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Molecular Methods for the characterization of foodborne pathogens *E. coli* and *Campylobacter*

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ABSTRACT

Foodborne pathogens have long been recognized as a major source of food poisoning worldwide, sometimes leading to serious illness and even fatalities. Microorganisms encountered in daily life can generally be classified into two categories based on their impact on health: beneficial microorganisms and pathogenic (disease-causing) microorganisms. Beneficial microorganisms play key roles in food fermentation processes, producing products such as cheese, fermented meats (e.g., pepperoni), vegetables, dairy items, and ethnic foods like sauerkraut, idli, and kimchi. In contrast, pathogenic microorganisms cause acute or chronic illness, either through infection during their life cycle in human hosts or due to their metabolic by-products. These pathogens can be contracted via air, water, or contaminated food. Waterborne pathogens, in particular, often lead to gastrointestinal disorders but have also been implicated in a range of other health issues. Common foodborne pathogens include Shigella, Salmonella, E. coli, Enterobacter, and Bacillus species. To effectively detect harmful foodborne pathogens, we aim to develop a Polymerase Chain Reaction (PCR) analysis method to identify microorganisms present in contaminated food samples. This approach involves identifying pathogen-specific genes and designing primers to amplify specific regions of bacterial DNA. The successful amplification of these genes serves as a reliable indication of the presence of the pathogen in the sample. PCR-based identification is both faster and more accurate than traditional microbiological or biochemical diagnostic techniques.

Keywords: *Shigella; Salmonell, E.coli; Enterobacter; characterization; detection; foodborne pathogens; genotyping*

Introduction

Foodborne illnesses have become a major topic of public concern and scientific research, primarily caused by three agents: microorganisms, chemicals, and parasites. Contaminated food poses significant health risks, especially to vulnerable groups with compromised immune systems. The rapid and accurate detection of foodborne pathogens is critical for ensuring food safety, both for food industries striving to maintain hygiene standards and for governments working to protect public health and consumer safety, these challenges, various rapid detection methods have been developed in recent years, with nucleic acid-based molecular techniques emerging as a preferred choice over traditional protein-based immunological methods. These molecular approaches offer advantages such as ease of operation, higher accuracy, and faster detection times [1]. The focus of this paper is to review recent advancements in nucleic acid-based detection methods and explore innovative molecular techniques currently under investigation for the reliable and swift identification of foodborne pathogens. Polymerase Chain Reaction (PCR) has become one of the most widely employed techniques for amplifying nucleic acids since its development by Kary Mullis [2-3]. PCR's efficiency and sensitivity make it ideal for detecting foodborne pathogens. The process involves a heat-stable DNA polymerase, template DNA from the target pathogen, and two complementary oligonucleotide primers that are specifically designed to flank the DNA region of interest [4]. During PCR amplification, 20 to 40 cycles are typically performed, allowing for the exponential amplification of the target DNA sequence, often increasing it by more than a billion-fold.

Each PCR cycle includes three key phases:

1. Denaturation: High temperatures separate the double-stranded DNA into single strands.

2. Annealing: The temperature is lowered, allowing the primers to bind to the target single-stranded DNA sequences.

3. Extension: The DNA polymerase synthesizes a complementary strand based on the template DNA.

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The simplicity, speed, and accuracy of PCR have led to its widespread adoption in numerous studies aimed at detecting and characterizing foodborne pathogens. Recent advancements in PCR technology, including real-time PCR (qPCR) and multiplex PCR, have further enhanced its utility by enabling the detection of multiple pathogens simultaneously and providing quantitative data on pathogen levels in contaminated food samples. These developments underscore PCR's role as a cornerstone in modern food safety diagnostics.

Escherichia coli (*E. coli*) is a Gram-negative bacterium belonging to the family *Enterobacteriaceae* and is commonly found in the lower intestines of warm-blooded organisms (endotherms). While most strains are harmless and part of the normal gut flora, certain strains, such as *E. coli* O157:H7, are pathogenic and can cause severe food poisoning in humans [5]. The beneficial strains of *E. coli* play important roles in the digestive system by producing essential nutrients like vitamin K2 and by inhibiting the colonization of harmful bacteria in the gut. Though typically found in the intestines, *E. coli* can survive for short periods outside the body, making it an excellent indicator organism for testing environmental samples for fecal contamination. Its ability to thrive in laboratory settings, coupled with its relatively simple and well-understood genetics, makes *E. coli* a model organism in biotechnology and microbiology research. It is widely studied due to its genetic manipulability, making it a cornerstone species for scientific advancements in these fields [6]. The transmission of *Escherichia coli* O157:H7 is primarily foodborne, with undercooked meat being the most common source of infection. However, secondary transmission through person-to-person contact is also significant. *E. coli* O157:H7 produces at least two Shiga-like toxins, which differ in their antigenic, physicochemical, and immunological properties, as well as their biological effects. These toxins are believed to play a crucial role in the pathogenicity of *E. coli* O157:H7 infections, contributing directly to the harmful effects experienced during infection [7].

