

Variations in the morphology of *Rhizomucor pusillus* in granulomatous lesions of a Magellanic penguin (*Spheniscus magellanicus*)

Fumiko SUZUTA¹⁾, Kumiko KIMURA^{2)*}, Ryo URAKAWA¹⁾, Yukio KUSUDA³⁾, Shogo TANAKA⁴⁾, Yasuko HANAFUSA²⁾ and Makoto HARITANI²⁾

¹⁾Nagasaki Prefecture Central Livestock Hygiene Service Center, Kaizumachi, Isahaya, Nagasaki 854-0063, Japan

²⁾National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Kannondai, Tsukuba, Ibaraki 305-0856, Japan

³⁾Nagasaki Penguin Aquarium, Shukumachi, Nagasaki, Nagasaki 851-0121, Japan

⁴⁾Kyushu Research Station, National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Chuzan, Kagoshima, Kagoshima 891-0105, Japan

(Received 7 October 2014/Accepted 18 March 2015/Published online in J-STAGE 18 April 2015)

ABSTRACT. This report presents a new case of mucormycosis encountered in penguin characterized by morphological variation of hyphae and presence of sporangia with numerous sporangiospores. A 4.5-year-old Magellanic penguin (*Spheniscus magellanicus*) died after exhibiting anorexia, poor nutritional condition and dyspnea. Multiple nodular lesions were observed in the thoracic and abdominal regions. Histopathologically, hyphae of various sizes were seen in the lungs, air sac and nodular lesions. Myriad sporangiospores and several sporangia were observed in/around the bronchi or parabronchi. The very narrow and short hyphae in the nodules were not consistent with the characteristics of *Mucorales*. However, for most hyphae, including those in the nodules, sporangiospores and sporangia, immunohistochemistry revealed *Mucorales*-positive reactions. In addition, these fungi were identified as *Rhizomucor pusillus* by gene analysis.

KEY WORDS: gene diagnosis, immunohistochemistry, penguin, *Rhizomucor pusillus*, sporangiospore

doi: 10.1292/jvms.14-0527; *J. Vet. Med. Sci.* 77(8): 1029–1031, 2015

Mycosis is one of the opportunistic infections affecting mammals and birds. Among the various species giving rise to this condition, aspergillosis, mucormycosis and candidiasis are considered major diseases. *Aspergillus* spp. are the most common cause of mycosis in birds, accounting for many cases of respiratory infections [1, 13]. There are also a few reports of candidiasis or mucormycosis in chickens, ostriches, ducks and parrots, affecting the lungs, air sacs, liver, spleen and skeletal muscle [4, 6, 8–10]. In penguins, several cases of aspergillosis have been reported [5, 15]. With respect to mucormycosis, only one case has been recorded so far [2], but the genus of *Mucoraceae* could not be identified. Here, we present a new case of mucormycosis encountered in a penguin and characterized by unique morphological variation of the hyphae and the presence of numerous sporangiospores. We investigated the case in detail using immunohistochemistry and genetic analysis.

A 4.5-year-old Magellanic penguin in a flock of 160 penguins showed anorexia, poor nutritional condition and dyspnea. Despite treatment with antibiotics, antifungals and gastrointestinal drugs, the penguin died within 6 weeks.

Necropsy revealed multiple nodules up to 5 mm in diam-

eter over the dorsal side of the lung surface, and multiple nodules of 1 to 2 mm in diameter were seen on organ sections (Fig. 1). In addition, multiple nodules of 5 to 10 mm in diameter were found in the abdominal region. The center of some nodules exhibited coagulative necrosis or cavitations. The air sacs were opaque.

For histopathological examinations, tissues were collected, fixed with 10% neutral buffered formalin and then embedded in paraffin wax. Paraffin sections were cut and stained with hematoxylin and eosin (HE), periodic acid-Schiff (PAS), Grocott-Gomori methenamine-silver and a Fungiflora Y staining kit (Biomate Co., Ltd., Tokyo, Japan).

