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College of Science
Department of Biology

Molecular Characterization of Carbapenems Resistant Genes among *Escherichia coli* Isolated from Urinary Tract Infections Patients in Hilla City-Iraq

A Thesis

Submitted to the Council of College of Science / University of Babylon
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of Philosophy of Science in Biology

By

Hayder Abdulameer Abdulredha Jaber Alassdy

B. Sc. College of Science / University of Al-Qadisyah (2006)

M.Sc. College of Science / University of BAMO\India (2014)

Supervised by

Prof. Dr. Eman Mohammed Jarallah

2023 A.D. **1445 A.H.**

بِسمِ اللهِ الرَحمنِ الرَحيم

﴿ يَا أَيُّهَا الَّذِينَ آمَنُوا إِذَا قِيلَ لَكُمْ تَفَسَّحُوا
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وَاللَّهُ بِمَا تَعْمَلُونَ خَبِيرٌ ﴾

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Supervisor Certification

I certify that this thesis, entitled **Molecular characterization of carbapenem resistant genes among *Escherichia coli* isolated from urinary tract infections patients in Hilla City-Iraq**, was prepared by **Hayder Abdulameer Abdulreda Jaber Alassdy** under my supervision at the Department of Biology, College of Science, University of Babylon as partial fulfillment of the requirement for the degree of doctor of Philosophy Of Science in Biology.

Professor

Dr. Eman Mohammed Jarallah

College of Science / University of Babylon

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In view of the available recommendation, I present this thesis for evaluation by the Examining Committee.

Asst. Prof. Dr. Adi Jassim Abd AL-Rezzaq

Head of Biology Department

College of Science / University of Babylon

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We, the examination committee, certify that we have read the thesis entitled (**Molecular Characterization of Carbapenems Resistant Genes Among *Escherichia coli* Isolated from Urinary Tract Infections Patients in Hilla City-Iraq**) and have examined the student (**Hayder Abdulameer Abdulreda Alassdy**) in its contents, and that in our opinion it is accepted as a thesis for the Degree of Doctorate of Philosophy of Science in Biology with excellent estimation.

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College of Science/University of Babylon
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College of Science /University of Babylon
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Assist. Prof. Dr. Sura Ihsan Abed Jabuk
College of Science /University of Babylon
(Member)

Signature
Assist. Prof. Dr. Marwa Fadhil Alsaffar
College of Food Science/Al-Qasim Green University
(Member)

Signature:
Prof. Dr. Eman Mohammad Jarallah
College of Science/University of Babylon
(Member and Supervisor)
Approved for the college committee of graduate studies

Signature:
Prof. Dr. Mohammed Hadi Shinen Alshammeri
(Dean of the College of Science – University of Babylon)
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Dedication

To…. My parents The kind heart and My dear wife and to everyone who supported me during my studies.

Hayder 2023

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Summary:

Urinary tract infections (UTIs) are one of the most widespread bacterial infections with a global extension. These infections are predominantly caused by uropathogenic *Escherichia coli* (UPEC). The relationship between multidrug resistance of *Escherichia coli* and resistance genes profiles among carbapenms resistance of uropathogenic *Escherichia coli* isolates that isolated from patients with in urinary tract infection in Hilla City . Therefore, the current survey was carried out in order to determine the phenotypically , genotypically and determine genes expressions among multidrug resistance uropathogenic *Escherichia coli* isolates that isolated from patients with UTIs in Hillah city.

The study included collection and analysis of 646 midstream urine specimens which is immediately examined by generals urine examination (GUE) after collection to detecting physical ,chemicals and microscopically examinations to identifying presence of infections due to the urinary tract infection occurs without any symptoms observed in the patient, followed by phenotypically ,genotypic and gene expression detecting methods.

The valuable midstream urine specimens were carefully collected from February to July\ 2022 from different hospitals in Hilla City. The clinical specimens from individuals with UTI symptoms were obtained from the following hospitals which is including : Al-Imam- Alsadiq teaching- hospital (377 urine clinical specimens), Marjan teaching hospital (175 urine clinical specimens), and Babylon for women's and children's hospital (94 urine clinical specimens). Subsequently, all midstream urine clinical specimens were examined by microscopically examination and cultured onto MacConkey agar , Eosin Methylene Blue (EMB) agar and blood agar plates followed by incubation for 24 hrs at 37°C to detect of lactose and non-lactose fermenting isolates. Out of total 646 midstream urine specimens, only 320

(49.5%) isolates were successfully identified as *Escherichia coli* bacteria through its growth on the following selective and differential cultures media which is including MacConkey and EMB agar media and the rest 326 (50.5%) isolates were belong to others gram negative bacteria.

Results indicated the presence of 320 clinical specimens, encompassing 273 (85.3%) females and 47 (14.7%) males. These *Escherichia coli* isolates were obtained from urinary tract infections (UTI) patients are subjected to antibiotics susceptibility testing (AST). The results of this study showed the highest incidence of UTIs was observed in the Adult (30-60) years old age group among the rest ages groups.

The biochemical results and Vitek2 System tests were also confirmed as confirmatory tests for these isolates, which showed that all the isolates were *E. coli* bacteria.

Antibiotics susceptibility testing (AST) revealed notable resistance trends among *E. coli* isolates. Notably, *β*-lactam combinations, including Amoxicillin-clavulanate and Ampicillin, exhibited resistance rates of 87.1% and 75.9%, respectively. Aminoglycosides (e.g., Gentamicin) demonstrated resistance in 66.8% of cases, while Sulfonamides (e.g., Trimethoprim) and Nitrofurans (e.g., Nitrofurantoin) exhibited resistance rates of 49.6% and 43.1% respectively. The susceptibility of isolates varied, with Ciprofloxacin, Cefapime, Ceftriaxone, and Piperacillin showing sensitivities in the 34.3% to 52.1% range.

Moreover, the results of AST showed only 20 *Escherichia coli* isolates (6.2%) were resistance to the meropenem and imipenem.

The results of phenotypically examinations which is including 20 carbapenms resistance *Escherichia coli* isolates showed positive result for amoxiclave double disk synergy test and modified hodge's test (MHT) which is confirms that these isolates have the ability to produce beta-lactamase enzymes.

The molecular detection methods has been used to identify carbapenems resistance genes among 20 *E. coli* isolates. The molecular detection methods were used to detect the following genes which is including $bla_{CTX\text{-}M\text{-}1}$ and bla_{DHA} were most prevalent with percentage levels (no=19) (95%), followed by bla_{TEM} (no=16) (80%), bla_{NDM} (no=15) (75%), bla_{IMP} (no=12) (60%), bla_{VIM} (no=10) (50%), and others with varying frequencies. While each of the genes of bla_{GES} and bla_{SME} were not detected during this study.

According to the study's findings, the majority isolates included at least two genes. Isolate number E.co-9 reveal a high percentage level which is carried 10 distinct genes, while others isolates which is including (E.co-2, E.co-3, E.co-11 and E.co-18) each carried nine different genes.

According to Amblar classification the results of molecular investigation of this study showed among 280 β-lactamase genes has been detected during this study that showed positive results for 126 (45%) genes whereas the rest of the genes totally 156 (55%) genes showed negative result .

The molecular characteristics of 20 carbapenms resistance *E. coli* isolates showed all of the genes belong to class A within n=53(42.1%) *β*-lactamase out of 126 positive genes followed by class B within n=37 (29.3%) *β*-lactamase genes, while class c was n=30 (23.8%) and finally class D showed the lowest percentage levels among *β*-lactamase genes that detected during this study it was n=6 (4.8%).

Finally this study assessed gene expression in the presence of Imipenem (IMP) inhibitors, revealing a 1.8 to 12.4 fold increase in bla_{IMP} mRNA levels in response to exposure to Imipenem antibiotics. The results of gene expression showed varying levels in the presence of the impenem inhibitor for all bacterial isolates. It is worth noting that isolate No. 13 showed the highest level of gene expression among the rest of the other isolates. It could be concluded that the investigation highlights the prominence of urinary

tract infections, particularly among uropathogenic *E. coli* , and demonstrates the prevalence of multi-resistance gene profiles among urinary tract infections patients in Hillah City.

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List of Abbreviations

Abbreviation	Full Name
A/E	Attaching and Effacing
ADC	*Acinetobacter*-Derived Cephalosporinase
Afa/Dr	Adhesions Factors Diarrhea
AMC	Amoxicillin
AMP	Ampicillin
AmpC	Amoxicillin, Clavulanic Acid
AMPs	Antimicrobial Peptides
APEC	Avian Pathogenic *E. coli*
AST	Antibiotics Susceptibility test
B.C.E	Before Christian Era
BAM	Barrel Assembly Machinery
BFP	Bundle-Forming Pilus
BHIB	Brain Heart Infusion Broth
bp	base pair
CDC	Center for Disease Control and Prevention
CDT	Cyclomodulin Toxin
CF	Colonization-Related Factors
CIP	Ciprofloxacin
CLSI	Clinical & Laboratory Standards Institute
CPT	Cefapime
CREC	Carbapenem-Resistant *E. coli*
CRO	Ceftriaxone
Ct	Cycle Threshold
CTD	C-Terminal Domain
CTX-1	Cefotaxime
CTX-M-1	Cefotaximase-Munchen
D.W	Distilled Water
DAEC	Diffusely Adhering *E. coli*
DEC	Diarrhea-Genic *E. coli*
DHA	Dhahran
DNA	Deoxyribonucleic Acid
dNTPs	Deoxynucleotide Triphosphates
dsDNA	Double-Stranded DNA
E. coli	*Escherichia coli*
EAEC	Enteroaggregative *E. coli*
EAF	*E. coli* Adherence Factor
EBC	*Enterobacter Cloacae*
EDTA	Ethylene-Diamine-Tetra-Acetic Acid
EHEC	Enterohemorrhagic *E. coli*

EIEC	Enteroinvasive *E. coli*
EMB	Eosin Methylene Blue
EPEC	Enteropathogenic *E. coli*
ESBLS	Extended-Spectrum ***β***-Lactamases
ETEC	Enterotoxigenic *E. coli*
ExPEC	Extraintestinal Pathogenic *E. coli*
GEN	Gentamicin
GES	Guiana Extended Spectrum
HUC	Hemorrhagic Colitis
IMP	Imepenem
IMpA	Inner Membrane Protein A
IMP-E	Imepenemase
INPEC	Intestinal Pathogenic *E. coli*
Kb	kilobases
kDa	Kilodaltons
KPC	*Klebsiella pneumoniae* Carbapenemase
LEE	Locus of Enterocyte Effacement
LPS	Lipopolysaccharides
LT	Heat Labile Enterotoxin
MBLs	Metalo ***β***-lactamase Enzymes
MDR	Multi-Drug Resistance
MEM	Meropenem
MHA	Muller Hinton Agar
MHB	Mueller-Hinton Broth
MHT	Modified Hodge's Test
MIC	Minimum Inhibitory Concentration
MR	Methyl Red
MRSA	Methicillin-Resistant *Staphylococcus aureus*
NBM	Neonatal Bacterial Meningitis
NDM	New Delhi metallo-***β***-lactamase
NFW	Nuclease Free Water
NIT	Nitrofurantion
NMEC	Neonatal Mingitis-Associated *E. coli*
NS	Normal Saline
NTD	N-terminal Domain
OmpA	Outer Membrane Protein A
OMPs	Outer Membrane Proteins
OXA	Oxacillinases
PAIs	Pathogenicity Islands
PBP	Penicillin-Binding Protein
PBS	Phosphate Buffered Saline

PCR	Polymerase Chain Reaction
PDR	Pan-Drug Resistant
PET	Plasmid-Encoded Toxin
pH	Power of H^+ Ion Concentration
PI	Piperacillin
PER	*Pseudomonas* Extended Resistance
Q-RT- PCR	Qualitative Real Time Polymerase Chain Reaction
QS	Quorum Sensing
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SAT	Secreted Auto-Transporter Toxin
SHET-1	*Shigella* Enterotoxin-1
SHV	Sulfhydryl Variable
SME	*Serratia marcenscens* Enzyme
ST	Heat-Stable Toxins
STEC	*Shiga*-Toxin Producing *E. coli*
STX	Trimethoprim
TBE	Tris Borate-EDTA Buffer
TEM	Temoneira
tEPEC	Typical Enteropathogenic *E. coli*
UPEC	Uropathogenic *E. coli*
UTIs	Urinary Tract Infections
UV	Ultraviolet
V	Volume
VAT	Vacuolating Auto-Transporter Toxin
VEB	Vietnamese Extended-Spectrum ***β***-lactamases
VFs	Virulence Factors
VIM	Verona integron-encoded metallo-***β***-lactamase
VP	Voges-Proskauer
W	Weight
WHO	World Health Organization
XDR	Extensively-Drug Resistant
XDR	Extensively-Drug Resistant

List of Measurement Units

Abbreviation	Key
bp	Base Pair
ºC	Degree Celsius
KDa	Kilo Dalton
µM	Micro Molar
µg	Microgram
µL	Microliter
µmol	Micromole
mg	Milligram
ml	Milliliter
mm	Millimeter
ng	Nanogram
g	Gram
kb	kilobases
kDa	Kilodaltons
RPM	Round Per Minute

Chapter One

Introduction

and

Literatures Review

1. Introduction and Literatures Review

1.1 Introduction

Recent scientific programs primarily focus on investigating into the intricate aspects of antibiotic resistance, including its genetic foundations, underlying mechanisms, and the complex pathways through which genes are transferred. This heightened interest has been fueled by the growing apprehension arising from the increasingly alarming trends in antibiotic resistance observed in bacteria (Tarín and Pérez., 2022).

A prominent trend that warrants ongoing attention is the escalating prevalence of carbapenem-resistant Enterobacteriaceae. The bacterial of carbapenems resistance strain has seen a surge in global reporting, largely attributed to the production of an enzyme catalyzing carbapenems and ***β***-lactamases (Ma *et al.*, 2023). A parallel concern emerges in latent carbapenem resistance within bacteria, a phenomenon where bacteria possessing resistance genes and positive phenotypic tests maintain responsiveness to carbapenem antibiotics (Drenjančević *et al.*, 2019). The aftermath of the COVID-19 pandemic, characterized by increased bacterial co-infections and suboptimal antibiotic use, anticipates a surge in antibiotic resistance rates (Ghosh *et al.*, 2021). Within the Enterobacteriaceae family, *Klebsiella pneumoniae* and *Escherichia coli* appeared as pivotal causative agents of a wide array of infections, encompassing pneumonia, urinary tract infections (UTIs), bacteremia, cystitis, and wound infections. Notably, these two pathogens, *E. coli* and *K.pneumoniae*, wield significant influence on the entire of urinary system and bloodstream-infections (Bitsori and Galanakis ., 2019).

The intricate landscape of carbapenem resistance within Enterobacteriaceae unveils a complicated situation. This landscape encompasses diverse mechanisms, ranging from the action of Metallo-***β***-lactamases operating independently to their collaboration with porin

protein loss. Furthermore, through existence of plasmid-mediated carbapenem-hydrolysis of Metallo-*β*-lactamases has been extensively documented across various geographical regions (Tilahun *et al.*, 2021).

Aim of Study:

The research conducted to determine molecular characterization of 20 carbapenms resistance *E. coli* isolates by using the following specific primers to detecting the following genes: class A (Extend spectrum *β*-lactamase (ESBLS) including :- [***bla*** $_{TEM}$ (972 bp), ***bla*** $_{SHV}$ (898 bp) , ***bla*** $_{CTX\text{-}M\text{-}1}$ (646 bp) ,***bla*** $_{PER}$ (520 bp) and ***bla*** $_{VEB}$ (961 bp)], while class A carbapenemase including : [***bla*** $_{GES}$ (371 bp) and ***bla*** $_{SME}$ (334 bp)]. Class B including: [***bla*** $_{IMP}$ (232 bp), ***bla*** $_{NDM}$ (550bp) and ***bla*** $_{VIM}$ (390 bp)]. Class C including: [***bla*** $_{ADC}$ (445 bp), ***bla*** $_{DHA}$ (405 bp) and ***bla*** $_{ECB}$ (302 bp)]. Finally Class D including only ***bla*** $_{OXA\text{-}1}$ (564 bp) respectively that assisted the genes identification process among UPEC isolated from UTI patients. The Objective of the current research has been achieved by the following steps :-

1-Midstream urine samples collected from UTI patients as well as isolation and identification of *E. coli* UTI patients by using different methods.

2-Antibiotics susceptibility test (AST) against these isolates.

3-Phenotypic detection of carbapenems resistance *E. coli* isolates by using ESBL tests including amoxiclave double disk Synergy test and modified hodge's test (MHT).

4- Genotypic detection of carbapenems resistance *E. coli* isolates by using conventional PCR techniques and real time PCR.

5-Evaluate the carbapenems inhibition antibiotics by using some inhibitory that used to detect the gene expression in response to some inhibitors (Impenem antibiotic) that effects on the *E. coli* isolates.

1. 2 Literatures Review

1.2.1 Enterobacteriaceae:

The Enterobacteriaceae family includes many Gram-negative bacteria that live as normal flora inside the intestines of humans and animals. This heterologous family includes *Escherichia*, *Salmonella*, *Shigella*, *Enterobacter*, *Klebsiella*, *Proteous*, etc (Riedel *et al.*, 2019). Members of this family can cause many diseases to humans and animals, including wound infections, nosocomial infections, respiratory tract infections, urinary tract infections (UTIs), and genital infections. They're rod-shaped, Gram-negative, aerobic or facultative anaerobe. Most of the members are lactose fermenters, oxidase test negative, indole test positive, non-spore forming, flagellated, and the optimal temperature for their growth is 37 °C. They have a wide range of virulence factors like toxins, enzymes, capsules, flagella, etc (Oliveira *et al.*, 2017).

1.2.2 *Escherichia coli:*

Escherichia species are integral members of the Enterobacteriaceae bacterial family, encompassing a diverse range, consist such as *E. hermanii., E. fergusonii, E. vulneris, E. albertii, E. blattae,* and the widely recognized *E. coli*. Each species manifests unique biochemical distinctions (Olowe *et al*., 2017).

Notably, *E. coli* is a prevalent constituent like the natural flora in the gastrointestinal tract, adopting a dual nature as an opportunistic pathogen capable of triggering distinct clinical outcomes. It can induce diarrhea, a diarrhea-genic *E. coli* (DEC) condition (Levinson *et al*., 2018).

Initial detection of this bacterium can be traced back to 1885, when Theodor Escherichia first recognized its existence (Sussman., 1985). Originally referred to as "Bacterium Coli" - "Colon Bacterium,"

additional knowledge emerged in 1894 when it has been isolated from the stool of healthy children, leading to its categorization as nonpathogenic (Sussman ., 1985). A pivotal turning point emerged during (1945), when Bray's research confirmed specific *E. coli* strains as the primary causative agents of infantile diarrhea in England (Bray ., 1945). *Escherichia coli* is situated within the Enterobacteriaceae family in the bacterial taxonomy, aligning with the classifications detailed by (Faner *et al*., 2017) as follows:

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Enterobacterales

Family: Enterobacteriaceae

Genus: Escherichia

Species: coli

1.2.3 *E. coli* Features :

Escherichia coli none spore forming, Gram-negative. Its motility is facilitated by flagella, enabling movement. Colonies of *E. coli* usually have a convex shape and are smooth on the surface. Notably, on MacConkey agar, colonies adopt a flat and dry appearance, showcasing a distinctive pink hue. Conversely, on Eosin Methylene Blue (EMB) agar, they present a vibrant and green metallic sheen. *Escherichia. coli* O157:H7 can be differentiated from other *E. coli* serotypes by utilizing sorbitol MacConkey agar since O157:H7, unlike other serotypes, cannot ferment sorbitol. *Escherichia coli* strains do not produce H_2S in Triple Sugar Iron agar (Wanger *et al*., 2017). Predominantly, *E. coli* strains exhibit the capacity to generate the *β*-glucuronidase enzyme. An optimal temperature of 36 - 37°C is conducive to growth conditions, while a pH

range of 4.4 to 9 sustains growth. *Escherichia coli* typically provides negative results in oxidase, urease, and nitrite tests, but it yields a positive catalase test outcome. Moreover, Indole test generally produces as positive-results in the majority of *E. coli* strains (Wanger *et al*., 2017).

1.2.3.1 Commensal *E. coli*

Commensal *E. coli* strains are members of the gastrointestinal micro-biota of maximum mammalian host, inclusive of human beings (Blyton *et al*., 2013). They're considered to play a position in helping digestion and to offer protection mechanisms towards enteric pathogens with the aid of effectively competing with different microbiota and hindering colonization of pathogenic agents and also produce vitamin K that is useful for the host (Schierack *et al*., 2009). Commensal *E. coli* are rarely associated with disease except in immune compromised hosts and make their area of interest in the mucous layer of the mammalian colon (Kaper *et al*., 2004). Commensal strains of human beginning introduction are specially derived from phylogenetic institution A and B1, generally missing specialized virulence attributes which are determined in intestinal pathogenic and extra-intestinal pathogenic *E. coli* (Micenkova *et al*., 2016).

1.2.3.2 Pathogenic *E. coli*

1.2.3.2.1 Extraintestinal Pathogenic *E. coli* (ExPEC)

Extraintestinal Pathogenic *E. coli* are the ones *E. coli* strain that cause extra-intestinal infections and are answerable for a huge spectrum of illnesses like urinary tract infections (UTI), neonatal bacterial meningitis (NBM),sepsis and septicemia (Ron, 2006). Moreover, an animal pathotype of extraintestinal pathogenic *E. coli* is avian pathogenic *E. coli* (APEC) causing respiratory contamination, septicemia in human and together with other ExPEC lines account for huge fitness and monetary lost (Kaper *et al*., 2004).

Phylogenetically and epidemiologically ExPEC are potentially exclusive from the ones of intestinal pathogenic and commensal lines (Smith *et al*., 2007). Most of the ExPEC lines Phylogenetically belong to B2 and to a lesser volume D companies and are ready with diverse virulence elements that help these lines in the course of special mode of infection mechanisms like adhesion, invasion of host tissues, escape host defense mechanisms, signaling and production of extraordinary toxins interfering host cellular capabilities thereby promoting extra-intestinal infection in both regular and immune compromised hosts (Dobrindt and Hacker 2008 ; Wiles *et al*., 2009).

1.2.3.2.1.1 Uropathogenic *Escherichia coli* (UPEC)

Strains of uropathogenic *E. coli* (UPEC) are the primary cause of urinary tract infections, including both cystitis and pyelonephritis (Wiles *et al* ., 2009). These bacteria have evolved a multitude of virulence factors and strategies that facilitate bacterial growth and persistence within the adverse settings of the host urinary tract (Wiles *et al* ., 2009). Expression of adhesive organelles like type 1 and P pili allow UPEC to bind and invade host cells and tissues within the urinary tract while expression of iron chelating factors (siderophores) enable UPEC to pilfer host iron stores , deployment of an array of toxins, including hemolysin and cytotoxic necrotizing factor 1, provide UPEC with the means to inflict extensive tissue damage, facilitating bacterial dissemination as well as releasing host nutrients and disabling immune effector cells. These toxins also have the capacity to modulate, in more subtle ways, host signaling pathways affecting myriad processes, including inflammatory responses, host cell survival, and cytoskeletal dynamics (Wiles *et al* ., 2009).Virulence factors are specific traits enabling *E. coli* to overcome host immune system and cause various diseases (Hacker *et al* ., 1997). Virulence genes are located on transmissible genetic elements and/or in

particular regions on the chromosome that are called pathogenicity islands (Johnson and Stell, 2000). Pathogenicity islands are associated with the genome of pathogenic strains and led to coordinate horizontal transfer of virulence genes between strains of one species or even related species (Joanna *et al.*, 2011). Uropathogenic *Escherichia coli* strains also have other various types of virulence factors such as adhesions, toxins and iron uptake systems that facilitate colonization and persistence of the bacteria in the urinary tract (Chedi *et al.*, 2009). Attachment of the bacterium to the uroepethelium is the main step to initiate and develop UTI. Adhesions, such as p-fimbriae, help the bacteria to resist against urinary lavage and invade epithelial cells. P-fimbriae are one of the most important adhesions encoded by pap (pyelonephritis-associated pili) genes and could act as predictors of pyelonephritis (Ejrnæs, 2011).

1.2.3.2.1.2 Pathogenicity Mechanisms of UPEC

Uropathogenic *Escherichia coli* preferentially colonizes the bladder and causes cystitis, but can also ascend through the ureters into the kidneys, causing pyelonephritis (Bower *et al*., 2005). In response to the breach by UPEC into the normally sterile urinary tract, host inflammatory responses are triggered leading to cytokine production, neutrophil influx, the exfoliation of infected bladder epithelial cells, and the generation of reactive nitrogen and oxygen species along with other antimicrobial compounds (Mulvey *et al*., 2000). Uropathogenic *Escherichia coli* have evolved a number of strategies to evade these innate immune responses, enabling the pathogens to more effectively colonize the urinary tract and persist. The ability of UPEC to bind host tissues is one of the paramount factors that facilitate UPEC colonization of the urinary tract, allowing the bacteria to withstand the bulk flow of urine and promoting UPEC invasion of urothelial cells. Within bladder epithelial cells, UPEC are trafficked into membrane-bound, acidic compartments with features similar to late

endosome or lysosomes (Eto *et al*., 2007). In the large, terminally differentiated superficial umbrella cells that line the lumen of the bladder UPEC are able to break into the host cell cytosol and rapidly multiply, forming large intracellular biofilm-like communities that can contain several thousand bacteria. This phenomenon is observed in human patients with UTI (Rosen *et al*., 2007).

1.2.3.3 Intestinal Pathogenic *E. coli* (InPEC)

Intestinal pathogenic *E. coli* (InPEC) are liable for health troubles in mammals , such as human beings (Muller *et al*., 2007). Primarily based at the virulence elements, severity of clinical implications and prognosis, presently intestinal or diarrheagenic *E. coli* are grouped into six foremost pathotypes Enteropathogenic *E. coli* (EPEC), Enterohemorrhagic *E. coli* (EHEC), Enterotoxigenic *E. coli* (ETEC), Enteroaggregative *E. coli* (EAEC), Enteroinvasive *E. coli*, (EIEC) and diffusely adherent *E. coli* (DAEC) (Nataro and Kaper., 1998). Strains from those pathotypes show range in phylogenetic groupings however are related specifically with the A and B1 phylogenetic corporations (Smith *et al*., 2007).

1.2.3.4 *E. coli* Pathological Patterns:

Escherichia coli pathotypes encompass a diverse group of strains, each characterized by unique features, virulence factors, and mechanisms of action. These pathotypes have been meticulously categorized into six distinct groups based on their distinctive characteristics (Rivas *et al*., 2015) and (Malema *et al*., 2018).

1.2.3.4.1 Enterohemorrhagic *E. coli* (EHEC):

Among foodborne pathogens, Shiga like toxin-producing *E. coli* (STEC), also referred to as Enterohemorrhagic *E. coli* (EHEC), stand out as significant agents capable of inducing a spectrum of infections, ranging from mild diarrhea to severe conditions such as hemorrhagic colitis (HUC). Notable human-infecting serotypes include O24, O103,

O111, O45, O121, O145, and O157 (Gould *et al*., 2013). Enterohemorrhagic *Escherichia coli* isolates are defined by their ability to synthesize Shiga toxins, comprising an active A subunit and a B5 sub-unit involved in binding receptors of glycolipid on target cells, leading to systemic absorption (Melton., 2014). Shiga toxins disrupt protein production by removing adenine residues of rRNA 28S within ribosome-60S (Moxley *et al*., 2020).

1.2.3.4.2 Enteropathogenic *E . coli* (EPEC):

Enteropathogenic *Escherichia coli* (EPEC) represents as well-defined pathotype responsible for causing diarrhea and intestinal tissue damage through the formation of linking as well as effacing lesions. These lesions disrupt natural microvilli construction (Mare *et al*., 2020). Unlike certain *E. coli* pathotypes, EPEC strains lack the ability to produce Shiga toxins LT or ST enterotoxins. Within the EPEC category, two subtypes are identified as atypical EPEC (*aEPEC*) and typical EPEC (*tEPEC*) (Mare *et al*., 2020). Strains tEPEC, associated with human diarrhea, carry the *E. coli EAF* plasmid containing the *bfp* operon, while *aEPEC* strains lack this operon (Mare *et al*., 2020).

1.2.3.4.3 Enterotoxigenic *E. coli* (ETEC):

Distinguished through their capacity to create colonization-related factors (CF) and enterotoxins, specifically L-T also S-T, Enterotoxigenic *E. coli* (ETEC) strains set themselves apart from other pathotypes (Buuck *et al*., 2020). Enterotoxigenic *Escherichia coli* is a major contributor to childhood diarrhea in developing countries and is associated with travel-related diarrhea, causing watery diarrhea due to significant ion and water loss. Enterotoxins disrupt trans-membrane channels within the intestinal epithelium, leading to diarrhea (Buuck *et al*., 2020).

1.2.3.4.4 Enteroinvasive *E. coli* (EIEC) :

Enteroinvasive *Escherichia coli* is responsible for dysentery, a condition prevalent in developing countries. Dysentery arises from EIEC's ability to invade and penetrate intestinal cells, resulting in cellular destruction (Michelacci *et al*., 2020). The bacterium adheres to the mucus layer via endocytosis, facilitating its spread from one cell to another. Recent outbreaks, such as the O96:H19 serotype outbreak in Sweden, highlight the significance of EIEC (Lagerqvist *et al*., 2020).

1.2.3.4.5 Enteroaggregative *E. coli* (EAEC):

Enteroaggregative *Escherichia coli* (EAEC) can be characterized by distinctive "stacked brick" pattern on epithelial cells in tissue cultures (Ellis *et al*., 2020). Enteroaggregative *Escherichia coli* induces a distinct form of diarrhea, characterized by its watery nature, mucus presence, potential blood, stomach pain, low-grade fever, and vomiting (Ellis *et al*., 2020). An exceptional hybrid strain was responsible for a major outbreak in Germany in 2011, contributing to thousands of diarrhea situations as well as multiple fatalities (Ellis *et al*., 2020).

1.2.3.4.6 Diffusely Adhering *E. coli* (DAEC):

Diffusely Adhering *Escherichia coli* (DAEC) is responsible for distinctive alterations in epithelium tissue cells associated with diarrhea also other gastrointestinal disorders (Omolajaiye *et al*., 2020).DAEC comprises a variety of strains of *E. coli* carrying Afa/Dr adhesions genes, potentially causing diarrhea in humans. This pathotype predominantly affects children and significantly contributes to child mortality, ranking as secondary reason for death among children below five years old (Omolajaiye *et al*., 2020).

1.2.4 Pathogenicity of *E. coli* :

A variety of human illnesses, including gastroenteritis, newborn meningitis, peritonitis, mastitis, and septicemia, are caused by pathogenic

E. coli strains (Tauschek *et al*., 2002). Additionally, these microorganisms are involved in foodborne illnesses, diarrheal conditions, and urinary tract infections (Foxman ., 2010). Urinary tract infections, in particular, exhibit a higher prevalence in women due to anatomical differences in the urinary tract. Uropathogenic *Escherichia coli* establish residence in the urethra, ascend to the bladder and kidneys, leading to conditions like pyelonephritis (Nicolle., 2008). The virulence of these bacteria is enhanced by the production of toxins such as alpha and beta-hemolysin, which disrupt urinary tract cells (Nicolle., 2008). Uropathogenic *Escherichia coli* strains possess a remarkable ability to evade the host's immune system through biofilm formation, thereby augmenting. *E. coli* is additionally implicated in various nosocomial infections affecting hospital patients (Nicolle., 2008).

1.2.5 *E. coli* Epidemiology:

Escherichia coli is a natural component of the gut flora, can colonize the intestines and transition to a pathogenic state under specific conditions (Gurtler *et al*., 2017). Urinary tract infections affect millions of individuals worldwide, with an annual incidence surpassing 150 million cases and significant economic burdens, costing approximately 3.5 billion annually in United States of America (Gharajalar and Sofiani, 2017). Recent *E. coli* outbreaks, such as one documented by the Center for Disease Control and Prevention (CDC) on March 11, 2021, involving 22 cases and one fatality, underscore the consistent rise in annual outbreak occurrences (Foxman ., 2014). The prevalence of infections varies based on individual health, geography, and environmental factors. Women, particularly those aged 30 to 39, are more susceptible to urinary tract infections (Hadi *et al*., 2014; Forsyth *et al*., 2018; Matsukawa *et al*., 2019).

1.2.6 Virulence Factors and Genetics:

Virulence is the bacteria's ability to cause disease, and it's a measurement of pathogenicity. *Escherichia coli* species have many virulence factors that allows the bacteria to cause infection and disease, the most important of these diseases being urinary tract infections (UTIs). The ability of certain bacterial strains to evade the immune system and trigger illness can be attributed to genes encoded within specific regions known as pathogenicity islands (PAIs). These islands span a range of 10-200 kb in size and diverge from other genomic segments due to variations in G+C content (Frankel and Ron, 2018).

Pathogenicity islands are present in pathogenic *E. coli* strains more than nonpathogenic strains and they code virulence factors such as adherence, toxins, siderophores, capsules, lipopolysaccharides and enzymes with each factor having its own encoding genes (Parvez and Rahman, 2018). While the majority *E. coli* strains share a genetic core of approximately 4.1Mb, pathogenic strains, fueled by horizontal gene transfers, can boast genomes maximum 1Mb more than those of commensal *E. coli*, thereby bestowing upon them potential to incite diverse spectrum of diseases across various hosts (Frankel and Ron, 2018)

Escherichia coli employs various mechanisms for DNA transmission, including bacterial conjugation, the horizontal transmission of genetic material from one bacterium to another (Riedel *et al*., 2019). In a related transduction method, *E. coli* leverages genetic recombination with bacteriophage genomes, culminating in initiating infection cycles in other bacteria (Nair *et al*., 2019).

1.2.6.1 Enzymes:

The *β*-lactamase-producing *E. coli* strains (ESBLs) have an impressive resistance profile and can survive a variety of *β*-lactam

medicines, including penicillin. Among these antibiotic-resistant *E. coli* strains, the culprit often bears the bla_{TEM} gene, the mastermind behind ESBL production. These enzymes rupture the amide link within the *β*-lactam ring of antibiotics, making them defenseless against bacterial attack. This is the deceptively straightforward yet devastatingly powerful mechanism by which ESBLs work (Frankel and Ron, 2018). But ESBLs are just one facet of *E. coli* resistance repertoire. Another menacing character in the bacterial saga is carbapenemase, frequently partnered with non-*β*-lactam antibiotic resistance genes. This sinister enzyme, often plasmid-mediated for ease of transmission, imparts a multi-drug resistant (MDR) profile to its host bacteria. Carbapenemase specialize in disarming carbapenem antibiotics, a last resort against many bacterial foes. This includes formidable antibiotics such as ertapenem, meropenem, imipenem, and doripenem when carbapenemase is on the scene, these antibiotics lose their efficacy, leaving clinicians with limited options in the battle against infection. The understanding of these resistance mechanisms owes much to the pioneering work of researchers (Frankel and Ron, 2018). Their insights into the enzymatic machinery behind antibiotic resistance are essential in the ongoing fight against multi-drug resistant bacteria, emphasizing the need for judicious antibiotic use and the creation of new treatment techniques to fight against these tough microbial foes (Frankel and Ron, 2018).

1.2.6.2 Toxins:

Toxins are proteins or substances produced by some strains of uropathogenic *E. coli* that plays a vital role in causing UTIs. These toxins can modify cell signal pathways and modulate the host's inflammatory response. In 1987, cyclomodulin toxin (CDT) was first described as a virulent toxin in uropathogenic *E. coli* then other toxins were described like cytotoxic necrotizing factor 1 which is encoded by the *CNF1* gene,

secreted autotransporter toxin (SAT), cytolysin A, plasmid-encoded toxin (PET), vacuolating autotransporter toxin (VAT), *Shigella* enterotoxin-1 (SHET-1) and *α*-hemolysin (*hly*A), which is the most important toxin. Hemolysins are lipoproteins that cause pores to form on cells' surfaces that it attaches to causing them to release ATP and leading to their deaths. When it attaches to red blood cells, this causes hemoglobin to be released into the environment which is then metabolized by the bacteria (Parvez and Rahman, 2018).

