NEAR EAST UNIVERSITY

INSTITUTE OF GRADUATE STUDIES

DEPARTMENT OF MEDICAL MICROBIOLOGY AND CLINICAL MICROBIOLOGY

BIOFILM FORMATION TENDENCY AND ANTIMICROBIAL SUSCEPTIBILITY OF ESCHERICHIA COLI ISOLATED FROM VARIOUS CLINICAL SAMPLES OF PATIENTS WITH UTI

M.Sc. THESIS

Qusai ALAZZEH

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DEDICATION

I hereby declare that the work in this thesis entitled “Biofilm formation tendency and antimicrobial susceptibility of Escherichia coli isolated from various clinical samples of patients with UTI” is the product of my own research efforts undertaken under the supervision of Assoc. Prof. Dr. AYSE ARIKAN SARIOGLU. No part of this thesis was previously presented for another degree or diploma in any university elsewhere, and all information in this document has been obtained and presented in accordance with academic ethical conduct and rules. All materials and results that are not original to this work have been duly acknowledged, fully cited and referenced.

QUSAI NUMAN ABDALLAH ALAZZEH

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Date:

STATEMENT (DECLARATION)

The data presented in this thesis was obtained in an experiment carried out in the microbiology laboratory/ King Abdullah University Hospital. I played a major role in the preparation and execution of the experiment, and the data analysis and interpretation are entirely my own work.

I am aware of and understand the NEAR EAST UNIVERSITY'S policy on plagiarism and I certify that this thesis is my own work I had no unethical behavior in all stages from the planning of the thesis until writing, except were indicated by the references, and the work presented in it has not been submitted in support of another degree or qualification from this or any other university or institute of learning.

QUSAI NUMAN ABDALLAH ALAZZEH

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QUSAI NUMAN ABDALLAH ALAZZEH

Biofilm Formation Tendency and Antimicrobial Susceptibility of Escherichia Coli Isolated from Various Clinical Samples of From Patients With UTI.

Qusai Numan Abdallah Alazzeh.

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Abstract

Objective: The aim of this study was to distribution pattern of *E. coli* organisms and evaluate Biofilm formation capacity of *E. coli* isolates previously isolated from UTI patients determine the prevalence and antimicrobial susceptibility of *E. Coli*.

Materials and Methods: A total of 150 samples of different organisms like *Klebsiella pneumoniae*, *p. aeruginosa*, *Acinetobacter baumannii*, and *E. coli* for the study were taken from different clinical specimens of hospitalized patients from various hospital departments. 49 samples were identified and the discovery of Escherichia coli was worked on in this research were analyzed for isolation and identification of bacteria and antimicrobial susceptibility testing and Preparation of Congo Red Agar (CRA) to do Qualitative biofilm formation assay and Quantitative Assessment of Biofilm Formation and Minimal biofilm eradication concentration (MBEC).

Results: *E. coli* was isolated from 49 samples. In the AST experiment, we noticed that (CIP (ciprofloxacin) (95.1%), TOB (tobramycin) (83.6%), LEV (levofloxacin) (79.5%), CN (gentamycin) (73.4%), S (streptomycin) (65.3%), and AMC (amoxicillin) (55.1%) have the highest resistance ration among the isolates with a ratio higher than 50%. On the other hand, AK (amikacin) (4%), CT (colistin sulphate) (2%) and MEM (meropenem) (0%) have the highest ratio in susceptibility among the isolates. We chose meropenem and amikacin for further investigation in the biofilm sensitivity test, and the percentage of the biofilm former isolates was 32.6%, and the intermediate biofilm former

isolates, 34.6%, in addition to the non-biofilm former isolates, also 32.6%. The black color indicates biofilm formation.

Conclusion: *E. coli* isolates from UTI patients can form biofilms in most cases. The Congo-Red method is a good screening method for biofilm formation in order to shorten the time needed to get results. Treating biofilm-forming *E. coli* with Meropenem and Amikacin is a good option.

Key Words: Escherichia coli, biofilm, antimicrobial susceptibility testing.

İdrar Yolu Enfeksiyonu Olan Hastaların Klinik Örneklerinden İzole Edilen Escherichia Coli'nin Biyofilm Oluşum Eğilimi ve Antimikrobiyal Duyarlılığı.

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ÖZET

Amaç: *E. coli* organizmalarnn dalm paterni ve daha önce idrar yolu enfeksiyonu hastalarndan izole edilen *E. coli* izolatlarnn Biyofilm oluşturma kapasitesini deerlendirmek, *E. coli* prevalansn ve antimikrobiyal kapasitesini deerlendirmek, *E. coli* prevalansn.

Gereç ve Yöntem: Klebsiella pneumoniae gibi farklı organizmalardan toplam 150 örnek, s. aeruginosa, Acinetobacter baumannii ve *E. coli*, çeşitli hastane bölümlerinden hastaneye yatırılan hastaların farklı klinik örneklerinden yürütülmüştür. 49 örnek tespit edilmiş ve bu araştırmada bakteri ve antimikrobiyal duyarlılık testinin izolasyonu ve tanımlanması için Escherichia coli keşfi üzerinde çalışılmış ve Kalitatif biyofilm oluşumu tahlili ve Biyofilm Oluşumunun Kantitatif Değerlendirmesi için Congo Red Agar (CRA) Hazırlanması ve Minimal biyofilm yok etme konsantrasyonu (MBEC).

Bulgular: *E. coli* 49 örnekten izole edilmiştir. AST deneyinde (CIP (siprofloksasin) (%95.1), TOB (tobramisin) (%83.6), LEV (levofloksasin) (%79.5), CN (gentamisin) (%73.4), S (streptomisin) (65.3) olduğunu fark ettik. %), AMC (amoksisilin) (%55.1) %50'den yüksek oran ile izolatlar arasında en yüksek direnç oranına sahipken, AK (amikasin) (%4), CT (kolistin sülfat) (%2) ve İzolatlar arasında duyarlılık açısından en yüksek orana MEM (meropenem) (%0) sahiptir.Biyofilm duyarlılık testinde daha fazla araştırma için meropenem ve amikasin seçtik ve biyofilm oluşturan izolatların yüzdesi %32.6 ve ara biyofilm oluşturan izolatların yüzdesi %34,6 idi. biyofilm oluşturmayan izolatlara ek olarak %32.6 idi.siyah renk biyofilm oluşumunu gösterir.

Sonuç: İYE hastalarından alınan *E. coli* izolatları çoğu durumda biyofilm oluşturabilir. Kongo-Kırmızı yöntemi, sonuçların alınması için gereken süreyi kısaltmak için biyofilm oluşumu için iyi bir tarama yöntemidir. Meropenem ve Amikasin, biyofilm oluşturan *E. coli'yi* tedavi etmek için iyi bir seçenektir.

Anahtar Kelimeler: Escherichia coli, biyofilm, antimikrobiyal duyarlılık testi

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LIST OF ABBREVIATIONS AND SYMBOLS

