Original Research Article   https://doi.org/10.20546/ijcmas.2017.607.276

Evaluation of Protective Capacity of Glycoprotein Based DNA Vaccine against Rabies Virus

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A B S T R A C T

Rabies is a re-emerging and fatal infectious disease in Asia mainly caused by exposure to rabid dogs. In spite of great advances in virology there is yet no cure for rabies. In recent times newer modality of disease prevention like genetic vaccines and other advanced vaccines have been taken into account. Prevention of dog rabies would be the most effective way to stop rabies transmission to humans. However, vaccinating stray dogs in urban and rural areas using conventional vaccines is always difficult and is not cost-effective for use in most areas. In this study, DNA vaccine encoding the antigenic glycoprotein under the CMV promoter and Kozak sequence was developed and tried in mice by i. m. inoculation. Results showed that 87.5% (7/8) of the immunized mice had developed virus neutralizing antibodies (VNA). The antibody titer after 30 days was 4624. This vaccine afforded 87.5% protection against challenge dose 50 LD_{50} of CVS-24 after 30 days post challenge. This demonstrated that the DNA vaccine pcDNA-RVG could be administered and it could be effective for the vaccination of dogs to prevent the canine rabies.

Introduction

Rabies is one of the oldest recognized and most important zoonotic diseases of India affecting man and animals. Rabies is a rapidly fatal neurological disease and to date therapeutic efforts in humans have proved futile except in rare cases in which rabies vaccine was administered prior to the onset of clinical disease (Jackson *et al.,* 2003).

Rabies virus (RV) almost always causes a fatal encephalomyelitis in several species of mammals including humans (Dietzschold, 1996). This virus is prototype of the *Lyssavirus* genus of the family *Rhabdoviridae* and is an enveloped virus having non-segmented, negative stranded RNA as genome. The genome of virus is about 12 kb in size and encodes five proteins; the nucleoprotein (N), the phosphoprotein (P), the matrix protein (M), the glycoprotein (G), and the RNA-dependent RNA polymerase (L). The viral RNA, which is always encapsidated by N, forms the ribonucleoprotein (RNP), which is the template for viral replication and transcription (Conzelmann, 2004). The RNP together with P and L forms the viral

replication complex, which is surrounded by the host cell-derived membrane that also contains glycoprotein (G). Matrix protein has been proposed to bridge the RNP and the cytoplasmic domain (CD) of RVG to form the bullet-shaped virion (Mebatsion, 1999). The RVG, which is organized as a trimer, is the major contributor to pathogenicity. The glycoprotein G of the virus is the major antigen responsible for the induction of protective immunity being the sole protein exposed on the surface of the virion. The RVG is predominant viral antigen that induces the production of virus-neutralizing antibodies (VNA), the major effectors against rabies (Foley, 2000). The disease is transmitted through the bite of an infected animal, usually from a dog, and can be prevented by the timely administration of rabies immune globulins and post-bite vaccination. In most developing countries, dogs represent the major rabies reservoir. The failure to eliminate dog rabies in most developing countries stresses the necessity of improving rabies control programs by applying new vaccines or new vaccination strategies. There is a need for development of alternative vaccine strategies to overcome the shortcomings of the current conventional vaccines.

In last two decades, the genetic vaccine (also called DNA vaccine) concept has been tested and applied against different pathogens and tumor antigens. Improvements in gene optimization strategies and effective delivery approaches have made DNA vaccine more effective and reliable. Now it is possible to elicit protective immune response by using DNA vaccine forms. It is supported by approval of four veterinary DNA vaccines for commercial production (Kutzler and Weiner, 2008). The DNA vaccine is safe, technically simple and elicits immune response against various dreaded pathogens. Further this technique affects both humoral and cell-

mediated immunity (CMI). The latter plays an important role in protection against intracellular pathogens. In particular, DNA vaccines induces killer cytotoxic T-lymphocytes (CTLs). DNA vaccine is relatively safer, as it is not live, which reduces the risk of its reversion to virulent form as observed in live, attenuated form of vaccines. Since rabies glycoprotein is the major antigen responsible for inducing protective immunity, it is mostly used in genetic vaccines for producing antigenic protein.

