



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



A.P.P. Teh<sup>a</sup>, T. Hirai<sup>a,\*</sup>, S. Ito<sup>a</sup>, Y. Hidaka<sup>b</sup>, Y. Goto<sup>c</sup>, H. Furukawa<sup>d</sup>, J. Sawada<sup>d</sup>, R. Yamaguchi<sup>a</sup>

<sup>a</sup> Department of Veterinary Pathology, Faculty of Agriculture, University of Miyazaki, Gakuen-kibanadai-nishi-1-1, 889-2192 Miyazaki, Japan

<sup>b</sup> Department of Veterinary Surgery, Faculty of Agriculture, University of Miyazaki, Gakuen-kibanadai-nishi-1-1, 889-2192 Miyazaki, Japan

<sup>c</sup> Department of Veterinary Microbiology, Faculty of Agriculture, University of Miyazaki, Gakuen-kibanadai-nishi-1-1, 889-2192 Miyazaki, Japan

<sup>d</sup> NOSAI Miyakonojo Agricultural Insurance Association, Kamikawahigashi 3-10-8, Miyakonojo-shi, 885-0012 Miyazaki, Japan

### ARTICLE INFO

#### Keywords:

Bovine  
*Lichtheimia corymbifera*  
Lymphogenous route  
Mucormycosis

### 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 entomophthoromycosis usually occur as opportunistic fungal infections mainly seen in immunosuppressed hosts [1]. Due to clinicopathological and histomorphological different between mucormycosis and entomophthoromycosis, 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 *Mycocladius corymbifera*) in zygomatous 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  $\gamma$ -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

\* Corresponding author.

E-mail address: [t-hirai@cc.miyazaki-u.ac.jp](mailto:t-hirai@cc.miyazaki-u.ac.jp) (T. Hirai).

<https://doi.org/10.1016/j.mmcr.2018.04.001>

Received 8 March 2018; Received in revised form 19 March 2018; Accepted 16 April 2018  
Available online 17 April 2018

2211-7539/ © 2018 The Authors. Published by Elsevier B.V. on behalf of International Society for Human and Animal Mycology. This is an open access article under the CC BY-NC-ND license (<http://creativecommons.org/licenses/by-nc-nd/4.0/>).



**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.

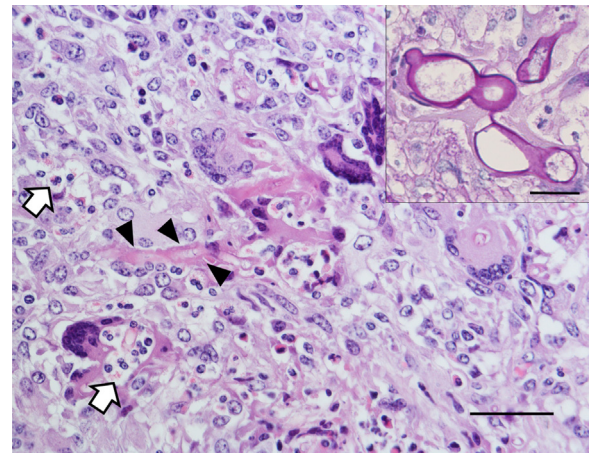
on the morphological features of hyphae, a tentative diagnosis of fungal granuloma caused by zygomycetes was made.

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



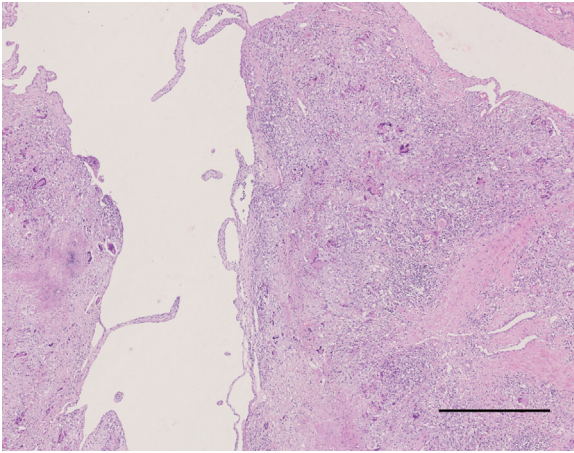
**Fig. 2.** Thin-walled, non-parallel and sparsely septate hyphae are seen scattered throughout granulomatous lesions, especially in the cytoplasm of multinucleated giant cells with infiltration of eosinophils (arrows), lymphocytes and plasma cells. Some eosinophils are phagocytosed by giant cells. Degranulation 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.