%: PERCENT SİGN

°C: CELSİUS

ESBL: EXTENDED SPECTRUM BETA-LACTAMASES

***E. COLI*:** ESCHERICHIA COLI

ASTS: ANTİBİOTİC SUSCEPTİBİLİTY TESTS

DDST: DOUBLE-DİSC SYNERGY TEST

CDT: COMBINED DISC TEST

TDT: THREE-DIMENSIONAL TEST

EXPEC: EXTRAİNTESTİNAL PATHOGENİC STRAİNS OF *E. COLİ*

INPEC: INTESTİNAL PATHOGENİC STRAİNS OF *E. COLİ*

CFU: COLONY-FORMİNG UNİT

G: GRAM

UTI: URİNARY TRACT İNFECTİON

3RD GC: THİRD-GENERATİON CEPHALOSPORİN

MIC: MİNİMAL INHİBİTORY CONCENTRATİON

NCCLS: NATİONAL COMMİTTEE FOR CLİNİCAL LABORATORY STANDARDS

EHEC: ENTEROHEMORRHAGIC ESCHERICHIA COLI

DNA: DEOXYRIBONUCLEIC ACID

ETEC: ENTEROTOXIGENIC *E. COLI*

EPEC: ENTEROPATHOGENIC *E. COLI*

EIEC: ENTEROINVASIVE *E. COLI*

EAGGEC: ENTEROADHERENT AGGREGATIVE *E. COLI*

DAEC: DIFFUSELY-ADHERENT *E. COLI*

NAM: NUTRIENT AGAR MEDIUM

CLED: CYSTEINE LACTOSE ELECTROLYTE DEFICIENT AGAR

EMB: EOSIN METHYLENE BLUE AGAR

TSB: TRYPTIC SOY BROTH

UPEC: UROPATHOGENIC *E. COLI*

MNEC: MENINGITIS / SEPSIS

QIRS: QUIESCENT INTRACELLULAR RESERVOIRS

IBCS: BLADDER INTRACELLULAR BACTERIAL COMMUNITIES

LPS: LIPOPOLYSACCHARIDE

OMPS: OUTER MEMBRANE PROTEINS

BBB: BLOOD–BRAIN BARRIER

APEC: AVIAN PATHOGENIC *E. COLI*

DEC: DIARRHEAGENIC *E. COLI*

VTEC / STEC: TOXIN-PRODUCING VERO/SHIGA *E. COLI*

LEE: LOCUS OF ENTEROCYTE EFFACEMENT

ST: HEAT-STABLE

LT: HEAT-LABILE

CFS: COLONIZATION FACTORS

AMP: ADENOSINE MONOPHOSPHATE

GMP: GUANOSINE MONOPHOSPHATE

VT: VEROTOXIN

HC: HEMORRHAGIC COLITIS

HUS: HEMOLYTIC UREMIC SYNDROME

GB3S: GLOBOTRIAOSYLCERAMIDES

HR: HOURS

HIV: HUMAN IMMUNODEFICIENCY VIRUS

CPS: CAPSULAR POLYSACCHARIDE

SAT: SECRETED AUTOTRANSPORTER TOXIN

CDT: CYTOLETHAL DISTENDING TOXIN

VAT: VACUOLATING AUTOTRANSPORTER TOXIN

AST: ARGININE SUCCINYL TRANSFERASE

SHET-1: SHIGELLA ENTEROTOXIN-1

INOS: INDUCIBLE NITRIC OXIDE SYNTHASE

PBPS: PENICILLIN-BINDING PROTEINS

AMES: AMINOGLYCOSIDE MODIFYING ENZYMES

PBP: PENICILLIN-BINDING PROTEIN

NAG: N-ACETYL GLUCOSAMINE

NAM: N-ACETYLMURAMIC ACID

DAP: DIAMINOPIMELIC ACID

EPE: ESBL-PRODUCING ENTEROBACTERIACEAE

TEM: TEMONEIRA

SHV: SULPHYDRYLVARIABLE

OMP: OUTER MEMBRANE PROTEINS

CMY: CEPHAMYCINS

FOX: CEFOXITIN

MOX: MOXALACTAM

IMP-1: IMIPENEM-HYDROLYZING B -LACTAMASE 1

VIM: VERONA INTEGRONS-ENCODED METALLO-B-LACTAMASES

NDM-1: NEW DELHI METALLO-B-LACTAMASE 1

HPA: HEALTH PROTECTION AGENCY

ARMRL: ANTIBIOTIC RESISTANCE MONITORING AND REFERENCE LABORATORY

CTX-M: CEFOTAXIME-MUNICH

ICU: INTENSIVE CARE UNIT

WHO: WORLD HEALTH ORGANIZATION

HRI: HEALTH-RELATED INFECTIONS

CLSI: CLINICAL AND LABORATORY STANDARDS INSTITUTE

EUCAST: EUROPEAN COMMITTEE ON ANTIMICROBIAL SUSCEPTIBILITY TESTING

PCR: POLYMERASE CHAIN REACTION

PFGE: PULSED-FIELD GEL ELECTROPHORESIS

LAMP: LOOP-MEDIATED ISOTHERMAL AMPLIFICATION

TRNC: TURKISH REPUBLIC OF NORTHERN CYPRUS

CAZ: CEFTAZIDIME

CRO: CEFTRIAXONE

CTX: CEFOTAXIME

ATM: MONOCYCLIC BETA-LACTAM AZTREONAM

AMC: AMOXICILLIN-CLAVULANIC ACID

CTC: CEFOTAXIME/CLAVULANIC ACID

SD: STANDARD DEVIATION

SPSS: STATISTICAL PACKAGE FOR THE SOCIAL SCIENCES

CDC: CENTERS FOR DISEASE CONTROL AND PREVENTION

CW: CELL WALL

ET AL.: AND OTHERS

IGA: IMMUNOGLOBULIN A

IGG: IMMUNOGLOBULIN G

IGM: IMMUNOGLOBULIN M

KG: KILOGRAM

L: LITER

LBS: A POUND

MCG /MG: MICROGRAM

MG: MILLIGRAM

MG/L: MILLIGRAMS PER LITER

MH: MUELLER-HINTON AGAR

ML: MILLILITER

MM: MILLIMETER

N: NUMBER

NO: NUMBER

PH: POTENTIAL FOR HYDROGEN

R: RESISTANT

S: SENSITIVE

U: UNIT

UT: URINARY TRACT

MG/ML: MICROGRAM PER MILLILITER

MM: MICROMETER

AMI: ANTIBODY-MEDIATED IMMUNITY

LOS: LIPOOLIGOSACCHARIDE

CHAPTER ONE

1. INTRODUCTION

Depending on the fundamental deference in the cell wall structure, Gram-negative bacteria are one of the two major groups in the domain Bacteria in the taxonomic tree. The Gram-negative bacteria cell wall is composed of three layers, mentioned in order from the extracellular region of the cell to the intracellular region (the outer membrane (OM), a thin layer of peptidoglycan (PG) with a gel like matrix periplasm between the OM and PG, and between the PG and the OM. This general structure is strictly conserved in all gram-negative bacteria (Beveridge, 1999).

Escherichia coli is a rod-shaped gram-negative, facultative anaerobic bacterium. *E. coli* natural environment is the human and animal intestinal tract, and it's part of the normal flora (Ishii & Sadowsky, 2008). Despite the fact that most *E. coli* strains are harmless and live in symbiosis with their hosts, they can cause severe and fatal diseases such as urinary tract infections, diarrheal disease, sepsis, and meningitis in some cases. (Kaper et al., 2004).

This virulence phenotype was caused by acquiring virulence genes via horizontal gene transfer from other types of bacteria (J. R. Johnson, 1991). *E. coli* strains had acquired resistant genes, rendering them resistant to the antibiotics used, which is a major concern for human health and is associated with high morbidity. of the acquired factors are associated with enhancing extracellular survival, which is facilitated by bacterial motility with the aid of the flagella, in addition to structural features such as the type-1 pili system (Olorunmola et al., 2013).

The virulence and resistance of pathogenic *E. coli* are not the only notable features; biofilm development protects the bacteria from adverse environmental conditions and causes infection (Sharma et al., 2016). In biology, biofilm is a collection of bacteria that

has been permanently adhered to a surface and encased in a slimy extracellular polymeric material (EPS) made up of proteins, DNA, and polysaccharides (Sharma et al., 2016).

Chemicals such as EPS help bacteria survive and grow. Escherichia coli biofilms have been shown to be the primary causal agent in many intestinal illnesses, according to numerous studies. Quorum sensing (QS) is a chemical signaling mechanism used by bacteria in biofilms to communicate. QS uses tiny signaling molecules to control proliferation and gene expression based on the density of the bacterial population (Sharma et al., 2016; Zuo et al., 2006).

Cells in the biofilm generate AI (auto inducer chemicals) for the extracellular environment and upregulate biofilm development and maturation when the required high density is achieved. AI substances are produced by cells in the biofilm. As a result of AI, bacteria in biofilms are better able to produce virulence factors, alter the host immunological response, and modify their genetic make-up. Biofilm also makes cells more resistant to antibiotics by preventing them from penetrating the cells. In order to combat illnesses caused mostly by *E. coli* biofilm development, it is necessary to find alternative therapeutic agents (Sharma et al., 2016; Zuo et al., 2006).

One of the most common causes of urinary tract infections (UTIs) has been identified as *E. coli*, and many of these isolates have been shown to be resistant to various medicines, including ampicillin and amoxicillin/clavulanic acid. In addition, there are many variables linked to UTI, including diabetes, renal illness, and the use of an intrauterine device, which may lead to more complex infections and increase mortality. Biofilm formation contributes to infection complications by increasing the number of antimicrobial-tolerant cells inside the biofilm compared to cells that develop planktonically. Currently, medical equipment such as catheters, ventilators, and contact lenses have been discovered to develop biofilms, and as a result, these devices as a result, treating an infection caused by a medical device is difficult.

Also, it has been observed that disrupted biofilms when grown planktonically directly become susceptible to antimicrobial agents (Sharma et al., 2016). So, one of the

important methods of killing those biofilm-forming bacteria is by using a combination of antibiotics and antimicrobial agents that could remove biofilm and kill bacteria.

1.2.1. Aims

Distribution pattern of *E. coli* organisms isolated from various clinical specimens and evaluate Biofilm formation capacity of *E. coli* isolates previously isolated from UTI patients and check if some antibiotics is capable of killing bacteria when they form biofilm.

1.2.2. Objectives

The study's basic objectives were to investigate:

1. Isolate and identify *E. coli* species in collected specimens.

2. Study the distribution of *E. coli* species in different clinical specimens.

3. Provide swift and correct diagnosis of *E. coli* species.

4. Evaluate Biofilm formation capacity of *E. coli* and check if some antibiotics is capable of killing bacteria when they form biofilm

CHAPTER TWO

2. GENERAL INFORMATION

2.1 History of *E. Coli*

The German-Austrian bacteriologist and pediatrician, Theodore von Escherich, described *E. coli* in 1885. His research focused on infant gut microbes and their function in illness and digestion. In the feces of children, which he called Bacterium coli commune, he identified a fast-growing bacterium. Dr. Escherich concluded in 1988 that this microorganism may be commensal since the digestive tract activity appeared undisturbed in breastfeeding neonates. Scientists dubbed this bacterium *E. coli* in honor of its discoverer after he retired (Escherich, 1886, p. 188; J. R. Johnson & Stell, 2000).

At the beginning of the 20th century, *E. coli* became the first bacteria for microbiology studies and laboratory selection teaching, and it was later developed as a bacterial standard for the revolution in molecular biology in the 1950s (Clermont et al., 2000). Francis's, H. C. Crick, senior scientist, H. C. Crick, who was given the Nobel Prize in Physiology in Medicine in 1962, used *E. coli* for his genetic code experiments. *E. coli* is also commonly employed in a number of areas of medicine, including commercial genetic modification, pharmaceutical research, and the biotechnology industry (Andreu et al., 2003). Many scientists have done extensive research on characterizing the isolates of *E. coli*. In 1919, Castellani and Chalmers reformed the definition of this species based on some additional characteristics.

In 1982, the first *E. coli* contributed to the epidemic, which came to light in the USA. Oregon and Michigan in the USA. These were reported from people that developed bloody diarrhea, with severe stomach cramps, after eating hamburgers in a food chain (Besser et al., 1999; Pennington, 2010, p. 157). EHEC serotype outbreaks have mainly included uncooked ground meat products and occasionally raw milk (Abd El Tawab et al., 2015). Doyle and Schoeniin, who tested *E. coli* in 1987 and identified this strain as 3.7% of 164 beef, 1.5% of 264 pork, 1.5% of 263 poultry and 2.0% of 205 lamb samples, conducted the first research on the occurrence of EHEC strains in meats in 1987

(Dulo, 2014). Each organism's unique characteristics are shaped by the characteristics of its cellular structure, metabolism, and heredity. For example, the bacterium *E. coli* in the domain Bacteria may be found in the kingdom Eubacteria and the phylum Proteobacteria can be found in the class Gammaproteobacterial.

They combined the facts that Escherich bacteria grow well in ordinary laboratory media, in addition to milk clotting, and do not form endospores. These are mostly optional anaerobes that can ferment glucose and lactose fully with acid production. Based on all the new evidence, they renamed the Escherich bacterium to *E. coli* (Castellani & ALBERT, 1919; Escherich, 1886, p. 188). Little did they all realize that once it was established, this bacterium would play a vital role in the advancement of science and civilization.

In the United States of America, these bacterial pathogens (animal and human) were first isolated and defined in 1975 by a California woman who had severe bleeding diarrhea. In 1977, an Argentina livestock pathogen was found in and 1982, respectively (Fernandez, 2008). In North America, when the ECCO (Escherichia coli O157: H7) outbreak was correlated to the import of infected cattle from Argentina, a human infection rate was found that was nearly three times higher than those that had been found previously in North America (McMichael, 2001).

