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ONRAB® oral rabies vaccine is shed from, but does not persist in, captive mammals

Kirk G. Sobey [a,1], Sarah E. Jamieson [a,*], Aaron A. Walpole [b], Rick C. Rosatte [a,2], Dennis Donovan [a,2], Christine Fehlner-Gardiner [c], Susan A. Nadin-Davis [c], J. Chris Davies [a,2], Christopher J. Kyle [d]

[a] Wildlife Research and Monitoring Section, Ontario Ministry of Natural Resources and Forestry, 2140 East Bank Drive, Trent University, Peterborough, Ontario K9L 0G2, Canada
[b] Wildlife Section, Ontario Ministry of Natural Resources and Forestry, 300 Water Street, Peterborough, Ontario K9J 8M5, Canada
[c] Canadian Food Inspection Agency, Ottawa Laboratory Fallowfield, PO Box 11300, Station H, Nepean, Ontario K2H 8P9, Canada
[d] Natural Resources DNA Profiling and Forensics Centre, 2140 East Bank Drive, DNA Building, Trent University, Peterborough, Ontario K9J 7B8, Canada

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ABSTRACT

ONRAB® is a human adenovirus rabies glycoprotein recombinant vaccine developed to control rabies in wildlife. To support licensing and widespread use of the vaccine, safety studies are needed to assess its potential residual impact on wildlife populations. We examined the persistence of the ONRAB® vaccine virus in captive rabies vector and non-target mammals. This research complements work on important rabies vector species (raccoon, striped skunk, and red fox) but also adds to previous findings with the addition of some non-target species (Virginia opossum, Norway rats, and cotton rats) and a prolonged period of post vaccination monitoring (41 days). Animals were directly inoculated orally with the vaccine and vaccine shedding was monitored using quantitative real-time PCR applied to oral and rectal swabs. ONRAB® DNA was detected in both oral and rectal swabs from 6 h to 3 days post-inoculation in most animals, followed by a resurgence of shedding between days 17 and 34 in some species. Overall, the duration over which ONRAB® DNA was detectable was shorter for non-target mammals, and by day 41, no animal had detectable DNA in either oral or rectal swabs. All target species, as well as cotton rats and laboratory-bred Norway rats, developed robust humoral immune responses as measured by competitive ELISA, with all individuals being seropositive at day 31. Similarly, opossums showed good response (89% seropositive; 8/9), whereas only one of nine wild caught Norway rats was seropositive at day 31. These results support findings of other safety studies suggesting that ONRAB® does not persist in vector and non-target mammals exposed to the vaccine. As such, we interpret these data to reflect a low risk of adverse effects to wild populations following distribution of ONRAB® to control sylvatic rabies.

1. Introduction

Globally, rabies has attracted a great deal of public and scientific interest given its significant human health and mortality risks [1], the economic costs of post-exposure treatment for humans and control in domestic animals [2,3], and the potential threats that it poses to rare wildlife populations [4,5]. Understanding of the rabies virus and development of effective measures to control its spread in wildlife populations has grown considerably over the past few decades (reviewed by Rosatte [6]). Initial attempts by wildlife managers and government agencies to control rabies included invasive and costly approaches such as vector species depopulation and capturing of live animals for parenteral vaccination. While some of these efforts were effective (e.g., Rosatte et al. [7]), in many situations these strategies have proven less successful, publicly unpopular, and/or impractical at large spatial scales [8]. In recent years, evolving rabies control strategies have coalesced around oral rabies vaccination of wildlife populations using vaccine baits. Oral vaccination strategies operate on the premise that if a sufficient proportion of the target population consumes

Abbreviations: Ct values, cycle threshold value; ONRAB®, Ontario Rabies Vaccine Bait (AdRG1.3 baits); ERA, Evelyn Rokitnicki Abelseth; SAD strains, Street Alabama Dufferin strains.

* Corresponding author.
E-mail addresses: sarah.jamieson@ontario.ca (S.E. Jamieson), aaron.walpole@ontario.ca (A.A. Walpole), rcr99@nexicom.net (R.C. Rosatte), ddonovan2421@gmail.com (D. Donovan), christine.fehlner-gardiner@canada.ca (C. Fehlner-Gardiner), susan.nadin-davis@canada.ca (S.A. Nadin-Davis), jchris.davies@bell.net (J.C. Davies), christopherkyle@trentu.ca (C.J. Kyle).

[1] This research was directed by our friend and colleague, Kirk Sobey, who passed away suddenly during the spring of 2011.

[2] Dr. Rick Rosatte, Dennis Donovan, and Chris Davies retired from the Ontario Ministry of Natural Resources and Forestry in 2014, 2016, and 2018, respectively.

