Sayfa

Anahtar Kelimeler: *Klebsiella. Pneumoniae* , Biyofilm oluşumu, Antibiyofilm, Virülans genleri

1. INTRODUCTION

Klebsiella pneumoniae is considered a Gram-negative, encapsulated bacterium that may be naturally found, in water, soil, or among animals. These bacteria have the ability to colonize medical equipment as well as the surroundings of healthcare facilities. Within the healthcare context, infections are a particular concern for newborn babies, people who are old or immunocompromised, as well as patients who have both of these characteristics [1]. In addition to this, it is responsible for a considerable number of illnesses that are acquired in communities all over the globe. In healthy humans, the bacterium *Klebsiella pneumoniae* may be found in the gastrointestinal system, the urinary tract, as well as the respiratory tract. It is a common hospital-acquired bacterium that may cause serious respiratory infections like pneumonia [2].

Biofilms are colonies of microorganisms that are highly organized and demonstration enhanced resistance to the antibiotics agents as well as the host immune-response. They are constituted of bacteria that are enclosed in an extracellular matrix that is consisting of proteins, polysaccharides, and the genetic material that is produced by the bacteria alone. The structure of these organisms is very complicated and diverse, in addition to coming from the host, and they operate as a reservoir for microbes throughout host colonization as well as via sticking to abiotic surfaces, promoting the pathogenicity of an infinite number of different kinds of bacteria [3].

Building up of resistance to antibiotics is a common phenomenon that has been identified as a significant risk to the health of people all over the world [4]. As a direct consequence of their capacity to reduce sensitivity to antibiotic drugs, biofilms have gained significant importance in the field of medicine. Inside a biofilm, a significantly reduced susceptibility to microbial agents is caused by many different factors. These factors include a physical impairment of the dispersion of antibacterial drugs, reduced bacterial rate of growth, as well as any regional changes that may have occurred within the pathogens that might have an impact on the antibacterial agent's effectiveness. Together, these factors contribute to the biofilm's reduced susceptibility [5].

There are two basic forms of fimbriae that are generated by *K. pneumoniae*. These fimbriae are known as type I and type III fimbriae, respectively. Fimbriae function as adhesins, which enhance adhesion to biological surfaces (which may then lead to future tissue intrusion) as well as to abiotic surfaces, such as hospital devices,on which bacteria can create biofilm. This can lead to subsequent tissue invasion. [6]. Type I fimbriae are the ones that bind to the receptors that possess mannose, which are located in a variety of tissues throughout the human body [7]. In vitro studies have revealed that type III fimbriae may form binding interactions with a diversity of cell kinds, such as kidney, lung, and bladder epithelial cells [8].

At least ten gene clusters that code for chaperones, ushers, as well as adhesin proteins for the construction of fimbriae are originate in the genome of *K. pneumoniae*. These gene clusters include *fim, mrk.ecp plus Kpa toKpg genes* [9]. These proteins are encoded via the *FIM* gene cluster, which is responsible for their production. The primary structural component is encoded by a *fim A* gene, whereas the tiny tip adhesion subunit is encoded by the *fim H* gene. Both genes are responsible for their respective coding functions [10]. An essential component in the attachment of the microbe to collagen fibers is the MrkD protein, which is provided by the *MerK* gene [10].

Within regions of *K. pneumoniae* chromosome that are essential for capsule production, the virulence gene *wcaG* may be detected. This gene is necessary for the conversion of mannose in to fructose, which may increase the resistance of bacteria to phagocytosis at the hands of macrophages [11]. Eugenol as well as *Andrographis paniculata,* two naturally occurring plant chemicals, demonstrated significant inhibitory action on the biofilm that *K. pneumoniae* formed [12].

Eugenol, sometimes referred to as 4-allyl-2-methoxyphenyl, is one of them. Eugenol is a colorless to light yellow aromatic oily liquid. It is made from numerous essential oils, most prominently clove as well as cinnamon oil [13]. It has a range of biological effects, including biofilm inhibition and eradication, antibacterial, antioxidant activity, as well as anti-inflammatory effect [14]. One of the most common disinfectants is sodium hypochlorite. It is a potent oxidizing agent that also has broad-ranging antibacterial activities. The breadth of its antibacterial activity is considerable [15].

1.1. Aims of the study

1- Detection the prevalence of *K. pneumoniae* isolated from different site of infections.

2- Studying the expansion of *K. pneumoniae* in different age categories in both sexes.

3- Investigating the biofilm forming ability of *K. pneumoniae* isolates.

4- Molecular detection of the presence of *fimH, wcaG* and *mrKD* genes.

5- Detection the antibiofilm formation by sodium hypochlorite and cloves extract.

2. LITERATURE REVIEW

2.1. The Enterobacteriaceae

Family Enterobacteriaceae is considered the biggest varied group of the Gram-negative bacteria that are significant to the medical field. At the moment, it is composed of fifty different genera, not to mention dozens of identified species and also subspecies. Primarily, they were categorized based on their biochemical characteristics and structure of antigens. Afterward, this was accomplished using DNA-DNA hybridization as well as sequencing of the 16S rRNA gene. The majority of the family's members are creatures that may be found almost everywhere; in addition to their human then animal hosts, who serve as regular flora, they can be discovered in water and soil. They are rod-shaped, Gram-negative bacteria that do not produce spores and range in size from 0.3 to 0.3 to 1.0 by 1.0 to 6.0 micrometers. While certain genera are mobile due to the presence of peritrichous flagella, others lack this characteristic and are immobile. There is a spectrum of degrees of pathogenicity among the members of the Enterobacteriaceae family. Those that are considered to be part of the normal flora may only cause diseases if they move to other parts of the body (for example, Escherichia coli may go from the gastrointestinal system to the urinary system.). Another, such members of the Salmonella genus, are considered to be strict pathogens, but many of them are primarily responsible for opportunistic infections, meaning that they only affect people whose immune systems are severely impaired (e.g. *Serratia marcescens*) [16].

2.2. *Klebsiella pneumoniae*

The bacteria known as *Klebsiella pneumoniae* are highly infectious. that is rather widespread in addition to prevalent factors that might lead to nosocomial infections, such as infections of the respiratory system, central nervous system, blood, and urinary tract [17]. furthermore, healthy people may get community-associated infections produced via *K. pneumoniae*, such as liver abscesses, endophthalmitis, as well as meningitis [18]. Edwin Klebs, a German microbiologist who lived from 1834 to 1913, is honored with the naming of the genus Klebsiella. It is additionally recognized as Friedlander's bacillus,

which is in honor of Carl Friedlander, a German researcher who proposed that this bacterium was the cause of infectious for pneumonia, which is often observed in immunocompromised persons such as alcoholics and those who suffer from chronic disorders. Friedlander postulated that this bacterium was the agent responsible for the case of pneumonia that was observed in immunocompromised patients. Friedlander's bacillus is a phrase that can be employed to describe community-acquired pneumonia which is produced via *Klebsiella pneumoniae*. This kind of pneumonia can be spread by coughing and sneezing [19].

In addition to being linked to the *Salmonella*, *Escherichia*, and *Shigella* genera, the bacterium known as *Klebsiella pneumoniae* belongs to the family known as the *Enterobacteriaceae.*, which is located under phylum proteobacteria and the Enterobacterial order of the bacterial kingdom [20]. It is a Gram-negative bacillus that is facultatively anaerobic and typically measures 0.3 until 1.5 m broad by 0.5 to 5.0 m long. It is also non-motile, ferments lactose, and does not produce spores. In contrast to other species of bacteria belonging to the *Enterobacteriaceae* family, it is shorter and thicker. They are also sometimes seen in pairs, chains, or joined end to end with one another. The optimal temperature for growth of *K. pneumoniae* is between 35 and 37 degrees Celsius, and the optimal pH level is somewhere around 7.2. *K. pneumoniae* may grow on any standard laboratory medium and does not have any specific growth needs [21]. It is present in a vast majority of ecological 'niches,' and these niches also include the environmental (like soil, water, and drainage) and host-associated niches (such the respiratory and gastrointestinal tracts, and skin). It is found everywhere [22]. *Klebsiella pneumoniae* is among the most prevalent bacterial species that has the potential to produce biofilm-related nosocomial infections, such as urinary tract infections as well as bloodstream infections that are linked with the utilization of catheters [23].

The infectious potential of the bacteria is influenced by a diverse set of conditions, any of which may result in infection or resistance to antibiotic. The polysaccharide capsule is still the greatest essential virulence component in addition to gives the bacteria the ability to escape being killed by the host's opsonophagocytosis and serum defense mechanisms [24]. Additional part of the bacterium's pathogenicity is termed fimbriae, and it is responsible for the bacterium's capacity to adhere on its own to host cells. The bacterium also requires siderophores, which are a type of virulence factor in order for it to be able to obtain iron from the host and hence continue its infection of subsequent hosts [25].

2.2.1. Scientific classification

Every kind of *Klebsiella* is a gram-negative bacterium that lacks the ability to move about on its own. In contrast to other types of bacteria belonging to the *Enterobacteriaceae* family, they are often shorter and thicker in appearance. There are more than eight species as well as subspecies of *Klebsiella s*pp. that have been reported in the Bergey's Manual. Some of them include: "*K. pneumoniae* subsp. *pneumoniae*, *K. pneumoniae* subsp. *ozaenae*, *K. pneumoniae* subsp. *rhinoscleromatis*, *K. mobilis*, *K. oxytoca*, *K. planticola*, *K. terrigena*, *K. ornithinolytica"* [26] .

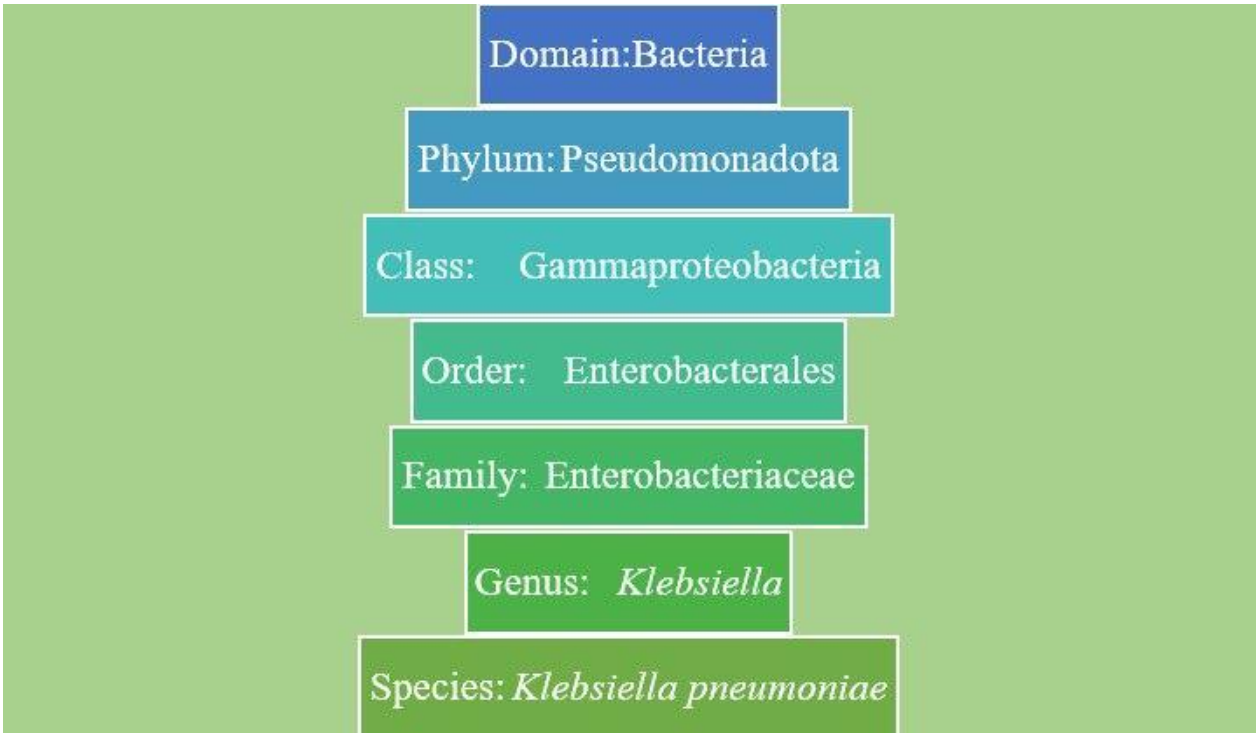

Figure 2.1. Scientific classification of *K. pneumoniae* (REF) [27]

2.2.2. Epidemiology

The primary source of *K. pneumoniae* is found in human beings as a reservoir [28]. There are between five and thirty-eight percent of people in the population who have the organism in their intestine, and there are between one and six percent who have it in their nasopharynx [29]. The digestive system and the people working in the medical field are the most common causes of infection. It has the potential to cause an epidemic of nosocomial infections [30]. Comparatively to the broader populace, the proportion of hospitalized patients who are carriers of *K. pneumoniae* is much greater [31]. One research found that patients brought to the hospital had carrier concentrations in their stools that reached up to

77%, and these concentrations are considered to be related to the quantity of antibiotics they were given [32]. *K. pneumoniae* infections were split into two categories: those that spread via the general population and those that spread through hospitals [33]. Although pneumonia that was acquired in the community is the most common diagnosis, infection of the *K. pneumoniae* is extremely uncommon [34]. It is believed that around three to five percent of wholly instances of pneumonia in Western culture are caused by *K. pneumoniae,* but it accounts for around fifteen percent of all incidence rate in developing nations for instance Africa [35].

The accounts of *K. pneumoniae* for about 11.8 percent of entirely cases of pneumonia acquired within hospitals around the globe [36]. *K. pneumoniae* causes among eight and twelve percent of deaths in people with pneumonia who are on a ventilator, but only seven percent of deaths in people with pneumonia who do not use a ventilator, despite the fact that mortality rates for patients with alcoholism as well as septicemia range from fifty to one hundred percent [37]. There is a high death rate associated with *Klebsiella pneumoniae*; this mortality rate is more than fifty percent in individuals who have illnesses such as alcoholicism, diabetes, nosocomial infections, or septicemic disorders [38]. In addition, *K. pneumoniae* is capable of causing bacteremia, lung abscesses, as well as the development of empyema, which may all lead to pneumonia [39]. One is the ecosystem in which they are found, and the other is the colonized the mucosal regions of the humans, also horses, as well as swine on the surface waters, sewage, soils, and plants. *Klebsiella* most likely exists in two distinct settings in the wild: the first being the environment in which it is discovered, and also the second is the mucosal regions in which it has colonized. Both of these environments are likely shared by *Klebsiella* [40]. In this regard, the Klebsiella group is analogous to *Enterobacter* as well as *Citrobacter*. On the other hand, it is distinguishable from the *Shigella spp*. & E. coli genus in that it is common among people but not from environmental settings due to the fact that it is more abundant in human populations. As a commensal organism, *K. pneumoniae* may be establish in the nasopharynx and also the digestive system of human beings [41]. There is a significant amount of variety in the carrier rates from one research to the other. Stool samples might vary anywhere from 5 percent to 38 percent positive for the presence of the pathogen., while the range of detection in nasopharyngeal samples is between 1% and 5%. (1 percent and 6 percent). *Klebsiella spp* should not be allowed to develop on human skin since gram-negative bacteria do not thrive in human skin's natural environment. Because it is so

uncommon, it is referred to be a member of the transitory flora [42]. These carrier rates fluctuate considerably in hospital settings, where colonization rates are directly proportional to the length of a patient's stay. The proportion of patients who have *Klebsiella* is also alarmingly high among hospital employees [43]. Hospitalized individuals have been found to have carrier rates of 77 percent in their feces, 19 percent in their pharynxes, and 42 percent in their hands [44]. It shows that the high prevalence of nosocomial *Klebsiella* colonization is related with the usage of antibiotics rather than variables connected with the provision of care in hospitals [43].

A substantial association has been found between the patient's acquisition of *Klebsiella* with the use of antibiotics in the patient's past [45]. The most common of those who saw the rise were those who were taking antibiotics, and more specifically, those who were on broad-spectrum as well as combination antibiotics [46]. Within the context of the hospital, the local antibiotic policy is a vital issue in decisive the kind of the colonization [47]. It was discovered that the patients who carried *Klebsiella* intestinal infection had an occurrence rate for the *Klebsiella* nosocomial that was four fold greater than the occurrence rate for patients who had acquired the infection via the hospital [48]. Besides this, the widespread utilize of the antibiotics in medical facilities has been shown to contribute to the disseminated of *Klebsiella* isolates that are resistant to management [49].

The patients' digestive systems as well as the hands of medical staff members, which are polluted due to improper hygiene measures, are the primary sources of *Klebsiella* transmission in hospitals. This is along with medical tools as well as products of the blood, which are secondary causes [50]. The ability of the organism to quickly spread, especially in neonatal units, is a common factor in the development of nosocomial infections [51]. The likelihood of contracting these strains is often proportional to the length of time spent in hospitals as well as the level of success achieved during invasive operations [52]. The careful observation of the fundamental epidemiological concepts of urinary catheter treatment, injection, plus the tracheostomy, wound, conservation, as well as protocols of hand-washing is helpful in the treatment of the development of nosocomial *Klebsiella* infections [53]. Another phase in the management infections of *Klebsiella* is the control of usage the antibiotic in hospitals, with the goal of preventing the misuse and overuse of antibiotics. In addition, monitoring for nosocomial infections is essential for the collection of data necessary for the reduction and management of the incidence of nosocomial *Klebsiella* infections [54].

