Vaccinating vampire bats against rabies: studies of vaccine efficacy, field application, and social perceptions in México

By

Elsa María Cárdenas Canales

A dissertation submitted in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy
(Comparative Biomedical Sciences)

at the
UNIVERSITY OF WISCONSIN-MADISON
2021

Date of final oral examination: 08/27/2021

The dissertation is approved by the following members of the Final Oral Committee:
Jorge E. Osorio, Professor, Pathobiological Sciences
Kristen Bernard, Professor, Pathobiological Sciences
Keith Poulsen, Clinical Assistant Professor, Medical Sciences
Hernando Rojas, Professor, Professor, School of Journalism and Mass Communications
Tonie E. Rocke, Research Epizootiologist, USGS National Wildlife Health Center

Dedication

Hoping that someday this work becomes a helpful tool, it is dedicated to the people in Latin America whose livelihoods continue to be affected by rabies and vampire bats

Acknowledgments

I consider myself the luckiest for having a research project that took me to México (my birth country) and allowed me to study one of the coolest bat species, the vampire bat. I knew that if I was to return to school for an advanced graduate degree, it had to be for a very special project. This was it! I am so grateful to Tonie Rocke and Jorge Osorio for giving me this opportunity and mentoring me during this time. Thank you immensely for your trust and support and for making me feel welcomed.

Most research projects are collaborative in nature, but this one went beyond the academic circle and included many people (family, friends, colleagues) in the United States and México. I had the fortune to get to know and to be surrounded by amazing people who were always helpful, providing me with advice on so many topics: laboratory techniques, statistics, bats and diseases, import of live animals, how to navigate the government system, presentation skills…you name it! Thanks to all of you.

In the U.S.: to my committee members Dr. Kristen Bernard, Dr. Keith Poulsen, and Dr. Hernando Rojas, thanks for taking an interest in this project and my growth as a scientist. Thank you for your valuable comments and questions during our meetings. My lab mates and colleagues at UW-Madison: Lex, your love for molecular biology is only a fraction of my gratitude to you for helping me with PCR and all things molecular. Jordan Mandli, Bieneke Bron, Cristhian Salas, Emily Medina, Jaime Lopera, thanks for providing conversations, advice, and help during the time we spent around the office. Folks at the School of Veterinary Medicine who helped me countless times: Jason Brenner (IT lifesaver), Linda Gauer, and Kendra Kearney (admin and travel rock stars), and Susan Thideman (incredible source of support). At the National Wildlife Health Center, thanks to Carly Malave, Susan Smith, Judith Williamson, Ariel

Leon, Liz Falendysz, and Tina Egstad. Learning my ways around the center and getting bat and lab work done would not have been possible without you. Many thanks! Andrés Velasco-Villa became a mentor and a good friend. His enthusiastic conversations about rabies and science were always on point. Thanks to you and Nadia for being my role models and sincere friends. Doris Olander has been

In México: Baltazar Cortes, Alejandro Zaldivar, Toñito Rivas, Ignacio Amezcua, José Blanco, all my gratitude for helping me coordinate fieldwork and logistics, and for making sure I ended up with the right people everywhere I went. Thank you as well for sharing your knowledge with me. There is no way I could have achieved any of my goals without your help and guidance.

My most tremendous gratitude goes to my parents, as always. Your presence and support are such a wonderful gift. You drove a truck to the border to help me start the vampire bat fieldwork trip and were my "representatives" with the Mexican government dealing with permits and paperwork. That was amazing! I am so fortunate to have you by my side in every milestone of my life. Gracias papá y mamá, los amo mucho.

I am also extremely lucky to have a wonderful group of friends and family that have joined me throughout my schooling years (and the time in between). You know the whole story, the good and bad and the fun and stressful. Your friendship and support are a treasure to me. Sending my gratitude from Wisconsin (where I´m now) to Monterrey, Kingsville, Fort Collins, and New Haven (the places where I met you all).

This research was supported by the American Zoo of Wildlife Veterinarians, The University of Wisconsin-Madison (IRIS and Fall Competition grants), and the UW-Madison Global Health

Institute. A two-year fellowship from The Morris Animal Foundation (DZO19-404) supported me with a stipend during my studies.

Table of contents

DISSERTATION ABSTRACT

Vaccinating vampire bats against rabies: studies of vaccine efficacy, field application, and social perceptions in México

Elsa María Cárdenas Canales

Under the supervision of Dr. Jorge E. Osorio
At the University of Wisconsin-Madison

Vampire bats are responsible for most of the rabies outbreaks in livestock across Latin America. As with other wildlife species, the use of oral vaccines to control rabies in vampire bats offers an opportunity to manage the disease at the reservoir without relying on lethal practices. In this dissertation, I report the results of my studies on the potential of vaccinating wild vampire bats against rabies. This project included 1) testing the efficacy of RCN-MoG, a recombinant rabies vaccine for vampire bats, 2) studying the perception toward a bat-focused vaccination strategy of those individuals involved in controlling vampire bats in México, and 3) a small pilot study to assess the application of a topical biomarker as a proxy for vaccination in colonies of vampire bats. Conducting research with wild-caught animals is always challenging. During the study, a natural outbreak of rabies occurred within the colony of vampire bats, complicating the vaccine experiment. Still, this incident allowed us to observe first-hand the natural progression of rabies

in vampire bats and the potential effect of vaccination during an outbreak. The incident also hinted at the possibility that RCN-MoG could block rabies transmission. At the end of the study, I found that RCN-MoG was safe for use in vampire bats and protective against rabies. Most importantly, the potential blocking effect against excretion of rabies virus in the saliva of vaccinated bats that succumbed to rabies was confirmed. This finding re-shaped the original question about vaccinating bats: what if vaccination not only protects some vampire bats from rabies but also could stop transmission by rabid bats within their colonies and to other species? Last, a survey from the personnel of the Mexican program for rabies control in livestock showed that the participants are very supportive of vaccination of bats as a potential strategy for rabies control. The results reported in this dissertation provide valuable insights for future planning of studies on rabies vaccination (in the lab and the field) of the common vampire bat.

CHAPTER 1

Introduction

Bats have recently gained much attention as they are the reservoirs for viruses of zoonotic interest [1] and the source of diseases with striking global consequences. As a result, many scientists have focused their research efforts to the ecology of bat-borne diseases and pathogen discovery, especially viruses. Additionally, there is a keen interest in understanding the mechanisms by which bats can withstand an array of viruses without becoming sick, a particular trait of these mammals [2,3]. The common goal of this research is to prevent outbreaks of zoonotic potential. The task is monumental, as more than 1,400 species of bats in the world [1] live in a wide gradient of ecological niches and are associated with >200 viruses [4]. While most of the concern is about novel pathogens, others, such as those in the genus *Lyssavirus,* have affected humans and animals for centuries, yet the biology in the bat reservoir remains poorly understood.

Lyssaviruses are negative-sense, single-stranded RNA viruses that cause rabies, a fatal neurologic disease that affects predominantly mammals worldwide [5]. The genus Lyssavirus is classified into 3 phylogroups that are characteristically and geographically distinct [6,7]. According to genetic analysis, Lyssaviruses have a bat origin and are present throughout the world in bat hosts [6,8]. Within the genus *Lyssavirus*, the rabies virus (RABV) is the most significant [8]. RABV is established globally in many reservoir species (wild and domestic) but commonly associated with its canine reservoir, the domestic dog. However, in the Americas,

RABV is the only Lyssavirus that circulates in bats [7,9,10], and different RABV lineages appear to be restricted to specific bat host species [8,11].

In the Americas, one bat species is of great interest given its association with RABV: the common vampire bat (*Desmodus rotundus*). This bat species is found only in Latin America, spanning from northern México to Argentina and Chile [12]. Currently, the common vampire bat is considered the main reservoir of RABV in Latin America [13], especially after the elimination of the canine-associated rabies in some countries through systematic vaccination of dogs [14,15].

Rabies in vampire bats was first described in 1936 in Trinidad, when wild-caught individuals were observed displaying the furious (aggressive) and paralytic forms of the disease, as well as suddenly dying without clinical signs [16]. While the natural progression of rabies in the common vampire bat is not fully understood, information based on field observations and experimental studies have provided a baseline understanding of this disease. Similar to other mammals, the main route of RABV transmission among vampire bats is by direct inoculation (e.g., bites) inflicted by a rabid individual [8]. Bats excrete RABV in their saliva, sometimes several days before the onset of clinical signs [17,18]. Transmission of RABV among individuals can occur during interactions that follow their natural behavior [8] (e.g., they are a highly social species, known to display food sharing and allogrooming [19]). Moreover, disease induced behaviors (e.g., aggression during the furious presentation of rabies) could increase the probability of viral transmission [8]. Long incubation periods (of up to 3 months), have been observed in vampire bats [18] before the virus reaches the central nervous system [20]. From the brain, RABV disseminates through the nerves to reach the salivary gland where it is finally being excreted in the saliva during the latter stages of the disease [14,20], prior to

death. Still, the mechanisms by which rabies is maintained in the vampire bat in nature, and what triggers outbreaks in this species, remain unresolved.

The role of vampire bats as vectors of RABV is based partly in their unique feeding behavior. Vampire bats are obligated blood feeders and must bite their prey to obtain blood [21]. A rabid vampire bat will transmit RABV via its infectious saliva during feeding. Vampire bats must feed every night, otherwise they starve to death [19]. Consequently, every night, susceptible hosts are at risk of being exposed to vampire-associated rabies (VBR). Livestock is the preferred prey of vampire bats, but occasionally they feed from other domestic animals or even humans [16]. Habitat suitability and availability of prey species contribute to the maintenance of vampire bats populations and, thus, perpetuate the occurrence of VBR throughout their natural range. In fact, increased reports of rabies in humans are strongly associated with the incidence of rabies in livestock species in South America [22]

Vampire-associated rabies is a burden to both animal health and public health in Latin America. Annually, VBR is believed to cost over USD 30 million in losses due to animal deaths and loss in production [23], although this amount may be underrepresented [24]. To combat VBR, many Latin countries rely on two main strategies 1) vaccination of livestock and 2) the control of vampire bat populations [22]. Livestock vaccination is an effective measure, but it is not always performed due to many socio-economic conditions that make it difficult to achieve, especially in rural areas. Vampire bat population control (i.e., culling) is accomplished by treating individuals in a colony with a topical poison (anticoagulants, known as “vampiricide”). The social behavior of vampire bats permits the transfer of the vampiricide from those treated to the rest of the colony through contact, thus resulting in multiple deaths. The use of the vampiricide, usually regulated by the animal health authorities, is very effective in reducing bat

colonies [25], and replaced the more devastating practices of gassing or burning roosts of bats. However, culling vampire bats can have negative effects on other bat species if used indiscriminately. More importantly, culling has not shown a sustainable effect in controlling VBR [26].

With the goal of devising alternatives for VBR control beyond lethal methods, vaccination of the common vampire bat against rabies has been explored since the late 1990s [27–30]. Rabies has been controlled before in other wildlife with the use of oral viral-vectored vaccines [31,32], as demonstrated by the success of the oral rabies vaccine program (ORVP) in eliminating fox rabies in some countries in Europe and coyote rabies in the U.S. [22,33]. Recombinant viral-vectored vaccines, such as those used in the ORVP, have certain advantages over other vaccine types (e.g., attenuated or killed virus), because the vectors can be manipulated to increase their safety and immunogenicity. Also, since viral-vectored vaccines act by infection and replication in the host, an additional cell-mediated immunity may be stimulated and confer greater protection [34]. But perhaps the most important advantage is that they can be delivered orally, which is important when attempting to vaccinate wildlife species [35].

For vaccination of wildlife two main characteristics should be addressed: 1) high coverage across a population and 2) vaccine efficacy [35]. But vaccination of vampire bats bears greater challenges, as the biology of the species and the ecological features where they live, differ greatly from those of successfully vaccinated carnivores.

Aware of these challenges, we resolved to further study rabies vaccination of vampire bats building upon previous research on a raccoon pox (RCN) virus-vectored rabies vaccine (RCN-MoG). The immunogenicity and efficacy of RCN-MoG was widely tested in several rodent animal models under controlled laboratory conditions and other bat species abundant in

domestic and peri-domestic environments (*Tadarida brasiliensis* and *Eptesicus fuscus*) [36,37]. Moreover, it was found that glycerin jelly was a suitable product for the topical delivery of a vaccine to bats, as it maintained the viral vector stably at elevated temperatures over time [38]. The next obvious step was to begin similar trials in the common vampire bat.

Under a One Health philosophy, this project was shaped by two different disciplines: social and biomedical sciences, each with a specific aim, merging with a third aim in applied field ecology. The vision of this project is to vaccinate wild vampire bats using RCN-MoG following the exact application mechanism as the topical vampiricide. To achieve the goal, the project covered a broad process, 1) testing the efficacy of a recombinant rabies vaccine for vampire bats, 2) assessing the field application of a product in lieu of vaccination (placebo) in colonies of vampire bats, and 3) studying the perception toward a bat-focused vaccination strategy in México. Following a "jigsaw" approach, the three elements validate and support each other to constitute a compelling, novel strategy. However, the outcome of each component is independently valuable. Moreover, each one provides insights for future planning of studies and development of technology and knowledge about rabies in the common vampire bat.

Chapter Introductions

Chapter 2 was published in the Journal of Tropical Medicine and Infectious Disease in March 2020. The publication is a case report based on a natural rabies outbreak in our wild-caught captive colony of vampire bats in December 2018 during the vaccine efficacy study. This chapter describes the clinical progression of rabies in a group of vampire bats and their serological response upon infection. During this investigation, we found that vampire bats that

were vaccinated with RCN-MoG during the outbreak, before they eventually succumbed to rabies, did not have detectable rabies nucleic acid in their saliva. Conversely, unvaccinated bats shed RABV in their saliva before or at their day of death. This finding motivated a more thorough investigation about the effect of RCN-MoG in RABV shedding during the vaccine efficacy study (chapter 3).

In **chapter 3**, I describe our findings regarding the safety, immunogenicity, and efficacy of our candidate vaccine (RCN-MoG), both orally and topically, in vampire bats and the effect of vaccination on RABV shedding in the saliva of vaccinated bats. For this experiment, we used vampire bats captured in Mexico in July-August 2018 and transferred to Madison, Wisconsin. We conducted the experiment from December 2018 – June 2019. We vaccinated bats orally or topically with RCN-MoG, collected serial samples for serology assays and finally challenged the bats with RABV. We found that vaccination partially protects bats against rabies and confirmed the lack of RABV shedding in bats vaccinated with RCN-MoG that succumb to the disease, as described previously in Chapter 2. The results from chapter 3 are encouraging and should stimulate further research, especially under field conditions.

A crucial step after finding a good rabies vaccine candidate is to test the feasibility of delivering the vaccine to individuals in the wild. We envision to vaccinate wild bats in the same way as the vampiricide is applied (i.e., topically). To follow up on this, we conducted a field pilot study to measure the dispersion of a topical product (made of glycerin jelly and Rhodamine B, a biomarker) in colonies of vampire bats in place of vaccination. We conducted vampire bat captures at two field sites to treat bats with biomarker and collect samples for further uptake analysis. The use of a biomarker allows the assessment of "vaccine" uptake by proxy [39] and the calculation of transfer rates in colonies of vampire bats. Field sites included the Mexican

states of Yucatán and Jalisco, both diverse ecological regions and with high populations of vampire bats (as reported by the Mexican rabies control program). The observations of this pilot study are included in **chapter 4** in a technical note format. Unfortunately, the vehicle of choice for this study (glycerin jelly) was not an ideal product for field use. When tested in the laboratory, glycerin jelly maintained the viral vector [38] and was applied without major issues to vampire bats. In the field however, the glycerin jelly/RB biomarker crystalized after 25 days of being prepared, thus affecting proper uptake between use. Uptake assessment (a qualitative measure based on the presence of biomarker in hair samples) was affected by the properties of the product and the differences in results are evident between the two field sites.

For **chapter 5**, we conducted a survey among the personnel from the Mexican rabies campaign regarding their perception about the program´s activities and attitudes toward vampire bats and vaccination. The aim of the survey was to identify factors that may influence the adoption of a vampire bat-focused rabies vaccine. The results from this section are important to tailor disease control strategies and to develop information programs that will ease adoption of novel technology (i.e., vaccination). We found that the adoption of (or support for) a vampire bat vaccine among the personnel surveyed was positive. There are two main uncorrelated drivers that influence the adoption of a vampire bat rabies vaccine. One is to have a positive belief that vaccination helps control rabies, the other is the availability of a vampire bat rabies vaccine with desired characteristics. We had hypothesized that some variables, such as demographic information, or the perception about the efficacy of culling vampire bats, would affect the adoption of a bat vaccine, but this was not the case. The results from this chapter show important factors to consider (e.g., the delivery of accurate information to address concerns regarding a bat vaccine) when advocating for the use of a rabies vaccines for vampire bats in México.

Chapter 6 summarizes the results of this work and provides some insights about the main challenges during the vaccine study. I also address some potential explanations for some of the challenges before adding recommendations for future work of this nature.

In **Appendix 1,** I describe the design and construction of the transport cages and feeding system used to transfer wild-caught vampire bats from México to Madison. Because there are no commercially available containers (or services) to transport vampire bats, my first task was to solve this logistical issue. This piece of the project resulted in a successful product. I transported 93 wild-caught vampire bats from México to Madison (including a feeding session) during a >40-hour trip by land, while complying with biosafety and transportation guidelines. A manuscript describing the cages is in preparation.

References

1. Wang LF, Gamage AM, Chan WOY, Hiller M, Teeling EC. Decoding bat immunity: the need for a coordinated research approach. Nat Rev Immunol [Internet]. 2021;21(5):269–71. Available from: http://dx.doi.org/10.1038/s41577-021-00523-0
2. Baker ML, Schountz T, Wang L-F. Antiviral Immune Responses of Bats: A Review. Zoonoses Public Health [Internet]. 2013 Feb;60(1):104–16. Available from: http://doi.wiley.com/10.1111/j.1863-2378.2012.01528.x
3. Subudhi S, Rapin N, Misra V. Immune system modulation and viral persistence in bats: Understanding viral spillover. Viruses. 2019;11(2).
4. Calisher CH. Viruses in bats: a historic review. In: Bats and Viruses: A New Frontier of Emerging Infectious Diseases. 1st ed. 2015. p. 23–45.
5. Rupprecht C, Kuzmin I, Meslin F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. F1000Research [Internet]. 2017 Feb 23;6:184. Available from: https://f1000research.com/articles/6-184/v1
6. Badrane H, Tordo N. Host Switching in. J Virol. 2001;75(17):8096–104.
7. Velasco-Villa A, Mauldin MR, Shi M, Escobar LE, Gallardo-Romero NF, Damon I, et al. The history of rabies in the Western Hemisphere. Antiviral Res [Internet]. 2017;146:221–32. Available from: https://doi.org/10.1016/j.antiviral.2017.03.013

8. Banyard AC, Hayman D, Johnson N, McElhinney L, Fooks AR. Bats and Lyssaviruses. Vol. 79, Advances in Virus Research. 2011. 239–289 p.

9. Velasco-Villa A, Gómez-Sierra M, Hernández-Rodríguez G, Juárez-Islas V, Meléndez-Félix A, Vargas-Pino F, et al. Antigenic diversity and distribution of rabies virus in Mexico. J Clin Microbiol. 2002;40(3):951–8.

10. Hayman DTS, Fooks AR, Marston DA, Garcia-R JC. The Global Phylogeography of Lyssaviruses - Challenging the "Out of Africa" Hypothesis. PLoS Negl Trop Dis. 2016;10(12):1–13.

11. Kuzmin I V., Bozick B, Guagliardo SA, Kunkel R, Shak JR, Tong S, et al. Bats, emerging infectious diseases, and the rabies paradigm revisited. Emerg Health Threats J. 2011;4(1):1–17.

12. Arellano-Sota C. Biology and control of the vampire bat. Rev Infect Dis. 1988;10(Issue supplement 4):S615–9.

13. Vigilato MAN, Clavijo A, Knobl T, Silva HMT, Cosivi O, Schneider MC, et al. Progress towards eliminating canine rabies: policies and perspectives from Latin America and the Caribbean. Philos Trans R Soc B Biol Sci. 2013 Aug 5;368(1623):20120143.

14. Banyard AC, Davis A, Gilbert AT, Markotter W. Bat rabies. In: Rabies. Elsevier; 2020. p. 231–76.

15. WHO-FAO-OIE. WHO | Zero by 30: the global strategic plan to end human deaths from dog-mediated rabies by 2030. Who. 2018.

16. Banyard AC, Hayman DTS, Freuling CM, Müller T, Fooks AR, Johnson N. Bat Rabies. In: Rabies. 2013. p. 215–67.

17. Moreno J, Baer GM. Experimental rabies in the vampire bat. Am J Trop Med Hyg. 1980;29(2):254–9.

18. Aguilar-Setien A, Loza-Rubio E, Salas-Rojas M, Brisseau N, Cliquet F, Pastoret PP, et al. Salivary excretion of rabies virus by healthy vampire bats. Epidemiol Infect. 2005;133(3):517–22.

19. Carter G, Leffer L. Social Grooming in Bats: Are Vampire Bats Exceptional? Russo D, editor. PLoS One [Internet]. 2015 Oct 7;10(10):e0138430. Available from: https://dx.plos.org/10.1371/journal.pone.0138430

20. Begeman L, GeurtsvanKessel C, Finke S, Freuling CM, Koopmans M, Müller T, et al. Comparative pathogenesis of rabies in bats and carnivores, and implications for spillover to humans. Lancet Infect Dis [Internet]. 2018 Apr;18(4):e147–59. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1473309917305741

21. Rocha F, Dias RA. The common vampire bat Desmodus rotundus (Chiroptera: Phyllostomidae) and the transmission of the rabies virus to livestock: A contact network approach and recommendations for surveillance and control. Prev Vet Med. 2020;174(August 2019):104809.

22. Meske M, Fanelli A, Rocha F, Awada L, Soto PC, Mapitse N, et al. Evolution of Rabies in South America and Inter-Species Dynamics (2009–2018). Trop Med Infect Dis. 2021 Jun 9;6(2):98.

23. Childs, James E., Real LA. Epidemiology. In: Jackson, Alan C., Wunner WH, editor. Rabies. 2nd ed. Elsevier Inc.; 2007.

24. Benavides JA, Rojas Paniagua E, Hampson K, Valderrama W, Streicker DG. Quantifying the burden of vampire bat rabies in Peruvian livestock. PLoS Negl Trop Dis. 2017;11(12):1–17.

25. Linhart SB, Flores Crespo R, Mitchell GC. Control of Vampire bats by topical application of anticoagulant, Chlorophacinone. Bol Of Sanit Panam. 1972;1(2).

26. Streicker DG, Recuenco S, Valderrama W, Benavides JG, Vargas I, Pacheco V, et al. Ecological and anthropogenic drivers of rabies exposure in vampire bats: Implications for transmission and control. Proc R Soc B Biol Sci. 2012;279(1742):3384–92.

27. Aguilar-Setién A, Campos YL, Cruz ET, Kretschmer R, Brochier B, Pastoret PP. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J Wildl Dis. 2002;38(3):539–44.

28. Sétien AA, Brochier B, Tordo N, De Paz O, Desmettre P, Péharpré D, et al. Experimental rabies infection and oral vaccination in vampire bats (Desmodus rotundas). Vaccine. 1998;16(11–12):1122–6.

29. Almeida MF, Martorelli LFA, Aires CC, Sallum PC, Massad E. Indirect oral immunization of captive vampires, Desmodus rotundus. Virus Res. 2005;

30. Almeida MF, Martorelli LFA, Aires CC, Barros RF, Massad E. Vaccinating the vampire bat Desmodus rotundus against rabies. Virus Res. 2008;137(2):275–7.

31. Del Médico Zajac MP, Garanzini D, Pérez OR, Calamante G. Recombinant Veterinary Vaccines Against Rabies: State of Art and Perspectives. Emerg Reemerging Viral Pathog. 2020;225–42.

32. Sidwa TJ, Wilson PJ, Moore GM, Oertli EH, Hicks BN, Rohde RE, et al. Evaluation of oral rabies vaccination programs for control of rabies epizootics in coyotes and gray foxes: 1995-2003. J Am Vet Med Assoc. 2005;227(5):785–92.

33. Maki J, Guiot AL, Aubert M, Brochier B, Cliquet F, Hanlon CA, et al. Oral vaccination of wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): A global review. Vet Res. 2017;48(1):1–26.

34. García-Sastre A, Mena I. Novel vaccine strategies against emerging viruses. Curent Opin Virol. 2013;3:210–6.

35. Cross ML, Buddle BM, Aldwell FE. The potential of oral vaccines for disease control in wildlife species. Vet J. 2007;174(3):472–80.

36. Stading BR, Osorio JE, Velasco-Villa A, Smotherman M, Kingstad-Bakke B, Rocke TE. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox

vectored rabies vaccine in the Brazilian Free-tailed bat (Tadarida brasiliensis). Vaccine. 2016;34(44):5352–8.

37. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.

38. Stading author BR. Development of a novel rabies mosaic antigen and the use of attenuated poxviruses as vaccine vectors in bats. 2016.; 2016.

39. Fernandez JR-R, Rocke TE. Use of Rhodamine B as a Biomarker for Oral Plague Vaccination of Prairie Dogs. J Wildl Dis. 2011 Jul;47(3):765–8.

CHAPTER 2

Clinical presentation and serologic response during a rabies epizootic in captive common vampire bats (*Desmodus rotundus*)

Elsa M. Cárdenas-Canales, Crystal M. Gigante, Lauren Greenberg, Andres Velasco-Villa, James A. Ellison, Panayampalli S. Satheshkumar, Lex G. Medina-Magües, Richard Griesser, Elizabeth Falendysz, Ignacio Amezcua, Jorge E. Osorio, and Tonie E. Rocke

As published in Trop Med Infect Dis. 2020 Mar 1;5(1):34.

doi: 10.3390/tropicalmed5010034

Abstract

We report mortality events in a group of 123 common vampire bats (*Desmodus rotundus*) captured in México and housed for a rabies vaccine efficacy study in Madison, Wisconsin. Bat mortalities occurred in México and Wisconsin, but rabies cases reported herein are only those that occurred after arrival in Madison (n = 15). Bats were confirmed positive for rabies virus (RABV) by the direct fluorescent antibody test. In accordance with previous reports, we observed long incubation periods (more than 100 days), variability in clinical signs prior to death, excretion of virus in saliva, and changes in rabies neutralizing antibody (rVNA) titers post-infection. We observed that the furious form of rabies (aggression, hyper-salivation, and hyper-excitability) manifested in three bats, which has not been reported in vampire bat studies since 1936. RABV was detected in saliva of 5/9 bats, 2–5 days prior to death, but was not detected in four of those bats that had been vaccinated shortly after exposure. Bats from different capture sites were involved in two separate outbreaks, and phylogenetic analysis revealed differences in the glycoprotein gene sequences of RABV isolated from each event, indicating that two different lineages were circulating separately during capture at each site.

Keywords: rabies virus; outbreak; vampire bat; clinical signs; neutralizing antibody

1. Introduction

Rabies is an ancient infectious disease with one of the highest case fatality rates, causing tens of thousands of human fatalities and millions of cattle deaths annually, worldwide [1]. Since the elimination of canine rabies from most of Latin America, the common vampire bat *(Desmodus rotundus*) has become the primary rabies virus (RABV) reservoir. Vampire bat-associated rabies is a major burden to the livestock industry and a significant public health concern across the tropical and subtropical Western Hemisphere [1–4]. Our research groups at University of Wisconsin—Madison (UW) and U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC) have been developing a recombinant rabies vaccine for use in bats [5,6]. The vaccine candidate is composed of raccoon pox (RCN) as a viral vector for a mosaic gene expressing rabies glycoprotein (MoG) and has conferred protection against rabies challenge in laboratory studies in the big brown bat (*Eptesicus fuscus*) when delivered orally and topically [6]. Given the importance of vampire bats in transmitting rabies in Latin America, the next step in our research was to test the efficacy of the RCN–MoG vaccine candidate in D. rotundus. We captured wild vampire bats in the state of San Luis Potosí, located in central México, where rabies is endemic in the southern part of the state [7], and transported them to NWHC in Madison, WI, USA.

The main route of RABV transmission to naive animals is by direct inoculation (e.g., bites) inflicted by a rabid host [1,2]. Vampire bats aggregate in large colonies and are highly social, facilitating RABV transmission to conspecifics [8]. Additionally, the virus itself can drive changes in the behavior of infected animals, allowing for an increased probability of transmission to others. It has been demonstrated that vampire bats excrete RABV in their saliva during late stages of infection and prior to death [9,10]. Previous research has also suggested that

infected bats can shed the virus and later recover from the disease, thus suggesting a carrier state is possible [11]. However, this hypothesis was not supported by later experiments [9]. The natural progression of rabies in the vampire bat and its immune response to RABV has not been described in sufficient detail. During the time of capture and temporary housing in México, and also during transport to and final housing at NWHC, numerous vampire bats died, and RABV was detected in their brains. Here, we describe the course of the infection, presentation of clinical signs, and changes in serologic status of captive vampire bats that succumbed to natural RABV infection during captivity prior to experimental activities.

2. Case Report

2.1. *Vampire Bats and Capture Sites*

A total of 123 common vampire bats were captured from seven sites throughout San Luis Potosí (Figure 1) during a 6-week period in the summer of 2018 (Jul–Aug). The majority of the bats (n = 75) were captured in the two southern capture locations (Loma and Catedral), where rabies had been reported during activities conducted by the Mexican national campaign for prevention and control of rabies in livestock [7,12]. The remaining bats came from the other 5 locations throughout the state.

We captured bats during the night using mist nets placed at cave entrances, natural or man-made roosting areas (e.g., abandoned water well), around livestock corrals, or known flight pathways. We focused on capturing males and did not collect visibly pregnant or lactating females. Sex and age category (juvenile, adult) were judged by development of external reproductive organs and body size. Following field health inspection, bats were housed

temporarily in a room within an animal facility of the Universidad de Matehuala, School of Veterinary Medicine, in Matehuala, San Luis Potosí, until transport to Madison, Wisconsin. The animal room had natural light and ventilation, and humidity was manipulated daily with humidifiers. Bats were grouped by sex and location of origin in mesh cages and were kept on a diet of citrated beef blood and water *ad lib*.

Most bats were held in captivity in México for 4–6 weeks, but the last group captured was held for only 1-week prior to departure. On September 4, 2018, a total of 93 bats (23 females and 70 males) were transported to Madison, Wisconsin, in custom-made cages built in compliance with biosafety and transport guidelines. During the 2-day travel time, bats were fed once with citrated bovine blood at night.

Upon arrival at the NWHC, bats were placed in a biosafety level 3 animal room with controlled temperature (28–34 °C) and humidity (>40%). The bats were combined into new cages of up to 20 animals per cage, but separated by sex and by site of capture, as availability of cages permitted. Husbandry practices remained similar to those in México, and all animal procedures for transport and captivity were approved by the NWHC animal care and use committee under appropriate permits as listed below. After arriving at the NWHC, bats were given an acclimation/quarantine period time of 104 days prior to the experimental vaccination.

2.2. *Rabies Serology*

We obtained an initial (baseline) plasma sample from 88 available bats within days 34–57 after arrival at the NWHC, and periodically thereafter, to determine rabies virus neutralizing antibodies (rVNA). Baseline samples were sent to the Centers for Disease Control and

Prevention (CDC, Atlanta, Georgia) and were tested using the micro-neutralization test, a modified version of the Rapid Fluorescent Focus Inhibition Test (RFFIT). Subsequent samples were tested at the NWHC following the same micro-neutralization protocol [13]. When possible, at death, serum was collected as a terminal sample for serology. Evidence of neutralization in >50% of the fields at a 1:10 dilution of the test serum was considered positive for rVNA (or equivalent to reciprocal titers ≥ 1:13). Intermediate samples were those at the cut off for 50% neutralization (i.e., reciprocal titers of 1:10). International units (IU/mL) were calculated as per the protocol; but, given the inherent variability of the test and the lack of a cut off value for wildlife species, we opted to assess serological response based on reciprocal titers and evidence of neutralization [13,14].

2.3. *Rabies Testing, Typing and Phylogenetic Analysis*

In México, a subset of dead bats was sent to the rabies reference laboratory in the city of San Luis Potosí (SPHL) for rabies diagnosis by direct antigen detection (DFA) following the official Mexican standard for rabies diagnosis [12]. In Wisconsin, all dead bats were sent to the Wisconsin State Laboratory of Hygiene (WSLH, Madison, Wisconsin) where brain impressions were tested using the DFA for detection of nucleoprotein antigen following the national standard protocol [15]. Brain samples tested at the WSHL were later submitted to CDC for molecular diagnostic confirmation and sequencing (Appendix 1). Sequences were deposited in GenBank under accession numbers MN968374-MN968401. Sequence alignments were generated using MAFFT in Geneious (https://www.geneious.com). Sequence alignments were trimmed to include only the coding region of the nucleoprotein and glycoprotein genes. Maximum likelihood phylogenetic analysis implemented in MEGA software 7.0.26 [16] was conducted by

using the GTR+G+I substitution model with 1000 bootstrap replicates to assess the statistical robustness of the tree topology.

We collected oral swabs from bats from Loma after the death of 2 bats from that site occurring within a week of arrival at the NWHC. We sent this set of samples to a commercial laboratory for RABV diagnosis using RT-PCR (Zoologix, Inc., Chatsworth, CA, USA). After collection, saliva samples were placed in vials containing 0.5 mL of viral transport media and stored at −80 °C until further testing. More oral swabs were serially collected from a group of bats involved in a natural outbreak of rabies that peaked in late December. We started swabbing these bats regularly (every 3–5 days) after the outbreak began and for 2 months thereafter. These swabs were tested at the NWHC following the LN34 pan-lyssavirus real-time RT-PCR [17,18], with some modifications to the protocol, such as using 8.5 µL of RNA per reaction and using an iCycler instrument (BioRad, Hercules, CA, USA). Cycle threshold (Ct) values ≤ 35 were considered positive (i.e., RABV RNA present), whereas values 35–40 were considered inconclusive and may indicate a low virus load, insufficient sample, or possible cross contamination. RNA extraction was performed using Direct-zol RNA Miniprep kit (Zymo Research, Irvine, CA, USA).

2.4. *Description of Mortality Events: Suspected and Confirmed for Rabies*

In México, bat mortalities were first recorded during capture at the Loma site on Aug 4–5, 2018 (Figure S1 in Supplementary Materials). A total of 64 bats were captured in one night, and 10 bats died before arrival at the animal facility in Matehuala 2 days later. Three of the 10 bats submitted to the SPHL were RABV positive by DFA, and the rest were negative. Trauma or

stress during capture and transport were suspected as likely causes of death. During Aug 7–25, 13 bats from Loma, 2 from Guadalcázar, and 1 from Catedral were found dead in their cages. An additional three bats from Loma were euthanized with unhealed wounds that impaired mobility. The last mortality event recorded in San Luis Potosí was on Aug 26, a bat from Catedral that died in good body condition without obvious clinical signs (Figure S1 in Supplementary Materials). Although some bats showed signs (e.g., aggressive behavior and bite wounds) suggestive of rabies infection, testing was not performed, and we cannot assume rabies was the cause of death. A total of 30 vampire bats died while in San Luis Potosí.

Just prior to departure, all surviving animals (n = 93) were given a health check and administered subcutaneous fluids to avoid dehydration while in transit. No signs of disease were observed, and all bats were placed in their correspondent cage for transportation.

During transport, one male bat from Loma (#563, caged with 11 other male bats from the same site) was lethargic and did not survive the 48 h drive to Wisconsin; it tested positive for rabies by DFA at WSLH. On Sept 24, 18 days after arrival at NWHC, one of the 11 bats (#565) died and was also positive for RABV (Figure 2). The other ten bats transported in the same cage showed no signs of rabies. Within a few days of arrival at NWHC on Sept 6, 2018, two of the 10 females (#573 and #641) from Loma died and were found to be RABV positive. One of the females (#641) was euthanized due to an unhealed fracture, and the other bat died suddenly. None of the 3 bats from Loma that died showed signs of rabies prior to death. Bats from Loma were kept separate from all other bats, and no other mortalities occurred in either the males or females from Loma during the observation period presented in this case report.