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the vaccine baits and subsequently develop immunity through the generation of specific immune responses against rabies virus proteins, then virus transmission networks will be disrupted, and rabies can be eliminated from an area [9].

In North America, attempts to control terrestrial rabies are challenged by the multiple rabies virus variants that circulate sympatrically and the numerous species occupying broad and diverse landscapes that are vulnerable to the disease [8]. While rabies virus can infect a wide range of mammalian species, certain species including the red fox (*Vulpes vulpes*), Arctic fox (*V. lagopus*), gray fox (*Urocyon cinereoargenteus*), coyote (*Canis latrans*), raccoon (*Procyon lotor*), small Indian mongoose (*Herpestes auropunctatus*) and striped skunk (*Mephitis mephitis*: herein referred to as skunks) act as disease reservoirs for one or more particular viral variants that are present in North America (e.g., raccoon rabies virus variant; [10]). Consequently, successful control of wildlife rabies requires a vaccine that can effectively immunize a suite of target species, can be distributed across a large geographic scale, and is economically practical. Several attenuated live virus vaccines, such as ERA and SAD strains, have been used successfully to control fox rabies both in Canada [11] and Europe [12] but due to their residual pathogenicity in target and non-target animals [13,14] alternative vaccines are now preferred. In Europe, safer live attenuated vaccines such as SAG2 have also been employed in addition to a vaccinia virus rabies glycoprotein recombinant vaccine, Raboral V-RG® [15]. However, recently in the European Union, Rabitec (SPBN GASGAS) has been licensed for control of rabies in foxes and raccoon dogs (*Nyctereutes procyonoides*) [16]. While application of Raboral V-RG® in the United States has contributed to prevention of the westward expansion of the raccoon virus variant [8] and has been effective in eliminating the dog-coyote virus variant and controlling the gray fox virus variant in southern states [17], variable antibody response to this vaccine has been observed in other campaigns [18–20]. For example, 3.6 million Raboral V-RG® baits were distributed at densities of 75 and 150 baits/km^2 in eastern Ontario between 1999 and 2006, and the seropositivity rate for rabies virus-binding antibodies was between 7% and 28% in raccoons and 2% and 16% in skunks, when a competitive enzyme-linked immunosorbent assay (cELISA) cut-off value corresponding to a minimum neutralizing titer of 0.5 IU/ml was used (cELISA cut-off = 20%) [20]. A recent review of Raboral V-RG® for oral vaccination of wildlife found that in USDA Rabies Management Program raccoon vaccination campaigns from 2008 to 2011, the proportion of seropositive raccoons (rabies virus neutralizing antibody titer $\geq$ 0.05 IU/mL) ranged annually from 29% ± 14% to 37% ± 17%, with wide variation in ranges [17]. Additionally, concerns regarding the safety of Raboral V-RG® for human subjects who are either immunocompromised or contra-indicated for vaccination against smallpox have been raised in response to rare incidents of human infection by V-RG [21]. These concerns around use of Raboral V-RG® and the limited efficacy of some attenuated live vaccines for some host species [22], have prompted research into other vaccines that can effectively immunize a suite of mammals against multiple rabies virus variants without residual negative impacts to wildlife and humans [9].

Adenoviruses are non-enveloped viral pathogens, that have a linear double stranded DNA genome, and that can infect a wide range of species and tissues [23]. Over 70 human adenovirus (HAd) types have been identified [24], most of which are generally limited in their pathogenicity [23,24]. Accordingly, the application of adenoviruses as vectors for transduction of foreign genetic material has attracted significant interest due to their relative safety and the potential for extensive engineering of their genomes [24]. Furthermore, their application for oral rabies vaccination is enhanced by their genetic stability and their ability to infect via the oral route [25]. Previous studies on several HAd5 constructs expressing a rabies virus glycoprotein demonstrated their potential utility as a vaccine to control rabies [25–28]; one of these, previously known as AdRG1.3, and subsequently named ONRAB® [29], is the subject of this study.

