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Isolation and Molecular Characterization of Enterotoxigenic Strains of *Staphylococcus aureus* in Raw Milk from District Kasur, Punjab, Pakistan

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Abstract

This research aimed to isolate and molecularly characterize Staphylococcus aureus (S. aureus) from raw milk. We collected 600 raw milk samples from commercial dairy farms, milk collection centers, and Gawalas (200 samples each) in Kasur District, Punjab, Pakistan. S. aureus was identified in 270 (45%) of the samples using culture techniques. Raw milk from Gawalas had the highest prevalence, with 110 (55%) positive samples, followed by 90 (45%) from milk collection centers and 70 (35%) from commercial dairy farms. The S. aureus isolates that tested positive for enterotoxin genes were further characterized using PCR. In raw milk from commercial dairy farms, the enterotoxin C gene was detected in 42.86% of samples, while the A and B genes were present in 28.57% each. In raw milk from collection centers, the enterotoxin A gene was found in 42.86% of samples, and the B and C genes in 28.57% each. In Gawala's raw milk, the enterotoxin A gene was present in 50% of samples, while the B and C genes were present in 30% and 20% of samples, respectively. Overall, enterotoxin genes A, B, and C were identified in 41.67%, 29.17%, and 29.17% of the samples, respectively. This study found a significant incidence of enterotoxigenic S. aureus in raw milk from Gawlas, indicating that unsanitary milking practices increase the risk of milk-borne diseases, and recommends implementing pasteurization.

1. Introduction

Pakistan's gross milk production in 2022-23 was 67.873 million tonnes, comprising 25.151 million tonnes from cows, 40.678 million tonnes from buffaloes, 0.042 million tonnes from sheep, 1.046 million tonnes from goats, and 0.956 million tonnes from camels (Government of Pakistan, 2023). In industrialized countries, 90-98% of milk is processed in modern dairy facilities. In contrast, Pakistan sells 97% of milk raw without adequate hygiene, with only 3% undergoing professional processing (Ajmal *et al*., 2022).

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Immediately after being released from the udder of healthy animals, raw milk is often considered safe for human consumption because of its low microbial load. However, spoilage bacteria and pathogens from a variety of sources, such as animal excrement, dirt, air, feed, water, bedding, animal skins, sick udders, milk containers, or humans, can quickly contaminate milk after it exits the udder (Guo *et al*., 2024; Owusu-Kwarteng *et al*., 2020).

The degree of microbial contamination in raw milk at the farm significantly influences product quality throughout the dairy chain (Burtscher *et al*., 2023). According to Elmoslemany *et al*. (2010), three main sources of microbial contamination in bulk milk at the farm level are mastitis organisms in the udder, milking equipment, and bacteria on the outside of the udder and teats. In Pakistan, staphylococcal mastitis—both clinical and subclinical is quite common. However, subclinical mastitis can be present and may not be detectable in the milk, even though the udder is infected (Jooste & Anelich, 2008).

S. aureus is a prominent pathogen related to bovine mastitis, leading to substantial financial losses in milk output and quality (Gonçalves *et al*., 2018). *S. aureus* poses a public health threat since it may generate thermostable enterotoxins in pre-prepared foods, leading to foodborne illness characterized by nausea, vomiting, abdominal cramps, and diarrhoea (Balaban & Rasooly, 2000). Five classical enterotoxins of *S. aureus* are SEA, SEB, SEC, SED, and SEE (Orwin *et al*., 2003). Staphylococcus generates many enterotoxins, including SEG, SEH, SEI, SEJ, SEK, SEL, SEM, SEN, SEO, SEP, SEQ, and SEU. *S. aureus* has been detected in milk and dairy items, exhibiting antibiotic resistance, resulting in financial detriment. Numerous efforts have been made to develop vaccines against S. aureus, although most have shown unsatisfactory outcomes (Tenhagen *et al*., 2001).

Microbiological examinations assess the sanitary conditions of raw milk and their implications for public health. Conventional culture techniques for detecting *S. aureus* in raw milk are often time-consuming. Consequently, modern methods such as polymerase chain reaction (PCR) are used to detect *S. aureus* more effectively. This technique is rapid, sensitive, and specific for identifying various bacteria. Therefore, this project was designed to isolate and identify *S. aureus* from raw milk using morphological and biochemical tests, and to detect enterotoxigenic *S. aureus* strains A, B, and C using a PCR-based method.

