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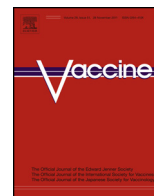
Fry, Tricia L.; Van Dalen, Kaci; Shriner, Susan A.; Moore, Susan M.; Hanlon, Cathleen A.; and Vercauteren, Kurt C., "Humoral immune response to oral rabies vaccination in raccoon kits: Problems and implications" (2013). *USDA National Wildlife Research Center - Staff Publications*. 1228.

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Humoral immune response to oral rabies vaccination in raccoon kits: Problems and implications

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ARTICLE INFO

Article history:

Received 15 January 2013

Received in revised form 4 April 2013

Accepted 7 April 2013

Available online 18 April 2013

Keywords:

Raccoon kits

Oral rabies vaccination

Procyon lotor

Rabies

Raboral V-RG

Maternal antibodies

ABSTRACT

Little is known about the immunogenicity of RABORAL V-RG[®] (V-RG), an oral rabies vaccine, in raccoon kits (*Procyon lotor*). The objectives of this study were to characterize the immunogenicity of V-RG in young kits and investigate the potential impact of maternal antibodies on response to vaccination of nursing raccoon kits. Raccoon kits ($n = 30$) were vaccinated at either 3 weeks of age, 7 weeks of age, or assigned as contact controls. Nineteen kits (73%) that were whelped by unvaccinated mothers responded to V-RG exposure (orally or indirect contact) by production of detectable rabies virus neutralizing antibodies (RVNA) while 7 (27%) kits did not respond to V-RG exposure. Four kits were whelped by a mother with high levels of RVNA and all four kits acquired maternal rabies antibodies. At approximately 9 months of age, all kits were inoculated with a killed rabies vaccine, IMRAB3[®]. The kits which initially responded to V-RG oral vaccination or contact with vaccinated littermates demonstrated a rapid anamnestic response. In contrast, the V-RG non-responders and those with acquired maternal antibodies exhibited a primary immune response to IMRAB3[®], where RVNA levels were substantially lower on days 5 and 7 than the levels in the animals with an anamnestic response. These findings suggest that the naïve contact kits and the nonresponsive kits most likely remained susceptible to rabies virus infection whereas the ones demonstrating response to V-RG would not have been susceptible to a rabies virus infection.

Published by Elsevier Ltd.

1. Introduction

Rabies is one of the oldest diseases described with records of clinical manifestations in humans dating back to 2300 B.C. It is estimated that more than 55,000 people die of rabies each year and another 20 million receive rabies post exposure prophylaxis [1]. Throughout most of the world, domestic dogs remain the most common reservoir for local transmission of canine-associated rabies virus variants; however, in the United States the dominant virus variants are associated with wildlife species [2]. Rabies viruses consist of a number of unique variants that are adapted to specific hosts [3]. The animals most frequently reported with rabies in the United States are raccoons (*Procyon lotor*), insectivorous bats (*Chiroptera spp.*), striped skunks (*Mephitis mephitis*), foxes of several

species (*Vulpes lagopus*, *Vulpes vulpes*, and *Urocyon cinereoargenteus*), and coyotes (*Canis latrans*) [4,5]. As an augment to traditional reactive public health measures, oral rabies vaccination (ORV) campaigns have been implemented on an experimental basis in strategic locations in an attempt to control the spread of expanding terrestrial epizootics. The effectiveness of ORV programs, most notably those directed toward control of rabies virus variants in Europe and North America has been previously reviewed [6,7].

