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Original Research Article

Molecular Studies in the genetic variability of verotoxin producing strain of *E.coli* from clinical isolates

M.S.Leelavathi*, Apoorva, Jaya Bansal and M.P.Prasad[2]

[1]PG Department of Microbiology, CMR Institute of Management Studies, 6th 'A' Main, HRBR Layout, 2nd Block, Kalyanagar, Bangalore-560043, India
[2]Sangenomics Research Labs, Domlur, Bangalore, India
******Corresponding author*

A B S T R A C T

E.coli are opportunistic pathogens mostly causing diarrheal diseases they are seen as pink short rods under the microscope by Gram staining. *E.coli* were isolated from clinical samples cultured in LB broth and streaked on Macconkey agar and nutrient agar slants. The appearance of cheery red colour in the alcohol layer confirmed the result for IMViC tests. Catalase test and Motility test was found to be positive. The confirmatory test was done in Eosin Methylene Blue Agar. The genomic DNA from 24 Hour old culture was extracted by phenol chloroform method. The Extracted DNA was quantified by nanodrop spectrophotometerand quantified by nanodrop spectrophotometer and its quality assessed in 1% agarose gel, the DNA was selected for RAPD PCR reaction. The PCR products were checked on 2% agarose gel. Amplification of DNA Arbitrary primers involving whole genome analysis conformed that all four samples showed DNA polymorphism.

Keywords

E coli; verotoxin; PCR, Arbitrary Primers; Polymorphism

Introduction

Microbes are everywhere in the biosphere, and their presence invariably affects the environment that they are growing in. The effects of microorganisms on their environment can be beneficial or harmful or in apparent with regard to human measure or observation. *E.coli* is one of several types of bacteria that normally inhabit the intestine of humans and animals (commensal organism). Some strains of *E.coli* are capable of causing immune system is compromised or disease may result from an environmental exposure. *E.coli* bacteria may give rise to infections in wounds, the urinary tract, biliary tract, and abdominal cavity (Mashood *et al.*, 2007).

This organism may cause septicemia, neonatal meningitis, infantile gastroenteritis, travellers diarrhea, and

hemorrhagic diarrhea. The numbers of bacteria that are necessary to reproduce infectious levels of bacteria are quite small, estimated at 10-100 viable bacteria. These toxins are lethal for intestinal cells and those that line vessels (endothelial cells), inhibiting protein synthesis causing cell death. *E. coli* are widespread intestinal parasites of mammals, birds and humans and are present wherever there is fecal contamination. Various media can then be inoculated with the culture and then growth can be observed by carefully formulating the various media, the biochemical and growth characteristics of the organism can be determined (Mashood *et al*, 2007). Previously determined morphological characteristics can be combined with biochemical data to properly classify the organism.

The organisms are known to cause enteric infections and diarrhea (gastroenteritis) in humans and animals, and many strains have been identified to produce verotoxins or shiga toxins. These toxins are responsible for lethal acute bloody diarrhea (haemolytic colitis and haemolytic uremic syndrome) in humans (Karmali 1989).

These toxins have RNA N-glycosidase activity, which inactivates protein synthesis of eukaryotic cells and causes cell death Stx1 and Stx2 share a common structure and 58% amino acid sequence homology (Endo *et al*., 1988, Saxena *et al.,* 1989, Ogasawara *et al.,* 1988). It has been reported that two amino acid residues of these toxins, 167 Glutamine and 170 Arginine are most important for toxic activity, and mutations in these sites greatly reduce their cytotoxicity (Hovde *et al.,* 1988, Jackson *et al.,* 1990, Yamasaki *et al*., 1991).

Molecular characterization of the genes can be done by different molecular biology tools such as RAPD, RFLP, ISSR, SCAR or specific gene markers. Since molecular biology works at genetic level, the use of these techniques is much more specific and accurate (Endo *e .al*., 1988).

RAPD (pronounced "rapid") stands for **random amplification of polymorphic DNA**.