Materials and Methods

Preparation of rabies virus glycoprotein DNA vaccine construct

Rabies virus glycoprotein gene along with Kozak sequence was cloned under CMV promoter which was designated as pCDNA.RVG. Glycoprotein gene was successfully transcribed and translated as described in our previous study (Tomar *et al.,* 2011). This construct was used as DNA vaccine to immunize mice in present study.

Determination of protective capacity of pCDNA.RVG DNA vaccine against rabies after challenge with rabies CVS in immunized mice

Before starting the experiments, necessary permissions were obtained from Institute ethics committee. Experiments using live virus handling were done in BSL-3 containment facility of Institute of Biotechnology, Patwadangar, G. B Pant University of Agriculture and Technology, Nainital, Uttarakhand.

Calculation of LD_{50} for challenge

To determine challenge dose (LD_{50}), the mice brain infected with rabies CVS was triturated and diluted 10 fold in 2% horse serum. The

mice were injected by intracerebral route with 0.03 ml of 10 fold diluted rabies CVS. The inoculated mice were observed for 15 days post-inoculation for rabies specific symptoms like paralysis and death. The LD_{50} was calculated as per the method of Reed and Muench (1938).

Preparation of plasmid DNA vaccine

The plasmid DNA vaccines *viz.* pcDNA.RVG and control plasmid pcDNA were isolated from LB broth (500 ml) with ampicillin, inoculated with 1/100 volume of overnight grown inocula using PureLink™ HiPure Plasmid DNA Purification Kit for maxi preparation (Invitrogen) following the manufacturer's instructions.

Immunization of mice with plasmid DNA vaccine

Three groups (Group I and Group II and Group III) of mice each containing eight mice were immunized with none, control plasmid [pcDNA3.1(+)] and DNA vaccine construct (pcDNA.RVG), respectively, by inoculating intramuscularly into quadriceps muscle in the hind leg each with 100 μg of DNA dissolved in 150 mM NaCl.

The mice were kept for 30 days in normal conditions. The blood from immunized mice was collected from the tail vein on 30th day post immunization and sera separated for determination of immune status of mice. After 30 days, these were challenged intracerebrally with 30 μl of $50LD_{50}$ of virus titre. All the mice were observed for 15 days for the development of rabies specific symptoms/death and percent protection of the vaccine was calculated.

Calculation of percent death

Both the groups (group II and III) of mice were challenged with 50 LD_{50} of rabies CVS

by intracerebral route on 30th day post-immunization. Immunized mice were challenged with rabies CVS and observed for 15 days post-inoculation for rabies specific symptoms like, paralysis and death. Percent protection was calculated by the formula given below.

$$\% \; death = \frac{number \; of \; mice \; showing \; symptoms \; or \; death}{total \; number \; of \; mice \; in \; the \; group} \times 100$$

$$= \frac{1}{8} \times 100 = 12.5$$

Detection of immune response by ELISA

The wells of ELISA plate were coated with purified rabies virus diluted in 0.05M carbonate-bicarbonate buffer (pH 9.6). The plate was incubated at 4^0C overnight. After washing once with wash buffer (PBS-Tween 0.05%), the coated wells were blocked with 2% BSA in PBS-T for two hours at 37^oC.

The plates were washed with PBS-T (PBS-Tween 0.05%) and various dilutions of serum starting from 1:50 to 1:3200 were added in 100 μl volume to the wells and incubated at 37^oC for 1 hour. The wells were washed thrice with PBS-T.

Then 100 μl of 1:2500 dilution of anti-mouse HRPO conjugate (sigma) was added to each well and incubated for 45 minutes at 37^oC. Washing was done 3 times with PBS-T and the colour was developed with OPD substrate.

After 20-30 minutes of incubation in dark, at room temperature, enzyme activity was stopped by adding 1N sulphuric acid and O.D was taken at 492 nm in ELISA plate reader. The ELISA titre was defined as the reciprocal of the highest serum dilution positive in ELISA.

The cut-off value for ELISA titre determination was calculated by the following formula:

Cut-off value = Mean OD value of negative control + 3 x standard deviation value of negative control

The antibody titre was calculated by the following formula:

$$\log \text{Ab titre} = X - \frac{A-C}{A-B} \times D$$

Where *A* is the A_{492} of the test sample dilution immediately higher than the cut-off A_{492} value, *B* the A_{492} of the test sample dilution immediately lower than the cut-off A_{492} value, *C* the cut-off A_{492} value [*C* the mean A_{492} of the negative samples + 3 x S.D.], D the log dilution factor, i.e. 2, X the log dilution of the test sample having A_{492} immediately higher than the cut-off value.