1.2.6.3 Capsule*:*

Escherichia coli capsules are classified into 4 major groups based on the way their gene clusters are organized and the mechanism of their biosynthesis and assembly. Group 2 capsules includes many K antigens (K1, K2, K5, etc.) (Frankel and Ron, 2018). K1 is associated with meningitis. K2 is a polysaccharide complex made of repeating units of oligosaccharide that provide protection from the immune system by its anti-phagocytic property and serum resistance provided by the alteration of the classical complement pathway by the bacterial outer membrane protein A (OmpA) (Croxen and Finlay, 2010). The genes responsible for the biosynthesis of the group 2 capsules are located on three different regions: Regions I (*kpsFEDUCS*) and III (*kpsMT*) encode a transmembrane complex involved in the export and the assembly of capsular polysaccharides. Region II presence is serotype-dependent and it encodes enzymes that synthesize the needed capsular polysaccharides (Goh *et al.*, 2017).

1.2.6.4 Adhesion:

Adhesion is one of the most important virulence factors the bacteria *E. coli*, and the bacteria's adherence to uroepithelial cells is considered the first step for invasion (Kallas *et al.*, 2020). Adherence depends on the genes encoding the necessary adhesion factors, and these

genes include *eae*, *fim*, *sfa*, *pap*, etc. Fimbriae (pili) include 3 types: F-fimbriae, S-fimbriae and P-fimbriae, and they're considered very important virulence factors as they allow the bacteria to adhere to host's cell which can then initiate colonization and allow the formation of the biofilm, which in turn, allows the bacteria to increase its antimicrobial resistance (Neamati *et al.*, 2015; Spaulding *et al.*, 2017).

1.2.6.4.1 Fimbriae:

Fimbriae are activators of host immune system, extending from the cell surface and promoting bacterial colonization of the susceptible host. Fimbriae play an important role in the invasion and colonization of the host by bacteria (Croxen *et al.*, 2013). There many different types of fimbriae, for example type 1 fimbriae help the bacteria adhere to the intestinal epithelium, while long polar fimbriae play a crucial role in cell invasion (Gurtler *et al.*, 2017). Different fimbriae have different assembly proteins, for example type I fimbriae are assembled by *FimC* and *FimD*, S fimbriae are assembled by *SfaE* and *SfaF*, and P fimbriae are assembled by *papC* and *papD* assembly proteins (Volkan *et al.*, 2015).

A. Type 1 Fimbriae:

Type 1 pili are expressed by 90% of uropathogenic *E. coli* strains and are encoded by *fimH*. Found on the surface of the bacteria, it is 2μm in length and 10 nm in width. The structure of the fimbriae is made of *FimA*, *FimF*, *FimG* and *FimH* (Kallas *et al.*, 2020). This type of fimbriae has a variety of functions, ranging from promotion of biofilm formation on abiotic surfaces to attachment to uroepithelial cells through a mannose-mediated attachment. This function of type 1 pili depends on the *fimH* adhesion found at the tip. *FimH* is made up of 279 amino acids, with two terminal domains: the N-terminal domain (NTD) and the C-terminal domain (CTD). NTD carries a lectin domain (*FimHLD*) while CTD carries a pilin domain (*FimHPD*). The interaction between these

two terminal domains determines how affinitive FimH will be with the related molecule or receptor. FimH is in low affinity mode (T-state) but in the present of high amounts of stress, it can dynamically change into high affinity mode (R-state) (Sarshar *et al.*, 2020).

B. S Fimbriae:

S fimbriae is another type of fimbriae at the disposal of *E. coli* that is present in more than 22% of the uropathogenic type of *E. coli*. Encoded by the *sfa* gene, this type of fimbriae helps *E. coli* adhere to the urinary bladder and kidneys (Behzadi, 2018). S fimbriae is related genetically to F1C fimbriae, but only S fimbriae has been related to more severe meningitis and sepsis infections caused by UPEC (O'Rouke and Cadieux, 2019).

C. P Fimbriae:

Recognized as important virulence factors for uropathogenic *E. coli* and its first recognized virulence factor, P fimbriae are encoded by the *pap* gene and are present in 70% of UPEC (Lane and Mobley, 2007). These type of fimbriae are composed of *PapA* at the base, stretching out to form a stalk which is made of *PapE* and *PapF*, and found at the tip is the adhesion *PapG*. The receptor epitopes for P fimbriae are present on the entirety of the human urinary tract uroepithelial cells, including renal epithelial cells (Behzadi, 2018). Attachment to renal epithelial cells induces an inflammatory response which could lead to more tissue damage, opening more ways for UPEC to invade the urinary tract epithelial layer. It is also worth mentioning that P fimbriae expression is associated with inhibition or reduction of some immune functions of the host, such as the movement of IgA to the renal lumen and neutrophils bactericidal capabilities which in turn elevates UPEC chances of survival and causing infection (O'Rouke and Cadieux, 2019).

1.2.6.5 A/E Lesions:

Escherichia coli attaching and Effacing gene (*eae* gene) is located on the locus of Enterocyte Effacement (LEE), which is a ~35-kb pathogenicity island where the main virulence genes of all strains of *E. coli* capable of inducing A/E lesions (Attaching/Effacing) are located. Locus of Enterocyte Effacement (LEE) is organized into 5 operons (LEE1 through LEE5) located on LEE5, the *eae* gene is about 2800 nucleotides and encodes the adhesion protein intimin, a 94-kDa protein required for the adherence of *E. coli* to host cells at the site of A/E lesion (Gomes *et al.*, 2016; Yang *et al.*, 2020).

Attaching and Effacing gene lesion are characterized by intimate adhesion of *E. coli* to the surfaces of enterocytes, on raised pedestals (pseudopodia), and destruction of nearby microvilli (Sperandio and Hovde, 2015). The intimin protein has an N-terminus and a C-terminus. The C-terminus is highly diverse between different sources and based on differences found in the C-terminus, at least 30 intimin subtypes have been identified (Croxen *et al.*, 2013). The N-terminus fixes intimin in the outer membrane while the C-terminus extends from the cell's surface and binds to Tir, the cellular receptor for the bacterial transmembrane protein intimin. The intimin-Tir interaction makes way for intimate adherence and pedestal formation (Gomes *et al.*, 2016). Huge amounts of intimate attachment of *E. coli* to intestinal cells cause diverse signal transduction pathways a process by which genetic material is transferred from one cell to another by the bacteria's plasmid leading to the bringing down of many cellular process for the benefit of the bacteria (Yang *et al.*, 2020).

1.2.6.6 Siderophores:

Escherichia coli need iron for growth, DNA synthesis, electron transport and metabolism but due to the fact that iron in the human body is mostly present as transferrin, bacteria secrete what is called

'siderophores' substances that chelate Fe and then attach to special receptor on the bacteria's surface (Levinson *et al.*, 2018) Utilization of iron acquired by this method is vital for the colonization process in UTI caused by UPEC. There are 4 systems of siderophores in *E. coli*: *Yersiniabactin*, *aerobactin* which is encoded by the *iuc* gene, enterobactin which is encoded by the *ent* gene, and salmochelin (Riedel *et al.*, 2019). Named "salmochelin" because it was first discovered in *Salmonella* species, it is a strongly hydrophilic siderophore encoded by the *iroBCDEN* gen cluster. These systems are expressed under low-iron conditions (Sarowksa *et al.*, 2019).

1.2.7 Outer Membrane Proteins (OMPs):

Escherichia coli that are Gram-negative are surrounded by 2 membranes: an inner membrane (IM) and an outer membrane (OM). The outer membrane's outer leaflet (outward facing side) is composed of lipopolysaccharides (LPS), a highly negatively charged molecule that protrudes into the bacteria's environment (Rollauer *et al.*, 2015). The outer membrane's ability to stop hydrophobic molecules from entering the bacteria protecting the bacteria in environments such as the intestines but due to its lipid nature, it also excludes hydrophilic substances. To overcome this, bacteria use special protein channels called pirons which allow low molecular weight hydrophilic substances into the cell (Riedel *et al.*, 2019). Outer membrane proteins (OMPs) are an integral part of the outer membrane with many diverse roles, acting as adhesion factors, mediators for the uptake of nutrients, siderophore receptors and enzymes such as proteases and lipases, etc. The LptD is a 26-stranded *β*-barrel which is responsible for the fundamental process of insertion of LPS into the outer leaflet of the OM, maintaining the asymmetry of the bilayer. *β*-barrel assembly machinery (BAM) complex is responsible for the insertion of *β*-barrel proteins (OMPs) into the OM (Rollauer *et al.*, 2015).

The *ompT* protein is a 10-strand antiparallel *β*-barrel outer membrane protease inserted into the OM by BAM complex because it plays a role in the cleavage of antimicrobial peptides (AMPs) like colicin. AMPs are substances secreted by organisms into the extracellular environment due to their antimicrobial (bactericidal) activity (Urashima *et al.*, 2017).

1.2.8 Biofilm Formation:

When faced with unfavorable environmental circumstances, nutritional shortages, or a high local cell density, *E. coli* produces biofilms. These biofilms are complex structures made of polysaccharides and substances derived from the bacterial environment, including nutrients, minerals, amino acids, and components of cell walls (Billings *et al.*, 2015).

This phenomenon, observed as early in 2019 by (Mukherjee and Bassler ., 2019) was characterized by thick bacterial communities on submerged slides across various water sources. The intricate biofilm formation process transpires through a series of stages, each serving a distinct purpose:

1. Reversible Attachment: Bacteria employ flagella and chemotaxis to access and affix onto the host's surface.
2. Irreversible Attachment: Cells transition into the stationary phase, curtailing elongation. Elements like type 1 pili, curli fibers, and antigen 43 are produced during this stage.
3. External Matrix Formation: Bacterial polysaccharide production initiates to facilitate surface attachment, generating a matrix inclusive of cellulose, poly glucosamine, and colonic acid. This matrix incorporates nucleic acids, proteins, and other components.
4. Three-Dimensional Biofilm Structure Formation: Bacterial colonies are established within a complex amalgamation of nutrients, water, and metabolic byproducts.

5. Detachment: Biofilms are formed and detached, commencing the cycle anew on a different the surface (Soto, 2014; Mukherjee and Bassler., 2019). (Figure 2.1).

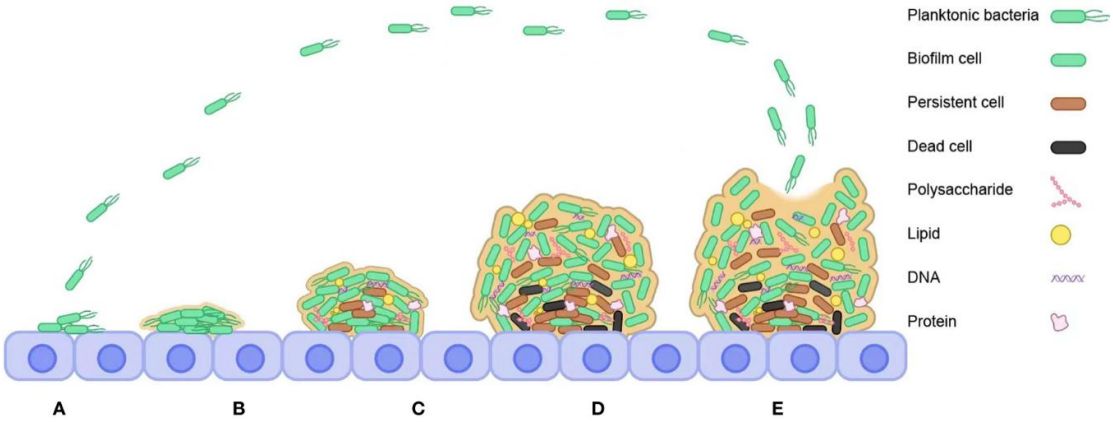

Figure 2.1 Steps of *E. coli* Biofilm Formation. **(A)** Reversible attachment. **(B)** Irreversible attachment. **(C)** Bacterial cells synthesize and secrete EPS. **(D)** Maturation. **(E)** Dispersal (Mukherjee and Bassler., 2019).

Intriguingly, bacterial colonies that foster complex, multicellular communities are more prone to causing infections than those growing individually. The research underscores that biofilms account for over 80% of human urinary tract bacterial infections. Biofilm growth can be impeded by various plant extracts and chemical compounds, manifesting efficacy in thwarting the quorum sensing (QS) system, a mechanism by bacteria communicate chemically. Notably, antimicrobial resistance tends to be heightened in bacteria-producing biofilms compared to their planktonic counterparts (Poursina *et al.*, 2018).

1.2.9 Treatment of UTIs:

Based on the general European susceptibility patterns and according to the European Association of Urology guidelines, the following antimicrobial agents are recommended for treatment of uncomplicated cystitis in premenopausal women and uncomplicated pyelonephritis in all European countries: nitrofurantoin, fosfomycin trometamol and trimethoprim-sulfamethoxazole (TMP-SMZ) (Bartoletti

et al. 2016). Nitrofurantoin and fosfomycin trometamol are recommended as first-line therapy for uncomplicated cystitis (Bonkat *et al*. 2017; Asadi *et al*. 2019). TMP-SMZ is not indicated as the empirical treatment due to the high prevalence of bacterial resistance and can be considered only for the patients with a low prevalence of resistant *E. coli* (< 20%) (Bartoletti *et al*. 2016; Bonkat *et al*. 2017). Fluoroquinolones (ciprofloxacin and levofloxacin) play an important role in the treatment of more severe infections and septicemia, and thus, ciprofloxacin should be considered as an alternative, not as a first-line antibiotic, in the treatment of uncomplicated cystitis (Bartoletti *et al.* 2016). Ciprofloxacin could be used as second-line empiric therapy in cases of mild and moderate pyelonephritis or complicated UTI treatment, and as third-line empiric treatment for uncomplicated cystitis. Amoxicillin-Clavulanic acid is recommended as first line-therapy for mild and moderate pyelonephritis or complicated UTI, as well as alternative empiric therapy for uncomplicated cystitis (Bonkat *et al*. 2017). For complicated UTI (high fever, sepsis, vomiting) or severe pyelonephritis, amoxicillin with gentamicin or a second-generation cephalosporin with an aminoglycoside are recommended as first-line empiric therapy, and third-generation cephalosporin applied intravenously as alternative empiric therapy. The choice of antimicrobials for the treatment of UTI is also based on local resistance profiles of the pathogen (Cheung *et al*. 2017).

1.2.10 Antibiotic Resistance:

In the realm of global health concerns, one issue looms ominously antibiotic resistance. Defined by the World Health Organization (WHO) as the diminishing medications effectiveness against health conditions caused by microorganisms, antimicrobial resistance has unleashed a wave of challenging infections. Urinary tract infections (UTIs), respiratory system infections, also a myriad of further health issues have become

increasingly a challenge to treat because of the heightened resistance exhibited by these microbial adversaries (Adhikari, 2020).

1.2.10.1 Evolution of *E. coli* Bacterial Resistance:

The emergence of antimicrobial resistance is a complex battle, waged through various mechanisms, including mutations and inter-organism transfers. Conjugation, transduction mediated by plasmids or transposons, and transformation serve as conduits for resistance transfer from other organisms. Mutations, on the other hand, are the subtle architects of change, altering an mode action of antimicrobial's, Changing the target, as well as ultimately undermining its efficacy. This can entail the activation of efflux pumps or the perturbation of metabolic pathways via regulatory operons (Vilalta, 2018).

This evolution and dissemination of antimicrobial resistance pose pervasive threats to global public health. An array of mechanisms and resistance determinant genes contributes to this phenomenon, presenting a formidable challenge. Bleak estimates forecast a future where over 3 million lives could be lost by 2050 due to multidrug-resistant (MDR) *E. coli* strains. Particularly concerning are the carbapenem-resistant strains of *E. coli*, spreading globally with the added complexity of dwindling treatment options, such as colistin (Vilalta, 2018). The battle against antimicrobial resistance is pivotal, demanding comprehensive strategies to mitigate its far-reaching impact (Vilalta, 2018).

1.2.10.2 Mechanisms of Resistance :

Resistance can be gained through mutations, or from other organism through processes like conjugation, transduction (plasmids or transposons) or transformation (Basak *et al.*, 2016). Mutations usually lead to a change in the antimicrobial’s mode of action by modifying its target and thusly, reducing its effectiveness and the activation of efflux pumps or by shifting the metabolism by regulatory operons (Basak *et al.*,

2016). Resistance can be classified into multiple levels, including: multi-drug resistant (MDR) (resistance to at least one antibiotic from 3 or more groups of antibiotics), extensively-drug resistant (XDR) (resistance to at least one antibiotic in 4 or more groups), and pan-drug resistant (PDR) (resistance to all antibiotics from all different groups) (Munita and Arias, 2016). The evolution and spread of antimicrobial resistance bacteria by different mechanisms and genes that are determinants of resistance is a major threat to public health globally (Tang *et al.*, 2014). It is estimated that more than 3 million individuals will lose their lives by 2050 through MDR *E. coli* strains, especially carbapenem-resistant strains, which are already spreading across the globe, and the only treatment available for eradicating them (colistin) is already losing effectiveness (Vilalta, 2018).

1.2.10.3 *E. coli* Antimicrobial Resistance:

Antibiotics are compounds that target specific locations in bacterium and to be effective, it targets a vital operation in the bacterium leading to either a stop to the cell's growth (bacteriostatic) or its death (bactericidal). There are many antibiotics available differing from each other by principle and mechanic of action (Sebastian *et al.*, 2021).

One of the most important antibiotics in medicine are *β*-lactam antibiotics. It includes amoxicillin and cephalosporin's. Used to treat UTI by interrupting the creation of the cell wall by attaching to the protein associated with penicillin, and thusly, inhibition of peptidoglycan formation for the bacterial cell wall (Ny, 2019). Many reports have documented the high rates of *β*-lactam antibiotics' resistance in *E. coli* and this is due to the production of *β*-lactamase, the most prevalent and evident resistance mechanism in *E. coli* (Furuya and Lowry, 2006; Pitout and Laupland, 2008; Wright, 2011; Tang *et al.*, 2014). There are other antibiotics which target the production of DNA or RNA and protein synthesis, and this includes fluoroquinolone which inhibits duplication

by attaching to DNA gyrase and topoisomerase IV which play a role in the uncoiling of DNA before duplication and induce double strand breaks before DNA polymerase (Ny, 2019). Fluoroquinolones have become the favorite antibiotics used in the treatment of upper and complicated UTIs due to their wide spectrum of effectiveness. Recently, it was shown that Fluoroquinolones and quinolones antibiotics' resistance have increased between UPEC strains in the United States and Europe due to mutations or by acquiring plasmids that have the resistance determinant genes (Al-Ghoribi, 2015). Aminoglycosides target protein synthesis by attaching to 30S subunit which leads to misreading by mRNA which eventually leads to early termination of protein synthesis (Kapoor *et al.*, 2017). Resistance to aminoglycosides by *E. coli* has several causes and they include target modification (16S rRNA and ribosomal mutations), use of efflux pumps to reduce the intracellular concentration of the antibiotic and enzymatic drug modification (Kumar and Singh, 2013).

Upon transfer of resistance genes, bacteria demonstrate diverse mechanisms of antibiotic resistance:

1-Target Modification: -

This intricate mechanism disrupts the construction of nucleic acids, leading to heightened bacterial resistance against Fluoroquinolones like ciprofloxacin and rifamycin. Simultaneously, it impacts ribosomal function, inducing disturbances in protein synthesis, resulting in an augmented bacterial resistance to anti-erythromycin and rifamycin (Kotsyuba. *et al.*, 2014).

In addition, this mechanism involves hindering antibiotic access to the cell wall's binding site, known as penicillin-binding protein (PBP). This interference leads to the reduced affinity between bacterial antibiotic molecules and PBPs. Although scarcely detailed, this mechanism plays a significant role in bacterial resistance. Notably, alterations in penicillin-

binding proteins in gram-negative bacteria contribute to carbapenem resistance. In contrast, dye-positive bacteria resist beta-lactam antibiotics, except for *Staphylococcus aureus*, through modifications in penicillin-binding protein (Munita and Arias, 2016).

2-Alteration of cell Membrane Permeability: -

Modifying the composition of proteins in the cell membrane alters the structure of the membrane pores and its transport systems. This modification impacts the antibiotic's efficacy by preventing it from passing through the membrane transport systems. This phenomenon is observed in certain aminoglycosides, such as gentamicin, as well as antibiotics and sulfonamide antibiotics (Kapoor *et al*., 2017).

3-Production of Enzymes: -

These protein carriers within the cell membrane are pivotal in transporting diverse substances, extricating them from the cell to mitigate their detrimental impact. Consequently, these transporters emerge as pivotal contributors to bacterial resistance against antibiotics. Encompassing toxic hydrophilic compounds, hydrophobic and amphipathic molecules, and dyes like acriflavine, crystal violet, and Red safe dye. Moreover, they enhance the efflux of disinfectants, antiseptics, fatty acids, heavy metals, organic solvents (Kotsyuba *et al*., 2014).

4- Metabolic Pathways Alteration : -

Bacteria use preformed folic acid from their surroundings to change their metabolic pathways. This modification permits them to become resistant to the antibiotics trimethoprim and sulfa, allowing them to avoid their inhibitory effects (Kapoor *et al.*, 2017). Bacterial resilience is bolstered by efflux pumps, specialized molecular machinery capable of expelling detrimental substances from within bacterial cells. These pumps facilitate the extrusion of harmful compounds, allowing bacteria to persist

in environments that might otherwise prove inhospitable (Paltansing, 2015).

5-Efflux Pumps: -

Protein carriers assume a pivotal function within the cellular membrane by facilitating the extrusion of various substances within the cell, thereby mitigating their potential harm. These carriers serve as a crucial mechanism for bacterial antibiotic resistance, effectively expelling diverse compounds beyond the cell membrane. "The substances transported encompass a wide range, including toxic hydrophilic compounds, hydrophobic and amphipathic molecules, and agents like acriflavine, as well as disinfectants, antiseptics, fatty acids, heavy metals, organic solvents, such as *β*-lactams, macrolides, tetracycline, chloramphenicol, and novobiocin (Venter *et al*., 2015).

1.3 *β*-Lactams Antibiotic:

Beta-lactam antibiotics encompass four fundamental categories, each sharing a similar structure. These antibiotics feature the beta-lactam ring and diverge based on the supplementary ring affixed to this core structure, resulting in Penicillin's, Cephalosporin's, Monobactams, and Carbapenems. A thiazolidine-5 ring is present among penicillin's, while cephalosporin's exhibit an additional cephem-6 ring. Monobactams retain solely the beta-lactam ring, and variations within each group arise from dissimilar side chains affixed to the beta-lactam ring (Samaha and Araj, 2003). *β*-lactam antibiotics' primary function is to impede bacterial cell wall synthesis, inducing cell lysis and fatality by binding to penicillin-binding proteins (PBPs) (Kawai *et al*., 2018). These beta-lactam antibiotics are the cornerstone of antibacterial agents, significantly extending human lifespan by approximately a decade. They enjoy widespread utilization due to their broad spectrum efficacy and minimal adverse effects (Ali *et al*., 2018).

1.3.1 *β*-lactamase Inhibitors:

Addressing the pressing concerns posed by the prevalence and implications of antibiotic resistance due to beta-lactamase enzyme synthesis, the development of beta-lactamase inhibitors is underway (Tehrani and Martin, 2018). Over the past three decades, only a select few *β*-lactamase inhibitors-referred to as first-generation beta-lactamase inhibitors have gained clinical approval. This group includes Clavulanic acid, Tazobactam, and Sulbactam, all of which are derivatives of beta-lactam compounds. Their primary objective is to thwart Class A *β*-lactamases and specific serine (C) beta-lactamases. However, novel generations of beta-lactamase inhibitors, characterized by non-*β*-lactam structures like Avibactam and Vaborbactam, are emerging. Notably, these inhibitors exhibit a broader spectrum of inhibition encompassing KPC enzymes (Tehrani and Martin, 2018). Nonetheless, it is worth acknowledging that many *β*-lactamase enzymes in class D and all those in class B demonstrate resistance against established and clinically employed inhibitors (Tehrani and Martin , 2018).

1.3.2 Carbapenems:

Antibiotics of ***β***-lactam belonging to the carbapenem class have outstanding efficacy against both Gram (positive and negative) bacteria, including both aerobic and anaerobic species (Duedu *et al.*, 2017). These antibiotics are vital for protecting against infections caused by multidrug-resistant Gram-negative bacilli because of their potent action against a wide variety of *β*-lactamase enzymes. (El-Gamal *et al.*, 2017). Their discovery in 1976 was spurred by the emergence of *β* -lactamase enzymes conferring resistance to penicillin (Papp *et al.*,2011). A distinctive feature of this class is the hydroxyethyl side chain (Papp *et al.*, 2011). Prominent members of the carbapenem family include imipenem, meropenem, doripenem, ertapenem, panipenem, and biapenem. These antibiotics have

gained global usage in response to heightened cephalosporin resistance (Patel and Bonomo, 2013). Imipenem, with its broad-spectrum activity encompassing both aerobic and anaerobic Gram-(negative and positive) bacteria, holds particular significance for *Enterobacteriaceae* and *Pseudomonas aeruginosa* but does not effectively counter MRSA (Papp *et al.*,2011). Meropenem and ertapenem demonstrate wide-ranging activity against non-lactose-fermenting Gram-negative bacilli, with ertapenem displaying relatively less activity against *P. aeruginosa* when compared to imipenem and meropenem (Papp *et al.*,2011). Carbapenem antibiotics have shown remarkable efficacy against severe infections caused by bacteria producing broad-spectrum beta-lactamase enzymes (Hawkey and Livermore, 2012). They are particularly effective against infections stemming from multidrug-resistant *P. aeruginosa* bacteria (Matsukawa *et al.*,2019). Regrettably, resistance to carbapenems has surfaced, with a troubling global increase and dissemination that necessitates the relentless pursuit of novel and more potent antibiotics (Etebu and Arikekpar, 2016). Research has indicated that metal *β*-lactamase enzymes (MBLs) exhibit high efficiency in degrading carbapenems, and the spread of MBL-encoding genes through mobile genetic elements has further facilitated their dissemination (Yang *et al.*, 2020). Consequently, therapeutic options have become limited, underscoring the urgency for innovative solutions (Palzkill, 2013).

1.3.3 Carbapenems Mode of Action:

Gram-negative bacteria's outer membrane acts as a powerful barrier against the entry of numerous substances. However, it contains proteins that facilitate the penetration of antibiotic molecules through porin pathways (Maier *et al.*, 2015). Type 2 outer membrane proteins, notably OmpD, are necessary for carbapenem antibiotics to penetrate the outer membrane of Gram-negative bacteria. Notably, these antibiotics employ

distinct outer membrane proteins compared to penicillin's and cephalosporin's, which engage *OmpC* and *OmpF* (Maier *et al.*, 2015).

Penicillin-binding proteins (PBPs) found in the bacterial cell wall interact with *β*-lactam antibiotics to cause the bacteria to die. These enzymes take involvement in significant peptidoglycan production processes. Upon binding, a complex known as the acyl-enzyme complex is formed, disrupting the elongation of peptide chains. Consequently, the final step of peptidoglycan synthesis is impeded, culminating in cell wall lysis and eventual bacterial cell death (Zervosen *et al.*, 2012).

1.3.4 Production of Carbapenemase:

Carbapenemase represent a diverse and extensive category of *β*-lactamase enzymes, which can degrade *β*-lactam antibiotics, including carbapenems, through disrupting of core *β*-lactam ring. Their emergence is pivotal in conferring resistance to carbapenem antimicrobial (Ye *et al.*, 2018). Among the many mechanisms contributing to carbapenem resistance, the escalating prevalence of carbapenemase is the most prominent (Cantón *et al.*, 2012).

While the discovery of β-lactamases in bacteria predates the clinical utilization of antibiotics by over 30,000 years, the focus has shifted to newer *β*-lactamases since the early 1980s. These include *AmpC* β-lactamases, extended-spectrum *β*-lactamases (ESBLs), and carbapenemase enzymes. Among these, the carbapenemase enzymes hold heightened significance due to their extensive spectrum of resistance, surpassing that of other *β*-lactamase enzymes. Notably, these carbapenemase can be acquired from other sources (Acquired Carbapenemase), representing a highly virulent subset with the greatest potential to undermine infection control efforts (Cantón *et al.*, 2012).

1.3.5 History of Carbapenemase:-

Initially observed in Gr-positive bacteria, carbapenems exhibit distinct characteristics compared to other ***β***-lactamases. Notably, they are susceptible to inhibition by EDTA. They are categorized as metallo-enzymes because their active site contains the minimum of one zinc atom, which is necessary for enabling the hydrolysis of the *β*-lactam ring (Frere *et al.*, 2005). During the mid to late 1980s, many carbapenemase enzymes emerged within the Enterococcus genus. Subsequent investigations revealed that these enzymes employ serine within their active site and remain unaffected by EDTA, yet can be countered with Clavulanic acid and Tazobactam (Yang *et al.*, 1990).

The *NmcA* chromosomal carbapenemase enzymes were shown to be associated in 1993 with the evolution of carbapenem antimicrobial resistant in Enterobacteriaceae. (Naas and Nordmann, 1994). Plasmids carrying metallo ***β***-lactamases (IMP-1) were also detected in *Pseudomonas aeruginosa* after identifying all chromosomal carbapenemase enzymes (Watanabe *et al.*, 1991). The discovery *Klebsiella pneumoniae* carbapenemase (KPC) emerged in the USA (Yigit *et al.*, 2001). Subsequently, the dynamic patterns of carbapenemase dissemination, plasmid-driven propagation, and the wide-ranging distribution among bacterial species garnered global attention as its expansion accelerated. Strains of *P. aeruginosa* that produce these enzymes have been isolated globally (Queenan and Bush, 2007).

1.4 Classification of Carbapenemase :

1.4.1 Functional Classification:

Scientists have devised a functional classification system for carbapenems based on fundamental principles (Bush *et al.*, 1995). This classification categorizes *β*-lactamase enzymes according to the substrate they influence and their hydrolysis rate and final charge. The enzymes are

grouped into four major clusters based on their sequence numbers (Drawz and Bonomo, 2010). Cluster II is the largest among these clusters, encompassing six sub-clusters designated as 2a, 2b, 2c, 2d, 2e, and 2f (Queenan and Bush, 2007). While this classification is comprehensive, it must completely address the diversity of *β*-lactamase enzymes, which exhibit considerable variability (Bush and Jacoby, 2010).

1.4.2 Molecular Classification:

Amplair's molecular classification of carbapenemase is based on their amino acid sequences, resulting in three primary classes: Class (A) and Class (B) Metallo *β*-lactamases, as well as Class (D) Oxacillinase, which possesses the ability to hydrolyze carbapenem compounds (Nordmann *et al.*, 2012). Serine carbapenems are additionally categorized into two major groups, contingent on the enzyme's active site. These groups are Class A and Class D enzymes, subject to regulation and potential inhibition by β-lactamase inhibitors. In contrast, the Class (B) Metallo *β*-lactamase enzymes feature a diploid zinc-dependent metallo carbapenemase (Zn^{2++}) in their active site, rendering them unaffected by *β*-lactamase inhibitors but susceptible to inhibition by EDTA (Busch and Jacoby, 2010).

1-Class A Carbapenemase (Serine *β*-Lactamases)

Following the taxonomy established by Bush and Jacoby , Class A carbapenemase were initially described in the early 1980s (Yang *et al.*, 1990), falling within the 2F subgroup (2010). The hydrolytic action of antibiotics is contingent upon the presence of serine in the enzyme's active region. This enzyme family can degrade carbapenems and other beta-lactams and be rendered inactive by Clavulanic acid (Bedenić , *et al.*, 2014).

Class A carbapenemase can be encoded on chromosomes or carried by transferable genetic elements like plasmids. Notably, this category

encompasses KPC enzymes (KPC-2 to KPC-13) and GES enzymes (GES-1 to GES-20), while chromosomally encoded enzymes encompass IMI, SFC, NmcA, and SME enzymes (Bedenić *et al.*, 2014). The discovery of GES enzymes occurred in *K. pneumoniae* (Coana and France., 2000). Initially classified as broad-spectrum beta-lactamases due to their capacity to hydrolyze extended-spectrum penicillin's and cephalosporin's, GES enzymes later exhibited an expanded hydrolysis spectrum to include imipenem antibiotics in *P. aeruginosa* (Poirel *et al.*, 2001). *Acinetobacter* and *Pseudomonas* have been identified as hosts for GES-2, GES-11, and GES-14 enzymes (Nordmann *et al.*, 2012; Bonnin *et al.*, 2013), with these enzymes dispersing to various regions such as South Africa, Greece, Japan, France, and Korea (Patil *et al.*, 2019).

Enzymes such as SME were initially identified in the bacterium *Serratia marcescens* in the United Kingdom in 1982, encompassing three types: SME-1, SME-2, and SME-3 (Queenan and Bush , 2007).

2- Class B Carbapenemase Metallo *β*-Lactamases:

Class B carbapenemase encompass metallo-*β*-lactamases and rely on a zinc ion within the enzyme's active site to catalyze the cleavage of the β-lactam ring (Queenan and Bush, 2007). These carbapenemase are classified based on the number of zinc ions in their active site, categorized as B-1 (containing two ions), B-2, and B-3 (each having one ion). Most Metallo-β-lactamase enzymes fall under class B-1 (Diene and Rolain, 2014; Meletis, 2016). Metallo-*β*-lactamases possess the capacity to hydrolyze all *β*-lactam antibiotics except for Monobactams. They also exhibit resistance to commercially available *β*-lactamase inhibitors for example Clavulanic acid, Tazobactam, and Sulbactam, effectively inhibited only by removing the metal ion using EDTA (Drawz and Bonomo, 2010).

Metallo-*β*-lactamases pose the highest clinical threat among all carbapenemase (Thaden *et al.,* 2017). They serve as the primary enzymes responsible for carbapenem resistance in Gram-negative bacilli (Karn *et al.*, 2016), and their hydrolytic activity extends to penicillin's and cephalosporin's as well (Jeon *et al.*, 2015). Currently, twelve distinct metallo-β-lactamase enzymes have been identified, including VIM, SIM, GIM, SPM, IMP, NDM, DIM, AIM, KHM, SMB, TMP, and FIM (El-Domany *et al.*, 2017). The most significant among these metallo-*β*-lactamases are the acquired forms, notably enzymes like SPM, GIM, VIM, IMP, and NDM. Through mobile genetic components like plasmids and introns, the genes encoding these enzymes are often transferred from one bacterial species to another (Nordmann *et al.*, 2012).

3-Class C Carbapenemase:-

The Ambler class C *β*-lactamases (*AmpC* cephalosporinase) confer resistance to penicillin, oxyiminocephalosporins, cephamycins (cefoxitin and cefotetan), and, variably, to aztreonam (Jeon *et al.*, 2015). An exceptional member with different properties is ACC-1, a plasmid-encoded class C *β*-lactamase identified in clinical isolates of *K. pneumoniae*, *P. mirabilis*, *Salmonella enterica*, and *E. coli*. Due to conformational alterations in this enzyme structure, ACC-1-producing bacteria are susceptible to cefoxitin, whereas they are resistant to oxyiminocephalosporins (Bae *et al.*, 2019). AmpC cephalosporinase are not significantly inhibited by other *β*-lactamase inhibitors such as Clavulanic acid, but may be inhibited by boronic acid and cloxacillin (Hammoudi *et al.*, 2014). They may be encoded by the chromosome of many *Enterobacteriaceae* like *E. cloacae* and *S. marcescens*, and a few non-enterobacterial organisms like *P. aeruginosa*. In such instances, they are inducible by antibiotics or expressed at high levels by mutation. Nevertheless, *AmpC* enzymes may also be acquired on transmissible

plasmids, which, consequently, makes them appear in bacteria by lacking or poorly expressing a chromosomal bla_{AmpC} gene, such as *E. coli*, *K. pneumoniae*, and *P. mirabilis* (Jacoby ., 2009). Studies describe only a few AmpC cephalosporinase with carbapenemase activity. For example, plasmidic CMY-2-type, ACT-1-type, and DHA-1-type AmpC enzymes may promote the emergence of carbapenem resistance in porin-deficient clinical isolates of *Enterobacteriaceae* (Jousset *et al.*, 2019). Very recently, Jousset et al. showed that *E. cloacae* can chromosomally encode an *AmpC* enzyme of the type ACT-28. Kinetic parameters of purified ACT-28 revealed a slightly increased imipenem hydrolysis compared to that of ACT-1 (Jousset *et al.*, 2019). CMY-10 was the first reported carbapenemase among plasmidic class C *β*-lactamases, and this enzyme was also a class C ESBL with extended substrate specificity for extended-spectrum cephalosporin's (Kim *et al.*, 2006). In 2014, ADC-68 was reported in *A. baumannii* from Korea as a chromosomal class C *β*-lactamases that possesses class C extended-spectrum *β*-lactamase and carbapenemase activities (Jeon *et al.*, 2014). Many reports showed that such rare AmpC with carbapenemase activity may aggravate carbapenem resistance when coupled with outer membrane permeability and/or efflux pump overproduction (Hammoudi *et al.*, 2014).