It is a type of PCR reaction, but the segments of DNA that are amplified are random. The scientist performing RAPD creates several arbitrary, short primers (8–12 nucleotides) then proceeds with the PCR using a large template of genomic DNA, hoping that fragments will amplify. By resolving the resulting patterns, a semi-unique profile can be gleaned from a RAPD reaction (Fevzi Bardakci., 2001).

Considering the clinical significance of the VTEC, rapid, specific, and sensitive detection methods are required to identify toxin-producing isolates and replace expensive and time-consuming tissue culture techniques, as reported for enterotoxigenic *E.coli* (Pollard *et.al*, 1990). The use of specific gene primers is a rapid and accurate technique for characterizing the *E.coli* which can produce verotoxins. A number of works have been done using VT specific primers. Polymerase chain reaction is the technique used to amplify the specific genes.

Primers (short DNA fragments) containing sequences complementary to the target region along with a DNA polymerase (after which the method is named) are key component to enable selective and repeated amplification. As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template

is exponentially amplified. PCR can be extensively modified to perform a wide array of genetic manipulations (Maurer *et al.*, 1998). In this study, we have made an attempt by collecting 4 *E.coli* clinical samples. Biochemical characterization of the samples showed to be positive. Molecular characterization was done by RAPD to check out the difference between the samples and specific genes were used to characterize whether they have verotoxin gene .For the later, we used to specific primers namely, VT_1 and VT2 (Pollard *et al.*, 1990).

Materials and Methods

The samples taken for this study was verotoxin producing clinical samples. (replicates from diaaharial Patients) was collected from Hospitals in and around Kalyannagar, Bangalore. The isolated colonies were identified based on morphology (shape, size, structure, texture, appearance, elevation and color). Further identification (genus level) was done on the basis of Gram staining and Biochemical tests. The isolated colonies were examined and the organisms were identified based on Morphological and Biochemical characterization and sub cultured on LB agar plates for further examination (Mashood *et.al*, 2007).

The samples were then centrifuged at 12,000 rpm for 10 minutes. The supernatant was discarded and the pellet was air dried. The DNA pellet was then dissolved in 20-50µl of 1X TE buffer, stored at 4^0C. The DNA isolated was then quantified by Nanodrop Spectrophotometer and in 1% Agarose gel.

Quantitative estimation of DNA by nanospectrophotometer

The computer was turned on the Nanodrop software was opened. Type of measurement was selected as Nucleic Acid. After initializing the machine by loading 1.5 ul of millipore-filtered water, 1.5µL water or other appropriate blank (e.g. TE buffer) was loaded on the pedestal surface. After each step, the top and bottom of the measurement pedestal was cleaned with clean tissue paper. After blanking, 1.5µL of the samples was loaded and the quantity and quality analysis was done.

DNA Fingerprinting by RAPD

The standard RAPD technology utilizes short synthetic oligonucleotides (10 bases long) of random sequences as primers to amplify nanogram amounts of total genomic DNA under low annealing temperatures by PCR. PCR amplification with primers shorter than 10 nucleotides [DNA amplification fingerprinting (DAF)] has also been used producing more complex DNA fingerprinting profiles. Although these approaches are different with respect to the length of the random primers, amplification conditions and visualization methods, they all differ from the standard PCR condition in that only a single oligonucleotide of random sequence is employed and no prior knowledge of the genome subjected to analysis is required. Nucleotide variation between different sets of template DNA will result in the presence or absence of bands because of changes in the priming sites. (Fevzi Bardakci., 2001).