During the summers of 2006 and 2007, Rosatte and colleagues [29] initiated field trials to test the efficacy of ONRAB® in free-ranging raccoons and skunks in Ontario, Canada. Since that time, similar field trials were conducted in Québec, Canada [30], New Brunswick, Canada [18], the Northeastern USA [31], and West Virginia, USA [32]. Furthermore, captive trials exploring persistence and shedding of ONRAB® were conducted on a range of avian and mammalian species common in North America and found that when ONRAB® was orally instilled into captive animals vaccine DNA became undetectable in oral and rectal swabs usually by day 7, or earlier [27,33]. In a study investigating the persistence of ONRAB® in free-ranging mammals collected from ONRAB®-treated areas in Ontario, Sobey and colleagues [34] concluded that the vaccine persisted for only a short period in wild mammals since none of the samples collected contained measurable quantities of ONRAB® DNA; however it should be noted that contact with the vaccine by the animals sampled could not be established in that study. While these studies build on previous findings (e.g., Charlton et al. [35], Prevec et al. [25]), suggesting that there would be minimal risk of negative impacts to wildlife populations following distribution of ONRAB® oral rabies vaccine baits in the natural environment, further studies in target and non-target mammals are important to inform potential licensing of the vaccine for distribution in other jurisdictions.

This work expands upon these studies by examining ONRAB® persistence and shedding by target and non-target species over a prolonged temporal scale (41-day period versus four to 29 days by Knowles and colleagues [27] and Fry and colleagues [33], depending on species and type of sampling) and with more frequent swab sampling (oral and rectal). Experiments were conducted on three common rabies vector species- raccoon, skunk, and red fox and three non-vector species - Norway rats (*Rattus norvegicus*), Virginia opossums (*Didelphis virginiana*, herein referred to as opossums), and cotton rats (*Sigmodon hispidus*). Of these species, the Norway rats is the only one not tested in previous safety studies and it was included here because these animals can reach very high densities in North America [36] and are thus expected to encounter baits in the field. Moreover, the recent outbreak of raccoon strain rabies in Ontario, Canada, has resulted in high numbers of ONRAB® baits being distributed in many suburban and urban areas [37], where, due to their commensal nature, Norway rats are one of the more likely non-target species to encounter the baits. Opossums were included in the study as they are expanding their distribution northward [38,39], thereby posing an increased risk of virus and disease spread into Canada and have been observed consuming oral baits in the field [40,41]. Lastly, cotton rats were of interest as they have been developed as an animal model of infection by human adenovirus [42].

2. Materials and methods

2.1. Animal care and handling

Three rabies vector species, raccoon, skunk, and red fox, and three non-target species, opossum, Norway rat, and cotton rat, were included in the study (Table 1). Each species was represented by a group of ten individuals, with the exception of cotton rats (N = 9), and Norway rats for which two groups (laboratory sourced and wild caught, N = 10 and 9, respectively) were studied (Table 1). Animals were housed individually in sheltered species appropriate cages at outdoor ambient temperatures during September to

Table 1
Origin of animals treated with ONRAB® oral rabies vaccine.

Species	Source	N
Raccoon (*Procyon lotor*)	Wild trapped[1]	10
Striped skunk (*Mephitis mephitis*)	Ruby's Fur Farm[2]	10
Red fox (*Vulpes vulpes*)	Yurick Fur Farm[3]	10
Virginia opossum (*Didelphis virginiana*)	Wild trapped[1]	10
Norway rat (*Rattus norvegicus*)	Wild trapped[1]	9
Norway rat (*R. norvegicus*)	Charles River Laboratories[4]	10
Cotton rat (*Sigmodon hispidus*)	Harlan Laboratories[5]	9

[1] Captured near Toronto, Ontario, Canada and released at capture site.
[2] New Sharon, Iowa, U.S.A.
[3] Lindsay, Ontario, Canada.
[4] St-Constant, Quebec, Canada.
[5] Dublin, Virginia, U.S.A.

November 2010 at the Ontario Ministry of Natural Resources and Forestry's Animal Care Facility in Codrington, Ontario. All animal handling, husbandry, and maintenance procedures were approved by the Ontario Ministry of Natural Resources and Forestry's Animal Care Committee in accordance with the Canadian Council on Animal Care guidelines (permit # #10–222).

2.2. Vaccine administration

The vaccine, ONRAB®, was prepared at the National Research Council Biotechnology Research Institute in Montréal, Québec as described by Shen and colleagues [26] and Rosatte and colleagues [29]. The vaccine was extracted from bait packages and contained a minimum titer of $10^{10.3}$ Tissue Culture Infectious Dose 50% (TCID50/mL). Using a catheter attached to a syringe, raccoons, opossums, skunks, and red foxes were orally administered 1.8 mL of vaccine, corresponding to the volume in a bait. Norway rats and cotton rats were orally administered a 1/10 dose (0.18 mL) due to their much smaller size. Animals were manually restrained during vaccine administration.

