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## Antibody response of cattle to vaccination with commercial modified live rabies vaccines in Guatemala



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### ABSTRACT

Vampire bat rabies is a public and animal health concern throughout Latin America. As part of an ecological study of vampire bat depredation on cattle in southern Guatemala, we conducted a vaccine seroconversion study among three dairy farms. The main objectives of this cross sectional and cohort study were to understand factors associated with bat bites among cattle, to determine whether unvaccinated cattle had evidence of rabies virus exposure and evaluate whether exposure was related to bat bite prevalence, and to assess whether cattle demonstrate adequate seroconversion to two commercial vaccines used in Guatemala. In 2012, baseline blood samples were collected immediately prior to intramuscular inoculation of cattle with one of two modified live rabies vaccines. Post vaccination blood samples were collected 13 and 393 days later. Sera were tested for rabies virus neutralizing antibodies (rVNA) by the rapid fluorescent focus inhibition test (RFFIT). Across two years of study, 36% (254/702) of inspected cattle presented gross evidence of vampire bat bites. Individual cattle with a bat bite in 2012 were more likely have a bat bite in 2013. Prior to vaccination, 12% (42/350) of cattle sera demonstrated rVNA, but bite status in 2012 was not associated with presence of rVNA. Vaccine brand was the only factor associated with adequate rVNA response of cattle by day 13. However, vaccine brand and rVNA status at day 13 were associated with an adequate rVNA titer on day 393, with animals demonstrating an adequate titer at day 13 more likely to have an adequate titer at day 393. Our findings support stable levels of vampire bat depredation and evidence of rVNA in unvaccinated cattle. Brand of vaccine may be an important consideration impacting adequate rVNA response and long-term maintenance of rVNA in cattle. Further, the results demonstrate that initial response to vaccination is associated with rVNA status over one year following vaccination.

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## 1. Introduction

Rabies is caused by infection with negative sense single stranded RNA viruses in the genus *Lyssavirus*. Rabies virus is the most relevant member from an epidemiological perspective, due to an estimated global human burden in excess of 55,000 cases annually (Knobel et al., 2005). While the global human burden is principally associated with transmission cycles involving domestic dogs, bats are an important reservoir and vector of rabies in the Americas. Rabies outbreaks in cattle have been reported since the early 20th century (Carini, 1911; Haupt and Rehaag, 1921). Carini (1911) linked the outbreaks in cattle to wildlife, as cases in dogs were rare at that time and canine population reduction had no effect on the incidence of cases in cattle. Haupt and Rehaag (1921) were able to isolate the virus from a fruit bat (*Artibeus lituratus*), providing the first evidence linking rabies in cattle with bats. However, it was not until an outbreak in Trinidad that rabies virus (RABV) was isolated from several naturally infected common vampire bats (*Desmodus rotundus*) and linked to the disease in cattle and man (Pawan, 1936). Thereafter, cattle rabies was widely recognized in Latin America, with estimated annual mortality of 0.5 million cattle and annual economic losses of \$47 million 1967 USD (~\$335 million 2014 USD; [www.bls.gov](http://www.bls.gov)) despite estimated annual vaccination of 2.7 million cattle (Acha, 1967). Cattle continue to be the primary sentinel animal associated with RABV circulation in vampire bats and outbreaks throughout Latin America. One study from Mexico demonstrated a mean mortality rate of 10.3% among affected herds with 50 or more animals, with a range of 1.3% to 29% (Prieto and Baer, 1972). As vaccination campaigns have reduced the burden of canine rabies in Latin America, *D. rotundus* has become the primary reservoir and vector of rabies (Schneider et al., 2009). These obligate blood feeders prefer cattle as a prey resource (Greenhall, 1988), though there can be dietary flexibility where cattle are scarce (Delpietro et al., 1992).

Experimental evidence supporting the utility of vaccination to protect cattle against bat rabies appear as early as 1955 (Carneiro et al., 1955), and modified live or nervous tissue vaccines were shown to reduce mortality of cattle on affected farms during outbreaks (Prieto and Baer, 1972). However, vaccination coverage among cattle, as a preventative measure against rabies infection, tends to be low (<5%) throughout most of Latin America (OIE, 2013), likely due to the relatively high cost of vaccinating large numbers of animals, turnover in herd animals across years, and low perceived threat of rabies among farmers. In Guatemala, the cattle population reported to OIE from 2005 to 2012 fluctuated between 2.0 and 4.5 million animals, with highest estimate in 2009 and lowest in 2012 (OIE, 2013). During all years except 2009, vaccination coverage was estimated to be between 0 and 2% of the cattle population. In 2009, 0.5 million doses were administered to cattle and coverage was estimated to be 11% of the cattle population. A recent study from Mexico has demonstrated that pre-exposure vaccination of cattle may be more efficient and economically beneficial than control efforts focused on depopulation of vampire bats

(Anderson et al., 2012). However, even if rabies risk was removed from the equation, the secondary infections that commonly result from vampire bat depredation still poses a serious economic hardship for farmers in lost production value of affected herds (Flores-Crespo and Arellano-Sota, 1991).

