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The Occurrence and Molecular Characterization of Feline Cryptococcosis in Korea

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ABSTRACT : A spayed female domestic short-hair cat of unknown age was admitted with a large proliferative mass in the face. Cytology and biopsy results suggested infection with *Cryptococcus spp*. A latex cryptococcal antigen agglutination test and an ALPHA cryptococcal antigen enzyme immunoassay yielded positive results. Results of canavanine-glycine-bromothymol blue agar test, serotyping and molecular typing by URA5 - RFLP and MLST analysis identified the isolates as *C. neoformans var. grubii* VNI/ST31. Two other cats were also diagnosed with the same methodology showing Crytococcosis with VNI/ST31. Cats presenting with facial or respiratory signs should be assessed for cryptococcosis in Korea.

Key words **:** Cryptococcosis, Feline, Korea, VNI/ST31.

Introduction

Feline cryptococcosis is a systemic fungal infection caused by *Cryptococcus* organisms acquired from contaminants in the environment, including in soil, avian guano, and tropical trees such as eucalyptus leaves. Because animals may be infected by inhalation of airborne pathogens, the most frequent site of primary infections is the upper respiratory tract. Severe infection may manifest as solitary masses protruding from one or both nostrils, resulting in facial distortion (4).

Cryptococcus is a basidiomycetous yeast of the genus *Cryptococcus* which includes species such as *C. neoformans* and *C. gatti*. In Korea, *C. neoformans var. grubii* (serotype A) was first detected in pigeon droppings in 2005 (2). The most frequently detected molecular types are *C. neoformans* VNI/ST5 and VNI/ST31, which are genetically homogeneous; moreover, clinical and environmental isolates are closely related genetically (11). To date, however, infection with *C. neoformans var. grubii* has been rarely reported in domestic animals (14). To our knowledge, this report is the first molecular characterization of feline Cryptococcosis with *C. neoformans var. grubii.* in Korea.

Case

A spayed female domestic short-hair cat of unknown age (Cat 1), weighing 3.1 kg, was rescued by the Korean Animal Rights Advocates (KARA) and admitted to the Western Animal Medical Center in Seoul, Korea. Physical examination showed a large proliferative mass (7 × 9 cm) over almost the entire face, including the right eye and nose (Fig. 1). Results of blood tests were all within normal range, including serum aspartate transaminase (AST; 76 U/L; normal range, 28-106 U/L) and globulin (4.9 g/dL; normal range 2.8-5.1 g/dL) concentrations, although the total protein concentration was high (8.6 g/dL; normal range 6.6-8.4 g/dL). Serological tests showed that the cat was negative for feline leukemia virus (FeLV) antigen and antibody against feline immunodeficiency virus (FIV). Findings on thoracic and abdominal radiology and abdominal ultrasonography were not specific. Skull radiography revealed soft tissue masses in the right orbital and extra nasal levels with increased opacity of right nasal cavity. Computed tomography (CT) of the head and neck showed an extensive, aggressive nasal mass with heterogeneous enhancement in both nasal cavities and in the

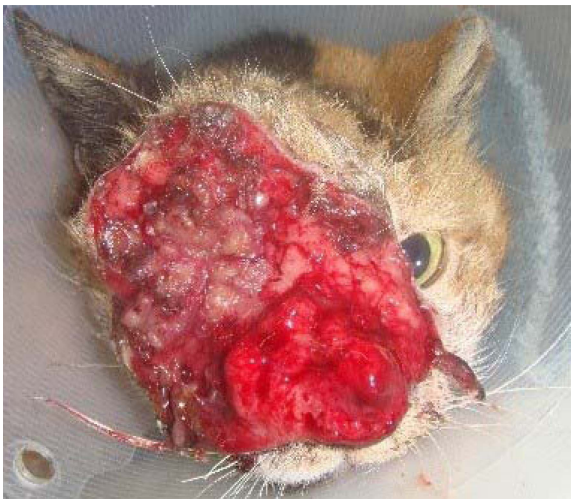

Fig 1. Photographs of Cat 1 showing the facial mass shortly after being rescued from the street.

