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Prevalence of Milk-Borne Bacterial Pathogens via Touchdown Multiplex PCR to Unravel Incidence trends and Associated Risk Factors

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ABSTRACT

Foodborne illnesses represent a substantial challenge for global public health. Milk, being a fundamental component of our dietary intake, offers crucial nutrients such as lipids, proteins, and various vitamins and minerals, notably calcium and phosphorus. However, milk also holds the potential to harbor harmful bacteria such as *Staphylococcus aureus* and *Salmonella* etc. posing significant health threats to consumers. This project aims to establish a reliable and sophisticated detection method to identify diverse pathogens in milk samples, along with focusing particularly on their prevalence rate in district Faisalabad, of province Punjab, Pakistan. Additionally, the study seeks to assess the antibiotic susceptibility profiles of these pathogens and some of the potential risk factors (such as animal herd size at the farm, age of animal, type of floor of the farm, farm hygiene, milking type i.e., manual or machine, udder hygiene, animal's health condition and farm type etc.) that might affect the prevalence of food-borne pathogens. Milk samples (n=381) were procured from five distinct subdivisions (Faisalabad City, Chakk Jhumra, Jaranwala, Samundri and Tandlianwala) of district Faisalabad.. Initially, all the pathogens were isolated using traditional methods (bacterial culturing on media), and their characteristics were determined using several biochemical techniques, *in silico* analysis and individual PCR analysis. The accuracy of TD-PCR was evaluated based on its specificity, sensitivity, and validity, demonstrating robust performance with 100% specificity and sensitivity. Analysis of the samples unveiled the presence of pathogens including *E. coli, Salmonella, L. monocytogenes, S. aureus,* and *Campylobacter* species, confirmed through *in silico* assessment via the Vitek-2 system, alongside DNA extraction and TD-PCR. 52% samples showed positive results for bacterial growth. Antibiotic susceptibility testing revealed varying levels of resistance among the isolates. *Staph. Aureus* and *Campylobacter spp*

showed highest resistance (71% & 67%, respectively) to tetracycline, *E. coli O157:H7* (78%) to streptomycin, *Listeria monocytogenes* (32%) to -Trimethoprim and *Salmonella enterica* (81%) to kanamycin. Through an exhaustive risk analysis, eight potential risk factors associated with milk sources and dairy farms were identified. Following the optimization of PCR assay, an examination of 231 raw milk samples was conducted from diverse sources disclosed the highest prevalence in Faisalabad city, reaching 57%. Risk factor analysis revealed that herd size, age, type of floor, farm hygiene, milking type udder hygiene, and animal's health condition were found statistically in-significant with a *p* value higher than 0.05 whereas the risk factor of farm type was found statistically highly significant with a *p* value of 0.004. These findings carry significant implications for food safety protocols, commercial practices, and economic evaluations. The advancement of TD multiplex PCR as a dependable tool for scrutinizing raw milk samples marks substantial progress in bolstering food safety efforts in Pakistan.

Key words: Milk-borne pathogens, Conventional isolation, Characterization, TD-PCR, Antibiotic sensitivity, Prevalence, Risk factors.

INTRODUCTION

Foodborne infections represent a significant public health concern and have substantial economic ramifications globally. Liu et al. (2022) reported 48 million cases of foodborne illnesses annually, leading to 128,000 hospitalizations and over 3,000 deaths. Similarly, Yap et al. (2022) found approximately 76 million reported cases each year, resulting in 325,000 hospitalizations and over 5,000 deaths worldwide.

Within the realm of foodborne illnesses, milkborne infections hold particular importance. Pakistan's economy relies heavily on the livestock sector, which provides essential sources of nutrition through milk and meat. According to Belkhemas et al. (2021), buffalo, cattle, and sheep/goats are the primary sources of milk production in Pakistan. While raw milk is nutritionally rich, it can also serve as a favorable environment for harmful bacteria such as *E. coli, Mycobacterium tuberculosis, Salmonella, Listeria monocytogenes, Campylobacter, Bacillus,* and *Micrococcus* (Ahmed et al., 2022). Additionally, in the Indo-Pakistan subcontinent, the consumption of raw milk and various raw milk products significantly contributes to the transmission of foodborne *Staphylococcus enterotoxin* (Verma et al., 2023).

In recent years, significant attention has been directed toward developing reliable, sensitive, and efficient methods for detecting foodborne microbes (Foddai and Grant, 2020) due to increased public awareness of associated health risks (Panwar et al., 2023). While these methods are both time-consuming and demanding, molecular techniques such as Polymerase Chain Reaction (PCR) have been extensively used for microbial identification and characterization in dairy and meat products (Chin et al., 2021). Typically, pathogen identification requires 48 to 72 hours, with specific selective enrichment methods necessary for recovering microbes from food samples. A comprehensive approach involves

spreading microbes onto specialized enrichment media and conducting a series of biochemical and microbiological tests, although this process can be laborious (Foddai and Grant, 2020). The single cell identification process is crucial for a range of bio-analysis applications, and the droplet microfluidics method is widely recognized as a powerful tool in this regard. The global impact of *Salmonella* infection on public health is significant, with devastating consequences for individuals. To stop *Salmonella* outbreaks and get rid of the need for complicated pre-treatment tests (Saravanan et al., 2021) it is important to come up with reliable, effective, and useful ways to find these infections in food. Prior research has demonstrated the precise and meticulous operation of a microfluidic chip investigative technique, which enables the accurate monitoring of microbes in food. This study not only identifies *Salmonella* in milk samples but also updates microfluidics-based analytical methods. This method has several benefits in terms of ensuring food safety and human health. Additionally, they contribute to improving the efficiency and accuracy of detecting food-borne microbes (Yan *et al*., 2021).

This study endeavors to formulate a protocol for the concurrent identification of various milk-borne pathogens directly from milk specimens. Additionally, it seeks to collect up-to-date information on bacterial pathogens present in milk within the Faisalabad district, while assessing their susceptibility to antibiotics.

METHODOLOGY

Sample collection

In this particular investigation, five distinct representative tehsils within the district of Faisalabad (comprising Faisalabad city, Chakk Jhumra, Jaranwala, Samundri, and Tandlianwala) were specifically chosen for the purpose of sampling raw milk. The duration of this research spanned from January 2021 to December 2021. Raw milk samples were methodically collected from various sources including dairy farms, bulk tanks (milkman pots), retail milk shops, and local households, utilizing a random selection approach. These samples were meticulously contained within sterile containers and were sourced from diverse locations across the Faisalabad district. Following collection, the samples were promptly transported to the laboratory under refrigerated conditions to maintain their integrity. Consistent with the findings delineated in previous literature (Moezi et al., 2019; Zastempowska et al., 2015; Claeys et al., 2013), the target bacterial strains for analysis encompassed *E. coli, L. monocytogenes, S. aureus, Campylobacter*, and *S. enterica*.