4-Class D Carbapenemase

Class D carbapenemase constitute the second-largest family of *β*-lactamases, trailing behind the SHV and TEM *β*-lactamases (Bush and Jacoby , 2010). This group stands among the earliest-discovered beta-lactamase enzymes, showcasing a broader range of activity beyond penicillin's and cephalosporin's. Specifically, these enzymes excel at hydrolyzing oxacillin due to their remarkable efficiency, earning them the label of Oxacillinases (Potron *et al.*, 2013). Their clinical significance arises from their capability to combat antibiotic-resistant bacteria, albeit

with a lower potency compared to other carbapenemase classes, including metallo-*β*-lactamases (Patel and Bonomo, 2013; Stewart *et al.*, 2018).

Like class (A) *β*-lactamases, Oxacillinases feature a serine residue within the enzyme's active site (Alfredson and Korolik , 2005). This category further divides into 12 subfamilies: OXA-23, OXA-24, OXA-48, OXA-51, OXA-58, OXA-143, OXA-253, OXA-211, OXA-213, OXA-214, OXA-229, and OXA-235 (Evans and Amyes, 2014). In 1980, *A. baumannii* isolates demonstrating carbapenem resistance were identified to carry plasmids encoding enzymes such as OXA-23, OXA-40, and OXA-58. Subsequently, isolates with chromosomal coding for the OXA-51 enzyme were also discovered (Evans and Ames, 2014).

The Class (B) carbapenemase encompass metallo-β-lactamases, relying on a zinc ion within the enzyme's active site to catalyze the breakdown of the *β*-lactam ring (Queenan and Bush, 2007).

1.5 Polymerase-Chain Reaction (PCR)

The Polymerase Chain Reaction (PCR) is a transformative technology that has revolutionized the field of molecular biology. It offers an efficient and precise method for amplifying specific segments of DNA for further analysis, replacing the cumbersome and time-consuming process of bacterial cloning (Biassoni and Raso, 2020). This technique relies on an enzymatic reaction involving primers, a mixture of DNA polymerase enzymes, buffer, and magnesium solution, all interacting with a target DNA sequence. Primers typically have 18 to 24 nucleotide base pairs that surround the area to be amplified (Disotell, 2017).

The PCR process consider orchestrated throughout a thermo-cycler, which meticulously controls the temperature of the reaction tubes. It operates through three fundamental stages:

The polymerase-chain reaction (PCR) is a technology used to multiply a specific part of DNA to be further studied instead of using the bacterium

cloning method that is both complex and time-consuming. It is an enzyme-based reaction between a primer (a mix of DNA-polymerase enzymes with some buffer and magnesium solution) and a target DNA. Primers are usually 18- to 24-nucleotide bp that surround the region that needs to be amplified (Biassoni and Raso, 2020). This is controlled by a thermocycler that heats and cools the reaction tubes in three basic steps:

1.Denaturation: This step involves the denaturation of the double-stranded DNA into single-strands by reaching a temperature of usually around 94 – 98°C.

2. Annealing: In this step, the temperature of the reaction tubes is cooled down to allow the 2 primers to anneal the newly-formed single-stranded DNA (Disotell, 2017).

3. Extension: The tube is heated again, this time to usually around 72°C. At this stage, dNTPs (Deoxynucleotide triphosphates) is being added to the double-stranded DNA extending from the space between the primers and template DNA by the DNA-polymerase (Biassoni and Raso, 2020).

1-Primers

Primers are short oligonucleotides, approx. 20 base pairs (bp) in length. They are often used in pairs, where one corresponds to the 3’ end of the coding stand, and the other to the 3’ end of the template strand. They are integral to PCR as without them the polymerase would have nowhere to start amplification. To design primers, one has to know the sequence of the site, meaning primer sites can’t be un-know, but the sequence between a pair can be unknown. PCR is therefore not ideal for entirely novel sequences. Primer design will not be discussed in depth as it was not part of this project, but some important things to consider are: length of primers, placement of primers which determines product length, melting temperature, GC content, 3’end stability, and amplification efficiency (Navarro *et al.*, 2015). Some other factors to consider is what

one wants to detect, DNA, RNA, or both, and for what the amplification product is to be used for (Navarro *et al.*, 2015).

2-SYBR Green

In Real-Time PCR, amplification of template is detected by fluorescent techniques after each cycle, visualizing the reaction progress continuously. Hydrolysis probes such as Taq-Man are specific and will only fluoresce when its specified target has undergone amplification. SYBR Green is a non-specific dye, adhering to any double-stranded DNA (dsDNA) (Store *et al.*, 2020). When unbound, SYBR Green will not fluoresce. When bound to DNA it will absorb blue light (λmax= 497 nm), and emit green light (λmax= 520 nm) (Navarro *et al.*, 2015). In Real-Time PCR the SYBR Green fluorescence signal is measured in the extension phase for each cycle (Navarro *et al.*, 2015). SYBR Green being a non-specific dye, will bind to the minor groove of all dsDNA with no regard to its source (Navarro *et al.*, 2015). Primer dimers and other non-specific products are components SYBR Green will bind to that might skew the results. It can seem as if there is more target amplification than there actually is. There is therefore a need for optimization of the assay to ensure minimal production of non-specific amplification products (Valones *et al.*, 2009).

As the concentration of dsDNA increases with each cycle, the fluorescent signal from the dye increases proportionally. The reaction is exponential, each cycle doubling the number of amplification products. The increase in fluorescent signals is displayed as an amplification plot. The horizontal green line indicates the threshold, separating noise from fluorescence from synthesized amplification product. Lines under the threshold are the baseline, where fluorescence from synthesized amplification product can’t be distinguished from noise. The point where a plotline crosses the threshold indicates its Cq-value. Cq-value is the

number of cycles it takes for a sample to be amplified above the background noise. Compared to other methods like probes, SYBR Green is usually the cheaper option (Valones *et al.*, 2009). SYBR green can also be used with different sets of primers since it is not specific to one sequence unlike hydrolysis probes. Despite its popularity SYBR Green I is not the most stable dye, contributing to dye-dependent PCR inhibition (Valones *et al.*, 2009).

1.6 Real-Time PCR

Real-Time PCR has a place in the molecular microbiology lab at the present. It can detect and characterize viral, bacterial, fungal, and protozoan nucleic acids (Kralik *et al.*, 2017). With the increasing amount of available data, it could be possible to develop a PCR for every microorganism. It is also fast and has a high-throughput, with detection of nucleic acid sequences from different matrices (Kralik *et al.*, 2017). The technique is especially useful for viruses, as they are notoriously hard to grow in many cases, and Real-Time PCR eliminates the need for culturing with viruses. It is also very advantageous to be able to quickly determine if an infection is viral or not, which can lead to better targeted therapy (Valones *et al.*, 2009). In bacteriology Real-Time PCR has been used to detect bacteria, type strains and isolates, look for the presence of specific genes and alleles, like resistance genes and toxin production (Kralik *et al.*, 2017). They can in many cases be faster than the standard method of culture and biochemical testing, leading to an early recognition and treatment (Valones *et al.*, 2009). For public health, early identification for optimized treatment is of tremendous value (Valones *et al.*,2009).

1-Melt Curve Analysis

Melt curve analysis can be done as an additional step in a Real-time PCR, as an alternative to gel electrophoresis for product analysis (Wienken *et al.*, 2011). The amplification products are put through a range of temperatures, including the melting temperature of the product, whilst the fluorescence is continuously monitored (Wienken *et al.*, 2011). The product's GC contents, length and sequence determine their melting points. This can be used to separate amplification products by their melting temperature, with less difference than 2 °C (Wienken *et al.*,2011). Non-specific products and amplicon can be differentiated based on these results (Navarro *et al.*, 2015). Negative controls are added to compare is there are contaminants in any of the reagents (Valones *et al.*, 2009).

2- Broad Range *16S rRNA* Gene PCR

The *16S rRNA* gene being universally present and conserved in all bacteria, has made it a popular universal target for bacterial PCRs. The conserved regions have made it possible to design universal primers that will detect every bacterium, whilst sequencing of the variable regions has the power to differentiate between a large amount of bacterial species/families (Chakravorty *et al.*, 2007). Due to the universal design, 16S PCR assays are sensitive to contamination (Millar *et al.*, 2002). Especially when working with low concentration samples, small amounts of contamination might overshadow the sample due to PCR core ability, nuclei acid amplification. A universal 16S PCR would pick up any contamination by other bacterial DNA, simply because it is designed to work with as many bacterial species as possible. Contamination has led to false-positive results, and thus to a more complicated clinical interpretation, with little benefit for the patient. It is therefore important to limit the opportunities where contamination might happen (Millar *et*

al.,2002). There have been examples of contamination by bacterial DNA found in commercially available BC bottles, even though they are considered sterile (Millar *et al.*, 2002). To reduce the chances of contamination, sample preparation should be as simple as possible. Reagents should be of high quality and might be pre-treated with UV-radiation or filtering to remove any possible contamination. Results from these assays should be interpreted in a relevant biological setting to determine if they can be clinically plausible (Millar *et al.*, 2002).

Chapter Two

Materials and Methods

2. Materials and Methods

2.1 Materials

The instruments and equipment which were used in the present study are listed in Table (2-1).

Table (2-1): Laboratory Equipment's and Instruments

Type of equipment	Manufacturer
Autoclave	Hirayama (Japan)
Benchtop centrifuge	Memmert (Germany)
Benson burner	Satorins (Germany)
Centrifuge	Hettich (Germany)
Compound light microscope	Zeiss (Germany)
Conical flask	HDA (China)
Disposable and glassware	Cito (China)
Eppendorf centrifuge	Hettich (Germany)
Eppendorf tube	Sigma (UK)
Flat bottomed 96 well microplates	Biohit (Finland)
Freezer	Kelon (China)
Gel Electrophoresis System	Cleaver Scientific (UK)
Hot Plate with magnetic Stirrer	Heidolph (Germany)
Incubator and oven	Memmert (Germany)
Laboratory distillation unit	GFL (Germany)
Laminar flow cabinet	Capp (Germany)
Latex gloves	Broche (Malaysia)
Light microscope	Kruss (Germany)
Micropipette (1-10 µl,1-200 µl,100-1000 µl)	Sartorius (Germany)
Microwave	Panasonic (Japan)
Nano drop	Implen (Germany)
PCR tube 1.5 ml and 200µl	Biobasic (Canada)
Petri dishes 9 cm	Afco-Dispo (Jordan)
PH-meter	WTW (Germany)
Plain tube.	Afco-Dispo (Jordan)
Platinum wire loop	Himedia (India)
Real time PCR	Techno lab (UK)
Refrigerator	Concord (Lebanon)
Sensitive balance	Denver (USA)
Sterilized cotton	Afco-Dispo (Jordan)
Sterilized needles	Shanchuan (China)
Swab	Lab. Servic (Spain)
Thermal cycler	Techne (UK)
Tips	Sterellin Ltd (UK)
Transport collection swabs	Citotest Lab ware-(China)
UV-trans illuminator	Clever (USA)

UV-Vis Spectrophotometer	Analytic Jena (Germany)
Volumetric cylinder	HDA (China)
Vortex	Gemmy (Taiwan)
Water bath, oven, incubator	Memmert (Germany)

2.1.1 Chemical Compounds: -

The chemical and biological compounds mentioned in Table 2.2 were used in the study.

Table (2-2): Chemical Material

Material	Manufacturer
Absolute ethanol (C_2H_5OH) 95%	Bioneer (South Korea)
Agarose	Promega (USA)
Alcohol (70%)	BDH (UK)
Ethylene Diamine Tetra Acetic acid (EDTA)	
Glucose	
Glycerol ($C_3H_8O_3$)	
Hydrogen peroxide	
Nuclease-free water	
Sodium chloride (NaCl)	
K_2HPO_4	
Urea 40%	
Normal saline solution	Promega (USA)
DNA ladder (100 bp)	
Tris-EDTA buffer (TE)	
Tris-Borate-EDTA (TBE) buffer	

2.1.2 Stains and Reagents: -

The following stains and reagents which were used in the current study are mentioned in Table 2.3.

Table (2-3): Stains and Reagents Used in The Study

Stain and Reagents	Manufacturer
Catalase reagent	Promega (USA)
Catalase reagent	
Gram stain	
Kovac's indole reagent	
Loading dye	
Methyl Red	
Oxidase reagent	
Red safe dye	
Voges-Proskauer (VP) reagent: **1.** VP1: Potassium hydroxide (KOH) and distilled water (40%) **2.** VP2: Alpha Naphthol and absolute ethanol (5%)	

2.1.3 Culture Media

Table 2-4 provides a detailed of the culture media utilized in this investigation. Strict adherence to the manufacturer's (HiMedia-India and Oxoid -UK) instructions was maintained while preparing all culture media.

Table (2-4): Cultures Used in The Study and Their Corresponding Manufacturers

Type of Media	Purpose	Reference
Blood base agar	Used to determine whether the bacteria can lyse blood cells and the lyse type	Forbes *et al.*, (2007)
Brain-heart infusion broth	Used as long-term storage after the addition of 15mL of glycerol to every 85mL of the broth	MacFaddin, (2000)
Eosin Methylene Blue agar	Used to differentiate *E. coli* from other Enterobacteriaceae members	Forbes *et al.*, (2007)
MacConkey agar	Used in the initial *E. coli* specimen collection and to determine whether the bacterium can ferment lactose	
Methyl Red (MR)/Voges-Proskauer (VP) media	Used to determine whether the bacteria can ferment lactose and if it can produce acetoin	Hemraj *et al.*, (2013)
Muller-Hinton agar	Used to test susceptibility and beta-lactamase production	MacFaddin, (2000)
Nutrient agar	Used to activate the isolates and as short-term storage	
Peptone water	Is used extensively in molecular biology (DNA extraction) procedures	
Simmon citrate agar	Used to test the bacteria's ability to use citrate as the only source for carbon	Hemraj *et al.*, (2013)
Urea agar base	Used to determine whether the bacteria can produce urease	Atlas and Snyder, (2015)

2.1.4 Laboratory Kits:-

The kits used in the current study are mentioned in Table 2-5.

Table (2-5): DNA Kits Used in This Study

Kit	Components	Manufacturer
DNA Extraction kit	1. Nuclei lyses solution 2. RNase solution 3. Protein precipitation solution 4. Ethanol 70% 5. Isopropanol 6. TAE buffer 7. TBE buffer 8. CL buffer	Promega (USA)
Primers	1. Vitek strip 2. Normal saline 3. Vitek 2 GN card 4. Vortex 5. Vitek 2 Densichek	
Vitek 2 kit	Macrogen-DNA ladder 100 bp-Master mix.	Korea

2.1.5 Antibiotics Disks

The antibiotics disc and their concentration which were used for determining the susceptibility of *E. coli* bacterial isolates according to the (CLSI, 2022) are listed in Table 2.6.

Table (2-6): Antibiotic Discs and Their Related Information

Antibiotic disc	Conc. (mg /disc)	Inhibition zone diameter (mm)			**Company/Origin**
		S	I	R	
Carbapenems	CLSI (2022)				
Meropenem (MEM)	10	≥ 18	15-17	≤ 14	Condalab/Spain
Impenem (IMP)	10	≥ 25	21-24	≤ 20	
Penicillin	CLSI (2022)				
Piperacillin (PI)	100	≥ 21	18-20	≤ 17	
Amoxicillin (AMC)	30	≥ 18	14-17	≤ 13	
Ampicillin (AMP)	30	≥ 17	14-16	≤ 13	
Aminoglycosides	CLSI (2022)				
Gentamicin (GEN)	10	≥ 15	13-14	≤ 12	
Sulfonamide	CLSI (2022)				
Trimethoprim (STX)	25	≥ 16	11-15	≤ 10	
Cephems	CLSI (2022)				
Cefapime (CPT)	30	≥ 25	19-24	≤ 18	
Ceftriaxone (CRO)	30	≥ 23	20-22	≤ 19	
Nitrofurans	CLSI (2022)				
Nitrofurantoin (NIT)	300	≥ 17	15-16	≤ 14	
Fluoroquinolone	CLSI (2022)				
Ciprofloxacin (CIP)	5	≥ 26	22-25	≤ 21	

2.1.6 Primer Sequences

The primers mentioned in Table 2-7 were used in the current study for detection of some *β*-lactams resistance genes.

Table (2-7): Primers Used in This Study

Primer Name	Class	Sequence	Annealing Temp. (°C)	Product size (bp)	Manufacturer
CTX-M-1	A	F-CAGAGATTTTGCCGTCTAAG R-GGCCCATGGTTAAAAAATCACTGC	55 °C for 40 s	946	Xiao *et al.*, (2019)
GES	A	F-GTTTTGCAATGTGCTCAACG R-TGCCATAGCCAATAGGCGTAG		371	
SHV	A	F-GCCTTTATCGGCCTTCACTCAAG R-TTAGCGTTGCCAGTGCTCGATCA		898	
SME	A	F-GAGGAAGACTTTGATGGGAGGAT R- TCCCCTCAGGACCGCCAAG		334	Guido *et al* ,. (2011)
PER	A	F-GCTCCGATAATGAAAGCGT R-TTCGGCTTGACTCGGCTGA		520	Gatya *et al*,. (2022)
VEB	A	F-GCGGTAATTTAACCAGA R-GCCTATGAGCCAGTGTT		961	Linlin *et al.*, (2019)
TEM	A	F- TCGGGGAAATGTGCG R- TGCTTAATCAGTGAGGCACC	60 °C for 40 s	972	Ibrahim and Nihad ,. (2015)
IMP	B	F-GGAATAGAGTGGCTTAAYTCTC R- GGTTTAAYAAAACAACCACC		232	Gantasala *et al.*, (2022)
NMD	B	F-GGTTTGGCGATCTGGTTTTC R-CGGAATGGCTCACGATC	55 °C for 40 s	550	Mohammed *et al*,. (2015)
VIM	B	F-GATGGTGTTTGGTCGCATA R- CGAATGCGCAGCACCAG		390	Gantasala *et al.*, (2022)
ADC	C	F-GGTATGGCTGTGGGTGTTATTC R- CTAAGACTTGGTCGAAAGGT		445	Linlin *et al.*, (2019)
DHA	C	F-AACTTTCACAGGTGTGCTGGGT R-CCGTACGCATACTGGCTTTGC	61 °C for 40 s	405	Yusser and Ali ., (2021)
ECB	C	F-TCGGTAAAGCCGATGTTGCGG R-CTTCCACTGCGGCTGCCAGTT	63 °C for 40 s	302	Yusser and Ali ., (2021)
OXA-1	D	F-GGCACCAGATTCAACTTTCAAG R-GACCCCAAGTTTCCTGTAAGTG	55 °C for 40 s	564	Mohamed *et al*,. (2018)
Housekeeping gene	*16 S rRNA*	F-GGAGGAAGGTGGGGATGACG R- TCCAATCCGGAGTACGACGC	60 °C for 40 s	138	Samarasinghe and Al-Bayati ., (2022)

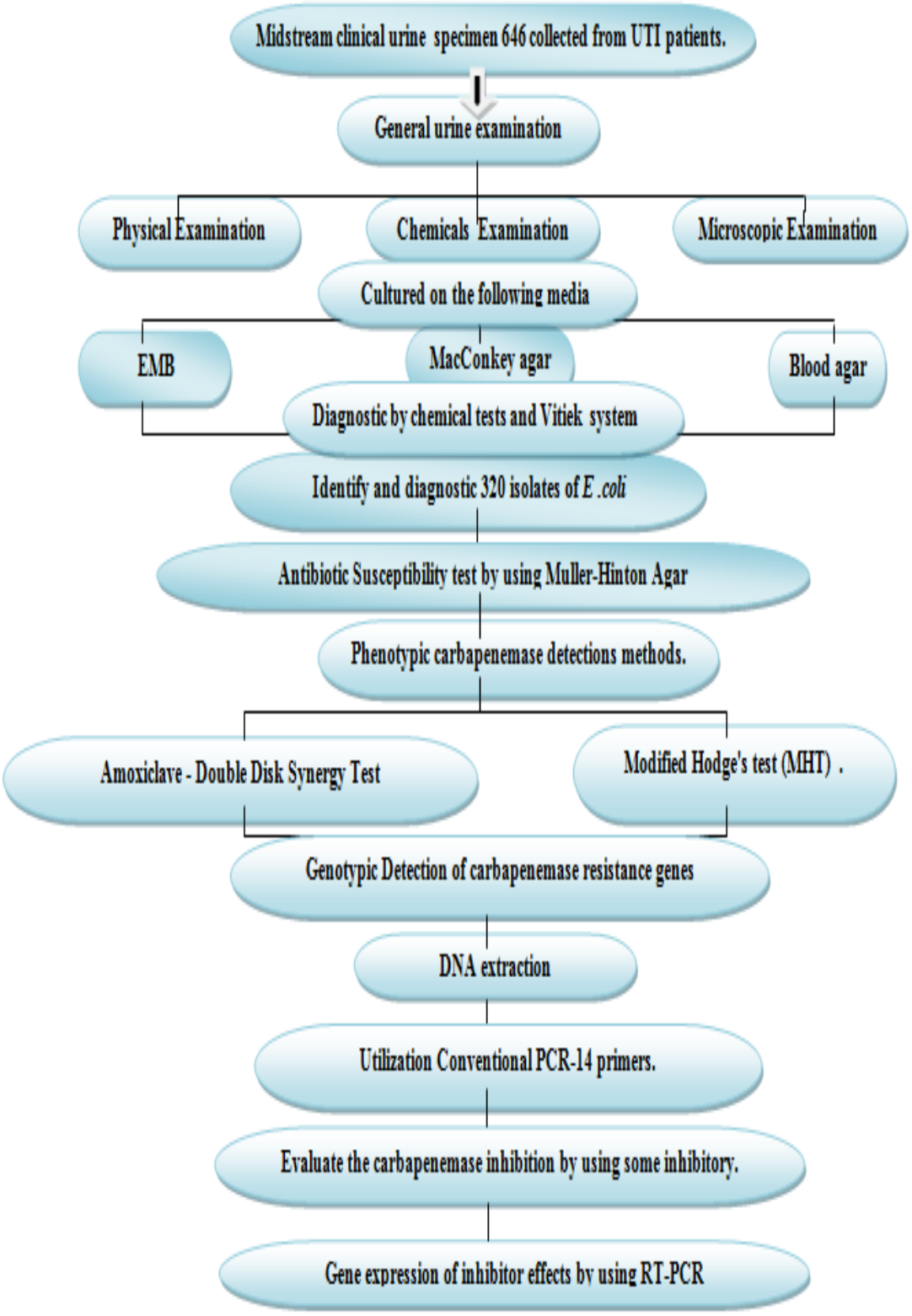

Figure 2.2 Study Design

2.2 Methods

2.2.1 Specimens Collection:-

The study included collection and analysis of 646 midstream urine specimens which is immediately examined by generals urine examination (GUE) after collection to detecting physical ,chemicals and microscopically examinations to identifying presence of infections due to the urinary tract infection occurs without any symptoms observed in the patient, followed by phenotypically ,genotypic and gene expression detecting methods.

The valuable specimens were carefully collected from February to July\ 2022 from different hospitals in Hilla City. The clinical specimens from individuals with UTI symptoms were obtained from the following hospitals which is including : Al-Imam Al-Sadiq Hospital (377 urine clinical specimens), Marjan teaching hospital (175 urine clinical specimens), and Babylon Women's and Children's Hospital (94 urine clinical specimens). Subsequently, all midstream urine clinical specimens were examined by microscopically examination and cultured onto MacConkey agar , Eosin Methylene Blue (EMB) agar and blood agar plates followed by incubation for 24 hrs at 37°C to detect of lactose and non-lactose fermenting isolates.

2.2.2 Sterilization Methods

The following guidelines were used to sterilize culture medium and solutions (Brown and Smith ., 2017):-

2.2.2.1 Sterilization Methods By Heat:-

Most culture media and solutions that are not affected by high temperatures were sterilized using this method by utilizing an autoclave at 121°C with a pressure of 15lbs/in2 for 15 minutes. As for glassware,

they were sterilized using an electric oven at 180 °C for 2 hours (MacFaddin, 2000).

2.2.2.2 Sterilization Methods By Filtration:-

Solutions and materials that are affected by heat like sugars and urea were sterile using Millipore filters with varying pore diameters (0.22μm) (MacFaddin, 2000).

2.2.3 Preparation of Culture Media:

2.2.3.1 Culture Media

The cultures; MacConkey agar, Eosin methylene blue, Nutrient agar, Peptone water, Simmon's citrate agar, Methyl Red-Voges-Proskauer (MR-VP), Muller Hinton agar, Trypton soya broth and Brian-Heart Infusion broth were prepared and Blood agar each according to their manufacturer's instructions. Sterilization was done using the autoclave at 121 °C and a pressure of 15 lbs/in2, then poured into sterile petri dishes and tubes and incubated at 37 °C for 24 hours to make sure the media weren't contaminated, then they were stored at 4 °C till needed (MacFaddin, 2000).

2.2.3.2 Laboratory Prepared Media

2.2.3.2.1 Urea Agar Base Media

Urea agar base media was prepared according to the manufacturer's instructions by adding 24g of urea agar base media to 950mL of distilled water, then the mixture was sterilized by utilizing the autoclave. After sterilization, the mixture was left to cool until it reached 50 °C then 50mL of 40% urea solution which was prepared by adding 40g of urea to 100mL of distilled water then filtered by Millipore filters with a pore diameter of 0.22μm was added to the mixture. It was then poured into sterile tubes and stored at 4 °C till needed. This media was

used to determine whether the bacteria was capable of producing urease (Tille, 2017).

2.2.3.2.2 Blood Agar Base Media

This media was prepared according to the manufacturer's instructions then sterilized using the autoclave. After that, it was cooled to 45 °C then blood with a concentration of 5% was added to the media, stirred carefully, poured into sterile petri dishes then left so that it can solidify (Forbes *et al.*, 2007).

2.2.3.2.3 Methyl Red (Voges-Proskauer Media)

Prepared according to the manufacturer's instructions found on the package by dissolving 7g of peptone, 5g of glucose and 5g of K2HPo4 in 950mL of distilled water then its pH was modified to 7.2 and its volume was completed to 1000mL. This was followed by sterilization of the media then it poured into sterile test tube, 5mL each (Hemraj *et al.*, 2013).

2.2.4 Solutions Preparation:

2.2.4.1 Tris-Boric-EDTA Buffer (TBE)

By combining 5mL of Tris-Boric-EDTA buffer (TBE) with 95mL of DW, a solution of TBE was created. TBE found its application in the electrophoresis process (Babapour *et al.*, 2016).

2.2.4.2 Normal Saline

Ready to use, sterile normal saline (NS) was used for the preparation of culture suspension (Babapour *et al.*, 2016).

2.2.4.3 Phosphate Buffer Saline (PBS):

One tablet of ready to use phosphate buffer saline (PBS) was dissolved in 100 ml of D.W. The PBS was sterilized by autoclave and then the pH was adjusted to 7.2 .

2.2.4.4 McFarland's Turbidity Standard

The 0.5 McFarland's standard tube (1.5×10^8 CFU/ml) was prepared by adding 0.5 ml of 1.175% barium chloride [$BaCl_2.2H_2O$: H_2O (w/v, 1.175g: 98.825 ml)] to 1% sulfuric acid [H2SO4: H2O (v/v, 1 ml: 99 ml)] in order to obtain a barium sulfate precipitate. The solution was used to visually compare the turbidity of a suspension of bacteria with the turbidity of the 0.5 McFarland's standard. The McFarland's standard tubes were sealed with parafilm to prevent evaporation and stored in the dark for up to 6 months at room temperature. The accuracy of a prepared 0.5 McFarland's standard was checked by using a spectrophotometer. The optical density was measured at 625 nm that should be between 0.08 and 0.1 (Murray *et al*., 2003).

2.2.4.5 1X TBE Buffer Preparation :

1X TBE buffer was prepared by dilution of concentrated 10X TBE buffer. This solution was used to dissolve agarose and in electrophoresis process. Each 100ml of 10X TBE added to 900ml of sterile distal water to give final concentration,1X TBE (Green and Sambrook, 2012).

2.2.4.6 6X DNA Loading Blue Buffer

It is provided in a premixed, ready-to-use form containing 100 mMTris-HCl (pH 8.0), mix of dyes (Xylene Cyanol and Bromophenol Blue), 30% glycerol,100 mM EDTA (pH 8.0). 6 x Loading Buffer BLUE is designated for preparation a DNA samples for loading on agarose and polyacrylamide gels. The glycerol content increases the density of the sample and ensures that DNA form a layer at the bottom of the well. EDTA is known for inhibition of metal dependent nucleases. Tracking dyes permit monitoring of progress of electrophoresis. Only 1µl of loading dye were mixed with 5 µl DNA sample to check DNA after extraction (Green and Sambrook, 2012).

2.2.4.7 Catalase Reagent

The reagent was prepared by adding 3ml of H_2O_2 to 100 ml of D.W, then stored in a dark bottle .The reagent was used to recognize bacterial capability to produce catalase enzyme (Forbes *et al*., 2007).

2.2.4.8 Gram Stain

Gram stain solution was supplied from Syrbio company. The solution was used to study Gram stain reaction and their arrangement (Forbes *et al*., 2007).

2.2.4.9 Urease Production Test

Stabbing was used to inoculate the bacteria into slant urea agar media. The tubes were incubated at 37 °C for 24 hours. The change of color from yellow to pink indicates a positive result; the bacterial isolates produced urease and lysed urea (Biswas and Rather, 2019).

2.2.5 Isolation and Identification of Bacterial Isolates

2.2.5.1Characteristic of Cultural Isolation

Samples were cultured and diagnosed using the appropriate culture media then the right characteristics of *E. coli* grown colonies were noticed, on MacConkey agar, shiny pink colonies were noted (Rajeshwari *et al.*, 2010). Pure colonies were taken and cultured on EMB (Eosin Methylene Blue) which is a differentiating media for *E. coli*. The grown of the bacteria gives colonies with a green metallic sheen (Forbes *et al.*, 2007).

2.2.5.2 Microscopic Identification

Small amount of the grown bacterial colonies was taken and spread on microscopic slides mixed with a drop of distilled water, the smear then stained with Gram stain and checked under light microscope, observing the bacteria's color, and under oil immersion, cells' shape and arrangement were observed.

2.2.5.3 Biochemical Tests

1. Oxidase Test

Grown colonies on MacConkey agar were transported onto filter papers using sterile wooden sticks, then a drop of oxidase reagent was added. Change of color to purple in 20 – 30 seconds results in a positive reaction, and indicates the production of oxidase enzyme (Biswas and Rather, 2019).

2. Catalase Test

Grown colonies were transferred onto sterile microscopic slides by sterile wooden sticks, then a drop of 3% catalase reagent was added. The appearance of bubbles on the surface of the slides indicates a positive result (Biswas and Rather, 2019).

4. Indole Production Test

This test was done by inoculated the isolated bacteria in a tubes containing peptone water then incubated at 37 °C for 24 hours. After that, 10 drops of Kovac's reagent were added, presence of the indole ring after a few seconds indicates a positive result (Hemraj *et al.*, 2013).

5. Methyl Red Test

Tubes containing methyl red-Voges-Proskauer media were inoculated with bacterial colonies then incubated at 37 °C for 24 hours, after that 4 – 5 drops of methyl red reagent were added. Shift of color to red indicates a positive result (Hemraj *et al.*, 2013).

6. Voges-Proskauer's Test

Tubes with Methyl Red-Voges-Proskauer's (MR-VP) media were inoculated with bacterial colonies then incubated at 37 °C for 24 hours. After that, 10 drops of VP1 (alpha-naphthol) were added then 5 drops of VP2 (KOH) followed up by a vigorous shaking of the tubes. The tubes then were observed for color shifting within 30 minutes. The shift of

color from yellow to red was an indicator of a positive result. This test was used to test the bacteria's ability to produce acetoin as result of sugars' fermentation (Brown and Smith, 2017).

7. Citrate Utilization Test

Tubes containing Simmon's citrate media were inoculated with bacterial colonies by streaking and stabbing followed by incubation for 24 hours at 37 °C. The shift of color from green to blue is an indicator of a positive result. This test was done to test the bacteria's capability to utilize citrate as its only source for carbon and ammonium salts as a source for nitrogen (Brown and Smith, 2017).

2.2.5.4 Vitek 2 System

Vitek 2 System was used to confirm *E. coli* from a diagnostic group specific to the system, and this requires a diagnostic card specific to Gram-negative bacteria containing 64 slots and in each slot, a dried color-indicator. These indicators react to the sample given, and the System records these changes that were happening due to bacterial growth on the slots. According to the given changes in color, the System identifies the bacterial sample according to the guidance given by bioMerieux (Pincus, 2011).

2.2.6 Bacterial Isolates Preservation :

2.2.6.1 Short-Term Preservation:

Tubes containing slant nutrient agar were inoculated by streaking then they were incubated at 37 °C for 24 hours and then at 4 °C till needed. Every month, isolates were continued and cultured on nutrient agar and then on slant nutrient agar again (MacFaddin, 2000).

2.2.6.2 Long-Term of Preservation

A media to preserve isolates for long-term was prepared by adding 15mL of glycerol to 85mL of brain-heart infusion broth which

was then spread across multiple sterile tubes that were not affected by heat to then be sterilized by incubation. After being left to cool down to room temperature, the tubes were inoculated with colonies grown on nutrient agar and stored at 37 °C for 24 hours followed by storage at -20 °C, knowing that the isolates could survive for about 6-8 months (McFadden, 2000).

2.2.7 Antibiotic Susceptibility Testing

2. 2.7.1 Antibiotic Disks

The susceptibility of the isolates to selected antibiotics was tested according to the guides of CLSI (2020) using Kirby-Bauer method and as follows (Vandepitte *et al.*, 2003):

1- Three to five colonies grown on nutrient agar were transported to tubes contain normal saline till the turbidity of MacFarland's standard ($1.5x10^8$ cell/mL) was achieved.

2- A sterile cotton swab was inserted into a tube containing bacterial suspension then it was rotated around and pressed against the inner walls of the tube to remove excess feed, then spread across petri dishes containing Muller-Hinton agar, in different directions to ensure a homogenous growth.

3-Antibiotic disks used in the study mentioned in table 3.6 were placed on the surface of the cultured media and with equal distance from each other and were pressed in using sterile forceps then stored at 37 °C for 24 hours. Results were read by measuring the diameter of the inhibition zone around the antibiotic disks then comparing them to the standards mentioned in CLSI, 2022.

2.2.7.2 Determination of Minimum Inhibitory Concentration By Utilizing VITEK System\2 (MIC):

Test Principle:

Optics systems utilize visible light to quantify the organism growth. This transmittance optics works on the principle on an initial light reading of a well before considerable growth has begun. Measurement of the light transmittance of the same well measure organism growth is done periodically, by the amount of light not permitted from going through the well An interpretive call is made between 4 and 16 hours for a rapid read, but may go upto18 hours sometimes. The VITEK 2 AST Inducible Imipenem Resistance consists of two wells at the following concentrations in the card: Clindamycin 0.5µg/mL and Clindamycin/Erythromycin 0.25/0.5µg/mL, with the 11 antibiotics as the following: Imipenem, Meropenem, Gentamicin, Piperacillin, Ciprofloxacin, Ampicillin, Cefapime, Nitrofurantoin, Ceftriaxone, Amoxicillin, and Trimethoprim (Macrolide, 2008). These antibiotics were selected based on their common use for treating *E. coli* infections (CLSI, 2022) Table (3-8) and Table (2-9).

