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Safety and immunogenicity of orally administered poxvirus vectored constructs in the white-footed mouse (*Peromyscus leucopus*)

Jordan T. Mandli [a,*], Susan M. Paskewitz [b], Jorge E. Osorio [a]

[a] University of Wisconsin – Madison, School of Veterinary Medicine, 1656, Linden Dr., Madison, WI 53706, USA
[b] University of Wisconsin-Madison, Department of Entomology, College of Agricultural and Life Sciences, 1630, Linden Drive, Madison, WI 53706, USA

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ABSTRACT

Globally, zoonotic spillover is becoming more frequent and represents a growing public health concern. Reservoir-targeted vaccination offers an intriguing alternative to traditional vaccine practices by establishing protection in wild populations that maintain the natural pathogen cycle. As an important pathogen reservoir, *Peromyscus leucopus* Rafinesque or the white-footed mouse has been the target of several experimental vaccines. However, strategies are limited by the method of administration, need for repeated dosing, or safety of constructs in the field. To address these concerns, we evaluated two highly attenuated poxviruses, raccoonpox virus (RCN) and modified vaccinia Ankara (MVA) virus as potential oral vaccine vectors in white-footed mice. Following oral administration, *P. leucopus* showed no adverse signs. A single oral dose elicited robust immune responses in mice to the foreign influenza hemagglutinin protein expressed by poxvirus vaccine vectors. Serum hemagglutinin inhibition antibody titers were detected by day 7 post immunization and persisted until study termination (77 days post immunization). This study establishes the safety and immunogenicity of recombinant MVA and RCN poxviruses in *P. leucopus* and demonstrates the suitability of these vectors as part of a reservoir-targeted vaccine strategy for white-footed mice.

1. Introduction

Zoonotic diseases are an increasing threat to global health. Between 1940 and 2004, emerging infectious disease events were predominately of zoonotic origin (60.3 %) with over 70–75 % derived from wildlife species [1,2]. *Peromyscus leucopus* Rafinesque, commonly known as the white-footed mouse, is a highly competent pathogen reservoir often found in high abundance in nature [3–5]. Widely distributed across North America, the white-footed mouse is an important reservoir for several human pathogens, including *Borrelia burgdorferi* sensu stricto (s.s) and *B. mayonii* (Lyme disease), *B. miyamotoi*, *Anaplasma phagocytophilum*, *Babesia microti*, Powassan virus, *Ehrlichia muris*, and Sin Nombre-like hantaviruses [5]. These pathogens are transmitted as part of an enzootic cycle through arthropod intermediaries (tick) or by direct exposure to rodent excrement as in the case of hantavirus.

Reservoir-targeted vaccination (RTV) has a growing record of successful inhibition of zoonotic disease [6,7]. By vaccinating reservoirs in their natural habitat, RTV can disrupt the transmission cycle and lower the probability of pathogen transmission to incidental hosts. In *P. leucopus*, RTV has been used to combat the Lyme disease causing spirochete *B. burgdorferi* s.s.. Field experiments performed by Tsao et al. (2004) reported that needle inoculation of mice with recombinant outer surface protein A (OspA) from *B. burgdorferi* [8,9] lowered the spirochete prevalence of nymphal blacklegged nymphs in treated sites the following year [10]. However, the magnitude of effect was contingent on local mouse density and availability of alternative reservoirs. Because needle delivery of RTV is unfeasible as a large-scale control method, alternative delivery methods are critical for RTV success [10]. Subsequent studies of a recombinant vaccinia virus vector expressing OspA confirmed the efficacy of vaccine delivery through an oral route using a food bait [11,12]. However, concerns about the safety of unattenuated vaccinia deployed in the wild, especially in non-target hosts, has since hindered further development of this RTV candidate [13]. Another vaccine construct has been developed using recombinant *Escherichia coli* as a recombinant OspA antigen amplification system, with delivery of the heat-killed bacteria

Abbreviations: RCN, Racoonpox virus; MVA, modified vaccinia Ankara.

* Corresponding author.
E-mail addresses: mandli@wisc.edu (J.T. Mandli), smpaskew@wisc.edu (S.M. Paskewitz), jorge.osorio@wisc.edu (J.E. Osorio).

