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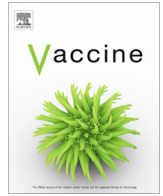Follow this and additional works at: http://digitalcommons.unl.edu/icwdm_usdanwrc

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Efficacy of Ontario Rabies Vaccine Baits (ONRAB) against rabies infection in raccoons

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ABSTRACT

In the US, rabies lyssavirus (RABV) only circulates in wildlife species and the most significant reservoir from a public and animal health perspective is the raccoon (*Procyon lotor*). Management of wildlife rabies relies principally on oral rabies vaccination (ORV) strategies using vaccine-laden bait delivery to free-ranging target hosts, in order to reduce the susceptible population to prevent the spread of and eliminate RABV circulation. Our objective was to evaluate efficacy of the Ontario Rabies Vaccine Bait (ONRAB) against a lethal RABV challenge in captive raccoons. Sham or live vaccine baits were offered to 50 raccoons and efficacy was evaluated in 46, split into two trials of 17 and 29 raccoons. Raccoons were challenged with a lethal dose of RABV 180 days post-vaccination and observed for 90 days post-infection. Raccoon bait interactions were assigned increasing integer scores for approach, oral manipulation, puncture, and consumption behaviors. Higher bait interaction scores were observed in the fall compared to the spring trial, indicating that more raccoons consumed baits in the fall. Although animal age did not explain variation in bait interaction scores, the geometric mean rabies virus antibody titers among juvenile vaccinates were higher than adults at all pre-challenge time points. The prevented fraction associated with ONRAB delivery was 0.73 (8/11, 95% CI 0.39–0.94) in the spring trial and 0.91 (21/23, 95% CI 0.72–0.99) in the fall trial. All sham-vaccinated raccoons (12/12) succumbed to rabies infection, in contrast to 15% (5/34) mortality among vaccinated raccoons. Our results indicate a high efficacy of ONRAB bait vaccination in protecting adult and juvenile raccoons against RABV infection for a minimum of six months. These data complement experimental field trials that have also demonstrated the potential of ONRAB for the control and prevention of RABV circulation in free-ranging raccoon populations in the US.
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1. Introduction

In the United States (US), rabies lyssavirus (RABV) only circulates in wildlife species and the most significant reservoir from a public and animal health perspective is the raccoon (*Procyon lotor*). The human exposures and animal case burden associated with raccoon RABV is due in part to the ubiquitous nature and high population densities of this peri-domestic species in suburban and urban habitats [1–3]. The current enzootic focus of raccoon RABV extends from a historical area in the southeastern US north to the border with Canada. Management of wildlife rabies historically involved population reduction strategies (e.g. culling), but now focuses on oral rabies vaccination (ORV) strategies to deliver

vaccine-laden baits to free-ranging target hosts, in order to reduce the susceptible population [4]. The National Rabies Management Program (NRMP), administered by the US Department of Agriculture (USDA), Animal and Plant Health Inspection Service, Wildlife Services program, has the mission to control and eliminate specific RABV variants circulating in wild carnivores in the US. Coordinated ORV programs to target raccoons have been operational since the 1990s [5]. While ORV has been successful in preventing westward spread of raccoon RABV in the US, post-ORV population immunity levels have averaged 30% across several years and have led to concern about the ability of current ORV products to eliminate RABV circulation in raccoons [5].

The Raboral V-RG® product (Boehringer Ingelheim Animal Health, Athens, Georgia, USA) is the only oral rabies vaccine currently licensed for use with free-ranging raccoons and coyotes (*Canis latrans*) in the US [6]. However, another product has shown promising results for the control of RABV in raccoons and striped skunks (*Mephitis mephitis*) in southern Ontario, Canada [7,8]. The Ontario Rabies Vaccine Bait (ONRAB; Artemis Technologies, Inc.,

Guelph, Ontario, Canada) is comprised of a sweet attractant matrix that coats a blister pack containing a live recombinant human adenovirus expressing the RABV glycoprotein [9], and is licensed for use with free-ranging striped skunks in Canada. One study demonstrated immunogenicity and efficacy of ONRAB baits for raccoons, but did not meet prevented fraction standards for animal rabies vaccines in the US [10].

We conducted a randomized and blind evaluation of the efficacy of ONRAB ultralite baits (ULBs) in protecting raccoons against lethal RABV infection, to re-assess its potential for meeting efficacy standards for animal rabies vaccines in the US.

