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NOTE

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A clinical case of neosporosis in a 4-week-old holstein friesian calf which developed hindlimb paresis postnatally

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ABSTRACT. A 4-week-old female Holstein Friesian calf presented with hindlimb paresis. Neurologic examination of spinal reflexes revealed depressed or absent reflexes of the hindlimbs. Menace responses on both sides disappeared on examination of cranial nerves. The calf was finally diagnosed with *Neospora caninum* infection by pathological findings including nonsuppurative inflammation associated with cysts in the cerebrum and spinal cord. High levels of antibody against recombinant surface antigen 1 of *N. caninum* (NcSAG1) were detected by ELISA from both serum and cerebrospinal fluid (CSF) samples. This result suggests that detection of antibodies against *N. caninum* by NcSAG1-ELISA in serum and CSF could be useful for the clinical diagnosis of neosporosis in calves with acquired neurological signs.

KEY WORDS: astasia, calf, NcSAG1-ELISA, *Neospora caninum*

Neosporosis is caused by the protozoan parasite *Neospora caninum* [10]. Common symptoms of neosporosis in cattle are abortion and stillbirth [2–4]. Congenital infection with limb paresis or dysfunction at birth as a result of encephalomyelitis may occasionally occur [10]. Neurological symptoms may also occur several weeks after birth [2–4, 10]. In such cases, clinical diagnosis of neosporosis is difficult due to clinical signs that are similar to acquired neurological diseases such as bacterial infection or injury, and the limitations of diagnostic methods in cattle. Here we report a case of neosporosis in a 4-week-old Holstein Friesian calf that acquired hindlimb paresis, which was confirmed by histological examination and immunohistochemistry. We also report on the utility of detecting *Neospora*-specific antibodies in the diagnosis of neosporosis in calves.

A 33-day-old female Holstein Friesian calf presented with self-standing difficulty for a few days at a local veterinarian. On day 1, the calf was suspected to have spinal cord injury and was treated with dexamethasone and thiamine, but the clinical signs did not improve. On day 2, the calf was sent to the Animal Medical Center at Obihiro University of Agriculture and Veterinary Medicine for a diagnostic work-up. At that time, body temperature, heart rate, and respiratory rate were 39.0°C, 117/min, and 92/min, respectively. The calf was able to drink milk, stand up by itself, and maintain the standing position with a wide-based stance. As soon as the calf attempted to walk, however, it fell down from the right hindlimb and adopted a dog-sitting posture (Fig. 1). Neurologic examination of spinal reflexes revealed normal forelimbs and depressed or absent cranial tibial muscle and gastrocnemius muscle reflexes, as well as patellar and withdrawal reflexes in both hindlimbs. Menace responses were lacking bilaterally. Cerebrospinal fluid (CSF) was colorless and transparent and contained no cell components. Hematological examination showed no abnormalities. Although the general condition of the calf was stable, it could not stand on its own from day 3.

On day 9, the calf was euthanized for necropsy, which revealed a segment from the seventh cervical cord to the first sacral cord with multifocal color change. The segment was brown and transparent (Fig. 2). Tissue samples were collected and fixed in 15% neutral-buffered formalin. Fixed samples were trimmed, embedded in paraffin, and cut into sections. Selected sections were subjected to immunohistochemical staining using polyclonal goat anti-*N. caninum* antibody (1:2,000; VMRD, Pullman, WA, U.S.A.) and a simple stain MAX-PO polymer reagent (Nichirei Bio-science, Tokyo, Japan). As a positive control, a pancreas section of a mouse experimentally infected with *N. caninum* was used. As a negative control, a brain section of a calf without neurologic disease was used. Histologically, mild or moderate perivascular cuffing was scattered throughout the central nervous system. Multifocal aggregation of mononuclear cells with necrosis was observed in the cerebrum and spinal cord (Fig. 3). Small

