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Efficacy Test in Mice of Two Novel Rabies Vaccine Candidates Using a Pakistan Rabies Virus Glycoprotein Gene

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Abstract

Purpose: Pakistan is one of the few countries where Rabies is endemic and a great threat to humans as well as live stocks. A large number of individuals died of rabies exposure due to either the limited access to rabies vaccines or the side-effects of sheep brain -originated Semple vaccine. Cell culture-derived rabies vaccines are mostly unaffordable to the needed population under rabies threat. Development of a safer and more affordable vaccine is necessary in Pakistan. Here, we developed two novel recombinant rabies virus vaccines using a local Pakistan rabies virus glycoprotein gene, and tested the efficacy of vaccines in mice.

Methods: The glycoprotein gene (RVG) of vector ERAg$_3$p and ERAg$_3$m was substituted, respectively, with a modified Pakistani RVG. The resulting recombinant vectors were applied for reverse genetics to recover two vaccine viruses, PK-SG and PK-DG. The efficacy of PK-SG and PK-DG were tested in mice by intramuscular injection and oral delivery.

Results: All mice survived the challenge after intramuscular vaccination using PK-SG, or PK-DG. In the oral vaccination groups, 80% mice with PK-SG and 90% mice with PK-DG survived the challenge. Meanwhile, 80% of the unvaccinated control mice succumbed after challenge. The mean rabies virus neutralizing antibody titers was ≥ 0.5 IU/ml in all vaccinated groups.

Conclusion: Our results demonstrated the efficacy of PK-SG and PK-DG in rabies vaccination in mice. The two recombinant virus strains may be good vaccine candidates for the target animals and humans in Pakistan. Detailed investigations are necessary in the future.

Keywords: Pakistani rabies vaccines; Reverse genetics; Cell culture-derived rabies vaccines; Rabies in Pakistan; Pakistan rabies virus isolate

Introduction

Rabies is an important international zoonotic disease and is the eleventh biggest cause of human death in the reportable infectious diseases [1-3]. The global human rabies death toll reaches more than 55,000 each year, mostly in the developing countries. Rabid dog bites attribute to about 99% of human rabies death toll, and more than 3.3 billion people are living under the threat of a potential exposure to the deadly rabies virus in the endemic regions [4]. By contrast, most European countries have achieved rabies free status mainly through mass vaccination of dogs and wild life [5]. Therefore, an affordable and potent rabies vaccine that can be used in vaccination campaign is of prime importance in rabies control and prevention.

The first nerve tissue-derived rabies vaccines (NTVs) were developed in 1880s by Louis Pasteur and Emile Roux [4,6]. Unfortunately, more than a century after the NTVs' introduction, Pakistan is still one of the few countries where the NTVs are still being used in humans. The NTVs have been tested less immunogenic, more reactogenic, life threatening and caused neurological adverse reactions in 0.3-0.8 per 1000 vaccinated people [7,8]. The Semple vaccine (sheep brain-derived) is produced by Pakistan NIH using the fixed rabies virus strain. Due to its low potency in human rabies post-exposure prophylaxis (PEP), the vaccine is administrated daily for 14 consecutive subcutaneous shots and then booster on day 24, 34 and 104 [9]. Because of the painful nature of the vaccination regimen, there is a high ratio of dropouts in rabies PEP. The reports indicate about 31% patients who do not complete the whole rabies PEP procedure [9,10]. Among those that completed the whole procedure, only about 50% of the individuals showed detectable rabies virus neutralizing antibodies [11]. Furthermore, the Semple vaccine is not always available in Pakistan, and cell culture-derived rabies vaccines are mostly unaffordable to the needed population under rabies threat. Development of a safer and more affordable vaccine is necessary in Pakistan.

