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ORIGINAL ARTICLE

Zoonotic Alphaviruses in Costa Rica: Long-Term IgM Serosurveillance in Horses from 2009–2019

Graphical abstract

Highlights

- VEEV was detected consistently each year throughout the 11-year study period with 23.4% of samples testing positive. The highest concentration occurred in Guanacaste province, accounting for 65.6% of all VEEV cases.
- EEEV was detected mainly in the northwestern region (Guanacaste), while VEEV was more widely distributed. VEEV peaks occurred cyclically, especially in 2009 and 2015.
- Seasonal trends were noted with 60% of VEEV-positive cases occurring from October to December; November accounted for 29% of all VEEV-positive cases.
- Juvenile horses were 3.73 times more likely to be VEEV IgM-positive than adults. No significant difference was found between sexes.
- Horses in premontane zones had nearly twice the risk of VEEV-positivity. ENSO/La Niña events were associated with a 4.6–6.4-fold higher risk of VEEV detection.
- Ataxia and blindness were the most common neurologic symptoms in VEEV-positive horses.
- Eleven human alphavirus cases were reported between 2013 and 2019, including 1 VEEV case and 4 EEEV cases confirmed by international reference labs.

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Brief statement

This 11-year IgM serosurveillance study of equine encephalitis in Costa Rica revealed the endemic circulation of Venezuelan and Eastern equine encephalitis viruses with significant temporal, geographic, and climatic associations. The findings underscore the importance of long-term monitoring and ecologic analysis to understand alphavirus transmission dynamics and guide public and veterinary health responses.

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ORIGINAL ARTICLE

Zoonotic Alphaviruses in Costa Rica: Long-Term IgM Serosurveillance in Horses from 2009–2019

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Abstract

Objective: This study aimed to determine the annually circulation of Venezuelan (VEEV), Eastern (EEEV), and Western (WEEV) equine encephalitis viruses, identify patterns in case occurrence, and assess the associations with environmental and geographic factors.

Methods: Serum samples from 548 ill equines exhibiting fever, depression, and/or neurologic symptoms were analyzed over 11 years (2009–2019) using IgM MAC ELISA. Logistic models were used to determine the associations between VEEV seropositivity and environmental conditions.

Results: VEEV was detected annually in 23.4% (128/548) of the cases, while EEEV appeared intermittently (1.5% [8/548]). No WEEV cases were detected, likely due to avian migratory flyway routes. VEEV exhibited a 6-year peak pattern linked to La Niña in Guanacaste province. Eleven human alphavirus cases were confirmed between 2013 and 2019, including 1 fatal VEEV case in a child.

Conclusion: These findings underscore the need for continued equine alphavirus surveillance within the One Health framework.

Key words: Venezuelan equine encephalitis, Eastern equine encephalitis, vector-borne diseases, alphavirus, One Health, surveillance, zoonotic pathogens

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INTRODUCTION

Some arboviruses in the Togaviride family are of particular concern to public and animal health in the Americas, while others remain neglected and largely unknown [1–4]. Within the Togaviridae family, three zoonotic species of the genus *Alphavirus* (Eastern equine encephalitis virus [EEEV], Western equine encephalitis virus [WEEV], and Venezuelan equine encephalitis virus [VEEV]) cause various disease stages ranging from non-specific clinical signs, such as fever, headaches, and nausea, to severe and deadly encephalitis in humans and equines [5–8].

The VEEV subtype I complex includes subtypes considered both enzootic/non-pathogenic (VEEV ID-IE) and epizootic/pathogenic [VEEV IAB-IC] [9], which can generate isolated cases or massive epidemics in horses with the potential for zoonotic spillover epidemics in humans [1].

VEEV, EEEV, and WEEV can cause isolated cases or limited outbreaks [10]. The South American variant of EEEV, known as the Madariaga virus (MADV), has been associated with outbreaks in

humans and equines in Panama [11,12]. WEEV caused 41 outbreaks affecting 27% (106/390) of symptomatic equines with a 13% fatality rate on the Pacific coast of Mexico in March 2019 [13]. WEEV is transmitted in enzootic and epizootic transmission cycles by *Culex* and *Aedes* mosquitoes among birds and lagomorphs, which can lead to sporadic spillover to equids and humans [14].

Serologic evidence of these arboviruses in Costa Rica has been reported since the early 1970s [15]. In 2013 a national IgG-seroprevalence ELISA study confirmed by a plaque reduction neutralization test (PRNT80) revealed that 36% and 3% of 217 horses were positive for VEEV and EEEV, respectively. Both viruses circulated in the lowlands and highlands of Costa Rica with rainfall and altitude identified as risk factors associated with VEEV seropositivity [16].

Because arboviruses are primarily mosquito-borne, climatic conditions are considered an important driver of arboviral diseases [17,18]. Specifically, heavy rainfall has been associated with EEEV outbreaks [19]. Additionally, it has been suggested that the intensity of global disease activity in some regions is 2.5–28% higher during years with compared to years without El Niño and these outbreaks could be related to El Niño-Southern Oscillation (ENSO) events [20]. However, little is known about how the different ENSO events (e.g., El Niño vs. La Niña) might drive arboviral outbreaks in equines.

Previous studies on equine encephalitis viruses have not investigated temporal patterns or the effect of climatic conditions. The aim of this study was to determine the frequency of VEEV, WEEV, and EEEV circulation during an 11-year surveillance period (2009–2019) in serum samples from equines exhibiting fever, depression, or neurologic signs using a IgM antibody capture enzyme-linked immunosorbent assay (MAC IgM ELISA). Additionally, ENSO climate events and other variables such as sex, age, month of sample collection, year, province were investigated as potential drivers of alphavirus occurrence in Costa Rica.

METHODS

Passive surveillance study

Costa Rica is a small tropical country (51,100 km^2) located in Central America. The Virology Laboratory of the Veterinary School of the National University of Costa Rica conducted a passive surveillance study from 2009–2019 in collaboration with the National Animal Health Service (SENASA). Serum samples were collected from 590 equines with fever, depression, and/or neurologic symptoms by SENASA or private veterinarians across all 7 provinces of Costa Rica. No approval was needed by appropriate human or animal subjects research review boards according to law 8495 (General Law of the National Animal Health Service).

