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Research Article

DETECTION OF SENSITIVE FOOD PATHOGENS IN BANANA, COLD MEAT AND MILK BY PCR AMPLIFICATION BASED TECHNIQUE

Brindha Durairaj^{1*}, Kirupa Devaki S², Santhoshkumar Muthu³¹Associate Professor and Head, Department of Biochemistry, PSG College of Arts and Science, Coimbatore, India²PG Student, Department of Biochemistry, PSG College of Arts and Science, Coimbatore, India³Research Scholar, Department of Biochemistry, PSG College of Arts and Science, Coimbatore, India

*Corresponding Author Email: brindhavenkatesh@gmail.com

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ABSTRACT

This study was carried out to detect *Escherichia coli*, *Klebsilla spp.* and *Streptococcus spp.* isolated from food samples (banana, cold meat and milk). The homogenized food samples were subjected to biochemical and molecular techniques. Specific primers for virulent genes of three different bacterial strains were designed and subjected to polymerase chain reaction. PCR reaction was performed with human primers also in order to confirm the nonspecific amplification. Varying concentrations of DNA (100 ng, 50 ng, 20 ng, 5 ng and 1 ng) were chosen from each bacterial strain and PCR was carried out. It was observed that *E. coli* and *Streptococcus* were active in all the concentrations including 1 ng. However, *Klebsilla* did not produce amplification at 1 ng. The results obtained suggest that *E. coli* and *Streptococcus* could be detected and remain active even in 1 ng of the DNA sample.

Keywords: *Escherichia coli*, *Klebsilla* and *Streptococcus*, PCR, banana, cold meat and milk.

INTRODUCTION

Food-borne diseases are widespread and represent a serious threat to health both in developing and developed countries, most severely affecting children, pregnant women and others¹. Many high risk pathogens that cause diseases in human are transmitted through food items². Bacteria that cause these illnesses go undetected because they have no odour and cause no change in the colour or the texture of the food that they are growing on. Therefore it would be fairly easy to cause an epidemic in a fairly large population using bacteria³. Most reported food borne disease outbreaks from consumption of contaminated products were caused by pathogens such as *E. coli* 0157:H7, *Klebsilla* and *Streptococcus*. However, *E. coli* 0157:H7 is considered to be one of the utmost important food borne pathogens⁴. The protection of the food supply requires analysis of the microbiologic quality and safety of commodities that are available for public consumption. As food items frequently contain pathogenic micro-organisms that cause immediate risks to human health, there is a growing interest in the commensal micro-biota associated with food⁵. One of the inherent difficulties in the detection of food pathogens is that these are generally present in very low numbers in the midst of millions of other bacteria⁶. Hence, there is a need for novel, more rapid and specific detection, quantification and characterization methods for food related micro-organisms. Recent advances in molecular cloning and recombinant DNA techniques have revolutionized the detection of pathogens in food⁷. PCR amplification method to detect microorganisms provides

an alternative approach to the conventional microbiological culture based assays. This method has the ability to detect the targeted microorganism in various biological and environmental samples with high specificity and sensitivity within hours instead of days⁸. There are very few systematic scientific evidences available for the detection of food pathogens at very low concentration. The objective of the study was to detect extremely low concentrations of microbes (*Escherichia* 0157:H7, *Streptococcus* and *Klebsilla* species) from food samples using molecular methods.

