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RESEARCH ARTICLE

Evaluation of safety and immunogenicity of a genetically modified rabies virus for use as an oral vaccine in several non-target species

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Highlights

- The G_{333E} mutation significantly reduces the virulence of the ERA strain.
- Oral rabies vaccine $rERAG_{333E}$ is safe in non-target species such as rhesus monkeys, foxes, raccoon dogs, piglets, goats, and sheep.
- The $rERAG_{333E}$ is immunogenic in rhesus monkeys, foxes, raccoon dogs, and piglets.

Abstract

Oral immunization is an alternative or supplementary approach that can significantly improve dog vaccination coverage, especially for free-roaming dogs. Safe and effective oral rabies vaccines for dogs are still being sought. In our previous studies, we generated a genetically modified rabies virus (RABV) ERA strain, $rERAG_{333E}$, containing a mutation from arginine (Arg, R) to glutamic acid (Glu, E) at residue 333 of the G protein (G_{333E}). Our previous results demonstrated that $rERAG_{333E}$ was safe for adult mice and dogs, and oral vaccination with $rERAG_{333E}$ induced a strong and long-lasting protective immune response in dogs. Here, we further investigated the safety and immunogenicity of $rERAG_{333E}$ in non-target species, including suckling mice, rhesus monkeys, foxes, raccoon dogs, piglets, goats, and sheep. Suckling mice studies demonstrated that the G_{333E} mutation significantly reduced the virulence of the ERA strain. All of the suckling mice aged 10 days and above survived and showed no apparent signs of disease after intracerebral inoculation with $rERAG_{333E}$. Animal studies demonstrated that $rERAG_{333E}$ was safe in rhesus monkeys, foxes, raccoon dogs, piglets, goats, and sheep. None of those animals inoculated orally with 10 times the intended field dose of $rERAG_{333E}$ showed abnormal clinical signs before and after the booster immunization with Rabvac 3, an inactivated rabies vaccine. Meanwhile, oral inoculation with $rERAG_{333E}$ induced strong neutralizing antibody (NA) responses to RABV in rhesus monkeys, foxes, raccoon dogs, and piglets. These results demonstrated that $rERAG_{333E}$ has the potential to serve as a safe oral rabies vaccine for dogs.

Keywords: rabies, genetically modified rabies virus, oral vaccine.

1. Introduction

Rabies is a widespread zoonotic infectious disease caused by the rabies virus (RABV), which is a member of the genus *Lyssavirus* of the family *Rhabdoviridae* (Jackson and Wunner 2007). RABV infects a wide range of mammals and causes acute, progressive, and fatal encephalitis with an almost 100% fatality rate (Osinubi *et al.* 2009). Rabies is still a serious public health problem worldwide, especially in the developing world. The World Health Organization (WHO) reports that rabies is responsible for approximately 55,000 human deaths per year, worldwide (Martinez 2000; WHO 2005), and approximately 95% of human deaths occur in Asia and Africa (Ge *et al.* 2011). Dogs, especially free-roaming, play an important role in rabies transmission in

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developing countries, including China (Shuai *et al.* 2015). According to the WHO, almost all global human deaths attributed to rabies are caused by dog bites (WHO 2007). However, in developed countries, human rabies cases primarily originate from infected wild animals. Therefore, the most efficient strategy for preventing human rabies is to eliminate the disease in dogs, especially free-roaming dogs, and wild animals through a vaccination program. In China, all rabies vaccines are inactivated vaccines and require to be administered by intramuscular injection. Although veterinary authorities require all pet dogs to be vaccinated against rabies, the overall vaccination coverage is relatively low in the entire dog population due to the high cost of vaccination and the difficulty of catching free-roaming dogs, especially in rural areas. Oral immunization is one of the most practical approaches to prevent the spread of RABV in free-roaming dogs and wild animals (Faber *et al.* 2005; Winkler and Bogel 1992). Over the past few decades, oral rabies vaccines have played a crucial role in the control of rabies in wild animals in North America and Europe (Muller *et al.* 2015; Rosatte 2011).

