The Journal of Veterinary Medical Science

NOTE

Pathology

Striatal necrosis caused by *Lichtheimia ramosa* in a neonatal calf

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J. Vet. Med. Sci.
83(12): 1943–1947, 2021
doi: 10.1292/jvms.21-0499

Received: 14 September 2021
Accepted: 14 October 2021
Advanced Epub:
28 October 2021

ABSTRACT. A 12-day-old male calf that did not want breast milk from birth died following neurological signs such as staggering. Postmortem examination revealed bleeding and encephalomalacia in the left striatum and frontal lobe. Histopathologically, necrotic granulomatous encephalitis with numerous fungi was detected. The fungi were positively stained with anti-*Rhizomucor* mouse monoclonal antibodies. *Lichtheimia ramosa* was detected in formalin-fixed paraffin-embedded samples of the affected tissue by molecular methods. To the best of our knowledge, striatal necrosis caused by *L. ramosa* in a neonatal calf has not been reported. This study provides the first evidence of striatal necrosis caused by *L. ramosa* in a neonatal calf.

KEY WORDS: encephalomalacia, in utero infection, *Lichtheimia ramosa*, neonatal calf, striatal necrosis

Mucormycosis is one of the most important mycoses in humans and animals. Mucormycosis is mainly caused by members of the two orders of the phylum Mucoromycota: *Mucorales* (Mucoromycotina), which includes *Mucor*, *Rhizomucor*, *Rhizopus*, and *Lichtheimia* (formerly *Absidia*), and *Mortierellales* (Mortirellomycotina), which includes *Mortierella* [3]. According to the distribution of lesions, mucormycoses are classified into rhinocerebral, pulmonary, cutaneous, gastrointestinal, and disseminated types [10]. In cattle, these pathogens have been reported to cause gastroenteritis [6], meningoencephalitis, and endometritis [2], but the identification of causative fungi is limited. There is rarely any raw material for fungal isolation at diagnosis, and the fungal strain required for identification is not cultured in many cases. The progression of mucormycosis is rapid. Mucoromycotina is highly invasive in the vascular system and forms fungal embolisms and suppurative necrotic lesions [3]. Neonatal mucormycosis has not been diagnosed before birth, and most cases result in poor prognosis [2].

Lichtheimia ramosa is a popular thermotolerant soil fungus that is abundant in the environment around cattle, such as hay, grain, bedding, and silage [7]. *L. ramosa* is a common member of the gastric fungal flora [9]. In humans, nasal *L. ramosa* infection is caused by the inhalation of asexual spores, subsequently leading to cerebral lesions [12].

The present study reports a rare case of striatal necrosis in a neonatal calf. For molecular biological examination, DNA was extracted from formalin-fixed paraffin-embedded (FFPE) sections, and the internal transcribed spacer (ITS) region DNA sequences were determined. Consequently, the causative fungus was identified as *L. ramosa*, and its ITS region showed a 100% match of the sequences of the strain from the first case of rhinocerebral mucormycosis in a 12-day-old calf [14]. Therefore, this is the second reported case of bovine encephalitis caused by *L. ramosa*.

In December 2020, a 12-day-old male calf died following neurological signs at a breeding farm with 50 Japanese Black cattle in Fukushima, Japan. The main clinical presentation was the loss of desire to suckle milk, slow and staggering movement, and

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(Supplementary material: refer to PMC https://www.ncbi.nlm.nih.gov/pmc/journals/2350/)

repeated hitting of the head from birth. The calf had no history of treatment. The mother cow had given birth 12 times before and was introduced to the farm as a pregnant cow. Four hours after the calf died, the calf was moved to Fukushima Prefectural Chuou Livestock Hygiene Service Center from the farm and autopsied.