Campylobacter is a genus of Gram-negative, spiral, microaerophilic bacteria belonging to the family *Campylobacteraceae*. These bacteria are characterized by their corkscrew-like shape and motility, using uni- or bi-polar flagella for movement. They are oxidase-positive and known for their ability to thrive in low-oxygen environments. The genus includes several species that are important in both human and animal health. *Campylobacter jejuni* is one of the most common bacterial causes of foodborne illness in developed countries, primarily linked to the consumption of undercooked poultry and contaminated water. Along with *C. jejuni*, *Campylobacter coli* is frequently implicated in human gastrointestinal infections. These infections typically cause symptoms such as diarrhea, fever, and abdominal cramps, and in some cases, can lead to more severe conditions like Guillain-Barré syndrome. *Campylobacter* fetus is a significant pathogen in livestock, causing spontaneous abortions in cattle and sheep [8]. It is also known as an opportunistic pathogen in immunocompromised individuals. The ability of Campylobacter species to evade host immune responses and persist in various environments underscores the importance of continued research and improved detection methods to prevent outbreaks and reduce the public health burden associated with these bacteria.

Materials and Methods

Preparation of Genomic DNA from Bacteria

Bacterial genomic DNA was extracted from a saturated liquid culture. The bacterial cells were lysed, and the proteins were degraded using Proteinase K to ensure efficient protein removal. To eliminate cell wall debris, polysaccharides, and any residual proteins, selective precipitation was performed using cetyltrimethylammonium bromide (CTAB). The high-molecular-weight DNA was then purified from the supernatant through isopropanol precipitation, yielding a concentrated genomic DNA sample suitable for downstream molecular analyses. To prepare genomic DNA from bacteria, several key solutions are required. Begin with TE Buffer (pH 8.0) by mixing 1 ml of 1M Tris with 200 µl of 0.5M EDTA and adjusting the volume to 100 ml with deionized water. A 10% Sodium Dodecyl Sulfate (SDS) solution is made by dissolving 10 g of SDS in 100 ml of water, which aids in cell lysis. For protein digestion, prepare a 20 mg/ml Proteinase K solution, using 5 µl for each 1 ml of bacterial culture. Additionally, create a 5M NaCl solution by dissolving 29.25 g of NaCl in 100 ml of water, which is essential for DNA precipitation. A CTAB/NaCl solution is prepared by dissolving 4.1 g of NaCl in 80 ml of water, then slowly adding 10 g of CTAB while heating, adjusting the final volume to 100 ml. This solution helps eliminate polysaccharides and cell debris. Chloroform/isoamyl alcohol is prepared in a 24:1 ratio for DNA purification, while isopropanol is used for DNA precipitation. Finally, a 70% ethanol solution, made by mixing 70 ml of ethanol with 30 ml of deionized water, is used to wash the DNA pellet, ensuring the removal of salts and contaminants.

DNA Isolation by CTAB Method

To isolate DNA using the CTAB method, begin by inoculating a 5-ml liquid culture with the bacterial strain of interest. Incubate under appropriate growth conditions until the culture reaches saturation, which may take several hours to a few days depending on the strain's growth rate. Once saturated, centrifuge 1.5 ml of the culture in a microcentrifuge for 2 minutes, or until a compact pellet forms, and discard the supernatant. Resuspend the pellet in 567 µl of TE buffer by repeated pipetting. Add 30 µl of 10% SDS and 3 µl of 20 mg/ml Proteinase K to achieve a final concentration of 100 mg/ml Proteinase K in 0.5% SDS. Mix thoroughly and incubate for 1 hour at 37°C. After incubation, add 100 µl of 5M NaCl and mix well, followed by the addition of 80 µl of the CTAB/NaCl solution. Incubate at 65°C for 10 minutes. Next, add an approximately equal volume (0.7 to 0.8) of chloroform/isoamyl alcohol, mix thoroughly, and centrifuge for 4 to 5 minutes. Carefully remove the aqueous, viscous supernatant to a fresh microcentrifuge tube, leaving the interface behind. Add an equal volume of phenol/chloroform/isoamyl alcohol, mix well, and centrifuge for 5 minutes. Transfer the supernatant to a fresh tube. To precipitate the nucleic acids, add 0.6 volume of isopropanol and shake the tube gently until a stringy white DNA precipitate becomes visible. At this point, transfer the pellet to a fresh tube containing 70% ethanol by hooking it onto the end of a heat-sealed and bent micropipette, or by pelleting the precipitate briefly at room temperature. Wash the DNA pellet with 70% ethanol to remove residual CTAB, then centrifuge for an additional 5 minutes at room temperature to repellet it. Carefully discard the supernatant and briefly dry the pellet in a lyophilizer. Finally, redissolve the pellet in 100 µl of TE buffer for storage or further analysis.

QIAGEN Kit Method for DNA Isolation

To isolate DNA using the QIAGEN kit method, begin by centrifuging a 5 ml bacterial culture at 10,000 rpm for 10 minutes to obtain a pellet.

Discard the supernatant and resuspend the pellet in 200 µl of Buffer. Add 40 µl of Proteinase K (20 mg/ml) and 20 µl of RNase A (10 mg/ml), then incubate the mixture at 56°C for about 10 minutes to digest proteins and degrade RNA. Next, add 200 µl of 96-100% ethanol and mix by pulse vortexing for 15 seconds. Transfer the mixture to a spin column and centrifuge at 8,000 rpm for 1 minute, discarding the flow-through. Subsequently, add 500 µl of Wash Buffer 1 to the column and centrifuge again at 8,000 rpm for 1 minute, discarding the flow-through once more. Then, add 500 µl of Wash Buffer 2 and centrifuge at 13,000 rpm for 3 minutes to wash the DNA. After discarding the flow-through, centrifuge the empty column for an additional minute at 13,000 rpm to remove any residual wash buffer. Finally, transfer the column to a fresh tube and elute the DNA by adding 50 µl of deionized water. Centrifuge at 8,000 rpm for 1 minute to collect the purified DNA for further analysis or storage.