On Dec 6, 2018, increased vocalization, uneasy behavior, and apparent fighting were recorded in a cage that housed 17 males from Catedral, Milagro, and Guadalcázar. Four days

later (day 95 post arrival), Bat #576 (from Catedral) was found dead without prior manifestation of clinical signs and tested positive for RABV (Figure 2). On day 98, bats from this group (n = 16) were split into 2 cages (Cages 4 and 5, Table 1) in preparation for treatment as part of our vaccine study. The next death to occur was Bat #677 from Cage 5 on Dec 30, 2018 (day 115); increased vocalization of the group had been recorded 4 days prior. Bat #677 (from Milagro) showed recent multiple wounds, dehydration, and aggressiveness 2 days before its death. After this death, and because of evidence of aggression within the group, we started collecting oral swabs periodically for RABV testing and bats were examined closely for signs of rabies. One week later (Jan 4, 2019, Day 120) all bats from Cage 5 (n = 10; 5-rVNA negative at baseline, 1-intermediate, 4-positive) were orally vaccinated with RCN-MoG as previously scheduled but also in an attempt to determine if vaccination might stop the outbreak. Bats in Cage 4 (n = 5, all rVNA negative) had received RCN expressing a neutral antigen (luciferase) as a control on Dec 19, 2018.

During Days 121–146, 8 more bats died in Cages 4 and 5 and all tested positive for RABV by DFA. Vocalization was often noticed 3–4 days prior to a new mortality in both México and the United States (Figure 2). Overall, at NWHC 4 bats that succumbed to rabies displayed the paralytic form of the disease: isolation, non-responsiveness, respiratory distress, lethargy, and dull mentation (#584, #676, #601, #679). The furious rabies presentation was observed in three bats only (#677—Milagro, and #605, #610—Guadalcázar), including signs of aggression, hyper-salivation, teeth chattering, irritability to sound and light sources, and excessive vocalization. Eight other bats did not show any clinical signs or evidence of sickness before they were found dead (including #563 that died during transport). In consultation with the

NWHC attending veterinarian, bats showing severe distress from either form of RABV infection were euthanized (n = 5).

RABV nucleic acid was detected in oral swabs from 5 (#677, #605, #584, #610, #676) of the 9 rabies-positive bats sampled 2–5 days prior to their death (Table 1). Bat #582 (Cage 4) suffered bite wounds from its cage-mates on Day 115 (one day before RABV was detected in the saliva of Bat #605), yet it did not become ill. An oral swab obtained from Bat #582 on Day 140 was inconclusive for RABV by real-time RT-PCR; all of its remaining samples were negative. The rest of its cage-mates tested positive for RABV in saliva on that same day and eventually succumbed to rabies (Table 1).

All bats that died at NWHC on Days 95–146 (n = 10) were negative for rVNA at baseline; half of these had rVNA at the time of death (Table 2). None of these bats had contact with any of the bats from Loma during capture, transportation, or housing. Surviving bats from Catedral (except #582 and #579) had detectable rVNA at baseline, and titers increased in 5 of the 7 within 25–32 days after the index case died (Table 2). Of the 6 surviving bats that received RCN-MoG, 4 showed an increase in antibody titer 28 days after vaccination.

Vaccination with RCN-MoG had no impact on survival of seronegative bats in Cage 5 as 4/4 vaccinates (#601, #678, #679, and #604) succumbed to rabies, compared to unvaccinated bats in Cage 4 (4/5). However, RABV nucleic acid was not detected in the saliva of 4 vaccinated bats that succumbed to rabies, unlike the saliva samples from unvaccinated individuals (#605, #584, #610, and #676) that were consistently RABV-positive. Seven bats (all from Catedral, one unvaccinated in Cage 4, and six vaccinated in Cage 5) survived up to 231 days, despite being co-housed with bats shedding RABV in saliva, as confirmed by detection of RABV nucleic acid in oral swabs by real-time RT-PCR (Table 1). For the purposes of this case report, the observation

or survival period lasted 231 days, when all bats were challenged with a heterologous strain of RABV as part of the vaccine study on Apr 25, 2019.

Nucleoprotein (N) and glycoprotein (G) sequences were determined for RABV isolated from brain samples sent to CDC after rabies testing at the WSLH. Phylogenetic analysis revealed that these sequences were consistent with RABV circulating in vampire bats in México. It also revealed two clusters of vampire bat sequences (Figure 3), here called Group 1 and Group 2. Group 1 corresponded to bats from Loma (magenta), which all died shortly after arrival at NWHC. Group 2 corresponded to bats from Catedral, Milagro, and Guadalcázar sites (blue). This lineage showed up at 95 days after arrival at NWHC in a rabid bat (#576) from Catedral. Three Group 1 G sequences were identical, and sample #573 (female) had one change. For Group 1 (n = 4), three sequences had identical N gene sequences and the sample from Bat #641 (female) had one nucleotide change. In Group 2 (n = 10), all G gene sequences were identical, and the N gene sequence was identical for 9/10 samples; one nucleotide change was observed in the sample from Bat #610. Between Groups 1 and 2, there were 4–5 changes throughout the entire coding region of the G gene nucleotide sequence and 2–4 changes in the N gene sequences. These results demonstrate a single lineage introduction likely caused by Bat #576 from Catedral. RABV sequences from subsequent rabid bats co-housed with #576 present the same viral lineage, and all formed a distinctive lineage from those obtained from rabid bats from Loma (Figure 3).

3. Discussion

In this report, we describe two separate rabies mortality events that occurred in a group of common vampire bats captured in Central México and transported to NWHC as part of a study to test the efficacy of a recombinant rabies vaccine candidate. We identified mortality events as independent introductions of RABV lineages from bats collected at Loma and Catedral. This was possible by integrating information obtained from a phylogenetic analysis and a detailed mortality timeline, which traced contacts among bats (co-housing history) and potential incubation periods between cases.

Lengthy incubation periods are known to occur after RABV exposure in vampire bats and other species of bats that usually undergo torpor in temperate climates [7,10,19,20]. We observed variable incubation lengths between exposure and rabies confirmation in our captive vampire bats (18 to more than 100 days post primary exposure). Bat #576 (from Catedral), the index case in the rabies mortality event at NWHC, was found to be RABV positive more than three months after bats from Catedral, Milagro, and Guadalcázar were merged into a single cage upon arrival at NWHC. We suspect that the index case or possibly one of the other bats was infected at capture or was exposed to RABV during captivity in México (100–130 days prior to the death of the index case). We also observed shorter incubation periods (18–26 days), similar to those observed in other RABV challenge studies in vampire bats [9,10]. Secondary transmission likely occurred in the event, as we observed bats shedding RABV (e.g., #677 and #605) followed by additional rabies mortalities 20–33 days later (Table 1). We have estimated incubation periods based on the timing between observed events (e.g., mortalities, RABV excretion in saliva samples, and presence of wounds) and exposure to other rabid bats; however, we recognize the lack of sufficient evidence to determine actual incubation periods.

Catedral and Loma sites are located in the southern (rabies endemic) region of San Luis Potosí, whereas Milagro and Guadalcázar are located farther north in areas without or with scarce reports of rabies cases. We observed differences in both survival and clinical presentation after RABV infection between bats from sites considered "rabies-free" and endemic sites. None of the known exposed bats from Guadalcázar and Milagro survived infection, whereas 18/22 Loma bats (males and females combined that arrived at NWHC) and 7/9 from Catedral bats survived co-housing with rabid bats. All bats that died from Guadalcázar and Milagro (n = 8) showed clinical signs (including three furious), whereas bats from endemic sites died without any visible clinical signs. One of the three deaths from Loma (one euthanized female with unhealed wounds) was not suspected of RABV infection and was considered an incidental death. It was somewhat surprising to us that it was positive for RABV by DFA.

The presence of clinical signs in experimentally infected vampire bats is not always observed [21–24]. In a rabies study with vampire bats, only 47% of the subjects that succumbed to the challenge with different doses of RABV isolated from a cow [21] showed clinical signs, including ataxia, tremors, and paralysis. Mortality and the presence of clinical signs can be dose-dependent [9,19]. The furious presentation of RABV infection in vampire bats has been described in experimental studies in the past [11] but has not been reported in more recent experiments [10,21–24]. We recorded the aggressive form of the disease (furious) in 3/15 rabies-positive cases in our vampire bats, all from the "rabies-free" sites, but most of the RABV-positive bats had the paralytic form of the disease. Moreover, we detected the presence of RABV nucleic acid in saliva samples of 5/9 bats that succumbed to rabies during the outbreak. Salivary excretion of RABV coincided with the presence of clinical signs. The three bats that presented the furious form of rabies (#677, #605, #610) tested positive for RABV nucleic acid 3–5 days

before death in at least one sample tested. We recorded aggressions (i.e., bite wounds) within the groups during days in which salivary excretion of RABV from individuals was confirmed. The presence of vocalization 3–4 days before a death record may indicate a behavioral response from the group (e.g., to avoid or expel a rabid bat from the group). With our limited observations and number of samples, we can only speculate about the rabies transmission history of the bats involved in these mortality events.

Antibody response to RABV and other immune mechanisms (e.g., cell mediated immune response) that drive survival after rabies infection are poorly understood in bats [2,25]. Notably, individuals with pre-existing rVNA did not succumb to rabies infection, although they had known multiple exposures to rabid bats while in captivity. We detected an increased rVNA response in Catedral bats that survived (5/7) approximately 25 days after the start of the second mortality event, which likely represents an anamnestic response [19,21]. Despite being seronegative at baseline, Bat Bat #582 (Catedral) seemed to have an abortive RABV infection that apparently elicited rVNA ~25 days after the first case of rabies occurred that was detected again on day 137 post-outbreak. Conversely, Bat #579 (Cage 5) was negative for rVNA at baseline, and it remained seronegative after vaccination with RCN–MoG and challenge with a heterologous RABV as part of the vaccine study (data not shown). None of the vampire bats that survived showed any indication of disease at any point during the rest of the time in captivity, even those with evident aggression from cage mates during the peak of the outbreak. Previous experiments also report survival of vampire bats after infection with a homologous RABV without the presence of rVNA [22,23], suggesting that some individuals may clear peripheral rabies virus infection by cell-mediated mechanisms [22,26]. Sub-lethal, or abortive, RABV

infections have been proposed as a potential explanation for the resistance of bats to rabies, as well as for the sporadic presence of rVNA [19,25–27].

As part of our scheduled vaccine study, and in an attempt to halt the ongoing rabies epizootic, we vaccinated bats (n = 10) from Cage 5 on Jan 4, 2019. These bats were collected from the Catedral, Guadalcázar, and Milagro locations, and some had detectable rVNA prior in their initial sample (Table 2). Other investigators have reported that the interaction between a vaccine-induced humoral immune response (neutralizing antibodies) and RABV infection may result in an "early death" phenomenon (i.e., shorter incubation period in vaccinated individuals after infection) [28]. However, this response may also prolong the time to onset of disease [28,29]. A study of foxes vaccinated with a recombinant rabies vaccine after infection with a homologous RABV illustrates these phenomena [29]. Subjects vaccinated early (0 and 3 days post-inoculation, p.i.) had shorter incubation periods than those receiving the vaccine later (14 days p.i.) with all succumbing to the rabies challenge. The range of time to death after vaccination (on Day 120) of our vampire bats was 13–26 days, suggesting an "early death" effect. These bats had been housed with Bat #677 who died on Day 115; RABV was detected in its saliva two days before death. Although our numbers are small for a definitive conclusion, post-exposure vaccination with RCN–MoG did not appear to improve survival. The central nervous system (CNS) of vaccinated animals that died may have already been compromised with RABV infection. Thus, any immune response elicited, regardless of its intensity, would be futile to clear RABV from the CNS, as observed in most rabies cases in mammals [30]. However, vaccination appeared to be associated with suppression of RABV shedding in saliva (Tables 1 and 2). Future research should focus on corroborating this observation since vaccine-induced

suppression of viral shedding in individuals already infected with RABV would help to disrupt the rabies transmission cycle in bat populations.

The genetic differentiation of two RABV lineages circulating within a single State in México was surprising; particularly, considering the close geographic distance between Loma and Catedral (~7 miles). It is likely that the RABV lineage identified in the index case from Catedral (#576) had been recently introduced from a more distant site, since a spillover of such lineage was not observed in bats captured in Loma. Our results indicate a complex dissemination dynamic of RABV associated with vampire bats in San Luis Potosí. More robust sampling is encouraged to further understand these dynamics in wild populations of bats.

The natural transmission dynamics of rabies in vampire bats and their serologic response after exposure to RABV is not entirely understood [2] but certainly has implications for the design and outcome of vaccine efficacy and challenge experiments, in addition to wildlife management strategies. In our captive colony of vampire bats, baseline serology (the absence of rVNA) was a criterion for allocating bats into treatment groups. However, it is possible their rVNA were below detectable limits of the micro-neutralization test, and thus we cannot be certain of their prior exposure history to rabies. The occurrence of a natural rabies epizootic likely caused changes in the immune status of our bats, not to mention the loss of experimental animals. In retrospect, we should have kept bats quarantined for longer periods of time (≥4 months) but given the cost of housing vampire bats in a biosafety level 3 facility as required by 42 CFR § 71.54, that is a difficult proposition. Fortunately, the mortality events at NWHC were confined to bats co-housed in three cages and did not spread to the three other cages in the same room. Our report reveals some of the intricacies of the natural progression of RABV infection in vampire bats and support previous reports on variable incubation periods, presentation of clinical

signs, occurrence of abortive infections linked to production of rVNA, and survival after exposure to RABV with no imminent presence of rVNA. In addition, our observations raise questions regarding underlying mechanisms of viral clearance and survivorship of vampire bats to RABV infection.

4. Ethical statement

Capture of wild vampire bats was conducted under a permit from the Mexican Secretariat of Environment and Natural Resources (SEMARNAT, permit number: SGPA/DGVS/003242/18). Permits for exporting bats from México and importing them into the United States were issued by SEMARNAT (permit number 44333), the Centers for Disease Control and Prevention (CDC, permit number 2018-04-108), and the Division of Wildlife for the Wisconsin Department of Agriculture, Trade, and Consumer Protection (permit number 356MO7168-BPI). All protocols for capture, husbandry, and transport of vampire bats were in compliance with USGS—National Wildlife Health Center Institutional Animal Care and Use Committee (protocol number EP180418).

Table 1. Rabies mortality during outbreak in captive vampire bats by capture site in México (Guadalcázar—GUAD; Catedral—CAT; Milagro—MIL). Bats were placed together in a single cage on Day 0 and then split into 2 cages on Day 98 (calendar date indicated above). Oral swabs collected periodically were tested for rabies virus by real-time RT-PCR (Pos = PCR positive, Neg = PCR negative; Inc = inconclusive). Bats that died and were confirmed rabies positive by DFA are indicated (M) on day of death. Clinical signs present at time of death (F = furious, P = paralytic, NS = no clinical signs, - = did not die).

Day after Arrival at National Wildlife Health Center PCR Result on Saliva and Rabies Mortality Confirmed by DFA																
Bat ID	**T Site**	**12/10 95**	**12/13 98**	**12/28 113**	**12/30 115**	**1/2 118**	**1/4 120**	**1/5 121**	**1/7 123**	**1/9 125**	**1/17 133**	**1/24 140**	**1/26 142**	**1/29 145**	**1/30 146**	**- Death**
576	CAT	M	-	-	-	-	-	-	-	-	-	-	-	-	-	NS
605	GUAD	-		-	-	Pos	-	M, Pos	-	-	-	-	-	-	-	F
584	CAT	-		-	-	Neg	-	-	Neg	-	Neg	Pos	M	-	-	P
610	GUAD	-	To Cage 4	-	-	Neg	-	-	Neg	-	Neg	Pos	-	M	-	F
676	MIL	-		-	-	Neg	-	-	Neg	-	Neg	Pos	-	M, Pos	-	P
582	CAT	-		-	-	Neg	-	-	Neg	-	Neg	Inc	-	Neg	-	-
677	MIL	-		Pos	M	-	-	-	-	-	-	-	-	-	-	F
601†	GUAD	-		-	-	-	Neg	-	Neg	Neg	M	-	-	-	-	P
678†	MIL	-		-	-	-	Neg	-	-	Inc	Neg	M	-	-	-	NS
679	MIL	-		-	-	-	Inc	-	-	-	-	-	-	M, Neg	-	P
604	GUAD	-		-	-	-	Neg	-	-	-	-	-	-	Neg	M	NS
579	CAT	-	To Cage 5‡	-	-	-	Neg	-	-	-	-	-	-	Neg	-	-
583	CAT	-		-	-	-	Neg	-	-	-	-	-	-	Neg	-	-
575‡	CAT	-		-	-	-	-	-	Neg	Neg	Neg	Neg	-	Neg	-	-
585†	CAT	-		-	-	-	Neg	-	Neg	Neg	Neg	Neg	-	Neg	-	-
580	CAT	-		-	-	-	Neg	-	-	-	-	-	-	Neg	-	-
581	CAT	-		-	-	-	Neg	-	-	-	-	-	-	Neg	-	-

† Four bats with signs of bites were moved to a separate cage for closer observation on Day 116.

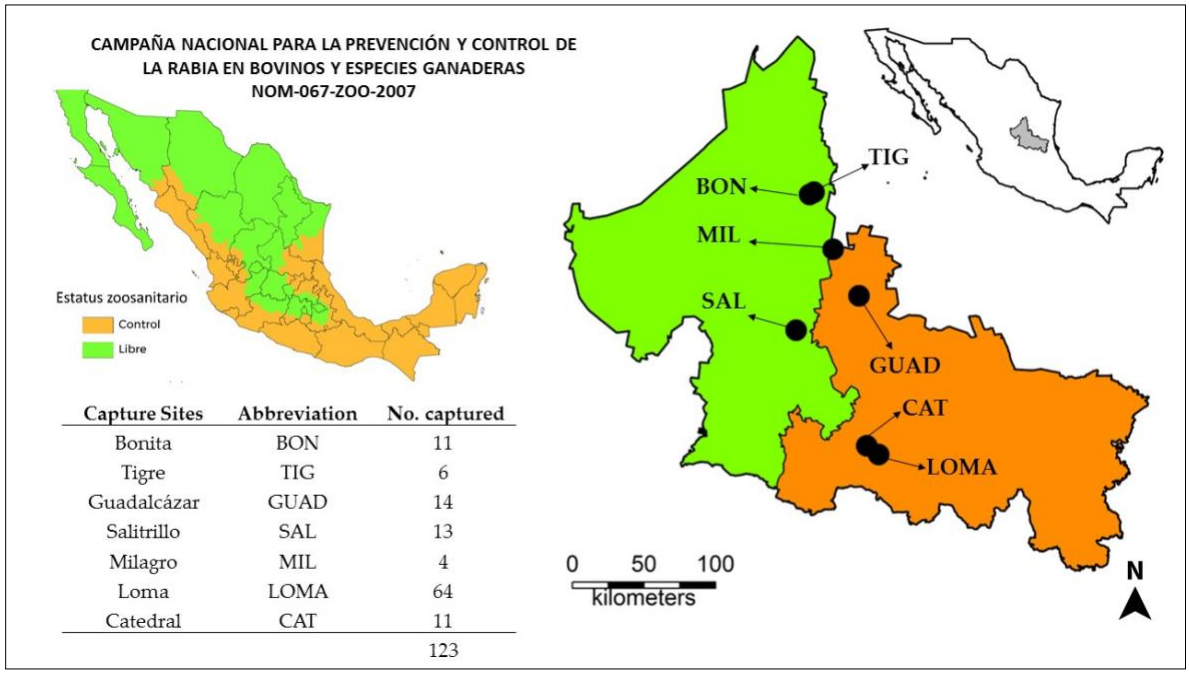
‡ Ten bats in Cage 5 were vaccinated against rabies with a raccoon poxvirus expressing rabies glycoprotein (RCN-MoG) on Day 120.

Table 2. Antibody titers of vampire bats collected from sites in México (Guadalcázar—GUAD; Catedral—CAT; Milagro—MIL) involved in natural rabies outbreak at baseline, post-outbreak (pre-vaccination; Days 119–127), post-vaccination (Day 148), and terminal for those bats that died of rabies during the outbreak. Treatments included RCN-expressing mosaic glycoprotein (RCN–MoG), a rabies vaccine candidate, and RCN-luciferase (*luc*), a placebo. Age group is indicated as A = adult, J = juvenile.

Bat ID	Capture Site	Age	Treatment	Baseline	Post-Outbreak	Post-Vaccination	Terminal	Outcome
576	CAT	A	-	<1:10	-	-	<1:10	-
605	GUAD	J	RCN-l*uc*	<1:10	-	-	1:1635	-
584	CAT	A	RCN-*luc*	<1:10	<1:10	-	<1:10	-
610	GUAD	A	RCN-*luc*	<1:10	<1:10	-	<1:10	-
676	MIL	J	RCN-*luc*	<1:10	<1:10	-	1:102	-
582	CAT	A	RCN-*luc*	<1:10	1:158	1:611[§]	-	survived
677	MIL	A	-	<1:10	-	-	1:3418	-
601	GUAD	J	RCN-MoG	<1:10	<1:10	-	<1:10	-
678	MIL	J	RCN-MoG	<1:10	<1:10	-	1:38	-
679	MIL	A	RCN-MoG	<1:10	<1:10	-	<1:10	-
604	GUAD	A	RCN-MoG	<1:10	<1:10	-	1:559	-
579	CAT	J	RCN-MoG	<1:10	<1:10	<1:10	-	survived
583	CAT	A	RCN-MoG	1:10	<1:10	1:49494	-	survived
575	CAT	A	RCN-MoG	1:22	1:3418	1:13975	-	survived
585	CAT	A	RCN-MoG	1:22	1:27	1:112	-	survived
580	CAT	A	RCN-MoG	1:18	1:3418	1:2286	-	survived
581	CAT	J	RCN-MoG	1:22	1:559	1:2287	-	survived

[§] #582 was bled on Day 177.

Figure 1. Location of seven capture sites in the State of San Luis Potosí in relation to areas recognized as free (green) or under control (orange) for rabies in livestock species according to the Mexican National Service for Agrifood Health, Safety and Quality (SENASICA) in 2018.



Capture Sites	Abbreviation	No. captured
Bonita	BON	11
Tigre	TIG	6
Guadalcázar	GUAD	14
Salitrillo	SAL	13
Milagro	MIL	4
Loma	LOMA	64
Catedral	CAT	11
		123

Figure 2. Timeline of vampire bat observations and mortalities recorded in Madison, Wisconsin, from September 2018 to January 2019. All bats were captured in México in August and transported to Madison on September 4–6. Bats from Groups 1 (A) and 2 (B) were never in contact since capture and presented two distinct RABV lineages based on phylogenetic analysis of N and G gene sequences. Bats in Group 1 with "(f)" are females and were never in contact with males from the same site. Sites of capture are color coded: Loma (blue), Catedral (gray), Guadalcázar (green), and Milagro (orange). Group 2 bats moved to Cage 4 after the first rabies mortality in that group (#576) are indicated with a circle and those moved to Cage 5 with a triangle. Bold/underline lettering indicates bats that were vaccinated. "V+" indicates RABV shedding detected in oral swabs prior to mortality.

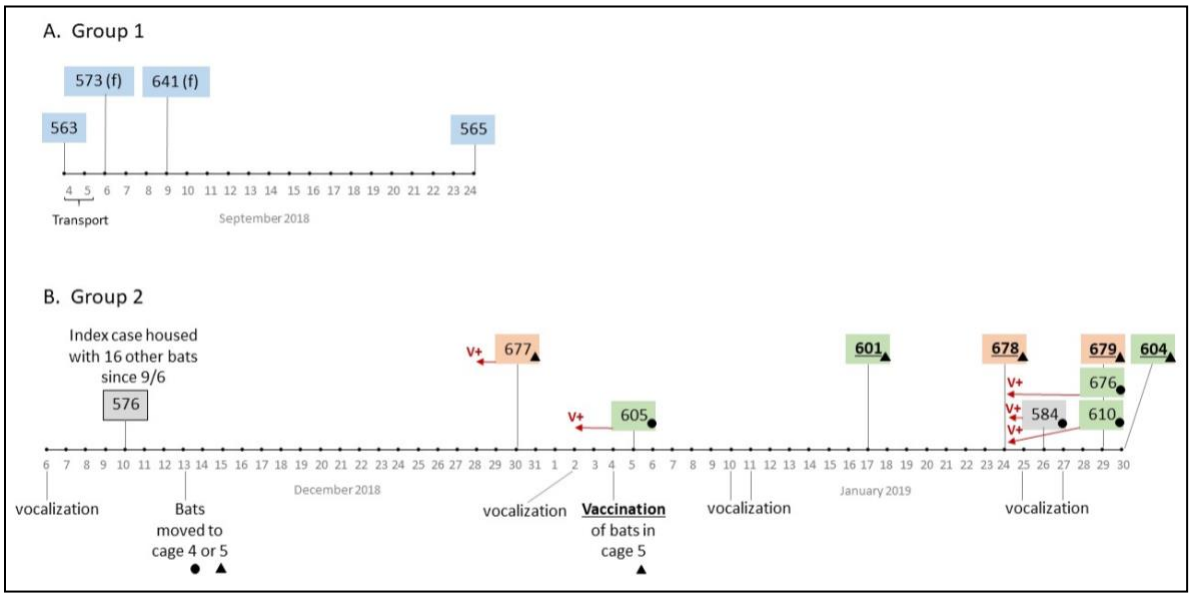
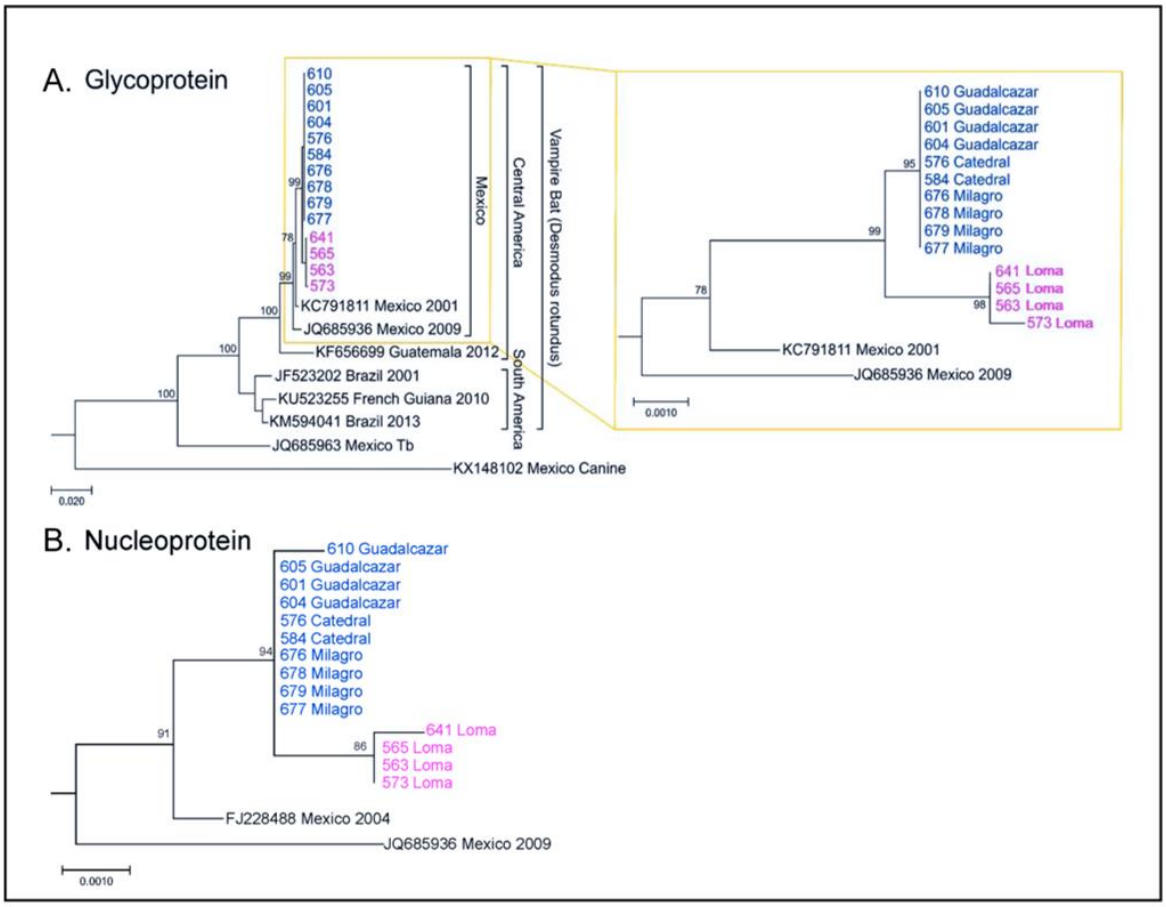
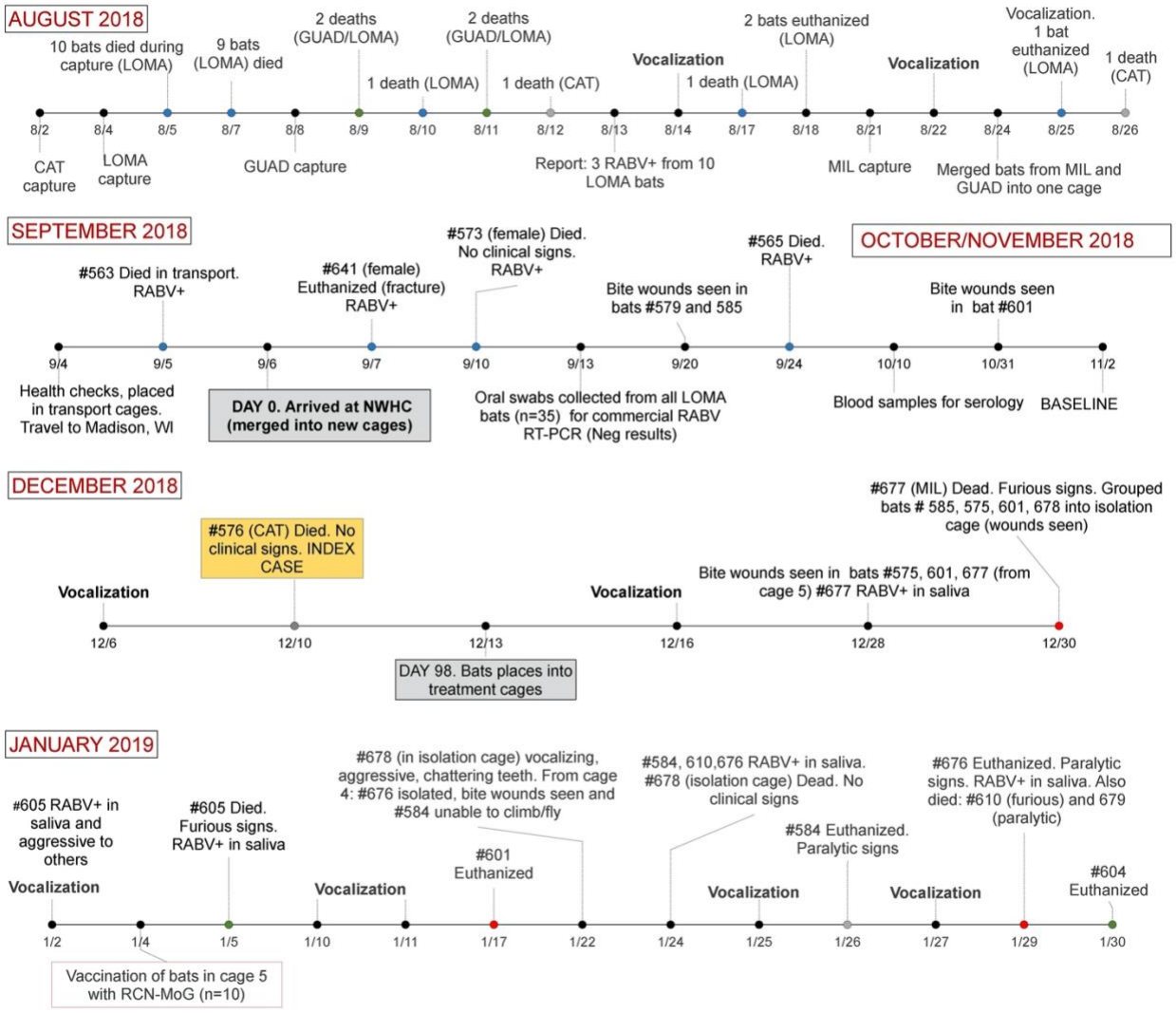


Figure 3. Phylogenetic analysis of glycoprotein (A) and nucleoprotein (B) nucleotide sequences. Phylogenetic trees were estimated by Maximum Likelihood in Mega7. Newly generated sequences from Mexican Desmodus rotundus (magenta, Group1 and blue, Group 2) clustered with isolates of RABV circulating in Desmodus rotundus in Mexico (A, left). The yellow box highlighted on the glycoprotein gene tree is enlarged on the right to show more detail. Numbers at branch points indicate bootstrap value (out of 1000 replicates). Scale bars indicate number of changes per site. Reference sequences from RABV strains circulating in vampire bats in México, Guatemala, Brazil, and French Guiana were included; references from RABV isolated from a Mexican free-tailed bat (*Tadarida brasiliensis)* and a dog (historical) were included as outgroups. Reference sequences are labeled with accession number, country, and year of isolation.



Supplemental figure S1. Timeline of observations and mortalities recorded in San Luis Potosí and Madison, Wisconsin, from August 2018 to January 2019. Sites are indicated CAT = Catedral (gray), LOMA = Loma (blue), GUAD = Guadalcázar (green), MIL = Milagro (red).



Supplemental information

Sequencing and Analysis (by Crystal M. Gigante)

RNA was extracted from rabies-positive brain samples using the Directzol miniprep kit (Zymo). cDNA was generated using random hexamer oligonucleotides and AMV reverse transcriptase (Roche, Sigma-Aldrich, St. Louis, MO, USA). Complete nucleoprotein and glycoprotein coding sequences were amplified using Takara long amplicon taq polymerase (Takara Bio USA, Mountain View, CA, USA) Samples were multiplexed using a 96 barcoding PCR kit (Oxford Nanopore Technologies, Oxford, United Kingdom) and Takara long amplicon TaqLibrary preparation was performed following instructions for Oxford Nanopore Ligation Sequencing Kit 1D (SQK-LSK108). Sequencing was performed overnight on a MinION sequencer using MIN107 R9 SpotON flow cells. Basecalling and demultiplexing was performed using Albacore version 2.3.0 Sequences were aligned to reference rabies virus genomes using bwa mem –x ont2d. Consensus sequences were generated from sam mapping files in CLC genomics workbench version 11 using a minimum threshold of 50× coverage. Consensus sequences were corrected using Nanopolish version 0.10.2 (https://github.com/jts/nanopolish/). The three single nucleotide differences within Group 1 or Group 2 were confirmed by Sanger sequencing.

Single nucleotide insertions and deletions were manually corrected using the following method. Nucleoprotein and glycoprotein coding regions of consensus and polished consensus sequences were aligned to rabies virus reference sequences using mafft v7.308 [31,32] in Geneious 9.1.4 (Biomatters, Inc., Newark, NJ, USA). Alignments were manually inspected for indels relative to the reference sequence. If an indel occurred in only the consensus or the polished corrected sequence, the sequence that had no indel relative to the reference was used. For insertions in a homopolymer, one of the homopolymer bases was removed. For a deletion in a homopolymer,

the base to insert was determined by the homopolymer present in the consensus sequence. In cases where indels were detected between two neighboring homopolymers, an ambiguous base was used. When a non-homopolymer nucleotide difference was observed between the consensus and the polished consensus, the nucleotide in the polished consensus was chosen.