MATERIALS AND METHODS

Preparation of bacterial strains

Meat, milk and banana were selected as the sources of *Klebsilla*, *Streptococcus* and *E. coli* respectively. Luria Bertoni agar was used for maintaining and cultivating recombinant strains of *Escherichia coli*⁹. Blood agar was chosen as non-selective medium for the isolation and cultivation of many pathogenic and non-pathogenic microorganisms like *Neisseria* and *Streptococcus*¹⁰. 1 g of each sample was homogenized in a mortar and pestle prior to the use. Eight tubes were arranged; 10 ml of saline was taken in the first tube and 9 ml of saline was taken in the remaining 7 tubes. Simultaneously LB agar, nutrient agar and blood agar were prepared for *E. coli*, *Klebsilla* and *Streptococcus* respectively and sterilized. 1 ml of the ground sample was added in the tube having 10 ml of saline (stock). Then 1 ml of sample was transferred from the stock to the first test tube. The dilutions were continued till the last tube. Pour plate method was

followed by transferring 1 ml of required dilution onto the Petri plates. Then the plates were incubated for 24 hours at 37°C. The colonies were found on the agar plates after 24 hours; sub cultured in the broth and was used for DNA isolation.

Biochemical tests

To identify the bacteria strains, IMVIC biochemical tests comprising Indole test, Methyl red test, Voges proskaur test, Citrate utilization test, Catalase test was carried out¹¹.

Mini preparation of bacterial genomic DNA

1.5 ml of the liquid culture was spun in a micro centrifuge for 2 minutes. The supernatant was discarded and re suspended the pellet in 567 µl TE buffer by repeated pipetting. 30 µl of 10 % SDS and 3 µl proteinase K were added. The solution was mixed thoroughly and incubated 1 h at 37 °C. 100 µl of 5 M NaCl was added to the solution and mixed thoroughly. 80 µl of CTAB/NaCl solution was added, mixed thoroughly and incubated for 10 minutes at 65° C. Approximately an equal volume of chloroform/isoamyl alcohol was added and spun for 4 to 5 minutes in a micro centrifuge at 12,000 rpm. The aqueous and viscous supernatant was removed leaving the interface behind. An equal volume of phenol/chloroform/isoamyl alcohol was added and spun at 12,000 rpm for 5 minutes. The supernatant was transferred to a fresh tube. 0.6 volume isopropanol was added to precipitate the nucleic acids (there is no need to add salt since the NaCl concentration is already high) and centrifuged at 12,000 rpm for 5 minutes to pellet out DNA. The DNA was washed with 70 % ethanol to remove residual CTAB and again spun for 5 minutes at room temperature. Supernatant was discarded and the pellet was dissolved in 100 µl of TE buffer¹².

Quantification of DNA

Isolated DNA was quantified by measuring the absorbance at 260 nm and 280 nm. The ratio of absorbance 260/280 was used to determine the quality of the isolated DNA.

PCR amplification

Primers were designed with the online available software Primer 3. The primers were designed for STX1 gene of *E. coli* 0157: H, CELA gene of *Klebsiella* and CPS gene of *Streptococcus* (Table 1). The primers were synthesized commercially (Bio serve India Pvt, Ltd). The primers were designated and named as Forward primer, Reverse primer which gives a product length of 100 bp. *Escherichia coli*- adulterated milk specimens were prepared as follows (Table 3). An *E. coli* culture was grown in Luria-Bertani (LB) Broth at 37°C for 16 h with shaking. A 25 ml aliquot of the culture was centrifuged at 3200 g for 15 minutes. After removal of the supernatant fluid, the pellet was re-suspended in 1 ml fresh pasteurized milk, raw milk or phosphate-buffered saline (0.05 % (v/v) Tween- 20 and PBST and each sample serially diluted in unadulterated matrix to determine the sensitivity of PCR amplification. A 100 µl aliquot of each dilution was also plated, in triplicate, on LB Agar and colony counting was performed using an Eagle-Eye II gel documentation system (Stratagene, La Jolla, CA, USA).

Fresh pasteurized milk and raw milk were individually plated to determine the presence of naturally contaminating micro flora¹⁰.

Reproducibility test

The sensitivity tests were repeated after two days in order to find the activity of the gene. RAPD (Random Amplification of Polymorphic DNA) was carried out in PCR machine where segments of DNA were amplified in random manner¹³. Several arbitrary short primers (8-12 nucleotides) were created and then continued with the PCR using a large template of genomic DNA.

