Antibiotic Sensitivity and Characterization of β-Lactamase Producing *E. coli* Isolates from Commercial Poultry

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DECLARATION

I hereby declare that the work presented in this thesis entitled is my own effort and the material contained in this thesis is original. I have not previously presented any part of this work elsewhere for any other degree.

Umama Fayyaz

CERTIFICATE

This dissertation submitted "**Antibiotic Sensitivity and Characterization of β -Lactamase Producing *E. coli* Isolates from Commercial Poultry**" by **Umama Fayyaz** is accepted in its present form by the Department of Zoology, Faculty of Biological Sciences, Quaid-i-Azam University, Islamabad as satisfying the thesis requirements for the degree of master of Philosophy in Animal Microbiology.

Supervisor:

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Dated:

DEDICATION

I would like to pay my special dedication TO **ALLAH ALMIGHTY & HOLY PROPHET HAZRAT MUHAMMAD (P.B.U.H)**

&

My Parents

Mr. and Mrs. Muhammad Fayyaz, their efforts, love, affection, prayers, and wishes, facilitated me with tremendous opportunities to explore myself in every part of life in this world and will after this world **IN SHA ALLAH**

&

My Teachers

The teacher is a way for a person through which someone can see him/herself into delight way of life. A special dedication for **Dr. Farhat Hashmi** who invokes love for Qur‘an, who changes all the prospective of life for me. I am very lucky to have you in my life.

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Abstract

This study was aimed at studying the antibiotic sensitivity and β-lactamase (ESBL and AmpC) production in E. coli isolated from local commercial poultry. An objective was to study the phylogenetic grouping of these isolates. Thirty *E. coli* isolates were recovered from the local commercial chicken and were confirmed through growth characteristics on respective medias and *uidA* gene PCR. Antibiotic susceptibility pattern of the *E. coli* isolates was determined against 13 different antibiotics. Tetracycline was found to be the least effective (80% resistance), followed by Ampicillin (76.66%) and Trimethoprim (63.3 %). On the other hand, Fosfomycin (86.66%), Gentamicin (80%) and Ceftiofur (73.3%) were found to be most effective drugs against *E. coli* isolates. The isolates that showed resistance to Cefpodoxime and Cefoxitin were considered potential producers of ESBLs and AmpC β-lactamases respectively. The five isolates were further tested for production of ESBLs and AmpC using a commercial D68C AmpC and ESβL Detection Disc Set (MAST Group Ltd, UK). The two isolates were positive for both AmpC and ESBL, two isolates were positive for AmpC, the remaining one was positive for ESBL only. The results of MAST kit showed agreement with the results of PCRs. *bla* CTX-M (ESBL gene) presented in three isolates, showed higher prevalence followed by *bla*-TEM in two isolates. All the isolates were negative for *bla*SHV gene. The AmpC genes were presented by AmpC-MOX and AmpC-FOX were present in three of isolates. A triplex PCR results depicted presence of 4 phylo-groups in the chicken *E. coli* isolates; most prevalent was phylo-group A (34%), followed by group B2 (23%), group D (23%) and group B1 (20%). Group A showed complete absence of ESBL and AmpC genes, while group D sowed absence of AmpC genes only. *bla*-TEM gene was found in isolates belonging to phylogroup D only, while *bla*-CTX-M was found in isolates belonging to phylogroup B2, D and B1. On the other hand, AmpC-MOX was found in phylogroup D, B2 and B1, while AmpC-FOX was found in isolates belonging to phylogroup D and B2 only.

From the results of this study, it is evident that ESBL and AmpC producing *E. coli* in poultry meat is low as compared to other sources, so there are need for practices or

actions that will keep this ratio low or even disseminate it. However, resistance towards drugs such as Tetracycline, Ampicillin and Trimethoprim is quite alarming. So, there is a need to emphasize the rational use of antibiotics in poultry practice.

INTRODUCTION

1.1 Escherichia coli

Escherichia coli are rod-shaped bacteria with rounded ends that are gram negative and have a length of 2.0–6.0 mm and a width of 1.1–1.5 mm., these bacteria have a variety of morphologies from spherical (cocci) cells to elongated or filamentous rods. *E. coli* due to the action of peritrichous flagella is motile and are non-spore forming. Polysaccharide capsules or microcapsule are produced by several strains (Farmer and Fanning, 1985). Mucoid *E. coli* strains produces extracellular slime that is made up of either a polysaccharide with distinct K antigen specificities or a common acid polysaccharide (also known as M antigen) made up of colanic acid (Jimenez *et al*., 2012).

E. coli are facultatively anaerobic and gas is mostly produced from fermentable carbohydrates, as demonstrated by gas and acid production from lactose at 37° C and 44°C.In *E. coli* fimbriae (or pili) of various structure and antigenic specificity can be found, and they also contribute host- or organ-specific adhesion features due to their hydrophobicity.

The German pediatrician Theodor Escherich first identified *E. coli* during his research on infants‘ gut flora. The organism in 1885 as *Bacterium coli commune* was described by him (Escherich, 1885) and demonstrated its pathogenic potentials in extra intestinal infection (Esherich, 1894). Until 1919, when Castellani and Chalmers define the genus *Escherichia* and established E. coli as the type species of *E. coli,* the name *Bacterium coli* was extensively used (Catellani and Chalmers, 1919).

There are many *E. coli* serogroups, the majority of which are non-pathogenic; however, some groups can produce severe diarrheal disease, with fatal consequences on rare occasions. *E. coli* is a bacterium that is found chiefly in the digestive tracts of warm-blooded animals, especially humans. As a result, the coliform index (the detection of *E. coli* in potable water) is used to detect animal or human excreta contamination (Leclerc *et al.***,** 2001). It lives in a mutually beneficial relationship with its hosts as a commensal, and it rarely causes disease (Ramos *et al.,* 2020)

The enterotoxigenic (ETEC), and Vero cytotoxigenic (VTEC), enteroinvasive (EIEC), enteropathogenic (EPEC) all are virulent *E. coli* (Percival and Williams, 2014). After spreading from the intestines of people with an underlying problem, *E. coli* is associated with various of human infections. Urinary tract infections (UTIs) caused by direct transmission from the rectum to the urethra due to *E. coli.* Infections in other parts of body are generally spread haematogenously (via the bloodstream), as evidenced by the prevalence of meningitis in infants. A prominent cause of postsurgical infection is *E. coli*, either directly contaminating the wound (when the bowel was opened) or indirectly contaminating the patient's fingers with feces (Percival and Williams, 2014)

1.2 Colibacillosis in Chicken

The natural microbiota of the digestive tract and other mucosal surfaces of farmed poultry and wild birds include avian pathogenic Escherichia coli (APEC). Pathogenic and nonpathogenic serotypes can also be isolated from the bird's surroundings. Colibacillosis in poultry production is caused primarily by APEC (Solà-Ginés, 2015). It's a widespread disease in poultry flocks all over the world, mainly in intensive farming (Chansiripornchai, 2009). Birds of various ages are affected. This disease has a significant economic impact on the global chicken industry (Schouler, 2012). It is one of the most common causes of deaths, in commercial poultry industry. Colibacillosis in birds manifests itself in a variety of ways from sudden death to off-color birds with their necks drawn inward (Johnston, 2007). APEC strains are the causal agents of colibacillosis in birds (Kwon *et al.*, 2008). Swollen head syndrome, omphalitis, coli granuloma, cellulitis, egg peritonitis, and colisepticaemia are all *E. coli* infections in poultry, with the latter being the most serious. Pericarditis, perihepatitis, airsacculitis, synovitis, peritonitis, conjunctivitis, and enteritis are all symptoms of colisepticaemia in ducks, poultry and turkeys, aged 4–12 weeks (Nolan *et al*., 2013). Domesticated, wild, and ornamental fowl can all be infected, usually as a secondary infection after a primary bacterial (Mycoplasma sp.) or viral (e.g., Newcastle Disease Virus, Infectious Bronchitis Virus) infection that causes blood and internal organ infection (Gross, 1991). With the advent of the broiler business, colisepticaemia became more well-known (Wassermann *et al*., 1954).

1.3 Antibiotic resistance mechanisms in bacteria:

Antibiotic drug discovery was one of the most significant advancements in modern medicine, and it improved our lives. Antibiotics are antimicrobial medications which are used to treat and prevent bacterial infections. They work either through killing or suppressing bacterial growth. Some antibiotics also have antiprotozoal effects. Antibiotics are ineffective against viruses like influenza, so, they should not be used to treat them. Antibiotics are also used to support growth in livestock, preserve building materials from contamination, and treat orchard blight (Richardson, 2017)

Based on their structure and mode of action, the different major groups of antibiotics have been described. These major mechanisms of actions of antibiotics include:

i. Inhibit cell wall synthesis (e.g., β-lactams., Glycopeptides)

ii. Protein synthesis inhibitors (e.g., Macrolides, Aminoglycosides, Tetracyclines)

iii. Cell membrane inhibitor (e.g., Polymyxins)

iv. Fatty acid biosynthesis inhibitor (e.g., Platensimycin)

Antibiotic resistance (AMR) develops over time as antibiotics become more widely available and are used extensively. In AMR a microorganism may survive the effects of antibiotics. The rapid emergence and spreading of antimicrobials have made AMR a global problem (WHO, 2014). According to Center of Disease Control and Prevention (CDC) of USA, infectious and parasitic diseases account for about 15.5 million doctor‘s visit and 3.7 million emergency room visits each year. AMR causes tremendous economic losses, because of irrational overuse use of costly antibiotics. Excess medical expenditures related to antimicrobial resistance in the United States cost over $20 billion per year, while antibiotic resistant organisms cause more than 2 million infections and around 23,000 deaths each year (Marston *et al*., 2016; CDC, 2015). In Europe, however, resistance is responsible for 25,000 deaths every year (Marston *et al*., 2016).

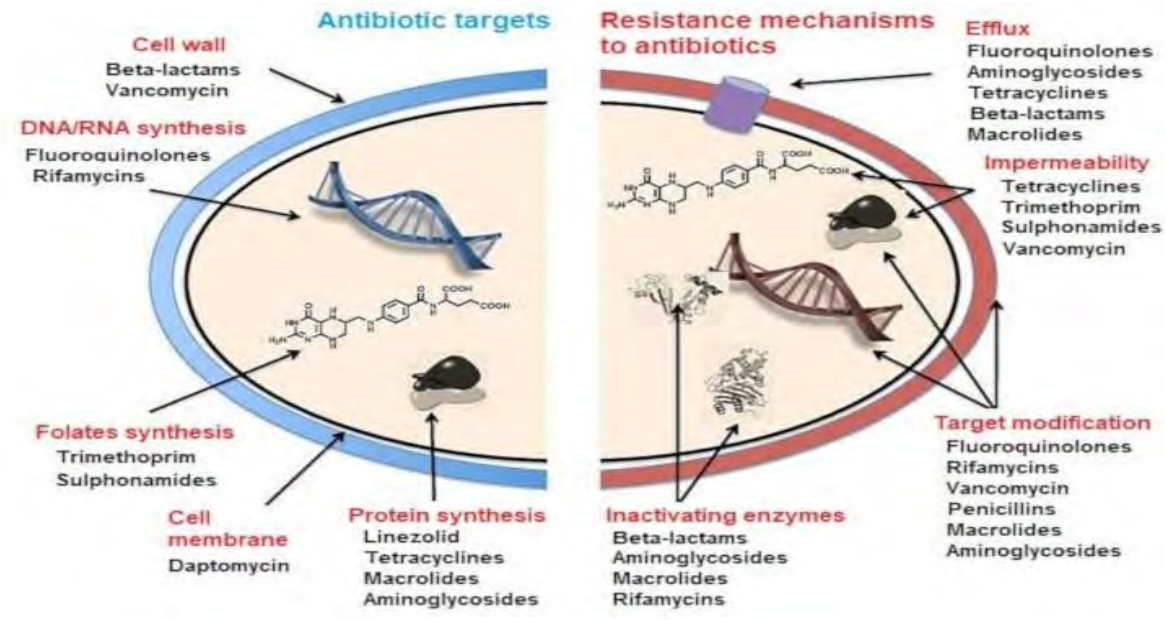


Figure 1.1. Antibiotic resistance vs. antimicrobial activity mechanism.

