Research Article

Assessment of Coliform Contamination in Sheep Milk: A Public Health Perspective

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Abstract | Accurate and prompt detection of coliform bacteria is essential for evaluating environmental contamination and managing bovine mastitis. This study investigated the presence of coliforms in milk samples using both VITEK 2 and standard microbiological techniques. Out of 78 clinical mastitic milk samples, 53 (67.95%) tested culture-positive, with 22 isolates (41.51%) identified as coliforms. In contrast, among 274 subclinical mastitic milk samples, 41 (14.96%) were culture-positive, and only 3 isolates (7.32%) were coliforms. *Escherichia coli* was the most prevalent coliform (56.52%), followed by *Klebsiella pneumoniae* (26.09%), *Enterobacter cloacae* complex (13.04%), and *Klebsiella aerogenes* (4.35%). A statistically significant difference (P<0.05) was observed between clinical and subclinical cases, emphasizing a higher prevalence of coliforms in clinical mastitis. *E. coli* was identified as the dominant pathogen, underscoring the need for pathogen-specific treatment strategies especially considering the rise in antimicrobial resistance. Antimicrobial susceptibility testing revealed complete resistance (100%) to neomycin, tetracycline, doxycycline, azithromycin, and clindamycin. In contrast, gentamicin, ciprofloxacin, and trimethoprim/sulfamethoxazole demonstrated full efficacy (100% sensitivity). These findings confirm the significant role of coliform bacteria, particularly *E. coli* as environmental pathogens contributing to acute clinical mastitis. The antimicrobial resistance results raise serious concerns for regulatory bodies due to the reduced efficacy of several antimicrobials that were previously effective.

Keywords | Coliform mastitis, Clinical mastitis, Environmental pathogens, Ovine milk, Prevalence

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INTRODUCTION

Mastitis is an inflammatory infection of the udder, primarily caused by pathogens such as *Escherichia coli*, *Klebsiella* spp., and *Staphylococcus aureus* (Sharun *et al.*, 2021b; Киркимбаева *et al.*, 2022c). It manifests in both clinical and subclinical forms, with the latter often going undetected yet playing a significant role in the transmission of infection within a herd (Fthenakis, 2019). Mastitis results in considerable reductions in milk yield and adversely affects lamb health, posing a major management challenge in sheep production systems (Anueyiagu *et al.*, 2020b).

Microbial contamination in raw milk exhibits seasonal variability, with higher bacterial loads typically observed during the summer months (Abbas *et al.*, 2018). Common causative agents also include *Mannheimia haemolytica* and *Streptococcus* spp. (Tassi *et al.*, 2024; Dalaka *et al.*, 2022). Accurate and timely diagnosis is critical for effective mastitis control. However, conventional culture-based methods are often limited by low sensitivity and slow turnaround times (Mitter *et al.*, 2023), and many environmental microbes remain unculturable due to specific growth requirements (Dione *et al.*, 2015).

Modern diagnostic techniques, such as PCR, enable rapid and precise pathogen detection (Algrooni *et al.*, 2024), while innovative tools like respirometric sensors offer non-contact monitoring of coliform bacteria in milk (Abdallah *et al.*, 2025). The advent of MALDI-TOF MS has further revolutionized microbial profiling (Chen *et al.*, 2021; Nonnemann *et al.*, 2019b), and automated systems like VITEK enhance diagnostic accuracy and efficiency. Nonetheless, integration with traditional microbiological methods remains essential for comprehensive diagnostics (Emad *et al.*, 2024). Additionally, somatic cell count (SCC) and differential somatic cell count (DSCC) analyses are valuable tools in identifying subclinical mastitis infections (Fonseca *et al.*, 2024).

This study was conducted using microbiological and VITEK 2 methods to determine the prevalence and diversity of coliform bacteria strains in raw sheep milk and assess their antibiotic resistance patterns.

MATERIALS AND METHODS

Chemicals and reagents

Bacteriology media were used: Nutrient agar, MacConkey agar, Müller-Hinton agar, blood agar media base, Eosin methylene blue (EMB), MR-VP broth, and Pepton water were purchased in powder form from Accumix –Spain, Scharlau-china, HIMEDIA-India, OXOID-England, Liofilchem-Italy, and Salucea-dutch. The media for culture were prepared according to instructions of the manufacturer the sterilized by autoclaved for 15 minutes at 15 lbs pressure and 121°C, in the case of blood media for the preparation of 5% sheep blood agar media, cooled the media after sterilization to 45–50°C, 5 ml of aseptically collected with EDTA tube blood was added to each 100 ml of media prepared. For the biochemical test, H_2O_2 was prepared at 3% for the catalase test, 1% of the oxidase reagent (1g in 100 ml D.W) for the oxidase test, Kovac's reagent for the indole test, methyl red,40% KOH (40g in 100 ml D.W), and Alpha-Naphthol, 5% (5 g in 100 ml Absolute Ethanol) for MR-VP test.

