Evaluation of Viral Migration of Different Variants of Equid Alphaherpesvirus 1 in the Central Nervous System of Hamsters by Immunohistochemistry

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Abstract: The intranasal inoculation of equid alphaherpesvirus1 (EHV-1) Brazilian variants A4/72, A9/92, A3/97, Iso72/10 and the Argentine variant AR4 in a Syrian hamster model *Mesocricetus auratus* induced severe encephalitis. Clinical signs included weight loss, lethargy, somnolence, anorexia, and intense salivation two days post-inoculation (dpi), followed by neurological signs such as loss of proprioception, walking in circles, spastic paralysis, seizures, recumbency and death at 3rd dpi (A9/92 and A4/72 variants) and 4th dpi. Respiratory signs such as dyspnea and serosanguinous nasal discharge were also observed. Histopathological changes in brain included mixed inflammatory infiltrate with predominance of mononuclear cells, neuronal degeneration, liquefactive necrosis, hemorrhagic foci, leptomeningitis, perivascular edema, mononuclear infiltration, and perivascular cuffing. Immunohistochemical examination showed viral replication in neurons restrict predominantly to olfactory bulb and frontal cortex (variants AR4 and A3/97) and in groups of cells from distant regions, such as the caudal diencephalon and rostral mesencephalon (variants Iso72/10) and absence of viral antigen labeling of variants A9/92 and A4/72 despite these variants were the most neurovirulent, so new experiments not staggered in days but in hours post inoculation are needed to better understand the viral migration of these variants.

Keywords: EHV-1, Neurovirulence, Neuropathogenicity, Immunohistochemistry, Myeloencephalopathy

1. Introduction

Equid alphaherpesvirus1 (EHV-1) is an important pathogen endemic in equine populations worldwide, leading to significant losses due to respiratory disease, abortion, neonatal death, and myeloencephalopathy [1-3]. Equine herpesvirus myeloencephalopathy (EHM), classified as potentially emerging by the US Department of Agriculture [4-7], is a neurological dysfunction producing clinical signs ranging from proprioceptive deficiency, ataxia, limb weakness, swaying, stumbling, and falling to paralys is affecting mainly the hind limbs [7, 8].

Necropsy shows brain congestion and focal areas of malacia or hemorrhage in the spinal cord and brain [8, 9]. Histological changes in brain of horses infected with EHV-1 include vasculitis of small blood vessels or spinal cord with perivascular mononuclear and polymorphonuclear cuffing, degeneration of neurons, glial cell reaction, hemorrhagic foci, thrombosis with resulting ischemic neuronal necrosis, congestion, diffuse gliosis, perineuronal and perivascular edema [7, 10-12].

Studies of EHV-1 pathogenicity have usually employed murine and equine models [13-18]. However, studies involving adult horses are costly, labor intensive, and limited to a small number of animals [19]. Besides this experimentally inoculated horses may have antibodies to EHV-1 (immune memory) that can neutralize virus replication and, consequently, influence the type and magnitude of lesions produced and serological cross-reaction between EHV-1 and EHV-4 may interfere in the results [17].

Experiments using suckling Syrian hamsters have been conducted for virus isolation and studies of the pathogenesis of EHV-1 infection since the 1950s, when the detection of intranuclear inclusion bodies in the hepatic cells of hamsters inoculated intraperitoneally became possible [20]. Equid alphaherpesvirus 1 was first isolated in Brazil in the 1960s from a sample of aborted equine fetus liver inoculated intraperitoneally into suckling hamsters [1].

The first neurological signs of EHV-1 infection in suckling hamsters in Brazil were reported after intraperitoneal inoculation with a suspension of liver, spleen, and lung from an aborted equine fetus from Campinas, São Paulo [21]. The isolate, designated A4/72, was shown to cause tonic-clonic convulsions and paralysis in the infected hamsters. After the 1980s, isolation and study of EHV-1 in Brazil was accomplished predominantly via VERO cell culture and in equine dermal cells [22-24].

In subsequent years, the model of EHV-1 infection in hamster was used for the study of antiviral agents [25] and immune response to the virus [26, 27]. Recently, hamster models have been used to study the neuropathogenesis of EHV-9 [28-31], but there are few studies about neuropathogenicity of EHV-1 in hamster models [32].