Laboratory analysis

All sera samples were analyzed in the Virology Laboratory of the Universidad Nacional de Costa Rica. The reference sera and antigens, including normal control suckling mouse brain antigen (catalog no. 0006), inactivated antigens prepared in newborn mice for EEEV (NJ-60), WEEV (Fleming), and VEEV (TC-83), negative normal equine serum, and positive Venezuelan, Western, and Eastern equine sera, were donated by the Arbovirus Reference Collection (ARC) at the Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Diseases, Division of Vector-Borne Diseases (Fort Collins, CO, USA). The antibody used for pre-treating the plate was goat anti-horse IgM (code E170; Hennessy Laboratories, Shawnee, KS, USA). An Immulon 1B plate (Thermo Scientific, Rochester, NY, USA) was pre-coated with a 1:400 dilution of the anti-horse IgM antibody in carbonate buffer (pH 9.6) and incubated overnight at 4°C in a humidified chamber. Following incubation, the plates were washed twice with PBS containing 0.05% Tween (PBST), then blocked with PBS containing 1% albumin and 0.02% casein for 30 min at 37°C in a humidified chamber. After blocking, the plates were washed twice again before proceeding with further steps.

Each equine virus-positive serum sample was diluted 1:400, assessed by a capture MAC IgM ELISA provided by the arbovirus Reference Collection (ARC) from the Centers for Disease Control and Prevention, National Center for Emerging and Zoonotic Infectious Disease, Fort Collins, CO, USA, then 50 μl/well was transferred to each plate and incubated for 90 min at 37°C in a humidified chamber The viral antigens (EEEV [NJ-60], WEEV [Flemming], VEEV [TC-83]) and negative control antigens (normal control suckling mouse brain antigen) were diluted in dilution buffer according to the CDC instructions and added to the plates (50 μL/well). The plates were then incubated overnight at 4°C in a humidified chamber. After 5 washes with PBST, 50 μl/well of alphavirus group-specific conjugated Mab 2A2C3/HRP was added to the plates. The conjugate Mab 2A2C3/HRP (catalog No. 0009) was donated by the ARC. Alphavirus group-specific conjugated Mab 2A2C3/HRP was diluted 1:4000 in PBST before addition to the plates (50 μL/well). The plates were incubated at 37°C for 90 min in a humidified chamber, then washed 5 times. Then, 50 μL/well of TMB substrate (T0440-1L; Sigma-Aldrich, Burlington, MA, USA) was added and the plates were incubated in the dark for 20–30 min. The reaction was stopped by adding 2M H_2SO_4 (100 μL/well) and absorbance was measured at 450 nm [21]. A serum sample was considered positive if the OD value was at least twice the mean OD of the negative control serum sample.

Climate data

ENSO data were obtained from the National Oceanic and Atmospheric Administration (NOAA). The monthly Niño-3.4 index, based on the Oceanic Niño Index in the Niño 3.4 region, was used because the monthly Niño-3.4 index appears to have the highest association with weather anomalies in Central America [22]. ENSO is defined as no anomalies/La Nina/El Nino, where anomalies < -0.5,

> 0.5, and between -0.5 and 0.5 are considered La Nina, El Nino, and no anomalies, respectively. Monthly rainfall information was obtained from the National Meteorological Institute of Costa Rica (IMN) through 65 weather stations located in 7 regions. Each sample was associated with the rainfall recorded at the closest weather station.

Data analysis

Information on age, gender, breed, and location was voluntarily provided by horse owners. Of the 590 equine samples submitted, 42 were excluded due to prior vaccination against these viruses or incomplete/missing location data. Vaccination against any of the studied viruses can produce antibodies that may be detected by the IgM MAC ELISA test, potentially leading to false-positive results [21]. Significant variables were prioritized over non-significant variables based on the significant findings from the National Seroprevalence Study [16]. Consequently, data lacking information on key variables, such as location (which is associated with province, altitude, and weather), rather than variables, such as gender, were excluded. Excluding data based on gender or age could have inadvertently resulted in the loss of valuable location-related information. Of the remaining 548 cases from 301 establishments, 29 lacked gender or location information and 100 had missing age or location records. Although all animals were clinically symptomatic, only 220 owners reported symptoms in the brief questionnaire.

Crosstab analyses were performed to evaluate the presence or absence of IgM antibodies against the studied viruses in relation to selected variables (year, month of sample collection, province, and animal gender) using Pearson's chi-square test. All analyses were performed using Infostat Statistics (version 2018; R/National University of Cordoba, Cordoba, Argentine). Additionally, open-source GIS software (QGIS 3.6.1-Noosa) was used to map the geographic distribution of positive and negative samples for each virus [23].

To identify potential demographic and ecologic factors influencing VEEV, the virus with the highest number of seropositive cases, two logistic regression models ('glm' function in the R package) with seropositivity as the response variable (i.e., positive or negative) were used. Both models determined the association between VEEV seropositivity in the Guanacaste province only, where most cases originated. The impact of weather is strongly influenced by geography in Costa Rica (e.g., differences between the Pacific and Atlantic regions, as well as the interactions with dry and wet seasons). Temporal variables (year and quarter of collection: Q1 = January–March, Q2 = April–June, Q3 = July–September, and Q4 = October–December) and environmental variables (altitude, monthly rainfall, season, ENSO index, and life zones) were considered for these models. Costa Rica has 12 life zones [24], which were included as explanatory variables.

These life zones are defined by variations in temperature and precipitation within a given area. Two ecozones (Basal and Premontane) in Guanacaste were considered the most appropriate altitude metrics. Altitude was determined using GPS data provided by the owner or veterinarian; if altitude information was unavailable (351 cases), Google Earth was used to estimate the closest point to the reported address or village. Only district-level coordinates could be assigned in 63 cases.

The Holdridge life zones (shapefile: AtlasCR2008) were integrated with GIS points for each animal in the study to create a dataset linking life zones to individual cases. This spatial analysis was performed using ArcGIS 10.8.1 software (ArcGIS® and ArcMap™; Esri, city, state, country). Season (rainy or dry) was defined by Hutter and colleagues [25] and included as an interaction term with the ENSO index to account for potential seasonal differences in ENSO effects.

The two logistic regression models differed with respect to inclusion of individual-level variables. The first model incorporated additional variables, such as gender and age category (juveniles < 48 months vs. adults > 48 months), with the previously mentioned temporal and environmental factors. In contrast, the second model excluded individual-level variables due to missing data on gender and age for some horses. Consequently, the first model was developed using a subset of the data (n = 201), while the second model utilized the full dataset (n = 282) for the Guanacaste province.

To identify the best-fit model, a backward selection process to the full models was used, which initially included all possible variables. The variable with the highest *P*-value was iteratively removed and models were compared using ANOVA and the Akaike information criterion (AIC) until the optimal model was achieved using the 'step' function in R. If the AIC difference between models was < 2, the simpler model was preferred for parsimony. Model fit was further assessed through residual diagnostics and dispersion analysis using the R package 'DHARMa' [26].

