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Outbreaks of Eastern equine encephalitis in northeastern Brazil

Maria L. C. R. Silva, Glauco J. N. Galiza, Antônio F. M. Dantas, Rafael N. Oliveira, Keila Iamamoto, Samira M. Achkar, Franklin Riet-Correa

Abstract. Outbreaks of eastern equine encephalitis observed from May 2008 to August 2009 in the Brazilian states of Pernambuco, Ceará, and Paraíba are reported. The disease occurred in 93 farms affecting 229 equids with a case fatality rate of 72.92%. Main clinical signs were circling, depression or hyperexcitability, ataxia, and progressive paralysis with a clinical manifestation period of 3–15 days. Main histologic lesions were a diffuse lymphocytic encephalomyelitis with neuronal death, satellitosis, neuronophagia, and hemorrhages being more severe in the cerebral gray matter of the telencephalon, diencephalon, and mesencephalon. Some animals also had areas of malacia in the telencephalon, thalamus, and basal nuclei. From 1 case, the virus was isolated by mice inoculation, and in other 13 cases was identified as Eastern equine encephalitis virus by semi-nested reverse transcription polymerase chain reaction. After DNA sequencing, all samples were identified as eastern equine encephalitis through the BLASTn analysis, but samples from the Ceará and Paraíba states corresponded to the same cluster, while the sample from the state of Pernambuco corresponded to a different cluster.

Key words: Brazilian semiarid; equine encephalomyelitis; laboratory diagnosis; semi-nested reverse transcription polymerase chain reaction.
Figure 1. Map of the Brazilian states of Paraíba, Ceará, and Pernambuco showing the municipalities where eastern equine encephalitis occurred.

Fourteen necropsies were performed. Traumatic lesions, including corneal opacity, subcutaneous periocular edema, and alopecia and erosions of the skin in different parts of the body were observed in 9 horses. Five animals showed congestion of the meningeal vessels. After fixation, red or brown areas were observed in the temporal and occipital cortex of 1 horse (Fig. 2).

Samples of the organs of the thoracic and abdominal cavities, and the CNS were collected for histologic examination. The samples were fixed in 10% buffered formalin solution. After fixation, the brain and spinal cord specimens were cut transversely into 3–5 mm thick sections, and samples of frontal, temporal, parietal, and occipital lobes, hippocampus, basal nuclei, thalamus, rostral and caudal colliculi, pons, cerebellum, medulla oblongata, and cervical, were less frequently observed. The clinical manifestation period was 3–15 days.

Figure 2. Cerebrum. Horse with equine encephalomyelitis from the Brazilian municipality of Jaguaribe, state of Ceará, showing red or brown areas of malacia in the occipital cortex.
titers against EEE virus were 1:10 and 1:10 in the first sample, increasing to 1:320 and 1:160 in the second.

Direct fluorescent antibody test\textsuperscript{10} and mouse inoculation test\textsuperscript{10} were negative for \textit{Rabies virus}. A litter of 5-day-old mice inoculated with CNS suspension collected from 1 horse immediately after euthanasia began to show signs 24 hr after inoculation. The inoculated mice were isolated from the other litters, failed to nurse, and had difficulty breathing and paresis progressing to flaccid paralysis. Death occurred within 6 hr after the onset of first clinical signs. Forty-eight hours after inoculation of the newborn mice, the mother presented nervous signs including circling movement, head pressing on the box, and paresis of the hind limbs progressing to flaccid paralysis, and died 72 hr after inoculation. The virus isolated from mice was confirmed as EEE by the semi-nested reverse transcription polymerase chain reaction (RT-PCR).

Identification of the virus was performed by semi-nested RT-PCR on 13 horse specimens collected from the municipality of Exú, in the state of Pernambuco (1); from the municipalities of Jaguaribe (1) and Várzea Alegre (2), both in the state of Ceará; and from the municipalities of São João do Rio do Peixe (2), Coremas (2), Poço de José de Moura (2), Uirâna (1), Paulista (1), and Patos (1), all in the state of Paraíba. RNA extraction was carried out using TRIZol\textsuperscript{b} according to the guidelines of the manufacturer, from the original specimens of horses. In 10 samples, the extraction was performed in a pool of brain stem and cerebellum, and in 3 samples, in a pool of cervical cord, hippocampus, and frontal cortex. As a positive control, a sample of fixed virus of Western equine encephalitis virus (WEEV), assigned by the Instituto Evandro Chagas (IEC), state of Pará, and main­