Study area and sample collection

The study was conducted in Al-Fallujah city, Iraq, from August 28, 2024, to February 25, 2025, involving different species of lactating ewes aged between 1 and 6 years. The animals were raised in under-resourced rural areas. Notably, ten of the ewes, each approximately three years old, had experienced twin births. The number of total parturitions per ewe ranged from one to four. A total of 352 milk samples were collected in sterile tubes from cases of both clinical (n=78) and subclinical (n=274) mastitis. Prior to sampling, a complete physical examination of each ewe was performed. The udder examination included visual inspection and manual palpation, and any abnormalities or changes in the udder or milk were recorded. Udders and teats were initially washed with tap water, dried, and disinfected by dipping in iodine solution. Teat orifices were further sanitized using 70% ethanol. The initial streams of milk were discarded, and midstream milk samples were aseptically collected into sterile test tubes. Samples were immediately transported to the laboratory in an ice box to maintain sample integrity.

Methods of mastitis diagnosis

California mastitis test (CMT) for subclinical cases

The test designed to detect subclinical mastitis was performed following the guidelines provided by Markey *et al.* (2014). Using a white plastic paddle with four containers, equal volumes of milk and California reagent were gently mixed with two milliliters of milk and two milliliters of reagent. The California Mastitis Test (CMT) was scored as follows: Negative no precipitate forms and the mixture stays liquid; Trace a small precipitate appears that disappears with continued movement of the paddle; + a distinct precipitate without gel formation; ++ the mixture's thickness indicates gel formation; +++ a unique gel forms and agglutinates at the bottom of the paddle.

Laboratory isolation and identification of the coliform bacteria

After sampling and performing the California Mastitis Test (CMT) for subclinical cases, the milk samples were incubated at 37 °C for 4 hours. Subsequently, the samples were cultured on three primary media: Nutrient agar, MacConkey agar, and blood agar, followed by overnight incubation at 37 °C. Pure colonies were then isolated and further incubated overnight under the same conditions. Gram staining was conducted to classify the bacteria, and a series of biochemical tests were performed to identify members of the Enterobacteriaceae family, including the oxidase test, indole test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test, and triple sugar iron test, as described by Markey *et al.* (2014). Additionally, EMB agar was employed as a selective and differential medium. Due to the presence of methylene blue, EMB agar is toxic to Gram-positive bacteria, thereby selectively promoting the growth of Gram-negative bacteria (Tankeshwar and Tankeshwar, 2024).

Antibiotic sensitivity test

This test was performed according to Markey *et al.* (2014) as follows: A bacterial colony was transferred to test tubes containing sterile saline 0.45% NaCl, pH 4.5 to 7.0, then diluted until it reached turbidity equivalent to tube number five of the McFarland solution, which has a bacterial count of 1.5 × 10^8, prepared according to Zapata and Ramirez-Arcos (2015). The suspension of bacteria was spread with

a sterile cotton swab on Mueller-Hinton agar in a grid pattern. The Petri dishes were then left to dry for a few minutes. Some isolations required 10% sheep blood agar for testing. In this study, the antibiotic discs (Bioanalyse/Turkey) used include gentamycin 10 µg, neomycin 10 µg, doxycycline 10 µg, ciprofloxacin 10 µg, azithromycin 15 µg, trimethoprim/sulfamethoxazole 1.25/23.75 µg, and tetracycline 10 µg. Using sterile forceps, the antibiotic disc was placed on the agar surface, ensuring a 24 mm distance between each disc to prevent overlapping inhibition zones. Additionally, there was about 1 cm between the disc and the edge of the Petri dish. The dishes were incubated at 37°C for 24 hours. The inhibition zone diameter for each antibiotic disc (mm) was measured with a Vernier caliper and compared to the standard diameter of the antibiotic inhibition zone listed in CLSI (2023).

