

Research paper

PCR Based Method for Detection of Pathogenic Bacteria in Food

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ABSTRACT

PCR-based detection method for three major foodborne pathogens i.e. *Salmonella* spp., *S. aureus*, and *B. cereus* found in milk was established. *Salmonella* spp., *S. aureus*, and *B. cereus* were detected in the singleplex PCR method. *Salmonella* spp. and *S. aureus* were also detected in multiplex PCR. Primers of *Inv-A*, *hbl*, and *ile S* genes were employed for the detection of *Salmonella* spp., *B. cereus*, and *S. aureus* respectively. *Salmonella* was detected at the genus level and the remaining two pathogens were detected at the species level in PCR tests.

KEYWORDS Multiplex PCR, *Salmonella* spp. *S. aureus*, *B. cereus*

INTRODUCTION

Foodborne pathogens are responsible for food poisoning which is regarded as a serious and ongoing issue in the public health sector all over the world. It is responsible for approximately 76 million illnesses and 5000 deaths annually [1]. *Salmonella* spp., *Escherichia coli*, and *Staphylococcus aureus* are among the ten most common bacteria causing food-borne diseases globally [2]. In recent years, most illnesses due to drinking contaminated milk are attributed to *Salmonella* spp., *Listeria monocytogenes*, *Campylobacter jejuni*, and *Escherichia coli* O157:H7 [3].

Salmonella is regarded as one of the most common pathogens causing food poisoning worldwide [4]. These bacteria are second only to *Campylobacter* spp. for causing gastrointestinal human infections [5]. More than 3000 serotypes of *Salmonella* have been discovered now [6]. Out of which ~100 serotypes cause infections in humans. *Salmonella typhi* is regarded as a major health burden in South Asia [7]. *S. aureus*, is an opportunistic pathogen and is able to cause human diseases ranging from toxin-mediated infections to pyogenic and invasive infections. It can cause community / hospital-acquired infections. *S. aureus* can develop resistance quickly to broad-spectrum antibiotics, such as quinolones,

aminoglycosides, and β -lactam antibiotics [8].

Bacillus cereus has also been associated with food poisoning and causes vomiting and diarrhea in infected people. It can even grow at refrigeration temperatures [1]. Conventional methods for foodborne pathogens detection are labor-some and time-consuming. Preliminary identification of pathogens takes 2 to 3 days while more than a week is needed for confirmation [9]. ISO standardized culture methods take up to ten days for results generation. The steps involved in conventional culture and sensitivity tests are the preparation of pre-enrichment media and selective differential media. It follows culturing and then identification. However conventional methods are well-established and simple, and their raw materials are easily available. Rapid and accurate detection of pathogens in food matrixes is needed for efficient food safety surveillance in society. Early detection of pathogens paves the way for early intervention and prevention of contaminated food products in the market, hence improving food security. Rapid and high throughput bacteria detection methods can be used as efficient tools in quality control and quality assurance (QC/QA) programs in the food industry. Nucleic acid-based methods like multiplex PCR,

qPCR, NASBA (nucleic acid sequence-based amplification), and LAMP (loop-mediated isothermal amplification) are increasingly used for the detection of pathogens. These methods are sensitive, specific, less time-consuming, and labor-saving. They avoid nonspecific, false negative results thus preventing community transmission of pathogenic bacteria. Multiplex PCR was used in this study as it is a fast, easy-to-perform, and high-throughput technique for the simultaneous detection of many pathogens. We aimed to develop a method for the rapid detection of major food pathogens by multiplex PCR.

MATERIALS AND METHODS

Cultures acquisition and preservation

Pure cultures of *Salmonella spp.*, *B. cereus*, and *S. aureus* were acquired from the Laboratory of Microbiology, Mohammad Ali Jinnah University, Karachi, Pakistan. All cultures were sub-cultured and maintained on LB agar.

Artificial inoculation of milk with pathogen culture

250 ml of pasteurized milk (different brands) packets were cut at the tip with sterile scissors and inoculated with one colony of fresh cultures of pathogens with a sterile wire loop. Sealed with tape and placed in a non-shaking incubator overnight. The next day, 15 ml of milk was taken aseptically and used for the enrichment of bacteria.

