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A Simplified Analysis of Different *Escherichia coli* Strains by Using RAPD Technique

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Abstract

Characterization of different strains of *Escherichia coli* were analyzed for which samples were collected from different areas of Bangalore and cultured in MacConkey agar. Strains were finally isolated and their biochemical characterization was done for their proper identification, extraction of the nuclear DNA and its purity was tested. Quality testing with the help of spectrophotometer has been carried out and finally the application of a random amplified polymorphic DNA (RAPD) analysis for molecular genetic typing of pathogenic *Escherichia coli* strains was performed. The RAPD technique was shown to be highly reproducible. Stable banding patterns with a high discriminatory capacity were obtained using two different primers. Overall, 06 *E. coli* strains were analyzed with the RAPD technique. The RAPD analysis showed that the *E. coli* strains isolated from different locality in Bangalore (India) could be grouped into different RAPD types by using these two different primer sets. Most of these different *E. coli* RAPD types were not geographically restricted. There was, as expected, a tendency of higher genetic relationship among *E. coli* strains isolated from the same locality, which was finally obtained, while some other strains differ from each other due to the environmental impact. In conclusion it may be suggested that the RAPD technique may provide a rapid, low cost, simple and powerful tool to study the clonal epidemiology of rapid *E. coli* infections. RAPD technique also helps in identifying phylogenic relationship among species.

Keywords: *E. coli*, phylogenic relationship, PCR, genetic typing, DNA

Introduction

Escherichia coli is a common inhabitant of human and animal intestines of all warm blooded animals. It is a facultative anaerobe. The temperature range is 10-40^{0}C (optimum 37^{0}C) is good for the growth of E.coli. Colonies are large, thick, grayish white, moist, smooth opaque or partially translucent discs. From birth, *E. coli* colonizes in the gastrointestinal tract where it interacts in symbiosis with its host. It is a wide indicator for fecal pollution (Whitman, 2005).

Four biochemical tests are widely employed in the classification of enterobacter, the Indole, Methyl red (MR), Voges-proskauer (VP) and Citrate utilization tests. *E. coli* is indole and MR positive and VP and citrate negative. Flores Abuxapqui JJ (1999). *E. coli* produces two kinds of toxins – hemolysens and enterotoxins. Hemolysens do not appear to be relevant in pathogenesis though they are produced more commonly by virulent strains. Thompson J S (1990).

Enterotoxins are important in pathogenesis of diarrhea. Three distinct types of *E. coli* enterotoxins have been indentified – heat labile toxin (LT), heat stable toxin (ST) and verotoxin (VT) (Pickett, 2004). The science of molecular biology has passed through many interesting events. Before, it used to take a lot of time to arrive at a conclusion regarding genetic problems and queries. But now the time taken to solve the genetic problems has been rapidly shrinking because of the availability of different advanced techniques. Histories of science repeat with examples of obvious and simple discoveries being missed. When these discoveries have been made at a later date many of them are turned out to be measure advances, out of such example is that Polymerase Chain Reaction (PCR).

The American scientist Kary Mullis of Ceutus Corporation, USA is the inventor of this precious instrument, PCR. This technique carries out different reactions equivalent of the reaction carried out in the molecules of Eukaryotic cell by DNA Polymerase Reaction. PCR can be defined as recurring replication amplification of a perpendicular region of the genome pre-selected with the help of primers flanking the region of genome to be amplified. Within a test tube the isolated dsDNA is denatured through heating at 90-98^{o}C to separate two strands at sites bordering the sequence to be amplified, which are mixed and the tube is cooled to 40-60^o C for primer annealing. Then the extension of DNA strand is maintained giving 70-80^{0}C for cycles and a final extension given for 5-7 minutes. Then the amplified product may be further used (Fromenty, 2000).