Conventional Isolation

Raw milk samples underwent culturing and assessment to detect the presence of pathogens, utilizing standardized microbiological methodologies. Five distinct types of media were employed to target the optimal growth conditions for each bacterium, including *Salmonella Shigella* (SS) agar for *Salmonella*, CCDA for *Campylobacter*, Blood agar for Staph aureus, *Listeria*-specific agar for *Listeria monocytogenes*, and MacConkey sorbitol agar for *E. coli* (*O157:H7*) (Ito et al., 2019). In sterile tubes containing 225 ml of Buffered Peptone Water

(BPW), 25 ml of raw milk samples were introduced. Subsequently, the tubes were aerobically incubated at 37 °C for 24 hours. After this initial incubation period, 1 ml of the incubated BPW was inoculated into 5 ml of MacConkey broth. Following another 24-hour incubation, a small volume of the broth was streaked onto agar plates and allowed to further incubate at 37 °C (Berhe et al., 2020).

Identification of microorganisms

Following isolation, confirmation of the predominant milk-borne pathogens was conducted using various methodologies. *E. coli* and *Salmonella* were confirmed utilizing the Vitek-2 system, while *Campylobacter*, *Listeria*, and *Staphylococcus aureus* were identified through culturing on appropriate selective media. Subsequent identification involved assessment of cultural, microscopic, biochemical, and serological traits, as described by Mohamed et al. (2017). Morphological characteristics of the isolated bacteria were initially employed for detection, followed by presumptive identification through gram staining and other standard biochemical assays.

Antibiotic sensitivity testing

The antibiotic profiling of the isolated bacteria was determined by the Kirby Bauer disk diffusion method (Venkadesan and Sumathi, 2015).

DNA Extraction

Overnight-cultivated bacteria were introduced into a nutrient broth, and their concentration was quantified using a spectrophotometer at a wavelength of 600nm. Genomic bacterial DNA was obtained using the Thermo Fisher Genomic DNA Purification Kit. Subsequently, three to four loops of fresh colonies from the pure culture were suspended in nuclease-free water within a 2 ml micro centrifuge tube. The bacteria were then harvested by centrifugation at 5000 rpm for 10 minutes, and the resulting pellet was suspended in 180 µl of digestion solution. Following the addition of 20 µl of Proteinase-K, the mixture was vigorously mixed using a vortex mixer and Incubated at 56°C for 30 minutes with intermittent vortexing. Afterward, 20 µl of rNase-A solution was added, and the mixture was incubated for 10 minutes at room temperature before 200 µl of lysis solution was added. The suspension was vortexed for 10 seconds to ensure homogeneity before the addition of 400 µl of a 50% ethanol solution, followed by another round of vortexing. The lysate was then applied to a Gene-jet genomic DNA purification column and centrifuged at 6000 rpm for 1 minute. The flow-through solution was discarded, and the column was transferred to a fresh 2 ml tube. A wash buffer containing 50 µl of ethanol was applied to the column and centrifuged at 8000 rpm for 1 minute, with subsequent removal of the flow-through. This washing step was repeated for 3 minutes at 12000 rpm centrifugation, and the purification column was finally placed in a sterile 1.5-ml micro centrifuge tube. Elution buffer (200 µl) was added to the center of the purification column to elute the sample DNA, followed by centrifugation at 8000 rpm for 1 minute after 2 minutes of room-temperature incubation. To maximize DNA yield, the elution step was repeated with an additional 200 µl of buffer. The purification column was then discarded,

and the DNA was immediately utilized. The concentration of isolated genomic DNA was determined using 1.5% agarose gel electrophoresis.

Primers Design

The molecular detection of the pathogenic microorganisms *E. coli O157:H7, L. monocytogenes, S. aureus, S. enterica*, and *Campylobacter* genus was done through the multiplex PCR technique. The amplification of the targeted bacteria was done by using the appropriate primers specifically targeting

- The rfbE gene in *E. coli* (FP 5'GCCACCCCCATTTTCGTTG3', RP 5'TCCTCTCTTTCCTCTGCGGT3') (Moezi *et al.,* 2019)
- the hlyA gene in *L. monocytogenes* (FP 5' ATCATCGACGGCAACCTCGGAGAC3', RP 5' CACCATTCCCAAGCTAAACCAGTGC3') (Wu *et al.,* 2004),
- the nuc gene in *S. aureus* (FP 5' GCGATTGATGGTGATACGGTTAG3', RP 5' CCAAGCCTTGACGAACTAAAGC3') (Brakstad *et al.*, 1992),
- the invA gene in *S. enterica* (FP 5' TCGTCATTCCATTACCTACC3', RP 5' AAACGTTGAAAAACTGAGGA3') (Hoorfar *et al.,* 2000), and
- the 16s rDNA gene in *Campylobacter* (FP 5' ATCTAATGGCTTAACCATTAAAC3', RP 5' GGACGGTAACTAGTTTAGTATT3') (Han *et al.,* 2016), respectively.

Optimization of Multiplex TD-PCR

Genomic DNA confirmation involved a two-step process. Initially, genomic DNA was verified using optimized multiplex TD-PCR, where each isolate underwent a separate TD PCR. Subsequently, we transitioned to simultaneous detection via multiplex. The PCR reaction mixture, totaling 50 ul, comprised 25 ul of Master Mix, 1 ul of purified DNA template (100–200 ng/ul) per isolated bacterium, 2 ul (0.2 umol/l) of forward (F) and reverse (R) primers for each bacterium, and nuclease-free water to complete the volume. Utilizing Bio-Rad c1000 thermocycler equipment, the procedure employed settings tailored for touchdown PCR. This entailed 35 cycles in two phases: 20 cycles featuring a descending annealing temperature, initially above 10 °C, decreasing by 0.5% per cycle, followed by 15 cycles at a constant annealing temperature of 55 °C. The reactions incorporated both a positive control (*E. coli* ATCC 25922) and a negative control (lacking DNA template) (Moezi et al., 2019; Yosilia et al., 2023).

