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Author(s): Dennis Slate, Richard B. Chipman, Timothy P. Algeo, Samuel A. Mills, Kathleen M. Nelson, Christopher K. Croson, Edward J. Dubovi, Kurt Vercauteren, Randall W. Renshaw, Todd Atwood, Shylo Johnson, and Charles E. Rupprecht

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SAFETY AND IMMUNOGENICITY OF ONTARIO RABIES VACCINE BAIT (ONRAB) IN THE FIRST US FIELD TRIAL IN RACCOONS (*PROCYON LOTOR*)

Dennis Slate,[1,7] Richard B. Chipman,[1] Timothy P. Algeo,[1] Samuel A. Mills,[2] Kathleen M. Nelson,[1] Christopher K. Croson,[2] Edward J. Dubovi,[3] Kurt Vercauteren,[4] Randall W. Renshaw,[3] Todd Atwood,[4,6] Shylo Johnson,[4] and Charles E. Rupprecht[5]

[1] US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Rabies Management Program, 59 Chenell Drive, Concord, New Hampshire 03301, USA
[2] US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, 730 Yokum Street, Elkins, West Virginia 26241, USA
[3] Animal Health Diagnostic Center, College of Veterinary Medicine, Cornell University, PO Box 5786, Ithaca, New York 14851, USA
[4] US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center, Rabies Research Project, 4101 LaPorte Avenue, Fort Collins, Colorado 80521-2154, USA
[5] Global Alliance for Rabies Control, 529 Humboldt Street, Suite 1, Manhattan, Kansas 66502, USA
[6] Current address: US Geological Survey, Alaska Science Center, Polar Bear Project, 4210 University Drive, Anchorage, Alaska 99508, USA
[7] Corresponding author (email: Dennis.Slate@aphis.usda.gov)

ABSTRACT: In 2011, we conducted a field trial in rural West Virginia, USA to evaluate the safety and immunogenicity of a live, recombinant human adenovirus (AdRG1.3) rabies virus glycoprotein vaccine (Ontario Rabies Vaccine Bait; ONRAB) in wild raccoons (*Procyon lotor*) and striped skunks (*Mephitis mephitis*). We selected ONRAB for evaluation because of its effectiveness in raccoon rabies management in Ontario and Quebec, Canada, and significantly higher antibody prevalence rates in raccoons compared with a recombinant vaccinia-rabies glycoprotein (V-RG) vaccine, Raboral V-RG®, in US–Canada border studies. Raccoon rabies was enzootic and oral rabies vaccination (ORV) had never been used in the study area. We distributed 79,027 ONRAB baits at 75 baits/km^2 mostly by fixed-wing aircraft along parallel flight lines at 750-m intervals. Antibody prevalence was significantly higher at 49.2% (n=262) in raccoons after ONRAB was distributed than the 9.6% (n=395) before ORV. This was the highest antibody prevalence observed in raccoons by US Department of Agriculture Wildlife Services for areas with similar management histories evaluated before and after an initial ORV campaign at 75 baits/km^2 with Raboral V-RG. Tetracycline biomarker (TTCC) was significantly higher among antibody-positive raccoons after ONRAB baiting and was similar among raccoons before ORV had been conducted, an indication of vaccine-induced rabies virus–neutralizing antibody production following consumption of bait containing TTCC. Skunk sample size was inadequate to assess ONRAB effects. Safety and immunogenicity results supported replication of this field trial and led to a recommendation for expanded field trials in 2012 to evaluate safety and immunogenicity of ground-distributed ONRAB at 150 baits/km^2 in residential and commercial habitats in Ohio, USA and aerially distributed ONRAB at 75 baits/km^2 in rural habitats along US–Quebec border.

Key words: AdRG1.3, ONRAB, oral rabies vaccination, rabies, raccoon, skunk.

INTRODUCTION

Coordinated oral rabies vaccination (ORV) with a recombinant vaccinia-rabies glycoprotein (V-RG) vaccine Raboral V-RG® (Merial, A SANOFI Company, Athens, Georgia, USA) has resulted in canine rabies elimination in coyotes (*Canis latrans*; Fearneyhough et al. 1998; Sidwa et al. 2005), and the US was declared canine rabies free in 2008 (Velasco-Villa et al. 2008). Oral rabies vaccination has been successfully applied in gray foxes (*Urocyon cinereoargenteus*) in Texas, USA (Sidwa et al. 2005), with no reported cases since May 2009 (Blanton et al. 2011), until a reported case in a cow in May 2013 (E. Oertli pers. comm.). ORV has prevented appreciable spread of raccoon (*Procyon lotor*) rabies to the west in the US (Slate et al. 2009).

Although these accomplishments are important, the inability to eliminate raccoon rabies from high-risk corridors