RESULTS

IgM-seropositive WEEV, EEEV, and VEEV cases

A total of 114 establishments (114/303 [37.6%]) were positive for at least 1 virus, while 1 animal exhibited a VEEV/EEEV co-infection in Guanacaste in 2010 highlighted as a red star symbol (Fig 1). Of the 548 analyzed samples, 128 were positive for VEEV (23.4%, CI 19.9%–27.1%) and 8 were positive for EEEV (1.5%, CI 0.6%–2.9%). Details of the positive sample distribution categorized by province, year, and month are shown in Tables 1–3 and Fig 1. The base map of Fig 1, which is shaded to indicate topographic relief (darker areas representing higher elevations), provides a geographic context for understanding viral spread. Notably, EEEV cases were concentrated in the northwestern region (Guanacaste), while VEEV cases were more widely distributed.

Only 1 of 35 samples from San Jose province tested positive for VEEV (2.58%) during 11 years of passive surveillance (Table 1, column 1). Column 2 presents the percentage of positive cases relative to the total horse

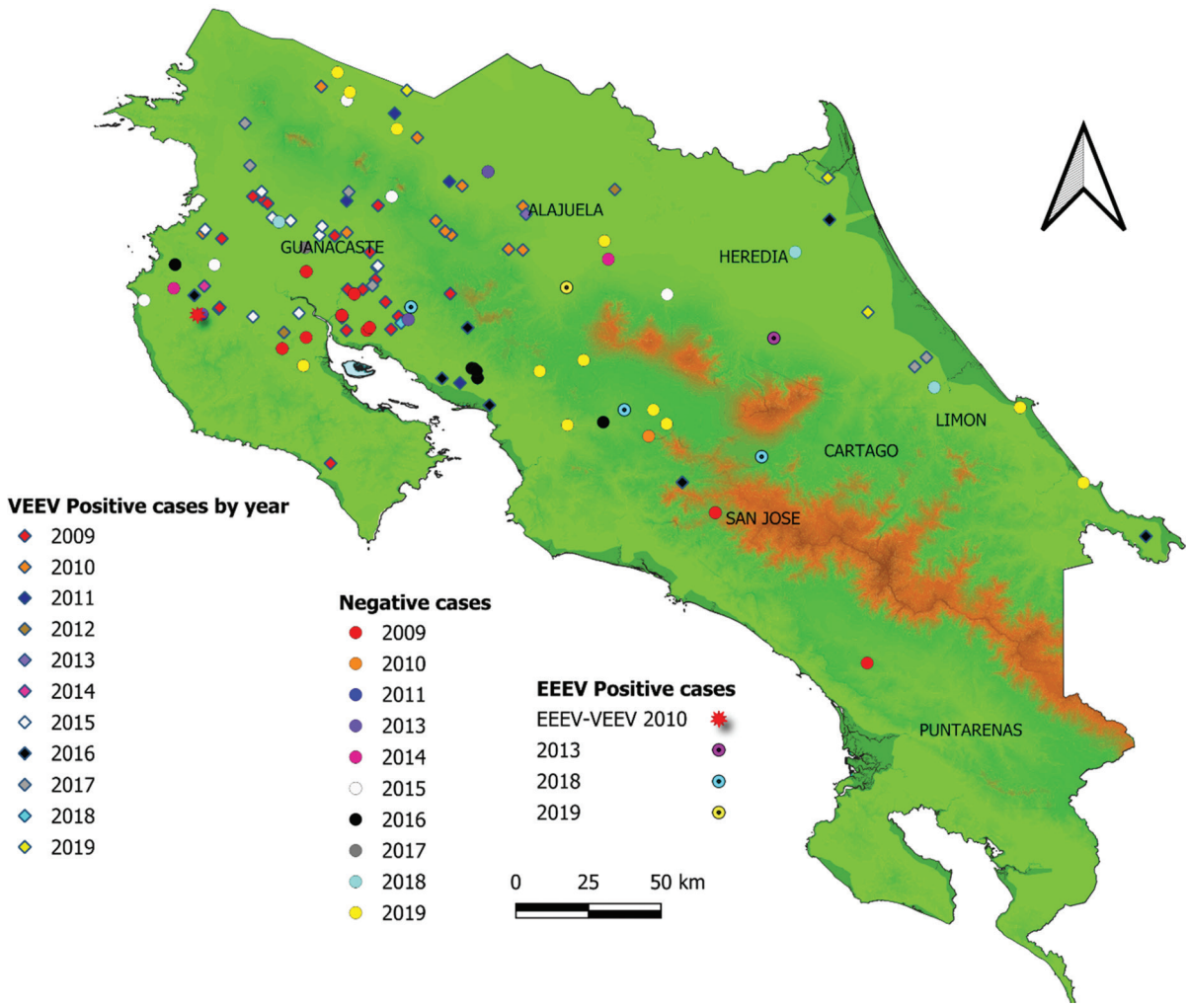

FIGURE 1 | Illustrates the geographic distribution of Eastern equine encephalitis virus (EEEV) and Venezuelan equine encephalitis virus (VEEV) cases in Costa Rica collected between 2009 and 2019. VEEV-positive samples are represented as diamonds, while EEEV-positive samples are depicted as circles with a dot. Negative samples are shown as dots. A red star indicates a sample that tested positive for VEEV and EEEV. The colors of the symbols vary according to the year the samples were collected. Provinces are also represented and only samples with georeferenced data are included.

population by province, as reported in the 2014 agricultural census, which was 0.015% in San Jose. Column 3 shows the percentage of positive cases relative to the total 128 VEEV-positive samples with San Jose accounting for 0.08% (Table 1).

Most of the tested samples were from Guanacaste, which is located in the northern region of Costa Rica. Guanacaste also had the highest VEEV-positive rate, with 84 of 286 samples testing positive (29.4%). Guanacaste was the province with the largest number of registered horses in the 2014 census (18,905) and had the highest percentage of positive cases for all studied viruses (0.44%). Additionally, Guanacaste had the highest proportion of positive cases relative to the total number of VEEV- (65.6% [84/128]) and EEEV-positive samples (50% [4/8]).

Of the 130 tested samples in Alajuela province, 21.5% were positive for VEEV, which was similar to the VEEV-positive cases in Limon (19.4% of 36 samples) and Puntarenas (19% of 42 samples) on the Caribbean and Pacific coasts, respectively (Table 1, column 1). These positive rates aligned with the percentages based on the total number of horses registered by province in the 2014 census (Table 1, column 2).

Cartago had the highest positive rate for EEEV (8.3% [1/12]; Table 1, column 1). An EEEV-positive rate equated to 0.032% (1/3031) when considering the total horse population (Table 1, column 2). However, Guanacaste had the highest absolute number of EEEV-positive horses (4/8 [50%]). A statistically significant difference in the distribution of both viruses across provinces was detected using the chi-square test ($P < 0.001$).

