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Feline herpesvirus vectored-rabies vaccine in cats: A dual protection

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ABSTRACT

In China, cats cause about 5% of human rabies cases. Rabies control in cats plays a role in achieving the ultimate goal of elimination of dog rabies-mediated human deaths. However, there is no cat-specific rabies vaccine in China yet. In this study, we constructed a recombinant rabies vaccine by using a felid herpesvirus 1 (FHV-1) isolate, and deleted the gI/E in the FHV-1 and replaced the region with a glycoprotein (G) of rabies virus (RABV) strain BD06 through homologous recombination. The recombinant virus FHV-RVG was recovered and purified, and the expression of RABV glycoprotein was verified by indirect immunofluorescent assay. For potency in cats, each animal was inoculated intranasally with 1 ml FHV-RVG at $10^{6.5}$ $TCID_{50}$. Blood samples were collected at defined intervals for antibody titration. The animals were challenged by herpes and rabies after completion of vaccination on day 180 and day 194, respectively. Our results demonstrated all vaccinated cats generated antibodies against both FHV-1 and RABV, and reached an arbitrary protective titer > 0.5 IU/ml for rabies viral neutralizing antibody (VNA) by day 14 post inoculation (dpi) and titer peaked on 30 dpi with VNA at 24.5 ± 10.23 IU/ml. All vaccinated cats presented no clinical signs of FHV-1 infection and survived rabies challenge, while the control cats had severe rhinotracheitis and died from rabies after challenge. All this demonstrated that the FHV-based recombinant vaccine is effective in protection against both FHV-1 and RABV infections.

1. Introduction

Dog rabies causes the vast majority, ~95%, of human rabies death in China, and the remaining 5% of human rabies death attributes mainly to exposures from cats and other animal species as reported by China CDC [1,2]. Cats contract rabies and transmit rabies to humans, but do not support rabies circulation in their own population. This could be an easy success for rabies control in cats in contrast to the reservoir animal species. Besides dogs, current important rabies reservoirs in China include Chinese ferret badgers (FDs) in Southeast China [3–5], and raccoon dogs (RDs) in Inner Mongolia [6,7]. A bat-related human rabies death was anecdotally described [8], and an Ikuta-like lyssavirus has been isolated in a bat (Murina leucogaster) in Jilin province where the bat-related human death occurred [8,9]. Rodents and domestic animals/livestock are opportunistic hosts for rabies, and related human rabies deaths have been infrequently reported [2].

Human rabies in China is a notifiable disease since 1950, and hundreds to thousands of human rabies deaths are captured in the reporting system each year. In 2005, a national surveillance program was introduced to systemically monitor rabies outbreaks in China. In contrast to human rabies monitoring, the system for dog rabies surveillance has not been fully established yet. However, vaccinating dogs for rabies control is an accepted practice, and a number of dog rabies vaccines have been licensed and produced in China. There is no rabies vaccine licensed specifically for cats yet. To maximize efficiency, our goal was to develop a cat rabies vaccine by using a cat-derived herpesvirus vector, providing dual protective effects against both viruses.

Felid herpesvirus 1 (FHV-1) is an alpha-herpesvirus, causing rhinotracheitis in cats, and accounts for about half of feline viral upper respiratory infections. There are available vaccines for disease prevention, including the modified-live and inactivated FHV-1. By deletion of the glycoprotein gI/E, the FHV-1 becomes attenuated and safe enough to be used as a live vaccine for cats [10,11].

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Rabies virus (RABV) is a member of *Lyssavirus* family, *Rhabdoviridae*, and has five structural proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and an RNA dependent RNA polymerase (L) [12]. The G is the only outer membrane protein responsible for stimulating the production of viral neutralizing antibodies (VNAs) [13]. Recombinant virus-vectored vaccines based on the G protein have been reported to be effective both in laboratory animals and in field studies [14–18]. Rabies control in wildlife has been successful through oral vaccination campaigns in the North Americas and Europe [19–22]. There are significant concerns regarding adoption of the wildlife oral rabies vaccines for use in cats, including risk for people who have contact with the vaccine baits to develop opportunistic infections, and the concern for vaccine efficacy, which has not been fully evaluated in cats. In this study, we explored the possibility of using FHV-1 as a vector to express the RABV G protein, creating a recombinant vaccine FHV-RVG. Our results demonstrated the FHV-RVG not only protected cats from feline herpesvirus challenge, but also stimulated high viral neutralizing antibodies (VNAs) against RABV and protected the cats from rabies challenge. The FHV-RVG vaccine provides dual protection against infections by both FHV-1 and RABV. The dual protection of this vaccine against two serious diseases within felines may increase consumer interest.

2. Materials and methods

2.1. Cells and antibodies

Feline kidney cells (F81) and baby hamster kidney cells (BHK) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% and 2% of heat-inactivated fetal bovine serum (FBS, Gibco), respectively, under 5% CO_2 at 37 in an incubator.

The FITC-conjugated monoclonal antibodies against RABV N protein and monoclonal antibodies against the RABV G protein were made in the Laboratory of Epidemiology, Veterinary Institute (Changchun, China). DyLight™ 594-conjugated goat anti-mouse IgG was purchased from Thermo Fisher Scientific.

2.2. Viruses

Feline herpesvirus 1 strain Z (FHV-Z) is a field isolate from a sick kitten with severe rhinotracheitis in our previous studies (unpublished). To propagate the virus, cat kidney cells F81 were infected with the virus at multiplicity of infection (MOI) of 0.1, and the cell culture supernatants were harvested for titration when ~90% of cytopathic effect (CPE) occurred. RABV strain BD06 is a street virus isolated from a rabid dog in 2006, Hebei province, China [18]. The virus was propagated in suckling mice by intracerebral inoculation, and the MIC LD_{50} was $10^{5.4}$/0.03 ml.

2.3. Plasmids, genes and primers

Plasmid peGFP-C3 was purchased from BD Biosciences, Clontech. The pMD18-T vector cloning kit was purchased from Takara. The primers (Supplementary Table 1) for FHV homologous recombination and polymerase chain reactions (PCR) were designed based on a reference sequence of feline herpesvirus (GenBank accession number: KR381803). The RABV G sequence was from BD06 strain (GenBank accession number: EU549783).

