Suppurative necrotizing bronchopneumonia caused by *Nocardia cyriacigeorgica* infection in a stranded striped dolphin (*Stenella coeruleoalba*) in Japan

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ABSTRACT. On a coastline in Miyazaki Prefecture, Japan, a wild subadult female striped dolphin was found dead. Necropsy revealed poor nutritional status and bilateral pneumonia, which was histologically diagnosed as severe suppurative necrotizing bronchopneumonia. Special staining detected numerous intralesional filamentous, branching bacteria, which was identified as *Nocardia cyriacigeorgica* by sequencing of 16S ribosomal RNA and gyrB genes. Other main histological findings included lymphoid depletion in the spleen and superficial cervical and pulmonary lymph nodes. Suppurative nocardiosis without a granulomatous reaction is uncommon, and it is assumed its pathogenesis was related to the host's immune status. This paper discusses the variable inflammatory response to nocardiosis and describes the first case of *N. cyriacigeorgica* infection in a wild striped dolphin in Japan.

KEY WORDS: dolphin, immunodeficiency, Japan, *Nocardia cyriacigeorgica*, suppurative bronchopneumonia

Nocardia spp. are aerobic, nonmotile, Gram-positive to Gram-variable actinomycetes. They are widely distributed in aquatic and terrestrial habitats, notably in soil, marine sediments, and wastewater systems. They form branching filaments that can fragment into pleomorphic rods or cocci. *Nocardia* partially stains positive with acid-fast stains due to the presence of mycolic acids in its cell wall [2, 9]. Nocardiosis is an uncommon disease and may occur in a wide range of terrestrial and aquatic animals, such as cats, dogs, cattle, dolphins, and fishes [9, 14]. Pathologically, pyogranulomatous lesions are the best-known pathogenicity in human and animal nocardioses [14]. Occasionally, clusters of epithelioid macrophages and multinucleated giant cells can be seen [20]. However, neutrophil infiltration remains the characteristic reaction [8]. Here, we report a case of pulmonary nocardiosis in a wild dolphin represented by non-granulomatous suppurative inflammation. In addition, we focus on the development of inflammatory lesions and discuss the pathogenesis along with a literature review. To our knowledge, this is the first report of *Nocardia cyriacigeorgica* infection in a wild striped dolphin in Japan.

A subadult wild striped dolphin (*Stenella coeruleoalba*) (female, body length: 207.3 cm) was found dead on a coastline in Mimitsu, Hyuga, Miyazaki, Japan (32°33'20" N, 131°61'7" E) in February 2015. Necropsy was performed at the Department of Veterinary Pathology, University of Miyazaki, the next day. According to the code system established by Geraci and Lounsbury to evaluate the carcass quality, the carcass of this case was assigned CODE 3- Fair based on the following gross findings: bloating, blubber blood-tinged, soft and black muscle, and gut distended by gas [13]. According to the body condition scoring (BCS) system for all dolphin species established by Joblon *et al.*, the dolphin was assigned to BCS 2- Thin based on the following gross findings: mild

concavity ventrolateral to dorsal fin and moderate depression posterior to blowhole [12]. At necropsy, approximately 80% of both lungs were diffusely affected by hard, whitish nodules, which were solid on cut surface (Fig. 1). Whitish pus was found in the bronchus and trachea. The pulmonary lymph nodes were enlarged. The thoracic cavity contained a small amount of dark reddish fluid (<50 ml). Small white cysts (approximately 5 mm in diameter) in the blubber around the back and urogenital opening and a large white cyst (approximately 40 mm in diameter) in the peritoneum of the rectum, which were the larval stage of tapeworms, were observed. Gastrointestinal contents were minimal and milky fluid. A few nematodes were also found in the pyloric stomach (length 50–90 mm). The abdominal cavity contained dark reddish fluid (approximately 100 ml). The central nervous system, including the brain and spinal cord, was not examined. Specimens of the lungs, liver, heart, intestine, spleen, kidneys, and superficial cervical and pulmonary lymph nodes were collected, fixed in 10% neutral buffered formalin, routinely processed, and embedded in paraffin wax. Sections (2–4 µm) were stained with hematoxylin and eosin. Selected tissue sections were also stained with Gram stain,

Fig. 1. Diffuse, whitish nodules are observed on the cut surface of the lung. Demarcation of the nodules is clear. Bar=5 cm.