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Main histologic lesions were a diffuse multifocal non­suppurative lymphoplasmacytic encephalomyelitis with perivascular infiltration by lymphocytes, plasma cells, macrophages, and some neutrophils, vasculitis with endothelial cells, swelling, neuronal death, satellitosis, neuronophagia, hemorrhages, edema, and multifocal lymphocytic infiltration of the neuropil (Fig. 3). These lesions were more severe in the gray matter of the telencephalon, diencephalon, and mesencephalon. Similar but mild lesions were observed in the cerebellum and spinal cord, mainly in the gray matter. The distribution and severity of the inflammatory lesions in 14 horses are presented in Table 1. In 5 cases, rare eosinophils were observed within the lymphoplasmacytic exudates. Nine cases showed lymphoplasmacytic menigitis with perivascular cuffing in the cerebral meninges, and in 4 of these horses, the menigitis also affected the cerebellum. Focal areas of malacia, with vacuolation of the neuropil and presence of macrophages, neutrophils, cellular debris, and occasional gitter cells were observed mainly in the telencephalon, thalamus, and basal nuclei. Axonal spheroids were occasionally observed. No significant lesions were observed in other organs examined.

Serum samples, 1 taken during the course of the clinical disease and 1 taken 15 days later, were collected from 2 horses that were not vaccinated against equine encephalitis, and sent to the Biological Institute of São Paulo (Brazil) to determine the antibody titers for equine eastern and western encephalitis by means of neutralization test in cell culture. The antibody

Figure 3. Brain. Horse with equine encephalomyelitis from the Brazilian municipality of Exú, state of Pernambuco. A, basal nuclei showing perivascular cuffing of lymphocytes, plasma cells and macrophages. Hematoxylin and eosin (HE). Bar = 20 μm. B, hippocampus showing neuronophagia. HE. Bar = 10 μm. C, basal nuclei showing neuronal necrosis with infiltration of the neuropil by lymphocytes, plasma cells and neutrophils. HE. Bar = 10 μm. D, brain stem showing severe hemorrhages. HE. Bar = 50 μm.
Outbreaks of eastern equine encephalitis

Table 1. Distribution and intensity of inflammatory histologic lesions observed in 14 cases of eastern equine encephalitis in northeastern Brazil.*

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*FC = frontal cortex; PC = parietal cortex; TC = temporal cortex; OC = occipital cortex; BN = basal nuclei; HY = hippocampus; TA = thalamus; RC = rostral colliculi; CC = caudal colliculi; PO = pons; MO = medulla oblongata; CE = cerebellum; MC = cervical spinal cord; MT = thoracic spinal cord; ML = lumbar spinal cord; NE = not examined; – = absent; + = mild; ++ = moderate; +++ = severe.

Table 2. Description of the primers used in the current study for semi-nested reverse transcription polymerase chain reaction (RT-PCR).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sense</th>
<th>Use</th>
<th>Sequence</th>
<th>Nucleotide position in the genome</th>
</tr>
</thead>
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<td>A1</td>
<td>Sense</td>
<td>RT-PCR</td>
<td>5'-AGAGCDTTTTCGCAAYSTRGCHW-3'</td>
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<td>A2</td>
<td>Anti-sense</td>
<td>RT-PCR/semi-nested PCR</td>
<td>5'-ACATRAPKGNNTGNGTRCTRAANCCDAYCC-3'</td>
<td>563–592</td>
</tr>
<tr>
<td>A3</td>
<td>Sense</td>
<td>Semi-nested PCR</td>
<td>5'-TGYYCNVTGMDNWSYVCNGARGAYCC-3'</td>
<td>283–308</td>
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</tbody>
</table>

purification, the sequences were generated in automatic genetic analyzer. The confirmation of the sequencing and determination of viral species was performed using the application BLASTn (http://www.ncbi.nlm.nih.gov/BLAST).

Of the 13 CNS samples submitted to semi-nested RT-PCR, all were positive for the genus Alphavirus. After DNA sequencing, all positive samples were identified as EEEV through the BLASTn analysis. Sequencing showed that the samples from the states of Ceará and Paraíba corresponded to the same cluster, while the sample from the state of Pernambuco corresponded to a different cluster. Detailed results of the sequencing of these samples will be published elsewhere.

