Vaccine 35 (2017) 3938–3944

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Oral vaccination of wildlife against rabies: Differences among host species in vaccine uptake efficiency

Ad Vos [a], Conrad M. Freuling [b], Boris Hundt [a], Christiane Kaiser [a], Sabine Nemitz [b], Andreas Neubert [a], Tobias Nolden [b,c], Jens P. Teifke [d], Verena te Kamp [b], Reiner Ulrich [d], Stefan Finke [b], Thomas Müller [b,*]

[a] IDT Biologika GmbH, 06861 Dessau-Rosslau, Germany
[b] Institute of Molecular Virology and Cell Biology, Friedrich-Loeffler-Institut, WHO Collaborating Centre for Rabies Surveillance and Research, OIE Reference Laboratory for Rabies, 17493 Greifswald-Insel Riems, Germany
[c] ViraTherapeutics GmbH, Exlgasse 20a, 6020 Innsbruck, Austria[1]
[d] Department of Experimental Animal Facilities and Biorisk Management, Friedrich-Loeffler-Institut, 174 93 Greifswald-Insel Riems, Germany

ARTICLE INFO

Article history:
Received 2 May 2017
Received in revised form 1 June 2017
Accepted 3 June 2017
Available online 19 June 2017

Keywords:
Rabies
Wildlife
Fox
Skunk
Oral vaccination
Vaccine uptake efficiency
Tonsil

ABSTRACT

Oral vaccination using attenuated and recombinant rabies vaccines has been proven a powerful tool to combat rabies in wildlife. However, clear differences have been observed in vaccine titers needed to induce a protective immune response against rabies after oral vaccination in different reservoir species. The mechanisms contributing to the observed resistance against oral rabies vaccination in some species are not completely understood. Hence, the immunogenicity of the vaccine virus strain, SPBN GASGAS, was investigated in a species considered to be susceptible to oral rabies vaccination (red fox) and a species refractory to this route of administration (striped skunk). Additionally, the dissemination of the vaccine virus in the oral cavity was analyzed for these two species. It was shown that the palatine tonsils play a critical role in vaccine virus uptake. Main differences could be observed in palatine tonsil infection between both species, revealing a locally restricted dissemination of infected cells in foxes. The absence of virus infected cells in palatine tonsils of skunks suggests a less efficient uptake of or infection by vaccine virus which may lead to a reduced response to oral vaccination. Understanding the mechanisms of oral resistance to rabies virus vaccine absorption and primary replication may lead to the development of novel strategies to enhance vaccine efficacy in problematic species like the striped skunk.

1. Introduction

Oral vaccination against rabies using modified live rabies virus vaccines has been highly successful in different reservoir species. The first animal targeted was the red fox (*Vulpes vulpes*) followed by the raccoon dog (*Nyctereutes procyonoides*) [1,2]. Subsequently, the concept of oral rabies baiting was investigated for other animal species, like raccoons (*Procyon lotor*) [3,4], coyotes (*Canis latrans*) [5–7], gray foxes (*Urocyon cineroargenteus*), striped skunks (*Mephitis mephitis*) [8], small Indian mongooses (*Herpestes auropunctatus*) [9,10], and domestic dogs (*Canis lupus domesticus*) [11–13].

It became evident that not all animal species were equally susceptible for vaccination by the oral route; some species like the striped skunk seem to be extremely refractory to oral rabies vaccination, irrespective of the construct used even when high virus titres were administered [4,14–20]. Oral virus vaccines for veterinary use, e.g. rabies [21–28] and classical swine fever (CFS) virus [29–32] or recombinant poxvirus against lethal plaque [33–35] have been developed and used under field conditions in Europe and North America. However, it remains largely unknown how and where the vaccine viruses are transported across the epithelium in the oral cavity.

Several studies have revealed that the vaccine viruses are predominantly present in the tonsils and less pronounced in the oral mucosal epithelium after oral administration as shown for both attenuated and recombinant oral rabies virus vaccines [36–41] as well as for attenuated CFS vaccine virus constructs [42–44]. Lymphoreticular tissues of the pharynx assumed to be involved in efficient oral immunization, also called Waldeyer's tonsillar ring, variably comprise *Tonsilla* (*T.*) *lingualis, T. palatina, T. veli palatini, T. paraepiglottica, T. pharyngea,* and *T. tubaria* in a species-dependent pattern. Furthermore, tonsils can be subdivided based

* Corresponding author at: Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Molecular Virology and Cell Biology, Südufer 10, D-17493 Greifswald-Insel Riems, Germany.
E-mail address: Thomas.Mueller@fli.de (T. Müller).
[1] Current affiliation.

http://dx.doi.org/10.1016/j.vaccine.2017.06.022
0264-410X/

on their histoarchitecture into those containing epithelial crypts or those covered by a smooth epithelium as well as those bulging above the mucosal surface versus those covered within a mucosal fossa [45–48]. Because of this complexity, the exact locations of entry of the attenuated rabies virus vaccines within these tissues and the pharmacokinetics have not been investigated in detail. Hence, even after 35 years of oral vaccination of wildlife against rabies there is limited knowledge on how oral vaccination in target species actually works. Considering the difficulties in inducing a protective immune response against rabies in reservoir species other than foxes and raccoon dogs, prompted us to elucidate the mechanisms behind. Therefore, the objectives of this study were to (i) examine the immunogenicity of an oral rabies virus vaccine construct in two species that show extreme differences in susceptibility to oral vaccination; the red fox (*Vulpes vulpes*) and striped skunk (*Mephitis mephitis*), after direct oral administration, and (ii) investigate the dissemination of the vaccine virus construct in the oral cavity of these two species by establishing the hypothesis that differences in virus presence and replication in lymphoreticular tissues, in particular the palatine tonsils between the two species could contribute to the vaccine uptake efficiency.

2. Material and methods

2.1. Vaccine virus

The SPBN GASGAS vaccine virus was constructed as previously described [49]. The parental vaccine of SPBN GASGAS is the SAD B19 oral rabies vaccine virus. The SPBN construct lacks the pseudogene (ψ) and the G-gene is flanked by a Sma/XmaI and PacI-restriction enzyme cleavage site. Furthermore, the SPBN-virus contains a linker to express a (foreign) gene with two restriction enzyme sites (BsiWhI/NheI) for subsequent introduction of additional genetic information. The construct contains two glycoprotein genes with the following two modifications (site-directed mutagenesis); a change from asparagine to serine and one from arginine to glutamic acid at position 194 and 333 of the glycoprotein, respectively [49,50]. The antigen, SPBN GASGAS was produced according to the protocol given by Vos et al. [51]. Antigen with titers $>10^{8.0}$ focus forming units (FFU)/ml was concentrated via tangential flow filtration using ultrafiltration flat sheet cassettes with a Molecular Weight Cut Off (MWCO) of 300 kDa.

