

## Sudden death of a Siamese crocodile (*Crocodylus siamensis*) due to systemic aspergillosis

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**ABSTRACT.** A 4-year-old female Siamese crocodile (*Crocodylus siamensis*) housed at a zoo died without any prior clinical signs. During necropsy, numerous scattered, well-demarcated, yellowish-white, firm nodules were observed throughout the liver and lungs. Microscopic examination with periodic acid-Schiff staining revealed granulomatous inflammation in the liver and lungs. Liver granulomas were characterized by the presence of a connective tissue barrier and hyphae, and the centers of the granulomas showed signs of necrosis. Lung samples showed characteristics similar to those observed in the liver samples. The fungus was identified as *Aspergillus fumigatus* based on its appearance on Sabouraud dextrose agar, microscopic examination with lactophenol cotton blue staining and genetic sequencing. Therefore, zoo veterinarians should pay close attention to fungal infections in captive animals.

**KEY WORDS:** *Aspergillus fumigatus*, *Crocodylus siamensis*, fungus, siamese crocodile

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Members of the genus *Aspergillus* are ubiquitous, and their spores are widely distributed [5]. *Aspergillus* spp. cause disease by invading body tissues and producing toxins [16]. In reptiles, systemic aspergillosis often originates from the respiratory and gastrointestinal tracts and is commonly associated with opportunistic saprophytic fungi [21]. In previous cases, aspergillosis was found in reptiles kept in environments with suboptimal conditions, such as improper temperatures, inappropriate humidity levels, poor hygiene and chronic stressors [5, 15, 16, 21, 23].

In crocodiles, fungal dermatitis and pneumonia are frequently associated with *Fusarium* spp., *Candida* spp. and *Aspergillus* spp. [16]. Fungal pneumonia associated with *A. fumigatus* and *A. ustus* has been reported in juvenile American alligators (*Alligator mississippiensis*) [6, 8]. Systemic fungal infections caused by *F. solani* have been described in captive saltwater crocodiles (*Crocodylus porosus*) and freshwater crocodiles (*Cr. johnstoni*) [3, 16, 17]. This is the first report of systemic aspergillosis causing the sudden death of a Siamese crocodile in Korea.

A 4-year-old female Siamese crocodile (*Cr. siamensis*) housed at a zoo died without any prior clinical signs. The animal was housed with a 3-year-old male Siamese crocodile in an enclosed facility at Daejeon O-World Theme Park, located in the middle of Korea (36°17'N, 127°23'E), and she was fed a diet of chicken and pork.

During necropsy, multifocal nodules were observed in the lung lobes (Fig. 1A). The examination of the cut surface of the liver parenchyma indicated the presence of scattered white nodules of approximately 1 cm in diameter (Fig. 1B). In the stomach, there were several ulcerations with greenish watery exudates. The remaining organs did not show any remarkable findings.

Lung and liver samples were submitted to the zoo laboratory for microbiological examination, and additional samples were fixed in 10% neutral buffered formalin for 2 weeks. Paraffin sections were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination and with periodic acid-Schiff (PAS) for differential diagnosis of fungal infection. The sampled organs were cultured on blood agar and MacConkey agar at 37°C for 12–18 hr. In addition, a culture was performed on Sabouraud dextrose agar (SDA) at 37°C for 5 days under aerobic conditions to cultivate the fungi [10].

The histopathological examination of the liver and lungs was unremarkable. When the liver was examined with PAS staining, the proliferation of fungal hyphae within the parenchyma and granulomas were observed (Fig. 1C). The granulomas were well demarcated and surrounded by fibrous connective tissue, and the centers of the granulomas displayed signs of necrosis. The fungal elements consisted of long, branched, septate hyphae and were surrounded by heterophils, macrophages, epitheloid cells and multinucleated giant cells (Fig. 1D). Similar granulomatous changes were found in the lungs.

