J. Comp. Path. 2012, Vol. 147, 94–99

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DISEASE IN WILDLIFE OR EXOTIC SPECIES

Pulmonary Zygomycosis with *Cunninghamella bertholletiae* in a Killer Whale (*Orcinus orca*)

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Summary

An adult female killer whale (*Orcinus orca*) was transported to the Port of Nagoya public aquarium in June 2010. While the animal was being maintained in the aquarium there was a gradual decrease in body weight. On October 1st, 2010 the whale exhibited signs of gastrointestinal disease and died on January 14th, 2011. At necropsy examination the gastric compartments were filled with a large number of variably-sized rocks (total weight 81.4 kg) and there was marked ulceration in the third compartment. There were multifocal tubercle-like nodules within the lungs and on sectioning there were numerous abscesses and pulmonary cavities. Microscopically, there was severe suppurative pneumonia associated with fungal hyphae that were infrequently septate and often branched. Numerous bacterial colonies were also present. The hyphae demonstrated immunohistochemical cross-reactivity with *Rhizomucor* spp. and *Cunninghamella bertholletiae* was cultured. Bacteriological culture revealed the presence of *Proteus mirabilis*, *Pseudomonas aeruginosa* and *Pseudomonas oryzihabitans*. This case represents the first documentation of zygomycosis associated with *C. bertholletiae* in a marine mammal.

Keywords: Cunninghamella bertholletiae; killer whale; pulmonary zygomycosis

Fungi may be primary or secondary pathogens in cetaceans (Robeck and Dalton, 2002). *Candida* spp. and *Aspergillus* spp. are the most frequently recovered and best-known causes of mycotic infections in marine mammals (Migaki and Jones, 1983), but there have been several reports of zygomycosis (Wünschmann *et al*., 1999; Thomas *et al*., 2001; Robeck and Dalton, 2002). *Cunninghamella bertholletiae* (class Zygomycetes, order Mucorales) is a saprobic fungus found in the soil in temperate climates. Pulmonary infections caused by this fungus are being identified with increasing frequency among human patients receiving immunosuppressive therapy and usually have a fatal outcome (Mazade *et al*., 1998). Angioinvasion is often seen in zygomycosis, although the mechanism by which this process occurs is unknown (Frater *et al*., 2001). The infection often progresses rapidly due to fungal invasion of the blood vessels, thrombosis and subsequent tissue infarction (Honda *et al*., 1999).

A 28-year-old female killer whale (*Orcinus orca*) had been kept at the Taiji Whale Museum (2934-2 Taiji, Higashimuro-gun, Wakayama, Japan) for over 20 years after having been captured in the Pacific Ocean off the Taiji coast in Wakayama Prefecture. The whale was moved to the Port of Nagoya public aquarium at the beginning of June, 2010. The animal exhibited gradual loss of body weight over several months after its arrival at the new location. Signs of anorexia first appeared at the beginning of October 2010. The animal became severely anorexic in mid-December 2010.

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0021-9975/$ - see front matter
doi:10.1016/j.jcpa.2011.08.012

Haematological and serum biochemical examinations were performed and the results were compared with earlier historical data from the same animal (Table 1). The total white blood cell (WBC) count was elevated and there was an increased fibrinogen concentration with decreased serum alkaline phosphatase (ALP). Follow-up blood tests revealed the same results with additional evidence of anaemia.