RAPD polymorphism is detected by using oligonucleotides (20 bases) of various sequences as primers in a

PCR reaction. In a strain which has in its genomic DNA a sequence complimentary to the primer oligonueleotide, PCR products will be detected in the gel, while in those strains that do not have the complementary sequence, the products will not be detected (Vogel, 2000) The RAPDs may be of different types, AP-PCR (Arbitrary Primer-PCR), AFLP (Amplified fragment Length Polymorphism) these are PCR amplified fragment lengths consisting of VNTRs). RAPDs analysis is facilitated by the availability of base sequence of a short segment of genomic DNA (Tseng, 1999).

Variation in this segment can now be detected by generating oligonueleotides having any base sequence that can be used in these studies. The oligonueleotides can be prepared by using an oligonueleotide synthesizer. It is important that RAPDs approach does not utilize restriction enzymes and probes of any kind. It utilizes in the PCR a DNA polymerase which is resistant to the high temperatures needed for DNA denaturation, so that it is reused repeatedly during DNA amplification. Thus RAPDs represent savings in both cost effort and their use is expanding rather rapidly (Niwat, 2001).

The RAPDs have the same applications as RFLPs. RAPD analysis uses a short (about 10 nucleotide long) primers to amplify the genomic DNA. Each primer can generate a specific DNA profile per sample (Kilic, 2009).

Materials and methods

The research was been carried out at Sangene institute of Bioscience, Bangalore, India. The samples are collected from possible distinct environmental conditions. Five water samples and five soil samples were taken initially for analysis of *E.coli* strains: such as from hospital area, park area, residential area, playground area, temple area, sewage area etc.

Media for the culture of microorganism were prepared with required precaution such as in aseptic conditions. After the media were prepared, they were sterilized with the help of autoclave at 1210C and 15lb pressure for 15 minutes. The glassware was also placed under the autoclave for proper sterilization. The Laminar airflow chamber (LAF) was properly sterilized in order to avoid contamination. The base of laminar airflow chamber was swabbed with alcohol and hands were rinsed with rectified spirit before performing any work under laminar airflow chamber.

Different culture techniques are adopted for samples such as serial dilution method for soil samples, spread plate methods and poor plate methods for water samples. MacConkey agar and EMB (Eosin Methylene Blue) agar have been used for the culture of *E. coli* as these are considered differential medium and can differentiate between lactose fermentive and non-fermentive organisms.

Several staining techniques were performed on the isolated colonies of microorganisms to distinguish them properly. A loop full of cultured organism was taken on a clean glass slide, adding a drop of water, while a smear is prepared. The prepared smear is fixed there by passing the slide over fumes or flames. Then the smear was stained with Crystal violet and kept for 30 seconds. The slide rinsed under tap water avoiding washing up the smear. The smear was stained with gram's iodine and allowed to stand for 30 seconds. The steps are repeated again and rinsed under tap water. The smear was then flooded with 95% alcohol for 5 minutes and treated with counter stain, saffranin for 30 seconds and washed under tap water finally allowed to dry and then observed under oil immersion objective microscope by adding a drop of immersion oil on smear.

Several biochemical tests have been performed to find out the responses of cultured bacteria to different biochemical agents such as:

Indole test, which has been performed to test the microbes whether, can produce tryptophanase enzyme or not. 5 ml of tryptone broth is taken into a test tube. The sample (microbe) is inoculated to the broth and incubated at 27^0C for 36 – 48 hours. After 48 hours 0.5 µl of kovac's reagent is added and shaken gently.

Methyl red test was done for the detection of acid production. For this test MR-VP broth was taken in a test tube. The tubes were inoculated with bacteria. Then one inoculated test tube was maintained as control. All the test tubes were incubated at 37^0C for about 48 hrs. After 48 hrs 5 – 6 drops of Methyl red indicator was added to each tube and shaked well.

Voges proskauker test was performed to detect the production of aceto toxin or acetyl methyl and carbonic acid from glucose. For this test 5 ml of MR-VP broth was taken in each test tube and the bacterial cultures were isolated to it. One tube was maintained as control. The tubes were incubated at 37^0C for 24 – 48 hours then 0.6 µl of α-naphol and 0.2 µl of KOH were added to all the tubes, finally allowed to stand for 45 minutes.

