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Efficacy of the oral rabies virus vaccine strain SPBN GASGAS in foxes and raccoon dogs

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ABSTRACT

To test the immunogenicity and efficacy of a new oral rabies virus vaccine strain SPBN GASGAS in wildlife target species, one group of foxes and two groups of raccoon dogs were offered a bait containing 1.7 ml of the vaccine ($10^{6.6}$ FFU/ml; $10^{6.8}$ FFU/dose) and subsequently challenged approximately 180 days later with a fox rabies virus isolate. One group of raccoon dogs (n = 30) received the same challenge dose ($10^{0.7}$ $MICLD_{50}$/ml) as the red foxes (n = 29). The other group with raccoon dogs (n = 28) together with 8 animals that received the vaccine dose by direct instillation into the oral cavity (DIOC) were infected with a 40-fold higher dose of the challenge virus ($10^{2.3}$ $MICLD_{50}$/ml). All but one of the 29 vaccinated foxes survived the challenge infection; meanwhile all 12 control foxes succumbed to rabies. Twenty-eight of 30 vaccinated raccoon dogs challenged with the same dose survived the infection, however only six of 12 control animals succumbed. When the higher challenge dose was administered, all 12 control animals died from rabies and all 36 vaccinated animals (28 baited plus 8 DIOC) survived. Blood samples were collected at different time points post vaccination and examined by both RFFIT and ELISA. The kinetics of the measured immune response was similar for both species, although in RFFIT slightly higher values were observed in foxes than in raccoon dogs. However, the immune response as measured in ELISA was identical for both species. The oral rabies virus vaccine SPBN GASGAS meets the efficacy requirements for live rabies virus vaccines as laid down by the European Pharmacopoeia.

1. Introduction

Fox-mediated rabies has been eliminated from most of Western Europe and parts of Canada (i.e., eastern Ontario) through large-scale oral rabies vaccination (ORV) programs. Several vaccines have been used in ORV campaigns and with the exception of two, a recombinant vaccinia virus and a human adeno virus expressing the rabies virus (RABV) glycoprotein (G), all constructs are based on live replication-competent attenuated rabies virus strains [1–5]. While in Canada, ERA-BHK21 was the only vaccine virus strain (>13 million baits) deployed in initial campaigns that eliminated cases from eastern Ontario [1], a recombinant oral rabies vaccine was employed in southwestern Ontario starting from 2006 [6]. In contrast, at least eleven different vaccine virus strains were applied for ORV throughout Europe (>650 million baits) between 1978 and 2014, of which the attenuated vaccine strains SAD Bern and SAD B19 were the most widely used [3].

The use of first generation vaccines has long been questioned due to observed residual pathogenicity in rodents [7–10], vaccine-associated rabies cases in target and non-target species [1,11–13], and high genetic diversity within certain commercial vaccine strains [14–16]. To eliminate some of these concerns [17,18], second generation oral RABV vaccines have been developed using monoclonal antibody selection mutants, for example SAD VA1 and SAG 2 [19–21]. The latter is characterized by a mutation from arginine to glutamic acid at residue 333 of the RABV G (R333E) that increases safety [19]. An alternative strategy is the use of recombinant vaccines based on heterologous replication competent virus vectors like vaccinia virus and human adenovirus

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type 5 that express the RABV G and cannot induce rabies [22–25]. Both recombinant oral rabies vaccines have found a wide range of application in ORV programs to combat rabies in foxes, raccoons and skunks in western Europe, the United States and Canada [26–30]. However, all commercially available recombinant rabies vaccines are based on human pathogens, hence a construct using a virus non-pathogenic for humans could eliminate some of the safety concerns. For example, different constructs based on canine-adenovirus type 2 (CAV2) expressing the RABV G were developed. Although capable of inducing a protective immune response [31,32], pre-existing immunity against CAV2 interfered with the immune response against rabies [33]. Other highly attenuated constructs, like Modified Vaccinia Ankara virus, are not able to induce an adequate immune response after oral administration [34].