In our previous studies, we generated a genetically modified RABV strain, rERAG$_{333E}$, containing a mutation from arginine (Arg, R) to glutamic acid (Glu, E) at residue 333 of the G protein (G$_{333E}$) in the genome background of the ERA strain using reverse genetics (Shuai *et al.* 2015). Our results demonstrated that the mutation decreased the virulence of the ERA strain in adult mice, and all of the adult mice survived and showed no apparent signs of disease after intracerebral inoculation with rERAG$_{333E}$. Meanwhile, the use of rERAG$_{333E}$ as an oral rabies vaccine (ORV) was deemed safe and immunogenic in dogs. Oral vaccination, at a dose of 1 mL 10^8 focus-forming unit (FFU) of rERAG$_{333E}$, induced a significant long-lasting RABV neutralizing antibody (NA) response in dogs, achieving a 100% RABV NA seroconversion. The ratios of RABV NA titers above 0.5 international unit (IU) mL^{-1} were 9/10, 7/9, 3/4, and 2/4 at 4, 52, 104, and 156 wk after a single vaccination dose. Moreover, a second vaccine dose at 1 year later induced a significant RABV NA booster response. These results indicated that rERAG$_{333E}$ was a promising oral rabies vaccine candidate in dogs, especially in stray dogs.

Dogs, especially stray dogs, are often in close association with non-target species such as humans, domestic animals, and wild animals, which may be exposed to oral vaccines during oral vaccination campaigns. Therefore, the safety of the oral rabies vaccines in non-target species must be evaluated. In this study, we further evaluated the safety and immunogenicity of rERAG$_{333E}$ in non-target species, including suckling mice, nonhuman primates, sheep, goats, pigs, foxes, and raccoon dogs.

2. Materials and methods

2.1. Laboratory facility

The experiments involving rhesus monkeys and suckling mice were conducted in the biosafety level 3 facilities and other animals were conducted in the biosafety level 2 facilities at Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS). These facilities have obtained approval for conducting such experiments from both the Ministry of Agriculture and Rural Affairs of the People's Republic of China and the Animal Experimentation and Laboratory Animal Welfare Committee of HVRI.

2.2. Viruses and cells

Baby hamster kidney (BHK-21) cells were grown in Dulbecco's Modified Eagle's MEM (DMEM) supplemented with 5% FBS. RABV strain CVS-11 received from the China Veterinary Culture Collection was then propagated in the BHK-21 cells.

2.3. Vaccine

The rERAG$_{333E}$ vaccine is a genetically modified RABV strain containing a mutation from Arg to Glu at residue 333 of the G protein in the genome background of the ERA strain. In our previous studies, we generated virus strain rERAG$_{333E}$ using reverse genetics (Shuai *et al.* 2015).

2.4. Pathogenicity assessment

To assess the pathogenicity of the rERAG$_{333E}$ vaccine in suckling mice, litters of different (1–12-d-old) BALB/c suckling mice were inoculated intracerebrally with 30 μL PBS containing 10^7 FFU rERAG$_{333E}$. The mice were observed daily for signs of disease or death for 4 wk. Mice that showed definitive clinical signs of rabies, such as paralysis, tremors, and spasms, were euthanized by CO_2 intoxication. Survival rates obtained for the different age groups were compared.