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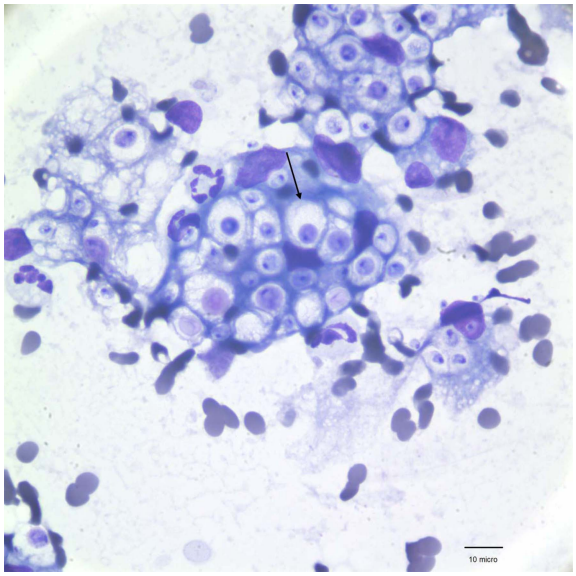

Fig 2. Fine needle aspiration cytology of the facial mass in Cat 1. Note the macrophages phagocytizing numerous encapsulated yeast cells having a clear thick capsule (arrow) (Diff Quick, x400).

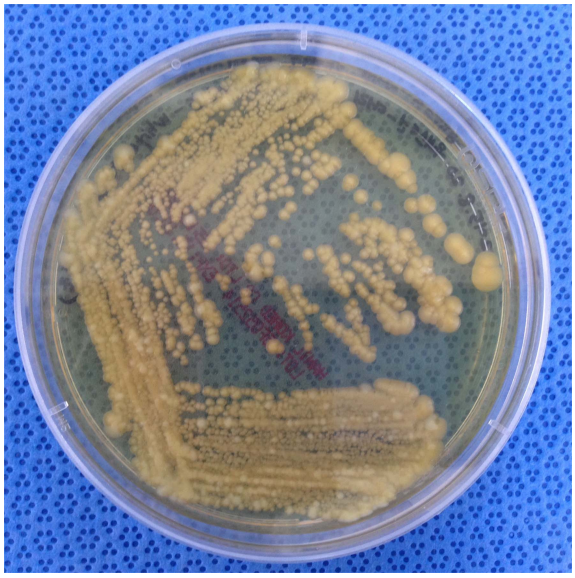

Fig 3. *Cryptococcus* spp. cultures. An isolate from Cat 1 was aspirated with a 23-gauge needle and spread onto SDA medium. Colonies were visible within 2 days after inoculation, and had a cream-white color and mucoid appearance.

right frontal and maxillary sinuses. Multiple bone lysis was observed in the nasal septum, the entire concha and adjacent (nasal, frontal, maxillary) bones. CT also showed distinct enlargement of the bilateral submandibular and retropharyngeal lymph nodes and of the right prescapular lymph nodes.

Fine needle aspiration (FNA) of the lesion was performed for cytological examination. Staining of FNA smears stained with Diff-Quik (International Reagents Corp., Kobe, Japan) showed numerous macrophages containing basophilic round yeast organisms (3-12 μm) having a clear, thick halo capsule (Fig 2). Moreover, some cells showed narrow budding, a finding consistent with cryptococcal infection. Histologic analysis of biopsy samples collected during surgery and fixed in 10% buffered formaldehyde showed that the mass was comprised of very dense granulomatous inflammation extending to the connective tissue and the margins. Large numbers of irregular transparent and/or clear yeast-like organisms, suspected of being *Cryptococcus spp.*, were also observed. Mayer's mucicarmine method specifically stained the capsule of these cryptococci.