Agarose gel electrophoresis and imaging

The TD-PCR-generated products underwent 2% agarose gel electrophoresis, followed by analysis using the Gel Documentation System Fluor-shot Vision Biosens Sc750 (Shanghai, China) for product visualization.

Multiplex TD-PCR evaluation in inoculated raw milk samples

To assess the effectiveness of the optimized Multiplex TD-PCR, raw milk samples underwent a preliminary step wherein they were subjected to boiling at 100°C for 30 seconds to eliminate milk-borne bacterial pathogens. Following this treatment, the milk

samples were cultured on specified media, including MacConkey agar, *Listeria* selective agar, Blood agar, SS agar, and mCCDA, each tailored to the detection of targeted bacteria: *E. coli O157:H7*, L. monocytogenes, *S. aureus*, *S. enterica*, and *Campylobacter*. Once the absence of bacteria in the milk samples was confirmed, deliberate inoculation with the desired bacteria was performed. The artificially contaminated milk underwent centrifugation to remove the supernatant, followed by rinsing of the pellet with phosphate-buffered saline (PBS) prior to its addition to the ELSS pre-enrichment medium. The ELSS suspension containing the pellet was then incubated for 16 hours at 37°C in a shaking incubator set to 150 revolutions per minute. The choice of ELSS pre-enrichment medium in this step was based on its demonstrated efficacy in facilitating simultaneous growth of *E. coli O157:H7*, *S. aureus*, *S. enterica*, *L. monocytogenes*, and *Campylobacter*. Following the incubation period, genomic DNA was extracted from the culture broth using Thermo Fisher's Genomic DNA Purification Kit. Subsequently, the isolated genomic DNA was employed in multiplex TD-PCR to directly identify the targeted bacteria present in the milk samples at molecular level. This comprehensive approach enables an accurate evaluation of the detection capabilities of the optimized Multiplex TD-PCR in complex matrices such as raw milk.

Specificity analysis

The specificity of the assay was evaluated through multiplex TD PCR amplification of DNA extracted from each bacterial strain, employing their respective primer pairs. This evaluation involved assessing the protocol's capability to discern non-target bacterial strains from the five designated target pathogens.

Sensitivity analysis

To evaluate the sensitivity of the multiplex TD-PCR assay with pure cultures, five standard strains including *E. coli O157:H7*, *L. monocytogenes*, *S. aureus*, *Campylobacter*, and *S. enterica* were introduced into raw milk. Subsequently, the milk samples underwent overnight incubation in ELSS at 37°C. Following this, the samples were subjected to a 10-fold dilution (104–100 cells per ml) with sterile saline solution. DNA extraction was then performed separately on each dilution. Utilizing one microliter of genomic DNA from each dilution of the representative sample, multiplex TD-PCR was conducted.

Evaluation of naturally contaminated raw milk by multiplex TD-PCR

The examination of specific milk-borne pathogens presents in raw milk, which became contaminated through natural processes at dairy farms, milkmen pots, and local stores, was undertaken by acquiring 50 raw milk samples randomly over a span of 30 days. These raw milk specimens were procured in sterile containers and kept at 4 °C throughout transportation and storage. Upon arrival at the UAF laboratory, the samples were introduced into the pre-enrichment medium ELSS and allowed to incubate for 16 hours. Subsequently, genomic DNA extraction was conducted, followed by optimized multiplex TD-PCR analysis. (Reference: UAF Laboratory Protocols Manual, Section 3.2.1)

Statistical Analysis

Descriptive statistical methods such as chi square, odds ratio, and relative risk were used to describe the results and analyze the significance of the obtained data.

RESULTS

Pathogen isolation and characterization

In the current investigation, a comprehensive analysis was conducted on a cohort of 381 samples to identify the predominant pathogens commonly associated with raw milk. The morphological attributes of the isolated bacteria were examined on selective agar, revealing diverse growth patterns, as illustrated in Figure 1(a). Notably, the colonies of L. monocytogenes observed on *Listeria* agar manifested as black against a yellowish background, with occasional darkening of the agar itself. Conversely, the growth of *S. enterica* on SS agar appeared colorless. Additionally, *S. aureus* presented as shiny, convex, β-hemolytic, creamy-whitish colonies. Similarly, *E. coli* exhibited a distinct pink hue on selective media such as Sorbitol-MacConkey agar, indicating lactose fermentation. Among the samples positive for *E. coli*, the presence of *E. coli O157:H7* was confirmed through latex agglutination serological testing (Fig. 1b), employed solely for screening purposes. Furthermore, *Campylobacter* species displayed an off-white colony morphology on CCDA agar, delineating their growth pattern.

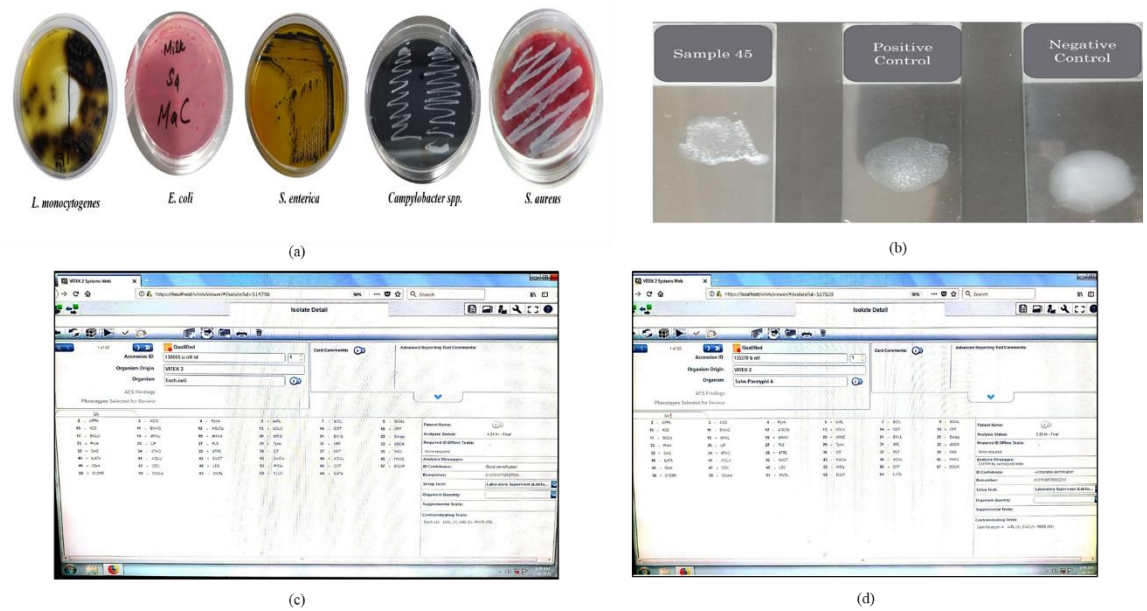

Figure 1: Isolation and characterization of milk-borne pathogens. (a) Culture plates were utilized for the isolation of raw milk-borne pathogens obtained from diverse sources conventionally. (b) The confirmation of *E. coli O157:H7* was performed using the latex agglutination test. (c) The VITEK-2 system was employed for the identification of *E. coli.* (d) *Salmonella* was identified utilizing the VITEK-2 system.

