INNOVARE JOURNAL OF LIFE SCIENCE

Vol 11, 2023

ISSN - 2321-550X

Research Article

OPTIMIZATION OF COLONY POLYMERASE CHAIN REACTION FOR THE 16SRRNA OF DIFFERENT STRAINS OF *ESCHERICHIA COLI*

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Received: 30 April 2023, Revised and Accepted: 13 July 2023

ABSTRACT

Objective: This work aimed to enhance colony polymerase chain reaction (PCR) for the 16S rRNA of several *Escherichia coli* strains.

Methods: The isolation of *E. coli* is done from the gut of the chicken and soil. Then, we optimized the condition for colony PCR for the amplification of 16s ribosomal RNA. We successfully designed primer 3 for 16s ribosomal RNA and made the dilution solution with PCR grade water that is 1:10. Moreover, finally, we made a 20 µL solution that contains the master mix of our isolated colony and forward and reverse base primer for amplification. After the conventional PCR, the amplified 16s ribosomal RNA was then run on Gel to obtain the desired bands. And finally saw the bands in the Gel Doc picture.

Results: Our result shows that the technique of colony PCR is an efficient and quick method than other existing methods that are too costly, tedious, and time-consuming procedures that deter their exploitation in various experimentations and for the identification of *E. coli* strains.

Conclusion: This study concluded that 16s ribosomal RNA can be amplified without the extraction and purification of total genomic DNA from a bacterial colony using colony PCR. Therefore, by designing rRNA primers for *E. coli* species, we can evaluate their various types of mutations, strain detection, and antibiotic resistance.

Keywords: 16SrRNA, Colony PCR, Eosin methylene blue agar, Indole, methyl red, voges proskauer, and citrate.

 DOI: http://dx.doi.org/10.22159/ijls.2023v11i4.48219. Journal homepage: https://innovareacademics.in/journals/index.php/ijls

INTRODUCTION

Escherichia coli, belonging to the *Enterobacteriaceae* family, encompass commensal and pathogenic strains [1]. Virulence traits are encoded on mobile genetic elements, leading to distinct strain variations [2]. The emergence of new *E. coli* strains necessitates the development of innovative diagnostic methods. A novel technique utilizing rRNA primers enables the amplification of the 16SrRNA gene for mutation evaluation, strain detection, and antibiotic resistance analysis. Gel Electrophoresis aids in the visualization of the amplified gene, facilitating rapid identification [3]. *E. coli* is categorized into intestinal pathogenic, extra-intestinal pathogenic and commensal classes [3]. Enteropathogenic *E. coli* (EPEC) is responsible for infant diarrhea in developing countries [4]. EPEC attaches to epithelial cells through the LEE gene, resulting in cytoskeletal changes [5].

Enterotoxigenic *E. coli* (ETEC) strains produce heat-labile enterotoxins (LTs) and/or heat-stable enterotoxins (STs) [6] ETEC variants generate heat-sensitive enterotoxin (LTs) and/or heat-resistant enterotoxin (STs) [6]. LTs, akin to cholera enterotoxin, are primarily found in human isolates (LT-I), while related proteins (LT-II) are present in animal isolates. STs comprise two classes, STa and STb, with STa being responsible for human disease. ETEC predominantly causes pathology in developing countries, exhibiting lower rates of colon cancer [7,8].

Enteroaggregative *E. coli* (EAEC) is a notable contributor to prolonged diarrhea in individuals of all ages, spanning across global populations [9,10]. It colonizes the intestinal mucosa, secretes enterotoxin and cytotoxin, and induces mild but notable mucosal damage, primarily in the colon. Attachment is facilitated by aggregative adherence fimbriae. EAEC synthesizes toxins, including the autotransporter protease referred to as pic, moreover, *Shigella flexneri* encompasses diverse strains that harbor the oligomeric enterotoxin, recognized as shigella enterotoxin (SHET1) [9,10].