2.2.3. Virulence factors

The potential of an organism to cause illness in connection to a particular host is referred to as its "pathogenicity," while the quantitative manifestation of this capacity is referred to as its "virulence." Both phrases describe the ability of a bacterium to reproduce, spread across its environment, and either infect its host with sickness or kill it. Virulence factors are chemicals and structural components of cells that have roles that are vital in the interface among the pathogen as well as the host when the infection is taking place. These characteristics provide bacteria an advantage in their capacity to bring about pathogenic alterations in their host [16].

2.2.3.1. Capsular antigens

Both the capsular polysaccharide (CPS; similarly identified as the K antigen) as well as the lipopolysaccharide (LPS; also known as the O antigen) are key components that are expressed on the surface of *K. pneumoniae*. Both of these components are necessary for *K. pneumoniae's* pathogenicity [55]. It is widely agreed upon that the thick capsule layer enclosing the bacterium that *K. pneumoniae* has (which measures 160 nm) is a significant contributor to the pathogenicity of the organism. In order for bacteria to produce illnesses, the primary step is for the bacterium to come in as well as colonize its host, then a vital part in this process is played by the capsule. The material that makes up the capsular envelope condenses into a dense mass of fibrillous structures that coat the surface of the bacterium. The capsule has a strong antiphagocytic effect, which helps to shield the bacteria from the bactericidal effects of serum factors as well as phagocytosis. In addition to this, it is capable of serving as a barrier against harmful hydrophobic compounds, such as detergents [16]. Different *K. pneumoniae* strains may produce a variety of antigenically different capsules, and some of these capsules are thought to be linked with a greater capacity for pathogenicity. It is possible that the formation of type 1 fimbriae, which is an adhesin that is unique to mannose receptors, is connected to the level of virulence that is supplied by a certain K antigen. Antigens K1, K2, K4, and K5 on the capsule are almost always linked with a high level of pathogenicity [56]. Initially, the gene clusters within *K. pneumoniae* that are accountable for the production of capsules were described to as cps gene clusters, Moreover, their locations on the bacterial chromosome were shown to be rather similar to his. The cloning of the cps cluster resulted in the discovery that each gene

contains approximately 15 kilobase pairs (kb) of DNA and includes all of the predictors that are required to identify the serotype of the given strain. [57].

According to findings of the investigations, there are three alleles of a separate gene known as rmpA/A2 that are known to have a role in the activation of the expression of the cps gene [58]. There is variation in the alleles depending on the serotype as well as the geographic location of the isolates. Plasmid DNA is responsible for encoding two of the alleles, whereas chromosomal DNA is responsible for the third allele. *K. pneumoniae* develops a hypermucous phenotype as a result of excessive synthesis of rmpA, Consequently, this results in a rise in the expression of transcriptional fusions that include cps-promoter regions [59].

Not just is the Fur protein involved in repressing rmpA transcription in *K. pneumoniae*, but so are other genes involved in iron uptake mechanisms [60]. Because of this, the amount of accessible iron within surroundings of *Klebsiella* might performance a significant part through development of the virulence genes, which includes the quantity of the capsule generated via clinical isolates. According to the findings of the investigations, K1 serotypes of the *K. pneumoniae* are linked with development of severe infections that spread throughout the body. The mucoviscosity-associated gene, also known as magA, was shown to be a component of the cps gene cluster in each of these particular isolates. This gene is essential for the production of K1. Previously, the polymerase chain reaction technique (PCR) was utilized to recognize K1- positive bacteria, but only lately, a similar allele was identified in additional K-serotypes. This gene has also been used to identify K1- positive strains [61].

In general, the greatest dangerous strains of the *K. pneumoniae* specific that form a capsule that is comprised saccharides, and since these saccharides do not promote attachment to the phagocytic cells, the pathogens are less susceptible to becoming ingested by the immune system [62].

2.2.3.2. Lipopolysaccharide (LPS)

Similar to the LPSs of many other members of the *Enterobacteriaceae* family, the lipopolysaccharides (LPSs) produced by *K. pneumoniae* are organized into three distinct structural domains. These domains include: (i) a hydrophobic lipid A, that is not only a key virulence component but is also essential for endotoxin action; This lipid is a critical

virulence factor in addition to being a large portion of the outer flap of the membrane that surrounds Gram-negative bacteria, (ii) the core oligosaccharide that serves as the binding domain and is joined to lipid A, (iii) the extended-chain polysaccharide, often known as the O antigen or the O chain [63]. Along with endotoxic properties of LPS, the O antigen of *K. pneumoniae* also makes a considerable contribution to the pathogenicity of the bacteria. Because the host cell has receptors on cell wall, *K. pneumoniae* is able to adhesion itself to the cell of host. In a same manner, lipopolysaccharide (LPS) has been presentation to have a part in the *K. pneumoniae* pathogenesis, It has been demonstrated in a variety of research's that the O-antigen of *K. pneumoniae* blocks complement components from gaining access to activators (such as porins and rough LPS), which is one of the factors that leads to resistance of complement-mediated death via bacteria [64].

2.2.3.3. Pili (fimbriae) and adhesion

Pili or fimbriae are hairlike appendages that are projecting from the surface of bacteria. They have a length of 10 micrometers and a width of 1 to 11 nanometers, in addition to this, they are composed of the spherical polymeric protein components known as pilin. Fimbriae, which play a role in adhesion, are a key element in colonization because of their role [16]. The types 1 and 3 fimbriae, together with the Kpc fimbriae, are the three varieties of *K. pneumoniae* fimbrial adhesins that have been described in substantial detail. In addition, the Kpc fimbriae have indeed been described. [65]. Because of their capacity to attach soluble mannose like such a competitive inhibitor to the cross - linking process, type 1 fimbriae are also known as mannose-sensitive fimbriae. Type 1 fimbriae are encoded by the *fim* gene cluster, which is also important for generating type 2 fimbriae. Type 1 fimbriae are often found in bacteria. The *mrk* gene cluster is responsible for encoding the type 3 fimbriae, and these fimbriae may be found in the *K. pneumoniae* either on the plasmid or within a chromosomal gene cluster. It has been demonstrated that mrkA is the most essential structural element of the fimbrial core and that mrkD acts as the fimbrial adhesion molecule in addition to helping to improve adherence to extracellular matrix proteins [62].

According to the results of the research, the Kpc fimbriae are frequently associated with K1-positive strains bacteria *K. pneumoniae*, which are the agents that are responsible for causing disseminated pyogenic illnesses [66]. The *kpcABCD* gene cluster is accountable for mediating the synthesis of these fimbriae; however, the circumstances necessary for the

highest possible level of expression of these genes are not yet completely known. The plasmid-encoded CF29K adhesin is the other form of adhesin that may be produced by *Klebsiella spp*. This fimbrial type is distinguished from others via its adherence to the cell lines of the digestive tract [67].

2.2.3.4. Iron acquisition

The amount of free ferric iron (Fe3+) within host is often rather little, despite the fact that iron is a necessary component for the growth of bacteria. As a result, pathogens like *K. pneumoniae* should be designed such that they may get their iron source from host. This is accomplished via a number of processes, the furthermost prevalent of which are either the use of ABC transporters or the construction of "low-molecular-weight, high-affinity iron chelators known as siderophores" [68]. Although it is very probable that the presence of siderophores is required in all systemic infections, the function of siderophores has been extensively examined in a group of *K. pneumoniae* strains known as hypervirulent *K. pneumoniae*. There are perhaps a dozen distinct mechanisms that have been uncovered that are involved in the process of providing iron to *K. pneumoniae* [69]. These genes that code for siderophores may be found in clinical isolates in a variety of different combinations. Attenuation occurs in mutants that are missing them [62].

2.2.3.5. Outer Membrane Proteins (OMPs)

It has been shown that the virulence of *K. pneumoniae* depends on the presence of a number of outer membrane proteins, such as "outer membrane protein A (OmpA), peptidoglycan-associated lipoprotein (Pal), and murein lipoprotein (LppA)". These OMPs are all encoded via genes with same names [70]. At the very least in part, the ability of OmpA to guard against the innate immune response help through pathogenicity of the *K. pneumoniae*. However, research employing OmpA isolated from *K. pneumoniae* or, conversely, OmpA in the context of complete *K. pneumoniae* bacterium has given findings that seem to be in disagreement with one another. Through the use of isolated OmpA, researchers were able to see that OmpA attaches to the cells of the bronchial epithelium, in addition to dendritic cells (DCs), and macrophages, which ultimately results in increased cytokine production [71]. This attachment of OmpA with DCs as well as macrophages takes place via the scavenger receptor (LOX-1), then which subsequently results in signaling via TLR2 in the cell [72]. When pure OmpA is inhaled into an animal's lungs, it causes an elevation in the production of cytokines and chemokines, which

ultimately results in a transitory increase in the number of neutrophils [73]. Alternatively, the OmpA of the bacterium lead to decrease the synthesis of cytokines, in addition to increasing microbial resistance against the antimicrobial peptides like alpha-defensin [74]. For instance, as a comparison to an isogenic strain that expressed OmpA, and the ompA of the *K. pneumoniae* with deletion mutant produced enhanced IL-8 generation throughout epithelial cells of the bronchial within vitro as well as raised TNF-alpha and also IL-6 levels through lung of mice, in addition to this, the ompA deletion mutant shown a reduced level of severity in a mouse model with pneumonia [75].

2.2.3.6. Porins

Porins OmpK35, as well as OmpK36, are fascinating due to the fact that antibiotic-resistant forms of *K. pneumoniae*, like those that generate ESBL and therefore are resistant to carbapenem, either express them insufficiently or fail to generate them at all [76]. The downregulation among these porins appears to give a benefit to these bacteria in the specific situation of antibiotic option, where the porins may actually function as a path that facilitates drugs to access the bacterium. This advantage may be due to the fact that the porins are able to allow drugs to gain entry into the bacterium [77]. Re-expression of ompK35 as well as ompK36 through carbapenem-resistant *K. pneumoniae* isolates led to a significant decrease in antibiotic resistance, but deletion in ompK36 in some kind of a "K2 HV *K. pneumoniae*" strain resulted to a rise in resistance to particular antibiotics when tested in laboratory [78]. Nevertheless, the simultaneous deletion in ompK35 plus ompK36 drove antibiotic resistance that was larger than that seen with the ompK36 single-deletion mutant. In furthermore, the deletion of ompK35 did not have an impact on the sensitivity of *K. pneumoniae* to specific antibiotics. This was the case despite the fact that the vulnerability of *K. pneumoniae* to particular antibacterial agents was not altered by t deletion of ompK35 [79]. Porin downregulation may originate at a cost to the fitness of the organism with respect to virulence [78]. In the absence of OmpK35, the existence of the OmpK36 boosted the virulence of *K. pneumoniae* in vivo mouse intraperitoneal infection model, nonetheless a *K. pneumoniae* isolates producing equally the OmpK35 as well as the OmpK36 was much further virulent[79] . After further research, it was shown that deletion mutant in the ompK36 may take possession of the liver, but it is unable to stay there after being injected intraperitoneally. Additional research comparing a traditional *K. pneumoniae* isolates whether or not with the ompK36, found that the mice given an intraperitoneal or intranasal infection along with the deletion mutant had a considerably

lower death rate. One way in which OmpK36 may aid in the development of virulence in vivo is by blocking phagocytosis. This idea is supported by the observation that human neutrophils were better able to absorb an ompK36 deletion mutant. This is presumably owing to a modification in the bacterial attachment to the neutrophils that is imparted through lack of the OmpK36 [79].

2.2.3.7. Pumps in addition to Transporters

AcrAB is already known to function as an efflux pump and has been connected with both the pathogenicity of *K. pneumoniae* in addition to its resistance against antibiotic therapy [80]. In such an animal model of pneumonic infection, infection with only an acrB deletion mutant in a K2-expressing *K. pneumoniae* strain resulted in a reduced bacterial hardship with in lungs as particularly in comparison to infection with WT strain, which suggests that AcrB improves bacterial wellness throughout the lungs. This was seen in comparison to infestation with WT strain. In addition, this acrB loss mutant was more susceptible to the effects of being exposed to human bronchoalveolar evacuation material as well as antibacterial compounds such as HBD-1 as well as HBD-2. This implies that this protein is crucial for giving protection against antibacterial humoral ingredients. A *K. pneumoniae* strain with an acrB deletion is more susceptible to a variety of medicines, particularly beta-lactams, when compared to the wild type strain. This is in contrast to the situation with the WT strain [81]. In a similar vein, the expression of AcrR throughout clinical strains has been linked to antibiotic resistance [82]. In the grand scheme of things, these AcrAB activities are probably mediated by the release of potentially harmful host chemicals or antibiotics from the bacterial cell. The production of this factor and the HV *K. pneumoniae* strains that are present has been shown to have a substantial link with one another. Kfu is a component of the ABC iron transportation system and has been linked to *K. pneumoniae's* capacity to take up iron. The significance of this gene to pathogenicity in vivo was shown using an animal model of lethal peritonitis caused by an HV *K. pneumoniae* deletion bacterium, in which a kfu deletion mutant that was isogenic to the original strain was unable to cause death [83]. Within the context of this specific model, the presence of kfu was necessary for the formation of abscesses within both the liver as well as the brain. In humans, Invasive clinical variants, particularly those that originate from the liver abscess to produce meningitis in addition to endophthalmitis, are more likely to include kfu than noninvasive strains do. This is certainly relevant of those strains that produce

endophthalmitis. This really is due to the fact that invasive strains have a higher probability of spreading to other areas of the body [84].

2.2.4. Genome of *Klebsiella pneumoniae*

According to genomic research, the population of *K. pneumoniae* is both varied and highly organized. This usual structure offers a helpful foundation for appreciative the epidemiology in addition to development of the clinically significant genetic dissimilarity. Intended for the appropriate strategy as well as evaluation of investigational research aiming at explaining procedures of antimicrobial resistance, pathogenicity of *K. pneumoniae,* as well as in lieu of the effective proposal of regulator methods, such an understanding is essential [14]. The genomes of typical *K. pneumoniae* bacteria are between 5 and 6 Mbp in size and encode between 5,000 and 6,000 genes. A total of around 1,700 genes, referred to as core genes, are existing in every member of species. The presence of the remaining genes, referred to as accessory genes, which encode a variety of non-essential tasks, might vary significantly across strains [85]. The sum of all core and auxiliary genes makes up the overall pan-genome, which is extraordinarily varied and almost certainly contains more than 100,000 protein-coding sequences [86].

The massive mainstream of accessory genes is very uncommon in the general population; more specifically, their presence may be found in less than 10 percent of all genomes. The G+C content of the *K. pneumoniae* genome is 57.27 percent, and it is composed of a single circular chromosome and four to six distinct plasmids that vary in size from 3 to 270 kb, with up to 10 recorded instances [87]. Plasmids are very critical carriers for the transmission of the "antimicrobial resistance (AMR)", pathogenicity, then further auxiliary genes from one bacterial cell to another. Whereas the overwhelming bulk of laterally obtained *AMR* genes throughout *K. pneumoniae* are conducted on huge conjugative plasmids that seem to be self-transmissible as well as pertain to a tiny proportion of conflicting groups (IncFII and IncR), small plasmids that are portable but still not self-transmissible also can possess *AMR* genes in *K. pneumoniae* [88]. Genes for the "extended-spectrum beta-lactamase", often known as *ESBL*, may be located on chromosomes or on transferable genetic elements like as plasmids. Both of these locations are possible. They are most often found in the family *Enterobacteriaceae,* but you may also find them in other Gram-negative rods that do not ferment their food, such as *P.*

aeruginosa otherwise *Acinetobacter* species. The bulk of genes that code for CTX-M proteins are found upon mobile genetic components [89].

Certain genes that influence resistance to carbapenemase, including "*blaOXA, bla NDM,* and *blaKPC",* have been linked to the development of resistance to the carbapenems like imipenem besides meropenem. This resistance may partially have explained by the existence of these genes. It has been shown that the *blaOXA* gene may be found in *K.pneumoniae* in both plasmid and integron sites, and that it is commonly found to be coupled with genes that code for "extended-spectrum beta-lactamases (ESBLs)" [90]. There is a connection between the 16S rRNA methylases that are encoded on the plasmids and the aminoglycoside resistance of *Klebsiella pneumoniae*. The *armA* gene was first discovered on the plasmid *pCTX-M3* in *Citrobacter freundii* and the plasmid *pIP1204* in *Klebsiella pneumoniae*. Both of these plasmids also contained an ESBL gene called *blaCTX-M-3* [91]. The *armA* gene may be found in these plasmids positioned downstream of the ISCR1 insertion sequence. The aminoglycoside resistance methyltransferase, or *armA*, was the first 16S rRNA methylase that was discovered in a *K. pneumoniae* clinical isolate [92]. Which protects the organism against the antibiotics kanamycin, amikacin, isepamicin, gentamicin, as well as fortimicin. The *armA* gene, which codes for the methylase, was found in a composite transposon called Tn1548, which was contained in a conjugative plasmid that was around 90 kb in size [93]. rmtC is being assimilated via MDR/XDR *K. pneumoniae*, especially those generating NDM kind carbapenemase. The *rmtC* gene has also been discovered in *K. pneumoniae* clinical isolates. Gene *rmtC* is responsible for coding resistance to gentamicin, tobramycin, & amikacin; however, it does not encode resistance to neomycin or apramycin [91].

