Antibiotic Sensitivity and Antibiotic Resistance Genes in Extended Spectrum Beta Lactamases (ESBL) Producing Strains of Escherichia coli Recovered from Small Ruminants

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2019-2021

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A thesis entitled

Antibiotic Sensitivity and Antibiotic Resistance Genes in Extended Spectrum Beta Lactamases (ESBL) Producing Strains of Escherichia coli Recovered from Small Ruminants

Submitted in partial fulfilment of the requirements for the

Degree of

Master of Philosophy

In

Zoology

By

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Quaid-i-Azam University, Islamabad

2021

“In the name of **ALLAH**, the Most *Gracious*, the Most Merciful”

I Heartily Dedicate my Dissertation Work to,

Allama Khadim Hussain Rizvi

Who increase my Love & Affection for,

Last Prophet Hazrat Muhammad ﷺ

Who makes me able to understand,

Allama Muhammad Iqbal (R.A.)

DECLARATION

I hereby declare that this thesis entitled "*Antibiotic Sensitivity and Antibiotic Resistance Genes in Extended Spectrum Beta Lactamases (ESBL) Producing Strains of Escherichia coli Recovered from Small Ruminants*" is my personal effort. I have not previously presented any part of this work elsewhere for any other degree.

Muhammad Zeeshan Malik

CERTIFICATE

This dissertation "Antibiotic Sensitivity and Antibiotic Resistance Genes in Extended-Spectrum Beta-Lactamases (ESBL) Producing Strains of Escherichia coli Recovered from Sheep and Goats" submitted by **Muhammad Zeeshan Malik,** 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 Animal Microbiology.

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Date: 06.07.2021

ACKNWOLEDGEMENT

First and foremost, I would like to praise and thank ***Allah Almighty*****,** Who has granted countless blessing knowledge and opportunity to me, so that I have been finally able to accomplish the thesis. My humble gratitude to the Last Prophet ***Hazrat Muhammad (S.A.W.)*** for whom all the universe is created and decorated.

I want to express my gratitude to my ***parents*** for their love and support throughout my life. I heartily admire the sacrifices they made for me and never allow any hurdle to come between me and my dreams. I also want to express my love & care for my siblings i.e. my younger brothers and pieces of my heart ***Muhammad Arsalan Malik*** and ***Meer Fadi Malik***; my only sister, first ever friend and an apple of my eyes ***Alina Nousheen Malik.***

I would like to thank my supervisor, ***Dr. Saeed ul Hassan Khan*** (Assistant Professor at QAU Islamabad), for the patience guidance, encouragement and advice he has provided throughout my time as his student.

I am deeply grateful to ***Dr. Hamid Irshad*** (Principal Scientific Officer at NARC) for his support and encouragement during this research work. At many stages in the course of this research I benefited from his advice. I am indebted to ***Dr. Aitazaz Ahsan*** (Scientific Officer at NARC) and ***Dr. Armaghan Shehzad*** for their cooperation and support during my research work.

Heartiest gratitude to my lab fellows at QAU & NARC, ***Dr. Faisal Rasheed Qaisarani, Aimen Fatima Naqvi, Naheed Khaskheli, Umama Fayyaz, Dr. Safeerullah, Haqnawaz Khan, Ayesha Javed, Mariyam Dilshad, Tayyaba Shahid*** and ***Aimen Rasheed.***

Some special words of gratitude go to my friends gifted by QAU ***Dr. Shakeel Ahmad, Dr. Muhammad Kabir, Muntazir Tanvi, Muhammad Ihrar, Shehryar Sandhu*** and ***Muhammad Danish*** who share moments of laughter and sorrow and have always been a major source of support when things would get a bit discouraging.

I also want to thank my uncle ***Malik Ghazanfar Awan*** for his support and encouragement throughout my stay at QAU Islamabad.

At the end, my special thanks to ***Dr. Rashid Abbas Khan*** for being there whenever I needed endless support.

Muhammad Zeeshan Malik

Table of Contents

List of Tables

List of Figures

List of Abbreviations

Abbreviation	Full Name
NARC	National Agricultural Research Centre
ASI	Animal Sciences Institute
AHP	Animal Health Program
CDC	Centre for Disease Control and Prevention
WHO	World Health Organization
CLSI	Clinical Laboratory Standard Institute
ESBL	Extended Spectrum β-Lactamases
MAC-CEF	MacConkey Agar + Cefotaxime
CTX-Na	Cefotaxime Sodium Salt
IMViC	Indole, Methyl Red, Voges Proskaur, Simmons Citrate
TSI	Triple Sugar Iron
DDDT	Double Disk Diffusion Test
DDST	Double Disk Synergy Test
AST	Antibiotic Susceptibility Test
MHA	Muller Hinton Agar
PCR	Polymerase Chain Reaction
CZC	Ceftazidime + Clavulanic Acid
CTC	Cefotaxime + Clavulanic Acid
AUG	Amoxcillin + Clavulanic Acid
SXT	Sulphamethoxazole+ Trimethoprim

Abstract

This study was conducted at Animal Health Program of National Agricultural Research Center Islamabad (NARC), and Zoology Department of Quaid-i-Azam University, Islamabad. The aim of the study was to find out prevalence of *ESBL* producing *E.coli* among the ESBL screened *E. coli* isolates recovered from small ruminants. The study was also performed to know their antibiotic susceptibility pattern and to detect *bla* genes in these *ESBL* producing *E.coli* isolates. A total of 112 samples of *E.coli* isolated from Pakistani sheep and goats were processed for the confirmation of *E.coli* and *ESBL* producing *E.coli* isolates. Out of these 112 samples, 84 were found to be positive for *E.coli* and 39 (46.4%) were confirmed to be *ESBL E.coli* by colonial morphology on specific media, biochemical and phenotypic testing as well as through the *bla* genes PCR. The 39 confirmed *ESBL E.coli* isolates were subjected to antibiotic sensitivity testing. Highest resistance (100%) against Penicillin and Lincosamides, (90%) against Cefixime and Cefotaxime while relatively low insensitivity was observed against Flouroquinolones (69%) and Tetracycline (54%) in the *ESBL E.coli* isolates. The most efficacious drugs were found to be Aminoglycosides and Ceftazidime against which 77% and 64% of the isolates were noted to be sensitive respectively. Results of PCRs for *bla* antibiotic resistance genes revealed that TEM gene was highly prevalent and found in 37 out of a total of 39 *ESBL E.coli* (94.8%). *Bla* CTX-M was present in 84.6% of the isolates, however, the *bla*SHV gene was not found in any of the isolated *ESBL E.coli*. A prevalence of 46.4% of *ESBL producing E.coli* in small ruminants depicts an alarming situation both for the livestock as well as public health perspectives.

INTRODUCTION

1.1. Escherichia coli

Escherichia coli (*E. coli*) belongs to the family Enterobacteriaceae. It is the best known species of the normal intestinal tract (GIT) of human, reptiles and other warm-blooded animals (Kaper *et al.,* 2004). It is a Gram-negative, rod shape, facultative anaerobe, motile, non-fastidious and non-sporulating bacterium (Dash *et al.,* 2012).

For the first time in 1885, Theodor Escherich described this bacterium. Most of the *E. coli* strains remain asymptomatic in gastrointestinal tract of human and animals, while some strain adopted pathogenic lifestyle and become virulent. *E. coli* are categorized on the basis of serogroups, their mechanisms of pathogenicity, clinical symptoms as well as virulence factor (Kaper *et al.,* 2004).

It is an oxidase negative bacterium. It can ferment glucose, lactose and sucrose. It requires 37°C temperature and 6.0 – 7.0 pH as optimum growth conditions. It can also tolerate the acidic pH (2.0) (Molina *et al.,* 2015).

The members of Enterobacteriaceae mostly remain commensals in the intestinal tract of animals (Carattoli, 2009). *E. coli* strains, are categorized into three major groups. It includes commensal *E. coli*, intestinal pathogenic (IPEC) and extra intestinal pathogen (ExPEC) (Díaz-Agero Pérez *et al.,* 2019).

1.2. Diseases caused by *E. coli*:

As *E. coli* is considered as cause of food borne diarrheal disease, but the significance of extraintestinal infection caused by *E. coli* cannot be underestimated. As these extraintestinal *E. coli*, mostly cause infection in the urinary tract (UTIs), on surgical wound site and meningitis in new born. Due to rapid increase in antibiotics resistance, the treatment of these infection become a major problem throughout the world. *E. coli* infections are also responsible for significant economic loss on this planet. In United States, a total cost of $94-252 million per annum lost to treat only surgical wound infection (Russo and Johnson, 2003).

Some strains remain without causing disease, while other such as shiga toxin (STEC) producing *E. coli* may cause food borne diseases. Different contaminated foods, including milk, not properly cooked meat and vegetables are primarily source of transmission. The name Shiga toxin, is due to their resemblance with Shigella dysenteriae toxin. The optimum temperature for STEC is 37 °C. It can also grow in temperature limits from 7°C - 50 °C with up to pH of 4.4. The symptoms including fever, vomiting and diarrhea having blood which is known as hemorrhagic colitis. Their incubation time is 3 to 8 days. Normally within 10 days the disease recover, but sometime it could lead to life threatening stage (especially in elderly age people and children) the haemolytic uraemic syndrome. The haemolytic uraemic syndrome progress into renal failure, thrombocytopenia (platelets count low) and haemolytic anemia (WHO, 2018).

Entero-invasive *E. coli* strains cause septicemia when it breaches the intestinal barrier, particularly in young lambs. They cause pathological alteration when colonize in various organs of animals. Small ruminants such as Sheep and goat have been reported as a major reservoir of *E. coli*. The bacterium lives in gastro-intestinal tract of animal and excreted into their feces. The feces of sheep and goats considered as a major source of food contamination. From food the bacterium access to human and cause different infections (Kiranmayi *et al.,* 2010).

Infection due to *E. coli* becomes challenge for people who live in close contact with animals especially with sheep and goat. They don't know about the severity of infection and their transmission (Ashraf, 2016). The pathogen may be attached any part of animal skin or it may contaminate the carcass during evisceration process. Among the other disease of goat, diarrhea is the most common gut associated disease around the world (Bist *et al.,* 2014).

E. coli harbor intestinal microflora of both animals and human. It causes different infection in their host, which could lead to fetal disease in human as well as animals (Bélanger *et al.,* 2011). Presence of *E. coli* in sheep and goat can act as a carrier of infection to human, as the meat of these small ruminants can transmit disease to human. During slaughtering, the meat contaminates with intestinal content permit it to enter the food chain while consuming contaminated meat. The transmission of this bacterium can occur through contact with animals (direct and indirect contact), but it may be transmitted from one to others in human. Transmission within humans has played a pivot role in the infection transmission (Lim *et al.,* 2010).

1.3. Resistance against antibiotics

Antibiotics are used against variety of infectious disease in human and animals. Extensive use of antibiotics in medical practices and in animal food adversely affect both pathogenic and commensal bacteria and these become antibiotics resistance bacteria (Tivendale *et al.,* 2009). Microorganisms known as multi-drug resistance (MDR), which acquired resistance against different classes (three or more) of antimicrobial agents (Santo *et al.,* 2007).

Mostly bacterial infections are treated with antibiotics which are classified according to mode of mechanism against bacteria. Antibacterial agents inhibit the bacterial growth by interfering in protein synthesis, cell wall synthesis and nucleic acid synthesis, also by stopping the metabolic pathway (Paterson DL *et al.,* 2003).

There are different methods by which gram negative organisms develop resistance to different antibiotics. Some bacteria produce Beta Lactamases (β-Lactamases), which hydrolyse and inactivate β-Lactam antibiotics. In some bacteria mutation can occur in the genes which are specific for binding to antibiotics, which result in alteration on target site and their function as well. Similarly, the alteration in the porins proteins and efflux pumps also result in reduce permeability of antibiotics into bacterial cells (Ho *et al.,* 2010).

