

CLONING AND MOLECULAR CHARACTERIZATION OF GAG GENE FROM HIV-1 INTO *E. COLI* DH5A HOST

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ABSTRACT

Considering the worldwide increasing prevalence of human immunodeficiency virus type 1 (HIV-1) infection, World Health Organization (WHO) has intensified the access to the antiretroviral treatment. In spite of that one of the major issues to eradicate HIV-1 is the persistence of proviral human immunodeficiency virus type 1 (HIV-1) DNA reservoir. Although PCR detects HIV-1 DNA, the diagnosis of early, post exposure HIV infection prior to seroconversion can be achieved by the detection of proviral DNA by RTPCR. In the present study HIV-1 DNA were isolated from patient with HIV and using specific primer designed using Primer 3 plus software for the HIV-1 gag gene. The amplified gene was ligated with T vector and transformed into DH5 α cells. The plasmid DNA obtained was then confirmed by restriction digestion and sequence analysis. The sequence was found to be 98% similar to that obtained in GenBank. Further research is required to express the gene to get the protein antigen for the production antibodies or effective vaccine for HIV-1.

Keyword: GAG gene, HIV, RNA, Cloning, RT-PCR.

INTRODUCTION

HIV, one of the major global public health issues constantly, has affected greater than 36 million lives so far. About 35.3 million people globally are living with HIV infection as per the World Health Organization (WHO) reports [1, 2, 3]. Globally, one of the challenges to eradicate HIV-1 is the persistence of proviral human immunodeficiency virus type 1 (HIV-1) DNA reservoir. In order to quantitate the proviral HIV-1 DNA load particularly when the HIV-1 RNA levels decreases below the threshold during the highly active retroviral therapy (HAART) treatment, HIV-1 proviral DNA remains as an important diagnostic marker in the evaluation of HIV-1 infection of newborns of HIV-1 seropositive women [4].

The increasing use of polymerase chain reaction (PCR), the method of choice for diagnosis of HIV-1 infection, especially when anti-HIV-1 antibodies are present in low levels or may be passively acquired, has become a significant tool in the field of biotechnology. However, owing to its more time consuming process, less sensitivity, highly sophisticated and labour intensiveness, currently, there is a lesser reliability on this conventional PCR for the diagnosis of HIV-1 infection [5, 6, 7]. Several studies on PCR-based diagnosis of HIV-1 genes in clinical samples have underpinned the needs for optimization of this technique for diagnosis and characterization of the virus. In the present study, our objective is to isolate DNA and RNA of HIV-1 type virus using RTPCR from clinical samples and to clone and identify the HIV gag gene [8, 9].

MATERIALS AND METHODS

Sample collection and extraction of RNA

Twenty HIV infected blood samples were collected from the AIDS patients at Govt. Hospitals around Bangalore. Out of which 3 samples were selected for further analysis. The sample blood collected from the HIV patient is taken and out of which 100 μ l blood is taken into separate tube container. The RNA is extracted from the sample and the quantity of the isolated RNA was checked in UV-VIS spectrophotometer (Vivaspec Biophotometer, Germany).

Primer Designing and PCR amplification

The specific primers were designed using Primer3 Plus software (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>) and the designed oligonucleotides were synthesized in Sigma Corporation USA. The Initial denaturation is done at 94°C for 2 min which is followed by denaturation, annealing and extension at 94°C for 50 s, 52°C for 30 s and 72°C for 1 min respectively. This step of denaturation, annealing and extension is continued for 30 cycles.

The final extension is done at 72°C for 6 min. The sequence (5'-3') of the forward primer is (ACA GGA CAC AGC AAT CAG GTC) and the % of GC is 52.0. The product size is 1000bp. The sequence (5'-3') of the reverse primer is (GCT CTG GTC TGC TCT GAA GAA) and the % of GC is 52.0. The product size is 1000bp. The products of the PCR are loaded on to the wells of the gel and the gel is allowed to run after the samples had run sufficiently, the gel was removed and visualized under U. V. light, photographed and documented using UV trans-illuminator.