1.4 Mechanisms Involved in Bacterial Antibiotic Resistance:

a. Efflux pumps

Antibiotic efflux pumps are membrane proteins that transport antibiotics out of cells to keep intracellular concentrations low (Džidic *et al*., 2008). Before antimicrobials reach their target, efflux systems draw them out at the same rate as they enter the cell. Unlike porins, which are found in the outer membrane, these pumps are present in the cytoplasmic membrane (OM). With the exception of polymyxin, all antibiotic classes are susceptible to efflux system activation (Lambert, 2002). and thus, result into the bacteria that are multidrug resistant

b. Decreased permeability

By limiting the antimicrobial molecule's absorption, Antibiotics are unable to reach intracellular or periplasmic targets because bacteria have developed methods to avoid them. Porins are water diffusion channels that allow them to penetrate through the outer

membrane. hydrophilic chemicals such as β-lactams, Tetracyclines, and certain Fluroquinolones are particularly susceptible to changes in permeability.

c. Modification of target molecule

Antimicrobial resistance is caused by a change in the antimicrobial target site that prevents medication binding. The target location will change as a result of a spontaneous mutation in a bacterial gene. Because antibiotics are very selective for their target molecule, even little changes in the target molecule can have a significant influence on antibiotic binding.

d. Inhibition of protein synthesis

For example, changes in the ribosome 30S or 50S subunit might lead to resistance to medications that interfere with protein synthesis, such as Macrolides, Tetracycline, Chloramphenicol, and Aminoglycosides. Aminoglycosides bind to the 30S ribosomal subunit, whereas Lincosamides, Chloramphenicol, and Macrolides bind to the 50S ribosomal subunit and inhibit protein synthesis (Lambert, 2002; Tenover, 2006).

e. Antibiotic resistance via horizontal gene transfer

Horizontal gene transfer is the process of transferring genes between two bacteria, either from the same species or from separate species. Antibiotic resistance genes are caused by variety of factors dissemination among bacteria species is through this mode of genetic material transfer. Several genetic components, including as gene cassettes, transposons, and plasmids, play a key role in carrying those resistance genes. Horizontal gene transfer is accomplished through three main mechanisms: conjugation, transformation, and transduction (McDermott *et al*., 2003; Wright, 2005). Through these mechanisms, the bacteria can acquire genes for β-lactamases, chloramphenicol acetyltransferase (AACs), and aminoglycoside-modifying enzymes etc. These enzymes inactivate antimicrobial drugs and lead to bacterial resistance (Dockrell *et al*; 2004).

i. β-lactamases

Cephalosporins, penicillin, monobactams, and carbapenems are all hydrolyzed by β-lactamases which hydrolyzes all β-lactams with ester and amide bonds. To date, around 300 β-lactamases have been identified. β-lactamases are enzymes that are often found and are classified using two different classification methods Ambler (structural) and Bush–Jacoby–Medeiros (functional) (Alekshun and Levy, 2007).

ii. Aminoglycoside-modifying enzymes (AME's)

Phosphoryl-transferases, also known as nucleotidyl transferases or adenylyl-transferases, and acetyl-transferases (AACs), are enzymes that primarily target aminoglycosides (AG). These AMEs are unable to bind to the 30S ribosomal subunit due to the decreased affinity of a modified molecule by these AMEs (Strateva *et al*., 2009) and provide broad protection against aminoglycosides and fluoroquinolones (Strateva *et al*., 2008). AMEs is recognized in *E. faecalis, S. aureus,* and *S. pneumoniae* strains.

1.5 β- Lactamases

Penicillin G was the first B-lactam antibiotic to enter clinical use (benzylpenicillin). As a result of their exceptional clinical success, four major groups of β-lactam antibiotics have emerged: There are three types of bicyclic structures: penicillins (1), in which the four-membered -lactam ring is fused to a thiazolidine ring; cephalosporins (2), in which the fusion partner is a six-membered dihydrothiazine; and carbapenems (3), with a five-membered pyrroline completing the bicyclic system. The monobactams (6) are a fourth class of monocyclic systems (Tooke *et al.,* 2019)

Tipper and Strominger (1965) The antibacterial activity of β-lactams is owing to their resemblance to the peptidoglycan stem pentapeptide's terminal D-Ala–D-Ala moiety, with the -lactam amide and neighboring carboxylate. (Or sulfonic acid in the case of monobactams) groups that serve to mimic the peptide bond and terminal carboxy. The -lactam ring combines with the target penicillin-binding proteins' (PBPs) nucleophilic serine, causing the ring to open and irreversible PBP acylation, which precludes the formation of peptidoglycan trans peptide cross-links (Sauvage**,** *et al*., 2008). As a result, antibacterial action can be retained by modifying the β-lactam scaffold at various points: C6 of penicillins, C7 and C3 of cephalosporins, C2 of carbapenems, and C3 of monobactams.

1.5.1 Resistance to β-Lactams

β-lactamases are enzymes that deactivate these medications by hydrolyzing the β-lactam molecule. This is the most common mechanism of resistance to antibiotics in this class (Kotra and Mobashery, 1999)

β-Lactamases, on the other hand, quickly had become therapeutically important as penicillin resistance in S. aureus caused by synthesis of the PC1 enzyme (encoded by the blaZ gene) harmed penicillin efficiency. This, along with the successful countering of PC1-producing organisms through the introduction of methicillin, signaled the start of a "arms race" between medicinal chemists and bacterial evolution that has seen the introduction of new -lactams lead to the emergence of new -lactamases via mutation of previously known families and the dissemination of genes encoding new enzymes.

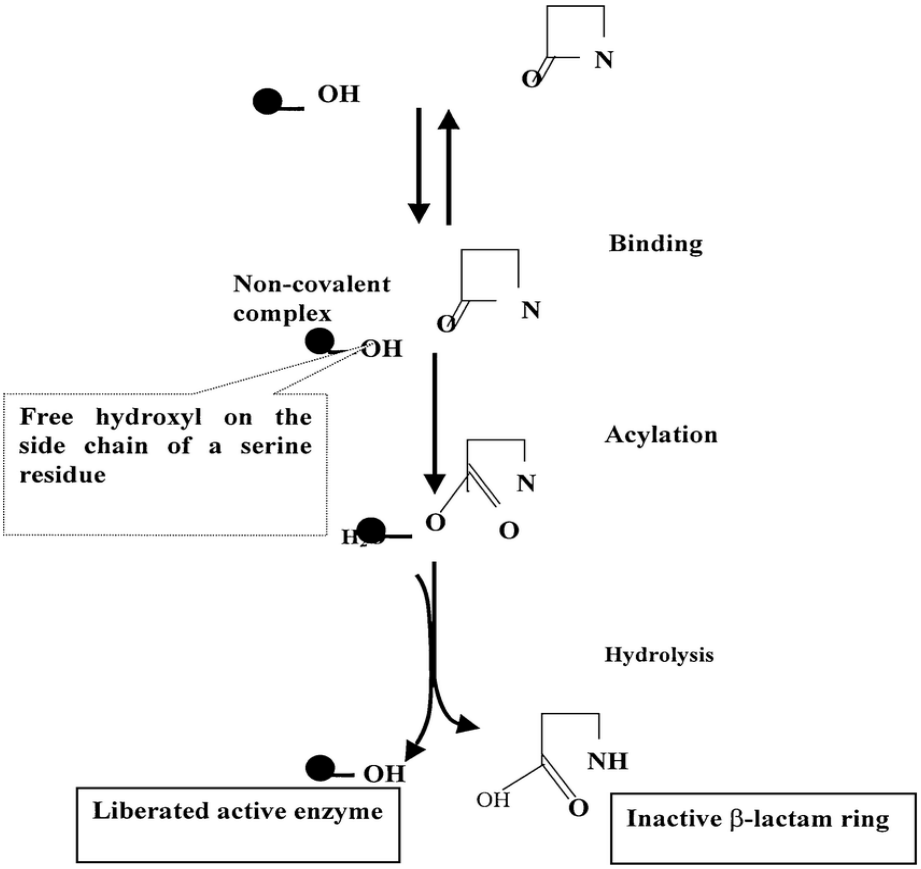


Figure 1.2: Action of β-lactamase

1.5.2 Classification of β-lactamases

a. Ambler molecular classification

The grouping of β-lactamases in this categorization scheme is based on protein sequence similarity. 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 them (Bush and Jacoby, 2010). Classification of β- lactamase enzyme is summarized in Table 1.1. Enzymes like β-lactamases, ESBLs (Extended spectrum β-lactamases), Carbapanemases, and plasmid mediated AmpC β-lactamases are developing inside Enterobacteriaceae to become even more problematic (Pitout, 2012).

1.5.3 Extended Spectrum β- lactamases (ESBLs)

According to the Ambler categorization, ESBLs are classified as class A. ESBLs are a class of enzymes that break down and render useless antibiotics from the penicillin and cephalosporin families. They cannot, however, degrade carbapenems and cephamycins. Some inhibitors of β-lactamases have been found to be capable of degrading these enzymes. Clavulanic acid, tazobactam, and sulbactam are examples of inhibitors encoded by genes that can be transmitted between bacteria (Paterson and Bonomo, 2005; Walsh, 2003). TEM (excluding TEM-1, 2 and 13), SHV (except SHV-1), and CTX-Ms are some of the most common and current examples of ESBLs. TEM-1, 2, and 13 as well as SHV-1 are parent ESBLs that can hydrolyze first-generation cephalosporins and penicillin. Clavulanic acid, on the other hand, has the ability to inhabit them, but because to their narrow range of activity, they are not called ESBLs. Due to their broad-spectrum activity, a variety of distinct mutations of these parent enzymes have been found and are now known as TEM and SHV type ESBLs (Paterson and Bonomo, 2005; Rubin and Pitout, 2014). According to the data up to March 2017, 223 TEM and 193 SHV variants have been characterized (http://www.lahey.org). The CTX-M family of ESBL enzymes is a newly found ESBL enzyme family with a broad spectrum achieved through horizontal gene transfer from Kluvyera *sp* rather than mutations acquired from existing TEM and

SHV parent enzymes (Pitout, 2012; Rubin and pitout, 2014). The CTX-M enzyme gets its name from the fact that it prefers to hydrolyze cefotaxime over ceftazidime. The ―M stands for Munchen, which is where enzymes were discovered (Paterson and B0nomo, 2005; Rubin and Pitout 2014). The total number of CTX-M variants is growing day by day and 172 variants of CTX-M are known worldwide as of M arch 2017 (Ramadan *et al*., 2019). The CTX-M family of enzymes can also be classified based on clustered sequences of amino acids into phylogenetic subgroups including CTX-M-1, CTX-M-3, CTX-M-8, CTX-M-9, CTX-M-15, and CTX-M-25) (Hawkey and Jones, 2009).

1.5.4 Plasmid mediated AmpC β-lactamases

According to Ambler classification and Bush-Jacoby-Medieros classification, plasmid-mediated AmpC β-lactamases belong to class C and Group 1 (Jacoby, 2009). AmpC enzymes are related to class A β-lactamase due to their similar structure, but they differ from class A in that class-C enzymes contain additional open binding sites for cephalosporins (Jacoby, 2009). Cephalosporins, penicillins, cephamycins, and monobactams are among the broad-spectrum antibiotics that this enzyme family is resistant to (Jacoby, 2009). Aztreonam, cloxacillin, and oxacillin have excellent inhibitory activity against AmpC enzymes; however, these enzymes are not inhibited by Class A enzyme inhibitors such as sulbactam, clavulanic acid, and tazobactam (Jacoby, 2009). AmpC β-lactamase was first discovered in *E. coli* and demonstrated penicillin resistance, whereas plasmid-mediated AmpC (pAmpC) genes were discovered in Enterobacter cloacae in the 1980s (Philippon *et al*., 2002). AmpC β-lactamase such as CMY, ACT, DHA and FOX are derived from a number of different bacteria‘s chromosomally encoded AmpC cephalosporins such as *Aeromonas* species *Enterobacter* species, *Morganella morganii, Hafnia alvei* as well as *Citrobacter freundii* (Rubin and Pitout, 2014).

1.5.5 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). Serine- β -lactamase and Metallo- β -lactamase are the two main

molecular families that make up carbapenemases (Queenan and Bush, 2007). Metallo-β-lactamase (MBLs) enzymes are structurally distinct from other kinds of β-lactamases in that they require at least one zinc atom at their active sites, which is responsible for the inhibition of MBLs by metal ion chelators, but cannot be inhibited by clavulanic acid, tazobactam or sulbactam. MBLs belong to Ambler class B and is the functional Group 3 (Queenan and Bush, 2007). Presently some of the most common types of MBLs includes Imipenemase (IMP), New Delhi metallo β-lactamase (NDM) and Verona integron encoded metallo β-lactamase (VIM) (Nordmann *et al*., 2011). In contrast to MBLs, Serine β-lactamases comprises of a serine at their active sites and metal ion chelators such as EDTA do not have the ability to inhibit this family of enzymes (Queenan and Bush, 2007). Serine β-lactamases belongs to Ambler class A and D. A number of different enzymes have been classified in class A carbapenemases in which some are chromosome-mediated like NMC-A, SME and IMI-1, where others are plasmid mediated including the KPC, GES and IMI-2 (Queenan and Bush, 2007). However, the most commonly identified across the world is plasmid-mediated KPC (Nordmann *et al*., 2011). Oxacillinase (OXA) have been reported all over the world, consists of carbapenemase activity and are characterized as class D enzymes (Nordmann et al.,2011). The OXA family includes a number of different enzymes such as OXA-181, OXA-48 and is very diverse family of β-lactamases (Nordmann *et al*., 2011; Queenan and Bush, 2007).

