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Foodborne Risks Associated with Multidrug-Resistant *Escherichia coli* and *Staphylococcus aureus* Isolated from Milk

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Abstract

Although milk is a vital part of the human diet, foodborne bacteria, especially those with antibiotic resistance, may spread through it. The purpose of this study was to assess the foodborne risks related to *Staphylococcus aureus* and multidrug-resistant (MDR) *Escherichia coli* that were isolated from retail milk samples. 360 milk samples in all were gathered from retail establishments and subjected to conventional bacteriological analysis. In compliance with CLSI recommendations, isolates were identified phenotypically and tested for antimicrobial susceptibility using the Kirby-Bauer disk diffusion method. Polymerase chain reaction (PCR) was used to molecularly detect the antimicrobial resistance genes (*blaTEM, mecA, tetA, and tetK*). *E. Coli* and *S. aureus* were isolated from the analyzed samples at rates of 38.9% and 33.3%, respectively, and co-contamination was found in 18.9% of the samples. While *S. aureus* isolates showed notable resistance to penicillin (85.0%), oxacillin (60.0%), and tetracycline (73.3%), *E. coli* isolates showed high levels of resistance to ampicillin (72.1%) and tetracycline (76.4%). In 63.3% of *S. aureus* isolates and 65.7% of *E. coli* isolates, multidrug resistance was found. *E. coli* isolates had *blaTEM* and *tetA* genes, while *S. aureus* isolates had *mecA* and *tetK* genes, according to PCR analysis. Phenotypic resistance and genotypic determinants were significantly correlated ($p < 0.05$). The results show that MDR foodborne bacteria can be found in retail milk, which could be dangerous for the general public's health. To reduce the spread of antimicrobial resistance across the food chain, dairy production must employ sensible antimicrobial usage, enhanced hygienic standards, and ongoing surveillance.

Keywords: Milk; Foodborne Pathogens; *Escherichia coli*; *Staphylococcus aureus*; Antimicrobial Resistance; Multidrug Resistance

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Introduction

Milk is widely recognized as a nutritionally rich food that supplies essential proteins, lipids, vitamins, and minerals necessary for human health. However, contamination during production, processing, or retail handling can transform milk into a vehicle for foodborne pathogens, posing a significant public health risk [22]. Among the microorganisms frequently associated with dairy-borne infections, *Escherichia coli* and *Staphylococcus aureus* are particularly important due to their capacity to cause illnesses ranging from mild gastrointestinal disturbances to severe systemic diseases [33].

Escherichia coli is a Gram-negative bacterium commonly present in the intestinal microbiota of humans and animals, but certain pathogenic strains, such as O157:H7, are well-known etiological agents of foodborne outbreaks linked to contaminated milk and dairy products [17, 26]. The occurrence of these pathogens in milk is often associated with inadequate hygienic measures during milking and handling, especially within small-scale or informal dairy production systems where sanitation practices may be suboptimal [9, 28].

The emergence of multidrug-resistant (MDR) *E. coli* strains has further intensified these concerns, as many isolates harbor extended-spectrum β-lactamase genes such as *bla* variants, which reduce the efficacy of commonly used antimicrobial agents and complicate therapeutic management

[12, 23]. Consequently, infections caused by resistant strains are more difficult to treat and may lead to prolonged illness and increased healthcare burden.

Similarly, *Staphylococcus aureus* is a Gram-positive pathogen frequently introduced into raw milk through mastitic udders or improper milking hygiene [4]. This organism is capable of producing heat-stable enterotoxins responsible for foodborne intoxications, and the emergence of methicillin-resistant *S. aureus* (MRSA) strains carrying resistance genes such as *mecA* and *blaZ* has become an increasing concern in both veterinary and public health contexts [25]. The dissemination of these resistant strains threatens the effectiveness of standard antimicrobial therapies and highlights the need for continuous monitoring.

The global spread of antimicrobial resistance (AMR) among foodborne pathogens, including *S. aureus* and *E. coli*, has been largely attributed to the excessive and inappropriate use of antibiotics in livestock production systems [7]. Such practices create selective pressure that enables resistant bacteria to survive, multiply, and ultimately disseminate throughout the food chain. In addition to compromising treatment outcomes, MDR bacteria present in milk may act as reservoirs of transferable resistance genes that can be exchanged with other microbial populations and potentially transmitted to humans [10, 32].