Historically in Guatemala, livestock farming was most prolific in the southeastern region of the country. However, an increase in sugar cane and rubber production in the southeastern region has spurred significant land use conversion, and livestock farming and the associated burden of rabies has primarily shifted to the northwestern region of the country, though smaller dairy operations remain active in the southeast. While the number of rabies cases in dogs in Guatemala appears to be declining, the number of cases in cattle has been rising steadily over the past decade (OIE, 2013), though the impact of testing effort is unclear due to the absence of reported denominator data. As virus typing is not performed on positive cases, it has not been possible to link the rise in cattle cases with vampire bat rabies. However, this scenario is most likely given the strong association of vampire bats with cattle rabies outbreaks elsewhere throughout Latin America and the high number of cattle cases and rare occurrence of canine cases in some Departments (e.g., Petén, Alta Verapaz). Despite a lack of conclusive laboratory evidence linking rabies in cattle to bats, vampire bat control activities (i.e., poisoning or culling) are conducted in Guatemala in response to suspected outbreaks in cattle, although these are reactive strategies and sporadically applied. While they may eliminate local colonies of bats, and reduce bite incidence to cattle within a short time frame, these strategies are ineffective at controlling rabies virus circulation in vampire bats at a landscape scale. Thus, vaccination is necessary to protect livestock against rabies infection. During past suspected outbreaks, mass vaccination of cattle was initiated with assistance from the Ministry of Agriculture (MAGA), but suspect clinical cases of rabies in vaccinated cattle have raised concerns about the efficacy of the vaccines used – as reported elsewhere (Oliveira et al., 2000). There are several possibilities regarding such cases, including scenarios that animals are incubating the disease prior to vaccination, that the vaccine was improperly stored or administered, that the animal did not actually die of rabies (i.e., no lab confirmation of case), or potential for reversion of virulence of the modified live vaccine (Whetstone et al., 1984). One recent paper has highlighted the complexity of diagnosing rabies based on clinical signs in areas where other neurologic diseases of cattle can be present (Ramirez-Romero et al., 2014).

The objectives of this study were to understand factors associated with bat bites among cattle, to determine whether unvaccinated cattle had evidence of rabies virus exposure and evaluate whether exposure was related to bat bite prevalence, and to assess whether cattle demonstrate adequate seroconversion to commercial modified live rabies vaccines licensed in Mexico and used in Guatemala. This study compares both the short-term response to two different rabies vaccines and the maintenance of antibody titers over one year post vaccination.

## 2. Materials and methods

### 2.1. Animal sampling and treatments

The study design had elements of a cross sectional and cohort nature. Bat bite prevalence among all animals inspected at the farms, and seroprevalence of antibodies in unvaccinated cattle, were investigated in a cross-sectional design. Bat bite prevalence to individual animals across years, and antibody response to vaccination, were investigated in a cohort design. Three dairy farms in the municipality of Patulul, located in the Department of Suchitepéquez, Guatemala, were enrolled in the vaccination study as a result of ongoing ecological studies of vampire bats in the same area and capture of bats at or near some of the farms. Farms were identified as A, B, and C (Fig. 1). Each farm has a standing herd ranging from 100 to 200 cattle, and animals are bought, sold, and born in the herds each year, though the rates of turnover vary across the farms (e.g., C has the highest turnover rates and A the lowest). All cattle over six months of age at each farm were included in the study. Sex ratios in the herds tend to be heavily skewed to females, with one or two breeding bulls and a variable number of juvenile males. No animals in the herds were reported as vaccinated against rabies by the farm owners prior to the study. Protocols for animal restraint and sampling were approved by the Centers for Disease Control and Prevention (USA) and the Universidad del Valle de Guatemala (Guatemala) Animal Care and Use Committees. During February 7–9, 2012, cattle were corralled and manually restrained to permit sampling and vaccination. Cattle were visually inspected to determine relative age, sex, and gross evidence of vampire bat bites (e.g., open and/or bleeding lesions approximately 2–3 cm in diameter). Approximate ages of cattle were available at farm A, but in other cases relative ages were assigned such that ‘juvenile’ refers to an animal less than 2 years of age and ‘adult’ refers to an animal equal to or greater than 2 years of age. A 3–5 ml blood sample was collected by venipuncture of the jugular or tail (coccygeal) vein immediately prior to intramuscular vaccination with 2 ml of (one of two) commercially available modified live rabies vaccines. Hereafter, these products are referred to as vaccine ‘A’ and vaccine ‘B’ to protect the identity of the manufacturers. Vaccine A is made with the Evelyn Rokitnicki Ableseth (ERA) strain of rabies virus, and vaccine B is made with the Street Alabama Dufferin (SAD) strain. Vaccines were reconstituted on site using supplied sterile water according to the manufacturer’s instructions, and were maintained on ice packs in a styrofoam cooler until use (i.e., within 6 h of reconstitution). Both products are licensed in Mexico and labeled for use in large animals. Animals were arbitrarily assigned to one of the two vaccination treatment groups during initial sampling, which resulted in greater treatment applications of vaccine B ( $N_A = 121$ ,  $N_B = 229$ ). No unvaccinated control animals were assigned or followed in the study.

