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Molecular typing of of *E.coli* strains isolated from various water samples

V Srihari[1], N Mallikarjuna Rao[2], B Vinusha[3], Muneera Bhanu[4], S V Prasad[5], M Prasad Naidu[6]

1. M.Sc Dravidian University, Kuppam, Andhra Pradesh, India
2. M.Sc Acharaya Nagarjuna Univerity, Andhra Pradesh, India
3. M.Sc biotechnology, S. V university, Andhra Pradesh, India
4. M.Sc Biochemistry, S. V university, Andhra Pradesh, India
5. PhD S. V University, Andhra Pradesh, India
6. M.Sc Medical Biochemistry, Narayana Medical College, Andhra Pradesh, India

Abstract

In the present study Random Amplification of Polymorphic DNA (RAPD) have been described as powerful molecular typing methods for microorganisms. Isolated bacterial species (*Escherichia* sp) were Biochemically characterized by Indole, Methyl red , Citrate and catalase tests. Genomic DNA was isolated and purified by enzymatic digestion methods for polymorphic studies by RAPD-PCR and plasmid DNA was isolated by using alkaline Lysis method for RFLP analysis. Both Genomic and Plasmid DNA were separated by using 1% Agarose gels and Quantified by using UV Visible Spectrophotometry at OD260 nm/OD280nm.

Keywords: Bacterial isolates, RAPD-PCR, RFLP, UV-Visible Spectrophotometry.

*Corresponding Author: M Prasad Naidu, M.Sc Medical, Department of Biochemistry, Narayana Medical College, Nellore, Andhra Pradesh, India. E.mail: m.prasadnaidu@ymail.com

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Introduction

Escherichia coli is a gram negative bacterium that is commonly found in the lower intestine of warm-blooded animals. Most *E. coli* strains are harmless, but some, such as serotypeO157:H7, can cause serious food poisoning in humans, and are occasionally responsible for costly product recalls. The harmless strains are part of the normal flora of the gut, and can benefit their hosts by producing vitamin K_2, or by preventing the establishment of pathogenic bacteria within the intestine. *E. coli* are not always confined to the intestine, and their ability to survive for brief periods outside the body makes them an ideal indicator organism to test environmental samples for contamination. The bacteria can also be grown easily and its genetics are comparatively simple and easily-manipulated, making it one of the best-studied prokaryotic model organisms, and an important species in biotechnology. *E. coli* was discovered by German pediatrician and bacteriologist Theodor Escherich in 1885, and is now classified as part of the Enterobacteriaceae family of gamma-Proteobacteria.

E. coli is the most frequent urinary pathogen isolated from 50-90 % of all uncomplicated infections. *E.coli* being the dominant pathogen. *E.coli*, the most common member of the family Enterobacteriaceae accounts for 75-90 % of all infections Identification of *E. coli* strains requires that these organisms be differentiated from nonpathogenic members of the normal flora. The identification of nonpathogenic members also needs to detect factors those determine virulence of this

organism. Antimicrobial resistance has become an important problem worldwide. Bacterial resistance to antimicrobial agents has been emerging and rapidly disseminating among many nosocomial and community acquired pathogens. These organisms have wide variety of antibiotic sensitivity patterns. The development of antibiotic resistance in *E. coli* has important clinical implications. The development of resistance to older agents such as ampicillin, gentamycin and ciprofloxacin resistance, may substantially limit our antibiotic choices.

Bacterial infections are usually treated with antibiotics. However, the antibiotic sensitivities of different strains of *E. coli* vary widely. As Gram-negative organisms, *E.coli* are resistant to many antibiotics that are effective against Gram-positive organisms. Antibiotics which may be used to treat *E. coli* infection include amoxicillin as well as other semi-synthetic penicillins, many cephalosporins, carbapenems, aztreonam, trimethoprim-sulfamethoxazole, ciprofloxacin, nitrofurantoin and the aminoglycosides.

E.coli often carry multidrug resistant plasmids and under stress readily transfer those plasmids to other species. Indeed, *E. coli* is a frequent member of biofilms, where many species of bacteria exist in close proximity to each other. This mixing of species allows *E. coli* strains that are piliated to accept and transfer plasmids from and to other bacteria. Thus *E.coli* and the other enterobacteria are important reservoirs of transferable antibiotic resistance. Resistance to beta-lactam antibiotics has become a particular problem in recent decades, as strains of bacteria that produce extended-spectrum beta-lactamases have become more common. These beta-lactamase enzymes make many, if not all, of the penicillins and cephalosporins ineffective as therapy. Extended-spectrum beta-lactamase producing *E.coli*are highly resistant to an array of antibiotics and infections by these strains.

