Original Article

A Comparative Approach between Heterologous Prime-Boost Vaccination Strategy and DNA Vaccinations for Rabies

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Abstract

Background and aim: Rabies is a widespread neurological zoonotic disease causing significant mortality rates, especially in developing countries. Although a vaccine for rabies is available, its production and scheduling are costly in such countries. Advances in recombinant DNA technology have made it a good candidate for an affordable vaccine. Among the proteins of rabies virus, the Glycoprotein (RVG) has been the major target for new vaccine development which plays the principal role in providing complete protection against RV challenge. The aim of this study is to produce recombinant RVG which could be a DNA vaccine candidate and to evaluate the efficiency of this construct in a prime-boost vaccination regimen, compared to commercial vaccine.

Methods: Cloning to pcDNA3.1(+) and expression of rabies virus glycoprotein gene in BSR cell line were performed followed by SDS-PAGE and Western blot analysis of the expressed glycoprotein. The resulting genetic construct was used as a DNA vaccine by injecting 80 µg of the plasmid to MNRI mice twice. Prime-Boost vaccination strategy was performed using 80 µg plasmid construct as prime dose and the second dose of an inactivated rabies virus vaccine. Production of rabies virus neutralizing antibody (RVNA) titers of the serum samples were determined by RFFIT.

Results: In comparisons between heterologous prime-boost vaccination strategy and DNA vaccinations, the potency of group D that received Prime-Boost vaccine with the second dose of pcDNA3.1(+)-Gp was enhanced significantly compared to the group C which had received pcDNA3.1(+)-Gp as first injection.

Conclusion: In this study, RVGP expressing construct was used in a comparative approach between Prime-Boost vaccination strategy and DNA vaccination and compared with the standard method of rabies vaccination. It was concluded that this strategy could lead to induction of acceptable humoral immunity.

Keywords: DNA vaccine, Pasteur virus, prime-boost, rabies

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Introduction

Rabies is well-known as a widespread neurological zoonotic disease which affects almost all kinds of mammals, including humans, leading to 100% mortality rate. Nearly 60,000 humans die of rabies all over the world each year, out of which more than 95% occurin Asia and Africa.[1] Current rabies vaccines and vaccination regimens are costly to many countries which have the highest demands, demonstrating the need for more economical rabies vaccines.[2] DNA vaccines introduced by Wolff JA in 1990 appear to be highly promising in this context.[3] Later, other researchers have found DNA vaccine as a potential eliciting cellular and humoral immune response against a variety of infections.[4–8] This potency, combined with the inherent advantages provided by DNA vaccine models such as low cost, ease of design and administration, and relatively fast production make them suitable for immunization purposes.[9] However, DNA vaccines' implementation as therapeutic or prophylactic agents cannot translate small animal success to large animal models and human clinical trials. As a result, several studies have found that parameters such as plasmid dosage and inoculation route are factors capable of enhancing RABV-specific virus-neutralizing antibody (VNA) titers in mice. Rabies virus glycoprotein (RVGP) has been the major target for new vaccines development as it is responsible for induction of virus neutralizing antibodies that provide protection against RV challenge.[10] Heterologous prime-boost vaccination has been shown to be an efficient way of responses in animals and humans.[11] In this study, the constructed RVGP expression plasmid was used in a eukaryotic system in a comparative approach between Prime-Boost vaccination strategy and DNA vaccinations. The application of this construct as a prospective DNA vaccine has also been evaluated.

Materials and Methods

Rabies virus Glycoprotein (RVGP) gene cloning

Pasteur virus strain (PV) was obtained from the Pasteur Institute of Iran. The original PV strain was isolated in 1882 in France from a rabid cow infected by a rabid dog, passaged in rabbits and mice and then in BHK cells at different levels.[12] The glycoprotein gene

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of this strain with the expected size of 1575 bp was synthesized and cloned into pBluescript vector (GENE-RAY, Hong Kong, China). The synthesized plasmid as well as pcDNA3.1(+) (Invitrogen) were linearized with *NheI* and *EcoRI* restriction enzymes (Fermentas UAB, Vilnius, Lithuania) and the target fragment was ligated into the linearized pcDNA3.1(+) plasmid using T4 DNA ligase (Fermentas UAB, Vilnius, Lithuania). Then, the constructed plasmid was checked by *NheI* and *EcoRI* restriction enzymes digestion (Fermentas UAB, Vilnius, Lithuania).