Cattle were corralled and manually restrained during February 20–22, 2012 to collect a 13 day post vaccination blood sample. Farms were sampled in an identical sequence. No visual inspection of bat bites was performed

during the day 13 follow up visit. During March 5–7, 2013, 393 days after vaccination, cattle were again corralled and manually restrained for collection of a blood sample prior to booster vaccination with 2 ml of vaccine B. Selection of vaccine B for booster vaccination was due to constraints of product availability from the Guatemalan supplier of vaccine A at the time of booster vaccination. During 2013 sampling, cattle were visually inspected to determine relative age, sex, and gross evidence of vampire bat bites.

At farm A, cattle were identified by permanently branded numbers, providing a unique and consistent form of identification. At farm B, cattle were identifiable by name, providing a unique and consistent form of identification. At farm C, 25 [milk] cattle were identifiable by name, and temporary chemical brands of a two or three-digit unique number were applied to all other farm C cattle by MAGA personnel during the initial sampling in 2012. Long-term re-sampling was not initially anticipated during activities in 2012. While it was possible to read the chemically branded numbers of farm C cattle at 13 days post vaccination, it was no longer possible to read the numbers at 393 days post vaccination. Due to this factor, and because it was not possible to re-sample the 25 cattle identified by name in 2013, farm C cattle were excluded from day 393 analyses. Other animals lost during follow up were necessarily excluded from day 13 or day 393 analyses.

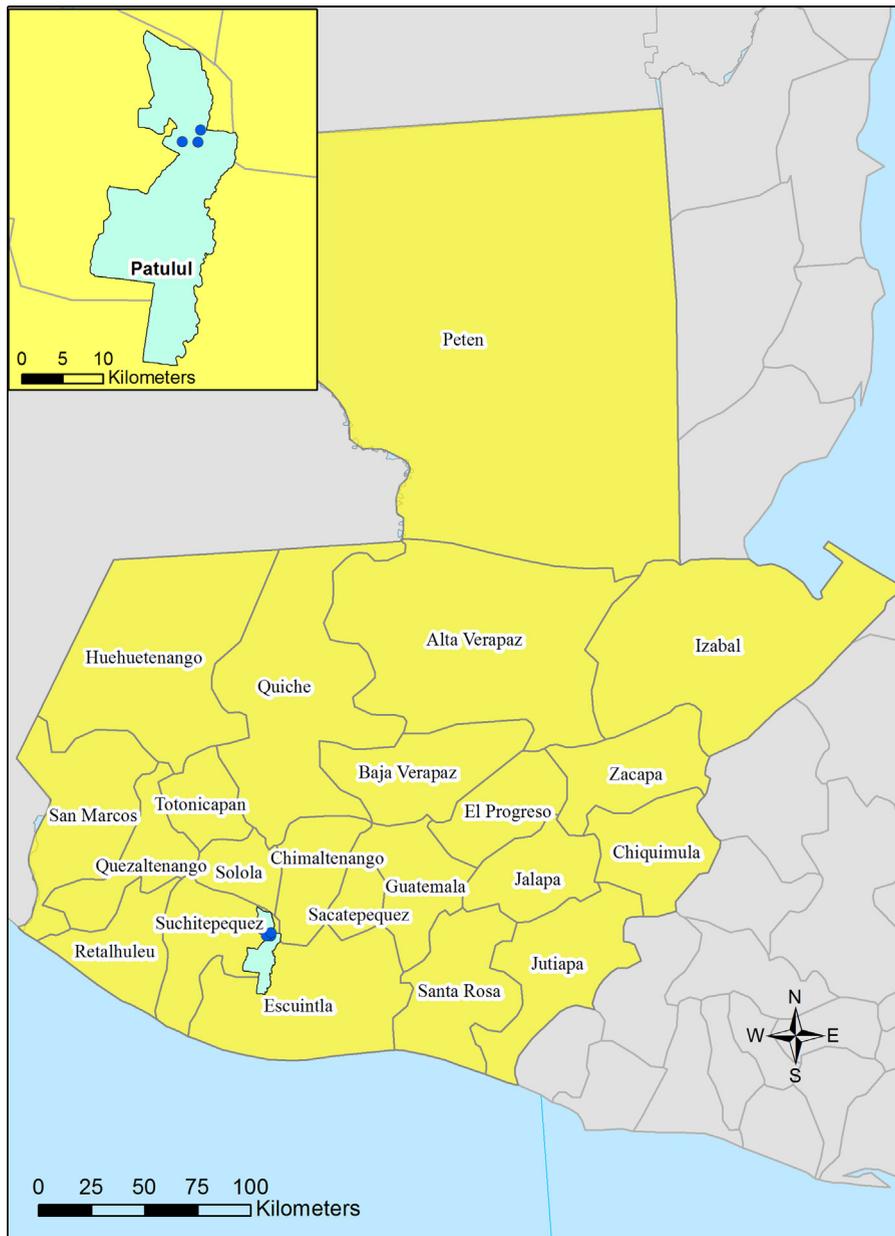
Blood samples were kept out of direct sunlight during sampling, and within 10 h of collection they were separated by low-speed centrifugation for 10 min. Sera were transferred into 2 ml cryovials using sterile pipettes, and stored at  $-70^{\circ}\text{C}$  until shipment to the Rabies Laboratory at CDC in Atlanta, GA, USA.

