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Research Article

AMPLIFICATION AND CLONING OF RABIES VIRUS VIRULENT GLYCOPROTEIN GENE IN pALPHA VECTOR

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ABSTRACT

Rabies virus virulent glycoprotein gene was cloned in pAlpha vector in right orientation which was confirmed by restriction enzyme analysis, colony PCR and gene sequencing.

Key Words: Rabies virus, glycoprotein gene, cloning, RT-PCR

INTRODUCTION

Ever since the discovery of Wolff *et al* (1990) that naked DNA injection into the muscle of mice led to expression of the encoded protein, many groups have successfully applied this technique to develop DNA vaccine against infectious pathogens in different animal models (Ulmer *et al.*, 1993; Lagging *et al.*, 1995; Lowrie *et al.*, 1994; Major *et al.*, 1995; Michel *et al.*, 1995; Gupta *et al.*, 2001). With the help of recombinant DNA technology, plasmid expression vector carrying gene of interest is delivered and expressed in animal muscle. The DNA as a vaccine assumes great significance since it can offer protection against dreadful infectious diseases in developing countries besides being temperature stable and

affordable for the poor when compared to recombinant/ cell culture vaccines. Rabies virus G protein cloned in mammalian expression vector was shown to protect mice on challenge with 15LD₅₀ of rabies virus CVS 14 days post vaccination. (Rai and Yadav, 2001; Rai *et al.*, 2002, 2005, 2006; Gupta *et al.*, 2005; Ahi *et al.*, 2008; Kaur *et al.*, 2009). Similarly, pre-exposure vaccination with DNA vaccine has been shown to induce protection in mouse by many workers (Xiang *et al.*, 1994; Ertl *et al.*, 1995; Bahloul *et al.*, 1998, 2003, 2006; Lodmell *et al.*, 1998; Biswas *et al.*, 2001). Post exposure vaccination with DNA vaccine against rabies has also produced successful results in mouse (Lodmell *et al.*, 2001; Bahloul *et al.*, 2003). Lundstrom (2014) advocated the use of alphavirus

based DNA vaccines for enhanced immunity and protection. The most commonly used delivery vectors are based on three single-stranded encapsulated alphaviruses- Semliki Forest virus, Sindbis virus and Venezuelan equine encephalitis virus. Alphavirus vectors have been applied as replication-deficient recombinant viral particles and, more recently, as replication proficient particles. A large number of highly immunogenic viral structural proteins expressed from alphavirus vectors have elicited strong neutralizing antibody responses in multispecies animal models. Furthermore, immunization studies have demonstrated robust protection against challenges with lethal doses of virus in rodents and primates. The present work was undertaken to clone the G gene from virulent strain of rabies virus in pAlpha vector.

MATERIALS AND METHODS

Vector

The pAlpha vector (10.799 kb) was available in the Biotechnology Lab of IBIT Bareilly and used for cloning the gene. It has Sind virus replicase gene, T7 promoter sequences, Bovine growth hormone (BGH) ply (A) signal sequences, CMV promoter sequences and ampicillin resistance gene.

Rabies virus

The rabies virulent virus RNA was available in the Biotechnology Lab, IBIT Bareilly and used for gene amplification.

Nucleotide sequence of G gene

The nucleotide sequence of the glycoprotein gene was downloaded from

NCBI GenBank data base Accession No. EF151231.

Primers

Forward Primer: 5' CAC CAA GGA AAG ATG GTT CCT CAG 3' (24 mer), Reverse Primer: 5' CCT CAC AGT CTG GTC TCA CC 3' (20 mer), GH Reverse Primer: 5' TAG AAG GCA CAG TCG AGG 3' (18 mer)

c-DNA preparation

The reaction mix was prepared using 5 µl of 0.1M DTT, 10 µl 5x 1st strand buffer, 2.5 µl of 10mM dNTPs, 1 µl hexamer primer and 18 µl DEPC treated distilled water which were mixed. The added 10 µl (2-5µg) rabies virulent virus RNA, vortexed, spin down and incubated at 65^o C for 5 min in a water bath. Then annealed the primer at room temperature for 10 min. Added 2.5 µl M-MLV reverse transcriptase (200 U/µl), 1 µl RNasin (10-40 U) to the reaction solution thus making the total volume 50 µl. Incubated at 37^o C for 1.5 h in an incubator and then kept at 94^oC for 2 min in water bath to denature RNA-cDNA hybrid. Immediately put the tube on ice and then set up the PCR.

