Antibiotic Sensitivity and Antibiotic Resistance Genes in *E. coli* Isolated from Human Patients; Detection of Beta-Lactamase Producing *E. coli*

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2023

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF PHILOSOPHY

IN

ZOOLOGY

(Animal Microbiology)

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2023

DECLARATION

I hereby declare that the work presented in the following thesis is my own effort, except where otherwise acknowledged, and that the thesis is my own composition. No part of this thesis has been previously presented for any other degree.

Rabbia Ali

CERTIFICATE

This is certified that the dissertation " Synergistic effect of commercial pesticides and entomopathogenic fungi on *Coptotermes heimi* Wasmann (Blattodea: Rhinotermitidae) " by Muhammad Shoaib, is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirement for the degree of Master of Philosophy in Zoology with specialization in Entomology.

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MRTsoft

Dedication

I would like to praise Allah the Almighty, the Most Gracious, and the Most Merciful for His blessing given to me during my study and in completing this thesis. May Allah's blessing goes to His final Prophet Muhammad (peace be up on him), his family and his companions.

With profound love & deep respect, this dissertation is dedicated to my sweet and loving parents and my husband

Whose affection, love, encouragement and prayers of day and night made me able to get this success and honour.

LIST OF CONTENT

LIST OF ABBREVIATIONS

Sr no.	**Abbreviations**	**Full names**
1.	ESBL	Extended spectrum β-lactamase
2.	UTI	Urinary tract infection
3.	UPEC	Uropathogenic *E. coli*
4.	ETEC	Enterotoxigenic *E. coli*
5.	EPEC	Enteropathogenic *E. coli*
6.	EIEC	Enteroinvasive *E. coli*
7.	STEC	Shiga toxin-producing E. coli
8.	EIEC	Enteroinvasive E. coli
9.	HUS	Hemoluytic uremic syndrome
10.	GNB	Gram negative bacilli
11.	MRSA	Methicillin-resistant Staphylococcus aureus
12.	PCR	Polemerase chain reaction
13.	DEC	Diarrheagenic E. coli
14.	AMR	Antimicrobial resistance
15.	CDC	Centre for Disease Control and Prevention
16.	LPS	Lipo-polysaccharide
17.	MFS	Major facilitator superfamily
18.	RND	Resistance-nodulation-cell division
19.	SMR	Small multidrug resistance
20.	MFP	Membrane fusion protein
21.	PBP	Penicillin binding protein
22.	CLSI	Clinical and Laboratory Standards Institute
23.	EUCAST	European Committee on Antimicrobial Susceptibility Testing
24.	ELISA	enzyme-linked immunosorbent assays
25.	IDC	Islamabad diagnostic centre
26.	AST	Anti sensitivity test
27.	CPD	Cefpodoxime
28.	FOX	cefoxitin
29.	IDSA	Infectious Diseases Society of America

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGMENTS

All the acclamations and appreciations are for ***Almighty ALLAH***, the compassionates and generous that knows best the mysteries and secrets of the universe, and his ***Holy Prophet Muhammad (Peace Be Upon Him)*** who has guided His Ummah to seek knowledge from cradle to grave and enabled me to win the honor of the life and whose teachings have served as a signal of light for the humanity in the hours of despair and darkness. I offer my humble and sincerest words of thanks to ***ALLAH Almighty*** that despite all problems which I encountered during M. Phil enabled me to complete this dissertation.

It is a great pleasure to pen my acknowledgment towards all those from whom I have incurred my debts and obligations during the compilation of my thesis. My heartiest compliments go to my supervisor-***Dr. Sseed-ul-Hassan khan*****,** As for his guidance, cooperation, and knowledgeable help. I feel lucky that I availed the opportunity to work under her kind supervision. I pray for her success and peace in the world and hereafter.

My gratitude extends to the ***Department of Zoology***, Quaid-i-Azam University Islamabad and head of department ***Dr. Amina Zuberi*** for providing me with the necessary research environment.

I would like to acknowledge my senior teachers from IDC lab sir Zuhaib Ali, Ma'am Mehwish, and Ma'am hafsa for their guidance and help throughout my thesis lab work. With their knowledge I was able to overcome any shortcomings in my research work.

I am extremely grateful to my parents for their love, prayers, care and sacrifices for educating and preparing me for my future. I would like to express my deepest and heartfelt gratitude to my lab fellow ***Sundas shafqat***, ***shanzae Tariq*** and my lab senior ***Dr. Faisal Qaisrani*** for their timely advice, meticulous scrutiny, timely suggestion with kindness, enthusiasm and dynamism have enabled me to complete my thesis.

And friends for their company and moral support throughout this journey.

Rabbia Ali

Abstract

The main purpose of this research work was to study the antibiotic resistance and β-lactamase (ESBL and AmpC) production in *E. coli* isolates from human patients. Aother objective was to also study the phylogenetic groups of the recovered *E. coli* isolates. Forty urine samples were collected from patients suspected of having urinary tract infection (UTI) at the Islamabad Diagnostic Center (IDC), Islamabad. These samples were cultured on different laboratory media and from all the 40 urine samples only *E. coli* was recovered. This was confirmed by growth characteristics on laboratory media and by the uid*A* gene PCR. The antibiotic sensitivity pattern of the *E. coli* isolates was determined against 14 antibiotics. The highest resistance was recorded for Ampicilin (85%), Ciprofloxacin and Levofloxacin (62.5%). The isolates showed lowest resistance against Minocycline (0%), Meropenem (2.5%) and Sulzone (2.5%). Resistance of *E. coli* isolates against Cefpodoxime and Cefoxitin was used to screen potential producers of ESBLs and AmpC β-lactamases. Twenty-four isolates were resistant against Cefpodoxime of which five *E. coli* isolates were found to be resistant to Cefoxitin. Twenty-four isolates were further tested for production of ESBLs and AmpC using a commercial AmpC and ESBL Detection Disc Set (MAST Group Ltd, UK). Two *E. coli* isolates out of the 5 cefoxitin resistant was confirmed to be positive for production of AmpC; while 14 out of the 24 cefpodoxime resistant isolates were confirmed positive for ESBL production.

PCR were done to screen the isolates for some selected β-lactamases (ESBL & AmpC) genes in the *E. coli* isolates. *bla*CTX-M (ESBL gene) was found to be present in 16 isolates, while no *bla* SHV and *bla* TEM was found in *E. coli* samples. On the other hand, AmpC genes: AmpC-MOX, AmpC-CIT and AmpC-FOX were found to be present in 3 of the isolates. No AmpC-EBC, AmpC-ACC and AmpC-DHA genes were detected in any of the isolates. A triplex PCR was conducted to investigate the phylogroup of the *E. coli* isolates. The highest number of *E. coli* isolates were found to belong to group B1 (33%), followed by group A (32%), phylogroup B2 (30%) and phylogroup D (5%).

The growing reports on ESBL producing *E. coli* poses a major public health threat, because, the ESBL production genes can be transferred to other bacteria including pathogens of humans through horizontal transfer by plasmids, especially, conjugative ones which can lead to resistance against multiple antibiotics. Thus, many human pathogens can become resistant to antibiotics and treatment can become difficult. Finally, there is need of research to find novel antibiotics and their rational use is needed for the future.

Antibiotic Sensitivity and Antibiotic Resistance Genes in *E. coli* Isolated from Human Patients; Detection of Beta-Lactamase Producing *E. coli*

INTRODUCTION

1.1 *Escherichia coli*

Escherichia coli (*E. coli)* is gram negative facultative anaerobic bacteria (Liu *et al.,*2019). It is oxidase negative and grows using simple carbon sources (Batt, C.A. 2014). *E. coli* is a nonspore-forming bacteria from the family Enterobacteriaceae. *E. coli* is the most known species of the genus *Escherichia*. It was isolated by Theodor Escherich in 1884 hence genus is named after him. (Schaechter, 2009) *E. coli* is a facultative anaerobe ~110 organisms in one gram of fecal matter. *E. coli* resides in the colon of humans and many mammals, thus mostly found in the feces. Certain strains of *E. coli* cause intestinal and extraintestinal infections in mammals mainly of the urinary system and digestive system parts, CNS and blood (Bonten *et al.,*2021).

E. coli strains show significant biochemical activities, it mainly ferments lactose, does not grow on citrate, does not produce H_2S, lysine decarboxylase enzyme is present, exhibits the production indole, and no production of acetoin making it Voges–Proskauer negative. Strains of *E. coli* isolates are distinguished mainly by their antigenic composition O antigens and K antigens. Other properties that are used to define individual strains are F and H antigens (Baker *et al.,* 2014).

E. coli is the main ESBL-producing specie that is prevalent and found worldwide. *Klebsiella* and *E. col* are both organism that are found among the six drug resistant microbes in a repprt presented by Infectious Diseases Society of AmericaOteo J *et al.,*2010). The main site of bacterial infections in humans is urinary tract, and *E. coli* comes first in the list to infect this particular site causing about 80% of community acquired infections (hagan, 2010).

1.2 Pathotypes of *E. coli*

Uropathogenic *E. coli* (UPEC) includes a certain type of *E. coli* that colonizes the urinary tract epithelium, it stimulates cystitis and pyelonephritis. **Enterotoxigenic *E. coli*** (ETEC) strains cause Tourist's diarrhea in people who travel from different countries following poor sanitation. The main cause of ETEC is contaminated food or water or animal feces (Hagan *et*

*al.,*2010). **Enteropathogenic *E. coli*** (EPEC) strains are the cause of watery or bloody ileum.occasionally bloody colonization of ileum. The fimbria that is present on host cell tissue is recognized by EPEC. Enteropathogenic *E. coli* strain can cause life threatening watery diarrhea, particularly in children aged below 2 years. These strains are more prevalent in areas with limited resources. If the diarrhea persists, it can lead to stunted growth in children. To prevent infection by these *E. coli*, practicing good hygiene, avoid contaminated water and food item sources (Makvana *et al*., 2015).

The enterohemorrhagic *E. coli* (EHEC) are confined to small number of serotypes which causes bloody diarrhea reffered to as hemorrhagic colitis. O157:H7 serotype is found prevalent in USA and O26 is found in the world. Virulent strains of *E. coli* cause diarrheal infections along with UTIs and neonatal septicemia. Shiga toxin–producing *E. coli* (STEC) has a dominant strains *E. coli* O157:H7, is found in fecal materialof ruminants. Humans encounter the diarrheal infections through contaminated food or direct contact with infected person incubation periods for most of bacterial strains is about a week to 10 days, but O157:H7 strain of *E. coli* has incubation periods is 3-4 days (Makvana *et al*., 2015).

Enteroinvasive *E. coli* (EIEC) strain is another strain that causes dysentery, resembling Shigella (M Schaechter, 2009). EIEC strains share similarities with Shigella, both in terms of their biochemistry and their ability to invade intestinal epithelial cells and cause illness. Specifically, these strains can lead to the development of symptoms such as severe diarrhea, high temperature, and bad abdominal cramps much like Shigella can (Makvana *et al*., 2015).

1.2.1 Extraintestinal *E. coli* infections

variety of extraintestinal infections are caused by *E. coli*, but these types of infections don't seem to get as much attention as intestinal pathogenic *E. coli* illnesses. This is likely because extraintestinal *E. coli* infections don't typically occur in large outbreaks and aren't usually associated with contaminated food. Additionally, these infections often only cause minor symptoms (cystitis) or affect people with compromised immune systems. Unlike with intestinal *E. coli* infections, there isn't as much focus on detecting and reporting extraintestinal infections (Jhonson & Russo 2002).

1.2.2 Neonates septicemia and meningitis

Neonates right after their birth are at verge of getting infected. They can acquire the infection through vertical transmission in first two days. Later onset of infections is referred to nosocomial acquisition of infection. Species like Listeria *monocytogenes, E. coli, and, B Streptococcus* causes early onset of illness/infection is caused by but later infections are caused by GNB and staphylococcal species. Pathogenic *E. coli* is leading cause of meningitis in newborn known as neonatal meningitis, having K_1 capsule sialic acid helping it to cross bloodbrain barrier. Clinical signs of septicemia include fluctuating body temperature, fever, difficulty in breathing pattrens, cyanosis, low energy levels, and severe diarrhea (Makvana *et al*., 2015).

1.2.3 Diarrheal infections

ETEC strains can cause abdominal cramping and diarrhea causing loose motion that lasts for 5 days. It is infact more common in neonates but it's a major cause of illness in USA, it causes traveler's diarrhea and can be contracted through contaminated food or water. On the other hand, toxins are found in STEC strains that resembles the toxins present in *Shigella dysenteriae* and can lead to diarrhea, hemolytic uremic syndrome (HUS), and post diarrheal thrombocytopenic purpura (usually in adults). The most virulent strain of *E. coli* is STEC:H7 as it causes diarrhea that comes with blood and abdominal cramps.

1.2.4 Hemolytic Uremic Syndrome

HUS, which is characterized by microangiopathic hemolytic anemia, thrombocytopenia, and renal failure, is a serious condition that can occur as a result of STEC enteric infections. In North America, the serotype responsible for most cases is *E. coli: H*7, and it typically develops about in period of 14 days after acquiring diarrhea. Unfortunately, ~20% of children with *E. coli: H*7 diarrhea can develop HUS, and half of those patients acquire severe infection which involve dialysis. Tragically, 3-5% of affected persons with HUS expire as a result of its drastic effects (Makvana *et al*., 2015).