Based on blowhole swab culture, which grew *Morganella morganii* spp. (+), *Proteus* spp. (+), group C-β *Streptococcus* (++) and α-*Streptococcus* spp. (++), the animal was treated with amoxicillin–clavulanate (5 mg/kg, per os, q12h) for 1 month. Antifungal treatment with nystatin (7,000 IU/kg, per os, q12h) was administered for 2 weeks to prevent secondary fungal infection. Amoxicillin–clavulanate was discontinued and cephalexin (11 mg/kg, per os, q8h) was administered for 3 days. Faecal culture grew *Edwardsiella* spp. (+++) and *Proteus* spp. (++) at this point (33 days after the initial clinical signs) and so ofloxacin (2.5 mg/kg, per os, q12h) was administered for 8 days, then ofloxacin was discontinued and the animal was placed on cefdinir (3.75 mg/kg, per os, q12h) for 5 days on the basis of another faecal culture, which grew enterotoxigenic *Escherichia coli* (++). At 46 days from the onset of clinical signs, sputum culture grew *Vibrio alginolyticus*, *Proteus mirabilis* and *Enterococcus faecalis*, and so treatment was continued with levofloxacin (3.75 mg/kg, per os, q12h) for 13 days. At this time, there was transient improvement in the laboratory parameters (Table 1) and general demeanour; however, the appetite remained inconsistent. A further culture of secretion from the nasal cavity grew *Corynebacterium* spp. and the animal was given ciprofloxacin (8 mg/kg, per os, q12h) for 17 days. Amoxicillin–clavulanate (5 mg/kg, per os, q12h) was administered until the time of death.

Blood samples obtained on day 91 after the first onset of clinical signs showed marked elevation of the WBC count and fibrinogen concentration with anaemia (Table 1). At this time there was also a further decrease in serum concentration of ALP and an elevation in blood urea nitrogen (BUN). Blood analysis on day 98 after the first onset of clinical signs showed anaemia, a sudden decrease in WBC count and ALP and an increase in aspartate aminotransferase (AST), alanine amino transferase (ALT) and serum concentration of immunoglobulin (Ig) M, together with persistent elevation of fibrinogen and lactic dehydrogenase (LDH) (Table 1).

The animal died on January 14th 2011 and a complete necropsy examination was performed 4 h after death. The whale was in poor bodily condition (2,450 kg in body weight) and measured 589 cm in length. There was marked enlargement of the stomachs and three stomach compartments contained a large number of palpable stones measuring 1–15 cm in diameter (Fig. 1a). The wall of the first compartment was markedly thinned, with pale mucosa, and contained 474 variably-sized stones with a total weight of 69.2 kg. The second compartment contained 16 stones collectively weighing 11.5 kg. The third compartment

Table 1
Summary of laboratory data

Parameter	*Day 1*	*Day 10*	*Day 26*	*Day 51*	*Day 63*	*Day 83*	*Day 91*	*Day 98*	*Reference range*[*]
RBCs ($\times 10^{12}$/l)	4.78	4.39	**4.12**	**3.86**	**3.78**	**3.36**	**3.06**	**3.13**	439–473
Haemoglobin (g/l)	161	146	135	119	113	108	95	95	149–165
Haematocrit (%)	50.2	46.1	**39.1**	**39.6**	**36.5**	**32.7**	**28.6**	**30.0**	46.9–50.6
Platelets ($\times 10^{9}$/l)	66	76	57	60	97	72	60	69	53–76
WBCs ($\times 10^{9}$/l)	**12.5**	**14.0**	**15.8**	**16.1**	6.1	**14.1**	**21.25**	7.1	5.5–9.9
Neutrophils ($\times 10^{9}$/l)	**10.29**	**11.75**	**13.24**	**13.22**	4.52	**11.76**	**19.34**	5.90	3.96–8.47
Eosinophils ($\times 10^{9}$/l)	0.25	0.21	0.62	0.47	0.33	**0.68**	0.13	0.11	0.08–0.54
Lymphocytes ($\times 10^{9}$/l)	1.48	1.54	1.44	**2.08**	1.08	1.28	1.15	0.99	0.51–2.03
Monocytes ($\times 10^{9}$/l)	0.49	0.50	0.51	0.34	0.17	0.38	0.64	0.11	0.09–0.69
Fibrinogen (μmol/l)	**10.11**	**14.94**	**12.64**	**13.44**	**15.29**	**14.61**	**20.90**	**24.52**	6.20–9.91
Albumin:Globulin ratio	0.91	**0.74**	**0.61**	**0.58**	**0.56**	**0.46**	**0.48**	**0.43**	0.89–1.04
AST (U/l)	40	52	43	35	33	38	44	**109**	33–49
ALT (U/l)	15	12	9	9	8	9	8	**26**	10–18
ALP (U/l)	**145**	**92**	**78**	**91**	**93**	**81**	**93**	**86**	165–330
Creatinine (μmol/l)	212.16	194.48	194.48	185.64	194.48	256.36	176.8	229.84	176.8–291.72
BUN (mmol/l)	16.71	14.07	15.42	18.85	19.10	**23.35**	**29.85**	**29.31**	15.14–20.24
Glucose (mmol/l)	6.05	6.72	6.49	7.16	6.77	9.10	7.77	7.99	5.83–9.16
LDH (U/l)	401	401	378	346	339	370	**489**	**945**	338–456
Creatine kinase (U/l)	43	36	35	41	45	46	50	58	51–70
IgM (mg/dl)	121	ND	149	152	ND	ND	ND	222	107–149

