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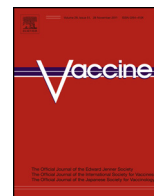
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## The safety of ONRAB<sup>®</sup> in select non-target wildlife



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### ABSTRACT

ONRAB<sup>®</sup> is a recombinant human adenovirus type 5 (HAd5) with the rabies glycoprotein gene incorporated into its genome. ONRAB<sup>®</sup> has been used in Canada as an oral rabies vaccine in target wildlife species such as: red fox (*Vulpes vulpes*), raccoon (*Procyon lotor*), and striped skunk (*Mephitis mephitis*). We evaluated the safety of ONRAB<sup>®</sup> in non-target wildlife species likely to contact the vaccine baits during oral rabies vaccine campaigns in the United States. We investigated the effects of oral inoculation of high titer ONRAB<sup>®</sup>, approximately ten times the dose given to target species, in wood rats (*Neotoma* spp.), eastern cottontail rabbits (*Sylvilagus floridanus*), Virginia opossums (*Didelphis virginiana*), eastern wild turkeys (*Meleagris gallopavo silvestris*), and fox squirrels (*Sciurus niger*). We performed real-time polymerase chain reaction (PCR) on fecal swabs, oral swabs, and tissues, including lung, liver, kidney, small intestine, large intestine, and when appropriate nasal turbinates, to detect ONRAB<sup>®</sup> DNA from inoculated animals. By seven days post-inoculation, turkeys, opossums, and cottontails had all stopped shedding ONRAB<sup>®</sup> DNA. One wood rat and one fox squirrel still had detectable levels of ONRAB<sup>®</sup> DNA in fecal swabs 14 days post-inoculation. Real-time PCR analysis of the tissues revealed some ONRAB<sup>®</sup> DNA persisting in certain tissues; however, there were no significant gross or histologic lesions associated with ONRAB<sup>®</sup> in any of the species studied. Our results suggest that many non-target species are not likely to be impacted by the distribution of ONRAB<sup>®</sup> as part of oral rabies vaccination programs in the United States.

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### 1. Introduction

Rabies viruses in the United States once affected domestic animals and wildlife alike, but since the 1970s have been effectively eliminated in domestic animals, a result of municipal mandates requiring pets be vaccinated for rabies. Now control of rabies virus in the United States focuses on controlling the pathogen in wildlife reservoirs. Since 1995, the United States National Rabies Management Program (NRMP), administered by the United States Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services (WS), has been working to control terrestrial rabies virus through wildlife vaccination. Oral rabies vaccines are distributed by hand and by fixed and rotary-wing aircraft in most states east of the Appalachian Mountains, as well as portions of Ohio, Texas, New Mexico, and Arizona. It is estimated that at least 60% of raccoons (*Procyon lotor*) in an infected population must be

immunized against the rabies antigen to eliminate raccoon rabies [1]. At present, it is estimated that 30% of raccoons are inoculated [2] by the Oral rabies vaccination (ORV) program in the United States.

Raboral V-RG<sup>®</sup> (Merial Ltd., Athens, GA), the vaccine presently used in the ORV program, is a recombinant vaccine comprised of a vaccinia virus with the rabies glycoprotein gene inserted into its genome. Presently, raccoons, gray foxes (*Urocyon cinereoargenteus*), coyotes (*Canis latrans*), and possibly other terrestrial mammals can be inoculated by ingesting this liquid vaccine, which is contained in a palatable sachet [2,3]. Ideally, when the animal bites into the sachet the liquid vaccine coats the buccal lining, entering and replicating in the oral cavity, tonsils, and other surrounding tissues, and stimulates an immune response. However, the immunogenicity of Raboral V-RG, as determined by measurements of circulating rabies virus neutralizing antibodies (rVNA), is limited. Brown et al. [4] reported that of 19 wild raccoons inoculated by Raboral V-RG<sup>®</sup> 14 succumbed to a rabies challenge. Additionally, a vaccine comparison study in Canada and the United States found that only 30% of raccoons inoculated in the United States by Raboral V-RG<sup>®</sup> demonstrated detectable rVNA  $\geq 0.5$  IU/mL, while nearly 75% of raccoons administered, ONRAB<sup>®</sup> (Artemis Technologies, Guelph, Ontario, Canada) produced rVNA titers  $\geq 0.5$  IU/mL [5,6].