Following the initial identification of bacteria relying on morphological traits, notable pathogens of clinical significance, including *E. coli* and *Salmonella* species, underwent additional confirmation through the Vitek-2 system (Fig. 1 c & d). Conversely, the remaining three microorganisms (*L. monocytogenes*, *S. aureus*, and *Campylobacter* species) were further characterized utilizing Gram staining and biochemical profiling, as delineated in Table 2.

Table 2: Biochemical testing profile of the isolated milk-borne pathogens.

	Urease	Catalase	Oxidase	Nitrate	Hippurate Hydrolysis	Citrate	Indole	MR	VP
E. coli	–	+	–	+	N/A	–	+	+	–
Staph	+	+	–	N/A	N/A	+	–	+	+
Salmonella	–	+	–	N/A	N/A	–	–	+	–
Listeria	N/A	+	–	–	N/A	N/A	–	N/A	N/A
Campylobacter	N/A	+	+	+	+	N/A	N/A	N/A	N/A

Determination of the isolated raw milk-borne pathogens

In the initial phase of the current investigation, a total of 100 milk samples were carefully transported to the laboratory under controlled temperature conditions over the course of one month. These samples were collected from various sources, as detailed in Table 3: 20 from milkmen pots, 20 from local households, 20 from retail milk shops, and the remaining 40 directly from dairy farms. Among the 100 samples analyzed, 52 (52%) tested positive for bacterial growth. Notably, certain agar plates exhibited multiple bacterial growths, necessitating subsequent pure culturing procedures. Subsequently, in the following step, a thorough examination of all 100 samples was conducted to detect five specific strains of raw milk-borne pathogens. This process yielded a total of 70 pathogens isolated from the samples.

Table 3: Overall distribution of raw milk-borne pathogens in district Faisalabad.

Milk N = 100	***E. coli O157 H7***	***Salmonella enterica***	***Listeria monocyto genes***	***Staph aureus***	***Campylo… bacter spp***	***Total No. of Pure Culture isolates***	***Total No. of Positive samples***
Dairy Farms (40)	8	4	3	5	3	23	20 (50%)
Milkmen Pots (20)	4	5	4	3	2	18	9 (45%)

Retail Milk Shops (20)	4	4	4	3	3	18	11 (55%)
Local Household (20)	3	3	2	2	1	11	12 (60%)
Total (100)	19	16	13	13	9	70	52 (52%)

DNA Extraction

The colonies derived from the pure culture underwent incubation in nutrient broth at 37°C for 24 hours before DNA extraction. Subsequently, the extracted DNA samples were loaded onto a 1.5% agarose gel for electrophoresis analysis. The findings of this process are dep, illustrating the outcomes of the extracted and purified DNA.

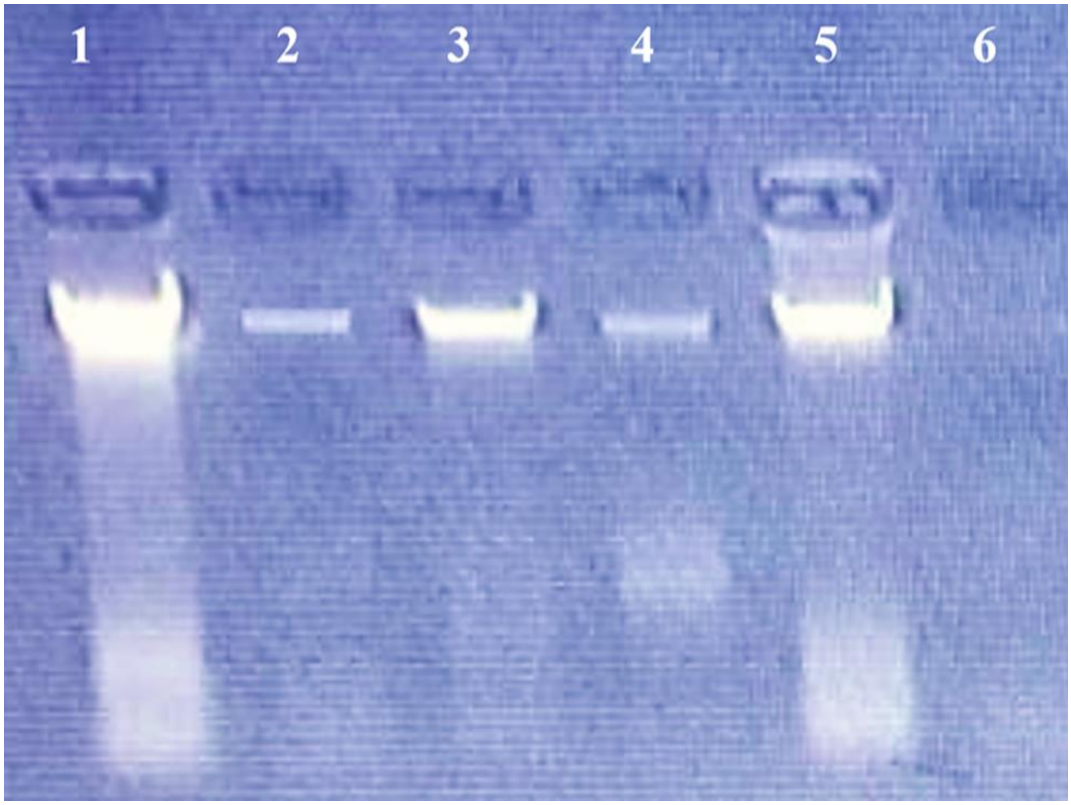

Figure 2: DNA confirmation via Agarose gel Electrophoresis. The wells **(1), (2), (3), (4), (5)** indicate the presence of purified DNA of *E. coli*, *L. monocytogenes*, *S. enterica*, *Campylobacter species* and *S. aureus*, respectively. **(6)** indicates the blank containing nuclease free water only.