RESULTS AND DISCUSSION

Extraction and identification of pathogens

Presence of bacteria was confirmed in all three food samples (milk, cold meat and banana) by observing the colonies prepared. The pinnate colonies on blood agar were the evidence for the presence of *Streptococcus* in milk sample. Individual colonies of bacteria were found on the Luria Bertuni agar plates which proved the presence of microorganism in the banana source. Large, spherical colonies of bacteria found on nutrient agar plates confirmed the presence of bacteria in cold meat (Figure 1).

IMVIC test

The culture from Luria Bertuni agar gave positive result for indole (Pink colour ring) and methyl red test (Red coloured solution). This confirmed the presence of *E. coli* in Luria Bertuni agar (Table 2). Positive result was obtained for Voges Proskaur test (pink colour solution) and citrate test (green) which confirmed the presence of *Klebsiella* in nutrient agar. The culture from blood agar showed negative results for all four tests. Catalase test developed no effervescence and hence the presence of *streptococcus* was confirmed. DNA isolated from bacteria was made to run on the agarose gel matrix and visible bands were found on the gel matrix. This confirmed the presence of DNA isolated by Cetyl Trimethyl Ammonium Bromide (CTAB) method (Figure 2).

Quantification of DNA

DNA of three different bacteria isolated with the CTAB method was quantified by UV Spectroscopy to check the purity of DNA. The value of purity was found between 1.7 -1.8 in *E. coli* genomic DNA and the concentration ranging from 1000 to 1250 µg/ml was its concentration. Purity of *Klebsiella* genomic DNA was found to be between 1.75 -1.8. The concentration of DNA ranged from 500-750 µg/ml. *Streptococcus* genomic DNA showed the purity between 1.73-1.8 and the concentration ranged from 700-1250 µg/ml.

Primer standardization

The optimum annealing temperatures were fixed by standardization method. The Optimum temperature for STX1 primer was found to be 54°C with 1.5 µl of magnesium chloride concentration. The primers were found to produce good amplification at 54°C and nonspecific bands were found at 56°C. Optimum temperature for protein celA and CPS gene was found to

be 56°C and 57°C respectively. Nonspecific bands were found at 58°C. Primers have been furnished in Table 3.

Molecular identification of bacterial strains

PCR products were run in agarose gel to identify the amplification of genes of *E. coli* (Figure 3). Lane 1 represents ladder DNA, lane 2 lab culture of *E. coli*, lane 3-5 DNA sample with STX1 primers and lane 6-8 sample DNA with human primers. Human primers did not amplify the genomic DNA of the bacteria. In the Figure 4(a), Lane 1 shows ladder DNA, lane 2- lab culture of *Klebsiella*, lane 3-5 sample DNA with *Klebsiella* primers and lane 6 and 7- sample DNA with human primer. Presence of *Klebsiella* gene was confirmed from the results.

Lane 1- ladder DNA, lane 2- lab culture of *Streptococcus*, lane 2-6 sample DNA with primers and lane 7 - sample DNA with human primer. The isolated DNA was amplified with human primers to visualize specific amplification. The results proved that the isolated DNA samples were specific to their respective primers. All the samples of DNA did not produce amplification with human primers which proved the elimination of nonspecific amplification. STX 1 primers were specific to *E. coli*. The serotype of the *E. coli* was found to be 0157:H7. This result was in accordance with previous results¹⁴. Protein cel A gene was specific to *Klebsiella* species. CPS primers were specific to *Streptococcus* species. Similar result was produced earlier¹⁵. The results also proved that the primers which were designed were species-specific and can only recognize particular strain of bacteria.