Table (2-8) Minimum Inhibitory Concentration by Using VITEK2 AST Card

Antibiotic Name	Treated Isolates of *E. coli*		The VITEK 2 AST ICR
	0.5µg/ml	0.25/0.5µg/ml	
Meropenem (MEM)	Growth	Growth	POSITIVE
Impenem (IMP)	Growth	Growth	POSITIVE
Piperacillin (PI)	No Growth	Growth	POSITIVE
Amoxicillin-clavulanate (AMC)	Growth	Growth	POSITIVE
Ampicillin AMP	Growth	Growth	POSITIVE
Gentamicin GEN	No Growth	No Growth	POSITIVE
Trimethoprim Sulfamethoxazole STX	No Growth	Growth	POSITIVE
Cefapime CPT	No Growth	Growth	POSITIVE
Ceftriaxone (CRO)	Growth	Growth	POSITIVE
Nitrofurantion NIT	No Growth	No Growth	NEGATIVE
Ciprofloxacin (CIP)	No Growth	Growth	POSITIVE

Table (2-9) Contents of VITEK 2 AST –P628 CARD

Antibiotic Name	CALLING	RANGE	CONCENTRATION
	≤	≥	
Meropenem (MEM)	**≤ 0.25**	**≥ 4**	**0.25, 0.5 , 1 , 2**
Impenem (IMP)	**≤ 0.25**	**≥ 4**	**0.25 , 0.5 , 1 , 2**
Piperacillin (PI)	**≤ 16**	**≥ 512**	**16 , 32 , 64 , 128**
Amoxicillin-clavulanate (AMC)	**≤ 0.5**	**≥ 32**	**1 , 2 , 4 , 8 , 16 , 32**
Ampicillin AMP	**≤ 0.5**	**≥ 32**	**1 , 2 , 4 , 8 , 16 , 32**
Gentamicin GEN	**≤ 0.5**	**≥ 16**	**8 , 16 , 64**
Trimethoprim Sulfamethoxazole STX	**≤10 (0.5/9.5)**	**≥320(16/304)**	**2/38, 8/152, 16/304**
Cefapime CPT	**≤ 16**	**≥ 64**	**16,32,64,128**
Ceftriaxone (CRO)	**≤ 16**	**≥ 64**	**16,32,64,128**
Nitrofurantion NIT	**≤ 16**	**≥ 512**	**16,32,64,128**
Ciprofloxacin (CIP)	**≤ 0.25**	**≥ 4**	**0.25,0.5,1, 2**

2.2.7.3 Hemolysis Test

Isolates were cultured by streaking on the surface of blood agar plates then incubated for 24 hours at 37 °C. Hemolysis types was observed then and as follows:

1- α-hemolysis: Colonies will be surrounded by a green hemolysis area.

2-β-hemolysis: Area surrounding colonies are clear.

3-ɣ-hemolysis: No lyse is observed around colonies.

2.2.8 Extended Spectrum *β*-lactamases Production

2.2.8.1 Amoxiclave - Double Disk Synergy Test

Double disk synergy disk method was implemented and as follows (Saha and Jhora, 2018):

1-Bacterial suspension was created by mixing the bacterial colonies with 15mL of normal saline and the turbidity was corresponded to MacFarland's standard solution.

2-A sterile cotton swab was rinsed into the bacterial suspension then spread across petri dishes with a diameter of 8.5cm containing Muller-Hinton agar to obtain a homogenous growth. The dishes were left for 5 minutes to dry and absorb the cultured bacteria.

3-Amoxicillin/Clavulanic acid antibiotic disc (20/10mg) with a concentration mentioned in Table 3.6 was placed in the center of the petri dish, and the antibiotic discs: aztreonam (30mg), ceftazidime (30mg), Cefotaxime (30mg), and ceftriaxone (30mg), were placed around the amoxicillin/Clavulanic acid disc and apart from each other by 20mm.

Then the inhibition zones were observed, and it was found that the inhibition zone of each disc of the 4 discs has been enhanced towards the amoxicillin/Clavulanic acid central disc, and this means a positive result (produce ESBL) (Saha and Jhora, 2018).

2.2.8. 2 Modified Hodge's Test (MHT)

This test was performed as described by Lee *et al*., (2016). A 0.5 McFarland's tube dilution of *E. coli* TOP-10 was prepared in 5 ml of brain heart infusion broth, a lawn was streaked to a Mueller-Hinton agar plate and allow to dry (3-5) min, 10 μg imipenem disk was placed in the center of the test area. In a straight line the test organism streaked from the edge of the disk to the edge of the plate, the plates were incubated at 37°C for overnight. Up to four organisms can be tested on the same plate with one disk. MHT positive test has a clover leaf-like indentation of the *E. coli* TOP-10 growing along the test organism growth streak within the disk inhibition zone. MHT negative test has no growth of the *E. coli* TOP-10 along the test organism (Lee *et al*., 2016).

2.2.9 Molecular Examination

2.2.9.1 DNA Extraction

Favor Prep^{TM} Genomic DNA Mini Kit was used to extract genomic DNA from *E. Coli* isolates following the manufacturer's protocol. Inoculate of *E. coli* were prepared at density up to 10^9Bacterial pellets were harvested via centrifugation at 14000 rpm for 1 min. The harvested cells were add 5μl from RNase and waited 3 minutes at room temperature , incubated for 5 min at room temperature, after this add 20

µl from proteinase K. Buffer FABG (200 µl) was added respectively to the bacterial cells. The resulting homogeneous cells suspension was incubated for 15 minutes at 70ºC and vortexed for 10 sec every 3 min. DNA was extracted from the homogeneous suspension by the addition (200µl) of 96~100% of ethanol and then transferred to the FABG column assembled inside a 2 ml collection tube, and centrifuged for 5 min. at 14000 rpm. The flow through was discarded. The FABG column was placed in a new 2 ml collection tube.W1 buffer (400µl)and Wash Buffer(750µl) were then added respectively to the FABG column assembled inside a new 2 ml collection tube, and centrifuged for 30 sec at 14000 rpm to wash column membrane. The flow through was discarded. The FABG column was dried via further centrifugation at 14000 rpm for 3 min. over a new 2 ml column collection tube to remove any residual ethanol solution. The dry FABG column was then transferred to a new 1.5 ml microcentrifuge tube, and 100µl of preheated elution buffer or TE was added directly to the FABG column membrane for 3~5 min, followed by centrifugation for 1 min at 14000 rpm to elute DNA. DNA quality and quantity were determined using a Nano-Drop 2000 spectrophotometer . The eluted genomic DNA was stored at -20ºC until use.

2.2.9.2 Quantification and qualification of DNA samples by NanoDrop :-

DNA quantity and quality was measured by reading the whole absorption spectrum (220–750 nm) with NanoDrop and calculating DNA concentration and absorbance ratio at both 260/280 and 230/260 nm (Lee *et al.*, 2010). NanoDrop ND-2000 is a spectrophotometer that uses two optical fibers installed in the pedestal (emitting light from a Xenon lamp) and a sample arm (spectrometer with linear charge-coupled device [CCD] array). Samples of 1 µl volume are measured without the need for

cuvettes or capillaries. The machine was calibrated and cleaned according to the NanoDrop 2000–2000c & 1000 Calibration Check procedure(Lee *et al.*, 2010).

2.2.9.3 Preparation of Primers:

The DNA that was extracted from the all *Escherichia coli* isolates examined under a PCR procedure to different target genes in the study. The final volume of each PCR reaction was 25 µL, and all the genes targeted and their appropriate size were appeared in table 2.7. The PCR mixture was composed of GoTag Green Master Mix (2x) solution that was melted at room temperature and mixed by using a vortex for homogenizing before use. The primer's solutions were also mixed by utilizing a vortex before use. **Table 2.9**

Table (2-10): Primers and Their Proper Volumes for Conventional PCR Mixture

Primers	CTX-M-1	ADC	DHA	EBC	GES	IMP	NMD	OXA-1	PER	SHV	SME	TEM	VEB	VIM
Vol. of master mix (2x)	**12**													
Primer F+R	**2**													
N.F.W.	**9**													
Vol. of template DNA	**2**													
Total volume	**25**													
The allocation per single RXN.	**12µL of Master mix per tube and add 2µL of Template.**													

2.2.9.4 Thermal Cycling Condition

In this study a vortex mixer has been utilized to ensure thorough mixing before loading samples into the thermo-cycler. The PCR procedure was conducted using a thermal-cycler, following the manufacturer's provided instructions. The process commenced with an initial denaturation phase at temperatures ranging from 94 to 95°C for varying durations in minutes. The next step involved performing a number of repeated cycles, with each cycle consisting of an extension phase at 72°C, a denaturation phase at 94–95°C, and an annealing phase

at a temperature determined by the primer utilized. As shown in Table 2-10, the procedure was completed with a final extension step at 72°C. Table 2-10.

Table (2-11): Genes and Their thermal Cycling Conditions

Primers			CTX-M-1	ADC	DHA	EBC	GES	IMP	NMD	OXA-1	PER	SHV	SME	TEM	VEB	VIM
Thermocycling conditions	Initial denaturation	Temp (C)	95													
		Time	4 min.													
	Denaturation	Temp (C)	94													
		Time	40 sec													
	Annealing	Temp (C)	55	55	61	63	55	60	55	55	55	55	55	60	55	55
		Time	40 sec													
	Extension	Temp (C)	72													
		Time	40 sec	1 min							40 sec.				1 min	
	Final extension	Temp (C)	72													
		Time	5 min													
	No. of Cycles		35													

2.2.9.5 Agarose Gel Electrophoresis:

In order verify existence amplification products, agarose gel electrophoresis was used after PCR amplification.

2.2.9.6 Preparation of Agarose :

1. 100 mL of 1X TAE buffer should first be added to a beaker.
2. Heat the solution until it reaches a boiling point, using a microwave, and continue heating until all agarose particles have completely dissolved.
3. 0.8 mg/mL of red safe day is added to the agarose solution.

4. Stir the agarose solution thoroughly to ensure uniform mixing while preventing the formation of bubbles.
5. Allow the solution to gradually cool down to a temperature range of 50-60°C.

2.2.9.7 DNA Loading

1. When loading PCR products, five µL of each product was added immediately to the wells.
2. Electrical-power was configured at 100 V/m–Amp for a duration of 75 minutes. During this process, DNA migrated from the cathode to the anode poles.
3. A Gel Imaging system was used to see the resultant gel and the red safe stained bands.

2.2.10 Minimum Inhibition Concentration of IMP:

Minimal inhibitory concentrations (MIC) of Imipenem (IMP) were measured to assess the impact of inhibitors on gene expression.

The prior experiment was repeated with the inhibitor (IMP) added to the culture medium (Mueller-Hinton broth) at a concentration of 0.25 mg/mL, and the outcomes were noted.

2.2.11 Antibiotic Resistance Gene Detection via PCR:

To confirm the existence of antibiotic resistance genes in *E. coli* isolates, a PCR experiment was conducted.

2.2.12 Total Ribonucleic Acid (RNA) Extraction:

Extraction of total RNA of *E. coli* cells were done by using (GENEzol TM TriRNA Pure kit) according to instruction of manufacturing company as steps below:

Sample Lysis:

1. Bacteria cells (up to 1 x 109) was transferred to a 1.5 ml micro-centrifuge tube (RNase-free).

2. The centrifugation was done at 12-16,000 x g for 2 minutes then remove the supernatant completely.

3. Lysis buffer (1 ml) was added to the micro-centrifuge tube containing 10 mg of lysozyme.

4. Dissolving the lysozyme powder by vortex the tube until it is completely dissolved.

5. The volume of 100 µl of bacteria lysis buffer that containing lysozyme was added to the bacteria cell pellet.

6. Vortex the cell pellet to make a suspension.

7. The sample was incubated for 5 minutes at room temperature.

8. Incubate at room temperature for 5 minutes after adding 700 µl of GENEzolTM Reagent and thoroughly mixing.

RNA Binding:

1. The sample was centrifuged at 12–16,000 g for 1 min to remove cell debris before transferring the clear supernatant to a new 1.5 ml microcentrifuge tube (RNase-free).

2. In GENEzolTM Reagent, add 1 volume of absolute ethanol to 1 volume of sample mixture (1:1).

3. Place an RB Column in a 2 ml collection tube after thoroughly mixing by vortexing.

4. Transfer 700 µl of the sample mixture to the RB Column then centrifuge at 14-16,000 g for 1 min after that discard the flow-through.

5. Transfer the remaining sample mixture to the RB Column, then centrifuge for 1 min at 14-16,000 g, discard the flow-through, and place the RB Column in a new 2 ml collection tube.

RNA Wash:

1. Pre-Wash Buffer (400 µl) was added then centrifuge at 14-16,000 x g for 30 seconds.

2. Remove the flow-through and replace the RB Column in the 2 ml Collection Tube.

3. Wash Buffer (600 µl) was added to the RB Column.

4. The centrifuge was run at 14-16,000 x g for 30 seconds and then discarded. Return the RB Column to the 2 mL collection tube.

5. Wash Buffer (600 µl) was added to the RB Column.

6. Centrifuge at 14-16,000 x g for 30 seconds then discard the flow-through.

7. Place the RB Column back in the 2 ml Collection Tube.

RNA Elution:

1. RB Column was put in a clean 1.5 ml microcentrifuge tube (RNase-free).

2. RNase-free Water (25-50 µl) was added into the center of the column matrix.

3. At least 3 minutes of waiting time to ensure the RNase-free water is completely absorbed by the matrix.

4. Centrifugation was done at 14-16,000 x g for 1 minute to gain the purified RNA.

2.2.13 Gene Expression Study :

Reverse transcriptase in quantitative form to determine relative quantification (gene expression analysis), Real-Time PCR was used. The investigation attempted to evaluate gene expression between two groups of *E. coli* isolates: the first group had nine carbapenems–resistance *E. coli* isolates without treatment, and the second group contained nine with treatment. Both groups contained the same gene, according to PCR results. The experiment evaluated the gene expression of the βla_{IMP} gene in the 9 carbapenems–resistance *E. coli* isolates from both groups, with and without antibiotics. At 37 °C overnight, all isolates were sub-cultured, incubated, and cultured in brain-heart infusion broth until an

OD600 of 0.6 to 0.8 was attained. Isolated, purified, and reverse-transcribed into cDNA from total RNA. Where specified, the antibiotic Imipenem (IMP; 0.25 µg/ml) was added at its MIC 30 minutes before harvesting cells. Gene segments of 232 bp (bla_{IMP}) forward were used as the target for the creation of quantitative real-time PCR (qPCR) primers: F({5′GGAATAGAGTGGCTTAAYTCTC-3′) and reverse primer: R(3′GGTTTAAYAAAACAACCACC 5′) , and a housekeeping gene of 138 bp (Samarasinghe *et al*., 2022) (*16s rRNA* gene) forward primer F (5′-GGAGGAAGGTGGGGATGACG-3′) and reverse primer-R (3′-TCCAATCCGGAGTACGACGC-5′). Melt curve studies, qRT-PCR reaction mixes, and amplification settings were carried out as previously described. Using the ΔΔC (t) technique and 2-ΔΔC (fold change), the expression levels of *16s rRNA* were standardized to the reference gene Bla_{IMP} (Samarasinghe *et al*., 2022).

2.2.14 Quantitative Real-Time RT-PCR :

The housekeeping gene *16s rRNA* was employed to standardize the quantification of relative values by Real-Time PCR, It was employed to evaluate the gene expression (mRNA transcript levels) of the bacterium investigated. This method employed TransScript® II Green One-Step qRT-PCR SuperMix for the genes, with the components' reaction volumes as outlined in Table (2.11).

Table (2-12): Master Mix Component Used to Prepare qRT-PCR Reaction

Component (Concentration)	Volume
RNA Template	2 µl
Forward primer (10 pmol/µl)	1 µl
Reverse primer (10 pmol/µl)	1 µl
2x perfect star ^{TM} Green One-Step qRT-PCR SuperMix	12.5 µl
TransScript® Green One-Step qRT-PCR SuperMix	0.5µl
RNase-Free Water	8 µl
Total volume	25 µl

2.2.15 Quantitative PCR Protocol:

Gene expression analysis was conducted through Real-Time PCR to determine the levels of mRNA transcripts for MDR genes in the tested bacteria, with normalization against a housekeeping gene. This methodology utilized the qRT-PCR SuperMix for both the bacterial genes of interest and the housekeeping genes. The necessary components, their respective reaction volumes, and preparation steps were followed as outlined.

Subsequently, the qPCR master mix, including the components mentioned earlier, was carefully dispensed in to standard plate tubes designed for qPCR. SYBR Green dye and other crucial PCR amplification components were added to these tubes specifically to meet their unique formulation. Utilizing an vortex centrifuge for three minutes, the plate was then thoroughly combined. After thorough mixing, the plate was placed into the QIAGEN Real-Time PCR apparatus.

Quantitative PCR plate underwent can be defined as thermo-cycler conditions as detailed in table 2-12. This carefully controlled temperature cycling approach allowed for the amplification of the DNA sample and the subsequent Real-Time PCR measurement of the levels of gene expression.

Table (2-13): The Program Used in qPCR for βla_{IMP} Gene and housekeeping Gene

Step	Temperature (°C)	Time	Repeat cycle
Denaturation	95	10 sec	40 cycles
Annealing	55	20 sec	
Extension	72	40 sec	
Final extension	60-95	5 sec	
Hold 1	45	10 min	
Hold 2	95	30 sec	

2.2.16 Data Analysis of qRT-PCR:

The data obtained from qRT-PCR experiments, targeting both the gene of interest and the housekeeping gene, were analyzed using the relative quantification method for assessing gene expression levels, commonly known as the ΔCt method concerning a control gene. This analytical approach, as Langendonk *et al*., (2021) described, involved calculating the ΔCt values for each condition using the following equation.

ΔCt (control) = (Ct) (target, control) – (Ct) (ref, control)

In this equation, (Ct) represents the cycle threshold, where "control" denotes the Ct value of the control measurement, "ref" refers to the reference gene's (Ct) value, and "target" corresponds to the target gene's (Ct) value.

Furthermore, the relative fold change in gene expression, denoted as 2-ΔΔCt, was estimated.

2.2.16 Ethical Approval :

Prior to beginning the sample collection for this research, the project received preliminary authorization. Additionally, the scientific research methodology ethics committee in the Babylon University's scientific college, Department of biology, approved the design of this study. Ref. No.7/17/1336 in 21/2/2022.

Chapter Three

Results and Discussion

3. Results and Discussion

3.1 Specimens Collection

The valuable specimens were carefully collected from February to July\ 2022 from different hospitals in Hilla City. The clinical specimens from individuals with UTI symptoms were obtained from the following hospitals which is including : Al-Imam Al-Sadiq teaching- hospital (377 urine clinical specimens), Marjan teaching hospital (175 urine clinical specimens), and Babylon Women's and Children's hospital (94 urine clinical specimens).

3.2 Isolation and Identification of *Escherichia coli*

Out of the total 646 midstream clinical urine specimens were carefully obtained from individuals with urinary tract infections, only 320 (49.5%) isolates diagnosed and identified as *E. coli* and the rest 326 (50.5%) isolates were belong to the others bacteria.

Primary identification of *E. coli* isolates were based on General urine examinations as diagnostic methods , cultural, morphological characteristics , while the confirmatory identification test was based on selective and differential media that including MacConkey agar , Eosin Methylene Blue (EMB) agar and Blood agar, additionally to the Vitek®2 system and IMVIC test. After isolates were cultured on brain Heart Infusion broth at 37◦C for 24 hours bacterial colonies developed on plate agar, were studied; on MacConkey agar the colonies appeared as a deep purple color due to lactose fermentation. It is also appeared as circular, flat and moist with entire margin on this medium. MacConkey agar is a selective plating medium used for the isolation of Enterobacteriaceae and related to gram negative rods.

On EMB agar Black, dark color colonies with green Metallic sheen, the aniline dyes (Eosin and Methylene Blue) in this medium combine to

form a precipitate of green metallic sheen at acidic pH serving as indicators of acid production from lactose (Leininger *et al*., 2001).
Microscopic examination after staining with gram stain is showing that the cells arrangement, appeared as pink rods, slender, or oval shaped organisms, short to medium length, straight or slightly curved, non-sporulating occurring as singles or in pairs. Identification of all 320 suspected isolates of *E. coli* was confirmed by Vitek®2-automated system.

The results demonstrated that there were full similarity between biochemical tests results, EMB, MacConkey agar and Vitek2 system results.

3.2.1 Sex-Based Distribution of Isolates:-

The distribution of isolates among the diagnosed cases of urinary tract infections exhibited distinctive patterns that clarification the presence of various bacterial. Specifically, *E. coli* accounted for a substantial portion of the distribution, constituting 320 (49.5%) cases. This *E. coli* subset further revealed a sex-based distribution, comprising 47 (14.7%) male cases and 273 (85.3%) female cases. In parallel, The others bacteria that including 326 (50.5%) isolates which is encompassing 60 (18.4%) male cases and 266 (81.6 %) female cases. These distributional insights are presented in detail in Table (3-1).

Table (3-1): Distribution of Bacterial Isolates According to Sex

Sex / Bacteria	Males No.(%)	Females No.(%)	Total No.(%)
E. coli (n=320)	47(14.7%)	273 (85.3%)	320 (49.5%)
Other G-ve bacteria (n=326)	60 (18.4 %)	266 (81.6 %)	326 (50.5%)
Total (%)	107 (16.6%)	539 (83.4%)	(646) (100%)

The results of the study investigation are consistent with other studies carried out by Iraqi scientists in Karkuk City. Similar findings were reported by Alsamarai and Ali (2016), who discovered that *E. coli*

was the most often detected bacterium (57.7%) from urine specimens that tested positive in cultures (41.6%). In Baghdad, Ghaima *et al*. (2018) observed comparable trends, with 57.9% of specimens exhibiting positive bacterial cultures.

These consistent findings extend beyond national borders, as demonstrated by (Rafalskiy *et al.,* 2020). According to their study, *E. coli* was a significant common species (49.1%) and made up 64.2% of the separated uropathogens. The elevated prevalence *E. coli* among patients with urinary system infections is consistent with global recognition. This bacterium is a normal constituent of human and animal intestinal microbiota, harboring strains that can lead to UTIs as well as other extra-intestinal infections. These kinds of strains stand out from commensal and enteric *E. coli* due to their virulence characteristics, which aid in their effective colonization (Wasiński., 2019). Uropathogenic *E. coli* possesses a range of virulence-associated factors (VFs), which empower it to attach to, invade, and damage the host cells (Wasiński., 2019).

The results of this study are consistent with other researchers, including Sharma *et al.* (2016), who reported *E. coli* recovery in 67.66% of UTI patients. Additionally, lower isolation percentages were documented in other studies: Goyal and Beniwal (2016) noted 53% of *E. coli*-caused UTIs.

Shanthi *et al.* (2018) identified *E. coli* in 45.60% of cases, Ghanbari *et al.* (2017) found *E. coli* to account for 58.28% of cases, While Paulo in (2016) identified *E. coli* as the most prevalent pathogen in (60.4%) among UTI patients.

While UTIs are not restricted by sex, women are particularly susceptible due to anatomical and reproductive differences.

Infection with UTI typically enters the urinary tract through the urethra, making women more vulnerable because of their shorter urethral

length. This anatomical discrepancy contributes to a higher incidence of infection among women than men (Foxman .,2014). The shorter female urethra (approximately 1.5 to 2 inches) increases the risk of pathogen invasion into the bladder, leading to bladder infections (Islam, 2018; Ibrahim *et al.*,2018).

The dominance of Uropathogenic *E. coli* (UPEC) in UTIs compared to other Gram-negative bacteria can be attributed to its array of virulence factors that facilitate colonization and disease causation (Terlizzi *et al.*,2017).

3.2.2 Age-Based Distribution of *E. coli* Isolates Causing UTI

The age-based distribution of *E. coli* isolates that causes of urinary tract infections was assessed within the framework of this study. Among the 646 isolates examined, only 320 isolates were positively identified as *E. coli*. The distribution of these isolates across various age groups is elucidated in Table (3-2)**.**

Table (3-2): Distribution of *E. coli* Isolates That Causes UTI According to The Age Groups

Age (years)	Sex		Total No.	Percentage
	Male	Female		
Young (< 30)	7 (2.2%)	67 (20.9%)	74	(23.1%)
Adult (30 - 60)	25 (7.8%)	134 (41.9%)	159	(49.7%)
Elderly (> 60)	15 (4.7%)	72 (22.5%)	87	(27.2%)
Total (%)	47 (14.7%)	273 (85.3%)	320	(100%)

The current study unveiled the demographic distribution of urinary tract infections (UTIs), with noteworthy trends emerging across different age groups. The highest incidence of UTIs was observed in the adult (30-60) years old age group, whereas the lowest incidence was recorded among the elderly (> 60) years old age group.

The study results showed the highest incidence of UTIs was observed in the adult (30-60) years old age groups among 320 *E. coli*

isolates is due to hormonal changes , nutritional and general health conditions that Which affects these ages.

These findings agreed with a study by Ojo. (2010), who reported a high prevalence of UTIs among individuals aged (30 - 60), while the lowest occurrence was within the (< 30) age group. Notably, the lowest prevalence was observed in the age group (> 60).

These results corroborate the investigation conducted by Neamati *et al.* (2015). Their study involved the collection of 150 urine samples from both genders. The most isolated pathogens were Uropathogenic *Escherichia coli* (UPEC), constituting 82.6% of the cases. Within this category, 78% of isolates were derived from females, whereas 22% were from males. A similar pattern was observed in a study conducted at Al-Karkh Surgery Hospital in Baghdad by Kareem and Rasheed , (2011). Among the 311 collected urine samples from individuals afflicted with UTIs, 68.75% of the isolates were identified as UPEC, with 100 isolates originating from females and 25 from males.

The increased vulnerability of women to UTIs can be attributed to anatomical factors, particularly the shorter length of the female urethra and its proximity to the anus. Additional factors such as sexual activity and spermicide use have been shown to heighten the risk of UTIs in women, potentially altering the vaginal microbial flora and facilitating the colonization of pathogenic Gram-negative bacteria, including *E. coli*. This assertion is supported by studies conducted by Scholes *et al.* (2000) and Walsh and Collyns , (2017).

The urinary tract infections prevalence in females is closely linked to sexual activity and childbirth. Many women are likely to experience UTIs during their lifetime, with postmenopausal women facing an increased risk. Conversely, UTIs are less common in men due to the elongated male urethra, which poses a challenge for bacteria to ascend to

the bladder. In young men, risk factors for UTIs may include sexual transmission from an infected partner, anal intercourse, or obstructions caused by conditions like kidney stones. An enlarged prostate can lead to UTIs in older men by inhibiting complete bladder drainage. Less frequently, urethral strictures, characterized by the formation of scar tissue within the urethra, can contribute to UTI occurrence (Sheerin , 2011 ; Johnson *et al.,* 2012 ; Pulipati *et al.,* 2017).

3.2.3 Morphological Identification

The identification process for *E. coli* isolates was predicated on their distinct morphological characteristics. Specifically, these isolates exhibited striking traits when cultured on both (MacConkey and EMB) agar. On MacConkey agar, *E. coli* isolate manifested as pink colonies.

This distinctive visual differentiation is attributable to the composition of MacConkey agar, which encompasses crystal violet and bile salts which allows Gram-negative bacteria to grow while inhibiting Gram-positive bacteria growth.

It is worth noting that the isolates showed their ability to ferment sugar, a characteristic that represents the basic diagnosis of *E. coli* bacteria. as depicted in Figure (3.1A).

while on Eosin Methylene Blue (EMB) media, their colonies showcased a distinct green metallic sheen.

The ability of *E. coli* to ferment sugar was proven by the Eosin Methylene Blue (EMB) medium, which was created as a differential medium to distinguish *E. coli* from other members of the Enterobacteriaceae family.

The explanation for this phenomena was reached by generating organic acids from the fermentation of glucose and lactose.

Eosin and methylene gave the colonies an eye-catching green metallic shine, as shown in Figure (3.1C), and this results agreed with studies (Singha and Prakash ,2008).

Furthermore, the isolates were cultured on blood agar to assess their propensity to lyse red blood cells. As evidenced in Figure (3.1B), the isolates demonstrated the ability to produce hemolysin enzymes, effectively lysing red blood cells. This capacity for hemolysis enhances the distinct profile of these *E. coli* isolates and their potential pathogenic attributes.

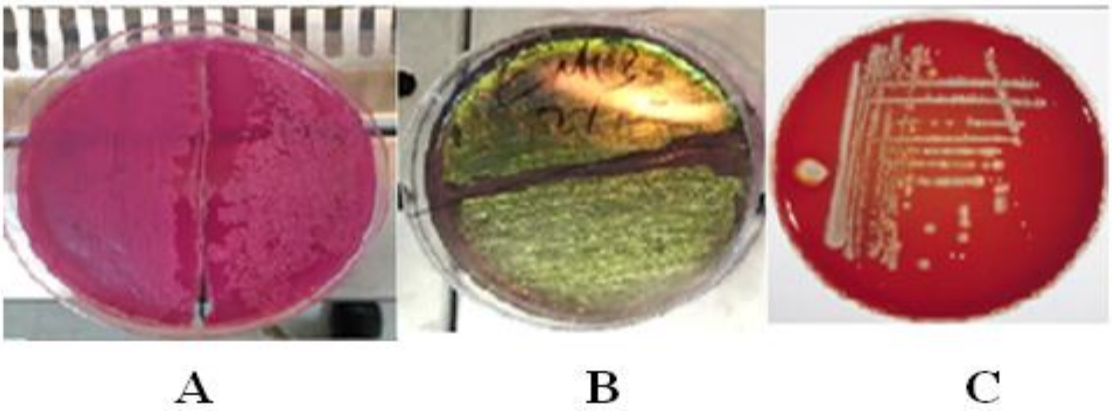

Figure (3.1): *Escherichia coli* Growth on 3 Different Mediums

A: Bacterial colonies on MacConkey agar, notice the bright pink colonies.

B: Green metallic sheen colonies on EMB media.

C: Hemolysis on blood agar plates, circular, convex and smooth colonies.

3.2.4 Vitek2 System Test:

All isolates of *E. coli* were identified by using Vitek2® system according to steps of manufacture Company, as shown in the appendix 2-Vitek 2 System report.

3.2.5 Microscopic Examination of *E. coli* Isolates:

The microscopic examination involved the creation of a smear from colonies cultivated on MacConkey agar, subsequently subjected to Gram's stain. Under this procedure, the bacteria materialized as compact, short

rods exhibiting a negative response to Gram's stain. Notably, these organisms were non-spore-forming, the results of this studies agreed with the findings reported in (Levinson *et al.*,2018).

3.2.6 Biochemical Tests of *E. coli* Isolates:

To determine the biochemical characteristics, comprehensive tests were conducted across all isolates. The outcomes of these assessments are comprehensively presented in Table (3.3). The catalase test yielded positive results, evident in dissolving the reagent into water and oxygen. Concurrently, the oxidase test outcomes were uniformly negative across the isolates, denoted by the absence of a shift to burgundy coloration. This negative response indicates the absence of cytochrome oxidase as a hydrogen receptor in the isolates. The urease test was also carried out, with all isolates reflecting negative results. This negative results signifies the lack of urease enzyme as shown in the appendix 1-C: Biochemical tests results.

Table (3.3): Identification of *E. coli* by Biochemical Tests.

Isolate	On MacConkey	Biochemical test						
E. coli-1	Lactose fermentation	Catalase	Indole	MR	Citrate utilization	Oxidase	Urease	VP
Results	+	+	+	+	+	-	-	-

In the biochemical examinations of IMVIC tests performed on the bacterial isolates, the indole test yielded a positive outcome. This was discerned by the appearance of a red ring on the surface of the medium, attributed to the enzymatic breakdown of tryptophan by the tryptophase enzyme. This specific test carries significance in distinguishing *E. coli* from other members of the Enterobacteriaceae family (Brown and Smith , 2017).

Furthermore, the methyl red test registered a positive result among the isolates. This was indicated by a color shift to red, a consequence of

the bacterial consumption of glucose and peptose. This consumption-led fermentation prompted a reduction in the medium's pH. However, the Voges-Proskauer test yielded a negative outcome. The media manifested a yellow-brown hue due to the bacteria's inability to convert glucose into acetoin (Tille *et al.*, 2017).

Regarding the citrate test, no shift to green blue occurred within the media. This lack of color alteration signifies that the bacteria could not rely solely on citrate as their carbon source. The absence of the citrate permease enzyme prevented the utilization of citrate. Consequently, the citric acid production didn't occur, resulting in an unchanged pH and no corresponding change in color (Brown and Smith , 2017).

These findings agreed with the conclusions presented by (Tille *et al.*, 2017) and (Sharmin *et al.*, 2010), further validating the outcomes of the current study.

3.3 Antibiotics Susceptibility Test (AST):

As total 320 *E. coli* isolates underwent testing against 11 antibiotic discs representing different classes of antibiotics through the disc diffusion method. The results revealed a spectrum of resistance and susceptibility among the isolates to the various antibiotics, as detailed in Table (3-4).

Table (3-4): Antibiotic's Susceptibility Test (AST) Results.

Antibiotics (mg/dL)	No. of resistant isolates	No. of Sensitive isolates	No. of Intermediated isolates	Total (%)
Carbapenems	**CLSI 2022**			
Meropenem (MEM) (10)	**20 (6.2%)**	**209 (65.3%)**	**91 (28.5%)**	**320 (100%)**
Impenem (IMP) (10)	**20 (6.2%)**	**255 (79.7%)**	**45 (14.1%)**	**320 (100%)**
Penicillin	**CLSI 2022**			
Amoxicillin (AMC)	**279 (87.1%)**	**27 (8.5%)**	**14 (4.4%)**	**320 (100%)**
Ampicillin (AMP)	**243 (75.9%)**	**54 (16.9%)**	**23 (7.2%)**	**320 (100%)**
Piperacillin (PI)	**98 (30.6%)**	**155 (48.4%)**	**67 (21%)**	**320 (100%)**
Aminoglycosides	**CLSI 2022**			
Gentamicin (GEN)	**214 (66.8%)**	**77 (24.1%)**	**29 (9.1%)**	**320 (100%)**
Sulfonamide	**CLSI 2022**			
Trimethoprim (STX)	**159 (49.6%)**	**126 (39.4%)**	**35 (11%)**	**320 (100%)**
Cephems	**CLSI 2022**			
Ceftriaxone (CRO)	**103 (32.2%)**	**167 (52.2%)**	**50 (15.6%)**	**320 (100%)**
Cefapime (CPT)	**85 (26.5%)**	**165 (51.6%)**	**70 (21.9%)**	**320 (100%)**
Nitrofurans	**CLSI 2022**			
Nitrofurantoin (NIT)	**138 (43.1%)**	**141 (44%)**	**41 (12.9%)**	**320 (100%)**
Fluoroquinolone	**CLSI 2022**			
Ciprofloxacin (CIP)	**110 (34.4%)**	**179 (55.9%)**	**31 (9.7%)**	**320 (100%)**

3.3.1 AST Results (Resistance *E. coli* Isolates):

As total 320 *E. coli* isolates was subjected for testing against 11 antibiotic discs belonging to various classes of antibiotics using the disc diffusion method. The results of this investigation showed that the *E. coli* isolates that resist for several drugs to diverse degrees. The resistance levels that were most prevalent were observed against certain antibiotic categories, specifically resistance to penicillin, represented by amoxicillin-clavulanate n=279 (87.1%) , ampicillin n=243 (75.9%) and Piperacillin was found in n=98 (30.6%) of the isolates. Resistance to Aminoglycosides, represented by Gentamicin, was noted in n=214 (66.8%) of the isolates. Sulfonamides, including Trimethoprim, exhibited resistance in n=159 (49.6%) of the isolates, while Nitrofurans, represented by Nitrofurantoin, showed resistance in n=138 (43.1%) of cases. Among the Fluoroquinolones, Ciprofloxacin resistance was observed in n=110 (34.4%) of the isolates. Resistance against Cephems , including Cefapime

and Ceftriaxone, was recorded at n=85 (26.5%) and n=103 (32.2%), respectively. Lastly, resistance to Carbapenems, which include Meropenem and Imipenem, was evident in n=20 (6.2%)of the isolates each. The detailed results are provided in figure (3.2).