The burden of MDR pathogens in milk is particularly alarming in low- and middle-income countries, where the consumption of raw milk is common and regulatory oversight of dairy hygiene may be limited [3]. Several investigations have reported high prevalence of MDR *E. coli* and *S. aureus* in dairy milk, often demonstrating substantial resistance to first-line antibiotics such as penicillin's and tetracyclines [11]. These findings emphasize the urgent need for continuous surveillance of antimicrobial resistance patterns in milk and for the implementation of effective hygienic practices throughout the dairy production and distribution chain to protect consumer health.

Materials and Methods

Study Area and Sample Collection

The presence of multidrug-resistant *Staphylococcus aureus* and *Escherichia coli* in milk meant for human consumption was examined using a cross-sectional investigation. From March to September 2025, 360 milk samples were gathered from retail establishments in the Kingdom of Saudi Arabia's Hafr Al-Batin Province. After being aseptically collected in sterile screw-capped containers, milk samples (about 50–100 mL each) were brought to the lab in an ice box at 4°C and processed within 24 hours of collection.

Isolation and Identification of *Escherichia coli*

For 18 to 24 hours, milk samples were incubated at 37°C after being pre-enriched in buffered peptone water. Eosin Methylene Blue (EMB) agar was used to further subculture lactose-fermenting colonies after enriched samples were streaked onto MacConkey agar. *E. coli* was assumed to be present in colonies that had a distinctive metallic shine. Using common tests such as the Indole, Methyl Red, Voges-Proskauer, Citrate (IMViC) assays, biochemical identification was carried out and verified in accordance with accepted microbiological procedures [15].

Isolation and Identification of *Staphylococcus aureus*

Egg yolk tellurite was added to Baird-Parker agar after milk samples were inoculated onto it, and the mixture was then incubated for 24 to 48 hours at 37°C. We chose and subculture typical black, glossy colonies with transparent halos. The coagulase test, catalase test, Gram staining, and growth on mannitol were used to confirm the suspected *S. aureus* isolates. salt agar, in accordance with accepted bacteriological techniques [24].

Antimicrobial Susceptibility Testing

The Kirby-Bauer disk diffusion method on Mueller-Hinton agar was used to conduct antimicrobial susceptibility testing (AST) in compliance with the standards set out by the Clinical and Laboratory Standards Institute [8].

Representatives of widely used antibiotic classes in both human and veterinary medicine were among the antimicrobial drugs evaluated, including:

- Amoxicillin-clavulanic acid and ampicillin are examples of β-lactams.
- Tetracyclines Gentamicin is one example of an aminoglycoside.
- Ciprofloxacin is one example of a fluoroquinolone.
- Trimethoprim-sulfamethoxazole, or sulfonamides.

Following 18–24 hours of incubation at 37°C, inhibition zone diameters were measured and classified as susceptible, intermediate, or resistant based on breakpoints [8] (Table 1).

Quality Control

To guarantee the precision and dependability of culture media

Table 1: Antimicrobial susceptibility test for *E. coli* and *S. aureus* isolates.

Antimicrobial Agent	Class	Disk concentration	Zone Diameter (nearest whole millimeter)		
			S	I	R
Amoxicillin-clavulanic A (AMX)	Penicillin's (Beta-lactam)	**30 µg**	≥20	15-17	≤19
Ampicillin (AMP)	Penicillin's (Beta-lactam)	**10 µg**	≥29	13-17	≤28
Ciprofloxacin (CIP)	fluoroquinolones	**5 µg**	≥22	16-21	≤15
Gentamycin (GM)	Aminoglycosides	**10 µg**	≥15	13-14	≤12
Penicillin G (P)	Penicillin's (Beta-lactam)	**10 µg**	≥17	12-14	≤16
Sulfa methoxazole-trimethoprim (SXT)	Sulfonamide-trimethoprim	**25 µg**	≥16	11-15	≤10
Oxytetracycline (OX)	Tetracyclines	**5 µg**	≥19	15-18	≤14

S: Susceptible, I: Intermediate, R: Resistance

Table 2: Primers used for PCR detection of antimicrobial resistance genes.

