



Prevalence, Antimicrobial Susceptibility, and Molecular Characterization of *Escherichia coli* isolated from Raw Milk in Dairy Herds

Amna Adnan¹, Muhammad Hassaan Hamza¹, Iqra Rafiq², Mengping Luo^{3*}

¹Department of Microbiology, Khwaja Fareed University of Engineering and Information Technology, Rahim Yar Khan-64200, Pakistan

²Department of Microbiology, The Islamia University of Bahawalpur, Bahawalpur-63100, Pakistan

³Guangdong Haid Institute of Animal Husbandry & Veterinary, Guangzhou-511400, China

*Correspondence: luomeng1283@gmail.com

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Abstract

Mastitis is one of the most endemic diseases in dairy cattle that has led to a huge economic losses, due to the decreased milk production, treatment costs and culling. *Escherichia coli* is an environmental pathogen and one of the major causes of clinical and subclinical mastitis in dairy animals and cows, which are a significant production factor in the dairy industry in Pakistan. The objectives of this study were to find out the abundance of *E. coli* in the raw milk of mastitis-infected cows in seven large dairy-producing districts in Punjab, Pakistan, and to assess the antimicrobial resistance profiles of the isolates. Around 235 samples were collected from mastitis-infected cows in 7 Punjab districts namely Lahore (n=34), Sheikhupura (n=37), Kasur (n=30), Nankana Sahib (n=42), Gujranwala (n=31), Sialkot (n=29) and Narowal (n=32). *E. coli* was confirmed via PCR in 39 out of 235 (16.6%) samples. District wise prevalence were Lahore (17.64%), Sheikhupura (18.92%), Kasur (20%), Nankana Sahib (19.04%), Gujranwala (16.12%), Sialkot (10.34%), and Narowal (12.5%). The disk diffusion method was used to test the antimicrobial susceptibility of confirmed isolates (n=39) against commonly used antimicrobial agents. All isolates displayed high resistance to ampicillin (72%), tetracycline (51%), streptomycin (49%), ceftriaxone (46%), and trimethoprim/sulfamethoxazole (46%). It is worth noting that meropenem and chloramphenicol (100% susceptibility) did not show any resistance, and only 3 % were colistin resistant. *E. coli* causes a significant number of cases of bovine mastitis in Punjab, Pakistan with moderate incidence and alarming rates of MDR, specifically among the most widely used β -lactams, tetracyclines, and aminoglycosides. The susceptibility to the reserve antibiotics such as meropenem and chloramphenicol shows opportunities of treatment, meanwhile highlighting the urgency of antimicrobial stewardship, farm hygiene measures, regular surveillance, and One Health strategy to reduce AMR spread and zoonotic risks in the dairy industry.

Introduction

Mastitis is a highly endemic disease in dairy cattle and buffaloes, that poses a serious risk to livestock welfare globally and leads to reduced milk production, increased operational costs, and the need for therapeutic measures

operational costs, and the need for therapeutic measures such as culling, collectively contributing to significant economic losses (Qiu et al., 2025). The disease negatively affects animal health, presenting symptoms such as udder

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inflammation and swelling, changes in the color and texture of milk, elevated body temperature, and general weakness. Pathogenic microorganisms that cause mastitis in dairy cows include bacteria, fungi, viruses, and mycoplasma, with bacteria being the most important (Dalanezi et al., 2020). Milk and dairy products are crucial sources of protein in lower-middle-income countries (LMICs). Buffaloes are the world's second-largest milk producers, accounting for 12% of global milk production, primarily from Pakistan (68%) and India (53%) nationally (Chowdhury et al., 2025; Preethirani et al., 2015). Pakistan's total buffalo milk production is estimated to be 7.27 million metric tons per year (Uddin et al., 2016). However, subclinical mastitis remains a major constraint, contributing to the decline in per capita milk production in the dairy sector (Preethirani et al., 2015). These symptoms arise from infections caused by pathogenic and opportunistic bacteria, including *Streptococcus* spp., *Staphylococcus* spp., *Escherichia coli*, *Enterococcus* spp., *Klebsiella* spp., and related species (Hoque et al., 2022; Salvador et al., 2012).