2.4. Construction and rescue of recombinant FHV-Z expressing eGFP through homologous recombination

In supplementary Fig. 1A, to replace the gI/E gene of FHV-Z with enhanced green fluorescent protein (eGFP) gene by homologous recombination, we first constructed an expression cassette having a left arm (Larm1) amplified from the FHV-Z genome DNA, the eGFP ORF flanked with the CMV early gene promoter and a poly A signal amplified from the peGFP-C3, and a right arm (Rarm1) amplified from the FHV-Z genome DNA. The 3 fragments, Larm1 (1.15 kb), eGFP and Rarm1 (1.05 kb), were joined together by an additional overlapping PCR using the primers LgI-F and RgE-R. The final fragment was cloned into the pMD-18T after sequence verification, and was renamed pT-eGFP.

The pT-eGFP was then co-transfected with genomic DNA of FHV-Z into F81 cells using TranslT-LT1 (Mirus Bio) according to the supplier's instructions. Briefly, cat kidney F81 cells were seeded to a 6-well-plate at ~90% confluency the day before transfection. In a vial, 3 μg of pT-eGFP in 100 μl DMEM, 5 μg FHV-Z viral genomic DNA in 100 μl DMEM and 16 μl TranslT-LT1 in 200 μl DMEM were mixed together and incubated at room temperature (RT) for 20 min. After incubation, the mixture was added dropwise to the cells in the 6-well-plate, and incubated under 5% CO_2 at 37 for 6 h. Then the cell culture medium was removed and replaced with fresh DMEM containing 2% FBS. The cells were maintained in an incubator under 5% CO_2 at 37 for 5–7 days. A "positive screening" was applied by selection of a single well of cells showing both CPE and eGFP and harvesting the cell supernatants for further infection in freshly cultivated F81 cells. After 4–5 rounds of selection through a limited dilution method (2–3 $TCID_{50}$/well in the 6-well-plate), the recovered virus FHV-ΔgI/E-eGFP became free from parental FHV-1, causing both CPE and eGFP expression in all infected cells. The plaque-purified virus FHV-ΔgI/E- eGFP was grown and further characterized in F81 cells.

2.5. Construction and rescue of recombinant FHV-Z expressing RABV G through homologous recombination

As shown in Supplementary Fig. 1B, to replace the eGFP gene of the virus FHV-ΔgI/E- eGFP obtained in the previous step with the G gene of RABV BD06, we first constructed an expression cassette having a left arm (Larm2) amplified from the FHV-△gI/E-eGFP genomic DNA, the G gene amplified from RABV BD06, and a right arm (Rarm2) amplified from the FHV-△gI/E-eGFP genomic DNA using the primers listed in Supplementary Table 1. The 3 fragments, Larm2 (1.8 kb), G gene, and Rarm2 (1.3 kb), were joined together by an additional overlapping PCR using primer CMV-F and polyA-R. The final fragment was cloned to the pMD18-T vector after sequence verification, and the plasmid was renamed pT-G.

The pT-G was then co-transfected with the genomic DNA of FHV-ΔgI/E-eGFP into F81 cells using TranslT-LT1 (Mirus Bio) according to the supplier's instruction. Briefly, 3 μg of pT-G in 100 μl DMEM, 5 μg FHV-ΔgI/E-eGFP viral genomic DNA in 100 μl DMEM, and 16 μl TranslT-LT1 in 200 μl DMEM were mixed together, and then transfected as described above. A "negative screening" was performed by selecting the "negative cells" without eGFP expression. In a similar process to the "positive screening", we recovered recombinant virus FHV-RVG free from FHV-ΔgI/E-eGFP, causing only CPE, but no eGFP expression. The pure virus FHV-RVG was further grown and characterized *in vitro*. The cloned genes were verified by size analysis and sequence verification.

2.6. Characterization of FHV-RVG

2.6.1. In vitro growth properties of the recombinant viruses

The cat kidney F81 cells were seeded in 24-well plates at 10^5 cells per well in 1 ml DMEM the day before virus infection. For

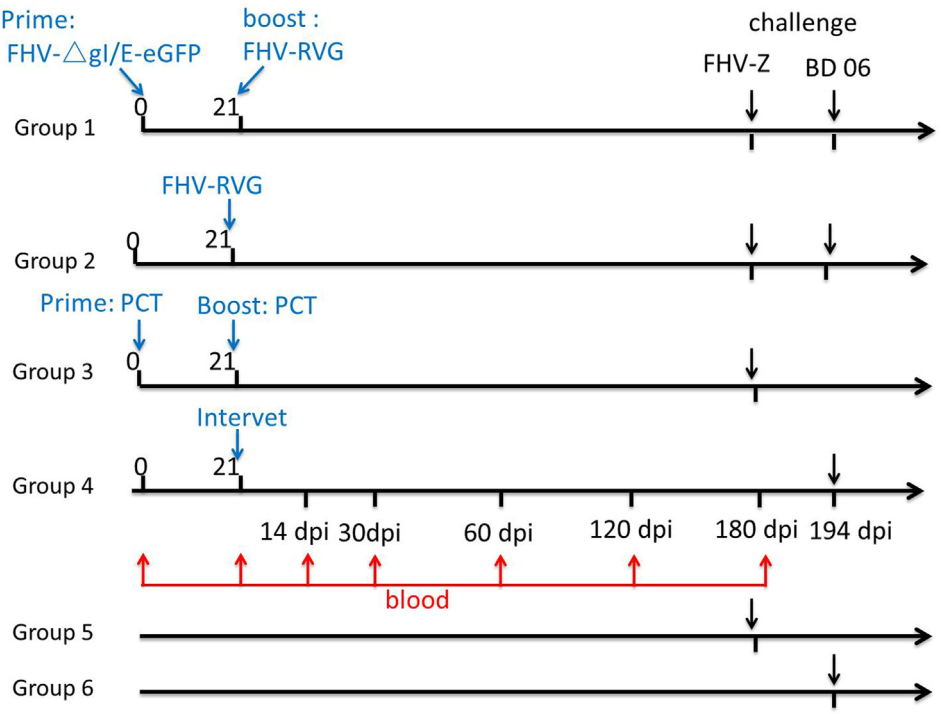

Fig. 1. Timeline of animal vaccinations, blood sampling and challenges. Group 1, vaccination by FHV-△gI/E-eGFP, followed by booster of FHV-RVG to investigate whether pre-existing Ab against FHV will affect vaccination efficacy of FHV-RVG. Group 2, FHV-RVG. Group 3, a commercial inactivated vaccine for felid herpesvirus (PCT). Group 4, a commercial inactivated vaccine for rabies (Intervet). Group 5, challenge control group for herpes FHV-Z. Group 6, challenge control group for rabies BD06.

one-step virus growth, we used an MOI of 1.0. For multiple-step virus growth, we used an MOI of 0.01. After virus absorption at 37 for 1 h, the inocula were removed and washed 3 times with PBS (pH7.2, 0.01 M). The cells were maintained in DMEM containing 2% FBS under 5% CO_2 at 37 in an incubator. At indicated intervals, the cell supernatants were collected for virus titration.