2.5. Safety and oral immunogenicity study in rhesus monkeys

To investigate the safety and immunogenicity of rERAG$_{333E}$ in nonhuman primates, a group of seven 1-year-old rhesus monkeys were inoculated orally (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe, with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$. This dose was 10 times the intended field dose for dogs. To evaluate the possibility of vaccine virus excretion from the saliva of the rhesus monkeys, oral swabs were collected from all rhesus monkeys prior to inoculation and at different times post-inoculation for vaccine virus isolation. The swab was placed into 1 mL PBS containing 1,000 IU mL^{-1} penicillin and 1,000 mg mL^{-1} streptomycin and then stored at –80°C until the virus isolation test was performed. At 14 wk after vaccination, all rhesus monkeys were inoculated intramuscularly (in the quadriceps muscle) with a dose of Rabvac 3 (an inactivated rabies vaccine, Zoetis Inc., USA). All rhesus monkeys were bled from the vein of the front leg prior to vaccination and at different times after inoculation for serological assessment. After inoculation, the rhesus monkeys were observed daily for signs of disease for 18 wk. None of the rhesus monkeys used in this study had a record of prior vaccination for rabies.

2.6. Safety and oral immunogenicity study in piglets, goats, sheep, foxes, and raccoon dogs

Groups of four 1-month-old piglets, five 1-year-old goats, five 1-year-old sheep, five 3-mon-old foxes, and five 3-mon-old raccoon dogs were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe. At 10 or 14 wk

after vaccination, all piglets, goats, sheep, foxes, and raccoon dogs were inoculated intramuscularly in the quadriceps muscle with a dose of Rabvac 3. All piglets, goats, sheep, foxes, and raccoon dogs were bled from the vein of the neck or the front leg prior to vaccination and at different times post-vaccination for serological assessment. After inoculation, all animals were observed daily for signs of disease for 14 or 18 wk. All piglets, goats, sheep, foxes, and raccoon dogs used in this study had no record of prior vaccination for rabies.

2.7. Serological tests

Animal sera were tested for RABV neutralizing antibodies (NAs) using the rapid fluorescent focus inhibition test as described previously (Wang *et al.* 2012), and titers of RABV NAs were expressed in IU mL^{-1} with a WHO standard as a reference.

2.8. Viral isolation

Each oral swab samples from Rhesus monkeys were thawed and mixed, and then centrifuged at 8,000 r min^{-1} for 5 min. The supernatant was then filtered with a 0.45 μm syringe-driven filter and incubated for 1 h on 80% confluent BHK-21 cells in 24-well plates and cultured using DMEM Medium supplemented with 2% FBS. In addition, a PBS-only negative control was run in parallel. All samples were passed on BHK cells for five successive generations. The cell supernatants were collected for titration on day 4 post-inoculation. The viral titers were determined in BHK-21cells by immunofluorescence assay (IFA) and expressed as FFU mL^{-1} as described previously (Tao *et al.* 2010).

3. Results

3.1. The G_{333E} mutation significantly reduced the virulence of the ERA strain

Litters of different (1–12-d-old) BALB/c suckling mice were inoculated intracerebrally with 30 μL PBS containing 10^7 FFU $rERAG_{333E}$. Suckling mice aged 7 d and below died within 17 d after intracerebral inoculation with $rERAG_{333E}$ (Fig. 1). Suckling mice aged 8 and 9 d were partially killed post-inoculation (survival rates 62.5 and 71.4%, respectively). However, the survival rate of suckling mice aged 10 d and above was 100% post-inoculation, and all suckling mice aged 10 d and above showed no abnormal clinical signs post-inoculation. These results demonstrated that the G_{333E} mutation significantly reduced the virulence of the ERA strain, and the $rERAG_{333E}$ vaccine was safe for suckling mice aged 10 d and above.