The incidence and development of mastitis have changed over recent years owing to variations in the scale and management of the dairy farming industry (Xu et al., 2022). Among the infectious agents of environmental origin that cause mammary infections, *E. coli* is the most common (Goulart & Mellata, 2022). Contagious pathogens are transmitted directly between animals, for example, via handlers or milking equipment, whereas environmental pathogens are ubiquitous in the farm environment and are found in bedding material, water, flies, and the bovine gastrointestinal tract (Orsi et al., 2023). *E. coli* is the most frequently isolated Gram-negative pathogen in mastitis cases. It is a facultative anaerobic, rod-shaped, flagellated, gram-negative bacterium belonging to the Enterobacteriaceae family. It is a normal inhabitant of the healthy human and ruminant gastrointestinal tract, with most strains categorized as non-pathogenic commensals, though some can cause disease (My et al., 2023).

Scientific and efficient detection of mastitis-causing agents is fundamental for diagnosing intramammary infections, enabling effective treatment, preventing recurrent infections, and improving control measures (Rifatbegović et al., 2024). *E. coli* is a leading cause of both subclinical and clinical mastitis. Recently observed key virulence factors in *E. coli* strains isolated from buffalo mastitis milk include Shiga toxins (stx1 and stx2) and extended-spectrum β -lactamase (ESBL)-producing capabilities in multidrug-resistant (MDR) strains (Abdlla et al., 2024; Mannan et al., 2021; Yadav et al., 2023). STEC virulence factors include Shiga

toxins (stx1 and stx2), which increase disease severity; intimin (eae), which facilitates host cell adherence; and hemolysin (hlyA), which promotes cell lysis and invasiveness. Given STEC's implication in food-borne epidemics, it holds significant public health relevance (Algammal et al., 2020).

Antimicrobial resistance (AMR) is a dynamic and multifaceted issue influenced by diverse factors (Benedict et al., 2015). *E. coli* serves as a key indicator for monitoring AMR in animals due to its role as a gut commensal, occasional pathogen, and reservoir for antibiotic resistance genes, which it can transfer to other bacteria (Sjölund et al., 2008). AMR in *E. coli* arises from selective pressure in various environments and host species (Ibrahim et al., 2016). Strains in animals and humans show resistance to antibiotics such as tetracyclines, aminoglycosides, phenicols, streptomycin, erythromycin, carbapenems, cephalosporins, sulfonamides, and β -lactams (Amézquita-López et al., 2016). Livestock can serve as sources of antibiotic-resistant *E. coli* in humans, with environmental contamination occurring via farm waste disposal and manure application (Manyi-Loh et al., 2018). ARGs in human pathogenic *E. coli* may resemble those in environmental dairy farm *E. coli* (e.g., from cow manure), suggesting transfer between environments and clinical pathogens (Rehman et al., 2017; Shoaib et al., 2025).

The purpose of current study was to determine the prevalence and isolation rate of *E. coli* in raw milk samples collected in seven major dairy producing districts of Punjab, Pakistan, with the help of the traditional biochemical tests and molecular methods based on the amplification of 16S rRNA gene. The antimicrobial susceptibility profile of the identified *E. coli* isolates against 12 regularly used antibiotics was analyzed to understand the magnitude and patterns of antimicrobial resistance of these mastitis-related strains.

Materials and Methods

Study Area and Sample Collection

A total of 235 raw milk samples from the mastitis-infected cows were randomly aseptically collected from 7 major dairy-producing districts of Punjab, Pakistan, namely Lahore (n=34), Sheikhpura (n=37), Kasur (n=30), Nankana Sahib (n=42), Gujranwala (n=31), Sialkot (n=29), and Narowal (n=32).

After the cow's teats were disinfected with 0.5% iodine solution and dried with a clean paper towel 25-mL milk sample was taken, and four to five foremilk streams were drawn before collection of sample into a sterile tube/bottle.

For the experiment, all milk samples were kept at 4°C and transported to the laboratory within 24 hours of the collection. For this study, all the experimental procedures used were approved by the Animal Care Advisory Committee at The Islamia University, Bahawalpur.