The National Rabies Management Program, administered by USDA-APHIS Wildlife Services, is tasked with controlling the spread of terrestrial wildlife-associated rabies in the United States. At present, the majority of ORV efforts involve the distribution of a live recombinant vaccine, RABORAL V-RG[®] (V-RG, Merial Inc., Athens, GA, USA). V-RG is currently the only effective oral vaccine licensed for use in free-ranging raccoons and coyotes in the United States [8]. In the eastern United States, the main focus of ORV campaigns is the raccoon rabies variant, which has spread and evolved since the 1970s into a widespread epizootic [9,10]. As a consequence of wildlife translocation from the southeast United States raccoon rabies was introduced into the Mid-Atlantic States and now extends north into Canada and across the Appalachian mountains

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as far west as Ohio [11]. Generally, raccoon ORV campaigns with V-RG commence in late summer or early fall when raccoon population density peaks [12]. While considerable research has been done on vaccination of adult raccoons, few published studies are available on the vaccine's immunogenicity and efficacy in young raccoons. Rupprecht et al. reported that three suckling raccoon kits (3–4 weeks olds) seroconverted within 28 days after their mother was orally vaccinated with V-RG, although the mode of transmission, either grooming or lactation was not ascertained. Subsequently, all three kits survived a rabies virus challenge [13]. Maternal antibodies can be passively transferred to young through the placenta, yolk sac, colostrum, and/or milk [14] depending upon the species and the type of placentation and other aspects of the species physiology. Maternal neutralizing antibodies to rabies virus (≥ 0.5 IU/mL) can circulate for several weeks in fox cubs [15–17]. Blasco et al. [15] observed the disappearance of maternal antibodies in fox cubs corresponded to approximately 10–20 days after weaning. The duration of maternally derived antibodies to rabies virus in raccoon kits has not been published but may be similar to the half-life of raccoon maternal antibodies to canine distemper virus, which is approximately 10.55 days post-weaning [18]. While the role of maternal antibodies is to protect a young animal with an immature immune system from harmful pathogens, it has been shown that the presence of circulating maternal antibodies in young animals may interfere with the response to some vaccinations [18–20]. Research examining the maternal antibody-rabies vaccination interference in wildlife has been limited to reports in red foxes with Blasco et al. [15] reporting that maternal immune status did not interfere with the oral rabies vaccination of cubs once maternal antibodies disappeared (<0.5 IU/mL) between 45 and 75 days old.

The specific objectives of this study were to: (1) monitor rabies virus neutralization antibody (RVNA) levels in very young raccoon kits following exposure to V-RG, (2) determine a raccoon kit's ability to produce an anamnestic response following a second exposure to the rabies antigen (in this case, the killed rabies vaccine IMRAB3[®] (Merial Inc., Athens, GA, USA)), (3) determine how long maternally derived antibodies circulate in young raccoons and, (4) demonstrate whether or not maternal antibodies to rabies virus interfere with V-RG vaccination.

2. Materials and methods

2.1. Study animals

As the result of another research project seven wild caught female raccoons became pregnant and whelped at least one kit. The female raccoons were trapped from within Larimer County, Colorado, USA and housed in the Outdoor Animal Research Facility at the USDA APHIS WS National Wildlife Research Center (NWRC), Fort Collins, CO, USA. Three of the females with litters had been previously vaccinated with IMRAB3[®], a killed rabies vaccine. Of the three vaccinated females, only one had detectable RVNA (14.4 IU/mL) one month prior to whelping. Four kits were born and weaned by this female. The four females which were not vaccinated and the two IMRAB3[®] vaccinates that did not have detectable RVNA (<0.1 IU/mL) whelped a total of 26 raccoon kits.

Raccoon kits were housed with their mothers for ≥ 14 weeks, but not >17 weeks. Raccoon kits remained with litter mates for three weeks after weaning, at which time they were moved to individual pens. Raccoons were held in accordance with IACUC guidelines, and fed a varying, age appropriate diet that included Omnivore Diet A (Mazouri, St. Louis, MO, USA), milk replacer, canned cat food, mackerel, eggs, and various fresh fruits. Water was provided ad libitum.