2. Materials and Methods

2.1 Collection of samples

A total of six hundred raw milk samples (n = 600) were collected from Commercial Dairy Farms (CDF; n = 200), Milk Collection Centers (MCC; n = 200), and Gawalas (G; n = 200) in District Kasur, Punjab, Pakistan, following National Mastitis Council guidelines (Liu *et al*., 2022; Adkins & Middleton, 2017). Samples were placed in sterile screw-capped tubes, kept in an icebox at 4°C, and transported within 2–4 h to the Dairy Technology Laboratory, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki, District Kasur.

2.2 Sterilization of media and glassware

All glass apparatus, including test tubes, Petri dishes, pipettes, and flasks, were meticulously cleaned and sterilized in a hot air oven at 180°C for 2 hours. All media and solutions were prepared in distilled water and autoclaved at 121°C for 15 minutes (AOAC, 2000).

2.3 Media preparation, isolation and purification of *S. aureus*

Serial dilutions were prepared by mixing 1 mL of raw milk with 9 mL of sterile phosphate-buffered saline (PBS, pH 7.4) to obtain a 10^{-1} dilution. We prepared the 10^{-2} and 10^{-3} dilutions identically. From each dilution, a total volume of 1.0 mL was spread across three separate Baird-Parker agar plates supplemented with Egg Yolk Tellurite Emulsion in aliquots of 0.3 mL, 0.3 mL, and 0.4 mL to avoid liquid overload on a single plate. A sterile glass spreader was used. Incubate plates at 37°C for 48 h. We counted characteristic black, convex, shiny colonies with clear zones and subcultured them onto Nutrient Agar and Mannitol Salt Agar (MSA) to obtain pure isolates (AOAC, 2000).

2.4 Preservation and identification of *S. aureus*

Pure cultures of *S. aureus* were grown on nutrient agar slants and stored at 4°C. Isolates were identified using colony morphology, Gram staining, mannitol fermentation on Mannitol Salt Agar (MSA), catalase testing, and coagulase testing. Gram-positive cocci that produced yellow colonies with a yellow zone on MSA were considered presumptive S. aureus. The catalase test differentiated staphylococci from streptococci (Hemraj *et al*., 2013). The tube coagulase test was performed using 0.5 mL rabbit plasma containing EDTA; 2–3 drops of broth culture were inoculated and incubated at 37°C for 6 h, with plasma clotting interpreted as a positive reaction (Sperber & Tatini, 1975).

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Table 1. Sequences of the primers used in the current study.

Primer Name	Description	Primer sequence (5'-3')	PCR product size
SA-U	Universal F-Primer	TGTATGTATGGAGGTGTAAC	270
SA-A	R-Primer for SEA	ATTAACCGAAGGTTCTGT	270
SA-B	R-Primer for SEB	ATAGTGACGAGTTAGGTA	165
SA-C	R-Primer for SEC	AAGTACATTTTGTAAGTTCC	69

(Sharma *et al*., 2000)

2.5 DNA isolation and detection of *S. aureus* via PCR

Bacterial DNA was extracted using the DNeasy Blood & Tissue Kit (QIAGEN, USA) according to the manufacturer's protocol for Gram-positive bacteria. DNA concentration and purity were assessed using a NanoDrop spectrophotometer (A260/A280 ratio of 1.8-2.0). PCR assays targeted the enterotoxin genes SEA, SEB, and SEC using the specific primers listed in Table 1, with expected products of 270 bp, 165 bp, and 69 bp, respectively. Each 25 µL reaction contained 12.5 µL of 2× Green PCR Master Mix, 1.0 µL of forward primer (10 µM), 1.0 µL of reverse primer (10 µM), 2.5 µL of template DNA (~50 ng), and 8.0 µL of nuclease-free water. Thermal cycling consisted of initial denaturation at 94°C for 5 min; 27 cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 30 s; and final extension at 72°C for 2 min. *S. aureus* ATCC 13565 (SEA), ATCC 14458 (SEB), and ATCC 19095 (SEC) served as positive controls, while nuclease-free water served as the non-template negative control (Khudor *et al*., 2012).

2.6 Agarose gel electrophoresis

Amplification products (10 µL) were analyzed on a 1.5% (w/v) agarose gel stained with ethidium bromide in 1× TAE buffer. Electrophoresis was performed at 75 V for 60 min. A DNA molecular weight ladder spanning 100–2000 bp was run alongside the samples to verify the expected product sizes (270 bp for SEA, 165 bp for SEB, and 69 bp for SEC). Amplicons were visualized under UV transillumination (Sharma *et al*., 2000).