PCR amplification of glycoprotein gene (G gene)

The PCR was carried out in Thermocycler (Quanta Biotech, UK) using its software installed in a laptop connected to the thermocycler. The reaction mix (50 µl) consisted of: distilled water 28 µl, c-DNA 5 µl, G gene F primer (50 pmol) 2 µl, G gene R primer (50 pmol) 2 µl, MgCl₂ (25 mM) 5 µl, dNTPs (10 mM) 2 µl, buffer (10X) 5 µl, Pfu DNA polymerase 1 µl. The cycling conditions were initial denaturation at 94^o C for 5 min then 35 cycles of 94^oC for

1 min, 57°C for 1 min, 72°C for 2 min and the final extension at 72°C for 10 min, then hold at 4°C. The expected gene amplified was 1642 bp. The PCR product was run on 0.7% agarose gel electrophoresis along with 1 kb DNA ladder. The gel was run at 50V for 5 min and then at 100V till the separation of bands and the bands was visualized under UV light and photographed (UVP Gel Doc with Imaging system, UVP USA).

DNA extraction from agarose gel

The gel extraction of DNA fragments was done using MinElute Gel extraction kit (Qiagen, Germany) as per manufacturer's instruction.

Plasmid DNA isolation

Plasmid DNA isolation was done using TELT method (Ausubel *et al*, 1990; He *et al*, 1990). A single *E. coli* colony was picked up from the plate with the help of a loop and then transferred into 3 ml of LB broth containing 100 µg/ml ampicillin in a wide mouth culture tube. The inoculated culture was incubated at 37°C overnight in an orbital shaking incubator at 210 rpm. Alternatively, scraped a 2 mm diameter *E. coli* transformant colony grown on LB agar plate and suspended in 1.5 ml TE buffer. Pelleted the cells at 10,000 rpm for 30 sec in a microcentrifuge and resuspended the pellet in 100 µl TELT solution. Added an equal volume of 1:1 phenol/chloroform and vortexed vigorously for 15 sec. Spun 1 min in microcentrifuge at 10,000 rpm at 22°C. Collected the upper phase of nucleic acid and mixed with 2 volumes of 100% of ethanol. After two min, spun at 10,000 rpm for 10 min in microcentrifuge at 4°C. Washed the pellet with 1 ml of 100%

ethanol, dried the pellet and then dissolved in 30 µl TE buffer or nuclease free distilled water. The expected yield is 2-3 µg DNA per 1.5 ml culture or colony. The DNA preparations are relatively pure and are suitable substrates for ligase and restriction endonucleases. The procedure can be scaled up for use with 5 to 200 ml cultures.

pAlpha vector preparation

pAlpha vector was revived in LB growth media. 2 µl plasmid was added to 200 µl competent *E. coli* DH5 cells. It was kept on ice for 1 h. The mixture was heat shocked at 45°C for 2 min and immediately chilled on ice till further use. 600 µl freshly prepared SOC media was added to heat shocked mixture of plasmid and competent cells and then incubated at 37°C for 1 h in shaking incubator at 110 rpm. Cells were plated on LB agar medium in Petri dish containing 100 µg/ml ampicillin and grown overnight. A few colonies were picked up and streaked on agar plate to store for use in future. The same loop was used to inoculate fresh LB broth (5ml). Plasmid was isolated from this freshly inoculated overnight grown culture and checked on 0.7% agarose gel electrophoresis against 1 kb DNA ladder marker for the presence and its size.

Creation of cloning site in vector

Among the available few restriction sites at MCS, *StuI* was chosen as preferred site to create blunt end. A reaction mixture (50 µl) was prepared with *StuI* enzyme (New England Biolabs 10,000U/ml) per the protocol: pAlpha vector DNA (100 ng/µl) 15 µl, NE buffer 4 (10X) 5 µl, nuclease free water 27 µl, *StuI* enzyme (NE Biolab 10,000U/ml) 3 µl.

The reaction mixture was incubated at 37°C overnight. The linearised plasmid was checked and quantitated on 0.7% agarose gel electrophoresis. The linearised plasmid was then gel extracted and quantified.

