Journal of Equine Veterinary Science 92 (2020) 103140

Contents lists available at ScienceDirect

Journal of Equine Veterinary Science

journal homepage: www.j-evs.com

Original Research

National Seroprevalence and Risk Factors for Eastern Equine Encephalitis and Venezuelan Equine Encephalitis in Costa Rica

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ARTICLE INFO

Article history:
Received 12 February 2020
Received in revised form
17 May 2020
Accepted 18 May 2020
Available online 2 June 2020

Keywords:
VEEV
EEEV
Costa Rica
PRNT
IgG ELISA
Seroprevalence

ABSTRACT

Eastern equine encephalitis and Venezuelan equine encephalitis are endemic neglected tropical diseases in the Americas, causing encephalitis in both horses and humans. In 2013, a cross-sectional study was performed in 243 horses located in the highlands and lowlands throughout Costa Rica. Serum samples were analyzed with an IgG ELISA and confirmed by the plaque-reduction neutralization test (PRNT80). Venezuelan equine encephalitis virus (VEEV) and Eastern equine encephalitis virus (EEEV) overall seroprevalences by the PRNT80 were 36% (95% confidence interval [CI]: 29.9–42.5; 78/217 horses) and 3% (95% CI: 1.3–5.9; 6/217 horses), respectively. Both the viruses occurred in the lowlands and highlands. Rainfall and altitude were associated with VEEV seropositivity in the univariate analysis, but only altitude <100 meters above sea level was considered a risk factor in the multivariate analysis. No risk factors could be identified for the EEEV in the multivariate analysis. This is the first study that estimates the seroprevalence of the EEEV and VEEV in Costa Rican horses. The VEEV is widely distributed, whereas the EEEV occurs at a much lower frequency and only in specific areas. Clinical cases and occasional outbreaks of both viruses are to be expected.

1. Introduction

In developing regions, zoonotic pathogens are often neglected and diseases are misdiagnosed or unrecognized because of their unspecific symptoms in the absence of specialized laboratory testing. In the Americas, several endemic and emerging arboviruses including the alphaviruses, Eastern equine encephalitis virus (EEEV), and Venezuelan equine encephalitis virus (VEEV) cause clinically indistinguishable systemic and neurologic disease in equids and humans [1–3]. The EEEV is unable to produce high viremia in either horses or humans (which are considered dead-end hosts for this virus), whereas epidemic subtypes of the VEEV may produce sufficient viremia in both species to allow them to act as amplifying hosts capable of transmitting the virus via infected mosquito vectors to other horses or humans [2].

Although these viruses impact public health and are notifiable diseases by the World Organization for Animal Health (OIE), their influence on animal health, their zoonotic potential [4], and their epidemiology are poorly described in many parts of the Americas [5,6]. In Central America, sporadic outbreaks of the EEEV and VEEV, influenced by weather conditions such as heavy rainfall and high temperatures [7], as well as the presence of known wildlife vectors and reservoirs have been reported [2,8,9]. Strains of the Madariaga virus (South American variants of the EEEV) have produced outbreaks in equines and humans in Panama [1,2]. In Costa Rica, in 1970, VEE-IAB subtype outbreaks killed 300 horses in the province of Guanacaste with a 64% case fatality ratio [8]. The overall equine

Animal welfare/ethical statement: The sampling was performed in accordance with Costa Rican General Law No. 8495 on the National Service of Animal Health from 2006. Horse owners were asked to participate voluntarily in the study and were informed that samples were being taken for research purposes only. No experimental animals were used in this study.

Conflict of interest statement: The authors have declared no conflict of interests.

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https://doi.org/10.1016/j.jevs.2020.103140

seroprevalence (neutralizing antibodies) was 43% at this time; 13 of 109 sampled humans also tested seropositive, six of whom presented fever and asthenia [8]. Another study in Guanacaste (located at an altitude of <250 meters above sea level [m.a.s.l], reported seroprevalence in 46 of 360 horses (13%) for the EEEV and 117 of 360 horses (32.5%) for the VEEV by IgG ELISA (N.B. these results were not confirmed by a seroneutralization test) [10]. Our study aimed to assess EEEV and VEEV seroprevalence in horses in highland and lowland areas across Costa Rica.