2.2.5. Pathogenicity

Klebsiella pneumoniae is the infectious agent that is accountable for a wide diversity of diseases. These infections mostly affect people who have compromised immune systems and have a propensity to cause hospital-acquired infections, commonly known as nosocomial infections [93]. *Klebsiella* is willing to take responsibility for six to seventeen percent over all nosocomial urinary tract infections (UTI), so it demonstrates an even higher prevalence in particular groups of individuals who are at risk, including such patient populations with neuropathic bladders as well as diabetes mellitus, alcohol abuse, pulmonary insufficiency, immune-suppressant patients, but also hospitalized patients,

particularly in intensive care facilities. As a result, accurate and timely diagnosis is required to avoid infection throughout hospitals [94] The infections of the urinary tract caused by drug-resistant *Klebsiella pneumoniae* isolates are a main problem for community health because the efficacy of several antimicrobial agents has been restricted. As a result, the numeral of therapeutic choices has been significantly reduced, and it is significantly more difficult to provide an appropriate antimicrobial therapy. Additionally, multidrug-resistant bacteria are to blame for urinary tract infections, which are notoriously difficult to treat. This contributes to a rise in both the expenditures of medical care as well as the rates of death and morbidity [95].

K. pneumoniae records for a greater proportion of pneumonia that is obtained throughout hospitals. This is because patients who are hospitalized are significantly likely to receive treatment with antibiotics, which allows this bacterium to monopolize the pharyngeal flora. Although *K. pneumoniae* is a relatively uncommon etiology of community-acquired pneumonia, it is responsible for a much greater proportion of cases of hospital-acquired pneumonia [96]. It is known to colonize respiratory mucosal surfaces and is classified as an opportunistic pathogen; yet, from the mucosa, it may spread to other tissues in addition to causing infections that are potentially fatal, including such "ventilator-associated pneumonia (VAP)". After Escherichia coli, *K. pneumoniae* is the bacteria responsible for the second greatest cases of the Gram-negative bacteremia. The *K. pneumoniae* bacteremia can either be primary as well as secondary bacteremias, which get up from the secondary propagation from the primary infection within lungs in addition to bladder, causing significant morbidity and mortality in general populations. Primary bacteremias occur when *K. pneumoniae* directly infects red blood cells, while secondary bacteremias arise when *K. pneumoniae* indirectly infect [97].The infections occur due to *K. pneumoniae* are described most prominently by their tendency to spread to other parts of the body, resulting in conditions like pyogenic brain abscess, meningitis, as well as endophthalmitis. In addition, *K. pneumoniae,* along with other bacteria, is capable of colonizing burns following surgical procedures [98].

2.2.6. Biofilm formation

K. pneumoniae is capable of forming biofilm, and once it does, the bacteria accumulate in cells that are embedded within a matrix that is made by themselves from an extracellular polymeric material. Polymeric compounds found outside of cells are made up of a variety

of different components, including polysaccharides, proteins, as well as DNA. The majority of *K. pneumoniae* biofilms that are visible in a clinical setting are found on catheters and the inner surfaces of internal devices [99]. It is possible for *K. pneumoniae* biofilm to cause colonization throughout the respiratory, digestive, and urinary tracts, in addition to the formation of infections caused, particularly in individuals who are immune-compromised. This is especially true in patients who have a weakened immune system. The institution of a *K. pneumoniae* biofilm upon surface of sharp things begins with the adherence of cells, which is followed by the creation of tiny colonies, maturity, and eventually the proliferation of free-living cells. The type 3 pili as well as the capsular polysaccharides (CPs) on the surface of the *K. pneumoniae* are the most essential surface structures that are involved in the development process [100].

Pili are responsible for maintaining adhesion, whereas CPs eventually have an effect on the structure of the biofilm as well as intercellular communication. Because the process of biofilm creation is dynamic and the environmental stimuli that it is exposed to might vary, the bacteria that are contained in the biofilm need to have the capacity to swiftly and comprehensively modify their gene expression. Quorum sensing is a system that coordinates the signals and reactions that govern gene expression in a microbial community. This system is responsible for regulating the transcriptional regulatory process. Although the potential quorum-sensing-related regulators as well as auto-inducers throughout *K. pneumoniae* have been found, the relevant data that is currently accessible is still lacking in certain important respects [62]. The steps of biofilm formation of *K. pneumoniae* show in Figure 2.1.

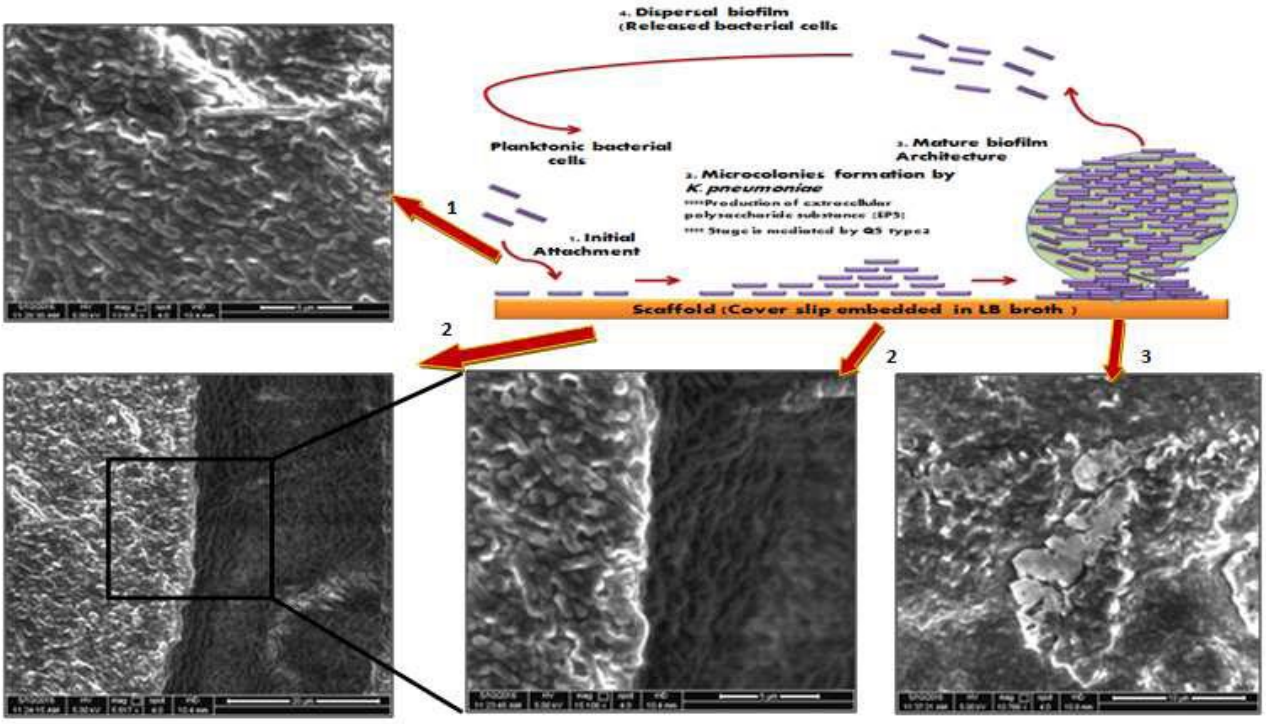

Figure 2.2. The Biofilm Formation of *Klebsiella pneumoniae* Extracted from Various Clinical Samples [101]

2.2.7. Genes responsible for biofilm formation in *K. pneumonia*

When *K. pneumoniae* is covered with thick layer of biofilm, it is partially shielded from the immunological response of the host. This matrix prevents antibodies plus antimicrobial peptides from coming into close contact with one another, and it also lessens the impact complement and phagocytosis have [62]. Alterations in the purpose of biofilm may also be caused via mutations through certain genes of *Klebsiella pneumoniae* [102]. Two primary adhesive pili complexes are responsible for the capacity of *Klebsiella pneumoniae* to cling with eukaryotic epithelial cells to a great degree. These structures are as follows: a mannose-sensitive type 1 pili (T1P), which are consists of a major fimbrial and include fimbrial isoform A (FimA) in addition to a minor tip adhesin fimbrial isoform H (FimH), and the mannose-resistant type iii pili (T3P or MR/K), which are constituted of the major pilus isoform, are the two types of pili that have been identified [103].

T1P are widely distributed across members of the Enterobacteriaceae, and their significance throughout the pathogenesis of human urinary tract infections (UTI) produced via *K. pneumoniae* has been studied extensively [104]. T1P is the mediator of the attachment of T1P with mannose-containing receptors that are found on the epithelial cells

that line the urinary tract as well as the trachea [105]. On the surface of bacterial cells are projections known as type I fimbriae. These fimbriae are thin, filament-like projections. The chaperone/usher pathway is responsible for collecting them, while the *FIM* gene cluster is responsible for encoding them [106]. The fim A gene is responsible for encoding the major structural subunit, while the *fim H* gene is responsible for encoding the small tip adhesin subunit [105]. The *fim K* gene is present in *K. pneumoniae*. It is very important to keep in mind that the absence of the *fim K* gene results in the inability to produce type 1 fimbriae. This gene plays a role through regulation of type 1 fimbriae, besides it is also vital to demonstrate that this gene is required for the production of type 1 fimbriae [107]. Because the genes for type 1 fimbria are expressed within urinary system, the fimbriae they encode are able to infiltrate the cells of the urinary bladder and create biofilms inside the bladder [105].

In comparison, the adhesive functions of the MR/K pili are not dependent on D-mannose. It is believed that most *K. pneumoniae* strains produce these adhesive functions in effort to enhance adherence to the tracheal epithelial cells, renal tubular cells,also extracellular matrix proteins, as well as basement membranes of lung tissue, as well as to assist in the formation of biofilm [108]. There is a high frequency of the *mrkD* gene in K. oxytoca strains, while it is quite uncommon in *K. pneumoniae* strains [109]. Even mrkD strains are able to form biofilms and adhere to host cells, which suggests that MrkA alone is sufficient to mediate the bacterial contacts that are necessary for the effective formation of biofilms [110].

It is possible for type 3 fimbrial adhesins to facilitate the binding of *K. pneumoniae* to a variety of human cells, including endothelium cells and epithelial cells found in the respiratory system as well as urinary tract, respectively. The MrkD protein is an essential component in the process by which the bacterium binds to the collagen molecules [111]. Additionally, the existence of adhesins (fimH as well as mrkD) associated with the production of biofilm was looked at [112]. The virulence gene *wcaG* is found in the sections of the *K. pneumoniae* chromosome that are important for capsule biosynthesis. This gene is required for the transformation of mannose into fructose which may boost the capacity of bacteria to avoid phagocytosis via macrophages [11]. Resistance as well as virulence are not two separate characteristics; rather, they are intertwined, and the dynamic between them may play a significant part in the pathogenesis of infections caused by *K. pneumoniae* [113].

2.2.8. Factors that influence on biofilm formation

The preliminary adhesion of bacterial cells to the substrate material is the beginning stage in the complex and unpredictable process of biofilm development, which also includes physiological changes inside the microbe, the factorial of adhered cells to establish micro-colonies, as well as the development of the biofilm itself [114]. Because they have a distinct physiology and a high resistance to the immune system as well as antibiotics, bacteria that are associated with biofilms are a source of chronic as well as persistent infections. Biofilms are a source of these infections because biofilms are a source of bacteria that are associated with chronic and persistent infections [115]. It is well established that the transition from the planktonic to the sessile type of the phenotypic takes place in response to changes in the circumstances of the environment [116]. The improvement of biofilm may be influenced by environmental variables such as the number of nutrients present, temperature, pH, as well as ionic strength [117].

Bacterial adhesion can be affected via a number of diverse aspects, including such as cell surface properties like hydrophobicity, flagellation, as well as motility, surface properties like hydrophobicity as well as unevenness, and ecological factors like temperature and pH, the accessibility of nutrients, in addition to the hydrodynamic conditions [118]. Each microorganism in a diverse microbial population may be given a competitive advantage by the existence of extracellular appendages like fimbriae as well as flagella, interactions during cell-to-cell contact, and EPS synthesis of surface-associated polysaccharides and proteins. The existence of extracellular extensions, including such fimbriae as well as flagella, is one characteristic of the cell surface that is of special note [119].

Bacteria that are hydrophobic are more probable to link to the surfaces than the bacteria that are hydrophilic; even so, the attachment of biofilm will happen easily and quickly on surfaces that are uneven, hydrophobic, as well as coated through surface conditioning films. Bacteria that are hydrophilic are less likely to attach to surfaces. Environmental variables such as pH, temperature, and nutrition levels may also have an effect on the physicochemical features of the substratum, like the texture, also hydrophobicity, as well as charge [120]. Within aquatic settings, the rate of microbial attachment may be enhanced through elevating the flow rapidity and temperature of water, or else the absorption of the nutrients, provided that these parameters do not reach critical limits [121].

2.3. Anti-biofilm compounds

Pathogens with in biofilm have a greater capacity for colonization as well as a lesser initiation of the immune system than the planktonic bacteria. This allows biofilm bacteria to assist other bacteria in evasion the immune response of body and also in forming an extracellular barrier that prevents antibiotics from penetrating [122]. creating conditions in individuals with biofilm bacteria that are similar to persistent infections or multiple infections [123]. As a result, preventing the development of biofilm or completely removing it has developed into an increasingly critical challenge in both therapy and prevention. The majority of anti-biofilm substances now available are antibiotics, metal ions, and bioactive chemicals According to the findings of a research, medical devices that have been coated using antibacterial agents are able to successfully prevent the colonization of biofilm; nevertheless, an excessive usage of the mediators may induce the microbial resistance [124].

Further research has revealed that silver has an antibacterial effect against both planktonic bacteria as well as biofilm bacteria. However, because large concentrations of silver as well as other metals are harmful to human cells and tissues, it is not safe to utilize metal ions [125]. Others researches demonstrated that naturally occurring plant compounds, such eugenol that extracted from cloves oil (*Syzygium aromaticum)* and *Andrographis paniculata,* had strong inhibitory effects on the biofilm produced by *K. pneumoniae* [12].

Disinfectants are a typical method of preventing and controlling nosocomial infections. Since disinfectants kill bacteria in vitro, they are able to cut off the transmission channel, which in turn helps prevent pathogen infection as well as transmission. In hospitals, sodium hypochlorite is a frequent disinfectant; nevertheless, the impact of this disinfectant on biofilms including *K. pneumoniae* strains with varying degrees of drug resistance is seldom examined. In particular, the majority of the recently published research on *K. pneumoniae* biofilm focuses only on describing the inhibitory or clearance impact. Sodium hypochlorite, often known as NaOCl, is a powerful oxidizing agent that also has antibacterial properties that are extensive in scope. This is what the “Scientific Committee on Emerging and Newly Identified Health Risks (SCENIHR)” says about it [126]. Depending on the concentration at which it is used, sodium hypochlorite may be either a low-level or an intermediate-level biocide. Despite this, NaOCl is a substance that is not only extensively utilized but also easily accessible in terms of the cost as well as benefit

[127]. It has been put to use for the disinfection of apparatus, surfaces, and laundry, in addition to being utilized for the decontamination of drinking water (Anonymous, 2013). However, bacterial resistance to chlorine-releasing substances has been observed, despite the fact that these compounds have bactericidal action throughout a wide range [128].

3. MATERIALS AND METHODS

3.1. Materials

3.1.1. Laboratory Instruments and Equipment

In the Table 3.1, every piece of equipment in addition to instrument used in the present investigation are listed

Table 3.1. Equipment and instrument

Items	Company	Origin
Autoclave	JR	Korea
Bunsen burner		China
Centrifuge	AL-rawan	China
Distillator	GFR	Germany
DNA extraction tubes	Eppendorf	China
Eppendrof centrifuge	HERMILE Labotechnic GmbH	Germany
Gel electrophoresis	Biometra	Germany
Hood	Memmert	Germany
Hot plate and magnetic stirrer	MS-300HS	Korea
Incubator	Hera Therm	Germany
Micropipettes	Eppendorf	Germany
Conventional PCR	Eppendorf AG	Germany
PCR tubes	Eppendorf	
Sensitive electron balance	WT3003CH	China
UV-transilluminator	Major science	Taiwan
VITEK 2- Compact	Biomerieux	France
Vortex	VM300	Taiwan
Cooling centrifuge	hanil	Japan
Freezer	hicool	China
Microscope	Lomo	Russia
Refrigerator	Concord	Lebanon
Water bath shaker	Jul abo	Germany
Water distillatory	Mocdwa	Russia
Elisa reader	Human	Germany

3.1.2. Materials containing both chemicals and biological substances

3.1.2.1. The chemical materials

Each of the biological and chemical substances that are included in the following Tables 3.2.

Table 3.2: The chemical materials

Chemicals	Company-Country
Agarose	MarLiJu/Korea
Alcohol (Ethanol) 99%, 95% and 70%	Fluka/ Switzerland
Crystal violet powder	Avonchem/UK
Glacial acetic acid	Himedia/India
Glucose	SDI/Iraq
Glycerol	SB/Turkey
Gram stain set	Fluka/ Switzerland
Phosphate buffer saline	Avonchem/UK
Tris-Borate EDTA buffer 10X (TBE)	Promega/USA

3.1.2.2. Biological materials

Each of the Culture Media that are included in the following Table3.3.

Table 3.3. The culture media

Culture Media	Company& Origin
BHI agar	Biomark/India
BHI broth	Biomark/India
MacConkey agar	Neogen/UK
Tryptic soy broth	DCM/Netherland
Blood agar	Biomark/India
Congo red agar	Biomark/India

3.1.3. Bacterial diagnostic kit

Table (3.4) below provides a list of the diagnostic kit used in this investigation.