Another approach to improve safety profile is to modify the RABV genome by directed mutagenesis using reverse genetics which allows targeting directly the genome by altering, deleting or inserting selected genome sequences [35]. In this way, the attenuated ERA 333 vaccine was generated containing the same mutations in G as SAG2 [36]. SPBN GAS is a SAD L16 (cDNA clone of the oral rabies virus vaccine strain SAD B19) [37] derived construct, which lacks the pseudogen (ψ) and shows alterations in the RABV G at both amino acid (aa) positions 194 and 333 where all three nucleotides were changed; position 194 – AAT [Asn] → TCC [Ser], position 333 – AGA [Arg] → GAG [Glu] [38]. Furthermore, increased efficacy and safety profiles could be achieved by overexpression of RABV G by insertion of additional one (SPBN GASGAS) and two (SPBN TriGAS) identical G genes containing the same genetic modifications [39,40]. In another construct, SAD dIND, codons specifying the aa 176–181 of the phosphoprotein (P) were deleted through site-directed mutagenesis, resulting in a loss of most of its inhibitory activity in preventing IRF-3 activities and thereby preventing IFN type 1 induction [41]. A recombinant SAD RABV variant called ORA-DPC encoded both, SAD and CVS G with the arginine residue of G at aa position 333 replaced with aspartic acid but in addition, also has a 7 aa deletion in the P-protein [42,43]. However, with the exception of ERA 333 [36], none of these newly developed constructs have been tested in potential wildlife target species for licensing.

Despite tremendous progress in wildlife rabies control, fox- and raccoon dog-mediated rabies continues to represent a major public health concern in Eastern Europe, Central Asia and the Far East, where at least seven out of nine different previously identified RABV strains belonging to the cosmopolitan lineage are still prevalent [44–47].

Therefore, the objective of these studies was to demonstrate the immunogenicity and efficacy of the oral RABV vaccine construct SPBN GASGAS both in foxes (*Vulpes vulpes*) and raccoon dogs (*Nyctereutis procyonoides*) according to the requirements of the Ph. Eur. monograph 'Evaluation of the efficacy of veterinary vaccines and immunosera' (04/2008; 5.2.7.) and 'Rabies vaccine (live, oral) for foxes and raccoon dogs' (Ph. Eur. Monograph 0746/2014) whereby baits containing the vaccine were offered to the target species in order to mimic natural conditions of bait consumption in the field.

2. Material and methods

2.1. Vaccine bait

The vaccine strain SPBN GASGAS [48] was propagated in BHK/BSR cells at IDT Biologika GmbH (IDT), Germany. In preliminary studies, a minimum effective vaccine dose for SPBN GASGAS of $10^{6.6}$ FFU/ml was established. Two different lots were used for efficacy trials (Table 1). The vaccine virus (1.7 ml) was filled in PVC-blisters and sealed with aluminium foil, subsequently each blister was incorporated in a bait matrix (40 × 40 × 12 mm) consisting of fish meal, vegetable fatty substances and paraffin. The bait matrix also contained 150 mg tetracycline used as bait marker. Following preparation, vaccine baits were stored at −20 °C.

2.2. Animals

For efficacy studies, foxes and raccoon dogs, i.e. 6–8 month-old, were purchased from a commercial registered breeder in Poland (Table 1). Adult NMRI-mice (Charles River, Germany), i.e. 4–6 week-old, were used for the determination of the mouse intracerebral lethal dose 50 (MICLD50) of the virus isolate used for challenge inoculation. Experimental studies in wildlife reservoirs and in mice were conducted at FLI, Greifswald-Insel Riems, Germany, and at IDT, Germany, respectively.

2.3. Challenge virus and determination of $MICLD_{50}$

The challenge virus (fox/148) was isolated in 1998 from the brain of a naturally infected red fox originating from North Rhine Westphalia, Germany (INSDC Accession#: LN879481) and passaged 3 times in murine neuroblastoma cells (Na42/13, Collection of Cell Lines in Veterinary Medicine (CCLV), FLI Riems) with a final titre of $10^{6.0}$ $TCID_{50}$/ml ($10^{5.4}$ FFU/ml). The $MICLD_{50}$/ml virus stock ($10^{6.7}$ raw material) was determined calculated as described [49,50].

2.4. Vaccination protocol and sampling

In total, three different studies were conducted (Table 1). Animals in vaccination groups were each offered a single vaccine bait during a 24 h presentation window following a 24 h fasting period. If animals did not consume a bait after two consecutive days, vaccine was administered by direct instillation into the oral cavity (DIOC). Serum samples (B) were collected at different time points prior and post vaccination (p.v.) to investigate the development and kinetics of rabies induced antibodies (Table 2).

Table 1
Group composition and study period.