Quantification of DNA

The concentration of isolated DNA was determined by measuring the absorbance at 260 nm and 280 nm using a spectrophotometer. The ratio of absorbance at 260 nm to that at 280 nm (A260/A280) was used to assess the quality of the DNA, with an optimal ratio typically around 1.8 indicating pure DNA. The concentration of the DNA was calculated using the following formula:

Concentration of the DNA = OD_{260} X 50 X dilution factor = µg of DNA /ml

This calculation provides the concentration of DNA in milligrams per milliliter, allowing for proper assessment and use in downstream applications.

Primer Designing

Primers are short synthetic oligonucleotides essential for various molecular techniques, including PCR and DNA sequencing. They are designed to be the reverse complement of a specific region of target DNA, allowing for precise annealing during amplification. When designing primers for applications such as sequencing or mutagenesis, it is crucial to predict key properties, including the melting temperature (Tm) and the likelihood of forming dimers with themselves or with other primers in the reaction. To facilitate this process, various software programs are available that can perform these calculations for any given primer sequence or pair. These tools help ensure optimal primer design, improving the efficiency and specificity of the intended molecular reactions.

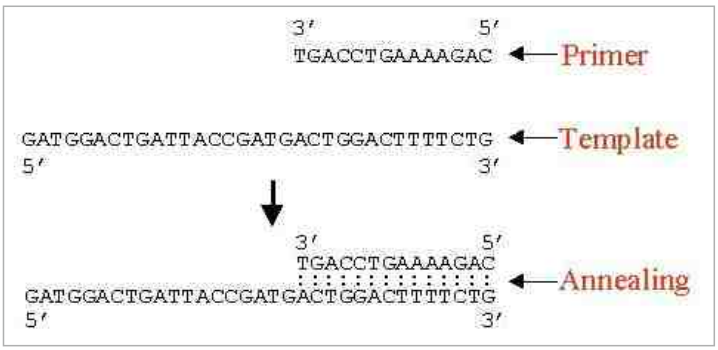


Figure:-01 Primer annealing principle

Multiplex PCR and Resolution of DNA Fragments on Agarose Gels

Multiplex PCR is a powerful technique that enables the simultaneous amplification of multiple target sequences in a single reaction by adding all relevant primers to the PCR mixture. This approach enhances efficiency by allowing for the detection of multiple DNA fragments in one assay. To analyze the resulting DNA fragments, agarose gel electrophoresis is employed. This process requires several key materials and equipment, including 1X TAE as the electrophoresis buffer, a 0.5 mg/ml solution of ethidium bromide for DNA visualization, and electrophoresis-grade agarose to create the gel matrix. Additionally, a 6X loading buffer is used to prepare the DNA samples, along with DNA molecular weight markers for size estimation. The horizontal gel electrophoresis apparatus, gel casting platform, gel combs for well formation, and a DC power supply complete the setup. By using these components, DNA fragments can be effectively separated based on size, allowing for thorough analysis and verification of the multiplex PCR results.

Gel Preparation for Electrophoresis

To prepare an agarose gel for electrophoresis, begin by dissolving 0.25 g of agarose in 25 ml of 1X TAE buffer to create a 1% gel solution. This mixture is heated to 70°C, usually in a microwave, to ensure the agarose dissolves completely. After cooling, 1 ml of ethidium bromide is added to the gel for DNA visualization, although this step requires caution due to ethidium bromide's toxicity. The gel is poured into a gel-casting tray with a comb in place to form wells. Once the gel solidifies, the comb is carefully removed, and the gel is placed in the electrophoresis tank. The tank is then filled with 1X TAE buffer until the gel is fully submerged. Sample loading involves mixing DNA samples with a loading dye containing bromophenol blue (a front dye) and xylene cyanol (a tracking dye), which help visualize the progress of electrophoresis. A reference size standard (DNA ladder) is also loaded to estimate the size of the fragments. The gel is run at 75 volts until the bromophenol blue migrates to about three-quarters of the gel, the DNA bands can be visualized using a UV light source and photographed using a gel documentation system. While this method is widely used for its simplicity and cost-effectiveness, the use of ethidium bromide presents health risks and environmental concerns, leading some labs to adopt alternative, safer DNA-staining options. Additionally, proper handling of UV light sources is critical to avoid exposure, though modern gel documentation units often mitigate this risk with shielding.