2.2. Blood collection

We collected blood from each adult female raccoon approximately one month prior to whelping and again at weaning. We used 5:1 ketamine (10 mg/kg) and xylazine (2 mg/kg) mixture [21] to anesthetize adult raccoons and collected blood [22]. Starting at 10–14 days old we collected blood weekly for 26 weeks from raccoon kits. Blood collection and restraint techniques varied based on the age of the raccoon kits. Raccoon kits were primarily physically restrained or anesthetized using isoflurane until they were housed individually. Once housed individually we anesthetized raccoons using a 5:1 ratio of ketamine: xylazine as described above. We collected blood samples via the jugular vein with volumes ranging from 0.4 mL to 3 mL ($\leq 1.5\%$ body mass) [22] in Vacutainer SST[®] serum separator tubes (Becton Dickinson, Franklin Lakes, NJ, USA). We stored sera at -80°C until it was sent to the Kansas State University Rabies Laboratory (Manhattan, KS, USA) for testing.

2.3. Primary vaccination

We arbitrarily assigned raccoon kits into one of three treatment groups: (1) vaccinated with V-RG at 3 weeks of age, (2) vaccinated at 7 weeks of age, or (3) unvaccinated (contact controls). Six litters had at least one representative of each group, except for one litter which consisted of a single kit that was vaccinated at 3 weeks of age. Vaccination consisted of 1.5 mL ($10^{8.2}$ PFU) V-RG administered orally via needleless syringe (Lot #576-151, Merial, Ltd., Athens, GA, USA). Immediately after vaccination, we returned kits to their mothers and litter mates.

2.4. Secondary vaccination to assess anamnestic responses

An anamnestic response to the rabies virus after oral vaccination has been previously shown to increase the likelihood of raccoon survival [23]. To evaluate young raccoons' responses to a second rabies antigen exposure (in this case, a killed rabies vaccine, IMRAB3[®]), we inoculated all raccoon kits with IMRAB3[®] (Serial: 12550, Merial, Ltd. Athens, GA, USA), between 38 and 41 weeks of age. We collected blood as previously described on days 0, 5, 7, and 31 post-IMRAB3[®] inoculation (time points were designated as B0, B5, B7, and B31). We used a Student's *t*-test to evaluate an anamnestic response by comparing the differences in median RVNA values between B0 and (1) day B5, (2) day B7, and (3) day B31.

2.5. Laboratory testing

We sent aliquots of serum to Kansas State University Rabies Laboratory for detection and quantification of RVNA. RVNA were characterized by the Rapid Fluorescent Focus Inhibition Test (RFFIT); the 50% end point titer of the test serum was converted to IU/mL values by comparison to the 50% end point titer of the U.S. Standard Rabies Immune Globulin, Lot R-3, 59 IU (1st WHO International Standard), which is measured in the same assay run, with an assigned value of 2.0 IU/mL [24,25]. Sero-status of raccoons was determined based on a detection threshold of 0.1 IU/mL to increase sensitivity [26].

3. Results

3.1. Antibody responses of raccoon kits after initial vaccination

Six litters ($n=26$ kits) were whelped by seronegative mothers and subsequently had no detectable RVNA 2–3 weeks after birth. Ten of these raccoon kits were vaccinated at 3 weeks of age and nine (90%) developed RVNA at an average of 5.2 weeks