2.7 Statistical Analysis

Data were compiled in Microsoft Excel and analyzed using SPSS Version 26.0. Differences in *S. aureus* prevalence rates and enterotoxin gene frequencies among sampling sources (CDF, MCC, and Gawalas) were evaluated using Pearson's Chi-square (χ^2) test. A p-value < 0.05 was considered statistically significant.

3. Results and Discussion

3.1 Microscopic identification of *Staphylococcus aureus*

S. aureus colonies exhibited distinctive morphological characteristics on Baird-Parker egg yolk tellurite medium, appearing as black, shiny, convex colonies, as shown in Figure 1. The clear differentiation of *S. aureus* colonies is critical for preliminary diagnostics, providing a reliable visual cue for microbiological evaluations. Moreover, biochemical tests on the isolates showed universal positivity for both coagulase and catalase, key markers for confirming *the* presence of *S. aureus*. The isolates' ability to thrive on MSA further corroborated their identity, as shown in . Following biochemical characterization, all positive isolates underwent molecular analysis via PCR targeting the genes responsible for enterotoxin production, specifically genes A, B, and C. Figures 2, 3, and 4 show the amplified products for each gene, demonstrating the efficacy of PCR in molecular diagnostics. This step provided critical insights into the potential virulence of the *S. aureus* isolates, as enterotoxins contribute significantly to foodborne illness.

3.2 Isolation and Identification of *Staphylococcus aureus*

Of the 600 raw milk samples evaluated, 270 (45.0%) tested positive for *S. aureus* based on selective culture and biochemical confirmation (Table 3). *S. aureus* occurrence differed significantly across collection points ($\chi^2 = 16.16$, $p < 0.001$). Gawalas had the highest isolation rate (110/200, 55.0%), followed by Milk Collection Centers (90/200, 45.0%) and Commercial Dairy Farms (70/200, 35.0%). All confirmed isolates were Gram-positive *Cocci*, coagulase-positive, catalase-positive, and fermented mannitol on MSA. This study characterized *S. aureus* isolated from raw milk obtained from three milk-source categories in District Kasur, Punjab, Pakistan. The samples were not collected from animals diagnosed with clinical or subclinical mastitis; therefore, the findings reflect contamination and the prevalence of enterotoxin genes in raw milk rather than a direct characterization of bovine mastitis isolates. The significantly higher prevalence of *S. aureus* in Gawala milk (55.0%) than in commercial dairy-farm milk (35.0%) highlights important differences in pre-harvest hygiene and

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Table 2. Biochemical tests for *S. aureus*.

Sources	Samples	Biochemical tests		
		Coagulase	Catalase	Growth on MSA
Commercial dairy farms	1	+	+	+
	2	+	+	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
	6	+	+	+
	200	+	+	+
Milk collection centers	1	+	+	+
	2	+	+	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
	6	+	+	+
	7	+	+	+
	8	+	+	+
	200	+	+	+
Gawalas	1	+	+	+
	2	+	+	+
	3	+	+	+
	4	+	+	+
	5	+	+	+
	6	+	+	+
	7	+	+	+
	8	+	+	+
	9	+	+	+
	10	+	+	+
	200	+	+	+

post-harvest handling. Gawalas represent traditional, unorganized milk distribution networks in which manual handling, limited access to rapid chilling, inconsistent teat sanitation, and variable water hygiene may increase the risk of contamination. These source-related differences plausibly explain the higher bacterial burden observed in Gawala milk.

3.3 Distribution and PCR Detection of Enterotoxin Genes

Among the 270 confirmed *S. aureus* isolates, 240 (88.89%) harbored at least one classical enterotoxin gene (SEA, SEB, or SEC) (Tables 4 and 5). In Commercial Dairy Farms, 70/70 isolates (100%) were enterotoxigenic: SEC was detected in 30/70 (42.86%), while SEA and SEB were each detected in 20/70 (28.57%). In Milk Collection Centers, 70/90 isolates (77.77%) were enterotoxigenic: SEA occurred in 30/70 (42.86%), and SEB and SEC in 20/70 (28.57%) each. In Gawalas, 100/110 isolates (90.91%) were enterotoxigenic: SEA occurred in 50/100 (50.0%), SEB in 30/100 (30.0%), and SEC in 20/100 (20.0%). Overall, among the 240 toxigenic isolates, SEA was most prevalent (100/240, 41.67%), followed by SEC (70/240, 29.17%) and SEB (70/240, 29.17%) ($\chi^2 = 12.00$, $p = 0.017$). The enterotoxin gene profile also has epidemiological and public health implications. Enterotoxin C (SEC) predominated among isolates from commercial dairy farms (42.86%). SEC is commonly associated with animal-adapted *S. aureus* and has been linked with bovine intramammary infection. In contrast, enterotoxin A (SEA) predominated in Gawalas (50.0%) and Milk Collection Centers (42.86%). SEA is a potent, heat-stable staphylococcal enterotoxin frequently implicated in classical food poisoning and may reflect

