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Expression of rabies virus glycoprotein gene into eukaryotic system and determination of potential T-cell epitopes

Neha R Tomar[1*], Rajesh Chandra[2], Rajiv kumar[3], A K Tiwari[4] & Anil Kumar[1]

[1]Department of Molecular Biology and Genetic Engineering,
College of Basic Sciences and Humanities, [2]Department of Veterinary
Microbiology and Immunology, College of Veterinary and Animal Sciences,
G.B Pant University of Agriculture and Technology, Pantnagar 263145, India.
[3]Animal Biotechnology Section, Central Sheep and Wool Research Institute, Avikanagar 304501, India.
[4]Molecular Biology Laboratory, Division of Animal Biotechnology, Indian Veterinary Research Institute,
Izatnagar 243122, India.

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The present study was undertaken to clone, express rabies virus glycoprotein (RVG) and to identify potential T-cell epitopes on it. RVG gene (1590 bp) was amplified using gene specific primers. The amplified product was cloned into pTZ57R/T cloning vector by TA cloning. RVG gene was subcloned into pcDNA3.1 (+) expression vector. In this study, cloning and expression of rabies virus glycoprotein gene was done under CMV promoter and an expression construct (pcDNA.RVG) was prepared and clones were confirmed by restriction digestion, colony PCR and nucleotide sequencing. The expression construct was further characterized by western blotting and indirect fluorescent antibody test (IFAT). *In silico* analysis of this protein was done to find out potential antigenic sites so that it can be further evaluated for its potential as candidate for epitope vaccine against rabies.

Keywords: DNA vaccine, Epitope mapping, Glycoprotein, Indirect fluorescent antibody test, Rabies virus

Rabies is one of the oldest recognized and most important zoonotic diseases of India affecting man and animals. It is caused by rabies virus (RV) which causes a fatal encephalomyelitis in several species of mammals including humans[1]. The dog is the main reservoir of rabies in India[2], though other animals such as monkeys, jackals, mongoose and rodents may bite incidentally on provocation and transmit the disease. Under such conditions it becomes mandatory for the victim to seek post exposure prophylaxis. At present, two types of vaccine are commonly used. A variety of cell culture-derived vaccines are available for prophylaxis against rabies[3]. However, the perceived high cost of these products may prohibit their wider use in developing countries. Clearly, new concepts are needed to preserve the record of high potency, purity, safety, efficacy, stability, and economy of rabies vaccine. As such, a number of alternative methods are being explored for rabies prevention and control through molecular applications. One of these approaches pertains to utilization of DNA vaccines. Glycoprotein (G) of the virus is the major antigen responsible for induction of protective immunity being the sole protein exposed on the surface of the virion. It interacts with cellular receptors[4], mediates *p*H-dependent fusion, and promotes viral entry from a peripheral site into the nervous system[5]. Moreover, RVG is involved in the transsynaptic spread within the central nervous system[6]. In this study, cloning and expression of glycoprotein gene was done under CMV promoter. The present study was conducted to predict and identify the promiscuous epitope peptide that binds to HLA molecules computationally.

*Correspondent author
Telephone: +91-9460675096, +91-9414045616
E-mail: nehatomar.biotech@gmail.com

Materials and Methods

Cells and virus culture—Mouse neuroblastoma cells (clone 2a) were used to propagate rabies virus CVS because CVS-N2a clone has a similar relative neurotropism index as CVS-24; the BHK-21 adapted CVS has a four fold lower relative neurotropism index. There is a difference of ten amino acids between glycoprotein of Neuro-2a adapted and BHK-21 adapted viruses. The differed amino acids may contribute to the altered neurotropism of rabies virus.

For expression studies, Baby hamster kidney-21 (BHK-21) cells were used. All cells were procured from National Centre for Cell Science (NCCS) Pune, India and grown at 37°C under 5% CO_2 in Eagle's modified minimum essential medium (EMEM, Himedia) and Glassgow's modified minimum essential medium (GMEM, Sigma) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 50 µg/ml gentamicin. Rabies virus, challenge virus standard (CVS) was propagated and maintained in mice brain.

