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Oral vaccination and protection of striped skunks (*Mephitis mephitis*) against rabies using ONRAB®

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ABSTRACT

Skunks are one of the most important rabies vector species in North America due to their wide geographic distribution, high susceptibility to the rabies virus, and tendency to inhabit areas around human dwellings and domestic animals. Oral vaccination is a cost-effective, socially acceptable technique often used to control rabies in terrestrial wildlife; however, control of rabies in skunks has proven especially challenging due to the lack of a vaccine effective by the oral route in this species. In this study, we examined the antibody response of captive striped skunks (*Mephitis mephitis*) to ONRAB® and tested the protection afforded by the vaccine against rabies virus. Thirty-one skunks were each offered one ONRAB® vaccine bait, 25 skunks were administered ONRAB® via direct instillation into the oral cavity (DIOC) and ten controls received no vaccine. A blood sample was collected from controls and vaccinates 6 weeks prior to treatment, and then 5 and 7 weeks post-vaccination (PV). A competitive ELISA was used to detect rabies antibody (RAb). Pre-vaccination sera for all skunks, and sera for all controls throughout the serology study, were negative for RAb. Fifty-eight percent (18/31) of skunks in the bait group and 100% (25/25) of skunks that received ONRAB® DIOC had detectable RAb by 7 week PV. All 10 controls succumbed to experimental rabies infection. In the group of skunks administered ONRAB® DIOC, 100% (23/23) survived challenge 247 days PV. Survival of skunks presented ONRAB® baits was 81% (25/31). In the bait group, all 18 skunks that had detectable RAb by 7 week PV survived challenge. Seven additional skunks without detectable RAb prior to week 7 PV also survived. Lack of any remarkable pathology in study animals, together with positive serology and challenge results, supports that ONRAB® is a safe and effective oral rabies vaccine for use in skunks.

1. Introduction

Rabies remains one of the most important zoonoses in North America as a variety of wildlife vectors continue to serve as virus reservoirs [1–5]. In Canada and the US, skunks are one of the most important rabies vector species due to their wide geographic distribution, high susceptibility to the rabies virus (RV), and tendency to inhabit areas around human dwellings and domestic animals. Skunks are the main rabies vector species in mid-continental North America and in California where three variants of skunk RV are enzootic [3,6,7]. They are also an important secondary host for raccoon RV variant (RRVV) along much of the US eastern seaboard. Historically, raccoons (*Procyon lotor*) have accounted for the largest portion of terrestrial wildlife rabies cases infected with RRVV, but recently some states have reported marked increases in rabid striped skunks (*Mephitis mephitis*), with numbers of RRVV-infected skunks often surpassing those of rabid raccoons [3,8]. During an epizootic of Arctic RV variant (ARVV) in southern Ontario that primarily involved red foxes (*Vulpes vulpes*), striped skunks were the second most important rabies vector species [4,9,10]; however, they may now serve as the main vector of this virus variant [11]. Skunks in Arizona were also shown to have maintained an unexpected variant of RV, one usually associated with big brown bats [12,13].

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Oral rabies vaccination (ORV) is a proven method of wildlife rabies control provided an effective oral vaccine exists for the target species. Several live-attenuated oral rabies vaccines have demonstrated utility in red fox [14–18] and at least one recombinant has proven efficacious in a variety of fox species, as well as in coyotes (*Canis latrans*) and raccoon dogs (*Nyctereutes procyonoides*) [19–25]; however, skunks have generally been refractory to immunization with these vaccines by the oral route. Commercial oral rabies vaccines have either proven completely ineffective in skunks [1,26–29] or effective only when administered per os [30]. An alternative recombinant rabies vaccine is in the experimental phase but has yet to be proven safe for wildlife other than skunks [31].