E.coli isolation and identification

For enrichment of *E. coli*, 25 mL of each raw milk sample was aseptically added to 225 mL of Tryptic Soy Broth (TSB). The mixture was then incubated for 16 h while being shaken at 150 rpm, and a loopful of broth was streaked onto Eosin Methylene Blue (EMB) and blood agar. Then, the purified presumed *E. coli* isolates were identified by observing their hemolysis and colony morphology, which appeared small green metallic sheen, with smooth, neat edges and moist surfaces.

Gram staining and biochemical identification

All the colonies were sub-cultured onto nutrient agar slants at 37°C for 16h, and then used for biochemical identification and Gram’s staining. Under the microscope, isolated *E. coli* were seen as short and pink rods. Biochemical tests including citrate utilization, Voges-Proskauer, methyl red, catalase, indole and oxidase were performed. All the suspected isolates were further confirmed as *E. coli* via 16S rRNA sequencing by PCR. The verified isolates were preserved in a tryptic soy broth medium with 10% glycerol at -80°C (Yu et al., 2020).

Molecular identification of E. coli

E.coli isolates were molecularly confirmed using polymerase chain reaction (PCR) with ECO-F and ECO-R primers that target the 16S rRNA gene, as indicated in Table 1. PCR is commonly employed for the specific identification of *E. coli*. Boiling was used to extract genomic DNA. A loopful of bacterial culture was suspended in 50 µL of sterile distilled water, heated for five minutes at 100°C, and then centrifuged for five minutes at 12,000 rpm. For the PCR, used 5 microliters of the supernatant. Molecular confirmation was performed by targeting the 16S rRNA gene.

Table 1. Primer sequence targeting 16S rRNA gene

Gene	Primer Sequence (5’ -3’)	Amplicon Size (bp)	Reference
ECO-F	GACCTCGGTTTAGTTCACAGA	585	(Tonu et al., 2012)
ECO-R	CACACGCTGACGCTGACCA		

The PCR reaction was carried out in a 20 µl volume, including 0.5 µl of forward primer, 0.5 µl of reverse primer, 10 µl of Master mix (2x), 2 µl of DNA template and 7 µl of Nuclease free water. Initial denaturation at 95 °C for 3 minutes was followed by 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 58 °C for 45 seconds, extension at 72 °C for 1 minute, and a final extension step at 72 °C for 3 minutes.

Antimicrobial Susceptibility Test

After the confirmation of the isolates by PCR, the antibiotic susceptibility test was done using the disk diffusion method (Bauer et al., 1966). The test was done to determine the antibiotic resistance of *E. coli* against the following antibiotics: Ampicillin (AMP, 10µg), Ceftriaxone (CRO, 30µg), Meropenem (MEM, 10µg), Gentamicin (CN, 10µg), Streptomycin (STR, 10µg), Nalidixic acid (NA, 30µg), Ciprofloxacin (CIP, 5µg), Trimethoprim/Sulfamethoxazole (TMP/SMX, 1.24µg/23.75µg), Colistin (10µg), Azithromycin (AZM, 5µg), Chloramphenicol (CHP, 30µg), and Tetracycline (TE, 30µg). Tryptic Soy Broth (TSB) was used to purify cultures of *E. coli* at 37°C overnight.

The concentration was adjusted to 0.5 McFarland turbidity. To prevent the overlapping zones of inhibition, antibiotic discs were plated on the surface of the inoculated plate at a distance according to the Clinical and Laboratory Standards Institute guidelines (Wayne, 2014). The results were interpreted after plates were incubated at 37°C for 24 h. Based on the zone diameter standards from the Clinical and Laboratory Standards Institute or antimicrobial disk manufacturer instructions, the results were classified as sensitive, intermediate, and resistant.

Statistical analysis

The data were analyzed using the Chi-square test to assess the occurrence of *E. coli* in different districts of Punjab, Pakistan. A *p-value* < 0.05 was considered positive.

Results

E. coli isolation from raw milk samples

Out of the 235 raw milk samples from mastitis-infected cows, 39 were positive for *E. coli* (Table 2). Six *E. coli* isolates were isolated from 34 samples in Lahore. Seven *E. coli* isolates were isolated from 37 samples in Sheikhpura. Six *E. coli* isolates were isolated from 30 samples in Kasur, which was the highest isolation rate (20%) from among the 7 major dairy-producing districts of Punjab. Eight *E. coli* isolates were isolated from 42 samples in Nankana Sahib. Five *E. coli* were from 31 samples in Gujranwala. Three were from 29 samples in Sialkot, and four were from 32 samples in Narowal.