RNA extraction—The isolate was first passaged in mouse neuroblastoma 2a cell line and total RNA was isolated using TRIZOL™ reagent (Invitrogen) according to the manufacturer's instructions.

Primer designing—To amplify complete ORF of rabies virus glycoprotein (RVG) forward 5′GATGGTTCCTCAGGCTCTCC3′ and reverse 5′GCCAGCTCTCACAGTCCGGTCTCAC3′ oligonucleotide primers were designed using Lasergene software (DNASTAR, Inc. Madison, WI, USA) from the sequences available at GenBank. Specificity of primers was checked by ncbi BLAST programme (http://www.ncbi.nlm.nih.gov/BLAST/). The KOZAK sequence ACCGCC was added to the 5' end of forward primer for proper expression into eukaryotic system.

RT-PCR and cloning—The designed set of primers was used to amplify a continuous 1590 bp segment of the RVG gene. The cDNA was synthesized using the random primers, nuclease free water (Invitrogen, USA) and RNA in a 34 µl reaction volume. The reaction mixture was heated in thermal cycler (Biometra, U.K) at 72°C for 10 min followed by 5 min incubation at 25°C and chilled on ice immediately. It was centrifuged for at 500 rpm for 5 sec (MiniSpin® plus, Eppendorf, India) and 5 × buffer, deoxynucleoside triphosphate (dNTP), RNase inhibitor and M-MLV reverse transcriptase were added to it (Invitrogen). The reaction mixture was spun at 500 rpm for 5 sec and incubated at 25°C for 5 min, 42°C for 1 h and then at 70°C for 10 min in a thermal cycler. The synthesized cDNA was used as a template for the polymerase chain reaction (PCR) with DNA polymerase [Taq DNA polymerase + DeepVent; (New England Biolabs), 14:1 ratio]. The reaction mixtures for PCR contained 10 × Taq buffer, dNTP mix at a concentration of 200 µ*M*, each primer at a concentration of 1 µ*M*, cDNA templates at a concentration of 1 *ng*/µl, and 1 *U* of DNA polymerase mix in a final volume of 25 µl. Gene amplification was performed using the following program: 5 min hot start at 94°C; followed by 39 cycles consisting of denaturation (60 sec at 94°C) annealing (60 sec at 48.2°C) and extension (90 sec at 72°C); and a final extension at 72°C for 10 min. PCR products were verified by the electrophoresis in 1% agarose gel and stained with ethidium bromide. The interested DNA fragment (1.5 kb) was purified by the agarose gel electrophoresis and then ligated into (pTZ57R/T) TA cloning vector (MBI, Fermentas) and the recombinant DNA was used to transform *E. coli* DH5α cells. The clones harboring recombinant plasmid pTZ57R/T.RVG were screened by α-complementation and verified by colony PCR using T7 primer and gene specific forward primer in order to check orientation. Plasmid DNA was isolated using the Hipure plasmid DNA purification kit (Invitrogen). The plasmids positive for RVG gene (designated as pTZ57R/T.RVG) were digested using the restriction enzymes *KpnI* and *SmaI* and then cloned into expression vector pcDNA3.1(+).

Construction of plasmid expression cassette (pcDNA.RVG)—The pcDNA3.1(+) expression vector (Invitrogen) was used to construct expression cassette. This vector contained multiple cloning site (MCS) located downstream to the cytomegalovirus (CMV) promoter. The RVG gene fragment was digested with *KpnI* and *SmaI* from pTZ57R/T.RVG and subcloned into the plasmid expression vector pcDNA3.1(+) between *KpnI* site and *EcoRV* sites to yield pcDNA.RVG, in which the expression of glycoprotein gene is under the control of CMV promoter. The recombinant *E. coli* DH5α/pcDNA.RVG were finally constructed by transforming pcDNA.RVG into the host strain *E. coli* DH5α. The correct cloning of the RVG gene was confirmed by colony PCR, by restriction enzyme analysis and by sequencing (Chromous Biotech Pvt. Ltd., Bangalore, India). The sequence thus obtained was submitted to NCBI database.