To advance current North American wildlife rabies control efforts, there is a need for a safe and efficacious oral rabies vaccine for skunks. One candidate new oral wildlife rabies vaccine is a human adenovirus serotype 5 rabies glycoprotein recombinant (AdRG1.3 – trade name ONRAB®) [32,33]. In laboratory studies, ONRAB® administered directly into the oral cavity (DIOC) resulted in seroconversion in 88–100% of skunks [33,34]. In early field trials in Ontario, live-trapped skunks and raccoons demonstrated serologic evidence of an immune response following aerial distribution of ONRAB® vaccine baits [35]. Recently, antibody response to ONRAB® baits, and protection from experimental RV infection, have been examined in detail under captive conditions for raccoons and foxes, and results were encouraging [36,37]. The current study was conducted to determine if similar results could be attained in skunks. The objectives of this study were to (1) examine the humoral immune response of striped skunks to ONRAB® and (2) test the protection afforded by the vaccine against street RV. Skunks were presented vaccine-filled baits or were administered ONRAB® via DIOC.

2. Methods

2.1. Study animals and housing

Sixty-six captive-reared striped skunks were purchased from a commercial breeder in July, 2010. The health of all skunks was assessed upon arrival at the Ontario Ministry of Natural Resources (OMNR) research facility and then all animals were de-wormed (Strongid*T; 50 mg/mL; Pfizer Animal Health, Quebec, Canada) and vaccinated against canine distemper (Galaxy*D; Schering-Plough Animal Health, NE, USA). For the serology study, skunks were housed in separate units within outdoor enclosures. During the challenge study at the Centers for Disease Control and Prevention (CDC) in the US, skunks were housed in kennel runs in an Animal Biocontainment Level-2 facility. Animals were provided a commercial feed once a day and water ad libitum. All animals were maintained in accordance with national guidelines (Canadian Council on Animal Care Guidelines [38] or Guide for the Care and Use of Laboratory Animals [39]) and protocols were approved by institutional Animal Care and Use Committees (OMNR, Canada or CDC, US).

2.2. Vaccination protocol

The Ultralite bait (UL) consisted of a wax-fat matrix surrounding a small plastic blister pack containing 1.8 mL (±0.1 mL) of ONRAB® oral rabies vaccine (10^{10} $CCID_{50}$/mL). UL were manufactured by Artemis Technologies Inc., Guelph, Ontario, Canada [35]. Skunks were randomly assigned to either a control group or one of two treatments (vaccinate groups) with minor adjustments to ensure similar age–sex distribution among the groups. At treatment, juveniles were 5 months old; adults were 15 months. Thirty-one skunks (14 adults, 17 juveniles) were each offered one ONRAB® bait on October 5, 2010 following a 24 h fasting period. Bait debris and vaccine spillage were collected on plastic sheets below each cage. The amount of bait ingested, vaccine loss during ingestion, condition of the blister pack (if found), and timing of ingestion were recorded every 10 min for the first 0.5 h then every 15 min for the next 1.5 h. Another 25 skunks (13 adults, 12 juveniles) were administered 1.8 mL ONRAB® via DIOC using a 3 mL needleless syringe. Ten controls (5 adults, 5 juveniles) received no vaccine.

2.3. Blood collection

A blood sample (3–4 mL) was collected from controls and vaccinates 6 weeks before treatment, and then 5 and 7 week post-vaccination (PV). Animals were anesthetised and blood collected as described in [36]. Blood samples were also taken immediately prior to rabies challenge (section 2.4) and 7 days post-inoculation (PI). Blood samples were refrigerated at 4 °C for up to 48 h and then centrifuged at 1000 × *g* for 12 min at 4 °C. Serum (1–2 mL) was collected and frozen at −20 °C until testing. Prior to testing, serum was thawed and then heat-inactivated at 56 °C for 30 min.

2.4. Rabies challenge

Sixty-four skunks (10 controls, 54 vaccinates) were subjected to rabies challenge on June 9, 2011. Two skunks from the serology study were not included in the challenge trial; one skunk was not shipped due to space limitations at CDC, another did not acclimate to new housing. ARVV was chosen as the challenge virus in this study as it is the virus variant currently circulating in southern Ontario, affecting skunks [4,9–11]. The challenge virus was isolated from the salivary glands of a naturally infected rabid red fox and identified as ARVV using a panel of anti-nucleocapsid monoclonal antibodies (mAb) and sequence analysis [13] (CDC SM1515; GenBank accession number JQ685990). The RV was amplified in murine neuroblastoma cells with 3 serial passages. The supernatant was harvested and clarified by low speed centrifugation (800 × *g*) for 10 min then stored at −120 °C before use.