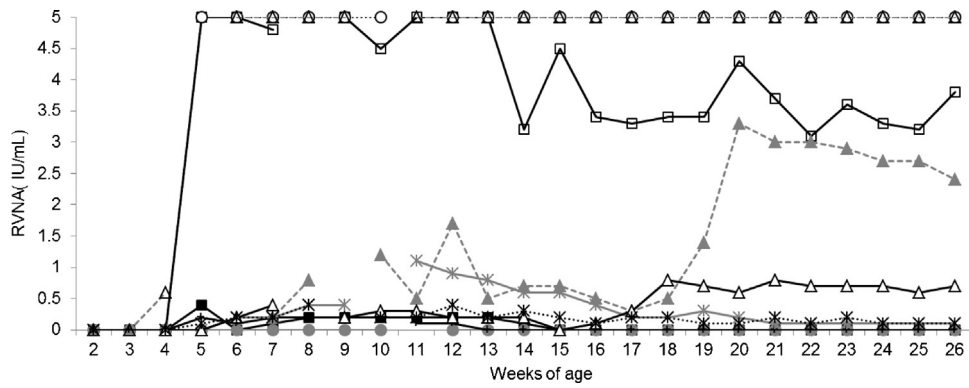


Fig. 1. RVNA profile of raccoon kits vaccinated with V-RG at 3 weeks of age. Nine of ten raccoon kits without maternal antibodies responded to VR-G vaccination by producing RVNA ≥ 0.1 IU/mL for at least five consecutive weeks. RVNA levels varied between individuals; seven kits still had detectable RVNA at 26 weeks post-vaccination.

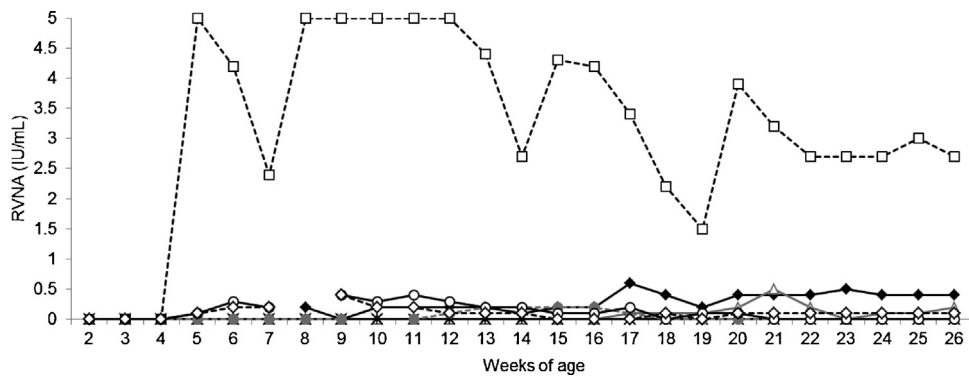


Fig. 2. RVNA profile of raccoon kits vaccinated with V-RG at 7 weeks of age. Six of eight raccoon kits without maternal antibodies responded to VR-G vaccination by producing RVNA ≥ 0.1 IU/mL for at least five consecutive weeks. RVNA levels varied between individuals; four kits still had detectable RVNA at 26 weeks post-vaccination.

of age (range = 4–6 weeks; Fig. 1). Eight raccoon kits were vaccinated at 7 weeks of age and 6 (75%) developed RVNA at an average of 8.6 weeks of age (Range = 5–17 weeks; Fig. 2). Eight raccoon kits were not orally vaccinated and served as contact controls; four of these kits (50%) developed RVNA at an average of 6.25 weeks of age (Range = 5–9 weeks; Fig. 3). All raccoon kits that seroconverted ($n = 19$) had detectable levels of RVNA for at least five consecutive weeks. Thirteen of these raccoon kits (68%) still had detectable RVNA at 26 weeks of age (one RVNA positive kit was euthanized on week 10 due to a pelvic deformity).

One litter ($n = 4$ kits) was whelped by a mother with detectable RVNA one month before whelping (14.4 IU/mL) and at weaning (4.3 IU/mL). All four kits had high levels of RVNA (≥ 8.0 IU/mL)

at the first blood sampling (12 days old; Fig. 4). After 3 weeks of age, RVNA levels of all four kits fluctuated between 8.0 IU/mL and 15 IU/mL for several weeks until levels steadily declined from 10 to 12 weeks through week 26. By 26 weeks (11 weeks after weaning), 3 of 4 kits with maternally acquired antibodies still had detectable RVNA values of 0.1 IU/mL, while 1 kit had no detectable RVNA.