2.2. Animals

A total of 14 and 4 foxes and 24 and 4 striped skunks were used for the vaccination and challenge and dissemination studies, respectively. All foxes and striped skunks used in this study were obtained from different commercial sources in Poland and the United States, respectively. Foxes were kept in individual cages during the entire observation period. Meanwhile, skunks were partially kept in small groups, if applicable, until challenge infection. All animals were sedated (mixture of 1.1 mg/kg Xylazin and 2 mg/kg Ketamin) during vaccine administration and challenge infection.

2.3. Ethics statement

All *in vivo* work was performed at IDT Biologika GmbH, according to European guidelines on animal welfare and care pursuant to the Federation of European Laboratory Animal Science Associations (FELASA). Study protocols were evaluated and approved by the responsible authorities (Landesverwaltungsamt Sachsen – Anhalt, Referat Verbraucherschutz, Veterinärangelegenheiten) in the federal state of Saxony-Anhalt, Germany; AZ42502-3-670 IDT (red fox – immunogenicity study), AZ 42505-3-669 IDT (striped skunk and red fox – dissemination study), AZ 42505-3-582 IDT (striped skunk – immunogenicity study).

2.4. Vaccination and challenge studies

To determine the minimum effective dose of SPBN GASGAS in striped skunks, different doses were administered by direct oral administration; $10^{7.3}$ FFU/ml (n = 3), $10^{8.0}$ FFU/ml (n = 5) and $10^{9.2}$ FFU/ml (n = 6). To mimic natural conditions, 1.5 ml (foxes) and 1 ml (skunks) of virus suspension was directly administered into the oral cavity but not targeted directly to the tonsils. Also, two skunks received the highest dose intra muscularly (i.m.). For foxes, a similar minimum effective dose as determined with the oral rabies vaccine strain SAD B19 was applied [52]. Hence, here only a single low dose of SPBN GASGAS ($10^{6.5}$ FFU/ml) by direct oral instillation was tested in 6 animals.

The vaccinated animals were inoculated with a challenge virus ($10^{5.1}$ MICLD50) between days 42 and 98 post vaccination together with control animals (N = 8). The challenge virus used was isolated from the salivary glands of a red fox (2nd passage) infected experimentally with an isolate from a naturally infected coyote (CVS/USA/TX Coyote/295/R/061893 – Centers for Disease Control and Prevention, Atlanta, USA).

2.5. Dissemination studies

For dissemination studies in each case four foxes and skunks were kept individually in groups of 2 animals each in an isolation unit within the Animal House at IDT. All animals received 1.0 ml SPBN GASGAS ($10^{7.5}$ FFU/ml) by direct oral instillation. Two animals of each species were euthanized at day 3 and 5 post vaccine administration. During necropsy different tissues (tonsils [*Tonsilla palatina, T. pharyngealis*], Supplementary Fig. 1), tongue, regional lymph nodes [*Lymphonodi (Lnn.) parotidei, Lnn. retropharyngei, Lnn. mandibulares*] and mucous membrane of the upper and lower oral cavity) were collected and examined for the presence of rabies virus vaccine construct by RT-PCR and RTCIT.

Saliva samples were collected prior to vaccine administration (S0) and 1 h (S1), 2 h (S2), 3 h (S3), 24 h (S4), 48 h (S5), 72 h (S6), if applicable, 120 h (S7) post vaccine administration. Saliva swabs were collected by swabbing of the oral cavity for 1–1.5 min. Subsequently, the cotton tips were placed in 2 ml MEM medium supplemented with antibiotics (gentamycin [50 mg/l] and amphotericin [2.5 mg/l]) and stored at −80 °C until further investigation using RT-PCR and RTCIT.

2.6. Diagnostic assays

Different regions of the brain, i.e. hippocampus, medulla oblongata and cerebellum, of animals challenged with street RABV were tested for the presence of viral antigen using the direct Immuno-Fluorescence Test (dIFT) [53]. For detection of SPBN GASGAS specific viral RNA in lymphopharyngeal tissues as well as in saliva swabs of foxes and skunks obtained during dissemination studies, RNA was extracted with TRIzol (Invitrogen)/TRIfast® (PEQLAB Biotechnologie GmbH, Erlangen, Germany) according to manufacturers' recommendations, followed by real-time RT-PCR (qRT-PCR) essentially as described [54]. The presence of viable rabies virus particles in qRT-PCR positive tissues was confirmed with the rabies tissue culture infection test (RTCIT) [55,56] using the mouse neuroblastoma cell line NA 42/13 (Collection of Cell Lines in Veterinary Medicine (CCLV), Friedrich-Loeffler-Institut, No. 411]. Three consecutive passages in cell culture were conducted to confirm a negative result.

Blood samples were taken prior to vaccination and challenge infection from foxes and skunks from veins (*V. cephalica antebrachii*

or V. saphena parva) and by claw clipping, respectively and, if possible, on day of death. Blood samples were examined for the presence of rabies virus neutralizing antibodies (VNA) using the Rapid Fluorescence Focus Inhibition Test RFFIT [57] with adaptations as described by Cox & Schneider [58]. VNA titres were defined as the dilution of test sera showing a 50% reduction of the test virus (50% neutralizing dose, ND50) and were calculated using inverse interpolation. Subsequently, VNA titres were compared to the titre of the reference serum [2nd WHO Standard for Rabies Immunoglobulin (Human) (National Institute for Biological Standards and Controls, NIBSC, Potters Bar, Hertfordshire, UK] and converted to IU/ml.

Palatine tonsils of red foxes and skunks were collected at necropsy immediately after euthanasia and fixed in 4% neutral-buffered formaldehyde. For immunocytochemical analysis, vibratome sections with a thickness of 100 μm were prepared and stained with a specific antibody against RABV N protein (polyclonal rabbit-α-RABV N 161-5, 1:5000 diluted in PBS) in combination with Alexa Fluor® 568 goat-α-rabbit secondary antibody (ThermoFisher Scientific, 0.7 μg/ml). For visualization of nuclei, Hoechst 33342 (ThermoFisher Scientific, 1 μg/ml) was used. The stained palatine tonsil sections were examined by confocal laser scanning microscopy (Leica SP5 confocal laser scan microscope, Microsystems, Germany). Images were processed with the ImageJ software version 1.48b [59].