With advances in molecular biology, hypotheses of the neuropathogenicity of EHV-1 have been proposed. Gene mutations of the open reading frame (ORF) 64, which encodes infected cell protein 4, could be linked to neuropathogenicity [33].

A mutation exchanging adenine for guanine at position 2254 in ORF 30 (A_{2254}–G_{2254}) that leads to an alteration in the aminoacid 752 sequence (from N_{752}–D_{752}) is also linked to the neuropathogenicity of EHV-1, but only the amino acid variation D_{752} is linked to inflammation of the central nervous system and ataxia, while the N_{752} amino acid mutation is not shown to cause neurological signs in the natural host [34, 35].

Recent studies have shown that EHV-1 with ORF30 mutation (N_{752}–D_{752}) did not lead to altered virus replication in FHK and mouse neuron cultured cells and residue 752 in the essential DNA Polymerase of EHV-1 was not required for virus growth [36]. The ORF37 (UL24) deletion has also been mentioned as a factor of lost of neuropathogenicity in a mouse model [37].

Despite the presented studies there is currently no consensus with respect to alterations that may make an EHV-1 variant neuropathogenic, so the aim of the present study was to characterize tissue damage and viral migration throughout the Central Nervous System of different variants of EHV-1 using a hamster model.

2. Materials and Methods

2.1. Virus Variants

The EHV-1 Brazilian variants A4/72, A9/92, Iso72/10, A3/97 (Rabies and Viral Encephalitis Laboratory, Biological Institute, SP, Brazil) [21, 23, 38] and AR4 Argentine variant isolated from aborted equine fetuses were used in this study [39, 40]. All isolates were confirmed to be EHV-1 by PCR amplification and DNA sequencing of the unique transcriptional regulator genes (Brazilian variants: GenBank accession number EU094655 to EU094657; Argentine variant: GenBank accession number EU366295).

The A9/92 (6th passage) and A4/72 (21th passage) viruses were propagated in VERO cells, A3/97 (5th passage) and Iso72/10 (1st passage) in ED cells and AR4 (2nd passage) in RK-13 cells. Cell lines were grown in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% fetal calf serum. After three cycles of freezing and thawing, the cells and infectious supernatant were centrifuged at 8000×g for 20 min to remove cell debris. The final infectious supernatant was fractionated into small volumes and stored at -70°C until use [8].

2.2. Experimental Animals

Twenty-four Syrian hamsters *Mesocricetus auratus* were obtained from the animal facility of the Department of Pathology, School of Veterinary Medicine and Animal Science, University of SãoPaulo, SãoPaulo, Brazil. Hamsters were separated into six groups, five experimental and an untreated control, with groups housed separately in polycarbonate micro-isolator cages, each connected to an individual ventilation system with HEPA filters on both the in- and out- flow. Filtered and autoclaved water and commercial pellets were provided *ad libitum*. All procedures were approved by the Ethics Committee on Animal Experiments of the Biological Institute (CETEA-IB) on registration protocol number CETEA-IB 106/10.

Five groups of four 3-week-old male hamsters, 40–50g, were anesthetized with inhaled Sevoflurane (Sevocris®, Cristália, Itapira, SP) and inoculated intranasally with 50μl EMEM containing 10^4 $TCID_{50}$ of one of the following variants of EHV-1: A4/72, A9/92, A3/97, Iso72/10 and AR4. A negative control group comprised four female 3-week-old Syrian hamsters, 40–50g, inoculated intranasally with 50μl non-infected EMEM.

After inoculation, the infected hamsters were weighed and evaluated twice daily to register of clinical signs that were recorded as absent, slight, moderate or severe by the same

operator of the experiment. At the onset of severe clinical signs they were euthanized along with the control group by an overdose of Sevoflurane and necropsied to collect samples of brain, lung, spleen, liver, thymus, heart, and kidney [18].

2.3. Histopathology

The collected organs were fixed for 48h in 10% buffered formalin then transferred to 70% ethanol, dehydrated in ethanol series, cleared in xylene, and embedded in paraffin. Sections were cut at 3µm, deparaffinized, rehydrated, and stained with H&E [11]. The histological score was classified in absent, slight, moderate or severe according to the intensity of microscopical lesions by the same pathologist of the experiment.

