RESEARCH ARTICLE

Molecular Characterization and Antimicrobial Resistance of *Escherichia coli* Isolated from Mastitis in Dairy Animals

Akash A. Bothikar*, Prashant P. Mhase, Pranita G. Manmod, Uma M. Tumlam, Prashant D. Pawar, Sameer N. Jadhav

Abstract

The present study investigated the prevalence of bovine mastitis and antimicrobial resistance patterns of *Escherichia coli* isolates from dairy animals in Western Maharashtra. A total of 400 lactating cows were screened for mastitis using the California Mastitis Test (CMT) and cultural examination. Of these, 210 (52.50%) animals were confirmed positive, comprising 19.52% (n=41) clinical and 80.47% (n=169) subclinical mastitis cases. Bacteriological analysis revealed *Staphylococcus* spp. as the predominant pathogen (40.95%), followed by other Gram-positive bacteria, while *E. coli* accounted for 22.85% (48/210) of the isolates. Antimicrobial susceptibility testing of all bacterial isolates demonstrated highest sensitivity to Amikacin (88.09%) and Ciprofloxacin (82.38%), whereas marked resistance was observed against Gentamicin (69.52%) and Ampicillin-Sulbactam (71.42%). Among *E. coli* isolates, high susceptibility was recorded for Amikacin (89.58%) and Cefotaxime-Clavulanic acid (83.33%), while resistance to Gentamicin was observed in 68.75% of isolates. MIC_{50} findings corroborated the disc diffusion results. Molecular confirmation using 16S rRNA PCR yielded a specific 401 bp amplicon. Resistance gene profiling revealed a high prevalence of *bla*TEM (70.83%), *tet*A (64.58%), *aac*(3)-IV (62.50%), *qnr*A (52.08%), and *bla*SHV (6.25%). Multidrug resistance was detected in 53.30% of all isolates and 22.91% of *E. coli* isolates, with common gene combinations including *bla*TEM+*tet*A (45.83%) and *bla*TEM+*qnr*A (35.41%). The study highlights the alarming emergence of multidrug-resistant *E. coli* in bovine mastitis and underscores the need for routine antimicrobial surveillance and rational antibiotic use in dairy farms.

Key words: Antimicrobial resistance, Bovine mastitis, *Escherichia coli*, MDR, Resistance genes

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Introduction

Bovine mastitis is an inflammatory condition of the mammary gland and remains one of the most economically important diseases affecting dairy animals. The disease results in reduced milk yield, altered milk composition, increased treatment costs, and premature culling. Mastitis occurs in clinical and subclinical forms, with subclinical mastitis being more prevalent and economically significant due to the absence of visible clinical signs (Youssif *et al.,* 2021; Alfakian *et al.,* 2023). Although Gram-positive bacteria, particularly *Staphylococcus aureus*, are traditionally regarded as the major mastitis pathogens, Gram-negative bacteria such as *Escherichia coli* have emerged as important causative agents. *E. coli* is an environmental pathogen capable of causing severe inflammatory reactions and systemic illness in dairy cattle (Abed *et al.,* 2021).

The indiscriminate use of antibiotics in dairy farming has led to the emergence of antimicrobial-resistant bacteria. Resistance genes such as *bla*TEM, *bla*SHV, *tet*A, *aac*(3)-IV, and *qnr*A confer resistance to β-lactams, tetracyclines, aminoglycosides, and quinolones, respectively, posing serious challenges for mastitis treatment and public health (Kour, 2016; Subhi *et al.,* 2023). Western Maharashtra is a major dairy-producing region; however, information on antimicrobial resistance patterns in mastitis-associated *E. coli* is limited. Therefore, the present study was undertaken to determine mastitis prevalence, identify bacterial pathogens, evaluate antimicrobial susceptibility patterns, and detect resistance genes among *E. coli* isolates.

Department of Veterinary Microbiology, KNP College of Veterinary Science, Shirwal, Satara- 412801, MAFSU, Maharashtra, India.