Histologically, granulomas were observed in the lungs, air sacs and abdomen nodules. There were two types of granu-

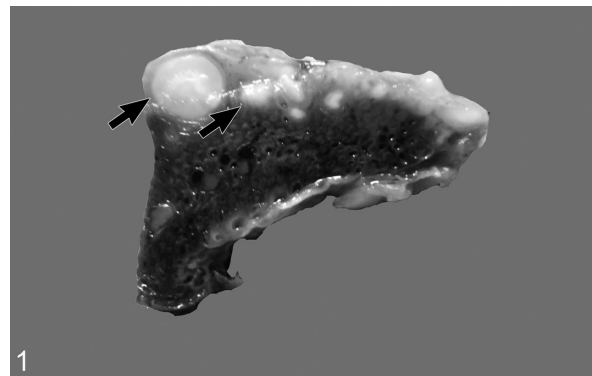


Fig. 1. The cut surface of the lung. There were multiple nodules (arrows) in the dorsal area.

*CORRESPONDENCE TO: KIMURA, K., National Institute of Animal Health, National Agriculture and Food Research Organization (NARO), Kannondai, Tsukuba, Ibaraki 305-0856, Japan.
e-mail: kumita@affrc.go.jp

©2015 The Japanese Society of Veterinary Science

This is an open-access article distributed under the terms of the Creative Commons Attribution Non-Commercial No Derivatives (by-nc-nd) License <<http://creativecommons.org/licenses/by-nc-nd/3.0/>>.