Cultures of lung and liver samples produced velvet-like, bluish-green fungal colonies on SDA (Fig. 2A); however, bacterial colonies were not observed in the culture on blood agar or MacConkey agar. Microscopic examination of samples stained with lactophenol cotton blue revealed that each fungus had a columnar conidial head and phialidae on the

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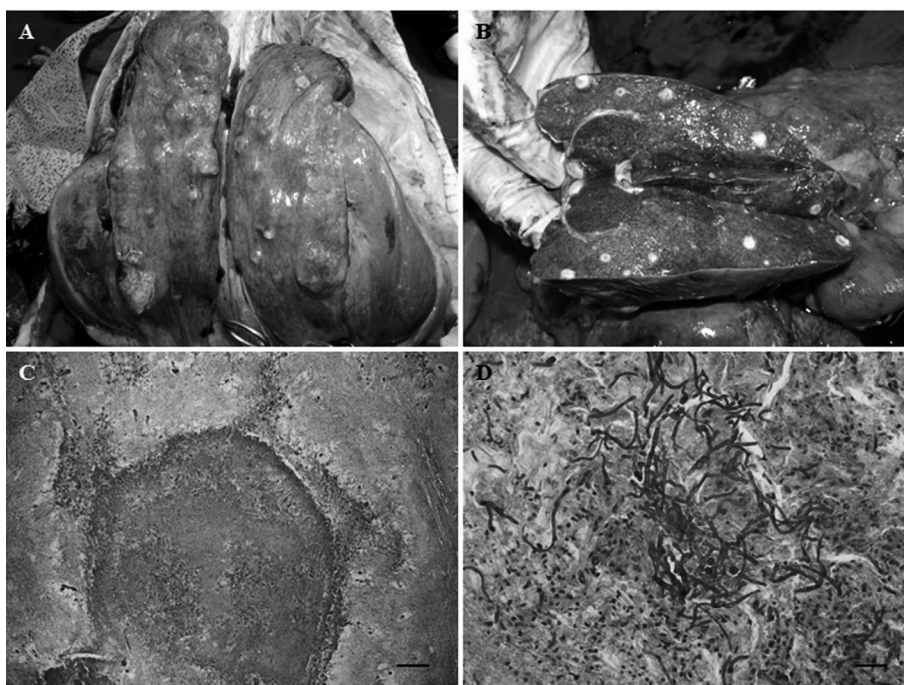


Fig. 1. Gross appearance of the lungs and liver in the diseased Siamese crocodile at necropsy and samples of the lungs and liver stained with periodic acid-Schiff. (A) Multiple scattered nodules in the lung lobes. (B) Many white nodules (approximately 1 cm in diameter) in the parenchyma of the dissected liver. (C) Well-demarcated granuloma in the liver ( $\times 40$ ). Bar=200  $\mu\text{m}$ . (D) Hyphae and inflammatory cells in the liver parenchyma ( $\times 400$ ). Bar=50  $\mu\text{m}$ .

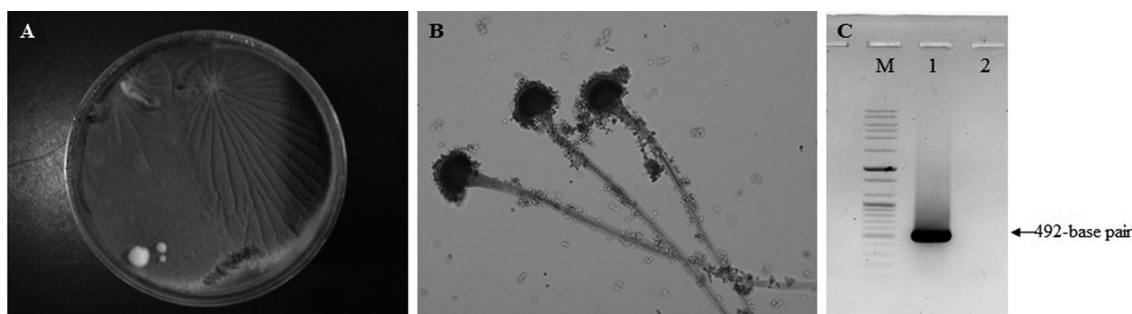


Fig. 2. Species identification of *Aspergillus fumigatus* cultured from the lesions was based on the appearance of colonies, microscopic observation and nucleotide sequencing. (A) Colonies cultured on Sabouraud dextrose agar appear velvet-like and bluish-green. (B) Dome-shaped colonies stained with lactophenol cotton blue show a columnar conidial head and phialidae on the upper half, which are typical of *A. fumigatus* ( $\times 200$ ). (C) Electrophoresis gel showing the 492-bp amplicon of an *A. fumigatus*  $\beta$ -tubulin gene fragment. Lanes: M, 100-bp DNA ladder; 1, *A. fumigatus* in this study; 2, a negative sample.

upper half, which are typical of *A. fumigatus* (Fig. 2B) [22].

