NOTE *Pathology*

Zygomycotic Mediastinal Lymphadenitis in Beef Cattle with Ruminal Tympany

Maki NISHIMURA[1)], Yumi TOYOTA[2)], Yoshikazu ISHIDA[2)], Hideki NAKAYA[5)], Kyohko KAMEYAMA[1)], Yoshifumi NISHIKAWA[1)], Kazuro MIYAHARA[3)], Hisashi INOKUMA[4)] and Hidefumi FURUOKA[2)]*

[1)]*National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan*
[2)]*Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan*
[3)]*Animal Medical Center, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan*
[4)]*Department of Clinical Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan*
[5)]*Tokachi Agricultural Mutual Aid Association, Kawanishi, Obihiro, Hokkaido 089–1182, Japan*

(Received 14 February 2013/Accepted 26 August 2013/Published online in J-STAGE 9 September 2013)

ABSTRACT. A 9-month-old steer was autopsied due to recurrent ruminal tympany. A macroscopic examination found an enlarged caudal mediastinal lymph node, and a section of the lymph node revealed necrosis with marked calcification, similar to tuberculous lymphadenitis. Histopathologically, the lesion consisted of multiple coagulative necrotic foci and fibrosis with macrophage, lymphocyte, eosinophil and multinucleated giant cell infiltration. Non-uniform width hyphae were detected in the necrotic area and within the cytoplasm of the multinucleated giant cells, and they were found to be anti-*Rhizopus arrhizus* antibody positive in an immunohistochemical examination. Therefore, the steer was diagnosed with necrotic caudal mediastinal lymphadenitis due to zygomycetes infection, and inhibition of eructation by the enlarged lymph node was the likely cause of the ruminal tympany.
KEY WORDS: cattle, necrotic lymphadenitis, ruminal tympany, zygomycosis.

doi: 10.1292/jvms.13-0084; *J. Vet. Med. Sci.* 76(1): 123–127, 2014

Zygomycosis is an opportunistic fungal infection caused by the fungi that used to belong to the class Zygomycetes, which includes *Mucor*, *Rhizopus* and *Absidia*. Recently, based on the results of molecular phylogenetic analysis, Zygomycota have been reclassified into a new division, Glomeromycota, and four subdivisions of *incertae sedis*, Mucoromycotina, Entomophthoromycota, Kickxellomycotina and Zoopagomycotina [8]. Although the term "zygomycosis" is commonly used for mucormycosis, zygomycosis was originally the name for two diseases, mucormycosis and entomophthoramycosis [8]. The term "zygomycosis" is preferred to mucormycosis when the diagnosis is made on tissue sections without culture confirmation, and the clinicopathology of mucormycosis and entomophthoramycosis overlap [8].

The pathogens are ubiquitous within the environment of cattle and can be isolated from air [10]. Moreover, zygomycetes, such as *Mucor pusillus* and *Lichtheimia corymbifera,* are members of normal rumen flora [9, 12]. Infection can occur with a disruption of the normal balance between animals and agents [5], which has been associated with several factors, such as ruminal acidosis and broad-spectrum antimicrobial drugs, that affect the normal flora in the forestomachs [1].

The main portal of entry for zygomycetes is the gastrointestinal mucosa in cattle [5], which results in alimentary mycosis and systemic mycosis. Zygomycotic lymphadenitis has been observed as a lesion of alimentary and systematic mycosis or detected incidentally in slaughtered cattle with no clinical signs [6]. The lesions have consisted of necrotizing granulomatous inflammation [11] and shown tuberculosis-like lymphadenitis. Although similar lesions have been reported mainly in mesenteric lymph nodes of slaughtered cattle [11], zygomycotic mediastinal lymphadenitis is rare in cattle, and there have been no reports of a clinical case of zygomycotic lymphadenitis in young cattle in Japan. Here, we report an additional case of a young beef steer with solitary caudal mediastinal lymphadenitis due to a zygomycetes infection that could result in ruminal tympany.

A 9-month-old Japanese black steer was submitted for examination to diagnose the cause of recurrent ruminal tympany. The steer had developed subacute ruminal tympany at the age of seven months and thereafter had several reoccurrences of severe ruminal tympany for 2 months. There were no abnormal findings using esophagography. After X-ray fluoroscopy, the problem was suspected to be a ruminal foreign body. The steer underwent a rumenotomy, and a mass of rope was removed; however, the cattle showed no improvement of its condition.