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.



**Fig. 3.** There are significantly dilated lymph vessels with marked granulomatous inflammation surrounding the vessels. Bar, 500  $\mu$ m.

DNA sequencing and molecular phylogenetic tree analysis on the detected fungi were carried out using the obtained amplification products, and estimation of related species was attempted. As a result, the specimen showed 100% homology with *L. corymbifera* within a 513-bp region of the 28S rRNA gene of strain AF113446 in particular (Fig. 4).

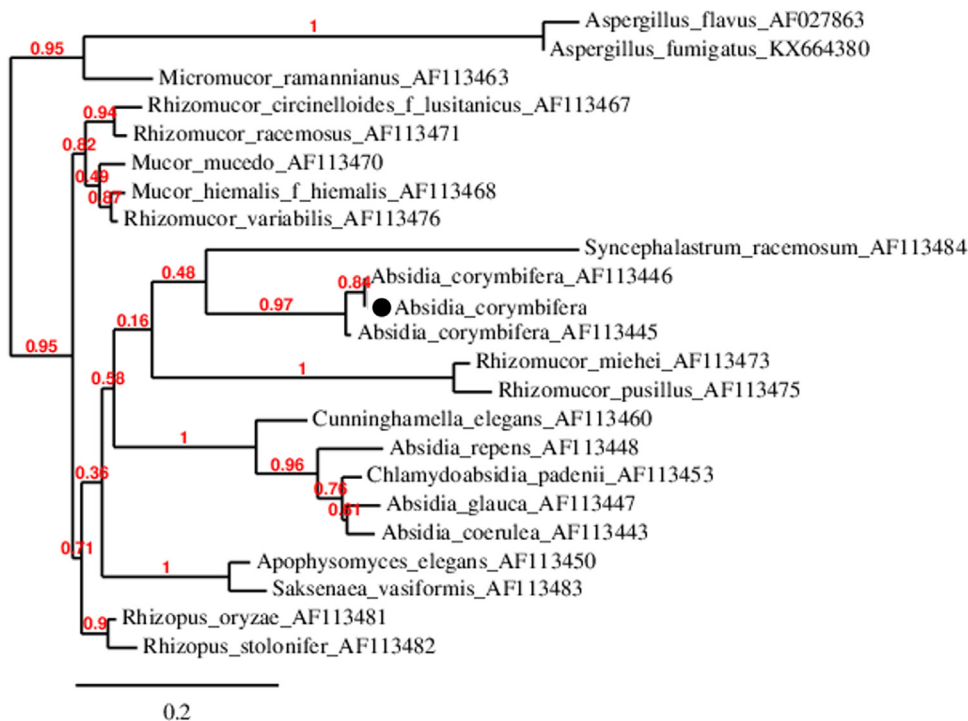
### 3. Discussion

Mucormycetes of clinical and veterinary importance, including *Rhizopus*, *Mucor*, *Lichtheimia*, and *Saksenaea*, have been reported in dogs, cats, horses, llamas, sheep, and cattle, and there is variance in pathogenicity. *L. corymbifera* has been reported to be a causative pathogen in cutaneous infection [14], abortion [12] and systemic infection [15] in horse; deep infection of the rhinocerebral and respiratory tract in human [13]; and mycotic abortion [11], zygomatic gastroenteritis [8] and lymphadenitis [9,10] in cattle. While mucormycosis usually occurs as an opportunistic infection in hosts with impaired immunity and underlying diseases, such infection can also occur in

healthy hosts. In this case, the calf was thought to have impaired immunity since birth due to stress connected with cold winter season. In addition, the farm was reported having a few cases of calves with low  $\gamma$ -globulin level. The factors that cause low  $\gamma$ -globulin level in calves can be due to inadequate consumption of colostrum, calf born to a heifer under stress condition and calf dam which fed with low crude protein diet prior to parturition [16]. Thus, these environmental and nutritional factors may have led to a compromised immune system in this calf and predisposed it to mucormycosis.