2.4. Immunohistochemistry

Sections of organs were dewaxed in xylol at 37°C for 30min, and endogenous peroxidase was blocked in a solution of 20ml of 30% hydrogen peroxide (H_2O_2) diluted in 80ml of methanol for 30min. Antigen retrieval was performed with citrate buffer (pH 6.0) by heating in a microwave oven (1000W for 15min) prior to incubation with the primary antibody in a humid chamber at 37°C for 30min with a 1:1000 dilution of goat antiserum specific for ERV/EHV-1 (VMRD® USA Inc. catalog 210-70-ERV, Pullman, Washington, USA). Finally, they were incubated with streptavidin-biotin complex (a combination of anti-mouse, -rabbit, and –goat immunoglobulins; LSAB+System-HRP, Dako® ref. K0690-Dako® Cytomation, Carpinteria, California, USA) for 45min in a humid chamber at room temperature. The reaction was revealed upon exposure to 3,3' diaminobenzidine chromogen solution (DAB, Dako® code ref K3468). The slides were counterstained with Mayer's hematoxylin and mounted with synthetic resin (Entellan-Merck®) [11]. The presence of viral antigen was classified in: -absent when no antigen was stained; + few when found less than 5 stained cells in 40 x field; ++ moderate when found 5-30 stained cells in 40 x field or +++ numerous when found more than 30 stained cells in 40 x field according to the evaluation of the same pathologist of the experiment.

3. Results

3.1. Clinical Signs

The hamsters inoculated with the A/92 and A4/72 variants presented high weight loss at 2 days post-inoculation (dpi), with severe disease at 3 dpi characterized by intense salivation and neurological signs such as loss of proprioception, walking in circles, spastic paralysis, seizures, and recumbency, followed by death. Those inoculated with the Iso72/10 variant showed similar neurological signs, but the acute phase started at 4 dpi and included respiratory signs such as dyspnea and serosanguinous nasal discharge.

Animals inoculated with A3/97 and AR4 variants showed weight loss, lethargy, somnolence, and respiratory signs, predominately dyspnea and serosanguinous nasal discharge. At 4 dpi, the hamsters exhibited neurological signs such as hyperexcitability in response to external stimuli, tremors, and seizures, but effects were less severe than observed with the other studied variants. The control group showed normal weight gain and did not exhibit behavior, respiratory, or neurological abnormalities (Figure 1; Table 1).
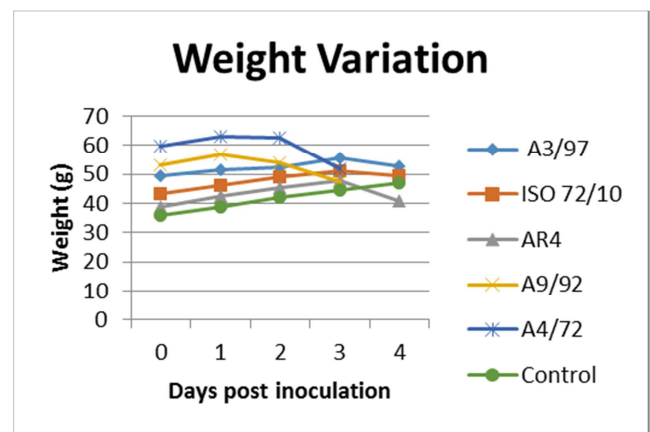

Figure 1. Weight variation of hamsters intranasaly inoculated with five variants of EHV-1, 0-4 days post-inoculation.

Table 1. Clinical signs linked to CNS damage in hamsters after inoculation with five EHV-1 variants.

Clinical Signs	A9/92	A4/72	ISO72/10	A3/97	AR4
Anorexia	+++*	+++	++	++	++
Lethargy	+++	+++	++	++	++
Weight loss	+++	+++	+	+	++
Nasal discharge	-	-	+++	+++	+++
Dyspnea	-	-	+++	++	+++
Tremors	+++	+++	+++	++	-
Intense salivation	+++	+++	+++	+++	++
Ataxia	+++	++	+	-	-
Spastic paralysis	++	++	-	-	-
Walking in circles	+++	++	-	-	-
Hypersensitivity to external stimulus	+++	+++	+++	++	+
Seizures	+++	+++	+++	++	-