Rabies challenge was scheduled to occur 90 days PV; however, various logistical factors delayed the trial 157 days. Two hundred and forty-seven days PV, skunks were anesthetised and inoculated bilaterally with 0.5 mL of RV ($10^{4.9}$ $MICLD_{50}$) into the masseter muscle. Animals were observed twice daily for clinical signs of rabies (paralysis, ataxia, acute behavioral change, lack of food consumption, hyper-salivation, vocalization, agitation, tremors, convulsions, unprovoked aggression) and euthanized upon manifestation of two or more clinical signs. All surviving animals were euthanized 142 days PI and submitted for rabies diagnosis using the direct fluorescent antibody test [40].

2.5. Serologic analyses

A competitive enzyme-linked immunosorbent assay (C-ELISA) [41] was used to detect rabies antibody (RAb) in sera from the humoral immune response study. C-ELISA measures the ability of a test serum to inhibit the binding of a neutralizing, peroxidase-labeled mAb, specific for a linear epitope in antigenic site I of the glycoprotein, to immobilized Evelyn-Rokitnicki-Abelseth virus. Results are expressed as the percent inhibition of binding of the mAb. Serum samples were considered positive for RAb if inhibition was ≥26%. At a cut-off of 26%, the C-ELISA had a sensitivity of 85% and a specificity of 96% when compared to a RV neutralization assay with a positive threshold value of 0.5 IU/mL. Detection of RAb by 7 week PV was considered a positive response to vaccination. A rapid fluorescent focus inhibition test [42] with endpoint titers determined [43] was used to detect rabies virus neutralizing antibody (RVNA) in sera from the challenge study. Titers are reported

Table 1
Numbers of striped skunks (*Mephitis mephitis*) in the control and vaccinate groups that demonstrated evidence of rabies antibody (RAb+) by week 7 post-vaccination (PV) and the number that survived rabies challenge 247 days PV. Numbers of skunks that had rabies virus neutralizing antibodies (RVNA) on the day of challenge and 7 days post-inoculation (PI) are summarized for skunks that were RAb+ and RAb− post-vaccination; also presented are numbers of skunks that survived challenge in each of the latter groups.

	RAb+ by week 7 PV (%)	Overall Survival (%)	RAb+ post-vaccination[a]				RAb− post-vaccination			
			Total	RVNA[b] on day of challenge	RVNA 7 days PI	Survived challenge	Total	RVNA on day of challenge	RVNA 7 days PI	Survived challenge
Control	0/10 (0%)	0/10 (0%)	0				10	0	0	0
Bait	18/31 (58%)	25/31 (81%)	18	10	17	18	13	0	8	7
DIOC	25/25 (100%)	23/23[c] (100%)	23	18	22	23	0			

[a] A competitive enzyme-linked immunosorbent assay was used to detect RAb in sera from the humoral immune response study.
[b] A rapid fluorescent focus inhibition test was used to detect RVNA in sera from the challenge study.
[c] Two skunks in the DIOC group were not included in the challenge study; one was not shipped due to space limitations at CDC, another did not acclimate to new housing at the challenge facility.

in IU/mL following normalization against the US Standard Rabies Immune Globulin (Laboratory of Standards and Testing, Food and Drug Administration, USA) diluted to 2 IU/mL; titers ≥0.5 IU/mL were considered positive for RVNA.

2.6. Statistical analyses

For skunks presented ONRAB® vaccine baits, a log-linear analysis was used to test for main and interaction effects among the following categorical variables: age (adult–juvenile), sex (male–female), and response to vaccination (positive–negative). For this same group of skunks, Fisher's exact tests (FET) were used to examine associations between sex and response to vaccination, and response to vaccination and survival following challenge. A Mann–Whitney test was used to determine whether the number of days until death post-challenge differed among the control group and vaccinates that received ONRAB® baits [44]. STATISTICA© (StatSoft, Tulsa, Oklahoma, USA) was used for all analyses (StatSoft, Tulsa, Oklahoma, USA).