The serum of this cat was positive on a latex cryptococcal antigen agglutination test (LCAT), with a titer of 1: 1,496. In addition, the serum was positive for capsular polysaccharide antigens of Cryptococcus species on an ALPHA cryptococcal antigen enzyme immunoassay (CrAg EIA). A sample obtained from the facial mass was homogenized in unbuffered saline and cultured on Sabouraud dextrose agar for 5 days at 27°C. Isolated colonies isolated were white to cream in color, with a smooth appearance and glistening surfaces (Fig 3). The colonies grew uniformly on Sabouraud agar, with the cultured yeast cells being identical in appearance to a Cryptococcus specimen. To distinguish between *C. neoformans* and *C. gatti,* the colonies were inoculated on canavanine-glycine-bromothymol blue agar and cultured at 25-30°C for 5 days (8). Because the colonies were light-green/blue in color (negative), they were confirmed as being *C. neoformans*.

The colonies were serotyped by slide agglutination (Crypto Check Kit; Iatron Laboratory, Tokyo, Japan) and compared with the reference strains WM148 (serotype A, VNI), WM626 (serotype A, VNII), WM628 (serotype AD, VNIII), and WM629 (serotype D, VNIV). All serotypes were confirmed as being *C. neoformans var. grubii* (serotype A).

Genomic DNA was extracted from each isolate using the Qiagen DNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. The URA5 gene was amplified by PCR, followed by digestion with the restriction enzymes HhaI and Sau96I, as previously

Table 1. The allelic profiles and sequence types of *Cryptococcus neoformans* isolates.

Strain	Molcular type	AT							ST
		CAP59	*GPD*1	*IGS*1	*LAC*1	*PLB*1	*SOD*1	*URA5*	
Cat 1	VNI	1	1	10	3	2	1	1	31
Cat 2	VNI	1	1	10	3	2	1	1	31
K52	VNIc/M5	1	3	1	5	2	1	1	5

AT, allele type; ST, sequence type; K52, VNIc/M5 strain included as a reference.

described (3). The digested PCR fragments were electrophoresed on 2.5% agarose gels and compared with four reference strains to determine their molecular types. MLST analysis was performed according to the ISHAM consensus scheme of seven unlinked genetic loci (CAP59, GPD1, LAC1, PLB1, SOD1, URA5, and IGS1). Using DNA from each isolate, each of these seven MLST loci was amplified by PCR in a 20 μL reaction volume using the primers and protocols described previously (10). Each locus was subsequently sequenced using the Applied Biosystems 3730 sequencer with the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA). Alleles at each locus were assigned numbers (allele types [ATs]) after comparisons with alleles previously identified in the global collection, resulting in a seven-digit profile for each isolate. Each unique allelic profile was concatenated and assigned a sequence type (ST) according to the MLST scheme (http://cneoformans.mlst.net). A phylogenetic tree encompassing the combined DNA sequences of these seven genes was inferred by neighbor-joining using MEGA 4 software (http://www.megasoftware.net) (12). The results of these analyses identified these isolates as *C. neoformans var. grubii* (serotype A) VNI/ST31 (Table 1).

This cat (Cat 1) was initially treated with antibiotics and steroids, but was later treated with fluconazole 50 mg and ketoconazole ointment after diagnosis with cryptococcosis. After 2 weeks of medical treatment, the large facial mass with erythema and ulceration was carefully removed under general anesthesia. This was followed by stent application of fluconazole to the wound and additional rounds of facial plastic surgery. Three months later, the cat's face was well healed and the animal's general health was excellent. Although the facial area remained excellent 44 days after discharge from the hospital, the cat remained positive on the Cryptococcus antigen test (titer 1:2048).

A second female cat of unknown age (Cat 2), living in an environment with more than 60 cats, presented with a chronic unilateral nasal discharge and a nasal mass with alopecia. This cat was positive on the LCAT and the ALPHA cryptococcal antigen enzyme immunoassay (CrAg EIA). Serotyping showed infection with *Cryptococcus neoformans var. Grubii* (serotype A) VNI/ST31. Treatment with itraconazole 10 mg/kg and ketoconazole ointment reduced the size of the nasal mass, but the cat died 3 months after admission of unknown causes.