* Intensity of clinical signs: (-) absent, (+) slight, (++) moderate, (+++) severe.
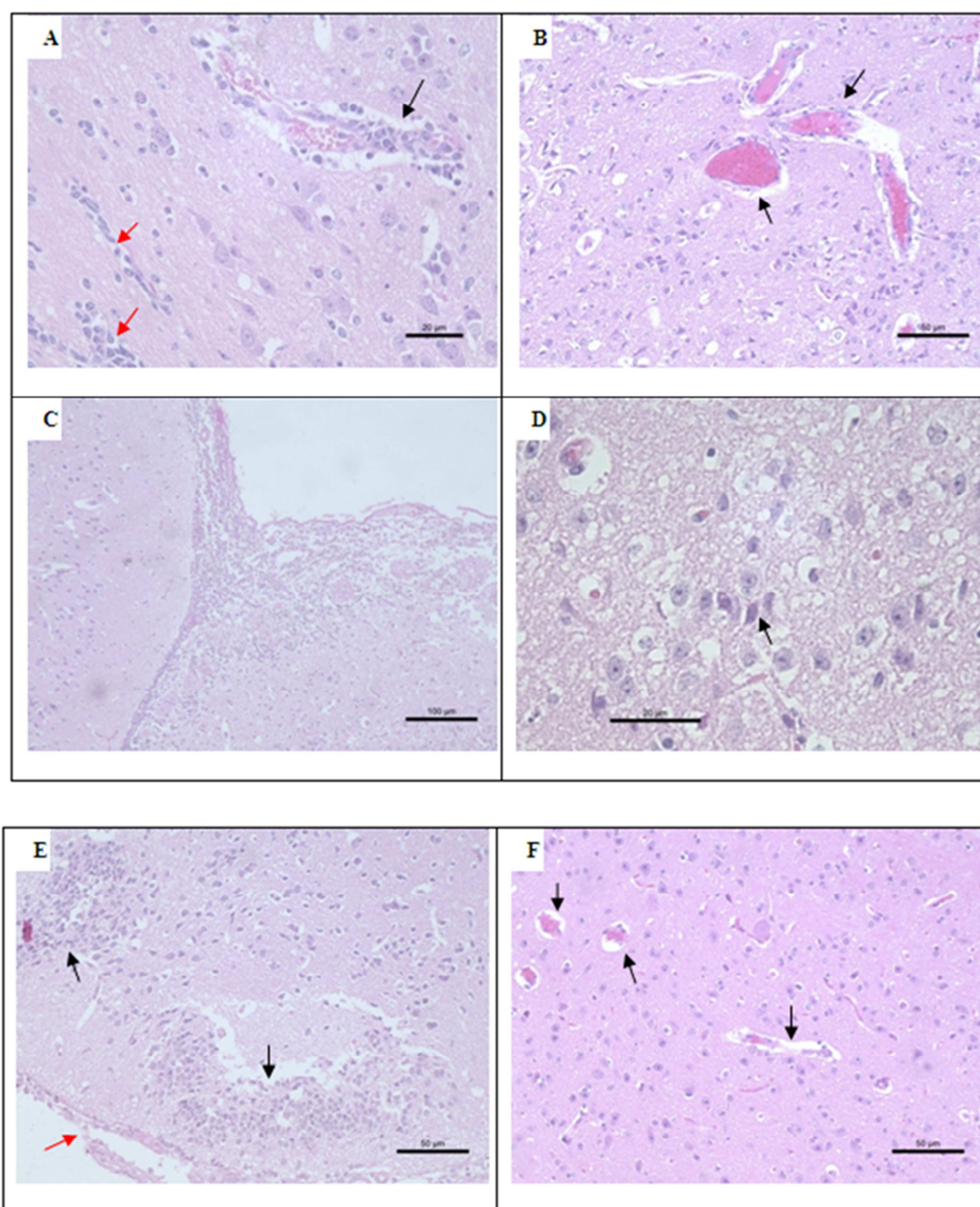
3.2. Pathological Findings

Figure 2. Brain; Cortex region of hamsters experimentally intranasally infected with equid alphaherpesvirus type 1 variants.(a) Iso72/10 variant; mononuclear perivascular cuff (arrow) and mononuclear foci (red arrow) (Bar20μm). (b) Iso72/10 variant; diffuse expansion of the meninges with a large number of macrophages and lymphocytes (leptomeningitis) (Bar100μm). (c) A4/72 variant;congestion of vessels (arrows) (Bar50μm). (d) A9/92 variant; neuronal degeneration (arrow) and vacuolization of the neuroparenchyma (Bar 20μm). (e) A3/97 variation; mononuclear foci (black arrows) and leptomeningitis (red arrow) (Bar 50μm). (f) AR4 variation; perivascular edema (arrows) (Bar 50μm). H&E stain.