Enteroinvasive *E. coli* (EIEC) shares genetic and biochemical similarities with Shigella species and can cause invasive inflammatory colitis, occasionally resulting in dysentery. EIEC and Shigella, both responsible for dysentery, exhibit similar symptoms including fever, abdominal cramps, and bloody and mucus-containing diarrhea. In many instances, the diarrhea caused by EIEC presents as a watery form, making it challenging to differentiate from diarrhea induced by other pathogenic strains of *E. coli* [11].

Extraintestinal pathogenic *E. coli* (ExPEC) differentiates from diarrheagenic and commensal *E. coli* strains [11]. ExPEC harbors virulent genes that enable it to cause infections beyond the gut, such as urinary tract infections (UTIs), sepsis, pneumonia, neonatal meningitis, surgical site infections, cellulitis, and osteomyelitis [12].

ExPEC is a major causative agent of UTIs, accounting for 75–95% of cystitis and pyelonephritis cases in the United States [13,14]. UTIs are categorized as lower (cystitis) or upper (pyelonephritis) infections [15]. Women are more susceptible to UTIs due to anatomical differences [16]. Commensal *E. coli* strains that colonize the intestinal tract have a symbiotic relationship with the host and confer health benefits [17-19].

Certain commensal *E. coli* strains, such as Nissle 1971, *E. coli* 83970, and AO 34/86, exhibit probiotic properties beneficial to humans [22,23]. Nissle 1917 is therapeutically used for gastrointestinal disorder treatment and lacks pathogenicity-related genes but possesses genes for protease, microcin M, iron synthesis, and fimbrial adhesion, promoting survival [24,25]. *E. coli* 83,970 inhibits acute UTIs by

preventing pathogen colonization in catheters and establishing growth in the urinary tract without triggering an immune response [26].

E. coli O157:H7 was identified as highly pathogenic in 1982 due to its production of cytotoxic toxins [27,28]. It can give rise to diverse clinical presentations, such as hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura [27]. Other *E. coli* serotypes can also produce Shiga-like toxins [28]. *E. coli* O157:H7 exhibits distinctive characteristics, including the inability to ferment sorbitol within 24 h and a negative reaction for β-glucuronidase, leading to colorless colonies on sorbitol MacConkey agar. It can be screened using antisera specific to O157:H7 [29-37].

In conclusion, *E. coli* encompasses a range of commensal and pathogenic strains that contribute to severe diseases such as intestinal disorders and watery diarrhea. Particular strains, such as *E. coli* O157:H7, are capable of producing verotoxins or Shiga-like toxins, which are cytotoxins encoded by bacteriophages [37]. These toxins can cause sporadic cases of diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome [37]. Verotoxin-producing *E. coli*/Shiga toxin-producing *E. coli* strains, characterized by the absence of the enterotoxin gene, carry a distinct fimbrial adhesion that is encoded by 60-megadalton plasmids. Effective detection methods, including hybridization probes and polymerase chain reaction (PCR)-based techniques, have been developed for their identification [38]. The utilization of nucleic acid-based tests, including PCR, has revolutionized bacterial pathogen detection in clinical laboratories, ensuring high sensitivity and specificity. Proper procedures and primer selection are crucial to achieve accurate results [38-42]. Colony PCR offers a rapid method for selecting specific DNA sequences without the need for DNA purification, making it useful for various applications in bacterial research [43-46].

METHODS

The study was conducted at the Centre of Biotechnology and Microbiology, University, and ethical permission was granted by the department's ethical committee.

Sample collection

Soil samples were collected from different regions in Peshawar University to isolate *E. coli* strains. The soil samples were wrapped in sterile Aluminum foil to prevent contamination.

Serial dilution

The collected soil samples were transferred to a Laminar Flow Hood (LFH) where sterile conditions were maintained to prevent contamination. Serial dilution of the samples was performed up to 10-5 in LFH.