E. coli is considered to be a part of the Enterobacteriaceae bacterial family. It is the commonest commensal inhabitant of human, even warm-blooded, gastrointestinal tracts and one of the most significant pathogens (Kaper et al., 2004b). As a commensal, it acts in a mutually advantageous relationship with hosts and seldom induces disease. However, it is also one of the most harmful human and animal pathogens, since it is responsible for a wide range of diseases. Due to the unique features of *E. coli*, it is a major biotechnology host organism, such as ease of processing, complete genome sequence availability, and its ability to extend both in aerobic and anaerobic conditions.

Each unique and adapted pathogen has developed a pathogen that adapts and develops forms to counter or Each unique and adapted pathogen has developed a pathogen that adapts and develops forms to counter or The pathogenicity of *E. coli* will

cause a broad variety of human diseases from the gastrointestinal tract to additional intestinal locations such as the urinary tract, bloodstream and central nervous system (Kaper et al., 2004b). Some forms of *E. coli* enter the stomach and seldom make patients ill, unless they are sick already.

Although rare, there are some cross-reactive pathogenic strains among healthy and immunocompromised individuals that can cause diarrhea or extra-intestinal diseases. Diarrheal diseases, such as rotavirus, are a serious public health issue and a main cause of mortality in infants and young children. Especially important in poor nations where *E. coli* bacteria may seem innocuous and just a handful of its Enteropathogenic strains have been discovered, this is especially important. Certain serotypes of the bacteria were then shown to possess both pathogenic and enterotoxigenic features, as well as a variety of virulence factors, leading to the discovery of a new pathogen. After World War II, researchers discovered the existence of *E. coli* serotypes that cause diarrhea. When toxins comparable to those produced by the Vibrio cholera toxin were discovered in the middle of the 1950s, the outlook for *E. coli* was altered significantly (Girão et al., 2006).

It has a wide range of industrial and medical uses. The most widely used microorganism in the field of recombinant DNA is *E. coli* (Yoon et al., 2009). Other researchers later identified the organism under several synonyms and iterations, and the term "*E. coli*" was not commonly accepted until 1954. More than 125 years later, in warm-blooded species, *E. coli* is remembered as a harmless gastrointestinal tract commensal and used as a colloquial laboratory workhorse (Cowan, 1954).

E. coli is a bacterium that may be found in several hundred different strains, each of which is classified according to its own serotype. Consider the bacteria *E. coli* O157:H7, which seems to generate Shiga-like toxins when cheese and contaminated meat are consumed. This strain of *E. coli* has been extensively researched and is considered to be a model strain. On the other hand, according to their virulence properties, enteric coli can be divided into six groups: Enterotoxigenic *E. coli* (ETEC), Enteroinvasive *E. coli* (EIEC), Enteropathogenic *E. coli* (EPEC), Enterohaemorrhagic *E. coli* (EHEC), Enteroadherent aggregated *E. coli* (EAggEC), and diffusely adherent *E. coli* (DAEC).

Enteric bacteria have the ability to cause a variety of intestinal and extra-intestinal diseases, including urinary tract infections and mastitis. The *E. coli* that resides within the digestive system can cause a number of illnesses and diseases, such as urinary tract infections and mastitis. On the other hand, it is not always an individual that can be affected by these bacteria. Most *E. coli* is in our intestines, helping to break down the food that we eat and to help reutilize nutrients and clean up garbage, producing vitamin K, and digesting food. *E. coli* bacteria are not found in nature in the same way as other coliform bacteria (Centers for Disease Control and Prevention (U.S.) & Centers for Disease Control and Prevention (U.S.), 2002, p. 20; Palmer & Parry, 2002).

E. coli O157: The H7 bacterium is Gram-negative, enterohaemorrhagic, and widespread in the food industry worldwide. The "O" in "O157" is also known as "Entero", and before the official name of the bacteria was determined, it was incorrectly thought to be "H57," meaning it could be on all continents except Antarctica, where there are 100,000 patients and 3,000 hospitalizations with 90 deaths every year in the United States (Croxen et al., 2013).

E. coli is most often transmitted through direct or indirect contact with infected feces, through contaminated or undercooked food, near proximity to the infected person, water, animal contact, or less often by laboratory exposure. Uncooked meat like ground beef, unwashed vegetables such as lettuce or slaw, and raw dairy products accounted for an estimated 50-60% of outbreaks in the United States, with waterborne cases accounting for around 15% of outbreaks and 5% of direct animal contact (Rangel et al., 2005, 1982–2002).

Most *E. coli* O157: H7 cases are related to contamination of food (Armstrong et al., 1996). An epidemiological study found that 41% of 183 outbreaks in the USA were the result of ground beef consumption from 1982 to 2002. Transmission from person to person is also reported as a source of infection, as 50 outbreaks (out of 183) reported in the United States have spread via the fecal-oral route. Although the ingestion of infected food and/or direct contact with contaminated fecal material is still the most frequent route

for *E. coli* O157: H7 transmission, new transmission routes are a major concern (Rangeletal.,2005,1982–2002).

2.2 Pathogenesis and Pathogenic Mechanisms

Within a few hours after birth, *E. coli* usually colonizes the gastrointestinal system of human infants. For decades, *E. coli* and its human host have coexisted peacefully and profitably. Unless the host is immunocompromised or conventional gastrointestinal barriers are broken, these commensal *E. coli* strains seldom cause illness. The mucosal surface of the mammalian colon harbors commensal Niche *E. coli*. As the most prevalent component of human intestinal microflora that is facultatively anaerobic, the bacterium is a strong competitor in this crowded arena (Sweeney et al., 1996). Like most mucosal pathogens, it can be said that *E. coli* practices the necessary technique of infection of the mucosal site. Colonization, avoidance of host protection, aggregation, and harm to the host. The most common feature of diarrheagenic *E. coli* strains is their potential to colonize the intestinal mucosal surface (Gomes et al., 2016)(Nataro, 2002).

Infectious *E. coli* strains are classified as enteral and parenteral diseases, in two groups. Both are *E. coli* extraintestinal pathogenic and *E. coli* intestinal (diarrheagenic). *E. coli* infection is likely to occur in three general clinical syndromes: enteric/diarrheal disorder, UTIs and sepsis/meningitis. Six well-described types of intestinal pathogens include: EPEC, EHEC, ETEC, EAEC, EIEC and DAEC (Nataro, 2002).

2.3 Morphology of *E. coli*

Bacteria are conducted as unicellular microorganisms that do not have chlorophyll pigments. There are no nuclei or membrane-bound organelles, so the composition of the cell is simpler than other organisms. There is a hard cell wall for all bacteria, which ensures they retain a definite cell structure. Rock-shaped bacillus *E. coli* cells, which usually occur individually and in large clumps (Barcella et al., 2016).

It was conducted as a gram-negative species, straight rod (1.1–1.5 m–2.0–6.0 m) organized in pairs or in pairs; it doesn't have the ability to form spores. In some species, *E. coli* is a motile or non-motile species, and the peritrichous flagella is the motility organ, and in some cases, capsules or microcapsules can be seen (Hilmi et al., 2019). Unlike other coliform bacteria, bacteria are not found in nature under natural conditions (Cengiz et al., 2004).

When it comes to filaments, they may be as varied as the letter Y in certain cultures, while in others, they can be as tiny and short as cocci (Robinson, 2014). Both forms can coexist if they choose to. Despite being able to move about by means of its lashes, it moves at a plodding pace (Cengiz et al., 2004). They may even seem to be immobile at times. Fimbriae are often formed by *E. coli* strains. Fimbriae help the virulence factor by clinging onto cells, which is a feature seen in all bacteria (Elbing & Brent, 2019).

Off-white or beige, with a glossy finish, is colored with an *E. coli* colony. It even looks like mucus on the plate's whole surface or a cloudy film. A slightly higher *E. coli* colony has a complete, established margin and a steady growth trend, which forms a focused expanding circle throughout the colony. The rings can be seen under a microscope. In the middle, older colonies are becoming darker (Shanahan & Tanner, 1948). *E. coli* has no specific dietary requirements and is readily applied to traditional media such as the Nutrient Agar Medium.

The nutritive Agar Medium and the MacConkey Agar Medium are two of the most often utilized media in *E. coli* culture labs. The ideal *E. coli* temperature varies between 10 and 40 degrees Celsius, but the optimum temperature for most strains is 37 degrees Celsius, and this is the temperature at which most strains are typically cultivated in labs. The pH ranges from 4.5 to 9.5, but the most significant development is seen around 7.0, which is neutral ph. The pH requirements for *E. coli* are often different depending on the strain (*E. Coli* | *CDC*, 2020).

Researchers in the lab work with a wide variety of culture media in order to conduct experiments. These media include Nutrient Agar medium and MacConkey Aga and others such as Sheen Blood Aga and Columbia Horse Blood Aga as well as Cysteine Red Bile Agar and Eosin Methylene Blue Aga and Sorbitol MacConkey Aga. Eosin Methylene Blue Agar is a selective medium that inhibits the growth of several gram-positive bacteria, inhibits the growth of Shigella and salmonella species, and encourages the growth of *E. coli* and MacConkey Agar Sorbitol, which also acts as a selective coli median. Columbia Blood Agar is more commonly used in laboratories to cultivate *E. coli*. *E. coli* prefers Blue Agar as a culture medium since it's a very selective environment for the bacteria.

Some strokes of *E. coli* display beta hemolysis in blood agar medium, particularly those which are excluded from conditions of pathology, while those that are extracted from normal people do not seem to have hemolysis on blood agar. The colonies of the MacConkey Agar are pink owing to the lactose fermentation, which is of considerable significance when it comes to separating *E. coli* from other specimen bacteria, in particular non-lactose gram and salmonella species, which gives the MacConkey agar medium colorlessly. Colonies of *E. coli* grow with a green metallic sheen in the Eosin Methylene Blue Agar medium as a result of the metachromatic presence of dyes in the medium and the lactose coli fermentation, which causes the pH of the media to change from alkaline to acidic conditions. Make the medium more selective for *E. coli* and easier to detect when it comes to *E. coli* (*E. coliInfection*, 2015) (Barcella et al., 2016; Barrick et al., 2009; Kaper et al., 2004b; Robinson, 2014).