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Table 3. Percentage of milk samples positive for *S. aureus* from Commercial dairy farms, Milk collection centers and Gawalas.

Growth on Baird-Parker medium	Commercial dairy farms	Milk collection centers	Gawalas	Total	χ^2	P.value
Positive	70 (35%)	90 (45%)	110 (55%)	270 (45%)	16.16	<0.001
Negative	130 (65%)	110 (55%)	90 (45%)	330 (55%)		

Table 4. Results of amplified Enterotoxin genes (SEA, SEB and SEC).

*Source	*S. aureus* positive Samples	Isolates of Enterotoxigenic gene	Source-specific distribution		
			A	B	C
CDF	70	70 (100%)	20 (28.57%)	20 (28.57%)	30 (42.86%)
MCC	90	70 (77.77%)	30 (42.86%)	20 (28.57%)	20 (28.57%)
Gawalas	110	100 (90.91%)	50 (50.0%)	30 (30.0%)	20 (20.0%)
Total	270	240 (88.89%)	100 (41.67%)	70 (29.17%)	70 (29.17%)

*CDF: Commercial dairy farms, MCC: Milk collection centers, G: Gawalas
A=SEA gene; B=SEB gene; C= SEC gene

Table 5. Percentage of the enterotoxigenic positive *S. aureus.*

*Source	*S. aureus* positive Samples	Isolates of Enterotoxigenic gene	Source-specific distribution			χ^2	P.value
			A	B	C		
CDF	70	70 (100%)	20 (28.57%)	20 (28.57%)	30 (42.86%)	12.00	0.017
MCC	90	70 (77.77%)	30 (42.86%)	20 (28.57%)	20 (28.57%)		
Gawala	110	100 (90.91%)	50 (50%)	30 (30%)	20 (20%)		
Total	270	240 (88.89%)	100 (41.67%)	70 (29.17%)	70 (29.17%)		

**CDF: Commercial dairy farms, MCC: Milk collection centers*
A=SEA gene; B=SEB gene; C= SEC gene

contamination during human handling. Its presence in raw milk is therefore of particular food-safety concern.

Molecular analysis of enterotoxins from *S. aureus* isolates in commercial dairy farms revealed that the gene for enterotoxin C was present at a rate of 42.86%, in contrast to enterotoxins A and B, which were each found at 28.57%. El Seedy *et al*. (2012) also found that the SEC gene in S. aureus isolated from buffaloes had the highest percentage, followed by SEA (15.7%) and SEB (11.8%) among other enterotoxin types. In a previous study, SEC was found to be more abundant in milk from mastitis-diseased positive animals (Jørgensen *et al*., 2005). The analysis of enterotoxins A, B and C of *S. aureus* from the milk collected from the Milk Collection Center showed that enterotoxin A was present in the milk in the highest percentage (42.86%), followed by B (28.57%) and C (28.57%). The *S*EA gene was present in about 55.7% of *S. aureus* isolates, the SEB gene in 14.8%, and the SEC gene in 6.5% (Manfredi *et al*., 2010). *S. aureus* was more prevalent in small farmers' milk (Gawala's milk), which might have been contaminated in a variety of ways. Prevalence was lower on dairy farms, possibly due to diligent hygiene and management of dairy animals. The outcomes resemble those of Gwida and El-Gohary (2013), in which 56.66% of *S. aureus* isolates were found in market milk and 18% in farm milk. The main differences in the results stem from variations in management systems and diagnostic tools across zones.