Expression of RVGP and Western blot analysis

BSR cells were cultured in a 96-well cell culture plate in Dulbeco modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) for 48 hrs at 37°C supplemented with 5% CO_2. Once 75%–90% confluency was observed, the cells were transfected with 4 µg of pcDNA3.1(+)/G using lipofectamine 2000 (Invitrogen™, USA). The cells were lyzed in a buffer containing 150 mM NaCl, 1% NP40, 50 mM Tris (pH 6.8) and protease inhibitor 48 hours after transfection. Protein extract was separated by SDS-PAGE using a 10% gel and the resolved proteins were transferred onto a nitrocellulose membrane for further analysis by Western blot. Membrane was blocked (TBS, 3% skimmed milk). Target protein was detected with HRIG (Human Rabies Immunoglubin, diluted 1:100 in TBS, 0.7% skim milk) as primary antibody. Whole human IgG conjugated with horseradish peroxidase (Sigma, UK) diluted 1:1000 in TBS, 0.7% skim milk was used as a secondary antibody and the reaction was visualized using chemiluminescence substrate (ECL).

DNA Vaccine constructs preparation

Recombinant vector pcDNA3.1(+)/G was grown in *Escherichia coli*, strain TOP10F´in Lauria Broth medium supplemented with Ampicillin. Large scale purification was conducted using Plasmid Mega Kit (Qiagen, Valencia, CA). The quality and quantity of purified plasmid were checked by agarose gel electrophoresis and spectrophotometer, respectively. 80 µg of the purified plasmid was used for each inoculation in MNRI mice.

Animals study

Recombinant plasmid pcDNA3.1(+)/G as well as Rabvet-PII, killed animal rabies (PV strain) produced in the Pasteur Institute of Iran were used for mice immunization. According to Table 1, five-week old NMRI mice were classified into five individual groups. Ethical aspects were applied to this study and handling or bleeding of mice were done in humane way. The mice were kept under daily surveillance. All groups received relevant booster for dose 10 days after primary immunization. In each injection, every mouse was vaccinated with either 80 µg of plasmid DNA subcutaneously or 1.5 IU of Rabvet-PII intraperitoneally. Group A was immunized and boosted with recombinant plasmid pcDNA3.1(+)/G, while both injections in group B were done using Rabvet-PII. The mice in group C were first immunized with recombinant plasmid pcDNA3.1(+)/G and boosted with Rabvet-PII. In contrast, group D was first vaccinated by Rabvet-PII and then boosted by recombinant plasmid pcDNA3.1(+)/G. As a negative control, both injections in group D were done using plasmid pcDNA3.1(+) alone (Table1).

Neutralizing titer determination and interpretation

To analyze humoral immune response, sera were collected 10 days after the second injection and tested for rabies antibodies using rapid fluorescent focus inhibition test (RFFIT). Briefly, each serum sample was heated at 56ºC for 30 min for decomplementation. Serial five-fold dilutions of the sera were made in 96-well plates and 100 focus forming dose (FFD50) of CVS (challenge virus standard) was added to each well. Serial dilutions (1/2, 1/5, 1/10) of the reference standard serum (anti-rabies immunoglobulin, Swiss serum and vaccine institute Bern 750 IU) were analyzed in parallel. Plates were incubated for 90 minutes at 37°C supplemented with 5% CO_2. BSR cells were added 50000 cells per well and plates were incubated at 37°C with 5% CO2. Twenty hours later, medium was removed and cells were fixed in 80% acetone for 30 min, then air dried and stained with FITC (fluorescein isothiocyanate) conjugated anti-rabies nucleoprotein antibody (Bio-Rad, Hercules, CA, USA). Fluorescent foci resulting from infected cells were detected and quantified by fluorescence microscopy. The results were compared with reference levels.