2.6.2. Indirect fluorescent assays

The F81 cells pre-cultured in a 24-well plate were infected individually with FHV-Z, FHV-ΔgI/E-eGFP and FHV-RVG at an MOI of 1.0 for 24 h. The cells were then fixed with 80% acetone at −20 for 30 min. After removal of acetone, the cells were air-dried and incubated with mouse anti-RABV G protein primary antibodies (1:400) at 37 for 1 h. The plate was washed 3 times using PBS (pH7.2, 0.01 M), and DyLight™ 594-conjugated goat anti-mouse IgG secondary antibodies (1:250) was added for incubation at 37 for 1 h. The cells were counterstained using DAPI (solarbio®) for 10 min. After air drying, the cell images were captured by using a laser scanning confocal microscope (Nikon).

2.7. FHV-RVG vaccination in cats

All animal experiments were approved in compliance with the Institutional Animal Care and Use guidelines by Changchun Veterinary Research Institute. Thirty 3-month-old cats prescreened FHV-1 negative were randomly divided into 6 groups at 5 cats per group.

In Group 1, each cat was administered intranasally (i.n.) 1 ml $10^{6.5}$ $TCID_{50}$ of FHV-ΔgI/E-eGFP on day 0, and the same dosage of FHV-RVG on day 21.

In Group 2, each cat was administered i.n. 1 ml $10^{6.5}$ $TCID_{50}$ of FHV-RVG on day 21.

In Group 3, each cat received subcutaneously (s.c.) one dose of inactivated feline parvovirus-calicivirus-herpesvirus tri-combined vaccine (Fel-O-Vax® PCT, zeotis, the USA) on day 0, and was boosted on day 21.

In Group 4, each cat was vaccinated s.c. with one dose of inactivated rabies vaccine according to the vendor's instruction (Nobivac, Intervet, The Netherlands).

Group 5 and 6 were unvaccinated controls for FHV and RABV, respectively.

Blood samples were separately collected from each animal before vaccination, and on days 14, 30, 60, 120 after the last vaccination (Fig. 1). Sera were stored at −20 for antibody testing.

2.8. Antibody assays

2.8.1. Virus neutralizing test for rabies

RABV neutralizing antibody titers were quantified by using the fluorescent antibody virus neutralization test (FAVN) as described elsewhere [18,23]. Briefly, 3-fold serial dilution of standard serum (0.5 IU/ml) and test serum samples were prepared in quadruplicate in a 96-well plate, and mixed with 50 μl of CVS-11 at 100 $TCID_{50}$. After incubation at 37 for 1 h, 2×10^4 BHK-21 cells in 50 μl DMEM was added to each well and incubated for another 48 h. The cells were fixed with 80% acetone at 4 for 30 min, and stained with FITC-conjugated mouse anti-RABV N monoclonal antibodies at 37 for 60 min. After washing with PBST for 3 times, fluorescence was observed by UV microscopy, and the VNA titers were calculated using the Spearman-Karber formula.

2.8.2. Virus neutralization test for feline herpesvirus

A modified microtiter neutralization assay was adopted for measuring neutralizing antibodies against FHV-1 [24]. In a 96-well plate, 50 μl serum was diluted in a 2-fold series, and 50 μl FHV-Z at 100 $TCID_{50}$ was added to each serum dilution. After incubation at 37 for 1 h, 10^4 F81 cells in 100 μl DMEM were added to each well for incubation at 37 for 72 h. The VNA titers were

expressed as the reciprocal of the highest serum dilution inhibiting CPE formation in 50% wells infected with 100 $TCID_{50}$ FHV-Z.

2.9. Virus challenges in cats

2.9.1. Feline herpesvirus challenge

One hundred and eighty days after vaccination, all cats except groups 4 and 6 were challenged i.n. with 1 ml $10^{7.0}$ $TCID_{50}$ of field isolate FHV-Z. Clinical signs of cough, sneezing, mucus and eye discharges were recorded daily for 2 weeks according to the scoring system shown in Supplementary Table 2.

After completion of herpes challenge, cats in group 5 were euthanized by CO_2 intoxication, and cats in other groups were kept for rabies challenge.

2.9.2. Rabies virus challenge

Each cat in groups 1, 2, 4 and 6 was challenged i.m. 4 weeks after completion of FHV challenge in the masseter muscle with 1 ml of mouse brain suspension containing 10^4 MIC LD_{50} of RABV BD06. The cats were observed daily for 90 days, and euthanized within 2–3 weeks once any rabies symptoms occurred. The animal brain tissues were examined for RABV by direct immunofluorescence assay (DFA) test as described elsewhere [25].

2.10. Statistical analyses.

Two-way analysis of variance (ANOVA) was used to compare statistical significance in neutralizing antibody response: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. One-way ANOVA was used to determine statistical significance in virus titer: not significant (ns); ns, $P > 0.05$; **, $P < 0.01$. Data are shown as the means ± standard deviations (SDs).