3. Results

3.1. Bait consumption

Vaccine baits were well consumed by both adult and juvenile skunks. Twenty-three skunks consumed baits within 10 min, two others within 20 min; the remaining six skunks took up to 1.5 h. Unconsumed vaccine (0.5 mL) and bait matrix (30%) were recovered from only one cage (#950). It would appear that all skunks offered a bait had some contact with the vaccine, as 17 blister packs were not found and presumed consumed by the animals, 11 blister packs were well masticated and a further two had a few bite marks. Blister pack information was not recorded for one skunk.

3.2. Serologic response to ONRAB®

Throughout the study, no vaccine-induced morbidity or mortality was observed among skunks. Pre-treatment sera for all skunks, and sera for all controls throughout the serology study, were negative for RAb (C-ELISA <26%).

By week 7 PV, RAb was detected in 58% (18/31) of skunks in the bait group and 100% (25/25) of skunks that received ONRAB® DIOC (Table 1). Fifteen skunks presented ONRAB® baits had RAb detectable at weeks 5 and 7; three others had RAb detectable only at week 7. Twenty-two skunks in the DIOC group demonstrated RAb at weeks 5 and 7. One skunk had RAb detectable only at week 5 PV, two others at week 7 only. For the bait group, only the two-way interaction among sex and response to vaccination was significant in the log-linear analysis ($P = 0.0202$) wherein more male skunks produced RAb following bait presentation than females ($P = 0.0325$, FET). Proportions of male and female responders were 76% (13/17) and 36% (5/14), respectively.

3.3. Challenge of skunks with RV

3.3.1. Unvaccinated controls

All control skunks ($n = 10$) succumbed to RV challenge as confirmed by the direct fluorescent antibody test. Among controls and vaccinates that did not survive challenge, there was no significant difference in the number of days to death or humane euthanasia PI (median = 13 days; range 10–29 days) ($U = 20$, $P = 0.2781$).

3.3.2. DIOC group

On the day of challenge, 18/23 skunks demonstrated RVNA (range 1.0–11.2 IU/mL). By seven days PI, 22/23 skunks appeared to have mounted an anamnestic response to rabies challenge (range 2.8–14.0 IU/mL) (Table 1). All skunks in this group survived challenge.

3.3.3. Bait group

Ten skunks in the bait group had RVNA on the day of challenge (range 1.0–11.2 IU/mL). These same skunks, as well as 15 others (seven responders, eight non-responders), had elevated RVNA 7 days PI (range 0.56–14.0 IU/mL) (Table 1). All 18 skunks that had RAb detectable by week 7 PV survived rabies challenge. Seven additional skunks without detectable RAb PV also survived challenge. There was a significant association between response to vaccination and survival following rabies challenge ($P = 0.0023$, FET) wherein more skunks with detectable RAb PV survived ARVV infection.

4. Discussion

Skunks have presented a challenge for North American wildlife rabies control efforts due to the lack of an effective oral rabies vaccine for this species. This is the first study to examine the antibody response of captive skunks to ONRAB® (AdRG1.3), a human adenovirus recombinant rabies vaccine, when presented in baits and administered DIOC, with subsequent RV challenge to test the protection afforded by the vaccine. In an early study by [45], skunks aged 4–36 months were immunized with various formulations of a prototype vaccine by DIOC and 75–100% of skunks were considered antibody positive (titer ≥0.13 IU/mL). In a later study, skunks were immunized with new variations of the adenovirus vector via DIOC and 88–100% seroconverted within 8 weeks [33]. More recently, a 100% seroconversion rate was observed for skunks administered the current formulation of ONRAB® DIOC [34]. Results obtained in the present study compare favorably with those of all studies in that 100% of skunks administered ONRAB® DIOC seroconverted by week 7 PV. In only one of the previous studies was vaccine

administered to skunks in baits; all five skunks presented sponge baits containing 4 mL of Ad5RG1 seroconverted [45]. In the present study, 58% of skunks offered baits containing 1.8 mL of ONRAB® demonstrated serologic evidence of an antibody response to vaccination. The lower vaccination rate in our study may be due to a lower vaccine volume and/or use of a higher seropositive threshold; however, the large sample size used in our study ($n = 31$) likely provided a more accurate estimate of immunization potential.