Ambler classification	Bush-Jacoby Medeirous Group	Active Site	Enzyme	Examples	Spectrum of Resistance	Inhibitors
A	2be, 2br, 2c, 2e, 2f	Serine	ESBLs	TEM, SHV, CTX-M	Penicillin, Cephalosporin, Monobactam, meropenem, Cephamycin	Clavulanic acid, Tazobactam, Sulbactam
			Carbapenemases (KPC type)	KPC	Penicillin, Cephalosporin Monobactam, Meropenem, Cephamycin	
B	3a	Zinc-binding thiol group	Carbapenemase (Metallo β-lactamase)	NDM, VIM, IMP	All β-lactamase	EDTA and other metal chelators
C	1	Serine	AmpC	CMY-2, FOX, ACT	Penicillins Cephalosporin Cephamycin, Monobactam	Cloxacillin, boronic acid
D	2df	Serine	Oxacillinase	OXA-1, OXA-48	Penicillin, Carbapenem	Nacl

Table 1.1: Classification of β-lactamase enzymes (Bush and Jacoby, 2010; Kanj and Kanafani, 2011; Rubin and Pittout, 2014).

1.1 Antibiotic resistance in *E. coli*.

E. coli is amongst the most common and the first microflora present in human gastrointestinal tracts as well as in poultry animals (Kmetova, 2009). They are essential for the health of digestive tract in the way that some strains of it, prevent infections caused by other bacteria present in intestines (Hudault *et al*., 2001). Apart from the beneficial effects of *E. coli*, some strains of it are pathogenic also. The percentage of pathogenic *E. coli* range from 10 to 15 percent. Almost 75 to 90 percent of the infections in urinary tract are caused by *E. coli*. Pathogenic *E. coli* cause formation of lesions in the gastrointestinal tract. The bowl of humans and animals contain a large amount of *E. coli* and is considered as the indicator of contaminations through feces in foods. The diseases caused by it are; infection in sac of yolk, endocarditis, septicemia, cellulitis and coli granuloma.

In poultry animals, antibiotics have been used as promoters for growth since 1960. This has caused increase in the growth and less mortality in animals, since, antibiotics cause reduction in the diseases. Other reasons for increase in growth are proposed to be:

1. The infections caused by other intestinal pathogens maybe reduced.
2. The destruction of bacteria inside the intestines may help in more absorption of nutrients.
3. The toxins produced by the intestinal pathogenic microbes maybe decreased by the antibiotics.
4. The nutrients itself may be protected due to the antibiotic‘s presence.

The growth of poultry animals has been observed to increase by 4 to 8 percent (Butaye *et al.,* 2003). Apart from these benefits, use of antibiotics went through a lot of criticism. In 1960, the use of antibiotics as growth promoters was applied and used with no limitation to the use of antibiotics. But later on, in UK, ‗Swann report‘ was issued. According to this report, there is an incidence of microbes especially, gram-negative bacteria such as *E. coli*, to acquire resistance against antibiotics due to their widespread use as therapeutics and as growth promoters (Van de Bogaard *et al.*, 2001). As a result of this report, following suggestions were provided:

1. The antibiotic being used for poultry, should not interact with drugs that are prescribed usually, and develop resistant strains.
2. The antibiotics that are used as therapeutics in humans, should not be used.
3. Penicillins, tetracyclines, sulfonamides and Tylosin were recommended not to be used as growth promoters.

The directive 70/524, of the European legislation contained clauses that were formed by using the above-mentioned report. In this legislation, the doses and the type of additives (e.g., antibiotics) that can be used was enlisted. Moreover, the poultry animals in which such products can be use and the period of withdrawal from the slaughter was described (Van de Bogaard *et al.*, 2001). The antibiotics that are considered safe for use in poultry by EC (European committee) were also listed.

Excessive use of antibiotics has led to the development of varieties of *E. coli* that are resistant to them. As a result of it, the resistance has travelled to other pathogenic microbes resulting in additional of antibacterial genes in their genomes (Van de Bogaard *et al.*, 2001; Schroeder *et al.,* 2002). A variant of *E. coli* which produces Shiga toxin (*E. coli* 0157), is nowadays considered as the public threat worldwide. It is transferred to the human population by the consumption of beef that is in the ground form and is half cooked or not properly cooked. This variant was first isolated and identified in 1982. As declared by CDC, among the category of *E. coli* 0157, the H7 and NM are the two types that are responsible for nearly 72,000 infected cases and 60 deaths in U.S. The syndromes in humans caused by these types are hemolytic-uremic and hemorrhagic colitis syndrome. A study (Schroeder *et al.,* 2002) showed that highest prevalence of Shiga-toxin producing *E. coli* strain is through cattle. As a result of the study, it was concluded that humans may get exposed to such strains through contact with contaminated food or drinking and using the contaminated water with animal waste. It was proposed that resistant bacteria may enter through these paths and cause lethal syndromes in humans. As evident from the data obtained through the study **(Table 1.2),** a resistance to Sulfamethoxazole and Tetracycline in *E. coli* is quite large. The latter is most commonly used to treat human intestinal infections, which results in no cure since, resistant strains has accumulated in

the intestines of humans. In another similar study, zero susceptibility towards Norfloxacin and Gentamicin was observed in poultry in Bangladesh (Kmetova, 2009).

Table1.2: Prevalence of *E. coli* 0157 resistant to various antibiotics in humans, cattle, swine and food (Schroeder *et al*., 2002).

Isolate type	**Antibiotics**	**E. coli (0157) Prevalence**
Human	Tetracycline	12%
	Sulfamethoxazole	12%
	Cephalothin	15%
	Ampicillin	21%
Total prevalence = 67.6%		
Cattle	Tetracycline	71%
	Sulfamethoxazole	74%
	Cephalothin	54%
	Ampicillin	24%
Total prevalence = 75.2%		
Swine	Tetracycline	71%
	Sulfamethoxazole	74%
	Cephalothin	54%
	Ampicillin	24%
Total Prevalence = 5.7%		
Food	Tetracycline	26%
	Sulfamethoxazole	26%

	Cephalothin	Susceptible
	Ampicillin	Susceptible
Total prevalence = 63%		

To alleviate the growing condition of antibiotic resistance in *E. coli* and HIV, several policies has been proposed. According to one study (Schrag *et al.,* 1997), resistance to antibiotics can be limited by reducing the use of such drugs (Darwinian fitness). Moreover, there are genes that are responsible for the creation of fitness in the resistant strain. The mutation in these genes reduce the fitness and increase sensitiveness of the strain towards drugs and without it, the sensitiveness can‘t be generated. These mutations are in rpsL genes. Another study (Okeke *et al*., 2000) proposed that use of nalidixic acid and trimethoprim can be sued to treat infections due to resistant *E. coli*. in Nigeria.

1.1.1 Prevalence of ESBLs and AmpCs in chicken worldwide:

Drug resistant bacteria are of major concern nowadays since, the infections caused by them result in higher mortality and are difficult to diagnose by simple laboratory methods. Among these resistant bacteria, there are some of that are multi-drug resistant. Such bacteria produce an enzyme i.e.; extended spectrum β-lactamase (ESBLs). These are a type of hydrolytic enzymes that are responsible for the cleavage of β-lactam ring present in antibiotics (β-lactam antibiotics). Since, the function of β-lactam is to reduce and inhibit the synthesis of cell wall, cleavage of this ring by gram-negative resistant bacteria results in the loss of antibiotic mechanism and hence act as antibiotic resistant bacteria. The bacteria with this capability are most virulent, more prevalent and are resistant to aminopenicillins, monobactams and cephalosporins (Pandey and Cascella, 2020). The patients in hospitals that are immunocompromised, intake a lot of antibiotics with its incomplete course, are at greater risk of getting infected with ESBL producing bacteria.

On the other hand, AmpCs are also β-lactamase enzymes that are cephalosporin in nature but they cause resistance towards penicillin, and second and third generation

cephalosporins and cephamycins. Moreover, they are produced in some *E. coli* naturally but not in salmonella species. The resistance is not limited to β-lactamase ring but also towards any substrate or a complex molecule that can inhibit the effect of β-lactamase. Fourth generation antibiotics have shown to have effects on such bacteria. In *E. coli* the genes for AmpCs are located on plasmids and hence named as plasmid AmpCs. This enzyme was first isolated from *E. coli* in 1940 with a molecular weight of 34 to 40 kilo Daltons.

Among important antibiotics for humans, cephalosporins play an important part. The use of these antibiotics in animals is considered to be causative agent for threat to public health. Chicken is the main diet of humans and is consumed excessively. A study (Casella *et al.,* 2017) declared chicken to the source of ESBL *E. coli*. A study in 2017, found that broiler chicken has more capability prevalence to contain resistant *E. coli*. strains than non-broiler chicken. The prevalence of broiler was found to be 2.2 times more than layer. Every community in the whole world has its separate distribution of ESBLs according to its environment. So, the systems to monitor such differences are different for each geographical region. Such systems are inadequate in some countries that are developing or are underdeveloped and thus there remains gap in the literature about prevalence of ay disease at the area (Alonso *et al.,* 2017). In New Delhi India, seventy chicken samples contained 40 percent of antimicrobial residues (Brower *et al.,* 2017)

Table 1.3: *E. coli* samples obtained from different poultry sources with ESBLs in them (Brower *et al*., 2017)

Poultry production (India) (2018)			
Broiler farms		**Layer farms**	
Independent operations	Contracted operations	Independent operations	Contracted operations
E. coli containing ESBLs isolated			
573	263	529	227

1.1.2 Prevalence in Pakistan:

In Various regions of Pakistan, ESBLs was calculated by a study held in 2018. According to the study, in Lahore, the prevalence for ESBLs produced by Enterobacteriaceae was determined from human urine samples (UTI patients). The results showed that, at 95% confidence interval 0.44 prevalence was found in Islamabad, 0.15, in Khyber Pakhtunkhwa, 0.19 and in Sindh, 0.30 was calculated (Abrar *et al*., 2018). Rehman *et al*., 2019 confirmed the presence of ESBL producing genes in *E. coli* from poultry chicken in Peshawar, Pakistan and determined their types. They reported 47.6% ESBL producing *E. coli* form chicken meat and among these genes, the *bla*-CTM, *bla*-CTM-1, bla-SHV2 were present in 30%, 10% and 20% respectively, indicating high occurrence of ESBL in *E. coli* isolated from chicken meat. Moreover, some studies also showed the prevalence of ESBL producing *E. coli* in Humans with UTI, which cause their treatment extremely difficult to cure through antimicrobials. A study by Hassan et al., 2011 reports 85%, 60% and 78% resistance against ciprofloxacin, gentamicin and cefotaxime in ESBL producing *E. coli* isolated from UTI patients in Karachi, Pakistan. Ejaz *et al*., 2011, reported 100% resistance towards cefotaxime in *E. coli* isolates from UTI patients in Children hospital, Lahore Pakistan.

In 2003, 87 out of 400 ESBLS positive strains were found in nosocomial and outpatients in kidney transplant center of Pakistan (Shah *et al.,* 2013). Ali *et al.,* 2004 found 366 out of 400 in clinical isolates in Rawalpindi, Mirza *et al.,* 2006 found ESBL among community isolates of urinary tract infection. 4 out of 59 in 2007 by Mumtaz *et al.,* in UTI patients. 15 out of 46 ESBL producing klebsiella pneumoniae isolated from UTI patients in North west Pakistan in 2009 by Ullah *et al*. Shafiq *et al*., 2013 found AmpC β lactamase producing *E. coli* and *Klebsiella* in care hospital, Islamabad

Table 1.4: Prevalence of ESBLs worldwide in Chicken (Abrar *et al*., 2018).

Sr.no	Prevalence of ESBLs worldwide in chicken 2018	
1.	Pakistan	40%
2	China	46%
3	East Africa	42%
4	Germany	15%
5	US	12%
6	Japan	6.3-20%
7	Egypt	50%
8	Spain	10%

The results from a number of studies show that the prevalence of ESBLs containing *E. coli* in Pakistan and China is very high. There is a need to develop method or variant for β -lactamase so that treatment can be provided to the patients (Akbar *et al*., 2018).

In African countries, the prevalence of β-lactamase and AmpC containing *E. coli* was observed through a study. In northern Africa, the prevalence through poultry was 16.5%, through beef was 16.2 % and through sheep was found to be 10.3%. In northeast regions, the poultry contributed towards 26.3% prevalence. In South African region, the prevalence was calculated to be 26.6%, 23.3% and 20.1% (Akbar *et al*., 2018).

The food products have also been studied and numerous researches have been conducted in Africa on food products obtained from animals such as milk, meat, eggs, processed foods such as cheese etc. to find pathogenic strain of *E. coli*. The main focus of such studies was to determine the presence or absence of β-lactamene containing *E. coli* in cattle (Ghotaslou *et al.,* 2018).