The β-Lactams are the most commonly used antibiotics include penicillin, cephalosporins, carbapenems, monobactams and cephamycin. All these antibiotics are characterized by having β-Lactam ring as a chemical base. In 1983, the first β-Lactamase SHV type was detected in *K. pneumoniae* in Germany. Similarly, the first TEM ESBLs (named after the patient Temoneira) were found in France and England. In 1989, another *ESBL* CTX family was described in Germany (named after the place of discovery, Munich) (Paterson and Bonomo, 2005). Different *ESBL* families have been studied, but most of the *ESBL* belong to SHV, TEM, CTX-M and OXA families (Pitout and Laupland, 2008).

Variety of antibiotics are resistance against *E. coli*. It is mainly due to the interference of efflux pumps. It may also be due to the presence of some genes, which show resistance against antimicrobial agents. These resistance genes are specifically present on the bacterial plasmids (Szmolka and Nagy, 2013). Plasmids are considered as vectors for transmission of resistance both phenotypically or genotypically (Ochman *et al.,* 2000). Commonly used class of antibiotics in clinical care, including the Extended-Spectrum Cephalosporins,

Fluoroquinolones, and Aminoglycosides are the plasmid encoded resistant antibiotics (Carattoli, 2013).

Antibacterial effects of tetracycline, aminoglycosides and macrolides are produced because these antibiotics inhibit the protein synthesis. This inhibition is mostly done by interfering with ribosomal structure as bacteria possess different ribosomal structure as compared to eukaryotes. Above mention antibacterial agent attach themselves with 30S ribosomal subunit to inhibit protein synthesis (McManus MC, 1997).

The β-Lactam antibiotics bind to penicillin binding protein (PBP) as a transpeptidases and carboxypeptidases, which are present on the inner membrane of cell wall. The penicillin binding protein helps both in the synthesis of bacterial cell wall as well as the synthesis of cytoplasmic membrane of bacteria. Any alteration in the function of penicillin binding protein, results in the bacterial cell wall lysis and cell shape disruption, which ultimately result in the cell death. The production of beta lactamases interferes with beta lactam ring, thus hydrolyze it and result in β-Lactam resistance among different bacteria (Livermore, 1995).

Quinolones target topoisomerase type II enzyme (DNA gyrase). This enzyme is responsible for the unwinding of DNA and the synthesis of mRNA. The inhibition of this enzyme prevents bacterial replication as well as their protein synthesis (Oliphant and Green, 2002). Quinolones become resistant due to point mutation. This mutation occurs in chromosomal quinolone resistance determinant region, the GyrA and the GyrB. The resistance occurs also due to mutation in the qnr protein and alteration in efflux pumps present in bacterial cell wall (Ranjbar and Farahani, 2017; Sharma *et al.,* 2009).

Aminoglycosides, which inhibit the bacterial growth by binding to 30S subunit of bacterial ribosome. Therefore, it alters the normal function of 30S ribosomal subunit, due to this reason its loss the capability to read the genetic code, which affect the translation process. Resistance to aminoglycoside, could also be due to mutation in bacterial chromosome as well as by acquiring the mobile genetic elements (Fux *et al.,* 2009).

1.4. β-Lactamases

β-Lactamases is the most investigated group than any other enzyme group which is shown through its citations i.e. 28900 in Medline. β-Lactam antibiotics are mostly inactivated by

these enzymes (Kirby, 1944). The β-Lactamases mainly mediate the resistance against beta lactams class of antibiotics in Gram-negative bacteria especially in Enterobacteriaceae. Inactivation of antimicrobial agents are done by hydrolysis of β-Lactam ring which is performed by β-Lactamases and this hydrolysis also prevent the β-Lactam to active against the enzyme which is involved in the synthesis of bacterial cell wall (Martínez-Martínez and González-López, 2014).

Variety of β-Lactamases are identified in different types of bacteria with minute changes of molecular structure. Chemical compounds having β-Lactam ring are mainly hydrolyzed by β-Lactamases (Knott-Hunziker *et al.,* 1979). On the basis of their hydrolyzing mechanism, they are divided into two main groups that they perform hydrolyzing either through metallo ions on serine active site or acyl enzyme formation on serine active site (Zhang and Hao, 2011). One of the most prominent antibacterial effect of β-Lactamases is that in penicillin binding proteins they acrylates the active site of serine, so as a result growth of replicating bacteria is inhibited (Frère *et al.,* 1976).

In recent studies variety of new β-Lactamases are identified and now they are over 2770 so proper nomenclature is the need of the time. Different schemes are prepared for their classification e.g. β-Lactamases as penicillinases or cephalosporinases (Sawai *et al.,* 1968).

The β-Lactamases are also divided into four molecular classes and simple named as A, B, C and D. This classification is done after sequence analysis in which their molecular sizes and homology between active sites are compared (Huovinen *et al.,* 1988). These four molecular classes of β-Lactamases have 17 different functional groups (Bush and Jacoby, 2010). But these enzymes are continuously differentiated the way they hydrolyse the β-Lactam substrates such as carbapenems, penicillin, monobactams and cephalosporin (Reading and Cole, 1977).

A research study in Lechuguilla cave of New Mexico (USA) revealed that β-Lactamases are originated around millions of years ago. It is also observed that 62% of recovered samples are capable of hydrolysing (Bhullar *et al.,* 2012). For the first time lactamases are noticed due to their antibacterial activities and they are also known as therapeutic agents (Flemming, 1929).

In case of gram negative, β-Lactamases play their role in resistance mechanism and neutralize the effect of lactam class of antibiotics. Lactamase activity is first time reported in 1940 in Bacillus coli (Abraham and Chain, 1988).

A six-year detailed study was conducted from 1979 to 1985 in which lactamases were evaluated from 1800 bacterial isolates. This research also revealed higher ratio of TEM-1 and TEM-2 pencillinases while very low ratio of SHV-1 and OXA enzymes were also noticed. Transfer of β-Lactam resistance between β-Lactamases were also observed through which future resistance patterns were easily predicted (Simpson *et al.,* 1980).

In Enterobacteriaceae, transferable cephalosporinases are observed whose hyperproduction lead to extended spectrum cephalosporins (Sanders and Sanders, 1988). Enterobacteriaceae is also become resistant cefotaxime and ceftazidime and it is extended toward aztreonam and other third generation cephalosporins which are known as extended spectrum beta lactamases (ESBL). This is all happening just because of transferable lactamases. There is difference of two or three amino acids between ESBL and parental enzymes and it is also described that ESBL were derived from TEM-1, TEM-2 and SHV-1 lactamases (Bradford, 2001).

In recent studies it is observed that in ESBL, CTX-M family replaced the SHV, TEM and IRT and currently CTX-M is considered having major contribution in developing multi drug resistance of Gram-negative bacteria. In gram-negative bacteria, high ratio of ESBL including plasmid coded cephalosporinases and carbapenamases is observed in hospitalized patients (Rahal et al., 1998).

New β-Lactamases are emerged rapidly and older fit enzymes are maintained by the environment created by both naturally occurring and clinically overused β-Lactams (Lopatkin *et al.,* 2017). TEM-1 and OXA-1 are older one lactamase but they are not disappeared yet and frequently identified in environmental as well as clinical isolates. It is concluded that β-Lactamases has the transmissible resistance factors and the way they evaluated they will arise as a major threat in near future (Poirel *et al.,* 2016).

1.5. Extended-Spectrum β-Lactamases (ESBLs)

In 1983, ESBL was described for the very first time and included in Enterobacteriaceae family. According to new taxonomy this family is known as Enterobacterales. ESBL are the enzymes which cause hydrolysis of β-Lactam antibiotics (Adeolu *et al.,* 2016; Knothe *et al.,* 1983).

An ESBL can precisely be defined as the β-Lactamase which can make bacteria resistance against pencillins, three generations of cephalosporins and aztreonam and this can be done

through hydrolysis of respective antibiotics as these antibiotics possess. Due to this extended spectrum these β-Lactamases are called ESBL (Bush *et al.,* 1995).

The ESBL genes are divided into various types in which most prevalent genes are CTX-M, TEM and SHV. But there are some other ESBL genes such as PER, VEB, OXA, BES, GES, TLA, IBS and SFO. For the treatment of many bacterial infection, β-Lactams are the common therapeutic agents especially against gram negative bacteria. So bacterial strains have continuous exposure to β-Lactams which induced mutation and over production of β-Lactamases in respective bacteria which ultimately expands their hydrolysis activity for newly developed β-Lactams. Due to this expanded activity against these β-Lactams the corresponding β-Lactamases are called as extended spectrum β-Lactamases (Pitout and Laupland, 2008; Paterson and Bonomo, 2005).
For the infections which are caused by the members of Enterobacteriaceae family, β-Lactam antibiotics are widely used all over the world. This wide spread usage of these antibiotics provide more exposure to bacteria which make bacteria resistant to these. In this specific case of resistant, main mechanism involves the production of β-Lactamases which in activates the β-Lactam drugs. Due to plasmid mediated nature of ESBL, it can easily be transmitted from one member of Enterobacteriaceae to others (Pitout JDLK, 2008).

ESBL *E. coli*, mediate resistant to β-Lactam class antibiotics. Since early 2000, an increase in prevalence rate of resistance have been noticed (Lee *et al.,* 2018 ; Shaikh *et al.,* 2015). ESBLs producing *E. coli* have become a major health threat around the globe due to their high resistance, but the resistance rate has been reported very high in underdeveloped countries. In Europe, the prevalence rate is lower than 10 % (Chervet *et al.,* 2018, Picozzi *et al.,* 2013).

The World Health Organization (WHO), described a list of important antibiotic-resistant pathogens for future research purpose, in which they place ESBL *E. coli* as the most prominent group (WHO, 2017). Instead of high resistance, the penicillin and third generation cephalosporins are most widely used antibiotics against these bacteria, due to their broad spectrum of action and low toxicity. ESBL is also found resistance to fluoroquinolones (Lee *et al.,* 2018 ; Livermore, 2012).

Traditionally, ESBL pathogens have also been linked to hospital acquired infection, but with the passage of time, the community acquired infection are increasing (Pitout *et al.,* 2005). In

2011 and 2016, the two systemic reviews on ESBL pathogens also showed a steady increase in the community prevalence rate. High mortality rate and prolonged hospital stay have also been noticed due to ESBL infections, which ultimately result high cost to treat these infections (Kim *et al.,* 2002; Schwaber *et al.,* 2006).

Enterobacteriaceae, including the *K. pneumoniae* and *E. coli*, are the most important ESBL producers, which are routinely isolated in different clinical laboratories. The Acinetobacter species also produce beta Lactamase enzymes and become resistance to different classes of beta Lactam antibiotics (Vahaboglu *et al.,* 2001). This resistance may be either plasmid or chromosomal mediated. PER-1, an ESBL, has been reported in Acinetobacter in Turkey, France and Korea, while 46% and 54.6% PER-1 also reported in Acinetobacter in Turkey and Korea respectively (Yong *et al.,* 2003).

In France 2003, VEB-1, another ESBL was reported in Acinetobacter baumannii (Poirel *et al.,* 2003). The routine detection of ESBL is difficult in Acinetobacter as compare to Enterobacteriaceae due to synergy between cephalosporin and clavulanic acid. Resistance to cefepime is due to presence of PER-1 production, as it is stable to AmpC but not to ESBL PER-1 (Yong *et al.,* 2003).

Since Extended-Spectrum ß-Lactamases (ESBLs) are increasingly detected in livestock environmental health risks of livestock farming are major debate among scientist (Huijbers *et al.,* 2013, Wielders *et al.,* 2017).

Variety of acute to chronic ESBLs associated diseases have been found in livestock, however today's discussion is about ESBLs infection in sheep and goats. Different studies documented infection of blood, lungs, intestine, and other organs and tissue caused by ESBL in livestock. For example, E. fergusonii harboring CTX-M-1 ESBL gene was isolated from sheep with clinical causes suggestive of diarrhea and salmonellosis (Rimoldi and Moeller, 2013). Same organism was found responsible for enteritis and septicemia in goats (Hariharan *et al.,* 2007).