Optimization of single and multiplex Touch down and PCR

Individual TD-PCR assays were conducted for each isolate to assess primer efficiency and validate the strains, with a 100-bp plus ladder serving as a standard. Analysis revealed distinctive bands corresponding to *Escherichia coli O157:H7* at 599 bp, *Listeria monocytogenes* at 404 bp, *Staphylococcus aureus* at 277 bp, *Salmonella* enterica at 119 bp, and *Campylobacter genus* at 857 bp.

To assess the specificity of the assay, individual TD-PCR runs were conducted using the designed primers separately. The outcome of these single TD-PCR runs revealed distinct bands corresponding to *Escherichia coli O157:H7* at 599 bp, *Listeria monocytogenes* at 404 bp, *Staphylococcus aureus* at 277 bp, *Salmonella enterica* at 119 bp, and *Campylobacter genus* at 857 bp. Furthermore, a combination of these primers was employed to detect all five targeted bacterial pathogens simultaneously through TD multiplex PCR. Nevertheless, multiple primer combinations were also utilized with DNA templates from non-target pathogens for thorough assessment via TD Multiplex PCR. The findings demonstrated successful amplification solely for the targeted bacterial pathogens, with no amplification observed for non-targeted pathogens. Consequently, the optimized assay exhibited a specificity of 100%.

Similarly, the sensitivity of the optimized TD multiplex PCR was gauged by diluting DNA from the five targeted bacterial isolates. A 5-fold serial dilution of the extracted DNA was prepared concurrently (104–100 cells/ml). These diluted DNA samples were then subjected to TD multiplex PCR using a combination of five primer pairs. The results revealed distinct bands corresponding to each of the five targeted bacterial strains. This concurrent detection demonstrated that the presence of even a single cell per ml of the target pathogens in the PCR reaction mixture was successfully amplified. Thus, the sensitivity of the assay was determined to be 100%.

Following the successful optimization of TD multiplex PCR, the validation of the assay was conducted by artificially contaminating sterile milk samples. Artificial contamination was achieved by adding 1 ml of enriched broth sample from each isolated organism, i.e., *E. coli O157:H7*, *L. monocytogenes*, *S. aureus*, *Campylobacter* spp., and *S. enterica* isolates, into 20 ml of sterile milk. The milk was then boiled, and its sterility was confirmed by inoculating it on all necessary culture media. Subsequently, a 25 ml representative milk sample was inoculated into 225 ml of pre-enrichment ELSS broth for 16 hours, followed by DNA extraction and multiplex TD-PCR. The results obtained indicated that the optimized TD multiplex PCR could detect the five targeted milk-borne pathogens simultaneously directly from the raw milk sample. This experiment was repeated three times.

Evaluation and comparison of natural contamination of raw milk by conventional and multiplex TD-PCR methods

To evaluate the practical applicability of the multiplex TD-PCR technique for simultaneous identification of five pathogenic bacteria in raw milk samples, a set of 50 raw milk samples

underwent testing using the optimized assay alongside the conventional method. This comparative analysis aimed to definitively ascertain the efficacy of the assay. All samples were sourced from local households, chosen due to their previously observed higher prevalence of positive samples. Prior to DNA extraction, each sample underwent a 16-hour enrichment period with ELSS. Results indicated similar detection rates for *L. monocytogenes, Staph aureus*, and *S. enterica* across both diagnostic methods. However, disparities were observed in the positive detection rates of *E. coli O157:H7* and *Campylobacter spp*., with one and two samples showing discrepancies, respectively (Table 4).

Table 4.: Practical application of multiplex TD-PCR and conventional culture in naturally contaminated raw milk

NO. of raw milk sample	*Escherichia coli O157:H7*		*Listeria monocytogenes*		*Staphylococcus aureus*		*Salmonella enterica*		*Campylobacter spp.*	
	mTD-PCR	Culture	mTD-PCR	Culture	mTD-PCR	Culture	mTD-PCR	Culture	mTD-PCR	Culture
3	+	+	-	-	-	-	+	+	+	+
5	-	-	+	+	-	-	-	-	-	-
8	-	-	-	-	+	+	-	-	-	-
10	+	+	-	-	+	+	-	-	-	-
11	+	+	-	-	-	-	-	-	-	-
13	-	-	+	+	-	-	-	-	-	-
17	-	-	+	+	-	-	-	-	+	-
21	+	+	-	-	-	-	+	+	-	-
24	+	+	-	-	+	+	+	+	-	-
26	-	-	-	-	+	+	-	-	-	-
29	-	-	+	+	-	-	-	-	-	-
32	-	-	-	-	+	+	-	-	+	-
34	+	-	+	+	+	+	-	-	-	-
37	-	-	-	-	+	+	+	+	-	-
40	-	-	-	-	-	-	+	+	-	-
42	-	-	-	-	+	+	+	+	+	+
45	+	+	-	-	-	-	+	+	-	-
47	-	-	-	-	-	-	-	-	-	-
49	+	+	+	+	-	-	-	-	+	+
Total samples	50	50	50	50	50	50	50	50	50	50
Positive Ratio	8(16%)	7(14%)	6(12%)	6(12%)	8(16%)	8(16%)	7(14%)	7(14%)	5(10%)	3(6%)

Antibiotic sensitivity profiles

Antibiotic susceptibility testing was conducted using appropriate antibiotic discs tailored to each isolate on Muller-Hinton agar plates, following CLSI guidelines (CLSI, 2020). Incubation of the plates took place at 37°C for 24 hours. Subsequently, inhibition zones on the plates were meticulously measured and categorized as either resistant or susceptible. Assessment of antimicrobial susceptibility test outcomes was carried out in accordance with CLSI-recommended intervals specific to each isolate. *E. coli* ATCC 25922 served as the quality control standard. The results are illustrated in Fig 3a.