Microorganisms like *E. coli* are anaerobic chemo-organic trophic, meaning they don't need oxygen to survive. To summarize, it has the following properties: oxidation is blocked; catalase is active; fermentation occurs; nitrate is reduced; and galactosidase activity is present. Over ninety-five percent of *E. coli* strains test positive for indole and methyl red, but test negative for Voges-Proskauer and citrate (a toxic compound) (Barnich et al., 2007)

2.4 Treatment, Prevention and Control

Treatment of *E. coli* O157: H7 infections with antibiotics may worsen the disease. The use of antibiotics may lead to the breaking up of the bacteria that produce Shiga toxin and secretion (Hiko et al., 2008). Data has shown that *E. coli* O157: H7 induces Shiga toxin production by ciprofloxacin or sub-inhibitory concentrations of trimethoprim-sulfamethoxazole. Therefore, diagnosis is mainly helpful in reducing the duration of symptoms and preventing systemic complications (Lim et al., 2010). For people with diarrhea, clear liquids are recommended to prevent dehydration and loss of electrolytes (Dulo, 2014). To accomplish the overall aim of avoiding and managing the spread of *E. coli*, we should concentrate on ensuring access to reliable sources of water, protecting our food supply from pollution, and informing the public to deter the spread of the pathogen (Mielke, 2010; Organization, 2001).

Measures to protect food products against infection involve sufficient temperatures for storage and preparation. Food irradiation technologies can be used in high-risk products to significantly reduce the bacterial load (Seib et al., 2012). In order to prevent the spread of multi-resistant pathogens in the hospital, there needs to be a strict focus and prevention of cross-contamination in the hospital environment, and the use of antimicrobial drugs needs to be completely regulated and managed (Mielke, 2010). Infections can spread easily whenever workers touch a contaminated surface with a contaminated hand. To prevent this, it is important to always wipe your hands and sterilize your work environment through the use of proper hand hygiene.

Antibiotics play a key function in both avoiding and managing human and animal (animal) Escherichia coli infections (De Vrese et al., 2008). As more and more antibiotics are misused, and over-prescribed, we have seen a rise in antibiotic resistant pathogens and a decrease in normal human bacterial flora (Van Duijn et al., 2011). Based on clinical proof, utilizing aerophagies in a manner that is healthy and reasonable should be a requirement for avoiding the production and spread of antibiotic-resistant bacteria. Probiotics are viable and stable microorganisms, primarily of the subgenus Lactobacillus and Bifid bacterium, that can infect the gastrointestinal tract and interact with infected

bacteria (Gillings, 2013). Probiotics are used for the prevention of infectious diarrhea. This has had positive benefits in reducing the incidence of diarrhea (Preidis et al., 2011). According to the report, the consumption of fermented milk and yogurt with the microorganism Lactobacillus in stable humans as part of the microbial environment decreases the probability of UTI and vaginal infections (Amdekar et al., 2011).

2.5 Biofilm

The biofilm is constructed in a tertiary model in which the bacteria are in direct contact with each other and embedded in a matrix of extracellular polymeric substances (EPS) that may incorporate exopolysaccharides, nucleic acids, proteins, and other macromolecules (Barraud et al., 2015; Branda et al., 2005). Biofilm is a collection of separate bacterial or combined cells that attach to abiotic or biotic plates (Hoiby et al., 2010). The primary factors behind the formation of bacterial biofilms are natural growth patterns for certain species, shielding from foreign harmful conditions, preferential colonization under conditions rich in nutrients, and cooperative effects as part of the community (Hall-Stoodley et al., 2004).

Biofilms were found in rivers on rocks and pebbles, contaminated water beds, ponds, waste and water tanks, military marine construction structures, boat hulls, etc. Microbial settling of human cells, such as heart plugs, teeth veneers, middle ear injuries, and surgical instruments, is of huge importance to human health. These clinical biofilms are responsible for 65 to 80% of human infections, which can result in morbidity and death. Within these biofilms, bacterial cells produce phenotypes that differ from native cells and are more resistant to current drugs, ultraviolet light, dry conditions, extreme pH, and the immune system (Singh et al., 2016).

2.6 Virulence Factors

As they are comparable in all gastrointestinal motions, *E. coli* bacillus has a complicated yet well-antigenic structure, as well as different antigen types (Robinson, 2014). He proposed the Kauffman serotype diagram in the 1940s, which included the somatic

lipopolymer known as the O antigen, capsular polysaccharide and flagellar H protein as markers for *E. coli* serotypes. (Akbar & Anal, 2011; Robinson, 2014).

2.6.1 Antigens

There are millions of lipopolysaccharides (LPS) molecules in the outer membrane of an *E. coli* organism, consisting of 1) O antigen, a polymer (1–40 units) of immunogenic repeated oligosaccharides. 2) The central area consists of non-repeating oligosaccharides that are phosphorylated. 3) Endotoxin (lipid A). These antigens have a heat-resistant somatic lipopolysaccharide structure. However, they aren't immune to formol despite their resistance to boiling and alcohol. There were 171 distinct choline O antigens identified in this study (Robinson, 2014).

O antigens react with other microorganisms of the same genus. Some O antigens and Salmonella spp. respond cross-reactively with *E. coli* O antigens, for example. Except for the O antigens found in certain Shigella sonnei, all O antigens respond to Shigella (Cengiz et al., 2004). Numerous antibody-based assays used to identify *E. coli* provide false positive findings because numerous bacteria respond with O antigens when exposed to this cross-reaction (Feng et al., 2002). *E. coli* is serologically divided into H and K serovars based on the antigens present in those serovars (Robinson, 2014).

2.6.2 H Antigens

The H antigen is a major component of flagella that is active in *E. coli* movement and is normally encoded by the fliC gene. Because of their thermolability and ability to be destroyed by heating to 100℃ as well as by ferments containing alcohol or protease, *E. coli* lash antigens have been discovered in mobile sources and have remained resistant to the antibiotic formol (Robinson, 2014). It is known that there are 53 H antigens in existence, which are sequentially numbered from H1 to H56, and that only around 20 of them are utilized to identify them. In contrast to other bacteria, H antigens do not induce cross-reactions with other H antigens (Wang et al., 2003).

2.6.3 K Antigens

Acid capsular polysaccharide is a dense, mucus-like polysaccharide found in several pathogenic *E. coli*; there are two distinct groups of K-antigen, group I and group II (while a minor intermediate subset (K3, K10, and K54/K96) was referred to as group III). K antigens are pieces of polysaccharide and cell capsules found in nature.

O antiserums do not bind to *E. coli* bacteria, even those that produce these antigens. Antigens of type K that have undergone agglutination analysis are identified based on structural differences. It's estimated that there are 80 different types of K antigens. For a few hours of boiling at 100 or even 120 degrees, they are heat-resistant and may be destroyed. Distemper in pigs is caused by antigens from the K88 and K99 species. Diarrhea and the K99 antigen are two different diseases (Don et al., 1984; Robinson, 2014).

2.6.4 Adhesion

In the *E. coli* attachment phase, it tends to completely overpower the regulation of urine flow; with some pathogenic bacteria, it is recognized as the initial stage in the colonization process, and in this process, both the host and the *E. coli* are working together (Bien et al., 2012; Riegman et al., 1988). Good surface adhesion of UPEC is dependent on various host cell gene expressions for UPEC's ability to colonize well. Fimbrial adhesions, UPEC expresses adherence components needed for attachment and thus are regarded as virulence factors. They are required for attachment. Many bacterial adhesives are found in a thin filamentous structure known as fimbriae or pili. During the attachment phase, fibrous adhesions are critical (Emody et al., 2003).

Pili is a short type of fimbriae and may be used interchangeably with fimbriae. (Winberg, 1984). These proteins are formed by a helical cylindrical structure that is both thinner and shorter than the flagellum. Fimbriae are composed of oligomeric pilin proteins; these protein complexes are expressed as virulence factors in uropathogenic strains of *E. coli*. The majority of *E. coli* receptors are carbohydrates for these fimbriae,

such as type 1 fimbriae, P fimbriae, and thin aggregative fimbriae. (Collinson et al., 1992).

2.6.5 Toxins

Toxins secreted by certain types of *E. coli* are largely responsible for their virulence. Moreover, toxins are capable of altering the signaling pathway of host cells and modulating inflammatory responses, and these toxins often lead to inducing host cell death and releasing the required nutrients (Agarwal et al., 2012). Cytolethal distending toxin (CDT) was first reported as a virulent toxin in UPEC in 1987, and then many other toxins, including: Shigella enterotoxin (VAT), vacuolating auto transporter toxin (VAT), alpha-hemolysin (HlyA), cytolysin A, cytotoxic necrotizing factor 1 (CNF1), arginine succinyl transferase (AST), plasmid-coded toxin (PET) and secreted auto transporter toxin (SAT) were identified in UPEC (ShET-1) (Soltani et al., 2018).

2.6.6 Capsule

When the capsule is shielding and defending the bacteria from external environments and the host immune response, the primary use is to shield the bacteria from different adverse conditions and the host immune response (Bien et al., 2012). The capsule also gives antimicrobial tolerance and antiserum activity defense in the host against swelling and complement-mediated bactericidal impact in the host (Bien et al., 2012; Jahandeh et al., 2015).

Several capsules, such as K1 and K5, inhibit the compromised hosts' proper humoral immune response by demonstrating molecular mimicry of tissue components (Bien et al., 2012). The K1 polysaccharide is a linear alpha2-8-linked sialic acid polymer, which plays a very significant role in both the production of IBC and the multiple phases of UTI pathogenesis (Nowicki et al., 1990; Olson et al., 2015).

2.6.7 Motility

The bacterium produces flagella and may be its way of getting around and also plays a role in the forming of biofilms in the formation of the initial adhesion (Bien et al., 2012). In research, it has been found that the bacteria are "moving" down towards the kidneys after they pass into the bladder (Lane et al., 2007). About 70–90% of all urinary tract infections arise because of flagellated Beta-Hemolytic uropathogens, and oral bacteria touch and epithelial cells of the urinary tract are a contributing factor throughout the pathogenesis (Bien et al., 2012). Since flagellar motility is a way for bacteria to adapt, it greatly improves the bacteria's capacity to react to some attractive or repellent environmental stimuli (Emody et al., 2003).