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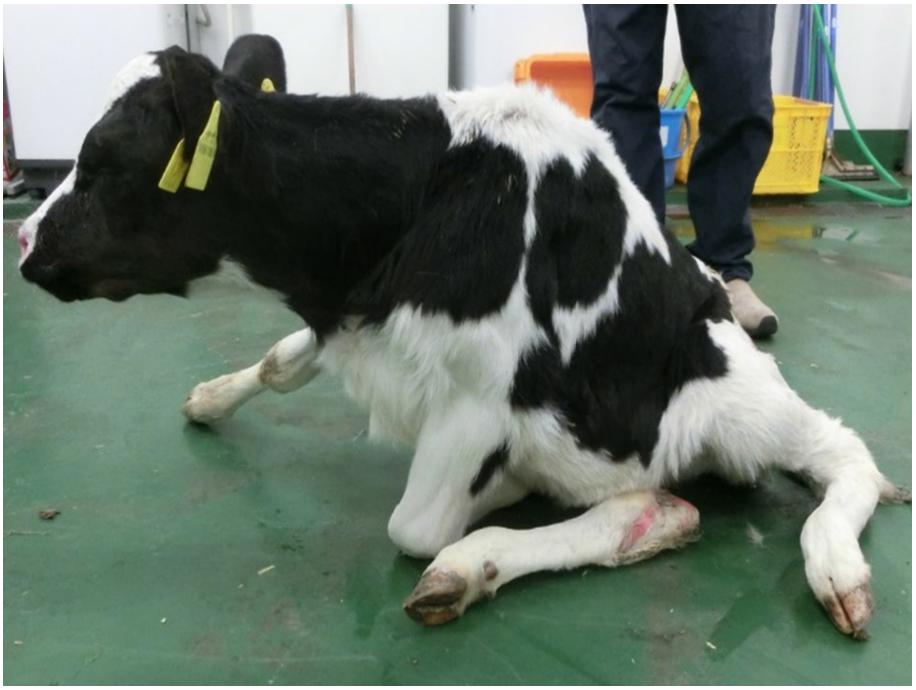

Fig. 1. As soon as the calf attempted to walk, it fell down from the right hindlimb and adopted a dog-sitting posture (Day 2).

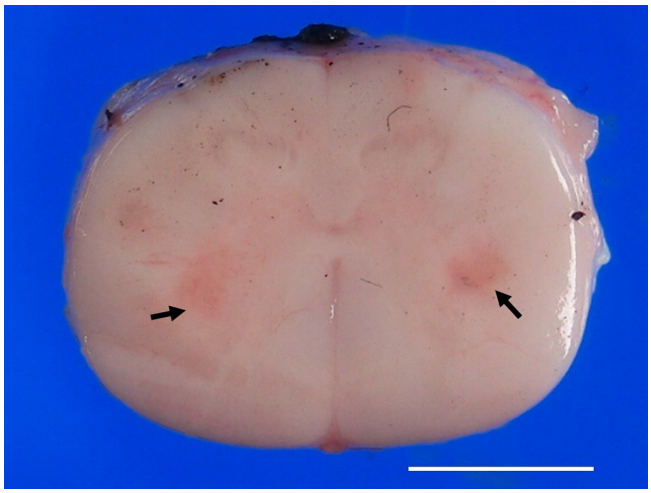

Fig. 2. Changes in color (arrows) were observed in a section of the spinal cord between C7 and T1. Bar=5 mm.

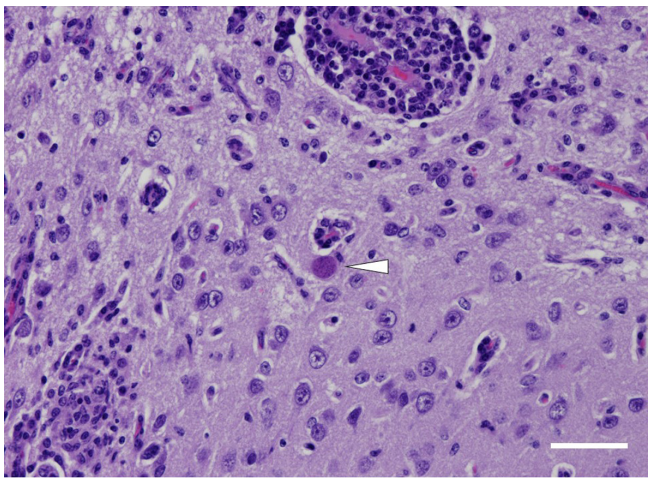

Fig. 3. A cyst (arrowhead) and perivascular cuffing were observed in the left frontal lobe of the cerebrum by H&E staining. Bar=50 μm.

basophilic protozoan cysts with distinct walls were detected in and/or around the multifocal lesion. These cysts stained strongly with anti-*N. caninum* antibody by immunohistochemistry. Based on these findings, the calf was diagnosed with neosporosis.