Bacterial identification (ID) using the Vitek 2 compact system

According to the manufacturer's instructions (Bio Mérieux-Germany), a sterile swab was used to transfer a sufficient number of colonies from a pure culture and to suspend the microorganism in 3.0 mL of sterile saline (aqueous 0.45% to 0.50% NaCl, pH 4.5 to 7.0) in a 12 x 75 mm clear polystyrene test tube.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) version 2019 was used to analyze the differences between the groups. The chi-square test was applied to compare percentages, with significance levels set at $p \leq 0.05$ and $p \leq 0.01$.

RESULTS AND DISCUSSION

Prevalence of coliform bacteria

A total of 78 clinical mastitis milk samples were analyzed, of which 53 samples (67.95%) were culture-positive. Among these, 22 isolates (41.51%) were identified as coliform bacteria. In contrast, out of 274 subclinical samples, only 41 samples (14.96%) yielded positive bacterial cultures, and a much smaller fraction 3 isolates (7.32%) were confirmed as coliforms (Table 1). The bacterial culture results showed that coliform bacteria typically form smooth, circular colonies that may appear opaque or translucent on blood agar and may exhibit beta-hemolysis, while others remain non-hemolytic. Characteristics of isolates on MacConkey agar are based on lactose fermentation; lactose fermenters like *E. coli* produce pink colonies, while *Klebsiella* produces pink, large, mucoid, and glistening colonies due to polysaccharide capsule production. *Enterobacter* spp. appeared smooth and less mucoid. EMB agar characteristics include that typical *E. coli* colonies exhibit a green metallic sheen due to lactose fermentation and acid production, which precipitates the dyes in the medium. Enterobacter may produce colonies of different colors or lack the sheen, aiding in differentiation. *Klebsiella* forms large mucoid colonies. Microscopically, bacteria stained with the Gram stain appear as short bacilli (Figure 1). Regarding biochemical tests, the findings of all strains of coliform bacteria are summarized in Table 2. Vitek system identification revealed an 82.9% positivity rate, with *E. coli* being predominant at 56.52%, followed by *Klebsiella pneumoniae*, *Enterobacter cloacae* complex, and *Klebsiella aerogenes* at 26.09%, 13.04%, and 4.35%, respectively (Table 3). There was a statistically significant difference ($P < 0.001$), with results showing a higher prevalence of coliforms in clinical mastitis compared to subclinical cases.

Table 1: Summary of sample numbers, positive culture results, and coliform species isolated from clinical and subclinical mastitis milk samples.

Sample type	Samples (n)	Positive in culture	Coliform bacteria (n)	Percentage %
Clinical milk sample	78	53	22[a]	41.51
Subclinical milk sample	274	41	3[b]	7.32
Chi-square test (p-value)	< 0.001**			

**Correlation is highly significant at the 0.05 level. [a-b] Superscript letters indicate the significant differences between proportions.

Table 2: Biochemical tests for coliform bacteria.

Tests*	*E. coli*	*Klebsiella* spp.	*Enterobacter* spp.
Oxidase	-	-	-
Catalase	+	+	+
O/F	F	F	F
Indole	+	-	-
Methyl red	+	-	-
Vogas-proskauer	-	+	+
Citrate	-	+	+
Urease	-	+	-
TSI	A/A/G[#]	A/A/G	A/A/G
KOH test	+	+	+

*TSI: O/F: Oxidation-Fermentation; Triple Sugar Iron test; KOH: Potassium Hydroxide test. [#]A/A/G: acid slant/acid butt/ gas production.

Table 3: Coliform species identified from clinical and subclinical mastitis milk samples.

Bacterial isolates	N	Percentage (%)
Escherichia coli	13[a]	56.52
Klebsiella pneumoniae	6[b]	26.09
Klebsiella aerogenes	1[d]	4.35
Enterobacter cloacae complex	3[c]	13.04
Total	23	100
Chi-square test (p-value)	< 0.001*	

* Correlation is significant at the 0.05 level. [a-b] Superscript letters indicate the significant differences between proportions.