Acknowledgments

We thank the School of Veterinary Medicine of the Universidad de Matehuala for providing the facility for temporary housing of the bats and the students: Yereida Ortíz; Mariana Amezcua; Israel Sandoval; Abigail Reyna; Luis Jasso; Samantha Amador; Obed Juárez for their assistance during captures and care of captive bats in México. We also thank staff at NWHC (Susan Smith; Judy Williamson; Rachel Abbot; Carly Malavé) and at University of Wisconsin-Madison (Jaime Lopera) for their help in sampling bats in Madison, Wisconsin and Michael Niezgoda at Centers for Disease Control for assistance with serology. Richard Carson donated material for the transport cages and Mat Bohne helped with design and construction. We thank Governmental agencies in México: Servicio Nacional de Sanidad, Inocuidad, y Calidad Agroalimentaria (SENASICA), Comité Estatal para el Fomento y Protección Pecuaria de San Luis Potosí, Secretaría de Desarrollo Agropecuario y de Recursos Hidráulicos de San Luis Potosí, Centro Nacional de Programas Preventivos y de Control de Enfermedades, and USDA APHIS-IS México for their support to obtain permits and with logistics during fieldwork in México. John Grider (NWHC); Baltazar Cortés and Miriam López (SENASICA) provided the maps for Figure 1.

Funding

This research was funded by the U.S. Geological Survey, American Association of Zoo Veterinarians (UW award number MSN209345), a Graduate Student Research Award from the University of Wisconsin—Madison Global Health Institute, a University of Wisconsin International Division IRIS Incubator Grant for Interdisciplinary Research in International Studies, and regular operations and the Advanced Molecular Detection Program of the Centers for Disease Control and Prevention (CDC). C.M.G. was supported in part by the Oak Ridge Institute for Science and Education through an interagency agreement between the U.S. Department of Energy and CDC.

Author contributions

Conceptualization, E.M.C.C., J.E.O., and T.E.R.; methodology, All; formal analysis, E.M.C.C., J.E.O., T.E.R.; investigation, All; resources, J.E.O., T.E.R., P.S., R.G.; data curation, E.M.C.C., C.M.G., T.E.R.; writing—original draft preparation, E.M.C.C., J.E.O., T.E.R.; writing—review and editing, All.; visualization, E.M.C.C., C.M.G., T.E.R.; supervision, J.E.O., P.S., T.E.R.; project administration, J.E.O., T.E.R.; funding acquisition, J.E.O., T.E.R.

References

1. Beltz, L.B. Rabies viruses and other bat Rhabdoviruses. In Bats and Human Health: Ebola, SARS, Rabies and Beyond, 1st ed.; John Wiley and Sons Inc.: Hoboken, NJ, USA, 2018; pp. 27–49.

2. Fisher, C.; Streicker, D.; Schnell, M. The spread and evolution of rabies virus: Conquering new frontiers. Nat Rev Microbiol. 2018, 16, 241–255, doi:10.1038/nrmicro.2018.11.

3. Johnson, N.; Aréchiga-Ceballos, N.; Aguilar-Setién, A. Vampire bat rabies: Ecology, epidemiology and control. Viruses 2014, 6, 1911–1928.

4. Banyard, A.C.; Hayman, D.; Johnson, N.; McElhinney, L.; Fooks, A.R. Bats and Lyssaviruses. Adv. Virus Res. 2011, 79, 239–289, doi:10.1016/B978-0-12-387040-7.00012-3.

5. Stading, B.; Osorio, J.; Velasco-Villa, A.; Smotherman, M.; Kingstad, B.; Rocke, T. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox vectored rabies vaccine in the Brazilian Free-tailed bat (*Tadarida brasiliensis*). Vaccine 2016, 34, doi:10.1016/j.vaccine.2016.08.088.

6. Stading, B.; Ellison, J.A.; Carson, W.C.; Panayampalli, S.S.; Rocke, T.E.; Osorio, J.E. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS. Negl. Trop. Dis. 2017, 11, e0005958.

7. Servicio Nacional de Sanidad, Inocuidad, y Calidad Agroalimentaria (SENASICA). Current Rabies Situation in Livestock. Available online: https://www.gob.mx/senasica/documentos/situacion-actual-de-la-rabia-en-bovinos-y-especies-ganaderas URL (accessed on 29 December 2019).

8. Wilkinson, G.S. Social grooming in the common vampire bat, Desmodus Rotundus. Anim. Behav. 1986, 34, 1880–1889.

9. Moreno, J.; Baer, G.M. Experimental rabies in the vampire bat. Am. J. Trop. Med. Hyg. 1980, 29, 254–259.

10. Aguilar-Setién, A.; Loza-Rubio, E.; Salas-Rojas, M.; Brisseau, N.; Cliquet, F.; Pastoret, P.P.; Rojas-Dotor, S.; Tesoro, E.; Kretschmer, R. Salivary excretion of rabies virus in healthy vampire bats. Epidemiol. Infect. 2005, 133, 517–522.

11. Pawan, J.L. The transmission of paralytic rabies in Trinidad by the vampire bat (Desmodus rotundus murinus Wagner 1840). Ann. Trop. Med. Parasitol. 1936, 30, 101–129.

12. Norma Oficial Mexicana, NOM-067-ZOO-2007. Campaña nacional para el control y la prevención de la rabia en bovinos y especies ganaderas. Diario Oficial de la Federación. [Official Mexican Standard 067-ZOO-2007, National campaign for the prevention and control of rabies in cattle and livestock species. Secretariat of the Interior]. Retrieved from http://www.

https://www.gob.mx/cms/uploads/attachment/file/203509/NOM-067-ZOO-2207_20may11_Ori.pdf

13. Smith, T.J.; Gilbert, A.T. Comparison of a Micro-Neutralization Test with the Rapid Fluorescent Focus Inhibition Test for Measuring Rabies Virus Neutralizing Antibodies. Trop. Med. Infect. Dis. 2017, 2, 24, doi:10.3390/tropicalmed2030024.

14. Moore, S.M.; Gilbert, A.; Vos, A.; Freuling, M.; Ellis, C.; Kliemt, J.; Müller, T. Rabies Virus Antibodies from Oral Vaccination as a Correlate of Protection against Lethal Infection in Wildlife. Trop. Med. Infect. Dis. 2017, 2, 31, doi:10.3390/tropicalmed2030031.

15. Centers for Disease Control and Prevention. Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing; A Minimum Standard for Rabies Diagnosis in the United States: 2011. Atlanta, Georgia.

16. Gadagkar, S.R.; Kumar, S. Maximum likelihood outperforms maximum parsimony even when evolutionary rates are heterotachous. Mol. Biol. Evol. 2005, 22, 2139–2141.

17. Wadhwa, A.; Wilkins, K.; Gao, J.; Condori, R.E.C.; Gigante, C.M.; Zhao, H.; Ma, X.; Ellison, J.A.; Greenberg, L.; Velasco-Villa, A.; et al. A pan-lyssavirus Taqman real-time RT-PCR assay for the detection of highly variable rabies virus and other lyssaviruses. PLoS. Negl. Trop. Dis. 2017, 11, e0005258, doi:10.1371/journal.pntd.0005258.

18. Gigante, C.M.; Dettinger, L.; Powell, J.W.; Seiders. M.; Condori, R.E.C.; Griesser, R.; Okogi, K.; Carlos, M.; Pesko, K.; Breckenridge, M.; et al. Multi-site evaluation of the LN34 pan-lyssavirus real-time RT-PCR assay for post-mortem rabies diagnostics. PLoS ONE 2018, 13, e0197074, doi:10.1371/journal.pone.0197074.

19. Turmelle, A.S.; Jackson, F.R.; Green, D.; McCracken, G.F.; Rupprecht, C.E. Host immunity to repeated rabies virus infection in big brown bats. J. Gen. Virol. 2010, 91, 2360–2366, doi:10.1099/vir.0.020073-0.

20. Bell, J.F. Latency and abortive rabies. In The Natural History of Rabies, 2nd ed.; Baer, G.M., Ed.; Academic Press, Inc.: New York, NY, USA, 1975; pp. 331–354.

21. Aguilar-Sétien, A.; Brochier, B.; Tordo, N.; De Paz, O.; Desmettre, P.; Péharpé, D.; Pastoret, P.P. Experimental rabies infection and oral vaccination in vampire bats (Desmodus rotundus). Vaccine 1998, 16, 1122–1126.

22. Aguilar-Setién, A.; Campos, Y.L.; Cruz, E.T.; Kretschmer, R.; Brochier, B.; Pastoret, P.P. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J. Wildl. Dis. 2002, 38, 539–544.

23. Almeida, M.F.; Martorelli, L.F.A.; Aires, C.C.; Sallum, P.C.; Dungun, E.L.; Massad, E. Experimental rabies infection in haematophagus bats *Desmodus rotundus*. Epidemiol. Infect. 2005, 133, 523–527.

24. Almeida, M.F.; Martorelli, L.F.; Aires, C.C.; Barros, R.F.; Massad, E. Vaccinating the vampire bat Desmodus rotundus against rabies. Virus Res. 2008, 137, 275–277.

25. Johnson, N.; Cunningham, A.F.; Fooks, A.R. The immune response to rabies virus infection and vaccination. Vaccine 2010, 28, 3896–3901.

26. Moore, S.M.; Wilkerson, M.J.; Davis, R.D.; Wyatt, C.R.; Briggs, D.J. Detection of cellular immunity to rabies antigens in human vaccinees. J. Clin. Immunol. 2006, 26, 533–545.

27. Katz, I.S.S.; Fuoco, N.L.; Chaves, L.B.; Rodrigues, A.C.; Ribeiro, O.G.; Scheffer, K.C.; Asano, K.M. Delayed progression of rabies transmitted by a vampire bat. Arch. Virol. 2016, 161, 2561–2566, doi:10.1007/s00705-016-2927-3.

28. Prabhakar, B.; Nathanson, N. Acute rabies death mediated by antibody. Nature 1981, 290, 590–591, doi:10.1038/290590a0.

29. Brochier, M.; Blancou, J.; Aubert, M.F.A.; Kieny, M.P.; Desmettre, P.; Pastoret, P.P. Interaction between Rabies Infection and Oral Administration of Vaccinia-Rabies Recombinant Virus to Foxes (Vulpes vulpes). J. Gen. Virol. 1989, 70, 1601–1604.

30. George, D.B.; Webb, C.T.; Farnsworth, M.L.; O'Shea, T.J.; Bowen, R.A.; Smith, D.L.; Stanley, T.R.; Ellison, L.E.; Rupprecht, C.E. Host and viral ecology determine bat rabies seasonality and maintenance. Proc. Natl. Acad. Sci. USA 2011, 108, 10208–10213, doi:10.1073/pnas.1010875108.

31. Katoh, K.; Misawa, K.; Kuma, K.; Miyata, K. MAFFT: A novel sequence alignment based on fast Fourier transform. Nucleic Acid. Res. 2002, 15, 3059–3066.

32. Katoh, K.; Standley, D.M. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol. Biol. Evol. 2013, 30, 772–780.

CHAPTER 3

Viral-vectored mosaic rabies vaccine increases survival after challenge and prevents viral shedding in rabid common vampire bats (*Desmodus rotundus*)

Elsa M. Cárdenas-Canales, Andrés Velasco-Villa, James A. Ellison, Panayampalli S. Satheshkumar, Jorge E. Osorio, Tonie E. Rocke

Manuscript under internal revision

Abstract

Vampire bat transmitted rabies (VBR) is a continuing burden to public health and agricultural sectors in Latin American countries, despite decades-long efforts to control the disease by culling bat populations. Culling has been shown to disperse bats in some situations, leading to increased spread of rabies, and thus, novel, non-lethal strategies to control VBR, such as vaccination, are desired. With this objective in mind, we tested the efficacy of a viral-vectored recombinant mosaic glycoprotein rabies vaccine candidate (RCN-MoG) in vampire bats (*Desmodus rotundus*) of unknown history of rabies exposure, captured in México and transported to a biosafety level 3 animal facility in the U.S. Vaccination with RCN-MoG protected vampire bats from rabies virus (RABV) challenge. The survival rate of 37 vaccinated adult bats (92%), seronegative at baseline, was significantly higher ($P=0.016$) than 27 seronegative adult controls (70%) and eight pups (1 – 2.5 months of age) used as naïve controls that all succumbed to challenge ($P<0.0001$). Importantly, we also found that vaccination with RCN-MoG hindered viral shedding, even when infection proved lethal. Using real-time PCR, we did not detect RABV nucleic acid in the saliva samples of 9 vaccinates that succumbed to rabies after challenge. In contrast, RABV nucleic acid was detected in 71% of unvaccinated bats (10/14) that died of the disease. A vaccine candidate that both lowers mortality from rabies and blocks RABV transmission by vampire bats that succumb to the disease provides an advantageous strategy for controlling VBR in Latin America beyond longstanding culling programs.

Keywords: rabies, vaccination, viral shedding, vampire bat

Introduction

Few diseases have haunted humanity as rabies has done for millennia[1,2]. Rabies is a fatal, zoonotic disease, caused by the rabies virus (RABV), an RNA-negative strand virus of the Lyssavirus family, capable of infecting a wide range of mammalian hosts [3]. Since early studies on rabies, numerous scientific advances have contributed to the control and prevention of this disease [1]. The most prominent is the development of rabies vaccines (for humans and wild and domestic animals). Examples of the effectiveness of multifaceted rabies vaccines (and their distribution strategies) are the reduction of rabies cases in wild carnivores in North America and Europe using bait-delivered recombinant vaccines [4,5]. Yet, RABV is maintained in numerous other reservoir species worldwide (e.g., bats) [6], contributing to the complexity of its epidemiology and control.

In Latin America, the common vampire bat (*Desmodus rotundus*) is currently the main reservoir of RABV [7]. Unique to this region of the continent, *D. rotundus* feeds on blood (via bite), a behavior that creates an ideal route of transmission for RABV. Every year, thousands of livestock animals and some humans are exposed to RABV by vampire bat bites, causing a substantial burden to public health and animal production sectors [6–9]. Culling vampire bat populations is a strategy widely used by Latin American countries to control vampire bat transmitted rabies (VBR). Unfortunately, several studies have concluded that this practice is not effective or sustainable [4,10,11], as evidenced by the expansion of rabies into new areas [10,12]. Moreover, beneficial bats are sometimes culled indiscriminately during these operations.

Motivated by the increasing need to identify novel control strategies, this study evaluated vaccination of vampire bats against RABV using a recombinant raccoon poxvirus (RCN) expressing a mosaic gene of the rabies glycoprotein G (MoG). This vaccine candidate (RCN-

MoG) was developed and tested previously in captive big brown bats (*Eptesicus fuscus*) [13,14] and shown to be safe and effective in protecting them against rabies via both oral and topical routes of administration, prerequisites for feasible application to wild bats [4]. While the concept of vaccinating vampire bats is controversial, it has been explored before using vaccinia rabies glycoprotein vaccines developed for terrestrial carnivores [15–19] but never advanced to field testing. The use of vaccinia as a vaccine vector has raised safety concerns for both humans [20,21] and livestock [22], the preferred prey of vampire bats. Although similar to vaccinia, raccoon poxvirus is a safer alternative, as it has shown to be more attenuated or avirulent, while capable of retain high immunogenicity [21].

We conducted rabies vaccination and challenge studies using common vampire bats captured in México and transported to a biosafety level 3 animal facility in the U.S. Our objectives were to assess the overall safety of RCN-MoG for vampire bats delivered orally or topically, humoral immunity elicited by vaccination, protection against RABV challenge, and viral shedding by rabid bats. We were particularly interested in the potential blocking effect of vaccination on RABV shedding, as documented previously in vampire bats vaccinated after exposure to RABV [23], and whether bats seropositive for rabies virus neutralizing antibody (RVNA), and thus likely exposed previously, survived RABV challenge at higher rates than seronegative bats.

Having a large cohort of wild-caught vampire bats from a diverse population available for vaccination studies is a unique opportunity. For this reason, we tried to leverage the study to address several additional questions beyond vaccine efficacy using specific cohorts of our captive population (e.g., vaccine safety during pregnancy, transfer of topically applied vaccine among cage mates, and antibody decline after RABV exposure), which required different

experimental parameters. We encountered challenges during the study, and it was difficult to control intrinsic factors, as expected when working with individuals of unknown disease history. We present results within broad categories (e.g., grouped by treatment received) and also indicate relevant information more specifically for some individuals or groups.

Methods

Ethics statement

The Mexican Secretariat of Environment and Natural Resources (SEMARNAT) approved the capture and export of bats under permit numbers SGPA/DGVS/003242/18 and 44333, and we imported the bats under the Centers for Disease Control and Prevention (import permit number 2018-04-108). The Institutional Animal Care and Use Committee at the U.S. Geological Survey National Wildlife Health Center (NWHC), Madison WI, USA, approved all husbandry and experimental procedures (protocol number EP180418).

Vampire bats

The common vampire bats used in this study were wild-caught and had an unknown history of exposure to RABV during their lifetime. Bats were captured in the state of San Luis Potosí, México in August 2018 using mist nets placed at cave entrances and known roosting areas after sunset. During a six-week field period, we captured 93 vampire bats (of mixed sex and age) for transport to NWHC where the experiment took place. We housed the bats in a biosafety level 3 animal facility for the duration of the study (September 2018 – June 2019). We followed recommended husbandry guidelines for vampire bats [24], feeding them a diet of

citrated cow blood and offering water *ad lib.* Bats were housed under controlled temperature (28-34 °C) and humidity (>40%), and males and females were kept in separate cages to avoid reproduction. However, eight females were captured at an early stage of pregnancy, and each gave birth later during the study, adding 8 animals to the study. The initial acclimation and quarantine period lasted approximately 100 days before the start of the vaccine study (Dec 2018) because rabies in vampire bats is known to have a long incubation period [25–27]. During this period, health checks were performed weekly, and on days 36-57 after arrival, we collected a blood sample (i.e., baseline) from all bats for serologic assays.

Detection of rabies virus neutralizing antibodies (RVNA)

To detect RVNA, we used the *in vitro,* cell-based functional assay Rapid Fluorescent Focus Inhibition Test (RFFIT) [28]. Due to the small volume of blood that can be obtained from bats (up to 200 μl), we performed a modified micro-RFFIT using plasma or serum samples as described in [29]. The test results are expressed in end reciprocal titers by the Reed-Muench method [30] and converted to International Units per milliliter (IU/ml) by comparison to a reference standard rabies immune globulin (SRIG) [28]. Given the lack of a defined cut-off value in bats [31], in this study we determined a sample "positive" based on the evidence of neutralization in >50% of the fields at a 1:10 dilution of the test sample (equivalent to a ≥1:10 reciprocal end titer or >0.06 IU/ml). Samples collected at baseline were screened for RVNA to determine which animals may have been previously exposed to rabies. Once all samples from individual bats were available at the end of the study, they were tested in the same micro-RFFIT run to allow comparisons over time, without inter-test variation.

Viral vector recombinant mosaic glycoprotein vaccine candidate, RCN-MoG

Our vaccine candidate, RCN-MoG, and its cultivation were described previously[14]. Briefly, MoG was designed *in silico* from a selection of 664 available sequences from the RABV Phylogroup I glycoprotein, representing strains most likely to occur in bats, thus providing a broader antigenic coverage. As a negative control, we used RCN expressing a luciferase gene (*luc*) instead of MoG. Stock cultures of both RCN-MoG and RCN-*luc* were cultivated in Vero cells (ATCC #CCL-18) and quantified by plaque assays [13,14]

Experimental Design

To evaluate the humoral immune response by vaccination, we first separated the bats based on the results from the RVNA screening at baseline into seropositive and seronegative groups. We then allocated them into subgroups according to treatment to be administered (e.g., vaccine-RCN-MoG, control-RCN-*luc*, no treatment) and route (direct, by instillation in the oral cavity, or indirect, in a topical vehicle) (Table 1). For oral delivery, $1x10^8$ plaque forming units (PFU) of RCN-MoG or RCN-*luc* were dropped directly into the mouth in 100 µl volumes by pipette. For topical delivery in the males, $2x10^8$ PFU of RCN-MoG or RCN- *luc* was mixed into 0.5 ml of laboratory grade glycerin jelly (Carolina Biological Supply, Burlington, North Carolina, USA) that included Rhodamine B (RB), a biomarker commonly used in wildlife [32], at a concentration of 0.06%. We applied the mixture onto the chest and back of the bats, to allow for its consumption during grooming. We collected hair as soon as 3 days (females) or 7 days (males) post-vaccination and examined it under fluorescent microscopy to detect the presence of

metabolized RB, observed as fluorescence. We assumed vaccine uptake if the hair sample was positive for RB.

We used the female group (n=22) to assess vaccine safety during pregnancy and lactation, and potential vaccination due to transfer of vaccine-laden jelly between bats. For this, we used a higher dose of RCN-MoG. Four females received $5x10^8$ PFU of RCN-MoG in 1 ml of glycerin jelly-RB mixture at a 0.09% concentration and were co-housed with eight untreated females. Hair was collected from all periodically to assess uptake of RB as described above. Untreated females were considered as "in-contact" (Table 1). Due to veterinary issues unrelated to the experimental protocols (chronic inflammation of an eye that required prolonged treatment), two other topically vaccinated females remained isolated for most of the study.

Seropositive bats were vaccinated orally, or received a placebo, and were used to identify the potential effect of vaccination in previously exposed bats (e.g., boost effect).

We vaccinated all groups of bats between December 2018 - February 2019. A subset of seronegative males (8 oral and 9 topically vaccinated) was boosted 100 days post-vaccination (dpv), using the same dose and route to determine if boosting would increase RVNA titers and enhance protection against challenge.

We periodically collected blood samples for serology at 21-28, 30, 71, and 113 dpv to follow up on antibody response and at day of death or at the end of the study (terminal sample), depending on the group. An additional blood sample was collected 30 days post rabies challenge (dpc) from 15 females (Table 2).

Given the large number of bats to bleed or process for a sampling session, we worked during consecutive days; thus, resulting in the different range of sampling dates mentioned above.

Challenge virus

For the challenge, we used 100 µl of a heterologous strain of RABV at a dose of $10^{4.3}$ tissue culture infective dose (TCID50/ml), injected intramuscularly into each masseter muscle (50 µl on each side). The RABV challenge virus (cRABV) we selected was a coyote variant, isolated from a naturally infected coyote in Texas and was provided by the CDC under a collaborative research agreement (D-615-15). Use of a heterologous strain allowed us to distinguish the challenge virus from naturally occurring VBR infections at the time of challenge.

Males and females were challenged with cRABV separately. Males (n=55) were challenged in April 2019 at 112 or 127 dpv, depending on when they received vaccine. Females (n=22) were challenged at 31 dpv in February 2019. One pup was challenged with this cohort, as it was volant and over one month old at the time. The rest of the pups (n =7; all unvaccinated) were challenged with cRABV in April 2019, at the same time as adult males (at an estimated age range of 2 to 2.5 months). After challenge, we observed bats for clinical signs of infection/mortality twice daily for 49- 50 days. Females with pups were kept alive longer and were euthanized if/when their pups succumbed to rabies. For some, the post-challenge observation period was of up to 99 days. Surviving bats at the end of the observation period were euthanized, necropsied, and tested for RABV as described below.

Rabies confirmation by Direct Fluorescent Antibody Test (DFA)

We performed the direct fluorescent antibody test (DFA) for RABV diagnosis in brain smear impressions from all bats, according to methods described elsewhere [28,33], to confirm death by rabies. Briefly, slides were fixed in acetone at -20º C and a rabies fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (Fujirebio Diagnostics, Malvern, Pennsylvania, USA) was added to each well and incubated at 37º C under 5% CO_2 for 30 minutes. Slides were washed with PBS and observed under fluorescent microscopy. A second conjugate (Light Diagnostics Rabies DFA Reagent 5200, Millipore, Burlington, Massachusetts, USA) was used as a negative control to confirm results and discriminate between background fluorescence.

Detection of RAVB in saliva and salivary glands

We used a real time RT-PCR LN34 pan-Lyssavirus assay as described elsewhere [34,35] to test for the presence of RABV nucleic acid in salivary glands and saliva (oral swabs) from bats that died or presented signs of rabies after challenge (Table 2). The saliva sample was obtained by swabbing the oral cavity with sterile polyester tipped applicators (Puritan, Guilford, Maryland, USA). Modifications to the RT-PCR protocol include using 8.5 µl of RNA per reaction and an iCycler instrument (BioRad, Hercules, California, USA). Cycle threshold (Ct) values ≤ 35 were considered positive. Values 35-40 were considered inconclusive, indicating low viral load, insufficient sample, or possible cross contamination. RNA extraction was performed using Direct-zol RNA Miniprep kit (Zymo Research, Irvine, California, USA) following manufacturer´s instructions.

We attempted virus isolation in cell culture from RT-PCR positive samples to detect infectious RABV. We followed standard methods for the rabies tissue culture infection test (RTCIT) [28]. Given the limited amount of tissue available, we used undiluted tissue homogenates to infect BHK-21 cells treated with DEAE-Dextran (Millipore-Sigma, St. Louis, Missouri, USA) at a concentration of 10mg/ml [36]. The cell-virus suspension was cultured in 25cm^2 flasks (Corning, Corning, New York, USA) at a density of 2.4x10^6 cells/ml and incubated at 37º C under 5% CO_2 for 48 hours. Along with the flasks, slides were prepared for each sample to monitor cell infection at 48 and 72 hours. Cell infection was determined by immunofluorescence as described above (DFA) and considered negative if no infection was detected after three cell passages performed every 72 hours.

Statistical analysis

To analyze humoral response to vaccination, we calculated geometric mean titers (GMT) and compared differences between groups vaccinated orally (n=23) or topically (n=21) and those unvaccinated or treated with a placebo (n=33) from the seronegative and seropositive groups using the Mixed-model approach with the Geisser-Greenhouse correction (recommended for missing data) in GraphPad Prism version 9.0 for Windows (GraphPad Software, San Diego, California, USA). We performed generalized linear models (GLM) to test for the effect of sex and treatment on survival (binomial, response variable) among groups classified by baseline serostatus using the Probit link function in R (R Core Team, 2020). To simplify analysis and report of results, we then grouped bats based on the preferred model using Akaike´s Information Criterion (AIC) model selection. We used Kaplan-Meier analysis in GraphPad Prism to calculate differences in survival among treatment groups (based on the GLM result mentioned above).

With Graph Pad Prism, we used Fisher´s exact test to analyze the viral shedding data from bats succumbing to challenge (n=27) and to determine the association of RABV shedding with vaccination (both binomial variables). A *P* value of <0.05 was considered significant.

Results

Using the micro-RFFIT, we detected evidence of RVNA in 13 of 88 available bats (12 males and 1 female) at the baseline timepoint and designated these bats as seropositive-at-baseline, likely exposed to RABV in the past. The rest were designated as seronegative-at-baseline, and the bats were assigned to their experimental groups based on this designation. Between December 10, 2018 (3 months after arrival in the US and prior to administering any vaccine to bats) and January 30, 2019 (after the vaccination experiment was already under course), we observed a natural rabies outbreak in two separate cages of males. Ten of 17 bats involved in the outbreak succumbed to infection with a RABV of vampire bat origin, including bats that were orally vaccinated with RCN-MoG as part of the planned experiment. Details of this outbreak, including clinical presentation, RABV shedding and RABV typing were published elsewhere [23]. Of the seven surviving bats, 4 had been seropositive-at-baseline and 3 seronegative-at-baseline, before the outbreak. Six were vaccinated orally and one received RCN-*luc* topically during the outbreak. These seven surviving bats were included in the subsequent challenge experiment. Another bat (#628, seropositive-at-baseline, control) died prior to RABV challenge of causes not related to rabies as confirmed by negative DFA in brain tissues; we only report serology results at baseline and 28 dpv for this bat.

To assess the safety of RCN-MoG in vampire bats, we observed bats daily after vaccination for signs of clinical disease or lesions (e.g., skin lesions) potentially caused by the viral vector. Apart from a slight decrease in blood consumption on the day of vaccination, no significant changes were reported in husbandry or behavior, and none of the bats showed any adverse effect to vaccination. Four pregnant females were vaccinated topically; all successfully weaned their pups later. Overall, we challenged 85 bats, including eight pups and the seven survivors of the natural outbreak.

RVNA development after vaccination

Bats seronegative-at-baseline

Thirty-seven of 65 seronegative-at-baseline bats were vaccinated either by the oral or topical route and ultimately challenged with cRABV (Table 1). All topically vaccinated bats (14 males and 6 females) were considered positive for vaccine uptake, as observed by the presence of RB (fluorescence) in the hair inspected. Three of eight "in-contact" bats had positive fluorescence in their hair indicative of vaccine uptake.

Only 9 bats (24%), all male, developed RVNA at some time point after vaccination. Of these, 8 received vaccine orally and one topically. Titers post-vaccination ranged between 0.1 and 2137 IU/ml (Fig 1). From the seventeen male bats that were boosted on 100 dpv (8 oral and 9 topical, which were seronegative by days 21 and 71 dpv), only two bats (orally boosted by instillation) seroconverted fourteen days later (at the pre-challenge time-point). One control male bat (#613, RCN-*luc*), seronegative at baseline, showed evidence of RVNA at the 21 and 113 dpv time points. The rest of the control males (n=12) remained without detectable RVNA throughout

the study. None of the unvaccinated (n=7), topically vaccinated (n=6), or the "in-contact" (n=8) females had detectable antibody responses after vaccination, despite detection of RB in the hair of all the topically vaccinated and 3 of the in-contact bats, indicative of vaccine uptake.

No significant differences in geometric mean titers (IU/ml) were detected among treatment groups, seronegative at baseline, after vaccination across time (P=0.32). This includes orally vaccinated males, topically vaccinated males and females, and control (RCN-*luc*) males and females with no treatment.

Bats seropositive-at-baseline

Seven of 13 bats, seropositive-at-baseline, were vaccinated (6 oral, 1 topical). All orally vaccinated bats had detectable increases in their RVNA titer by 28 dpv. The weakest response went from 0.08 to 0.69 IU/ml. Baseline titers of seropositive bats that received RCN-*luc* (n=5), or were not treated (1 female), decreased or were lost entirely by 28 dpv (Fig 1). No significant change was detected in mean geometric antibody titers (IU/ml) on days 28 (P=0.31) or 112 (P=0.64) post-vaccination in both groups.

Bats that survived the natural outbreak

Four bats that survived the natural rabies outbreak, seropositive-at-baseline measured before the outbreak had detectable increases in RVNA titers (ranging from 2 to 160-fold) approximately 28 days after the death of the index case and just prior to being orally vaccinated on 1/4/19. All remained seropositive on 28 dpv with only one bat showing a decrease in titer.

Three other bats, seronegative-at-baseline, also survived the outbreak. Bats #579 and #583, were negative for RVNA at 28 days after the death of the index case and just prior to being orally vaccinated. Bat #579 remained seronegative throughout the study. In contrast, bat #583 developed a very high RVNA titer (2137 IU/ml) at 28 dpv (approximately 56 days after contact with rabid bats). Bat #582 (topical control) had detectable RVNA at 28 dpv, but seroconversion is likely due to exposure during the natural outbreak. This bat maintained detectable RVNA throughout the sampling timepoints and it was moved to the seropositive group for the survival analysis. All seven bats also survived cRABV challenge in June 2019 (~5 months after the end of the natural outbreak).

RVNA development after cRABV challenge

We were able to obtain a blood sample from 15 female bats at 31 days post-challenge with cRABV. Seventy-three percent (11/15) demonstrated an increase in antibody titers ranging from 0.52 – 69.9 IU/ml, compared to baseline or post-vaccination time points, at which they were seronegative. The only female seropositive at baseline (#644, not treated) showed a highly elevated RVNA titers after challenge (69.9 IU/ml, 200-fold), although before challenge, her titer had dropped to undetectable levels from the baseline time point and would be considered seronegative by ~127 days from the baseline timepoint.

Collectively, 69% of the bats (58/84, one sample missed) had detectable RVNA on the terminal sample (i.e., death or end of study) stimulated after cRABV challenge. Bat #615 (seronegative, topically vaccinated) was the only bat that developed RVNA on 21 dpv (2 IU/ml). It maintained the response on 71 and 113 dpv (with a lower titer on both days, 0.14 IU/ml);

although RVNA were not detected at terminal sampling for this bat, it survived cRABV challenge. All pups were negative at both baseline (obtained few days prior to cRABV challenge) and terminal samplings. Seventeen other bats (9 unvaccinated, and 8 vaccinated) failed to show RVNA throughout the study, even after cRABV challenge and after three received a booster vaccination. Yet, eight of these bats (3 unvaccinated, and 5 vaccinated) survived cRABV challenge and were rabies negative by DFA.

Overall survival of vampire bats after challenge with heterologous cRABV

Of the 85 bats that were challenged with cRABV, 27 (32%) succumbed to the infection within the 49/50-day observation period, as confirmed by DFA of brain tissue samples. Mean incubation time was 16.3 days (range: 9 to 35), not including bat #598. Bat #598 (seronegative-at-baseline, topically vaccinated) was alive by the end of the challenge observation period (50 dpc) but had started showing progressive clinical signs consistent with rabies two weeks prior. It was confirmed RABV positive upon euthanasia and thus, we are counting this bat as a rabies mortality (n= 27). However, since it may have survived longer, we did not include this bat for the calculation of the mean incubation time.

We observed clinical signs corresponding to both furious and paralytic forms of rabies in our vampire bats, including self-mutilation, aggressive behavior, lack of grooming, incoordination, respiratory distress, and reluctance or inability to move. Most of the bats showing the furious form of rabies were not vaccinated (12/16) compared to 3/10 in the vaccinated group, this difference was significant (P= 0.043). All other surviving bats did not show clinical signs of the disease and were confirmed DFA negative upon euthanasia.

Our regression analyses provide no evidence for an effect of sex or treatment on survival (best-fit model included sex only AICc= 77.04, AIC weight= 0.44), nor did route (topical or oral) or boosting in vaccinated bats (Supp. Fig 1). We therefore combined bats that received one or two doses of vaccine either topically or orally into vaccine treatment groups and compared their survival to untreated groups (no treatment or placebo), by serostatus at baseline. Pups are reported alone and were considered a "RABV naïve" group. The 7 bats involved in the natural outbreak are included in their respective treatment groups, as removing them did not change the results.

The overall proportion of survival by treatment group was 100% (7/7) of seropositive-at-baseline vaccinated bats, 92% (34/37) of seronegative-at-baseline vaccinated bats, 83% (5/6) of seropositive-at-baseline control or no treatment bats, 70% (19/27) of seronegative-at baseline control or no treatment bats, and 0% pups (no treatment). Using Kaplan-Meier survival analyses, a significant difference among treatment groups was detected with all included ($P = <0.0001$) (Fig 2). When analyzed separately, significantly higher survival was detected in seronegative-at-baseline vaccinated bats compared to seronegative-at-baseline control or untreated bats ($P = 0.016$) and compared to the naïve control pups ($P < 0.0001$).

Vaccination with RCN-MoG hinders rabies virus shedding in saliva of infected vampire bats

To evaluate RABV shedding in saliva, we collected oral swabs periodically after challenge (e.g., each bat was swabbed every 3 days or daily if clinical signs were observed) and at time of death, if possible. Salivary glands were collected upon necropsy (Table 2). We used RT-PCR to detect the presence of RABV nucleic acid for both sample types. In Table 3, we

report results of oral and topically vaccinated bats in a grouped "vaccinated" category and control bats (RCN-*luc*) and those that did not receive any treatment (i.e., pups, no treatment, and in-contact females) are grouped as "non-vaccinated", irrespective of serostatus at baseline. Of the 27 bats confirmed rabies positive by DFA, four bats had missing saliva samples or the RT-PCR result was repeatedly deemed "inconclusive" and not included in subsequent analyses. Most bats in the vaccinated group underwent the paralytic form of rabies without visible clinical signs, so they were not swabbed daily, and they died suddenly. Hence, a single saliva test result is shown for this group (Table 3). Similarly, the RT-PCR results on salivary glands from three bats were consistently "inconclusive" and not included. For comparison, five bats (one control, one topically vaccinated, and two orally vaccinated and one control from the natural outbreak) confirmed negative by DFA were also tested by RT-PCR using the salivary gland. Their results (not shown in table) were negative.