A third female cat (Cat 3) was admitted for evaluation of a proliferating mass between the eyes of 3 months' duration. No improvement was observed after in treatment for herpes virus infection, with deterioration occurring after steroid administration. Cytological evaluation showed numerous round to oval yeast cells within the cytoplasm of macrophages, suggesting cryptococcal infection. The cat was positive on both LACT (titer 1:73) and ALPHA cryptococcal antigen enzyme immunoassay. Administration of fluconazole 50 mg for 3 months resulted in significant resolution of the mass. However, MLST analysis was not performed because the owner refused to allow further tests.

Discussion

Cryptococcus neoformans var. grubii (serotype A) was first detected in South Korea in 2005, when it was found in pigeon droppings (2). *C. neoformans* VNI/ST5 and VNI/ST31 are the serotypes most frequently found in South Korea. MLST results showed that ST5 was predominant in human patients (n = 131/137) and in contaminated environments (n = 7/10), whereas ST31 was present in five of 137 human patients and in three of 10 contaminated environments (11). Although VN/ST5 is the dominant serotype in Korea, ST31 was confirmed to be present in both genotyped cats in this report. The clinical and epidemiological significance of ST31 infection in cats remains unknown (1,13), and whether cats are more susceptible to the ST31 serotype, with more severe clinical manifestations, than ST5 should warrant a confirmation in a larger number of cats.

Most cats acquire *C. neoformans* and *C. gatti* infections by inhaling these pathogens from contaminated environments (13). Genetic studies of cryptococcal isolates from infected individuals and contaminated environments in Japan, China, Thailand and India have identified serotypes ST4, ST5, ST6 and ST93 of *C. neoformans* molecular type VNI. ST31, which is mainly found in India and Thailand, was isolated from a polluted environment, not from infected patients, similar to other STs in Korea (6,7). Thus, the ST31 identified in these animals may have originated from a contaminated environment, suggesting that these may have been the sources of infection of Cats 1 and 2, both of which were living on the road. In the third cat the route was uncertain because it was an indoor cat. It cannot be ruled out the possibility of obtaining the pathogen before she entered the country from the United States.

Although studies have reported that FIV/FeLV infection, glucocorticoid administration, tumors and diabetes mellitus increase feline susceptibility to these infections (1), whereas other studies have found no link to these factors (5). Cats 1 and 2 in this report were not infected with FelV/FIV, and no other underlying diseases were identified.

Cat 1, with a severe facial infection, showed marked improvement following treatment with an antifungal agent and surgery. Complete facial plasticity was not achieved, but quality of life was improved by preserving the right orbit. Clinical symptoms disappeared, but the LCAT still showed a high titer (1:2048), indicating that non-viable cryptococci and/or capsular materials remained in infected tissues and macrophages or indicating insufficient therapy. This could have been confirmed by re-culturing from the previously infected region, but this test was not performed. Therefore, it is necessary to monitor this animal periodically for recurrence and to assess LCAT (9). Cat 2 died of unknown causes after treatment. At the time of diagnosis, FIV/FeLV was excluded and there was no definite underlying disease. However, the persistence of cachexia and hypoglycemia suggests that other conditions may have had a negative impact on the immune system of this animal. Cat 3, which is still being treated with fluconazole for 6 months, has shown a marked reduction in the facial mass without any other complications.

Three cats with facial masses were diagnosed with cryptococcosis infection by cytology and Ag test, with MLST showing that two of these animals were infected with serotype VNI/ST31. All three animals were treated with antifungal agents to reduce the size of the mass, with one also undergoing facial plastic surgery. To our knowledge, this report is the first to identify VNI/ST31 in cats with cryptococcus infection in Korea. Most cats live in a strict indoor environment in Korea, but the numbers of stray cats are increasing (http://www.animal.go.kr/portal_rnl/ index.jsp). Therefore, cats presenting with facial or respiratory signs should be assessed for cryptococcosis.

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