On gross pathological examination, an elongated mass (20 × 5 × 5 cm) was observed between the thoracic aorta

*CORRESPONDENCE TO: FURUOKA, H., Department of Basic Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080–8555, Japan.
e-mail: furuoka@obihiro.ac.jp

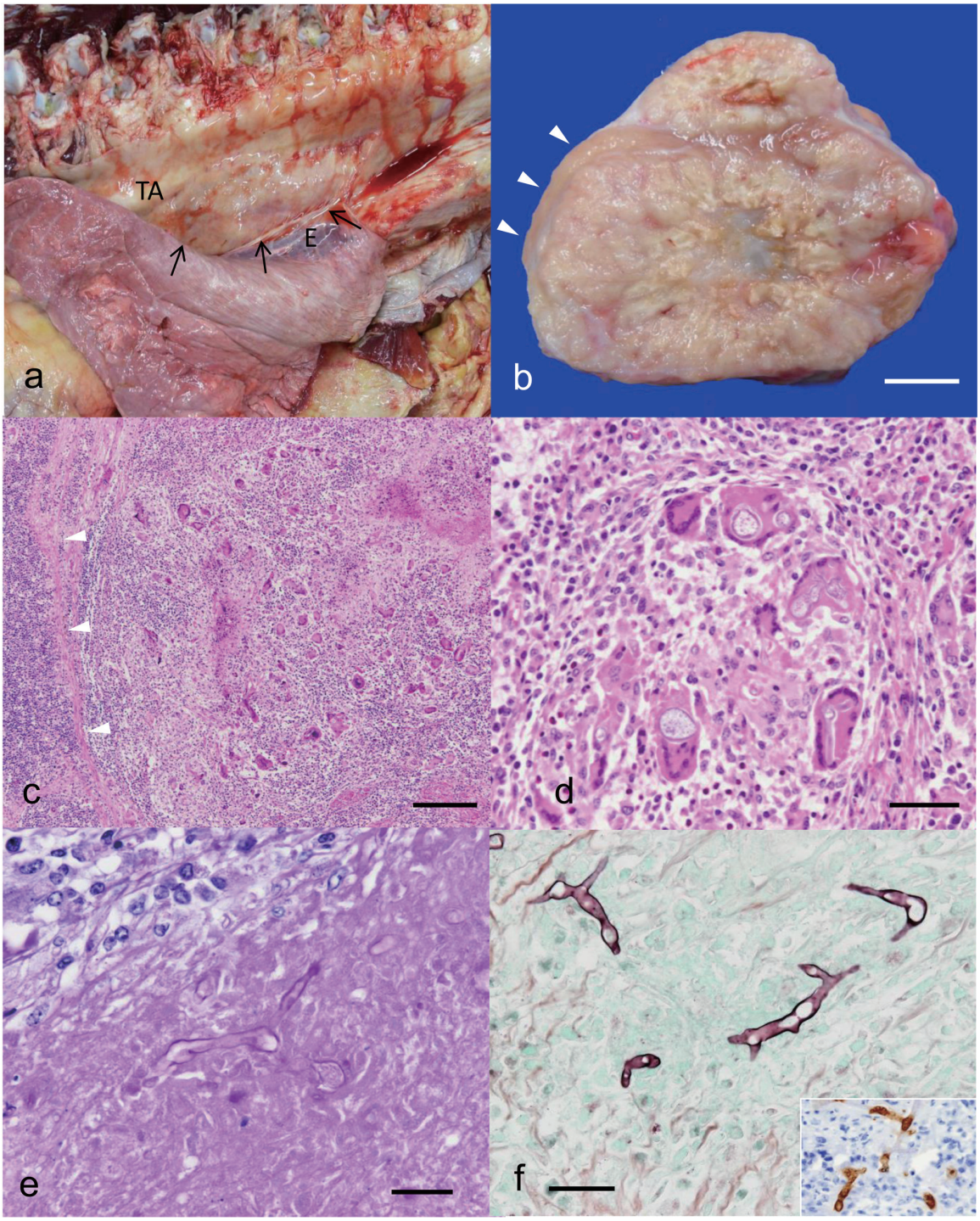

Fig. 1. Caudal mediastinal lymph node. (a) Enlarged caudal mediastinal lymph node (arrow) between the thoracic aorta (TA) and esophagus (E). (b) Cross section of the caudal mediastinal lymph node. Note the grayish white necrosis and yellow-white coarse calcification in the central area. A pale pink, solid area was found in the peripheral area of the cross section of the nodule (white arrowheads). Bar=1 cm. (c) Granulomatous lesions consisted of severe multinucleated giant cell infiltration and fibrous proliferation around the necrotic foci. Remains of the original structure of the lymph node was observed (white arrowheads). Hematoxylin and eosin. Bar=250 μm. (d) A severe infiltration of macrophages, lymphocytes, plasma cells, neutrophils, eosinophils and multinucleated giant cells around the necrotic foci. Phagocytized non-uniform width hyphae were seen in the cytoplasm of multinucleated giant cells. Hematoxylin and eosin. Bar=50 μm. (e) Fungal hyphae stained with PAS. Bar=25 μm. (f) Fungal hyphae stained with Grocott's methenamine silver. Bar=25 μm. Inset, fungal hyphae reacted with anti-*Rhizopus arrhizus* antibody. Immunohistochemistry, (DAB with hematoxylin counterstain).

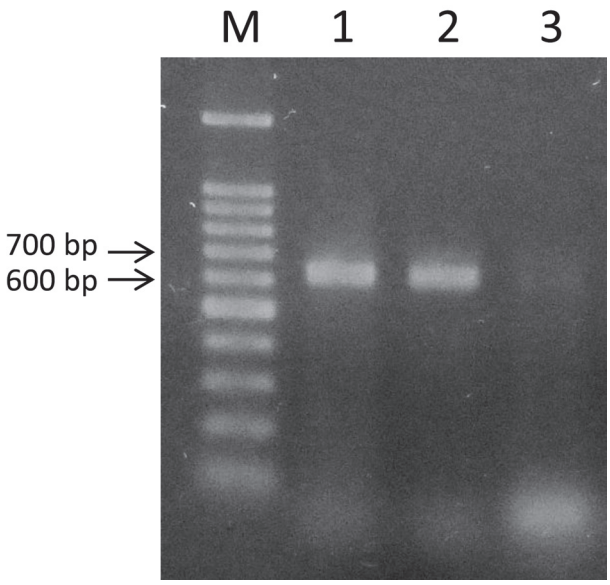

Fig. 2. PCR detection of fugal DNA in paraffin-embedded tissues with primers TW13 and Ctb6. Lane M, molecular weight marker; lanes 1, caudal mediastinal lymph node, lane 2, abomasum; lane 3, small intestine.