Corresponding Author: Dr. Bothikar Akash A., Department of Veterinary Microbiology, KNP College of Veterinary Science, Shirwal, Satara-412801, MAFSU, Nagpur, Maharashtra, India. e-mail: akashbothikar1111@gmail.com

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Materials and Methods

Sample Collection

A total of 400 milk samples were collected aseptically from lactating cows from organized and unorganized dairy farms in Western Maharashtra. Animals were screened for mastitis using the California Mastitis Test (CMT), and only CMT-positive samples were processed further at the Department of Veterinary Microbiology, KNP College of Veterinary Science, Shirwal, Satara, MAFSU, Maharashtra, India.

Isolation, Identification and Antibiotic Susceptibility Testing of *Escherichia coli*

Milk samples were enriched in nutrient broth and brain heart infusion broth and cultured on MacConkey agar and EMB agar. Lactose-fermenting colonies showing metallic sheen on EMB agar were presumptively identified as *E. coli* and confirmed by Gram staining and biochemical tests, including oxidase, catalase, and IMViC reactions.

Antimicrobial susceptibility testing was performed by the Kirby–Bauer disc diffusion method on Mueller-Hinton agar as per CLSI (2018) guidelines against eight standard routinely used antibiotics alone or in combinations. Minimum inhibitory concentration (MIC) was determined using a resazurin-based microbroth dilution assay.

Molecular Confirmation and Detection of Resistance Genes

Genomic DNA was extracted from confirmed *E. coli* isolates using a commercial DNA extraction kit and hot-cold lysis method according to Sambrook and Russell (2001).

PCR was performed for molecular confirmation of *E. coli* using 16S rRNA gene primers. Resistance genes *bla*TEM, *bla*SHV, *tet*A, *qnr*A, and *aac*(3)-IV were detected using gene-specific primers using conditions specified in the published literature (Table 1).

Results and Discussion

Prevalence and Risk Factors of Mastitis

Out of 400 milk samples screened, 210 (52.5%) were found positive for mastitis using the California Mastitis Test (CMT), and all of these were culture-positive, confirming the reliability of CMT as a rapid diagnostic tool (Sadashiv and Kaliwal, 2013; Kasa *et al.,* 2020). Breed-wise analysis revealed that Holstein Friesian (HF) cattle were most affected (168/210), followed by Gir (16), Jersey (12), and Khillar (11), indicating higher susceptibility of exotic breeds to mastitis, likely due to lower environmental resilience of HF cattle (Jagadeesh *et al.,* 2016) as well as total population of each of these breeds screened.

Bacteriological examination of mastitic milk samples revealed a predominance of Gram-positive organisms with *Staphylococcus* spp. being the most frequently isolated pathogen, accounting for 40.95% of the total isolates, followed by *Bacillus* spp. (9.52%) as shown in Table 2. Among Gram-negative bacteria, *Escherichia coli* constituted 22.85% of the isolates, while other lactose-fermenting organisms such as *Klebsiella* spp. and *Enterobacter* spp. together accounted for 20.47% (Table 2). The dominance of Staphylococcus species corroborated with the earlier reports identifying them as the principal causative agents of bovine mastitis, whereas the substantial presence of environmental Gram-negative bacteria highlights their important role in both clinical and subclinical mastitis cases (Kasa *et al.*, 2020; Janus *et al.,* 2023).

Milk yield, age, and parity were found to significantly influence the prevalence of mastitis, as summarized in Table 3. Cows producing ≥15 L of milk per day showed a higher incidence of mastitis (71%), with 21.93% of these cases associated with *Escherichia coli*, supporting the concept that high-producing animals are more susceptible due to increased metabolic and physiological stress (Fesseha *et al.,* 2021). Age-wise analysis revealed that animals in the 5-7year age group exhibited the highest prevalence of mastitis, which may be attributed to age-related alterations in immune competence (Mourya *et al.,* 2020). Parity also played a significant role, with cows having 4-6 calvings showing the greatest overall mastitis prevalence, whereas animals with ≥7 calvings exhibited fewer mastitis cases but a comparatively higher proportion of *E. coli*-associated infections, indicating that parity influences both disease occurrence and pathogen distribution (Janus *et al.,* 2023).