Materials and Methods

Sample Collection

Sugar plant water: Yes, sugar does help plants grow. However, excessive amounts of sucrose can be harmful to a plant. For plants growing hydroponically or in a selective medium such as in a petri dish, sucrose is often used as a carbon source for sprouting plants. Plants make sugars through photosynthesis by combining water and carbon dioxide. Plants use carbon dioxide as their main carbon source so they do not need sugar in their substrate to grow. But young plants and tissue plant clones that aren't yet efficiently producing sugars through photosynthesis can benefit from the extra carbon stored in sucrose. Sugar water used in a plant's natural environment can also attract other organisms and bacteria. Although some may be symbiotic (help the plant), many can interfere with the plant's growth or even cause it to die.

Pond water: Pond is a body of standing water, either natural or man-made, that is usually smaller than a lake. A wide variety of man-made bodies of water are classified as ponds, including water gardens designed for aesthetic ornamentation, fish ponds designed for commercial fish breeding, and solar ponds designed to store thermal energy.

Ponds and lakes are distinguished from streams via current speed. While currents in streams are easily observed, ponds and lakes possess thermally driven microcurrents and moderate wind driven currents. These features distinguish a pond from many other aquatic terrain features, such as stream pools and tide pools.

Laboratory Diagnosis: In microscopy water samples show Gram negative rods, with no particular cell arrangement. Then, either MacConkey agar or EMB agar (or both) are inoculated with the sample. On MacConkey agar, deep red colonies are produced as the organism is lactose positive, and fermentation of this sugar will cause the medium's pH to drop, leading to darkening of the medium. *E.coli* as its indole positive (red ring) and methyl red positive (bright red), but VP negative (no change-colorless) and citrate negative (no change-green color).

Gram staining

Gram staining (or Gram's method) is an empirical method of differentiating bacterial species into two large groups (Gram-positive and Gram-negative) based on the chemical and physical properties of their cell walls.

Gram-positive bacteria have a thick mesh-like cell wall made of peptidoglycan (50-90% of cell wall), which stain purple and Gram-negative bacteria have a thinner layer (10% of cell wall), which stain pink. Gram-negative bacteria also have an additional outer membrane which contains lipids, and is separated from the cell wall by the periplasmic space.

There are four basic steps of the Gram stain, which include applying a primary stain (crystal violet) to a heat-fixed smear of a bacterial culture, followed by the addition of a mordant (Gram's iodine), rapid decolorization with alcohol or acetone, and counterstaining with safranin or basic fuchsin.

After decolorization, the Gram-positive cell remains purple and the Gram-negative cell loses its purple color. Counter stain, which is usually positively-charged safranin or basic fuchsin, is applied last to give decolorized Gram-negative bacteria a pink or red color.

Genotyping refers to the process of determining the genotype of an individual by the use of biological assays. Current methods of doing this include PCR, DNA sequencing, ASO probes, and hybridization to DNA microarrays or beads. The technology is important in clinical research for the investigation of disease-associated genes.

Genotyping applies to a broad range of "individuals" including microorganisms. Viruses for instance, or bacteria, can be genotyped. Genotyping in this context may help in controlling the spreading of pathogens, by tracing the origin of outbreaks. This area is often referred to as molecular epidemiology or forensic microbiology.

Sample collection and transportation

A total number of water samples were collected equally from different origin. Samples were collected aseptically in sterile containers and brought to the laboratory within 30-45 minutes using ice box. After collection, bacteriological analyses of the samples were performed to assess the selected microbial attributes.

Isolation of bacteria from the water sample by serial dilution technique

This method is commonly used to obtain pure cultures of those microorganisms that have not yet been successfully cultivated on solid media and grow only in liquid media. A microorganism that predominates in a mixed culture can be isolated in pure form by a series of dilutions. The inoculum is subjected to serial dilution in a sterile liquid medium, and a large number of tubes of sterile liquid medium are inoculated with aliquots of each successive dilution. The aim of this dilution is to inoculate a series of tubes with a microbial suspension so dilute that there are some tubes showing growth of only one individual microbe.