2.3. ONRAB® virus detection in swab samples with quantitative real-time PCR (qPCR)

Following vaccine administration, animals were physically immobilized for the sample collection. Samples were collected from the oral mucosa and rectal area using cotton swabs at 6 hrs post inoculation, with subsequent swabbing 1, 3, 9, 17, 23, 34, and 41 days post inoculation, corresponding to three samplings within the first three days after inoculation, followed by approximately weekly sampling thereafter. Applicator tips were placed in sterile containers containing lysis buffer (4 M urea, 0.2 M NaCl, 0.5% n-lauroylsarcosine, 10 mM 1,2-cyclohexanediaminetetraacetic acid, 600 U/mL proteinase K (Roche Applied Science), 0.1 M Tris–HCl, pH 8) and stored at 4 °C until extraction. From each swab, DNA was extracted and presence of ONRAB® was determined by real-time PCR as previously described by Sobey and colleagues [34] and Knowles and colleagues [27]. All samples were tested in duplicate; if ONRAB® DNA was detected in one or both tests, then two additional replicates were tested. A mean value was calculated for each set of replicates.

Using 2 μL aliquots of a set of tenfold serial dilutions of the titrated ONRAB® stock (range $10^{8.4}$–$10^{9.7}$ $TCID_{50}$/mL; [27]), a standard curve for ONRAB® detection by qPCR was generated and included in all assays. The standard curve, which yielded R^2 values ranging between 0.986 and 0.994 and slopes ranging from −3.385 to −3.760 depending on the assay, was used to convert sample Ct values to $TCID_{50}$/μL equivalent units (EU) of virus. A sample was considered positive for ONRAB® detection if the value met or exceeded the limit of quantification of 1 EU/μL for this assay as reported previously [27,34]. Previous work on untreated target species showed no amplification of ONRAB® above the assay threshold [27]; therefore, to minimize animal handling and costs, pre-treatment swabs were not collected in the present study.

2.4. Rabies immunological response

Raccoons and opossums were wild-caught near Toronto, Ontario (Table 1), therefore blood was collected from them prior to vaccination with ONRAB® to ensure that they had no previous exposure to the vaccine. Baseline samples were not collected from the wild-caught rats as the probability of previous exposure was considered negligible since ONRAB® baits had never been distributed in the area from which the rats were collected (near Codrington, Ontario). Captive bred animals were not previously treated with the vaccine. Blood was sampled from all study animals at 31 days post inoculation. Cotton and Norway rats were anesthetized with a sevofluorane inhalant. The remaining species were anesthetized by intramuscular injection of ketamine hydrochloride (100 mg/mL, Animal Health Canada Inc., Belleville, Ontario) and medetomidine hydrochloride (1 mg/mL, Pfizer Canada Inc., Montreal, Québec). Once fully anesthetised, 3–5 mL of blood was drawn from the femoral or subclavian vein or via cardiac puncture. Samples were centrifuged (15 min @ 3500 rpm), after which sera were collected and stored frozen (−20 °C). Sera were assayed for the presence of rabies virus glycoprotein-specific antibodies by a competitive enzyme-linked immunosorbent assay (cELISA) as described by [43].

2.5. Statistical analyses

Due to the non-Gaussian distribution of the data, a generalized linear mixed model with a penalized quasi-likelihood approach was used to investigate relationships between the concentrations of DNA collected in swabs and the sample site (oral, rectal), species, and day of experiment (animal ID was used as the random variable). Post-hoc pairwise comparisons were made using Tukey tests. The analyses were performed in R version 3.4.2 using the packages *nlme* (https://CRAN.R-project.org/package=nlme>) and multcomp (https://cran.r-project.org/web/packages/multcomp/). Critical alpha was set at 0.05. Unless otherwise noted, medians are presented with interquartile ranges.

3. Results

3.1. ONRAB® virus detection

ONRAB® was detected in oral swabs from 60 to 100% of the animals in each group 6 hrs after inoculation (Table 2a). The median levels of virus were especially high in oral swabs collected from raccoons (2528.4 EU/μL) and red foxes (1805.4 EU/μL), and the remaining sample groups all had median measurements of less than 100 EU/μL (Table 2a). Type III test results indicated that all fixed effects were significant (species: $_{6,61}$ = 6.80, P < 0.001; sample site: $F_{1,1018}$ = 13.16, P < 0.001; day of experiment: $F_{1,1018}$ = 389.50, P < 0.001). The covariance parameter estimate for the random variable (animal ID) was 79.44.

In general, there was a strong decline in the quantity of ONRAB® DNA over time (t = −21.94, P < 0.001; Table 2). Oral swabs collected significantly more DNA than rectal swabs (t = 4.30, P < 0.001; Table 2).