Species (study)	Number of animals				Vaccination	Challenge					
	Female	Male	Vaccinated	Controls	Dose in FFU/ml	Day p.v.	Dose in $MICLD_{50}$/ml	Survival			
								Vaccinated	Percentage	Controls	Percentage
1 red fox	12	30	30*	12	$10^{6.6}$#	191	$10^{0.7}$	28/29	97%	0/12	0%
2 raccoon dog	15	27	30	12	$10^{6.6}$#	190	$10^{0.7}$	28/30	93%	6/12	50%
3 raccoon dog	24	24	28 + 8°	12	$10^{6.6}$†	183/184	$10^{2.7}$	36/36	100%	0/12	0%

* One vaccinated fox was removed from the study prior to challenge virus administration to due to animal welfare reasons unrelated to the vaccine.
° 28 animals were vaccinated by offering a bait and 8 additional animals received the vaccine by DIOC.
Lot VM 0010814-A.
† Lot VM0020814-A.

Table 2
Immune response in vaccinated and control animals pre- and post vaccination as well as post infection. Animals were challenged at days 190 (study 1 and 2) and 183 (study 3). Bleed date B9 (2 8 0) represents day 90 post infection.

Species (study)	Status	No.	Test		Day									
					Pre-vaccination	Post vaccination								
					B0	B1	B2	B3	B4	B5	B6	B7	B8	B9**
					−13	8	15	29	56	92	102	148	190	280
1 red fox	Baited	30	ELISA	% SC	0	70.0	96.6	100.0	100.0	100.0	100.0	100.0	100.0	100.0
				MPI	29.5	49,0	70,2	81,2	95,4	95,5	94,6	93,8	91,6	95,25
			RFFIT	% SC	0	26.6	90	83.3	80	70	66.7	53.3	65.5	62.1
				GMT	0.1	0.2272	2.221	1.162	1.281	0.8074	0.6923	0.6349	0.6137	0.729
	Control	12	ELISA	% SC	0	–	–	0	–	–	–	–	0	–
				MPI	21.3	–	–	24.2	–	–	–	–	20.8	–
			RFFIT	% SC	0	–	–	0	–	–	–	–	0	–
				GMT	0.1	–	–	0.1	–	–	–	–	0.1	–
2 raccoon dog	Baited	30	ELISA	% SC	0	20	86.6	86.6	86.6	90	90	83.3	83.3	100.0
				MPI	22.6	30.0	61.6	72.0	86.2	88.3	90.5	87.0	86.6	88.84
			RFFIT	% SC	0	0	60	66.7	66.7	66.7	73.3	60	66.7	70.0
				GMT	0.1	0.1	0.5	0.8	0.9	0.7	0.9	0.7	0.8	1.0
	Control	12	ELISA	% SC	0	–	–	0	–	–	–	–	0	–
				MPI	30.5	–	–	22.9	–	–	–	–	21.0	–
			RFFIT	% SC	0	–	–	0	–	–	–	–	0	–
				GMT	0.1	–	–	0.1	–	–	–	–	0.08	–
					B0		B1	B2		B3			B4	
					−6		13	27		92			183	
3 raccoon dog	Baited	28	ELISA	% SC	0	–	100.0	100.0	–	100.0	–	–	100.0	–
				MPI	22.7	–	66.8	92.5	–	98.1	–	–	99.9	–
			RFFIT	% SC	0	–	85.7	92.8	–	96.4	–	–	100	–
				GMT	0.1	–	1.6	2.1	–	3.3	–	–	3.0	–
	DIOC	8	ELISA	% SC	0	–	100.0	100.0	–	100.0	–	–	100.0	–
				MPI	22.07	–	69.5	94.7	–	99.7	–	–	100.6	–
			RFFIT	% SC	0	–	66.6	83.3	–	83.3	–	–	100	–
				GMT	0.1	–	2.3	2.3	–	6.5	–	–	5.5	–
	Control	12	ELISA	% SC	0	–	–	–	–	–	–	–	0	–
				MPI	18.9	–	–	–	–	–	–	–	27.7	–
			RFFIT	% SC	0	–	–	–	–	–	–	–	0	–
				MPI	0.1	–	–	–	–	–	–	–	0.1	–

B = bleed day.
%SC = percentage seroconversion.
MPI = mean percentage inhibition.
GMT = geometric mean titre.
** Survivors only.

2.5. Challenge protocol

After challenge dose-finding study with graded doses ($10^{2.0–4.7}$ $MICLD_{50}$/ml) in both species (unpublished results), all animals were challenged with the RABV challenge virus 6 months p.v. (table 1) by intra muscular (i.m.) inoculation with 0.5 ml into each masseter muscle (1.0 ml total) (Table 1). Survival of vaccinated and naïve control animals was followed over a study period of at least 90 days post infection (p.i.). Brains were examined for presence of virus.