### 2.2. Detection of rabies virus neutralizing antibodies

Sera were assayed for rabies virus neutralizing antibodies (rVNA) by rapid fluorescent focus inhibition test (RFFIT) as described by Smith et al. (1996), and screened at 1:5 and 1:25 dilutions. Raw titers were converted to international units (IU) by comparison to the positive control standard rabies immune globulin (SRIG) containing 2 IU/ml, evaluated in each test in five-fold dilutions up to 1:625. The cut-off threshold for seropositive rVNA status was taken as 100% neutralization of virus at a 1:5 serum dilution, corresponding to titers equal to or greater than 0.10 IU/ml. The threshold for adequate rVNA response was taken as 50% or greater neutralization of virus at a 1:25 serum dilution, which corresponded to titers equal to or greater than 0.20 IU/ml. All positive sera (i.e., titer greater than or equal to 0.10 IU/ml) were tested in duplicate or triplicate, and geometric mean titers were used for the final evaluation in relation to the cut-offs.

### 2.3. Statistical analyses

Contingency analyses were used to evaluate variation in bite prevalence among all cattle at farms A, B and C during 2012 and 2013. Generalized linear mixed models (GLMs) of a logistic nature were used to test for associations with five binomial response variables. Farm was treated as a random



**Fig. 1.** Map of the study area and departments of Guatemala. The map inset is the municipality of Patulul, located in the Department of Suchitepéquez. Solid circles show the locations of the three farms in the study.

effect in all GLMs to permit generalizing the results of this study and to control for confounding effects of farm variation on certain fixed effect variables. Fixed effects in the GLMs were binary, and included sex (male/female), relative age (adult/juvenile), rVNA status (0/1), bite status (0/1), and vaccine brand (A/B). Due to uneven sampling of relative age and sex, an interaction term was included in GLMs where applicable. For repeat observations of an individual animal, a factor of the earlier status of the individual was included as a fixed effect in the GLM to account for prior observations (e.g., bite or antibody status). Continuous age data measured in years were available from cattle at farm A, and logistic GLMs with age as the only fixed effect were tested

against response variables of interest when untreated or log-transformed age data were normally distributed. When untreated or log-transformed age data from farm A were not normally distributed, a nonparametric ANOVA on the ranks was used to test for an association of the response variable with age. Pending the results of logistic GLMs using a single fixed effect of age, multiple fixed effects were tested with farm A data to evaluate the effect of age while controlling for other binary variables described above. Logistic GLMs (PROC GLIMMIX) and other analyses were run using SAS v9.2 (SAS Institute, Cary, NC). Estimates and standard error for farm, the random effect covariance parameter, are shown for each mixed GLM evaluated. Odds ratios (OR)

**Table 1**

Demographic data for the cohort of cattle sampled at three farms in 2012 and re-sampled in 2013.

Farm	2012					2013				
	Adults		Juveniles		Total	Adults		Juveniles		Total
	Male	Female	Male	Female		Male	Female	Male	Female	
A	1	154	0	0	155	1	81	0	0	82
B	3	94	0	25	122	2	73	0	5	80
C	4	52	26	34	116	0	0	0	0	0
Total	8	300	26	59	393	3	154	0	5	162

**Table 2**

The number of cattle inspected and proportion demonstrating evidence of bat bites during cross sectional surveys in 2012, 2013, and cumulatively at three farms in Patulul, Guatemala.