1.3 Urinary tract infections

Urinary tract infections UTI (are caused by bacteria especially *E. coli* in humans, around 150 million people get infected by UTI per annum. Women are supposed to get infected with UTI once a lifetime (G. Bergsten *et al.,*2005). UTI can cause different types of infections ranging

from acute, and chronic to recurrent infections along with asymptomatic bacteriuria (M.R. Asadi Karam, *et al.,*2019). UTIs are caused by bacteria belonging to the Enterobacteriaceae family (*Klebsiella pneumoniae, Escherichia coli, Proteus mirabilis, Enterobacter* and *Citrobacter*), *Pseudomonas aeruginosa, Staphylococcus aureus, Streptococcus* and *Enterococcus faecalis*. Extraintestinal pathogenic *E. coli* (ExPEC) consists of the heterogenous group Uropathogenic Escherichia coli (UPEC), that are found in the gut and disseminated through contaminated food, oral-fecal route, or sexual contact. There are seven phylogroups of *E. coli* A, B1, B2, C, D, E, and F, these groups are defined on the basis of genetic and phenotypic properties. These properties put UPEC strains in groups B2 and D (Karam, *et al.,*2019). Various studies showed that UTI patients were the main source of UPEC isolates (Tabasi *et al.,*2015, 2016).

Bacterial virulence factors involve adhesion, growth, infection, invasion, and metabolism. Bacteria colonize in the epithelium of urinary tract, and virulence genes play an important role in the severity of infection. In 2021 Makkie and its associates performed a series of studies in UTI patients to check for the virulence factors in UPEC strains, their studies were confined to Iran. They collected 40 samples of UTI patients and performed a series of metabolic tests and extracted the DNA. After checking the purity and concentration of DNA, PCR was done to check the virulence factor involved in UTI. Among all detected factors, fimh, pap, cnf1, hlyA , afa, iron, and iuc harbored the higher distribution. Their studies were based locally in different regions of iran that showed that the prevalence of UPEC gene is geographically dependent and even the climate change in different regions. The customs, food preferences, public health, methods involving samples handling and anitory conditions of hospitals affected the prevalence pattern of the virulent genes of UPEC *E. coli* (makkie *et al.,*2021).

1.3.1 Uropathogenic *Escherichia coli* (UPEC)

Escherichia coli strains causes diseases outside the gastrointestinal tract and belong to *ex*traintestinal *p*athogenic *E. coli* (ExPEC). Uropathogenic *E. coli* is the prominent member of ExPEC family, and it is responsible for 90% of UTI worldwide. UTI infection occurs when contamination in periurethral area via fecal route. Bacteria ascends the urethra and resides in the bladder causing cystitis. In rare cases this bacterium transfer contamination to kidney via ureters causing pyelonephritis. Foxman and colleagues reported that 10.8% of women older

than 18 reported oof having UTI in that year while majority of women went through the UTI at least two or more times in a lifetime (Lloyd *et al.,*2007)

1.4 Prevalence of UTI in Pakistan

A study in Peshawar showed the gender wise prevalence of UTI in men and women, being 49.3% and 50.7% respectively. The most recognized isolates were 68.9% *Escherichia coli* (Muhammad *et al.,*2020).

Table 1.1 Bacterial isolates islated from UTI patients in Peshawar (JAN –JUN 2019)

Bacterial isolates	**Female**	**Male**	**total**
E. coli	72.7	63.6	68.3
K. pneumonia	6.1	12.6	9.3
Staph aureus	4.1	5.6	4.8
MRSA	3.4	7.7	5.5
Pseudomonas	3.4	4.9	4.1
Enterococcus	4.1	1.4	2.8
Proteus	1.4	2.8	2.1
Satph. Spp	4.8	1.4	3.1
Total	50.7	49.3	100

Another study was done at Lahore General Hospital, Pakistan, about 72% of UTI were caused by gram negative bacteria *E. coli* followed by *Klebsiella* (11%), *Enterobacter* (7%) and *Enterococcus* (4%). The pathogenic isolates had highest resistance against ampicillin, cotrimoxazole, and cephalexin (72%–95%), intermediate sensitivity to cephalosporins, and highly sensitivity to amikacin, nitrofurantoin, and ciprofloxacin (Sohail *et al.,*2023).

A study done in 2019 comprised of genetic virulence factors of UTEC and DEC strains involved in UTIs. UPEC and DEC strains (173) were studied to check for the virulence genes and to check the phylogenetic profile of *E. coli* done by the PCR. The most prevalent phylogenetic group was A both in UPEC and DEC strains, after that B2 group, contrary to most of studies done before. Amplification assays showed that UPEC (89.32%) and DEC (94.29%) strains, was found to have the virulence genes for causing UTI, the most prevalent genes were *fim H* gene for UPEC and DEC strains respectively (Khairy *et al.,*2019).

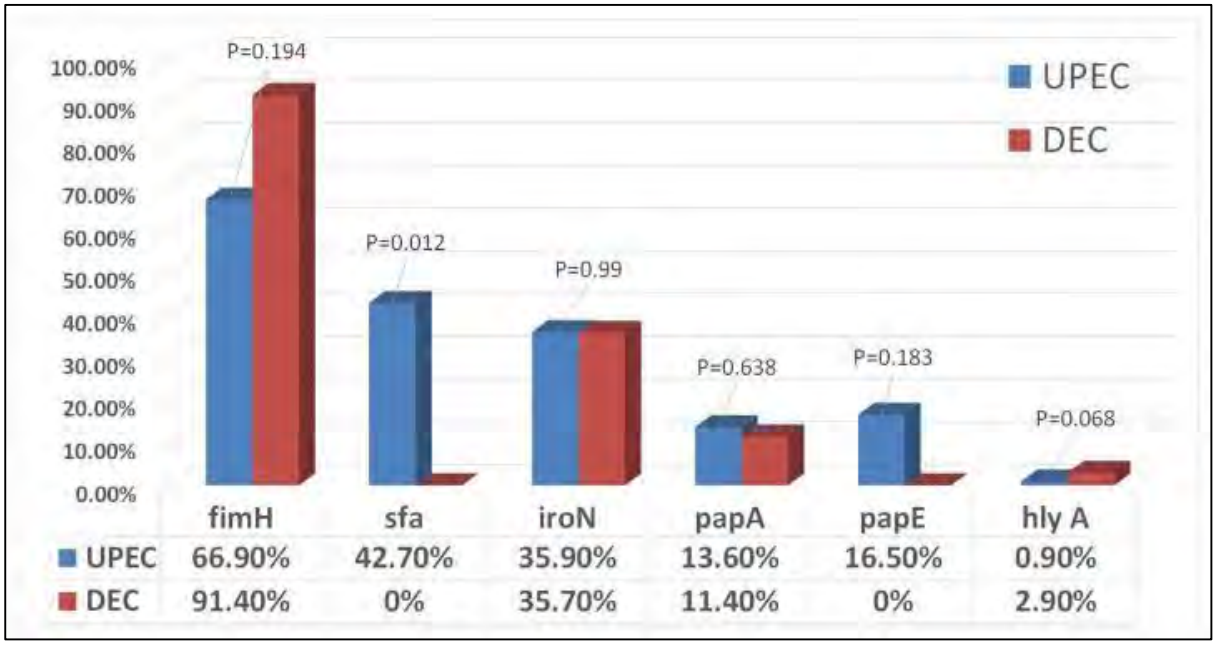


Figure 1.1 Percentage of different virulence genes in both UPEC and DEC isolates.

In 2021 the prevalence of gram-negative bacilli (GNB) in UTIs was performed along with the antibiotic sensitivity. The study showed the main GNB responsible for UTI was *E. coli*. The antibiotic assay GNB resistant against number of antibiotics. (Rasool *et al.,*2019).

1.5 Antibiotic resistance in bacteria

Excessive and unmonitored use of antibiotics for the past 80 years has caused an immense amount of antibiotic resistance. It is not possible even today to find out the phenotype of resistant genes in pathogenic strains that will cause disease. The knowledge regarding factors involved in resistance of bacteria can help coping with resistance mechanism and develop better target sites via antibiotics (Christaki *et al.,*2020). Seven hundred thousand deaths are assigned to antimicrobial resistance per annum globally, at this rate the death toll per year would upsurge to a billion in near future (O'Neill 2014).

Antibiotic resistance is of three types**,** intrinsic, adaptive or acquired (Joon-Hee 2019).

As evident from the name Intrinsic resistance is already present resistance pattern in bacteria. Examples include, Gram-negative bacteria showing glycopeptide resistance because of its inability to penetrate cell envelope of GNB.

Acquired resistance is the resistance that takes place when previously sensitive bacterium either by mutation or new genetic makeup shows resistance (Holmes *et al.,* 2016).

When one or more antibiotics are introduced due to any environmental factors it causes **Adaptive resistance**, it is transient and once the inducing signal is removed the bacteria go back to its original state (Joon-Hee 2019)

1.5.1 Mechanism of resistance in bacteria

Antimicrobial resistance (AMR) in microorganisms develops after they have been exposed to medication. AMR poses threat to public health throughout the world. As per the data of Centers for Disease Control and Prevention (CDC) annually 20 billion dollars go to healthcare department, and it doesn't include the 35 billion dollars expenditure in production (Porooshant Dadgostar, 2022).

The mechanism of antimicrobial resistance falls into four main groups.

- limiting uptake of a drug
- modifying a drug target
- inactivating a drug active drug efflux

- Gram negative bacteria uses all main mechanisms, on the other hand gram positive bacteria due to lack of outer LPS membrane doesn't use drug efflux mechanism and uses limiting the uptake of a drug (Chancey ST *et al.,*2014).

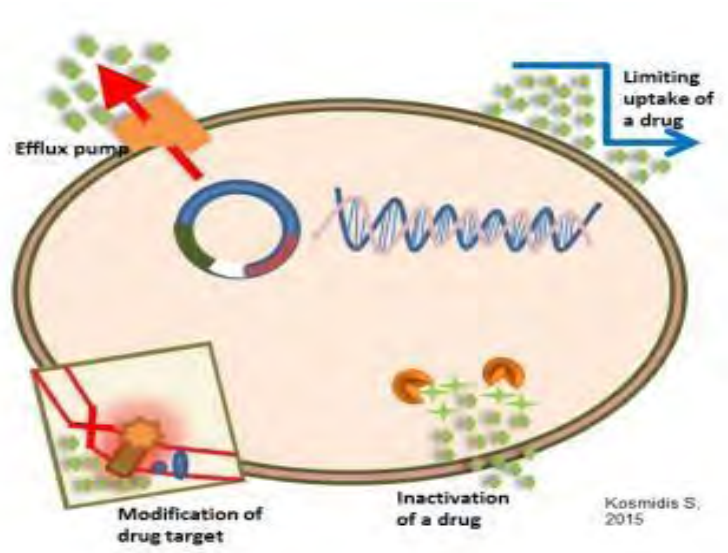


FIGURE 1.2 General mechanism of bacterial antibiotic resistance

1.5.2 Limiting drug uptake

Gram negative bacteria contains a Lipid polysaccharide layer whose function is to provide barrier from harmful substances. This LPS layer gives resistance to bacteria to large range of antimicrobial drugs (Blair JM *et al.,*2014). Outer layer of mycobacteria has high lipid content which allow hydrophobic drugs (rifampicin and the fluoroquinolones) entry while hydrophilic drugs have limited access. *Mycoplasma* doesn't have a cell wall which makes it resistant to drug that effect the cell wall which includes β-lactams and glycopeptides. Gram positive bacteria have no outer membrane so inhibiting medication is not that rampant. *Staphylococcus aureus* has established resistance against vancomycin through an unexplained mechanism of developing a thick outer cell wall making it impossible for drug to get entry. (Reygaert *et al*.,2018).

In bacteria which have larger outer membranes there is a substance that gives entry to molecules known as porin channels. These porin channels are present in GNB which only allow substances that are hydrophilic in nature (Blair *et al.,*2014). Due to the changes in the porin channels there can be limitation in the drug uptake, by two means Either there is decrease in the number of the porin present or there is some mutation that causes change in the porin channel. *Enterobacteriaceae* reduces the number of porins against carbapenems (Cornaglia ***et al.,***1996). Mutations leading to change in the porin channel are evident in *E. aerogenes*, making it resistant to imipenem and cephalosporins (Reygaert *et al.,*2018).

Biofilm development in pathogenic bacteria protects it from the immune system of host and antimicrobial agents. This biofilm contains polysaccharides proteins and DNA that makes it difficult for the antimicrobial agents to surpass it. These biofilms are great for horizontal transfer of genes that makes it ready easier for them to pass antimicrobial resistant genes (Van Acker H *et al.,*2014).

1.5.3 Modification of drug targets

Bacterial cell wall contains layer of peptidoglycan, a molecule that is a polymer of glycan molecules linking through transglycosidases. There is a cross linking between D-alanyl-alanine and glycine, this linkage strengthens cell wall. This strong cross linking is the cause of resistance. β-lactam targets PBPs, it mimics the D-alanyl-alanine region linking with glycine. Hence no new peptidoglycan is formed, and its disruption causes lysis of bacterium (Džidic S, *et al.,*2008). Glycoproteins binds with D-alanyl-alanine region causing drugs like vancomycin to prevent this linkage hence inhibiting cell wall synthesis (Grundmann H *et al.,*2006).

1.5.4 Drug inactivation

There are two mechanisms for drug inactivation.

i.Degradation of drug ii.Transfer of chemical groups (acetyl, phosphoryl, and adenyl groups)

β-lactamase make extensive cluster of enzymes that are hydrolyzing agents. The most common drug inactivation through is done through acetylation against aminoglycosides, chloramphenicol, and fluoroquinolones (Blair *et al*., 2015).

1.5.5 Drug efflux

chromosomal genes are present in Bacteria for efflux pumps; some are induced or overexpressed under the stimulus of environment or mutation causing modification in transport channel. The central task of efflux pumps is to get rid of any toxic substance. Many of the efflux pumps will transport variable quantity of multiple compounds (MDR). Carbon source has a greater role in activity of efflux pumps (Blair *et al.,*2014).

Efflux pumps are of many types but mainly important families of efflux pumps are five. These families are categorized on their energy source and structure.

i) ABC family ii) MATE family iii) SMR family iv) MFS

v) RND family.