2. Materials and Methods

2.1. Study Area

Costa Rica is a tropical country located in Central America, between Nicaragua and Panama. Altitude ranges from sea level up to 3819 m.a.s.l in the mountain regions [11]. Based on the alphavirus vector distribution in Costa Rica [8], the country was divided into two areas: highlands (>900 m.a.s.l) and lowlands (≤900 m.a.s.l).

Costa Rica was administratively divided into seven provinces and 81 cantons when sampling was carried out [11].

2.2. Study Population and Design

To estimate the sample size, two scenarios using EpiTools (https://epitools.ausvet.com.au/) were considered: (1) freedom from disease using an imperfect test (absence or low prevalence, in highlands) and (2) apparent prevalence with specific precision in lowlands. In both cases, the sample size estimation was based on the seroprevalence reported for the EEEV (7%) [12]. Because there are no studies from the highlands and only two studies from the lowlands [10,12] in which the EEEV had a lower prevalence than the VEEV, the apparent prevalence was based on the EEEV considering this virus as the limiting factor. From the two scenarios, we selected the scenario that estimated the largest sample size. We assumed an infinite population with a 95% confidence level, a statistical power of 80%, a specificity of 97%, and a sensitivity of 95% for the plaque-reduction neutralization test (PRNT80). Assuming a 7% prevalence and 5% precision, 101 horses should be sampled in the highlands and in the lowlands, respectively (scenario 2).

We used altitude (m.a.s.l) to stratify the country into highlands (>900 m.a.s.l) and lowlands (≤900 m.a.s.l). As no information was available on nationwide horse distribution, a systematic sampling approach was selected, with official veterinarians collecting serum from three horses per canton by convenience sampling. Horses had to be sampled at three different locations in each canton to include horses throughout the whole country and obtain a reasonable level of representability. Because there are 40 cantons in the highlands and 41 cantons in the lowlands, we selected 120 samples in the highlands and 123 in the lowlands.

Predefined inclusion criteria included >12 months of age, no previous history of alphavirus vaccination, and never moved between the highlands and lowlands.

2.3. Blood Sample Collection

Sampling was performed between mid-October and mid-November 2013 by official personal of the Costa Rican National Veterinary Service, SENASA, in accordance with national law [13]. Horse owners were asked to participate voluntarily in the study and were informed that samples were being taken for research purposes only. For each horse, two 5-mL blood samples were collected from the jugular vein in vacuum serum tubes and transported to the laboratory at ~4°C within 24–48 hours, where they were centrifuged on arrival. Information on each horse (age, sex, and breed), sampling location, and potential risk factors were collected from the horse caretaker/owner. This included perceived mosquito density, previous fumigations, proximity to forest areas, presence of wild mammals, observed clinical signs (specifically fever and central nervous system [CNS] signs), horse management practices, as well as the total number of horses on the premises. Horses with unknown age data were considered as a missing value, horses > four years were considered adults, horses ≤ four years were considered juveniles. In 21 records, the information was >4 years or ≤ 4 years instead of a specific number.

2.4. Laboratory Analysis

All analyses were performed at the Laboratory of Virology at the Universidad Nacional and the Virology Laboratory (LSE-LANASEVE) of SENASA in Costa Rica.

