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MOLECULAR CHARACTERIZATION OF MULTI DRUG RESISTANT *ESCHERICHIA COLI* CAUSING BOVINE MASTITIS

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Abstract *Different antibiotics are used to treat mastitis in dairy cows that is caused by Escherichia coli (E. coli). Addressing this threat requires a combination of a multidisciplinary approach involving human, animal, and environmental health. Because antibiotic agents used in veterinary medication may be the same or similar to those in human medication. Mastitis is a common and economically important disease in dairy cattle. It remains one of the most common reasons for the extensive use of antimicrobials in dairy farms, leading to the emergence of antimicrobial-resistant pathogens. The current study aimed to investigate the incidence of multidrug-resistant E. coli in dairy milk samples collected from Mastitis and healthy milk in the district of Dir Lower, characterize the multi-drug-resistant (MDR) E. coli, and molecular detection of the predominant ESBL-encoding genes by using PCR assay. All confirmed (17) samples of ESBL-encoding E. coli strains were tested via conventional PCR for the molecular screening of blaCTX-M gene, blaTEM gene, and blaSHV gene from mastitic milk isolates. Out of 17 isolates, 6 (35.29%) isolates were positive for co-existence of blaCTX-M gene and blaTEM gene, 5 (29.41%) isolates were positive for single existence of blaCTX-M, 2 (11.76%) isolates were positive for single existence of blaTEM, 4 (23.52%) isolates were positive for blaSHV gene. In this study, the most predominant gene was blaCTX-M gene, showing (64.70%) followed by blaTEM gene (47.05%) and blaSHV gene (23.52%). Eleven different antibiotics were used in this study to check their susceptibility pattern. The isolated strains were highly resistant to Ampicillin (100 %) and highly sensitive to Levofloxacin (100%), Ciprofloxacin (100%), and Chloramphenicol (100%). The incidence of these genes in indicator organisms from milk samples, enforced the potential of food-producing animals as sources of MDR organism's infection in humans via the food chain. Thus, there is a need for the adoption of a tripartite One Health approach in surveillance to control antimicrobial resistance, and antibiotic sensitivity must be checked before treatment of any infection.*

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Keywords: *mastitis; Escherichia coli; multi-drug resistant; antibiotics; infection*

Introduction

In the twenty-first century, antibiotic resistance is a serious health issue, causing a huge problem for humans, animals, the environment, and threatening life on the globe. The phenomenon occurs when pathogenic microbes are nonresponsive to the killing or inhibitory properties of standard doses of antimicrobials. The cow and buffalo take a significant share of the antibiotic resistance as reservoirs of the resistant strains. Globally in the dairy industry, bovine mastitis is one of the largest production related disease in cows and buffalo. The main plentiful bacteria, *E. coli* is collected from the mammary gland of mastitis cows *(*Kempf et al., 2016*)*. However, some strains of *E. coli* are pathogenic via virulence factors such as transposons, plasmids, pathogenicity islands, and bacteriophages (Kaper et al., 2004). The strains of *E. coli* mostly colonize in the GIT of animals and humans as a normal flora.

For preventive and therapeutic procedures, different antimicrobial agents that are used against mastitis in animal farms, for example, amoxicillin and ampicillin, are the first choice of drugs in veterinary medicine, but an increase in medicine resistance against these drugs has been observed. Because of this resistance, in China, extended-spectrum antibiotics such as cephalosporins (ESC) have been approved for the treatment of animal infections. To overcome these bacteria throughout illness, antibiotics are used for the cure, to promote livestock growth and prevent bacterial infections in domestic animals; these drugs might also be utilized in

prophylactic methods. Extended-spectrum cephalosporins are often favored for cure to decrease mastitis-related financial losses in dairy cows. This drift might be related to the appearance of antibiotic resistance of bacteria, as indicated by the increasing occurrence of ESBL-producing bacteria (Xu et al., 2015). Multidrug-resistant bacterial infections could be difficult or delayed to treat (Roberts et al., 2009). Experiential treatment using antibiotics has led *E. coli* to become resistant to a variety of drugs. In *E.coli*, resistance against carbapenem improved from human clinical settings, food-producing animals, and the community (Ali et al., 2017; Ali et al., 2016). At an alarming rate, bacterial resistance against Beta-lactams antibiotics has increased because of their proven efficiency and safety. This resistance is highly achieved via Beta-lactamase enzymes that can dissolve most Beta-lactam ring antibiotics, such as 3rd, 4th generation, and even monobactams (Peirano et al., 2014). ESBLs are considered a key mechanism confer resistance to cephalosporins that are predominantly produced in gram-negative bacteria, particularly in *E. coli* (Perez et al., 2007). In most ESBL-producers, multidrug resistance has been commonly observed, and more frighteningly, co-resistance to common use of other drugs such as tetracycline, aminoglycosides, and fluoroquinolones has been frequently reported (Timofte et al., 2014; Xu et al., 2015).). Previous conducted studies have indicated the rate of ESBL-encoding *E. coli* strains from 5 to 8% in Singapore, Japan, Malaysia, and Korea, from 12 to 24 % in Taiwan, Thailand, Indonesia and Philippines (Paterson and Bonomo, 2005), from 62 to 100 % in India (Liu et al., 2015). By contrast, the rate was nearly up to 60 % in China (Xiao et al., 2011) A meta-analysis of ESBL from various parts of Pakistan, indicated that the total pooled proportion of ESBL-producing bacteria was 40%. The majority of cases (41.8 %) have been reported from Islamabad/Rawalpindi, followed by Punjab (25.4 %) and KPK (16.4%), with no cases reported from Baluchistan (Abrar et al., 2018). However, these Figures are conservative. In Pakistan, migratory birds were found to have a similar incidence of ESBL-encoding *E. coli* (17%) (Mohsin et al., 2017). Many vaccine-based studies were carried out over the past few years as controlled experimental and field trials. Most of these experimental and marketable vaccines are bacterins that inactivate the whole microorganism, and some are composed of subparts of the microorganism like membrane proteins, polysaccharides, or toxins (Merrill et al., 2019).