Farm	2012		2013		Cumulative	
	N	Proportion with bites	N	Proportion with bites	N	Proportion with bites
A	155	0.43	115	0.71	270	0.55
B	122	0.09	127	0.31	249	0.20
C	116	0.20	67	0.49	183	0.31
Total	393	0.25	309	0.50	702	0.36

were calculated for significant fixed effects ( $\alpha = 0.05$ ), with 95% confidence limits.

### 3. Results

A total of 393 cattle were sampled and vaccinated during the initial visit in 2012 (Table 1). Based on unique permanent identification present at farms A and B in 2012, it could be determined that 58% of 277 cattle sampled from those two farms were resampled in 2013. Due to temporary chemical brand identification applied to cattle at farm C in 2012, and inaccessibility of the uniquely named farm C cattle in 2013, it was not possible to determine whether any of the 116 cattle sampled at farm C in 2012 were resampled in 2013.

#### 3.1. Bat bite prevalence among cattle

Prevalence of bat bites among cattle at the farms varied between years (Table 2). Bite prevalence across farms varied in 2012 ( $\chi^2 = 43.3$ ,  $P < 0.0001$ ) and in 2013 ( $\chi^2 = 39.8$ ,  $P < 0.0001$ ) (Table 2). In comparisons of cross-sectional bite data from 2012 to 2013 (Table 2), year was associated with bite prevalence (year  $F_{1,698} = 53.4$ ,  $P < 0.0001$ , OR 3.7 [95% CL 2.6–5.3], farm  $0.83 \pm 0.86$ ) and cattle in 2013 were more likely to be bitten. In the 2012 cohort, sex was the only factor associated with bite status and males were more likely to be bitten compared to females (Table 3, Model 1; sex  $F_{1,387} = 4.3$ ,  $P = 0.04$ , OR 3.0 [95% CL 1.1–8.7], farm =  $1.13 \pm 1.20$ ). From the 2012 cohort, 162 cattle were re-inspected in 2013 (Table 3, Model 2). Animals bitten in 2012 were more likely to have evidence of bites in 2013 (2012 bite status  $F_{1,159} = 9.5$ ,  $P = 0.002$ ; OR = 4.0 [95% CL 1.6–9.5], farm =  $0.88 \pm 1.34$ ).

In the farm A cross sectional data from 2012 to 2013 (Table 2), neither age nor log-transformed age data were normally distributed. Although none of the farm A cattle sampled were juveniles (range<sub>2012</sub>: 2–16 years, median<sub>2012</sub> = 7.5 years; range<sub>2013</sub>: 2.5–17 years,

**Table 3**

A description of generalized linear mixed models evaluated in the cohort study. Farm was treated as a random effect in each model. Fixed effects in bold text were significant for a given model.

Model	N	Response variable	Fixed effects
Predictors of bite status			
(1)	393	2012 bite status	Relative age, sex, relative age $\times$ sex
(2)	162 <sup>a,b</sup>	2013 bite status	2012 bite status
Predictors of rVNA in unvaccinated animals			
(3)	350	Day 0 rVNA <sup>c</sup>	Relative age, sex, 2012 bite status
Predictors of response to rabies vaccination			
(4)	318	Day 13 rVNA <sup>d</sup>	Relative age, sex, 2012 bite status, vaccine brand, day 0 rVNA status, relative age $\times$ sex
(5)	133 <sup>a,b</sup>	Day 393 rVNA <sup>d</sup>	2013 bite status, vaccine brand, day 0 rVNA status, day 13 rVNA status

<sup>a</sup> Less than five males sampled; sex not included.

<sup>b</sup> Five juveniles sampled total; relative age not included.

<sup>c</sup> Evaluated at rVNA greater than or equal to 0.10 IU/ml.

<sup>d</sup> Evaluated at rVNA greater than or equal to 0.20 IU/ml.

median<sub>2013</sub>: 7 years), age was associated with bite status in the cross sectional 2012 data and younger animals were more likely to be bitten in a nonparametric ANOVA on the ranks ( $F_{1,142} = 12.4$ ,  $P = 0.0006$ ). In the cross sectional data from farm A in 2013 ( $n = 115$ ), a similar trend was observed with younger animals more likely to be bitten in a nonparametric ANOVA on the ranks ( $P = 0.09$ ).