PCR product extraction from Agarose gel

About 300mg agarose gel fragment is excised with scalpel. The gel slice is transferred to 1.5 ml or 2 ml tube. 650 μ l gel solubilised was added. Sample was incubated for 10 min at 50°C to dissolve slice and vortexed. 50 μ l binding optimizer was added and vortexed. 750 μ l of sample was transferred to spin column A placed in collection tube. Sample was centrifuged at 10000g for 1 min. Filtrate was discarded and collection tube was reused. 700 μ l wash buffer A was added. Sample is centrifuged at 10000g for 1 min. Filtrate is discarded and collection tube is reused. Sample was centrifuged at max speed for 2 min and collection tube was discarded. Spin column A is placed in 1.5 ml elution Tube. 30-50 μ l elution buffer is added directly to spin column membrane. Sample was incubated at room temperature for 1 min. Sample was centrifuged at 6000g for 1 min to elute DNA. The eluted DNA was stored at 4°C, until further use.

Ligation of the eluted gene product into T-vector

The eluted PCR products were ligated separately into the pTZ57R/T cloning vector (Fermentas, USA). The amount of PCR product to be used for 1:3 vectors to insert ratio for each ligation reaction. Ligation mixture was incubated at room temperature (25°C) for one hour after a short spin. The ligated product was later kept on ice until the transformation experiment started. *E. coli* strain DH 5- α was used in the cloning work. The competent *E. Coli* cell is prepared by using sambrook and Russel method 2001 [10]. After the preparation of the competent cell the bacterial cells were subjected to transformation and from the suspension of transformed bacterial culture, 100 μ l was spread on LB agar plate using a bent sterile glass rod. The plates were incubated at 37°C overnight.

Screening of positive clones and Plasmid isolation by alkaline lysis method

White colonies containing recombinant plasmids due to the insertional inactivation of the *lacZ* gene were selected and streaked on a fresh LB plate containing Ampicillin and incubated overnight

and served as a master plate for each transformants. All colonies from the master plate were subjected to plasmid DNA isolation and restriction analysis to identify the positive recombinants. The plasmid isolation protocol included harvesting and lysis of the cells by alkali. Each of the selected clones were inoculated separately into 2 ml of LB medium containing Ampicillin (50 µg/ml) in a 15 ml sterile tube. The cultures were incubated overnight at 37°C with vigorous shaking. To a new sterile micro centrifuge tube, 1.5 ml of culture was poured and centrifuged at 12000 rpm for 30 seconds at 4°C in a micro centrifuge. The medium was removed and the bacterial pellet was dried. The bacterial pellet was resuspended in 100 µl of ice cold solution I by vigorous vortexing. 200 µl of freshly prepared solution II was added and closed tightly. The contents were mixed by inverting the tube rapidly for five times, stored on ice and added 150 µl of ice cold solution III. The tubes were closed again and vortexed gently in an inverted position for 10 seconds. Then the tubes were incubated on ice for 3-5 minutes and centrifuged at 12000rpm for 5 minutes at 4°C in a micro centrifuge. The supernatant was transferred to a fresh tube and an equal volume of chloroform: isoamylalcohol (24:1) was added. The mixture was vortexed and centrifuged at 12000rpm for 2 minutes at 4°C in a micro centrifuge. Plasmid DNA was precipitated with 0.6 volume of ice cold isopropanol and centrifuged at 12000rpm for 10 minutes at 4°C in a micro centrifuge. The supernatant was discarded. The pellet was washed using 500µl of 70% ethanol and centrifuged at 12000rpm for 10 minutes. The pellet was re-suspended in 50µl of deionized sterile distilled water.