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again through oral baits [14]. Results of field studies using this vaccine system have demonstrated some effectiveness in reducing *B. burgdorferi* infection in *P. leucopus* and tick vectors [15,16]. Unlike the vaccinia virus, the recombinant *E. coli* does not actively infect hosts, therefore protection is dependent on mice consuming a sufficient amount of antigen in multiple RTV baits [15–17].

Poxviruses offer several features that make them suitable reservoir-targeted vaccine vectors. As part of a wildlife oriented RTV strategy, both RCN and MVA offer significant advantages compared to other viruses. They can be delivered by mucosal routes [18–20]. This is made possible through redundant mechanisms of attachment which facilitate infection across a variety of cell types and hosts [21,22]. Furthermore, with a genome tolerant of several large DNA insertions (up to 20 kb), recombinant poxviruses can be engineered to express multiple proteins in a single virus construct [23–25]. Infection of host cells facilitates strong humoral and cellular immune responses that often provide life-long protection [26]. Recombinant poxviruses are also well suited for field use. Lyophilization can be used to improve stability in long-term storage or in extreme environmental conditions [27,28]. Moreover, the relative ease of production of poxviruses enables simpler scaling for broader use [27]. Attenuated strains like modified vaccinia Ankara (MVA) and raccoonpox virus (RCN) have value as vaccine vectors with high safety profiles [29–34]. Severe attenuation of MVA through serial passage in non-target cells has resulted in a loss of capacity to productively infect mammals without inhibiting vaccine antigen expression due to a defect in the later stages of viral DNA replication and viral morphogenesis [30,35]. In the case of RCN, the virus naturally circulates as an attenuated poxviruses in North America [32]. Its broad seropositivity in wild animals and safety in numerous animal models suggests an eco-friendly vaccine vector [18,29,36–40]. Further attenuation can also be achieved through the deletion of the thymidine kinase gene; however, the exact mechanism of debilitation remains unknown [32].

To address the concerns identified by previous RTV efforts in *P. leucopus*, we evaluated RCN and MVA as vaccine vectors for mucosal delivery. Here, we administered, in separate groups, two recombinant poxviruses expressing an influenza hemagglutinin 5 (H5) protein, a model antigen with extensive study in a variety of animal models [19,41,42], to white-footed mice either orally or parenterally. Safety was assessed using non-invasive means including clinical observation and weight change. Immunogenicity was determined using a hemagglutinin inhibition assay. We hypothesize that recombinant MVA and RCN are highly safe and immunogenic in *P. leucopus*, providing support for the development of reservoir targeted vaccines using these two vaccine vectors in white-footed mice.

2. Materials and methods

2.1. Ethics statement

This study was carried out with strict adherence with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol (#V005220) was approved by the Institutional Animal Care and Use Committee of the University of Wisconsin-Madison.

2.2. Viral and mouse strains

African Green Monkey kidney cells (VERO, ATCC #CCL-81) and Baby Syrian Hamster kidney cells (BHK-21 [C-13], ATCC #CCL-10) were propagated in Dulbecco's modified Eagle medium, (DMEM; Gibco, Carlsbad, CA) augmented with 3–5 % fetal bovine serum (FBS) and antibiotics and incubated at 37 °C in 5 % CO2. Two recombinant poxvirus constructs were used; a recombinant MVA virus expressing a mosaic hemagglutinin protein (H5M) constructed from a wild-type MVA obtained through BEI resources (NIAID, NIH, ref# NR-727) (described in [42]); and a recombinant RCN virus expressing influenza hemagglutinin protein (H5) constructed from a wild-type RCN obtained through the Centers for Disease Control (described in [19]). Virus isolation was facilitated by the inclusion of fluorescent proteins as part of gene insertions. Purification of recombinant virus was completed by ultracentrifugation through a sucrose cushion [43]. Protein production was verified by western blot. Outbred *P. leucopus* mice were purchased from the University of South Carolina Peromyscus Genetic Stock Center (Columbia, SC). Mice were an even split of male and female mice aged 46 – 76 days when experiments began.

2.3. Oral bait preparation

Oral baits were constructed from a bait matrix developed by Foodsource Lures Corp. (Alabaster, AL). Briefly, dry bait powder was mixed with peanut butter and distilled water at 70 °C. Ingredients were thoroughly mixed and allowed to cool to 40 °C. The desired dose of recombinant poxvirus was incorporated into the mixture which was then cooled in a plastic mold. Phosphate buffered saline (PBS) was used in place of viruses for negative controls. Assuming homogeneous distribution of the poxvirus, baits were cut to reflect a concentration of approximately 1×10^7 plaque forming units (pfu)/bait. Separate materials and equipment were used for each virus construct and negative control.