Enrichment of bacteria in milk

Raw milk samples and artificially inoculated milk from pasteurized 250 ml packets were processed according to the following procedure for the enrichment of bacteria. 15 ml of milk taken from sample containers/packets and diluted with 50 ml of autoclaved distilled water in a flask. Mixed well and then 60 ml of the mixture was transferred in 15 ml plastic falcon tubes. These falcon tubes were centrifuged in a macro centrifuge machine at 4000 rpm for ten minutes. The supernatant was

discarded and 1-2 ml residue from each tube was collected in a single tube and used for DNA extraction.

Bacterial DNA extraction and purification:

Bacterial DNA extraction and purification was done from pure cultures of bacteria and from milk samples by GJC Bacterial DNA Purification Kit according to manufacturer's instructions. Briefly, 1.5 ml fluid was taken into a 1.5/2 ml tube and centrifuged for 10 minutes at 14,000 rpm. Supernatant were discarded. Then added 400 μ L "S1" Buffer from the kit and re-suspended the cells in the pellet. 20 μ L of proteinase K was added and mixed thoroughly by vortexing and incubated at 56 °C for 30 minutes. 200 μ L "S1P" Buffer was added to the sample and mixed thoroughly by pulse-vortexing for 30 seconds. Incubated at 70 °C for 10 minutes. 200 μ L ethanol (96–100%) was added and mixed thoroughly by vortexing for 30 seconds. 600 μ L of the mixture was transferred to the spin column with a 2 ml collection tube. Centrifuged at 8000 rpm for 3 minutes. Filtrate in collection tube discarded. 600 μ L "S3" solution was added and centrifuged for 3 minutes at 10,000 rpm and flow-through discarded. 600 μ L "S4" Solution was added and centrifuged for 03 minutes at 14,000 rpm to dry the membrane in the spin column. Flow-through with collection tube discarded. Spin Column was placed into a new 2 mL collection tube. Incubated the assembly at 56°C for 3 minutes with an open lid to dry the membrane. 20-100 μ L "EB" buffer was added onto the membrane in the spin column, incubated at room temperature for 2 minutes, then centrifuged for 1 minute at 10,000 rpm to elute the DNA.

DNA Gel electrophoresis

Gel electrophoresis was done for viewing DNA and PCR product bands after DNA extraction/PCR. The gel is prepared with 1 % agarose in TAE buffer.

DNA estimation

The quantity of DNA was estimated by using the Quantus™ Fluorometer. A procedure for measurement of ds DNA quantity in single tube format was used.

Polymerase chain reaction (PCR):

PCR reactions were carried out in a PCR machine (PeQ STAR Universal 96 Well Gradient, Manufacturer: PeQLab Biotechnologie GmbH). Primer sequences were prepared by performing in-silico PCR. Further parameters of primer sequences were confirmed by the Oligo analyzer tool at the website of Integrated DNA Technologies IDT. Stock solutions of primers were prepared by adding nuclease-free water in primer tubes as per supplier instructions. Working solutions of primers were prepared to obtain a final primer concentration of 10 pmol/ uL.

Various combinations of reaction mixtures tried to optimize results. GoTaq® G2 Green master Mix and nuclease-free water (Promega Corp., USA) were used. 25 µL total reaction mixture used with 12.5 µL of

master mix for Singleplex PCR reactions. Multiplex PCR was carried out with total reaction mixtures of 50 µL and 25 µL with the master mix.

RESULTS

Here, we report rapid single-plex and multiplex PCR-based methods for the detection of pathogenic bacteria in milk. The polymerase chain reaction test was first set with DNAs extracted from pure bacterial cultures of three bacteria, *Salmonella spp.*, *S. aureus*, and *Bacillus cereus*. Single-plex PCR carried out with DNA extracted from *B. cereus* pure culture resulted in an amplicon of 437 bp (Figure 1a). Likewise, PCR test was carried out using DNA extracted from a pure culture of *S. aureus* and as expected PCR product of 227 bp was obtained (Figure 2b). Single-plex PCR of *Salmonella spp.* carried out with two different primer pairs (Primer pair-I & Primer pair-II) of Inv-A gene having amplicon sizes of 284 bp & 338 bp respectively (Figure 1c&d).

Table 1: Primer sequences with bacterial target genes, and amplicon sizes used during this study.