All these families of efflux pump consitutue of one component pump which is needed to help the substrate to pass through the cytoplasmic membrane of cell. Rnd family of efflux family that have multi component pumps (Reygaert, 2018).

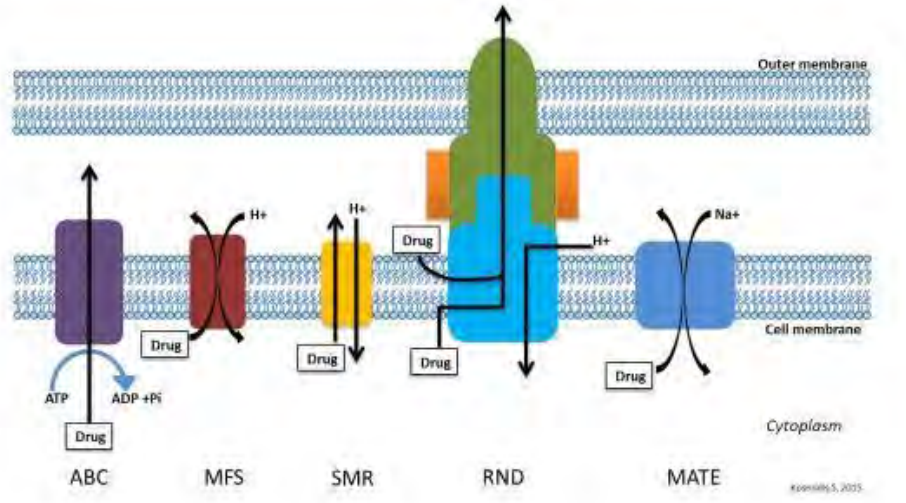


Figure: 1.3 structure of efflux pump families

MacB which extrudes macrolide drugs working as tripartite pump (MacAB-TolC) is the member of the ABC family (Jo I *et al*., (2017).

Penicillin was the first B-lactam antibiotic for commercial use (benzylpenicillin). As a result of their exceptional clinical success, four majogr groups of β-lactam antibiotics have emerged: There are three types of bicyclic structures: penicillins cephalosporins , and carbapenems. The monobactam is referred fourth class of monocyclic systems (Tooke *et al.,*2019)

1.6 ß-lactamases

Most commonly used antibiotics include β-lactam drugs, they have a main structure enclosed in 4 sided β-lactam rings. Three mechanisms are responsible for the bacteria to show resistance against β-lactam drugs.

(1) by modifying the drug binding to PBPs, altering existing PBPs or acquiring new PBPs.

(2) β-lactam drugs thrown out by efflux pumps

(3) β-lactamases (hydrolyzing the drug) (Pfeifer Y *et al.,* 2010)

The β-lactamases enzymes work by change in the β-lactam ring structure, at a particular site by process of hydrolyzing, it makes the ring to break and open . This doesn't allow binding to any PBPs. Gram negative bacteria uses the β-lactamases to inactive the β-lactam drugs. (Reygaert , 2018).

1.7 Classification of ß-lactamases

The β-lactamase enzymes are organized based on their molecular and functional characteristics. On the basis of structure there are four main groups (A, B, C, and D). βlactamase are composed of β & α-pleated sheets, having similar features making them strucullay alike.

Beta-lactamases are characterized on the basis of amino acid sequence similarity (Ambler classes A through D) or on substrate and inhibitor profile (Bush-Jacoby-Medeiros Groups 1 through 4) (BABIC *et al.,*2006).

a. Ambler molecular classification

The plan consists of four classes: A, B, C, and D. Metallo β-lactamases are classified as class B, while serine β-lactamases are classified as classes A, C, and D (Paterson and Bonomo, 2005; Ambler *et al.,*1991; Bush *et al.,*1995).

b. Bushacoby Medieros functional classification:

The functional similarities (substrate and inhibitor profiles) of enzymes are used to classify the beta lactamases as shown in table. (Bush and Jacoby, 2010).

Ambler classification system		
class A	penicillinases	TEMs, SHVs, PC1, CTX-Ms, SME-1, KPC-1
class B	metallo-beta-lactamases (zinc)	IMP-1, VIM-1, Ccr A
class C	cephalosporinases	AmpCs, CMY-2, ACT-1
class D	oxacillinases	OXA-1
Bush-Jacoby-Medeiros classification		
Group 1	cephalosporinases hydrolyze extended-spectrum cephalosporins; clavulanate resistant	AmpCs, CMY-2, ACT-1, MIR-1
Group 2	all clavulanic acids susceptible	
2a	penicillinase	PC1 from S. aureus
2b	broad-spectrum penicillinase	TEM-1, SHV-1, TEM-2
2be	ESBLs	SHV-2, TEM-10, CTX-Ms
2br	inhibitor resistant	TEMs, IRTs TEM-30, TEM-31
2c	carbenecillin hydrolyzin	PSE-1
2d	oxacillin hydrolyzing	OXA-10, OXA-1
2e	cephalosporinases inhibited by clavulanate	FEC-
2f	carbapenemases	KPC-1, SME-1
Group 3	metallo-beta-lactamases hydrolyze imipenem, inhibited by EDTA, resistant to clavulanate	IMP-1, VIM-1, Ccr A
Group 4	miscellaneous	

Figure 1.4 β-lactamases groups by Ambler and Bushacoby classification

On the basis of functional characteristics substrate specificity divides them into three groups.

i.Cephalosporins ii.Serine β-lactamases iii.Mettalo β-lactamases

i.TEM ii.SHV (sulphydryl

variable) iii.CTX (hydrolyzes

cefotaxime)

All structural groups in GNB produces β-lactamases while the gram positive bacteria produce β-lactamases only using Group A and Group B. These enzymes are present on bacterial chromosomes or acquired through a plasmid. Members of the *Enterobacteriaceae* family (gram negative bacteria) possess chromosomal β-lactamase genes. the *Enterobacteriaceae* family most commonly carry the Plasmid β-lactamase genes but can also be found in some species of gram-positive bacteria such as *Staphylococcus aureus*, *Enterococcus faecalis*, and *Enterococcus faecium* (Reygaert WC, 2018).

β-lactamase in *E. coli is* encoded for the ampicillin resistance *ampC* gene. Low level of AmpC gene is expressed in *E. coli* but mutation in AmpC causes overexpression.

1.7.1 Plasmid borne β-lactamases

Plasmid carried β-lactamases have a range of β-lactamase genes. If these plasmids carried βlactamase is cephalosporins resistant then this class β-lactamase is called ESBLs, and along with OXA SHV, CTX-M and TEM. *E. coli* exhibits CTX-Ms in UTI isolates. The ESBLs are sensitive towards β-lactamase inhibitors. Whearas they are ressiatant to most of variable range of drugs available in market. β-lactamase administered in combination with a β-lactam drug synergistically works best (Bevan *et al.,*2017).

1.7.2 Carbapenemases

Carbapenemases are an enzyme family that can hydrolyze all -lactam drug classes, including carbapenems, which are regarded antibacterial medications of last resort (Nordmann *et al.,*2011). In recent times β-lactamases have become vigorous against carbapenems, which are found in the *Enterobacteriaceae*. Carbapenemases types are as follows;

i. *Klebsiella pneumoniae* carbapenemases (KPCs) ii.

Carbapenem-Resistant Enterobacteriaceae (CRE) enzymes)

iii. lactamases (MBLs) in Class B, and they have the ability to

hydrolyze all β-lactam drugs but acannot inactivate the β-

lactamase inhibitors (Pfeifer *et al.,*2010).

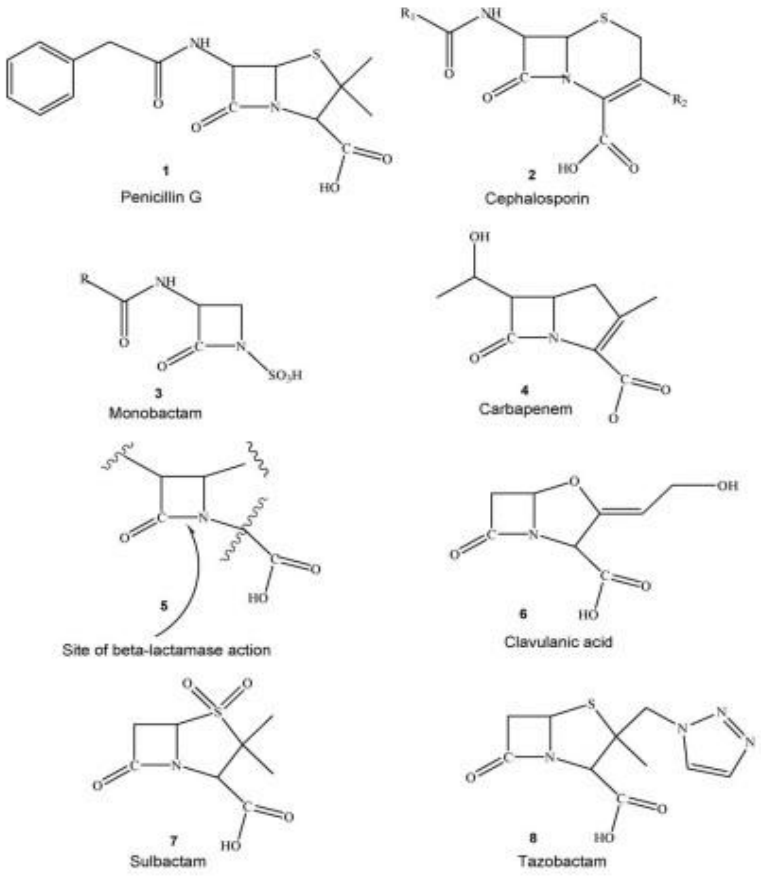


Figure 1.5 Types of β-lactamases

1.7.3 β-lactamases

CTX-M enzymes are the most credited among other ESBLs, and widespread amongst *Enterobacteriaceae*. *E. coli* was the first organism which exhibited the presence of geneCTXM was as stated in late 20th century. After that *E. coli* strains have been reported to isolate from community-acquired infections and nosocomial infections, carrying the CTX-M gene (Smet *et al.,*2010).

Genetic assessment of CTX-M genes showed that gene expression and its dissemination was controlled by the gene present in up-stream part of promoter region in gene. While the downstream region of *bla*$_{\text{CTX-M}}$ region showed very little to none in selection of the gene.

showed in the study that most of the CTX-Ms hydrolyze cefotaxime better than the ceftazidime *(*Bajaj *et al.,*2016).

1.7.4 AmpC β-Lactamases

The AmpC resembles the ESBLs β-lactamases; because of hydrolytic properties. AmpC has a upper hand as they show better hydrolytic activities against cefoxitin and cefotetan. Both these drugs belongs to Cephamycins. cloxacillin and boronic acid inhibits the AmpC activity (Helmy and Wasfi, 2014). Regulation of AmpC gene in *E. coli* is regulated by AMC gene, AMR is absent. *E. coli* have non induceable AmpC phenotype unlike all the other members of Enterobacteriaceae Jaurin *et al (*1981); They suggested that in all other Enterobacteriaceae family members the hyper production of ampC gene in greater number than 1. According to Haenni *et al.,*2014. hyper production is directed by mutations in the promoter region–42 to +81 in regard with ampC open reading frame.

1.7.5 Carbapenemases (metallo β-Lactamases)

The first & foremost options being ESBL and AmpC producing *E. coli* strains Carbapenemaes comes last when treating any infection. The mechanism of carbapenems resistance involves production of the carbapenemases which shows the most diverse hydrolytic potential (Bajaj *et al.,*2016).KPC, MBLs and OXA have been found in different *E. coli* strains.

1.8 Detection of β-lactamases

Phenotypic detection

1.8.1 Conventional laboratory methods

The methods used in the antibiotic susceptibility tests and elucidation of the results are presented from (CLSI) & (EUCAST). (Clinical and Laboratory Standards Institute [CLSI], 2014 European Committee on Antimicrobial Susceptibility Testing [EUCAST], (2014). Fire detection ESBL producing *E. coli* is done by using cephalosporins cefotaxime or ceftazidime as indicators by these organisations. Other than this E test are also available for the testing of the ESBL producing *E. coli*. These ESBL methods are incubated overnight and, but these have limitations, false negatives being one, specificity is affected second being false positive.

There are no standardized procedure for the AmpC β-lactamases from CLSI, but use of cefoxitin is highly effective in phenotypic detection of AmpC. Agar dilution procedure is also considered quite accurate but disk diffusion methods have proved to more precise and accurate. (Tenover *et al.,*2009).

Disk methods giver better precise results as compared to agar dilution methods.

1.8.2 Automated rapid detection

Commercially available methods to detect ESBL is VITEK 2, Phoenix ESBL test, and Microscan WalkAway-96 System. All automated system shows equally accurate results in detecting the ESBLs, but since its machines and there is to be about 5% of error expected hence to be more careful with results.

While using the automated system for ESBL, combination disk method have proved to be best choice, and further screening through the E-tests (Wiegand *et al.,*2007). The combination disk method is not that accurate and more consideration in result interpretation is to be given as there may be strains of AmpC β-lactamases (Bajaj *et al.,*20160

For AmpC β-lactamases the commercially available methods include Etest and MAST kit detects both plasmid borne and chromosomal AmpC.

1.9 Detection of β-Lactamases genes

1.9.1 PCR

There are many phenotypic methods available for the detection of ESBLs but molecular biology methods like PCR and sequencing are still the best methods for identifying and detecting as well as their differentiation of ESBL. Molecular methods show results even if βlactamase are expressed very low. these methods show exact mechanism of resistance. (Gazin *et al.,*2012). Improved speed, sensitivity, and specificity is achievable by multiplex and real time PCR. different families of AmpC β-lactamases are detected by Multiplex PCR (PérezPérez and Hanson, 2002).