2. Materials and methods

2.1. Animals, housing, and restraint

A total of 65 naïve captive-bred raccoons (19 adult males, 20 adult females, 14 juvenile males, 12 juvenile females) were obtained from Ruby Fur Farm (New Sharon, Iowa, USA). Animal use procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the USDA National Wildlife Research Center (NWRC) under protocol 2278. The import and housing of raccoons at the NWRC facility was authorized under Colorado Parks and Wildlife permits 14TR2056A1, 15TR2143, and 16TR2143. During quarantine, individual animal health was inspected by a veterinarian and passive integrated transponders (PIT tags; Avid Identification System, Inc., Norco, California, USA) were subcutaneously injected into each raccoon under anesthesia for unique identification (ID). A total of 50 raccoons (30 adults, 20 juveniles) were randomly assigned to the vaccine efficacy study, which was conducted as two consecutive trials (trial 1, n = 21, March 2015–December 2016; trial 2, n = 29, November 2015–August 2016). At the time of vaccination, juveniles in the first trial were ten months old and in the second trial were six months old. A total of 15 raccoons (9 adults, 6 juveniles) were used in a challenge virus titration study, conducted as two consecutive trials during November 2014 (n = 10) and January 2016 (n = 5). All raccoons were housed in individual elevated pens (1.2 × 2.4 × 1.8 m) in an open-air outdoor building during quarantine and vaccination. Raccoons were moved to individual pens on concrete (3 × 3 × 2.5 m) in an open-air outdoor building during post-vaccination (pv) monitoring. Raccoons were housed in individual cages (0.7X1X1m) in an Animal Biosafety Level 2 room during RABV challenge and post-infection (pi) monitoring. Each pen or cage had a den box attached to the outer edge, and contained other forms of enrichment. Raccoons were fed a daily ration of 200 g of Mazuri omnivore diet (PMI Nutrition International, St. Louis, Missouri, USA) except during vaccination and were provided water *ad libitum*. Raccoons were anesthetized using inhalation delivery of isoflurane gas or intramuscular (IM) injection a 5:1 ratio (20 mg/kg and 4 mg/kg respectively) of ketamine (Ketaset®; Zoetis, Inc., Florham Park, New Jersey, USA) to xylazine (AnaSed®; Akorn, Inc., Lake Forest, Illinois, USA) for the purpose of blood sample collection from a jugular vein and inoculation. For isoflurane anesthesia, induction was accomplished by delivery of 5% isoflurane in oxygen at 5L/min in the den box containing the animal [11]. Upon induction, a cone was then fitted over the oronasal region for maintenance at 2–3% at 1–2 L/min during sample collection. Upon completion of procedures, animals were returned to den box for arousal under ambient conditions, and monitored until bright, alert and responsive to stimuli.

2.2. Challenge virus titration study

A RABV challenge virus was obtained from the USDA Center for Veterinary Biologics. The 92-5A RABV is a New York City dog variant that was most recently passaged in red foxes (*Vulpes vulpes*). This virus was selected for study because it met regulatory requirements for purity, potency, and purpose. The neat titer was $10^{7.9}$ mouse intracerebral lethal doses per mL (MICLD$_{50}$/ml). Virus was diluted using sterile phosphate buffered saline, supplemented with 2% fetal bovine serum (Invitrogen, Carlsbad, California, USA). As data were not available regarding pathogenicity of this virus for raccoons, an initial titration trial was designed so that five raccoons each were randomly assigned, while blocking for sex and age, to receive a dose of $10^{6.9}$ or $10^{5.9}$ MICLD$_{50}$/mL. In a second trial, five additional raccoons received the $10^{5.9}$ MICLD$_{50}$/mL dose to test the repeatability of the initial trial outcome at this dose. Baseline blood samples were collected from each raccoon prior to IM inoculation with 0.5 mL of diluted RABV into each masseter muscle (1.0 mL total). Animals were monitored daily, or twice-daily during the expected clinical period (days 7–35 pi), for 90 days pi. Upon display of two or more clinical signs of rabies, raccoons were anesthetized with an IM injection of ketamine/xylazine. Under heavy anesthesia, a terminal blood sample was collected prior to intracardiac administration of pentobarbital sodium and phenytoin sodium (VetOne Euthanasia Solution; Med-Pharmex, Inc., Pomona, California, USA). Surviving raccoons were euthanized on or after day 90 pi. Brainstem and cerebellum tissues were collected postmortem from individual raccoons.

2.3. Vaccine immunogenicity and efficacy study

Fifty raccoons were randomly assigned, while blocking for sex and age, to one of two treatment groups, live or sham, for presentation of a single ONRAB ULB (lots AdRG1.3 14-01, AdRG1.3 14-01P). Raccoon treatments were assigned by the cooperator (Artemis), and the NWRC research team was blind to the assignments until the conclusion of each efficacy trial. Food was withheld from raccoons for 24 h prior to bait offering. A total of 12 raccoons received a sham bait, and 38 raccoons received a live bait containing 1.8 mL of vaccine at a titer of $10^{9.6}$ TCID$_{50}$/mL during a 24 h presentation window. Bait offering was monitored using motion-activated trail cameras (Reconyx Silent Image, Holmen, Wisconsin) and video cameras (Supercircuits model PC161IR, Supercircuits, Inc., Austin, Texas, USA; Zodiac model CAMZ836IR, Zodiac Light Waves Inc., Ontario, Canada; Polaris model EZ-380VF, Polaris USA, Norcross, Georgia, USA). Plastic sheets were placed underneath the elevated cages of each animal prior to bait offering to collect bait debris and vaccine spillage, and were inspected every 4 hrs to assess bait remains and estimate spillage to the nearest 0.1 mL using a pipette.

Scores were assigned for each raccoon-bait interaction as follows: (0) animal does not approach bait, (1) animal approaches bait, but without oral contact, (2) oral manipulation of bait by animal, but no puncture of blister pack, (3) oral manipulation and puncture of the blister pack, but incomplete consumption, or (4) animal consumes entire bait. Camera and video footage were analyzed post-trial to verify scores.

Baseline blood samples were collected from raccoons prior to vaccination, and then on days 30, 60, 90, and 176 pv. Raccoons were challenged on day 180 pv by IM inoculation with 0.5 mL of diluted RABV into each masseter muscle (1.0 mL total). Animals in the first trial (n = 17) were inoculated with $10^{6.2}$ MICLD$_{50}$/mL of RABV. Based on results of the first trial, the animals in the second trial (n = 29) were inoculated with a lower dose of $10^{5.9}$ MICLD$_{50}$/mL. Animals were monitored as described previously, and blood samples were collected on days 15, 30, and 60 pi. Surviving raccoons were euthanized on days 90 or 91 pi. The procedures for euthanasia and tissue collection were the same as described for the challenge virus titration study.