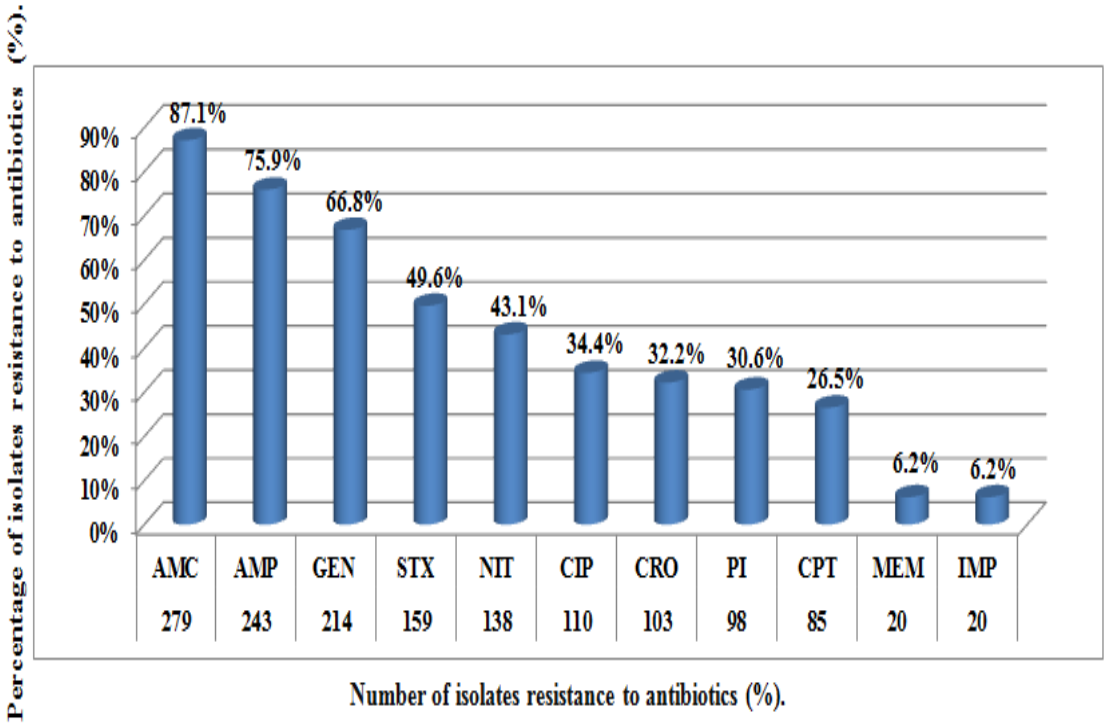

Figure (3.2): Resistance *E .coli* Isolates to Word Antibiotics among 320 *E. coli* Isolates

3.3.2 AST Results (Sensitive *E. coli* Isolates):

As total 320 *E. coli* isolates was subjected for testing against 11 antibiotic discs belonging to various classes of antibiotics using the disc diffusion method. The results revealed diverse sensitivity among the isolates to distinct antibiotics, as depicted in Figure (3.3).

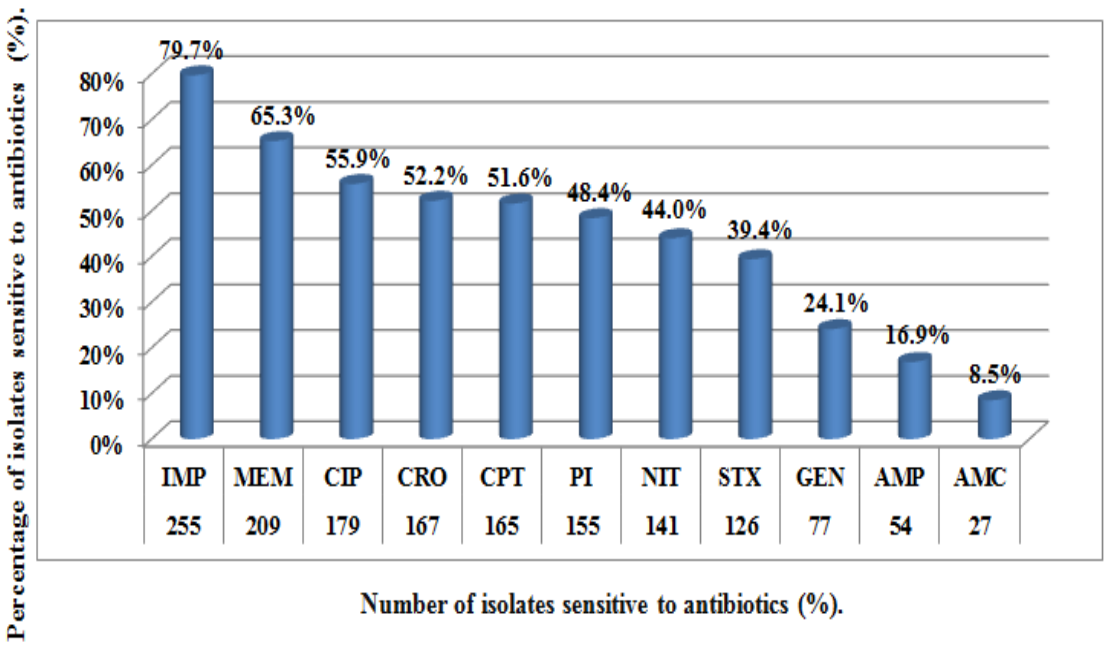

Figure (3.3): Sensitive *E. coli* Isolates to Word Antibiotics among 320 *E. coli* Isolates

The antibiotic susceptibility test (AST) results of this study, following the CLSI guidelines (2022), indicated the following antibiotics with the greatest percentage of sensitivity that including: penicillin, represented by amoxicillin-clavulanate n=27 (8.5%), ampicillin n=54 (16.9%) and Piperacillin was found in n=98 (30.6%) of the isolates. Aminoglycosides, represented by gentamicin, exhibited sensitivity in n=77 (24.1%) of cases. Sulfonamides, specifically Trimethoprim, displayed a sensitivity rate of n=126 (39.4%). Nitrofurans, including nitrofurantoin, showed a sensitivity rate of n=141 (44%). Fluoroquinolones, such as Ciprofloxacin, demonstrated sensitivity in n=179 (55.9%) of cases. Cephalosporin's, encompassing Cefapime and ceftriaxone, revealed n=165 (51.6%) and n=167 (52.2%) sensitivity rates, respectively. Finally, Carbapenems, which include meropenem and imipenem, displayed sensitivity rates of n=209 (65.3%) and n=255 (79.7%) , respectively (Table 3-4)

The susceptibility test for 11 antimicrobials was conducted on all 320 *E. coli* isolates. The results revealed higher resistance rates against

antibiotics such as Amoxicillin, Ampicillin, Gentamicin, Trimethoprim, Nitrofurantoin, Ciprofloxacin, Ceftriaxone, Piperacillin, Cefapime, and Carbapenems (Imipenem and Meropenem) respectively . Conversely, the isolates demonstrated higher sensitivity to Carbapenems (Imipenem and Meropenem), Ciprofloxacin, Ceftriaxone, Cefapime, Piperacillin, Nitrofurantoin, Trimethoprim, Gentamicin, Ampicillin, and Amoxicillin (Table 3-4)

The characteristic of multi-resistance to antibiotics prevailed in most of the isolates of *E. coli* bacteria under study. The reason for this may be due to a mutation in the chromosomal gene in the bacterium, or the characteristic of multi-resistance may be carried on the plasmid. The multi-resistance characteristic of bacterial isolates is considered to be more than one antibiotic Teawtrakul *et al.*,(2015).

Gram-negative bacteria, most of their species possess a gene encoded (*β*-Lactamase) chromosomal and another plasmid, and this confirms the possibility of transmission of resistance between the species of the family Enterococcus, and transmission may be through bacterial conjugation.

The bacterial production of beta-lactamase enzymes leads to the breaking of the beta-lactam ring, or gives the affinity between the antibiotics and the target sites penicillin-binding proteins (PBPs), or changes the permeability barrier. The isolates of *E. coli* bacteria are Gram-negative, the outer membrane that envelops the cell wall contains channels called (Porins) in which a mechanism that changes the permeability barrier that prevents the entry of antibiotic molecules into the bacterial cell is activated (Poirel *et al.*, 2001).

Recent findings from Iraqi researchers (Mhawesh *et al.*,2021) align with this result of the current study's resistance patterns. Antimicrobial resistance in UPEC poses a significant global concern across human and

animal populations, as it extends its resistance to various antibiotic classes (Poirel *et al.*,2018). The overuse and inappropriate administration of antibiotics, coupled with empirical treatment without proper bacterial identification, have contributed to heightened resistance among uropathogens, thereby diminishing the efficacy of oral therapies (Mazzariol *et al.*,2017). The rise in antimicrobial resistance necessitates a comprehensive understanding of epidemiological data to guide appropriate empirical treatment (Miotla *et al.*,2017). Resistance to novel drugs is acquired by horizontal gene transfer of plasmids encoding resistance determinants across bacteria, even across species (Lee *et al.*,2018).

The development of broad-spectrum *β*-lactamases by gut bacteria is thought to be responsible on resist to *β*-lactam antimicrobial (Al-Hilali., 2015). The rise of strains that produce ESBL has been related to the overuse of expanded-spectrum cephalosporin's (Hussein ., 2019). Appropriate antibiotic use is necessary to stop rise and spread out multidrug-resistance Gram-negative bacteria (Lin *et al.*,2018).

Notably, all UPEC isolates in this study exhibited 79.6% and 65.3% were sensitivity to Imipenem and Meropenem. These antibiotics serve as reliable last-resort treatments for bacterial infections (Meletis *et al.*,2016). Additionally, the lower rates of resistance to aminoglycosides (Gentamicin: 18.4%, Amikacin: 2.6%) may be related to their less frequent use because they are more expensive than *β*-lactams. (Hussein ., 2019). Resistance to nitrofurantoin, found among 7.8% clinical isolates of *E. coli*, may be a result of its less frequent use. All of the isolates being studied lacked either complete or broad drug resistant (XDR) or pan-drug resistance (PDR), and all multi-drug resistant (MDR). This indicates how quickly MDR in *E. coli* developed due to the bacteria's resistance to multiple antibiotic classes (Shaik *et al.*,2017). In order to avoid the

establishment of multidrug-resistant organisms, it is crucial to use antibiotics wisely and to follow approved doses.

Antibiotic overuse by healthcare workers, unskilled practitioners, and the general population are factors causing high resistance rates. Indiscriminate over-the-counter sale of antibiotics fosters self-medication, while preferences for certain antibiotics without susceptibility testing contribute to resistance (Mohamed *et al.*,2018).

The findings of this study emphasize the urgent may be need to prevent antibiotic misuse and implement appropriate antimicrobial stewardship practices to counteract the escalating issue of antimicrobial resistance. The responsible use of antibiotics is crucial to preserve the effectiveness of existing treatments and inhibit further prevalence of MDR.

3.3.3 Results of MIC by Utilizing VITEK System\2 :

Antibiotic powders were bought from Sigma in Germany in order to conduct the expression analysis. In order to achieve MIC in accordance with the CLSI (2022), the MICs were established using the broth dilution technique by utilization VITEK system-2 enriched in magnesium (W=12.5 mg/L) also calcium (W=25 mg/L). During this study, MICs utilization for 20 carbapenem-resistant *E. coli* (CREC) isolates were assessed against (11) effective antibiotics, including Imipenem, Meropenem, Gentamicin, Piperacillin, Ciprofloxacin, Ampicillin, Cefapime, Nitrofurantoin, Ceftriaxone, Amoxicillin, and Trimethoprim. These antibiotics were selected based on their common use for treating *E. coli* infections (CLSI, 2022) Table (3-5).

Table (3-5): MIC Values and Percentages of Antibiotics Resistance and Sensitivity *E. coli* Isolates Using VITEK-2

Antibiotic Name	Isolates *E. coli*				MIC (Mg/ml)
	(R)		(S)		
	No.	(%)	No.	(%)	
Meropenem (MEM)	20	100%	0	0%	≥ 4
Impenem (IMP)	20	100%	0	0%	≥ 4
Piperacillin (PI)	14	70%	6	30%	≥ 128
Amoxicillin-clavulanate (AMC)	20	100%	0	0%	≥ 32
Ampicillin AMP	20	100%	0	0%	≥ 32
Gentamicin GEN	12	60%	8	40%	≥ 16
Trimethoprim Sulfamethoxazole STX	10	50%	10	50%	≥320
Cefapime CPT	8	40%	12	60%	≥64
Ceftriaxone (CRO)	20	100%	0	0%	>= 64
Nitrofurantion NIT	9	45%	11	55%	≥16
Ciprofloxacin (CIP)	20	100%	0	0%	≥4

All isolates were collected and incubated in test tubes overnight at a temperature of 37°C. The lowest antibiotic dose at which the tested bacteria's growth was clearly inhibited served as the MIC. The antibiotic concentrations varied from 0.25 g/ml to 256 g/ml, and the antibiotic resistance breakpoints, according to the CLSI 2022 recommendations, which went into effect in 2022 were summarized in Table (3-5). The result of MIC by using VITEK\2 for all of the 20 carbapenems resistance isolates showed the resistance to 11 antibiotics as the following:- (100%) IMP and MER antibiotics with (MIC ≥ 4), (70%) PI MCI ≥ 128, (100%) AMC and AMP with (MIC ≥ 32), (60%) GEN with MIC (≥ 16), (50%) STX with (MIC ≥ 320) , (40%) CPT and (55%) CRO with (MIC ≥ 64) , (45%) NIT with (MIC ≥16) and finally 100% CIP with (MIC ≥ 4), as shown in Table (3-5) as shown in the appendix 3 Table detailing MIC test results by using VITEK-system -2.

The results of the current study agreed with the results of Abdul-Ghaffar in 2017, which isolated *E. coli* from the urine of patients with urinary tract infections in Baghdad governorate, as it became clear that all

isolates were resistant to antibiotics and to more than three antibiotics, meaning that they fall under Multi-drug resistant bacteria were labeled with resistance to Ceftriaxone (72.09%), Ceftazidime (62.79%), Ciprofloxacin (60.46%) and Gentamicin (46.51%) (Abdul-Ghaffar, 2017). With regard to Nitrofurantoin, it was found that it is the best treatment that can be used against *E. coli* (UPEC), as the current study was compatible with the local studies Al-Tamemi, Al-Saadi, Abdul-Ghaffar with percentages (87.5%, 96%, 100%) on relay (AL-Saadi, 2019).

The VITEK-2 system's methodology is utilized to determine the Minimal Inhibitory Concentrations (MICs) for each antibiotic. This method involves standardizing data and normalizing each bacterium's growth characteristics. This is accomplished by comparing the growth characteristics found in each well that contains an antibiotic with those found in a well that serves as a positive control. Changes in urine specimen's turbidity, leukocytes, erythrocytes, and other components that influence bacterial growth (such as pH, proteins that restrict bacterial growth, or antimicrobial compounds) had an identical effect on the control and antibiotic-containing wells. Therefore, these traits are irrelevant to the MIC results since the VITEK\2 system's methodology analyzes the trend of bacterial growth curves in the presence of antibiotics while neutralizing and normalizing the sample specifics (AL-Tamemi, 2021).

3.3.4 Extend Spectrum *β*-lactamases Phenotype Identification:

The usual disk diffusion technique was used to initially confirm that each *E. coli* isolates that showed antimicrobial resistant result as depicted in Figures (3-5) and (3-6). Two phenotypic confirmatory screening tests were applied to the *E. coli* isolates to determine ESBLS production by using two different methods including amoxiclave- double disk Synergy test and modified Hodge's tests.

3.3.4.1 Amoxiclave- Double Disk Synergy Test:

Testing the synergy of double disks using amoxiclave was utilized to identify extend spectrum *β*-lactamase enzymes. The findings showed that 20 isolates (62.5%) had an increased diameter zone surrounding the Amoxiclave disk, which suggested the presence of extend spectrum *β*-lactamase enzymes. Genuine carbapenemase have hydrolysis activity against several other *β*-lactam antibiotics, including imipenem and meropenem. This can be observed in Figure (3-4).

Figure (3-4): Amoxiclave - Double Disc Synergy Test (DDST) for ESBL Confirmation.

A- Positive result B-Negative result.

The KPC (*Klebsiella pneumoniae* carbapenemase) type of carbapenemase is the most therapeutically relevant one in Ambler class A (Baraniak *et al.*,2011). These enzymes can hydrolyze all *β*-lactams, and substances like boronic acid can inhibit their activity. Tazobactam and Clavulanic acid also partially block them. Ambler class B *β*-lactamases, however, have the highest carbapenemase activity. These enzymes predominantly comprise Enterobacteriaceae IMP, VIM, and NDM types. All penicillin's, cephalosporin's, and carbapenems with the exception of Monobactams aztreonam are hydrolyzed by them, as are a large variety of other antibiotics. Importantly, *β*-lactamase inhibitors that are readily accessible on the market such Clavulanic acid, Tazobactam, or Sulbactam do not stop their action (Nordmann *et al.*,2011).

Class B enzymes' hydrolysis process depends on how the β-lactam reacts with the zinc ions in their active site. This clarifies why substances like EDTA, a divalent cation chelator, or dipicolinic acid can inhibit their activity. OXA-48 and OXA-181 are the most common Ambler class D enzymes in Enterobacteriaceae with carbapenemase activity. These enzymes reveal a distinct hydrolysis profile, sparing ceftazidime, exhibiting limited Cefotaxime hydrolysis, and indicating resistance to Clavulanic acid and Tazobactam inhibition. Clinically isolates frequently contain additional co-existing *β*-lactamases such broad-range *β*-lactamases (ESBLs), therefore they do not always just display the carbapenemase expression pattern for resistance (Nordmann *et al.*, 2012).

3.3.4.2 Modified Hodge's Test (MHT):

Modified Hodge's test exhibited a distinctive cloverleaf-like indentation around the imipenem disk, signifying a positive outcome for all 20 isolates displaying carbapenem resistance genes. Among this study's 320 *E. coli* isolates, only 20 (62.5%) were identified as carriers of the carbapenemase enzyme through the MHT. In contrast, the remaining isolates were confirmed as carbapenemase-negative, as depicted in Figure (3-5).

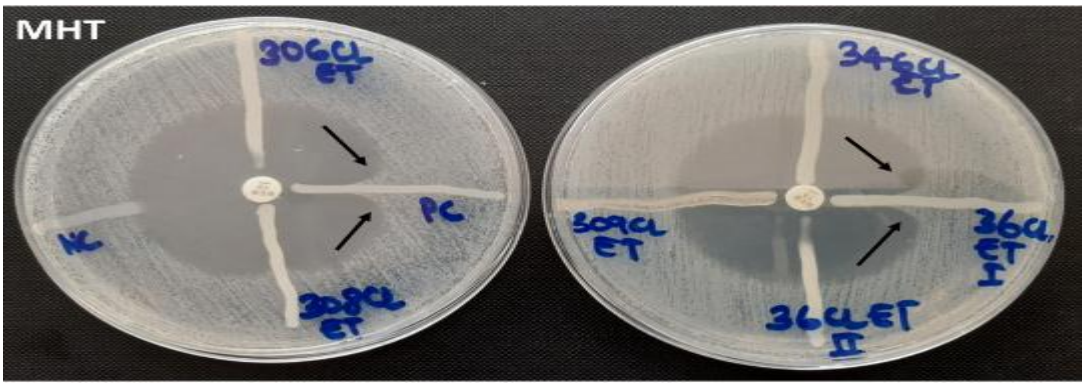

Figure (3-5): Modified Hodge Test. The results showing negative and positive controls (left) and negative and positive test organisms (right).

The CLSI 2022 now supports the modified Hodge test (MHT), which is used often for epidemiological reasons as a phenotypic screening

technique for identifying carbapenemase. Despite its ease execution, varying specificity values have been reported, and concerns about false-positive outcomes exist (Cury *et al.*, 2012).

Zinc in the culture medium has a substantial impact on sensitivity MHT for detecting NDM producers. This test performs well as a preliminary evaluation of the carbapenemase activity of potential isolates. Additionally, it is an essential component of infection control strategy used during outbreaks brought on by strains that produce carbapenemase. Although this method, utilizing imipenem, demonstrates efficacy in detecting metallo-*β*-lactamase (MBL) producers showcasing high-level resistance, it might not always recognize MBL producers manifesting low-level resistance to imipenem (Girlich *et al.*, 2012).

In identifying and differentiating carbapenemase, molecular techniques continue to be the golden standard. However, these methods carry certain drawbacks, including cost implications, the need for skilled personnel, and the inability to identify novel carbapenemase genes (Nordmann *et al.*, 2012).

3.4 Molecular Detection of *β*-Lactamase Genes:

In the Ambler classification, *β*-lactamases are grouped into four classes A, B, C, and D by motifs composed of primary sequences constituting the protein molecules. *β*-lactamases of classes A, C, and D use a serine at the enzyme active center, whereas *β*-lactamases of class B use metal zinc ions (Ambler , 1980).

The result of AST showed only 20 *E. coli* isolates were determined to be positive for carbapenemase resistance isolates, attributing to urinary tract infections (UTIs), as indicated by their ESBL phenotypes and beta-lactamase production. Employing the conventional polymerase chain reaction (PCR) technique, verified bacterial DNA extracts were templates to identify *E. coli* isolates harboring *β*-lactamases-encoding genes.

Specific primers, including class A (Extend spectrum *β*-lactamase (ESBLS) including :- [***bla***$_{TEM}$ (972 bp), ***bla***$_{SHV}$ (898 bp) , ***bla***$_{CTX-M-1}$ (646 bp) ,***bla***$_{PER}$ (520 bp) and ***bla***$_{VEB}$ (961 bp)], while class A carbapenemase including : [***bla***$_{GES}$ (371 bp) and ***bla***$_{SME}$ (334 bp)]. Class B including: [***bla***$_{IMP}$(232 bp), ***bla***$_{NDM}$ (550bp) and ***bla***$_{VIM}$ (390 bp)]. Class C including: [***bla***$_{ADC}$ (445 bp), ***bla***$_{DHA}$ (405 bp) and ***bla***$_{ECB}$ (302 bp)]. Finally Class D including only ***bla***$_{OXA-1}$(564 bp) respectively that assisted the gene identification process in this study.

3.4.1 Molecular Detection of Class A Serine *β*-lactamase (ESBLS) Production:

The Presence serine-*β*-lactamase enzymes in carbapenem resistant *E. coli* isolates can be observed through conventional PCR techniques. Detection focused on genes encoding five distinct types of these enzymes, facilitated by their respective primers (mentioned in Table 2-7). The results of the molecular detection revealed a considerable frequency of these enzymes among the isolates under investigation. Among the identified genes, which is including the following genes [***bla*** $_{TEM}$ (972 bp), ***bla*** $_{SHV}$ (898 bp) , ***bla*** $_{CTX-M-1}$ (646 bp) ***bla***$_{PER}$ (520 bp) and *bla*$_{VEB}$ (961 bp)], while class A carbapenemase including : [***bla***$_{GES}$ (371 bp) and ***bla***$_{SME}$ (334 bp)].

3.4.1.1 Molecular Detection of ***bla*** $_{TEM}$ (972 bp) :-

The gene of *bla*$_{TEM}$ (972 bp) have been identified by utilizing conventional PCR. Results revealed that only 16 out of the 20 total isolates of *E. coli* carried *bla*$_{TEM}$ (80%), which is provides important information on the nature of ***β-lactamase*** enzymes and how they relate to the resistance profile, as illustrated in Figure (3.6).

The current study showed only 16 isolates positive to the *bla*$_{TEM}$ and only 4 isolates showed negative results that is may be due to their ability

to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps.

Aligning with the findings of Ahmed, who concluded that approximately 56% of UPEC isolates exhibited the bla_{TEM} gene (Ahmed., 2013). The result of this study served a significant correlation between bla_{TEM} and β-lactamase enzymes. Globally, TEM β-lactamase enzymes, including TEM1, TEM2, TEM3, and TEM4, have been explored by various researchers (Gundran *et al.*, 2019). The findings of this study confirm that the increased prevalence of TEM among other ESBL types. The high percentage of resistant of UPEC to the third generation of β-lactam is due to the excessive uptake of these antibiotics, improper usage of drugs as prescribed by a physician, as well as a lack of personal education exhibited by an incomplete full course of antibiotics to destroy the pathogen in order to increase infection cure rates and avoid the formation of any resistance or treatment failures, and in addition to the fact that the majority of the isolates were beta-lactamase producers (ESBL) (Al-khikani *et al.*, 2020).

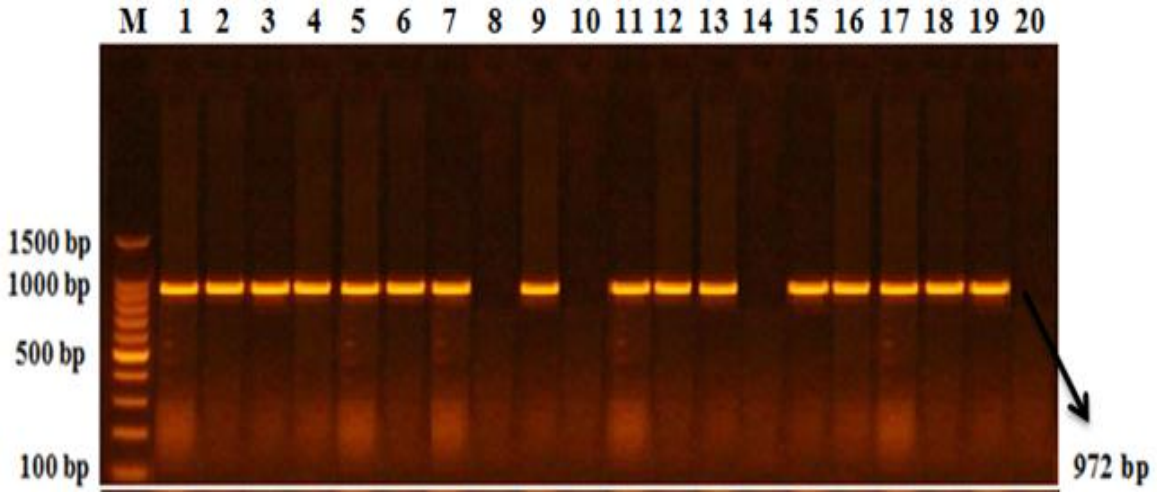

Figure (3-6): Electrophoresis of bla_{TEM} (972 bp). Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (1,2,4,5,6,7,9,11,12,13,15,16,17,18 and 19) show positive result (972-bp). Lines (8,10,14 and 20) show negative results.

3.4.1.2 Molecular Detection of *bla* $_{SHV}$ (898 bp):-

The gene of bla_{SHV} (898 bp) have been identified by utilizing conventional PCR. results revealed that only **5** isolates out of the 20 total isolates of *E. coli* carried bla_{SHV} (25%) as showed in Figure (3.7).

The current study showed only **5** isolates positive to the bla_{SHV} and only **15** isolates showed negative results that is perhaps due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps.

Correspondingly, the study of Manoharan and colleagues yielded similar findings (Manoharan *et al.*, 2011). On the other hand Khalaf and Al-Ouqaili *et al* (2018) in Baghdad, during a period one year demonstrated that SHV gene was detected only in 12.5% *E. coli*. These outcomes highlight require for Amikacin and Ciprofloxacin were found to be the most to the least active antimicrobial agents in vitro.

Therefore, isolation and detection of ESBL producing strains are essential for the selection of the most effective antibiotic for treatment and also infection control. Overall, continuous monitoring and effective infection control measures, rational use of drugs and also the use of carbapenems instead of extended-spectrum cephalosporin’s, quinolones, and aminoglycosides for treating infections in which ESBL-producing strains are likely to emerge could be effective (Manoharan *et al.*, 2011).

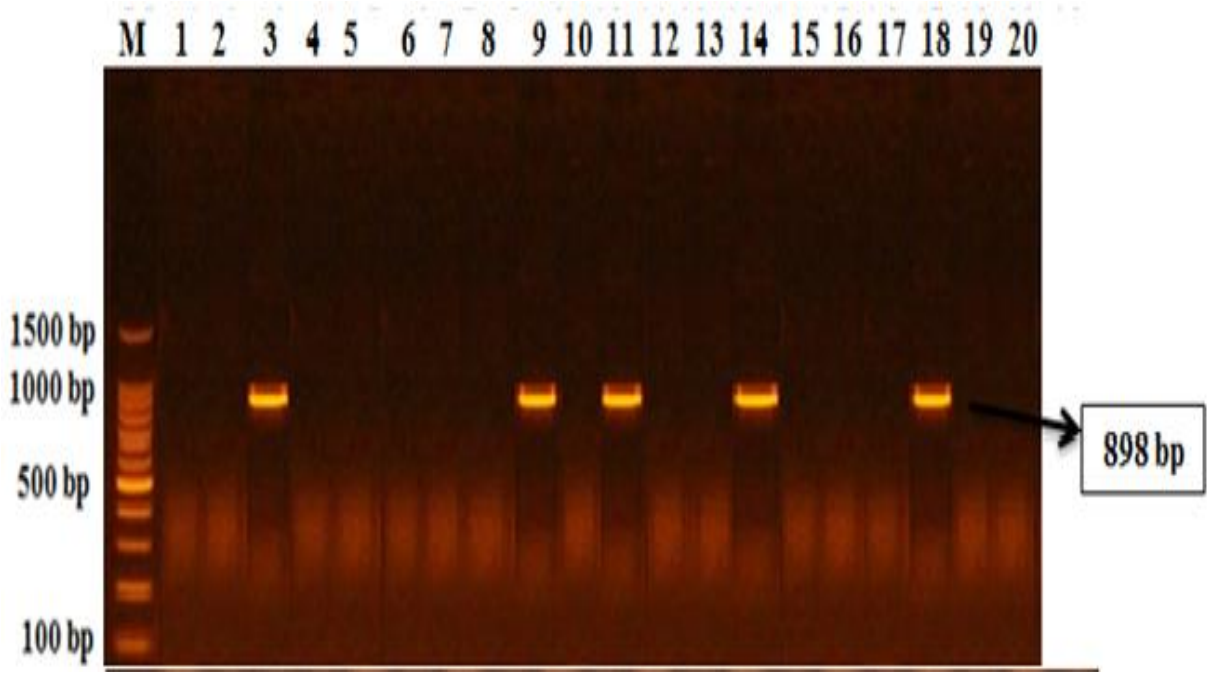

Figure (3-7): Electrophoresis of *bla*$_{SHV}$ (898 bp). Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (3,9,11,14 and 18) show positive result (898-bp) while the lines (1,2,4,5,6,7,8,10,12,13,15, 16,17,19 and 20) show negative results.

3.4.1.3 Molecular detection of *bla* $_{CTX-M-1}$ (646 bp):-

The gene of *bla*$_{CTX-M-1}$ (646 bp) have been identified by utilizing conventional PCR. results revealed that 19 isolates out of the 20 total isolates of *E. coli* carried *bla*$_{CTX-M-1}$ (95%) as illustrated in Figure (3.8).

The current study showed only 19 isolates positive to the *bla*$_{CTX-M-1}$ and only 1 isolates showed negative results that is probably due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. It may also have the ability to change permeability.

The incidence and molecular characteristics of ESBL genes, especially the *bla*$_{CTX-M}$ gene, were examined in a comparable research carried out in Zakho, Iraq. Their findings highlighted that out of 169 isolates examined, a substantial 159 carried CTX-M type enzymes, constituting a prevalence rate of 94.1% (Polse *et al.*, 2016). Drawing from

the PCR outcomes, it was observed that all *E. coli* isolates harbored one or more ESBL genes, and among these, CTX-M type ESBL was predominant, accounting for 87.2% of the isolates in the region of Duhok, Iraq (Merza *et al.*, 2016). The results of this study were similar to others studies that showed the most globally common type of ESBL appeared to be CTX-M-type ESBLs with their higher incidence in most locations compared to SHV and TEM ESBLs (Jorgensen *et al.*, 2010).

The high prevalence of CTX-M gene in the current study, agreed with some earlier studies that carried out by Seputien *et al*. (2010), that showed the high prevalence of CTX-M among *E. coli* (96%) and *K. pneumoniae* (71%) isolates, showing the ESBL phenotype (Seputien *et al.*,2010). In a study which was done on 181 unduplicated *E. coli* strains isolated in nine different hospitals in three Portuguese regions in 2007, it showed that CTX-M producer strains were prevalent among UTIs (76%) (Mendonça *et al.*,2007). Another study that was conducted in Turkey (2013) showed that CTX-M was the most prevalent ***β***-lactamase enzymes among ESBL-producing *E. coli* isolates (83.18%) (Copur *et al.*,2013). On the basis of current results, it can be argued that current ESBL epidemiology in this study is consistent with some neighboring countries and Europe (Hassan and Abdalhamid ., 2014). These higher rates of CTX-M among study isolates may be associated with high mobilization of the encoding genes. Barlow *et al*. reported increased tenfold in movement of $bla_{CTX\text{-}M}$ genes via plasmid in compare to other class A β-lactamase (Barlow *et al.*, 2008).

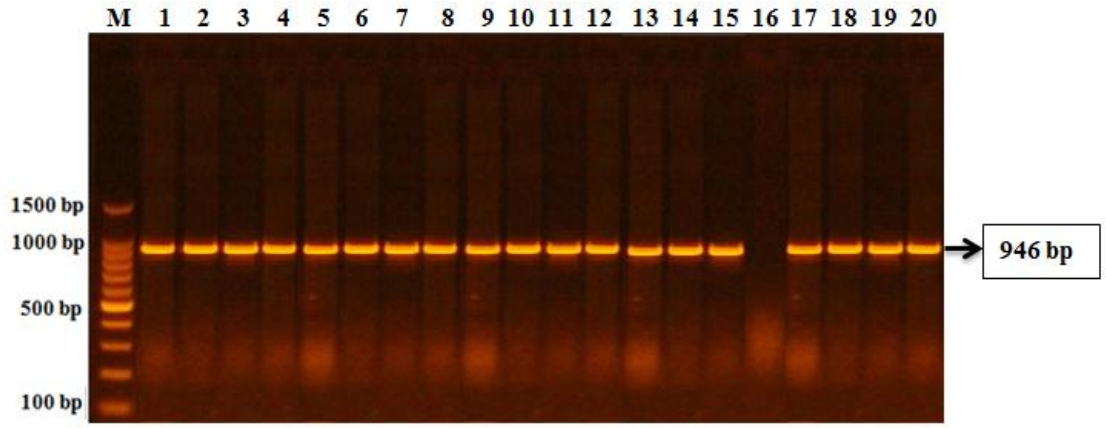

Figure (3-8): Electrophoresis of *bla* $_{CTX-M-1}$ (946 bp). Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Line (1,2,3,4,5,6,7,8,9,10,11,12,13,14,15,17,18,19 and 20) show positive result (946 bp). lines (16) show negative results.

3.4.1.4 Molecular detection of *bla* $_{PER}$ (520 bp):-

The gene of bla_{PER} (520 bp) have been identified by utilizing conventional PCR. Results revealed that only 7 out of the 20 total isolates of *E. coli* that carried bla_{PER} (35%), as illustrated in Figure (3.9).

The current study showed only 7 isolates positive to the bla_{PER} and only 13 isolates showed negative results that is possibly due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to PER may be due to a resistance mechanism other than the production of PER.