The microbiological investigation was conducted to determine the level of contamination of water samples processed at different origin. A total number of water samples were collected. After collection, bacteriological analysis of the samples were performed to assess the selected microbial attributes in water samples of different sources by using MacConkey (MC) agar or nutrient agar medium to find out the sanitary quality and identification of bacterial strain in different water samples.

Genomic DNA is located in the cell nucleus of eukaryotes, as well as small amounts in mitochondria and chloroplasts. In prokaryotes, the DNA is held within an irregularly shaped body in the cytoplasm called the nucleoid. The genetic information in a genome is held within genes, and the complete set of this information in an organism is called its genotype. A gene is a unit of heredity and is a region of DNA that influences a particular characteristic in an organism. Genes contain an open reading frame that can be transcribed, as well as regulatory sequences such as promoters and enhancers, which control the transcription of the open reading frame.

In many species, only a small fraction of the total sequence of the genome encodes protein. For example, only about 1.5% of the human genome consists of protein-coding exons, with over 50% of human DNA consisting of non-coding repetitive sequences. The reasons for the presence of so much non-coding DNA in eukaryotic genomes and the extraordinary differences in genome size, or *C-value*, among species represent a long-standing puzzle known as the "C-value enigma." However, DNA sequences that do not code protein may still encode functional non-coding RNA molecules, which are involved in the regulation of gene expression.

- Collection of various water samples(sewage water, sugar plant water, pond water)
- Isolation, identification and Biochemical characterization of Isolated Bacteria from various water samples
- Extraction of DNA from microbial population

- Visualization of DNA fragments by Gel documentation system
- Purification of DNA by Alcohol precipitation
- Qualitative and quantitative estimation of UV spectrophotometer
- Amplification of gene by RAPD-PCR
- Various in the DNA sequence of a genome by restriction fragment length polymorphism(RFLP)

The microbiological investigation was conducted to determine the level of contamination of water samples. A total number of water samples were collected from different sources. After collection, bacteriological analysis of the samples were performed to assess the selected microbial attributes in water samples of different sources by using MacConkey (MC) agar or nutrient agar medium to find out the sanitary quality and identification of bacterial strains in water samples.

Extraction is an easy and quick way to purify DNA from a mixture of proteins, lipids and nucleic acids (e.g., a cell or bacterial lysate). The mixture is extracted with phenol or a 50/50 mixture of phenol and chloroform. The organic solvents have two effects: 1.They dissolve hydrophobic molecules and 2. They denature proteins (which makes them insoluble in water). As a result, cell membranes and cellular proteins are either dissolved in the phenol/$CHCl_3$ (which is then discarded) or trapped in the interface between the two phases. DNA and RNA remain in the aqueous phase, and are easily separated.

To obtain the purified DNA from cells, the DNA must first be separated from the rest of the cellular material. This involves destruction of the cell membrane (cell wall), elimination of structural materials, and separation of proteins and RNA from the DNA. Because the extra cellular material differs among the major groups of organisms, the techniques for this are varied. *E. coli*, which is a prokaryote and unicellular, removes its cell wall with the enzyme lysozyme. Then we will eliminate the cellular RNA with the enzyme ribonuclease, the proteins with the enzyme protease. Finally, we will remove these with chloroform and cause the DNA to precipitate out of solution with alcohol.

DNA Extraction

Water samples were collected from different ecological sub zones. Isolate a high molecular weight DNA from the indigenous bacterial communities present in the water. Indigenous bacterial populations are more difficult to lyse than the seeded bacteria in the water sample.

Qualitative and quantitative estimation of genomic DNA and of plasmid DNA is done.

It indicates that, quantification of the extracted DNA and its subsequent utilization for determination of the optimum template DNA concentration required for PCR initiation. This was also confirmed by spectrophotometric analysis. The absorbance ratios (260/280) for the crude DNA extracts in all samples were found (2.0 is indicative of pure DNA).

Gene Amplification

The reason for this procedure is to separate the DNA from its associated proteins so that further manipulations can be done to it. Enzymes added to purify DNA *in vitro* can have unhindered access to it. A poorly purified DNA preparation will only be partially accessible to the enzymes. For this reason, special care must be taken to ensure a pure DNA preparation.