Gene	Target organism	Primer sequence (5′–3′)	Amplicon size (bp)	Reference
bla TEM	*E. coli*	R: CCCCGAAGAACGTTTTC F: ATCAGCAATAAACCAGC	516 bp	[6]
*mec*A	*Staphylococcus aureus*	R: AGTTCTGCAGTACCGGATTTGC F: AAAATCGATGGTAAAGGTTGGC	533 bp	[20]
*tet*A	*E. coli*	R: CTGTCCGACAAGTTGCATGA F: GGTTCACTCGAACGACGTCA	577 bp	[21]
*tet*K	*Staphylococcus aureus*	R: GTAGTGACAATAAACCTCCTA F: GTAGCGACAATAGGTAATAGT	360 bp	[30]

and antimicrobial susceptibility testing protocols, reference strains of *E. Coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were employed as quality control organisms.

Molecular Detection of Antimicrobial Resistance Genes

DNA Extraction

The genomic DNA of verified isolates of *Staphylococcus aureus* and *Escherichia coli* was extracted in accordance with the manufacturer's instructions using a commercial bacterial DNA extraction kit (e.g., QIAamp DNA Mini Kit, Qiagen, Germany). Cell lysis, protein digestion, and DNA purification were performed on the pellets that were obtained from overnight bacterial cultures cultivated in nutrient broth after they had been centrifuged at 10,000 × g for five minutes. A NanoDrop spectrophotometer was used to measure the concentration and purity of extracted DNA, and DNA samples were kept at -20°C until they could be examined further (Table 2).

PCR Amplification of Antimicrobial Resistance Genes

Polymerase chain reaction (PCR) assays were performed to detect selected antimicrobial resistance genes associated with β-lactam, methicillin, and tetracycline resistance.

The following genes were targeted:

- ***bla* genes (e.g., *blaTEM*)** for β-lactam resistance in *E. coli*
- ***mecA*** gene for methicillin resistance in *S. aureus*
- ***tet* genes (*tetA* and/or *tetK*)** for tetracycline resistance in both organisms

PCR reactions were carried out in a total volume of 25 µL, containing:

- 12.5 µL of PCR master mix (2×)
- 1 µL of each forward and reverse primer (10 pmol)
- 2 µL of template DNA
- Nuclease-free water to final volume

PCR Cycling Conditions

PCR amplification was performed in a thermal cycler under the following conditions:

- Initial denaturation at **94–95 °C for 5 min**
- 30–35 cycles of:
  1. Denaturation at **94–95 °C for 30 s**
  2. Annealing at **[55–60] °C for 30 s** (depending on the primer set)
  3. Extension at **72°C for 45–60 s**
- Final extension at **72°C for 5–7 min**

Positive and negative controls were included in each PCR run. DNA from reference strains known to harbor the target genes served as positive controls, while nuclease-free water was used as a negative control.

Agarose Gel Electrophoresis

Ethidium bromide or a safe substitute dye was used to stain a 1.5% agarose gel produced in Tris-acetate-EDTA (TAE) buffer for the analysis of PCR products utilizing agarose gel electrophoresis. The size of the amplified products was estimated using a 100 bp DNA ladder. A UV transilluminator was used to view the gels, and the anticipated amplicon sizes were used to validate the presence of resistance genes.

Interpretation of Molecular Results

When a distinct band that matched the target gene's anticipated molecular size was seen, the isolates were deemed to be positive for antimicrobial resistance genes. To evaluate the relationship between genotypic and phenotypic resistance patterns, molecular results were contrasted with phenotypic antimicrobial susceptibility data.

Statistical Analysis

For analysis, data were imported into [SPSS / Excel / R]. Patterns of antibiotic resistance and isolate prevalence were ascertained using descriptive statistics. Results were presented as percentages, and when appropriate, differences were deemed statistically significant at $p < 0.05$.

Results

Prevalence of *Escherichia coli* and *Staphylococcus aureus* in Retail Milk Samples

Of the 360 milk samples that were gathered from retail establishments, 192 samples (53.3%) tested positive for *Staphylococcus aureus* or *Escherichia coli* bacterial contamination. *Staphylococcus aureus* was found in 120 samples (33.3%), but *Escherichia coli* was identified from 140 samples (38.9%). In 68 samples (18.9%), co-contamination with both species was found (Table 3).