In vitro expression analysis of the construct—Expression of glycoprotein gene from pcDNA.RVG was analyzed in BHK-21 cells after transfection using Lipofectamine™ 2000 transfection reagent (Invitrogen) following manufacturer's instructions. Control cells were mock-transfected with pcDNA 3.1 (+) vector. Cells were then grown for further 48 h with growth medium. Whole cell lysate of transfected and mock-transfected BHK-21 cells were prepared by lysing the cell monolayer in SDS-PAGE sample

buffer and separated on 10% SDS-PAGE gel. For Western blotting, proteins after SDS-PAGE were transferred onto PVDF membrane and probed with anti-rabies polyclonal serum. The bound antibodies were detected using secondary antibodies conjugated with Horse radish peroxidase (Santacruz) and visualized with DAB substrate solution (Invitrogen). For the detection of intracytoplasmic expression of glycoprotein gene transfected cells were also subjected to indirect fluorescence antibody test as described[7].

Identification of epitopes—The sequence from accession number FJ979833 was used to identify the epitopes with human relevance from the IEDB and analysis resource. Artificial neural network MHC class I binding prediction method was used to find out the most potent binders towards class I MHC molecules. Peptides showing highest scores for binding as well as for processing and TAP transport towards class I MHC molecules were considered positive.

ClustalW alignment of deduced epitopes—The peptides deduced from IEDB and analysis resource by ANN method were further subjected to clustal W alignment to check their conservancy among different strains of rabies viruses. The peptides showing highest scores for class I MHC binding as well as for processing and conserved among different strains of rabies may be inferred as good epitopes.

Results and Discussion

Total RNA was extracted using TRIZOL™ reagent (Invitrogen) and glycoprotein gene was amplified using cDNA templates at a concentration of 1 ng/µl and designed primers. Specific single band of approximately 1590 bp as seen in ethidium bromide stained 1% agarose gel (Fig. 1) confirming the amplification of RVG. Amplified PCR product was gel purified and ligated to pTZ57R/T plasmid cloning vector. The concentration of purified insert was 30 ng/µl. Correct cloning into pTZ57R/T vector was confirmed by colony PCR using T7 and gene specific forward primers and restriction digestion with *kpnI* and *smaI* restriction enzyme. In general, virally-derived promoters have provided greater gene expression *in vivo* than other eukaryotic promoters. In particular, the CMV immediate early enhancer-promoter (known as the CMV promoter) has often been shown to direct the highest level of transgene expression in eukaryotic tissues when compared with other promoters. So attempts were made to clone the glycoprotein gene downstream to CMV promoter by forced cloning to get the gene in correct orientation. The plasmid expression cassette encoding rabies glycoprotein was constructed using pcDNA3.1(+) vector (Invitrogen). RVG from pTZ57R/T was subcloned into pcDNA3.1(+) vector between *KpnI* site and *EcoRV* sites. The subcloning of RVG gene into pcDNA3.1(+) was confirmed by colony PCR using T7 and gene specific reverse primers (Fig. 2a) and restriction digestion of construct pcDNA.RVG by restriction enzymes *NheI* and *PmeI* (Fig. 2b). The recombinant plasmid pcDNA.RVG was sequenced from the sequencing facility, Chromous Biotech Pvt. Ltd., Bangalore. The sequenced data was stored in the "Editseq" programme of Lasergene software (DNASTAR). The sequence was submitted to GenBank and is now available with accession number FJ979833. The plasmid pcDNA.RVG expressed the protein in transfected cells, as observed in Western blot (Fig. 3). In cell lysate, the protein of approximately 66 kDa reacted with anti-rabies specific hyperimmune sera in Western blot. The size of expressed protein bands indicated (approximately 66 kDa) that the protein retained the authentic confirmation. Also they underwent the post translational modification as confirmed by increased molecular weight. In this study, it was deduced from the bioinformatics tool protparam that the protein produced from sequenced gene was of approximately 60 kDa but from Western blot analysis it was found that the expressed protein was having a molecular weight of approximately 66 kDa, which could be because RVG expressed by BHK cells are highly glycosylated and sialylated. So our results are in