Table 3.4. Diagnostic kit

Kit	Company –origin
Grams stain kit	SyBio Syria
VITEK 2 GN	Biomerieux- France

3.1.4. Polymerase Chain Reaction (PCR) Materials

Table (3.5) lists the PCR materials and it is appendices utilized in the current investigation

Table 3.5. Manufactured kits were employed in this investigation

Item	Company	Origin
Diamond nucleic acid dye	Promega	USA
DNA extraction kit	Geneaid	Taiwan
DNA Ladder 100 bp	BIONEER	Korea
Loading dye	Promega	USA
PCR master mix solution	Promega	USA
Green master mix	Promega	USA
Primers of *wcaG, fimH and mrkD*	BIONEER	Korea

- **DNA extraction kit components**
  1- GT Buffer
  2- proteinase K
  3- GB Buffer
  4- RNase solution
  5- W1 Buffer
  6- Wash Buffer
  7- Elution Buffer
  8- GD Column

3.2. Methods

3.2.1. Subjects

During the time period of June 2021 through January 2022, isolates were got from a diversity of clinical specimens obtained from hospitals located in the Medical city of

Baghdad, Iraq. These samples were gathered from 300 samples taken from patients with various diseases who visited various hospitals and medical institutions in the province of Baghdad. These samples included blood, urine, sputum, plus swabs from burn as well as wound cases. Bacteria were grown in an aerobic environment at 37 degrees Celsius for 18 to 24 hours on agar plates containing blood as well as MacConkey agar. After that, they established their identities by means of a VITEK 2 automated system with (GN) cards, as is suggested via the original equipment maker. Table displays, both in terms of kind and quantity, the clinical specimens that were collected for this investigation (Table 3.6)

Table 3.6. The various clinical samples that were obtained for this investigation, as well as the total number of samples

Type of specimen	Number
Sputum	23
Blood	31
Urine	21
Burn	12
Ear	6
Pus	5
Wood	2
Total	100

3.2.2. The Preparation of the Solutions as well as the Reagents

3.2.2.1. One percent glucose solution

A glucose solution with a concentration of one percent was made by dissolving one gram of glucose in one hundred milliliters of distilled water. In order to identify the creation of biofilm, it was added to the tryptic soy broth.

3.2.2.2. Phosphate buffer saline(PBS)

This solution was being utilized for washing in the biofilm detection technique. It was made by mixing together 50 milliliters of phosphate buffer saline and 950 milliliters of the distilled water [129].

3.2.2.3. Glacial acetic acid 33%

This solution was used to eliminate the excess stain that was produced during the biofilm detection technique. It was created by combining 33 ml from the glacial acetic acid and 67 ml of distilled water to produce 33 percent glacial acetic acid.

3.2.2.4. Crystal violet 0.25%

To make it, 0.25 milligrams of crystal violet material were dissolved into 100 ml of distilled water, and it was utilized in the process of staining the biofilm that bacteria form.

3.2.2.5. Tris Borate-EDTA-buffer solution (TBE)

The buffer had a concentration of 10X when it was received, and in order to get it down to 1X, 100 ml of 10X buffer was combined with 900 ml of distilled water. Both the agarose gel as well as the electrophoresis tank were filled with 1X buffer before to running the experiment [130].

3.2.3. Preparation of culture media

The culture medium was produced in accordance with the directions provided by the firm and then sterilized by placing them in an autoclave for 15 minutes at 121 degrees Celsius.

3.2.3.1. MacConkey agar medium

The preparation of the MacConkey agar medium was carried out in accordance with the procedure that was advised by the manufacturing business. It was used as a selective medium that grew exclusively Gram-negative bacterial species; it had the capability of further differentiating Gram-negative organisms based on its ability to digest lactose that was included in the media [129].

3.2.3.2. BHI agar

The preparation of the brain heart infusion agar was carried out in accordance with the instructions provided by the manufacturing business. When it was required to cultivate bacteria, this nutrient-rich media was utilized as the medium.

3.2.3.3. BHI broth

The preparation of the brain heart infusion broth followed the instructions provided by the firm that manufactured the product. This medium, which was reinforced by the addition of 15–20 percent glycerol, was used in the process of sustaining the development of bacteria as well as ensuring their preservation.

3.2.3.4. Tryptic Soy broth

This medium was made by dissolving 29.77 grams of media containing tryptic soy broth powder into 1000 milliliters of distilled water. After being sterilized by autoclaving at 121 degrees Celsius for fifteen minutes, this medium was used to detect the development of biofilm by *K. pneumonia*.

3.2.3.5. Congo red agar

The medium included Brain heart infusion broth at a concentration of 37 gm/l, sucrose at a concentration of 5 gm/l, agar number 1 at a concentration of 10 gm/l, and Congo red dye at a concentration of 0.8 gm/l. The Congo red stain was made as a determined aqueous solution before being autoclaved for 15 minutes at a temperature of 1210 degrees Celsius. After that, it was combined with autoclaved Brain heart infusion agar that contained sucrose at 550 degrees Celsius [131].

3.2.4. Preservation and Maintenance of Bacterial Isolates

- **Preservation for the near term**

Solitary, uncontaminated colonies derived from individual bacterial strains were kept alive in a limited week through sub-culturing them on BHI agar plates, making sure the plates were firmly wrapped with parafilm, and storing them at 4 degrees Celsius.

- **Preservation for the medium term**

The bacterial isolate was placed in sterile tubes that already included BHI agar slants. The tubes were then incubated around 37 degrees Celsius for one full day, after which they may be kept at 4 degrees Celsius.

- **Preservation for a very long time**

sterile tubes were filled with 5 ml of BHI broth, it was then injected with a single organism from *K. pneumoniae* The tubes were then placed in an incubator at 37 degrees Celsius for 24 hours, after which 800 l of the cell suspension was mixed with 200 l of 15 percent glycerol and stored at -20 degrees Celsius over many months.

3.2.5. Identification of Bacterial Isolates n by VITEK 2 – Compact

Because the VITEK2- Compact is a sophisticated colorimetric technology for the identification of bacteria and since it comprises of 64 biochemical tests as well as 20 antibiotic tests, it was used for the purpose of confirming diagnosis of the *Klebsiella. pneumoniae* by utilizing a Gram-negative card and also an Antibiotic sensitivity test card (AST-GN327) for the purpose of determining whether or not these isolates are susceptible to antibiotics. This card is able to define the Minimum Inhibitory Concentration (MIC) of *Klebsiella. pneumoniae* isolates.

- **Procedure**

  The following procedures were carried out in strict accordance with the manufacturer's instructions

1- Vortexing was used to suspend a single pure colony that had been picked up in three milliliters of physiological normal saline that had been put in a polystyrene tube.
2- For Gram-negative bacteria, the optical density of the suspension has to be verified using Densi check, and the result should fall somewhere in the range of (0.5-0.63) McF.
3- In order to determine the MIC, three milliliters of physiological normal saline were poured into a polystyrene tube. After that, 145 microliters of ID suspension were added to the mixture, and the tube was vortexed to ensure thorough mixing.
4- Following the insertion of the standardized inoculum into the cassette, a specimen identity card was then input into the computer program using a barcode.
5- After that, the barcode that had been put on the card during manufacturing was scanned, and the VITEK-2 card type was determined. The card was then linked to a sample identity connection.

6- It first cut the cassettes and provided the order (burden), which is a digital signal that has been retained since then. All of the tubes & cards in the cassette were put in the filter module. When the cards were full, the cassette was transferred to the reader/incubator module. A computer that is connected to the VITEK system receives the information on each sample from a rack that contains tubes and travels away from the device.
7- The remaining processes, such as the incubation temperature, as well as the optical scanning of the cards, are all controlled by the device, which also continually monitors and uploads test results to a computer for further analysis.

3.2.6. Detection of Biofilm Production

1- Micro-titer plate by using glucose and tryptic soy broth

The microtiter plate (MtP), which is also known as the 96-well plate, is a quantitative approach that is used by the microplate reader to quantify the amount of biofilm produced. Single bacterial colony was grown in a culture consisting of 5 milliliters of BHI broth, which was then heated to 37 degrees Celsius for a period of 24 hours. After that, the bacterial culture was diluted by adding 50 uL of bacterial broth to 950 uL of tryptic soy broth (TSB) that was supplemented with 1% glucose inside an Eppendorf tube. This was done so that the final concentration of bacteria was lower. The micro titer plate in the form of a U was employed. The first three wells were just filled with TSB as a negative control, and then 200 µl of bacterial suspension was put to the wells, 3 wells for each isolate.

The plate was incubated at 37 degrees Celsius for 24 hours. The micro titer plate was kept in an incubator at 37 degrees Celsius for a whole day. After the contents of each well were discarded and removed by tapping, the wells were washed three times with PBS in order to remove free-floating bacteria and planktonic cells without compromising the integrity of the biofilm. Finally, the micro titer plate was left to dry in an inverted position for an entire day. It was possible to evaluate the biofilm that had grown by staining the wells with 210 I of crystal violet solution containing 0.25 percent, which was kept there for 15 minutes before being discarded and rinsed 3 times via distilled water. After adding 210 l of glacial acetic acid at a concentration of 33 percent, the surplus CV stain was removed, and the absorbance was measured at 630 nm using a micro ELISA auto-reader.

2- Micro-titer plate by using glucose and brain heart infusion broth

After the bacterial cultures were titrated in the (BHIB) broth and the McFarland tube was used to adjust the concentration to be 0.5, 125 l was deposited in the wells of a sterile 96-well polystyrene microtiter plate. The plate was then placed in an incubator at 37 degrees Celsius for 24 hours with constant conditions. The following constituted the detection of the biofilm:

1- Aspiration was used to remove the contents of the wells once the incubation process was complete. The wells were then rinsed with distilled water four times to remove any cells that had not attached themselves.
2- After adding 125 µL of a solution of crystal violet (CV) diluted to a concentration of 0.1 percent to every well of microtiter plate, the plates were left to incubate at room temperature for 10-15 minutes.
3- In order to remove any remaining cells and dye from the plate, it was submerged in water, shaken quickly, and then blotted on a stack of paper towels. This process was repeated three to four times using D.W.
4- After drying for a few hours or overnight, the microtiter plate was inverted and placed on its upside down.
5- In order to dissolve the CV, a total volume of 125µL of glacial acetic acid with a concentration of 30 percent was applied to every well of the microtiter plate.
   Incubation of the microtiter plate took place at room temperature for ten to fifteen minutes.
6- A total volume of 125 µL of the CV that had been solubilized was then transferred into a clean microtiter plate with a flat bottom.
7- The absorbency was determined by using acetic acid at a concentration of 30 percent and reading the plate in a reader set to 630 nanometers.

- **Biofilm degree was calculated as follows:**

  Biofilm = OD1 – ODc

  - OD1 stands for experiments in which crystal violet was bound to wells that had been treated to culture media containing bacteria;
  - ODc stands for controls in which wells had only been exposed to culture medium devoid of bacteria were used.

3- Congo red agar method

The medium was streaked with a single colony of the bacteria, and then it was heated to 37 degrees Celsius for twenty-four hours. The results showed that biofilm formation had occurred when the colonies were black and crystalline in consistency, whereas non-slime producing bacteria typically remain pink. Isolates that produced black colonies with a black media were given results of +++ or ++++, but isolates that produced red colonies without a black medium were given results of ++ or +. The experiment was carried out three times for accuracy.

3.2.7. Molecular detection of *wcaG, fimH and mrkD* genes

3.2.7.1. Isolation of Genomic DNA

The genomic DNA extraction Kit was added by the manufacturing, and all of the subsequent processes were completed in accordance with its instructions.

1- After placing a single bacterial colony in 180 µL of GT buffer that was put in a 1.5 ml microcentrifuge tube, this pellet was resuspended via aggressively vortexing the contents of the tube.
2- After adding twenty microliters of proteinase K and incubating the mixture at a temperature of sixty degrees Celsius for at least ten minutes, the tube was turned upside down once every three minutes in order to ensure proper mixing.
3- To guarantee that the sample lysate is transparent, 200 µL of GB buffer was added, the mixture was agitated by vortexing for ten seconds, and then it was heated to 70 degrees Celsius for ten minutes. During the incubation process, the tube was turned upside down at regular intervals of three minutes.
4- The elution buffer has to be heated to 70 degrees Celsius before moving on to the next stage.
5- After incubation, 200 µL of 100% ethanol was placed in the sample lysate, and the mixture was well shaken to combine the two substances. The precipitate was divided up using a pipette at this point.
6- GD column was put inside of a collecting tube that had a capacity of 2 ml. The mixture, including any insoluble precipitate, was loaded to GD column and centrifuged at 14000 xg for two minutes. After the centrifugation, the 2 ml

collection tube that contained the flow-through was thrown away, and GD column was put in a fresh 2 ml collection tube.

7- After adding 400 µL of W1 buffer to the GD column and centrifuging it at 14000 xg for 30 seconds, the stream was discarded, and the GD column was reinserted into the 2 ml collecting tube.
8- In order to dry the column matrix, GD column was put back into a 2 ml collection tube after being centrifuged for 3 min at 14000 xg with 600 µLof Wash buffer added to it.
9- Transferring the GD column to a fresh, clean 1.5 microcentrifuge tube, adding 100 µL of pre-heated elution buffer into the middle of the column matrix, and then waiting three minutes for the elution buffer to absorb entirely. then 30 seconds of centrifugation at 14000 x g to elute the pure DNA.
10- At 4 oC, the DNA was kept.

3.2.7.2. Preparation of primers suspension

Following spinning down using nuclease-free water, the lyophilized primers were dissolved in order to resuspend the primers so that they could be used First, a stock solution was created by adding 10 times as much water as nmol of the primer mean; this resulted in a stock solution with a concentration of 100 µM. After that, a 1:10 dilution of the stock solution was created by combining ten microliters of the stock solution with ninety microliters of nuclease-free water. After that, this functional solution was kept at -20 degrees Celsius.

3.2.7.3. Genes detection

A- Primers

The primers that were used in the process of amplifying the genes are detailed in the following Table (3.7).

Table 3.7. Primers sequences for detection of *wcaG*, *fimH*, and *mrkD* genes

Gene		Primer sequence 5´---- 3´	Size of product
wcaG	F	GGTTGGTTCAGCAATCGTA	169 bp
	R	ACTATTCCGCCAACTTTTGC	
fimH	F	TGCTGCTGGGCTGGTCGATG	550 bp
	R	GGGAGGGTGACGGTGACATC	
mrkD	F	AAGCTATCGCTGTACTTCCGGCA	340 bp
	R	GGCGTTGGCGCTCAGGATAGG	

B- The reaction mixture

The DNA was amplified in a total volume of 25 µL, which included the following ingredients:

Table 3.8. The reaction mixture's components

Reaction mixture's components	Volume
PCR master mix	12.5
Forward primer	2
Reverse primer	2
DNA template	5
Nuclease free water	3.5

C- The PCR conditions for genes

PCR condition for *mrkD, fimH* and *wcaG* genes are detailed in the table (4-9, 4-10 and 4-11), respectively.

Table 3.9. PCR conditions for *mrkD* gene used in this study

Temperature	Time	Cycles
95°C	15 min	1
94°C	30 sec	29
60°C	90 sec	
72°C	60 sec	
72°C	10 min	1

Table 3.10. PCR conditions for *fimH* gene used in this study

Temperature	Time	Cycles
94°C	4 min	1
94°C	45 sec	35
55°C	45 sec	
72°C	1 sec	
72°C	7 min	1

Table 3.11. PCR conditions for *wcaG* gene used in this study

Temperature	Time	Cycles
95℃	15 min	1
94℃	30 sec	35
58℃	90 sec	
72℃	90 sec	
72℃	10 min	1

3.2.7.4. Amplified product detection by use agarose gel electrophoresis

The agarose gel was made by dissolving 1.5 grams of agarose powder throughout 100 milliliters of 1X TBE buffer that had been previously prepared in either a microwave for two min or maybe in a boiling water bath. After the mixture was allowed to cool to 55 degrees Celsius, 3 microliters of diamond nucleic acid dye were incorporated. The comb was fastened to one end of the tray so that it could be used to make wells, which were then used for loading DNA samples. After carefully pouring the agarose onto the tray, it was let to cool down at ambient temperature for half an hour so that it could become solid. After that, the comb was carefully taken away from the tray. After the tray was secured in the electrophoresis chamber and filled with TBE buffer so that it coated the surface of the gel, 3 ul of amplified PCR product was injected into each well of the agarose gel, and 5 ul of DNA ladder mixture was placed in one of the wells. The electric charge was let to remain at Ninety volts for a period of half an hour. In order to see the DNA bands, a UV transilluminator was used, and a digital camera was utilized in order to picture the gel.

3.2.8. Anti-biofilm

- **Clove extract procedure**

In order to make the clove aqueous extract, first clean the cloves to eliminate any dirt or other impurities, then weigh out 50 grams of ground cloves, and last combine this powder with 500 milliliters of distilled water. After the fumigation process, the rotary evaporator is used, and the temperature is maintained at less than 40 degrees Celsius until a viscous liquid is transferred to the hatchery for 2 or 3 days to be dried. After that, 5 grams are dissolved in 100 milliliters of distilled water so that the final concentration will be 5 percent (Al-Sodanii, 2004).