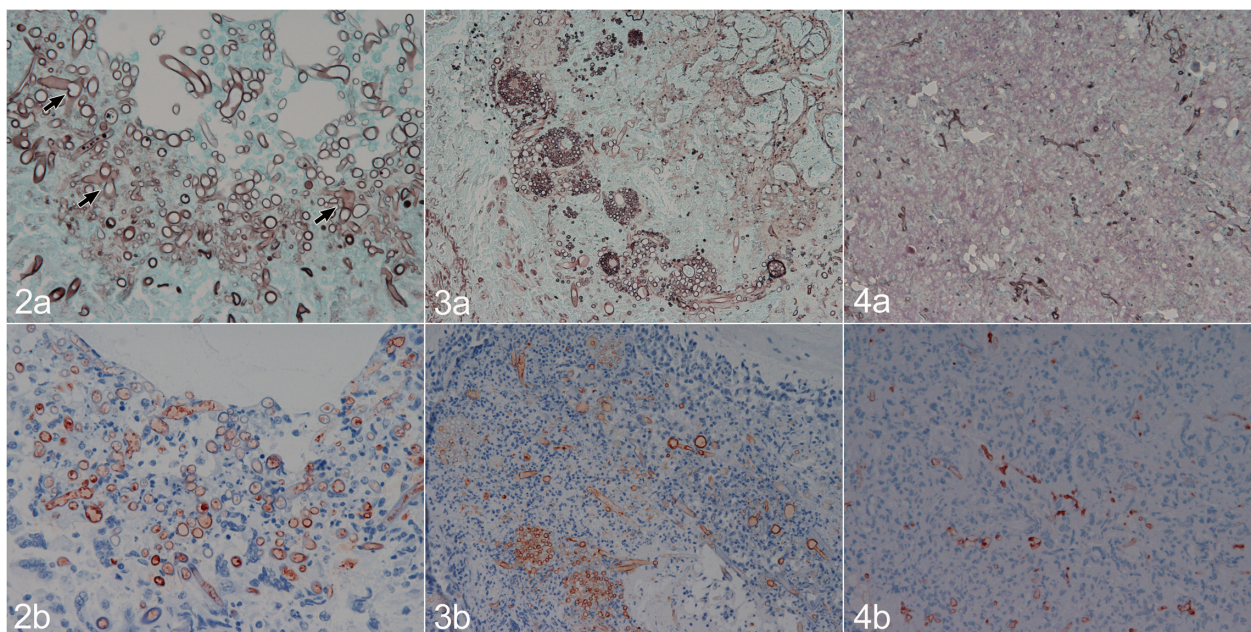


Fig. 2. Fungal granuloma in the lung. (a) Many broad hyphae and sporangiospores were observed in the parabronchi. A few right angle branchings of hyphae (arrows) were also seen. Grocott-Gomori methenamine-silver stain. (b) Broad hyphae and sporangiospores reacted with anti-*Rhizopus* antibody. Immunohistochemistry for *Mucoraceae*.

Fig. 3. Fungal granuloma in the lung. (a) Many sporangia and sporangiospores were observed in the parabronchi. Grocott-Gomori methenamine-silver stain. (b) Sporangia, sporangiospores and broad hyphae reacted with anti-*Rhizopus* antibody. Immunohistochemistry for *Mucoraceae*.

Fig. 4. Fungal granuloma in the lung. (a) Narrow and short hyphae were scattered throughout the nodular necrotic lesion in the lung. Grocott-Gomori methenamine-silver stain. (b) The narrow hyphae reacted with anti-*Rhizopus* antibody. Immunohistochemistry for *Mucoraceae*.

lomas in the lungs. One type was seen around bronchi or parabronchi, and the other type, which was macroscopically identified as nodules, was composed of necrosis encapsulated with fibrous tissues. In the former granulomas, bronchi and parabronchi were obliterated or necrosed with many hyphae and sporangiospores with a few sporangia. Macrophages, epithelioid cells, multinucleated giant cells, lymphocytes and fibrous tissue surrounded them. Some granulomas bridged with each other. The hyphae were heterogeneous and had relatively broad widths ranging normally from 3 to 8 μm , with some having widths of more than 10 μm , although this was rare. Most of the hyphae were not septate. There were a few right angle branchings (Fig. 2a). Sporangiospores were 2 to 8 μm in diameter. There were several round sporangia of about 50 μm in diameter with round columellae (Fig. 3a). The latter granulomas were composed of severe necrosis with scattered hyphae, which were narrow (1 to 3 μm in width) and short with irregular branching (Fig. 4a). The hyphae were 5 to 6 μm in width and were rarely seen in the periphery of the nodular lesions. No sporangiospores were seen. A few conidial heads of *Aspergillus* spp. (*aspergilla*) and hyphae with parallel walls and septa and foreign bodies were found in bronchi or parabronchi. However, they did not invade the parenchyma.

The lesions of the air sacs were similar to bronchial and parabronchial lesions. Lesions in the nodules in the abdomen were similar to nodular lesions of the lungs. No vascular le-

sions were seen at any of these sites.

In sections stained with the Fungiflora Y staining kit, the narrow and short hyphae seen in the necrotic tissue of nodular lesions in the lungs and abdominal nodules had a positive reaction. However, broad hyphae and sporangiospores showed a negative reaction. *Aspergilla* were extremely rare and exhibited positive reactions with Fungiflora Y.

Immunohistochemistry was carried out using mouse anti-*Aspergillus* spp. monoclonal antibody (WF-AF-1; AbD Serotec, Oxford, U.K.), mouse anti-*Rhizopus arrhizus* monoclonal antibody (WSSA-RA-1; AbD Serotec) and rabbit anti-*Candida albicans* type A polyclonal antibody (AbD Serotec) together with a Simple Stain Multi kit (Nichirei, Tokyo, Japan). Antigen retrieval pretreatment was done by immersing the sections in 0.1% actinase E (Kaken Pharmaceutical, Tokyo, Japan) for 5 min at 37°C (anti-*Aspergillus* monoclonal Ab) or for 20 min at 37°C (anti-*Rhizopus* monoclonal Ab and anti-*Candida* polyclonal Ab). As positive controls, one slide of a paraffin-embedded culture fungi array including *A. fumigatus*, *Lichtheimia corymbifera* (*Absidia corymbifera*), *Rhizomucor miehei*, *C. albicans*, *C. tropicalis*, *Lecytophora hoffmannii* and *Trichosporon asahii* was used.