3.2. $rERAG_{333E}$ is safe and immunogenic in rhesus monkeys

To assess the safety and immunogenicity of $rERAG_{333E}$ in nonhuman primates, a group of seven 1-year-old rhesus monkeys were inoculated orally with 1 mL PBS containing 10^9 FFU $rERAG_{333E}$ using the inoculation schedules detailed in the Materials and Methods section. At 2 wk post-inoculation with $rERAG_{333E}$, RABV NA was detected in the blood of rhesus monkeys (Fig. 2). The mean RABV NA titer was 2.47 IU mL^{-1} (0.5, 1.97, 1.97, 3.42, 1.5, 3.42, and 4.5 individually) at 2 wk post-inoculation. At 8 wk post-inoculation, RABV NA gradually declined to a mean titer of 1.25 IU mL^{-1} (0.66, 1.97, 0.66, 0.66, 0.87, 1.97, and 1.97 individually). At 14 wk post-inoculation, the mean RABV NA titer was 0.56 IU mL^{-1} (0.66, 0.87, 0.38, 0.38, 0.38, 0.87, and 0.38 individually). After receiving a dose of Rabvac 3 at 14 wk post-inoculation, the rhesus monkeys showed substantial re-boost responses. The mean RABV NA titer for the group increased to 38.13 IU mL^{-1} (53.3, 40.5, 17.8, 30.8, 40.5, 53.3, and 30.8 individually) at 1 wk after the second vaccine dose (Fig. 2).

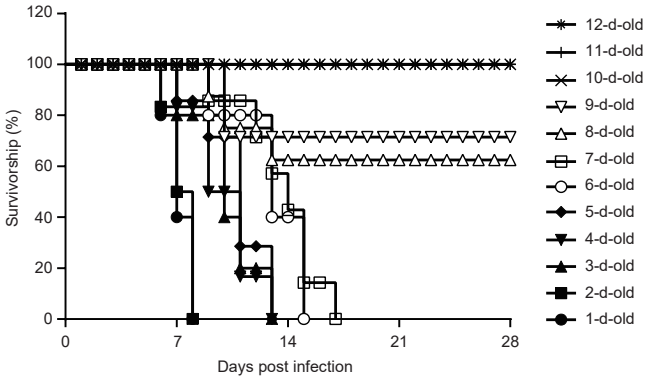


Fig. 1 Pathogenicity evaluation of $rERAG_{333E}$ in suckling mice. Litters of different (1–12-d-old) BALB/c suckling mice were inoculated intracerebrally with 30 μL PBS containing 10^7 FFU $rERAG_{333E}$. The mice were observed daily for signs of disease or death for 4 wk.

To evaluate the possibility of vaccine virus excretion from the saliva of the rhesus monkeys, the vaccine virus isolation and titration from oral swab samples were performed in BHK cells. At 2 h post-inoculation, the vaccine virus isolation of oral swab samples from all rhesus monkeys were positive (Table 1). Moreover, the oral swab samples in five of seven rhesus monkeys had detectable vaccine virus at 4 h post-inoculation. Attempts at vaccine virus isolation for oral swab samples obtained beyond 4 h post-inoculation were negative for all rhesus monkeys.

No abnormal clinical signs were observed during the course of the study, which suggests that the $rERAG_{333E}$ is safe for rhesus monkeys.

3.3. $rERAG_{333E}$ is safe and immunogenic in foxes and raccoon dogs

The safety and immunogenicity of the $rERAG_{333E}$ were further assessed in foxes and raccoon dogs using the inoculation schedules described in the Materials and Methods section. At 2 wk post-inoculation, RABV NA was detected in the blood of foxes inoculated with $rERAG_{333E}$ (Fig. 3). The mean RABV NA titer was 1.65 IU mL^{-1} (3.42, 3.42, 0.38, 0.66, and 0.38 individually) at 2 week post-inoculation and rose to 5.10 IU mL^{-1} (10.26, 5.92, 7.79, 0.38, and 1.14 individually) at 8 wk post-inoculation. A gradual decline in RABV NA to 3.14 IU mL^{-1} (7.79, 2.6, 4.5, 0.17, and 0.66 individually) was observed at 14 wk post-inoculation. After receiving a dose of Rabvac 3 at 14 wk post-inoculation, foxes showed substantial re-boost responses. The mean RABV NA titer of the group increased to 20.6 IU mL^{-1} (40.5, 10.26, 40.5, 10.26, and 1.5 individually) at 1 wk after the second vaccine dose (Fig. 3).