Ziehl-Neelsen (ZN) stain, Fite's stain, and Grocott's methenamine silver (GMS) stain. Immunohistochemistry (IHC) was performed using primary antibodies listed in Table 1. Horseradish peroxidase (EnVision+ Dual Link System, Dako North America Inc.) was used to detect the primary antibodies and developed with 3,3′-diaminobenzidine solution (Sigma-Aldrich, St. Louis, MO, USA). Positive and negative control tissues were included in all staining procedures.

Parts of the excised lung lesion were submitted for microbiological analysis at the Department of Veterinary Microbiology, University of Miyazaki. Brain heart infusion agar plates, tryptic soy agar (TSA) plates, and TSA with 5% sheep blood (blood agar) plates were used for inoculation. Inoculated plates were incubated for 2–3 days at 37°C under aerobic and anaerobic conditions. DNA was extracted from cultured colonies by the boiling method, and polymerase chain reaction (PCR) and sequencing were performed. DNA was amplified by primers for the 16S ribosomal RNA gene, which amplifies a 661-bp fragment of 16S ribosomal RNA, and primers for the DNA gyrase subunit B (gyrB) gene, which amplifies a 691-bp fragment of gyrB [11, 23]. Tissue samples of the lung and spleen were also examined for morbillivirus nucleic acid by reverse transcription (RT)-PCR. A universal morbillivirus primer set, which is based on highly conserved regions of the morbillivirus phosphoprotein (P) gene and amplifies a 429-bp DNA fragment, was applied [1]. RT-PCR cycling conditions were as described by Stone *et al.* (2011) [19]. RNA extracted from canine distemper virus was used as a positive control, and nuclease-free water was used as a negative control.

Microscopic examination of the lung revealed severe necrosis with complete loss of the normal pulmonary structure and diffuse infiltration of degenerated or necrotized neutrophils into the bronchus, bronchiole, and alveoli (Fig. 2). Neutrophils also infiltrated into destructed bronchial cartilages (Fig. 2, inset). Fibrinous exudate was observed multifocally. Granulomatous lesions, composed of epithelioid macrophages, multinucleated giant cells, or lymphocytes, were not seen. In the less affected area around the lesions, slight infiltration of alveolar macrophages was observed (Fig. 3). The pulmonary lesions were histologically diagnosed as severe suppurative necrotizing bronchopneumonia. GMS staining revealed numerous filamentous branched organisms, which were distributed throughout the lesion including destructed bronchial cartilages (Fig. 4). They were positive for Gram staining, negative for ZN staining, and focally positive for Fite's staining. These organisms were not observed in alveolar macrophages. IHC examination revealed that there were very few macrophages expressing Iba-1 in inflammatory regions. Alveolar macrophages around the lesion were positive for Iba-1. T cells expressing CD3 were also rare, and B cells expressing CD20 were not seen. In the spleen and superficial cervical and pulmonary lymph nodes, severe lymphoid depletion was confirmed by a comparison with those in a normal cetacean (*Feresa attenuata*) (Fig. 5). Mild neutrophil infiltration was observed in the spleen and pulmonary lymph nodes, and bacteria same as those in the lungs were detected in the expanded lymph sinus by GMS staining. No significant changes were observed histologically in the other organs examined.

