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Molecular detection of pathogenic bacteria in bovine milk samples collected from local vendors in Jabalpur

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Abstract

Milk provides essential nutrients for growth but also serves as a medium for various microbial growth. The milk gets contaminated with some pathogenic microbes, either from infected udder or due to poor milking practices like uncleaned udder and teat, unhygienic hands, uncleaned milk containers, improper sanitation of milking equipment, contaminated water source etc. The pathogenic microbes that contaminate milk Poses serious threats to public health and produce milk-borne diseases. It is essential to study the quality of milk supplied by the local vendors to avoid outbreaks of any food-borne illness. Identification of pathogens in milk samples is one of the important quality parameters. Conventional detection methods like bacterial culture and microscopy are time-consuming and less accurate. Molecular techniques like polymerase chain reaction offers rapid detection of pathogens with high sensitivity and specificity. This study utilized polymerase chain reaction to detect the presence of *Staphylococcus aureus*, *Brucella* spp., and *Escherichia coli* in milk samples collected from vendors in Jabalpur, India. A total of 40 samples were analyzed and results showed contamination rates of 37.5% for *Staphylococcus aureus*, 7.5% for *Brucella* spp., and 15% for *Escherichia coli*. The findings underscore the importance of implementing proper hygiene measures and routine microbial surveillance in dairy practices to ensure public health safety and to prevent the development of foodborne illnesses due to contaminated pathogenic microorganisms.

Keywords bovine milk, *Escherichia coli*, milk vendors, PCR, *Staphylococcus aureus*

Introduction

Milk has long been considered as the ideal nourishment for humans since it provides all the nutrients essential for accelerated and healthy development of the body. At the same time, milk supports the growth and multiplication of various pathogenic microbes and causes foodborne illness. Among microbes, bacterial pathogens are predominately found in contaminated milk and include *Staphylococcus aureus* (*S. aureus*), *Brucella* spp., toxin producing *Escherichia coli* (*E. coli*), *Streptococcus* spp., *Salmonella* spp., *Listeria monocytogenes*, *Mycobacterium* spp., *Campylobacter* spp., *Yersinia enterocolitica*, *Coxiella burnetii* [1]. The sources of milk contamination with bacterial pathogens include infected mammary tissue (i.e. mastitis), body discharges from infected animals, unhygienic milking practices like unhygienic hands, uncleaned milking equipment, dirty udder and teat, uncleaned milk containers for

storage and transportation, contaminated water sources etc. [2]. The contaminated milk can spread pathogenic bacteria rapidly among consumers under the right circumstances and cause outbreaks of foodborne diseases. Therefore, the detection of milk-borne bacterial pathogens in raw milk is critical for the safety of human health. Again, timely and accurate detection of milk borne pathogens is the crucial factor in the prevention of the occurrence of any foodborne illness, for efficient health management of humans and cattle, and to reduce economic losses to the dairy industry.

The conventional method used for detecting bacteria in various food items including milk is bacterial isolation, followed by microscopic and biochemical identification [3]. However, these methods are laborious, may need selective enrichment growth media for growth, are time-consuming, less sensitive, and involve risk to laboratory workers in the case of *Brucella* spp. [4-5]. Since the last three decades the molecular technique, of polymerase chain reaction (PCR) and its recent advancements, have been used in the detection of different pathogens contaminating food items including milk and other dairy products by targeting the pathogen's genomic nucleic acid sequences for identification [6,7]. Simple PCR and its variants can also detect several pathogens simultaneously in less time, with high sensitivity, and high specificity as compared to the conventional bacterial culture method [8]. Considering the advantages of PCR, it is being employed in routine microbial surveillance of animal origin food.