prompted the evaluation of vaccine baits potentially capable of producing population immunity levels necessary to achieve this goal (USDA 2010b). A live, recombinant human adenovirus–rabies glycoprotein vaccine (AdRG1.3; Charlton et al. 1992), designated Ontario Rabies Vaccine Bait (ONRAB; Artemis Technologies, Guelph, Ontario, Canada; Fig. 1), was selected on the basis of 1) its use along the US–Canada border without untoward events and success in reducing and eliminating raccoon rabies (Rosatte et al. 2009b; Mainguy et al. 2012); 2) lack of timely access to other vaccines for field testing; 3) absence of reported rabid striped skunks (*Mephitis mephitis*) infected with the raccoon rabies virus variant in Ontario since 2004 and Quebec since 2009 (D. Donovan and J. Mainguy pers. comm.), suggesting dependence on virus spillover from the raccoon reservoir; 4) published and unpublished ONRAB data from Canada (USDA 2008a; Rosatte et al. 2009a; Rees et al. 2011); 5) results of border studies comparing ONRAB and Raboral V-RG under similar environmental and rabies management conditions (Fehlner-Gardiner et al. 2012; Mainguy et al. 2013); and 6) effectiveness in controlling rabies in residual foci in skunks through high bait densities (300 baits/km^2) and narrow flight lines (250 m; Rosatte et al. 2011). We summarize key safety and immunogenicity findings of this ONRAB trial in raccoons and skunks, the first study of this type in the US in >20 yr (Hanlon et al. 1998; Roscoe et al. 1998), and discuss management implications and recommendations.

MATERIALS AND METHODS

Study area

The 1,460 km^2 field trial site in West Virginia, USA (37°44′N, 80°35′W) consisted of hardwood, mixed coniferous-hardwood, and evergreen forests (61%); livestock agriculture characterized by pasture/hay (23%); small towns (2%); and other habitats (14%; USDA 2011a; USGS 2014). Habitats were characterized by Strausbaugh and Core (1978) and Fleming et al. (2006). No ORV had been conducted in this study area, which was enzootic for raccoon rabies (Fig. 2).

FIGURE 1. Ontario Rabies Vaccine Bait (ONRAB) distributed in the oral rabies vaccine field trial in West Virginia, USA, 2011. (Photo: Wildlife Services' Rabies Management Program Photo Library)

Field trial design

The design included four 127-km^2 sampling cells separated by a 5-km buffer (Fig. 3). An 11-km buffer was established from the western edge of the ONRAB sampling cells to the Raboral V-RG zone baited at 75 baits/km^2 since 2001 (USDA 2005) to reduce the chance of confounded serologic results. A 5-km buffer was established from the sampling cell perimeters within the ONRAB baited area to reduce potential dilution effect from sampling raccoons with home ranges that extended into unbaited areas.

Oral rabies vaccine bait

The virus vector is well characterized (Graham and Prevec 1992). The Ultralite bait and live AdRG1.3, constituting ONRAB, have been described (Rosatte et al. 2009b). The matrix contained 100 mg of tetracycline hydrochloride (TTCC) biomarker (Rosatte et al. 2009b). There were 77,447 ONRAB baits aerially distributed along parallel flight lines at 750-m intervals at 75 baits/km^2; 1,580 ONRAB baits were distributed at the same bait density by ground means near and within towns where aerial baiting was deemed unsafe. Baits had a warning label with a toll-free number (Fig. 1) to facilitate communication of potential bait and vaccine exposures to the West Virginia Department of Health and Human Resources (WVDHHR).

Animal capture, handling, and sampling

Biologic and serologic sampling: Animal sampling included live (cage) trapping (Tomahawk

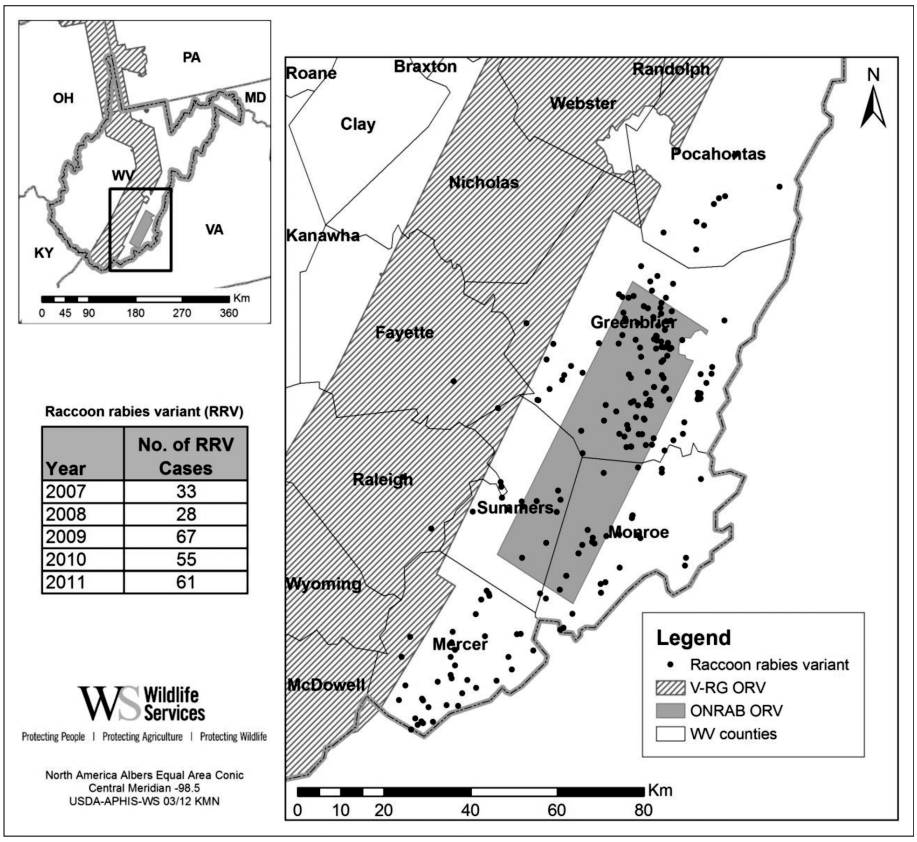

FIGURE 2. Raccoon (*Procyon lotor*) rabies virus variant (RRV) case history in West Virginia (WV), USA, 2007–11 within an 83-km radius of the center of the 2011 Ontario Rabies Vaccine Bait (ONRAB) oral rabies vaccination (ORV) zone (gray shading). Each dot represents a confirmed case of RRV in an animal, and the hatched area represents the 2011 vaccinia-rabies glycoprotein (V-RG) ORV zone.