Result

Production of Recombinant Plasmid and RVGP expression

In order to verify glycoprotein insertion in the expression vector, plasmid was treated by the restriction enzyme. Figure 1 shows rabies glycoprotein containing plasmid pcDNA3.1(+)/G digested by restriction enzymes NheI and EcoRI. The result demonstrates a band migrating on agarose gel higher than 1500 bp that could correspond to the glycoprotein gene. Following transfection of BHK cells with pcDNA3.1(+)/G, the protein content was extracted and resolved on acrylamide gel. The glycoprotein expression was analyzed using Western blot technique and visualized with ECL detection (Figure 2). Location of virus glycoprotein appeared as a single band with molecular mass of 66 kDa.

Neutralizing antibody titers from vaccinated mice

Ten days after the second injection, antibody titer of vaccinated mice was determined by RFFIT. In group A where both injections were done using recombinant plasmid pcDNA3.1(+)/G, an average of 6 foci were observed in each microscopic field (Figure 3).

Table 1. Mice inoculation protocol.

Group	First injection	Second injection	Production occulted	Booster interval
A	pcDNA3.1(+)-GP	pcDNA3.1(+)-GP	80µg	10 days
B	Rabvet-PII*	Rabvet-PII	1.5 IU	10 days
C	pcDNA3.1(+)-GP	Rabvet-PII	80µg	10 days
D	Rabvet-PII	pcDNA3.1(+)-GP	80µg	10 days
E	pcDNA3.1(+)	pcDNA3.1(+)	80µg	10 days
Rabvet-PII* = killed animal rabies (PV strain) produced in the Pasteur Institute of Iran.				

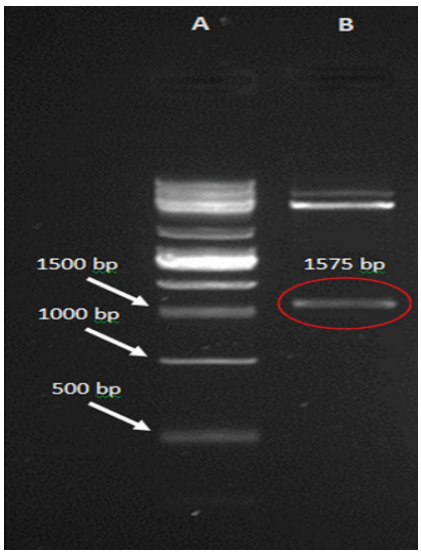

Figure 1. Restriction enzyme analysis of recombinant plasmid. A) DNA Ladder 1kb (Vivantis). B) Double digestion of recombinant plasmid pcDNA3.1(+)/G by NheI and EcoRI restriction enzymes resulted in a 1575 bp Fragment.

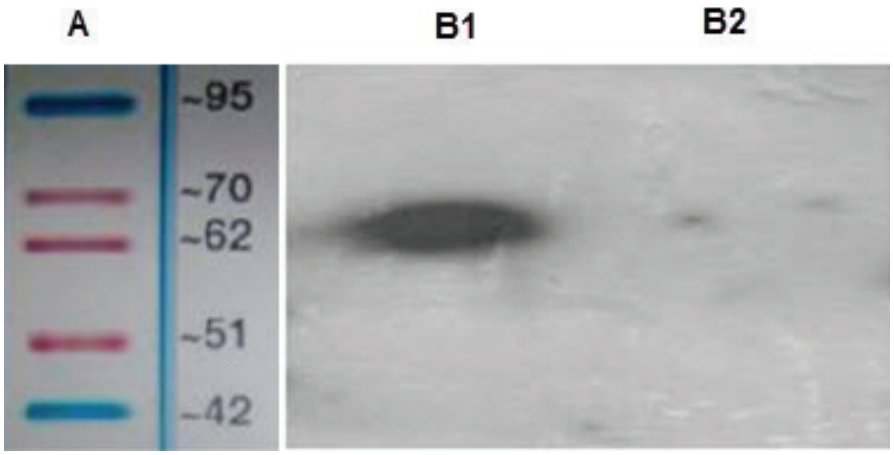

Figure 2. Western blot analysis of the expressed recombinant Gp protein. Part A shows protein marker by Sinaclon (Tehran – Iran). Part B1 shows recombinant glycoprotein and B2 is negative control.