2.5. Indirect ELISA

Each serum sample was diluted 1:100 in phosphate buffered saline with Tween-20 (PBST); replicates of 50 μL of each sample, as well as positive and negative controls were dispensed in sensitized plates with Sindbis/EEE chimeric [14], TC83, and negative antigen. Plates were incubated at 25°C for 90 minutes in a WellMixx3 shaker. Subsequently, they were washed five times with 300 μL of PBST. The conjugate (anti-horse IgG peroxidase [Sigma A 6917]) was diluted 1:10,000 (PBST containing 0.3% bovine serum albumin, and 0.006% casein), 50 μL of the conjugate was added to each well, and incubated at 37°C for 1 hour in a wet chamber; the plate was washed as aforementioned. Finally, after adding 50 μL of 3,3′,5,5′−tetramethylbenzidine substrate (Sigma cat.T0440-1L) to each well, the reaction was stopped with 100 μL of 2M H_2SO_4 solution. Results were read at 450 nm in a Multiskan AscenT (Thermo Electron Co.) spectrophotometer. Youden's index and receiver operating characteristic (ROC) analysis were carried out for selecting the optimum ELISA cutoff point [15].

2.6. Plaque-Reduction Neutralization Test

Serum samples diluted 1:5 were inactivated at 56°C for 1 hour. A total volume of 150 μL of each inactivated sample was divided into two sets of six 2-fold serial dilutions in Dulbecco minimum essential medium (DMEM). Each set of dilutions (75 μf) was mixed with a solution of 75 μL of each alphavirus strain at 800 Plaque-forming unit/mL (TC83, VEEV-IAB), which could detect other VEEV subtypes [8,16,17] and Sindbis/EEE chimeric virus (both used in the ELISA) and was incubated overnight at 4°C or alternatively at 37°C for 1 hour. A total volume of 100 μL of the virus-serum mix were inoculated in two 12-well plates onto monolayers of confluent Vero cells and then incubated at 37°C for 1 hour. For the control plate, five replicates of 100 μL of each virus positive control (800 Plaque-forming unit/mL) diluted 1:2 in DMEM were used to estimate the 80% reduced plaques; two wells were used as negative controls (no virus was added). Finally, 1 mL of DMEM containing 0.4% agarose supplemented with 2% fetal bovine serum was added to each well in the plates. After 48 hours, the media was removed, the plates were stained with 0.25% crystal violet in 20% ethanol, and plaques were counted. Neutralization titers were considered the highest dilution of sera reduced plaques by 80% [18].

2.7. Data Analysis

Descriptive statistics were performed to evaluate the seroprevalence of the EEEV and VEEV as per the results of the PRNT80. The rainfall variable was created from the climate data organization

database (https://es.climate-data.org/info/sources/). Altitude was arbitrarily classified into five categories and was also dichotomized into elevations ≤900 m.a.s.l and >900 m.a.s.l. All independent variables were analyzed as categorical. In the first step, the association between potential risk factors and seropositive outcomes was tested using Fisher's exact test for independent samples. Variables with a significance level $P \leq .25$ were tested in a backward binary logistic unconditional regression analysis [19]. The odds ratio (OR) for each variable in the final model was the outcome of interest. A scatter plot was used to evaluate the degree of covariation among the variables, altitude (meters), rainfall (mm), and the number of horses (#) by premises. When a trend line was observed, a correlation test was performed. Data analyses were performed using Infostat Statistics, version 2018 [20], and Stata I/C 13 [21].

QGIS 3.6.1 software [22] was used to generate maps of the presence or absence of IgG antibodies against each virus in lowlands and highlands.

3. Results

Of 243 blood samples collected, data were analyzed from 217 horses. No horses could be located in the canton of Tibás (highlands), and these samples were substituted randomly from three different cantons (two in lowlands Garabito and San Carlos and one in highlands Escazú). Twenty-three horses from 243 did not meet the inclusion criteria: three had been moved between highlands and lowlands, 12 had been vaccinated against equine encephalitis, and eight were <12 months of age. Three samples (orange diamonds in Figs. 1 and 2) were positive for both viruses (PRNT80); however, because they did not reach a 4-fold titer to differentiate between them, they were considered undefined and were not analyzed further [23]. Data from the remaining 217 samples (95 highland and 122 lowland samples) were analyzed. Age ranged from 1 to 32 years (mean 7.5 years; median 7.0). Fig. 3 shows the distribution of the animal age by EEEV and VEEV serostatus (when exact age was reported), the outlier (a horse of 32 years of age) was not included in this graphic. The number of horses on the premises ranged from 1 to 80 (mean 7.2; median 4.0). The factor "breed" was not evaluated because most horses were classified as Criollo (n = 187; 85%).