Citrate utilization test was performed to detect whether the given bacterial culture can utilize citrate as a carbon source or not. For this test About 2 ml of Simon citrate agar was taken into each test tube and test tubes were allowed to form slants. To each of the slant, a bacterial culture was inoculated and incubated at 37^0C for 24 - 48 hours.

Finally, after several biochemical analyses and staining processes, six out of ten samples were identified as containing *E. coli* colonies. Then the isolation of DNA from the samples of *E. coli* has been performed, as DNA was extracted from bacterial cultures at mid to late logarithmic growth phase. 1.5 ml of culture was taken in an eppendorff tube and centrifuged at (6000 rpm for 10 min) for three times. Discarding supernatant 1 ml of lysine buffer was added and incubated at 65^0C (water bath) for 10 minutes then 1 ml of phenol chloroform mixture to added and centrifuged (10,000 rpm, 10 min). The top aqueous layer was transferred and an equal volume of chloroform. Then sodium acetate was added to the solution: 1/20th part of 3M and centrifuged (10.000 rpm for 10 min.). The aque-

ous layer is collected and double volume of chilled ethanol is added. Then it was incubated at (-20^{0}C) for 15–20 minutes. It was followed by centrifuging at (10,000 rpm for 10 min). The supernatant was discarded, the pallet is air dried and dissolved in 20 -50 µl of TE buffer.

The purification of the extracted DNA was performed to identify the purity of the DNA from contaminants like RNA, protein, carbohydrates etc., the DNA was treated with RNAase at 37^{0}C for 1 hr. Then the DNA was purified by phenol: chloroform (1:1) extraction and precipitated in chilled ethanol (volume 2.5 ml), then preserved in 0.3 M sodium acetate (pH 5.2). The DNA was pooled out, washed in 70% ethanol; air dried and dissolved in Tris buffer.

The quantification of DNA was performed to assess the amount of DNA present. The concentration of the DNA in the buffer was determined by the instrument UV-VIS spectrophotometer. The 10 µl of distilled water and 10 µl of DNA sample taken. Then observed optical density was recorded.

The quality of DNA is tested by two methods. The UV-VIS spectrophotometeric method and agarose gel electrophoresis method. In Spectrophotometry method the OD of surplus are recorded at 260 nm and 280 nm and purity value calculated. In the agarose gel electrophoresis method 1% agarose gel is prepared, EtBr (ethidium bromide) was added to it, the gel was casted the samples are loaded finally observed under UV-Transilluminator

By the use of PCR, RAPD profiles were generated by using single decamer primers (Openon technologies, alameda, USA) in polymerase chain reaction. The reaction mixture ware prepared such as each reaction mixture contained 10 µl of template DNA, 28µl of d NTPs, 30 µl of tris buffer (50 µm kcl, 10 µm Tris-HCl, 1.5 µm MgCl2, pH – 9.0), Nuclear free water 151 µl, 14 µl of Taq DNA polymerase (Bangalore Genie Pvt. Ltd., Bangalore, India) and mineral Oil 142 µl in final cocktail solution of 280 µl. It is divided into seven tubes, where six tubes with different template DNAs and one tubes without template DNA with mineral oil was used as marker. In cocktail solution the 18 µl of each, two different types of primers were added having the sequence 5' GTTTCGCTCC3' and 5' AAGAGCCCGT 3'. The reaction was carried out in (Gene Amp PCR System 2400 thermal cycler). The initial phase consisted of denaturation 95^{0}C for 5 minutes. Then the cycle starts with denaturation phase at 95^{0}C for 1 minute, primer annealing phase at 360C for 1 minutes and DNA extension phase at 72^{0}C for 2 minutes and repeated for 45 cycles. At the end of cycles, a final extension period of 5 minutes was given at 72^{0}C. The amplified product were stored at 4^{0}C and separated by electrophoresis on 1% agarose gel in 1% TAE buffer EtBr (ethidium bromide) was added to it the gel was run for 3 hours at 50 V. The gel was finally visualized by UV- transilluminator.