Most hyphae and sporangiospores were positively stained only with anti-*Rhizopus* monoclonal antibody (Figs. 2b–4b). *Aspergilla*, which are rarely seen in bronchi or parabronchi, exhibited positivity for anti-*Aspergillus* monoclonal antibody.

For isolation of fungi, the air sacs, lungs and abdominal nodules were cultured on potato dextrose agar at 36°C and 52°C. The white fluffy colonies were observed a few days after the start of incubation. Microscopically, the hyphae were broad and infrequently septate, with sporangia and rhizoids, which were characteristic features of *Rhizomucor*.

In addition, the DNA of isolated fungi was extracted using a QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) for identification of the fungi. The internal transcribed spacer (ITS) region of the rRNA gene was amplified using two primers: ITS1, 5'-TCC GTA GGT GAA CCT GCG G-3', and ITS4, 5'-TCC TCC GCT TAT TGA TAT GC-3' [14]. The PCR products were sequenced using an Applied Biosystems 3130xl genetic analyzer (Life Technologies Corp., Carlsbad, CA, U.S.A.), and a BLAST search of the GenBank database was used for analysis of the sequences. The sequences of isolated fungi had 100% homology with *Rhizomucor pusillus* strain CNRMA03.1205 in GenBank (DQ119000.1).

In the present case of mucormycosis, histopathologically, there were various widths of hyphae from narrow to broad. In close-packed regions, most of the hyphae were narrow; a few broad hyphae, which were characteristic of mucormycetes, were scattered throughout. In the present case, no broad hyphae were observed in nodular lesions. Narrow hyphae with short irregular branching were 1 to 3 µm in width and reacted positively to Fungiflora Y staining, which is a sensitive method to detect the cell wall components of most fungi, except for those of Mucoraceae. Although the possibility of mixed infection of fungi was suspected, because some case reports of mycosis indicated mixed infection with other fungi or bacteria [4, 10, 11], immunohistochemical examination revealed that all the fungi, excluding the rarely seen aspergilla, were Mucoraceae. The degeneration of fungi resulting from caseous necrosis may lead to an ambiguous reaction, as most of the narrow hyphae that exhibited positive reactions with Fungiflora Y were observed in or around the areas of necrosis.

Another characteristic finding of this case was that a great many sporangiospores and several sporangia were seen in the bronchi and parabronchi. Although many cases of mucormycosis of the lungs have been reported [3, 4, 8, 9, 12], sporangiospores have been seen in only a very few birds [3, 4] and in no domestic animals, except for poultry. The shapes of sporangiophores, sporangia and columellae are key to the identification of Mucoraceae [7], and the round sporangia and round columellae in the present case were consistent with those of *Rhizomucor* sp. The more abundant airflow in the respiratory systems of nonmammalian avian species might provide a more aerobic environment that encourages the fungi to produce sporangia. It is also probable that certain fungi tend to form sporangia in tissue.

In the present case, a *Rhizomucor* sp. was isolated in culture and was identified as *Rhizomucor pusillus* by gene analysis. Histopathologically, it was impossible to identify the genus of Mucoraceae, and therefore, the pathological features of each zygomycete were obscure. Pathological analysis, together with immunohistochemistry and gene analysis, might be able to clarify the characteristic lesions of

various zygomycetes.

ACKNOWLEDGMENTS. We are grateful to all staff of the Chuou Livestock Hygiene Center, Nagasaki, Japan. We would also like to thank Mr. K. Kawasaki for his technical help.