3.1. Comparison of ELISA Results With the Reference Test Plaque-Reduction Neutralization Test

The cutoff criteria were defined in accordance with the Youden's J index in the ROC analysis for both ELISA tests. The sensitivity and specificity of both tests are shown in Table 1.

A total of 117 (54%) samples were negative to both the viruses in both tests, and 133 (61%) samples were negative to both the viruses by the PRNT80. Two samples were positive in both ELISAs and negative in both PRNT80s.

Table 2 shows the distribution of the positive and negative samples by test and virus.

3.2. National Seroprevalence and Distribution of the Venezuelan Equine Encephalitis Virus and Eastern Equine Encephalitis Virus

Venezuelan equine encephalitis virus overall prevalence by the PRNT was in 78 of 217 horses (35.9%) (confidence interval [CI]: 29.9–42.5): 27 of 95 horses (28.4%) (CI: 19.3–37.5) were positive in the highlands and 51 of 122 horses (41.8%) (CI 33.1–50.5) in the lowlands. The difference in seroprevalence between highlands and lowlands was statistically significant ($P = .04$).

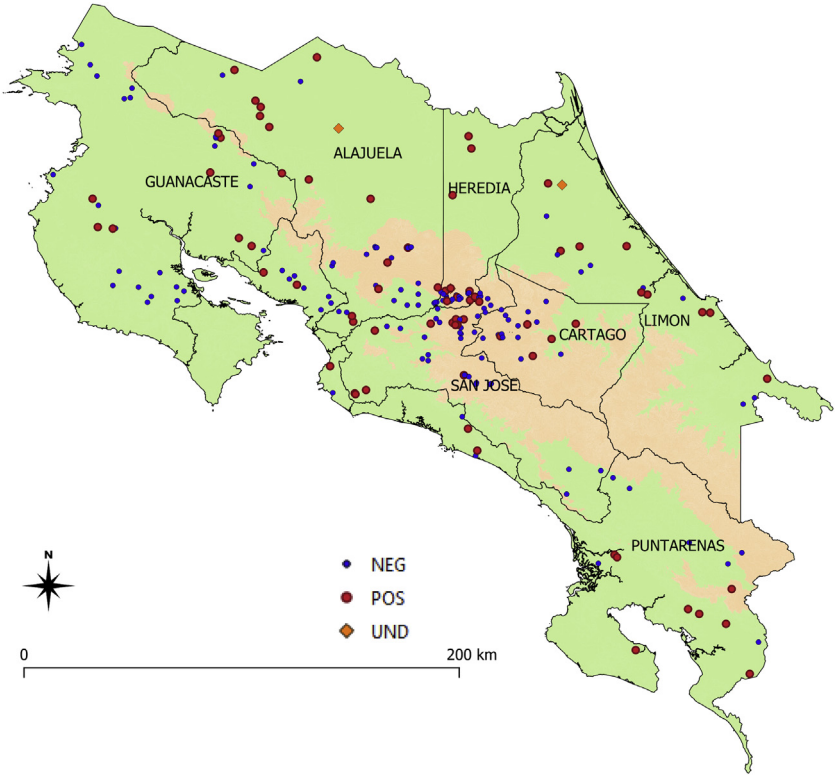

Fig. 1. Seroprevalence of the VEEV by the PRNT80. Positive samples: red dots, negative samples: blue dots, undefined samples: diamonds; the lowlands are in green and the highlands in peach. PRNT80, plaque-reduction neutralization test; VEEV, Venezuelan equine encephalitis virus.

