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CHARACTERIZATION OF ANTIBIOTIC SUSCEPTIBILITY TESTING AND EFFLUX PUMP OF MULTI-DRUG RESISTANT *ESCHERICHIA COLI* ISOLATED FROM RAW MILK SAMPLES

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Abstract

Background: *Escherichia coli is increasingly exhibiting antimicrobial resistance in communities and hospital settings, therefore triggering a growing public health emergency. Pakistan's high population density, poor sanitary infrastructure, and filthy food and water sources make it more prone to illness than other countries. In numerous circumstances, antibiotic overuse and abuse have caused bacterial resistance.* ***Aim and Objective:*** *In this study we have assessed the prevalence of multi-drug resistance and its associated demographic variables Escherichia coli was extracted from raw milk of cow in the Peshawar area of the Khyber Pakhtunkhwa (KP).* ***Methods:*** *Eighty percent of 80/100 raw milk samples proved negative for culture growth, while 20% (n=20) tested positive. The Study consists of a total of 100 Raw milk sample collected from both urban and rural area of district Peshawar 61% were collected from rural area and 39% sample were collected from urban area. The colonies were subjected to double-disc synergy test (DDST) to detection Extended-spectrum beta-lactamases (ESBLs) producing enzyme.* ***Results:*** *Within our investigation, we evaluated the antibiotic resistance profiles of 20 isolates, finding notable diversity in their susceptibility. The isolates showed significant resistance to Meropenem (95%), Gentamicin (95%), and Ciprofloxacin (80%), but seemed to be most susceptible to Ampicillin (95%) and Amoxicillin (90%). Resistance of moderate intensity was detected for Ceftazidime (35%), Cefoxitin (30%), and Tetracycline (30%).* ***Conclusion:*** *These findings highlight the essential need for antibiotic stewardship which denotes systematic approaches to enhance antibiotic use, guaranteeing efficient treatment while reducing resistance. Practical applications include the formulation of prescription recommendations, the education of healthcare personnel and patients, the monitoring of use trends, and the promotion of infection prevention. These initiatives seek to improve patient safety and maintain antibiotic effectiveness for future generations. In the case of DDST, a total of eight isolates have been found to be resistant (R) to one or more antibiotics, which indicates the possibility of having produced (ESBL).*

Keywords: *Antibiotic susceptibility testing, Antimicrobial resistances, Double disc synergy test, Extended spectrum beta-Lactamase, Raw milk*

INTRODUCTION

Milk is rich in nutrients. About equal proportions of lipids, carbohydrates, proteins, and energy components are organic substances. Besides phosphates, nitrates, and chlorides of calcium, magnesium, potassium, and sodium, it contains vitamins, enzymes, and dissolved gasses in minor amounts. Dissolved gases make up about 5% of the volume, principally nitrogen (N), oxygen (O_2), and carbon dioxide (1). About 4% of foodborne diseases worldwide are connected to dairy products.More common in low-income and middle-income countries. Economic impacts exceed $4 billion yearly (2). When discovered in milk, *E. coli* is a common illness that signals feces contamination. *E. coli's* presence in milk is largely due to its abundance in cow's gastrointestinal tract GITs. Some *E. coli* strains in the gastrointestinal tract (GIT) may cause enteritis, although most are innocuous (3). A research has shown that most milk storage facilities are antiquated, posing health risks (4). Considering these conditions, milk-borne illness is a major public concern. *Escherichia coli* is more prevalent in less developed nations characterized by inadequate sanitation and hygiene.

Quantifying the worldwide impact of *E. coli* infections is challenging due to variations in monitoring and reporting systems. The standards for food safety and quality control include the analysis of food for pathogenic bacteria that are responsible for most gastrointestinal diseases (5).

Milk-related foodborne outbreaks have implicated *Listeria monocytogenes, Salmonella, Campylobacter, Staphylococcus aureus, B. cereus, and Cl. botulinum*. Recently, multiple outbreaks of *E. coli* in affluent nations have caused moderate diarrhea to the potentially deadly hemolytic uremic syndrome, threatening the dairy sector (6). *E. coli* is rod-shaped. Gram-negative bacteria have lipopolysaccharide-based cell walls. *E. coli* is classified as a facultative anaerobe, which means it can survive and grow in both oxygen-rich and oxygen-poor conditions (7).