In microbiological analysis, white raised colonies were found on all inoculated plates. Gram-positive and weakly staining

Table 1. Primary antibodies used for immunohistochemistry in this study

| Antibody	Host	Dilution	Antigen retrieval	Catalog No.	Source
CD3	Rabbit	RU	Heat, pH 6.0	IR503	Dako, Tokyo, Japan
CD20	Rabbit	1:400	None	RB-9013-P1	Thermo Fisher Scientific, Waltham, MA, USA
Iba-1	Rabbit	1:250	Heat, pH 6.0	019–19741	Wako, Osaka, Japan

RU, ready to use.
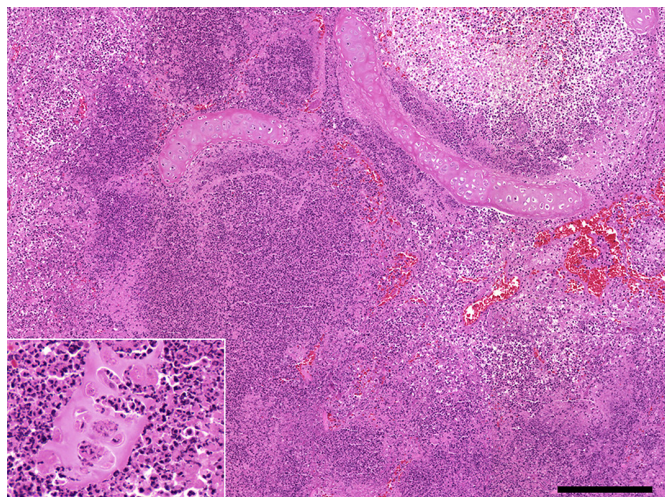
Fig. 2. Histological feature of lung. Lung is characterized by severe and diffuse neutrophilic infiltration in the bronchus, bronchiole, and alveoli. The normal structure is destroyed. Inset shows destructed bronchial cartilage. Hematoxylin and eosin staining. Bar=250 µm.
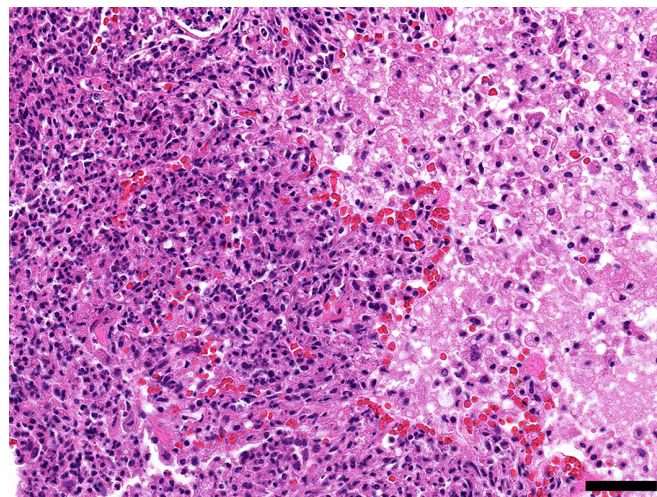
Fig. 3. Histological feature of peripheral area of the pulmonary lesions. This image shows infiltration of numerous neutrophils (left side), and infiltration of alveolar macrophages and fibrinous exudate in alveolar space around the lesion (right side). Hematoxylin and eosin staining. Bar=50 µm.
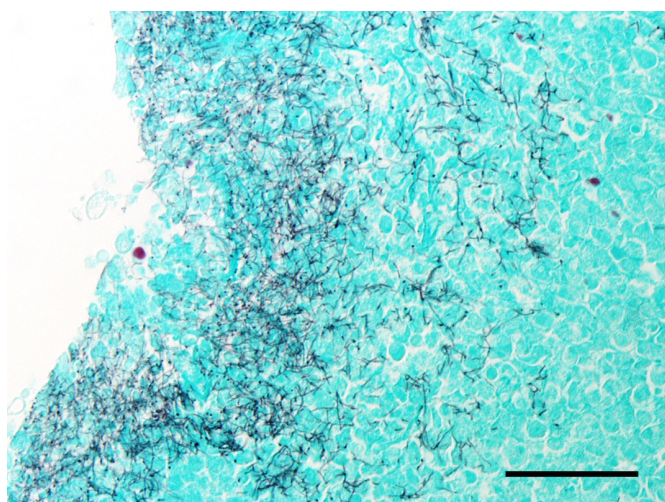
Fig. 4. Grocott's methenamine silver staining reveals numerous filamentous organisms (*i.e.*, *Nocardia cyriacigeorgica*) in pulmonary lesions. The organisms are radially distributed from the remained bronchial airways (space on the left). Grocott's methenamine silver staining. Bar=50 µm.
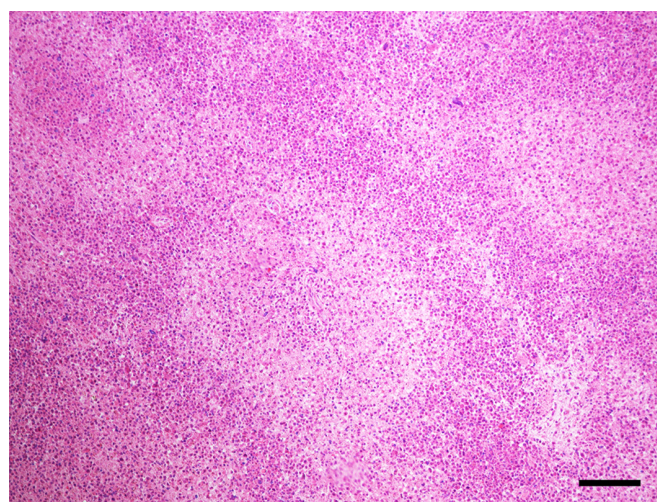
Fig. 5. Histological feature of spleen. Severe lymphoid depletion with ambiguous lymphoid follicles and an overall decrease in the numbers of lymphocytes are observed. Hematoxylin and eosin staining. Bar=100 µm.