#### 3.2. Rabies antibodies in unvaccinated cattle

Among 393 cattle sampled prior to vaccination in 2012, rVNA titers were determined from 89% of 393 animals. Of 350 cattle sera tested, 12% of sera presented evidence of rVNA prior to vaccination (Table 4). The rVNA

**Table 4**

The proportion of cattle in the cohort study with pre and post vaccination rabies virus neutralizing antibody titers of cattle at three farms in Patulul, Guatemala.

Farm	Day 0		Day 13			Day 393		
	N	≥0.10 <sup>a</sup>	N <sup>b</sup>	≥0.10	≥0.20 <sup>a</sup>	N <sup>b</sup>	≥0.10	≥0.20
A	114	0.06	102	0.57	0.40	54	0.24	0.13
B	120	0.04	111	0.61	0.47	71	0.31	0.20
C	116	0.26	73	0.67	0.49	0	–	–
Total	350	0.12	286	0.61	0.45	125	0.28	0.17

<sup>a</sup> Rabies virus neutralizing antibody titer cut-off (IU/ml).

<sup>b</sup> Includes only animals seronegative at baseline (day 0).

seroprevalence in unvaccinated cattle varied across farms ( $\chi^2 = 31.8, P < 0.0001$ ), with farm C (26%) having higher antibody prevalence compared to farm A (6%) and farm B (4%). However, the presence of rVNA in unvaccinated cattle was not associated with the animal's bite status in 2012, sex nor relative age (Table 3, Model 3; farm =  $1.01 \pm 1.13$ ). Among cattle sera tested at the farm A, log age data were normally distributed, but the presence of rVNA in unvaccinated cattle was not associated with log age.

### 3.3. Antibody response of cattle to vaccination

Day 13 post vaccination rVNA titers were determined from 318 cattle with a known baseline titer (Table 4). Among cattle that were rVNA negative prior to vaccination and sampled at day 13 ( $n = 286$ ), 45% demonstrated an adequate rVNA response 13 days post vaccination (i.e., titer  $\geq 0.20$  IU/ml), whereas 61% were rVNA seropositive (i.e., titer  $\geq 0.10$  IU/ml). Among cattle that were rVNA seropositive prior to vaccination and sampled at day 13 ( $n = 32$ ), 59% demonstrated an adequate rVNA response 13 days post vaccination, whereas 78% were rVNA seropositive. Among all 318 cattle with sera evaluated at day 0 and day 13 post vaccination (Table 3, Model 4), vaccine brand was the only factor associated with an adequate rVNA response at day 13 (vaccine brand  $F_{1,309} = 24.0, P < 0.0001$ , OR 3.6 [95% CL 2.2–6.0], farm =  $0.06 \pm 0.12$ ), and cattle treated with vaccine A were more likely to demonstrate an adequate titer by day 13 compared to cattle treated with vaccine B. An adequate response by day 13 was marginally associated with baseline rVNA status ( $P = 0.08$ ), and cattle seropositive at baseline were more likely to demonstrate an adequate rVNA response by day 13. An adequate rVNA response at day 13 was not associated with relative age, sex, a relative age by sex interaction term, nor 2012 bite status. Among cattle at farm A with titers evaluated at day 0 and day 13 post vaccination, log age data were normally distributed, but an adequate rVNA response was not associated with log age.

Among cattle that were seronegative prior to vaccination in 2012 and sampled at days 13 and 393 ( $n = 125$ ), 17% demonstrated an adequate rVNA titer at day 393, whereas 28% were seropositive (Table 4). Among cattle that were seropositive prior to vaccination in 2012 and sampled at days 13 and 393 ( $n = 8$ ), 13% demonstrated an adequate rVNA titer at day 393, whereas 63% were seropositive. Among cattle sampled longitudinally across all time points (Table 3, Model 5), vaccine brand (vaccine brand  $F_{1,127} = 6.0,$

$P = 0.02$ , OR 3.6 [95% CL 1.3–10.4]) and rVNA status at day 13 post vaccination (day 13 status  $F_{1,127} = 7.6, P = 0.007$ , OR 4.4 [95% CL 1.5–12.8]) were associated with an adequate rVNA titer at day 393 post vaccination (farm =  $0.40 \pm 0.81$ ). Similar to the day 13 results, cattle that received vaccine A were more likely to have an adequate rVNA titer at day 393. Cattle which demonstrated an adequate rVNA titer at day 13 post vaccination were more likely to have an adequate titer at day 393. Cattle with evidence of a bat bite in 2013 were marginally more likely to have an adequate rVNA titer at day 393 ( $P = 0.10$ ). Baseline rVNA status was not associated with an adequate rVNA response at day 393. At farm A, log age data were normally distributed, but an adequate rVNA response at day 393 was not associated with log age.