No.	Pathogen	Gene	Primer Oligonucleotide sequences	Amplicon size
1	<i>Salmonella spp.</i> (primer-pair-I)	Inv-A	139-GTG AAA TTA TCG CCA CGT TCG GGC AA 141-TCA TCG CAC CGT CAA AGG AAC C	284 bp
2	<i>Salmonella spp.</i> (primer-pair-II)	Inv-A	F-TTCTCTTGGCGCCACAATGCGAG R-TCCATCAGCAAGGTAGCAGTC	338 bp
3	<i>B. cereus</i>	Hbl	F-TCATTGATTTGCCGTTGCGTAT R-GTCACATCCATTGTA ACTGGAGGA	437 bp
4	<i>S. aureus</i>	ileS	F-CATACAGCACCAGGTCACGGGGAA R-GTTCTCCAGTCGTGTGGATAGC	227 bp

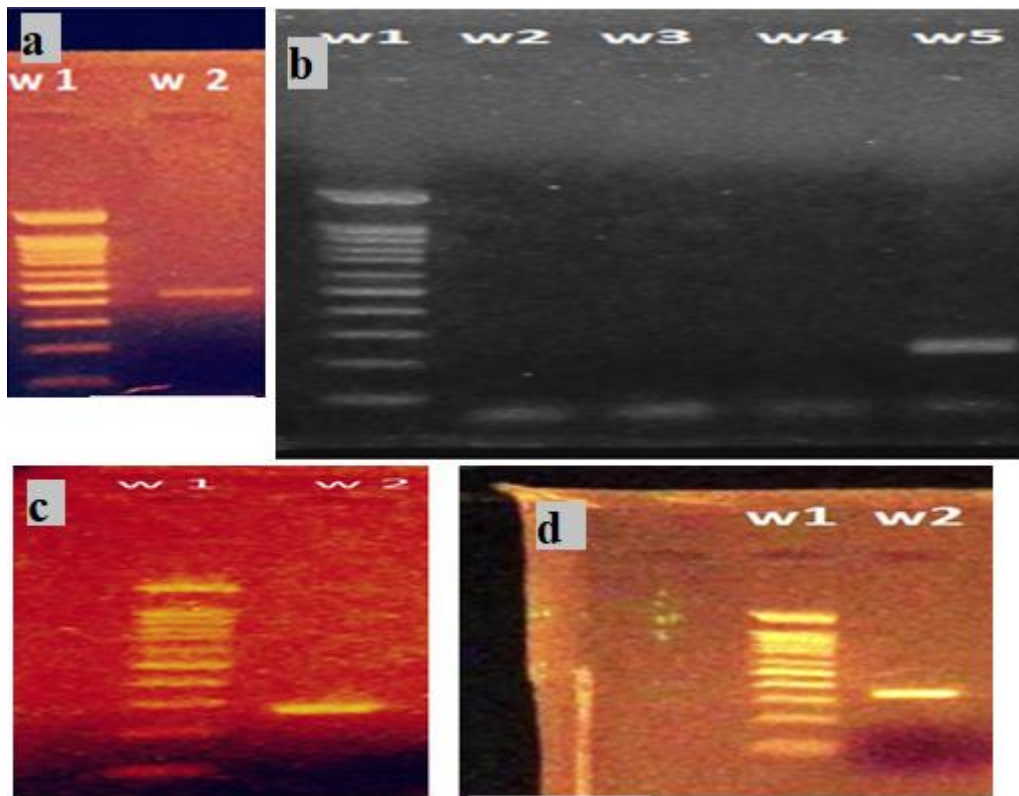


Figure 1: (a) Band of PCR amplicon showing amplification of *Hbl* gene of *B. cereus* (w1= 100 bp ladder; w2=*Hbl* gene amplicon). (b) DNA of PCR amplicon of *ileS* gene of *S. aureus*. (w1: 100 bp ladder, w5: DNA band of *ileS* gene). (c) Band of PCR amplicon of gene *Inv-A* of *Salmonella species* amplified by Primer pair-I (284 bp band; w1= 100 bp ladder and w2=*Inv-A* gene amplicon); (d) Band of PCR amplicon of gene *Inv-A* of *Salmonella species* amplified by Primer pair-II (338 bp band; w1=100 bp ladder and w2=*Inv-A* gene amplicon).