Table 2: Bacteria isolated from milk samples of dairy animals

Isolated organism	Name of organism	No. of isolates	% Prevalence
Gram-positive bacteria (n=119)	*Staphylococcus* spp.	86	40.95
	Bacillus spp.	20	9.52
	Streptococcus spp.	13	6.19
Gram-negative bacteria (n=91)	*Escherichia coli.*	48	22.85
	Klebsiella spp or *Enterobacter* spp	43	20.47
TOTAL		**210**	**100%**

Table 1: Sequence of primers used for the detection of antibiotic resistance genes (*bla*TEM, *bla*SHV, *qnr*A, *tet*A, and *aac (3)-iv*) in *Escherichia coli*

Name of Primer Gene	Sequence of oligonucleotide (5′ to 3′)	Amplicon Size (bp)	Reference
*bla*TEM	F: TCG CCG CAT ACA CTA TTC TCA GAA TGA R: ACG CTC ACC GGC TCC AGA TTT AT	445	Ombarak *et al.* (2021)
*bla*SHV	F: TCA GCG AAA AAC ACC TTG R: TCC CGC AGA TAA ATC ACCA	472	Karczmarczyk *et al.* (2011)
*tet*A	F: GGCCTCAATTTCCTGACG R: AAGCAGGATGTAGCCTGTGC	372	Ashraf *et al.* (2018)
*qnr*A	F:ATTTCTCACGCCAGGATTTG R:GATCGGCAAAGGTTAGGTCA	516	Vansia *et al.* (2021)
aac(3)-iv	F: CTTCAGGATGGCAAGTTGGT R: TCATCTCGTTCTCCGCTCAT	285	Momtaz *et al.* (2012)

Seasonal trends revealed the highest mastitis incidence during the monsoon (70.47%), consistent with increased environmental contamination and humidity favouring bacterial proliferation (Kurjogi and Kaliwal, 2014).

Antimicrobial Susceptibility of *E. coli* Isolated from Bovine Mastitis

In this study, 48 (*22.85%*) *Escherichia coli* isolates recovered from 210 bovine mastitis milk samples were subjected to antibiotic susceptibility testing using eight antibiotics, commonly used for mastitis treatment in the study region. The results revealed varying degrees of resistance among the isolates (Table 4) Antimicrobial susceptibility testing indicated emerging multidrug resistance. Among 210 isolates, 53.33% of *E. coli* were resistant to three or more antibiotics. Gentamicin and Ampicillin+Sulbactam showed the highest resistance (68.75%), while Amikacin and Cefotaxime+Clavulanate had low resistance rates (6.25%), suggesting their continued therapeutic efficacy. Moderate resistance to Ciprofloxacin (22.91%) and Enrofloxacin (16.66%) highlights the need for judicious antibiotic use (Srinivasan *et al.,* 2007; Emon *et al.,* 2024).

The highest resistance was observed against Gentamicin (Aminoglycoside) and Ampicillin+Sulbactam (beta-lactam) at 68.75%, with 33 isolates resistant to each, indicating that these antibiotics are largely ineffective for treating *E. coli*-associated mastitis in this population. High resistance to beta-lactam antibiotics has been widely reported in bovine mastitis pathogens, primarily due to their excessive and indiscriminate use (Srinivasan *et al.,* 2007; Emon *et al.,* 2024). Moderate resistance was observed for Ciprofloxacin (Quinolone, 22.91%), Amoxicillin+Clavulanate (beta-lactam, 25%), and Enrofloxacin (Quinolone, 16.66%). These findings suggest that while these antibiotics retain some efficacy, emerging resistance could compromise treatment outcomes if usage continues without proper monitoring (Janus *et al.,* 2023).

Conversely, low resistance rates were recorded for Amikacin (Aminoglycoside, 6.25%), Cefotaxime+Clavulanate (Cephalosporin, 6.25%), and Ceftriaxone+Tazobactam (Cephalosporin, 10.41%), indicating that these agents remain largely effective against *E. coli* isolates. The retained susceptibility to aminoglycosides and third-generation cephalosporins aligned with previous reports, suggesting their potential as reliable therapeutic options for mastitis treatment (Srinivasan *et al.,* 2007; Kasa *et al.,* 2020).

The high resistance to Gentamicin in the present study contrasts with some previous reports where aminoglycosides showed moderate susceptibility, highlighting potential regional differences in antimicrobial resistance patterns and the dynamic nature of AMR over time (Srinivasan *et al.,* 2007). Such variations emphasize the necessity of continuous local surveillance to inform empirical therapy and reduce the risk of treatment failure.