Recent progress in molecular biology and vaccinology makes the development of highly attenuated rabies virus strains possible, with improved efficacy and safety profiles [12,13]. Rabies virus glycoprotein is responsible for viral pathogenicity, and induces virus neutralizing antibody responses [8,14-17]. Unlike traditional methods for virus attenuation, rabies virus reverse genetics provides a defined

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modification by introducing site mutations, gene insertion and gene deletion into the virus genome. Studies have shown the amino acid residue 333 of the glycoprotein is important in pathogenicity, with arginine or lysine in many street rabies virus isolates [14,18]. Studies also demonstrated a positive relationship between the level of glycoprotein expression and immune protection [16,17,19-24]. Therefore, we hypothesized that by choosing a representative Pakistan rabies virus isolate, through glycoprotein gene mutation and insertion of an extra glycoprotein gene copy, an ideal vaccine candidate will be developed. A recent street rabies virus was isolated from a cow died of rabies in Harichand, Pakistan in 2007. Based on two established plasmid vectors ERAg$_3$p and ERAg$_3$m, we substituted the ERA glycoprotein with the Pakistan rabies virus glycoprotein, and recovered virus PK-SG (with a single glycoprotein gene) and PK-DG (with double glycoprotein genes) by reverse genetics [13,22,25]. Thereafter, we tested the efficacy of PK-SG and PK-DG in mice.

Methods

RNA extraction and amplification of a Pakistan rabies virus glycoprotein gene

Total RNA was extracted from the cow brain tissue using Trizol Reagent according to the manufacturer's instruction (Invitrogen, CA, USA). The Pakistan rabies virus glycoprotein (PKG) cDNA was transcribed by using RAVGF primer and Transcriptor Reverse Transcriptase (Roche, Germany) at 55°C for 30 minutes. The primer set RAVGF and RAVGR was employed to amplify the entire glycoprotein gene (RVG) under the PCR conditions: 94°C for 4 minutes, followed by 30 cycles of 94°C for 50 seconds, 53°C for 90 seconds, 72°C for 120 seconds, and the final extension at 72°C for 15 minutes. The Taq DNA polymerase and dNTPs were obtained from Roche, Germany. The amplified RVG was analyzed by electrophoresis on 1% gel, cloned into pCR®-XL-TOPO® vector (Invitrogen, CA, USA), and sequenced as described previously [22,26]. The primers are listed in table 1.

Site directed mutagenesis of the PKG

Two restriction enzyme sites *Kpn*I and *Pst*I were introduced at the terminal end of RVG using primers PSTPK5 and KPNPK3. The Accu Prime Pfx Super Mix was used for high fidelity amplification according to the manufacturer's instruction (Invitrogen, CA, USA). The PCR was carried out under the cycle program: 95°C for 3 minutes, 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, 68°C for 90 seconds and final termination 68°C for 7 minutes. The amino acid arginine (CGA) at amino acid residue position 333 of RVG was mutated to glutamic acid (GAG), as previously described [27]. The primer set PFG5 and PKG5 was used for site directed mutagenesis.

Cloning the PKG into the ERAg$_3$p and ERAg$_3$m backbone

The PKG was cloned into the reshuffled rabies virus vectors, and replaced the g$_3$ gene with the PKG in the ERAg$_3$p (gene order N- g$_3$- P - M- L) and ERAg$_3$m (gene order N- P - g$_3$ – M – G - L) to create the recombinant vector PK-SGV and PK-DGV, respectively, as described [22].

Virus recovery from PK-SGV and PK-DGV by reverse genetics

The recombinant vector PK-SGV and PK-DGV, along with other T7 helper plasmids, were co-transfected to the BSR cell lines (a clone of BHK cells) to recover rabies virus as described [28]. The presence of PKG in the recovered recombinant viruses was confirmed by RT-PCR. The recovered virus PK-SG and PK-DG were further passaged in the BSR cell culture, and titrated before animal testing.

Name	Primer sequence	Location	Purpose
RAVGF*	5′ CAAGGAAAGATGGTTCCTCAG 3′	3309-3329	RT-PCR
RAVGR	5′ TCACAGTCTGGTCTCACCTCCAC 3′	4870-4892	RT-PCR
GS3*	5′ GTACCCGGACTACCACTGGCTCAG 3′	3721-3744	Sequencing
GS4*	5′ GGAACGTGGGTTGCGATGCAAACA 3′	4088-4111	Sequencing
GS5*	5′ GGTATAATACTGGGTCCTGACGGC 3′	4460-4483	Sequencing
M13 F*	5′ GTAAAACGACGGCCAG 3′		Sequencing
M13 R	5′ CAGGAAACAGCTATGAC 3′		Sequencing
PSTPK5*	5′ CTTCAA**CTGCAG**ATGGTTCCTCAGGTTCTTTTG 3′		*Pst*1 site
KPNPK3	5′ GCCCTA**GGTACC**TCAGAGTCTGGTCTCACCTCC 3′		*Kpn*1 site
PFG5	5′ GATGCTCATTACAAGTCAGTCGAGACCTGGAATGAGATCATC 3′		Mutagenesis
PKG3	5′ GACTGACTTGTAATGAGCATCTGCTTCCATCAAGGT 3′		Mutagenesis