There were differences in the amounts of ONRAB® DNA collected from the various species. Samples collected from raccoons had significantly more ONRAB® DNA than samples collected from skunks (t = 4.68, P < 0.001), opossums (t = 3.52, P = 0.001), labora-

Table 2
The median and interquartile range of quantities of ONRAB® DNA recovered from (a) oral swabs and (b) rectal swabs collected from study animals that tested positive at various times after oral instillation of the vaccine as determined using quantitative real-time PCR. DNA quantities are expressed in equivalent units (EU) per μL; 1 EU/μL, which corresponds to a viral titre of 1 TCID50/μL, represents the limit of quantification of the assay.

a) Oral	***Raccoon***		***Striped Skunk***		***Red Fox***		***Virginia Opossum***	
Time post- inoculation	*% positive (n = 10)*	*DNA quantity*	*% positive (n = 10)*	*DNA quantity*	*% positive (n = 10)*	*DNA quantity*	*% positive (n = 10)*	*DNA quantity*
6 hrs	100	2528.4 (1063.2–8884.1)	60	26.4 (9.8–143.0)	100	1805.4 (298.8–4013.7)	90	45.3 (14.1–1513.3)
1 day	100	45.5 (21.2–139.2)	70	28.6 (24.1–431.8)	100	30.5 (8.3–65.4)	70	11.2 (10.2–16.6)
3 days	90	1.4 (1.1–4.9)	20	2.4 (2.0–2.8)	60	4.7 (2.5–10.0)	40	8.3 (4.1–16.1)
9 days	10	2.3 (.)	0	.	10	2.4 (.)	10	3.1 (.)
17 days	70	9.3 (2.5–18.5)	60	4.0 (2.7–15.7)	0	.	0	.
23 days	60	2.4 (2.1–9.0)	40	8.3 (2.8–14.3)	40	2.4 (2.0–2.8)	10	1.5 (.)
34 days	40	2.7 (1.6–4.6)	80	13.6 (9.6–58.5)	20	2.8 (2.4–3.1)	0	.
41 days	0	.	0	.	0	.	0	.

	Norway Rat (wild)		***Norway Rat (lab)***		***Cotton Rat***	
Time post-inoculation	*% positive (n=9)*	*DNA quantity*	*% positive (n=10)*	*DNA quantity*	*% positive (n=9)*	*DNA quantity*
6hrs	78	5.7 (3.3–10.1)	70	4.3 (3.4–10.3)	89	13.2 (7.2–24.7)
1 day	78	2.0 (1.5–6.7)	100	5.1 (3.2–6.1)	100	25.0 (14.8–46.6)
3 days	11	5.2 (.)	0	.	11	1.5 (.)
9 days	0	.	0	.	0	.
17 days	0	.	0	.	11	1.7 (.)
23 days	0	.	0	.	0	.
34 days	0	.	0	.	0	.
41 days	0	.	0	.	0	.

b) Rectal	***Raccoon***		***Striped Skunk***		***Red Fox***		***Virginia Opossum***	
Time post-inoculation	*% positive (n=10)*	*DNA quantity*	*% positive (n=10)*	*DNA quantity*	*% positive (n=10)*	*DNA quantity*	*% positive (n=10)*	*DNA quantity*
6 hrs	100	5.4 (2.8–11.1)	70	11.0 (2.5–21.4)	40	16.8 (8.4–23.4)	20	6.2 (4.4–8.0)
1 day	60	14.7 (4.0–28.8)	80	8.2 (4.6–24.0)	100	6.2 (2.8–57.5)	70	9.2 (4.1–18.9)
3 days	30	1.7 (1.5–2.2)	10	9.5 (.)	70	13.2 (4.5–54.5)	80	1.8 (1.3–2.6)
9 days	0	.	0	.	0	.	0	.
17 days	20	1.7 (1.6–1.7)	0	.	0	.	0	.
23 days	20	1.6 (1.5–1.7)	20	1.2 (1.2–1.2)	10	8.4 (.)	0	.
34 days	0	.	0	.	0	.	0	.
41 days	0	.	0	.	0	.	0	.

	Norway Rat (wild)		***Norway Rat (lab)***		***Cotton Rat***	
Time post-inoculation	*% positive (n=9)*	*DNA quantity*	*% positive (n=10)*	*DNA quantity*	*% positive (n=9)*	*DNA quantity*
6hrs	100	2.2 (1.9–6.6)	100	13.0 (5.1–25.2)	100	78.5 (28.0–156.3)
1 day	100	10.2 (7.9–30.1)	100	26.7 (6.7–51.9)	100	15.9 (14.4–23.5)
3 days	22	3.1 (2.5–3.7)	10	3.0 (.)	11	1.6 (.)
9 days	0	.	0	.	0	.
17 days	0	.	0	.	0	.
23 days	0	.	0	.	0	.
34 days	0	.	10	1.1(.)	0	.
41 days	0	.	0	.	0	.