Boldface entries indicate altered parameters when compared with the reference ranges. ND, not determined; RBCs, red blood cells.
[*]Reference ranges are derived from historical samples taken from the same killer whale.

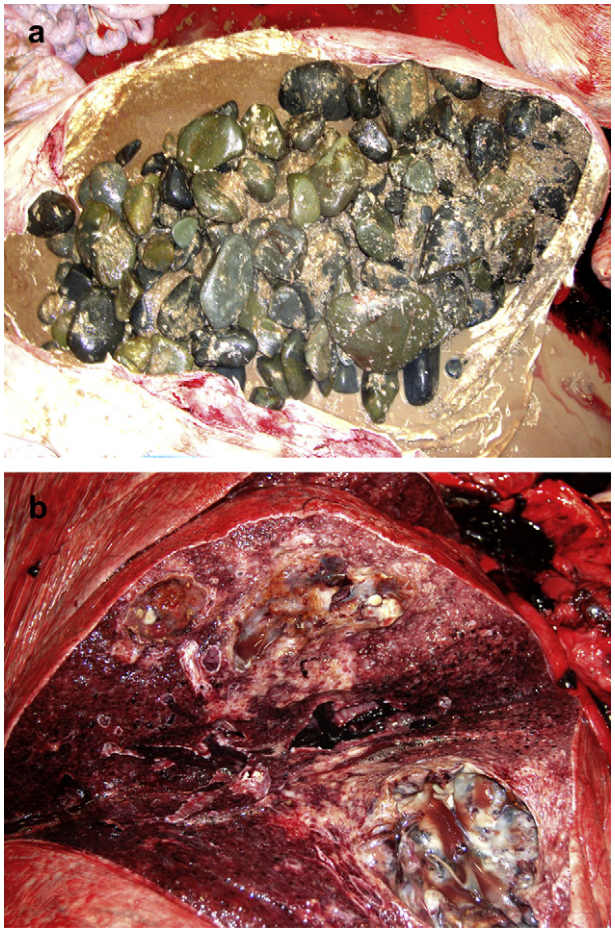

Fig. 1. (a) First stomach showing impaction caused by a large number of ingested stones. (b) Lungs showing multifocal cavitation and consolidation with necrotic areas and abscesses.

contained a single stone 0.7 kg in weight. The stones ranged in size from a few cm to 17 cm in diameter and in shape from round to oval, and all had smooth surfaces. Multiple petechial haemorrhages with severe erosions were observed in the mucosa of the second and third stomachs.

The thoracic cavity contained a small amount of clear red fluid and the bronchial lymph nodes were markedly enlarged and oedematous. The trachea and mainstem bronchi were filled with white froth. Both lungs were consolidated, with numerous nodules, especially in the dorsal half of the lungs. On cut surface, there were multiple areas of consolidation or liquefaction in addition to multiple cavities measuring 3–5 cm in diameter (Fig. 1b). Some cavities had coalesced to form larger cavities and these were generally filled with variable amounts of dark-coloured, caseous material.