Confirmation of clones by restriction digestion, Sequencing and BLAST analysis

The purified plasmid was subjected to restriction digestion using restriction endonucleases (Merck, India). The products of restriction digestion were resolved in 1% agarose gel for confirming the release of the insert by the restriction endonucleases. The released gene insert was eluted from the Agarose gel using gel extraction kit (BioLine USA). The insert in the purified plasmid was sequenced using ABI PRISM Big Dye Terminators v1.1 cycle sequencing kit (Applied Biosystems Foster city, CA, USA). The comparison of the nucleotide sequences of the unique fragment with the sequences available in the GenBank database was carried out using the NCBI BLAST program (<http://www.ncbi.nlm.nih.gov/blast>).

RESULTS AND DISCUSSION

RNA isolation from blood

The total RNA was isolated from blood using phenol (chloroform method). The isolated RNA was electrophorized in 1% Agarose gel (Fig.1).

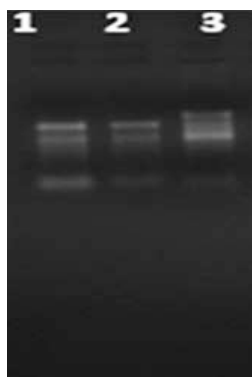


Fig. 1: Isolation of total RNA from AIDS patient blood

Reverse Transcriptase PCR (RTPCR)

Isolated RNA was quantified and cDNA was synthesized using MMLV Reverse Transcriptase enzyme. The single stranded cDNA was used as the template for the specific PCR based detection of HIV. Among three samples we tested one sample show the expected specific band at 1070bp (Fig.2). To confirm the RTPCR product, the band

was cut and eluted from the gel and cloned in to T vector and sequenced for further confirmation.

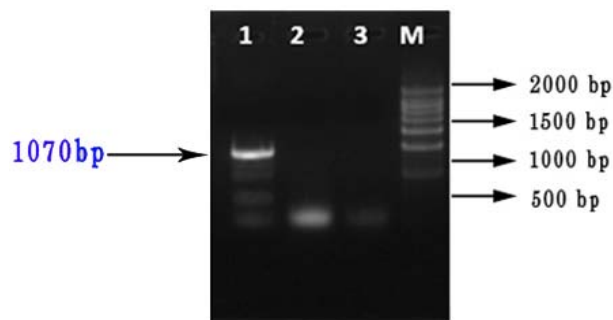


Fig. 2: RTPCR of the HIV blood samples

Cloning of PCR product in to T vector

PCR yielded a specific amplicon of ~1000-bp in one of the samples (Fig. 2). The fragment was separated in gel and purified the product using gel extraction kit. The purified PCR product was quantified and ligated with cloning vector (Fig. 3) using T4 DNA ligase enzyme. The ligated plasmid was transformed in to *E. coli* bacterial strain DH5α. The transformation was done by heat shock method and transformed cell was cultured in the Xgal-IPTG-Ampicillin-LB Agar plate at 37°C for overnight (Fig. 4). The white colonies were picked up from the plates and cultured in Ampicillin containing LB broth.

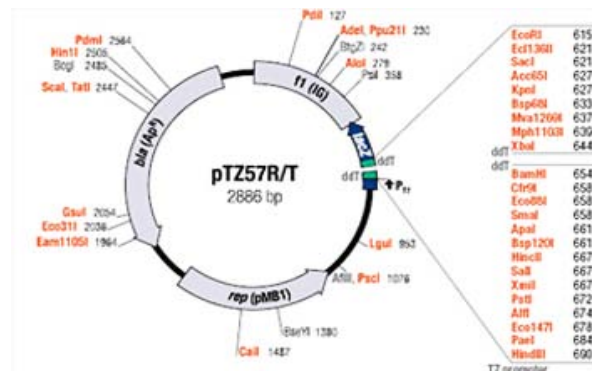


Fig. 3: Map of T vector pTZ57R/T (Fermentas, Germany)