Bacteriological identification of *E. coli* and *S. aureus* isolated from milk samples

Cultural characteristics: Lactose-fermenting *E. coli* growth is reddish-pink on MacConkey agar, as seen in Photo 1. *S. aureus*

Table 3: The Prevalence of *Escherichia coli* and *Staphylococcus aureus* isolated from retail milk samples (n = 360).

Isolate	Number of positive samples	Prevalence (%)
Escherichia coli	140	38.9
Staphylococcus aureus	120	33.3
Co-contamination (*E. coli* + *S. aureus*)	68	18.9
Total contaminated samples	192	53.3

Photo 1: Growth of *E. coli* on MacConkey blue agar medium.

Photo 2: Growth of *S. aureus* on mannitol salt agar medium.

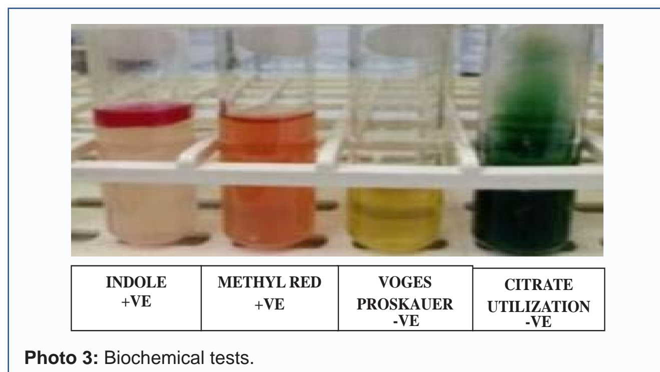

Photo 3: Biochemical tests.

growth on mannitol appears to be fermentable in a salt agar media. generating acid, as seen in Photo 2, which causes the indicator's color to shift from red to yellow.

Biochemical identification of *E. coli* and *S. aureus* isolated from the positive samples

See Table 4 and Photo 3.

Antimicrobial Susceptibility Patterns

Escherichia coli: High resistance rates among *E. coli* isolates were found by phenotypic antimicrobial susceptibility testing. Most often, resistance was found to ampicillin (72.1%), tetracycline (76.4%), and trimethoprim-sulfamethoxazole (58.6%). Both gentamicin (21.4%) and ciprofloxacin (32.1%) showed moderate resistance. 92 *E. Coli* isolates (65.7%) were categorized as multidrug-resistant (MDR) due to their resistance to three or more antibiotic classes.

Staphylococcus aureus: Penicillin resistance was highest among *Staphylococcus aureus* isolates (85.0%), followed by tetracycline (73.3%) and oxacillin (60.0%). Ciprofloxacin (28.3%) and gentamicin (20.0%) showed lower resistance rates. Multidrug resistance was found in 76 *S. aureus* isolates (63.3%) (Table 5).

Molecular Detection of Antimicrobial Resistance Genes

Escherichia coli: Using PCR analysis, it was found that 90 isolates (64.3%) had *tetA* and 84 isolates (60.0%) had *blaTEM* gene. Strong genotype–phenotype connection was indicated by the majority of gene-positive isolates showing comparable phenotypic resistance (Table 6).

Staphylococcus aureus: The existence of methicillin-resistant *S. aureus* (MRSA) was confirmed by the detection of the *mecA* gene in 64 isolates (53.3%) of *S. aureus*. 76 isolates (63.3%) had the *tetK* gene, mostly from bacteria that were resistant to tetracycline.

Phenotype–Genotype Correlation

Phenotypic resistance and the existence of resistance genes were found to be very consistent. While oxacillin-resistant *S. aureus* isolates were primarily *mecA*-positive, the majority of ampicillin-resistant *E. coli* isolates had *blaTEM*. The identification of *tetA* and *tetK* genes also showed a substantial correlation with tetracycline resistance in both organisms. *BlaTEM* and *tetA* gene detection in *E. coli* was substantially correlated with resistance to ampicillin and tetracycline, respectively (χ^2 test, $p < 0.05$). Similarly, the presence of *mecA* and *tetK* genes was significantly correlated with *Staphylococcus aureus's* resistance to oxacillin and tetracycline ($p < 0.05$).