Histologically, lesions in mucormycosis and entomophthoromycosis are generally quite similar, and the hyphae in the coagulative necrosis foci appear “ghost-like” [3]. Morphologically, it is considered impossible to distinguish the type of hyphae by HE staining alone. However, Entomophthorales such as *Conidiobolus* spp. tend to produce a thicker (2.5–25  $\mu$ m) eosinophilic sleeve (Splendore-Hoeppli substance) [17]. There are no clear reports about Splendore-Hoeppli features in mucormycosis. It is possible that eosinophilic sleeves formed in mucormycosis are thin or absent, such as demonstrated in this case. Thus, this may be an important feature to differentiate mucormycosis from entomophthoromycosis.

Eosinophilic infiltration was thought to be remarkable in this case as previous studies noted neutrophilic infiltration as the main defense mechanism in mucormycosis [7]. The role of eosinophils in fungal infections is thought to be degranulation of recruited eosinophils caused by interleukin (IL)-4 and IL-10 secreted by CD4-positive T lymphocytes [18]. Hyphae are coated by granules from eosinophils and form a thin eosinophilic sleeves. The thin eosinophilic sleeves have two main opposing effects: either it will aid in phagocytosis of hyphae by multinucleated giant cells or it will lead to chronicity of infection due to prevention of phagocytosis and intracellular killing. Phagocytosed eosinophils were also seen in multinucleated giant cells in this case. One previous study of emphysematous eosinophilic lymphangitis in cattle revealed that phagocytic removal of apoptotic eosinophils promoted the resolution and repair process of eosinophilic inflammation of allergic origin [19]. However, the exact roles and mechanisms of eosinophils in fungal infection are still in debate. Additionally, the compromised immune status in this case may have also contributed to the altered inflammatory response.



**Fig. 4.** Phylogenetic analysis of fungal sequences obtained from tissue samples from the Japanese Black calf with extensive swelling in the neck region compared to sequences of representative zygomycetes showed high homology with *Lichtheimia (Absidia) corymbifera*. Numbers at nodes represent bootstrap percentages from 1000 replications. The isolate from this investigation is indicated by a black circle (●).

Mucormycosis is generally considered to have high affinity for blood vessels and it is said to be more likely to cause vascular lesions and vascular invasion than other fungal diseases [3]. However, in this case, although vasculitis and vascular invasion were seen, typical fungal emboli were not observed. The calf had no history of skin trauma and no significant contamination of skin wounds. Most of the masses had no existing normal structures left because of extensive granulomatous inflammation. However, since lymphoid follicle-like structures and dilated lymph vessels were found in part of the lesion, a lymph node origin was highly considered. Some masses resected during the second surgery were presumed to be part of existing lymph nodes such as lateral retropharyngeal lymph nodes. Based on a previous study, most cattle with mycotic lymphadenitis had involvement of mesenteric lymph nodes, and lymphatic spread of hyphal elements through direct invasion or phagocytosis by the mucosal defense system from intestinal mucosa was speculated [10]. Zygomycetes also tend to be found in the lymphatic system in healthy young cattle [9]. Lymphangitis and lymph vessel proliferation were prominent and thought to be the main route of fungal spread in this case. We speculated that fungi were orally engulfed from the environment or among normal flora that was regurgitated to the oral cavity through oral mucosa to lateral retropharyngeal lymph nodes via the lymphatic vessels. Subsequently, extensive granulomatous inflammation occurred along the efferent lymphatic pathway and the lesions spread to adjacent neck muscles and tendons, leading to the characteristic nodular appearance in this case. On day 120, the calf has completely recovered after the second surgery, thus lesions in other organs or involvement of other lymph nodes and blood vessels remain unclear.

In conclusion, lymphatic spread of mucormycosis caused by *Lichtheimia* might play a greater role in disease spread than dissemination through blood vessels. However, various factors such as host immune response may have contributed to the difference in pathogenesis observed in this case.

#### Conflict of interest

None declared.

