ABSTRACT

**Background:** Bovine encephalitis herpesvirus, or bovine herpesvirus type 5 (BoHV-5), a member of the family Herpesviridae, subfamily *Alphaherpesvirinae*, is long recognized as the causative agent of bovine herpesvirus encephalitis. The disease caused by BoHV-5 is characterized by signs of nervous impairment, consequent to non-suppurative meningoencephalitis. Although bovine herpetic encephalitis is a rare event in herds from the Northern Hemisphere, BoHV-5 infections are an important cause of central nervous system disease in cattle in Brazil and Argentina. Recovery of animals from clinical illness has been documented before, both in naturally infected animals and experimentally infected individuals.

**Case:** During an experiment of experimental inoculation of a virulent isolate of BoHV-5, clinical signs of neurological disease were detected in three out of four calves experimentally inoculated with bovine encephalitis herpesvirus (bovine herpesvirus type 5; BoHV-5; strain EVI88/95). Clinical signs varied from slight prostration (1 calf) to severe signs of nervous impairment which lasted from 3 to 14 days (in 2 calves) from the beginning of clinical signs to death. Despite the neurological signs, one of the calves with mild clinical signs recovered: on day 14 p.i, this animal showed apathy, bruxism, dysphagia, pressing of the head against the walls of the bay, hyper salivation, tongue paralysis, hypermetria, and transient blindness but the signs become gradually milder and the health status of the animal improved until day 21 p.i. Recovery was complete ten days after the development of clinical signs and no evident sequelae were noticed up to day 180 when the remaining calves were culled. At necropsy, the prominent macroscopical finding was a large atrophic area at the left frontal lobe of the cortex. Another atrophic area was evident on the parietal lobe, at the level of the mamillary bodies, revealing a yellow-orange cystic area on the basis of cerebrum, involving the nucleus caudatus and the putamen. At histopathology, large areas on the affected frontal cortex were infiltrated by macrophages containing haemosiderin and some plasma cells BoHV-5 infection was confirmed by viral isolation from nasal and ocular swabs during acute infection from day 1 p.i. until day 19 p.i., with titres up to $10^4$ TCID 50/50 µL, form all inoculated animals. BoHV-5 was also isolated from many organs, especially from the brain, of the animals which died on the acute phase of infection; however, no infectious virus could be recovered from tissues collected at necropsy from the calf at 180 p.i.

**Discussion:** These findings suggest the possible association of BoHV-5 with atrophic lesions in the brain, a finding which had not been previously linked to BoHV-5 infections. Therefore, animals that recover from clinically evident BoHV-5 infection under field conditions may also bear brain lesions that would remain undetected if the calf is not culled; yet these may be detected only months or years later, at slaughter. These results are of interest for the South American countries where infections with BoHV-5 are highly prevalent.

**Keywords:** BoHV-5, bovine encephalitis herpesvirus, cortical necrosis, atrophic brain lesions.
INTRODUCTION

Bovine encephalitis herpesvirus, or bovine herpesvirus type 5 (BoHV-5), a member of the family Herpesviridae, subfamily Alphaherpesvirinae, is long recognized as the causative agent of bovine herpesvirus encephalitis [1,12]. Clinical disease associated to BoHV-5 is characterized by signs of nervous impairment, consequent to non-suppurative meningoencephalitis [7]. The disease usually affects calves up to 2 years old, although occasionally older animals may be involved [9]. Mortality rates usually approach 100% [5], but some cattle may recover [9]. BoHV-5 disease can be easily misdiagnosed as rabies, botulism, polioencephalomalacia, NaCl intoxication and other causes of encephalitis [9].