The results of this investigation closely align with those of Zhang *et al.* (2014), who detected positive PER genes in *E. coli* isolates prevalent in five hospitals. Also the result of this study agreed with some finding studies carried out by Sareaa whose study of 38 *E. coli* isolates resistant to carbapenem that revealed presence bla_{PER} in only 4 isolates, corresponding to a prevalence rate of 47.3% (Gatya *et al.*, 2022). The results of this study showed only 7 isolates encoding for bla_{PER} genes, while the rest of the 13 isolates showed negative results for detecting presence of bla_{PER} and that is due to these isolates carrying multiple virulence factors that capable to

resist multiple drugs or may be these 13 isolates dose not encoding for bla_{PER}. Additionally these 13 isolates required specific primers to detect presences bla_{PER} genes among these isolates. The presence of multiple genotypes in a single isolate might be the result of complex antibiotic resistance pattern (Kaftandzieva *et al.*, 2011).

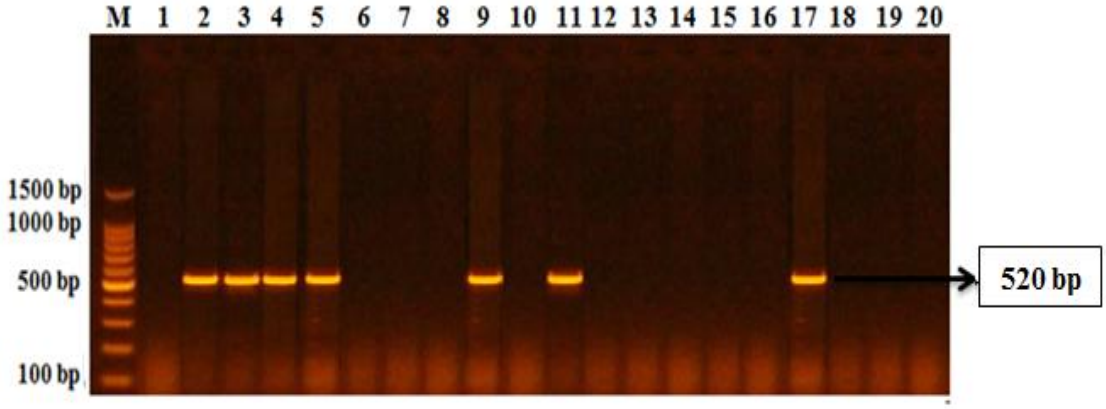

Figure (3-9): Electrophoresis of bla_{PER} (520 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (2,3,4,5,9,11 and 17) show positive result ,while lines (1,6,7,8,10,12,13,14,15,16,18,19and 20) show negative results.

3.4.1.5 Molecular detection of bla_{VEB} (961 bp):-

The genes of bla_{VEB} (961 bp) have been identified utilizing conventional PCR. Results revealed that only 6 of the 20 total isolates of *E. coli* carried bla_{VEB} (30%), as illustrated in figure (3.10).

The current study showed only 6 isolates positive to the bla_{VEB} and only 14 isolates showed negative results that is probably due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to VEB may be due to a resistance mechanism other than the production of VEB.

There may be a rapid spread of *E. coli*, especially in hospital settings where various antimicrobials are largely used and thus may support the dissemination of these bacteria.

In Thailand, *β*-lactamase genes expressing VEB-1 were discovered in 8.5% of ESBL-producing *E. coli* isolates as part of a similar investigation that focused on healthcare-associated infections (Patil *et al.*, 2019). The study's findings did not match those of a study done in Iran, where UPEC isolates did not include any bla_{VEB} genes (Alizade *et al.*,2015). These findings are consistent with a study from northern Iran, where a prevalence of 8% for the VEB gene was observed in UPEC isolates (Govindaswamy *et al.*,2019). Currently, the clinical microbiology laboratories do not have a reliable method of detecting and confirming AmpC β-lactamases. For this reason, the molecular characterization of *β*-lactamases, in particular the *AmpC* family, is of crucial importance in in terms of molecular epidemiology and genetic contributing factors (Dolatyar *et al.*, 2021).

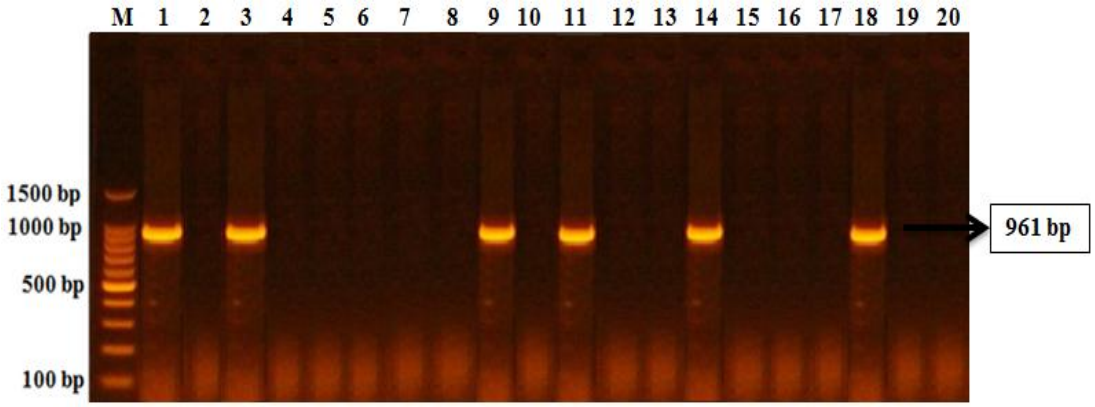

Figure (3-10): Electrophoresis of *bla* $_{VEP}$ (961 bp). Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (1,3,9,11,14 and 18) show positive result (961-bp). Lines (2,4,5,6,7,8,10, 12,13,15,16,17,19 and 20) show negative results.

3.4.2 Molecular detection of Carbapenemase *bla* $_{GES}$ (371 bp) and *bla* $_{SME}$ (334 bp) :-

The both genes of bla_{GES} (371 bp) and bla_{SME} (334 bp) have been were detected by utilizing conventional PCR. Results revealed that targets

genes was not observed out of the total 20 isolates of *E. coli* as illustrated in Figure (3.11).

The current study showed negative results for bla_{GES} and bla_{SME} among 20 carbapenms resistance isolates is due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. It may also have the ability to change their permeability.

The main reason for the non-appearance of these genes (bla_{GES} and bla_{SME}) is may be due to these isolates possess multiple resistant factors, including capsule, the ability to form a biofilm, and their production of various types of enzymes, as well as changing the permeability of the membrane and modifying the target site, which enables them to resist several types of antibiotics. Also may be the isolates dose not encoding for the genes or may be the primer non-specifics to detect for this genes and required specific primers to detect for bla_{GES} and bla_{SME} genes (Garza-Ramos *et al*., 2015).

The Guiana-Extended Spectrum (GES-1) ESBL, first discovered in *Klebsiella pneumonia* isolated from a patient in Guyana, has been gradually isolated from Enterobacteriaceae and other bacteria from various geographical areas (Garza-Ramos *et al*., 2015).

In some of studies that detected on GES gene which is not observed within *E. coli* isolates in recent Egyptian investigations (El-Domany *et al.,* 2007). Also the results of the current studies that carried out by (Gatya *et al.*, 2022) that showed no PCR-amplification products were noticed with bla_{GES}.

Whereas the current results were agreed with a study by Linlin *et al.* (2019), which observed negative results for bla_{SME} among 50 *E. coli* isolates.

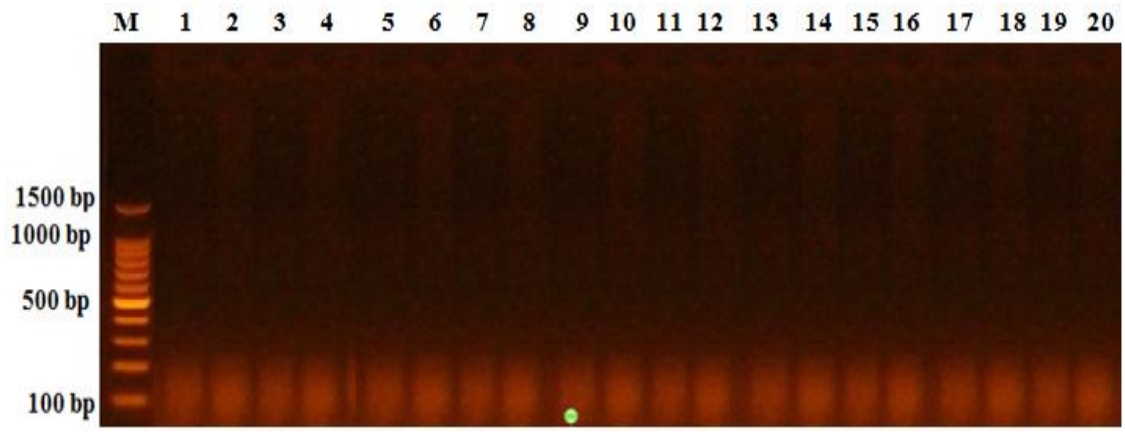

Figure (3-11) : Electrophoresis of *bla* $_{SME}$ (334 bp) and *bla* $_{GES}$ (371 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (1-20) show negative result (334-bp and 371 bp).

3.4.3 Molecular detection of Class B Metallo *β*-lactamase production:-

The Presence Metallo-β-lactamase enzymes in carbapenem resistant *E. coli* isolates can be observed through conventional PCR techniques. Detection focused on genes encoding 5 distinct types of these enzymes, facilitated by their respective primers (mentioned in Table 2-7). The results of the molecular detection revealed a considerable frequency of these enzymes among the isolates under investigation. Among the identified genes that consist Class B including: [***bla*** $_{IMP}$(232 bp), ***bla*** $_{NDM}$ (550bp) and ***bla*** $_{VIM}$ (390 bp)].

3.4.3.1 Molecular detection of *bla* $_{IMP}$ (232 bp) :-

The gene of bla_{IMP} (232 bp) have been identified by utilizing conventional PCR. Results revealed that only 12 out of the 20 total isolates of *E. coli* carried bla_{IMP} (60%) as illustrated in Figure (3.12).

The current study showed only 12 isolates positive to the bla_{IMP} and only 8 isolates showed negative results that is perhaps due to their ability to resist the antibiotics by various mechanisms such alter the target's

position or by use efflux pumps. Thus, non-susceptibility to IPM may be due to a resistance mechanism other than the production of IMP.

IMP is the second most prevalent metallo-beta-lactamase enzyme identified. Most common MBL-gene among *E. coli* isolates in a Sudanese investigation was bla_{VIM} (16.7%), which was followed by bla_{IMP} (8.3%) and bla_{NDM} (2.8%) (Adam and Elhag ., 2018).

The production of all kinds of carbapenemase by *E. coli* isolates represent a major issue with further problem in UTI treatment (Shaik *et al.*,2017). From a therapeutic perspective, CRE represent a threat as only a few antibiotics retain activity against them. This is due to the ability of carbapenemase to hydrolyze most other ***β***-lactam antibiotics, and to frequent coexistence in CRE isolates of additional mechanisms of resistance against other antibiotics such as Fluoroquinolones and aminoglycosides (Moxon and Paulus ., 2016). In addition, Sahin *et al* studied 43 strains of *Klebsiella* and *E. coli* and found that 35 strains were positive for the MHT, which were not reported in any of the VIM and IMP genes after the PCR (Sahin *et al.*,2015). In this study 20 isolates were positive for producing MBLs by the MHT test while the PCR results showed only 12 isolates were positive to bla_{IMP} and the rest of 8 isolates showed negative result. The reasons of negative results is due to these 8 isolates possessing other antibiotic resistance factors or may be these 8 isolates dose not encoding the bla_{IMP} genes. Additionally the negative results may be is due to utilized none specific primers.

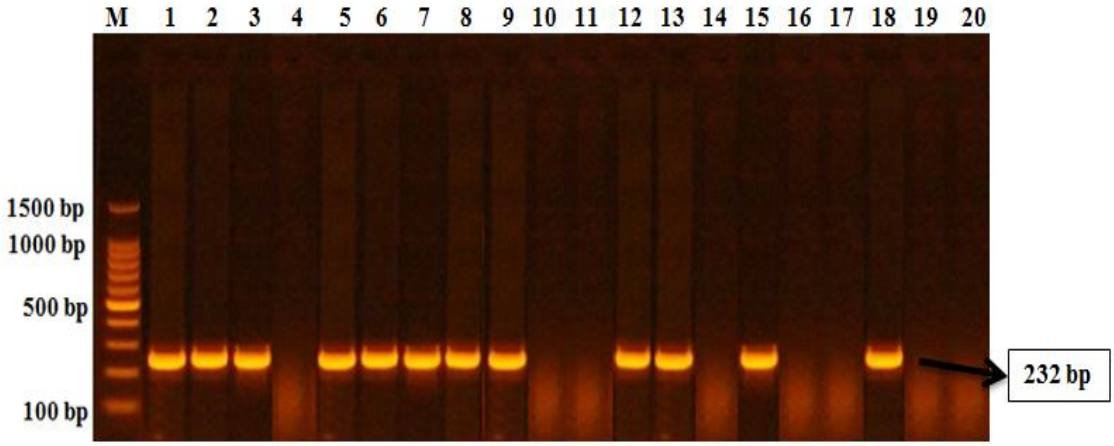

Figure (3-12): Electrophoresis of *bla* $_{IMP}$ (232 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (1,2,3,5,6,7,8,9,12,13,15 and 18) show positive result (232-bp). lines (4,10,11,14,16,17,19 and 20) show negative results.

3.4.3.2 Molecular detection of *bla* $_{VIM}$ (390 bp) :-

The gene of bla_{VIM} (390 bp) have been identified by utilizing conventional PCR. Results revealed that only 10 of the 20 total isolates of *E. coli* carried bla_{VIM} (50%) as illustrated in Figure (3.13).

The current study showed only 10 isolates positive to the bla_{VIM} and only 10 isolates showed negative results that is possibly due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to VIM may be due to a resistance mechanism other than the production of VIM.

The bla_{NDM}, bla_{KPC}, bla_{IMP} and bla_{VIM} types are more important clinically and have been detected in clinical Gram-negative bacteria particularly in the Mediterranean region and the far east. (Nordmann and Poirel., 2002).

The current study results agreed with study carried out by a Jordanian study has proved the presence of bla_{VIM} in uropathogenic *E. coli* (Nairoukh *et al.*, 2018).

The emergence of *E. coli* isolates that can cause UTIs and produce MBL raises concerns due to their resistance to therapy. The most common MBL gene among clinical *E. coli* isolates was similarly found to be bla_{VIM} (16.7%), according to a Sudanese investigation carried out by Adam and Elhag (2018). In a research from southwest Iran, 8.3% of uropathogenic *E. coli* isolates that harbored bla_{VIM} genes (Matin *et al.*,2021). Deldar *et al*. (2020) also observed lower frequencies of bla_{VIM} (9.8%) in UTI-causing *E. coli* from Iran.

A recent study from Taiwan reported a prevalence rate of 8.7% for VIM-1 (Huang *et al.*,2021). The results of this study showed that only 10 isolates were harbored bla_{VIM} and the rest showed negative results due the ability of this isolates having another way to resist of the antibiotics such as increase their membrane permeability, secretion various enzymes, and other methods of resistance that microorganisms demonstrate to survive. The rapid emergence and spread of antibiotic resistance makes it vital to keep track of antibiotic use and develop therapeutic solutions to decrease antibiotic misuse (Hetta *et al.*, 2020; Algammal *et al.*, 2021). As a result, it's vital to continue researching the genes that drive bacteria to become antibiotic resistant (Farhan *et al.*,2020).

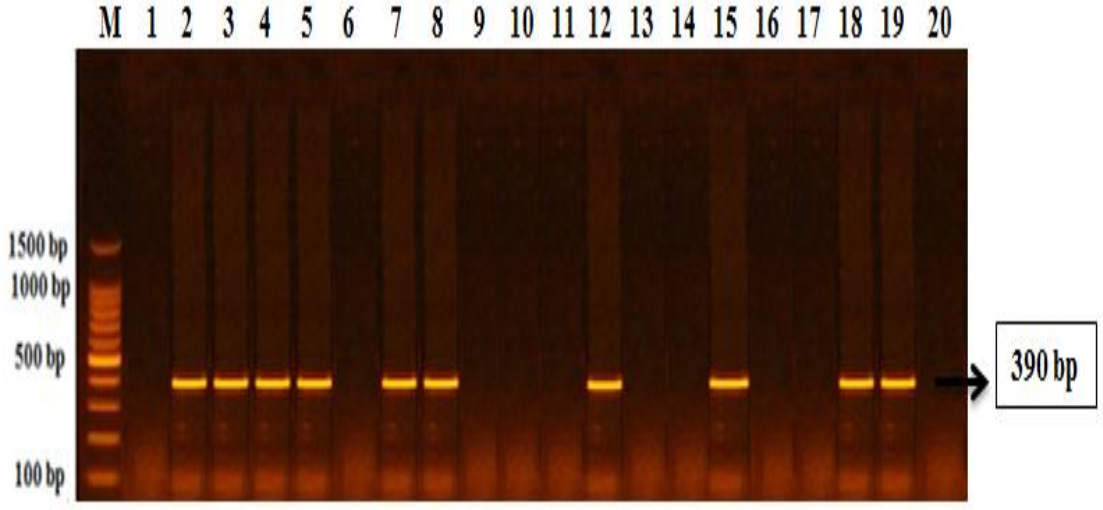

Figure (3-13): Electrophoresis of *bla* $_{VIM}$ (390 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (2,3,4,5,7,8,12,15,18 and 19) show positive result (390-bp). lines (1,6,9,10,11,13,14,16,17 and 20) show negative results.

3.4.3.3 Molecular detection of *bla* $_{NDM}$ (550 bp) :-

The gene of bla_{NDM} (550 bp) have been identified by utilizing conventional PCR. Results revealed that only 15 of the 20 total isolates of *E. coli* carried bla_{NDM} (75%) as illustrated in Figure (3.14).

The current study showed only 15 isolates positive to the bla_{NDM} and only 5 isolates showed negative results that is may be due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to NDM may be due to a resistance mechanism other than the production of NDM.

Metallo-lactamase enzymes, NDM, is an emerging carbapenem-resistant β-lactamase that is of major public concern due to its high medical and economic burden (Otter *et al*., 2017), especially for developing countries such as India, Pakistan, and the Balkan countries. As the most populous country in the world, there are major difficulties in preventing the dissemination of multidrug resistant genes in China. Therefore, comprehensive, extensive studies on diverse bla_{NDM} variant-

positive *E. coli* are needed to provide clear information to optimize antibiotic policy in endemic areas. Generally, the prevalence of the bla_{NDM} gene has continuously increased worldwide. As of now, the NDM enzyme has been identified in almost all of the world, including many countries in Asia, Africa, Europe, the Americas, and Australia (Berrazeg *et al*., 2014). A study from India also analyzed the occurrence of the bla_{NDM} gene among carbapenem resistant isolates, and it accounted for 45.4% of them (Rahman *et al*., 2018).

In a research conducted in India, isolates that were carbapenem-resistant and made up 45.4% of the sample were examined for the presence of the bla_{NDM} gene (Rahman *et al.,* 2018). Meanwhile, a survey conducted by the French National Reference Center revealed about 21% out of 140 isolates that were carbapenem-resistant were NDM producers (Gauthier *et al.,* 2018).

NDM manufacturers made up 49% of the carbapenem-resistant *E. coli* strains in China, according to a national research (Zhang *et al.*,2017). Furthermore, a multicenter study of the China CRE network revealed that among 39 carbapenem-resistant *E. coli* isolates, 74.4% were NDM producer, suggesting that there is a serious challenge in combating infections caused by this "superbug" in China (Zhang *et al*., 2018).

Among 54 CREC, another investigation found 53 bla_{NDM}-carrying isolates, outnumbering earlier results from China (Liang *et al.,* 2017), signifying the escalating prevalence of the bla_{NDM} gene in the area.

This study was conducted to identify carbapenem-resistant uropathogenic *E. coli* and possible acquisition of MBL carbapenemase genes (NDM variants) among such isolates so that this study could become a valuable reference to figure out overall prevalence of drug resistance in the study region (Liang *et al.*, 2017).

To the best of our knowledge, this is also the first report on the bla_{NDM} gene in Northern Jiangsu Province. Moreover, the emergence of such a high prevalence of bla_{NDM} variants indicates that the bla_{NDM} gene is increasing in this area. (Jennifer and Adrian., 2011).

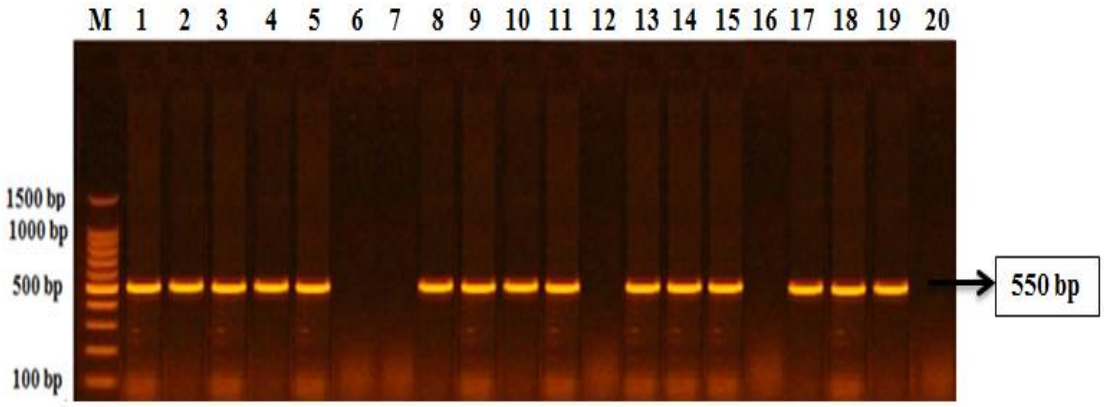

Figure (3-14): Electrophoresis of *bla* $_{NDM}$ (550 bp). Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (1,2,3,4,5,8,9,10,11,13,14,15,17,18, and 19) show positive result (550-bp). Lines (6,7,12,16 and 20) show negative results.

3.4.4 Molecular detection of Class C (*Ampc*) Serine *β*-lactamase Production:-

The presence of serine-*β*-lactamase enzymes in carbapenem resistant *E. coli* isolates can be observed through conventional PCR techniques. Detection focused on genes encoding 5 distinct types of these enzymes, facilitated by their respective primers (mentioned in Table 2-7). The results of the molecular detection revealed a considerable frequency of these enzymes among the isolates under investigation. Among the identified genes which is belong to the Class C (*Ampc*) including: [***bla*** $_{ADC}$ (445 bp), ***bla*** $_{DHA}$ (405 bp) and ***bla*** $_{ECB}$ (302 bp)].

3.4.4.1 Molecular Detection of *bla* $_{ADC}$ (445 bp):-

The genes of bla_{ADC} (445bp) have been identified by utilizing conventional PCR. Results revealed that only 3 out of the 20 total isolates of *E. coli* carried bla_{ADC} (15%), as illustrated in Figure (3.15).

The current study showed only 3 isolates positive to the bla_{ADC} and only 17 isolates showed negative results that is possibly due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to ADC may be due to a resistance mechanism other than the production of ADC.

These findings exhibit partial alignment with those of Rima *et al*. (2021) and their colleagues, who observed that approximately 93% of isolates within the Enterobacteriaceae family exhibited the presence of the bla_{ADC} enzyme, suggestive of resistance to various antibiotics (Rima *et al.*, 2021).

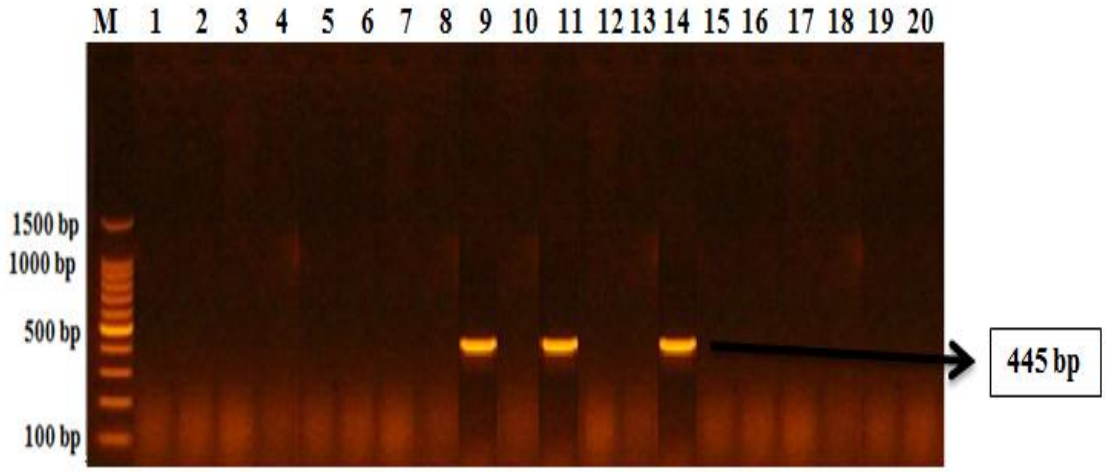

Figure (3-15): Electrophoresis of *bla* $_{ADC}$ (445 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (9,11 and 14) show positive result (445-bp). Lines (1,2,3,4,5,6,7,8,10,12,13,15,16,17,18,19and 20) show negative results.

3.4.4.2 Molecular Detection of *bla* $_{DHA}$ (405 bp) :-

The gene of bla_{DHA} (405 bp) have been identified utilizing conventional PCR. Results revealed that only 19 of the 20 total isolates of *E. coli* carried bla_{DHA} (95%) as illustrated in Figure (3.16).

The current study showed only 19 isolates positive to the bla_{DHA} and only 1 isolates showed negative results that is possibly due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to DHA may be due to a resistance mechanism other than the production of DHA.

Remarkably, this study marks the initial documentation of the presence of the bla_{DHA} gene in the Babylon province. The findings of this research closely correlate with a previous investigation that unveiled a noteworthy presence of the bla_{DHA-1} gene among *AmpC* β-lactamase producers, accounting for 5 isolates at a rate of 27.8%. Importantly, this study signifies the inaugural recognition of the bla_{DHA} gene in the Najaf region (Abeer *et al.*,2013). Conforming to the observations by (Mohamudha *et al*., 2012). DHA group genes exhibited a dominant presence in *E. coli*, with 24 out of 60 isolates (40%) carrying these genes (Mohamudha *et al.*, 2012).

In a recent study from Iran, were bla_{DHA}, and bla_{ECB} found in uropathogenic *E. coli* isolates were detected in 73.6%, 10.5%, 10.5%, and 15.8% of *E. coli* isolates, respectively (Dolatyar *et al.*, 2021). Currently, the clinical microbiology laboratories do not have a reliable method of detecting and confirming *AmpC* β-lactamases. For this reason, the molecular characterization of β-lactamases, in particular the *AmpC* family, is of crucial importance in in terms of molecular epidemiology and genetic contributing factors (Dolatyar *et al.*, 2021).

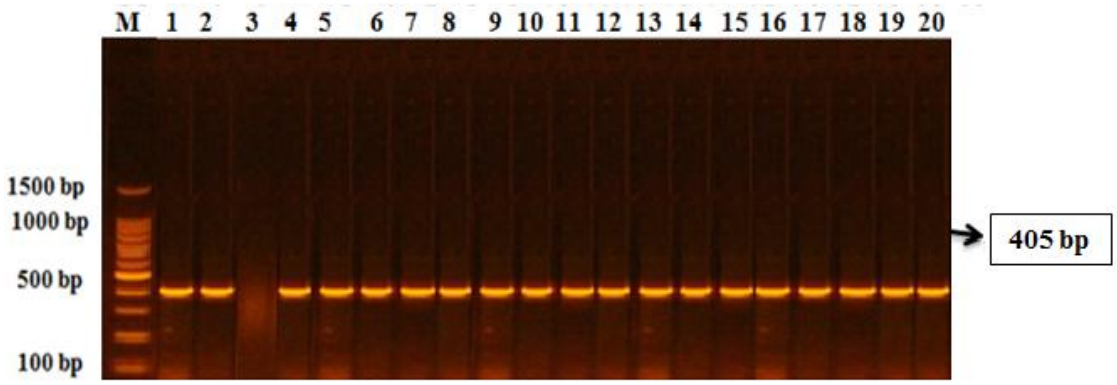

Figure (3-16): Electrophoresis of *bla* $_{DHA}$ (405 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (1,2,4,5,6,7,8,9,10,11,12,13, 14,15,16,17,18,19 and 20) that show positive result whereas lines (3) show negative results.

3.4.4.3 Molecular Detection of *bla* $_{ECB}$ (302 bp):-

The gene of bla_{ECB} (302 bp) have been identified utilizing conventional PCR. Results revealed that only 8 of the 20 total isolates of *E. coli* carried bla_{ECB} (40%), as illustrated in Figure (3.17).

The current study showed only 8 isolates positive to the bla_{ECB} and only 12 isolates showed negative results that is due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by use efflux pumps. Thus, non-susceptibility to ECB is may be due to a resistance mechanism other than the production of ECB.

This result is concordant with research conducted by Sadeghi *et al*. in Iran, where they reported bla_{ECB} (8.3%) (Sadeghi *et al.*,2022). Moreover, a study conducted in Najaf Hospitals detected a prevalence of bla_{ECB} in 50% of isolates (Al-jubouri *et al.*,2014). Variability was also observed in Turkey, About 3.03% of *E. coli* isolates that carrying the ECB gene. Also the results of this study agreed with study carried out by some researchers were the found out only 5 isolates that encoding bla_{ECB} genes among the rest of *E. coli* isolates (Helmy *et al.*,2014).

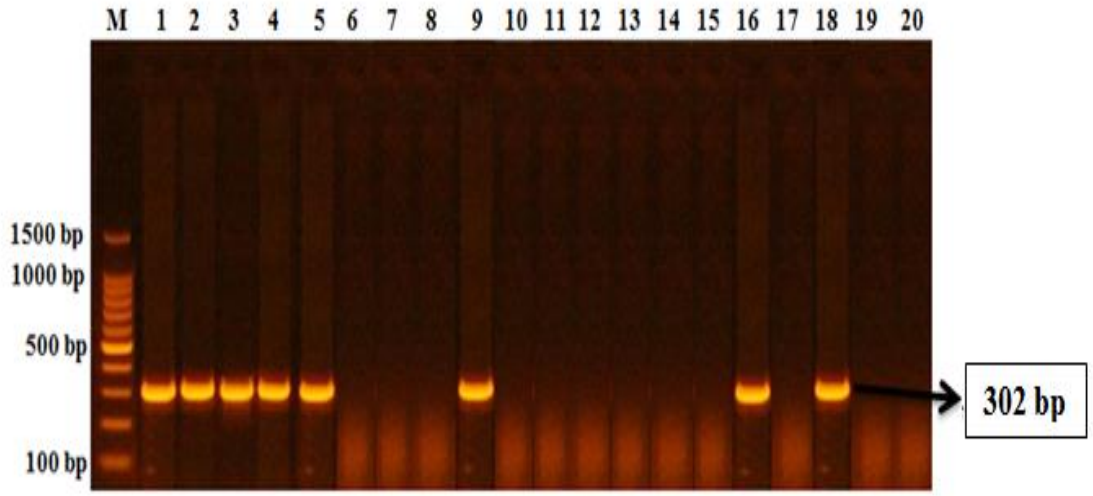

Figure (3-17): Electrophoresis of *bla* $_{ECB}$ (302 bp). Utilizing 1% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the Lines (1,2,3,4,5,9,16 and 18) show positive result (302-bp). Lines (6,7,8,10,11,12,13,14,15,17 ,19 and 20) showed negative results.

3.4.5 Molecular Detection of Class D Serine *β*-lactamase Production

The Presence serine-*β*-lactamase enzymes in carbapenem resistant *E. coli* isolates can be observed through conventional PCR techniques. Detection focused on genes encoding five distinct types of these enzymes, facilitated by their respective primers (mentioned in Table 2-7). The results of the molecular detection revealed a considerable frequency of these enzymes among the isolates under investigation. Among the identified genes, [(Carbapenemase ***bla*** $_{OXA}$ **(**564 bp)].

3.4.5.1 Molecular Detection of *bla* $_{OXA}$ (564 bp):-

The gene of bla_{OXA} (564 bp) have been identified utilizing conventional PCR. Results revealed that only 6 of the 20 total isolates of *E. coli* carried bla_{OXA} (30%) as illustrated in Figure (3.18).

The current study showed only 6 isolates positive to the ***bla*** $_{OXA}$ and only 14 isolates showed negative results that is due to their ability to resist the antibiotics by various mechanisms such alter the target's position or by

use efflux pumps. Thus, non-susceptibility to OXA may be due to a resistance mechanism other than the production of OXA.

Noteworthy, diverse subtypes of OXA-1 have been reported in *E. coli* isolates from various global regions (Alizade *et al.*, 2015).

In the current study, all OXA-type ESBLs detected were found to coexist with CTX-M-1. This observation is consistent with similar findings in Asia and Africa, where OXA-type ESBLs and CTX-M-1 co-occurrence were documented (Park *et al.*, 2012; Al-Agamy *et al.*, 2014).

Comparatively, the outcomes of our present study closely parallel those of a study conducted in Egypt, which detected the bla_{OXA-1} gene in 3 out of 50 UPEC isolates, corresponding to a prevalence of 6% (Mazzariol *et al.*, 2017).

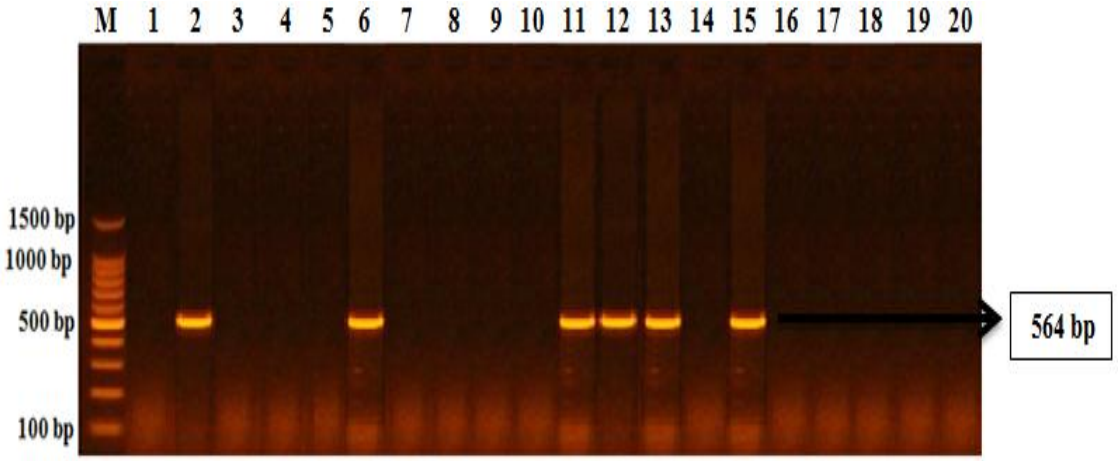

Figure (3-18): Electrophoresis of *bla* $_{OXA-1}$ 564 bp. Utilizing 1.5% agarose gel at 90V for 60 min. in 1x TBE buffer, and visualized under transilluminator UV after staining by red safe. Lane M: 100-1500 bp DNA ladder. All of the lines (2,6,11,12,13 and 15) show positive result (564-bp). All of the lines (1,3,4,5,7,8,9,10,14,16,17,18,19 and 20) show negative results.

The result of molecular detection among the carbapenem-resistant *E. coli* isolates (20, accounting for 62.5%) revealed that positive results for specific genes. Notably, the $bla_{CTX-M-1}$ and bla_{DHA} genes were the most prevalent (95%), followed by bla_{TEM} (80%), bla_{NDM} (75%), bla_{IMP} (60%), bla_{VIM} (50%), bla_{ECB} (40%), bla_{PER} (35%), bla_{OXA} & bla_{VEB} (30%), bla_{SHV}

(25%), and bla_{ADC} (15%). Importantly, no PCR-amplification products were observed for the bla_{GES} and bla_{SME} genes, as depicted in Figure (3.19).