1.2 Detection and susceptibility testing

Several automated approaches for detecting and AmpC and ESBL phenomenon have been developed. The phenotypic and genotypic characteristics of ESBL and/or AmpC-producing Gram negative bacteria have us with the most common detection methods which include E-test, MAST discs, DDDT, DDST, PCRs etc.

1.2.1 Double Disk Synergy Test (DDST)

Gram-negative bacteria are typically isolated and identified using cultural characteristics, oxidase test, Gram stains, and biochemical tests. Gram-negative bacteria‘s antimicrobial susceptibility was studied and it was discovered that over time, the hyperproduction of ESBL and AmpC enzymes resulted in enhanced resistance (Sari *et al*., 2021). This phenomenon was also seen in *E. coli, K. pneumoniae* and other Gram-negative bacteria strains. ESBL and AmpC β -lactamases producing bacteria are also detected by Double –disk –synergy-test (DDST). DDST was the first test exactly designed for the finding of ESBL in *Enterobacteriaceae.* For this study, all strains of bacterium are subjected additionally to an

expanded double disk diffusion synergy test. One disc containing co-amoxiclav 20+10 g is placed in the center of an ISO agar plate between ceftazidime 30 g and cefotaxime 30 g discs in the British Society for Antimicrobial Chemotherapy (BSAC) disc susceptibility test. The first two discs are used on a 25-30mm part (Livermore and Williams, 1996) Plates are examined after overnight examination at 37°C. When cefotaxime susceptibility is reduced and ceftazidime and clavulanate have a synergistic effect, the double-disc synergy test is considered positive. The distribution of ESBL producers varies between Enterobacteriaceae species. The rapid increase in the number of ESBLs results in more severe infections due to their continuous mutation and multidrug resistance, making treatment difficult. As a result, a reliable, sensitive, and low-cost method for detecting ESBLs producers is of great interest

1.2.2 E-TEST

After DDST, the E-test (also known as the epsilometer test) is a method of determining antimicrobial sensitivity by placing an antimicrobial-impregnated strip onto an agar plate. Cefotaxime/ cefotaxime + clavulanic acid (CT/CTL), TZ/TZL, and PM/PML are used in this, which are two-sided strips with CT, TZ, or PM either alone on one side of the strip or in combination with clavulanate at a concentration of 4mg/L on the other side. The presence of ESBL is confirmed when the minimum inhibitory concentration value (MIC ratio 8) is reduced more than three doubling dilution steps in the presence of clavulanate (test positive) (Cormican *et al.*, 1996) In microbiology laboratories around the world, the E-test is a well-established in vitro method for determining minimum inhibitory concentration (MIC). E-tests are widely recognized as a versatile, cost-effective, and accurate tool for detecting and validating emerging resistance.

1.2.3 Double Disk Diffusion Test (DDDT)

Another disc diffusion strategy for measuring resistance of strains include Double-Disk-Diffusion-Test (DDDT). These methods have been found to be unreliable for a number of reasons, including the presence of various classes of β-lactamases and difficulties in identifying ESBL production due to AmpC producing bacteria (Oberoi *et al*., 2013). Similarly, there is a scarcity of skilled clinical microbiology laboratory personnel to detect ESBL producing bacteria. This leads to untrustworthy testing, which has a direct impact on antimicrobial therapy (Oberoi *et al*., 2013).

Testing ESBL production is a two-stage process that includes bacterial screening and confirmation. Antibiotics such as CAZ, CTX, CRO, and ATM are tested on the bacteria. The bacteria that produce ESBL are identified by a specific clear zone diameter around the antibiotic disc. More than one antibiotic is recommended to improve the sensitivity of ESBL detection. CTX is generally recommended because of its consistent susceptibility to CTX-M, CAZ, TEM, and SHV (Livermore and Paterson, 2006)

1.2.4 Combination Disk Method

The inhibition zone around the cephalosporin disc, as well as the same cephalosporin in combination with the clavulanate disc, are measured in this test. A difference of ≥ 5mm between the diameters of two discs, or approximately 50% zone expansion of the antibiotic disc, indicates that the bacteria are ESBL producers (Carter et al., 2000). This test's interpretation is simple, and it's simple to perform. For the first time, the test's sensitivity and specificity were reported to be high, at 96 percent and 100 percent, respectively (Linsott and Brown, 2005).

Carter et al. reported on ESBL detection in Klebsiella using the same (combination disc) method. They compare the effectiveness of cefpodoxime and cefpodoxime in combination with clavulanate, with doses of 10 mg and 10 mg plus 1 mg, respectively. The large zone appeared for all ESBL producing Klebsiella against clavulanate disc with cefpodoxime, which showed a 5mm diameter zone. Similarly, AmpC overproducers and *K. oxytoca* isolates expressing high K1 enzyme showed a 1mm diameter zone (Carter *et al*., 2000).

1.2.5 MAST Disc test (MDD)

A low-cost tool for differentiating resistant enzyme types with simple comparative interpretations is MAST disc method, a technically simple method for ESBLs detection. The MAST double- disc test (MDD), is composed of a disc containing antibiotic with disc having resistant bacterial strains. M'Zali *et al*., (2000) assessed the MAST disc in national committee for clinical laboratory standards (NCCLS)-types and attained detection of 93% of ESBL producer in Gram negative Bacteria., the most major contributing cause to resistance to β lactam antibiotics is the formation of β lactamases.

Mast Group Ltd.'s ESBL detection discs provide laboratories with a simple, dependable, and low-cost method of identifying and detecting by double disc diffusion using paired and combination disc sets. When antibiotic and antibiotic plus inhibitor combinations are tested simultaneously, the presence of an ESBL and/or AmpC is easily determined by zone size comparison. D68C detects ESBL-positive strains, AmpC-positive strains (derepressed/hyperproduced and plasmid-mediated), and AmpC-ESBL enzyme co-production.

1.2.6 Molecular identification and PCRs

Phenotypic screening and confirmation have become a routine strategy for detecting ESBLs. However, the approach is time-consuming and ineffective in distinguishing between different ESBL genotypes, Low levels of drug class expression and the presence of more than one AMR gene may result in erroneous susceptibility or resistance results. Several molecular techniques, such as PCR, real-time PCRs, microarrays, and next-generation sequencing, require expensive equipment and well-trained personnel to operate and interpret results quantitatively. Additionally, resistant organisms commonly have multiple ESBL and/or AmpC genes from different resistance gene families. This can lead to the identification of ESBL and AmpC β -lactamase genes, which can then be detected quickly using PCRs. According to phenotypic detection by PCR screening of Pseudo aeruginosa-produced ESBL, a pathogen commonly implicated in serious nosocomial infections reported in Taiwan, 22 isolates were PCR-negative and 35 isolates were PCR-positive for β-lactamase genes of all 57 P. aeruginosa isolates (Lin et al., 2012). Polymerase Chain Reaction (PCR) is a widely used method for scientists to make millions to billions of copies of DNA and amplify it to a large enough amount for further study. This is a genotypic confirmation method that uses DNA sequencing to identify Gram negative strains that produce ESBL and AmpC. Bacteria are isolated using PCR and confirmed using DNA sequencing, and each sequence obtained is then compared to known genotypic sequences. PCR is typically carried out with gene-specific primers and has the ability to make small nucleotide sequence changes directed by mutagenesis.

The PCR is a highly accurate and fast method for duplicating genetic material. Many PCR variants, including digital PCRs, are constantly being developed, reducing the amount of manpower required to conduct these experiments. The shortcomings of PCRs include a lack of standardization, limited availability, and the possibility of contamination, which can result in false-positive results. Despite the existence of other nucleic acid amplification technologies, PCR is by far the most commonly used. With a wide range of applications, PCR is considered a must-have tool in the geneticist's toolbox.

1.3 Phylogroups in *E. coli*

E. coli strains can be classified into four groups based on their phylogenetic relationships (A, B1, B2, and D) (Clermont *et al*., 2000). In a sophisticated way, drug resistance in *E. coli* could predict its evolutionary background and virulence profile (Johnson, *et al*., 2003). Extra intestinal *E. coli* isolates are predominantly from phylogenetic groups B2 and, to a lesser extent, D, whereas commensal *E. coli* isolates are mostly from phylogenetic groups A or B1 (Clermont, *et a*l 2000). The pathogenic strains have various pathogenicity linked islands and express multiple virulence factors such as biofilm development and high surface hydrophobicity, toxin (hemolysin and CNF) and siderophore synthesis, yet they are more antibiotic resistant (Smith *et al*., 2007). However, with growing drug resistance, a shift away from the most virulent group B2 and toward the less virulent groups A, B1, and D was found in some human *E. coli* isolates from UTIs (Moreno *et al*, 2006).

Diverse natural populations of pathogenic *E. coli* and Shigella strains have multiple origins, according to phylogenetic analysis (Pupo *et al*., 1997). Shigella clones are thus produced from *E. coli* strains outside of the B2 and A phylogenetic groupings (Rolland *et al*., 1998; Pupo *et al*., 2000), whereas verotoxin-producing *E. coli* O157: H7 clones could belong to phylogenetic group D (Clermont et al., 2000). Human extraintestinal infection clones are frequently found in the anciently divergent B2 phylogenetic group (Picard *et al*., 1999).

The organization of commensal and pathogenic strain populations is poorly understood. To develop a background database for future investigations on harmful strains, it is necessary to define the intraspecies phylogenetic relationships of *E. coli* isolates from commercial poultry. In this investigation, we used a recently established approach for determining the phylogenetic group of *E. coli* quickly and easily (Clermont *et al*., 2000).

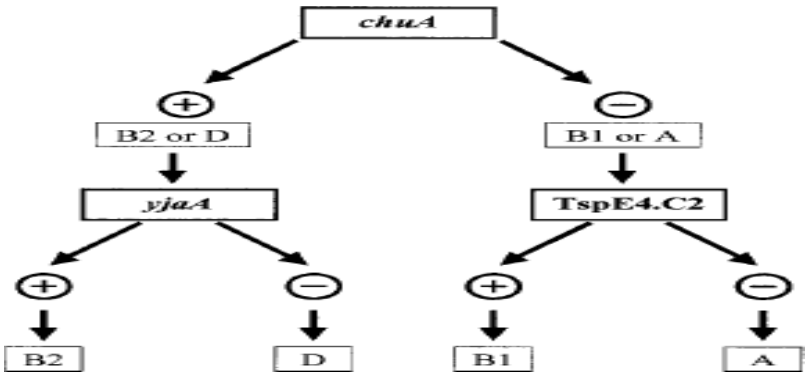


Figure 1.3: Dichotomous Diagram for Rapid phylogenetic Grouping of *E. coli* (Clermont *et al*., 2000)

Aims and Objectives

1. To study the prevalence of antibiotic resistance in local *E. coli* isolates recovered from commercial chicken.
2. To study the prevalence of ESBLs and AmpCs in these *E. coli* isolates.
3. To investigate the phylogroups of these *E. coli* isolates.

Material and Methods

The Disease Diagnostic Section of the Poultry Research Institute of Rawalpindi (PRI) and the Animal Microbiology lab of the Department of Zoology at Quaid-i-Azam University Islamabad collaborated on this research and the sampling was done between November 2020 to May 2021. A total of 50 tissue samples of poultry birds suspected of having colibacillosis, including liver and spleen tissues, were collected at PRI from various commercial poultry farms in Pakistan. These birds were brought for diagnosis at PRI. These tissue samples were sent to the Bacteriology Laboratory of the Disease Section for further identification of E. coli.

2.1 *E. coli* isolation and identification

After collecting the samples, the isolates were recovered after culturing on Selenite broth (REF 610145, Liofilchem, Italy). incubated for 18–24 hours at 37∘C. After 24 hours, a loopful of inoculums from Selenite broth were streaked onto the **MacConkey agar,** (CM0007, Oxoid, UK) the medium used for the initial isolation of E. coli, and then streaked on **Eosin methylene blue (EMB),** (CM0069, Oxoid, UK) for 24 hours, which produces a metallic green sheen 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 tests (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 Iso-sensitest agar (CM0471, Oxoid, UK) and 13 antimicrobial agents as listed in table 2.13. The ISO agar plate was then allowed to come to room temperature. The plate of isolates to be tested was clearly labelled. The colonies of the test isolates were selected using an inoculating loop and mixed in normal saline to create a smooth 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. To remove excess fluid, the swab was pressed against the tube's side. Inoculated on agar plate 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

Name of Antibiotics	Concentration	S	I	R	Source
Cefpodoxime	10µg	≥21	18-20	≤17	CLSI, 2017
Co-amoxiclav (Amoxicillin-clavulanate)	20/10 µg	≥18	14-17	≤13	CLSI, 2015
Cefoxitin	30 ug	≥18	15-17	≤17	CLSI, 2017
Ampicillin	10 ug	≥17	14-16	≤13	CLSI, 2015
Trimethoprim	5 ug	≥16	11-15	≤10	CLSI, 2017

Trimethoprim-sulfamethoxazole	1.25/23.7 ug	≥16	11-15	≤10	CLSI, 2015
Ceftiofur	30 ug	≥21	18-20	≤17	CLSI, 2015
Doxycycline	30 ug	**≥14**	**11-13**	**≤10**	CLSI, 2017
Chloramphenicol	30 ug	≥18	13-17	≤12	CLSI, 2015
Tetracycline	30 ug	≥15	12-14	≤11	CLSI, 2015
Enrofloxacin	5 ug	≥23	17-22	≤16	CLSI, 2015
Fosfomycin	200 ug (with G6P)	21≥		21<	EUCAST
Gentamicin	10 ug	≥16	13-15	≤12	CLS1, 2015

Table 2.1 Reference Values for AST.