Details on animal husbandry during experimental studies, monitoring of animals p.i., euthanasia and sampling are provided elsewhere (supplementary information).

2.6. Ethics statement

All animals were kept in accordance with the prevailing guidelines and the in vivo work was performed according to European guidelines on animal welfare, clinical endpoints [51] and care according to the Federation of European Laboratory Animal Science Associations (FELASA). Studies in mice to establish the $MICLD_{50}$ of the challenge virus and challenge dose finding studies in foxes and raccoon dogs were evaluated and approved by the responsible authorities in the federal state of Saxony Anhalt (IDT A 5a/2012), while immunogenicity and efficacy studies in foxes and raccoon dogs were evaluated and approved by responsible authorities in the federal state of Mecklenburg-Western Pomerania (FLI 7221.3-2-007/14; 7221.3-2-018/15). General care was provided as required.

2.7. Diagnostic assays

The presence of RABV antigen in the brain (Ammons' horn, cerebellum and Medulla oblongata) was diagnosed using the fluorescent antibody test (FAT) using FITC-marked monoclonal anti-rabies antibodies (SIFIN, Berlin, Germany) essentially as described [52]. Defined positive (PC) and negative controls (NC) were included in every test run. In the case of FAT inconclusive results RABV was isolated using the rabies tissue culture infection test (RTCIT) [53] with three consecutive passages to confirm a negative result.

Sera were tested for the presence of virus neutralizing antibodies (VNAs) in a modified rapid fluorescence focus inhibition test (RFFIT) as described previously [54], using RABV (CVS-11) as test virus and BHK21-BSR/5 (CCLV-RIE 0194/260) cells. B4 serum samples (studies 1 and 2, Table 2) were also tested using the homologous vaccine virus strain SPBN GASGAS as test virus. The calibrated WHO international standard immunoglobulin (2nd human rabies immunoglobulin preparation, National Institute for Standards and Control, Potters Bar, UK) adjusted to 0.5 international units

