

EVALUATION OF ORAL RABIES VACCINATION: PROTECTION AGAINST RABIES IN WILD CAUGHT RACCOONS (*PROCYON LOTOR*)

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ABSTRACT: Oral rabies vaccination (ORV) is an effective tactic for wildlife rabies control, particularly for containment of disease spread along epizootic fronts. As part of the continuing evaluation of the ORV program in free-ranging raccoons (*Procyon lotor*) in the US, 37 raccoons from ORV-baited areas in Pennsylvania were live-trapped and transferred to captivity to evaluate protection against rabies in animals with varying levels of existing neutralizing antibodies, expressed in international units per milliliter (IU/mL). Among the 37 raccoons at the date of capture, 24% (9/37) of raccoons were seronegative (<0.05 IU/mL), 22% (8/37) were low positive (≥ 0.05 – <0.11 IU/mL), 27% (10/37) were medium positive (>0.11 – <0.5 IU/mL), and 27% (10/37) were high positive (≥ 0.5 IU/mL). Raccoons were held for 86–199 d between the date of capture and rabies virus challenge. At challenge, 68% (25/37) raccoons were seronegative. The overall survival rate among challenged animals was 46% (17/37). Based on the antibody titers at the time of challenge, survivorship was 24% (6/25) among seronegative animals, 100% (4/4) among low positive animals, 83% (5/6) among medium positive animals, and 100% (2/2) among high positive animals. Evidence of high-titer seroconversion after vaccination is a good surrogate indicator of rabies survival; however, survival rates of approximately 45% (15/35) were found among raccoons with detectable titers below 0.5 IU/mL. In contrast, any detectable titer at the time of challenge (>3 mo after vaccination) appeared to be a surrogate indicator of survival. Overall, we illustrated significant differences in the value of specific titers as surrogates for survival based on the timing of measurement relative to vaccination. However, survivorship was generally greater than 45% among animals with any detectable titer regardless of the timing of measurement. These findings suggest that lower titer cutoffs may represent a valid approach to measuring immunization coverage within ORV management zones, balancing both sensitivity and specificity for estimating herd immunity.

Key words: Neutralizing antibodies, *Procyon lotor*, rabies, raccoon, vaccination, virus.

INTRODUCTION

Rabies is an invariably fatal encephalitis caused by a *Lyssavirus* (Family *Rhabdoviridae*). Globally, rabies is responsible for more than 60,000 human deaths each year, largely due to uncontrolled canine rabies circulating in Africa and Asia (Hampson et al. 2015). While canine rabies has been eliminated in the US, wildlife rabies remains a significant source of exposure to humans and domestic animals and represents a high financial and social cost of coexistence (Uhaa et al. 1992; Kemere et al. 2000; Velasco-Villa et al. 2008). The expansive raccoon (*Procyon lotor*) rabies epizootic along the eastern coast of the US is associated with most animal rabies cases and human exposures (Christian et al. 2009; Monroe et al. 2016). While raccoon rabies

was largely restricted to the southeast US prior to the 1970s, the translocation of infected animals into the Mid-Atlantic region resulted in its rapid spread throughout the Northeast. By the early 2000s, the distribution of this rabies virus (RV) variant ranged from Alabama and Florida northward along the Appalachian Mountains into Maine and southeastern Canada (Nettles et al. 1979; CDC 2000; Blanton et al. 2008).

While live attenuated oral rabies vaccines had been developed in the US in the 1960s and were successfully used to control rabies in red foxes (*Vulpes vulpes*) in Europe by the late 1970s, they were not approved for use in the US at the time raccoon rabies began to expand into the Mid-Atlantic states (Wandeler et al. 1988). Subsequently, large-scale interventions to control rabies in the raccoon

population did not begin until the recombinant vaccinia-rabies glycoprotein (V-RG) vaccine became available for oral rabies vaccination (ORV) in the 1990s (Rupprecht et al. 1988; Hanlon et al. 1998). Since then, the raccoon ORV program in the US has expanded to include more than 16 states, and, along with special contingency actions, it is credited with preventing appreciable westward expansion of the raccoon RV variant (Slate et al. 2009).