2.4. Detection of rabies virus antigen

Brainstem and cerebellum tissues from individual raccoons were stored in conical vials on ice packs and submitted the same day or refrigerated and submitted within 72 h to the Veterinary Teaching Hospital Diagnostic Lab at Colorado State University for rabies diagnosis using the direct fluorescent antibody test [12].

2.5. Detection of rabies virus antibodies

Blood samples were centrifuged at 4000g for 15 min and serum was separated into cryovials. Sera were stored at −80 °C until shipment to the Rabies Laboratory at Kansas State University (KSU). Sera were analyzed to titrate the level of RABV neutralizing antibodies (RVNA) to endpoint by rapid fluorescent focus inhibition test (RFFIT) [13]. Titers were converted to international units per mL (IU/mL) by comparison to a positive control standard rabies immune globulin containing 2 IU/mL. Titers less than 0.1 IU/mL were considered negative and titers greater than or equal to 0.1 IU/mL were considered RVNA positive. The vaccine efficacy trial sera were also evaluated for RABV binding antibodies (RVBA) at KSU by a commercial indirect ELISA (BioRad Platelia Rabies Kit II, Marnes-la-Coquette, France) using the Bio-Rad Evolis instrument per the manufacturer's instructions. Sample test results were reported in equivalent units per mL (EU/mL) calculated by comparison of the sample optical density against a standard curve of positive standards supplied in the kit. Sample EU/mL values of less than or equal to 0.125 EU/mL were considered negative, and values greater than 0.125 EU/mL were considered RVBA positive.

2.6. Detection of rabies virus RNA

Brainstem tissue from rabid animals was tested by PCR analysis to confirm a match to the challenge inoculum. RNA extraction, reverse transcription, amplification, cleanup and sequencing were performed as previously described [14]. Forward and reverse sequences were aligned and visually inspected using Sequencher (Gene Codes Corporation, Ann Arbor, Michigan, USA). Alignment of consensus sequences and identity comparisons were performed using BioEdit v.7.2.0. [15].

2.7. Analyses

A generalized linear model was used to analyze the bait interaction score data across 50 raccoons. Bait treatment (live/sham), age (adult/juvenile), sex (male/female), and trial (one/two) were evaluated as fixed effects in a multivariable model. A multinomial distribution with cumulative logit link function was used to model the relationship of fixed effects with ascending interaction scores (i.e., scale of 0–4). The antibody response data were natural log transformed and checked for normality. The difference in mean antibody response between adult and juvenile vaccinates across pv time points was compared by t-test. The pooled variance method was used for t-tests when the variance of the antibody response was equal between adult and juvenile raccoons, whereas the Satterthwaite method was used when the variance of the antibody response between adult and juvenile raccoons was unequal. The t-test p-values were adjusted for multiple comparisons (i.e., time points) using the Holm method [16]. The prevented fraction and 95% exact confidence intervals from the vaccine efficacy experiments were calculated from 46 challenged raccoons. A survival analysis was performed to test for homogeneity in survival curves pi, using a log-rank test on data stratified by bait treatment and age and the Šidák correction to adjust for multiple pairwise comparisons. SAS v.9.4 was used to perform all analyses (SAS Institute, Cary, North Carolina, USA) and significance was assessed at $\alpha = 0.05$.

Geometric mean titers (GMT) and 95% confidence intervals were calculated among juvenile and adult vaccinates at specific time points. For the purpose of GMT calculations, RVNA titers of less than 0.1 IU/mL were treated as 0.05 and RVBA titers of less than or equal to 0.125 EU/mL were treated as 0.063. Also for GMT calculation purposes, seven vaccinate sera on day 15 pi with RVBA values exceeding the detection limit (i.e., 20.000 EU/mL) were coded as 20.000 EU/mL.

3. Results

3.1. Challenge virus titration

Thirteen of 15 naïve challenged raccoons developed rabies, and sequencing of brain tissue demonstrated 100% identity to the RABV inoculum. Mortality was 100% and incubation periods were 10–15 days (median: 11 days) for five raccoons inoculated with $10^{6.9}$ MICLD$_{50}$, and mortality was 80% with incubation periods of 10–13 days (median: 12 days) for ten raccoons inoculated with $10^{5.9}$ MICLD$_{50}$ (Table 1). Clinical signs most commonly included paresis, ataxia, tremors, and lethargy or irritability. Neither of the two surviving raccoons presented a terminal RVNA titer at day 90 pi. However, among 12 animals which succumbed with sera available, terminal RVNA titers were detected from five

Table 1
Results of rabies virus titration with 15 captive-bred naïve raccoons (*Procyon lotor*).

Raccoon ID	Sex	Age	Trial	Virus Dose (log 10 MICLD50)	Baseline RVNA (IU/ml)	Result (incubation period)	Terminal RVNA (IU/ml)
1-007	F	A	1	6.9	<0.1	D (15)	<0.1
3-800	F	A	1	6.9	<0.1	D (10)	<0.1
1-600	M	A	1	6.9	<0.1	D (12)	0.1
8-342	M	A	1	6.9	<0.1	D (11)	n.d.[a]
3-515	M	J	1	6.9	<0.1	D (10)	0.2
7-618	F	A	1	5.9	<0.1	S	<0.1
1-786	M	A	1	5.9	<0.1	D (12)	<0.1
7-298	M	A	1	5.9	<0.1	D (11)	0.3
3-115	M	J	1	5.9	<0.1	D (11)	0.5
0-840	M	J	1	5.9	<0.1	D (12)	<0.1
9-269	F	A	2	5.9	<0.1	S	<0.1
4-378	F	A	2	5.9	<0.1	D (13)	0.3
9-566	F	J	2	5.9	<0.1	D (12)	<0.1
6-074	F	J	2	5.9	<0.1	D (10)	<0.1
9-108	F	J	2	5.9	<0.1	D (12)	<0.1

F = female, M = male, A = adult, J = juvenile, MICLD50 = mouse intracerebral lethal doses, RVNA = rabies virus neutralizing antibodies, D = succumbed and tested rabies positive, S = survived 90 days and tested rabies negative.