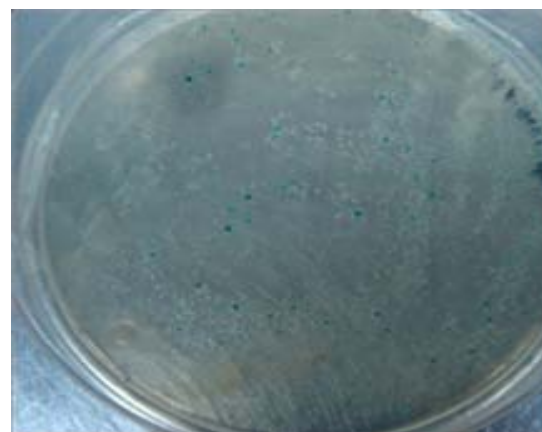


Fig. 4: Blue white selection of the transformed bacterial cells in the Xgal-IPTG Ampicillin-LB Agar.

Plasmid Isolation

Plasmid was isolated from the transformed cells by using alkaline lysis method. The isolated plasmid was electrophorized on 1% Agarose gel (Fig. 5).

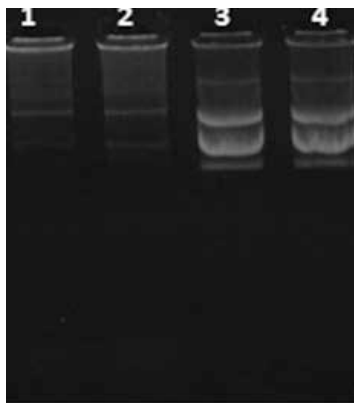


Fig. 5: Plasmid isolated from the transformed bacterial cells

Confirmation of clone by restriction digestion

The purified plasmid was subjected to restriction digestion using *Bam* H1 and *Eco*R 1 (Merck, India). After incubation at 37°C for 4 hours the restricted product was electrophorized on 1% Agarose gel. The release of the gene product was visualized in the gel (Fig. 6)

Sequence data

The gene insert was identified by sequencing of plasmid. An approximately ~1000-bp region of the, *HIV* specific region was

sequenced at Euro fins, Bangalore. The sequence data was shown below. Nucleotide sequence analysis of the insert was used to investigate the identity of HIV gene. To demonstrate the quality and accuracy of results provided from a public database, we compared sequences to their corresponding GenBank sequences. The sequence had "perfect" match (similarity, 98%) with sequences of HIV type 1 from GenBank as determined by using BLAST (version 2.7).

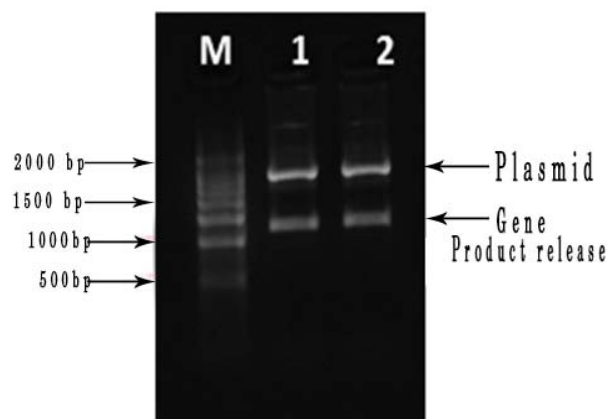


Fig. 6: Restriction digestion of ligated plasmid using *Bam* H1 and *Eco*R 1 (Lane 1, 2- restricted product, M-200bp ladder)