An important public health worry is the increase in ESBL-encoding *E. coli* among community-onset UTIs, as these organisms are resistant to numerous antibiotics (Pitout and Laupland, 2008; Pallett and Hand, 2010). Extended-spectrum beta-lactamase enzymes are capable of hydrolyzing β-lactam ring antibiotics, including 3rd generation of cephalosporins (Pitout and Laupland, 2008)

The drug of choice for the cure of ESBL-producing *E. coli* infections is usually known as carbapenems. Ertapenem may be a good choice because it is administered only once every day and it has a half-life of 4 hours, unlike the rest of the carbapenems (Prakash et al., 2009). Ertapenem could also be administered intramuscularly instead of intravenously, which permits for the usage in patients without intravascular access.

There are limited oral choices for the cure of ESBL-encoding *E. coli* cystitis. Some oral antimicrobial agents, such as Fosfomycin, an antibiotic that has wide-ranging activity against multi-drugs-resistance bacteria, including ESBL-producing *E. coli*. Fosfomycin is an excellent antimicrobial agent for the treatment of cystitis due to attaining very high concentrations within the urine, but should not be recommended for the treatment of pyelonephritis or patients with bacteremia because of insufficient concentrations inside the blood vessels. While Fosfomycin antibiotic is currently approved by the Food and Drug Administration for the treatment of uncomplicated cystitis in women at a daily dose of 3 grams (Pullukcu et al., 2007; Neuner et al., 2012).

Another oral antibiotic that can be considered for the cure of ESBL-encoding *E. coli* cystitis is nitrofurantoin. Another study showed clinical treatment rates of 69 percent in patients with ESBL-encoding *E. coli* cystitis, in which all bacterial isolates were also shown to be resistant to ciprofloxacin and SMX/TMP.

The key components of mastitis economic effects are linked to the decrease in milk production caused by clinical and subclinical cases, milk disposal, the cost of drugs used in clinical cases, the costs associated with clinical cases, the drop in the selling price of milk, and animal culling. The factor with the greatest economic impact on the overall cost of mastitis has been described as a decrease in milk production (Tasbakan et al., 2012).

Objectives

The aims and objectives of the planned study are:

1. To investigate the incidence of multidrug-resistant *Escherichia coli* in dairy milk samples collected from mastitis and healthy milk in the District Dir Lower.
2. To characterize the multi-drug resistant (MDR) *Escherichia coli* recovered from the milk samples.
3. Molecular characterization of the predominant ESBL-encoding genes.

Material and methods

Study area

This research work was conducted from December 2020 to June 2021 in the laboratory of Microbiology, College of Veterinary Science and Animal Husbandry, Abdul Wali Khan University, Mardan, KPK, Pakistan. In this study total of 90 milk samples were collected from different villages of the district

Dir lower. Milk samples were taken in single-use disposable plastic vials with tight-fitting or sterile glass and brought to the laboratory for screening of ESBL-producing *E. coli.*

Samples collection

Milk samples were collected from the domestic cows, along with a history recorded on a questionnaire. For the collection of samples, first, every type of dirt, garbage, or bedding particles was removed from the teats and udder, and each teat was dried carefully. These milk samples were screened in order to check the predominant ESBL-encoding genes in *E. coli.*

Isolation of E. coli

Different culturing media

Different kinds of culture media were used in this study, such as MacConkey agar, Eosin methylene blue agar (EMB), Muller Hinton agar (MHA), and Lysogeny Broth (LB).

On MacConkey agar

MacConkey media is mostly used to differentiate *E. coli* Strains from other gram-negative bacterial species. MacConkey and EMB media inhibit the growth of gram-positive bacteria, and lactose-fermenting bacteria produce pink-colored colonies (Mac Faddin, 1985). MacConkey agar was prepared for culturing according to the given protocol and then autoclaved for about 15 minutes at 121 °C. Then the media was poured into the sterile plates and allowed to solidify. Samples were streaked on MacConkey agar for screening of ESBL-encoding *E. coli* as per the guidelines of the Clinical and Laboratory Standards Institute 2014 and incubated at 37 °C overnight. After overnight incubation results were examined on the plates, and the pink colour colonies appeared, and some had no growth, as shown (Figure 1). The same procedure was followed for all collected milk samples.

Figure 1. Pink colour colonies indicate *E. coli* growth on MacConkey agar

Eosin Methylene Blue Medium

Eosin methylene blue medium provides a rapid and accurate way for distinguishing *E. coli* from some other gram-negative mastitis-causing pathogenic bacteria. On EMB agar, *E. coli* produces a metallic green sheen (Schalm et al., 1971). The pink-colored colonies representing *E. coli are* on the plates of MacConkey agar medium. These pink colonies of bacteria were further confirmed through culturing them on Eosin methylene blue agar according to the guidelines of clinical and laboratory standard institute 2016. A single colony was picked and transferred to EMB agar with the help of a sterile wire loop. The plates were incubated for 24 hours at 37°C. A green metallic sheen was observed on EMB agar, which indicated *E. coli* (Figure 2).

