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Original Research

Causes of Encephalitis and Encephalopathy in Brazilian Equids

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ABSTRACT

The etiology of diseases that affect the central nervous system (CNS) of equids was investigated. Samples (n = 218) collected from equids showing clinical signs of nervous or behavioral changes were analyzed, of which 37 (17.0%) were positive for rabies, 13 (6.0%) for the presence of protozoans (one *Sarcocystis neurona*, 12 *Toxoplasma gondii*), three (1.4%) for equine herpesvirus type 1 myeloencephalopathy, and 24 (11%) for bacterial encephalitis. Histopathology of the CNS revealed one (0.4%) case of cryptococcal myelomeningoencephalitis and 20 (9.2%) cases of equine leukoencephalomalacia. Central nervous system samples were positive for *Sarcocystis neurona* and *Toxoplasma gondii* by nested PCR-ITS1 followed by nucleotide sequencing. Diagnosis of equine herpesvirus 1 was confirmed by cell isolation and polymerase chain reaction followed by sequencing of the *GD* and polymerase (ORF 30) genes in three samples. No case of equine encephalomyelitis was diagnosed in samples analyzed by isolation in mice, VERO cell cultures, and RT-PCR for the *nsP1* gene. Bacterial agents (*Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., Enterobacteriaceae spp., *Corynebacterium* spp., and nonfermenting gram-negative bacillus) were detected in pure or preponderant cultures. Diagnosis was conclusive in 45% of samples, indicating that other infectious and noninfectious etiologies of encephalitis and encephalopathy should be considered for investigation.

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1. Introduction

Brazil has the world's third largest horse herd, with over 5,000,000 heads that, together with mules and asinines, comprise a stock of approximately 7,500,000 animals [1]. The country also exports considerable numbers of live horses and horse meat [2]. Rabies, Eastern equine encephalitis virus (EEEV), Western equine encephalitis virus (WEEV), Venezuelan equine encephalitis virus (VEEV), West Nile virus, and equine herpesvirus type 1 myeloencephalopathy (EHM) are among the neurologic diseases of equids listed by the World Organization for Animal Health (OIE), and these conditions take on central importance in the context of public health and of the international trade of live animals and animal-based products [3]. However, other diseases also affect the central nervous system (CNS) of equids, such as tetanus, listeriosis, botulism, equine protozoal myeloencephalitis (EPM), mycoplasmosis, degenerative myeloencephalomalacia, leukoencephalomalacia (mycotoxicoses), besides an array of different intoxications. The monitoring and the differentiation of the CNS syndromes that share similar signs are essential measures to identify outbreaks, screen natural

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reservoirs, and improve the quality of feed given to animals. In light of the outcomes of neurologic diseases in the context of equid breeding and public health, the etiology of other causes that affect the CNS has to be investigated. In this sense, the present study evaluated the etiology of CNS disorders in biological samples of equids.

2. Materials and Methods

2.1. Samples

The samples analyzed in the present study were collected between 2007 and 2012 by private veterinarians and veterinary health authorities from the state of São Paulo and other Brazilian states. Samples were refrigerated or frozen and fixed in 10% of buffered formalin and sent to the Laboratory of Rabies and Other Encephalitis, Biological Institute, São Paulo, Brazil. A total of 218 CNS samples from equids presenting clinical signs of neurologic disorders or behavioral changes were analyzed by macroscopic inspection of brains and histopathology. In addition, fragments of these materials were used to detect the presence of viral, bacterial, and protozoan pathogens.

2.2. Diagnosis of Rabies

Direct immunofluorescence was carried out using the antirabies conjugate based on fluorescein isothiocyanate–labeled monoclonal antibodies as described by Dean et al. [4]. Recently weaned Swiss mice (11–15 g, approximately 21 days old) were used in virus isolation [5].

2.3. Histopathology

Brain fragments were cut down to smaller pieces that were fixed in 10% buffered formalin [6] and analyzed in the Laboratory of Pathological Anatomy, Biological Institute, São Paulo, Brazil. Specimens were paraffin embedded, sectioned, and stained with hematoxylin–eosin as previously described [7].