In the present study, three bacteria important for public health namely, *S. aureus*, *E. coli*, and *Brucella* spp. were targeted for their detection in raw milk samples. *S. aureus* is a gram-positive, facultative anaerobic bacterium and is responsible for one-third of cases of bovine mastitis [9]. It releases several heat-stable enterotoxins and causes food poisoning [10]. *E. coli* is a gram-negative, facultative anaerobic bacterium that is normally found in human and animal intestinal tracts as commensals. However, some of its pathogenic strains produce several toxins and cause food poisoning upon consuming contaminated food materials [11]. *Brucella* spp. is gram-negative, intracellular facultative pathogens, which cause disease brucellosis in domesticated livestock. Some strains of *Brucella* spp. cause intermittent fever, malaise, and anorexia conditions in humans [12].

India is one of the world's biggest milk producers. In India, small dairy farms are the main source of milk production. These small dairies don't follow hygienic practices during milking and its distribution to consumers. Milk vendors are an important factor in the origin of foodborne diseases [13].

Combining all these facts, this study was designed to assess the presence of *S. aureus*, *Brucella* spp., and *E. coli* in milk samples collected from vendors of the Jabalpur region by using PCR techniques. Several reports are available where PCR has been used in detecting *S. aureus* [14-15]. *E. coli* [16-17], and *Brucella* spp. [18-19].

Methodology

Area under study and place of study

A total of 40 milk samples were collected in the month of May and June 2023 from different locations of Jabalpur city (approximate location 23o 10' 13'' N and 79o 56' 00'' E) which include Kanchanpur, Adhartal (approximate location 23° 12' 52'' N and 79° 57' 17'' E), Gwarighat (approximate location 23° 06' 29.02" N and 79° 55' 41.99" E), Garha (approximate location 23.1616° N and 79.8994° E), Vehicle Factory Jabalpur (VFJ) (approximate location 23°12'44" N and 79°58'41" E), Tilwara (approximate location 23.1079° N and 79.8758° E), Panhera (approximate location 23° 11' 23" N and 79° 58' 23" E) and Sadar (approximate location 23°9'10" N and 79°56'46" E).

Sample collection

The milk samples were collected from the milk vendors at their doorstep in sterile 15 ml screw-cap centrifuge tubes. The milk samples were collected from the bulk tank containing pooled milk. Immediately, the tubes were transported in an ice box to the laboratory, Animal Biotechnology Centre, Nanaji Deshmukh Veterinary Science University (NDVSU), Jabalpur, Madhya Pradesh. The milk samples were kept in the refrigerator and further DNA isolation process was performed within two hours of collection. Proper sterilization and hygienic conditions were maintained during the whole process. The samples that were

found positive in PCR for target bacteria were further confirmed by re-collection of milk samples followed by retesting. Before milk sample collection, hygienic conditions like udder washing, hand sterilization, use of cleaned utensils, etc. were strictly followed to minimize any exogenous microorganism contamination.

Extraction of genomic DNA

The extraction of DNA from the pure culture of bacteria was performed by using a QIAamp DNA mini kit (Qiagen). Isolation of *S. aureus*, *Brucella* spp., and *E. coli* was performed performed as per the protocol given by K. Wilson [20], but with a pre-extraction sample processing step [20]. Genomic DNA extraction from milk samples was performed by the conventional method of cell lysis followed by the phenol-chloroform phase separation. Briefly, 1 ml of TE (10mM Tris-Cl and 1mM EDTA, pH=8.0) buffer was added to 1 ml of milk sample and vortexed for 1 minute. The microcentrifuge tube was centrifuged at 5000g for 5 min at 4°C.

The upper cream layer and supernatant (whey part) layer were discarded. The pellet in the tube was resuspended in 1ml of TE buffer and centrifuged at 5000g for 3 min at 4°C. The supernatant was discarded, and this step was repeated for another two times. After three washing, the pellet was resuspended in 560µl of lysozyme solution (20mg/ml lysozyme, Tris-Cl 10mM, 1mM EDTA, and 1% triton) and incubated at 37°C for 1 hour. Then, 30 µl of 10% SDS and 10 µl of proteinase K (20mg/ml) were added to the tube, mixed by vortexing, and incubated at 55°C for 1 hour. To the cell lysate, 100µl of 5M NaCl (Sodium chloride) was added and mixed well. To this mixture, 80 µl of preheated (65ºC) CTAB (Cetyltrimethylammonium bromide) /NaCl solution was added, mixed well, and incubated at 65°C for 10 minutes. After cooling to room temperature, an equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) was added to the lysate and mixed properly.