Preparation of media

Selective Eosin Methylene Blue Agar (EMB) media was used to culture the samples to grow *E. coli* strains. Media was prepared according to the manufacturer's instructions and autoclaved at 121°C and 15psi pressure. The media was poured into sterile petri plates and incubated at 37°C for 24 h to check for sterility. LFH was sterilized by washing it with 70% ethanol followed by turning on the ultraviolet (UV) light for 15 min. A sterile environment was maintained throughout the research to prevent contamination.

Sample inoculation

The collected samples were inoculated on EMB media to obtain the growth of *E. coli* on selective media. After streaking the inoculums, the plates were kept in an incubator for 24 h at 37°C to observe growth.

Biochemical tests for identification of *E. coli*

Biochemical tests were performed to identify *E. coli* strains. *E. coli* strains as shown in Table 1. *E. coli* strains are Gram-negative and give pink-colored colonies that are arranged either singly or in pairs [47]. They are lactose fermenters and catalase positive. For quick identification, "IMViC" tests were performed. These bacteria are indole and methyl red positive and Voges Proskauer and citrate utilization negative [48]. They do not produce H2S gas while other species of this genus produce gas using indole, glucose, mannitol, lysine, arabinose, xylose, and trihalose [49]. Thomas in 1988 carried out biochemical tests of *E. coli* species and found that most of the *E. coli* species ferment lactose; however, 10% of the species are late lactose fermenters while some of the species are non-lactose fermenters [50].

Detailed procedure

PCR Amplification of Bacteria 16s rRNA Genes Directly from a Colony:

Equipment required

Thermal cycler, Vortex mixture, 1–10 μL and 10–100 μL pipette with tips, PCR tubes, gloves.

Materials required

PCR-grade water, Master mix, Forward and Reverse base primers, Template (*E. coli* colony), Falcons tubes, PCR tube rack.

Primer designing and dilution

Specific primer 3 was designed for 16s ribosomal RNA amplification. Primer dilution was necessary because primers are highly concentrated. To dilute, 10 μL of forward and reverse primers were mixed with 90 μL of PCR-grade water at a 1:10 dilution.

PCR mixture

To make the absolute mixture up to 20 μL, 13 μL of PCR-grade water was taken, and 4 μL of the master mix was added to it. Then, 1 μL of the forward base primers and 1ul of the reverse base primers were added to the PCR tubes. A colony (1 μL) from the EMB plate containing the *E. coli* culture was picked, and the 20 μL mixture was made. The PCR tubes were placed in a thermal cycler and run on the specific desired conditions required for denaturation, annealing, and amplification. After completion of the PCR, the PCR tubes were transferred to a freezer and kept at 4°C.

Gel electrophoresis

Materials required for gel electrophoresis

Agarose powder, TAE buffer, Ethidium bromide, Loading dye, DNA ladder, Gel casting tray and comb, Gel electrophoresis apparatus, UV transilluminator.

Gel preparation

We prepared a 1% of agarose gel by adding 1 g of agarose powder to 100 mL of 1X TAE buffer in a flask. The mixture was heated in a microwave until the agarose dissolved completely and then allowed to cool to 60°C. We added 1 μL of ethidium bromide to the gel mixture and poured the mixture into the gel casting tray with the comb inserted. The gel was allowed to solidify for 30 min at room temperature.

Sample loading

We mixed 5 μL of loading dye with 10 μL of the PCR product and loaded the mixture into the wells of the gel using a micropipette. We also loaded 5 μL of DNA ladder into one of the wells as a size reference.

Electrophoresis

The gel was run in the electrophoresis apparatus for 45 min at 100 V. After the run was complete, the gel was removed from the apparatus and placed on a UV transilluminator. The DNA bands were visualized by UV light and photographed using a gel documentation system.

RESULTS

E. coli culture

Sample of *E. coli* is cultured on MacConkey agar media which are obtained from soil (Fig. 1) and from the gut of chicken (Fig. 2) and stored at 4°C in the freezer. The cultured plates of *E. coli* are then processed for 16s r RNA extraction through colony PCR. Moreover, after the completion of PCR, we run it on agarose gel and then finally saw the bands on the Gel Doc picture (Fig. 3).