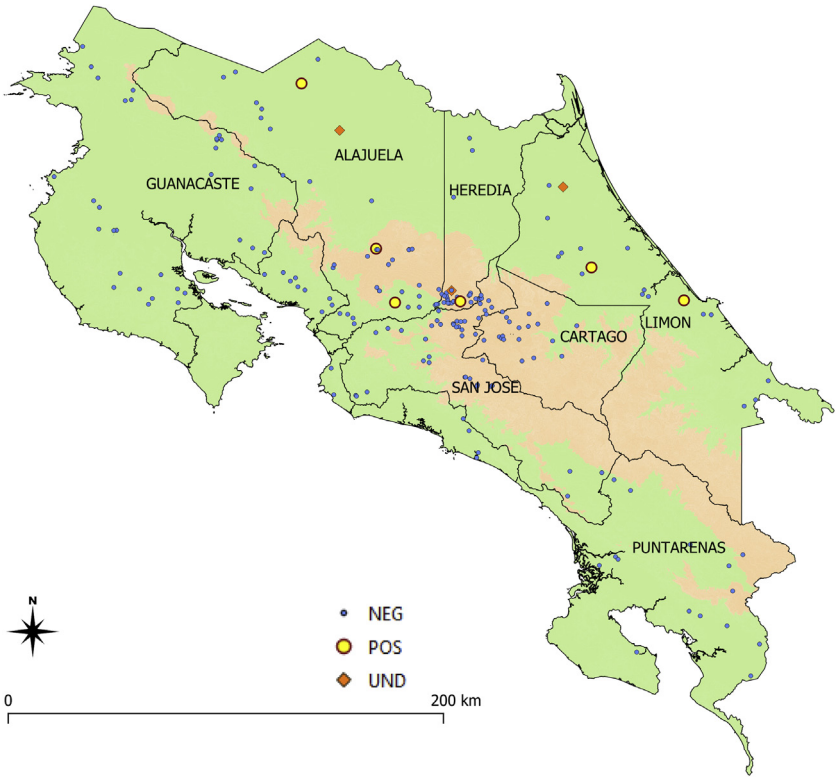

Fig. 2. Seroprevalence of the EEEV by the PRNT80. Positive samples: yellow dots, negative samples: blue dots, undefined samples: diamonds. EEEV, Eastern equine encephalitis virus; PRNT80, plaque-reduction neutralization test.

Eastern equine encephalitis virus overall prevalence by the PRNT was in 6 of 217 horses (2.8%) (CI: 1.3–5.9): 2 of 95 horses (2.1%) (CI: 0.58–7.4) in the highlands and 4 of 122 horses (3.3%) (CI: 1.28–8.13) in the lowlands, ($P = .6$).

3.3. Risk Factors Associated With Seropositivity

The PRNT80 results of the significant univariate analysis for each virus are shown in Table 3. The variables altitude, rainfall, and

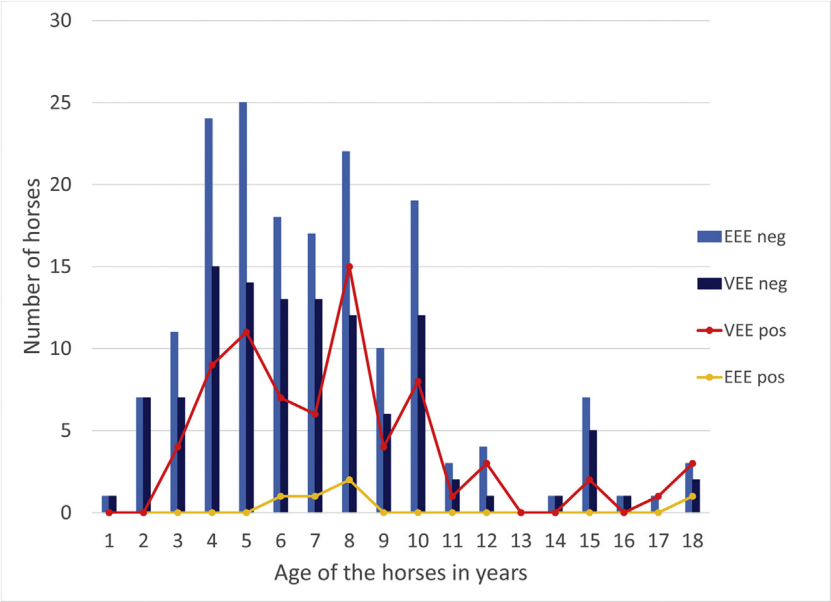

Fig. 3. Distribution of the horses' age by their VEEV and EEEV serological status. EEEV, Eastern equine encephalitis virus; neg, negative; pos, positive; VEEV, Venezuelan equine encephalitis virus.