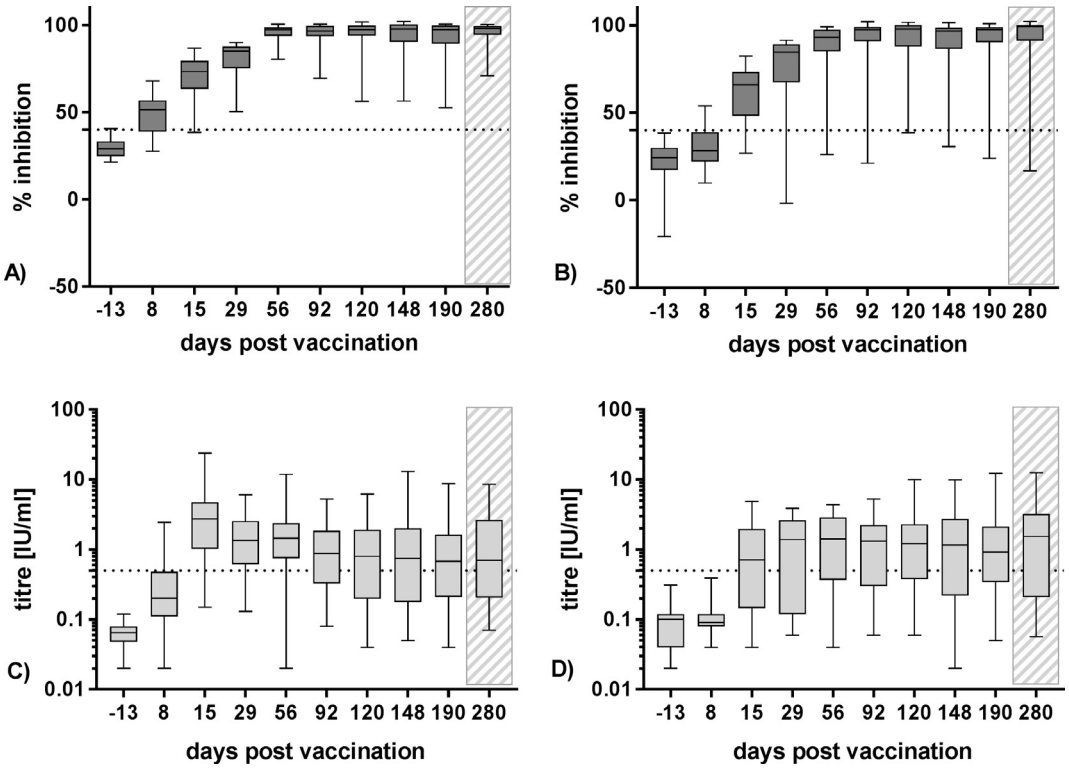

Fig. 1. Boxplots of ELISA data (A, B) and VNA titres (C, D) of blood samples taken at different days post vaccination for studies 1 (A, C) and 2 (B, D) (only vaccinated animals). For better visualization, the ELISA values (percent inhibition) and VNA titres per sampling are displayed as whiskered boxplots, whereby the bottom and top of the box corresponds to the first and third quartiles and the band inside the box is the median. The whiskers indicate the minimum and maximum of all data. The dotted lines represent cut-off and threshold of positivity in ELISA and RFFIT, respectively. Box plots in dashed boxes represent ELISA and RFFIT values of animals that survived challenge at the end of the observation period. At the day of challenge, 11 vaccinated foxes (study 1) were RFFIT negative (VNA < 0.5 IU/ml) but ELISA positive. Contrastingly, 10 vaccinated raccoon dogs from study 2 were RFFIT negative of which 5 were negative in ELISA, too. The challenge did not represent a booster dose for the majority of vaccinated animals. Only 2 of 11 and 1 of 10 foxes and raccoon dogs, respectively, with RFFIT titres below 0.5 IU/ml at challenge had an increased titre above this threshold after challenge. Also, only one out of five raccoon dogs that were negative in ELISA at challenge presented a positive result at the end of the studies.

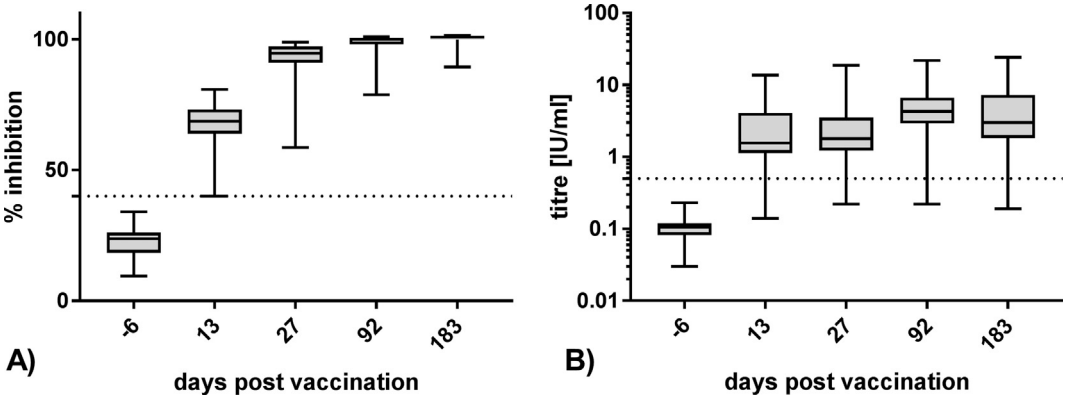

Fig. 2. Boxplots of ELISA data (A) and VNA titres (B) of blood samples taken at different days post vaccination for study 3 (only vaccinated animals). For better visualization, the ELISA values (percent inhibition) and VNA titres per sampling are displayed as whiskered boxplots, whereby the bottom and top of the box corresponds to the first and third quartiles and the band inside the box is the median. The whiskers indicate the minimum and maximum of all of the data. The dotted lines represent cut-off and threshold of positivity in ELISA and RFFIT, respectively.

(IU) and a naive bovine serum served as PC and NC, respectively. The VNA titres were calculated using inverse interpolation as described and subsequently converted into concentrations expressed in international units (IU/ml) [55].

Presence of rabies specific binding antibodies was tested using a commercial blocking ELISA (BioPro Rabies ELISA, Czech Republic) [56,57] essentially as described [56]. Cut-off for positivity (40% inhibition of the test serum compared to the NC), validity parameters and characteristics were followed as stated in the kit insert.