After diagnosis of the disease, nearly all equidae from the affected regions were vaccinated and new outbreaks were not observed during 2010. In the 2008 and 2009 outbreaks, the presumptive diagnosis of EEE was made by the epidemiological, clinical, and pathological features of the disease, and confirmed by virus isolation, semi-nested RT-PCR, and the increased antibody titer in 2 horses that had recovered from the disease. The results of the sequencing of the isolates proved that the outbreak in 2008 in Pernambuco was caused by a distinct EEEV variant different from the virus variant causing the outbreak in Ceará and Paraíba in 2009. The diagnosis of 2 outbreaks of EEE in the semiarid regions of 3 northeastern states represents the first full description of the disease in Brazil, including epidemiology, clinical signs, pathology, and virus identification.

During the visits to the farms, some experienced owners and veterinarians mentioned that this disease is known as roda (circling) and occurs periodically in the region. The occurrence of both outbreaks of EEE at the end of the rainy season suggests that the disease is seasonal and epidemic in the Brazilian semiarid region.

The semiarid region is characterized by a warm climate with an average temperature of 26°C and rainfall of approximately 800 mm per year, and in some regions of only 500–600 mm of rain per year. The rains are irregular, with insignificant to low rainfall occurring for some years. The rainy season is short, ranging from June-July to April-May. The humidity is low, ranging from 60% to 75%, and the vegetation is typical of caatinga (“white forest” in the Tupi-Guarani language), an exclusive Brazilian biome, occupying almost 11% of the country. This vegetation is characteristic of the arid conditions (xerophytic) with a strong presence of bushes with twisted branches and deep roots, and the presence of cacti and bromeliads. Bushes lose almost all of their leaves in times of drought.
The seasonal variation of EEE, as well as other arboviral diseases, is associated with a particular temperature range, rainfall, and other environmental factors that determine the density of vectors. The rainfall of Paraíba, in 2009 (1,345 mm), was among the highest in the last 15 years (mean of 864 mm), suggesting that the increased rainfall is an important factor for the occurrence of outbreaks, which occur preferentially at the end of the rainy season (Fig. 4) when there is an increase in vector population.

The morbidity and fatality rates suggest that the isolate from the first outbreak was more pathogenic than the isolate from the second outbreak. *Eastern equine encephalitis virus* is considered more pathogenic than WEEV and *Venezuelan equine encephalomyelitis virus*, although considerable variation in pathogenicity may occur. Epidemiologic characteristics (epidemic and seasonal) as well as clinical signs suggesting mainly cerebral and brain stem involvement allowed differentiation of EEE from rabies, *Crotalaria retusa* poisoning, and leukoencephalomalacia, which are common diseases of horses in the Brazilian semiarid.

It is concluded that EEE is an important disease of horses in northeastern Brazil. Although the epidemiology, clinical signs, and pathology allow the presumptive diagnosis of the disease, it is necessary to submit specimens for laboratory diagnosis to determine the etiologic agent of the encephalitis. Such identification is very important for the surveillance of equine arboviruses because of their public health importance, in addition to any economic loss.

**Sources and manufacturers**

a. TRIZol, Invitrogen Brasil Ltda., São Paulo, Brazil.
b. QIAquick®, QIAGEN Biotecnologia Brasil Ltda., São Paulo, Brazil.
c. Low DNA Mass™ Ladder, Invitrogen Brasil Ltda., São Paulo, Brazil.
e. Sephadex™ G-50, GE Healthcare do Brasil Ltda., São Paulo, Brazil.
f. ABI 3130, Applied Biosystems do Brasil, São Paulo, Brazil.

**Declaration of conflicting interests**

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**References**


**Figure 4.** Mean monthly rainfall, from January to December 2009, in 16 Brazilian municipalities (Belém do Brejo do Cruz, Cajazeiras, Catolé do Rocha, Condado, Coremas, Itaporanga, Malta, Patos, Sousa, São Francisco, Vista Serrana, Poço de José de Moura, São Bentinho, São José do Brejo do Cruz, Uiraúna, São João do Rio do Peixe) in the state of Paraíba where eastern equine encephalitis occurred. The monthly distribution of cases observed in the State is also presented.
Outbreaks of eastern equine encephalitis