and the esophagus (Fig. 1a). The mass was at the site of the caudal mediastinal lymph node and did not adhere to adjacent tissues. It consisted of large and small nodules, and grayish white necrosis and yellow-white coarse calcification were observed in the central area of cross sections of both nodules, which resembled tuberculous lymphadenitis (Fig. 1b). A pale pink, solid area was found in a peripheral area of a cross section of a nodule (Fig. 1b, white arrowheads). The other mediastinal lymph nodes showed only mild enlargement. Also, there were no significant findings in other organs, except for an ulcerating lesion about 2 cm in diameter in the abomasum, mild thickening of the small intestine, mild enlargement of the mesenteric lymph node and a yellow-green fibrin clot formation in the anterior part of the thoracic cavity. The fibrin clot was likely the result of stimulation by catheter insertion into the rumen to remove the gas.

The major organs, including the mediastinal lymph nodes, were collected, fixed in 15% formalin, embedded in paraffin and sectioned at 4 μm. Sections were routinely stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Grocott's methenamine silver and Ziehl-Neelsen methods. Immunohistochemistry was performed on the sections of the caudal mediastinal lymph node with a mouse anti-*Rhizopus arrhizus* monoclonal antibody (MCA2577, AbD Serotec, Oxford, U.K.) as the primary antibody and a secondary antibody conjugated with horseradish peroxidase-labeled polymer (EnVision+ kit, Dako, Denmark A/S). The chromogen was developed with 3,3′-diaminobenzidine (DAB; ImmPACT DAB®, Vector Laboratories Inc., Burlingame, CA, U.S.A.).