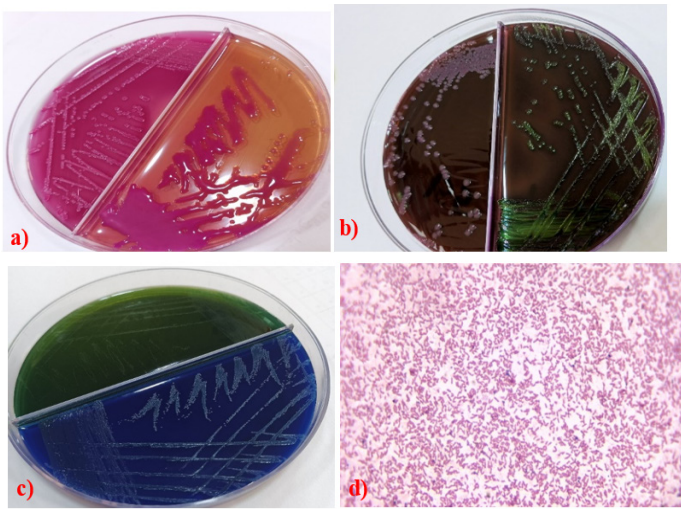

Figure 1: (a) MacConkey agar plate: The left half displays numerous pink colonies of *E. coli*, while the right half shows large, mucoid, and glistening colonies of *Klebsiella* spp. (b) Eosin Methylene Blue (EMB) agar plate: Growth of *Enterobacter* spp. on the left side producing pink colonies, and *E. coli* on the right side exhibiting characteristic green metallic sheen colonies. (c) Simmons citrate agar plate: The left half remains green, indicating *E. coli* (negative citrate utilization), whereas the right half turns blue, indicating positive citrate utilization by *Klebsiella* spp. (d) Microscopic examination (100x oil immersion): Gram-negative short bacilli observed.

Results in Table 1 showed that coliform detection was significantly higher in clinical mastitis cases compared to subclinical ones, both in absolute numbers and relative frequency (41.51% vs. 7.32%). The chi-square test yielded a statistically significant result ($P < 0.001$), indicating a strong association between mastitis type (clinical vs. subclinical) and the presence of coliform bacteria. Coliforms likely play a more prominent role in the pathogenesis of clinical mastitis, possibly due to their endotoxin production and rapid induction of inflammatory responses, whereas their role in subclinical cases appears minimal. High levels of coliforms in sheep milk can lead to spoilage and compromise dairy product quality, underscoring the need for proper handling and sanitation practices (Tonamo *et al*., 2019; Gaya *et al*., 1987). This finding supports the hypothesis that coliforms, particularly *E. coli* and other *Enterobacteriaceae*, are more frequently associated with acute clinical mastitis manifestations, consistent with Rahma (2024). *E. coli* was the predominant isolate, accounting for 13 cases (56.52%), followed by *Klebsiella pneumoniae* with 6 cases (26.09%), *Klebsiella aerogenes* in 1 case (4.35%), and *Enterobacter cloacae* complex identified in 3 subclinical cases (13.04%). The p-value (< 0.001) indicates a highly significant difference in the frequencies of these bacterial isolates (Table 3). These results align with findings reported by Nsaif (2025), Bogdanovičová *et al*. (2016), Sarba *et al*. (2023), Rahma (2024), and Kasa *et al*. (2020). Yu *et al*. (2019) found significant regional differences but no seasonal variation. Notably, in some cases, clinical signs of mastitis associated with coliform bacteria occur without isolation of these bacteria; this may be explained by the presence of *Acinetobacter* spp., which possess multiple contact-dependent inhibition (CDI) systems. These systems use CdiA proteins to bind target bacteria and deliver toxic domains that disrupt cellular functions for example, a toxin from *A. baumannii* can induce DNA damage in *E. coli*, halting its growth (De Gregorio *et al*., 2019; Roussin *et al*., 2019). The significant predominance of these pathogens ($a \neq b + c$) highlights the urgent need for pathogen-specific therapeutic strategies, especially in light of increasing antimicrobial resistance (Espina-Ávila *et al*., 2021).

Susceptibility test results according to the Kirby-Bauer method

The antibiotic sensitivity test results revealed multidrug resistance in *E. coli*, which exhibited 100% resistance to Neomycin, Tetracycline, Doxycycline, Azithromycin, and Clindamycin. High sensitivity was observed for Gentamicin (100%), while Trimethoprim/sulfamethoxazole showed 84.62% resistance, 7.69% intermediate sensitivity, and 7.69% sensitivity. Both *K. pneumoniae* and *K. aerogenes* demonstrated consistent resistance, exhibiting 100% resistance to Neomycin, Tetracycline, Doxycycline, Azithromycin, and Clindamycin. In contrast, all isolates of *Enterobacter cloacae* complex (3/3) showed 100% susceptibility to Gentamicin, Tetracycline, Ciprofloxacin, and Trimethoprim/sulfamethoxazole.