Table 2. Isolated *E. coli* from different sources

Districts of Punjab	Total No. of samples	Collected samples	Positive <i>E. coli</i> isolates	Percentage
Lahore	n=235	34	06	17.64%
Sheikhupura		37	07	18.92%
Kasur		30	06	20%
Nankana Sahib		42	08	19.04%
Gujranwala		31	05	16.12%
Sialkot		29	03	10.34%
Narowal		32	04	12.5%

Gram Staining and Biochemical Identification

Gram staining and biochemical identification were performed on 235 samples for confirmed *E. coli* isolates. After Gram staining under a light microscope, 39 isolates were shown as short, pink rods, which confirmed the Gram-negative *E. coli*. Based on these features, the isolates were presumed to be *E. coli* which were chosen for further phenotypic and biochemical identification. Their results are shown in Table 3.

Table 2. Biochemical tests for confirmed *E. coli* isolates

Biochemical tests	Results
Catalase	+
Oxidase	-
Indole	+
Methyl red	+
Voges-Proskauer	-
Citrate	-
Sugar Fermentation (Glucose, lactose, and sucrose)	+

Molecular confirmation via PCR

DNA extraction was performed for the isolates by the Simple Boiling method. 39 isolates of *E. coli* were positive for genomic DNA. PCR amplification targeting the 16S rRNA gene produced a 585 bp specific band in all tested isolates, confirming their identity as *Escherichia coli*. (Tonu et al., 2012).

Antimicrobial susceptibility test

AST was performed on 39 samples using Kirby Bauer method (Bauer et al., 1966), according to the Clinical and Laboratory Standards Institute guidelines (Wayne, 2014). These samples were tested against 12 antibiotics that are commonly used in human and veterinary medicines (Table 4).

High resistance rates were observed in Ampicillin (72%), Tetracycline (51%), Streptomycin (49%), Trimethoprim/Sulfamethoxazole (46%), and Ceftriaxone (46%). Meanwhile, Meropenem and Chloramphenicol show no resistance and were considered highly sensitive for *E. coli*, and only 3% resistance to Colistin and Meropenem.

Discussion

The study critically describes the prevalence, isolation, and antimicrobial resistance (AMR) trends of *Escherichia coli* in raw milk of mastitis-affected cows in seven large dairy producing districts in Punjab, Pakistan. Using a multi-tiered strategy of phenotypic, biochemical and molecular identification techniques, *E. coli* was confirmed in 39 out of 235 samples with an overall prevalence of 16.6%. The total isolation rate stood at 16.6 % (39/235 samples) while the district wise occurrence ranges between 10.34-20 %. These observations are in line with previous reports that have also identified *E.coli* as an environmental pathogen most prevalent in bovine mastitis, especially in areas with intensive dairy production (Goulart & Mellata, 2022; Orsi et al., 2023). It is possible that the environmental determinants, including the poor hygiene of bedding materials and manure

Table 4. Resistance pattern of *E. coli* against antibiotics

Antibiotics used	Resistant (%)	Intermediate (%)	Sensitive (%)
Ampicillin	72%	15%	13%
Ceftriaxone	46%	0%	54%
Meropenem	0%	0%	100%
Gentamicin	3%	31%	67%
Streptomycin	49%	31%	21%
Nalidixic acid	26%	18%	56%
Ciprofloxacin	21%	28%	51%
Trimethoprim/Sulfamethoxazole	46%	0%	54%
Colistin	3%	0%	97%
Azithromycin	26%	0%	74%
Chloramphenicol	0%	0%	100%
Tetracycline	51%	8%	41%