There was a large volume (approximately 2 l) of tan-coloured watery pericardial fluid. Impression smears from the lungs revealed numerous bacteria and frequent non-septate fungal hyphae. Other organs appeared grossly normal.

Tissue samples were taken from the lungs in addition to the heart, skeletal muscle, liver, spleen, kidney, different stomach compartments, small and large intestines, pancreas, uterus, bladder and the parotid, submandibular, inguinal, axillary, mediastinal, bronchial, mesenteric and iliac lymph nodes. These samples were fixed in 10% neutral buffered formalin and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE) and selected sections were stained with periodic acid–Schiff (PAS) and Grocott's methenamine silver (GMS).

Microscopically, there was severe suppurative bronchopneumonia. The bronchi and alveoli were markedly dilated and packed with purulent and mucopurulent exudates consisting of neutrophils, macrophages, mucus and cellular debris containing thin-walled, randomly-branched basophilic hyphae as well as numerous bacterial colonies (Fig. 2a). With GMS staining, the hyphae were seen to be

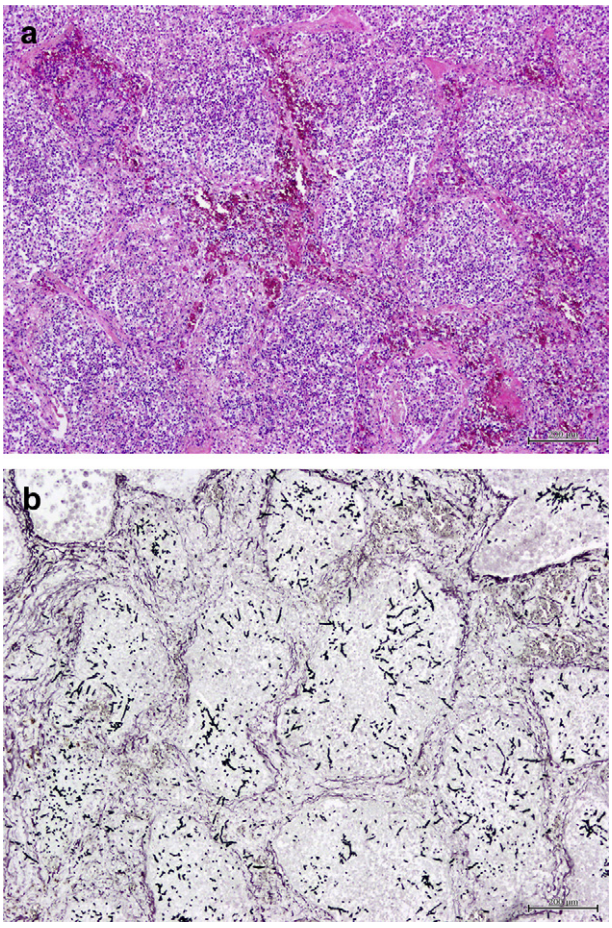

Fig. 2. (a) Lung showing severe purulent bronchopneumonia. HE. Bar, 200 μm. (b) Lung showing a large number of fungal hyphae. GMS. Bar, 200 μm.

3–4 μm in diameter, sparsely septate, thin-walled, irregularly-branched and tangled. There were frequent focal bulbous dilations (Fig. 2b). Extensive areas of infarction and haemorrhage were often associated with necrotizing vasculitis. Numerous fungal hyphae were detected within the vessel walls and lumina (Fig. 3a). Other areas showed abscess formation surrounded by numerous fungal hyphae.