acid-fast bacteria were detected on smears. Most bacteria were rod-shaped, while few bacteria were filamentous. Genetic analysis confirmed that colonies grown under aerobic conditions showed 99% nucleotide identity with *N. cyriacigeorgica*. In addition, three other bacterial species were identified from colonies grown under anaerobic conditions (Table 2). The result of RT-PCR for morbillivirus examination was negative.

Microbiological analysis supported histological findings of the lung. The filamentous bacteria within the lesions had characteristics consistent with *Nocardia* spp. Pier *et al.* (1970) and St Leger *et al.* (2009) reported 13 cases (11 captive and 2 wild) of nocardiosis in cetaceans [16, 18]. Of these cetacean cases, *N. asteroides* was isolated from 3 cases, *N. farcinica* from 2 cases, *N. brasiliensis* from 2 cases, *N. caviac* from 1 case, *N. levis* from 1 case, and *N. cyriacigeorgica* from 1 case. In the other 3 cases, the isolated bacteria are described only as *Nocardia* spp. Most cases had pulmonary involvement, and it was considered that inhalation and aspiration are the major infection routes for cetaceans [18]. *N. cyriacigeorgica* was first isolated from bronchial secretions of a human with chronic bronchitis in Germany in 2001 and forms an extensively branched substrate mycelium that fragments into rod-shaped elements at a late stage of growth [9, 25]. This morphological variation was confirmed in this case; the organisms formed

Table 2. Bacterial species identified from each cultural condition and culture medium

Cultural condition	Culture medium	Identified bacterial species
Aerobic	BHIA	*Nocardia cyriacigeorgica*
	TSA	
	TSA with blood	
Anaerobic	BHIA	*Clostridium* sp.
	TSA	*Clostridium sordellii*
	TSA with blood	*Shewanella* sp.

BHIA, brain heart infusion agar; TSA, tryptic soy agar.

filamentous shapes in tissue sections and rods in smears.