While ORV has been in place for 20 yr in some areas, there has not been a sufficient combination of efficacious vaccine, bait matrix, and application strategies to move toward elimination of the raccoon RV variant within the raccoon population. The US Department of Agriculture monitors post-ORV rabies virus neutralizing antibody (RVNA) titers in baited regions as an operational metric of performance for the national ORV program. Despite multiple applications of ORV across these regions, the overall seroprevalence of RVNA among trapped raccoons has averaged around 30%, with some spatiotemporal variability (Slate et al. 2009).

While the efficacy of V-RG has been documented in captive animals to comply with regulatory requirements, many issues may impact the effectiveness of the vaccine when distributed in the field. These factors include design of the bait, timing of vaccine-bait distribution, nontarget species uptake, animal nutrition levels, and exposure to other infections such as wild orthopoxviruses that might interfere with response to V-RG (Root et al. 2008; Gallardo-Romero et al. 2016). The relative usefulness of serologic evaluation in relation to protection against RV challenge is not well understood for animals receiving ORV under natural conditions. Following one field administration of ORV, survival against RV challenge of wild-caught raccoons was reported in 78% of raccoons captured (Rupprecht et al. 1993). In that study, approximately 40% of the animals had high RVNA titers (>0.5 IU/mL) when challenged nearly 7 mo post-ORV (Rupprecht et al. 1993). Antibodies developing following ORV exposure and the subsequent anamnestic response to

infection are presumed to be correlates of survival and have been used as markers of ORV effectiveness. We evaluated survivorship among raccoons captured in an ORV zone with a range of RVNA titers to evaluate correlations between titer level and survival against RV challenge. The objective was to identify potential antibody titer cutoffs that might represent a valid surrogate marker of protection when monitoring seroprevalence of adequate response in ORV management areas.

MATERIALS AND METHODS

Raccoons were cage-trapped (Model 54130 live traps, Safeguard Products, Inc., New Holland, Pennsylvania, USA) during June 2005 in an ORV zone in western Pennsylvania (Westmoreland, Somerset, and Indiana counties, approximately $40^{\circ}15'0.12''\text{N}$, $79^{\circ}7'51.04''\text{W}$) where RABORAL V-RG[®] (Merial Ltd., Athens, Georgia, USA) had been distributed. Traps were tended to daily. Nontarget species and juvenile raccoons were immediately released.

All raccoons sampled for blood and other biologic information were sedated based on weight with an intramuscular injection with a mixture of 10.0 mg/kg ketamine hydrochloride (Fort Dodge Laboratories, Inc., Overland Park, Kansas, USA) and 2.0 mg/kg xylazine hydrochloride (Mobyay Corp., Shawnee, Kansas, USA). Five-milliliter to 7-mL samples of blood were collected from a jugular vein from each sedated raccoon. Sex, reproductive status, relative age, weight, and other pertinent information were recorded. Blood was centrifuged ($1,000 \times G$), and serum was collected, stored in labeled cryovials the day of capture, and shipped by express mail on dry ice to the Centers for Disease Control and Prevention (CDC) Rabies Laboratory to determine baseline RVNA titers. Test results were returned within 48–72 h, and 37/195 raccoons with a range of RVNA titers were selected for further study. OpenEpi was used to calculate study sample size assuming a survival rate following challenge with rabies virus of $<20\%$ in the animal cohorts with no detectable titer and $>80\%$ in animal cohorts with detectable titers (Dean et al. 2009). We estimated a sample size of approximately 10 animals in each group to find statistical significance between experimental groups. The primary effect measured was survival after challenge with rabies virus. The Fisher's exact test was used to assess differences in survival between study groups. A probability value of <0.05 was consid-

ered significant. Rate ratios (RR) and 95% confidence intervals (CIs) of survival were calculated for comparisons between groups.

The remaining raccoons were released at their original site of capture. Raccoons held for one night or longer were placed in dog kennels at a site secure from public access and monitored to ensure water was available ad libitum, and they were fed a commercial dry dog food. Kennels were cleaned daily. Selected animals were transported to the CDC Rabies Laboratory animal holding facility for RV challenge studies. Raccoons transferred to CDC custody were individually caged and offered commercial food and water ad libitum for a minimum quarantine period of 30 d for general health observations.