Figure 2. Green metallic sheen indicates the presence of *E. coli* on EMB

Screening of ESBL-producing Escherichia coli via combined disc method

A combined disc method (Legese *et al.*, 2017) was performed for the phenotypic recognition of extended-spectrum beta-lactamase-encoding *E. coli* according to CLSI. Ceftazidime, Amoxicillin, and Ceftazidime, their combinations with clavulanic acid, were used for confirmations of extended-spectrum beta-lactamase-encoding bacteria. Bacterial colonies were mixed in normal saline, and this saline was inoculated on MHA agar plates with the help of sterile ear swabs. The selected antibacterial disc was placed on the medium using sterile forceps or syringe and incubated the medium overnight at 37 ˚C. A ≥5 mm increase in zone diameters for cephalosporin/clavulanate discs compared to cephalosporin discs alone was interpreted as extended spectrum beta-lactamase enzyme producer, as shown in Figure 3.

Figure 3. Combined Disc method for the detection of ESBL-producer

Preparation of glycerol stocks

Pure glycerol was added to distilled water in equal volume to make a 50% glycerol solution and then

autoclaved. After that, glycerol solution was added to sterile Eppendorf tubes. Bacterial colonies were picked from EMB fresh culture with the help of a sterile wire loop, added into LB broth, and incubated for 24-48 hours at 37 ˚C. After incubation, 50 microliters of liquid bacterial culture (LB broth culture) was added to the 50% glycerol solution and stored at -20 ˚C.

Antibiotic sensitivity testing profile

Muller-Hinton agar medium is a non-selective culture and a non-differential medium. MHA allows a wide variety of microorganisms to grow, particularly used for antimicrobial susceptibility tests of several kinds of bacterial species, including *E. coli.* Antibiotic sensitivity tests for ESBL-producing *E. coli* were checked out on Mueller-Hinton agar and using antibiotic discs (Sparks, Becton Dickinson, MD, USA) following the standard Kirby-Bauer disk and interpreted as in Clinical and laboratory standard institute (Rahman et al., 2018)

Testing method and Antibiotics

Bacterial colonies were mixed in normal saline, and this saline was inoculated on Muller-Hinton agar with the help of sterile cotton swabs. Antibiotic discs were placed on the medium using forceps or a syringe and incubated at 37 °C for 24 hours. After overnight incubation, the zone of inhibition surrounding the antibiotic discs was measured and compared with CLSI guidelines, and antibiotic-resistant strains of bacteria were identified as shown in Figure 4 (Mac Faddin, 1985). In this research work, each isolate of *E. coli* was tested against 11 commercially available antibiotics, including Ciprofloxacin (5µg), Cephalexin (30µg), Streptomycin (5µg), Chloramphenicol (10µg), Lincomycin (10µg), Gentamycin (10µg), Aztreonam (30µg), Cefaclor (30µg), Tetracycline (30µg), Ampicillin (10µg), Levofloxacin (5µg). The results of antibiotics were recorded in three categories: sensitive, resistant, and intermediate according to CLSI. The inhibition zone around antibiotic discs was measured from the middle of antibiotics discs to the end of the clear zone of inhibition, and each inhibition zone was recorded in diameter (mm) as shown in Table 1.

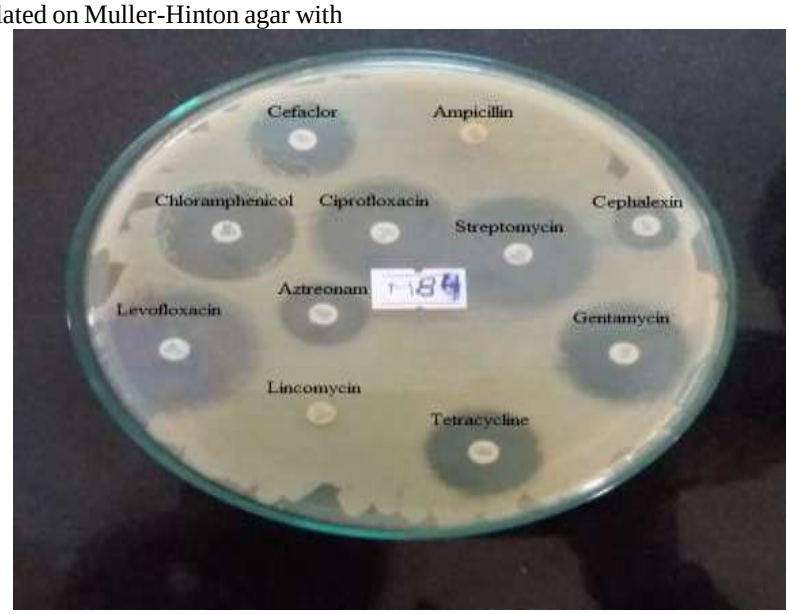

Figure 4. Disc diffusion method of antibiotics susceptibility testing

Table 1. Zone of inhibition of selected antibiotics

S. N0.	Antibiotics (Potency)	Resistant mm	Intermediate mm	Susceptible mm
1	Ciprofloxacin (5µg)	≤15	16-20	≥21
2	Cephalexin (30µg)	≤15	-	≥16
3	Streptomycin (5µg)	≤11	12-14	≥15
4	Chloramphenicol(10µg)	≤12	13-17	≥18
5	Lincomycin (10µg)	≤11	12-17	≥22
6	Gentamycin (10µg)	≤12	13-14	≥15
7	Aztreonam (30µg)	≤17	18-20	≥21
8	Cefaclor (30µg)	≤14	15-17	≥18
9	Tetracycline (30µg)	≤11	12-14	≥15
10	Ampicillin (10µg)	≤13	14-16	≥17
11	Levofloxacin (5µg)	≤13	14-16	≥17

Genotypic screening of major ESBL-encoding genes

Extraction of DNA from Bacterial cells via WizPrep™ gDNA Mini kit.