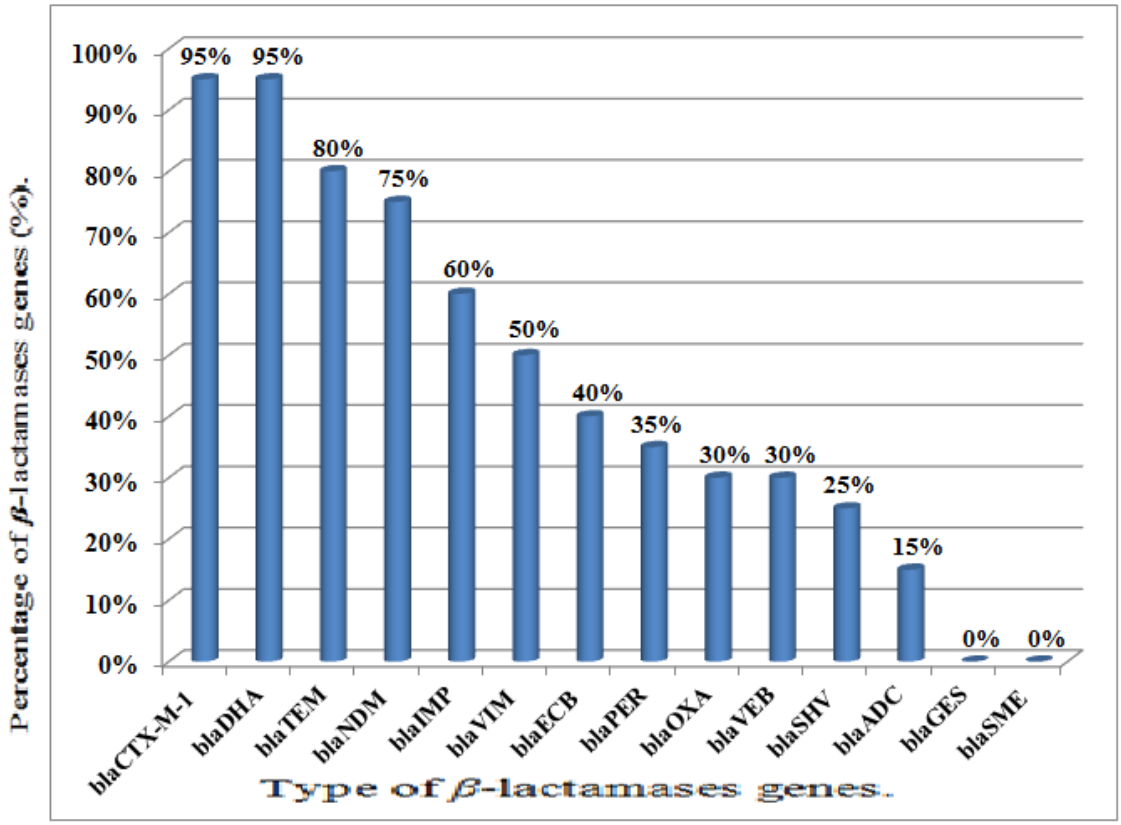

Figure (3-19): *β*-lactamases Genes Distribution Used in This Study among n=20 Carbapenms Resistance *E. coli* Isolates.

3.5 Distribution of *β*-lactamases Resistance Genes among *E. coli* Isolates.

The present study findings investigated the presence of *β*-lactamase genes, which included genes like ESBLS enzymes that including:- bla_{TEM}, bla_{SHV} and $bla_{CTX\text{-}M\text{-}1}$, bla_{PER} and bla_{VEB}, while carbapenemase enzymes including bla_{GES} and bla_{SME} that all of which fall within class (A). Genes like bla_{IMP}, bla_{VIM}, and bla_{NDM} were also identified in class (B). The study protocol also encompassed the examination of broad-spectrum Ampc genes, such as bla_{ECB}, bla_{DHA}, and bla_{ADC} was assigned to class (C). At the same time, $bla_{OXA\text{-}1}$ was assigned to class (D).

The molecular investigations revealed the genetic composition of various among *E. coli* isolates. For instance, **Eco-1** isolate contained a total of **7** distinct genes encoding enzymes that including bla_{TEM} , $bla_{CTX\text{-}M\text{-}1}$ and bla_{VEB} that belong to class (A) bla_{IMP} and bla_{NDM} class (B) as well as other genes like bla_{DHA} and bla_{ECB} (class C). These genes may be responsible to the multidrug resistance observed among these isolates.

In a similar results, **Eco-2** isolate exhibited a more complex genetic composition, with **9** different genes encoding enzymes such as bla_{TEM} , $bla_{CTX\text{-}M\text{-}1}$ and bla_{PER} that belong to class (A), also bla_{IMP}, bla_{NDM}, and bla_{VIM} class (B), alongside, bla_{DHA} and bla_{ECB} class (C) and other gene like bla_{OXA} class (D) that known for their significant roles in causing multidrug resistance among *E. coli* isolates.

Eco-3 isolate was found harbored for **9** distinct genes that encode enzymes. Specifically, it carried the $bla_{TEM,}$ bla_{SHV}, $bla_{CTX\text{-}M\text{-}1,}$ bla_{PER} and bla_{VEB} genes that associated with class (A) as well as $bla_{IMP,}$ bla_{VIM} and bla_{NDM} genes that classified under class (B). Furthermore, other genes were identified within this isolate, including only bla_{ECB} gene that classified under class (C) which plays a significant role in developing multidrug resistance in *E. coli* isolates.

Eco-4 isolate was found harbored for **7** distinct genes that encode enzymes. Specifically it carried the $bla_{TEM,}$ $bla_{CTX\text{-}M\text{-}1}$ and bla_{PER} associated with class (A). Also bla_{VIM} and bla_{NDM} genes that classified under class (B). Furthermore other genes were identified within this isolate including bla_{ECB} and bla_{DHA} genes classified under class (C), which plays a significant role in formation multidrug resistance in *E. coli* isolates.

Eco-5 isolate was found harbored for **8** distinct genes that encode enzymes. Specifically, it carried the $bla_{TEM,}$ $bla_{CTX\text{-}M\text{-}1}$ and bla_{PER} genes, associated with class (A). Also $bla_{IMP,}$ bla_{VIM} and bla_{NDM} genes classified under class (B). Furthermore other genes were identified within this isolate

including bla_{ECB} and bla_{DHA} genes classified under class (C), which plays a significant role in formation of multidrug resistance in *E. coli* isolates.

Eco-6 isolate was found harbored for **5** distinct genes that encode enzymes. Specifically, it carried the bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes, associated with class (A). Also bla_{IMP} gene that classified under class (B). While bla_{DHA} gene classified under class (C). Furthermore other gene were identified within this isolate, including bla_{OXA} gene that classified under class (D) which plays a significant role in formation multidrug resistance in *E. coli* isolates.

Eco-7 isolate was found harbored for **5** distinct genes that encode enzymes. Specifically, it carried the bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes, associated with class (A). Also bla_{IMP} and bla_{VIM} genes classified under class (B). Furthermore other gene were identified within this isolate, including bla_{DHA} gene classified under class (C), which plays a significant role in formation multidrug resistance in *E. coli* isolates.

Eco-8 isolate was found harbored for **5** distinct genes that encode enzymes. Specifically, it carried the $bla_{CTX\text{-}M\text{-}1}$ gene that associated with class (A). Also the bla_{IMP} , bla_{NDM} and bla_{VIM} genes, classified under class (B). Furthermore, other gene were identified within this isolate including bla_{DHA} gene classified under class (C), which plays a significant role in formation multidrug resistance in *E. coli* isolates.

Eco-9 isolate was found harbored for **10** distinct genes that encode enzymes. Specifically, it carried the following genes $bla_{TEM,}$ bla_{SHV}, $bla_{CTX\text{-}M\text{-}1,}$ bla_{PER} and bla_{VEB} that associated with class (A). Also bla_{IMP} and bla_{NDM} genes, classified under class (B). Furthermore, other genes were identified within this isolate, including $bla_{DHA,}$ bla_{ADC} and bla_{ECB} genes classified under class (C), which plays a significant role in formation multidrug resistance in *E. coli* isolates.

Eco-10 isolate was found to host **3** distinct genes that encode enzymes. Specifically, it carried the $bla_{CTX\text{-}M\text{-}1}$ gene, classified under class (A). Also bla_{NDM} gene, associated with class (B). Furthermore, other gene were identified within this isolate, including bla_{DHA} gene classified under class (C) , which plays a significant role in developing multidrug resistance in *E. coli* isolates.

Eco-11 isolate displayed a more intricate genetic makeup, encompassing **9** genes encoding enzymes. Notably, it harbored of the following genes that including $bla_{TEM,}$ bla_{SHV}, $bla_{CTX\text{-}M\text{-}1,}$ bla_{PER} and bla_{VEB} that classified under class (A). Also others gene like bla_{NDM} gene within class (B). Alongside these, the isolate carried genes such as bla_{ADC} and bla_{DHA} genes within class(C). As well as the bla_{OXA} gene within class (D), that contributing to the emergence of MDR among *E. coli* isolates.

Eco-12 isolate, a combination of **6** genes was detected, including bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes within class (A). Also bla_{IMP} and bla_{VIM} genes within class (B). While bla_{DHA} gene within class (C). Moreover, the bla_{OXA} gene within class (D) that known for its involvement in multidrug resistance among *E. coli* isolates , was also observed.

Eco-13 isolates exhibited the presence of **6** different genes encoding enzymes. Among these were bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes within class (A). Also bla_{IMP} and bla_{NDM} genes within class (B). As well as bla_{DHA} gene within class (C). Additionally, the isolate contained the bla_{OXA} gene within class (D), that contributing to its multidrug resistance profile among *E. coli* isolates.

Eco-14 isolates genetic composition included **6** genes encoding enzymes. The bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$ and bla_{ECB} genes within class (A) was noted, along with bla_{NDM} gene within class (B), and other genes like bla_{ADC} and bla_{DHA} genes within class (C), collectively responsible for the emergence of multidrug resistance in *E. coli* isolates .

Eco-15 isolate possessed **7** genes that encoded enzymes, including bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes within class (A). While bla_{IMP}, bla_{VIM} and bla_{NDM} genes within class (B), as well as the bla_{DHA} gene within class (C). Furthermore, the presence of bla_{OXA} gene within class (D) that contributed to the isolates of multidrug resistance characteristics.

Eco-16 isolates carried **3** genes encoding enzymes, including bla_{TEM} gene within class (A), and additional genes like bla_{DHA} and bla_{ECB} within class (C), which have been linked to multidrug resistance in *E. coli* isolates.

Eco-17 isolates displayed **5** genes encoding enzymes, including the bla_{TEM} $bla_{CTX\text{-}M\text{-}1}$ and bla_{PER} genes within class (A) and gene like bla_{NDM} within class (B). Similarly, gene such as bla_{DHA} within class (C) that contributed among *E. coli* isolate's that responsible of multidrug resistance characteristics.

Eco-18 isolate was characterized by the presence of **9** different genes encoding enzymes. Noteworthy among these were $bla_{TEM,}$ bla_{SHV}, $bla_{CTX\text{-}M\text{-}1}$ and bla_{VEB} genes within class (A). While bla_{IMP}, bla_{VIM}, and bla_{NDM} genes within class (B). Genes like bla_{DHA} and bla_{ECB} genes within class (C) that contributed among *E. coli* isolates which responsible of multidrug resistance profile.

Eco-19 isolate exhibited **5** genes encoding enzymes, encompassing bla_{TEM} and $bla_{CTX\text{-}M\text{-}1}$ genes within class (A). Also bla_{VIM} and bla_{NDM} genes within class (B). While the presence of the bla_{DHA} gene was within class (C) that contributing among *E. coli* isolates and responsible of multidrug resistance attributes.

Finally **Eco-20** isolate carried only **2** genes encoding enzymes, specifically the $bla_{CTX\text{-}M\text{-}1}$ gene within class (A), and the presence of the bla_{DHA} gene within class (C), contributing among *E. coli* isolates that responsible of multidrug resistance properties.

The study's results indicated that most isolates contained multiple genes. Notably, isolates E.co-9 consisted the highest number of genes, totaling 10. Similarly, the following isolates (E.co-2, E.co-3, E.co-11 and E.co-18) were carried 9 different genes for each one. Finally the isolate number 20 was carried the lowest numbers of genes which including only 2 genes.

Furthermore, the co-production of these enzymes was observed across all 20 isolates, suggesting a complex interplay. The most prevalent combination was $bla_{CTX\text{-}M\text{-}1}$ and bla_{DHA}, accounting for 95% of cases among the 20 isolates. Refer to Table (3-6) for a detailed overview.

The outcomes of this study indicate a level of complexity that exceeds initial expectations. The findings underscore that the situation is multifaceted. Specifically, the study results revealed that all 20 isolates were classified under class C (43\280) and class A (40\280) followed by class B (37\280) and finally class D showed lowest genes that including (6\280) according to the Ambler classification. Intriguingly, these isolates exhibited not just singular gene presence but rather combinations of genes alongside their extend-spectrum *β*-lactamase synthesis. Notably, all carbapenemase-producing isolates (100%) demonstrated a multidrug-resistant (MDR) profile.

In conclusion, the Ambler classification system provides insights into the diversity of *β*-lactamase enzymes, and the study's molecular investigations shed light on the genetic complexity underlying multidrug resistance among *E. coli* isolates. Several genes from several classes demonstrate the complexity of antimicrobial resistance pathways. By identifying these genes and their associations, the study contributes to a better understanding of resistance patterns, thereby opening the way for more informed approaches to combat multidrug-resistant infections.

Table (3-6) Distribution of *β*-lactamase Genes among *E. coli* Isolates.

Symbol of isolates	*β*-lactamases distributions genes among carbapenms resistance *E. coli* isolates														Total of present genes	Total of missing genes
	A							B			C			D		
	Carba		ESBLS													
	bla GES	*bla* SME	*bla* TEM	*bla* SHV	*bla* CTX-M-1	*bla* PER	*bla* VEB	*bla* IMP	*bla* NDM	*bla* VIM	*bla* ADC	*bla* DHA	*bla* ECB	*bla* OXA		
E.co-1	-	-	+	-	+	-	+	+	+	-	-	+	+	-	7	7
E.co-2	-	-	+	-	+	+	-	+	+	+	-	+	+	+	9	5
E.co-3	-	-	+	+	+	+	+	+	+	+	-	-	+	-	9	5
E.co-4	-	-	+	-	+	+	-	-	+	+	-	+	+	-	7	7
E.co-5	-	-	+	-	+	+	-	+	+	+	-	+	+	-	8	6
E.co-6	-	-	+	-	+	-	-	+	-	-	-	+	-	+	5	9
E.co-7	-	-	+	-	+	-	-	+	-	+	-	+	-	-	5	9
E.co-8	-	-	-	-	+	-	-	+	+	+	-	+	-	-	5	9
E.co-9	-	-	+	+	+	+	+	+	+	-	+	+	+	-	10	4
E.co-10	-	-	-	-	+	-	-	-	+	-	-	+	-	-	3	11
E.co-11	-	-	+	+	+	+	+	-	+	-	+	+	-	+	9	5
E.co-12	-	-	+	-	+	-	-	+	-	+	-	+	-	+	6	8
E.co-13	-	-	+	-	+	-	-	+	+	-	-	+	-	+	6	8
E.co-14	-	-	-	+	+	-	+	-	+	-	+	+	-	-	6	8
E.co-15	-	-	+	-	+	-	-	+	+	+	-	+	-	+	7	7
E.co-16	-	-	+	-	-	-	-	-	-	-	-	+	+	-	3	11
E.co-17	-	-	+	-	+	+	-	-	+	-	-	+	-	-	5	9
E.co-18	-	-	+	+	+	-	+	+	+	+	-	+	+	-	9	5
E.co-19	-	-	+	-	+	-	-	-	+	+	-	+	-	-	5	9
E.co-20	-	-	-	-	+	-	-	-	-	-	-	+	-	-	2	12
Total positive isolates	0% 0	0% 0	80% 16	25% 5	95% 19	35% 7	30% 6	60% 12	75% 15	50% 10	15% 3	95% 19	40% 8	30% 6	126	154
Total negative isolates	100% 20	100% 20	20% 4	75% 15	5% 1	65% 13	70% 14	40% 8	25% 5	50% 10	85% 17	5% 1	60% 12	70% 14	280	

It is evident that multidrug resistance does not stem from a specific set of genes but rather arises from the intricate combination of various genes, particularly Broad-spectrum ***β***-lactamase synthesis (Nikaido., 2009).

Several studies highlight the significance of carbapenemase-producing Enterobacteriaceae in the context of drug resistance, with some

reporting higher resistance rates than other multidrug-resistant (MDR) Enterobacteriaceae. The production of these enzymes emerges as a principal contributor to drug resistance within this bacterial family (Jain *et al.*,2021). In order to improve the effectiveness of therapy, Jain *et al*. concluded that the development of antibiotic resistance caused by *β*-lactamases is a significant problem in urinary tract infections (UTIs). They emphasized the need for frequent surveillance of these *β*-lactamases. The World Health Organization emphasizes the essential importance of developing new medicines to combat bacteria that produce ESBLs and are carbapenem-resistant (Martinez ., 2021).

A recent study conducted in Iraq by Mhawesh *et al.* (2021) identified carbapenemase resistance in a subset of isolates. A few isolates demonstrated genotypic positivity for carbapenemase, notably bla_{OXA}-48 and bla_{IMP}, coexisting in three isolates. While several investigations revealed the prevalence OXA-1 in *E. coli* strain, notably B_2 strain (Ssekatawa *et al.,* 2021). Studies conducted in Iraq and elsewhere have showed existence several carbapenemase‒genes in *E. coli* (Hojabri *et al.,* 2019; Al-Sa'ady *et al.,* 2020). Other researchers noted such discrepancies, attributing the challenge of detecting carbapenemase-producing *E. coli* to low carbapenem minimum inhibitory concentrations (MICs) that can fall within the susceptibility range (Gauthier *et al.*,2018). The suggestion to employ ertapenem disc diffusion for phenotypic detection and the poor hydrolytic activity of OXA-48 enzymes towards carbapenems in particular were noted (Oueslati *et al.,* 2015; Ortega *et al.,* 2016).

Notably, a Spanish study found that the development of carbapenemase-producing *E. coli* followed a rise in resistant against Fluoroquinolones and 3^{d}-generation‒cephalosporin's (Ortega *et al.,* 2016). Diverse production carbapenemase isolates of *E. coli* poses significant challenge, particularly in UTI treatment (Shaik *et al.*,2017). Since most *β*-

lactam drugs may be hydrolyzed by carbapenemase and since carbapenem-resistant Enterobacteriaceae frequently display other ways that some medicines are resistant that including (Fluoroquinolones as well as aminoglycosides) implications are noteworthy from a clinical perspective (Moxon and Paulus ., 2016). Furthermore, indiscriminate carbapenem use may exacerbate the emergence of resistance and give rise to substantial therapeutic issues in the future. Preventing the selection of these extremely resistant organisms and their dissemination requires effective infection control and preventive measures, such as antibiotic rotation (Rawat and Nair ., 2010). The globally fear about this issue is highlighted by the transfer of OXA-1-producing Enterobacteriaceae from Middle Eastern countries to other regions of the world (Halat and Ayoub .,2020).

According to Amblar classification the results of molecular investigation of this study showed among 280 *β*-lactamase genes has been detected during this study that showed positive results for 126 (45%) genes whereas the rest of the genes totally 156 (55%) genes showed negative result .

The molecular characteristics of 20 carbapenms resistance *E. coli* isolates showed all of the genes belong to class A within n=53(42.1%) *β*-lactamase out of 126 positive genes followed by class B within n=37 (29.3%) *β*-lactamase genes, while class c was n=30 (23.8%) and finally class D showed the lowest percentage levels among *β*-lactamase genes that detected during this study it was n=6 (4.8%) as indicated in the figure (3.20) There may be a rapid spread of *E. coli*, especially in hospital settings where various antimicrobials are largely used and thus may support the dissemination of these microorganisms.

The rest of 156 *β*-lactamase genes that showed negative result may be is due to the *E. coli* isolates has lost the resistance feature for

carbapenms resistance genes phenotypically and genotypically due to the plasmid curing by serial sub-culturing and incubation in a high temperature and DNA extractions. So this observation showed that carbapenms resistance genes can be carried on plasmid but not on chromosome.

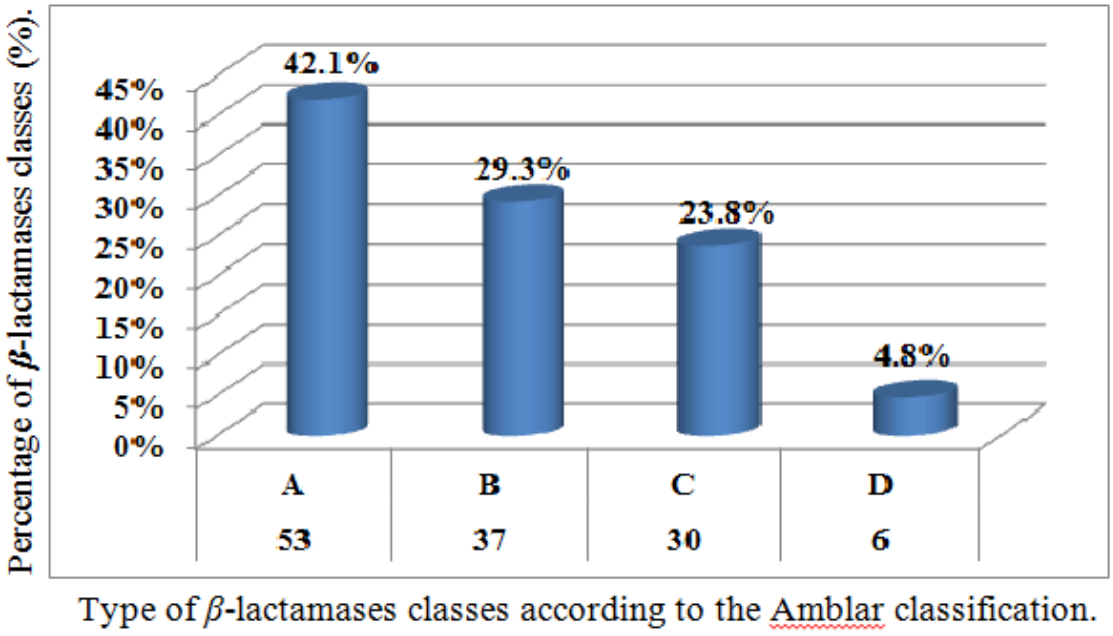

Figure (3-20): Distribution *β*-lactamase Genes among *E. coli* Isolates According to Amblar Classification.

3.6 Detection *bla* $_{IMP}$ By Using Real Time PCR:-

The current results of real time PCR for this study to detection presence ***bla*** $_{IMP}$ among 20 carbapenms resistance *E. coli* isolates that showed only 12 isolates harboring for ***bla*** $_{IMP}$ as the following according to melting curve:-Ct-IPM-**1**=34.3, Ct-IPM-**2**=30.65, Ct-IPM-**3**=36.03, Ct IPM-**5**=34.76, Ct-IPM-**6**=40.64, Ct-IPM-**7**=34.55, Ct-IPM-**8**=37.63, Ct IPM-**9**=36.99, Ct-IPM-**12**=34.30, Ct-IPM-**13**=36.03, Ct-IPM-**15**=38.98 and finally Ct-IPM-**18**=36.72. While the others isolates were showed negative results as the following :- IPM-**4**=No Ct, IPM-**10**=No Ct, IPM-**11**=NO Ct, IPM-**14**=No Ct, IPM-**16**=No CT, IPM-**17**=No Ct , IPM **19**=No Ct and finally IPM 20 showed also No Ct as detailed in the table (3.7). The negative results for those 8 isolates (4,10,11,14,16,17,19 and

20) is due to their possesses other virulence factors that enable it to resist imipenem, such as possessing efflux pumps, capsules, and other means of resistance.

Table (3-7): Melting Curve of Rt PCR for IMP Detection.

Well		Sample name	Sample type	Dye	Gene	Ct	Mean Ct
A1		2 IMP	Unknown	FAM		30.65	30.65
A2		4 IMP	Unknown	FAM		No Ct	
A3		5 IMP	Unknown	FAM		34.76	34.76
A4		6 IMP	Unknown	FAM		40.64	40.64
A5		7 INP	Unknown	FAM		34.55	34.55
A6		8 IMP	Unknown	FAM		37.63	37.63
A7		9 IMP	Unknown	FAM		36.66	36.66
A8		10 IMP	Unknown	FAM		No Ct	
A9		11 IMP	Unknown	FAM		No Ct	
B1		12 IMP	Unknown	FAM		34.3	34.3
B2		13 IMP	Unknown	FAM		36.03	36.03
B3		14 IMP	Unknown	FAM		No Ct	
B4		15 IMP	Unknown	FAM		38.96	38.96
B5		16IMP	Unknown	FAM		No Ct	
B6		17IMP	Unknown	FAM		No Ct	
B7		18IMP	Unknown	FAM		36.72	36.72
B8		19IMP	Unknown	FAM		No Ct	
B9		20IMP	Unknown	FAM		No Ct	
B10		1 IMP	Unknown	FAM		34.3	34.3
B11		3 IMP	Unknown	FAM		36.03	36.03

Ct=Cycle threshold line.

The real-time results were consistent with the results of conventional PCR .

Real-time PCR was used as a prior step in the gene expression process, as well as a confirmation step for previous tests. The results are interpreted by the real-time PCR instrument software by the crossing or not crossing of the threshold line by the fluorescence curve (in the middle of the linear section of the fluorescence curve for the positive control (C+) in logarithmic coordinates). The result of amplification is considered positive if the fluorescence curve is characteristic of real-time PCR (S-shaped) and crosses the threshold line once in the significant fluorescence increase section and if the Ct value detected in the channel is below the

threshold value specified in the below table. The result of amplification is considered negative if the fluorescence curve is not S shaped and if it does not cross the threshold line (the Ct value is absent) as detailed in the figure (3.21).

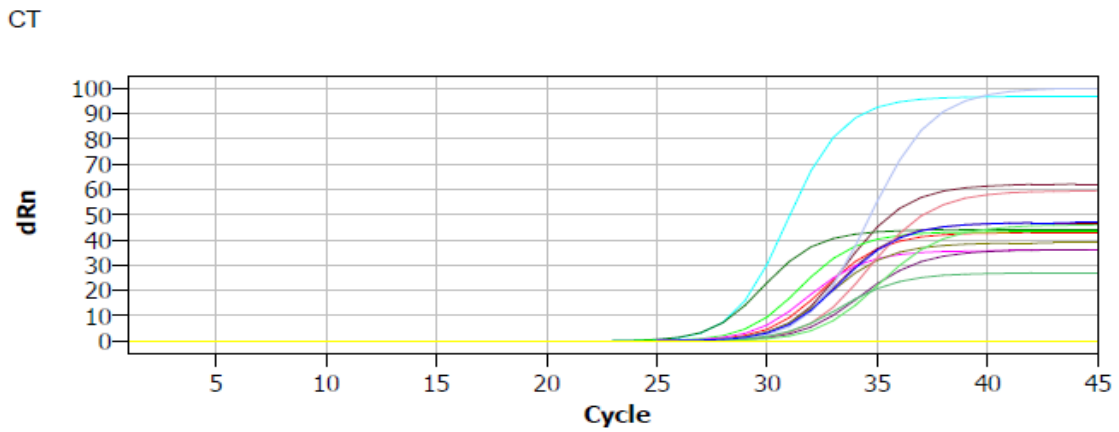

Figure (3-21): Real Time PCR Result for Detection *bla*$_{IMP}$ Genes among 20 Carbapenms Resistance *E. coli* Isolates

Several multiplex real-time PCR methods, which further reduce the detection time for carbapenemase genes, were developed by research groups at the same time. Real-time PCR methods can be followed by a melting curve step, allowing the accurate differentiation of carbapenemase gene variants. In 2007, Mendes *et al*. who described the first multiplex real-time PCR assay for the detection of genes encoding MBL-type enzymes (IMP and VIM types, SPM-1, SIM-1, and GIM-1) identified up to that point (Mendes *et al.*, 2007). The MBL identification was based on the characteristic amplicon melting peak. In a study published a few months later, the specific detection of *bla*$_{VIM}$ and *bla*$_{IMP}$ genes in Gram-negative bacteria was achieved in <1 hr with a real-time PCR assay (Bisiklis *et al.*,2007). The authors showed that melting curve analysis of the real-time PCR products clearly differentiated the genes into four groups: (i) *bla*$_{VIM-1}$-like; (ii) *bla*$_{VIM-2}$-like; (iii) *bla*$_{IMP-1}$-like; and (iv) blaIMP-2-like. In 2011, Chen *et al.* (2011) developed a multiplex real-time PCR scheme that could identify *bla*$_{KPC}$ gene variants (Chen *et*

al.,2011). In contrast to other PCR-based methods for the detection of bla_{KPC} genes, such as two real-time PCR assays (Cole *et al.*,2009) and a method that uses PCR/electrospray ionization–MS (Endimiani *et al.*,2010) , the described protocol made use of the technology of molecular beacon probes, which are able to detect the presence of single-nucleotide polymorphisms. Thus, the described real time PCR could distinguish between different bla_{KPC} variants (Chen *et al.*,2011) , and therefore provides information of both epidemiological and evolutionary significance. Later, Chen *et al*. (2012), using the technology of molecular beacon probes, designed a multiplex real-time PCR assay capable of identifying both the epidemic *K. pneumoniae* ST258 clone and bla_{KPC} carbapenemase genes in a single reaction (Chen *et al.*,2012). That assay showed excellent sensitivity (100%) and specificity (100%), and seemed to be a useful tool for screening of *K. pneumoniae* isolates and surveillance of the epidemic ST258 clone in both community and healthcare settings. Although the initial cost of investment for the molecular beacons is significant, it is offset by the reduced requirements for template quality, reaction volume, and time (Chen *et al.*,2009). Recently, Monteiro *et al*. (2012) who developed a single multiplex real-time PCR assay for the identification of the most common types of serine carbapenemase (KPC, GES, and OXA-48) and metallo-carbapenemase (IMP, VIM, and NDM), already described in enterobacterial isolates, using high-resolution melting curves (Monteiro *et al.*, 2012). The entire assay, including DNA extraction, sample preparation, a multiplex PCR run, and analysis of results, was performed in 3 hr. In the two studies that followed: (i) the performance of a real-time PCR for identification of bla_{VIM}-type and bla_{KPC} carbapenemase in an ultra-rapid single reaction was compared with the performance of other PCR-based methods (Frasson *et al.*, 2012) and (ii) a multiplex real-time PCR assay for the detection of

OXA-48, VIM, IMP, NDM and KPC carbapenemase genes was evaluated in a multi-center study (Van der Zee *et al*.,2014). Both the sensitivity and the specificity of all of the real-time protocols presented above were 100%. These results indicate that real-time PCR assays are robust, reliable and rapid protocols for the detection of the most prevalent carbapenemase genes (Van der Zee *et al.*, 2014).

Therefore, molecular methods should be used for screening for the presence of the carbapenemase genes, so that their epidemiological spread can be monitored (Van der Zee *et al.*, 2014).

3.7 Gene Expressions

Quantitative PCR (qPCR) stands out among gene expression analysis methods due to its accuracy, sensitivity, and rapid results. This technology has become the golden standard for gene expression assessment. This study involved a relative quantification, the primary interest lies in comparing the expression levels of specific genes across different samples. In this study, qRT-PCR was employed to measure the gene expression of IMP among *E. coli* isolates, which is responsible for beta-lactamase enzyme production. A housekeeping gene (H.K) served as a control. The findings exhibited a reduction in gene expression following treatment with antibiotics at the 0.25µg/mL minimum inhibitory concentration (MIC) of Imipenem (IMP), as presented in Tables (3.8 and 3.9).

3.8 Results of RT‑PCR

The impact of a 0.25 µg/mL MIC of Imipenem (IMP) on the expression of the bla_{IMP} gene was assessed by monitoring the expression within cultures of 9 isolates grown both with and without antibiotics, using *16S rRNA* as a normalization gene.

The average of cycle threshold (Ct) results for the gene expression (group-1) for *16sRNA* and isolates DNA extracted before antibiotics

treatment for the all isolates was (E.1=20.12, E.2=35.01, E.3=35.04, E.5=20.05, E.6=34.31, E.9=34.04, E.12=20.05, E.13=35.1 and E.15=35.5) while the average cycle threshold (Ct) results of the target gene (group-1) before antibiotics treatment for the all isolates was (E.1=18.51, E.2=30.45, E.3=33.53, E.5=18.51, E.6=30.45, E.9=33.53, E.12=18.51, E.13=31.12 and E.15= 33.53). The delta cycle threshold (dCT) for both 16sRNA and target gene before antibiotics treatment for the all isolates was (E.1=-1.61, E.2=-4.56, E.3=-1.51, E.5=-1.54, E.6=-3.86, E.9=-0.51, E.12=-1.54, E.13=-3.98 and E.15= -1.97). The delta-delta cycle threshold (ddCT) for both 16sRNA and target gene before antibiotics treatment for the all isolates it was (0). The Fold change for all of the isolates was 1 and the average fold change for all isolates it was (1) Table (3-8).

The average of cycle threshold (Ct) results for the gene expression (group-2) for 16sRNA with isolates RNA extracted before antibiotics treatment for the all isolates was (E.1=34.75, E.2=32.21, E.3=31.33, E.5=31.55, E.6=32.06, E.9=33.24, E.12=34.81, E.13=32.33 and E.15=32.01) while the average cycle threshold (Ct) results of the target gene (group-2) before antibiotics treatment for the all isolates was (E.1=31.55, E.2=26.64, E.3=25.66, E.5=26.05, E.6=27.11, E.9=30.03, E.12=31.11, E.13=26.35 and E.15= 28.41). The delta cycle threshold (dCT) for both 16sRNA and target gene before antibiotics treatment for the all isolates was (E.1=-3.2, E.2=-5.57, E.3=-5.67, E.5=-5.5, E.6=-4.95, E.9=-3.21, E.12=-3.7, E.13=-5.98 and E.15= -3.6). The delta-delta cycle threshold (ddCT) for both 16sRNA and target gene before antibiotics treatment for the all isolates it was (E.1=-0.8577778, E.2=-3.2277778, E.3=-3.3277778, E.5=-3.1577778, E.6=-2.6077778, E.9=-0.8677778, E.12=-1.3577778, E.13=-3.6377778 and E.15= -1.2577778). The Fold change for all of the isolates it was (E.1=1.81224471, E.2=9.368238324, E.3=10.04062923, E.5=8.924539802, E.6=6.095640321,

E.9=1.824849869, E.12=2.562901047, E.13=12.44744536 and E.15= 2.391271231), and finally the average fold change for all isolates it was (6.163084433).Table (3-9).

The data in Table 3.9 demonstrates fold changes ranging from 1.8 to 12.4 in the presence of Imipenem (IMP) inhibitors, in comparison to the control (no antibiotics), as determined by paired testing ($P < 0.05$). The exposure to 0.25 µg/mL MIC of antibiotics increased bla_{IMP} mRNA levels, ranging from 1.8-fold to 12.4-fold in the presence of Imipenem (IMP) inhibitors, varying with the isolates. Notably, isolate E.13 exhibited a more substantial enhancement in bla_{IMP} expression when exposed to Impenem (IMP) inhibitors at 0.25 µg/mL MIC compared to the other isolates. The average fold change before treatments was 1, while the average change following treatments was 6.163084433. These findings suggest that these isolates exhibit elevated gene expression compared to the control groups. The comprehensive results for the 9 isolates are summarized in tables 3-8 and 3-9 and further depicted in Figures (3-22 and 3-23).

Table (3-8): Melting Curve before Antibiotics Treatment.

Control	16sRNA average Ct	Target gene bla_{IMP} average Ct	dCT	ddCT	Fold change	Average fold change
E.1	20.12	18.51	-1.61	0	1	1
E.2	35.01	30.45	-4.56			
E.3	35.04	33.53	-1.51			
E.5	20.05	18.51	-1.54			
E.6	34.31	30.45	-3.86			
E.9	34.04	33.53	-0.51			
E.12	20.05	18.51	-1.54			
E.13	35.1	31.12	-3.98			
E.15	35.5	33.53	-1.97			
		Average	-2.3422222			

Table (3-9): Melting Curve after Antibiotics Treatment.