3. Results

3.1. Construction and characterization of recombinant virus FHV-ΔgI/E-eGFP and FHV-RVG

We applied a "positive screening" and a "negative screening" methodology in the recombinant virus recovery using the eGFP as a positive tag. In Fig. 2A Lane 4, the PCR using primers ΔIE-F and ΔIE-R identified a clear band of ~2.6 kb after electrophoresis, consistent with the expected size for eGFP deletion and replacement with the G from RABV BD06. The FHV-RVG was pure and stable without loss of the insert G gene after 9 passages in cells, as shown by PCR in Fig. 2A Lanes 4–8. By confocal microscopy, eGFP expression was detected in the FHV-ΔgI/E- eGFP infected F81 cells, while rabies virus G was expressed in the FHV-RVG infected cells (Fig. 2B).

In the virus growth dynamics, both multi-step (Fig. 2C) and single-step (Fig. 2D) curves demonstrated that recombinant viruses grew slower than the parental FHV-Z, and needed more time (~15 h at an MOI of 0.01, and ~10 h at an MOI of 1) to reach complete CPE in the infected wells. In the multiple-step (Fig. 2E), the highest titer for the recombinant viruses were ~$10^{7.0}$$TCID_{50}$/ml, which was significantly lower than FHV-Z at ~$10^{7.5}$$TCID_{50}$/ml ($P < 0.01$). However, no difference in growth was observed in the single step (Fig. 2F), all viruses reached a titer of ~$10^{7.5}$$TCID_{50}$/ml ($P > 0.05$), suggesting deletion of gI/E and replacement with either eGFP or BD06 G did not affect virus growth.

3.2. FHV-RVG vaccination induced neutralizing antibodies against herpesvirus and rabies virus in cats

To investigate if pre-existing FHV-1 antibodies in cats compromises FHV-RVG vaccination efficacy, one cohort (Group 1) of cats were administered 1 ml of FHV-ΔgI/E-eGFP at $10^{6.5}$ $TCID_{50}$ i.n. on day 0 to mimic a natural infection, and were boosted with FHV-RVG via the same route and dosage on day 21. Blood samples were collected on days 14, 30, 60, 120 and 180 after the last vaccination. In Fig. 3A, all animals in Group 1 seroconverted for antibodies against FHV-Z, and the viral neutralizing antibodies reached 1:50 on day 14, maintaining neutralization at 1:25 180 days post-vaccination. Rabies virus neutralizing antibodies peaked on day 60 in Group 1 at 13.81 ± 8.29 IU/ml, and maintained well above the arbitrary protective threshold of 0.5 IU/ml by termination of the experiment (Fig. 3B). Although the maximal neutralizing antibody response against rabies was not as robust in Group 1 (13.81 ± 8.29 IU/ml) compared to Group 2(24.5 ± 10.23 IU/ml), which only received the FHV-RVG, Group 1 displayed greater neutralization against rabies than Group 4 (7.09 ± 3.00 IU/ml), which received the licensed Rabies vaccine. Therefore, pre-existing antibodies against FHV-1 in cats did not interfere with FHV-RVG immunization against rabies.

We also compared FHV-RVG with a commercial vaccine for FHV protection, parvovirus-calicivirus-herpesvirus tri-combined vaccine (Fel-O-Vax® PCT). We did not have a monovalent vaccine for FHV in China. In Group 3, the viral neutralizing antibodies against FHV were <1:10. The Group 2 cats had a titer between 1:10 and 1:20, and the Group 1 cats presented a titer between 1:20 and 1:50 (Fig. 3A, C). Therefore, one dose of our experimental vaccine FHV-RVG generated higher viral neutralizing antibodies against FHV than two doses of the commercial vaccine ($P < 0.01$ on day 30 and $P < 0.05$ on day 60).

To compare FHV-RVG with a rabies vaccine (Nobivac, Intervet, The Netherlands), we tested the commercial vaccine in Group 4 cats. In Fig. 3B and D, rabies virus neutralizing antibodies were lower in Group 4 than in Groups 1 and 2, especially than group 2 on day 30 ($P < 0.0001$), suggesting our experimental vaccines induced sufficient protective antibodies after immunization.

3.3. FHV-RVG protected cats from challenges by both herpesvirus and rabies virus

Cats in Groups 1, 2, 3 and 5 were challenged i.n. with FHV-Z at $10^{7.0}$ $TCID_{50}$ after completion of the vaccination studies (see Fig. 2 for timeline). All 5 unvaccinated cats in control Group 5 developed severe clinical symptoms 7–14 days after challenge, including coughing, sneezing with thick mucus, and secretions in the eyes with a mean clinical score of 8 (see Supplementary Table 2 for clinical scores). The vaccinated cats in Groups 1 and 2 looked healthy, and did not develop any clinical signs. Nonetheless, the cats in Group 3 vaccinated by a commercial vaccine developed mild clinical symptoms 7–14 days after FHV-Z challenge, including infrequent light coughing and sneezing, with a mean clinical score of 2 (Fig. 4A).

After completion of the FHV challenge, we further challenged the cats (Groups 1 and 2) using RABV BD06, along with Groups 4 and 6. All 5 cats in control Group 6 died of rabies with obvious clinical signs 8–15 days post-challenge. The brain tissues from the succumbed animals were confirmed rabies positive by direct fluorescent assay following a standard protocol [25]. The cats in Groups 1, 2, and 4 survived the challenge and looked healthy throughout the observation period of 3 months (Fig. 4B).

4. Discussion

In contrast to more than ten licensed dog rabies vaccines, there is only one exported recombinant canary poxvirus-vectored rabies vaccine for cats in China. Although the dog rabies vaccines could be effective and applicable for cat vaccination, the "off-label" use