- **Modify procedure of anti-biofilm formation (sodium hypochlorite and carnation)**

1- Single bacterial colony cultured in 5 milliliters of BHI broth, which was then heated to 37 ^{o}C for a period of 24 hours.
2- In an Eppendorf tube, 950 µl of TSB that was going to be supplemented with 1% glucose was produced.
3- As a means of conducting a negative control experiment, a U-shaped microtiter plate was utilized, with each of its first three wells filled just with TSB.
4- Following the addition of 10 µl of bacterial suspension, another 100 µl of TSB was put on top of the bacterial suspension in the well.
5- Above each of them, 100 UI of sodium hypochlorite and\or 100 UI of carnation were added to the wells.
6- Incubation of the microtiter plate was place at 37 ^{o}C for 48 hours.
7- Wells were washed three times with PBS in order to remove free-floating bacteria and planktonic cells without compromising the integrity of the biofilm.
8- the microtiter plate was left to dry in an inverted position for an entire day.
9- Staining the wells with 210 I of crystal violet solution containing 0.25 percent, which was kept there for 15 minutes before being discarded and rinsed 3 times via distilled water.
10- The absorbance was measured at 630 nm using a micro ELISA auto-reader.

- **Evaluation the anti-bioflim activity after treatments**

The anti-biofilm effectiveness of the two different kinds of extract was evaluated based on the optical density value (Table 3-4 and figure 1), as well as the percentage of the biofilm that was still present after treatment with extract from each type was determined using the equation below: “Percentage of biofilm remaining= (1- mean of treatment- mean of control/ mean of control×100%)” [132]

3.2.9. Statistical analysis

To analyze the relationship between categorical variables and percentages, researchers used either Fisher's exact test or Pearson's chi-square test. The test used a significance threshold of a= 0.05, which was determined by the results. (Programs from SPSS version 23 that were used to analyze.

4. RESULTS

4.1. Isolation and Identification of *K. pneumoniae*

This study included 300 samples collected from patients suffering from different infections. These samples were collected from blood, sputum, burn, urine, ear, pus, and fluid 100 *Klebsiella pneumoniae* isolates were obtained from various clinical specimens are cultured overnight incubation at 37 °C on Blood agar and MacConkey agar show white large, mucoid, non hemolytic colony in blood agar and pink, large, mucoid and lactose ferment colony in MacConkey agar as shown in appendix(1) *K. pneumoniae* isolate were identify by different biochemical reactions such as VITEKE compact 2 system as shown in appendix (2).

4.2. Demographic Characteristics of Study Group

4.2.1. Distribution of gender according to age groups

In Table 4.1 and Figure 4.1, observe 20 (62.5%) cases out of 100 under the age groups (45-59) years were infected *K. pneumoniae* were female groups versus 12 (37.5%) were among male groups, while the less cases of *K. pneumoniae* infection under the age groups were (25-34) years among female groups 1 (16.7%), and 2 (20.0%) under the age group (15-24) years among male groups, statistically these differences were non- significant (Chi-square= 8.7, P-value= 0.06), during this current study.

Table 4.1. Distribution of gender infected with *K. pneumoniae* according to age groups

Age group (Years)	Gender (N=100)		Total
	Male (N=47)	Female (N=53)	
(15-24)	2 (20.0%)	8 (80.0%)	10 (100.0%)
(25-34)	5 (83.3%)	1 (16.7%)	6 (100.0%)
(35-44)	10 (47.6%)	11 (52.4%)	21 (100.0%)
(45-59)	12 (37.5%)	20 (62.5%)	32 (100.0%)
(60-74)	18 (58.1%)	13 (41.9%)	31 (100.0%)
Chi-square	8.7		
P-value	0.06 (N.S)		

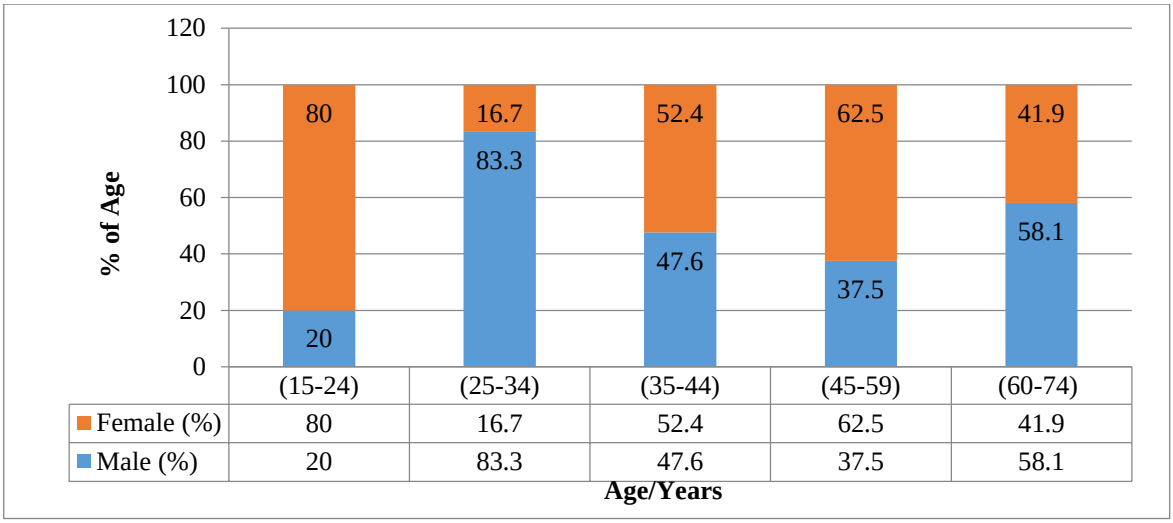

Figure 4.1. Distribution of gender infected with *K. pneumoniae* according to age groups

4.2.2. Distribution of gender according to clinical samples

In Table 4.2 and Figure 4-2, showed that most cases of *K. pneumoniae* infections among male group 17 (54.8%) out of 100 cases were picked up from blood samples, while among female groups were picked up from urine samples 15 (71.4%), less cases of *K. pneumoniae* infections were 0 (0.0%) from fluid sample among male groups and 2 (100.0%) among female groups under the same samples, these differences among the groups were non-significant (Chi-square= 7.4, P-value= 0.2). throughout this current study.

Table 4.2. Distribution of gender infected with *K. pneumoniae* strains according to clinical samples

Age group (Years)	Gender (N=100)		Total
	Male (N=47)	Female (N=53)	
Blood	17 (54.8%)	14 (45.2%)	31 (100.0%)
Urine	6 (28.6%)	15 (71.4%)	21 (100.0%)
Burn	5 (41.7%)	7 (58.3%)	12 (100.0%)
Sputum	13 (56.5%)	10 (43.5%)	23 (100.0%)
Ear	4 (66.7%)	2(33.3%)	6 (100.0%)
Pus	2 (40.0%)	3 (60.0%)	5 (100.0%)
Fluid	0 (0.0%)	2 (100.0%)	2 (100.0%)
Chi-square	7.4		
P-value	0.2 (N.S)		

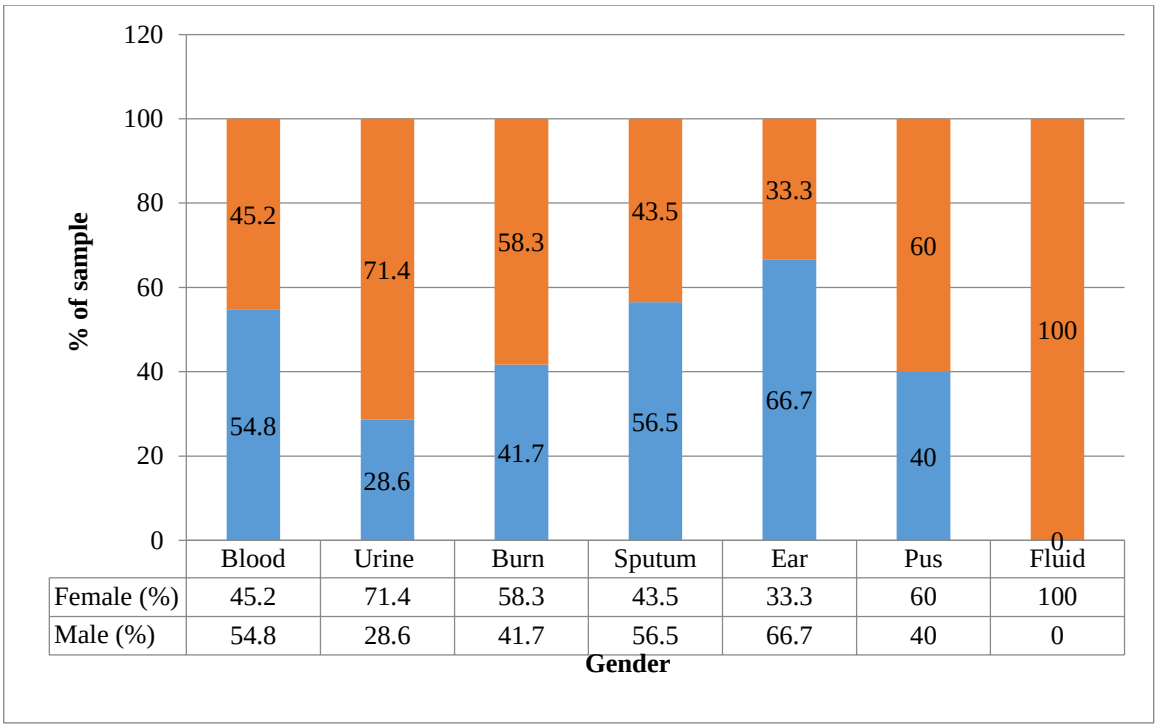

Figure 4.2. Distribution of gender infected with *K. pneumoniae* strains according to clinical samples

4.3. Biofilm Formation by *K. pneumoniae* Isolates

4.3.1. Biofilm formation by used Congo red agar

According to our results, one hundred percent of the *K. pneumoniae* isolates developed biofilms. According to the results of the biofilm study, the strains were classified into four distinct groups. 13 of the 100 *K. pneumoniae* strains that were examined produced biofilms with a score of (+++), 19 strains created biofilms with a score of (++), 52 strains produced biofilms with a score of (+), and 16 of the strains did not develop biofilms. In comparison to the isolates acquired from other sources, the *K. pneumoniae* strains that were recovered from blood, urine, as well as sputum samples had a strikingly higher capacity to produce score (+++) and (++) developed biofilms. high (+++) biofilm-formation capacity was seen in the blood isolates, which was observed to be greater than in other isolates. As seen in Table (4.3), Figures (4.3, 4.5) show results of biofilm formation of Congo red agar, tryptic soy broth and Brain- Herat infusion broth methods.

Table 4.3. Relationship between sample types and biofilm development using the Congo red agar procedure

Clinical sample	(N.%)	Congo red ager				Total
		Positive(+++)	Positive(++)	Positive(+)	Negative	
Blood	Count	5	6	15	5	31
	%	38.5%	31.6%	28.8%	31.3%	31.0%
Urine	Count	3	5	12	1	21
	%	23.1%	26.3%	23.1%	6.3%	21.0%
Burn	Count	0	5	7	0	12
	%	0.0%	26.3%	13.5%	0.0%	12.0%
Sputum	Count	4	1	11	7	23
	%	30.8%	5.3%	21.2%	43.8%	23.0%
Ear	Count	1	2	2	1	6
	%	7.7%	10.5%	3.8%	6.3%	6.0%
Pus	Count	0	0	4	1	5
	%	0.0%	0.0%	7.7%	6.3%	5.0%
Fluid	Count	0	0	1	1	2
	%	0.0%	0.0%	1.9%	6.3%	2.0%
Total	Count	**13**	**19**	**52**	**16**	**100**
	%	100.0%	100.0%	100.0%	100.0%	100.0%

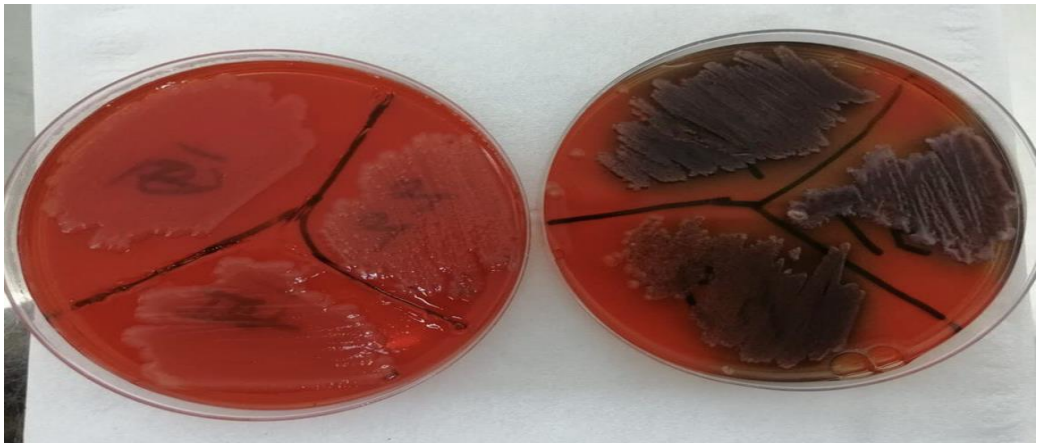

Figure 4.3. Biofilm formation by congo red agare

4.3.2. Biofilm formation by used tryptic soy broth

Our study revealed that 100% of the *K. pneumoniae* isolates produced biofilms. The strains were divided into four unique groups based on the findings of the biofilm investigation. Of the 100 *K. pneumoniae* strains tested, 17 strains generated strong biofilms, 14 strains produced moderate biofilms, 61 strains produced weak biofilms, and 8 strains did not

produce any biofilms at all. The *K. pneumoniae* isolates that were collected from sputum, blood, as well as urine samples, in addition to the samples themselves, exhibited a markedly increased potential to create strong and moderately developed biofilms when compared to the isolates that were collected from other sources. It was discovered that the sputum isolates had a significant ability for the development of biofilm, which was shown to be higher than that of other isolates. As may be seen in the Table 4.4.

Table 4.4. Relationship between sample types and biofilm development using tryptic soy broth procedure

Clinical sample		**(N.%)**	**Tryptic soy broth with glucose method**				**Total**
			Non-Biofilm	Weak	Moderate	Strong	
	Blood	Count	3	19	5	4	31
		%	37.5%	31.1%	35.7%	23.5%	31.0%
	Urine	Count	0	15	3	3	21
		%	0.0%	24.6%	21.4%	17.6%	21.0%
	Burn	Count	0	10	0	2	12
		%	0.0%	16.4%	0.0%	11.8%	12.0%
	Sputum	Count	4	7	6	6	23
		%	50.0%	11.5%	42.9%	35.3%	23.0%
	Ear	Count	0	4	0	2	6
		%	0.0%	6.6%	0.0%	11.8%	6.0%
	Pus	Count	1	4	0	0	5
		%	12.5%	6.6%	0.0%	0.0%	5.0%
	Fluid	Count	0	2	0	0	2
		%	0.0%	3.3%	0.0%	0.0%	2.0%
Total		Count	**8**	**61**	**14**	**17**	**100**
		%	100.0%	100.0%	100.0%	100.0%	100.0%

Figure 4.4. Biofilm formation by Tryptic soy

4.3.3. Biofilm formation by used Brain-Heart Infusion broth

According to our research, all *K. pneumoniae* isolates generated biofilms. On the basis of the results of the biofilm research, the strains were split into four distinct groups. Out of the 100 *K. pneumoniae* strains studied, 14 strains produced no biofilms at all, 68 strains produced weak biofilms, and 9 strains formed strong and moderate biofilms. The *K. pneumoniae* isolates obtained from sputum, blood, and urine samples showed a considerably enhanced propensity to form strong and moderately established biofilms in comparison to the isolates obtained from other sources. The potential of the sputum isolates to produce biofilm was found to be considerable and to be greater than that of other isolates. As seen in the Table 4.5.

Table 4.5. Relationship between sample types and biofilm development using Brain-Heart infusion procedure

Clinical sample	**(N,%)**	**Brain heart infusion broth method**				**Total**
		Non-Biofilm	Weak	Moderate	Strong	
Blood	Count	4	22	3	2	31
	%	28.6%	32.4%	33.3%	22.2%	31.0%
Urine	Count	3	13	3	2	21
	%	21.4%	19.1%	33.3%	22.2%	21.0%
Burn	Count	3	8	0	1	12
	%	21.4%	11.8%	0.0%	11.1%	12.0%
Sputum	Count	1	15	3	4	23
	%	7.1%	22.1%	33.3%	44.4%	23.0%
Ear	Count	2	4	0	0	6
	%	14.3%	5.9%	0.0%	0.0%	6.0%
Pus	Count	1	4	0	0	5
	%	7.1%	5.9%	0.0%	0.0%	5.0%
Fluid	Count	0	2	0	0	2
	%	0.0%	2.9%	0.0%	0.0%	2.0%
Total	Count	**14**	**68**	**9**	**9**	**100**
	%	100.0%	100.0%	100.0%	100.0%	100.0%

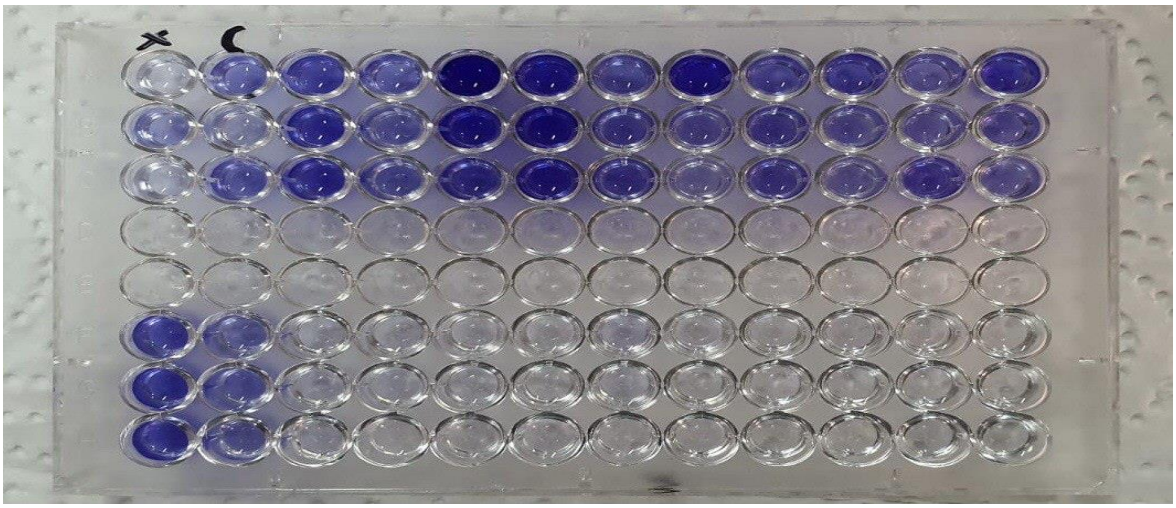

Figure 4.5. Biofilm formation by Brain-Heart infusion

4.3.4. Prevalence of biofilm-related genes among *K. pneumoniae* isolates

In Figure 4.6, observed there were 95 (95%) and 78 (78%), cases out of 100 of *K. pneumoniae* bacteria were produced *mrkD* and *fimH* gene respectively, while no *K. pneumoniae* strains could not produce *wcaG* gene 0(0.0%).