Although this analysis yields important findings, it is vital to identify its shortcomings. A key limitation is that sampling was restricted to a single district, despite 600 raw milk samples being assessed. This may restrict the generalizability of the findings to a broader population or different geographic regions. Future investigations should include a more extensive and heterogeneous sample group, incorporating samples from farms across different regions to ensure a comprehensive evaluation of *S. aureus* prevalence and associated enterotoxin profiles. Additionally, studies

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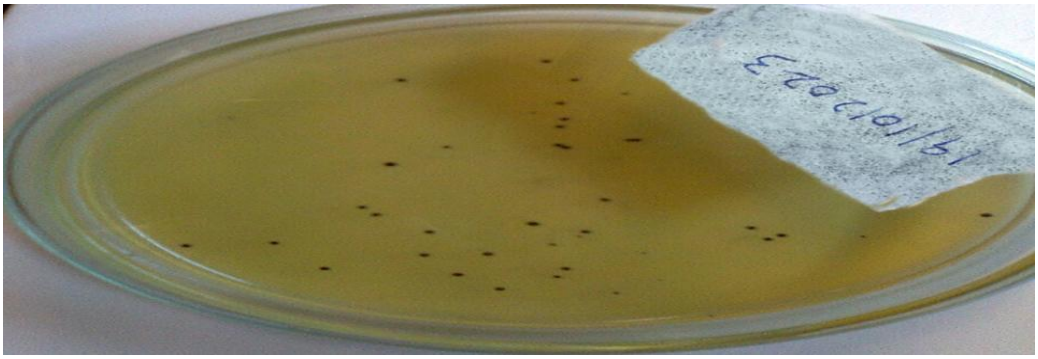


Figure 1. Colonies of *S. aureus* appeared as black, shiny, convex colonies on Baird-Parker egg yolk tellurite medium.

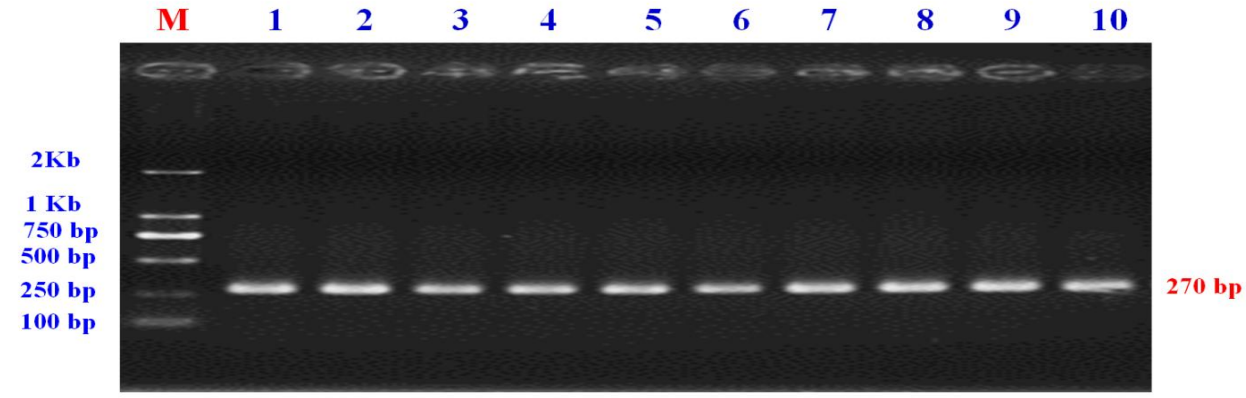


Figure 2. Gel electrophoresis in 1.5% agarose. M: DNA molecular-weight marker (100–2000 bp); lanes 1–10: PCR-amplified enterotoxin A (SEA) products of *S. aureus*; expected product size: 270 bp.

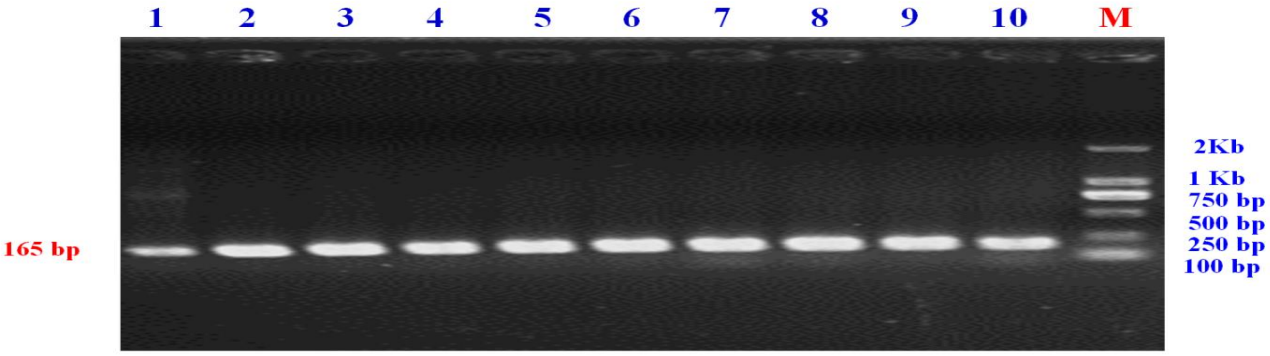


Figure 3. Gel electrophoresis in 1.5% agarose. M: DNA molecular-weight marker (100–2000 bp); lanes 1–10: PCR-amplified enterotoxin B (SEB) products of *S. aureus;* expected product size: 165 bp.