Antimicrobial resistance (AMR) *Escherichia coli* in food borne and research pathogens is a big challenge for public health and animal food processing systems, according to One Health. Milk-surviving AMR strains may spread resistance strain germs from animals to humans. Recent investigations have shown ESBL-producing microbes in raw milk, suggesting that consumers may be exposed to them (8). Antimicrobial resistance is further compounded by the presence of multidrug-resistant bacteria, which possess diverse resistance mechanisms to several antimicrobial agents. Antimicrobial resistance, including sulphonamides (sulfamethoxazole-trimethoprim), penicillin (Ampicillin), tetracyclines (tetracycline), aminoglycosides (kanamycin), and cephalosporins, impacts both veterinary and human antimicrobials concurrently (9).

ESBL-producing *Escherichia coli* bacteria threaten global health. These bacteria resist beta-lactam antibiotics and other drugs. Treating resistant ESBL-producing *E. coli* is tough. ESBL-producing *E. coli* is hard to cure. Limited options lead clinicians to utilize carbapenems, which are more powerful (10). Enterobacteriaceae produce ESBL enzymes, a key cause of antibiotic resistance. Ambler divides ESBL into A, B, C, and D. *E. coli* produces group A ESBL enzymes such as *TEM, SHV*, and *CTX-M*, as indicated. These enzymes break down several cephalosporin antibiotics, including ampicillin and carbenicillin. Oxacillin-ceftazidime. (11)

The aim of our research was to ascertain the prevalence and population characteristics of *Escherichia coli* in unpasteurized milk in both urban and rural areas of the Peshawar district in KP. The goal is to identify and evaluate the resistance of *Escherichia coli* to many drugs. The Double Disc Synergy Test is employed for the identification of *Escherichia coli* strains that exhibit the production of Extended Spectrum Beta-Lactamase (ESBL).

METHODOLOGY

SAMPLE COLLECTION

Sterile glass or a disposable plastic tube with a tight lid was used to collect raw milk samples, samples were collected before milking by cleaning cow udder and teats with water. After collecting the milk sample, the lid of the container was closed. 100 raw milk specimens were taken to the Microbiology laboratory at Khyber Medical University Peshawar for processing.

SPECIMEN ISOLATION

The specimens were streaked on an EMB culture plate before being subjected to processing, each sample of raw cows' milk and shops will undergo inoculation to get a pure culture and distinct morphology. The frozen samples were then thawed at room temperature for a period of 4 to 6 hours. For each milk sample, one milliliter was combined with nine milliliters of buffered peptone water in a test tube, making a 1:9 ratio. The combination was thereafter subjected to homogenization for duration of two minutes, followed by an incubation period of 20 to 24 hours at a temperature range of 35-37°C. The enrichment technique was conducted to augment the retrieval of the organisms. To separate *E. coli*, the culture was placed on Eosin Methylene Blue agar and kept in an environment with sufficient oxygen at a temperature of 37 °C for duration of 24 hours. The detection of a metallic sheen color on the EMB agar plates showed the proliferation of *E. coli*, which led to further processing. Microscopy, biochemical analysis, and gram staining

were later used to confirm the growth of *E. coli*. The bacterial isolates were subsequently preserved at a temperature of -80 °C in a liquid broth solution supplemented with 30% glycerol (12). Further these colonies were confirm using biochemical test and such as indole, catalase, and the Triple Sugar Iron Test.

CATALASE TEST

Place precisely 4 to 5 droplets of 3% hydrogen peroxide (H2O2) into a test tube measuring 12 x 75 millimeters. Using a wooden applicator stick, get a tiny quantity of organism from a separate colony that has been growing for 18 to 24 hours, and transfer it into the test tube. Exercise caution to avoid handling any agar. This is especially crucial if the colony isolate was cultivated on agar that includes red blood cells. Red blood cells remaining in the test may lead to a false-positive response. Position the tube in contact with a dimly lit surface and carefully examine for the prompt emergence of bubbles (resulting from the reaction between O2 and water) at the tip of the wooden applicator stick (13).