[a] n.d. = not determined; the animal was found dead during health checks, and terminal blood collection was unsuccessful.

(0.1–0.5 IU/mL), whereas the remaining seven were seronegative. The median dose of the two tested ($10^{6.2}$ MICLD$_{50}$) during the initial titration was selected for the first vaccine efficacy trial.

Observation of an inadequate prevented fraction during the first efficacy trial was the rationale for lowering the dose to $10^{5.9}$ MICLD$_{50}$ for the second efficacy trial.

3.2. Bait trials

Overall, 92% (46/50) of raccoons interacted with the ULBs and punctured the blister pack or consumed the bait in its entirety. Raccoons exhibited slightly higher interaction scores with sham compared to live ULBs (Table 2), and bait treatment was a marginal predictor of interaction scores (F = 2.8, p = 0.10) in the multivariable generalized linear model. Animals in the second trial exhibited higher interaction scores compared to the first trial (F = 17.0, p = 0.0002). Spillage of the sham or live vaccine liquid was noted in 66% (33/50) of the individual bait offerings, whereas no spillage was observed in 26% (13/50) and not applicable in 8%

Table 2
The mean (standard error) bait interaction score by trial and bait treatment for 50 captive-bred raccoons (*Procyon lotor*) offered Ontario Rabies Vaccine Baits (ONRAB). Interactions were scored on a scale of 0 to 4, with increasing integer values for animal approach, oral manipulation, puncture and consumption behaviors.

Trial	Treatment	N	Mean Score
1	vaccinate	15	2.80 (0.24)
1	sham	6	3.33 (0.21)
Subtotal		*21*	*2.95 (0.19)*
2	vaccinate	23	3.87 (0.07)
2	sham	6	4.00 (0.00)
Subtotal		*29*	*3.90 (0.06)*

Table 3
Results of immunogenicity and efficacy experiments evaluating delivery of Ontario Rabies Vaccine Baits (ONRAB) across two consecutive trials with 46 captive-bred naïve raccoons (*Procyon lotor*). Immunogenicity was measured by quantitation of rabies virus neutralizing antibodies (RVNA) post-vaccination. Efficacy was measured by challenge of raccoons with a lethal dose of rabies virus 180 days post-vaccination.

Trial	ID	Sex	Age[a]	Treatment	RVNA (IU/mL)							Terminal	Fate
					Day 0	Day 30	Day 60	Day 90	Day 176	Day 195	Day 270		
1	4-297	F	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (13)
1	2-883	M	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (13)
1	6-308	M	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			0.2	D (10)
1	6-864	M	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (11)
1	7-838	M	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			n.d.[b]	D (13)
1	5-788	M	J	sham	<0.1	<0.1	<0.1	<0.1	<0.1			0.2	D (11)
2	3-337	F	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (13)
2	0-785	F	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (12)
2	1-844	F	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			n.d.	D (14)
2	9-797	F	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (12)
2	4-096	M	A	sham	<0.1	<0.1	<0.1	<0.1	<0.1			<0.1	D (11)
2	5-591	M	J	sham	<0.1	<0.1	<0.1	<0.1	<0.1			0.1	D (11)
1	7-363	F	A	vac	<0.1	0.1	0.1	1.1	0.1			74	D (11)
1	7-123	F	A	vac	<0.1	<0.1	<0.1	<0.1	<0.1			22	D (11)
1	4-611	F	A	vac	<0.1	0.1	<0.1	0.1	<0.1			6.6	D (11)
2	7-565	M	A	vac	<0.1	<0.1	<0.1	<0.1	<0.1			1.9	D (12)
2	2-370	M	A	vac	<0.1	<0.1	<0.1	<0.1	<0.1			24	D (14)
1	0-032	M	A	vac	<0.1	2.7	16.5	5.5	0.7	8.5	0.9		S
2	0-553	F	A	vac	<0.1	<0.1	0.2	0.1	<0.1	0.7	<0.1		S
2	1-278	F	A	vac	<0.1	3.6	3.7	3.0	2.5	4.4	2.2		S
2	5-863	F	A	vac	<0.1	0.2	0.9	1.0	0.5	88	13.1		S
2	9-854	F	A	vac	<0.1	1.0	0.2	0.6	0.3	11.1	0.6		S
2	0-823	M	A	vac	<0.1	<0.1	0.3	0.2	0.1	4.2	0.4		S
2	1-382	M	J	vac	<0.1	2.2	2.2	0.7	3.4	3.8	1.1		S
2	9-534	M	A	vac	<0.1	1.5	0.7	0.2	0.1	11.1	1.3		S
2	0-109	M	A	vac	<0.1	0.2	0.2	0.2	0.1	11.1	1.1		S
2	2-066	M	A	vac	<0.1	0.2	0.5	1.5	0.2	44	3.3		S
2	2-285	M	A	vac	<0.1	1.3	0.4	0.6	0.2	7.1	1.3		S
2	4-270	F	A	vac	<0.1	0.7	0.4	1.0	0.2	37	3.5		S
2	7-343	M	A	vac	<0.1	0.1	0.2	0.2	0.2	2.5	0.2		S
2	5-795	F	A	vac	<0.1	0.5	7.5	11.5	5.0	59	16		S
2	9-026	F	J	vac	<0.1	1.5	3.0	2.2	1.7	14.9	0.6		S
2	2-622	F	J	vac	<0.1	3.0	3.7	3.4	3.2	27	5.5		S
2	1-524	F	J	vac	<0.1	2.5	7.0	3.7	1.0	10.0	0.7		S
2	6-839	F	J	vac	<0.1	6.6	8.0	12	10	28	1.2		S
2	7-117	F	J	vac	<0.1	1.0	4.2	3.5	0.7	10.0	2.6		S
2	7-036	F	J	vac	<0.1	3.4	6.6	6.2	3.4	24	4.0		S
2	6-527	F	J	vac	<0.1	2.0	1.0	1.7	0.3	10.0	1.0		S
2	3-773	F	J	vac	<0.1	3.2	3.5	3.2	0.9	13.0	3.3		S
1	6-526	F	J	vac	<0.1	0.8	0.9	2.7	0.2	4.1	21		S
1	8-789	M	J	vac	<0.1	1.1	4.3	3.7	3.4	16	1.8		S
1	5-577	M	J	vac	<0.1	0.8	3.9	4.3	3	21	4.5		S
1	1-572	M	J	vac	<0.1	0.1	0.1	<0.1	<0.1	3.4	<0.1		S
1	8-780	M	J	vac	<0.1	0.9	3.7	3.5	4.4	20	4.4		S
1	9-633	M	J	vac	<0.1	0.8	1.1	0.5	0.5	5.4	0.8		S
1	2-291	M	J	vac	<0.1	4.7	11.5	3.3	4.4	17	5.9		S