According to van der Zee *et al.,*2014 multiplex PCR is used to detect genes that encodes different carba penemases from gram negative bacteria.

Sequencing done by PCR not only helps in identifying the genes, but it also helps in discovery of new genes differing in nucleotide sequence of β-lactamase.

1.9.2 High-throughput analyses

(ELISA and DNA microarrays) are used in high throughput analysis. ELISA is enzyme linked immunoaborbent assay, DNA microarrays that are commercially available are Check-Points assays (Check-Points Health, Wageningen, The Netherlands) and the Identibac AMR-ve assays (Alere GmbH, Cologne, Germany) enzyme-linked immunosorbent assay (ELISA) (Hyplex assays, Amplex Diagnostics, Gars, Germany) (Gazin *et al.,*2012).

Up-to-date of the Check-Points assays available in marketshows 100% sensitivity and specificity for the detection of ESBLs, plasmid-mediated AmpCs and carbapenemases (Bajaj *et al.,*2016).

In Hyplex assay multiplex PCR is done, after the PCR amplification the amplified DNA is hybridized to reverse probes by immobilizing the Amplified DNA on ELISA plates. This process makes the hybridization complexes are viewed peroxidase-conjugated antibodies. It's a prompt process so this method can be used directly on the samples but specificity when it comes to find a bla gene can be time taking process (Gazin *et al.,*2012).

1.10 Phylogenetic groups of *E. coli*

In 2000, Clermont and his associates worked up on a triplex PCR strategy which could help quickly identify *E. coli* isolates. The strategy utilized three markers, chuA and yjaA , and the TSPE4.C2 DNA sequences. The *E. coli* samples were allocated to a phylogroup based on the different combinations of the three amplicons. This method is still used in *E. coli* research today due to its effectiveness (Doumith *et al.,* 2012). *chuA* gene is crucial for iron transport in enterohemorrhagic *E. coli* enterohemorrhagic O157:H7 *E. coli*, *yjaA* gene identified in complete genomic sequence of *E. coli* K-12 (function is unknown), an anonymous DNA fragment designated TSPE4.C2 (Clermont *et al.,* 2000)

Based on the presence or absence of three genes, chuA, yjaA, and TspE4.C2, *E. coli* can be categorized into 4 phylogroups. These phylogroups are A, B1, B2 and D phylogenetic analysis of *E. coli* showed that all the extraintestinal virulent *E. coli* designated to group B2 worldwide. Most of the commensal strains are associated with either A group or B1. In 2013 Clermont and

colleagues discovered gene arpA, along with other the three genes and a quadruplex PCR was designed to classify an *E. coli* isolate from phylogroups A, B1, B2, C, D, E, F, and clade I (Clermont *et al.,*2013).

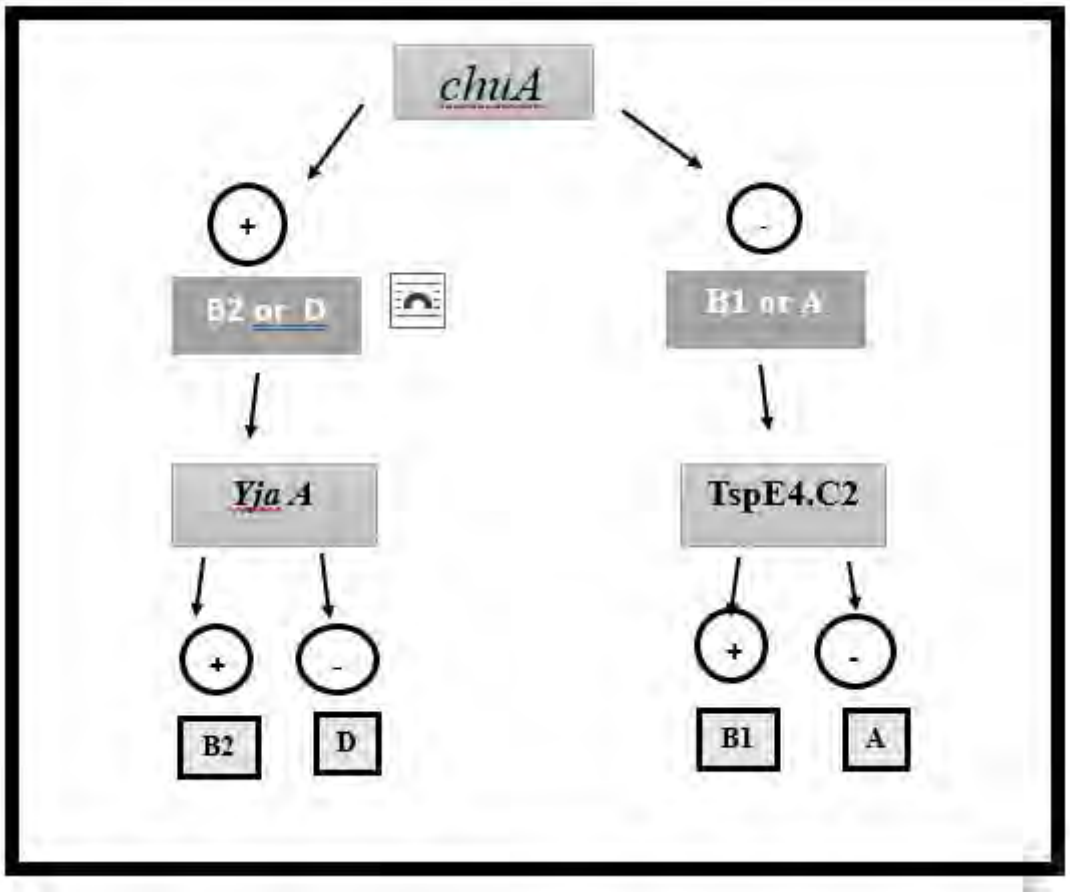


Figure 1.6 diagram of phylogenetic Grouping of *E. coli* (Clermont *et al*., 2000

Aims and Objectives

i. To study the antibiotic sensitivity and antibiotic resistance genes in the *E. coli* isolates responsible for causing infections in local human population

ii. To assess the phylogroups of these *E. coli* isolates.

Materials and Methods

Sample collection

The work was done at the Islamabad diagnostic center (IDC) and at the Animal Microbiology lab of the Department of Zoology at Quaid-i-Azam University Islamabad. The sampling was done between September 2022 to March 2023. A total of 40 urinary tract infection samples of patients were collected at IDC. These samples of UTI infections constituted of urine samples of patients of variant age periods, very few samples were obtained from body fluids. Further identification of *E. coli* was done through chemical and phenotypic means as described down below.

2.1 Identification and isolation of *E. coli*

After collecting the isolates, the urine samples were streaked onto CLED media agar directly using a sterile loop by using quantitative streak plate technique, in which a horizontal line is streaked and then multiple continuous verticle lines are streaked onto CLED agar to get isolated colonies. Yellow colonies with opaque edges were visible 18–24 hours of incubation at 37 C. After 24 hours, isolated colonies from CLED were streaked onto the **MacConkey agar,** (CM0007, Oxoid, UK) the medium used for the initial isolation of *E. coli* , which turns the medium pink and the colonies are also of pink color.

2.2 Grams' staining

Bacterial colony smears were prepared, and heat fixed. Gram staining was used, and the slides were examined under a microscope with an oil immersion at 100X magnification.

2.3 Antibiotic susceptibility test (Kirby –Bauer Disk Diffusion Method)

The antibiotic susceptibility test AST was performed using the Kirby –Bauer Disk Diffusion Method for determining the antibiotic resistance pattern of all *E. coli* isolates and the interpretation of results was done as described in the Clinical Laboratory Standards Institute Manual (CLSI, 2017; CLSI, 2015). The disc diffusion method was performed using Mueller Hinton agar (CM0471, Oxoid, UK) and 14 antimicrobial agents as listed in table 2.13. Fourteen different antibiotics were used to assess the pattern of *E. coli* antibiotic sensitivity. Out of 40

antibiotic drugs used no drug is proven 100% effective against *E. coli.* Based on the size of the zone formed around the antimicrobial drugs used, *E. coli* isolates were classified as sensitive, resistant, or intermediate.

Out of 40 antibiotic drugs used no drug is proven 100% effective against *E. coli* . Based on the size of the zone formed around the antimicrobial drugs used, *E. coli* isolates were classified as sensitive, resistant, or intermediate The Mueller Hinton agar plate was then allowed to come to room temperature. The isolated colonies were inoculated using a loop and mixed in normal saline to make a solution in clear suspension. The suspension turbidity was then adjusted to the McFarland standard value of 0.5. A sterile swab used for inoculation, was dipped into an inoculum tube. The swab was pressed against the tube's side to remove excess fluid. Inoculation on agar plate was done by "streaking the swab over the entire agar surface three times." For an even distribution of inoculums, the plate was rotated 60 degrees each time. After inoculating an agar plate with bacterial suspension, antibiotic discs (Bioanalyse/Oxoid, UK) were applied to the agar surface with forceps and incubated at 37°C for 24 hours. Following incubation, measurement of zones of inhibition was done by using a ruler and the results were interpreted as sensitive, intermediate and resistant (CLSI, 2015; CLSI, 2017). Following

CLSI guidelines, isolates showing zones of inhibition of ≤17 mm for cefpodoxime is potential ESBL producers and the isolates showing ≤17 mm zones of inhibition for cefoxitin are potential AmpC producers.

Table 2.1 Reference Values for AST

Name of antibiotic	Concentration in disc	S	I	R	Source
Cefpodoxime	10µg	≥21	18-20	≤17	CLSI
Augmentin	20/10 µg	≥18	14-17	≤13	CLSI
Cefoxitin	30 ug	≥18	15-17	≤17	CLSI
Ampicillin	10 ug	≥17	14-16	≤13	CLSI
Fosfomycin	200 ug	≥21		<21	CLS1

Gentamicin	10 ug	≥16	13-15	≤12	CLS1
Imipenem	10 ug	≤16	14-15	≥13	CLS1
Meropenem	10 ug	≤16	14-15	≥13	CLS1
Minocycline	30 ug	≥16	13-15	≤12	CLS1
Norfloxacin	10 ug	≥16	13-16	≤12	CLS1
Sulzone	250 ug	≥17	13-16	≤12	CLS1
Trimethoprim-sulfamethoxazole	1.25/23.75 ug	≥16	11-15	≤10	CLS1
Ciprofloxacin	5 ug	≥21	16-20	≤15	CLS1
Levofloxacin	5 ug	≥17	14-16	≤13	CLS1

2.4 Phenotypic confirmation of AmpC and ESBLs producers by MAST Discs:

MAST discs (D68C AmpC and ESBL Detection Disc Set (MAST Group Ltd, UK) were used to confirm the AmpC and ESBL enzyme producers after the initial screening for the production of these enzymes. A pure, fresh 3,4 colonies of the test organism in saline water were used, and saline solution having McFarland 0.5 opacity standard. sterile swab was used to evenly spread the suspension across the surface of MHA agar plate or Iso sensitest agar (CM0471, Oxoid, UK), in accordance with clinical and Laboratory standard Institute guidelines (CLSI).

Using a sterilized syringe or The MAST DISCMASTER, insert one MASTDISCS ® Combi AmpC and ESBL enough space between discs allowed for the formation of clearly defined zones of inhibition. Incubation was done at the temperature of 37°C for a day. Measuring and recording the diameter of any inhibition zones to the nearest whole millimeter. Discs showing no zones of inhibition were measured as 6 millimeters

2.5 Polymerase Chain Reactions (PCRs)

2.5.1 Genomic DNA Isolation

The DNA of *E. coli* isolates was extracted using **the boiling method.** Four to five colonies were taken from with the help of sterile loop from the agar culture/ a sterile pipette tip and suspended in 200 µl nuclease free PCR water in a PCR tube and gently mix with pipette. For 10 minutes, heated in a heating block (Shaking Micro incubator, ESCO, USA) at 100 °C. Immediately placed the tubes in ice for 5 minutes to cool. For 10 minutes, centrifuged PCR tubes at 14000 rpm. Supernatant was removed and used as template DNA (DNA prepared by this method is supposed to contain genomic as well as plasmid DNA) in PCRs.

2.5.2 PCR for confirmation of *E. coli*

PCR was used to confirm the *E. coli* after the phenotypic confirmation of *E. coli* . *uidA* gene described previously Tantawiwat *et al* (2005) was used for the *E. coli* isolates.

Table.2.2. Reagents used for PCR of *uidA* gene.

Sr. No	Reagents	Volume
1.	Template DNA	2.5 µl
2.	Forward primer (*uidA-F*) (10 µM)	2.5 µl (1 µM)
3.	Reverse primer (*uidA-R*) (10 µM)	2.5 µl (1 µM)
4.	10X PCR buffer	2.5µl
5.	25 Mm $MgCl_2$ (Thermo Scientific)	3 µl (1.5 mM)
6.	*Taq* DNA polymerase (Thermo Scientific)	0.5 µl (2.5 U)
7.	10 mM dNTPs	0.5 µl (0.2 mM)

8.	Water	11 µl (up to 25 µl)
9.	Total	25 µl

2.5.3 PCR conditions for *uidA* gene

PCR amplification was performed in a thermal cycler (REF 4483636, Applied biosystem, ProFlex PCR system, Thermo fisher scientific, USA) with 5 minutes of initial denaturation at 94°C, 30 cycles of denaturation at 94°C for 30 seconds, 30 seconds of annealing at 62.5°C, and 1 minute of extension at 72°C. The final cycle of extension was carried out for 10 minutes at 72°C.

Following the PCR, the PCR product was electrophoresed for 40-50 minutes at 100 volts in 1% agarose gel using 50bp DNA ladder (SM0373, Thermo scientific) as the DNA size marker. Under UV light, bands of the expected size were viewed and photographed using a gel documentation system (OmniDOC)

2.6 PCRs for detection of antibiotic resistance genes

2.6.1 Multiplex PCR for detection of AmpC genes

Table.2.3. Reagents used in the multiplex PCR for detection of AmpC *bla* genes.