Table 3: Influence of milk yield, age and number of parity of animals on the occurrence of mastitis

Risk factor	Level	No. of animals tested	CMT positive Samples	Culturally positive samples		
				Other than *E. coli* (%)	*E. coli* (%)	Total (%)
Milk yield	≥ 15 ltr	220	155 (71.00%)	121 (78.06)	34 (21.93)	155
	<15 ltr	180	55 (30.56%)	41 (74.54)	14 (25.45)	55
Age (year)	2-5	175	96 (54.85%)	79 (82.29)	17 (17.70)	96
	≥6	225	114 (50.66%)	83 (55.26)	31 (27.19)	114
Parity	1-3	165	94 (56.96%)	75 (79.78)	19 (20.21)	94
	4-6	180	107 (59.44%)	87 (81.30)	20 (18.69)	107
	≥ 7	55	09 (16.36%)	0	09 (100)	09
	Total	**400**	**210**	**162 (77.14)**	**48 (22.85)**	**210**

Table 4: Antimicrobial resistance pattern of *Escherichia coli* (n=48) isolates from mastitis in dairy animals

Class of antibiotic	Antibiotic	Concen-tration	S	I	R	Rate of resistance (%)
Quinolones	Enrofloxacin	30 mcg	34	6	8	16.66
	Ciprofloxacin	30 mcg	32	5	11	22.91
Aminoglycosides	Gentamicin	50 mcg	14	1	33	68.75
	Amikacin	30 mcg	43	2	3	6.25
Beta–lactam	Amoxycillin+clavum	30+10 mcg	23	13	12	25
	Ampicillin+Sulbactum	30+15 mcg	9	6	33	68.75
Cephalosporin	Ceftriaxone+Tazobactum	80+10 mcg	38	5	5	10.41
	Cefotaxim+Clavum	30+10 mcg	40	5	3	6.25

Table 5: Antimicrobial resistance gene in *Escherichia coli* isolates (n=48) from mastitis in dairy animals

Antibiotic Resistance gene	*bla*TEM No.(%)	*tet*A No.(%)	*bla*SHV No.(%)	*qnr*A No.(%)	*aac(3)*-iv No.(%)
No. of isolates positive	34 (70.83)	31 (64.58)	04 (8.33)	25 (52.08)	30 (62.5)
No. of isolate negative	14 (29.16)	17 (35.41)	44 (91.66)	23 (47.91)	18 (37.5)

The growing prevalence of multidrug-resistant *E. coli* in bovine mastitis presents a significant challenge for the dairy industry, contributing to chronic and recurrent infections. These findings reinforce the importance of judicious antibiotic use, routine susceptibility testing, and exploring alternative treatment strategies such as nanotechnology or immunotherapy to mitigate the escalating threat of antimicrobial resistance (Fesseha *et al.,* 2021; Emon *et al.,* 2024).

Molecular Characterization of *E. coli* Isolated from Bovine Mastitis

In molecular characterization of *Escherichia coli* isolated from bovine mastitis the presence of *E. coli* was confirmed by observing a single band of 401 bp, corresponding to the expected size compared with a 100 bp DNA ladder (Sambrook and Russell, 2001). In the present study, all 48 milk samples examined showed a band at 401 bp (Fig. 1), indicating that all samples were positive for *E. coli* isolates.

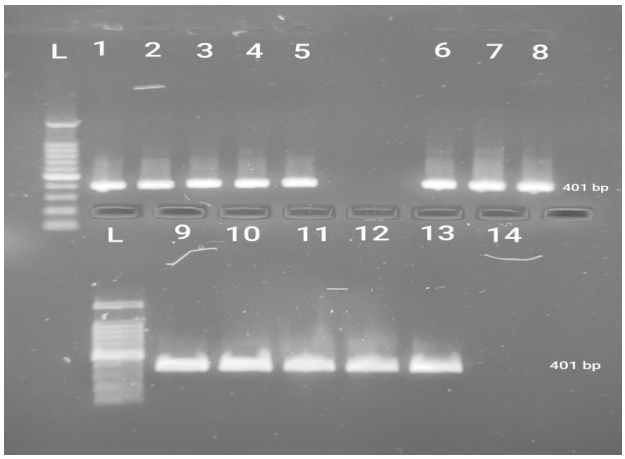

Fig. 1: Polymerase chain reaction species specific detection of *Escherichia coli* isolated from mastitis milk samples by 16sRNA

