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Tissue Distribution of *Neospora caninum* in Experimentally Infected Cattle

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Histopathology and quantitative PCR (qPCR) were used to determine the tissue distribution of *Neospora caninum* in calves at 80 days postinfection. Our findings revealed that the most appropriate brain areas for researching *N. caninum* pathogenesis were the amygdala and hippocampus for qPCR and the corpus striatum and diencephalon for histopathology.

Neospora caninum is an apicomplexan parasite and a major cause of abortion in cattle (1). Infection with *N. caninum* causes severe economic losses through fetal death and lower milk production (2, 3); therefore, it is desirable to develop a safe and effective vaccine against bovine neosporosis. The efficacy of vaccine candidates against *N. caninum* is evaluated by various approaches; these include analysis of antibody production, clinical signs, gamma interferon (IFN-γ) production, mortality, and pathology in animal models (4). Although qualitative or quantitative analysis of parasites in tissues is often performed by PCR (5–8) or quantitative PCR (qPCR) (9–11), little is known about the tissue distribution of *N. caninum*. The aim of our study was to determine the distribution of *N. caninum* in experimentally infected cattle and to identify the most suitable organs and tissues for the evaluation *N. caninum* infection intensity.

Male Holstein calves ($n = 8$), ages 2 to 4 months, were assessed to investigate tissue distribution of *N. caninum*. Animals were seronegative for *N. caninum* antibodies. The systemic distribution of *N. caninum* was investigated in calves 1 to 4. The distribution of *N. caninum* in the brain was conducted for calves 5 to 8. Animals were intravenously inoculated with 5×10^7 (calves 1 and 3) or 1×10^7 (calves 2 and 4 to 8) tachyzoites of the *N. caninum* Nc-1 isolate. Animals were euthanized at 77 days postinoculation (dpi) (calves 1 to 4) or 85 dpi (calves 5 to 8). For qPCR assays, liver, spleen, kidney, heart, lung, adrenal gland, thyroid gland, pancreas, thymus, tongue, parotid gland, mandibular salivary gland, skeletal muscle, brachial and sciatic plexus, sympathetic trunk, cerebrum, cerebellum, spinal cord, eye, optic nerve, pituitary gland, gastrointestinal tract, and lymph nodes were collected. Brain and spinal cord were collected for use in histopathological examination. For further analysis of brain distribution, the prefrontal cortex, caudate putamen, amygdala, hippocampus, hypothalamus, periaqueductal gray, pons, and medulla oblongata were collected from calves 5 to 8 and subjected to qPCR analysis. Samples of liver, kidney, spleen, thymus, skeletal muscle, mesenteric lymph node, cerebrum, cerebellum, and spinal cord from cattle that were seronegative for *N. caninum* were used as negative controls in the qPCR assays. DNA was extracted from 1 g of tissue using a DNeasy Blood & Tissue kit (Qiagen, Santa Clarita, CA). The DNA concentration was adjusted to 50 ng/μl for each sample, and 50 ng of DNA was used as a template. The qPCR assays specifically targeted parasite DNA (Nc5) and were carried out as previously described (12). Results are expressed as the number of parasites in 50 ng of DNA. The limit of detection was 0.1 parasites in 50 ng of tissue DNA. In some regions, such as the cerebellum, negative controls showed relatively high values. Under our experimental conditions, cell-rich tissue samples, including lymph nodes, showed higher qPCR values despite being negative controls. In the brain, the cell density of the cerebellum is much higher than that of any other region (13). We hypothesize that the increased background in the qPCR result may be due to increased cell density in certain tissue samples. Test samples were considered positive when the parasite number was greater than 0.1 and higher than the values for the negative-control samples.

For histopathological analysis, tissues were fixed in 10% formalin solution, and brains were cut in coronal sections. The frontal lobe, corpus striatum, diencephalon, and mesencephalon included each area that was evaluated by qPCR: the prefrontal cortex, caudate putamen, hippocampus and hypothalamus, and periaqueductal gray. Tissues were embedded in paraffin, cut into sections that were 4 μm thick, and then stained with hematoxylin and eosin (HE). Immunohistochemistry (IHC) for *N. caninum* was performed with anti-*N. caninum* polyclonal antiserum (210-70 NC; VMRD, Pullman, WA) as the primary antibody. The secondary antibody was conjugated with horseradish peroxidase-labeled streptavidin biotin (LSAB+ kit, universal; Dako, Burlingame, CA). The chromogen was developed with 3,3′-diaminobenzidine (DAB) (Impact DAB; Vector Laboratories, Burlingame, CA). At least two sections were observed for each area.

Parasite DNA was detected only in the central nervous system (CNS) of calves 1 to 4. For the cerebellum and spinal cord, there was little difference between infected and control tissues (Fig. 1A).