model 608, Tomahawk Live Trap LLC, Hazelhurst, Wisconsin, USA) and release of target species apparently not rabid based on behavior and gross examination. Rabies suspects were tested using a direct rapid immunohistochemistry test (Lembo et al. 2006), with rabies confirmation and virus variant typing by the Centers for Disease Control and Prevention (CDC). Nontarget species were released or collected for future histopathologic analysis. We set 150 live traps within 800 m of 25 preselected random points within sampling cells (Fig. 3). Traps were checked for 10 consecutive days. The same random points were used pre- and post-ORV sampling, 2–26 August 2011 and 21–31 October 2011, respectively.

Captured rabies reservoir species were sedated using an intramuscular injection of a 5:1 ketamine:xylazine (Kreeger 1999), to collect blood for rabies virus–neutralizing antibody (RVNA) determination, and first premolar tooth samples for age and biomarker analysis. Sex, reproductive status, and general condition were recorded. Serum samples were collected from blood centrifuged the day of capture and stored in labeled cryovials at −25 C to −70 C before shipment for analysis. Raccoons and skunks were released at their capture site after recovery from sedation.

Oral swab sampling: Oral swab samples were collected 1–6 days after ONRAB distribution through live trapping in the three spaces between the four sampling cells (Fig. 3). This scheme was used to reduce the chance of trap shyness among raccoons and skunks sampled post-ORV, which began 5 wk after bait distribution, approximating the minimum time for serologic response from ONRAB consumption (Brown et al. 2012).

RVNA determination

Labeled cryovials containing serum were shipped to CDC frozen on dry ice after pre-

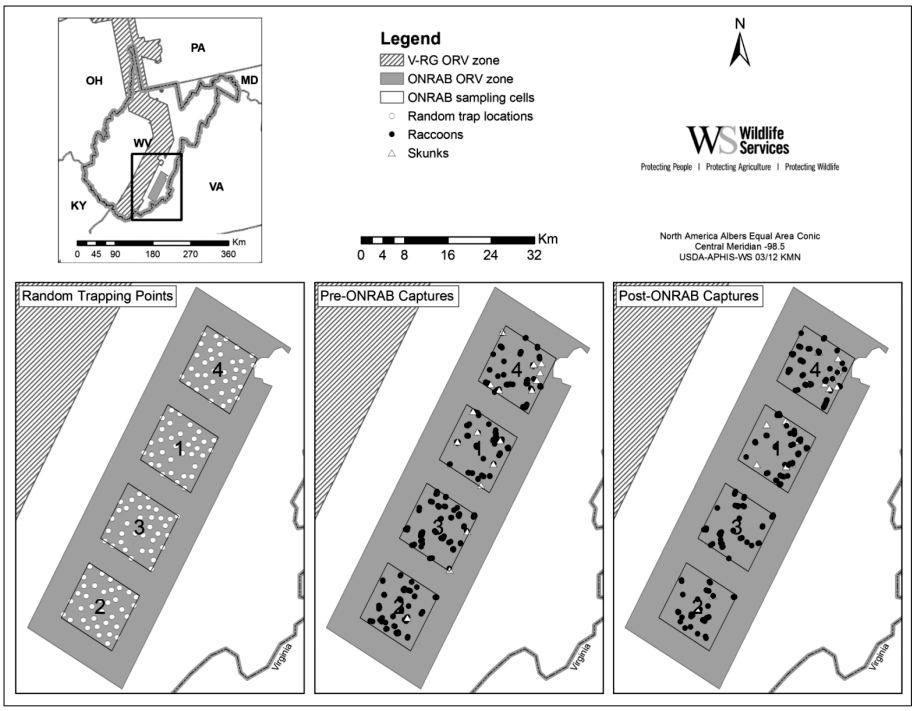

FIGURE 3. Preselected random live-trapping points and locations of raccoon (*Procyon lotor*) and striped skunk (*Mephitis mephitis*) captures during pre– and post–oral rabies vaccination (ORV) sampling periods in the Ontario Rabies Vaccine Bait (ONRAB) field trial in West Virginia, USA, 2011. The hatched area represents the 2011 vaccinia-rabies glycoprotein (V-RG) ORV zone.

and post-ORV sampling. The rapid fluorescent focus inhibition test (Smith et al. 1973; CDC 2011) was used to determine RVNA titer. Samples with international units (IUs) ≥0.06 were considered RVNA positive.

AdRG1.3-neutralizing antibody determination

Frozen serum was shipped in labeled cryovials on dry ice to the Animal Health Diagnostic Center, Cornell University (Ithaca, New York, USA), to determine AdRG1.3 virus–neutralizing antibody (VNA) pre- and post-ORV sampling. The adenovirus virus neutralization assay followed standard procedures. Twofold serum dilutions (50 μL) in duplicate were mixed with 100–300, 50% tissue culture infectious doses of Ad-5 (VR-5, American Type Culture Collection [ATCC]) in a 50-μL volume. Mixtures were incubated for ≥1 hr at room temperature. A 100-μL volume of indicator cells (A549-ATCC) was added to each well, and the plates were placed in a CO_2 incubator at 37 C for 6 days. Wells were scored for the presence or absence of typical Ad-5 cytopathology. Positive serum controls were an equine anti–Ad-5 (CDC, Atlanta, Georgia, USA) and a bovine anti–Ad-5.