Meanwhile, RABV NA was detected in the blood of raccoon

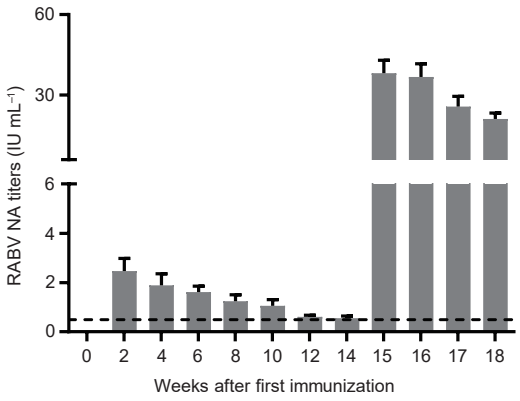


Fig. 2 Immunogenic efficacy of rERAG$_{333E}$ in rhesus monkeys. A group of seven 1-year-old rhesus monkeys were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe. At 14 wk post-inoculation, all rhesus monkeys were inoculated intramuscularly in the quadriceps muscle with a dose of Rabvac 3. The serum samples from rhesus monkeys were collected at indicated time-points to individually detect rabies virus (RABV) neutralizing antibody (NA). The titers of RABV NAs were determined by the FAVN test and expressed in IU mL^{-1} using the WHO standard.

dogs inoculated with rERAG$_{333E}$ at 2 weeks post-inoculation (Fig. 3). The mean RABV NA titer of the group was 0.46 IU mL^{-1} (0.29, 0.66, 0.38, 0.29, and 0.66 individually) at 2 wk post-inoculation and rose to 7.54 IU mL^{-1} (1.5, 3.42, 1.5, 0.5, and 30.77 individually) at 8 wk post-inoculation. A gradual decline in RABV NA to 2.4 IU mL^{-1} (1.14, 1.97, 0.87, 0.22, and 7.79 individually) was observed at 14 wk post-inoculation. After receiving a dose of Rabvac 3 at 14 wk post-inoculation, the raccoon dogs showed substantial re-boost responses. The mean RABV NA titer of the group increased to 28.61 IU mL^{-1} (40.5, 17.77, 13.5, 30.77, and 40.5 individually) at 1 wk after the second vaccine dose (Fig. 3).

No abnormal clinical signs were observed during the course of the study, which suggests that the rERAG$_{333E}$ is safe for foxes and raccoon dogs.

3.4. rERAG$_{333E}$ is safe in piglets, goats and sheep

To assess the safety and immunogenicity of the rERAG$_{333E}$ in piglets, goats, and sheep, groups of four 1-mon-old piglets, five 1-year-old goats, and five 1-year-old sheep were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ using the inoculation schedules detailed in the Materials and Methods section. At 1 wk post-inoculation, RABV NA was detected in the blood of piglets inoculated with rERAG$_{333E}$ (Fig. 4). The mean RABV NA titer of the group was 0.55 IU mL^{-1} (0.22, 0.66, 0.66, and 0.66 individually) at 1 wk post-inoculation. A gradual decline in RABV NA to 0.22 IU mL^{-1} (0.22, 0.22, 0.22, and 0.22 individually) was observed at 14 wk post-inoculation. After receiving a dose of Rabvac 3 at 14 wk post-inoculation, the piglets showed substantial re-boost responses. The mean RABV NA titer of the group increased to 8.05 IU mL^{-1} (17.77, 2.6, 5.92, and 5.92 individually) at 1 wk after the second vaccine dose (Fig. 4).