For saliva samples, RABV nucleic acid was detected in 71% of the non- vaccinated group (10/14 bats) in at least one sample. In contrast, none of the vaccinated bats (n=9) had detectable RABV nucleic acid in any samples tested post challenge. The proportion of shedding between vaccinated and non-vaccinated bats is significantly different (P=0.0016). For salivary gland, we detected RABV in 94% of the non-vaccinated group (16/17), and the one bat with the negative result (#608, female, in-contact) had an RABV positive saliva sample. In contrast, we detected RABV nucleic acid in 43% of salivary glands from vaccinates (3 of 7 available samples), and the proportion of RABV nucleic acid detection between vaccinated and non-vaccinated groups was significantly different (P=0.014) for this tissue sample. Bat #598 (seronegative, topically vaccinated, and DFA positive) was repeatedly sampled after showing signs of rabies; however, RABV was not detected from a total of 9 saliva samples from this bat

or from its salivary gland. We were unable to isolate RABV from saliva or salivary glands using RTCIT from any of the sampled bats.

Discussion

An ideal vaccine candidate against rabies would not only protect susceptible individuals from infection (e.g., by eliciting a protective immune response) but also stop the spread of RABV within susceptible populations. We found that vaccination of vampire bats against rabies using RCN-MoG did both: preventing rabies disease among susceptible individuals and blocking RABV shedding in saliva (the main mode of transmission of RABV). Of the 85 bats that we challenged with heterologous cRABV, 27 from all treatment groups and including all pups, were confirmed rabid and succumbed to infection (Table 3). Although survival was high among all adult treatment groups (70-100%), in bats designated seronegative-at-baseline, survival was higher among vaccinates compared to controls, indicating a positive effect of vaccination. Of the 9 vaccinated bats that succumbed to infection, detection of RABV nucleic acid in salivary glands was significantly lower (43%) compared to non-vaccinated bats (94%). More importantly, RABV nucleic acid was not detected in any saliva samples of vaccinated bats, whereas it was detectable in 10 of 14 unvaccinated bats, suggesting shedding of the virus was blocked. This is similar to results observed during a natural outbreak of rabies in our vampire bats in December 2018. Bats vaccinated orally after RABV exposure did not have detectable RABV nucleic acid in their saliva prior to or at death [23], whereas RABV was detected in bats that were not vaccinated.

While other studies on RABV infection of vampire bats have reported discrepancies in the detection of RABV in the salivary gland and saliva [26,37–39], our results suggest that lack of detection in our bats was associated with RCN-MoG vaccination instead. Timing of sample collection and RVNA titers may be responsible for the discrepancies in RABV detection in different tissues. For instance, a rapid death after infection (evidenced by short incubation times) may not allow RABV to reach the salivary gland and be excreted in the saliva [26]; or sampling may not coincide with viral excretion (e.g., during intermittent shedding) [40]. In our study, we serially swabbed bats when they showed signs of illness and at the time of death (Table 2), and mean incubation times (time to death) between vaccinated and non-vaccinated groups was not significantly different ($t= 1.71$, $df= 25$, $P= 0.099$). Interestingly, most of the bats that suffered the furious type of rabies (80%, n=15) were not vaccinated, including 6 out of the 7 pups that had clinical signs recorded (Table 3). The mechanism by which RCN-MoG may hinder shedding of RABV through saliva remains unclear. Recombinant poxvirus vectors have shown the potential to induce strong mucosal humoral and cellular immune responses [41,42]. Possibly, this cellular response may prevent replication of RABV in the salivary gland before excretion into the saliva.

Unexpectedly, in our study, seronegative, non-vaccinated bats survived rabies challenge in high proportions (70%). It is possible these bats were previously exposed to RABV, as some of our capture sites in México were in rabies endemic areas, but, if so, we failed to detect RVNA with our neutralization assays at baseline. Abortive infections occur occasionally [27,43] in the wild, allowing individuals to resist rabies infection even after repeated exposure to the virus [23,27]. Other studies [14,15,17] have also demonstrated that survival after RABV challenge occurs in the absence of detectable neutralizing antibodies (elicited by vaccine or natural infection), suggesting alternative immune response mechanisms operate in vampire bats against

RABV not mediated by neutralizing antibodies. In total, we observed eight bats that survived RABV challenge and failed to show RVNA throughout the study. Bat #598 remained alive throughout the 50-day observation period after RABV challenge. However, it showed progressive signs indicative of rabies beginning day 20 post-challenge and was confirmed rabid upon euthanasia 30 days later. Its RVNA titer at the terminal sample was 9898 IU/ml, despite never showing evidence of seroconversion after vaccination. To determine with certainty the baseline immune status against RABV is a challenge for any study that involves wild bats. We acknowledge that other immune mechanisms (i.e., cell-mediated immunity or innate) may play a role in surviving RABV infection [15,44,45] but this remains poorly understood in bats [9,46].

Vaccination with RCN-MoG elicited RVNA production in only 24% of bats designated seronegative-at-baseline, only males and predominantly those that received the vaccine orally. The two bats that seroconverted after receiving a booster on day 113 dpv had higher titers than those not boosted. All but one (8/9) of these bats that seroconverted after vaccination survived the challenge, but so did 19 of 28 that did not develop RVNA, supporting that survival is not necessarily correlated with RVNA presence [15,27]

On the other hand, 14.7% (13/88) of bats exhibited RVNA at the beginning of the study. This group (seropositive-at-baseline) provided the opportunity to assess if vaccination could stimulate the production of higher titers of RVNA, comparable to an anamnestic response. Collectively, bats from this group that were involved in the initial outbreak (vaccinated and survived) had the highest levels of antibody titers throughout the sampling timeframe. However, for both seronegative-at-baseline and seropositive-at-baseline bats, the RVNA mean geometric titer after vaccination was not different between treatment groups.–Others have observed highly variable seroconversion rates (e.g., 25-95%) in vampire bats after vaccination with recombinant

vaccines[15,17,18], and in another bat species, *E. fuscus*, using RCN-MoG topically and orally (0 and 22%, respectively)[14]. In accordance with other studies [27,38,47], we also observed a decline in RVNA over time. For instance, bat #634 (control) had a notable high titer at baseline (683.8 IU/ml) which declined to 5.85 when measured 91 days later. Individually, females had detectable RVNA 30 days after challenge, but most of them (10/17) declined by the end of the study (approximately 3 months later).

Our study was confounded by the occurrence of a natural rabies outbreak in our captive animals 3 months after capture and the possibility that bats testing seronegative for RVNA at baseline had probably been exposed to RABV previously. Despite that, our results suggest that vaccination could be used to manage and control VBR without culling; not only in a conventional manner (i.e., by protecting susceptible populations from RABV infection), but also by potentially blocking the main route of RABV transmission (saliva, via a bite) and stopping the spread among individuals. For future work, ideally, we suggest establishing colonies of vampire bats that can be guaranteed "free" of rabies, e.g., pups born in captivity.

Even though rabies vaccination programs have significantly lowered the incidence of rabies in other wild animal populations [5] and could be useful in vampire bats, we recognize this is a controversial prospect. In addition to the risk of transmitting rabies, vampire bat predation on livestock, in the absence of rabies, can be a major cause of economic losses in most Latin American countries [48]. Some believe that vaccinating bats to protect them from rabies will increase their abundance, but little evidence exists to support this concern. A high proportion of vampire bats survive rabies naturally, as evidenced by high seroprevalence in wild populations, and modeling based on available data has estimated that vampire bats rarely develop lethal rabies infection (~10% of exposures) [11]. It is unlikely, then, that rabies acts as a form of

population control. Other factors have been shown to be associated with vampire bat abundance, such as prey availability (especially livestock)[49] or additional suitable habitat (e.g., man-made roosting sites). The general consensus among farmers and other stakeholders is to eliminate vampire bats [50], even when rabies outbreaks do not occur, and culling vampire bat populations has been, for decades, the main practice to mitigate VBR. Thus, introducing vampire bat vaccination as an alternative strategy, even to prevent livestock losses, will require breaking longstanding paradigms. In addition to vaccination, other strategies (non-lethal) aimed at controlling bat abundance would be needed, such as the use of contraceptives, which ideally could be delivered topically along with rabies vaccine.

The results of our study should encourage further investigation on vaccination of vampire bats and motivate field trials with wild populations on a manageable-sized scale. This approach, in combination with other strategies aimed at reducing bat abundance and predation, may show promise in significantly reducing the burden of rabies in Latin America.

Table 1. Individual common vampire bats grouped by rabies virus neutralizing antibodies serostatus at baseline time point, sex, treatment received, route and dose. Eight captive-born pups did not receive treatment but were included in the RABV challenge. For this group F= female and M=male, one pup was not sexed.

Baseline serostatus	Sex	Treatment	Route	Dose (PFU/ml)[a]	Total individuals (n)[b]	Total challenged[c]
Seronegative	Male	RCN-MoG (vaccine)	oral	$1x10^8$	21(8 [d])	17
			topical	$2x10^8$ (in 0.5 ml of glycerin jelly)	14 (9 [d])	14
	Male	RCN-*luc* (control)	oral	$1x10^8$	12	12
			topical	$2x10^8$	5	1[e]
Seronegative	Female	RCN-MoG (vaccine)	topical	$5x10^8$ (in 1 ml of glycerin jelly)	6	6
	Female[f]	No treatment	in-contact	na	8	8
		No treatment	na	na [g]	8	8
	2M / 5F (pups)[f]		na	na	8	8
Seropositive	Male	RCN-MoG	oral	$1x10^8$	6	6
			topical	$2x10^8$	1[g]	1
	Male	RCN-*luc*	oral	$1x10^8$	5	4

[a] Vaccine dose is measured in plaque forming units per ml of the raccoon pox viral vector.

[b] (n) is the initial number bats that received treatment, this number changed later due to the death of subjects and change in serostatus after the natural rabies outbreak occurring in December 2018.
[c] Bats were challenged in February (females) or April 2019 with $10^{4.3}$ tissue culture infective dose (TCID50/ml) of a heterologous (coyote) RABV strain.
[d] A subset of bats from the oral and topically vaccinated groups (8 and 9 bats respectively) were boosted 100 dpv, using the same dose and route as previously administered
[e] The only remaining bat from this group (#582) seroconverted after exposure during the outbreak. It was moved to the seropositive group for the challenge and considered seropositive for the survival analysis
[f] Sixteen females considered "no treatment" did not receive vaccine or control, but eight were housed with other four females that received RCN-MoG topically. Eight pups born during the study did not receive treatment but were included in the rabies challenge. One pup was not sexed.
[g] The "no treatment" female was the only female bat that was seropositive. The topically vaccinated male bat was initially seronegative at baseline and grouped accordingly, but on retesting several times was found to be seropositive, therefore is alone in a treatment group.

Table 2. List of available samples obtained from each bat in treatment groups for serology and RABV confirmation

Baseline serostatus	Sex	Treatment	Sample type	Sampling schedule (days post-vaccination)
Seronegative	Male	RCN-MoG (oral or topical) or RCN-*luc*	blood	21, 71, 113, at death or end of study (terminal sample)
			oral swab	every 3 days, daily if clinical signs observed, at death
	Female[a]	RCN-MoG (topical) or No treatment	blood	30, at death or end of study (terminal sample). And at 31 days post-challenge
			oral swab	weekly, daily if clinical signs observed, at death
	Pups	No treatment	blood	before RABV challenge (considered baseline) (and death or end of study (terminal sample)
			oral swab	weekly, daily if clinical signs observed, at death
Seropositive	Males	RCN-MoG (oral) or RCN-*luc*	blood	28, 112, at death or end of study (terminal sample)
			oral swab	weekly, daily if clinical signs observed, at death

[a] Females have a blood sample obtained 30 days after the group was challenged with heterologous RABV isolated from a coyote.

Table 3. Detection of RABV in salivary gland and RABV shedding in saliva by LN34 RT-PCR in bats confirmed positive for rabies in brain tissue by the direct fluorescent antibody test. Saliva samples were obtained on day of death (0) if possible, daily if clinical signs were observed, or 1-3 days prior to death according to a rotating group schedule. Symbols are: + "positive", – "negative", and "inc" inconclusive. Inconclusive results are those with a Ct value from 35-40 and could indicate low virus load, insufficient sample, or possible cross contamination. Ct values with n/d is "not detected", n/a means sample was not available. Age groups are J= juvenile, A= adult, and p=pup. Clinical signs are categorized as furious (F) or paralytic (P), and time to death is the number of days from RABV challenge until the bat died or was euthanized.

Bat ID	Sex	Age	Group	Treatment	RT-PCR salivary gland		RT-PCR saliva (day before death)				RVNA terminal (IU/ml)	Clinical signs	Time to death (days)
					result	Ct value	0	-1	-2	-3			
608	fem	J	Non-vaccinated	contact	–	n/d	+				0.06	F	11
696[a]	fem	J		contact	+	27.08	n/a				0.06	F	11
893[a]	male	p		none	+	26.48	n/a				0.06	F	12
561	male	A		control	+	29.12	–			inc	1.53	P	15
617	male	A		control	+	32.76	–			inc	0.06	P	14
891		p		none	+	32.06	–				0.06	F	13
894	fem	p		none	+	32.62	–				0.06	F	35
692 [a]	fem	A		none	+	31.19	inc				0.06	P	20
560	male	J		control	+	29.32	+				0.06	F	9
594	male	J		control	+	29.6	+	+			0.16	P/F[c]	14
636	male	A		control	+	33.92	+		inc		2.62	F	15
690	male	J		control	+	31.77	+				0.06	F	9
890	fem	p		none	+	26.15		+			0.06	n/a	15

892	male	p		none	+	26.5	+			0.06	F	18
895	fem	p		none	+	32.89	+	+		0.06	F	10
899	fem	p		none	+	29.48	+	+		0.06	P	18
669	fem	p		none	+	30.53		+		0.06	F	17
598	male	A	Vaccinated	topical	–	n/d	–			9898	P[b]	50
599	male	J		topical	–	n/d	–			na	P	18
614	male	A		topical	–	n/d	–			0.06	P/F[c]	23
687[a]	male	A		oral	–	n/d	inc			0.4	F	12
638[a]	male	A		oral	inc	38.05	–			0.08	P	22
595[a]	male	A		topical	inc	37.27	–			0.06	P	15
691[a]	male	A		topical	inc	37.14	–			3.42	P	29
597	male	A		oral	+	31.74			–	390.8	P	11
639	male	A		oral	+	31.68	–			0.11	P	18
586	male	A		topical	+	29.99	–			0.06	F	11

[a] Indicates bats that were excluded from the Fisher´s exact test to compare proportion of RABV shedding in saliva or salivary gland of bats by vaccination group

[b] This bat showed progressive loss of movement and coordination, consistent with rabies but remained alive by the 50-day observation period. It was confirmed RABV positive. We tested nine of his oral swabs collected between the initial observation of clinical signs until euthanasia, The results were all negative.

[c] Bats 594 and 614 initially displayed signs corresponding to paralytic presentation (incoordination, unresponsiveness) but prior to death behaved furiously (self-inflicted wounds, aggressive behavior, and excitability).

Figure 1. Detection of rabies virus neutralizing antibodies (RVNA) in individual vampire bats at different time points after vaccination and challenge with a heterologous (coyote) strain of RABV in A) vampire bats seronegative at baseline and B) vampire bats seropositive at baseline. Events such as vaccination, occurrence of a natural rabies outbreak within the captive colony, and end of the study are indicated. Yellow markers indicate the seven bats that were involved in the outbreak and survived the rest of the study.

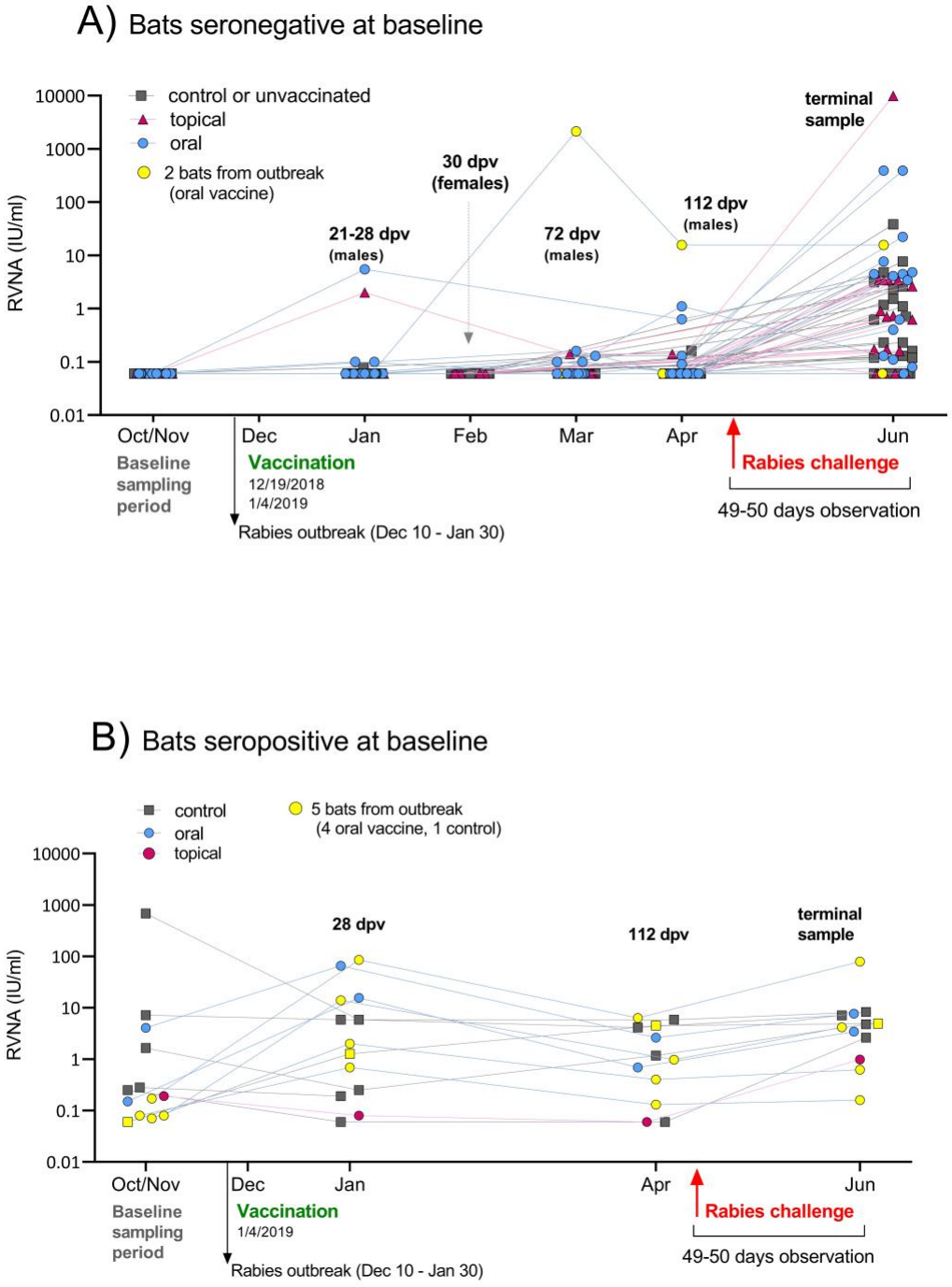
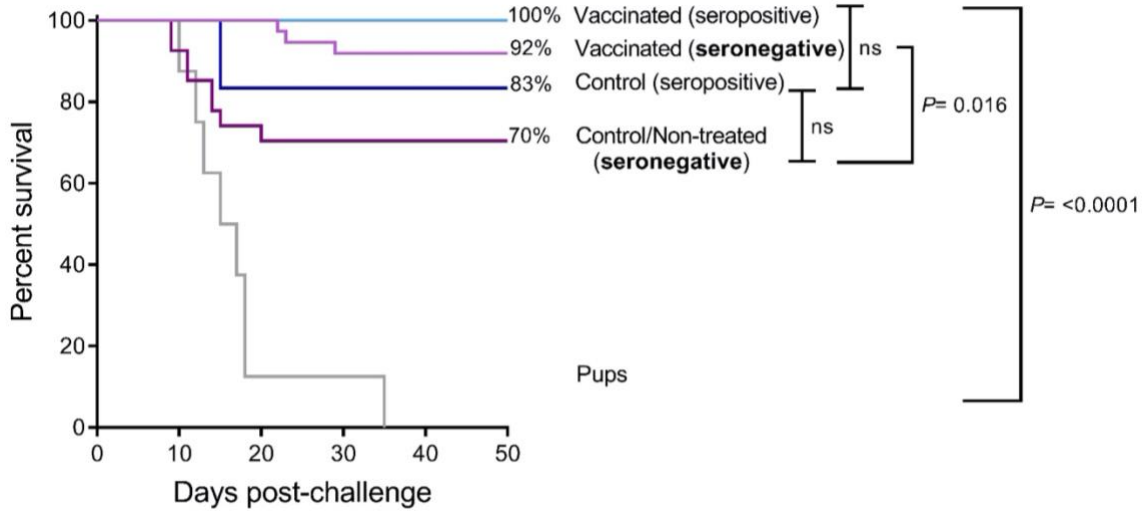


Figure 2. Kaplan-Meier analysis of survival of vampire bats grouped by initial serostatus (seropositive/seronegative) and treatment received (vaccinated, orally and topically, or non-vaccinated (control, RCN-*luc* or not treated) after challenge with heterologous RABV strain and observed for 50 days post-challenge. Eight captive born pups are RABV naïve controls. A P value of < 0.05 was set as significant and "ns" indicates no significance



Supplemental figure 1.

Model selection based on Akaike Information Criterion testing for the effect of sex and/or treatment on survival, and boost and/or route of common vampire bats after challenge with heterologous strain of RABV (n=65). All bats were seronegative at baseline. Models for boost/route included vampire bats that received treatment (n=37)

Model name	AICc	Delta AICc	AICc weight	k
Test the effect of sex and treatment in all seronegative bats				
Sex	77.04	0.00	0.44	2
Sex + vaccination	78.00	0.96	0.27	3
Sex * vaccination	78.98	1.94	0.17	4
Vaccination	80.19	3.15	0.09	2
Sex + vaccination + route	82.18	5.15	0.03	6
Test the effect of route, boost, and sex in all seronegative, vaccinated bats				
Sex	43.34	0	0.52	2
Sex + route	44.40	1.06	0.31	3
Route	47.34	4.00	0.07	2
Boost	47.44	4.10	0.07	2
Boost + route	49.62	6.28	0.02	3
Boost * route	52.13	8.79	0.01	4

Acknowledgments

We thank staff at NWHC (Susan Smith; Judy Williamson; Rachel Abbot; Carly Malavé, and Elizabeth Falendysz) and at University of Wisconsin-Madison (Lex Medina-Magües) for their help in sampling bats and laboratory assistance in Madison, Wisconsin. We thank Robin Russell and Ariel Leon, NWHC, for providing statistical assistance and reviewing the manuscript. We thank Michael Niezgoda, Crystal Gigante, Edgar Condori, Lillian Orciari, and Lauren Greenberg at Centers for Disease Control for assistance with serology. We are also grateful to Governmental agencies in México: Servicio Nacional de Sanidad, Inocuidad, y Calidad Agroalimentaria (SENASICA), Comité Estatal para el Fomento y Protección Pecuaria de San Luis Potosí, Centro Nacional de Programas Preventivos y de Control de Enfermedades, and USDA APHIS-IS México for their support to collect vampire bats in México.

Funding

This research was funded by the U.S. Geological Survey, American Association of Zoo Veterinarians (UW award number MSN209345), a Graduate Student Research Award from the University of Wisconsin—Madison Global Health Institute, a University of Wisconsin International Division IRIS Incubator Grant for Interdisciplinary Research in International Studies and a Fall Competition Research Award. Elsa M. Cárdenas Canales was supported by a fellowship from the Morris Animal Foundation (D19ZO – 404). Any use of trade, firm, or product names does not imply endorsement by the U.S. Government.

Author Contributions:

Conceptualization, All; data curation, E.M.C.C.; formal analysis, E.M.C.C and T.E.R.; funding acquisition: J.E.O., and T.E.R.; Investigation, E.M.C.C., A.V.V., T.E.R.; methodology, All; project administration, J.E.O. and T.E.R.; resources, P.S.S., J.E.O. and T.E.R.; supervision, P.S.S., J.E.O. and T.E.R.; writing- original draft, E.M.C.C.; writing- review and editing, All.

References

1. Baer GM. The History of Rabies [Internet]. Second Edi. Rabies. Elsevier Ltd; 2007. 1–22

2. Steele JH. History of Rabies [Internet]. The Natural History of Rabies. Academic press, inc.; 1975. 1–29.

3. Davis BM, Rall GF, Schnell MJ. Everything You Always Wanted to Know about Rabies Virus (But Were Afraid to Ask). Annu Rev Virol. 2015;2:451–71.

4. Cross ML, Buddle BM, Aldwell FE. The potential of oral vaccines for disease control in wildlife species. Vet J. 2007;174(3):472–80.

5. Maki J, Guiot AL, Aubert M, Brochier B, Cliquet F, Hanlon CA, et al. Oral vaccination of wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): A global review. Vet Res. 2017;48(1):1–26.

6. Banyard AC, Hayman D, Johnson N, McElhinney L, Fooks AR. Bats and Lyssaviruses. Vol. 79, Advances in Virus Research. 2011. 239–289 p.

7. Johnson N, Aréchiga-Ceballos N, Aguilar-Setien A. Vampire bat rabies: Ecology, epidemiology and control. Viruses. 2014;6(5):1911–28.

8. Beltz LB. Rabies viruses and other Rhabdoviruses. In: Bats and human health: Ebola, SARS, Rabies and beyond. 1st Ed. John Wiley & Sons, Ltd.; 2018. p. 27–49.

9. Fisher CR, Streicker DG, Schnell MJ. The spread and evolution of rabies virus: Conquering new frontiers. Nat Rev Microbiol [Internet]. 2018;16(4):241–55.

10. Benavides JA, Valderrama W, Streicker DG. Spatial expansions and travelling waves of rabies in vampire bats. Proc R Soc B Biol Sci. 2016;283(1832):1–9.

11. Blackwood JC, Streicker DG, Altizer S, Rohani P. Resolving the roles of immunity, pathogenesis, and immigration for rabies persistence in vampire bats. Proc Natl Acad Sci [Internet]. 2013 Dec 17 [cited 2018 Nov 4];110(51):20837–42.

12. Bárcenas-Reyes I, Loza-Rubio E, Zendejas-Martínez H, Luna-Soria H, Cantó-Alarcón GJ, Milián-Suazo F. Comportamiento epidemiológico de la rabia paralítica bovina en la región central de México, 2001-2013. Rev Panam Salud Publica/Pan Am J Public Heal. 2015;38(5):396–402.

13. Stading BR, Osorio JE, Velasco-Villa A, Smotherman M, Kingstad-Bakke B, Rocke TE. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox vectored rabies vaccine in the Brazilian Free-tailed bat (Tadarida brasiliensis). Vaccine. 2016;34(44):5352–8.

14. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.

15. Aguilar-Setién A, Campos YL, Cruz ET, Kretschmer R, Brochier B, Pastoret PP. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J Wildl Dis. 2002;38(3):539–44.

16. Sétien AA, Brochier B, Tordo N, De Paz O, Desmettre P, Péharpré D, et al. Experimental rabies infection and oral vaccination in vampire bats (Desmodus rotundas). Vaccine. 1998;16(11–12):1122–6.

17. Almeida MF, Martorelli LFA, Aires CC, Sallum PC, Massad E. Indirect oral immunization of captive vampires, Desmodus rotundus. Virus Res. 2005

18. Almeida MF, Martorelli LFA, Aires CC, Barros RF, Massad E. Vaccinating the vampire bat Desmodus rotundus against rabies. Virus Res. 2008;137(2):275–7.

19. Serrano H, Pérez-Rivero JJ, Aguilar-Setién A, De-Paz O, Villa-Godoy A. Vampire bat reproductive control by a naturally occurring phytooestrogen. Reprod Fertil Dev. 2007

20. Moss B. Vaccinia virus vectors. [Internet]. Vol. 20, Biotechnology (Reading, Mass.). Butterworth-Heinemann, a division of Reed Publishing (USA) Inc.; 1992. 345–362 p.

21. Jones GJB, Boles C, Roper RL. Raccoonpoxvirus safety in immunocompromised and pregnant mouse models. Vaccine [Internet]. 2014;32(31):3977–81.

22. Matos ACD, Rehfeld IS, Guedes MIMC, Lobato ZIP. Bovine vaccinia: Insights into the disease in cattle. Viruses. 2018;10(3).

23. Cárdenas-Canales EM, Gigante CM, Greenberg L, Velasco-Villa A, Ellison JA, Satheshkumar PS, et al. Clinical presentation and serologic response during a rabies

epizootic in captive common vampire bats (desmodus rotundus). Trop Med Infect Dis. 2020;5(1):1–13.

24. Barnard SM. Bats in captivity. Krieger Publishing Company; 2011.

25. Bell JF. Latency and Abortive Rabies [Internet]. The Natural History of Rabies. Academic Press, Inc; 1975. 331–354 p.

26. Aguilar-Setien A, Loza-Rubio E, Salas-Rojas M, Brisseau N, Cliquet F, Pastoret PP, et al. Salivary excretion of rabies virus by healthy vampire bats. Epidemiol Infect. 2005;133(3):517–22.

27. Turmelle AS, Jackson FR, Green D, McCracken GF, Rupprecht CE. Host immunity to repeated rabies virus infection in big brown bats. J Gen Virol. 2010;91(9):2360–6.

28. Rupprecht CE, Fooks AR, Abela-Ridder B. Laboratory techniques in rabies. Fifth edition. World Health Organization, Geneva. 2018; Available from: http://www.who.int/rabies/resources/WHO-CDS-NTD-NZD-2018.03-Draft.pdf?ua=1

29. Smith, T.J.; Gilbert AT. Comparison of a Micro-Neutralization Test with the Rapid Fluorescent Focus Inhibition Test for Measuring Rabies Virus Neutralizing Antibodies. Trop Med Infect Dis. 2017;2(24).

30. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints12. Am J Epidemiol. 1938 May;27(3):493–7.

31. Moore SM, Gilbert A, Vos A, Freuling CM, Ellis C, Kliemt J, et al. Rabies virus antibodies from oral vaccination as a correlate of protection against lethal infection in wildlife. Trop Med Infect Dis. 2017;2(3).

32. Fernandez JR-R, Rocke TE. Use of Rhodamine B as a Biomarker for Oral Plague Vaccination of Prairie Dogs. J Wildl Dis [Internet]. 2011 Jul;47(3):765–8.

33. CDC. Protocol for Postmortem Diagnosis of Rabies in Animals by Direct Fluorescent Antibody Testing, A Minimum Standard for Rabies Diagnosis in the United States. http://www.cdc.gov/ncidod/dvrd/rabies/Professional/publications/DFA_diagnosis/DFA_protocol-b.htm. 2006

34. Gigante CM, Dettinger L, Powell JW, Seiders M, Condori REC, Griesser R, et al. Multi-site evaluation of the LN34 pan-lyssavirus real-time RT-PCR assay for postmortem rabies diagnostics. PLoS One. 2018;13(5):1–25.

35. Wadhwa A, Wilkins K, Gao J, Condori Condori RE, Gigante CM, Zhao H, et al. A Pan-Lyssavirus Taqman Real-Time RT-PCR Assay for the Detection of Highly Variable Rabies virus and Other Lyssaviruses. Kading RC, editor. PLoS Negl Trop Dis. 2017 Jan 12;11(1):e0005258.

36. Larghi OP, Nebel AE, Lazaro L, Savy VL. Sensitivity of BHK 21 cells supplemented with diethylaminoethyl dextran for detection of street rabies virus in saliva samples. J Clin Microbiol. 1975;1(3):243–5.

37. Vieira LFP, Sílvia SRFG, Galante AC, Castilho JG, Oliveira RN, Brandão PE, et al. Detection of rabies virus nucleoprotein-RNA in several organs outside the central nervous system in naturally-infected vampire bats. Pesqui Vet Bras. 2011;31(10):922–5.

38. Almeida MF, Martorelli LFA, Aires CC, Sallum PC, Durigon EL, Massad E. Experimental rabies infection in haematophagous bats Desmodus rotundus. Epidemiol Infect. 2005;133(3):523–7.

39. Moreno J, Baer GM. Experimental rabies in the vampire. Am J Trop Med Hyg. 1980;29(2):254–9.

40. Hanlon CA. Rabies in Terrestrial Animals [Internet]. Second Edi. Rabies. Elsevier Ltd; 2013. 179–213 p.

41. Gherardi MM, Esteban M. Recombinant poxviruses as mucosal vaccine vectors. J Gen Virol. 2005;86(11):2925–36.

42. Kingstad-Bakke B, Kamlangdee A, Osorio JE. Mucosal administration of raccoonpox virus expressing highly pathogenic avian H5N1 influenza neuraminidase is highly protective against H5N1 and seasonal influenza virus challenge. Vaccine. 2015 Sep;33(39):5155–62.

43. Katz IS, Fuoco NL, Chaves LB et al. Delayed progression of rabies transmitted by a vampire bat. Arch Virol. 2016;161(9):2561–6.

44. Moore, S.M.; Wilkerson, M.J.; Davis, R.D.; Wyatt, C.R.;Briggs DJ. Detection of cellular immunity to rabies antigens in human vaccinees. J Clin Immunol. 2006;26:533–45.

45. Lafon M. Immunology. In: Jackson AC, Wunner WH, editors. Rabies. Elsevier; 2007.

46. Johnson N. Interplay between rabies virus and the mammalian immune system. World J Clin Infect Dis. 2015;5(4):67.

47. Jackson FR, Turmelle AS, Farino DM, Franka R, McCracken GF, Rupprecht CE. Experimental rabies virus infection of big brown bats (Eptesicus fuscus). J Wildl Dis. 2008;44(3):612–21.

48. Anderson A, Shwiff S, Gebhardt K, Ramírez AJ, Shwiff S, Kohler D, et al. Economic evaluation of vampire bat (Desmodus rotundus) rabies prevention in Mexico. Transbound Emerg Dis. 2014;61(2):140–6.

49. Rocha F, Ulloa-Stanojlovic FM, Rabaquim VCV, Fadil P, Pompei JC, Brandão PE, et al. Relations between topography, feeding sites, and foraging behavior of the vampire bat, Desmodus rotundus. J Mammal. 2020;101(1):164–71.

50. Shapiro HG, Willcox AS, Tate M, Willcox E V. Can farmers and bats co-exist? Farmer attitudes, knowledge, and experiences with bats in Belize. Human–Wildlife Interact. 2020;14(1).

CHAPTER 4

Assessing the transfer rate of a topical “sham vaccine” among wild common vampire bats using a fluorescent biomarker

Elsa M. Cárdenas-Canales, Tonie E. Rocke, Jorge E. Osorio

Abstract

The common vampire bat is the main reservoir of rabies in Latin America. Topical poisons (applied directly to one bat and transferred through contact to others) have been widely used to reduce vampire bat populations for rabies control in livestock or humans. The development of rabies vaccines for use in bats has been tested with success in the laboratory, but no vaccine is available for field use yet. One of the challenges for vaccinating bats is the ability to deliver the vaccine to individuals in colonies. We used a biomarker to test the feasibility of vaccinating wild vampire bats topically (in the same way as with the poison). We attempted to measure its transferability in two colonies of vampire bats in Yucatan and Jalisco, Mexico. Bats treated with a biomarker (in a mix of glycerin jelly) ingest the product and pass it through contact to others who ingest it. Biomarker uptake is assessed in hair samples observed under fluorescent microscopy, a positive sample being fluorescent. From an estimated colony size of 346 vampire bats in Yucatan, 76 bats (20.9%) were treated topically. We recorded 45 non-treated bats showing fluorescent hair samples for an overall marked transfer rate of 34%. We could not detect meaningful evidence of the transfer rate in Jalisco, as the topical vehicle failed to maintain homogeneity and the biomarker crystallized in it. Others have used glycerin jelly successfully, and our work with glycerin jelly in the laboratory has not shown complications. Nevertheless, other products (e.g., nanoparticles) can also be considered options for future field studies and delivery of vaccines to bats.