CASE REPORT

Four 3 to 5 months old calves of mixed European breeds, previously tested to ensure that they had not been exposed to BoHV-1, BoHV-5 or bovine viral diarrhoea virus (BVDV) were kept in isolation units. The calves were infected intranasally with 10^6.50 fifty percent tissue culture infective doses (TCID50) of BoHV-5 strain EVI 88/95. Two other calves were inoculated with sterile cotton swabs deeply introduced into each nostril or conjunctival sac, daily, until day 21 p.i.. After sampling, swabs were dipped in 2 mL of MEM supplemented with 100 mg/mL enrofloxacin and 2.5 mg/mL Amphotericin B (MEM AB 10X) and stored at -70°C for subsequent processing. Inoculation of cell cultures was performed with 10^-1 to 10^-4 dilutions of the initial suspension. Newly formed monolayers of CRIB-1 and MDBK cells were inoculated with 200 mL of inoculum and incubated at 37°C in a 5% CO2 atmosphere. Solid tissues (spleen, liver, adrenal glands, thymus, trachea, lung, trigeminal ganglia, and various lymph nodes), brain specimens (frontal cortex, pons, cerebellum, hippocampus, medulla, and cortex) and cerebrospinal fluid (CSF) were collected from the two necropsied calves. Suspensions of approximately 10% of tissues were prepared in MEM AB 10X, clarified by centrifugation and the supernatant inoculated onto CRIB-1 monolayers. After 1 h for adsorption at 37°C, the inoculum was removed, cells washed 3 times with MEM AB 10X and overlaid with the same medium. CSF was directly inoculated onto cell cultures, in volumes of 100 mL per well in 6 well culture dishes. Cells were monitored daily for cytopathic effect (CPE). The virus recovered was confirmed as BoHV-5 by an immunoperoxidase assay with BoHV-5-specific monoclonal antibodies.

Tissue samples were collected from all organs listed above. Calves were necropsied soon after death or culling. Samples were fixed in 10% buffered formalin, processed and stained with haematoxylin-eosin (HE) following routine procedures.

Fixed tissue sections were deparaffinized in xylol at 60°C for 10 to 30 min and washed twice in basic Tris buffer (BTB, 1M Tris Buffer Solution, pH 9.5). Endogenous peroxidase activity was blocked with 3% H2O2 in methanol for 30 min, followed by washing in BTB. Subsequently, the slides were incubated with 0.1% trypsin for 30 min. Sections were then incubated with 10% normal rabbit serum for ten minutes and then incubated with the primary antibody (anti-BoHV-5 monoclonal antibodies). After three new washes in BTB, sections were overlaid with anti-mouse IgG/peroxidase conjugate for 10 min at room temperature and again washed three
times in BTB. Subsequently, the substrate 3-amino-9-ethyl carbazole with 0.03% \( \text{H}_2\text{O}_2 \) was added. The reaction was stopped after 15 min at room temperature, by another wash with BTB and the slide counterstained with haematoxylin. After a final wash in running water, the slides were mounted and examined at the microscope. Sections were also stained with a commercially available avidin-biotin complex-peroxidase kit following the manufacturer’s instructions.

Three of the four BoHV-5 infected calves presented two febrile peaks on days 2 (40.1°C) and 4 (40.8°C) p.i. Two of them died presenting a set of severe clinical signs of nervous impairment (Table 1). On day 14 p.i, one calf showed apathy, bruxism, dysphagia, pressing of the head against the walls of the bay, hyper salivation, tongue paralysis, hypermetria, and transient blindness but the signs become gradually milder and the health status of the animal improved until day 21 p.i.

Recovery was complete ten days after the development of clinical signs and no evident sequels were noticed up to day 180 when the remaining calves were culled. At necropsy, the prominent macroscopic finding was a large atrophic area at the left frontal lobe of the cortex (Figure 1). Another atrophic area was evident on

(a)

(b)

Figure 1. a) Large atrophic lesions at the left cortical frontal lobe. b) Close up of the same lesion, detailing the pronounced meningeal sequestration.

<p>| Table 1. Clinical signs observed in calves experimentally infected with Bovine herpesvirus type 5. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|</p>
<table>
<thead>
<tr>
<th><strong>A</strong></th>
<th><strong>B</strong></th>
<th><strong>C</strong></th>
<th><strong>D</strong></th>
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<tbody>
<tr>
<td><strong>De-ath</strong></td>
<td>Recovered</td>
<td>Not observed</td>
<td>Yes</td>
</tr>
<tr>
<td><strong>Fever</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Anorexia</strong></td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>Mucous-sanguinolent nasal secretion</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td><strong>Dyspnoea</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
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<tr>
<td><strong>Hyper-metria</strong></td>
<td>++</td>
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<td>++</td>
</tr>
<tr>
<td><strong>Hyper-reflexia</strong></td>
<td>++</td>
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<td>++</td>
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<tr>
<td><strong>Incoordination</strong></td>
<td>++</td>
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<tr>
<td><strong>Walk in circles</strong></td>
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<td>++</td>
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<tr>
<td><strong>Transient Blindness</strong></td>
<td>++</td>
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Signals of nervous impairment: (•) unavailable; (+) mild; (++) moderate; (+++) severe.
the parietal lobe, at the level of the mammillary bodies, revealing a yellow-orange cystic area on the basis of cerebrum, involving the nucleus caudatus and the putamen (Figure 2). At histopathology, large areas on the affected frontal cortex were infiltrated by macrophages containing haemosiderin and some plasma cells (Figure 3). Small foci of malacia in the trigeminal ganglia were also evident. No other alterations were evident at necropsy.