In addition to the examination of morphological features, genetic sequencing was also used for species identification. DNA of *A. fumigatus* cultured on SDA was extracted by using the DNeasy<sup>®</sup> Blood & Tissue Kit (Qiagen, Hilden, Germany), and PCR was performed to amplify the  $\beta$ -tubulin gene of *A. fumigatus* as described in a previous study [1]. Agarose gel electrophoresis of PCR products showed a 492-bp amplicon of *A. fumigatus*  $\beta$ -tubulin gene fragment (Fig. 2C), and the amplified PCR products were sent to Sol-

gent (Daejeon, Korea) for nucleotide sequencing. Use of the Basic Local Alignment Search Tool revealed a high degree of homology between this sequence and those of *A. fumigatus* deposited in GenBank database: 98.8% (DQ438512) to 100% (KJ527255). On the basis of morphological assessments and genetic sequencing results, the causative agent was identified as *A. fumigatus*.

Only a few studies have reported aspergillosis in free-living reptiles, but numerous cases have been reported in captive chelonians and crocodiles [5, 7]. Most cases of

systemic aspergillosis in reptiles were diagnosed at necropsy [5]. The prognosis of aspergillosis is poor, particularly when it involves a major organ or the infection has spread to multiple locations [5, 21].

The species of many fungal genera, such as *Aspergillus*, *Penicillium*, *Basidiobolus*, *Fusarium* and *Mucor*, have been isolated from the intestine and feces of healthy reptiles [13, 16]. *A. niger*, *A. fumigatus*, *Pseudallescheria boydii*, *Penicillium* spp., *Paecilomyces* spp. and *Cladosporium* spp. (all of which belong to the normal fungal population of the lungs) have been isolated from pulmonary lesions [11, 16]. These species usually cause opportunistic infections, but can occasionally act as primary pathogens [19]. Typically, fungal infections are due to immunosuppression, overexposure to fungal spores or stressors within the captive environment [14, 21]. In field situations, inadequate husbandry practices, poor sanitation, overcrowding, stressful conditions and heating system failure were identified as potential predisposing factors [21] that might have suppressed the immune system and caused a secondary systemic fungal infection. Because reptiles are ectotherms, their core body temperatures are more prone to fluctuations compared to those of mammals or birds. The wider variation in body temperature may favor the development of mesophilic fungi in reptiles [16].

In the present study, *A. fumigatus*, which causes aspergillosis, was identified by the appearance of the colonies on SDA, by microscopic examination of samples stained with lactophenol cotton blue and by genetic sequencing. Microscopic observation revealed that the fungal spores were dome-shaped, which is characteristic of *A. fumigatus* and *A. terreus*, and does not radiate as in *A. niger*, *A. flavus* and *A. glaucus* [22]. The color of the colonies on SDA was blue-green, which is consistent with the presence of *A. fumigatus*, and was not brown, as in *A. terreus* [22]. However, identification of *A. fumigatus* can be difficult, because the morphological features within that species vary and depend on growth conditions [1, 2]. Usually, the recommended treatment for systemic aspergillosis in reptiles has been a systemic antifungal chemotherapeutic, such as amphotericin B, fluconazole, mycystatin, itraconazole, ketoconazole, fluorocytosine or terbinafine [5]. Because *A. fumigatus* resembles other species within the genus, such as *A. lentulus* and *A. udagawae*, that have low susceptibility to multiple antifungal drugs [2], it is important to identify the species by using molecular techniques.

This study and previous studies [12, 18, 20] revealed that the skin, respiratory tract and the gastrointestinal tract, are the usual routes of entry for the fungi that cause aspergillosis in reptiles. In this case, stress, improper temperature and inappropriate humidity may have predisposed the crocodile to systemic aspergillosis by facilitating mycotic invasion.

In this study, we described for the first time the sudden death of a Siamese crocodile due to systemic aspergillosis in Korea. Usually, *A. fumigatus* is not considered as a primary pathogen in crocodiles. However, aspergillosis in captive animals, including monkeys, vultures and penguins, has been reported in several studies [4, 9, 10]. Therefore, zoo veterinarians should pay close attention to fungal infections

in captive animals and to the environmental conditions under which they are kept.

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