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ONRAB<sup>®</sup> is a recombinant oral rabies vaccine with human adenovirus type 5 (HAD5) as the backbone and the rabies virus glycoprotein gene inserted into its genome. HAD5 is a relatively safe and well-studied virus, which is used in many vaccine formulations [7–9], but does not replicate well in most non-human species [10]. The addition of the rabies virus glycoprotein allows the virus to infect many different species and triggers the host's immune system to produce antibodies against the rabies virus [10,11]. Studies on the efficacy and safety of the ONRAB<sup>®</sup> vaccine in animals have been completed in Canada [6,11]. Results of field and laboratory studies indicated that persistence of the virus in animals is short-lived and that ONRAB<sup>®</sup> is unlikely to have an overall negative impact on wildlife [6,10–12]. Rabies virus seroconversion rates for raccoons in areas baited with ONRAB<sup>®</sup> averaged 80% in the first two years [13], well above the seroconversion rates detected when the vaccine Raboral V-RG<sup>®</sup> was used [3,4].

Knowles et al. conducted vaccine safety and immunogenicity studies on 18 species: striped skunks, red foxes (*V. vulpes*), raccoons, meadow voles (*Microtus pennsylvanicus*), deer mice (*Peromyscus leucopus*), gray squirrels (*Sciurus carolinensis*), laboratory rabbits (*Oryctolagus cuniculus*), ground squirrels (*Marmota monax*), cattle (*Bos taurus*), horses (*Equus ferus*), domestic swine (*Sus domesticus*), domestic chickens (*Gallus domesticus*), sheep (*Ovis aries*), dogs (*Canis familiaris*), cats (*Felis domesticus*), cotton rats (*Sigmodon hispidus*), SCID mice and nude mice (*Mus musculus*) [11]. For their non-target safety trials, Knowles et al. [11] administered an ONRAB<sup>®</sup> dose approximately 10 times greater than that needed to vaccinate target species. Animals were monitored daily and all tolerated exposure to the vaccine. Analyses of tissues resulted in viral DNA detected in some lung and kidney samples. Following histopathological analysis, the most marked findings were in the lungs with pulmonary congestion possibly due to vaccine aspiration. Oral swabs and/or fecal samples were collected from skunks, raccoons, foxes, cotton rats, cats and dogs. Viral shedding, if any, was generally complete by 4 days post-inoculation (dpi) except for one skunk, which continued to shed virus in feces through 14 dpi [11].

Our research expands on the species evaluated by Knowles et al. [11] and investigates the vaccine as it relates to its safety in wildlife species likely to come in contact with ONRAB<sup>®</sup> as part of WS NRPV ORV operations. We investigated the effects of oral inoculation of high titer ONRAB<sup>®</sup> vaccine in 21 eastern wild turkeys (*Meleagris gallopavo silvestri*), 17 opossums (*Didelphis virginiana*), 14 cottontail rabbits (*Sylvilagus floridanus*), 21 fox squirrels (*Sciurus niger*), and 15 wood rats (*Neotoma* spp.). We performed post-mortem examination to determine any gross or histological pathology that may be linked to the vaccine. We assessed immunogenicity of ONRAB<sup>®</sup> in the non-target animals and evaluated the duration of viral DNA shed into the environment via fecal and oral shedding. We also investigated the persistence of ONRAB DNA in select tissues of vaccinated animals.

## 2. Materials and methods

### 2.1. Study animals

All animals were wild caught with the exception of the eastern wild turkeys which were purchased from a commercial breeder (McMurry Hatchery, Webster City, IA, USA) and shipped at 2 days old. All animal trapping, handling, maintenance, and sampling techniques were approved by the WS National Wildlife Research Center (NWRC) Institutional Animal Care and Use Committee (QA1882). Animals were trapped and transported under permits issued by the State of Colorado (Permit #11TR1232A1, Permit # 12TR1232) and individually housed throughout the study.

### 2.2. Vaccination

Artemis Technologies Inc. (Guelph, Ontario, Canada) supplied us with bulk ONRAB<sup>®</sup> oral rabies vaccine under the United States Veterinary Biological Product Permit No. VB-118764. The 50% cell culture infectious dose (CCID<sub>50</sub>) of the vaccine was 10<sup>10.44</sup> CCID<sub>50</sub>/mL. We followed the methods described by Knowles [11] et al. and orally inoculated animals with 1.8 mL dose of vaccine, which resulted in an infectious virus titer approximately 10 times higher than found in a standard ONRAB<sup>®</sup> bait.

### 2.3. Sample collection

We evaluated the safety of the ONRAB<sup>®</sup> vaccine via numerous tests and observations following the methods of Knowles et al. [11]. On a daily basis we monitored animals for changes in behavior and eating habits. We looked for shedding of ONRAB<sup>®</sup> DNA via oral swabs on 0, 4, and 7 days post infection (dpi) in turkeys, cottontail rabbits, opossums and fox squirrels and on 0 and 4 dpi in wood rats. We also collected feces on 0–7, 14, 21, and 28 dpi for all species. We collected paired blood samples on 0 and 28 dpi to evaluate sera for Rabies virus neutralizing antibodies (rVNA).