For the extraction of DNA (WizPrep™ gDNA Mini kit) was used according to the manufacturer.

Step 1: Take 150ml of overnight LB broth culture in an Eppendorf tube and centrifuge at about 7500 rpm for 10 minutes. After centrifugation, the supernatant was removed and the pellet in the GT1 buffer, about 180µl.

Step 2: 200µl GT2 Buffer was added, and 20 µl proteinase K to the solution, and mixed via vortex, and the solution was incubated at 56 °C for 10 minutes.

Step 3: Then 200µl of 100 percent ethanol was added to the samples and mixed via vortex. The mixture of the sample was added into the spin column and centrifuged for one minute at 13000 rpm, the flow-through was removed from the spin column.

Step 4: 500 µl of W1 was added to the column, and it was centrifuged at 13000 rpm for 1 minute. Again, discarded the flow through and reconnected the spin column.

Step 5: W2 Buffer was added up to 700µl in the spin column and centrifuged for one minute at 13000 rpm. Again, discarded the flow through and reconnected the spin column and centrifuge for two minutes at 13000 rpm.

Step 6: The spin column was attached to the new Eppendorf tube and elution buffer about 70µl then incubated for one minute at room temperature. After the incubation sample was centrifuged for 1 minute at 13000 rpm.

Furthermore, the spin column was removed, and the DNA was stored at −20 °C for the next process.

PCR and its conditions

All the confirmed isolates were analysed for the existence of ESBL genes via polymerase chain reaction, finding different classes of ESBL genes Such as *blaCTX-M* gene, *blaSHV* gene, and *blaTEM* gene. The mixture and PCR conditions were used for molecular screening of *blaCTX-M* gene are

CTX-M F-CGCTTTGCGATGTGCAG

CTX-M-R-ACCGCGATATCGTTGGT (Villegas et al., 2004)

PCR mixture included forward and reverse primers, DNA sample, master mix, and double-distilled water. The mixture was loaded into PCR tubes and processed in a thermocycler. The condition for gene amplification was followed as denaturation temperature set at 94 °C for 5 minutes, 35 cycles for 1 minute at 94 °C, annealing temperature at 55 °C for 30 sec, extension temperature of 72 °C for 30 sec, and final extension was set for 5 minutes at 72 °C (Kanokudom et al., 2021)

The mixture and PCR conditions used for the molecular detection of *SHV* gene are

SHV F- GGG TTA TTC TTA TTT GTC GC

SHV R-TTAGCGTTGCCAAGTGCTC (Chang et al., 2001)

Same as the above-mentioned PCR mixture, and condition were followed.

The mixture and PCR conditions were used for the detection of *TEM* gene, which are described below:

TEM F- ATA AAA TTC TTG AAG ACG AAA

TEM R-GAC AGT TAC CAA TGC TTA ATC (Yao et al., 2007)

PCR mixture included Forward and Reverse primers, DNA sample, master mix, and double-distilled water. The mixture was loaded into PCR tubes and processed in a thermocycler. The condition for gene amplification was followed as denaturation temperature set at 94 °C for 5 minutes, 35 cycles for 1 minute at 94 °C, annealing temperature 54 °C for 30 sec, extension temperature 72 °C for 30 sec, and final extension was set for 5 minutes at 72 °C (Kanokudom et al., 2021)

Gel Electrophoresis

PCR products were run via gel electrophoresis for the detection of desired genes. For this purpose, 1% agarose solution was prepared in 40 ml of TAE buffer by adding 0.4 agarose and boiling for 1 minute, and after that, ethidium bromide dye was added about 2µl. The gel tray was prepared and ready by placing a comb in order to form wells for the loading of PCR products. The prepared agarose gel was poured into the tray and allowed to solidify completely. After the solidification agarose gel was shifted to the gel tank full of TAE buffer. After that, 5µl of ladder was loaded in the first wells, and 3.5 µl of PCR products were added to the remaining wells. Both electrodes of the gel tank were set at 100 Ampere electricity and 60 volts for 35 minutes (Al-Agamy et al., 2009)

Results and discussion

The current work was carried out in the laboratory of Microbiology, College of Veterinary Science and Animal Husbandry, AWKUM, KP-Pakistan. A total of 90 samples were collected from different villages of the district Dir lower i.e., Munda, Samar Bagh, Mayaro, Badin, Kambat, and Barchoni, as described in Figure 5. In this study, domestic cows were chosen for sample collection. Out of them, 63 samples showed positive results on MacConkey agar, 43 samples showed positive results on EMB ager and 17 were confirmed as ESBL-encoding *E. coli* when tested with the combined disc method test according to the instructions of the Clinical Laboratory of standard institute and the PCR assay.