Dephosphorylation of 5' ends

The calf intestinal alkaline phosphatase (CIAP) was used to remove 5' phosphate group from both the ends of the linearized plasmid. The reaction mixture (50µl) contained linearized vector pAlpha (100ng/µl) 10µl, CIAP enzyme (20 U/µl, Promega) 1µl, buffer (10X) 5 µl, nuclease free water 34 µl. The reaction mixture was incubated at 37°C for 30 min. But to be on safer side 1µl CIAP was added again and re-incubated at 37°C for 30 min. Enzyme was then inactivated by heating the reaction mixture at 75°C for 10 min.

Purification of dephosphorylated linearized vector

The Phenol chloroform precipitation was used following the protocol of (Sambrook and Russell, 2001). In brief, 50 µl of TE buffer was added to the reaction mixture followed by addition of equal volume of phenol chloroform Isoamyl alcohol (25:24:1) i.e. 100 µl for above reaction mixture. It was centrifuged at 10000 rpm for 10 min. The aqueous layer was transferred into a different tube. Again 50 µl of TE buffer was added to the tube containing phenol chloroform isoamyl alcohol and centrifuged at 10000 rpm for 10 min. aqueous layer was transferred to the collection tube. 1/10 volume (5µl) of 3M

sodium acetate was added to the aqueous collection. Then 2.5 times of 100% ethanol was added to it. The tube was kept at -20°C overnight. It was centrifuged at 10000 rpm for 20 min. supernatant was carefully discarded and pellet was washed in 1 ml of 70% ethanol by centrifuging at 10000 rpm for 10 min. the supernatant was discarded and the pellet was air dried. The dried pellet was then dissolved in 12 µl of nuclease free water. Again the presence of purified blunted DNA was checked by running 1 µl DNA on 0.7% agarose gel.

Blunt end ligation of pAlpha vector and rabies G gene

A 10 µl reaction mixture was standardized with 30% PEG8000 for blunt end ligation. The components were mixed in the following concentrations: pAlpha vector (100 ng/µl) 0.5 µl, rabies G gene (150 ng/µl) 4 µl, Ligation buffer (10X) 1 µl, 30% PEG 8000 (Amresco) 1.5 µl, T4 DNA ligase (Fermentas 20U/µl) 2 µl. The ligation mixture was incubated at 14°C overnight.

One step competent cell preparation & transformation

It was done as per protocol of Chung *et al.*, (1989). Fresh overnight culture of bacteria was diluted 1:100 into LB broth and the cells were incubated at 37°C with shaking at 225 rpm to an OD₆₀₀ of 0.3 - 0.4. An equal volume of ice-cold 2x TSS was added and the cell suspension was mixed gently. For transformation 0.1 ml aliquot of cells was pipetted into a cold polypropylene tube containing 1 µl (100 pg) of ligation mixture and the cell/DNA suspension was

mixed gently. The cell DNA mixture was incubated at 4°C for 5-60 min. A 0.9 ml aliquot of LB broth plus 20 mM glucose was added, and the cells were incubated at 37°C with shaking at 225 rpm for 1h to allow expression of antibiotic resistance gene. The transformants were selected by plating cells (in triplicate) on agar plates containing 100 µg/ml ampicillin.

Screening of recombinant clones

About 12 colonies were picked from the overnight grown transformants. The individual colonies were inoculated in fresh ampicillin (100 µg/ml) containing LB broth and allowed to grow overnight at 37°C in shaking incubator at 225 rpm. Plasmid DNA was isolated from these colonies by TELT method and checked on 0.7% agarose gel electrophoresis. The concentration and purity of plasmid DNA was done spectrophotometrically by taking absorbance at 260 nm and 280 nm in Biophotometer using microcuvette G1. 0 (Eppendorf, Germany) and using the formula:

$$\text{Plasmid DNA (} \mu\text{g/ml)} = \text{OD (260nm)} \times 50 \times \text{dilution factor}$$

$$\text{Purity of plasmid DNA} = \frac{A_{260}}{A_{280}} \times 1.8$$

Isolated plasmids were checked for the presence of insert by digestion with restriction enzyme XhoI. The digestion mixture (total 15 µl) contained: Plasmid DNA 2.5 µl, XhoI (10 U/µl, Promega) 1 µl, buffer (10X) 1.5 µl, nuclease free water 10 µl. The reaction mixture was vortexed, spin and incubated in a water bath at

37°C overnight. The digested mixture was then electrophoreses in 1% agarose (Low EEO Himedia). The released fragments after digestion were compared against 1 kb DNA ladder.