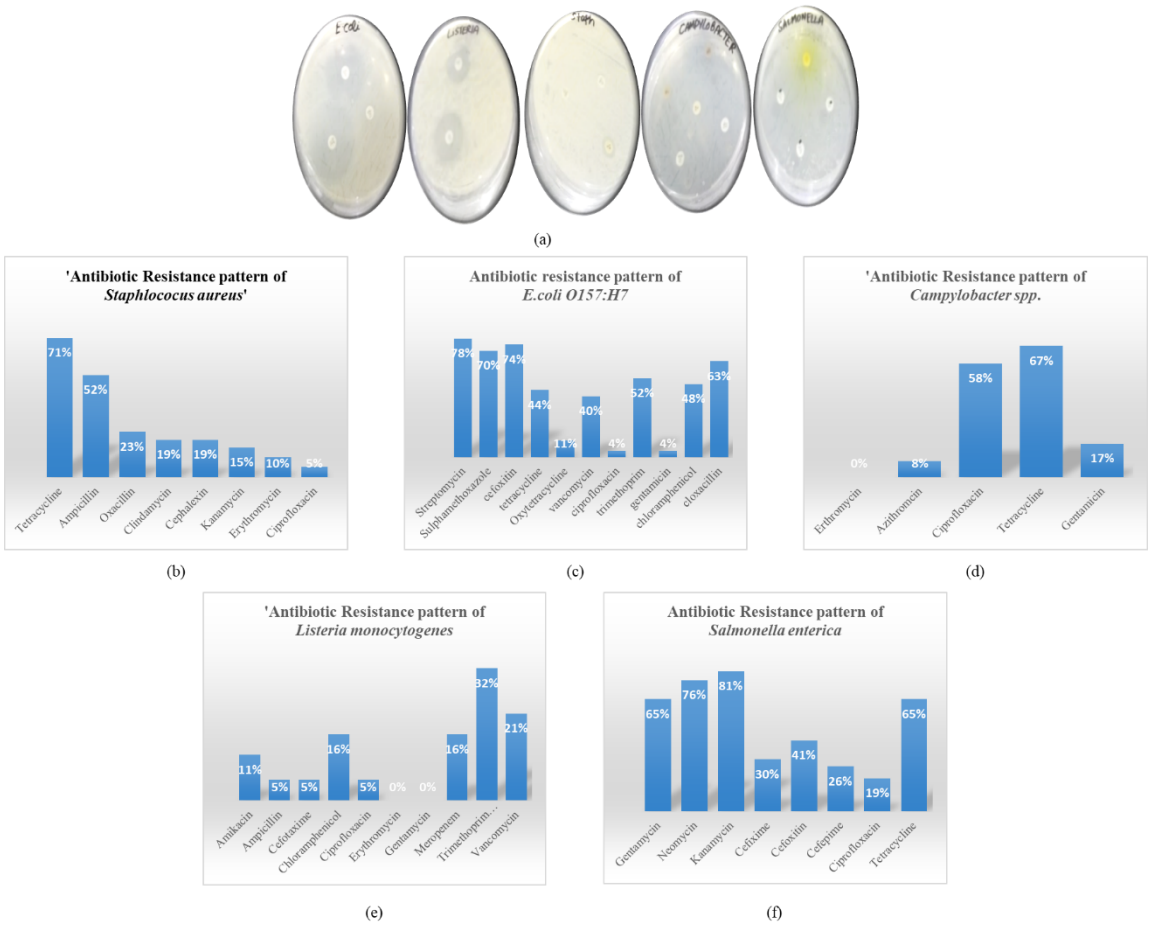

Figure 3: Antibiotic resistance pattern of five targeted bacteria. (a) Antibiotic resistance pattern of five targeted bacteria (b) Antibiotic resistance pattern of *Staph. aureus* (c) Antibiotic resistance pattern of *E. coli O157:H7* (d) Antibiotic resistance patterns of *Campylobacter spp* (e) Antibiotic resistance patterns of *Listeria monocytogenes* (f) Antibiotic resistance patterns of *Salmonella enterica*

The findings predominantly indicated high levels of resistance among *S. aureus* to tetracycline and ampicillin. Similarly, *E. coli O157:H7* exhibited notable resistance to

streptomycin, sulfamethoxazole, and cefoxitin. *Campylobacter spp*. displayed significant resistance to tetracycline and ciprofloxacin. *Listeria monocytogenes* exhibited the highest resistance trends against vancomycin and trimethoprim. Additionally, *Salmonella enterica* demonstrated marked resistance to gentamycin, kanamycin, and neomycin.

Prevalence of raw milk samples with the optimized assay

A comprehensive dataset comprising 231 random samples of raw milk was gathered from various sources including dairies, retail milk shops, local households, and milkmen pots across different subdivisions of district Faisalabad. Of these samples, 117 yielded positive results. Ensuring strict adherence to sterile protocols, the samples were collected in sterile containers and promptly dispatched to the laboratory, maintaining a temperature range of 2-8°C using insulated iceboxes to minimize any potential degradation. These samples were then promptly examined upon arrival. The consolidated findings from all surveyed areas are presented in Table 5 for reference.

Table 5: An overall prevalence of all the pathogens from the targeted territories

Milk samples N = 231	***E. coli O157 H7***	***Salmonella enterica***	***Listeria monocyte genes***	***Staph aureus***	***Campylo… bacter spp***	**Total No. of Positive isolates**	**Total No. of Positive samples**
Dairy Farms (100)	**24**	**17**	**9**	**11**	**8**	**69**	**45 (45%)**
Milkmen Pots (50)	**14**	**11**	**4**	**4**	**3**	**36**	**26 (52%)**
Retail Milk Shops (50)	**17**	**10**	**5**	**7**	**4**	**43**	**27 (54%)**
Local Household (31)	**13**	**5**	**4**	**6**	**2**	**30**	**19 (61%)**
Total (231)	**68**	**43**	**22**	**28**	**17**	**178**	**117 (51%)**

The primary urban center, Faisalabad, exhibited the highest prevalence at 57%, with Jhumra (54%) and Samundri (52%) subdivisions following closely. Additionally, Table 6 provides a detailed breakdown of bacterial species load across all subdivisions. Statistical

analysis revealed no significant correlation between the sampling location within the city and the ratio of positive samples.

Table 6: Prevalence of bacteria in locally prepared dairy products

Subdivision	Total Screened	Positive samples	Prevalence	Chi-Square	P-value
Faisalabad	150	86	57%	0.646	0.9578
Samundri	60	31	52%		
Jaranwala	60	30	50%		
Jhumra	60	32	54%		
Tandlianwala	51	24	47%		

Risk factors associated with the occurrence of miscellaneous milk-borne pathogens

In this investigation, we evaluated four distinct sources of milk (dairy farms, milkmen pots, retail milk shops, and local households) as potential reservoirs for a substantial presence of pathogens (refer to Table 7). A chi-square test of independence was conducted to assess the association between milk sources and the prevalence of bacterial contamination. The results of the chi-square analysis indicated a lack of significance in the relationship between these factors. Consequently, no noteworthy correlation was observed between the sampling source and the presence of bacterial contamination.