Sr. No	Reagents	Volume
1.	Template DNA	5 µl
2.	**20 uM each)** Primers MOXF, MOXR, CIT-F, CIT-R,DHA-F and DHA-R:	0.75 µl (4.5 µl total)
3.	**(20 uM each)** Primers ACC- F, ACC-R, EBC-F, EBC-R:	0.625 ul each (2.5 ul total)
4.	**(20 uM each)** Primers FOX-F, FOX-R	0.5 ul each (1 ul total)
5.	10X PCR buffer using KCl	2.5µl

6.	25 mM $MgCl_2$ (Thermo Scientific)	1.5 µl (1.5 mM)
7.	*Taq* DNA polymerase (Thermo Scientific)	0.5 µl (2.5 U)
8.	10 mM dNTPs	0.5 µl (0.2 mM)
9.	Water	7 µl (up to 25 µl)
10.	Total	25 µl

2.6.2 Cycling conditions

Initial denaturation at 94℃ for 3 minutes, followed by 25 cycles of denaturation at 94 ℃for 30 sec, annealing at 64℃ for 30 sec and extension at 72℃ for 1 min. Then one-time final extension at 72℃for 7 min (Pérez-Pérez and Hanson, 2002).

2.7 PCRs for detection of ESBLs genes (*bla* TEM, *bla* SHV, *bla* CTX)

Table 2.4: The reagents used in the PCR for *bla* TEM, *bla* CTX, R, and *bla* SHV.

Sr. No	Reagents	Volume
1.	Genomic DNA	5 µl
2.	10X buffer (NH_4SO_4)	2.5 µl
3.	10 uM Forward Primer: **(**ESBL-TEM-F / ESBL- SHV-F / ESBL-CTX-M-F**)**	2.5 ul
4.	10 uM Reverse Primer: (ESBL-TEM-R / ESBL- SHV-R/ ESBL-CTX-M-R)	2.5 ul
6.	25 mM $MgCl_2$	0.8 µl

7.	*Taq* DNA polymerase (Thermo Scientific)	0.5 µl (2.5 U)
8.	10 mM dNTPs	0.5 µl
9.	Water	10. 7 µl
10.	Total	25 µl

2.7.1 Cycling conditions

All three PCRs done separately; it is not multiplex PCR. Initial denaturation at 94℃ for 5 minutes, followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing at 64℃ for 30 sec and extension at 72℃ for 2 min. Then one-time final extension at 72℃ for 10 minutes and hold at 22℃ in the PCR machine for infinite (Chen *et al*., 2010).

2.7.2 Agarose gel electrophoresis for visualization of PCR products

Just after the PCR, gel electrophoresis had been used to visualize the PCR amplification of the targeted gene. A 100bp DNA marker (Thermo scientific) was used, as well as the PCR product was electrophoresed for 60 minutes at 100 volts on a 1 percent agarose gel that included ethidium bromide. Under UV light, bands of the expected size were visualized and photographed using a gel documentation system (OmniDOC).

2.8 PCR for phylogrouping of *E. coli*

The phylogrouping of the *E. coli* was achieved by means of a previously described method (Clermont *et al*, 2000). Following the positive and negative PCR results, four groups were formed and interpreted. Phylo-grouping was done using the chu A, YjaA, and Tspe42c genes.

Table 2.5: The reagents used in PCR for phylogrouping

Sr. No	Reagents	Volume
1.	Genomic DNA	5 µl
2.	10 uM ChuA-R primer	1 µl

3.	10 uM ChuA-R primer	1 µl
4.	10 uM YjaA-F primer	1 µl
5.	10 uM YjaA-R primer	1 µl
6.	10 uM TspE4C2-F Primer	1 µl
7.	10 uM TspE4C2-R primer:	1 µl
8.	10X PCR buffer	2.5µl
9.	25 mM $MgCl_2$	1.5 µl
10.	*Taq* DNA polymerase (Thermo Scientific)	0.5 µl
11.	10 mM dNTPs	0.5 µl (0.2 mM)
12.	Water	9 µl
	Total	25 µl

2.8.1 Cycling conditions

Initial denaturation of DNA was done for 5 minutes at the temperature of 94℃ , this denaturation was followed by denaturation cycles 30 in total at 94℃ for 30 sec, annealing of DNA was done at 55℃ for 30 sec and extension at 72℃ for 30 sec. Then one-time final extension at 72℃ for 7 minutes and hold at 22℃ in the PCR machine for infinite time.

2.8.2 Agarose gel electrophoresis for visualization of PCR products

After PCR, gel electrophoresis was carried out to visualize the PCR amplification of the targeted gene. 50bp DNA marker (Thermo scientific) was used and product of PCR was electrophoresed on 1% agarose gel having ethidium bromide for 180 minutes at 110 volts. Bands of the expected size were visualized under UV light and photographed by using gel documentation system (OmniDOC).

2.9 Primers and the amplicon size of *uidA*

Table 2.6: Primers and amplicon size of *uidA*

Name Of Isolate	Gene	Sequence (5′ → 3′)	Fragment Size (bp)
E. coli	*uidA-F* *uidA-R*	5'- TGGTAATTACCGACGAAAACGGC3 '- 5'-ACGCGTGGTTACAGTCTTGCG3'-	147bp

2.10 AmpC Primers

Table 2.7: The AmpC Primers and their PCR product sizes

Gene	Sequence (5′ → 3′)	Product size (bp)
AmpC-MOX-F **AmpCMOX-R**	GCTGCTCAAGGAGCACAGGAT CACATTGACATAGGTGTGGTGC	520
AmpC-CIT-F **AmpCCIT-R**	TGGCCAGAACTGACAGGCAAA TTTCTCCTGAACGTGGCTGGC	462
AmpC-DHA-F **AmpC-DHA-R**	AACTTTCACAGGTGTGCTGGGT CCGTACGCATACTGGCTTTGC	405
AmpC-ACC-F **AmpCACC-R**	AACAGCCTCAGCAGCCGGTTA TTCGCCGCAATCATCCCTAGC	346
AmpC-EBC-F **AmpCEBC-R**	TCGGTAAAGCCGATGTTGCGG CTTCCACTGCGGCTGCCAGTT	302
AmpC-FOX-F **AmpCFOX-R**	AACATGGGGTATCAGGGAGATG CAAAGCGCGTAACCGGATTGG	190

2.11 ESBL Primers

Table 2.8: The ESBL Primers and their PCR product sizes.

Gene	Sequence (5′ → 3′)	Product Size (bp)
ESBL-TEM-F **ESBL-TEM-R**	ATAAAATTCTTGAAGACGAAA GACAGTTACCAATGCTTAATC	1086
ESBL-SHV-F **ESBL-SHV-R**	GGGTTATTCTTATTTGTCGC TTAGCGTTGCCAGTGCTC	567
ESBL-CTXM- F **ESBL-CTXM- R**	CGCTTTGCGATGTGCAG ACCGCGATATCGTTGGT	550

2.1 Table 2. *E. coli* Phylo Primers

Table 2.9: The *E. coli* Phylo Primers and their PCR product sizes

Gene	Sequence (5′ → 3′)	Product size
***E. coli* -chuA-F *E. coli* -chuA-R**	GACGAACCAACGGTCAGGAT TGCCGCCAGTACCAAAGACA	279bp
***E. coli* -yjaA-F *E. coli* -yjaA-R**	TGAAGTGTCAGGAGACGCTG ATGGAGAATGCGTTCCTCAAC	211bp
***E. coli* -TspE4.C2F** ***E. coli* -TspE4.C2R**	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152bp

2.2 Antibiotics used in AST against *E. coli* isolates

AST was carried out using amoxicillin-clavulanic acid (20/10µg), Cefoxitin (30 µg), Cefpodoxime

Table 2.10: Antibiotics, their Class, concentrations and Symbols used in AST against *E. coli* isolates

Sr. No.	Antibiotics	Class	Symbol	Concentration (µg)
1.	Cefoxitin	Cephalosporin	FOX	30µg
2.	Cefpodoxime	Cephalosporin	CPD	10µg
3.	Augmentin	Penicillin	AMC	30 µg
4.	Ampicillin	Penicillin	AMP	10 µg
5.	Imepenem	Carbapenums	IPM	30µg
6.	Meropenem	Carbapenums	MEM	30µg
7.	Minocycline	Tetracycline	MH	30µg
8.	Gentamicin	Aminoglycoside	CN	10µg
9.	Fosfomycin	……	FOS	50µg
10.	Norfloxacin	Flouroquinolones	NOR	10µg
11.	Sulzone	Cephalosporin	SCF/SUL	105µg
12.	Trimethoprim-sulfamethoxazole	Sulphonamides	SXT	2.5 µg
13.	Ciprofloxicin	Qunilones	CIP	5 µg
14.	Levofloxacin	Qunilones	LEV	5 µg

2.3 The contents and formulation of MAST Discs

Table 2.11 The contents and Formulation of MAST Disc

Disc Name	Antibiotics
A	Cefpodoxime 10µg
B	Cefpodoxime 10 µg + ESBL inhibitor disc
C	Cefpodoxime 10 µg + AmpC inhibitor discs
D	Cefpodoxime 10 µg + ESBL inhibitor + AmpC inhibitor discs

RESULTS

3.1 Cultural characterization and colony morphology of *E. coli*

All the clinical urine samples were found to contain *E. coli* and n other bacteria were recovered from the urine samples of patients having some UTI infection. The isolates were categorized according to gender and age distribution as given in tables 3.1& 3.2.

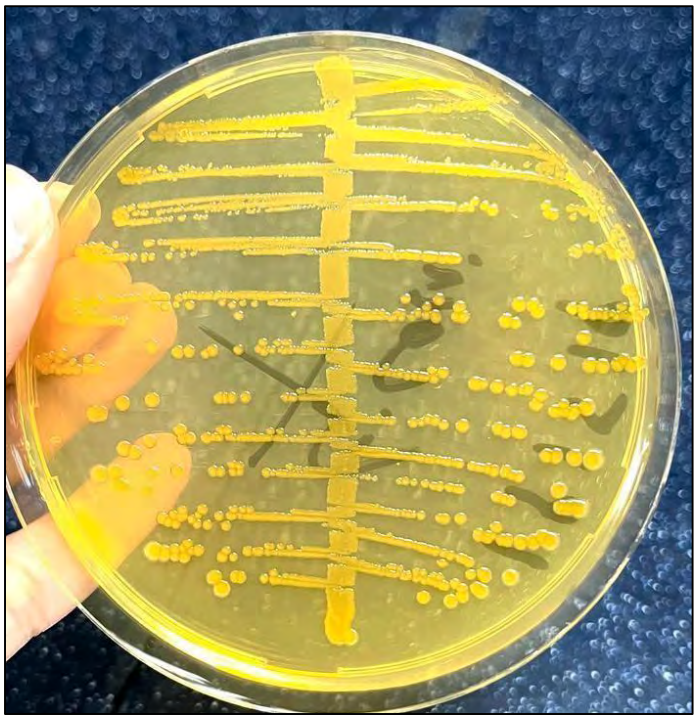


Figure 3.1 *E. coli* colonies round and yellow with opaque edges on CLED agar

Figure 3.2 Pink and round colonies of *E. coli* on MacConkey agar

3.2 Grams' staining:

By picking the pink colonies from MacConkey agar, the Gram 's staining was done. The results showed, pink color rod shaped bacteria when viewed under 100X lens under an oil immersion.

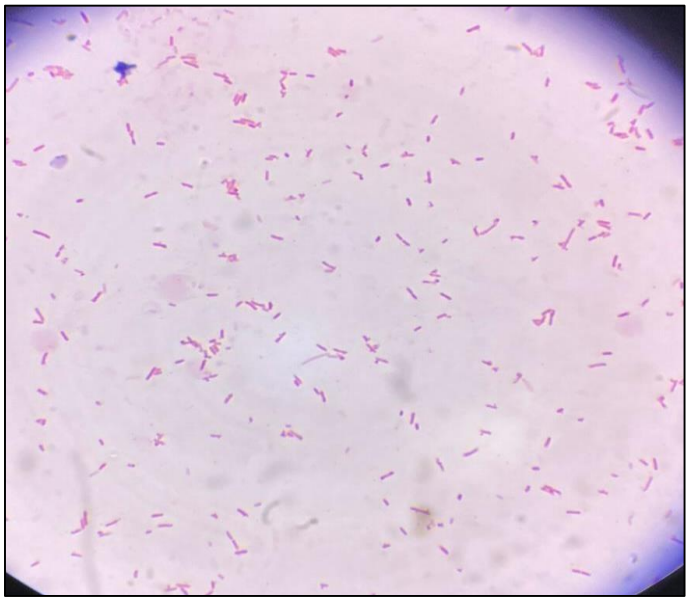


Figure 3.3 The results of Gram's staining showing pink rods

3.3 Confirmation *E. coli* through PCR

All the 40 isolates found to be *E. coli* through staining and growth characteristics were confirmed to be *E. coli* through the *uidA* PCR (Fig. 3.4).