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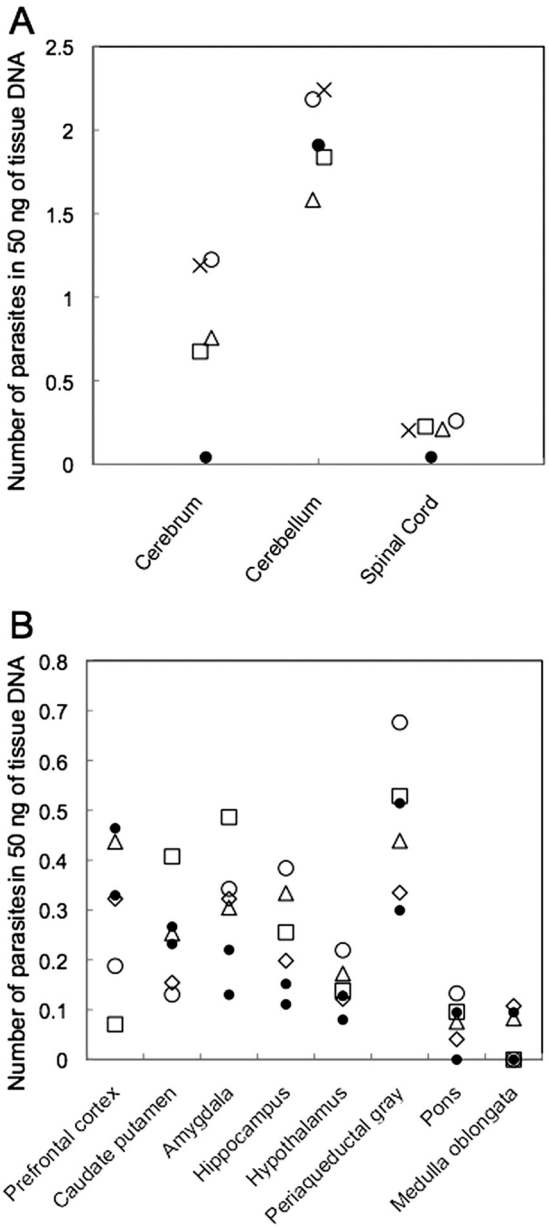

FIG 1 Quantitation of parasites in tissues from cattle infected with *N. caninum* tachyzoites by qPCR. Samples were considered positive when the number of parasites was greater than 0.1 and was higher than those for negative-control samples. (A) *N. caninum* distribution in the CNS. Open circle, calf no.1; open triangle, no. 2; open square, no. 3; cross, no. 4; filled circle, control. (B) *N. caninum* distribution in the brain. Open circle, calf no. 5; open triangle, no. 6; open square, no. 7; open diamond, no. 8; filled circle, control.

Calves 5 to 8 tested positive for the presence of *N. caninum* in the amygdala and hippocampus. Although the parasite load was relatively low in the hypothalamus, three of four animals were positive (Fig. 1B). Our results suggest that the cerebrum, in particular the amygdala, hippocampus, and hypothalamus, can be used to evaluate the level of *N. caninum* infection in cattle.

Histopathologically, mild lesions, such as focal necrosis, glial activation, and perivascular cuffing, were observed in the cerebrum of infected cattle (Fig. 2A). These changes were frequently found in sections from the corpus striatum and diencephalon

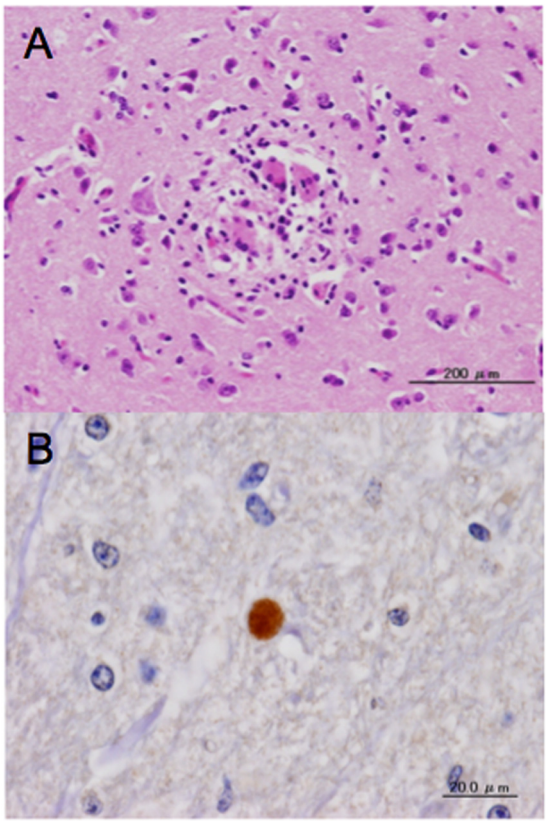

FIG 2 Histological sections of brain from cattle infected with *N. caninum* tachyzoites. (A) A section of corpus striatum from animal 7. Focal reactive glial cell infiltration as indicated by HE staining (magnification, ×20). (B) A section of mesencephalon from calf 4, showing a cyst as determined by IHC with anti-*N. caninum* polyclonal antiserum (magnification, ×60).