F = female, M = male, A = adult, J = juvenile, sham = placebo vaccine bait, vac = live vaccine bait, RVNA = rabies virus neutralizing antibodies, D = succumbed and tested rabies positive, S = survived 90 days and tested rabies negative.

[a] Juveniles in cohort 1 were approximately 10 months of age at vaccination, juveniles in cohort 2 were approximately 6 months of age at vaccination.

[b] n.d. = not determined; the animal was found dead during health checks, and terminal blood collection was unsuccessful.

(4/50). Among the 33 cases of spillage, a volume of less than 0.1 mL was estimated in all but one case, where an estimate of 0.5 mL was noted. Neither animal age (F = 0.01, p = 0.92) nor sex (F = 1.5, p = 0.23) were associated with higher bait interaction scores.

3.3. Vaccine immunogenicity and efficacy

Four raccoons (one adult male, one adult female, two juvenile males) which had been assigned live vaccine baits in the first trial were removed from the efficacy test because they neither interacted with nor punctured the blister pack of the ULBs during the 24 h trial. No adverse reactions were noted pv during the observation period prior to challenge. The RVNA GMT of vaccinated raccoons remained equal to or above 0.5 IU/mL during all pv time points prior to challenge, and the RVBA GMT remained equal to or above 0.646 EU/mL. Peak antibody response among vaccinated animals prior to challenge was observed at day 60 pv (RVNA

GMT = 0.9 IU/mL, RVBA GMT = 0.786 EU/mL). Among sham-vaccinated animals, neither RVNA nor RVBA were detected at any time point prior to challenge (Tables 3 and 4). The RVNA and RVBA GMTs of juvenile vaccinates were higher than adults at all pv time points prior to challenge (Fig. 1, Table S1).

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.vaccine.2018.06.052.

Mortality to RABV infection among sham-vaccinated raccoons was 100% (12/12). The prevented fractions among vaccinates were 0.73 (8/11, 95% CI 0.39–0.94) in first (spring) trial and 0.91 (21/23, 95% CI 0.72–0.99) in the second (fall) trial. The incubation period of animals that developed rabies was 10–14 days (median of 12 days) (Table 3). Heterogeneity in survival curves of adult and juvenile raccoons was detected between sham and vaccine bait treatments (adults $\chi^2 = 11.3$, p = 0.005, juveniles $\chi^2 = 16.2$, p = 0.0003; Fig. 2). Heterogeneity in survival curves between juvenile and adult vaccinates was not detected ($\chi^2 = 1.4$, p = 0.80).

Table 4

Rabies virus binding antibody (RVBA) titers, as measured by Bio-Rad Platelia assay, associated with Ontario Rabies Vaccine Bait (ONRAB) vaccination and lethal rabies virus challenge of 46 captive-bred naïve raccoons (*Procyon lotor*).

