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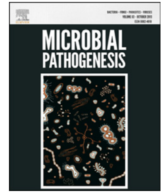

Respiratory and neurological disease in rabbits experimentally infected with equid herpesvirus 1

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ABSTRACT

Equid herpesvirus type 1 (EHV-1) is an important pathogen of horses worldwide, associated with respiratory, reproductive and/or neurological disease. A mouse model for EHV-1 infection has been established but fails to reproduce some important aspects of the viral pathogenesis. Then, we investigated the susceptibility of rabbits to EHV-1 aiming at proposing this species as an alternative model for EHV-1 infection. Weanling rabbits inoculated intranasal with EHV-1 Kentucky D (10^7 $TCID_{50}$/animal) shed virus in nasal secretions up to day 8–10 post-inoculation (pi), presented viremia up to day 14 pi and seroconverted to EHV-1 (virus neutralizing titers 4 to 64). Most rabbits (75%) developed respiratory disease, characterized by serous to hemorrhagic nasal discharge and mild to severe dyspnea. Some animals (20%) presented neurological signs as circling, bruxism and opisthotonus. Six animals died during acute disease (days 3–6); infectious virus and/or viral DNA were detected in the lungs, trigeminal ganglia (TG), olfactory bulbs (OBs) and cerebral cortex/brain (CC). Histological examination showed necrohemorrhagic, multifocal to coalescent bronchointerstitial pneumonia and diffuse alveolar edema. In two rabbits euthanized at day 50 pi, latent EHV-1 DNA was detected in the OBs. Dexamethasone administration at day 50 pi resulted in virus reactivation, demonstrated by virus shedding, viremia, clinical signs, and increase in VN titers and/or by detection of virus DNA in lungs, OBs, TGs and/or CC. These results demonstrate that rabbits are susceptible to EHV-1 infection and develop respiratory and neurological signs upon experimental inoculation. Thus, rabbits may be used to study selected aspects of EHV-1 biology and pathogenesis, extending and complementing the mouse model.

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1. Introduction

Equid herpesvirus type 1 (EHV-1) is an enveloped, double stranded DNA virus, member of the family *Herpesviridae*, subfamily *Alphaherpesvirinae* and genus *Varicellovirus*, closely related to EHV-4 [1,2]. EHV-1 is distributed worldwide and infection of susceptible horses often results in respiratory disease and, occasionally, in neurological signs and abortions [3]. Like other alphaherpesviruses, EHV-1 establishes lifelong latent infections mainly in trigeminal ganglia (TGs) and in lymphoid tissues [4,5]. Latency may be naturally reactivated, resulting in virus replication, shedding and transmission to susceptible animals [1,6].

EHV-1 infection and respiratory disease in young horses is often associated with important economic losses due to impair in athletic training. In addition, fetal losses caused by late-term abortions and outbreaks of neurological disease causing deaths and restrictions to animal movement contribute for the economic impact of EHV-1 infection [7]. Thus, the diverse manifestations of EHV-1 infection have an important impact in the equine industry.

During acute infection, EHV-1 generally penetrates by the respiratory route and replicates in the epithelium of the respiratory tract, spreading to regional lymph nodes and promoting cell-associated viremia [8,9]. Viremia is important for virus spread to

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target organs. In addition, infection of endothelial cells results in vasculitis, thrombosis and ischemic neuronal injury, often leading to neurological disorders and abortions [10,11].

Mouse models have been used to investigate different aspects of EHV-1 infection, including respiratory disease [12], latency and reactivation [13], neurological infection [13–15], reproductive failure [16] and the function of specific gene products in the viral pathogenesis [14]. These models, however, often fail to reproduce some important aspects of the infection and disease in horses. Thus, we investigated the susceptibility of rabbits to EHV-1 infection and whether rabbit infection would reproduce the main aspects of EHV-1 infection in horses.

2. Material and methods

New Zealand white rabbits (30 days-old), weighing approximately 400–500 g were used. The inoculated rabbits and controls were allocated in separated cages to avoid cross-contamination and were given food and water *ad libitum*. All procedures of animal handling and experimentation were performed under veterinary supervision and according to the recommendations of the Brazilian Committee on Animal Experimentation (COBEA, law # 6.638 of May, 8th, 1979). The experiments were approved by the Institutional Committee on Ethics and Animal Welfare and Experimentation (UFSM, Comitê de Ética e Experimentação Animal: process 047/2012).

The rabbits (n = 20) were inoculated intranasal (IN) into the paranasal sinuses [17] with EHV-1 Kentucky D (10^7 $TCID_{50}$/animal) or with RPMI (n = 5, mock-infected controls), after sedation with ketamine (50 mg/kg) and xylazine (5 mg/kg). The EHV-1 strain Kentucky D was kindly provided by Dr. Rodrigo Franco (Butantan Institute, São Paulo, SP, Brazil). After virus inoculation, rabbits were monitored for clinical signs, weight and body temperature on a daily basis during 14 days pi (dpi). Nasal swabs collected on a daily basis up to day 14 pi were submitted to three passages in Vero cells (*African Green Monkey)* for virus isolation. Cell cultures and virus growth were performed at 37 °C with CO_2 at 5%. Peripheral blood obtained daily from the experimental animals was pooled and submitted to DNA extraction for PCR using phenol-chloroform protocol. Animals euthanized or dying during acute infection were submitted to necropsy. Fragments of lungs, cerebral cortex (CC), olfactory bulbs (OB), trigeminal ganglia (TG) and submandibular lymph nodes (SLN) were submitted to virus isolation in Vero cells, PCR for viral DNA and histopathology. Tissue fragments were homogenized in MEM (10% w/v) and submitted to virus isolation as described above.