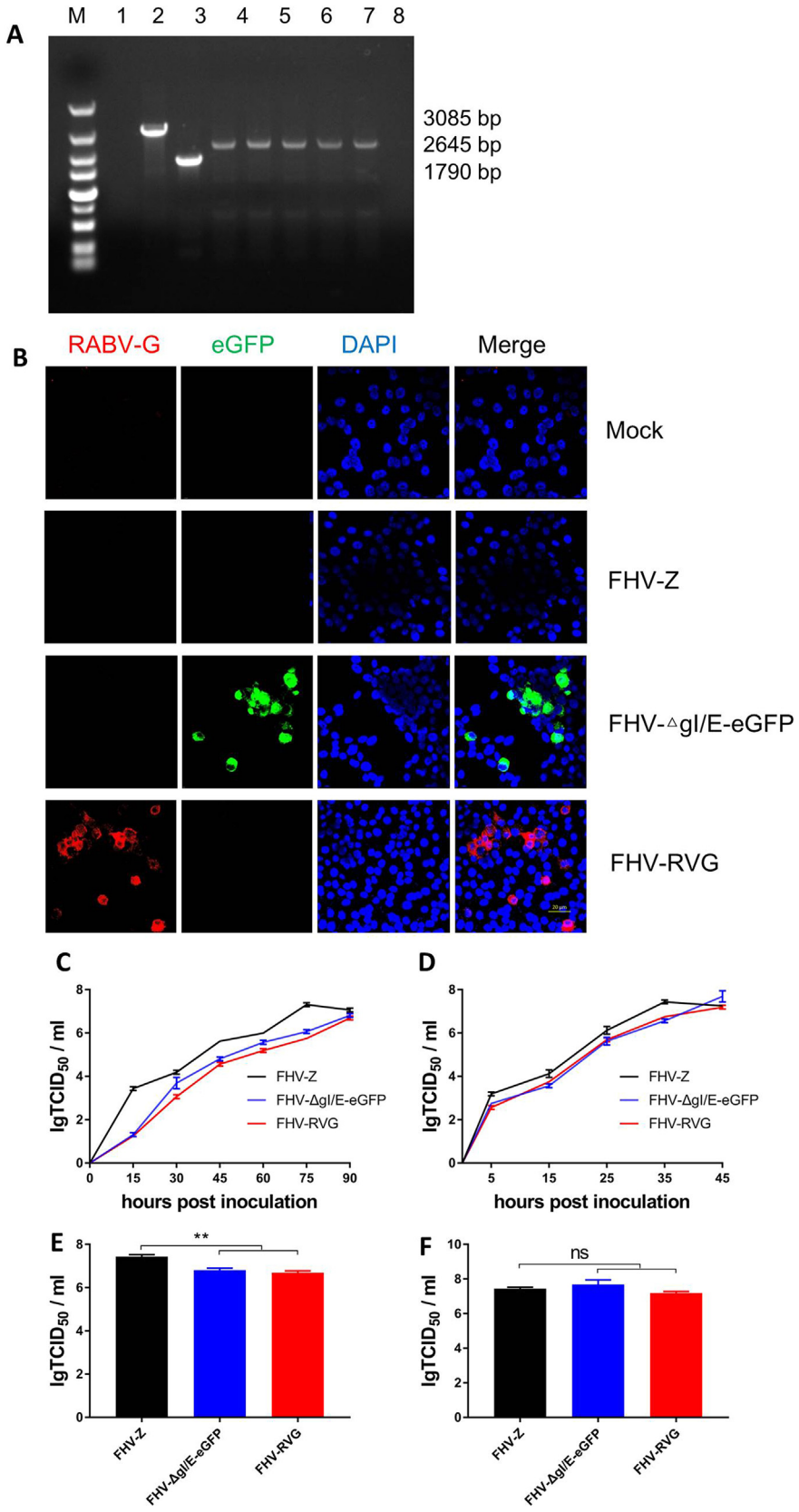

Fig. 2. Characterization of FHV-ΔgI/E- eGFP and FHV-RVG. A: presence of gI/E fragment in the parenteral FHV by PCR (Lane 2, 3085 bp), deletion and replacement of the counterpart by eGFP in the FHV-ΔgI/E- eGFP (Lane 3, 1790 bp), and substitution of eGFP by BD06 G in the FHV-RVG of passages 1, 3, 5, 7 and 9, respectively (Lanes 4–8, 2645 bp). M: DNA Marker 5000. Lane1: blank control. B: eGFP expression in the FHV-ΔgI/E-eGFP infected cells and rabies virus G expression in the FHV-RVG infected F81 cells by incubation with mouse-anti-G Mab (6B12, made in the Veterinary Research Institute), followed by staining with 594-labeled goat-anti-mouse IgG Ab. The eGFP and BD06-G expression were observed by laser confocal microscopy. C: multiple-step virus growth curve (MOI of 0.01). D: one-step virus growth curve (MOI of 1.0). E: highest virus titers at MOI of 0.01. F: highest virus titers at MOI of 1.0. One-way ANOVA was used to determine statistical significance in virus titer: not significant (ns); ns, $P > 0.05$; **, $P < 0.01$. Data are shown as the means ± SDs.