Colony PCR

The transformant clones were screened by colony PCR as well (Sambrook and Russell, 2001) using G gene specific forward primer and BGH reverse primer. The PCR reaction mixture (25 µl) contained 100 ng of recombinant plasmid, 50 pmol each of gene specific forward and BGH reverse primer and 3 units of Taq DNA Polymerase in 1X PCR buffer: distilled water 17.5 µl, plasmid DNA 1 µl, G Forward primer 1 µl, BGH Reverse primer 1 µl, dNTPs (10mM) 1.5 µl, Buffer (10X) 2.5 µl, Taq DNA polymerase 0.5 µl. The cycling conditions were same as described for G gene amplification PCR.

Sequencing of gene using BGH reverse primer

The sequencing of the gene insert in pAlpha vector was got done using BGH reverse primer to further confirm the gene insert as well as its orientation.

RESULTS AND DISCUSSION

Amplification of glycoprotein gene

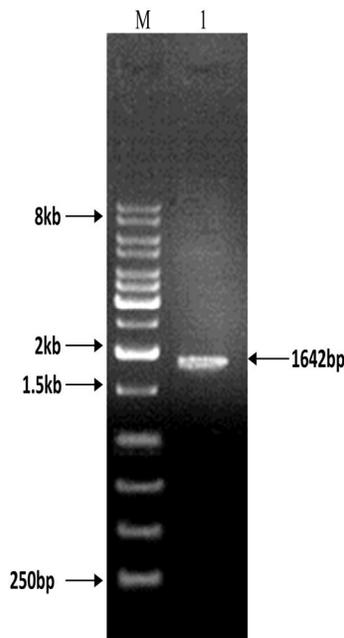
The cDNA was amplified using rabies virulent virus RNA as a template, random hexamer primer, M-MLV reverse transcriptase and RT-PCR reaction. Using amplified cDNA as a template, glycoprotein gene (G gene) of the rabies virulent virus was successfully amplified using gene specific forward and reverse primers. The

PCR product obtained was run on 0.7% agarose gel electrophoresis along with 1kb DNA molecular weight marker and the PCR product of 1642 bp confirmed the successful amplification of rabies glycoprotein gene (G gene) (Fig.1).

Cloning of rabies virulent virus glycoprotein gene (G gene)

The recombinant pAlpha-rvvg was transformed in *E. coli* DH5 competent cells and plated on LB agar plates containing 100 µg/ml ampicillin. After 16 hours incubation at 37°C, the plates were found to contain colonies from which 12 discrete colonies were selected randomly

and grown in LB broth containing ampicillin. The recombinant pAlpha-rvvg plasmids were isolated (Fig.2).The recombinant pAlpha-rvvg plasmid was digested with *XhoI* enzymes to confirm the rabies glycoprotein (G gene) insert as well as its orientation. (Fig 3, 4)For screening of clone, the recombinant plasmid obtained was used as a template and gene specific G gene forward primer and BGH reverse were used and the product of 1642 bp was obtained which confirmed the glycoprotein gene in recombinant plasmid as well as its right orientation (Fig.5, 6). Sequencing of the insert using BGH reverse primer showed that G gene was in right orientation.



**Fig.1. Amplification of 1642 bp rvvg gene
Lane M: 1kb DNA ladder,
1: rabies virus G gene**

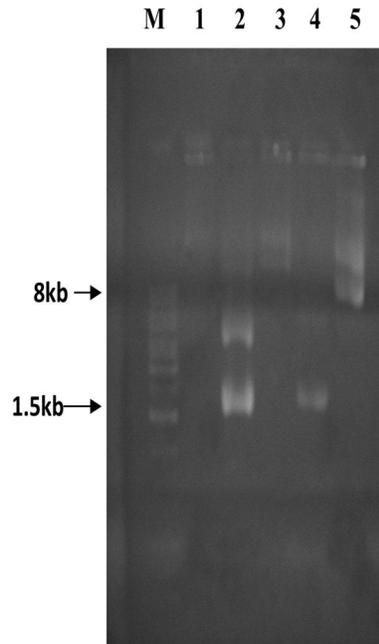


Fig. 2. pAlpha-rvvg plasmid DNA isolated from transformed colonies.