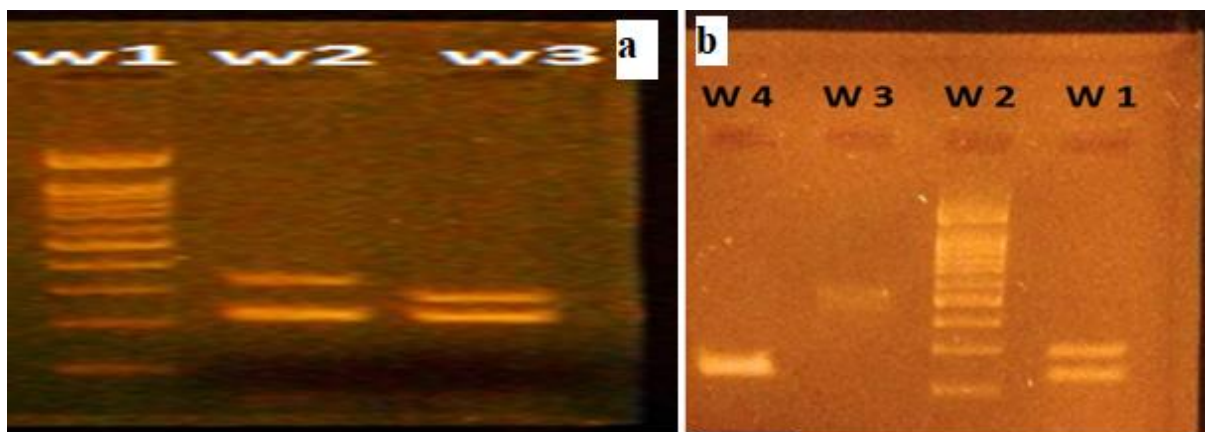


Figure 2: (a) Gel picture of multiplex PCR of DNA purified from *Salmonella spp.* and *S. aureus*. (w1: ladder 100 bp, w2: *S. aureus* (227 bp band) and *Salmonella spp.* (338 bp band) in primer pair-I, w3: *S. aureus* (227 bp band) and *Salmonella spp.* (284 bp band) with primer pair-II. (b) Gel picture of multiplex PCR after inoculation of *S. typhi*, *S. aureus* and *B. cereus* in milk. (w1: *Salmonella spp.* (284 bp band, Primer pair-I) and *S. aureus* (227 bp band) whereas *B. cereus* gene could not be amplified, w3: *B. cereus* gene amplified in singleplex PCR (437 bp band), w4: *S. aureus* gene amplified in singleplex PCR (227 bp band).

Multiplex PCR (mPCR) was carried out with two pathogens *Salmonella spp.* and *S. aureus*. *Salmonella* primer pair-I was used in one set of mPCR, and *Salmonella* primer pair-II was used in the second set of mPCR (annealing temperature = 54 °C). PCR products were observed for both bacteria in both mPCR assays (Figure 2a).

In another set of assays, bovine milk was artificially inoculated with bacteria (i.e. *S. typhi*, *S. aureus*, *B. cereus*). The next day, metagenomic DNA was extracted and purified from milk followed by mPCR with an annealing temperature of 55 °C. Multiplex PCR (mPCR) resulted in amplicons specific for *Salmonella spp.* and *S. aureus* and *B. cereus* could not be amplified (Figure 2b).

DISCUSSION

This study intended to set the PCR method for the simultaneous detection of food-borne pathogens i.e. *Salmonella spp.*, *S. aureus*, *B. cereus*. Primers were synthesized for DNA amplification of all these bacteria. Pure cultures of *B. cereus*, *Salmonella spp.*, and *Staphylococcus aureus* were used to establish singleplex and multiplex PCR assays. After successfully achieving PCR product bands from the bacterial DNA obtained from overnight cultures, the PCR assays were carried out using artificially inoculated milk with *Salmonella spp.*, *S. aureus*, and *B. cereus*. Thermo-cycling conditions were set for 90 minutes only.

Salmonella spp. specific PCR bands obtained with two primer pairs of Inv-A genes of *Salmonella spp.* PCR products of two different amplicon sizes (284 bp & 338 bp) were obtained for same the pathogen (Figure 1).

PCR for *B. cereus* was troublesome. Initial PCR attempts either failed to produce the required size bands of PCR product (or a very light band). However sufficient PCR band of *B. cereus spp.* (437 bp) achieved after improving PCR parameters.