At necropsy, the brain, liver, spleen, kidneys, heart, lungs, superficial cervical lymph node, subiliac lymph node, and umbilical cord tissue samples were fixed in 20% neutral-buffered formalin. Fixed tissues were embedded in paraffin, sectioned (~3 µm), and stained with hematoxylin and eosin (H&E) for histological examination. Periodic acid-Schiff (PAS) reaction, Grocott's silver, Gram, and phosphotungstic acid-hematoxylin (PTAH) staining were also performed on the sections of the cerebrum and cerebellum.

For immunohistochemical examination, FFPE sections of the left striatum were deparaffinized and incubated with 3% hydrogen peroxide in methanol solution to suppress endogenous peroxidase activity. Antigen retrieval was conducted using 0.1% actinase E solution in phosphate-buffered saline at 37°C for 20 min. After adding 10% normal goat serum to block non-specific reactions, sections were incubated with anti-*Rhizomucor* (1:256, Clone WSSA-RA-1; Dako, CA, USA) and anti-*Aspergillus* (1:1,024, Clone Mab-WF-AF-1; Dako) mouse monoclonal antibodies, and anti-*Candida albicans* (1:2,048, Biogenesis, Dorset, UK) rabbit polyclonal antibody for 30 min at room temperature. Sections were incubated with secondary antibody (Histofine Simple Stain MAX-PO Multi; Nichirei Bioscience Inc., Tokyo, Japan) for 30 min at room temperature, and then treated with aminoethyl carbazole (AEC) substrate solution (Histofine Simple Stain AEC solution; Nichirei Bioscience Inc.) at room temperature. Finally, the sections were counterstained with hematoxylin. Sections with fungal solution were used as a positive control. The fungi used were *Rhizomucor pusillus*, *Aspergillus fumigatus,* and *Candida lusitaniae*.

For the identification of fungal species in the FFPE section of the left striatum, genomic DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. To amplify the ITS region, the primer pairs ITS1 and ITS4 [15] were used. The PCR product was sequenced using an Applied Biosystems 3130×l genetic analyzer (Applied Biosystems 3130*xl* genetic analyzer; Life Technologies Co., Carlsbad, CA, USA), and underwent a Basic Local Alignment Search Tool (BLAST) search of the GenBank database.

The ITS region sequence of genomic DNA used for phylogenetic analysis was D35097Fukushima2241. The evolutionary history was inferred using the maximum likelihood method and the Tamura-Nei model [13]. The tree with the highest log likelihood (−5,195.66) is shown. The initial tree (s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach, and then the topology with the superior log likelihood value was selected. The tree was drawn to scale, with branch lengths measured as the number of substitutions per site. This analysis involved 53 nucleotide sequences from previous data [1] and an additional 7 datapoints (Table 1). There were 913 positions in the final dataset. Evolutionary analyses were conducted using MEGA X [8].

The right parietal lobe of the cerebrum, cerebrospinal fluid (CSF), liver, spleen, kidneys, heart, and lungs were sampled and used for isolation of bacteria. All samples were stamped onto 5% sheep blood supplemented with blood agar base (BD BBL; (Becton, Dickinson and Co., Franklin Lakes, NJ, USA) and MacConkey agar (BD Difco; (Becton, Dickinson and Co.) and incubated at 37°C for 72 hr under air plus 5% CO_2 under anaerobic and aerobic conditions, respectively. Anaerobic cultivation was performed using the Anaero Pack system (Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan). The cerebrum and CSF were used to isolate the fungus. All samples were stamped and placed onto potato dextrose agar with chloramphenicol (Nissui Pharmaceutical Co., Ltd.) and incubated at 37°C or 25°C for 1 week under aerobic conditions.

Reverse transcriptase polymerase chain reaction (RT-PCR) was performed with the cerebrum and spleen to detect bovine viral diarrhea virus (BVDV). In addition, virus neutralization tests of BVDV types 1 and 2, Akabane virus (AKAV), Aino virus (AINOV), and Chuzan virus (CHUV) were performed with calf serum.