Table 1
Diagnostic characteristics of the ELISA compared with the reference test PRNT80.

Parameter/Optical Density	ELISA VEEV /0.244 (95% Confidence Interval [CI])	ELISA EEEV /0.433 (95% CI)
Sensitivity	0.88 (0.78–0.95)	0.83 (0.35–0.99)
Specificity	0.86 (0.79–0.92)	0.87 (0.81–0.91)
Positive predictive value	0.78 (0.70–0.84)	0.16 (0.10–0.28)
Negative predictive value	0.93 (0.88–0.96)	0.99 (0.96–0.99)
Kappa coefficient	0.72	0.23

EEEV, Eastern equine encephalitis virus; PRNT80, plaque-reduction neutralization test; VEEV, Venezuelan equine encephalitis virus.

number of horses by premises were statistically significant to the VEEV (*P*-value <.05). Province was the only variable with a significant difference observed between positive and negative samples to the EEEV, $P = .02$ (Table 3). No significant differences were observed for the age of the animals and other variables (Table 1S).

Variables with a *P*-value of ≤.25 were included in the logistic regression model. Animals sampled at ≤900 m.a.s.l had a 1.81-fold (CI: 1.0–3.2) higher odds of being seropositive to the VEEV than horses at higher elevations. However, the animals kept <100 m.a.s.l had the highest OR with a 7.2-fold increase (CI: 2.24–23.17) of being VEEV seropositive ($P = .0001$). Horses in areas with >4000 mm of annual precipitation had 6.42-fold (CI: 1.76–23.41) higher odds of possessing antibodies to the VEEV than animals exposed to lower rainfall. The number of horses by premises was a protective factor for the presence of antibodies against the VEEV with an OR of 0.37 (CI: 0.16–0.84). A Pearson correlation between the variables altitude and rainfall and altitude versus horses by premise showed a negative and low relationship, −0.38 ($P = 4.6 \times 10^{-9}$) and −0.18 ($P = .01$), respectively.

When altitude, rainfall, and horses by premise were included in the logistic regression model, only altitude remained significant with a decreased OR of 5.37 (CI: 1.59–18.12), showing that the animals kept at <100 m.a.s.l. have significantly more VEEV IgG antibodies ($P = .006$) (Table 2S).

4. Discussion

As expected, VEEV prevalence was higher in the lowlands (41.8%) than in the highlands (28.4%). This result is practically identical to the 43% overall seroprevalence reported in 1970 in lowland areas [8], whereas in the study carried out by Fuentes in 1971, the seroprevalence to the VEEV was 21.2% in the lowland area in the north of the country [12]. The differences observed show that even though the VEEV is widely distributed in Costa Rica, its seroprevalence depends on the region sampled, these variations are also related to the sensitivity and specificity of the different diagnostic tools used [24–26].

VEEV ELISA was more reliable than EEEV ELISA based on Cohen's kappa coefficient 0.72 (substantial agreement) versus 0.23 (fair agreement), owing to the higher cross-reactive cases observed in the EEEV (false positives) [24,25]. A hamster study in the 1970s demonstrated cross-reactive protective immunity between the VEEV, Western Equine Encephalitis Virus, and EEEV and this could explain some of the false positive results determined in the ELISA used in the present study [26].