2.4 Phenotypic Confirmation of AmpC and ESBLs producer 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 a suspension equivalent in density to the McFarland 0.5 opacity standard was used. Using a sterile swab, evenly spread the suspension across the surface of a suitable susceptibility test agar plate, such as Iso sensitest agar (CM0471, Oxoid, UK), in accordance with clinical and Laboratory standard Institute guidelines (CLSI). Using a sterile needle, forceps, or The MAST DISCMASTER, insert one MASTDISCS ® Combi AmpC and ESBL Detection on the inoculated medium, leaving enough space between them to allow for the formation of clearly defined zones of inhibition. Incubated at 35 to 37°C for 18 to 24 hours. 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 Molecular characterization of *E. Coli*

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 an overnight agar culture with a sterile loop (cooled to room temperature) or 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 Polymerase chain reaction (PCR) for detection of *E. Coli*

The isolates shown to be *E. Coli* by growth characteristics were confirmed as *E. Coli* by a PCR for the *uidA* gene described previously (Tantawiwat *et al*., 2005).

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

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

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.

2.5.4 Agarose gel electrophoresis for visualization of PCR products

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 Detection of antibiotic resistance genes

2.6.1 Multiplex PCR for detection of AmpC 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

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

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 Amplification of ESBLs genes (*bla* TEM, *bla* SHV, *bla* CTX)

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

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

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; Ali *et al.*, 2016).

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 E. coli Phylo-grouping PCR (ChuA, YjaA, TspE4C2 genes)

The phylo-grouping of the E. Coli isolates was performed using 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.

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

Table 2.5: The reagents used in PCR for phylogenetic grouping

2.8.1 Cycling conditions

Initial denaturation at 94℃ for 5 minutes, followed by 30 cycles of denaturation at 94℃ for 30 sec, annealing 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 PCR product size of *uidA*

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

Table 2.6: The Primer and the PCR product size of *uidA*

2.10 AmpC Primers

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

Table 2.7: The AmpC Primers and their PCR product sizes

2.11 ESBL Primers

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

Table 2.8: The ESBL Primers and their PCR product sizes

2.1 Table 2. *E. coli* Phylo Primers

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.C2-F *E. coli*-TspE4.C2-R	GAGTAATGTCGGGGCATTCA CGCGCCAACAAAGTATTACG	152bp

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

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

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

Sr. No.	Antibiotics	Class	Symbol	Concentration (µg)
1.	Cefoxitin	Cephalosporin	FOX	30µg
2.	Cefpodoxime	Cephalosporin	CPD	10µg
3.	Co- Amoxiclav (Amoxicillin-clavulanate)	Penicillin	AMC	20/10 µg
4.	Ampicillin	Penicillin	AM	10 µg
5.	Chloramphenicol	Chloramphenicol	C	30µg
6.	Ceftiofur	Cephalosporin	FUR	30µg
7.	Tetracycline	Tetracycline	TE	30µg
8.	Gentamicin	Aminoglycoside	CN	10µg
9.	Fosfomycin	……	FFC	200µg (with G6P)
10.	Doxycycline	Tetracycline	DO	30µg
11.	Enrofloxacin	Fluoroquinolone	ENR	5µg
12.	Trimethoprim-sulfamethoxazole	Sulphonamides	SXT	1.25/23.7µg
13.	Trimethoprim	Sulphonamides	TMP	5 µg

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

2.3 The contents and Formulation of MAST Discs

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

Table 2.11 The contents and Formulation of MAST Disc

Results

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

The samples brought by Farmers from all over Pakistan in the PRI Rawalpindi. Firstly, they were inoculated in selenite broth at 37°c for 24 hours, then be streaked on MacConkey agar. All of the *E. coli* isolates produced bright pink, rounded colonies on MacConkey agar, pure colonies taken by sterilized loop streaked on Eosin Methylene Blue agar. The EMB showed distinguished metallic sheen colonies. there was also a slight variation in colony character on EMB agar, with greenish red colonies with a faint metallic sheen.

Fig 3.1 Rounded pinkish colonies of *E. coli* on MacConkey agar

Fig 3.2 *E. coli* colonies producing green metallic sheen on EMB 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.

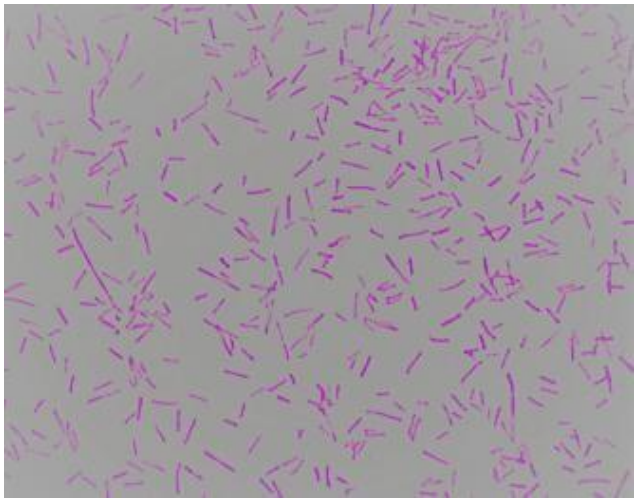


Figure 3.3 The results of Gram's staining showing pink rods

3.3 Molecular Characterization of *E. coli*

The 30 isolates that were confirmed to be *E. coli* by showing characteristic growth on MacConkey and then on EMB agar were further be confirmed by polymerase chain reaction by using *uidA* primers and cyclic conditions used by Tantawiaat *et al*., (2005).

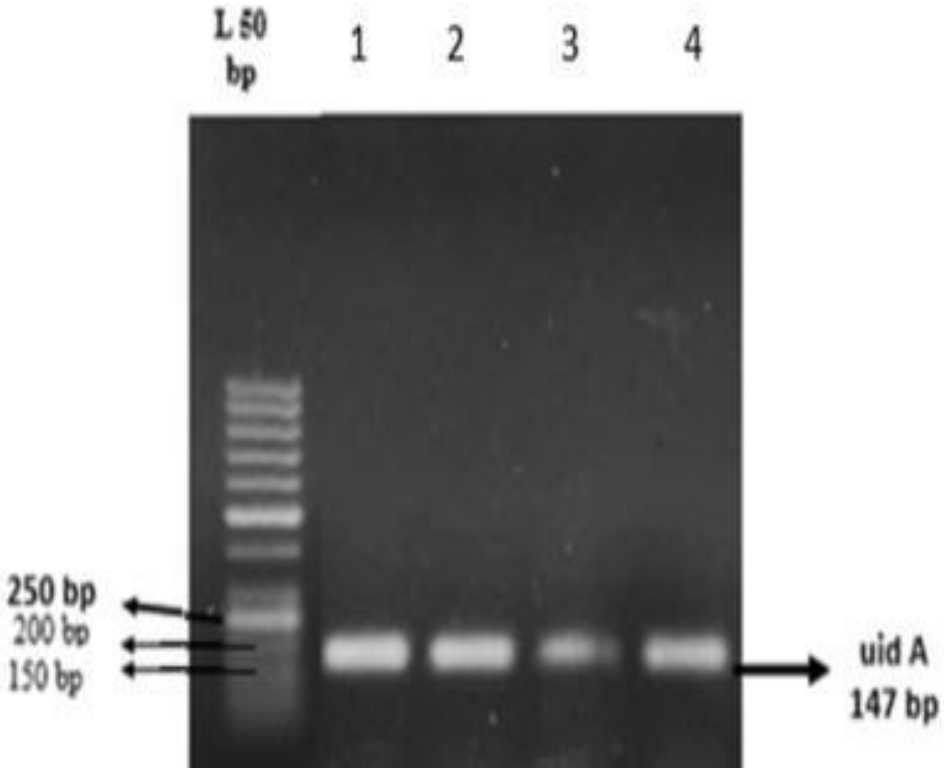


Fig 3.4 Agarose gel Electrophoresis for detection of *uidA* gene. L: 50 bp DNA ladder,Lane 1, 2, 3, 4: *uidA* gene PCR amplicons.

3.4 Antibiotic Sensitivity Test

Thirteen different antibiotics were used to assess the pattern of *E. coli* antibiotic sensitivity. There was no drug that has been proven to be 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 zone sizes of antimicrobial agents used are listed in the table 3.1 below.

Antibiotic sensitivity testing against different antimicrobial drugs was performed on 30 *E. coli* isolates. All the isolates that were resistant to Cefoxitin and Cefpodoxime were declared as ESBL producer. Five (16.66%) out of 30 isolates showed resistant to the Cefoxitin and Cefpodoxime and they are further confirmed by using MAST discs.

E. coli antibiotic sensitivity pattern isolated from poultry flock showed that they had high resistance for Tetracycline (80%), Ampicillin (76.6%) and Trimethoprim (63.3%). The percentage of antimicrobial resistant of *E. coli* isolates against Trimethoprim-sulfamethoxazole and Chloramphenicol was (57% and 53.3% respectively) while the percentage of resistant isolates against Co-amoxiclav was (36.6%), Enrofloxacin (50%), Doxycycline (16.66%) Fosfomycin (6.66%) detected. The isolates showed lower resistance against Gentamicin and Ceftiofur that is (6.6% and 6.66%) respectively. Antibiotic which showed more sensitivity against *E. coli* was Gentamicin (80%) Ceftiofur (73.3%) and Fosfomycin (86.66%) sensitivity. The resistant pattern of different antimicrobials is given in table 3.3.

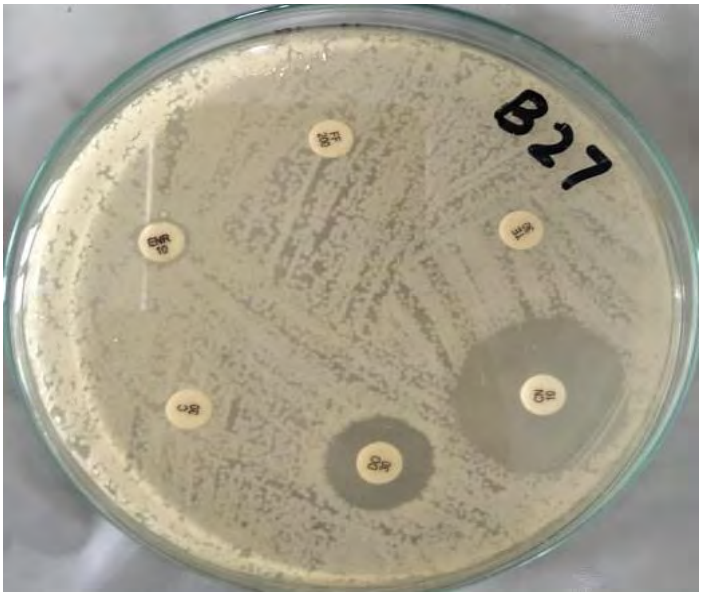


Fig 3.5 A representative AST result.