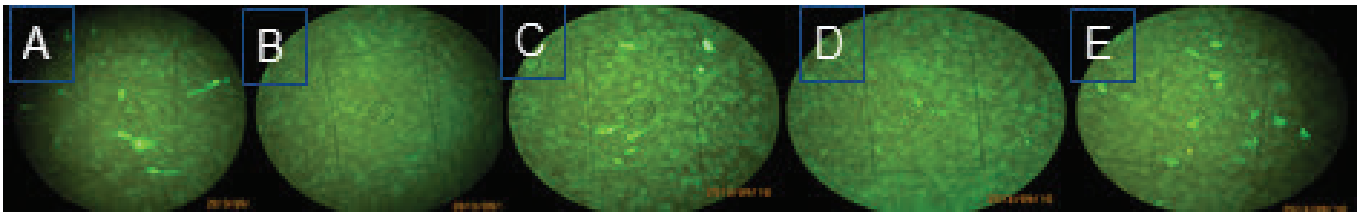

Figure 3. RFFIT test for A to E group. Group A (recombinant plasmid pcDNA3.1(+)-Gp) :6 foci, group B (Rabvet-PII: killed animal rabies (PV strain)): 0 focus, group C (recombinant plasmid pcDNA3.1(+)-Gp → Rabvet-PII: killed animal rabies (PV strain)): 5 foci, group D (Rabvet-PII: killed animal rabies (PV strain) → recombinant plasmid pcDNA3.1(+)-Gp): 2 foci, group E (pcDNA3.1(+) as negative control): 12 foci.

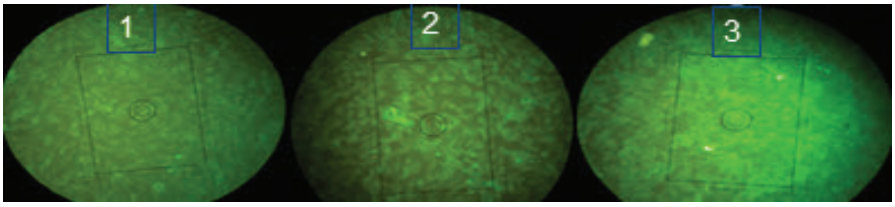

Figure 4. RFFIT test for reference serum. Group 1) 1:2 dilution of serum: 0 focus. Group 2) 1:5 dilution of serum: 1 focus. Group 3) 1:10 dilution of serum: 4 foci.

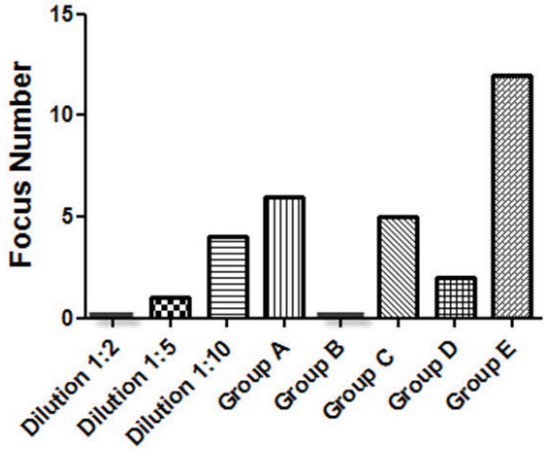

Figure 5. Results of comparison between Prime-Boost vaccination strategy and DNA vaccinations. Group A (recombinant plasmid pcDNA3.1(+)-Gp) : 6 foci, group B (Rabvet-PII: killed animal rabies (PV strain)): 0 foci, group C (recombinant plasmid pcDNA3.1(+)-Gp → Rabvet-PII: killed animal rabies (PV strain)): 5 foci, group D (Rabvet-PII: killed animal rabies (PV strain) → recombinant plasmid pcDNA3.1(+)-Gp): 2 foci, group E (pcDNA3.1(+)) as negative control: 12 foci. And reference serum; Group 1) 1:2 dilution of serum: 0 focus. Group 2) 1:5 dilution of serum: 1 focus. Group 3) 1:10 dilution of serum: 4 foci. The result showed that serum neutralizing antibody response in DNA prime boosted group is similar to that of PVRV injected group.