Table 2. Histopathological changes observed in the CNS of hamsters inoculated with five EHV-1 variants.

Microscopic lesions in CNS (Histopathology)	A9/92	A4/72	ISO72/10	A3/97	AR4
Inflammatory infiltrate areas	++*	++	+++	+++	+++
Leptomeningitis	++	++	+++	++	++
Hemorrhagic foci	++	+	+	-	-
Neuronal degeneration	+++	+++	++	++	++
Liquefactive necrosis	+++	++	++	+	+
Intranuclear inclusion body	+	-	-	-	-
Neuropil edema	+++	+++	++	+	+
Perivascular cuff	+	+	++	+	+

* Intensity of microscopic lesions: (-) absent, (+) slight, (++) moderate, (+++) severe.

The lungs were edematous and did not collapse upon opening of the thoracic cavity. They presented diffuse reddish coloration in the pulmonary lobes corresponding to hemorrhagic areas. The histological examination showed intense and diffuse mixed inflammatory infiltrate with predominance of mononuclear cells, areas of moderate to intense congestion, hemorrhagic foci, thickened alveolar walls, moderate to intense foamy macrophage infiltration in the alveolar lumen, presence of free red blood cells and alveolar edema, intact bronchiolar epithelium, and slight to moderate mucous secretion. The pleura showed discrete swelling and moderate emphysema.

Cellular debris and free red blood cells in the bronchiolar lumen were observed in hamsters inoculated with the A9/92 and AR4 variants. In hamsters inoculated with Iso72/10 and AR4 variants, syncytia and intranuclear inclusion bodies were observed in the parenchyma. No alterations were found in spleen, liver, thymus, kidney, or heart of inoculated hamsters. No macroscopic or histological changes were observed in any organ of the control group.

3.3. Immunohistochemistry

Positive immunohistochemical labeling was found in a group of neurons from a necrotic foci in the frontal pole region of hamsters inoculated with A4/72. The hamsters inoculated with Iso72/10 presented immunolabeling in neurons of the olfactory bulb and in groups of cells of the caudal diencephalon and rostral mesencephalon (Figure 3a, b).