2.4. Mouse vaccination and sample collection

Groups of *P. leucopus* mice were administered a single dose of RCN-HA or MVA-H5M by footpad injection using a 30-gauge, 12.7 mm insulin syringe (FP: IRCN or IMVA) or offered as oral baits (OB: BRCN or BMVA) to be ingested ad libitum (Table 1). Negative controls were sham injected with an equal volume of PBS or given an equivalent weight of OB lacking virus (IPBS or BPBS). Briefly, mice vaccinated by OB were individually caged without an alternative food source for 48 h and were each supplied a dose of 5 baits (~8.0 g) containing a total of $\sim 5 \times 10^7$ pfu of recombinant poxvirus. Following isolation, mice were returned to communal treatment group cages and remaining bait from each individual cage was massed. The FP group received 5×10^7 pfu of a recombinant poxvirus construct suspended in a total of 0.025 mL PBS. This dose was selected based on our laboratory's previous experience with poxvirus systems in mice [19,42,44,45]. Weekly blood samples were collected by a *retro*-orbital blood draw and serum was isolated and stored at −20 °C until analysis. Mouse weight was measured weekly prior to blood draw.

2.5. Serology

Detection of antibodies against H5N1 influenza virus was measured by means of hemagglutinin inhibition (HI) assays with 1.0% chicken erythrocytes and inactivated whole virion influenza (A/Vietnam/1203/2004) from BEI Resources (cat# NR-12147) in accordance with established procedures [46]. Samples were assayed in duplicate. Serum samples were tested at serial twofold dilutions from 1:25 to 1:3200. HI antibody titers lower than 1:3200 were assigned a value of 1:3200 and values >1:25 were assigned a value of 1:10. Samples from a specific date were run simultaneously with corresponding negative controls.

Table 1
Experimental design including treatment groups as defined by construct (RCN-HA or MVA-H5M) or control (PBS) and route of administration (foot pad injection: IRCN, IMVA and IPBS, oral bait: BRCN, BMVA, and BPBS). Provided dose is reported as virus plaque forming units (pfu) in either liquid inoculate or bait. Mean dose administered represents the pfu received. Number of mice per group is reported with gender breakdown (Male/Female). Serum collection schedule in days post immunization (dpi).

Treatment	Group	Provided Dose (volume/mass)	Mean Dose Administered	N (M/F)	Serum Collection
RCN-HA	IRCN	5.0×10^7 pfu - (0.025 mL)	5.0×10^7 pfu	7(4/3)	Once prior to study
	BRCN	5.0×10^7 pfu - (8.0 g)	4.3×10^7 pfu	7(4/3)	(-1 dpi) and weekly ending at 77 dpi
MVA-H5M	IMVA	5.0×10^7 pfu - (0.025 mL)	5.0×10^7 pfu	7(4/3)	
	BMVA	5.0×10^7 pfu - (8.0 g)	3.8×10^7 pfu	7(4/3)	
PBS	IPBS	0.0 pfu - (0.025 mL)	0.0 pfu	3(2/1)	
	BPBS	0.0 pfu - (8.0 g)	0.0 pfu	2(1/1)	

2.6. Statistical analysis

Statistical analyses were performed in GraphPad version 8.0 software (La Jolla, CA). Welch's *t*-test was used to evaluate differences in baseline sample population characteristics. A linear mixed effect analysis (REML) was performed to analyze the effect of time (week) and treatment (RCN-HA, MVA-H5M, PBS) on mouse percent of initial weight (weight at current week/weight from baseline) subdivided by route of administration (OB vs FP). One-way analysis of variance (ANOVA) was used to assess the effect of treatment combinations on HI titers at select time points. Follow-up analysis was completed using a Fisher's LSD test as necessary.