Following the quarantine period, raccoons were routinely sedated, and blood (2 to 4 mL) was sampled as mentioned earlier. Serum was separated at low-speed centrifugation ($1,000 \times G$), collected, and stored at $-20\text{ }^{\circ}\text{C}$ until testing. Levels of RVNA were determined by use of the rapid fluorescent focus inhibition test (RFFIT; Smith et al. 1973) and expressed in international units per milliliter (IU/mL).

All captured raccoons were grouped based on their RVNA titers at capture. Cutoff points for these groups were based on existing recommended levels by the US Advisory Committee on Immunization Practices (0.11 IU/mL) and World Health Organization (0.5 IU/mL) and are commonly used for determining human and animal vaccination recommendations (Manning et al. 2008; WHO 2013). These values are also approximately equivalent to complete neutralization of virus at a 1:5 serum dilution (0.12 IU/mL) or 1:25 serum dilution (0.5 IU/mL) in the RFFIT. Seronegative animals were designated as animals with RVNA below the threshold of detection for the RFFIT test at CDC (<0.05 IU/mL); a value of 0.01 IU/mL was used for these animals in geometric mean titer calculations.

Raccoons were challenged in three groups between 86 to 199 d after capture. At challenge, raccoons were inoculated in the right and left masseter muscle with 0.5 mL each of submandibular salivary gland suspensions of RV obtained from naturally infected eastern raccoons ($1 \times 10^{4.9}$ mouse intracerebral lethal dose 50%). After inoculation, raccoons were observed daily for onset of clinical signs of rabies. A postchallenge titer was determined 7 d after challenge. When signs consistent with RV infection were observed, raccoons were sedated and then euthanized by intracardiac administration of a phenytoin-pentobarbital mixture (Beuthansia©-D, Merck Animal Health, Madison, New Jersey, USA). Postmortem tissue collection included brain stem and serum. A diagnosis of rabies was confirmed by the direct fluorescent antibody test on fresh brain tissue

samples, done at CDC. All animal care and experimental procedures were performed in compliance with the CDC Institutional Animal Care and Use Guidelines (no. 1364RUPRACL).

RESULTS

In total, 24% (9/37) of the raccoons were seronegative (<0.05 IU/mL), 22% (8/37) had a low RVNA titer (≥ 0.05 – 0.11 IU/mL), 27% (10/37) had a medium RVNA titer (>0.11 – <0.5 IU/mL), and 27% (10/37) had a high RVNA titer (≥ 0.5 IU/mL) at time of capture (Table 1). The mean length of time between baseline serum collection and challenge with RV was 141 d (SD: 33.7, range: 86–199 d). Between baseline titer collection and challenge, 100, 82, and 9% of the low, medium, and high RVNA titer animals became seronegative, respectively (Fig. 1). Overall, the median RVNA titer decline among raccoons with a detectable titer at baseline was 0.81 IU/mL (range: 0.04–10.39 IU/mL). At challenge, 68% (25/37) of the raccoons were seronegative, 11% (4/37) had a low RVNA titer, 16% (6/37) had a medium RVNA titer, and 5% (2/37) had a high RVNA titer (Table 1).

All nine raccoons that were seronegative at capture (baseline) developed signs of rabies and were euthanized. Overall, 46% (17/37) of the raccoons survived challenge. Regardless of the timing of titer determination in relation to RV challenge, higher titers were correlated with survival (Table 2). A total of 16 raccoons had a detectable RVNA titer at baseline but had become seronegative at challenge. Among those, 37% (6/16) survived rabies challenge. In comparison, 92% (11/12) of the raccoons that still had a detectable titer at challenge survived.

Raccoons with a detectable titer (≥ 0.05 IU/mL) at capture were significantly more likely to survive challenge (Fisher exact test, $P=0.001$) after an average of 145 d after capture. Assuming animal vaccination status was accurately classified based on serologic status at baseline (i.e., a detectable titer is associated with history of vaccination), this would represent a vaccine efficacy of approximately 61%. Based on the titer measurement

TABLE 1. Rabies virus neutralizing antibodies at baseline, challenge, and 7 d after challenge among raccoons (*Procyon lotor*) captured in an area where oral rabies vaccine was distributed.