The *bla*CTX-M-15 and DHA ESBLs producing *K. pneumoniae* strains were identified in feces of livestock sheep. The bacterium is well known as causative agent of pneumonitis in both human and animals. Another ESBL producers and most commonly isolated bacteria from livestock is *E. coli*. It is capable of producing CTX-M-1 and TEM-20 ESBLs are most recovered bacterial strains from sheep and goat meat, feces and blood. The *E. coli* in said livestock is responsible for gastrointestinal infections including acute/chronic inflammatory

bowel disease, colibacillosis, diarrhea and septicemia (Kempf *et al.,* 2013). Studies found that these infections spread among live stock through open wound, contaminated water, feces, and food (Poirel *et al.,* 2013; Rimoldi and Moeller, 2013).

1.6. Prevalence of ESBLs

The worldwide prevalence of ESBL producing *E. coli* is increasing. Since the last two decades, a high prevalence rate of ESBL in human gut has been noticed. This rate is very high in South-East Asia than Europe (Bezabih *et al.,* 2021). Similarly, a high prevalence rate has also been investigated in different animals. A recent study published in Saudi Arabia show a high prevalence rate in sheep and goat with high ESBL producing and multi drug resistance strain (Shabana and Al-Enazi, 2020). Similarly, a study published in Turkey on goat and lamb kids, showed 7.7% ESBL producing strain among other entero-virulent including shiga toxigenic, enterotoxigenic and enteropathogenic strain (Turkyilmaz *et al.,* 2013).

In Pakistan, the prevalence rate of ESBL is also very high. Different geographic region shows different prevalence rate. According to (Ullah *et al.,* 2009), 56.9% of *E. coli* isolates were ESBL positive in patients suffering from urinary tract infection. This study also showed that, 83% of isolates also resistant to more than four antibiotics. The prevalence of ESBL producing strain of *E. coli* is continuously increasing. A study conducted in 2016 shows upward increase in prevalence rate from 37% to 60% in 2005 and 2009-2010 respectively (Habeeb *et al.,* 2013) A study conducted by (Sabir *et al.,* 2014), show highest prevalence 80% of *E. coli* strain, from patient suffering with urinary tract infection (UTI). These strains were resistance to various antibiotics. The highest resistance was against the penicillin and amoxicillin (100), and cefotaxime (89.7%) respectively.

Another study shows 23.56% rate of ESBL producing isolates in hospitalized patients. After PCR confirmation it was found that 59.45% isolates were positive for bla CTX-M-1 gene and 40.54% for bla TEM-1. In this study aztreonam antibiotic showed the highest prevalence rate of 97.29%, while imipenem shows 2.7% resistance against ESBL producing *E. coli* (Rahman *et al.*, 2016).

Umair *et al.,* 2019 found 31% and 13.7% prevalence of *ESBL E.coli* in cattle and poultry respectively. They also reported the high occurrence of CTX-M with 96.5% in isolates

recovered from both cattle and poultry. In rapid phylogenetic grouping B1 was dominant with 44 percentage.

In a research study it is reported that in Pakistani cows there is higher prevalence percentage is noticed for CTX-M i.e. 77.78% while TEM and SHV are 55.56% and 16.67% prevalent respectively (Rehamn *et al.,* 2018)

1.7. Phenotypic Tests for ESBL Detection

The **disk diffusion and broth micro-dilution method** are described to detect the production of ESBLs in gram negative bacteria (Drieux et al., 2008). These methods have been found unreliable due to different reasons including, the presence of different class of beta lactamases, difficulties in identifying ESBL production due to AmpC β-Lactamases over production, masking of ESBL production due to AmpC producing bacteria (Oberoi *et al.,* 2013 ; Shoorashetty *et al.,* 2011). Similarly, the lack of skillful clinical microbiology laboratory staff to perform the detection of ESBL producing bacteria. This results in unreliable testing which directly affects antimicrobial therapy (Oberoi *et al.,* 2013).

Testing ESBL production is two stage process, which involve screening and confirmation of bacteria. The bacteria are tested against different antibiotics including, CAZ, CTX, CRO and ATM. The ESBL producing bacteria are identified by specific clear zone diameter around antibiotic disk. To improve the sensitivity of ESBL detection, more than one antibiotic is recommended. Mostly the CTX is recommended, because it show consistent susceptibility against CTX-M and CAZ, TEM as well as SHV (Livermore and Paterson, 2006).

As recommended by the Clinical Laboratory Standards Institute (CLSI), the resistance against bacteria of these agent required further phenotypic conformation of ESBLs (CLSI, 2018). It is achieved by using cephalosporin/clavulanate combination disk diffusion method. The use of DDS and modified DDS method confirmed the production of ESBL. It also differentiates the ESBL production and the overexpression of AmpC- derepressed mutant.

For the first time **Double-disk synergy test** was used to identify the ESBLs producing bacteria. Initially, the test was designed on the basis to differentiate between cefotaxime resistant strain, specifically for those bacteria which producing more cephalosporinase and the ESBLs. The test is performed on Mueller-Hinton agar, with 30-lg disk of cefotaxime and amoxicillin-clavulanate disk (10 lg clavulanate) having 30mm center to center distance

between the disks. The test result is considered positive, when the inhibition zone diameter of clavulanate in combination with cefotaxime is greater as compare to cefotaxime alone (Jarlier *et al.,* 1988). This test was used in French hospitals for the first time in epidemiological based studies, to find out presence of ESBLs producing bacteria (Legrand *et al.,* 1989 ; Buré *et al.,* 1988).

Generally, the DDST is regarded the most simple and reliable technique for the detection of extended spectrum beta lactamases. It is necessary, to adjust the correct distance among the antibiotic's disks, as by reducing the space up to 20 milli meter between the clavulanate containing disk and cephalosporin disk (3^{rd} generation) improve the test sensitivity. (Tzelepi *et al.,* 2000).

E-test have been develop to confirm the clavulanic acid resistance in ESBLs producing bacteria such as *E. coli*, Klebsiella pneumonia and K. oxytoca. In this, cefotaxime/ cefotaxime + clavulanic acid (CT/CTL), TZ / TZL and PM / PML are used, which are two sided strips having CT, TZ or PM either alone on one side of strip or in combination with clavulanate having concentration of 4mg/L on other side. The presence of ESBL confirmed (test positive), when the minimum inhibitory concentration value is reduced (MIC ratio ≥ 8) more than three doubling dilution steps in the presence of clavulanate (Cormican *et al.,* 1996).

The test result also considered as positive when phantom zone (round zone) appears or deformation of the CT, TZ or PM ellipse. This indicates (phantom zone and ellipse deformation) presence of ESBL production. Result interpreting by ESBL E-test is delicate, it requires skilled trained personnel in microbiology. Some study also reported that laboratories fail to interpret correct result, especially inhibition ellipse in 30% of cases (Leverstein-van Hall *et al.,* 2002). Similarly, the detection of ESBL by E-test may fail, when the MIC value for cephalosporins fall beyond the MIC value available on the strip (Linscott and Brown, 2005).

Combination disk method is based on the measurement of inhibition zone around the cephalosporin disk as well as the same cephalosporin in combination with clavulanate disk. The difference of ≥5 mm between the diameter of two disk or about 50 % zone expansion of antibiotic disk indicating the bacteria as ESBL producers (Carter *et al.,* 2000). The interpretation of this test is straightforward and it is easy to perform. The sensitivity and

specificity of the test was reported high for the first time, 96% and 100% respectively (Linscott and Brown, 2005).

A study reported by Carter et al. on ESBL detection in Klebsiellae by using same (combination disk) method. They evaluate the performance of cefpodoxime and cefpodoxime in combination with clavulanate, having 10 mg and 10 mg plus 1 mg respectively. The large zone seemed for all ESBL producing Klebsiellae against clavulanate disk with cefpodoxime, which show ≥ 5 mm diameter zone. Similarly, ≤1 mm diameter zone was reported for AmpC overproducers and K. oxytoca isolates expressing high K1 enzyme (Carter *et al.*, 2000).

Thomson and Sander proposed the **three-dimensional test** (direct or indirect). The direct test is the modification of disk diffusion test. It generates data both on the antimicrobial susceptibility of tested strain as well as the profile of beta lactamase produced by the same strain. While performing this test, the tested strain (0.5 McFarland standard) is inoculated onto the surface Mueller Hinton agar plates. After this, a circular slit cut in the agar is filled with a higher inoculum density of 109- 1010 cell per ml.

Next, the antibiotics disks are placed in such a way on the agar plate that these are at 3 mm outside the slit, having strain into it. Inactivation of each antibiotic due to enzymatic action is detected, by examination the margin of zone of inhibition in its intersecting vicinity with the strain containing slit. Test is considered as a positive due to inactivation of antibiotics. It occurs when antibiotic diffuse through the slit, which result in a distortion or discontinuity in that specific circular inhibition zone or it produce discrete colonies in the vicinity of the inoculated slit (Thomson and Sanders, 1992).

The **indirect test** is the modification of direct three-dimensional test. In this test, the only difference is the inoculation of the surface of agar plates with an indicator strain which is fully susceptible (ATCC 25922). With the exception of this modification, the method is the same as that described for the direct three-dimensional test. A study design by Thomson and Sander on the comparison of direct and indirect three-dimensional test and DDST. In this study 32 strains of *E. coli* and K. pneumoniae were examined, 28 out of 32 produced ESBL. It shows that detection rate was high 93% for three-dimensional test than DDST which was 82% (Thomson and Sanders, 1992).

1.8. Rapid Phylogenetic Grouping of *E. coli*

Different studies show that strain of *E,coli* can be divided in to four groups i.e. A, B1, B2 and D (Clermont et al,. 2000). In these groups high percentage of virulence is detected in B2 group and high ratio of commensals *E.coli* are present in A group (Johnson *et al.,* 2000).

Phylogenetic grouping of *E.coli* can also be done by ribotyping and multilocus enzyme electrophoresis, but these methods take a lot of time to perform. So this can be done through the triplex PCR described by Clermont et al (2000) very quickly. In this specific PCR two genes (chu & yja) and a DNA fragment (TspE4) are used and given strains can be divided to their respective groups by following the dichotomous diagram given below:

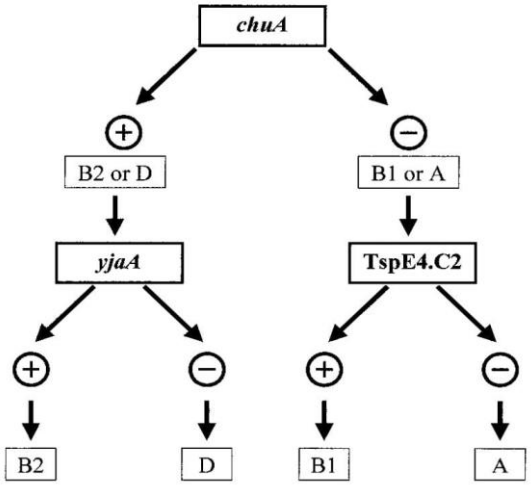

Fig 1.1: Dichotomous Diagram for Rapid phylogenetic Grouping of *E.coli* (Clermont *et al.,* 2000)

Aims and Objectives of the Study

a. To study the prevalence of ESBL *E. coli* in sheep and goats
b. To study the prevalence of important ESBL genes i.e. CTX-M, TEM and SHV and antibiotic sensitivity in *E. coli* isolates

MATERIALS AND METHODS

The experiments were performed at the Animal Health Program of the Animal Sciences Institute, National Agricultural Research Centre Islamabad (AHP, ASI, NARC).

2.1. Specimen collection

Recto Anal Mucosal Swabs (RAMS) samples from sheep and goat were collected from the Ratta Amraal Slaughter House Rawalpindi and kept chilled. Thirty samples were collected on each visit and ten samples were processed per week.

2.2. Transport and storage

All collected samples were returned to the laboratory (AHP, ASI, NARC) in cold conditions on the same day of sampling and processed as soon as possible. After enrichment in buffer peptone water for 24 hours all the samples were preserved in glycerol and stored at -20°C.