Nocardiosis is an opportunistic infection that usually occurs in immunocompromised hosts [8]. Previously, *N. cyriacigeorgica* infection was reported in a neonate beluga whale [18]. This report and lymphoid depletion in the spleen and lymph nodes support the possibility that immunosuppression participated in the onset of nocardiosis in our case. In general, nocardiosis pathologically induces pyogranulomatous inflammation, which is a feature used to aid in its diagnosis. A recent report concluded that *N. cyriacigeorgica* should be considered a potential cause of systemic pyogranulomatous lesions in dogs [6]. Even in cetacean nocardiosis, granulomatous reactions with suppuration are considered essential features [15]. The inflammatory pathogenesis in nocardiosis has been studied experimentally, as described below [2, 7, 17]. After the organism enters a host, an early inflammatory response with mobilization of neutrophils begins to inhibit growth of the organisms and limit the spread of infection. Subsequently, cell-mediated immunity (CMI) is triggered by activated macrophages and T cells cause direct lymphocyte-mediated toxicity to the organism. The host ultimately releases antibodies and/or lymphocyte signals, enabling phagocytic cells to kill the organism. Pathologically, suppurative inflammation dominated by neutrophils is observed in the acute stage, and granulomatous inflammation characterized by predominantly macrophage and T cell infiltration is observed in subacute and chronic stages. Because the CMI response following neutrophil recruitment is rapidly induced, spontaneous cases of nocardiosis are usually encountered during the subacute or chronic stage.

Pneumonia in the current case, however, was characterized by a lack of granulomatous reaction. IHC revealed that there were only a few macrophages and lymphocytes in the lesion. This finding is particular to this case and is not seen in typical nocardiosis. Although a few reports described nocardiosis in which neutrophil infiltration was prominent, rather than granulomatous inflammation [4, 5, 10, 24], most of them did not mention or discuss its histogenesis. Interestingly, experimental studies of nocardiosis demonstrate that, unless antimicrobial agents are given or CMI takes over, the infection progresses with predominantly neutrophil infiltration, as seen in early lesions [2, 7, 17]. A study of experimental infections of mice with *N. asteroides* indicated that, in CMI-deficient mice caused by administration of cyclosporin A or cortisone acetate, the early inflammatory response with infiltration of neutrophils and few mononuclear cells was continuously maintained, and the numbers of viable bacteria remained significantly greater than those in control mice [7]. CMI is important for effective eradication of *Nocardia* from the lungs, especially after the first few days of infection. For hosts in which the CMI response is inadequate, the presence of neutrophils may account for the characteristic indolent nature of nocardiosis [15].

In the present case, chronic histological changes such as fibrosis and angiogenesis were not observed. Thus, the pneumonia seems to be in the acute or subacute stage histologically. In general, even in an immunocompromised state, CMI is triggered to eliminate the organism in nocardiosis. However, in the present case, severe immunodeficiency characterized by lymphoid depletion occurred and induction of CMI could be impaired. Considering the pathogenesis of nocardiosis mentioned above, severe immunodeficiency with CMI suppression could participates in the presentation characterized by non-granulomatous suppurative inflammation and the spread to most of the lung in the present case. Furthermore, it is assumed that the host could not eliminate *Nocardia* due to inadequate CMI, resulting in exacerbation of pneumonia and death. An *in vitro* study has demonstrated that activated macrophages kill substantial numbers of *Nocardia*, whereas resident (unstimulated) macrophages have little or no effect [2]. This evidence supports the findings that phagocytosis for *Nocardia* by alveolar macrophages was not seen in our case. In addition, other bacterial agents isolated in this case may be involved in this unique presentation as another potential cause. Both *Clostridium* and *Shewanella* spp. cause pneumonia in dolphin [3, 22], and *Shewanella* spp. in particular are a common organism in seawater [21]. Co-infection with these bacteria may affect the inflammatory response dominated by neutrophils in this case.

Morbillivirus infections and toxicants such as heavy metals and organochlorines are considered well-known underlying causes of immunosuppression in marine mammals. Environmental stressors could be another possible cause of impaired immune function [18]. In this case, infection of morbilliviruses was rejected by RT-PCR analysis.

CONFLICTS OF INTEREST. The authors declare that they have no conflicts of interest related to the subject materials discussed in this article.

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