2.8. Statistical analysis

Weight development between vaccinated and control animals at the start and end of the studies as well as rabies antibody

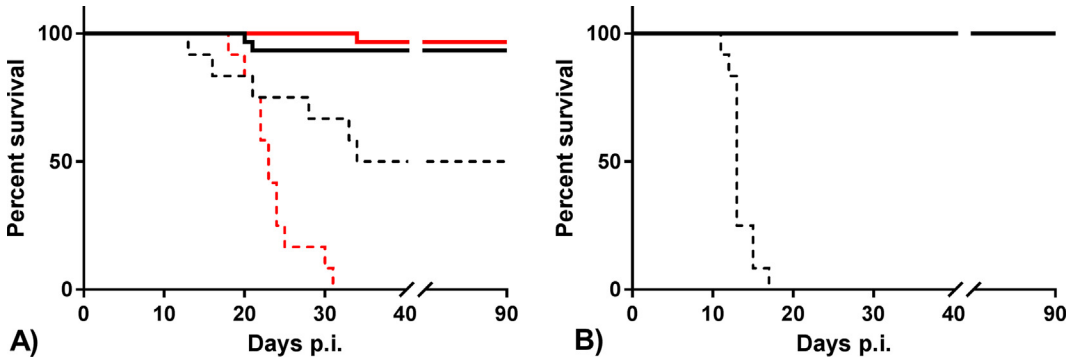

Fig. 3. Survival curves of vaccinated (solid line) and control (dashed line) foxes (study 1 - red) and raccoon dogs (study 2 - black) (A) and raccoon dogs of study 3 (B) after challenge. The median incubation period for control foxes and raccoon dogs (A) was 24 days (range: 18–31 days) and 24 days (range 12–34 days), respectively. Control animals in study 3 succumbed on average 12 days p.i. (range 11–15).

dynamics and kinetics over the observation period were assessed using *t*-test with p-values < 0.05 considered significant. Statistical testing was done in GraphPad Prism version 7.00 (GraphPad Software, La Jolla California USA.

3. Results

3.1. Vaccine immunogenicity

All animals were seronegative at the start of the experimental studies (Table 2). Vaccination with SPBN GASGAS by vaccine bait delivery and DIOC induced a rabies specific primary immune response. ELISA mean percent blocking values in animal sera of vaccine bait treatment groups were above the cut-off (40%) after 8–15 days (Fig. 1A, B and 2A). All treated animals were ELISA positive 29 days p.v. except in study 2. Here, seroconversion in raccoon dogs as measured by ELISA peaked only at 90% 92 days p.v. (Table 2). Primary immune response as measured by RFFIT revealed a differentiated picture. Median VNA titres of treatment groups had values above 0.5 IU/ml from 13 to 15 day p.v. onwards (Table 2, Fig. 1C, D and 2B). However, only in study 3 all raccoon dogs developed VNAs in response to vaccination (Table 2). There was a significant difference in the proportion of seropositives and the VNA levels on all the bleed dates in common between study 2 and 3 (*t*-test, $p < 0.05$) (Table 2, Figs. 1D and 2B). In study 1, two (6.9%) of 29 foxes that were ELISA positive from day 4 p.v. onwards did not develop any measurable VNAs throughout the pre-challenge phase. In study 2, seven (23.3%) of 30 raccoon dogs were negative in RFFIT at all times pre-challenge. Five of the seven VNA-negative raccoon dogs were at least at one sampling point positive in ELISA between days 29 and 190 p.v.

Blood samples from foxes (study 1) and raccoon dogs (study 2) taken at day 56 p.v. and tested against SPBN GASGAS showed 13.7 and 6.6 times higher VNAs compared to CVS-11, respectively. Six of 30 foxes were seronegative against CVS-11 but were seropositive against the vaccine virus. By comparison, four of 30 raccoon dogs neither had VNAs against CVS-11 nor against SPBN GASGAS at this bleed day. Contrastingly, no mock treated animals developed rabies specific binding antibodies or VNAs at any pre-challenge time point (Table 2).