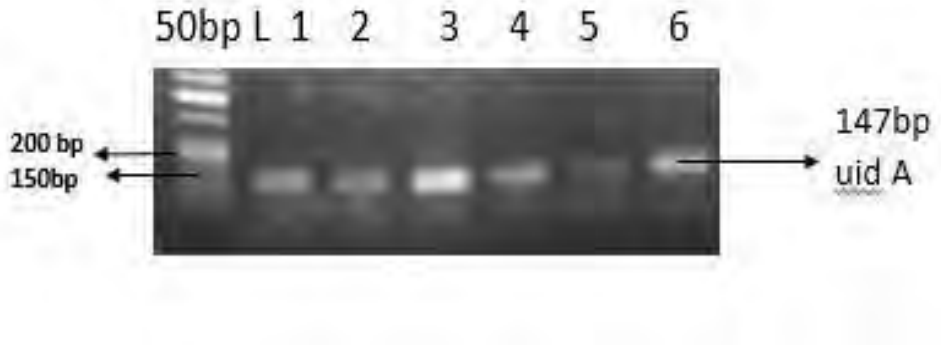
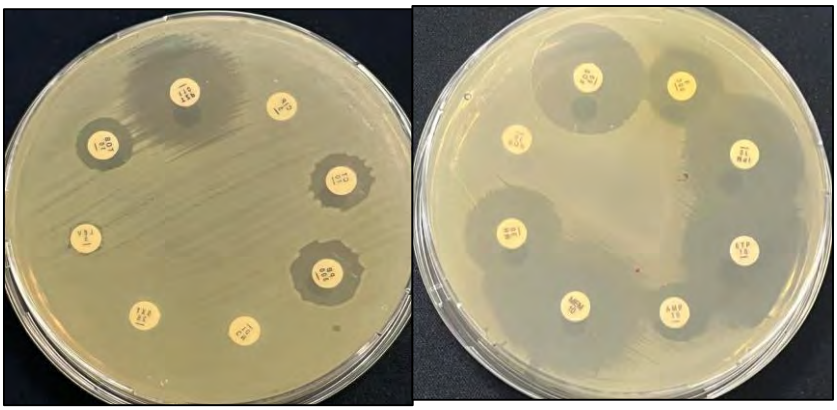


Figure 3.4 Agarose gel Electrophoresis for detection of *uidA* gene. 1-6 represent isolates positive for uid*A* gene L: 50 bp DNA ladder, (50 bp;L ThermoFisher)

3.4 Results of AST

Results of AST against different antimicrobial drugs was performed on 40 *E. coli* isolates. *E. coli* antibiotic sensitivity pattern isolated from human urine samples showed that they had high resistance for Ampicillin (85%), Ciprofloxacin and Levofloxacin (62.5%). The isolates showed lowest resistance against Minocycline (0%) Meropenem and Sulzone that is (2.5%). Antibiotics which showed more sensitivity against *E. coli* was Sulzone and Meropenem (97.5%) and Imepenem (95%) sensitivity. The resistant pattern of different antimicrobials is given in table 3.2.



3.5 AST results showing sensitivity and resistant against different antimicrobial drugs used.

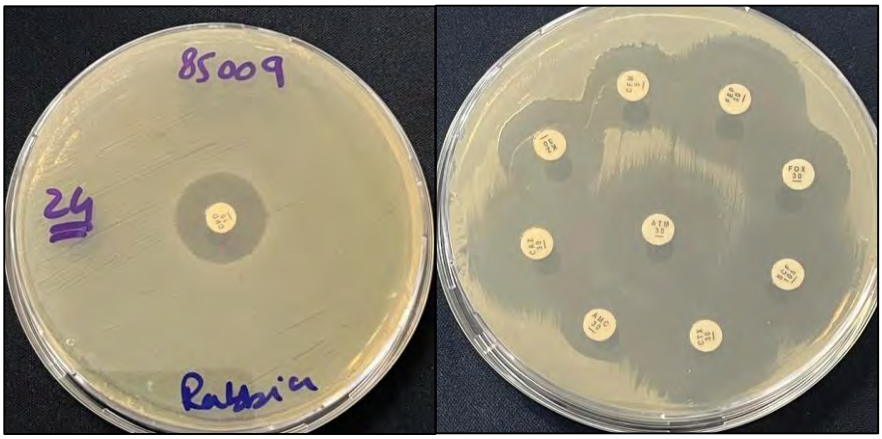


Figure 3.6 AST results, showing sensitivity towards Cefpodoxime and Cefoxitin

Table 3.3 *E. coli* Isolates AST Results

Sr. No.	Antibiotics	Total no. of isolates	Sensitive(%)	Intermediate (%)	Resistant(%)
1.	Cefpodoxime	40	13(32.5%)	3(7.5%)	24(60%)
2.	Augmentin	40	26(65%)	6(15%)	8(20%)
3.	Cefoxitin	40	35(87.5%)	0	5(12.5%)
4.	Ampicillin	40	6(15%)	0	34(85%)

5.	Fosfomycin	40	32(80%)	0	8(20%)
6.	Gentamicin	40	19(47.5%)	8(20%)	13(32.5%)
7.	Imipenem	40	38(95%)	0	2(5%)
8.	Meropenem	40	39(97.5%)	0	1(2.5%)
9.	Minocycline	40	30(75%)	10(%)	0
10.	Norfloxacin	40	15(37.5%)	1(2.5%)	24(60%)
11.	Sulzone	40	39(97.5%)	0	1(2.5%)
12.	Trimethoprim-sulfamethoxazole	40	9(22.5%)	1(2.5%)	30(75%)
13.	Ciprofloxacin	40	12(30%)	3(7.5%)	25(62.5%)
14.	Levofloxacin	40	11(27.5%)	4(10%)	25(62.5%)

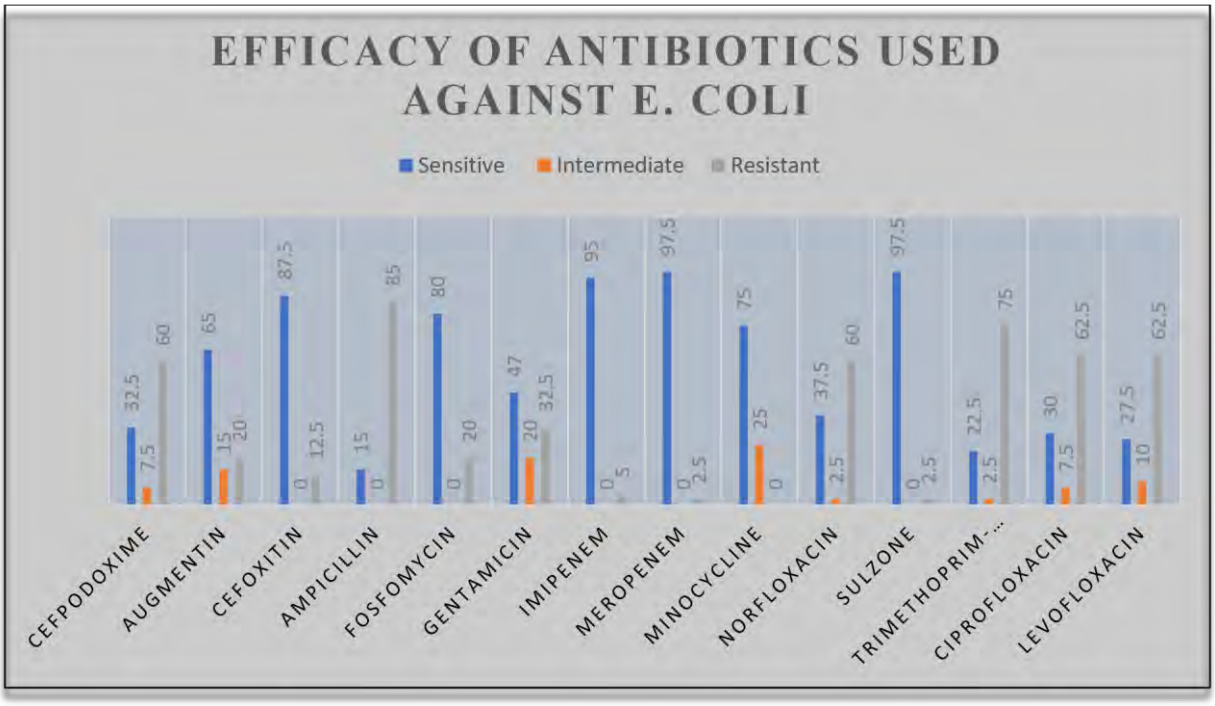


Figure 3.7 Graphical representation of the AST results.

3.5 Phenotypic confirmation of AmpC/ ESBL *E. coli* using MAST disks:

After the initial screening of the isolates for potential AmpC and ESBL by resistance to Cefoxitin and Cefpodoxime respectively, the isolates were further subject to confirmation of ESBL and AmpC production using the MAST kit. Five samples showed resistance to FOX and 24 *E. coli* samples showed resistance against CPD. These *E. coli* isolates were further confirmed by using MAST discs. Four discs were used: A (CPD 10µg), B (CPD10 + ESBL inhibitor discs), C (CPD 10 µg + AmpC inhibitors) and D (CPD 10µg + ESBL inhibitor + AmpC inhibitor discs). Included in the MAST disc pack were used. Interpretation was performed according to the manufacturer's direction. Two isolates were positive for AmpC and 14 isolates were positive for ESBL producers through the MAST kit analysis.

Figure 3.8 A representative sample positive for AmpC

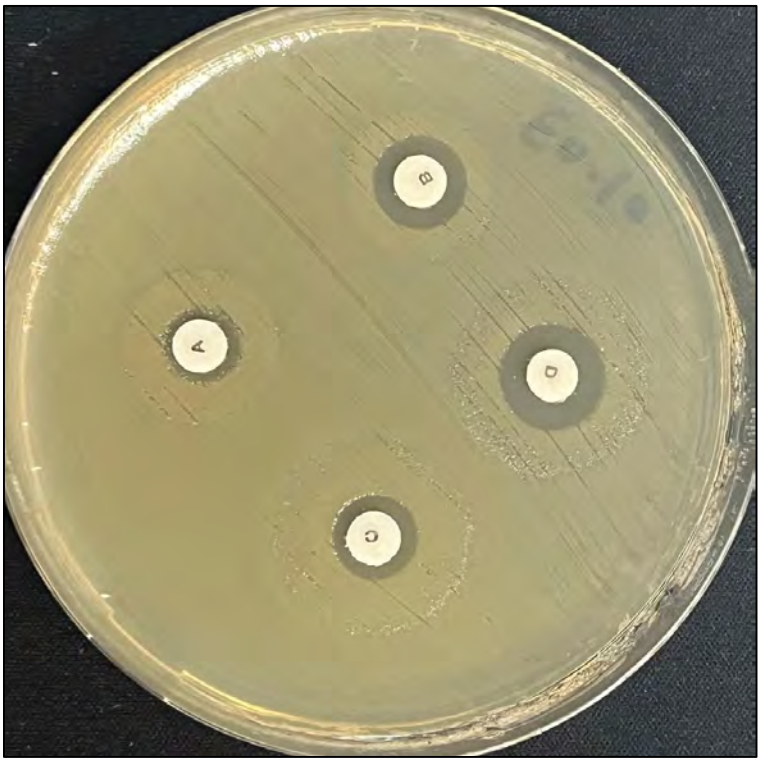


Figure 3.9 A representative sample positive for ESBL

3.6 Molecular detection of ESBL genes through PCR:

Based on the results of AST, and the results by phenotypic confirmation by using MAST discs, the isolates that were found to produce ESBL were further screened for some important selected ESBL genes through PCR. In total 23 samples were processed for ESBL genes. ESBL gene *bla* CTX-M showed positivity in 14 out of 23 isolates. All samples showed negativity for *bla* SHV and *bla* TEM.

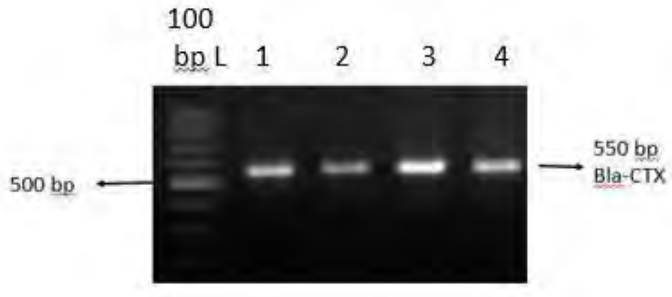


Figure 3.10 Agarose gel electrophoresis of PCR for detection of *bla*CTX-M (550 bp) L: 100 bp DNA ladder (1kb bp;L ThermoFisher), 1-4 represent isolates positive for *bla*CTX-M gene.

Gel electrophoresis showed the gene positive for *bla*-CTX-M in sixteen (16) isolates. Isolate that was positive for both AmpC and ESBL didn't show any *bla*-CTX-M gene. Gel picture in figure 3.10 shows *bla*-CTX-M gene.