There was severe lymphadenitis characterized by marked macrophage infiltration of the dilated sinuses with prominent erythrophagocytosis. The liver showed severe congestion and hepatic degenerative changes in the form of multifocal centrilobular eosinophilic foci associated with focal areas of coagulative necrosis, in addition to slight to moderate periportal lymphocytic infiltration (Fig. 3b). The spleen was markedly congested, with subcapsular multifocal haemorrhage and moderate lymphoid depletion in addition to diffuse extramedullary haematopoiesis represented by the presence of erythroblasts and megakaryocytes. Both kidneys showed slight congestion. The mucosa of the gastric compartments showed multifocal haemorrhage and there were ulcers in the third stomach, with frequent bacterial colonies seen on the surface of the ulcerated mucosa. The small and large intestines showed features of chronic enteritis, with moderate to severe lymphoplasmacytic infiltration of the lamina propria and degenerative changes in the epithelium. A cross-section of a nematode of undetermined species was seen in the intestinal lumen. The pancreas showed marked depletion of zymogen granules in the acinar cells, with mild interstitial fibrosis. The heart had multiple eosinophilic foci in the myocardium of the right ventricle with mild myocardial fibrosis. A fibrinous thrombus was seen in the lumen of the right ventricle.

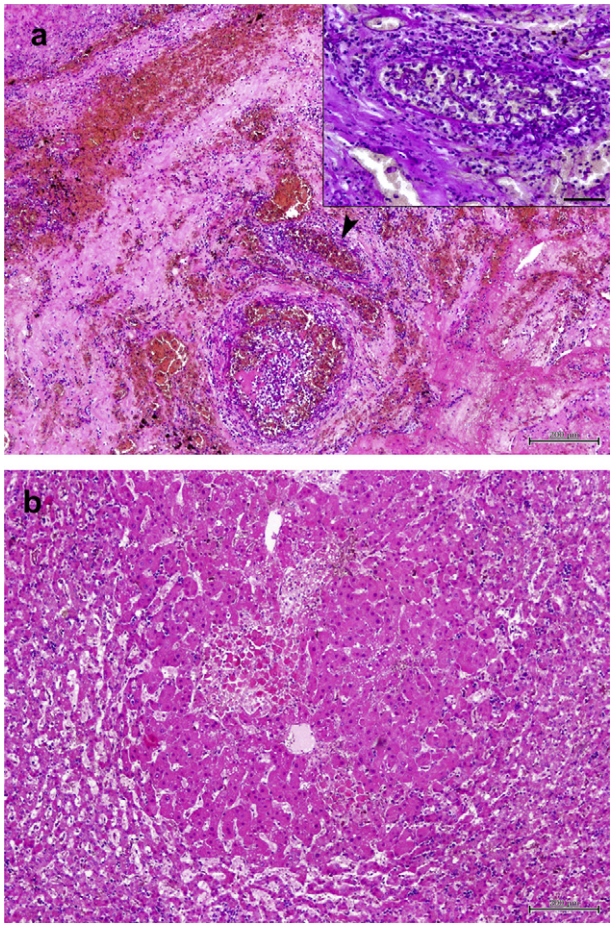

Fig. 3. (a) Lung showing necrosis and haemorrhage associated with necrotizing vasculitis. HE. Bar, 200 μm. (Inset) There are mycotic emboli within the blood vessels (arrow). PAS. Bar, 50 μm. (b) Liver showing centrilobular eosinophilic foci with mid-zonal coagulative necrosis. HE. Bar, 200 μm.