3.2. Vaccine efficacy

In all studies, overall survival following lethal challenge was >90% among bait delivery treatment groups and 50% and 100% in control groups (Table 1, Fig. 3). While one ELISA and VNA positive fox (study 1) was removed on day 171 p.v. for animal welfare reasons (rabies was excluded), another vaccinated fox expired 34 days p.i., together with all 12 control foxes. The latter fox was ELISA positive but seronegative against CVS in RFFIT at any time point. Also, with 0.6 IU/ml it was the only fox weakly seropositive against SPBN GASGAS at day 56 p.v.. The two vaccinated raccoon dogs (study 2) that had to be terminated because of clinical signs 20 and 21 days p.i. were ELISA and VNA negative at all time points pre challenge. In contrast, five vaccinated, VNA-negative but ELISA-positive raccoon dogs survived challenge. Meanwhile, only six of 12 control animals died or had to be euthanized p.i. (Table 1, Fig. 3A). Of these six survivors, three were ELISA positive and one had a VNA titre of 0.5 IU/ml at the end of the 90 days observation period p.i.. When using a higher challenge dose (study 3) all vaccinated raccoon dogs survived the challenge infection meanwhile all control animals succumbed p.i. (Table 1, Fig. 3B). All animals that succumbed to disease p.i. were FAT positive. There was no difference in body weight between vaccinated and control animals at the beginning and end of the study (*t*-test, $p > 0.05$).

4. Discussion

The use of modified live virus vaccines has been successful in eliminating fox-mediated rabies in western Europe and parts of Canada [2,3,58,59] and controlling rabies in raccoons and coyotes in North America [4,60,61]. Efficacy trials of modified live rabies vaccines have been published for foxes, e.g. ERA, SAD B19, SAD P5/88, SAG2, ERA 333, and VRG [20,36,62–67] and raccoon dogs, e.g. SAD P5/88, SAG2, and VRG [66–68]. Here, we tested the immunogenicity and efficacy of a genetically engineered rabies virus vaccine construct (SPBN GASGAS) for oral use in both foxes and raccoon dogs.

The immunogenicity and efficacy trials demonstrated absence of residual virulence of SPBN GASGAS in both target species when given field dose. The vaccine construct was well-tolerated and all treated animals but one fox (see above) remained healthy throughout the 273–280 day observation period. Although there was strong immune response as measured by ELISA (Fig. 1A, B and 2A), in general, the immune responses in terms of VNA observed in both foxes and raccoon dogs was less pronounced (Fig. 1C, D and 2B). VNA titres (Fig. 1C, D and 2B) were not as high as described for efficacy studies with first and second generation attenuated rabies vaccines, i.e. SAD B19, SAD P5/88 and SAG2 [65–68]. Rather, VNA titres were similar to those observed in target species vaccinated with lyophilized SAG2 [69], SAG1, VRG [70] and ERA333 [36] (Fig. 1C, D and 2B). Although gene duplication in the

SPBN GASGAS construct resulted in increased G protein levels compared to the progenitor SPBN virus [71] and correlated with a more efficient protection even in orally immunized raccoons [72], G levels of SPBN GASGAS have not been directly compared to current oral vaccine strains. It remains to be clarified whether currently used and effective live vaccines indeed produce less G than SPBN GASGAS and whether this has an impact on VNA titre levels. In view of an intrinsic genetic variability of conventional live vaccines like SAD B19 [16], differences in G expression levels between cell culture passaged vaccine strains and recombinant virus clones cannot be excluded. Also, differences in observed VNA titres could be a result of serological test protocols since the RFFIT sometimes is sensitive to multiple sources of variation [73]. Also, the virus strain used in biological assays to measure rabies VNA p.v. can profoundly influence test results. Assays using homologous test viruses reveal higher VNA titre values than heterologous testing systems (Moore et al., 2005). This is strongly supported by the observation that seroconversion and VNA titres measured in vitro against the homologous SPBN GASGAS vaccine strain were many times higher than against the heterologous strain CVS-11. This could also partially explain the differences observed between ELISA and VNA data (Table 2). The kinetics of VNA development in raccoon dogs was comparable to SAG2 [68], while those in foxes was quite similar to what was seen with juvenile foxes vaccinated with ONRAB®, a recombinant human adenovirus type 5 expressing the RABV glycoprotein [27]. Explanations for the significant difference in immune response between study 2 and 3 (Table 2, Fig. 1D and 2B) remain elusive. Parameters that changed in study 3 were the vaccine and animal lot. Although titres of vaccine lots were identical, due to the nature of the assay a +/− 0.3 log standard deviation cannot be excluded. Also, individual differences in animals from the same supplier in terms of general health conditions, food preference (bait acceptance) and immune response may have had an influence (unpublished data).