A low level of RABV NA was detected in the blood of sheep and goats (Fig. 5) inoculated with rERAG$_{333E}$ at 2 wk post-inoculation. Moreover, the RABV NA titers for groups of sheep and goats, inoculated with rERAG$_{333E}$, were always kept at a low level until 10 wk post-inoculation. After receiving a dose of Rabvac 3 at 10 wk post-inoculation, both sheep and goats showed substantial re-boost responses. The mean RABV NA titer in groups of sheep and goats increased to 5.01 IU mL^{-1} (4.5, 7.79, 5.92, 3.42, and 3.42 individually) and 1.1 IU mL^{-1} (1.14, 1.5, 0.87, 1.5, and 0.5 individually (Fig. 5)), respectively, at 1 wk after the second vaccine dose.

No abnormal clinical signs were observed during the course of the study, which suggested that the rERAG$_{333E}$ is safe for piglets, sheep, and goats.

4. Discussion

Dogs (especially free-roaming dogs) play an important role in rabies transmission in developing countries, especially in Africa and Asia (Tang *et al.* 2005). In order to control and eventually eliminate of dog-mediated rabies, recurrent mass dog vaccination campaigns must be conducted improve overall vaccination coverage, to at least 70% (Coleman and Dye 1996; Hampson *et al.* 2009). In developed countries, such efforts have been effective in controlling rabies in dogs. Oral immunization is an alternative or supplementary approach that can significantly improve the overall vaccination coverage in the entire dog population (Ben Youssef *et al.* 1998).

In our previous studies, we generated a genetically modified RABV strain, rERAG$_{333E}$, containing a mutation from Arg to Glu at residue 333 of the G protein in the genome background of the ERA strain using reverse genetics (Shuai *et al.* 2015). Our results demonstrated that the rERAG$_{333E}$, serve as a safe oral rabies vaccine, induced a long-lasting protective immune

Table 1 Evaluation of the vaccine virus excretion from the saliva in rhesus monkeys post-inoculation[1)]

Monkeys ID	Vaccine virus isolation and titration from oral swab samples at indicated time-points post-inoculation (log_{10}FFU mL^{-1})										
	0 h	2 h	4 h	6 h	1 d	2 d	3 d	4 d	5 d	6 d	7 d
1	–	3.48	1.30	–	–	–	–	–	–	–	–
2	–	3.30	1	–	–	–	–	–	–	–	–
3	–	2.70	–	–	–	–	–	–	–	–	–
4	–	3.70	2	–	–	–	–	–	–	–	–
5	–	3	–	–	–	–	–	–	–	–	–
6	–	3	1	–	–	–	–	–	–	–	–
7	–	3.70	1.85	–	–	–	–	–	–	–	–

[1)] Seven 1-year-old rhesus monkeys were inoculated orally with 1 mL 10^9 FFU of rERAG$_{333E}$. The oral swabs were collected from all rhesus monkeys for virus titration at indicated time-points post-inoculation. The viral titers were determined in BHK-21 cells by IFA and expressed as FFU mL^{-1}. –, no vaccine virus was detected from the oral swab samples.

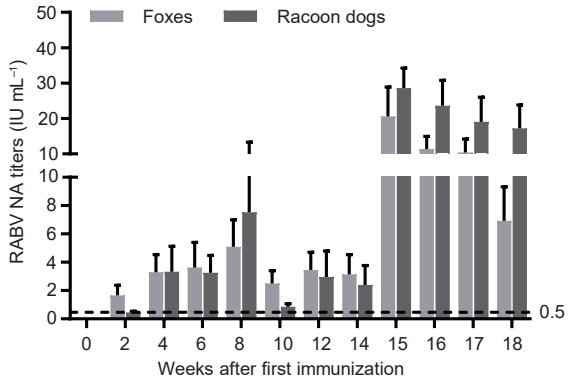


Fig. 3 Immunogenic efficacy of rERAG$_{333E}$ in foxes and raccoon dogs. A group of five 3-mon-old foxes and a group of five 3-mon-old raccoon dogs were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe. At 14 wk post-inoculation, all foxes and raccoon dogs were inoculated intramuscularly in the quadriceps muscle with a dose of Rabvac 3. The serum samples from foxes and raccoon dogs were collected at indicated time-points to individually detect rabies virus (RABV) neutralizing antibody (NA). The titers of RABV NAs were determined by the FAVN test and expressed in IU mL^{-1} using the WHO standard.