Fig. 1: *Escherichia coli* isolated from soil

Fig. 2: *Escherichia coli* isolated from the gut of a chicken

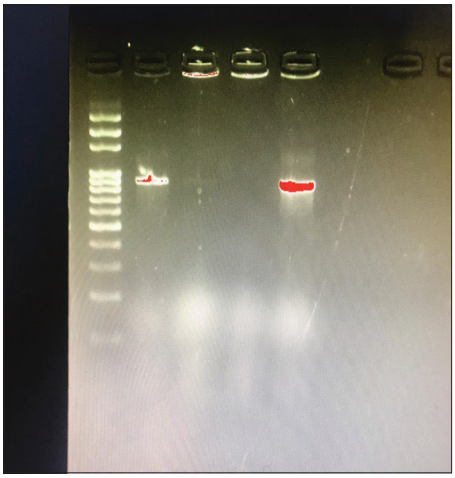

Fig. 3: Bands of 16s rRNA

PCR results

After the completion of conventional PCR, the sample was then run on Gel to obtain the desired bands, and then finally see the bands on the Gel Doc picture.

Gel doc picture

The sample was loaded to wells on the gel, in well no 1st 100bp DNA ladder was loaded to find the number of nucleotides as a positive control. After the completion of Gel electrophoresis, the Gel is kept in Gel Doc to see the bands on the Gel Doc picture (Fig. 3).

Table 1: Biochemical tests for identification of *Escherichia coli*

Tests	Results
Gram staining	Gram-negative
Catalase test	Positive
Oxidase test	Negative
Indole test	Positive
Methyl red test	Positive
Citrate utilization test	Negative
Lactose, glucose, sucrose, and maltose fermentation	Yes
H2s production	No
Gas production	Yes
Voge's Proskauer test	Negative

DISCUSSION

E. coli, a Gram-negative, facultative anaerobe, belongs to the *Enterobacteriaceae* family and is known to colonize a human infant's gastrointestinal tract a few hours after birth. While *E. coli* strains are less likely to cause intestinal illness, certain strains such as EPEC and enterohemorrhagic *E. coli* can cause diarrhea and hemolytic uremic syndrome.

Several techniques are used to detect pathogenic *E. coli*, including biochemical tests, PCR-based diagnosis, and 16s ribosomal RNA [40]. The most effective method for identifying specific DNA sequences is colony PCR, which involves picking a bacterial colony and using PCR to identify the desired sequence. The 16s ribosomal RNA sequence is frequently used as a marker for taxonomy categorization and phylogenetic study of microorganisms due to its highly conserved primer design and hypervariable region [39].

Shigella and EIEC share many characteristics and cause dysentery and invasive inflammatory colitis, respectively. The most prevalent pathogen, ExPEC, is responsible for 75–95% of instances of cystitis and pyelonephritis in the US [14].

In conclusion, identifying specific strains of *E. coli* is essential for treating and preventing the conditions caused by them. Colony PCR and 16s ribosomal RNA sequencing is effective techniques for identifying pathogenic *E. coli* and studying their taxonomy and phylogenetic characteristics.

CONCLUSION

This study demonstrates that 16s ribosomal RNA can be amplified directly from bacterial colonies using colony PCR without the need for extraction and purification of total genomic DNA. Our findings suggest that this technique can potentially be used for the direct identification of bacterial strains without studying the genetic protocol of DNA extraction and purification. We have successfully designed rRNA primers for *E. coli* species that can be amplified to evaluate various types of mutations, strain detection, and antibiotic resistance. The three-step quick and novel method for the amplification of *E. coli* 16SrRNA gene sequences can be a convenient technique for biomedical personnel to rapidly identify the *E. coli* variants. This study contributes to the development of a faster and simpler technique for bacterial identification and characterization.

ACKNOWLEDGMENT

We would like to thank Centre of Biotechnology and Microbiology, University of Peshawar for generous help in research.

CONFLICTS OF INTEREST STATEMENT

None.

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