Table 1: Primers used for RT-PCR, sequencing, creation of restriction enzyme sites and mutagenesis.

Mice number	PK-SG IM	Mice number	PK-DG IM	Mice number	PK-SG PO	Mice number	PK-DG PO	Mice number	Control PO
1	8.80	11	2.24	21	2.24	31	0.49	41	<0.04
2	2.56	12	2.88	22	<0.04	32	2.24	42	<0.04
3	2.24	13	0.56	23	0.56	33	0.60	43	<0.04
4	1.84	14	8.80	24	2.88	34	7.20	44	<0.04
5	8.00	15	8.80	25	2.88	35	1.16	45	<0.04
6	0.10	16	0.56	26	<0.04	36	0.60	46	<0.04
7	1.68	17	2.00	27	0.56	37	7.20	47	<0.04
8	2.16	18	2.16	28	2.88	38	1.16	48	<0.04
9	1.68	19	5.60	29	2.24	39	2.24	49	<0.04
10	2.16	20	2.16	30	2.88	40	0.49	50	<0.04
Mean Titer	3.12	Mean Titer	3.58	Mean Titer	1.71	Mean Titer	2.34	Mean Titer	<0.04

Table 2: Rabies virus neutralizing antibodies in the immunized mice.

Vaccination trials in mice

The animal test was performed under CDC approved protocol 1651. Fifty 3-weeks old mice (ICR, Charles River Laboratories MA, USA) were equally distributed into five groups, with 5 mice per cage. Both PK-SG and PK-DG were administrated intramuscularly (IM) in the gastrocnemius muscle, or per os (PO) at 50 µl (6.0 ×1010ffu / ml) per mouse. The control mouse group was given 50 µl PBS (0.01 M, pH 7.2).

Blood was collected in all mice at 14 day post-vaccination through retro-orbital route. Rabies virus neutralization antibody was measured by Rapid Fluorescent Focus Inhibition Test (RFFIT) following the standard protocol [29]. The mice were checked daily for any sign of illness. The mice were challenged on day 28 post-vaccination using a street Chad dog rabies virus [21].

Statistical analysis

The antibody titers between the vaccinated and control groups were analyzed statistically by performing ANOVA and DNMRT with the help of computer-aided program DASTAT® (UPB, Perugia, Italy).

Results and Discussion

The Pakistan rabies virus glycoprotein (RVG) presented the arginine (CGA) residue at position 333, was mutated to GAG (glutamic acid), resulted in PKG. The mutation of arginine to glutamic acid generally attenuates most rabies virus isolates, and improves immunogenicity [14,19,30,31]. Rabies virus ERA strain is attenuated empirically and has been used for vaccination in wild life [22]. The genome of ERA strain was reshuffled to create two vectors ERAg$_3$m and ERAg$_3$p [22,28]. The PKG was successfully cloned into the ERAg$_3$m and ERAg$_3$p backbone, creating the recombinant vector PK- SGV and PK-DGV. Subsequent sequencing confirmed the presence of a single glycoprotein gene (PKG) in the PK-SGV and a double copy of glycoprotein genes in the PK-DGV.

The recombinant virus PK- SGV and PK-DGV were recovered (Figure 1). The viruses were further adapted to cell culture, and were harvested when the titer reached ≥ 10^6 ffu/ml.