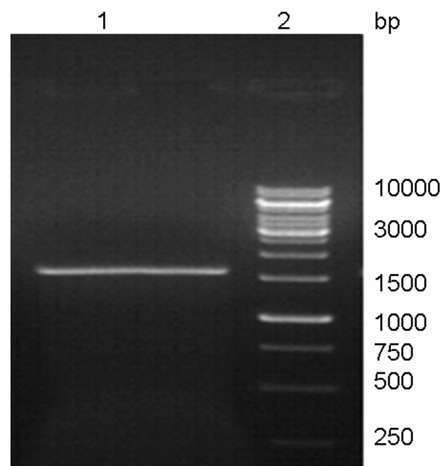

Fig. 1—Agarose gel electrophoresis showing amplification of rabies virus glycoprotein gene

agreement with those observed earlier[8]. Expression of glycoprotein was also detected in transfected cells by indirect immunofluorescent test (Fig. 4). The cytoplasmic fluorescence was observed for glycoprotein in IFAT analysis from outer nuclear membrane to the plasma membrane indicating the movement of glycoprotein in endocytic vesicles from endoplasmic reticulum to golgi apparatus and subsequently to the plasma membrane. Although the present assay was not quantitative as the amount of expressed protein in each cell was not determined, but its presence in each cell was observed. Since large number of cells showed expression of rabies glycoprotein, it was concluded that pcDNA.RVG plasmid was capable of expressing the protein efficiently in transfected cells. The construct can be directly used as DNA vaccine as it is of great potential value for protecting humans against rabies and rabies-related viruses by immunizing the canine reservoir against the disease. Studies by many workers have revealed that vectors containing CMV promoters work well in DNA vaccination[9,10]. There are many reports where pcDNA3.1(+) has been used as a vector for developing DNA vaccines[11]. DNA vaccinations are highly effective in rabies pre-exposure experiments, but post-exposure protection has not been achieved. This failure is likely due to the slow onset of DNA vaccine induced antibody

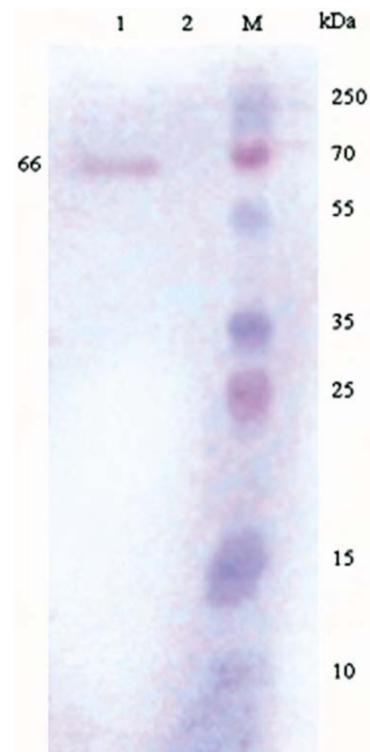

Fig. 3—Western blot showing expressed glycoprotein from cell lysate of BHK-21 cells transfected with pcDNA.RVG. Lane 1-66kDa expressed glycoprotein from cell lysate of BHK-21 cells transfected with pcDNA.RVG; Lane 2-Cell lysate from mock transfected BHK-21 cells; and Lane 3-PageRuler™ plus prestained protein ladder