Serum and CSF collected from the calf on day 2 were tested for antibodies against *N. caninum* with an enzyme-linked immunosorbent assay (ELISA) on the basis of recombinant surface antigen 1 of *N. caninum* (NcSAG1) [7]. Recombinant NcSAG1 (rNcSAG1) was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein and purified using Glutathione Sepharose 4B (Amersham Pharmacia Biotech, Sweden) as described previously [1, 6]. Fifty microliters of purified rNcSAG1 (55 kDa) and control GST (25 kDa) at a final concentration of 0.1 μM (rNcSAG1: 5.5 μg/m*l*, GST: 2.5 μg/m*l*) were coated onto ELISA plates (Nunc, Denmark) overnight at 4°C in a carbonate–bicarbonate buffer (pH 9.6). Plates were washed once with PBS containing 0.05% Tween20 (PBS-T), and blocked with PBS containing 3% skim milk (PBS-SM) for 1 hr at 37°C. Plates were then washed once with PBS-T, and 50 μ*l* of serum samples diluted at 1:250 with PBS-SM were added to duplicate wells. Plates were incubated at 37°C for 1 hr. After six washes with PBS-T, plates were incubated with horseradish peroxidase (HRP)-conjugated anti-bovine total IgG (Bethyl

Laboratories, Montgomery, TX, U.S.A.) diluted at 1:10,000 with PBS-SM at 37°C for 1 hr. Plates were further washed six times, and substrate solution (0.1 M citric acid, 0.2 M sodium phosphate, 0.003% H_2O_2, and 0.3 mg/m*l* 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid); Sigma–Aldrich, St. Louis, MO, U.S.A.) was added to each well in 100 μl aliquots. Absorbance was read at 415 nm after 1 hr of incubation at room temperature using an ELISA reader (Corona Microplate Reader MTP-120; Corona, Tokyo, Japan). Absorbance values were determined as the difference in mean optical density at 415 nm (OD415 nm) between the recombinant antigen (rNcSAG1) and GST protein. As negative controls, serum samples were obtained from 20 healthy calves (1-week to 2-months of age, 16 males and 4 females). Nine CSF samples were also collected from 9 other healthy calves (1-week to 1-month of age, 8 males and 1 female). Mean OD_{415nm} values ± standard deviation of serum and CSF from the calf were 1.669 and 1.006, respectively, while those from negative controls were 0.014 ± 0.010 and 0.001 ± 0.004, respectively (Fig. 4).

Antibodies against *N. caninum* in serum were evaluated with a commercial indirect immunofluorescence assay (IFA) kit (*Neospora caninum* FA Substrate Slide; VMRD, Pullman). The result was positive with a titer of 1:1,280. However, antibodies in CSF could not be evaluated due to an insufficient amount of sample. An attempt to detect DNA fragments of internal transcribed spacer 1 (ITS1) of *N. caninum* by polymerase chain reaction (PCR) from paraffin-embedded brain tissue of the calf [13] failed.