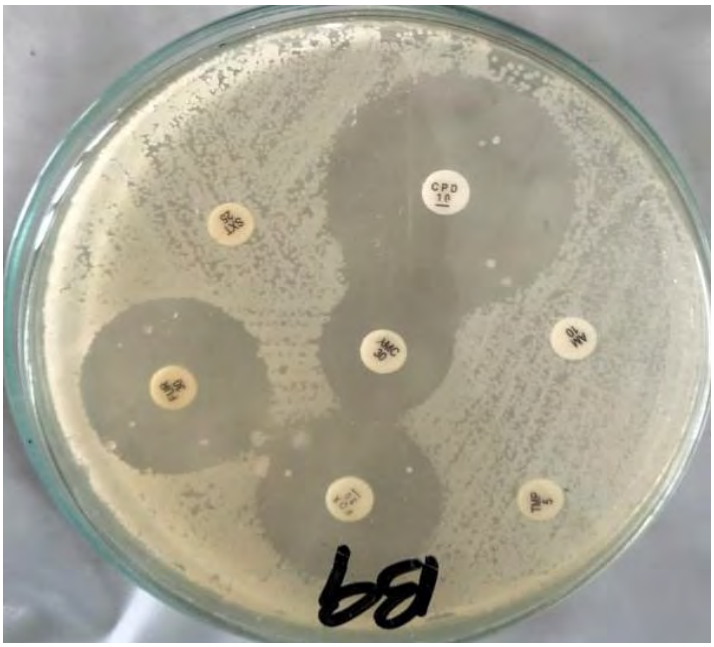
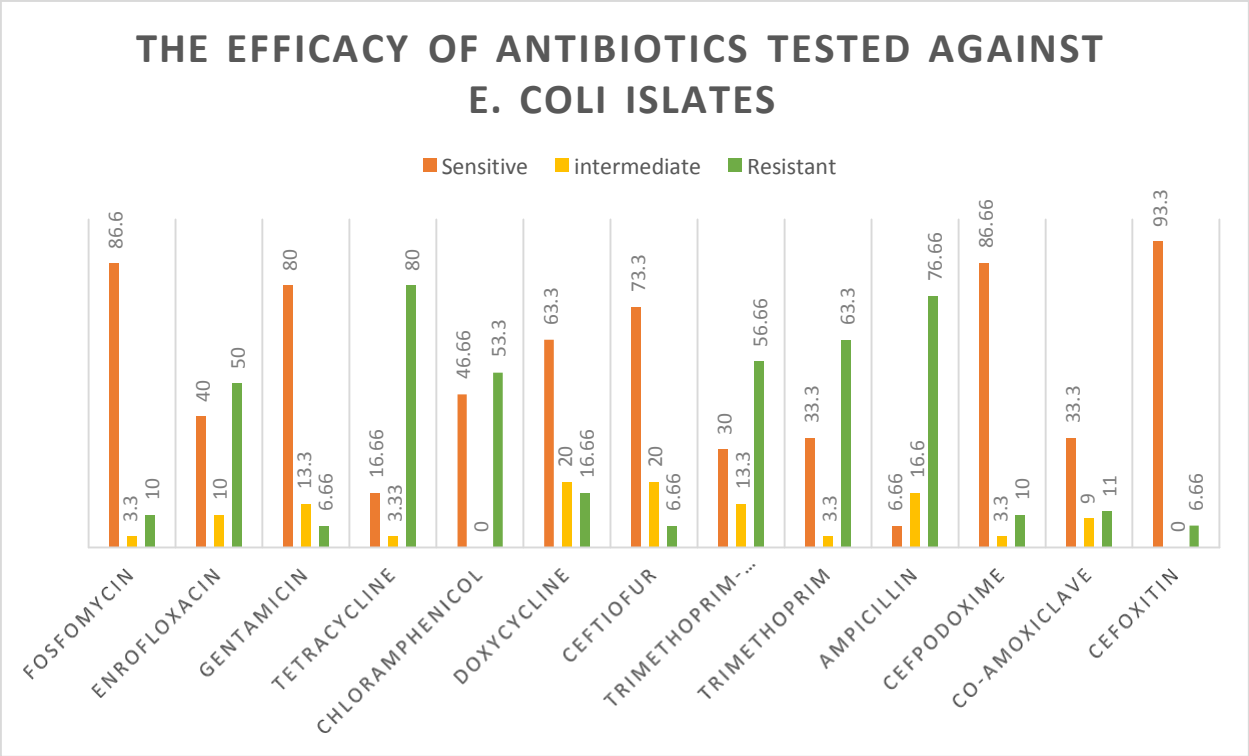


Fig 3.6 Results showing sensitivity to Cefoxitin and Cefpodoxime.

Name of Antibiotics	Total no. of *E. coli isolates*	Sensitive Isolates (%)	Intermediate isolates (%)	Resistant isolates (%)
Cefoxitin	30	28(93.3%)	0	2(6.66%)
Co-amoxiclav (Amoxicillin-clavulanate)	30	10(33.3%)	9(30%)	11(36.66%)
Cefpodoxime	30	26(86.66%)	1(3.3%)	3(10%)
Ampicillin	30	2(6.66%)	5 (16.6%)	23(76.66%)
Trimethoprim	30	10(33.3%)	1(3.3%)	19(63.3%)
Trimethoprim-sulfamethoxazole	30	9(30%)	4(13.3%)	17(56.66%)
Ceftiofur	30	22(73.3%)	6(20%)	2(6.66%)
Doxycycline	30	19(63.3%)	6(20%)	5(16.66%)
Chloramphenicol	30	14(46.66%)	0	16(53.3%)
Tetracycline	30	5(16.66%)	1(3.33%)	24(80%)
Gentamicin	30	24(80%)	4(13.3%)	2(6.66%)
Enrofloxacin	30	12(40%)	3(10%)	15(50%)
Fosfomycin	30	26(86.66%)	1(3.3%)	3(10%)

Table 3.1 *E. coli* Isolates AST Results



Figures 3.7 Percentage of sensitivity and resistance pattern of different antibiotics

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

After the initial screening of AmpC and ESBL *E. coli* done by Cefoxitin and Cefpodoxime. The isolates further evaluated by the commercial D68C AmpC and ESβL Detection Disc Set (MAST Group Ltd, UK). This system is based on a combination disc method that incorporates cefpodoxime (CPD10) as the screening agent in the presence of AmpC- inhibiting and ESBL inhibiting agents which enable to detect both plasmid-mediated and chromosomal AmpC. 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).

A total of 5 previously characterized isolates (showed resistance to Cefoxitin and Cefpodoxime) were tested using the detection kit. Interpretation was performed according to the manufacturer's direction. The isolate E10 and E13 were positive for

AmpC. The isolate E9 and E43 were positive for both AmpC and ESBL while E36 were positive for ESBL only.

The isolate E9, E10 and E13 that showed resistant to CPD only, by treating with MAST discs showed positive results both for AmpC and ESBL according to instructions by MAST UK. The E36 that was resistant to FOX showed only ESBL production surprisingly on interpreting MAST discs. E43 was resistant to FOX and CPD both, showed similarity to its results with MAST kit interpretations as it showed positivity for AmpC and ESBLs.

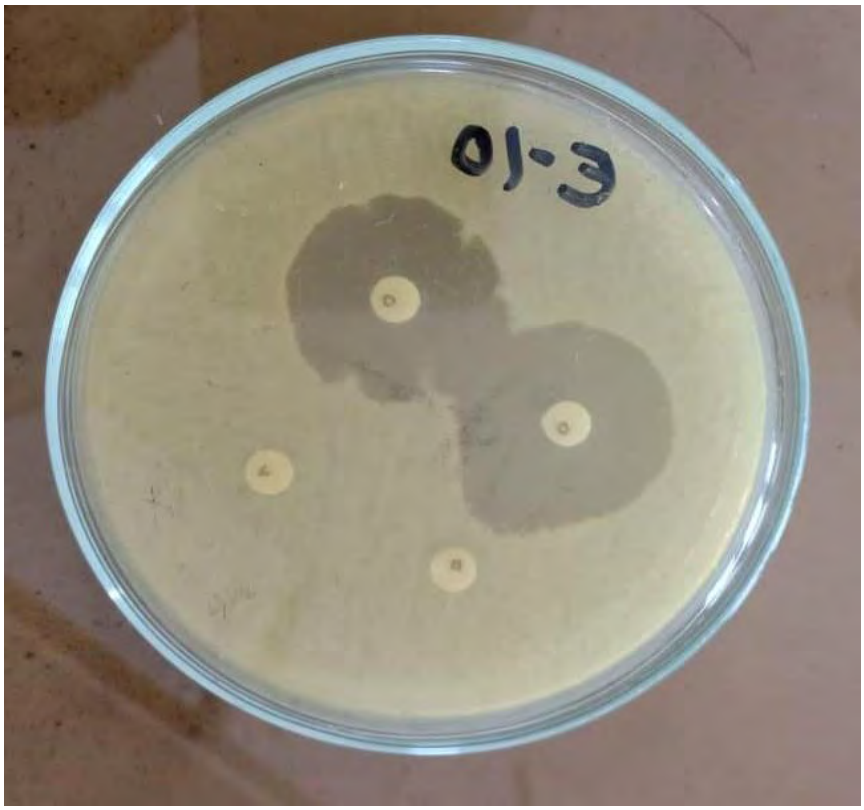


Figure 3.8 Results of MAST discs showing AmpC positive.

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 the type of the ESBL by PCR. Isolate no 9 and 36 were positive for *bla* TEM.

ESBL gene *bla* CTX-M showed positivity in isolate no E9, E10 and E43. All samples showed negativity for *bla* SHV.

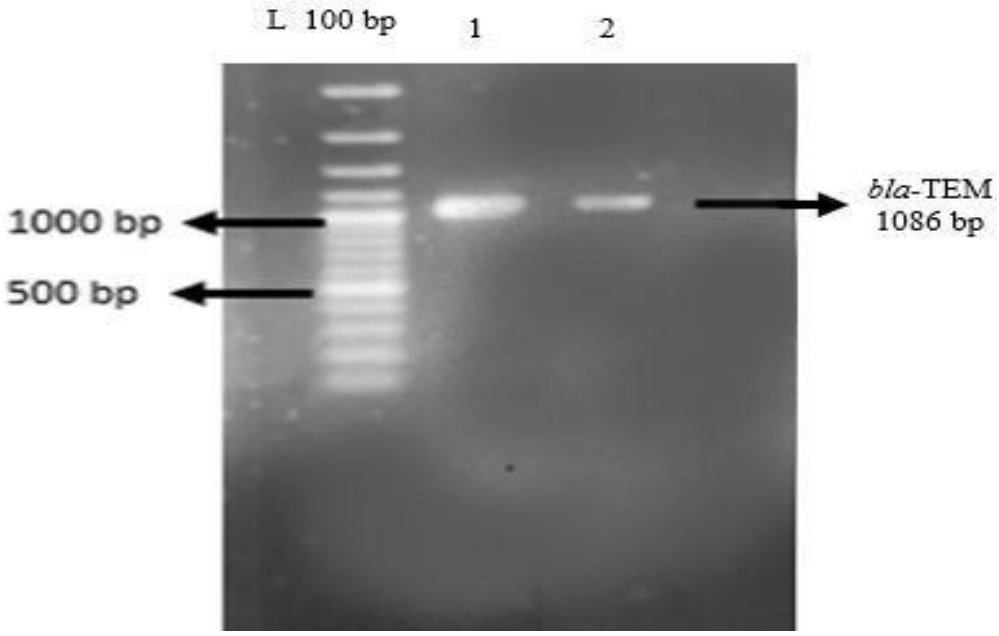


Fig 3.9 Agarose gel electrophoresis for detection of *bla*-TEM gene. L: 100 bp. 1: E9, 2: E36. E9 and E36 are *bla* TEM gene PCR positive samples.

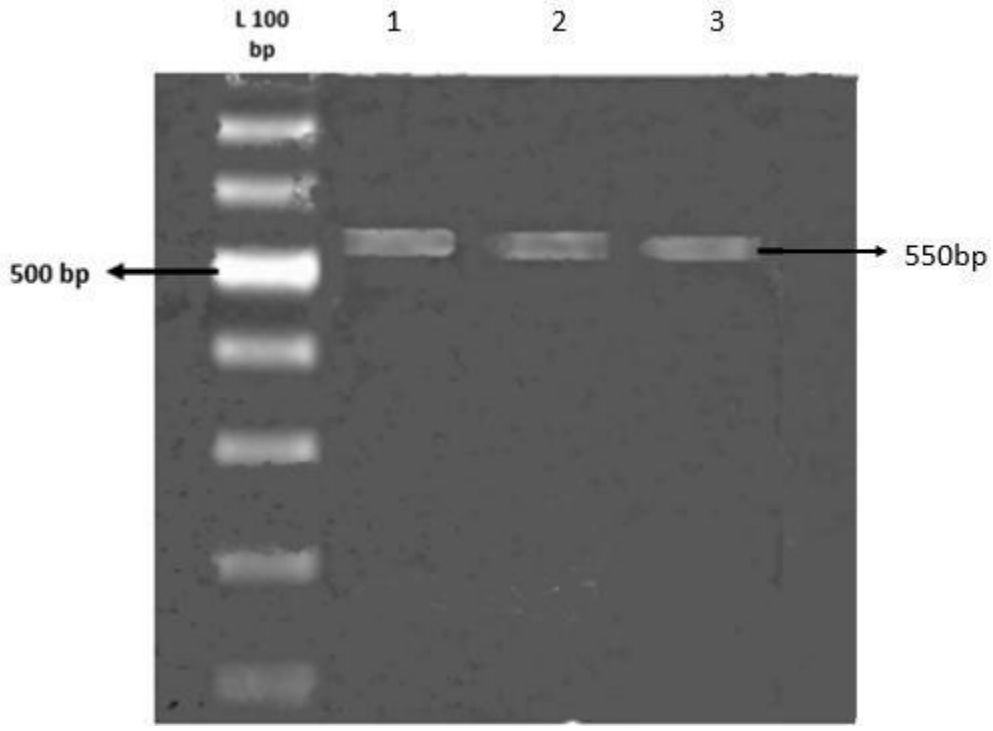


Figure 3.10 Agarose gel electrophoresis for detection of *bla*CTX-M (550 bp). L: 100 bp.