Trial	ID	Sex	Age[a]	Treatment	RVBA (EU/mL)							Terminal
					Day 0	Day 30	Day 60	Day 90	Day 176	Day 195	Day 270	
1	4-297	F	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
1	2-883	M	A	sham	n.d.[b]	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
1	6-308	M	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
1	6-864	M	A	sham	n.d.	n.d.	≤0.125	≤0.125	≤0.125			n.d.
1	7-838	M	A	sham	≤0.125	n.d.	≤0.125	≤0.125	≤0.125			n.d.
1	5-788	M	J	sham	n.d.	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	3-337	F	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	0-785	F	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	1-844	F	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	9-797	F	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	4-096	M	A	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	5-591	M	J	sham	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
1	7-363	F	A	vac	≤0.125	0.156	≤0.125	0.546	≤0.125			n.d.
1	7-123	F	A	vac	≤0.125	n.d.	≤0.125	≤0.125	≤0.125			n.d.
1	4-611	F	A	vac	≤0.125	n.d.	0.369	0.228	0.226			n.d.
2	7-565	M	A	vac	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
2	2-370	M	A	vac	≤0.125	≤0.125	≤0.125	≤0.125	≤0.125			n.d.
1	0-032	M	A	vac	0.133	n.d.	8.350	2.005	≤0.125	n.d.	1.753	
2	0-553	F	A	vac	≤0.125	0.138	0.267	0.145	0.151	1.895	0.135	
2	1-278	F	A	vac	≤0.125	3.959	3.515	3.798	2.333	2.138	2.179	
2	5-863	F	A	vac	≤0.125	≤0.125	0.425	0.535	≤0.125	>20.00	3.434	
2	9-854	F	A	vac	≤0.125	0.594	0.681	0.245	0.152	6.722	0.464	
2	0-823	M	A	vac	≤0.125	n.d.	0.845	0.329	0.207	4.488	0.670	
2	1-382	M	J	vac	≤0.125	1.240	1.671	1.361	1.170	9.137	1.819	
2	9-534	M	A	vac	≤0.125	1.251	0.599	0.180	≤0.125	7.770	1.393	
2	0-109	M	A	vac	≤0.125	n.d.	0.229	0.139	≤0.125	12.109	1.997	
2	2-066	M	A	vac	≤0.125	0.496	0.560	n.d.	≤0.125	>20.00	2.589	
2	2-285	M	A	vac	≤0.125	0.159	0.244	0.198	≤0.125	2.483	0.909	
2	4-270	F	A	vac	≤0.125	0.587	0.234	0.266	≤0.125	15.554	1.602	
2	7-343	M	A	vac	≤0.125	n.d.	≤0.125	≤0.125	≤0.125	1.080	≤0.125	
2	5-795	F	A	vac	≤0.125	0.990	6.938	3.106	2.907	>20.00	17.649	
2	9-026	F	J	vac	≤0.125	0.745	1.767	1.650	0.834	7.536	0.529	
2	2-622	F	J	vac	≤0.125	3.709	2.285	1.920	1.039	>20.00	3.274	
2	1-524	F	J	vac	≤0.125	1.875	4.610	3.758	0.591	6.106	0.578	
2	6-839	F	J	vac	≤0.125	4.878	9.132	7.549	5.531	>20.00	0.555	
2	7-117	F	J	vac	≤0.125	1.007	1.451	1.427	1.140	6.542	2.514	
2	7-036	F	J	vac	≤0.125	3.202	4.328	3.581	1.967	>20.00	3.722	
2	6-527	F	J	vac	≤0.125	0.707	1.217	1.281	0.175	8.264	1.572	
2	3-773	F	J	vac	≤0.125	1.494	1.786	1.848	0.906	11.657	2.362	
1	6-526	F	J	vac	≤0.125	0.680	1.082	0.941	0.308	2.333	13.900	
1	8-789	M	J	vac	≤0.125	0.877	3.708	2.787	1.554	>20.00	2.845	
1	5-577	M	J	vac	≤0.125	0.568	3.291	2.281	1.268	11.992	2.195	
1	1-572	M	J	vac	≤0.125	n.d.	≤0.125	≤0.125	≤0.125	1.192	≤0.125	
1	8-780	M	J	vac	≤0.125	1.384	1.880	2.178	≤0.125	9.191	1.899	
1	9-633	M	J	vac	≤0.125	n.d.	0.817	0.546	≤0.125	3.857	1.479	
1	2-291	M	J	vac	≤0.125	n.d.	2.756	2.251	≤0.125	n.d.	2.459	