should also examine potential seasonal effects on mastitis prevalence and *S. aureus* occurrence, as climatic variations may influence pathogen growth and animal health.

Another limitation is the reliance on conventional culture methods and PCR to detect *S. aureus* and its enterotoxins. While these methods are effective, they may not capture the full spectrum of microbial diversity and virulence factors present in the samples. Future investigations should use advanced molecular methods, such as metagenomics or whole-genome sequencing, to better understand the microbial populations associated with mastitis. This comprehensive approach would help identify novel

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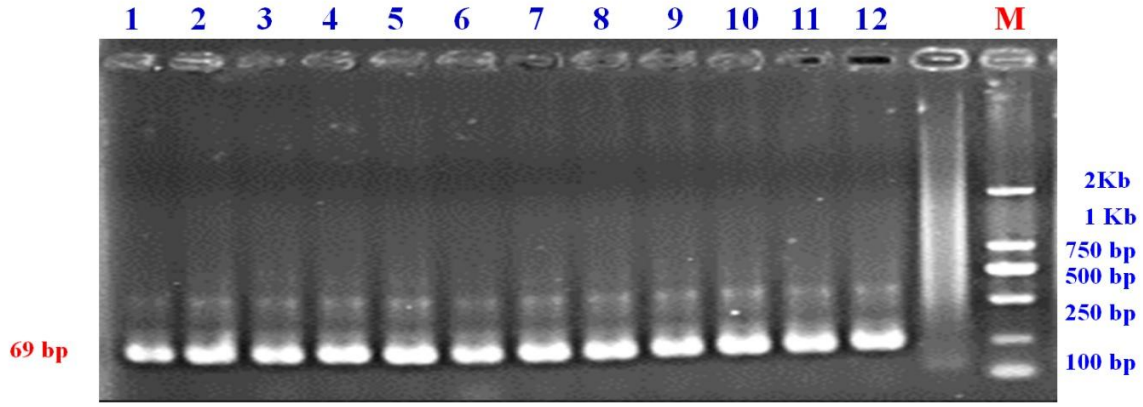


Figure 4. Gel electrophoresis in 1.5% agarose. M: DNA molecular-weight marker (100–2000 bp); lanes 1–12: PCR-amplified enterotoxin C (SEC) products of *S. aureus*; expected product size: 69 bp.

pathogens and clarify interactions between different microbial species. Furthermore, it would help identify more precise correlations between management practices and the prevalence of mastitis-causing organisms, ultimately supporting the development of tailored interventions to minimize S. aureus contamination in dairy farms.

Conclusions

The findings underscore substantial concerns about the microbiological quality and safety of raw milk. *S. aureus* was detected in 270 of 600 samples (45.0%), and 240 of the 600 samples (40.0%) yielded S. aureus isolates carrying at least one of the tested enterotoxin genes. Gawala milk had the highest *S. aureus* prevalence, underscoring the importance of hygienic milking, handling, rapid cooling, and pasteurization. The presence of enterotoxigenic *S. aureus* in raw milk poses a potential food safety risk, particularly when consumed without heat treatment. Strengthened hygiene practices and routine microbiological monitoring are therefore recommended to protect public health.

Author contributions

Ibrar Hussain: Conceptualization, methodology, research trial, and data collection; Abdul Jabbar: Conceptualization, methodology, supervision, formal analysis, investigation, and writing and editing; Muhammad Shuaib: Software, formal analysis, data curation and visualization, and writing-original draft preparation; Obaid Ullah, Talat Bilal Yasoob, Najeeb Ullah, Shoaib Sultan, Shahzad Hussain, Muhammad Shahkar Uzair, Zuhaib Hassan Bughio: Data curation, writing-review & editing. All authors have read and approved the final manuscript.

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Data Availability Statement: All data created or analyzed during this investigation are included in this paper.

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