1: E9, 2: E10, 3: E43 are bla CTX-M gene PCR positive samples.

The isolate E9 that show resistant to CPD, when treated with MAST disc show both AmpC and ESBL producer, matched with PCR results. The isolate E10 that was AmpC positive only showed genes both for AmpC and ESBLs, So, here in this case the genotypic results were better as compared to MAST discs. The isolate E13 that was also AmpC positive when treated with MAST discs show all together negative PCR results, might be false negative results. The isolate E36 was ESBL positive showed resemblance with PCR results. The PCR results of E43 were coordinated with MAST kit results as showed positivity for both AmpC and ESBLs. So, the results of MAST kits resemble with PCR results except one case. Thus, the detection of β-lactamase genes using Mast discs is the better option.

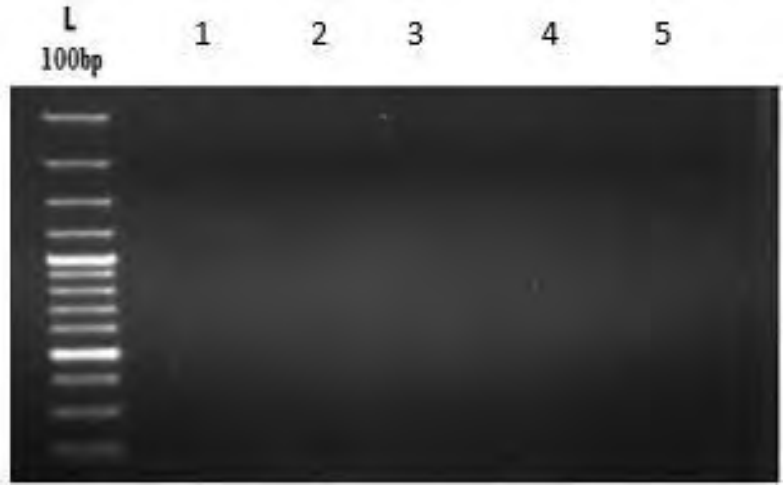


Figure 3.11 Agarose gel electrophoresis for detection of PCR *bla* SHV. L: 100 bp. Nopositive results were observed.

3.7 Molecular detection of AmpC genes through PCR:

The results showed only the amplification in AmpC-FOX and AmpC-MOX genes. Sample E9 and E10 were positive with AmpC-MOX and FOX. E43 was positive for AmpC-MOX only. Isolate E13 and E36 were negative for all. There was no amplification for all other AmpC genes.

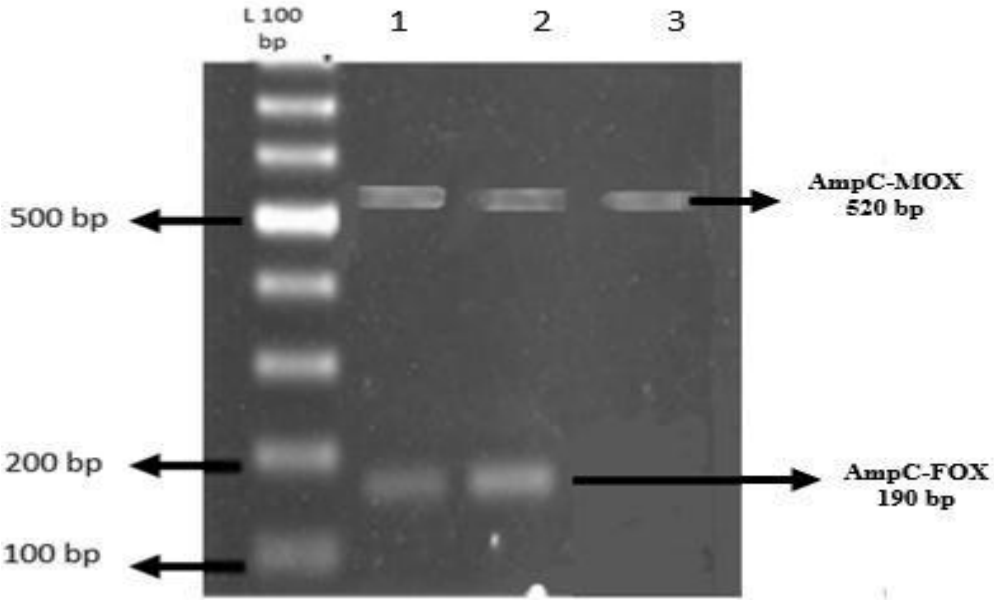


Figure 3.12: Agarose gel electrophoresis for detection of AmpC-MOX (520bp), AmpC-CIT (462bp), AmpC-DHA (405 bp), AmpC-ACC (346bp), AmpC- EBC (302bp), AmpC-FOX (190bp). L: 100 bp. 1: E9, 2: E10, 3: E43. E9 and E10 are positive for AmpC-FOX and AmpC-MOX. E43 is positive for AmpC-MOX.

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 4. The results showed that the commensal phylogroup A was found to be the most prevalent (34%), followed by virulent extra-intestinal group B2 (23%) and phylogroup D also showed the same frequency (23%). The phylogroup B1 showed lesser prevalence and that is (20%).

Strains were grouped according to Clermont *et al*., (2000).

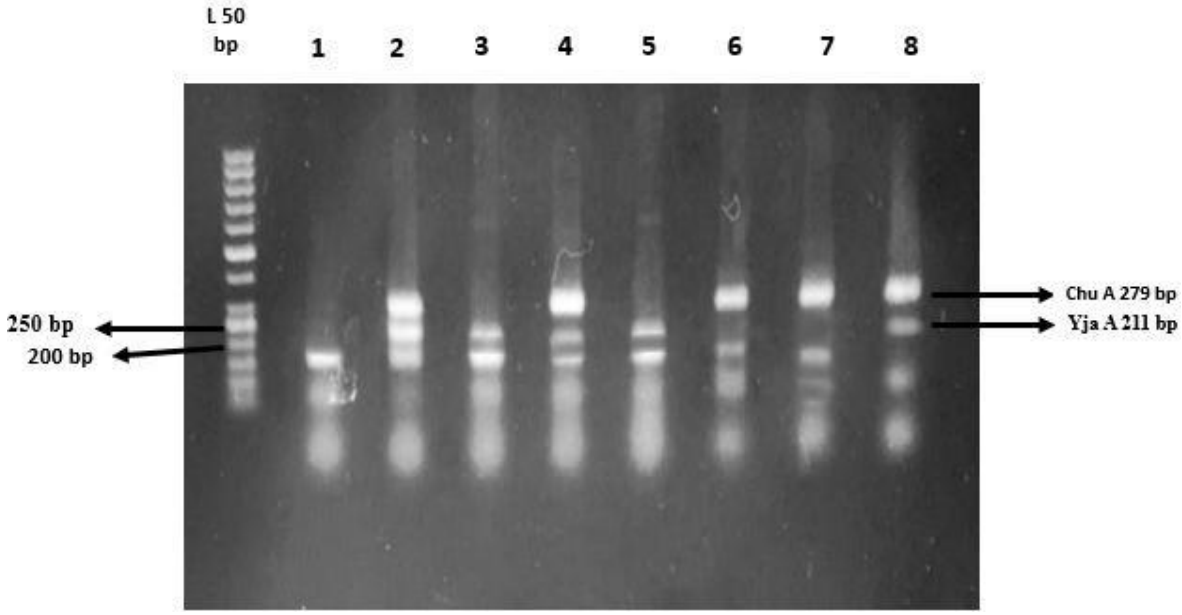


Figure 3.13 The DNA amplification of chu A, yjaA, TspE4C2 genes showed 279bp, 211bp and 152 bp respectively, L; Ladder 50 bp. Lane 1: B1, Lane 2: B2, lane 3: B1, Lane 4: B2, Lane 5: B1, Lane 6, 7: D and 8: B2

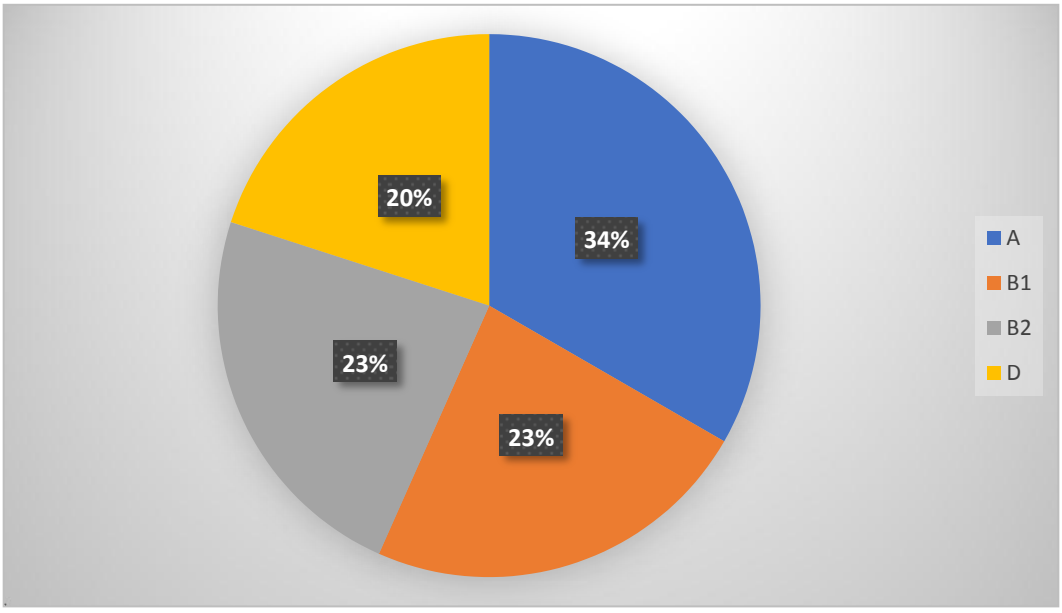


Figure 3.14 A pie chart representing the percent occurrence of different phylogenetic groups in the *E. coli* isolates.

3.9 The phylogenetic typing and molecular Characterization of AmpC/ ESBL *E. coli.*

A comparison was made between resistance patterns of antibiotics with the antibiotic resistant genes as given in table 3.2. It revealed that all the isolates belong to different phylogenetic groups. Almost all the isolates showed resistance against TE, TMP, SXT, DO and ENR. The PCR results showed that E9, E10 and E43 represents both AmpC and ESBL producers which were also phenotypic positive. E36 was ESBL producer alone. Surprisingly the sample E13 which showed phenotypic resistance when performed initial screening by antibiotics and then by using MAST discs were negative when PCR was performed.

SampleID	Phylogroup	MAST Kit Result	ESBL genes	AmpC genes	AST Results		
					S	I	R
E1	B1	-	-	-	DO, C, CN, FFC, FOX, AMC, CPD, TMP, SXT		AM, TE, ENR
E2	B2	-	-	-	DO, C, TE,CN, ENR, FFC, FOX, CPD,	AMC	AM, TMP, SXT
E4	B1	-	-	-	DO, C, CN, FFC, FOX, AMC, CPD, TMP, SXT	AM, FUR	TE
E5	D	-	-	-	C, FOX, CPD, FUR, FFC	DO, AM	TE, ENR, AMC, TMP, SXT

E6	D	-	-	-	FOX, CPD, TMP, SXT,C, CN, FFC	ENR, AMC,FUR	DO, TE,AM
E7	B2	-	-	-	DO, C, TE,CN, ENR, FFC, FOX, CPD, TMP,SXT	AMC	AM
E9	D	AmpC +ive ESBL +ive	*bla*- TEM, *bla* CTX-M	AmpC-MOX, AmpC-FOX	C, CN, FOX	DO, ENR, AMC	TE, FFC, CPD, AM,TMP, SXT
E10	B2	AmpC +ive	*bla* CTX-M	AmpC-MOX, AmpC-FOX	CN, FFC,CPD,		DO, C, TE, ENR, FOX AMC, AM, TMP, SXT, FUR,
E11	B1	-	-	-	DO, CN, FFC, FOX,CPD,		C, TE,ENR, AMC, AM, TMP, SXT
E13	A	AmpC +ive	-	-	FFC, CPD	CN, FUR	AMC, FOX, AM, TMP, SXT,DO, C, TE, ENR
E14	A		-	-	FOX, CPD, TMP, SXT, DO, C, FFC,FUR	AMC,AM	C, TE,ENR,