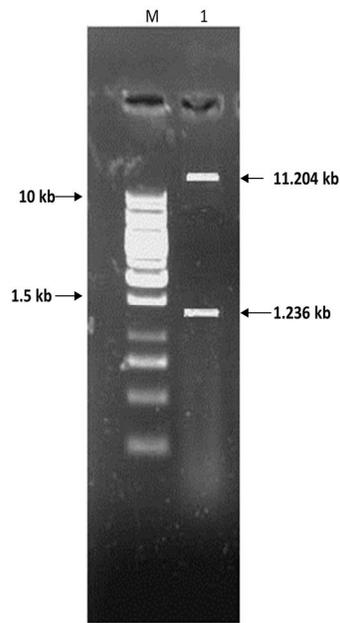


Fig.3.pAlpha-rvvg digested with XhoI yielding fragments 11.204 kb and 1.236 kb confirming right orientataion of the gene.

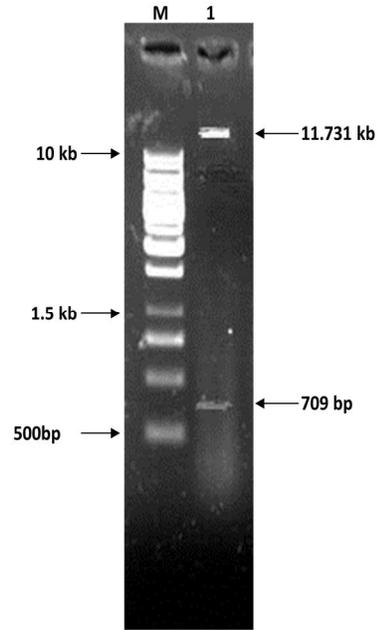


Fig.4.4.pAlpha-rvvg plasmid DNA digested with XhoI yielding fragments 11.731 kb and 709 bp showing wrong orientataion of the gene.

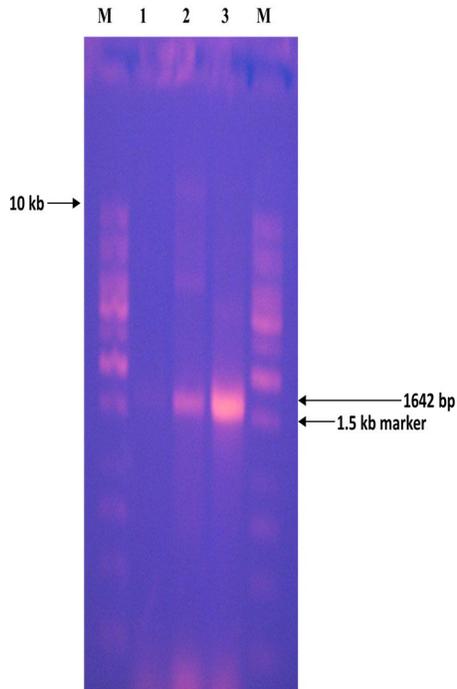


Fig. 5. Colony PCR from transformed clone using G gene specific F and BGH R primers.

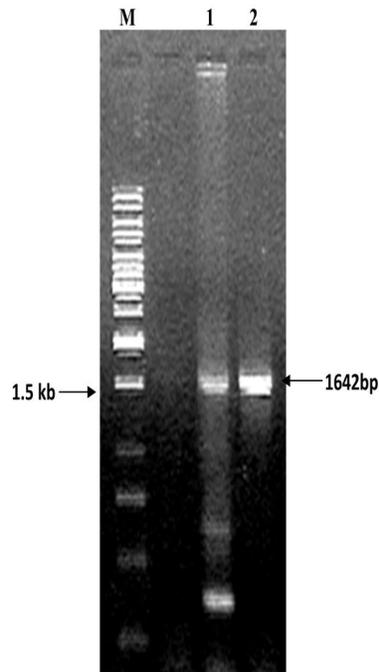


Fig. 6. PCR Amplification of rabies virus G gene. Lane M: 1 kb DNA ladder,1,2: Rabies virus virulent G gene 1642 bp

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