VEEV-positive rates (> 40%) were recorded from 2009–2012 and in 2015, while the percentages ranged from 33%–4% in the remaining 6 years. Interestingly, EEEV-positive cases were only detected in 2010, 2013, 2018, and 2019.

TABLE 1 | Positive samples distributed by province according to each virus.

Processed samples by province/horse population	VEEV-positive cases $P = 0.002$ (1) $P = 0.000$ (2)			EEEV-positive cases $P = 0.00$ (1) $P = 0.00$ (2)		
	1	2	3	1	2	3
	Positive #/%	%	%	Positive #/%	%	%
San Jose						
35/6493	1/2.58	0.015	0.8			
Alajuela						
130/15588	28/21.5	0.18	21.9	2/1.5	0.012	25
Cartago						
12/3031	0			1/8.3	0.032	12.5
Heredia						
7/3261	0					
Guanacaste						
286/18905	84/29.4	0.44	65.6	4/1.4	0.021	50
Puntarenas						
42/13958	8/19	0.057	6.25	0		0
Limon						
36/5706	7/19.4	0.12	5.46	1/2.8	0.018	12.5
Total						
548/66942	128	0.19	100	8		100

1 Number of positive samples by province/percentage of positive samples over the samples processed by province, **2** Percentage of positive samples over the number of horses at risk by province **3** Percentage of positive samples by province over the total of the positive samples during the surveillance (n = 128).

The highest percentage of VEEV-positive samples (27.3% [35/128]) occurred in 2009. In subsequent years the number of positive samples and the proportion decreased until a new peak occurred in 2015 when 17.2% (22/128) were positive (Table 2 and Fig 2).

The proportions of VEEV- and EEEV-positive samples by month were also statistically significant. Whereas 15.6% of all positive samples occurred during the first 6 months of the year, 60% (77/128) of the positive samples occurred between October and December with most cases occurring in November (29% [37/128]; Fig 3).

No statistically significant difference was detected in the number of positive cases based on animal gender; specifically, 58 of 248 females tested positive compared to 59 of 271 males ($P > 0.53$, chi-square test). However, the mean age of positive cases (39.3 months) was significantly lower than the mean age of negative cases (73.6 months) with a P-value < 0.05 (two-tailed t-test assuming unequal variances) according to Fisher's exact test ($P < 0.05$). Of the 548 horses exhibiting neurologic signs, information on the type of symptoms was only reported for 220 animals, 156 of which tested positive for at least 1 of the 44 viruses studied.

S1 Table displays the clinical signs for VEEV-positive horses. The initial symptoms were non-specific in 17 horses but 14 subsequently exhibited ataxia and 12 were blind (S1 Table). S2 Table presents the main clinical signs observed in the 72 animals positive for any of the studied viruses with ataxia the most frequent symptom for all three viral infections, followed by blindness in VEEV-related cases.

Factors associated with VEEV occurrence based on climatologic variables

The first model included the following variables: year; altitude (Basal vs. Premontano); age category; and ENSO (ΔAIC of best and full model = 6.93). The second model retained the same variables (year, altitude [Basal vs. Premontano], and ENSO (ΔAIC of best and full model = 7.72), but with stronger correlations. The results from both models (Table 4) suggested that VEEV seropositivity decreased slowly over the years (Table 4). The first model indicated that juvenile horses were 3.73 times more likely to be IgM-positive than adults. The premontane ecozone, which was used as a proxy for altitude in the second model, had nearly twice the risk of VEEV seropositivity than the basal ecozone. ENSO/La Niña was considered a risk factor for VEEV seropositivity in both models (OD increasing from 4.6 to 6.4) compared to times with no ENSO anomalies, while ENSO/El Niño was significant

TABLE 2 | Frequency of the positive samples by year of collection.

Year/processed samples	VEEV-positive cases $P = 0.0001$			EEEV-positive cases $p = 0.0007$		
	1	2	3	1	2	3
	Positive #	%	%	Positive #	%	%
2009/75	35	46.7	27.3			
2010/52	25	48.1	19.5	1	1.9	12.5
2011/18	8	44.4	6.3	0		
2012/11	5	45.5	4	0		
2013/22	1	4.5	0.8	1	4.5	12.5
2014/6	2	33.3	1.6	0		
2015/49	22	44.9	17.2	0		
2016/90	13	14.4	10.2	0		
2017/33	8	24.2	6.3	0		
2018/51	3	5.9	2.3	5	9.6	62.5
2019/141	6	4.3	4.7	1	0.7	12.5
Total						
548	128		100	8		100

1 Number of positive samples by year, **2** Percentage of positive samples by year over the total samples processed in that year, **3** Percentage of positive samples by year over the total of the positive samples (n = 128).

TABLE 3 | Frequency of the positive samples by month of collection.

Month/processed samples	VEEV-positive cases $P = 0.0001$			EEEV-positive cases $P = 0.0001$		
	1	2	3	1	2	3
	Positive #	%	%	Positive #	%	%
Jan/56	3	5.4	2.3	0		
Feb/13	0					
Mar/12	0			2	16.7	25
Apr/13	1	7.7	0.8	0		
May/24	2	8.3	1.6	3	12.5	37.5
Jun/53	14	26.4	10.9	0		
Jul/29	11	37.9	8.6	0		
Aug/61	13	21.3	10.2	0		
Sep/62	7	11.3	5.5	0		
Oct/84	24	28.2	18.8	1	1.2	12.5
Nov/106	37	34.9	28.9	1	0.9	12.5
Dec/35	16	45.7	12.5	1	2.9	12.5
Total						
548	128	100		8		100

1 Number of positive samples month, **2** Percentage of positive samples by month. **3** Percentage of positive samples by month over the total of the positive samples (n = 128).

only in the second model (OD = 4.3; Table 4). All other variables related to weather or ecologic regions, such as rain and life zone, were excluded after model selection.

DISCUSSION

The purpose of the surveillance reported herein was to establish if VEEV and EEEV circulate annually and if the occurrence was more frequent in some months.

The prevalence and geographic distribution of these viruses in Costa Rica were previously determined through a national IgG study using ELISA, which was confirmed by PRNT80. In that study, 36% and 3% of 217 horses tested positive for VEEV and EEEV, respectively [16]. During the surveillance reported herein no horses tested positive for WEEV. WEEV is less prevalent in the Americas compared to VEEV and EEEV. However, recent cases occurred in Argentina, Brazil, and Uruguay from 2023–2024 [27]. Outbreaks have also been reported in the the Caribbean islands [28]. These outbreaks may be associated with the Atlantic migratory flyway of wild birds, which extends from Canada and the U.S. east coast through the Caribbean islands to Argentina, Brazil, and Uruguay. In contrast, only the wild bird flyways of the Pacific and Central regions pass through Costa Rica, as suggested by phylogenetic studies conducted on avian influenza virus H5N1 sequences in 2023 (unpublished results). This finding could be the reason that no VEEV or EEEV cases have been reported in Costa Rica. Additionally, 1086 equines were affected during the 2024 outbreak of WEEV in Uruguay, which resulted in 388 deaths and 5 human cases. Genomic analyses revealed that the virus strains from this outbreak are closely related to a 1958 Argentine strain, suggesting that the virus may have remained enzootic in the region for an extended period of time [29]. A genome sequence obtained from an equine in Costa Rica that tested positive for VEEV by the MAC IgM ELISA in 2015 was phylogenetically linked to cases reported in Nicaragua and Guatemala in 1968. All VEEV cases that were successfully sequenced corresponded to subtype IE, which is considered an endemic strain of VEEV [6,30].