The low specificity of the IgG ELISA by cross reactions (to other alphaviruses) compared with that of the PRNT80 has been widely reported [3,27–29]. The presence of PRNT80 VEEV-positive samples in ELISA negative results could be false negatives (observed in other alphaviruses, e.g. O'nyong'nyong virus and Chikungunya virus) [29] or a PRNT80 antibodies cross-reaction to another VEEV subtype [16]. The PRNT is considered a reference test for its high sensitivity and specificity for arbovirus [16,30]. The OIE recommended test is the PRNT90 [4], whereas we used the PRNT80, which might have slightly reduced test specificity, nevertheless, PRNT80 is widely used [14,31].

The three undefined samples (positive by PRNT80 and ELISA) could be dual infections [1] or positive for another alphavirus or a different subtype of the same species [29]. Unfortunately, we did not have access to chimeras of other VEEV subtypes or another alphavirus to exclude this possibility. Seven horses in our study were reported to have been febrile in the recent past. One of these samples was positive to the EEEV in both the ELISA (0.477 OD) and PRNT80 (1:80). IgG antibodies to EEEV can persist for many years and it is unknown when this male, 7-year-old horse was infected. Of 78 VEEV-seropositive animals, only four reported clinical signs: three had fever (only two had a specific age reported: 18 and 8 years) and another presented CNS signs. A 5-year-old male must have been infected between 2009 and 2013. The low rate of animals with CNS signs may have been due to a mild VEEV strain, such as subtype IE (considered a nonpathogenic and endemic in Costa Rica). However, natural [32] or experimental equine infection with this subtype can produce CNS signs and death [27,32,33]. The reasons why some variants of subtype IE may or may not induce CNS signs are unclear. However, it should be noted that subtype IE may also cause disease in humans [27], and, as such, more cases would be expected considering the relatively high seroprevalence (42%) in horses in the lowlands. Possible reasons for underreporting could be that VEE is indistinguishable from dengue and other arboviral diseases in people, humans and horses did not share the same mosquitoes, or most VEEV infections did not cause severe clinical signs in humans. The second explanation is unlikely given that the mosquitoes, *Deinocerites pseudes* and *Aedes taeniorhynchus*, transmit the VEEV, and both feed from mammals including humans in

Table 2
Contingency table showing the distribution of positive and negative results as per ELISA and PRNT80.

	VEEV PRNT80			EEEV PRNT80		
	Positive	Negative	Total	Positive	Negative	Total
ELISA						
Positive	68	19	87	5	27	32
Negative	9	119	128	1	182	183
N/A	1	1	2	0	2	2
Total	78	139	217	6	211	217

EEEV, Eastern equine encephalitis virus; N/A: ELISA not performed; PRNT80, plaque-reduction neutralization test; VEEV, Venezuelan equine encephalitis virus.

Table 3
Univariate analyses of significant variables.

Risk Factor	Category	# Horses	EEEV			VEEV		
			Antibody-Positive Horses		Significance Fisher's Exact Test	Antibody-Positive Horses		Significance Fisher's Exact Test
			#	%		#	%	
Altitude (meters above sea level)	0–100	50	2	4		30	60	
	101–500	40	1	3		16	40	
	501–1000	35	1	3		5	14	
	1001–1500	70	2	3		22	31	
	>1500	22	0	0	$P = .944$	5	23	$P = .000$
Altitude								
	≤900	122	4	3.3		51	42	
	>900	95	2	2.1	$P = .698$	27	28	$P = .047$
Province	Alajuela	38	3	8		15	39	
	Cartago	21	0	0		6	29	
	Guanacaste	33	0	0		9	27	
	Heredia	28	1	4		10	36	
	Limón	17	2	12		10	59	
	Puntarenas	31	0	0		14	45	
	San Jose	49	0	0	$P = .027$	14	29	$P = .257$
Rainfall (mm)	≤2000	50	0	0		15	30	
	>2000–<3500	138	4	3		44	32	
	≥3500	29	2	7	$P = .185$	19	66	$P = .002$
Number of horses on the premises	1–3	106	2	2		41	39	
	4–8	60	3	5		14	23	
	>8	51	1	2	$P = .557$	23	45	$P = .040$

EEEV, Eastern equine encephalitis virus; VEEV, Venezuelan equine encephalitis virus.

the lowlands [8]. The first and the last hypotheses are related and could be the key to understanding the situation of the VEEV in humans but need to be investigated further. Venezuelan equine encephalitis virus diagnosis requires the use of specialized laboratory tests, which are difficult to obtain in resource-limited regions [34]. In Costa Rica, the diagnostic tests for equine encephalitis viruses in people are not done routinely and only carried out at hospitals or in the reference laboratory in patients with central nervous system symptoms.