Bioinformatics Result

S. No	Organism	Name of the Genes
1	*E. coli*	eaeA, aggR, bfpA, astA, rpeA
2	*Campylobacter*	flaA, ftsZ, ceuE, hipuricase gene

Specific gene selected for primer designing

S.no	Organism	Selected genes
1	***E. coli***	*BfpA*
2	***Campylobacter***	*FlaA*

Primers designed

Organism	Gene	Primer length
E coli	***BfpA***	107bp
Campylobacter	**FlaA**	290bp

Primer Designing Results for *E. coli*
· No mispriming library specified
· Using 1-based sequence positions

Oligo	Start	Length	Tm (°C)	GC%	Any Compl.	3' Compl.
Left Primer	127	20	58.87	45.00	3.00	3.00
Sequence:	TCCAATAAGTCGCAGAATGC					
Right Primer	233	21	59.42	38.10	3.00	2.00
Sequence:	AAAATCGTTGAGTCCAATCCA					

Sequence Size: 582 bp
Included Region Size: 582 bp
Product Size: 107 bp
Pair Any Complementarity: 3.00
Pair 3' Complementarity: 1.00

1ATGGTTTCTAAAATCATGAATAAGAAATACGAAAAAGGTCT
GTCTTTGATTGAATCTGCA61

ATGGTGCTTGCGCTTGCTGCCACCGTTACCGCAGGTGTGATGTTT
TACTACCAGTCTGCG121

TCTGATTCCAATAAGTCGCAGAATGCTATTTCAGAAGTAATGAGC
GCAACGTCTGCAATT
>>>>>>>>>>>>>>>>>>>>
181AATGGTCTGTATATTGGGCAGACCAGTTATAGTGGATTGGAC
TCAACGATTTTACTTAAC

<<<<<<<<<<<<<<<<<<<<
241ACATCTGCAATTCCGGATAATTACAAAGATACAACAAACAAA
AAAATAACCAACCCATTT

301GGGGGGGAATTAAATGTAGGTCCAGCAAACAATAACACCGCA
TTTGGTTACTATCTGACG

361CTTACCAGGTTGGATAAAGCGGCATGTGTTAGTCTTGCAACC
TTGAACTTAGGTACTT

421GCGAAAGGCTACGGTGTTAATATCTCTAGCGAAAATAACATT
ACATCATTTGGTAATAGC

Campylobacter
No mispriming library specified
Using 1-based sequence positions
OLIGO start len tm gc% any 3' seq
LEFT PRIMER 1135 20 59.53 50.00 2.00 2.00
GCTATGGGATTTGGTTCTGC
RIGHT PRIMER 1424 23 59.32 43.48 2.00 0.00
CCTGCTGTTTCATCTTTTACTCC
SEQUENCE SIZE: 1719
INCLUDED REGION SIZE: 1719

PRODUCT SIZE: 290, PAIR ANY COMPL: 3.00, PAIR 3' COMPL: 0.00

1ATGGGATTTCGTATTAACACCAATGTTGCAGCTTTAAATGCAAA
AGCAAACGCTGATTTA

61AATAGTAAAAGTTTAGATGCTTCTTTAAGCAGACTTAGTTCAG
GTCTTAGAATCAACTCC

121GCAGCAGATGATGCTTCAGGGATGGCGATAGCAGATAGTTTA
AGATCTCAAGCTAATACT

181TTAGGTCAAGCTATATCTAATGGTAATGATGCTTTAGGTATCT
TACAAACTGCTGATAAG

241GCTATGGATGAGCAACTTAAAATCTTAGATACAATCAAAACT
AAGGCAACTCAAGCGGCT

301CAAGATGGACAAAGTTTAAAAACAAGAACCATGCTTCAAGCA
GATATCAACCGTTTAATG

361GAAGAACTTGACAATATTGCAAATACTACTTCATTTAACGGT
AAACAACTTTTAAGTGGG

421AATTTTATCAATCAAGAATTTCAAATCGGTGCAAGTTCAAAT
CAAACTGTAAAAGCTACT

481ATAGGAGCAACTCAATCTTCTAAGATAGGTTTAACACGCTTT
GAAACAGGAGGAAGAATT

Results of *E.coli*

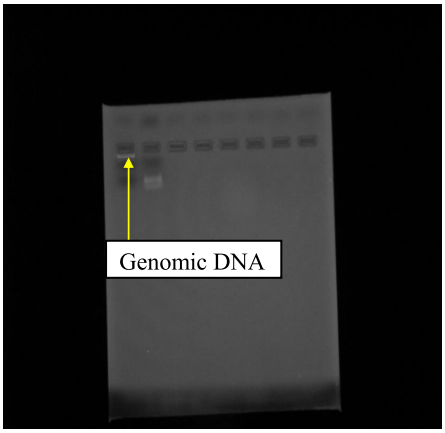
DNA isolation
The DNA isolated with CTAB method and the kit method was analyzed by the gel electrophoresis to check the integrity of the DNA as shown in figure

Spectroscopic results of isolated DNA
After isolation of DNA, we have taken the O.D values to identification and purification of DNA.