One-half of the study animals for each species were humanly euthanized on 4 dpi, when viral replication should be at or near its peak [11–12,14]. The remaining individuals were euthanized at the conclusion of the study (28 dpi). Turkeys were euthanized by an intravenous injection of an overdose of barbiturates. Opossums were anesthetized with 10 mg/kg ketamine HCl and 2 mg/kg xylazine via intramuscular injection before receiving an intracardiac injection of an overdose of barbiturates. Cottontail rabbits and fox squirrels were anesthetized with isoflurane then given an overdose of barbiturates via intracardiac injection. Wood rats were euthanized by an inhaled overdose of isoflurane.

### 2.4. Post-mortem examination

Following euthanasia on either 4 dpi or 28 dpi, a standard post-mortem examination was conducted by a veterinary pathologist. Any abnormal tissue and routine sections of lung, liver, kidney, small intestine, and large intestine were preserved in 10% neutral buffered formalin for histology. Tissues were embedded in paraffin, cut at 5 μm, stained with hematoxylin and eosin stain and examined histologically. Fresh tissues were also collected for ONRAB<sup>®</sup> DNA detection by real-time PCR.

### 2.5. Serological methods

Serum samples were heat inactivated at 56 °C for 30 min. Rabies virus neutralizing antibodies were detected in the sera by use of the rapid fluorescent focus inhibition test (RFFIT), as described by Smith et al. [15]. Murine neuroblastoma cells were used for infection by the CVS rabies virus strain. Sera with rVNA activity ≥0.2 IU/ml were considered positive samples.

### 2.6. Real-time polymerase chain reaction

We used the MagMAX<sup>™</sup> Viral RNA isolation kit (Life Technologies, Grand Island, NY, USA), which is also recommended for isolating DNA, to extract ONRAB<sup>®</sup> DNA from fecal and oral swabs. The DNeasy Blood & Tissue kit (Qiagen, Valencia, California, USA) was used to isolate DNA from 10 to 25 mg of lung, liver, kidney, small intestine, large intestine, and nasal turbinates as directed by the manufacturer. Real-time PCR was performed on the extracted DNA using published primers and a dual-labeled probe for an ONRAB target sequence [11]. Twenty-five milliliter PCR reactions containing 1× TaqMan Universal Master mix (Life Technologies,

**Table 1**The frequency of oral and fecal samples positive for ONRAB<sup>®</sup> DNA by real-time PCR, by species, following vaccination with ONRAB<sup>®</sup>.

dpi	Positive fecal swabs (n <sup>a</sup> )								Positive oral swabs (n)	
	1	2	3	4	5	6	7	14	4	7
Eastern wild turkey	14(21)	6(21)	1(21)	0(21)	0(10)	0(10)	0(10)	0(10)	1(21)	0(10)
Opossum	9(17)	17(17)	12(14)	5(17)	5(17)	2(8)	0(8)	0(8)	3(17)	0(8)
Eastern cottontail	4(13)	0(14)	2(14)	0(14)	1(7)	0(7)	0(7)	0(7)	1(14)	0(7)
Fox squirrel	17(21)	16(21)	17(20)	12(21)	8(11)	9(11)	8(11)	1(11)	19(21)	3(11)
Wood rat	1(15)	2(15)	1(15)	9(15)	–	–	1(7)	1(7)	0(15)	–

<sup>a</sup> Sample size includes number of inoculated individuals tested.**Table 2**The frequency of tissue samples positive for ONRAB<sup>®</sup> DNA by real-time PCR, by species, following vaccination with ONRAB<sup>®</sup>.

	dpi	Lung (n)	Liver (n)	Kidney (n)	Small intestine (n)	Large intestine (n)	Nasal turbinates (n)
Eastern wild turkey	4	2(11)	0(11)	0(11)	0(11)	4(11)	
	28	0(10)	0(10)	0(10)	0(10)	0(10)	
Opossum	4	0(9)	0(9)	0(9)	0(9)	0(9)	0(9)
	28	0(8)	1(8)	1(8)	0(8)	0(8)	1(8)
Eastern cottontail	4	4(7)	0(7)	0(7)	1(7)	2(7)	0(7)
	28	2(7)	0(7)	0(7)	0(7)	1(7)	1(7)
Fox squirrel	4	9(10)	6(10)	8(10)	3(10)	5(10)	0(10)
	28	4(11)	1(11)	1(11)	0(11)	0(11)	1(11)
Wood rat	4	0(8)	3(9)	0(9)	0(9)	0(9)	0(9)
	28	0(7)	1(7)	0(7)	0(7)	0(7)	0(7)