The hamsters inoculated with A3/97 and AR4 variants showed positive immunolabeling only in the olfactory bulb region (internal granule cells) and in groups of neurons of the frontal pole cortex (Figure 3 c, d). The meninges and other areas of the CNS such as striated septum, pons, and medulla oblongata showed no positive labeling with any variant (Table 3). No immunoreactivity was observed in the organs of hamsters inoculated with A9/92 variant and from negative control group.
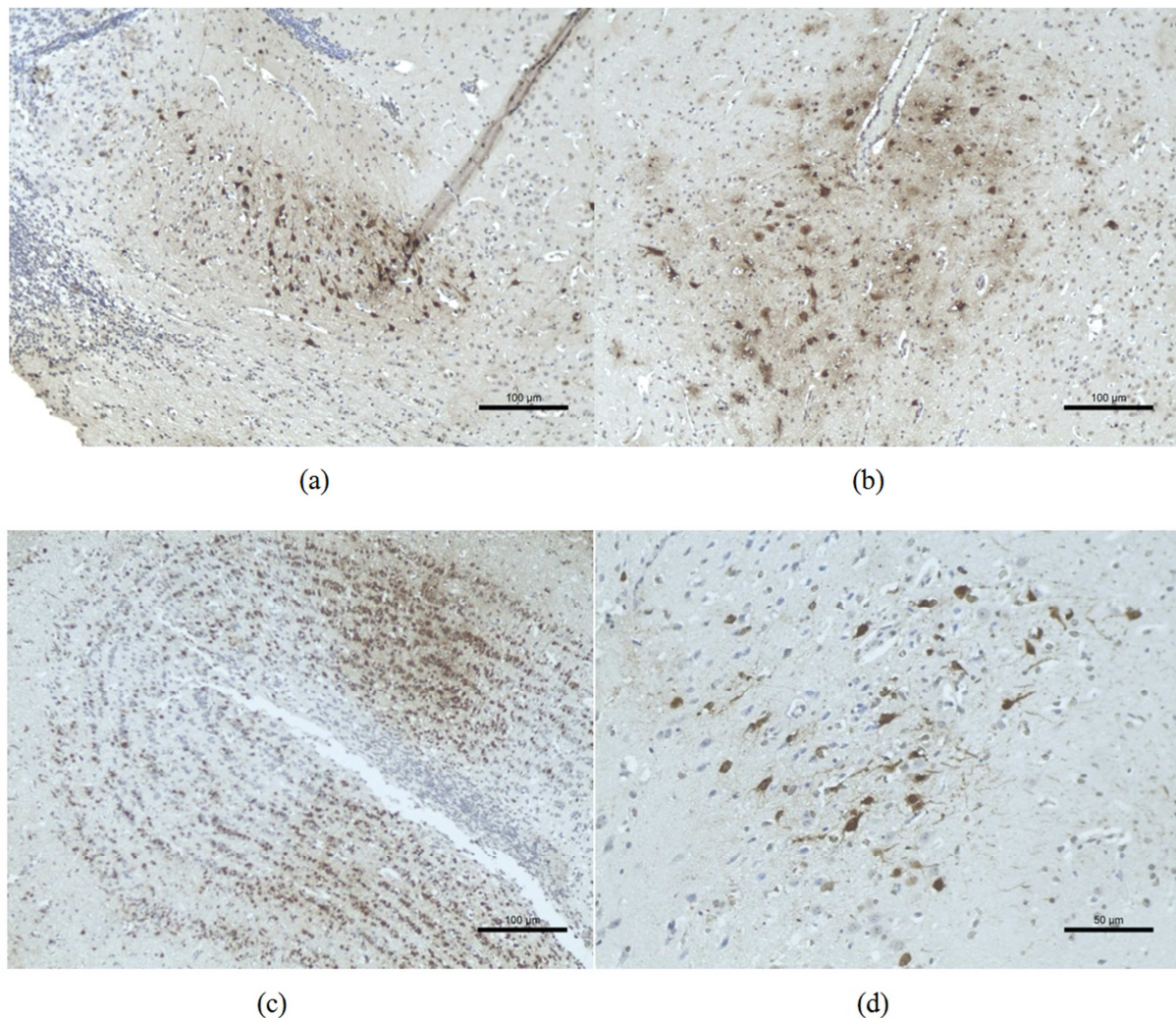

(a)

(b)

(c)

(d)

Figure 3. *Brain of hamsters intranasally infected with equid alphaherpesvirus type1 variants.(a) Iso72/10 variant; frontal cortex region with many neurons exhibiting strong cytoplasmic and nuclear immunolabeling for EHV-1 antigens (Bar 100µm). (b) Iso72/10 variant;rostral mesencephalon region presenting a group of neurons exhibiting strong cytoplasmic and nuclear immunolabeling for EHV-1 antigens (Bar 100µm). (c) A3/97 variant; olfactory bulb region with internal granule cells exhibiting strong cytoplasmic and nuclear immunolabeling for EHV-1 antigens (Bar 100µm). (d) A3/97 variant; frontal pole region with groups of neurons exhibiting strong cytoplasmic and nuclear immunolabeling for EHV-1 antigens (Bar 50µm). Immunohistochemistry for EHV-1.*

Table 3. Viral detection by immunohistochemistry in brain of hamsters intranasally inoculated with five EHV-1 variants.

Brain Region	Viral detection of EHV-1 by IHC				
	A9/92	A4/72	ISO72/10	A3/97	AR4
Meninges	-*	-	-	-	-
Olfactory bulb	-	-	+++	+++	+++
Frontal pole	-	+	+++	++	++
Striated septum	-	-	-	-	-
Caudal diencephalon	-	-	+	-	-
Rostral mesencephalon	-	-	++	-	-
Pons and medulla oblongata	-	-	-	-	-

*Presence of viral antigen: -absent; +few (<5/40xfield); ++moderate (5-30/40xfield); +++numerous (>30/40xfield).

4. Discussion

The neurological signs observed in hamsters inoculated with the EHV-1 variants tested were similar to those described after natural infection in the host equine species: lethargy, anorexia, and ataxia reaching peak intensity at 2-3 dpi [41-43]. Meningeal congestion was the only macroscopic alteration in brain of the inoculated hamsters, this alteration has also been observed in mice and horses as a result of EHM, associated with congestion and hemorrhagic foci in spinal cord [7, 8, 18].