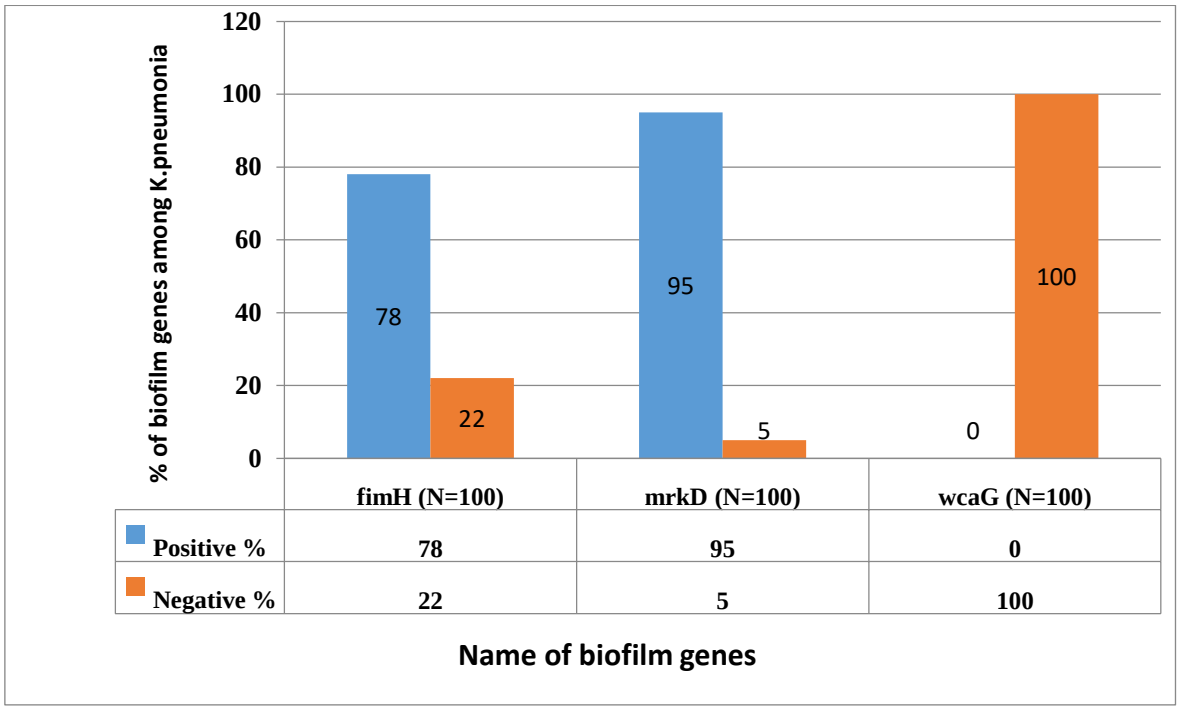

Figure 4.6. Prevalence of biofilm genes among *K. pneumoniae* strains (N=100)

In Figure 4.7 and Figure 4.8, demonstrated the gel electrophoresis for both fimH and mrkD genes PCR products.

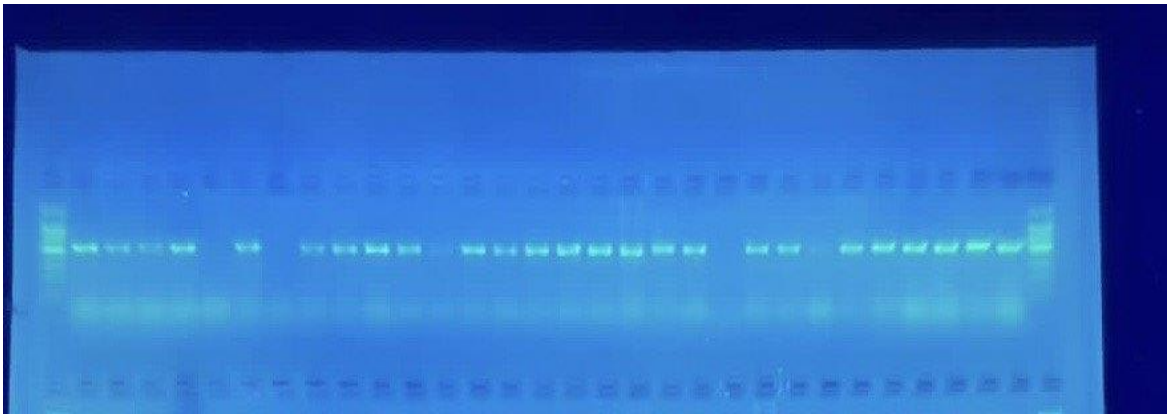

Figure 4.7 Gel electrophoresis for *fimH* gene PCR product with 550 bp

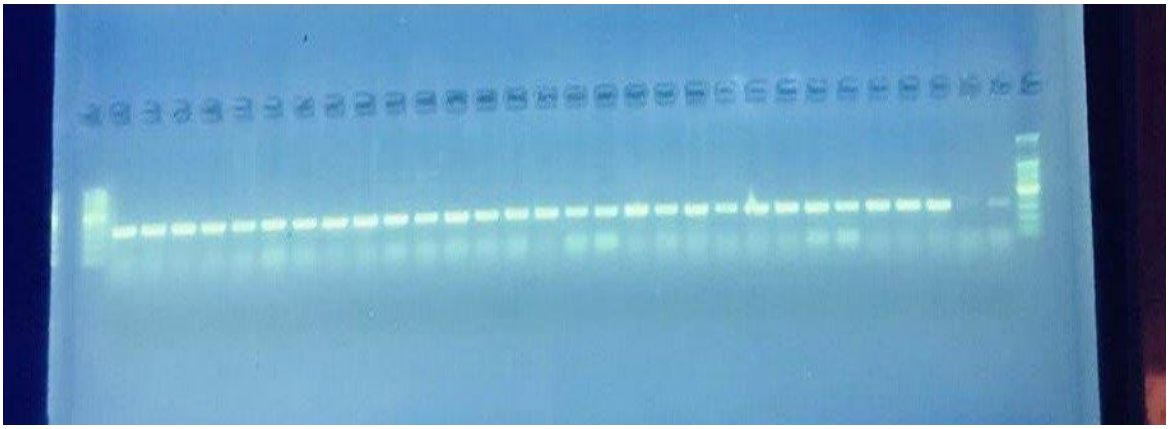

Figure 4.8. Gel electrophoresis for *mrkD* gene PCR product with 340 bp

4.3.5. Comparison between the results of biofilm genes patterns with results of Congo red agar method

In Table 4.6 and Figure 4.9, demonstrated that high percentage of *K. pneumoniae* isolates which contains fimH gene were 42 (53.8%) with positive score (+), while low percentage 11(14.1%) were with positive score (+++), in addition, 9 (11.5%) were negative biofilm producing. 49 (51.6%) were high percentage with positive score (+), while 13 (13.7%) were low percentage with positive score (+++), in contrast 14 (14.7%) were with negative biofilm producing in respect to *K. pneumoniae* contains mrkD gene. As well as there were no biofilm producing score in respect to *K. pneumoniae* contains wuca gene.

Table 4.6. Comparison between the results of biofilm genes patterns with results of Congo red agar method (n=100)

Name of genes	Result	Congo red agar method				*P-value
		Positive (+++)	**Positive (++)**	**Positive (+)**	**Negative**	
fimH (n=100)	Positive	11 (14.1%)	16 (20.5%)	42 (53.8%)	9 (11.5%)	0.1 (N.S)
	Negative	2 (9.1%)	3 (13.6%)	10 (45.5%)	7 (31.8%)	
mrkD (n=100)	Positive	13 (13.7%)	19 (20.0%)	49 (51.6%)	14 (14.7%)	0.2 (N.S)
	Negative	0 (0.0%)	0 (0.0%)	3 (60.0%)	2 (40.0%)	
WcaG (n=100)	Positive	-	-	-	-	-
	Negative	**13 (13.0%)**	**19 (19.0%)**	**52 (52.0%)**	**16 (16.0%)**	
***Chi-square, a. No statistics are computed because wuca is a constant, N.S, non-significant.**						

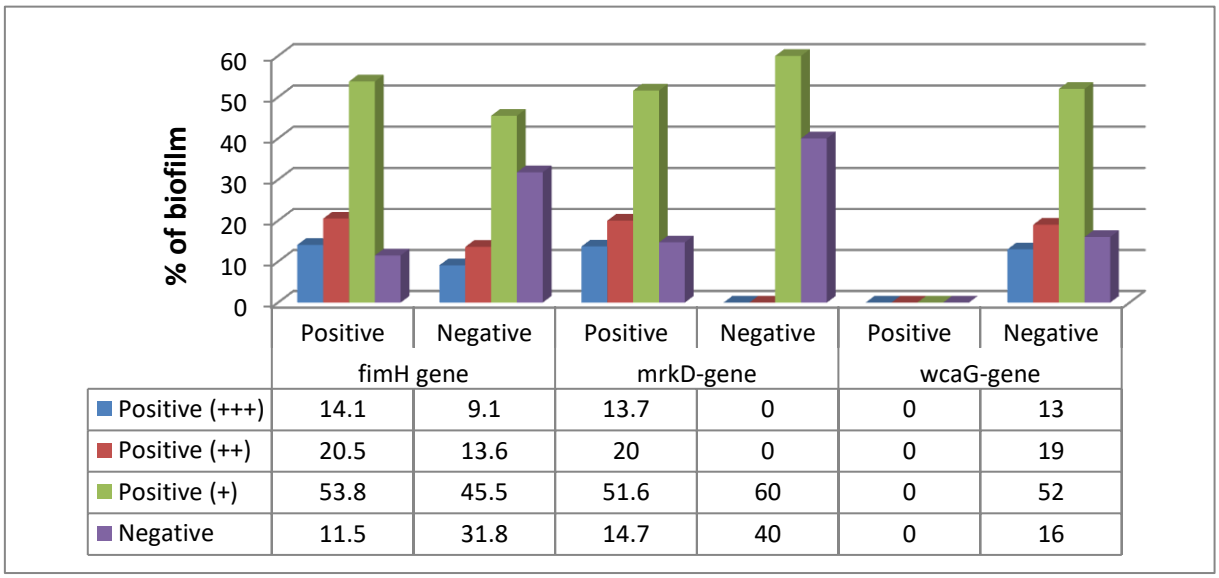

Figure 4.9. Comparison between the results of biofilm genes patterns with results of Congo red agar method (n=100)

4.3.6. Comparison between the results of biofilm genes patterns with results of Tryptic soy broth with glucose (TSBG) method

In Table 4.7 and Figure 4.10, demonstrated that high percentage of *K. pneumoniae* isolates which contains fimH gene were 47 (60.3%) with weak, and 15 (19.2%) in strong biofilm

formation, while low percentage 12(15.4%) were with moderate, in addition, 4 (5.1%) were negative biofilm producing. 57 (60%) were high percentage with weak, and 17 (17.9%) in strong biofilm formation, while 14 (14.7%) were low percentage with moderate, in contrast 7 (7.4%) were with negative biofilm producing in respect to *K. pneumoniae* contains mrkD gene. As well as there were no biofilm producing score in respect to *K. pneumoniae* contains wuca gene.

Table 4.7. Comparison between the results of biofilm resistance genes patterns with results of Tryptic soy broth with glucose (TSBG) method (n=100)

Name of genes	Result	Tryptic soy broth with glucose (TSBG) method				*P-value
		Non-Biofilm	Weak	Moderate	Strong	
fimH (n=100)	Positive	4 (5.1%)	47 (60.3%)	12 (15.4%)	15 (19.2%)	0.1 (N.S)
	Negative	4 (18.2%)	14 (63.6%)	2 (9.1%)	2 (9.1%)	
mrkD (n=100)	Positive	7 (7.4%)	57 (60.0%)	14 (14.7%)	17 (17.9%)	0.2 (N.S)
	Negative	1 (20.0%)	4 (80.0%)	0 (0.0%)	0 (0.0%)	
wcaG (n=100)	Positive	-	--	-	-	-
	Negative	8 (8.0%)	61 (61.0%)	14 (14.0%)	17 (17.0%)	
***Chi-square, a. No statistics are computed because wcaG is a constant, N.S, non-significant.**						

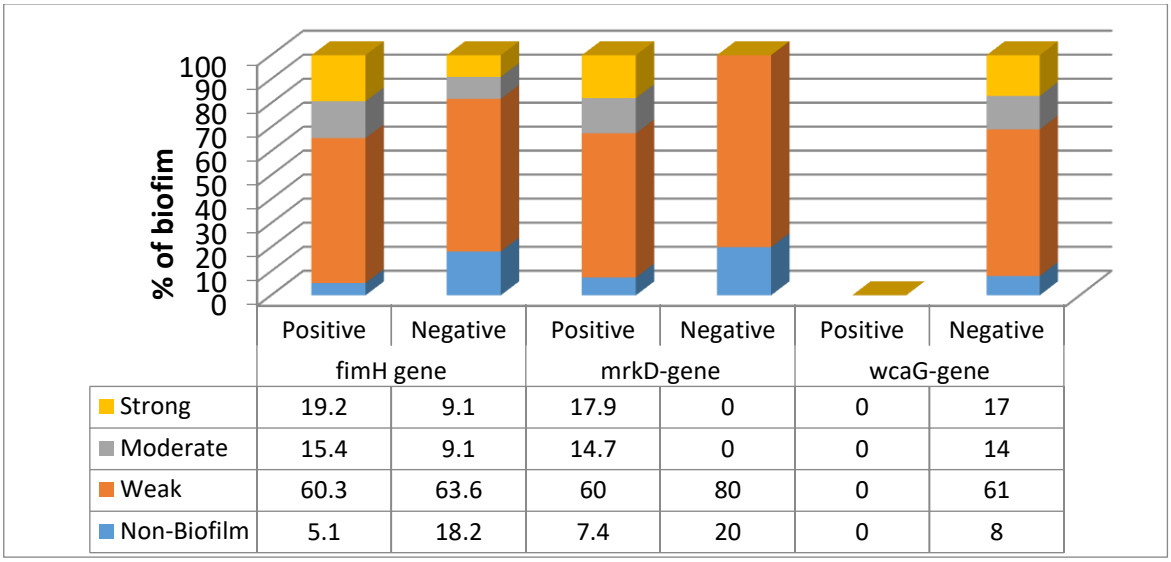

Figure 4.10. Comparison between the results of biofilm genes patterns with results of Tryptic soy broth with glucose (TSBG) method (n=100)

4.3.7. Comparison between the results of biofilm genes patterns with results of Brain heart infusion broth with glucose (BHIG) method

In Table 4.8 and Figure 4.11, demonstrated that high percentage of *K. pneumoniae* isolates which contains *fimH* gene were 52 (66.7%) with weak, and 9 (11.5%) in strong biofilm formation while low percentage 7(9.0%) were with moderate, in addition, 10 (12.8%) were negative biofilm producing. 67 (70.5%) were high percentage with weak, and 9 (11.5%) in strong biofilm formation while 8 (8.4%) were low percentage with moderate, in contrast 11 (11.6%) were with negative biofilm producing in respect to *K. pneumoniae* contains *mrkD* gene. As well as there were no biofilm producing score in respect to *K. pneumoniae* contains *wcaG* gene.