COAGULASE TEST

Using a pipette, transferred 0.5 ml of the reconstituted plasma into the test tube in an aseptic manner. With the sterile loop or applicator stick, collected two or three distinct colonies of the microorganisms that will be analyzed. Following the process of emulsifying the bacteria in 0.5 milliliters of plasma, proceed to place the resulting mixture in the incubator. Before proceeding, sanitize the loop that may be used again and discard the loop and applicator stick that are meant for one-time use in the suitable container. During the next four hours, regularly monitor the culture for any indications of a clot. The formation of blood clots is a desirable consequence. If no clot is seen after 4 hours, the test may be repeated by incubating overnight at room temperature and doing a final inspection after 24 hours (14).

TSI (TRIPLE SUGAR IRON) TEST

A minute amount of the experimental bacterium, derived from a 24-hour-old uncontaminated culture, was introduced into the tubes by piercing and spreading with a sterilized inoculating needle, following strict aseptic procedures. The tubes were incubated at a temperature of 37°C for a whole day with the screw caps not fully fastened. The slope and darkening of the butt of the Triple Sugar Iron (TSI) agar media indicate the occurrence of fermentation. The presence of gas resulting from fermentation would be visibly shown in the agar by the cracking or formation of bubbles in the butt (15). Further these colonies were subjected to antibiotic Susceptibility testing following the Kirby Bauer disc diffusion technique as specified by the Clinical and Laboratory Standards Institute (CLSI 2022) (16). Further the bacteria that were shown to have extended-spectrum beta-lactamase (ESBML) were used in the Double Disc Synergy test for isolation. The suspension's turbidity will be measured using McFarland's standard (0.5). The willing suspension will be injected on an MHA (Muller Hinton agar) plate done in lawn culture. The culture plate will have the disc containing Amoxiclav placed precisely in its middle. Ceftazidime (CAZ) and ceftriaxone (CI) shall be placed 15mm apart from Amoxiclav. The culture plates will be incubated for a duration of 24 hours. At a temperature of 37 degrees Celsius. An increase in the size of the area the presence of extended-spectrum beta-lactamase (ESBL) will be determined by measuring the inhibition of bacterial growth caused by the antibiotics ceftazidime (CAZ) or ceftriaxone (CI) (17).

RESULTS

ESCHERICHIA COLI GROWTH ISOLATION PREVALENCE IN RAW MILK

A total of 100 raw milk samples were obtained. Out of which 80% (n=80) tested negative for culture growth, whereas 20% (n=20) tested positive shown in the Fig. 1 (a). The distribution of sample military farmland in Sarak (n=39) is as follows: n=22 is owned by Ram Kishan near Bakhshi, n=21 is owned by Essa Khel Topchian, and n=40 within the Peshawar district shown in the Table I and Fig. 1 (b).

Analysis of antibiotic resistance in bacteria from urban and rural environments demonstrating the prevalence of positive and negative growth responses to different antibiotics. The data reveals significant

disparities in resistance patterns, with rural regions exhibiting elevated rates of positive growth for the majority of antibiotics assessed shown in the Fig. 2.