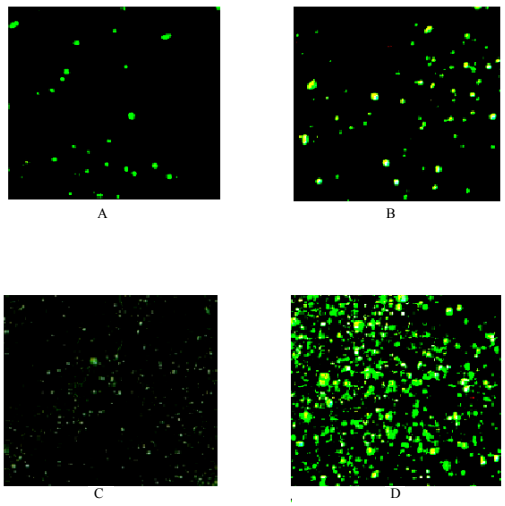

Figure 1: Direct fluorescent antibody testing of rabies virus at different days after virus recovery. A: Day 4; B: Day 7; C: After reinfecting cells with harvested virus at Day 10; D: At Day 15.

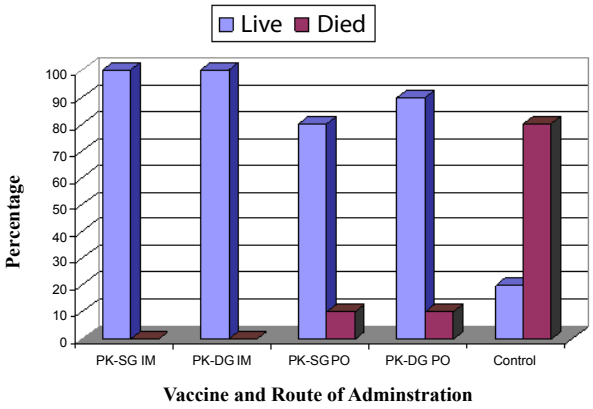

Figure 2: Survivorship of the vaccinated mice after challenge.

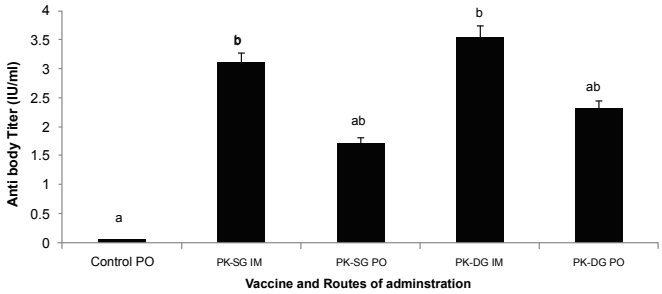

Figure 3: Mean rabies virus neutralizing antibody titers summarized from each mouse group.

The efficacy test of PK-SG and PK-DG in mice showed very promising results. All mice survived the challenge in the IM groups, vaccinated with either PK-SG or PK-DG. Meanwhile, 90% in the PK-DG and 80% in the PK-SG survived the challenge after oral vaccination (Figure 2). The highest mean virus neutralizing antibody titer was observed in the PK-DG IM group, followed by the PK-SG IM group, PK-DG PO group and PK-SG PO group. The average antibody titer in each group was greater than 0.5 IU/ml, an arbitrary value suggestive of rabies protection *in vivo*. The control mice presented base antibody titers (Table 2). Oral vaccination is a valid tool for rabies control in wild life [21,22]. Our rabies vaccine candidates are of potential to administer through oral route to control rabies in free-ranging dogs in Pakistan. The increased virus neutralizing antibody titers in the PK-DG group agreed with the previous report, since more copies of RVG induce higher protection in rabies (Figure 3) [12,32].

The NTVs need to be replaced by cell culture-derived rabies vaccines in Pakistan. The benefit of a cell culture-derived rabies vaccine to human rabies PEP in Pakistan could be tremendous. The PK-SG and PK-DG viruses grow to very high titers in cell culture, and are promising candidates for rabies vaccine development. Detailed characterization on the two vaccine strains will be investigated in the target animal species, such as dogs and domestic animals. Based on the results of various animal models, it is also possible to develop the candidates into a cell culture-derived vaccine for humans in Pakistan in the future.

Conflict of Interest

Use of trade names and commercial sources are for identification only and does not imply endorsement by the U. S. Department of Health and Human Services. The findings and conclusions in this report are those of the authors and do not necessarily represent the views of the funding agency.

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