2.7 Resistance of *E. coli*

We as human beings are becoming immune to the medicinal properties of antimicrobial agents. Antibiotic resistance has been a significant concern. Many bacteria had responded to the usage of antibiotics before the use of penicillin was introduced and their ability to evolve and expand antimicrobial susceptibility to other species. Many antibiotics are being obtained at an increased rate and are being used improperly, which has led to these species being more resistant to these medications. Going along with disease, the social (travel) activities of moving between countries as well as travel by international tourists and businessmen has an effect on the likelihood of the spread of multidrug-resistant strains of disease (van der Bij & Pitout, 2012).

Antimicrobial tolerance was also observed in animals, and is when antimicrobials are sprayed, ointments are added, or administered to cure infections and other medical issues (Szmolka & Nagy, 2013). While in humans, the development of antimicrobial agents in pathogenic bacteria and endogenous bacteria was related to the increased frequency of drug resistance, The bacteria from animals could specifically reach through the human skin and food items, as well as indirectly through animal products.

Resistance to multiple antifungals is characterized as resistance to more than three antibiotic classes that may not have the intrinsic potential of the bacterium or are immune

to it (Magiorakos et al., 2012). It is concerning that multi-resistant bacterial strains are rapidly spreading across the globe. The reason for this is that these strains are acquired for many pathogenic bacteria, as well as multiple other microorganisms, by the multiple methods of zoonosis, such as infection via contaminated water or food. Therefore, it is imperative that the global community work to prevent the propagation of these bacterial strains. There are carriers of these genes that have the ability to resist the all-common classes of antibiotics. In addition, this combination often leads to species of bacteria that are resistant to all major classes of antibiotics (J. R. Johnson & Stell, 2000; Kaper et al., 2004b).

E. coli was naturally resistant to the therapeutic doses of penicillin G, the first β-lactam antibiotic put through clinical practice, referring to the outer membrane barrier. *E. coli* is also able to resist a lot of types of antibiotics and has a unique sort of resistance mechanism (T. J. Johnson et al., 2012). The creation of the -lactamase for the narrow-spectrum of the -lactams is the most important role in initiating the resistance of the broad-spectrum of the -lactams. It is proven that bacterial β-lactamases are found in pathogenic and commensal bacteria. Investigators have found at least 30 distinct phenotypic and genetic patterns among Escherichia coli. However, the types and degrees of plasmid-encoded β-lactamase genes in *E. coli* are unknown. Samples of pathogenic and commensal *E. coli* from 11 different clinical sources were characterized.

DNA from clinical isolates was extracted and amplified using nested forward and reverse primers specific for each β-lactamase. Xenografts from a chronic pancreatitis survivor were examined in a clinical laboratory setting to establish colony types that harbor identified β-lactamase genes and to screen clinical isolates for additional plasmid-encoded β-lactamase genes following a 2-month exposure. Thermostability criteria were utilized toidentifyd enzymes in a recombinant, plasmid-encoded--lactamase system (Poirel et al., 2012). β-lactamases confer penicillin and cephalosporin resistance and are an emerging cause of multidrug resistance in Gram-negative bacteria (Poirel et al., 2012). It alludes to asystemc that looks to decode conditions of lactamases, ESBLs, and their species that likely enhance conformity to cephalosporins and mono-lactams of the third and fourth centuries(Cantón & Coque, 2006).

The most prevalent type of cluster variant is the CTX-M-1, though the change to the CTX-M-15 is one that is rapidly increasing around the world. There are several types of CTX-M-14, even for CTX-M-15, which are mostly found in humans (Ewers et al., 2012).

CHAPTER THREE

3. MATERIAL AND METHODS

3.1 Design of the Study

The microbiology laboratory at King Abdullah University Hospital in Jordan was used for the investigation. For the study, researchers collected 150 clinical specimens from hospitalized patients from various hospital departments, each containing a different strain of bacteria such as *Klebsiella pneumoniae*, *P. aeruginosa*, *Acinetobacter baumannii*, and *E. coli*. The finding of *E. coli* was part of this study, which included 49 samples.

The King Abdullah University Research Assessment Committee approved the research protocol on September 29th, 2015, and it now has the accession number ref. 19/90/2015. Tests were performed on *E. coli* organisms isolated from several clinical specimens of the same patient, and the same patient's clinical material was utilized for repeated isolations.

3.2. Specimens Collection

Samples were collected between May 2016 to October 2016. 150 of different organisms stored samples were used from Dr.Samer Swedan. 49 of them is *E. coli* which is isolated from UTI patients and the samples were used in previous research and the type of all *E. coli* samples were urine samples.

3.3. Culture

The stored samples of *E. coli* isolates were inoculated on Blood Agar (Merck KGaA, Darmstadt, Germany) to get pure colonies. Blood Agar was prepared as per the manufacturer's directions. *E. coli* colonies were produced after a 24-48-hour incubation period at 37 °C. Colonies include Circular, Convex, Smooth (fresh isolation); Rough (repeated subculture); Mucoid, and greyish white (capsulated strains). After that, on EMB

agar, we do subculture (Becton Dickinson, Sparks, MD 211 52, USA). Colonies appeared after an incubation period of 24 to 48 hours at a temperature of 37°C. Round, convex, and smooth (fresh isolation) colonies are rough (repeated culturing), mucoid (capsulated strains), and greyish white in color.

MacConkey agar (MCM20500, Biolab Diagnostics Laboratory) cultures of *E. coli* were performed overnight at 37 C to look for contamination in the glycerol preserved stock. Mueller-hinton agar (CM0337, OXOID) was used to cultivate a single colony, which was incubated at 37 C overnight. After that, single colonies were cultured for 24 hours at 37 degrees Celsius in mueller-hinton broth (CM0405, OXOID). This was followed by incubating the bacteria in glycerol for 666 hours at -80 C before further testing.

3.4 Antibiotic sensitivity test

To find out which isolates were resistant to the chosen antibiotics, researchers utilized the Kirby-bauern disk diffusion technique. The 28th edition of the Clinical and Laboratory Criteria Institute (CLSI) performance standards for antimicrobial susceptibility testing was used to choose antibiotics and interpret the findings (2018).

A loopful of overnight bacterial culture on Mueller-Hinton agar was suspended in 5 mL of sterile normal saline. After the solution was mixed well by vortex, the absorbance (OD600) was measured using a spectrophotometer and was diluted to 0.5 Mcfarland (0.08 to 0.123).

After that, a sterile cotton swab was dipped in the solution and inoculated on Mueller-Hinton agar in 3 directions. The plates were allowed to dry afterward. The appropriate antibiotic disks were applied to the agar and incubated at 37 C for 20–24 hours. The inhibition zone for each antibiotic disk was measured in millimeters.

3.5 Preparation of Congo Red Agar (CRA)

Biofilm formation was studied through the cultivation of all *E. coli* clinical isolate strains on the reported Modified Congo Red Agar (MCRA) consisting of 0,4 g of Congo

red dye (Alfa Aesar, ThermoFisher GmbH, Erienbachweg 2, 768 70 Kandel, Germany), 10 g of glucose (Merck, KgaA, Germany), and Blood Agar Base as nutrient agar.

A 100-ml solution of the dye was prepared in distilled water and autoclaved for 15 minutes at 121°C. Blood Agar Base (Merck, KgaA, Germany) was autoclaved at 121°C for 15 minutes after the glucose had been dissolved in 900 ml of deionized water. Before injecting the glucose into the plates, the dye was added to the Blood Agar Base and well mixed. Critics have labeled the color red as representing a harmful biofilm. Black and Strong Black have been identified for the positive formation of biofilms (Mariana et al., 2009).

3.6 Qualitative biofilm formation assay

Congo Red Agar (CRA) was used to detect biofilm isolates. CRA media were prepared using 37g per liter of brain heart infusion broth (BHIB), 50g/L sucrose, 10g/L bacteriological agar, and 0.8g/L Congo red indicator, which had been prepared previously as a concentrated solution and were sterilized by autoclave. (121 degrees Celsius for 15 minutes) and were added to the prepared BHIB after autoclaving at 55 degrees Celsius and pouring into sterile petri dishes.

The isolates were plated on agar and cultured at 37c for twenty-four hours before being analyzed. Biofilm formation was detected by the presence of black colonies on the incubated plates, which is a positive indication of biofilm formation. On the other hand, any white, orange, or red colonies were treated as negative biofilm formation isolates. Any gray or near black isolates were incubated for another 24 hours to check for any weak biofilm formation.

3.7 Quantitative Assessment of Biofilm Formation

The ability of *E. coli* to form biofilm was measured using a micro titer plat assay as described previously. Macconkey agar was used to cultivate *E. coli* isolates overnight at a temperature of 37c. Then a loop full (1l loop) of the bacteria was inoculated into 5 ml of TSB (Oxoid, UK) and incubated overnight at 37 C. The overnight broth culture was diluted 1:100 in TSB, and 200 l of the diluted culture was inoculated on a polystyrene

microtiter plate. Each isolate was run in triplicate. Each plate has its own negative control and is incubated for 24 hours aerobically at 37 C.

After the incubation, the media was aspirated from each well using a multichannel pipette. And the wells were washed twice using 200 µl Phosphate Buffer Saline PBS to remove non-adherent cells. The remaining attached biofilm was stained with 200 l of crystal violet 1% for 30 min. After staining to remove excess, the plates were washed twice with 200 l of distilled water, and then, after aspiration, were air dried at room temperature. 200 liters of pure ethanol makes The optical density of the suspension was measured using an ELISA reader at 570 nm (OD570).

3.8 Minimal biofilm eradication concentration (MBEC)

The test was carried out using MBEC plates (innovotech). Samples with the criteria specified above were cultured on MHA, then after 37 C for overnight, one colony was inoculated on MHB and incubated for 37 C overnight. (OD600) was measured using a spectrophotometer and diluted to 0.5 mcfarlan (0.08 – 0.0123) and further diluted 100-fold using MHB.

Then 150 l of each sample were inoculated onto the MBEC. For each sample, 2 rows were used (A and B, C and D) after the inoculation plate was incubated at 37 C for 24 hours. Then two 96-well plates were prepared, one for washing and the other for the challenge plate. The washing plate was filled with 200 l of 1x PBS and the challenge plate was filled with 200 l of the appropriate dilution of the antibiotic. Each dilution was filled into one column. In total, 11 dilutions were prepared as in Table 1, and column 12 was filled with sterile MHB to serve as a negative control.