O.D values of *E.coli*
1) 30.5 260/280 = 1.29
2) 30.9 260/280 = 1.31
3) 34.3 260/280 = 1.30

Average
(30.5+30.9+34.3)/3 = 31.9µg/ml
For 1000µl = 31.9 µg/ml
For 60 µl = (6031.9)/1000
= 1.914 µg
For 1 µl = 1.914/60
= 0.031 µg/µl

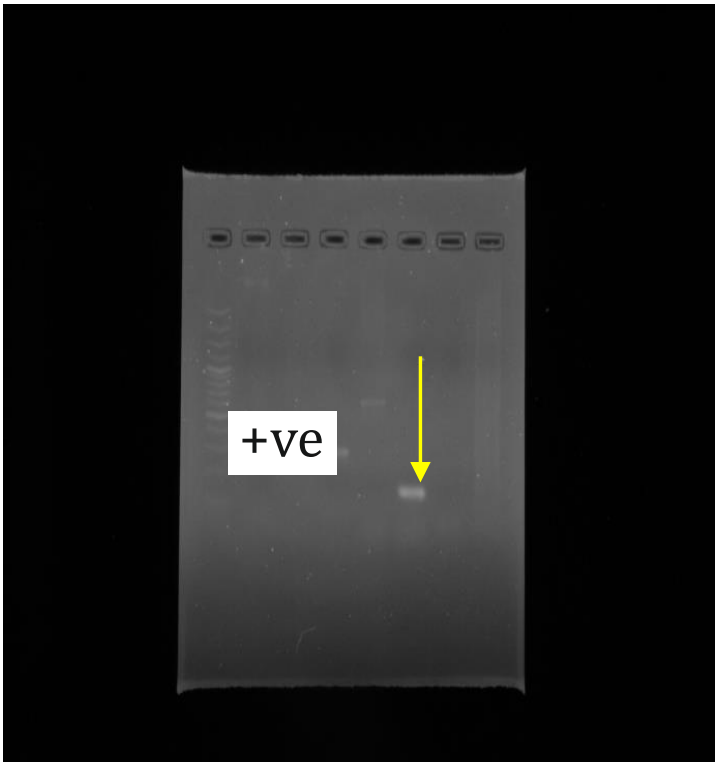
For the identification of DNA present in the isolated sample from E.coli culture, we performed the gel electrophoresis. After performing gel electrophoresis, the bands obtained as follows.



Showing DNA bands at 58^{0}C
Reaction mixture
Reaction volume - 25µl
No. of Reactions - 1

We perform the PCR after identification of DNA
• Annealing temperature: 58^{0}C
• Now we perform the PCR reaction with this reaction mixture.
• After performing the PCR reaction we obtained the PCR product.
• By using this PCR product we conduct the gel electrophoresis.
After the gel electrophoresis we observed the bands and are showed as follows.

S. No	Components	Volume
1	Buffer	2.5µl
2	Mgl_2	1.5 µl
3	dNTP's	2.5 µl
4	Forward primer	2 µl
5	Reverse primer	2 µl
6	Template	6 µl
7	Enzyme	1 µl
8	Water	8.5 µl



Result of specificity test
Sensitivity detection
To identify the sensitivity and efficiency of the PCR for amplification of DNA at low template concentrations, so template can be diluted to minimal concentrations as follows,
Template dilution was 5 µl templates in 45 µl water (diluted)

Reaction mixture
Reaction volume - 25 µl
No. of reactions - 2

S.no	Componens	Volume
1	Buffer	2.5 µl2 = 5 µl
2	Mgcl2	1.5 µl2 = 3 µl
3	dNTP's	2.5 µl2 = 5 µl
4	Forward primer	2 µl2 = 4 µl
5	Reverseprimer	2 µl2 = 4 µl
6	Enzyme	1 µl2 = 2 µl

We had taken the template in different volumes, in above two reactions and finally make the reaction volume to 25 µl with water.

Sample 1

S.no	Components	Volume
1	template	5 µl
2	Water	8.5 µl

Reaction mixture:
Reaction volume - 25 µl
Number of reactions - 2

S.no	Components	Volume
1	Buffer	2.5 µl2 = 5 µl
2	Mgcl2	1.5 µl2 = 3 µl
3	dNTP's	2.5 µl2 = 5 µl
4	Forward primer	2µl2 = 4 µl
5	Reverse primer	2 µl2 =4 µl
6	Enzyme	1 µl2 = 2 µl

We had taken the template in different volumes, in above two reactions and finally make the reaction volume to 25 µl with water.

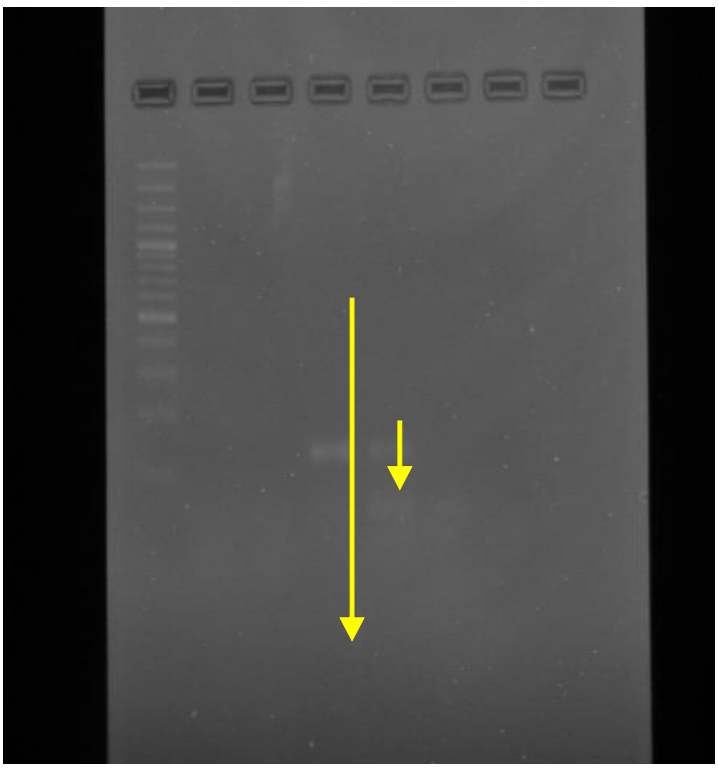
Sample: 1

S.no	Components	Volume
1	Template	2.5µl
2	Water	11µl

Sample: 2

S.no	Components	Volume
1	Template	5µl
2	Water	8.5µl

• Now we perform the PCR with this reaction mixture for DNA amplification.
• After performing the reaction we obtained the PCR product.
• By using this PCR product we conduct the gel electrophoresis.
• After the gel electrophoresis we observed the bands as follows