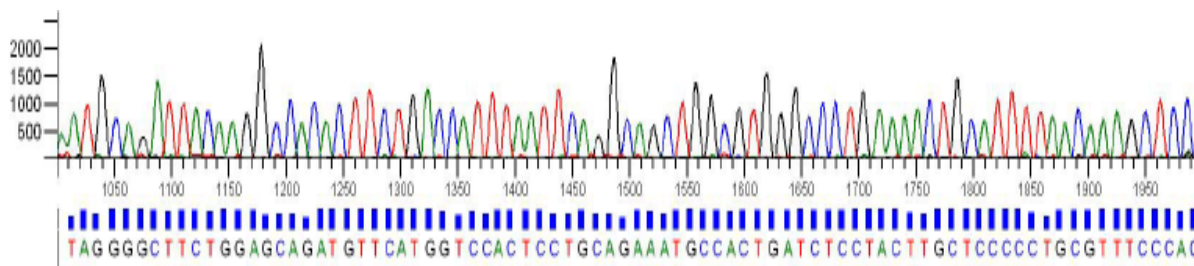


Fig. 7: Showing the sequencing Dendrogram

Sequence

>HIV 1 specific sequence obtained

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5'CAGCCAAAATTACCTATAGTGCAGAACATCCAGGGGCAAATCGTAC
ATCAGGCCATATCACCTAGAACCTTTAAATGCATGGGTAAAAGTAGTAG
AAGAGAAGGCTTTTCAGCCAGAAGTGATACCCATGTTTTTCAGCATTAT
CAGAAGGAGCCACCCACAAGATTTAAACACCATGCTAAACACAGTGG
GGGACATCAAGCAGCCATGCAAATGTTAAAAGAGACCATCAATGAGG
AAGTGCAGAATGGGATAGAGTGCATCCAGTGCATGCAGGGCCTATTG
CACCAGCCAGATGAGAGAACCAAGGGGAAGTGACATAGCAGGAACCTA
CTAGTACCCCTTCAGGAACAAAATAGGATGGATGACAAATAATCCACCTA
TCCAGTAGGAGAAATTTATAAAGATGGATAATCCTGGGATTAATA
AAATAGTAAGAATGTATAGCCTACCAGCATTCTGGACATAAGACAAG
GACCAAAGGAACCCCTTAGAGACTATGTAGACCGGTTCTATAAACTC
TAAGAGCCGAGCAAGCTTACAGGAGATAAAAAATTTGGATGACAGAAA
CCTTGTGGTCCAAAATGCGAACCAGATTGTAAGACTATTTTAAAAG
CATTGGGACCAGCGGCTACACTAGAAGAAATGATGACAGCATGTCAGG
GAGTAGGAGGACCCGGCCATAAGGCAAGAGTTTTGGCTGAAGCAATGA
GCCAAGTAACAAATTCAGCTACCATAATGATGCAGAGAGGCAATTTA
GGAACCAAAGAAAGATTGTTAAGTGTTCATTGTTGGCAAAGAAGGGC
ACACAGCCAGAAATTCAGGGCCCTAGAAAAAGGGCTGTTGGAAT
GTGAAAGGAAGGACACCAATGAAAGATTGACTGAGAGACAGGGTA
AATTTTTAGGAAGATCTGGCCTTCTACAAGGGAAGGCCAGG3'
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CONCLUSION

Systematic methodological development had significantly improved the sensitivity of assays of plasma HIV-1 RNA, so that detection of lesser levels of HIV-1 RNA can now be achieved. This higher

sensitivity has raised the following questions on the importance of transient increases in levels of plasma HIV-1 RNA in patients receiving antiretroviral therapy, the appropriate virologic definition of antiretroviral failure, and the cellular and compartmental origins of virus in patients receiving antiretroviral therapy who have persistent low-level viremia but no evidence of antiretroviral- drug resistance [11, 12]. It is probable that important insights into HIV-1 pathogenesis will come from studies that deal with these criteria [13]. In the present study HIV-1 DNA were isolated from patient with HIV and a recombinant plasmid DNA which contains the gene encoding the protein gag gene of HIV in *E. coli* strain has been successfully constructed. The results of the present study possibly remain as a basis for further studies on the usefulness of the gene and its expression product in the development of new vaccine against HIV.

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