Grand Island, New York, USA), 400 nM forward primer, 400 nM reverse primer, 400 nM dual-labeled probe, and 3  $\mu$ l DNA template were analyzed with an ABI 7900HT real-time PCR system (Life Technologies, Grand Island, New York) under the following conditions: 50 °C for 2 min, 95 °C for 10 min and then 45 cycles of 95 °C for 10 s and 60 °C for 1 min. The working range of the assay was approximately 10<sup>3</sup>–10<sup>8</sup> TCID<sub>50</sub>/mL PCR equivalents [9] and samples with cycle threshold (Ct) values less than 37 (approximately 10<sup>3</sup> TCID<sub>50</sub>/mL or greater) were considered positive for ONRAB<sup>®</sup> DNA.

### 3. Results and discussion

We did not observe any behavior changes in any of the animals inoculated with ONRAB<sup>®</sup>. All gross and histologic lesions seen in all species were interpreted as secondary to natural infection or experimental protocol. Numerous parasites were considered incidental findings in inoculates. Pneumonia and lymphoid hyperplasia seen in the lungs of a single wood rat were interpreted as secondary to aspiration and antigenic stimulation.

We did not detect rVNA in any day 0 samples, regardless of species (<0.06 IU/ml). We used the RFFIT to determine that the majority of animals (91%) responded with the induction of rVNA from comparison of paired sera samples, sera collected at 0 and 28 dpi from the same individual. Eight of 10 inoculated turkeys had evidence of rVNA, and all positive samples were >0.2 IU/ml. In inoculated opossums, 8 of 8 had evidence of rVNA, and all positive samples were >0.2 IU/ml. For the inoculated cottontail rabbits, 6 of 7 day had evidence of rVNA, and all positive samples were >0.2 IU/ml. Eleven of 11 inoculated fox squirrels had evidence of rVNA, and all positive samples were >17 IU/ml. Six of 8 inoculated woodrats had evidence of rVNA, and all positive samples were >2.7 IU/ml. These results show that nearly all animals, 91%, developed a positive rabies antibody response after being orally inoculated with ONRAB<sup>®</sup>.

By 7 dpi no ONRAB<sup>®</sup> DNA was detected in the fecal swabs of turkeys, opossums, or cottontails (Table 1). One wood rat and eight fox squirrels still had detectable levels of ONRAB<sup>®</sup> DNA in fecal swabs on 7 dpi. The high number of fox squirrels still shedding ONRAB<sup>®</sup> DNA at 7 dpi was unexpected and may be explained by more efficient viral replication in fox squirrel cells or some false-positive detection by PCR. Additionally, at necropsy it was

determined that at least 50% of fox squirrels were co-infected with *Leptospira interrogans* [16]. This co-infection may be a confounding factor, compromising an animal's immune system and ability to clear the virus, resulting in the prolonged detection of ONRAB<sup>®</sup> DNA in fecal swabs from these animals. At 14 dpi, we recovered ONRAB<sup>®</sup> DNA from fecal swabs of one wood rat and a single fox squirrel; by 21 dpi no ONRAB<sup>®</sup> DNA from fecal swabs was detected in any individuals.

Oral shedding of ONRAB<sup>®</sup> DNA on 4 dpi was only detected in 3 opossums, and one turkey and one cottontail, but nearly all fox squirrels (Table 1). At 7 dpi, only three fox squirrels exhibited oral shedding. With the exception of our fox squirrels, we considered the potential persistence of ONRAB<sup>®</sup> in many non-target species to be low given the limited ONRAB DNA recovery through both oral and fecal sampling. Because we only tested for the presence of ONRAB DNA, we cannot assess whether or not infectious virus was being shed.

Although no pathology was noted, we detected ONRAB<sup>®</sup> DNA in all tissue types sampled (Table 2). ONRAB<sup>®</sup> DNA was detected most often in lung tissue, followed by liver and large intestine, kidney, small intestine, and nasal turbinates. There appeared to be some species variation as ONRAB<sup>®</sup> DNA was isolated in tissues of 14 fox squirrels, six cottontails, four turkeys, four wood rats, and only one opossum. Although varied, our results likely show individual variation as opposed to widespread shedding and persistence of ONRAB<sup>®</sup>.

Our results were not unexpected, and we concur with previous research [6,11,12] suggesting a low likelihood of persistence of ONRAB<sup>®</sup> in the environment or in individual animals that contact the vaccine even at ten times the recommended dose. To date there have been no known negative impacts on target or non-target animals following large-scale distributions of ONRAB in Canada [12]. Based on the results of our study, ONRAB<sup>®</sup> is not expected to have detrimental effects on non-target wildlife species if incidental ingestion occurs following ORV campaigns in the United States.

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