Animal no.	Titer group ^a	Days from capture to challenge	Rabies virus neutralizing antibody titer (IU/mL)			
			At baseline ^b	At challenge ^b	At 7 d postchallenge ^b	Survival ^c
125	Negative	105	0.01	0.01	0.01	N
5199	Negative	105	0.01	0.01	0.01	N
5142	Negative	107	0.01	0.01	0.01	N
5143	Negative	107	0.01	0.01	0.01	N
5162	Negative	107	0.01	0.01	0.01	N
5714	Negative	107	0.01	0.01	0.01	N
5038	Negative	151	0.01	0.01	0.01	N
5044	Negative	198	0.01	0.01	0.10	N
123	Negative	108	0.01	0.01	0.01	N
5198	Low	105	0.05	0.01	0.01	N
5716	Low	107	0.06	0.01	0.01	N
5718	Low	107	0.06	0.01	9.00	Y
5667	Low	86	0.08	0.01	0.68	Y
122	Low	108	0.07	0.01	0.01	N
5672	Low	129	0.08	0.01	0.07	Y
5745	Low	129	0.11	0.01	0.01	Y
5167	Low	151	0.11	0.01	0.01	N
5227	Medium	128	0.13	0.01	0.55	Y
5938	Medium	128	0.13	0.07	0.55	Y
5749	Medium	128	0.14	0.01	0.55	Y
5164	Medium	151	0.19	0.01	0.55	N
5459	Medium	152	0.22	0.01	0.07	N
5047	Medium	149	0.23	0.01	0.01	N
5160	Medium	151	0.36	0.01	0.10	N
5032	Medium	152	0.37	0.01	0.01	N
5195	Medium	149	0.43	0.10	0.13	Y
5043	Medium	149	0.40	0.13	0.05	N
5936	High	129	0.50	0.16	0.01	Y
5668	High	130	0.50	0.55	0.55	Y
4104	High	152	0.62	0.22	0.55	Y
5145	High	151	0.76	0.40	0.55	Y
5194	High	198	0.90	0.08	37.50	Y
5186	High	198	1.70	0.45	37.50	Y
5046	High	198	2.20	0.36	37.50	Y
5040	High	199	2.50	2.00	37.50	Y
5183	High	198	4.30	0.10	1.16	Y
5189	High	198	10.40	0.01	0.01	N

^a Group categories based on animal's rabies virus neutralizing antibody level at baseline: negative: <0.05 IU/mL, low: ≥0.05–0.11 IU/mL, medium: >0.11–<0.5 IU/mL, high: ≥0.5 IU/mL.

^b Animal blood sampling time periods: baseline = day animal captured in the wild, challenge = day animal challenged with rabies virus.

^c Survival status of animals: Y = survived rabies challenge, N = developed signs consistent with rabies and was euthanized. All euthanized animals were confirmed rabid by direct fluorescent antibody.

at challenge, the rate of survival for animals with a titer ≥0.05 IU/mL was nearly four times higher than those without a measurable RVNA titer (RR=3.8, 95% CI=1.9–7.8). The

rate of survival in animals that demonstrated an anamnestic response to RV challenge (≥2-fold increase in titer) 7 d after challenge was three times higher (RR=3.2, 95% CI=1.3–7.1).