Serial sections were prepared for immunohistochemistry (IHC) with the universal immunoenzyme polymer method using a Histofine simple stain MAX-PO™ Kit (Nichirei Corp., Tokyo, Japan). The sections were pre-treated with 0.1% trypsin for antigen retrieval and endogenous peroxidase activity was blocked by H_2O_2 3% in methanol. Primary antibodies used in this study were murine monoclonal antibodies specific for *Aspergillus fumigatus* wall fractions (monoclonal antibody WF-AF-1; catalogue number M3564; Dako, Carpinteria, California, USA) and water-soluble somatic antigens from *Rhizopus arrhizus* (monoclonal antibody WSSA-RA-1; catalogue number M3565; Dako) and rabbit polyclonal antibodies to *Candida albicans* (1750-5507 Biogenesis, Poole, Dorset, UK). Sections were lightly counterstained with haematoxylin and assessed by light microscopy. Simultaneously, bovine or human tissues infected with *Aspergillus* spp., *Zygomycetes* and *Candida* spp. were labelled as positive controls (Yokota *et al*., 2004a, b; Ogawa *et al*., 2008). Negative controls were performed by replacing the primary antibody with phosphate buffered saline or non-immune mouse or rabbit serum.

IHC of the lung lesions revealed strong and uniform labelling of the hyphae with antibody specific for *R. arrhizous* (Fig. 4), but these structures were negative when tested with reagents specific for *A. fumigatus* and *C. albicans*.

Fungal culture and identification of the fungus was performed in the Department of Pathobiology, Nihon University School of Veterinary Medicine. A zygomycete, *C. bertholletiae*, was identified by morphological characteristics (Kwon-Chung and Bennet, 1992) and confirmed by molecular techniques using the internal transcribed spacer (ITS) region of the ribosomal DNA (Kano *et al*., 2011). Comparison of the sequence of the clinical isolate with the ITS region in GenBank showed 100% identity to *C. bertholletiae* (GenBank accession number FJ345351).

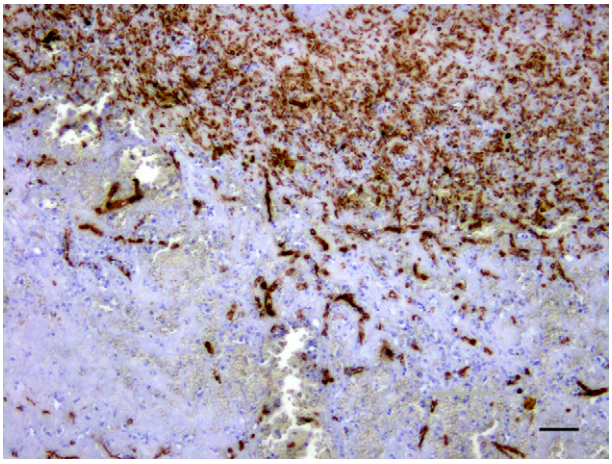

Fig. 4. Immunolabelling of the hyphae by antiserum specific for Mucorales. IHC. Bar, 200 μm.

Microbiological analysis and identification of the isolated bacteria were performed by the Microbiology Department at Gifu University. Culture for aerobic and anaerobic microorganisms yielded *P. mirabilis*, *Pseudomonas aeruginosa* and *Pseudomonas oryzihabitans* from the lungs. Most of the isolates were resistant to the antibiotics that had been used for therapy.

Compared with a number of wildlife species, there is little information available concerning diseases in free-ranging and captive killer whales, which makes the identification of potential pathogens more difficult. In a review of 143 captive and wild marine mammals (of 24 species) with mycotic infections, 19 different fungal species were identified (Reidarson *et al.*, 1999). Sixty-nine (48%) of these animals were found stranded and 45 of these had an underlying non-mycotic disease. The other 74 animals were from various oceanaria and 31 (42%) of these also had some underlying non-mycotic illness. Pulmonary mycotic infections are reported to be the most common mycoses in cetaceans (Reidarson *et al.*, 1999). Zygomycosis has been recorded in a variety of species, including infections with *Apophysomyces elegans*, *Saksenaea vasiformis* (Thomas *et al.*, 2001; Robeck and Dalton, 2002), *Rhizomucor pusillus* (Thomas *et al.*, 2001), *Entomophthora coronata* (Sweeney *et al.*, 1976) and *Rhizopus* spp. (Wünschmann *et al.*, 1999; Naota, *et al.*, 2009). Most cases of zygomycosis have revealed systemic involvement, including the brain and heart, as reported in a harbour porpoise and a finless porpoise, respectively (Wünschmann *et al.*, 1999; Naota, *et al.*, 2009).

