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Local extensive granulomatous inflammation of the neck region and lymphangitis caused by *Lichtheimia corymbifera* infection in a Japanese Black calf

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ABSTRACT

A 7-month-old female Japanese Black calf developed elongated, nodular mass measuring 30 × 16 cm extended from the retropharyngeal region to mid lateral neck region. Histological examination revealed granulomatous lymphangitis with non-septate fungal hyphae recognized throughout the lesions. Fungal culture, DNA sequencing and molecular phylogenetic tree analysis confirmed the sequence of *Lichtheimia corymbifera*. The lymphogenous route was speculated to be the main route of fungal spread leading to the characteristic nodular appearance of this case.

1. Introduction

Mucorales and Entomophthorales are the two main orders of fungi of veterinary importance that belong to the class of Zygomycete fungi. Mucormycosis and entomophthoramycosis usually occur as opportunistic fungal infections mainly seen in immunosuppressed hosts [1]. Due to clinicopathological and histomorphological different between mucormycosis and entomophthoramycosis, the term “mucormycosis” is more specific than zygomycosis [1,2]. Mucormycetes such as those in the genera *Mucor*, *Rhizopus* and *Lichtheimia* are common causative fungi of systemic and deep fungal infections in domestic animals [3]. These agents are ubiquitous in the housing environment of cattle such as in air, soil and feed and can also be found in normal rumen flora [4].

To date, the forms of mucormycosis that have been identified in domestic animals include gastrointestinal, lymph nodal, cutaneous, pulmonary, rhinocerebral and disseminated forms [5–7]. In cattle, causative pathogens identified were mainly *Lichtheimia corymbifera* (formerly known as *Absidia corymbifera* and *Mycocladus corymbifera*) in zygomatic gastroenteritis [6,8]; *Rhizopus microsporus* var. *microsporus* in rumenitis and omasitis [5]; *L. corymbifera*, *R. microsporus* and *Rhizomucor pusillus* in mycotic lymphadenitis [9,10]; and *L. corymbifera*, *Mortierella*, *Rhizomucor* and *Rhizopus* in mycotic abortion [11,12]. The hematogenous route of spread is thought to be the systemic route of mucormycete dissemination due to their angioinvasive capability [3,13]. To our knowledge, there are no clear reports of *L. corymbifera* spread through the lymphogenous route with localized deep tissue involvement in cattle. We herein present the first case of suspected spread of *L. corymbifera* through the lymphogenous route and local extensive granulomatous inflammation in multiple neck tissues such as the muscle and tendon in a Japanese Black calf.

2. Case

A 7-month-old female fattening Japanese Black calf developed a slow-growing mass in the left lateral neck region (Fig. 1). The calf was born in midwinter and was from a farm which reported to have a calf with low γ-globulin levels occasionally. On day 0, an elongated, nodular mass measuring 30 × 16 cm (Fig. 1, inset) extending from the retropharyngeal region to mid lateral neck region was surgically resected. The calf was treated with penicillin (20,000 IU/kg) -streptomycin (25 mg/kg) solution SID for 5 days, and sulpyrine 62.5 mg/kg on day 1 and 37.5 mg/kg on day 4 respectively. Microscopic examination revealed granulomatous inflammation characterized by extensive necrotic foci and dystrophic calcification with severe infiltration of macrophages, eosinophils, lymphocytes, plasma cells and multinucleated giant cells. Non-septate fungal hyphae with non-parallel walls and irregular branching were recognized throughout the lesions and remarkably found in the cytoplasm of multinucleated giant cells. Based

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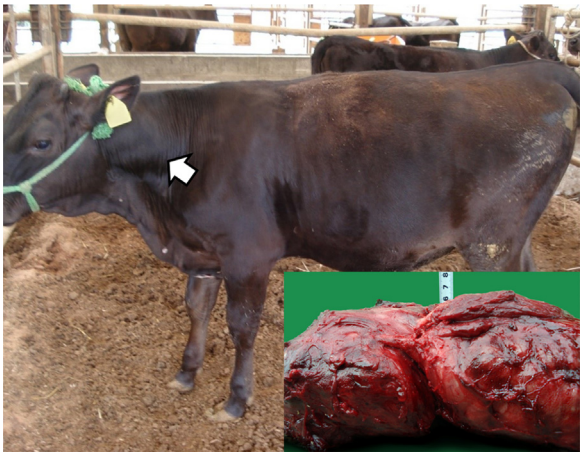

Fig. 1. An elongated neck mass caudal to the left ear pinna extends to the mid lateral neck area (arrow). Inset: an elongated, nodular mass measuring 30 × 16 cm extends from the retropharyngeal region to mid lateral neck region.

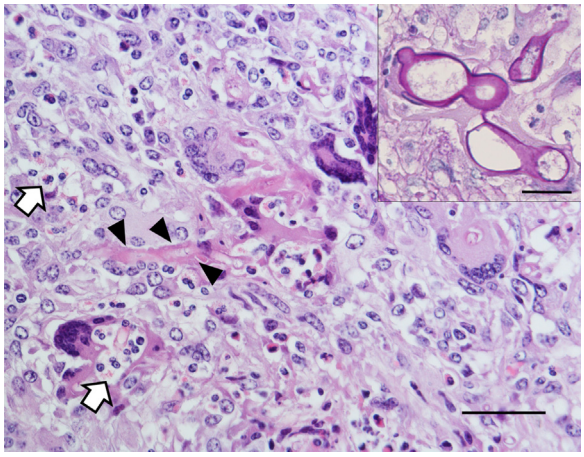

Fig. 2. Thin-walled, non-parallel and sparsely septate hyphae are seen scattered throughout granulomatous lesions, especially in the cytoplasm of multinucleated giant cells with infiltration of eosinophils (arrows), lymphocytes and plasma cells. Some eosinophils are phagocytosed by giant cells. Degranulation of eosinophils is seen surrounding fungal hyphae, leading to a thin (2.5–3.0 μm) eosinophilic sleeves (arrowheads). Bar, 50 μm. Inset: PAS staining reveal numerous hollowed, non-pigmented, thin-walled and sparsely septate filamentous hyphae with occasional ballooning dilatations throughout the lesions. Bar, 25 μm.

on the morphological features of hyphae, a tentative diagnosis of fungal granuloma caused by zygomycetes was made.