Treated	16sRNA average Ct	Target gene *bla*$_{IMP}$ average Ct	dCT	ddCT	Fold change	Average fold change
E.1	34.75	31.55	-3.2	-0.8577778	1.81224471	6.163084433
E.2	32.21	26.64	-5.57	-3.2277778	9.368238324	
E.3	31.33	25.66	-5.67	-3.3277778	10.04062923	
E.5	31.55	26.05	-5.5	-3.1577778	8.924539802	
E.6	32.06	27.11	-4.95	-2.6077778	6.095640321	
E.9	33.24	30.03	-3.21	-0.8677778	1.824849869	
E.12	34.81	31.11	-3.7	-1.3577778	2.562901047	
E.13	32.33	26.35	-5.98	-3.6377778	12.44744536	
E.15	32.01	28.41	-3.6	-1.2577778	2.391271231	

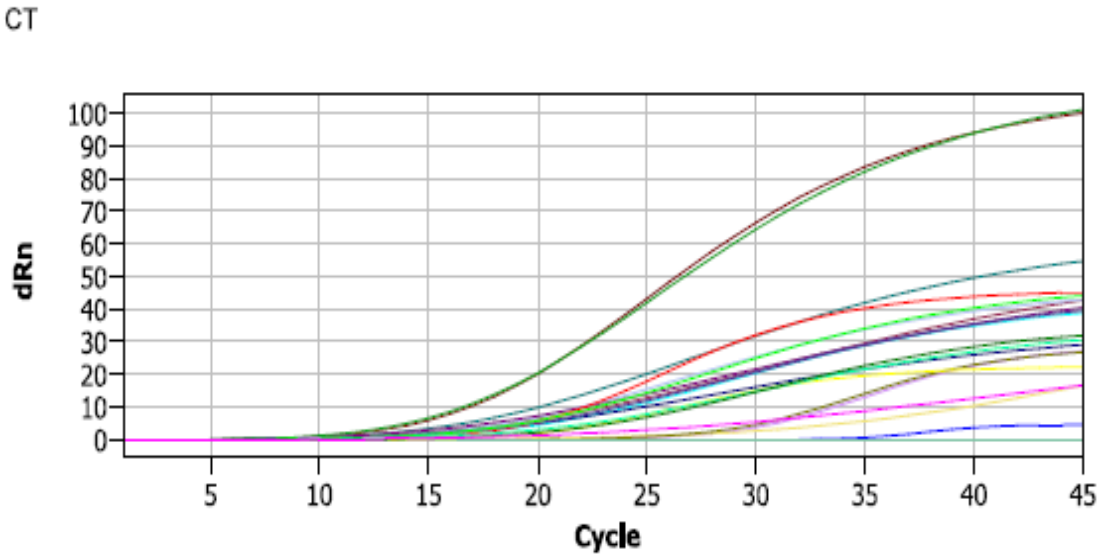

Figure (3-22): Gene Expressions of *bla*$_{IMP}$ Gene among Carbapenems Resistance *E. coli* Isolates

ΔRn :- is an increment of fluorescent signal at each time point. The ΔRn values are plotted versus the cycle number.

Threshold:- is an arbitrary level of fluorescence chosen on the basis of the baseline variability. A signal that is detected above the threshold is considered a real signal that can be used to define the threshold cycle (Ct) for a sample. Threshold can be adjusted for each experiment so that it is in the region of exponential amplification across all plots.

Ct :- is defined as the fractional PCR cycle number at which the reporter fluorescence is greater than the threshold. The Ct is a basic principle of real time PCR and is an essential component in producing accurate and reproducible data.

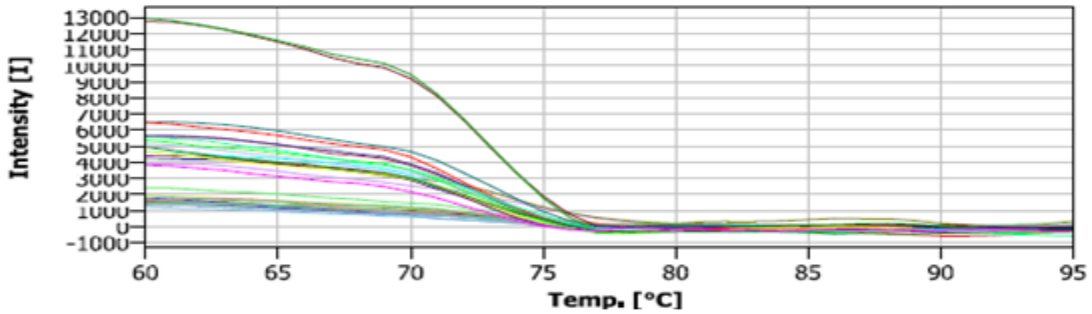

Figure (3-23): Melting Curve of *bla*$_{IMP}$ Gene among Carbapenems Resistance *E. coli* Isolates for 9 *E. coli* isolates

Escherichia coli that is resistant to IMP antibiotics and is known as carbapenem-resistant Enterobacteriaceae (CRE), which is the most worrisome, is a serious health problem. These isolates contribute to worldwide CRE outbreaks by being connected to common community and hospital-acquired illnesses (Hussein *et al.*,2017). Despite the fact that medical indicators of risk for CRE infections are largely established, bacterial risk factors, such as antibiotic sensitivity, resistance mechanisms, and biofilm development, have not been sufficiently investigated. Understanding bacterial characteristics such as the creation of biofilm is therefore essential for treating biofilm-associated illnesses brought on by CRE pathogens. These infections are concerning due to their adverse clinical outcomes and limited therapeutic interventions (Samarasinghe and Al-Bayati.,2022).

According to these results, the current study is consistent with a other studies which is including investigation that discovered that the stress-responsive gene *soxS* was up-regulated in *E. coli* IMP. This increased regulation is thought to protect bacteria against oxidative damage in response to growth circumstances (Samarasinghe and Al-Bayati.,2022). Similar findings were carried out by some of researchers

about the high-level up-regulation of bla_{IMP}, which is in charge of carbapenem resistance. This phenomenon might be linked to hyper-virulence/resistant capabilities exhibited by the strain during biofilm maturation. Such attributes might contribute to its successful dissemination across various infections (Zhang *et al.*,2018).

Conclusions and Recommendations

Conclusions and Recommendations:

Conclusions:

1-Through this study, it was observed that the infection rate of *E. coli* bacteria is higher than that of other bacteria among people with urinary tract infections.

1-Females represent 85.3% of the population and are more infected by UTI than males also age groups most effected between (30-60) years old. Antibiotic susceptibility test results showed highly prevalence resistance to Amoxicillin and Ampicillin and less resistance to Meropenem and Imipenem, while sensitivity test results showed a high prevalence to Meropenem and Imipenem and less sensitivity to Amoxicillin and Ampicillin.

3-All 20 *E. coli* isolates showed positive results for phenotype identification test that capable to produce β-lactamases and ESBLS .

Among the remaining genes included in this investigation, $bla_{CTX\text{-}M\text{-}1}$ and bla_{DHA} have a high prevalence distribution. Additionally No bla_{GES} or bla_{SME} PCR-amplification products were not observed.

4-According to the study's findings, the majority isolates included at least three genes . Isolates 9 in particular carried 10 distinct genes, while isolates (E.co-2, E.co-3, E.co-11 and E.co-18) each carried nine different genes, also the result of this study showed most of the *E. coli* isolates belonged to classes A and B followed by C as well as less frequency of class D.

5-In addition, gene expression in the E.13 isolate dramatically raised the levels of bla_{IMP} mRNA from 1.8 to 12.4 fold in the presence of imipenem (IMP) inhibitors in response to 0.25 g/mL imipenem (IMP) MIC.

Recommendations:

1. Reducing antibiotic uses and taken consulting a specialist that will reduce the chances of increasing antibiotic resistance.
2. An antimicrobial susceptibility test must be performed before prescribing any antibiotic.
3. The proper administration of antibiotics and avoiding overuse of medications will limit the bacteria's rising antibiotic resistance.
4. Investigating the genes of multiple antibiotic resistance factors that are important for the bacteria that cause urinary tract infections to determine the epidemiology of *E. coli* bacteria from a genetic standpoint using PCR technology at the molecular level and limit its spread.
5. Early detection of UTI is essential to avoid exacerbation of UTI.
6. Using modern techniques, such as q-RT-PCR, to investigate the genes responsible for MDR among UTI patients.

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Part 1:Patients' Questionnaire Form

Name :

Age :

Sex:

Address:

Part 2: Result of urine examination

Color :

Reaction :

Albumin :

Specific gravity :

Albumin :

Pus cell:

Ketone Bodies:

Red Blood Cell:

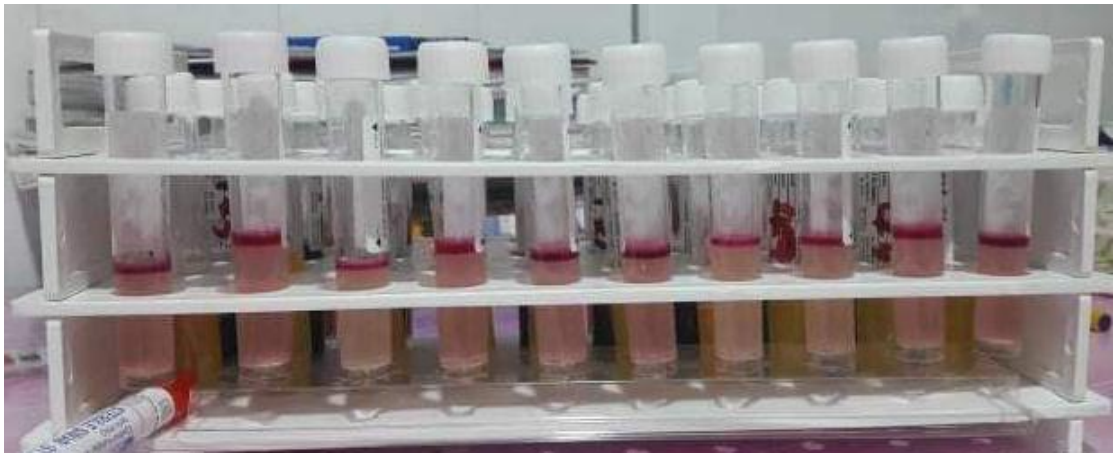
Epithelial cell:

Sugar:

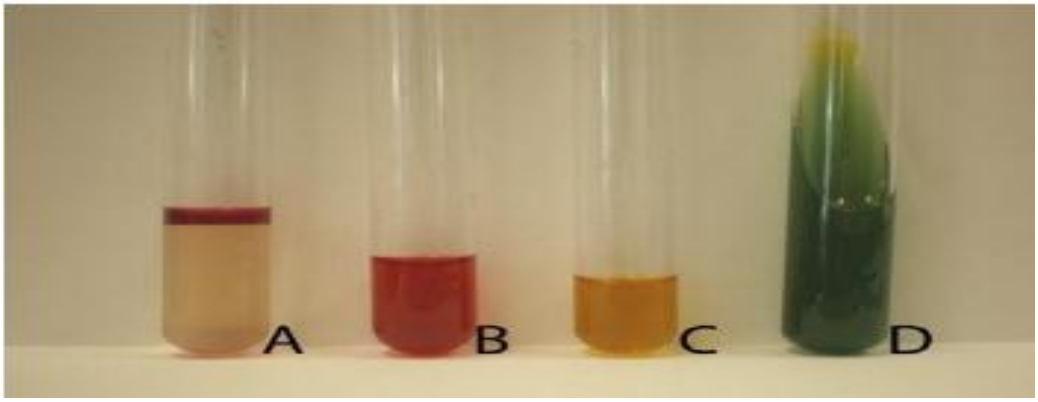
Appendix

Appendix 1-A: General Urine Examination -Biochemical tests results

Appendix 1-B: Biochemical tests results



Appendix 1-C: Biochemical Characterization by iMViC tests for *Escherichia coli* isolate.



Results of an IMViC series done on *E.coli* after 24-hour incubation at 37°C. Tube A shows a positive indole test in tryptone broth. The positive result is indicated by the red layer at the top of the tube after the addition of Kovács reagent. Tube B shows a positive methyl red test as indicated by the red color after the addition of methyl red reagent. Tube C shows a negative Voges-Proskauer test as indicated by the lack of color change after the addition of Barritt's A and Barritt's B reagents. Tube D shows a negative citrate result as indicated by the lack of growth and color change in the tube.

Appendix 2- Vitek 2 System report.

bioMerieux Customer

System#:11789 Printed
January 18,2023

Patient Name: Printed by Lab
Admin

Isolate Group:178-1 Patient
ID:44

Card Type GN Testing Instrument:0000158FF4C7(12152)

Biomumber:0043053003500240

Organism Quantity:

Comments:			
Identification Information	Card: GN	Lot Number: 241330940	Expires: Jan 18,2023 12:00 CDT
	Completed:Jan 3,2016 6: 19 CST	Status: Final	Analysis Time: 4.75 hours
Slected Organism	99% Probability Escherichia coli Biomumber: 04056104545266l0		Confidence:Excellent Identification
SRF Organism			
Analysis Organisms and Tests to Separate:			
Analysis Messages:			
Contraindicating Typical Biopattern (s)			

Biochemical Details																	
2	APPA	-	3	ADO	-	4	PyrA	-	5	IARL	-	7	Dcel	-	9	BGAL	+
10	H2S	-	11	BNAG	-	12	AGLTp	-	13	dGLU	+	14	GGT	-	15	OFF	+
17	BGLU	-	18	dMAL	+	19	dMAN	+	20	dMNE	+	21	BXYL	-	22	BAlap	-
23	ProA	-	26	LIP	-	27	PLE	-	29	TyrA	-	31	URE	-	32	dSOR	+
33	SAC	+	34	Dtag	-	35	dTRE	+	36	CIT	-	37	MNT	-	39 ≈	5KG	+
40	ILATk	+	41	AGLU	-	42	SUCT	+	43	NAGA	-	44	AGAL	+	45	PHOS	-
46	GlyA	-	47	ODC	+	48	LDC	+	53	IHISa	-	56	CMT	+	57	BGUR	+
58	O129R	+	59	GGAA	-	61	IMLTA	-	62	ELLM	-	64	ILATa	-			

Appendix

Appendix 3 Table detailing antibiotic resistance test results by using Vitek 2 System.

No.	Isolates	MEM	IMP	PI	AMC	AMP	GEN	STX	CPT	CRO	NIT	CIP
1	E.co1	R	R	R	R	R	R	R	R	R	S	S
2	E.co2	R	R	R	R	R	R	S	R	R	S	S
3	E.co3	R	R	R	R	R	R	R	R	R	S	S
4	E.co4	R	R	R	R	R	R	R	R	R	R	R
5	E.co5	R	R	R	R	R	S	S	R	S	R	R
6	E.co6	R	R	R	R	R	S	S	S	S	R	R
7	E.co7	R	R	R	R	R	S	R	S	S	S	R
8	E.co8	R	R	S	R	R	R	R	S	S	S	S
9	E.co9	R	R	R	R	R	R	R	R	S	S	S
10	E.co10	R	R	R	R	R	R	S	R	S	S	S
11	E.co11	R	R	R	R	R	R	R	S	R	R	S
12	E.co12	R	R	S	R	R	R	R	S	R	R	S
13	E.co13	R	R	R	R	R	R	S	S	R	R	S
14	E.co14	R	R	S	R	R	S	S	R	S	S	R
15	E.co15	R	R	R	R	R	S	S	R	S	S	R
16	E.co16	R	R	S	R	R	S	R	R	R	S	S
17	E.co17	R	R	R	R	R	S	R	S	R	R	S
18	E.co18	R	R	R	R	R	S	S	S	R	R	R
19	E.co19	R	R	R	R	R	R	S	S	R	R	R
20	E.co20	R	R	R	R	R	R	S	S	S	S	S
	Total R	100%	100%	70%	100%	100%	60%	50%	40%	55%	45%	45%
	Total S	0%	0%	30%	0%	0%	40%	50%	60%	45%	55%	55%
	Total I	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%	0%

Appendix 3: MIC by using Vitek 2 System.

bioMérieux Customer:

Microbiology Chart Report

Printed January 18, 2023 7:49:33 AM CST

Patient Name: Haider Alazdy, 1
Location:
Lab ID: 17120231

Patient ID: 17120231
Physician:
Isolate Number: 1

Organism Quantity:
Selected Organism : Escherichia coli

Source:

Collected:

Comments:	

Susceptibility Information	Analysis Time: 8.05 hours	Status: Final

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
ESBL	NEG	-	Imipenem	>= 4	R
+Amoxicillin		R	+Meropenem	>= 4	R
Ampicillin	>= 32	R	+Panipenem		
+Piperacillin/Sulbactam		R	Amikacin	<= 2	S
Piperacillin/Tazobactam	>= 128	R	Gentamicin	<= 1	S
Cefazolin	>= 64	R	+Isepamicin		S
+Cefotetan			+Tobramycin		S
Cefoxitin	>= 64	R	Ciprofloxacin	>= 4	R
+Cefoperazone			Levofloxacin	>= 8	R
Ceftazidime	>= 64	R	+Sparfloxacin		R
+Ceftizoxime			Tigecycline	<= 0.5	S
Ceftriaxone	>= 64	R	Nitrofurantoin	<= 16	S
Cefepime	>= 64	R	Trimethoprim/ Sulfamethoxazole	>= 320	R

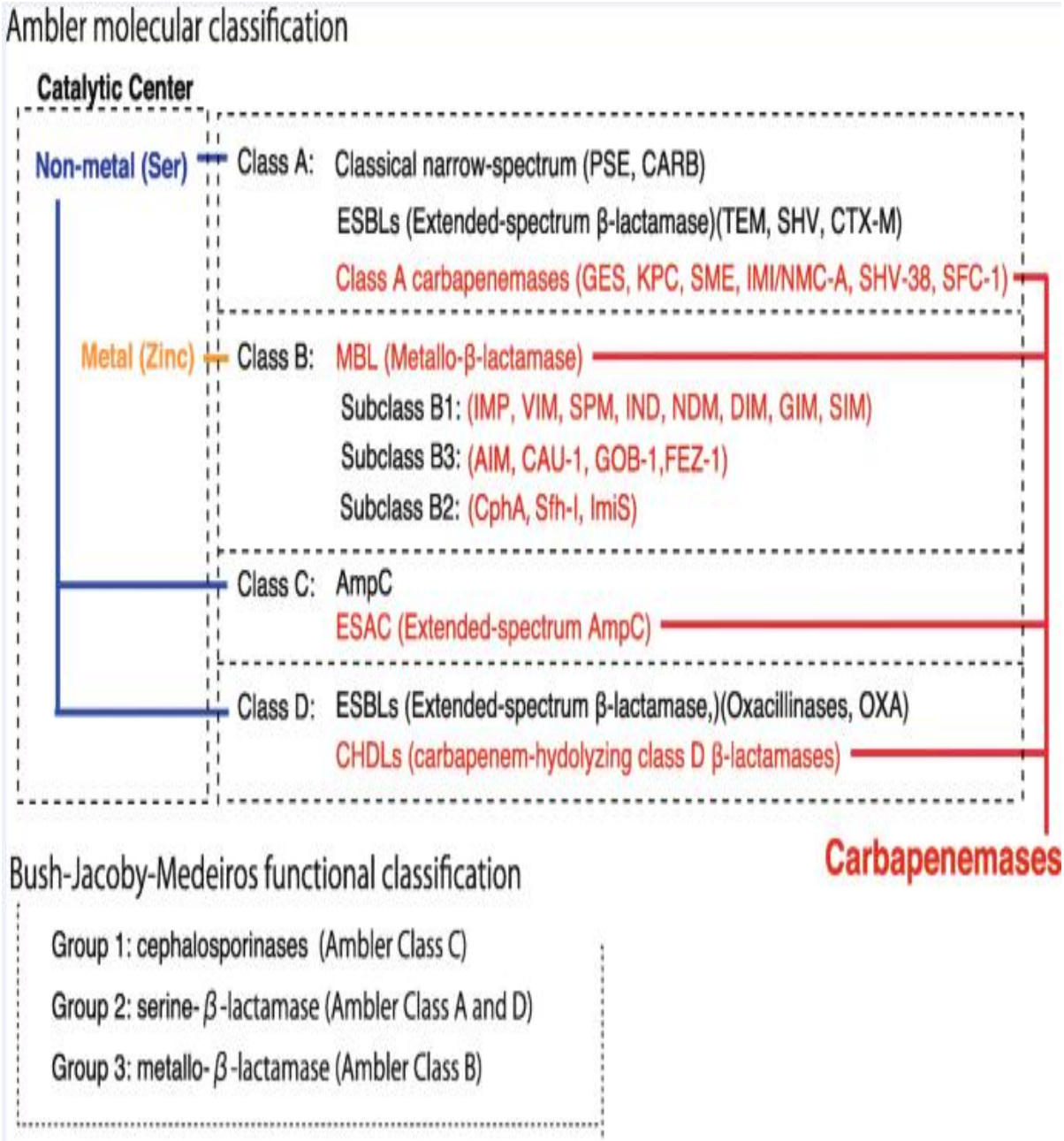
AES Findings	
Confidence:	Consistent

Appendix 4: Isolates missing the gene and Isolates carrying beta-lactam genes

No.	Genes	Isolates missing the gene	Isolates carrying beta-lactam genes
1	***bla*** **GES**	All 20 isolates	None
2	***bla*** ***SME***	All 20 isolates	None
3	***bla*** ***TME***	E.co-8 , E.co-10, E.co-14, E.co-20	E.co-8 , E.co-10, E.co-14, E.co-20, E.co-4 , E.co-5, E.co-6, E.co-7, E.co-9 , E.co-11, E.co-12, E.co-13, E.co-14 , E.co-15, E.co-16, E.co-17, E.co-18 and E.co-19.
4	***bla*** ***SHV***	E.co-1 , E.co-2, E.co-4, E.co-5, E.co-6 , E.co-7, E.co-8, E.co-10, E.co-12 , E.co-13, E.co-15, E.co-16, E.co-17 , E.co-19 and E.co-20	E.co-3, E.co-9, E.co-11 , E.co-14 and E.co-18.
5	***bla*** ***CTX-M-1***	E.co-16	E.co-1 , E.co-2, E.co-3, E.co-4, E.co-5 , E.co-6, E.co-7, E.co-8, E.co-9 , E.co-10, E.co-11, E.co-12, E.co-13 , E.co-14, E.co-15, E.co-17, E.co-18, E.co-19 and E.co-20.
6	***bla*** ***IMP***	E.co-4 , E.co-10, E.co-11, E.co-14, E.co-16 , E.co-17, E.co-19, and E.co-20.	E.co-1 , E.co-2, E.co-3, E.co-5, E.co-6 , E.co-7, E.co-8, E.co-9, E.co-13 , E.co-15 and E.co-18.
7	***bla*** ***VIM***	E.co-1 , E.co-6, E.co-9, E.co-10, E.co-11 , E.co-13, E.co-14, E.co-16, E.co-17 and E.co-20.	E.co-2 , E.co-3, E.co-4, E.co-5, E.co-7 , E.co-8, E.co-12, E.co-15, E.co-18 and E.co-19.
8	***bla*** ***NDM***	E.co-6 , E.co-7, E.co-12, E.co-16, E.co-20.	E.co-1,E.co-2, E.co-3, E.co-4, E.co-5, E.co-8, E.co-9 , E.co-10, E.co-11, E.co-13 , E.co-14, E.co-15 E.co-17, E.co-18 and E.co-19.
9	***bla*** ***PRE***	E.co-1 , E.co-6, E.co-7, E.co-8, E.co-10, E.co-12 , E.co-13, E.co-14, E.co-15, E.co-16, E.co-18 , E.co-19 and E.co-20.	E.co-2, E.co-3, E.co-4, E.co-5, E.co-9, E.co-11 and E.co-17
10	***bla*** ***ADC***	E.co-1 , E.co-2, E.co-3, E.co-4, E.co-5 , E.co-6, E.co-7, E.co-8, E.co-12 , E.co-13, E.co-15, E.co-16, E.co-17 , E.co-18, E.co-19 and E.co-20	E.co-9, E.co-10 and E.co-11.
11	***bla*** ***VEB***	E.co-2, E.co-4, E.co-5, E.co-6 , E.co-7, E.co-8, E.co-10, E.co-12 , E.co-13, E.co-15, E.co-16, E.co-	E.co-1, E.co-3 , E.co-9, E.co-11, E.co-14 and E.co-18.

		17 , E.co-19 and E.co-20	
12	***bla*** $_{DHA}$	E.co-3.	E.co-1 , E.co-2, E.co-4, E.co-5 , E.co-6, E.co-7, E.co-8, E.co-9 , E.co-10, E.co-11, E.co-12, E.co-13 , E.co-14, E.co-15, E.co-16,E.co-17, E.co-18, E.co-19 and E.co-20.
13	***bla*** $_{EBC}$	E.co-6 , E.co-7, E.co-8, E.co-10, E.co-11 , E.co-12, E.co-13, E.co-14, E.co-15 , E.co-17, E.co-19 and E.co-20	E.co-1 , E.co-2, E.co-3, E.co-4, E.co-5, E.co-9, E.co-16 and E.co-18.
14	***bla*** $_{OXA}$	E.co-1 , E.co-3, E.co-4, E.co-5, E.co-7, E.co-8 , E.co-9, E.co-10, E.co-14, E.co-16, E.co-17 , E.co-18, E.co-19 and E.co-20.	E.co-2, E.co-6, E.co-11, E.co-12, E.co-13 and E.co-15.

Appendix 5: Amblar molecular classification.


Ambler molecular classification
Catalytic Center
Non-metal (Ser)
Class A:
Classical narrow-spectrum (PSE, CARB)
ESBLs (Extended-spectrum β-lactamase)(TEM, SHV, CTX-M)
Class A carbapenemases (GES, KPC, SME, IMI/NMC-A, SHV-38, SFC-1)
Metal (Zinc)
Class B:
MBL (Metallo-β-lactamase)
Subclass B1: (IMP, VIM, SPM, IND, NDM, DIM, GIM, SIM)
Subclass B3: (AIM, CAU-1, GOB-1,FEZ-1)
Subclass B2: (CphA, Sfh-I, ImiS)
Class C:
AmpC
ESAC (Extended-spectrum AmpC)
Class D:
ESBLs (Extended-spectrum β-lactamase,)(Oxacillinases, OXA)
CHDLs (carbapenem-hydolyzing class D β-lactamases)
Carbapenemases
Bush-Jacoby-Medeiros functional classification
Group 1: cephalosporinases (Ambler Class C)
Group 2: serine-β-lactamase (Ambler Class A and D)
Group 3: metallo-β-lactamase (Ambler Class B)

جمهورية العراق

وزارة التعليم العالي والبحث العلمي

جامعة بابل

كلية العلوم

قسم علوم الحياة

التوصيف الجزيئي للجينات المقاومة للكاربابينم بين عزلات بكتريا الإشريكية القولونية المعزولة من مرضى التهاب المجاري البولية في مدينة الحلة – العراق

أطروحة مقدمة الى

مجلس كلية العلوم / جامعة بابل وهي جزء من متطلبات نيل شهادة

الدكتوراه فلسفة في العلوم / علوم الحياة

من قبل

حيدر عبد الامير عبد الرضا الاسدي

بكالوريوس / علوم حياة / احياء مجهرية/ **2006**

ماجستير علوم حياة – أحياء مجهرية/ **2014**

بأشـــراف

الاستاذ الدكتور

ايمان محمد جار الله

1445هـ 2023م

الخلاصـــــــــــة:

تعد التهابات المسالك البولية (UTIs) واحدة من أكثر أنواع العدوى البكتيرية انتشارًا ولها امتداد عالمي. تحدث هذه الالتهابات في الغالب بسبب الإشريكية القولونية المسببة للأمراض البولية (UPEC). العلاقة بين المقاومة للأدوية المتعددة لبكتيريا الإشريكية القولونية وملامح جينات المقاومة لدى عزلات الكاربابينمز من بكتيريا الإشريكية القولونية المسببة للأمراض المعزولة من المريض المصاب بالتهاب المسالك البولية في مدينة الحلة. لذلك، تم إجراء المسح الحالي من أجل تحديد النمط المظهري والنمط الوراثي وكذلك تحديد التعبيرات الجينية بين عزلات الإشريكية القولونية المقاومة للأدوية المتعددة والمعزولة من المرضى الذين يعانون من التهابات المسالك البولية في مدينة الحلة.

تضمنت الدراسة جمع وتحليل 646 عينة ادرار وسطية والتي تم فحصها فوراً عن طريق فحص الادرار العام (GUE) بعد جمعها للكشف عن الخصائص الفيزيائية والكيميائية وكذلك الفحص المجهري لتحديد وجود التهابات المجاري البولية وذلك لان التهاب المجاري البولية تحدث دون أي أعراض ملحوظة تظهر على المريض، تليها طرق الكشف عن النمط الظاهري والوراثي والتعبير الجيني.

تم جمع العينات الوسطية للإدرار خلال الفترة من شباط إلى حزيران (2022) من مستشفيات مختلفة في مدينة الحلة. تم الحصول على العينات السريرية من الأفراد الذين يعانون من أعراض التهاب المسالك البولية من المستشفيات التالية وهي: مستشفى الامام الصادق التعليمي (377 عينة بول سريرية)، مستشفى مرجان التعليمي (175 عينة بول سريرية)، ومستشفى بابل للنسائية والأطفال (94 عينة بول سريرية). بعد ذلك، تم فحص جميع العينات الوسطية للإدرار عن طريق الفحص المجهري وتم زراعتها على أجار ماكونكي وأجار الأيوسين ميثيلين الأزرق (EMB) وأجار الدم تليها الحضانة لمدة 24 ساعة عند 37 درجة مئوية للكشف عن عزلات تخمر اللاكتوز وغير اللاكتوز. من إجمالي 646 لعينات الادرار الوسطية ، تم تحديد 320 عزلة فقط (49.5%) بنجاح على أنها بكتيريا الإشريكية القولونية من خلال نموها على اوساط زرعيه الانتقائية والتفاضلية والتي تشمل وسط أجار المكونكي والايوسين المثيل الازرق فيما كانت بقية العزلات والبالغة 326 (50.5%) عزلة كانت تنتمي إلى انواع اخرى من البكتريا السالبة لصبغة غرام.

أشارت النتائج إلى وجود 320 عينة سريرية، تشمل 273 (85.3%) إناث و47 (14.7%) ذكور. تم الحصول على عزلات الإشريكية القولونية من مرضى التهابات المجاري

البولية (UTI) والتي خضعت لاختبار الحساسية للمضادات الحيوية (AST). أظهرت نتائج هذه الدراسة أن أعلى نسبة حدوث عدوى المجاري البولية والتي لوحظت في الفئة العمرية بين (30-60) سنة بين بقية الفئات العمرية.

كما تم تأكيد النتائج بواسطة الفحوصات الكيموحيوية واختبارات نظام Vitek2 كفحوصات تأكيدية لهذه العزلات والتي أظهرت أن جميع العزلات هي بكتيريا الإشريكية القولونية المسببة لالتهاب المجاري البولية.

كشف اختبار الحساسية للمضادات الحيوية (AST) عن اتجاهات مقاومة ملحوظة بين عزلات الإشريكية القولونية. ومن الجدير بالذكر أن مجموعات بيتا لاكتام، بما في ذلك الأموكسيسيلين-كلافولانيت والأمبيسيلين، أظهرت معدلات مقاومة قدرها 87.1% و75.9% على التوالي. أظهرت مجموعة أمينوغليكوزيد (مثل الجنتاميسين) مقاومة في 66.8% من الحالات، في حين أظهرت مجموعة السلفوناميد (مثل تريميثوبريم) والنيتروفوران (مثل النيتروفورانتوين) معدلات مقاومة قدرها 49.6% و43.1% على التوالي. تباينت حساسية العزلات، حيث أظهرت مجموعة سيبروفلوكساسين، سيفابيم، سيفترياكسون، وبيبراسيللين حساسيات في نطاق 34.3٪ إلى 52.1٪.

علاوة على ذلك، أظهرت نتائج فحوصات الحساسية الدوائية أن 20 عزلة فقط من الإشريكية القولونية (6.2%) كانت مقاومة للميروبينيم و الإيميبينيم.

اظهرت نتائج الفحوصات المظهرية والتي شملت مقاومة 20 عزلة مقاومة لمضادات الكاربابينم من عزلات بكتريا الإشريكية القولونية نتيجة إيجابية لاختبار الاموكسيكلاف لتآزر القرص المزدوج واختبار الهودج المعدل (MHT) مما يؤكد أن هذه العزلات لديها القدرة على إنتاج إنزيمات بيتا لاكتاماز .

استخدام طرق الكشف الجزيئي للتعرف على جينات مقاومة الكاربابينيمات في 20 عزلة من بكتيريا الإشريكية القولونية. تم استخدام طرق الكشف الجزيئي للكشف عن الجينات التالية التي تشمل $bla_{CTX-M-1}$ وbla_{DHA} والتي كانت الأكثر انتشارًا بمستويات النسبة المئوية (n= 19) (95%)، تليها bla_{TEM} (n= 16) (80%)، bla_{NDM} (n= 15) (75%)، bla_{IMP} (n= 12) (60%)، bla_{VIM} (n=10) (50%)، و جينات اخرى بترددات متفاوتة. بينما لم يتم الكشف عن كل من جينات bla_{GES} و bla_{SME} خلال هذه الدراسة.

ووفقا لنتائج الدراسة، فإن غالبية العزلات شملت اثنان من الجينات الوراثية على الأقل. كشفت العزلة ذات العدد E.co-9 عن نسبة مئوية عالية وهي تحمل 10 جينات متميزة، في حين

أن العزلات الأخرى والتي تشمل (E.co-2، E.co-3، E.co-11، E.co-18). يحمل كل منهم تسعة جينات مختلفة.

حسب تصنيف أمبلار أظهرت نتائج الفحص الجزيئي لهذه الدراسة أنه تم الكشف خلال هذه الدراسة عن 280 جين يشفر الى انزيمان البيتا لاكتاماز والتي أظهرت نتائج إيجابية لـ 126 (45%) جين بينما أظهرت باقي الجينات 156 (55%) جيناً نتيجة سلبية .

أظهرت الخصائص الجزيئية لـ 20 عزلة من الإشريكية القولونية المقاومة للكاربابينم أن جميع الجينات تنتمي إلى الفئة A ضمن البتالاكتمايز (42.1٪) 53 = nمن بين 126 جينًا موجبًا تليها الفئة B ضمن البتالاكتمايز (29.3٪) 37 = n جينات البتالاكتمايز ، بينما كانت الفئة C = (23.8٪) 30 = n وأخيرًا أظهرت الفئة D أقل مستويات النسبة المئوية بين جينات بيتا لاكتاماز التي اكتشفت خلال هذه الدراسة كانت بتأثير قليل يتراوح بنسبة (4.8٪) 6 = n .

أخيرًا، قامت هذه الدراسة بتقييم التعبير الجيني في وجود مثبطات الإيميبينيم (IMP)، وكشفت عن زيادة بمقدار 1.8 إلى 12.4 ضعفًا في مستويات mRNA bla_{IMP} استجابةً للتعرض للمضادات الحيوية للإيميبينيم. أظهرت نتائج التعبير الجيني مستويات متفاوتة في وجود مثبط الامبينيم لجميع العزلات البكتيرية. ومن الجدير بالذكر أن العزلة رقم 13 أظهرت أعلى مستوى في التعبير الجيني بين بقية العزلات الأخرى. يمكن الاستنتاج أن هذا البحث يسلط الضوء على بروز التهابات المسالك البولية، وخاصة بين الإشريكية القولونية المسببة للأمراض التهاب المجاري البولية، ويبين مدى انتشار الملامح الجينية متعددة المقاومة بين مرضى التهابات المسالك البولية في مدينة الحلة.