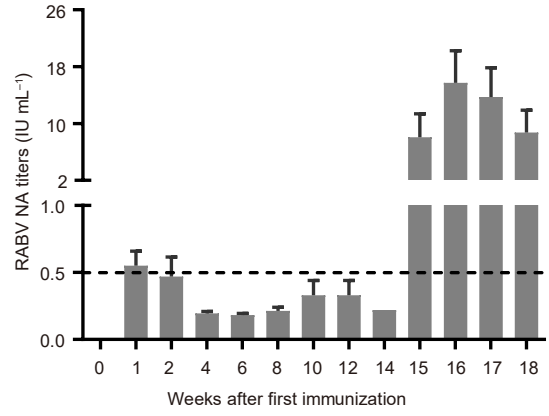


Fig. 4 Immunogenic efficacy of rERAG$_{333E}$ in piglets. A group of four 1-mon-old piglets were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe. At 14 wk post-inoculation, all piglets were inoculated intramuscularly in the quadriceps muscle with a dose of Rabvac 3. The serum samples from piglets were collected at indicated time-points to individually detect rabies virus (RABV) neutralizing antibody (NA). The titers of RABV NAs were determined by the FAVN test and expressed in IU mL^{-1} using the WHO standard.

response in adult mice and dogs. In single-dose vaccinated dogs, the mean RABV NA titer of dogs inoculated with 10^8 FFU of rERAG$_{333E}$ can still reach 1.25 IU mL^{-1} at 104 wk after oral inoculation. For two-dose vaccinated group, a group of dogs inoculated orally with 10^8 FFU of rERAG$_{333E}$ received the same dose of oral booster vaccination at 55 weeks after inoculation. The mean RABV VNA titer of 10^8 FFU vaccinated dogs was 1.54 IU mL^{-1} at 55 wk p.v. and soared to 6.30 IU mL^{-1} at 56 wk p.v. (1 wk post oral booster vaccination), and gradually declined to 1.61 IU mL^{-1} at 156 wk. These findings suggested

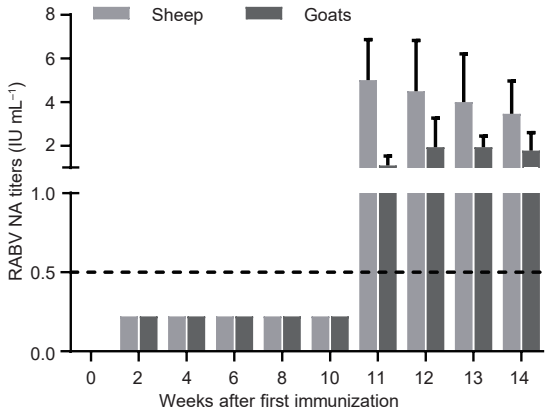


Fig. 5 Immunogenic efficacy of rERAG$_{333E}$ in sheep and goats. A group of five 1-year-old sheep and a group of five 1-year-old goats were inoculated orally with 1 mL PBS containing 10^9 FFU rERAG$_{333E}$ (drop by drop in the center of the tongue surface) using a 1.0 mL needle-less syringe. At 10 wk post-inoculation, all sheep and goats were inoculated intramuscularly in the quadriceps muscle with a dose of Rabvac 3. The serum samples from sheep and goats were collected at indicated time-points to individually detect rabies virus (RABV) neutralizing antibody (NA). The titers of RABV NAs were determined by the FAVN test and expressed in IU mL^{-1} using the WHO standard.

that rERAG$_{333E}$ is a promising oral rabies vaccine candidate in dogs. In the present study, we further investigated the safety and immunogenicity of the rERAG$_{333E}$ in non-target species, including suckling mice, rhesus monkeys, foxes, raccoon dogs, piglets, goats, and sheep.