F = female, M = male, A = adult, J = juvenile, sham = placebo vaccine bait, vac = live vaccine bait, RVBA = rabies virus binding antibodies.
  [a] juveniles in cohort 1 were approximately 10 months of age at vaccination, juveniles in cohort 2 were approximately 6 months of age at vaccination.
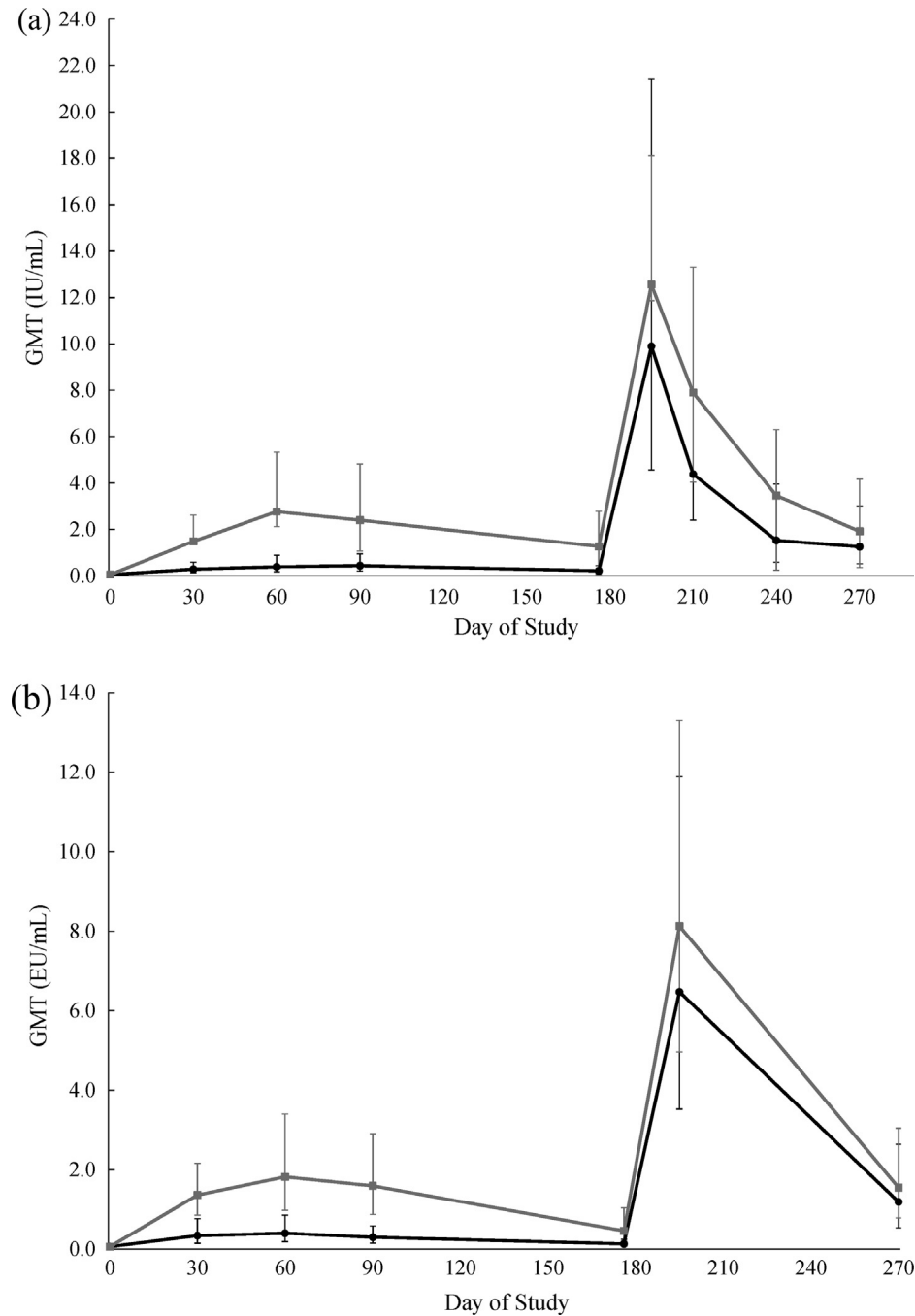 [b] n.d. = not determined.

Fig. 1. The (a) geometric mean rabies virus neutralizing antibody (RVNA) and (b) rabies virus binding antibody (RVBA) titers of vaccinated raccoons pre- and post-infection. Vaccinates included 19 adult (black) and 15 juvenile (gray) raccoons. Error bars reflect the 95% confidence limits on age specific geometric mean titers.

Terminal blood samples were obtained from ten of 12 sham-vaccinated raccoons and weak RVNA titers were observed in three (0.1–0.2 IU/mL), whereas the seven remaining control raccoons were seronegative. The terminal sera of these ten control animals were not tested for RVBA. However, among 25 raccoon sera with RVNA values of 0.1–0.2 IU/mL, and tested by both assays, approximately half (13) were RVBA negative.

A total of five vaccinated raccoons developed rabies, all adults (Table 3, Fig. 2). Three of these raccoons (7-123, 7-565, 2-370) never seroconverted pv for RVNA nor RVBA, yet presented terminal RVNA titers (1.9, 22, and 24 IU/mL respectively). Two of the three had partially eaten baits, whereas the other had fully consumed the bait. One vaccinated female (4-611) that fully consumed a bait

and succumbed demonstrated an RVNA titer of 0.1 IU/ml on day 30 and day 90 pv, but was seronegative at other time points, yet presented a terminal RVNA titer of 6.6 IU/mL. The same animal demonstrated positive RVBA values on days 60, 90, and 176 pv (0.369, 0.228, and 0.226 EU/mL respectively). Only one vaccinated female (7-363) that partially consumed a bait and succumbed demonstrated an RVNA response up to 1.1 IU/mL on Day 90 pv, with levels of 0.1 IU/mL at other time points, and a terminal titer of 74 IU/mL. The same animal demonstrated positive RVBA values on days 30 and 90 pv (0.156 and 0.546 EU/mL respectively), but was seronegative on days 60 and 176 pv.

All other 29 vaccinated raccoons mounted RVNA responses prior to challenge and survived challenge, including all juveniles.
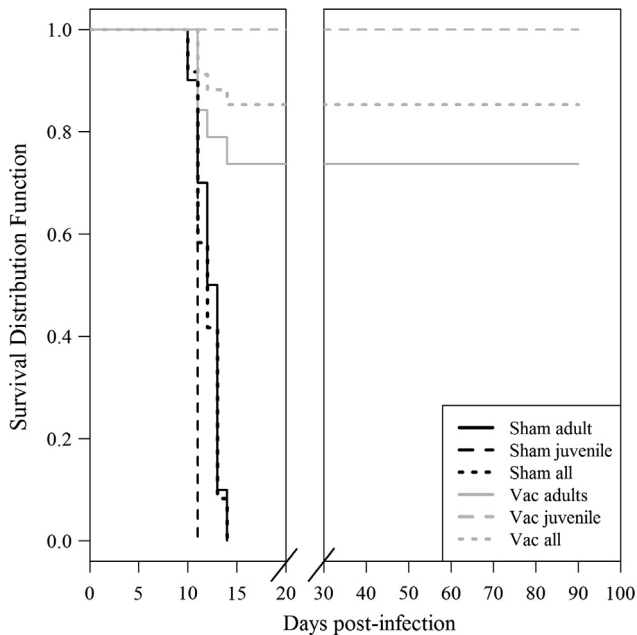
Fig. 2. Survival curves for raccoons by bait treatment and age combined across two trials (n = 46). Bait treatments were sham or vaccinated (vac), and age was adult or juvenile.