At necropsy, the surfaces of the left olfactory bulb and tract were mildly hemorrhaged and necrotized (Fig. 1a), but the bottom and dorsal sides were normal (Supplementary Fig. 1). In addition, the cerebral parenchyma of the left striatum and frontal lobe showed bleeding and encephalomalacia (Fig. 1b). The thymus was atrophied (weight, 18 g). No abnormalities were detected in the

Table 1. Additional data for phylogenetic analysis

No.	Species strain	Country/ place	Source	Accession No.	Reference
1	*Lichtheimia ramosa* D35097Fukushima2241	Fukushima, Japan	Brain, 12-day-old Japanese Black calf/male	LC643024	https://www.ncbi.nlm.nih.gov/nuccore/LC643024
2	*L. ramosa* OBIHIRO	Obihiro, Japan	Rhinocerebral zygomycosis, 5-day-old Japanese Black calf/ female	None (perfectly matched with a strain CA-A2, GeneBank accession No. HQ285637)	[14]
3	*L. ramosa* CA-A2	Korea	Traditional starter cultures (Nuruks) used for rice wine	HQ285637.1	https://www.ncbi.nlm.nih.gov/nuccore/HQ285637
4	*L. ramosa* B17Q226	Korea	Pregnant cow	MH675478.1	https://www.ncbi.nlm.nih.gov/nuccore/MH675478.1
5	*L. ramosa* FSU10175*		Cattle gut		[11]
6	*L. ramosa* FSU10566*		Stork lung	JQ775400	[11]
7	*L. ramosa* FSU10568*		Stork lung	JQ775401	[11]

*FSU: Jena Microbial Resource Collection (formerly: Fungal Centre of the Friedrich Schiller University Jena, Germany).

nasal speculum, nasal cavity, palate, or tongue. No other gross lesions were observed (Supplementary Fig. 2). The mother cow had no clinical signs, but the placenta could not be examined.

The primary histopathological finding in the cerebrum was severe necrotic granulomatous encephalitis with numerous fungi. In the cerebral parenchyma of the left striatum, perivascular cuffing (Fig. 1c) was also detected with numerous fungi. Inflammatory cells, fungal hyphae and thrombi were also seen in the blood vessels (Fig. 1d). Fibrin thrombi were positive for PTAH staining. The lesions also contained nerve cell necrosis and reduction. Similar moderate to slight localized lesions were detected from the left olfactory bulb to the left frontal lobe, diencephalon, and right striatum.