In the current study equine serum samples from clinically symptomatic horses tested positive in 23.4% of cases for VEEV and 1.5% for EEEV. The percentage of positive cases among equines ranged from 14%–90% and the percentage increased with age [0.6–6 months to 24% >10 years] [7]. In another historic study of a region in the north province of Costa Rica, Fuentes and colleagues (year) determined the IgG prevalence for VEEV to be 21.2% using a microneutralization test, while the prevalence of EEEV was 7.2%. The antibody titers for WEEV were considered to be cross-reactions with VEEV or EEEV. Additionally, during the IgG ELISA analysis of EEEV- and

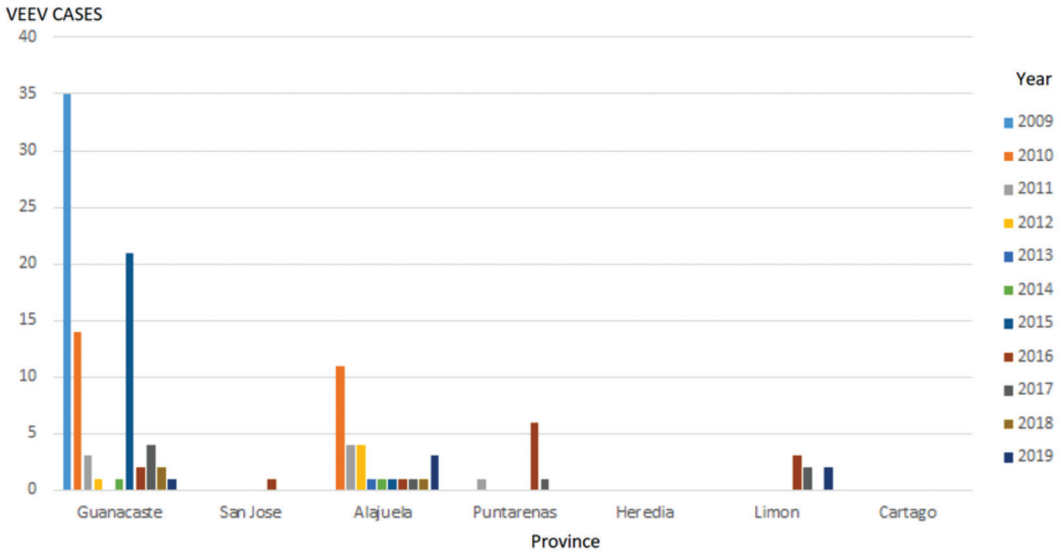

FIGURE 2 | VEEV-positive samples for every province by year. The y-axis shows the number of cases by year and province.

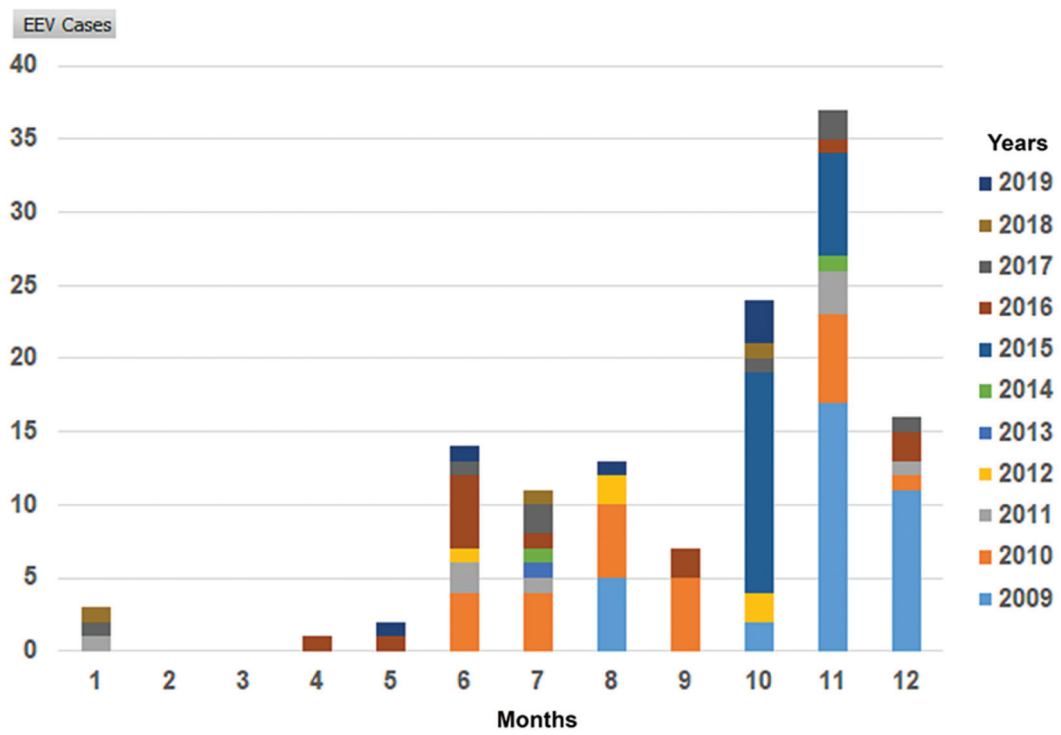

FIGURE 3 | Distribution of the positive samples of each virus by month and year. The y-axis shows the number of cases.

VEEV-positive samples, 12% of EEEV-positive samples cross-reacted with VEEV. Furthermore, 8.7% of the IgG-positive samples were deemed to cross-react with other alphaviruses [15]. These prevalences were similar to the percentage of positive cases recorded during the IgM MAC ELISA surveillance.

This is the first report of passive surveillance of IgM antibodies in clinically-symptomatic equines, some of which exhibited neurologic signs, to determine the presence of alphaviruses in Central America.