Discussion

The present study demonstrated notable contamination of retail

Table 4: Biochemical identification of *E. coli* and *S. aureus* isolated from the positive milk samples.

Test	*E. coli*	*S. aureus*
Catalase	**+ve** (Bubble formation).	**+ve** (Bubble formation).
Oxidase	**-ve** (Absence of deep-purple coloration or coloration later than 60 seconds).	**-ve** (Absence of deep-purple coloration or coloration later than 60 seconds.)
Coagulase	**-ve**	**+ve** (Slide Coagulase test: formation of clumps within 10-15 seconds) (Tube Coagulase test: clotting)
Triple sugar iron agar (TSI agar)	**+ve** Acid/Acid, Gas (Yellow slant/yellow butt) reaction	-ve
Indole (I)	**+ve** (Red ring formation)	-ve
Methyl red (MR)	**+ve** (Bright red color)	-ve
Voges Proskauer (VP)	**-ve** (No color change)	-ve
Citrate utilization	**-ve** (Slant remains green)	-ve

Table 5: Antimicrobial susceptibility patterns of *E. coli* and *S. aureus* isolated from milk.

A. *Escherichia coli* (n = 140)

Antibiotic	Antibiotic class	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Ampicillin	β-lactam	101 (72.1)	12 (8.6)	27 (19.3)
Tetracycline	Tetracycline	107 (76.4)	9 (6.4)	24 (17.2)
Trimethoprim-sulfamethoxazole	Folate inhibitor	82 (58.6)	14 (10.0)	44 (31.4)
Ciprofloxacin	Fluoroquinolone	45 (32.1)	18 (12.9)	77 (55.0)
Gentamicin	Aminoglycoside	30 (21.4)	10 (7.1)	100 (71.5)

Multidrug-resistant (MDR) *E. coli*: 92 isolates (65.7%)

B. *Staphylococcus aureus* (n = 120)

Antibiotic	Antibiotic class	Resistant n (%)	Intermediate n (%)	Susceptible n (%)
Penicillin	β-lactam	102 (85.0)	6 (5.0)	12 (10.0)
Oxacillin	β-lactam	72 (60.0)	10 (8.3)	38 (31.7)
Tetracycline	Tetracycline	88 (73.3)	9 (7.5)	23 (19.2)
Ciprofloxacin	Fluoroquinolone	34 (28.3)	12 (10.0)	74 (61.7)
Gentamicin	Aminoglycoside	24 (20.0)	8 (6.7)	88 (73.3)

Multidrug-resistant (MDR) *S. aureus*: 76 isolates (63.3%)

Table 6: Distribution of antimicrobial resistance genes detected by PCR among *E. coli* and *S. aureus* isolates.

A. *Escherichia coli* (n = 140)

Resistance gene	Function	Positive isolates n (%)
blaTEM	β-lactam resistance	84 (60.0)
tetA	Tetracycline resistance	90 (64.3)

B. *Staphylococcus aureus* (n = 120)

Resistance gene	Function	Positive isolates n (%)
mecA	Methicillin resistance (MRSA)	64 (53.3)
tetK	Tetracycline resistance	76 (63.3)

milk samples with *Staphylococcus aureus* (33.3%) and *Escherichia coli* (38.9%), which agrees with findings reported in different regions indicating that milk and dairy products frequently harbor foodborne pathogens [29]. Comparable prevalence rates have been described in several studies conducted in Arabian countries, where *E. coli* and *S. aureus* remain among the most commonly detected bacterial contaminants in raw milk and dairy products, posing considerable public health risks due to their pathogenic potential and antimicrobial resistance profiles [2].

The detection of these bacteria in retail milk suggests that dairy products may serve as reservoirs for multidrug-resistant (MDR) microorganisms, thereby representing an important food safety concern. The high resistance observed among *E. coli* isolates to β-lactams and tetracyclines corresponds with earlier epidemiological investigations in bovine milk, where similar resistance patterns were associated with frequent exposure to these antimicrobial classes [31]. Likewise, the marked resistance of *S. aureus* isolates to penicillin, oxacillin, and tetracycline is consistent with previous reports highlighting the widespread occurrence of resistance to β-lactams and tetracyclines in staphylococcal isolates from raw milk [18].