Detection of Antibiotic Resistance Genes (AMR Genes)

A total of 48 *E. coli* isolates, confirmed through genus-specific PCR, were screened for antibiotic resistance genes using PCR. The distribution of AMR genes in these isolates and their specific gene amplification PCR with band size is summarized in Table 5 and Figures 2-6. The *bla*TEM gene, which confers resistance to beta-lactam antibiotics, was detected in 70.83% of the isolates. This prevalence was slightly lower than the 100% positivity reported in previous studies by Youssif *et al.* (2021) and Alfakian *et al.* (2023), but still indicates significant potential for beta-lactam resistance in *E. coli* strains causing bovine mastitis.

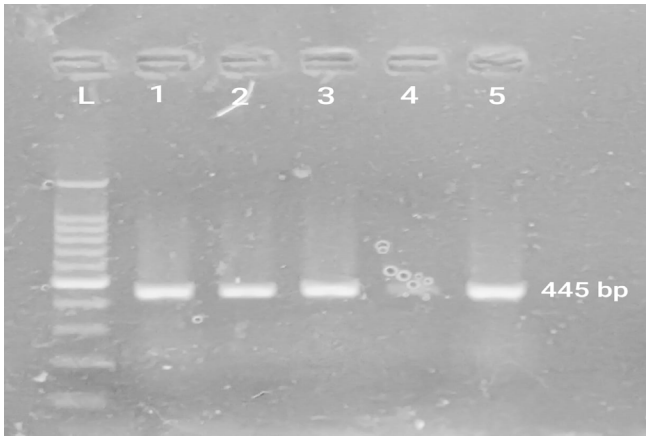

Fig. 2: PCR amplification product of *bla*TEM gene (445 bp) in *E. coli* isolated from mastitis milk samples

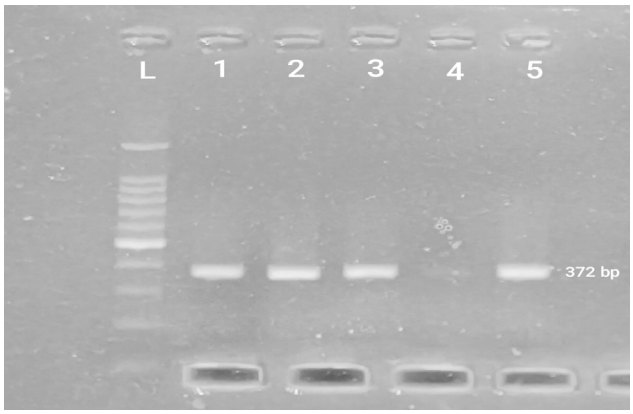

Fig. 3: PCR amplification product of *tet*A gene (372 bp) in *Escherichia coli* isolated from mastitis milk samples

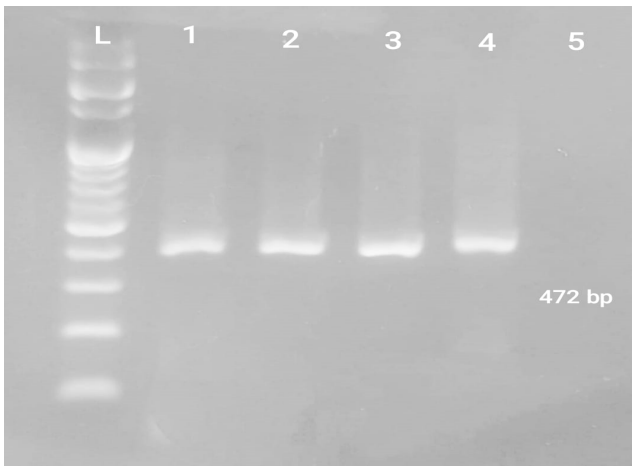

Fig. 4: PCR amplification product of *tet*A gene (472 bp) in *Escherichia coli* isolated from mastitis milk samples