The microscopic lesions were similar to those reported in hamsters infected with EHV-9 and in mice and hamsters inoculated with EHV-1, with the olfactory bulb and frontal cortex being the primary affected regions after intranasal inoculation [8, 16, 28, 29, 31, 32]. In hamster and murine models, the intranasally inoculated virus migrates from the nasal mucosa by neural dissemination via the olfactory neuroepithelium throughout the olfactory bulb, olfactory nerve, and ventricular surface, leading to neuronal degeneration, mainly in cortical areas and the hippocampus, with associated generalized ventriculitis [16, 18, 28, 30].

As reported by other authors, we also found variation in severity of clinical signs, according the pathogenicity of the virus variant [15, 30]. Variants A9/92 and A4/72 showed high neurovirulence, compared to the other tested variants, with results similar to those reported in mice after intranasal inoculation [18]; the Iso72/10 variant showed lighter neurological signs with acute phase starting only at 4 dpi and presented also respiratory signs while A3/97 and AR4 variants caused predominantly respiratory signs [32]. In a study using several line ages of mice, the A3/97 variant caused no weight loss or apparent clinical signs of disease, although it was possible to recover the virus from lung of all infected mice and from brain of a single BALBc nude mouse [18].

Hamsters infected with Iso/72, A3/97 and AR4 presented CNS lesions predominantly inflammatory (meningo-encephalitis) while hamsters inoculated with A4/72 and A9/92 showed predominantly neuronal necrosis with vacuolization of the neuroparenchyma, perineuronal and perivascular edema, with liquefaction necrosis in many areas of the CNS instead of inflammatory changes [32].

In adult horses, EHM induces microvascular damage to the central nervous system via initiation of an inflammatory cascade followed by degenerative thrombotic vasculitis of small blood vessels in the brain or spinal cord with resultant ischemic neuronal necrosis, perivascular mononuclear cuffing, congestion, hemorrhage, diffuse gliosis, perineuronal and perivascular edema [7, 12, 44]. In hamsters is suggested that these lesions may be the result of the viral replication within neurons, as opposed to vascular changes [32].

Effects of the less pathogenic EHV-1 variants have been reported to remain confined to the olfactory bulb region, while the more virulent variants migrates via neurons throughout the brain by anterograde axonal transport and slow axonal flow, infecting neurons and causing severe tissue damage throughout the CNS [16]. In contrast, in the present study, one less neuropathogenic variant (Iso72/10) were immunohistochemically detected even in distant regions from the olfactory bulb such as caudal diencephalon and rostral mesencephalon, while A4/72 one of the more virulent variant were detected only in a group of cells of necrotic area from the frontal pole region, so further studies are needed to better understand EHV-1 migration.

The route of inoculation seems to be an important factor in the development and progress of experimental herpes infection and migration. In a study of EHV-9, clinical signs of encephalitis including salivation, tremors, uncoordinated movement, convulsions, and severe moribund states were present in 100% of hamsters intranasally inoculated but in only 25% of those inoculated by oral and peritoneal routes [30]. On the other hand, noneurological signs were observed in hamsters inoculated via intravenous or ocular routs [30].

The time course of infection in hamsters seems also to be important in the migration of the herpes virus throughout the CNS. EHV-9 viral antigens were detected in the olfactory epithelium, nerve and bulb in the first 48 hours post inoculation, while in the cerebral cortex and areas such as those connecting the trigeminal sensory nerve root to the brain stem, pons and medulla oblongata, positive detection was found at 60h PI [29]. Recent studies in mice have shown the EHV-1 and 9 migration from the olfactory epithelium to the olfactory bulb on day 3 post inoculation, but without viral migration to other areas of the CNS, probably due to the higher susceptibility to infection of the hamster in comparison to mice [16, 45].

The dose of the inoculums and the type of anesthetic used must also be considered in pathogenicity and virulence studies. Intranasal inoculation of hamsters with EHV-9 at 10^1 PFUs was reported to not cause death, whereas with 10^4 PFUs, hamsters became severely ill at 3 dpi and died at 4 dpi [28]. In a study with mice intranasally inoculated with EHV-1 (AR4 variant) it was observed that clinical signs were more severe in

animals anesthetized with ether; ketamine/xylazine – anesthetized animals presented the highest cell death rates, while those anesthetized with isoflurane showed the highest proliferation rates, despite this isoflurane was considered a good anesthetic for experimental assays, since it produced few known side effects, animal suffer and showed a better post anesthesia recovery [46].