The development of BoHV-5 neutralizing antibodies detected in the recovered calf is shown in Figure 4.

BoHV-5 infection was confirmed by viral isolation from nasal and ocular swabs during acute infection from day 1 p.i. to day 19 p.i., with titres up to $10^4.5$ TCID50/50 mL, form all inoculated animals. BoHV-5 was also isolated from many organs, especially from the brain, of the animals which died on the acute phase of infection; however, no infectious virus could be recovered from tissues collected at necropsy from the calves killed 180 p.i., even in the case of the recovered calf and no viral antigens were found during immunohistochemical assays.

No virus could be recovered from the control calves throughout the experiment.

**DISCUSSION**

Recovery of animals from clinical illness has been documented before both in naturally infected animals and experimentally infected calves [9]. Here, it was observed that the recovered animal showed no clinically visible sequelae. Most likely, after a natural infection such calves would recover and any possible brain lesions would only be detected at necropsy, being most likely never undetected if the calf is not culled during the acute phase of infection; yet these may be detected only months after the period of clinically acute disease. However, at necropsy, the massive atrophic lesion observed in the brain was the most striking finding. To the knowledge of the authors, no similar lesions had been previously associated to herpesvirus infections in cattle. Herpesviruses have been associated to malacia in other hosts, such as pseudorabies (Aujeszky’s disease) virus in swine[1]. Herpesviruses have also been considered as possible causes for large atrophic lesions occasionally found in human brains, associated with previous necrotic temporobasal herpetic encephalitis [13]. In addition, areas of malacia in trigeminal ganglia are commonly found in cases of BoHV-5 infection, resembling the zosteric neuritis in humans, as well as the ganglioneuritis associated to pseudorabies or Aujeszky’s disease. Therefore, these atrophic lesions are probably originated from the initial malacia associated to a consequent vascular ischemia.

It has been demonstrated here that the BoHV-5 strain EVI 88/95 is capable of inducing clinically apparent encephalitis in experimentally infected calves. The cortical necrosis showed to be a consistent and reproducible finding on affected calves, although other regions within the brain also occasionally presented malacia. During field outbreaks, BoHV-5 disease has been often lethal [2,4,5,8]. However, the number of infected animals without clinically noticeable disease – like for many other herpesvirus infections – is likely to be much higher.

Evidence was also provided here to show that BoHV-5 may be associated to the development of atrophic lesions in the brain of infected calves. Thus, surviving calves may bear brain lesions that would remain undetected if the calf is not culled during the acute phase of the infection; yet these may be detected only months
Figure 2. Atrophic cystic area involving the nucleus caudatus and putamen, with a yellow-orange color and a cutting surface resembling foci of malacia (arrow).

Figure 3. Collapsed meningeal area with many haemosiderin-containing macrophages (arrow). Haematoxylin-eosin staining (Bar = 30 µm).

Figure 4. Virological and serological findings for the calf which recovered (a) Nasal viral excretion during the acute phase of infection. Infectious titres expressed in $\log_{10} \text{TCID}_{50}/50 \mu l$. (b) Kinetics of anti-BoHV-5 specific antibody titers until the day of euthanasia. Antibody (Ab) titres expressed as $\log_2$ of the reciprocal of the neutralizing antibody titre.
or years later, at slaughter. These results are of interest for those countries where infections with BoHV-5 strains are frequent, like Brazil and Argentina.

SOURCES AND MANUFACTURERS
1 FCS, Cultilab, Campinas, SP, Brazil
2 Dako, Glostrup, Denmark.
3 AEC, Sigma, MO, USA
4 ABC kit, Vector Laboratories, Burlingame, CA, USA.

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REFERENCES