Keywords: biomarker, vaccination, vampire bats

Introduction

Vaccination has been an effective strategy to control infectious diseases in animal populations across the globe [1–3]. For rabies, a significant viral zoonotic disease, distributing baits containing recombinant rabies vaccine to wild carnivores in Europe and North America has made possible the suppression of the disease [2,4,5]. The success behind rabies vaccination relies on the availability of both effective rabies vaccines and feasible delivery methods to the target populations [1]. Since the ecology of the target species to be vaccinated plays a vital role in devising the best strategy for vaccine delivery [2,3], some reservoirs of the rabies virus (RABV) are more challenging to vaccinate than others (e.g., bats) [5].

The common vampire bat (*Desmodus rotundus*) lives only in Latin America. Its geographic range spans from northern México to southern Argentina and Chile [6,7]. Currently, the vampire bat is the main vector for rabies in livestock species in these regions [8,9]. Additionally, in many rural areas, the rise in human cases of rabies is associated with vampire bats [10–12]. Unlike wild carnivores, no commercially available rabies vaccines exist for vampire bats (or any bat species) [5]. Currently, control strategies for vampire bat rabies (VBR) rely mainly on reducing vampire bat populations by culling.

For decades, the most efficient method for vampire bat population control has been the use of a topical poison known as “vampiricide”. Developed in the 1970s, the vampiricide is a mixture of warfarin, an anticoagulant that, upon ingestion, causes death by internal bleeding [6,13]. The vampiricide is delivered to bats topically. Vampire bats are captured, treated by applying the poison to the skin, and released back to their colony or roost.

Vampire bats can congregate in small groups (<100) or colonies of tens to thousands of individuals. Females with their offspring usually roost together with a dominant male, and other satellite or bachelor roosts are within the vicinity [14]. Because vampire bats are highly social and active groomers [15–17], the poison is transferred to others via close contact. Ingestion by several bats results in multiple deaths due to ingestion of the poison. While this practice explicitly targets vampire bats, its indiscriminate use (sometimes by non-trained people) can indirectly harm other bat species of ecological importance or conservation status. Moreover, culling has not been effective at curbing VBR, as the disease continues to spread and emerge in new areas [18]. In fact, the World Health Organization considers this practice obsolete [19]. Therefore, the development of alternative strategies for VBR control is advised, and considerable effort has been made to assess the feasibility of vaccinating vampire bats against rabies [9,20], especially since the development of different experimental rabies vaccine candidates for bats [21–28]. Recently we tested, in vampire bats, the protective efficacy of a vaccine candidate that utilizes raccoon pox (RCN) as a viral vector for a mosaic gene expressing rabies glycoprotein (MoG) [25,28] previously shown to protect big brown bats (*Eptesicus fuscus*) from rabies

As with other wildlife reservoirs, ensuring proper delivery and uptake of a rabies vaccine by vampire bats will be critical for vaccination to be effective. Considering the efficient use and applicability of vampiricide, a rabies vaccine for vampire bats could be easily delivered to wild bats following this same mechanism of application and dispersion. Recent studies demonstrated that vaccination of vampire bats using a transferrable vaccine could increase population level immunity against rabies [20]. Using Rhodamine B as a biomarker, this study in Perú estimated that per every bat treated (topically), 0.23 – 1.25 other bats could potentially be vaccinated via contact and transfer within the colony [20]

The overall goal of this pilot study was to assess the potential delivery of vaccines to populations of wild vampire bats using a topical mixture of glycerin jelly and a biomarker (Rhodamine B). Specifically, we wanted to measure the transfer rate (via contact) of the topical mixture by quantifying biomarker uptake in treated and un-treated (but in contact) bats.

These results could serve as the basis for a field vaccine trial in wild populations of vampire bats using RCN-MoG.

Methods

Selection of biomarker, topical vehicle, and preparation of the biomarker gel

Rhodamine B (RB) is a xanthene dye used as a biomarker for wildlife research. It is a safe and non-toxic product and has been used successfully as a method to assess the uptake of oral vaccine baits [20,29–32]. RB can be readily detected in tissues such as hair and whiskers within 24 hours of consumption as fluorescent bands when examined using fluorescent microscopy (with an excitation wavelength of 540 nm and an emission wavelength of 625 nm)(Figure 1). Based on different doses tested during the vaccine efficacy study (Chapter 3), we observed that a bat consuming at least 0.3 mg of RB would show strong fluorescence in the hair follicle. We used laboratory-grade glycerin jelly (Carolina Biological Supply, Burlington, NC, USA) as the topical vehicle mixed with RB, since previous studies demonstrated its superiority over other substances (e.g., petroleum-based paste) in maintaining RCN viral titers adequately at different ambient conditions, proper adhesion to bats´ fur, and palatability [33]. For field application, a total volume of 400 ml of biomarker gel (for ~ 300 bats) was prepared as follows: 178 ml of glycerin jelly, 600 mg of Rhodamine B (≥95% (HPLC, Sigma-Aldrich, Darmstadt,

Germany) for a concentration of 0.15%, and 222 ml of commercially available distilled water. Each bat treated received 1.2 ml of the gel applied on the chest and back using a ¼ teaspoon measuring spoon.

Study field sites

We needed field study sites with the following characteristics: locations with known colonies of vampire bats of >100 individuals, relatively easy access, and where rabies control activities (i.e., vampiricide application) had not been performed in at least the previous six months. We relied on the Mexican National Service for Agro-alimentary Public Health, Safety and Quality (SENASICA), who oversees the Mexican rabies campaign, to select appropriate regions to work. They designated the states of Yucatán and Jalisco, which are located in the southern peninsula (Yucatan) and by the Pacific coast (Jalisco). The rabies program coordinator located the ideal sites based on previous monitoring as part of their control and surveillance activities (Fig 2).

Vampire bat captures, treatment, and sampling

Fieldwork was divided into two sessions: one session for estimating the colony size and applying the biomarker, and the second session for sample collection only. Bats were captured using mist or butterfly nets and placed in a paper bag until processed. Processing of bats included determining sex and age, placing a metal band on the wrist for identification (4mm, Porzana, Inc.) [34], and obtaining a hair sample (10-20 hairs) plucked from the rump. The hair samples were placed in a plastic bag, identified with the bat´s number, and stored at ambient temperature

until analysis. The biomarker was applied at the end of the work night, before releasing the bats. We captured on two consecutive nights in the first session and estimated the colony size using the Lincoln-Petersen index based on mark-recapture techniques [35]. The aim was to treat 20-40% of the estimated population (over the two nights). On the second night, as we reached the application target goal, we stopped applying biomarker but continued processing all bats and collecting hair samples. For the second session, starting at least one week later, we conducted one or more capture nights to obtain enough hair samples for analysis. For reference, we estimated the prevalence of biomarker based on the assumption that 1.25 bats would be treated by contact for every bat treated directly, as observed by Bakker et al. [20] and calculated a sample size required using an online calculator (www.epitools.ausvet.com.au/prevalence). Because we captured more than two nights in each site, we re-calculated the colony size using the Schnabel method (which allows for a more precise estimation of population size [36,37] and report the proportion of bats treated and results based on this estimation.

Results and conclusion

In Yucatán, we conducted fieldwork in a private cattle ranch in the municipality of Huhí (location 20.7050589 -89.2006095). We captured the vampire bats at night by placing the mist nets around a cattle corral (holding eight adult cows). The bats roosted in a cenote (natural sinkholes with underground cavern systems) next to the corral and foraged daily on the cattle. We conducted captures for four nights on days 0, 1, 6, and 14 post-application of the biomarker. On days 0 and 1, we followed the “capture session one” protocol as described above. We captured 142 individuals (sex ratio 56% male, 70% adults) and applied treatment (1.2 ml of biomarker) to 76 bats over the first two nights. The proportion of males and females receiving

treatment was 53.9% and 46.1%, respectively. Based on the Schnabel method, the adjusted colony size was estimated to be ~346 vampire bats. Therefore, 21.9% of the estimated colony size received treatment. Upon analyzing the hair samples at the NWHC, of 167 samples collected, 67 were positive, 97 were negative, and 3 were indeterminate. Of the positive samples, 45 were attributed to transfer, as they were obtained from bats that did not receive direct application of RB. Adding the samples positive by transfer to the known number of bats treated (76), then an estimated proportion of 34.9% bats was positive for RB uptake either by direct application or by transfer from another treated bat. Most of the bats with RB transfer were adults (32/45), and the proportion of male (44.4.%) and female (55.6%) was similar (p=0.35). Three species of non-hematophagous bats were captured and sampled, but none showed fluorescence in the hair sample.

In Jalisco, the field site was located in the municipality of Unión de Tula. The colony of vampire bats was located inside an abandoned tunnel that served as drainage for a nearby reservoir (Presa Tacotán) years before (location 20.036918, -104.323043). On this site other bat species (*Leptonycteris* spp. and *Pteronopus* spp.) and groups of *Desmodus* roosted close to each other. The tunnel had only one entrance and an estimated distance of 400 meters to the end. Given the characteristics of this site, we were able to capture during the day and use butterfly nets instead of mist nets. This approach was ideal since we were able to target the roosts of vampire bast and avoid bycatching other bat species. Our captures consisted of 2-3 periods of 15 minutes each, using two butterfly nets to trap vampire bats. We captured on days 0, 1, and 8 post-application of the biomarker. We captured 371 (sex ratio 37% male and 81% adults) vampire bats and treated with RB a total of 124 over the first two nights. We also captured 19 non-hematophagous bats unintentionally, from which we collected hair samples, but no other

treatment was given. Since the male/female ratio was uneven, we tried to apply RB to many male bats to have a similar proportion. Overall, we treated 48.4% of males and 51.6% of females. The adjusted colony size using Schnabel was estimated to be ~552 vampire bats. Therefore, the proportion of treated bats in the estimated colony was 22.4%. A total of 482 hair samples were collected (not accounting for those from non-hematophagous bats) and analyzed. Based on microscopic analysis, 165 were positive, 83 were indeterminate, and 164 were negative. The number of positive samples due to transfer was 37. Therefore, an estimated proportion of 29.1% of the bats ingested RB by direct application or by transfer. Most of the bats positive due to transfer were adults (28/35), and almost all were females (94.5%). For this site, we observed a high number of samples designated as "indeterminate", meaning that the strength of fluorescence was not enough to consider it positive but was distinguishable brighter than background fluorescence. A total of 23 "indeterminate" bats were observed, and none had received RB directly. If the indeterminate samples were considered transfer positive, then the overall proportion of bats marked (RB uptake) would increase to 33.3%. None of the 19 non-hematophagous bats captured showed fluorescence in their hair sample.

The difference in the proportion of the overall RB uptake between the two sites was not significant ($p=0.068$). In Jalisco, the higher rate of females captured and showing positive RB transfer may be explained by the colony's higher proportion of this sex. Males were only 24% (137/552) of the colony based on the estimated colony size. Possible explanations are that the tunnel could have been a maternal colony, or our capture technique was biased.

This pilot study aimed to assess the feasibility of using a topical vehicle as a means to deliver oral vaccines to wild vampire bats. Unfortunately, due to the failure of the biomarker, our

pilot study did not provide meaningful data to assess the transfer of the glycerin jelly topical "sham" vaccine. We observed the crystallization of the Rhodamine B in the glycerin jelly (Figure 3). This crystallization likely affected the uptake of the biomarker, as evidenced in the high proportion of "indeterminate" samples in Jalisco but not for Yucatán. We used glycerin jelly as the topical vehicle, based on previous experience. The mixture of glycerin jelly and Rhodamine B has been used before in bat species by our research group. In the laboratory setting, other than losing consistency on contact with the bats due to the warm body temperature, no other complications have been common with the glycerin jelly. However, for this field study, the topical vehicle was not ideal. Some considerations, such as handling and storage of the delivery medium could be considered. The product used in Jalisco was prepared at the same time as the one for Yucatán. However, it was not used until ~3 weeks later. Both mixtures were prepared from a single batch.

Other products have been of interest as suitable vaccine delivery systems. For example, nanoparticles (such as chitosan) have been used in studies for rabies vaccines [38–41] and offer possible options. The observations made in this pilot study highlight the challenges of translating results from controlled experiments in the laboratory to field conditions and should encourage further research on the applicability of vaccines for bats.

Ethical statement

Permit for vampire bat captures were granted SEMARNAT (permit 19/LW-0120/11/20). All handling and procedure protocols were reviewed and approved by the USGS ACUC (protocol #EP190528.A1).

Figure 1. Hair sample obtained from common vampire bat. Fluorescence is as expected for . The technique is subjective and a is only determined as negative, positive (from mild – strong fluorescence), or indeterminate.

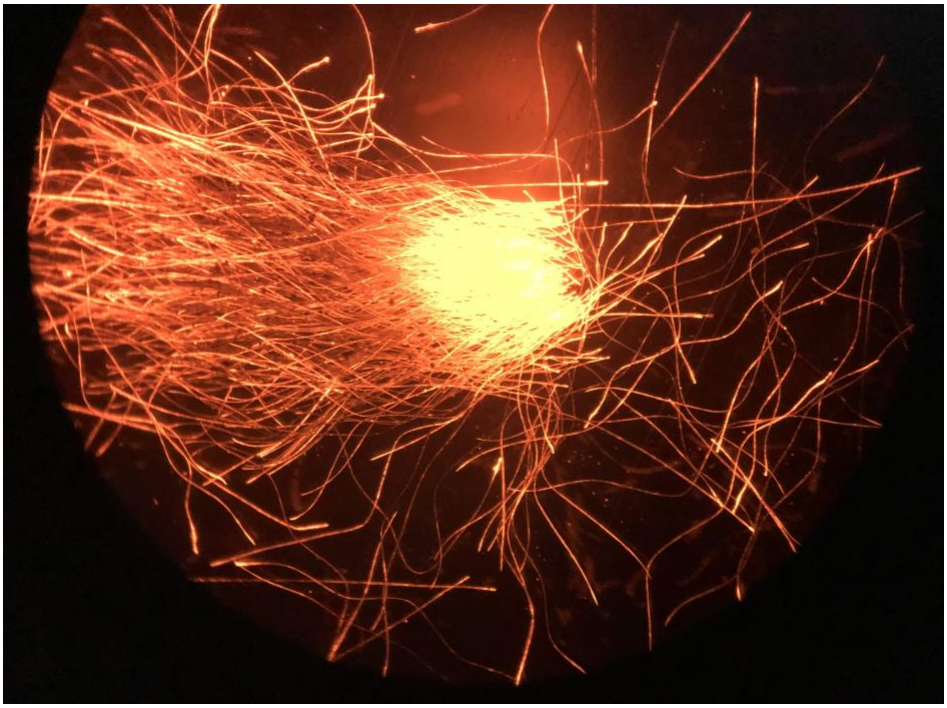
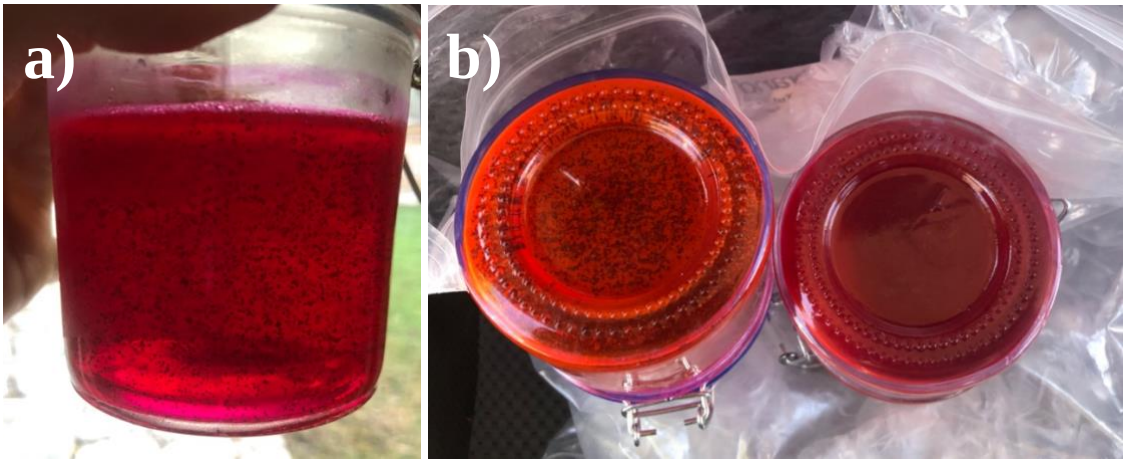


Figure 2. Study sites for the assessment of biomarker in common vampire bats in the Mexican states of Jalisco and Yucatan.

	Captured	Treated
Yucatán	142	76
Jalisco	371	124

Figure 3. A mixture of glycerin jelly and Rhodamine B was used to assess the transfer rate of a "sham" vaccine in colonies of common vampire bats (*Desmodus rotundus*), showing crystallization of the product causing the failure to assess transfer rates among vampire bats accurately.



a) The biomarker gel in the left shows RB crystallized and precipitated.
b) The color difference between the two jars shows the lack of homogenization of RB that could have influenced the detection in hair samples of vampire bats after ingestion of the biomarker gel. The right jar shows the appropriate color and homogenous mix of the RB. This product was used in the Yucatán field site

Acknowledgments

Special thanks to the field teams in Mexico for their invaluable support. In Yucatán: Alfredo Colín, Daniel Córdova, Juan Córdova, Ariadna Beb, Sabino Frías, Víctor Frías, Fernando and Brayan Balan. In Jalisco: Roberto Jáuregui, Sergio Baltazar, Jorge García, María Magdalena Ramírez, Nancy López. This work was supported by funds from a Graduate Student Research Award from the University of Wisconsin—Madison Global Health Institute, and a University of Wisconsin Fall Competition Research Award. Elsa M. Cárdenas Canales was supported by the Morris Animal Foundation Fellowship Training Grant (#D19ZO – 404).

Author contributions

Conceptualization, E.M.C.C., T.E.R.; methodology, E.M.C.C.; writing—original draft preparation, E.M.C.C.; writing—review and editing, All.; visualization, E.M.C.C.; supervision, J.E.O., and T.E.R.; project administration, J.E.O., T.E.R.; funding acquisition, E.M.C.C., J.E.O., and T.E.R.

References

1. Cross ML, Buddle BM, Aldwell FE. The potential of oral vaccines for disease control in wildlife species. Vet J [Internet]. 2007;174(3):472–80.

2. Rosatte RC, Tinline RR, Johnston DH. Rabies Control in Wild Carnivores. Rabies. 2007. 595–634 p.

3. Barnett KM, Civitello DJ. Ecological and Evolutionary Challenges for Wildlife Vaccination. Trends Parasitol [Internet]. 2020 Dec;36(12):970–8.

4. Maki J, Guiot AL, Aubert M, Brochier B, Cliquet F, Hanlon CA, et al. Oral vaccination of

wildlife using a vaccinia-rabies-glycoprotein recombinant virus vaccine (RABORAL V-RG®): A global review. Vet Res. 2017;48(1):1–26.

5. Rupprecht CE, Hanlon CA, Slate D. Oral vaccination of wildlife against rabies: opportunities and challenges in prevention and control. Dev Biol. 2004;119:173–84.

6. Arellano-Sota C. Biology and control of the vampire bat. Rev Infect Dis. 1988;10(Issue supplement 4):S615–9.

7. Lee DN, Papeş M, van Den Bussche RA. Present and potential future distribution of common Vampire bats in the Americas and the associated risk to cattle. PLoS One. 2012;7(8):1–9.

8. Banyard AC, Davis A, Gilbert AT, Markotter W. Bat rabies. In: Rabies [Internet]. Elsevier; 2020. p. 231–76.

9. Benavides JA, Valderrama W, Recuenco S, Uieda W, Suzán G, Avila-Flores R, et al. Defining New Pathways to Manage the Ongoing Emergence of Bat Rabies in Latin America. Viruses. 2020 Sep 8;12(9):1002.

10. Ruiz M, Chávez CB. Rabies in Latin America. Neurol Res. 2010;32(3):272–7.

11. Escobar LE, Peterson T, Favi M, Yung V, Medina-Vogel G. Bat-borne rabies in latin America. Rev Inst Med Trop Sao Paulo. 2015;57(1):63–72.

12. Meske M, Fanelli A, Rocha F, Awada L, Soto PC, Mapitse N, et al. Evolution of Rabies in South America and Inter-Species Dynamics (2009–2018). Trop Med Infect Dis. 2021 Jun 9;6(2):98.

13. Linhart SB, Flores Crespo R, Mitchell GC. Control of Vampire bats by topical application of anticoagulant, Chlorophacinone. Bol Of Sanit Panam. 1972;1(2).

14. Arthur M. Greenhall US, editor. Natural History of Vampire Bats . Boca Raton, FL: CRC Press; 1988. 231–232 p.

15. Wilkinson GS. The social organization of the common vampire bat - I. Pattern and cause of association. Behav Ecol Sociobiol. 1985;17(2):111–21.

16. Wilkinson GS. Reciprocal food sharing in the vampire bat. Nature. 1984 Mar 8;308:181.

17. Thompson RD, Mitchell GC, Burns RJ. Vampire Bat Control by Systemic Treatment of Livestock with an Anticoagulant. Science. 1972 Sep 1;177(4051):806–8.

18. Streicker DG, Recuenco S, Valderrama W, Benavides JG, Vargas I, Pacheco V, et al. Ecological and anthropogenic drivers of rabies exposure in vampire bats: Implications for transmission and control. Proc R Soc B Biol Sci. 2012;279(1742):3384–92.

19. WHO Expert Consultation on Rabies. Second report. World Health Organ Tech Rep Ser. 2013;(982):1–139.

20. Bakker KM, Rocke TE, Osorio JE, Abbott RC, Tello C, Carrera JE, et al. Fluorescent biomarkers demonstrate prospects for spreadable vaccines to control disease transmission in wild bats. Nat Ecol Evol. 2019;3(12):1697–704.

21. Sétien AA, Brochier B, Tordo N, De Paz O, Desmettre P, Péharpré D, et al. Experimental rabies infection and oral vaccination in vampire bats (Desmodus rotundas). Vaccine. 1998;16(11–12):1122–6.

22. Aguilar-Setién A, Campos YL, Cruz ET, Kretschmer R, Brochier B, Pastoret PP. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J Wildl Dis. 2002;38(3):539–44.

23. Almeida MF, Martorelli LFA, Aires CC, Sallum PC, Massad E. Indirect oral immunization of captive vampires, Desmodus rotundus. Virus Res. 2005.

24. Almeida MF, Martorelli LFA, Aires CC, Barros RF, Massad E. Vaccinating the vampire bat Desmodus rotundus against rabies. Virus Res. 2008;137(2):275–7.

25. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.

26. Gilbert AT, Wu X, Jackson FR, Franka R, McCracken GF, Rupprecht CE. Safety, immunogenicity, and efficacy of u¡intramuscular and oral delivery of ERA-G333 recombinant rabies virus vaccine to big borwn bats (Eptesicus fuscus). J Wildl Dis. 2020 Jul 2;56(3):620.

27. Griffiths ME, Bergner LM, Broos A, Meza DK, Filipe A da S, Davison A, et al. Epidemiology and biology of a herpesvirus in rabies endemic vampire bat populations. Nat Commun. 2020;11(1):1–12.

28. Stading BR, Osorio JE, Velasco-Villa A, Smotherman M, Kingstad-Bakke B, Rocke TE. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox vectored rabies vaccine in the Brazilian Free-tailed bat (Tadarida brasiliensis). Vaccine. 2016;34(44):5352–8.

29. Fernandez JR-R, Rocke TE. Use of Rhodamine B as a Biomarker for Oral Plague Vaccination of Prairie Dogs. J Wildl Dis. 2011 Jul;47(3):765–8.

30. Rocke TE, Tripp DW, Russell RE, Abbott RC, Richgels KLD, Matchett MR, et al. Sylvatic Plague Vaccine Partially Protects Prairie Dogs (Cynomys spp.) in Field Trials. Ecohealth. 2017;14(3):438–50.

31. Fry TL, Atwood T, Dunbar MR. Evaluation of rhodamine B as a biomarker for raccoons. Human-Wildlife Interact. 2010;4:275–82.

32. Weerakoon MK, Price CJ, Banks PB. Hair type, intake, and detection method influence Rhodamine B detectability. J Wildl Manage. 2013;77(2):306–12.

33. Stading author BR. Development of a novel rabies mosaic antigen and the use of attenuated poxviruses as vaccine vectors in bats. 2016.; 2016.

34. Barnett A, Dutton J. Expedition Field Techniques: bats. Vol. 22, Journal of Zoology. 1995. 1–140 p.

35. Lettink M, Armstrong DP. Mark-recapture analysis for monitoring threatened species. Dep Conserv Tech Ser 28. 2003;28:5–32.

36. Krebs CJ. Ecological methodology. New York, NY: Harper and Row Publishers Inc.; 1989.

37. O'Donnell C, Sedgeley J. Inventory and monitoring toolbox: bats. New Zealand; 2012.

38. Wu X, Yang Y, Kling C, Seigler L, Gallardo-Romero NF, Martin BE, et al. Inactivated rabies virus-vectored immunocontraceptive vaccine in a thermo-responsive hydrogel induces high and persistent antibodies against rabies, but insufficient antibodies against gonadotropin-releasing hormone for contraception. Vaccines. 2019;7(3).

39. Del Médico Zajac MP, Garanzini D, Pérez OR, Calamante G. Recombinant Veterinary Vaccines Against Rabies: State of Art and Perspectives. Emerg Reemerging Viral Pathog. 2020;225–42.

40. Bansal A, Gamal W, Menon IJ, Olson V, Wu X, D'Souza MJ. Laser-assisted skin delivery of immunocontraceptive rabies nanoparticulate vaccine in poloxamer gel. Eur J Pharm Sci [Internet]. 2020;155(July):105560.

41. Mendoza-Guevara CC, Ramon-Gallegos E, Martinez-Escobar A, Alonso-Morales R, Ramos-Godinez M del P, Ortega J. Attachment and in vitro transfection efficiency of an anti-rabies Chitosan-DNA nanoparticle vaccine. IEEE Trans Nanobioscience. 2021;1–1.

CHAPTER 5

Identifying factors that influence the adoption of a vampire bat rabies vaccine among the Mexican rabies control program personnel

Elsa M. Cárdenas-Canales, Hernando Rojas

Abstract

In Latin America, rabies transmitted by the common vampire bat (*Desmodus rotundus*) is a burden to the public health and veterinary sectors. In Mexico, vaccination of livestock and culling of vampire bats are the two methods employed by the National rabies control program to prevent the disease in livestock. Despite these control efforts, rabies continues to spread, threatening livestock and humans, and the need for alternative control strategies has increased. Vaccination of vampire bats has been proposed for this purpose, and the recent development of rabies vaccines for bats is promising. However, the existence of a novel vaccine does not guarantee that it will be readily adopted for use. To understand what factors can influence the adoption of vampire bat vaccination as a potential disease control strategy , we surveyed the personnel of the Mexican rabies control program (n=86). The goal was to assess their perception on various topics: vaccination of vampire bats, the efficacy of the current rabies program, and attitudes toward vampire bats. We found that the participants are very supportive of using rabies vaccination for vampire bats (a score of 7.4 on a scale of 1 - 10). Their support was influenced by two main factors: the availability of vaccines with some desired characteristics (e.g., price and efficacy) and the belief that vaccination helps control rabies. The perceived impact of rabies in the participant's region, or demographics, such as position or education level, did not have a role in predicting the support of vaccination. More research is needed before the vaccination of vampire bats becomes a reality (e.g., field trials of vaccination efficacy in wild populations). However, the importance of studies like ours, conducted at the early stages in the process of integrating a novel strategy such as vaccination, will help identify the challenges in ensuring support from key stakeholders.

Keywords: rabies, vaccine adoption, vampire bats

Introduction

Rabies is a zoonotic viral disease with one of the highest mortality rates that affects humans and animals worldwide[1]. In the majority of the cases, the transmission of rabies is associated with rabid domestic dogs. For decades, dog-focused mass vaccination programs have led to a dramatic reduction, or the elimination, of dog-mediated rabies in humans [2]. In Latin America, many countries have employed this strategy to control rabies [3,4]. However, throughout the central and southern part of the continent, the common vampire bat (*Desmodus rotundus*) is currently the main reservoir of the rabies virus (RABV) and represents the major threat for transmission to humans and livestock. The burden of vampire-associated rabies (VBR) on Latin American countries can be overwhelming [5,6]. Therefore, many have established national programs to manage VBR based on measures aimed at protecting species at risk with vaccination and reducing populations of vampire bats from problem geographic areas [7].

The Mexican national campaign for the prevention and control of rabies in bovines and livestock species (hereafter "MRC", Mexican Rabies Campaign) was formally established in 2007 for this purpose [8]. Directed by the National Service for Agro-alimentary Public Health, Safety and Quality (SENASICA), the MRC currently operates in 26 states of México, where rabies is endemic or under control. The other six states are considered "rabies-free". The program relies on three core activities for controlling VBR in Mexico: 1) vaccination of livestock, 2) culling populations of vampire bats, and 3) providing workshops to the communities regarding rabies preventative measures. But these activities have a "reactive" approach since they are executed after a rabies outbreak in livestock is confirmed. Likewise, the activities are accomplished if and when resources (human and economic) are available. Many regional offices can be understaffed, and some suspect rabies reports do not receive attention [9]. Moreover,

many areas where rabies occurs are rural, inaccessible, or unsafe due to the illicit drug trade. Thus, a successful rabies control operation is not always possible, and the resulting consequence is the high prevalence of rabies cases, despite the program's annual effort.

Our research groups at the University of Wisconsin—Madison (U.W.) and U.S. Geological Survey (USGS) National Wildlife Health Center (NWHC) have been developing a recombinant rabies vaccine for use in bats [10,11]. Given the importance of the common vampire bat as a reservoir of rabies, we became interested in testing this novel rabies vaccine, with a future aim of implementing vaccination of bats as an alternative tool to control VBR. Since the initial stages of our proposed vaccine study (Chapters 2 and 3), we established a collaborative relationship with the MRC [12], which has been instrumental in the completion of our goals. Our intention is to continue this collaboration and, in the future, establish field trials in México to test the effect of vaccinating vampire bats against rabies. Consequently, our interest in conducting a study about the perception of vampire bat vaccination is intended to include this group.

We recognize, though, that factors such as the personnel's technical knowledge and engagement in the current control strategies also affect the success of the MRC program (besides the epidemiology of VBR). Therefore, any novel strategy proposed to control VBR, such as vaccination, must also consider these factors and not only the scientific merit of its innovation. In this regard, the local rabies control programs' willingness to adopt the vaccination of vampire bats will be essential to implementing this strategy in the field.

This study aims to identify the factors influencing the perception and adoption of a rabies vaccine for vampire bats among the personnel of the MRC. We will use the results of this study to produce tailored information materials regarding the development and use of a vampire bat vaccine and its potential role in controlling rabies in livestock. This will help guide the design of

vampire bat vaccination field studies implemented in the most suitable regions (e.g., willing to vaccinate) and with an epidemiological need.

Methods

Ethical statement

We received approval for this study from the University of Wisconsin-Madison, Education and Social Behavioral Science Institutional Review Board (IRB #2019-0708-CP001).

Population of interest

Our target population was the personnel of the MRC from the states that currently operate under the program, including some considered "rabies-free". The main requirement for inclusion was to be actively enrolled as an employee of the program, even if unpaid (e.g., volunteers with regular field participation). We procured the agreement of the SENASICA central office in Mexico City for this study, which provided us with a list of emails for each state's personnel. We received a list of 129 participants that fit the inclusion criteria, including state coordinators, technicians, student volunteers, and state program managers (Table 1).

Survey administration

This cross-sectional survey was first distributed online to participants on November 25, 2020, via an automated email using the distribution channel of the software Qualtrics (Qualtrics, CA). We sent eight reminders to those that had not completed the survey before closing the response collection on April 8, 2021. Participation was voluntary and those agreeing to

participate in the study completed and signed a written consent form before continuing with the survey questions. Of the 129 participants invited, we collected 96 responses for an overall response rate of 74.4%. However, of these 96 responses, only 85 were complete. Our final sample size was 86 responses because we included one participant that responded partially (78% progress). Nuevo León was the only state that did not respond to the survey (two invitees).

Questionnaire design

We designed the online survey to assess, among other variables, the perception of the current rabies control practices, attitudes toward vampire bats and the potential use of a bat-focused rabies vaccine (Table 1). The survey was created using the software Qualtrics and included 64 questions; the format was adapted from other knowledge, attitudes, and practices (KAP) surveys regarding rabies [5,13–15]. The researcher (a native Spanish speaker) developed the survey in Spanish and translated it into English for review of the IRB (appendix 1). We pilot tested the survey with nine coordinators of the MRC from different states. Only minor changes were made after piloting, mainly word choice and option selection.

We divided the survey into six sections: 1) demographics, 2) perception of current activities for VBR control practices, 3) perception about vampire bats, 4) opinion on the use of vaccination to control VBR, and 5) communication.

The survey included Likert scale questions, multiple-choice (single and multiple responses), and ranked questions. We measured the personnel's perception about the impact of rabies and vampire bats in livestock production, efficacy and importance of vaccination using a 10-point Likert scale (minor –great impact, not a problem–big problem, not efficient –extremely

efficient, not important –extremely important). Attitudes toward vaccination of vampire bats were asked in a 10-point Likert scale (completely against –entirely in favor). We also included a combination of matrix and 5-point Likert scale for questions regarding the perception about the current rabies program activities (e.g., rating effectiveness, success factors, vaccine characteristics, etc.). We used different question types, such as yes/no or multiple-choice, to capture personnel experiences regarding compliance with the program activities, VBR epidemiology (e.g., seasonality, trends), and vaccine knowledge. Section 6 (communication) included matrix/Likert, multiple-choice, and ranked questions. The aim was to assess the personnel's preferred and most efficient method for delivering them information regarding the development and use of a vampire bat-focused rabies vaccine.

To detect specific issues that cannot be captured in structured surveys, we prompted participants to expand on complex topics such as attitudes toward vampire bats or rabies vaccines for wildlife by using open-ended questions (e.g., "can you tell us why…"). Open-ended responses were revised and tabulated into categories for analysis.

Question q4.4 asked participants how often they perform rabies control activities during the year. However, to accommodate the impact on the frequency of work due to the ongoing COVID-19 pandemic, we modified the question to consider work during previous years and estimated the response.

Statistical analysis and variables of interest

We used the in-built statistical software in Qualtrics for descriptive statistics of demographic factors and exploratory data analysis. SPSS software (IBM SPSS Statistics for

Windows, Version 27.0, released 2020) was used for the statistical analyses (e.g., regression, ANOVA, reliability test).

Two of the questions in the survey were considered our criterion variables. The rest of the variables were grouped by themes and used in the exploratory model selection process (Table 1).

Questions using a Likert scale were tabulated, and the average was calculated as a whole. If a specific demographic group was of interest, the average for this group was calculated separately. One-way analysis of variance (ANOVA) was used for comparing group differences in variables of interest. Simple and multiple linear regression were used to predict effects between response variables (attitude to vampire bat vaccination and perception of vampire bat vaccine) and particular variables of interest as described in Table 1. Kendall's coefficient of concordance was used for assessing agreement in ranked questions.

A set of variables were created to simplify exploring the possible factors affecting the criterion variables. One was "State reports", which combined the participant states into a category (low/medium/high) based on the average number of rabies reports they received during 2018-2020. The information to create this variable was obtained from the MRC's open database. Interquartile ranges were used to designate the states into the low/medium/high categories (Table 1 and Figure 1). Variables such as perception of the efficacy of the program's activities and difficulty to perform the program's activities (each having a matrix with a set of Likert 5-point scales for each activity) were grouped into an "overall" variable after being tested for score reliability using Cronbach's alpha of > 0.5 [16]. The average of the new overall variable (in a 1 to 5 scale, too) was then used for analysis.