Table 3
Serological response to oral instillation of ONRAB® by rabies vector and non-vector species as measured by a competitive enzyme linked immunosorbent assay (cELISA) 31 days after instillation.

Species	cELISA positive threshold (% inhibition)[a]	Mean % Inhibition ± SE	Percentage of seropositive animals
Raccoon	25	67.4 ± 5.4	100 (10/10)
Striped skunk	26	78.7 ± 6.2	100 (10/10)
Red fox	20	65.9 ± 5.0	100 (10/10)
Virginia opossum	26	46.4 ± 7.3	89 (8/9)
Wild Norway rat	26	9.4 ± 4.5	11 (1/9)
Lab Norway rat	26	82.6 ± 4.3	100 (10/10)
Cotton rat	26	89.1 ± 3.2	100 (7/7)

[a] Fehlner-Gardiner and Wandeler [38].

tory raised Norway rats ($t = 2.21$, $P = 0.031$), and cotton rats ($t = 4.47$, $P < 0.001$). Furthermore, fox samples had significantly more DNA than those collected from skunks ($t = 3.43$, $P = 0.001$), opossums ($t = 2.09$, $P = 0.041$), and cotton rats ($t = 3.37$, $P = 0.001$). All other pairwise comparisons were not significant ($P > 0.050$).

There was a significant effect of sample site ($t = -4.31$, $P < 0.001$) and the proportion of samples that tested positive for ONRAB® DNA in oral and rectal swab samples of vector species varied over time (Table 2). For example, between 10 and 90% of the oral swabs collected from raccoons tested positive between days 3 and 34 of the trials (Table 2). In contrast, non-vector species values dropped below the limit of quantification between day 3 and 9 of the experiment (Table 2), with the exception of a single opossum on day 23 (oral = 3.0 EU/μL), a cotton rat on day 17 (oral = 1.7 EU/μL), and a Norway rat on day 34 (rectal = 1.1 EU/μL). In the vector species (raccoons, skunks and foxes), small quantities of ONRAB® DNA were detectable in 20–70% of oral swabs and 10–20% of rectal swabs between 17 and 34 days post inoculation. Forty-one days after vaccine administration, no ONRAB® DNA was detectable in any sample (Table 2).

3.2. Rabies immunological response

Competitive ELISA was used to assess antibody response to the rabies virus glycoprotein. Raccoons and opossums were all negative for rabies virus antibody prior to ONRAB® administration. The majority of animals in each group were seropositive 31 days after treatment (89 – 100%) with the exception of wild Norway rats (only one of nine animals was seropositive; Table 3). Of the rabies vector species, skunks displayed the highest mean inhibition rate (78.7%, 95% CI: 64.6–92.8%) and cotton rats had the highest mean inhibition rate of the non-target species (89.1%, 95% CI: 81.2–97.1%; Table 3). Of the seropositive individuals, the measured rabies antibodies responses were very high, mean cELISA inhibition rates ranged from 65.9% (95% CI: 54.6–77.2%) to 89.1% (95% CI: 81.2–97.1%), with the exception of opossums (mean inhibition = 50.3%, 95% CI: 33.6–66.8%).

4. Discussion

This study was conducted to evaluate the shedding of ONRAB® vaccine virus and potential for environmental persistence in target (raccoon, skunk, red fox) and non-target (opossum, Norway rat, cotton rat) species as one of the requirements for vaccine licensure. While not the first study to examine ONRAB® viral DNA shedding in wildlife, it is the first to examine ONRAB® shedding up to six weeks following oral instillation and to include Norway rats as a non-target species.

Throughout the duration of the current study there was a general pattern of decline in the number of samples with quantifiable amounts of ONRAB® DNA, as well as a decline in the average quantity of ONRAB® DNA detected. Not surprisingly, given the route of inoculation, the quantity of ONRAB® DNA recovered from oral swabs was much higher than that from rectal swabs. While examining shedding of the oral rabies virus vaccine strain SPBN GASGAS, Vos and colleagues [44], also reported longer detection in oral swabs compared to faecal samples. All species exhibited at least one sampling time point commencing day 9 or day 17 where ONRAB® DNA was undetectable in either oral or rectal swabs, consistent with results of previous studies [27,33]. However, in the target species, there was a period between days 17 and 34 when both oral and rectal swabs were once again qPCR-positive, albeit at much lower levels than detected in the first 24 h post-inoculation. These data highlight the utility of monitoring for viral shedding over longer durations of time, as previous studies of ONRAB® shedding concluded at four days to four weeks, depending on the species under test and the anatomic site sampled.