Our previous results demonstrated that the mutation at residue 333 of the G protein decreased the virulence of the ERA strain in adult mice. Adult mice inoculated intracerebrally with rERA showed typical clinical signs of rabies and died within 14 d post-inoculation. However, all of adult mice inoculated intracerebrally with rERAG$_{333E}$ survived post-inoculation and showed no abnormal clinical signs. Here, the pathogenicity of rERAG$_{333E}$ was further investigated in different (1–12-d-old) BALB/c suckling mice that are not fully immunocompetent until approximately 6 wk of life. Our results showed that all of the suckling mice aged 10 d and above survived post-inoculation and showed no abnormal clinical signs. The results demonstrated that this mutation significantly reduced the virulence of the ERA strain in suckling mice, and the rERAG$_{333E}$ was apathogenic for suckling mice aged 10 d and above. In fact, SPBN GASGAS, a 3rd generation oral rabies vaccine recently authorized in the European Union for oral vaccination of foxes and raccoon dogs against rabies, is pathogenic to suckling mice aged 10 d and below, which inoculated intracerebrally with 10^3 FFU of SPBN GASGAS died within 10 d post-inoculation (Faber *et al.* 2009). In addition, SAG2, one of two oral rabies vaccines recommended by WHO, is also pathogenetic for 3-d-old suckling mice (Schumacher *et al.* 1993). So far, we have not found any evaluation report on the pathogenicity of SAG2 and SPBN GASGAS to suckling mice of other ages.

For oral rabies vaccine, one concern that requires investigation is whether the vaccine virus is safe for the target

and non-target animals (WHO 2007). Dogs (especially free-roaming dogs) are often in close association with humans, domestic animals, and wild animals. In this study, no abnormal clinical signs were observed in those non-target animals inoculated with $rERAG_{333E}$ during the course of the study and the vaccine virus could not be isolated from oral swab samples of rhesus monkeys after 6 h post-inoculation, which suggested that the vaccine virus was not excreted from rhesus monkey saliva. In a few previous studies, some humans or animals received post-exposure rabies vaccine, and clinical features of rabies developed at a shorter time compared to humans or animals that were exposed to the circulating rabies virus but did not receive a vaccine. This phenomenon is called the "early death" (Prabhakar and Nathanson 1981; Sadeghi *et al.* 2015; Willoughby 2009). Our results showed that no abnormal clinical signs were observed in non-target animals, inoculated orally with $rERAG_{333E}$, after receiving a dose of Rabvac 3 at 10 or 14 wk post-inoculation, which suggested that the vaccine virus, $rERAG_{333E}$, does not induce a persistent infection in these animals. These results confirmed that $rERAG_{333E}$ was safe for rhesus monkeys, sheep, goats, piglets, foxes, and raccoon dogs.

Meanwhile, animal studies showed that oral inoculation with $rERAG_{333E}$ induced strong RABV NA responses in rhesus monkeys, foxes, and raccoon dogs. After oral inoculation, the mean RABV NA titer of rhesus monkeys, foxes, and raccoon dogs reached a peak of 2.47, 5.10, and 7.54 IU mL^{-1}, respectively. Piglets inoculated orally with $rERAG_{333E}$ developed a significant RABV NA response at 1 wk post-inoculation, and the mean RABV NA titer was 0.55 IU mL^{-1}. However, oral inoculation with $rERAG_{333E}$ induced a relatively low level of RABV NA response in goats and sheep.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical approval

All animal experiments were approved by the Committee on the Ethics of Animal Experiments of the Harbin Veterinary Research Institute (HVRI) of the Chinese Academy of Agricultural Sciences (CAAS). All institutional and national guidelines for the care and use of laboratory animals were followed. All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China.

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