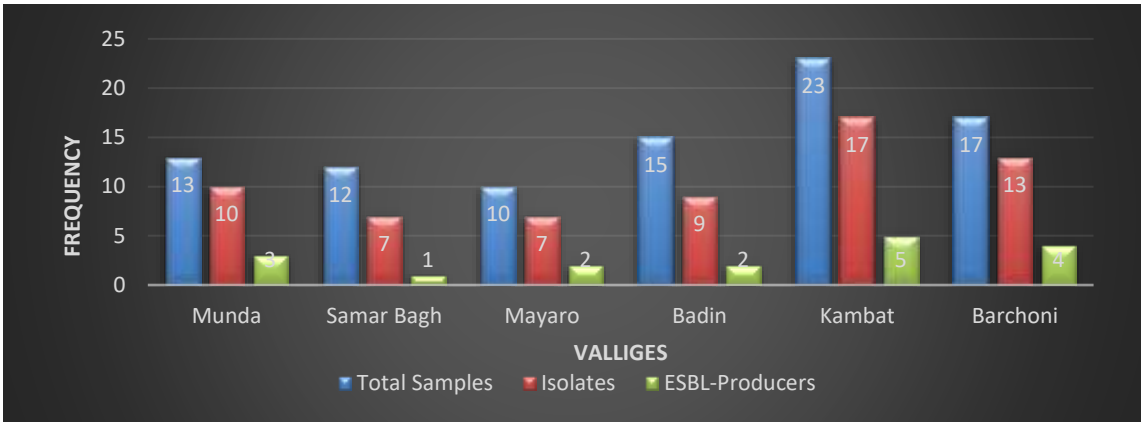

Figure 5. Graphical representation of positive ESBL-Producer on the Base of area

Morphological isolation of Escherichia coli

All collected samples were inoculated on MacConkey agar; among these, 63 (70%) samples showed growth, and 27 (30 %) samples did not develop and appearance of colonies. Among 63 isolates, 43 (68.25%) were positive (*E. coli*) and 20 (31.74%) were negative when tested for screening and confirmation via Eosin Methylene Blue agar, as shown in Figure 6.

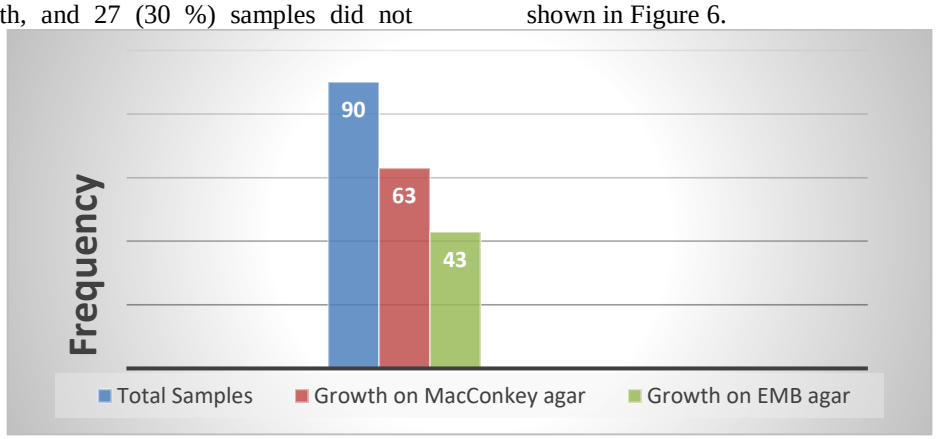

Figure 6. Frequency *E. coli* isolates on the basis of morphological characteristics

Confirmation of ESBL-Producing Escherichia coli via combined disc method

Out of 43 Isolates, 17 (39.53%) were positive, and 26 (60.46%) were negative for ESBL production when tested through the combined discs method (Figure 7).

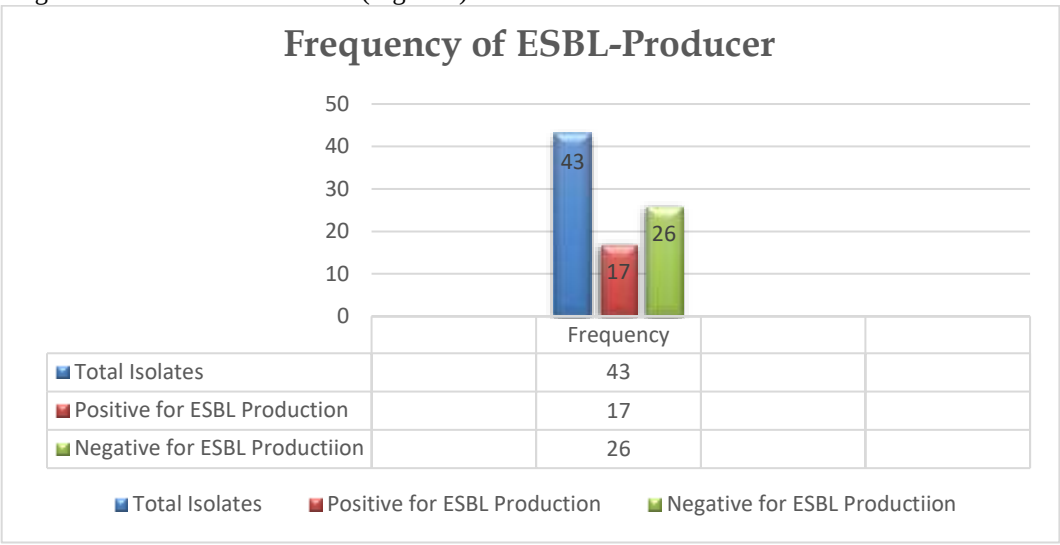

Figure 7. Graphical representation of ESBL-Producing *Escherichia coli* Strains

Antibiotics susceptibility testing

All confirmed ESBL-encoding *E. coli* strains were tested against 11 commercially available antibiotics that showed different effects as described below.

Resistance, Sensitive, and Intermediate patterns of antibiotics susceptibility testing

Eleven different antibiotics were used in this study to check their susceptibility pattern. The isolated strains of *E. coli* highly resistant to Ampicillin (100%) followed by Lincomycin (92.85%), Cephalexin (35.71%), Aztreonam (28.57%), Tetracycline (21.14%), Cefaclor (21.14%), Gentamycin (7.14%), Streptomycin (7.14%), Levofloxacin (0%), Ciprofloxacin (0%) and Chloramphenicol (0%) as described in (Figure 8). The isolated strains of *E. coli* highly sensitive to Levofloxacin (100%), Ciprofloxacin (100%) Chloramphenicol (100%) and followed by Streptomycin (85.71%), Gentamycin (78.57%), (Tetracycline 78.57%), Cefaclor (71.42%), aztreonam (64.28%), Cephalexin (57.14%), Lincomycin (0%) and Ampicillin (0%) shown in (Figure 9). Some antibiotics showed intermediate effects against tested bacterial isolates, Gentamycin (14.18%), Lincomycin (7.14%), Cephalexin (7.14%), Aztreonam (7.14%) Streptomycin (7.14%), and the remaining others shown Zero intermediate effects shown on (Figure 10) and over all effects of Resistance, Sensitive and intermediate as described in (Table 2).