To the best of our knowledge, the present case is the first report of zygomycosis caused by *C. bertholletiae* infection in marine mammals. In man, *C. bertholletiae* is becoming a serious pathogen affecting immunocompromised patients (Cohen-Abbo *et al.*, 1993). *Cunninghamella* spp. infections most frequently involve the lungs and have been reported in two cases of endocarditis and three cases of major epicardial artery thrombosis, as well as in 17 cases of cardiac involvement due to other zygomycetes. The infection is known for its very rapid, refractory course, and mortality is around 60–82% (Cohen-Abbo *et al.*, 1993; Ng *et al.*, 1994; Lee *et al.*, 1999).

Mycoses in cetaceans may also be indicative of immunosuppression. A large number of stones were found in the stomach compartments of this killer whale and could have been a factor facilitating mycosis. The stones were consistent with those found at the bottom of the lagoon at the Taiji Whale Museum where the animal had been living for over 20 years. It is uncommon for killer whales to ingest foreign objects; however, the number and weight of the rocks found in the stomach of this animal is unprecedented. The killer whale must have ingested these stones over a long period of time. Immunosuppression may have permitted both *C. bertholletiae* and opportunistic bacteria to colonize the lungs.

Based on the histopathological findings, the possible cause of death in this animal was severe bronchopneumonia; however, degenerative changes in the liver and systemic lymphadenitis may have been a result of subsequent septicaemia caused by opportunistic microflora. The observed pulmonary cavitation and necrosis is suspected to have been caused by *C. bertholletiae*. The most significant pathological findings involving *C. bertholletiae* have been described as angioinvasion accompanying haemorrhage and necrosis (Honda *et al.*, 1999).

The primary site of infection with zygomycetes is often the subcutaneous tissues and skeletal musculature (Robeck and Dalton, 2002); however, the lungs, nasal sinus and alimentary tract are obvious entry portals for the fungus (Migaki and Jones, 1983; Thomas *et al.*, 2001).

The compromised immune status of the killer whale could have resulted in secondary infection by common microflora. *P. mirabilis*, *P. aeruginosa* and *P. oryzihabitans* are ubiquitous in the environment and are considered as opportunistic pathogenic microorganisms. These organisms may cause suppurative infection in immunocompromised individuals (Watanakakunakorn and Perni, 1994; Marin *et al.*, 2000). In marine mammals, *P. aeruginosa* has been isolated from abscessing pneumonia in a killer whale that died after transportation stress (Rozanova *et al.*, 2007).

The clinical pathology investigation of this case revealed increased total WBC count and fibrinogen concentration. Killer whales have a strong leucocytic response to infection (Thomas and Reidarson, 1999). Fibrinogen is a non-specific acute phase reactant, but

a reliable marker of inflammation (Thomas and Reidarson, 1999). It can also simply be an indicator of zygomycosis (Robeck and Dalton, 2002). Two days before death, the killer whale exhibited anaemia, a sudden decrease in WBC count and increases in most of the serum enzymes, which could possibly indicate systemic involvement and/or toxaemic and septicaemic states caused by the opportunistic bacteria. In marine mammals, a decreased WBC count is sometimes noted in fatal infectious diseases (Shirai and Sakai, 1997). Increased AST and ALT are generally related to both hepatic and muscular damage (Bossart *et al*., 2001).

The present case emphasizes the pathogenic potential of *C. bertholletiae* zygomycosis in marine mammals, as well as the potential for infection with opportunistic microflora, particularly *P. mirabilis*, *P. aeruginosa* and *P. oryzihabitans*, in an immunocompromised killer whale.

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[*Received, May 30th, 2011*
Accepted, August 29th, 2011]