Because of the simplicity and low cost of the RAPD technique, it has found a wide range of applications in many areas of biology. PCR mixture was prepared as follows (25µl reaction volume). Dendrogram Analysis was done for the samples using Alpha Imager Software and similarity was calculated by plotting Similarity Matrix. For Frequency

Table.1 Showing the Verotoxin specific primers VT1 and VT2

Primer	Sequence	Tm (^{0}C)
VT1 Forward	5'-CAGTTAATGTGGTGGCGAAGG-3'	56.3
VT1 Reverse	5'-CACCAGACAATGTAACCGCTG-3'	56. 2
VT2 Forward	5'-ATCCTATCCCCGGGAGTTTACG-3'	58.1
VT2 Reverse	5'-GCGTCATCGTAT ACACAGGAGC-3'	57. 5

Fig 1 & 2 Nanodrop spectrophotometer report for samples A,B,C and D

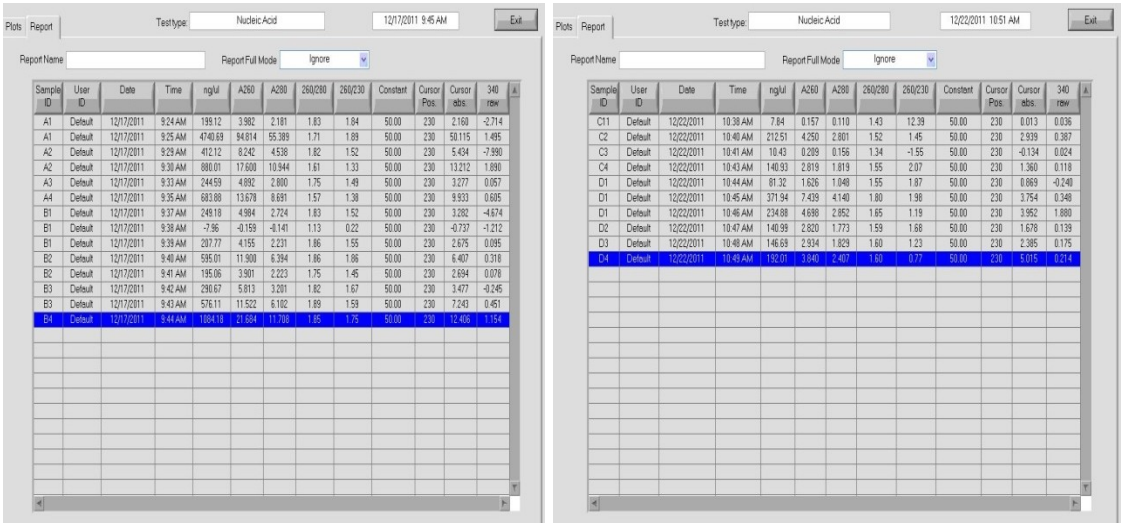


Fig 3: Plot of A1 & **Fig 4:** Plot of A3

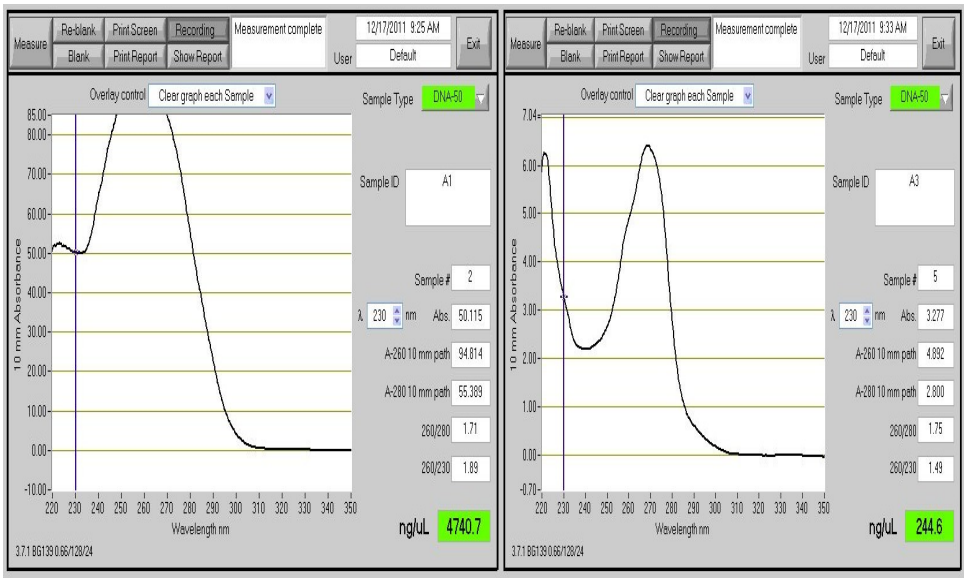
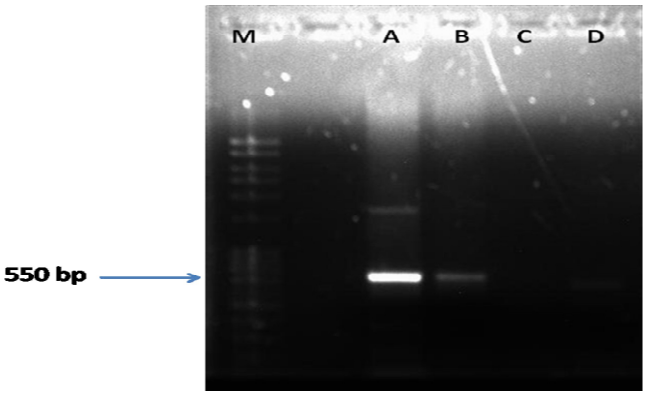