Test procedures

All the procedures were performed by following standard protocols. References of these standard protocols are given below with test procedures'.

2.3. Enrichment broths

Enrichment broth i.e. Buffer Peptone Water (Oxoid, UK; Cat. No.X4227B) were used to increase the number of required bacteria (in this case ESBL *E. coli*) in the sample and to facilitate the isolation of bacteria.

Method

RAMS were placed in separate universal bottles one by one. All the bottles were properly labelled i.e. date and sample ID. All the samples were incubated for 24 hours at 37°C.

After 24 hours of incubation, the enriched broth were streaked immediately for primary culture or were stored at 4°C to prevent further growth.

2.4. Isolation of *E. coli*

Enriched samples were inoculated on MacConkey agar (Oxoid, UK; Cat. No.X4236D) in which cefotaxime Sodium salt solution (CTX-Na salt solution) (Toku-E, Cat. No.C011-1G) was added to make it selective for ESBL E.coli (MAC-CEF agar plates) (Bortolaia and Hendriksen, 2017)

Method

- MacConkey agar media (Oxoid, UK; Cat. No.X4236D) were prepared by Suspending 51.5g powder in 1L of distilled water. Then autoclaved at 121° C for 15 minutes.
- After the media were cooled down up to 50°C CTX-Na salt solution were added and the flask was shaken gently to completely dissolve the CTX-Na salt solution in media. Then 25 ml media were poured per petri plate which was pre-sterilized. All the media plates were incubated overnight at 37°C to check contamination.
- Isolation of colonies was performed by four-way streaking method.

2.5. Purification of Suspected Colonies of *E. coli*

Suspected isolated colonies of *E. coli* were re-streaked on MAC-CEF media plates for purification. A separate single colony of *E. coli* was suspected and inoculated on fresh MAC-CEF media plate for purification. It was done to get uniform colonies.

2.5.1. Cefotaxime (CTX) stock Solution

Antibiotic solution that was added in MacConkey Agar for specificity.

Method

- Cefotaxime (CTX) stock solution with concentration (5 mg/ml) was prepared. This was done by dissolving 10mg powder of Cefotaxime Sodium salt (Toku-E, Cat. No.C011-1) in 2ml of distilled water, OR 0.1g Cefotaxime NA salt powder in 20 ml distilled water.

2.6. Biochemical Tests

Indole, Methyl Red, Voges Proskaur & Simmon Citrate (IMViC) and Triple Sugar Iron (TSI) biochemical tests were performed for the confirmation of E.coli.

2.6.1. Indole test

Indole test was performed for confirmation of *E. coli* (Alves *et al.,* 2006)

Method

- First of all 15g of peptone water media were dissolved in 1 L distilled water through boiling. Then 5 ml peptone water was poured per tube and autoclaved at 121°C for 15

minutes. The broth test tubes were incubated overnight at 37°C to check for any contamination.

- Each 5ml tube of peptone water was inoculated with 3 to 4 colonies from purified culture growth. Then all the tubes were incubated at 37ºC for 24 hrs.
- Than few drops of Indole Reagent (Kovac's Reagent) (Scharlau,Spain; Cat. No.RE0007G100) was added directly to the tube and agitated. Tube was allowed to stand for 5-10 minutes.
- Formation of red colored ring at the top was taken as positive indole reaction for *E. coli* and absence of red color ring was indicated as negative reaction.

2.6.2. Methyl Red Test

Methyl red was performed for confirmation of *E. coli (*McDevitt *et al.,* 2009).

Method

- First of all 17g powder of MR-VP Medium (Oxoid, UK; Cat. No.X5279D) was dissolved in 1 L of distilled water and then dispensed 5ml/tube and autoclaved at 121ºC for 15 minutes. Broth test tubes were incubated overnight at 37ºC to check for any contamination.
- Each 5ml tube of MR-VP broth was inoculated with 2-3 colonies from purified growth culture of *E. coli* and all the tubes were incubated at 37ºC for 24hrs.
- Than 2-3 drops of Methyl Red indicator were added. Red coloration was indicated as positive reaction for *E. coli.*

2.6.2.1. Methyl Red Indicator

This indicator was used in the methyl red test.

Method

- First of all, 0.2g of Methyl Red powder (Daejung, Korea; Cat. No.5565-4125), 500ml 95% ethyl alcohol and 500ml distilled water was taken and mixed. Then the funnel was placed on the reagent bottle and the filter paper was folded, so it could fit in the funnel. Then the solution was poured for filtration and after filtration stored in reagent bottle at 4ºC to 10ºC. Working solution was made by taking 20 to 25 ml of prepared solution in falcon tube.

2.6.4. Voges Proskauer Test

Voges Proskauer test was performed for *E. coli* confirmation (McDevitt *et al.,* 2009)

Method

- In a reagent bottle 17g powder of MR-VP Medium (Oxoid, UK; Cat. No.X5279D) was dissolved in 1 L of distilled water and then dispensed 5ml/tube and autoclaved at 121ºC for 15 minutes. Broth test tubes were incubated overnight at 37ºC to check for any contamination.
- Each 5 ml tube of MR-VP broth were inoculated with 2-3 colonies from purified growth culture of *E. coli* and all the tubes were incubated at 37ºC for 24hrs.
- Than 15 drops of Reagent A and 5 drops of Reagent B were added in each tube.
- Development of distinct red/pink color within 5 minutes was taken as indication of positive reaction but *E. coli* was negative for Voges Proskauer.

2.6.4.1. Voges Proskauer Reagent A

This was first reagent of Voges Proskauer test.

Method

- First of all 6.0g Alpha Naphthol powder (Daejung, Korea; Cat. No.5597-4425) and 100ml 95% Ethyl Alcohol was taken and mixed well. Then the funnel was placed on the reagent bottle and the filter paper was folded, so it could fit in the funnel. Then the solution was poured for filtration and after filtration stored in reagent bottle at 4ºC to 10ºC. Working solution was made by taking 20 to 25 ml of prepared solution in falcon tube.

2.6.4.2. Voges Proskauer Reagent B

This was second reagent of Voges Proskauer test.

Method

- First of all 16g potassium hydroxide powder and 100ml distilled water was taken and mixed well. Then the funnel was placed on the reagent bottle and the filter paper was folded, so it could fit in the funnel. Then the solution was poured for filtration and after filtration stored in reagent bottle at 4ºC to 10ºC. Working solution was made by taking 20 to 25 ml of prepared solution in falcon tube.

2.6.5. Citrate Test

Citrate test is performed for *E. coli* confirmation (Silva *et al.,* 1980)

Method

- In a reagent bottle 23g powder of Simmons Citrate agar (Oxoid, UK; Cat. No.X4544G) was dissolved in 1L of distilled water and dispensed 5ml in each tube and autoclaved at 121° C for 15 minutes. The tubes were allowed to cool down by kept them in slanted position. The slanted citrate test tubes were incubated overnight at 37°C to check for any contamination.
- Inoculum were taken from purified culture growth and inoculated the citrate tubes by stabbing the butt and streaked the slant using straight inoculation needle. All the tubes were incubated at 37°C for 24 hrs.
- No change in color (un-inoculated dark green) was indicated as negative reaction. Blue color of slant was indicated as positive reaction.

2.6.6. Triple Sugar Iron (TSI)

TSI test was performed for *E. coli* confirmation (Dash *et al.,* 2012)

Method

- In a reagent bottle 65g of TSI (Oxoid, UK; Cat. No.X4398E) was dissolved in 1L of distilled water and dispensed 5ml in each tube then autoclaved it at 121°C for 15 minutes. The tubes were allowed to cool down by kept them in slanted position. The slanted TSI test tubes were incubated overnight at 37°C to check for any contamination.
- Inoculum was taken from purified culture growth to inoculate triple sugar iron agar tubes by stabbing the butt and streaked the slant using straight inoculation needle. Then all the tubes were incubated at 37°C for 24 hrs.
- Yellow color of slant and butt, gas production, cracks and motility were indicated as positive reaction for *E. coli*.

2.7. ESBL Confirmation Tests

2.7.1. Double Disk Diffusion Test

This test was performed for the confirmation of ESBL (Thomson and Sander, 1992))

Method

- In a flask, 38g Muller Hinton Agar Media (Himedia, India; Cat. No.M173-500G) was suspended in 1L of distilled water. Suspension was boiled to dissolve the powder completely then autoclaved at 121° C for 15 minutes. Then media was poured in the plates at 25ml/plate of 90mm diameter. All the media plates were incubated overnight at 37°C to check for any contamination.
- Then 5ml of Normal Saline was poured in test tube as per sample and dispensed 2-3 colonies and compare the turbidity to the 0.5 McFarland standard (Remel, UK; Cat. No.R20421). This preparation was used to swab the Muller Hinton Agar plate to make a lawn of the inoculums on the medium for antibiotic sensitivity testing. After swabbing all four-antibiotic disks i.e. Ceftazidime (CAZ 30), Ceftazidime with Clavulanic acid CAL 40), Cefotaxime (CTX 30) and Cefotaxime with Clavulanic Acid (CTL 40) were applied at the distance of 25-30mm apart and incubated the plates for 18hrs at 35°C.
- The sample was considered positive for ESBL if the diameter of inhibition zone of the disk with clavulanic acid is 5mm larger than the diameter of inhibition zone of the disk without clavulanic acid.

2.7.2. Double Disk Synergy Test

This test was performed for the confirmation of ESBL (Kaur *et al.,* 2013)

Method

- In a flask, 38g Muller Hinton Agar Media powder (Himedia, India; Cat. No.M173-500G) was suspended in 1L of distilled water. Suspension was boiled to dissolve the powder completely and then autoclaved at 121°C for 15 minutes. After that media was allowed to cool down and then poured in the plates at 25ml per plate of 90 mm diameter. All the media plates were incubated overnight at 37°C to check for any contamination.
- Then 5ml of Normal Saline was poured in test tube as per sample and dispensed 2-3 colonies and compare the turbidity to the 0.5 McFarland standard (Remel, UK; Cat. No.R20421). This preparation was used to swab the Muller Hinton Agar plate to make a lawn of the inoculums on the medium for antibiotic sensitivity testing. After swabbing Amoxycillin + Clavulanic Acid (antibiotic disk) was applied in the center of petri plate. All five-antibiotic disks i.e. Ceftazidime (CAZ), Aztreonam (ATM), Ceftriaxone (CRO), Cefixime (CFM) and Cefepime (FEP) mentioned above

were applied at the distance of 20mm apart each other and from central disk. All the plates were incubated for 24hrs at 37ºC.

- After incubation synergism was checked between the Amoxycillin + Clavulanic Acid (antibiotic disk) and other antibiotic disks. The synergistic effect was considered when any antibiotic disk out of five antibiotic disks mentioned above gave zone of inhibition towards Amoxycillin + Clavulanic Acid (antibiotic disk) in the center.

2.7.2.1. Normal Saline

- In a reagent bottle 9g of NaCl (Scharlau, Spain; Cat. No.S0-2271000) was added in 1L distilled water.
- Autoclaved at 121º C for 15 minutes and stored at 4^{O}C.

McFarland standards

- McFarland standards (Remel, UK; Cat. No.R20421) were used as a reference to adjust the turbidity of bacterial suspensions so that the number of bacteria was within a given range to standardize microbial testing. If standard was not used then a suspension can be too heavy or too dilute, an erroneous result (either falsely resistant or falsely susceptible) for any given anti-microbial agent could occur.
- For antibiotic sensitivity test 0.5 no. McFarland standard (Remel, UK; Cat. No.R20421) is recommended.