## 4. Discussion

Vampire bat depredation is a well-recognized public and veterinary health concern throughout Latin America, and the risk of rabies, a highly fatal zoonosis, is clearly the most high profile infection risk posed by vampire bat bites. Other relevant health risks associated with vampire bat depredation of cattle include secondary infections that result from the open wounds left by bats after feeding, although data on lost production value due to blood loss alone have been equivocal (Flores-Crespo and Arellano-Sota, 1991). Despite this, relatively low proportions of farmers in Latin America vaccinate their cattle against rabies. Literature has suggested that vampire bats may be loyal to stable and reliable food sources (de Verteuil and Ulrich, 1936). While the feeding behaviors of individual vampire bats were not monitored during the study period, individual bat fidelity to foraging grounds (i.e., cattle pastures) was observed during a three week radiotracking study in 2011 at the farm A involving 16 *D. rotundus* (AG, unpublished data). Vampire bat roosts (est. 20–50 bats) were visually located within 2 km proximity to farms A and C during 2012, but no bat roost was visually located in proximity to B. The results of the current study demonstrate nonrandom attacks on cattle across years, where certain cattle suffer repeated depredation by vampire bats over time. Where approximate cattle ages were known at farm A, younger cattle were more likely to be bitten compared to older cattle, although all farm A cattle sampled were considered adults.

Despite the relatively high prevalence of vampire bat bites on cattle during the two-year study period, it is equally noteworthy that only two cases of cattle rabies

were reported to OIE during 2005–2012 from the Department of Suchitepéquez (one in 2010, one in 2011), whereas 102 cases of cattle rabies were reported from the northern Department of Petén and 50 cattle cases reported from the northern Department of Alta Verapaz during the same time period (OIE, 2013). While geographic area or population size of the Departments may be a factor (Fig. 1), area-corrected cattle rabies incidence is still three to six times as high in the northern Departments of Petén ( $2.8 \times 10^{-3}$  cases/km<sup>2</sup>) and Alta Verapaz ( $5.1 \times 10^{-3}$  cases/km<sup>2</sup>) in comparison to Suchitepéquez ( $8.3 \times 10^{-4}$  cases/km<sup>2</sup>). The population corrected incidence, using the average projected population from 2005 to 2010 of the three Departments (I.N.E., 2008), shows similar trends (Petén – 10 cases per 100,000 pop, Alta Verapaz – 9 cases per 100,000 pop, Suchitepéquez – 0.42 cases per 100,000 pop). However, during the same time period, 45 cases of dog rabies were reported from Suchitepéquez, in contrast to two dog cases from Alta Verapaz and five dog cases from Petén, suggesting that canine rabies is of greater concern in the southwestern Departments. As a result, we cannot rule out that some rabies virus exposures in unvaccinated cattle that were detected in this study might also be due to contact with dogs, although the ecological link with vampire bats is clearly stronger. Despite this, reports of cattle rabies in Guatemala appear to be on the rise in recent years, though a lack of variant typing has precluded direct association with vampire bat RABV, and the absence of reported rabies cases in bats leaves many questions. A low proportion of clinically suspect cattle are subject to diagnostic testing by the national laboratories in Guatemala, primarily due to difficulties with sample transport and cold chain maintenance, as likely occurs elsewhere in Latin America (Baer, 1991). Thus, the current passive surveillance system underestimates the true burden of cattle rabies in the country. Although independent surveillance of bats in this study area (i.e., at farm A) did not provide serologic evidence of rabies virus circulation in 2011, there is strong evidence of rabies virus circulation among bats in nearby localities based on rVNA seroprevalence and virus isolation (Ellison et al., 2014). The detection of rVNA in unvaccinated cattle suggests prior exposure of cattle to RABV in the study area, and may be associated with the repeated vampire bat depredation as observed during the study. None of the cattle were reported by farm owners to have been vaccinated prior to the study and, with a single exception (i.e., the bull), all animals sampled at farm A were born and raised on that farm. It is unclear why there were lower proportions of unvaccinated cattle with rVNA detected in 2012 at farms A (6%) and B (4%) compared to farm C (26%), but an unrecognized history of vaccination may have been possible at farms B and C. However, at farm A, where animal records, identification and animal retention were highly reliable, we can confirm that all seven unvaccinated animals with rVNA at day 0 had been on that farm for the past 5–11 years (i.e., since birth).