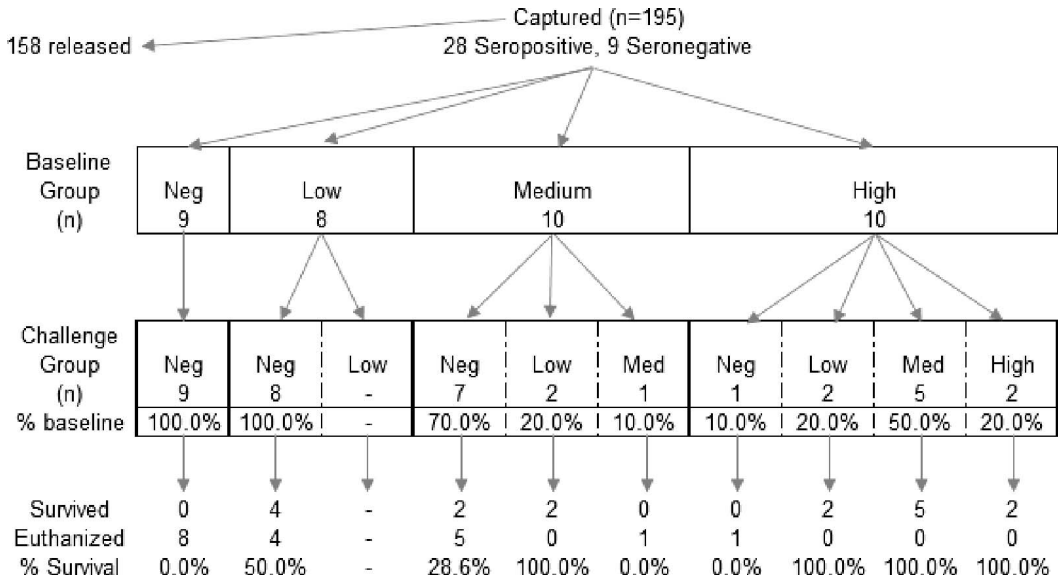


FIGURE 1. Sampling of wild-caught raccoons (*Procyon lotor*) from an area where oral rabies vaccine was distributed for inclusion in study, selected animals by rabies virus neutralizing antibody category, and change in group distribution between animal capture and challenge with rabies virus. Categories based on animal's rabies virus neutralizing antibody level: negative: <0.05 IU/mL, low: ≥ 0.05 – 0.11 IU/mL, medium: >0.11 – <0.5 IU/mL, high: ≥ 0.5 IU/mL. Blood sampling time periods occurred at baseline (when animal was captured in the wild) and at challenge (when animal was challenged with rabies virus).

DISCUSSION

The raccoons in this study were likely vaccinated during the ORV period ending on 29 April 2005. Approximately 2 mo passed between the period when the raccoons may have consumed ORV baits and when they were captured, and baseline antibody titers were determined. This is consistent with the

period during which the US Department of Agriculture routinely conducts serologic monitoring after distributing baits. Animals were challenged over three periods, resulting in a total of 7–10 mo between possible oral vaccination and challenge with RV. Approximately 68% of the seropositive animals became negative over this period. Prior

TABLE 2. Survivorship following rabies virus challenge among wild-caught raccoons (*Procyon lotor*) by rabies virus neutralizing antibody level.

Titer group ^a	Titer group at baseline ^b			Titer group at challenge ^b		
	Survived	Total	% Survival	Survived	Total	% Survival
Negative	0	9	0	6	25	24
Low	4	8	50	4	4	100
Medium	4	10	40	5	6	83
High	9	10	90	2	2	100
All seropositive	17	28	61	11	12	92
Total	17	37	46	17	37	46

^a Group categories based on animal's rabies virus neutralizing antibody level: negative: <0.05 IU/mL, low: ≥ 0.05 – 0.11 IU/mL, medium: >0.11 – <0.5 IU/mL, high: ≥ 0.5 IU/mL.

^b Animal blood sampling time periods: baseline = day animal captured, challenge = day animal challenged with rabies virus.

studies of vaccination of dogs with parenterally administered modified live virus vaccines found high antibody titers persisted for most animals for more than 1 yr (Coyne et al. 2001). A study of oral vaccination of foxes with an adenovirus vectored rabies vaccine similarly reported duration of immunity over 1 yr, but it also identified peak immune response at 7 wk followed by a general decline, resulting in more than 50% of the titers of vaccinated animals falling below 0.5 IU/mL by 6 mo postchallenge (Brown et al. 2014).