handling which allow the opportunistic pathogens to spread between the farm environment and the udder, cause the high prevalence in the districts, including Kasur and Nankana Sahib (My et al., 2023). On the other hand, reduced rates in Sialkot and Narowal could be an indication of better farm management or changes in herd density which still needs additional epidemiological research to ascertain such relationships. Comparative to the literature on other low- and middle-income countries (LMICs) in terms of prevalence of subclinical mastitis, including Bangladesh and India, where it is common to find the prevalence of isolated *E. coli* in buffaloes and cows to be over 20-30 % (Chowdhury et al., 2025; Preethirani et al., 2015). The colony morphology in EMB agar (green metallic sheen) and blood agar (non-hemolytic growth) and positive catalase, indole, methyl red and carbohydrate fermentation findings corresponded to the typical *E. coli* phenotype. These isolates were further identified at the molecular level by PCR amplifying the 16S rRNA gene gave a 585-bp amplicon that is more specific than the traditional methods when it comes to isolation (Tonu et al., 2012). This multi-level identification is essential in proper diagnosis in the mastitis case as it reduces false positives due to morphologically similar Enterobacteriaceae and gives specific interventions (Rifatbegović et al., 2024).

The misuse and overuse of antibiotics on livestock farmers in Pakistan has contributed to the rise of AMR rates and this has been worsened by the lack of an

effective surveillance system which translates to lack of sufficient data. One of the most significant findings that we cannot overlook is the AMR profile of the isolated *E. coli* strains, which were highly resistant to various types of generally used antibiotics. It is important to note that the resistance to ampicillin (72 %), tetracycline (51 %), streptomycin (49 %) and ceftriaxone and trimethoprim/sulfamethoxazole (46 % each) are alarming indicators of multidrug resistance (MDR). These findings support the worldwide trends of *E. coli* in the dairy setting, where β -, tetracycline-, aminoglycoside-, and sulfonamide-resistance is progressively becoming more common under the influence of excessive veterinary medicine application (Amézquita-López et al., 2016; Ibrahim et al., 2016; Manyi-Loh et al., 2018). The high rate of resistance to ampicillin and ceftriaxone can be attributed to the high usage of the antibiotics for the treatment of mastitis in Pakistan, which provides a selective pressure and propagates the spread of resistance genes like those that encode extended-spectrum β -lactamases (ESBLs) (Abdlla et al., 2024; Mannan et al., 2021; Yadav et al., 2023). Likewise, high tetracycline and streptomycin resistance is consistent with their widespread use in livestock feed and prophylaxis, which induces horizontal gene transfer in commensal *E. coli* groups (Rehman et al., 2017; Sjölund et al., 2008). Conversely, a 100 % sensitivity to meropenem and chloramphenicol and 97 % sensitivity to colistin indicate that these antibiotics will continue being viable treatment

methods but their careful use is necessary to prevent the development of resistance. MDR strains in raw milk represent serious threats to the community, since *E. coli* is capable of harboring transferable ARGs, which can then infect the food chain and cause human infections (Benedict et al., 2015; Shoaib et al., 2025). It is especially concerning in LMICs like Pakistan, where raw milk use are the primary sources of protein and sources of zoonotic transfers of resistant pathogens, including Shigella toxin causing *E. coli* variants (STEC) (Algammal et al., 2020; Hoque et al., 2022).

The spread of resistance could be reduced by means of strategies like programs of antimicrobial stewardship, farm hygiene and surveillance systems similar to the GLASS of WHO (Kasanga et al., 2024). In Pakistan, the dependency on antimicrobials might be decreased by implementing the regulation of antibiotic usage in veterinary practice and promoting alternatives like vaccines or probiotics according to the National Action Plans on AMR.

Conclusion

E. coli is the predominant cause of bovine mastitis in Punjab Pakistan, and isolates of *E. coli* (17%) demonstrate extensive resistance to antimicrobials used routinely. These results confirm the relevance of the necessity of enhanced hygienic practice, routine surveillance of dairy farms, and rational use of antibiotics. One Health under alternative approaches to mitigate the emergence of resistance and safeguard the health of animals and human beings involves the adoption of alternative mastitis management measures and antimicrobial stewardship programs.

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Conflict of Interest

There is no conflict of interest by the authors.

Authors' Contribution

Conceptualization, Iqra Rafiq; Writing—original draft preparation, Amna Adnan, Muhammad Hassaan Hamza; Writing—review and editing, Amna Adnan, Muhammad Hassaan Hamza, Iqra Rafiq, Mengping Luo; All authors

have read and agreed to the published version of the manuscript

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