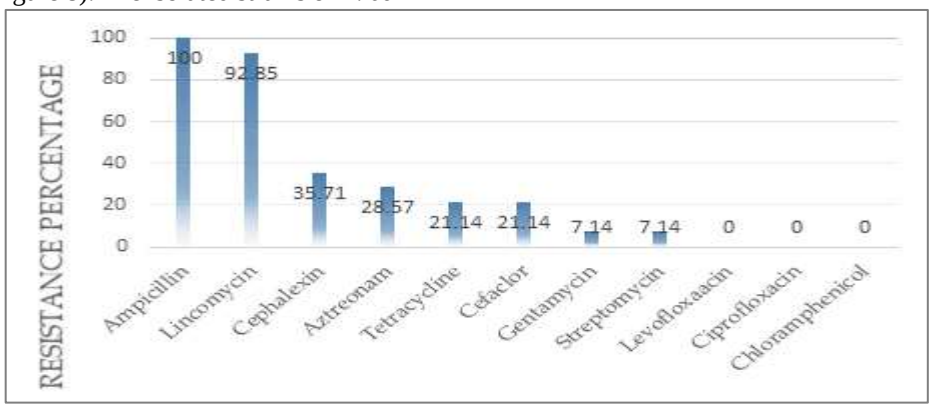

Figure 8. Graphical representation of the resistance percentage

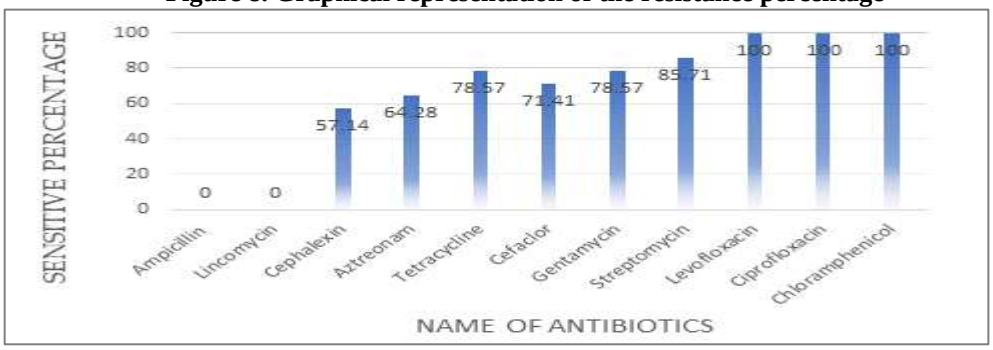

Figure 9. Graphical representation of the sensitivity percentage

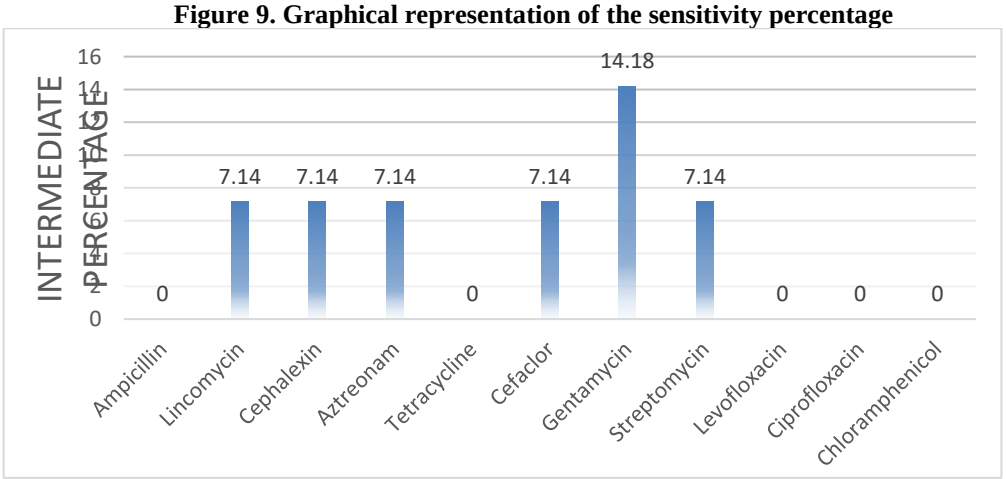

Figure 10. Graphical representation of the intermediate percentage

Table 2. Antibiotics susceptibility testing

S. No.	Name of Antibiotics	Antibiotics Potency	Percentage of antibiotics susceptibility		
			% Resistance	% Sensitive	%Intermediate

1	Ampicillin	10µg	100 %	0%	0%
2	Lincomycin	10µg	92.85%	0%	7-14%
3	Cephalexin	30µg	35.71%	57.14%	7.14%
4	Aztreonam	30µg	28.57%	64.28%	7-14%
5	Tetracycline	30µg	21.14%	78.57%	0%
6	Cefaclor	30µg	21.14%	71.42%	7.14%
7	Gentamycin	10µg	7.14%	78.57%	14.18%
8	Streptomycin	5µg	7.14%	85.71%	7.14%
9	Levofloxacin	5µg	0%	100%	0%
10	Ciprofloxacin	5µg	0%	100%	0%
11	Chloramphenicol	10µg	0%	100%	0%