In group B, which was immunized and boosted using Rabvet-PII, no foci were observed. In contrast, the result of RFFIT in group C that was immunized by recombinant plasmid pcDNA3.1(+)/G and boosted by Rabvet-PII showed 5 foci in each field. In the case of group D, there were about 2 foci in each field. This group was immunized by Rabvet-PII and boosted by recombinant plasmid pcDNA3.1(+)/G. In group E, both injections were done with plasmid pcDNA3.1(+) as negative control and about 12 foci were observed in each microscopic field after RFFIT. On the other hand, reference standard serum was diluted in 1:2, 1:5, and 1:10 ratio and numbers of the observed foci for these samples were 0, 1 and 4, respectively (Table 2).

Discussion

Most of the current human rabies vaccines contain inactivated whole virus particles and induce immune response by eliciting the production of neutralizing antibodies against rabies virus glycoprotein. The expensive production processes of available vaccines are one of the major drawbacks of these vaccines. Since these vaccines originate from the whole virus particles, they are also prone to transmitting the theoretical risk of live virus if inactivated improperly.[13] However, there is no infectious agent in DNA vaccines. In addition, DNA vaccination has been proposed as a cheaper and easily producible vaccine that requires a few doses. Besides, these vaccines are reasonably stable at room temperature which makes them highly desirable in endemic countries.[14] Multiple immunizations are often required for many vaccines.[15,16] Over the past decade, studies have shown that prime-boost immunizations can be given with unmatched vaccine delivery methods, while using the same antigen, in a heterologous prime-boost format. The results of the study conducted by Lu *et al.* showed that heterologous prime-boost is more effective than the homologous prime-boost approach.[16] Furthermore, Hu *et al.* demonstrated that priming with a live recombinant virus and boosting with a subunit recombinant protein can be more effective than immunization by either immunogenic alone.[17] Actually, DNA prime and recombinant protein boost with primary HIV-1 Env antigens became the first approach to elicit positive neutralizing antibodies in rabbit sera.[18,19] Heterologous approach has been successfully investigated for malaria, tuberculosis and HSV-2.[20–22]

In this study, the RVGP expressing construct was used in a comparative approach between prime-boost vaccination strategies and compared with the standard method of rabies vaccination. The recombinant plasmid pcDNA3.1(+)/G was transfected to the BSR cells. NMRI mice were inoculated with DNA plasmid, while prime-boost vaccinations were done in other groups by prime dose of the plasmid construct and the second dose of PVRV. Among rabies virus antibody assay methods, RFFIT and Mouse Neutralization Test (MNT) are the most common. Rapid fluorescent focus inhibition test (RFFIT) described in 1973 by Smith, Yager & Baer appeared promising because of its economical use of reagents and test sample, its rapidity and suitability for testing large numbers of samples.[23] In this study, RFFIT was performed due its rapidity compared to the MNT method. Moreover, according to other studies, RFFIT could also substitute the standard MNT.[24] Hence, Rabies virus neutralizing antibody (RVNA) titration of the serum samples was determined by RFFIT. Analysis of the antibodies in different groups indicated that group B which received two doses of Rabvet-PII induced very high levels of RVNA which could also provide protection against rabies virus challenge. However, the potency of group D that received Prime-Boost vaccine with the second dose of pcDNA3.1(+)/G was enhanced more significantly than group C. These results suggest that the described strategy used in group D can lead to better immunization in laboratory animals compared to group C.