3. Results

3.1. Animal health and safety

At baseline, 33 *P. leucopus* (16 female and 17 male) were randomly assigned to one of six treatment combinations (treatment and route of administration) (Table 1) with a total mean age of 61.1 days (95 % CI: 58 – 64) and mean weight of 16.0 g (95 % CI: 15–17). No significant difference in age (t = 0.39, df = 34, p-value = 0.70) or weight (t = 1.05, df = 34, p-value = 0.30) was detected between sexes at baseline. As the study progressed, mouse mean percent of initial weight trended upward (Fig. 1) as reflected by the statistically significant effect of time ($P_{OB} < 0.001$, $P_{FP} < 0.001$). Simple main effects analysis showed that the effect of treatment on weight was not statistically significant for either route of administration ($P_{OB} = 0.11$, $P_{FP} = 0.10$). Furthermore, percent of initial weight was statistically indistinguishable between treatments and controls administered in the same manner at each time point (ex: weight of IRCN-H5 vs IPBS on day 49).

3.2. Recombinant poxvirus administration

Peromyscus leucopus consumption of oral bait was variable, indicating incomplete dosing. Surprisingly, residual oral bait was present in fourteen of the sixteen bait-inoculation cages following the 48 hours of isolation. On average, 85 % of the RCN-HA bait was consumed with an approximate mean dose of 4.3×10^7 pfu (median = 4.2×10^7 pfu, min/max = 3.8×10^7– 5.0×10^7 pfu), 76 % of the MVA-H5M bait was consumed with an approximate mean dose of 3.8×10^7 pfu (median = 3.9×10^7 pfu, max/min = 2.9×10^7 – 4.6×10^7 pfu), and 75 % of the PBS bait was consumed (Table 1).

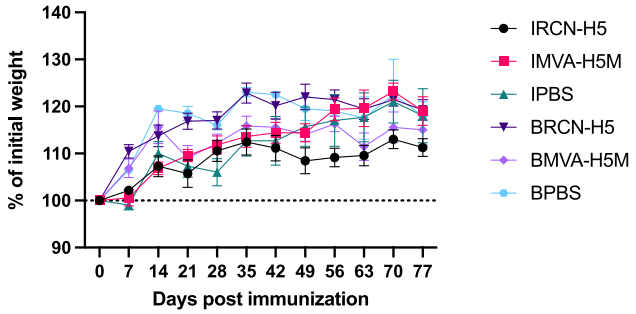

Fig. 1. Mean percent of initial weight of mice from treatment groups with SEM. Values presented by days post immunization. No significant differences were detected between treatments at all time points by Fisher's LSD test.

3.3. Immunogenicity

A single dose of the RCN-HA or MVA-H5M construct produced potent H5 antibody immune responses in treated mice by both routes of administration associated with protection against lethal infection [47]. At baseline −1 days post immunization (dpi), antibodies against H5 were not detected in serum samples as measured by HI assay (titer > 1:25) and therefore assigned a titer of 1:10. All treated mice developed a detectable HI titer by day 7 (Fig. 2) perhaps representative of developing IgM antibody titers. Hemagglutinin inhibition in RCN-HA treated mice peaked at day 49 (>3 logs) and declined thereafter whereas titers in MVA-H5M treated mice peaked at day 21 (>2 logs) and then gradually deceased throughout the remainder of the study. HI titers between parenteral and oral administered counterparts were not significantly different by sampling period with the exception of IRCN and BRCN at day 49 (Fig. 3). At this time, the HI assay threshold of detection surpassed (>1:3200) for IRCN and complete titers remain unknown and likely underestimate the difference between routes. PBS control mice displayed no detectable HI activity during the study.

4. Discussion

This study demonstrates a proof of concept for recombinant MVA and RCN poxviruses as vaccine vectors in *P. leucopus*. As

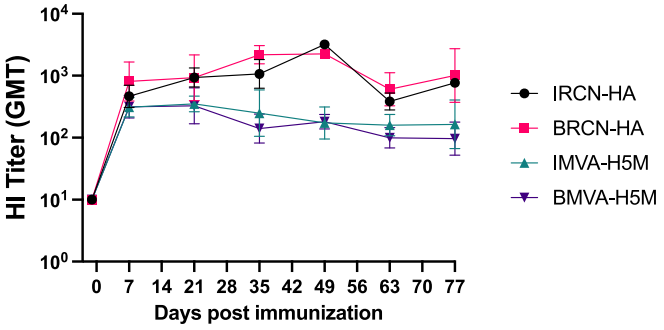

Fig. 2. Kinetics of RCN-HA, MVA-H5M antibody response post-immunization. *P. leucopus* received 5×10^7 pfu or equal volume of PBS by foot-pad injection, or a 5×10^7 pfu oral bait dose, or control sham bait. The geometric mean hemagglutinin inhibition titer and 95 % confidence interval of serum antibody titers are shown over the course of the study.