Studies have suggested that genotype alterations may be responsible for EHV-1 neuropathogenicity. EHV-1 variants with the exchange of adenine for guanine at position 2254 in ORF30 (A_{2254}–G_{2254}), leading to alteration in the aminoacid 752 sequence (from N_{752}–D_{752}), have been associated with the potential to cause neurological disease. This genotype alteration was associated with 30 of 32 outbreaks of neurological disease for EHV-1 in 2001-2006 in United Kingdom and United States [34, 47].

Prevalence of EHV-1 variants considered neuropathogenic (G_{2254}/D_{752}) increased from 3.6% in the 1960s to 13.3% in the 1990s in central Kentucky's thoroughbred broodmare population [19]. This number increased to 19.4% in 2000–2006, suggesting that viruses of the neuropathogenic genotype were increasing in prevalence in the latent reservoir, leading to greater risk of outbreak of equine herpesvirus neurological disease [19].

The amount of EHV-1 nasal shedding significantly increased in animals infected with the D_{752} variant compared with N_{752} variant (p=0.001); the D_{752} variant led, on average, to a four-fold higher amount of nasal EHV-1 shedding compared to infections with the N_{752} variant, suggesting that neuropathogenic variants could have a selective advantage and are systematically increasing in prevalence in domestic horse populations [48].

A experiment comparing the partial nucleotide sequences of ORF72 (glycoprotein D-gD), ORF64 (ICP4), and ORF30 (DNA polymerase) genes to corresponding sequences of EHV-1, found that the reference variant showed no molecular variation in the ICP4, gD, or viral DNA polymerase gene regions from the evaluated variants, in the experimental the EHV-1 Brazilian variants analyzed, including A3/97, A4/72, and A9/92, were classified as non-neuropathogenic variants (N_{752}) based on the ORF30 analysis, suggesting that other factors, such as the immuneresponse of the host species, could be involved in EHV-1 neuropathogenicity [49].

We found Brazilian (A4/72, A3/97, A9/92, Iso72/10) and Argentine (AR4) variants to demonstrate neurotropism and neurovirulence, and were capable of causing neurological disorders and acute brain lesions (cell death and diffuse encephalitis) of inoculated hamsters, despite their classification as non-neuropathogenic variants [40, 49]. Mice inoculated with an EHV-1 mutant variant (Ab4pΔORF37) in which ORF37 was deleted, did not show neurological symptoms, death and body weight loss indicating that ORF37 may be one of the neuropathogenicity factors of EHV-1 [37].

Recent studies have shown neuropathogenic variants 01c1 to exhibit growth kinetics similar to those of non-neuropathogenic variants in fetal horse kidney and cultured neurons and suggest that the D/N_{752} dissimilarity in ORF30 may not be related to replication ability in fetal horse kidney and neural cell lines [36].

In a study to evaluate the ability of neuropathogenic (EHV-1) and non-neuropathogenic (Jan-E and Rac-H) EHV-1 variants to infect neuronal cells, real-time PCR analysis demonstrated that both neuropathogenic and non-neuropathogenic EHV-1 variants replicated in primary murine neurons and ED cells at a similar level [50].

Analyzed together, these data reinforcing the hypothesis that factors other than only a specific genotype mutation can also be involved in the neuropathogenicity of EHV-1, such as innate response, glycoproteins (gI, gE) and different cellular immune markers, including alpha-interferon (IFN-α), gamma-interferon (IFN-γ), interleukin-10 (IL-10) and interleukin-1 beta (IL-1β) that were identified to play a role during the course of the disease in experimental animal models [19, 37, 50-55].

5. Conclusions

The five EHV-1 variants tested induced severe encephalitis in the hamster model; viral replication in neurons restrict predominantly to olfactory bulb and frontal cortex were observed in variants AR4 and A3/97, more related with respiratory clinical signs than neurological; while the variant Iso72/10, related to both neurological and respiratory signs presented migration in groups of cells of caudal diencephalon and rostral mesencephalon and the most neurovirulent strains A9/92 and A4/72 presented no labeling, suggesting that further studies, not staggered in days but in hours post inoculation, are needed to better understand the viral migration of these neurovirulent variants.

Acknowledgements

Conflicts of Interest

The authors declare no conflict of interest.

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