E15	B2		-	-	FOX, CPD, TMP, SXT, DO, C, FUR, AMC, TE, ENR, CN,		FFC, AM
E17	A	-	-	-	FOX, CPD, FUR, CN,FFC	AMC,DO	C, TE, ENR, AM,TMP, SXT
E19	A	-	-	-	FUR, FOX, CPD, DO,FFC, CN		C, TE,ENR, AMC, AM, TMP, SXT
E20	A	-	-	-	FOX, CPD, TMP, FFC,FUR	CN, DO	AMC, AM, SXT, C,TE, ENR
E22	B2	-	-	-	CN, DO,FOX, CPD	FUR	C, TE,ENR, FFC,AMC, AMP, TMP, SXT,
E23	A	-	-	-	FOX, CPD,FFC, FUR	CN	DO, C, TE, ENR, SXT, TMP,AMC, AM
B23	A	-	-	-	FOX, AMC,CPD, DO, CN, ENR, FFC, FUR		C, TE, AM, TMP, SXT
E26	A	-	-	-	DO, ENR, FFC, FOX,		C, CN, TE, AM, TMP,

					AMC, CPD, FUR		SXT
E27	B1	-	-	-	DO, ENR, FFC, FOX, AMC, CPD,FUR		C, CN, TE, AM, TMP,SXT
E29	D	-	-	-	FOX, AMC,CPD, DO, ENR, FFC,FOX, CN	AM, TE	C, TMP,SXT
E34	B2	-	-	-	CN, FFC, FOX, CPD,FUR	AMC,SXT	C, TE, DO,AM, TMP, ENR
E35	A	-	-	-	DO, CN, ENR, FFC,FOX, AMC, CPD		C, TE, AM, TMP, SXT
E36	D	ESBL +ive	*bla*-TEM	-	C, CN, FUR,FOX,	DO, ENR,	CPD, TE, FF, AMC,AM, TMP, SXT
E37	D	-	-	-	FOX, CPD, AM, SXT, CN, ENR,FFC	AMC,DO	TMP, DO,C, TE
E38	B1	-	-	-	DO, C, TE,CN, FFC, FOX, CPD, FUR		ENR, AMC, AM, TMP, SXT

E42	B2	-	-	-	DO, C, FFC, CN, FOX,AMC, CPD	FUR, SXT,	TE, ENR,TMP, AM
E43	B1	AmpC +ive ESBL +ive	*bla* CTX-M	AmpC-MOX	DO, C, CN, ENR, FFC, FUR, TMP, SXT	CPD,	TE, FOX, AMC, AM,
E50	B1	-	-	-	DO, ENR, FOX, AMC,CPD	CN, TE,	C, FFC, AM, TMP,SXT
E53	A	-	-	-	FOX, CPD. TMP, SXT,DO, C,	CN, FUR, ENR, FFC	TE, AMC,AM

Table 3.2 The molecular characterization, phylogenetic grouping and phenotypic AST results against different antibiotics of β-lactamase producing and non- producing *E. coli.*

Discussion

Anti-microbial resistance is one of the most alarming public health hazards in recent year. Initially, ESBL/AmpC -producing bacteria were exclusively seen in human medical practice, but the recent discovery of these bacteria, first in companion animals and then increasingly in livestock, has prompted livestock monitoring investigations. According to the risk assessment studies, the majority of human cases (80%) can be related to the chicken reservoir, which includes broiler meat handling, preparation and consumption, as well as their development circumstances.

With an investment of more than 1,190 billion rupees, Poultry is one of the largest agro based sectors. Pakistan‘s Poultry Industry is making a significant contribution to closing the gap between supply and demand for meat protein. With the red meat supplies continually depleting, poultry is the lowest accessible animal protein source for our general public (https://pakistanpoultrycentral.pk/) However, antibiotic usage in the poultry business is unfortunately not properly monitored and thus is a raising concern of emergence of AMR microorganisms (Mitema, 2010).

The present study was aimed 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 poultry production. For this purpose, 30 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 13 antibiotics, and our results revealed *E. coli* as highly resistant pathogen.

In current study, susceptibility pattern of *E. coli* revealed highest resistance that was observed against Tetracycline (80%). Jahantigh *et al* (2020) in Iran found 95.0 % resistance in *E. coli* against Tetracycline during their investigation in broiler chicken. In another study in Iran on commercial broiler chicken revealed the frequency of Tetracycline resistance of *E. coli* isolated from colibacillosis was reported as 96.0%.

In our findings, second most drugs against which most resistance was observed were Ampicillin (76.6%) and Trimethoprim (63.3%). Lee *et al* (2001) reported 63.1% resistance to Trimethoprim and 98.7% resistance to Ampicillin in urinary *E. coli* isolates. Bogaarrd *et al* (2010) reported 68% resistant to Trimethoprim in laying eggs and 80% in Broiler. Mulder *et al* (2019) found 31.1 % resistance to Trimethoprim in human UTI patients in their study.

E. coli was found to be most sensitive to Fosfomycin (86.66%), Gentamicin (80%), Ceftiofur (73.3%) and Doxycycline (63.3%). Miles *et al* (2006) reported 100% sensitivity towards Gentamicin and 64.7% sensitive towards Ciprofloxacin in fecal samples of poultry chicken and human sources (urine and wound specimen). Abbasi *et al* (2021) reported moderate resistance of *E. coli* isolated from feces of broiler chicken towards Ciprofloxacin (32.5%), lowest resistance towards Kanamycin (8.4%), Cefotaxime (3.6%) and Ceftazidime (2.4%) and no resistance towards Gentamicin. Hassan *et al* (2011) reported 85%, 60% and 78% resistance against Ciprofloxacin, Gentamicin and Cefotaxime in ESBL producing *E. coli* isolated from UTI patients in Karachi, Pakistan. Ejaz *et al* (2011) reported 100% resistance towards Cefotaxime in *E. coli* isolates from UTI patients in Children hospital, Lahore Pakistan.

Adator *et al* (2020) found that ESBL containing *E. coli* obtained from beef samples had 22.5% resistance against Ampicillin, whereas, in our study, 76.66% isolates obtained from poultry chicken were resistant towards Ampicillin.

Bebora *et al* (1994) reported 100% resistance of *E. coli* isolated from chicken towards Trimethoprim- sulfamethoxazole, while the current study demonstrates the 63.3% of resistance of *E. coli* isolated from chicken towards Trimethoprim-sulfamethoxazole. Muhammad *et al* (2011) reported 45.54% resistant genes (sull) against sulfamethoxazole in UTI patients in Islamabad, Pakistan.

Persoons *et al* (2011) reported resistance in *E. coli* obtained from Belgian broilers towards Ceftiofur. The resistance was found to be 34%. On the other hand, in the current study, resistance towards Ceftiofur was found to be 6.66%. Jiang *et al* (2011) reported 95.6% resistance towards Doxycycline and 50.2% resistance towards chloramphenicol in

poultry chicken and pigs, while the present study found 16.66% and 53.3% resistance towards Doxycycline and Chloramphenicol respectively, in *E. coli* isolated from chicken.

Donado-odoy *et al* (2015) found the prevalence of Enrofloxacin and Fosfomycin resistance in *E. coli* isolated form chicken meat. The resistance of these drugs was 50.3% and negligible for Enrofloxacin and Fosfomycin respectively. While in present study, 50% resistance was found towards Enrofloxacin and only 10% against Fosfomycin. Moreover, Donado-odoy *et al* (2015) also reported 8.5% resistance towards Amoxicillin-clavulanate (co-amoxiclav) in chicken meat, while the present study reports 36.66% resistance towards Amoxicillin-clavulanate (co-amoxiclav).

According to the results obtained from present study, the resistance towards Ampicillin, trimethoprim, Co-amoxiclav (Amoxicillin-clavulanate) and Tetracycline was found to be alarming.

Antimicrobial resistance (especially through ESBLs and AmpC genes) is an emerging threat in Pakistan, not only to humans but also animals (El Salabi *et al*., 2013). The drugs such as beta-lactams have lost their effect due to ESBL and AmpC genes. According to Hassan *et al* (2011) in Karachi, Pakistan, 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. According to Rehman *et al* (2019) there are 3 main types for ESBL genes. These are *bla*-TEM, *bla*-CTM and *bla*-SHV. Among these genes, *bla*-CTM is commonly found in *E. coli* isolates from poultry and dairy animals in Pakistan (Rehman *et al*., 2019). 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).

In this study, phenotypic confirmation of 5 *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, 2

isolates were positive for AmpC (i-e; they had gene for AmpC), one isolate had gene for ESBL and other 2 isolates had both AmpC and ESBL genes.

Rehman *et al* (2019) confirmed the presence of ESBL producing genes in *E. coli* from poultry chicken in Peshawar, Pakistan and determined their types. They reported 47.6% ESBL producing *E. coli* form chicken meat and among these genes, the *bla*-CCTM, *bla*-CTM-1, *bla*-SHV2 were present in 30%, 10% and 20% respectively indicating high occurrence of ESBL in *E. coli* isolated from chicken meat.

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* TEM in 2 isolates. Another ESBL gene i-e; *bla* CTX-M was also found in 3 isolates, while no *bla* SHV was found in any of the isolate. On the other hand, AmpC-MOX and AmpC-FOX were found to be present in 3 of the isolates.

The phylogenetic results (done through triplex PCR) of ESBL gene containing *E. coli* obtained from tertiary care hospitalized patients in Mangalore, 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%. Ali *et al* (2020) found the isolates from table eggs form markets of Peshawar, Pakistan. The phylogenetic analysis revealed by triplex PCR showed that major ESBL and carbapenemase producer *E. coli* belonged to phylogroup D and A. eggs from poultry farms contained *E. coli* that belonged to group D and no isolates was found that belonged to group A. group A was present in eggs from supermarket and grocery stores only and not poultry farms. 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. To Group A, *E. coli* isolates were found to be most prevalent and isolates belonging to B1 were found to be least prevalent. Phylogenetic typing and molecular characterization of genes that impart resistance to *E. coli* isolates was performed in order to determine the presence/absence or prevalence of antibiotic resistance genes in 4 phylo-groups. Group A showed complete absence of ESBL and AmpC genes, while group D sowed absence of AmpC genes only. *bla*-TEM gene was

found in isolates belonging to phylogroup D only, while *bla*-CTX-M was found in isolates belonging to phylogroup B2, D and B1. On the other hand, AmpC-MOX was found in phylogroup D, B2 and B1, while AmpC-FOX was found in isolates belonging to phylogroup D and B2 only. Moreover, *bla*-TEM was present in *E. coli* isolates that had AmpC-MOX and AmpC-FOX genes and *bla* CTX-M are present in isolates that had both AmpC-MOX and AmpC-FOX genes also.

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). Several other studies have suggested the transmission of ESBLs from retail poultry meat to humans in which 12% of the meat samples contained ESBL producing *E. coli* (Voets *et al*., 2013). Apart from this factor, the lack of proper surveillance and monitoring of antibiotic usage also contribute to the current situation. Poultry industry in Pakistan shares a 32.7% of total meat produced (http://www.finance.gov.pk/survey_1718.html) but the monitoring and regulation of antibiotics used in this industry is not under strict rules, which has caused increase in resistance towards various antibiotics in ESBL producing *E. coli* (Mitema, 2010). 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.

At the end of this study, it is concluded that ESBL producing *E. coli* in poultry meat is low as compared to other sources. However, high resistance towards drugs such as Tetracycline and Trimethoprim is quite alarming, so there is a need to create actions and ways that will either stop its usage or which can no longer cause resistance in microbes. The increasing 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 and mobile

elements, which will lead towards resistance against many antibiotics. As a result of it, many human pathogens can become resistant to antibiotics and treatment can become difficult. Rational use of antibiotics and observing proper hygienic measures can help in reduction of ESBL/AmpC *E. coli* in the poultry industry.

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Conclusions

- Antimicrobial resistance (especially through ESBLs and AmpC genes) is an emerging threat in Pakistan, not only to humans but also animals.
- In this study, Tetracycline was found to be the least effective (80% resistance), followed by Ampicillin (76.66%) and Trimethoprim (63.3 %).
- The results of MAST kit showed agreement with the results of PCRs so an effective method to detect β- lactamases.
- *bla* CTX-M (ESBL gene) presented in three isolates, showed higher prevalence followed by *bla*-TEM in two isolates.
- All the isolates were negative for *bla*SHV gene. The AmpC genes were presented by AmpC-MOX and AmpC-FOX were present in three of isolates
- A triplex PCR results depicted presence of 4 phylo-groups; Group A (34%), followed by group B2 (23%), group D (23%) and group B1 (20%).
- Group A showed complete absence of ESBL and AmpC genes, while group D showed absence of AmpC genes only.
- High resistance towards drugs such as Tetracycline and Trimethoprim and presence of β-lactamases is quite alarming, so there is a need to create actions and ways that will either stop its usage or wisely use
- Rational use of antibiotics and observing proper hygienic measures can help in reduction of ESBL/AmpC *E. coli* in the poultry industry.

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