Extreme resistance (100%) was observed against Neomycin, Tetracycline, Doxycycline, Azithromycin, and Clindamycin in *E. coli*, consistent with findings by Mahmood and Ahmed (2021), Hussein *et al*. (2025), and Hammoudi and Hussein (2023), who reported 97.5% resistance to Tetracycline and 80% resistance to Ciprofloxacin. Gentamicin remained highly effective, showing 100% sensitivity, aligning with previous studies indicating 92.5% susceptibility (Mahmood and Ahmed, 2021). Trimethoprim/sulfamethoxazole resistance was high at 84.62%, contrasting with Roșu *et al*. (2024), who found only 2.1% resistance in some strains, suggesting geographical or strain-specific variation. Ciprofloxacin resistance is emerging (15.38% resistant, 69.24% intermediate), likely due to overuse in veterinary medicine.

Uniform resistance was also noted in *K. pneumoniae* and *K. aerogenes*, with both species showing 100% resistance to the same antibiotics as multidrug-resistant *E. coli* (Al-Khfaji *et al*., 2022), highlighting a widespread problem among Gram-negative bacteria isolated from sheep milk. However, Gentamicin, Ciprofloxacin, and Trimethoprim/

sulfamethoxazole were fully effective (100% sensitivity), making them reliable treatment options for *Klebsiella* infections. Extended-spectrum beta-lactamase (ESBL) genes were detected in 16 isolates, representing 17.0% (Klaper *et al*., 2021). According to Liu *et al*. (2022), *Klebsiella* species showed the highest resistance to sulfonamides, followed by tetracyclines, aminoglycosides, and β-lactams, while quinolones and polypeptides exhibited the lowest resistance rates.

E. cloacae complex (ECC) isolates demonstrated excellent sensitivity, with 100% susceptibility to Gentamicin, Tetracycline, Ciprofloxacin, and Trimethoprim/sulfamethoxazole, suggesting minimal resistance development in this species. However, ECC exhibits high resistance rates to third-generation cephalosporins, with ceftriaxone resistance at 39.9% and ceftazidime at 36.7% (Han *et al*., 2025). The uniform susceptibility to other antibiotics makes *E. cloacae* infections easier to manage clinically, contrasting sharply with *E. coli* and *Klebsiella*, and reflecting species-specific resistance patterns. This high resistance may be attributed to indiscriminate antibiotic use and lack of standardized treatment protocols, leading to widespread resistance.

CONCLUSION

This study underscores the higher prevalence of coliform bacteria, particularly *E. coli*, in clinical mastitis cases than in subclinical infections. The isolates exhibited typical cultural, microscopic, and biochemical profiles consistent with those of *Enterobacteriaceae*. This study emphasizes the importance of routine bacteriological screening in clinical mastitis cases to detect coliform pathogens early, which often require different management strategies than contagious pathogens such as *Staphylococcus aureus*. Moreover, the significant difference in coliform prevalence between clinical and subclinical samples aligns with previous studies that reported that coliforms are more commonly implicated in acute, severe mastitis than in chronic or subclinical forms. Gentamicin is the most reliable antibiotic for treating infections caused by these species. Trimethoprim/sulfamethoxazole should be used cautiously (high resistance in *E. coli* but effective in *Klebsiella* spp.). Tetracyclines, macrolides (azithromycin), and clindamycin are ineffective and should be avoided. Emerging resistance to Ciprofloxacin in *E. coli* calls for stricter antibiotic use policies in livestock. Further surveillance is required to track the resistance trends of foodborne bacteria.

Future research should explore genomic resistance mechanisms and alternative therapies to mitigate antimicrobial resistance in dairy herds. Herd management practices should focus on preventing coliform mastitis through improved hygiene and udder health monitoring.

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NOVELTY STATEMENET

The VITEK system plays a crucial role in the diagnosis of coliform bacteria and the identification of specific resistance genes, which is essential for containing the spread of antibiotic-resistant bacteria.

AUTHOR'S CONTRIBUTION

DGF was responsible for sample collection, practical work, and writing the manuscript.
SMAA-K: Contributed by editing and reviewing the manuscript.

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Data availability

Data available upon request.

Generative AI and AI-assisted technology statement

Some AI tools were used and declared.

Conflict of interest

The authors have declared no conflict of interest.

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