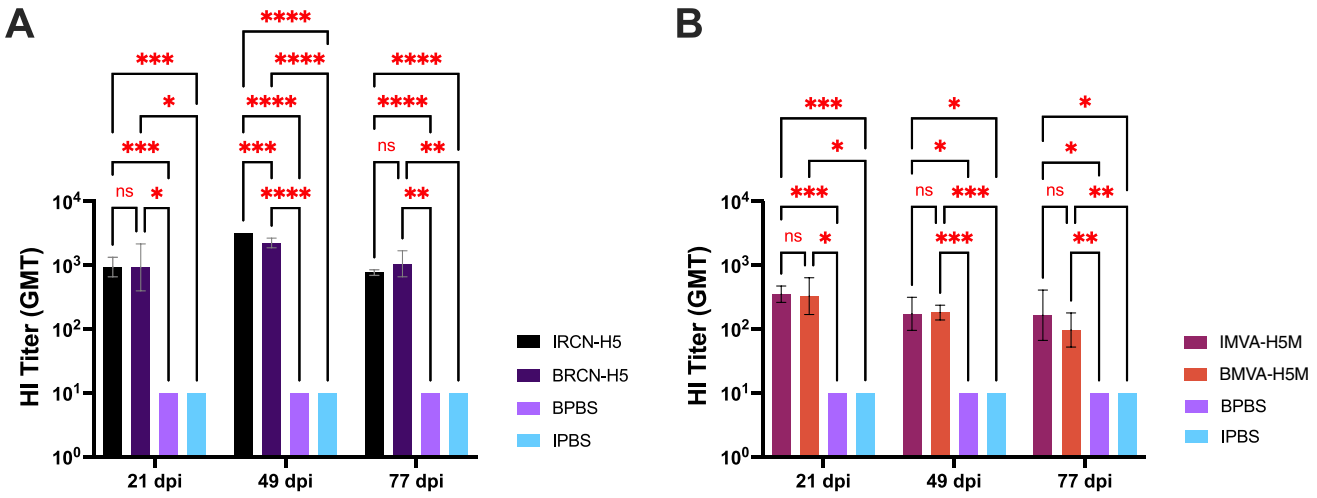

Fig. 3. Antibody responses in *P. leucopus* exposed to MVA-H5M, RCN-HA, and PBS administered by oral bait (OB) or footpad injection (FP). HI titers in *P. leucopus* mice at 21, 49, and 77 days post immunization (dpi) are shown. Horizontal bars represent geometric mean hemagglutinin inhibition of A) RCN constructs + PBS controls and B) MVA constructs + PBS controls at select time points with a 95 % confidence interval. Pips indicate significant difference by Fisher's LSD test; *, p-value < 0.05; **, p-value < 0.01; ***, p-value < 0.001.

expected, a single dose of recombinant RCN-H5 or MVA-H5M was highly immunogenic when delivered to white-footed mice by oral or parenteral route. Elevated HI titers were established early and persisted for 77 days, suggesting oral delivery as an efficient means of inducing extended antibody immunity. Mice exhibited no observable health complications following immunization and changes in weight between treated and untreated mice were not statistically significant thereby supporting the safety of recombinant poxvirus vectors in *P. leucopus*.

Mucosal delivery of vaccines is an essential requirement for RTV in wild mammal populations [10,48]. Here we show that two recombinant poxvirus constructs induce strong anti-H5 antibody responses by oral bait. While *P. leucopus* is not a reservoir for avian influenza, these findings show the utility of RCN and MVA as alternative vaccine vectors for the immunization of white-footed mice with foreign antigens. While not the subject of direct comparison, RCN-H5 and MVA-H5M elicited different levels of anti-H5 activity in *P. leucopus*. Varying antigen immunogenicity between H5 and H5M and MVA's lack of replicative ability both likely contributed to the MVA-H5M treatment group's reduced titers and may be distinguished with alternative study design [32,44,49]. Within poxvirus constructs, similar HI titers between FP and OB indicate that oral vaccination with recombinant poxviruses may provide an efficient alternative to needle immunization. However, antigen specific characteristics may drive additional variation between routes of administration and should be studied more thoroughly.