2.4. Sample Preparation for Isolation of Bacterial Agents

Fragments (2 g) of the CNS of equids were suspended in 20-mL brain heart infusion broth (Difco), placed in sterile plastic bags, and homogenized in a mechanical paddle blender (Stomacher 80, Lab System) for 4 minutes for use in the growth of culture media.

2.5. Sample Preparation for Isolation of Viral Agents and DNA Detection

A 20% suspension of CNS fragments was used for virus isolation in mice, VERO and ED cells, as well as for nucleic acid extraction.

2.6. Isolation and Identification of Bacterial Agents

Listeria spp. was identified by adding 10 mL of the homogenate obtained as described in Section 2.4 to a thioglycollate medium. The mixture was maintained in cryoenrichment for up to 4 weeks. Growth was carried out in Mueller-Hinton agar containing 5% sheep blood under microaerophilic incubation at 30°C for 14 hours. Aerobic microorganisms were identified seeding 10 μL of the homogenate in Mueller-Hinton agar containing 10% sheep blood under incubation at 37°C for 48 hours.

2.7. Virus Isolation in Cell Cultures

Equine herpesvirus type 1 (EHV-1), EEEV, WEEV, and VEEV were isolated using VERO and ED lineages grown in 24-well plates with 3×10^5 cells/mL in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. After 24 hours, when monolayer confluence was achieved, the culture medium was discarded and the cells were washed using phosphate-buffered saline buffer (pH 7.2) and inoculated with 2.0 mL of the suspension of material suspected to contain virus. Ninety minutes later, the inoculum was discarded and the cells were once again washed with phosphate-buffered saline. Next, 2-mL Eagle's minimum essential medium was added. The plates were maintained in a stove in a 5% CO_2 atmosphere at 37°C for 7 days. Daily readings were carried out to visualize the cytopathic effect (CE). The results were considered negative after three blind readings without detectable CE. Cells that exhibited CE were collected and submitted to polymerase chain reaction (PCR) or reverse transcription polymerase chain reaction (RT-PCR) to identify the viral agent.

2.8. Nucleic Acid Detection

Equine herpesvirus type 1 was detected by PCR followed by nucleotide sequencing of the GD genes and ORF 30 [8,9], whereas EEEV, WEEV, and VEEV were detected by RT-PCR followed by nucleotide sequencing of the *nsP1* gene [10]. Protozoans were identified by nested PCR and nucleotide sequencing of the gene *ITS1* [11]. DNA of *Listeria monocytogenes* was identified by PCR for the *hly* gene [12].

3. Results

Of the 218 samples analyzed, 37 (17.0%) were positive for rabies, three (1.4%) for EHV-1, and 24 (11.0%) for bacterial encephalitis. In addition, 13 (6.0%) were positive for protozoans using nested PCR-ITS1 assay. Nucleotide sequencing identified one (0.4%) *Sarcocystis neurona* and 12 (5.6%) *Toxoplasma gondii.* Histopathologic analysis indicated meningeal or neuropil inflammation, congestion or edema, hemorrhage, necrosis, or malacia in these positive samples. Moreover, histopathology demonstrated one (0.4%) case of cryptococcal myelomeningoencephalitis and 20 (9.2%) cases of equine leukoencephalomalacia (ELEM). No cases of EEEV, WEEV, or VEEV were identified. A neuropathogenic variant G2254/D752 was detected after nucleotide sequencing of the polymerase gene in one of the three EHV-1 isolates from positive samples of myeloencephalopathy. In total, six different bacterial isolates (*Staphylococcus* spp., *Streptococcus* spp., *Bacillus* spp., Enterobacteriaceae spp., *Corynebacterium* spp., and nonfermenting gram-negative bacillus) were isolated from the 24 (11%) of the 218 CNS samples analyzed in pure or

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