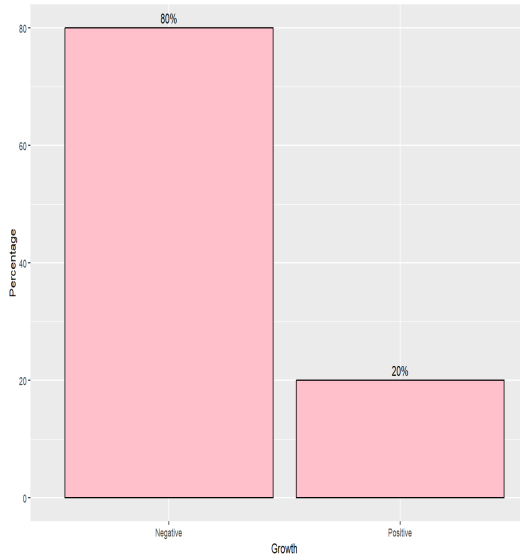

Fig. 1 (a). Distribution of bacterial growth

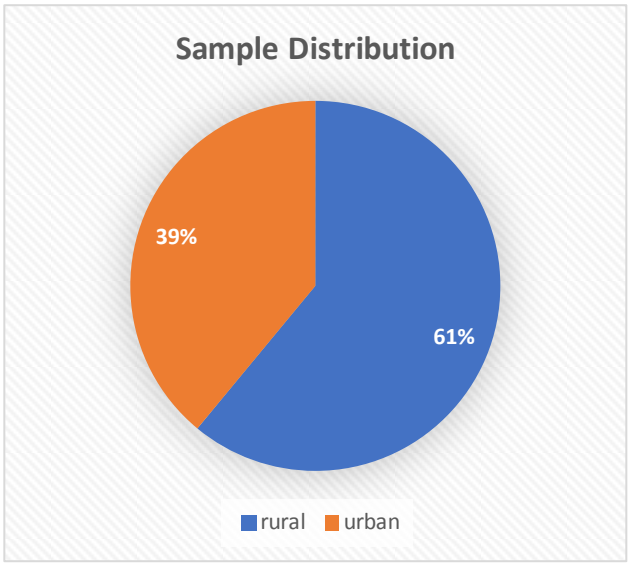

Fig. 1 (b). Distribution of sample from urban and rural area

Table I. Collection of sample from different areas of Peshawar

S. No	Sample Area		Number of samples	Percentage (%)
	Urban	Rural		
1	Military farm Landi Sarak		39	39
2		Ram Kishan near Bakhshi full	21	21
3		Essa Khel Topchian	40	40
	Total		100	100%