In a study performed in Trinidad and Tobago, the EEEV seroprevalence was higher than the VEEV seroprevalence [23], contrary to our findings and studies in other Latin American countries [6,12]. Although we cannot explain this difference, we believe that it might be related to a difference in vectors and/or reservoirs for these two viruses. Until now, EEEV has been reported in the northern areas (Alajuela) [12], on the Caribbean coast (Limón), and in the Central Valley (Heredia) of Costa Rica, but this virus has not yet been confirmed on the Pacific coast. Positive EEEV areas are among the rainiest provinces of Costa Rica with > 4000 mm annual precipitation/m^2 [35] and the lowest altitudes (<100 m.a.s.l), both variables which are related to mosquito habitat. Environmental factors [36] related to altitude, such as temperature and humidity level, can certainly be considered relevant along with precipitation, resulting in more mosquito breeding sites and mosquito abundance and a higher likelihood of disease transmission [8,37]. The province of Limón had the highest seroprevalence for VEEV (59%) and EEEV (12%) (Table 3; Figs. 1 and 2), suggesting that competent vectors for these viruses cohabit there. The reason for a higher prevalence for the VEEV than EEEV might be because of a higher abundance and diversity of vectors [38] and the presence of its vertebrate reservoirs [39,40]. Further research into the vectors and reservoirs of alphaviruses in Costa Rica and their modes of transmission is highly recommended, and it should be determined whether other subtypes are circulating in the country besides subtype IE.

The distribution of the samples by cantons was a study limitation, as cantons with a low number of horses might have adversely influenced the prevalence estimation, whereas the prevalence of cantons with a high horse density might be underrepresented.

In our study, alphavirus prevalence was higher in the lowlands than in the highlands; however, the difference was only statistically significant for the VEEV, probably due to the low number of positive cases of EEEV.

To the authors' knowledge, this is the first national study of equine encephalitis alphaviruses in Costa Rica or in Central America. Our findings show that the VEEV and EEEV circulate in both the highlands and the lowlands. In addition, the seroprevalence detected and the distribution of the VEEV and EEEV throughout the country suggest the important role of these diseases in public health, and occasional clinical cases or outbreaks of both VEEV and EEEV should be expected. Veterinarians, horse owners, and public health officials should be aware of these viruses and their zoonotic potential. Appropriate surveillance and preventive actions, such as vaccinating horses and avoiding mosquito bites, are highly recommended in most areas of Costa Rica.

Further (modeling) studies of outbreak data and environmental factors could help improve predictions of such disease outbreaks.

Acknowledgments

We would like to thank Consenjo Nacional de Rectores (CONARE) for funding this research (Acuerdo-VI-177–2012) and Servicio Nacional de Salud Animal (SENASA) for its operative efforts in this study. Particular thanks also go to Drs. Sandra Lopez, Yaneth Pitti, Jean-Paul Carrera and Lisseth Saenz from the Department of Research in Virology and Biotechnology, Gorgas Memorial Institute of Health Studies, who gave us the viral chimeric controls for the PRNT80, as well as the opportunity for an internship within their department. In addition, we would like to show our gratitude to Lourdes Fuentes for her help with the analysis and to Dr. Ronaldo

Chaves for his expert horse knowledge (SENASA) and Dr. Carlos Loría from Universidad Nacional (UNA) for his advice on the python language to modify variable altitude in Qgis software.

Financial disclosure: This research was funded by CONARE funding (Acuerdo-VI-177−2012), University-government cooperation MIDEPLAN-CONARE 2017 and SENASA 2013−2018.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jevs.2020.103140.

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