Similar to the previous work of Fry and colleagues [33], the lack of virus isolation data in the current study makes it impossible to know with certainty whether the swab samples contained infectious virus. In the study by Knowles and colleagues [27], limited comparison was made between virus isolation from tissues (not feces) recovered from immunized animals and a standard PCR-southern blot method (PCR-SB; presumed to perform similarly to qPCR). In that study, two thirds (14/21) of PCR-SB positive tissues were also positive by virus isolation, suggesting that qPCR positives in the present study could have also contained infectious virus.

In construction of the ONRAB® vaccine, the early region 3 (E3) of HAV5 was deleted and replaced with the rabies virus glycoprotein gene. While E3 is not required for viral replication, E3-encoded proteins have been shown to have many immunomodulatory effects which could impact the ability of the host to clear infection (reviewed in [45]). Deletion of the E3 region would be expected to result in diminished capacity for persistence of the vaccine virus in an animal. Indeed, passage of ONRAB® in the established cotton rat model for human adenovirus infection has proven challenging [27]. Nevertheless, in humans, adenovirus can persist in a latent form in different tissues following primary infection and can be shed intermittently (reviewed in [24]). Thus, it is possible that the detection of qPCR-positive samples in target species between days 17 and 34 in the current study was due to persistence and replication of virus below the detection limit of the qPCR assay during the quiescent period.

Regardless of whether the detections represented genome fragments or infectious virus, the failure to detect ONRAB® DNA in the oral and rectal swabs of non-target species after day 9 (with the exception of swabs from one opossum, one cotton rat, and one laboratory bred Norway rat on days 23, 17, and 34, respectively) strongly suggests little risk of persistence of the ONRAB® virus in these species if baits are encountered in the field. In a preliminary study of a canine adenovirus-2 recombinant rabies vaccine, vector DNA could be detected in anal swabs from 3 of 11 skunks seven days after oral administration of the vaccine [46], whereas in studies of V-RG in foxes [47], and chimpanzees [48], no virus could be detected in feces by PCR or virus isolation, respectively. Similarly, in studies of the highly attenuated, live rabies virus vaccine strain SPBN GASGAS, viable virus could not be isolated from fecal swab samples from a range of target and non-target species sampled up to two weeks after oral immunization, although some samples up to day 7 were PCR-positive [44]. Unlike vaccinia- and rabies virus-based oral vaccines, adenoviruses can survive in the gastrointestinal tract, which likely accounts for the differences observed in fecal shedding studies among these vaccine strains. In the present study, ONRAB® DNA was readily detected in 70–100% of samples

from target species one day after vaccine administration, but only low levels of ONRAB® DNA were detected in relatively few raccoons (2/10), skunks (2/10) or foxes (1/10) on day 17 or 23. Even if all the ONRAB® DNA detected by PCR was indicative of viable virus, these results still suggest little risk of environmental contamination with vaccine virus from the target species through the fecal route.

The ONRAB® detections in oral swabs from target species could be more concerning if they represented infectious virus. It is not known if ONRAB® can cause human illness, however, human infection with wildtype HAV5 is common and generally causes only a mild, self-limiting respiratory illness [49]. Such infections often occur in children younger than the age of 5 years and result in development of anti-HAV5 antibodies and life-long immunity [50]. Given that the second wave of ONRAB® shedding observed in target species was of low quantities of DNA compared to the detections early in the study, and that no DNA was detected by day 41, even if there was some residual viral replication it seems unlikely that ONRAB® will persist long-term in these species and present an ongoing risk of human exposure. Furthermore, direct contact between people and the target wildlife species is rare.

Dogs and cats that eat a bait might be a more plausible route for human exposure if they were to subsequently shed vaccine virus. While ONRAB® virus shedding in companion animals was not the focus of this study, Knowles and colleagues [27] found that following oral administration of a vaccine dose 10-fold higher than present in a typical bait, no ONRAB® DNA could be detected in oral swabs of four cats, and only three oral swabs from four dogs were positive up to day 2, with sampling conducted for 18 days.

Another source of human exposure to vaccine virus could be contact with a bait shortly after distribution in the field. During three ONRAB® field efficacy trials in Canada and the USA, the rate of reporting of found baits ranged from 0.6 to 5.5 per 100,000 baits distributed, however no adverse events in dogs or people were detected [29,51,52]. Inhalation of as few as five infective adenovirus particles has been reported to be sufficient to cause disease in adults who lack pre-existing antibody [53]. The lack of adverse events observed in individuals exposed to full-titered vaccine in the field evaluations (including three individuals considered "higher risk" in the US study), strongly suggests that even if there was vaccine shed from a dog following ingestion of an ONRAB® bait, it would be in a quantity insufficient to cause an adverse event [51].