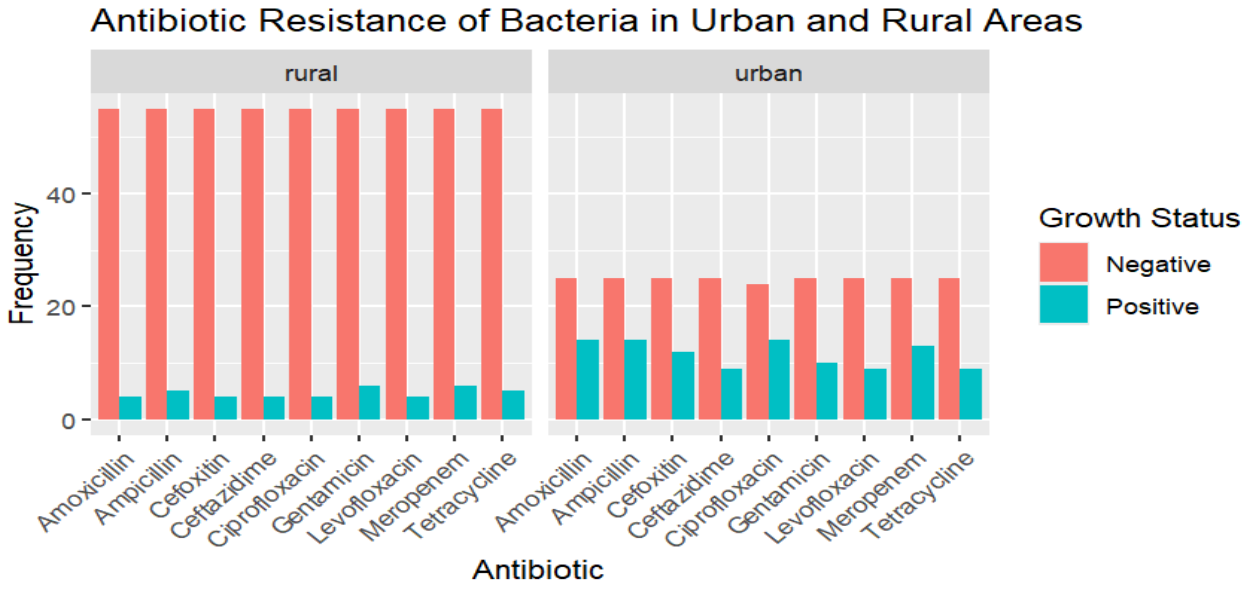

Fig. 2. Antibiotic resistance on the basis of urban and rural area

After overnight incubation on EMB agar, streaking colonies were smooth, round, black, and metallic shine. Under a microscope, EMB stained smears revealed Gram-negative, pink-tinted bacteria. Microscopic, rod-shaped bacteria were found alone, in pairs, or in short chains. After confirming bacterial growth on the culture plate, *E. coli* tested negative for Coagulase and positive for Catalase.

ANTIMICROBIAL RESISTANCE PATTERN

The results of the antimicrobial susceptibility (AST) are shown in the Fig. 3, test for 20 isolates indicated that n=17 (95%) of the isolates are highly susceptible to Gentamicin and Meropenem. Additionally, n=7 (35%) of the isolates are susceptible to Ceftazidime, n=6 (30%) are susceptible to Tetracycline, n=16 (80%) are susceptible to Ciprofloxacin, n=6 (30%) are susceptible to Cefoxitin, n=9 (45%) are susceptible to Levofloxacin, n=2 (10%) are susceptible to Amoxicillin, and only one n=1 (5%) is susceptible to Ampicillin. The resistance pattern shown by *E. coli* in raw milk demonstrates a significant degree of resistance to Ampicillin, with n=19 isolates (95%) displaying resistance. Amoxicillin shows resistance in n=18 (90%) isolates, Tetracycline in n=14 (70%) isolates, Ceftazidime in n=13 (65%) isolates, and Ciprofloxacin in n=14 (70%) isolates shown in Table II.

Table II. Antibiotic susceptible and resistant pattern of different against *Escherichia coli* from raw milk

S. No	Antibiotic	Susceptible		Resistant		Total isolates
		Number (n)	Percentage (%)	Number (n)	Percentage (%)	
1	Ceftazidime	13	65	7	35	20
2	Tetracycline	14	70	6	30	20
3	Amoxicillin	18	90	2	10	20
4	Ciprofloxacin	4	20	16	80	20
6	Ampicillin	19	95	1	5	20
7	Cefoxitin	14	70	6	30	20
8	Meropenem	1	5	19	95	20
9	Levofloxacin	11	55	9	45	20
10	Gentamicin	1	5	19	95	20

DOUBLE DISC SYNERGY TEST CONFIRMATION FOR *ESCHERICHIA COLI* THAT PRODUCE ESBL

For each of the 20 isolates, a double-disc synergy antibiotic test verified that the bacteria were ESBL enzyme-producing *E coli* or not. For ESBL ceftazidime (CAZ) disc near clavulanic acid disc in amoxicillin + clavulanic (AMC) acid test. A positive outcome is noted if a disc containing clavulanic acid crosses the Ceftriaxone (CRO) disc inhibitory zone. All 8 isolates demonstrate resistance to one or more antibiotics, indicating the potential presence of Extended-Spectrum Beta-Lactamase (ESBL) synthesis. *E. coli*, in accordance with the recommendations outlined by the Clinical and Laboratory Standards Institute (CLSI-2022) shown in the Fig. 3 (b). Fig. *4 s*hows that each species has a different antibiotic-resistant pattern in which isolate number 17 has shown a different resistance pattern from the rest of the *E. coli* that were collected from raw milk.