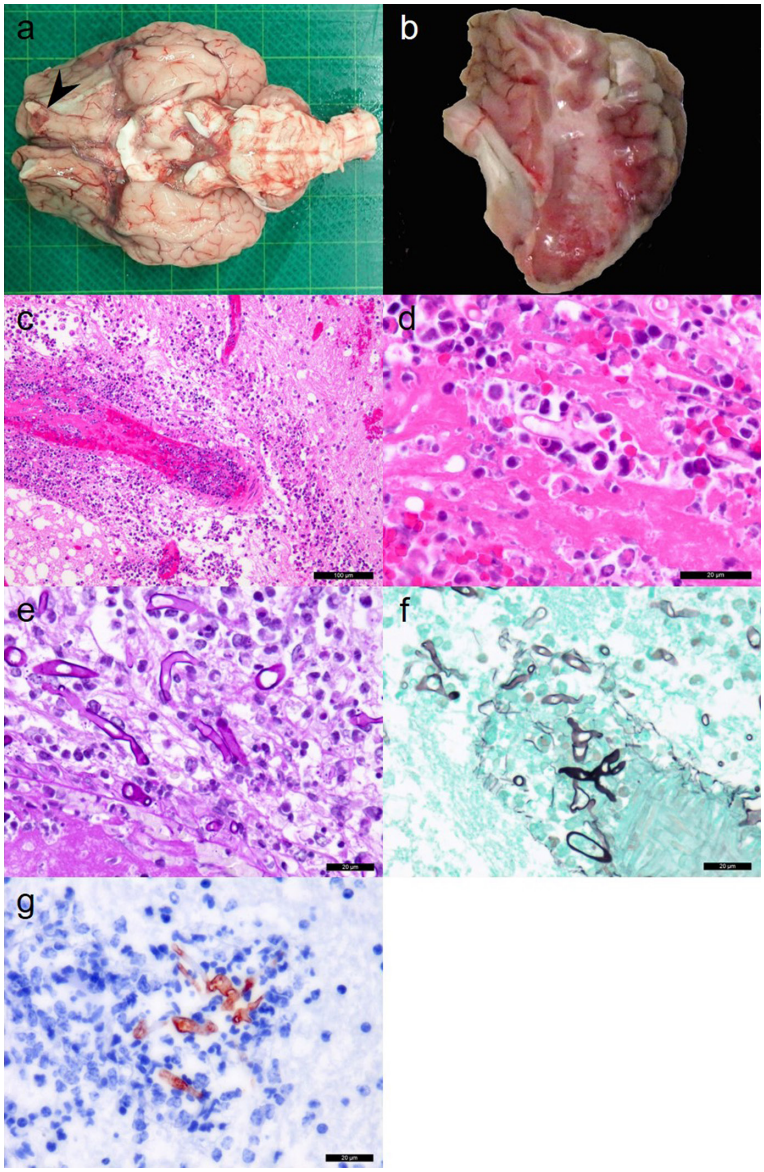

Fig. 1. **a**. Ventral surface of brain. The left olfactory bulb and tract were mildly hemorrhaged and necrotized (arrowhead). The bottom of the cerebrum was normal. **b**. Bleeding and encephalomalacia were detected in the left striatum. **c**. Perivascular cuffing was detected in the left striatum. Hematoxylin and eosin staining. Scale bar=100 µm. **d**. Inflammatory cells and fungal hyphae and thrombi were seen in the blood vessel. Hematoxylin and eosin stain. Scale bar=20 µm. **e**. Fungal hyphae, 5–10 µm wide, were surrounded by inflammatory cells and strongly stained. Periodic acid-Schiff reaction. Scale bar=20 µm. **f**. Fungal hyphae, 5–10 µm wide, were thin-walled, hollow, tubular structures and branched irregularly in the blood vessel. No septa were observed. Grocott's silver stain. Scale bar=20 µm. **g**. Immunohistochemical staining revealed positive reactions to anti-*Rhizomucor* mouse monoclonal antibody against the fungi in the lesions. Scale bar=20 µm.

The fungal hyphae were 5–10 µm wide, surrounded by inflammatory cells, and strongly stained by PAS reaction (Fig. 1e) and Grocott's silver stain (Fig. 1f). The hyphae were thin-walled, hollow, tubular structures and branched irregularly. No septa were observed. No bacteria were detected in the cerebrum or cerebellum by Gram staining. No other lesions were detected in other organs.

Immunohistochemical staining revealed positive reactions to only anti-*Rhizomucor* mouse monoclonal antibodies against the fungi in the lesions (Fig. 1g). No reactions were observed with the other antibodies.

The fungus was identified as *L. ramosa* subgroup I using molecular methods (Fig. 2). The sequence of the ITS region showed a 100% match with that of the *L. ramosa* strain CA-A2 (accession no. HQ285637.1) isolated from traditional starter cultures (Nuruks) used for rice wine in Korea, and OBIHIRO isolated from the brain of Japanese Black calves at Obihiro, Hokkaido, Japan in 2019 [14]. The ITS region sequence was deposited in the DNA Data Bank of Japan (accession no. LC643024). In addition, the sequence of the present material was placed in the clade of *L. ramosa* and was clearly distinguished from the sequences of other *Lichtheimia* species. There were no human or animal-specific clusters.

No bacteria or fungi were isolated from these organs.

The RT-PCR results for BVDV were negative. In addition, the neutralizing antibody titers of AKAV, AINOV, and CHUV were less than double, and BVDV was 32 times.