The rVNA response to vaccination with rabies vaccines is well studied in livestock (Prosperi et al., 1984; Cortes et al., 1993; da Silva et al., 2000; Oliveira et al., 2000), and most prior work has compared responses to live versus inactivated rabies vaccines. An [adjuvanted] inactivated vaccine

produced more robust rVNA responses than a modified live vaccine following a single dose regimen in cattle in one study (Prosperi et al., 1984), but a subsequent study found weak stimulation of antibodies from a single dose administration of inactivated or modified live vaccines (Oliveira et al., 2000). However, stimulation of cell-mediated immunity by [replication of] modified live vaccines may confer additional resistance to rabies challenge even where rVNA induction appears suboptimal. The RABV glycoprotein (G) is the primary antigen responsible for induction of rVNA (Wiktor et al., 1973). The peak rVNA response to vaccination with rabies vaccines typically is observed 14–28 days following vaccination, and thus the timing of sampling periods in this study may have precluded detection of maximal proportions of animals with adequate rVNA, and potential underestimation of rVNA response to vaccination. Despite this, an adequate rVNA response was detected on day 13 in nearly half of the animals, and closer to two-thirds showed at least some evidence of rVNA seroconversion on day 13. Although this study did not quantify endpoint titers to facilitate analysis of an anamnestic response, cattle that were seropositive prior to vaccination seroconverted in greater proportions than seronegative cattle.

There was no association of relative age or sex on the development of an adequate rVNA response by cattle at day 13. Although outside the scope of the current study, additional investigation is warranted to determine whether physiological parameters of an animal's health could explain the individual variation in serologic response observed in this study. One potential confounding factor for the low proportion of study animals with adequate responses at day 13 may relate to the lack of randomization in the treatment schema, as two-thirds of 393 cattle received vaccine B on day 0. Results unequivocally indicated an effect of vaccine brand on development of adequate rVNA among cattle on days 13 and 393 post vaccination. Among 286 cattle seronegative on day 0, 63% of 99 cattle treated with vaccine A had an adequate titer, whereas 36% of 187 cattle treated with vaccine B had an adequate titer on day 13. Although vaccine B appears to be less immunogenic to cattle in this study, warranting follow-up comparative investigations of potency, we cannot conclude from these data that vaccine B is less efficacious in conferring resistance to RABV infection in vaccinated cattle. The form of G presentation was found to be an important factor affecting immunogenicity in mice (Dietzschold et al., 1983). A study in Brazil also demonstrated the importance of the form of G presentation for rVNA response in cattle, where virion-attached G was the only significant predictor of rVNA response, when compared with total G or free-soluble G (Piza et al., 2002). The same study also did not find a correlation between vaccine potency, measured in vivo by the standard NIH (i.e., mouse inoculation) test, and rVNA induction in cattle (Piza et al., 2002). More laboratory studies of the composition of the vaccines utilized in this study are needed to understand the variation due to vaccine brand observed in this study.

Among 133 animals in the study cohort through 2013, 40% of 57 animals with an adequate titer on day 13 were rVNA seropositive over one year later. However, one confounding factor for the long-term follow up was the

absence of an unvaccinated seronegative control group to evaluate the impact of potential natural RABV exposures from vampire bats or other animals in the study area. Given the detection of rVNA in unvaccinated cattle and the marginal association of 2013 bite status with presence of adequate titers on day 393, natural exposures to RABV from bat (or dog) bites to cattle may have influenced rVNA titers on day 393. The low proportions of animals with adequate titers at day 13 and over one year later are not surprising given other studies which have shown weak rVNA response and maintenance to single doses of modified live vaccines (da Silva et al., 2000; Oliveira et al., 2000), and collectively these studies underscore the importance of annual booster vaccination. Moreover, rVNA titers may not correlate with protection against rabies and there is no agreed upon level deemed protective, as there may be other immunological factors impacting resistance or susceptibility to rabies (Moore and Hanlon, 2010).

## 5. Conclusions

Vaccination of incidental hosts such as livestock and companion animals is a key tool to protect against RABV infection, especially in areas where there is evidence of repeated depredation by vampire bats. Given the apparent rise in significance of vampire bat rabies for Latin America as a whole, and as observed from increasing numbers of laboratory confirmed cases in Guatemala in particular, the importance of vaccinating valuable production animals should not be overlooked. Enhanced [active] rabies virus surveillance among bats in Guatemala also demonstrated that the risk of RABV infection associated with bats is geographically widespread. The risk of RABV infection is clearly highest among cattle that are repeatedly bitten by bats, and in this study bite status in one year was associated with bite status the following year, although bite status was not associated with the presence of rVNA in unvaccinated animals. Vaccine brand was an important factor both in the initial response and long-term maintenance of an adequate rVNA response, though additional laboratory studies are needed to understand the basis for these differences. The initial rVNA response to vaccination was associated with the long-term maintenance of adequate rVNA titers in cattle sampled longitudinally. The results of this study demonstrate that cattle respond to intramuscular vaccination with commercial modified live vaccines in Guatemala, and that relative age and sex of the animals are not associated with the initial rVNA response to vaccination.

## Conflict of interest

No competing financial interests exist.

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