REFERENCES

1. Arné, P., Thierry, S., Wang, D., Deville, M., Le Loc'h, G., Desoutter, A., Féménia, F., Nieguitsila, A., Huang, W., Chermette, R. and Guillot, J. 2011. *Aspergillus fumigatus* in Poultry. *Int. J. Microbiol.* **2011**: 1–14. [Medline] [CrossRef]
2. Bigland, C. H., Graesser, F. E. and Pennifol, K. S. 1961. An osteolytic mucor mycosis in a penguin. *Avian Dis.* **5**: 367–370. [CrossRef]
3. Dawson, C. O., Wheeldon, E. B. and McNeil, P. E. 1976. Air sac and renal mucormycosis in an African gray parrot (*Psittacus erithacus*). *Avian Dis.* **20**: 593–600. [Medline] [CrossRef]
4. Desmidt, M., De Laender, P., De Groote, D., Rysselaere, M., De Herdt, P., Ducatelle, R. and Haesebrouck, F. 1998. *Rhizomucor pusillus* mucormycosis combined with chlamydiosis in an African grey parrot (*Psittacus erithacus erithacus*). *Vet. Rec.* **143**: 447–448. [Medline] [CrossRef]
5. Flach, E. J., Stevenson, M. F. and Henderson, G. M. 1990. Aspergillosis in gentoo penguins (*Pygoscelis papua*) at Edinburgh Zoo, 1964 to 1988. *Vet. Rec.* **126**: 81–85. [Medline]
6. Jeffrey, J. S., Chin, R. P., Shivaprasad, H. L., Meteyer, C. U. and Droual, R. 1994. Proventriculitis and ventriculitis associated with zygomycosis in ostrich chicks. *Avian Dis.* **38**: 630–634. [Medline] [CrossRef]
7. Kern, M. E. and Bleiviins, K. S. 1997. Common fungal opportunists. pp. 75–113. *In: Medical Mycology: A Self-instructional text*, 2nd ed., F. A. Davis Company, Philadelphia.
8. McCaskey, P. C. and Langheinrich, K. A. 1984. Zygomycosis in the duck. *Avian Dis.* **28**: 791–798. [Medline] [CrossRef]
9. Migaki, G., Langheinrich, K. A. and Garner, F. M. 1970. Pulmonary mucormycosis (phycomycosis) in a chicken. *Avian Dis.* **14**: 179–183. [Medline] [CrossRef]
10. Muir, M. and Raidal, S. R. 2012. Necrotising ventriculitis due to combined infection with *Rhizopus microsporus* var. *chinensis* and *Candida krusei* in an eclectus parrot (*Eclectus roratus*). *Aust. Vet. J.* **90**: 277–280. [Medline] [CrossRef]
11. Mukaratirwa, S. 2006. Outbreak of disseminated zygomycosis and concomitant pulmonary aspergillosis in breeder layer cockerels. *J. Vet. Med. B Infect. Dis. Vet. Public Health* **53**: 51–53. [Medline] [CrossRef]
12. Munday, J. S., Laven, R. A., Orbell, G. M. B. and Pandey, S. K. 2006. Meningoencephalitis in an adult cow due to *Mortierella woifli*. *J. Vet. Diagn. Invest.* **18**: 619–622. [Medline] [CrossRef]
13. Tell, L. A. 2005. Aspergillosis in mammals and birds: impact on veterinary medicine. *Med. Mycol.* **43** Suppl 1: S71–S73. [Medline] [CrossRef]
14. White, T. J. B. T., Lee, S. B., Taylor, J. 1990. Amplification and Direct Sequencing of Fungal Ribosomal RNA Genes for Phylogenetics., pp. 315–324. *In: In PCR Protocols: A Guide to Methods and Applications*, 1st ed., Academic Press Inc., San Diego.
15. Xavier, M. O., Soares, M. P., Meinerz, A. R. M., Nobre, M. O., Osorio, L. G., da Silva, R. P. and Meireles, M. C. A. 2007. Aspergillosis: A limiting factor during recovery of captive Magellanic penguins. *Braz. J. Microbiol.* **38**: 480–484. [CrossRef]