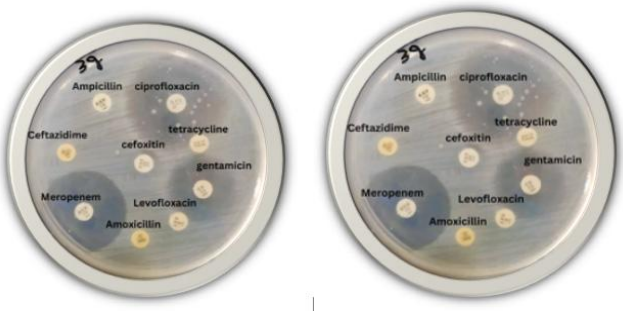

Fig. 3 (a). AST plates of *Escherichia coli*

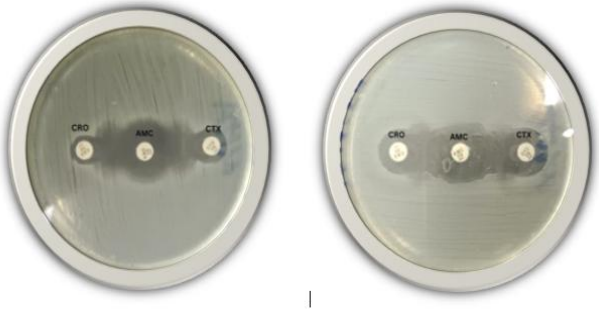

Fig. 3 (b). DDST plat of *Escherichia coli*

DISCUSSION

This study assessed the frequency of antimicrobial resistance (AMR) in *E. coli* samples obtained from unpasteurized milk in Peshawar, Pakistan. (18) Reported that the incidence of *E. coli* detected in milk was 70.4%. This data illustrates the inadequate hygiene practices employed by farmers during the milking

procedure. In our study antimicrobial susceptibility testing (AST) of the 20 *E. coli* isolates showed that (95%) had a high susceptibility to Gentamicin and Meropenem. The resistance profile exhibited a notable pattern, shown resistance to Ampicillin and Amoxicillin up to 90% to 95%. Tetracycline had a resistance rate of 70%, whereas Ceftazidime and Ciprofloxacin showed resistance rates of 65% and 70% respectively. This is lower than India's 81.1% *E. coli* in raw milk (Shakya *et al*. 2016), and 75% in Bangladesh(19). This outcome is intricately connected to the (19, 20). The antibiotic susceptibility *of E. coli* played a crucial role in determining the appropriate antibiotic for sepsis (21). The *E. coli* isolates that were studied exhibited a notable level of resistance to amoxicillin. Specifically, 32.2% of the *E. coli* organisms obtained from cows with mastitis were shown to be resistant to Ampicillin (22).

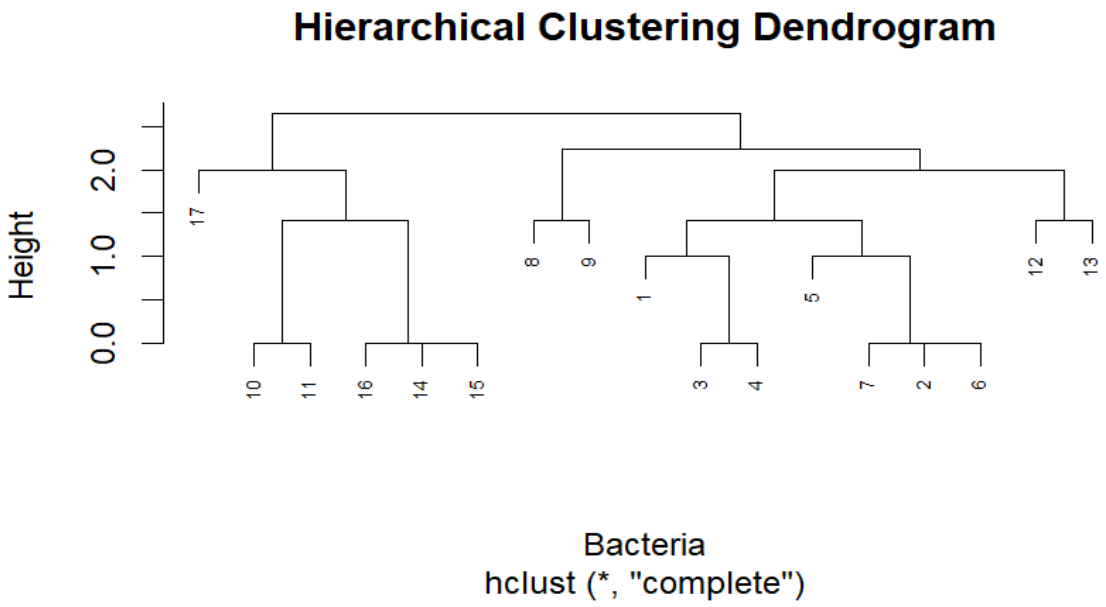

Fig. 4. Hierarchical clustering dendogram shows different antibiotic-resistant pattern of species