3. Results

3.1. Seroconversion and protection against RABV require higher infectious SPBN GASGAS vaccine doses in striped skunk than in red fox

In order to assess whether increased immunogenicity of SPBN GASGAS virus, as observed in mouse [49], raccoon [60] and dog [13] models, affects the minimal effective dose in skunks, seroconversion and protection against RABV challenge was compared after oral immunization of red foxes with $10^{6.5}$ FFU SPBN GASGAS/animal or of striped skunks with dosis ranging from $10^{7.3}$ FFU up to $10^{9.2}$ FFU SPBN GASGAS/animal. All foxes seroconverted and survived the challenge infection (Table 1). In contrast, seroconversion in skunks, which was measured by the detection of VNAs, was not detected at doses of $10^{7.3}$ and $10^{8.0}$ FFU. Furthermore, even at the highest dose of $10^{9.2}$ FFU SPBN GASGAS/animal only 5 out of 6 (83.3%) animals seroconverted. Notably, even though seroconversion was not detected after immunization with $10^{8.0}$ FFU, partial protection against RABV was observed, as 2 out of 5 skunks survived the challenge infection. However, even at the highest dose of $10^{9.2}$ FFU there was incomplete protection as only 5 out of 6 animals survived.

These data showed that similar to previous oral live vaccines the immunogenicity of SPBN GASGAS at lower doses is limited in skunks and that those limitations can be overcome partially by application of almost 1000x higher vaccine virus doses than required for efficient red fox immunization. In contrast to direct oral administration of SPBN GASGAS, two skunks that received $10^{9.2}$ FFU vaccine virus by the intramuscular (i.m.) route of infection developed high levels of rabies VNAs with 47.5 IU/ml both at day 28 post vaccination. All control foxes and striped skunks succumbed to the challenge infection as confirmed by dIFT. These data confirmed that requirement for vaccine virus doses was not a result of low immunogenicity of SPBN GASGAS in skunks in general but more likely represents unknown host specific constraints for oral vaccination route.

3.2. Dissemination of SPBN GASGAS in the oropharynx differs in skunks and foxes

As the low immunogenicity of SPBN GASGAS relied on the oral administration route and restriction in the immunization process could be at least partially overcome by increased infectious virus titers, we hypothesized that dissemination of the oral rabies vaccine virus in the oropharynx and infection of particular target cells or tissues is a determinant for vaccination efficacy. Therefore, dissemination of SPBN GASGAS in the oropharynx of 4 skunks and 4 foxes after oral vaccination with $10^{7.5}$ FFU vaccine virus/animal was investigated. With $10^{7.5}$ FFU a virus dose was chosen that was one log above the 100% protective doses in red foxes and still two logs below protective doses in skunks (Table 1).

For both, vaccinated skunks and foxes, virus detection in saliva swabs by RT-PCR was only possible within the first 3 h after direct vaccine administration (Table 2), with exception of one skunk specimen taken at day 1 after vaccination. Interestingly, none of

Table 1
Results of challenge study after direct oral administration of SPBN GASGAS in foxes and striped skunks. (day p.v.) – day post vaccination; (n/N) – number of animals seroconverted and surviving rabies virus challenge, respectively.

Species	Dose (FFU)	No. animals	Challenge (day p.v.)	No. animals that seroconverted (>0.5 IU/ml)	VNA		Survived
					GMT	SD	
Red fox	$10^{6.5}$	6	98	6/6	3.12	2.81	6/6
Striped skunk	$10^{7.3}$	3	42	0/3	0.08	0.08	0/3
Striped skunk	$10^{8.0}$	5	56	0/5	0.16	0.09	2/5
Striped skunk	$10^{9.2}$	6	56	5/6	1.22	7.45	5/6

Table 2
Detection of SPBN GASGAS rabies virus in saliva swabs from red foxes and striped skunks by qRT-PCR and RTCIT. Red foxes and striped skunks received $10^{7.5}$ FFU SPBN GASGAS by direct oral instillation. (−) – negative; (+) – positive; blank space – not determined.

	PCR (Ct-values)								RTCIT							
Time post vacc.	0 h	1 h	2 h	3 h	24 h	48 h	72 h	96 h	0 h	1 h	2 h	3 h	24 h	48 h	72 h	96 h
Fox 1	–	28.3	33.4	31.1	–	–	–	Not taken	–	–	–	–				Not taken
Skunk 1	–	34.1	33.2	–	–	–	–		–	+	–					
Fox 2	–	31.4	33.0	34.4	–	–	–		–	–		–				
Skunk 2	–	31.9	34.4	31.5	–	–	–		–	+	–	–				
Fox 3	–	32.0	32.6	34.5	–	–	–	–	–	–	–	–				
Skunk 3	–	25.8	31.9	33.0	–	–	–	–	–	+	–	–				
Fox 4	–	24.9	35.3	–	–	–	–	–	–	–	–					
Skunk 4	–	27.9	29.3	31.2	34.3	–	–	–	–	+	+	+	–			

Table 3
Detection of SPBN GASGAS rabies virus in tissue samples of oropharyngeal tract from red foxes and striped skunks by qRT-PCR (ct-values) and RTCIT. Red foxes and striped skunks received $10^{7.5}$ FFU SPBN GASGAS by direct oral instillation. (−) – negative; (+) – positive; blank space – not determined.

		Day 3 post vaccination				Day 5 post vaccination			
		Fox 1	Skunk 1	Fox 2	Skunk 2	Fox 3	Skunk 3	Fox 4	Skunk 4
T. palatina	PCR	31.19	37.55	33.5	–	–	36.33	32.31	–
	RTCIT	+	+	+			–	+	
T. pharyngica	PCR	–	–	35.19	–	–	–	–	–
	RTCIT			–					
Tongue	PCR	37.74	–	20.83	–	27.59	–	–	31.80
	RTCIT	–		–		–			–
Ln. retropharyngeus	PCR	–	–	36.68	–	–	–	–	–
	RTCIT			–					
Ln. mandibularis	PCR	36.92	–	–	–	–	–	–	–
	RTCIT	–							
Parotis	PCR	–		–	–	–	–	–	–
	RTCIT								
Mucosa (d)	PCR	–	–	–	–	–	–	–	–
	RTCIT								
Mucosa (v)	PCR	26.91	–	–	–	–	–	–	–
	RTCIT	–							

the PCR positive saliva specimens from foxes was positive for viable virus detection by RTCIT, whereas 6 out of 12 saliva specimens from skunks were RTCIT positive. Detection of more infectious virus in skunks than in foxes within the first 3 h after vaccination indicated, that rapid virus inactivation by unknown skunk specific factors was not responsible for the low immunogenicity of the live virus vaccine. Notably, with the exception of one PCR positive saliva specimen from skunks at day 1 (24 h p.i.), neither in foxes nor in skunks positive saliva samples were detected at 24 h, 48 h, 72 h, and 96 h after vaccine administration, showing that no or very low virus amounts that remained below detection levels were shed into the saliva of immunized animals. Lack of virus in the saliva at 24 h to 96 h post vaccine administration raised the question whether vaccine virus did not efficiently infect cells in the oropharynx of both, fox and skunk, or whether dissemination in different organ tissues still occurred. Therefore, dissemination of the virus was followed by RT-PCR detection in organ tissues taken after day 3 and 5 post vaccination from 2 animals per host species and time point.