Four of 29 vaccinated survivors (1-572, 0-553, 0-109, 7-343) were weak RVNA responders (equal to or less than 0.2 IU/mL across all time points pv) and two were seronegative prior to challenge, yet all demonstrated an anamnestic response on day 15 pi (RVNA 0.7–11.1 IU/mL). Two of the four (7-343, 1-572) were RVBA negative across all time points pv until day 15pi (1.080 and 1.192 EU/mL), and then seronegative upon terminal sampling. The other two (0-109, 0-553) demonstrated RVBA across multiple time points pv (range 0.139–0.267 EU/mL), anamnestic responses on day 15 pi (12.109 and 1.895 EU/mL respectively) and were RVBA positive at terminal sampling (1.997 and 0.135 EU/mL respectively).

Even though it was inadequate to protect against lethal RABV infection, there did appear to be a level of immune priming conferred by vaccination among raccoons which ultimately developed rabies, as all presented evidence of an anamnestic response at terminal sampling and with RVNA titers that were notably higher than control animals.

4. Discussion

The control of RABV circulation in raccoons, and other wildlife, is principally achieved using ORV. To date, existing ORV products have effectively halted appreciable westward spread of raccoon RABV in the US, but suboptimal levels of herd immunity in raccoons may be insufficient for RABV elimination [5,17]. Novel ORV products for target wildlife may be needed in the US, and our study provides evidence of acceptable efficacy among raccoons for one candidate product. The prevented fractions observed, 0.73 in first trial and 0.91 in second trial, are comparable to or higher than an earlier study evaluating the same product in raccoons. Brown et al. [10] observed 75% survival among vaccinated raccoons compared to 89% mortality among control animals, for a prevented fraction of 0.72 when animals were challenged with RABV 350 days pv. It is unclear whether the prevented fractions observed in our study would have been lower under an extended interval between vaccination and challenge. We reduced the challenge dose between the first and second trial of the current study, yet

the mortality of sham-vaccinated control animals for both trials was 100%. The higher dose of challenge virus may have negatively impacted the prevented fraction observed in the first trial, although bait interaction scores were also lower.

In the Brown et al [10] study, 100% of 42 wild-caught raccoons that were offered ONRAB baits punctured the blister pack and/or consumed the entire bait, compared to 92% (46/50) of captive-bred raccoons in our study. Collectively, these data indicate high acceptability of the ONRAB baits by raccoons, similar to what was observed with other ORV products [18]. In our study, higher interaction with baits was observed during the trial in mid-November compared to mid-March; there may be unidentified seasonal cues that influence attractiveness of ORV baits to free-ranging raccoons. One controlled field study examining ONRAB bait visitation and removal by free-ranging raccoons demonstrated higher bait contact rates in the fall (October) when compared to late spring (June) and summer (August) [19]. The timing of ORV targeting raccoons typically occurs in the late summer and early fall, and so we expect field acceptability to be more similar to the second trial of our study.

Brown et al [10] did not observe a marked difference in the immunogenicity of the baits between adult and juvenile cohorts, in contrast to our results. Nevertheless, both studies collectively indicate that neither immunogenicity nor efficacy of ONRAB ULB for juvenile raccoons is inferior compared to adults, which is critical given the importance of targeting susceptible young of the year during annual baiting campaigns. Peak antibody response at day 60 pv in this study appears delayed in comparison to prior ONRAB studies in striped skunks [14] and raccoons [10], although the latter study reported a peak response at day 56 pv among raccoons receiving partial vaccine doses. In contrast, timing shifts of peak antibody response were not evident among striped skunks receiving different doses of vaccine by direct instillation [14].

A prior study reported a strong non-linear correlation ($R^2 = 0.8$) of ELISA and RFFIT values across raccoon sera tested in this study, but a poor correlation ($R^2 = 0.3$) considering the subset with RFFIT values equal to or less than 1.0 IU/mL [20]. Some of the RVNA titers reported in this study as weak (0.1–0.2 IU/mL) could be false positives, given our estimate that half of such samples tested negative for RVBA. We observed two vaccinates with weak RVNA and RVBA responses which succumbed to challenge, and two other vaccinates with weak RVNA and RVBA below detection limits that survived challenge. Nevertheless, mean vaccinate RVNA and RVBA kinetics in this study were remarkably similar and anamnestic responses to challenge were routinely observed among vaccinates, even those which succumbed to rabies. The phenomenon of anamnestic response among oral vaccinates that ultimately succumb to RABV challenge was observed in earlier studies with striped skunks [14], raccoons [10,18], and red foxes [21].

The efficacy of ONRAB ULB for raccoons in our second trial was within recommended prevented fraction standards for animal rabies vaccines in the US (9 CFR 113.312). These data complement experimental field trials that have also demonstrated the potential of ONRAB for the control and prevention of RABV circulation in free-ranging raccoon populations in the US [17,22].

the authors and do not necessarily represent the official position of their respective institutions or funding source.

Conflict of interest

AB is the owner of a pharmaceutical company manufacturing oral rabies vaccine baits.

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