Tetracycline biomarker and age determination

First premolars from raccoons and skunks were shipped to Matson's Laboratory (Milltown, Montana, USA) for TTCC and age determination. TTCC presence was determined with ultraviolet light at 100× magnification with a Leitz™ compound microscope (Leica Microsystems GmbH, Wetzlar, Germany; G. Matson pers. comm.). Ages were determined through the cementum method (Johnston et al. 1987). When ages could not be determined, individuals were assigned as juvenile or adult based primarily on body size and tooth characteristics (Grau et al. 1970).

ONRAB detection from oral swabs

An assay was developed for the AdRG1.3 vaccine in ONRAB according to Knowles et al. (2009a, b). A positive amplicon control (PAC) was generated using primers that flanked the assay target region (Ad5E3-F, GCGGACG-GCTACGACTGAATGTTA; RVG-R, TTGT-TTGGGCAGCTGAGGTGATGT). The PAC

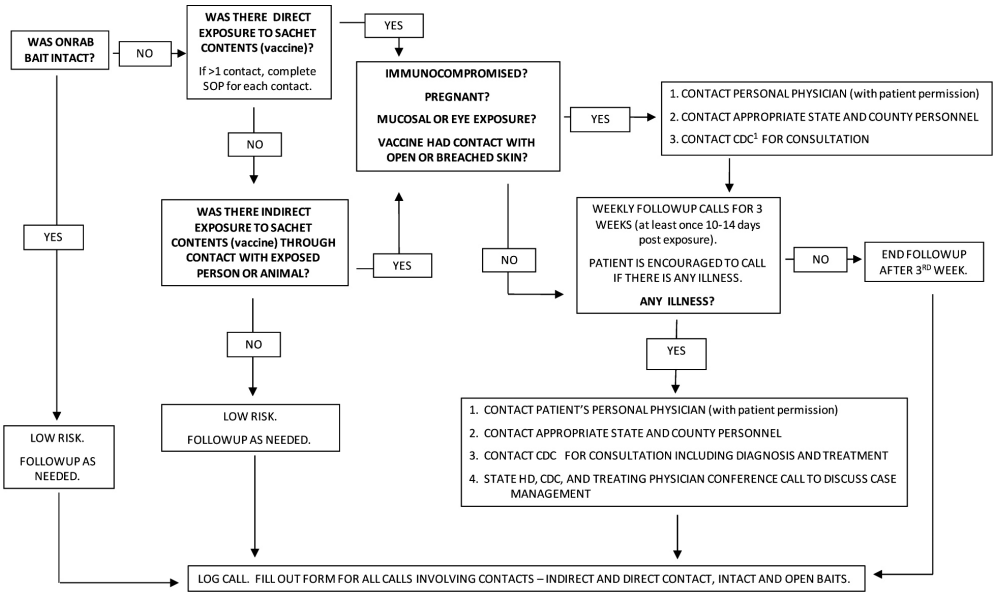

FIGURE 4. Suggested standard operating procedure (SOP) for documenting and managing human contacts with Ontario Rabies Vaccine Baits (ONRABs) during field trial in West Virginia, USA, 2011. This is a suggested SOP. State and county health departments were not required to follow this SOP and may have altered the components to accommodate their program. This document was originally created for vaccinia-rabies glycoprotein (V-RG) oral rabies vaccination (ORV) distribution by Andrea McCollum and Brett Petersen in 2010 and modified by Richard Chipman for ONRAB in 2011. The content is based on information and SOPs from the US Department of Agriculture, Texas Department of State Health Services, and Ohio Department of Health. CDC Emergency Operations Center phone number: (770) 488-7100.

dilution amplification series showed the assay to be consistently sensitive to a level of detection of approximating 10 copies of viral DNA.

Oral Swab samples stored in universal viral transport tubes (Becton Dickinson, Sparks, Maryland, USA) were shipped for analysis to the Animal Health Diagnostic Center. Nucleic acid was purified from 175 μL of the medium using a MagMax Kit (ABI 1840). The ABI Path-ID mastermix (ABI, Loughborough, UK) was used for PCR amplification. Forward and reverse primers were used at a concentration of 400 nM and the probe was used at a concentration of 120 nM. Purified nucleic acid (4 μL) was used per reaction in a total volume of 25 μL. The quantitative (q)PCR reactions were run on an ABI 7500 thermocycler. Cycling conditions were 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 1 min. In addition to the test samples, each set of reactions included two or more negative control wells, a positive extraction control well with AdRG1.3 vaccine diluted at 1×10^{-5} in transport medium, a negative extraction control consisting of transport medium only, and a PAC well with a calculated copy number of 100.

ONRAB vaccine-bait contact

A communications campaign to raise public awareness of the field trial, ORV and the risks of bait and vaccine contact, and the need to report potential vaccine exposures included a National Environmental Policy Act, Environmental Assessment 30-day public comment period (August–September 2011); talking points and a flow chart algorithm (Fig. 4) for responding to calls and categorizing vaccine vs. bait contacts (August–September 2011); a press release (September 2011); and a presentation on the field trial to the Greenbrier County Commission that included two television interviews (September 2011). We provided ONRAB information and safety guidelines, the material safety data sheet, and the ONRAB ground baiting protocol to Wildlife Services (WS) staff responsible for ground baiting (September 2011).