Results and discussion

The ten samples which have been taken from different parts of Bangalore after being cultured in an agar medium, only six samples have provided the colonies which can be suspected as being *E. coli* colonies from the morphological point of view, as usually *E. coli* colonies provides a greenish overlapping colony with a metallic shine, hence only these culture petri plates were taken for subculture and biochemical analysis.

Biochemical characterization of different strains of *E. coli* showed similar results for all the tests (Tab. 1). In gram's iodine test the non retention of primary stain showed that the strains are gram negative. In the Indol test after addition of kovac's reagent the cherry red colour appeared showing indol positive test. In Methyl red test all the samples showen MR positive and in Voges proskauker test all the tested samples showed VP negative tests. In the citrate utilization test all the tubes showed the green colour after the incubation of 48 hours which also showed the citrate negative for all strains.

The quality of DNA obtained from the research work was found to be good as it was (≤ 1.8) in purity reading (Tab. 2).

The gel profile study showed the result of similarity in the banding pattern of sample 1 and 2, which are obtained along with sample 4 and 5 and all the other samples were different from each other in there banding pattern (Fig. 1). Due to the diversity of the environmental condition, the species or different strains of the same species such as *E.*

Tab. 1. Result of biochemical characterization of different samples of *E.coli*

Serial number of samples	Gram staining	Indole test	Methyl red test	Voges proskauker test	Citrate utilization test
1	Negative	Positive	Positive	Negative	Negative
2	Negative	Positive	Positive	Negative	Negative
3	Negative	Positive	Positive	Negative	Negative
4	Negative	Positive	Positive	Negative	Negative
5	Negative	Positive	Positive	Negative	Negative
6	Negative	Positive	Positive	Negative	Negative

Tab. 2. Result of Optical density (OD) value of tested samples showing their purity value

Serial number of samples	Absorbency at 260nm	Absorbency at 280nm	A260/A280
1	0.437	0.248	1.76
2	0.322	0.201	1.6
3	0.355	0.212	1.67
4	0.429	0.239	1.79
5	0.278	0.171	1.62
6	0.249	0.142	1.75

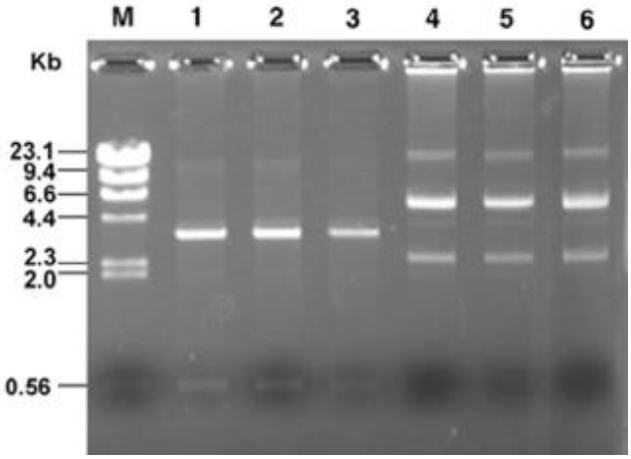

Fig. 1. Gel profile study showing M (marker) bands and the similarity in the banding pattern of sample 1 and 2 with each other and sample 4 and 5 with each other data shown with reference to the molecular weight

coli were not showing the same sort of phylogenic relationship among each other.

Conclusions

The gel profile study suggested that although the bacteria are found in a same locality or different areas, the environmental impact on their growth and development will be always found. As such, it is difficult to find out the phylogenic relationship among species of the same genus of bacteria found in a certain environmental condition.

From the detailed study it was concluded that this E.coli, which is considered as a harmful gram negative pathogen is very commonly found in different localities of Bangalore and strains are prominent in public places as well as in parking and school areas, which is a major cause of infection by *E .coli*. The contamination of food products should be avoided from this harmful pathogen in order to control the infectious diseases caused by *E. coli* strains.

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