Immunization with higher dilutions of PVRV rabies vaccine (1/625) induced lower levels of RVNA. Inoculation of mice with PVRV 1/5, PVRV 1/25, PVRV 1/125 and PVRV 1/625; consisting of 100 μg of DNA and diluted vaccine preparations resulted in higher levels induction of RVNA than inoculation with DRV (DNA rabies vaccine) or PVRV 1/625 alone.[25] Furthermore, its potency is at least 2-fold higher than that of undiluted vet RV. In another study, the highest RVNA titer was elicited by injection of protein as additional dose of PV vaccine.[26] In the present study, data obtained from immunization in the prime-boost approach showed comparable results by previous studies.[25,26] According to Figure 5, the prime-boost vaccination induces a higher antibody response than DRV alone. It is noteworthy that humoral immunity in the rabies virus neutralization is of pivotal importance. Inactivated vaccine (PVRV) induces production of more antibodies compared to DNA vaccines. This study demonstrated that immunogenicity of the rabies DNA vaccine can increase by using heterologous prime-boost strategy of vaccination in a small animal model. Nevertheless, further experiments with different animal models should be repeated with a decreased amount dose of PV to confirm the present results. Modification of vaccine formulation and use of appropriate adjuvants along with DNA vaccines could also be considered. Altogether, these strategies could lead to better induction of humoral immunity and thus improve the immunogenicity of a DNA vaccine.

Conflict of interest

The authors of this article declare that they have no conflict of interest related to the material in the manuscript.

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Submission declaration

This article has not been published previously and is not under consideration for publication elsewhere, either.

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Reference

1. Knobel DL, Cleaveland S, Coleman PG, Fèvre EM, Meltzer MI, Miranda MEG, et al. Re-evaluating the burden of rabies in Africa and