Statistical methods

Contingency tables and the Cochran-Mantel-Haenszel (CMH; Agresti 1996) test were used to determine whether the proportions of individuals pre- and post-ORV differed among

TABLE 1. Prevalence (%) of antibody to rabies virus by species sampled for pre– and post–Ontario Rabies Vaccine Bait (ONRAB) bait distribution in a field trial in West Virginia, USA, 2011.[a]

Species	Pre-ONRAB		Post-ONRAB	
	%	*n*	%	*n*
Raccoon (*Procyon lotor*)	9.6	395	49.2	262
Striped skunk (*Mephitis mephitis*)	5	20	7	28
Gray fox (*Urocyon cinereoargenteus*)	0	1	100	2
Red fox (*Vulpes vulpes*)	—	0	100	1
Coyote (*Canis latrans*)	—	0	100	1
Total	9.4	416	45.9	294

[a] One skunk and one coyote were collected as roadkill within sampling cells during the post-ONRAB field trial period.

sampling cells (1–4). The CMH test was used to determine whether age, sex, and antibody status were conditionally independent across sampling cells. McNemar's test for dependent samples was used to determine whether the proportion of antibody-positive individuals differed pre- and post-ORV. The CMH test was used to determine whether relationships existed between biomarking, serology, age, and sex. Among post-ORV samples, we explored differences by multiple logistic regression. Significance was set at $P<0.05$. All statistical analyses were conducted in SAS 2009 (SAS Institute Inc. 2009).

RESULTS

Target species biomarker and rabies serology

Antibody prevalence was 9.6% among raccoons ($n=395$) before ONRAB baiting (Table 1) in the field trial area. Cell-to-cell proportion of antibody-positive raccoons during pre-ORV sampling did not differ relative to sex (CMH=1.92, df=1, $P=0.166$) or age (CMH=2.51, df=1, $P=0.113$). Cell-to-cell proportion of antibody-positive demographic classes ranged from 0% to 25% (Table 2). Adults comprised a greater proportion of individuals than juveniles.

By contrast, 49.2% of raccoons ($n=262$) sampled post-ORV were antibody positive (S=102.68, df=1, $P<0.001$). Cell-to-cell proportion of antibody-positive demographic classes ranged from 16% to 89% (Table 2) and did not differ by sex (CMH=3.75, df=1, $P=0.053$) but did by age (CMH=14.99, df=1, $P=0.0001$) due to a lower proportion of antibody-positive juveniles in cells 1 ($\chi^2=6.28$, df=1, $P=0.012$) and 2 ($\chi^2=7.06$, df=1, $P=0.008$).

TTCC presence was similar ($P=0.7303$) at 10% (3/31) among raccoons during

TABLE 2. Rabies virus antibody prevalence (%) in raccoons (*Procyon lotor*) and corresponding sample size for pre– and post–Ontario Rabies Vaccine Bait distribution by sex and relative age and sampling cell in a field trial in West Virginia, USA, 2011.[a]

Interval	Cell	Adult male		Adult female		Juvenile male		Juvenile female	
		%	*n*	%	*n*	%	*n*	%	*n*
Prebait	1	13	32	7	30	0	21	0	18
	2	10	40	15	27	11	19	0	10
	3	10	49	13	32	7	14	12	17
	4	22	23	4	24	25	12	0	20
Postbait	1	68	25	38	21	39	18	16	19
	2	56	9	89	9	31	16	27	7
	3	86	21	67	18	64	11	44	9
	4	54	26	38	21	32	19	39	13

[a] Adult (≥1 yr old) and juvenile (<1 yr old) raccoons were based on specific ages determined by cementum annuli. Fifty raccoons for which there were no tooth or usable samples were assigned to juvenile or adult age classes based on size and tooth characteristics (Grau et al. 1970).

TABLE 3. Rabies virus–neutralizing antibody (RVNA) and tetracycline (TTCC) biomarker results for 341 and 248 raccoons (*Procyon lotor*) sampled before and after the distribution of Ontario Rabies Vaccine Bait in a West Virginia, USA, field trial in 2011.[a]

			TTCC negative		TTCC positive	
Interval	Antibody status	Age[b]	Male	Female	Male	Female
Prebait	RVNA negative	Adult	114	85	5	5
		Juvenile	41	45	11	4
	RVNA positive	Adult	15	8	1	1
		Juvenile	3	2	1	0
Postbait	RVNA negative	Adult	23	28	2	3
		Juvenile	28	26	9	7
	RVNA positive	Adult	39	30	9	5
		Juvenile	3	2	22	12

[a] Four raccoons from the prebait interval that were RVNA negative and TTCC negative were excluded because sex was not recorded; one raccoon from the prebait interval that was RVNA positive and TTCC negative was also excluded because sex was not recorded.

[b] Specific ages were used when they were available from cementum annuli to assign to adult or juvenile age classes. Otherwise, raccoons were assigned to juvenile or adult age classes based on size and tooth characteristics (Grau et al. 1970).

pre-ORV but was significantly higher ($P<0.0001$) at 39.2% (48/122) among antibody-positive raccoons post-ORV (Table 3). No relationships were detected pre-ORV between biomarking and RVNA ($P=0.5300$), age ($P=0.1716$), or sex ($P=0.1657$). However, differences were noted post-ORV relative to age and RVNA ($P<0.0001$), but not for sex ($P=0.6074$).