Bands after dilutions at 5 template in 45 µl water

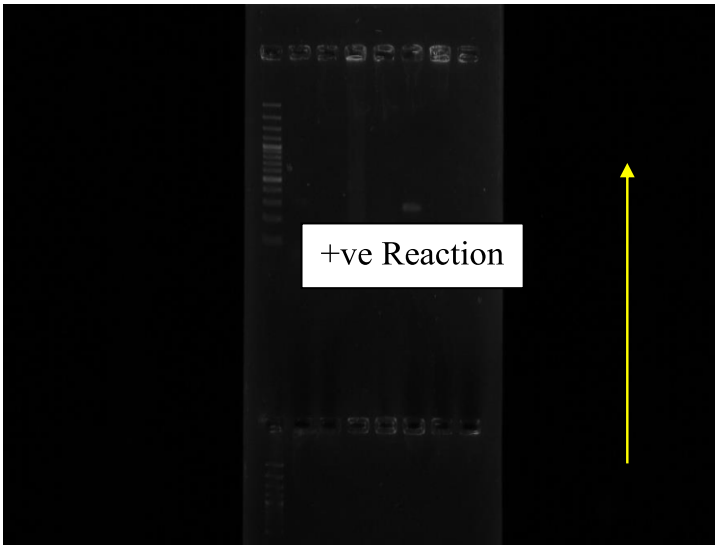
Results of *Campylobacter*
DNA Isolation
The DNA isolated with CTAB method and the kit method was analyzed by the gel electrophoresis to check the integrity of the DNA as shown in figure.

DNA isolated and their spectroscopic Results
After isolation of DNA, we have taken the O.D values to identification and purification of DNA.

O.D values of Campylobacter:
1) 82.9 260/280 = 1.10
2) 80.3 260/280 = 1.31
3) 81.0 260/280 = 1.30

Average
(82.9+80.3+81.0)/3 = 81.4µg/ml
For 1000µl = 81.4 µg/ml
For 60 µl = (3081.4)/1000
= 2.442 µg
For 1 µl = 2.442/30
= 0.081 µg/µl

For the identification of DNA present in the isolated sample from campylobacter culture, we performed the gel electrophoresis. After performing gel electrophoresis the bands obtained as follows:



Band formed at 58^0c
We performed the PCR reactions, after identification of DNA

Reaction 1:
Reaction volume - 25µl:
Number of reactions - 1

Buffer	2.5µl
Mgcl2	1.5µl
dNTP's	2.5µl
Forward primer	2µl
Reverse primer	2µl
Template	5µl
Enzyme	0.33µl
Water	9.17µl

- Annealing Temperature: 58^0
- Now we perform the PCR reaction with this reaction mixture.
- After performing the PCR reaction we obtained the PCR product.
- By using this PCR product we conduct the gel electrophoresis.
- After the gel electrophoresis we observed the bands and are shoed in below.

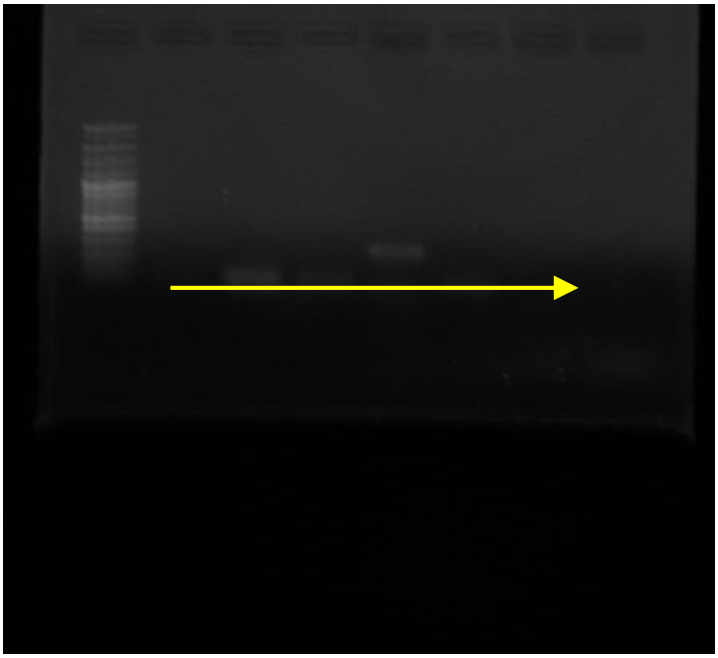


Figure shows the result of specificity analysis shows no amplification in human genomic DNA (lane3), other bacterial DNA (lane4) and negative control (lane6). Amplification is present in only lane 5 which is specific.