Antibody titer level as a surrogate value for protection is not well defined and can be difficult to determine given multiple sources of variation (e.g., host immune response, environmental factors, and diagnostic test variability). To reflect this variability and to account for concerns over false-positive serology results, higher cutoffs to document seroconversion have been suggested for seroprevalence studies and post-ORV monitoring (Bahloul et al. 2005). In the current study, approximately 45% of animals with low and medium level titers at baseline survived challenge 7–10 mo later, suggesting that the use of high titer cutoff values for seroprevalence surveys may misclassify many animals that would be protected against rabies challenge. However, the use of a very low cutoff value (i.e., ≥ 0.05 IU/mL) should be construed as an upper threshold of population immunity at most.

Few studies (Rupprecht et al. 1993) have measured longitudinal changes in titers for raccoons exposed to ORV under field conditions, but the current study suggests that titer levels may decay quickly (e.g., less than 1 yr). This could have a significant impact on cross-sectional surveys of RVNA titers and dynamics related to the critical vaccination coverage necessary to eliminate rabies. Measurement of high antibody titers (i.e., >0.5 IU/mL) 2 mo after ORV appears to be a strong indicator of protection; however, maintenance of any detectable titer (i.e., ≥ 0.05 IU/mL) at the point of RV exposure has a higher correlation with survival. If serologic monitoring is conducted at periods greater than 2 mo after ORV distribution, the use of lower RVNA

cutoffs as a surrogate of protection appears to be warranted.

We encountered a few limitations during the study. The most notable limitation was the assumption regarding the study animals' vaccination and rabies exposure histories. While these raccoons had the potential to be vaccinated by ORV, we cannot exclude that some may have received parenteral vaccination, been exposed to rabies, or had ingested multiple ORV baits over multiple years. Serologic surveillance among raccoons in enzootic areas of the US Southeast where ORV had not been applied varied between 2% and 22% seropositive depending on the epizootic cycle (McLean 1971). No noted epizootics had been reported in the areas where these Pennsylvania raccoons were collected.

In addition to the assumptions regarding the vaccination status of animals, there are underlying assumptions regarding the specificity of neutralization observed in the RFFIT, particularly at low titers. The RFFIT is a cell culture-based assay, and performance is susceptible to variation from laboratory and operator conditions, cytotoxicity, and other factors (e.g., presence of complement) that might impact interpretation of results. In this case, it is possible that low positive titers (≥ 0.05 – 0.1 IU/mL) were false or nonspecific and did not represent virus neutralization due to RVNA in the serum. Previous studies have suggested higher thresholds up to 1 IU/mL be used to assess serum collected from field animals. These conclusions were based in part on studies that identified high rates of seropositive dogs in areas with low vaccination rates or that were believed to be free of rabies (Cleaveland et al. 1999; Bahloul et al. 2005). In these previous studies, there was concern about the interpretation of lower titers as an adequate surrogate for immunity. However, in the current study, nearly 50% of the animals with a detectable titer (≥ 0.05 IU/mL) at capture survived RV challenge. Of the 10 raccoons that did not survive, three produced an anamnestic response within 7 d of challenge. Overall, this would suggest that specific RVNA was detected in 43% of the low, 73% of

the medium, and 82% of the high titer groups as indicated by survival or anamnestic response postchallenge.

The detection of RVNA antibodies from raccoons in an ORV zone appears to be an adequate surrogate marker of protection and may help with the assessment of ORV coverage and effectiveness. Many factors affect antibody levels in a free-ranging population. A critical consideration is the timeliness of sample collection following bait distribution, because antibody kinetics from ORV in raccoons may lead to a narrow window of detection (Brown et al. 2012). The presence of any level of antibody indicates a reasonable probability of immunity to a lethal RV challenge for the purposes of serologic monitoring of ORV programs. Increasing the cutoff to >0.11 IU/mL may improve specificity while maintaining a high sensitivity.

Additional research regarding the use of other assays for serologic monitoring (e.g., enzyme-linked immunosorbent assay) should be conducted to determine if they are also closely correlated with protection against RV challenge. These tests are frequently easier to perform than the RFFIT and may be more cost-effective for monitoring ORV in wildlife. In addition, further evaluations of the antibody decay rate in wild raccoons where ORV is distributed should be further explored.

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