Results and Discussion

Determination of immune responses against rabies glycoprotein in mice immunized with DNA vaccine

Immunization of mice

The plasmid DNA for DNA Vaccine with kozak sequence was prepared using Hipure plasmid DNA purification kit (Invitrogen, USA) for maxi preparation and DNA was checked for purity and quantified by U.V spectrophotometer. The DNA in prepared plasmid vaccine was quantified by taking absorbance at 260 nm using the following formula:-

Plasmid DNA (μg) = OD (260nm) × 50 μg/ml × dilution factor

From 500 ml LB culture volume, the plasmid yield was 1139 μg (1.139 mg) and the concentration was 5695 μg/ml. The ratio of OD_{260}/OD_{280} came out to be 1.81, which indicated the good level of purity to use this construct as a DNA vaccine. Each mice was

immunized with 100 μg of plasmid DNA vaccine intramuscularly.

Detection of rabies glycoprotein specific antibodies in ELISA

The rabies-specific antibody titre in DNA vaccine immunized mice was detected by ELISA (Table 1).

The antibody titre was calculated by the following formula:

$$\log \text{Ab titre} = X - \frac{A-C}{A-B} \times D$$

$$= \log 800 - \frac{1.394 - 1.24}{1.394 - 1.20} \times \log 2$$

log Ab titre = 2.6650

Antibody titre = antilog 2.6650 = 4624

The vaccine pcDNA.RVG produced the antibody titre of 4624 at 31st day post vaccination in a single dose of 100μg.

Determination of protective capacity of vaccine against rabies after challenge with rabies CVS in immunized mice

Calculation of LD50

For calculating LD_{50}, mice were injected with different dilutions of rabies CVS intra-cerebrally and were observed for 15 days post injection for rabies specific symptoms or death. The LD_{50} was calculated by the method of Reed and Muench (1938), as shown in (Table 2).

$$Proportionate\ distance = \frac{\%\ mortality\ at\ dilution\ above\ 50\% - 50\%}{\%\ mortality\ at\ next\ dilution\ above\ 50\% - \%\ mortality\ at\ next\ dilution\ below\ 50\%}$$

$$= \frac{100 - 50}{100 - 16.6}$$

= 0.59 or 0.6 (*i.e.* proportional distance

between 10^{-6} and 10^{-7} at which 50% of the host system is affected)

$\log_{10}$ 50% end point dilution = $\log_{10}$ of dilution showing a mortality next above 50% - (P.D × logarithm of dilution factor)

= log 10^{-6}- (0.6 × log 10)
= -6-0.6
= -6.6

50 % end point dilution= $10^{-6.6}$

Titre of the virus = $10^{6.6}$ LD$_{50}$/ 30 µl

Calculation of percent protection

The protection in pcDNA.RVG immunized mice was found to be 87.5% however, no protection with pcDNA3.1 (+) immunized mice was recorded.

Immunizaton of mice with plasmid DNA vaccine

After analyzing the expression of glycoprotein from the construct *in vitro* it was used *in vivo* for the induction of immune response against to rabies. Previous studies have shown that DNA-based immunization with plasmids encoding the rabies virus glycoprotein (CVS, ERA and PV strains) protects mice against rabies (Bahloul, 1998; Jallet, 1999; Lodmell, 1998; Xiang, 1994). Different groups of mice were immunized with pcDNA.RVG construct and pcDNA3.1 (+) vector. Almost all the mice showed seroconversion. This revealed that the glycoprotein was expressed in a fully immunogenic form and presented to the immune cells. In this study, intramuscular (i. m.) inoculation was done for several reasons: (i) Needle injection seems more appropriate than gene gun delivery for mass vaccination campaigns for animals in developing countries and (ii) Intramuscular injection of DNA vaccines has been demonstrated to be efficient in both rodents and monkeys. When the plasmid DNA vaccine was injected into mice, immunized mice showed a high percentage of survival and it was observed that single i. m. injection of 100 µg of plasmid (pcDNA.RVG) encoding the rabies virus glycoprotein, was sufficient to induce high VNAb titers (4624) against the standard virus (CVS strain). Similarly, other workers have reported that antigens encoded by a single plasmid (Wild *et al.,* 1998; Kwissa *et al.,* 2000) or multiple plasmids successfully induce immune responses to all components.