To discover the point of entry of the fungi, the mediastinal lymph node, abomasum and small intestine were analyzed by PCR. Briefly, the fungal-specific DNA was extracted from the formalin-fixed paraffin-embedded tissue using a commercial kit (QIAamp® DNA FFPE Tissue Kit; Qiagen, Hilden, Germany) and amplified with the previously described fungal primers TW13 (5′- GGT CCG TGT TTC AAG ACG- 3′) and Ctb6 (5′-GCA TAT CAA TAA GCG GAG G-3′) [11, 15]. The PCR mixture (25 μl total volume) contained 2.5 μl of 10× PCR Buffer with 7.5 mM $MgCl_2$, 0.7 U of polymerase (Expand High Fidelity PCR System; Roche Diagnostics, Tokyo, Japan) and 200 μM concentrations of each deoxynucleoside triphosphate (dNTP Mixture; Takara Bio, Tokyo, Japan) and 800 nM of each primer and 1 μl of DNA extract as a template. Amplification was performed under the following conditions: one cycle of 95°C for 10 min; 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 2 min; and a final extension at 72°C for 7 min followed by a hold at 4°C (iCycler; Bio-Rad, Hercules, CA, U.S.A.). The PCR product was electrophoresed with 1.5% agarose gel, and the size of the amplification product was determined. Additionally, to identify the fungal species in the lymph node, the fungal DNA sequence was analyzed. Amplicons from the lymph node were purified using a QIAquick Gel Extraction kit (Qiagen) and sequenced with the same primer used for amplification (ABI 3730 DNA Analyzer; Applied Biosystems, Carlsbad, CA, U.S.A.). The obtained sequences were identified using BLAST (Basic Local Alignment Search Tool) on the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov).

A histopathological examination showed that a large portion of both nodules was occupied by an extensive necrotic lesion with severe fibrosis and calcification; both lesions were considered to be necrotizing granulomatous lesions. A lymph node structure with follicular hypertrophy was found in the peripheral part of the mass (Fig. 1b and 1c, white arrowheads); the mass was found to have been the caudal mediastinal lymph node (Fig. 1c). However, the original structure of the lymph node was almost entirely lost (Fig. 1c). A severe infiltration of macrophages, lymphocytes, plasma cells, neutrophils, eosinophils and multinucleated giant cells of a foreign body type was around the necrotic foci (Fig. 1d). Furthermore, the necrotic foci with inflammatory cell infiltration were surrounded by severe fibrosis. Eosinophils and multinucleated giant cells dominated in the periphery of the granuloma. Poorly septate hyphae with a non-uniform width and irregular and wide-angle branching were detected in the necrotic foci and within the cytoplasm of the multinucleated giant cells. Only a few hyphae were surrounded by Splendore-Hoeppli material, and no sporulation or conidiation was observed. No obvious blood vessel invasion was found in the lymph node. The hyphae showed a positive periodic acid-Schiff (PAS) reaction (Fig. 1e) and were stained with Grocott's methenamine silver stain (Fig. 1f). Furthermore, they were anti-*Rhizopus arrhizus* monoclonal antibody positive in immunohistochemistry (Fig. 1f, inset). No acid-fast organisms were seen in the affected lymph node stained with Ziehl-Neelsen stain. Although follicular hypertrophy and hyperplasia of the medullary cord

were observed in the other mediastinal lymph node, no necrotic foci and no hyphae were found. Only focal lymphocyte infiltration was found in the proper gastric region of the abomasum and mild macrophage and eosinophil infiltration was observed in the small intestine submucosa. However, no hyphae were detected in either of the tissues histopathologically. In a molecular biological analysis, approximately 600- to 700-base pair PCR products were amplified from samples of the mediastinal lymph node and abomasum as well as a previous report [11], but were not amplified from samples of the small intestine (Fig. 2). However, a full-length sequence was not obtained, and no fungal species with high homology to the sequence of the product were found. Thus, the steer was diagnosed histopathologically as having necrotic lymphadenitis with foreign body giant cell infiltration due to zygomycetes infection, and it is likely that inhibition of eructation by the enlarged caudal mediastinal lymph node resulted in recurrent ruminal tympany.

Zygomycetes tend to invade blood vessels, and the infection tends to spread in necrotic or granulomatous lesions systemically [5]. In addition to the gastrointestinal tract, the respiratory tract could be a main portal of entry for zygomycetes in cattle subjected to inhalation of spores from moldy feedstuffs [5]. Zygomycotic lymphadenitis has been found in domestic animals including cattle and pigs [3]. In several earlier studies in cattle, most of the lesions were detected in the mesenteric lymph nodes, and mediastinal lymphadenitis merely accounted for 7 of 94 [6] and 4 of 194 [11] cattle and 9 of 105 [3] lymph nodes with granulomatous mycotic lesions. These cases were detected in slaughtered cattle, and no detailed information, including age, clinical condition or pathological findings in other organs, was described. In the present case, the lesion was confined to the mediastinal lymph node and could have resulted in clinical ruminal tympany. The caudal mediastinal lymph nodes drain lymph from the pleura, the lungs, the esophagus, the pericardium, the mediastinum, the diaphragm, the peritoneum, the liver and the spleen [7], and thus, both intrathoracic and intra-abdominal organs could be the portal of entry of fungi in the lymph node. Meanwhile, zygomycetes tend to be confined to the lymphatic system in healthy young cattle; granulomatous lesions have been found only in mediastinal and bronchial lymph nodes in calves aged 4 to 6 months [3]. Hence, respiratory organs may be the main portals of entry of fungi in young cattle. In the present case, although no hyphae were observed in the tissue section of abomasum and the small intestine, fungal DNA was detected in the affected lymph node and abomasum but not in the small intestine. Therefore, the possibility of prior infection in the abomasum cannot completely be rejected, even though hyphae were not seen in the tissue sections. Though, since zygomycetes are members of the normal rumen flora [9], fungi attached to the abomasal mucosa may be detected.