The pronounced immune response of SPBN GASGAS and the correlation between the induction of binding and neutralizing antibodies and protection was also reflected in survival rates. Survival of SPBN GASGAS ($10^{6.6}$ FFU/ml) vaccinated foxes and raccoon dogs (studies 1, 3) and lack thereof in control animals (Table 1, Fig. 3) is in agreement with efficacy studies using SAG2 [$10^{8.0}$ and $10^{8.15}$ Cell Culture Infective Dose 50 ($CCID_{50}$)], SAD B19 ($10^{6.3}$ FFU/ml), and SAD P5/88 ($10^{5.9}$ FFU/ml) [20,65,66]. In contrast, a comparatively low efficacy rate (approximately 48–50%) was reported when foxes were offered an ONRAB bait or three different oral rabies vaccine baits (SAD B19, SAG1, V-RG) and challenged 547 and 34 days p.v., respectively. However, in the latter study one of 8 control foxes also survived challenge infection [27,74].

How VNAs neutralize RABV is not entirely understood [75,76]. It is assumed that antibodies that neutralize virus in vitro are more efficient in the process that leads to protection against virus infection in vivo than antibodies that do not neutralize virus in vitro [77]. Our data show that SPBN GASGAS vaccinated foxes and raccoon dogs (studies 1, 2) with rabies specific antibodies other than VNAs survive challenge (Tables 1 and 2; Fig. 3). Survival after challenge without presence of detectable VNAs has also been reported from efficacy studies using SAG1 and SAG2 [20,68,70]. It cannot be excluded that those animals may have developed VNAs but below the level of detection (<0.5 IU/ml). The latter was established as an indication of adequate vaccination rather than protection in humans at risk of rabies exposure. Therefore, when rabies seroneutralisation assays are employed for other species, the accepted level of 0.5 IU/ml may not apply [78]. However, if not it may indicated that contrary to earlier assumptions [75,76] VNAs are not necessarily needed for protection even if they are a considered indicator.

To mimic natural conditions of an infection we chose a RABV isolated from a fox in Germany in 1998 as challenge virus. The Ph. Eur. Monograph 0746/2014 does not specify a defined challenge dose to be used in efficacy studies for licensing of oral rabies vaccines. The outcome of the challenge using $10^{0.7}$ $MICLD_{50}$ in vaccinated and control foxes (study 1) clearly met international standards [79] (Table 1, Fig. 3A) and confirmed previous observations showing that when using a RABV isolate of a naturally infected fox a challenge dose of less than $10^{0.5}$ lethal dose 50 (LD_{50}) was highly pathogenic [80,81]. A similar effect was seen during a low dose challenge ($10^{2.3}$ $MICLD_{50}$) of dogs with a RABV of a naturally infected dog [82]. In contrast, the low dose ($10^{0.7}$ $MICLD_{50}$) was not able to induce rabies in at least 80% of unvaccinated control raccoon dogs as required for a valid challenge study (study 2, Table 1, Fig. 3A). This shows that the high pathogenicity of the chosen challenge virus (fox/148) even at low dose infections is rather indicative of its high genetic adaptation to the fox as the principle reservoir hosts rather than to the raccoon dog [80]. Because in efficacy studies with SAG2 in raccoon dogs even field RABV isolates from same species were unable to induce at least 90% mortality in controls, it was decided to use a high titred ($10^{3.6}$ $MICLD_{50}$) coyote strain as challenge virus [68]. Only when a higher challenge dose ($10^{2.7}$ $MICLD_{50}$) of RABV fox/148 (but still lower than the one used for SAG2) was used, all unvaccinated raccoon dogs (study 3) succumbed to disease (Table 1, Fig. 3B).

The duration of protective immunity of at least 6 months induced by SPBN GASGAS when offered baits containing field dose fits the bi-annual bait distribution scheme as practiced in Europe [83,84]. Oral rabies virus vaccines, however, have shown to be extremely immunogenic in foxes and depending on doses, volumes and bait system applied to induce long lasting protective immunity for more than a year or even longer [20,64,85]. The duration of immunity induced by SPBN GASGAS in foxes and raccoon dogs beyond 6 months remains to be investigated.

5. Conclusions

The efficacy studies demonstrated that SPBN GASGAS is immunogenic in both foxes and raccoon dogs inducing a long-lasting humoral response and protection after a highly virulent RABV challenge, and therefore, meets the requirements of European Pharmacopoeia monograph No. 0746/2014.

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Conflict of interest

AV, AK, CK, CK, PS, SO are full-time employees of IDT Biologika GmbH, Germany, a company manufacturing oral rabies vaccine baits including SPBN GASGAS. TM, CMF and SF from the Friedrich-Loeffler-Institute received funding from IDT Biologika GmbH for research into mechanisms of oral rabies vaccination and serological response.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2017.09.093.

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