Rabies virus RNA was detected in 10 tissue samples from foxes including mucosa, lymph nodes, tongue and tonsils (Table 3). Notably, viable virus detection by RTCIT was only possible from the Tonsilla palatina at day 2 (both foxes) and day 3 (one of two foxes) post vaccination, indicating that the main site of vaccine virus replication is located in the Tonsilla palatina.

In striking contrast, RT-PCR screening for virus RNA in skunks was negative in most tissues (Table 3). Only one PCR positive tongue sample at day 5 post infection and two positive tonsils (one Tonsilla palatina at days 3 and 5, respectively) indicated that virus infection of screened tissues in the oropharynx was less efficient in skunks than in foxes. However, virus isolation from the RT-PCR positive skunk tonsil at day 3 showed that vaccine virus infection occurs in skunks, but obviously on a much lower level.

3.3. Vaccine virus infection is restricted to peripheral areas of the palatine tonsils

As palatine tonsils were identified as a most reliable replication site for SPBN GASGAS with presence of infectious virus even at days 3 and 5 post vaccination, the tropism of SPBN GASGAS in tonsil tissues from foxes was further investigated by immunofluorescence analysis of 100 μm thick vibratome slices stained with RABV specific antibodies. At day 3 post vaccination, foci of infected cells were detected in peripheral areas of the tonsils (Fig. 1A). Blue nuclei staining further indicated that the foci were restricted to the outer epithelium cell layer and were excluded from subepithelial and inner-/extra-follicular regions. Comparable distribution of SPBN GASGAS in the palatine tonsil of the second fox (not shown) indicated that the observed focal infection pattern in peripheral epithelium was characteristic for vaccine virus infection at day 3 after oral administration.

Analysis of fox tonsils at day 5 post infection (Fig. 1B) revealed loss of most infection foci at the epithelial region. Only single cells or smaller sized foci could be detected by immunofluorescence against RABV N protein. Similar to day 3, no infected cells were detected in inner tonsil areas of lymphoid follicles and extrafollicular regions. These data show that virus infected cells are largely removed from tonsil tissues until day 5 post infection.

4. Discussion

All available oral rabies vaccines are based on replication-competent viruses. It seems that for the induction of a protective long-lasting immune response, primary virus replication is needed. Due to the instability of the rabies virus, the gastro-intestinal tract will cause rapid antigen degradation. Hence the rabies virus vaccine must be taken up in the oral cavity for the development of an immune response. The first animal species targeted for oral vaccination against rabies was the red fox and coincidentally a relative low dose was required to induce protective immunity. However, the efficacy of oral rabies virus vaccines constructs like SPBN GASGAS is much less pronounced in other species like skunks [61], which was confirmed by our efficacy study. Only when an extreme high number of virus particles were administered, a detectable and protective immune response could be achieved in skunks (Table 1). The different time points of challenge chosen for foxes and skunks post vaccination in our studies are unlikely to have an influence on the outcome of the study as the immune response after oral vaccination with attenuated rabies virus vaccines was shown to be fully developed within 3–4 weeks [52,62,63]. Because the studies had a pilot character, the number of animals had to be kept to a minimum to follow the 3Rs principles. Therefore, our results could not be statistically evaluated for significance. However, the differences are in agreement with other published studies. This less

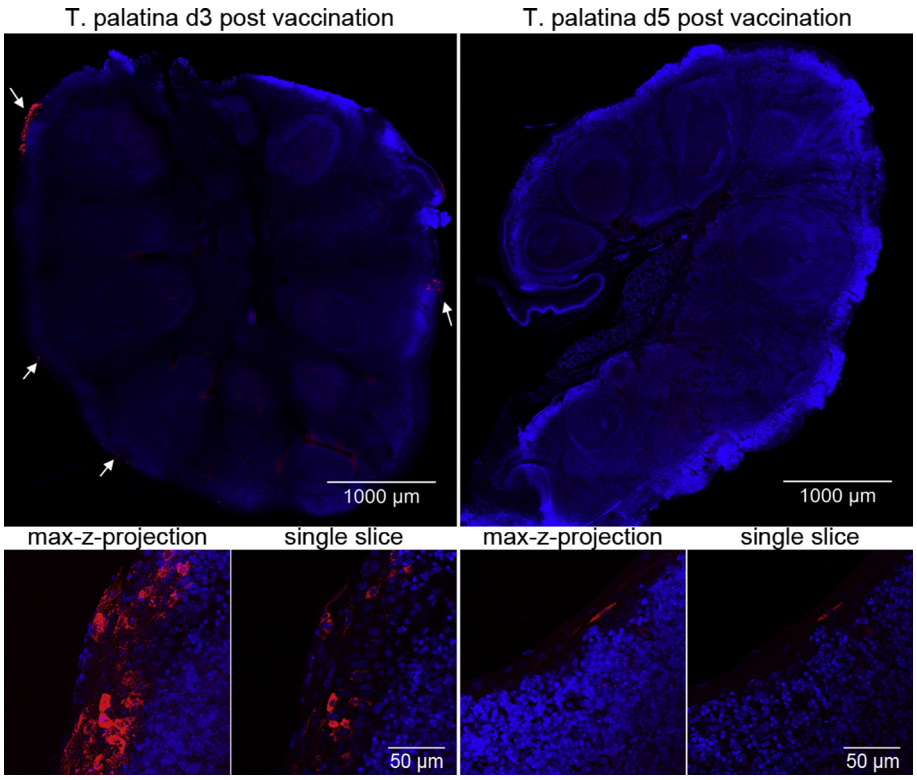

Fig. 1. Dissemination of SPBN GASGAS infected cells in palatine tonsils of red foxes. (A) Fluorescent images of red fox palatine tonsil sections (100 µm) reveal infection foci in epithelial regions (white arrows) at day 3 after oral inoculation with SPBN GASGAS. (B) At day 5 post vaccination, only single infected cells are present. RABV N protein: red, cell nuclei: blue.