3.2. Immune responses of raccoon kits after second exposure to rabies vaccine

Overall, 19/26 kits (73%) that were whelped by seronegative mothers and exposed to V-RG produced RVNA levels of ≥ 0.1 IU/mL

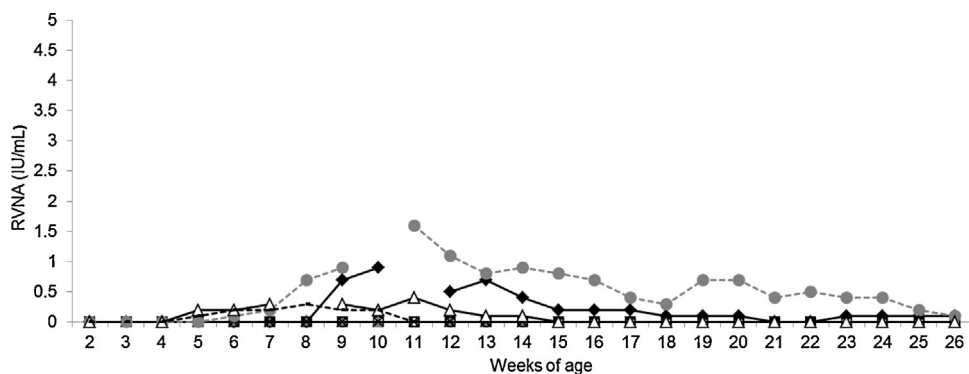


Fig. 3. RVNA profile of contact raccoon kits. Four of eight raccoon kits without maternal antibodies produced RVNA ≥ 0.1 IU/mL for at least five consecutive weeks after exposure to vaccinated littermates. RVNA levels varied between individuals; two kits still had detectable RVNA at 26 weeks post-vaccination.

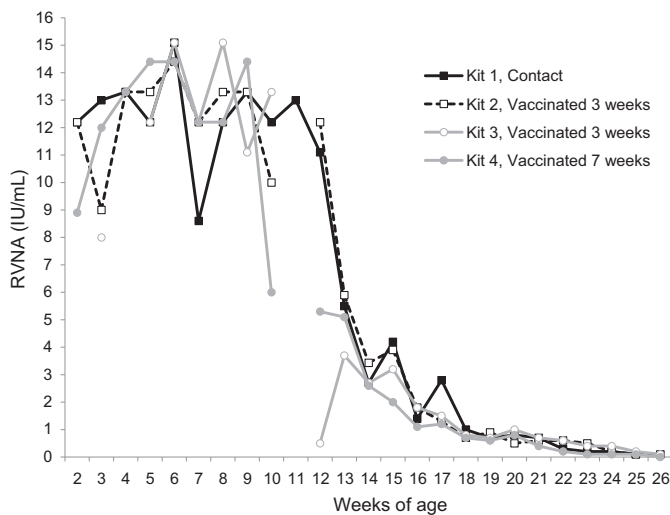


Fig. 4. RVNA profile of raccoon kits with maternally acquired antibodies. All four individuals exhibit a similar RVNA curve regardless of V-RG vaccination. At 26 weeks three raccoon kits still had detectable RVNA of 0.1 IU/mL. The raccoon kit vaccinated at 7 weeks had undetectable RVNA at 26 weeks.