The MBEC plate cover was taken from the original plate and dipped in the washing plate 3 times to remove planktonic cells from the biofilm. It was then incubated in the challenge plate for 24 hours at 37 C. After the incubation, another washing plate was prepared and the recovery plate was filled with sterile 200 l MHB. The MBEC cover pigs were washed 3 times and then dipped in the recovery plate and incubated at 37 C in a shaker incubator at 220 RPM for 5 min to get the remaining alive cells in the media. Then

the MBEC cover was discarded and the 96 well plate was covered with its normal cover and incubated at 37 C for 24 hours. Clear wells indicate that the antibiotic could successfully kill the bacteria at that concentration after the incubation.

3.9 Statistical Analysis

In order to do statistical analysis, we utilized IBM SPSS ver. 26 (IBM Inc., Armonk, New York, USA). To compare frequency data, researchers utilized Pearson's Chi-square test. Significant results were those with a p value lower than 0.05.

Note: Congo Red Codes are as follows: 0=Negative; 1=Weak; 2=Moderate; 3=Strong.

In this analysis, the AST results (S, I, and R) are used.

CHAPTER FOUR

4. RESULTS

4.1 Biofilm percentage

After the culturing of bacterial isolates on Congo Red plate, black growth colonies were counted as biofilm former isolates. The percentage of the biofilm former isolates was 32.6% and the intermediate biofilm former isolates were 34.6%, in addition to the non-biofilm former isolates, which were also 32.6%.

4.2 Antibiotic sensitivity test

The antimicrobial agent's sensitivity test results of *E. coli* samples were chosen and interpreted based on CLSI 2018 recommendations and listed in table 2. The highest rate of resistance was categorized as followed (CIP (ciprofloxacin) (95.1%), TOB (tobramycin) (83.6%), LEV (levofloxacin) (79.5%), CN (gentamycin) (73.4%), S (streptomycin) (65.3%), AMC (amoxicillin) (55.1%), FEP (cefepime) (48.9%), ATM (aztreonam) (46.9%), CAZ (ceftazidime) (46.9%), CTX (cefotaxime)) (51%), AK (amikacin) (4%), CT (colistin sulphate) (2%) and MEM (Meropenem) (0%)). The highest level of resistance was observed against (ciprofloxacin, tobramycin, levofloxacin, gentamycin, streptomycin and amoxicillin) which more than 50% of the samples were resistance to those antibiotics. On the other hand, all the samples were sensitive to (Meropenem) and more than 90% of the samples were susceptible to (colistin sulphate, amikacin). The results of the antimicrobial sensitivity test of the *E. coli* samples that have been worked on are shown in Table 1.

Antimicrobial agent	Resistance		Intermediate		Sensitive	
	count	Percentage	count	percentage	Count	percentage
Cefotaxime	25	38.7%	5	10.2%	19	51%
colistin sulphate	1	2%	0	0%	48	98%
Meropenem	0	0%	0	0%	49	100%
Streptomycin	32	65.3	12	24.5%	5	10.2%
Amikacin	2	4%	5	10.2%	42	85.7%
Ceftazidime	23	46.9%	1	2%	25	51%
Aztreonam	23	46.9%	0	0%	26	53%
Gentamycin	36	73.4%	0	0%	13	53%
Ciprofloxacin	47	95.9%	0	0%	2	4%
Tobramycin	41	8%	4	8%	4	83.6%
Levofloxacin	39	6.1%	7	14.2%	3	79.5%
Amoxicillin	27	16.3%	14	28.5%	8	55.1%
Cefepime	24	44.8%	3	6.1%	22	48.9%

Table 1. The antimicrobial agent's sensitivity test results of *E. coli* samples.

4.3 Qualitative biofilm formation assay

The ability to form biofilms of our isolates was detected using the Congo Red agar plate method. After the culturing of bacterial isolates on Congo Red plate, black growth colonies were counted as biofilm former isolates. The percentage of the biofilm former isolates was 32.6% and the intermediate biofilm former isolates were 34.6%, in addition to the non-biofilm former isolates, which were also 32.6%. The black color indicates biofilm formation as in figure 1.

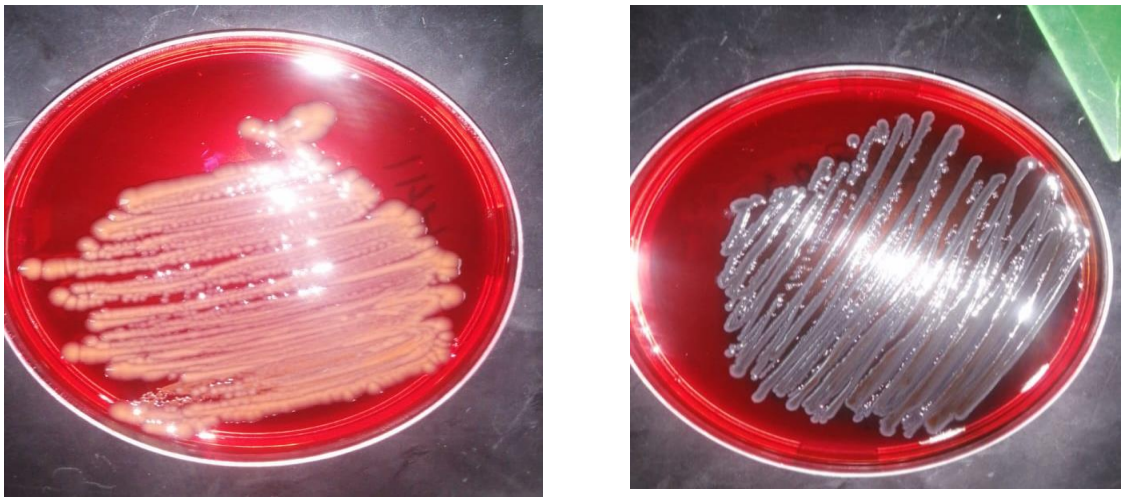

Figure 1: Biofilm formation on Congo Red media.

		Congo Red							
		0		1		2		3	
		Count	Row N %	Count	Row N %	Count	Row N %	Count	Row N %
CTX (cefotaxime)	I	0	0.0%	5	100.0 %	0	0.0%	0	0.0%
	R	7	31.8 %	13	59.1 %	1	4.5%	1	4.5%
	S	5	33.3 %	10	66.7 %	0	0.0%	0	0.0%
CT (colistin sulphate)	S	12	28.6 %	28	66.7 %	1	2.4%	1	2.4%
MEM (meropenem)	S	12	28.6 %	28	66.7 %	1	2.4%	1	2.4%
S (streptomycin)	I	3	25.0 %	7	58.3 %	1	8.3%	1	8.3%
	R	8	30.8 %	18	69.2 %	0	0.0%	0	0.0%
	S	1	25.0 %	3	75.0 %	0	0.0%	0	0.0%
AK (amikacin)	I	1	50.0 %	1	50.0 %	0	0.0%	0	0.0%
	R	1	50.0 %	1	50.0 %	0	0.0%	0	0.0%
	S	10	26.3 %	26	68.4 %	1	2.6%	1	2.6%
CAZ (ceftazidime)	R	7	33.3 %	12	57.1 %	1	4.8%	1	4.8%
	S	5	23.8 %	16	76.2 %	0	0.0%	0	0.0%
ATM (aztreonam)	R	6	30.0 %	12	60.0 %	1	5.0%	1	5.0%
	S	6	27.3 %	16	72.7 %	0	0.0%	0	0.0%
CN (gentamycin)	R	6	18.8 %	24	75.0 %	1	3.1%	1	3.1%
	S	6	60.0 %	4	40.0 %	0	0.0%	0	0.0%

CIP (ciprofloxacin)	**R**	12	29.3 %	27	65.9 %	1	2.4%	1	2.4%
	S	0	0.0%	1	100.0 %	0	0.0%	0	0.0%
TOB (tobramycin)	**I**	0	0.0%	4	100.0 %	0	0.0%	0	0.0%
	R	11	31.4 %	22	62.9 %	1	2.9%	1	2.9%
	S	1	33.3 %	2	66.7 %	0	0.0%	0	0.0%
LEV (levofloxacin)	**I**	4	57.1 %	3	42.9 %	0	0.0%	0	0.0%
	R	8	24.2 %	23	69.7 %	1	3.0%	1	3.0%
	S	0	0.0%	2	100.0 %	0	0.0%	0	0.0%
AMC (amoxicillin)	**I**	3	25.0 %	8	66.7 %	0	0.0%	1	8.3%
	R	7	30.4 %	15	65.2 %	1	4.3%	0	0.0%
	S	2	28.6 %	5	71.4 %	0	0.0%	0	0.0%
FEP (cefepime)	**I**	0	0.0%	2	100.0 %	0	0.0%	0	0.0%
	R	6	28.6 %	13	61.9 %	1	4.8%	1	4.8%
	S	6	31.6 %	13	68.4 %	0	0.0%	0	0.0%

Table 2: No significant associations were observed between biofilm formation according to the Congo Red assay and antimicrobial susceptibility results.

		Biofilm assay					
		Intermediate		Negative		Positive	
		Count	Row N %	Count	Row N %	Count	Row N %
CTX (cefotaxime)	I	1	20.0%	2	40.0%	2	40.0%
	R	11	47.8%	6	26.1%	6	26.1%
	S	6	33.3%	5	27.8%	7	38.9%
CT (colistin sulphate)	R	0	0.0%	0	0.0%	1	100.0%
	S	18	40.0%	13	28.9%	14	31.1%
MEM (meropenem)	S	18	39.1%	13	28.3%	15	32.6%
S (streptomycin)	I	6	50.0%	5	41.7%	1	8.3%
	R	10	33.3%	8	26.7%	12	40.0%
	S	2	50.0%	0	0.0%	2	50.0%
AK (amikacin)	I	1	33.3%	2	66.7%	0	0.0%
	R	1	50.0%	0	0.0%	1	50.0%
	S	16	39.0%	11	26.8%	14	34.1%
CAZ (ceftazidime)	R	11	50.0%	4	18.2%	7	31.8%
	S	7	29.2%	9	37.5%	8	33.3%
ATM (aztreonam)	R	11	52.4%	4	19.0%	6	28.6%
	S	7	28.0%	9	36.0%	9	36.0%
CN (gentamycin)	R	13	37.1%	11	31.4%	11	31.4%
	S	5	45.5%	2	18.2%	4	36.4%
CIP (ciprofloxacin)	R	18	40.0%	12	26.7%	15	33.3%
	S	0	0.0%	1	100.0%	0	0.0%
TOB (tobramycin)	I	1	25.0%	2	50.0%	1	25.0%
	R	17	43.6%	11	28.2%	11	28.2%
	S	0	0.0%	0	0.0%	3	100.0%
LEV (levofloxacin)	I	3	42.9%	3	42.9%	1	14.3%
	R	15	40.5%	9	24.3%	13	35.1%
	S	0	0.0%	1	50.0%	1	50.0%
AMC (amoxicillin)	I	8	57.1%	1	7.1%	5	35.7%
	R	9	36.0%	9	36.0%	7	28.0%
	S	1	14.3%	3	42.9%	3	42.9%
FEP (cefepime)	I	1	33.3%	1	33.3%	1	33.3%
	R	11	50.0%	5	22.7%	6	27.3%
	S	6	28.6%	7	33.3%	8	38.1%

Table 3: No significant associations were observed between biofilm formation according to the Biofilm assay and antimicrobial susceptibility results.