pronounced efficacy does not seem to be restricted to this particular construct or other rabies virus vaccines but a more general observation, irrespective of the used virus backbone [14–20]. An exception could be a recombinant canine adenovirus 2 (CAV2) construct expressing the rabies virus glycoprotein. Almost all skunks that received a relatively low dose of this construct ($10^{7.0}$ TCID50) by direct oral administration seroconverted and were protected against a challenge infection [61]. However, pre-existing immunity against CAV2 has been shown to interfere with vaccination success [64]. This lack of efficacy seems to be associated specifically with the oral route of administration because skunks developed consistent high levels of rabies virus neutralizing antibodies when the vaccine construct SPBN GASGAS was administered intramuscularly (i.m.). Also, when vaccinated with an inactivated commercial rabies vaccine, striped skunks developed a typical rabies VNA response as observed in other carnivores [65]. To proof whether requirement for vaccine virus doses is not a result of low immunogenicity of SPBN GASGAS in skunks in general but more likely represents unknown host specific constraints for oral vaccination route, dissemination studies were conducted. The results showed, that using the same dose, vaccine absorption in oral cavity was much less pronounced in striped skunks than in red foxes (Table 3), which could be a possible explanation why skunks are extremely refractory to oral vaccination against rabies. Uptake by the oral route may also be influenced by the duration of exposure in the oral cavity, for example as a result of high saliva secretion and flow that may dilute the antigen or lead to swallowing of the antigen before it is absorbed [66,67]. However, it was actually shown that viable virus in the oral cavity is much longer detectable in the saliva of skunks than in foxes (Table 2). Possibly, a very early infection of lymphoreticular tissues of the oropharynx is decisive for subsequent vaccine efficacy, which suggests that those tissues of foxes are more susceptible regardless of the quick inactivation of viable vaccine virus. This might explain why none of the PCR positive saliva specimens from foxes, even with a relatively low ct-value, was positive for viable virus detection by RTCIT (Table 2). Therefore, it can also be excluded that potential viral inhibitory factors (microbial peptides, pH) in the oral cavity of skunks play a role in the reduced vaccine absorption. Considering the natural predominant route of rabies transmission, whereby the virus loaded saliva of an infected animal is transmitted during a biting incident, the presence of antimicrobial peptides and proteins in the oral cavity of skunks acting as rabies virus inhibitors, seems unlikely. However, the presence of salivary inhibitors including acidic proline-rich-proteins, albumins, lactoferrin, mucins and salivary agglutinins that may block cell binding and fusion through interaction in the saliva of skunks has not been investigated and can therefore not be excluded.

Like for oral CSF vaccine virus constructs [42–44] the results of this study confirm previous studies that identified the palatine tonsils as the primary site of rabies vaccine virus uptake compared to the oral mucosal epithelium [39–41], although rabies vaccine virus was isolated on some occasion from the oral mucosa in this and other studies [40]. Direct virus vaccine transport across the oral mucosa is possible though (1) transcytosis through epithelial cells, (2) epithelial transmigration of infected donor cells, or (3) uptake during physical breaches. The latter mechanism can be excluded, as an artificial disruption of the oral mucosa epithelial barrier in skunks, providing the vaccine virus a direct access to the mucosal microcirculation prior to direct oral RABV vaccine administration, did not improve the immune response (unpublished results). The palatine tonsils as primary site for vaccine uptake seem evident, since the tonsils are a major component of the mucosa-associated lymphoid tissue (MALT) [68,69].

It remains to be resolved, why rabies virus vaccine uptake is relatively inefficient in skunks compared to other animal species like

foxes. The demonstration of a comparable anatomic configuration of Waldeyer's tonsillar ring with a dominating palatine tonsil [45,47], as well as the histologic confirmation that it represents a crypt-free tonsil covered within its mucosal fossa in both, red foxes and skunks (Supplementary Fig. 1), suggests that the morphology of the lymphoreticular tissue of the pharynx cannot explain the observed difference in vaccine uptake.

Hence, further studies will have to focus on vaccine virus entry and replication in the palatine tonsils of different reservoir species. For this purpose, *in vivo* tracking studies should be conducted to identify the main target cells of virus entry in the palatine tonsils. It is claimed that the covering epithelium of the palatine tonsils is unsuitable for antigen uptake and that specialized cells similar to M-cells of the Peyers' patches play an important role [70]. Understanding the mechanisms of antigen uptake, transport and initial replication may lead to concepts improving rabies virus vaccine uptake in reservoir species, refractory to the oral route of vaccine administration and to improved vaccine formulation containing potent and safe mucosal adjuvants or mucoadhesives [67,71].

Author contributions

Drafted the concept and design of the study: AV, AN, SF and TM; acquired, analysed, and interpreted data: AV, CMF, BH, CK, SN, TN, JPT, VtK, RU, SF, TM; drafted the article and revised it critically for important intellectual content: all; all authors have approved the final manuscript to be submitted.

Conflict of interest

This study is part of a research cooperation on the mechanisms of oral vaccination of wildlife and derived immunity between the Friedrich-Loeffler-Institut and the IDT Biologika GmbH, which has been developing the SPBN GASGAS used in this study as a novel oral rabies virus vaccine.

Acknowledgements

The authors would like to acknowledge the technical assistance of Jeannette Kliemt, Christian Kaiser, Antje Kretzschmar and Steffen Ortmann. At the Friedrich-Loeffler-Institut, this study was financially supported by research grant Ri-0360 through IDT Biologika GmbH and by an intramural collaborative research grant. The continuous support by the German Federal Ministry of Food and Agriculture is gratefully acknowledged.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2017.06.022.