Fig 5: PCR product of VT1 primer (Specific gene seen at 550bp in A and B).



similarity development Distance Matrix was used and UPGMA was the cluster method used (Atienzar *et al*., 2002; Atak *et al*., 2004).

PCR Analysis of verotoxin specific gene

After RAPD verotoxin specific gene analysis was done by PCR by using two sets of Verotoxin specific primers namely, VT1 and VT2. The melting temperature (Tm) of primers the primers were found by using Oligoanalyzer software.

The presence of verotoxin gene was confirmed by running the PCR product on 1.2% Agarose Gel (Pollard *et al.*,1990).

Results and Discussion

The samples taken for this study was verotoxin producing clinical samples. These samples were then identified by Biochemical Characteristics such as IMVIC, Motility test and then the Selective Medium (EMB Agar). And finally four samples were selected and given the codes A, B, C, D respectively. The samples were then inoculated on to LB broth and incubated for 48 hours at 37^0C. After Biochemical characterization, molecular characterization was done for the samples. For further studies DNA was isolated from the samples by Phenol: chloroform Extraction method. The isolated DNA was quantified by Agarose gel Electrophoresis and Nanodrop Spectrophotometer.

Random Amplified Polymorphic DNA is a PCR based technique that is widely used in molecular biology. PCR selectively amplifies the specific segments of DNA of an organism. RAPD was done using 2 bacterial specific RAPD primers (Table 1). Including the 4 clinical samples, a non pathogenic *E.coli* DNA was also used. The non pathogenic *E.coli* was labeled as K and the 4 clinical ones as A, B, C and D respectively. After performing RAPD, PCR specific gene primers (two) which are verotoxin gene specific, namely VT1 and VT2 was analyzed. (Guillermo *et.al*., 1995). (Pollard *et al.,* 1990).

The study was done to isolate verotoxin producing strains of *E.coli* from clinical samples and study their genetic variation. These are opportunistic pathogens mostly causing diarrheal diseases. The PCR products were checked on 2% agarose gel. 5 RAPD primers specific for

bacterial genome was used. Amplification of DNA with arbitrary primers involving whole genome analysis confirmed that all four samples showed DNA polymorphism. Except Primer 3, amplification of the bacterial DNA was seen with other four primers. Verotoxin gene of the pathogenic *Escherichia coli* samples were amplified using verotoxin specific primers VT1 (sequence) and VT2 (sequence) (Fevzi Bardakci., 2001).Amplification was seen with primer VT1 at 550 bp in A and B samples (Fig5).

From the study it is concluded that the verotoxin producing strains of *E.coli* isolated from the clinical samples of diaaharial patients and their study of genetic variation based on the RAPD markers showed the whole genome analysis. And it is confirmed that all four samples showed the DNA polymorphism.

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