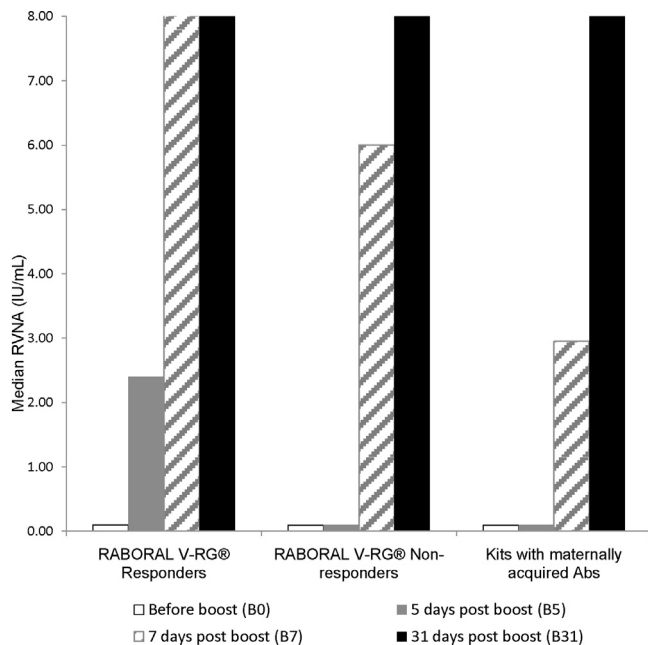


Fig. 5. RVNA profile of raccoon kits after a secondary rabies antigen exposure (IMRAB3®). All kits had previously been exposed to V-RG (orally or contact) and were designated as “Responders”, “Non-responders”, and “Kits with maternally acquired Abs” based on their primary response. Kits that responded initially to the V-RG exposure (RVNA ≥ 0.1 IU/mL) appeared to have an anamnestic response to the IMRAB3®, while the other kits may have had a primary response.

for at least five consecutive weeks (designated as “responders”). Of the 19 responders, 15 were orally vaccinated and 4 were contact controls. Seven of 26 kits (30%) failed to produce RVNA levels of ≥ 0.1 IU/mL (designated as “non-responders”). Following the subsequent vaccination with the killed rabies vaccine, IMRAB3®, we measured RVNA levels to assess any possible anamnestic responses. “Responders” and “non-responders” differed significantly between day B0 and B5 and between day B0 and B7 ($P < 0.05$). No difference was observed between “responders” and “non-responders” by day B31. The 4 kits with maternally acquired antibodies had a similar RVNA profile as the “non-responders” (Fig. 5).

4. Discussion

Vaccination of young animals can be problematic. First, depending on species and age, their immune system may still be relatively immature and secondly, they may still have circulating antibodies acquired from their mothers that may interfere with vaccinations [16,18–20]. This is the first study to monitor the RVNA responses of very young raccoons (3–7 weeks of age) to V-RG and subsequent rabies vaccine exposure.

Twenty-six raccoon kits were whelped by mothers without detectable RVNA. While 90% of kits vaccinated at 3 weeks of age developed detectable RVNA (≥ 0.1 IU/mL) and 75% of kits vaccinated at 7 weeks of age developed detectable RVNA, the relatively low sample size does not have the statistical power to differentiate these two treatment groups. However, it appears that very young kits vaccinated at 3 weeks old responded as well as or better than kits vaccinated at 7 weeks old. In fact, of the 6 responders that were vaccinated at 7 weeks, three (50%) developed RVNA at 5 weeks of age, suggesting that they were actually “vaccinated” by contact with 3 week vaccinates. Interestingly, 50% of our non-vaccinated contact kits also developed detectable levels of RVNA. These contact kits were most likely exposed to V-RG from suckling or playing with vaccinated kits. Limited horizontal transmission has previously been documented in other oral rabies vaccine experimental studies [13,27,28]. We did not observe any signs of disease or adverse effects associated with vaccination of these young animals and the overall seroconversion rate was similar to what we have observed in experimentally vaccinated adult raccoons [29]. Regardless of vaccination treatment, kits that developed RVNA also showed an anamnestic response after exposure to IMRAB3® at 38–41 weeks of age. Although we did not challenge the young raccoons with live rabies virus in this study, an anamnestic response to the rabies virus after oral vaccination has been previously shown to increase the likelihood of raccoon survival [23].