References

[1] Steck F, Wandeler A, Bichsel P, Capt S, Hafliger U, Schneider L. Oral immunization of foxes against rabies. Laboratory and field studies. Comp Immunol Microbiol Infect Dis 1982;5(1–3):165–71.
[2] Nyberg M, Kulonen K, Neuvonen E, Ek-Kommonen C, Nuorgam M, Westerling B. An Epidemic of sylvatic rabies in Finland – descriptive epidemiology and results of oral vaccination. Acta Vet Scand 1992;33(1):43–57.
[3] Rupprecht CE, Wiktor TJ, Johnston DH, Hamir AN, Dietzschold B, Wunner WH, et al. Oral immunization and protection of raccoons (Procyon lotor) with a vaccinia-rabies glycoprotein recombinant virus vaccine. Proc Natl Acad Sci U S A Biol Sci 1986;83:7947–50.
[4] Rupprecht CE, Dietzschold B, Cox JH, Schneider LG. Oral vaccination of raccoons (procyon-lotor) with an attenuated (Sad-B19) rabies virus-vaccine. J Wildl Dis 1989;25(4):548–54.
[5] Meehan SK. Rabies epizootic in coyotes combated with oral vaccination program. J Am Vet Med Assoc 1995;206(8):1097–9.
[6] Fearneyhough MG, Wilson PJ, Clark KA, Smith DR, Johnston DH, Bradley N, et al. Results of an oral rabies vaccination program for coyotes. J Am Vet Med Assoc 1998;212(4):498–502.
[7] Sidwa TJ, Wilson PJ, Moore GM, Oertli EH, Hicks BN, Rohde RE, et al. Evaluation of oral rabies vaccination programs for control of rabies epizootics in coyotes and gray foxes: 1995–2003. J Am Vet Med Assoc 2005;227(5):785–92.
[8] Rosatte RC, Power MJ, MacInnes CD, Campbell JB. Trap-vaccinate-release and oral vaccination for rabies control in urban skunks, raccoons and foxes. J Wildl Dis 1992;28(4):562–71. http://dx.doi.org/10.7589/0090-3558-28.4.562. PubMed PMID: 1474654.
[9] Blanton JD, Meadows A, Murphy S, Manangan JS, Hanlon CA, Faber ML, et al. Vaccination of small asian mongoose (herpestes javanicus) against rabies. J Wildl Dis 2006;42(3):663–6.
[10] Vos A, Kretzschmar A, Ortmann S, Lojkic I, Habla C, Muller T, et al. Oral vaccination of captive small Indian mongoose (Herpestes auropunctatus) against rabies. J Wildl Dis 2013;49(4):1033–6. http://dx.doi.org/10.7589/2013-02-035. Epub 2014/02/08. PubMed PMID: 24502736.
[11] Haddad N, Khelifa RB, Matter H, Kharmachi H, Aubert MFA, Wandeler A, et al. Assay of oral vaccination of dogs against rabies in Tunisia with the vaccinal strain Sad(Bern). Vaccine 1994;12(4):307–9.
[12] Aylan O, Vos A. Efficacy studies with SAD B19 in Turkish dogs. J ETLIK Veterin Microbiol 1998;9(Special Issue Oral vaccination of dogs against rabies):93–102.
[13] Rupprecht CE, Hanlon CA, Blanton JD, Manangan JS, Morrill P, Murphy S, et al. Oral vaccination of dogs with recombinant rabies virus vaccines. Virus Res 2005;111(1):101–5.
[14] Brown LJ, Rosatte RC, Fehlner-Gardiner C, Ellison JA, Jackson FR, Bachmann P, et al. Oral vaccination and protection of striped skunks (Mephitis mephitis) against rabies using ONRAB(R). Vaccine 2014;32(29):3675–9. http://dx.doi.org/10.1016/j.vaccine.2014.04.029. PubMed PMID: 24814554.
[15] Charlton KM, Artois M, Prevec L, Campbell JB, Casey GA, Wandeler AI, et al. Oral rabies vaccination of skunks and foxes with a recombinant human adenovirus vaccine. Arch Virol 1992;123:169–79.
[16] Grosenbaugh DA, Maki JL, Rupprecht CR, Wall DK. Rabies challenge of captive striped skunks (mephitis mephitis) following oral administration of a live vaccinia-vectored rabies vaccine. J Wildl Dis 2007;43(1):124–8.
[17] Rupprecht CE, Charlton KM, Artois M, Casey GA, Webster WA, Campbell JB, et al. Ineffectiveness and comparative pathogenicity of attenuated rabies virus-vaccines for the striped skunk (mephitis-mephitis). J Wildl Dis 1990;26(1):99–102.
[18] Tolson ND, Charlton KM, Lawson KF, Campbell JB, Stewart RB. Studies of Era/Bhk-21 rabies vaccine in skunks and mice. Can J Vet Res 1988;52(1):58–62.
[19] Tolson ND, Charlton KM, Stewart RB, Casey GA, Webster WA, MacKenzie K, et al. Mutants of rabies viruses in skunks: immune response and pathogenicity. Can J Vet Res 1990;54(1):178–83.
[20] Vos A, Pommerening E, Neubert L, Kachel S, Neubert A. Safety studies of the oral rabies vaccine SAD B19 in striped skunk (Mephitis mephitis). J Wildl Dis 2002;38(2):428–31.
[21] Freuling C, Hampson K, Selhorst T, Schröder R, Meslin FX, Mettenleiter TC, et al. The elimination of fox rabies from Europe: determinants of success and lessons for the future. Philos Trans R Soc Lond B Biol Sci 2013;368.
[22] Müller TF, Schroder R, Wysocki P, Mettenleiter TC, Freuling CM. Spatio-temporal use of oral rabies vaccines in fox rabies elimination programmes in Europe. PLOS Negl Trop Dis 2015;9(8):e0003953. http://dx.doi.org/10.1371/journal.pntd.0003953. PubMed PMID: 26280895; PubMed Central PMCID: PMC4539187.
[23] 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(1):157–67. http://dx.doi.org/10.7589/0090-3558-48.1.157. Epub 2012/01/17. PubMed PMID: 22247384.
[24] Mainguy J, Fehlner-Gardiner C, Slate D, Rudd RJ. Oral rabies vaccination in raccoons: comparison of ONRAB(R) and RABORAL V-RG(R) vaccine-bait field performance in Quebec, Canada and Vermont, USA. J Wildl Dis 2013;49(1):190–3. http://dx.doi.org/10.7589/2011-11-342. Epub 2013/01/12. PubMed PMID: 23307388.
[25] MacInnes CD, Smith SM, Tinline RR, Ayers NR, Bachmann P, Ball DG, et al. Elimination of rabies from red foxes in eastern Ontario. J Wildl Dis 2001;37(1):119–32. http://dx.doi.org/10.7589/0090-3558-37.1.119. PubMed PMID: 11272485.
[26] Rosatte RC, Power MJ, Donovan D, Davies JC, Allan M, Bachmann P, et al. Elimination of arctic variant rabies in red foxes, metropolitan Toronto. Emerg Infect Dis 2007;13(1):25–7. http://dx.doi.org/10.3201/eid1301.060622. Epub 2007/03/21. PubMed PMID: 17370512; PubMed Central PMCID: PMC2725809.
[27] Slate D, Algeo TP, Nelson KM, Chipman RB, Donovan D, Blanton JD, et al. Oral rabies vaccination in north america: opportunities, complexities, and challenges. PLOS Negl Trop Dis 2009;3(12):e549. http://dx.doi.org/10.1371/journal.pntd.0000549. Epub 2009/12/23, PubMed PMID: 20027214; PubMed Central PMCID: PMC2791170.
[28] Sobey KG, Walpole AA, Rosatte R, Fehlner-Gardiner C, Donovan D, Bachmann P, et al. An assessment of ONRAB oral rabies vaccine persistence in free-ranging mammal populations in Ontario, Canada. Vaccine 2013;31(17):2207–13. http://dx.doi.org/10.1016/j.vaccine.2013.02.057. Epub 2013/03/19, PubMed PMID: 23499602.