Antibiotics List for DDDT & DDST

Sr. No.	Antibiotics	Class	Symbol	Concentration (µg)	Company Name	Catalogue No.
1.	Ceftazidime	Cephalosporins	CAZ	30µg	Oxoid, UK	CT0412B
2.	Ceftazidime with clavulanic acid	B-Lactams Inhb.	CZC	40µg	Bioanalyse, Turkey	ASD02110

3.	Cefotaxime	Cephalosporins	CTX	30µg	Oxoid, UK	CT0166B
4.	Cefotaxime with clavulanic acid	B-lactam Inhb.	CTC	40µg	Bioanalyse, Turkey	ASD01820
5.	Amoxycillin with clavulanic acid	B-lactam Inhb.	AUG	40µg	Bioanalyse, Turkey	ASD09048
6.	Cefixime	Cephalosporins	CFM	5µg	Oxoid, UK	CT0653B
7.	Cefipime	Cephalosporins	FEP	30µg	Oxoid, UK	CT0771B
8.	Ceftriaxone	Cephalosporins	CRO	30µg	Oxoid, UK	CT0417B
9.	Aztreonam	Monobactams	ATM	30µg	Oxoid, UK	CT9008B

Table 2.1: List of antibiotics discs used for phenotypic tests with their symbols and concentrations

2.8. Antibiotic Susceptibility Tests

The antibiotic susceptibility test was performed to find out the antibiotic resistance pattern of all *ESBL E.coli* isolates (CLSI, 2017). AST was conducted using Muller-Hinton agar (Himedia, India) and 14 antimicrobial agents.

Method

- In a flask, 38g Muller Hinton Agar Media powder (Himedia, India; Cat. No.M173-500G) was suspended in 1L of distilled water. Suspension was boiled to dissolve the powder completely and then autoclaved at 121ºC for 15 minutes. After that media was allowed to cool down and then poured in the plates at 25-30ml per plate. All the media plates were incubated overnight at 37ºC to check for any contamination.

- Than 5ml of Normal Saline was poured in test tube as per sample and dispensed 2-3 colonies and compare the turbidity to the 0.5 McFarland standard (Remel, UK; Cat. No.R20421). This preparation was used to swab the Muller Hinton Agar plate to make a lawn of the inoculums on the medium for antibiotic sensitivity testing.
- After swabbing, antibiotic disks were applied in petri plate. All antibiotic disks mentioned above were applied at the distance of 20mm apart each other. All the plates were incubated for 18hrs at 35ºC.
- After incubation results were noted as measurement of inhibition zones.

List of Antibiotics used for AST

Sr. No.	Antibiotic Agents	Class	Symbol	Concentration	Company Name	Catalogue No.
1.	Pencillin G	Penicillin	P	10µg	Oxoid, UK	CT0043B
2.	Oxytetracycline	Tetracycline	OT	30µg	Liofilchem, Italy	$9065
3.	Gentamicin	Aminoglycoside	CN	10µg	Oxoid, UK	CT0024B
4.	Lincomycin	Lincosamides	MY	10µg	Oxoid, UK	CT0123B
5.	Ceftazidime	Cephalosporin	CAZ	30µg	Oxoid, UK	CT0412B
6.	Cefixime	Cephalosporin	CFM	5µg	Oxoid, UK	CT0653B
7.	Ampicillin	Penicillin	AMP	10µg	Oxoid, UK	CT0003B
8.	Doxycycline	Tetracycline	DO	30µg	Oxoid, UK	CT0018B
9.	Ciprofloxacin	Fluoroquinolone	CIP	5µg	Oxoid, UK	CT9056B
10.	Enrofloxacin	Fluoroquinolone	ENR	5µg	Oxoid, UK	CT0639B

11.	Cotrimoxazole	Sulfonamide	SXT	25µg	Oxoid, UK	CT9042B
12.	Streptomycin	Aminoglycosides	S	10µg	Oxoid, UK	CT0047B
13.	Aztreonam	Monobactams	ATM	30µg	Oxoid, UK	CT9008B
14.	Cefotaxime	Cephalosporins	CTX	30µg	Oxoid, UK	CT0166B

Table 2.2: List of Antibiotics discs used for AST with their symbols and concentrations

Reference Values for Antimicrobial Sensitivity Tests

Sr. No.	**Antimicrobial Agent**	**Resistance (mm)**	**Intermediate (mm)**	**Sensitivity (mm)**
1	**AMP**	≤13	14-16	≥17
2	**P**	28	-	29
3	**CFM**	≤15	16-18	≥19
4	**CAZ**	≤17	18-20	≥21
5	**CTX**	≤22	23-25	≥26
6	**ATM**	≤17	18-20	≥21
7	**CN**	≤12	13-14	≥15
8	**S**	≤11	12-14	≥15
9	**DO**	≤10	11-13	≥14
10	**OT**	≤11	12-14	≥15
11	**MY**	-	-	-
12	**CIP**	≤21	22-25	≥26
13	**ENR**	≤16	17-22	≥23
14	**SXT**	≤10	11-15	≥16

Table 2.3: Reference Values for Antimicrobial Sensitivity Tests (CLSI: Wayne, Pennsylvania, 2017)

2.9. DNA Extraction

- DNA of all the isolates was extracted through conventional boiling method.
- For one isolate, 200 µl autoclaved distilled water was taken in an Eppendorf and 2-3 colonies from pure culture was added through wire loop after sterilization of wire loop.
- After labelling, vortex (Maxi Max II) was done for 30 seconds to mix the colonies in autoclaved distilled water properly.
- Then cap of the Eppendorf was punctured by hot wire and placed it in hot plate (Labnet International) which had temperature of 96°C for 10 minutes.
- After that Eppendorf was placed at 4°C for 5 minutes and then centrifuged at 12,500 rpm for 5 minutes.
- At the end 150µl supernatant was collected in a separate Eppendorf and stored at -20°C.

2.10. PCR Mastermix for CTX-M Gene

Sr. No	Reagents	Volume
1	dNTPs	0.5µl
2	Taq Buffer	2.5µl
3	Taq DNA polymerase	0.3µl
4	Forward Primer	1.0µl
5	Reverse Primer	1.0µl
6	$MgCl_2$	1.5µl
7	Nuclease free water	13.2µl
8	DNA	5.0µl
	Total	**25µl**

Table 2.4: Recipe of PCR Master-mix for the amplification of CTX Gene.

2.10.1. Thermal Profile for CTX-M gene

PCR was performed as described by Kaftandzieva *et al (*2011). PCR conditions for *CTX-M* gene were: 5 min at 94º C and 32 cycles of amplification consisting of 30 s at 95º C, 1 min at 54º C, and 2 min at 72º C, with 5 min at 72º C for the final extension After PCR, gel electrophoresis was carried out to affirm the PCR amplification of the targeted gene. PCR product was taken from each tube and poured into gel wells.100bp DNA marker was used and PCR product was electrophoresed on 1%

agarose gel containing ethidium bromide at 100 volts for 60 minutes. Bands of the expected size were visualized under UV light.

2.11. PCR Mastermix for TEM Gene

Sr. No	Reagents	Volume
1	dNTPs	0.5µl
2	Taq Buffer	2.5µl
3	Taq DNA polymerase	0.3µl
4	Forward Primer	1.0µl
5	Reverse Primer	1.0µl
6	$MgCl_2$	1.5µl
7	Nuclease free water	13.2µl
8	DNA	5.0µl
9	**Total**	**25µl**

Table 2.5: The tabular depiction of the reagents used for mastermix of TEM Gene with their volume.

2.11.1. Thermal Profile for TEM gene

PCR was performed as described by Gangoue *et al (*2005). PCR conditions for *TEM* gene were: 5 min at 94º C and 32 cycles of amplification consisting of 30 s at 95º C, 1 min at 54º C, and 2 min at 72º C, with 5 min at 72º C for the final extension After PCR, gel electrophoresis was carried out to affirm the PCR amplification of the targeted gene. PCR product was taken from each tube and poured into gel wells. 100bp DNA marker was used and PCR product was electrophoresed on 1% agarose gel containing ethidium bromide at 100 volts for 60 minutes. Bands of the expected size were visualized under UV light.

2.12. PCR Mastermix for SHV Gene

Sr. No	Reagents	Volume
1	dNTPs	0.4µl
2	Taq Buffer	2.0µl
3	Taq DNA polymerase	0.3µl
4	Forward Primer	1.0µl
5	Reverse Primer	1.0µl

6	$MgCl_2$	1.2µl
7	Nuclease free water	11.1µl
8	DNA	3.0µl
9	**Total**	**22µl**

Table 2.6: Recipe of PCR Master-mix for the amplification of SHV Gene.

2.12.1. Thermal Profile for SHV gene

PCR was performed as described by Colom *et al (*2003). PCR conditions for *SHV* gene were: 5 min at 94º C and 32 cycles of amplification consisting of 30 s at 94º C, 30 s at 54º C, and 1 min at 72º C, with 10 min at 72º C for the final extension. After PCR, gel electrophoresis was carried out to affirm the PCR amplification of the targeted gene. PCR product was taken from each tube and poured into gel wells.100bp DNA marker was used and PCR product was electrophoresed on 1% agarose gel containing ethidium bromide at 100 volts for 60 minutes. Bands of the expected size were visualized under UV light.

Gene	**Sequence (5′ → 3′)**	**Fragment Size (bp)**
blaCTX-F	ACCGCCGATAATTCGCAGAT	588bp
blaCTX-R	GATATCGTTGGTGGTGCCATAA	
blaTEM-F	TTGGGTGCACGAGTGGGTTA	465bp
blaTEM-R	TAATTGTTGCCGGGAAGCTA	
blaSHV-F	AGGATTGACTGCCTTTTTG	392bp
blaSHV-R	ATTTGCTGATTTCGCTCG	

Table 2.7: List of Primers used for PCR

RESULTS

3.1. Cultural and Morphological Characteristics

From the 112 suspected RAMS samples, 84 isolates of *E.coli* were recovered by culturing on MAC-CEF agar. MAC-CEF agar gives round or star shape pinkish colonies of *ESBL E.coli.* (Fig 3.1)

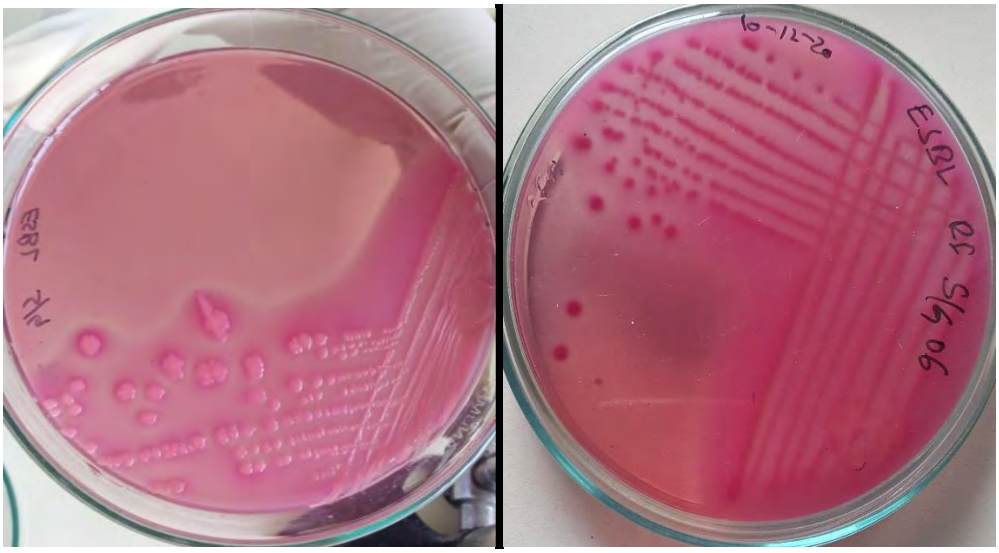

Fig 3.2: Round and Star shape colonies of *E.coli* on MacConkey Agar Plates

3.2. Biochemical Testing For *E.coli*

After obtaining pure culture from enriched broth, all samples were processed through biochemical tests for the confirmation of *E.coli* Out of total 112 samples, 84 samples were confirmed *E.coli* through these tests. All 84 samples were tested positive for indole and methyl red tests while negative for Voges Proskaur and Simmon Citrate tests. In case of TSI test due to dextrose or lactose fermentation these 84 samples produced gas in the slant with yellow butt (not H2S as it produced black color in slant).