We asked participants to rate the importance of some characteristics of rabies vaccines for livestock, including price, efficacy, and route of administration. To assess if the same

characteristics would be desired in a vampire bat rabies vaccine, we repeated the question but for a bat vaccine instead and calculated the average score on a Likert scale of 1 (not important) to 5 (extremely important). Additionally, "overall" variables for both bat and rabies livestock vaccines were created as described above (reliability test), and the means of both were compared, segregated by demographics.

Results

Demographics

Sociodemographic variables are summarized in Table 2. Most of the respondents were males (89.5%, n=86). Project specialists (38%) and state coordinators (31%) account for the majority of the respondents. At least one person from each state (except Nuevo León) responded to the survey; all state coordinators participated. Regarding education level, most of the participants (82.6%) have a bachelor's degree. There is a strong relationship between the highest level of education and the position held, with those in higher-ranked positions (state committee managers) having graduate level education ($P<0.001$, $X^2=39.7$, $df=12$). Sixty-two percent of the respondents have been working for the MRC for 1-10 years. The mean age of respondents was 43.1 years old (median: 41.5, range: 23 to 65). Forty-one percent of the participants (n=35) owned some type of livestock. None of the demographics were significant factors predicting the support of vampire bat vaccination, or other variables of interest that contributed to explain vaccine support.

Criterion variable: support of vaccinating vampire bats

The average score for the support of vampire bat vaccination (question 6.14) was 7.52 (± 2.48 SD) in a Likert scale of 1-10, where 10 was "completely in favor" (Figure 2). Based on the open-ended responses, for those in favor of vaccinating vampire bats (with a Likert score of 7 or higher), the most frequent answer (mentioned by 27 of 47) was that "vaccinating bats would help prevent rabies cases in livestock since the virus would be controlled in the main vector". For respondents with a neutral or negative attitude to vaccination of vampire bats (Likert ≤6), additional costs, the lack of resources, and the complexity to vaccinate wild bats were frequent reasons to disagree with vaccination. This group mentioned that vaccination would not help control the frequency of vampire bat aggression (bites) in livestock, or it may even increase the problem. Lack of scientific evidence was a reason to doubt, or to be against vaccination, stated by some (n=8) as well as the feasibility of vaccinating vampire bats (e.g., "it is going to be difficult to capture so many bats to deliver the vaccine to them").

Following a systematic revision of our survey, we identified a final model that served as a predictor for supporting vaccination of vampire bats (Figure 3). The two variables included in this model were also tested against other variables to find potential factors explaining their importance and indirectly influencing vaccine support.

Overall, the support of vaccinating vampire bats was driven by the belief that this strategy can be helpful to control VBR and by the characteristics of a vampire bat vaccine candidate ($p=0.001$, $R^2=0.32$). Vaccine characteristics stands alone as a predictor, while the belief in vaccination is explained by variables such as plausible scenarios to vaccinate vampire bats and factors for accomplishing the program´s activities successfully ($p=0.004$, $R^2=0.166$).

We found that 61% of the participants believe that vaccination of vampire bats can help control rabies in livestock, compared to 8% that do not. The rest of the respondents (31%) did

not know if vaccination would be helpful (Figure 2). At the same time, two variables (perceived risk of the job and “after an outbreak” as a possible scenario for vaccinating vampire bats) were part of a model that helped explain the belief in a vampire bat vaccine being helpful to control VBR.

Next, we describe findings specific to our variables of interest and how/if they affected either the criterion variable or the belief in vampire bat vaccination.

Perception of the Mexican rabies program for rabies control in livestock

A new variable rating the overall perception of the program's effectiveness was created (Cronbach's alpha= 0.69). It grouped the scores of the three core activities plus an additional activity related to "case completion". The program was regarded as "very effective" in a Likert scale of 1 – 5 ($\bar{x}$ =4.08, ± SD 0.56). The position of the participant was a factor that favored a higher rate; with those of a higher rank (e.g., coordinators or regional managers) rating higher scores for the effectiveness of the program (p=0.035) (Table 3).

Similarly, a new variable rating the overall perception of the difficulty to complete the program's activities was created (Cronbach's alpha= 0.71). In a Likert scale of 1 (extremely easy) to 5 (extremely difficult), the overall difficulty was rated with a 2.9 (± SD 0.65), corresponding to a neutral response. None of the demographics impacted this response. Neither the efficacy nor the difficulty variables had an effect on supporting vampire bat vaccination or in believing that a vampire bat vaccine would help control VBR.

We also asked participants to rate how important were a list of factors for them to successfully complete the activities required by the program. The most important factor (ranked

in a Likert scale of 1 to 5) was the availability of resources ($\bar{x}$= 4.28, ± SD 0.92) followed by the level of safety in the areas where the job is performed ($\bar{x}$= 4.08, ± SD 0.93). Table 4 shows the average scoring for each factor and the total percent of the participants that scored them as "extremely important" (a score of 5). To note, the rest of the variables (e.g., access to communities, support from locals, safety, etc.) were significantly correlated among each other and could be considered important to successfully fulfill the rest of the program´s tasks. The belief that vaccination of vampire bats can be helpful to control VBR was predicted by a model including "labor risk", the risk associated with performing the program´s activities (p=0.004, R^2=0.166). Those rating the importance of labor risk higher tend to believe that vaccination can help control VBR (*B*=0.268).

Attitudes toward vampire bats

The perception of vampire bats as a problem for the livestock industry scored high in a Likert scale of 1 – 10, where the average score was 7.91 (±SD 2.1). Similarly, a high proportion of participants think that vampire bats should be eliminated (60%), while only 28% think they should be protected, and 12% did not know. The relationship between the negative perception of vampire bats and thinking that they should be eliminated is significant (p=0.025, R^2=0.065, *B*=-0.255). Cattle ownership was the only demographic variable that had an effect on the negative perception of vampire bats. Respondents who own cattle (41%) tend to consider bats more as a problem (p=0.037, R^2=0.052, *B*=-0.227).

When testing for any effect of both perception of and attitude toward vampire bats, these were not important predictors for supporting vaccinating vampire bats or believing that vaccination could be helpful for VBR control.

Perceptions of rabies vaccines for livestock and vampire bats

The mean scores for a list of desired characteristics (e.g., accessible price, efficacy) in rabies vaccine for livestock and vampire bats are summarized in Table 5. Overall, participants rated the importance of both vaccine characteristics equally, and no differences were detected by demographic variables. Scoring high on a Likert scale of 1 (not important) to 5 (extremely important) for the vampire bat vaccine characteristics resulted in a strong predictor for supporting the use of a vampire bat rabies vaccine ($p=0.001$, $R^2=0.32$, $B=0.341$). However, vaccine characteristic was not related to the belief that a vampire bat rabies vaccine will help control VBR ($p=0.386$).

In an open question, the participants listed the most important information they want to receive about a vampire bat vaccine (Figure 4). “Application method” of the vaccine (n=42) accounted for 32% of the responses. Vaccine use and handling accounted for 17% of the mentions and it referred to the appropriate conditions for optimal use (e.g., maintenance under cold chain, storage). Efficacy was mentioned 13 times (10%). Other desired information was safety for humans and non-target species, and the long-term effect of the vaccine.

Burden of work

We asked participants how many vampire bat culling operations they usually perform. Overall, 47% of the respondents execute over 20 vampire bat culling operations in a regular year. A summary of the overall number of captures performed in a year by the low/med/high "State report" categories is in Table 6. Figure 6 shows the proportion of each frequency of capture by state category. Most vampire bat culling operations are performed by teams of 2-5 people (78%). Participants were asked if they preferred to have more people participating in the captures; 51% said the number of people is enough and 48% wanted more participants.

The variables that comprised the "burden" of work (Table 1) were included in a model to test their effect on both bat vaccine support and belief in a bat vaccine, but no significant effects were detected.

Perception of the impact of rabies and trends in vampire bat activity

Overall, respondents said that rabies has a somewhat big impact on livestock in their regions. In a Likert scale of 1 (little impact) to 10 (big impact), the average score was 7.45 (± SD 2.04). This answer was influenced by the frequency in which the respondents receive notifications of vampire bat aggressions to livestock ($p=0.001$). Congruently, the frequency of aggression notifications stated by the participants is strongly correlated with the low/med/high category we created for the states based on the average rabies reports from 2018-2020 ($p= 0.002$, $r=0.35$). While the low/med/high category for the average number of reports is not a significant predictor of rabies's impact, it is positively correlated with it ($r=0.28$, $p=0.006$) (i.e., high average of reports –bigger impact).

The model to test for the effect of the frequency of aggression notifications, the perceived trend of vampire bat aggressions in the last five years, and the rate of the rabies impact in the region did not provide a significant result. Although marginally significant, there was a positive correlation of the perceived bat aggression trend (i.e., an increase) with both the belief that a vampire bat vaccine can be helpful ($r=0.18$, $p=0.058$) and the support to adopt its use ($r=0.17$, $p=0.063$).

Knowledge and perception about wildlife rabies vaccines

We asked participants if they considered it important to vaccinate wildlife species against rabies. The mean score in a Liker scale of 1 (not important) to 10 (very important) was 7.33 (± SD 2.77), with 32% of participants choosing the highest score. Meanwhile, 49% of the respondents had heard/learned about the development of a rabies vaccine for wildlife species before. There was no significant relationship between the respondent's position and knowledge of a wildlife rabies vaccine ($p=0.07$). The knowledge about the existence of a rabies wildlife vaccine was a significant predictor for considering it important to vaccinate wildlife species ($p<0.001$, $R^2=0.452$) but not for believing that vaccinating vampire bats could be helpful ($p=0.238$) or for supporting the vaccination of vampire bats ($p=0.206$). Similarly, considering it important to vaccinate other wildlife species had no effect on the criterion variables.

Perception of the challenges and possible effect of vaccinating vampire bats

Participants were asked if they believed if vaccinating vampire bats against rabies would impact vampire bat populations. Forty-five percent of them said "yes", while the rest were evenly

divided between not knowing and thinking there would not be an effect (29% and 26%, respectively). From those who think that vaccination will have an effect, 72% believe that the population of vampire bats will increase, followed by 67% saying that vampire bat aggressions to livestock will also increase.

The perception about the possible effect of vaccination on vampire bat populations did not predict the respondents' support to vaccinate bats ($p=0.227$), nor their belief that vaccination of vampire bats could be helpful to control VBR ($p=0.261$).

Regarding some of the perceived challenges of vaccinating vampire bats, 82% of the participants chose a higher need for resources (i.e., funding and personnel). Other challenges were the complicated logistics for vaccination (61%) and the difficulty in locating vampire bat colonies (54%) (Figure 5). Only 16% of the participants commented on additional challenges, including vaccine-related qualities (e.g., effectiveness) and the need to obtain the agreement and collaboration of the livestock producers. The sum of the challenges selected by each person was used to create an "overall challenging score". For example, participants selecting fewer options (thus with a lower score) may be more willing to adopt a bat vaccine than those perceiving vaccination of vampire bats as more challenging. However, this variable did not have an effect on vaccine adoption or belief, nor was the demographic information correlated with considering vaccination of vampire bats challenging.

Scenarios to vaccinate vampire bats and other preferences

We provided the participants with a series of scenarios they would consider pertinent to vaccinate vampire bats (Figure 6). We found that 53% of them considered vaccinating vampire

bats after a rabies outbreak in livestock species. Thirty-two percent considered vaccination in areas where it is difficult to access vampire bat colonies, and 29% in rabies-free areas that border with rabies endemic areas. Thirty-five percent of the participants indicated that bat vaccination would be pertinent after receiving reports of vampire bats biting humans. However, while 54% of the respondents have been notified of vampire bat aggressions to humans; this response did not significantly correlate with choosing to vaccinate bats after bat aggressions to humans ($p=0.36$). When all the scenarios were included in a model to test for their effect on the criterion variables, we found that vaccinating vampire bats after an outbreak is a predictor for believing that vaccinating vampire bats can help control VBR ($p=0.004$, $R^2=0.166$, $B=0.264$) (in addition to labor risk).

Participants were asked to select the ideal route(s) to vaccinate vampire bats. We found that 62% preferred an aerosol delivery method, 48% a topical vaccine, 27% an oral vaccine and only 8% chose an injectable vaccine.

Access and preference to communication media

Most participants have access to a mobile telephone (93%) and a computer with an internet connection (82%). Communication platforms such as WhatsApp (92%) and email (82%) are used regularly by the participants.

According to their response, the most efficient method in which to receive official information (e.g., new activities, notifications, etc.) is by e-mail ($\bar{x}= 3.82$, ± SD 0.91), followed by WhatsApp ($\bar{x}= 3.76$, ± SD 0.89), both rated more efficiently than personal communication or newsletters. Although the participants' ranking showed a low degree of concordance ($W=0.344$,

p=0.142), an in-person workshop was ranked as the preferred method for receiving information about a rabies vaccine for vampire bats, followed by an instructional video.

Based on a Likert scale of 1 (not important) to 5 (extremely important), the participants stated that it would be "very important" ($\bar{x}$ =4.00, ± SD 0.9) to collaborate with the State and Municipal Governments if implementing the use of a rabies vaccine for vampire bats. It was rated as equally important to collaborate with the Ministry of Natural Resources (SEMARNAT) and the livestock owners ($\bar{x}$ =3.94). Collaboration with International Agencies or with universities was ranked in the lower ($\bar{x}$ =3.38, ± SD 1.07 and $\bar{x}$ =3.72, ± SD 0.9, respectively).

Discussion

It is encouraging to find that overall, the Mexican rabies program's personnel favor vaccinating vampire bats against rabies as a strategy to control VBR. Among those in favor, the most common reason for supporting the vaccination of vampire bats is that “rabies will be eliminated from the reservoir ”. This exact reason motivates us to study and develop a rabies vaccine for vampire bats [2,7,11,17–19]. The fact that the concept is already important for the MRC personnel is advantageous for advocating using an alternative strategy (controversial for some) to control VBR beyond lethal practices.

Our overall model showed that two paths might lead people to support (adopt) vaccination of vampire bats as a strategy to control VBR in México. One is the belief that vaccination of vampire bats can be helpful, and the other is the availability of a vampire bat vaccine that fulfills some desired characteristics.

We found that 61% of the respondents believe that vaccinating vampire bats will be helpful to control VBR. Moreover, this belief was partly explained by two factors: the influence of the job-related risk for accomplishing the program's tasks and the possibility to vaccinate vampire bats after a rabies outbreak in livestock. However, a concrete explanation of how the belief in vaccination of vampire bats is shaped (or not) is difficult [20]. As mandated by the MRC, livestock vaccination occurs after a case of rabies in livestock is confirmed. Perhaps familiarity with the reactive approach of the program influences people to believe the same for a vampire bat vaccine. We did not find that demographic variables (e.g., education level or position of participants) to influence this result.

Interestingly, the knowledge about other rabies vaccines for wildlife species was a predictor for thinking that it is important to vaccinate wildlife species. Yet, the importance of vaccinating wildlife species had no relationship with the belief in vampire bat vaccination.

The second significant factor for vaccine adoption was the availability of a rabies vaccine for vampire bats that satisfies a set of desired characteristics, including efficacy and protection, easy route of administration, safety, stability in field conditions, price, etc. (Table 5). The rabies vaccines used in México for livestock are two different types: modified live and inactivated virus [8]. Vaccine safety and strict handling conditions (e.g., maintaining a cold chain) are top concerns for appropriate and effective use. Recombinant vaccines (like our vaccine candidate RCN-MoG) are fundamentally different from live modified and modified rabies vaccines that the personnel are accustomed to use [21,22]. Some of the characteristics of recombinant vaccines (e.g., use recommendations, delivery approaches, and handling requirements) offer a range of advantages for their use, especially in wildlife species. However, the use of recombinant vaccines is not widespread in the livestock industry [23]. In our survey, we found that most of

the personnel are concerned about some of the characteristics of the bat vaccine based on their familiarity with livestock rabies vaccines. In fact, we asked participants if they knew what a recombinant vaccine was. Half of them said “yes”; however, most had an incorrect answer when asked to elaborate. Therefore, if vaccination adoption correlates with knowledge of vaccines, we should direct our efforts to effectively communicate the specifics about our vaccine candidate and the advantages that RCN-MoG offers for bats. Respondents mentioning that a “lack of evidence” is a reason for not being in favor of vaccinating vampire bats also reinforces the importance of transferring accurate information.

In the study, we expected other variables would impact the adoption of a vampire bat vaccine but did not. For example, we expected to see lower scores for supporting the vaccination of vampire bats if the participants thought that culling vampire bat populations was effective. However, even those participants who think that culling vampire bats is “extremely effective” support vampire bat vaccination. Another variable that we considered important for supporting vampire bat vaccination was how participants think rabies impacts livestock in their regions. While rabies was overall rated as causing a “somewhat big” impact on livestock, this perception was not related to supporting vampire bat vaccination. As we explored the variables included in the category of “perception of the impact of rabies”, we found that (with marginal significance), if participants thought that bat aggressions were increasing over the past few years, they would tend to believe that vaccination of vampire bats could help. This finding supports an initial reasoning that, perhaps, as VBR becomes more of a problem, people would start to open up to novel strategies regarding VBR control. Unfortunately, the evidence provided by this exploratory analysis is not sufficient for demonstrating that.

Additionally, none of the demographics (Table 2) seemed to have an effect on the criterion variable or on other variables that indirectly affected the criterion variables (e.g., important characteristics of a vampire bat vaccine). While this finding was unexpected, it could be an advantage in our case. For example, suppose vampire bat vaccination support is not segregated by position or education level. In that case, a single communication strategy can be enough to deliver a compelling message about the importance of vaccinating vampire bats across the MRC personnel. Although comprising most of the MRC personnel, our sample size was small and homogeneous (e.g., male-dominated, and all mostly holding the same education level); therefore, the lack of significance might be related to the sampled population.

Delivering information about vampire bat vaccination

While we cannot control people´s belief on vaccinating vampire bats, we can focus our efforts on delivering an accurate message about the potential of vaccinating vampire bats as a strategy for controlling VBR. Since we found that having a satisfactory rabies vaccine for bats predicts the adoption of vaccination, we should emphasize communicating the characteristics of our vaccine candidate and the logistics of a proposed field trial, if possible.

Some of the challenges perceived by the respondents regarding vaccinating vampire bats are that “it will be difficult to capture all bats” and not having specifics about “how the vaccine is going to be applied to bats”. Another critical challenge is the perception that more resources will be needed. These concerns reflect the most practical aspects of the current program´s operation but should not restrict the possibility of implementing bat vaccination as an additional control strategy. For example, most respondents said that the state and municipal governments are the

most important institutions to have good collaboration with. Even though the initiative of using a bat vaccine is coming from an international university, collaborating with universities and international agencies was perceived as less important. This exposes an area of opportunity for us to promote a multidisciplinary approach to combat VRB with the MRC and other stakeholders. Ultimately, support, not only from the government but also from the communities, will be needed for a successful implementation of bat vaccinations field trials.

As indicated by the respondents, a workshop would be the ideal method to deliver information about our vaccine candidate. Nonetheless, the respondents were also keen to receive information by video. Therefore, we can take advantage of the availability of communication tools (e.g., Zoom) to initiate communication with them regarding vaccine information.

Are we vaccinating the enemy?

The vaccination of vampire bats can be a controversial topic. A common complaint of resistance is the idea that vaccination will "protect vampire bats from rabies" leading to an increase of vampire bat abundance and thus resulting in more aggressions to livestock species or human attacks. Our survey confirmed this notion, as 72% and 67% of the participants think bat abundance and livestock aggression will increase, respectively. These proportions come from a total of 38 participants responding that vaccination will have an effect on bat populations. However, it is encouraging to find that the prevalence of these perceptions among the respondents was not a reason to be against vaccination or the belief that vaccination can help control VBR. Moreover, there is no evidence to allege that rabies acts as a natural suppressor of vampire bat populations.

In wild carnivores, rabies has been controlled (or eliminated) in many regions of Europe and the United States, thanks to the Oral Rabies Vaccination Program (ORVP). Since its conception in the early 1970s, the ORVP promoted a change in the approach to control rabies. Similar to VBR, the control of rabies in wild carnivores relied on culling. The program faced many challenges, similar to what can be expected with the proposal of vaccinating vampire bats. It took years to go from an experimental concept to a widespread and successful strategy for rabies control that continues to operate to date. As evidenced by the ORVP over the years, crucial elements make a program of such nature thrive: well-defined collaboration and planning, continuous communication exchange between agencies, policymakers, and stakeholders, and monitoring and surveillance of the program's outcome.

Implementing the vaccination of vampire bats as a strategy to control VBR will be challenging. It will require the approval for field studies and the assessment of the effect of vaccination at a large scale to justify its use. Yet, the promising results from our vaccine study (Chapter 3) should warrant further research of vaccination in the field. For this, we will need support from agencies such as the Mexican rabies control program and similar agencies in other countries. Based on the observations in this study, it is reasonable that we will be able to design a plan for future field trials based on regional needs and a higher index of vaccine acceptance by the Mexican Rabies control program employees.

Table 1. List of criterion variables and other variables of interest, grouped by theme, from questions included in the survey

Variable topic/category	Description of question	Question number
Agreement with vaccination of vampire bats (Criterion variables)	• Do you think vaccination will help control VBR?	6.12
	• Would you be in favor of or against vaccinating vampire bats?	6.14
Demographics	• Sex, age, job position, years working in the program, highest education level	2.3 –3.4
Perception on current activities for VBR control practices	• How effective are the activities of the program to control VBR?	4.3
	• How easy or difficult is it to perform the program activities to control VBR?	4.5
	• Influence of different reasons (e.g., availability of resources) to successfully complete their job	4.9
Burden of work	• How many captures/year?	4.4
	• How many team members participate per capture?	4.7
	• Would you prefer to have more team members during captures?	4.8
Perception of the impact of rabies and trends in vampire bat activity	• What is the impact of rabies on livestock in your region?	4.2
	• How often do you receive notifications of vampire bat aggressions?	5.1
	• Have you received a report of bat aggression to a human?	5.3
	• Have the aggressions of vampire bats decreased, remained the same, or increased?	5.6
Perception and attitude toward vampire bats	• Do you think vampire bats are a problem?	5.8
	• What should be done to populations of vampire bats?	5.9

Knowledge and perception about wildlife rabies vaccines	• How important is it to vaccinate wildlife against rabies?	6.9
	• Have you heard of a rabies vaccine for wildlife?	6.11
Perception about vaccination of vampire bats	• What would be the challenges of vaccinating vampire bats?	6.17
	• Do you think vaccination will have an effect on vampire bat populations?	6.18
Desired characteristics of a rabies vaccine (livestock or bats)	• What characteristics are desired in a rabies vaccine for livestock?	6.2
	• What characteristics are desired in a rabies bat vaccine?	6.20

Table 2. Demographics of the participating personnel from the Mexican rabies control program

Sex	Percent (n=86)	Frequency
Male	89.5%	77
Female	10.5%	9
Position		
State committee manager	7%	6
State project coordinator	31.4%	27
Project specialist	38.4%	33
Field technician	20.9%	18
Other	2.3%	2
Education status		
Junior High	1.2%	1
Highschool	7%	6
Bachelor'sBachelor's degree	82.6%	71
Graduate degree	9.3%	8
Years working in the program		
< 1 year	7%	6
1 – 10 years	62.8%	54
11 – 20 years	23.2%	20
> 20 years	7%	6

Table 3. Overall perception of the current activities for the control of rabies of the Mexican rabies control program (n=86) based on position held (excluding state regional managers) in the program. Perceptions about the activities were measured in a 5-point Likert scale from 1= not effective at all, to 5= extremely effective.

Perception	Overall score (mean ± S.D.)	Position		
How effective are the program activities to control rabies in livestock?		State Coordinators	Project specialists	Field technicians
Vaccination of livestock	4.24 ± 0.61	4.37 ± 0.63	4.09 ± 0.68	4.22 ± 0.43
Culling of vampire bats	4.07 ± 0.75	4.19 ± 0.75	3.94 ± 0.75	4.11 ± 0.83
Providing workshops to farmers	3.73 ±1.0	3.96 ± 0.96	3.67 ± 0.85	3.35 ± 1.28
Attention to case reports	4.28 ± 0.48	4.58 ± 0.5	4.12 ± 0.82	4.22 ± 0.65

Table 4. Perception of the importance of each factor for successfully completing the program's activities and percent of the participants rating each factor with the highest score (n=85)

Likert scores:

List of factors and their importance to perform the activities of the program	Mean	SD	% Participants rating "Extremely important"
Availability of resources	4.28	0.92	53%
Safety issues in the areas to work at	4.08	0.93	36%
Risk associated with performing program's activities (occupational hazard)	3.93	0.93	29%
Support and collaboration from communities and livestock owners	3.81	1.1	34%
Difficulty to locate and reach colonies of vampire bats	3.71	1.0	22%
Difficulty to access the local communities	3.60	1.1	19%
Number of personnel available to participate in the activities	3.59	0.93	13%
Number of reports	3.25	1.1	11%

1= not important
2= little importance
3= important
4= very important
5= extremely important

Table 5. Perception of the importance of some desired characteristics in rabies vaccines for livestock and vampire bats.

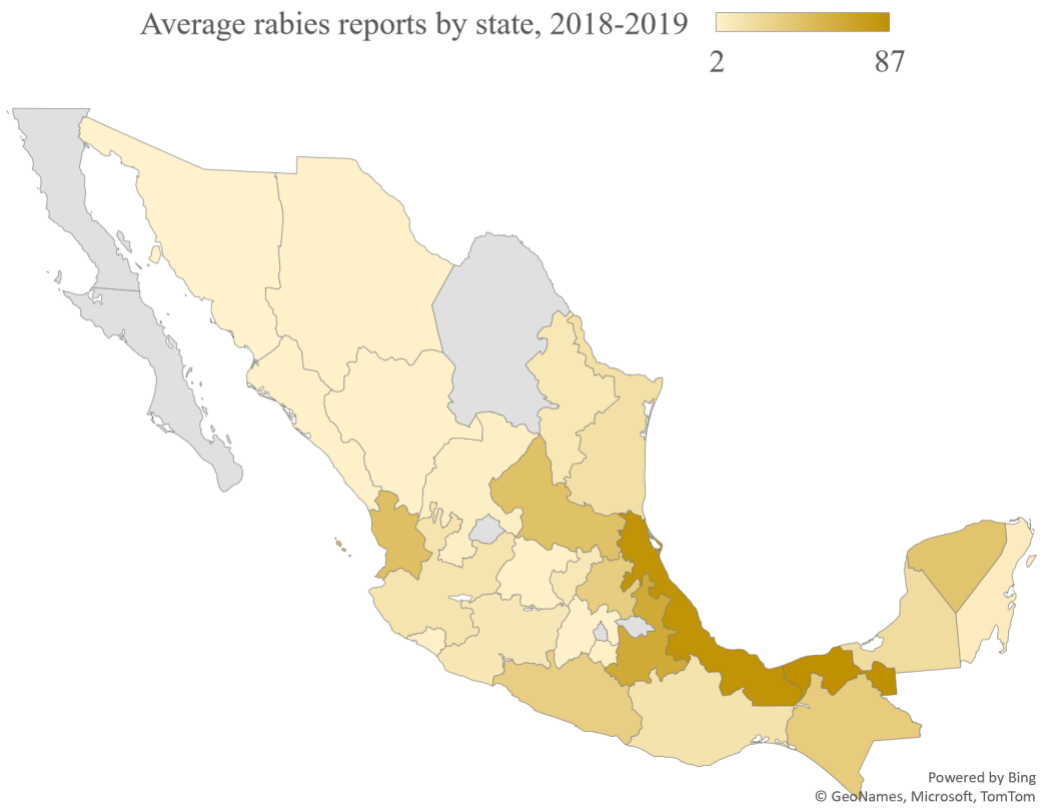
Desired characteristic	Livestock vaccine			Vampire bat vaccine		
	Mean	SD	n	Mean	SD	n
Efficacy and protection	4.62	0.57	85	4.46	0.72	82
Stability and durability in field conditions	4.27	0.81	84	4.27	0.83	82
Safe and harmless	4.21	0.77	85	3.70	1.03	83
Easy to obtain	4.14	0.67	85	4.06	0.82	82
Easy route of administration	3.88	0.76	85	4.28	0.79	82
Reasonable price	3.75	0.84	84	3.96	0.78	80
Safe for other bat species	-	-	-	4.27	0.83	82
Authorized by SENASICA	-	-	-	4.17	.87	82
Overall score	**4.15**	**0.52**	**85**	**4.15**	**0.63**	**83**

Likert scores:
1= not important
2= little importance
3= important
4= very important
5= extremely important

Table 6. Number of vampire bat captures executed in a year by category of rabies report "State report" (n=82)

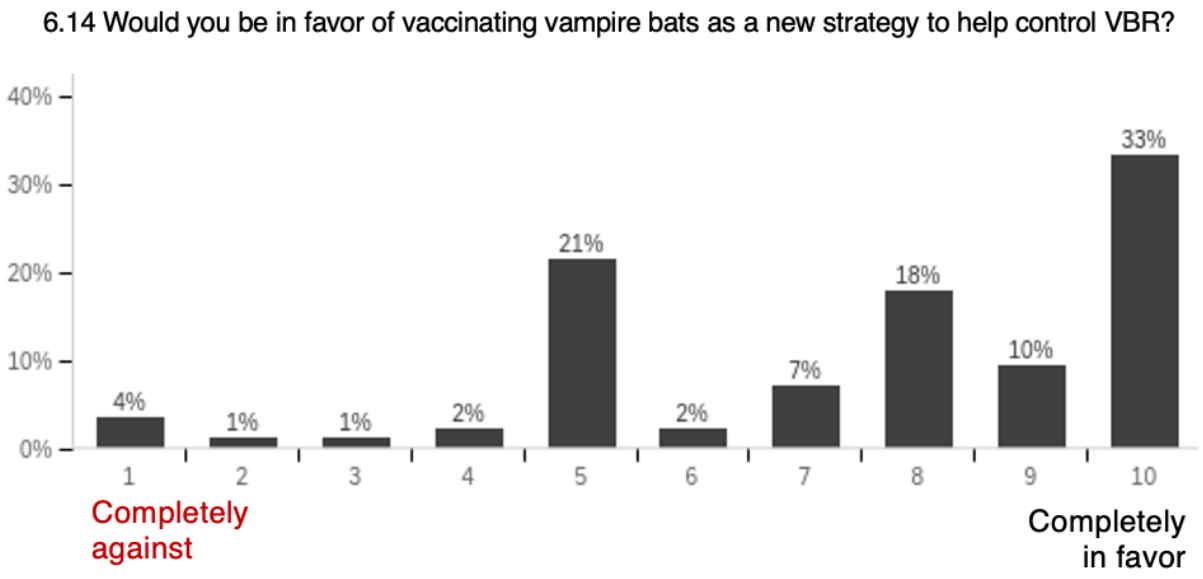
		States categorized by the average rabies reports received from 2018-2020		
Captures/year	Overall percent of capture frequency	Low (29)	Medium (32)	High (21)
none	10 % (8)	63% (5)	25% (2)	12% (1)
1 – 10	29 % (24)	42% (10)	29% (7)	29% (7)
11 – 20	13 % (11)	46% (5)	36% (4)	18% (2)
> 20	48 % (39)	23% (9)	48% (19)	28% (11)

Figure 1. "States reports": a variable created to group the states in a Low, Medium, or High category based on their mean number of cases reported between 2018 and 2020. Each state is individually colored based on the average rabies report.



Category	# Reports	States
Low	≤ 25	Colima, Chihuahua, Durango, Estado de México, Guanajuato, Morelos, Sinaloa, Sonora, Zacatecas
Medium	26-74	Campeche, Chiapas, Guerrero, Hidalgo, Jalisco, Michoacán, Oaxaca, Querétaro, Quintana Roo, Tamaulipas
High	≥ 75	Nayarit, Puebla, Tabasco, San Luis Potosí, Veracruz, Yucatán

Figure 2. Description of the response to the two criterion variables



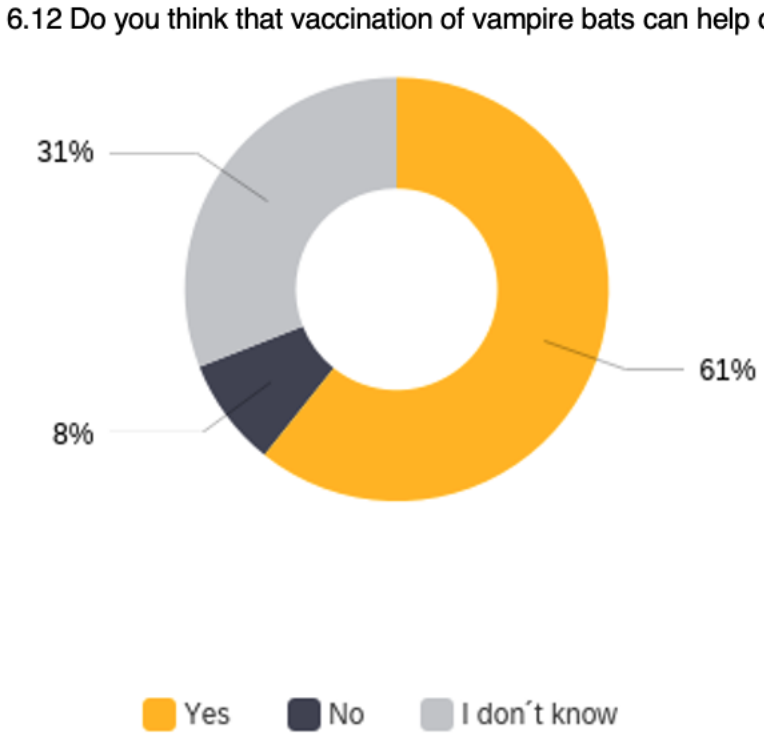


Figure 3. Map of variables that can have an effect on the adoption of a vampire bat vaccine

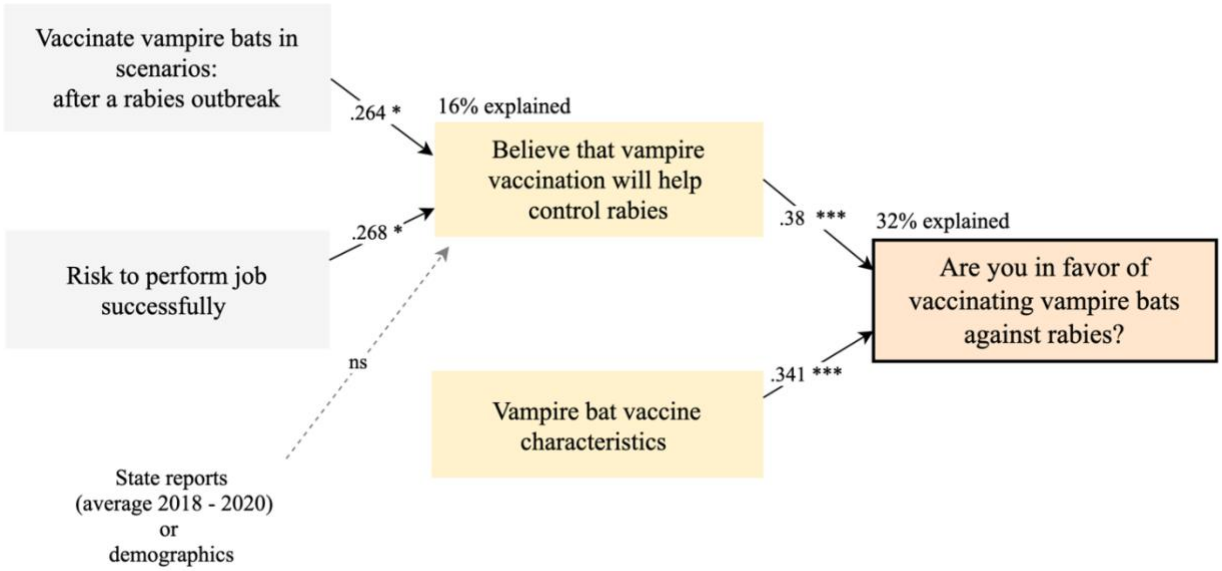


Figure 4. Participants' responses regarding what information they want to receive about a rabies vaccine for vampire bats (n=68 participants providing 132 mentions)

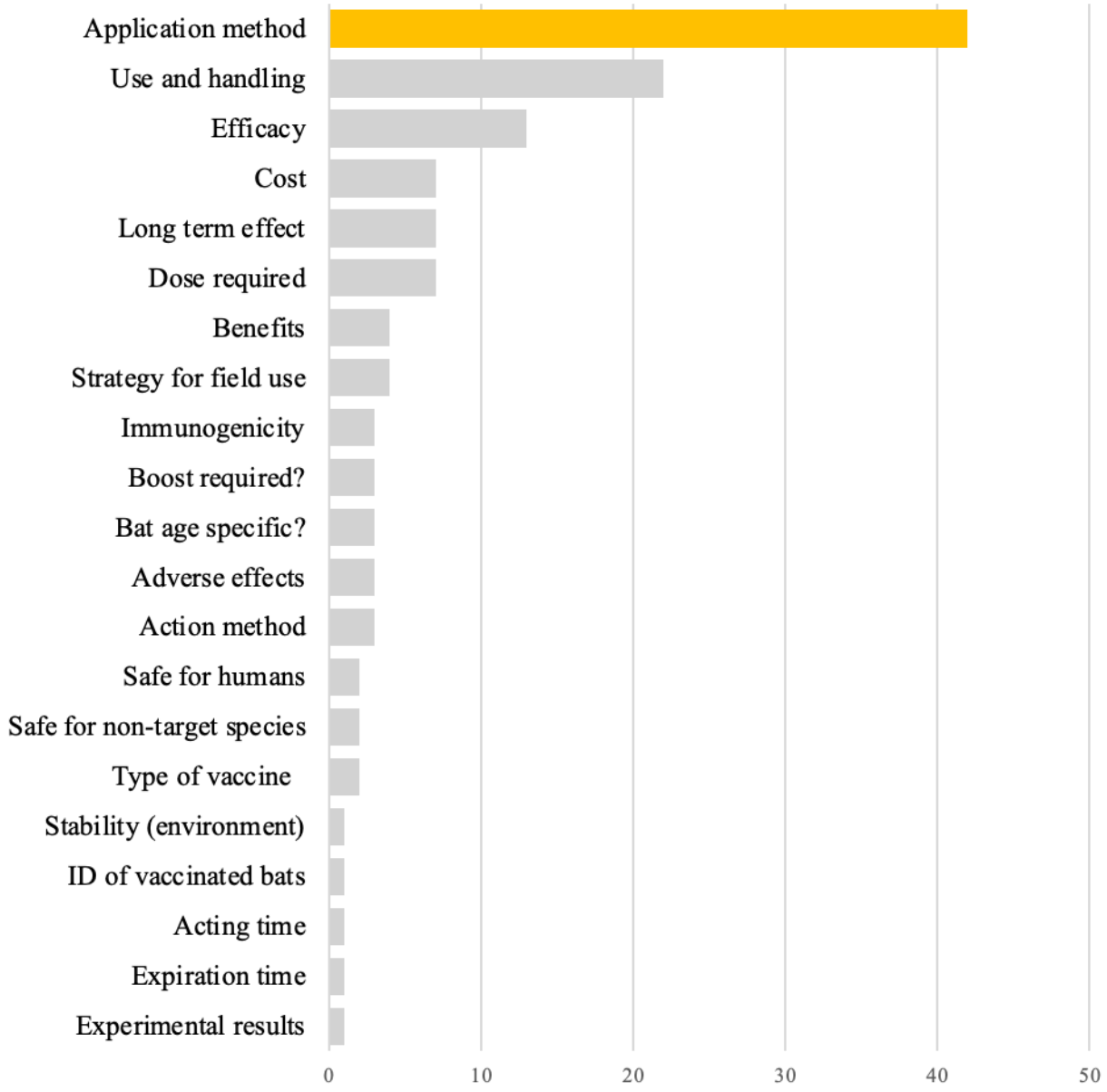


Figure 5. Perception on the main challenges to vaccinate vampire bast, percent selected form multiple choice question (n=86).