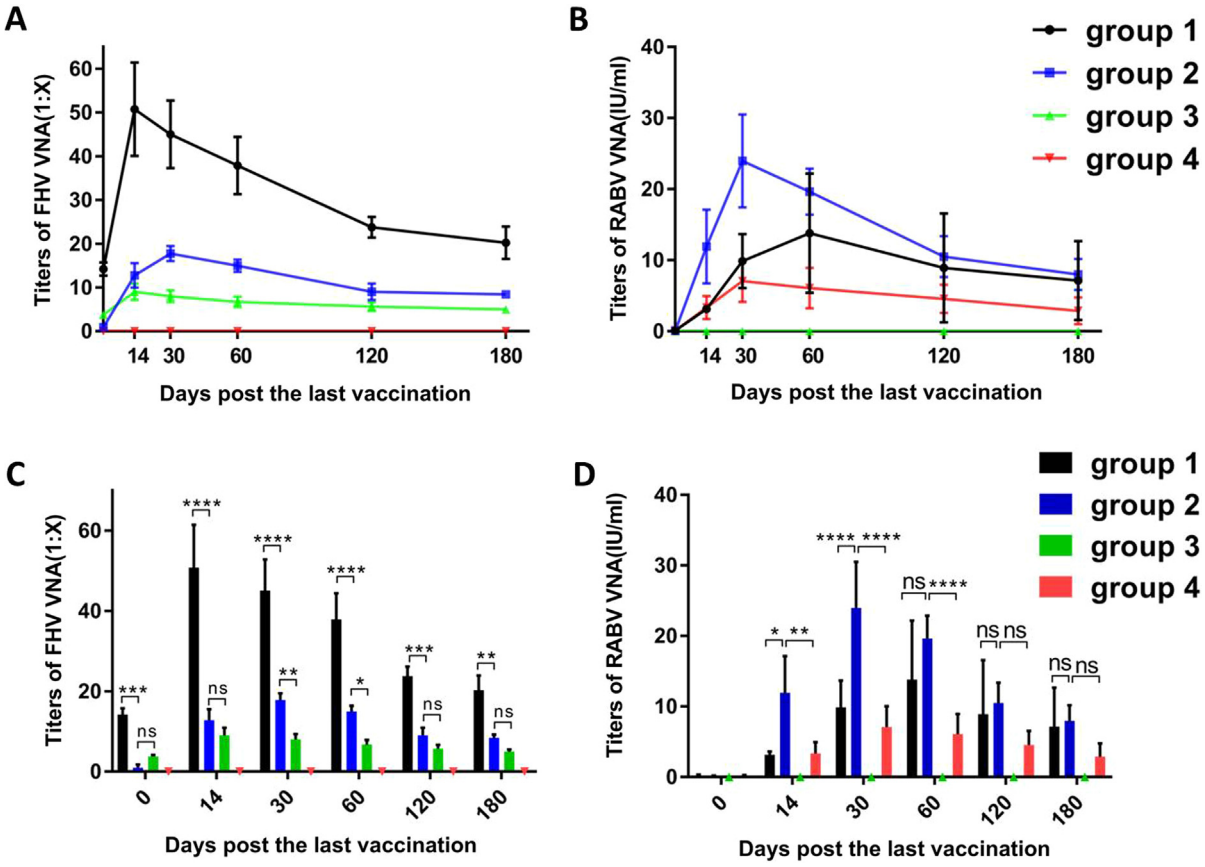

Fig. 3. Viral neutralizing antibodies against FHV and RABV after vaccinations. Sera in Groups 1–4 were collected on six time points: 0 dpi, 14 dpi, 30 dpi, 60 dpi, 120 dpi and 180 dpi after the last vaccination. A and C: viral neutralizing antibodies against FHV, the reciprocal of the highest serum dilution inhibiting CPE formation in 50% wells infected with 100 $TCID_{50}$ FHV-Z. B and D: viral neutralizing antibodies against RABV by fluorescent antibody virus neutralization test (FAVN). Two-way analysis of variance (ANOVA) was used to compare statistical significance in neutralizing antibody response: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. Data are shown as the means ± SDs.

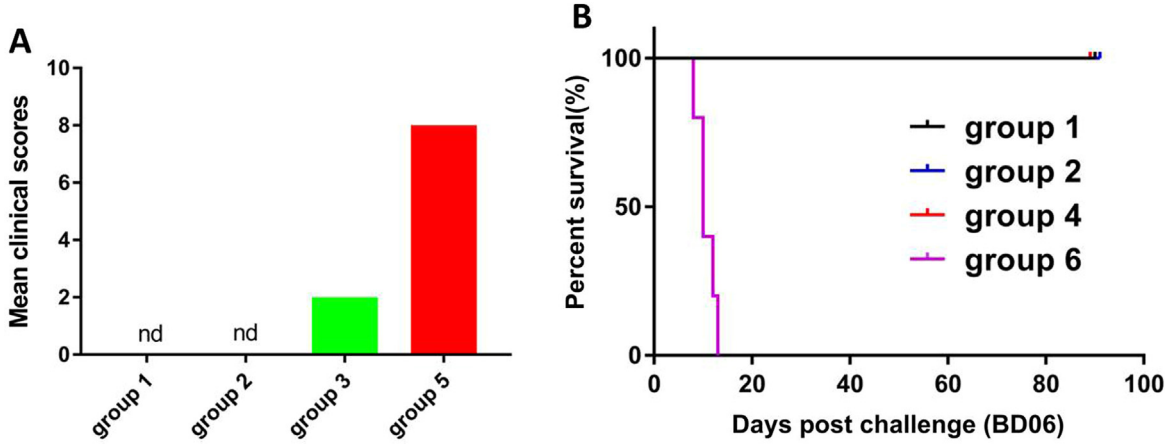

Fig. 4. Animal symptoms and survival after challenges by FHV and RABV. Animals in Groups 1, 2, 3 and 5 were challenged with the FHV-Z on 180 dpi after the last vaccination. The symptoms were recorded daily for 14 days and mean clinical scores per group were compared. After completion of FHV-Z challenge, animals in Groups 1, 2, 4 and 6 were challenged again with RABV BD-06. A: Mean clinical scores per group after FHV-Z challenge. B: Animal survivorship after challenge by RABV BD-06. nd: not detected.

without clinical trials and reapplication for licensing will be against vaccine regulations. To develop a cat virus vectored rabies vaccine, we focused on the FHV-Z which was isolated 2 years ago. The FHV-Z causes feline rhinotracheitis, and is one of the most serious respiratory diseases in cats. The virus is shed in saliva, eyes and nasal secretions, and is transmitted through direct contact. Previously, we studied different routes of inoculation for FHV-Z infection in cats, including i.n, oral, s.c, and i.m administrations, and found the i.n. route was the optimal for infection [unpublished data]. The cats contracted rhinotracheitis and recovered after clinical signs within 15 days post i.n. inoculation. Therefore, we applied the i.n. administration in the FHV-RVG studies.

To test if our virus isolate FHV-Z was a suitable vector for accepting the RABV G protein, we introduced an eGFP tag into the viral genome to replace the gI/gE region. The gI/gE- deleted FHV-1 became attenuated in kittens (unpublished data) with a slightly slower growth dynamics in cells, and induced a robust immune response against the parental virus challenge after intranasal delivery. Our FHV-RVG was recovered and purified through two cycles of screening: firstly, the eGFP in an expression cassette was introduced to the FHV-1 genome to replace the gI/E region for generation of FHV-ΔgI/E-eGFP virus. Then the eGFP was replaced with the G protein from RABV strain BD06 to create the dual vaccine candidate FHV-RVG. The recombinant virus can reach a final titer as high as the FHV-1. *In vivo*, the FHV-RVG stimulated high VNAs to rabies in cats after i.n. vaccinations, and protected the cats from challenges by both the virulent herpesvirus and the street RABV. The VNAs to rabies in vaccinated cats were monitored for 6 months, and preexisting antibody to FHV did not significantly affect immunization against rabies by FHV-RVG.