Sensitivity detection
To identify the sensitivity and efficiency of the PCR for amplification of DNA at low template concentrations, so template can be diluted to minimal concentrations as follows-
Template dilution:
- 5 µl template in 45 µl water (diluted)

Buffer	2.5 µl2 = 5 µl
Mgcl2	1.5 µl2 = 3 µl
dNTP's	2.5 µl2 = 5 µl
Forward primer	2µl2 = 4 µl
Reverse primer	2µl2 = 4 µl
Enzyme	0.33µl2 = 0.66µl

Reaction:
Reaction volume - 25 µl
Number of reactions - 2

We taken the template in different volumes, in above two reactions and finally make the reaction volume to 25 µl with water.

Sample 1:

Template	5 µl
Water	9.17 µl

Sample 2:

Template	2.5 µl
Water	11.67 µl

- 1µl template in 49 µl water (diluted)

Reaction:
Reaction volume - 25 µl
Number of reactions - 2

Buffer	2.5 µl2 = 5 µl
Mgcl2	2.5 µl2 = 5 µl
dNTP's	l2.5 µl2 = 5 µl2.5 µl2 = 5 µ
Forward primer	2 µl2 = 4 µl
Reverse primer	2 µl2 = 4 µl
Enzyme	0.33µl2 = 0.66µl

We taken the template in different volumes, in above two reactions and finally make the reaction volume to 25 µl with water.

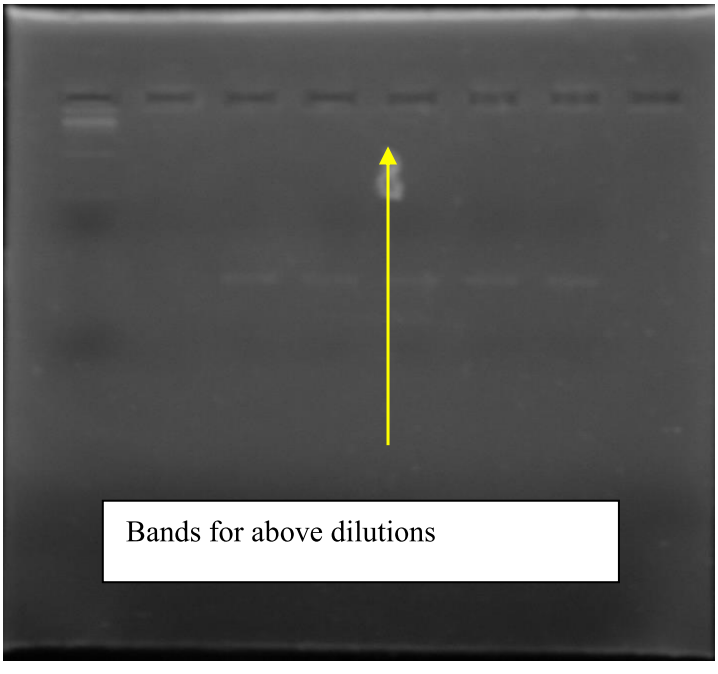
Sample 1:

Template	10 µl
Water	4.17 µl

Sample 2:

Template	5 µl
Water	9.17 µl

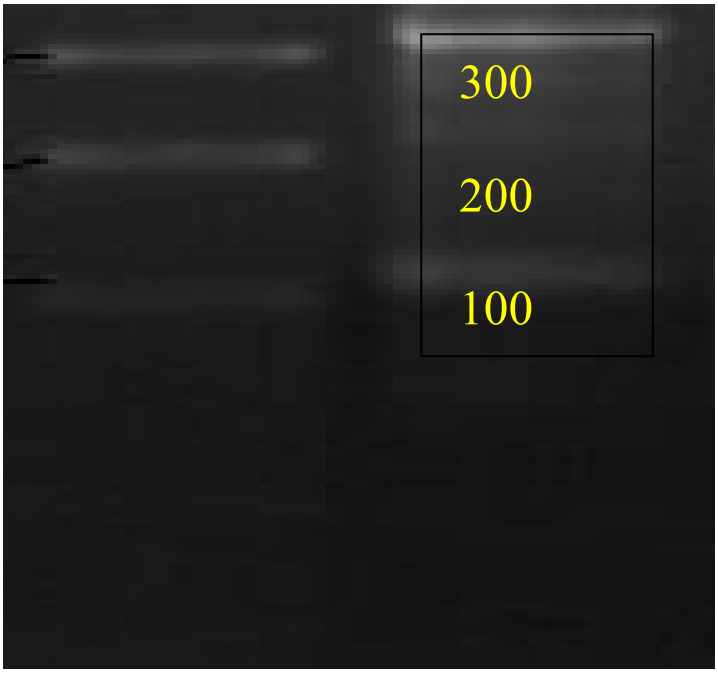
- Now we perform the PCR with this reaction mixture for DNA amplification.
- After performing the reaction we obtained the PCR product.
- By using this PCR product we conduct the gel electrophoresis.
- After the gel electrophoresis we observed the bands as follows.



Result of sensitivity

Multiplex-PCR result

As all the primers worked at 58ºC we tried multiplex also at 58ºC. The PCR conditions and reaction setup was similar as mentioned earlier. The result of the PCR has shown below.