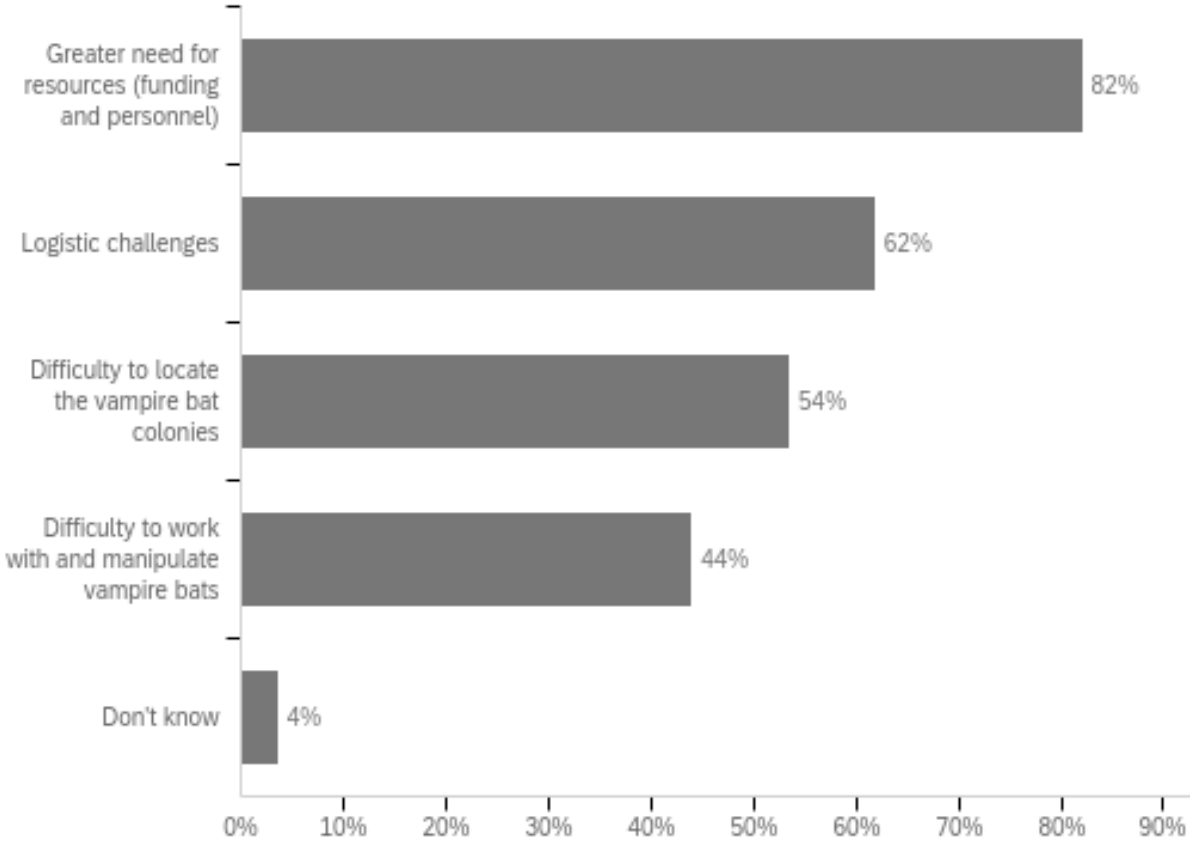


Figure 6. Possible scenarios in which it would be pertinent to vaccinate vampire bat. Percent selected by multiple choice (n=86).

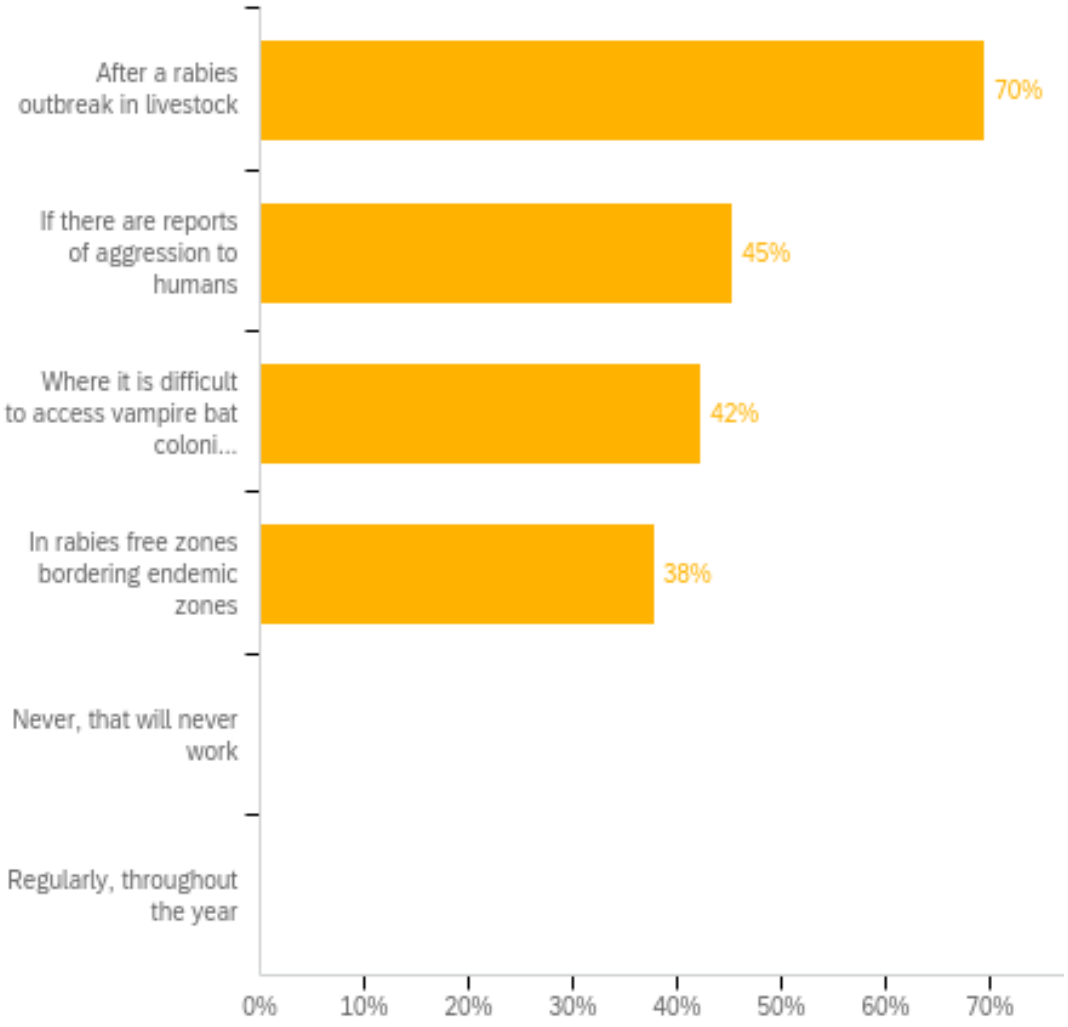
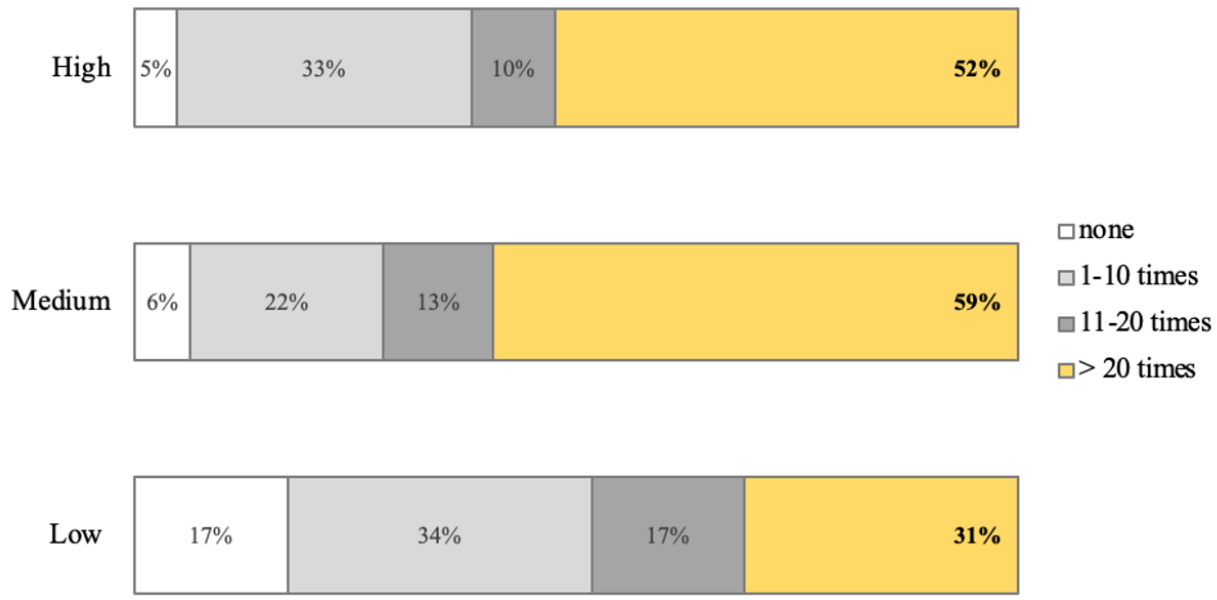


Figure 7. Percent captures performed by states, based on a category of the average number of reports received from 2018 – 2020.



Category	# Reports	States
Low	≤ 25	Colima, Chihuahua, Durango, Estado de México, Guanajuato, Morelos, Sinaloa, Sonora, Zacatecas
Medium	26-74	Campeche, Chiapas, Guerrero, Hidalgo, Jalisco, Michoacán, Oaxaca, Querétaro, Quintana Roo, Tamaulipas
High	≥ 75	Nayarit, Puebla, Tabasco, San Luis Potosí, Veracruz, Yucatán

Acknowledgments

We thank SENASICA for their support in conducting this study and granting permission to distribute the survey among its personnel. Special thanks to MVZ. Baltazar Cortes Garcia who was instrumental for the completion of the surveys and provided the list of emails from the rabies campaign personnel directory.

Author contributions

Conceptualization, All; data curation, E.M.C.C.; formal analysis, E.M.C.C and H.R.; writing-original draft, E.M.C.C.; writing- review and editing, All.

References

1. Jackson AC. Rabies virus. Curated Ref Collect Neurosci Biobehav Psychol. 2016;1027–30.
2. Rupprecht C, Kuzmin I, Meslin F. Lyssaviruses and rabies: current conundrums, concerns, contradictions and controversies. Research. 2017, 23;6:184.
3. Organización Panamericana de la Salud. Eliminación de la rabia humana transmitida por perros en América Latina: análisis de la situación, año 2004. Organ Panam la Salud Elimin la rabia humana Transm por perros en América Lat análisis la situación, año 2004 Washington, USA, PAHO [Internet]. 2005;8–10. Available from: http://bibliotecadigital.puc-campinas.edu.br/services/e-books/OPS-rabia-humana2004.pdf
4. Vigilato MAN, Clavijo A, Knobl T, Silva HMT, Cosivi O, Schneider MC, et al. Progress towards eliminating canine rabies: policies and perspectives from Latin America and the Caribbean. Philos Trans R Soc B Biol Sci. 2013 Aug 5;368(1623):20120143.
5. Benavides JA, Rojas Paniagua E, Hampson K, Valderrama W, Streicker DG. Quantifying the burden of vampire bat rabies in Peruvian livestock. PLoS Negl Trop Dis. 2017;11(12):1–17.
6. Johnson N, Aréchiga-Ceballos N, Aguilar-Setien A. Vampire bat rabies: Ecology, epidemiology and control. Viruses. 2014;6(5):1911–28.
7. Benavides JA, Valderrama W, Recuenco S, Uieda W, Suzán G, Avila-Flores R, et al.

Defining New Pathways to Manage the Ongoing Emergence of Bat Rabies in Latin America. Vol. 12, Viruses. MDPI AG; 2020.

8. NOM. Norma Oficial Mexicana, NOM-067-ZOO-2007. Campaña nacional para el control y la prevención de la rabia en bovinos y especies ganaderas. Diario Oficial de la Federación. [Official Mexican Standard 067-ZOO-2007, National campaign for the prevention and cont.

9. Bárcenas-Reyes I, Loza-Rubio E, Zendejas-Martínez H, Luna-Soria H, Cantó-Alarcón GJ, Milián-Suazo F. Comportamiento epidemiológico de la rabia paralítica bovina en la región central de México, 2001-2013. Rev Panam Salud Publica/Pan Am J Public Heal. 2015;38(5):396–402.

10. Stading BR, Osorio JE, Velasco-Villa A, Smotherman M, Kingstad-Bakke B, Rocke TE. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox vectored rabies vaccine in the Brazilian Free-tailed bat (Tadarida brasiliensis). Vaccine. 2016;34(44):5352–8.

11. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.

12. Cárdenas-Canales EM, Gigante CM, Greenberg L, Velasco-Villa A, Ellison JA, Satheshkumar PS, et al. Clinical presentation and serologic response during a rabies epizootic in captive common vampire bats (desmodus rotundus). Trop Med Infect Dis. 2020;5(1):1–13.

13. Shapiro HG, Willcox AS, Tate M, Willcox E V. Can farmers and bats co-exist? Farmer attitudes, knowledge, and experiences with bats in Belize. Human–Wildlife Interact. 2020;14(1).

14. Alie A, Assefa A, Derso S, Ayele B. Assessment of Knowledge, Attitude and Practice on Rabies in and Around Debretabor, South Gondar, Northwest Ethiopia. Int J Basic Appl Virol. 2015;4(1):28–34.

15. WHO/HTM/STB. Advocacy, communication and social mobilization for TB control: a guide to developing knowledge, attitude and practice surveys. 2008;46(1.Tuberculosis).

16. Pallant author J. SPSS survival manual : a step by step guide to data analysis using IBM SPSS. 7th edition. Crows Nest, NSW : Allen & Unwin, 2020.

17. Nuismer SL, Bull JJ. Self-disseminating vaccines to suppress zoonoses. Nat Ecol Evol. 2020 Sep 27;4(9):1168–73.

18. Aguilar-Setién A, Campos YL, Cruz ET, Kretschmer R, Brochier B, Pastoret PP. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J Wildl Dis. 2002;38(3):539–44.

19. Obregón-Morales C, Aguilar-Setién Á, Perea Martínez L, Galvez-Romero G, Martínez-Martínez FO, Aréchiga-Ceballos N. Experimental infection of Artibeus intermedius with a vampire bat rabies virus. Comp Immunol Microbiol Infect Dis. 2017;

20. Moon K, Blackman D. A Guide to Understanding Social Science Research for Natural Scientists. Conserv Biol. 2014;28(5):1167–77.

21. Dreesen DW. Animal Vaccines [Internet]. Second Edi. Rabies. Elsevier Ltd; 2007. 517–530 p. Available from: http://dx.doi.org/10.1016/B978-0-12-369366-2.50016-6

22. Marín-López A, Ortego J. Vaccine Technologies for Veterinary Viral Diseases. Methods Mol Biol. 2016;1349:137–50.

23. Jorge S, Dellagostin OA. The development of veterinary vaccines: a review of traditional methods and modern biotechnology approaches. Biotechnol Res Innov. 2017;1(1):6–13.

CHAPTER 6

Conclusions and future directions

Millions of dollars are spent every year to combat rabies globally. The World Health Organization has set the goal to eliminate human rabies transmitted by dogs by 2030. The fundamental approach is to fight rabies at its source. Thus, mass vaccination of dogs in rabies endemic regions worldwide has been the best strategy, given that dog-mediated rabies is responsible for more than 90% of human cases. Dog vaccination and education of the public have already proven efficient at reducing or eliminating the disease from certain regions (Mexico, Chile, Argentina, Brazil, Colombia, Peru) [1]. Since its inception, the WHO initiative has gained support from governments, non-profit organizations, and stakeholders committed to fight this lethal disease and protect humanity from suffering rabies. Because RABV is a cosmopolitan pathogen that steadily circulates in many reservoir species, besides the domestic dog, eliminating rabies from all its natural sources is complicated [2].Nevertheless, vaccination remains a powerful strategy to combat rabies, even in reservoir species that are challenging to manage, such as free-ranging wild carnivores. The development of effective vaccines, accompanied by practical vaccine delivery methods specific to reservoir species, has been crucial for the success of specific control and elimination programs in terrestrial carnivores (e.g., fox rabies in Europe, coyote and gray fox rabies in Texas) [3,4]. Could vaccination be used to prevent rabies in wild bats in a similar manner? Specifically, can we vaccinate the common

vampire bat, currently the main reservoir of rabies in Latin America? If bat vaccination is a realistic, feasible strategy, rabies eradication will become a more attainable goal.

The studies described in this dissertation show that RCN-MoG, a raccoon poxvirus vectored mosaic-glycoprotein rabies vaccine, protects vampire bats against rabies infection when administered orally and topically. More importantly, it showed a potential blocking effect of viral shedding in the saliva among rabies-infected bats that ultimately succumbed to rabies. Blocking viral shedding in the saliva is critical to slowing or stopping rabies virus dissemination within bat colonies and other susceptible species.

A vaccine candidate targeting a wild reservoir must be both efficacious and practically delivered to the target species to become an applicable product in the field. Under laboratory settings, the topical application of RCN-MoG using a glycerin jelly vehicle was successful, and vaccine uptake was corroborated by observing a biomarker in hair samples obtained from vaccinated bats. However, this same product failed to perform as expected when applied to vampire bats in the field to measure its dispersion and transfer rate within colonies. The topical application remains the most viable mechanism to vaccinate vampire bats in the wild to date [5]. Until other vaccine delivery methods are developed (e.g., aerosol sprays), searching for a topical substance capable of maintaining the vaccine vector and allowing bat uptake should be prioritized in future field studies. Additionally, behavioral studies on wild and captive vampire bat populations will be essential to help find more efficient ways to deliver vaccines across entire populations [6].

The most challenging element of this project was the vaccine efficacy trial using wild-caught vampire bats. Attempting to control all the variables that can influence the outcome of the experiment was nearly impossible. However, there is no laboratory-adapted animal model

that would ever provide the opportunity to observe how a vaccine will perform in the target species. Thus, in a study of this nature, results should be interpreted with a broad ecological perspective. The limitations of the currently available laboratory tools to assess immune responses in bats, as well as the limited knowledge on the breadth and nature of the immune response of bat species to known pathogens, must be considered as well. Using a heterogenous population of bats (such as ours) is likely to better represent what can be expected in nature when vaccinating vampire bats against rabies and can shed light on potential mechanisms for disease dissemination and persistence. In Figure 1, I provide a visual outline of the vaccine efficacy experiment, along with a list of challenges encountered, our observations during the study, and some suggestions for future studies for reference in this text.

Challenges in assessing previous exposure to RABV in wild-caught bats

The unknown history of exposure to rabies in wild bats complicates an accurate assessment of their basal immune status [7], which no doubt impacts their response to vaccination (i.e., humoral immune response) and survival to challenge. Vampire bats are a long-lived species which increases their chances of being exposed to different variants of RABV circulating endemically in the wild at any time during their lifespan. Moreover, a lack of RVNA based on microRFFIT is no warrant for a naïve status. Rabies neutralizing antibodies are known to fluctuate and decline over time [8], and the timing of sampling for a basal assessment is critical. Additionally, because of the variation between tests, perhaps due to the test sensitivity (e.g., due to small volumes or dilution factors [9]), we recommend more than one baseline screening for the detection of RVNA previous to treatment.

The detection of a pre-existing anamnestic immune response, either humoral or cellular, is difficult to assess with the currently available functional neutralization assays optimized for use in bats. In the absence of more sensitive assays such as mesoscale ELISA-like assays for both antibody and cytokines or cell activation assays to detect cellular response, we will continue to rely on functional tests such as the microRFFIT.

The only alternative to guarantee a naïve basal immune status in a group of bats for future studies is to have individuals born in captivity from uninfected mothers. This study had eight captive-born pups birthed by females from both the vaccinated and unvaccinated groups. Given the timeline of the experiment and the dates of birth of the pups, none were vaccinated but were included in the rabies challenge. There was no evidence of maternally derived RVNA (screened using microRFFIT) in any of the pups (earliest age when tested was 1.2 months). None of the pregnant females showed RVNA throughout the study either. The dynamics of maternally transferred immunity in vampire bats have not been described before. We assumed the age of the pups at the time of challenge corresponded to that of a developed immune system. Challenging the pups confirmed the lethality of the heterologous RABV strain that we used (from canine origin) since all succumbed to rabies. In contrast, adult vampire bats without evidence of RVNA, when screened at baseline, survived the RABV challenge; thus, suggesting previous exposure to RABV in the wild that remained under the microRFFIT minimal detection threshold.

Logistical challenges of studying wild-caught vampire bats

It is difficult to control all of the variables that can affect the outcome (e.g., immunogenicity and survival evaluations) of any rabies experimental study, especially when

working with wild-caught bats. Even with careful planning and strict protocols, it has been challenging to obtain reproducible results between experiments of different Lyssaviruses in several bat species [10].

The presence of RABV should be assumed as a constant risk in any captive population from wild origin [11,12], since it is enzootic in most populations. As we experienced (Chapter 2), a natural rabies outbreak affected our experimental design and protocols. Fortunately, it was localized, and we were able to contain it and identify the individuals involved. However, the full effect of an active infection of RABV in our group of bats before vaccination remains unknown. For example, one seronegative bat at baseline in the control group developed RVNA after the outbreak. The bat was censored from its group (control, RCN-*luc*), which compromised the sample size for this group. The additional sampling timepoint after the outbreak allowed us to identify the changes in the serostatus of the bats involved before vaccination. However, bleeding bats frequently is not always possible and demands flexibility in the research plan and prior approval. Potential unexpected changes to experimental protocols can include segregating bats into smaller groups. This, however, would have to comply with IACUC requirements and animal welfare guidelines.

Because the natural outbreak affected the immune status of our bats, we used a heterologous RABV strain of canine origin. This was the right decision since it would allow us to discern better the cause of death between a vampire bat or coyote strain at the end of the challenge. However, prior to challenge, the lethality of this strain in vampire bats was unknown. Previous work has shown that the route, dose, and inoculation site of RABV affect the consistency of disease progression and mortality rates in bats [10]. For the challenge, using a homologous rabies strain (of vampire bat origin in this case) in naïve bats would have been ideal.

However, that would have required establishing the lethal dose first. For our vaccine efficacy study in vampire bats that would have required lower sample sizes in treatment groups or the need to capture more individuals.

Our recommendations are meant to minimize potential variations in future experiments. The costs associated with adopting them can be a constraint; however, their implementation could guarantee more robust and reproducible results in the long run.

Challenges in evaluating the immune response of bats and vaccine performance based on antibodies only

If we measured the efficacy of vaccines in wild vampire bats based on neutralizing activity only, we would be neglecting the relative contribution of other essential components of the immune response elicited by vaccination or RABV infection. Especially in bats, the role of innate and anamnestic cell-mediated immune response effectors against RABV has been suggested to explain survival to infection in the absence of RVNA. Many experiments have reported non-seroconverting bats that survive RABV challenges [13,14], concluding that survival may be due to protection conferred by a cell-mediated immune response and not by humoral immunity. Studies in rabies-vaccinated humans have demonstrated the presence of cellular immunity to RABV thanks to the availability of activation assays monitored by flow cytometry [15]. These types of assays, however, have not been implemented in bats yet. Therefore, developing more specific tests (e.g., functional assays and specific antibody detection assays) for bat species of particular interest would better characterize the immune response to RABV infection or other pathogens of interest.

The rapid fluorescent foci inhibition test (RFFIT) has been the gold standard for rabies serology and detects broad neutralizing activity, mainly mediated by neutralizing antibodies [16]. Because the RFFIT can be modified to allow for the use of very small samples of test serum (e.g., those from bats) [17], we used it to evaluate the immune response elicited by vaccination. However, the RFFIT cannot differentiate between antibodies and other effectors of the immune system (i.e., cellular) that can play a critical role in the immune response developed by some individuals against rabies.

Additionally, the RFFIT uses a rabies virus challenge standard strain (CVS-11), of canine-origin, to detect neutralizing antibodies. While exposure to RABV elicits robust neutralization and cross-protective humoral responses, subtle antigenic variations among different RABV variants may affect the detection thresholds and the analytical sensitivity of serologic assays [18]. At a finer scale, these differences may be playing a role in our ability to detect a weak neutralizing response in vampire bats. Using a homologous strain (e.g., vampire bat strain) as a challenge virus in the RFFIT may increase detection thresholds and allow a better evaluation of the protection against enzootic RABV strains circulating within populations of vampire bats. The effort and cost entailed in making an adequate vampire bat-RABV challenge strain (e.g., maintenance of viral stocks, adaptation to cellular culture, consistency in viral titers) should be considered though. As mentioned above, this suggestion could result in more standardized methods.

Adequate vaccination of wild vampire bats

To date, the most suitable method to deliver a vaccine to wild vampire bats is the topical application of a product for oral ingestion. In this study, we vaccinated bats orally and topically. Based on the presence of a biomarker (mixed in the topical vaccine preparation) in hair samples of vaccinated bats, we corroborated ingestion of the topical vaccines. However, we cannot guarantee a complete uptake of the vaccine dose, even in laboratory/controlled settings. It is possible that the lack of detection of RVNA in some individuals may be due to the partial uptake of the ideal volume of a vaccine dose.

The RCN-MoG vaccination study in big brown bats demonstrated protection of bats to rabies challenge (83% survival) but lack of RVNA when vaccinated topically. On the other hand, vampire bats vaccinated topically (in Vaseline paste) with V-RG topically showed an increase in RVNA 16 days after vaccination [19]. Previous studies of vampire bats [14,20] used other routes (IM) showing that the vaccine (V-RG) was immunogenic. In wild carnivores, oral vaccination using recombinant vaccines in a bait formulation has effectively eliminated rabies circulation [3]. High uptake rates and seroconversion by both ELISA and RFFIT have been demonstrated [21]. Besides the use of different vaccine vectors, a species-specific relationship may be another reason why the immunogenicity of RCN-MoG in vampire bats was unlike that of wild carnivores. It would be advisable to address if the nature of the vector may be responsible for deflecting the immune response either to a cell or antibody-mediated for future studies of vaccination in bats both in the laboratory and field settings.

Challenges to interpret variation in the immune responses and development of RVNA

In this study, we observed a broad spectrum of outcomes to vaccination and challenge; without surprise, given that we had a heterogeneous group of individuals obtained from a free-ranging, healthy population.

For example, two bats (seronegative at baseline) were involved in the rabies outbreak at the beginning of the study. They were likely exposed to RABV during the event, as evidenced by the presence of wounds. However, neither bat had detectable RVNA in a second sample obtained after baseline, right before vaccination. Twenty-one days post-vaccination, one bat responded with RVNA titers of 2357 IU/ml, while the other bat never showed any neutralizing activity throughout the rest of the study. The latter is considered a non-responder bat. Nonetheless, both bats survived the rabies challenge (using a heterologous strain of RABV of canine origin). The remarkable outcome of the non-responder bat raises the question of the mechanisms involved in survival (e.g., cell-mediated immunity, which RFFIT cannot detect) and the frequency in which this outcome can be expected. In contrast, the only bat of the study (seronegative at baseline) that developed and maintained a neutralizing response following topical vaccination with RCN-MoG succumbed to rabies challenge.

One seropositive bat (RVNA detected at baseline) succumbed to rabies challenge, too, challenging the assumption of long-term immunity after a non-lethal exposure or abortive infection and protection from rabies as a result of previous exposure. How often should death upon challenge be expected when the presence of RVNA is continuously detected? Moreover, what are the specific characteristics of these individuals that cause such different outcomes? Answering these questions would improve our understanding of rabies and help reveal the implications of heterogenous response to RABV infection in wild populations. Genomic

characterizations of vampire bats can provide an insight into the molecular mechanisms that contribute to their immune responses [22].

Remarks on observations and possible explanations

The wide variation in the responses to vaccination within experimental groups of bats did not allow for significant conclusions regarding the immunogenicity of RCN-MoG. Nonetheless, the information collected provides valuable insights for future studies.

Models on the natural progression of infection and viral dissemination are based on few experimental and field studies; the rest is best assumed [2]. Our observations will contribute to the current knowledge and help refine compartmentalized models for better predictions of rabies persistence and geographic dissemination for the control and management of rabies [23]. The ability to estimate the proportions of non-RVNA responders in a population (extrapolated from our results) can provide helpful information for future modeling. Furthermore, what role do these bats play in the maintenance and transmission rates of RABV in the wild? In our case, at least 6% of the bats did not show any neutralization activity. Still, they survived the challenge, while others seroconverted and succumbed to the challenge. The awareness of the broad spectrum of outcomes to rabies infection is essential for refining these disease ecology models.

Future directions for the use of topical vaccines in the field

For field studies of vaccination, other compounds as vehicles to deliver the vaccine need to be investigated. Glycerin jelly seemed to be a good vehicle for delivering topical vaccines,

especially those based on recombinant vectors such as RCN. However, this was true in laboratory settings, with somewhat controlled temperature and conditions, but not during use in the field. Flavored, petrolatum-based pomades (like the vampiricide) might be more suitable to deliver vaccines under tropical and subtropical settings (based on better thermostability), however these products did not maintain viral titer [24]. The ability to maintain vaccine titer is one of the most critical features of any topical vehicle. This is yet to be tested with other substances in field conditions.

Recommendations for the adoption of a bat-focused vaccine at the government level

This work included evaluating people's (the Mexican rabies control program personnel) attitude toward vaccination of vampire bats, an unusual component in the biomedical sciences.

We found that the belief about the usefulness of a vampire bat vaccine and the availability of a bat vaccine with desired characteristics are important drivers for supporting the adoption of a vampire bat-focused rabies vaccine. A future goal should include conducting this same type of evaluation in other Latin American countries. Nevertheless, as promising as a vaccine might be, their immediate adoption by those responsible for rabies control, usually a country's governmental agency, is not guaranteed. As more strategies are devised, it will be essential to consult with regional and local institutions across countries.

Final thoughts

This dissertation presents a promising avenue for controlling VBR by stopping the maintenance, proliferation, and dissemination of RABV at its reservoir host and primary vector for human transmission. Unfortunately, VBR is only one aspect of the problem that vampire bats represent in Latin America. Bat predation on livestock species causes secondary infections, losses in production, anemia, or the death of the animals, thus affecting the livestock industry [25]. For many people, culling will remain the best option to manage both rabies and bat predation. This work left unaddressed options to reduce bat predation, such as non-lethal population control measures using contraceptives.

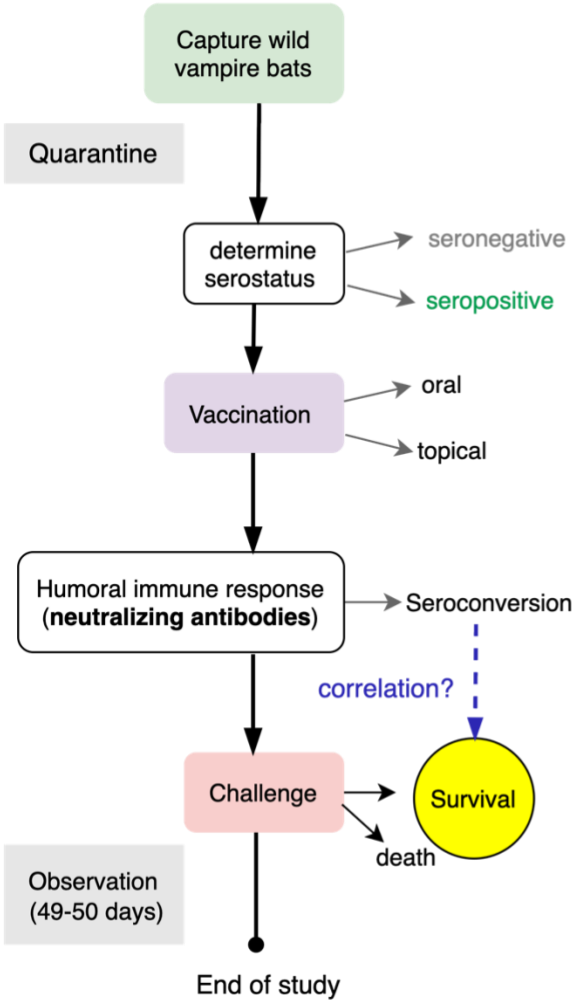
Under a One-Health context, the combination of disease and population control should be sought. Other novel ideas such as anti-vampire vaccines have been proposed as well. This idea expands on the observation of livestock's resistance to vampire bat salivary anticoagulants [26,27]. Ideally, substances for population control could be integrated into rabies vaccines for vampire bats [28] . The need for novel strategies, especially non-lethal ones, should be more favorable to preserve biodiversity and avoid collateral damage to bat species of ecological benefit during culling operations of vampire bats. In fact, in their latest document on rabies, the WHO no longer recommends culling as the primary method to control VRB [29,30].