[29] Kaden V, Lange E, Fischer U, Strebelow G. Oral immunisation of wild boar against classical swine fever: evaluation of the first field study in Germany. Vet Microbiol 2000;73(2–3):239–52. PubMed PMID: 10785331.

[30] Romelt M, Klingelhefer I, Konig A, Braun B, Reiner G. Classical swine fever in wild boar in rhineland-palatinate: evaluation of the official control measures from 2005–2011. Berl Munch Tierarztl Wochenschr 2015;128(11–12):507–16. PubMed PMID: 26697717.

[31] Moennig V. The control of classical swine fever in wild boar. Front Microbiol 2015;6:1211. http://dx.doi.org/10.3389/fmicb.2015.01211. PubMed PMID: 26594202; PubMed Central PMCID: PMC4635204.

[32] von Ruden S, Staubach C, Kaden V, Hess RG, Blicke J, Kuhne S, et al. Retrospective analysis of the oral immunisation of wild boar populations against classical swine fever virus (CSFV) in region Eifel of Rhineland-Palatinate. Vet Microbiol 2008;132(1–2):29–38. http://dx.doi.org/10.1016/j.vetmic.2008.04.022. PubMed PMID: 18534790.

[33] Rocke TE, Kingstad-Bakke B, Berlier W, Osorio JE. A recombinant raccoon poxvirus vaccine expressing both yersinia pestis F1 and truncated V antigens protects animals against lethal plague. Vaccines 2014;2(4):772–84. http://dx.doi.org/10.3390/vaccines2040772. PubMed PMID: 26344891; PubMed Central PMCID: PMC4494250.

[34] Rocke TE, Pussini N, Smith SR, Williamson J, Powell B, Osorio JE. Consumption of baits containing raccoon pox-based plague vaccines protects black-tailed prairie dogs (Cynomys ludovicianus). Vector Borne Zoonotic Dis 2010;10(1):53–8. http://dx.doi.org/10.1089/vbz.2009.0050. PubMed PMID: 20158332.

[35] Mencher JS, Smith SR, Powell TD, Stinchcomb DT, Osorio JE, Rocke TE. Protection of black-tailed prairie dogs (Cynomys ludovicianus) against plague after voluntary consumption of baits containing recombinant raccoon poxvirus vaccine. Infect Immun 2004;72(9):5502–5. http://dx.doi.org/10.1128/IAI.72.9.5502-5505.2004. PubMed PMID: 15322054; PubMed Central PMCID: PMC517477.

[36] Baer GM, Broderson JR, Yager PA. Determination of the site of oral rabies vaccination. Am J Epidemiol 1975;101(2):160–4.

[37] Rupprecht CE, Hamir AN, Johnston DH, Koprowski H. Efficacy of a vaccinia-rabies glycoprotein recombinant virus vaccine in raccoons (procyon lotor). Rev Infect Dis 1988;10(4):803–9.

[38] Thomas I, Brochier B, Languet B, Blancou J, Peharpe D, Kieny MP, et al. Primary multiplication site of the vaccinia - rabies glycoprotein recombinant virus administered to foxes by the oral route. J Gen Virol 1990;71:37–42.

[39] Orciari LA, Niezgoda M, Hanlon CA, Shaddock JH, Sanderlin DW, Yager PA, et al. Rapid clearance of SAG-2 rabies virus from dogs after oral vaccination. Vaccine 2001;19(31):4511–8.

[40] Vos A, Neubert A, Aylan O, Schuster P, Pommerening E, Müller T, et al. An update on safety studies of SAD B 19 rabies virus vaccine in target and non-target species. Epidemiol Infect 1999;123(1):165–75.

[41] Zhou M, Wang L, Zhou S, Wang Z, Ruan J, Tang L, et al. Recombinant rabies virus expressing dog GM-CSF is an efficacious oral rabies vaccine for dogs. Oncotarget. 2015;6(36):38504–16. http://dx.doi.org/10.18632/oncotarget.5904. PubMed PMID: 26436700.

[42] Koenig P, Hoffmann B, Depner KR, Reimann I, Teifke JP, Beer M. Detection of classical swine fever vaccine virus in blood and tissue samples of pigs vaccinated either with a conventional C-strain vaccine or a modified live marker vaccine. Vet Microbiol 2007;120(3–4):343–51. http://dx.doi.org/10.1016/j.vetmic.2006.10.034. PubMed PMID: 17147979.

[43] Tignon M, Kulcsar G, Haegeman A, Barna T, Fabian K, Levai R, et al. Classical swine fever: comparison of oronasal immunisation with CP7E2alf marker and C-strain vaccines in domestic pigs. Vet Microbiol 2010;142(1–2):59–68. http://dx.doi.org/10.1016/j.vetmic.2009.09.044. PubMed PMID: 19857935.

[44] Drager C, Petrov A, Beer M, Teifke JP, Blome S. Classical swine fever virus marker vaccine strain CP7_E2alf: Shedding and dissemination studies in boars. Vaccine 2015;33(27):3100–3. http://dx.doi.org/10.1016/j.vaccine.2015.04.103. PubMed PMID: 25980427.

[45] Zimmermann G. Über den Waldeyer'schen lymphatischen Rachenring.. Arch Wiss Prakt Tierheilk 1934;67(141–153).

[46] Vollmerhaus B. Zur vergleichenden Nomenklatur des lymphoepithelialen Rachenringes der Haussäugetiere und des Menschen. Zbl Vet Med 1959;6:82–9.

[47] Graff S. Descriptive anatomy of the pharyngeal tonsil and its place in Waldemyer's fauces. Anat Anz. 1954;100(8–10):120–33. PubMed PMID: 13148631.