Asia. *BullWorld Health Org*. 2005; **83:** 360 – 368.
2. Kaur M, Saxena A, Rai A, Bhatnagar R. Rabies DNA vaccine encoding lysosome-targeted glycoprotein supplemented with Emulsigen-D confers complete protection in preexposure and postexposure studies in BALB/c mice. *FASEB J*. 2010; **24:** 173 – 183.
3. Wolff JA, Malone RW, Williams P, Chong W, Acsadi G, Jani A, Felgner PL. Direct gene transfer into mouse muscle *in vivo*. *Science*. 1990; **247:** 1465 – 1468.
4. Kaur M, Rai A, Bhatnagar R. Rabies DNA vaccine: no impact of MHC Class I and Class II targeting sequences on immune response and protection against lethal challenge. *Vaccine*. 2009; **27:** 2128 – 2137.
5. Alves AMB, La´saro MO, Almeida DF, Ferreira LCS. Epitope specificity of antibodies raised against enterotoxigenic Escherichia coliCFA/I fimbriae in mice immu-nized with naked DNA. *Vaccine.* 1998; **16:** 9 –15.
6. Wang R, Doolan DL, Le TP, Hedstrom RC, Coonan KM, Charoenvit Y, et al. Induction of Antigen-Specific Cytotoxic T Lymphocytes in Humans by a Malaria DNA Vaccine. *Science*. 1998; **282:** 476 – 480.
7. Kim N, Kwon S, Lee J, Kim S, Yoo T. Protective effect of the DNA vaccine encoding the major house dust mite allergens on allergic inflammation in the murine model of house dust mite allergy. *Clin Mol Allergy.* 2006; **4:** 4.
8. Timmerman JM, Singh G, Hermanson G, Hobart P, Czerwinski DK, Taidi B, et al. Immunogenicity of a Plasmid DNA Vaccine Encoding Chimeric Idiotype in Patients with B-Cell Lymphoma. *Cancer Res.* 2002; **62:** 5845 – 5852.
9. Kenji Okuda YWMS. Recent Developments in Preclinical DNA Vaccination. *Vaccines.* 2014; **2:** 89 – 106.
10. Ramya R, Mohana Subramanian B, Sivakumar V, Senthilkumar RL, Sambasiva Rao KRS, Srinivasan VA. Expression and solubilization of insect cell-based rabies virus glycoprotein and assessment of its immunogenicity and protective efficacy in mice. *ClinVaccine Immunol*. 2011; **18:** 1673 – 1679.
11. Donald L, Lodmell LCE. Rabies vaccination: comparison of neutralizing antibody responses after priming and boosting with diferent combinations of DNA,inactivated virus, or recombinant vaccinia virus vaccines. *Vaccine*. 2000; **18:** 2394 – 2398.
12. Meslin FX, Kaplan MM, Koprowski H. *Laboratory Technique in Rabies*. World Health Organization, Geneva. 4th ed; 1973.
13. Warrell MJ, Warrell DA. Rabies and other lyssavirus diseases. *Lancet*. 2004; **363:** 959 – 969.
14. Ullas PT DA, Madhusudana SN. Rabies DNA vaccines: current status and future. *World J Vaccines*.2012; **2:** 36 – 45.
15. Petrovsky, Nikolai, and Julio César Aguilar. Vaccine adjuvants: current state and future trends. *Immunol Cell Biol.* 2004; **82(5):** 488 – 496.
16. Lu S. Heterologous prime-boost vaccination. *NIH.* 2009; **21:** 346 – 351.
17. Hu S, Klaniecki J, Dykers T, Sridhar P, Travis B. Neutralizing antibodies against HIV-1 BRU and SF2 isolates generated in mice immunized with recombinant vaccinia virus expressing HIV-1 (BRU) envelope glycoproteins and boosted with homologous gp160. *AIDS Res Hum Retroviruses*. 1991;**7:** 615 – 620.
18. Richmond JFL, Lu S, Santoro JC, Weng J, Hu S-L, Montefiori DC, et al. Studies of the Neutralizing Activity and Avidity of Anti-Human Immunodeficiency Virus Type 1 Env Antibody Elicited by DNA Priming and Protein Boosting. *J Virol*. 1998; **72:** 9092 – 9100.
19. Beddows S, Schülke N, Kirschner M, Barnes K, Franti M, Michael E, et al. Evaluating the immunogenicity of a disulfide-stabilized, cleaved, trimeric form of the envelope glycoprotein complex of human immunodeficiency Virus Type 1. *J Virol.* 2005; **79:** 8812 – 8827.
20. Dunachie SJ, Walther M, Epstein JE, Keating S, Berthoud T, Andrews L, et al. A DNA prime-modified vaccinia virus Ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naive adults against plasmodium falciparum sporozoite challenge. *Infect Immun.* 2006; **74:** 5933 – 5942.
21. Magalhaes I SD, Ahmed R, Mueller S, Wehlin L, Scanga C, Weichold F, et al. rBCG induces strong antigen-specific T cell responses in rhesus macaques in a prime-boost setting with an adenovirus 35 tuberculosis vaccine vector. *PLoS ONE.* 2008; **3:** e3790.
22. Sin J BM, Pachuk C, Weiner D. DNA priming-protein boosting enhances both antigen-specific antibody and Th1-type cellular immune responses in a murine herpes simplex virus-2 Gd vaccine model. *DNA Cell Biol.* 1999; **18:** 771 – 779.
23. Louie RE, Dobkin MB, Meyer P, Chin B, Roby RE, Hammar AH, et al. Measurement of rabies antibody: comparison of the mouse neutralization test (MNT) with the rapid fluorescent focus inhibition test (RFFIT). *J Biol Stand.* 1975; **3:** 365 – 373.
24. Simani S. Comparison of three Serological tests for titration of rabies in immunized individuals, WHO-Collaborating Center for Reference and Research on Rabies. *Arch Iran Iran*. 1999; **2:** 106.
25. Biswas S, Reddy G, Srinivasan V, Rangarajan P. Preexposure efficacy of a novel combination DNA and inactivated rabies virus vaccine. *Hum Gene Ther.* 2001; **12:** 1917–1922.
26. Rath A, Choudhury S, Batra D, Kapre SV, Rupprecht CE, Gupta SK. DNA vaccine for rabies: Relevance of the trans-membrane domain of the glycoprotein in generating an antibody response. *Virus Res*. 2005; **113:** 143 – 152.