Only 27 raccoons marked during the pre-ORV period (6.8%) were recaptured post-ORV (10.3%). One raccoon recapture had detectable RVNA pre- (0.06 IU) and post-ORV (0.32 IU). This individual was TTCC negative. Eight of these 27 raccoons had RVNA post-ORV (including the one previously mentioned), but only two were biomarker positive. Four of six raccoons ($n=25$, two were not tested for biomarker) that were biomarker positive post-ORV were antibody negative. Two of 23 raccoons with suitable pre-ORV tooth samples were TTCC positive.

One of 20 skunks had detectable RVNA pre-ORV; two of 28 had RVNA post-ORV (Table 1). No skunks were recaptured. Other species tested for RVNA represented incidental captures of rabies reservoir species in North America (Table 1; Blanton et al. 2011).

There was no evidence of adenovirus antibodies in 416 animals from the pre-ORV period. Seven of 296 (2.4%) samples demonstrated AdRG1.3 VNAs post-ORV: six raccoons and one skunk. Adenovirus antibodies were detected in 10 of 23 cattle serum samples collected within the study area prior to ONRAB distribution. Fewer (three of 23) cattle (different individuals from the pre-ORV sample) had VNA to adenovirus post-ORV.

ONRAB detection from oral swabs post-ORV

Oral swab samples were collected from 125 target and nontarget individuals live-trapped from days 1 to 6 post-ORV. Individual raccoons on days 2, 3, and 4 and a Virginia opossum (*Didelphis virginianus*) on day 4 had *q*PCR values <35, which are considered strong positives (Knowles et al. 2009a). An additional nine raccoons and four opossums with >35 *q*PCR values <40 were captured on days 1–4. No ONRAB was detected on days 5 or 6. Two of 15 raccoons and two of 12 opossums had >35 *q*PCR values <40 on day 1. These are not strong positives, but they represent the only animals sampled that could have had vaccine contact during day 1 after ONRAB distribution. It was

not possible to determine the day of vaccine contact for the animals deemed positive from days 2–4.

ONRAB vaccine bait contact

No human or domestic animal bait or vaccine contacts were reported to the WVDHHR or WS during this field trial.

DISCUSSION

North American rabies management goals include elimination of rabies at its source in wildlife reservoirs (USDA 2008a, b). Raccoon rabies has not spread appreciably in the USA since ORV intervention began in the late 1990s, but contingency actions in the US and point infection control in Ontario (Rosatte et al. 2001), Quebec (Mainguy et al. 2012), and New Brunswick have been integrated into control to achieve this goal (Slate et al. 2009). Lack of progress toward elimination of raccoon rabies in the US has been attributed in part to relatively low population immunity with ORV strategies involving a single annual late summer aerial baiting with 750-m parallel flight line spacing at 75 baits/km^2 with Raboral V-RG.

Antibody prevalence of 49.2% among raccoons (unadjusted for the 9.6% pre-ORV level) after ONRAB distribution was the highest WS has observed for a first baiting at 75 baits/km^2 and 750-m flight line spacing where the RVNA baseline had been measured. Field trial replication was recommended and initiated in 2012 to determine whether raccoon population immunity becomes asymptotic after a second successive annual baiting, similar to the pattern observed for Raboral V-RG, but at antibody prevalence levels necessary to pursue raccoon rabies elimination more aggressively. Additionally, replicating this trial will allow for further investigation of differences noted in juvenile antibody prevalence across sampling cells. Population immunity levels approaching 70% have been cited as a general threshold for rabies elimination–based modeling (Anderson et al. 1981) and expertise (Coleman and Dye 1996); however, 60% (or between 50% and 60%) may be sufficient to break the rabies transmission cycle, with higher population immune responses increasing certainty of success (Thulke and Eisinger 2008).

Although this field trial was not specifically designed to compare ONRAB and Raboral V-RG, a mean antibody prevalence of 17.0±9.9% (SD; n=11), uncorrected for mean RVNA of 4.9±4.8% (n=13) was observed after a first baiting with Raboral V-RG in naïve areas under essentially the same initial ORV baiting characteristics during 2001–09 in Alabama, Florida, Georgia, Maine, North Carolina, Pennsylvania, Tennessee, and Virginia and similar raccoon density indices (USDA 2011c) as in this field trial, except in Florida (Tables 4, 5). Although comparative antibody prevalence samples were not collected in the adjacent Raboral V-RG zone in West Virginia post-ORV during 2011, in the 4 yr prior to this ONRAB field trial, RVNA prevalence ranged from 18.5% (n=135; 2008) to 46% (n=84; 2007). These represent RVNA levels for areas that have been subject to annual ORV campaigns at 75 baits/km^2 with Raboral V-RG since 2001.

A RVNA prevalence of 9.6% in the pre-ORV sample was higher but fell within 1 SD of the mean for areas of similar status before ORV with Raboral V-RG (Table 4). Background RVNA is likely related in part to naturally acquired immunity from sublethal exposures (Bell et al. 1972; Botros et al. 1979) to raccoon rabies virus (Fig. 2) or bat rabies virus variants enzootic in the study area (Blanton et al. 2011). Although raccoon rabies was enzootic in the West Virginia field trial zone, previous studies have reported higher RVNA prevalence in raccoon populations in areas that were naïve to the raccoon rabies virus variant and ORV (McLean 1971; Ramey et al. 2008). Movement of orally vaccinated raccoons across the

TABLE 4. Rabies virus–neutralizing antibody prevalence before and after initial oral rabies vaccination (ORV) with Raboral V-RG, in areas never previously subjected to ORV. Raccoon population density indices associated with these regions are shown for post-ORV sampling periods (2001–09).