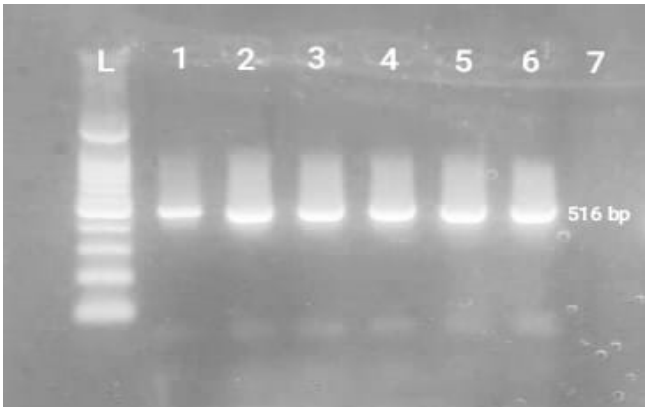

Fig. 5: PCR amplification product of *qnr*A gene (516 bp) in *Escherichia coli* isolated from mastitis milk samples

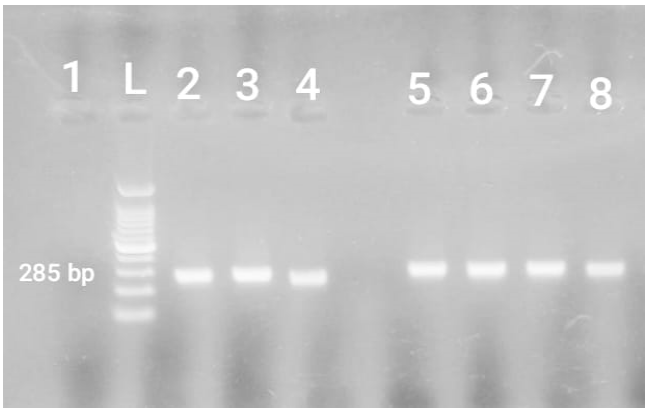

Fig. 6: PCR amplification product of *aac(3)*-iv gene (285 bp) in *E. coli* isolated from mastitis milk samples

The *tet*A gene, associated with tetracycline resistance, was found in 64.58% of isolates. Previous studies reported varying prevalence: Maynard *et al.* (2003) found 25%, while Abed *et al.* (2021) and Youssif *et al.* (2021) reported 20% in multidrug-resistant *E. coli* isolates. The high prevalence of *tet*A in the current study indicates that tetracycline resistance remains a major concern.

The *bla*SHV gene, responsible for extended-spectrum beta-lactam resistance, was present in 6.25% of isolates. This is consistent with Subhi *et al.* (2023), who reported 14% prevalence, suggesting that extended-spectrum beta-lactam resistance occurs at lower levels but is still present in *E. coli* from bovine mastitis.

The *qnr*A gene, a marker for quinolone resistance, was detected in 52.08% of isolates. This finding aligned with Alfakian *et al.* (2023), who reported 50% prevalence, highlighting the potential for quinolone resistance in these strains.

The *aac*(3)-IV gene, which confers resistance to aminoglycosides, was observed in 62.5% of isolates. This was higher than the 26.92% prevalence reported by Kour (2016), indicating a significant contribution of aminoglycoside resistance in the isolates studied.

The remaining isolates were negative for these genes as follows: 29.16% for *bla*TEM, 35.41% for *tet*A, 93.75% for *bla*SHV, 47.91% for *qnr*A, and 37.5% for *aac*(3)-IV (Table 5). These findings highlight the presence of multiple resistance mechanisms in *E. coli* isolates from bovine mastitis, representing significant challenges in antibiotic therapy. The high prevalence of *bla*TEM and *tet*A genes suggests considerable resistance to beta-lactams and tetracyclines in these isolates.

Conclusion

The study revealed a high prevalence of bovine mastitis in Western Maharashtra, with subclinical mastitis being the dominant form. *Escherichia coli* constituted a significant proportion of mastitis pathogens and exhibited high levels of antimicrobial resistance. The detection of multiple resistance genes highlights the urgent need for rational antibiotic use, routine antimicrobial susceptibility testing, and continuous AMR surveillance to control the spread of multidrug-resistant *E. coli* in dairy herds.

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