Showing Multiplex PCR amplified products

Discussion

The characterization of foodborne pathogens, particularly Escherichia coli and Campylobacter, is critical for ensuring food safety and public health. Both pathogens are significant contributors to foodborne illnesses worldwide, and their rapid and accurate identification is essential for effective monitoring and control measures. *E. coli* encompasses a diverse group of strains, some of which are harmless and part of the normal gut flora, while others, such as *E. coli* O157:H7, are pathogenic and can cause severe gastrointestinal disease [9]. The pathogenic strains can be distinguished based on virulence genes, such as eaeA, aggR, and astA, which are associated with their ability to adhere to and invade intestinal cells. The use of molecular methods, including multiplex PCR, allows for the simultaneous detection of these virulence genes, providing a comprehensive profile of the pathogenic potential of E. coli strains present in food samples [10]. This is particularly valuable in outbreak investigations, where rapid identification can inform public health responses and prevent further spread.

Campylobacter, particularly Campylobacter jejuni and Campylobacter coli, is another leading cause of foodborne illness, often associated with the consumption of undercooked poultry and unpasteurized dairy products [11]. The pathogenicity of Campylobacter is linked to specific genes, such as flaA and hipuricase, which contribute to its virulence and survival in hostile environments.

Molecular characterization techniques, including PCR, enable precise identification of these pathogens, even in complex food matrices, facilitating better control measures in food production and processing [12]. The application of molecular methods, particularly multiplex PCR, offers numerous advantages over traditional microbiological techniques. These include increased sensitivity, specificity, and the ability to process multiple samples simultaneously. The successful amplification of target genes indicates that the primers designed for this study are effective for both E. coli and Campylobacter, demonstrating high specificity and minimal cross-reactivity. This efficiency not only accelerates the diagnostic process but also enhances the reliability of the results, the advancements in molecular characterization, challenges remain [13]. The presence of inhibitors in food matrices can affect the PCR process, leading to false negatives. Thus, optimizing DNA extraction protocols, such as those utilizing the CTAB method or commercial kits like QIAGEN, is crucial to ensure high-quality DNA is obtained for analysis, the emergence of new strains and the continuous evolution of existing pathogens necessitate ongoing surveillance and refinement of molecular methods [14-17]. Future research should focus on expanding the range of detectable pathogens, improving the robustness of the assays, and integrating these techniques into routine food safety protocols, the use of molecular methods for the characterization of *E. coli* and *Campylobacte*r represents a significant advancement in food safety diagnostics. By enabling rapid and accurate identification of these pathogens, we can enhance food safety measures, reduce the incidence of foodborne illnesses, and protect public health more effectively. The multiplex PCR (mPCR) system is highly reproducible and sensitive, enabling the identification of pathogenic strains regardless of the location of virulence genes. This method can be performed with high throughput, facilitating the presumptive identification of pathogens present in food by designing and incorporating primers specific to virulence gene sequences found in 40% of the tested strains [18-20]. The mPCR assay demonstrated 100% reproducibility, with no amplification observed in any of the non-pathogenic strains tested in this study. Furthermore, the mPCR could successfully detect as few as 100 colony-forming units (cfu) of pathogens per reaction mixture in spiked fecal samples following preincubation (Nashwa et al.).

Conclusion

The multiplex PCR (mPCR) system demonstrates excellent reproducibility and sensitivity, effectively identifying pathogenic strains regardless of the location of virulence genes. This method facilitates high-throughput analysis, allowing for the presumptive identification of causal pathogens. The current primer sets designed for Escherichia coli and Campylobacter exhibit remarkable specificity and sensitivity for all tested organisms. The results of the multiplex PCR are promising, significantly minimizing diagnosis time by amplifying any of the three target pathogens present in the sample. Food contamination is a significant public health concern, often leading to illnesses such as diarrhea and other serious conditions. To determine pathogenic microorganisms, we employ multiplex PCR, which utilizes multiple primers simultaneously for rapid identification. The consumption of contaminated food is a leading cause of mortality, as various diseases arise from pathogens including bacteria, viruses, prions, and parasites.

Key foodborne pathogens include Escherichia coli, Campylobacter, Klebsiella, Salmonella spp., and Shigella spp. *Escherichia coli* is an enteropathogenic bacterium responsible for diseases like diarrhea and kidney failure, particularly in children. This rod-shaped, gram-negative bacterium is commonly found in the lower intestines of warm-blooded animals. Salmonella, another genus of rod-shaped, gram-negative, non-spore-forming bacteria, causes diseases such as typhoid, paratyphoid, and salmonellosis. Shigella, also a gram-negative rod, is an intracellular pathogen that causes dysentery and shigellosis. The objective of this project is to develop a PCR-based kit for the identification of pathogenic bacteria in samples. This involves several steps: isolating pathogenic bacteria from food samples, designing specific primers for each organism, standardizing individual PCR protocols, running multiplex PCR, and verifying the specificity and sensitivity of the designed primers. Isolation of pathogenic bacteria utilizes methods such as the CTAB method for genomic DNA preparation, where bacteria from a saturated liquid culture are lysed, and proteins are removed through proteinase K digestion. This is followed by selective precipitation with CTAB to recover high-molecular-weight DNA. An alternative method for DNA isolation involves using the QIAGEN kit.

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