At day 50 pi, two inoculated and one control rabbits were submitted to euthanasia for tissue collection. At necropsy, OBs, CC, TGs and SLNs were collected and, after cleaning and processing, the tissues were submitted to virus isolation and DNA extraction for PCR. The remaining animals (n = 11) were submitted to Dexamethasone (Dx) administration (Dx, 4 mg/kg/day × 5 days, Azium, Coopers Brazil Ltda) and monitored subsequently. Fifteen days later (day 15 pDx) these rabbits were submitted to euthanasia for tissue collection. Serum samples collected at the day of virus inoculation, at 50 dpi and 65 dpi (15 dpDx) were submitted to virus neutralization (VN) assay for EHV-1 antibodies, testing two-fold dilutions of sera against a fixed dose of virus (100–200 $TCID_{50}$/well). VN readings were performed after 96 h. VN titers were considered as the reciprocal of highest dilution of sera that prevented the production of cytopathic effect in Vero cells. Serum from a horse naturally infected with EHV-1 and from an EHV seronegative horse was used as positive and negative controls, respectively.

The presence of virus or viral DNA in neural and non-neural tissues (SLNs, blood) was determined by PCR. For this, total DNA (approximately 200 ng) of each sample was submitted to PCR to amplify a sequence of EHV-1 glycoprotein B (gB) gene. The primers utilized to amplify a product of 311bp were: forward 5′- TAC CTG AAC GAG CTT GTG -3′ and reverse 5′- TTG ACG TGG GAT TGG ATG -3’. PCR reactions were performed in a 25 µl volume, using 2 µl of template DNA, 12.5 µM of each primer, 2.5 mM $MgCl_2$, 10 mM of dNTPs, 1 × reaction buffer and 1 unit of Taq polymerase (Life Technologies®). PCR conditions were: initial denaturation (95 °C for 10 min), followed by 30 cycles of 95 °C–60s; 50 °C–60s for primer annealing and 72 °C–60s for primer extension; and a final extension of 7 min at 72 °C. Products were visualized in an 1.5% agar gel, stained with Gel Red (Biotium®) and visualized under UV light. In all reactions, DNA extracted from Vero cells infected with EHV-1 Kentucky D was used as positive control; sterilized ultrapure water was used as negative control.

Histological analysis was performed in sections of brains and lungs collected at necropsy. Tissue fragments were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin (H&E) and submitted to microscopic examination.

3. Results and discussion

Fifteen out of twenty rabbits (75%) inoculated with EHV-1 Kentucky D developed respiratory disease following virus inoculation. The disease was characterized by nasal discharge (serous to hemorrhagic) and mild to severe dyspnea. Additionally, four animals also developed neurological signs, presenting bruxism, circling and opisthotonus. The sick animals developed severe apathy and six died during the acute phase, between days 3 and 6 pi (Table 1). One rabbit (#10) died at 25 dpi of unknown causes. The onset of clinical signs occurred around days 2–4 pi but some rabbits became sick by days 5–7 pi (Table 1). The rabbits that survived acute infection generally recovered by days 10–13 dpi, but three animals recovered faster (around days 6–8 pi).

Early after virus inoculation (days 1 and 2 pi), inoculated animals had fever (around 41.5 °C) and near to death the animals usually developed hypothermia (approximately 34 °C). The body temperature remained stable therefore (not shown). No weight loss was recorded among inoculated animals (not shown).

Virus shedding in nasal secretions was first detected at days 1–2 pi and, thereafter, was detected in alternate days until day 10 pi. Viremia was detected in pooled blood of inoculated rabbits up to day 14 dpi (Table 1). No virus shedding or viremia were detected in mock-infected animals (negative controls). Lungs, brain (cortex) and TG of rabbits that died during the acute phase were positive for viral DNA by PCR and/or in virus isolation (Table 1).

Gross examination of lungs of rabbits that died during acute infection revealed marked pulmonary changes, characterized by multifocal hemorrhagic areas and interstitial pneumonia (Fig. 1B). Severe proliferative necrohaemorrhagic neutrophilic and lymphoplasmacytic broncointersticial pneumonia with thrombus, vasculitis and diffuse alveolar edema were observed at histopathology (Fig. 1D). Furthermore, one rabbit had mild lymphocytic inflammatory infiltrate in the meninges of the frontal lobe of the brain.

Tissues (OBs, CC, TG, lungs and SLN) from rabbits (#3 and #9) euthanized at day 50 pi were negative in virus isolation after three passages in culture cells. Nonetheless, the BOs of both rabbits were positive for EHV-1 DNA by PCR, indicating latent infection. In the animals submitted to Dx administration (n = 11), virus shedding was detected in four animals (4/11); six rabbits presented viremia (days 5–14 dpDx) and presented clinical signs (respiratory distress, neurological signs) in the days following Dx administration (Table 2). In five rabbits (#1, #4, #6, #7 and #8), no virus shedding, viremia or clinical signs were observed following Dx treatment.

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