Histopathological and immunohistopathological results confirmed mucormycosis in a neonatal calf and necrotic granulomatous encephalitis in the cerebrum. The anti-*Rhizomucor* mouse monoclonal antibody reacted positively not only to *Rhizomucor* spp. but also to *Lichtheimia*, *Mucor*, and *Rhizopus* [5]. Therefore, we could not identify the genera and species of the mucorales taxa based on these results.

Because there were severe lesions in the left striatum, the neurological signs in the present case were suspected to be due to encephalitis and subsequent nerve cell necrosis and reduction. The striatum, a part of the basal ganglia, influences action selection and modulation of movement vigor [4]. In agreement with this information, the present calf had slow movement, occasionally hit its head against walls, and showed neurological signs such as staggering.

In a previous case, a 3-day-old calf diagnosed with necrotic cerebellitis caused by *Mortierella wolfii* was suspected to be a case of placental

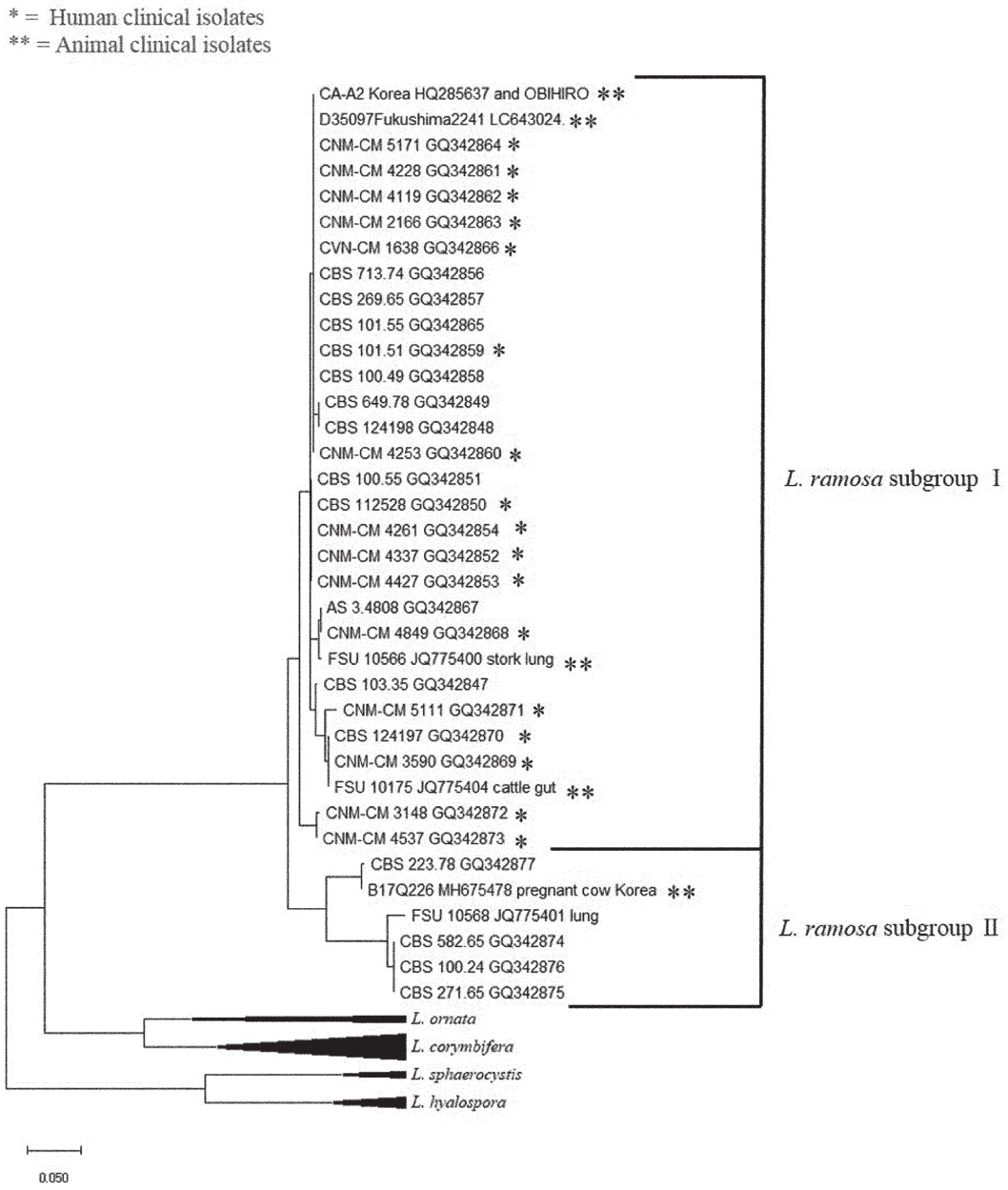

Fig. 2. Phylogenic tree based on internal transcribed spacer region sequences. The scale bar represents genetic distances among strains. The sequence was classified as the same clade of *Lichtheimia ramosa* and different from the clade of *L. corymbifera*.

infection [16]. In another previous case, a 12-day-old calf was diagnosed with rhinocerebral mucormycosis due to *L. ramosa* infection, and postnatal infection was suspected [14]. In our case, the presence of granulomatous lesions may indicate a chronic course. In addition, the neonatal calf showed neurological signs from birth. Therefore, the pathogen would have invaded the calf before birth and finally reached and formed the lesions in the striatum. There were two possible entry routes to the striatum. The first possible route was hematogenous dissemination from the placenta. However, there were no lesions containing fungal hyphae in the kidneys, liver, or spleen, like the previous case [16]. Unfortunately, the placenta could not be examined in the present case. The second possible entry route was rhinocerebral, or through the olfactory nerves from the amniotic fluid. However, the lesions were too small, localized, and mild for this to be the cause compared to the previously reported case [14]. Thus, the accurate entry route could not be identified in the present case. If an affected animal presents with neurological signs immediately after birth, examinations of the placenta, vaginal swab, and lochia are essential to accurately diagnose the route of infection.