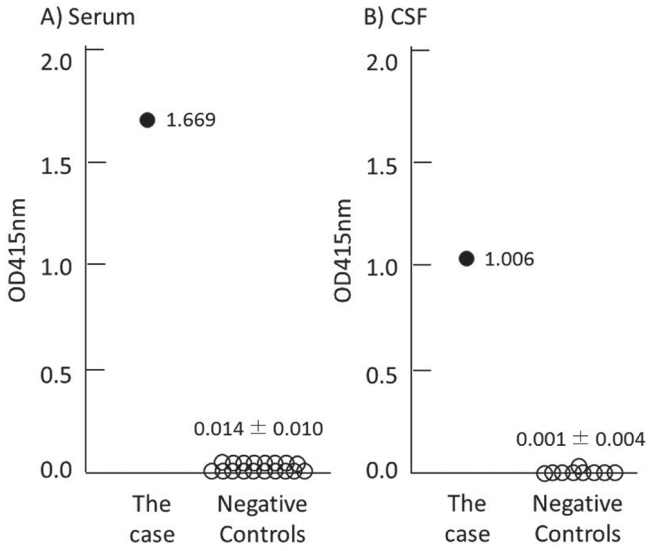

Fig. 4. OD_{415nm} values of NcSAG1-ELISA for serum (A) and CSF (B) in the present case and negative controls. The present case had higher *N. caninum* antibody levels in serum and CSF compared to those of healthy control calves. Mean OD_{415nm} values ± standard deviation of negative controls for serum (N=20) and CSF (N=9) were 0.014 ± 0.010 and 0.001 ± 0.004, respectively.

N. caninum causes abortion in both dairy and beef cattle [2–4]. Most neosporosis-induced abortions occur at 5 to 6-months gestation [2–4, 10]. In such cases, fetuses may die in utero, be resorbed, mummified, autolyzed, or stillborn [2–4]. However, most calves from dams chronically-infected with *N. caninum* are born alive with or without clinical signs [2–4, 10]. Clinical signs of neosporosis in such calves include being underweight, weakness, or neurological signs such as hyperextended hindlimbs and/or forelimbs [2–4, 10]. These clinical signs have only been reported in calves younger than 2 months of age [2, 4]. A pathologically confirmed case of neosporosis was reported in a Hereford calf which was born clinically normal, but showed weakness and pica at 2 weeks of age and acquired paralysis at 4 weeks of age [5]. Therefore, congenital neosporosis should be considered in the differential diagnosis of calves with acquired neurological signs.

In the present case, the lumbosacral cord was suspected as the main lesion at the first step of diagnosis due to the history and clinical signs of acquired hindlimb paresis at 4 weeks of age and depressed reflexes of both hindlimbs. Degenerative or traumatic lesions were suspected from the history and laboratory findings. However, the definitive diagnosis was confirmed by histological examination and immunohistochemistry as nonsuppurative encephalomyelitis caused by *N. caninum* infection. Attempts at detecting *N. caninum* DNA in the brain by PCR failed, possibly due to degeneration of DNA in the sample or the inability to obtain parasites from the paraffin-embedded sample, which corresponded only to a portion of the brain.

The calf had higher *N. caninum* antibody levels in both serum and CSF compared to those from healthy control calves. In the present study, NcSAG1 was used as the antigen for ELISA. NcSAG1 is an antigen expressed in the tachyzoite and its expression is down-regulated during the tachyzoite-to-bradyzoite conversion stage [15]. It is reportedly a useful antigen for detecting both acute and chronic *N. caninum* infection [7, 14]. The high antibody levels found in the present case suggest that detection of *N. caninum*-specific antibodies could be useful for diagnosing bovine *N. caninum* infection. In small animals, a presumptive diagnosis of neosporosis can be made by combining appropriate clinical signs of disease and positive serology or presence of antibodies in CSF [9, 11]. IgG antibody titers of at least 1:200 have been detected in most dogs with clinical neosporosis with the immunofluorescent assay test [9, 11]. In cattle, examination of serum from an aborting cow is performed to determine exposure to *N. caninum*. According to previous studies, a clinically healthy calf infected with *N. caninum* had high *N. caninum* antibody levels at birth and the high levels were maintained for approximately 2 months [8, 16]. Moreover, antibodies against *N. caninum* from a congenitally infected calf without clinical signs were reported to increase dramatically and peak within 1–2 months after calving [12]. However, there is currently insufficient data to determine appropriate cut-off levels for serological tests meant to diagnose neosporosis in calves with neurological signs [3].

The present findings highlight the utility of detecting antibodies against *N. caninum* with NcSAG1-ELISA for both serum and CSF samples in the clinical diagnosis of neosporosis in calves with acquired neurological signs. More cases should be examined to analyze the specificity, sensitivity, and clinical significance of NcSAG1-ELISA for diagnosing neosporosis.

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