The RABV G gene was derived from a street strain BD06, which was demonstrated to elicit a stronger humoral response than the fixed strains when expressed by type 5 human adenovirus [18]. Since FHV-RVG was delivered live, FHV-1 alone infects cat sensory ganglion neurons [26], and street RABV is high neurotrophic [27]. We had concerns regarding the safety/neurotoxicity of FHV-RVG in cat vaccination. Fortunately, neither rhinotracheitis nor rabies encephalitis occurred in the vaccinated cats. However, we still do not know at what infection stage or in which infection site the FHV-RVG was cleared, or whether the FHV-RVG became persistent in neurons after vaccination. Further studies are needed to address those speculations. Preexisting antibodies to FHV in cats did not significantly interfere FHV-RVG vaccination and protection, although the VNAs to RABV did decrease under that scenario (Fig. 3).

In conclusion, we demonstrated the FHV-RVG served as a dual vaccine for protection against both felid herpesvirus and rabies infections in cats. Though the cat is not a reservoir in the rabies transmission cycle, China CDC reports about 5% of human rabies death are due to cat bites or scratches [1,2]. Rabies in cats is usually a spillover event originating from rabid dogs and reported cat rabies cases were acquired from contacts with wildlife or bats where dog rabies has been well controlled, or eliminated [28–31]. In the U.S where canine rabies has been eliminated since 1997, cat rabies traces back to contact with raccoons, skunks or bats [32], substantiating the purported explanation of rabies as a spillover event in felines. Further support of this hypothesis is found in Brazil, where bat-related cat rabies became well recognized after successful control of dog rabies [28].

Although mass dog vaccination is the most efficient and cost-effective approach to prevent transmission of rabies to humans [33–36], rabies vaccination in cats can be a short-term preventive measure until canine rabies is eliminated in China. Elimination of cat-mediated human rabies transmission is gaining attention by the international rabies community. However, cat vaccination against rabies is not mandatory in China despite the large population of cats estimated at 10–20 million. Our FHV-RVG may have a niche in providing dual protection to two high consequence diseases in felines (FHV and Rabies).

5. Disclaimer

Use of trade names and commercial sources are for identification only and do 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 or the Centers for Disease Control and Prevention.

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Interest conflict declaration

We claim no conflict of interest.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.03.008.

References

[1] Qi L, Su K, Shen T, Tang W, Xiao B, Long J, et al. Epidemiological characteristics and post-exposure prophylaxis of human rabies in Chongqing, China, 2007–2016. BMC Infect Dis 2018;18:6. https://doi.org/10.1186/s12879-017-2830-x.
[2] Song M, Tang Q, Rayner S, Tao XY, Li H, Guo ZY, et al. Human rabies surveillance and control in China, 2005–2012. BMC Infect Dis 2014;14:212. https://doi.org/10.1186/1471-2334-14-212.
[3] Zhang S, Liu Y, Hou Y, Zhao J, Zhang F, Wang Y, et al. Epidemic and maintenance of rabies in chinese ferret badgers (Melogale moschata) indicated by epidemiology and the molecular signatures of rabies viruses. Virol. Sin. 2013;28(3):146–51. https://doi.org/10.1007/s12250-013-3316-7.
[4] Zhao J, Liu Y, Zhang S, Zhang F, Wang Y, Mi L, et al. Molecular characterization of three ferret badger (Melogale moschata) rabies virus isolates from Jiangxi province, China. Arch Virol 2014;159(8):2059–67. https://doi.org/10.1007/s00705-014-2044-0.
[5] Zhao J, Zhang S, Liu Y, Zhang F, Hu R. Complete Genome Sequence of a Rabies Virus Isolate from a Ferret Badger (Melogale moschata) in Jiangxi. China. Genome announcements 2013;1. https://doi.org/10.1128/genomeA.00192-13.
[6] Liu Y, Zhang HP, Zhang SF, Wang JX, Zhou HN, Zhang F, et al. Rabies outbreaks and vaccination in domestic camels and cattle in northwest china. PLoS NeglTrop Dis 2016;10:e0004890. https://doi.org/10.1371/journal.pntd.0004890.
[7] Liu Y, Zhang S, Zhao J, Zhang F, Li N, Lian H, et al. Fox- and raccoon-dog-associated rabies outbreaks in northern China. Virol. Sin. 2014;29(5):308–10. https://doi.org/10.1007/s12250-014-3484-0.
[8] Liu Y, Zhang S, Zhao J, Zhang F, Hu R. Isolation of Irkut virus from a Murina leucogaster bat in China. PLoS NeglTrop Dis 2013;7:e2097. https://doi.org/10.1371/journal.pntd.0002097.
[9] Liu Y, Chen Q, Zhang F, Zhang S, Li N, Lian H, et al. Evaluation of rabies biologics against irkut virus isolated in china. J. Clin. Microbiol. 2013;51(11):3499–504. https://doi.org/10.1128/JCM.01565-13.
[10] Kruger JM, Sussman MD, Maes RK. Glycoproteins gI and gE of feline herpesvirus-1 are virulence genes: safety and efficacy of a gI-gE deletion mutant in the natural host. Virology 1996;220:299–308.
[11] Sussman MD, Maes RK, Kruger JM, Spatz SJ, Venta PJ. A Feline Herpesvirus-1 recombinant with a deletion in the genes for glycoproteins gI and gE Is effective as a vaccine for feline rhinotracheitis. Virology 1995;214(1):12–20. https://doi.org/10.1006/viro.1995.9959.
[12] Wunner WH, Larson JK, Dietzschold B, Smith CL. The molecular biology of rabies viruses. Rev Infect Dis 1988;10(4):S771–84.
[13] Benmansour A, Leblois H, Coulon P, Tuffereau C, Gaudin Y, Flamand A, et al. Antigenicity of rabies virus glycoprotein. J Virol 1991;65:4198–203.
[14] Amann R, Rohde J, Wulle U, Conlee D, Raue R, Martinon O, et al. A new rabies vaccine based on a recombinant orf virus (parapoxvirus) expressing the rabies virus glycoprotein. J. Virol. 2013;87(3):1618–30. https://doi.org/10.1128/JVI.02470-12.
[15] Astray RM, Jorge SA, Pereira CA. Rabies vaccine development by expression of recombinant viral glycoprotein. Arch Virol 2017;162(2):323–32. https://doi.org/10.1007/s00705-016-3128-9.
[16] Huang Y, Chen Z, Huang J, Fu Z, He B. Parainfluenza virus 5 expressing the G protein of rabies virus protects mice after rabies virus infection. J. Virol. 2015;89(6):3427–9. https://doi.org/10.1128/JVI.03656-14.
[17] Li Z, Wang J, Yuan D, Wang S, Sun J, Yi B, et al. A recombinant canine distemper virus expressing a modified rabies virus glycoprotein induces immune responses in mice. Virus Genes 2015;50(3):434–41. https://doi.org/10.1007/s11262-015-1169-x.
[18] Wang S, Sun C, Zhang S, Zhang X, Liu Y, Wang Y, et al. Glycoprotein from street rabies virus BD06 induces early and robust immune responses when expressed from a non-replicative adenovirus recombinant. Arch Virol 2015;160(9):2315–23. https://doi.org/10.1007/s00705-015-2512-1.
[19] Fehlner-Gardiner C, Rudd R, Donovan D, Slate D, Kempf L, Badcock J. Comparing ONRAB(R) AND RABORAL V-RG(R) oral rabies vaccine field performance in raccoons and striped skunks, New Brunswick, Canada, and