Many cases of zygomycotic lymphadenitis produce no clinical symptoms, and the lesions are detected incidentally in many cases at slaughter [6]. In the present case, the enlarged mediastinal lymph node likely resulted in chronic ruminal tympany, which suggests that the present case was a rare pathological condition. The enlargement of the caudal mediastinal lymph node has been reported to be one of the important causes of chronic ruminal tympany, because the enlarged lymph node may press on the esophagus or interfere with vagal control of gastric function [4]. One of three pathogenic bacteria, *Arcanobacterium pyogenes*, *Actinobacillus* spp. or *Mycobacterium* spp., could cause enlargement of the mediastinal lymph node [13]. Although antibiotics, such as penicillin-streptomycin mixtures, are prescribed for the treatment of unexplained chronic ruminal tympany, these pathogens do not always respond to treatment [13]. In the present case, penicillin was administered after the rumenotomy; however, ruminal tympany persisted, despite the treatment. Therefore, the response to antibiotic treatment cannot assist in the diagnosis of unexplained chronic ruminal tympany, so mycotic infection in the mediastinal lymph node should be considered as a differential diagnosis of chronic ruminal tympany that is unresponsive to antibiotic treatment.

Histopathologically, the lesions of mycotic lymphadenitis have been characterized as amorphous necrosis with calcification, fibrosis and infiltration of neutrophils, macrophages, lymphocytes and multinucleated giant cells [3, 11]. The lymphadenitis in the present case was a necrotizing granulomatous inflammation, and the histopathological features of the lesion were similar to those in the earlier studies. Additionally, multinucleated giant cell and eosinophil infiltration was observed dominantly. When filamentous fungi are observed in tissue sections, a major differentiation must be made among the zygomycetes and other filamentous fungi, such as *Aspergillus* spp. and *Candida* spp. [14]. While zygomycetes produce wide and aseptate hyphae with wide-angle branching, *Aspergillus* spp and *Candida* spp. present consistently thin hyphae or pseudohyphae that branch at acute angles. Additionally, sporulation and blastoconidia may be seen in tissues infected with *Aspergillus* spp. and *Candida* spp., respectively [14]. In the present case, the fungal hyphae presented the morphological features of zygomycetes, such as poor septate, non-uniform width and wide-angle branching hyphae. In differentiation of mucomycosis and entomophthoramycosis, blood vessel invasion, thrombosis, tissue necrosis, acute inflammation and dissemination are characteristically seen in mucomycosis, while these features are not typically observed in entomophthoramycosis; furthermore, chronic inflammation is generally seen, and phagocytized hyphae or hyphae surrounded by Splendrore-Hoeppli material can be seen in entomophthoramycosis [14]. Although the two diseases, mucomycosis and entomophthoramycosis, present different clinical manifestations in human infections, it is difficult to differentiate the 2 orders of fungi based on histopathological examination or epidemiologic background [2]. In the present case, the histopathological findings included features of both mucormycosis and entomophthoramycosis. Additionally, the fungal species was not identified, so we used the term “zygomycetes” in the diagnosis. In the present case, no fungal species with high homology to the sequence of the sample were found. Possible causes include a defect in primer or template due to extraction from formalin-fixed paraffin-embedded tissue,

because a full-length sequence was not obtained.

Although the portal of entry of the pathogen was not identified, we showed a case of tuberculous-like lymphadenitis due to zygomycetes infection in the caudal mediastinal lymph node of a steer. The clinical and pathological findings in the present case suggest that zygomycetes infection in the caudal mediastinal lymphadenitis should be considered as a possible cause of recurrent ruminal tympany.

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