Because ORV baits are often dispersed in late summer/early autumn and because RVNA can still be detected in adult raccoons for many months after vaccination [30,31] it is possible that females would pass on maternal rabies antibodies to their young in the spring/summer. Three previous studies demonstrated that some fox cubs (*Vulpes vulpes*) born of rabies vaccinated mothers had detectable levels of RVNA in their serum for months [15–17], probably dependent on time of weaning. Two of our goals were to monitor the levels of maternally acquired antibodies in raccoon kits and investigate possible interference with oral rabies vaccination. Only a single mother had detectable RVNA one month prior to whelping so our ability to determine the importance of maternal antibody interference was limited to the analysis of four raccoon kits. We were unable to collect blood from the mother while she was nursing the kits. However, we were able to determine her RVNA levels one month prior to whelping (14.4 IU/mL) and when kits were weaned at 15 weeks (4.3 IU/mL), levels adequate to allow antibody transfer during lactation. Regardless of vaccination treatment, the four kits whelped by this vaccinated mother had similar RVNA curves that fluctuated between 8.0 IU/mL and 15 IU/mL for several weeks until levels steadily declined through week 26. Three out of four kits still had detectable RVNA at nine weeks post-weaning. If these RVNA were solely maternally derived (and not affected by active vaccination) then the half-life of maternal RVNA was approximately 10.5 days after weaning. This finding would be consistent with the duration of maternally acquired canine distemper virus (CDV) neutralizing antibodies, which had a half-life of 10.55 days after weaning [18].

Pare et al. [18] also showed that CDV neutralizing antibodies will nullify or interfere with subsequent CDV vaccination of raccoons at 8 weeks of age. Similarly, fox cubs with maternal RVNA (≥ 0.5 IU/mL) showed a significantly lower response to vaccination

with an oral live attenuated vaccine SAD B19 (at 4–7 weeks) than fox cubs without maternal RVNA, suggesting that the maternal antibodies interfered with vaccination [16]. Another study demonstrated that fox cubs vaccinated with V-RG at 30 days or 90 days of age mounted similar immune responses regardless of the age at vaccination or immune status of their mothers suggesting that no interference existed between passive immunity of maternal origin and active immunity conferred by V-RG oral vaccination [15]. However, in this study [15] all but one pup had maternally acquired RVNA values less than the threshold of the assay (0.5 IU/mL) at the time of vaccination.

To determine whether or not maternally acquired antibodies interfered with V-RG vaccination of raccoon kits, we looked for an anamnestic response to a second vaccination with IIMRAB3[®]. While kits that responded to the first vaccination (RVNA levels of ≥ 0.1 IU/mL) showed a marked increase in median RVNA levels by 5 days post-IMRAB3[®] exposure, kits that did not respond to the first vaccination (RVNA levels of < 0.1 IU/mL throughout sampling) and kits that had maternally acquired antibodies exhibited a slower increase in median RVNA levels. Although the data from this study are limited, the results indicate that maternally acquired antibodies may interfere with a kit's immune response to vaccination and that further investigation is warranted.

Overall, our data suggest that raccoon kits (3–7 weeks of age) are able to produce RVNA after oral vaccination with V-RG. These same kits are also able to mount an anamnestic response to a second rabies antigen exposure, which may be indicative of surviving rabies virus infection. However, it is possible that circulating maternally derived antibodies may interfere with active V-RG vaccination. If RVNA circulates in adult female raccoons through whelping and maternally acquired antibodies interfere with active immunizations then ORV programs must be cognizant of the timing ORV operations to account for this population segment of young raccoons unaffected by vaccination. The current timeline used by USDA APHIS Wildlife Services for ORV campaigns begins each year in early September when most young of the year are nearing 26 weeks old and no longer exhibiting maternally transferred antibodies, thus increasing the likelihood of success of the vaccine at the initial exposure to V-RG. There are of course exceptions, as it is possible that raccoon kits born later in the spring or early summer may experience interference between maternally acquired antibodies and vaccination, decreasing the success of ORV campaigns.

Acknowledgements

The authors would like to thank Dan Gossett and the animal care staff at NWRC for their assistance with the research. We would also like to thank Rebecca Riesen-Kruger for her guidance in blood collection from the neo-nates. Many NWRC technicians also assisted on the project including C. Ellis, J. Ellis, C. Minor, N. Mooers, R. Pleszewski, and T. Rigg.

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