Maine, USA. J Wildl Dis 2012;48:157–67. https://doi.org/10.7589/0090-3558-48.1.157.
[20] Gilbert AT, Johnson SR, Nelson KM, Chipman RB, VerCauteren KC, Algeo TP, et al. Field trials of ontario rabies vaccine bait in the Northeastern USA, 2012-14. J Wild Dis 2018. https://doi.org/10.7589/2017-09-242.
[21] Mainguy J, Rees EE, Canac-Marquis P, Belanger D, Fehlner-Gardiner C, Seguin G, et al. Oral rabies vaccination of raccoons and striped skunks with ONRAB(R) baits: multiple factors influence field immunogenicity. J Wild Dis 2012;48:979–90. https://doi.org/10.7589/2011-12-316.
[22] Shwiff S, Aenishaenslin C, Ludwig A, Berthiaume P, Bigras-Poulin M, Kirkpatrick K, et al. Bioeconomic modelling of raccoon rabies spread management impacts in Quebec Canada. Transboundary Emerg Dis 2013;60:330–7. https://doi.org/10.1111/j.1865-1682.2012.01351.x.
[23] Cliquet F, Aubert M, Sagne L. Development of a fluorescent antibody virus neutralisation test (FAVN test) for the quantitation of rabies-neutralising antibody. J Immunol Methods 1998;212:79–87.
[24] Spatz SJ, Rota PA, Maes RK. Identification of the feline herpesvirus type 1 (FHV-1) genes encoding glycoproteins G, D, I and E: expression of FHV-1 glycoprotein D in vaccinia and raccoon poxviruses. J General Virol 1994;75 (Pt 6):1235–44. https://doi.org/10.1099/0022-1317-75-6-1235.
[25] Woldehiwet Z. Clinical laboratory advances in the detection of rabies virus. Clinica Chimica Acta; Int J Clin Chem 2005;351:49–63. https://doi.org/10.1016/j.cccn.2004.09.018.
[26] Card JP, Enquist LW, Miller AD, Yates BJ. Differential tropism of pseudorabies virus for sensory neurons in the cat. J Neurovirol 1997;3:49–61.
[27] Fooks AR, Cliquet F, Finke S, Freuling C, Hemachudha T, Mani RS, et al. Rabies. Nat Rev Dis Primers 2017;3:17091. https://doi.org/10.1038/nrdp.2017.91.
[28] Castilho JG, de Souza DN, Oliveira RN, Carnieli Jr P, Batista H, Pereira PMC, et al. The epidemiological importance of bats in the transmission of rabies to dogs and cats in the State of Sao Paulo, Brazil, between 2005 and 2014. Zoonoses Public Health 2017;64:423–30. https://doi.org/10.1111/zph.12320.
[29] De Lucca T, Rodrigues RC, Castagna C, Presotto D, De Nadai DV, Fagre A, et al. Assessing the rabies control and surveillance systems in Brazil: an experience of measures toward bats after the halt of massive vaccination of dogs and cats in Campinas Sao Paulo. Prevent Veterinary Med 2013;111:126–33. https://doi.org/10.1016/j.prevetmed.2013.03.007.
[30] Dyer JL, Yager P, Orciari L, Greenberg L, Wallace R, Hanlon CA, et al. Rabies surveillance in the United States during 2013. J Am Vet Med Assoc 2014;245:1111–23. https://doi.org/10.2460/javma.245.10.1111.
[31] Morikawa VM, Ribeiro J, Biondo AW, Fellini A, Bier D, Molento MB. Cat infected by a variant of bat rabies virus in a 29-year disease-free urban area of southern Brazil. Rev Soc Bras Med Trop 2012;45:255–6.
[32] Singh AJ, Chipman RB, de Fijter S, Gary R, Haskell MG, Kirby J, et al. Translocation of a stray cat infected with rabies from north carolina to a terrestrial rabies-free county in Ohio, 2017. MMWR Morb. Mortal. Wkly. Rep. 2018;67(42):1174–7. https://doi.org/10.15585/mmwr.mm6742a2.
[33] Taylor LH, Hampson K, Fahrion A, Abela-Ridder B, Nel LH. Difficulties in estimating the human burden of canine rabies. Acta Trop 2017;165:133–40. https://doi.org/10.1016/j.actatropica.2015.12.007.
[34] Meslin F-X, Briggs DJ. Eliminating canine rabies, the principal source of human infection: what will it take? Antiviral Res 2013;98(2):291–6. https://doi.org/10.1016/j.antiviral.2013.03.011.
[35] Shwiff S, Hampson K, Anderson A. Potential economic benefits of eliminating canine rabies. Antiviral Res 2013;98(2):352–6. https://doi.org/10.1016/j.antiviral.2013.03.004.
[36] Hampson K, Coudeville L, Lembo T, Sambo M, Kieffer A, Attlan M, et al. Estimating the global burden of endemic canine rabies. PLoS NeglTrop Dis 2015;9:e0003709. https://doi.org/10.1371/journal.pntd.0003709.