As *L. ramosa* is closely related to *L. corymbifera*, their ITS region sequences are very similar. To avoid confusion, careful phylogenetic analysis is desirable and effective to distinguish between the two species in many cases [1]. Rapid and cost-effective development of species-specific PCR will be useful for diagnosis in the future.

Two samples of *L. ramosa* in Japan, which would also cause cerebrum lesions, interestingly showed the same ITS region

sequences [14]. Therefore, this result suggests a possible relationship between certain genetic groups of *L. ramosa* and their pathogenicity to Japanese Black calves. However, information is currently limited to isolates from calves with encephalitis in Japan. In the future, the accumulation of case data is expected to verify this hypothesis.

Although the main lesion was on the left side of the cerebrum in the present case, the right parietal lobe of the cerebrum was sampled and used to isolate fungi. Subsequently, living fungal culture was not isolated. Therefore, antibiotic susceptibility tests were not performed. We need to realize the importance of these analyses and improve the sampling methods in the future.

An increase in the BVDV neutralizing antibody titer was suspected because of the maternal antibodies. No viral involvement was observed.

In conclusion, striatal necrosis in a neonatal calf due to *L. ramosa* was observed. Rhinocerebral mucormycosis due to suspected postnatal infection by *L. ramosa* has been reported [14], but there are no reports of calves being infected with *L. ramosa* in the uterus. In addition, the ITS region sequence of the causative fungus showed a perfect match to that of the fungus in the first case. Our study provides important insights into striatal necrosis of neonatal calves that should benefit both veterinarians and farmers.

CONFLICT OF INTEREST. The authors declare no conflict of interest.

ACKNOWLEDGMENTS. We thank Mr. M. Kobayashi and Ms. M. Shimada for their histopathological assistance, and Dr. Ban Sayaka (Chiba University Medical Mycology Research Center) for helpful discussions and advice.

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