(Table 1). Additionally, many lesions were found in the cortex and in the white matter adjacent to the cortex. However, the distribution of lesions was not concentrated in areas that were found to be positive by qPCR. No lesions were seen in the mesencephalon, pons, medulla oblongata, and cerebellum. Our IHC results showed that a small tissue cyst was detected only in calf 4 (Table 1 and Fig. 2B).

In fetuses naturally infected with *N. caninum*, PCR of brain

TABLE 1 Histopathological changes in central nervous system

Area	Finding(s) for calf no.[a]:							
	1	2	3	4	5	6	7	8
Frontal lobe	M	C		N				
Corpus striatum	C, G			M, C, GN	C, S		C, G	M
Amygdala	*	*	*	*				G
Diencephalon	M, C, G			M			C	C, G
Occipital lobe	C, G					C		
Mesencephalon				P				
Pons								
Medulla oblongata								
Cerebellum								
Spinal cord				S				

[a] M, mononuclear cell infiltration in meninges; C, perivascular cuffing; G, glial cell activation or aggregation; GN, glial nodule; N, focal necrosis; S, spheroid; P, parasite detected by IHC; *, not examined.

samples is considered most suitable for diagnosis of bovine neosporosis (14). In experimentally infected calves, Kritzner et al. (15) showed that parasite DNA could be detected in the brain, muscle, and heart but not in the liver, spleen, lung, pancreas, popliteal lymph node, and gastrointestinal tract. In another study, parasite DNA was detected in the brain, spinal cord, heart, lung, diaphragm, and skeletal muscle (16). In the present study, parasite DNA was detected only in the CNS, emphasizing the importance of evaluating the brain in cattle experimentally infected with *N. caninum*. Additionally, we demonstrated that parasites were detected mainly in the amygdala and hippocampus of the limbic area. In the case of *Toxoplasma gondii*, closely related to *N. caninum*, the limbic area is a region where the cysts are known to localize at a high prevalence in murine models (17). Therefore, *N. caninum* may exhibit similar brain topology.

Brain lesions were also frequently found in the cerebrum, particularly the gray matter in aborted *Neospora*-infected fetuses (18). The cerebrum showed a significantly higher frequency of lesions than the medulla oblongata and cerebellum (18). Our findings are in accordance with previously published results. Most lesions were found in superficial gray and white matter and to a lesser extent in deeper areas. Lesions indicative of glial and inflammatory cell activation suggest some form of immune reaction against the parasites. It is also possible that the parasites could have already been eradicated, thereby leading to differences in the distribution of lesions and parasite DNA. *N. caninum* is believed to be disseminated hematogenously, with perivascular cuffing a common tissue response in neosporosis. This was actually observed in seven of the eight experimentally infected animals. Perivascular spaces around the artery in the brain have been shown to play an immunological role. The structure of the perivascular space in basal ganglia, including the amygdala, differs markedly from that seen in the cortex (19). Although it is not clear why few lesions were found in the deeper areas of the brain, the differences in anatomical structure, including the vascular system of each area, possibly affects invasion of the parasite or the host immune reaction.

Using IHC, the parasite was detected in only one experimentally infected animal. In approximate calculation from the qPCR results, the number of parasites per 1 g of tissue sample of amygdala was about 110 parasites on average. Assuming a specific gravity of brain 1, calculated simply, 1 g of tissue sample could provide 2,500 pieces of 1-cm^2 tissue sections when sliced 4 μm thick. This means that at least 23 sections are needed to find one parasite even when the parasites are distributed evenly in tissue. In general, the parasites would be sparsely distributed in the tissue as colonies of tachyzoites or cysts. Therefore, detection of the parasite by histopathological and immunohistochemical techniques is difficult with low sensitivity. In contrast, qPCR is highly sensitive and specific, especially in tissues with low levels of parasites.

In the present study, the importance of sampling and analyzing brain tissue was confirmed in cattle experimentally infected with *N. caninum*. Our results show that the amygdala, hippocampus, and hypothalamus samples are most appropriate for qPCR assays. Additionally, sections from the corpus striatum and diencephalon were most useful in histopathological analysis. Our findings show that these areas are most useful for evaluating the extent of *N. caninum* infection, investigating the pathogenesis of neosporosis, and evaluating antiparasitic drugs and vaccines against such organisms. Although the Nc-1 isolate has a lower ability for cyst formation, this isolate has been used in many studies of the pathogenesis of neosporosis and can induce fetal death in experimentally infected cattle (20–22). On the other hand, cystogenic isolates, such as Nc-Liv, may show some differences in brain parasite burdens and pathological changes. Thus, in future work, comparative study of different isolates of *N. caninum* will be important to understand the pathogenesis.

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