The primers (BioServe Biotech. Ind. Ltd.) used in the present study were the universal primers. The amplification mixture for each sample DNA contained 10 pmol of each primer. Template DNA used was in the range of 100 to 150 ng in a total final reaction volume. Control PCR included DNA of a pure *E. coli* culture. PCR was performed in an automated thermal cycler with an initial 94ºC denaturation for 3 min, followed by 30 cycles of 94ºC for 30 sec, 50ºC for 35 sec, 72ºC for 45 sec and a final extension at 72ºC for 7 min. Crude DNA extracts were loaded on 1% Agarose gel prepared in 1 X TAE buffer containing ethidium bromide. Gels were electrophoresed in 1 X TAE buffer at constant voltage of 100 volts, 20-30 min and visualized using gel documentation system. For PCR amplified products, 1.5% Agarose was used with the similar buffer concentration at constant voltage of 100 volts, 30-45min.

There are 3 basic steps in PCR that are carried out at different temperatures to create conditions optimal for:

1. DNA denaturation (meaning to separate the double-stranded DNA into single strands).

2. Primer binding or hybridization to each of the single strands of DNA at either the beginning or the end of the target sequence, depending upon the single-strand of DNA. Hybridization combines complementary, single-stranded DNA into a single molecule. This process is called annealing.
3. DNA polymerase elongation. The enzyme attaches to the primer-single-stranded DNA duplex and synthesizes the complementary strand of DNA, using the existing single-strand as a template.
4. The PCR was performed using the primer sets. To find out the optimum template concentration for a PCR reaction, a wide range of DNA templates were used (100 to 150 ng).

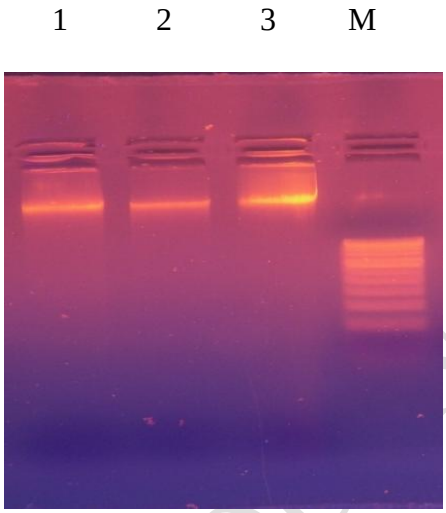
Newly synthesized DNA strands can serve as additional template for complementary strand synthesis. PCR rapidly amplifies DNA; because both strands are copied, there is an exponential increase in the number of copies. Assuming there is only a single copy of the target gene before cycling starts

Random amplified product of *E.coli* strains isolated from various water samples

The microbial diversity studies conducted in complex ecosystems, such as soil and sewage have been often found to be biased. Essentially this has been due to the inability to culture many microorganisms. It indicates that only 1 to 4% of the microbes can be cultivated under the standard laboratory conditions.

In case of molecular analysis of microbial communities, the bottleneck remains the isolation of pure community DNA in reasonable quantity. To analyze the efficiency of methods for extraction and purification of DNA, revealing that these methods suffer from low efficiency, mainly due to incomplete cell lysis and DNA adsorption to particles. This studyshows how the modified direct lysis method can actuallygive good yield of DNA from a large number of variedsources.

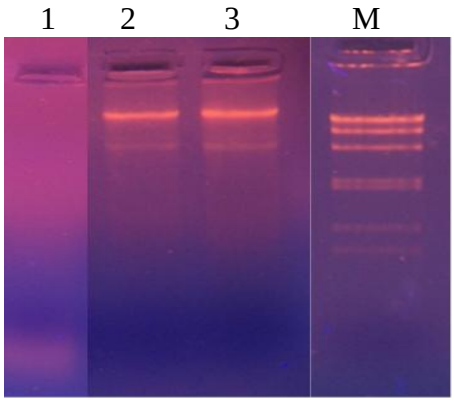
- The microorganisms isolates were characterized using the biochemical tests indole, methyl red ,citrate tests, catalase tests.
- Different DNA banding patterns ,depending on the number and size of amplified products were observed by using the RAPD-PCR with random primer:OPC-12.



Genomic DNA separted on 1% agrosegel

Lane 1: Extracted Dna From Sewage Water Sample.
Lane 2: Extracted DNA from sugar plant water Sample.
Lane 3: Extracted DNA from pond water Sample.
Lane 4: 1kb DNA Marker.

Pasmid DNA separated on 1% agrosegel



The fragmentation of plasmid DNA by a restriction enzyme *BamH1*, which can recognized by the RFLP analysis.

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