State (US)	Year	Bait density (baits/km^2)[a]	Prevalence (%)	n	Region[b]	Raccoon density index[c]	No. of studies
Alabama	2002	0	3	68	Georgia-Alabama-Tennessee	—	—
	2003	75	33	67	Georgia-Alabama-Tennessee	6.5	2
	2002	0	11.3	133	Selma	—	—
	2004	75	13	99	Selma	8.9	2
	2005	0	1	84	Birmingham	—	—
	2005	75	2.7	113	Birmingham	n/a	0
Florida	2003	0	9	99	Mainland (not Pinellas County)	—	—
	2003	75	9	65	Mainland (not Pinellas County)	17.0	5
Georgia	2003	0	0	19	Georgia-Alabama-Tennessee	—	—
	2004	75	30.2	116	Georgia-Alabama-Tennessee	n/a	0
Maine	2002	0	14	21	Hodgdon	—	—
	2003	0	0	11	Hodgdon	—	—
	2003	70	20	82	Hodgdon	3.6	2
	2007	0	1	74	Caribou	—	—
	2009	75	26.4	144	Caribou	12.1	2
North Carolina	2005	0	3	30	Appalachian Ridge	—	—
	2005	75	7.5	120	Appalachian Ridge	n/a	0
Pennsylvania	2002	0	9.9	192	Appalachian Ridge	—	—
	2002	75	13	39	Appalachian Ridge	n/a	0
Tennessee	2002	0	3	30	Appalachian Ridge	—	—
	2002	75	11	74	Appalachian Ridge	9	1
Virginia	2001	0	9	33	Appalachian Ridge	—	—
	2002	0	3	29	Appalachian Ridge	—	—
	2002	75	22	68	Appalachian Ridge	4.7	2
Total[d]		0	=4.9±4.8	841		—	—
Total[d]		70/75	=17.0±9.9	987		—	—

[a] 0 = sampling conducted in an ORV-naïve area (i.e., has never been baited for ORV).

[b] Regions represent distinct ORV zones within a given state where sampling was conducted independent of other regions.

[c] n/a = not applicable (no density studies were conducted).

[d] Means and standard deviations of unbaited (naïve = 0) vs. baited areas were generated from 13 and 11 events, respectively (n=13, n=11).

established buffers for this field trial from the nearby Raboral V-RG ORV zone (Fig. 3) cannot be completely ruled out as a potential contributing factor. There are no known wildlife rehabilitation facilities in the immediate area that could have released hand-inoculated raccoons as a source of RVNA pre-ORV.

The significant relationship between TTCC and RVNA adds confidence that antibody prevalence was, in large part, attributable to ONRAB bait consumption (Johnston and Voight 1982; Rupprecht et al. 1987; Rosatte et al. 2009b). That TTCC was not closer to unity than 48 of 122 for antibody-positive raccoons may be largely a function of using first premolar teeth for biomarker detection. Canine teeth and mandibular bone are superior tissues for TTCC biomarking (Linhart and Kennelly

TABLE 5. Raccoon (*Procyon lotor*) population density indices on the basis of 50 cage traps set for 10 consecutive nights and moved to cover a 3-km^2 study area within the area that served as the Ontario Rabies Vaccine Bait field trial study area in West Virginia, USA, 2011.

Year	Study name	No. of skunks	County	Density (raccoons/km^2)	Area habitat class
2006	WVGREENB06	0	Greenbrier	8.2	Rural (mixed forest)
2007	WVGREENB07	2	Greenbrier	12.2	Rural (mixed forest)
2010	WV00310	0	Greenbrier	12.0	Agriculture (pasture/hay)
2010	WV00410	2	Greenbrier	10.7	Rural (mixed forest)
2010	WV00510	0	Monroe	6.6	Rural (mixed forest)
2010	WV00610	0	Monroe	7.0	Rural (mixed forest)

1967; Algeo et al. 2013), but we collected first premolar teeth as a less intrusive procedure so that raccoons could be released after full recovery from sedation. Canine tooth sampling would have required euthanasia and eliminated the opportunity to obtain valuable biologic information in future field trials through recaptures.

Background sources could have contributed to the TTCC level of 48 of 122 RVNA-positive raccoons post-ORV. The most likely background TTCC sources may have included consumption of medicated feeds sometimes used for cattle production and nonspecific fluorescence that may be found naturally (Chopra and Roberts 2001). TTCC was detected in 28 of 341 (8%) pre-ORV (3/31 antibody-positive raccoons and 25/310 antibody-negative raccoons were biomarker positive pre-ORV) and 69 of 248 (28%) of post-ORV raccoons irrespective of RVNA status. However, there was significantly higher biomarker post-ORV irrespective of antibody status compared with pre-ORV. TTCC exposure through bait contact only (i.e., the raccoon failed to puncture the blister pack containing the vaccine) may account for a portion of TTCC-positive, antibody-negative raccoons post-ORV. Only one of 27 raccoons recaptured during post-ORV trapping that also had detectable RVNA pre-ORV had an elevated titer suggestive of an anamnestic response to ONRAB consumption, but this was not supported by TTCC marking.