Sensitivity results

The sensitivity result of *E.coli* produced amplification at all concentrations. The STX 1 gene was active at all concentrations including 1 ng. This evidences that STX 1 gene was active at 1 ng also (Figure 5). The results produced by Rambabu Naravaneni in 2004 used 4 ng of DNA concentration. The sensitivity result of *Klebsiella*, produced amplification at all concentrations except at 1 ng. Protein cel A was active till 5 ng of DNA and the gene lost its activity at 1ng concentration (Figure 6). The molecular weight of *Klebsiella*, was found to be 500 bp. The sensitivity result of *Streptococcus* produced amplification at all concentrations. The CPS gene was found to be active at all concentrations including (Figure 7). The results produced by Martinez (2000) revealed that CPS gene was active in 50 ng of DNA concentration¹⁶.

Reproducibility

The sensitivity tests were repeated after 2 days which proved the reproducing ability of the gene. The virulent genes did not lose their activity even after several days indicating that environmental factors did not influence the activity of DNA. The reproducibility tests were proved positive and thus a rapid and standardized technique for isolation of DNA has been developed. Rambabu Naravaneni in 2004 developed a rapid technique for isolation of pathogens, but only lab cultures were used instead of food samples. Molecular techniques like transformation, ligation and restriction digestion are used to detect a microbe. It may take about 10-14 to produce

results. But by this PCR based technique, the pathogens could be detected within 4 – 5 hours. PCR amplification method provides an alternative approach to conventional methods⁸.

In Figure 8 (a), Lane 1 – ladder DNA, lane 2-6- varying concentrations of DNA namely 1 ng, 5 ng, 10 ng, 25 ng and 50 ng. These primers were standardized to find the optimum annealing temperature. The optimum temperature was found to be 58°C. These primers were designed by choosing the common sequences from the pool of *E. coli* 0157:H7 genes. These primers were not used in any of the research works so far (Bioserve India Ltd). RAPD results for *E. coli* produced positive results. The DNA samples were able to produce amplicons with arbitrary primers also. The amplicons were produced using the random sites of template without a target gene. The molecular weight of *E. coli* was found to be 420 bp. Satyajit Kanungo in 2009 produced results in *E. coli* strains using 100 ng of template²¹. Likewise, RAPD results for *Streptococcus* produced positive results. The molecular weight of *Streptococcus* was found to be 240 bp.

DISCUSSION

The food samples namely banana, cold meat and milk were taken to isolate bacteria. The bacterial culture was subjected to biochemical tests for identification of species. The DNA was isolated using the culture. The isolated DNA from different microorganisms was examined for purity using UV-Visible Spectroscopy and quantification of the DNA was also carried out. Mary Cooke *et al* in 1979 reported the presence of *Klebsiella* in cold meat provided in the hospitals¹⁷. *Streptococcus* was isolated from milk and similar result was obtained by Gabriella Martinez¹⁸. Direct PCR detection was introduced by Gabriella Martinez and the organism was found to produce septicemia in human. Tarak Parekh and Rema Subash in 2008 used phenol-chloroform method in for DNA isolation but it resulted in large number of protein contaminants in DNA¹⁹. But in this method it was found that the isolated DNA was free from protein contaminants. The primer standardization results for *E. coli* found by Rambabu Naravaneni in 2004 was 56°C²⁰. The result found for *E. coli* in this study was 54°C. Suitable primers were designed for the virulent genes of bacteria and they were standardized with 1.5 microliters of magnesium chloride. The optimum annealing temperatures for *E. coli*, *Klebsiella* and *Streptococcus* were 54°C, 56°C and 57°C respectively. PCR reactions were carried out for isolated DNA with specific primers and also with human primers. *E. coli* produced amplification for the primers designed for STX 1 gene, *Klebsiella* for protein cel A gene and *Streptococcus* for CPS gene. All three isolated DNA produced no amplification with human genomic DNA. This infers that the isolated DNA is specific for its own primers. Varying concentrations of DNA such as 100 ng, 50 ng, 20 ng, 5 ng and 1 ng were chosen for PCR reaction. Both *E. coli* and *Streptococcus* DNA were active at all concentrations including 1ng. But, *Klebsiella* did not produce amplification at 1 ng. The optimum temperature for amplification of *E. coli* DNA for RAPD analysis was 58°C and for *Streptococcus* it was 57°C.