Sr. No.	Biochemical Test	Observation	Result
1.	Indole Test	Red Ring on the Top	Positive
2.	Methyl Red Test	Red Coloration	Positive
3.	Voges Proskaur Test	No Change	Negative
4.	Simmon Citrate Test	No Change	Negative
5.	Triple Sugar Iron Test	Gas with yellow butt	Positive

Table 3.8: E.coli Confirmation through Biochemical Tests

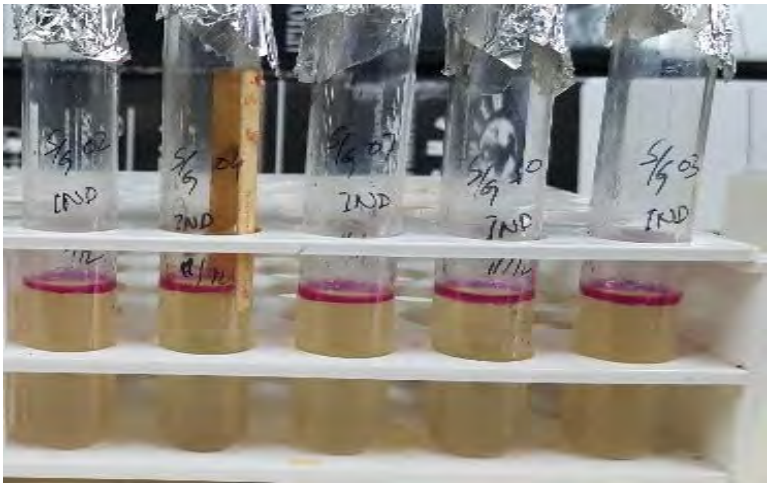

Fig 3.3: Indole test, showing Positive Results for E.coli

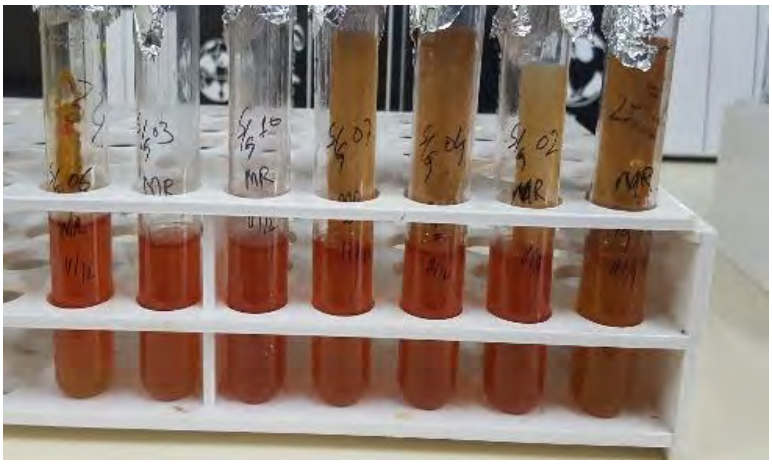

Fig 3.4: Methyl Red, showing Positive Results for E.coli

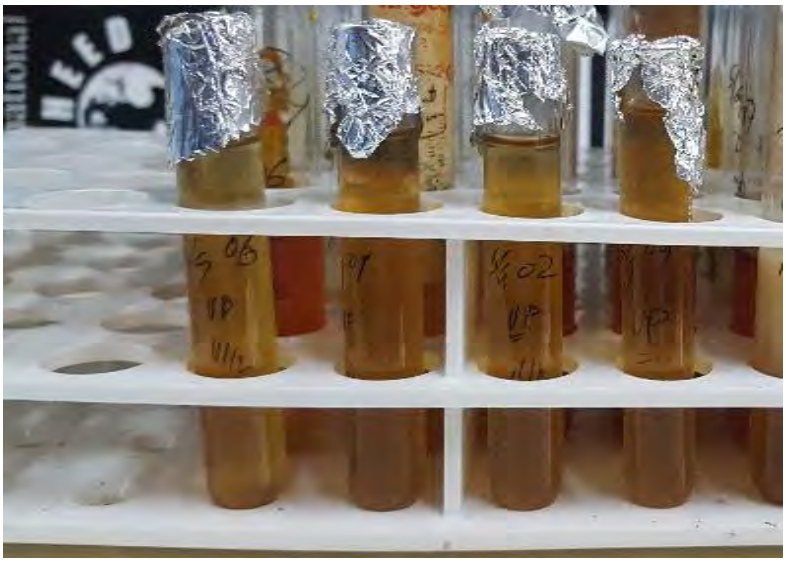

Fig 3.5: Voges Proskaur, indicating Negative Result for E.coli

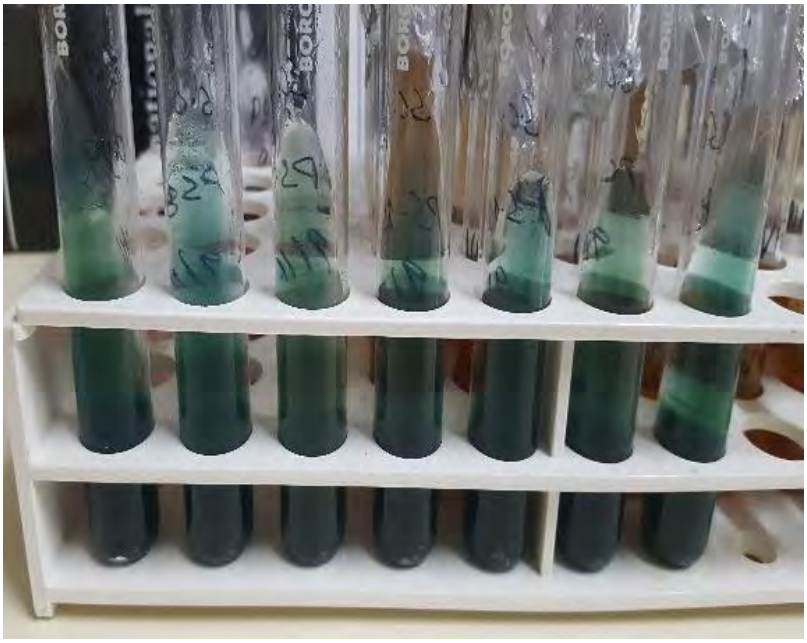

Fig 3.6: Simmon citrate, indicating Negative results for E.coli

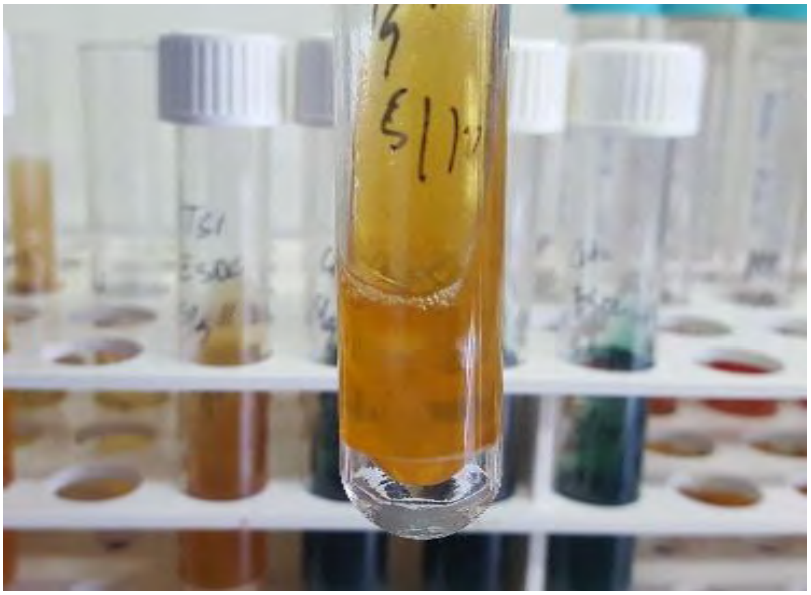

Fig 3.7: Triple Sugar Iron Test, showing Positive Result for E.coli

3.3. Results of Phenotypic Tests for ESBLs

Out of 84 *E.coli* confirmed samples, 39 samples were tested positive for Double Disk Diffusion Test and Double Disk Synergy Test. These 39 samples were considered *ESBL* producing *E.coli* isolates.

Sr. No.	Phenotypic Test	Observation	Result
1	Double Disk Diffusion Test	Discs with clavulanic acid had more than 5mm zone of inhibition as compared to disc without clavulanic acid subject to the isolate was found resistant to either of the normal cephalosporin disc (CTX/CAZ)	Positive
2	Double Disk Synergy Test	Discs around central disc showed synergy toward centre	Positive

Table 3.9: ESBL E.coli Confirmation through Phenotypic Tests

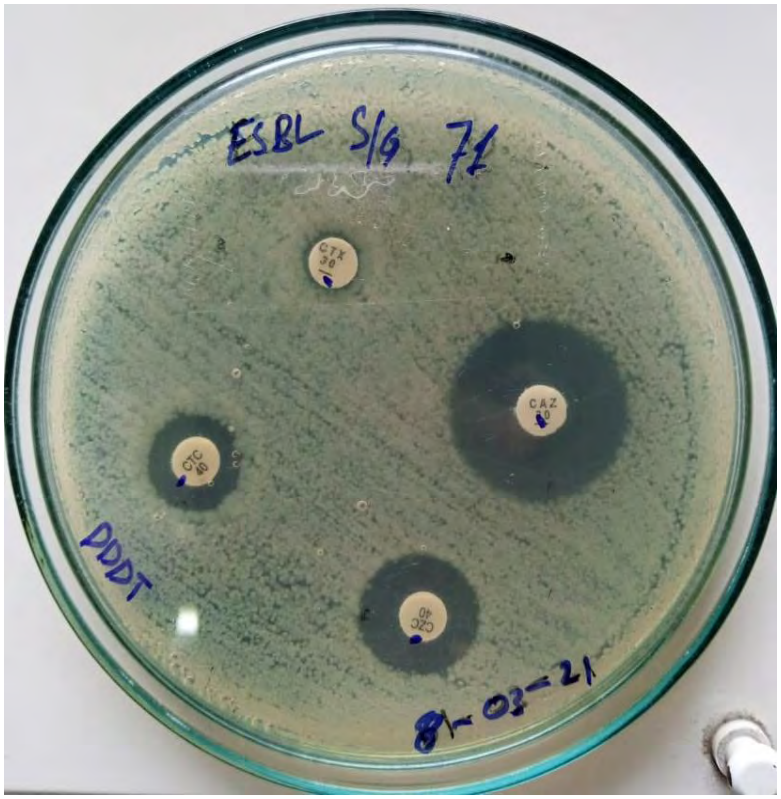

Fig 3.8: Positive Result for DDDT.

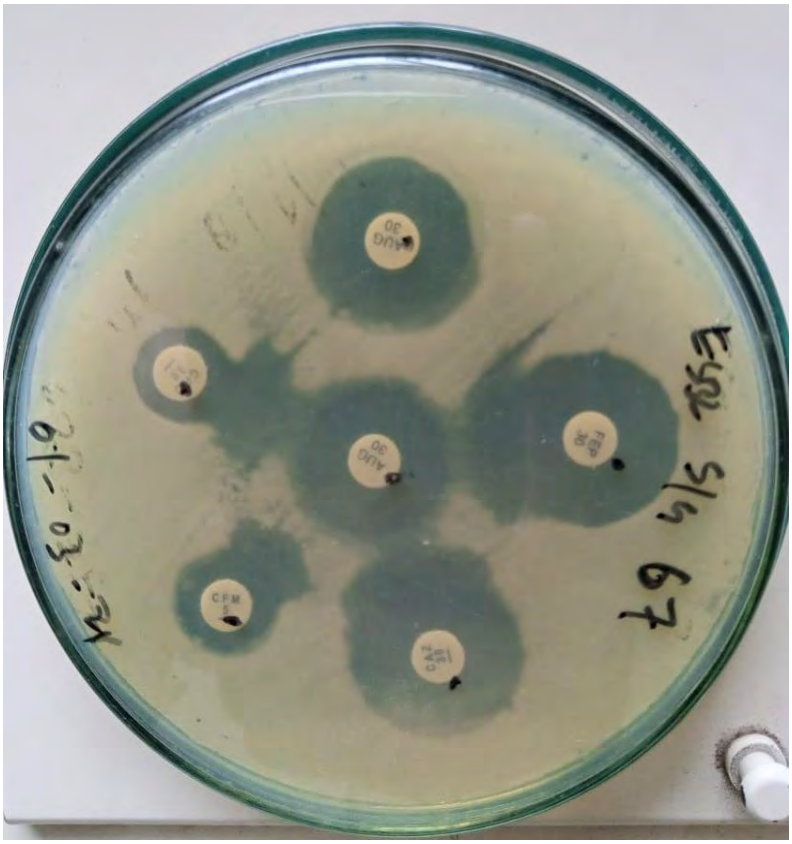

Fig 3.9: Positive Result for DDST

3.4. Antibiotic Sensitivity Test

To evaluate the antibiotic sensitivity profile of *ESBL E. coli* isolates, 14 different antimicrobial agents were used. The 39 pure isolates were tested for antibiotic sensitivity testing against different antimicrobials.