Table 7: Association of milk source with the occurrence of bacterial contamination

	N. Examined	Positive	Positive %	Chi- square	p-value
Dairy Farms	140	65	46.42	2.36	0.501
Milkmen Pots	70	35	50		
Retail Milk Shops	70	38	54.28		
Local Household	101	65	64.35		
Total	381	203	53.28		

Moreover, the study examined eight (8) potential risk factors associated with the transmission of milk-borne pathogens originating from dairy farms. These factors encompassed herd size, farm type, flooring material, milking method, cattle body condition, age, farm sanitation practices, and udder hygiene (see Table 8 for details). Statistical evaluation, outlined in Table 8, revealed that herd size, age, flooring material, farm sanitation practices, milking method, and udder hygiene demonstrated no statistical significance, with p-values exceeding 0.05, whereas farm type emerged as statistically significant. Analysis of odds ratios and relative risks predominantly indicated values exceeding 1, suggesting a heightened likelihood of pathogenic presence under these circumstances.

Table 8: Association of dairy farms associated risk factors with the occurrence of bacterial contamination in the raw milk

Variables	Category	N. examined	Positive	Chi-square	p value	Relative Risk	Odds Ratio
Herd Size	<10 10-16 >16	57 63 20	23 32 10	0.53016	0.767	1.03	1.09
Type of Floor	Concrete Soil	93 47	33 32	0.0015	0.969	1.24	1.92
Miking Type	Hand Machine	91 49	45 20	0.3558	0.551	0.94	0.83
Body Condition	Good Moderate Bad	45 59 36	11 29 25	3.8274	0.147	1.22	1.81

Age in years	3 4 5 6 7	19 34 40 27 20	9 16 19 13 8	0.1499	0.997	0.98	0.94
farm hygiene	Poor Good	106 34	52 13	0.4614	0.496	0.93	0.78
Udder hygiene	Washing & Drying Washing Only	49 91	16 49	2.21	0.137	1.16	1.65
Type of Farm	*Semi Intensive **Intensive	81 59	51 14	8.218	0.004	0.76	0.38

*Semi Intensive: Small Scale farms (< 50 Animals)

**Intensive: Large Scale farms (> 50 Animals)

DISCUSSION

The focus of this study was also the detection of prominent pathogenic diarrheal bacteria that hold significant implications for human health, including *E. coli*, *Salmonella*, and *Campylobacter*, alongside toxin-producing bacterial strains such as *S. aureus* and *L. monocytogenes*. These pathogenic microorganisms not only pose a threat to public health but also contribute to substantial economic losses. Despite the critical importance of microbiological hazard analysis across various stages of food production, the detection of bacterial pathogens at the on-site production unit remains a significant challenge in achieving a dependable level of accuracy (Gupta et al., 2022).

Furthermore, this optimized protocol could be considered a reliable method for detecting food-borne pathogens in raw milk samples. Notably, this study marks the first attempt in Pakistan to optimize the TD multiplex PCR assay for the simultaneous detection of five predominant raw milk-borne bacterial pathogens directly from milk samples. Its implementation stands to significantly benefit the food industry due to its accuracy and robustness (Ali et al., 2024).

The secondary aim of this investigation was to assess the antibiotic susceptibility patterns and prevalence rates of specific pathogens across five distinct Tehsils within district Faisalabad. As identified globally, key foodborne pathogens in animal-origin foods encompass *Salmonella*, *Campylobacter*, *E. coli*, and *Staphylococcus* (Shafiq et al., 2019), with comparable trends evident in our current findings. Consistent with prior studies

conducted domestically and internationally (Al Amin et al., 2022; Bag et al., 2021; Rahman et al., 2020), our results highlighted predominant resistance of *S. aureus* to tetracycline (71%) and ampicillin (52%). Similarly, resistance was observed in *E. coli O157:H7* against streptomycin (78%), sulfamethoxazole (70%), and cefoxitin (74%). *Campylobacter spp.* exhibited notable resistance to tetracycline (67%) and Ciprofloxacin (58%), while *Listeria monocytogenes* showed elevated resistance to Vancomycin (21%) and Trimethoprim (32%). Furthermore, *Salmonella enterica* demonstrated heightened resistance to Gentamycin (65%), Kanamycin (81%), and Neomycin (76%), consistent with findings by Rafiq et al. (2022).

Regarding the prevalence rates of milk-borne pathogens across Faisalabad district, Faisalabad city exhibited the highest incidence (57%). Plausible explanations for this heightened milk contamination include the utilization of unpasteurized milk for commercial purposes, suboptimal hygiene practices, inadequate cooling, and the absence of standard facilities for milk milking, storage, and transportation. Substantial evidence indicates that microbial contamination within the milk market chain can stem from diseased cattle, unhygienic milking practices, poor personal hygiene, unsanitary utensils, and/or milking equipment, as well as inadequate storage conditions and a lack of access to pure water (Berhe et al., 2020; Asfaw et al., 2023). Studies have reported inadequate hygienic practices throughout the dairy production system in developing nations like Ethiopia, where standard milking protocols are lacking, exemplified by the absence of teat disinfection before milking and insufficient handwashing practices (Berhe et al., 2020; Birhanu et al., 2022).

The risk factor analysis indicated that variables such as herd size, age, type of floor, farm hygiene, milking type, udder hygiene, and animal health condition were statistically insignificant, with a p-value greater than 0.05. However, the risk factor associated with farm type was deemed statistically highly significant, with a p-value of 0.004.

With the continual progress in science and technology, there arises an imperative in the contemporary era to devise innovative, resilient, and efficient molecular methodologies for identifying microorganisms pivotal to public health. Polymerase Chain Reaction (PCR) stands out as a precise and expeditious molecular technique, replicating specific DNA segments to generate multiple copies. Key parameters including total cycle count, primer length, purification concentration of the DNA template, dNTPs, and Mg2+ concentration are meticulously optimized to craft an effective PCR protocol (Tao et al., 2020). Consequently, protocol optimization for PCR proves to be a laborious, time-consuming endeavor.