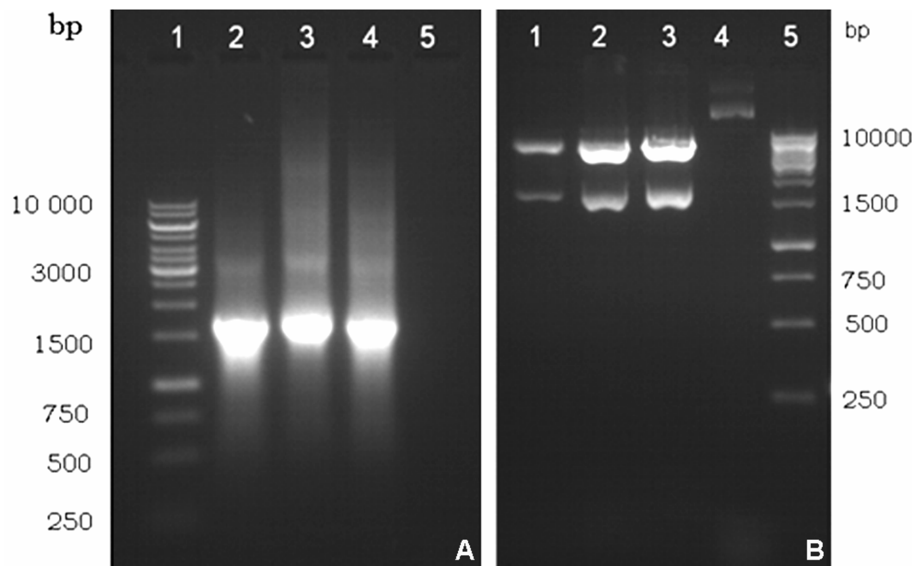

Fig. 2—Characterization of expression construct pcDNA.RVG : (A)-Agarose gel electrophoresis confirming correct orientation of RVG gene by colony PCR from recombinant clones using reverse primer and T7 primer. Lane.1—1 kb DNA ladder, Lane, 2, 3, 4 – amplified G gene from recombinant colonies, Lane-5- -ve control (PCR from non-recombinant cells). (B)-Agarose gel electrophoresis showing presence of RVG gene insert in pcDNA3.1(+) eukaryotic expression vector through restriction enzyme analysis. Lane.1,2,3- recombinant clones releasing 1590 bp insert on digestion with *NheI* and *PmeI* from recombinant colonies, Lane.4- undigested recombinant clone, Lane.5- 1 kb DNA ladder

production. It has been shown that post-exposure DNA vaccination protects mice against rabies virus. The onset of the antibody response was accelerated by manipulating variables, such as the route of vaccination and booster frequency[12]. It has been investigated whether recombinant vaccinia viruses expressing either the glycoprotein (G), the nucleoprotein (N), or both the G and N (GN) of the challenge virus strain (CVS) of rabies virus will cross-protect mice against 17 rabies virus isolates representing the spectrum of rabies virus variants found worldwide. After comparing results with the commercially available human diploid cell vaccine (HDCV) it has been found that among mice injected with any of the 17 viruses, >95% are protected by vaccination with recombinant viruses expressing G or GN[13]. These studies encouraged us to use glycoprotein sequence for finding conserved epitope for the possible candidates of synthetic anti-rabies vaccine so that it might target all the rabies virus strains found worldwide. The sequence of glycoprotein was analysed with IEDB analysis and resource server for binding with HLA molecules to find out T-cell epitopes. The deduced peptides from ANN method are shown in Table 1. The affinities of peptides towards class I MHC were represented by IC_{50} (n*M*). The lower is the IC_{50} value of epitope the stronger is the binding of epitope to the class I MHC molecule. Therefore, a lower number indicates higher affinity. From the selected peptides, peptide from region 54-62 in glycoprotein sequence had the highest affinity (i.e 31.4n*M*), while peptide from region 228-236 had affinity of 305.1n*M* and petide from region 283-291 had affinity of 110.1n*M*. These three peptides were further analyzed for proteasomal cleavage, TAP transport and MHC score. The total score was calculated by adding all the three scores. Peptide from amino acid 54 to 62 had total score of 0.51, peptide from amino acid 228 to 236 had total

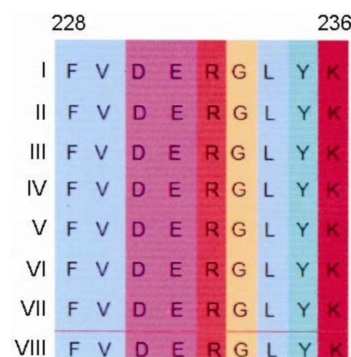

Fig. 5—Clustal W alignment of the epitopes from 228-236 [(I) AB009663 Rabies virus/1-1070; (II) AB009097 Rabies virus strain PG/1-524; (III) RVU03767 Rabies virus/ 1-524; (IV) U11752 Rabies virus/1-524; (V) AF 325465 Rabies virus/1-524; (VI) AF 325470 Rabies virus/1-524; (VII) AF 325475 Rabies virus USA2-SP/1-524; and (VIII) FJ 979833 RVCVSPATWADANGAR/1-524]