		Biofilm assay					
		Intermediate		Negative		Positive	
		Count	Row N %	Count	Row N %	Count	Row N %
CTX (cefotaxime)	NS	12	42.9%	8	28.6%	8	28.6%
	S	6	33.3%	5	27.8%	7	38.9%
CT (colistin sulphate)	NS	0	0.0%	0	0.0%	1	100.0%
	S	18	40.0%	13	28.9%	14	31.1%
MEM (meropenem)	S	18	39.1%	13	28.3%	15	32.6%
S (streptomycin)	NS	16	38.1%	13	31.0%	13	31.0%
	S	2	50.0%	0	0.0%	2	50.0%
AK (amikacin)	NS	2	40.0%	2	40.0%	1	20.0%
	S	16	39.0%	11	26.8%	14	34.1%
CAZ (ceftazidime)	NS	11	50.0%	4	18.2%	7	31.8%
	S	7	29.2%	9	37.5%	8	33.3%
ATM(aztreonam)	NS	11	52.4%	4	19.0%	6	28.6%
	S	7	28.0%	9	36.0%	9	36.0%
CN (gentamycin)	NS	13	37.1%	11	31.4%	11	31.4%
	S	5	45.5%	2	18.2%	4	36.4%
CIP (ciprofloxacin)	NS	18	40.0%	12	26.7%	15	33.3%
	S	0	0.0%	1	100.0%	0	0.0%
TOB (tobramycin)	NS	18	41.9%	13	30.2%	12	27.9%
	S	0	0.0%	0	0.0%	3	100.0%
LEV (levofloxacin)	NS	18	40.9%	12	27.3%	14	31.8%
	S	0	0.0%	1	50.0%	1	50.0%
AMC (amoxicillin)	NS	17	43.6%	10	25.6%	12	30.8%
	S	1	14.3%	3	42.9%	3	42.9%
FEP (cefepime)	NS	12	48.0%	6	24.0%	7	28.0%
	S	6	28.6%	7	33.3%	8	38.1%

Table 4: In this study, there was a statistically significant relationship between biofilm development and non-susceptibility to tobramycin (P=0.036). According to the results of the Biofilm test, no statistically significant relationships were found between biofilm development and the remaining antimicrobial agents.

4.4 Quantitative Assessment of Biofilm Formation

The amount of biofilm produced by the bacteria was measured using the biofilm micro titer plate method. Most of the isolates were weak biofilm formers with a percentage of 66% and the rest of the samples were non-biofilm formers with a percentage of 34%. As shown in figure two, a biofilm microtiter plate with weak biofilm former and non-biofilm former isolates.

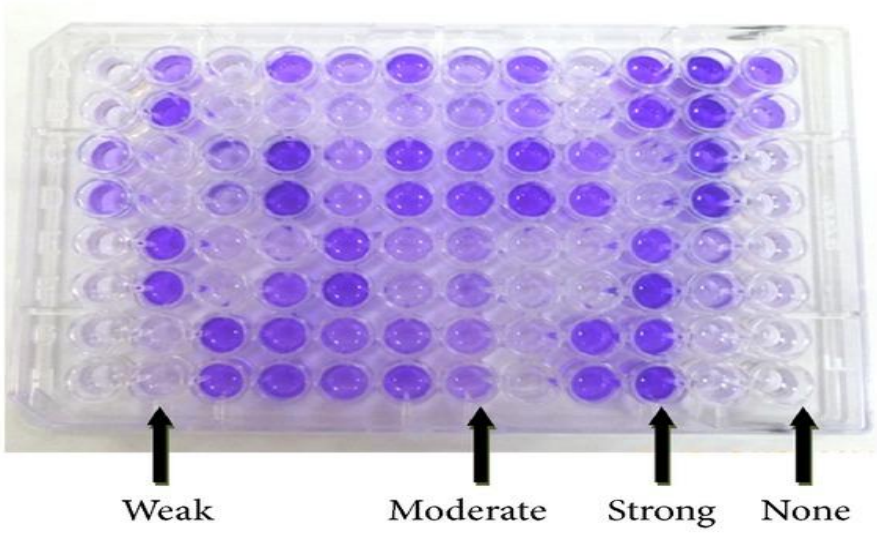

Figure 2: Micro titer plate for biofilm formation.

4.5 Minimum biofilm eradication concentration (MBEC)

They performed antibiotic sensitivity tests on the biofilms that make up Escherichia coli, and the minimum concentration that could eliminate the biofilm was measured using the MBEC panel assay for two antibiotics (Amikacin and Meropenem). These were the two strongest antibiotics in our study, and our isolates were mostly resistant to those antibiotics. The minimum concentration that removes the biofilm and kills the bacteria is given.

CHAPTER FIVE

5. DISCUSSION

Infections of *E. coli* species have intensified and become more complicated to manage owing to the rise of immunogenic diseases, misuse of broad-spectrum antibiotics, increasing usage of immunosuppressive drugs, malnutrition, endocrine diseases, extensive use of medical devices, aging, and an increase in the number of patients. In addition, in the last two decades, the whole evolution of infectious diseases has undergone significant changes. In patients that are immunocompromised and chronically ill, bacteria that were previously deemed nonpathogenic or less virulent are now recognized as a major cause of morbidity and mortality.

Beta-lactamase activity is the most significant and widespread cause of resistance to beta-lactam antibiotics. The effectiveness of broad-spectrum cephalosporins against the Enterobacteriaceae family is challenged by newly emerging enzymes and innovations in the production of old enzymes. Especially in the enzyme group called ESBL. It is responsible for the development of resistance to beta-lactams such as aztreonam, ceftazidime, ceftriaxone, and cefotaxime. In Europe, these enzymes were first identified, but it was known that they appeared in several different countries, such as the United States of America and Japan. This has been associated with the increased use of third-generation cephalosporins in particular (Paterson & Bonomo, 2005, p. 20). Epidemiological studies of the presence of ESBL in *E. coli* strains are more common and are important factors for public and hospital infections, especially in urinary tract infections (Henshke-Bar-Meir et al., 2006)

Escherichia coli is a normal flora bacterium that has a symbiotic relationship with the human body. In some cases, these bacteria might cause some severe illness in humans.

Bacterial biofilm-associated infections are one of the major problems worldwide, accounting for 65% of all infections. In addition, bacteria show decreased susceptibility to antibiotics when inside biofilms because biofilms form a protective structure around the bacteria (Ito et al., 2009).

In this study, the percentage of the former isolates was 32.6% and the former biofilm intermediate was 34.6%, in addition to the previous non-biofilm isolates of 32.6%. The black color indicates the formation of biofilms.

The fact that bacterial biofilm may potentially play a role in disease pathogenesis has prompted researchers to pay more attention to diseases that may be linked to biofilms. It is common for biofilm infections to recur because the microorganisms that live in biofilms are often resistant to the immune system, antibiotics, and other therapies. In the majority of instances, the biofilms could be detected using a variety of imaging techniques as well as other research methods. Research shows that biofilms may play a role in a number of different diseases and pathologies.

This ranges from the biofilm simply serving as a bacterial reservoir to the biofilm playing a more active function, such as causing inflammation. Research further shows biofilm formation is not limited to external environments; it may potentially take place inside live cells. In addition, biofilms have been linked to cancer development. After everything is said and done, it's clear that biofilms are a part of many chronic illnesses, if not all of them. In the development of efficient treatment techniques for such illnesses, data like these may be essential (Vestby et al., 2020).

Biofilms may also serve as environments that collect a wide range of microbial species and bacterial counts in constrained areas. This bacteria's concentrated, sequential, and/or synergistic activity will have detrimental consequences on host cells. Persistent biofilms may also alter the local immune response in a number of ways, such as by triggering an inflammasome that damages or irritates nearby tissues. It has also been suggested that these biofilm-mediated processes might be of interest in the start and/or progression of malignancies, such as CRC. To better understand illness severity and prognosis, researchers will look at whether or not a patient has a biofilm present.

A sub-clinical reservoir for infectious agents prior to clinical infection with organism bacteria may also operate within the biofilm type of organization, as was shown for S. pneumoniae in the cavum and UPEC in the urinary bladder. Nonpathogenic bacterial biofilms may even provide protection against pathogen infection, as has been shown in

the upper airways with biofilms of S. oralis and S. salivarius. Because of this generously funded study, it's now apparent that the significance of biofilm should be included in the diagnosis and treatment of a wide range of chronic illnesses. These diagnostic criteria have been recommended for a few illnesses, including monogenic conditions and persistent wounds, so they're essential for biofilm infections. It is also critical to have access to appropriate treatment for such illnesses.

The CRA technique was used to find the earliest signs of biofilm growth. The results revealed that 10% of the bacteria produced biofilms, which was in line with previous research (Freeman et al., 1989). When it came to bacteria, Hassan and his colleagues found that just 49% were capable of forming strong, healthy biofilms, while the remainder were either unable to produce biofilms or had their ability to do so reduced (Hassan et al., 2011). As a consequence, Kaleem's findings and the results from the conventional microtiter plate are virtually identical. 63% of the bacteria in his research formed biofilms, whereas the remaining 33% were unable to do so for various reasons (Hassan et al., 2011).