The CDC laboratory criteria for arboviral infection require the following: isolation of virus from or demonstration of specific viral antigen or nucleic acid in tissue, blood, CSF, or other body fluids; or four-fold or greater change in virus-specific quantitative antibody titers in paired sera; or virus-specific IgM antibodies in serum with confirmatory virus-specific neutralizing antibodies in the same or a later specimen; or virus-specific IgM antibodies in CSF or serum [31]. It is important to note that interpreting serologies can be complicated by antibody

TABLE 4 | Results of the two logistic regression models describing VEEV seropositivity.

Covariates	Odds ratio	95% CI	*P*-value
MODEL 1 (n = 201)			
Year	0.830	0.746–0.923	**0.00059**
Enso [No anomaly]			
Altitude [Basal]			
Premontane	2.030	0.920–4.477	0.07930
La Niña	4.600	1.209–17.502	**0.02519**
El Niño	3.032	0.799–11.499	0.10297
Juveniles	3.732	1.576–8.833	**0.00274**
MODEL 2 (n = 282)			
Year	0.863	0. 788–0.945	**0.00150**
Altitude [Basal]			
Premontane	1.858	0.978–3.529	**0.02231**
Enso [no anomaly]			
La Niña	6.477	1.838–22.816	**0.00364**
El Niño	4.307	1.231–15.068	**0.02231**

cross-reactivity with viruses from the same serogroup, especially in the case of IgG due to persistent antibodies from prior infections. Thus, a positive first-line serologic test requires confirmation by a PRNT, detection of seroconversion, or a four-fold rise in virus-specific IgM [32]. We did not use the PRNT as a confirmatory test for the IgM ELISA results due to the possibility of false-positives caused by prior exposure to the viruses and subsequent development of IgG antibodies as evidenced by Sahu et al. [21], in which 132 of 381 samples tested positive for EEEV using an IgM capture alphavirus test, while 195 were positive for EEEV using the hemagglutination inhibition (HI) test (1:40) and 205 were positive based on a virus neutralization test [VNT] (1:10) for EEEV [21], indicating that HI and VN methods that detected previous virus infections or vaccinations and the IgM ELISA are less sensitive than neutralization tests. Indeed, when IgM-negative sera were compared in that study, 75 (75.8%) had VNT antibodies to ≥ 2 viruses and 21 sera (22%) had antibodies to EEEV, WEEV, or VEEV, demonstrating that these horses were previously vaccinated or had been infected with encephalomyelitis viruses in the past. Anti-EEEV IgM antibodies were monospecific and did not cross-react with WEEV or VEEV antigens based on ELISA [21]. Alternatively, IgM cases could be confirmed using molecular methods, such as RT-PCR and sequencing. However, the primary limitation of molecular tests is that viremia is too brief, with peaks typically occurring within the first 7 d of symptom onset. The virus is no longer detectable in the blood when IgM is detected. Neurologic signs were reported in only 60 of the 128 VEEV-positive animals. Only 1 brain sample that was collected from a mare in 2015 was submitted to the laboratory. This sample was VEEV-positive by RT-PCR and was sequenced [6], confirming the IgM MAC ELISA results.

IgM capture ELISAs are highly sensitive and can detect IgM antibodies against EEEV as early as 1 d after the reported onset of illness in humans. Anti-EEEV IgM antibodies do not react with heterologous viruses. IgM antibodies can be detected as early as the 3rd d after infection with WEEV in horses [33]. Importantly, the anti-EEEV IgM antibodies in horses do not cross-react with WEEV and VEEV antigens, in agreement with previous findings of anti-EEEV IgM antibodies in humans and chickens and anti-WEEV IgM antibodies in horses [33–35]. Detection of anti-VEEV and -EEEV IgM antibodies is considered conclusive evidence of a recent infection. However, a single determination of antibodies by tests, such as HI, complement fixation, NT, PRNT, or virus-specific IgG assays, is not sufficient to confirm a recent infection because these antibodies may persist in the body for years after recovery, while IgM levels decline 90 d post-infection [35].

The MAC ELISA reduces false-positives caused by non-specific antibody binding and false-negatives resulting from competition with pre-existing IgG. MAC ELISA minimizes IgG competition and non-specific binding compared to standard ELISAs, thereby enhancing sensitivity and specificity. A systematic review and meta-analysis reported that IgM detection tests, including ELISA-based tests, immunofluorescence assays (IFAs), and in-house developed assays, achieved > 90% diagnostic accuracy, especially for samples collected > 7 d after symptom onset [32,36,37].

Of the positive cases detected by the IgM ELISA, only 1 horse sample reacted to EEEV and VEEV in a horse from Guanacaste in December 2010, which could represent the unique cross-reaction observed in the study or a co-infection. Co-infection with EEEV and VEEV has also been reported in a human patient in Panama [38] and in horses [15]. Another possible explanation is an ecologic overlap event, which is relatively rare but biologically plausible, especially in regions where both viruses co-circulate. Notably, the first detected case of EEEV was located 35 km from a site where 3 VEEV cases were diagnosed on the same farm in 2018, indicating the presence of both viruses in the area. Another reason could be a recent vaccination response but this is not likely because the encephalitis vaccines are not commonly distributed in the country due to the low demand.

It is unlikely that heterologous IgM is produced in the event of a hypothetical superinfection of EEEV in a VEEV-immune individual or vice versa because pre-existing IgG, a result of the primary infection, would neutralize the heterologous virus [34]. Therefore, rapid, sensitive, and specific diagnostic methods are necessary to support surveillance activities and epidemiologic studies. The IgM test is a simple and rapidly implemented test, that when used in conjunction with animals with neurologic signs,