Overall incidence and frequency of distribution of ESBL-genes

All confirmed (17) samples of ESBL-encoding *E. coli* strains were tested via conventional PCR for the molecular screening of *blaCTX-M* gene, *blaTEM* gene, and *blaSHV* gene from milk isolates. The overall incidence of ESBL-encoding *E. coli* was 39.53% (n=17) and 43 recovered isolates of *E. coli*. The finding of this study agree with similar study was conducted in China, that reports showed the incidence 23.53% (*n* = 36) out of 153 *Escherichia coli* isolates from the milk samples of mastitic cows (Ali et al., 2016). Reports of our study showed higher prevalence of ESBL-producing *Escherichia coli* as compared to similar study was conducted in China, that showed the prevalence (5.3%) 0f ESBL-encoding *E. coli*, only 13 isolates positive for ESBL production among 245 collected samples (Yu et al., 2015)The results of this study do not agree with similar studies were conducted in France (Dahmen et al., 2013) and Switzerland (Geser et al., 2012). Out of 17 isolates, 6 (35.29%) isolates were positive for co-existence of *blaCTX-M* gene and *blaTEM* gene, 5 (29.41%) isolates were positive for single gene presence of *bla*CTX-M, 2 (11.76%) isolates were positive for the single existence of *blaTEM*, 4 (23.52%) were positive for *blaSHV* gene. The *blaCTX-M* gene was observed at 550 bp shown in Figure 11.

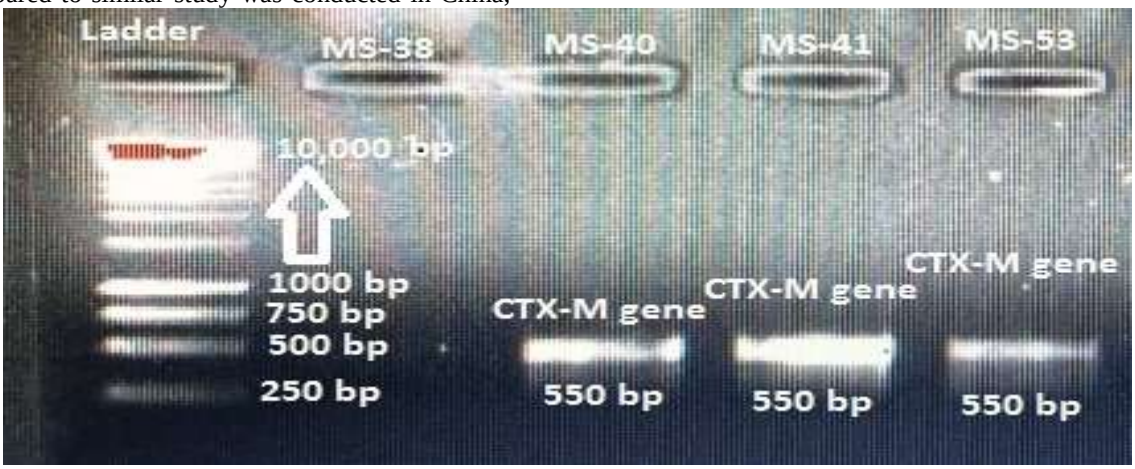

Figure 11. Ladder showing 250-10,000 bp marker, Lane MS-40, MS-41, and MS-53 showing 550 bp amplified PCR product of CTX-M gene.

The *blaTEM* gene was observed at 1086 bp shown in Figure 12 and *blaSHV* observed at 567 bp shown in Figure 13.

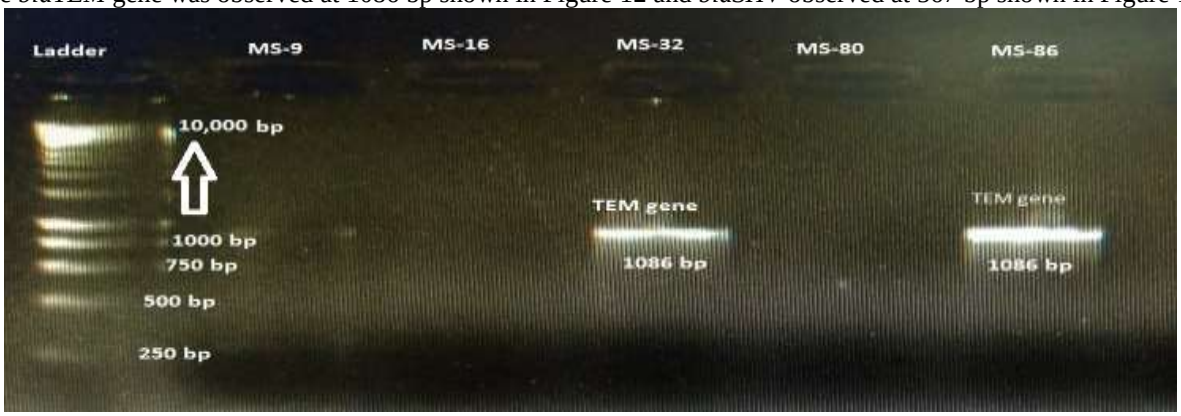

Figure 12. Ladder showing 250-10,000 bp marker, Lane MS-32 and MS-86 showing 1086 bp amplified PCR product of TEM genes.