Dheepa et al. investigated the formation of biofilms and discovered that sixty-five percent of the fifty isolates they tested formed biofilms (Dheepa, 2011). Another study showed that 62 percent of the 51 isolates formed biofilms, which is indisputable (Rao et al., 2008). Finally, they all agree with the findings of this research. Dheepa et al. conducted research in 2011 on 50 A. baumannii isolates and found that biofilm tests were carried out using three different methods: the tube technique, the conventional microtiter plate, and the altered microtiter plate. Our study's chances of biofilm development matched those found by Dheepa et al. to an alarming degree. Biofilm manufacturing victimization tubes and new microtiter plate methods were described by Abdi-Ali et al. in their study (Abdi-Ali et al., 2014).

When they worked with 100 isolates, they got the following results by using the check tube technique: 17% had no power, 42% had weakness, 17% had a modicum of strength, and 22% had a lot of power. In the absence of biofilm development, the modified microtiter plate technique yielded the following results: 25% of the samples had

weak, 41% of the samples had moderate, and 18% had high power levels. These findings contrasted with those from the previous research. This may be due to the fact that there are many clinical isolates from various origins.

Here in this study, we are trying to test for bacterial susceptibility within the biofilm forming isolates. In the AST experiment, we noticed that (CIP (ciprofloxacin) (95.1%), TOB (tobramycin) (83.6%), LEV (levofloxacin) (79.5%), CN (gentamycin) (73.4%), S (streptomycin) (65.3%), and AMC (amoxicillin) (55.1%) have the highest resistance ration among the isolates with a ratio higher than 50%.

On the other hand, AK (amikacin) (4%), CT (colistin sulphate) (2%) and MEM (meropenem) (0%) have the highest ratio in susceptibility among the isolates. From these, we chose meropenem and amikacin for further investigation in the biofilm sensitivity test.

In a study conducted with materials taken from patients coming from Acıbadem Hospital in Istanbul with UTI examination, 1456 urine samples were collected, 1100 of them were identified as *E. coli*, and 356 as Klebsiella spp. isolates. The ESBL rates of the isolated isolates were 12% (n: 132) for *E. coli* and 12% (n: 41) for Klebsiella spp. In *E. coli* strains, amikacin 3%, nitrofurantoin 3%, cefoxitin 5.3%, piperacillin-tazobactam 21.2%, gentamicin 34.8%, trimethoprim / sulfamethoxazole 68.9%, tobramycin 75.9%, ciprofloxacin and norfloxacin 80.3% (Akyar, 2008).

In a study conducted at Eskişehir Osmangazi University, 82 clinical samples were evaluated. 49 of them were reported as ESBL (+) *E. coli* isolates, and 33 as ESBL (+) *Klebsiella pneumoniae* isolates. In both isolates and resistance levels, strong resistance rates were found; ciprofloxacin 50.9%, cefepime 81.7%, trimoxazole 47.4%, tetracycline 75.0%, and gentamicin 48.7%. Meropenem and imipenem were 100% susceptible and in *K. pneumoniae* isolates, ertapenem was 100% susceptible and in *E. coli* isolates, 95.9%. Just two *E. coli* isolates were confirmed to be ertapenem-resistant (Zafar et al., 2014).

In the Mykhalko study (Mykhalko, 2018), amoxicillin/clavulanic acid showed the lowest resistance to *E. coli* in youngsters, with a susceptibility of just 2.27 percent, with a

95 percent confidence interval of 0.00 to 12.89 percent. Cephalosporin susceptibility ranged from 34.09 percent (cefuroxime, 95 percent CI 21.82-48.92 percent) to 65.91 percent (cefepime, 95 percent CI 51.08-78.18 percent) for the antibiotics studied. Adults were the most susceptible to Gemifloxacin and ceftriaxone (80.00 percent [95 percent CI 70.19-87.22 percent] for both], while amoxicillin/clavulanic acid was the least susceptible (2.35 percent, 95 percent CI 0.14-8.68 percent).

In this study (Kibret & Abera, 2011) . Of the 446 samples tested, *E. coli* was found in 14.2% of them. The piddle samples had the highest isolation rate (203). 45.5 percent Perimycin (89.4%), Larotid (86.0%), and antibacterial medicines (72.6%) also had high resistance rates. A significant amount of resistance was found, however, with respect to antibacterial (96.4 percent) as well as norfloxacin (90.6 percent) and antibiotics (79.5 percent) (p 0.001). In addition, there was a 74.6 percent incidence of multiple antimicrobial resistance and hyperbolic resistance to all antimicrobials except ciprofloxacin.

Isolates were characterized as biofilm formers, intermediates, and non-biofilm formers according to the Cong-Red test. Interestingly, all the samples classified as biofilm former were intermediate biofilm former when quantitative biofilm microtiter plate was carried out. This indicates the Congo-red test is less sensitive than the microtiter plate.

Lastly, all the isolates were tested using minimal biofilm eradication concentration to check if the biofilm could affect the sensitivity of the isolate to the susceptible antibiotic. As previously known, most of the isolates are resistant to low concentrations of the two antibiotics when they form biofilm.

6. CONCLUSION

E. coli isolates from UTI patients can form biofilms in most cases. The Congo-Red method is a good screening method for biofilm formation in order to shorten the time needed to get results. Treating biofilm from *E. coli* is a good option.

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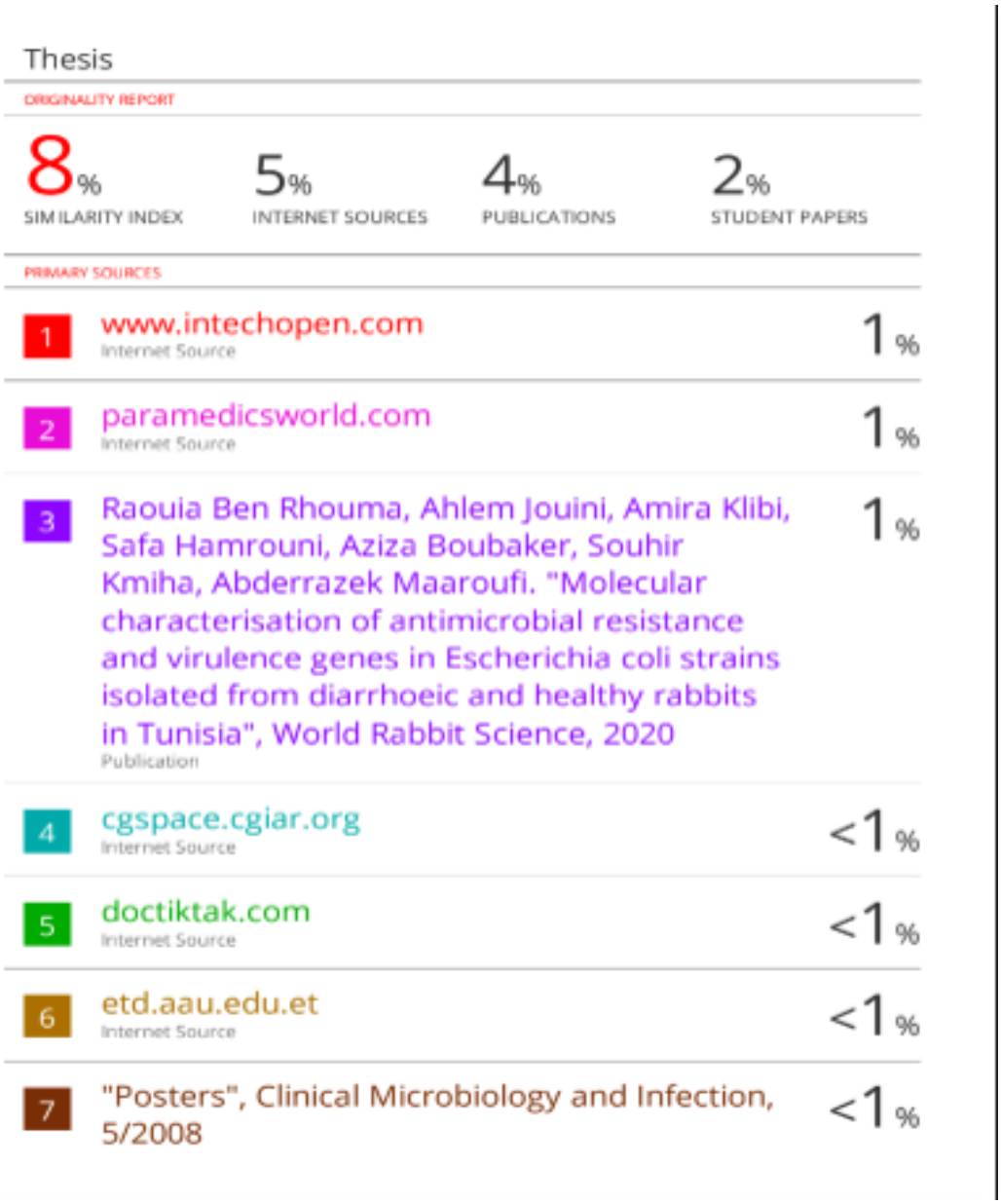

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Title:	**Biofilm formation tendency and antimicrobial susceptibility of Escherichia coli isolated from various clinical samples of patients with UTI**
Supervisor:	Assoc. Prof. Dr. Ayşse ARIKAN SARIOGLU

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Duty	Institution	Duration (Year - Year)
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Mathematical Modeling in Health Certificate (Attendance)	DESAM Institute, Near East University, Nicosia	2019
Parasitology Academic Course: Essential and Application	Turkish Microbiology Society, TMC-KKTC Microbiology Platform, Nicosia	2020

Foreign Languages	Reading comprehension	Speaking *	Writing*
English	Excellent	Excellent	Excellent

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•			•	•				

Computer Knowledge

Program	Use proficiency
SPSS	Excellent
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Common Computer Programs and Skills	Excellent

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Dean of Faculty of Applied Medical Sciences

Jordan University of Science and Technology

In reference to the scientific research which is presented by Dr. Samer Swedan, and Nadia Alyahya, entitled:

Resistance mediated by 16S rRNA methylases and beta-lactamases among aminoglycoside-resistant Gram-negative clinical isolates

We would like to inform you that the IRB Committee has granted the researchers the approval to conduct this proposal in the Jordanian Community for the purpose mentioned above, under the following conditions:

1. Committment to the Scientific Research Policy at Jordan University of Science and Technology and King Abdullah University Hospital.
2. Maintaining data confidentiality and using it only for scientific purposes.
3. Consent form is not required.
4. This approval will be cancelled if the principal investigator doesn't provide IRB with the final report about the results of the research after twelve months.

Regards,

Prof. Yousef Al-Gaud

Chairman of the Institutional Review