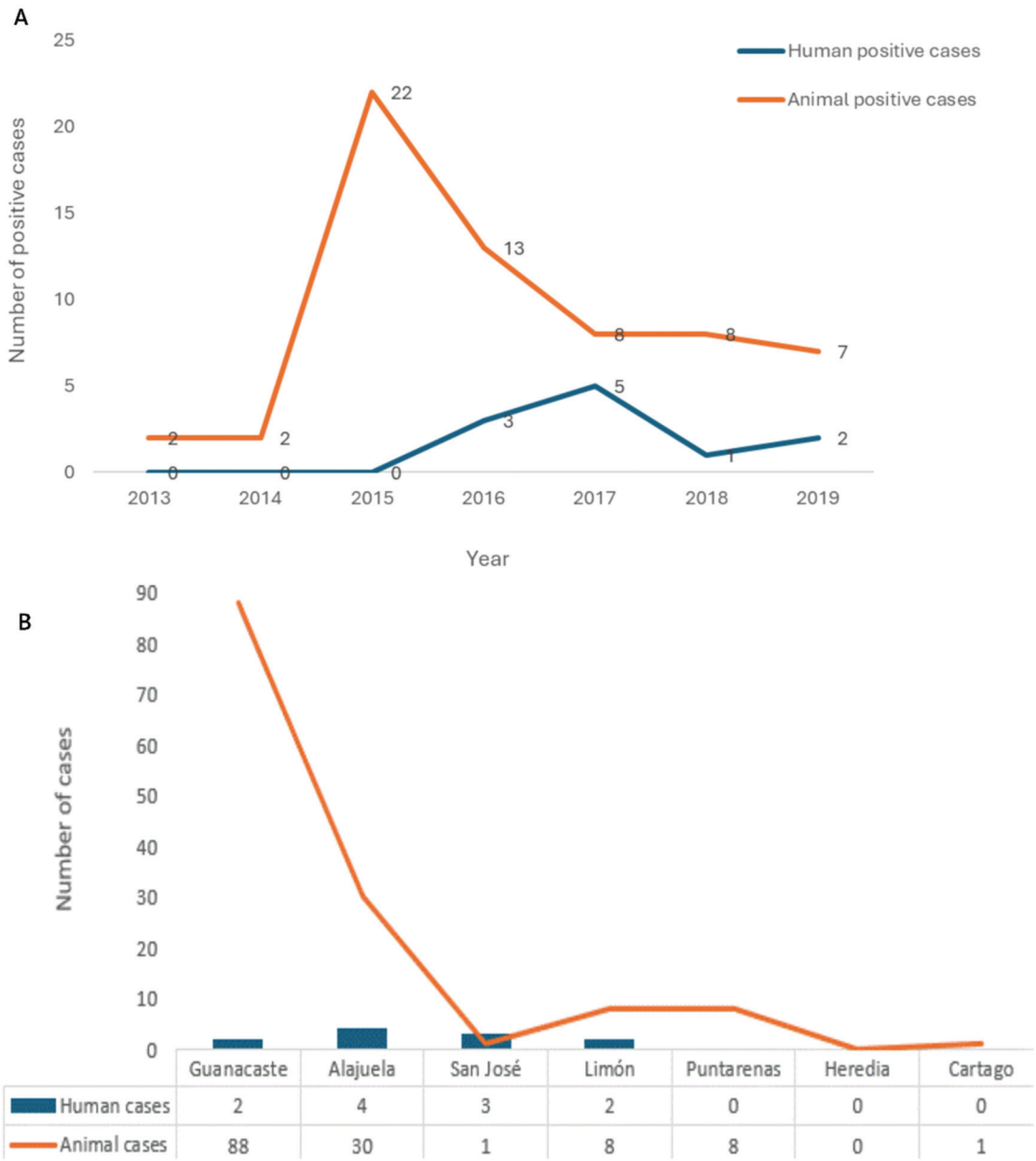

FIGURE 4 | (A) Number of positive animal and human cases of alphaviruses by year in Costa Rica (2013–2019). (B) Number of positive animal and human cases of alphaviruses by province in Costa Rica (2013–2019).

can produce quick and reliable results. IgM capture ELISA is more sensitive and specific than classical HIT and VNT tests and can detect IgM classes in whole serum, making IgM capture ELISA the recommended test of choice for a rapid, clinically, and epidemiologically relevant serodiagnosis of EEEV or WEEV infections in humans [34] and horses [21].

Without a doubt, VEEV is the most prevalent of the studied arboviruses, probably because VEEV is more efficient in inducing higher viremia titers than EEEV [39], potentially making VEEV more available to hosts. The density of some competent mosquito species could also have a role in the higher prevalence of VEEV.

Ochlerotatus (formerly *Aedes*) *taeniorhychus*, *Anopheles punctimacula*, *Culex nigripalpus, Mansonia indubitans, Mansonia titillans, Psorophora cilipes, Ptorophora confinnis, Deinocerites pseudes* were suspected vectors of VEEV captured during the 1970 outbreak in Costa Rica. However, VEEV could only be isolated from *O. taeniorhychus and D. pseudes* [7].

The density of reservoirs could also impact the higher frequency of positive cases and the circulation of VEEV compared to EEEV. VEEV reservoirs are primarily small rodents, while some passerine bird species serve as reservoirs for EEEV. Rodents tend to live in resident groups with limited displacement, which contributes to a higher local population density, facilitating VEEV transmission.

In contrast, birds exhibit seasonal migration patterns and can spread viruses over long distances but may not contribute to local transmission as effectively as rodents.

Small mammals, especially rodents of the genera *Proechimys, Sigmodon*, *Oligoryzomys*, and *Oryzomys*, serve as reservoir hosts for enzootic VEEV strains [40–42]. In addition, *Sigmodon hispidus* (cotton rat), is widely distributed from the USA to Venezuela [43]. These animals develop moderately high levels of viremia for 2–4 d and have short-lived populations with high reproductive rates, resulting in a nearly continuous supply of susceptible hosts.

However, in a study in which 72 wild rodents were collected in Chiapas, only 2.7% were seropositive for VEEV, this low percentage was associated with extensive floods in those areas that could interrupt VEEV transmission [44]. Interestingly, in a study conducted in northern Costa Rica in 2014, 3 of 100 birds captured (*Columbina talpacoti, Protonotaria citrea*, and *Vireo flavifrons*) were EEEV-positive [45], which confirmed the low prevalence of EEEV in Costa Rica.

The current study established that the number of arbovirus cases changes every year and the number of cases is higher in some areas than in others. According a VEEV prevalence study [16], horses that had been in contact with VEEV were detected in all provinces. However, in the present study more VEEV-positive cases were recorded in Guanacaste and Alajuela (provinces located in northern Costa Rica). The majority of cases in Guanacaste occurred in 2009 (n = 35) and in 2010 (n = 14), which was 58% of 84 cases. If the 21 cases in 2015 are added, 71% of the cases in Guanacaste occurred in those 3 years. Of the 28 cases in Alajuela, 11 occurred in 2010 and only 1 occurred in 2015 for 42% of the cases. This finding shows that between 2009 and 2010 there was an outbreak of VEEV in northern Costa Rica. VEEV cases were also reported in Belize, Guatemala, and Panama in 2009, 2010, 2014, and 2015 (World Organization for Animal Health OIE WAHIS). This finding coincides with the years that had the highest number of cases in Guanacaste province, Costa Rica, suggesting that epidemics are not restricted to one region but involve larger areas.

In addition, 2 possible VEEV case peaks that appeared to occur every 6 years were noted, 1 in 2009 and 1 in 2015 in Guanacaste province. Two years (2009 and 2015) were moderate-to-very strong El Niño years. In fact, the climate was drier and hotter on the Pacific side and wetter on the Caribbean side of Costa Rica in those years and cases were only detected on the Pacific side. La Niña typically has the opposite effect by increasing rainfall slightly on the Pacific side and sometimes drying out the Caribbean side. La Niña was partially responsible for flooding in 2017 in Costa Rica.