The tube was centrifuged at 10000 g for 10 minutes at room temperature. The upper aqueous phase was transferred to a fresh micro-centrifuge tube and to it equal volume of chloroform-isoamyl alcohol (24:1) was added. The content was mixed thoroughly and centrifuged at 10000 g for 10 minutes. The upper aqueous phase was transferred to a fresh 1.5ml micro-centrifuge tube and mixed with 350 µl (0.7 × volume of aqueous phase) of 100% isopropanol. The tube was placed at -20ºC overnight in an upright condition. The next day, the tube was centrifuged at 12,000 rpm for 15 minutes. The supernatant was discarded. The DNA pellet was washed twice with 70% ethanol. After washing, the DNA pellet was air-dried inside the laminar airflow cabinet for about 30 minutes. The dried DNA pellet was dissolved in 50µl of TE buffer.

Quantity and quality assessment of genomic DNA

The genomic DNA purity and quantity were determined by using the nanodrop spectrophotometer (ND-2000, Thermo Scientific). The instrument manufacturer's instruction was followed for quality and quantity measurements. Two microlitres of DNA solution were loaded onto the instrument pedestal to initiate the process. The genomic DNA concentration was determined from the UV absorbance value at 260nm (A_{260}). The purity of isolated genomic DNA was assessed from the ratio of UV absorbance values at 260nm and 280nm ($A_{260/280}$).

Additionally, the integrity of extracted genomic DNA was checked on 0.7% of the agarose gel matrix containing DNA binding fluorescent dye. Five microlitres of DNA solution of each sample was loaded into wells of agarose gel bed. Visualization of genomic DNA bands and image documentation was done in the gel documentation system (SynGene). Irrespective of their visibility on agarose gel, genomic DNA extracted from all milk samples underwent the molecular screening process by PCR.

Polymerase chain reaction assay optimization

Reported primers were used for the detection of *S. aureus*, *E. coli*, and *Brucella* spp. in milk samples. The primer sequences are listed in Table 1.

Table 1. Details of primers

Target pathogen	Primer Sequence (5'-3')	Amplicon length	References
Brucella spp.	forward: GTTCCGGCTCCGGTTGAAGTA	523 bp	21
	Reverse: CGCGGATATCCTGCGTGTC		
E. coli	forward: GACCTCGGTTTAGTTCACAGA	585 bp	22
	Reverse: CACACGCTGACGCTGACCA		
S. aureus	Forward: GTAGGTGGCAAGCGTTATCC	229 bp	23
	Reverse: CGCACATCAGCGTCAG		

The PCR assays for three targets were optimized. The parameters like MgCl2 concentration, DNA template quantity, and primer annealing temperature were considered during PCR optimization. Diluted genomic DNA extracted from a pure culture of bacteria was used as a DNA template in PCR optimization reactions.

In brief, the 25µl of PCR reaction mixture contained 2.5µl of 10X PCR buffer, 2µl $MgCl_2$ (25mM), 0.5 µl dNTPs mixture (10mM), 0.5µl forward primer (20µM), 0.5µl reverse primer (20µM), 0.4 µl Taq DNA polymerase (5U/ µl, Sigma), 4 µl DNA template and final volume adjusted with nuclease-free water. In the NTC (Non-template control) tube, nuclease-free water was added instead of the DNA template. The details of optimized PCR thermal conditions for each target are given in Table 2. The PCR reaction products were subjected to electrophoretic analysis on 1.2% agarose gel followed by image documentation.