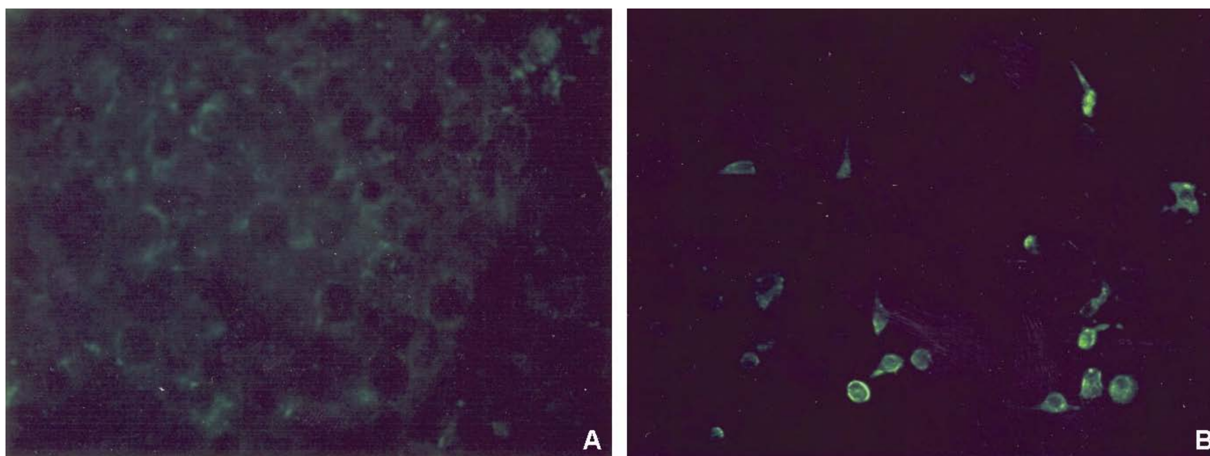

Fig. 4—Presence of intracytoplasmic fluorescence in BHK-21 cells transfected with pcDNA.RVG, indicating the expression of glycoprotein gene (IFAT, 400X). (a) Negative control cells; (b) BHK-21 cells transfected with pcDNA.RVG

Table 1—Prediction of class I MHC binding peptides by ANN method

Allele	Position	Peptide length	Sequence	IC50[n*M*]
HLA A*0101	1:54-62	9	CTNLSEFSY	31.4
HLA A*0101	1:283-62	9	QSDEIEHLV	110.1
HLA A*0101	1:228-236	9	FVDERGLYK	305.1
HLA A*0101	1:57-65	9	LSEFSYMEL	2721.7
HLA A*0101	1:156-164	9	PSVTDLDPY	4762.9

score of -1.32 and peptide from amino acid 283 to 291 had total score of-1.56. From total score these three peptides were found to be processed in proteasome and presented by class I MHC molecules computationally. These three epitopes were further subjected to conservancy analysis by clustal W alignment, the only epitope from amino acid 228 to 236 was found to be completely conserved among different rabies viruses (Fig. 5). Although two epitopes (from amino acid 54 to 62 and from amino acid 283-291) showed a high level of conservancy, some amino acid substitutions have been observed on these sites in some strains. From the 8 analyzed naturally occurring rabies virus isolates, only two isolates (AF325465 and FJ979833) contained nonconserved amino acid changes at key residues within the epitopes that can eliminate antibody binding. If a peptide based vaccine is derived from this region of the sequence, the probable effectiveness would be poor. It is clear from the MHC binding, processing predictions and clustal W alignment of different peptides that the epitopes selected from 228-236 may be the promising T cell epitope.

In this study, a novel conserved epitope was found in rabies virus glycoprotein by scanning the complete sequence for peptide recognition that may be used as promising target for safe epitope vaccine against the rabies virus as well as a diagnostic reagent for rabies. It would be beneficial to include N in vaccines because N has been shown to have protective activity *in vivo*[14-17] and anti-N antibody protects mice against *in vivo* challenge and also inhibits rabies virus replication *in vitro*[15]. Furthermore, vaccination with GN may result in a longer primary neutralizing antibody response and/or an enhanced memory neutralizing antibody response than vaccination with G alone.

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