Results of this study support the hypothesis that ONRAB® is not only immunogenic in skunks, but as previously shown with raccoons and red foxes [36,37], also confers protection against virulent RV challenge, with no deaths in the group administered vaccine DIOC and only 19% mortality in the bait group. Interestingly, although 58% of skunks in the bait group seroconverted, 81% of these animals survived challenge, suggesting that in skunks, antibody production alone is not an absolute correlate of protection. These results differ from those observed in the captive raccoon and red fox ONRAB® studies where there was a relatively strong association between serologic response and survival following rabies challenge [36,37]. It is possible that cellular immune responses triggered by the vaccine contributed more to protection in skunks than in the other species. Alternatively, the C-ELISA positive threshold was selected to correspond to a neutralizing titer of 0.5 IU/mL; thus, levels of RAb may have been produced that were below this threshold, but a memory immune response that was protective still developed. This is supported by the observation that eight RAb negative skunks had detectable RVNA 7 d PI, suggestive of an anamnestic response, and seven of these animals survived challenge.

In previous vaccine efficacy studies in skunks, most challenge trials were performed 30–90 days PV. In this study, the duration between vaccination and challenge was longer than the planned 90 days; however, it provided a great opportunity to test the long-term protective potential of ONRAB® in skunks. Duration of immunity provided by ONRAB® in baits (and DIOC) was at least 247 days. Based on results of this study, skunks that consume ONRAB® baits during fall ORV campaigns should be protected through to spring and early summer when intra-species interactions are heightened during territorial defence, mating, and parturition.

Field immunization rates in target species are usually estimated by collecting blood samples from live-trapped animals post-ORV and testing sera for RAb. Various ONRAB® field trials have been conducted in the provinces of Ontario, Quebec and New Brunswick that have, for the most part, used bait densities and flight line spacings designed to target raccoon populations. In these studies, RAb prevalence in sampled skunks, measured by C-ELISA, ranged from 7 to 25% [35,46,47]. When baits were distributed at higher densities and flight lines spaced closer together to better target skunk populations, immunization estimates were improved (20–34%) [48]. If results of the present captive study translate to the field, then the proportion of "protected" wild skunks may actually exceed that estimated by post-ORV sampling. In Ontario, ORV targeting foci of rabid skunks began in 2008. Between 1998 and 2007, 17–57 rabid skunks were reported in the province each year. In 2011 and 2012, there was only one confirmed case of skunk rabies each year, and none were reported in 2013 [2,5]; thus it would appear a field immunization rate sufficient for disease control was achieved.

The higher seroprevalence observed in this captive study, as compared to the field trials, cannot be explained by differences in bait characteristics as the same bait format was used in all trials. However, in the captive trials, skunks were withheld food for 24 h prior to bait presentation which may have improved bait consumption, with most individuals (25/31) doing so within 20 min. In contrast, it is possible that the availability of more attractive and/or abundant food items, and consumption of baits by other wildlife, may lower bait uptake by free-ranging skunks. The sampling schedule of 5–6 weeks post-ORV in all field trials would appear adequate for detection of RAb as 48 and 58% of captive skunks tested seropositive at 5 and 7 weeks PV, respectively. However, in field trials, the exact length of time between bait uptake and serologic sampling is unknown and so it is possible that some animals were sampled prior to (or after) the development of maximal RAb titers. Together these considerations may have contributed to the lower seroprevalence rates observed in the ONRAB® field trials.

In summary, we have reported on the effectiveness of ONRAB® vaccine in striped skunks, an important North American rabies vector species that has previously been refractory to oral immunization. Survival rates of 81% and 100% for skunks that ate vaccine baits and that received ONRAB® DIOC, respectively, are tremendous achievements in the field of wildlife ORV. We present these results and suggest that ONRAB® is certainly worthy of consideration in efforts to control rabies in skunks. Previous investigations have also shown ONRAB® to be safe and effective by the oral route in raccoons and red foxes [35–37]; thus, it may now be possible to immunize several important North American rabies vector species with the same vaccine. ONRAB® is currently licensed for use in Canada, for government wildlife rabies control programs targeting skunks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vaccine.2014.04.029.

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