Table 4.8 Comparison between the results of biofilm genes patterns with results of Brain heart infusion broth with glucose (BHIG) method (n=100)

Name of genes	Result	Brain heart infusion broth with glucose (BHIG) method.				*P-value
		Non-Biofilm	Weak	Moderate	Strong	
fimH (n=100)	Positive	10 (12.8%)	52 (66.7%)	7 (9.0%)	9 (11.5%)	0.1 (N.S)
	Negative	4 (18.2%)	16 (72.7%)	2 (9.1%)	0 (0.0%)	
MrkD (n=100)	Positive	11 (11.6%)	67 (70.5%)	8 (8.4%)	9 (11.5%)	0.03 (S)
	Negative	3 (60.0%)	1 (20.0%)	1 (20.0%)	0 (0.0%)	
wcaG (n=100)	Positive	-	--	-	-	-
	Negative	14 (14.0%)	68 (68.0%)	9 (9.0%)	9 (9.0%)	

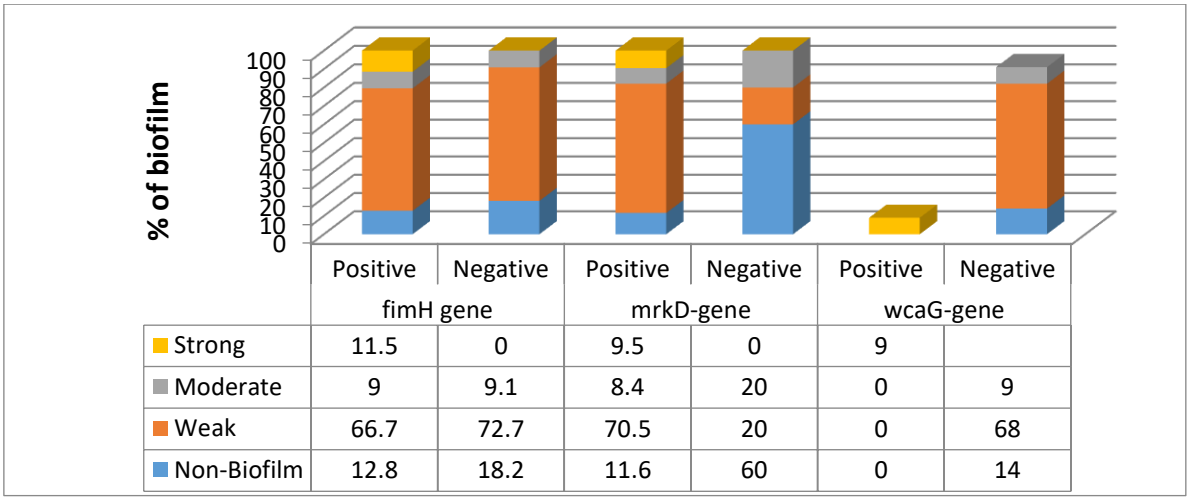

Figure 4.11. Comparison between the results of biofilm genes patterns with results of Brain heart infusion broth with glucose (BHIG) method (n=100)

4.4. Evaluation the anti-biofilm formed by *K. pneumoniae* isolates after treatment with sodium hypochlorite and carnation extract

In this study, the biofilm remaining ratio (%) after treatment of the formed biofilm was calculated as shown in tables (4-9,4-10) and figure (4-12); the results revealed that the both extracts exhibited anti-biofilm activity which indicated by reduced the amount of biofilm (biofilm remaining) after treatments with syzygies aromatic (carnation extract) and sodium hypochlorite solution both in 5% concentration respectively.

Table 4.9. Biofilm remaining ratio (%) after treatment of biofilm formed by *K. pneumoniae* isolates (N=10) with sodium hypochlorite solution concentration (5%)

Isolates that produce strong biofilm formation	Positive control	biofilm formation after treatments	% of biofilm remaining
K. pneumonia-1	0.295	0.019	6.44
K. pneumonia-2	0.312	0.0105	3.36
K. pneumonia-3	0.179	0.013	7.26
K. pneumonia-4	0.517	0.0035	0.67
K. pneumonia-5	0.438	0.023	5.25
K. pneumonia-6	0.267	0.023	8.61
K. pneumonia-7	0.305	0.021	6.88
K. pneumonia-8	0.282	0.029	10.28
K. pneumonia-9	0.453	0.0215	4.74
K. pneumonia-10	0.594	0.036	6.06

Table 4.10. Biofilm remaining ratio (%) after treatment of biofilm formed by *K. pneumoniae* isolates (N=10) with syzygies aromatic extract concentration (5%)

Isolates that produce strong biofilm formation	Positive control	biofilm formation after treatments	% of biofilm remaining
K. pneumonia-1	0.295	0.0275	9.32
K. pneumonia-2	0.312	0.0115	3.68
K. pneumonia-3	0.179	0.016	8.93
K. pneumonia-4	0.517	0.0055	1.06
K. pneumonia-5	0.438	0.001	0.22
K. pneumonia-6	0.267	0.081	30.33
K. pneumonia-7	0.305	0.027	8.85
K. pneumonia-8	0.282	0.0195	6.91
K. pneumonia-9	0.453	**0.001**	**0.22**
K. pneumonia-10	0.594	**0.0155**	**2.60**

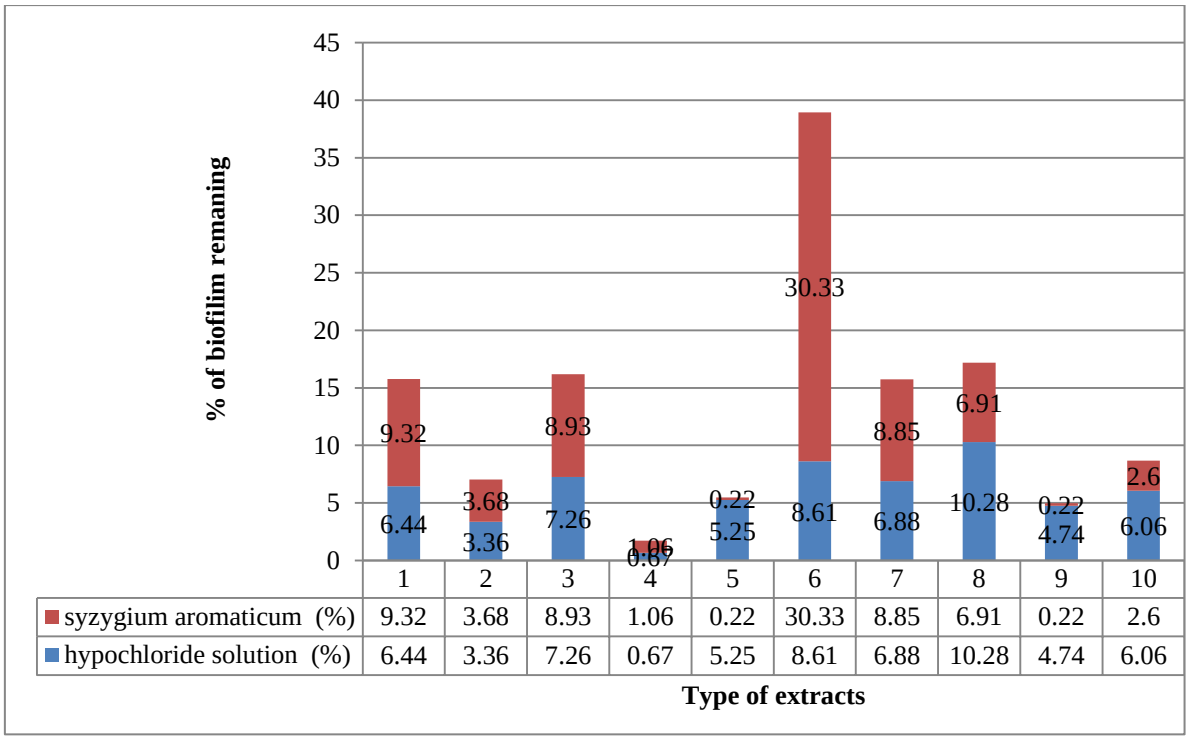

Figure 4.12. Biofilm remaining ratio (%) after treatment of biofilm formed by *K. pneumoniae* isolates (N=10) with syzygium aromaticum extract and sodium hypochlorite concentration (5%)

5. DISCUSSION

5.1. Study Group's Demographic Characteristics

5.1.1. A statistical representation of gender over the age spectrum

One hundred samples were isolated from those infected with the bacteria *Klebsiella pneumoniae*, where fifty-three of these isolates were taken from infected females, in contrast, forty-seven isolates were taken from male infected. And according to the sex distribution of patients according to age groups. This study showed that 20 (62.5%) cases out of 100 under the age groups (45-59) years were infected *K. pneumoniae* were female groups versus 12 (37.5%) were among male groups, while the less cases of *K. pneumoniae* infection under the age groups were (25-34) years among female groups 1 (16.7%), and 2 (20.0%) under the age group (15-24) years among male groups, statistically these differences were non- significant in respect to gender distribution according age groups during this current study.

Another research conducted in the same way included 159 patients and was divided into four age groups. This study discovered that 33 (56.9 percent) out of 159 isolates were men under the age group 65-74 years, whereas 30 (69.77 percent) of the isolates under the age group 75-84 years were females. While the less instances under the age group over 85 years 7 (33.33 percent) were from men, the fewer cases from females under the same age group were 14 in comparison to the fewer cases from males (66.67 percent). In addition, there were substantial disparities between men and females with regard to age groups [133]. In contrast, according to the findings of a number of research, the incidence of *K. pneumoniae*, which is the causative agent of community-acquired pneumonia, is more common in men (usually aged 65-74) [134]–[136]. However, the results of our research showed that it is more often correlated with females than with males. This is most likely due to the fact that the number of females living in our nation outnumbers the number of males.

5.1.2. Proportions of males and females based on clinical samples

During the course of this investigation, bacteria were isolated from a variety of clinical locations, including blood, urine, and sputum. In addition, bacteria were isolated from additional sites, such as pus, bodily fluids, as well as burn regions. With regard to the distribution of gender according to clinical samples, this study showed that the majority of cases of *K. pneumoniae* infections among the male group 17 (54.8 percent) out of 100 cases were collected from blood samples, while the majority of cases among female groups were picked up from urine samples 15 (71.4 percent). Fewer cases of *K. pneumoniae* infections were 0 (0.0 percent) from fluid sample among male groups, and 2 (100.0 percent) among female groups within the same samples. These variations between the groups were not statically important in any manner.

In a prior research that was carried out in Duhok City in the Kurdistan Region of Iraq, it was discovered that the prevalence of *K. pneumoniae* was found to be greater in females than in men in the urine, blood, wound, and oral samples. This was the case regardless of the sample type. On the other hand, the dissemination of the isolates was more widespread in the men than in the females, both in the central venous line and in the sputum sample [137]. Another study in Saudi Arabia also showed that the highest percentage of bacterial isolates was taken from sputum, while the percentages were equal for other clinical sites in males, while the highest percentage was taken from urine and the lowest percentage of sputum for females (ref). In contrast to the findings of our research, an additional investigation was carried out in Bangladesh, and the findings of that study suggested that male patients were more vulnerable to *K. pneumoniae* infection isolated from urine as well as wound samples than female patients were [138]. This disagreement is fairly difficult to understand and might be related to variances in the collection of samples, research design, questionnaire design, inclusion criteria of participants, environmental conditions, or personal cleanliness. Both of these explanations are rather complicated.

5.2. Biofilm Formation by *K. pneumoniae* Isolates

The ability of *K. pneumoniae* isolates acquired from clinical specimens to form biofilm was tested, as was the relationship between the capacity of biofilm production and sites of infection or the type of clinical sample. The purpose of these tests was to compare the various methods that were used for detecting biofilm during the course of this particular

study. According to results of biofilm formation by used Congo red agar methods. Thirteen of the 100 different *K. pneumoniae* strains that were studied developed biofilms with a score of (+++), Nineteen of the strains developed biofilms with a score of (++), fifty-two of the strains developed biofilms with a score of (+), and Sixteen of the strains did not grow biofilms. The *K. pneumoniae* strains that were collected from blood, urine, and sputum samples showed a dramatically greater potential to create scores (+++) and (++) formed biofilms as compared to the isolates collected from other sources. This was the case regardless of the type of sample. It was discovered that the blood isolates had a significant ability for the development of biofilm, which was shown to be higher than that of other isolates. It is currently believed that *K. pneumoniae* is a biofilm-forming bacteria. This bacterium has the potential to cause nosocomial opportunistic infections, and it also reduces the effectiveness of antibiotic therapies [139].

Using the Congo red agar technique, a different prior research also evaluated the biofilm development caused by *K. pneumoniae*. indicated that all of the isolates were capable of producing slime, of which 91.7 percent [140] were classed as strong biofilm developers and 8.3 percent [133] were categorized as moderate biofilm producers [141]. In addition, further research evaluated the biofilm development among *K. pneumoniae* isolates taken from a variety of clinical samples by using procedures including Congo red agar. According to the findings of the biofilm production test, 71% of the isolates that produced biofilm were classified as strong biofilm formers, 20% were classified as moderate biofilm formers, and 14% of the isolates produced weak biofilm. When compared to other kinds of specimens, there was a statistically significant link between the production of biofilm in isolates that were collected from urine [142].

In relation to the Congo red agar test, a different research was conducted for the purpose of performing a qualitative analysis of the pathogenic biofilm. After culturing of *K. pneumoniae* isolates on the medium at 37 °C for twenty-four hours, the results revealed that only the four biofilm-producing *K. pneumoniae* isolates were capable of changing the color of the medium from red to black, whereas the other isolates did not change the color of the medium from red to black. After allowing all of the isolates to develop for lengthy periods of time throughout the incubation process, it was discovered that the isolates were incapable of create biofilms [143]. In spite of this, a test revealed that there is a correlation between the color of the colonies and the robustness of the biofilm base. whereby dense black colonies indicate a significant amount of biofilm development, whilst red colonies

announce that there is no biofilm creation [140]. They proposed Congo red agar as a prescribed technique to morphologically analyze bacteria that create biofilms. When the findings of our research were compared to those of previous studies, we discovered that a significant proportion of *K. pneumoniae* isolates are capable of forming biofilms. The findings of our investigation showed that one hundred percent of the *K. pneumoniae* isolates were capable of producing biofilms when subjected to the tryptic soy broth procedure. In a study including 100 different strains of *K. pneumoniae*, only 8 of the strains did not form any biofilms at all, whereas 17 of the strains generated strong biofilms, 14 produced moderate biofilms, and 61 produced weak biofilms. When compared to the *K. pneumoniae* isolates that were collected from other sources, the *K. pneumoniae* isolates that were collected from sputum, blood, and urine samples displayed a significant elevated potential to create strong and moderately developed biofilms. This was the case for all three of these types of samples. It was found that the sputum isolates had a considerable aptitude for the creation of biofilm, which was proved to be greater than that of other isolates. In a recent research, the biofilm formation caused by *K. pneumoniae* isolates was evaluated using the technique of tryptic soy broth, which was quite similar to the data that we obtained. According to the results of this investigation, 62 (or 74.5%) of the isolates produced biofilm. There were four distinct groups that the strains fell into. According to the findings of the biofilm study, 27 (32.5%) of the isolates developed biofilm in a weak manner, 18 (21.6%) of the isolates created biofilm in a moderate manner, and 17 (20.4%) of the isolates were strong creators of biofilms [144].

An examination of the creation of biofilms by *K. pneumoniae* strains that were isolated from several samples was carried out by Yang and colleagues, who found that 62.5% of the isolates produced biofilms [145]. There were a total of 167 *K. pneumoniae* isolates, and of them, 143 (85.63%) were biofilm producers. The remaining 24 (14.37%) isolates were not biofilm producers. There were 50 (29.94%) isolates that were classified as weak biofilm producers, 45 (26.95%) isolates that were strong biofilm producers, and 48 (28.74%) isolates that were recognized as intermediate biofilm producers [139]. According to the findings of the research carried out by Hassan et al., out of the 110 *K. pneumoniae* strains that were examined, 70 of the isolates (64.7%) were found to be high or moderate biofilm producers, whereas 40 of the isolates (35.3%) were found to be weak biofilm producers [146].

According to brain heart infusion methods, the results of the biofilm formation demonstrated out of the 100 *K. pneumoniae* strains studied, 14 strains produced no biofilms at all, 68 strains produced weak biofilms, and 9 strains formed strong and moderate biofilms. The isolates obtained from sputum, blood, and urine samples showed a considerably enhanced propensity to form strong and moderately established biofilms in comparison to the isolates obtained from other sources. The potential of the sputum isolates to produce biofilm was found to be considerable and to be greater than that of other isolates. In the prior investigation, 94 *K. pneumoniae* isolates came from two hospitals throughout Tehran, Iran. These facilities are located in Iran. An altered version of the brain-heart infusion test was used to investigate the production of biofilm. The results of the study showed that 93.6% of the isolates produced biofilms. The strains were categorized into the following four groups: According to the results of the biofilm analysis, 52.1% of the isolates were classified as strains that produced moderate amounts of biofilm, 33% of the strains formed fully established biofilms, 8.5% of the strains produced minimal biofilms, and 6.4% of the strains did not produce any biofilm at all. In comparison to the isolates that were acquired from blood samples, the *K. pneumoniae* strains that were collected from sputum, surgical-wound swabs, and urine samples exhibited a remarkable increase in their capacity to produce biofilms that were both completely formed and moderately developed [147]. Differences in the findings of various research may be attributed to a variety of factors, including the region, kind, and quantity of samples used, as well as the features of the bacterial isolates themselves, which may include antibiotic resistance trends.

In comparison among Congo red agar, tryptic soy broth and brain-heart infusion methods in order to evaluation of biofilm formation by *K. pneumoniae* isolates obtained from several clinical samples during this current study. And according to results from all methods, this results demonstrated the highest count 17 of 100 isolates were categorized belong strong biofilm formation detected on tryptic soy broth, followed by 13 isolates belong same categorized was detected on Congo red agar, and lowest count was detected on brain-heart infusion 9 isolates with strong biofilm formation. These findings suggested that the Congo red agar technique and the brain-heart infusion method are neither as accurate or as quantitative as the tryptic soy broth method for detecting the formation of biofilms. Instead, the tryptic soy broth approach was shown to be the most effective method. In addition to the techniques of preparation and standards used in the

measurements, the nature of the interaction that occurs between bacteria and the fundamental components that are employed in each of these three approaches is a potential contributor to the discrepancies that have been observed. Another studies compared the biofilm formation among different microorganisms by using various methods and demonstrated that the tissue culture plate method is a more quantifiable and dependable technique for the detection of biofilm-forming microorganisms as compared to the tube and Congo red agar methods. This conclusion was reached as a result of the fact that the tissue culture plate method detected a greater number of biofilm-forming microorganisms [146], [148].