Complete resistance was observed against Pencillins, Ampicillin and Lincosamides each with 100% of *ESBL E. coli* isolates were found resistant to it. Second highest resistance was found against Cefotaxime with 98% resistance. Third highest resistance was found against Cifixime with 90% resistance.

Highest antimicrobial sensitivity was observed against Gentamicin (Aminoglycosides) and Ceftazidime with 77% and 64% sensitivity respectively while it is 44% in case of Aztreonam and Doxycycline.

Enrofloxacin and Doxycycline had 74% and 44% isolates with of intermediate sensitivity respectively while Ceftazidime, Stereptomycin and Aztreonam had 26% intermediate sensitive isolates.

In combination of both *ESBL* producers i.e. CTX-M and TEM, all *ESBL* positive isolates were 100% resistant against Penicillin, Ampicillin and Lincosamides while Enrofloxacin and Doxycycline showed intermediate efficacy. Same results were found for five isolates which were only TEM positive.

In case of cephalosporin, third generation cephalosporins were found totally inefficacious for both CTX-M and TEM positive isolates which means that none of isolate found sensitive against third generation cephalosporins.

Sr. No.	**Antimicrobial Agents**	**Total Isolates of ESBL E.coli**	**Resistant Isolates**	**Intermediate Isolates**	**Sensitive Isolates**
1	AMP	39	39 (100%)	-	-
2	P	39	39 (100%)	-	-
3	CFM	39	35 (90%)	04 (10%)	-
4	CAZ	39	04 (10%)	10 (26%)	25 (64%)
5	CTX	39	38 (98%)	01 (02%)	-

6	ATM	39	12 (30%)	10 (26%)	17 (44%)
7	CN	39	02 (5%)	07 (18%)	30 (77%)
8	S	39	28 (72%)	10 (26%)	01 (2%)
9	DO	39	05 (12%)	17 (44%)	17 (44%)
10	OT	39	21 (54%)	03 (8%)	15 (38%)
11	MY	39	39 (100%)	-	-
12	CIP	39	27 (69%)	09 (23%)	03 (8%)
13	ENR	39	07 (18%)	29 (74%)	03 (8%)
14	SXT	39	19 (49%)	04 (10%)	16 (41%)

Table 3.10: Result of Antimicrobial Susceptibility Test for *ESBL E. coli*

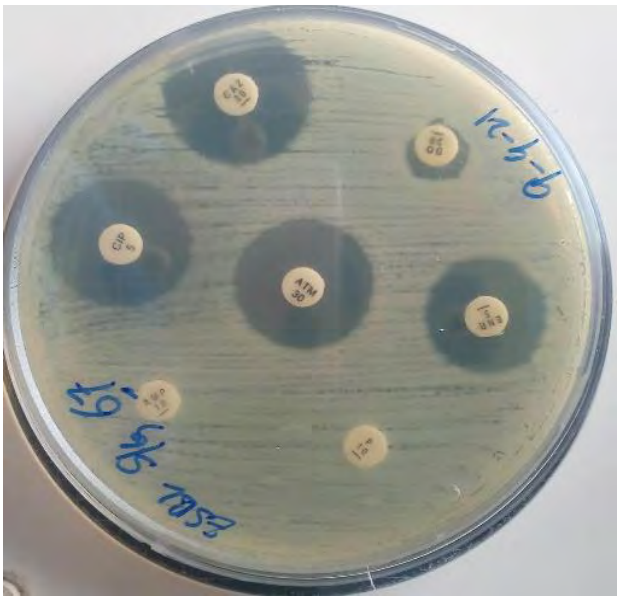

Fig 3.10: Petri Plate showing Result for AST

3.5. Molecular Detection of *bla* genes through PCR

bla genes were detected through PCR in 39 *ESBL E. coli* isolates. Highest prevalence was noticed for TEM i.e. 94.8%. There were 37 out of 39 isolates found positive for TEM. CTX-M had 84.6% prevalence rate and 33 out of 39 isolates were found positive for CTX-M. None of the isolate was positive for SHV.

3.5.1. *bla*TEM Gene Result

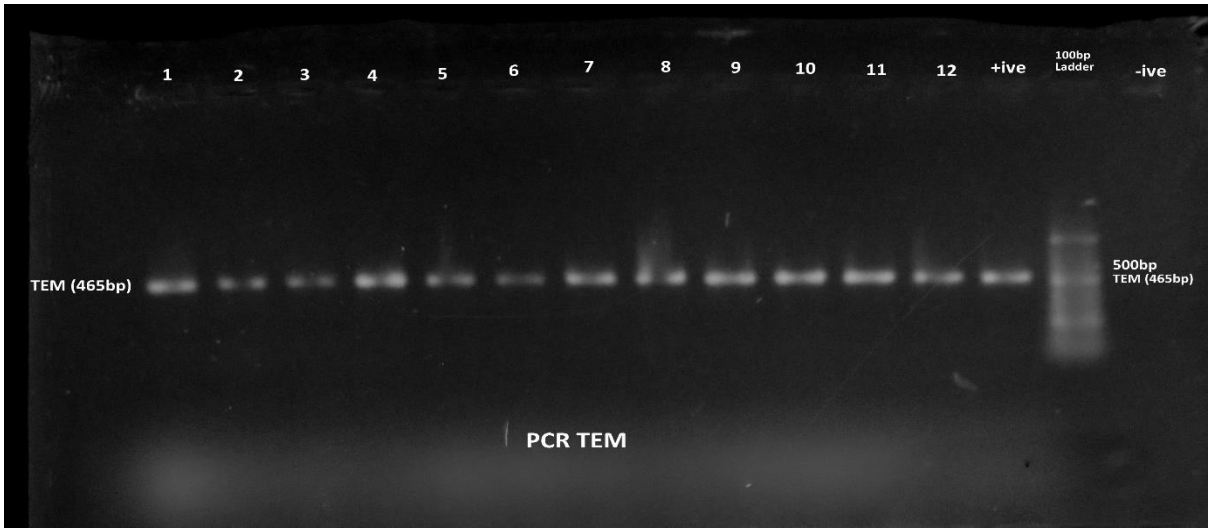

Fig 3.11: Agarose gel electrophoresis of PCR product for detection of *bla*TEM gene (465bp). Molecular weight marker of Gene Ruler 100 bp (Thermofisher Scientific) was used, Lane (-ve) and Lane (+ve) represent negative and positive control respectively. Lane 1-12 represent *ESBL* isolates

3.5.2. *bla*CTX-M Gene Result

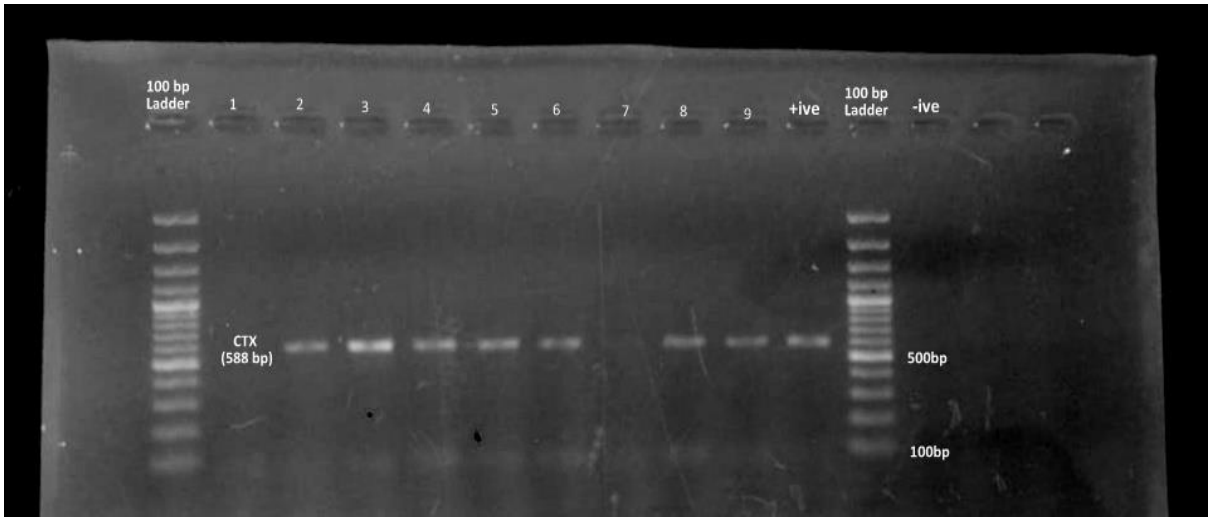

Fig 3.12: Agarose gel electrophoresis of PCR product for detection of *bla*CTX-M gene (588bp). Molecular weight marker of Gene Ruler 100 bp (Thermofisher Scientific) was used, Lane (-ve) and Lane (+ve) represent negative and positive control respectively. Lane 1 and 7 indicate negative result while Lane 2, 3, 4, 5, 8 and 9 denote the positive result for CTX-M.

Sr. No.	Isolate ID	Phenotypic Resistance Pattern	Number of Antibiotics	*Bla* Genes Present
1	ESBL S/G 04	AMP,P,MY,CTX,CFM,ATM,SXT	07	TEM
2	ESBL S/G 10	AMP,P,MY,CTX,CFM,CIP	06	CTX-M,TEM
3	ESBL S/G 11	AMP,P,MY,CTX,CFM,OT,SXT,S	08	CTX-M,TEM
4	ESBL S/G 13	AMP,P,MY,CTX,CFM,CIP,S	07	CTX-M,TEM
5	ESBL S/G 19	AMP,P,MY,CTX,CFM,CN,CIP,S	08	CTX-M,TEM
6	ESBL S/G 21	AMP,P,MY,CTX,CFM,OT,SXT,S	08	CTX-M,TEM
7	ESBL S/G 22	AMP,P,MY,CTX,CFM,DO,OT,SXT,CIP,S	11	TEM
8	ESBL S/G 24	AMP,P,MY,CTX,CFM,DO,OT,SXT,S	09	CTX-M,TEM
9	ESBL S/G 25	AMP,P,MY,CTX,CFM,OT,SXT,CIP,S	09	CTX-M,TEM
10	ESBL S/G 26	AMP,P,MY,CTX,CFM,OT,SXT,S	08	CTX-M,TEM
11	ESBL S/G 29	AMP,P,MY,CTX,CFM,OT,SXT,S	08	CTX-M,TEM
12	ESBL S/G 36	AMP,P,MY,CTX,CFM,OT,SXT,CIP	08	CTX-M,TEM
13	ESBL S/G 41	AMP,P,MY,CTX,CFM,DO,OT,CIP,S	09	TEM
14	ESBL S/G 50	AMP,P,MY,CTX,CIP,S	06	CTX-M,TEM
15	ESBL S/G 51	AMP,P,MY,CTX,CFM,CIP,S	07	TEM
16	ESBL	AMP,P,MY,CTX,CFM,OT,SXT,CIP,S	09	CTX-M,TEM