On day 90, swelling recurred at the same location and the calf was referred to the University of Miyazaki Veterinary Teaching Hospital for surgical resection and further examination. Upon surgery, the mass was severely adhered to surrounding neck tissue and extensively invaded the deep muscle layer and tendon of the neck region. Masses with a total weight of 1.75 kg were resected and sent for histopathological examination, fungal culture and isolation, DNA extraction and fungal identification. There were no therapeutic interventions in addition to surgery.

For histopathological examination, special stains and immunohistochemical (IHC) examination, resected samples were fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 4 μm. Sections were stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Grocott's methenamine silver (GMS), Gram stain and Ziehl-Neelsen stain. IHC was performed using primary antibodies against CD3 (Dako, Glostrup, Denmark; prediluted), CD20 (Dako, Glostrup, Denmark; prediluted), von Willebrand factor (vWF) (Nichirei Biosciences Inc., Tokyo, Japan; prediluted) and Iba-1 (Wako Chemicals, Tokyo, Japan; 1:200 dilution) on selected sections. For fungal growth and isolation, fresh material sampled during the second surgery was incubated on potato dextrose agar medium with chloramphenicol at room temperature for 3 days.

For molecular biological analysis, DNA was extracted from 50 μl of 10% emulsion made from fresh tissues by treatment with 2 μl of proteinase K (20 μg/ml) at 37 °C for 2 h, followed by incubation in a warm water bath (100 °C) for 20 min, and centrifugation at 15,000 revolutions per minute. Then, supernatants that contained DNA products were amplified using previously described fungal primers TW13 (5′-GGT CCG TGT TTC AAG ACG-3′) and Ctb6 (5′-GCA TAT CAA TAA GCG GAG G-3′) [4]. Fungal DNA amplicons which contained a 700-base pair fragment from the 50 end of the large subunit of the rRNA gene were sent for sequencing.

Consensus sequences were generated from individually sequenced strands from each amplicon and a basic local alignment search tool search was performed (BLAST; Vector NTI Advance 10.3, Invitrogen Corporation, Carlsbad, CA). A multiple-sequence alignment was constructed with reference sequences of representative zygomycetes obtained from the GenBank database. Sequence alignments were manipulated using a multiple-sequence alignment editor (GeneDoc, http://www.nrbsc.org/gfx/genedoc/index.html), and a tree showing sequence relationships was constructed. The Jukes-Cantor model was used for distance estimation; phylogenetic dendrograms were built by the neighbor-joining method and tree topologies were evaluated by performing bootstrap analysis with 1000 replications.

The histopathological examination showed that resected masses comprised extensive granulomatous lesions involving several deep tissue structures in the neck such as muscles and tendons. Thin-walled, non-parallel and sparsely septate hyphae with occasional ballooning dilatations were seen scattered throughout the granulomatous lesions, especially in the cytoplasm of multinucleated giant cells, granulation areas and necrotic areas. Some eosinophils were also phagocytosed by giant cells along with the hyphae (Fig. 2). Degranulation of eosinophils was seen surrounding some of the fungal hyphae, leading to a thin (2.5–3.0 μm) eosinophilic sleeve. A marked infiltration of macrophages and eosinophils, and occasionally lymphocytes and plasma cells, was present around the muscle and tendon layers. GMS and PAS staining revealed numerous hollowed, non-pigmented, thin-walled and sparsely septate filamentous hyphae throughout the lesions. The hyphae measured 5–8 μm in diameter with irregular branching. Some hyphae showed ballooning dilation (10–30 μm) (Fig. 2, inset) and yeast-like spore structures. Conversely, Gram staining and Ziehl-Neelsen staining were negative.

Numerous necrotic, coalescing foci of calcium lakes surrounded by extensive granulation tissues and fibrotic tissues were also found all over the masses. Proliferation of capillary blood vessels and dilatation of lymph vessels were prominent in these areas. Areas with multiple lymphoid follicle apparatus were also seen. In these areas, CD20-positive B lymphocytes were found at the center of the lymphoid follicle, surrounded by CD3-positive T lymphocytes. Iba-1-positive cells were found throughout the granulomatous lesions. In addition, vWF-positive high endothelial venules were present in the lymphoid-like follicles. In between the resected mass, there were significantly dilated lymph vessels with marked granulomatous inflammation surrounding the lymph vessels (Fig. 3b). In comparison, angioinvasion was rarely noted. Proliferation of endothelial cells in vasculitis was noted, however, no fungal emboli were found in blood vessel lumens.

For fungal culture and isolation, white fluffy peculiar colonies similar to the order Mucorales were isolated after 72-h cultivation at room temperature. Microscopically, the isolates showed hypha without septum and oval sporangia typified by genus *Lichtheimia*. As a result of PCR analysis using universal primers TW13 and Ctb6, an amplified band was detected in an approximate 700-bp region by electrophoresis.