5.3. The Prevalence Of Genes Associated To Biofilm Production Among *K. pneumoniae* Isolates

It has been shown that the production of biofilm is influenced by a variety of fimbrial adhesins. The majority of *K. pneumoniae* isolates have been shown to exhibit type 3 fimbrial adhesion [149]. During the course of this investigation, the distribution of genes that are thought to play a part in the process of biofilm development was analyzed. According to the findings of this research, there were 95 (95 percent) and 78 (78 percent) instances out of 100 of *K. pneumoniae* bacteria that generated the *mrkD* and *fimH* genes, respectively, but none of the *K. pneumoniae* strains were unable to create the *wcaG* gene 0 gene (0.0 percent).

Another study investigated the distribution of *mrkD* and fimH among biofilm formation isolates and found that the fimbrial (*mrkD* and *fimH* genes) among these isolates were 100 percent and 86.95 percent respectively. This was shown by the fact that the study found that the biofilm formation isolates contained both of these genes [150]. Moreover, the *mrkD* gene was found to be present in 38.9 percent of the isolates when research was conducted that also looked at the incidence of fimbrial genes associated with *K. pneumoniae.* in addition, *fimH* genes are found in 27.8 percent of the isolates [151]. On the other hand, the encoding genes *mrkD* and *fimH* were discovered in 38.9 percent and 27.8 percent of isolates, respectively, in the research that was previously reported [152].

In an additional investigation that had been conducted in Iraq in the past, the presence of the *mrkD* gene in all biofilm-producing *K. pneumoniae* was investigated All of these isolated specimens tested positive for the *mrkD* gene, as was discovered [5]. Numerous

studies have shown that type 3 fimbriae, which are controlled by the *mrkD* gene, are essential for the production of biofilm by *K. pneumoniae* [153], [154]. Therefore, providing evidence that there is a connection between the presence of the *mrkD* and *fimH* genes and the production of biofilm. In contrast to the findings of this investigation, *wcaG* gene was found in over half of the specimens, and there was a statistically massive disparity between the production of biofilm and the presence of this gene [155]. The capacity to create biofilm was discovered to be linked to the *wcaG* gene, according to the findings of a research that was carried out by Zheng and colleagues. Additionally, inhibiting this gene might result in a decrease in biofilm [156]. Differences in the results of prior investigations may be attributable to a variety of circumstances, including variations in the expression of virulence determinants, biofilm development, or the source of sample isolation. It is recommended that more research be conducted on the processes of biofilm development in *K. pneumoniae*.

5.4. Comparison Between the Results of Biofilm Genes Patterns With Results of Congo Red Agar, Tryptic Soy Broth and Brain-Heart Infusion Methods

The development of biofilm by bacterial pathogens has garnered a lot of interest recently due to the fact that it is a significant virulence element that makes infection difficult to eradicate. Earlier studies have shown that there is a connection between the presence of certain virulence genes in certain bacteria and their propensity to produce biofilms [156], [157]. Throughout the whole of this present research, an inquiry was carried out with the goal of determining whether or not the *FimH, merkD,* or *wucaG* genes have a part in the production of biofilm in *K. pneumonia*. These findings demonstrated a high frequency of *fimH* and *merkD* among biofilm-forming isolates, compared with negative biofilm-forming isolates when utilizing all three techniques. The frequency of *fimH* and *mrkD* was 78 and 95 out of 100, respectively, of the total isolates.

Isolates that were positive for *wcaG, fimH,* and *mrkD* were shown to display more biofilm formation than isolates that were negative for these genes. This was the conclusion of another research that was conducted in the past [158]. Another research found that the *mrkD* and *fimH* genes, which code for type 1 and type 3 fimbrial adhesion involved in biofilm formation, were present in all strains of biofilm formers. This confirms the findings of our investigation, which indicated that these genes are present in all biofilm formation

strains. The prevalence of genes related with biofilm, such as *fimH* and *mrkD*, was consistently 88% across all bacteria that were investigated [142].

Also there was no detection of *wcaG* gene among *K. pneumoniae* isolates during this current study, in contrast to our results, the authors of a second research revealed that biofilm development was more widespread in isolates that were positive for the genes *magA, wcaG, iutA,* and *rmpA* and *allS* than in isolates that were negative for the identical genes. Although they stated that *wcaG* only had an important role in the production of *K. pneumoniae* biofilm based on several logistic analyses, they found that *wcaG* was necessary [156]. These variations may be attributable to a variety of variables, such as the locations of sample collection, the procedures for collecting samples, and the storage conditions. Therefore, more research is required to understand the function that these genes play in the production of biofilm and to identify the underlying factors that contribute to the differences in findings across studies.

5.5. Impact Sodium Hypochlorite and Carnation on Anti-Biofilm Formed by *K. pneumoniae* Isolates

In recent times, *K. pneumoniae* has emerged as one of the principal pathogens responsible for nosocomial infections. These infections are associated with a high rate of morbidity and death. *K. pneumoniae* possesses a wide array of antimicrobial resistance genes, which allows it to gain resistance to many drugs. This may make it difficult to treat infections caused by this bacterium [159]. *K. pneumoniae* in vivo can avoid as well as resist the clearance of the immunity of the host and the majority of antimicrobial agents due to the formation of biofilms, which is a significant part in the mechanism of antimicrobial resistance. This results in a chronic infection and the inability of antibiotic treatment to cure the infection [160].

As a result, the management and prevention of biofilm infections has developed into a substantial challenge. In this work, we evaluate how the use of sodium hypochlorite as well as carnation affects the production of biofilm by *K. pneumoniae* that has been isolated from a variety of clinical locations. In this study, the biofilm remaining ratio (%) after treatment of the formed biofilm was calculated and the results revealed that the both extracts exhibited anti-biofilm activity which indicated by reduced the amount of biofilm

(biofilm remaining) after treatments with carnation (syzygium aromaticum) and sodium hypochlorite solution both in 5% concentration.

Similar to this one, a prior study looked into the impact of sodium hypochlorite on the development of biofilms by *K. pneumoniae* isolates. The findings indicate that sodium hypochlorite has an inhibitory as well as clearance effect on the development of *K. pneumoniae* biofilms with various drug resistance, and that the effect was amplified with an increase in concentration within the range of bactericidal and bacteriostatic concentration. The same research also examined its suppressive impact on *K. pneumoniae* biofilm development and discovered that, after the addition of various sodium hypochlorite concentrations, the biofilm production steadily rose from day 1 to day 5. Nevertheless, it significantly dropped when compared to the positive control group, showing that the addition of sodium hypochlorite had a negative impact on the capacity of bacteria to build biofilms [161].

Studies conducted in the past have shown that sodium hypochlorite inhibits the development of bacteria. These studies also discovered that concentrations of sodium hypochlorite of 0.25 percent, 0.5 percent, and 1 percent had the most effective antibacterial impact on the bacteria that were examined. Unfortunately, the focus of these investigations was only on the lethal impact that sodium hypochlorite has on plankton; no consideration was given to the role it plays in the creation of biofilm (ref, ref). Certain researchers have documented the impact of sodium hypochlorite on the biofilm of S. aureus, while others have examined the activities of different disinfectants and found that the activity of biofilm greatly reduced [162]–[164]. The research done by Tiwari and colleagues demonstrated that sodium hypochlorite had a substantial impact on inhibiting the development of biofilm by S. aureus [165]. Cai and colleagues discovered that sodium hypochlorite considerably decreased the activity of Enterococcus faecalis biofilm, and they discovered that the treatment effect was significantly enhanced with the rise of sodium hypochlorite concentration [166].

There are two possible explanations for sodium hypochlorite's effectiveness against bacterial biofilm: first, the development of most natural biofilms may begin at a pH that is near to neutral (Fukuzaki, 2006). Any major change in pH that occurs either below or above 7 will have a direct and immediate influence on the metabolic processes of microbes as well as the characteristics of solid surfaces. It does this by either increasing or

decreasing the electrical repulsion that exists between the two entities, and as a result, it disrupts the ability of germs to adhere to the surface. Hypochlorite solution, which has an alkaline pH (pH > 11), is capable of dissolving biofilm cells by increasing the repulsive forces among bacterial cells as well as the surface of the substance [168]. Second, Line back put out the hypothesis that sodium hypochlorite breaks down proteins inside the biofilm matrix and hinders the action of key enzymes in bacterial cells, hence causing the death of bacterial cells in biofilms in an irreversible manner [169]. On the other hand, there is not enough information to explain how certain components undermine the integrity of biofilms.

According to investigation of carnation impact on biofilm formation by *K. pneumoniae*, there was no previous study utilized this compound on this bacterium. On the other hand, earlier research that was carried out in Egypt used various amounts of eugenol, and it was tested against biofilms that were created by 50 distinct MRSA strains. It indicated that the successful eradication of the formed biofilms was detected at MIC or 2x MIC when contrasted to the control specimens [170]. Antonia et al., who reported that the formation of biofilms was inhibited in vitro by the presence of phenol-containing phytocompounds like as eugenol [171]. This was also documented in another investigation, which found that eugenol demonstrated substantial action against MRSA clinical strains biofilms at either the MIC or the 2 MIC concentrations. It prevented new biofilms from forming, helped detach any existing biofilms, and eradicated any MRSA germs that were present in biofilms [171].

In the further investigation, Rosemary Officinalis. L (EO) was utilized to treat *K. pneumoniae*; the results showed that there were differences in the MIC value of EO across the different isolates [172]. Although Rosemary was utilized against *K. pneumoniae*, similar effects were found when the ethyl acetate extract of Rosemary was applied. The reported differences in MIC value across isolates might be due to the existence of varied inherent degrees of resistance to antimicrobials among the tested bacteria. On the other hand, aqueous as well as crude extracts were able to inhibit the bacteria at greater concentrations [173]. Due to the presence of the capsule, the Quercus brantii ethanol extract did not inhibit *K. pneumoniae*. On the other hand, cinnamon and green tea showed the strongest antibacterial activity against this particular bacterium [174]. Therefore, more research is required so that we can analyze and comprehend the mechanism of influence that eugenol has on the development of biofilm by *K. pneumoniae*.

6. CONCLUSIONS AND RECOMMENDATIONS

- The investigation revealed that a substantial member of *K. pneumoniae* strains isolated from sputum, burn, urine, as well as blood samples were able to form fully developed biofilms. In contrary, *K. pneumoniae* strains that were obtained from samples of pus, ear fluid, and fluid had a reduced propensity to create significant biofilms. In light of these results, there is sufficient indication to backing the suggestion that there is a connection between infection and the production of biofilm.
- This study found that the utilize of tryptic soy broth is easily quantifiable and may be a dependable method to detect biofilm formation when compared to the use of Congo red agar and the brain-heart infusion methods. The use of the tryptic soy broth method can be suggested as a assay technique for the recognition of bacteria that produce biofilms.
- The presence of the *mrkD* and *fimH* genes at high frequency in isolates of *K. pneumoniae* suggested that these genes may be crucial in the pathogenesis of this bacterium. Additionally, the correlation among the capacity to produce biofilm and presence of *mrkD* and *fimH* genes among the *K. pneumoniae* bacteremia isolates implies that these genes might be effective treatment targets.
- The production of biofilm by *K. pneumoniae* strains with varying levels of antibiotic resistance may be inhibited and cleared by sodium hypochlorite as well as carnation, in particular Syzygium aromatics. Additionally, this impact was amplified with increasing concentrations that fell between the range of those that were bacteriostatic and those that were bactericidal.

6.1. Recommendations

- Additional studies regarding the use of different methods to determine and accurately measure biofilm production in order to avoid factors that play a role in inhibiting biofilm production, such as the method of tissue culture and biofilm gene

expression using polymerase reaction methods such as real time- “polymerase chain reaction”.

- Recommendation for other studies to identify other virulence genes and reveal their role through the biofilm formation, in addition to their effect on pathogenesis and antibiotic resistance by bacteria.
- The use of chemicals with a small toxic effect in addition to plant materials and extracts as anti-biofilms with the purpose of diminish the extent of bacteria and reduce their resistance to antibiotics, especially in health institutions and hospitals.

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APPENDIXES

Appendix 1: Ethics Committee Report

Republic of Iraq
Ministry of Health/Environment.

Consent form of a research project
The initial Consent form of a research project to collect the samples from Ministry of Heath
website www.moh.gov.iq

Title in English language:

MOLECULAR DETECTION OF FIM H, MRKD AND WCAG GENES RESPONSIBLE FOR BIO-FILM FORMATION IN KLEBSIELLA PNEUMONIAE ISOLATED FROM DIFFERENT SITES OF INFECTION(

1- Details of the main author/researcher:

Name	The scientific title/ Career Title	Work address	Phone number	Email
NOOR ABDUL JABBAR KHALAF	Master student	KIRSEHIR AHI EVRAN UNIVERSITY HEALTH SCIENCES INSTITUTE DEPARTMENT OF MOLECULAR MEDICINE	7710893909 00964	MAYTHEMQASSIM@GMAIL.COM

Details of the contributed author/researcher:

Name	The scientific title/ Career Title	Work address	Phone number	Email
A. Dr. Estabraq Ali MAKLEF	Assistant prof Doctor, lecturer of MEDICAL LAB	COLLEGE OF HEALTH AND MEDICAL TECH	07711428804	ASTABRAQALS124@GMAIL.COM
Prof. Dr. Ergin KARİPTAŞ	Assistant prof Doctor, lecturer Department of Medical Microbiology	,Samsun University ,Faculty of Medicine Department of Medical Microbiology	00905052904114	ergin.kariptas@samsun.edu.tr

16/6/2021

2- The Scientific Supervisor/ if found

Name	The scientific title/ Career Title	Work address	Phone number	Email
None				

Work background:

mrkD and fimH genes in K. pneumoniae are associated with biofilm formation, making them interesting appropriate treatment optionsand more

A. The importance of the research and its objectives:

1- Detection the prevalence of K. pneumonia isolated from different site of infections.

2- Studying the expansion of K. pneumonia in different age categories in both sexes.

3- Investigating the biofilm forming ability of K. pneumonia isolates.

4- Molecular detection of the presence of fimH, wcaG and mrKD genes.

5- Detection the antibiofilm formation by sodium hypochlorite and cloves extract. Conclusion:

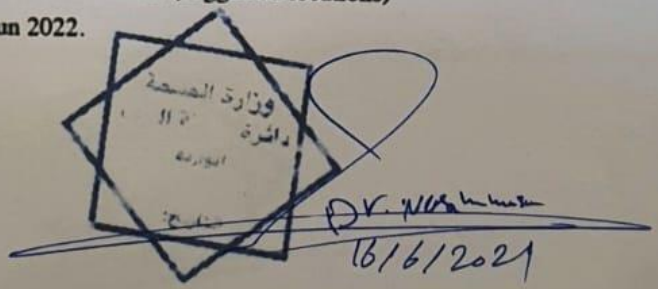
3- People or martials that are required for this research from the Ministry of Health.?

Martials	Type
Laboratories samples included blood, urine, sputum, plus swabs from burn as well as wound cases	Bacteria were grown in an aerobic envi- . ronment at 37 degrees
Martials/ equipment's	(No need (Privet
information from the patients Records	No need
Patients or other staff members	The study included a total of 100 male AND FEMALE
Others	No

Time and the date to perform the research: (suggested locations)

Time: 1st April 2021- 1st Jun 2022.

Locations:



Name of institute	Approval
Medical city department	

4- Fund: None.

5- Methodology:

A. Study design: Laboratories samples included blood, urine, sputum, plus swabs from burn as well as wound cases collected from 100 patients

B. Studying the expansion of K. pneumonia in different age categories in both sexes..

C. Investigating the biofilm forming ability of K. pneumonia isolates

D. Molecular detection of the presence of fimH, wcaG and mrKD genes..

E. Detection the antibiofilm formation by sodium hypochlorite and cloves extract. mention

F. Signed Commitment:

This is **NOOR ABDUL JABBAR KHALAF** I signed below to commit that I perform the research according to this protocol. Also, I commit that I will never change or modify it after it is being approved unless agreed with research committee in the health institute. Moreover, I commit following the laws, rules and instructions of Iraqi health ministry and any other official parties that follow the scientific and ethical commitment for research.

Name and the signature of the main researcher: **NOOR ABDUL JABBAR KHALAF**

The name and the signature of the supervisor/ if the research is performed to obtain a BSc, MSc or PhD etc: **ESTABRAQ ALI MEKLEF**

Approval of the research committee at the health institution (or the body authorized to approve this form)

وزارة الصحة

Dr.

6/6/2021

CURRICULUM VITAE

Personal Information	
Name and surname	NOOR ABDUL JABAR
Place of birth	
Date of birth	
nationality	❒ T.C. ☑ Diğer:

Education Information	
License	
University	Middle Technical University
Faculty	College of Health And Medical Tech.
department	Department of Analysis
Graduation Year	2011

Master	
University	Kirsehir Ahi Evran University
Institute Name	Health Sciences Institute
Department	Molecular Medicine
Program	
Date of graduation	2020- Currently

Articles and Papers

1. NOOR ABDUL JABAR, Ergin Kariptas, ESTABRAQ ALI MAKLEF "MOLECULAR DETECTION OF fim H ,mrkD ,and wcaG genes responsible for biofilm formation in *Klebsiella pneumoniae* isolated from different sites of infection international symposium on current developments in science ,technologyand social sciences septemper 16-18 ,2022 /malatya,turkiye page 598