This work is the first to examine ONRAB® viral shedding in Norway rats - a non-target species that, due to its high population densities and commensal nature, is very likely to encounter baits in the field. In both laboratory-raised and wild-caught Norway rats we found that the vaccine virus was cleared quickly - with the exception of a single rectal swab from a laboratory-sourced Norway rat (day 34, 1.1 EU/μL), no ONRAB® DNA was detected in any Norway rat after the third day of sampling. The immune response to ONRAB® differed considerably between laboratory- and field-sourced rats; only one of the nine wild rats had detectable antibodies at day 31, whereas all captive-bred rats were seropositive. One possibility for this difference is that environmental exposure of wild rats to HAV5 may have resulted in production of anti-HAV5 antibodies that impaired the immune response to ONRAB®. A similar effect has been seen in animal models of HAV5-vectored gene therapy following parenteral administration of transgene-carrying vectors [54,55], however, in other studies pre-existing immunity to HAV5 did not impair oral vaccination of mice with E1-deleted HAV5-rabies virus glycoprotein recombinant viruses [56,57]. Furthermore, an unpublished pilot project conducted by Ontario's Ministry of Natural Resources and Forestry suggests that a relatively low proportion urban raccoons have detectable levels of HAV5 and there is no reason to suspect Norway rats to have a much higher environmental exposure rate. Numerous endoparasites are found in rats [58] and helminths have been shown to produce a variety of immunomodulatory mediators [59,60]. Further research might examine whether the response to ONRAB® is influenced by endoparasites found in the natural environment. Nevertheless, these data suggest that immune response to vaccine does not influence the rate of viral clearance in Norway rats.

Humoral immune response to ONRAB® in vector species has been previously examined, with skunks, foxes and raccoons all exhibiting seroconversion and sustained rabies glycoprotein antibody production following vaccine bait consumption [61–64]. We observed that all animals tested were seropositive following ONRAB® instillation, with the exceptions of opossums (89%, 8/9) and wild Norway rats (11%, 1/9). These results are consistent with previous research demonstrating that ingestion of ONRAB® vaccine leads to antibody response in target and non-target mammals [27,29,33,61]. Knowles and colleagues [27] observed a 100% seroconversion rate in rabies vector species (12 each of raccoons, skunks and foxes), with lower rates in non-target rodents, ranging from 50% (4/8) in deer mice (*Peromyscus maniculatus*), to 83% (5/6) in cotton rats, and 88% (7/8) in meadow voles (*Microtus pennsylvanicus*). Fry and colleagues [33] observed a similar pattern of vaccination response, with seroconversion rates in non-target species ranging from 75% (6/8 wood rats; *Neostoma* spp.) to 100% (8/8 opossums). The results of this current study, when combined with the findings of other experiments on captive and free-ranging animals, suggest that ingestion of ONRAB® vaccine baits is an effective method of inducing a serological response in rabies vectors and non-target wildlife as a tool for controlling the spread of rabies.

In conclusion, ONRAB® vaccine virus was shed from all species tested but could not be detected in any samples by completion of the 41-day experiment. Overall, both the quantity of ONRAB® DNA and the number of samples exceeding the quantification limit followed a steep declining trend following vaccination. However, ONRAB® DNA could be detected in target species between 17 and 34 days after vaccination, after which the quantities of DNA in samples fell outside of the working range of the assay. This study contributes to previous research that suggests ONRAB® does not persist in the systems of rabies vector and non-target mammal species and, therefore, the risk of horizontal transmission is low.

Authors' contributions

KGS was the project lead until his passing. SEJ and AAW drafted the manuscript, analysed the data, and produced the figure and tables. RCR was involved with project design, supervised the experiment, and provided feedback on the manuscript. DD coordinated the project, was involved with project design, and provided feedback on the manuscript. CFG performed the serologic testing, evaluated the laboratory results, and contributed significantly to the writing process. SAND developed the real-time PCR method used for ONRAB detection, and provided feedback on the manuscript. JCD was involved in the coordination of the program and provided feedback on the manuscript. CJK assisted with study design and implementation and contributed to data analysis and writing.

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Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The Ministry of Natural Resources and Forestry, for which many of the authors work or have worked, receives royalties from Artemis from their sales of ONRAB® baits. None of the authors, however, gain anything personally from this arrangement.

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