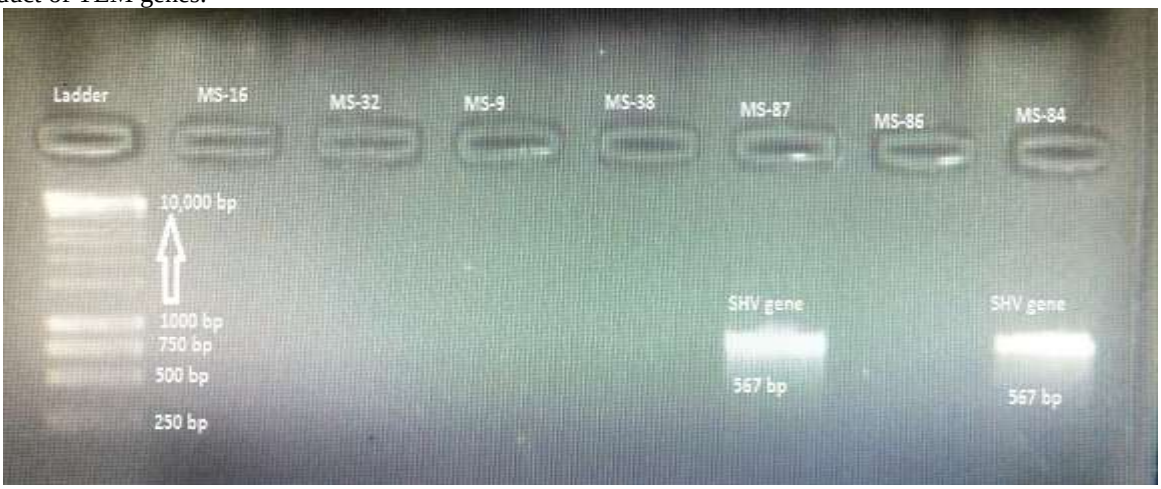

Figure 13. Ladder showing 250-10,000 bp marker, Lane MS-84 and MS-87 showing 567 bp amplified PCR product of SHV genes.

In this study, the most predominant genotype was *blaCTX-M* gene, showing (64.70%) followed by *blaTEM* gene (47.05%) and *blaSHV* gene (23.52%). The findings of the current study agree with other prevalent studies conducted in China and other countries that also testified *blaCTX-M* gene as the predominant ESBL-producing genotype. A study conducted in China, which reports showed *blaCTX-M* gene was the most predominant ESBL-producing gene (28/36) 77.78%, while *blaTEM* gene and *blaSHV* gene were present in (20/36) 55.56% and (6/36) 16.67% of ESBL-positive isolates, respectively (Ali et al., 2016). The results of a previous study also confirmed that the *blaCTX-M* was the most predominant ESBL gene, followed by *blaTEM* (Feng et al., 2018). Besides the above-mentioned studies, other studies also conducted around the world that revealed the *CTX-M* gene was the predominant in ESBL-encoding *Escherichia coli* from mastitis (Freitag et al., 2017; Pehlivanoglu et al., 2016). The majority of isolates in current study was resistant to Ampicillin (100 %) followed by Lincomycin (92.85%), Cephalexin (35.71%), Aztreonam (28.57%), Tetracycline (21.14%), Cefaclor (21.14%) and highly sensitive to (Levofloxacin 100%, Ciprofloxacin 100% Chloramphenicol 100%) and followed by Streptomycin (85.71%), Gentamycin (78.57%), (Tetracycline 78.57%), Cefaclor (71.42%), aztreonam (64.28%) and Cephalexin (57.14%).

A study (Freitag et al., 2017; Pehlivanoglu et al., 2016) concerning antibiotic resistance in *E. coli* and *Shigella* strains isolated from children in Hanoi, Vietnam. Among 162 E. coli isolates, 86.4% were resistant to ampicillin, and 3.7% were resistant to ciprofloxacin.

Conclusion and recommendation

This research showed that the most predominant gene was *blaCTX-M* gene, followed by *blaTEM* gene and *blaSHV* gene in ESBL-encoding *E. coli*. This work showed that ESBL-encoding *Escherichia coli* were highly resistant to Ampicillin (100 %) followed by Lincomycin (92.85%), Cephalexin (35.71%), Aztreonam (28.57%), Tetracycline (21.14%), Cefaclor (21.14%), Gentamycin (7.14%), Streptomycin (7.14%), Levofloxacin (0%), Ciprofloxacin (0%) and Chloramphenicol (0%) and highly sensitive to Levofloxacin (100%), Ciprofloxacin (100%) Chloramphenicol (100%) and followed by Streptomycin (85.71%), Gentamycin (78.57%), (Tetracycline 78.57%), Cefaclor (71.42%), aztreonam (64.28%), Cephalexin (57.14%), Lincomycin (0%) and Ampicillin (0%). So, it is mandatory that antibiotic sensitivity be checked before the treatment of any infection.

Future prospective

1. Awareness among people about the usage of antibiotics.
2. The resistant genes are developing due to the improper use of antibiotics, so they should be taken in an appropriate dosage for animals and humans.
3. Further molecular recognition of other antibiotics-resistant genes in *E. coli.*

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Statements and Declarations

Data Availability Statement

All data are fully available and can be found within the manuscript file.

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Conflicts of Interest

The authors declare no conflict of interest.

Ethics Approval Statement

Not applicable.

Consent to Participate

Not applicable.

Consent to Publish
Not applicable.
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Conceptualization by RAHMAN SU; methodology by RAHMAN SU. and ULLAH N; software by ULLAH N; validation by AHMAD S; resources by RAHMAN SU and Riaz, S; data curation by ULLAH N; writing—original draft preparation was prepared by ULLAH N., writing—review and editing by AHMAD S., Riaz, S., Adil, MM., and Mansoor S; visualization by ULLAH N and Riaz, S; supervision, RAHMAN SU and AHMAD S; project administration RAHMAN SU.; All authors have read and agreed to the published version of the manuscript.
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