RCN and MVA represent two poxviruses with enhanced safety profiles without transmissibility already approved (FDA and USDA) for human (MVA) and wildlife (RCN) use [32,34,50,51]. Throughout our study, treated animals demonstrated no adverse outcomes associated with the administration of either recombinant poxvirus construct. Moreover, treated mice actively gained weight at a rate similar to untreated mice. These data support the safety of recombinant RCN and MVA in *P. leucopus*. However, future studies are warranted to investigate the histological and hematological impacts of other expressed foreign antigens and how repeated or variable administration, which are more likely to occur in the field, may affect mouse health. With additional scrutiny of releasing a virus into the wild, there may be additional value of using MVA as a nonreplicating vaccine vector. As reported, RCN elicited a robust immune response, but its replicative nature would likely require additional study in other non-target animal species who may unintentionally become exposed [13]. Selective baiting strategies (bait box, repellents) could also offer better control of vaccine consumption [16].

Complex ecological and behavioral factors may introduce inconsistency in orally delivered vaccines in the field [52]. Protection achieved through repeated dosing is unreliable when faced with competing food interests, food caching, short life expectancy, and high reproductive rates [53]. Based on this study, RCN and MVA vaccine constructs quickly elicited robust immune responses that lasted up to 77 days after a single oral dose. This suggests that recombinant poxviruses could be used to vaccinate a *P. leucopus* population rapidly and efficiently in minimal doses thereby reducing risk of zoonotic spillover. For an extended immune response (>77 days), RCN is suspected of being better suited to maintaining antibody titers due to its ability to briefly replicate *in situ* after vaccination unlike its nonreplicating MVA counterpart [49]. Future studies comparing immune responses between vaccine constructs beyond 77 days could provide further insight into the resilience of protection and offer a vaccine candidate best suited to the pathogen of interest. Regardless, additional deployments may be required to adjust for seasonal white-footed mouse population growth, turnover, and movement. Pathogen-specific strategies will need to be tailored to achieve ideal expression and immunogenicity in target hosts while considering logistical and ecological aspects of field deployment.

Successful vaccination of a wild reservoir population requires a means of administration that limits the chance for incomplete treatment. Even under ideal laboratory conditions lacking alternative food sources, our *P. leucopus* did not consume all the bait allotted. The number of baits provided was selected to achieve the desired collective dose (5.0×10^7 pfu) but likely surpassed mouse digestive capacity or interest for the 48-hour isolation period. Using the same bait matrix and a peanut butter bait attractant, but at a smaller size (2.0 g), Bhattacharya et al. (2001) reported complete bait uptake by *P. leucopus* within 48 hours [12]. Furthermore, the bait matrix and attractant were readily consumed by mice in the field [54]. Collectively these data demonstrate mouse preference for this bait setup even in the presence of alternative food sources. By adjusting recombinant poxvirus concentration in baits, we can elicit protection in a single minimum dose bait.

Vaccination of humans is a critical public health strategy and can be an effective means to prevent zoonotic disease; however

safe and highly effective vaccines for humans may not be available [48]. Now more than ever, public perception and misinformation of vaccination has hindered the development of population immunity, derailed global vaccine campaigns, and allowed for pathogen reemergence [55–57]. RTV offers an alternative approach that bypasses humans entirely. Moreover, the lower complexity, shorter timeline, reduced cost of development, and abridged regulatory guidelines for product approval make animal vaccination more appealing [48,53]. Developing a new animal vaccine costs approximately 10 % of a typical human vaccine ($200–$500 million) [58]. Therefore, an intervention based on the immunization of wildlife reservoirs could lead to rapid and relatively cost-effective improvements to public health.

Our data demonstrates that mucosal immunization with recombinant RCN or MVA is safe and immunogenic after a single oral dose in white-footed mice. When addressing the zoonoses directly or indirectly transmitted by *P. leucopus*, these vaccine vectors embody qualities lacking from previous RTV candidates. Therefore, recombinant poxviruses warrant further investigation as potential vaccine vectors in white-footed mice.

CRediT authorship contribution statement

Jordan T. Mandli: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Susan M. Paskewitz:** Conceptualization, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition. **Jorge E. Osorio:** Conceptualization, Methodology, Validation, Resources, Writing – review & editing, Supervision, Project administration.

Data availability

Data will be made available on request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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