Table 2. PCR thermal conditions

Target pathogens	Initial denaturation	Denaturation	Primer annealing	Extension	Final extension
B. abortus	95°C / 5 min	95°C / 30 sec	54°C / 35 sec	72°C /45 sec	72°C / 7 min
E. coli	95°C / 5 min	95°C / 30 sec	56°C / 45 sec	72°C /45 sec	72°C / 7 min
S. aureus	95°C / 3 min	95°C / 30 sec	55°C / 30 sec	72°C /30 sec	72°C / 5 min
	1 cycle	30 cycles			1 cycle

Molecular screening of milk samples by PCR

A total of 40 raw milk samples collected from local vendors were screened by PCR for the presence of *S. aureus*, *Brucella* spp., and *E. coli*. Genomic DNA extracted from milk samples were used as a DNA template for PCR reactions. NTC (negative control) and genomic DNA from pure culture (positive control) were included in every PCR experiment. Ten microliters of PCR product were loaded to 1.2% of agarose gel for electrophoretic analysis.

The milk samples which were found positive for any target bacteria under study were again collected by visiting the vendor's house under strict hygienic conditions. The animal's history of past mammary tissue infection (mastitis) and abortion were collected. The genomic DNA was extracted from all recollected milk samples and subjected to a screening process by PCR assays.

Results

Quality and quantity of extracted genomic DNA

The purity of extracted genomic DNA was determined from their A260/280 ratio. The quantity and purity values of all genomic DNA solutions are given in Table 3. The DNA concentration values of samples were found in between 1.7 to 248.5 µg/ml. The A260/280 values of DNA samples varied between 1.05 to 2.03. The extracted genomic DNA from milk samples was observed as a single band on agarose gel.

Table 3. Quantity and purity ($A_{260/280}$ value) of genomic DNA extracted from milk samples

S.N.	DNA Quantity (µg/ml)	$A_{260/280}$ value
1	11.7	1.36
2	8.0	1.41
3	10.0	1.72
4	11.3	2.0
5	6.4	1.1
6	10.9	1.47
7	5.6	1.63
8	9.6	1.39
9	2.0	1.38
10	5.4	1.5
11	1.7	1.91
12	10.6	1.46
13	2.5	1.87
14	8.6	1.43
15	5.0	1.32
16	9.4	1.50
17	10.0	1.69
18	10.1	1.87
19	13.9	1.81
20	13.0	1.99
21	16.0	1.70
22	7.9	1.99
23	20.2	1.99
24	23.0	1.5
25	59.1	1.7
26	4.0	1.09
27	106.7	1.48
28	6.5	2.03
29	30.0	1.5
30	40.9	1.4
31	6.9	1.1
32	1.9	1.05
33	28.3	1.4
34	17.5	1.47
35	248.5	1.89
36	63.4	1.94
37	39.0	1.65
38	30.5	1.81
39	11.0	1.70
40	39.12	1.90

Polymerase chain reaction optimization

The PCR reaction condition was optimized for all three targets to obtain maximal amplification. The optimized PCR reaction mixture includes; 1X PCR reaction buffer, 0.2mM dNTP mixture, 2mM Magnesium chloride, 0.4 µM forward primer, 0.4 µM reverse primers, 2.5 units Taq DNA polymerase, and 5ng of DNA template. The optimized thermal condition for each target is given in Table 2.

Agarose gel electrophoresis analysis of PCR products

The PCR products were visualized on 1.2% agarose gel. The PCR products formed bands of size 229 bp for *S. aureus* (Figure 1a), 585 bp for *E. coli* (Figure 1b), and at 523bp for *Brucella* spp. (Figure 1c). There were no amplified products observed in NTC (negative control) thus negating any environmental contamination during reaction set-up.