#### References

- [1] L. Mendoza, R. Vilela, K. Voelz, A.S. Ibrahim, K. Voigt, S.C. Lee, Human fungal pathogens of mucorales and entomophthorales, *Cold Spring Harb. Perspect. Med.* 5 (2015) a019562.
- [2] K.J. Kwon-Chung, Taxonomy of fungi causing mucormycosis and entomophthoromycosis (zygomycosis) and nomenclature of the disease: molecular mycologic perspectives, *Clin. Infect. Dis.* 54 (2012) 8–15.
- [3] E.A. Mauldin, J. Peters-Kennedy, Subcutaneous fungal infections, in: M.G. Maxie (Ed.), *Jubb, Kennedy & Palmer's Pathology of Domestic Animals Volume 1, 6th ed.*, Elsevier, Missouri, 2016, pp. 653–661.
- [4] M. Nishimura, Y. Toyota, Y. Ishida, H. Nakaya, K. Kameyama, Y. Nishikawa, et al., Zygomycotic mediastinal lymphadenitis in beef cattle with ruminal tympany, *J. Vet. Med. Sci.* 76 (2014) 123–127.
- [5] T. Ikeda, K. Tabuchi, K. Shiota, Y. Ue, Y. Nomura, Mucormycosis in a cow, *Jpn. J. Vet. Sci.* 49 (1987) 527–530.
- [6] Y. Chihaya, K. Matsukawa, S. Mizushima, Y. Matsui, Ruminant forestomach and abomasal mucormycosis under rumen acidosis, *Vet. Pathol.* 25 (1988) 119–123.
- [7] Y. Chihaya, H. Okada, K. Matsukawa, Y. Matsui, Disseminated mycoses in cattle. A study on nine autopsy cases, *J. Vet. Med. Sci.* 54 (1992) 485–491.
- [8] H.E. Jensen, S.N. Olsen, B. Aalbek, Gastrointestinal aspergillosis and zygomycosis of cattle, *Vet. Pathol.* 31 (1994) 28–36.
- [9] W.M. Dion, A. Bundza, T.W. Dukes, Mycotic lymphadenitis in cattle and swine, *Can. Vet. J.* 28 (1987) 57–60.
- [10] J. Ortega, F.A. Uzal, R. Walker, H. Kinde, S.S. Diab, F. Shahriar, et al., Zygomycotic lymphadenitis in slaughtered feedlot cattle, *Vet. Pathol.* 47 (2010) 108–115.
- [11] C. Piancastelli, F. Ghidini, G. Donofrio, S. Jottini, S. Taddei, S. Cavirani, et al., Isolation and characterization of a strain of *Lichtheimia corymbifera* (ex *Absidia corymbifera*) from a case of bovine abortion, *Reprod. Biol. Endocrinol.* 7 (2009) 138.
- [12] D.H. Schlafer, R.A. Foster, Female genital system, in: M.G. Maxie (Ed.), *Jubb, Kennedy & Palmer's Pathology of Domestic Animals Volume 1, 6th ed.*, Elsevier, Missouri, 2016, pp. 417–419.
- [13] B. Spellberg, J.J. Edwards, A. Ibrahim, Novel perspectives on mucormycosis: pathophysiology, presentation, and management, *Clin. Microbiol. Rev.* 18 (2005) 556–569.
- [14] J. Lopez-Sanroman, M.J. Paya, M.T. Cutuli, J.L. Gonzalez, Cutaneous mucormycosis caused by *Absidia corymbifera* in a horse, *Vet. Dermatol.* 11 (2000) 151–155.
- [15] J. Guillot, C. Collobert, H.E. Jensen, M. Huerre, R. Chermette, Two cases of equine mucormycosis caused by *Absidia corymbifera*, *Equine Vet. J.* 32 (2000) 453–456.
- [16] E. Toghyani, A. Moharrery, Effect of various levels of dietary protein in transition period on colostrum quality and serum immunoglobulin concentration in Holstein cows and their newborn calves, *Ann. Anim. Sci.* 15 (2015) 493–504.
- [17] P.J. Quinn, B.K. Markey, F.C. Leonard, E.S. Fitzpatrick, S. Fanning, E.S. Fitzpatrick, Zygomycetes of veterinary importance, in: P.J. Quinn, et al. (Ed.), *Veterinary Microbiology and Microbial Disease, 2nd ed.*, Wiley-Blackwell, Iowa, 2011, p. 450.
- [18] M.R. Hussein, Mucocutaneous Splendore-Hoeppli phenomenon, *J. Cutan. Pathol.* 35 (2008) 979–988.
- [19] S. Ohfuji, Emphysematous eosinophilic lymphangitis in the ruminal submucosa of cattle, *Vet. Pathol.* 52 (2015) 1163–1166.