In response to this challenge, an alternative method is introduced, leveraging the optimization of Touchdown PCR (TD-PCR) for amplifying target DNA segments. TD-PCR distinguishes itself from conventional PCR techniques by employing a range of annealing temperatures rather than a fixed temperature. This variance in annealing temperatures serves to mitigate non-specific binding, such as primer-dimer formation. The

efficacy of PCR for pathogen detection hinges significantly on the purity of the extracted DNA template and the abundance of target molecules (Kim et al., 2020).

Detecting pathogens in raw milk presents a formidable challenge among food-borne bacteria due to the myriad of benign microbiota inherent in milk's complex composition. Furthermore, food items harbor PCR inhibitors, thereby impeding the effectiveness of PCR-based assays, diminishing sensitivity, and yielding false negative outcomes. Consequently, efficient DNA extraction and the removal of inhibitory elements are essential for the successful detection of food-borne pathogens (Kim and Oh, 2021).

The detection of pathogens in raw milk samples presents challenges due to the inhibitory effects of milk fats and proteins on DNA extraction. Additionally, the complex composition of raw milk complicates DNA extraction, affecting both the quantity and quality of DNA. In this study, alongside optimizing the TD multiplex PCR assay, a novel method was employed to extract purified DNA from milk. Prior to DNA extraction, low-speed centrifugation of raw milk was conducted to reduce the formation of the cream layer, which contains PCR inhibitors. Manual removal of this layer proved beneficial.

As discussed previously, in TD-PCR, annealing temperature is adjusted to enhance specificity by initiating cycles at a slightly higher temperature than primer Tm, minimizing nonspecific binding and unwanted amplification. However, this may lead to reduced PCR yield. To address this, annealing temperature is gradually decreased by 0.5 to 1°C per cycle, achieving an optimal temperature for desirable amplicon production. Consequently, the desired amplicon is amplified without encountering nonspecific binding challenges during PCR cycles (Nischala et al., 2022; Okulmus and Şimşek, 2023; Achyar et al., 2022; Asif et al., 2021).

In this study, both single and multiplex TD-PCR were optimized and applied for detecting five targeted bacterial pathogens in raw milk. A comparison between single and multiplex TD-PCR results was established by analyzing an unknown raw milk sample with five specific primers for bacterial pathogens. Molecular characterization revealed the presence of only two bacterial pathogens in the naturally contaminated raw milk samples. In multiplex TD-PCR, 35 cycles were conducted with a gradual decrease of 0.5°C in annealing temperature for the first 20 cycles. In contrast, single PCR maintained a constant annealing temperature, often lower than primer Tm, resulting in nonspecific bindings. Therefore, multiplex TD-PCR is advantageous for specific amplification due to its higher annealing temperature. Results indicated the detection of one or two additional bacteria through multiplex TD-PCR compared to traditional microbiological techniques, suggesting that current molecular techniques cannot differentiate between dead and viable cell counts, leading to false-positive results. These findings corroborate those of Waldman et al. (2020) and Lechner et al. (2021).

Prior to DNA extraction, a preliminary stage known as "pre-enrichment" in Enrichment and Lysis Selective Saponin (ELSS) media aids in the recovery of damaged cells and enhances bacterial yield within the sample (Prabhakara et al., 2022; Sharma et al., 2022).

Conversely, conventional microbiology-based culture techniques fail to detect viable but non-culturable bacteria. Additionally, the presence of competitive bacteria may impede the growth of target bacteria by utilizing the nutrients in the culture medium (Prabhakara et al., 2022; Sharma et al., 2022). These challenges are circumvented by TD multiplex PCR and pre-enrichment, resulting in increased detection of positive pathogens through molecular assays compared to culture-based methods.

Traditionally, multiplex PCR has been employed to simultaneously detect multiple pathogens in a single assay, offering time-saving and simplified procedures. However, previous studies have shown unsatisfactory sensitivity in conventional multiplex PCR, with results indicating 104 CFU/ml for *S. aureus* and *L. monocytogenes*, and 103 CFU/ml for *Bacillus cereus* (Wei et al., 2019). Similarly, multiplex PCR sensitivity for detecting *L. monocytogenes, E. coli O157:H7,* and *Salmonella* species is reported as 106 CFU/ml (Kim and Oh, 2021), and 103 CFU/ml for *Salmonella* species such as *S. agona* and *S. enteritidis* (Silva et al., 2011). However, Yue et al. (2021) demonstrated a higher detection sensitivity of 200 CFU/ml for five targeted bacteria using an enriched multiplex PCR assay, including *E. coli O157:H7, Salmonella, L. monocytogenes,* and *S. aureus*. Similarly, Bundidamorn et al. (2021) showed a detection sensitivity of 5 CFU/ml for three specific foodborne bacterial pathogens, namely *E. coli O157:H7, L. monocytogenes,* and *Salmonella* species, using multiplex PCR on food samples.

The primary aim of elucidating the various potential outcomes regarding the detection sensitivity of the conventional multiplex PCR assay was to establish a comparative framework with the multiplex TD PCR utilized in the current investigation. A detection sensitivity of at least 5 CFU/mL was documented for the concurrent identification of food-borne bacterial pathogens. However, the present study has determined a detection sensitivity of 1 cell/mL through both single and multiplex TD-PCR methodologies, thus ensuring heightened accuracy. Consequently, it can be inferred that this assay demonstrates superior efficiency, time-saving attributes, and enhanced sensitivity when contrasted with previous techniques (Smith et al., 2023).

Presently, efforts are directed towards addressing two primary challenges in order to mitigate the risk of raw milk-borne infections among consumers. This encompasses the development and optimization of innovative bioassays and molecular techniques for pathogen detection. Likewise, in line with technological advancements, there is a need for the introduction of improved strategies aimed at enhancing the robustness of existing molecular techniques and implementing effective methodologies within the field (Jones & Brown, 2021).

Conclusion

At present, it is evident that significant strides have been achieved in the molecular detection of bacterial pathogens within the food industry. However, there exists a gap for gradual uptake of these enhanced nucleic acid-based detection methodologies within the

commercial sector and regulatory bodies overseeing food quality. Furthermore, a void persists within the research area, particularly concerning the robustness, sensitivity, specificity, and efficacy of diagnostic methods pertaining to distinct food systems. This study conducted comparative analyses of molecular technique against traditional microbiological techniques and suggests replacing them with novel molecular approaches, which offer rapid and notably precise outcomes. In the future, the risk factors study could be expanded to explore how different risk factors affect the quality of the food so that the root cause could be interventioned at the grass root level.

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