Potential contribution of this work beyond rabies

As a final remark, the scope of this work could reach beyond rabies. Other diseases of bat-origin may be controlled with the use of vaccination as a strategy. The development of mosaic-recombinant vaccines could provide the framework technology to incorporate multiple

antigen sequences of different zoonotic pathogens of current concern (Ebola, Nipah, Hendra, and Coronaviruses, to name a few), thus allowing for the design of multivalent vaccines targeting pathogens of regional concern with a bat-origin [31]

Figure 1. A visual summary of our vaccine experiment with vampire bats: challenges, observations, and future recommendations.



Challenges	Our study	Future recommendations
Heterogeneity = variation Unknown if previous exposure to RABV	true	Use naive populations
Risk of outbreaks, deaths, maladaptation	Natural outbreak, death of bats assigned to groups	Longer quarantine times
True serostatus?	Single baseline measure Underestimated serostatus?	Multiple sampling (entry and pre-vax)
Confirmed uptake? (especially topical vaccine)	Assumed Rely on uptake observation and biomarker readings (for topical vaccine)	Improved vaccine delivery vehicles
Adequate replication of vector?		Use of in-vivo imaging for viral infection
Low immunogenicity? Assay issues?	Only 24% seroconversion	Complementary assays: - homologous strain, RFFIT - proliferation assays (measure cellular immune responses)
Other (cellular immunity) important for survival?	Cellular immunity not explored	
Effect of challenge virus and lethal dose	Heterologous RABV strain available for challenge	Use homologous challenge virus and known lethal dose
Variable incubation periods	One rabid bat alive by day 50	Longer observations times

References

1. Organización Panamericana de la Salud. Eliminación de la rabia humana transmitida por perros en América Latina: análisis de la situación, año 2004. Organ Panam la Salud Elimin la rabia humana Transm por perros en América Lat análisis la situación, año 2004 Washington, USA, PAHO [Internet]. 2005;8–10.

2. Fisher CR, Streicker DG, Schnell MJ. The spread and evolution of rabies virus: Conquering new frontiers. Nat Rev Microbiol. 2018;16(4):241–55.

3. Slate D, Rupprecht CE, Rooney JA, Donovan D, Lein DH, Chipman RB. Status of oral rabies vaccination in wild carnivores in the United States. Virus Res. 2005 Jul;111(1):68–76.

4. Velasco-Villa A, Reeder SA, Orciari LA, Yager PA, Franka R, Blanton JD, et al. Enzootic Rabies Elimination from Dogs and Reemergence in Wild Terrestrial Carnivores, United States. Emerg Infect Dis. 2008 Dec;14(12):1849–54.

5. Bakker KM, Rocke TE, Osorio JE, Abbott RC, Tello C, Carrera JE, et al. Fluorescent biomarkers demonstrate prospects for spreadable vaccines to control disease transmission in wild bats. Nat Ecol Evol. 2019;3(12):1697–704.

6. Roberts G. Cooperation: How Vampire Bats Build Reciprocal Relationships. Curr Biol. 2020 Apr;30(7):R307–9.

7. Gilbert AT, Fooks AR, Hayman DTS, Horton DL, Müller T, Plowright R, et al. Deciphering serology to understand the ecology of infectious diseases in wildlife. Ecohealth. 2013;10(3):298–313.

8. Turmelle AS, Jackson FR, Green D, McCracken GF, Rupprecht CE. Host immunity to repeated rabies virus infection in big brown bats. J Gen Virol. 2010;91(9):2360–6.

9. Timiryasova TM, Luo P, Zheng L, Singer A, Zedar R, Garg S, et al. Rapid fluorescent focus inhibition test optimization and validation: Improved detection of neutralizing antibodies to rabies virus. J Immunol Methods. 2019;474(April).

10. Banyard AC, Davis A, Gilbert AT, Markotter W. Bat rabies. In: Rabies. Elsevier; 2020. p. 231–76.

11. Abbott RC, Saindon L, Falendysz EA, Greenberg L, Orciari L, Satheshkumar PS, et al. Rabies Outbreak in Captive Big Brown Bats (Eptesicus fuscus) Used in a White-Nose Syndrome Vaccine Trial. J Wildl Dis. 2020 Jan 6;56(1):197.

13. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.

14. Aguilar-Setién A, Campos YL, Cruz ET, Kretschmer R, Brochier B, Pastoret PP. Vaccination of vampire bats using recombinant vaccinia-rabies virus. J Wildl Dis. 2002;38(3):539–44.

15. Moore, S.M.; Wilkerson, M.J.; Davis, R.D.; Wyatt, C.R.;Briggs DJ. Detection of cellular immunity to rabies antigens in human vaccinees. J Clin Immunol. 2006;26:533–45.

16. Rupprecht CE, Fooks AR, Abela-Ridder B. Laboratory techniques in rabies. Fifth edition. World Health Organization, Geneva. 2018; Available from: http://www.who.int/rabies/resources/WHO-CDS-NTD-NZD-2018.

17. Smith, T.J.; Gilbert AT. Comparison of a Micro-Neutralization Test with the Rapid Fluorescent Focus Inhibition Test for Measuring Rabies Virus Neutralizing Antibodies. Trop Med Infect Dis. 2017;2(24).

18. Moore SM, Ricke TA, Davis RD, Briggs DJ. The influence of homologous vs. heterologous challenge virus strains on the serological test results of rabies virus neutralizing assays. Biologicals. 2005;33(4):269–76.

19. Almeida MF, Martorelli LFA, Aires CC, Barros RF, Massad E. Vaccinating the vampire bat Desmodus rotundus against rabies. Virus Res. 2008;137(2):275–7.

20. Sétien AA, Brochier B, Tordo N, De Paz O, Desmettre P, Péharpré D, et al. Experimental rabies infection and oral vaccination in vampire bats (Desmodus rotundas). Vaccine. 1998;16(11–12):1122–6.

21. Moore SM, Gilbert A, Vos A, Freuling CM, Ellis C, Kliemt J, et al. Rabies virus antibodies from oral vaccination as a correlate of protection against lethal infection in wildlife. Trop Med Infect Dis. 2017;2(3).

22. Jebb D, Huang Z, Pippel M, Hughes GM, Lavrichenko K, Devanna P, et al. Six reference-quality genomes reveal evolution of bat adaptations. Nature. 2020 Jul 23;583(7817):578–84.

23. Benavides JA, Valderrama W, Streicker DG. Spatial expansions and travelling waves of rabies in vampire bats. Proc R Soc B Biol Sci. 2016;283(1832):1–9.

24. Stading author BR. Development of a novel rabies mosaic antigen and the use of attenuated poxviruses as vaccine vectors in bats. 2016.; 2016.

25. Johnson N, Aréchiga-Ceballos N, Aguilar-Setien A. Vampire bat rabies: Ecology, epidemiology and control. Viruses. 2014;6(5):1911–28.

26. Delpietro HA, Russo RG, Rupprecht CE, Delpietro GL. Towards development of an anti-vampire bat vaccine for rabies management: Inoculation of vampire bat saliva induces immune-mediated resistance. Viruses. 2021;13(3).

27. Delpietro HA, Russo RG. Acquired Resistance to Saliva Anticoagulants by Prey Previously Fed upon by Vampire Bats (Desmodus rotundus): Evidence for Immune Response . J Mammal. 2009;90(5):1132–8.

28. Wu X, Yang Y, Kling C, Seigler L, Gallardo-Romero NF, Martin BE, et al. Inactivated rabies virus-vectored immunocontraceptive vaccine in a thermo-responsive hydrogel induces high and persistent antibodies against rabies, but insufficient antibodies against gonadotropin-releasing hormone for contraception. Vaccines. 2019;7(3).

29. WHO Expert Consultation on Rabies. Second report. World Health Organ Tech Rep Ser. 2013;(982):1–139, back cover.

30. WHO. WHO expert consultation on rabies. World Health Organization - Technical Report Series. 2018.

31. Lauer KB, Borrow R, Blanchard TJ. Multivalent and Multipathogen Viral Vector Vaccines. Papasian CJ. Clin Vaccine Immunol. 2017 Jan;24(1).

APPENDIX 1.

Transport of vampire bats by land: design of a transport container and care during transit

Elsa M. Cárdenas Canales, Mat Bohne, Elizabeth Falendysz, Tonie E. Rocke, Jorge Osorio

Introduction

Bats have gained much attention, given their role as reservoir hosts of pathogens of human interest [51,52]. There is an increased interest in studying the biology of bat diseases in controlled settings and establishing captive colonies for this purpose [53].

The common vampire bat (*Desmodus rotundus*) is a species of concern, as they are the main vector of rabies in Latin America. Currently, there is a need to develop strategies, such as vaccination, to control vampire transmitted rabies beyond the conventional use of lethal practices (i.e., culling bat populations) [7].

Recent work by our research groups at the University of Wisconsin-Madison and the U.S. Geological Survey- National Wildlife Health Center (NWHC), Madison, WI, includes developing a rabies vaccine candidate for bats. The vaccine candidate, a viral vector-recombinant mosaic glycoprotein vaccine, showed protection against rabies in big brown bats (*Eptesicus fuscus*) [10,11]. Encouraged by the results, our next objective was to conduct a similar study with vampire bats as the target species. Since there are no available colonies of vampire bats for such studies in the U.S., it was necessary to collect vampire bats from the wild and transport them to a Biosafety Level 3 (BSL-3) animal facility at NWHC.

In this paper, we describe the design of a transport container designed to safely transport vampire bats in a trip requiring > 24 hours of driving and a feeding session. We considered the following for the design: 1) compliance with biosafety and animal welfare regulations, 2) ability to feed bats during transport while maintaining the seal on the containers and 3) custom dimensions to fit in the available vehicle.

Materials and Methods

Ethics statements and required permits

Our capture activities were conducted under permits from the Mexican Secretariat of Environment and Natural Resources (SEMARNAT, permit number: SGPA/DGVS/003242/18). Permits for exporting bats from México to the U.S. were issued by SEMARNAT (permit number 44333). All protocols for capturing, husbandry, and transporting vampire bats complied with NWHC Institutional Animal Care and Use Committee (protocol number EP180418).

To legally import vampire bats into the U.S., we complied with the Centers for Disease Control and Prevention (CDC) requirements to transport the bats under category B infectious material and maintain them long-term in NWHC's BSL-3 animal facility (CDC, permit number 2018-04-108). We declared the transaction with U.S. Fish and Wildlife Services. They are responsible for inspecting any shipments into the United States to verify the conservation/protection status of the type of wildlife being transported. We also notified the Division of Wildlife for the Wisconsin Department of Agriculture, Trade, and Consumer Protection (permit number 356MO7168-BPI).

We followed the International Air Transport Association (IATA) general regulations for the transport of bat species and modified them according to our needs for vampire bats. IATA requires a primary and a secondary containment device to prevent escape and ensure that handling during transport does not affect the shape of the containers and harm animals inside [54,55]. Other requirements include adequate ventilation, access to food, appropriate dimensions to allow the animals to move and turn in a natural manner, and proper labeling for identification

Vampire bat captures

In July - August 2018, we captured 93 vampire bats (of mixed sex and age) in the state of San Luis Potosí, México. Bats were trapped using mist nets and then transported from the field in small mesh cages to the temporary holding facility in a restricted building property of the Universidad de Matehuala, in Matehuala, Mexico. During containment, bats were housed in custom-made mesh cages of ~ 60" (long) by 20" (wide) by 50" (height) in groups of no more than 20, separated by sex and, as cage availability allowed, by location of capture. During this period, bats were given an individual metal forearm band (4 mm, Porzana, Inc.) and were treated topically with selamectin (dose 6 mg/kg, Zoetis, Florham Park, NJ, USA) for ectoparasites. After a six-week field period and temporary containment of the bats, we drove the bats across the border at Nuevo Laredo, México- Laredo, Texas, to the NWHC. The trip required a total of ~1750 miles and one stop overnight to rest and feed the bats.

Vampire bat care requirements and care during transit

Vampire bats feed solely on blood and need to eat daily. Fasting periods of >24-48 can result in mortality [56,57]. Since vampire bats rarely drink water, prolonged periods without feeding lead to rapid dehydration. In captivity, blood meals are offered once a day, usually at night. Vampire bats ingest an average of 15-30 ml of blood per day. The blood, generally from cattle and obtained in established abattoirs, is in liquid form and treated either manually to defibrinate, or chemically to prevent coagulation [58]. During feeding, the blood is quickly digested, and excess water is eliminated in the form of urine for vampire bats to take flight once they are finished. Their feces contain an excess of ammonia, which produces a strong,

characteristic smell and can be a respiratory irritant if not adequately ventilated [57]. For this reason, after feeding the bats overnight, we needed to keep the container as clean as possible to avoid additional irritants in the enclosed environment. Additionally, uneaten blood spoils quickly and can be a source of bacterial infection or intoxication for vampire bats. Thus, we considered it necessary to remove uneaten blood within 7 hours/the following morning.

The average size of a vampire bat is 9-10 cm of body size and 30-40 cm wingspan. Typical behavior of vampire bats includes congregating in small groups of the same sex or females with their offspring [59]. Vampire bats rest by hanging from their hind limbs and actively move, stretch, and groom. We planned to allocate bats into the transport containers in same-sex, small groups (no more than ten individuals) to avoid disrupting their natural behavior but also provide enough space for comfortable movement.

Transport container design

Primary container

To comply with the requirements for the primary container, we searched for durable and non-toxic materials resistant to cleaning and disinfection of appropriate dimensions to allow the shipping of ten bats per container. We used Taconic Transit Cages™ (TTC™) ordinarily used to transport laboratory rats and mice (Taconic, Rensselaer, NY, U.S., www.taconic.com). With some modifications to these cages, we built ten primary containers. The TTC™ cages are made of molded polypropylene #5 plastic and have air vents on the sides made of a filter material that allows airflow while also preventing contamination by pathogens. The cages are autoclavable and reusable. The cage dimensions are 22″ (long) by 16″ (wide) by 7″ (height).

However, the TTC™ dimensions were not large enough to let ten bats move freely inside the primary container (e.g., open their wingspan, perch upside down with room to flip onto the floor). Thus, we used two TTC™ bottoms, one inverted and on top of the other, for a total height of ~13". We joined the two bottoms by placing a set of hinges on the long side, allowing us to open/close the container up to 180º degrees. We glued a waterproof, high-density foam tape strip along the rim of each container to provide a sealing surface when the cage was closed. To fasten the container closed, we drilled two holes through the longer rim (opposite to the hinged side) to insert two 3" screws with butterfly wing nuts (Figure 1). We used a thermoplastic film tape (Parafilm® M, P7793, Millipore Sigma, Burlington, MA, U.S.) to wrap around the junction of the two container parts to provide an additional layer for sealing. To enable the inspection of the bats during transit, we placed a window on the side of the wide face of the top part of the container by cutting a 4" diameter hole (Figure 2). We covered the window hole by gluing a square of clear polycarbonate fiberglass, fastened with four screws on each corner. As per welfare requirements, we provided the bats with a surface to hang from the inside of the top section (i.e., the ceiling). We cut a piece of a ¼" galvanized hardware cloth to the dimensions of the ceiling and attached it with screws and washers, trimming and smoothing the edges off to avoid harming the bats while perching.

Secondary container

IATA requires a secondary containment unit as a second line of defense. We built two wooden containers using commercial plywood sheets. The dimensions of each allowed us to stack six primary containers (2 columns of 3 containers) in one (Figure 3) and three containers in the other. Each container was placed over a stainless-steel tray (commercially available for

kitchenware) as a liquid-proof divider surface between the containers. To provide adequate ventilation and to access the primary container during feeding, we included two lateral windows and a front door into the wooden box. All were shielded with a ¼" galvanized hardware cloth mesh. We also installed a 12-volt fuse plug, electric fan on the back face of the larger secondary container that served as an air extractor. The tenth, single container was placed inside a commercially available pet carrier with dimensions 36″ (long) by 28″ (wide) by 32″ (height) made of plastic and metal wire (Petmate Traditional Vari kennel).

Feeding systems

The most critical aspect of transporting vampire bats over the ~ 48-hour planned travel time was to have the capacity to feed the bats while maintaining biosecurity measures (e.g., not opening the primary container and avoiding spillage or aerosolization of particles from inside the cage). To overcome this challenge, we devised a feeding system using a set of enteral feeding bags set (feeding bag set, The Kangaroo™ Gravity Feeding Bag, Product #8884702500). We made some modifications that allowed the delivery of blood to a feeding receptacle (trough) placed inside the primary transport container. To build the trough, we cut in half (transversally) a 2" PVC pipe, measured it to fit within the length of the container, and glued it fixed onto the bottom section (i.e., floor) by the opening side. The size of the trough allowed for a filling capacity of ~200-250 ml of liquid without spilling. We drilled a small hole of the bottom section (same side as the window) that exited through the wall, as close as possible to the center bottom of the trough. We then inserted an "L" shaped hard plastic tube with proper diameter to fit tightly into the container and allow the flow of blood or liquids from the outside into the trough. We glued and sealed the joining of the tube to the container wall using silicon glue. The tube faced

downward into the trough and protruded ~½" to the outside, to which end we connected an extension line connector set with a male luer lock end (container connector). This connector was the only port to move liquids (blood or cleaning solutions) into or out from the trough and always remained attached to the container, tightly capped. To fill the trough with blood and to clean/rinse it after the feeding session, we used two sets of 3-way stopcock valves, one feeding bag set, two extension line connectors with male luer lock, and two 60 ml needleless syringes that would connect to the main tube/connector, per container.

Feeding and cleaning process

We fed the vampire bats with citrate-treated bovine blood [56], following the same procedures as during captivity in Matehuala. The blood had been previously packed in the feeding bags or 1L Nalgene bottles and stored frozen at -20C until use. A few hours before the feeding session (and resting stop) overnight, we thawed the blood under warm water to ensure a liquid state and easy flow through the feeding bag set lines and the connectors (Figure 4).

After inspecting the bats inside, we opened the doors of the secondary container to access each primary container connector and begin the feeding process. Afterward, we hung the feeding bags at an elevated height (allowing gravity to facilitate blood flow), removed the cap from the container connector, and attached a 3-way stopcock valve onto the male luer lock. We then connected the feeding bag line to the second port of the 3-way stopcock valve and opened it to allow the blood to flow into the trough. The filling time per container was approximately 5-7 minutes (for ~200 ml of blood). Once each trough was filled to capacity, we disconnected the feeding bag line and either filled another cage or discarded the empty feeding bag. Since the

blood was considered “clean”, we only sprayed-cleaned the IV lines and bags with 70% ethanol. When finished, each container extension line was secured again with the male luer lock cap.

The cleaning process after the feeding period included rinsing the lines and the trough with saline solution and chlorhexidine (a disinfectant), retrieving the liquids using suction, and discarding them into waste containers. For the rinsing, we attached to the container connector a 3-way stopcock valve with one of its ports connected to a 60 ml needleless syringe and the other to a second set of a 3-way stopcock valve and extension connector line. A bag of saline solution was connected to the end and delivered into the trough (using the same procedure to dispense the blood). To retrieve the rinsing solution, we redirected the 3-way stopcock valve and suctioned it using the 60 ml syringe. When the syringe was full, we redirected the valve again and pushed the recovered solution into a 1L Nalgene waste bottle containing 100 ml of bleach that was connected to a port of the second 3-way stopcock valve. To avoid pressurizing the waste bottle, we placed and sealed a 0.22160 µm filter (Cole-Parmer, UX-02915-60, Vernon Hills, Illinois, U.S.) through the bottle cap that let air out. This process was repeated 2-3 times until the saline solution was retrieved clear. In the final rinse step, we used a 2%solution of chlorhexidine (Phoenix Pharmaceuticals, Phoenix, AZ) to disinfect the trough and the extension lines. Upon completion of the cleaning, we closed the first 3-way stopcock valve and secondarily occluded the line using the attached pinching device to avoid any entry of liquid into the trough and using a syringe, we passed a solution of 10% bleach through the connectors and extension lines into the waste bottle. Last, we disconnected all lines and disinfected by spraying 70% ethanol. We placed all waste into biohazard labeled plastic bags first, then into screw-top buckets.

Transport description

We transported bats to the NWHC from Matehuala on September 5, 2018. We fed the bats 2-3 hours earlier than usual the night before the trip to ensure that all ate completely. At 2:00 am we began placing bats in cages for transport. All bats received a health checkup and a subcutaneous fluid administration of lactated Ringer´s solution. We distributed the bats into the travel containers and tried to maintain the groups already formed during captivity to avoid stress; males and females always remained separate.

We rented a vehicle (15 passenger van) to drive the bats from Matehuala, Mexico, to the International Bridge in Laredo, Texas, U.S. (~330 mi), where Mexican and U.S. authorities inspected the export/import of the vampire bats. Once in Laredo, Texas, we transferred the containers into our agency vehicle (minivan) to complete the rest of the trip. Driving, and all the processes involved, were performed by two people. During the trip, the temperature was controlled with the vehicle A/C system at an ambient temperature of 25º C; we kept the back windows open to allow airflow. Twenty-two hours after placing the bats in the travel containers, we stopped and fed them overnight for ~6 hours. It took about one hour to complete the feeding session, while the cleaning process lasted ~1.5 hours the next morning. After visually inspecting the bats through the container windows, we resumed the driving to the NWHC.

Results

During most of the trip, the bats remained apparently calm and roosted as a single group in each transport container. Upon stopping in the evening, bats were observed moving around the container more than during transport. Once beef blood had been provided, the bats immediately left their roost to feed from the trough. All bats appeared to be eating. Bats could be heard

chattering to each other during and after eating, but no significant aggression or food guarding was observed.

The following morning, one male bat was observed roosting outside of the group and appeared depressed. Because the CDC transport requirements require complete containment of the bats during transport, the bat could not be examined or treated. It was observed that he appeared dead during a visual check later that afternoon. The bat was confirmed to be dead upon arrival in the late afternoon the second day. This vampire bat was confirmed as positive for rabies virus by brain IFA at the Wisconsin State Laboratory of Hygiene. No other bats showed signs of rabies or were injured. However, later in our captive study at the NWHC, we experienced a rabies outbreak two months after arrival, linked to the bat that died in transport [12]. Upon arrival at NWHC, the remaining 92 bats were visually examined and placed in mesh housing cages. None appeared significantly dehydrated or lethargic. The bats were fed more citrated beef blood shortly after arrival and some were observed eating immediately. Over the following week, bats were examined in groups of 20-40 per day. One female bat was humanely euthanized for a chronic tendon injury to the right stifle. It was submitted for rabies IFA and was also positive, despite not showing any clinical signs typical of rabies infection. This bat had not been co-housed with the first rabies case before or during transport and thus was likely infected naturally in the wild prior to capture.

Discussion and Future recommendations

While our prototype container was efficient, we have suggestions to improve the design. We recommend using a softer material for the perching area (ceiling), as the metal mesh may

irritate the foot padding of the bats. We also recommend designing the cage opening with a limited range (compared to the 180º). In our case, the two Taconic™ pieces opened wide enough and from all sides that bats could have potentially escaped; however, they were never opened while in transit. Another setback of the transport cages was the susceptibility of some materials (e.g., foam padding, silicon glue around the bolts) to high temperatures during autoclaving. Therefore, these parts must be replaced if the transport cages are to be reused.

Additionally, some of the beef blood was stored frozen inside the enteral feeding bags. During storage and transport of the frozen bags, the plastic cracked on several bags, leading to the leaking of beef blood during transport. We recommend freezing the blood in hard-sided containers such as Nalgene bottles and transferring it to the enteral feeding bags just before use.

The feeding system we designed was effective, and we were able to provide ~200 ml of bovine blood per cage (of 10 bats maximum) for one overnight feeding session. We successfully rinsed and flushed the trough and tubes with disinfectant using the same tubing system and valves. We did not experience any blood spills (clean or waste) or disinfecting liquid and the time to complete both tasks was reasonably fast, performed by two people. In our experience, we noticed that vampire bats were very adaptable to a novel surrounding and feeding settings as they readily figured out how to perch and feed from the trough.

Overall, we safely transported by vehicle 93 bats from San Luis Potosí, México to Madison, Wisconsin, U.S. over a total of ~1,750 miles. To our knowledge, this is the first time that vampire bats have been imported through the México-U.S. border by land. The trip was completed in a total of 47 hours from the moment the bats were placed in their transport containers until they were placed in cages at the NWHC on September 6, 2018. The transport

containers and feeding system described herein provided safe and bio secure transportation for vampire bats during extended land travel.

Figure 1. Vampire bat transport container prototype using modified Taconic Transit Cages™

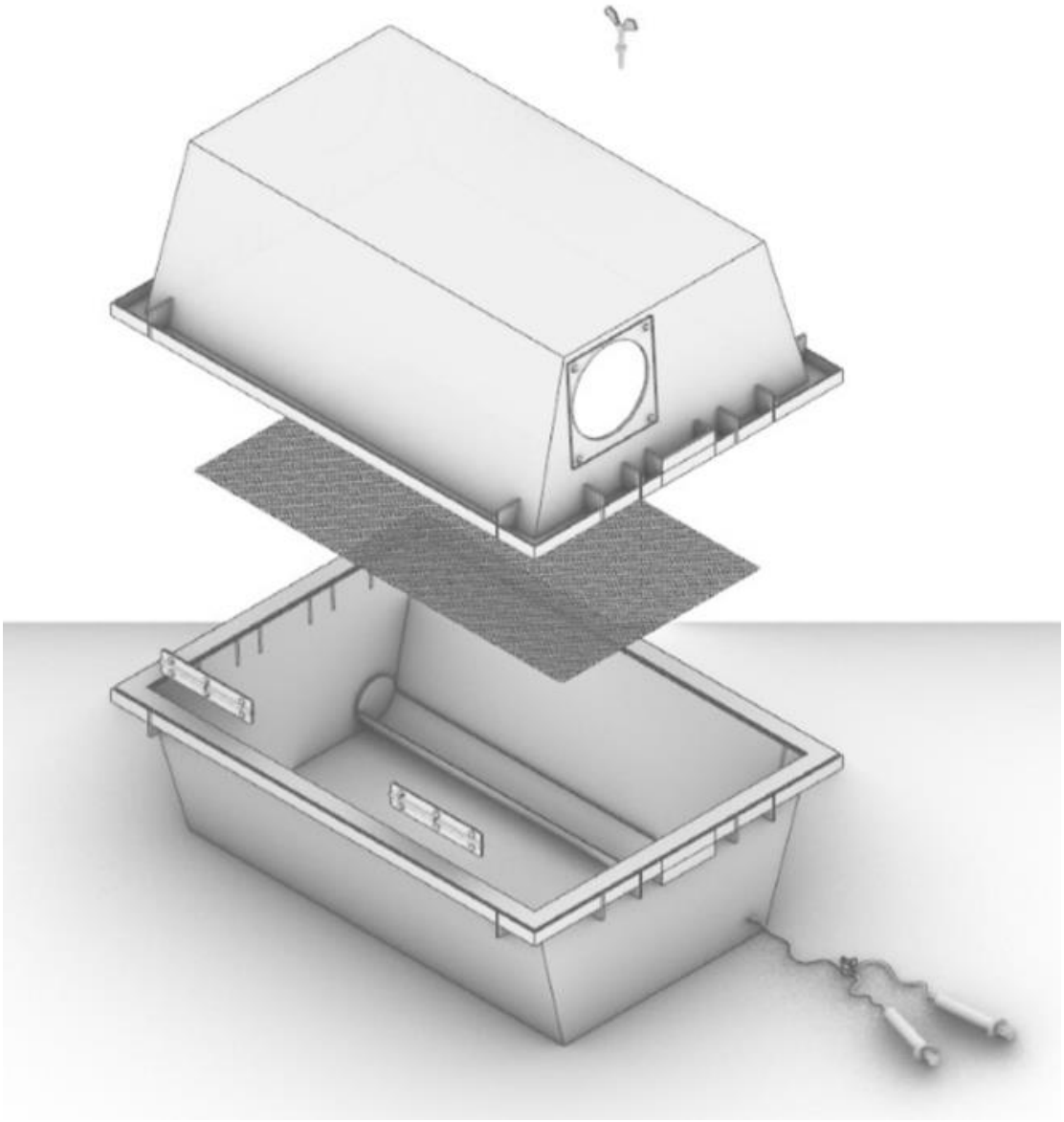


Figure 2. Use of the side windows for inspection of the vampire bats at the Mexico-U.S. border

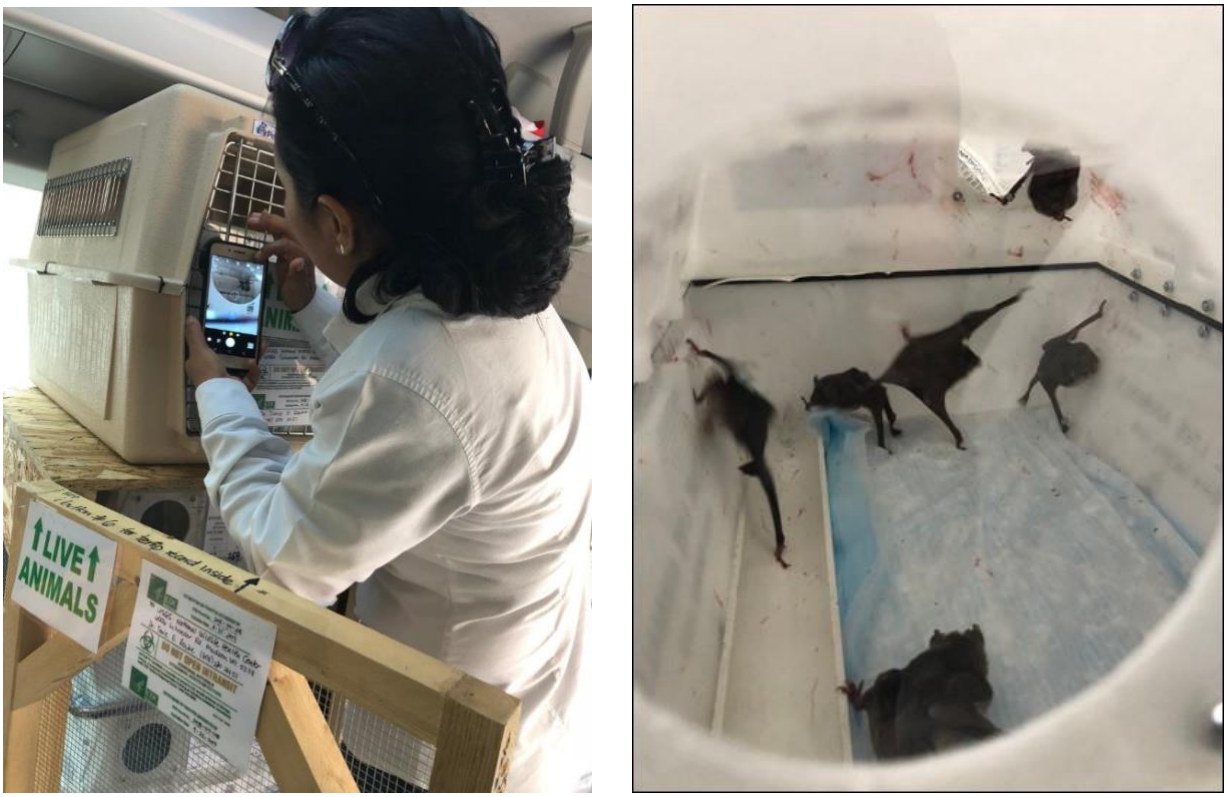


Figure 3. Final setup of the transport cages inside the minivan during transport within the U.S. The largest secondary container (shown here) held six primary containers. A smaller secondary container held three primary containers, and a tenth primary container was placed inside a commercial dog kennel cage.

Figure 4. Feeding session. Bovine blood (thawed and placed in the feeding bag set) was delivered into the container´s trough using the extension lines.

Acknowledgments

We thank Richard Carson for providing material to build the primary containers. The University of Wisconsin-Madison shop provided us with the space to construct the cages. Emily Russel, Lex Medina, Jordan Mandli, Nichole Kuha, and Dean Dischler helped assemble the transport containers. José Ignacio Cárdenas designed Figure 1.

Author contributions

Conceptualization, E.M.C.C., M.B., and E.F.; methodology, E.M.C.C., and M.B.; resources, J.E.O., and T.E.R.; writing—original draft preparation, E.M.C.C.; writing—review and editing, All.; visualization, E.M.C.C.; supervision, J.E.O., and T.E.R.; project administration, J.E.O., T.E.R.; funding acquisition, E.M.C.C., J.E.O., and T.E.R.

References

1. Mandl JN, Schneider C, Schneider DS, Baker ML. Going to bat(s) for studies of disease tolerance. Front Immunol. 2018;9:1–13.
2. Olival KJ, Epstein JH, Wang LF, Field HE, Daszak P. Are bats exceptional viral reservoirs? In: Aguirre AA, Ostfeld RS, Daszak P, editors. New directions in conservation medicine Applied cases of ecological health. Oxford University Press; 2012. p. 195–212.
3. Letko M, Seifert SN, Olival KJ, Plowright RK, Munster VJ. Bat-borne virus diversity, spillover and emergence. Nat Rev Microbiol. 2020;18(8):461–71.
4. Benavides JA, Valderrama W, Recuenco S, Uieda W, Suzán G, Avila-Flores R, et al. Defining New Pathways to Manage the Ongoing Emergence of Bat Rabies in Latin America. Vol. 12, Viruses. MDPI AG; 2020.
5. Stading B, Ellison JA, Carson WC, Satheshkumar PS, Rocke TE, Osorio JE. Protection of bats (Eptesicus fuscus) against rabies following topical or oronasal exposure to a recombinant raccoon poxvirus vaccine. PLoS Negl Trop Dis. 2017;11(10):1–19.
6. Stading BR, Osorio JE, Velasco-Villa A, Smotherman M, Kingstad-Bakke B, Rocke TE. Infectivity of attenuated poxvirus vaccine vectors and immunogenicity of a raccoonpox

vectored rabies vaccine in the Brazilian Free-tailed bat (Tadarida brasiliensis). Vaccine. 2016;34(44):5352–8.

7. Prescott MJ. Guidelines for the Human Transportation of Research Animals. Vol. 28, International Journal of Primatology. 2007. 505–506 p.

8. Lanza N. Keeping the Live in Live Animal Air Cargo Transport. J Air L Com [Internet]. 2011;76(2).

9. Barnard SM. Bats in captivity. Krieger Publishing Company; 2011.

10. Arthur M. Greenhall US, editor. Natural History of Vampire Bats . Boca Raton, FL: CRC Press; 1988. 231–232 p.

11. De Almeida MF, Aires CC, Martorelli LFA, De Barros RF, Massad E. Maintenance of the haematophagous bat (Desmodus rotundus) in captivity for experimental studies on rabies. Chiropt Neotrop. 2009;15(1):434–40.

12. Wilkinson GS. The social organization of the common vampire bat - I. Pattern and cause of association. Behav Ecol Sociobiol. 1985;17(2):111–21.

13. Cárdenas-Canales EM, Gigante CM, Greenberg L, Velasco-Villa A, Ellison JA, Satheshkumar PS, et al. Clinical presentation and serologic response during a rabies epizootic in captive common vampire bats (desmodus rotundus). Trop Med Infect Dis. 2020;5(1):1–13.