Table 1: Primers used to detect three different pathogens

Primer	Nucleotide sequence
STX1 (<i>E. coli</i> 0157:H)	5'TGTCCAATGAAGAGCAAGACTTGACAGTAGCAATAATGTCACGCCCGCAAGGGCAAGT3'
CELA (<i>Klebsiella</i>)	5'AATTTTGGTGTGATTGATGAGTCCTGAAAGCATGAATAGT3'
CPS (<i>Streptococcus</i>)	5'GTCTTCAAAAGAAAAATGACACATCAGAAAATATGAACAACACATT3'

Table 2: IMVIC test for identification of bacteria

Culture	Indole test	Methyl red test	Voges Proskaur	Citrate test
LB agar	+	+	-	-
Nutrient agar	-	-	+	+
Blood agar	-	-	-	-

IMVIC test confirms the presence of Bacteria isolated from the food samples

Table 3: Forward and reverse primers for virulent genes of bacterial strains

Species	Target gene	Primers (forward and reverse)
<i>E. coli</i>	STX 1	TGTCCAATGAAGAGCAAGACTTGACAGTAG/CAATAATGTCACGCCCGCAAGGGCAAGT
<i>Klebsiella</i>	Protein cel A	AATTTTGGTGTGATTGATGA/GTCCTGAAAGCATGAATAGT
<i>Streptococcus</i>	CPS	GTCTTCAAAAGAAAAATGACAC/ATCAGAAAATATGAACAACACATT

Forward and reverse primers for each bacterial strain were standardised

Figure 1: Colonies represent the presence of microbial pathogen from food sample

Figure 2: DNA isolated by CTAB method from bacterial strains was confirmed by Gel electrophoresis

Figure 3: Amplification of DNA isolated from bacterial strains by specific primers

Figure 4: Amplification of DNA using *Klebsiella* specific primers (4a.left), Amplification of DNA using *Streptococcus* specific primers (4b.right)

Figure 5: Lane 1 represents the ladder DNA. Ladder 2- 6 represents *E. coli* the DNA of varying concentrations (1,5,10, 25 and 50 ng)

Figure 6: Lane 9 represents the ladder DNA. Ladder 2- 8 represents *E. coli* the DNA of varying concentrations (1,5,10, 20,25,50,75 and 100 ng)

Figure 7: Lane 1 represents the ladder DNA. Ladder 2- 6 represents *E. coli* the DNA of varying concentrations (1,5,10,25, and 50 ng)

Figure 8: RAPD results of *E. coli* DNA (8a.left), RAPD results of *Streptococcus* (8b.right)

The size of the isolated DNA was also found using ladder DNA. The size of *E. coli*, *Klebsiella* and *Streptococcus* was found to be 420, 500 and 250 bp, RAPD analysis was carried out for *E. coli* and *Streptococcus* at 1 ng concentration. Both the organisms were found produce amplification with arbitrary primers also. From the above results, it is concluded that an efficient and rapid PCR based technique can be used for the detection of food pathogens which had high specificity and sensitivity. Therefore, this technique will be helpful in detecting emerging food borne pathogens and avoiding epidemic food borne outbreaks. This study has also proved that

minimal concentrations of the virulent genes are enough to cause infectious diseases in human.

CONCLUSION

In conclusion, the findings of the present investigation revealed that *E. coli* and *Streptococcus* were detected at minimum DNA concentration (1 ng) using PCR based amplification. However, gene of *Klebsiella* was not amplified at 1 ng. We suggest that *E. coli* and *Streptococcus* remain active even in 1 ng of the DNA sample and the same can be detected.

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