Table.1 Average O.D values at different dilutions of primary antibodies

Dilution	A$_{492}$
1:50	3.920
1:100	2.922
1:200	2.14
1:400	1.65
1:800	1.394
1:1600	1.200
1:3200	1.02
-ve sera	0.76
-ve sera	0.81
-ve sera	0.79
-ve sera	0.48
-ve sera	0.38

Table.2 Calculation LD_{50}

Virus % dilution	Died (D)	Survived (S)	Cumulative Dead (P)	Cumulative Survived (N)	Ratio D/S	P/P+N %
10^{-4}	6	0	19	0	6/6=1	100
10^{-5}	6	0	13	0	6/6=1	100
10^{-6}	6	0	7	0	6/6=1	100
10^{-7}	1	5	1	5	1/6=0.166	16.6
10^{-8}	0	6	0	11	0/6=0	0.00

Table.3 Calculation of % protection in all immunized groups after challenge with 50 LD_{50} rabies CVS

Groups	Plasmid DNA vaccine	No. of mice challenged	No. of mice survived	No. of mice died	% protection
Group I	Uninoculated –ve control	8	0	8	0.0
GroupII	pcDNA3.1(+)	8	0	8	0.0
GroupIII	pcDNA.RVG	8	7	1	87.5

After challenging the mice with rabies virus CVS, the vaccinated mice showed considerable high degree of protection, 87.5 %. Slightly lower level of protection was observed previously using different monocistronic DNA vaccine constructs (Patial *et al.,* 2008). Bahloul *et al.,* (2006) obtained 40% and 53% protection with cell culture derived and DNA vaccines, respectively, in post exposure therapy against rabies. Among previous workers, Rai *et al.,* (2002) has reported 88.88 % protection, whereas Biswas *et al.,* (1999) has reported 64 % protection against rabies using a monovalent rabies vaccine (Table 3).

Our studies revealed that the glycoprotein was expressed in a fully immunogenic form. This indicated that the DNA vaccine was able to induce virus neutralizing antibody response in vaccinated mice more efficiently than DNA vaccines described earlier. The increased level of protection might be due to the added kozak sequence, which helps in increasing the expression of protein in mammalian systems. These results highlight the positive influence of Kozak sequence in a DNA vaccine against rabies, using the glycoprotein gene, whose vaccinated group showed better seroconversion results. So it can be speculated that this vaccine showed significant level of protection. However, protection studies in dog still need to be done.

Immunization with DNA vaccine has great potential for improving the strategy for vaccination against lyssaviruses. It would be most useful if a strong immune response could be obtained after a single shot, because it is difficult to recover dogs for booster injections in developing countries. It is demonstrated that mice immunized with the plasmid pcDNA.RVG were protected against an intracerebral challenge with 50LD_{50} dose of CVS strain of rabies virus. This study has shown that a single dose of DNA vaccine encoding the G protein of CVS rabies virus delivered by i. m. route elicited neutralizing antibody and protected the mice against a highly lethal dose of a CVS strain of rabies virus. Our results correlates well with Nancy *et al.,* (1997) who described that expression of

G gene with the added kozak sequence under a strong promoter like CMV can protect mice against lethal rabies virus infection after giving single dose of nanograms of plasmids. The use of such DNA vaccine for immunizing dogs would be beneficial.

The results for mice protection demonstrate that immunization with DNA vaccine can be promising for protecting humans working in high risk area against rabies and for immunizing canids.

In conclusion, it was attempted to evaluate the protective capacity of an optimized glycoprotein expression construct (pcDNA.RVG) as DNA vaccine. It is evident from the present study that the vaccine pcDNA.RVG is capable of expressing fully functional immunogenic protein in mammalian cells. Glycoprotein specific antibodies were produced in mice immunized with this construct and the immunity developed in mice demonstrated protection against rabies. Although, the results of the present study were satisfactory, this construct need to be further optimized with the addition of nucleoprotein gene to get 100 % protection.

Acknowledgement

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