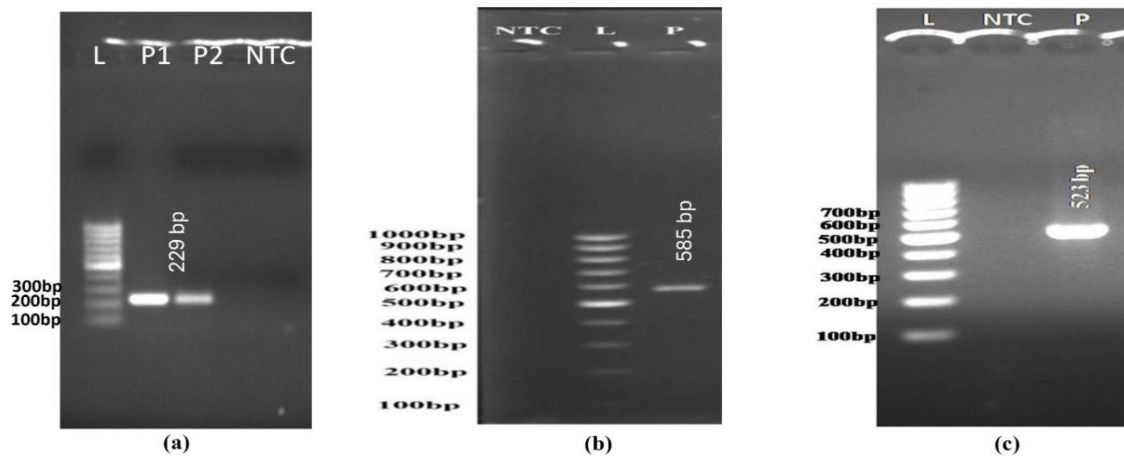

Figure 1. Agarose gel electrophoresis of PCR amplified region of targeted bacteria
(a) *S. aureus* (229 bp amplified product) (b) *E. coli* (585 bp amplified product) (C) *Brucella* spp. (523bp amplified product)
L: 100bp DNA ladder; P, P1&P2: PCR amplified products, NTC: Non template control

Screening of milk samples for targeted bacteria

Out of forty samples, the PCR detected *S. aureus* genomic DNA in fifteen samples, *Brucella* spp., genomic DNA in three samples, and *E. coli* genomic DNA in six samples.

Discussion

For ages, milk has been regarded as the perfect food for humans due to its wholesome nutrient content needed for a body's fast and healthy growth. Due to its excellent nutrient content, milk acts as a unique growth medium for several microbes, both non-pathogenic and pathogenic. Contamination of milk with pathogenic microorganisms is responsible for various milk-borne diseases in humans. Animal diseases where causative microorganisms are secreted into milk, the uncleaned udder and teat skin surface, unhygienic human hands, uncleaned milking machines, uncleaned milk containers, water, etc. are the reasons for milk contamination. The pathogenic microorganism-contaminated raw milk supplied by local vendors creates serious health hazards among consumers. The evaluation of the microbial quality of raw milk by suitable diagnostic tools could minimize the development of foodborne illnesses.

In the present study, the raw milk supplied by local vendors to consumers was screened for the presence of three bacteria; *S. aureus*, *Brucella* spp., and *E. coli* by using the molecular tool polymerase chain reaction.

At the beginning of the study, forty raw milk samples were collected from different vendors in Jabalpur. Appropriate precautionary measures like sterile sample containers, sanitized hands etc. were taken to minimize external contamination of raw milk. Further to prevent the growth of milk spoilage microbes, the milk samples were transported to the laboratory in ice boxes immediately and stored inside the refrigerator till genomic DNA extraction.

The fat layer and the milk proteins pose notable challenges to efficient DNA extraction from milk [24]. It is the DNA quantity that represents the bacterial population in the milk sample and is the crucial parameter for PCR-based detection of target bacteria. Therefore, in this study, additional steps like removal of the fat layer and washing of the cell pellet with TE buffer were included.