	S/G 52			
17	ESBL S/G 53	AMP,P,MY,CTX,DO,CIP,S	07	CTX-M,TEM
18	ESBL S/G 54	AMP,P,MY,CTX,CFM,OT,SXT,CIP,S	09	CTX-M,TEM
19	ESBL S/G 56	AMP,P,MY,CTX,CFM,ATM,CAZ,DO, OT,S,SXT,ENR,CIP	13	CTX-M,TEM
20	ESBL S/G 58	AMP,P,MY,CTX,ENR,CIP,S	07	TEM
21	ESBL S/G 59	AMP,P,MY,CTX,CFM,ATM,OT,ENR, S,SXT,CIP	11	CTX-M,TEM
22	ESBL S/G 60	AMP,P,MY,CTX,CFM,ATM,OT,ENR, CIP,S	10	CTX-M,TEM
23	ESBL S/G 62	AMP,P,MY,CTX,CFM,ATM,CIP,S	08	CTX-M,TEM
24	ESBL S/G 64	AMP,P,MY,CTX,CFM,ATM,OT	07	CTX-M,TEM
25	ESBL S/G 67	AMP,P,MY,CTX,CFM,OT,CIP	07	CTX-M,TEM
26	ESBL S/G 68	AMP,P,MY,CTX,CFM,ATM,OT,ENR, SXT,S,CIP	11	CTX-M,TEM
27	ESBL S/G 70	AMP,P,MY,CTX,CFM,ATM,CAZ,OT, ENR,S,CIP,SXT	12	CTX-M,TEM
28	ESBL S/G 71	AMP,P,MY,CTX,CFM,ATM,CIP,S	08	CTX-M,TEM
29	ESBL S/G 73	AMP,P,MY,CTX,CFM,ENR,CIP,S	08	TEM
30	ESBL S/G 75	AMP,P,MY,CTX,CFM,S	06	CTX-M,TEM
31	ESBL S/G 76	AMP,P,MY,CTX,CFM,ATM,CIP,CAZ	08	CTX-M,TEM
32	ESBL S/G 77	AMP,P,MY,CTX,CFM,OT,SXT,CIP,S	09	CTX-M,TEM

33	ESBL S/G 79	AMP,P,MY,CTX,CFM,SXT	06	TEM
34	ESBL S/G 82	AMP,P,MY,CTX,CFM,ATM,CIP	07	CTX-M,TEM
35	ESBL S/G 86	AMP,P,MY,CTX,CFM,ATM,SXT,CAZ,CIP,S	10	CTX-M,TEM
36	ESBL S/G 87	AMP,P,MY,CTX,CFM	05	CTX-M,TEM
37	ESBL S/G 89	AMP,P,MY,CTX,CFM	05	CTX-M
38	ESBL S/G 90	AMP,P,MY,CTX,OT,SXT,S	07	CTX-M,TEM
39	ESBL S/G 92	AMP,P,MY,CTX,CFM,OT,CIP	07	CTX-M

Table 3.11: Antibiotic Resistance and Resistant Genes Pattern of ESBL Isolate

DISCUSSION

ESBL E.coli is considered as major threat in antibiotic sensitivity especially to third generation antibiotics these days. It is also a causative agent of different infections such as urinary tract infections and diarrhoea. *E. coli* is more dangerous than any other opportunistic pathogen as it is commensal and found naturally in the gut micro-flora of the animals. Current study was performed to check the prevalence of ESBL *E.coli* and comparison of the resistance genes available in those isolates through phenotypic and genotypic confirmation against different antibiotics.

To obtain the antibiotic sensitivity and resistance pattern of *ESBL E.coli* 39 isolates were tested against 14 different antibacterial agents. *ESBL E.coli* was found completely (100%) resistant against Penicillin, Ampicillin and Lincomycin. Second highest was Cefotaxime and Cefixime against which *ESBL E. coli* were 98% and 90% resistant respectively.

For the detection of *bla* resistant genes, PCR was performed on *E.coli* isolates which were confirmed as *ESBL* through phenotypic assays i.e. DDDT and DDST. The PCRs revealed the presence of the *bla*TEM and *bla*CTX-M genes in these isolates.

In Pakistan Amin et al. (2009) reported 47.5% prevalence rate of *ESBL E.coli* in their findings working on patients admitted in tertiary care hospitals and similar to these results after meta-analysis of different studies on ESBL in Pakistan Abrar et al. (2018) revealed 41.8% and 16.4% prevalence of *ESBL E. coli* in Punjab and Islamabad respectively. In two different research studies (Ullah et al., 2009a; Ullah et al., 2009b) found 36% and 56.9% of prevalence for *ESBL E.coli* in UTI patient from human hospitals. In a research study on migratory birds Mohsin et al. (2017) reported 17.3% prevalence of *ESBL E.coli* with 92.3% prevalence rate of *bla*CTX-M. These all results have shown much similarities with findings of present study where prevalence rate for *ESBL E.coli* was 46.4% while it was 84.6% and 94.8% for *bla*CTX-M and *bla*TEM respectively.

In China, a study was conducted in Chinese secondary hospitals in which Quan et al. (2016) found 55.5% prevalence for *ESBL E.coli* while in same year Zhang et al. (2016) reported 31% prevalence rate for *ESBL E.coli*. Similarly in India a study was conducted in tertiary care teaching hospital and according to Umadevi et al. (2011) prevalence rate for ESBL *E. coli* was 81%. In a research study conducted in urban hospitals of Bangladesh Rahman et al. (2004) found 39.5% prevalence rate for *ESBL E.coli*.

Ahmed et al. (2021) investigated the antimicrobial resistance of *ESBL*-producing *E. coli* in cattle and observed that all the isolates were resistant to Penicillin and Cotrimoxazole as it was also found that *ESBL E.coli* was 100% resistant against penicillin but in case of Cotrimoxazole, it showed 49% resistance in present study. They also observed that Tetracycline, Ceftazidime, Ciprofloxacin, and Gentamicin were 85.7 % effective while in present study 44%, 64% and 77% sensitivity rate was noticed for Tetracycline, Ceftazidime and Gentamicin respectively.

In contrary to present study Adator et al. (2020) found that *ESBL E.coli* recovered from beef samples had 51.1%, 22.6% and 22.5% resistant against Oxytetracycline, Streptomycin and Ampicillin respectively whereas in our study we observed 100% and 72% resistance against Streptomycin and Ampicillin respectively while 12% were found to be resistant against Oxytetracycline.

Roldan-Masedo et al. (2019) recovered *E. coli* from urine samples during the years 2014, 2015 and 2016 and resistance rates in these against Gentamicin were found to be 15%, 5.3% and 10.6% during these years respectively. In agreement we also found 5% resistance in *ESBL E. coli* against Gentamicin in present study, which was the lowest resistance observed in the ESBL isolates.

Prevalence for CTX-M was 84.6% in present study, similar to this Umair et al. (2019) reported 86.2% prevalence rate for CTX-M in *ESBL E.coli* isolates which were recovered from cattle and poultry.

Higher percentages of *ESBL E.coli* was found by Ahmed et al. (2013) i.e. 65.6% in which CTX-M and TEM were prominent with 52.3% which is contrary to results of present study but like us they found very low percentage of SHV 6%.

In another study on prevalence rate of bla genes, results of Jena et al. (2017) were quite similar to our results. According to them TEM had highest prevalence rate of 93.5% which was 97% in our findings, while second most prevalent gene was CTX-M (82.6%) which was 93% in our results. SHV was at very low prevalence rate of 4% which was not detected even in a single ESBL isolate in present study showing 0% prevalence.

In contrary to present findings, Gundran et al. (2019) and Cicek et al. (2013) found 58% and 44% prevalence for TEM respectively while CTX-M were found in higher percentages like our results i.e. 74% and 83% respectively. But both were contrary to each other in case of SHV having 27 and 1.8 percentages respectively.

Diagbouga et al. (2016) found in their study 20.6% prevalence for TEM and 62% for CTX-M which is totally contrary to present study while in case of SHV like all above mentioned

researches they were also found low prevalence percentage i.e. 4.8%. Phenotypically they observed higher resistance percentages of *ESBL E.coli* against Streptomycin and Ciprofloxacin i.e. 72% and 69% respectively as we found in present study.

Parvin et al. (2020) screened antimicrobials for susceptibility in frozen chicken meat and reported higher percentage of resistance for Ciprofloxacin and Cotrimoxazole (88.4%), Ampicillin (89.5%), followed by Oxytetracycline (93%) resistant against *E. coli* in contrary to present study

In contrary to present study Lin et al. (2017) documented declined resistance rates of *ESBL E.coli* (recovered from pigs) to Ceftriaxone, Ciprofloxacin, and Cefotaxime in the control group while high-level resistance rates in test period for Ampicillin, Cotrimoxazole, Tetracycline, and Streptomycin as we noticed.

Lai et al. (2016) reported that they found 30% *ESBL*-producing *E. coli* susceptible to doxycycline while in case of present study we found *ESBL E. coli* 12% resistant against Doxycycline.

In line with the results of the present study Najjuka et al. (2016) also detected high rates of antimicrobial resistance in *ESBL E. coli* isolates. They found 40% resistant rate for Cotrimoxazole. But in present study resistance rate for Ciprofloxacin (69%) was high as they found 11% resistance for Ciprofloxacin in their study.

Pourakbari et al. (2012) described resistance rates for *E. coli* in young patients against Cotrimoxazole, Gentamicin, Cefixime, and Ceftriaxone. These antibiotics presented the resistance rates of 84%, 24%, 46%, and 45% respectively.

One decade ago in urinary tract infections, Azap et al. (2010) recorded the percentages of isolates with resistance to Ciprofloxacin, Gentamicin and Cotrimoxazole were 39.2% in the *ESBL*-positive group while 4.6% in the negative group which was contrary to results of present study.

Al-Tawfiq and Anani (2009) revealed the susceptibility rates of *ESBL E. coli* to Ceftriaxone, Nitrofurantoin, Cotrimoxazole, and Ciprofloxacin in community acquired and health care associated isolates were 92.7% and 62.3%, 97.0% and 32.5%, 62.7% and 42.2% and 74.1% and 53.3% respectively.

Duttaroy and Mehta (2005) tested all the *ESBL*-positive isolates in clinical isolates for their susceptibility to Cefotaxime, Ceftriaxone, and Ceftazidime. The combined results of all the three drugs yielded 73% of ESBL *E. coli* to be resistant. Ceftazidime was found most effective in detecting resistance with the rate of 85%.

Goldstein (2000) observed the antibiotic susceptibility rates for *ESBL E. coli* in community acquired patients. He found Gentamicin 98.4%, Ciprofloxacin (98.3%), Ceftazidime (99%), Cefotaxime (99.8%) and Cefixime (83.6%) susceptible but now after 21 years according to our results *ESBL E.coli* is almost resistant against Cefotaxime, Cefixime and Ciprofloxacin while sensitivity percentage of Gentamicin and Ceftazidime is reduced to 77% and 64% respectively.

In a recent research study Mahamat et al. (2020) examined the samples from different animals to find out prevalence of *ESBL E.coli* and they found 72%, 20% and 36% prevalence of ESBL in human, chicken and cattle respectively. In agreement they also found higher prevalence rate for CTX-M while lower for SHV i.e. 98% and 2% respectively.

At the end of this study it is concluded that the *ESBL E.coli* strains which are isolated from small ruminants were resistant against most of the antibiotic groups including Penicillin, Cephalosporin (cefotaxime and cefixime) and Lincosamides which is an alarming situation. Two antibiotics including Gentamicin (Aminoglycosides) and Ceftazadime show higher sensitivity towards ESBL isolates. The high prevalence of *bla* genes i.e. *bla*TEM and *bla*CTX-M is predicted to be responsible for high resistance of *ESBL E.coli*. Furthermore, these *ESBL E. coli* also pose a threat to public health, because they can get transmitted from livestock to humans through the food chain. A comprehensive strategy is required to be implemented in semi-advanced, low and middle income countries for effective control of increasing AMR threat to the public as well as animal health.

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