Sample size was inadequate to evaluate the immune response of ONRAB in striped skunks. The four skunk captures during raccoon density indexing (Table 5) suggests low population densities. However, it may be possible to improve skunk capture rates in future trials using baits more attractive to skunks (Rosatte et al. 1992).

There was no evidence of AdRG1.3 VNAs in target species pre-ORV. That only 2.4% (six raccoons and one skunk) of target species post-ORV had AdRG1.3 VNAs is likely a function of the virus vector being adapted to human hosts. Additional serologic surveys are planned for the replicate 2012 West Virginia trial to monitor AdRG1.3 VNA patterns. However, the presence of higher levels of adenovirus VNAs in cattle surveyed pre-ORV than post-ORV might be due to cross-reactivity to natural exposure to a ruminant adenovirus (10 known species in two genera), as there are no licensed ruminant adenovirus vaccines.

Absence of reported ONRAB bait contacts in humans was not surprising given that the 79,027 baits were distributed in predominantly rural areas. As field trials expand to include urban-suburban environments where contacts would be expected to increase, contact rates may be compared with those reported for Raboral V-RG (CDC 2013). About 120 million Raboral V-RG baits have been distributed from 1995 to 2011, with 1,464 human-bait contacts (i.e., picking up bait, finding bait in a yard, or removing bait from a pet dog's [*Canis familiaris*] mouth, feces, or vomit), equating to a human contact rate

of 1/68,521 baits distributed (USDA 2011b; Roess et al. 2012), resulting in only two known adverse reactions (CDC 2009; USDA 2010a).

Between 2006 and 2011, ≥3.9 million ONRAB baits with an affixed phone label were distributed in Ontario with a call rate of 1/65,140 baits (64 calls) distributed. Fifteen calls involved possible vaccine contact (unconfirmed) as a result of handling a punctured bait or removing a bait from a dog's mouth, or a contact rate of 1/260,560 baits distributed. There were no reports of adverse events associated with reported human-bait contacts (Ontario Ministry of Natural Resources 2007, 2008, 2009, 2010, 2011). Between 2007 and 2009, only 16 reports of human exposure occurred in Quebec despite the distribution of almost 2 million baits (J. Mainguy unpubl. data). In 2010, ≥1 million ONRAB baits were distributed in Quebec, with no human bait contacts reported (Ontario Ministry of Natural Resources 2011).

From 1995 to 2008, 1,327 pet or other domestic animal Raboral V-RG bait contacts were documented, equating to a contact rate of 1/75,596 baits distributed (USDA 2011b). There were no reports of adverse reactions among the 261 pets with bait contacts, except eight dogs experienced vomiting or diarrhea after ingesting several baits. Animals that contact Raboral V-RG or ONRAB vaccine will most likely be immunized against rabies or receive a boost from a previous rabies vaccination.

Using a liberal interpretation of oral swab analysis, 17 (12 raccoons and five opossums) of 125 samples (14%) were positive for AdRG1.3 the first 4 days after ONRAB distribution. No AdRG1.3 was detected in wild-captured animals within the ONRAB field trial zone on days 5 or 6. Knowles et al. (2009b) reported excretion of the vaccine in the oral cavity at 0.8% of oral swabs and 6.8% of fecal specimens from experimental animals subjected to oral instillation. One skunk shed a small amount of virus in feces on day 14. Although fecal swab analysis was not conducted in this field trial, it will be considered in future ONRAB field trials. However, AdRG1.3 has been observed to remain stable through serial passage of infected lung tissue in the cotton rat (*Sigmodon* sp.), one of the few species susceptible to human adenovirus infection (Knowles et al. 2009a).

Knowles et al. (2009b) reported no adverse histologic effects for AdRG1.3 PCR-Southern Blot positives (18/1,280; 1.4%) and negative tissue samples obtained from experimental vaccinates or contact animals. Likewise, no histopathologic lesions were observed in a companion captive study for this field trial in the Virginia opossum, eastern cottontail rabbit (*Sylvilagus floridanus*), turkey (*Meleagris gallopavo*), eastern fox squirrel (*Sciurus niger*), and wood rat (*Neotoma* sp.) that received a 10× dose of AdRG1.3 per os (Fry et al. 2013). Although these animal safety findings support continuation of field trials, samples (pre-ORV, $n=290$; post-ORV, $n=300$) from wild-caught target and nontarget species collected from the study area will be analyzed and reported separately to expand the ONRAB animal safety database.

Results from this field trial led to a recommendation to initiate a safety and immunogenicity trial in Ohio in 2012 to evaluate ground baiting at 150 baits/km^2 in urban-suburban environments where ORV with Raboral V-RG has occurred since 2004 at high bait densities, often twice annually. A field trial was also initiated in northern Vermont and New Hampshire and northeastern New York along the Quebec border in 2012. Site selection was based on the absence of raccoon rabies in Quebec since May 2009, with extensive enhanced surveillance and ONRAB ORV central to their elimination strategy.

Given the long-term goal of raccoon rabies elimination, the absence of cases is the ultimate measure of success. However,

indices of bait consumption from biomarker and population immunity from RVNA represent key metrics of ORV performance for achieving success. Based on those indices in this first ONRAB field trial, positive results from comparative border studies between Raboral V-RG and ONRAB (Fehlner-Gardiner et al. 2012; Mainguy et al. 2013), and raccoon rabies control successes in Canada, successive year field trials with ONRAB should reveal whether immune levels necessary to eliminate raccoon rabies are attainable, recognizing that ORV strategies will need to be adapted to specific environments.

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