Mosquito population cycles are well-known to depend on weather conditions with rainfall and higher temperatures commonly associated with peaks in mosquito populations. However, our VEEV models did not demonstrate an association between rainfall and VEEV cases. The mosquito presence in Costa Rica is generally higher during the dry season when rivers carry little water and puddles in the riverbeds form breeding grounds for mosquitoes. The findings herein demonstrate that VEEV seropositivity risk increases during ENSO/La Niña and El Niño. Although the quarterly variable was not retained in the final models, likely due to confounding factors with ENSO, the trend in the data suggests that VEEV might be higher in the second half of the year. In a previous study to determine possible niches of VEEV using MaxEnt, variables that contributed the most to explaining the VEEV cases were the mean temperature of the coldest quarter (32.5%), precipitation of the driest quarter (16.9%), the annual mean temperature (15.1%), altitude (m.a.s.l [6.6%]), and precipitation of the warmest quarter [6.2%] [46]. In this study the suitability of VEEV cases increased from 34%–94% with the presence of the first rainfall. Epidemic peaks produced by epidemic or enzootic strains require explosions of mosquitoes as a result of unusually rainy seasons [47] as well as virus strains adapted to produce viremia and mosquitos that feed from equines and humans [47]. Variables, such as temperature, precipitation, humidity, and ENSO, have been closely correlated to the occurrence of cases and the seasonality of another disease transmitted by mosquitoes [dengue virus] [48].

No association was demonstrated between VEEV seropositive cases and Holdridge life zones because these zones were established in 1971 and the Costa Rica environment has changed due to climate [49] and probably land use.

The initial VEEV clinical signs are quite generic and could be confused with a range of possible etiologies. It was not until the neurologic signs develop that the sample was sent to the laboratory to confirm an arboviruses case, so the number of VEEV cases could be higher than reported herein given that those horses that did not develop neurologic signs. It is important to highlight that clinical disease presentation was not associated with vaccination despite equine vaccines existing for all agents investigated in this study because all the samples from equines with vaccine records were discarded. The fact that VEEV and EEEV are circulating in Costa Rica with variable frequencies over the years has important public health implications, especially because human cases of VEEV are often misdiagnosed [1].

During the equine encephalitis surveillance study, 11 human alphavirus infections were detected between 2013 and 2019 by Inciensa, the reference laboratory of the Ministry of Health in Costa Rica. Seven cases tested positive for alphavirus by PCR but were negative for Chikungunya. Additionally, four cases were positive for EEEV and one case was positive for VEEV by IgM MAC ELISA. These results were confirmed by HI testing performed by Dr.Thomas Ksiazek and collaborators from the University of Texas (Galveston, TX, USA), and others tested positive by convectional PCR for alphavirus.

Fig 4A presents a comparison of alphavirus-positive cases in humans and horses, while Fig 4B illustrates the distribution of alphavirus cases in humans and horses

across the Costa Rica provinces (Guanacaste, Alajuela, San José, and Limón). Of the confirmed human cases, 64% (7/11) were children, 27% (3/11) were adults > 48 years of age, and 9% (1/11) were young adults in their 20s. Two alphavirus cases were detected in Liberia, Guanacaste with the patient's symptoms beginning in November 2017 and another patient was reported in February 2019. A 54-year-old female was diagnosed with viral meningoencephalitis in November 2017. VEEV-positive cases were diagnosed in horses in Liberia, Guanacaste in June and July (1 case in Bagaces, Guanacaste in November of the same year [27 km from Liberia] and the other case in Cañas, Guanacaste in October [46 km from Liberia]).

A seven-year-old girl from the southern Caribbean coast presented with fever, lethargy, loss of appetite, vomiting episodes, green diarrhea, and seizures in 2016. Unfortunately, this child died in the Children's National Hospital. The child was initially diagnosed with dengue and later with dengue hemorrhagic fever. However, she tested positive for VEEV by the IgM capture ELISA used in this study and subsequently confirmed by the HI test (1:640). Other possible etiologies, such as dengue, were discarded by the University of Texas (Galveston, TX, USA). As a consequence of this case, between 29 April and 5 May 2016, the National Service of Animal Health (SENASA) performed an active sampling in the area of the child´s residence and 13 of the 62 horses sampled (from 8 different premises in the local area) were positive for VEEV by the IgM capture ELISA.

We conclude that VEEV circulates more widely in the country than EEEV, while no evidence of WEEV circulation was detected. Guanacaste province exhibited the highest VEEV positivity rate (29.4%) and accounted for 65.6% of all VEEV-positive cases. Guanacaste was also the source of 50% of the EEEV-positive cases. VEEV seropositivity peaked in 2009 (27.3%) and again in 2015 (17.2%), with the majority of positive cases (60%) occurring between October and December, especially in November (29%). Younger horses (mean age, 39.3 months) were significantly more likely to be seropositive than older horses (mean age, 73.6 months). Our findings confirm the importance of considering VEEV as a differential diagnosis for dengue virus [1] and recognizing the role of VEEV in encephalitis cases, especially in children and the elderly. VEEV seropositivity was significantly associated with La Niña events (OR = 4.6–6.4) and the premontane ecozone (up to twice the risk compared to basal zones). Even more importantly, within the One Health framework, encephalitis outbreaks in horses should be taken into account and can serve as an early warning system for vector-borne encephalitis cases in humans.

DATA AVAILABILITY STATEMENT

Not applicable.

ETHICS STATEMENT

No ethics approval was required in accordance with the criteria of the Scientific Ethics Committee of the Costa Rican Institute for Research and Education in Nutrition and Health (CEC-INCIENSA). Studies conducted by INCIENSA as part of its epidemiological surveillance activities are exempt from submission to a scientific ethics committee for approval. Additionally, the Animal Bioethics and Welfare Commission of the School of Veterinary Medicine at the National University of Costa Rica has thoroughly reviewed the study and concluded that, as it involves collaboration with SENAS, a legally authorized entity for conducting animal health research, no further ethical approval procedures are necessary. Nevertheless, the commission identified no ethical concerns regarding the conduct or publication of the study and fully endorses the research protocol and methodology employed.

AUTHOR CONTRIBUTIONS

CJ and BL contributed to the conception and initial drafting of the article. BL, SH, and AIR participated in editing the manuscript. MV and SH collaborated on data analysis; CJ was responsible for processing the samples. BL, MB, CLF, AK also contributed to editing and reviewing the manuscript. All authors approved the final version for submission.

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CONFLICT OF INTEREST

The authors declare no conflicts of interest.

SUPPLEMENTARY MATERIAL

Supplementary Material can be downloaded from https://zoonoses-journal.org/wp-content/uploads/2025/06/zoonoses20240062_Suppl.pdf.

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