Multiplex PCR was tried with the DNA of three pathogens, *Salmonella spp.*, *S. aureus*, and *B. cereus*. Specific bands of *Salmonella spp.* and *S. aureus* were obtained in multiplex PCR. We were unable to obtain the *B. cereus* band in multiplex PCR. Multiplex PCR of *Salmonella spp.* and *S. aureus* was also successfully carried out with artificially inoculated milk with these bacteria. Multiplex PCR of *Salmonella spp.* and *S. aureus* was done at three different DNA amounts (17 ng, 35 ng and, 70 ng) of both bacteria and, intact dark bands were obtained.

CONCLUSION

The high prevalence of Gram-negative bacteria and antibiotic-resistant bacteria in raw milk in Karachi is concerning as several Gram-negative bacteria are pathogenic. We established rapid PCR based bacterial detection methodology for application in dairy industry. By using the method adopted during this study (i.e. pre-enrichment of bacteria, DNA extraction by spin column method, optimized thermo-cycling conditions) results can be obtained within 2-3 hours.

REFERENCES

1. Razei A, Sorouri R, Mousavi SL, Nazarian S, Amani J, Aghamollaei H. Presenting a rapid method for detection of *Bacillus cereus*, *Listeria monocytogenes* and *Campylobacter jejuni* in food samples. Iran J Basic Med Sci. 2017 Sep;20(9):1050-1055. doi: 10.22038/IJBMS.2017.9275. PMID: 29085601; PMCID: PMC5651459.
2. Lopes ATS, Albuquerque GR, Maciel BM. Multiplex Real-Time Polymerase Chain Reaction for Simultaneous Quantification of *Salmonella spp.*, *Escherichia coli*, and *Staphylococcus aureus* in Different Food Matrices: Advantages and Disadvantages. Biomed Res Int. 2018 Sep 25;2018:6104015. doi: 10.1155/2018/6104015. PMID: 30356394; PMCID: PMC6176325.

3. PARVEEN, B., Junejo, Y., Safdar, M., & ÖZASLAN, M., 2021. Molecular characterization of *Escherichia coli* isolated from raw cow milk samples collected from district Bahawalpur, Pakistan. *Zeugma Biological Science*, 2(2), 1-13.
4. Chang YJ, Chen CL, Yang HP, Chiu CH (2022). Prevalence, Serotypes, and Antimicrobial Resistance Patterns of Non-Typhoid *Salmonella* in Food in Northern Taiwan. *Pathogens*. doi: 10.3390/pathogens11060705. PMID: 35745560; PMCID: PMC9229336.
5. Fulgione A, Cimafonte M, Della Ventura B, Iannaccone M, Ambrosino C, Capuano F, Proroga YTR, Velotta R, Capparelli R (2018). QCM-based immunosensor for rapid detection of *Salmonella* Typhimurium in food. *Sci Rep*. 2018 Oct 31;8(1):16137. doi: 10.1038/s41598-018-34285-y
6. El-Sharkawy H, Tahoun A, El-Gohary AEA, El-Abasy M, El-Khayat F, Gillespie T, Kitade Y, Hafez HM, Neubauer H, El-Adawy (2017). Epidemiological, molecular characterization and antibiotic resistance of *Salmonella enterica* serovars isolated from chicken farms in Egypt. *Gut Pathog*. doi: 10.1186/s13099-017-0157-1. PMID: 28203289; PMCID: PMC5301364
7. Havelaar AH, Kirk MD, Torgerson PR, Gibb HJ, Hald T, Lake RJ, Praet N, Bellinger DC, de Silva NR, Gargouri N, Speybroeck N, Cawthorne A, Mathers C, Stein C, Angulo FJ, Devleeschauwer B (2015). World Health Organization Foodborne Disease Burden Epidemiology Reference Group. *PLoS Med*. 2015. doi: 10.1371/journal.pmed.1001923. PMID: 26633896; PMCID: PMC4668832.
8. Amir Afzal Khan, Asad Ali, Nagendran Tharmalingam, Eleftherios Mylonakis, Rabaab Zahra (2020). First report of *mecC* gene in clinical methicillin-resistant *S. aureus* (MRSA) from tertiary care hospital Islamabad, Pakistan. *Journal of Infection and Public Health*.
9. Law, J. W. F., Ab Mutalib, N. S., Chan, K. G., & Lee, L. H. (2015). Rapid methods for the detection of foodborne bacterial pathogens: principles, applications, advantages and limitations. *Frontiers in microbiology*, 5, 770.