The substantial proportion of MDR isolates identified in this study (65.7% for *E. coli* and 63.3% for *S. aureus*) reflects the escalating global problem of antimicrobial resistance among foodborne bacteria. Similar or even higher MDR rates have been documented in studies conducted on dairy milk in Bangladesh, where resistance reached up to 80% in *E. coli* and 88.5% in *S. aureus*, emphasizing the widespread nature of this issue [1]. The emergence and persistence of such resistant strains are likely driven by the extensive use and misuse of antibiotics in dairy cattle, which create selective pressure that favors the survival and dissemination of resistant bacteria along the food chain [14].

Molecular detection of resistance genes, including *blaTEM*, *tetA*, *tetK*, and *mecA*, provided genotypic confirmation of the phenotypic resistance patterns observed among the isolates. The presence of these genes indicates that milk can act as a reservoir for transferable antimicrobial resistance determinants. Similar findings have been reported in other investigations of raw milk, where high frequencies of β-lactamase and tetracycline resistance genes were closely associated with MDR phenotypes in dairy-associated bacterial populations [19]. Furthermore, the detection of *mecA* and *tetK* genes in *S. aureus* isolates aligns with reports from various regions documenting the molecular basis of methicillin and tetracycline resistance in dairy environments [16].

The strong correlation observed between phenotypic resistance and the presence of corresponding resistance genes supports the reliability of linking molecular markers with antimicrobial resistance behavior. This finding highlights the importance of integrating molecular diagnostic tools with conventional antimicrobial susceptibility testing to improve the surveillance and control of AMR in foodborne pathogens [5].

From a public health perspective, the occurrence of MDR *E. coli* and *S. aureus* in retail milk is alarming. The consumption of raw milk or milk processed under inadequate hygienic conditions may facilitate the transmission of resistant bacteria and their genetic determinants to humans, thereby complicating treatment options and contributing to the growing burden of antimicrobial resistance at the community level. These observations are consistent with global reports emphasizing the accelerating spread of resistant infections due to inappropriate antibiotic use in both veterinary and human sectors [13].

The detection of multidrug-resistant *Escherichia coli* and *Staphylococcus aureus* in retail milk has important public health implications for Saudi Arabia, where dairy consumption is high and raw or minimally processed milk is still consumed in some communities [32]. The presence of MDR foodborne pathogens in

milk suggests a potential route for the dissemination of antimicrobial resistance to humans through the food chain. In Saudi Arabia, antimicrobial resistance has been increasingly recognized as a national health priority, with reports indicating rising resistance rates among both clinical and foodborne bacterial isolates. This trend is likely influenced by the extensive use of antimicrobials in livestock production, which can select for resistant strains capable of persisting in dairy environments and reaching consumers [31]. Consequently, contaminated milk may serve as a reservoir for transferable resistance genes, facilitating their spread within the human population and complicating treatment of bacterial infections. These findings support national and global calls for strengthened surveillance of antimicrobial resistance in food products, stricter regulation of antibiotic use in animal husbandry, and improved hygiene practices across the dairy value chain. Implementing coordinated One Health strategies that integrate veterinary, food safety, and public health sectors is therefore essential to limit the transmission of MDR pathogens in Saudi Arabia and to protect consumer health [27].

Overall, the findings of this study underscore the urgent need for strengthened antimicrobial stewardship in dairy production, improved hygienic milking and processing practices, and continuous monitoring of resistance trends. Implementing such measures within a One Health framework is essential to limit the dissemination of MDR bacteria from dairy systems to the human population and to mitigate the ongoing expansion of antimicrobial resistance.

Conclusion

Stricter antibiotic stewardship in livestock production, better milking practices, regular milk microbiological testing, and consumer education regarding the dangers of raw milk should all be included of this study. To reduce the transmission of MDR bacteria from dairy systems to the general public, regulatory control must be strengthened and a One Health strategy that incorporates perspectives on animal, human, and environmental health must be implemented.

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