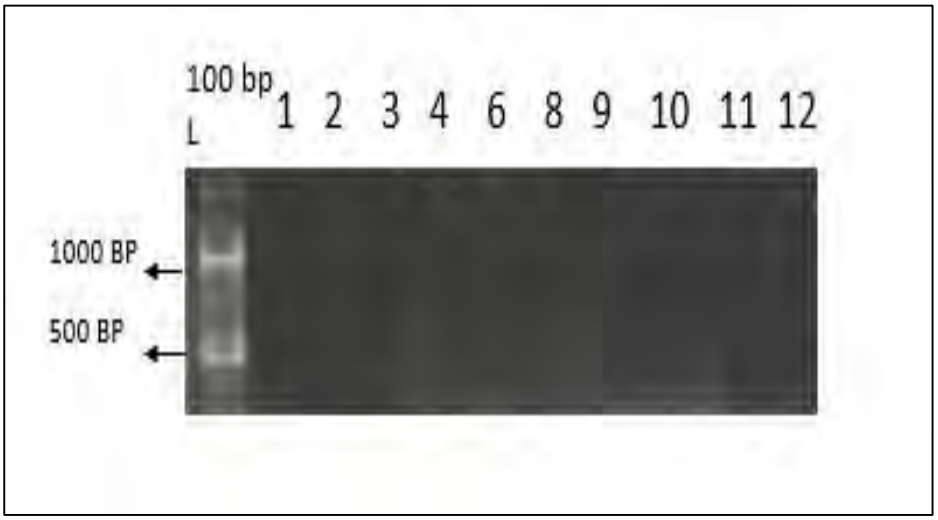


Figure 3.11 Agarose gel electrophoresis for detection of *bla*-TEM gene. L: 100 bp (100 bp L ThermoFisher) no positive isolates were observed

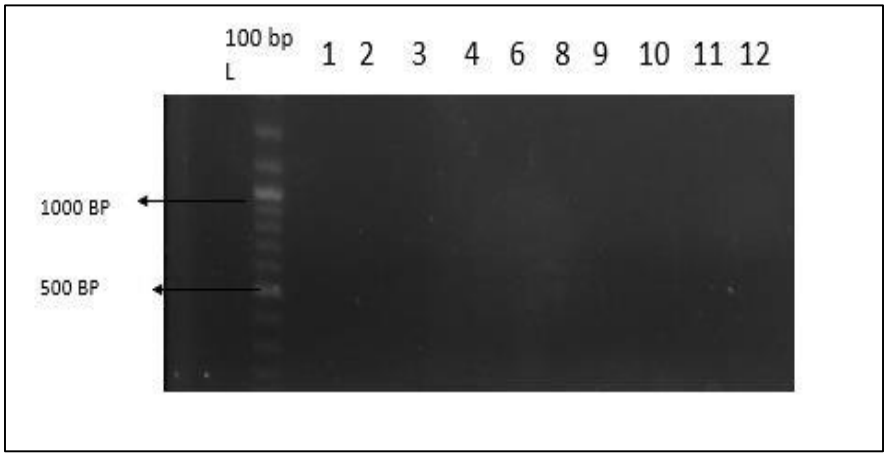


Figure 3.12 Agarose gel electrophoresis for detection of *bla*-SHV gene. L: 100 bp.

(100 bp L ThermoFisher) No positive results were observed.

3.7 Molecular detection of AmpC genes through PCR:

The results showed amplification in AmpC-FOX AmpC- CIT and AmpC-MOX. Three samples showed the presence of AmpC-FOX AmpC- CIT and AmpC-MOX genes here was no amplification for all other AmpC genes.

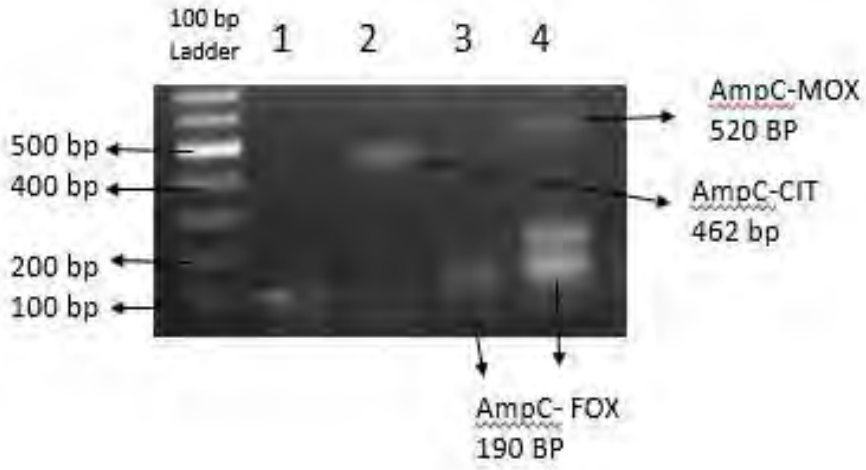


Figure 3.13 Agarose gel electrophoresis for detection of AmpC-MOX (520bp), AmpC-CIT (462bp), AmpC-DHA (405 bp), AmpC-ACC (346bp), AmpC- EBC (302bp), AmpC-FOX (190bp). L: 100 bp. (1kb bp L ThermoFisher). 1-4 represent isolates positive for AmpC genes.

3.8 Molecular Phylogenetic Analysis of *E. coli* isolates

E. coli were divided into four phylogroups using a triplex PCR for phylogenetic grouping, as indicated in Table 3.4. The results showed that the phylogroup B1 was found to be the most prevalent (33%), followed by group A (32%) and phylogroup B2 virulent extra-intestinal showed the frequency (30%). The phylogroup D showed lesser prevalence and that is (5%). Strains were grouped according to Clermont *et al*., (2000).

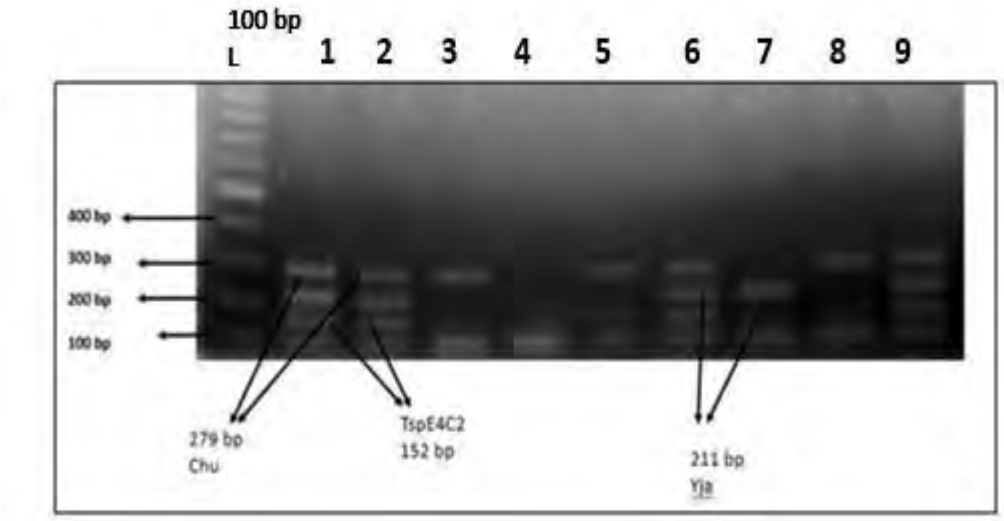


Figure 3.14 The DNA amplification of chu A, yjaA, TspE4C2 genes showed 279bp, 211bp and 152 bp respectively, L; Ladder 100 bp. (1kb bp L ThermoFisher)

Table 3.1 Genderwise distribution of UTI samples collected from IDC lab

Gender	**Number of samples (n)**	**Percentage %**
Male	13	32.5%
Female	22	55%%
Children	5	12.5%
Total	40	100%

n= number %= percentage

Table 3.2 Agewise distribution of UTI samples collected from IDC lab

Age	Male N (%)	Female N (%)	Percentage N (%)
<18	1(2.5)	7(17.5)	8(20)
18-35	2(5)	3(7.5)	5(12.5)
35-55	1(2.5)	7(17.5)	8(20)
55-65	2(5)	5(12.5)	7(17.5)
>65	7(17.5)	5(12.5)	12(30)
Total	13(32.5%)	27(67.5%)	40(100%)

n= number %= percentage

Table 3.4. Cumulative results showing phyo-group, B-lactamase production, AMR genes and AST results of the 40 *E. coli* isolates.

Sample ID	Phylo-group	MAST Kit Result	ESBL Genes	AmpC genes	AST Results		
					S	I	R
EU1	B2	ESBL+	-	-	CPD, AMC, CN, CIP, AMP, FOS, NOR, LEV, MH		FOX, IPM, MEM, SCF
EU2	B2	-	-	-	FOX, AMC, CN,CIP,IPM,FOS, NOR,SCF, LEV, MEM, MH	SXT	CPD, AMP
EU3	**D**	**-**	**-**	**-**	MEM, IPM, FOS, SXT	MH, CN	CPD, FOX, AMC,CIP, AMP NOR, SCF, LEV
EU4	B2	ESBL+	-	-	CPD, CN, CIP,AMP, SXT, LEV, NOR, FOD		MEM, MH, SCF, IPM, AMC, FOX
EU5	**B2**	**-**	**-**	**-**	CN, IPM, MEM, MH, FOS, SCF,	FOX, AMC,	CPD, CIP, NOR, SXT, LEV, AMP
EU6	B2	AmpC+	bla CTX-M	AmpC-CIT	MEM, MH, SCF,		CPD, FOX, CIP, AMP, FOS, NOR, LEV, SXT, IPM, AMC, CN
EU7	**A**	**-**	**-**	**AmpC-FOX**	CPD, IPM, MEM, SCF, MH		AMC, AMP, FOX, FOS, NOR, LEV, SXT,

EU8	D	ESBL+	-		FOX, AMC, AMP, FOX, SCF, MEM, IPM, MH		CPD, FOS, CN, CIP, NOR, LEV, SXT,

Antibiotic Sensitivity and Antibiotic Resistance Genes in

EU9	**B2**	**ESBL+**	**bla CTX-M**	**AmpC-FOX AmpC-MOX**	MEM, MH, SCF	AMC	CPD, FOX, AMP, IPM, CN, CIP, NOR, LEV, SXT, FOS
EU10	B2	AmpC+ ESBL+	-	-	CN, CIP, IPM, MEM, FOS, NOR, SCF, LEV, SXT	MH	CPD, FOX, AMC, AMP,
EU11	**A**	**ESBL+**	**-**	**-**	FOX, IPM, MEM, SCF, MH		CPD, AMC, AMP, NOR, SXT, CN, CIP, FOS, LEV
EU12	A	-	bla CTX-M	-	FOX, CN, MEM, IPM, SCF, FOS, SCF	AMC, MH	CPD, CIP, NOR, LEV, SXT,
EU13	**A**	**ESBL+**	**-**	**-**	FOX, AMC, MH, MEM, IPM, SCF	CN	CPD, AMP, CIP, NOR, LEV, SXT, FOS
EU14	A	-	-	-	CPD, FOX, AMC, AMP, IPM, MEM, MH, FOS, SCF		CN, CIP, NOR, LEV, SXT
EU15	**A**	**ESBL+**	**-**	**-**	FOX, AMC, CN, MEM, CIP, FOS, LEV, SXT, SCF, NOR	MH,	CPD, IPM, AMP,

EU16	A	ESBL+	bla CTX-M	-	MEM, AMC, IPM, FOS, SCF, NOR, MEM,	CN, CIP	CPD, FOX, SXT, AMP, LEV, MH
EU17	**A**	**-**	**-**	**-**	CPD, FOX, AMC, CIP, IPM, FOS, NOR, SCF, LEV, MEM	CN, MH,	AMP, SXT

EU18	A	-	-	-	CPD, FOX, AMC, CN, IPM, FOS, SCF, MEM	CIP, NOR, LEV	AMP, SXT, MH
EU19	**B1**	**ESBL+**	**bla CTX-M**	**-**	MH, MEM, SCF, NOR, IPM, CIP, AMC, FOX		CPD, AMP, FOS, SXT, LEV, CN
EU20	B1	ESBL+	bla CTX-M	-	FOX, IPM, FOS, SCF, MEM, MH	AMC,	CPD, CN, CIP, AMP, NOR, SXT, LEV,
EU21	**B1**	**-**	**-**	**-**	CPD, FOX, AMC, CN, IPM, FOS, NOR, SCF, MEM,	CIP, LEV, MH	AMP, SXT,
EU22	B1	-	bla CTX-M	-	FOX, AMC, CIP, IPM, NOR, SCF, LEV, MEM,	CN	CPD, AMP, MH, FOS, SXT
EU23	**B1**	**ESBL+**	**bla CTX-M**	**-**	FOX, AMC, CN, IPM, MEM, SCF, SXT, MH, FO		CPD, CIP, NOR, LEV, AMP,
EU24	B1	-	-	-	FOX, CN, IPM, FOS, SCF, MEM, MH	CPD	AMC, CIP, AMP, NOR, LEV, SXT,

EU25	**B1**	**-**	**-**	**-**	CPD. FOX, AMC, CN, IPM, AMP, FOS, SCF, MEM, MH		CIP, NOR, LEV, SXT,
EU26	B1	-	-	-	CPD, FOX, AMC, CIP, IPM, AMP, FOS, NOR, SCF, LEV, MEM, MH	CN	SXT
EU27	**B1**	**ESBL+**	**bla CTX-M**	**-**	FOX, AMC, MH, MEM, SCF, FOS		CPD, CIP, CN, AMP, NOR, LEV, SXT, IPM,
EU28	B2	-	-	-	CPD, FOX, AMC, CN, CIP, IPM, AMP, FOS, NOR, SCF, LEV, MH, MEM		SXT
EU29	**A**	**-**	**-**	**-**	CPD, FOX, AMC, CN, MH, MEM, SXT, SCF, FOS, IPM		CIP, NOR, LEV, AMP
EU30	B2	-	bla CTX-M	-	FOX, IPM, CIP, FOS, NOR, SCF, LEV, MEM, CN	MH	CPD, AMC, AMP, SXT
EU31	**B2**	**-**	**bla CTX-M**	**-**	FOX, CN, IPM, FOS, SCF, MEM, MH		CPD, AMC, CIP, AMP, NOR, SXT, LEV
EU32	B1	ESBL+	-	-	FOX, IPM, SCF, MEM, MH	AMC,	CPD, CN, CIP,

							AMP, NOR, LEV, SXT, FOS,
EU33	**B1**	**-**	**-**	**-**	CPD, FOX, AMC, CN, SCF, SXT, MEM, MH, IPM		CIP, AMP, FOS, NOR, LEV
EU34	B1	-	-	-	FOX, AMC, CPD, CIP, IPM, MEM, NOR, SCF,SXT, MH		CN, AMP,
EU35	**B1**	**-**	**-**	**-**	FOX, AMC, CN, CIP, IPM, MEM,		CPD, CIP, AMP, NOR, LEV, SXT
					FOS, NOR, SCF, MH		
EU36	A	-	-	-	FOX, AMC, CPD, CN, CIP, IPM, MEM, FOS, NOR, SCF, LEV, MH		AMP, NOR, SXT
EU37	**B2**	**-**	**-**	**-**	FOX, AMC, CPD, CN, CIP, IPM, MEM, AMP, FOS, NOR, SCF, LEV, SXT, MH		
EU38	A	-	bla CTX-M	-	CN, FOX, AMC, IPM, MEM, FOS, NOR, SCF, LEV	CIP, LEV	CPD, AMP, SXT, MH

EU39	**B2**	**-**	**-**	**-**	CN, FOX, AMC, CPD, CIP, IPM, MEM, AMP, FOS, NOR, SCF, LEV, SXT, MH		
EU40	A	-	bla CTX-M	-	FOX,, IPM, MEM, FOS, SCF, MH		CPD, AMC, CN, CIP, NOR, SXT, AMP, LEV

DISCUSSION

Anti-microbial resistance is the most alarming public health concerns in recent years. Antibiotic resistance in its new forms makes it difficult to tackle as it now easily crosses the continents. These antibiotic resistance bacteria are termed as 'nightmare bacteria' having a catastrophic threat to man's health worldwide. Report from the IDSA listed ESBL producing *Klebsiella* spp. and *E. coli* as among the six drug resistant microbes. (Oteo, Pérez-Vázquez, and Campos 2010).