Milk provides an ideal environment for the rapid growth of many bacteria, including potentially harmful kinds. It is known that fresh milk includes both pathogenic and non-pathogenic germs that may be transmitted to people during the milking process and consumption of milk. Noncompliance with appropriate hygiene protocols during the milking process might lead to the spread of infections. The presence of *E. coli* in unpasteurized milk and dairy products is concerning owing to its connection with fecal contamination and the consequent risk of enteric pathogenic bacteria in food (6). The antimicrobial susceptibility (AST) test results for n=20 isolates show that n=17 (95%) of the isolates exhibit a high level of susceptibility to Gentamicin and Meropenem. Furthermore, out of the isolates, n=7 (35%) showed susceptibility to Ceftazidime, n=6 (30%) show susceptibility to Tetracycline, n=16 (80%) show susceptibility to Ciprofloxacin, n=6 (30%) showed susceptibility to Cefoxitin, n=9 (45%) showed susceptibility to Levofloxacin, n=2 (10%) showed susceptibility to Amoxicillin, and one n=1 (5%) showed susceptibility to Ampicillin. This outcome was intricately connected to the Islam *et al.,* (2016) and Somro *et al.,* (2002) (19, 20). The antibiotic susceptibility *of E. coli* played a crucial role in determining the appropriate antibiotic for sepsis (21).

The existence of ESBL-producing *E. coli* in unpasteurized milk poses a significant risk to public health, and it is crucial to increase public knowledge on the hazards associated with these disease-causing microorganisms (18). In our study all eight isolates exhibited resistance to one or more antibiotics, indicating the presence of ESBL development (23). show in has study that three isolates of *E. coli* that produce extended-spectrum beta-lactamase (ESBL) were found in raw milk, accounting for 1.7% of the total isolates. The identification of ESBL *Enterobacteriaceae* (*E. coli*) in milk indicates the existence of environmental contamination and inadequate sanitation practices during the milking process. *E. coli* is a bacteria that can serve as a reservoir for several antibiotic resistance genes (24). ESBL enzymes are synthesized by numerous species within the Enterobacteriaceae family. These bacteria possess the ability to break down penicillins

and third-generation cephalosporins, monobactam, and various other antibiotics, with the exception of carbapenems (25). In this investigation, although there was a wide range of phenotypic resistance patterns among *E. coli* isolates, a notable correlation was found between isolates that were resistant to a particular strain and their phenotypic co-resistance to Ampicillin, and tetracycline which is similarity to Rehman *et al.*, (2017), who showed strong correlations between resistance characteristics and some virulence genes found in *Escherichia coli* from cattle in China (26).

To discover taxonomic difference among bacterial species, whole genome or Sanger sequencing is used to detect antimicrobial resistance (AMR). Subsequent research will examine differences in antibiotic resistance using genomic sequencing.

CONCLUSION

The research investigated the prevalence of multi-drug resistant, ESBL-producing bacteria, specifically *Escherichia coli* isolated from bovine milk. Findings of the study suggest that unprocessed foods, like raw milk, can facilitate the spread of drug-resistant microorganisms in the environment. These microorganisms may then enter the food chain, posing a potential risk to human health. According to AST testing, Gentamicin and Meropenem showed high sensitivity, while Amoxicillin and Tetracycline were resistant. This study established a clear link between efflux capabilities and resistance, potentially paving the way for developing new combination therapies.

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Conflict of interest:

The authors declare no conflicts of interest.

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