[48] Schummer A, Wilkens H, Vollmerhaus B, Habermehl KH. The circulatory system, the skin, and the cutaneous organs of the domestic mammals. US: Springer; 1981.

[49] Faber M, Pulmanausahakul R, Hodawadekar SS, Spitsin S, McGettigan JP, Schnell MJ, et al. Overexpression of the rabies virus glycoprotein results in enhancement of apoptosis and antiviral immune response. J Virol 2002;76(7):3374–81. PubMed PMID: 11884563; PubMed Central PMCID: PMC136034..

[50] Faber M, Bette M, Preuss MAR, Pulmanausahakul R, Rehnelt J, Schnell MJ, et al. Overexpression of tumor necrosis factor alpha by a recombinant rabies virus attenuates replication in neurons and prevents lethal infection in mice. J Virol 2005;79(24):15405–16. http://dx.doi.org/10.1128/Jvi.79.24.15405-15416.2005. PubMed PMID: WOS:000234276700042.

[51] Vos A, Neumann G, Hundt B, Neubert A. Attenuated vaccines for veterinary use. In: Rupprecht CE, Nagarajan T, editors. Current laboratory techniques in rabies diagnosis, research and prevention, vol. 1. San Diego: Elsevier Academic Press; 2015. p. 237–44.

[52] Neubert A, Schuster P, Muller T, Vos A, Pommerening E. Immunogenicity and efficacy of the oral rabies vaccine SAD B19 in foxes. J Veter Med Ser B-Infect Dis Veterin Public Health 2001;48(3):179–83.

[53] Dean DJ, Abelseth MK, Athanasiu P. The fluorescence antibody test. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. Geneva: World Health Organization; 1996. p. 88–93.

[54] Hoffmann B, Freuling CM, Wakeley PR, Rasmussen TB, Leech S, Fooks AR, et al. Improved safety for molecular diagnosis of classical rabies Viruses by use of a TaqMan real-time reverse transcription-PCR "Double Check" Strategy. J Clin Microbiol 2010;48(11):3970–8. http://dx.doi.org/10.1128/Jcm.00612-10. PubMed PMID: ISI:000283588500021.

[55] Webster WA. A tissue culture infection test in routine rabies diagnosis. Can J Vet Res 1987;51:367–9.

[56] Webster WA, Casey GA. Virus isolation in neuroblastoma cell culture. In: Meslin FX, Kaplan MM, Koprowski H, editors. Laboratory techniques in rabies. Geneva: World Health Organization; 1996. p. 93–104.

[57] Smith JS, Yager PA, Baer GM. A rapid reproducible test for determining rabies neutralizing antibody. Bull World Health Organ 1973;48:535–41.

[58] Cox JH, Schneider LG. Prophylactic immunization of humans against rabies by intradermal inoculation of human diploid cell culture vaccine. J Clin Microbiol 1976;3(2):96–101.

[59] Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods 2012;9(7):671–5. PubMed PMID: 22930834.

[60] Blanton JD, Self J, Niezgoda M, Faber ML, Dietzschold B, Rupprecht C. Oral vaccination of raccoons (Procyon lotor) with genetically modified rabies virus vaccines. Vaccine 2007;25(42):7296–300.

[61] Henderson H, Jackson F, Bean K, Panasuk B, Niezgoda M, Slate D, et al. Oral immunization of raccoons and skunks with a canine adenovirus recombinant rabies vaccine. Vaccine 2009;27(51):7194–7. http://dx.doi.org/10.1016/j.vaccine.2009.09.030. PubMed PMID: 19925952.

[62] Cliquet F, Guiot AL, Munier M, Bailly J, Rupprecht CE, Barrat J. Safety and efficacy of the oral rabies vaccine SAG2 in raccoon dogs. Vaccine 2006;24(20):4386–92.

[63] Schuster P, Müller T, Vos A, Selhorst T, Neubert L, Pommerening E. Comparative immunogenicity and efficacy studies with oral rabies virus vaccine SAD P5/88 in raccoon dogs and red foxes. Acta Vet Hung 2001;49(3):285–90.

[64] Wright N, Jackson FR, Niezgoda M, Ellison JA, Rupprecht CE, Nel LH. High prevalence of antibodies against canine adenovirus (CAV) type 2 in domestic dog populations in South Africa precludes the use of CAV-based recombinant rabies vaccines. Vaccine 2013;31(38):4177–82. http://dx.doi.org/10.1016/j.vaccine.2013.06.089. Epub 2013 Jul 16.

[65] Rosatte RC, Howard DR, Campbell JB, MacInnes CD. Intramuscular vaccination of skunks and raccoons against rabies. J Wildl Dis 1990;26(2):225–30.

[66] Nittayananta W. Transmission of HIV-1 via oral route: why is it difficult? J Dent Assoc Thai 2009;59(4):201–12.

[67] Kraan H, Vrieling H, Czerkinsky C, Jiskoot W, Kersten G, Amorij JP. Buccal and sublingual vaccine delivery. J Control Release: Off J Control Release Soc 2014;190:580–92. http://dx.doi.org/10.1016/j.jconrel.2014.05.060. PubMed PMID: 24911355.

[68] Casteleyn C, Breugelmans S, Simoens P, Van den Broeck W. The tonsils revisited: review of the anatomical localization and histological characteristics of the tonsils of domestic and laboratory animals. Clin Dev Immunol 2011;2011:472460. http://dx.doi.org/10.1155/2011/472460. PubMed PMID: 21869895; PubMed Central PMCID: PMC3159307.

[69] Cho KA, Kim JY, Kim HS, Ryu KH, Woo SY. Tonsil-derived mesenchymal progenitor cells acquire a follicular dendritic cell phenotype under cytokine stimulation. Cytokine 2012;59(2):211–4. http://dx.doi.org/10.1016/j.cyto.2012.04.016. PubMed PMID: 22578801.

[70] Bernstein JM, Yamanaka N, Nadal D. Immunology of the tonsils and adenoids. In: Ogra PL, Mestecky J, Lamm ME, Strober W, McGhee JR, Bienenstock J, editors. Handbook of mucosal immunology. San Diego: Academic Press Inc.; 1994. p. 625–40.

[71] Zhou M, Zhang G, Ren G, Gnanadurai CW, Li Z, Chai Q, et al. Recombinant rabies viruses expressing GM-CSF or flagellin are effective vaccines for both intramuscular and oral immunizations. PLoS ONE 2013;8(5):e63384. http://dx.doi.org/10.1371/journal.pone.0063384. PubMed PMID: 23700422; PubMed Central PMCID: PMC3658976.