UTIs are quite common, second only to respiratory tract infections. It's important to treat UTIs with antimicrobial therapy, but it's concerning that *E. coli* is often the cause of these infections. Even more concerning is the fact that *E. coli* and other uropathogens are becoming increasingly resistant to antimicrobials, which limits treatment options for those who suffer from UTIs. It's important that we continue to research and develop new treatments so that we can effectively combat these infections. UTI patients over 60 years are mostly related to factors associated with ESBL. To detect antimicrobial resistance, it is highly needed to identify the β-lactamases involved in infections (Tabar *et al*., 2016).

The current study was directed at monitoring ESBL/AmpC-producing *E. coli,* comparison of the resistance genes available in those isolates through phenotypic and genotypic confirmation against different antibiotics and phylogenetic grouping of *E. coli* in UTI in human samples. For this purpose, 40 isolations were made and molecular identification of ESBL and AmpC genes that provides resistance against antimicrobial drugs was carried out. We tested resistance in *E. coli* against 14 antibiotics, and our results revealed *E. coli* a resistant pathogen.

Recent study revealed the susceptibility pattern of *E. coli* revealed highest resistance that was detected against Ampicillin (85%). Sohail *et al.,* 2021 observed antibiotic resistance of Ampicillin (93.7%) in their study on UTI prevalence in infants and children in Lahore Pakistan. Saira *et al*., 2011 studied multi drug resistance (MDR) pattern of uropathogenic *E. coli* in Faisalabad, Pakistan. In their study *E. coli* isolates showed the resistance of 91% against Ampicillin of. In another study done by Lee *et*

al., 2015 in South Korea 84.4% of the uropathogenic *E. coli* were resistant to ampicillin.

With these findings it becomes evident that ampicillin has become less effective in UTIs over the years.

In this study the lowest antibiotic resistance by *E. coli* in urine samples was demonstrated by two antibiotics Meropenem and Sulzone, which 2.5% against each, this research study is in agreement with Muhammad *et al* (2020) they reported the resistance pattern of *E. coli* in urine samples in Peshawar, their results showed lowest *E. coli* resistance against carbapenums. Our findings are in harmony with study done by Molina-López *et al* (2011) in which they studied the drug resistance of *E. coli* in UTI, its serotypes, and phylogenetic groups. Their study reported that 0.85% resistance of *E. coli* samples was against Meropenem. In present study resistance of *E. coli* isolates against 0% Minocycline and 62.5% Ciproflaxicin was observed but Suzuki *et al* (2008) reported 12.5% and 33.3% resistance of *E. coli* against Minocycline and Ciproflaxacin respectively. Muhammad *et al* (2020) reported 82.8% *E. coli* resistance against Ciproflaxicin.

With these results at hand we can conclude that *E. coli* isolates became more resistant against Quinolones. Our study reported 60% resistance of *E. coli* isolates against Flouroquinolones but Bashir, S. *et al* (2011) reported 18% resistance of *E. coli* towards Flouroquinolones, another study done by Shigemura *et al* (2008) has reported an emergence of fluoroquinolne resistant *E. coli* responsible for UTIs. Our study also highlights this increace in *E. coli* resistance against Flouroquinolnes. Fosfomycin showed 20% resistance according to the AST assay performed in our study. Muhammad *et al*., 2020; Suzuki *et al*., 2008 reported *E. coli* resistance of 59.6% and 0% towards Fosfomycin respectively.

Current study revealed the antibiotic resistance of *E. coli* isolates against Cefoxitin to be 12.5% and Trimethoprim- sulfamethoxazole to be 75%. A study done by Suzuki *et al*., 2008 showed resistance of *E. coli* isolates in Cefoxitin to be 22.2% and 100% against Trimethoprim- sulfamethoxazole, which shows that Cefoxitin and Trimethoprim- sulfamethoxazole are becoming more effective antibiotics in UTI over

the years. In this study *E. coli* was most sensitive against carbapenums and Meropenem (97.5%) and Imepenem (95%) sensitivity. This study is in consistent with study reported by Suzuki *et al*., 2008 in which ESBL producing *E. coli* showed Imepenem showed 0% resistance making carbapenems strong antibiotic drugs against UTI.

Imipenem is a carbapenem antibiotic, which is highly active against Enterobactereaceae producing ESBL (Ko *et al*., 2007). The resistance of *E. coli* isolates against imipenem was found to be 9% (Hassan *et al*., 2011); In our findings Gentamicin showed resistance of 47.5% towars *E. coli* but Sohail *et al*., 2021 reported 35.1% resistance of *E. coli* against Gentamicin.

Results obtained from present work, the resistance towards Ampicillin, Trimethoprim-sulfamethoxazole and Quinolones (Levoflaxicin & Ciproflaxicin) are found to be alarming. Our study showed highest sensitivity towards Meropenem. Infection caused by both the Gram negative bacteria and Gram positive bacteria can be treated with carbapenems, which proves it to very notable result in recent study. This result can be a cause of low use of these certain antibiotics locally. These results match the previously done study by Tabar *et al*., 2016. There is high resistant pattern shown by *E. coli* isolates in present study gainst Cephalosporins, penicillin, quinolones and sulphonamides, this high resistance can be a consequence of misuse or overuse of of antibiotics; it is causing MDR infections in UTI patients. Consistent with our results in study by Soltani *et al* (2014) antibiotic resistance of GNB is associated with ESBL production. Antimicrobial resistance (especially through ESBLs and AmpC genes) is an emerging threat in Pakistan (El Salabi *et al*., 2013).

According to Rehman *et al* (2019) there are 3 main types for ESBL genes. These are bla-TEM, bla-CTM and bla-SHV. Day by day, the subtypes for these genes are increasing due to increase in resistance towards antimicrobials. For example, the subtype for bla-CTM has subtypes; bla-CTM-1, bla-CTM-2, bla-CTM-8, bla-CTM-9 and bla-CTM-25 and has now increased to more than 160 subtypes (Abrar *et al*., 2017).

Demographic data of our study shows that females acquire more UTI than men, children also being affected after the age of one year. This study is in accordance with results of previous study performed by Sohail *et al* (2021). Muhammad *et al*., 2020 did

a study on antibiotic resistance pattern of bacterial isolates from urine samples, according to their results females were being more affected with UTI than men.

our study showed higher prevalence of UTI in age group from 50 years to more than 65 year old patients (47.5%), it can be due to factors like genito-urinary atropy, and menupause prolapse, vaginal pH or decrease in vaginal flora that can contribute to UTI in older women, Age 18-30 years patients affected with UTI maybe resulted due to increased sexual activity; these results are in agreement with Muhammad *et al*., 2020; Seifu and Gebissa 2018). A. C. Gales *et al*., also discussed the demographic pattern of UTI patients and their results showed higher prevalence of UTI infection in elder people (98%) women being (72%).

In this study, phenotypic confirmation of 24 *E. coli* isolates (that showed resistance to Cefoxitin and Cefpodoxime) for AmpC/ESBL was performed using commercial (D68C)AmpC and ESβL Detection Disc Set (MAST Group Ltd, UK). Among these isolates, 5 isolates were positive for AmpC (i-e; they had gene for AmpC), 14 isolates were positive ESBL genes.

In current study, in order to further screen the type of ESBL and AmpC genes in identified isolates, PCR was performed which resulted in the identification of bla CTXM in 16 isolates, while no bla TEM and bla SHV was found in any of the isolates. On the other hand, AmpC-MOX, AmpC-CIT and AmpC-FOX were found to be present in 3 of the isolates. No AmpC-EBC, AmpC-ACC and AmpC-DHA genes were detected.

Tabar *et al*., 2016 reported a study in their study revealed PCR results of the bla CTXM gene in ESBL producing isolates, 20 out of 29 isolates were positive for CTX-M. Hassan *et al*., 2011 used combination disk method to detect the ESBL producing *E. coli*, their results showed 46 positive ESBL producing *E. coli* isolates out of 54 isolates.

In our study the multiplex PCR for AmpC genes detection showed the positive results for AmpC-MOX, AmpC-CIT and AmpC-FOX, The AmpC-encoding genes were reported by Mansouri *et al* (2009). Tan *et al*., 2009 found that screening to find cefoxitin resistance has a lower sensitivity toward blaACC family.

Phylogenetic results (done through triplex PCR) of ESBL gene containing *E. coli* obtained from tertiary care hospitalized patients in India, found by Chakraborty *et al* (2015) showed that 67% of the isolates belonged to group A and 81.50% belonged to B1 group (commensal strain). B2 and D group (virulent strain) contributed by 64.5% and 76%. Bashir *et al*., 2011 reported phylogenetic grouping of uropathogenic bacteria via disk diffusion method, 50% *E. coli* samples showed the phylogroup B2, 12% isolates were designted to group D, 19% to both group B and A.

In the current study, the phylogenetic analysis of *E. coli* isolates done by using triplex PCR, revealed 4 groups i.e.; A, B1, B2 and D. The results showed that phylogroup B1 was found to be (33%), followed by group A (32%) and phylogroup B2 was found to be (30%). The phylogroup D was (5%) in the *E. coli* Isolates. With use of one class of antibiotic having lesser effect against an infection can cause increase in use of another antibiotic class. Proper administration of cephalosporins or β-lactamase inhibitor combinations are helping with decrease in ESBL producing *E. coli* (Taneja *et al*., 2008).

The high resistance of *E. coli* against antibiotics was seen in group B2, and B1 group mostly showed sensitivity towards antibiotic, group A also showed resistance against most of antibiotics; whereas group D showed resistance to Quinolones. Takahashi *et al* (2009) results are in partial agreement with present study; the reason can be the geographical difference, samples collected or other clinical sources.

The AMR in recent years caused due to ESBLs has limited the use of antibiotics which are beta-lactam and are used against *E. coli* in nature (Nordmann and Poirel, 2002). AMR in developing countries such as Pakistan, is more serious as compared to in developed countries because, in developing countries, the antimicrobials especially, antibiotics are not used under control or a particular set of regulations in animals especially (Mitema, 2010). Bacteria will find ways to develop resistance against antibiotics, strong and immediate actions are required to keep new resistance action from developing.

The high prevalence of resistance in *E. coli* due to ESBL is a serious matter and cannot be neglected, so, proper monitoring of the production of resistant genes in *E. coli* in a region along with the AST of the UTI patients is necessary to prevent future treatment

failures in them. *E. coli* isolates causing UTI shows greater resistance as it contains the AmpC genes, genotypic study of AmpC resistance and prevalence in bacterial isolates is the utmost need of today (Dehkharghani D. *et al*., 2021). ESBL detection in routine laboratory practices is crucial to limit the spread of multidrug-resistant organisms and ensure patients receive appropriate treatment for their infections. (Shakya *et al*., 2017).

The growing reports on ESBL producing *E. coli* poses a major public health threat, since, these features can be transferred to other bacteria including pathogens of humans through horizontal transfer by plasmids, especially, conjugative ones which will lead towards resistance against many antibiotics. Finally, there is need of research to find novel drugs and their rational use is needed for the future.

The drugs like beta-lactams have lost their effect due to ESBL and AmpC genes. Hassan *et al* (2011) studied the occurrence of MDR and ESBL producing *E. coli;* their study showed that 54% of the *E. coli* isolates were ESBLs producing. ESBL are mainly produced in *E. coli* and *Enterobacteriaceae* (Rehman *et al*., 2019). From both these bacteria, ESBL produced in *E. coli* are resistant to more than one antibiotic and hence makes *E. coli* a multidrug resistant. This feature of *E. coli* makes it harmful for the healthcare.

Conclusion

Forty isolates were recovered from human UTI patients and subjected to AST, ßlactamase production tests and antibiotic resistance genes screening. In this study, Ampicillin was found to be the least effective (85% resistance), followed by Trimethoprim (75%) and Levoflaxicin and Ciproflaxicin (62.5%). Twenty-four samples were positive for the ESBL production and 5 samples were positive for AmpC. All the isolates were negative for bla-TEM and blaSHV genes. The AmpC genes AmpC-CIT, AmpCMOX and AmpC-FOX were present in three of isolates. A triplex PCR results depicted presence of 4 phylo-groups; group B1 (33%). Group A (32%), followed by group B2 (30%), group D (5%). Rational use of antibiotics and observing proper hygienic measures can help in reduction of ESBL/AmpC *E. coli* in UTIs. The rising number of ß-lactamase producing *E. coli* and other bacteria are being reported and this depicts an alarming situation. ß-lactamase genes can be transferred from *E. coli* to other bacteria resulting in spread of antibiotic resistance in both the humans and livestock. This necessitates rational use of antibiotics.

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