Assuming that the collected milk samples contain both Gram-positive (*S. aureus*) and Gram-negative (*E. coli* and *Brucella* spp.) bacteria; treatment of cell pellet with enzyme solution (containing lysozyme, triton-X, and Tris-EDTA) was performed. There is no need to follow separate methods of DNA extraction for Gram-positive and Gram-negative bacteria and hence saves both labor and time. Conventional phenol-chloroform-based liquid phase separation followed by DNA precipitation methodology was used for total DNA extraction from milk samples. The solid phase spin column-based

DNA extraction kit (Qiagen) was used for the extraction of DNA from the pure culture of bacteria. The spectrophotometric analysis revealed that the $A_{260/280}$ value for most of the extracted DNA samples found between 1.7-2.0 with 1.05 as the lowest and 2.03 highest ratio value. The pure DNA generally has an absorbance ratio ($A_{260/280}$) value of nearly 1.8. The lower $A_{260/280}$ value of some purified DNA samples indicated the presence of protein, phenol, and other additives [25], suggesting the need for further improvement in the DNA purification process. The concentration of purified DNA, from milk samples, was found in between 1.7 to 248.5 µg/ml. The purified DNA may contain the genomic DNA of both targeted bacteria and non-targeted cells (exfoliated mammary epithelial cells and other microorganisms). However, the concentration of DNA was found to be in sufficient quantity for downstream applications such as PCR-based detection of pathogens. The primer sequences used to detect targeted pathogens in this study were already employed in separate studies to detect several toxicogenic *S. aureus* strains, prevalence and identification of *E. coli* in rectal swabs of cattle, and development of multiplex PCR for *Brucella* spp. (references mentioned in table 1). In the present study, these primers were used to detect the above bacteria in milk samples. The target sequence amplification conditions (reaction mixture and thermal) were optimized for each target by using these reported primers. The PCR-amplified products, of each target sequence, on agarose gel formed bands of the same size as reported in earlier studies and analyzed in *silico*. In NTC, no target sequence amplified band was observed and thus, demonstrated the absence of environmental aerosol contamination of the template. After PCR optimization, purified DNA from each milk sample was used as a DNA template and subjected to PCR for the detection of bacteria species under study. Both NTC and positive controls were included in each PCR experiment to negate false positive and false negative results. It was found that 37.5%, 7.5%, and 15% of milk samples were found positive for *S. aureus*, *Brucella* spp., and *E. coli*. On-site re-collection of milk samples at the vendor's house and retesting by PCR also produced the same result. The inclusion of positive and negative controls during PCR validated the PCR experiments. Additionally, the collected animal's disease history supported and validated the PCR result. The cows found positive for *Brucella* spp., had a history of abortions. History of mastitis and continuing subclinical mastitis in some cows could be the reason for the presence of S. aureus and E. coli in some milk samples. The previous studies reported by Shrivastava et. al., [26-27], on the characterization of prevalent *S. aureus* demonstrated the presence of 16.47 % of methicillin resistant *S. aureus* (MRSA) in mastitis milk samples and 10.53% panton-valentine leucocidin positive pathogenic *S. aureus* collected from dairy farms in Jabalpur [26-27]. The present study targeted conserved sequence regions of genomic DNA so that all types of *S. aureus* in milk samples can be detected. This could be the reason for the higher prevalence (37.5%) of *S. aureus* in Jabalpur as compared to previous studies. Available reports showed the occurrence of 23.33% of Shiga toxin producing *E. coli* in milk and dairy products collected in and around Jabalpur [28].

Conclusion

Exploiting polymerase chain reaction (PCR) for detecting milk borne pathogens in raw milk from local vendors in Jabalpur, India, has revealed the presence of *S. aureus*, *Brucella* spp., and *E. coli* with prevalence rates of 37.5%, 7.5% and 15% respectively. The previous reports and present study clearly indicated the presence of *S. aureus*, *E. coli*, and *Brucella* spp. in milk supplied to consumers of Jabalpur and thus highlighted the need for screening of milk and dairy products at regular intervals, for common food borne pathogens. The presence of these pathogens in milk emphasizes how important it is to enforce strict hygiene regulations and routine monitoring in dairy operations to protect the public's health.

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